Virus life cycle and the pathogenesis of malignant catarrhal fever

By

Osama Kumati

BVSc & MSc

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Division of Infection and Immunity
School of Veterinary Medicine and Science
The University of Nottingham

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Abstract

Malignant catarrhal fever (MCF) is caused by two closely associated gamma herpes viruses namely alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2) and characterised with lymphocyte infiltration in non-lymphoid tissues, vasculitis and epithelial damage. The mechanism by which the viruses cause the disease is not fully understood. The hypothesis of this project was that MCF is initiated by aberrant gene expression in endothelium, epithelium and infected T cells of susceptible animals, because they are not the natural hosts for the viruses and the viruses will not have evolved in them. The first goal was to examine whether rabbit epithelium and bovine endothelium can be infected *in vitro* and *in vivo* with AlHV-1 using q PCR and, if infected whether viral transcripts could be identified in these tissue cells using q PCR and *in situ* hybridisation (ISH). The results revealed that endothelium and epithelium can be infected and latent infection can be established in them. This suggests the likelihood of establishing a similar type of infection *in vivo*. Secondly, the trial to identify latency-associated transcripts using 5-azacitidine treatment on bovine turbinate fibroblast (BT) cells and rabbit large granular lymphocytes (LGLs) was only partially successful. However, pan T antigen was expressed in 5-azacitidine treated but not untreated LGLs cells. This may indicate a function of the drug either directly or

through the latency state. Transcriptome analysis in the infected and treated LGLs and BT cells showed that several pathways were affected by 5-aza although a possible latency (low transcript levels) was only seen in the BTs. Transcriptome analysis revealed similar pathways to those described for MCF in the tissues *in vivo*, and an effect of 5-aza on these. Viral transcripts analysis showed that genes related to productive/lytic cycles were higher than latent ones on day 17 of the *in vivo* experiment demonstrating that the virus may replicate at this stage of the disease. The attempt to localize the viral transcripts on the rabbit infected tissues using ISH was unsuccessful due to a lack of time.

Declaration

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Nottingham.

The work is original and has not been submitted for any other degree at the University of Nottingham or elsewhere.

Name: Osama Kumati

Signature:

Date:

Conferences, posters, and oral presentations:

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Table of contents

List of tables

List of Figures

List of abbreviations

µl	Microliter
5-aza	5-azacitidine
AlHV-1	Alcelaphine herpes virus 1
APHA	Animal and plant health agency
BAC	Bacterial artificial chromosome
BAE	Bovine aortic endothelium
BLAT	Blast-like alignment tool
BT	Bovine turbinate fibroblast cells
CPE	Cytopathic effect
CpHV-2	Caprine herpes virus 2
CsA	Cyclosporine A
DEFRA	Department of environment, food and rural affairs
DEPC	Diethyl bicarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ECACC	European Collection of Authenticated Cell Cultures
EHV-1	Equine herpesvirus 1
ELIZA	enzyme-linked immunosorbent assay
EM	Electron microscope
ET	Electron tomography

H-DNA	High guanine-cytosine (GC) content of DNA
HVS	Herpes virus saimiri
IFA	Immunofluorescence assay
IFN-γ	Interferon gamma
IL-2	Interleukin-2
IPA	Ingenuity Pathway Analysis
ISH	In situ hybridisation
Kbp	Kilo base pair
KSHV	Kaposi sarcoma-associated herpes virus
LANA	Latency associated nuclear antigen
LATs	Latency associated transcripts
L-DNA	Low guanine-cytosine (GC) content of DNA
LGLs	Large granular lymphocytes
MCF	Malignant catarrhal fever
MCFV	Malignant catarrhal fever virus
MCF-WTD	Malignant catarrhal fever- white tailed deer
ml	Millilitre
mRNA	Messenger Ribonucleic acid
NK	Natural killer cells
ORF	Open reading frame
OvHV-2	Ovine herpes virus 2
PCR	Polymerase chain reaction
RNA	Ribonucleic acid

RTA	R trans-activator protein
RT-PCR	Reverse- transcriptase PCR
SA-MCF	Sheep associated malignant catarrhal fever
SIRC	Serum institute rabbit cornea
TCR	T cell receptor
TGN	Transit Golgi network
WA-MCF	Wildebeest associated malignant catarrhal fever

Chapter 1

Introduction

1.1 Malignant catarrhal fever (MCF)

1.1.1 General comments:

Malignant catarrhal fever (MCF) is acute, systemic, lethal lymphoproliferative disease of cattle and even-toed ungulates including pigs, deer, and bison (Buxton, 1984; Plowright, 1960; Schultheiss, 2000). The disease is caused by group of viruses belonging to the *Herpesviridae* family, subfamily *Gammaherpesvirinae*, genus Macavirus (Li et al., 2005). Two viruses of this group that are known to cause MCF, alcelaphine herpes virus 1 (AlHV-1) and ovine herpes virus 2 (OvHV-2), are widely distributed and extensively studied (Russell et al., 2009). Wildebeest (*Connochaetes taurinus*) is the reservoir host for AlHV-1 in the sub-Saharan area, hence the name wildebeest-associated MCF (WA-MCF) for disease susceptible-animals that contract MCF from wildebeest. Sheep (*Ovis aries*) are the reservoir host for OvHV-2 worldwide; hence the name sheep associated MCF (SA-MCF) (Dewals et al., 2008, Russell et al., 2009). There are no clinical signs of MCF in the reservoir hosts, however, often-fatal MCF is seen when virus is transmitted to the disease-susceptible species (cattle, deer, bison, pigs and water buffalo) (Russell et al., 2009). The clinical signs of MCF range from per-acute to mild form and the outcome are usually fatal although recovery has been recorded. The most common symptoms recorded are high fever; salivation, nasal and ocular discharge, diarrhoea, and enlarged lymph nodes.

Pathologically, the disease is characterised with erosions and haemorrhages in the intestinal tract, vasculitis, and infiltration of lymphoid cells in non-lymphoid organs (kidney, lung, and liver) (CFSP, 2012, OIE, 2013). The importance of MCF lies in the fact that the disease is a major cause of death in many disease-susceptible animals worldwide that come into contact with reservoir hosts. It is also a serious welfare issue (Lankester et al., 2015a, Li et al., 2014). MCF occurs worldwide wherever reservoir and disease-susceptible animals are close to each other, and can affect a wide range of naturally-susceptible species within the Artiodactyl families Cervidae, Suidae, Bovidae, and Geravidae. Most of the well-adapted carrying hosts are within the subfamilies Hippotraginae, Alcellaphinae, and Caprinae, containing roan antelope, wildebeest, sheep, and goats (Li et al., 2005, Russell et al., 2009). Based on their susceptibility to MCF, animals can be classified as relatively resistant (such as European cattle to OvHV-2 MCF) intermediate susceptibility (e.g. water buffalo and most deer) to the highly susceptible ones like Pere David's deer, bison and Bali cattle (CFSP, 2012). Transmission of MCF is brought about by wildebeest calves and lambs between two to nine months through aerosol and close contacts (Li et al., 2011a, Mushi et al., 1981). MCF is associated with a high mortality rate (often 100% in outbreaks where clinical signs are observed) and low morbidity rate (sporadic) (Schultheiss et al., 1998). Rabbits and hamsters are good animal models for the

disease as they show similar clinical signs and pathological changes to those exhibited in the susceptible animals (Anderson et al., 2007, Buxton and Reid, 1980, Buxton et al., 1984).

Gross diagnosis of MCF can be achieved through noting the characteristic clinical signs and pathological changes. Nevertheless, an accurate (specific) diagnosis is necessary as well. Molecular tools such as the polymerase chain reaction (PCR) and serological tests like the enzyme-linked immunosorbent assay (ELISA) have become bench standards for detecting MCF viral DNA and antigen/antibody respectively (Fraser et al., 2006, Li et al., 2001). To date, there is no effective treatment for MCF (Li et al., 2014, Russell et al., 2009). However, there have been vaccine trials by several researchers most without success (Mirangi, 1991, Rossiter et al., 1977) until recently (Haig et al., 2008). Despite the fact that the disease is generally sporadic (affecting small numbers of animals) the incidence of MCF fluctuates year on year and continues to be a problem (Anderson et al., 2007). In some outbreaks MCF can affect up to 40 % of a herd (Russell et al., 2009). The details of the virus life cycle and the pathogenesis (the mechanism by which the disease happens) of MCF is still not well understood (Russell et al., 2009).

1.2 Aetiology of MCF

1.2.1 Herpes viruses

Herpes viruses comprise a large family of DNA viruses that induce disease in both animals and humans (Mettenleiter et al., 2009). The herpesvirion, an infectious virus particle, comprises an icosahedral capsid (Figure 1) consisting of 162 capsomeres (Mettenleiter et al., 2009) which contain a large double stranded linear DNA genome (125 to 240 kilo base pair Kbp), and encircled with an envelope holding glycoprotein spikes on its surface. The structure between the capsid and the envelope is called the tegument which is made up of proteins that are related to the capsid proteins (Pellett and Roziman, 2007). The aim of the productive/lytic infection is to produce progeny virions that are able to spread and infect new cells. In this type of infection, there are regulated viral genes expressions, and genome replication, virion assembly, egress and transmission. Early in this cycle, successive sets of genes are transcribed including α immediate early (IE) that are involved in initiation of virus DNA replication, β early (E) genes that act to DNA replication and control cellular activities such as RNA polymerase II (Estes *et al.*, 2007). After that, the transcription program switches to γ late genes expressing γ proteins. The newly replicated viral DNA is integrated into immature capsids and tegument proteins in the nucleus (Mettenleiter & Minson, 2006). The virus also gets its primary envelope in the nucleus, nuclear membranes play role in virus

capsid nuclear egress (Peng *et al.*, 2010). The virus gains its secondary membrane when buds through Golgi apparatus derived vesicles (Mettenleiter *et al.*, 2006). Eventually, mature virion fuse with the cell membrane and buds to the extracellular spaces (Mettenleiter, 2006).
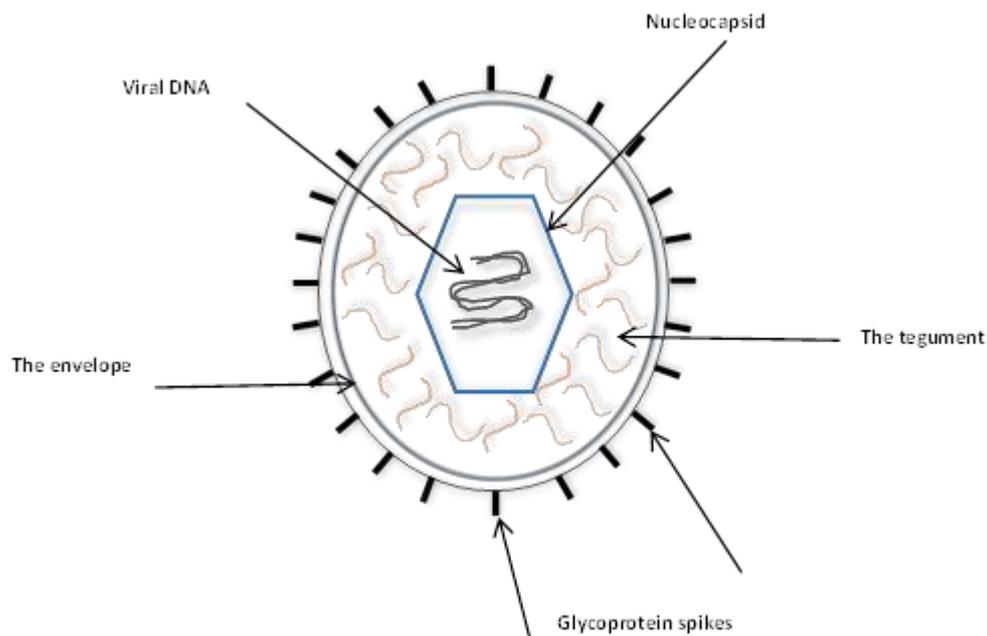
Figure 1   schematic figure illustrates the simple structure of the herpes virus virion (stdgen.northwestern.edu/stdgen/bacteria/hhv2/herpes.html).

The viruses of the family Herpesviridae have common biological features, i.e. they contain groups of enzymes that are implicated in metabolizing nucleic acid, DNA synthesis, and processing of proteins as well as the ability to establish latent infection in their natural hosts (Pellett and Roziman, 2007). The family Herpesviridae is classified into three subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gamaherpesvirinae*) according to their

biological properties and the viruses within these subfamilies further categorised to genera on the basis of resemblances in the DNA sequence (Pellett and Roziman, 2007).

Herpesviridae are well distributed in nature in a way that most animals have at least one herpesvirus and some viruses have more than one definitive host. To date more than 200 herpesviruses have been recognized, nine of which are human viruses (Pellett & Roizman, 2013). Viruses of veterinary importance are found in mammals, birds and fish. The range of diseases caused by herpesvirus infections is quite large. Moreover, the same virus can provoke different diseases, depending on the viral strain, the age of the animal or the route of infection. Bovine herpesvirus 1 (BHV-1) and Suid herpesvirus 1 (SHV-1) are good examples: BHV-1 infection of cattle causes rhinotracheitis, vulvovaginitis, conjunctivitis, abortion in pregnant cows, encephalitis in young calves, metritis; BHV-1 is also associated with enteritis and mastitis (Pastoret et al., 1982). SHV-1 infection of pigs is followed by abortion in pregnant sows, by septicaemia in neonates, by encephalomyelitis in piglets or by respiratory diseases in young pigs and adults. In domestic animals, clinical disease is usually produced following a primary infection; recrudescent disease, i.e. caused by a reactivated virus, seems to be rare. The situation differs in humans: labial and genital recrudescent lesions are commonly observed in humans infected with herpes simplex virus type 1 and 2 (HSV 1 and 2), even in the

presence of a high level of specific immunity (Nahmias et al., 1981).

Shingles is a painful expression of reactivation of varicella-zoster

virus, another human herpesvirus.

1.3 Malignant catarrhal fever viruses (MCFV)

Malignant catarrhal fever viruses (MCFV) belong to the genus Macavirus in the subfamily *Gammaherpesvirinae* within the family *Herpesviridae* (Davison et al., 2009, Gasper et al., 2012). The classification of these viruses within the genus Macavirus came after being categorised previously under the genus Rhadinovirus, and is based on the existence of the 15A antigenic epitope and also due to the resemblance in in the conserved regions of the DNA polymerase gene among MCFV (Li et al., 2003, Russell et al., 2009). At least two of these viruses within the MCFV can give rise to the disease in naturally susceptible species and have been studied in detail- AlHV-1 and OvHV-2 (Davison et al., 2009). The MCFV group comprises ten viruses (Table 1). Six of these viruses have been recognised as pathogenic (capable of inducing disease in the susceptible host) (Li et al., 2003). The pathogenic ones consist of alcelaphine herpes virus 1 (AlHV-1), ovine herpes virus 2 (OvHV-2), alcelaphine herpes virus 2 (AlHV-2), ibex-MCFV, caprine herpes virus 2 (CpHV-2), and MCF- white tailed deer (WTD) (Gasper et al., 2012, Li et al., 2003). Wildebeest associated MCF (WA-MCF) caused by AlHV-1 poses an important problem in cattle in Eastern and Southern Africa and zoos where natural and susceptible species are kept together in the same vicinity. Sheep associated MCF (SA-MCF) is considered as a major problem in many parts of the world, in particular, to the highly vulnerable animals, like Bali cattle, Pere David's deer, and bison as

well as in zoological collections where sheep and susceptible animals

co-mingle (Heuschele, 1988, Plowright et al., 1960, Schultheiss et

al., 1998).

Table 1 shows malignant catarrhal fever viruses and their resevoir and susceptible species (Li et al., 2014).

The virus	Disease documented	natural host	Susceptible host
AlHV-1	√	Wildebeest	cattle
AlHV-2	√	Hartebeest	Deer and bison
OvHV-2	√	Sheep	Cattle, deer, bison, pigs, and giraffe
CpHV-2	√	Goat	Sika deer and white tailed deer
Ibex-MCF	√	Ibex	Bongo and anoa
MCF-WTD	√	Unknown	White tailed deer
HiHV-1	X	Roan antelope	Unknown
Oryx-MCF	X	Oryx	Unknown
Muskox-MCF	X	Muskox	Unknown
Aoudad-MCF	X	Aoudad	Unknown

Abbreviations: AlHV-1, alcelaphine herpes virus 1. AlHV-2; alcelaphine herpes virus 2. OvHV-2; ovine herpes virus 2, CpHV-2; caprine herpes virus 2, Ibex-MCF; ibex9-malignant catarrhal fever, MCF-WTD; MCF-white tailed deer, HiHV-1; hippotrogine herpes virus 1, Oryx-MCF; Oryx-malignant catarrhal fever, Muskox-MCF; Muskox-malignant catarrhal fever, Aoudad-MCF; Aoudad-malignant catarrhal fever. √: Yes, X: NO.

The reservoir host for MCFV-WTD is still unidentified. However, the

virus can infect and give rise to MCF in white tailed deer in North

America and the virus was found to be genetically closely related to

AlHV-1 and OvHV-2 (Li et al., 2000). Domestic and wild goats are

the reservoir hosts for CpHV-2 and the virus is endemic in them. In addition, it has been reported that the virus can cause MCF in a wide range of species including pronghorn antelope, white tailed deer, and sika deer. The most common symptoms associated with the CpHV-2 MCF are emaciation, alopecia, and dermatitis (Keel et al., 2003, Li et al., 2011b). AlHV-2, initially, was classified as apathogenic under natural circumstances. Nevertheless, the virus was isolated from Jackson's hartebeest which induced MCF in Barbary red deer leading to the observation that the virus can be pathogenic (Klieforth et al., 2002, Li et al., 2003, Li et al., 2005). Ibex MCF virus found in ibex is capable of inducing MCF in bongo, an American forest antelope and in anoa (midget buffalo) (Li et al., 2011b). The non-pathogenic MCFV are Hippotragine herpes virus 1 (HiHV-1) and Oryx-MCFV in roan antelope and Oryx respectively with no recognisable susceptible hosts as well as Muskox and Aoudad MCFV in Muskox and Aoudad also with no susceptible hosts recognised to date (Li et al., 2005).

1.4 Genomic structure of AlHV-1 and gene function

AlHV-1 can easily be grown in cell culture. In contrast, it has been difficult to propagate OvHV-2 *in vitro*. This has hindered the study of OvHV-2 virology, infection, and pathogenesis. However, the use of T-lympho-blastoid cell lines with the morphology of large granular lymphocytes (LGL) derived from organs from MCF-affected animals has enabled researchers to study the host-virus communication (Hart et al., 2007a). These large granular lymphocytes are large lymphoblasts that can grow in the absence of exogenous cytokines and are indiscriminately cytotoxic, killing various target cells in a non-MHC-dependent manner (swa et al., 2001). The two viruses have had their genomes sequenced (Figure 2) (Ensser et al., 1997a, Hart et al., 2007a). The OvHV-2 genome is very similar to that of AlHV-1 and herpesvirus saimiri (HVS), a *gamaherpesvirus* categorised under the genus Rhadinovirus. Both viruses have a unique sequence of around 131 kbp and these are flanked by terminal repeats of 4.2 kbp and 1.1 kbp for OvHV-2 and AlHV-1 respectively. OvHV-2 has 73 open reading frames (ORFs), 62 of which have homologues in other *gammaherpesviruses* whereas AlHV-1 contains 70 ORFs, 61 of which are conserved among other *gammaherpesviruses*. Eight out of the 10 unique genes, designated A1 to A10 in AlHV-1, have homologues in OvHV-2 while OvHV-2 includes 3 completely unique ORFs, i.e. Ov2.5, Ov3.5, and Ov8.5 (Ensser et al., 1997a, Hart et al., 2007a). All ORFs of the two

viruses were named after those within the other *gamaherpesviruses*. As such, ORFs with similarity in herpesvirus saimiri were given the number of the related herpesvirus saimiri gene. Those which are unique to each virus were designated prefixes with A and Ov for AlHV-1 and OvHV-1 respectively followed by numbers one to ten. The unique genes of OvHV-2 assigned a nomenclature showing the relative position of the adjacent genes, for example, Ov2.5 locates between OV2 and Ov3 and so on and so forth for the other genes (Hart et al., 2007a, Russell et al., 2009).
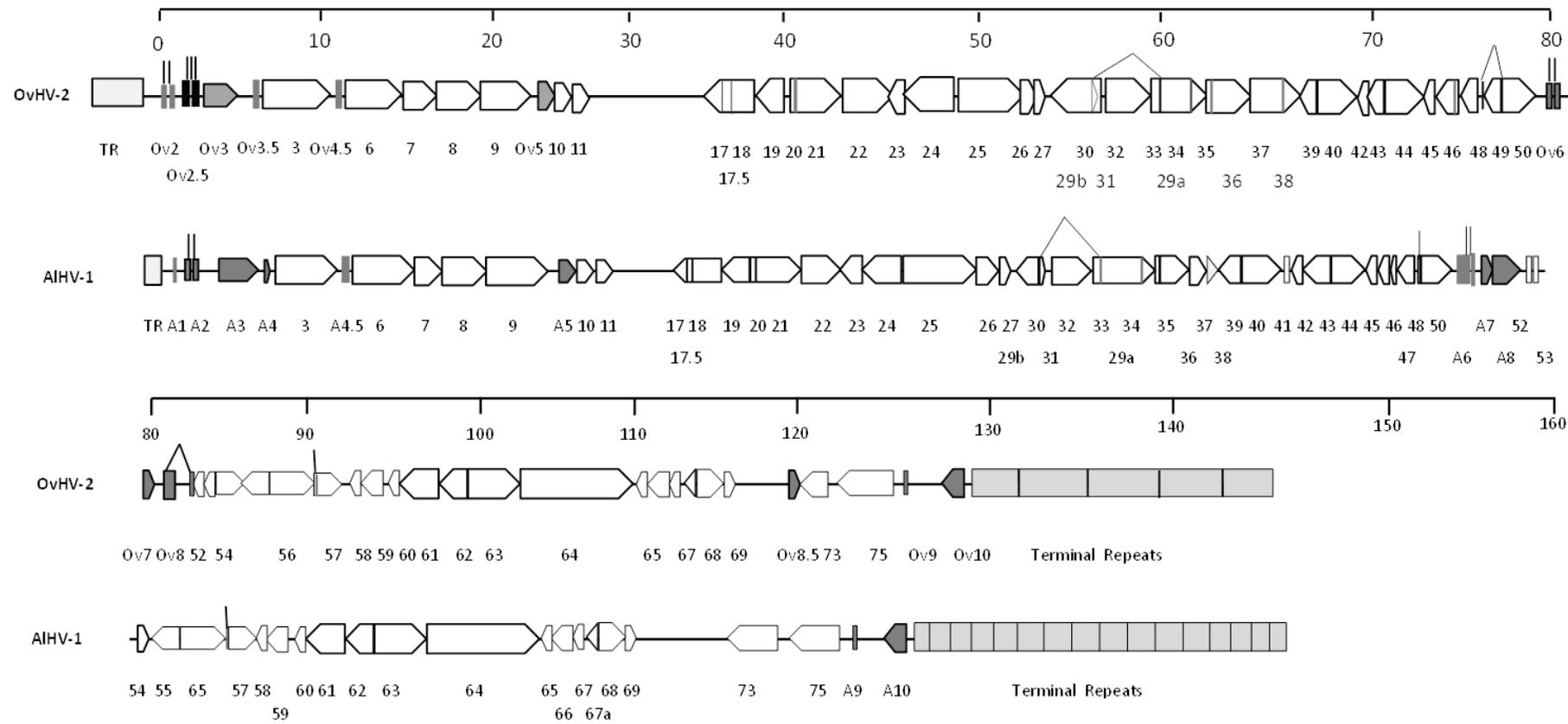
Figure 2 demonstrates the genome structure of AlHV-1 and OvHV-2.
Genes are shown as block arrows representing their orientation and position of open reading frames. Gene designations are written below according to the numbering system adopted in Herpes virus saimiri. The unique genes in both viruses are shaded grey and named beneath each gene with a prefix A for AlHV-1 virus and Ov for OvHV-2 whereas conserved genes are shown as white arrows. The terminal repeats (TR) are given grey shaded blocks. (Russell et al., 2009).

1.5 AlHV-1 genome sequence classification

Following the complete AlHV-1 DNA genome characterized by Ensser and co-researchers in 1997, Coulter et al. (2001) arranged the Herpesvirus genome sequences in five blocks, designated the roman numbers, based on the gene similarity among the viruses within this family. Blocks I, II, IV include ORFs that are conserved within all herpesviruses subfamilies while blocks III and V comprise ORFs that are conserved between the subfamily *Gamaherpesvirus* only. Block I contains ORFs 6 to 9. Block II contains ORFs 17 to 47. Block III contains ORFs 48 to 50. Block IV contains ORFs 52 to 69. Block V contains ORF75.

There are changeable areas which can be assigned A-F disseminated between these conserved blocks of genes throughout the family of Herpesviruses. In AlHV-1 area A comprises A1 to A4 and ORF3. Area B comprises A5 and ORFs 10 and 11. Area D/E comprises A6, A7, A8, ORFs 73, 75, A9, and A10 (Coulter et al., 2001).

1.6 AlHV-1 genome sequence

As in Herpesvirus saimiri, the low guanine-cytosine (GC) content (L-DNA) (46.17%) in AlHV-1, which contains the coding ORFs is surrounded by high GC content area of nucleotide repeats (H-DNA) (71.83%) which is considered as predominately a non-coding region (Ensser et al., 1997a). The L-DNA region of around 131,000 Kbp nucleotide sequence comprises (Figure 2) 70 ORFs as previously found by Ensser et al. (1997a). However, Mills et al. (2003) and Russell et al. (2013) discovered two new ORFs in AlHV-1 virus located in the inter-genic spaces between ORF 6 and ORF3 (designated ORF A4.5) and between ORF A9 and ORF A10 which is assigned ORF A9.5. This brings the number of open reading frames in the L-DNA region to 72 ORFs instead of 70 as previously stated. Consequently, the number of the unique genes (shared by AlHV-1 and OvHV-2 plus unique to AlHV-1) in AlHV-1 virus is twelve to date. Ten of these have homologues in OvHV-2 and two are unique to AlHV-1. Ovine herpes virus genes Ov2 and Ov3 which have homologues in AlHV-1 lie in the left end of the unique region whereas Ov2.5 and Ov3.5 locate in the same area but with no homology with AlHV-1 genes (Hart et al., 2007a, Russell et al., 2009).

The ORFs A1 to A4 are located at the left end of L-DNA genome and at the right end of the H-DNA region whereas ORF A4.5 and Ov4.5 lie between ORF3 and ORF6 (Coulter et al., 2001, Hart et al., 2007a,

Russell et al., 2009). ORF A5 and Ov5 locate between ORFs 9 and 10 (Coulter et al., 2001, Hart et al., 2007a). ORFs A6, A7, A8 and Ov6, Ov7, and Ov8 locate amongst ORF50 and ORF52. Ovine herpes virus Ov8.5 with no homology in AlHV-1 locates between ORF73 and ORF69 (Hart et al., 2007a, Russell et al., 2009). ORF A9 and Ov9 locate after ORF75 towards the right H-DNA genome while A10 and Ov10 locate adjacent to and to the left of the right H-DNA repeat (Coulter et al., 2001, Hart et al., 2007a, Russell et al., 2009). ORF A9.5 and its homologues Ov9.5 lie between A9 and A10, and Ov9 and Ov10 respectively (Russell et al., 2014, Russell et al., 2013). Figure 2 shows the location of the unique genes on both AlHV-1 and OVHV-2 genomes.

1.7 Spliced genes in AlHV-1 virus

Splicing is a process that happens in the nucleus after transcription to RNA in which the introns are removed and the exons are joined together to make mRNA which can then be translated to a protein within the ribosomes (House, 2008; Poulos, 2011). This process is important as it removes the introns (non-coding sequence) and joins exons (coding-sequence) to produce mature mRNA.

In the two MCF viruses that have been sequenced (AlHV-1 and OvHV-2) splicing takes place in both conserved and unique ORFs (Ensser et al., 1997a, Hart et al., 2007a). ORF A2 encodes a spliced product and ORF50 the R-transactivator gene is expected to show

splicing due to the fact that herpesviruses equivalent genes exhibit splicing (Ensser et al., 1997a).

1.8 Functions of non-conserved genes

ORF A1 has no homology to other herpes virus ORFs and its putative protein product is not similar to any known proteins with no known function. It is therefore assumed to be non-functional or a pseudogene (Russell et al., 2009). Table 2 lists the known and predicted functions of the twelve unique genes of AlHV-1. ORF A2 and ORF Ov2 are similar to ATF3 which is considered as stress-induced transcription factor. A2 gene and its product is a member of the basic leucine zipper proteins implicated in altering host genes expressions. Therefore, they may play a role as transcription factor (Hart et al., 2007a, Parameswaran et al., 2014). Nevertheless, AlHV-1 ORFA2 is dispensable for the induction of MCF (Parameswaran et al., 2014). Semaphorins are huge family of glycoproteins that are found in all vertebrates and invertebrate's species in addition to viruses (Jongbloets et al., 2013). It has been found that these proteins are involved in many biological activities like cancer, bone haemostasis, and immune system (Xu et al., 1998). Semaphorin 7A (SEMA7A) is encoded by AlHV-1 ORF A3 and OvHV-2 ORF Ov3 homologue (Myster et al., 2015). However, AlHV-1 ORF A3 has not been implicated in lymphocytes infiltration in MCF but is essential for avoiding immune evasion (Myster et al., 2015).

Ovine herpes virus Ov3.5 is a unique gene with no similarity to any other viral genes encoding a putative signal peptide. Therefore, it thought to be secreted by the infected cells (Hart et al., 2007a, Thonur et al., 2006). ORF A4 has no obvious role with no homology to other herpes virus ORFs (Russell et al., 2009). ORF A4.5 is similar to OvHV-2 Ov4.5 which encodes a protein similar to Epstein-Barr virus EBV BALF1 and cellular B-cell lymphoma genes of the Bcl-2 family that regulate programmed cell death (apoptosis). It seems therefore that this gene may be involved in inhibiting/supporting cell death, hence encouraging cell survival and establishment of productive and / or latent infection cycles (Hart et al., 2007a, Russell et al., 2009).

Table 2 Function of unique genes in AlHV-1 and OvHV-2 (Russell et al., 2009).

AlHV-1 ORF	OvHV-2 ORF	Function
A1		Unknown
A2	Ov2	Leucine zipper protein; transcription regulation
	Ov2.5	Viral IL-10
A3	Ov3	Semaphorin homologue; intracellular signalling
	Ov3.5	Unknown, signal peptide
A4		Unknown
A4.5	Ov4.5	Bcl-2 homologue; cell death regulators
A5	Ov5	GPCR (G-protein coupled receptor); intracellular signalling
A6	Ov6	Similar to Epstein-Barr virus BZLF1; viral transactivator
A7	Ov7	Virus glycoprotein
A8	Ov8	Virus glycoprotein
	Ov8.5	Unknown
A9	Ov9	Bcl-2 homologue; cell death regulators
A9.5	Ov9.5	Secreted glycoprotein with unknown function
A10	Ov10	Unknown, glycoprotein

ORF A5 is similar to OvHV-2 Ov5 encodes a G protein-coupled-receptors orthologue (GPCRs), hence it may play a role in modulating the host immune defence or any of the other functions of this diverse family of molecules (Ensser et al., 1997a, Hart et al., 2007a). However, the role of this ORF was investigated using the bacterial artificial chromosome AlHV-1BAC to produce a recombinant virus with a deleted A5 gene. Animals infected with this clone developed MCF-like syndrome indicating that A5 has no function in the development of MCF (Boudry et al., 2007). ORF A6 is similar to OV6 encodes a protein similar to EBV BZLF1 which encodes a protein considered as a transcriptional factor that is responsible for

the activation of early genes leading to latent / lytic cycle switch (Coulter et al., 2001, Hart et al., 2007a). ORF A7 and A8 and their homology Ov7 and Ov8 in OvHV-2 virus are viral glycoproteins and may have a role in cell attachment and entry to the host cell (Coulter et al., 2001, Hart et al., 2007a, Russell et al., 2009). ORF A9 and Ov9 has similar predicted function to ORF A4.5 as it has homology to bcl-2 anti-apoptosis family genes and therefore may inhibit/induce the host cell death (Coulter et al., 2001, Russell et al., 2009) to enhance productive and /lytic infections. ORF A10 is putative (unknown gene function) glycoprotein and may be involved in cell attachment and entry to the host cell (Coulter et al., 2001). ORF A10, similar to Ov10, is putative (unknown gene function) glycoprotein and may be involved in cell attachment and entry to the host cell (Coulter et al., 2001, Hart et al., 2007a, Russell et al., 2009). Ovine herpes virus Ov8.5 with no similarity to any genes in AlHV-1 genome has no identified function (Hart et al., 2007a, Thonur et al., 2006). ORF A9.5 is similar to Ov9.5 encoding secreted glycoprotein that is similar to IL-4 (Lankester et al., 2015b, Russell et al., 2014, Russell et al., 2013). However, the role of this gene in inducing MCF is still unknown.

1.9 Gene similarities to other herpes viruses

AlHV-1 ORF73, encodes latency associated nuclear antigen (LANA), is homologous to latency associated transcripts of other herpes

viruses including HHV-8- also known as Kaposi's sarcoma-associated herpesvirus (KSHV LANA) ORF73 (Palmeira et al., 2013, Taus et al., 2007), herpes saimiri ORF73 (Blake, 2010), And EBV Epstein Barr nuclear antigen (EBNA1) (Blake, 2010). These are important for the viral latency cycle (Hu et al., 2002). It looks likely therefore that AlHV-1 ORF73 has the same function (Dewals et al., 2008, Palmeira et al., 2013). The R transactivator (RTA) ORF50 has a role in switching from latency to lytic replication responsible for the productive life cycle (Frame and Dalziel, 2008, Goodwin et al., 2001, Staudt and Dittmer., 2007). ORF 25 homologue is conserved in all herpesviruses and encodes a major capsid protein (Ensser et al., 1997a). This ORF product is expressed during the lytic cycle demonstrating viral replication (Cunha et al., 2008, Dewals et al., 2008). ORF63 is conserved among other herpes viruses and encodes a tegument structural protein (Boyle and Monie, 2012, Ensser et al., 1997a). The presence of these proteins is associated with the expression of ORF50/ORF25 responsible for productive/lytic cycle (Gailbreath et al., 2008, Meier-Trummer et al., 2009b).

1.10 Gene expression of the MCF viral genome, latency and lytic cycles

Based on the genome sequence of OvHV-2, Thonur et al. (2006) studied the transcription of OvHV-2 genes and its conformation in lymphocytes from both natural (wildebeest and sheep) and

susceptible hosts (cattle, bison, pigs, deer, water buffalo). OvHV-2 viral genome from peripheral blood mononuclear cells derived from sheep (natural host reservoir species) was primarily circular as measured using Gardella gel analysis, indicating a latent conformation and mRNA for only Ov3.5 unique gene, encoding a putative signal peptide which thought to be secreted by the infected cells, was identified. In contrast, cultured large granular lymphocytes (LGL), have natural killer (NK)/T cells morphology, contain enriched-population of infected cells and are good source to study virus-host interaction, from the rabbit (a MCF-susceptible species following experimental infection) had mainly a linear viral genome indicating productive cycle whereas bovine (natural disease-susceptible host) LGL cultures contained mainly circular viral DNA indicating latency dominance. Both these susceptible species genomes expressed nearly all of the virus unique genes. Their expression in this experiment indicates that they may be involved some way in MCF pathogenesis. Treating the LGLs from the susceptible species with doxorubicin, a drug used for cancer treatment that drives the productive life cycle (AbuHammad and Zihlif, 2013, Feng et al., 2004, Rahman et al., 2001), stimulated predominantly linear genome from latent circular ones, while treatment with 5-azacytidine, a drug utilised to stimulate latency in EBV-infected tissues (Feng et al., 2004, Mossman et al., 1989, Schaefer et al., 1997), supported circular genome production in LGL

lines and a range of putative latency transcripts (Thonur et al., 2006). In spite of productive cycle gene expression, no virions were detected by electron microscopy (EM) in the cultures, indicating an incomplete virus life cycle (Thonur et al., 2006). These results are in agreement with the previous work of Rosbottom and co-researchers who made the same conclusion (Rosbottom et al., 2002).

In particular, OvHV-2 and AlHV-1 gene expression in the tissues of infected cattle or rabbits indicated that the latency transcript ORF 73 could be detected, leading to a belief that MCF is a disease of latency (Meier-Trummer et al., 2009a, Palmeira et al., 2013). In contrast, other researchers have shown evidence of viral replication and productive virus associated with this (Cunha et al., 2012, Cunha et al., 2013, Li et al., 2008a, Nelson et al., 2013). The structural viral proteins (as mRNA transcripts) were identified in organs within SA-MCF virus-infected rabbits indicating viral replication was occurring (Gailbreath et al., 2008). In addition, it was found that there was low expression of ORF50 in lymphoid tissues extracted from WA-MCF-affected rabbits, which might indicate productive infection (Dewals et al., 2008).

1.11 Virulent and attenuated forms of AlHV-1

AlHV-1 C500 strain derived from a cow (Plowright et al., 1960, Plowright et al., 1975) that developed MCF can be cultured in bovine turbinate (BT) cells (Handley et al., 1995). After several passages

(up to 5 times) the virus exists as free virus that is virulent and can cause disease when injected into MCF-susceptible animals (Dewals et al., 2011, Haig et al., 2008, Palmeira et al., 2013, Parameswaran et al., 2014) but as culture passages proceed, the proportion of virus that is attenuated (i.e. cannot induce disease when injected into MCF-susceptible animals) increases. Virus can exist as free in culture or as a proportion that is cell-associated and not excreted into the cultures (Wright et al., 2003). Handley et al. (1995) conducted the first study of these changes. They found out that an area in the middle of AlHV-1 DNA was deleted that results in virus attenuation. This region contains the distal part of ORF 50b, proximal part of A6 and distal part of A10.

The AlHV-1 C500 strain was completely sequenced by Ensser et al,. (1997a), allowing more detailed study. Wright et al. (2003) discovered that after multiple passages *in vitro* there was evidence of gene deletion, translocation, and duplication that could be involved in attenuation process. Candidate ORFs included ORF50 and ORF A6 that were truncated and translocated (distal part of ORF 50b, proximal part of A6 and distal part of A10). ORF48 and ORF A7 might have been affected which locate to either side of the translocated fragments (Figure 3) (Wright et al., 2003).
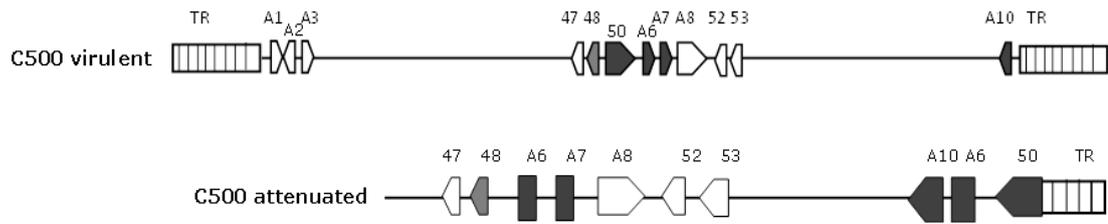
Figure 3 schematic map shows the changes in genes due to passage resulting in attenuation (Wright et al., 2003).

A further proteomic study identified differences in protein expression between the virulent and attenuated forms of AlHV-1 (Dry et al., 2008). This revealed that 23 virus proteins were expressed in both virus forms. This means that there was no change in the structural composition of the virulent and attenuated viruses detectable by this method. It was concluded therefore that the attenuation of the virus was not due to the virus particle structure, but perhaps a result of altered viral gene expression in the infected cell (Dry et al., 2008).

1.12 Virus life cycle

The life cycle (Figure 4) of viruses of the herpesvirus family is initiated when the virus engages with the host cell surface, binding to specific receptors, for example, it was shown that gD of herpes simplex virus-1 (HSV-1) bound to the herpes virus entry mediator A (HveA receptor) whereas gp42 of Epstein-Barr virus (EBV) bound to HLA-DR (Flint et al., 2009). The receptors used by MCF-related viruses to enter the host cell are still not known (Russell et al., 2009). After binding of viral envelope glycoproteins to cell

membrane receptors, entry is brought about by membrane fusion proteins. By contrast, a study carried out to identify the different stages of *Gammaherpesvirus* life cycle using murine gammaherpesvirus 68 (MHV 68) and utilizing dual-axis electron tomography (ET) revealed that the entry occurs by endocytosis (Peng et al., 2010). Once the virion is internalised, un-coating or disassembling occurs and the DNA genome is released and then transported *via* transit Golgi network (TGN) to the nucleus where the linear double stranded DNA genome is circularised by covalent bonds before replication. After that, viral DNA is transcribed into early mRNAs which are then transported to the cytoplasm for early protein synthesis. These early proteins are imported into the nucleus and promote viral DNA replication. The viral DNA is then transcribed into the late mRNAs which are responsible for late viral protein synthesis (capsid and envelope proteins). The viral envelope proteins are processed in the endoplasmic reticulum (ER) and Golgi complex. The capsid proteins are imported into the nucleus and then encapsidate the newly replicated genomes. The assembly of the progeny virions partially occurs in the nucleus and in the cytoplasm by the Golgi membranes. The release of the enveloped virus from the host cell occurs by budding (Flint et al., 2009).
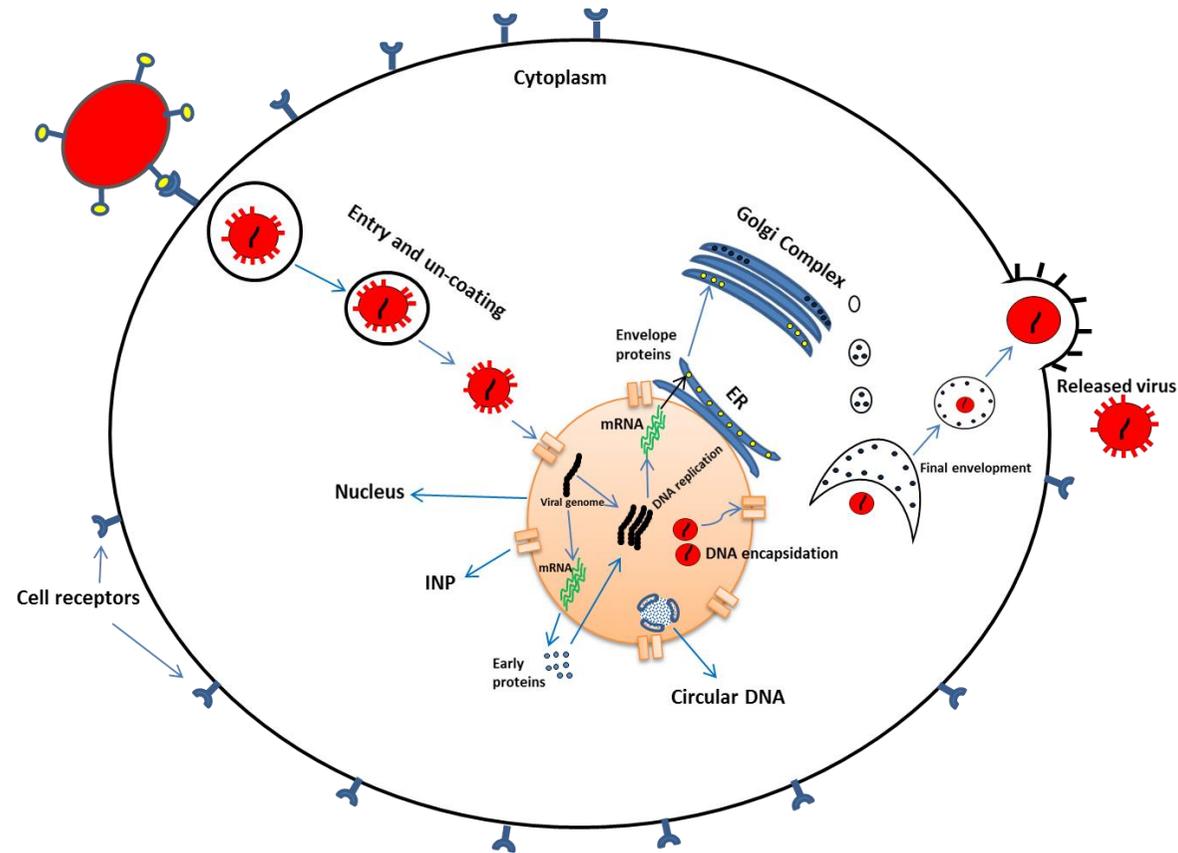
Figure 4 illustrates the herpesvirus life cycle steps from entry to egress. The entry happens when virus glycoprotein attaches to specific cell receptors. Un-coating of the virus takes place in the cytoplasm and linear DNA transferred to the nucleus where the replication and transcription occur. The virus is transported to the cytoplasm through inter-nuclear pore (INP) from the nucleus where complete assembly of the progeny virus takes place. The release of the Herpes virion occurs by budding.

There have been studies carried out on MCF viruses' replication, in particular, OvHV-2. These studies were able to identify the stages through which OvHV-2 replicate in both reservoir and susceptible host (Li et al., 2008a, Rosbottom et al., 2002, Taus et al., 2010). In sheep, the reservoir host for SA-MCF, the virus initially replicates in particular in the alveolar epithelium of the lung. This takes place in the early stage of the disease course. Afterwards, the virus disseminates into the blood (tropism) favouring the lymphocytes where it can establish latent infection (Li et al., 2008a, Taus et al., 2010). Ultimately, the virus targets the respiratory system, especially the lung and nasal sinuses where it departs the host in a cell-free form (Cunha et al., 2012). In susceptible species such as rabbits and bison that have been experimentally infected, the pattern of infection, initially, is similar to the reservoir host where the virus replicates in the lung tissue and then disseminates into the blood. However, the pattern here varies from the one in the reservoir host. The expression of ORF50, ORF25, and ORF73 is marked in most of the tissues (Cunha et al., 2012). The expression of ORF63 and ORF43 genes encoding structural proteins in the tissues of the susceptible species supports the incidence of viral replication (Meier-Trummer et al., 2009b). With regard to AlHV-1 virus, the details of virus life cycle are still unknown. Nonetheless, recent work has shown that the virus replicates in the lung of the susceptible species in very early clinical stage and then disseminate

into the blood where it establishes latency in T lymphocytes (mainly CD8^{+} T lymphocyte) (Myster et al., 2015, Palmeira et al., 2013).

1.13 Latency

MCF-associated viruses (AIHV-1 and OvHV-2) are assumed similar to the viruses of the family *Herpesviridae* in terms of their ability to become latent. The herpes viruses are able to establish latency in natural host tissues and sometimes in tissue culture (Flint et al., 2009). The hallmark of this stage is that the viral genome is preserved as a circular episome inside the nucleus, few viral genes are expressed (Virgin et al., 1999), and no virions produced. Moreover, the cytopathic effects (cell lysis due to the lytic virus cycle) are inhibited and the virus can avoid the host immune control (Blake, 2010). Reactivation occurs when the host immune system is stressed or compromised (Russell et al., 2009). Different herpesviruses have the ability to establish latency in certain cells. For example, Herpes simplex virus (HSV) remains latent in peripheral sensory ganglia (Flint et al., 2009) whereas the Epstein-Barr virus undergoes latency in B lymphocytes and epithelial cells (Mesri et al., 1996). In regard to the MCF-associated viruses AlHV-1 or OvHV-2, the site in which the viruses establish latency it is still unknown (Russell et al., 2009).

The mechanism by which Herpes viruses establish latency is complex and not completely known (Flint et al., 2009, Nicoll et al.,

2012). In herpes simplex virus 1 (HSV 1), supporting of latency goes through two important steps. In the first one the virus suppresses the expression of lytic cycle genes and secondly prevent programed cell death (apoptosis) (Nicoll et al., 2012). The expression of latency associated transcripts (LAT) is important in establishing latency in HSV1 and HSV 2 (Nicoll et al., 2012). LAT is a set of RNAs encoded from the virus genome resulting in the construction of minor LAT transcripts. These transcripts inhibit the immediate early genes (IE), such as ICP0 (transcriptional activator) and ICP4 (transcriptional regulator), required to initiate lytic/productive cycle in the host cell (Nicoll et al., 2012). In addition, LATs are involved in repression of proteins production such as glycoprotein gD and gJ, ICP27 (transcriptional factor), ICP10 (apoptotic regulator), which is responsible for the promotion of cell survival (Nicoll et al., 2012).

The role of micro-RNA (miRNA) in regulating viral latency has been highlighted. Micro-RNA is non-coding sequence belongs to the RNA family and found in plants, animals, algae and viruses (Griffiths-Jones, 2008). EBV and KSHV which belong to *gammaherpes* virus subfamily, have been found to encode miRNAs (Pfeffer, 2005) that are associated with immune response (Grundhoff, 2011) and also have anti-apoptotic role (Seto, 2010).

1.14 Symptoms and pathology of MCF

1.14.1 Clinical signs

Animals affected include cattle deer, bison, and pigs. The signs are similar for AlHV-1 and OvHV-2 MCF. The clinical signs of MCF are diverse as the disease can affect many systems of the infected animals including the digestive system, respiratory system, head and eye, nervous system, and uro-genital system (Liggitt and DeMartini, 1980a, Liggitt and DeMartini, 1980b, Russell et al., 2009). MCF has many forms ranging from peracute to mild stages. The symptoms are few in the peracute phase, which is characterised by sudden death. However, depression, weakness, dysentery may develop 12 to 24 hours before the onset of death. This is mostly seen in the highly susceptible species (CSFH, 2012; OIE, 2013). In the acute form, the clinical signs are more obvious and are associated with the emergence of high fever, inappetence, lachrymation, nasal discharge which progresses to mucopurulent, corneal opacity, decrease in milk production, and skin lesions in the form of ulceration and exudation that are mainly found in perineum, udder and teats. Salivation, erosions of the tongue and the buccal papillae are detected. Superficial lymph nodes are enlarged and limb joints may be swollen. Head pressing, hyperesthesia, tremors and nystagmus are the most obvious nervous signs of the MCF (Russell et al., 2009, Zemljic et al., 2012). The severity of the disease depends on the causative agent, species infected and the course of

31

disease (Russell et al., 2009). Although the clinical signs are similar in other species to those found in cattle, there is some variance in some aspects. For instance, in bison, the animals usually die in the acute stage without developing the head and eye form, diarrhoea, neurological signs as well as lymph nodes only slightly enlarged. Acute MCF is predominant; however, chronic disease and recovery of some cases have also been documented (Milne and Reid, 1990, O'Toole et al., 1997).

1.14.2 Pathology of MCF

The macroscopic pathological changes are commonly diffuse and mirror the intensity of the clinical signs, and may include most body systems (OIE, 2013). Erosions and petechial haemorrhages are seen in the gastrointestinal tract and echymotic haemorrhages (a haemorrhagic spots forming rounded and irregular patch) of the gall bladder epithelium. In the respiratory tract the development of a diphtheric membrane and erosions are often recognized. Lymph nodes are enlarged, in particular in cattle and can be solid, and white when cut. Kidneys' surfaces are, generally, characterised by the presence of white foci (OIE, 2013; CSFH, 2012; Russell et al, 2009).

Epithelial degeneration, vasculitis, hyperplasia and necrosis of lymphoid organs, and the accumulation of lymphoid cells in nonlymphoid organs are the main obvious microscopic appearances

in MCF (OIE, 2013). Vascular lesions exist and are obvious in the arterioles, venules, arteries and veins of tissues including the brain. They are identified by the infiltration of the lymphoid cells in the tunica adventia and media of the blood vessels. The accumulation of the lymphoid cells is pronounced, especially, in the renal cortex, periportal regions of the liver, and extremely large in the kidney (OIE, 2013).

 The infiltration of lymphoid cells in both SA-MCF and WA-MCF are mostly T cells, the majority of which are CD8$^+$ T cells along with very small numbers of CD4$^+$ T cell (Anderson et al., 2007, Dewals et al., 2008).

1.15 Epidemiology of MCF

1.15.1 MCF Transmission

Although MCF viruses (AlHV-1 and OvHV-2) show no clinical signs in the reservoir host, they can induce the disease in susceptible species. Both viruses can be propagated horizontally from reservoir to susceptible host by either direct contact or respiratory route, mostly from lambs and wildebeest calves under 12 months old (Li et al., 1998, Mushi and Rurangirwa, 1981, Mushi et al., 1980). The horizontal transmission between the susceptible hosts does not occur and they are considered as dead end hosts (Ackermann 2006). Nonetheless, some cases of transmission among infected deer were documented (Reid et al., 1986). Also, it has been shown that the inability of animals to transmit the disease horizontally is owing to the cell-associated trait of the viruses as well as the failure to shed cell-free viruses by the animals (Li et al., 2014). However, vertical transmission has been documented in susceptible host animals. In study carried out by Plowright et al, (1972) a cow was inoculated with AlHV-1. After 84 months the cow was killed and the virus was grown in a culture from its organs. It was found that four out of the six calves that the cow yielded throughout its life were infected with the virus after parturition. In addition, OvHV-2 genome was identified in specimens of digestive tract, respiratory and urogenital tract of a sheep as well as semen of rams (Hussy et al, 2001; CFSPH, 2012). These observations have defined a possible

role for vertical transmission for passing the infection to the progeny after birth. Although the presence of both reservoir and susceptible hosts within the same vicinity is fundamentally required to transmit MCF, some cases of infection have been recorded when both types of hosts were at some distance up to five Km (CFSPH, 2012). The role of external parasites (Plowright, 1965) and urine (Michel, 1993) in transmitting MCF is ruled out.

1.15.2 Geographical distribution

Malignant catarrhal fever is distributed all over the globe; however, the incidence of the disease is dependent on both the causative agent and the susceptible host (CFSPH, 2012). WA-MCF is found in the sub-Saharan region in Africa when cattle and wildebeest are reared together in the same proximity. In addition, many cases of WA-MCF have been reported from wild parks and zoological gatherings (Russell et al., 2009). Sheep associated malignant catarrhal fever is found mainly in Europe and North America (Russell et al., 2009). However, the disease has been reported from various areas of the world in the Middle East (Abu Elzein et al., 2003) in New Zealand (Russell et al., 2009). Moreover, the disease is considered as a serious problem in Bali cattle in Indonesia (Wiyono et al., 1994).

1.15.3 Susceptible species

Malignant catarrhal fever viruses can infect a wide range of animals worldwide and exhibit clinical signs in these species. These animals belong to subfamily Bovinae including, cattle, bison, and water buffalo as well as family Cervidae containing, deer and moose. It has also been found that MCF viruses can induce the disease in animals belong to other species such as pigs and giraffes (Russell et al, 2009; CSFH, 2012). Some species are more sensitive to the infection of certain type of MCF virus than other species. For example, European cattle are more easily infected with OvHV-2 and are somewhat more resistant to AlHV-1. In contrast, animals such as Pere David's deer, wild-tailed deer, and bison are extremely susceptible to OvHV-2 infection (CFSPH, 2012).

1.16 Disease Impact

The lack of an effective reporting approach along with misdiagnosis of the clinical MCF has almost certainly led to the under-diagnosis of MCF (O'Toole et al., 1997). The significance of MCF lies in the fact that the disease is considered as a major cause of death in farmed bison in North America (Li et al., 2006), farmed deer and Bali cattle as well as a cause of death wherever the reservoir and host species for the virus cohabit. In the USA, an outbreak of MCF in 2003 led to the death of about 800 bison resulting in a loss of millions of dollars to the owners (Li et al., 2006). This has led the United States department of agriculture (USDA) placing MCF research in its priority programme. MCF is also reported as a serious problem in farmed deer in variety of locations and Bali cattle in Indonesia (Wiyono et al., 1994). The disease is mainly sporadic in Europe and the UK. However, outbreaks range (60-300) occurs annually in the UK. In addition, MCF may contribute to Bovine respiratory disease (Parameswaran et al., 2014). This has led organisations such as Animal and Plant Health Agency (APHA), Department for Environment, Food and Rural Affairs (DEFRA), and Global Alliance for Livestock Veterinary Medicines (GALVMED) to support research on MCF control (personal comment, David Haig). In Africa, it has been estimated that about 7% of the cattle exposed to wildebeest calves during migration perish due to MCF (Cleaveland et al., 2001). Consequently, MCF has been placed as one of the most significant

cattle diseases in certain parts of eastern and southern Africa (Cleaveland et al., 2001). MCF is a major cause of death in zoological gatherings where susceptible and reservoir hosts cohabit, hence loss of some expensive animals (Russell et al., 2009). The significance of MCF does not only lie on the fact that the disease can cause death in many animals around the world but also due to serious welfare issues that this brings and also socio-economic problems for farmers and pastoralists (Li et al., 2014, Palmeira et al., 2013). Eventually, the research on MCF and studying the virus life cycle and how it reacts with the host may contribute scientifically towards opening opportunities for researchers studying herpes viruses to gain more knowledge about the virus behaviour.

1.17 Diagnosis of MCF

Diagnosis of MCF depends, initially, on the clinical signs and the pathology of the disease (Li et al., 2011b). The existence of the susceptible species close to the reservoir hosts in the same vicinity could be a valuable approach for diagnosing MCF (CFSP, 2012). Tissues such as liver, kidney, intestines, lung, spleen, and lymph nodes should be collected from animals developing MCF clinical signs for diagnostics (Anderson et al., 2007, Parameswaran et al., 2014). Histologically, lesions such as vasculitis and accumulation of lymphocytes in the lymphoid tissues are suggestive of MCF incidence (Anderson et al., 2007, Cunha et al., 2012, Palmeira et al., 2013). MCF should be differentiated from other viral diseases such as mucosal disease, bovine viral diarrhoea virus, and infectious bovine rhinotracheitis (OIE, 2013; CFSP, 2012). One of the effective methods for diagnosing MCF with regard to AlHV-1 virus infection is to infect bovine turbinate fibroblast (BT) cells with the isolated virus and monitor any cytopathic effect (CPE) development. This is characterized by the absence of monolayer and the accumulation of cell debris (Wright et al., 2003). However, the advent of molecular based techniques like the polymerase chain reaction (PCR) assays has considerably enhanced MCF laboratory diagnostics (Li et al., 2011a, Russell et al., 2009). Especially, real time quantitative PCR (RT q-PCR) and nested PCR which are considered powerful and reliable tools for detecting viral DNA in the samples of MCF infected

animals (Hussy et al., 2001, Traul et al., 2005). This is due the fact that MCF affected animals have detectable levels of Viral DNA in their blood (at certain stages of the disease) and organs at post-mortem (Dewals et al., 2008). There are serological approaches to detecting MCF virus antibodies including the virus neutralisation test (VN), enzyme-linked immunosorbent assay (ELISA), immunoperoxidase test (IPT), immunofluorescence assay (IFA) and immunoblotting introduced for the diagnosis of MCF (OIE, 2013). These techniques depend upon identifying antibodies for specific MCF virus antigens (CSFH, 2012). Neutralisation test (VN) could be effective for detecting antibodies from animals infected with AlHV-1 virus or viruses associated with AlHV-1 such as AlHV-2 and HiHV-1 whereas it is non-specific for detecting antibodies in animals infected with OvHV-2 or other related viruses (CSFH, 2012). The use of competitive enzyme-linked immunosorbent assay (cELISA), monoclonal antibody based technique utilizing 15A antibody targeting a conserved epitope among all MCF viruses or an indirect ELISA using virus extract are the methods of choice for identifying antibodies in MCF affected animals (Fraser et al., 2006, Li et al., 1994).

1.18 Treatment and prevention

There is currently no effective remedy or licensed vaccination for treating or preventing MCF (Russell et al., 2012b, Russell et al., 2009). The only current approach to control the disease is to separate the reservoir host from the susceptible species (CSFH, 2012; OIE, 2013). The efficiency of this method depends upon the reservoir host, susceptible species, separation distance as well as the age of the carrying host (CSFH, 2012; OIE, 2013). With regard to WA-MCF, separation of cattle from wildebeest is crucially important in preventing MCF transmission especially around the calving season whereas in SA-MCF this approach is of less significance in particular in the European cattle, the relatively resistant species (CSFH, 2012; OIE, 2013). However, isolation of highly susceptible animals to SA-MCF such as bison, Pere David's deer, and Bali cattle is highly recommended (CSFH, 2012; OIE, 2013). The MCF viruses can be transmitted over several km (Li et al., 2008b), with one report of disease in bison located 5km from the nearest sheep farm (CFSH, 2012). The age of wildebeest calves and lambs should be avoided ranges from two to nine months as these animals have higher cell-free virus titrations in their nasal secretions (CSFH, 2012; OIE, 2013). Avoiding stress factors such as starvation, high stock density, travelling could be effective in controlling MCF as such factors can induce virus reactivation (CSFH, 2012; OIE, 2013). Equine herpesvirus 1 (EHV-1) latently infected

horses can reactivate if the appropriate conditions such as stress, immunosuppression, transportation, sale barns and competitions appear (Dunowska., 2014).

1.19 Prospective vaccine development

The lack of effective treatment for MCF has encouraged scientists to develop a strategic vaccination approaches to protect susceptible species against the disease. Therefore, many studies (Plowright et al., 1975, Ferris et al., 1976, Edington and Plowright, 1980, Haig et al., 2008, Russell et al., 2012a) have been carried out in order to fulfil this target. The first trial to protect cattle utilising live or attenuated WC11 strain of AlHV-1 was not effective (Plowright et al., 1975). Similarly, study using inactivated cell free virulent C500 AlHV-1 virus in rabbits challenged parentally to generate immunity against cell free virus did not provide complete protection (Russell, 1980). However, another study using rabbits inoculated systemically with cell-associated virus and vaccinated with live cell-associated virus produced protection against the virus while inactivated cell-associated vaccination did not generate immunity (Edington and Plowright, 1980).

In order to examine whether cattle immunised with AlHV-1 C500 strain naturally and challenged with the attenuated virus could develop protective immunity Haig et al. (2008) conducted a study to address this hypothesis. In this trial cattle challenged with virulent cell-free virus naturally (intranasal inoculation) and immunised with attenuated AlHV-1 C500 strain in Freund's adjuvant (intramuscular route). The data obtained revealed that nine out of ten of the cattle systemically administrated developed a high level of neutralizing

antibodies in their nasal discharges as well as generating protective immunity. Moreover, animals challenged systemically did not develop protection against the virus (Haig et al., 2008).

The role of licensed adjuvant (Emulsigen, MVP Technologies) in helping protective immunity in cattle challenged with AlHV-1 (intranasal route) has been examined. The results indicated that considerable number of cattle were protected from the virulent virus at around three months post immunisation, however, the disease is developed when cattle further challenged at later time from 26 to 39 weeks post primary immunisation (Russell et al., 2012a). In addition, the high level of neutralising antibodies in the nasal secretions and blood from vaccinated and survived animals highlighted their role as a mucosal barrier for blocking the virus entry (Russell et al., 2012a).

The bulk of the vaccine development work has been done on AlHV-1 rather than OvHV-2. This is due to the absence of an effective propagating system for OvHV-2 in culture (Hart et al., 2007b). However, there are many factors such as the high similarity between AlHV-1 and OvHV-2 (Hart et al., 2007b, Ensser et al., 1997b), the existence of good reliable experimental animals (Anderson et al., 2007), and the use of large granular lymphocytes (LGLs) generated from the tissues of infected animals (Thonur et al., 2006), which can pave the way for researchers to develop a promising vaccine for MCF protection.

1.20 Chemicals for the experiments

1.20.1 5-azacitidine

5-azacitidine is a chemical analogue of cytidine, a nucleotide in DNA and RNA. The drug is a chemotherapeutic agent and used in the treatment of cancer (Brodovsky et al., 2013). The drug works through two mechanisms- at low doses, by inhibiting of DNA methyltransferases leading to hypo methylation of DNA, and high doses through its incorporation to the nucleic acids causing cell death (Kaur et al., 2014). The drug has wide spectrum of side effects ranging from anaemia, renal failure, foetal damage, fever, vomiting, and diarrhoea. Due to its hypo methylation activity, it has been found that 5-azacitidine can reduce the stability of silencing signals (stabilized by methylation) and hence affecting gene expression (Navada et al., 2014).

1.20.2 Cyclosporine A

Cyclosporine A is an immunosuppressant agent used in organ transplantation to prevent tissue rejection (Mott, Zhang et al. 2004). The drug decreases the activity of the immune system by interfering with the development and activity of T cells. Beside its use in tissue transplantation, it has been used for the treatment of rheumatoid arthritis, acute ulcerative colitis, and dry eyes. The adverse effects of cyclosporine A include fever, diarrhoea, vomiting, convulsions, hypertension, thrombosis in renal vasculature and

weakening the immune response through lowering the growth of T cells (Henry et al., 1995, Robert et al., 2010). The mechanism of action of cyclosporine A is not well known but it is thought that cyclosporine binds to cyclophilin of T cells. This cyclosporine and cyclophilin complex inhibits calcineurin required for the transcription of IL-2 (Youn et al., 2002).

1.21 Pathogenesis

Malignant catarrhal fever is a lymphoproliferative disease characterised by the proliferation of lymphocytes in lymphoid tissues (lymph nodes, spleen, and appendix) and interstitial infiltration of lymphoid cells in non-lymphoid organs (Liver, kidney, and lung) and this is often connected with tissue necrosis (Buxton et al., 1984). Many hypotheses have been suggested and many studies have been conducted in order to interpret the pathogenesis (mechanism by which the disease occurs) of MCF (Dewals et al., 2008, Anderson et al., 2007, Buxton et al., 1984). However, it is yet unknown.

1.21.1 Direct versus indirect mechanism of MCF

Buxton et al. (1984) infected rabbits with sheep-associated malignant catarrhal fever virus (SA-MCF) in lymphoid cells intravenously; it was found that the systemic pathological changes were analogous to those found in rabbits similarly infected with WA-MCF virus. It was also discovered that there was augmentation in T-

lymphocytes which was due to hyperplasia instead of neoplasia. Moreover, after treating the infected rabbits with cyclosporine (lymphocyte proliferation suppressor), the T cell proliferation ceased but areas of necrosis and disease were unaffected suggesting to the authors that tissue necrosis was due to autoimmunity induced by cytotoxic activity of uninfected cells under the control of a small number of infected cells. This indicated that the development of MCF was not associated with cell infiltration as such but to the tissue necrotic lesions. This hypothesis was strengthened by the paucity of virus or viral antigen in infected tissues, supporting this indirect mechanism (Bridgen et al., 1992). However, further studies have shown that both viruses induce lymphoid cell accumulations comprised mainly of T-lymphocytes, of which CD8$^+$ T-cells predominate (Anderson et al., 2007, Simon et al., 2003). Using *in situ* PCR, Simon et al. (2003) confirmed that larger numbers of the CD8$^+$ T-cells were infected with OvHV-2 in the brain of a cow and bison than previously thought indicating that the pathogenesis of MCF may not be by bystander cell activity after all.

Dewals and Vanderplasshen continued examining the pathogenesis of WA-MCF (AlHV-1) in rabbits using multi-colour flow cytometry staining to phenotype mononuclear leucocytes in both lymphoid (during the infection and at time of euthanasia) and lymphoid tissues (at time of euthanasia). They found that the prevalent T cell subsets were CD3$^+$ CD8$^+$ CD4$^-$ and the majority of these cells

produced gamma interferon (IFN-γ) and perforin (Dewals and Vanderplasschen, 2011). In addition, the identification of CD8 perforin[+] γδ^{-} T cells has been reported (Nelson et al., 2010) in the perivascular spaces throughout experimental SA-MCF (OvHV-2) in bison. These outcomes show that MCF is associated with the extension and infiltration of infected activated and cytotoxic CD8^{+} T cells expressing high levels of IFN-γ and perforin.

1.21.2 Experimental MCF

The use of rabbits as experimental animals has facilitated the study of the pathogenesis of MCF as they develop the same symptoms and systemic changes that occur in the naturally-susceptible hosts (Anderson et al., 2007, Buxton et al., 1984, Cunha et al., 2013, Gailbreath et al., 2008). These studies have demonstrated certain variations between MCF caused by AlHV-1 and OvHV-2. AIHV-1-related lesions were more abundant in the peripheral lymph nodes, while lesions associated with OvHV-2 were more obvious in visceral lymph nodes such as mesenteric lymph nodes. Moreover, AIHV-1-associated lesions showed less necrotic regions than those seen in OvHV-2 (Anderson et al., 2007, Buxton et al., 1984).

In an experiment carried out by Gailbreath et al. (2008) cell-free OvHV-2 removed from nasal secretions of virus-shedding sheep was inoculated by intranasal nebulisation into eight rabbits. Five of these, developed clinical signs and systemic pathological changes

which were similar to those changes in rabbits infected intravenously by cell-associated OvHV-2 in earlier studies.

1.21.3 Large granular lymphocytes in MCF and their phenotype

Large granular lymphocytes (LGL) are the T/NK cells that grow in culture from the tissues (usually lymphoid tissue) of MCF-affected animals. They are enriched for virus-infected cells that are difficult to detect *in vivo*. They possess cytotoxic activity and seem to express either natural killer or T cell phenotypes (Burrells and Reid, 1991). The phenotype of LGLs has been studied in cells from various species infected with either OvHV-2 or AIHV-1 (Burrells and Reid, 1991, Schock et al., 1998, Schock and Reid, 1996). Schock et al. (1998) examined the phenotype of LGLs in five different cell lines derived from some cattle infected with OvHV-2. It was shown that surface antigen CD2 and CD5 expressed on greater than 97.5 % of cells in all cell lines. CD4 was expressed on the majority of cells in three cell lines while CD8$^+$ was expressed on one cell line. However, CD8$^+$ T cell was the predominant cell type accumulated in the brain of a cow and bison infected with OvHV-2 (Simon et al., 2003). It was also demonstrated that CD25, the interleukin-2 (IL-2) alpha chain receptor, was expressed on all the cell lines. The growth of LGLs infected with OvHV-2 is enhanced in the presence of IL-2 and

high cell density (Schock et al., 1998). However, these cells can develop in IL-2-free culture (Swa et al., 2001).

Greater than 90% of LGL cell lines are virus infected as identified by immunocytochemistry or by *in situ* hybridisation for viral DNA or mRNA, despite the scarcity of cells containing virus in MCF affected organs *in vivo*. LGL cell lines are able to induce MCF when transferred to rabbits (Russell et al., 2009). LGLs are able to destroy tissue cells in an MHC-unrestricted manner and do not grow after stimulation with mitogens, while control T cells do (Schock et al., 1998, Swa et al., 2001). The interpretation of the activated cytotoxic phenotype of infected LGLs, in IL-2 free culture, includes constitutively activated Lck and Fyn kinases. These kinases are normally significant for the primary stimulation of T cells via the T cell receptor and co-receptors on the cell surface (Swa et al., 2001). LGLs produce various cytokines including TNF-alpha, IFN-gamma but are usually deficient in IL-2 either before or after stimulation by mitogen (Schock et al., 1998). LGLs have been used intensively to study the pathogenesis of MCF *in vitro* (Hart et al., 2007a, Thonur et al., 2006).

1.21.4 The role of cytokines in MCF pathogenesis

The role of cytokines, in particular, interleukin 15 (IL-15) and interleukin 2 (IL-2) in the pathogeneses of MCF has been examined (Anderson et al., 2008, Meier-Trummer et al., 2009a) IL-15 is a

cytokine which has a structural resemblance with IL-2. It is secreted by a wide range of cells and tissues containing, monocytes, macrophages, dendritic cells (DC), epithelium, and endothelium (Grabstein et al., 1994). Its function is to recruit, activate the proliferation of, and regulate cytotoxic cells including CD8$^+$ cytotoxic T lymphocytes, natural killer cells (NK), NK T cells, and CD4$^+$ cytotoxic lymphocytes. Therefore, it plays a significant role in both innate and adaptive immunity. Also, it has an anti-apoptotic function (Anderson et al., 2008, Lodolce et al., 2002). Moreover, the involvement of IL-15 in the pathogenesis of various autoimmune diseases has been documented. It has been found that, in mouse paradigms of autoimmunity, inhibiting IL-15 abolished the intensity of the diseases (Smith et al., 2000).

It was discovered; utilizing *in situ* hybridisation, after infecting rabbits with either AlHV-1 or OvHV-2 that IL-15 was abundant in the lymphoid tissues. The abundance of IL-15 is associated with tissue lesions indicating that the cytokine may be implicated in the pathogenesis of MCF (Anderson et al., 2008). In addition, it was found in the same experiment that the intensity of BLT esterase, (that detects tryptase) measuring cytotoxicity, was preserved in LGLs when stimulated with IL-15 in comparison with IL-2. These observations demonstrate that IL-15 may play a role in the regulation of the active cytotoxic cells in MCF (Anderson et al., 2008).

IL-2 is a cytokine which plays a significant role in the growth of T cells as well as the development of T regulatory cells (Tregs). It is expressed by T cells, mainly CD4$^+$ T lymphocytes during immune responses (Boyman and Sprent, 2012, Meier-Trummer et al., 2009a). IL-2 transcript abundance was low in lymphoid tissues derived from cows infected with OvHV-2 using microarray RNAseq assay. Although, the abundance of CD28 and T cell receptor (TCR), co-stimulatory enhancer for IL-2 production, was very high. It was also demonstrated that the ratio of CD4$^+$ was less than CD8$^+$ lymphocytes. These observations indicate that IL-2 deficiency may have a significant role in the pathogenesis origin of MCF (Meier-Trummer et al., 2009a).

1.21.5 The site of infection and MCF pathogenesis

A reverse-transcription PCR was used as the technique to detect OvHV-2 in organs in which OvHV-2 replication takes place *in-vivo*. Samples (turbinate, lungs, and trachea) were collected from naturally-infected sheep (reservoir species) and samples including kidney, bladder, brain, and intestine were obtained from cattle and bison infected with OvHV-2. OvHV-2 major capsid protein gene (ORF25) was utilised as a marker of viral replication. The data obtained revealed that the ORF25 transcript was present in 30% of the respiratory tract-related samples, 66% of which were turbinate, in sheep, indicating that this is the site of virus production in the

reservoir host. In contrast, most of the bovine and bison samples contained ORF25 transcripts indicating that replication or at least partial productive cycle appears to takes place in most tissues (Cunha et al., 2008). Of course in the MCF-susceptible animals this productive cycle is incomplete as discussed above. *In situ* hybridisation and immuno-histochemistry using antibodies for capsid and tegument structural proteins (ORF43and ORF63) of OvHV-2 were used to identify regions of viral replication in six OvHV-2 infected rabbits. The results showed that all infected rabbits developed pathological lesions in the samples. However, virus structural proteins were identified solely in the appendix, in particular, in the epithelial cells and M-cells, while ORF63 mRNA was only found in the epithelial cells but not in the M-cells suggesting that these cells may have a role in the pathogenesis of MCF and may represent an entry site for the virus (Meier-Trummer et al., 2009b).

It may be worth mentioning that the researchers in both experiments have used different techniques, different virus administration approaches (intravenous and aerosol), and different viral strains. This may have an impact on the data obtained as, for example, the OvHV-2 virus used was from European and American strains (Meier-Trummer et al., 2009b). Although it has been found in earlier work that the two strains are highly similar 94-100% (Hart et al., 2007b, Taus et al., 2007), variations in the genomic

sequences between the two strains may interpret their biological diversity (Meier-Trummer et al., 2009b).

1.21.6 Recombinant AlHV-1

In order to study the role of virus genes in pathogenesis, Researchers have exploited the availability of virulent AIHV-1 virus in low passage tissue culture to construct a stable virus genome as a bacterial artificial chromosome (BAC), for this, a modified *loxP*-flanked BAC cassette was integrated in one of the two large non-coding sites of AlHV-1 DNA. The integration led to the generation of an AlHV-1 BAC clone in bacteria and capable of producing infectious particles when transfected into permissive cells. AlHV-1 virions originated from BAC were able to replicate and generate MCF in rabbits that were similar to that of the AlHV-1 virulent parental strain. This discovery will greatly assist our understanding of MCF pathogenesis by allowing gene knockout and reinsertion recombinant viruses to be made (Dewals et al., 2006a).

1.22 Hypothesis

Malignant catarrhal fever is initiated by aberrant gene expression in infected T cells, epithelium and endothelium.

1.23 Objectives

1. **Objective 1:** To determine whether bovine endothelium and rabbit epithelium can be infected with AlHV-1 C500 strain *in vitro* (cell lines, rabbit and cattle) and *in vivo* (in rabbits detected by *in situ* hybridisation). Also to determine the life cycle stage of the virus in the infected cells (by RT-qPCR and *in situ* hybridisation) using known latent and lytic transcript analysis.

2. **Objective 2:** to attempt to switch the virus life cycle from lytic to latent in large granular lymphocytes (LGLs, mixed latency and lytic cycle) and BT fibroblasts (lytic cycle only) infected with AlHV-1 C500 strain after treatment with 5-azacitidine (a drug used to support latency). In addition, RNAseq will be used to map the host and viral gene transcripts in these cells to look for pathways associated with latency and the lytic cycle.

3. **Objective 3:** To determine viral gene expression in the tissues of animals infected with AlHV-1 and treated with cyclosporine-A (CSA) to attempt removal of the infiltrating

infected T cells. The effect of this on disease outcome and virus gene expression will be monitored.

Chapter 2

General materials and methods

2.1 Introduction

This chapter describes the approaches which have been done in most of the work and common for all of the chapters while more specific methodology will be described in the results chapters.

Virus: The C500 strain (Plowright et al., 1960, Plowright et al., 1975) was used. For preparation of virus stocks, see section (2.2.4) below.

2.2 Tissue culture

Three types of cultured plate adherent cells were grown for the study; bovine turbinate fibroblast (BT) cells were kindly donated by Dr George Russell of Moredun institute (Palmeira et al., 2013, Parameswaran et al., 2014), bovine aortic endothelium (BAE) cells (cat# 87022601, ECACC) the official name is BFA but BAE is used throughout the project, (Pollock et al., 1991), and statens seruminstitut rabbit cornea (SIRC) cells (Cat# 89090404, ECACC), (Parameswaran et al., 2014). The media used were Iscove's Modified Dulbecco's Medium (IMDM, Gibco® ThermoFisher Scientific, UK) for the growth of BT cells and Dulbecco's Modified Eagle Media (DMEM, Gibco® ThermoFisher Scientific, UK) for the culture of BAE and SIRC. All the manipulations were undertaken under aseptic conditions in a laminar air flow tissue culture hood. The cells were split when they reached 80% confluence using 1%

trypsin in phosphate buffer saline (PBS) for up to 10 minutes (2ml for a 25 cm^2 flask and 4ml for a 75cm^2 flask) in 37° C, 5% CO2 in air incubator. Trypsin reaction was stopped by adding the same volume of warmed media containing 10% foetal calf serum (FCS). Supernatant was removed by centrifugation at the 1500 rpm for 5 minutes at 21 °C. The cell pellet was re-suspended by adding media (5ml for 25 cm^2 and 14 ml for 75 cm^2 flasks). Cells were incubated at 37 °C, 5% CO$_2$ in air and checked regularly (every 3 days) until cells become confluent.

2.2.1 Large granular lymphocytes

Large granular lymphocytes (LGLs) derived from rabbit mesenteric or popliteal lymph nodes are used in the study. Briefly, lymph node tissues were teased apart using sterile forceps and scissors. To give a single cell suspension the tissues were then passed through a 70 μm filter using PBS (using back of 2ml syringe and 50ml centrifuge tube). The cells were washed twice with IMDM medium (containing Glutamax, 10% FCS, 100μg/ml penicillin, 50μg/ streptomycin). All manipulations were undertaken using sterile solutions, equipment and aseptic technique under the laminar flow hood. Cells were culture in upright 25cm^2 flasks at 37°C, in 5% CO$_2$ in air. For maintaining the cultures, the cells were fed with fresh IMDM media (contain 10% special FCS and 50 μl interleukin-2 (IL-2) 50ng/ml media (Cat# 202-IL, Recombinant Human IL-2, R&D Systems))

added up to 10 ml total volume of media by remixing the top ~one third of culture medium where most of the cells are at the bottom region of the flasks)) when the medium colour changes to yellow/orange or once every seven days. After that, cells are checked under the microscope for growth and stored back in the incubator in the upright position.

2.2.2 Counting cells

A haemocytometer was used to count cells and trypan blue exclusion used as a measure of cell viability.

2.2.3 Cryopreservation of cells

Adherent cells (BT, BAE and SIRC) were cryopreserved at $5x10^6$- $1x10^7$ cells per ml medium containing 50% FCS in 10% v/v dimethyl sulfoxide (DMSO) (Cat# 472301, Sigma-Aldrich) and the LGLs and lymphoid cells at $1x10^7$- $1x10^8$ cells per ml. Adherent cells were detached from cultured plates by adding 1% trypsin in 1xPBS, centrifuged at 1200 rpm for 5 min. The cell pellet was re-suspended in freezing medium consists of 90% IMDM or DMEM media (containing 10% FCS, 1% penicillin/streptomycin) and 10% filtered (DMSO) according to the type of the cells. The cell suspension was then transferred to labelled cryo-tubes, placed in Mr frosty (freezing container, ThermoFisher Scientific, UK) in -80 ºC freezer overnight and then in liquid nitrogen.
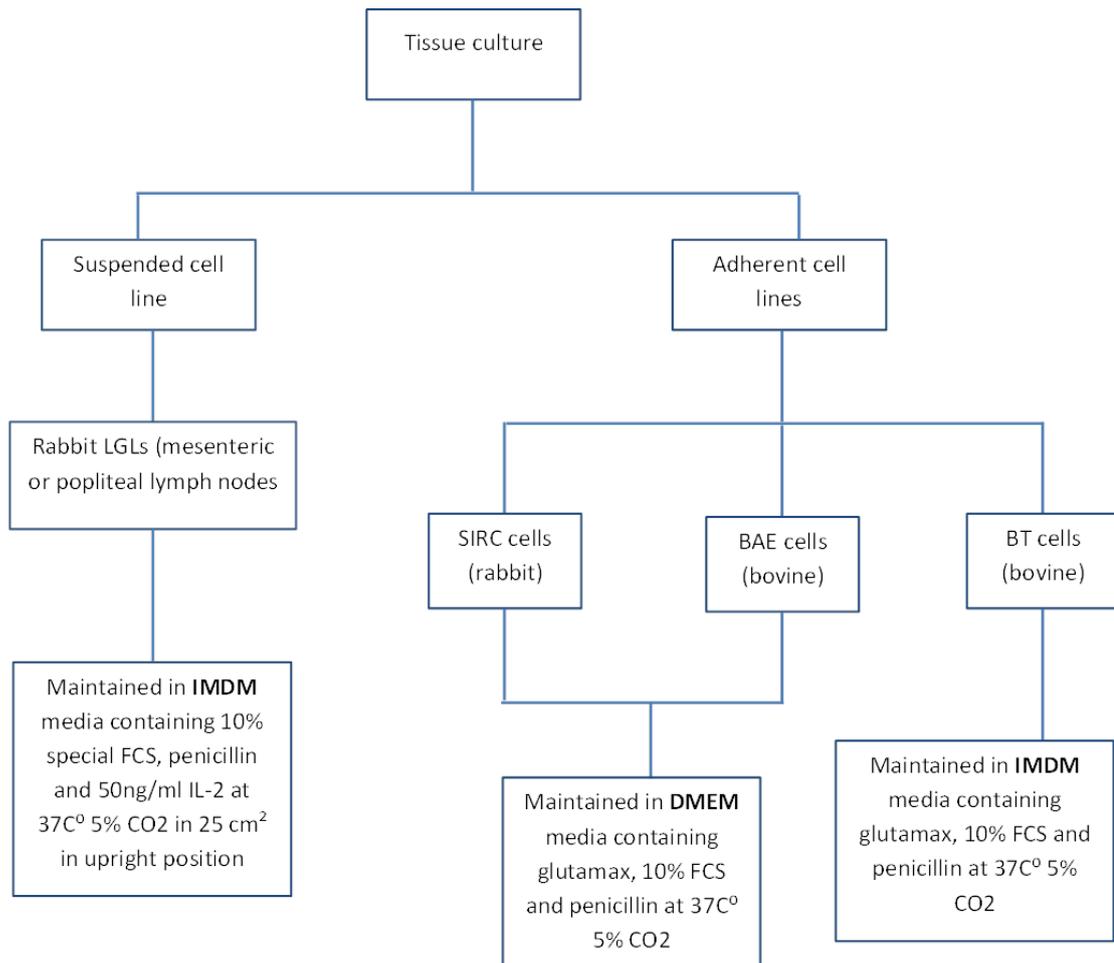
2.2.4 AlHV-1 virus stocks

Low culture pass (virulent) AlHV-1 C500 strain (Plowright et al., 1975) virus stock was obtained from infected rabbit lymphoid cells incubated with BT cells where a mixture of cell-associated virulent and cell-free virulent viruses obtained from up to five passages in BT cells were harvested and frozen away as stock virus. The AlHV-1 C500 strain used to infect BT cells to make the virus stock was obtained from AlHV-1 infected rabbit's mesenteric lymph node (MLN) cells. The approach applied was as follows: All the

manipulations undertaken were under aseptic conditions in the laminar flow hood. BT cells were washed twice with PBS. Vials of infected rabbits MLN cells were taken out of the liquid Nitrogen storage and placed on dry ice. The vials were thawed and the MLN cells were added to a centrifuge tube containing 15 ml IMDM media and centrifuged then re-suspended two and half millilitres of the medium were added to each flask of BT cells (4 flasks in total) and one left as a BT only control. The flasks were incubated at 37 ^{o}C, in 5% CO_2 in air incubator overnight.

The following day, the media were discarded and the cells were washed with 1xPBS twice. Fifteen millilitres of IMDM media were added to each flask and then kept at 37 ^{o}C, in a 5% CO_2 air incubator. The cells were monitored daily for any cytopathic effect (CPE) which is characterised by the accumulation of cell debris and the absence of monolayer.

The supernatant from the cultures were collected when the BT cells showed approximately 50% CPE, centrifuged and the pellet re-suspended in 4 ml medium. The supernatant collected in 75 cm^2 flask and placed on Ice. Four ml 1xPBS was added to the attached cells which were mechanically detached from all the flasks, centrifuged and the pellet mixed with the previously obtained pellet. The mixture was then freeze-thaw three times in liquid nitrogen and spun down. The supernatant carefully collected and finally mixed with the supernatant initially collected in 75 cm^2 flask. The

supernatant was aliquot in labelled cryo-tubes (1ml each), placed in

-80 ^{o}C freezer overnight and then stored in liquid nitrogen. Applying

the same technique P_1, P_2, P_3, and P_4 were finally made. With regard

to high pass attenuated AlHV-1 virus stock was made by passaging

the virus in BT cells more than 5 times up to thirty passages.

2.2.5 Virus titration : 50% tissue culture infective dose (TCID50)

$TCID_{50}$ is the approach used to measure virus titration. This measure the infective dose of the virus required to kill 50 % of infected BT cells measured as a cytopathic effect (CPE).

The procedure was undertaken under aseptic conditions in the laminar flow hood. Seven Eppendorf tubes were labelled 10^1 to 10^7 for the virus to be titrated. A volume of 100 µl of BT cell suspension ($2x10^5$ cells/ml in IMDM medium containing 10% FCS) was added to wells of a 96-well plate designated A1-4 to H1-4 (Figure 5). The plate was kept at 37 ºC, in 5% CO_2 in air in an incubator overnight. The following day the media were discarded and the cells were washed twice with 1xPBS. A volume of 900 µl serum-free IMDM medium was loaded into each labelled Eppendorf tubes. AlHV-1 C500 virus for titration was thawed from frozen storage. A volume of 100 µl of neat AlHV-1 virus was added to the tube labelled 10^1, and mixed thoroughly. A volume of 100 µl of 10^1 dilutions was then transferred to 10^2 Eppendorf tube and so on up to the 10^7 tube giving a number of serially diluted samples. A volume of 100 µl of dilution 10^1 was added to the wells B1-4 and dilution 10^2 to wells C1-4 and so on to H1-4. A volume of 100 µl of serum free medium was added to the wells A1-4 used as control (Figure 5).
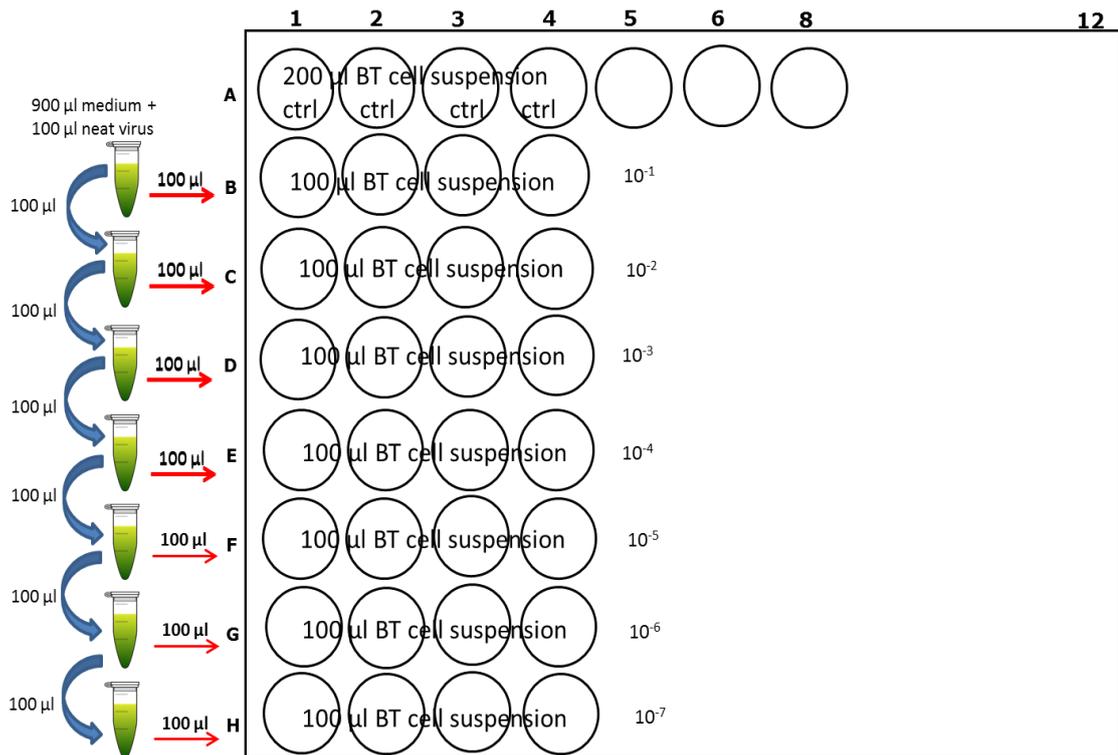
Figure 5 Virus titration: 50% tissue culture infective dose (TCID50).
The figure shows the steps were taken to carry out TCID50 for virus titration.

The plate was finally placed in the 37 ^{o}C, 5% CO_2 incubator for two hours. After two hours, all the wells including the control were topped up with 100 µl IMDM medium containing 10% FCS. The plate was then placed in the 37 ^{o}C, 5% CO_2 incubator and read for % CPE after 4-5 days.

Wells were positive if CPE was present. The virus titre is calculated by Spearman/Karber (http://www.cureffi.org/2015/09/20/the-math-behind-spearman-karber-analysis/) Calculation as follows: Log TCID50-L-d (S-0.5) where

L   = negative log of lowest dilution

D   = difference between log dilution steps

S   = sum of proportion of positive tests (wells showing CPE from

virus infection)

Example of virus titration

Table 3 example of virus titration

Virus dilution	Positive wells				Proportion of infected culture
10⁻¹	+	+	+	+	4/4 = 1
10⁻²	+	+	+	+	4/4 = 1
10⁻³	+	+	+	+	4/4 = 1
10⁻⁴	+	+	-	-	2/4 = 0.5
10⁻⁵	-	-	+	-	1/4 = 0.25
10⁻⁶	-	-	-	-	0/4 = 0
					3.75

2.3 Molecular techniques

2.3.1 DNA extraction

The total cellular and viral DNA was extracted using DNeasy blood and tissue kit (cat# 69506, Qiagen) according to manufacturer's instructions. DNA was extracted from different tissues, blood and cultured cells.

Cultured cells: appropriate number of cells was centrifuged (maximum $5x10^6$) for 5 min at 300 g. The pellet was re-suspended in 200 µl phosphate buffered saline (PBS) and 20 µl proteinase K was added. A volume of 200 µl of buffer AL was added, mixed thoroughly by vortexing, and then incubated at 56 ^{o}C for 10 min. After that, 200 µl of absolute ethanol was added to the sample and mixed thoroughly by vortexing. The mixture was then pipetted into DNeasy Mini spin column placed in a 2 ml collection tube, centrifuged at 6000g for 1 min, and the flow-through and the collection tube were completely discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube, 500 µl of buffer AW1 was added and centrifuged at 6000 g for 1 min. The flow-through and the collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube, 500 µl of buffer AW2 was added and centrifuged at 20,000 g for 3 min. The flow-through and the collection tube were discarded. The DNeasy Mini spin column was placed in a clean 1.5 ml, 100 µl of buffer AE was directly loaded onto DNeasy membrane, incubated at room

temperature for 1 min, and then centrifuged for 1 min at 6000 g to elute.

From blood: A volume of 20 µl of proteinase K was loaded into a 1.5 ml Eppendorf tube. A volume of 50-100 µl anti-coagulated blood was added and the volume was adjusted to 220 µl with PBS. A volume of 200 µl of buffer AL was added, mixed thoroughly by vortexing, and then incubated at 56 ⁰C for 10 min. After that, the steps were exactly similar to the ones done for cultured cells.

Animal tissues: A piece of 25mg tissue was cut into small pieces, and then placed in a 2 ml micro-centrifuge tube. A volume of 200 µl of buffer ATL was then added. A stainless steel bead was added to the tube and the tube was placed in a tissue lyser (TissueLyser II Qiagen, Uk) to help disrupting the tissue. A volume of 20 µl of proteinase K was added, mixed thoroughly by vortexing, and incubated at 56 ⁰C until the tissue is completely lysed. The next steps are similar to the ones done for cultured tissues.

2.3.2 RNA extraction

Total RNA was extracted using RNeasy Plus mini kit (cat# 74134, Qiagen). A maximum of 1×10^7 cells were harvested and appropriate volume (600 µl) of Buffer RLT Plus was added and mixed by vortexing for 30 s. The homogenised lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 s at 8000 g. the column was discarded and the flow-through was saved. One volume (600 µl) of 70% ethanol was added to the flow-through and mixed well by pipetting. Up to 700 µl of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 s at 8000 g. the flow-through was discarded.   A volume of 350 Buffer RWT was added to the RNeasy spin column and was centrifuged for 15 s at 8000 g to wash the membrane. The flow-through was discarded. A volume of 10 µl DNase I stock (cat# 79254, Qiagen) solution was added to 70 µl Buffer RDD (cat# 79254, Qiagen), mixed gently by inverting the tube, and centrifuged briefly. The DNase incubation mix (80) was directly added to the RNeasy spin column membrane, and placed on the benchtop for 15 min. A volume of 350 Buffer RWT was added to the RNeasy spin column and was centrifuged for 15 s at 8000 g to wash the membrane. The flow-through was discarded. A volume of 500 µl Buffer RPE was added to RNeasy spin column, and centrifuged for 15 s at 8000 g to wash the membrane. The flow-through was discarded.   A volume of 500 µl Buffer RPE was added

to RNeasy spin column, and centrifuged for 2 min at 8000 g to wash the membrane. The RNeasy spin column was placed in a new 1.5 ml collection tube. A volume of 30 µl RNase-free water was directly added to the spin column membrane, centrifuged for 1 min at 8000 g to elute the RNA.

Animal tissues: a piece of an animal tissue (less than 50 mg) was excised and loaded into a 2 ml collection tube. 600 µl of Buffer RLT Plus were added and a stainless steel bead was added to the tube and the tube was placed in a tissue-Lyser (Tissue Lyser II, Qiagen, UK) to disrupt the tissue. The tube containing the lysate was placed on the benchtop at room temperature for 5 min. The next steps are similar to the ones done for extraction of the RNA from the cultured cells.

2.4 Nucleic acid amplification and quantitation

2.4.1 Complementary DNA (cDNA) synthesis

Complementary DNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (cat # 04897030001, Roche Applied Sciences, UK). The template-primer mixture for 20 µl reaction was prepared in a sterile, nuclease-free, thin-walled PCR tube on ice by adding the components in the following order; the equivalent of 1 µg total RNA, 2 µl random hexamer primer, and up to 13 µl RNase free water. After that, the RT (reverse transcriptase) mix was prepared by adding the following reagents; 4µl Transcriptor Reverse

Transcriptase Buffer, 0.5 µl Protector RNase Inhibitor, 2 µl Deoxynucleotide Mix, and 0.5 µl Transcriptor Reverse Transcriptase (storage buffer; 200 mM potassium phosphate, 2 mM dithiothreitol, 0.2 % Triton X-100 (v/v), 50% glycerol (v/v), pH approx. 7.2). The RT mix was then added to the tube containing the template-primer mix. The mixture was mixed carefully by flicking the tube, centrifuged briefly, and finally placed in a thermal block cycler with a heated lid for 10 min at 25 °C followed by 35 min at 55 °C. The Transcriptor Reverse Transcriptase was inactivated by heating to 55 °C for 5 min using XP Thermal Cycler (Bioer, UK). The reaction was stopped by placing the tube on ice and sample was stored at -20 °C.

2.4.2 Polymerase chain reaction (PCR)

2.4.2.1 PCR condition

The procedure was undertaken under aseptic conditions in the ultra violet cabinet. The equipment (pipettes, tips, and nuclease-free water) were exposed to ultra violet light for 15 min. All the reagents except the Taq polymerase were thawed and placed into the ice box. The master mix mixture for 24 µl reactions was prepared in a sterile, nuclease-free, thin-walled PCR tube on ice by adding the components in the following order; 17,875 µl nuclease free water, 2.5 µl 10x buffer, 1 µl MgCl2 (25mM), 0.5 µl dNTPs (10mM), 1 µl forward primer (5pmol/ µl), 1 µl reverse primer (5pmol/ µl), and 0.125 µl Taq polymerase (5 u/µl). A volume of 1 µl sample/s was added to the tube containing the master mix mixture outside the UV hood. The tube was centrifuged and placed in the PCR machine to run according to the following programme; 1 cycle (initializing) x 94 ᵒC, 35 (denaturation) x 94 ᵒC for 1 min, 60 ᵒC (annealing) for 1 min, 72 ᵒC (elongation) for 1 min, and 1x 4 ᵒC hold.

2.4.2.2 Agarose gel electrophoresis

The PCR products were resolved to agarose gel (Sigma-Aldrich, UK). Certain amount of agarose powder, according to the concentration required, was dissolved in 100 ml 1xTAE buffer in a beaker. The beaker was then heated in the microwave oven for sometimes until completely dissolved, cooled down, and 3.8 µl Nancy-520 (nucleic

acid stain) (Sigma-Aldrich, Uk) was added. The gel solution was slowly poured into the gel tray and the air bubbles were removed using disposable tips. The gel was left for sometimes to set. The gel tray was placed in the gel tank and 1xTAE buffer (Tris base, acetic acid and EDTA) was added to the gel tank until the wells were covered. A volume of 1 µl 6% loading dye (New England BioLabs) was thoroughly mixed with 5 µl sample and then loaded into the gel wells along with 6 µl DNA ladder (New England BioLabs). The voltage was adjusted to 110 V for 1 hour and the DNA was viewed by the UV illuminator (ImageQuant 300 imager, GE healthcare, UK).

2.4.3 Viral load measurement

Viral load is used to quantify the viral copy number in specific cells and tissues. q PCR is the device by which the viral load is identified. AlHV-1 virus copy number is measured by comparing the C_T value with standard curve by normalizing AlHV-1 ORF3 gene with 10^5 copy number of rabbit β globin used as a reference gene as described in (Boudry, 2007). The forward primer sequence for ORF3 used 5'-GGGCTAATTTGTGCAGTTTGTGA-3' and reverse primer 5'-AGGTGTTTCTGAAAAGAGGGGAA-3' and the probe used for the assay was FAMAlHV-1 6FAM ACAGGCTCCTCGTCCTCGTCGTGT TAMRA. The rabbit β globin forward primer used 5'-GGTATCCTTTTTACAGCACAAC-3' and the reverse primer 5'-CAGGTCCCCAAAGGACTCG-3', in the presence of the fluorescent probe 5'-FAM-CCTGGGCTGTTTTCATTTTCTCAGG-TAMRA-3'.

AlHV-1 BAC plasmid was utilized as standard as described in (Dewals et al., 2006a). The data obtained analysed using the absolute quantitative approach utilizing Light Cycler 480 software analysis.

2.4.4 Quantitative real-time PCR (q RT-PCR)

The quantitative PCR, using the Light Cycler 480 System (Roche Applied Science, UK), is the method used to measure the viral copy number and detection of the virus DNA in the infected cells and tissues as well as measuring the viral gene expression profiles throughout the project work. The technique is utilized to amplify and quantify a target DNA based on the sequence-specific probes tagged with florescence probe upon hybridisation with its complementary DNA sequence in the presence of flanked primers. The probes and primer sequences for q RT-PCR used in this study are shown in table 4. The probes and primers were designed using Universal Probe Library (UPL) System Assay Design

 (https://lifescience.roche.com/shop/products/universal-probelibrary-system-assay-design). Probes were labelled with fluorescent reporter dye 5 carboxyfluorescein (FAM) at the 5' end and quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMARA) at the 3' end. The technique was carried out utilizing the Light Cycler 480 Probes Master Kit (Roche Applied Science, UK) with the universal cycle profile. The 96-well plates were used for q RT-PCR with 20 µl added per reaction (well). For the test sample, each q RT-PCR mixture contained 1x light cycler probe master, 900 nM forward primer, 900 nM reverse primer, 100 nM probe, 2 µl (10-50) template DNA, made up to 20 µl with RNase free water. Each q RT-PCR reaction was carried out in duplicate. In addition, each q RT-

74

PCR trial comprises two no template controls (NTC), test samples, a control from non- infected cells along with a standard $\log_{10}$ dilution series. The standard $\log_{10}$ dilution series were used to produce standard curves. The efficiency of the standard curve was measured using the Light Cycler 480 (LC480) software. The PCR reaction efficiency, ideally, should be 100% meaning that the amount of product doubles each cycle ($E=2$) (Figure 5 & 6). The normalised values for the gene expression profile were measured using the advanced relative quantification method utilizing the Light Cycler 480 (LC480) analysis software. The genes selected for viral transcripts analysis (Table 4) are known with their involvement in latent and productive/lytic cycle in other herpesviruses. ORF73 encodes latency associated nuclear antigen (LANA), homologue to Epstein Barr virus nuclear antigen (EBVNA1) and and KSAH ORF73 LANA protein involved in latent infection, ORF50 encodes RTA protein the transcriptional and replication activator responsible for the switch to lytic cycle, ORF25 encodes a major capsid protein highly expressed during viral replication, A4.5 similar to Bcl-2 family of apoptosis and virus cycle expression not known and A9.5 encodes a secreted glycoprotein that is similar to IL-4 and virus cycle expression not known.

Table 4 primers and probes used for target and reference genes throughout the study.

Gene symbol	Oligonucleotide sequence (5'-3')
ORF50 F	GCCACAGCCTCAATCTGAAT
ORF50 R	TTCTGGACGAACCTGGCTAT
ORF50 P	UPL23
ORF73 F	TGGGCCTTCTCCTTCAGAT
ORF73 R	AAGTGGACCAGGTTCGTCTG
ORF73 P	UPL32
ORF25 F	TTCACCGTTATGCACAATAAGC
ORF25 R	CTTGTGCTTTGCCTGCACT
ORF25 P	UPL155
ORF A4.5 F	TTTGACCCGGGTAGTCAATC
ORF A4.5 R	GCTAGAATACATTCTGTGACCGTTAG
ORF A4.5 P	UPL92
ORF A9.5 F	CAACCAGTTTTGTTGTTATTCAATG
ORF A9.5 R	AAGAGAGTTAAAGTTAAATTTGGAGCA
ORF A9.5 P	UPL83
ORF63 F	GCTGTAATTGGCACTCTCAGG
ORF63 R	TCATGAGGCGGAAGTTGC
ORF63 P	UPL40
Rabbit SDHA1 F	ACCGTGAAGGGCTCTGAC
Rabbit SDHA1 R	TTTCTAGCTCGACCACAGAGG
Rabbit SDHA1 P	UPL158
Bovine RPS9 F	GCCTCGACCAAGAGCTGAAG
Bovine RPS9 R	GGGCAGCCTTTCGGATCT
Bovine RPS9 P	TGATCGGCGAGTATGGGCTCCG
Bovine G actin F	CACCTTCCAGCAGATGTGGA
Bovine G actin R	CTAGAAGCATTTGCGGTGGAC
Bovine G actin P	AGCAAGCAGGAGTACG
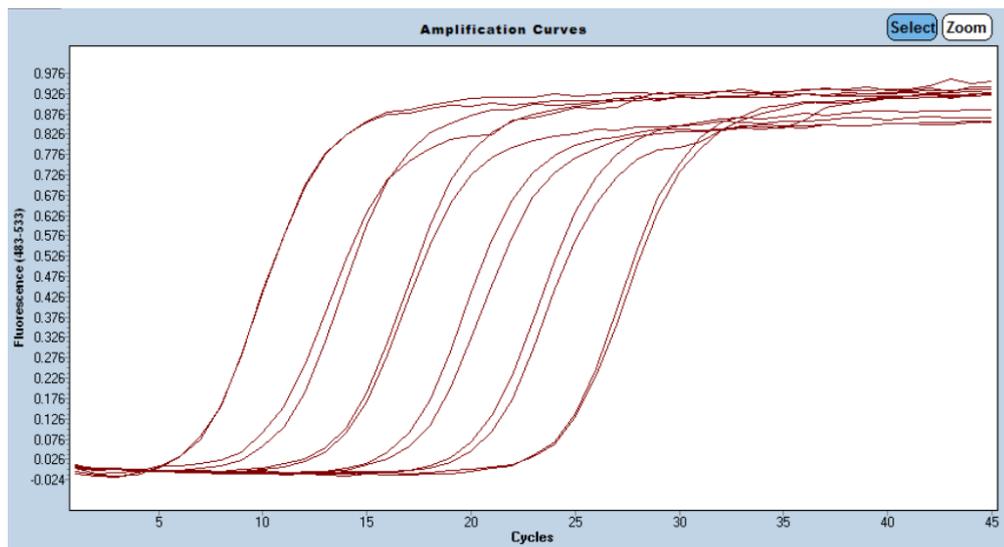
Figure 5 snapshot of q RT-PCR amplification curve.
The figure illustrates the amplification curves generated using Light Cycler 480 for determination of AlHV-1 DNA load from infected rabbit tissues utilizing ORF3 gene as a standard.
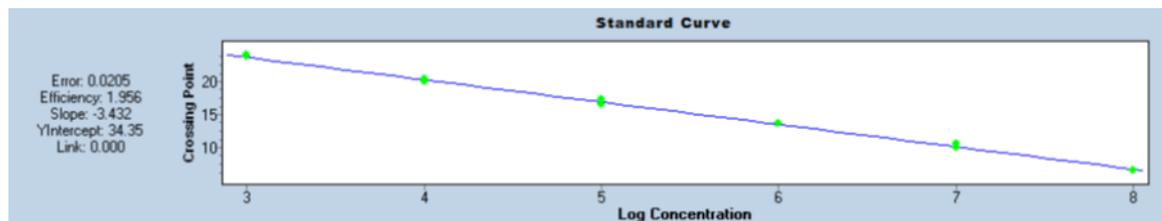
Figure 6 snapshot of q RT-PCR standard curve.
The figure shows the standard curve generated using Light Cycler for determination of AlHV-1 DNA load from infected rabbit tissues utilizing ORF3 gene as a standard and seven $\log_{10}$ serial dilutions 10^{-3} to 10^{-8} and PCR efficiency of 1.95.

2.4.5 Quantification of DNA and RNA by spectrophotometer

DNA and RNA concentrations were assessed by a NanoDropTM ND-8000 spectrophotometer (ThermoScientific, UK) according to the manufacturer's instructions using the software program NanoDrop ND-8000 version 2.2.0. To make the data as precise as possible, 1.5 µl blank was used for reading. After that, 1.5 µl of samples were loaded onto each lower measurement pedestal and read. The ratio of absorbance at 260 was used to measure the purity of nucleic acids. A ratio of ~1.8-2.0 showed pure nucleic acids and values outside this range were presumed to indicate the presence of proteins or phenols or other contaminants.

2.5 Flow cytometry

Flow cytometry was used to phenotype rabbit LGL samples (recognise the cell type and cell subset). Ten Eppendorf tubes labelled as non-stained, CD4 FITC, CD4 isotype control, CD8, CD11B (monocyte), B cell (CD19), Pan T (T cell), secondary antibody only FITC, secondary antibody only APC, and double staining (CD4&CD8) were used in this experiment. Labelling also includes rabbit LGL IDs (IYDY, IYJJ, LFLK, and LFKJ) for both 5-azacitidine-treated and non-treated LGL. After three weeks, RPMI (cat 61870-010 Gibco Life technologies) was used with 2% FCS supplement for cell labelling: The only direct conjugated antibodies used were the CD4 FITC and CD4 isotype control (primary antibodies/one step labelling) For indirect immunofluorescence, the secondary antibodies used were mouse IgG1 FITC (CD11B, B cell, Pan T, and secondary FITC only) and mouse IgG1 APC (CD8, CD4&CD8, and secondary APC only). Table 5 shows the antibodies and the dilution factors used in the experiment.

Table 5 Labelling steps

Antibody specificity	Primary antibody + dilution factor	Secondary antibody + dilution factor
CD4	mouse IgG2a anti-rabbit CD4 FITC (KEN-4, AbD Serotec)	NO
CD4 isotype control	mouse IgG2a CD4 isotype control-FITC (MCA929F, AbD Serotec)	NO
Non-stained	NO	NO
CD11b	Anti-rabbit CD11b (1:10) (MCA802GA, AbD Serotec)	Goat anti-mouse IgG1 FITC (1:20)
B cell	Anti-rabbit B cells (1:10)	Goat anti-mouse IgG1 FITC (1:20)
Pan T cell	Anti-rabbit Pan T cell (1:10) (RTH2A, 2BScientific)	Goat anti-mouse IgG1 FITC (1:20)
CD8	Anti-rabbit CD8 (1 :10) (MRB107A, 2BScientific)	Goat anti-mouse IgG1 APC (1:20) (A10530, Invitrogen, UK)
CD8&CD4	Anti-rabbit CD8 (1 :10) and mouse IgG2a anti-rabbit CD4 FITC (1:10)	Goat anti-mouse IgG1 APC (1:20)
APC	NO	anti-mouse IgG1 FITC (1:20)
FITC	NO	anti-mouse IgG1 FITC (1:20)

Ten tubes were labelled for each cell line and four cell lines (IYJJ, IYDY, LFLK, and LFKJ) were used in the experiment.

2.5.1 Direct Immunofluorescence labelling of cells

For this purpose, cells were spun at 2000 rpm for 5 minutes and pelleted then RPMI culture medium was added. Cells were counted to 5 X 10^5 cells/45 µl and placed in Eppendorf tubes. Antibodies of 5µl mouse IgG2a anti-rabbit CD4 FITC (KEN-4, AbD Serotec) to CD4 and CD4 & CD8 tubes, and 5µl mouse IgG2a CD4 isotype control-FITC (MCA929F, AbD Serotec) were added to CD4 iso tube. 5µl of anti-rabbit CD8 (ISC29E, WS0768U-100, USA) (1:10) to CD8 and CD4 & CD8 tubes, 5µl of anti-rabbit CD11B (MCA802GA, AbD Serotec) (1:10) to CD11B tubes, 5µl of anti-rabbit Pan T cells (RTH2A, 2BScientific) (1:10) to Pan T tube, and 5µl of anti-rabbit B cells (MRB107A, 2BScientific) (1:10) to B cell tubes. Labelled Eppendorf tubes incubated in the dark in the fridge for 30 minutes.

2.5.2 Indirect Immunofluorescence labelling of cells

After that, cells were centrifuged at 2000 rpm for 2 minutes and washed twice with RPMI then 5µl (1:20 diluted) of goat anti-mouse IgG1 FITC (secondary Ab) was added to CD11b, B cell, and Pan T tubes, 5µl (1:20) of anti-mouse IgG1 APC (A10530, Invitrogen, UK) to CD8 and CD4 & CD8 tubes, 5µl (1:20) of anti-mouse IgG1 FITC secondary only tubes, 5µl (1:20) of anti-mouse IgG1 APC to APC secondary only tubes. Labelled Eppendorf tubes were again incubated in the dark in the fridge for 30 minutes. Cells were washed twice by spinning at 2000 rpm for 2 minutes leaving 100 µl

supernatant. Cells were fixed with 2% PFA and finally suspended in 0.5 ml of PBS.

2.5.3 Flow cytometry analysis of labelled cells

The cells were centrifuged and rinsed then directed to FACScan ™ flow cytometer system (Becton Dickinson, Bioscience, USA). The Cells population were gated out to appropriate forward scatter (FSC), side scatter (SSC), 280 and 400, respectively. Cell's samples were then acquired by CellQuest pro-software (BD Biosceince, USA). Finally, the fluorescence data analysis was performed by FACSDiva software (BD Biosceince, USA) and the quadrant sitting for labelled and unlabelled cells was based on antibody-isotype control fluorescence.

2.6 Animals

Ten three month old New Zealand white female rabbits were used (Purchased from Harlan UK). Animals were kept at the animal facility at Sutton Bonington, University of Nottingham. Animal experiments were under ASPA (UK) regulations with a project license held by David Haig at the University of Nottingham. Ethical review was obtained from the local SVMS ethics committee and the University of Nottingham AWERB committee. After infection (see section 2.6.1) animals were observed and temperature was recorded daily using a subcutaneous mini chip. Animals were

euthanized when high body temperature (40°C) developed for two consecutive days or more along with an accumulation of clinical signs typical of MCF that would progress the disease to a higher severity limit than moderate (limited by ASPA project license held by D Haig).

2.6.1 Inoculation

Wildebeest-associated MCF virus (AlHV-1) contained in mesenteric lymph node cells isolated from a rabbit previously infected with AlHV-1 virus C500 strain was used to infect the rabbits injected intravenously (IV, ear vein) with 2×10^6 cells in 1 ml PBS.

2.6.2 Experiment (1) design

Ten rabbits were infected with AlHV-1 C500 strain on day 0 of the experiment as described above. Six of the ten rabbits were treated with cyclosporine A (Cat# 239835, CALBIOCHEM, Germany) every other day starting at day 0 at dose of 0.25-0.5ml IV (depending on the weight of the rabbit) to give a dose of 20mg/kg body weight. The CsA was dissolved in 1XPBS D-α-Tocopherol 25mg/ml per animal in 1XPBS (Cat# 57668-25G, Sigma) containing 2% v/v ethanol in PBS. The remaining four rabbits were given vehicle (tocopherol- ethanol-PBS) only every other day intravenously.

2.6.3 Experiment (2) design

The second experiment was similar to the first one with the exception that the number of rabbit groups included an uninfected control group. Four were infected with the virus; four were infected and treated with cyclosporine A as before except that it was dissolved in PBS containing castor oil 25mg/ml per animal (Kolliphor® EL, cat# C5135-500G, Sigma) and two non-infected controls given vehicle only.

Experiment # 1	Inoculation	Treatment	Observations
10 rabbits	All animals infected with the virus from day 0 of the experiment IV	6 rabbits treated with CsA (20 mg/kg body weight) IV every other day	D-α-Tocopherol 25mg/ml per animal in 1XPBS containing 2% ethanol IV used as a vehicle
Experiment # 2			
16 rabbits	12 rabbits infected with the virus from day 0 of the experiment IV 4 rabbits were not infected used as a control	6 rabbits treated with CsA (20mg/kg body weight) IV every other day 6 rabbits no treatment	Castor oil 25mg/ml per animal IV used as a vehicle

2.6.4 Sample collection

Liver, kidney, lung, spleen, and appendix tissues about 1cm^3 were collected (four pieces per tissue) and one piece each placed in Eppendorf tubes containing 500 µl RNA-later (Cat# AM7020,

ThermoFisher Scientific, UK), and placed immediately in -20 C° freezer until RNA extraction. A second piece of each tissue was placed in Eppendorf tubes without adding RNA-later and then placed in -20 C° freezer for subsequent DNA extraction. Mesenteric (MLN) and popliteal lymph nodes were placed in 60ml tubes containing 40ml sterile 1XPBS. The lymph nodes were processed immediately after collection (see section 2.2.1). Blood samples ~ 1800µl were placed in Eppendorf tubes and then placed in -20 C° freezer. The third piece of liver, kidney, lung spleen, and appendix were placed in 7ml bijou tubes containing 5ml 4% paraformaldehyde (Cat# P/0840/53, Fisher Scientific) in PBS for 48 hours prior to paraffin embedding (see section 2.7.1) for *in situ* hybridisation work. Finally, the fourth piece of each tissue (liver, kidney, lung, spleen, and appendix) was placed in 60ml tubes containing 40ml 10% neutral-buffer-formalin (Cat# 361387P, VWR International) for 24-48 hours prior to paraffin embedding (see section 2.7.1) for H&E staining.

2.7 Histology

Histological approaches are used to investigate the structural organisation of tissue.

2.7.1 Paraffin embedding

Following fixation (see above), tissues were placed in an embedding cassette and dehydrated by processing with an increasing gradient of absolute ethanol in a tissue processor (Leica TP 1020, Leica microsystem. UK) as the following protocol:

- 70% (v/v) Ethanol, 1 hour.
- 80% (v/v) Ethanol, 1 hour.
- 95% (v/v) Ethanol, 1 hour.
- 100% (v/v) Ethanol, four changes, 30 min each.
- Histoclear, three changes, 1 hour.
- Paraffin wax (56-58 C°), two changes, 1 hour and half each.

Finally, the processed tissue samples were oriented and embedded into paraffin molds and kept on the cold plate for 30 minutes to set. Then the mold is removed and the excessive paraffin is trimmed off.

2.7.2 Tissue sectioning and staining

Sections, 6µm thickness, from kidney and appendix fixed in buffered formalin were cut, de waxed and then stained with haematoxylin and eosin stain (H&E) according to the standard histological approach (described below) to visualize the histological structure of the tissues. The slides of tissue sections were passed into histoclear (two changes for 5 min each), dabbed to remove excess fluid, then rehydrated in a series of alcohol concentrations (100%, 95%, and 70%) for 2 minutes each and then rinsed in running water. Slides were immersed in a bath comprising haematoxylin (Cat# H9627,

Sigma-Aldrich) for 3 minutes and then washed in running tab water. After that, slides were placed in 1% acid alcohol (300ml ethanol+ 3ml HCl) for few seconds, and then washed in water.

Slides were immersed in Eosin (cat# 318906-500 ml, Sigma-Aldrich) for 5 minutes and then briefly washed in water. Eventually, slides were dehydrated by passing through different concentrations of ethanol (Cat# E10650DF/17, Fisher Chemicals) (95% and 100% alcohol), dabbed to remove excess, and then cleared in xylene for 5 minutes in a fume hood before being mounted with 1,3-diethyl-8-phenlxanthine (DPX) (Fluka analytical, Sigma Aldrich, UK). Sections were visualized using upright microscopy Leica DM 5000B epifluoresence imaging system.

For the third experiment, sample preparation and fixation was performed manually applying the same parameters with the exception that xylene (cat# x10200/17, Fisher Chemicals) was used as an alternative to histoclear. In addition, DEPC-treated water was used in 1XPBS to make up 4% paraformaldehyde.

2.8.1 RNA *in situ* hybridisation

In situ hybridisation is the technique by which gene transcripts (mRNA) can be located on tissue using a combination of molecular and histological approaches providing that the tissue components (RNA and morphological features) are maintained.

2.8.1.1 Preparation of riboprobes for virus gene transcripts

RNA probe synthesis is one of the steps towards achieving comprehensive *in situ* hybridisation procedure. The other two main steps include tissue fixation and sectioning and hybridisation of probes.

2.8.1.2 Primers design

The PCR primers used in preparing the riboprobes for AlHV-1 ORF 65 and ORF A9.5 were designed by professor James Stewart (ORF65) and Dr George Russell and colleagues (Russell et al., 2013). Table 6 shows the primers sequence and their amplicon size.

Table 6 shows AlHV-1 ORF65 and ORF A9.5 primer sequences and amplicon size

AlHV-1 gene	Oligonucleotides sequence	Amplicon size
ORF 65 Forward ORF 65 Reverse	GCACGCTAGGCCTAAACTTCC GCCTTTGCCTAGTTTTCCTCTGG	730 bp
ORF A9.5 Forward ORF A9.5 Reverse	TGGTTGTGAGAATGAAACACTTG GCACAAACCGAGTTTGACAG	525 bp

2.8.1.3 Polymerase chain reaction (PCR) amplification

PCR was used to amplify the genes of interest according to the protocol described in the materials and methods, section (2.4.2) using Taq polymerase enzyme (NEB). The benefit of using Taq polymerase enzyme is to add a single deoxyadenosine A to the 3′

end of the PCR product. The PCR fragment of the gene of interest was then inserted into a linearized plasmid victor (TOPO-TA one shot cloning kit, Invitrogen) having single overhanging 3′ deoxythymidine T residues. This will provide efficient ligation of the PCR insert with the plasmid DNA victor. Complementary DNA (cDNA) from rabbit's kidney infected with AlHV-1 C500 strain was used to amplify the gene of interest. The reason for using cDNA is to obtain coding sequence without any introns (non-coding sequence), hence avoiding any complication while synthesising the RNA probe. Figure 7 shows the amplicon size for both genes.
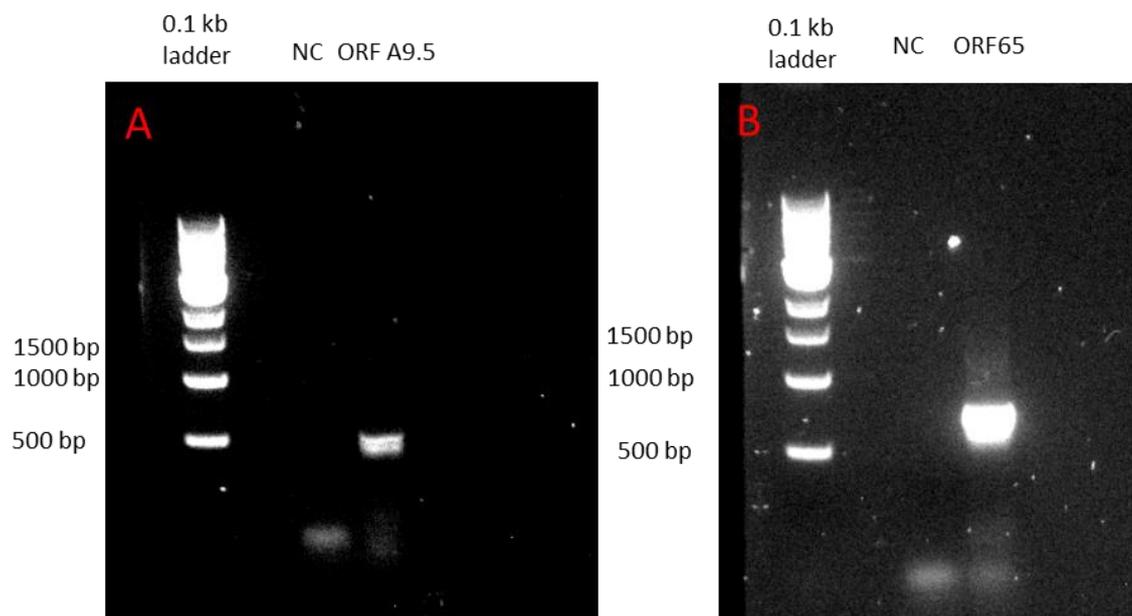
Figure 7 AlHV-1 PCR products for ORF A9.5 and ORF65 genes.

The figure illustrates the PCR products (cDNA) for two AlHV-1 genes used to generate RNA probes utilizing plasmid vector. Image (A) shows the product size (525 bp) of ORF A9.5 while image (B) shows the product size (730 bp) of ORF65. One kilo base pair ladder was used to analyse the genes product size. NC: negative control.

2.8.1.4 Gel extraction for ORF65 product

Gel extraction was performed on ORF65 PCR products as it did not give a clear single band using QIAquick Gel Extraction Kit (Cat# 28704, Qiagen). The protocol used was according to the manufacturer's instructions as the following. The DNA fragment was excised from the agarose gel with a clean, sharp scalpel, weighed in a colourless tube, and 3 volumes Buffer QG were added to 1 volume gel. The tube incubated at 50 °C for 10 min and 10 µl 3 M sodium acetate was added. One volume isopropanol was added to the sampled and mixed. The sample was applied to a QIAquick spin column in 2 ml collection tube and centrifuged for 1 min. The flow-through was discarded. A volume of 500 µl Buffer QG was added to the spin column and centrifuged for 1 min. The flow-through was discarded. A volume of 750 µl Buffer BE was added to QIAquick column and centrifuged for 1 min to wash the DNA. The flow-through was discarded. The QIAquick column was placed in a clean 1.5 ml micro-centrifuge tube. A volume of 30 µl Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the centre of the QIAquick membrane, let stand for 1 min, and then centrifuged for 1 min to elute the DNA.

2.8.2 Plasmid cloning, transformation and bacterial culture

The PCR products for the genes of interest were then cloned into TOPO vector (PCR II TOPO, Invitrogen) utilizing TOPO-TA one shot cloning kit (Invitrogen) according to the following steps: Two µl of fresh PCR product mixed with 1 µl salt solution (1.2 M NaCl, 0.06 M MgCl2) and 2 µl of RNase free water was added then 1 µl of plasmid (10 ng/µl linearized plasmid, 50 glycerol, 50 mM Tris, 1mM DTT, 0,1% 100x triton, 100 µg/ml BSA, phenol red) was added to make a final mixture of 6 µl cloning mix. The mixture was incubated for 5 minutes at room temperature. The mix then placed on ice.

Two µl of the TOPO cloning reaction was added to the chemically competent *E. coli* in a vial (one shot, Invitrogen), mixed gently and then incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42˚C in water bath without shaking and quickly transferred on ice. A 250µl of room temperature supplied SOC medium (Invitrogen) was added to the bacterial vial. The vial was caped tightly and placed horizontally (200 rpm) at 37˚C for 1 hour in a shaker.

Upon incubation, 40 µl of the transformed cells were spread on agar plate with 50 µg/ml kanamycin (Cat# 15160054, Thermo-fisher Scientific) and x gal (Cat# 15520-018, Thermo-fisher Scientific) for white or blue colony screening. The plates were incubated overnight at 37˚C. The following day six white colonies were chosen and

cultured in 5 ml nutrient broth containing 50 µg/ml kanamycin and then incubated for 24 hours at 37˚C in universal tubes in orbital shaker at 200 rpm.

2.8.2.1 Plasmid purification

2.8.2.1.1 Minimum isolation of plasmid DNA (Mini-prep)

The plasmid DNA was isolated using resource plasmid mini kit (cat # SBS27104, Source Bio Science, UK). One to five ml bacterial overnight culture was pelleted by centrifugation at 6000 g for 3 min at room temperature. Pelleted bacterial cells were re-suspended in 250 µl buffer 1 and then transferred to a micro-centrifuge tube. A volume of 250 µl Buffer 2 was added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes clear. A volume of 350 µl Buffer 3 was added, mixed immediately and thoroughly by inverting the tube 4-6 times, and then centrifuged for 10 min at 17,900 g in table-top micro-centrifuge. The supernatant was applied to spin column in a 2ml collection tube, washed by adding 0.5ml Buffer B and centrifuged for 1 min. The flow-through was discarded. The spin column was washed by adding 0.75ml Buffer E and centrifuged for 1 min. The flow-through was discarded. The spin column was further centrifuged for 1 min to remove residual wash buffer. The spin column was placed in a clean 1.5 ml micro-centrifuge tube and 50 µl Elution buffer was added to the

centre of the spin column, let stand for 1 min, and then centrifuged for 1 min to elute the DNA.

2.8.2.1.2 Maximum isolation of plasmid DNA (maxi-prep)

The maximum isolation of plasmid DNA was performed using plasmid maxi kit (Cat# 12362, Qiagen). The 150 ml overnight nutrient broth culture was harvested by centrifuging at 6000 g for 15 min at 4 ^{o}C and the bacterial pellet was completely re-suspended in 10 ml Buffer P1. A volume of 10 ml Buffer P2 was added mixed thoroughly by inverting 4-6 times, and incubated at room temperature for 5 min. During the incuation, the QIAfilter Cartridge cap was screwed onto the outlet nozzle of the QIAfilter Cartridge in a falcon tube. A volume of 10 ml chilled Buffer P3 was added and mixed thoroughly by inverting 4-6 times. The lysate was loaded into the barrel of the QIAfilter Cartridge, incubated at room temperature for 10 min. After removing the cap from the QIAfilter Cartridge outlet nozzle, a plunger was gently inserted into the QIAfilter Cartridge to filter the cell lysate into the falcon tube. A volume of 2.5 ml Buffer ER was added to the filtered lysate, mixed by inverting the tube 10 times, and then incubated on ice for 30 min. A QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT and allowed the column to empty by gravity flow into a falcon tube. The filtered lysate placed on ice was applied to the QIAGEN-tip and allowed to enter the tip. The QIAGEN-tip was washed twice with 30 ml Buffer

QC. The DNA was eluted with 15 ml Buffer QN into a 30 ml endotoxin free tube, precipitated by adding 10.5 ml room-temperature isopropanol, mixed, and then centrifuged at 15,000 g for 30 min at 4 °C. The supernatant was carefully discarded and the DNA pellet was washed with 5 ml of endotoxin-free room-temperature 70% ethanol, and centrifuged at 15,000 g for 30 min at 4 °C. the supernatant was carefully decanted without disturbing the pellet. The DNA pellet was air-dried for 5-10 min and re-dissolved in a suitable volume (100 µl) of endotoxin-free Buffer TE.

2.8.2.1.3 DNA sequencing

The plasmid DNA containing the PCR product inserts of the genes of interest were sent for a sequence check to Source Bioscience (Rochdale, UK) utilizing universal primers (M13 reverse and M13 forward) to sequence sense and anti-sense DNA strands. The data obtained from the sequence analysis were compared with the genes of interest sequence available at

(http://www.ncbi.nlm.nih.gov/nuccore/10140926/) using clustalW2 software programme.

2.8.2.1.4 Restriction digestion for plasmid DNA preparation

In order to prepare DNA template, plasmids were linearized using suitable restriction enzymes generating correct DNA cut. A volume of 8 µg of DNA was mixed with 8 µl restriction enzyme and 8 µl

restriction enzyme buffers (10X). The volume was then completed to 100 µl RNase free water and then incubated for 2 hours at 37˚C. A volume of 2.5 µl of the digested DNA were run on 1% agarose gel as described in section (2.4.2.2) to compare the size of plasmid and gene of interest. The restriction enzymes used were Bam H l (Ref# 10798975001, version 12, Roche) to prepare anti-sense probe and Xho l (Ref# 10899194001, version 22, Roche) to prepare sense probe. Digested DNA was purified utilizing Phenol:Chloroform: Isomyl (25:24:1) (P 3803-100, Sigma) Chloroform:Isomyl alcohol (24:1) (Cat# C0549-1QT, Sigma) and ethanol and then suspended in 50 µl RNase free water and kept in -20˚C freezer.

2.8.2.1.5 In vitro transcription of digoxygenin (dig)-labelled RNA probes

In order to produce digoxigenin-labelled RNA probes, the linearized DNA template was used for *in vitro* riboprobe synthesis utilizing Dig RNA labelling kit (Cat # 11175025910, Roche, USA) in line with the manufacturer's instructions. An amount of 1 µg of purified template DNA was added into Eppendorf tube and 2 µl of 10X NTP labelling mix, 2 µl of 10X transcription buffer, 1 µl of protector RNAse inhibitor, 2 µl RNA polymerase SP6 to generate sense probe or 2 µl RNA polymerase T7 to produce anti-sense probe and then the volume was made up to 13 µl with RNase free water. The mix was centrifuged briefly and then incubated for 2 hours at 37˚C. a

volume of 2 µl DNase I were added and incubated for 15 minutes at 37°C to degrade the template DNA. The reaction was then stopped by adding 2 µl of 0.2 M EDTA pH 8. A volume of 1 µl of 1 mg/ml carrier yeast tRNA was added to the DNase digested probes, precipitated by ethanol and then re-suspended in 50 µl RNase free water.

2.8.2.1.6 Dot blot analysis of generated probes

In order to check the excellence of the generated probes, serial dilutions of sense, antisense and T7 control were prepared in 10 µg/ml yeast tRNA (cat # VYAM7119, Fisher Scientific, UK) and tested by dot blot hybridization. A volume of 5 µl of each dilution was spotted on Amersham's nylon membrane (Amersham Pharmacia biotech, Hybond-N+, England) from 10^{-5} to 10^{-1} in a petri dish. Upon drying, the dish containing the membrane was placed in UV croslinker (stratalinker) to fix the probes. The membrane was then placed on a bigger dish on a shaker and washed in 100 ml washing buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5, 0.3 % v/v Tween 20) for 2 minutes. The membrane was soaked in 100 ml blocking solution (Dig-wash and block, cat # 11585762001, version 10, Roche) (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) on a shaker for 30 minutes. After that, the membrane was incubated with 20 ml labelled anti-DIG antibody solution (anti-Digoxigenin-AP FAb fragments, cat# 11093274910, Roche) diluted 1:5000 in blocking

solution) for 30 minutes. The membrane was then washed twice with 100 ml washing buffer for 15 minutes and equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 3 minutes. The colour substrate was prepared by dissolving one tablet Sigma fast BCIP/NBT (Cat# B5655-25TAB, Sigma) in 10 ml RNase free water. The dish was then placed in the dark until the dot blot coulure develop. The reaction was then stopped by adding 50 ml TE buffer for 5 minutes.
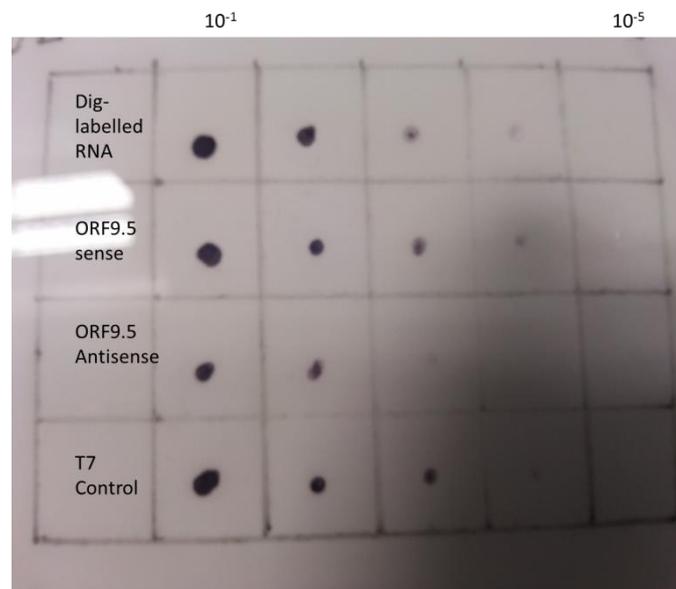
Figure 8 dot blot analysis of AlHV-1 ORF A9.5 RNA probe

The figure shows the $\log_{10}$ dilutions of sense, antisense, dig-labelled RNA and T7 control which were spotted on Amersham's nylon membrane from 10^{-1} to 10^{-5} concentrations. The membrane was washed and blocked using dig-wash and block solutions to develop the dot blot colour.

2.8.3 Deparaffinisation, proteolysis, and acetylation of tissue sections

Deparaffinisation was performed by placing tissue sections in xylene (Cat# X/0200/17, Fisher Scientific) twice in staining dishes for 5 minutes. The sections were then rehydrated by dipping twice in 100% ethanol for 5 minutes and 96% and 70% once for 5 minutes. The slides were dipped in DEPC treated water (diethyl pyrocarbonate, cat# E174-25G, Amresco) in staining dish for 5 minutes. The slides were then transferred to coplin jar and washed with 1XPBS for 5 minutes.

To achieve proteolysis, slides were incubated in 0.2 M HCl at room temperature for 30 minutes. Slides were then incubated twice 2X sodium saline citrate with 5 mM EDTA at 50ºC for 30 minutes, trailed by digestion in proteinase K solution ({proteinase K, recombinant PCR grade, cat# 03115836001, Roche}, with 1 M TRIS pH 8 and 0.1 M CaCl2 made up to 60 ml DEPC treated water) for 15 minutes at 37ºC. Fixation steps were performed in room temperature through incubation in 0.2% glycine-PBS for 5 minutes, followed by washing in 4% paraformaldehyde for 4 minutes and then washing in 1XPBS for 2 minutes and 15 minutes in 5mM MgCl2 in 1XPBS.

Acetylation was done through incubation with 0.25% acetanhydrid (Cat# 45830-250ML-F, Sigma) with 0.1 M triethanolamine (Cat# 1.08379.0250, VWR International Ltd) pH 7.5, followed by washing

in 1XPBS twice for one minute and once for 15 minutes in room temperature.

2.8.4 Hybridisation of probes

Pre-hybridisation, hybridisation, and post hybridisation wash:

The slides were prepared for hybridisation by pre-warming in pre-hybridisation buffer (0.1 mg/ml salmon sperm DNA [Sigma] and 0.25 mg/ml yeast tRNA in a pre-hybridisation stock mix of 30% v/v 20X SSC, 45% v/v 100% deionised formamide [cat# 11FORMD002, MP Biomedicals, LLC], 10% v/v 50x Denhardt's solution [Invitrogen] with 14% DEPC-treated dH20) in coplin jar at 52°C for 1 hour.

To perform the hybridisation step, slides were transferred to metal tray and covered with 30-40 µl/ tissue section hybridisation buffer consisting of 250µg/ml tRNA, 10mg/ml salmon sperm DNA, 80 µl dextransulfate, 2-6 µl RNA probe (1:100) dilution added to 700 µl hybridisation mix 100% deionised formamide, 20X of 0.5 M EDTA pH 8, 0.5 M piperazin-N,N'bis 2-ethanesulfate-acid-PIPES pH 7.0 (cat# P3768-25G, Sigma), 5 M NaCl, 50X Denhardt's solution, heparin 20,000 U (Cat# H 3393-25 KU, Sigma), and 10% Triton X-100. Slides were covered with hydrophobic surface of gel-bond film (Cat# LZ53734, Scientific Laboratory Supplies LTD, Uk), sealed with Fix-O-gum gel (Marabu, Tamm, Germany) and incubated at 52°C in a moist box overnight.

The following day gel bond was carefully removed and slides placed again in coplin jar for post hybridisation wash. Washes were performed twice in 6XSSC with 45% formamide (Cat# 1.09684.1000, VWR International LTD) at 42°C for 15 minutes, followed by two washes in 2XSSC for 5 minutes at room temperature and twice in 0.2XSSC at 50°C for 15 minutes.

2.8.5 Anti-DIG labelling for probe detection

Tissue sections were washed with buffer 1 (TRIS 100mM, NaCl 100mM, pH 7.5) for one minute at room temperature. Slides were then washed with blocking solution (sterile neutral sheep serum {NSS, cat# S2263-100ML, Signa}, 10% Triton X-100) for 30 minutes at room temperature. Slides were then placed on metal tray, carefully dried, and circled by water repellent pen (PAP-PEN). Antibody solution 400 µl/slide (30 µl AP-conjugated {Anti-DIG-AK-AP 1:200}, 42 µl NSS, 188 µl 10% Triton X-100, 6 ml buffer1) were added on top of the tissue sections for 2 hours at room temperature. Slides were then transferred to coplin jar and washed with buffer 1 twice for 15 minutes at room temperature. Tissue sections were further washed with buffer 3 (TRIS 100mM, NaCl 100mM, pH 9.5, $MgCl_2+6H_2O$) for two minutes at room temperature. Ultimately, slides were incubated in the dark with staining solution (three tablets of BCIP/NBT, 30 mg levamisole (Cleaveland et al. L9756-5G, Sigma), 60 ml buffer 3) for long time

up to 1 week. Slides were periodically inspected under light microscope for signal development (dark blue colour in cell cytoplasm). The reaction was stopped by incubating slides with buffer 4 (TRIS 10mM and EDTA 1mM, pH 8) in the dark for 10 minutes. Slides were kept in duple distilled water for 5 minutes. Counter staining was done by dipping slides in haematoxylin (Ref# HHS32-1L, Sigma Aldrich) for 10 seconds. After that, slides were blueing in tap water for 5 minutes. Slides were then dried and covered with cover slip using 1, 3-diethyl-8-phenlxanthine (DPX) (Fluka analytical, Sigma Aldrich, UK) and then stored in the dark.

2.9 RNA quality measurement using Agilent Bio-analyser Nano 6000 kit

Agilent Nano chips (Agilent Technologies, Stevens Creek Blvd, USA) comprise a set of micro channels used to separate nucleic acid fragments based on base sizes as they are driven through it electrophoretically. RNase ZAP (Cat# AM9780, ThermoFisher Scientific) which removes RNases and reduce nuclease contamination in RNA purification laboratories and RNase free water were used to decontaminate the electrodes by washing for 1 minute and 10 seconds respectively. All the reagents and samples used in the technique were equilibrated at room temperature for 30 minutes. A volume of 550 µl of Agilent 6000 Nano gel matrix (red colour tube in the kit) was placed in a spin filter and centrifuged for 10 minutes at 1500 g. A volume of 65 µl filtered gel was aliquoted in RNase free 0.5ml micro-centrifuge tubes. For single use, the gel dye mix was prepared by adding 1 µl of RNA 6000 Nano dye (blue colour) to 65 µl aliquot of pre-prepared filtered gel. The tube was mixed thoroughly by vortex and then centrifuged for 10 minutes at room temperature at 13000 g. The gel dye mix was loaded into the RNA Nano chip by pipetting 9 µl of the mix at the bottom of the wells marked**G** (Figure 9) after the chip was being placed on the chip priming station. The plunger was positioned at 1 ml position and the chip priming station was closed for 30 seconds. The plunger of the syringe was held down until it was held by the clip. After 30

seconds the plunger was released by a clip release mechanism. The chip priming station was opened and 9 µl of the gel dye mix was pipetted in the wells marked **G**(Figure 9). 5 µl of RNA 6000 Nano marker (green colour) was pipetted into the 12 wells and the wells marked ⚡ (Figure 9). The RNA samples and the ladder were heat denatured at 70ºC for 2 minutes to minimise secondary structure. 1 µl of the ladder was pipetted to the wells marked ⚡ (Figure 9) and 1 µl of each sample into each of the 12 sample wells. The chip was horizontally placed in the adapter of the available vortex mixer and vortexed for 1 minute at 2000 rpm. The chip was inserted in the Agilent 2100 Bioanalyzer and read. Figure 9 shows the Agilent 6000 Nano chip shape and its various loading wells.
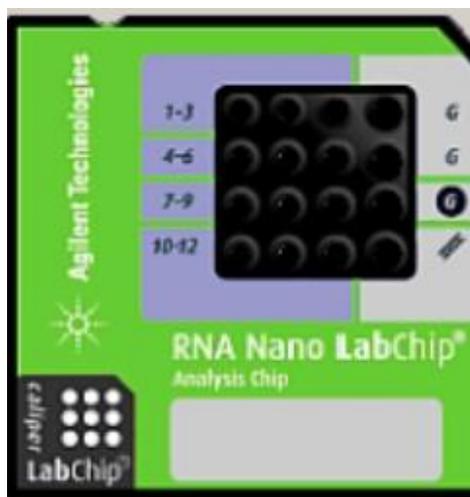
Figure 9 shows the Agilent 6000 Nano chip appearance and its different loading wells

2.10 RNA sequencing (RNA-Seq) methods

RNA-seq is also known as whole transcriptome shotgun sequencing (WTSS) and utilizes next-generation sequencing (NGS) to detect and quantify RNA molecule by way of DNA copies in a given biological sample (Morin et al., 2008, Wang et al., 2009). The use of this technique has enabled scientists to analyse cellular transcriptomes and simplified looking at alternative gene spliced transcripts, changes in gene expression, single nucleotide polymorphism (SNP), posttranscriptional modifications and the ability to look at RNA populations including mRNA, micro-RNA (miRNA), small RNA, total RNA, tRNA and ribosomal RNA (Ingolia et al., 2012, Maher et al., 2009). There are many approaches by which RNA sequencing can be achieved.

2.10.1 RNA poly (A) library

In this approach the polyadenylated (poly (A)) tail is targeted to guarantee that coding RNA is split from non-coding RNA. This can be done by using poly (T) magnetic beads (Wang et al., 2009). Because ribosomal RNA (rRNA) represent up to 80% of total RNA in a given cell resulting in dilution of mRNA species, tRNA is removed by either enzymatic degradation or hybridisation-based depletion approaches (Chen and Duan, 2011, Yi et al., 2011). Prior to cDNA synthesis, RNA samples are fragmented by either chemical hydrolysis or enzymatic digestion to approximately 200-250

nucleotide (nt) long (Mortazavi et al., 2008). After that, complementary DNA (cDNA) is synthesized by reverse transcriptase and oligonucleotides, used as adapters, are utilized to ligate to cDNA to assist amplification and allowing sequencing (Chu and Corey, 2012).

2.11 Illumina machine NextSeq500 sequencing platform work flow

The work flow of the Illumina sequencing machine comprises four main steps; sample preparation, cluster generation, sequencing and data analysis. In the first step, the sample of interest (total RNA) is subjected to a series of stages to fragment the RNA molecule, converted into cDNA and then amplified to produce cDNA library. This library is then placed in an eight-lane flow cell in which the cDNA fragments are attached to oligonucleotide primers complementary through its adapters. The cDNA fragments then undergo a series of amplification by which clusters of double stranded DNA are produced. The flow cell is placed in the sequencing machine where each cluster is sequenced and at each cycle four-fluorescence labelled nucleotides are added and the emitted signals are recorded. The intensity of the fluorescence is translated into base-calls. The cycle numbers show the length of the reads whereas clusters numbers define the number of reads (Bullard et al., 2010).

2.12 Bioinformatics tools

The activities that can be covered by bioinformatics include sequence alignment, prediction of gene expression, mapping and analysing DNA and protein sequencing and aligning DNA. All of these activities can be achieved by various bioinformatics tools (Luscombe et al., 2001).

2.12.1 Blast like alignment tool (BLAT)

BLAT is a sequence alignment tool introduced by Jim Kent in 2002 to facilitate the assembly and annotation of the human genome. BLAT was ~500 times faster with performing mRNA/DNA alignment and ~50 times faster with protein/protein alignments than any other alignment tools. BLAT can be used for aligning multiple RNA sequences onto a DNA assembly to deduce their genomic matches, aligning of a protein or mRNA sequence from one species onto a sequence data base from another species and identification of gene family members of certain gene query. BLAT and BLAST have many things in common however they vary in some aspects. While BLAT scans the query sequence for matches, BLAST searches the data base for matches. BLAT is less sensitive than BLAST. BLAT involves query sequence in FASTA format, whereas BLAST requires the query in accession number and FASTA-formatted sequence. BLAT can be utilized as server-client programme or as stand-alone programme (Kent, 2002).

2.12.2 Scythe-a Bayesian adapter trimmer

Scythe utilizes a Bayesian method categorising contaminant substrings in sequence reads. The software has the ability to remove the 3' –end adapters which contain low quality bases. The process of a poor-quality trimmer is required before mapping, analysis and assemblies for the next generation sequencing run to pick out low quality bases. The mechanism of scythe is based on two probabilities i.e. the likelihood of identifying the matches in a given contaminant or not given contaminant. Assuming that the read is contaminated, the likelihood of seeing the specific number of matches and mismatches is reliant on how good the quality of the sequence is. In the later model, the likelihood of seeing the specific number of matches and mismatches is an accidental. Scythe does not function on 5'-end contaminant or any other contaminants (Lindgreen, 2012).

2.12.3 Sickle- a windowed adaptive trimming tool for FASTQ files using quality

The majority of contemporary sequencing technologies generate reads that have relapsing quality towards the 3'-end and some towards the 5'-end which in turn can affect mapping, assembles, and data analysis. Sickle is a bioinformatics tool that utilizes sliding windows along with quality and length thresholds in order to define when quality is appropriately low to remove the 3'-end of reads and

also determine when the quality is adequately high to trim the 5'-end of the reads. Sickle also rejects reads based upon the length threshold. Sickle slides a window across quality values whose length is 0.1 times the length of the reads. Sickle backs up three sorts of quality standards i.e. Illumina, Solexa and Sanger. Sickle comprises two techniques to work with both paired-end reads and single-end reads sickle se and sickle pe (Joshi and Fass., 2011)

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2.12.4 HISAT

The first step in RNA-Seq downstream data analysis is to align the reads against a reference genome. The use of pre-existing and vastly utilised alignment programmes such as TopHat2 and GSNAP is time consuming. In order to overcome this deficit, HISAT, a spliced aligner tool, was produced with indexing approach based on the Burrows-Wheeler transform and the FM index. The programme algorithms occupy two various sorts of indexes: a global FM index that denotes the whole genome and several small FM indexes that collectively cover the entire genome. Each index exemplifies ~ 64,000 bp and ~ 48,000 bp are required to cover the human genome. These small indexes (local indexes) are stored in small set of files and other optimisations are applied to reduce the memory requirements. This has allowed the human genome to be indexed in almost 4 GB of space. The advantage of using these small indexes in combination with numerous alignment approaches is in allowing

effective alignment of RNA-seq reads, especially, reads spanning multiple exons. HISAT is a fast, accurate and sensitive alignment programme for mapping RNA-seq reads (Kim et al., 2015).

2.12.5 Sequence alignment map (SAM) tool

The sequence alignment map is a TAB-delimited text format comprising a header section which is optional and an alignment section. The header line begins with'@' whereas alignment lines do not. Each alignment line contains 11 compulsory fields for important alignment information like mapping location and changeable figure for optional fields. SAM is a universal format for saving sequence nucleotide alignments and easy to work with. SAM's file size is compact and permits the file to be indexed by genomic location to professionally recover all reads aligning to a locus. SAM permits most of the processes on the alignment to function on a stream without loading the whole genome into a memory. SAM tools deliver different advantages for handling alignments in the SAM format comprising merging, sorting and generating alignments in pre-located format (Li et al., 2009).

2.12.6 String Tie assembler tool

The approaches utilised to sequence the transcriptome such as RNA-seq generate more than 200 million short sequences. Each of these short reads is 100-150 bp. Due to the fact that various transcripts

have highly variable sequence coverage and alternative transcripts from the same locus can share exons, this has made assembling the short reads a hard task. String Tie is a computational approach enforcing a network flow algorithm initially designed in optimisation theory along with optional de novo assembly to assemble a complex data into transcripts. In comparison with other superior transcript assembly packages such as Cufflings, Isolasso, scripture and Traph, String tie generates comprehensive and precise reconstructions of genes and better evaluates the expression level. The software is faster on all data sets than other assembly softwares (Pertea et al., 2015).

2.12.7 Ballgown

Ballgown is a software package to assist the analysis of RNA-Seq data. It supplies assignments to organise, visualize and analyse the expression quantities for transcriptome assembly. There are a number of pre-processing stages required prior to the use of ballgown R package i.e. the RNA-Seq reads should be aligned to a reference genome, a transcriptome should be assembled and expression for the traits (transcript, intron and exon junctions) ought to be evaluated in ballgown readable format. There are two sample pipelines for pre-processing and they are pipeline one (tophat2 + stringtie) and pipeline two (tophat2 + cufflinks + table maker). Certain files should be produced by stringtie and

tablemaker for ballgown to load and some of these are e_data.ctab, i_data.ctab and t_data.ctab. Data is then loaded utilizing ballgown function. The soft contain six slots to access the assembly data: structure, expr, indexes, dirs, mergedDate, and meas. Ballgown delivers broad bundles of simple, fast statistical approaches such as limma and stattest for examining whether transcripts are differentially expressed between experimental conditions or across a continuous covariate (such as time) (Frazee et al., 2014).
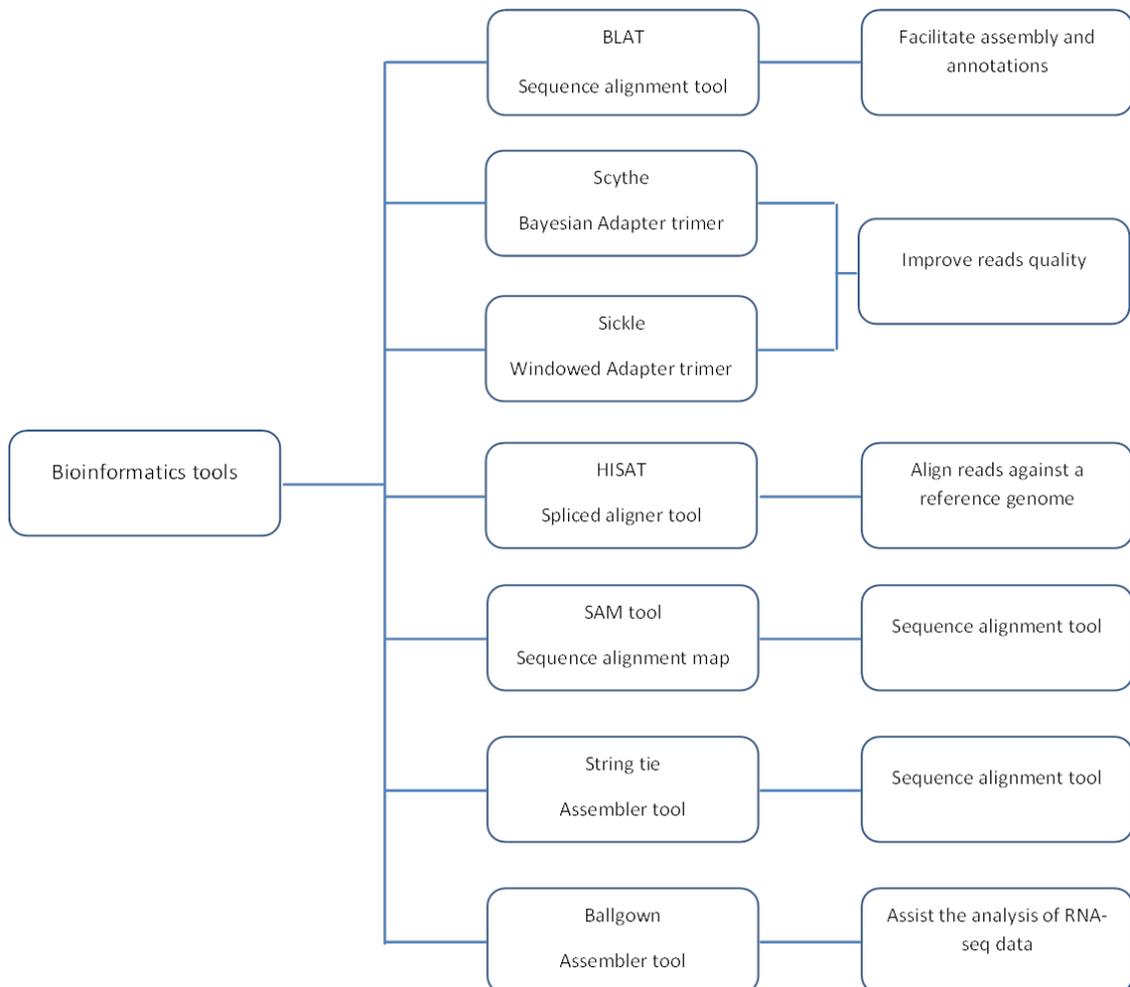
2.13 Statistical analysis

Deferential statistical analysis was performed by ball gown (Frazee et al., 2014) and the experiment replicate was in duplicate (N=2). The gene expression analysis was done with q PCR LightCycler 480 software and Microsoft Office Excel 2010 applying Pfaffl method (Pfaffl., 2004). LGL viral load was calculated using LightCycler 480 software the experiment replicate was done in duplicate (N=2) and two-way ANOVA used to analyse the data followed Sidak's multiple comparisons test.

Chapter 3

AlHV-1 infection of epithelial and endothelial cell lines

3.1 Abstract

Malignant catarrhal fever is characterised by accumulation of lymphocytes in non-lymphoid tissues, vasculitis and epithelial damage. The pathogenesis of MCF is not well understood. It could be due to the cytotoxic activity of infiltrated T cells or direct infection and destruction of tissues (particularly epithelium or endothelium) or a mixture of both. It is also not clear whether infection of these cell types establishes latent or productive virus life cycle or both. To address this, rabbit epithelium and bovine endothelium cell lines were used to attempt infection and measure virus life cycle gene transcripts. Bovine turbinate fibroblasts were included as a positive control for productive virus infection. The cells were infected with low pass AlHV-1 (fully virulent virus) and high pass AlHV-1 C500 strain (attenuated with gene deletions and translocations, Handley et al., 1995, Wright et al., 2003). Polymerase chain reaction and q PCR were used to detect the viral DNA and measure the viral gene transcript profiles in the infected cells. It was found that the virus infected the epithelial and endothelial cells (as measured by viral DNA). There was, however, no CPE and very low level gene expression such that it can only be concluded that there was no or limited productive infection in the infected epithelium and endothelium *in vitro* compared to a productive transcript

profile seen in the control BT Cells, or alternatively, that the cells were latently-infected. Importantly though, when the virus extracted from the BAE and SIRC cells was used to infect BT cells, a productive infection (with CPE) was recorded. This indicated that virus from the BAE and SIRC cells was infective and intact (expressed genes).

3.2 Introduction

Malignant catarrhal fever is characterized by infiltration of lymphocytes in non-lymphoid tissues, lymphoid hyperplasia vasculitis and epithelial and endothelial degeneration (Buxton et al., 1984, Jacoby et al., 1988, Liggitt and DeMartini, 1980a, Liggitt and DeMartini, 1980b, Simon et al., 2003). The severity of the lesions in particular in the epithelium of the oral cavity, gastrointestinal tract, urinary bladder, liver, biliary ducts, and kidneys as well as damage to the endothelium of arteries and veins is associated with an increase in T lymphocyte (mainly CD8$^+$ T cells) infiltration a proportion of which are infected with virus in these tissues (Dewals et al., 2008, Dewals et al., 2011, Palmeira et al., 2013). Furthermore, this damage becomes more pronounced towards the end of the disease course (Liggitt and DeMartini, 1980a, Liggitt and DeMartini, 1980b). In addition, the paucity of viral transcripts supporting productive virus cycle as well as for the latent cycle (where this is expected possibly) in the tissues of animals infected with AlHV-1 has been reported (Dewals et al., 2008, Dewals et al., 2011) leading to the hypothesis that MCF is a disease of latency. However, there is evidence of productive virus cycle transcripts in infected cells and *ex vivo* LGLs as well as antibody responses to envelope and capsid proteins that would indicate productive virus infection. The mechanism of epithelium and endothelium damage is not proved. It could be due to the cytotoxic activity of the

accumulating infected T cells in the tissues or may be due to direct infection of these cells and destruction of them (cytopathic effect) or possibly a combination of both mechanisms. The aim of this study is to examine whether AlHV-1 (low culture passage, virulent C500 AlHV-1 or high pass attenuated virus) can infect epithelial and endothelial cells and cause cell damage, and also to investigate whether the virus is able to establish a productive/lytic infection or latent one in the infected cells. BT cells were used as controls where productive virus infection occurs.

3.3 Materials and methods

3.3.1 Virus

Low culture pass (virulent) AlHV-1 C500 strain and high pass attenuated C500 AlHV-1 were obtained from inoculated bovine turbinate fibroblasts (BT cells) and titrated as described in M&M chapter 2 (section 2.2.5). Titres obtained were ~ 10^3 TCID$_{50}$ per ml for low pass and ~10^6 TCID$_{50}$ per ml for high pass virus. The cells were infected with the virus (see virus dose below) when they become 80% confluent (confluency is the percentage of the cells covering the vessel). This is carried out by discarding the old media, washing the cells with 1x PBS and then inoculating the virus directly onto the cell surface with serum free media until the surface is covered.

3.3.2 Virus dose

Statens seruminstitut rabbit cornea (SIRC) epithelial cells, BT fibroblasts and bovine aortic endothelium (BAE) cells were cultured as described in Chapter 2 (section 2.2). The cells were infected with high pass AlHV-1 virus C500 strain at a dose of 5x 10^4 TCID$_{50}$ per 50 µl inoculum for SIRC and BAE and 5x10^3 TCID$_{50}$ per 5 µl for BT cells. For low pass virus 2x10^3 TCID$_{50}$ per 2 ml inoculum was used to infect both SIRC and BAE cells and 1x10^3 TCID$_{50}$ per 1 ml inoculum for BT cells.

3.4 In vitro infection

3.4.1 Direct virus infection

Bovine turbinate fibroblast (BT) cells, SIRC, and BAE were cultured in two types of media IMDM or DMEM as described in chapter two (2.2). The cells were infected as described (3.3.1) with either low pass or high pass AlHV-1 virus C500 strain with the doses described above and DNA was extracted from the cells on 2, 5, and 10 days after infection. This was to determine the presence or absence of the virus as viral DNA detected by PCR. BT cells at 50% CPE were harvested as a productive virus control (around day 5 post infection).

In order to determine whether the BAE and SIRC cells infected as described above contained infective virus, the cells and supernatant were harvested 7 days after infection and cell sonicate (to release cell-associated virus) and the supernatant were then added to uninfected cultures of BAE, SIRC and BT cells, which were analysed at different time points afterwards for the presence of virus DNA. For the harvest of the indirect (or secondary) infected cells, medium was collected from centrifuged cells placed into falcon tubes on ice. The cell pellet was re-suspended in medium after being centrifuged at 300xg for 10 minutes. The supernatant was collected and added to the collected supernatant placed on ice. The cell pellets were re-suspended in medium, freeze/thawed once in liquid nitrogen, sonicated in water bath sonicator and then spun down at 300xg for

10 minutes. This supernatant was added to the already collected medium and supernatant to make up viral product from the cells which was used to infect healthy SIRC and BAE cells at 80% confluence in 75cm^3. The flasks were placed in a 37 C^o 5% CO2 incubator for seven days. During this time cells were monitored for CPE development under the microscope. Finally, (day 7), cells were harvested and DNA and RNA extracted for further analysis of viral DNA and viral gene transcripts (as shown in Table 7) by PCR and Q RT PCR (as described in chapter 2 sections 2.3.1 and 2.3.2).

Table 7 Viral gene transcripts representing either productive or latent cycle or involved in the virus life cycle.

AlHV-1 genes (ORF)	Putative function/homology to productive or lytic cycle herpesvirus genes.
ORF50	Encodes RTA the transcriptional and replication activator responsible for the switch to lytic cycle.
ORF25	Major capsid protein expressed during lytic cycle.
ORF63	Encodes structural protein during lytic cycle.
ORF73	Encodes LANA protein involved in latent infection.
A4.5	Similar to Bcl-2 family of apoptosis. Virus cycle expression not known.
A9.5	Encodes a secreted glycoprotein that is similar to IL-4. Virus cycle expression not known.

3.5 Results

3.5.1 Infection of the epithelial and endothelial cell lines

Three cell lines, BAE bovine endothelium, SIRC rabbit epithelium, and control BT bovine fibroblasts were infected with low and high pass AlHV-1 C500 strains. Bovine turbinate (BT) fibroblast cells showed a cytopathic effect (CPE) when infected with either low or high pass AlHV-1 virus five to six days after inoculation, indicating that both virus preparations contained infective virus capable of replicating and generating a CPE as part of the productive/lytic virus life cycle (Figure 10).
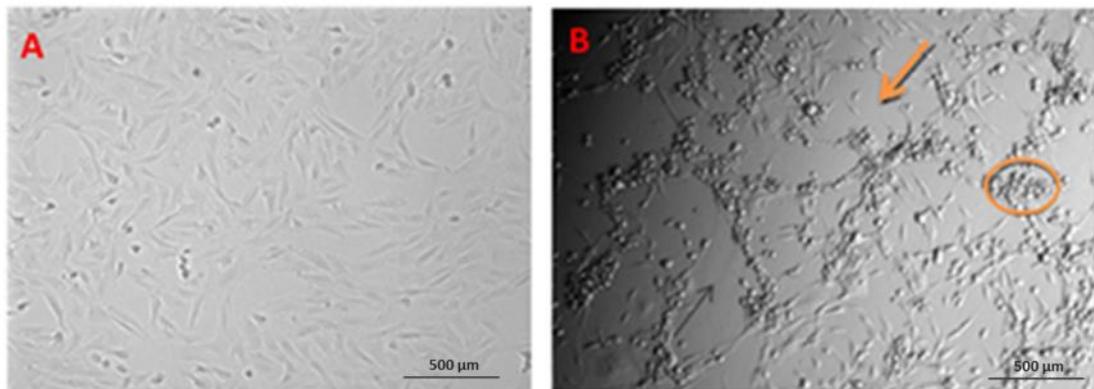
Figure 10 infection of bovine turbinate fibroblast (BT) cells
A: healthy BT cells used as a control, image B: infected BT cells with low pass AlHV-1 C500 strain. The arrow indicates the absence of monolayer and the circle shows the accumulation of dying or dead cells and debris. The pictures represent scale bar of 500 µm.

3.5.2 BAE endothelial cells

Infection of BAE cells directly or after secondary transfer of infected cell sonicates and supernatants to uninfected BAE cells did not show any signs of CPE and the cells remained intact up to ten days post

121

infection (Figure 11). Conversely, BT cells infected with cell sonicates and supernatant from directly infected BAE cells displayed CPE around day 5 post infection (Figure 12). This indicates that BAE cells were successfully infected and generated virus able to induce CPE in BT reporter cells.
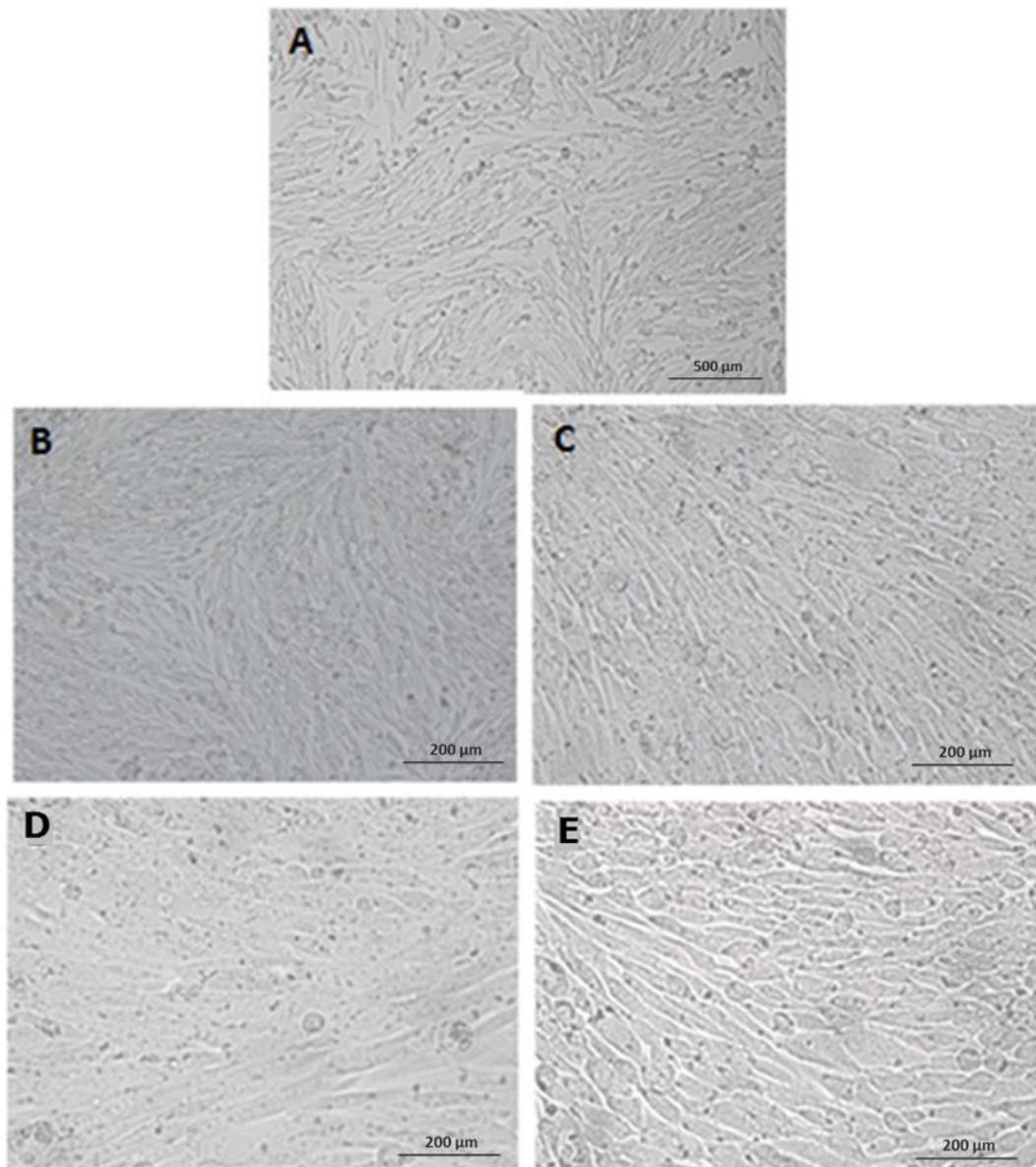
Figure 11 Infection of bovine aortic endothelial (BAE) cells
A: control uninfected BAE cells, B and C: BAE cells infected directly with low pass or high pass AlHV-1 C500 respectively (day 10 of culture), Serial infection, D and E: BAE infected with sonicate and supernatant material from directly-infected BAE

cells. No CPE seen in the cells up to ten days post infection but cells look unhealthy at this point. Picture A represents scale bar of 500 µm whereas pictures B, C, D and E represent scale bar of 200 µm.
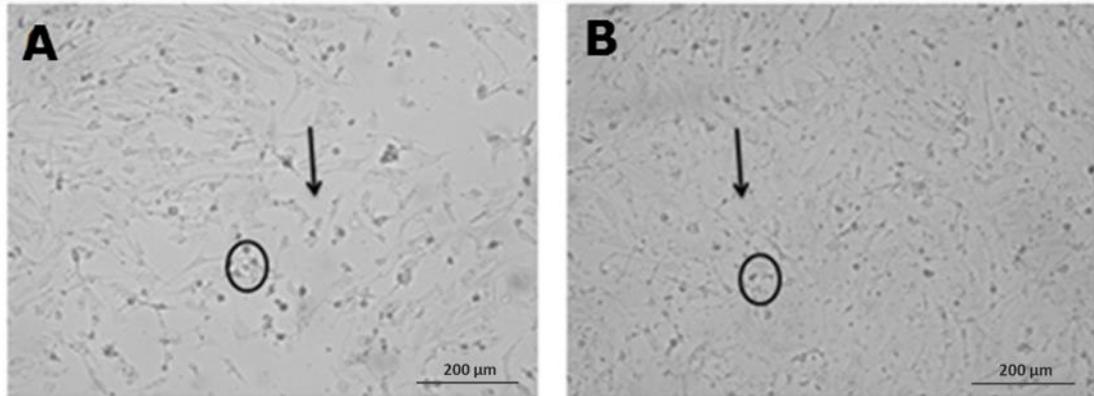
Figure 12 Infection of bovine turbinate BT cells with infected BAE
Serial infection, A and B: BT infected with directly-infected BAE and secondary BAE infected with cell sonicate and supernatant materials from directly infected cells, showing CPE of the BT cells. Arrows indicate absence of monolayers whereas circles indicate accumulation of cell debris. Pictures represent scale bar of 200 µm.

3.5.3 SIRC epithelial cells

There was no evidence of CPE in SIRC cells infected either directly or in a serial/ secondary infection using cell sonicates and supernatant, up to 10 days after infection (Figure 13). However, BT cells infected with cell sonicates and supernatant from directly-infected SIRC cells displayed CPE within a few days post inoculation (Figure 14), indicating that SIRC cells had supported infection and generated infective virus capable of inducing CPE in BT reporter cells.
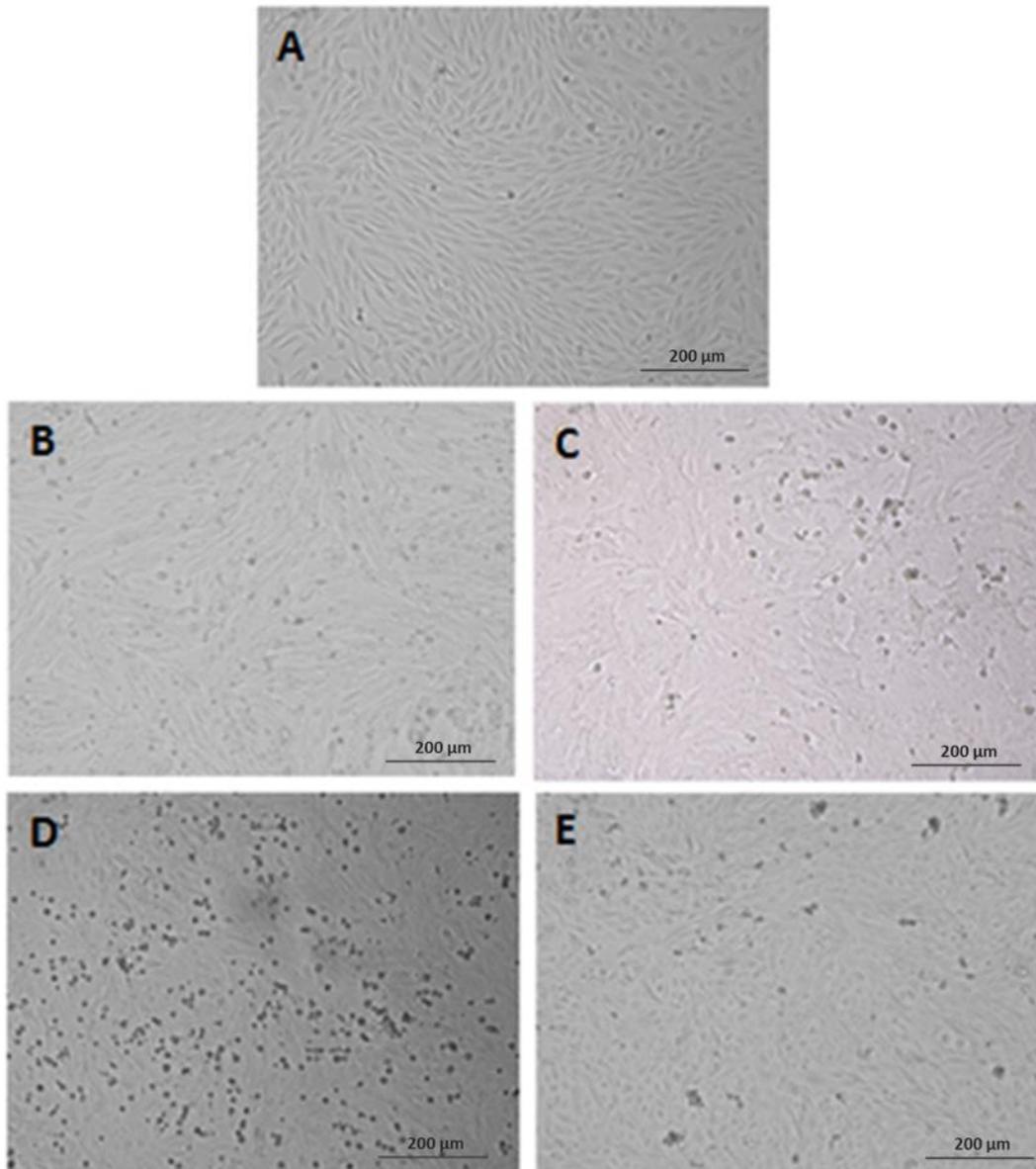
Figure 13 Infection of SIRC epithelial cells
A: SIRC uninfected control. B and C: SIRC cells infected directly with low or high culture pass AlHV-1 C500 strain respectively. D and E: Serial infection: SIRC cells infected with cell sonicates and supernatant derived from directly-infected SIRC cells. No CPE was seen in the cells up to ten days post infection but cells look unhealthy. Pictures represent scale bar of 200 μm.
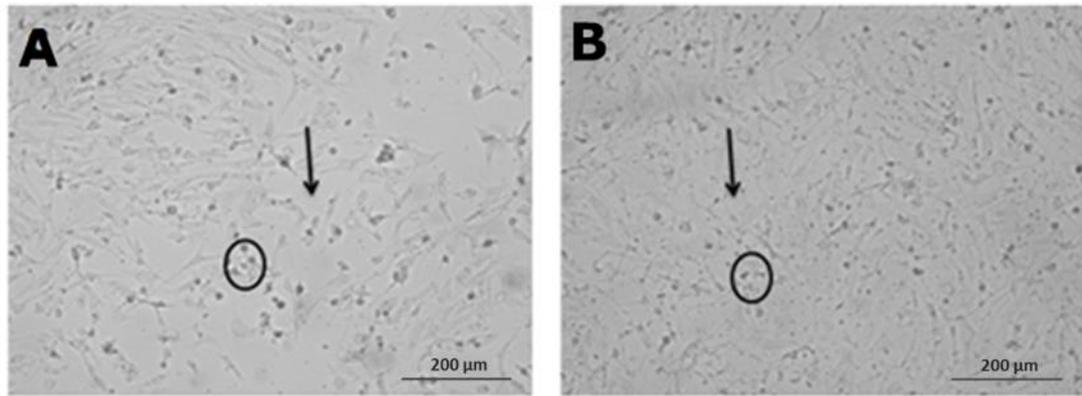
Figure 14 Infection of bovine turbinate BT cells with infected SIRC
A and B, BT cells show CPE after infection with cell sonicates and supernatant derived from directly-infected SIRC and with SIRC infected with cell sonicate and supernatant materials from directly infected cells. Arrows indicate absence of monolayers and circles show accumulation of dying/dead cell and debris. Pictures represent scale bar of 200 μm.

3.5.4 Detection of viral DNA in the infected cells

Viral DNA was detected in SIRC, BAE and BT cells infected with either low pass AlHV-1 or high pass AlHV-1 at different time points (Figure 15 and 16) after infection. Viral DNA was not identified in non- infected cells (Figure 17).
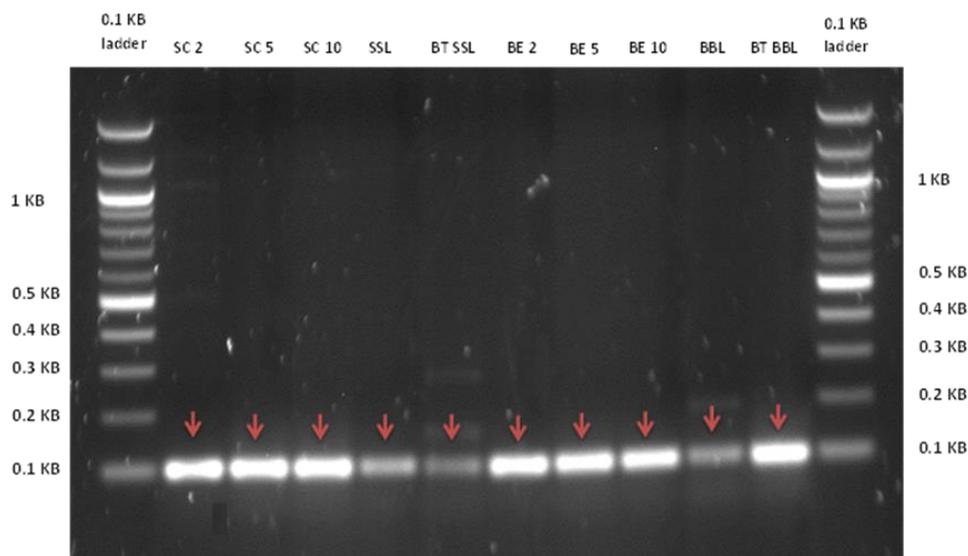
Figure 15 qPCR products for low-pass AlHV-1 ORF3 gene visualised on agarose gel.

SC2; DNA extracted from SIRC cells 2 days post infection with AlHV-1 low pass virus, SC5; DNA extracted from SIRC cells 5 days post infection with AlHV-1 low pass virus, SC10; DNA extracted from SIRC cells 10 days post infection with AlHV-1 low pass virus, SSL; Serial infection: DNA extracted from SIRC cells infected with whole cell sonicate plus supernatant collected from directly-infected SIRC cells infected (AlHV-1 low pass virus), BT SSL; serial infection: DNA extracted from BT cells infected with whole cell sonicate plus supernatant collected from directly–infected SIRC cells (AlHV-1 low pass virus), BE2; DNA extracted from BAE cells 2 days after infection with AlHV-1 low pass virus, BE5; DNA extracted from BAE cells 5 days after infection with AlHV-1 low pass virus, BE 10; DNA extracted from BAE cells 10 days after infection with AlHV-1 low pass virus, BBL; serial infection: DNA extracted from BAE cells infected with whole cell sonicate plus supernatant collected from directly–infected BAE cells (AlHV-1 low pass virus). BT BBL; DNA extracted from bovine turbinate cells infected with whole cell lysate collected from BAE cells infected with whole cell sonicate plus supernatant collected from directly–infected BAE cells (AlHV-1 low pass virus), 0.1KB ladder; 100 base pair ladder, red arrows; indicates PCR product.
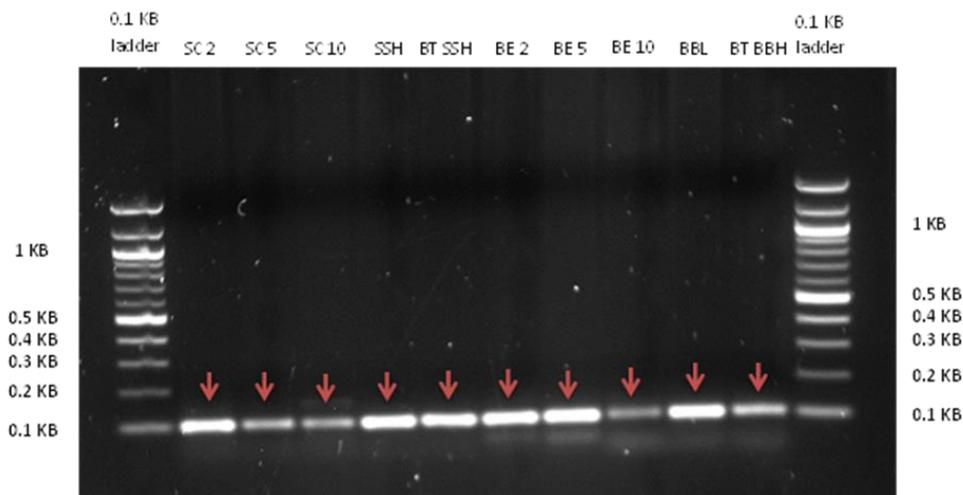
Figure 16 qPCR products for high-pass AlHV-1 ORF3 gene visualised on agarose gel

SC2; DNA extracted from SIRC cells 2 days post infection with AlHV-1 high pass virus, SC5; DNA extracted from SIRC cells 5 days post infection with AlHV-1 high pass virus, SC10; DNA extracted from SIRC cells 10 days post infection with AlHV-1 high pass virus, SSL; serial infection: DNA extracted from SIRC cells infected with whole cell sonicate plus supernatant collected from directly–infected SIRC cells (AlHV-1 high pass virus), BT SSL; serial infection: DNA extracted from BT cells infected with whole cell sonicate plus supernatant collected from directly–infected SIRC cells (AlHV-1 high pass virus), BE2; DNA extracted from BAE cells 2 days post infection with AlHV-1 high pass virus, BE5; DNA extracted from BAE cells 5 days post infection with AlHV-1 high pass virus, BE 10; DNA extracted from BAE cells 10 days post infection with AlHV-1 high pass virus, BBL; serial infection: DNA extracted from BAE cells infected with whole cell sonicate and supernatant collected from directly-infected BAE cells (AlHV-1 high pass virus), BT BBL; serial infection: DNA extracted from BT cells infected with whole cell sonicate and supernatant collected from directly-infected BAE cells (AlHV-1 high pass virus), 0.1KB ladder; 100 base pair ladder, red arrows; indicate the PCR products.
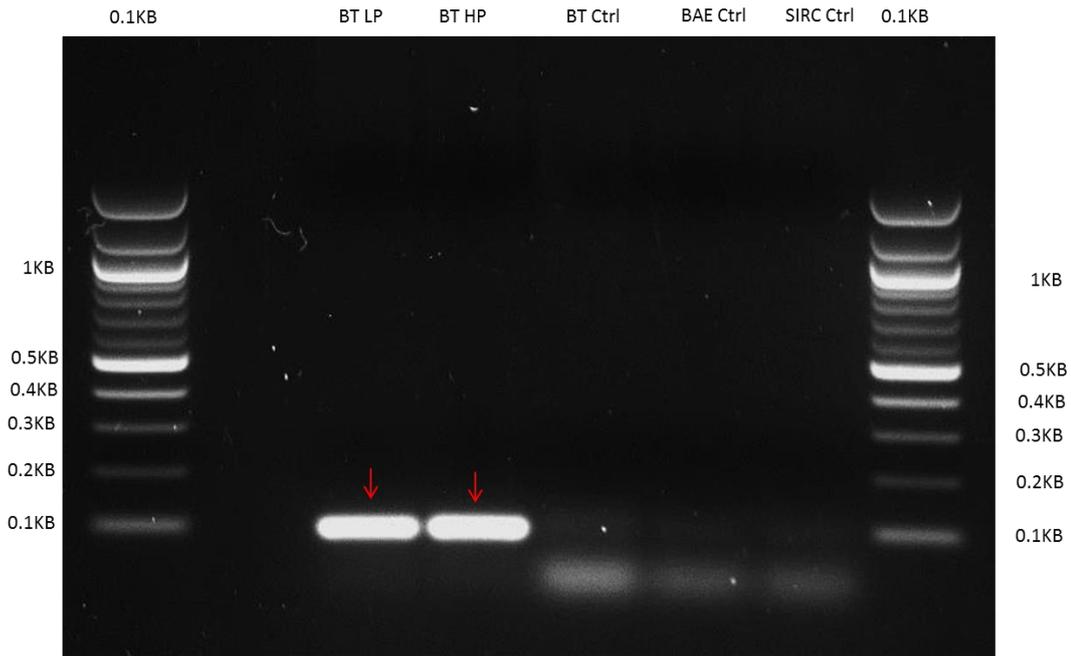
Figure 17 qPCR products for low and high pass AlHV-1 ORF3 gene visualised on agarose gel. Infected BT cells and controls uninfected BT, SIRC and BAE cells

BT LP; bovine turbinate cells infected with AlHV-1 low pass virus, BT HP; bovine turbinate cells infected with high pass virus, BT ctrl; non-infected bovine turbinate cells. BAE ctrl; non-infected bovine aortic endothelium cells, SIRC ctrl, non-infected statens seruminstitut rabbit cornea cells; 0.1 KB ladder; 100 base pair ladder, red arrows; indicate PCR products corresponding to ORF3 DNA.

3.5.5 Viral gene expression in the infected cells

The transcripts of six AlHV-1 genes (A4.5, ORF50, ORF25, ORF73, ORF63, and A9.5) were measured using qPCR. In BT cells infected with low pass virus, ORF50 and ORF25 genes were highly expressed compared to the others that were present at very low level (Figure 18A) indicating that these transcripts were associated with the productive/lytic cycle and as such they can be used as productive cycle positive control transcripts. The other transcripts were very poorly expressed (Figure 18A). There was no detectable expression of the transcripts in BT cells infected with serial/secondary infection

from BAE endothelium and SIRC epithelium cell sonicates and supernatant after infection with low pass virus (Figure 18A). On the other hand, in BT cells infected with high pass AlHV-1, we detected ORF50 transcript and the A4.5 gene transcript in all BT cells infected, although levels were variable between different cultures (hence the large error bars) (Figure 18B). In BT cells infected with cell sonicates and supernatant from directly-infected SIRC or BAE cells, variable but low levels of A4.5 and ORF 50 were also detected. There was no virus gene expression in non-infected BT cells (Figure 18 A and B).
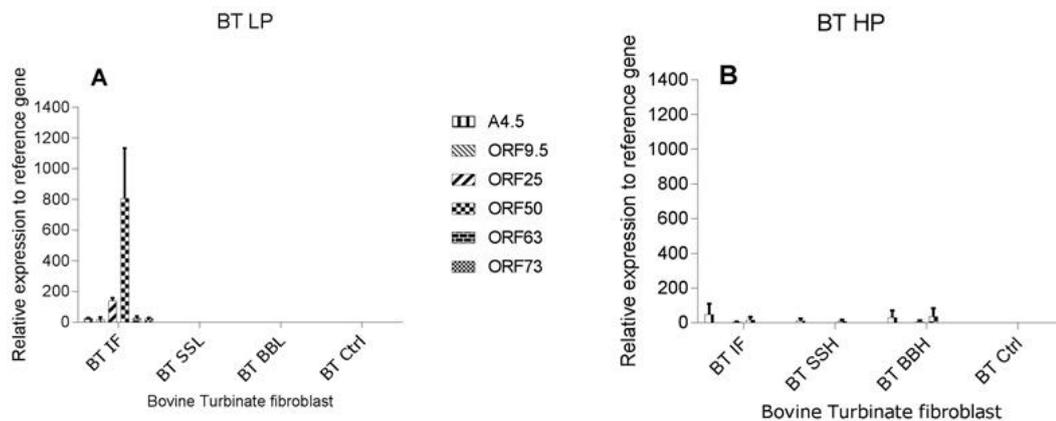
Figure 18 AlHV-1 transcripts in infected BT cells

Viral transcripts of six AlHV-1 C500 strain genes (A4.5, ORF9.5, ORF25, ORF50, ORF63, and ORF73) in BT cells infected with low and high pass virus. BT INF; BT cells infected directly with low culture pass virus (A) and high pass virus (B), BT SSL (A) and BT SSH (B); BT cells infected with whole cell sonicate and supernatant derived from SIRC cells directly-infected with low and high pass virus respectively, BT BBL and BT BBH; BT cells infected with whole cell sonicate and supernatant derived from directly-infected BAE cells (low culture pass (A) and high pass virus (B)), BT Ctrl; non-infected BT cells. Q RT PCR was used to measure the viral transcripts by normalizing the target genes with bovine ribosomal protein S 9 (RPS9) reference gene (chapter 2, section 2.4.4). The error bars indicate SD of duplicate samples from infected BT cells.

The viral gene transcript profile of the six virus genes revealed that they were expressed at very low levels in both endothelium (Figure 19) and epithelium (Figure 20) infected with either low culture passage AlHV-1 or high pass virus. There was no gene expression in endothelial and epithelial cells serially-infected with directly-infected endothelium and epithelium-derived virus. The use of different reference genes in the bovine cells (BT and BAE) make the gene expression comparison between the two cell lines difficult. This is because the expression rate varies between the two genes.
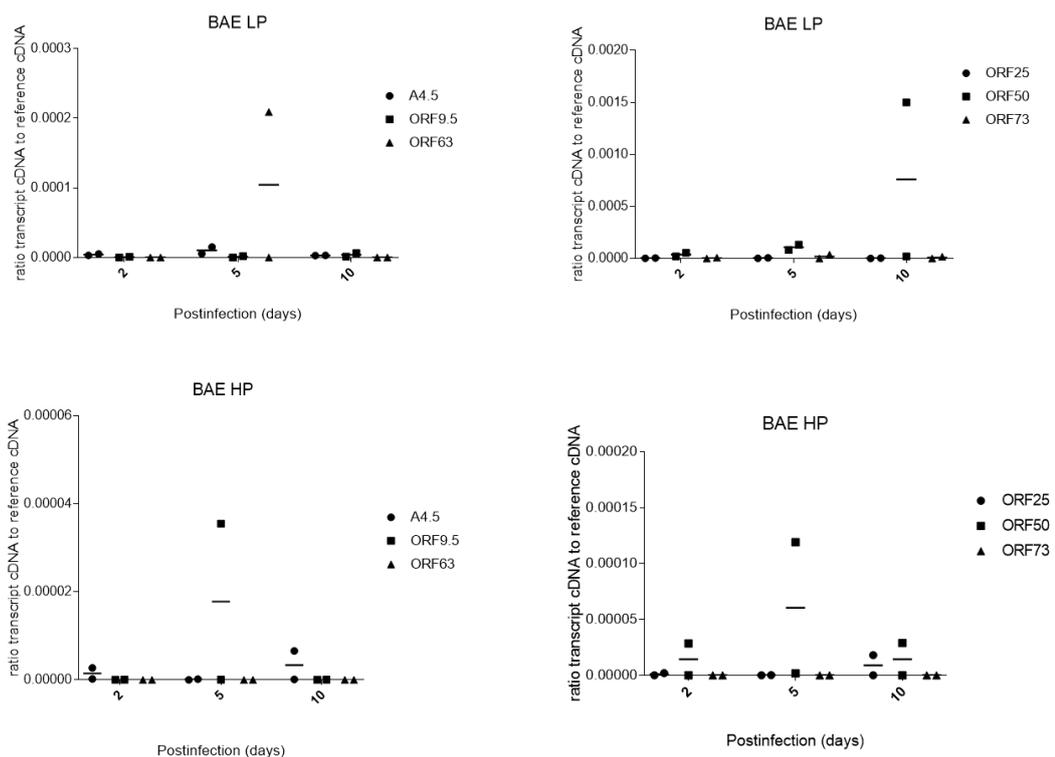
Figure 19 AlHV-1 transcripts in infected endothelial cells

Viral transcripts of six AlHV-1 C500 strain genes (ORF25, ORF50, ORf73, A4.5, ORF9.5, and ORF63) in bovine aortic endothelial (BAE) cells infected with low and high pass virus. BAE was infected directly on day 0 and transcripts were measured at different time point two, five, and ten days post infection for both

low and high pass virus. Error bar, represented by continuous black dashed line, represents the variation between samples. q PCR was used to measure the viral transcripts by normalizing the target genes with bovine G actin reference gene (chapter 2 section 2.4.4).
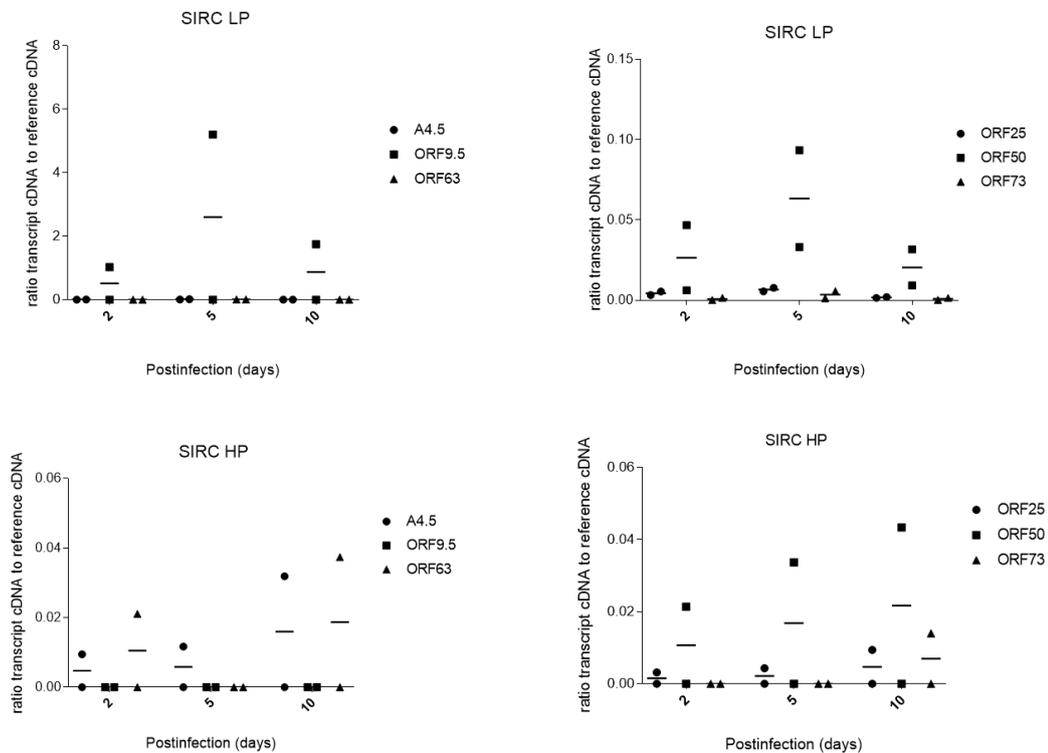
Figure 20 AlHV-1 transcripts in infected epithelial cells

Viral transcripts of six AlHV-1 C500 strain genes (ORF25, ORF50, ORf73, A4.5, ORF9.5, and ORF63) in statens seruminstitut rabbit cornea (SIRC) cells infected with low and high pass virus. SIRC was infected directly on day 0 and transcripts were measured at different time point two, five, and ten days post infection for both low and high pass virus. Error bar, represented by continuous black dashed line, represents the variation between samples. q PCR was used to measure the viral transcripts by normalizing the target genes with rabbit SDHA1 reference gene (chapter 2 section 2.4.4).

Table 8 Summary of results.

Virus	Cell type/ infection[1]	Viral DNA	CPE	Transcripts
Low pass	BT	+	+	ORF50; ORF25
AlHV-1	SIRC	+	-	Low level ORF50, A9.5
	BAE	+	-	V low level ORF50
	BT+SIRCv[2]	+	+	-
	BT+BAEv[2]	+	+	-
	SIRC+SIRCv[2]	+	-	-
	BAE+BAEv[2]	+	-	-
High pass	BT	+	+	Low level ORF50, A4.5
AlHV-1	SIRC	+	-	V low ORF50, A4.5, A9.5
	BAE	+	-	V low ORF50
	BT+SIRCv[2]	+	+	V low ORF50, A4.5
	BT+BAEv[2]	+	+	V low ORF50, A4.5
	SIRC+SIRCv[2]	+	-	-
	BAE+BAEv[2]	+	-	-

[1]BT fibroblasts support productive virus infection; SIRC epithelial cells and BAE endothelial cells. [2]Serial infection: uninfected cells were inoculated with cell sonicates and supernatant from directly-infected SIRC (SIRCv) or BAE (BAEv) after ~10 days in culture. Transcripts analysed: ORF50; ORF25; ORF63; ORF73; A4.5 and A9.5. Inoculated cells analysed on days 2, 5 and 10 after inoculation. CPE occurred around day 5 In BT cells. Viral DNA scored positive and transcripts listed only if present at all-time points after inoculation.

3.6 Discussion

In this series of experiments (summarised in table 8), rabbit epithelial corneal SIRC cells, bovine aortic endothelium (BAE) cells and bovine turbinate fibroblasts (BT) cells were infected with either low culture passage virus or high pass AlHV-1 C500 strain to determine whether the endothelial cells and /or the epithelial cells could be infected, and if so whether they could induce CPE in the cells or not. A selection of viral gene transcripts was analysed, representing either productive cycle (ORF50, ORF 25, ORF 63), latent cycle (ORF 73) or where their involvement in the virus life cycle was unknown (A4.5, A9.5).

The results indicate that low pass and high pass virus were able to infect SIRC and BAE cells although there was no CPE. This might indicate establishment of the latent cycle, and this was supported by the results of the gene transcription profiles that were of low abundance in these cells in spite of good evidence of viral DNA, indicating the presence of virus (Table 8 summary). The virus was intact and infective as, when directly-infected SIRC and BAE cell sonicates and culture supernatant were used to infect BT cells that support the productive life cycle, the BT cells were infected and showed CPE, evidence of virus replication and the productive life cycle. However, there was little or no viral gene transcription, possibly indicating a low level of gene expression.

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To speculate, a consequence of the study could be that BAE and SIRC represent endothelium and epithelium *in vivo* but a lack of direct virus-induced CPE *in vitro* may reflect a similar lack of CPE *in vivo* and that the destruction of epithelium and endothelium is the result of infected T cell damage. The BAE and SIRC cells may be in a latent state (low gene expression and lack of CPE in spite of infective virus present in the cells). This requires further study.

The lack of knowledge of latency transcripts in particular in MCF is hampered by difficult access to the natural reservoir species for AlHV-1 – the wildebeest. This is where the latent and lytic cycles will have evolved and not in the disease-susceptible species where the virus is less likely to persist. Recent work from the Stewart laboratory may be challenging this view though, where evidence of subclinical infections is seen in cattle (unpublished results).

The high expression of ORF50 and ORF25 in BT cells infected with low pass virus indicates their involvement in productive cycle, which given what they code for is expected. Why ORF63 was not seen here is a mystery, but may reflect the low abundance of the viral transcripts and lack of sensitivity of the q PCR used. The low expression and absence of productive/lytic ORFs has also been reported in lymphoid and non-lymphoid tissues from animals infected with AlHV-1 C500 strain (Dewals et al., 2008, Palmeira et al., 2013).

In BT cells infected with high pass virus and epithelium and endothelium derived virus, there was an expression of ORF50 and ORF A4.5 but no expression of the other genes, indicating that A4.5 is probably a productive virus life cycle gene. The absence of the expression of the other genes is probably due to the deletion, translocation, and rearrangement that the high pass virus undergoes where ORF50 and ORFA6 were truncated and translocated (distal part of ORF50b, proximal of A6 and distal part of A10) (Wright et al., 2003). ORF A4.5 may be involved in preventing cell death, hence supporting cell survival and the establishment of either productive and/or latent infection. This is because the gene encodes a protein similar to Epstein-Barr virus EBV BALF1 and cellular B-cell lymphoma genes of the BCl-2 family that controls apoptosis (Hart et al., 2007b, Russell et al., 2009). The respiratory system including turbinate cells are the primary site of infection in OvHV-2 MCF-affected animals (Cunha et al., 2008, Myster et al., 2015) and this is likely to be the case with AlHV-1 MCF as well.

Recent work has shown that AlHV-1 establishes latency in infected tissues and cells (Dewals et al., 2008, Dewals et al., 2011, Palmeira et al., 2013) and that MCF is therefore a disease of latency. The epithelial and endothelial cell lines infected with the virus did not show any CPE. It was possible though that the virus may support latent infection in these cells. However, the undetectable levels of ORF73, a latency-associated transcript make it difficult to be clear

on this point. It would be interesting to establish sensitive detection methods for the transcripts and co-culture cells with uninfected activated T cells to see if this forces a latency programme in the cultured epithelial and endothelial cells. These cells may play, *in vivo*, a role in enforcing the virus to establish latency in order to avoid detection by the immune system (Blake, 2010). In addition, the longevity of infection may be required for the virus to support latency.

To conclude, Endothelial and epithelial cells were infected with AlHV-1 but there was no evidence of productive infection and although there was some indication that there may be a latent state (lack of CPE). The low level of gene transcripts makes a definitive conclusion currently difficult.

Chapter 4

The effect of 5-azacitidine on AlHV-1 viral gene expression in large granular lymphocytes (LGLs) and infected bovine turbinate (BT) cells

4.1 Abstract

Malignant catarrhal fever is fatal lymphoproliferative disease of even-toed ungulates. Although the tissue damage leading to animals' death is linked to lymphocytes accumulating within these tissues, the pathogenesis is still unknown. Culture of infected tissues cells can be achieved *ex vivo* to develop large granular lymphocytes (LGL) that represent the infected cells *in vivo*. These cells enrich for MCF virus-infected cells and are useful to study virus-host interactions. In this series of experiments, AlHV-1 virus genes, known for their involvement in productive/lytic and latent life cycles, were examined in LGLs in the presence and absence of 5-azacitidne, a drug used to drive latency programming from a productive cycle one (Thonur et al, 2006). In addition, bovine turbinate fibroblast (BT) cells were studied as they support the productive virus life cycle and virus genes expressed in these represent important ones in the productive virus life cycle. Flow cytometry was used to phenotype the LGL, which were predominantly CD8$^+$ T cells. The results showed that LGL show gene expression representative of a mixture of productive/lytic and latent life cycle and the effect of 5-azacitidine on gene expression was irregular and inconsistent with respect to identifying key latency genes. Interestingly, 5-azacitidine had an impact on LGL phenotype where it increased the percentage of pan T cells in the treated LGLs group. There was also a slight increase in CD8+ cells in 5-aza

treated group in comparison to non-treated group. In the BT cells infected with the virus, productive/lytic associated genes ORF50, ORF25, and ORF A4.5 were highly expressed. The expression of these genes was reduced when BT cells were treated with 5-azacitidine but there was no elevation in ORF73 latency-associated transcript. Importantly, there was no CPE in the infected and treated cells.

4.2 Introduction

The mechanism by which the MCF related viruses (AlHV-1 and OvHV-2) induce disease is not yet well known (Li et al., 2014, Russell et al., 2009). Nonetheless, the pathogenesis of MCF is attributed *in vivo* to T cells accumulating in the tissues, many of which are infected with MCF virus (Dewals et al., 2011, Palmeira et al., 2013). Infected cells (LGL) can be developed in culture and are useful to study host-pathogen interactions (Reid et al., 1989, Reid et al., 1983). LGL are found to be capable of transmitting MCF when inoculated into naive rabbits (Reid et al., 1989). The viral genes profile, especially those responsible for productive/lytic and latent infection, from LGL derived from tissues of both reservoir and susceptible species, has been studied and found to be variable (Rosbottom et al., 2002). The ability to manipulate the productive/latent virus cycle, using drugs such as doxorubicin, which was found to stimulate productive virus cycle in lymphocytes infected with Epstein Barr virus (EBV) and 5-azacitidine, which had been found to drive latency in gamma herpes viruses (Szyf et al., 1985, Feng et al., 2004) has motivated researchers to study virus life cycle in OvHV-2 MCF-affected animals (Thonur et al., 2006). Here we study the effect of 5-azacitidine to drive the AlHV-1 latent virus cycle in rabbits LGL and in BT cells.

4.3 Materials and methods

4.3.1 Samples collection

Popliteal lymph nodes were obtained from rabbits infected with AlHV-1 C500 strain virus (see general materials and methods (2.6.4)). Samples were collected from rabbits IYDY, IYJJ, LFLK and LFKJ which were euthanized on day 17 of the experiment. The lymph node samples were placed in 1XPBS and processed immediately (see materials and methods section 2.2.1).

4.3.2 Tissue culture and infection

Large granular lymphocytes (LGL) were cultured and BT cells were cultured and infected as described in materials and methods chapter 2 sections (2.2.1 and 2.2). Quantitative PCR and PCR were performed as described in materials and methods, sections (2.4.4 and 2.4.2) to detect the virus and measure the viral DNA load.

4.3.3 Drug treatments

LGL derived from AlHV-1-infected rabbits were cultured as described in materials and methods chapter, section (2.2.1) and treated with 25µM 5-azacitidine (CALBICHEM, cat# 189825, Germany) over a period of three weeks with culture medium removal and drug replacement. This dose of drug was determined to be the highest concentration that did not affect the viability and growth of the LGLs or BT cells. Cultured LGL were exposed to the above mentioned

concentration for three days after which the drug was diluted by changing two thirds of the medium with fresh IL-2 IMDM medium every three days and adding fresh 5-azacitidine. Infected BT cells were treated with 5-azacitidine exactly in the same manner as the LGLs with the exception that the medium was completely discarded and replaced with fresh containing 5-azacitidine.

4.3.4 Flow cytometry

The protocol is as described in the materials and methods (chapter2, section 2.5.3).

4.4 Results

4.4.1 Viral DNA detection

Quantitative PCR was used to detect AlHV-1 DNA in LGL and BT

cells. AlHV-1 ORF3 gene was utilised to perform the procedure. This

is due to the fact that AlHV-1 ORF3 is validated for AlHV-1 DNA

detection and quantitation (Traul et al., 2005). The results showed

that AlHV-1 DNA was identified in all samples (Figures 21, 22 and

23) demonstrating that LGL were infected with the virus. The virus

DNA was not identified in non-infected BT cells (Figure 23).
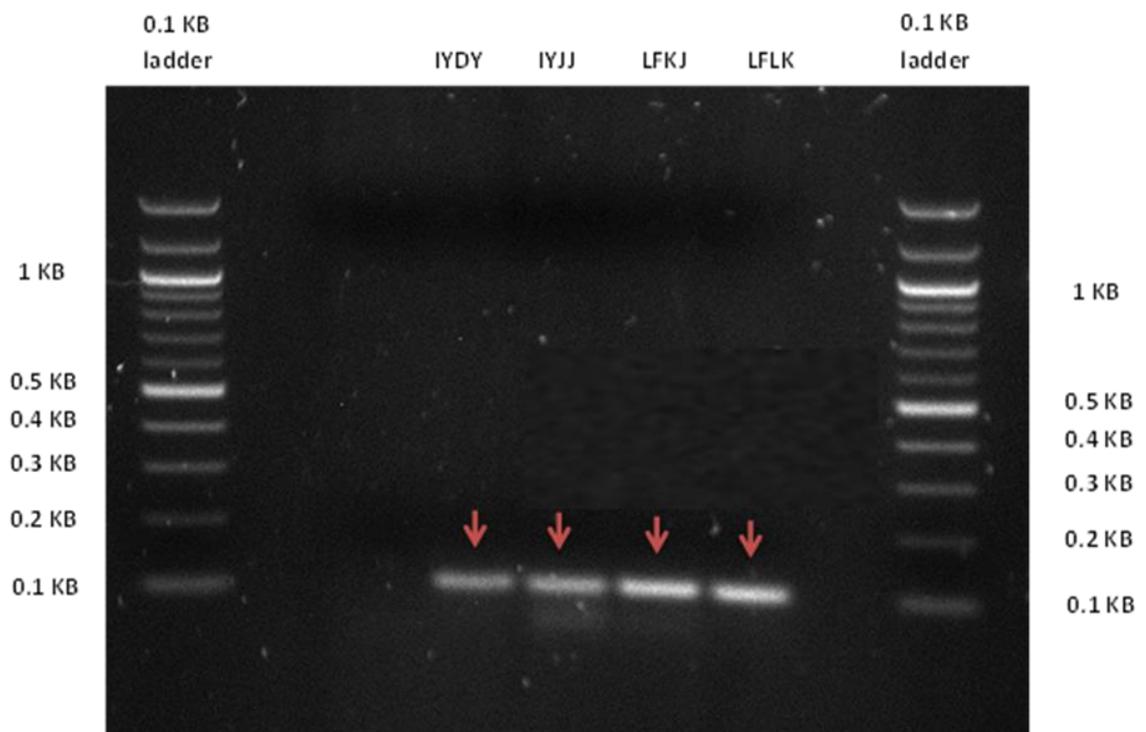
Figure 21 Q PCR and PCR products for AlHV-1 ORF3 gene
The figure shows qPCR and PCR products of AlHV-1 ORF3 in the rabbit LGL (110 bp) visualised on agarose gel. PCR product was estimated by comparison with the standard DNA (0.1 kb) ladder. AlHV-1 ORF3 was detected in LGL samples treated with 5-azacitidine drug. IYDY, IYJJ, LFKJ, and LFLK; different rabbit IDs from different cell lines, 0.1 KB ladder, 100 bp ladder, red arrows; indicate to the PCR product of the corresponding cell line.
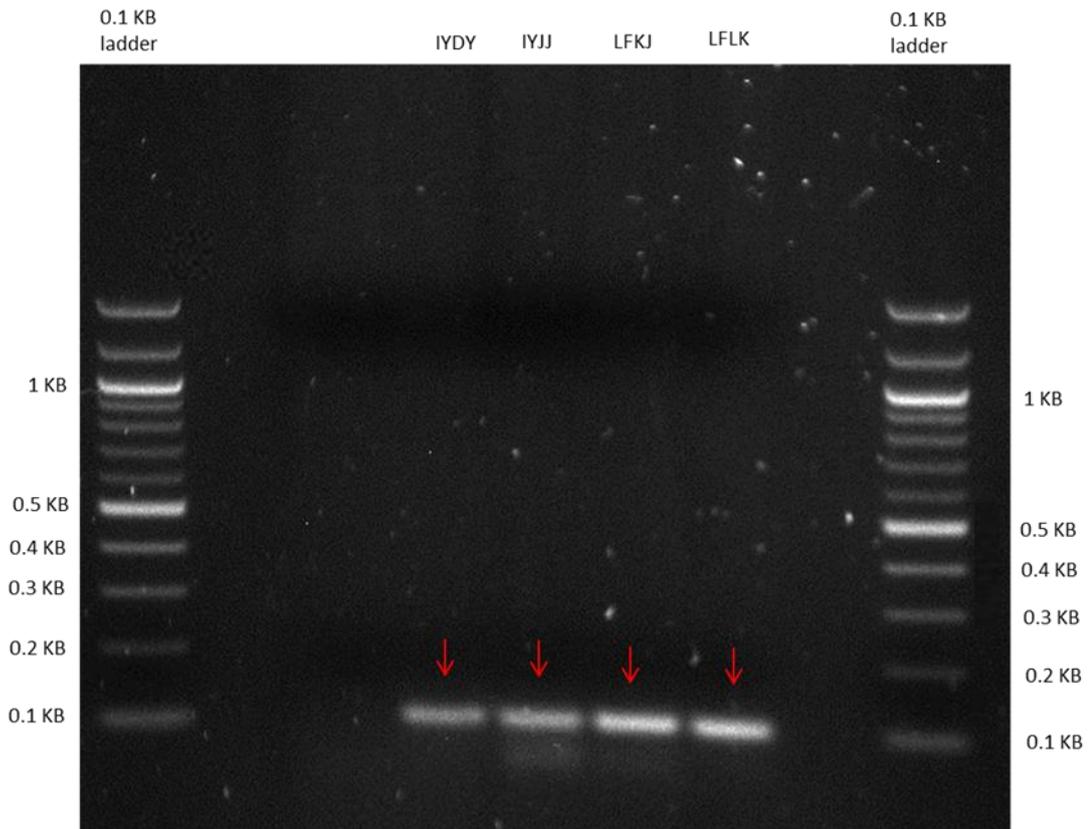
Figure 22 Q PCR and PCR products for AlHV-1 ORF3 gene
The figure shows qPCR and PCR products of AlHV-1 ORF3 in the rabbit LGL (110 bp) visualised on agarose gel. The size of the gene was estimated by comparison with the standard DNA (0.1 kb) ladder. AlHV-1 ORF3 was detected in non-treated LGL samples. IYDY, IYJJ, LFKJ, and LFLK; different rabbit IDs from different cell lines, 0.1 KB ladder, 100 bp ladder, red arrows; indicate to the PCR product of the corresponding cell line.
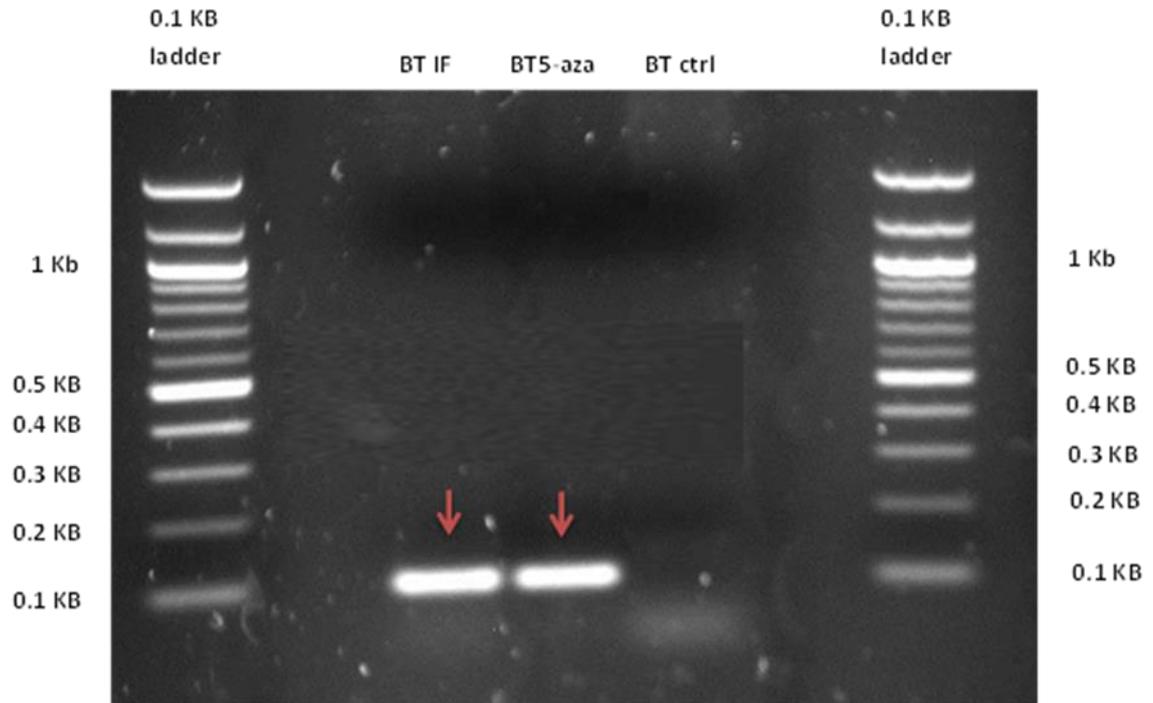
Figure 23 Q PCR and PCR products of AlHV-1 ORF A3 gene
The figure shows q PCR and PCR products of AlHV-1 ORF3 (110 bp) visualised on agarose gel. The size of the gene was estimated by comparison with the standard DNA (0.1 kb) ladder. AlHV-1 ORF3 was detected in BT cells samples (infected only and infected and treated with 5-azacitidine) whereas the viral DNA was not detected in uninfected BT control. BT IF; bovine turbinate cells infected with AlHV-1 virus, BT 5-aza; bovine turbinate cells infected with AlHV-1 virus and treated with 5-azacitidine drug, BT ctrl; bovine turbinate cells not infected with the virus and not treated with 5-azacitidine; 0.1 KB ladder; 100 base pair ladder; red arrows; indicate to the PCR products of the corresponding cells.

4.4.2 Viral DNA load in LGLs

To evaluate AlHV-1 copy number in LGL (treated and non-treated samples), q PCR assay was performed on LGL samples. The viral DNA copy number was estimated by normalising AlHV-1 ORF3 gene with 10^5 rabbit β globin reference gene as described in Dewals et al. (2008). The data obtained showed that the viral load is nearly

similar in both groups (Figure 24) with values between 1-1.6x 10^5

AlHV-1 copy numbers per 10^5 rabbit β globin.
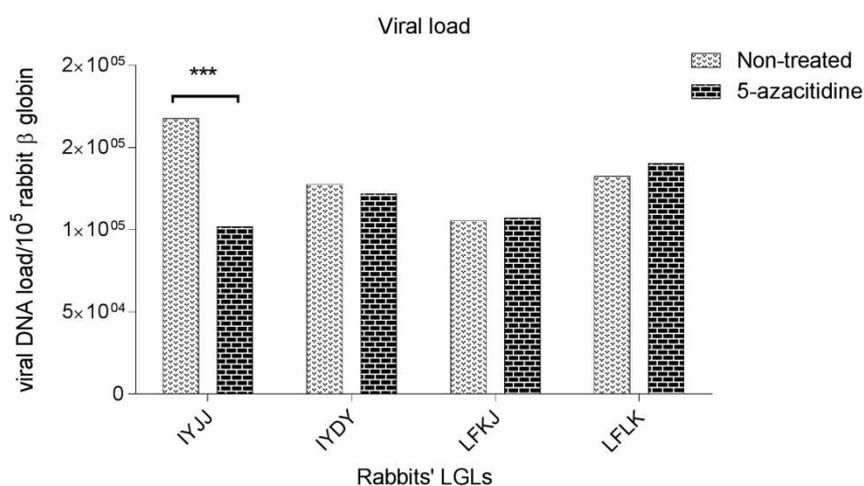
Figure 24 AlHV-1 DNA copy number in rabbits' large granular lymphocytes.
The figure illustrates the viral load (as copy numbers per 105 beta globin DNA copies) in large granular lymphocytes (LGL) from rabbits infected with AlHV-1 C500 strain. Four lines (IYJJ, IYDY, LFKJ, and LFLK) were treated with 5-azacitidine drug or kept without treatment as a control. The viral load was estimated by normalizing the viral DNA copy number per 105 rabbit β globin reference gene using qPCR. The primers and probe (FAMAlHV-1) for AlHV-1 ORF3 were used inside the standard sequence and then analysed using Roche Light Cycler 480 system. Two-way ANOVA was performed to do the statistical analysis using Sidak's multiple comparisons test. The primers and probe (FAM) for rabbit β globin reference gene was utilized within the standard sequence.

4.4.3 The effect of 5-azacitidine on BT cells

There was an obvious cytopathic effect (CPE) in BT cells infected with the virus. However, the development of CPE was not seen in BT cells infected and treated with 5-azacitidine. Figure 25 shows the difference between infected BT cells in the presence and absence of 5-azacitidine.

144
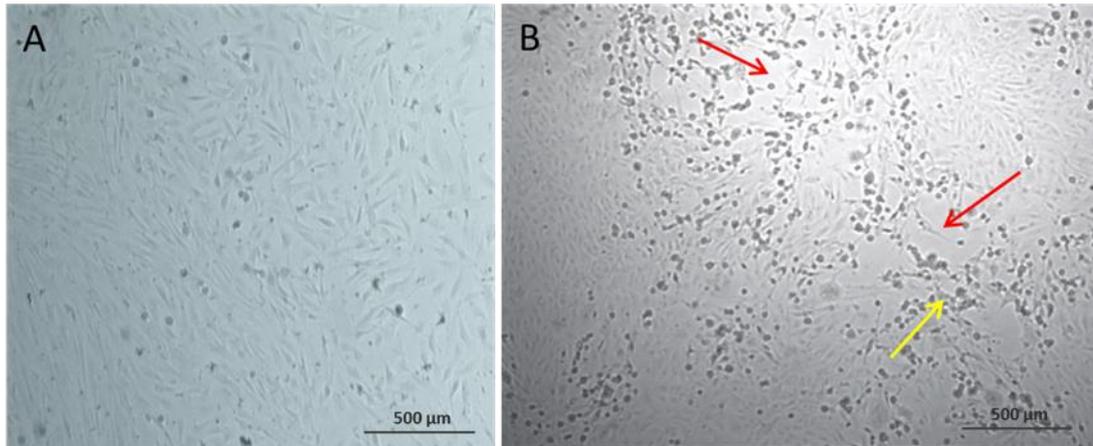
Figure 25 BT cells infected with AlHV-1 C500 strain and infected and treated with 5-azacitidine

The figure depicts BT cells infected with AlHV-1 and treatment of one of the samples with 5-azacitidine. Image (A) shows BT cells infected with the virus and treated with the drug the following day and the drug was changed every three days up to day 17 post infection. No CPE was seen after treatment. Image (B) BT infected with the virus without treatment and there was CPE detected from around day 5 post infection. The red arrows indicate the absence of monolayer and the yellow arrow show the accumulation of cell debris.

4.4.4 Measuring viral gene transcripts

In order to examine the viral gene profiles in LGL and BT cells, cDNA was synthesised from RNA for q PCR as described in chapter two, section (2.4.1). The results showed that all viral gene transcripts examined were expressed at very low level in two of the LGL lines (LFLK and LFKJ) and were not detected in the other two lines (IYDY

and IYJJ) (Figure 26). AlHV-1 ORF73 was expressed at a higher level in the 5-azacitidine treated group than non-treated in LFLK cell line. However, ORF50 expression is also identified. The expression of ORF 25, ORF63 and ORF A4.5 varied in each cell line in both groups.

In infected BT cells there was high expression of ORF50, ORF25, and ORF A4.5 in comparison with the other genes (Figure 27). In BT cells infected and treated with 5-azacitidine there was an obvious reduction in the genes supporting productive/lytic infection namely ORF50 and ORF25, but no increase in ORF73 transcripts (Figure 27) even though the cells did not show any CPE illustrating that the drug has had an effect on the expression of these genes. No virus gene expression was seen in non-infected and non-treated control BT cells (Figure 27).
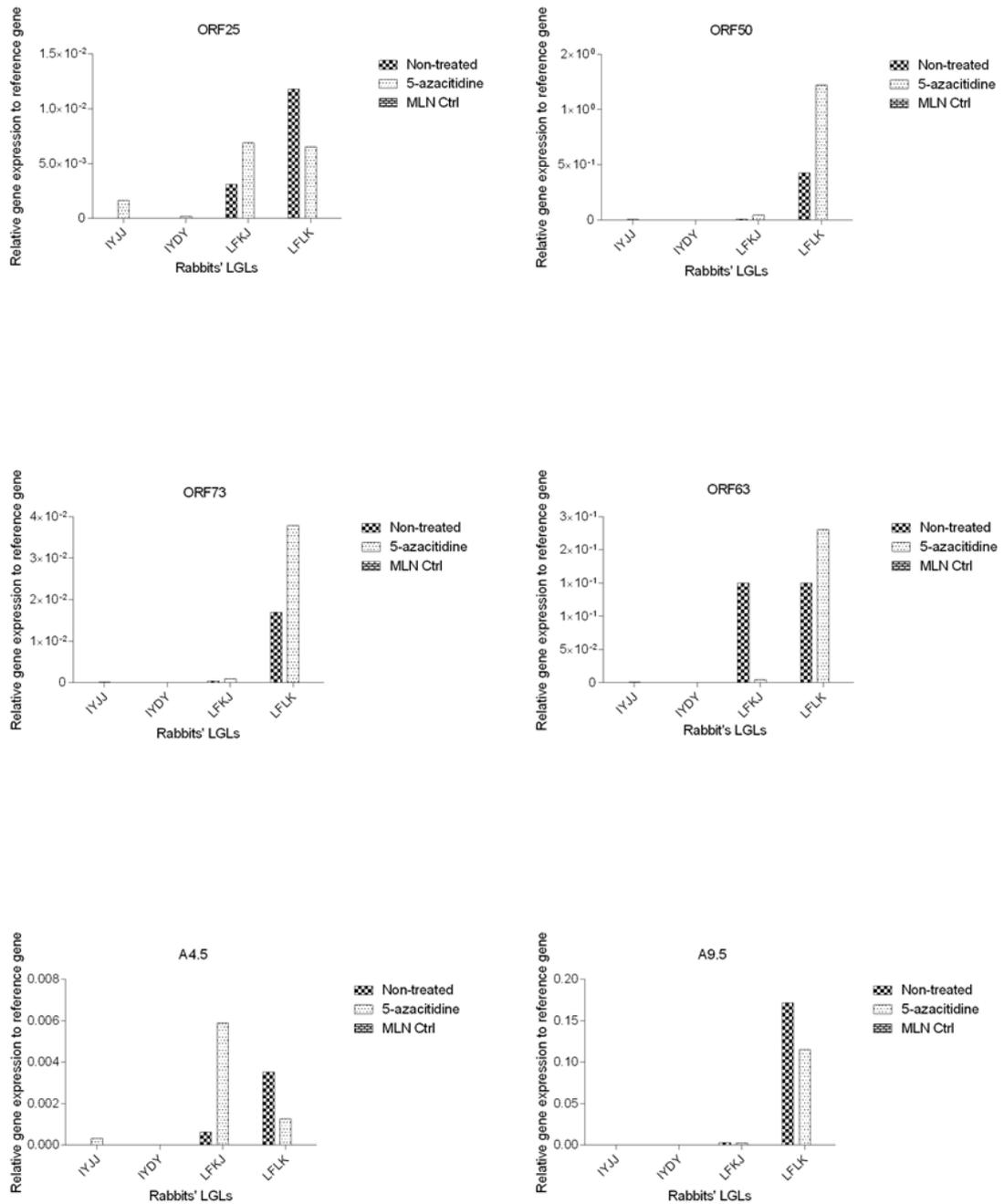
Figure 26 viral gene transcripts of AlHV-1 C500 in rabbit LGLs
The figure shows the viral gene profiles of AlHV-1 C500 strain genes (ORF25, ORF50, ORF73, ORF63, ORF A4.5, and ORF A9.5) in rabbit LGLs treated with 5-azacitidine or not-treated. Rabbit mesenteric lymph node cells from a non-infected rabbit were used as a negative control. Q PCR was used to measure the gene expression by normalizing the target genes with rabbit SDHA1 reference gene (chapter 2 section 2.4.4).

147
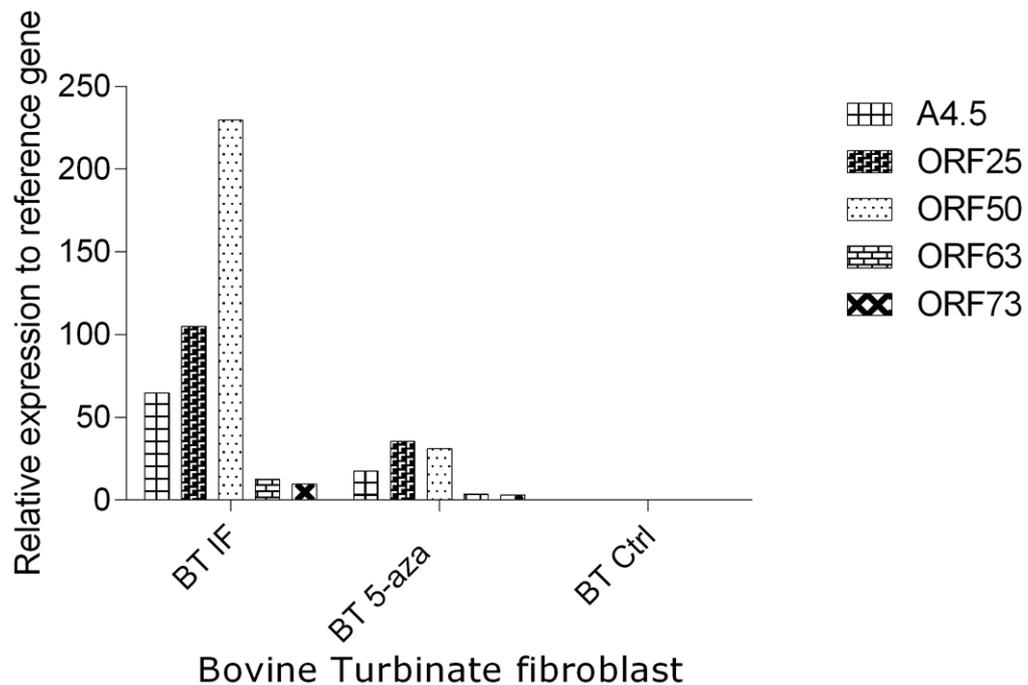
Figure 27 viral gene transcripts of AlHV-1 C500 strain in BT cells
The figure shows the viral gene transcripts of five AlHV-1 C500 genes (ORF A4.5, ORF25, ORF50, ORF63, and ORF73) in BT cells. QPCR was used to measure the gene expression by normalizing the target genes with bovine ribosomal protein S9 (RPS9) used as a reference gene (chapter 2, section 2.4.4). BT INF; infected BT without treatment, BT 5-aza; infected BT and treated with 5-azacitidine, BT Ctrl; non-infected and non-treated BT cells.

4.4.5 Flow cytometry analysis of LGL

Monoclonal antibodies directed against CD4, CD8, CD11b, B cell, and Pan T cells were used for flow cytometry analysis of the rabbit LGLs (material and methods (2.5.3)). The results revealed that the predominant cell type in the LGLs was CD8$^+$ whereas CD4$^+$ T cells were not detected. Figure 28 depicts the percentage of mononuclear cells in LGLs in the absence or presence of 5-azacitidine. In addition, the results showed that the percentage of Pan T cells is low in non-treated LGL but the percentage was high in 5-azacitidine treated

cells. The cell lines in both groups were devoid of both CD11b cells (expressed on leukocytes, particularly most monocyte/macrophage series cells) and CD19+ B cells. There was a bias towards CD8$^+$ in the double staining samples used for all cell lines (Figure 29). Figure 29 shows the typical results in one cell line (IYDY) in both treated and non-treated groups.
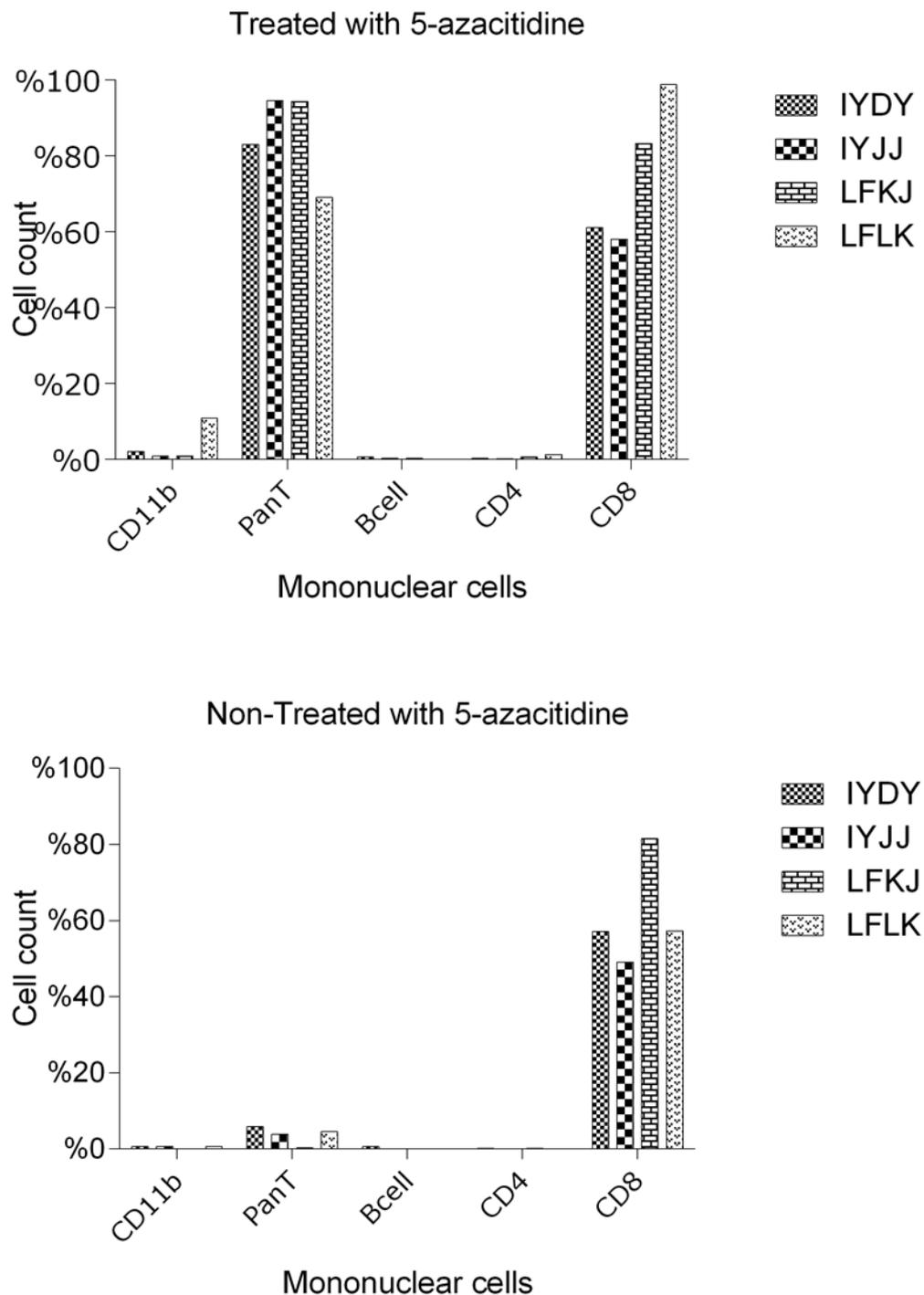
Figure 28 flow cytometry analysis of mononuclear cells in LGL non-treated and treated with 5-azacitidine
The figure illustrates the percentage of cell types within LGL upon analysis by flow cytometry. CD8 represents the majority of expressed T cell subset in both 5-azacitidine treated group and non-treated group whereas Pan T cell was high in 5-azacitidine treated group and low in non-treated group. CD4, CD11b and B cell were not detected in both groups.
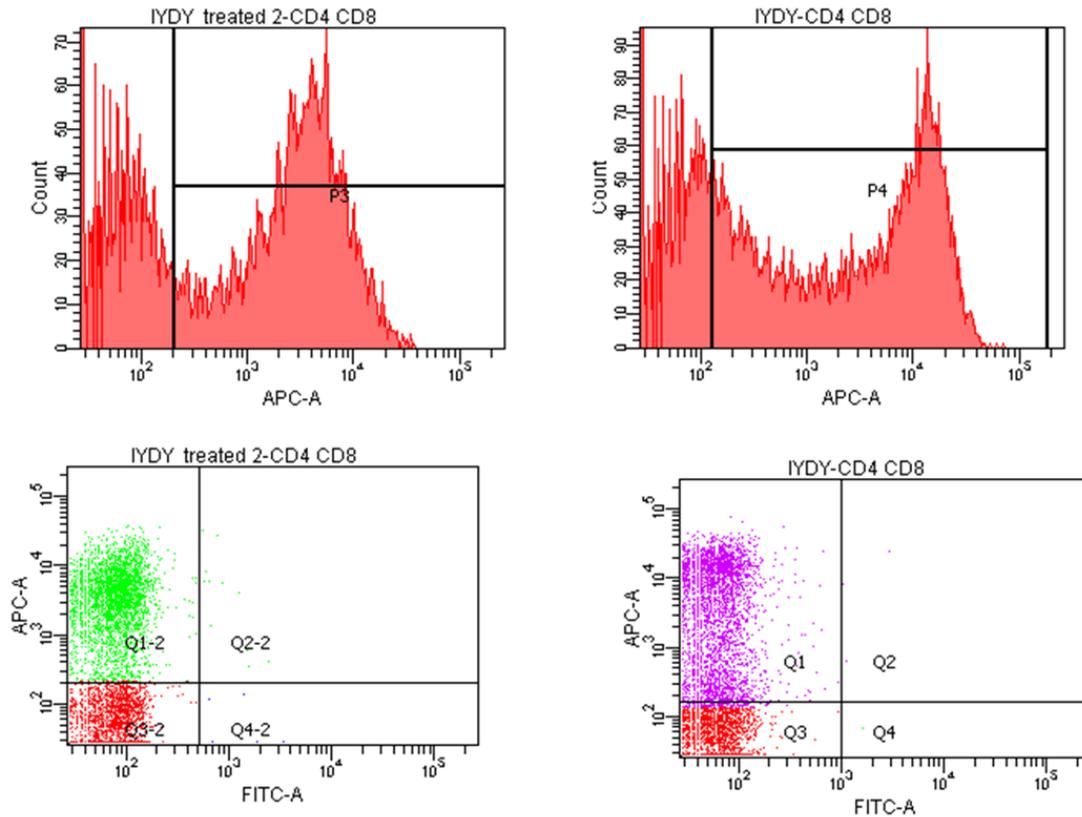
Figure 29 flow cytometry analysis of LGL double stained with two fluorescence antibodies in 5-azacitidine treated and non-treated IYDY cell lines

The figure shows the flow cytometry dot blot double staining of IYDY LGL lines with secondary antibody APC for CD8 and primary antibody conjugated with FITC CD4. High percentage of labelled cells lie in the quadrants 1-2 and 1 representing APC fluorescence for CD8 whereas no cells labelled with FITC for CD4 in quadrants 4-2 and 4 in both treated and non-treated IYDY cell lines. The figure also shows high percentage of CD8 when parameter of count used versus fluorescence in both groups. For the calculation of cell percentage, CD4 isotype control was used as a negative control for CD4 samples. Therefore, the figures of CD4 isotype control were subtracted from the values of CD4 for each sample. Due to the fact that the secondary antibodies have a role in the detection and do not bind directly to the cells; the figures for secondary APC and secondary FITC can be subtracted from the figures of each cell type to give percentage of cell count. For example, secondary FITC must be subtracted from CD11b, Pan T, B cell and FITC only. Secondary APC must be subtracted from CD8, double stained and APC only.

4.5 Discussion

In this study, Rabbit LGLs were shown to be infected as determined by viral DNA presence, but expressed only a low abundance of transcripts. In a few, a mixture of known productive cycle (ORF50, ORF25, ORF63) and latent cycle (ORF73) transcripts were detected,

but not in all the LGLs. ORF50 is the R transactivator (RTA) which plays a role in driving the productive viral programme from latency (Frame and Dalziel, 2008, Goodwin et al., 2001); ORF 25 encodes a major capsid protein that is expressed during lytic cycle indicating viral replication (Dewals et al., 2008); ORF 73 encoding the latency associated nuclear antigen (LANA) is important for driving latent infection (Dewals et al., 2011, Hu et al., 2002, Palmeira et al., 2013). ORF63 encodes a tegument protein which is involved in preventing apoptosis (Boyle and Monie, 2012). This indicates that ORF63 may serve a function within the lytic life cycle. ORF A4.5 (a bcl-2 anti-apoptosis orthologue) (Mills et al., 2003) and A9.5 (secreted cytokine orthologue) (Lankester et al., 2015a, Russell et al., 2013) were included in the study to see if they could be identified as either productive cycle or latent cycle expressed. ORF73, ORF50, ORF63 and ORF25 were expressed in two LGL lines, indicating possibly predominance of productive cycle, but with no indication of a latent cycle within some of the cells which remains a possibility. This is in consistent with the observation made by Thonur et al., (2006) in cattle and rabbits using LGL infected with OvHV-2. There was also expression of ORF A4.5 in two of the rabbit lines (LFLK and LFKJ). Thonur et al. (2006) found similar results with Ov4.5 in LGL cell lines infected with OvHV-2 virus. ORF A9.5 was expressed in one of the lines (LFLK). However, the role of this gene in latent and productive/lytic life cycle remains unclear.

Upon treatment with 5-azacitidine, there was an increase in ORF73 expression in one cell line (LFLK) in comparison to the non-treated control. This is consistent with 5-azacitidine driving latency and was also seen in LGL infected with OvHV-2 virus and treated with 5-azacitidine (Thonur et al., 2006). It is worth mentioning that ORF73 can be 'leaky' with respect to expression in the different virus life cycles such that absence of ORF73 in productive infection is not necessarily absent (Thonur et al., 2006). The expression also of ORF50 indicates that the cells support both latent and lytic infection. The expression of the other genes: ORF25, ORF63 and ORF A9.5 were different in each cell line in 5-aza treated and non-treated groups. This may indicate that LGL support both productive/lytic and latent virus cycle. In addition, the viral gene expression may be irregular in these cells. ORF A4.5 was expressed to a much lower level in 5-aza treated LGLs than the untreated control cells, indicating a role in the productive virus life cycle. ORF A9.5 was not investigated because of the time limit and lab authorisation required to repeat the experiment.

5-azacitidine is a DNA hypo-methylating agent that functions by inhibiting DNA methyltransferase. It has also been found that 5-azacitidine has different effects on different virus gene expression patterns (Chang et al., 2014, Qiu et al., 2010). In herpes-virus saimiri, 5-azacitidine encourages productive cycle from latency (Mossman et al., 1989) while it appears that it changes the form of

EBV expression from type I latency to type III latency in lymphoma cell lines (Schaefer et al., 1997). It seems therefore that the effect of 5-azacitidine on gene expression is possibly virus and cell-type specific (Thonur et al., 2006). Thonur et al. (2006) also discovered differences in viral gene expression between cattle and rabbit LGLs samples. As defined by Gardella analysis (Gardella et al., 1984), bovine LGLs infected with OvHV-2 supports predominately latent infection (circular DNA) and only a weak band (smear) of linear DNA. OvHV-2 -infected rabbit LGLs showed productive infection (linear DNA) and only a small amount of circular DNA. Unfortunately, time ran out before Gardella gels could be performed on the samples in this study. However, the match between Gardella analysis and productive and latent gene expression was high (Thonur et al., 2006).

The high level of expression of ORF50, A4.5 and ORF25 in infected BT cells confirms the support of the virus productive/lytic cycle in these cells, which undergo a CPE and release infectious virions into the culture medium. However, when treated with 5-azacitidine, there was a lack of CPE and a sharp decline in the expression of these genes (ORF50, A4.5 and ORF25) consistent with a latency programme. This confirms the latency-inducing activity of 5-aza in this study with AlHV-1. ORF73 expression however was not increased, but this in itself may not be diagnostic of the latent state (i.e. an increase in expression rather than just expression).

MCF viruses (OvHV-2 and AlHV-1), initially, replicate in the respiratory system, in particular in the nasal turbinate (Li et al., 2008, Myster et al., 2015) where ORF25 is identified. Afterwards, the virus disseminates to the blood where, in the case of one set of studies with AlHV-1 infection, it establishes latency in lymphocytes (mainly CD8^{+} T cells) and ORF73 expression is detected (Dewals et al., 2008, Palmeira et al., 2013). ORF A4.5 may be involved in preventing cell death, hence supporting cell survival and the establishment of productive infection. This is because the gene encodes a protein similar to Epstein-Barr virus EBV BALF1 and cellular B-cell lymphoma genes of the BCl-2 family that controls apoptosis (Hart et al., 2007, Mills et al., 2003).

Flow cytometry analysis of the rabbit LGLs showed that the predominant cell type was CD8^{+} in both 5-azacitidine-treated and non-treated cells. The results are in line with the observations made in other studies that also found that the main cell type in LGL and infected tissues *in vivo* was CD8^{+} cells (Dewals et al., 2008, Dewals et al., 2011). The results showed that the LGL were devoid of CD4^{+} cells. Dewals et al. (2008) discovered that the rise in CD8^{+} cells is correlated with a reduction in CD4^{+} cells. The observation that T cells proliferating in MCF affected tissues outnumbered CD4^{+} cells was also highlighted by other researchers (Anderson et al., 2007). LGL are indiscriminately cytotoxic and have the morphology of T/natural killer (NK) cells (Swa et al., 2001).

However, in this study there was a surprising result. Very few LGL cells reacting with the Pan-T marker were detected in the LGLs not treated with 5-aza, whereas in the treated cells, the pan T marker was present on the majority of the cells. The actual antigenic target of the Pan-T antibody used is not known (Parameswaran et al., 2014). However, a recent study showed that MCF virus infection is associated with an effect on T cell phenotype of infected cells. The A2 gene (transcription regulator) had an effect on the regulation of LGL T cell phenotype where there was downregulation of γδ TCR receptors and upregulation of αβ TCR receptors in the absence of A2 gene (Parameswaran et al., 2014). This may explain the reduction in pan T cells in the non-treated group if A2 is involved in latency versus productive cycle gene expression affecting this phenotype. The effect of 5-azacitidine on human T cells in particular CD4 and CD8 has been reported. It was established that 5-azacitidine treatment of Human T cells reduced $CD8^+$ T cells while $CD4^+$ T cells increased (Stübig et al., 2014).

In conclusion, 5-azacitidine affected the latency programme as evidenced in BT cells, where it prevented CPE and inhibited markedly productive virus gene transcription. In LGLs the effect of 5-aza was less clear due to the low level of viral transcripts although in some lines there appeared to be a small effect. The drug clearly affected the LGLs as there was a marked increase in the pan T cell

marker on T cells compared to infected untreated controls. At this stage it is not clear what this means.

Chapter 5

Cellular and viral gene expression analysis (RNA-Seq) in rabbit LGLs and BT cells infected with AlHV-1 and treated with 5-azacitidine

5.1 Abstract

The goal of this study was to determine the molecular-biochemical pathways affected by treatment of AlHV-1 infected rabbit large granular lymphocytes (LGLs) and bovine turbinate (BT) cells infected with the virus, with 5-azacitidine (5-aza). The hypothesis is that 5-aza will suppress productive virus infection and aid identification of virus latency transcripts and the host transcriptome in latently-infected cells. In order to achieve this objective, RNA-Seq was performed on the cell RNA (as copy DNA) samples. The results showed differences in gene expression patterns between treated and untreated cells, both LGLs and BT cells. Pathways identified using David® and Ingenuity Pathway analysis software showed that cell death (apoptosis), T cell chemo-attraction, proliferation and immune response pathways affected in 5-aza-treated LGLs compared to non-treated ones. In addition, the analysis indicated that mRNA translation, gene expression, cell cycle, DNA replication and post transcriptional modification pathways were affected in 5-aza treated BT cells in comparison to non-treated BT cells. In conclusion the pathway analysis showed that there were variations between rabbit LGLs and cattle BT cells. While LGLs have pathways that aid T cell proliferation and accumulation and immune responsiveness in 5-aza-treated groups compared to untreated ones, BT cells contain pathways that support gene expression and mRNA translation in treated groups compared to untreated. In

addition, it is unclear whether rabbit LGLs support productive or latent virus cycle. RNA-Seq was not sensitive enough to pick up any other than a few viral transcripts.

5.2 Introduction

In the previous chapter, the issue of 5-azacytidine effect on the viral productive and latency programmes was studied. In this chapter, the global transcriptome of LGLs and BT cells was compared in treated versus untreated cells using RNAseq. Although Gamma herpes virus latency, generally, is not connected with disease development, latency in lymphocytes can produce malignant lymphocyte activation and proliferation dependent upon the viral species and the infected host (Barton et al., 2011, Ensser and Fleckenstein, 2005). Latency is regulated by the control of a viral gene expression programme in the infected cells enabling the virus to avoid the detection by the host immune system and persist in the infected cells. One of the approaches the virus adopts to induce latency is the control of the expression of transcriptomes including mRNA and non-coding RNAs such as micro-RNAs (miRNA) (Bartel, 2009, Kincaid and Sullivan, 2012, Pfeffer et al., 2004). The advent of sequence-based approaches such as deep sequencing, in particular RNA-Seq have opened opportunities to researchers to look at post transcriptional modification, mutations and gene expression alterations (Kumar et al., 2012, Wang et al., 2009, Wilhelm and Landry, 2009). Here, RNA-Seq technique is used to map host (and viral) gene transcripts in LGLs derived from rabbits infected with AlHV-1 virus and bovine turbinate (BT) cells infected with AlHV-1 virus to look for host pathways associated with latency and the lytic

cycle. LGLs represent the T cells naturally-infected *in vivo* and in the previous chapter, as with a previous study with OvHv-2 (Thonur et al., 2006) infected rabbit cells showed that the cells supported a mixture of latency and productive cycle genes, although in this study gene expression was low and the results not particularly clear cut. 5-aza could suppress the productive virus life cycle and reveal latency transcripts. Cattle BT cells support productive virus infection and they are a control for viral productive cycle transcripts. It was of interest to compare the host responses in 5-aza-treated and untreated cells for both species.

5.3 Materials and methods

5.3.1 Tissue and infection

Large granular lymphocytes (LGL) were obtained and cultured and BT cells were cultured and infected as described in materials and methods chapter 2 sections (2.2.1). Quantitative PCR was performed as described in materials and methods, sections (2.4.4) to measure the viral DNA load.

5.3.2 RNA extraction

Total (viral and cellular) RNA was extracted using RNeasy Plus Universal Mini Kit (cat # 73404, Qiagen) according to the manufacturer's instructions as described in materials and methods chapter, section (2.3.2).

5.3.3 RNA quality measurement using utilizing Agilent Bio-analyser Nano 6000 kit

The RNA quality measurement was as described in the materials and methods, section (2.9).

5.3.4 Complimentary (cDNA) DNA Library preparation and sequencing

This was done by Deepseq (University of Nottingham). Total RNA was measured using Qubit RNA BR assay kit (Life technologies, Q10210). 1μg of Total RNA was used for enrichment of mRNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490). Illumina stranded whole transcriptome sequencing libraries were prepared using NEBNext Ultra Directional RNA library prep kit for Illumina (NEB, E7420S). Library QC was performed using the bioanalyser HS kit (Agilent biotechnologies, 5067-4626). Libraries were quantified using qPCR (Kapa Biosystems, KK4824). Libraries were pooled at desired concentrations, denatured and loaded for sequencing according to the manufacturer's instructions. Sequencing was performed on the Illumina NextSeq500 sequencing platform to generate 2 x 75bp reads.

5.3.5 Sequence analysis, bioinformatics and statistics

This was done by Deepseq (University of Nottingham) with bioinformatics by ADAC (UoN). Ingenuity pathway analysis (IPA, www.ingenuity.com/products/pathway) was performed to look at relationships between the differentially-expressed gene lists (5-aza treated versus untreated for both cell types). The data from both species (cattle and rabbits) were interpreted utilizing the core analysis function included in IPA system in the context of biological

processes, pathways and networks. The up and down regulators were described as value parameters for the analysis. The significance of predicted bio-functions and canonical pathways was examined by the Fisher Exact test p-value (McKinney et al., 1989). Bio-functions were categorised in diseases and disorders, molecular and cellular functions. In a similar manner, canonical pathways were clustered in metabolic and signalling pathways. The bovine and rabbit (Cattle=Bos-taurus UMD3.1, rabbit=Orycorolagus-cuniculus 2.0 from ensemble genome version 82) as contained in BT cells and rabbit LGLs respectively were used to map transcript sequences. The viral genome annotation file was modified to include A9.5_ORF. BLAT (Kent, 2002) was used to identify the overlapping sequence regions. The viral genome sequence and their cosponsoring annotations were merged with the cow and rabbit files so that the viral genome sequence appeared to represent an additional contig within the host genome.

The reads were trimmed with scythe (Lindgreen, 2012) to remove residual adaptor contamination and with sickle (Joshi and Fass., 2011) to remove low quality bases. The trimmed Reads were aligned to the modified reference genomes using HISAT v2 (Kim et al., 2015). Technical replicates from different lanes were merged with SAM tools (Li et al., 2009). The merged alignments were assembled using string tie (Pertea et al., 2015) and differential expression analysis was performed using Ballgown (Frazee et al.,

2014). Scripts used in this analysis pipeline can be found in appendices in the soft copy version section (8.10).

5.4 Results

5.4.1 Viral DNA load in LGLs

To evaluate AlHV-1 copy number in LGL (5-azacitidine-treated and non-treated samples), q PCR assay was performed on LGL samples. The viral DNA copy number was estimated by normalising AlHV-1 ORF3 gene with 10^5 rabbit β globin reference gene copies as described in Dewals et al. (2008). The data obtained showed that the viral load is similar in both groups (Figure 30). This is the same as the figure shown in Chapter 2, as the same cells were used and analysed just prior to sending samples off for RNA-seq and doing the viral transcript studies in chapter 2. This is repeated here to provide context for this chapter.

5.4.2 RNA Integrity Number (RIN) measurement

The RNA integrity number is measured by a set of bio-analytical devices, which is a combination of microfluidic chips, voltage-induced size separation in gel filtered channels and laser-induced fluorescence detection and analysis of data using Agilent software tool (Schroeder et al., 2006). The RIN number ranges from 1 to 10. A RIN number of 1 show completely degraded RNA whereas a RIN number of 10 indicate totally intact RNA samples. Upon RNA extraction, the RNA integrity was estimated and samples with RIN

numbers 7 and more were used for further analysis. Figure 31

shows the RIN number in the some of the RNA samples sent for

RNA-Seq analysis. Table 9 shows sample number, sample name,

RNA concentrations and RNA integrity numbers (RIN) for samples

sent for RNA-Seq analysis.

Table 9 sample number, sample name, RNA concentrations and RNA integrity numbers (RIN) for LGL and BT samples sent for RNA-Seq analysis.

Sample number	Sample name	Sample type	Concentration ng/µl	RIN #
1	LGLLK5aza	Total RNA	297	10
3	LGLKJ5aza	Total RNA	291	10
4	LGLLKcon	Total RNA	655	9.90
5	LGLKJcon	Total RNA	1217	9.90
6a	BTinfa	Total RNA	1788	8.40
6b	BTinfb	Total RNA	1341	8.40
7a	BT5AZa	Total RNA	1335	9.60
7b	BT5AZb	Total RNA	916	9.60

LGLLK5aza and LGLKJ5aza: large granular lymphocytes derived from rabbit IDs

LFLK and LKKJ and treated with 5 azacitidine, LGLLK con and LGLKJ con: Large

granular lymphocytes derived from rabbits IDs LFLK and LFKJ without treatment,

BTinfa and b: Bovine turbinate infected only with AlHV-1 virus, BT5Aza and b: BT

treated only with 5-azacitidine.
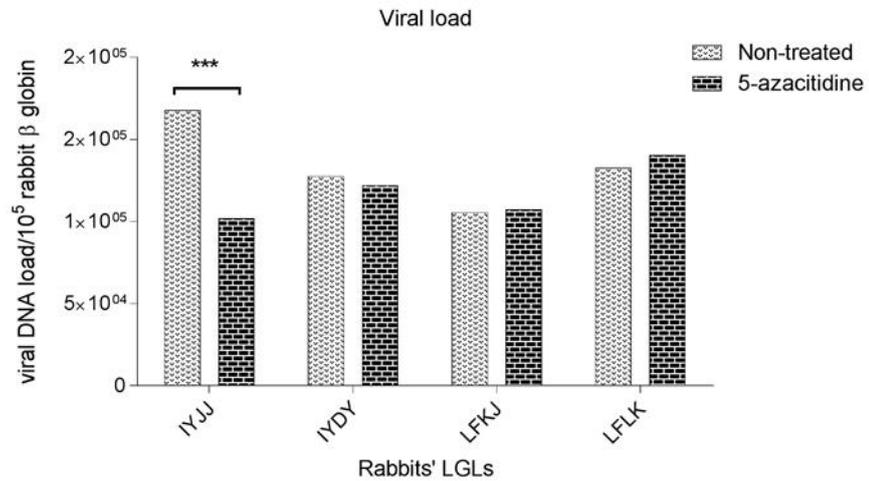
Figure 30 AlHV-1 DNA copy number in rabbit large granular lymphocytes
The figure illustrates the viral load (as DNA copy numbers per 10^5 beta globin DNA copies) in large granular lymphocytes (LGL) from rabbits infected with AlHV-1 C500 strain. Four lines (IYJJ, IYDY, LFKJ, and LFLK) were treated with 5-azacitidine drug or kept without treatment as a control. The viral load was estimated by normalizing the viral DNA copy number per 10^5 rabbit β globin reference gene using qPCR. The primers and probe (FAMAlHV-1) for AlHV-1 ORF3 were used inside the standard sequence and then analysed using Roche Light Cycler 480 system. Two-way ANOVA was performed to do the statistical analysis using Sidak's multiple comparisons test. The primers and probe (FAM) for rabbit β globin reference gene was utilized within the standard sequence.

Figure 31 RNA Integrity Number.
Samples with different RIN numbers show various RNA qualities. A RIN of 1 shows a completely degraded RNA sample while a RIN of 10 demonstrates a fully intact RNA sample. The two peaks in the electropherogram represent the 18S and 28S areas of the ribosomal RNA (rRNA) molecule during the single RNA cleavage. The height of 28S peak reflects the status of the RNA degradation as it dissolves faster than 18S peak. The highest peaks for categories 9 and 10 and the lowest for 1-3. (A), RIN value for LGLLK5aza sample; (B), RIN value for LGLKJ5aza sample; (C), RIN value for BT5Aza sample; (D), RIN value for BTinfa sample.

5.4.3 Mapping results

The raw data containing reads with low sequencing score were filtered and aligned to adapter sequences. All filtered reads were mapped against the reference genome (AlHV-1, Bos-tourus 8 and Oryctolagus-cuniculus 2) in line with known gene exon coordinates using HISAT2 (Kim et al., 2015). The data revealed that the number of trimmed reads ranged from 85.86 million reads for sample (LGLLFLKctrl) to nearly 112 million reads for (LGLLFLK5-aza) (Figure 32). In addition, the number of mapped reads ranged from 23

million reads for (LGL LFLK ctrl) with sample to 35 million reads for

sample (BTinfb) with percentage of 98% (Figure 33). Of those

mapped reads, the correctly mapped reads ranged from 22 million

counts to 35 million counts for the same samples respectively

(Figure 32). The percentage of mapped reads ranged from 92.93%

for sample (LGL LFLK ctrl) to 98.5% for ample (BT5Aza) and those

for correctly mapped reads ranged from 89.8% to 96.8% for sample

(LGL LFKJ5-aza) and (BT5Aza) respectively (Figure 33).
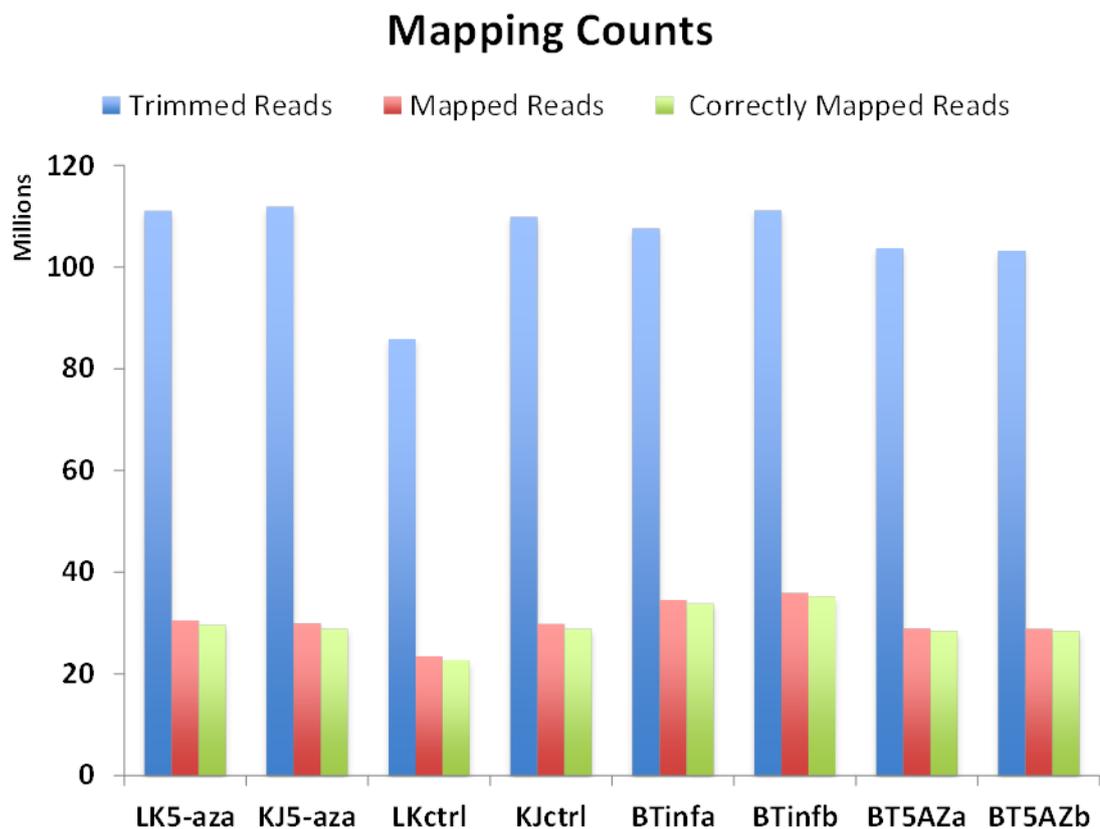
Figure 32 genome coverage for rabbits (Oryctolagus cuniculus2) and cattle (Bos tourus 8).
The figure shows the mapping count of trimmed reads, mapped reads and uniquely and correctly mapped reads for rabbit and cattle transcripts. The highest counts for trimmed reads were nearly 112 million reads and the lowest was 85 million reads. The highest counts for mapped reads were 35.9 million counts while the lowest was 28.9 million counts. The highest counts for uniquely and correctly

mapped reads were 35 million reads whereas the lowest was 22 million counts. Reads with low sequencing score and reads aligned to adapter sequences were filtered. These reads were mapped against the rabbit and cattle gene transcripts in the context of known gene exon coordinates by HISAT mapping tool. LK5-aza; LGLs LFLK treated with 5-azacitidine, KJ5-aza; LGLs LFKJ treated with 5-azacitidine, LKctrl; non-treated LGL LFLK, KJ ctrl; non-treated LGLs LFKJ, BTinfa; BT infected with AlHV-1 sample a, BTinfb; BT infected with AlHV-1 sample b, BT5Aza; BT infected with AlHV-1 virus and treated with 5-azacitidine sample a, BT5AZb; BT infected with AlHV-1 virus and treated with 5-azacitidine sample b.
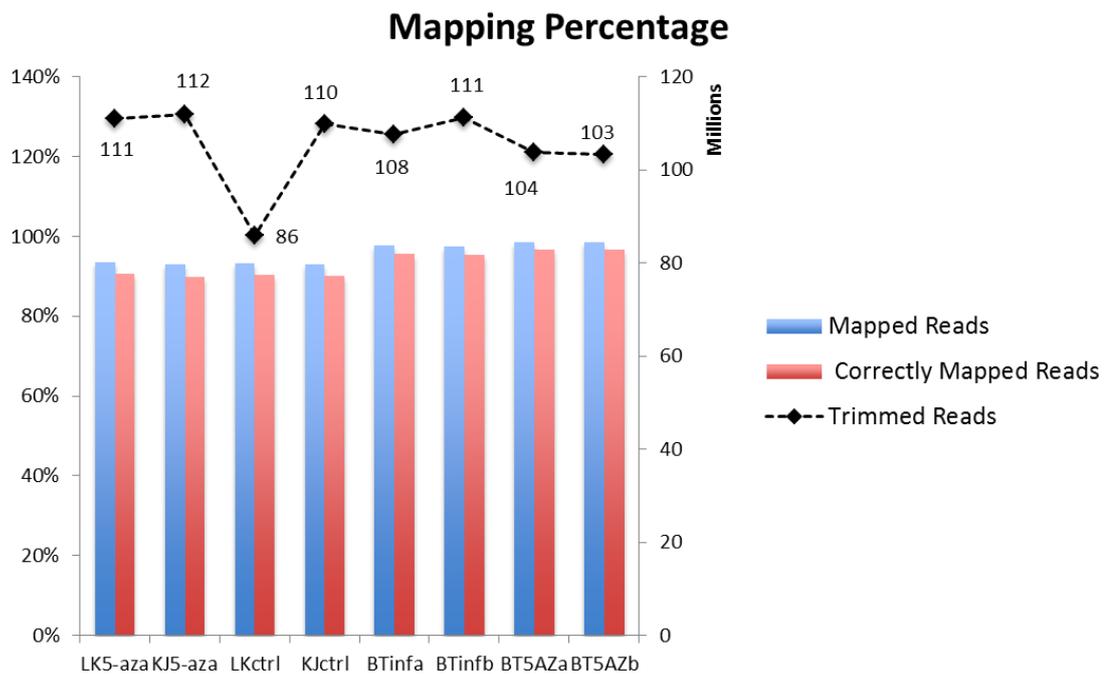
Figure 33 mapping percentage for rabbits (Oryctolagus cuniculus2) and cattle (Bos tourus 8) genomes.

The figure shows the mapping count of mapped reads and uniquely and correctly mapped reads for rabbit and cattle transcripts. The highest counts for trimmed reads were nearly 112 million reads and the lowest was 85 million reads. The highest percentage for mapped reads was 98.5 % while the lowest was 92.9 %. The highest percentage for uniquely and correctly mapped reads was 96.8 % whereas the lowest was 89.8 %. Reads with low sequencing score and reads aligned to adapter sequences were filtered. These reads were mapped against the rabbit and cattle reference transcripts in the context of known gene exon coordinates by HISAT mapping toll. LK5-aza; LGLs LFLK treated with 5-azacitidine, KJ5-aza; LGLs LFKJ treated with 5-azacitidine, LKctrl; non-treated LGL LFLK, KJ ctrl; non-treated LGLs LFKJ, BTinfa; BT infected with AlHV-1 sample a, BTinfb; BT infected with AlHV-1 sample b, BT5Aza; BT infected with AlHV-1 virus and treated with 5-azacitidine sample a, BT5AZb; BT infected with AlHV-1 virus

5.4.4 Differentially expressed host genes

The data obtained from RNA sequencing analysis were run in BioMart- ensemble www.enssembl.org/biomart to identify the transcript descriptions for both species. The excel look up function was used to annotate the differential transcript lists with the clusters identified by David analysis (see sections (8.6 and 8.8)). Five top clusters represented the most affected pathways based on their enrichment score (the highest values) and then the overlapped differentially-expressed genes were identified between the clusters for both species. Table 10 and 11 show the pathways most affected in both species and the differentially expressed host genes expressed in the presence of 5-azacitidine in comparison to infected control as well as the up-regulated and down regulated genes.

The data also revealed that the pathways that had the majority of genes that were down regulated in rabbits are nuclear lumen (cell structural components), lipid biosynthesis, DNA repair, apoptosis and cell cycle in the presence of 5-azacitidine in comparison to the infected control (Table 12-16). The data also showed that the principle affected pathways that had down-regulated genes overall in cattle (BT) cells are those involved in mitochondrion integrity, endosome (structural cell components), organelle lumen (structural cell components), lysosome (structural cell components), and purine and pyrimidine biosynthesis in the presence of 5-azacitidine in comparison to infected control (Table 17-21).

Some of the genes belong to the Bcl-2 family of apoptosis such as BCL2-Associated X Protein (BAX) and BCL2/Adenovirus E1B 19kDa Interacting Protein 2 (BNIP2), which were up-regulated in 5-aza-treated LGLs compared to untreated controls are considered as pro and anti-apoptotic genes respectively while BCL2/Adenovirus E1B 19kDa Interacting Protein 1 (BNIP1) and BCL2/Adenovirus E1B 19kDa Interacting Protein 3 (BNIP3), which were down regulated are thought to be cell death suppressor and cell death activator respectively. The upregulated genes known to inhibit apoptosis were Tumor Necrosis Factor Receptor Superfamily, Member 1B (TNFRSF1B) and X-Linked Inhibitor of Apoptosis, E3 Ubiquitin Protein Ligase (XIAP) (Table 12-16).

There were overlapped genes between the different pathways that are known to act as anti-apoptotic such as Mitogen-Activated Protein Kinase 7 (MAPK 7) and Pim-1 Proto-Oncogene, Serine/Threonine Kinase (PIM1) and genes acting as cell death activator like STE20-Like Kinase (SLK), Receptor (TNFRSF)-Interacting Serine-Threonine Kinase 1 (RIPK1) and Macrophage Erythroblast Attacher (MAEA) (Table 10). In addition, the overlapped genes involved in apoptosis with unknown function, either as pro or anti apoptotic, were Brain and Reproductive Organ-Expressed (TNFRSF1A Modulator) (BRE), Tubulin, Beta Class I (TUBB), Coagulation Factor II (Thrombin) Receptor-Like 3 (PAR4)

and Nuclear Receptor Subfamily 2, Group C, Member 2 (NR2C2) (Table 12-16).

The host differentially-expressed genes in BT cells that were down regulated in 5-aza treated BT cells compared to untreated controls included Polymerase (RNA) III (DNA Directed) Polypeptide F, 39 KDa (POLR3F), Proliferating Cell Nuclear Antigen (PCNA), GATA Binding Protein 2 (GATA2), Polyglutamine Binding Protein 1 (PQBP1), and Kruppel-Like Factor 4 (Gut) (KLF4) (Table 17-21). All of these genes are involved in transcription, translation, post-translational activity and DNA replication.

Table 10 conserved gene matrix of rabbit LGLs differentially expressed genes in the presence of 5-azacitidine in comparison to infected only control.

	Nuclear lumen	Lipid biosynthesis	DNA repair	Cell death and apoptosis	Cell cycle
Nuclear lumen	22 (red) 22 (green)	X	14 7	10 6	9 2
Lipid biosynthesis	X 7 (green)	2	1 0	X	X
DNA repair	14 7 (green)	1 1 (green)	11 9 (green)	11 8	6 3
Cell death and apoptosis	10 4 (green)	X	11 3 (green)	22 12 (green)	7 4
Cell cycle	9 7 (green)	X	6 3 (green)	7 3 (green)	10 15 (green)

The table contains the most affected pathways in rabbits in the presence of 5-azacitidine in comparison to the infected control. The total of differentially expressed genes in 5-aza compared to untreated controls in each cluster

is in bold black font, the up regulated genes are in red font, the down regulated genes are in green font and no overlapping is represented by X. The total number of the genes in the same cluster is split to halves. The line was drawn to split the genes in each pathway to up-regulated and down regulated genes.

Table 11 conserved gene matrix of cattle's differentially expressed genes in the presence of 5-azacitidine in comparison to infected control.

	Mitochondrion	Endosome	Organelle lumen	Lysosome	Purine and pyrimidine biosynthesis
Mitochondrion	20 34	**4** 2	**12** 5	**9** 5	**6** 2
Endosome	**4** 2	8 6	X	**7** 4	X
Organelle lumen	**12** 7	X	18 27	X	**2** 1
Lysosome	**9** 4	**7** 3	X	5 4	**1** 1
Purine and pyrimidine biosynthesis	**6** 4	X	**2** 1	**1** 0	9 8

The table contains the most affected pathways in rabbits in the presence of 5-azacitidine in comparison to the infected control. The total of differentially expressed genes in 5-aza compared to untreated controls in each cluster is in bold black font, the up regulated genes are in red font, the down regulated genes are in green font and no overlapping is represented by X. The total number of the genes in the same cluster is split to halves. The line was drawn to split the genes in each pathway to up-regulated and down regulated ones.

Table 12 up-regulated and down regulated of rabbit gene transcripts involved in lipid bio-synthesis

Gene ID	Gene name/description	p-value	Log FC
Lipid biosynthesis			
ENSOCUG00000025649	**TPI1** triosephosphate isomerase 1	0.003501	-2.1
ENSOCUG00000005722	**CERS6** Cyramide synthase 6	0.007421	1.2
ENSOCUG00000015384	**OXSM 3** Oxacyl-ACP-synthase, mitochondarial	0.018148	-1.2
ENSOCUG00000014801	Uncharacterised protein	0.035263	-3.5
ENSOCUG00000024832	Uncharacterised protein	0.02456	1.2
ENSOCUG00000003499	**FADS 2** fatty acid desaturase 2	0.011453	-3.5
ENSOCUG00000022356	Uncharacterised protein	0.016451	-0.9
ENSOCUG00000004549	**CYP51A1** cytochrome P450, family 51, subfamily A, polypeptide 1	0.040602	-0.8
ENSOCUG00000015843	**MCAT** Malonyl CO:CAP acyltransferase (mitochondorial)	0.040358	-1.2

The table shows the rabbits' genes name involved in the lipid bio-synthesis pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gene transcript up-regulation in 5-aza treated compared to untreated controls. P values are significant ≤ 0.04.

Table 13   up-regulated and down regulated LGL transcripts involved in the nuclear lumen pathway

Gene ID	Gene name/description	p-value	Log FC
Nuclear lumen			
ENSOCUG00000011841	**LIG1** ligase I, DNA, ATP-dependent	0.000739	-1.67635
ENSOCUG00000006346	**PSKH1** protein serine kinase H1 [Source:HGNC Symbol;Acc:HGNC:9529]	0.001056	0.527987
ENSOCUG00000014274	**MNAT1** MNAT CDK-activating kinase assembly factor 1	0.003235	0.0827
ENSOCUG00000015063	**URI1** URI1, prefoldin-like chaperone	0.008231	0.54297
ENSOCUG00000029326	serine/arginine-rich splicing factor 10	0.010133	3.421816
ENSOCUG00000007138	**EXOSC2** exosome component 2	0.01081	-0.1158
ENSOCUG00000003624	**RFC3** replication factor C (activator 1) 3, 38kDa	0.015444	-0.24525
ENSOCUG00000010418	**SAP30** Sin3A-associated protein, 30kDa	0.015697	-1.5082
ENSOCUG00000015154	**MAPK7** mitogen-activated protein kinase 7	0.018904	0.796661

ENSOCUG00000005172	**ZSCAN30** zinc finger and SCAN domain containing 30	0.019611	-0.59829
ENSOCUG00000017150	**HNRNPH1** heterogeneous nuclear ribonucleoprotein H1 (H)	0.022724	0.573093
ENSOCUG00000009275	**PPP1CC** protein phosphatase 1, catalytic subunit, gamma isozyme	0.023446	-1.71405
ENSOCUG00000006975	**NR2C2** nuclear receptor subfamily 2, group C, member 2	0.023524	-0.35353
ENSOCUG00000008735	**MAK16** MAK16 homolog	0.034845	0.145717
ENSOCUG00000005398	**CCNE2** cyclin E2	0.040346	-0.85486
ENSOCUG00000025072	Uncharacterized protein	0.041209	-0.08768
ENSOCUG00000005276	**NF2** neurofibromin 2 (merlin)	0.04231	0.231713
ENSOCUG00000002802	**FANCD2** Fanconi anemia, complementation group D2	0.042852	-0.59767
ENSOCUG00000027381	Uncharacterized protein	0.04345	-0.44872
ENSOCUG00000000322	**PARN** poly(A)-specific ribonuclease	0.043932	-0.19677
ENSOCUG00000007979	**TBP** TATA box binding protein	0.045047	0.407046
ENSOCUG00000024051	**IDH3B** isocitrate dehydrogenase 3 (NAD+) beta	0.045702	0.159479
ENSOCUG00000003848	**TCOF1** Treacher Collins-Franceschetti syndrome 1	0.04798	0.278411
ENSOCUG00000014124	**SENP5 SUMO1**/sentrin specific peptidase 5	0.048374	-0.53382

ENSOCUG00000002541	**BNIP3** BCL2/adenovirus E1B 19kDa interacting protein 3	0.010643	-2.36666
ENSOCUG00000011703	**PQBP1** polyglutamine binding protein 1	0.02809	1.548224
ENSOCUG00000004604	**EME1** essential meiotic structure-specific endonuclease 1	0.009054	0.278554
ENSOCUG00000000408	Uncharacterized protein	0.018583	-1.83834
ENSOCUG00000003879	**CTDP1 CTD** (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1	0.013843	0.268011
ENSOCUG00000010012	**AK4** adenylate kinase 4	0.01612	-3.43355
ENSOCUG00000006610	**MAEA** macrophage erythroblast attacher	0.02166	-0.85255
ENSOCUG00000012396	**ZMYM3** zinc finger, MYM-type 3	0.049674	0.179336
ENSOCUG00000014778	**MORF4L2** mortality factor 4 like 2	0.008387	0.524559
ENSOCUG00000021243	**EXOSC4** exosome component 4	0.026038	-2.99464
ENSOCUG00000024309	**SMAD7** SMAD family member 7	0.002216	0.707824
ENSOCUG00000016827	**ETV6** ets variant 6	0.032107	-0.57071
ENSOCUG00000006642	**XRCC5b** X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)	0.024364	0.140797

ENSOCUG00000014717	Uncharacterized protein	0.013059	2.937234
ENSOCUG00000010624	**POLR3G** polymerase (RNA) III (DNA directed) polypeptide G (32kD)	0.016503	0.121737
ENSOCUG00000017091	**MBIP** MAP3K12 binding inhibitory protein 1	0.028152	0.787543
ENSOCUG00000003587	**ZMIZ1** zinc finger, MIZ-type containing 1	0.048565	0.274723
ENSOCUG00000008568	**ANKRD28** ankyrin repeat domain 28	0.042355	-0.53151
ENSOCUG00000012218	**MRPS26** mitochondrial ribosomal protein S26	0.01552	-0.14257
ENSOCUG00000004933	**SUPV3L1** SUV3-like helicase	0.024445	-0.1046

The table shows the rabbits' genes name involved in the nuclear lumen pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gen up-regulation in 5-aza compared to untreated controls. P values are significant ≤ 0.04.

Table 14 up-regulated and down regulated of LGL transcripts involved in the DNA repair pathway

Gene ID	Gene name/description	p-value	Log FC
DNA repair			
ENSOCUG00000022356	Uncharacterized protein	0.016451	-0.96062
ENSOCUT00000006644	**XRCC5** X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break re-joining)	0.024364476	0.140797037
ENSOCUT00000021649	Uncharacterized protein	0.043449515	-0.448723692
ENSOCUG00000011841	**LIG1** ligase I, DNA, ATP-dependent	0.000739	-1.67635
ENSOCUG00000014274	**MNAT1** MNAT CDK-activating kinase assembly factor 1	0.003235	0.0827
ENSOCUT00000002540	**BNIP3** BCL2/adenovirus E1B 19kDa interacting protein 3	0.010643207	-2.36666053
ENSOCUG00000003624	**RFC3** replication factor C (activator 1) 3, 38kDa	0.015444	-0.24525
ENSOCUG00000002802	**FANCD2** Fanconi anemia, complementation group D2	0.042852	-0.59767
ENSOCUG00000004604	**EME1** essential meiotic structure-specific endonuclease 1	0.009054	0.278554

ENSOCUG00000014778	**MORF4L2** mortality factor 4 like 2	0.008387	0.524559
ENSOCUT00000010697	**BRE** Brain And Reproductive Organ-Expressed (TNFRSF1A Modulator)	0.040783077	0.323317894
ENSOCUG00000017091	**MBIP** MAP3K12 binding inhibitory protein 1	0.028152	0.787543
ENSOCUT00000005398	**CCNE2** Cyclin E2	0.040346282	-0.854855505
ENSOCUG00000012218	**MRPS26** mitochondrial ribosomal protein S26	0.01552	-0.14257
ENSOCUT00000012453	**EIF2AK3** Eukaryotic Translation Initiation Factor 2-Alpha Kinase 3	0.001836194	0.588992333
ENSOCUT00000009875	**XIAP** X-Linked Inhibitor Of Apoptosis, E3 Ubiquitin Protein Ligase	0.023859876	-1.292221674
ENSOCUT00000003039	**SLK** STE20-Like Kinase	0.013003841	2.362971241
ENSOCUT00000011792	**RIPK1** Receptor (TNFRSF)-Interacting Serine-Threonine Kinase 1	0.031130748	0.443478374
ENSOCUT00000014532	**PARP4** Poly (ADP-Ribose) Polymerase Family, Member 4	0.047262905	0.218631005
ENSOCUT00000024642	**BAX** BCL2-Associated X Protein	0.03360173	1.01942528

The table shows the rabbits' genes name involved in the DNA repair pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gene up-regulation in 5-aza treated to untreated controls. P values are significant ≤ 0.04.

Table 15   up-regulated and down regulated LGL transcripts involved in cellular death and apoptosis pathway

Gene ID	Gene name/ description	p-value	Log FC
Cell death and apoptosis			
ENSOCUG00000014274	**MNAT1** MNAT CDK-activating kinase assembly factor 1	0.003235	0.0827
ENSOCUG00000027381	Uncharacterized protein	0.04345	-0.44872
ENSOCUG00000002541	**BNIP3** BCL2/adenovirus E1B 19kDa interacting protein 3	0.010643	-2.36666
ENSOCUG00000006642	**XRCC5** X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break re-joining)	0.024364	0.140797
ENSOCUG00000012451	**EIF2AK3** eukaryotic translation initiation factor 2-alpha kinase 3	0.001836	0.588992
ENSOCUG00000009878	**XIAP E3** ubiquitin-protein ligase XIAP	0.02386	-1.29222
ENSOCUG00000011791	**RIPK1** receptor (TNFRSF)-interacting serine-threonine kinase 1	0.031131	0.443478
ENSOCUG00000006423	**BAX** BCL2-associated X protein	0.033602	1.019425
ENSOCUG00000014529	**PARP4** poly (ADP-ribose) polymerase family, member 4	0.047263	0.218631
ENSOCUG00000003035	**SLK** STE20-like kinase	0.013004	2.362971

ENSOCUG00000010695	**BRE** brain and reproductive organ-expressed (TNFRSF1A modulator)	0.040783	0.323318
ENSOCUG00000015154	**MAPK7** mitogen-activated protein kinase 7	0.018904	0.796661
ENSOCUG00000006975	**NR2C2** nuclear receptor subfamily 2, group C, member 2	0.023524	-0.35353
ENSOCUG00000005276	**NF2** neurofibromin 2 (merlin)	0.04231	0.231713
ENSOCUG00000007979	**TBP** TATA box binding protein	0.045047	0.407046
ENSOCUG00000006610	**MAEA** macrophage erythroblast attacher	0.02166	-0.85255
ENSOCUG00000014717	Uncharacterized protein	0.013059	2.937234
ENSOCUG00000001542	**RIPK2** receptor-interacting serine-threonine kinase 2	0.001105	-0.59341
ENSOCUG00000000172	**VAV3** vav 3 guanine nucleotide exchange factor	0.006063	1.138616
ENSOCUG00000007938	**ALDH1A3** aldehyde dehydrogenase 1 family, member A3	0.010062	0.170754
ENSOCUG00000012028	**PIM1** Pim-1 proto-oncogene, serine/threonine kinase	0.019968	0.398831
ENSOCUG00000004627	**APH1B** APH1B gamma secretase subunit	0.020786	0.424591
ENSOCUG00000015236	Uncharacterized protein	0.021786	-1.71366
ENSOCUG00000006277	**BNIP2** BCL2/adenovirus E1B 19kDa interacting protein 2	0.034452	0.307168
ENSOCUG00000000040	**BNIP1** BCL2/adenovirus E1B 19kDa interacting protein 1	0.025577	-3.16923

ENSOCUG00000014127	**TNFRSF1B** tumor necrosis factor receptor superfamily, member 1B	0.048762	-1.25577
ENSOCUG00000002187	**TUBB** tubulin, beta class I	0.038957	-0.37109
ENSOCUG00000006958	**TMEM173** transmembrane protein 173	0.039155	0.522144
ENSOCUG00000028156	Uncharacterized protein	0.020263	-0.21927
ENSOCUG00000017921	**BCAP31** B-cell receptor-associated protein 31	0.043509	-0.3856
ENSOCUG00000015961	**PPP2CA** Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	0.007485	0.092106
ENSOCUG00000015961	**PPP2CA** Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	0.007485	0.092106
ENSOCUG00000015413	**PDCD10** programmed cell death 10	0.008327	0.106757
ENSOCUG00000026354	**PGF** placental growth factor	0.032233	0.349872

The table shows the rabbits' genes name involved in the cellular death and apoptosis pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gene up-regulation in 5-aza treated in comparison to untreated controls. P values are significant ≤ 0.04.

Table 16 up-regulated and down regulated LGL transcripts involved in the cell cycle pathway

Gene ID	Gene name/ description	p-value	Log FC
Cell Cycle			
ENSOCUG00000014274	**MNAT1** MNAT CDK-activating kinase assembly factor 1	0.003235	0.0827
ENSOCUG00000006423	**BAX** BCL2-associated X protein	0.033602	1.019425
ENSOCUG00000015154	**MAPK7** mitogen-activated protein kinase 7	0.018904	0.796661
ENSOCUG00000006975	**NR2C2** nuclear receptor subfamily 2, group C, member 2	0.023524	-0.35353
ENSOCUG00000006610	**MAEA** macrophage erythroblast attacher	0.02166	-0.85255
ENSOCUG00000012028	**PIM1** Pim-1 proto-oncogene, serine/threonine kinase	0.019968	0.398831
ENSOCUG00000002187	**TUBB** tubulin, beta class I	0.038957	-0.37109
ENSOCUG00000011841	**LIG1** ligase I, DNA, ATP-dependent	0.000739	-1.67635
ENSOCUG00000005398	**CCNE2** cyclin E2	0.040346	-0.85486
ENSOCUG00000002802	**FANCD2** Fanconi anemia, complementation group D2	0.042852	-0.59767
ENSOCUG00000004921	**DCLRE1A** DNA cross-link repair 1A	0.009148	0.087371

ENSOCUG00000009275	**PPP1CC** protein phosphatase 1, catalytic subunit, gamma isozyme	0.023446	-1.71405
ENSOCUG00000014124	**SENP5** SUMO1/sentrin specific peptidase 5	0.048374	-0.53382
ENSOCUG00000007827	**SASS6** SAS-6 centriolar assembly protein	0.00895	-0.42226
ENSOCUG00000011298	**FAM83D** family with sequence similarity 83, member D	0.018536	-1.03649
ENSOCUG00000009879	**STAG2** cohesin subunit SA-2	0.021314	0.364604
ENSOCUG00000016706	**KIF11** kinesin family member 11	0.027452	-0.19417
ENSOCUG00000006348	**PSMB10** proteasome subunit beta 10	0.028911	0.594683323
ENSOCUG00000001418	**PSMD14** proteasome 26S subuni, non-ATPase 14	0.029403	-0.62192
ENSOCUG00000001097	**CGRRF1** cell growth regulator with ring finger domain 1	0.035752	-1.73356
ENSOCUG00000000791	**RPL24** ribosomal protein L24	0.043664	-1.61249
ENSOCUG00000003623	**ITGB1** integrin, beta 1	0.00548	4.106411
ENSOCUG00000014903	**RGCC** regulator of cell cycle	0.042739	0.620927
ENSOCUG00000010944	**NEDD1** neural precursor cell expressed, developmentally down-regulated 1	0.033882	-1.16131

ENSOCUG00000005880	**PDS5A** PDS5 cohesin associated factor A	0.048663	0.879025

The table shows the rabbits' genes name involved in the cell cycle pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gen up-regulation in 5-aza treated compared to untreated controls. P values are significant ≤ 0.04.

Table 17 up-regulated and down regulated of cattle BT transcripts involved in the mitochondrion integrity pathway

Gene ID	Gene name/description	p-value	Log FC
Mitochondrion			
ENSBTAG00000006272	**nth**-like DNA glycosylase 1	0.017064	0.52413
ENSBTAG00000015294	**COX10** heme A:farnesyltransferase cytochrome c oxidase assembly factor	0.016254	-0.62018
ENSBTAG00000045703	cytochrome c oxidase assembly homolog 15 (yeast)	0.018873	4.340498
ENSBTAG00000007332	Uncharacterized protein	0.029911	-7.33293
ENSBTAG00000005791	uroporphyrinogen III synthase	0.036102	-4.84748
ENSBTAG00000047462	Uncharacterized protein	0.045345	-2.40732
ENSBTAG00000046690	Uncharacterized protein	0.023888	1.060966
ENSBTAG00000011931	**CD63** antigen	0.028907	-0.32749
ENSBTAG00000006065	proliferating cell nuclear antigen	0.004827	-2.10674
ENSBTAG00000015006	mitochondrial ribosomal protein L11	0.009037	-1.45233
ENSBTAG00000047906	mitochondrial ribosomal protein L36	0.015561	0.068833

ENSBTAG00000015567	translocase of inner mitochondrial membrane 44 homolog (yeast)	0.016698	-0.84677
ENSBTAG00000015833	**DEAH** (Asp-Glu-Ala-His) box helicase 30	0.016792	-8.03291
ENSBTAG00000001780	serine--tRNA ligase, mitochondrial precursor	0.019816	-1.32708
ENSBTAG00000001780	serine--tRNA ligase, mitochondrial precursor	0.029509	-1.15327
ENSBTAG00000009091	ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)	0.036661	1.759817
ENSBTAG00000001107	calcyclin binding protein	0.040685	0.183786
ENSBTAG00000006978	hydroxysteroid (17-beta) dehydrogenase 4	0.046434	0.519715
ENSBTAG00000009839	glutathione S-transferase kappa 1	0.049364	-0.69816
ENSBTAG00000015098	Rab9 effector protein with kelch motifs	0.008312	7.027562
ENSBTAG00000004019	premelanosome protein	0.020502	-1.15137
ENSBTAG00000008135	SRA stem-loop-interacting RNA-binding protein, mitochondrial	0.004899	-3.087
ENSBTAG00000011760	mitochondrial ribosomal protein L14	0.011364	-2.85854
ENSBTAG00000002474	**MAD1** mitotic arrest deficient-like 1 (yeast)	0.012979	-1.7572
ENSBTAG00000011809	**nudix** (nucleoside diphosphate linked moiety X)-type motif 8	0.014434	4.202557
ENSBTAG00000020898	carbohydrate (chondroitin 4) sulfotransferase 12	0.014673	4.543596

ENSBTAG00000018770	hydroxysteroid dehydrogenase like 1	0.015835	2.683234
ENSBTAG00000014653	spinster homolog 1 (Drosophila)	0.016729	1.300334
ENSBTAG00000009682	LOC532995 protein; Uncharacterized protein	0.017564	-4.66056
ENSBTAG00000008730	sideroflexin 4	0.019208	0.333715
ENSBTAG00000008077	solute carrier family 37 (glucose-6-phosphate transporter), member 4	0.020471	-3.86726
ENSBTAG00000043959	platelet derived growth factor C	0.020528	-5.22392
ENSBTAG00000019854	ATP-binding cassette, sub-family E (OABP), member 1	0.021995	-1.79642
ENSBTAG00000021780	**SCO1** cytochrome c oxidase assembly protein	0.02314	-0.33666
ENSBTAG00000003177	solute carrier family 25 (pyrimidine nucleotide carrier), member 33	0.023753	-0.50344
ENSBTAG00000001463	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase	0.024295	1.709409
ENSBTAG00000015255	**ST6** (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	0.031701	0.68046
ENSBTAG00000024781	39S ribosomal protein L17, mitochondrial	0.031911	-0.41802
ENSBTAG00000008629	mitochondrial fission regulator 1	0.036049	-0.20923
ENSBTAG00000004240	thymopoietin	0.037698	-4.9725

ENSBTAG00000006398	translocase of outer mitochondrial membrane 7 homolog (yeast)	0.037962	-5.25928
ENSBTAG00000000301	Glycerol kinase; Uncharacterized protein	0.038167	-0.30485
ENSBTAG00000001962	mitochondrial ribosomal protein S27	0.040067	-0.25581
ENSBTAG00000021790	apolipoprotein O-like	0.040527	-2.04374
ENSBTAG00000047836	**NADH** dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	0.041881	2.67723
ENSBTAG00000008438	ADP-ribosylation factor interacting protein 1	0.042427	2.705246
ENSBTAG00000012107	solute carrier family 25 (mitochondrial iron transporter), member 28	0.042458	-4.15644
ENSBTAG00000005903	low density lipoprotein receptor-related protein 5	0.042508	3.191428
ENSBTAG00000012072	**NADH** dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa	0.046031	-2.11016
ENSBTAG00000030566	**GLE1** RNA export mediator	0.046476	-0.51529
ENSBTAG00000034827	platelet derived growth factor D	0.046738	6.932076
ENSBTAG00000006966	**nurim** (nuclear envelope membrane protein)	0.046957	-3.65053
ENSBTAG00000013282	**NECAP** endocytosis associated 2	0.048582	-0.20713
ENSBTAG00000046671	Wolfram syndrome 1 (wolframin)	0.049274	2.497423

The table shows cattle gene names involved in the mitochondrion pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gen up-regulation in 5-aza treated compared to untreated controls. P values are significant ≤ 0.04.

Table 18 up-regulated and down regulated of cattle BT transcripts involved in endosomal pathways

Gene ID	Gene name/description	p-value	Log FC
endosome			
ENSBTAG00000046690	Uncharacterized protein	0.023888	1.060966
ENSBTAG00000011931	**CD63** antigen	0.028907	-0.32749
ENSBTAG00000016094	solute carrier family 36 (proton/amino acid symporter), member 1	0.002693	-3.00111
ENSBTAG00000018202	cytochrome b561 family, member A3	0.013663	-3.80245
ENSBTAG00000039968	transmembrane protein 55A	0.023713	3.109691
ENSBTAG00000034689	zinc and ring finger 1, E3 ubiquitin protein ligase	0.036233	1.176685
ENSBTAG00000003745	**WD** repeat domain 48	0.037767	0.725065
ENSBTAG00000015098	**Rab9** effector protein with kelch motifs	0.008312	7.027562
ENSBTAG00000004019	premelanosome protein	0.020502	-1.15137
ENSBTAG00000046467	protein tyrosine phosphatase type IVA, member 3	0.009776	-2.61277
ENSBTAG00000018915	WASH complex subunit FAM21	0.01622	3.690243

ENSBTAG00000025297	membrane magnesium transporter 1	0.018348	10.41085
ENSBTAG00000003565	tripartite motif containing 3	0.022235	4.037245
ENSBTAG00000021639	activity-regulated cytoskeleton-associated protein	0.040311	-0.17954

The table shows cattle's genes name involved in the endosome pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gen up-regulation in 5-aza-treated compared to untreated controls. P values are significant ≤ 0.04.

Table 19 up-regulated and down regulated of cattle BT gene transcripts involved in the organelle lumen pathway

Gene ID	Gene name/description	p-value	Log FC
Organelle lumen			
ENSBTAG00000006272	nth-like DNA glycosylase 1	0.017064	0.52413
ENSBTAG00000008825	polymerase (RNA) III (DNA directed) polypeptide F, 39 kDa	0.038073	-1.69785
ENSBTAG00000006065	proliferating cell nuclear antigen	0.004827	-2.10674
ENSBTAG00000015006	mitochondrial ribosomal protein L11	0.009037	-1.45233
ENSBTAG00000047906	mitochondrial ribosomal protein L36	0.015561	0.068833
ENSBTAG00000015567	translocase of inner mitochondrial membrane 44 homolog (yeast)	0.016698	-0.84677
ENSBTAG00000015833	**DEAH** (Asp-Glu-Ala-His) box helicase 30	0.016792	-8.03291
ENSBTAG00000001780	serine--tRNA ligase, mitochondrial precursor	0.019816	-1.32708
ENSBTAG00000001780	serine--tRNA ligase, mitochondrial precursor	0.029509	-1.15327
ENSBTAG00000009091	ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)	0.036661	1.759817
ENSBTAG00000001107	calcyclin binding protein	0.040685	0.183786

ENSBTAG00000006978	hydroxysteroid (17-beta) dehydrogenase 4	0.046434	0.519715
ENSBTAG00000009839	glutathione S-transferase kappa 1	0.049364	-0.69816
ENSBTAG00000011360	pre-mRNA-splicing factor SYF1	0.00146	-1.66151
ENSBTAG00000003849	guanine nucleotide binding protein-like 2 (nucleolar)	0.005061	-3.48445
ENSBTAG00000016606	polycomb group ring finger 1	0.00592	7.051528
ENSBTAG00000019707	GATA binding protein 2	0.007854	-0.29559
ENSBTAG00000007323	cleavage and polyadenylation specific factor 6, 68kDa	0.008762	1.478988
ENSBTAG00000012777	serum response factor	0.008861	-1.61675
ENSBTAG00000009562	zinc finger protein 593	0.0127	-1.68797
ENSBTAG00000016332	inhibitor of growth family, member 3	0.015852	-2.50246
ENSBTAG00000012575	macrophage erythroblast attacher	0.016016	1.536743
ENSBTAG00000008190	sulfatase modifying factor 2	0.0175	-0.57121
ENSBTAG00000007480	cold inducible RNA binding protein	0.018909	8.773147
ENSBTAG00000038409	methyl-CpG binding domain protein 3	0.01934	-0.93366
ENSBTAG00000026585	transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)	0.019828	1.808792

ENSBTAG00000003697	**TAR** DNA binding protein	0.021319	-6.7882
ENSBTAG00000015946	retinoblastoma binding protein 5	0.021543	1.793306
ENSBTAG00000020490	**NACC** family member 2, BEN and BTB (POZ) domain containing	0.02163	-4.77528
ENSBTAG00000005076	synovial apoptosis inhibitor 1, synoviolin	0.025081	1.024949
ENSBTAG00000021192	glioma tumor suppressor candidate region gene 2	0.025745	0.595285
ENSBTAG00000006112	SprT-like N-terminal domain	0.027078	1.98873
ENSBTAG00000010384	cell division cycle 6	0.0276	-1.29413
ENSBTAG00000021845	striatin, calmodulin binding protein 3	0.028528	-1.55617
ENSBTAG00000016271	small nuclear ribonucleoprotein polypeptide F	0.029432	-1.43929
ENSBTAG00000003423	DEAD (Asp-Glu-Ala-Asp) box helicase 24	0.030951	-2.64543
ENSBTAG00000018498	polyglutamine binding protein 1	0.031957	-5.22647
ENSBTAG00000017582	checkpoint kinase 1	0.032011	4.752555
ENSBTAG00000008905	folliculogenesis specific bHLH transcription factor	0.03221	-0.81315
ENSBTAG00000012699	exosome component 1	0.034345	-1.84211
ENSBTAG00000008704	fidgetin	0.038805	-1.51006

ENSBTAG00000019846	alkB homolog 2, alpha-ketoglutarate-dependent dioxygenase	0.03964	11.41241
ENSBTAG00000017219	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	0.044658	3.336827
ENSBTAG00000020355	Kruppel-like factor 4 (gut)	0.045216	-9.77397
ENSBTAG00000003934	paraspeckle component 1	0.047011	5.469299

The table shows cattle's genes name involved in the organelle lumen pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gen up-regulation in 5-aza treated compared to untreated controls. P values are significant ≤ 0.04.

Table 20 up-regulated and down regulated of cattle BT gene transcripts involved in lysosomal pathways

Gene ID	Gene name/description	p-value	Log FC
Lysosome			
ENSBTAG00000011960	glutamic-oxaloacetic transaminase 1, soluble	0.009702	0.410793
ENSBTAG00000046690	Uncharacterized protein	0.023888	1.060966
ENSBTAG00000011931	CD63 antigen	0.028907	-0.32749
ENSBTAG00000016094	solute carrier family 36	0.002693	-3.00111
ENSBTAG00000018202	cytochrome b561 family, member A3	0.013663	-3.80245
ENSBTAG00000039968	transmembrane protein 55A	0.023713	3.109691
ENSBTAG00000034689	zinc and ring finger 1, E3 ubiquitin protein ligase	0.036233	1.176685
ENSBTAG00000003745	WD repeat domain 48	0.037767	0.725065
ENSBTAG00000000484	hyaluronoglucosaminidase 2	0.042501	-0.97983

The table shows cattle's genes name involved in the lysosome pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gen up-regulation in 5-aza-treated compared to untreated controls. P values are significant ≤ 0.04.

Table 21 up-regulated and down regulated of cattle BT gene transcripts involved in purine and pyrimidine biosynthesis pathways

Gene ID	Gene name/description	p-value	Log FC
Purine and pyrimidine biosynthèses			
ENSBTAG00000011960	glutamic-oxaloacetic transaminase 1, soluble	0.009702	0.410793
ENSBTAG00000006272	nth-like DNA glycosylase 1	0.017064	0.52413
ENSBTAG00000008825	polymerase (RNA) III (DNA directed) polypeptide F, 39 kDa	0.038073	-1.69785
ENSBTAG00000015294	**COX10** heme A:farnesyltransferase cytochrome c oxidase assembly factor	0.016254	-0.62018
ENSBTAG00000045703	cytochrome c oxidase assembly homolog 15 (yeast)	0.018873	4.340498
ENSBTAG00000007332	Uncharacterized protein	0.029911	-7.33293
ENSBTAG00000005791	uroporphyrinogen III synthase	0.036102	-4.84748
ENSBTAG00000047462	Uncharacterized protein	0.045345	-2.40732
ENSBTAG00000015509	nicotinamide phosphoribosyltransferase	0.00014	6.215473
ENSBTAG00000019274	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	0.006476	10.49776

ENSBTAG00000007758	phosphodiesterase 10A	0.011243	4.872506
ENSBTAG00000013776	inosine triphosphatase	0.013862	-0.93005
ENSBTAG00000003025	**NME/NM23** nucleoside diphosphate kinase 6	0.032176	5.09935
ENSBTAG00000016552	**NME/NM23** nucleoside diphosphate kinase 3	0.036335	14.84213
ENSBTAG00000004549	uracil phosphoribosyltransferase (FUR1) homolog	0.037439	-5.2164
ENSBTAG00000013825	branched chain amino-acid transaminase 1, cytosolic	0.044216	3.56528
ENSBTAG00000002689	**NME/NM23** family member 7	0.048514	-0.52516

The table shows cattle's genes name involved in the purine and pyrimidine biosynthesis pathway along with their gene IDs. (-) values of log FC indicates gene down regulation where positive values indicate gen up-regulation in 5-aza-treated compared to untreated controls. P values are significant ≤ 0.04.

5.4.5 Differentially-expressed virus genes detected by RNA-Seq

The sequence analysis revealed that one virus gene differentially expressed (ORF 8, down-regulated in 5-aza group compared to controls, figure 34) in the cattle samples whereas two virus genes (ORF 21 and ORF 7) were up-regulated in the rabbit samples in the presence of 5-azacitidine in comparison to infected control (Figure 34). Table 22 shows viral DNA load and viral gene transcripts in rabbit LGL and cattle BT cells in the presence and absence of 5-azacitidine as measured by PCR and qPCR.

Table 22 summary of viral gene transcripts and viral DNA load in rabbit LGL and cattle BT cells in the presence and absence of 5-azacitidine as measured by qPCR and PCR approaches.

Cell type/treatment	Viral DNA	Transcripts
Non-treated LGLs		
IYJJ	**+**	Undetectable level of all transcripts
IYDY	**+**	Undetectable level of all transcripts
LFKJ	**+**	Low level ORF25, ORF63, ORFA4.5 and undetectable level of ORF50, ORF73, ORF A.9.5
LFLK	**+**	Low level of all transcripts
5-aza treated LGLs		
IYJJ	**+**	Very low level of ORF25 and ORF A4.5
IYDY	**+**	Undetectable level of all transcripts
LFKJ	**+**	Low level ORF25, ORF A4.5 and undetectable level of ORF73, OR50, ORF63, ORF A9.5
LFLK	**+**	Low level of all transcripts
infected only BT cells	**+**	High level of ORF50, ORF25, ORF A4.5 and low level of ORF73, ORF63
5-aza treated and infected BT cells	**+**	Low level of ORF50, ORF25, ORF A4.5 and undetectable level of ORF73, ORF63
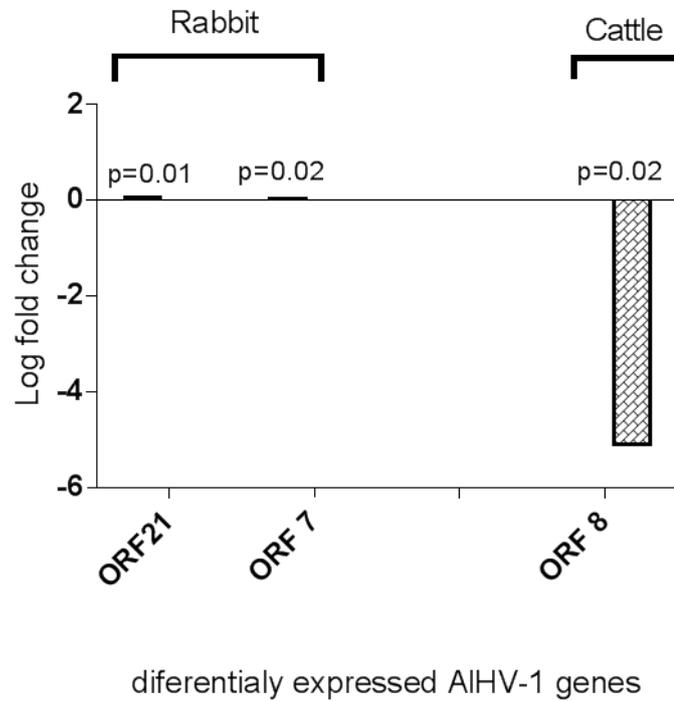
Figure 34 differentially expressed AlHV-1 virus in rabbits' LGLs and BT cells.
The figure shows the differentially expressed genes (ORF 8) in bovine turbinate (BT) cells and ORF21 and ORF 7 in rabbits' LGLs in the presence of 5-azacitidine in comparison to infected control. The figure also shows that ORF 8 gene was down regulated (log FC) whereas ORF 7 and ORF 21 were up-regulated. The genes were significantly expressed with p value < 0.05.

5.4.6 David clustering

The human orthologue gene ID's lists for both rabbit and cattle were uploaded in David's software http//david.ncifcrf.gov in order to cluster the functionally annotated genes. The results showed that there were 93 and 100 clusters in rabbit and cattle species respectively (see the sections (8.2 and 8.3) in appendix). These were reduced to 15 and 11 clusters for both rabbit and cattle

respectively (see sections (8.7 and 8.9) in appendix) based upon the enrichment score by which the clusters trimmed. These clusters with enrichment score of ≥1 were kept for further analysis.

5.4.7 IPA results

Ingenuity pathway analysis (IPA) was performed on the data obtained from RNA sequencing analysis. For this it was necessary to generate one to one orthologues between our species (cattle and rabbit) and human using BioMart ensemble www.ensemble.org . The data obtained is placed in sections 8.6 and 8.7 in the appendix. The IPA system was used to investigate the differentially regulated pathways in the comparison between the treated (5-aza) and untreated cells (cattle or rabbit). The entire data sets were uploaded to the IPA software for global analysis. The top five significant canonical pathways affected in LGLs in the presence of 5-azacitidine in comparison to control LGLs were related to cell death and T cell activation (Table 23). The principal networks/pathways recognised contained those connected with: cell death and survival; cellular growth and proliferation; lymphoid tissue structure and development; lipid metabolism and the humoral immune response (Table 23). In general, genes within the pathways were down regulated in 5aza treated LGLs compared to untreated controls and the overall impact of this was possible induction of apoptosis and immune response genes. In addition, the top five significant canonical pathways affected in BT cells in the presence of 5-azacitidine in comparison to control BT cells were related to: mRNA translation and mitochondrion structure (Table 24). The most significant networks/pathways observed are those associated with

cell cycle, gene expression, DNA replication and cancer (Table 24). The balance of genes in the majority of affected pathways were down regulated in 5aza treated in BT cells compared to untreated controls and the overall affect was a decrease in transcription and translation.

Table 23 summary of ingenuity pathway analysis of rabbit LGLs in the presence of 5-azacitidine

Top Canonical Pathways		
	p-value	overlap
Ceramide Signalling	9.18E-05	8.8 % 7/80
Induction of Apoptosis by AlHV-1	1.40E-04	10.0 % 6/60
Death Receptor Signalling	2.21E-04	7.6 % 7/92
Tec Kinase Signalling	2.51E-04	5.7 % 9/157
NF-B Activation by Viruses	4.12E-04	8.2 % 6/73
Molecular and cellular functions		
	p-value	#Molecules
Cell Death and Survival	1.32E-02 - 9.08E-06	98
Cellular Function and Maintenance	1.32E-02 - 9.08E-06	49
Cellular Development	1.32E-02 - 1.34E-05	66
Cellular Growth and Proliferation	1.32E-02 - 1.34E-05	95
Lipid Metabolism	1.32E-02 - 3.65E-05	31
ID Associated Network Functions		Score
Antimicrobial Response, Cell Morphology, Cellular Assembly		51

and Organization	
Cancer, Organismal Injury and Abnormalities, Gastrointestinal Disease	41
Auditory and Vestibular System Development and Function, Cellular Development, Cellular Growth and Proliferation	35
Lymphoid Tissue Structure and Development, Organ Development, DNA Replication, Recombination, and Repair	33
Cell Death and Survival, Cancer, Organismal Injury and Abnormalities	30

IPA uses Fisher's Exact test to calculate p value

Table 24 summary of ingenuity pathway analysis of bovine turbinate (BT) cells in the presence of 5-azacitidine

Top Canonical Pathways		
	p-value	Overlap
Regulation of eIF4 and p70S6K Signaling	2.35E-04	6.8 % 10/146
EIF2 Signaling	1.43E-03	5.4 % 10/184
mTOR Signaling	1.61E-03	5.3 % 10/187
2-oxobutanoate Degradation I	2.89E-03	40.0 % 2/5
Oxidative Phosphorylation	2.89E-03	6.4 % 7/109

Molecular and Cellular Functions		
	p-value	#Molecules
Cellular Assembly and Organization	2.41E-02 - 2.67E-06	43
Gene Expression	1.73E-02 - 1.52E-04	62
RNA Damage and Repair	1.33E-02 - 1.52E-04	7
RNA Post-Transcriptional Modification	1.52E-04 - 1.52E-04	6
DNA Replication, Recombination, and Repair	2.24E-02 - 1.71E-04	35

ID Associated Network Functions	Score
Cancer, Organismal Injury and Abnormalities, Gastrointestinal Disease	53
Cancer, Organismal Injury and Abnormalities, Gastrointestinal Disease	41
Cell Cycle, DNA Replication, Recombination, and Repair, Gene Expression	36
Ophthalmic Disease, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders	36
Cell Cycle, DNA Replication, Recombination, and Repair, Developmental Disorder	34

IPA uses Fisher's Exact test to calculate p value

5.5 Discussion

In this study the aim was to investigate the pathways affected by AlHV-1 in rabbit LGLs and cattle BT cells infected with AlHV-1 in the presence or absence of 5-azacitidine. The viral DNA copy number was used to identify the presence of AlHV-1 virus in LGLs samples. The RNA integrity number (RIN) results showed that all samples exhibited high quality RNA. This is important as the degradation of RNA can affect the gene expression level (Romero et al., 2014, Schroeder et al., 2006). The RNA sequencing analysis revealed that the mapping efficiency (the percentage of reads aligned to host genome or transcripts) was high (92% to 98%) indicating that high percentage of the RNA reads matched to rabbit and cattle transcripts (Benjamin et al., 2014, Mortazavi et al., 2008).

The pathway analysis indicated that cell death and T cell accumulation, proliferation and immune response pathways were augmented in 5-aza-treated LGLs in comparison to infected only control LGL suggesting that LGLs have selected genes involved in T cell activation, proliferation and apoptosis that are normally suppressed in infected untreated cells. This makes sense as the virus requires inhabiting the infected T cell without killing the cell or being removed by an activation process. From other published work (Swa et al., 2001) it is known that the infected LGLs do not exhibit significant apoptosis but do have a heightened activation state with respect to T cell cytotoxicity and constitutive activation of the T cell

receptor. Thus the RNA-Seq study is recording a net effect based on the genes differentially regulated by 5-aza, that may not affect other activation genes and pathways. This picture is also in agreement with observations made by other authors (Palmeira et al., 2013, Parameswaran et al., 2014, Russell et al., 2012) who found the similar pathways (in the *in vivo* study) from rabbit and cattle Lymph nodes infected with AlHV-1 and LGLs derived from rabbits infected with AlHV-1 virus. In addition, the results are in line with analysis of transcription profiles in lymph node tissues from OvHV-2 infected cattle (Meier-Trummer et al., 2009). As mentioned above the results showed that a number of genes involved in the various pathways were either up or down regulated in the presence of 5-aza in comparison to infected control, allowing a clue to overall function only when the activity of the particular genes is known. Among those genes were Bcl-2 family members, which can be either anti-apoptotic or pro-apoptotic (Czabotar et al., 2014, Tsujimoto, 1998). In this study, the overall effect of these changes was a net pro-apoptotic state in 5-aza-treated cells, but with no time course aspect to the analysis, it is difficult to see if there are any time related changes. However, the analysis was done when the LGLs were in a steady state of growth in IL-2 with a stable phenotype. The dysregulation of this gene family could also be under the effect of AlHV-1 ORF A4.5 or ORF A9 which has similarities to the Bcl-2 family (Hart et al., 2007, Mills et al., 2003). However, their role

whether they act as anti-apoptotic or cell death activator is yet unknown. The presence of the viral bcl-2 orthologues indicates that these are required in the virus life cycle. Importantly, the study only identifies changes associated with 5-aza treatment as both the control and treated cells were infected. Regarding the objective to this study it was hoped that the changes would represent those seen as part of a latent state induced by 5-aza. The impact of 5-azacitidine on the expression of OvHV-2 Ov4.5, the homologue of AlHV-1 A4.5, has been recorded where it increased in expression in cattle LGLs but not rabbit LGLs (Thonur et al., 2006). It is an advantage to the virus to escape the recognition by the immune system in order to survive and replicate within cells. Latency is the approach by which the virus can avoid the immune evasion by the expression of ORF73 LANA protein (Coscoy, 2007). 5-azacitidine is a nucleoside analogue which inhibits the methyltransferase enzyme and hence altering gene expression (Haaf, 1995, Jones, 1985). It was found that the drug can establish latency in LGLs from OvHV-2 infected rabbits (Thonur et al., 2006) but failed to control latency III in primary lymphoma cells infected with recombinant Epstein-Barr virus (Anastasiadou et al., 2005). Two genes (XIAP and TNFRSF1B) which are known as inhibitors of apoptosis (Morizane et al., 2005, Schall et al., 1990) were down regulated in the presence of 5-azacitidine indicating that the virus may affect these genes in order to alter cell survival. It was demonstrated that KSHV ORF73 LANA

was involved in tumour necrosis factor regulatory network which affects in the survival of latently infected cells (Si and Robertson, 2006, Uppal et al., 2014).

LGLs are known to be predominantly productive cycle programmed or latently programmed or have a mixture of both productive and latent virus cycles (Rosbottom et al., 2002, Thunor et al., 2006). The analysis demonstrated that many host genes involved in the various pathways; in particular cell death and cell cycle, acting as both cell death inhibitors and activators, were either up or down regulated in the presence of 5-aza compared to non-treated controls. In addition, two viral genes (ORF7 and ORF21), where their role in the life cycle and the pathogenesis of MCF is unknown, were up-regulated in the presence of 5-aza-treated group in comparison to non-treated controls. Moreover, the viral gene profiles from the previous chapter indicated that the expression of ORF73 and ORF50 supporting latent and productive virus cycle were very low (Table 22). Taking these observations together, one could extrapolate that it is not obvious that the cells support either productive or latent virus cycle or both.

The pathway analysis in treated BT cells versus infected controls is likely to identify productive cycle versus latency cycle changes better than in LGLs as the effect of 5-aza on infected BT cells was more profound (induction of a latency phenotype with respect to lack of CPE and reduction of productive cycle gene transcripts).

Overall, genes involved in mitochondrion integrity, mRNA translation, regulator of gene expression, cell cycle and DNA replication, post transcriptional modification and genes known to be associated with neoplasia were down regulated in 5-aza treated infected BT cells compared to untreated BT cells. These effects are compatible with the fact that the virus replicates in these cells and establishes a productive/lytic infection and may reveal the latency programme in the transcriptome of the cells treated with 5-aza. The results also showed that ORF8 gene was differentially expressed, being down regulated in the presence of 5-azacitidine in BT cells compared to non-treated control indicating that the virus may affect the expression of this gene. ORF8 is a glycoprotein required for viral entry to the host cell (Dry et al., 2016) and is also expressed during productive infection (Palmeira et al., 2013). This is therefore predicted as BT cells support productive virus infection (Dry et al., 2008, Dry et al., 2016, Parameswaran et al., 2014). However, upon treatment with 5-azacitidine, ORF 73 was not highly expressed (Table 22) even though BT cells did not show any CPE. The explanation of this is that the expression of ORF73 may not, in isolation, be diagnostic of the latent state (i.e. an increase in expression rather than expression per se). 5-azacitidine is a hypo-methylation agent (Christman, 2002) which may have not completely removed ORF50 gene in BT cells meaning that ORF50 may have repressed the expression of ORF 73 gene. Recombinant

murine Gama herpesvirus (MHV-68) was engineered to overexpress RTA, transcriptional and replication activator encoded by ORF50 of Gama herpesviruses, was found to inhibit the expression of ORF73 gene and induce lytic virus cycle (Hair et al., 2007). The approach one could think of to address the expression of ORF73 in BT cells therefore is to completely remove ORF 50, the productive gene, by knockout gene technique.

The work study revealed that there were differences between the gene expression profiles between 5-aza treated and non-treated cells and also between rabbit LGLs and cattle BT cells, which reflects the different types of cell under study.

Chapter 6

The effect of Cyclosporine-A on T cell recruitment, viral gene expression and disease pathogenesis in infected rabbits

6.1 Abstract

The mechanism by which MCF viruses (AlHV-1 and OvHV-2) cause the disease is not fully understood. The tissue damage seen in the affected tissues is attributed to the accumulation of T lymphocytes. However, it is not well established whether the damage is due to the direct effect of infiltrated T lymphocytes (infected or bystander non-infected) or due to the effect of small number of infected non T cells or both. Cyclosporine A drug, in rabbits infected with OvHV-2 prevented lymphocyte accumulation but not overt MCF (Buxton et al., 1984). This is a key observation and the basis of the aims of this thesis. Rabbits were infected with AlHV-1 and treated with cyclosporine A. Unfortunately; the rabbits reacted badly to the CsA and infection such that they had to be euthanized before full onset of MCF. However, these animals showed some pathological changes and these data were analysed. In a subsequent trial to examine the pathogenesis of MCF in rabbits infected only with AlHV-1, results were again inadequate due to the early termination of the experiment as the animals reacted unexpectedly to infection. The viral transcript profile analysis revealed that the productive/lytic genes were expressed at low level in the infected only group indicating the virus may replicate at this stage of the disease.

6.2 Introduction

There has been a controversy for long time over the mechanism by which MCF viruses (AlHV-1 and OvHV-2) cause the disease (Buxton et al., 1984, Dewals et al., 2011, Palmeira et al., 2013, Schock et al., 1998). The core of this debate has been over the issue whether the tissue damage is due to dysregulated immune response or direct viral impact, and whether the disease is one of viral latency or not (Palmeira et al., 2013). Rabbits are a good model for MCF because they exhibit clinical signs and pathological changes similar to those seen in susceptible species (Anderson et al., 2007, Cunha et al., 2013). Treating rabbits infected with OvHV-2 with cyclosporine A, an immunosuppressive drug, eliminated the lymphocytes infiltrating the infected tissues but did not prevent disease development (Buxton et al., 1984). There is a scarcity of the virus in the infected tissues (Bridgen et al., 1992, Thonur et al., 2006) making analysis difficult. The phenotype and cytokine profile of the lymphoblastoid cells infiltrating the infected tissues represent unregulated cytotoxic cells (Schock et al., 1998, Swa et al., 2001). Nonetheless, growing evidence demonstrates that lymphocytes accumulating in MCF-affected tissues are infected with the virus and the virus establishes latency in them (Palmeira et al., 2013, Simon et al., 2003). Furthermore, the cells are cytotoxic and secrete cytokines and proteins that are responsible for the tissue damage seen (Dewals et al., 2008, Dewals et al., 2011, Palmeira et al., 2013). I aim to treat

AlHV-1 infected rabbits with CsA to examine the role of T lymphocytes in the pathogenesis of AlHV-1 MCF and to study the gene expression using known productive/lytic and latent viral transcripts in rabbits during MCF disease course. Cyclosporine acts by inhibiting calcineurin which is responsible for triggering a significant signal transduction pathway of T cell activation. The outcome of this action is a reduction in T cell maturation (Matsuda and Koyasu, 2000, Mott et al., 2004). We hoped to see whether ablation of T cells would alter the course of MCF and whether there was an associated change or not in gene transcription profiles. For this, q PCR and *in situ* hybridisation techniques will be used.

6.3 Materials and methods

6.3.1 Animals

See the materials and methods chapter section (2.6).

6.3.2 Inoculum

As described in the materials and methods chapter section (2.6.1).

6.3.3 Experimental design

As described in the materials and methods chapter sections (2.6.2) and (2.6.3).

6.3.6 Molecular techniques

The molecular approaches as described in materials and methods chapter. Quantitative R-T PCR was used to measure both viral load and gene expression in the infected tissues as described in materials and methods sections (2.4.3) and (2.4.4).

6.3.4 Samples collection

As described in the materials and methods chapter section (2.6.4).

6.3.5 Histology

See the materials and methods chapter section (2.7).

6.3.7 In situ Hybridization

The *in situ* hybridisation method used as described in materials and methods sections (2.8.1) to (2.8.5).

6.4 Results

6.4.1 Experiment (1) results

10 rabbits split into two groups of 6 to get CsA and infection and 4 to get infection only with vehicle IV rather than CsA (See table 25). Two rabbits were culled from CsA treated group (IYTU and IYDI) without collecting samples from them as they died suddenly (see below). Tissue samples were collected from the remaining animals in both CsA treated group (4 animals, day 12) and infected only group (4 animals, day 17).

6.4.1.1 Clinical signs

None of the rabbits injected with cyclosporine A and infected with AlHV-1 developed clinical signs until around day 10 of the experiment two rabbits died from the CsA treated group (IYTU and IYDI) after exhibiting fever, weight loss, and conjunctivitis (Table 25). They then died suddenly overnight. In the control group (infected with the virus along with vehicle), one of the rabbits developed a diarrhoea with high temperature that reached 41.2C° on day 17 of the experiment. Nearly all of the rabbits in this group exhibited high temperature, in-appetence, lethargy and conjunctivitis just prior to euthanasia. No other clinical findings were recorded on the rabbits up to the seventeenth day of the experiment when all rabbits were euthanized.

Table 25 shows the design of two rabbit's experiments infected and treated with cyclosporine A as long as pathological changes

Exp # (1)	Clinical signs/euthanasia	Pathological changes
4 rabbits infected + CsA	Started on day 10 (2 animals). Animals developed fever, conjunctivitis, apathy, weight loss. Reminder euthanized due to severe reaction to CsA on day 12.	Small numbers white foci on the kidney, intestines, liver and spleen. Lung haemorrhage in both IYBM and IYKA.
4 rabbits infected only	On day 15 animals developed fever, conjunctivitis, apathy and weight loss and were euthanized on day 17 of the experiment.	Multiple white foci on the kidney, liver, lung, enlarged lymph nodes and enlarged spleen. Some micro-haemorrhage in the intestines and lung.
Exp #(2)	Clinical signs/euthanasia	Pathological changes
4 rabbits infected + CsA	Adverse reaction to CsA seen days 9-10. Fever, apathy, and rapid demise. Animals euthanized on day 10	Slight lung haemorrhage, slight lymph nodes enlargement and small numbers of white foci on the liver and kidney.
4 rabbits infected not treated.	Day 9 animals exhibited fever and weight loss. Animals euthanized on day 10 of the experiment.	Enlarged spleen, white foci on the liver, haemorrhage in the intestines and lung.
2 rabbits, uninfected controls	No clinical signs seen. Animals euthanized on day 10.	No pathological changes seen.

6.4.1.2 Gross appearance of MCF lesions

In the cyclosporine A treated group there were some small white foci on the kidney, liver, intestines and spleen along with haemorrhage in the lung of the rabbit IYBM as well as haemorrhagic lung and red eyes in the rabbit IYKA (Table 25). In the infected control group, the lesions were diverse affecting many body systems and more pronounced in three rabbits (LFKJ, LFLK, and IYDY) ranging from multiple white foci on the kidneys, liver, and lung, enlarged lymph nodes (in particular popliteal lymph nodes) and slight enlargement in mesenteric lymph nodes (MLN), congested intestines, enlarged and dark spleen, and dark and enlarged urinary bladder (Table 25).

6.4.1.3 Viral DNA load measurement

Viral DNA load was identified in both blood and lymph nodes of three animals from each group (Figure 35). In the tissues (Liver, kidney, lung, spleen and appendix), the viral load was detected in the infected control group confirming infection. The viral copy number was below the detection level in the cyclosporine A treated group in these tissues (Figure 36).
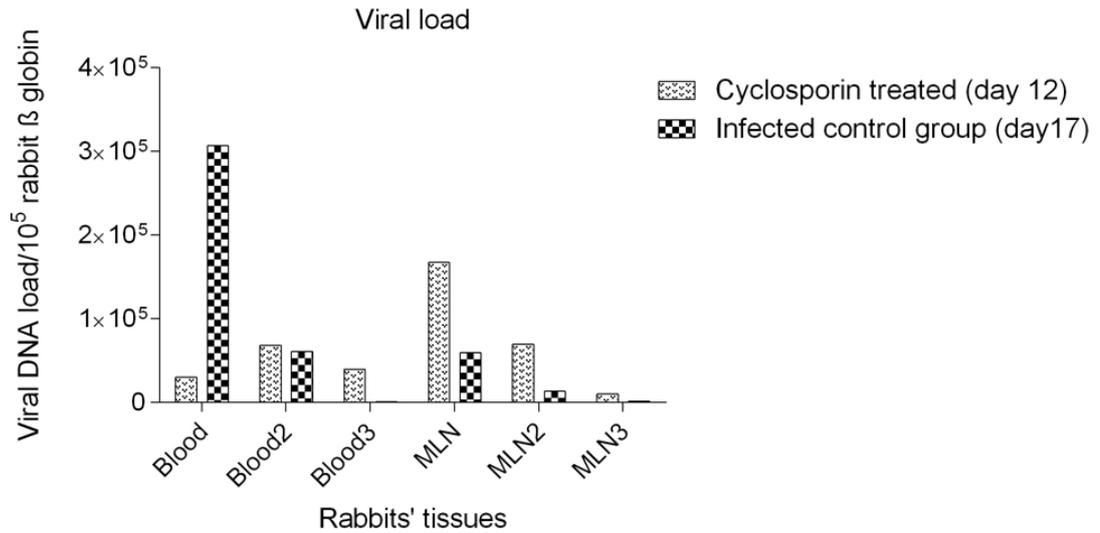
Figure 35 viral DNA load in lymph nodes and blood from rabbits tissues infected and treated with cyclosporine and infected only control animals.

The figure shows viral DNA load in the blood and lymph nodes from rabbits infected with AlHV-1. Samples were collected on day 12 and 17 of the experiment. The viral load was estimated by normalizing the viral DNA per 10^5 rabbit β globin using q PCR.
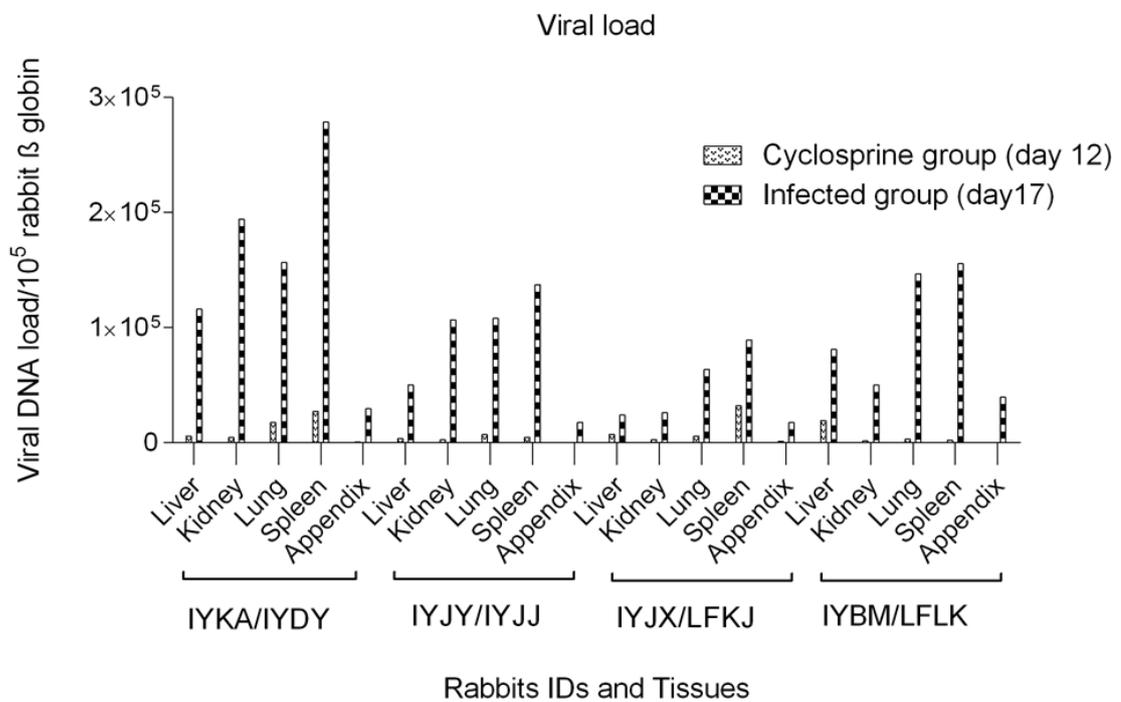
Figure 36 viral DNA load in rabbits' tissues infected and treated with cyclosporine A and infected only controls.

228

The figure demonstrates viral DNA load in rabbit tissues infected with AlHV-1 virus. Samples were collected on day 12 and 17 of the experiment. The viral load was estimated by normalizing the viral DNA copy number to 10^5 rabbit β globin gene copies using q PCR.

6.4.1.4 Histological analysis of AlHV-1 in rabbits

The major histological findings in regard to MCF lesions in rabbits in both groups (infected control and infected plus cyclosporine A treated) are summarised in Table 26. The non-lymphoid tissues in the control group (Liver, kidney, and lung) illustrated marked lymphocyte infiltration (accumulation of lymphoid cells) especially around the blood vessels, which showed evidence of vasculitis. In addition, there has been marked hyperplasia in the lymphoid follicles in the lymphoid tissues (appendix and spleen). In the cyclosporine A treated group there was not any change in either lymphoid or non-lymphoid tissues.

Table 26 cyclosporine A experiment: AlHV-1 MCF histology on lymphoid and non-lymphoid tissues.

Exp (1)	Exp (2)	Lymphoid Tissues		Non-lymphoid Tissues		
		Hyperplasia/Necrosis[1]		Lymphoid cell accumulation[2]		
animal	animal	Appendix	spleen	Kidney	Liver	lung
Infected only	**Infected only**					
LFKJ	JYDR	+/0 0/0	+/0 0/0	++ 0	++ +	++ 0
LFLK	JYBU	+/0 0/0	+/0 0/0	++ 0	+ +	++ 0
IYJJ	JYFC	+/0 0/0	+/0 0/0	++ 0	++ +	++ 0
IYDY	JYJE	+/0 0/0	+/0 0/0	++ 0	+ -	++ 0
CsA+infection	**CsA+infection**					
IYKA	JYIS	-/- -/-	-/- -/-	0 0	0 0	0 0
IYBM	JXXS	-/- -/-	-/- -/-	0 0	0 0	0 0
IYJY	JXTS	-/- -/-	-/- -/-	0 0	0 0	0 0

IYJX	JXJJ	-/-	-/-	-/-	-/-	0	0	0	0	0	0
IYTU (Culled)											
IYDI (Culled)											
Uninfected control	**Uninfected control**										
C1	JXXL	0	0	0	0	0	0	0	0	0	0
C2	JXXR	0	0	0	0	0	0	0	0	0	0

Lymphoid tissues: + = change present, 0 = change not present (hyperplasia = increased lymphoid cell accumulation /proliferation compared to the controls).

[2]Non- lymphoid tissues: Interstitial and perivascular lymphoid cell accumulations. ++ = marked accumulation, + = moderate accumulation, 0 = no accumulation. There was evidence of vasculitis (vascular endothelium thickening) in the infected control animal tissues. The rabbits were not marked in the table are those which were thrown away without samples being collected.

Kidney: there was perivascular lymphocyte infiltration in the cortex in the kidneys of the control group while in the cyclosporine A (CsA) treated group there was not any evidence of infiltration around the blood vessels (Figure 37).

Liver: in the infected control group there was an obvious perivascular lymphoid accumulation in the periportal area whereas there was no infiltration in the cyclosporine A treated group (Figure 38).

Lung: no lymphocyte accumulation seen in the lungs from the cyclosporine A group, however, there was a clear lymphocyte infiltration in the peri-bronchial region as well as disperse infiltration throughout the parenchyma (Figure 39).

Kidney
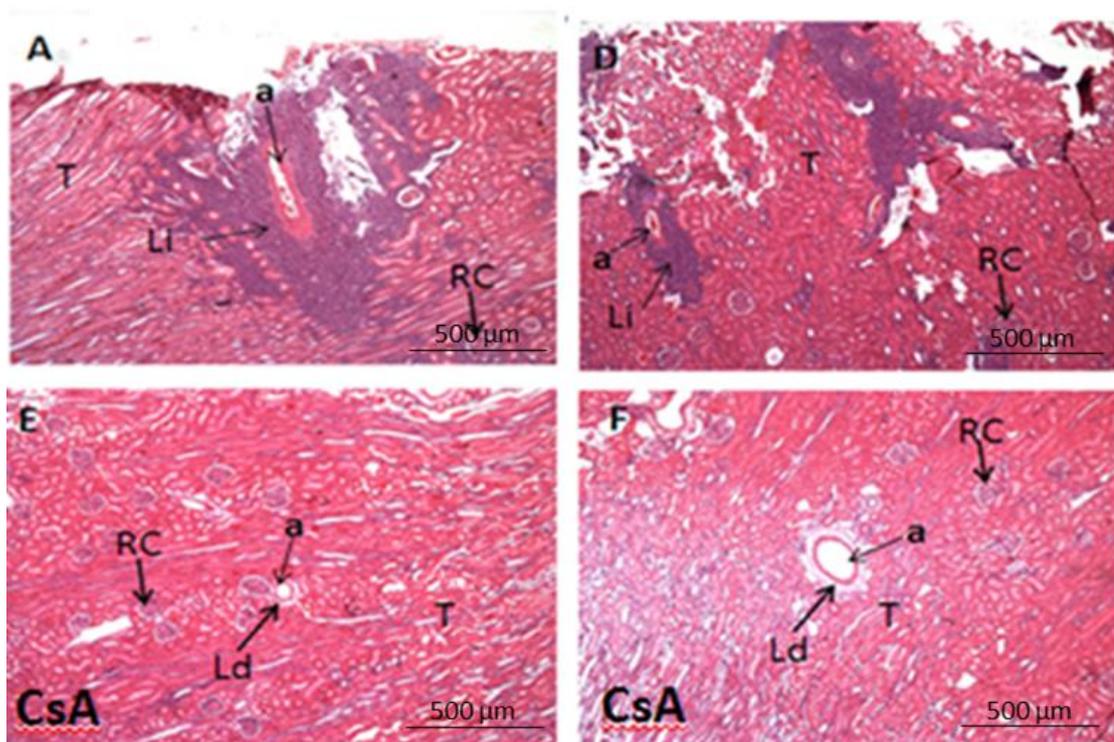
Figure 37 haematoxylin and eosin stain of rabbits' kidney

The images show the formalin fixed, haematoxylin and eosin stained histological sections of rabbits' kidney (A, D, E, and F). Slides A and D show typical histological lesions of MCF after infection with AlHV-1 C500 strain. The most obvious appearance in these slides is the infiltration of lymphocytes around the blood vessels in the cortex area. Slides (E and F) are from the rabbits infected with AlHV-1 C500 strain and treated with cyclosporine A. There is an absence of lymphocyte infiltration. (a) Blood vessel, (Li) lymphocyte infiltration, (Ld) No evidence of lymphocyte accumulation, (RC) renal corpuscle, (T) uriniferous tubules. Pictures represent scale bar of 500 μm.
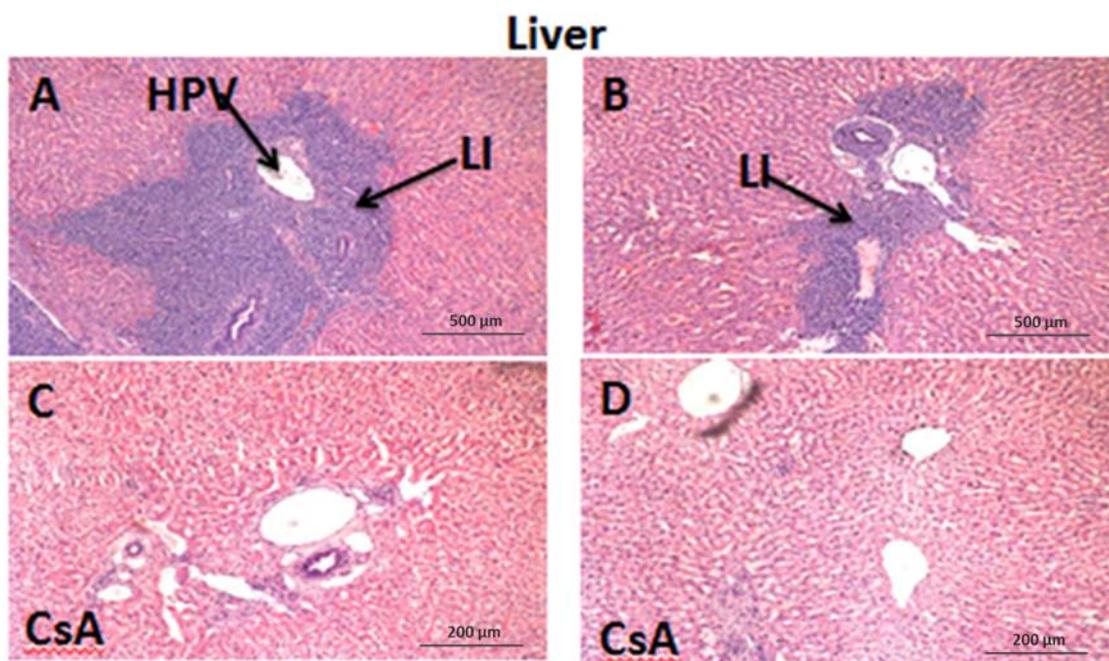
Figure 38 haematoxylin and eosin stain of rabbit's liver

The images depict the Formalin fixed, haematoxylin and eosin stained histological sections of rabbits' liver (A, B, C, D). There is obvious lymphoid infiltration around the blood vessels in the periportal area in the infected tissues only with AlHV-1 C500 strain virus (slid A and B) whereas no infiltration in the tissues infected with the same virus and treated with cyclosporine A drug (slides C and D). CsA: cyclosporine A treated group, LI: lymphoid infiltration, HPV: hepatic portal vein. Pictures A and B represent scale bar of 500 μm whereas pictures C and D represent 200 μm.

233
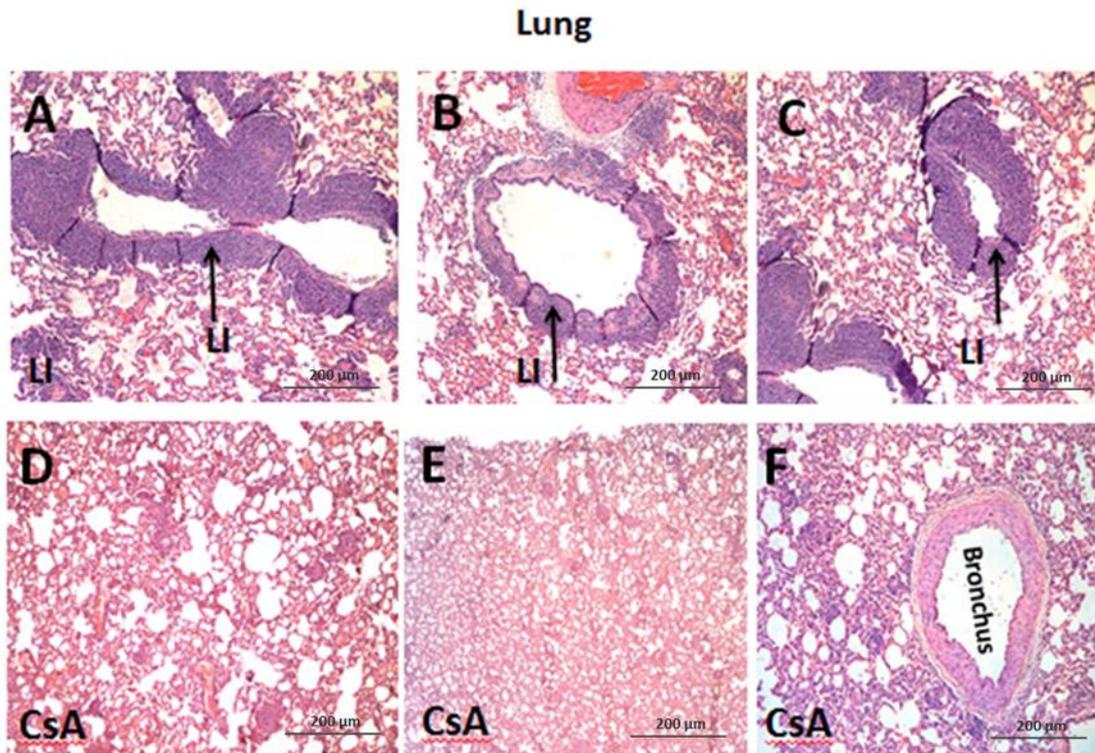
Figure 39 haematoxylin and eosin stain of rabbit's lung
The images depict the Formalin fixed, haematoxylin and eosin stained histological sections of rabbits' lung (A, B, C, D, E, F). There is obvious evidence of peribronchial lymphoid infiltration as well as dispersed lymphocyte infiltration throughout the parenchyma slides (A, B,C) in the tissues infected only with AlHV-1 C500 strain virus. No lymphocyte infiltration in the tissues infected with AlHV-1 C500 strain virus and treated with cyclosporine A drug slides (D, E, F). LI: lymphocyte infiltration. Pictures represent scale bar of 200 µm.

Appendix: there was an obvious expansion of the lymphatic nodules, absence of the inter-follicular space, haemorrhage in the blood vessels, and loss of the tissue architecture in the infected control group. No apparent changes in the cyclosporine A treated group (Figure 40).

Spleen: there were no remarkable changes in the tissues from the cyclosporine A treated group. However, there was hyperplasia in the

white bulb nodules, intensive scattered lymphocyte infiltration, and

loss of architecture in the infected control group (Figure 41).
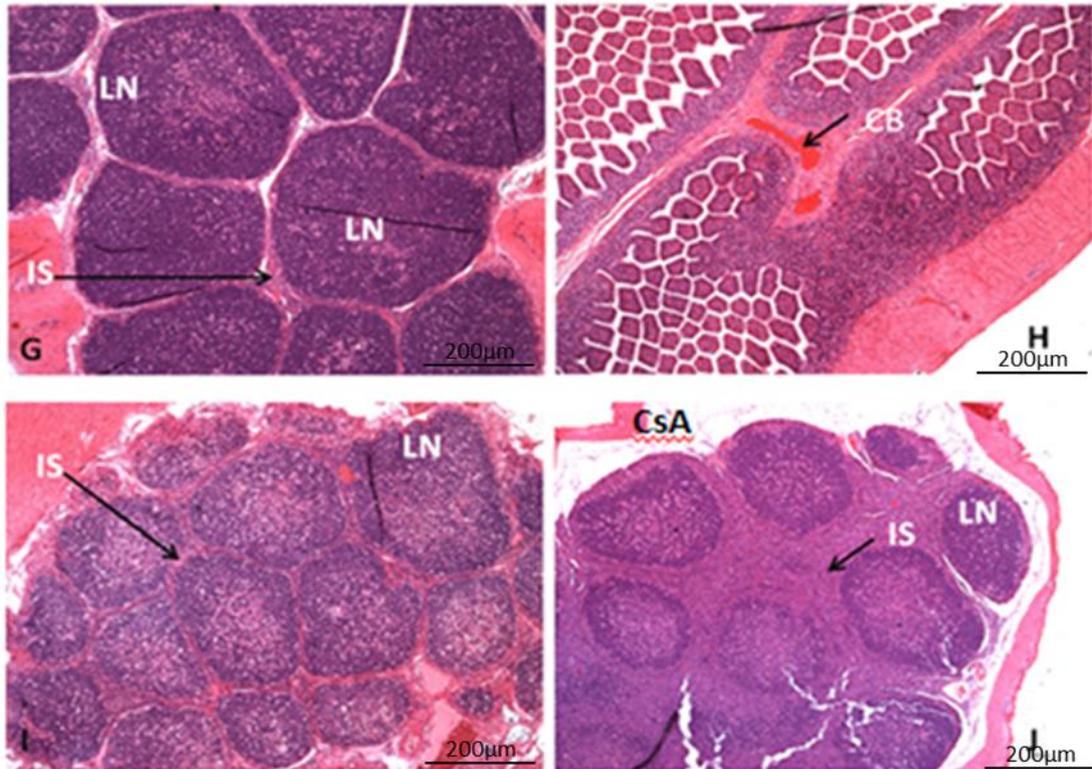
Figure 40 haematoxylin and eosin stain of rabbit's appendix
The images show the formalin fixed, haematoxylin and eosin stained histological

sections of rabbit's appendix (G, H, I, and J). Slides G, H, and I are from rabbits

infected with AlHV-1 virus without treatment with CsA. Slides illustrate expansion

of follicles, relative absence of inter-follicular space, loss of tissue architecture as

well as congested blood vessels. Slide J is from CsA treated group where the

appearance is more normal. LN, lymphatic nodule; IS, inter-follicular space; CB,

congested blood vessel. Pictures represent scale bar of 200 μm.
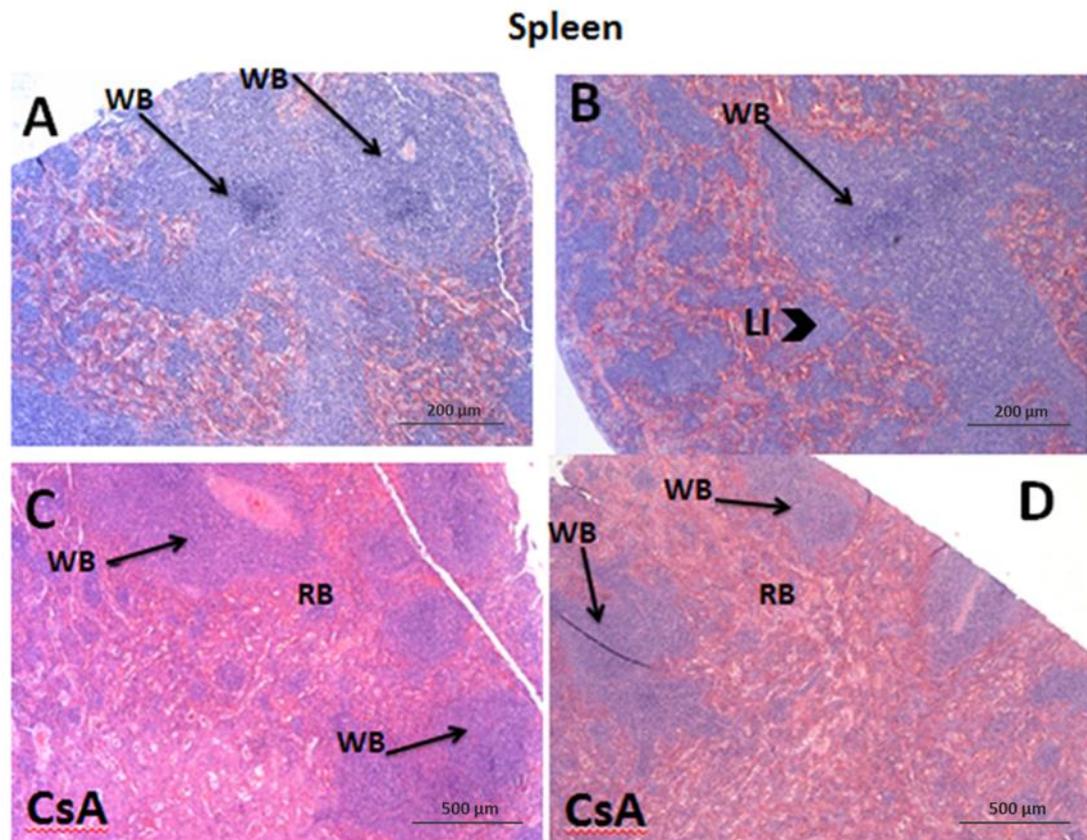
Figure 41 haematoxylin and eosin stain of rabbit's spleen
The images show the Formalin fixed, haematoxylin and eosin stained histological sections of rabbit's spleen (A, B, C, D). There was apparent hyperplasia in the white bulb (black arrow) as well as intensive dispersing of lymphoid cells throughout the tissue (arrow head) in the tissue infected only with AlHV-1 C500 strain virus slide (A and B). No clear and remarkable changes in the cyclosporine A treated group (C and D). CsA: cyclosporine A treated group, WB: white bulb, RB: red bulb, LI: lymphocyte infiltration. Pictures A and B represent scale bar of 200 µm whereas pictures C and D represent scale bar of 500 µm.

6.4.1.5 Gene expression

Gene expression in the infected tissues was measured by normalising viral target genes with rabbit SDHA1 reference gene using q PCR. The results showed that the gene expression of viral target genes was higher in infected controls than cyclosporine A treated group. Also, the pattern of expression was similar in nearly

236

all genes tested with highest values in spleen, lung, and kidney. ORF A4.5 recorded the highest gene expression in tissues of infected animals and in the spleen of the cyclosporine A treated group. The expression of ORF25, ORF50, and ORF63 was similar in the tissues of infected animals. The expression of ORF73 was lower than the other genes whereas ORF A9.5 was the lowest. Figure 42 shows the gene expression of 6 viral genes putatively responsible for productive/lytic and latent virus infection in rabbit tissues.
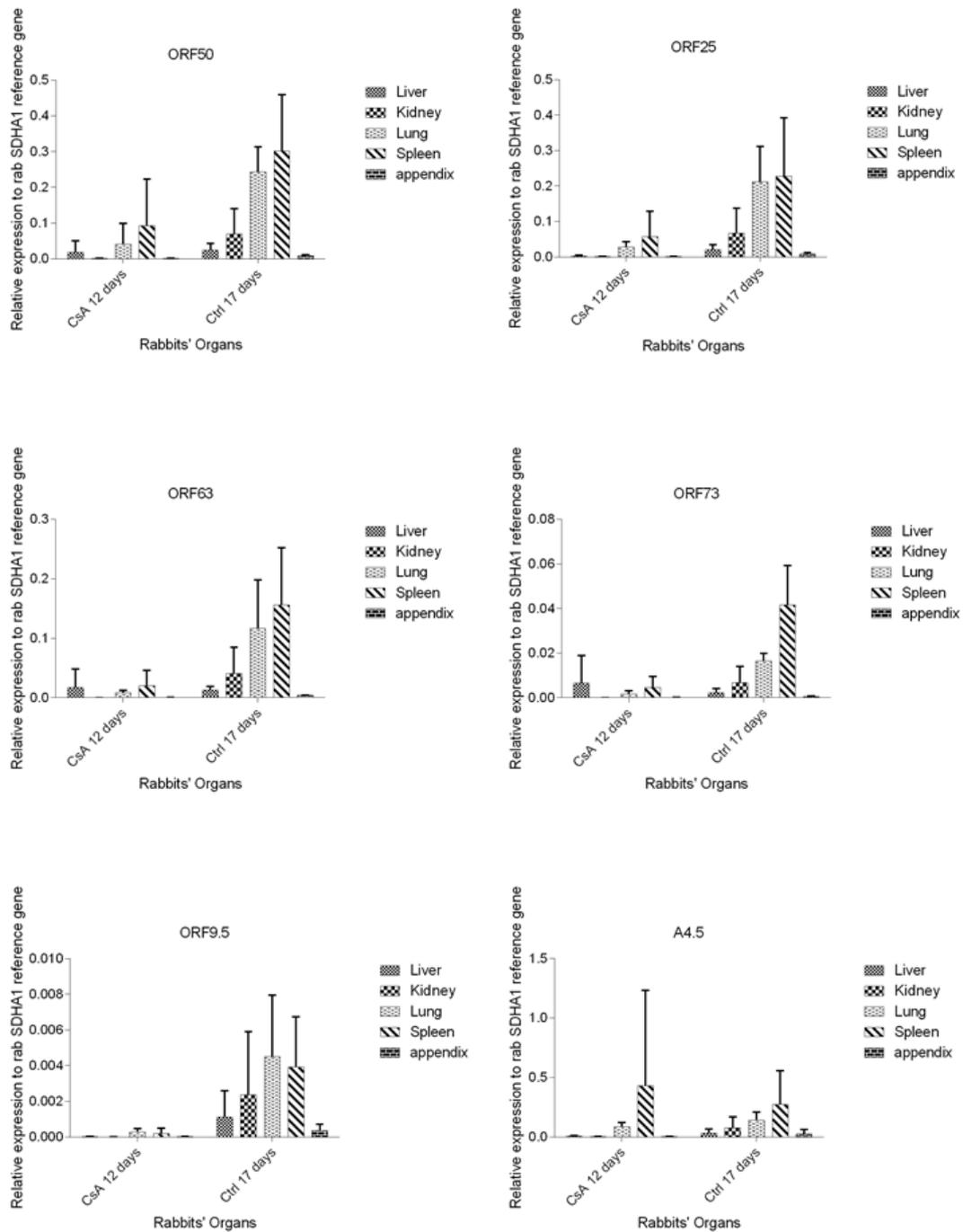
Figure 42 viral transcripts of six AlHV-1 genes in rabbits' tissues
The figure shows viral gene expression of six AlHV-1 genes (ORF50, ORF25, ORF63, ORF73, ORF9.5, and A4.5) in five rabbits' tissues (liver, kidney, lung, spleen, and appendix) from infected only and infected and treated with cyclosporine A groups euthanized on day 12 (CsA group) and 17 of the experiment. The transcripts were measured using qPCR and normalised with rabbit SDHA1 reference gene. CsA 12 day; cyclosporine A treated group euthanized on day 12 of the experiment, Ctrl 17 days; infected control group euthanized on day 17 of the experiment.

6.4.1.6 *In situ* Hybridisation

No results were obtained from the *in situ* hybridisation work despite

the fact that synthesis of RNA probe for ORF65 and ORF A9.5 genes

was unsuccessful.

6.4.2 Experiment (2) results

The clinical signs associated with MCF in the infected cyclosporine A treated group were fever, apathy, and rapid demise. At this time two CsA-treated rabbits showed a rapid decline with fever, respiratory distress and apathy, and were euthanized for humane reasons. In the infected control group number of animals developed fever, weight loss, enlarged spleen, white foci on the liver and haemorrhage in the intestines and lung. In the negative control group there were no clinical signs and pathological changes seen. All animals were euthanized on day 10.

In terms of the histological changes, the only changes were seen in the liver from infected group in form of small perivascular lymphocyte infiltration in the periportal area whereas there was no lymphoid infiltration in infected cyclosporine A treated group animals or the uninfected control group (Figure 43)
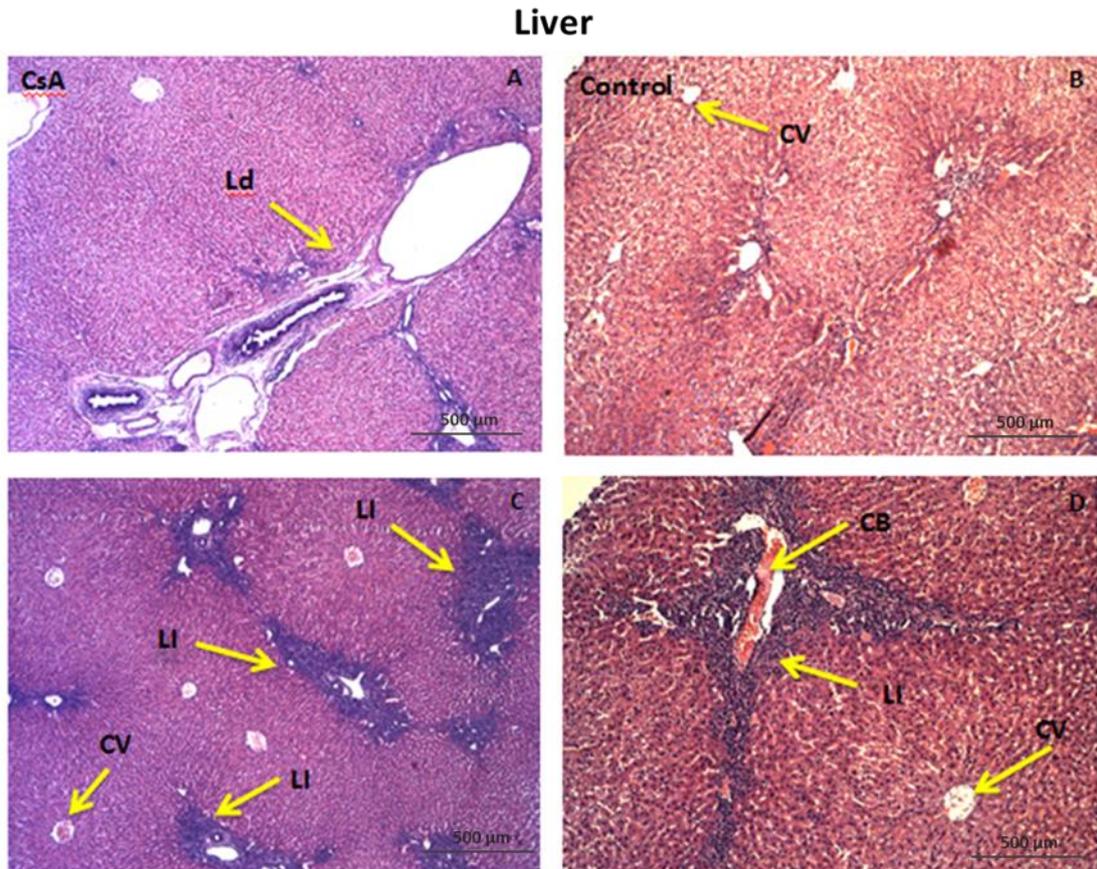
Figure 43 haematoxylin and eosin stain in rabbit's liver (1)

The figure illustrates formalin fixed, haematoxylin and eosin stained liver (A, B, C, D). There is obvious lymphoid infiltration around the blood vessels in the periportal area in the infected only group tissues (slides C and D) whereas no infiltration was seen in the tissues of the infected and CsA-treated animals (slide A) or the uninfected control (slide B). CsA: cyclosporine A treated group, LI: lymphoid infiltration, CV: central vein, CB: congested blood vessel, LD: infiltration disappearance. Pictures represent scale bar of 500 μm.

The viral DNA load data revealed that this was under detectable level in most animals' tissues except some liver and kidney samples from the CsA+infection group, the viral load was more than 3×10^6 in one of the kidneys (rabbit JXTS). There was no change recorded in the uninfected control group (Figure 44).
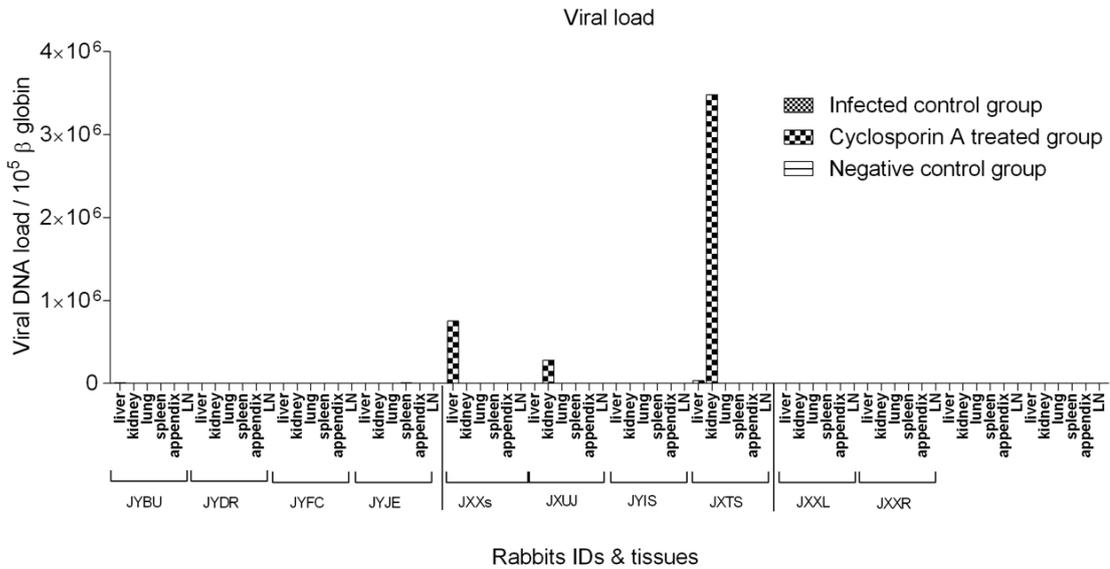
Figure 44 viral DNA load in tissues in infected only and infected and treated with cyclosporine A group animals (1)

The figure demonstrates the viral DNA load in infected tissues and from samples collected on day 10 of the experiment. Infected control group; rabbits infected only with the virus, Cyclosporine A treated group; rabbits infected with the virus and treated with cyclosporine A uninfected control group; non-infected and non-treated rabbits. The viral DNA load was calculated by normalizing the viral DNA per 105 rabbit β globin gene copy number using quantitative PCR.

No results were obtained from the gene expression experiment in any sample.

6.5 Discussion

Two *in vivo* experiments were carried out in order to investigate the role of CsA on MCF induction, and whether it would prevent T cell accumulation as shown previously (Buxton et al., 1984). The clinical signs and pathological changes observed in both experiments were typical for MCF in the control infected groups and consistent with other observations (Anderson et al., 2007, Dewals et al., 2008, Jacoby et al., 1988). There was a severe reaction to CsA in some animals necessitating the euthanasia of the entire group in each case. Thus, throughout this series of experiments the CsA-treated animal tissues examined represented an early stage of MCF and the infection only controls were not time matched to this in experiment 1.

Consequently, in terms of the histological findings, typical MCF-associated changes were seen in the infected only groups in all the tissues collected which is in agreement with previous observations (Anderson et al., 2007, Buxton et al., 1984, Palmeira et al., 2013). In the cyclosporine A treated group, however, the lymphocytes did not accumulate around the blood vessels. This is also in agreement with results recorded by Buxton et al. (1984) with OvHV-2 infection, but this is an early time in infection before control infection only animals got MCF and this most likely explains the result.

Similarly, possibly due to early euthanasia, it was noticed that the viral DNA load in the tissues of the control infection group was

considerably higher than the cyclosporine A treated group and in general although in some of the cyclosporine A animals viral DNA load was high in blood and MLN. However, detectable levels of MCF viral DNA (AlHV-1 and OvHV-2) can be identified as early as five days in the tissues and blood of infected animals (Cunha et al., 2012, Dewals et al., 2008, Taus et al., 2005).

All studied viral genes were expressed at low level in both groups and were higher in infected control groups than cyclosporine A treated group. Again this is due to the early death of the animals before MCF fully developed. The gene expression patterns were typical of AlHV-1 MCF with ORF73 (encoding latency associated nuclear antigen (LANA)) expressed at a lower level than most of the others. ORF A9.5 which encodes a secreted glycoprotein that is similar to IL-4 (Lankester et al., 2015, Russell et al., 2013) was expressed at very low level in the infected group. The role of this gene in the virus life cycle is still unknown.

It was hoped to perform in situ hybridisation to localise viral genes to tissues and cells *in vivo*. Much time was spent developing the technique. Although synthesis of RNA probes was achieved, there were no convincing results obtained from the *in situ* analysis of infected tissues. Time was not available to troubleshoot the technique further. *In situ* hybridisation is a powerful technique which is used to detect cellular location of certain genes representing latent or lytic cycle (Jin and Lloyd, 1997, Malarkey and

Maronpot, 1996, Nouri-Aria, 2008). This means that the viral mRNA copy numbers may have been below the *in situ* hybridisation threshold (Simon et al., 2003). The approach necessitates the presence of around 10-20 mRNA copy numbers in a single cell to make the signal detectible (Jin and Lloyd, 1997).

The second CsA experiment was terminated on the tenth day due to an adverse reaction to CsA once again in spite of changing the vehicle used to dissolve the drug. Consequently, there were no results obtained as MCF did not develop. We surmise that in both experiments the CsA may have not been fully dissolved and formed micro-precipitates causing vascular obstruction/damage. Buxton et al. (1984) injected rabbits with cyclosporine A intramuscularly which may delay the drug diffusion, hence its effect on the animals' tissues and the disease course. However, I applied intravenous route where the drug absorption time is fast.

Chapter 7

General discussion

7.1 General discussion

Although the pathological changes of MCF such as vasculitis, lymphocyte infiltration and tissue necrosis are well characterised, the pathogenesis of the disease is still not completely understood (Parameswaran et al., 2014, Russell et al., 2012). The tissue damage seen in MCF-affected tissues, especially epithelium and endothelium could be due to the infiltration and activity of cytotoxic T cells (either containing the virus or dysregulated non-infected cells) or virus infection of epithelium and endothelium or a mixture of both.

The main hypothesis of this project work was that malignant catarrhal fever is initiated by aberrant virus gene expression in infected T cells, epithelium and endothelium. Whether the disease was predominantly one of latency (as has been suggested (Palmeira et al., 2013, Dewals et al., 2008)) or productive virus infection still remains controversial. The first aim was to determine whether epithelium and endothelium can be infected *in vitro* using q PCR and, if so whether a latent or productive viral cycle is established. Rabbit epithelial (SRIC) cells, Bovine aortic endothelial (BAE) cells and bovine turbinate fibroblast (BT) cells (productive virus control) were infected with both low pass (virulent) and high (attenuated) AlHV-1. It was clear that both cell-types can be infected with either form of AlHV-1 as shown by the presence of viral DNA and the ability to transfer infection to uninfected fresh cells where, in BT

cells, a productive infection was detected. If these cells represent epithelial cells and endothelial cells *in vivo* then it is possible that these cell types can contain virus. Interestingly, a lack of CPE in the SIRC and BAE cells indicated a lack of productive viral life cycle, raising the possibility that the cells are latently infected. The viral gene transcription analysis of these cells did not confirm a latency pattern, but levels of transcripts were very low and there was no gene transcription pattern typically seen in productively-infected BT cells. The ability of virus isolated from the SIRC and BAE cells to induce a productive infection in BT cells (as measured by a CPE) strongly suggests the virus was latent in the SIRC and BAE cells. It is also possible that the low viral transcripts were due to the fact that the virus could not enter or penetrate the cells. Consequently, the effect in the serial infection may have been the result of transfer of virus direct from the supernatant or that was attached to the surface of the first cells, and not virus that had "infected" the first cells.

The question remains why ORF73 gene (the LANA equivalent latency-associated transcript) was not expressed in epithelium and endothelium in the *in vitro* model if the latent state was established. One explanation is that viral DNA load in the cells is present but transcript abundance is below detectable level. Another, explanation more speculative and less likely is that there might be biological factors in the *in vivo* system not available in the *in vitro* paradigm to

make the virus express ORF73 gene and hence establish true latency. One of these is a lack of immune cells in the *in vitro* system which may be important to establish latency to escape the detection by the immune system (Blake, 2010). Finally, an important point when studying latency in MCF, is that this will have evolved in the host reservoir species (wildebeest for AlHV-1) and not the disease-susceptible species, where the virus cannot transmit between animals (at least that we know of). Consequently, the virus life cycles may be altered or corrupted in some way in the MCF susceptible species cells, a possibility explored by Thonur et al., in studying cycles in LGLs from reservoir (sheep) and MCF susceptible animals in OvHV-2 MCF. A study of wildebeest cells infected with AlHV-1 was beyond the scope of this project.

So, what is in BT vs SIRC and BAE that causes the productive virus cycle to switch on. The RNA-Seq analysis of infected BT cells and LGLs with and without 5-aza did not reveal any obvious answer to this, at least from the host transcriptome analyses. There was not enough resource to do transcriptome analyses on infected SIRC or BAE cells versus infected BT cells to address this directly.

Large granular lymphocytes (LGLs), with T/natural killer cells morphology that are infected with AlHV-1 (Schock et al., 1998, Swa et al., 2001), were obtained from the infected rabbits and were treated with 5-azacitidine, a drug used to drive latency in gamma herpes viruses (Schaefer et al., 1997, Thonur et al., 2006). Bovine

turbinate fibroblast (BT) cells were infected with AlHV-1 and infected and treated with 5-azacitidine as productive cycle virus controls. The notion was to see if 5-aza could inhibit the productive cycle and establish a latent state and allow identification of latency-associated transcripts for AlHV-1. All infected cells treated or not were shown to have viral DNA using q PCR and PCR analysis indicating that the cells were infected with the virus. The most noticeable effect was in the infected BT cells where 5-aza treatment inhibited a CPE and this was associated with a marked reduction in gene transcripts associated with the productive viral life cycle (ORF50 and ORF25 and ORF A4.5 and low level expression of ORF 73 the latency-associated transcript). This confirms that 5-aza drives a latency-like state in AlHV-1-infected cells as it does with OvHV-2 infected cells (Thonur et al., 2006). The results for LGLs were less clear. 5-aza treatment caused a dramatic effect on the pan T antigen (uncharacterised to date) recognised by a monoclonal antibody, but although there was some evidence of changes in viral productive and latent gene expression this was at low level, near detection limits and not consistent between the LGL lines. It is known that the A2 gene of AlHV-1 is a leucine zipper transcriptional regulator that has multiple functions on gene regulation in rabbits infected with an A2 gene knock-out virus or wild-type /gene reinsertion controls (Parameswaran et al., 2014). One of these effects is to alter the T cell receptor phenotype, downregulating alpha-beta TCR and

augmenting the gamma-delta TCR. It is known that there is a gd T cell subset that expresses CD8 (the predominant T cell marker detectable on the LGLs), found particularly at mucosal sites (Nelson et al., 2010, Wilson et al., 2002, Davis et al., 1996).

Unfortunately, there is no gd T cell-specific antibody for the rabbit to test this theory out. Furthermore, the gd T cells lack the CD3 molecule that is a pan T cell marker (Owens et al., 2015).

Although the effect seen in this study is due to 5-aza treatment, it may be that this or the associated drive to a latent state may have stimulated this phenotypic change in the T cells and that A2 may be active in this way during latency. All of this speculation is open to further research. In contrast possibly, the impact of 5-azacitidine on (uninfected) human T cells especially CD4 and CD8 T cells has been documented. It was established that 5-azacitidine treatment of human T cells decreased CD8+ T cells whereas CD4+ T cells were augmented (Stübig et al., 2014). CD8$^+$ T cells were the prevalent cell type after flow cytometry analysis of rabbit LGLs in both 5-azacitidine and non-treated groups. The results are compatible with the observations made by other researchers who found that CD8$^+$ T cells were the main cell type in LGLs and infected tissues *in vivo* (Dewals et al., 2008, Dewals et al., 2011). Also, there was a lack of CD4$^+$ T cells in both groups. Anderson et al. (2007) also observed the reduction in CD4$^+$ T cells in MCF affected tissues. The target cell

in the T cell lineage for virus infection in MCF is unknown (Parameswaran et al., 2014).

The global transcriptome analysis by RNAseq on the 5-aza-treated and control infected LGLs and BT cells did not reveal an answer to the above speculation. However, the genes differentially expressed in 5-aza-treated versus untreated infected BT cells showed a pattern consistent with changes in mRNA translation, mitochondrion integrity, regulation of gene expression, cell cycle control, DNA replication, post transcriptional modification and in genes associated with neoplasia (see gene lists and pathway analyses in chapter 5). This is consistent with the fact that these changes are fully active in the untreated BT controls indicating productive/lytic virus cycle.

In LGLs, the effect of 5-aza treatment was less clear on viral gene transcripts and the host transcriptome when compared to untreated cells. Viral transcript profiles showed that there was low expression of ORF50, ORF25, ORF63 and ORF73 in LGLs in two cell lines (LFLK and LFKJ) and no evidence of expression in two others. This could be because the level of viral transcripts is low to start with, which is the most likely explanation, or that a latency state was in these cells. A Gardella gel analysis of viral genome configuration was not performed due to a lack of time left in the study. This would reveal whether the LGLs had closed circular DNA (latent configuration, tight band on the gel) or linear DNA (lytic cycle configuration, smear on the gel). In the Thonur et al., (2006) analysis of rabbit LGLs

infected with OvHV-2, a mixed latent and lytic configuration was seen. After 5-azacitidine treatment there was a rise in ORF73 expression in one cell line (LFLK) compared to the infected control but ORF50 was also detected. This is consistent with 5-azacitidine driving latency via increased ORF73 expression and was also seen in LGL infected with OvHV-2 virus and treated with 5-azacitidine (Thonur et al., 2006). It is worth mentioning that ORF73 can be 'leaky' with respect to expression in the different virus life cycles such that complete absence of ORF73 in productive infection is not usually recorded (Thonur et al., 2006). The expression of ORF50 confirms that the cells support at least productive infection and its transcript may be abundant. There was an increase in ORF A4.5 in one cell line due to 5-azacitidine. Thonur et al. (2006) found an increase in OvHV-2 Ov4.5 in cattle LGLs but not rabbit LGLs treated with 5-aza. The reason for the difference in this study is not known, but may be due to some difference between AlHV-1 and OvHv-2.

The expression of ORF73 may not, in isolation, be diagnostic of the latent state (i.e. an increase in expression rather than expression per se). 5-azacitidine is a hypo-methylation agent (Christman, 2002) which may have not completely removed ORF50 gene in BT cells meaning that ORF50 may have blocked the expression of ORF 73 gene. The suppression of ORF73 gene and the induction of lytic virus cycle had been observed when ORF50 overexpressed using an

engineered recombinant murine Gama herpesvirus (MHV-68) (Hair et al., 2007).

The pathway analysis showed that cell death and T cell accumulation, proliferation and immune response pathways were increased in 5-azacitidine treated LGLs compared to infected-only control LGL indicating that LGLs have genes associated in immunological, cell death and proliferation processes. The pathways present in the untreated LGLs are similar to those in lymphoid tissues from MCF-affected cattle or rabbits (Palmeira et al., 2013, Paramiswaran et al., 2014, Russell et al., 2012). This indicates that the LGLs are an accurate representation of the host changes *in vivo*. The result also shows that 5-aza affects the above pathways (increased in treated versus untreated LGLs). This may be as a consequence of inducing a latent viral state, but there could also be some productive cycle activity as well as the results on this were not clear. It will be of interest in future work to determine if this is as a consequence of forcing the latent state for the virus and that these changes are at least in part stimulated by the latent gene expression programme or a lack of productive cycle gene expression. These pathways do not fit with classical latency, where inducing cell death in particular is not a good strategy for the virus. But, the viral gene expression may not be typical (i.e. is aberrant) in MCF-susceptible species. This all requires further research.

Importantly, although the pathway analysis identified several pathways affected by 5-aza-treated LGLs compared to untreated cells, the contribution of up and down regulated genes within these pathways can only really be assessed for a net overall effect if the precise functions of the genes are known in the context of the LGLs. For the apoptosis (cell death) pathway, this is possible as the function of many of the genes is known and conserved in animals. For example, there are both cell death inhibitors (BNIP1 and BNIP2) and activators (BAX and BNIP3) that were differentially expressed in the presence of 5-aza compared to non-treated controls. The overall effect is a tendency towards increased cell death in the 5-aza-treated LGLs compared to control cells.

In the final set of experiments, it was hoped that the Buxton et al., 1984 study on the effect of CsA on OvHV-2-infected rabbits could be repeated for AlHV-1-infected ones. This is because the CsA prevented the accumulation of T cells in the tissues, but did not prevent MCF. This key paper was the main reason for the hypothesis and objectives of this thesis, namely that if the accumulating T cells (that we now know are infected with virus) are not present to cause epithelial and endothelial damage /tissue damage in MCF, what other possible mechanisms can cause disease? That is why we looked at infecting epithelial cells and endothelial cells in chapter 1.

Unfortunately, the rabbits reacted badly to IV CsA and had to be euthanized early in the development of MCF (around 10-12 days over two experiments). Changing the formulation for CsA delivery did not help (experiment 2). In the Buxton paper, the formulation was given intramuscularly, which may have avoided the problems experienced in this study. Nonetheless, the pathological changes and clinical signs seen in infected only rabbits represented early stages of MCF. These observations are compatible with results observed by other authors (Anderson et al., 2007, Jacoby et al., 1988, Liggitt and DeMartini, 1980a, Liggitt and DeMartini, 1980b).

There was not any accumulation of lymphocytes in the tissues of the cyclosporine A treated group in the non-lymphoid tissues. This was probably due to the early demise of the animals. However, and interestingly, in the control infected animals in experiment two (euthanased on day 10 after infection and therefore at a similar time to the CsA-treated animals) lymphoid accumulations were seen, mainly around blood vessels (e.g figure 43). So some effect on lymphocyte accumulation by the CsA treatment is implied. Also interestingly, in the CsA-tereated animals but not the infected controls, high levels of viral DNA were seen in kidney and liver samples in some animals. These could be sites of virus replication at this time in the presence of CsA.

The viral transcript profiles in the experiments revealed that the level of gene expression was low but higher in the infected control

group than cyclosporine A treated group. This may have been due to the early finish of the experiment and also due to the variable viral copy number between the two groups as it was higher in the infected control than the cyclosporine A group. In addition, the results showed that the pattern of expression in the infected only animals was similar. Most of the genes especially those supporting productive/lytic virus cycles were expressed in the lung and spleen of the infected animals as seen in experiment 1 (Figure 42). The expression of ORF50, ORF25, ORF63 and ORF A4.5 and the low expression of ORF73 gene indicating that the virus may replicate and not tend to establish latency at this stage of the disease.

The other main objective of this series of experiments was to use *in situ* hybridisation to localise putative productive virus cycle or latency cycle gene transcripts to particular cells in various tissues. This was to determine whether MCF was associated with predominantly latent viral gene expression or a productive cycle one, comparing animals with and without T cell accumulation (CsA-treatment).

Unfortunately, no results were obtained as time ran out to perform a comprehensive study. ORF 65 (productive cycle gene) and ORF A9.5 (secreted IL-4 orthologue) riboprobes were made and the technique developed and validated (with help from Dashty Amin and James Stewart in Liverpool). First attempts to detect the transcripts in the tissues of the infected animals were unsuccessful. *In situ*

hybridisation is a powerful approach which is utilized to localize certain genes signifying latent or lytic cycle along with the detection of cellular cytokine mRNA during viral infection (Anderson et al., 2008, Anderson et al., 2001, Bridgen et al., 1992). It was documented that for potential detection of certain transcripts the approach requires the existence of approximately 10-20 mRNA copy numbers in a single cell to make the signal measureable (Jin and Lloyd, 1997). This may explain the inability to detect the transcripts in the affected tissues as viral mRNA copy numbers may have been below the *in situ* hybridisation threshold (Simon et al., 2003).

7.1.1 Conclusion and recommendations

The results of the *in vitro* epithelial and endothelial cell cultures indicate that the cells can be infected and establish a latent state, indicating that this is also possible *in vivo*, which should be looked at when studying MCF pathogenesis. The identification of proven latency transcripts by using 5-aza treatment on BTs and LGLs was unsuccessful, and more viral genes should be measured or other means as stimulating latency explored. The effect of 5-aza/ possible latency-associated expression the pan T antigen in LGLs is interesting and deserves further research. The transcriptome analyses in the infected and treated BTs and LGLs revealed several changes that may help future work to focus in on latency and productive infections *in vivo*. Moreover, the use of Gardella gel

analysis would have been useful in analysing the DNA configuration and hence the virus life cycle. The development of the *in situ* hybridisation work using productive virus cycle and latency gene riboprobes should proceed and the technique is partially developed here.

The conclusion from this study using q PCR is that MCF in rabbits involves virus expressing both productive and putative latency transcripts. This raises some doubts that MCF is a disease of latency. Gene expression may be 'aberrant' but more work is required.

Two papers are in preparation from this work. The first is on the *in vitro* analyses of more BT samples and LGLs with 5-aza, and also the use of phosphonoacetic acid as a treatment to induce latency. This has worked more efficiently on BT cells and LGLs than 5-aza treatment and was used by Abbie Jones, a student in D Haig lab. The second will be on the *in vitro* infection of endothelial and epithelial cells after some further analyses and increased sensitivity and extended range gene transcription analyses by q PCR. A work to address the role of ORF A9, a second bcl-2-like gene in AlHV-1 (along with A4.5) in the pathogenesis of MCF is ongoing by a graduate student.

7.1.2 Project summary

The research work was carried out to investigate the virus life cycle and the pathogenesis of malignant catarrhal fever (MCF). Rabbits, which are good model for MCF, were infected with alcelphine herpesvirus 1 (AlHV-1) and treated with cyclosporine A to suppress the accumulation of lymphocytes in the infected tissues. Large granular lymphocytes (LGLs) with the morphology of T/natural killer cells derived from the infected only rabbits were treated with 5-azacitidine in order to drive the latency programme in them. RNA-Seq was performed on the cells RNA (as copy DNA) samples to identify virus latency transcripts and the host transcriptome in latently-infected cells. Epithelial (SIRC) and endothelial (BAE) cells were infected with the virus *in vitro* to identify whether the cells can be infected and if so what kind of infection can be established (latent or productive/lytic). The results obtained can be summarised as following:

- The *in vitro* epithelium and endothelium cell culture showed that the cells can be latently infected with the virus and this may be possible *in vivo*.
- The identification of latency transcripts on LGLs and BT cells using 5-azacitidine was unsuccessful.
- The flow cytometry analysis of LGLs showed that CD8$^+$ T cells was the predominant cell subset in both 5-aza treated and

non-treated LGLs and the percentage of Pan-T cells were higher 5-aza treated LGLs.

- The pathway analysis of LGLs and BT cells showed that there were variations between rabbit LGLs and cattle BT cells. LGLs have pathways supporting T cell proliferation and accumulation and immune responsiveness in 5-aza-treated groups compared to untreated ones, BT cells contain pathways that aid gene expression and mRNA translation in treated groups compared to untreated.

- The viral genes profile from the infected rabbits euthanized at early stage of the disease showed a mixture of both latent and productive virus cycle.

- Unfortunately, no results obtained from the *in situ* hybridisation work.

Appendices

8.1 SOLUTIONS

Use distilled water to make up solutions, then autoclave solution. When using distilled water within the protocol, use autoclaved distilled water.

8.1.1 DEPC water

(ddH$_2$O, diethylepyrocarbonate-treated)

     1 ml DEPC (Fluka, 32490) add 1000 ml ddH$_2$O

Dissolve overnight on magnetic stirrer. Autoclave at 0.4 atm, 120°C, 20 min. Care: This solution is regarded as carcinogenic!

8.1.2 ddH$_2$O

Autoclave ddH$_2$O without DEPC (like all other solutions) at 0.4 atm, 120°C, 20 min.

8.1.3 TrisHCl

(MW 121.14): 1 M, pH 8.0

12.11g Tris(hydroxymethyl)aminomethane (Fluka, 93352) in 100 ml ddH$_2$O

Adjust pH with concentrated HCl.

8.1.4 CaCl$_2$

(MW 147.02) for proteinase K: 0.1 M

1.47 g CaCl$_2$ (Merck, 102382) in 100 ml ddH$_2$O

8.1.5 MgCl₂-hexahydrate

(MW 203.3) for PBS: 5mM $MgCl_2$

1 M: 20.33 g $MgCl_2$-hexahydrate (Merck, 105833) in 100 ml ddH_2O

8.1.6 NaCl

(MW 58,44; Merck, 1.06400.5000)

For hybridisation salts: 5 M = 29.22 g/100 ml ddH_2O

For RNAse treatment: 3 M = 87.66 g/500 ml ddH_2O

8.1.7 0.2 M HCl (Sterilize prior to use; not 2 N HCl)

50 ml 2 N HCl (Merck, 1.09970. Titrisol®)

450 ml ddH_2O

8.1.8 PIPES

(Piperazin-N,N'bis(2-ethanesulfate-acid)), pH 7,0 (MW 346.3; Sigma, P3768)

For hybridisation salts: 0.5 M = 1.7315 g/10 ml ddH_2O (sterile preparation!)

8.1.9 10x PBS

(Phosphate buffered saline), 1x PBS:

Dilute some 10 x PBS 1:10 to 1x PBS (in ddH_2O without DEPC) and adjust the pH to 7.4 with 1M HCl or 1M NaCl.

8.1.10 Buffer 1, pH 7,5

Tris (MW 121,14)   12.11 g Tris (final concentration 100 mM)

NaCl (MW 58,44)   8.77 g NaCl (final concentration 100 mM)

add 1000 ml ddH$_2$O (without DEPC).

Adjust pH with concentrated HCl.

8.1.11 Buffer 3, pH 9.5

Prepare fresh! To 150ml ddH$_2$O without DEPC add;

Tris (MW 121,14)   2.422 g (3.633g) Tris   (final   concentration 100 mM)

NaCl (MW 58,44)   1.168 g (1.752g) NaCl   (final   concentration 100 mM)

Adjust pH to 9.5, then add

MgCl$_2$ + 6H$_2$0 (MW 203,3)   2.034 g (3.051g) MgCl$_2$ (final concentration 50 mM)

Make volume to 200 ml (300ml) with ddH$_2$O (without DEPC).

8.1.12 Buffer 4, pH 8.0

Tris (MW 121,14)   1.21 g   Tris (final concentration 10 mM)

EDTA (MW 372,3)   0.37 g   EDTA   (final concentration 1 mM; Serva, 11280.01)

add 1000 ml ddH$_2$O (without DEPC). Adjust pH with 2 N HCl.

8.1.13 1x PBS + 5 mM MgCl$_2$

10x PBS   10 ml

1 M MgCl$_2$ 0.5 ml

add 100 ml ddH$_2$O (without DEPC)

8.1.14 2x SSC + 5 mM EDTA-Na$_2$

20x SSC   50 ml

0.5 M EDTA-Na$_2$ 5 ml

add 500 ml ddH$_2$O (without DEPC)

8.1.15 0.2% Glycine in 1x PBS

1 g glycine

500 ml 1x PBS (adjust pH before!)

8.1.16 4% paraformaldehyde (PFA)

For 250 ml:

Dissolve 10 g paraformaldehyde in approx. 200 ml 1x PBS at approx. 60°C (on magnetic stirrer; do not increase the temperature above 70°C, as the solution will precipitate!); adjust pH to 7.35 – 7.4. Fill with 1x PBS to 250 ml. Solution can be stored for up to one month. N.B.: poisonous fumes so do this in hood. Takes about 2 hours.

Do not autoclave the prehybridisation mix, hybridisation mix or their components, but produce them under sterile, that is to say RNAse-free, conditions!! (You may autoclave: 20x SSC, Triton X-100, 5 M NaOH)

8.1.17 50x Denhardt`s solution

100x Denhardt` s solution   100 ml

ddH$_2$O (without DEPC)   100 ml

Store at −20°C.

8.1.18 20x hybridisation salts

EDTA pH 8.0   0.5 M   10 ml (final concentration 0.1 M)

PIPES pH 7.0   0.5 M   10 ml (final concentration 0.1 M)

NaCl,   5.0 M   30 ml

Produce the salt solutions with ddH$_2$O (without DEPC).

8.1.19 Prehybridisation mix (PHB-Mix)

20x SSC   150 ml

100% formamide, deion.   225 ml

50x Denhardts solution   50 ml

ddH$_2$O with DEPC   70 ml

store at −20°C.

8.1.20 50x TAE Electrophoresis Buffer

Tris free base 242 g

Disodium EDTA 18.61 g

Glacial Acetic Acid 57.1 ml

DDI H2O to 1 l

Add the Tris free base and EDTA to approximately 700 ml DDI H2O and stir until the Tris and EDTA are dissolved. Add the acetic acid and adjust the volume to 1 liter.

8.1.21 1x TAE Electrophoresis Buffer

The 1x TAE solution is 40mM Tris, 20mM Acetate and 1mM EDTA and typically has a pH around 8.6

(do not adjust).

Or add 480 ml DDI H2O to 20 ml 50x TAE solution.

Hybridisation mix (HB-Mix):

100% formamide, deion.   16 ml

20x hybridisation salts   8 ml

50x Denhardts solution   3.2 ml

Heparin   400 µl (Heparin (20,000 U), diluted in 1 ml ddH$_2$O)

10% Triton X-100   320 µl

27.84 ml = 40 aliquots a 696 µl; store at –20°C.

8.1.22 ssDNA

Dissolve ssDNA (Deoxyribonucleic acid (DNA) Sodium Salt Type XIV from Salmon sperm; Sigma, D6898, 250 mg) in **buffer 4**, pH 8.0 to a final concentration of 10 mg/ml.

8.1.23 Proteinase K solution:

Is supplied by company (diluted in 10 mM TrisHCl, pH 7.5 (15.6 mg/ml); Roche Diagnostics, 1413783).

8.1.24 Dextransulfate solution

250mg dextransulfate + 400µl ddH$_2$O with DEPC; pipette into Eppendorf tube, mix well and dissolve by placing eppendorf in boiling waterbath; **takes time**!

8.1.25 RNA stock solution

10 mg RNA (Sigma, R 7250)

1 ml ddH$_2$O (DEPC-treated)

Need 1.3ml per run (for steps V and VI); keep on ice

8.1.26 Dye solution

Nitrobluetetrazoliumchloride (NBT):

Sigma, Order No. N 6639

1 g NBT add 13.3 ml 70% dimethylformamide (DMF: = 30 ml Aqua ddH$_2$O + 70 ml DMF) = Stock solution with 75 mg/ml final concentration

5-bromo-4-chloro-3-indolylephosphate (X-phosphate, BCIP)

Sigma, Order.No. B6777

500 mg X-Phosphate add 10 ml 100% dimethylformamide (syock solution)

The solutions are prepared in their original container. N.B.: toxic.

8.2 Rabbit DAVID cluster

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DAVID Bioinformatics Resources 6.7
National Institute of Allergy and Infectious Diseases (NIAID), NIH

Help and Manual

Functional Annotation Clustering

Current Gene List: List_2
Current Background: Homo sapiens
288 DAVID IDs

Options   Classification Stringency   Medium ▼
Renun using options   Create Sublist

Download File

93 Cluster(s)

Annotation Cluster 1	Enrichment Score: 2.16		Count	P_Value	Benjamini
GOTERM_CC_FAT	nuclear lumen	G	38	1.5E-3	3.3E-1
GOTERM_CC_FAT	intracellular organelle lumen		44	1.7E-3	2.0E-1
GOTERM_CC_FAT	organelle lumen		44	2.7E-3	2.1E-1
GOTERM_CC_FAT	membrane-enclosed lumen		44	3.8E-3	2.3E-1
GOTERM_CC_FAT	nucleoplasm		21	5.5E-2	6.4E-1
GOTERM_CC_FAT	nucleolus		17	7.6E-2	6.9E-1

Annotation Cluster 2	Enrichment Score: 1.9		Count	P_Value	Benjamini
SP_PIR_KEYWORDS	Fatty acid biosynthesis		6	3.4E-4	2.6E-2
SP_PIR_KEYWORDS	lipid synthesis		8	4.9E-4	2.5E-2
GOTERM_BP_FAT	fatty acid biosynthetic process		6	7.2E-3	4.7E-1
GOTERM_BP_FAT	organic acid biosynthetic process		7	3.2E-2	7.5E-1
GOTERM_BP_FAT	carboxylic acid biosynthetic process		7	3.2E-2	7.5E-1
GOTERM_BP_FAT	fatty acid metabolic process		6	1.9E-1	9.5E-1
GOTERM_BP_FAT	lipid biosynthetic process		8	2.3E-1	9.6E-1

Annotation Cluster 3	Enrichment Score: 1.88		Count	P_Value	Benjamini
GOTERM_BP_FAT	DNA metabolic process		19	7.8E-4	6.7E-1
GOTERM_BP_FAT	response to DNA damage stimulus		15	1.8E-3	4.0E-1
GOTERM_BP_FAT	DNA repair		12	4.4E-3	4.3E-1
GOTERM_BP_FAT	cellular response to stress		18	6.2E-3	4.7E-1
GOTERM_BP_FAT	dna repair		8	2.0E-2	2.7E-1
GOTERM_BP_FAT	DNA damage		8	2.7E-2	3.2E-1
GOTERM_BP_FAT	dna recombination		3	9.7E-2	6.0E-1
GOTERM_BP_FAT	DNA recombination		3	4.8E-1	9.9E-1

Annotation Cluster 4	Enrichment Score: 1.84		Count	P_Value	Benjamini
SP_PIR_KEYWORDS	mitochondrion		25	8.2E-4	3.1E-2
SP_PIR_KEYWORDS	mitochondrion		28	9.6E-3	3.1E-1
UP_SEQ_FEATURE	transit peptide-Mitochondrion		12	7.3E-3	1.0E0
SP_PIR_KEYWORDS	transit peptide		12	7.8E-2	5.3E-1

Annotation Cluster 5	Enrichment Score: 1.82		Count	P_Value	Benjamini
SP_PIR_KEYWORDS	manganese		8	6.1E-3	1.4E-1
GOTERM_MF_FAT	manganese ion binding		8	6.9E-3	6.2E-1
UP_SEQ_FEATURE	metal ion-binding site-Manganese		3	8.0E-2	1.0E0

Annotation Cluster 6	Enrichment Score: 1.82		Count	P_Value	Benjamini
SP_PIR_KEYWORDS	Apoptosis		16	3.8E-4	2.3E-2
GOTERM_BP_FAT	programmed cell death		21	1.1E-3	5.5E-1
GOTERM_BP_FAT	cell death		23	1.5E-3	5.1E-1
GOTERM_BP_FAT	death		23	1.7E-3	4.4E-1
GOTERM_BP_FAT	apoptosis		20	2.3E-3	3.7E-1
GOTERM_BP_FAT	regulation of apoptosis		20	3.9E-2	7.8E-1
GOTERM_BP_FAT	regulation of programmed cell death		20	4.4E-2	7.8E-1
GOTERM_BP_FAT	regulation of cell death		20	4.6E-2	7.7E-1
GOTERM_BP_FAT	negative regulation of apoptosis		11	4.6E-2	7.7E-1
GOTERM_BP_FAT	negative regulation of programmed cell death		11	4.9E-2	7.8E-1
GOTERM_BP_FAT	negative regulation of cell death		11	5.1E-2	7.5E-1
GOTERM_BP_FAT	anti-apoptosis		6	2.1E-1	9.6E-1

https://david.ncifcrf.gov/term2term.jsp?annot=52,9,79,85,25,32,39,1,3,47,45,63,76&currentList=1

1/14

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	Category	Term				Count	P_Value	Benjamini
	GOTERM_BP_FAT	cell proliferation	G			8	5.0E-1	9.9E-1
	Annotation Cluster 7	Enrichment Score: 1.66				Count	P_Value	Benjamini
	GOTERM_CC_FAT	endoplasmic reticulum				26	7.2E-3	3.2E-1
	GOTERM_CC_FAT	endoplasmic reticulum membrane				11	9.2E-3	3.4E-1
	GOTERM_CC_FAT	nuclear envelope-endoplasmic reticulum network				11	1.3E-2	3.6E-1
	GOTERM_CC_FAT	endomembrane system				20	3.4E-2	5.7E-1
	GOTERM_CC_FAT	endoplasmic reticulum part				11	4.4E-2	6.0E-1
	GOTERM_CC_FAT	organelle membrane				24	8.3E-2	6.9E-1
	Annotation Cluster 8	Enrichment Score: 1.64				Count	P_Value	Benjamini
	SP_PIR_KEYWORDS	cell cycle	G			18	3.4E-4	3.4E-2
	GOTERM_BP_FAT	cell cycle				23	3.9E-3	4.6E-1
	SP_PIR_KEYWORDS	cell division				11	4.6E-3	1.2E-1
	GOTERM_BP_FAT	cell division				12	5.8E-3	4.7E-1
	GOTERM_BP_FAT	mitotic cell cycle				13	1.2E-2	5.4E-1
	GOTERM_BP_FAT	cell cycle process				16	2.7E-2	7.6E-1
	GOTERM_BP_FAT	cell cycle phase				12	5.3E-2	7.7E-1
	GOTERM_BP_FAT	M phase of mitotic cell cycle				8	5.7E-2	7.7E-1
	GOTERM_BP_FAT	organelle fission				8	6.2E-2	7.9E-1
	GOTERM_BP_FAT	mitosis				6	1.2E-1	6.3E-1
	SP_PIR_KEYWORDS	nuclear division				7	1.2E-1	9.1E-1
	GOTERM_BP_FAT	mitosis				7	1.2E-1	9.1E-1
	GOTERM_BP_FAT	M phase				9	1.3E-1	9.2E-1
	Annotation Cluster 9	Enrichment Score: 1.55	G			Count	P_Value	Benjamini
	GOTERM_BP_FAT	DNA catabolic process				4	6.4E-2	7.9E-1
	GOTERM_BP_FAT	apoptotic nuclear changes				3	6.0E-2	7.8E-1
	GOTERM_BP_FAT	DNA catabolic process, endonucleolytic				3	6.0E-2	7.8E-1
	GOTERM_BP_FAT	nucleus organization				4	4.5E-2	7.7E-1
	GOTERM_BP_FAT	DNA fragmentation involved in apoptosis	G			3	3.4E-2	7.6E-1
	GOTERM_BP_FAT	cellular component disassembly				5	8.7E-3	5.0E-1
	GOTERM_BP_FAT	cell structure disassembly during apoptosis				4	5.0E-3	4.4E-1
	GOTERM_MF_FAT	3'-5'-exoribonuclease activity				3	1.2E-2	6.5E-1
	Annotation Cluster 10	Enrichment Score: 1.54				Count	P_Value	Benjamini
	GOTERM_MF_FAT	ribonuclease activity				3	2.5E-1	9.6E-1
	GOTERM_MF_FAT	3'-5' exonuclease activity				3	6.1E-2	8.1E-1
	GOTERM_MF_FAT	exonuclease activity				4	4.1E-2	7.7E-1
	GOTERM_MF_FAT	exonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters				3	3.3E-2	7.6E-1
	SP_PIR_KEYWORDS	nuclease activity				7	2.7E-2	7.6E-1
	GOTERM_MF_FAT	exonuclease				4	2.3E-2	3.0E-1
	KEGG_PATHWAY	RNA degradation				5	2.0E-2	9.1E-1
	SP_PIR_KEYWORDS	nuclease				6	1.8E-2	2.7E-1
	GOTERM_MF_FAT	exoribonuclease activity				3	1.5E-2	6.4E-1
	GOTERM_MF_FAT	exoribonuclease activity, producing 5'-phosphomonoesters				3	1.5E-2	6.4E-1
	GOTERM_MF_FAT	3'-5'-exoribonuclease activity				3	1.2E-2	6.5E-1
	Annotation Cluster 11	Enrichment Score: 1.49				Count	P_Value	Benjamini
	UP_SEQ_FEATURE	domain:VWFA				4	5.9E-2	1.0E0
	INTERPRO	von Willebrand factor, type A				5	2.8E-2	9.9E-1
	SMART	VWA	G			5	2.1E-2	9.3E-1
	Annotation Cluster 12	Enrichment Score: 1.39	G			Count	P_Value	Benjamini
	GOTERM_BP_FAT	phosphorus metabolic process				28	1.9E-3	3.6E-1
	GOTERM_BP_FAT	phosphate metabolic process				28	1.9E-3	3.6E-1
	GOTERM_MF_FAT	protein serine/threonine kinase activity				15	3.8E-3	8.0E-1
	GOTERM_BP_FAT	phosphorylation				9	8.2E-3	1.7E-1
	SP_PIR_KEYWORDS	serine/threonine-protein kinase				13	8.5E-3	1.7E-1
	SP_PIR_KEYWORDS	kinase				19	9.7E-3	1.8E-1
	SP_PIR_KEYWORDS	phosphorylation				22	1.1E-2	1.8E-1
	GOTERM_BP_FAT	phosphorylation				22	1.1E-2	5.4E-1
	GOTERM_MF_FAT	nucleotide binding				46	1.1E-2	6.9E-1
	INTERPRO	Serine/threonine protein kinase, active site				12	1.5E-2	1.0E0
	INTERPRO	Serine/threonine protein kinase-related				12	1.6E-2	9.9E-1

270

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	Category	Term		Count	P_Value	Benjamini
☐	GOTERM_MF_FAT	protein kinase activity	RT	16	2.9E-2	7.5E-1
☐	SP_PIR_KEYWORDS	nucleotide-binding	RT	34	3.7E-2	3.8E-1
☐	UP_SEQ_FEATURE	binding site:ATP	RT	14	4.8E-2	1.0E0
☐	GOTERM_BP_FAT	protein amino acid phosphorylation	RT	17	5.0E-2	7.7E-1
☐	UP_SEQ_FEATURE	active site:Proton acceptor	RT	16	5.1E-2	1.0E0
☐	GOTERM_MF_FAT	purine nucleotide binding	RT	37	5.5E-2	8.4E-1
☐	SMART	S_TKc	RT	8	5.8E-2	9.8E-1
☐	UP_SEQ_FEATURE	nucleotide phosphate-binding region:ATP	RT	21	5.9E-2	1.0E0
☐	SP_PIR_KEYWORDS	atp-binding	RT	27	5.9E-2	5.0E-1
☐	SP_PIR_KEYWORDS	transferase	RT	28	6.2E-2	4.9E-1
☐	GOTERM_MF_FAT	purine ribonucleotide binding	RT	35	7.1E-2	8.4E-1
☐	GOTERM_MF_FAT	ribonucleotide binding	RT	35	7.1E-2	8.4E-1
☐	UP_SEQ_FEATURE	domain:Protein kinase	RT	12	7.5E-2	1.0E0
☐	GOTERM_MF_FAT	adenyl ribonucleotide binding	RT	27	1.8E-1	9.2E-1
☐	GOTERM_MF_FAT	nucleotide binding	RT	29	1.7E-1	9.1E-1
☐	SP_PIR_KEYWORDS	serine/threonine-specific protein kinase	RT	3	2.1E-1	7.7E-1
	KEGG_PATHWAY	MAPK signaling pathway	RT G	3	9.6E-1	1.0E0
	Annotation Cluster 13	Enrichment Score: 1.33		Count	P_Value	Benjamini
☐	GOTERM_BP_FAT	serine/threonine protein kinase	RT	8	8.4E-1	1.0E0
☐	INTERPRO	Protein kinase, core	RT	12	8.8E-1	1.0E0
☐	INTERPRO	Protein kinase, ATP binding site	RT	11	1.3E-1	1.0E0
☐	GOTERM_MF_FAT	adenyl nucleotide binding	RT	29	1.4E-1	9.2E-1
☐	GOTERM_MF_FAT	purine nucleoside binding	RT	29	1.6E-1	9.3E-1
☐	GOTERM_MF_FAT	ATP binding	RT	27	1.6E-1	9.2E-1
☐	GOTERM_MF_FAT	nucleoside binding	RT	29	1.6E-1	9.1E-1
	Annotation Cluster 14	Enrichment Score: 1.17		Count	P_Value	Benjamini
☐	GOTERM_BP_FAT	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	RT	3	7.7E-2	8.3E-1
☐	GOTERM_BP_FAT	nuclear-transcribed mRNA catabolic process	RT	3	1.0E-1	8.8E-1
☐	SP_PIR_KEYWORDS	nonsense-mediated mrna decay	RT	3	6.0E-2	4.9E-1
☐	GOTERM_BP_FAT	mRNA catabolic process	RT	4	2.8E-1	7.6E-1
☐	GOTERM_BP_FAT	RNA catabolic process	RT	5	1.7E-1	6.6E-1
	Annotation Cluster 14	Enrichment Score: 1.17		12	2.2E-1	7.1E-1
☐	GOTERM_BP_FAT	regulation of kinase activity	RT	12	2.2E-1	7.1E-1
☐	GOTERM_BP_FAT	regulation of phosphorylation	RT	14	2.7E-2	7.7E-1
☐	GOTERM_BP_FAT	regulation of transferase activity	RT	12	2.8E-2	7.5E-1
☐	GOTERM_BP_FAT	regulation of phosphorus metabolic process	RT	14	3.6E-2	7.7E-1
☐	GOTERM_BP_FAT	regulation of phosphate metabolic process	RT	14	3.6E-2	7.7E-1
☐	GOTERM_BP_FAT	regulation of protein kinase activity	RT	11	4.0E-2	7.7E-1
☐	GOTERM_BP_FAT	positive regulation of catalytic activity	RT	14	5.7E-2	7.8E-1
☐	GOTERM_BP_FAT	positive regulation of molecular function	RT	15	6.6E-2	8.0E-1
☐	GOTERM_BP_FAT	positive regulation of kinase activity	RT	6	2.8E-1	9.7E-1
☐	GOTERM_BP_FAT	positive regulation of transferase activity	RT	6	3.1E-1	9.7E-1
☐	GOTERM_BP_FAT	positive regulation of protein kinase activity	RT G	5	4.4E-1	9.9E-1
	Annotation Cluster 15	Enrichment Score: 1.03		Count	P_Value	Benjamini
☐	GOTERM_BP_FAT	regulation of apoptosis	RT	20	3.9E-2	7.8E-1
☐	GOTERM_BP_FAT	regulation of programmed cell death	RT	20	4.3E-2	7.8E-1
☐	GOTERM_BP_FAT	regulation of cell death	RT	20	4.4E-2	7.7E-1
☐	GOTERM_BP_FAT	induction of apoptosis	RT	9	1.2E-1	9.1E-1
☐	GOTERM_BP_FAT	induction of programmed cell death	RT	9	1.2E-1	9.1E-1
☐	GOTERM_BP_FAT	positive regulation of apoptosis	RT	11	1.2E-1	9.2E-1
☐	GOTERM_BP_FAT	positive regulation of programmed cell death	RT	11	1.3E-1	9.2E-1
☐	GOTERM_BP_FAT	positive regulation of cell death	RT	11	1.3E-1	9.2E-1
☐	GOTERM_BP_FAT	induction of apoptosis by extracellular signals	RT G	4	2.5E-1	9.6E-1
	Annotation Cluster 16	Enrichment Score: 0.94		Count	P_Value	Benjamini
☐	INTERPRO	Nucleotide-binding, alpha-beta plait	RT	8	3.6E-2	9.9E-1
☐	SMART	RRM	RT	7	6.3E-2	9.3E-1
☐	INTERPRO	RNA recognition motif, RNP-1	RT	7	8.8E-2	1.0E0
☐	UP_SEQ_FEATURE	domain:RRM 3	RT	3	1.6E-1	1.0E0
☐	UP_SEQ_FEATURE	domain:RRM 2	RT	4	1.8E-1	1.0E0

https://david.ncifcrf.gov/term2term.jsp?annot=52,79,85,25,32,39,1,3,47,45,63,78&currentList=1

271

2/29/2016

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Category	Term		Count	P_Value	Benjamini
UP_SEQ_FEATURE	domain:RRM 1	RT	4	1.8E-1	1.0E0
SP_PIR_KEYWORDS	methylation	RT	6	2.6E-1 8.2E-1	1.0E0

Annotation Cluster 17 — Enrichment Score: 0.93

Category	Term		Count	P_Value	Benjamini
KEGG_PATHWAY	Glycolysis / Gluconeogenesis	RT G	5	2.3E-1	7.6E-1
GOTERM_BP_FAT	hexose metabolic process	RT	8	2.8E-2	7.4E-1
SP_PIR_KEYWORDS	Gluconeogenesis	RT	3	2.9E-2	3.3E-1
GOTERM_BP_FAT	gluconeogenesis	RT	3	5.5E-2	7.8E-1
GOTERM_BP_FAT	monosaccharide metabolic process	RT	8	5.6E-2	7.8E-1
GOTERM_BP_FAT	hexose biosynthetic process	RT	3	8.1E-2	8.5E-1
GOTERM_BP_FAT	glucose metabolic process	RT	3	8.6E-2	8.5E-1
GOTERM_BP_FAT	cellular carbohydrate biosynthetic process	RT	6	8.9E-2	8.6E-1
SP_PIR_KEYWORDS	monosaccharide biosynthetic process	RT	4	1.1E-1	9.0E-1
GOTERM_BP_FAT	glycolysis	RT	3	1.3E-1	6.5E-1
GOTERM_BP_FAT	pyruvate metabolic process	RT	3	1.3E-1	9.2E-1
GOTERM_BP_FAT	alcohol biosynthetic process	RT	3	1.5E-1	9.3E-1
GOTERM_BP_FAT	glucose catabolic process	RT	3	1.6E-1	9.4E-1
GOTERM_BP_FAT	carbohydrate biosynthetic process	RT	3	2.2E-1	9.6E-1
GOTERM_BP_FAT	carbohydrate catabolic process	RT	4	2.3E-1	9.6E-1
GOTERM_BP_FAT	hexose catabolic process	RT	3	2.8E-1	9.7E-1
GOTERM_BP_FAT	monosaccharide catabolic process	RT	13	2.4E-1	9.5E-1
GOTERM_BP_FAT	alcohol catabolic process	RT	3	3.0E-1	9.7E-1
GOTERM_BP_FAT	cellular carbohydrate catabolic process	RT	3	3.5E-1	9.8E-1

Annotation Cluster 18 — Enrichment Score: 0.92

Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	cellular carbohydrate catabolic process	RT	3	3.7E-1	9.8E-1
GOTERM_BP_FAT	modification-dependent protein catabolic process	RT	12	2.6E-1	9.6E-1
GOTERM_BP_FAT	modification-dependent macromolecule catabolic process	RT	12	2.6E-1	9.6E-1
GOTERM_BP_FAT	protein catabolic process	RT	13	2.4E-1	9.5E-1
GOTERM_BP_FAT	cellular protein catabolic process	RT	13	2.1E-1	9.6E-1
GOTERM_BP_FAT	proteolysis involved in cellular protein catabolic process	RT	13	2.1E-1	9.6E-1
SP_PIR_KEYWORDS	ubl conjugation pathway	RT	11	1.9E-1	7.4E-1
GOTERM_BP_FAT	macromolecule catabolic process	RT	23	4.2E-3	4.5E-1
GOTERM_BP_FAT	cellular macromolecule catabolic process	RT G	22	3.7E-3	4.8E-1
GOTERM_BP_FAT	proteolysis	RT	17	5.5E-1	1.0E0

Annotation Cluster 19 — Enrichment Score: 0.89

Category	Term		Count	P_Value	Benjamini
GOTERM_MF_FAT	GTPase activity	RT	8	3.3E-2	7.2E-1
SP_PIR_KEYWORDS	GTP-binding	RT	10	8.9E-2	8.7E-1
GOTERM_MF_FAT	small GTPase mediated signal transduction	RT	10	9.7E-2	8.7E-1
GOTERM_BP_FAT	gtp-binding	RT	9	9.8E-2	5.9E-1
SP_PIR_KEYWORDS	guanyl ribonucleotide binding	RT	10	1.0E-1	8.8E-1
GOTERM_MF_FAT	guanyl nucleotide binding	RT	10	1.0E-1	8.8E-1
UP_SEQ_FEATURE	nucleotide phosphate-binding region:GTP	RT	7	2.5E-1	1.0E0
INTERPRO	Ras GTPase	RT	4	3.1E-1	1.0E0
INTERPRO	Small GTP-binding protein	RT G	4	4.4E-1	1.0E0

Annotation Cluster 20 — Enrichment Score: 0.84

Category	Term		Count	P_Value	Benjamini
GOTERM_CC_FAT	extrinsic to plasma membrane	RT	4	4.2E-2	6.2E-1
GOTERM_CC_FAT	heterotrimeric G-protein complex	RT	3	7.4E-2	7.0E-1
SP_PIR_KEYWORDS	transducer	RT G	6	1.0E0	1.0E0

Annotation Cluster 21 — Enrichment Score: 0.82

Category	Term		Count	P_Value	Benjamini
GOTERM_MF_FAT	phosphoprotein phosphatase activity	RT	6	9.1E-2	8.6E-1
SP_PIR_KEYWORDS	protein phosphatase	RT	5	1.1E-1	6.2E-1
GOTERM_MF_FAT	protein serine/threonine phosphatase activity	RT	3	1.4E-1	9.2E-1
GOTERM_BP_FAT	protein amino acid dephosphorylation	RT	5	1.5E-1	9.3E-1
GOTERM_MF_FAT	phosphatase activity	RT	7	1.5E-1	9.3E-1
GOTERM_BP_FAT	dephosphorylation	RT	5	2.1E-1	9.6E-1
SP_PIR_KEYWORDS	phosphatase	RT	5	2.6E-1	9.6E-1

Annotation Cluster 22 — Enrichment Score: 0.82

Category	Term		Count	P_Value	Benjamini
GOTERM_MF_FAT	phosphoric monoester hydrolase	RT	3	2.6E-1	8.2E-1

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Category	Term		Count	P_Value	Benjamini
INTERPRO	Nucleotide-binding, alpha-beta plait	RT	8	3.6E-2	9.9E-1
GOTERM_MF_FAT	RNA binding	RT	17	5.5E-2	8.2E-1
GOTERM_BP_FAT	mRNA metabolic process	RT	11	5.8E-2	7.7E-1
SMART	RRM	RT	7	6.3E-2	9.3E-1
INTERPRO	RNA recognition motif, RNP-1	RT	7	8.8E-2	1.0E0
GOTERM_BP_FAT	RNA splicing	RT	8	1.5E-1	9.3E-1
SP_PIR_KEYWORDS	rna-binding	RT	12	1.5E-1	6.9E-1
SP_PIR_KEYWORDS	mrna processing	RT	7	1.7E-1	7.2E-1
GOTERM_BP_FAT	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	RT	5	2.1E-1	9.6E-1
GOTERM_BP_FAT	RNA splicing, via transesterification reactions	RT	5	2.1E-1	9.6E-1
GOTERM_BP_FAT	nuclear mRNA splicing, via spliceosome	RT	5	2.1E-1	9.6E-1
GOTERM_BP_FAT	RNA processing	RT	12	2.2E-1	9.5E-1
GOTERM_BP_FAT	mRNA processing	RT	8	2.2E-1	9.6E-1
SP_PIR_KEYWORDS	mrna splicing	RT	5	3.5E-1	8.8E-1
KEGG_PATHWAY	Spliceosome	RT	4	4.1E-1	9.5E-1
UP_SEQ_FEATURE	domain:RRM	RT	3	5.1E-1	1.0E0
Annotation Cluster 23	**Enrichment Score: 0.81**		**Count**	**P_Value**	**Benjamini**
KEGG_PATHWAY	Small cell lung cancer	G	5	6.7E-2	9.4E-1
BIOCARTA	Role of Mitochondria in Apoptotic Signaling	RT	3	7.1E-2	8.8E-1
KEGG_PATHWAY	Apoptosis	RT	5	7.4E-2	9.0E-1
KEGG_PATHWAY	Colorectal cancer	RT	5	4.6E-1	9.5E-1
KEGG_PATHWAY	Pathways in cancer	RT	7	5.6E-1	9.7E-1
Annotation Cluster 24	**Enrichment Score: 0.76**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	regulation of binding	RT	6	8.6E-2	8.5E-1
GOTERM_BP_FAT	regulation of transcription factor activity	RT	4	2.1E-1	9.6E-1
GOTERM_BP_FAT	regulation of DNA binding	G	4	2.8E-1	9.7E-1
Annotation Cluster 25	**Enrichment Score: 0.74**		**Count**	**P_Value**	**Benjamini**
SP_PIR_KEYWORDS	er-golgi transport	RT	7	1.2E-3	3.5E-2
SP_PIR_KEYWORDS	protein transport	RT	9	3.9E-1	9.0E-1
GOTERM_BP_FAT	protein transport	RT	11	7.4E-1	1.0E0
GOTERM_BP_FAT	establishment of protein localization	RT	11	7.5E-1	1.0E0
GOTERM_BP_FAT	protein localization	RT	12	8.0E-1	1.0E0
Annotation Cluster 26	**Enrichment Score: 0.73**		**Count**	**P_Value**	**Benjamini**
GOTERM_CC_FAT	cytoskeleton	G	18	8.7E-1	1.0E0
GOTERM_CC_FAT	cytoskeletal part	RT	14	7.2E-1	9.9E-1
GOTERM_CC_FAT	centrosome	RT	6	2.7E-1	9.0E-1
GOTERM_CC_FAT	microtubule organizing center part	RT	3	2.2E-1	8.9E-1
GOTERM_CC_FAT	microtubule organizing center	RT	7	2.0E-1	9.0E-1
SP_PIR_KEYWORDS	cytoskeleton	RT	13	2.0E-1	7.5E-1
GOTERM_CC_FAT	microtubule cytoskeleton	RT	14	8.5E-2	6.8E-1
GOTERM_CC_FAT	centriole	RT	3	8.3E-2	7.0E-1
GOTERM_CC_FAT	spindle	RT	7	2.7E-2	5.2E-1
Annotation Cluster 27	**Enrichment Score: 0.73**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	regulation of cellular protein metabolic process	G	9	6.5E-3	4.6E-1
GOTERM_BP_FAT	negative regulation of protein metabolic process	RT	9	8.2E-3	4.9E-1
GOTERM_BP_FAT	negative regulation of protein amino acid phosphorylation	RT	4	1.3E-2	5.6E-1
GOTERM_BP_FAT	negative regulation of phosphorylation	RT	4	3.1E-2	7.7E-1
GOTERM_BP_FAT	negative regulation of protein modification process	RT	6	3.6E-2	7.6E-1
GOTERM_BP_FAT	negative regulation of phosphorus metabolic process	RT	4	3.7E-2	7.6E-1
GOTERM_BP_FAT	negative regulation of phosphate metabolic process	RT	4	3.7E-2	7.6E-1
GOTERM_BP_FAT	regulation of cell adhesion	RT	5	1.6E-1	9.4E-1
GOTERM_BP_FAT	negative regulation of catalytic activity	RT	7	2.5E-1	9.6E-1
GOTERM_BP_FAT	negative regulation of molecular function	RT	8	2.5E-1	9.6E-1
GOTERM_BP_FAT	negative regulation of ubiquitin-protein ligase activity	RT	3	2.7E-1	9.7E-1
GOTERM_BP_FAT	negative regulation of ligase activity	RT	3	2.7E-1	9.7E-1

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Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	regulation of protein amino acid phosphorylation	5	2.7E-1	9.6E-1
GOTERM_BP_FAT	regulation of protein modification process	7	3.0E-1	9.7E-1
GOTERM_BP_FAT	regulation of cellular protein metabolic process	10	3.1E-1	9.7E-1
GOTERM_BP_FAT	negative regulation of protein ubiquitination	3	3.1E-1	9.7E-1
GOTERM_BP_FAT	regulation of ubiquitin-protein ligase activity	3	3.4E-1	9.7E-1
GOTERM_BP_FAT	regulation of ligase activity	3	3.5E-1	9.8E-1
GOTERM_BP_FAT	positive regulation of protein ubiquitination	3	3.7E-1	9.8E-1
GOTERM_BP_FAT	positive regulation of protein kinase activity	3	3.9E-1	9.8E-1
GOTERM_BP_FAT	positive regulation of protein kinase activity	3	4.0E-1	9.8E-1
GOTERM_BP_FAT	negative regulation of transferase activity	3	4.3E-1	9.9E-1
GOTERM_BP_FAT	regulation of protein ubiquitination	3	4.5E-1	9.9E-1
GOTERM_BP_FAT	negative regulation of signal transduction	3	7.8E-1	1.0E0
GOTERM_BP_FAT	positive regulation of cellular protein metabolic process	3	8.6E-1	1.0E0
GOTERM_BP_FAT	positive regulation of protein metabolic process	3	8.8E-1	1.0E0
GOTERM_BP_FAT	negative regulation of cell communication	3	8.9E-1	1.0E0

Annotation Cluster 28 — Enrichment Score: 0.72

Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	structural molecule activity	7	9.0E-1	1.0E0
KEGG_PATHWAY	Ribosome	3	4.8E-1	9.5E-1
GOTERM_MF_FAT	structural constituent of ribosome	4	4.4E-1	1.0E0
GOTERM_BP_FAT	translation	8	2.5E-1	9.6E-1
GOTERM_CC_FAT	ribosome	6	2.4E-1	8.9E-1
GOTERM_BP_FAT	translational elongation	4	2.0E-1	9.6E-1
SP_PIR_KEYWORDS	ribosomal protein	6	1.3E-1	6.5E-1
GOTERM_CC_FAT	ribonucleoprotein complex	14	5.7E-2	6.3E-1
SP_PIR_KEYWORDS	ribonucleoprotein	10	1.9E-2	2.7E-1

Annotation Cluster 29 — Enrichment Score: 0.62

Category	Term	Count	P_Value	Benjamini
GOTERM_CC_FAT	non-membrane-bounded organelle	46	2.2E-1	8.8E-1
GOTERM_CC_FAT	intracellular non-membrane-bounded organelle	46	2.2E-1	8.8E-1
GOTERM_CC_FAT	nucleolus	17	7.6E-2	6.9E-1
GOTERM_CC_FAT	cytoskeleton	18	8.7E-1	1.0E0

Annotation Cluster 30 — Enrichment Score: 0.61

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	forebrain development	3	6.8E-1	1.0E0
GOTERM_BP_FAT	telencephalon development	3	2.6E-1	9.6E-1
GOTERM_BP_FAT	limbic system development	3	8.6E-2	8.5E-1

Annotation Cluster 31 — Enrichment Score: 0.6

Category	Term	Count	P_Value	Benjamini
GOTERM_CC_FAT	mitochondrial lumen	6	2.8E-1	9.0E-1
GOTERM_CC_FAT	mitochondrial matrix	6	2.8E-1	9.0E-1
GOTERM_CC_FAT	mitochondrial part	13	2.1E-1	9.0E-1

Annotation Cluster 32 — Enrichment Score: 0.59

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	spermatogenesis	6	5.1E-1	9.9E-1
GOTERM_BP_FAT	male gamete generation	6	5.1E-1	9.9E-1
GOTERM_BP_FAT	reproductive cellular process	5	2.4E-1	9.6E-1
GOTERM_BP_FAT	multicellular organism reproduction	11	2.1E-1	9.6E-1
GOTERM_BP_FAT	reproductive process in a multicellular organism	11	2.1E-1	9.6E-1
GOTERM_BP_FAT	sexual reproduction	11	1.7E-1	9.4E-1
GOTERM_BP_FAT	gamete generation	10	1.5E-1	9.3E-1

Annotation Cluster 33 — Enrichment Score: 0.58

Category	Term	Count	P_Value	Benjamini
KEGG_PATHWAY	RNA degradation	5	2.0E-2	9.1E-1
GOTERM_BP_FAT	ribonucleoprotein complex biogenesis	6	1.4E-1	7.1E-1
SP_PIR_KEYWORDS	rna processing	3	2.1E-1	7.7E-1
GOTERM_BP_FAT	ribosome biogenesis	4	2.9E-1	9.7E-1

Annotation Cluster 34 — Enrichment Score: 0.54

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	rRNA processing	3	4.1E-1	9.9E-1
GOTERM_BP_FAT	rRNA metabolic process	3	4.3E-1	9.9E-1
GOTERM_BP_FAT	ncRNA processing	3	7.8E-1	1.0E0
GOTERM_BP_FAT	ncRNA metabolic process	3	8.7E-1	1.0E0

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Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	protein kinase cascade	9	2.1E-1	9.6E-1
GOTERM_BP_FAT	MAPKKK cascade	5	3.1E-1	9.7E-1
GOTERM_BP_FAT	regulation of MAP kinase activity	4	3.7E-1	9.8E-1
Annotation Cluster 35	**Enrichment Score: 0.54**	Count	P_Value	Benjamini
GOTERM_BP_FAT	kidney development	4	1.8E-1	9.5E-1
GOTERM_BP_FAT	urogenital system development	4	2.4E-1	9.5E-1
GOTERM_BP_FAT	tissue morphogenesis	5	3.0E-1	9.7E-1
GOTERM_MF_FAT	protein homodimerization activity	6	5.3E-1	1.0E0
Annotation Cluster 36	**Enrichment Score: 0.53**	Count	P_Value	Benjamini
GOTERM_CC_FAT	mitochondrial outer membrane	4	1.6E-1	8.5E-1
GOTERM_CC_FAT	organelle outer membrane	4	2.2E-1	8.9E-1
GOTERM_CC_FAT	outer membrane	4	2.3E-1	8.9E-1
GOTERM_CC_FAT	mitochondrial envelope	8	4.7E-1	9.6E-1
GOTERM_CC_FAT	mitochondrial membrane	7	5.7E-1	9.7E-1
Annotation Cluster 37	**Enrichment Score: 0.53**	Count	P_Value	Benjamini
INTERPRO	potassium channel, voltage dependent, Kv, tetramerisation	4	4.0E-2	9.9E-1
GOTERM_CC_FAT	potassium channel complex	5	4.4E-2	5.8E-1
GOTERM_CC_FAT	voltage-gated potassium channel complex	5	4.4E-2	5.8E-1
GOTERM_MF_FAT	voltage-gated potassium channel activity	5	5.6E-2	8.0E-1
GOTERM_MF_FAT	potassium channel activity	5	1.3E-1	9.1E-1
UP_SEQ_FEATURE	domain:BTB	5	1.5E-1	1.0E0
INTERPRO	BTB/POZ fold	4	1.5E-1	1.0E0
GOTERM_CC_FAT	cation channel complex	5	1.5E-1	8.3E-1
GOTERM_MF_FAT	voltage-gated cation channel activity	5	1.6E-1	9.2E-1
SMART	BTB	5	2.2E-1	1.0E0
GOTERM_BP_FAT	potassium ion transport	5	2.3E-1	9.6E-1
INTERPRO	BTB/POZ-like	5	2.6E-1	1.0E0
GOTERM_MF_FAT	monovalent inorganic cation transport	6	5.4E-1	1.0E0
GOTERM_MF_FAT	cation channel activity	6	5.1E-1	1.0E0
GOTERM_BP_FAT	gated channel activity	5	5.6E-1	1.0E0
GOTERM_MF_FAT	ion channel activity	6	6.6E-1	1.0E0
GOTERM_MF_FAT	substrate specific channel activity	6	6.6E-1	1.0E0
GOTERM_MF_FAT	channel activity	6	6.8E-1	1.0E0
GOTERM_MF_FAT	passive transmembrane transporter activity	6	7.1E-1	1.0E0
GOTERM_MF_FAT	cation transport	6	7.1E-1	1.0E0
GOTERM_BP_FAT	metal ion transmembrane transporter activity	4	4.9E-1	1.0E0
GOTERM_BP_FAT	ion transport	8	7.5E-1	1.0E0
GOTERM_BP_FAT	metal ion transport	6	8.4E-1	1.0E0
SP_PIR_KEYWORDS	ionic channel	3	9.4E-1	1.0E0
Annotation Cluster 38	**Enrichment Score: 0.53**	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	metalloprotein	5	1.0E-1	5.8E-1
SP_PIR_KEYWORDS	iron	7	2.2E-1	7.7E-1
SP_PIR_KEYWORDS	chromoprotein	3	2.2E-1	7.7E-1
SP_PIR_KEYWORDS	iron ion binding	7	2.8E-1	9.8E-1
SP_PIR_KEYWORDS	heme	3	5.1E-1	9.5E-1
GOTERM_MF_FAT	heme binding	3	5.2E-1	1.0E0
GOTERM_BP_FAT	heme	3	5.6E-1	1.0E0
Annotation Cluster 39	**Enrichment Score: 0.51**	Count	P_Value	Benjamini
GOTERM_MF_FAT	protein heterodimerization activity	7	8.0E-2	8.6E-1
GOTERM_MF_FAT	cell activation	8	1.5E-1	9.3E-1
GOTERM_BP_FAT	lymphocyte activation	6	1.9E-1	9.5E-1
GOTERM_BP_FAT	T cell activation	4	3.0E-1	9.7E-1
GOTERM_BP_FAT	leukocyte activation	6	3.1E-1	9.7E-1
GOTERM_BP_FAT	B cell activation	3	3.2E-1	9.7E-1
GOTERM_BP_FAT	...	...	...	...

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Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	immune system development	RT G	6	4.7E-1	9.6E-1
GOTERM_BP_FAT	lymphocyte differentiation	RT	3	4.9E-1	9.9E-1
GOTERM_BP_FAT	hemopoiesis	RT	5	5.7E-1	1.0E0
GOTERM_BP_FAT	hemopoietic or lymphoid organ development	RT	3	6.0E-1	1.0E0
GOTERM_BP_FAT	leukocyte differentiation	RT			

Annotation Cluster 40 — Enrichment Score: 0.51

Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	activation of caspase activity	RT	3	2.0E-1	9.6E-1
GOTERM_BP_FAT	positive regulation of caspase activity	RT	3	2.3E-1	9.6E-1
GOTERM_BP_FAT	positive regulation of peptidase activity	RT	3	2.3E-1	9.4E-1
GOTERM_BP_FAT	positive regulation of hydrolase activity	RT	5	2.9E-1	9.7E-1
GOTERM_BP_FAT	regulation of caspase activity	RT	3	3.4E-1	9.7E-1
GOTERM_BP_FAT	regulation of endopeptidase activity	RT	3	3.6E-1	9.8E-1
GOTERM_BP_FAT	regulation of peptidase activity	RT	3	3.8E-1	9.8E-1
GOTERM_CC_FAT	regulation of hydrolase activity	RT G	6	5.9E-1	1.0E0

Annotation Cluster 41 — Enrichment Score: 0.5

Category	Term		Count	P_Value	Benjamini
GOTERM_CC_FAT	mitochondrial membrane	RT	7	5.7E-1	9.7E-1
GOTERM_CC_FAT	mitochondrial envelope	RT	8	4.7E-1	9.6E-1
GOTERM_CC_FAT	nuclear envelope	RT	5	3.9E-1	9.4E-1
GOTERM_CC_FAT	envelope	RT	12	3.7E-1	9.4E-1
GOTERM_CC_FAT	organelle envelope	RT	12	3.7E-1	9.4E-1
GOTERM_BP_FAT	mitochondrial part	RT	13	2.1E-1	9.0E-1
KEGG_PATHWAY	Huntington's disease	RT	7	1.1E-1	9.4E-1

Annotation Cluster 42 — Enrichment Score: 0.49

Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	generation of precursor metabolites and energy	RT G	10	5.2E-2	7.7E-1
KEGG_PATHWAY	Huntington's disease	RT	7	1.1E-1	9.4E-1
SP_PIR_KEYWORDS	electron transport	RT	4	1.7E-1	7.2E-1
SP_PIR_KEYWORDS	Parkinson's disease	RT	5	1.8E-1	9.5E-1
KEGG_PATHWAY	Alzheimer's disease	RT	4	2.5E-1	9.6E-1
KEGG_PATHWAY	respiratory chain	RT	3	2.7E-1	8.2E-1
GOTERM_CC_FAT	mitochondrial membrane part	RT	4	3.1E-1	9.2E-1
GOTERM_CC_FAT	mitochondrial inner membrane	RT	4	3.2E-1	9.3E-1
GOTERM_CC_FAT	Oxidative phosphorylation	RT	5	3.5E-1	9.5E-1
GOTERM_CC_FAT	mitochondrial membrane	RT	4	4.2E-1	9.5E-1
GOTERM_BP_FAT	oxidation reduction	RT	3	4.4E-1	9.9E-1
GOTERM_BP_FAT	electron transport chain	RT	3	4.7E-1	9.6E-1
GOTERM_CC_FAT	cellular respiration	RT	4	1.8E-1	9.5E-1
GOTERM_CC_FAT	oxidative phosphorylation	RT	4	1.9E-1	9.5E-1
GOTERM_BP_FAT	mitochondrial envelope	RT	11	5.2E-1	9.9E-1
GOTERM_BP_FAT	energy derivation by oxidation of organic compounds	RT	7	5.2E-1	9.9E-1
SP_PIR_KEYWORDS	respiratory chain	RT	3	6.9E-1	9.9E-1
GOTERM_CC_FAT	mitochondrial inner membrane	RT	3	9.5E-1	1.0E0
GOTERM_CC_FAT	organelle inner membrane	RT	3	9.7E-1	1.0E0

Annotation Cluster 43 — Enrichment Score: 0.47

Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	ribonucleoprotein complex biogenesis	RT G	6	1.4E-1	9.3E-1
GOTERM_BP_FAT	protein homooligomerization	RT	4	1.8E-1	9.5E-1
GOTERM_BP_FAT	ribonucleoprotein complex assembly	RT	3	2.8E-1	9.7E-1
GOTERM_BP_FAT	macromolecular complex subunit organization	RT	14	2.9E-1	9.7E-1
GOTERM_BP_FAT	macromolecular complex assembly	RT	13	3.2E-1	9.7E-1
GOTERM_BP_FAT	protein complex assembly	RT	10	3.7E-1	9.8E-1
GOTERM_BP_FAT	protein complex biogenesis	RT	10	3.7E-1	9.8E-1
GOTERM_BP_FAT	protein oligomerization	RT	4	3.7E-1	9.8E-1
GOTERM_BP_FAT	cellular macromolecular complex subunit organization	RT	5	5.0E-1	9.9E-1
GOTERM_BP_FAT	cellular macromolecular complex assembly	RT	6	6.4E-1	1.0E0

Annotation Cluster 44 — Enrichment Score: 0.44

Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	G1/S transition of mitotic cell cycle	RT G	5	7.2E-1	1.0E0
GOTERM_BP_FAT	interphase of mitotic cell cycle	RT	3	2.1E-1	9.6E-1
GOTERM_BP_FAT	interphase	RT	3	4.7E-1	9.9E-1

Annotation Cluster 45 — Enrichment Score: 0.43

Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	interphase	RT G	3	4.8E-1	9.9E-1
UP_SEQ_FEATURE					

https://david.ncifcrf.gov/term2term.jsp?annot=52,9,79,85,25,32,39,13,47,45,63,76&currentList=1

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Category	Term		Count	P_Value	Benjamini
	zinc finger region:RING-type		0	1.0E-1	1.0E0
SMART	RING	RT	6	3.6E-1	1.0E0
INTERPRO	Zinc finger, RING-type, conserved site	RT	6	3.9E-1	1.0E0
INTERPRO	Zinc finger, RING-type	RT	6	4.3E-1	1.0E0
INTERPRO	Zinc finger, C3HC4 RING-type	RT G	4	6.5E-1	1.0E0
Annotation Cluster 46	**Enrichment Score: 0.42**		**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	DNA-dependent ATPase activity	RT	3	2.0E-1	9.4E-1
SMART	AAA	RT	4	3.7E-1	9.8E-1
INTERPRO	ATPase, AAA+ type, core	RT	4	3.0E-1	9.8E-1
GOTERM_MF_FAT	ATPase activity, coupled	RT	5	3.5E-1	1.0E0
GOTERM_MF_FAT	ATPase activity	RT G	5	5.5E-1	1.0E0
Annotation Cluster 47	**Enrichment Score: 0.4**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	coagulation	RT	4	5.7E-1	1.0E0
GOTERM_BP_FAT	blood coagulation	RT	3	4.6E-1	9.8E-1
GOTERM_BP_FAT	regulation of body fluid levels	RT	4	4.5E-1	9.9E-1
GOTERM_BP_FAT	hemostasis	RT	4	3.7E-1	9.8E-1
GOTERM_BP_FAT	wound healing	RT G	4	5.6E-1	1.0E0
Annotation Cluster 48	**Enrichment Score: 0.38**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	positive regulation of signal transduction	RT	4	8.3E-1	9.5E-1
GOTERM_BP_FAT	positive regulation of protein kinase cascade	RT	4	4.7E-1	9.5E-1
GOTERM_BP_FAT	regulation of protein kinase cascade	RT	6	3.4E-1	9.7E-1
GOTERM_BP_FAT	regulation of I-kappaB kinase/NF-kappaB cascade	RT	4	2.3E-1	9.6E-1
GOTERM_BP_FAT	positive regulation of I-kappaB kinase/NF-kappaB...	RT G	4	1.9E-1	9.5E-1
Annotation Cluster 49	**Enrichment Score: 0.37**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	positive regulation of cell communication	RT	4	8.8E-1	1.0E0
GOTERM_BP_FAT	blood vessel morphogenesis	RT G	8	4.4E-2	7.8E-1
GOTERM_BP_FAT	blood vessel development	RT	8	6.7E-2	7.9E-1
GOTERM_BP_FAT	vasculature development	RT	8	8.2E-2	8.5E-1
GOTERM_BP_FAT	angiogenesis	RT	6	9.1E-2	8.6E-1
GOTERM_BP_FAT	regulation of hydrolase activity	RT	6	3.4E-1	9.5E-1
GOTERM_CC_FAT	extrinsic to membrane	RT	6	6.0E-1	9.4E-1
GOTERM_BP_FAT	response to toxin	RT	4	5.9E-1	1.0E0
GOTERM_BP_FAT	regulation of cell motion	RT	10	3.6E-1	9.4E-1
GOTERM_BP_FAT	regulation of locomotion	RT	5	3.4E-1	9.7E-1
GOTERM_BP_FAT	regulation of cell migration	RT	5	2.9E-1	9.7E-1
GOTERM_BP_FAT	di-, tri-valent inorganic cation homeostasis	RT	3	2.6E-1	9.6E-1
GOTERM_BP_FAT	positive regulation of hydrolase activity	RT	4	2.9E-1	9.7E-1
GOTERM_BP_FAT	regulation of cell migration	RT	3	2.6E-1	9.6E-1
GOTERM_BP_FAT	regulation of membrane potential	RT	5	1.9E-2	9.5E-1
GOTERM_BP_FAT	cation homeostasis	RT	4	6.1E-1	1.0E0
GOTERM_BP_FAT	metal ion homeostasis	RT	8	6.1E-1	1.0E0
GOTERM_BP_FAT	ion homeostasis	RT	5	6.0E-1	1.0E0
GOTERM_BP_FAT	regulation of hydrolase activity	RT	10	6.0E-1	1.0E0
GOTERM_CC_FAT	extrinsic to membrane	RT	7	5.9E-1	1.0E0
GOTERM_BP_FAT	response to toxin	RT	3	3.6E-1	9.4E-1
GOTERM_BP_FAT	cellular calcium ion homeostasis	RT	7	7.9E-1	1.0E0
GOTERM_BP_FAT	calcium ion homeostasis	RT	5	8.0E-1	1.0E0
GOTERM_BP_FAT	chemical homeostasis	RT	8	8.1E-1	1.0E0
GOTERM_BP_FAT	cellular metal ion homeostasis	RT	6	8.2E-1	1.0E0
Annotation Cluster 50	**Enrichment Score: 0.34**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	cellular ion homeostasis	RT	5	8.4E-1	1.0E0
GOTERM_BP_FAT	homeostatic process	RT	10	8.3E-1	1.0E0
GOTERM_BP_FAT	cellular chemical homeostasis	RT	5	8.4E-1	1.0E0
GOTERM_BP_FAT	cellular di-, tri-valent inorganic cation homeostasis	RT	3	8.7E-1	1.0E0
GOTERM_BP_FAT	negative regulation of gene expression	RT	6	8.9E-1	1.0E0
GOTERM_BP_FAT	cellular cation homeostasis	RT	3	9.0E-1	1.0E0
GOTERM_BP_FAT	cellular cation homeostasis	RT	3	9.0E-1	1.0E0
GOTERM_BP_FAT	cellular homeostasis	RT	5	9.3E-1	1.0E0
GOTERM_CC_FAT	DNA-directed RNA polymerase II, holoenzyme	RT G	4	1.3E-1	8.0E-1
GOTERM_BP_FAT	transcription initiation	RT	3	3.6E-1	9.8E-1
GOTERM_CC_FAT	transcription factor complex	RT	4	6.4E-1	9.8E-1
GOTERM_BP_FAT	transcription, DNA-dependent	RT	4	8.3E-1	1.0E0

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				Count	P_Value	Benjamini
GOTERM_BP_FAT	RNA biosynthetic process	RT	G	4	8.3E-1	1.0E0
Annotation Cluster 51	Enrichment Score: 0.33			Count	P_Value	Benjamini
GOTERM_BP_FAT	response to lipopolysaccharide	RT		3	3.3E-1	9.7E-1
GOTERM_BP_FAT	response to molecule of bacterial origin	RT		3	3.8E-1	9.8E-1
GOTERM_BP_FAT	response to bacterium	RT	G	3	8.0E-1	1.0E0
Annotation Cluster 52	Enrichment Score: 0.33			Count	P_Value	Benjamini
SMART	ANK	RT		5	3.9E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 1	RT		5	4.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 2	RT		5	4.2E-1	1.0E0
SP_PIR_KEYWORDS	ank repeat	RT		5	4.4E-1	9.3E-1
INTERPRO	Ankyrin	RT		5	4.6E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 3	RT		4	5.3E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 4	RT		3	6.6E-1	1.0E0
Annotation Cluster 53	Enrichment Score: 0.32			Count	P_Value	Benjamini
GOTERM_BP_FAT	cardiac muscle tissue development	RT		3	4.2E-1	9.6E-1
GOTERM_BP_FAT	heart development	RT	G	5	2.2E-1	9.6E-1
GOTERM_BP_FAT	striated muscle tissue development	RT		3	5.5E-1	1.0E0
GOTERM_BP_FAT	muscle tissue development	RT		3	5.7E-1	1.0E0
GOTERM_BP_FAT	muscle organ development	RT		3	8.4E-1	1.0E0
Annotation Cluster 54	Enrichment Score: 0.32			Count	P_Value	Benjamini
GOTERM_CC_FAT	cell fraction	RT	G	19	4.1E-1	9.4E-1
GOTERM_CC_FAT	membrane fraction	RT		14	4.9E-1	9.6E-1
GOTERM_CC_FAT	insoluble fraction	RT		14	5.4E-1	9.7E-1
Annotation Cluster 55	Enrichment Score: 0.31			Count	P_Value	Benjamini
GOTERM_CC_FAT	membrane-bounded vesicle	RT	G	11	3.9E-1	9.4E-1
GOTERM_CC_FAT	vesicle	RT		12	4.7E-1	9.6E-1
GOTERM_CC_FAT	cytoplasmic membrane-bounded vesicle	RT		10	4.8E-1	9.6E-1
GOTERM_CC_FAT	cytoplasmic vesicle	RT	G	10	6.7E-1	9.8E-1
Annotation Cluster 56	Enrichment Score: 0.27			Count	P_Value	Benjamini
INTERPRO	Tetratricopeptide-like helical	RT		4	4.0E-1	1.0E0
INTERPRO	Tetratricopeptide TPR-1	RT		3	4.3E-1	1.0E0
SMART	TPR	RT		3	4.6E-1	1.0E0
INTERPRO	TPR	RT		3	5.1E-1	1.0E0
INTERPRO	Tetratricopeptide repeat	RT		3	5.3E-1	1.0E0
UP_SEQ_FEATURE	repeat:TPR 3	RT		3	5.9E-1	1.0E0
UP_SEQ_FEATURE	repeat:TPR 2	RT		3	6.4E-1	1.0E0
UP_SEQ_FEATURE	repeat:TPR 1	RT		3	6.4E-1	1.0E0
SP_PIR_KEYWORDS	tpr repeat	RT	G	3	6.7E-1	9.8E-1
Annotation Cluster 57	Enrichment Score: 0.27			Count	P_Value	Benjamini
KEGG_PATHWAY	Chemokine signaling pathway	RT		5	4.5E-1	9.5E-1
KEGG_PATHWAY	Fc gamma R-mediated phagocytosis	RT		3	5.2E-1	9.6E-1
INTERPRO	Pleckstrin homology-type	RT	G	5	6.4E-1	1.0E0
Annotation Cluster 58	Enrichment Score: 0.27			Count	P_Value	Benjamini
GOTERM_BP_FAT	response to ionizing radiation	RT	G	3	2.3E-1	9.5E-1
GOTERM_BP_FAT	response to radiation	RT		3	8.1E-1	1.0E0
GOTERM_BP_FAT	response to abiotic stimulus	RT		5	8.2E-1	1.0E0
Annotation Cluster 59	Enrichment Score: 0.25			Count	P_Value	Benjamini
GOTERM_CC_FAT	Golgi membrane	RT	G	5	3.3E-1	9.2E-1
GOTERM_CC_FAT	Golgi apparatus part	RT		5	6.7E-1	9.9E-1
GOTERM_CC_FAT	Golgi apparatus	RT		12	8.0E-1	1.0E0
Annotation Cluster 60	Enrichment Score: 0.24			Count	P_Value	Benjamini
UP_SEQ_FEATURE	repeat:LRR 3	RT		6	4.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 1	RT		6	4.8E-1	1.0E0
SP_PIR_KEYWORDS	leucine-rich repeat	RT		6	4.8E-1	9.4E-1
UP_SEQ_FEATURE	repeat:LRR 2	RT		6	4.9E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 10	RT		3	5.0E-1	1.0E0
SMART	LRR_TYP	RT		3	5.2E-1	1.0E0
INTERPRO	Leucine-rich repeat, typical subtype	RT		3	5.7E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 9	RT		3	5.8E-1	1.0E0
INTERPRO	Leucine-rich repeat	RT		4	6.0E-1	1.0E0

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Category	Term	Count	P_Value	Benjamini
UP_SEQ_FEATURE	repeat:LRR 8	3	8.0E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 5	3	7.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 4	4	7.0E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 7	4	6.4E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 6	3	6.3E-1	1.0E0
Annotation Cluster 61	**Enrichment Score: 0.24**	Count	P_Value	Benjamini
SMART		3	5.9E-1	1.0E0
INTERPRO		3	5.5E-1	1.0E0
INTERPRO	C2 membrane-targeting protein	3	4.6E-1	1.0E0
GOTERM_MF_FAT	C2 calcium-dependent membrane targeting	3	7.3E-1	1.0E0
Annotation Cluster 62	**Enrichment Score: 0.24**	Count	P_Value	Benjamini
GOTERM_MF_FAT	phospholipid binding	4	4.8E-1	1.0E0
UP_SEQ_FEATURE	lipid moiety-binding region:S-palmitoyl cysteine	4	5.8E-1	9.7E-1
SP_PIR_KEYWORDS	palmitate	4	6.9E-1	9.8E-1
SP_PIR_KEYWORDS	lipoprotein	9	9.7E-1	9.8E-1
Annotation Cluster 63	**Enrichment Score: 0.24**	Count	P_Value	Benjamini
GOTERM_CC_FAT	cell junction	6	9.1E-1	1.0E0
GOTERM_CC_FAT	anchoring junction	4	5.0E-1	9.6E-1
GOTERM_CC_FAT	adherens junction	4	4.3E-1	9.5E-1
Annotation Cluster 64	**Enrichment Score: 0.23**	Count	P_Value	Benjamini
GOTERM_BP_FAT	response to hormone stimulus	4	3.9E-1	9.8E-1
GOTERM_BP_FAT	response to endogenous stimulus	5	8.2E-1	1.0E0
GOTERM_BP_FAT	cellular response to hormone stimulus	6	7.5E-1	1.0E0
GOTERM_BP_FAT	response to organic substance	5	7.0E-1	1.0E0
Annotation Cluster 65	**Enrichment Score: 0.2**	Count	P_Value	Benjamini
GOTERM_CC_FAT	microtubule	3	6.1E-1	1.0E0
GOTERM_CC_FAT	microtubule	13	4.2E-1	9.6E-1
GOTERM_BP_FAT	microtubule cytoskeleton organization	4	7.5E-1	1.0E0
GOTERM_BP_FAT	microtubule-based process	4	6.4E-1	9.7E-1
Annotation Cluster 66	**Enrichment Score: 0.2**	Count	P_Value	Benjamini
GOTERM_BP_FAT	eye development	3	6.0E-1	1.0E0
GOTERM_BP_FAT	camera-type eye development	3	8.7E-1	1.0E0
GOTERM_BP_FAT	sensory organ development	4	4.9E-1	9.9E-1
Annotation Cluster 67	**Enrichment Score: 0.19**	Count	P_Value	Benjamini
GOTERM_BP_FAT	cell motility	4	8.0E-1	1.0E0
GOTERM_BP_FAT	cell migration	5	7.0E-1	1.0E0
GOTERM_BP_FAT	localization of cell	5	4.4E-1	9.6E-1
GOTERM_BP_FAT	cell motion	9	4.2E-1	9.6E-1
Annotation Cluster 68	**Enrichment Score: 0.18**	Count	P_Value	Benjamini
GOTERM_BP_FAT	positive regulation of response to stimulus	3	8.7E-1	1.0E0
GOTERM_BP_FAT	positive regulation of immune response	3	6.5E-1	1.0E0
GOTERM_BP_FAT	positive regulation of immune system process	3	8.8E-1	1.0E0
Annotation Cluster 69	**Enrichment Score: 0.18**	Count	P_Value	Benjamini
GOTERM_MF_FAT	metallopeptidase activity	4	4.9E-1	1.0E0
GOTERM_MF_FAT	peptidase activity	9	5.9E-1	1.0E0
SP_PIR_KEYWORDS	metalloprotease	3	6.3E-1	9.7E-1
GOTERM_MF_FAT	peptidase activity, acting on L-amino acid peptides	8	6.8E-1	1.0E0
SP_PIR_KEYWORDS	protease	7	6.9E-1	9.8E-1
GOTERM_MF_FAT	endopeptidase activity	3	9.7E-1	1.0E0
Annotation Cluster 70	**Enrichment Score: 0.18**	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	lysosome	3	6.3E-1	9.7E-1
GOTERM_CC_FAT	lysosome	4	5.9E-1	9.8E-1
GOTERM_CC_FAT	lytic vacuole	4	6.4E-1	9.8E-1
GOTERM_CC_FAT	vacuole	4	6.4E-1	9.8E-1
Annotation Cluster 71	**Enrichment Score: 0.17**	Count	P_Value	Benjamini
GOTERM_BP_FAT	response to wounding	4	7.5E-1	9.9E-1
GOTERM_BP_FAT	defense response	9	5.7E-1	1.0E0
GOTERM_BP_FAT	inflammatory response	10	6.0E-1	1.0E0
Annotation Cluster 72	**Enrichment Score: 0.16**	Count	P_Value	Benjamini
INTERPRO		4	8.8E-1	1.0E0
		5	6.4E-1	1.0E0

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Category	Term	Count	P_Value	Benjamini
UP_SEQ_FEATURE	domain:PH	4	6.6E-1	1.0E0
SMART	PH	4	7.2E-1	1.0E0
INTERPRO	Pleckstrin homology	4	7.7E-1	9.3E-1
Annotation Cluster73	Enrichment Score: 0.15	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	microsome	3	4.5E-1	1.0E0
GOTERM_CC_FAT	microsome	3	8.9E-1	1.0E0
GOTERM_CC_FAT	vesicular fraction	3	9.0E-1	1.0E0
Annotation Cluster74	Enrichment Score: 0.15	Count	P_Value	Benjamini
GOTERM_BP_FAT	regulation of cell growth	4	5.8E-1	1.0E0
GOTERM_BP_FAT	regulation of cell size	4	6.1E-1	1.0E0
GOTERM_BP_FAT	regulation of cellular component size	4	7.9E-1	1.0E0
GOTERM_BP_FAT	regulation of growth	4	9.0E-1	1.0E0
Annotation Cluster75	Enrichment Score: 0.15	Count	P_Value	Benjamini
GOTERM_MF_FAT	transcription cofactor activity	6	6.0E-1	1.0E0
GOTERM_MF_FAT	transcription corepressor activity	3	6.2E-1	1.0E0
GOTERM_MF_FAT	transcription repressor activity	3	9.4E-1	1.0E0
Annotation Cluster76	Enrichment Score: 0.14	Count	P_Value	Benjamini
GOTERM_MF_FAT	GTPase activator activity	4	6.2E-1	1.0E0
GOTERM_MF_FAT	GTPase regulator activity	6	7.0E-1	1.0E0
GOTERM_MF_FAT	nucleoside-triphosphatase regulator activity	6	7.1E-1	1.0E0
GOTERM_MF_FAT	small GTPase regulator activity	4	7.6E-1	1.0E0
Annotation Cluster77	Enrichment Score: 0.14	Count	P_Value	Benjamini
GOTERM_MF_FAT	enzyme activator activity	4	8.7E-1	1.0E0
GOTERM_MF_FAT	transcription activator activity	5	8.5E-1	1.0E0
GOTERM_MF_FAT	transcription coactivator activity	3	8.2E-1	1.0E0
GOTERM_MF_FAT	transcription cofactor activity	6	6.0E-1	1.0E0
Annotation Cluster78	Enrichment Score: 0.13	Count	P_Value	Benjamini
GOTERM_BP_FAT	actin filament-based process	3	8.9E-1	1.0E0
GOTERM_BP_FAT	actin cytoskeleton organization	3	8.6E-1	1.0E0
GOTERM_CC_FAT	cytoskeleton organization	8	5.0E-1	9.9E-1
Annotation Cluster79	Enrichment Score: 0.12	Count	P_Value	Benjamini
GOTERM_CC_FAT	plasma membrane part	34	6.1E-1	9.8E-1
GOTERM_MF_FAT	intrinsic to plasma membrane	16	8.3E-1	1.0E0
GOTERM_CC_FAT	integral to plasma membrane	16	8.3E-1	1.0E0
GOTERM_MF_FAT	transcription coactivator activity	5	8.5E-1	1.0E0
Annotation Cluster80	Enrichment Score: 0.11	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	metal-binding	44	4.7E-1	9.4E-1
GOTERM_MF_FAT	transition metal ion binding	39	6.8E-1	1.0E0
SP_PIR_KEYWORDS	zinc-finger	23	7.3E-1	9.8E-1
SP_PIR_KEYWORDS	zinc	29	7.5E-1	9.9E-1
GOTERM_MF_FAT	cation binding	54	8.9E-1	1.0E0
GOTERM_MF_FAT	metal ion binding	53	9.0E-1	1.0E0
GOTERM_MF_FAT	ion binding	54	9.1E-1	1.0E0
GOTERM_MF_FAT	zinc ion binding	26	9.6E-1	1.0E0
Annotation Cluster81	Enrichment Score: 0.1	Count	P_Value	Benjamini
GOTERM_BP_FAT	positive regulation of transcription from RNA polymerase II promoter	6	6.7E-1	1.0E0
GOTERM_BP_FAT	regulation of transcription from RNA polymerase II promoter	11	6.8E-1	1.0E0
GOTERM_BP_FAT	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	9	7.4E-1	1.0E0
GOTERM_BP_FAT	positive regulation of transcription, DNA-dependent	8	7.7E-1	1.0E0
GOTERM_BP_FAT	positive regulation of gene expression	8	7.7E-1	1.0E0
GOTERM_BP_FAT	positive regulation of transcription	12	7.7E-1	1.0E0
GOTERM_BP_FAT	positive regulation of nitrogen compound metabolic process	8	7.9E-1	1.0E0
GOTERM_BP_FAT	positive regulation of macromolecule metabolic process	9	7.7E-1	1.0E0
GOTERM_BP_FAT	positive regulation of RNA metabolic process	8	8.6E-1	1.0E0
GOTERM_BP_FAT	positive regulation of macromolecule biosynthetic process	8	8.8E-1	1.0E0
GOTERM_BP_FAT	positive regulation of cellular biosynthetic process	8	9.1E-1	1.0E0

https://david.ncifcrf.gov/term2term.jsp?annot=52,79,85,25,32,39,1,3,47,45,63,76&currentList=1

12/14

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Category	Term		Count	P_Value	Benjamini
GOTERM_BP_FAT	positive regulation of biosynthetic process	G	8	9.1E-1	1.0E0
Annotation Cluster 82	**Enrichment Score: 0.1**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	negative regulation of macromolecule metabolic process	RT	14	3.3E-1	9.7E-1
GOTERM_BP_FAT	negative regulation of transcription from RNA polymerase II promoter	RT	4	7.8E-1	1.0E0
GOTERM_BP_FAT	negative regulation of RNA metabolic process	RT	5	8.1E-1	1.0E0
GOTERM_BP_FAT	negative regulation of macromolecule biosynthetic process	RT	7	8.5E-1	1.0E0
GOTERM_BP_FAT	negative regulation of cellular biosynthetic process	RT	7	8.6E-1	1.0E0
GOTERM_BP_FAT	negative regulation of gene expression	RT	6	8.8E-1	1.0E0
GOTERM_BP_FAT	negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	RT	6	9.0E-1	1.0E0
GOTERM_BP_FAT	negative regulation of nitrogen compound metabolic process	RT	6	9.0E-1	1.0E0
GOTERM_BP_FAT	negative regulation of transcription, DNA-dependent	RT	4	9.1E-1	1.0E0
GOTERM_BP_FAT	negative regulation of transcription	RT	4	9.7E-1	1.0E0
Annotation Cluster 83	**Enrichment Score: 0.08**		**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	transcription factor binding	G	10	3.2E-1	9.8E-1
GOTERM_MF_FAT	regulation of transcription from RNA polymerase II promoter	RT	11	6.8E-1	1.0E0
GOTERM_BP_FAT	regulation of RNA metabolic process	RT	26	7.4E-1	1.0E0
GOTERM_BP_FAT	regulation of transcription, DNA-dependent	RT	23	8.8E-1	1.0E0
GOTERM_BP_FAT	DNA binding	RT	27	9.4E-1	1.0E0
GOTERM_MF_FAT	transcription regulator activity	RT	32	9.5E-1	1.0E0
GOTERM_BP_FAT	regulation of transcription	RT	10	9.5E-1	1.0E0
GOTERM_MF_FAT	Transcription	RT	16	9.6E-1	1.0E0
GOTERM_MF_FAT	transcription factor activity	RT	22	9.6E-1	1.0E0
SP_PIR_KEYWORDS	Transcription	RT	21	9.7E-1	1.0E0
SP_PIR_KEYWORDS	transcription regulation	RT	24	9.7E-1	1.0E0
SP_PIR_KEYWORDS	transcription	RT	15	1.0E0	1.0E0
SP_PIR_KEYWORDS	dna-binding	RT	15	1.0E0	1.0E0
Annotation Cluster 84	**Enrichment Score: 0.08**		**Count**	**P_Value**	**Benjamini**
SP_PIR_KEYWORDS	membrane	G	86	7.1E-1	1.0E0
SP_PIR_KEYWORDS	transmembrane	RT	68	7.1E-1	9.8E-1
SP_PIR_KEYWORDS	transmembrane region	RT	67	7.4E-1	1.0E0
UP_SEQ_FEATURE	transmembrane region	RT	73	9.4E-1	1.0E0
GOTERM_CC_FAT	intrinsic to membrane	RT	75	9.5E-1	1.0E0
UP_SEQ_FEATURE	topological domain:Cytoplasmic	RT	36	9.9E-1	1.0E0
Annotation Cluster 85	**Enrichment Score: 0.05**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	intracellular protein transport	G	10	6.8E-1	1.0E0
GOTERM_BP_FAT	cellular macromolecule localization	RT	4	9.5E-1	1.0E0
GOTERM_BP_FAT	cellular protein localization	RT	4	9.5E-1	1.0E0
GOTERM_BP_FAT	intracellular transport	RT	3	9.8E-1	1.0E0
Annotation Cluster 86	**Enrichment Score: 0.05**		**Count**	**P_Value**	**Benjamini**
SP_PIR_KEYWORDS	vision	G	3	5.9E-1	9.7E-1
GOTERM_BP_FAT	sensory perception of light stimulus	RT	3	7.5E-1	1.0E0
GOTERM_BP_FAT	visual perception	RT	3	8.5E-1	1.0E0
SP_PIR_KEYWORDS	sensory transduction	RT	3	8.5E-1	1.0E0
GOTERM_BP_FAT	sensory perception	RT	4	1.0E0	1.0E0
GOTERM_BP_FAT	cognition	RT	4	1.0E0	1.0E0
Annotation Cluster 87	**Enrichment Score: 0.05**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	neurological system process	G	5	1.0E0	1.0E0
GOTERM_BP_FAT	in utero embryonic development	RT	3	7.5E-1	1.0E0
GOTERM_BP_FAT	chordate embryonic development	RT	3	9.5E-1	1.0E0
GOTERM_BP_FAT	embryonic development ending in birth or egg hatching	RT	3	9.7E-1	1.0E0
Annotation Cluster 88	**Enrichment Score: 0.03**		**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	chromosome organization	G	6	8.7E-1	1.0E0
GOTERM_BP_FAT	chromatin modification	RT	3	9.3E-1	1.0E0
GOTERM_BP_FAT	chromatin organization	RT	3	9.8E-1	1.0E0
Annotation Cluster 89	**Enrichment Score: 0.02**		**Count**	**P_Value**	**Benjamini**
UP_SEQ_FEATURE	zinc finger region:C2H2-type 1	G	6	8.9E-1	1.0E0

https://david.ncifcrf.gov/term2term.jsp?annot=52,79,85,25,32,39,1,3,47,45,63,76&currentList=1

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Category	Term		Count	P_Value	Benjamini
UP_SEQ_FEATURE	zinc finger region:C2H2-type 4	RT	6	9.2E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 2	RT	6	9.4E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 3	RT	6	9.5E-1	1.0E0
INTERPRO	Zinc finger, C2H2-type/integrase, DNA-binding	RT	5	9.8E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 5	RT	4	9.8E-1	1.0E0
SMART	ZnF_C2H2	RT	6	9.8E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 7	RT	3	9.9E-1	1.0E0
INTERPRO	Zinc finger, C2H2-type	RT	6	9.9E-1	1.0E0
INTERPRO	Zinc finger, C2H2-like	RT	6	9.9E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 6	RT	3	9.9E-1	1.0E0

Annotation Cluster 90   Enrichment Score: 0.01

Category	Term		Count	P_Value	Benjamini
SP_PIR_KEYWORDS	cell adhesion	RT G	4	9.4E-1	1.0E0
GOTERM_BP_FAT	cell adhesion	RT	6	9.8E-1	1.0E0
GOTERM_BP_FAT	biological adhesion	RT	6	9.8E-1	1.0E0

Annotation Cluster 91   Enrichment Score: 0

Category	Term		Count	P_Value	Benjamini
INTERPRO	Immunoglobulin-like fold	RT	4	9.9E-1	1.0E0
SP_PIR_KEYWORDS	Immunoglobulin domain	RT	3	9.9E-1	1.0E0
INTERPRO	Immunoglobulin-like	RT	3	9.9E-1	1.0E0

Annotation Cluster 92   Enrichment Score: 0

Category	Term		Count	P_Value	Benjamini
SP_PIR_KEYWORDS	transducer	RT G	6	1.0E0	1.0E0
GOTERM_BP_FAT	G-protein coupled receptor protein signaling pathway	RT G	8	1.0E0	1.0E0
INTERPRO	GPCR, rhodopsin-like superfamily	RT	3	1.0E0	1.0E0
INTERPRO	7TM GPCR, rhodopsin-like	RT	3	1.0E0	1.0E0
PIR_SUPERFAMILY	PIRSF800006::rhodopsin-like G protein-coupled receptors	RT	3	1.0E0	1.0E0
SP_PIR_KEYWORDS	g-protein coupled receptor	RT	3	1.0E0	1.0E0

Annotation Cluster 93   Enrichment Score: 0

Category	Term		Count	P_Value	Benjamini
GOTERM_CC_FAT	extracellular region part	RT G	5	1.0E0	1.0E0
SP_PIR_KEYWORDS	Secreted	RT	10	1.0E0	1.0E0
SP_PIR_KEYWORDS	disulfide bond	RT	20	1.0E0	1.0E0
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT	33	1.0E0	1.0E0
SP_PIR_KEYWORDS	signal	RT	23	1.0E0	1.0E0
UP_SEQ_FEATURE	signal peptide	RT	23	1.0E0	1.0E0
SP_PIR_KEYWORDS	glycoprotein	RT	34	1.0E0	1.0E0
GOTERM_CC_FAT	extracellular region	RT	12	1.0E0	1.0E0
UP_SEQ_FEATURE	disulfide bond	RT	15	1.0E0	1.0E0

217 terms   were not clustered.

8.3 Cattle DAVID clusters

Help and Manual

Functional Annotation Clustering

Current Gene List: List_1
Current Background: Homo sapiens
323 DAVID IDs

Options
Renun using options | Create Sublist
Classification Stringency [Medium ▼]

100 Cluster(s)

Download File

	Term	Count	P_Value	Benjamini
Annotation Cluster 1	**Enrichment Score: 2.27**			
GOTERM_CC_FAT	mitochondrion	33	3.6E-4	5.0E-2
SP_PIR_KEYWORDS	mitochondrion	28	4.6E-4	3.6E-2
GOTERM_CC_FAT	organelle envelope	22	7.2E-4	6.6E-2
GOTERM_CC_FAT	envelope	22	7.5E-4	5.2E-2
GOTERM_CC_FAT	organelle membrane	30	3.5E-3	1.8E-1
GOTERM_CC_FAT	mitochondrial membrane	15	3.6E-3	1.6E-1
GOTERM_CC_FAT	mitochondrial part	19	5.7E-3	1.5E-1
GOTERM_CC_FAT	organelle inner membrane	13	5.7E-3	1.4E-1
GOTERM_CC_FAT	mitochondrial envelope	15	6.2E-3	1.4E-1
GOTERM_CC_FAT	mitochondrial inner membrane	12	8.8E-3	1.8E-1
GOTERM_CC_FAT	transit peptide:Mitochondrion	13	8.0E-2	1.0E0
UP_SEQ_FEATURE	transit peptide	13	8.6E-2	7.5E-1
SP_PIR_KEYWORDS	mitochondrion inner membrane	6	2.0E-1	8.4E-1
Annotation Cluster 2	**Enrichment Score: 2.09**			
SP_PIR_KEYWORDS	endosome	12	6.0E-4	3.7E-2
GOTERM_CC_FAT	endosome	11	2.7E-2	3.6E-1
SP_PIR_KEYWORDS	lysosome	7	3.4E-2	5.1E-1
Annotation Cluster 3	**Enrichment Score: 2**			
GOTERM_CC_FAT	intracellular organelle lumen	43	3.7E-3	1.4E-1
GOTERM_CC_FAT	membrane-enclosed lumen	44	4.7E-3	1.5E-1
GOTERM_CC_FAT	organelle lumen	43	5.6E-3	1.6E-1
GOTERM_CC_FAT	nuclear lumen	35	1.0E-2	1.9E-1
GOTERM_CC_FAT	nucleoplasm	20	9.7E-2	6.5E-1
Annotation Cluster 4	**Enrichment Score: 1.98**			
GOTERM_CC_FAT	lytic vacuole	9	1.8E-2	2.8E-1
GOTERM_CC_FAT	lysosome	9	1.8E-2	2.8E-1
GOTERM_CC_FAT	vacuole	7	3.4E-2	5.1E-1
Annotation Cluster 5	**Enrichment Score: 1.37**			
SP_PIR_KEYWORDS	nucleotide metabolism	5	2.6E-4	2.7E-2
GOTERM_BP_FAT	pyrimidine nucleoside triphosphate metabolic process	4	1.7E-3	9.1E-1
KEGG_PATHWAY	Pyrimidine metabolism	7	3.0E-3	2.8E-1
UP_SEQ_FEATURE	active site:Pros-phosphohistidine intermediate	3	3.5E-3	9.2E-1
SMART	NDK	3	3.8E-3	9.7E-1
INTERPRO	Nucleoside diphosphate kinase, core	3	6.1E-3	9.7E-1
GOTERM_MF_FAT	nucleoside diphosphate kinase activity	3	6.8E-3	9.7E-1
GOTERM_BP_FAT	pyrimidine nucleoside metabolic process	3	8.8E-3	7.1E-1
GOTERM_BP_FAT	UTP metabolic process	4	9.2E-3	9.7E-1
GOTERM_BP_FAT	GTP biosynthetic process	3	1.0E-2	9.5E-1
GOTERM_BP_FAT	UTP biosynthetic process	3	1.0E-2	9.5E-1
GOTERM_BP_FAT	CTP metabolic process	3	1.0E-2	9.5E-1
GOTERM_BP_FAT	CTP biosynthetic process	3	1.3E-2	9.5E-1
GOTERM_BP_FAT	pyrimidine ribonucleoside triphosphate metabolic process	3	1.3E-2	9.5E-1
GOTERM_BP_FAT	pyrimidine ribonucleoside triphosphate biosynthetic process	3	1.3E-2	9.5E-1

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Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	pyrimidine nucleoside triphosphate biosynthetic process	3	1.3E-2	9.5E-1
GOTERM_BP_FAT	CTP biosynthetic process	3	1.8E-2	9.5E-1
KEGG_PATHWAY	GTP metabolic process	3	1.8E-2	7.9E-1
GOTERM_BP_FAT	Purine metabolism	7	2.8E-2	7.9E-1
GOTERM_BP_FAT	pyrimidine ribonucleoside triphosphate biosynthetic process	3	2.8E-2	9.8E-1
GOTERM_BP_FAT	pyrimidine ribonucleotide metabolic process	3	3.1E-2	9.6E-1
GOTERM_BP_FAT	pyrimidine ribonucleotide biosynthetic process	3	3.5E-2	9.5E-1
GOTERM_BP_FAT	pyrimidine nucleotide biosynthetic process	3	3.5E-2	9.5E-1
GOTERM_MF_FAT	nucleobase, nucleoside, nucleotide kinase activity	3	5.6E-2	9.7E-1
GOTERM_BP_FAT	nitrogen compound biosynthetic process	11	6.0E-2	9.7E-1
GOTERM_BP_FAT	phosphotransferase activity, phosphate group as acceptor	3	6.0E-2	9.0E-1
GOTERM_MF_FAT	pyrimidine nucleotide metabolic process	3	1.0E-1	9.8E-1
GOTERM_BP_FAT	nucleoside metabolic process	4	1.0E-1	9.8E-1
GOTERM_BP_FAT	purine ribonucleoside biosynthetic process	5	1.5E-1	9.8E-1
GOTERM_BP_FAT	purine ribonucleoside metabolic process	4	1.5E-1	9.3E-1
GOTERM_BP_FAT	nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	3	1.5E-1	9.8E-1
GOTERM_BP_FAT	ribonucleotide biosynthetic process	5	1.7E-1	9.8E-1
GOTERM_BP_FAT	ribonucleotide metabolic process	3	1.9E-1	9.9E-1
GOTERM_BP_FAT	nucleobase, nucleoside and nucleotide biosynthetic process	5	2.0E-1	9.9E-1
GOTERM_BP_FAT	nucleobase, nucleoside, nucleotide metabolic process	6	2.5E-1	9.9E-1
GOTERM_BP_FAT	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4	2.5E-1	9.9E-1
GOTERM_BP_FAT	ribonucleoside triphosphate metabolic process	6	2.5E-1	9.9E-1
GOTERM_BP_FAT	purine nucleoside triphosphate biosynthetic process	4	2.3E-1	9.9E-1
GOTERM_BP_FAT	nucleoside triphosphate biosynthetic process	4	2.7E-1	9.8E-1
GOTERM_BP_FAT	purine nucleotide metabolic process	4	2.6E-1	9.8E-1
GOTERM_BP_FAT	ribonucleoside triphosphate metabolic process	5	2.6E-1	9.8E-1
GOTERM_BP_FAT	nucleoside triphosphate metabolic process	5	2.6E-1	9.8E-1
GOTERM_BP_FAT	ribonucleotide biosynthetic process	4	2.5E-1	9.8E-1
GOTERM_BP_FAT	purine nucleoside triphosphate metabolic process	4	2.5E-1	9.9E-1
GOTERM_BP_FAT	purine ribonucleoside triphosphate metabolic process	6	2.5E-1	9.9E-1
GOTERM_BP_FAT	purine nucleotide biosynthetic process	5	3.6E-1	9.9E-1
GOTERM_BP_FAT	purine nucleoside triphosphate biosynthetic process	5	3.4E-1	9.9E-1
GOTERM_BP_FAT	purine ribonucleoside triphosphate biosynthetic process	4	3.4E-1	9.9E-1
GOTERM_BP_FAT	purine nucleoside triphosphate metabolic process	4	3.6E-1	9.9E-1
GOTERM_BP_FAT	purine nucleotide metabolic process	6	2.5E-1	9.9E-1
GOTERM_BP_FAT	purine nucleoside triphosphate metabolic process	4	4.1E-1	9.9E-1

Annotation Cluster 6   Enrichment Score: 1.26

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	cellular protein complex assembly	5	8.0E-3	9.8E-1
GOTERM_BP_FAT	respiratory chain complex IV assembly	3	6.6E-2	9.7E-1
GOTERM_BP_FAT	generation of precursor metabolites and energy	7	3.1E-1	9.9E-1

Annotation Cluster 7   Enrichment Score: 1.26

Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	transition metal ion binding	62	6.2E-3	7.3E-1
SP_PIR_KEYWORDS	metal-binding	65	8.3E-3	2.8E-1
SP_PIR_KEYWORDS	zinc-finger	40	1.8E-2	4.1E-1
SP_PIR_KEYWORDS	zinc	43	1.3E-1	7.7E-1
GOTERM_MF_FAT	zinc ion binding	45	1.4E-1	9.3E-1
GOTERM_MF_FAT	metal ion binding	76	1.4E-1	9.2E-1
GOTERM_MF_FAT	cation binding	76	1.6E-1	9.4E-1
GOTERM_MF_FAT	ion binding	76	2.0E-1	9.5E-1

Annotation Cluster 8   Enrichment Score: 1.18

Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	adenyl nucleotide binding	37	2.2E-2	8.4E-1
GOTERM_MF_FAT	purine nucleoside binding	37	2.6E-2	8.5E-1
GOTERM_MF_FAT	adenyl ribonucleotide binding	35	2.7E-2	8.1E-1
GOTERM_MF_FAT	nucleoside binding	37	2.9E-2	7.9E-1
UP_SEQ_FEATURE	binding site:ATP	16	3.2E-2	1.0E0
GOTERM_MF_FAT	ATP binding	34	3.6E-2	8.2E-1
SP_PIR_KEYWORDS	atp-binding	31	3.8E-2	5.3E-1
UP_SEQ_FEATURE	nucleotide phosphate-binding region:ATP	47	5.2E-2	8.7E-1
GOTERM_MF_FAT	nucleotide binding	23	6.5E-2	1.0E0
GOTERM_MF_FAT	purine nucleotide binding	40	7.9E-2	9.2E-1

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Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	purine ribonucleotide binding	38	9.5E-2	9.2E-1
GOTERM_MF_FAT	ribonucleoside binding	38	9.5E-2	9.2E-1
SP_PIR_KEYWORDS	nucleotide-binding	35	1.0E-1	7.4E-1
SP_PIR_KEYWORDS	kinase	16	1.5E-1	7.9E-1
GOTERM_MF_FAT	protein kinase activity	14	1.9E-1	9.4E-1
INTERPRO	protein kinase, ATP binding site	11	2.0E-1	1.0E0
GOTERM_MF_FAT	protein amino acid phosphorylation	15	2.8E-1	9.9E-1

Annotation Cluster 9 — Enrichment Score: 1.13

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	pigment biosynthetic process	4	3.1E-2	9.7E-1
GOTERM_BP_FAT	heme biosynthetic process	3	3.5E-2	9.5E-1
GOTERM_BP_FAT	pigment metabolic process	4	4.4E-2	9.6E-1
GOTERM_BP_FAT	porphyrin biosynthetic process	3	4.7E-2	9.6E-1
GOTERM_BP_FAT	tetrapyrrole biosynthetic process	3	4.7E-2	9.6E-1
GOTERM_BP_FAT	heme metabolic process	3	5.6E-2	9.7E-1
KEGG_PATHWAY	Porphyrin and chlorophyll metabolism	3	8.8E-2	9.7E-1
GOTERM_BP_FAT	cofactor biosynthetic process	5	9.1E-2	9.8E-1
GOTERM_BP_FAT	tetrapyrrole metabolic process	3	9.1E-2	9.8E-1
GOTERM_BP_FAT	porphyrin metabolic process	3	9.1E-2	9.8E-1
GOTERM_BP_FAT	cofactor metabolic process	6	2.6E-1	9.8E-1
GOTERM_BP_FAT	heterocycle biosynthetic process	3	2.8E-1	9.9E-1

Annotation Cluster 10 — Enrichment Score: 1.09

Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	Oxoglutarate and iron-dependent dioxygenase	4	9.2E-2	9.2E-1
UP_SEQ_FEATURE	domain:Fe2OG dioxygenase	3	3.0E-2	1.0E0
INTERPRO	domain:Fe2OG dioxygenase	3	4.5E-2	1.0E0
GOTERM_MF_FAT	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	4	9.5E-2	9.0E-1
GOTERM_MF_FAT	oxidoreductase activity, acting on single donors with incorporation of two atoms of oxygen	4	9.2E-2	9.2E-1

Annotation Cluster 11 — Enrichment Score: 1.05

Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	manganese ion binding	6	1.1E-1	9.0E-1
GOTERM_MF_FAT	manganese	6	1.0E-1	7.4E-1
SP_PIR_KEYWORDS	magnesium ion binding	13	6.6E-2	9.1E-1

Annotation Cluster 12 — Enrichment Score: 0.99

Category	Term	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	ribonucleoprotein	13	8.3E-2	7.8E-1
SP_PIR_KEYWORDS	ribosome	9	5.2E-2	5.2E-1
GOTERM_CC_FAT	ribosomal protein	8	3.3E-2	5.3E-1
GOTERM_CC_FAT	ribosome	8	2.9E-1	9.1E-1
GOTERM_BP_FAT	translation	13	1.4E-2	9.5E-1
GOTERM_MF_FAT	structural constituent of ribosome	6	1.4E-1	9.3E-1
GOTERM_CC_FAT	large ribosomal subunit	3	2.8E-1	9.1E-1
GOTERM_CC_FAT	ribosomal subunit	4	3.2E-1	9.2E-1
GOTERM_MF_FAT	structural molecule activity	12	4.6E-1	1.0E0

Annotation Cluster 13 — Enrichment Score: 0.99

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	regulation of fatty acid metabolic process	4	5.4E-2	9.7E-1
GOTERM_BP_FAT	regulation of cellular ketone metabolic process	4	7.8E-2	9.8E-1
GOTERM_BP_FAT	regulation of fatty acid oxidation	3	8.6E-2	9.8E-1
GOTERM_BP_FAT	regulation of lipid metabolic process	4	3.1E-1	9.9E-1

Annotation Cluster 14 — Enrichment Score: 0.99

Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	iron ion binding	12	1.2E-1	7.2E-1
SP_PIR_KEYWORDS	iron	10	4.2E-1	5.5E-1
GOTERM_BP_FAT	oxidation reduction	14	3.3E-1	9.9E-1

Annotation Cluster 15 — Enrichment Score: 0.97

Category	Term	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	oxidoreductase	9	6.9E-1	9.9E-1
GOTERM_BP_FAT	cellular response to stress	17	4.1E-2	9.5E-1
GOTERM_BP_FAT	DNA repair	10	6.2E-2	9.7E-1
GOTERM_BP_FAT	DNA metabolic process	14	1.1E-1	9.8E-1
GOTERM_BP_FAT	response to DNA damage stimulus	11	1.2E-1	9.8E-1
SP_PIR_KEYWORDS	dna repair	6	2.0E-1	8.6E-1

https://david.ncifcrf.gov/relatedTerms.jsp?id=790000437&currentList=0

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Category	Term	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	DNA damage	6	2.4E-1	8.7E-1
Annotation Cluster 16	**Enrichment Score: 0.94**			
GOTERM_MF_FAT	endoribonuclease activity, producing 5'-phosphomonoesters	3	7.1E-2	9.1E-1
GOTERM_MF_FAT	endonuclease activity	5	8.0E-2	9.0E-1
SP_PIR_KEYWORDS	Endonuclease	4	8.4E-2	7.6E-1
SP_PIR_KEYWORDS	nuclease	5	9.2E-2	7.6E-1
GOTERM_MF_FAT	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	3	1.1E-1	9.0E-1
GOTERM_MF_FAT	nuclease activity	6	1.2E-1	9.0E-1
GOTERM_MF_FAT	endoribonuclease activity	3	1.8E-1	9.4E-1
GOTERM_MF_FAT	ribonuclease activity	3	2.9E-1	9.7E-1
Annotation Cluster 17	**Enrichment Score: 0.92**			
SP_PIR_KEYWORDS	rna-binding	19	3.0E-3	1.3E-1
SMART	RRM	7	1.0E-1	9.9E-1
INTERPRO	RNA recognition motif, RNP-1	7	1.3E-1	1.0E0
INTERPRO	Nucleotide-binding, alpha-beta plat	4	3.0E-1	1.0E0
UP_SEQ_FEATURE	domain:RRM	7	1.3E-1	1.0E0
UP_SEQ_FEATURE	domain:RRM 2	3	4.9E-1	1.0E0
UP_SEQ_FEATURE	domain:RRM 1	3	4.9E-1	1.0E0
Annotation Cluster 18	**Enrichment Score: 0.9**			
SMART	Zinc finger, RING-type, conserved site	9	8.4E-2	1.0E0
INTERPRO	RING	9	8.4E-2	9.9E-1
INTERPRO	Zinc finger, RING-type	9	1.1E-1	1.0E0
INTERPRO	Zinc finger, RING-type	7	1.6E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:RING-type	21	6.9E-2	9.7E-1
INTERPRO	Zinc finger, C3HC4 RING-type	11	6.5E-2	1.0E0
Annotation Cluster 19	**Enrichment Score:**			
GOTERM_BP_FAT	phosphorus metabolic process	27	2.1E-2	9.5E-1
GOTERM_BP_FAT	phosphate metabolic process	27	2.1E-2	9.5E-1
UP_SEQ_FEATURE	binding site:ATP	16	3.2E-2	1.0E0
GOTERM_MF_FAT	ATP binding	34	3.6E-2	8.2E-1
SP_PIR_KEYWORDS	atp-binding	31	3.8E-2	5.3E-1
UP_SEQ_FEATURE	nucleotide phosphate-binding region:ATP	23	6.3E-1	1.0E0
INTERPRO	Serine/threonine-protein kin…	11	8.2E-1	1.0E0
INTERPRO	Serine/threonine-protein kin…	10	7.9E-1	1.0E0
GOTERM_BP_FAT	phosphorylation	16	1.6E-1	1.0E0
INTERPRO	Serine/threonine-protein kin…	12	1.5E-1	1.0E0
INTERPRO	Protein kinase, ATP binding site	12	1.4E-1	1.0E0
SMART	S_TKc	10	1.2E-1	1.0E0
INTERPRO	Protein kinase, ATP binding site	21	9.7E-2	9.7E-1
GOTERM_MF_FAT	protein kinase activity	11	1.1E-1	1.0E0
SP_PIR_KEYWORDS	kinase	14	1.9E-1	9.4E-1
SP_PIR_KEYWORDS	serine/threonine-protein kinase	10	1.6E-1	8.2E-1
KEGG_PATHWAY	Acute myeloid leukemia	3	2.2E-1	9.5E-1
UP_SEQ_FEATURE	Serine/threonine protein kinase	7	2.4E-1	1.0E0
GOTERM_MF_FAT	protein serine/threonine kinase activity	10	2.7E-1	9.6E-1
GOTERM_BP_FAT	protein amino acid phosphorylation	15	2.8E-1	9.9E-1
UP_SEQ_FEATURE	active site:Proton acceptor	11	1.5E-1	9.8E-1
KEGG_PATHWAY	Chemokine signaling pathway	5	1.5E-1	9.8E-1
GOTERM_BP_FAT	intracellular signaling cascade	16	9.6E-1	1.0E0
Annotation Cluster 20	**Enrichment Score: 0.82**			
GOTERM_BP_FAT	ncRNA metabolic process	8	1.1E-1	9.8E-1
GOTERM_BP_FAT	ncRNA processing	7	1.1E-1	9.8E-1
GOTERM_BP_FAT	rRNA processing	4	1.5E-1	9.8E-1
GOTERM_BP_FAT	rRNA metabolic process	5	1.5E-1	9.8E-1
SP_PIR_KEYWORDS	rna processing	3	2.9E-1	9.1E-1
Annotation Cluster 21	**Enrichment Score: 0.8**			
GOTERM_CC_FAT	mitochondrial membrane part	6	4.7E-2	5.1E-1
GOTERM_BP_FAT	mitochondrial ATP synthesis coupled electron transport	4	7.5E-2	9.8E-1

Tooltip: http://www.uniprot.org/keywords/?query=serine/threonine-protein%20kinase

https://david.ncifcrf.gov/term2term.jsp?annot=52,9,79,85,25,32,39,1,3,47,45,63,76&currentList=0   4/16

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Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	ATP synthesis coupled electron transport	4	7.5E-2	9.8E-1
GOTERM_BP_FAT	oxidative phosphorylation	5	9.3E-2	9.8E-1
GOTERM_BP_FAT	respiratory electron transport chain	4	1.0E-1	9.8E-1
KEGG_PATHWAY	Oxidative phosphorylation	5	1.3E-1	3.3E-1
GOTERM_BP_FAT	electron transport chain	5	1.4E-1	9.8E-1
GOTERM_BP_FAT	cellular respiration	4	2.4E-1	9.8E-1
GOTERM_BP_FAT	energy derivation by oxidation of organic compounds	5	2.5E-1	9.8E-1
GOTERM_CC_FAT	mitochondrial respiratory chain	3	2.6E-1	9.0E-1
GOTERM_BP_FAT	generation of precursor metabolites and energy	8	3.1E-1	9.9E-1
GOTERM_CC_FAT	respiratory chain	3	3.3E-1	9.2E-1
SP_PIR_KEYWORDS	electron transport	3	4.8E-1	9.5E-1

Annotation Cluster 22   Enrichment Score: 0.79

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	in utero embryonic development	8	3.5E-2	9.5E-1
GOTERM_BP_FAT	chordate embryonic development	9	2.3E-1	9.8E-1
GOTERM_BP_FAT	embryonic development ending in birth or egg hatching	9	2.3E-1	9.8E-1
GOTERM_BP_FAT	embryonic organ development	5	3.6E-1	9.9E-1

Annotation Cluster 23   Enrichment Score: 0.79

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	regulation of apoptosis	20	1.1E-1	9.8E-1
GOTERM_BP_FAT	regulation of programmed cell death	20	1.2E-1	9.8E-1
GOTERM_BP_FAT	regulation of cell death	20	1.2E-1	9.8E-1
GOTERM_BP_FAT	negative regulation of apoptosis	10	1.7E-1	9.8E-1
GOTERM_BP_FAT	negative regulation of programmed cell death	10	1.8E-1	9.8E-1
GOTERM_BP_FAT	negative regulation of cell death	10	1.8E-1	9.8E-1
GOTERM_BP_FAT	anti-apoptosis	6	2.9E-1	9.9E-1

Annotation Cluster 24   Enrichment Score: 0.78

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	oxidative phosphorylation	5	9.3E-2	9.8E-1
KEGG_PATHWAY	Oxidative phosphorylation	5	1.3E-1	3.3E-1
GOTERM_MF_FAT	hydrogen ion transmembrane transporter activity	4	1.8E-1	9.4E-1
GOTERM_MF_FAT	inorganic cation transmembrane transporter activity	5	2.3E-1	9.6E-1
GOTERM_MF_FAT	monovalent inorganic cation transmembrane transporter activity	4	2.4E-1	9.6E-1

Annotation Cluster 25   Enrichment Score: 0.78

Category	Term	Count	P_Value	Benjamini
GOTERM_CC_FAT	non-membrane-bounded organelle	50	8.4E-2	6.2E-1
GOTERM_CC_FAT	intracellular non-membrane-bounded organelle	50	8.4E-2	6.2E-1
GOTERM_CC_FAT	cytoskeleton	21	6.8E-1	9.6E-1

Annotation Cluster 26   Enrichment Score: 0.75

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	mitotic cell cycle	13	3.0E-2	9.8E-1
GOTERM_BP_FAT	cell cycle	22	3.2E-2	9.6E-1
GOTERM_BP_FAT	interphase of mitotic cell cycle	6	3.5E-2	9.6E-1
GOTERM_BP_FAT	interphase	6	3.9E-2	9.5E-1
GOTERM_BP_FAT	cell cycle phase	12	1.1E-1	9.8E-1
KEGG_PATHWAY	Cell cycle	5	1.2E-1	9.7E-1
GOTERM_BP_FAT	cell cycle process	15	1.2E-1	9.8E-1
GOTERM_BP_FAT	regulation of cell cycle	10	1.3E-1	9.7E-1
GOTERM_BP_FAT	organelle fission	7	2.1E-1	9.9E-1
GOTERM_BP_FAT	cell cycle checkpoint	4	2.1E-1	9.8E-1
GOTERM_BP_FAT	G1/S transition of mitotic cell cycle	3	2.6E-1	9.8E-1
GOTERM_BP_FAT	mitosis	6	3.4E-1	9.9E-1
GOTERM_BP_FAT	nuclear division	6	3.4E-1	9.9E-1
GOTERM_BP_FAT	M phase	6	3.5E-1	9.9E-1
GOTERM_BP_FAT	M phase of mitotic cell cycle	6	3.6E-1	9.9E-1
GOTERM_CC_FAT	spindle	4	4.1E-1	9.9E-1
GOTERM_BP_FAT	cell division	6	4.1E-1	9.9E-1
SP_PIR_KEYWORDS	cell division	7	4.2E-1	9.9E-1
GOTERM_BP_FAT	regulation of mitotic cell cycle	4	5.0E-1	1.0E0

Annotation Cluster 27   Enrichment Score: 0.73

Category	Term	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	mitosis	4	5.7E-1	9.8E-1

https://david.ncifcrf.gov/term2term.jsp?annot=52,9,79,85,25,32,39,1,3,47,45,63,76&currentList=0   5/16

2/29/2016 DAVID: Database for Annotation, Visualization, and Integrated Discovery (Laboratory of Immunopathogenesis and Bioinformatics (LIB); National Inst...

			Count	P_Value	Benjamini
SP_PIR_KEYWORDS	protein transport	RT	13	9.6E-2	7.4E-1
GOTERM_BP_FAT	cellular protein localization	RT	12	1.1E-1	9.8E-1
GOTERM_BP_FAT	cellular macromolecule localization	RT	12	1.1E-1	9.8E-1
GOTERM_BP_FAT	protein transport	RT	19	1.2E-1	9.8E-1
GOTERM_BP_FAT	intracellular protein transport	RT	11	1.2E-1	9.8E-1
GOTERM_BP_FAT	establishment of protein localization	RT	19	1.3E-1	9.7E-1
GOTERM_BP_FAT	protein localization	RT	20	2.1E-1	9.8E-1
GOTERM_BP_FAT	protein localization in organelle	RT	5	2.5E-1	9.8E-1
GOTERM_BP_FAT	protein targeting	RT	6	3.2E-1	9.9E-1
GOTERM_BP_FAT	intracellular transport	RT	14	3.7E-1	9.9E-1
Annotation Cluster 28	protein import	RT G	3	6.7E-1	1.0E0
Enrichment Score: 0.69			Count	P_Value	Benjamini
INTERPRO	Protein kinase, C-terminal	RT	3	1.4E-1	1.0E0
SMART	S_TK_X	RT	3	2.1E-1	9.7E-1
INTERPRO	S_TKc	RT	7	2.1E-1	9.5E-1
INTERPRO	AGC-kinase, C-terminal	RT	3	2.2E-1	1.0E0
UP_SEQ_FEATURE	domain:AGC-kinase C-terminal	RT	3	2.2E-1	1.0E0
INTERPRO	Serine/threonine protein kinase	RT	7	2.4E-1	1.0E0
Annotation Cluster 29			Count	P_Value	Benjamini
Enrichment Score: 0.67					
GOTERM_CC_FAT	microtubule cytoskeleton	RT	15	5.0E-2	5.2E-1
GOTERM_CC_FAT	microtubule organizing center	RT	4	5.7E-2	5.2E-1
GOTERM_CC_FAT	centrosome	RT	8	6.3E-2	5.4E-1
GOTERM_CC_FAT	microtubule organizing center part	RT	8	1.0E-1	6.4E-1
GOTERM_BP_FAT	microtubule-based process	RT	8	1.6E-1	9.8E-1
GOTERM_BP_FAT	microtubule cytoskeleton organization	RT G	5	2.6E-1	9.8E-1
SP_PIR_KEYWORDS	cytoskeleton	RT	13	3.3E-1	9.3E-1
GOTERM_BP_FAT	M phase	RT	8	3.5E-1	9.9E-1
GOTERM_BP_FAT	cell division	RT	7	4.1E-1	9.9E-1
GOTERM_CC_FAT	cytoskeletal part	RT	16	5.3E-1	9.7E-1
GOTERM_BP_FAT	cytoskeleton organization	RT	8	6.5E-1	1.0E0
GOTERM_CC_FAT	cytoskeleton	RT	21	6.8E-1	9.9E-1
Annotation Cluster 30			Count	P_Value	Benjamini
Enrichment Score: 0.67					
GOTERM_BP_FAT	programmed cell death	RT	14	2.7E-1	9.9E-1
GOTERM_BP_FAT	apoptosis	RT	14	2.6E-1	9.8E-1
GOTERM_BP_FAT	death	RT	17	2.0E-1	9.8E-1
GOTERM_BP_FAT	cell death	RT	17	1.9E-1	9.9E-1
SP_PIR_KEYWORDS	Apoptosis	RT G	10	1.6E-1	8.2E-1
Annotation Cluster 31			Count	P_Value	Benjamini
Enrichment Score: 0.66					
GOTERM_CC_FAT	chromosome	RT	11	8.2E-2	6.2E-1
GOTERM_CC_FAT	chromosomal part	RT G	11	1.8E-1	8.2E-1
GOTERM_CC_FAT	chromatin	RT	5	3.9E-1	9.3E-1
SP_PIR_KEYWORDS	chromosomal protein	RT	4	4.1E-1	9.4E-1
Annotation Cluster 32			Count	P_Value	Benjamini
Enrichment Score: 0.62					
GOTERM_CC_FAT	cellular macromolecular complex subunit organization	RT G	12	5.0E-2	9.6E-1
GOTERM_BP_FAT	cellular protein complex assembly	RT	7	6.6E-2	9.7E-1
GOTERM_BP_FAT	cellular macromolecular complex assembly	RT	10	1.1E-1	9.8E-1
GOTERM_CC_FAT	macromolecular complex subunit organization	RT	14	4.7E-1	1.0E0
GOTERM_BP_FAT	macromolecular complex assembly	RT	12	6.2E-1	1.0E0
GOTERM_BP_FAT	protein complex assembly	RT	9	6.6E-1	1.0E0
GOTERM_BP_FAT	protein complex biogenesis	RT G	9	6.6E-1	1.0E0
Annotation Cluster 33			Count	P_Value	Benjamini
Enrichment Score: 0.61					
SP_PIR_KEYWORDS	Chaperone	RT G	6	1.1E-1	7.6E-1
GOTERM_MF_FAT	heat shock protein binding	RT	3	3.4E-1	9.8E-1
GOTERM_BP_FAT	protein folding	RT	5	3.8E-1	9.9E-1
Annotation Cluster 34			Count	P_Value	Benjamini
Enrichment Score: 0.61					
GOTERM_BP_FAT	ncRNA metabolic process	RT G	8	1.1E-1	9.8E-1
GOTERM_BP_FAT	ncRNA processing	RT	7	1.1E-1	9.8E-1
GOTERM_BP_FAT	ribonucleoprotein complex biogenesis	RT	6	2.1E-1	9.8E-1
GOTERM_BP_FAT	ribosome biogenesis	RT	4	2.6E-1	9.8E-1

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288

2/29/2016　DAVID: Database for Annotation, Visualization, and Integrated Discovery (Laboratory of Immunopathogenesis and Bioinformatics (LIB); National Inst…

				Count	P_Value	Benjamini
GOTERM_BP_FAT				4	4.8E-1	1.0E0
GOTERM_BP_FAT	rRNA processing			3	1.0E-1	6.5E-1
GOTERM_BP_FAT	rRNA metabolic process			3	5.0E-1	1.0E0

Annotation Cluster 35		Enrichment Score: 0.6		Count	P_Value	Benjamini
GOTERM_CC_FAT	nuclear envelope			7	1.0E-1	6.5E-1
GOTERM_CC_FAT	nuclear pore			3	3.5E-1	9.2E-1
GOTERM_CC_FAT	pore complex			3	4.4E-1	9.5E-1

Annotation Cluster 36		Enrichment Score: 0.59		Count	P_Value	Benjamini
GOTERM_BP_FAT	regulation of apoptosis			20	1.1E-1	9.8E-1
GOTERM_BP_FAT	regulation of programmed cell death			20	1.2E-1	9.8E-1
GOTERM_BP_FAT	regulation of cell death			20	1.2E-1	9.8E-1
GOTERM_BP_FAT	induction of apoptosis			8	3.3E-1	9.9E-1
GOTERM_BP_FAT	induction of programmed cell death			8	3.3E-1	9.9E-1
GOTERM_BP_FAT	positive regulation of apoptosis			10	3.5E-1	9.9E-1
GOTERM_BP_FAT	positive regulation of programmed cell death			10	3.5E-1	9.9E-1
GOTERM_BP_FAT	positive regulation of cell death			10	3.5E-1	9.9E-1
GOTERM_BP_FAT	induction of apoptosis by extracellular signals			3	5.9E-1	1.0E0

Annotation Cluster 37		Enrichment Score: 0.59		Count	P_Value	Benjamini
GOTERM_BP_FAT	endocytosis			7	1.9E-1	9.9E-1
GOTERM_BP_FAT	membrane invagination			7	1.9E-1	9.9E-1
SP_PIR_KEYWORDS	Endocytosis			4	2.0E-1	8.5E-1
GOTERM_CC_FAT	vesicle-mediated transport			13	3.1E-1	9.9E-1
GOTERM_BP_FAT	membrane organization			8	5.0E-1	1.0E0

Annotation Cluster 38		Enrichment Score: 0.54		Count	P_Value	Benjamini
SMART	ZnF_C2H2			17	1.6E-1	9.8E-1
UP_SEQ_FEATURE	zinc finger region:C2H2-type 9			10	1.6E-1	1.0E0
PIR_SUPERFAMILY	PIRSF005559:zinc finger protein ZFP-36			5	1.7E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 1			13	1.7E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 2			14	1.9E-1	1.0E0
INTERPRO	zinc finger, C2H2-type/integrase, DNA-binding			14	2.0E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 3			14	2.1E-1	1.0E0
INTERPRO	Zinc finger, C2H2-like			17	2.2E-1	1.0E0
INTERPRO	Zinc finger, C2H2-type			17	2.4E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 8			10	2.4E-1	1.0E0
INTERPRO	Zinc finger, C2H2-type			16	2.8E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 7			10	3.1E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 4			12	3.4E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 11			7	3.4E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 5			11	3.7E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 12			6	3.9E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 6			10	4.0E-1	1.0E0
UP_SEQ_FEATURE	domain:KRAB			7	4.5E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 10			7	4.6E-1	1.0E0
SMART	KRAB			7	4.6E-1	1.0E0
INTERPRO	Krueppel-associated box			7	5.1E-1	1.0E0
UP_SEQ_FEATURE	zinc finger region:C2H2-type 13			4	6.4E-1	1.0E0

Annotation Cluster 39		Enrichment Score: 0.53		Count	P_Value	Benjamini
KEGG_PATHWAY	Wnt signaling pathway			5	1.9E-1	9.5E-1
SP_PIR_KEYWORDS	Wnt signaling pathway			4	3.1E-1	9.2E-1
GOTERM_BP_FAT	Wnt receptor signaling pathway			4	4.1E-1	9.9E-1

Annotation Cluster 40		Enrichment Score: 0.53		Count	P_Value	Benjamini
GOTERM_BP_FAT	response to amine stimulus			3	1.5E-1	9.8E-1
GOTERM_BP_FAT	response to organic nitrogen			3	3.0E-1	9.9E-1
GOTERM_BP_FAT	response to endogenous stimulus			8	5.7E-1	1.0E0

Annotation Cluster 41		Enrichment Score: 0.53		Count	P_Value	Benjamini
SMART	KISc			3	1.4E-1	9.9E-1
INTERPRO	Kinesin, motor region, conserved site			3	1.5E-1	1.0E0
INTERPRO	Kinesin, motor region			3	1.5E-1	1.0E0
UP_SEQ_FEATURE	domain:Kinesin-motor			3	1.5E-1	1.0E0
GOTERM_BP_FAT				3	1.5E-1	1.0E0

https://david.ncifcrf.gov/term2term.jsp?annot=52,9,79,85,25,32,39,1,3,47,45,63,76&currentList=0

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Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	microtubule-based process	8	1.6E-1	9.8E-1
GOTERM_BP_FAT	microtubule-based movement	4	3.2E-1	9.9E-1
GOTERM_MF_FAT	microtubule motor activity	3	3.6E-1	9.8E-1
GOTERM_CC_FAT	microtubule	5	6.2E-1	9.9E-1
SP_PIR_KEYWORDS	motor protein	3	6.3E-1	9.8E-1
GOTERM_MF_FAT	motor activity	3	6.8E-1	1.0E0
SP_PIR_KEYWORDS	microtubule	4	7.2E-1	9.9E-1
Annotation Cluster 42	**Enrichment Score: 0.53**	Count	P_Value	Benjamini
KEGG_PATHWAY	Prostate cancer	4	1.5E-1	9.3E-1
KEGG_PATHWAY	Melanoma	3	2.9E-1	9.7E-1
KEGG_PATHWAY	Focal adhesion	4	5.9E-1	9.9E-1
Annotation Cluster 43	**Enrichment Score: 0.52**	Count	P_Value	Benjamini
GOTERM_BP_FAT	cellular macromolecule catabolic process	18	1.4E-1	9.8E-1
GOTERM_BP_FAT	macromolecule catabolic process	19	1.4E-1	9.8E-1
SP_PIR_KEYWORDS	ubl conjugation pathway	12	2.0E-1	8.6E-1
GOTERM_BP_FAT	modification-dependent macromolecule catabolic process	13	3.1E-1	9.9E-1
GOTERM_BP_FAT	modification-dependent protein catabolic process	13	3.1E-1	9.9E-1
GOTERM_BP_FAT	proteolysis involved in cellular protein catabolic process	13	3.6E-1	9.9E-1
GOTERM_BP_FAT	cellular protein catabolic process	13	3.6E-1	9.9E-1
GOTERM_BP_FAT	protein catabolic process	13	4.1E-1	9.9E-1
GOTERM_BP_FAT	ubiquitin-dependent protein catabolic process	6	4.2E-1	9.9E-1
GOTERM_BP_FAT	proteolysis	17	7.6E-1	1.0E0
Annotation Cluster 44	**Enrichment Score: 0.52**	Count	P_Value	Benjamini
GOTERM_MF_FAT	DNA binding	49	4.4E-2	8.5E-1
SMART	ZnF_C2H2	17	1.6E-1	9.8E-1
INTERPRO	Zinc finger, C2H2-type/integrase, DNA-binding	14	2.0E-1	1.0E0
INTERPRO	Zinc finger, C2H2-like	17	2.2E-1	1.0E0
SP_PIR_KEYWORDS	Transcription	38	2.8E-1	9.0E-1
INTERPRO	Zinc finger, C2H2-type	16	2.8E-1	1.0E0
GOTERM_BP_FAT	transcription	41	3.1E-1	9.9E-1
SP_PIR_KEYWORDS	dna-binding	34	3.2E-1	9.7E-1
SP_PIR_KEYWORDS	transcription regulation	35	3.2E-1	9.3E-1
GOTERM_MF_FAT	transcription regulator activity	26	5.0E-1	1.0E0
GOTERM_BP_FAT	regulation of transcription	47	5.0E-1	1.0E0
GOTERM_BP_FAT	regulation of RNA metabolic process	28	8.4E-1	1.0E0
GOTERM_BP_FAT	regulation of transcription, DNA-dependent	27	8.6E-1	1.0E0
Annotation Cluster 45	**Enrichment Score: 0.51**	Count	P_Value	Benjamini
INTERPRO	ATPase, AAA-type, core	3	1.9E-1	1.0E0
SMART	AAA	4	3.8E-1	1.0E0
INTERPRO	ATPase, AAA+ type, core	4	4.1E-1	1.0E0
Annotation Cluster 46	**Enrichment Score: 0.48**	Count	P_Value	Benjamini
GOTERM_BP_FAT	protein amino acid dephosphorylation	5	2.0E-1	9.8E-1
GOTERM_MF_FAT	protein tyrosine phosphatase activity	4	2.4E-1	9.6E-1
UP_SEQ_FEATURE	domain:Tyrosine-protein phosphatase	5	2.7E-1	1.0E0
GOTERM_MF_FAT	phosphoprotein phosphatase activity	5	2.8E-1	9.7E-1
GOTERM_BP_FAT	dephosphorylation	5	2.8E-1	9.9E-1
SP_PIR_KEYWORDS	protein phosphatase	4	3.5E-1	9.3E-1
INTERPRO	Protein-tyrosine phosphatase	3	3.8E-1	1.0E0
INTERPRO	Dual-specific/protein-tyrosine phosphatase	3	3.8E-1	1.0E0
UP_SEQ_FEATURE	active site:Proton donor	5	3.9E-1	1.0E0
UP_SEQ_FEATURE	active site:Phosphocysteine intermediate	3	3.9E-1	1.0E0
INTERPRO	Protein-tyrosine phosphatase, active site	3	4.2E-1	1.0E0
GOTERM_MF_FAT	phosphatase activity	5	5.8E-1	1.0E0
Annotation Cluster 47	**Enrichment Score: 0.47**	Count	P_Value	Benjamini
GOTERM_BP_FAT	carboxylic acid catabolic process	4	3.1E-1	9.9E-1
GOTERM_BP_FAT	organic acid catabolic process	4	3.1E-1	9.9E-1
GOTERM_BP_FAT	cellular amino acid catabolic process	3	3.3E-1	9.9E-1

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	amine catabolic process	3	4.0E-1	9.1E-1
Annotation Cluster 48	**Enrichment Score: 0.47**	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	ubl conjugation	13	2.4E-1	8.8E-1
UP_SEQ_FEATURE	cross-link:Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin)	5	4.0E-1	1.0E0
SP_PIR_KEYWORDS	isopeptide bond	7	4.1E-1	9.4E-1
Annotation Cluster 49	**Enrichment Score: 0.46**	Count	P_Value	Benjamini
UP_SEQ_FEATURE	repeat:ANK 9	3	1.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 8	3	1.5E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 7	3	2.4E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 4	5	2.5E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 5	4	3.6E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 3	5	3.9E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 6	3	4.4E-1	1.0E0
SMART	ANK	5	5.0E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 1	5	5.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:ANK 2	5	5.4E-1	1.0E0
INTERPRO	Ankyrin	5	5.4E-1	1.0E0
SP_PIR_KEYWORDS	ank repeat	5	5.4E-1	9.7E-1
Annotation Cluster 50	**Enrichment Score: 0.46**	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	dendrite	6	1.1E-1	6.5E-1
GOTERM_CC_FAT	cell projection	5	4.3E-1	9.3E-1
GOTERM_CC_FAT	neuron projection	7	4.5E-1	9.5E-1
Annotation Cluster 51	**Enrichment Score: 0.45**	Count	P_Value	Benjamini
GOTERM_CC_FAT	cell projection	11	6.6E-1	9.9E-1
GOTERM_BP_FAT	embryonic morphogenesis	8	2.9E-1	9.9E-1
GOTERM_BP_FAT	embryonic organ development	5	3.6E-1	9.9E-1
GOTERM_BP_FAT	sensory organ development	6	3.7E-1	9.9E-1
GOTERM_BP_FAT	embryonic organ morphogenesis	4	4.1E-1	9.9E-1
Annotation Cluster 52	**Enrichment Score: 0.44**	Count	P_Value	Benjamini
UP_SEQ_FEATURE	zinc finger region:PHD-type	3	2.1E-1	1.0E0
INTERPRO	Zinc finger, PHD-finger	3	4.0E-1	1.0E0
INTERPRO	PHD	3	4.0E-1	1.0E0
SMART	PHD	3	4.3E-1	1.0E0
INTERPRO	Zinc finger, PHD-type	3	4.4E-1	1.0E0
INTERPRO	Zinc finger, PHD-type, conserved site	3	4.4E-1	1.0E0
Annotation Cluster 53	**Enrichment Score: 0.4**	Count	P_Value	Benjamini
KEGG_PATHWAY	Spliceosome	5	1.2E-1	9.5E-1
SP_PIR_KEYWORDS	mRNA processing	14	1.6E-1	9.8E-1
SP_PIR_KEYWORDS	mRNA splicing	5	1.6E-1	9.8E-1
GOTERM_BP_FAT	RNA processing	8	4.8E-1	1.0E0
GOTERM_BP_FAT	mRNA metabolic process	7	5.0E-1	1.0E0
SP_PIR_KEYWORDS	mRNA processing	5	6.1E-1	9.8E-1
GOTERM_BP_FAT	RNA splicing	4	6.6E-1	9.9E-1
GOTERM_BP_FAT	mRNA processing	4	6.6E-1	9.9E-1
Annotation Cluster 54	**Enrichment Score: 0.38**	Count	P_Value	Benjamini
GOTERM_BP_FAT	eye development	6	2.0E-1	9.8E-1
GOTERM_BP_FAT	camera-type eye development	5	2.9E-1	9.9E-1
GOTERM_BP_FAT	sensory organ development	6	3.7E-1	9.9E-1
GOTERM_BP_FAT	neuron differentiation	8	6.5E-1	1.0E0
GOTERM_BP_FAT	neuron development	5	8.5E-1	1.0E0
Annotation Cluster 55	**Enrichment Score: 0.35**	Count	P_Value	Benjamini
GOTERM_BP_FAT	glycoprotein biosynthetic process	6	1.4E-1	9.8E-1
GOTERM_BP_FAT	glycoprotein metabolic process	6	2.8E-1	9.9E-1
GOTERM_BP_FAT	biopolymer glycosylation	3	3.7E-1	9.9E-1
GOTERM_BP_FAT	protein amino acid glycosylation	3	6.6E-1	1.0E0
SP_PIR_KEYWORDS	glycosylation	6	6.6E-1	1.0E0
SP_PIR_KEYWORDS	Signal-anchor	3	6.8E-1	1.0E0
Annotation Cluster 56	**Enrichment Score: 0.35**	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	amino-acid transport	3	1.1E-1	7.5E-1
GOTERM_BP_FAT	amino acid transport	3	4.7E-1	1.0E0
GOTERM_BP_FAT	amine transport	3	5.0E-1	1.0E0

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	amine transport		9.2E-4	1.0E0
GOTERM_BP_FAT	carboxylic acid transport	3	7.3E-1	1.0E0
GOTERM_BP_FAT	organic acid transport	3	7.4E-1	1.0E0

Annotation Cluster 67   Enrichment Score: 0.35

Category	Term	Count	P_Value	Benjamini
KEGG_PATHWAY	Pancreatic cancer	3	3.0E-1	9.6E-1
KEGG_PATHWAY	Chronic myeloid leukemia	3	3.1E-1	9.6E-1
KEGG_PATHWAY	Pathways in cancer	6	5.6E-1	9.9E-1
KEGG_PATHWAY	MAPK signaling pathway	4	7.8E-1	1.0E0

Annotation Cluster 68   Enrichment Score: 0.34

Category	Term	Count	P_Value	Benjamini
UP_SEQ_FEATURE	repeat:10	4	2.0E-1	1.0E0
UP_SEQ_FEATURE	repeat:9	4	2.3E-1	1.0E0
UP_SEQ_FEATURE	repeat:7	4	3.5E-1	1.0E0
UP_SEQ_FEATURE	repeat:6	4	4.1E-1	1.0E0
UP_SEQ_FEATURE	repeat:5	4	4.7E-1	1.0E0
UP_SEQ_FEATURE	repeat:4	4	5.7E-1	1.0E0
UP_SEQ_FEATURE	repeat:8	4	5.8E-1	1.0E0
UP_SEQ_FEATURE	repeat:3	4	6.8E-1	1.0E0
UP_SEQ_FEATURE	repeat:1	4	7.6E-1	1.0E0
UP_SEQ_FEATURE	repeat:2	4	7.6E-1	1.0E0

Annotation Cluster 69   Enrichment Score: 0.33

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	embryonic morphogenesis	8	2.9E-1	9.9E-1
GOTERM_BP_FAT	embryonic appendage morphogenesis	3	4.5E-1	9.9E-1
GOTERM_BP_FAT	embryonic limb morphogenesis	3	4.5E-1	9.9E-1
GOTERM_BP_FAT	appendage morphogenesis	3	5.2E-1	1.0E0
GOTERM_BP_FAT	limb morphogenesis	3	5.2E-1	1.0E0
GOTERM_BP_FAT	di-, tri-valent inorganic cation transport	3	8.2E-1	1.0E0
GOTERM_BP_FAT	cation transport	8	8.6E-1	1.0E0
GOTERM_BP_FAT	monovalent inorganic cation transport	4	9.2E-1	1.0E0
GOTERM_BP_FAT	ion transport	9	9.6E-1	1.0E0
GOTERM_BP_FAT	metal ion transport	5	9.7E-1	1.0E0
GOTERM_BP_FAT	appendage development	3	5.4E-1	1.0E0
GOTERM_BP_FAT	limb development	3	5.4E-1	1.0E0

Annotation Cluster 60   Enrichment Score: 0.32

Category	Term	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	metal ion transport	5	9.8E-1	9.8E-1
GOTERM_BP_FAT	iron ion transport	3	9.1E-1	9.8E-1
GOTERM_BP_FAT	transition metal ion transport	4	1.3E-1	9.8E-1
GOTERM_BP_FAT	proton transport	3	2.9E-1	9.9E-1
GOTERM_BP_FAT	hydrogen transport	3	3.0E-1	9.9E-1

Annotation Cluster 61   Enrichment Score: 0.31

Category	Term	Count	P_Value	Benjamini
GOTERM_MF_FAT	microtubule binding	3	3.3E-1	9.8E-1
GOTERM_MF_FAT	tubulin binding	3	4.9E-1	1.0E0
GOTERM_MF_FAT	cytoskeletal protein binding	8	7.2E-1	1.0E0

Annotation Cluster 62   Enrichment Score: 0.31

Category	Term	Count	P_Value	Benjamini
GOTERM_CC_FAT	cytoplasmic vesicle	12	4.2E-1	9.4E-1
GOTERM_CC_FAT	vesicle	12	4.8E-1	9.6E-1
GOTERM_CC_FAT	cytoplasmic membrane-bounded vesicle	10	4.9E-1	9.6E-1
GOTERM_CC_FAT	membrane-bounded vesicle	10	5.3E-1	9.7E-1
SP_PIR_KEYWORDS	cytoplasmic vesicle	5	5.4E-1	9.7E-1

Annotation Cluster 63   Enrichment Score: 0.29

Category	Term	Count	P_Value	Benjamini
INTERPRO	Tetratricopeptide repeat	4	2.9E-1	1.0E0
INTERPRO	Tetratricopeptide region	4	3.1E-1	1.0E0
INTERPRO	Tetratricopeptide-like helical	4	4.6E-1	1.0E0
INTERPRO	Tetratricopeptide TPR-1	3	4.8E-1	1.0E0
SMART	TPR	3	5.3E-1	9.9E-1
UP_SEQ_FEATURE	repeat:TPR 3	3	6.6E-1	1.0E0
UP_SEQ_FEATURE	repeat:TPR 1	3	7.1E-1	1.0E0
UP_SEQ_FEATURE	repeat:TPR 2	3	7.1E-1	1.0E0
SP_PIR_KEYWORDS	tpr repeat	3	7.3E-1	9.9E-1

Annotation Cluster 64   Enrichment Score: 0.28

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Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	anterior/posterior pattern formation	4	4.5E-1	9.9E-1
GOTERM_BP_FAT	regionalization	5	4.5E-1	9.9E-1
GOTERM_BP_FAT	pattern specification process	5	6.9E-1	1.0E0

Annotation Cluster 65   Enrichment Score: 0.28

Category	Term	Count	P_Value	Benjamini
SMART	PH	6	4.2E-1	9.9E-1
INTERPRO	Pleckstrin homology	6	4.6E-1	1.0E0
UP_SEQ_FEATURE	domain:PH	5	5.4E-1	1.0E0
INTERPRO	Pleckstrin homology-type	5	7.2E-1	1.0E0

Annotation Cluster 66   Enrichment Score: 0.28

Category	Term	Count	P_Value	Benjamini
SP_PIR_KEYWORDS	helicase	4	3.7E-1	9.3E-1
GOTERM_MF_FAT	helicase activity	4	4.0E-1	9.9E-1
GOTERM_MF_FAT	purine NTP-dependent helicase activity	3	4.7E-1	1.0E0
GOTERM_MF_FAT	ATP-dependent helicase activity	3	4.7E-1	1.0E0
UP_SEQ_FEATURE	domain:Helicase ATP-binding	3	5.4E-1	1.0E0
INTERPRO	DEAD-like helicase, N-termin...	3	5.3E-1	1.0E0
INTERPRO	DNA/RNA helicase, C-termin...	3	5.2E-1	1.0E0
INTERPRO	Helicase, superfamily 1 and ...	3	5.2E-1	1.0E0
UP_SEQ_FEATURE	domain:Helicase C-terminal	3	5.1E-1	1.0E0
SMART	DEXDc	3	5.0E-1	9.9E-1
SMART	HELICc	3	4.9E-1	1.0E0
GOTERM_MF_FAT	ATPase activity, coupled	3	8.0E-1	1.0E0
GOTERM_MF_FAT	ATPase activity	5	9.4E-1	1.0E0

Annotation Cluster 67   Enrichment Score: ...

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	lipid localization	3	7.6E-1	1.0E0
GOTERM_BP_FAT	lipid transport	3	7.3E-1	1.0E0

Annotation Cluster 68   Enrichment Score: ...

Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	positive regulation of specific transcription from RNA polymerase II prom...	4	7.8E-2	9.8E-1
GOTERM_BP_FAT	positive regulation of gene-specific transcription	4	9.8E-1	1.0E0
GOTERM_BP_FAT	regulation of specific transcription	4	2.6E-1	9.0E-1
GOTERM_BP_FAT	regulation of gene-specific transcription	4	2.0E-1	9.5E-1
GOTERM_BP_FAT	RNA polymerase II promoter	3	2.3E-1	9.8E-1
GOTERM_BP_FAT	regulation of gene-specific transcription from RNA polymerase II promoter	4	4.2E-1	9.9E-1
GOTERM_BP_FAT	positive regulation of macromolecule metabolic process	16	5.4E-1	1.0E0
GOTERM_BP_FAT	positive regulation of macromolecule biosynthetic process	12	6.0E-1	1.0E0
GOTERM_BP_FAT	sequence-specific DNA binding	10	6.6E-1	1.0E0
GOTERM_MF_FAT	nucleoside, nucleotide and nucleic acid metabolic process	11	6.6E-1	1.0E0
GOTERM_BP_FAT	positive regulation of cellular biosynthetic process	12	6.6E-1	1.0E0
GOTERM_BP_FAT	positive regulation of nitrogen compound metabolic process	12	6.8E-1	1.0E0
GOTERM_BP_FAT	positive regulation of biosynthetic process	11	7.0E-1	1.0E0
GOTERM_BP_FAT	regulation of transcription from RNA polymerase II promoter	6	7.8E-1	1.0E0
GOTERM_BP_FAT	positive regulation of transcription, DNA-dependent	7	8.5E-1	1.0E0
GOTERM_BP_FAT	positive regulation of RNA metabolic process	8	8.5E-1	1.0E0
GOTERM_BP_FAT	positive regulation of transcription	8	8.7E-1	1.0E0
GOTERM_BP_FAT	positive regulation of gene expression	8	8.9E-1	1.0E0
GOTERM_MF_FAT	transcription factor activity	12	9.3E-1	1.0E0

Annotation Cluster 69   Enrichment Score: 0.27

Category	Term	Count	P_Value	Benjamini
INTERPRO	Helix-loop-helix DNA-binding	3	3.9E-1	9.9E-1
SMART	HLH	3	3.9E-1	9.6E-1
INTERPRO	Basic helix-loop-helix dimerisation region bHLH	3	5.2E-1	9.6E-1

Annotation Cluster 70   Enrichment Score: 0.26

Category	Term	Count	P_Value	Benjamini
UP_SEQ_FEATURE	domain:Helix-loop-helix motif	3	5.5E-1	1.0E0
GOTERM_BP_FAT	DNA-binding region:Basic motif	3	5.5E-1	1.0E0
GOTERM_BP_FAT	response to toxin	4	9.1E-1	9.8E-1
GOTERM_BP_FAT	amino...	4	3.0E-1	9.9E-1
GOTERM_BP_FAT	cell migration	7	3.5E-1	9.9E-1
GOTERM_BP_FAT	positive regulation of growth	3	4.1E-1	9.9E-1

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293

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Category	Term			Count	P_Value	Benjamini
GOTERM_BP_FAT	cell motility	RT		7	4.5E-1	9.9E-1
GOTERM_BP_FAT	localization of cell	RT		7	4.5E-1	9.9E-1
GOTERM_BP_FAT	regulation of growth	RT		7	5.5E-1	1.0E0
GOTERM_BP_FAT	regulation of cell growth	RT		4	6.6E-1	1.0E0
GOTERM_BP_FAT	regulation of cell size	RT		4	7.0E-1	1.0E0
GOTERM_BP_FAT	positive regulation of developmental process	RT		5	7.2E-1	1.0E0
GOTERM_BP_FAT	cell motion	RT		8	7.3E-1	1.0E0
GOTERM_BP_FAT	regulation of cellular component size	RT		4	8.6E-1	1.0E0
GOTERM_BP_FAT	blood vessel morphogenesis	RT		3	8.9E-1	1.0E0
GOTERM_BP_FAT	positive regulation of cell differentiation	RT		3	9.1E-1	1.0E0
GOTERM_BP_FAT	blood vessel development	RT		3	9.3E-1	1.0E0
GOTERM_BP_FAT	vasculature development	RT	G	3	9.4E-1	1.0E0
Annotation Cluster 71	**Enrichment Score: 0.26**			**Count**	**P_Value**	**Benjamini**
SP_PIR_KEYWORDS	lipase	RT	G	7	3.7E-1	9.3E-1
GOTERM_MF_FAT	acid-amino acid ligase activity	RT		4	6.4E-1	1.0E0
GOTERM_MF_FAT	ligase activity, forming carbon-nitrogen bonds	RT		4	7.3E-1	1.0E0
Annotation Cluster 72	**Enrichment Score: 0.23**			**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	histone modification	RT	G	4	3.6E-1	9.9E-1
GOTERM_BP_FAT	covalent chromatin modification	RT		4	3.8E-1	9.9E-1
SP_PIR_KEYWORDS	chromatin regulator	RT		4	6.7E-1	9.9E-1
GOTERM_BP_FAT	chromatin modification	RT		5	7.1E-1	1.0E0
GOTERM_BP_FAT	chromosome organization	RT		6	7.5E-1	1.0E0
GOTERM_BP_FAT	chromatin organization	RT		6	8.0E-1	1.0E0
Annotation Cluster 73	**Enrichment Score: 0.22**			**Count**	**P_Value**	**Benjamini**
GOTERM_CC_FAT	soluble fraction	RT	G	8	2.2E-1	8.6E-1
GOTERM_BP_FAT	response to insulin stimulus	RT		3	5.3E-1	1.0E0
GOTERM_BP_FAT	response to endogenous stimulus	RT		8	5.7E-1	1.0E0
GOTERM_BP_FAT	response to steroid hormone stimulus	RT		4	6.6E-1	1.0E0
GOTERM_BP_FAT	cellular response to hormone stimulus	RT		3	6.8E-1	1.0E0
GOTERM_BP_FAT	response to peptide hormone stimulus	RT		3	7.6E-1	1.0E0
GOTERM_BP_FAT	response to hormone stimulus	RT		6	7.7E-1	1.0E0
GOTERM_BP_FAT	response to organic substance	RT		9	9.4E-1	1.0E0
Annotation Cluster 74	**Enrichment Score: 0.17**			**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	regulation of protein transport	RT	G	3	6.0E-1	1.0E0
GOTERM_BP_FAT	regulation of establishment of protein localization	RT		3	6.3E-1	1.0E0
GOTERM_BP_FAT	regulation of protein localization	RT		3	7.0E-1	1.0E0
GOTERM_BP_FAT	regulation of cellular localization	RT		4	8.1E-1	1.0E0
Annotation Cluster 75	**Enrichment Score: 0.14**			**Count**	**P_Value**	**Benjamini**
INTERPRO	BTB/POZ	RT	G	3	6.1E-1	1.0E0
UP_SEQ_FEATURE	domain:BTB	RT		3	6.7E-1	1.0E0
SMART	BTB	RT		3	7.6E-1	1.0E0
INTERPRO	BTB/POZ-like	RT		3	7.9E-1	1.0E0
INTERPRO	BTB/POZ fold	RT		3	7.9E-1	1.0E0
Annotation Cluster 76	**Enrichment Score: 0.12**			**Count**	**P_Value**	**Benjamini**
UP_SEQ_FEATURE	repeat:WD 6	RT	G	4	6.1E-1	1.0E0
INTERPRO	WD40 repeat 2	RT		4	7.1E-1	1.0E0
INTERPRO	WD40/YVTN repeat-like	RT		5	7.1E-1	1.0E0
UP_SEQ_FEATURE	repeat:WD 5	RT		4	7.3E-1	1.0E0
INTERPRO	WD40 repeat region	RT		4	7.4E-1	1.0E0
UP_SEQ_FEATURE	WD40 repeat subgroup	RT		4	7.5E-1	1.0E0
UP_SEQ_FEATURE	repeat:WD 4	RT		4	7.8E-1	1.0E0
SMART	WD40	RT		4	7.8E-1	1.0E0
INTERPRO	WD40 repeat	RT		4	8.0E-1	1.0E0
UP_SEQ_FEATURE	repeat:WD 3	RT		4	8.1E-1	1.0E0
UP_SEQ_FEATURE	repeat:WD 2	RT		4	8.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:WD 1	RT		4	8.2E-1	1.0E0
SP_PIR_KEYWORDS	wd repeat	RT		4	8.2E-1	1.0E0
INTERPRO		RT		4		1.0E0

294

	Category	Term			Count	P_Value	Benjamini
	Annotation Cluster 77	**Enrichment Score: 0.11**			**Count**	**P_Value**	**Benjamini**
☐	SMART	W040_repeat_conserved_site	RT	G	4	8.3E-1	1.0E0
☐	SMART	LRR_TYP	RT		3	6.0E-1	1.0E0
☐	INTERPRO	Leucine-rich repeat, typical subtype	RT		3	6.3E-1	1.0E0
☐	INTERPRO	Leucine-rich repeat	RT		4	6.7E-1	1.0E0
☐	UP_SEQ_FEATURE	repeat:LRR 5	RT		4	7.3E-1	1.0E0
☐	UP_SEQ_FEATURE	repeat:LRR 4	RT		4	7.8E-1	1.0E0
☐	UP_SEQ_FEATURE	repeat:LRR 6	RT		3	8.6E-1	1.0E0
☐	UP_SEQ_FEATURE	repeat:LRR 3	RT		4	8.7E-1	1.0E0
☐	UP_SEQ_FEATURE	repeat:LRR 1	RT		4	8.9E-1	1.0E0
☐	UP_SEQ_FEATURE	repeat:LRR 2	RT		4	8.9E-1	1.0E0
☐	SP_PIR_KEYWORDS	Leucine-rich repeat	RT		4	8.9E-1	1.0E0
	Annotation Cluster 78	**Enrichment Score: 0.11**			**Count**	**P_Value**	**Benjamini**
☐	GOTERM_MF_FAT	transcription repressor activity	RT	G	6	5.9E-1	1.0E0
☐	GOTERM_BP_FAT	regulation of transcription from RNA polymerase II promoter	RT		13	6.3E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of transcription from RNA polymerase II promoter	RT		5	6.9E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of transcription, DNA-dependent	RT		6	7.5E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of RNA metabolic process	RT		6	7.6E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of gene expression	RT		7	7.9E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of macromolecule metabolic process	RT	G	9	9.5E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of biosynthetic process	RT		8	8.0E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of cellular biosynthetic process	RT		8	8.1E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of transcription	RT		7	8.2E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of macromolecule biosynthetic process	RT		8	8.5E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	RT		8	8.7E-1	1.0E0
☐	GOTERM_BP_FAT	negative regulation of nitrogen compound metabolic process	RT		8	8.8E-1	1.0E0
	Annotation Cluster 79	**Enrichment Score: 0.1**			**Count**	**P_Value**	**Benjamini**
☐	GOTERM_BP_FAT	secretion by cell	RT	G	5	6.0E-1	1.0E0
☐	GOTERM_BP_FAT	secretion	RT		3	8.8E-1	1.0E0
☐	GOTERM_BP_FAT	exocytosis	RT		4	8.5E-1	1.0E0
	Annotation Cluster 80	**Enrichment Score: 0.08**			**Count**	**P_Value**	**Benjamini**
☐	GOTERM_CC_FAT	microsome	RT	G	4	7.2E-1	9.9E-1
☐	GOTERM_CC_FAT	vesicular fraction	RT		4	7.4E-1	9.9E-1
☐	GOTERM_CC_FAT	cell fraction	RT		15	9.9E-1	1.0E0
☐	GOTERM_CC_FAT	membrane fraction	RT		8	8.1E-1	1.0E0
☐	GOTERM_CC_FAT	insoluble fraction	RT		9	9.4E-1	1.0E0
	Annotation Cluster 81	**Enrichment Score: 0.07**			**Count**	**P_Value**	**Benjamini**
☐	GOTERM_BP_FAT	reproductive cellular process	RT	G	9	9.6E-1	1.0E0
☐	GOTERM_BP_FAT	sexual reproduction	RT		6	9.1E-1	1.0E0
☐	GOTERM_BP_FAT	gamete generation	RT		6	9.2E-1	1.0E0
☐	GOTERM_BP_FAT	reproductive process in a multicellular organism	RT		5	9.7E-1	1.0E0
☐	GOTERM_BP_FAT	multicellular organism reproduction	RT		5	9.7E-1	1.0E0
	Annotation Cluster 82	**Enrichment Score: 0.07**			**Count**	**P_Value**	**Benjamini**
☐	GOTERM_BP_FAT	transcription from RNA polymerase II promoter	RT	G	5	7.8E-1	1.0E0
☐	GOTERM_BP_FAT	transcription, DNA-dependent	RT		4	8.9E-1	1.0E0
☐	GOTERM_BP_FAT	RNA biosynthetic process	RT		4	8.9E-1	1.0E0
	Annotation Cluster 83	**Enrichment Score: 0.07**			**Count**	**P_Value**	**Benjamini**
☐	GOTERM_BP_FAT	regulation of protein modification process	RT	G	5	7.6E-1	1.0E0
☐	GOTERM_BP_FAT	positive regulation of protein metabolic process	RT		4	8.0E-1	1.0E0
☐	GOTERM_BP_FAT	positive regulation of protein modification process	RT		3	8.4E-1	1.0E0
☐	GOTERM_BP_FAT	positive regulation of cellular protein metabolic process	RT		3	9.2E-1	1.0E0
☐	GOTERM_BP_FAT	regulation of cellular protein metabolic process	RT	G	5	9.7E-1	1.0E0
	Annotation Cluster 84	**Enrichment Score: 0.06**			**Count**	**P_Value**	**Benjamini**
☐	UP_SEQ_FEATURE	domain:Ig-like C2-type	RT		3	5.0E-1	1.0E0

http://www.ebi.ac.uk/QuickGO/GTerm?id=GO:0051247

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Category	Term			Count	P_Value	Benjamini
SMART	IG			3	9.6E-1	1.0E0
INTERPRO	Immunoglobulin subtype			3	9.7E-1	1.0E0
INTERPRO	Immunoglobulin-like fold			5	9.8E-1	1.0E0
SP_PIR_KEYWORDS	Immunoglobulin domain			3	9.8E-1	1.0E0
INTERPRO	Immunoglobulin-like		G	4	9.9E-1	1.0E0
Annotation Cluster 85	**Enrichment Score: 0.06**			**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	activation of protein kinase activity	RT		3	6.0E-1	1.0E0
GOTERM_BP_FAT	regulation of protein kinase activity	RT		5	7.6E-1	1.0E0
GOTERM_BP_FAT	regulation of protein modification process	RT		5	8.1E-1	1.0E0
GOTERM_BP_FAT	regulation of protein amino acid phosphorylation	RT		3	8.6E-1	1.0E0
GOTERM_BP_FAT	regulation of protein kinase activity	RT		5	8.8E-1	1.0E0
GOTERM_BP_FAT	regulation of kinase activity	RT		5	8.9E-1	1.0E0
GOTERM_BP_FAT	regulation of transferase activity	RT		5	9.1E-1	1.0E0
GOTERM_BP_FAT	positive regulation of protein kinase activity	RT		3	9.2E-1	1.0E0
GOTERM_BP_FAT	regulation of phosphorylation	RT		6	9.2E-1	1.0E0
GOTERM_BP_FAT	positive regulation of kinase activity	RT		3	9.3E-1	1.0E0
GOTERM_BP_FAT	positive regulation of transferase activity	RT		3	9.3E-1	1.0E0
GOTERM_BP_FAT	regulation of phosphorus metabolic process	RT		6	9.3E-1	1.0E0
GOTERM_BP_FAT	regulation of phosphate metabolic process	RT		6	9.3E-1	1.0E0
GOTERM_BP_FAT	regulation of cellular protein metabolic process	RT		5	9.7E-1	1.0E0
GOTERM_BP_FAT	positive regulation of catalytic activity	RT		5	9.8E-1	1.0E0
GOTERM_BP_FAT	positive regulation of molecular function	RT		5	9.9E-1	1.0E0
Annotation Cluster 86	**Enrichment Score: 0.06**			**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	cysteine-type peptidase activity	RT		3	6.7E-1	1.0E0
GOTERM_MF_FAT	peptidase activity, acting on L-amino acid peptides	RT		5	9.8E-1	1.0E0
GOTERM_MF_FAT	peptidase activity	RT	G	5	9.9E-1	1.0E0
Annotation Cluster 87	**Enrichment Score: 0.06**			**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	endopeptidase activity	RT		3	9.9E-1	1.0E0
GOTERM_MF_FAT	protein homodimerization activity	RT		4	9.1E-1	1.0E0
GOTERM_MF_FAT	identical protein binding	RT		8	9.0E-1	1.0E0
GOTERM_MF_FAT	protein dimerization activity	RT		5	8.7E-1	1.0E0
Annotation Cluster 88	**Enrichment Score: 0.06**			**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	GTPase activity	RT	G	3	8.6E-1	1.0E0
GOTERM_MF_FAT	guanyl ribonucleotide binding	RT		5	8.7E-1	1.0E0
GOTERM_MF_FAT	guanyl nucleotide binding	RT		5	8.7E-1	1.0E0
SP_PIR_KEYWORDS	GTP-binding	RT		4	9.0E-1	1.0E0
GOTERM_MF_FAT	GTP binding	RT		4	9.0E-1	1.0E0
UP_SEQ_FEATURE	nucleotide phosphate-binding region:GTP	RT		3	9.4E-1	1.0E0
Annotation Cluster 89	**Enrichment Score: 0.03**			**Count**	**P_Value**	**Benjamini**
GOTERM_CC_FAT	endoplasmic reticulum part	RT		4	9.1E-1	1.0E0
GOTERM_CC_FAT	endoplasmic reticulum membrane	RT		3	9.3E-1	1.0E0
GOTERM_CC_FAT	nuclear envelope-endoplasmic reticulum network	RT	G	3	9.4E-1	1.0E0
Annotation Cluster 90	**Enrichment Score: 0.02**			**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	metal ion transmembrane transporter activity	RT		4	9.1E-1	1.0E0
GOTERM_MF_FAT	cation channel activity	RT		3	9.4E-1	1.0E0
GOTERM_MF_FAT	ion channel activity	RT		4	9.5E-1	1.0E0
GOTERM_MF_FAT	substrate specific channel activity	RT		4	9.6E-1	1.0E0
GOTERM_MF_FAT	channel activity	RT		4	9.7E-1	1.0E0
GOTERM_MF_FAT	passive transmembrane transporter activity	RT		4	9.7E-1	1.0E0
Annotation Cluster 91	**Enrichment Score: 0.02**			**Count**	**P_Value**	**Benjamini**
SP_PIR_KEYWORDS	synapse	RT		3	8.6E-1	1.0E0
GOTERM_CC_FAT	synapse	RT		3	9.8E-1	1.0E0
GOTERM_CC_FAT	cell junction	RT		3	9.9E-1	1.0E0
SP_PIR_KEYWORDS	cell junction	RT		4	9.9E-1	1.0E0
Annotation Cluster 92	**Enrichment Score: 0.02**			**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	cellular di-, tri-valent inorganic cation homeostasis	RT	G	3	9.1E-1	1.0E0
GOTERM_BP_FAT	di-, tri-valent inorganic cation homeostasis	RT		3	9.2E-1	1.0E0
GOTERM_BP_FAT	cellular cation homeostasis	RT		3	9.4E-1	1.0E0

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Category	Term	Count	P_Value	Benjamini
GOTERM_BP_FAT	chemical homeostasis	6	9.5E-1	1.0E0
Annotation Cluster 93	**Enrichment Score: 0.02**			
GOTERM_BP_FAT	cellular ion homeostasis	4	9.6E-1	1.0E0
GOTERM_BP_FAT	cation homeostasis	3	9.6E-1	1.0E0
GOTERM_BP_FAT	cellular chemical homeostasis	4	9.6E-1	1.0E0
GOTERM_BP_FAT	cellular homeostasis	5	9.7E-1	1.0E0
GOTERM_BP_FAT	ion homeostasis	4	9.8E-1	1.0E0
GOTERM_BP_FAT	homeostatic process	8	9.8E-1	1.0E0
Annotation Cluster 94	**Enrichment Score: 0.01**			
SP_PIR_KEYWORDS	extracellular matrix	3	9.0E-1	1.0E0
SP_PIR_KEYWORDS	proteinaceous extracellular matrix	3	9.6E-1	1.0E0
GOTERM_CC_FAT	extracellular matrix	3	9.6E-1	1.0E0
GOTERM_CC_FAT	extracellular region part	7	9.7E-1	1.0E0
Annotation Cluster 95	**Enrichment Score: 0**			
SP_PIR_KEYWORDS	glycoprotein	38	1.0E0	1.0E0
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	36	1.0E0	1.0E0
UP_SEQ_FEATURE	topological domain:Cytoplasmic	36	9.9E-1	1.0E0
SP_PIR_KEYWORDS	transmembrane	63	9.9E-1	1.0E0
UP_SEQ_FEATURE	transmembrane region	63	9.9E-1	1.0E0
GOTERM_CC_FAT	integral to membrane	70	9.8E-1	1.0E0
GOTERM_CC_FAT	intrinsic to membrane	75	9.6E-1	1.0E0
SP_PIR_KEYWORDS	membrane	88	9.6E-1	1.0E0
Annotation Cluster 96	**Enrichment Score: 0**			
GOTERM_BP_FAT	biological adhesion	5	1.0E0	1.0E0
GOTERM_BP_FAT	cell adhesion	5	1.0E0	1.0E0
SP_PIR_KEYWORDS	cell adhesion	3	9.9E-1	1.0E0
Annotation Cluster 97	**Enrichment Score: 0**			
GOTERM_CC_FAT	plasma membrane	18	1.0E0	1.0E0
GOTERM_CC_FAT	intrinsic to plasma membrane	10	1.0E0	1.0E0
GOTERM_CC_FAT	integral to plasma membrane	10	1.0E0	1.0E0
Annotation Cluster 98	**Enrichment Score: 0**			
GOTERM_CC_FAT	extracellular region	13	1.0E0	1.0E0
SP_PIR_KEYWORDS	Secreted	13	1.0E0	1.0E0
GOTERM_CC_FAT	extracellular region part	7	1.0E0	1.0E0
GOTERM_CC_FAT	extracellular space	5	1.0E0	1.0E0
Annotation Cluster 99	**Enrichment Score: 0**			
GOTERM_BP_FAT	neurological system process	6	1.0E0	1.0E0
GOTERM_BP_FAT	sensory perception	3	1.0E0	1.0E0
GOTERM_BP_FAT	cognition	5	1.0E0	1.0E0
GOTERM_BP_FAT	behavior	5	1.0E0	1.0E0
Annotation Cluster 100	**Enrichment Score: 0**			
SP_PIR_KEYWORDS	transducer	5	9.9E-1	1.0E0
SP_PIR_KEYWORDS	G-protein coupled receptor	4	1.0E0	1.0E0
GOTERM_BP_FAT	G-protein coupled receptor protein signaling pathway	5	1.0E0	1.0E0
UP_SEQ_FEATURE	topological domain:Cytoplasmic	36	1.0E0	1.0E0
SP_PIR_KEYWORDS	signal	28	1.0E0	1.0E0
UP_SEQ_FEATURE	signal peptide	28	1.0E0	1.0E0
UP_SEQ_FEATURE	topological domain:Extracellular	28	1.0E0	1.0E0
UP_SEQ_FEATURE	disulfide bond	21	1.0E0	1.0E0
SP_PIR_KEYWORDS	disulfide bond	22	1.0E0	1.0E0
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	23	1.0E0	1.0E0
SP_PIR_KEYWORDS	glycoprotein	36	1.0E0	1.0E0
UP_SEQ_FEATURE	glycoprotein	38	1.0E0	1.0E0

234 terms were not clustered.

https://david.ncifcrf.gov/term2term.jsp?annot=52,9,79,85,25,32,39,1,3,47,45,63,7&&currentList=0

15/16

8.4 Rabbit IPA analysis

INGENUITY
PATHWAY ANALYSIS

Analysis Name: Rabbit - 2016-03-14 10:57 AM
Analysis Creation Date: 2016-03-14
Build version: 366632M
Content version: 26127183 (Release Date: 2015-11-30)

Analysis Settings

Reference set: Ingenuity Knowledge Base (Genes Only)

Relationship to include: Direct and Indirect

Includes Endogenous Chemicals

Optional Analyses: My Pathways My List

Filter Summary:

Consider only relationships where

confidence = Experimentally Observed

1

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298

Summary of Analysis - Rabbit - 2016-03-14 10:57 AM

Top Canonical Pathways

Name	p-value	Overlap	
Ceramide Signaling	9.18E-05	8.8 %	7/80
Induction of Apoptosis by HIV1	1.40E-04	10.0 %	6/60
Death Receptor Signaling	2.21E-04	7.6 %	7/92
Tec Kinase Signaling	2.51E-04	5.7 %	9/157
NF-B Activation by Viruses	4.12E-04	8.2 %	6/73

Top Upstream Regulators

Upstream Regulator	p-value of overlap	Predicted Activation
MAX	1.20E-05	
SREBF1	4.07E-05	Inhibited
SATB1	4.38E-05	
amiodarone	9.08E-05	
WHSC1	1.04E-04	

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	#Molecules
Gastrointestinal Disease	1.32E-02 - 1.05E-04	207
Hepatic System Disease	1.28E-02 - 1.05E-04	13
Inflammatory Disease	1.32E-02 - 1.05E-04	44
Organismal Injury and Abnormalities	1.32E-02 - 1.82E-04	245
Immunological Disease	1.32E-02 - 2.23E-04	58

Molecular and Cellular Functions

2

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Name	p-value	#Molecules
Cell Death and Survival	1.32E-02 - 9.08E-06	98
Cellular Function and Maintenance	1.32E-02 - 9.08E-06	49
Cellular Development	1.32E-02 - 1.34E-05	66
Cellular Growth and Proliferation	1.32E-02 - 1.34E-05	95
Lipid Metabolism	1.32E-02 - 3.65E-05	31

Physiological System Development and Function

Name	p-value	#Molecules
Embryonic Development	1.32E-02 - 9.08E-06	24
Hematological System Development and Function	1.32E-02 - 4.41E-05	41
Humoral Immune Response	1.32E-02 - 4.41E-05	19
Immune Cell Trafficking	1.32E-02 - 4.41E-05	13
Lymphoid Tissue Structure and Development	1.14E-02 - 2.09E-04	25

Top Tox Functions

Assays: Clinical Chemistry and Hematology

Name	p-value	#Molecules
Increased Levels of AST	1.81E-01 - 1.32E-02	1
Increased Levels of Alkaline Phosphatase	2.37E-01 - 1.32E-02	2
Decreased Levels of Albumin	5.19E-02 - 2.63E-02	1
Increased Levels of CRP	2.63E-02 - 2.63E-02	1
Increased Levels of ALT	2.34E-01 - 6.45E-02	1

Cardiotoxicity

3

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Name	p-value	#Molecules
Cardiac Inflammation	4.21E-01 - 2.53E-03	5
Cardiac Damage	1.02E-01 - 1.32E-02	2
Cardiac Necrosis/Cell Death	5.21E-01 - 1.32E-02	6
Congenital Heart Anomaly	4.80E-01 - 3.39E-02	6
Cardiac Stress Response	3.92E-02 - 3.92E-02	1

Hepatotoxicity

Name	p-value	#Molecules
Liver Damage	5.63E-01 - 1.82E-04	6
Liver Degeneration	2.63E-02 - 8.57E-03	3
Liver Necrosis/Cell Death	2.44E-01 - 8.61E-03	7
Liver Inflammation/Hepatitis	5.63E-01 - 1.24E-02	9
Liver Enlargement	1.03E-01 - 2.63E-02	4

Nephrotoxicity

Name	p-value	#Molecules
Renal Necrosis/Cell Death	3.02E-01 - 1.25E-03	14
Glomerular Injury	4.87E-01 - 3.42E-03	8
Renal Damage	3.25E-01 - 1.13E-02	6
Nephrosis	2.13E-01 - 1.32E-02	3
Renal Fibrosis	4.87E-01 - 2.63E-02	3

Top Regulator Effect Networks

ID	Regulators	Diseases & Functions	Consistency Score
1	SREBF1	synthesis of cholesterol ester	1.732
2	STAT1	cell death of tumor cell lines	-6.5

4

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3	ARNT	cell death of tumor cell lines	-7.0
4	STAT1	cell death of leukocyte cell lines	-8.083
5	SREBF1	necrosis	-11.431

Top Networks

ID	Associated Network Functions	Score
1	Antimicrobial Response, Cell Morphology, Cellular Assembly and Organization	51
2	Cancer, Organismal Injury and Abnormalities, Gastrointestinal Disease	41
3	Auditory and Vestibular System Development and Function, Cellular Development, Cellular Growth and Proliferation	35
4	Lymphoid Tissue Structure and Development, Organ Development, DNA Replication, Recombination, and Repair	33
5	Cell Death and Survival, Cancer, Organismal Injury and Abnormalities	30

Top Tox Lists

Name	p-value	Overlap	
Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane	1.67E-04	6.8 %	8/117
Increases Liver Hepatitis	6.69E-04	9.4 %	5/53
Anti-Apoptosis	8.06E-04	12.5 %	4/32
Increases Liver Steatosis	9.87E-04	7.0 %	6/86
Increases Permeability Transition of Mitochondria and Mitochondrial Membrane	4.64E-03	25.0 %	2/8

Top My Lists

Name	p-value	Overlap	
Colorectal cancer	1.57E-02	1.7 %	73/4364
dysregulated FDR p=0.05 010216	2.64E-01	4.3 %	1/23
Hu downreg	3.30E-01	2.0 %	3/153
EDN1 p=0.01	3.89E-01	2.7 %	1/37
CD40LG p=0.01	5.20E-01	1.8 %	1/55

5

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Top Analysis-Ready Molecules

Exp Fold Change up-regulated

Molecules	Exp. Value	Exp. Chart
CD3D	↑ 23.528	
ITGB1	↑ 17.225	
TMEM127	↑ 14.373	
RCAN3	↑ 12.205	
RAB40AL	↑ 11.558	
SRSF10	↑ 10.717	
MRPL38	↑ 10.485	
LMBR1	↑ 8.205	
TAF9B	↑ 7.659	
UCKL1	↑ 6.825	

Exp Fold Change down-regulated

Molecules	Exp. Value	Exp. Chart
SLC2A3	↓ -12.324	
FADS2	↓ -11.672	
SCD	↓ -11.341	
AK4	↓ -10.804	
BNIP1	↓ -8.996	
EXOSC4	↓ -7.970	
TNF	↓ -6.723	
PRKD3	↓ -6.407	
MIF	↓ -6.105	
PIK3AP1	↓ -5.433	

6

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303

8.5 Cattle IPA analysis

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PATHWAY ANALYSIS

Analysis Name: Cow2 - 2016-03-15 11:00 AM
Analysis Creation Date: 2016-03-15
Build version: 366632M
Content version: 26127183 (Release Date: 2015-11-30)

Analysis Settings

Reference set: Ingenuity Knowledge Base (Genes Only)

Relationship to include: Direct and Indirect

Includes Endogenous Chemicals

Optional Analyses: My Pathways My List

Filter Summary:

Consider only relationships where

confidence = Experimentally Observed

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Summary of Analysis - Cow2 - 2016-03-15 11:00 AM

Top Canonical Pathways

Name	p-value	Overlap	
Regulation of eIF4 and p70S6K Signaling	2.35E-04	6.8 %	10/146
EIF2 Signaling	1.43E-03	5.4 %	10/184
mTOR Signaling	1.61E-03	5.3 %	10/187
2-oxobutanoate Degradation I	2.89E-03	40.0 %	2/5
Oxidative Phosphorylation	2.89E-03	6.4 %	7/109

Top Upstream Regulators

Upstream Regulator	p-value of overlap	Predicted Activation
NUPR1	1.22E-06	Activated
E2f	6.76E-06	
TP53	1.23E-05	
ST1926	1.65E-05	Activated
RRP1B	4.15E-05	

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	#Molecules
Cancer	2.52E-02 - 1.10E-06	319
Organismal Injury and Abnormalities	2.52E-02 - 1.10E-06	324
Developmental Disorder	2.49E-02 - 2.98E-05	65
Endocrine System Disorders	1.73E-02 - 3.54E-05	22
Gastrointestinal Disease	1.73E-02 - 1.70E-04	273

Molecular and Cellular Functions

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305

Summary of Analysis - Cow2 - 2016-03-15 11:00 AM

Name	p-value	#Molecules
Cellular Assembly and Organization	2.41E-02 - 2.67E-06	43
Gene Expression	1.73E-02 - 1.52E-04	62
RNA Damage and Repair	1.33E-02 - 1.52E-04	7
RNA Post-Transcriptional Modification	1.52E-04 - 1.52E-04	6
DNA Replication, Recombination, and Repair	2.24E-02 - 1.71E-04	35

Physiological System Development and Function

Name	p-value	#Molecules
Embryonic Development	2.37E-02 - 2.98E-05	53
Organismal Survival	5.15E-03 - 2.98E-05	77
Connective Tissue Development and Function	1.73E-02 - 8.86E-04	24
Tissue Development	2.37E-02 - 8.86E-04	48
Organismal Development	2.37E-02 - 1.02E-03	55

Top Tox Functions

Assays: Clinical Chemistry and Hematology

Name	p-value	#Molecules
Decreased Levels of Hematocrit	1.30E-01 - 1.30E-01	1
Increased Levels of Hematocrit	4.91E-01 - 4.91E-01	2
Increased Levels of Creatinine	4.94E-01 - 4.94E-01	1
Increased Levels of Red Blood Cells	5.24E-01 - 5.24E-01	2

Cardiotoxicity

Name	p-value	#Molecules
Cardiac Degeneration	1.73E-02 - 1.73E-02	1
Congenital Heart Anomaly	5.75E-01 - 1.73E-02	2

3

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Summary of Analysis - Cow2 - 2016-03-15 11:00 AM

Cardiac Arrythmia	4.01E-01 - 3.43E-02	3
Cardiac Dilation	1.77E-01 - 6.98E-02	3
Cardiac Arteriopathy	5.18E-01 - 9.95E-02	6

Hepatotoxicity

Name	p-value	#Molecules
Liver Fibrosis	3.19E-01 - 8.76E-03	7
Hepatocellular Peroxisome Proliferation	1.73E-02 - 1.73E-02	1
Liver Hyperplasia/Hyperproliferation	5.97E-01 - 1.73E-02	128
Hepatocellular Carcinoma	5.97E-01 - 3.11E-02	124
Liver Inflammation/Hepatitis	5.55E-01 - 3.43E-02	6

Nephrotoxicity

Name	p-value	#Molecules
Nephrosis	1.73E-02 - 1.73E-02	1
Renal Dilation	1.73E-02 - 1.73E-02	1
Glomerular Injury	3.31E-01 - 4.62E-02	6
Renal Hyperplasia/Hyperproliferation	4.62E-02 - 4.62E-02	2
Renal Atrophy	1.75E-01 - 1.60E-01	1

Top Networks	
ID Associated Network Functions	Score
1 Cancer, Organismal Injury and Abnormalities, Gastrointestinal Disease	53
2 Cancer, Organismal Injury and Abnormalities, Gastrointestinal Disease	41
3 Cell Cycle, DNA Replication, Recombination, and Repair, Gene Expression	36
4 Ophthalmic Disease, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders	36
5 Cell Cycle, DNA Replication, Recombination, and Repair, Developmental Disorder	34

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Top Tox Lists

Name	p-value	Overlap	
Mitochondrial Dysfunction	3.39E-02	4.0 %	7/176
Long-term Renal Injury Anti-oxidative Response Panel (Rat)	3.81E-02	11.1 %	2/18
VDR/RXR Activation	4.64E-02	5.1 %	4/78
Mechanism of Gene Regulation by Peroxisome Proliferators via PPAR	8.29E-02	4.2 %	4/95
Increases Cardiac Dilation	8.41E-02	7.1 %	2/28

Top My Lists

Name	p-value	Overlap	
CCL5 p=0.01	3.46E-03	16.7 %	3/18
CSF2 p=0.01	6.87E-03	7.2 %	5/69
CSF1 p=0.01	2.41E-02	8.3 %	3/36
CXCL8 p=0.01	3.05E-02	12.5 %	2/16
CSF3 p=0.01	1.00E-01	6.5 %	2/31

Top My Pathways

Name	p-value	Overlap	
CCL5 p=0.01	3.46E-03	16.7 %	3/18
CSF2 p=0.01	6.87E-03	7.2 %	5/69
CSF1 p=0.01	2.41E-02	8.3 %	3/36
CXCL8 p=0.01	3.05E-02	12.5 %	2/16
HGF mechanistic network p0.01	5.81E-02	3.3 %	8/240

Top Analysis-Ready Molecules

Appendices

5

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308

Exp Fold Change up-regulated

Molecules	Exp. Value	Exp. Chart
CFHR3	↑ 34.156	
PSMD11	↑ 30.737	
NME3	↑ 22.407	
UNC5B	↑ 19.885	
MUM1	↑ 16.392	
WNT5A	↑ 13.694	
SLC36A4	↑ 12.970	
VASH1	↑ 12.130	
NATD1	↑ 11.199	
SMOC1	↑ 10.862	

Exp Fold Change down-regulated

Molecules	Exp. Value	Exp. Chart
HMGA2	↓ -80.890	
CEP55	↓ -12.382	
UHRF1	↓ -11.811	
KIF2C	↓ -8.982	
PBK	↓ -8.369	
ESPL1	↓ -8.023	
MT-CYB	↓ -7.237	
MT-ND3	↓ -7.147	
KIF22	↓ -7.069	
CCNF	↓ -7.000	

6

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8.6 Rabbit to human with gene description

#	Transcript_Name	Human Ensembl Gene ID	CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 6	CLUSTER 7	FC	pval	Log_FC	strand	start	end	num_exons	length	gene_id	Description	gene_name
2	ENSOCUT00000006948	ENSG00000110958		YES	YES			0.513835471	0.016451238	-0.960621609	-	40230879	40231361	1	483	ENSOCUG00000022356	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1SS21]	0
3	ENSOCUT00000023770	ENSG00000100294		YES				0.918355717	0.040357954	-0.122875018	+	16562	26899	4	4784	ENSOCUG00000015843	malonyl CoA:ACP acyltransferase (mitochondrial) [Source:HGNC Symbol;	MCAT
4	ENSOCUT00000024031	ENSG00000138381		YES				1.232731108	0.0245595	0.301858143	+	9303819	9305696	1	1878	ENSOCUG00000024832	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1TX10]	0
5	ENSOCUT00000015376	ENSG00000151093		YES				0.434145939	0.018148202	-1.203748005	+	12327109	12331612	2	2548	ENSOCUG00000015384	3-oxoacyl-ACP synthase, mitochondrial [Source:HGNC Symbol;Acc:HGI	OXSM
6	ENSOCUT00000024169	ENSG00000172292		YES			YES	1.270737712	0.007420982	0.34566628	+	108176894	108508100	11	1467	ENSOCUG00000005722	ceramide synthase 6 [Source:HGNC Symbol;Acc:HGNC:23826]	CERS6
7	ENSOCUT00000003499	ENSG00000134824		YES			YES	0.085672586	0.011453431	-3.545022558	-	2874101	2906618	12	1353	ENSOCUG00000003499	fatty acid desaturase 2 [Source:HGNC Symbol;Acc:HGNC:3575]	FADS2
8	ENSOCUT00000014794	ENSG00000099194		YES			YES	0.088172741	0.035263001	-3.503523475	-	48425105	48437117	6	1129	ENSOCUG00000014801	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1T847]	0
9	ENSOCUT00000004547	ENSG00000001630		YES			YES	0.560022688	0.040601664	-0.836442819	+	36634694	36654515	10	1527	ENSOCUG00000004549	cytochrome P450, family 51, subfamily A, polypeptide 1 [Source:HGNC Sy	CYP51A1
10	ENSOCUT00000025399	ENSG00000111669		YES				0.231695789	0.00350146	-2.109696273	+	32890155	32893009	7	929	ENSOCUG00000025649	triosephosphate isomerase 1 [Source:HGNC Symbol;Acc:HGNC:12009]	TPI1
11	ENSOCUT00000006644	ENSG00000079246	YES		YES	YES		1.102514047	0.024364476	0.140797037	+	157535923	157646113	21	5250	ENSOCUG00000006642	X-ray repair complementing defective repair in Chinese hamster cells 5 (do	XRCC5
12	ENSOCUT00000021649	ENSG00000172115	YES			YES		0.73269075	0.043449515	-0.448723692	-	82231495	82231812	1	318	ENSOCUG00000027381	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1TY21]	0
13	ENSOCUT00000014272	ENSG00000020426	YES		YES	YES		1.058998383	0.003234823	0.082700386	+	8315599	8542359	8	1415	ENSOCUG00000014274	MNAT CDK-activating kinase assembly factor 1 [Source:HGNC Symbol;Ac	MNAT1
14	ENSOCUT00000002540	ENSG00000176171	YES		YES	YES	YES	0.19389392	0.010643207	-2.36666053	-	90716	95350	5	1033	ENSOCUG00000002541	BCL2/adenovirus E1B 19kDa interacting protein 3 [Source:HGNC Symbol;	BNIP3
15	ENSOCUT00000005276	ENSG00000186575	YES			YES		1.174228086	0.042310157	0.23171267	+	2795414	2874309	16	2349	ENSOCUG00000005276	neurofibromin 2 (merlin) [Source:HGNC Symbol;Acc:HGNC:7773]	NF2
16	ENSOCUT00000026928	ENSG00000112592	YES			YES		1.325968243	0.045046504	0.407046223	-	21719	40912	7	3011	ENSOCUG00000007979	TATA box binding protein [Source:HGNC Symbol;Acc:HGNC:11588]	TBP
17	ENSOCUT00000015150	ENSG00000166484	YES			YES		1.73707655	0.018904004	0.796661332	-	323191	327527	6	2750	ENSOCUG00000015154	mitogen-activated protein kinase 7 [Source:HGNC Symbol;Acc:HGNC:68	MAPK7
18	ENSOCUT00000006977	ENSG00000177463	YES			YES		0.782668317	0.023524254	-0.353527049	+	10502766	10566683	16	2058	ENSOCUG00000006975	nuclear receptor subfamily 2, group C, member 2 [Source:HGNC Symbol;/	NR2C2
19	ENSOCUT00000006606	ENSG00000090316	YES			YES		0.553806453	0.021660036	-0.85254623	-	541	750	1	210	ENSOCUG00000006610	macrophage erythroblast attacher [Source:HGNC Symbol;Acc:HGNC:137	MAEA
20	ENSOCUT00000014714	ENSG00000187325	YES			YES		7.659413773	0.013058727	2.937233977	-	69877434	69884179	7	819	ENSOCUG00000014717	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1T7Y8]	0
21	ENSOCUT00000004604	ENSG00000154920	YES		YES			1.212978903	0.00905367	0.278554458	-	37333315	37340228	8	2171	ENSOCUG00000004604	essential meiotic structure-specific endonuclease 1 [Source:HGNC Symb	EME1
22	ENSOCUT00000014772	ENSG00000123562	YES		YES			1.438494192	0.008386517	0.524553396	+	86448916	86449782	1	867	ENSOCUG00000014778	mortality factor 4 like 2 [Source:HGNC Symbol;Acc:HGNC:16849]	MORF4L2
23	ENSOCUT00000011847	ENSG00000105486	YES		YES			0.312874143	0.000738829	-1.676345662	+	918	24350	22	5416	ENSOCUG00000011841	ligase I, DNA, ATP-dependent [Source:HGNC Symbol;Acc:HGNC:6598]	LIG1
24	ENSOCUT00000017089	ENSG00000151332	YES		YES			1.726132245	0.028151746	0.787542999	-	55912983	55935420	9	4896	ENSOCUG00000017031	MAP3K12 binding inhibitory protein 1 [Source:HGNC Symbol;Acc:HGNC:2	MBIP
25	ENSOCUT00000005398	ENSG00000175305	YES		YES			0.552920702	0.040346282	-0.854855505	-	110486503	110500646	12	1326	ENSOCUG00000005398	cyclin E2 [Source:HGNC Symbol;Acc:HGNC:1590]	CCNE2
26	ENSOCUT00000002807	ENSG00000144554	YES		YES			0.660821298	0.042852429	-0.59766791	-	159720	240687	44	5418	ENSOCUG00000002802	Fanconi anemia, complementation group D2 [Source:HGNC Symbol;Acc:	FANCD2
27	ENSOCUT00000003622	ENSG00000133119	YES		YES			0.843671168	0.015444366	-0.245247295	+	4595342	4615895	9	2898	ENSOCUG00000003624	replication factor C (activator 1) 3, 38kDa [Source:HGNC Symbol;Acc:HGN	RFC3
28	ENSOCUT00000012216	ENSG00000125901	YES		YES			0.905904761	0.015520212	-0.14256871	+	10039298	10041261	4	1304	ENSOCUG00000012218	mitochondrial ribosomal protein S26 [Source:HGNC Symbol;Acc:HGNC:14	MRPS26
29	ENSOCUT00000025560	ENSG00000113356	YES					1.088044048	0.016502701	0.121736964	+	10247401	10269842	6	724	ENSOCUG00000010624	polymerase (RNA) III (DNA directed) polypeptide G (32kD) [Source:HGNC S	POLR3G
30	ENSOCUT00000000324	ENSG00000140694	YES					0.872502785	0.043931808	-0.196788358	-	7493345	7667457	23	1905	ENSOCUG00000000322	poly(A)-specific ribonuclease [Source:HGNC Symbol;Acc:HGNC:8609]	PARN
31	ENSOCUT00000008034	ENSG00000163682	YES					0.941032153	0.041208972	-0.087684078	-	3788180	3788758	1	579	ENSOCUG00000025072	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1SUB9]	0
32	ENSOCUT00000027010	ENSG00000108175	YES					1.209761617	0.048564533	0.274722793	+	438368	538550	21	6902	ENSOCUG00000003587	zinc finger, MIZ-type containing 1 [Source:HGNC Symbol;Acc:HGNC:164S	ZMIZ1
33	ENSOCUT00000005170	ENSG00000186814	YES					0.66053788	0.01961141	-0.598286796	-	76619089	76634379	4	1799	ENSOCUG00000005172	zinc finger and SCAN domain containing 30 [Source:HGNC Symbol;Acc:H	ZSCAN30
34	ENSOCUT00000006345	ENSG00000159792	YES				YES	1.441915589	0.001056105	0.527986711	+	23455908	23493787	4	1712	ENSOCUG00000006346	protein serine kinase H1 [Source:HGNC Symbol;Acc:HGNC:9529]	PSKH1
35	ENSOCUT00000011703	ENSG00000102103	YES					2.924568738	0.028090071	1.548223898	+	33551942	33558604	6	827	ENSOCUG00000011703	polyglutamine binding protein 1 [Source:HGNC Symbol;Acc:HGNC:9330]	PQBP1
36	ENSOCUT00000009275	ENSG00000186298	YES				YES	0.30480252	0.02344638	-1.714053263	-	7001293	7024361	8	2703	ENSOCUG00000009275	protein phosphatase 1, catalytic subunit, gamma isozyme [Source:HGNC :	PPP1CC
37	ENSOCUT00000024194	ENSG00000147130	YES					1.132362549	0.049674327	0.17933594	-	49710474	49726020	25	6078	ENSOCUG00000012396	zinc finger, MYM-type 3 [Source:HGNC Symbol;Acc:HGNC:13054]	ZMYM3
38	ENSOCUT00000010009	ENSG00000162433	YES					0.092555	0.01612021	-3.433545267	-	101181454	101255102	6	1500	ENSOCUG00000010012	adenylate kinase 4 [Source:HGNC Symbol;Acc:HGNC:363]	AK4
39	ENSOCUT00000008576	ENSG00000206560	YES					0.691831054	0.042355193	-0.531508321	-	1755795	1868227	28	6250	ENSOCUG00000008568	ankyrin repeat domain 28 [Source:HGNC Symbol;Acc:HGNC:29024]	ANKRD28
40	ENSOCUT00000004934	ENSG00000156502	YES					0.930061083	0.024445079	-0.104602624	-	17539978	17568564	15	4206	ENSOCUG00000004933	SUV3-like helicase [Source:HGNC Symbol;Acc:HGNC:11471]	SUPV3L1
41	ENSOCUT00000003856	ENSG00000070814	YES					1.212858538	0.047980362	0.278411291	+	32115536	32150424	28	6245	ENSOCUG00000003848	Treacher Collins-Franceschetti syndrome 1 [Source:HGNC Symbol;Acc:H	TCOF1
42	ENSOCUT00000024418	ENSG00000101665	YES					1.633338546	0.002215582	0.707823853	-	89924494	89950849	4	3534	ENSOCUG00000024309	SMAD family member 7 [Source:HGNC Symbol;Acc:HGNC:6773]	SMAD7
43	ENSOCUT00000014123	ENSG00000119231	YES					0.690724499	0.048373815	-0.5338177	-	91888326	91929462	10	3238	ENSOCUG00000014124	SUMO1/sentrin specific peptidase 5 [Source:HGNC Symbol;Acc:HGNC:2	SENP5
44	ENSOCUT00000015263	ENSG00000105176	YES					1.456968656	0.008231044	0.54296984	+	730203	826058	11	3639	ENSOCUG00000015204	URI1, prefoldin-like chaperone [Source:HGNC Symbol;Acc:HGNC:13236]	URI1
45	ENSOCUT00000017149	ENSG00000169045	YES					1.487709194	0.022723742	0.573092547	-	1097171	1104740	13	1407	ENSOCUG00000017150	heterogeneous nuclear ribonucleoprotein H1 (H) [Source:HGNC Symbol;/	HNRNPH1
46	ENSOCUT00000034139	ENSG00000188529	YES					10.71690331	0.010132538	3.421816189	+	129365575	129366425	3	515	ENSOCUG00000029326	serine/arginine-rich splicing factor 10 [Source:HGNC Symbol;Acc:HGNC:	0
47	ENSOCUT00000010417	ENSG00000164105	YES					0.351549812	0.01569712	-1.508198972	+	51238221	51244206	4	2247	ENSOCUG00000010418	Sin3A-associated protein, 30kDa [Source:HGNC Symbol;Acc:HGNC:105	SAP30
48	ENSOCUT00000027951	ENSG00000178896	YES					0.125465243	0.026038242	-2.994640332	-	10554	11771	3	738	ENSOCUG00000021243	exosome component 4 [Source:HGNC Symbol;Acc:HGNC:18189]	EXOSC4
49	ENSOCUT00000003879	ENSG00000060069	YES					1.204146629	0.013843083	0.26801108	-	66143	102352	12	3665	ENSOCUG00000003879	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosph	CTDP1
50	ENSOCUT00000000407	ENSG00000152256	YES					0.279642846	0.013858212	-1.838342669	+	112251933	112291030	10	4275	ENSOCUG00000000408	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1SD87]	0
51	ENSOCUT00000016821	ENSG00000139083	YES					0.673286458	0.032107124	-0.570707648	-	27833373	28095975	8	1356	ENSOCUG00000016827	ets variant 6 [Source:HGNC Symbol;Acc:HGNC:3495]	ETV6
52	ENSOCUT00000008735	ENSG00000198042	YES					1.106280007	0.034845197	0.145716588	-	74283410	74293517	10	1005	ENSOCUG00000008735	MAK16 homolog [Source:HGNC Symbol;Acc:HGNC:13703]	MAK16
53	ENSOCUT00000024812	ENSG00000101365	YES					1.11688346	0.045702179	0.159478658	-	9687260	9692746	12	2082	ENSOCUG00000024051	isocitrate dehydrogenase 3 (NAD+) beta [Source:HGNC Symbol;Acc:HGN	IDH3B
54	ENSOCUT00000007134	ENSG00000130713	YES					0.922870689	0.010810012	-0.115739581	+	807	9417	9	882	ENSOCUG00000007138	exosome component 2 [Source:HGNC Symbol;Acc:HGNC:17097]	EXOSC2
55	ENSOCUT00000010637	ENSG00000158019			YES	YES		1.251204749	0.040783077	0.323317894	-	157715199	158205131	12	4382	ENSOCUG00000010695	brain and reproductive organ-expressed (TNFRSF1A modulator) [Source:	BRE
56	ENSOCUT00000012453	ENSG00000172071			YES	YES	YES	1.504195757	0.001836194	0.588992333	+	99078398	99118142	15	3856	ENSOCUG00000012451	eukaryotic translation initiation factor 2-alpha kinase 3 [Source:HGNC Sy	EIF2AK3
57	ENSOCUT00000009875	ENSG00000101966			YES	YES		0.408321751	0.023859876	-1.292221674	+	98384983	98406022	6	1494	ENSOCUG00000009878	E3 ubiquitin-protein ligase XIAP [Source:RefSeq peptide;Acc:NP_001164	XIAP
58	ENSOCUT00000003039	ENSG00000065613			YES	YES		5.144287384	0.013003841	2.362371241	+	51856128	51940694	19	3726	ENSOCUG00000003035	STE20-like kinase [Source:HGNC Symbol;Acc:HGNC:11088]	SLK
59	ENSOCUT00000011792	ENSG00000137275			YES	YES		1.359879081	0.031130748	0.443478374	-	2549274	2583836	10	2013	ENSOCUG00000011791	receptor (TNFRSF)-interacting serine-threonine kinase 1 [Source:HGNC S	RIPK1
60	ENSOCUT00000014532	ENSG00000102699			YES	YES		1.163628877	0.047262305	0.218631005	-	44952602	45044352	34	6201	ENSOCUG00000014529	poly (ADP-ribose) polymerase family, member 4 [Source:HGNC Symbol;Ac	PARP4

#	ENSOCUT ID	ENSG ID				Value 1	Value 2	Value 3	+/-	Start	End	n	Len	ENSOCUG ID	Description	Symbol
61	ENSOCUT00000024642	ENSG00000087088	YES	YES	YES	2.027111268	0.03360173	1.01942528	−	90719	93528	5	579	ENSOCUG00000006423	BCL2-associated X protein [Source:HGNC Symbol;Acc:HGNC:959]	BAX
62	ENSOCUT00000032048	ENSG00000119630		YES		1.274447604	0.032233476	0.349872061	+	30172447	30180160	6	489	ENSOCUG00000026354	placental growth factor [Source:HGNC Symbol;Acc:HGNC:8893]	PGF
63	ENSOCUT00000015230	ENSG00000145649		YES		0.304886331	0.021785852	-1.713656624	+	71073774	71082586	5	792	ENSOCUG00000015236	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1T902]	0
64	ENSOCUT00000031433	ENSG00000075790		YES	YES	0.859000883	0.020262515	-0.219268481	−	126566	127291	1	726	ENSOCUG00000028156	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1TPZ4]	0
65	ENSOCUT00000008309	ENSG00000100234		YES		2.512241051	0.003666106	1.328974698	−	88307960	88362257	5	5335	ENSOCUG00000008310	metalloproteinase inhibitor 3 precursor [Source:RefSeq peptide;Acc:NP_...	TIMP3
66	ENSOCUT00000007940	ENSG00000184254		YES		1.125646297	0.010062358	0.170753572	−	21	32016	12	6992	ENSOCUG00000007938	aldehyde dehydrogenase 1 family, member A3 [Source:HGNC Symbol;Ac...	ALDH1A3
67	ENSOCUT00000001542	ENSG00000104312		YES		0.662776092	0.001105467	-0.593406534	+	105172432	105208358	11	4818	ENSOCUG00000001542	receptor-interacting serine-threonine kinase 2 [Source:HGNC Symbol;Ac...	RIPK2
68	ENSOCUT00000006956	ENSG00000184584		YES	YES	1.436087749	0.039155328	0.522143905	−	21717532	21721600	6	1143	ENSOCUG00000006958	transmembrane protein 173 [Source:HGNC Symbol;Acc:HGNC:27962]	TMEM173
69	ENSOCUT00000000040	ENSG00000113734		YES	YES	0.111164894	0.025577372	-3.169226838	+	54179923	54197136	6	687	ENSOCUG00000000040	BCL2/adenovirus E1B 19kDa interacting protein 1 [Source:HGNC Symbol;A...	BNIP1
70	ENSOCUT00000000172	ENSG00000134215		YES		2.201697586	0.006062542	1.138616321	+	56144498	56550635	27	6134	ENSOCUG00000000172	vav 3 guanine nucleotide exchange factor [Source:HGNC Symbol;Acc:H...	VAV3
71	ENSOCUT00000006278	ENSG00000140299		YES	YES	1.237276578	0.034451892	0.307168034	+	12711305	12731422	11	1285	ENSOCUG00000006277	BCL2/adenovirus E1B 19kDa interacting protein 2 [Source:HGNC Symbol;...	BNIP2
72	ENSOCUT00000004623	ENSG00000138613		YES		1.342191638	0.020785756	0.424590674	−	8941946	8981823	6	774	ENSOCUG00000004627	APH1B gamma secretase subunit [Source:HGNC Symbol;Acc:HGNC:240...	APH1B
73	ENSOCUT00000015411	ENSG00000114209		YES		1.076805208	0.008326844	0.106757293	−	62397127	62443385	8	823	ENSOCUG00000015413	programmed cell death 10 [Source:HGNC Symbol;Acc:HGNC:8761]	PDCD10
74	ENSOCUT00000014132	ENSG00000028137		YES		0.418770231	0.048761698	-1.255769206	−	1901647	1932164	9	2458	ENSOCUG00000014127	tumor necrosis factor receptor superfamily, member 1B [Source:HGNC Syr...	TNFRSF1B
75	ENSOCUT00000012027	ENSG00000137193		YES		1.318433588	0.019968308	0.398831467	+	27027310	27031509	6	1514	ENSOCUG00000012028	Pim-1 proto-oncogene, serine/threonine kinase [Source:HGNC Symbol;A...	PIM1
76	ENSOCUT00000015956	ENSG00000113575		YES		1.065924802	0.007485036	0.092105664	−	17013509	17040696	7	1424	ENSOCUG00000015961	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform...	PPP2CA
77	ENSOCUT00000017921	ENSG00000185825		YES	YES	0.765459382	0.043508814	-0.38560227	+	694531	727417	8	831	ENSOCUG00000017921	B-cell receptor-associated protein 31 [Source:HGNC Symbol;Acc:HGNC:...	BCAP31
78	ENSOCUT00000002187	ENSG00000196230		YES		0.77320004	0.038956903	-0.371086382	+	22748030	22752315	4	2641	ENSOCUG00000002187	tubulin, beta class I [Source:HGNC Symbol;Acc:HGNC:20778]	TUBB
79	ENSOCUT00000006052	ENSG00000181938	YES			0.776087549	0.022884741	-0.365708686	+	14294137	14304532	3	1862	ENSOCUG00000006054	GINS complex subunit 3 (Psf3 homolog) [Source:HGNC Symbol;Acc:HGN...	GINS3
80	ENSOCUT00000025334	ENSG00000198924	YES			1.06243205	0.009148041	0.087370573	−	61409924	61437082	9	4172	ENSOCUG00000004321	DNA cross-link repair 1A [Source:HGNC Symbol;Acc:HGNC:17660]	DCLRE1A
81	ENSOCUT00000017632	ENSG00000174943	YES			0.773371366	0.006671638	-0.370766745	−	18314268	18329818	6	990	ENSOCUG00000017636	potassium channel tetramerization domain containing 13 [Source:HGNC S...	KCTD13
82	ENSOCUT00000000013	ENSG00000070950	YES			0.5860789	0.026107386	-0.770833196	+	42530	146086	13	1597	ENSOCUG00000000013	RAD18 E3 ubiquitin protein ligase [Source:HGNC Symbol;Acc:HGNC:1827...	RAD18
83	ENSOCUT00000031279					0.054120272	0.001699629	-4.207687089	−	119951100	119951563	1	464	ENSOCUG00000027293		0
84	ENSOCUT00000015392	ENSG00000015285			YES	1.089654141	0.002391647	0.123870293	+	33343571	33362270	13	2596	ENSOCUG00000015393	Wiskott-Aldrich syndrome [Source:HGNC Symbol;Acc:HGNC:12731]	WAS
85	ENSOCUT00000019646	ENSG00000277846				0.832794559	0.002532464	-0.263967453	+	1509980	1510048	1	69	ENSOCUG00000019646	Small nucleolar RNA SNORD30 [Source:RFAM;Acc:RF00088]	SNORD30
86	ENSOCUT00000017551					0.98037903	0.004855068	-0.028588469	+	71896473	71896981	1	509	ENSOCUG00000017552		0
87	ENSOCUT00000006965	ENSG00000136010				0.643275676	0.013769814	-0.636490958	+	91263358	91325818	23	6154	ENSOCUG00000006962	aldehyde dehydrogenase 1 family, member L2 [Source:HGNC Symbol;Acc...	ALDH1L2
88	AIHV1gp18.1					1.035905543	0.01510364	0.05089246	+	38345	40030	1	1686	AIHV1gp18	#N/A	0
89	ENSOCUT00000019581					0.942382699	0.021954861	-0.085615042	+	1509745	1509809	1	65	ENSOCUG00000019581	Small nucleolar RNA SNORD29 [Source:RFAM;Acc:RF00070]	SNORD29
90	ENSOCUT00000003374	ENSG00000107669				0.491244869	0.021964763	-1.025485757	−	68671085	68850046	13	5208	ENSOCUG00000003373	arginyltransferase 1 [Source:HGNC Symbol;Acc:HGNC:782]	ATE1
91	ENSOCUT00000008192	ENSG00000071054				0.498702868	0.02780959	-1.003747594	−	90545349	90731059	32	7249	ENSOCUG00000008180	mitogen-activated protein kinase kinase kinase kinase 4 [Source:HGNC S...	MAP4K4
92	ENSOCUT00000031018					0.009074761	0.030517064	-6.783324567	+	23171050	23171457	1	408	ENSOCUG00000027607	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1U116]	0
93	ENSOCUT00000028495					0.121375049	0.017050447	-3.042456211	−	242	336	1	95	ENSOCUG00000025865		0
94	ENSOCUT00000022824	ENSG00000240891				2.450640084	0.021877649	1.293158617	−	107473747	107533072	4	1630	ENSOCUG00000027805	phosphatidylinositol-specific phospholipase C, X domain containing 2 [So...	PLCXD2
95	ENSOCUT00000033691					1.484330878	0.015547678	0.569812725	−	249501	252516	4	1192	ENSOCUG00000029179	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:U3KNC9]	0
96	ENSOCUT00000025140					0.993587712	0.010742514	-0.009280763	−	22803292	22804049	1	758	ENSOCUG00000029563		0
97	AIHV1gp08.1					1.013526685	0.020804324	0.019384074	+	14725	16767	1	2043	AIHV1gp08	#N/A	0
98	ENSOCUT00000013565	ENSG00000173114				1.024956285	0.01126394	0.035562379	+	47981727	47983853	1	2127	ENSOCUG00000013571	leucine rich repeat neuronal 3 [Source:HGNC Symbol;Acc:HGNC:17200]	LRRN3
99	ENSOCUT00000023234	ENSG00000175857				1.022788321	0.008608298	0.032507593	+	74425245	74425718	1	474	ENSOCUG00000021351	GRB2-binding adaptor protein, transmembrane [Source:HGNC Symbol;A...	GAPT
100	ENSOCUT00000033736					1.03951913	0.041509753	0.055916307	+	143548	144798	1	1251	ENSOCUG00000029739	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:U3KNH4]	0
101	ENSOCUT00000028806	ENSG00000211535				1.027606111	0.002479401	0.039287375	−	16278486	16278558	1	73	ENSOCUG00000026051		0
102	ENSOCUT00000033317	ENSG00000112081				1.036278329	0.034092733	0.051411541	+	26545708	26553842	5	4519	ENSOCUG00000006684	serine/arginine-rich splicing factor 3 [Source:HGNC Symbol;Acc:HGNC:1...	SRSF3
103	ENSOCUT00000002404					1.034876887	0.012287232	0.049459149	+	14889862	14890171	1	310	ENSOCUG00000002404		0
104	ENSOCUT00000008995	ENSG00000182324				1.037876849	0.010078283	0.053635268	+	96090	98925	2	1302	ENSOCUG00000008998	potassium channel, inwardly rectifying subfamily J, member 14 [Source:HG...	KCNJ14
105	ENSOCUT00000023471	ENSG00000178878				1.038444187	0.010996465	0.054423678	−	26945542	26946288	1	747	ENSOCUG00000025481	apolipoprotein L domain containing 1 [Source:HGNC Symbol;Acc:HGNC:2...	APOLD1
106	ENSOCUT00000029987					1.03552974	0.000823618	0.050368988	+	10738755	110739432	1	678	ENSOCUG00000026237		0
107	ENSOCUT00000021607					1.034461539	0.009711978	0.048880008	+	6776859	6777764	1	906	ENSOCUG00000022196	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1TZU1]	0
108	ENSOCUT00000013264					0.964560403	0.022262767	-0.052056509	+	15896219	15897556	1	1338	ENSOCUG00000013266		0
109	ENSOCUT00000010785					1.035111862	0.035556711	0.049786713	−	1828855	1829584	1	730	ENSOCUG00000010788		0
110	ENSOCUT00000025731	ENSG00000201129				1.516043944	0.029816454	0.600311572	−	38289165	38289301	1	137	ENSOCUG00000024097	Small nucleolar RNA SNORA58 [Source:RFAM;Acc:RF00418]	SNORA58
111	ENSOCUT00000005887	ENSG00000121892				1.83913245	0.048663098	0.879025383	−	29073825	29206002	32	7044	ENSOCUG00000005880	PDS5 cohesin associated factor A [Source:HGNC Symbol;Acc:HGNC:29...	PDS5A
112	ENSOCUT00000033492	ENSG00000178338				1.174290484	0.01718789	0.231789332	−	464145	478206	3	5524	ENSOCUG00000029238	zinc finger protein 354B [Source:HGNC Symbol;Acc:HGNC:17197]	ZNF354B
113	ENSOCUT00000009889	ENSG00000101972				1.287528617	0.021314026	0.364604499	+	98528365	98628576	33	6281	ENSOCUG00000009879	cohesin subunit SA-2 [Source:RefSeq peptide;Acc:NP_001164797]	STAG2
114	ENSOCUT00000001597	ENSG00000125848				1.139067977	0.040041663	0.187853846	+	21887023	21888972	1	1950	ENSOCUG00000001599	fibronectin leucine rich transmembrane protein 3 [Source:HGNC Symbol;A...	FLRT3
115	ENSOCUT00000013823	ENSG00000180332				1.071056557	0.01540475	0.099034664	+	49910756	49911535	1	780	ENSOCUG00000013829	potassium channel tetramerization domain containing 4 [Source:HGNC S...	KCTD4
116	ENSOCUT00000026344	ENSG00000207789				1.074310545	0.015145841	0.103411086	−	918065	918148	1	84	ENSOCUG00000024507		0
117	ENSOCUT00000028659	ENSG00000180902				1.092672345	0.034080074	0.127860852	+	146845	171334	10	1836	ENSOCUG00000022982	D-2-hydroxyglutarate dehydrogenase [Source:HGNC Symbol;Acc:HGNC...	D2HGDH
118	ENSOCUT00000032544					1.106407644	0.045638356	0.145883029	+	13904701	13904739	1	99	ENSOCUG00000028549	U6 spliceosomal RNA [Source:RFAM;Acc:RF00026]	U6
119	ENSOCUT00000019053	ENSG00000206602				1.421494185	0.043512626	0.507408197	−	90428023	90428090	1	68	ENSOCUG00000019053	Small nucleolar RNA SNORD58 [Source:RFAM;Acc:RF00151]	SNORD58
120	ENSOCUT00000030273					1.040685963	0.01746062	0.057534788	+	50798372	50798851	1	480	ENSOCUG00000027750	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G1TNG5]	0

8.7 Rabbit DAVID analysis 1

Enrichment Score: 2.160388230158114

Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
ENSG00000159792, ENSG00000105176, ENSG00000101665, ENSG00000198042, ENSG00000112592, ENSG00000186575, ENSG00000105486, ENSG00000175305, ENSG00000206560, ENSG00...	GO:0031981~nuclear lumen	38	13.1944	0.001486427	1.683299255	0.327781027
ENSG00000159792, ENSG00000105176, ENSG00000162433, ENSG00000101665, ENSG00000198042, ENSG00000125901, ENSG00000172115, ENSG00000112592, ENSG00000186575, ENSG0000	GO:0070013~intracellular organelle lumen	44	15.2778	0.001714856	1.588628923	0.20477471
ENSG00000159792, ENSG00000105176, ENSG00000162433, ENSG00000101665, ENSG00000198042, ENSG00000125901, ENSG00000172115, ENSG00000112592, ENSG00000186575, ENSG0000	GO:0043233~organelle lumen	44	15.2778	0.002662996	1.552841129	0.211263371
ENSG00000159792, ENSG00000105176, ENSG00000162433, ENSG00000101665, ENSG00000198042, ENSG00000125901, ENSG00000172115, ENSG00000112592, ENSG00000186575, ENSG0000	GO:0031974~membrane-enclosed lumen	44	15.2778	0.003843962	1.522721365	0.226692771
ENSG00000159792, ENSG00000105176, ENSG00000060069, ENSG00000020426, ENSG00000101665, ENSG00000169045, ENSG00000112592, ENSG00000105486, ENSG00000108175, ENSG0000	GO:0005654~nucleoplasm	21	7.2917	0.055346149	1.529313233	0.637043939
ENSG00000151332, ENSG00000177463, ENSG00000178896, ENSG00000169045, ENSG00000198042, ENSG00000070814, ENSG00000154920, ENSG00000140694, ENSG00000186575, ENSG0000	GO:0005730~nucleolus	17	5.9028	0.075520078	1.564369124	0.688006168

Enrichment Score: 1.8962251240059185

Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
ENSG00000099194, ENSG00000111669, ENSG00000151093, ENSG00000100294, ENSG00000134824, ENSG00000110958	Fatty acid biosynthesis	6	2.0833	3.44E-04	9.795450687	0.025879519
ENSG00000099194, ENSG00000172292, ENSG00000111669, ENSG00000151093, ENSG00000100294, ENSG00000001630, ENSG00000134824, ENSG00000110958	lipid synthesis	8	2.7778	4.95E-04	5.730671831	0.024847861
ENSG00000099194, ENSG00000111669, ENSG00000151093, ENSG00000100294, ENSG00000134824, ENSG00000110958	GO:0006633~fatty acid biosynthetic process	6	2.0833	0.007210794	4.93962999	0.471999462
ENSG00000138381, ENSG00000099194, ENSG00000111669, ENSG00000151093, ENSG00000100294, ENSG00000134824, ENSG00000110958	GO:0016053~organic acid biosynthetic process	7	2.4306	0.031954613	2.937220844	0.750823103
ENSG00000138381, ENSG00000099194, ENSG00000111669, ENSG00000151093, ENSG00000100294, ENSG00000134824, ENSG00000110958	GO:0046394~carboxylic acid biosynthetic process	7	2.4306	0.031954613	2.937220844	0.750823103
ENSG00000099194, ENSG00000111669, ENSG00000151093, ENSG00000100294, ENSG00000134824, ENSG00000110958	GO:0006631~fatty acid metabolic process	6	2.0833	0.187788658	1.970862471	0.953077378
ENSG00000099194, ENSG00000172292, ENSG00000111669, ENSG00000151093, ENSG00000100294, ENSG00000001630, ENSG00000134824, ENSG00000110958	GO:0008610~lipid biosynthetic process	8	2.7778	0.226410393	1.610859729	0.956055431

Enrichment Score: 1.8771899367092622

Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
ENSG00000020426, ENSG00000181938, ENSG00000154920, ENSG00000172115, ENSG00000158019, ENSG00000087088, ENSG00000105486, ENSG00000175305, ENSG00000133119, ENSG0000	GO:0006259~DNA metabolic process	19	6.5972	7.80E-04	2.44215567	0.66731155
ENSG00000020426, ENSG00000101966, ENSG00000154920, ENSG00000125901, ENSG00000158019, ENSG00000087088, ENSG00000105486, ENSG00000133119, ENSG00000079246, ENSG0000	GO:0006974~response to DNA damage stimulus	15	5.2083	0.001820508	2.615487729	0.402248446
ENSG00000133119, ENSG00000079246, ENSG00000123562, ENSG00000065613, ENSG00000020426, ENSG00000144554, ENSG00000154920, ENSG00000070950, ENSG00000198924, ENSG0000	GO:0006281~DNA repair	12	4.1667	0.0043765	2.748104009	0.430511344
ENSG00000151332, ENSG00000020426, ENSG00000101966, ENSG00000154920, ENSG00000125901, ENSG00000158019, ENSG00000087088, ENSG00000105486, ENSG00000133119, ENSG0000	GO:0033554~cellular response to stress	18	6.2500	0.006218337	2.068360968	0.466940283
ENSG00000079246, ENSG00000123562, ENSG00000144554, ENSG00000154920, ENSG00000070950, ENSG00000198924, ENSG00000158019, ENSG00000105486	dna repair	8	2.7778	0.019538324	2.940344709	0.271484256
ENSG00000079246, ENSG00000123562, ENSG00000144554, ENSG00000154920, ENSG00000070950, ENSG00000198924, ENSG00000158019, ENSG00000105486	DNA damage	8	2.7778	0.027419441	2.73954068	0.319850019
ENSG00000079246, ENSG00000154920, ENSG00000105486	dna recombination	3	1.0417	0.096706767	5.691951075	0.598433049
ENSG00000079246, ENSG00000154920, ENSG00000105486	GO:0006310~DNA recombination	3	1.0417	0.479476128	1.858241758	0.991784059

Enrichment Score: 1.8365367441713873

Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
ENSG00000162433, ENSG00000204316, ENSG00000151093, ENSG00000184584, ENSG00000196449, ENSG00000205220, ENSG00000125901, ENSG00000172115, ENSG00000100294, ENSG0000	mitochondrion	25	8.6806	8.23E-04	2.109396333	0.030889374
ENSG00000204316, ENSG00000162433, ENSG00000151093, ENSG00000184584, ENSG00000196449, ENSG00000172115, ENSG00000125901, ENSG00000100294, ENSG00000100889, ENSG0000	GO:0005739~mitochondrion	28	9.7222	0.009634107	1.654528392	0.308750153
ENSG00000101365, ENSG00000204316, ENSG00000151093, ENSG00000152256, ENSG00000196449, ENSG00000125901, ENSG00000100294, ENSG00000125246, ENSG00000156502, ENSG0000	transit peptide:Mitochondrion	12	4.1667	0.072508119	1.792431892	0.998614995
ENSG00000101365, ENSG00000204316, ENSG00000151093, ENSG00000152256, ENSG00000196449, ENSG00000125901, ENSG00000100294, ENSG00000125246, ENSG00000156502, ENSG0000	transit peptide	12	4.1667	0.078422171	1.769766301	0.529900417

Enrichment Score: 1.8234411390579053

Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
ENSG00000137193, ENSG00000101365, ENSG00000113575, ENSG00000196236, ENSG00000170340, ENSG00000154237, ENSG00000186298, ENSG00000100889	manganese	8	2.7778	0.0060746	3.694775259	0.14347086 6
ENSG00000137193, ENSG00000101365, ENSG00000113575, ENSG00000196236, ENSG00000170340, ENSG00000154237, ENSG00000186298, ENSG00000100889	GO:0030145~manganese ion binding	8	2.7778	0.006934887	3.587455098	0.623405421
ENSG00000113575, ENSG00000186298, ENSG00000100889	metal ion-binding site:Manganese	3	1.0417	0.080374717	6.341406768	0.997508129

Enrichment Score: 1.8196504433657357

Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
ENSG00000184584, ENSG00000101966, ENSG00000075790, ENSG00000185825, ENSG00000172115, ENSG00000113734, ENSG00000158019, ENSG00000087088, ENSG00000065613, ENSG0000	Apoptosis	16	5.5556	3.81E-04	2.948062149	0.022988329
ENSG00000184584, ENSG00000101966, ENSG00000075790, ENSG00000185825, ENSG00000172115, ENSG00000134215, ENSG00000113734, ENSG00000158019, ENSG00000087088, ENSG0000	GO:0012501~programmed cell death	21	7.2917	0.001122499	2.235364472	0.547482611
ENSG00000184584, ENSG00000101966, ENSG00000075790, ENSG00000185825, ENSG00000172115, ENSG00000134215, ENSG00000152592, ENSG00000113734, ENSG00000158019, ENSG0000	GO:0008219~cell death	23	7.9861	0.001521007	2.080507115	0.511507997
ENSG00000184584, ENSG00000101966, ENSG00000075790, ENSG00000185825, ENSG00000172115, ENSG00000134215, ENSG00000152592, ENSG00000113734, ENSG00000158019, ENSG0000	GO:0016265~death	23	7.9861	0.001659925	2.066138972	0.443695976
ENSG00000184584, ENSG00000101966, ENSG00000075790, ENSG00000185825, ENSG00000172115, ENSG00000134215, ENSG00000113734, ENSG00000158019, ENSG00000087088, ENSG0000	GO:0006915~apoptosis	20	6.9444	0.002256924	2.160746231	0.366039366
ENSG00000100234, ENSG00000020426, ENSG00000101966, ENSG00000113575, ENSG00000172115, ENSG00000134215, ENSG00000113734, ENSG00000087088, ENSG00000079246, ENSG0000	GO:0042981~regulation of apoptosis	20	6.9444	0.039475165	1.617872178	0.776098843
ENSG00000100234, ENSG00000020426, ENSG00000101966, ENSG00000113575, ENSG00000172115, ENSG00000134215, ENSG00000113734, ENSG00000087088, ENSG00000079246, ENSG0000	GO:0043067~regulation of programmed cell death	20	6.9444	0.042909032	1.60193255	0.779175421
ENSG00000100234, ENSG00000020426, ENSG00000101966, ENSG00000113575, ENSG00000172115, ENSG00000134215, ENSG00000113734, ENSG00000087088, ENSG00000079246, ENSG0000	GO:0010941~regulation of cell death	20	6.9444	0.044251302	1.596035866	0.773774029
ENSG00000079246, ENSG00000137193, ENSG00000020426, ENSG00000101966, ENSG00000090316, ENSG00000104312, ENSG00000187325, ENSG00000113734, ENSG00000140299, ENSG0000	GO:0043066~negative regulation of apoptosis	11	3.8194	0.045932848	2.020969144	0.771317656
ENSG00000079246, ENSG00000137193, ENSG00000020426, ENSG00000101966, ENSG00000090316, ENSG00000104312, ENSG00000187325, ENSG00000113734, ENSG00000140299, ENSG0000	GO:0043069~negative regulation of programmed cell death	11	3.8194	0.049368123	1.992821941	0.781507992
ENSG00000079246, ENSG00000137193, ENSG00000020426, ENSG00000101966, ENSG00000090316, ENSG00000104312, ENSG00000187325, ENSG00000113734, ENSG00000140299, ENSG0000	GO:0060548~negative regulation of cell death	11	3.8194	0.050667131	1.987286325	0.769697344
ENSG00000101966, ENSG00000104312, ENSG00000113734, ENSG00000140299, ENSG00000176171, ENSG00000087088	GO:0006916~anti-apoptosis	6	2.0833	0.209216678	1.894324122	0.957428779
ENSG00000119630, ENSG00000079246, ENSG00000137193, ENSG00000020426, ENSG00000177463, ENSG00000104312, ENSG00000186575, ENSG00000087088	GO:0008283~cell proliferation	8	2.7778	0.503263476	1.193366267	0.993019773

#	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
60		Enrichment Score: 1.6608436214037252					
61	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
62	ENSG00000111885, ENSG00000099194, ENSG00000128699, ENSG00000159792, ENSG00000133056, ENSG00000145354, ENSG00000105823, ENSG00000198856, ENSG00000184584, ENSG000	GO:0005783~endoplasmic reticulum	26	9.0278	0.00719027	1.739593802	0.319785734
63	ENSG00000128699, ENSG00000101558, ENSG00000172071, ENSG00000198856, ENSG00000175826, ENSG00000185825, ENSG00000076258, ENSG00000113734, ENSG00000001630, ENSG000	GO:0005789~endoplasmic reticulum membrane	11	3.8194	0.009215732	2.626552839	0.337676699
64	ENSG00000128699, ENSG00000101558, ENSG00000172071, ENSG00000198856, ENSG00000175826, ENSG00000185825, ENSG00000076258, ENSG00000113734, ENSG00000001630, ENSG000	GO:0042175~nuclear envelope-endoplasmic reticulum network	11	3.8194	0.013119271	2.487826456	0.356448498
65	ENSG00000111885, ENSG00000128699, ENSG00000099194, ENSG00000105823, ENSG00000198856, ENSG00000175826, ENSG00000015285, ENSG00000185825, ENSG00000076258, ENSG000	GO:0012505~endomembrane system	20	6.9444	0.034355499	1.642740557	0.571970423
66	ENSG00000128699, ENSG00000101558, ENSG00000172071, ENSG00000198856, ENSG00000175826, ENSG00000185825, ENSG00000076258, ENSG00000113734, ENSG00000001630, ENSG000	GO:0044432~endoplasmic reticulum part	11	3.8194	0.043695379	2.036146149	0.600536669
67	ENSG00000111885, ENSG00000128699, ENSG00000099194, ENSG00000105823, ENSG00000198856, ENSG00000175826, ENSG00000184584, ENSG00000015285, ENSG00000185825, ENSG000	GO:0031090~organelle membrane	24	8.3333	0.083046583	1.406521659	0.685705995
68							
69		Enrichment Score: 1.6393495529309094					
70	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
71	ENSG00000121892, ENSG00000139350, ENSG00000020426, ENSG00000100532, ENSG00000156876, ENSG00000101972, ENSG00000105486, ENSG00000119231, ENSG00000186298, ENSG000	cell cycle	18	6.2500	3.36E-04	2.741026331	0.033613244
72	ENSG00000121892, ENSG00000150093, ENSG00000139350, ENSG00000020426, ENSG00000100532, ENSG00000114391, ENSG00000205220, ENSG00000156876, ENSG00000101972, ENSG000	GO:0007049~cell cycle	23	7.9861	0.003874097	1.92768636	0.456096337
73	ENSG00000121892, ENSG00000139350, ENSG00000138160, ENSG00000090316, ENSG00000198924, ENSG00000101972, ENSG00000105486, ENSG00000119231, ENSG00000186298, ENSG000	cell division	11	3.8194	0.004612683	2.925030414	0.120316432
74	ENSG00000121892, ENSG00000139350, ENSG00000177463, ENSG00000138160, ENSG00000090316, ENSG00000198924, ENSG00000101972, ENSG00000105486, ENSG00000119231, ENSG000	GO:0051301~cell division	12	4.1667	0.005789834	2.645632334	0.46777697
75	ENSG00000121892, ENSG00000139350, ENSG00000150093, ENSG00000020426, ENSG00000114391, ENSG00000205220, ENSG00000115233, ENSG00000137193, ENSG000	GO:0000278~mitotic cell cycle	13	4.5139	0.011568436	2.285135135	0.542680861
76	ENSG00000121892, ENSG00000139350, ENSG00000150093, ENSG00000020426, ENSG00000100532, ENSG00000114391, ENSG00000205220, ENSG00000156876, ENSG00000101972, ENSG000	GO:0022402~cell cycle process	16	5.5556	0.027121756	1.841797141	0.762587636
77	ENSG00000121892, ENSG00000139350, ENSG00000150093, ENSG00000137193, ENSG00000020426, ENSG00000144554, ENSG00000196230, ENSG00000114391, ENSG00000138160, ENSG000	GO:0022403~cell cycle phase	12	4.1667	0.052983019	1.885172798	0.771954016
78	ENSG00000121892, ENSG00000139350, ENSG00000196230, ENSG00000114391, ENSG00000138160, ENSG00000198924, ENSG00000101972, ENSG00000101447	GO:0000087~M phase of mitotic cell cycle	8	2.7778	0.056943224	2.322802198	0.771971136
79	ENSG00000121892, ENSG00000139350, ENSG00000196230, ENSG00000138160, ENSG00000198924, ENSG00000101972, ENSG00000087088, ENSG00000101447	GO:0048285~organelle fission	8	2.7778	0.062481589	2.272085993	0.786491585
80	ENSG00000121892, ENSG00000139350, ENSG00000138160, ENSG00000198924, ENSG00000101972, ENSG00000101447	mitosis	6	2.0833	0.119787823	2.301663276	0.631324027
81	ENSG00000121892, ENSG00000139350, ENSG00000196230, ENSG00000138160, ENSG00000198924, ENSG00000101972, ENSG00000101447	GO:0000280~nuclear division	7	2.4306	0.122116729	2.069405594	0.913880207
82	ENSG00000121892, ENSG00000139350, ENSG00000196230, ENSG00000138160, ENSG00000198924, ENSG00000101972, ENSG00000101447	GO:0007067~mitosis	7	2.4306	0.122116729	2.069405594	0.913880207
83	ENSG00000121892, ENSG00000139350, ENSG00000144554, ENSG00000196230, ENSG00000114391, ENSG00000138160, ENSG00000198924, ENSG00000101972, ENSG00000101447	GO:0000279~M phase	9	3.1250	0.132751141	1.779316741	0.921581745
84							
85		Enrichment Score: 1.5465483572493635					
86	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
87	ENSG00000145649, ENSG00000172115, ENSG00000176171, ENSG00000087088	GO:0006921~cell structure disassembly during apoptosis	4	1.3889	0.004989581	11.31103679	0.444883335
88	ENSG00000145649, ENSG00000172115, ENSG00000167862, ENSG00000176171, ENSG00000087088	GO:0022411~cellular component disassembly	5	1.7361	0.008687756	6.135703319	0.495649488
89	ENSG00000172115, ENSG00000176171, ENSG00000087088	GO:0006309~DNA fragmentation involved in apoptosis	3	1.0417	0.033586584	10.26923077	0.757994046
90	ENSG00000175826, ENSG00000172115, ENSG00000176171, ENSG00000087088	GO:0006997~nucleus organization	4	1.3889	0.045142418	5.00295858	0.772902966
91	ENSG00000172115, ENSG00000176171, ENSG00000087088	GO:0000737~DNA catabolic process, endonucleolytic	3	1.0417	0.059559976	7.50443787	0.775740772
92	ENSG00000172115, ENSG00000176171, ENSG00000087088	GO:0030262~apoptotic nuclear changes	3	1.0417	0.059559976	7.50443787	0.775740772
93	ENSG00000020426, ENSG00000172115, ENSG00000176171, ENSG00000087088	GO:0006308~DNA catabolic process	4	1.3889	0.064051622	4.335897436	0.789398586
94							
95		Enrichment Score: 1.5403676044959722					
96	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
97	ENSG00000178896, ENSG00000130713, ENSG00000140694	GO:0000175~3'-5'-exoribonuclease activity	3	1.0417	0.012386175	17.26462766	0.649862267
98	ENSG00000178896, ENSG00000130713, ENSG00000140694	GO:0016896~exoribonuclease activity, producing 5'-phosphomono	3	1.0417	0.014500733	15.93657938	0.641173819
99	ENSG00000178896, ENSG00000130713, ENSG00000140694	GO:0004532~exoribonuclease activity	3	1.0417	0.014500733	15.93657938	0.641173819
100	ENSG00000147687, ENSG00000178896, ENSG00000130713, ENSG00000154920, ENSG00000140694, ENSG00000138767	nuclease	6	2.0833	0.017629354	3.973626222	0.273206265
101	ENSG00000178896, ENSG00000130713, ENSG00000164167, ENSG00000140694, ENSG00000138767	hsa03018:RNA degradation	5	1.7361	0.019800024	4.745240761	0.912825018
102	ENSG00000178896, ENSG00000130713, ENSG00000140694, ENSG00000138767	exonuclease	4	1.3889	0.022385295	6.530300458	0.298557981
103	ENSG00000065613, ENSG00000147687, ENSG00000178896, ENSG00000130713, ENSG00000154920, ENSG00000140694, ENSG00000138767	GO:0004518~nuclease activity	7	2.4306	0.026898516	3.059554269	0.761865759
104	ENSG00000178896, ENSG00000130713, ENSG00000140694	GO:0016796~exonuclease activity, active with either ribo- or deoxyrib	3	1.0417	0.03307501	10.3587766	0.75731874
105	ENSG00000178896, ENSG00000130713, ENSG00000140694, ENSG00000138767	GO:0004527~exonuclease activity	4	1.3889	0.040739746	5.211963067	0.767581627
106	ENSG00000178896, ENSG00000130713, ENSG00000140694	GO:0008408~3'-5' exonuclease activity	3	1.0417	0.06108123	7.33912614	0.809552135
107	ENSG00000178896, ENSG00000130713, ENSG00000140694	GO:0004540~ribonuclease activity	3	1.0417	0.245885771	3.139023211	0.959688092
108							
109		Enrichment Score: 1.4872446537026687					
110	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
111	ENSG00000079246, ENSG00000150093, ENSG00000214078, ENSG00000102699, ENSG00000085719	SM00327:VWA	5	1.7361	0.020956463	4.710002075	0.930649017
112	ENSG00000079246, ENSG00000150093, ENSG00000214078, ENSG00000102699, ENSG00000085719	IPR002035:von Willebrand factor, type A	5	1.7361	0.027975905	4.338959212	0.993890957
113	ENSG00000150093, ENSG00000214078, ENSG00000102699, ENSG00000085719	domain:VWFA	4	1.3889	0.058906594	4.50035319	0.998914661

115		Enrichment Score: 1.3857313657555221					
116	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
117	ENSG00000081985, ENSG00000159792, ENSG00000133056, ENSG00000101665, ENSG00000175826, ENSG00000175354, ENSG00000206203, ENSG00000065613, ENSG00000152256, ENSG00	GO:0006793~phosphorus metabolic process	28	9.7222	0.001882304	1.871610404	0.358140752
118	ENSG00000081985, ENSG00000159792, ENSG00000133056, ENSG00000101665, ENSG00000175826, ENSG00000175354, ENSG00000206203, ENSG00000065613, ENSG00000152256, ENSG00	GO:0006796~phosphate metabolic process	28	9.7222	0.001882304	1.871610404	0.358140752
119	ENSG00000117020, ENSG00000159792, ENSG00000020426, ENSG00000103319, ENSG00000206203, ENSG00000065613, ENSG00000172071, ENSG00000137193, ENSG00000104312, ENSG00	GO:0004674~protein serine/threonine kinase activity	15	5.2083	0.00377395	2.409017813	0.796448943
120	ENSG00000159792, ENSG00000162433, ENSG00000137193, ENSG00000133056, ENSG00000152256, ENSG00000115825, ENSG00000137275, ENSG00000156958, ENSG00000067057	phosphotransferase	9	3.1250	0.008152184	3.143316265	0.174731619
121	ENSG00000117020, ENSG00000159792, ENSG00000103319, ENSG00000206203, ENSG00000065613, ENSG00000172071, ENSG00000137193, ENSG00000104312, ENSG00000166484, ENSG000	serine/threonine-protein kinase	13	4.5139	0.008538829	2.395300496	0.170409659
122	ENSG00000117020, ENSG00000159792, ENSG00000162433, ENSG00000133056, ENSG00000103319, ENSG00000198276, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00	kinase	19	6.5972	0.009718526	1.938682949	0.180102375
123	ENSG00000117020, ENSG00000081985, ENSG00000159792, ENSG00000133056, ENSG00000101665, ENSG00000103319, ENSG00000099795, ENSG00000206203, ENSG00000065613, ENSG00	GO:0016310~phosphorylation	22	7.6389	0.011015403	1.788557692	0.542511015
124	ENSG00000159792, ENSG00000133056, ENSG00000162433, ENSG00000112081, ENSG00000105486, ENSG00000067057, ENSG00000198276, ENSG00000100889, ENSG00000206203, ENSG00	GO:0000166~nucleotide binding	46	15.9722	0.011034434	1.415007345	0.688958591
125	ENSG00000206203, ENSG00000117020, ENSG00000159792, ENSG00000172071, ENSG00000065613, ENSG00000137193, ENSG00000104312, ENSG00000166484, ENSG00000115825, ENSG000	IPR008271:Serine/threonine protein kinase, active site	12	4.1667	0.014681945	2.323317137	0.999655093
126	ENSG00000206203, ENSG00000117020, ENSG00000159792, ENSG00000172071, ENSG00000065613, ENSG00000137193, ENSG00000104312, ENSG00000166484, ENSG00000115825, ENSG000	IPR017442:Serine/threonine protein kinase-related	12	4.1667	0.016084591	2.291550604	0.987349231
127	ENSG00000117020, ENSG00000159792, ENSG00000020426, ENSG00000103319, ENSG00000206203, ENSG00000065613, ENSG00000172071, ENSG00000137193, ENSG00000152256, ENSG00	GO:0004672~protein kinase activity	16	5.5556	0.028923303	1.823327014	0.746633856
128	ENSG00000159792, ENSG00000133056, ENSG00000162433, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000100889, ENSG00000206203, ENSG00000065613, ENSG00	nucleotide-binding	34	11.8056	0.036811308	1.415673083	0.379132786
129	ENSG00000117020, ENSG00000159792, ENSG00000105486, ENSG00000206203, ENSG00000065613, ENSG00000172071, ENSG00000137193, ENSG00000152256, ENSG00000104312, ENSG00	binding site:ATP	14	4.8611	0.048313181	1.801801923	0.999941291
130	ENSG00000117020, ENSG00000081985, ENSG00000159792, ENSG00000101665, ENSG00000103319, ENSG00000206203, ENSG00000065613, ENSG00000172071, ENSG00000137193, ENSG00	GO:0006468~protein amino acid phosphorylation	17	5.9028	0.050314389	1.657651943	0.774090827
131	ENSG00000117020, ENSG00000159792, ENSG00000111669, ENSG00000120437, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00000172071, ENSG00000137193, ENSG00	active site:Proton acceptor	16	5.5556	0.051222541	1.69618175	0.99974551
132	ENSG00000159792, ENSG00000133056, ENSG00000162433, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000100889, ENSG00000206203, ENSG00000065613, ENSG00	GO:0017076~purine nucleotide binding	37	12.8472	0.054728972	1.33220276	0.838414756
133	ENSG00000206203, ENSG00000117020, ENSG00000159792, ENSG00000065613, ENSG00000137193, ENSG00000166484, ENSG00000115825, ENSG00000071054	SM00220:S_TKc	8	2.7778	0.057641122	2.298626495	0.976251917
134	ENSG00000117020, ENSG00000159792, ENSG00000162433, ENSG00000103319, ENSG00000198276, ENSG00000067057, ENSG00000160404, ENSG00000206203, ENSG00000065613, ENSG00	nucleotide phosphate-binding region:ATP	21	7.2917	0.058549683	1.522728652	0.999634327
135	ENSG00000159792, ENSG00000162433, ENSG00000133056, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00000138160, ENSG00	atp-binding	27	9.3750	0.058821758	1.429426627	0.495815212
136	ENSG00000117020, ENSG00000159792, ENSG00000133056, ENSG00000162433, ENSG00000151093, ENSG00000103319, ENSG00000107669, ENSG00000169902, ENSG00000100294, ENSG00	transferase	28	9.7222	0.062049597	1.410057703	0.490189207
137	ENSG00000159792, ENSG00000133056, ENSG00000162433, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000100889, ENSG00000206203, ENSG00000065613, ENSG00	GO:0032555~purine ribonucleotide binding	35	12.1528	0.071360068	1.316474876	0.840136544
138	ENSG00000159792, ENSG00000133056, ENSG00000162433, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000100889, ENSG00000206203, ENSG00000065613, ENSG00	GO:0032553~ribonucleotide binding	35	12.1528	0.071360068	1.316474876	0.840136544
139	ENSG00000206203, ENSG00000117020, ENSG00000159792, ENSG00000172071, ENSG00000065613, ENSG00000137193, ENSG00000104312, ENSG00000166484, ENSG00000115825, ENSG000	domain:Protein kinase	12	4.1667	0.074538205	1.784788259	0.997748803
140	ENSG00000206203, ENSG00000117020, ENSG00000159792, ENSG00000065613, ENSG00000137193, ENSG00000166484, ENSG00000115825, ENSG00000071054	IPR002290:Serine/threonine protein kinase	8	2.7778	0.084258258	2.117546118	0.99963192
141	ENSG00000206203, ENSG00000117020, ENSG00000159792, ENSG00000172071, ENSG00000065613, ENSG00000137193, ENSG00000104312, ENSG00000166484, ENSG00000115825, ENSG000	IPR000719:Protein kinase, core	12	4.1667	0.087911849	1.728291317	0.999162789
142	ENSG00000206203, ENSG00000117020, ENSG00000159792, ENSG00000172071, ENSG00000065613, ENSG00000137193, ENSG00000104312, ENSG00000166484, ENSG00000115825, ENSG000	IPR017441:Protein kinase, ATP binding site	11	3.8194	0.127621323	1.657387057	0.999718881
143	ENSG00000159792, ENSG00000162433, ENSG00000133056, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00000138160, ENSG00	GO:0030554~adenyl nucleotide binding	29	10.0694	0.140928241	1.269940906	0.922543867
144	ENSG00000159792, ENSG00000162433, ENSG00000133056, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00000138160, ENSG00	GO:0001883~purine nucleoside binding	29	10.0694	0.159609543	1.250903691	0.926798568
145	ENSG00000159792, ENSG00000162433, ENSG00000133056, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00000138160, ENSG00	GO:0005524~ATP binding	27	9.3750	0.162892627	1.262410147	0.917517393
146	ENSG00000159792, ENSG00000162433, ENSG00000133056, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00000138160, ENSG00	GO:0001882~nucleoside binding	29	10.0694	0.168630494	1.242367747	0.9119375
147	ENSG00000159792, ENSG00000162433, ENSG00000133056, ENSG00000105486, ENSG00000198276, ENSG00000067057, ENSG00000206203, ENSG00000065613, ENSG00000138160, ENSG00	GO:0032553~adenyl ribonucleotide binding	27	9.3750	0.180354761	1.24554428	0.920918275
148	ENSG00000159792, ENSG00000137193, ENSG00000115825	serine/threonine-specific protein kinase	3	1.0417	0.209366335	3.510036496	0.768288114
149	ENSG00000117020, ENSG00000166484, ENSG00000071054	hsa04010:MAPK signaling pathway	3	1.0417	0.960443685	0.607817356	0.999982375
150							
151		Enrichment Score: 1.3309995554112992					
152	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
153	ENSG00000189266, ENSG00000090621, ENSG00000105771, ENSG00000138668, ENSG00000140694	GO:0006401~RNA catabolic process	5	1.7361	0.01744334	5.00295858	0.660512852
154	ENSG00000189266, ENSG00000105771, ENSG00000138668, ENSG00000140694	GO:0006402~mRNA catabolic process	4	1.3889	0.027831376	6.050089445	0.759105757
155	ENSG00000189266, ENSG00000105771, ENSG00000140694	nonsense-mediated mrna decay	3	1.0417	0.059573238	7.521506778	0.487807973
156	ENSG00000189266, ENSG00000105771, ENSG00000140694	GO:0000184~nuclear-transcribed mRNA catabolic process, nonsen:	3	1.0417	0.076645425	6.503846154	0.832577139
157	ENSG00000189266, ENSG00000105771, ENSG00000140694	GO:0000956~nuclear-transcribed mRNA catabolic process	3	1.0417	0.099839057	5.574725275	0.87653316
158							
159		Enrichment Score: 1.1742365547228661					
160	Genes	Term	Count	%	PValue	Fold Enrichment	Benjamini
161	ENSG00000151332, ENSG00000137193, ENSG00000020426, ENSG00000113575, ENSG00000102760, ENSG00000173114, ENSG00000115825, ENSG00000134215, ENSG00000157193, ENSG000	GO:0043549~regulation of kinase activity	12	4.1667	0.021643666	2.186166774	0.709414461
162	ENSG00000151332, ENSG00000020426, ENSG00000101665, ENSG00000113575, ENSG00000134215, ENSG00000186575, ENSG00000087088, ENSG00000175305, ENSG00000157193, ENSG000	GO:0042325~regulation of phosphorylation	14	4.8611	0.02697686	1.953345196	0.773537298
163	ENSG00000151332, ENSG00000137193, ENSG00000020426, ENSG00000113575, ENSG00000102760, ENSG00000173114, ENSG00000115825, ENSG00000134215, ENSG00000157193, ENSG000	GO:0051338~regulation of transferase activity	12	4.1667	0.028264051	2.098014888	0.752412034
164	ENSG00000151332, ENSG00000020426, ENSG00000101665, ENSG00000113575, ENSG00000134215, ENSG00000186575, ENSG00000087088, ENSG00000175305, ENSG00000137193, ENSG000	GO:0019220~regulation of phosphate metabolic process	14	4.8611	0.035775192	1.87733889	0.770010791
165	ENSG00000151332, ENSG00000020426, ENSG00000101665, ENSG00000113575, ENSG00000134215, ENSG00000186575, ENSG00000087088, ENSG00000175305, ENSG00000137193, ENSG000	GO:0051174~regulation of phosphorus metabolic process	14	4.8611	0.035775192	1.87733889	0.770010791
166	ENSG00000151332, ENSG00000137193, ENSG00000020426, ENSG00000113575, ENSG00000102760, ENSG00000173114, ENSG00000115825, ENSG00000157193, ENSG00000186575, ENSG000	GO:0045859~regulation of protein kinase activity	11	3.8194	0.039694851	2.073690078	0.769258928
167	ENSG00000205220, ENSG00000172115, ENSG00000134215, ENSG00000087088, ENSG00000115233, ENSG00000170989, ENSG00000172071, ENSG00000137193, ENSG00000138613, ENSG000	GO:0043085~positive regulation of catalytic activity	14	4.8611	0.056604213	1.751035503	0.775960686
168	ENSG00000205220, ENSG00000172115, ENSG00000134215, ENSG00000087088, ENSG00000115233, ENSG00000170989, ENSG00000172071, ENSG00000137193, ENSG000	GO:0044093~positive regulation of molecular function	15	5.2083	0.066266466	1.664807036	0.79548053
169	ENSG00000137193, ENSG00000173114, ENSG00000115825, ENSG00000134215, ENSG00000157193, ENSG00000137275	GO:0043674~positive regulation of kinase activity	6	2.0833	0.283675498	1.689310689	0.967887126
170	ENSG00000137193, ENSG00000173114, ENSG00000115825, ENSG00000134215, ENSG00000157193, ENSG00000137275	GO:0051347~positive regulation of transferase activity	6	2.0833	0.30540296	1.625961538	0.969816139
171	ENSG00000137193, ENSG00000173114, ENSG00000115825, ENSG00000157193, ENSG00000137275	GO:0045860~positive regulation of protein kinase activity	5	1.7361	0.444739283	1.458261469	0.988508554

8.8 Cattle to human with gene description

	Transcript_Name	Human Ensembl Gene ID	CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 4	CLUSTER 5	FC	pval	Log_FC	strand	start	end	num_exons	length	gene_id	Description	gene_name
2	ENSBTAT00000015873	ENSG00000120053				YES	YES	1.3294164	0.0097024	0.4107931	-	20285687	20310044	9	1974	ENSBTAG00000011960	glutamic-oxaloacetic transaminase 1, soluble [Source:HGNC Symi	GOT1
3	ENSBTAT00000049780	ENSG00000065057	YES		YES		YES	1.4380666	0.017064	0.5241305	-	1590252	1595934	6	1029	ENSBTAG00000006272	nth-like DNA glycosylase 1 [Source:HGNC Symbol;Acc:HGNC:802	NTHL1
4	ENSBTAT00000011619	ENSG00000132664			YES		YES	0.3082448	0.0380734	-1.697852	+	38841092	38855175	9	1839	ENSBTAG00000008825	polymerase (RNA) III (DNA directed) polypeptide F, 39 kDa [Source:	POLR3F
5	ENSBTAT00000020336	ENSG00000006695	YES				YES	6.6505892	0.0162541	-0.620181	+	32696437	32804796	7	2845	ENSBTAG00000015294	COX10 heme A:farnesyltransferase cytochrome c oxidase assemb	COX10
6	ENSBTAT00000064176	ENSG00000014919	YES				YES	20.259094	0.0188730	4.3404977	-	20533690	20550733	9	1585	ENSBTAG00000045703	cytochrome c oxidase assembly homolog 15 (yeast) [Source:HGNC	COX15
7	ENSBTAT00000009643	ENSG00000167283	YES				YES	0.0062025	0.0299109	-7.332933	+	29521980	29530109	3	474	ENSBTAG00000007332		0
8	ENSBTAT00000007615	ENSG00000188690	YES				YES	0.0347346	0.0361015	-4.847484	-	45635844	45660024	10	1714	ENSBTAG00000005791	uroporphyrinogen III synthase [Source:HGNC Symbol;Acc:HGNC:	UROS
9	ENSBTAT00000064252	ENSG00000128951	YES				YES	0.1885056	0.0453446	-2.40732	+	142679111	142679692	1	582	ENSBTAG00000047462	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G3N0R;	0
10	ENSBTAT00000020608	ENSG00000105835					YES	74.309429	0.0001398	6.2154734	-	47597860	47635332	11	1814	ENSBTAG00000015509	nicotinamide phosphoribosyltransferase [Source:HGNC Symbol;A	NAMPT
11	ENSBTAT00000025662	ENSG00000138363					YES	1445.9097	0.0064757	10.497762	+	103847180	103870755	16	2042	ENSBTAG00000019274	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferas	ATIC
12	ENSBTAT00000010204	ENSG00000112541					YES	29.293443	0.0112433	4.8725059	-	101987619	102068801	19	2382	ENSBTAG00000007758	phosphodiesterase 10A [Source:HGNC Symbol;Acc:HGNC:8772]	PDE10A
13	ENSBTAT00000018301	ENSG00000125877					YES	0.5248396	0.0138623	-0.930052	-	52449560	52462362	8	934	ENSBTAG00000013776	inosine triphosphatase (nucleoside triphosphate pyrophosphatase	ITPA
14	ENSBTAT00000003935	ENSG00000172113					YES	34.281313	0.0321761	5.0993505	+	52126504	52133264	6	1086	ENSBTAG00000003025	NME/NM23 nucleoside diphosphate kinase 6 [Source:HGNC Symi	NME6
15	ENSBTAT00000022012	ENSG00000103024					YES	29371.596	0.0363354	14.842134	-	1348415	1349600	5	813	ENSBTAG00000016552	NME/NM23 nucleoside diphosphate kinase 3 [Source:HGNC Symi	NME3
16	ENSBTAT00000005978	ENSG00000094841					YES	0.0268973	0.0374393	-5.216395	-	80939906	80970032	8	1131	ENSBTAG00000004549	uracil phosphoribosyltransferase (FUR1) homolog (S. cerevisiae) [S	UPRT
17	ENSBTAT00000018370	ENSG00000060982					YES	11.837396	0.044216	3.5652798	+	85480247	85537788	11	2258	ENSBTAG00000013825	branched chain amino-acid transaminase 1, cytosolic [Source:HG	BCAT1
18	ENSBTAT00000046902	ENSG00000143156					YES	0.6948802	0.0485144	-0.525164	-	37533775	37838172	12	1413	ENSBTAG00000002689	NME/NM23 family member 7 [Source:HGNC Symbol;Acc:HGNC:20	NME7
19	ENSBTAT00000065618	ENSG00000069974	YES	YES			YES	2.086328	0.0238884	1.060966	-	35101143	35101808	1	666	ENSBTAG00000046690	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G3MYK	0
20	ENSBTAT00000015829	ENSG00000135404	YES	YES			YES	0.7969207	0.0289074	-0.327492	+	57854278	57857485	8	850	ENSBTAG00000011931	CD63 antigen [Source:UniProtKB/Swiss-Prot;Acc:Q9XSK2]	CD63
21	ENSBTAT00000021426	ENSG00000123643		YES			YES	0.1249039	0.0026932	-3.00111	+	64650884	64684500	10	1531	ENSBTAG00000016034	solute carrier family 36 (proton/amino acid symporter), member 1 [S	SLC36A1
22	ENSBTAT00000024228	ENSG00000162144		YES			YES	0.0716718	0.0136631	-3.802451	-	40551223	40562584	7	1868	ENSBTAG00000018202	cytochrome b561 family, member A3 [Source:HGNC Symbol;Acc:H	CYB561A3
23	ENSBTAT00000028656	ENSG00000155099		YES			YES	8.6319793	0.0237126	3.1096914	+	75708345	75780623	7	1024	ENSBTAG00000033968	transmembrane protein 55A [Source:HGNC Symbol;Acc:HGNC:2!	TMEM55A
24	ENSBTAT00000049126	ENSG00000186187		YES			YES	2.2605669	0.0362335	1.1766846	+	2396335	2482363	4	1389	ENSBTAG00000034689	zinc and ring finger 1, E3 ubiquitin protein ligase [Source:HGNC Syi	ZNRF1
25	ENSBTAT00000004876	ENSG00000114742		YES			YES	1.6529749	0.0377671	0.7250648	+	12416919	12469477	19	3957	ENSBTAG00000003745	WD repeat domain 48 [Source:HGNC Symbol;Acc:HGNC:30914]	WDR48
26	ENSBTAT00000052079	ENSG00000068001					YES	0.5070393	0.0425012	-0.97983	+	50593627	50597453	4	1876	ENSBTAG00000000484	hyaluronoglucosaminidase 2 [Source:HGNC Symbol;Acc:HGNC:5	HYAL2
27	ENSBTAT00000007967	ENSG00000132646	YES		YES			0.2321709	0.0048274	-2.106741	-	47788530	47794602	6	1988	ENSBTAG00000006065	proliferating cell nuclear antigen [Source:HGNC Symbol;Acc:HGN	PCNA
28	ENSBTAT00000019979	ENSG00000174547	YES		YES			0.3654304	0.009037	-1.452331	-	45127205	45130554	5	827	ENSBTAG00000015006	mitochondrial ribosomal protein L11 [Source:HGNC Symbol;Acc:H	MRPL11
29	ENSBTAT00000064878	ENSG00000171421	YES		YES			1.0488679	0.0155609	0.068833	+	70995210	70996377	2	775	ENSBTAG00000047906	mitochondrial ribosomal protein L36 [Source:HGNC Symbol;Acc:H	MRPL36
30	ENSBTAT00000020681	ENSG00000104980	YES		YES			0.5560291	0.016698	-0.846768	-	17907314	17920304	13	1816	ENSBTAG00000015567	translocase of inner mitochondrial membrane 44 homolog (yeast) [	TIMM44
31	ENSBTAT00000030623	ENSG00000132153	YES		YES			0.0038182	0.0167916	-8.03291	-	52465093	52480017	19	3743	ENSBTAG00000015833	DEAH (Asp-Glu-Ala-His) box helicase 30 [Source:HGNC Symbol;A	DHX30
32	ENSBTAT00000032066	ENSG00000104835	YES		YES			0.3985734	0.0198164	-1.327083	+	48896564	48905591	16	1888	ENSBTAG00000001780	serine--tRNA ligase, mitochondrial precursor [Source:RefSeq pep	0
33	ENSBTAT00000002334	ENSG00000104835	YES		YES			0.449606	0.0295088	-1.153267	+	48896564	48905610	16	1907	ENSBTAG00000001780	serine--tRNA ligase, mitochondrial precursor [Source:RefSeq pep	0
34	ENSBTAT00000044248	ENSG00000135828	YES		YES			3.3865515	0.0366613	1.7598169	-	65097628	65115555	7	2779	ENSBTAG00000009091	ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent) [S	RNASEL
35	ENSBTAT00000001464	ENSG00000116161	YES		YES			1.1358608	0.040685	0.1837861	+	57634739	57705495	6	1200	ENSBTAG00000001107	calcyclin binding protein [Source:HGNC Symbol;Acc:HGNC:3042;	CACYBP
36	ENSBTAT00000033835	ENSG00000133835	YES		YES			1.4336715	0.0464338	0.5197145	-	35662593	35763653	24	2611	ENSBTAG00000006978	hydroxysteroid (17-beta) dehydrogenase 4 [Source:HGNC Symbol	HSD17B4
37	ENSBTAT00000012369	ENSG00000197448	YES		YES			0.6163584	0.0493638	-0.698159	+	107468164	107473271	8	951	ENSBTAG00000009839	glutathione S-transferase kappa 1 [Source:HGNC Symbol;Acc:HG	GSTK1
38	ENSBTAT00000015097	ENSG00000076924			YES			0.316109	0.0014603	-1.661506	-	17689653	17698338	19	2624	ENSBTAG00000011360	pre-mRNA-splicing factor SYF1 [Source:RefSeq peptide;Acc:NP_	XAB2
39	ENSBTAT00000005018	ENSG00000134697			YES			0.0893459	0.0050607	-3.484455	+	108892202	108916432	16	2302	ENSBTAG00000003849	guanine nucleotide binding protein-like 2 (nucleolar) [Source:HGN	GNL2
40	ENSBTAT00000022090	ENSG00000115289			YES			132.65435	0.0053198	7.0515282	+	10113121	10115411	9	906	ENSBTAG00000016606	polycomb group ring finger 1 [Source:HGNC Symbol;Acc:HGNC:17	PCGF1
41	ENSBTAT00000026265	ENSG00000179348			YES			0.8147415	0.007854	-0.295586	+	60016985	60024586	6	3021	ENSBTAG00000019707	GATA binding protein 2 [Source:HGNC Symbol;Acc:HGNC:4171]	GATA2
42	ENSBTAT00000050636	ENSG00000111605			YES			2.7875308	0.0087621	1.4789878	-	44765461	44793485	10	2123	ENSBTAG00000007323	cleavage and polyadenylation specific factor 6, 68kDa [Source:HG	CPSF6
43	ENSBTAT00000016981	ENSG00000112658			YES			0.3260686	0.0088613	-1.616752	+	16747958	16754754	7	1905	ENSBTAG00000012777	serum response factor [Source:HGNC Symbol;Acc:HGNC:11291]	SRF
44	ENSBTAT00000012578	ENSG00000142684			YES			0.3103642	0.0126996	-1.687966	-	127523710	127524790	3	748	ENSBTAG00000009562	zinc finger protein 593 [Source:HGNC Symbol;Acc:HGNC:30943]	ZNF593
45	ENSBTAT00000021725	ENSG00000071243			YES			0.1764751	0.0158515	-2.502464	+	86211497	86237574	12	1380	ENSBTAG00000016332	inhibitor of growth family, member 3 [Source:HGNC Symbol;Acc:H	ING3
46	ENSBTAT00000016693	ENSG00000090316			YES			2.9013876	0.016016	1.536743	+	109330259	109343607	9	1243	ENSBTAG00000012575	macrophage erythroblast attacher [Source:RefSeq peptide;Acc:N	MAEA
47	ENSBTAT00000010773	ENSG00000129103			YES			0.6730517	0.0175	-0.571211	-	27995066	28005720	9	1773	ENSBTAG00000008190	sulfatase modifying factor 2 [Source:HGNC Symbol;Acc:HGNC:20	SUMF2
48	ENSBTAT00000004477	ENSG00000099622			YES			437.50236	0.0189091	8.773147	+	45354486	45359172	7	3101	ENSBTAG00000007480	cold inducible RNA binding protein [Source:HGNC Symbol;Acc:HC	CIRBP
49	ENSBTAT00000033926	ENSG00000071655			YES			0.5235298	0.01934	-0.933657	-	45577377	45579729	4	615	ENSBTAG00000038409	methyl-CpG binding domain protein 3 [Source:HGNC Symbol;Acc	MBD3
50	ENSBTAT00000037847	ENSG00000011007			YES			3.503487	0.0198284	1.8087915	-	129743823	129752821	4	1255	ENSBTAG00000026585	transcription elongation factor B (SIII), polypeptide 3 (110kDa, elong	TCEB3
51	ENSBTAT00000065252	ENSG00000120948			YES			0.0090479	0.0213186	-6.788205	-	43481408	43486458	6	1351	ENSBTAG00000003697	TAR DNA binding protein [Source:HGNC Symbol;Acc:HGNC:11571	TARDBP
52	ENSBTAT00000021204	ENSG00000117222			YES			3.466082	0.0215429	1.7933058	-	2694854	2733979	14	2536	ENSBTAG00000015946	retinoblastoma binding protein 5 [Source:HGNC Symbol;Acc:HGN	RBBP5
53	ENSBTAT00000027302	ENSG00000148411			YES			0.0365173	0.0216296	-4.775275	-	103572295	103607342	5	1758	ENSBTAG00000020490	NACC family member 2, BEN and BTB (POZ) domain containing [Sc	NACC2
54	ENSBTAT00000006699	ENSG00000162298			YES			2.0348881	0.0250807	1.0249495	-	43997381	44003325	16	1914	ENSBTAG00000005076	synovial apoptosis inhibitor 1, synoviolin [Source:HGNC Symbol;Ac	SYVN1
55	ENSBTAT00000043382	ENSG00000105373			YES			1.5107708	0.0257453	0.5952848	+	55089143	55097794	13	1516	ENSBTAG00000021192	glioma tumor suppressor candidate region gene 2 [Source:HGNC §	GLTSCR2
56	ENSBTAT00000008030	ENSG00000010072			YES			3.968874	0.0270781	1.9887298	-	3346617	3358272	6	1908	ENSBTAG00000006112	SprT-like N-terminal domain [Source:HGNC Symbol;Acc:HGNC:25	SPRTN
57	ENSBTAT00000013706	ENSG00000094804			YES			0.4077818	0.0275998	-1.294131	+	41185975	41196948	12	2502	ENSBTAG00000010384	cell division cycle 6 [Source:HGNC Symbol;Acc:HGNC:1744]	CDC6
58	ENSBTAT00000029119	ENSG00000196792			YES			0.340053	0.0285277	-1.556169	-	41964003	42070978	18	4071	ENSBTAG00000021845	striatin, calmodulin binding protein 3 [Source:HGNC Symbol;Acc:H	STRN3
59	ENSBTAT00000021643	ENSG00000139343			YES			0.3687495	0.0294318	-1.439287	+	60556051	60564297	4	1299	ENSBTAG00000016271	small nuclear ribonucleoprotein polypeptide F [Source:HGNC Symi	SNRPF
60	ENSBTAT00000004441	ENSG00000089737			YES			0.159826	0.0309509	-2.645426	-	59256632	59275061	9	2887	ENSBTAG00000003423	DEAD (Asp-Glu-Ala-Asp) box helicase 24 [Source:HGNC Symbol;	DDX24

#	Transcript	Gene												ENSBTAG	Description	Symbol
61	ENSBTAT00000024621	ENSG00000102103			YES	0.0267102	0.031957	-5.226466	+	92092355	92098095	6	1397	ENSBTAG00000018498	polyglutamine binding protein 1[Source:HGNC Symbol;Acc:HGNC	PQBP1
62	ENSBTAT00000023380	ENSG00000149554			YES	26.956389	0.0320107	4.7525553	+	29402369	29420344	13	2261	ENSBTAG00000017582	checkpoint kinase 1[Source:HGNC Symbol;Acc:HGNC:1925]	CHEK1
63	ENSBTAT00000011723	ENSG00000183733			YES	0.569136	0.0322099	-0.813155	+	13612910	13619093	3	582	ENSBTAG00000008305	folliculogenesis specific bHLH transcription factor [Source:HGNC S	FIGLA
64	ENSBTAT00000016879	ENSG00000171311			YES	0.2789132	0.0343448	-1.842112	-	18522299	18528929	8	885	ENSBTAG00000012699	exosome component 1[Source:HGNC Symbol;Acc:HGNC:17286]	EXOSC1
65	ENSBTAT00000011468	ENSG00000182263			YES	0.3510973	0.0388047	-1.510057	+	32850155	32852428	1	2274	ENSBTAG00000008704	fidgetin [Source:HGNC Symbol;Acc:HGNC:13285]	FIGN
66	ENSBTAT00000026442	ENSG00000189046			YES	2725.705	0.0396397	11.412414	+	66234084	66239899	4	2047	ENSBTAG00000019846	alkB homolog 2, alpha-ketoglutarate-dependent dioxygenase [Sc	ALKBH2
67	ENSBTAT00000022893	ENSG00000185163			YES	10.103807	0.0446581	3.3368271	+	46015020	46021726	15	1660	ENSBTAG00000017219	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51[Source:HGNC Symb	DDX51
68	ENSBTAT00000027125	ENSG00000136826			YES	0.0011422	0.0452157	-9.773967	-	98843684	98847740	4	2343	ENSBTAG00000020355	Kruppel-like factor 4 (gut) [Source:HGNC Symbol;Acc:HGNC:6348	KLF4
69	ENSBTAT00000005134	ENSG00000121390			YES	44.301962	0.0470105	5.4692987	+	36529631	36579763	9	1983	ENSBTAG00000003934	paraspeckle component 1[Source:HGNC Symbol;Acc:HGNC:203	PSPC1
70	ENSBTAT00000020093	ENSG00000136333	YES	YES		130.46886	0.0083122	7.0275617	+	96092486	96113995	7	1149	ENSBTAG00000015098	Rab9 effector protein with kelch motifs [Source:HGNC Symbol;Acc	RABEPK
71	ENSBTAT00000005250	ENSG00000185664	YES	YES		0.4501991	0.0205022	-1.151365	+	57669835	57677941	11	2046	ENSBTAG00000004019	premelanosome protein [Source:HGNC Symbol;Acc:HGNC:10880	PMEL
72	ENSBTAT00000064455	ENSG00000184489		YES		0.1634848	0.0097756	-2.612772	-	3578346	3586015	5	1532	ENSBTAG00000046467	protein tyrosine phosphatase type IVA, member 3 [Source:HGNC S	PTP4A3
73	ENSBTAT00000025173	ENSG00000172661		YES		12.908443	0.0162201	3.6902431	+	44495476	44542213	31	4104	ENSBTAG00000018915	WASH complex subunit FAM21[Source:RefSeq peptide;Acc:NP_	0
74	ENSBTAT00000035505	ENSG00000169446		YES		1361.3805	0.018348	10.410855	-	19576179	19585346	5	923	ENSBTAG00000025297	membrane magnesium transporter 1[Source:HGNC Symbol;Acc:H	MMGT1
75	ENSBTAT00000004636	ENSG00000110171		YES		16.418434	0.0222347	4.0372446	+	47193828	47219561	12	2792	ENSBTAG00000003565	tripartite motif containing 3[Source:HGNC Symbol;Acc:HGNC:100	TRIM3
76	ENSBTAT00000028836	ENSG00000198576		YES		0.8829855	0.0403111	-0.179538	+	2892788	2893978	1	1191	ENSBTAG00000021639	activity-regulated cytoskeleton-associated protein [Source:HGNC	ARC
77	ENSBTAT00000043035	ENSG00000119705	YES			0.1176846	0.0048986	-3.087003	+	89914334	89923167	4	378	ENSBTAG00000008135	SRA stem-loop-interacting RNA-binding protein, mitochondrial [S	0
78	ENSBTAT00000015619	ENSG00000180992	YES			0.1378779	0.0113643	-2.858537	-	17644487	17647839	2	1270	ENSBTAG00000011760	mitochondrial ribosomal protein L14 [Source:HGNC Symbol;Acc:H	MRPL14
79	ENSBTAT00000003210	ENSG00000002822	YES			0.2958212	0.0129786	-1.757202	-	41535263	41778055	18	2711	ENSBTAG00000002474	MAD1 mitotic arrest deficient-like 1(yeast) [Source:HGNC Symbol;	MAD1L1
80	ENSBTAT00000044604	ENSG00000167799	YES			18.411771	0.0144342	4.2025565	-	46123752	46126289	4	917	ENSBTAG00000011809	nudix (nucleoside diphosphate linked moiety X)-type motif 8 [Sour	NUDT8
81	ENSBTAT00000027841	ENSG00000136213	YES			23.321624	0.0146728	4.5435963	-	41372194	41390545	2	1839	ENSBTAG00000025291	carbohydrate (chondroitin 4) sulfotransferase 12 [Source:HGNC Sy	CHST12
82	ENSBTAT00000024993	ENSG00000103160	YES			6.4229422	0.015835	2.6832343	-	10439489	10451245	6	3223	ENSBTAG00000018770	hydroxysteroid dehydrogenase like 1[Source:HGNC Symbol;Acc:H	HSDL1
83	ENSBTAT00000019510	ENSG00000169682	YES			2.4628588	0.0167286	1.3003339	-	26126253	26134744	12	2189	ENSBTAG00000014653	spinster homolog 1(Drosophila) [Source:HGNC Symbol;Acc:HGNC	SPNS1
84	ENSBTAT00000012771	ENSG00000100890	YES			0.0395397	0.0175643	-4.660555	+	45821718	45953661	7	1936	ENSBTAG00000009682	LOC532995 protein; Uncharacterized protein [Source:UniProtKB/	0
85	ENSBTAT00000011502	ENSG00000183605	YES			1.2602546	0.019208	0.3337152	-	39653443	39669981	14	1397	ENSBTAG00000008730	sideroflexin 4 [Source:HGNC Symbol;Acc:HGNC:16088]	SFXN4
86	ENSBTAT00000061244	ENSG00000137700	YES			0.0685236	0.0204713	-3.867255	-	30122001	30127150	8	2110	ENSBTAG00000008077	solute carrier family 37 (glucose-6-phosphate transporter), membe	SLC37A4
87	ENSBTAT00000061070	ENSG00000145431	YES			0.0267513	0.0205277	-5.223922	+	43348702	43457620	5	938	ENSBTAG00000043959	platelet derived growth factor C [Source:HGNC Symbol;Acc:HGNC	PDGFC
88	ENSBTAT00000048747	ENSG00000164163	YES			0.2878889	0.0219952	-1.796416	-	13333870	13364710	18	2391	ENSBTAG00000019854	ATP-binding cassette, sub-family E (OABP), member 1[Source:HC	ABCE1
89	ENSBTAT00000029032	ENSG00000133028	YES			0.7918704	0.0231404	-0.336664	-	30276156	30296404	6	3793	ENSBTAG00000021780	SCO1 cytochrome c oxidase assembly protein [Source:HGNC Sym	SCO1
90	ENSBTAT00000004127	ENSG00000171612	YES			0.7054251	0.0237531	-0.503435	-	44801770	44831904	7	1447	ENSBTAG00000003177	solute carrier family 25 (pyrimidine nucleotide carrier), member 33 [	SLC25A33
91	ENSBTAT00000001917	ENSG00000173273	YES			3.2702694	0.0244927	1.7094095	+	24632930	24789416	27	4308	ENSBTAG00000001463	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymera	TNKS
92	ENSBTAT00000042557	ENSG00000070731	YES			1.6026506	0.0317013	0.6804599	+	55803307	55822126	8	1825	ENSBTAG00000015255	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-ace	ST6GALNAC
93	ENSBTAT00000034505	ENSG00000158042	YES			0.7484515	0.0319107	-0.418019	+	47004019	47005492	3	831	ENSBTAG00000024781	39S ribosomal protein L17, mitochondrial [Source:UniProtKB/Swiss	0
94	ENSBTAT00000011379	ENSG00000066855	YES			0.864997	0.0360488	-0.209233	+	31735268	31794502	8	1852	ENSBTAG00000008629	mitochondrial fission regulator 1[Source:HGNC Symbol;Acc:HGNC	MTFR1
95	ENSBTAT00000005553	ENSG00000120802	YES			0.0318515	0.0376983	-4.972497	+	63027194	63052802	7	2356	ENSBTAG00000004240	thymopoietin [Source:HGNC Symbol;Acc:HGNC:11875]	TMPO
96	ENSBTAT00000008388	ENSG00000196683	YES			0.0261096	0.0379617	-5.259275	-	89692607	89692771	1	165	ENSBTAG00000006398	translocase of outer mitochondrial membrane 7 homolog (yeast) [S	TOMM7
97	ENSBTAT00000000378	ENSG00000198814	YES			0.8095261	0.0381665	-0.30485	+	111538716	111600550	1	1835	ENSBTAG00000000301	Glycerol kinase; Uncharacterized protein [Source:UniProtKB/TrEM	0
98	ENSBTAT00000022549	ENSG00000113048	YES			0.8375143	0.040067	-0.255814	+	9211237	9315274	11	2179	ENSBTAG00000001962	mitochondrial ribosomal protein S27 [Source:HGNC Symbol;Acc:H	MRPS27
99	ENSBTAT00000029044	ENSG00000155008	YES			0.2425345	0.0405268	-2.043738	-	75160536	75265102	9	1236	ENSBTAG00000021790	apolipoprotein O-like [Source:HGNC Symbol;Acc:HGNC:24009]	APOOL
100	ENSBTAT00000064188	ENSG00000131495	YES			6.3962646	0.0418814	2.6772296	-	64586967	64587266	1	300	ENSBTAG00000047836	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	0
101	ENSBTAT00000029827	ENSG00000164144	YES			6.5216893	0.0424265	2.7052457	-	4732242	4868678	9	3026	ENSBTAG00000008438	ADP-ribosylation factor interacting protein 1[Source:HGNC Symbc	ARFIP1
102	ENSBTAT00000016063	ENSG00000155287	YES			0.0560773	0.0424577	-4.15644	-	20466104	20476402	4	1146	ENSBTAG00000012107	solute carrier family 25 (mitochondrial iron transporter), member 28	SLC25A28
103	ENSBTAT00000077756	ENSG00000162337	YES			9.135145	0.0425081	3.1914276	+	46485172	46573460	22	5030	ENSBTAG00000005903	low density lipoprotein receptor-related protein 5 [Source:HGNC S	LRP5
104	ENSBTAT00000016013	ENSG00000110717	YES			0.2316219	0.0460312	-2.110156	+	46205045	46208795	7	886	ENSBTAG00000030375	NADH dehydrogenase [ubiquinone] Fe-S protein 8, 23kDa (NADH	NDUFS8
105	ENSBTAT00000020333	ENSG00000119392	YES			0.69965	0.0464764	-0.515295	+	99077978	99103590	16	2436	ENSBTAG00000030566	GLE1 RNA export mediator [Source:HGNC Symbol;Acc:HGNC:431	GLE1
106	ENSBTAT00000049292	ENSG00000170962	YES			122.11325	0.0467381	6.932076	+	4501435	4788300	7	2055	ENSBTAG00000034827	platelet derived growth factor D [Source:HGNC Symbol;Acc:HGNC	PDGFD
107	ENSBTAT00000035896	ENSG00000137404	YES			0.0796308	0.0469574	-3.650529	-	28111675	28114705	4	1538	ENSBTAG00000006966	nurim (nuclear envelope membrane protein) [Source:HGNC Symbc	NRM
108	ENSBTAT00000017663	ENSG00000157191	YES			0.8662606	0.0485823	-0.207127	-	136252384	136266397	8	2019	ENSBTAG00000013282	NECAP endocytosis associated 2 [Source:HGNC Symbol;Acc:HG	NECAP2
109	ENSBTAT00000066249	ENSG00000109501	YES			5.6467589	0.0492745	2.497423	-	104673701	104696495	7	2667	ENSBTAG00000046671	Wolfram syndrome 1 (wolframin) [Source:HGNC Symbol;Acc:HGNC	WFS1
110	ENSBTAT00000011044					10.977035	0.0002071	3.4564165	+	219743	220147	1	405	ENSBTAG00000032198	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:F1N150]	0
111	ENSBTAT00000065516					0.9621405	0.0003172	-0.055681	-	71676362	71677651	1	1290	ENSBTAG00000046463		
112	ENSBTAT00000010089	ENSG00000026652				0.0494256	0.0005049	-4.338597	-	98239672	98376380	9	1886	ENSBTAG00000007668	1-acylglycerol-3-phosphate O-acyltransferase 4 [Source:HGNC S	AGPAT4
113	ENSBTAT00000009283	ENSG00000110046				0.1667031	0.0012762	-2.584647	-	43752917	43757930	9	1224	ENSBTAG00000007058	autophagy related 2A [Source:HGNC Symbol;Acc:HGNC:29028]	ATG2A
114	ENSBTAT00000008606	ENSG00000171320				0.4819603	0.0013901	-1.053014	-	10859329	10885722	10	2972	ENSBTAG00000006551	ESCO2 protein; Uncharacterized protein [Source:UniProtKB/TrEM	0
115	ENSBTAT00000024759	ENSG00000095539				2.2374255	0.0019449	1.1618396	+	21677156	21690795	16	4269	ENSBTAG00000018604	sema domain, immunoglobulin domain (Ig), transmembrane domair	SEMA4G
116	ENSBTAT00000052263	ENSG00000158985				0.4781896	0.0018425	-1.064345	-	24318564	24436629	6	3306	ENSBTAG00000005961	CDC42 small effector 2 [Source:HGNC Symbol;Acc:HGNC:18547]	CDC42SE2
117	ENSBTAT00000044006	ENSG00000137720				5.1974376	0.0025845	2.3778005	+	22550859	22552378	3	609	ENSBTAG00000045821	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:G3N356	0
118	ENSBTAT00000022481	ENSG00000197860				6.0090595	0.0029296	2.5871392	+	13805928	13851473	11	1115	ENSBTAG00000016902	small glutamine-rich tetratricopeptide repeat (TPR)-containing, be	SGTB
119	ENSBTAT00000064601	ENSG00000180537				0.2878961	0.0030021	-1.79638	-	42467107	42467856	1	750	ENSBTAG00000045884	ring finger protein 182 [Source:HGNC Symbol;Acc:HGNC:28522]	RNF182
120	ENSBTAT00000045045					0.8290912	0.0030304	-0.270397	-	31497480	31497890	1	411	ENSBTAG00000031766		HIST1H3B

316

8.9 Cattle DAVID analysis 1

	A	B	C	D	E	F	G	H
1		Annotation Cluster 1	Enrichment Score: 2.273398306120239					
2	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
3	ENSG00000113048, ENSG00000183605, ENSG00000132153, ENSG00000119705, ENSG00000180992, ENSG00000174547, ENSG00000197448, ENSG00000133835, ENSG00000	GOTERM_CC_FAT	GO:0005739~mitochondrion	33	10.21672	3.60E-04	1.930577	0.049994
4	ENSG00000113048, ENSG00000183605, ENSG00000132153, ENSG00000119705, ENSG00000180992, ENSG00000174547, ENSG00000133028, ENSG00000171421, ENSG000001	SP_PIR_KEYWORDS	mitochondrion	28	8.668731	4.64E-04	2.081453	0.035555
5	ENSG00000137404, ENSG00000167283, ENSG00000155287, ENSG00000119392, ENSG00000131495, ENSG00000197448, ENSG00000116161, ENSG00000002822, ENSG0000001	GOTERM_CC_FAT	GO:0031967~organelle envelope	22	6.811146	7.15E-04	2.256492	0.065694
6	ENSG00000137404, ENSG00000167283, ENSG00000155287, ENSG00000119392, ENSG00000131495, ENSG00000197448, ENSG00000116161, ENSG00000002822, ENSG0000001	GOTERM_CC_FAT	GO:0031975~envelope	22	6.811146	7.45E-04	2.249236	0.051726
7	ENSG00000109501, ENSG00000137700, ENSG00000197448, ENSG00000133028, ENSG00000145431, ENSG00000185664, ENSG00000170962, ENSG00000173273, ENSG0000000	GOTERM_CC_FAT	GO:0031090~organelle membrane	30	9.287926	0.003514	1.740658	0.181807
8	ENSG00000167283, ENSG00000155287, ENSG00000131495, ENSG00000197448, ENSG00000006695, ENSG00000133028, ENSG00000171612, ENSG00000110717, ENSG00000	GOTERM_CC_FAT	GO:0031966~mitochondrial membrane	15	4.643963	0.003636	2.421017	0.158881
9	ENSG00000167283, ENSG00000132153, ENSG00000155287, ENSG00000131495, ENSG00000174547, ENSG00000197448, ENSG00000104835, ENSG00000006695, ENSG0000001	GOTERM_CC_FAT	GO:0044429~mitochondrial part	19	5.882353	0.005688	2.03067	0.150043
10	ENSG00000167283, ENSG00000155287, ENSG00000131495, ENSG00000197448, ENSG00000133028, ENSG00000171612, ENSG00000110717, ENSG00000155008, ENSG0000001	GOTERM_CC_FAT	GO:0019866~organelle inner membrane	13	4.024768	0.005723	2.512755	0.138174
11	ENSG00000167283, ENSG00000155287, ENSG00000131495, ENSG00000197448, ENSG00000006695, ENSG00000133028, ENSG00000171612, ENSG00000110717, ENSG000001	GOTERM_CC_FAT	GO:0005740~mitochondrial envelope	15	4.643963	0.00621	2.276565	0.137518
12	ENSG00000171612, ENSG00000133028, ENSG00000167283, ENSG00000110717, ENSG00000155287, ENSG00000158042, ENSG00000155008, ENSG00000169682, ENSG000000	GOTERM_CC_FAT	GO:0005743~mitochondrial inner membrane	12	3.71517	0.008799	2.493805	0.176141
13	ENSG00000119705, ENSG00000180992, ENSG00000174547, ENSG00000104835, ENSG00000006695, ENSG00000133028, ENSG00000110717, ENSG00000171421, ENSG000000	UP_SEQ_FEATURE	transit peptide:Mitochondrion	13	4.024768	0.079682	1.710783	0.998304
14	ENSG00000119705, ENSG00000180992, ENSG00000174547, ENSG00000104835, ENSG00000006695, ENSG00000133028, ENSG00000110717, ENSG00000171421, ENSG000000	SP_PIR_KEYWORDS	transit peptide	13	4.024768	0.085751	1.68915	0.753051
15	ENSG00000171612, ENSG00000167283, ENSG00000155287, ENSG00000169682, ENSG00000131495, ENSG00000104980	SP_PIR_KEYWORDS	mitochondrion inner membrane	6	1.857585	0.202068	1.922763	0.843293
16								
17		Annotation Cluster 2	Enrichment Score: 2.088331357171254					
18	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
19	ENSG00000162144, ENSG00000185664, ENSG00000172661, ENSG00000186187, ENSG00000184489, ENSG00000169446, ENSG00000069974, ENSG00000135404, ENSG000000	SP_PIR_KEYWORDS	endosome	12	3.71517	6.05E-04	3.551132	0.037042
20	ENSG00000162144, ENSG00000185664, ENSG00000186187, ENSG00000184489, ENSG00000169446, ENSG00000069974, ENSG00000135404, ENSG00000110171, ENSG000000	GOTERM_CC_FAT	GO:0005768~endosome	11	3.405573	0.026509	2.220674	0.362639
21	ENSG00000162144, ENSG00000186187, ENSG00000023643, ENSG00000069974, ENSG00000135404, ENSG00000155099, ENSG00000114742	SP_PIR_KEYWORDS	lysosome	7	2.167183	0.033889	2.905652	0.51184
22								
23		Annotation Cluster 3	Enrichment Score: 2.0042406789176077					
24	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
25	ENSG00000120948, ENSG00000183733, ENSG00000071243, ENSG00000139343, ENSG00000132153, ENSG00000011007, ENSG00000089737, ENSG00000174547, ENSG000000	GOTERM_CC_FAT	GO:0070013~intracellular organelle lumen	43	13.31269	0.0037	1.537076	0.140091
26	ENSG00000120948, ENSG00000183733, ENSG00000071243, ENSG00000139343, ENSG00000132153, ENSG00000011007, ENSG00000089737, ENSG00000174547, ENSG000000	GOTERM_CC_FAT	GO:0031974~membrane-enclosed lumen	44	13.62229	0.004653	1.50757	0.153068
27	ENSG00000120948, ENSG00000183733, ENSG00000071243, ENSG00000139343, ENSG00000132153, ENSG00000011007, ENSG00000089737, ENSG00000174547, ENSG000000	GOTERM_CC_FAT	GO:0043233~organelle lumen	43	13.31269	0.005565	1.502449	0.161984
28	ENSG00000120948, ENSG00000183733, ENSG00000071243, ENSG00000139343, ENSG00000011007, ENSG00000089737, ENSG00000185163, ENSG00000094804, ENSG000000	GOTERM_CC_FAT	GO:0031981~nuclear lumen	35	10.83591	0.010242	1.53498	0.189073
29	ENSG00000183733, ENSG00000117222, ENSG00000136826, ENSG00000071243, ENSG00000139343, ENSG00000149554, ENSG00000148411, ENSG00000011007, ENSG000000	GOTERM_CC_FAT	GO:0005654~nucleoplasm	20	6.19195	0.097056	1.441996	0.646254
30								
31		Annotation Cluster 4	Enrichment Score: 1.5772804042500475					
32	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
33	ENSG00000162144, ENSG00000186187, ENSG00000068001, ENSG00000123643, ENSG00000069974, ENSG00000135404, ENSG00000155099, ENSG00000120053, ENSG000000	GOTERM_CC_FAT	GO:0000323~lytic vacuole	9	2.786378	0.018001	2.712457	0.276436
34	ENSG00000162144, ENSG00000186187, ENSG00000068001, ENSG00000123643, ENSG00000069974, ENSG00000135404, ENSG00000155099, ENSG00000120053, ENSG000000	GOTERM_CC_FAT	GO:0005764~lysosome	9	2.786378	0.018001	2.712457	0.276436
35	ENSG00000162144, ENSG00000186187, ENSG00000023643, ENSG00000069974, ENSG00000135404, ENSG00000155099, ENSG00000114742	SP_PIR_KEYWORDS	lysosome	7	2.167183	0.033889	2.905652	0.51184
36	ENSG00000162144, ENSG00000186187, ENSG00000068001, ENSG00000123643, ENSG00000069974, ENSG00000135404, ENSG00000155099, ENSG00000120053, ENSG000000	GOTERM_CC_FAT	GO:0005773~vacuole	9	2.786378	0.044691	2.271144	0.515146
37								
38		Annotation Cluster 5	Enrichment Score: 1.3669326361639456					
39	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
40	ENSG00000128951, ENSG00000103024, ENSG00000143156, ENSG00000125877, ENSG00000172113	SP_PIR_KEYWORDS	nucleotide metabolism	5	1.547988	2.61E-04	15.46222	0.026828
41	ENSG00000128951, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009147~pyrimidine nucleoside triphosphate metabolic pr	4	1.23839	0.001675	16.2401	0.912949
42	ENSG00000132664, ENSG00000128951, ENSG00000103024, ENSG00000094841, ENSG00000143156, ENSG00000125877, ENSG00000172113	KEGG_PATHWAY	hsa00240:Pyrimidine metabolism	7	2.167183	0.002958	4.803644	0.280269
43	ENSG00000065057, ENSG00000128951, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0006220~pyrimidine nucleotide metabolic process	5	1.547988	0.003484	7.894491	0.921206
44	ENSG00000103024, ENSG00000143156, ENSG00000172113	UP_SEQ_FEATURE	active site:Pros-phosphohistidine intermediate	3	0.928793	0.003767	30.7283	0.966907
45	ENSG00000103024, ENSG00000143156, ENSG00000172113	SMART	SM00562:NDK	3	0.928793	0.006134	24.31875	0.513199
46	ENSG00000103024, ENSG00000143156, ENSG00000172113	INTERPRO	IPR001564:Nucleoside diphosphate kinase, core	3	0.928793	0.006772	23.22351	0.971193
47	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_MF_FAT	GO:0004550~nucleoside diphosphate kinase activity	3	0.928793	0.008778	20.41352	0.70984
48	ENSG00000128951, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0046213~pyrimidine nucleoside metabolic process	4	1.23839	0.009187	9.094454	0.965246
49	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0046051~UTP metabolic process	3	0.928793	0.010145	18.94678	0.948665
50	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0006183~GTP biosynthetic process	3	0.928793	0.010145	18.94678	0.948665
51	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0006228~UTP biosynthetic process	3	0.928793	0.010145	18.94678	0.948665
52	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0046036~CTP metabolic process	3	0.928793	0.012536	17.0521	0.953174
53	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009208~pyrimidine ribonucleoside triphosphate metaboli	3	0.928793	0.012536	17.0521	0.953174
54	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009209~pyrimidine ribonucleoside triphosphate biosynth	3	0.928793	0.012536	17.0521	0.953174
55	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0006241~CTP biosynthetic process	3	0.928793	0.012536	17.0521	0.953174
56	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009148~pyrimidine nucleoside triphosphate biosynthetic	3	0.928793	0.017968	14.21008	0.946776
57	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0046039~GTP metabolic process	3	0.928793	0.017968	14.21008	0.946776
58	ENSG00000132664, ENSG00000103024, ENSG00000112541, ENSG00000138363, ENSG00000143156, ENSG00000125877, ENSG00000172113	KEGG_PATHWAY	hsa00230:Purine metabolism	7	2.167183	0.02759	2.982655	0.788343
59	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009220~pyrimidine ribonucleotide biosynthetic process	3	0.928793	0.027619	11.36807	0.975453
60	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009218~pyrimidine ribonucleotide metabolic process	3	0.928793	0.031206	10.65756	0.963009

#	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
61	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0046131 pyrimidine ribonucleoside metabolic process	3	0.928793	0.034965	10.03065	0.952558
62	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0006221 pyrimidine nucleotide biosynthetic process	3	0.928793	0.056109	7.750955	0.965369
63	ENSG00000006695, ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000060982, ENSG00000105835, ENSG00000120053, ENSG00000014919, ENSG0000	GOTERM_BP_FAT	GO:0044271 nitrogen compound biosynthetic process	11	3.405573	0.059918	1.923827	0.968574
64	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_MF_FAT	GO:0016776 phosphotransferase activity, phosphate group a	3	0.928793	0.10001	5.567324	0.903171
65	ENSG00000128951, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009116 nucleoside metabolic process	4	1.23839	0.101857	3.552521	0.983692
66	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000143156, ENSG00000172113	GOTERM_MF_FAT	GO:0009152 purine ribonucleotide biosynthetic process	5	1.547988	0.149619	2.429074	0.976379
67	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_MF_FAT	GO:0019205 nucleobase, nucleoside, nucleotide kinase activ	3	0.928793	0.154345	4.272598	0.933764
68	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009260 ribonucleotide biosynthetic process	5	1.547988	0.173047	2.291949	0.982895
69	ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009119 ribonucleoside metabolic process	3	0.928793	0.192561	3.706978	0.985963
70	ENSG00000167283, ENSG00000128951, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009141 nucleoside triphosphate metabolic process	5	1.547988	0.197644	2.169478	0.985281
71	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009150 purine ribonucleotide metabolic process	5	1.547988	0.22323	2.059432	0.985413
72	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000105835, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009165 nucleotide biosynthetic process	6	1.857585	0.227444	1.833559	0.985325
73	ENSG00000167283, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009206 purine ribonucleoside triphosphate biosynthetic	4	1.23839	0.246274	2.320014	0.985012
74	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000105835, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0034404 nucleobase, nucleoside and nucleotide biosynt	6	1.857585	0.250047	1.767057	0.985475
75	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000105835, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0034654 nucleobase, nucleoside, nucleotide and nuclei	6	1.857585	0.250047	1.767057	0.985475
76	ENSG00000167283, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009145 purine nucleoside triphosphate biosynthetic pro	4	1.23839	0.250971	2.296579	0.984484
77	ENSG00000167283, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009201 ribonucleoside triphosphate biosynthetic proces	4	1.23839	0.250971	2.296579	0.984484
78	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009259 ribonucleotide metabolic process	5	1.547988	0.257286	1.933345	0.983829
79	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0006164 purine nucleotide biosynthetic process	5	1.547988	0.261135	1.920282	0.983726
80	ENSG00000167283, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009142 nucleoside triphosphate biosynthetic process	4	1.23839	0.265139	2.229033	0.984288
81	ENSG00000167283, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009205 purine ribonucleoside triphosphate metabolic pr	4	1.23839	0.336936	1.943259	0.988103
82	ENSG00000167283, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009199 ribonucleoside triphosphate metabolic process	4	1.23839	0.341737	1.926791	0.987867
83	ENSG00000167283, ENSG00000103024, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0009144 purine nucleoside triphosphate metabolic proce	4	1.23839	0.36091	1.863618	0.987778
84	ENSG00000167283, ENSG00000103024, ENSG00000138363, ENSG00000143156, ENSG00000172113	GOTERM_BP_FAT	GO:0006163 purine nucleotide metabolic process	5	1.547988	0.410751	1.527966	0.991877
85								
86		Annotation Cluster 6	Enrichment Score: 1.2634076030382662					
87	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
88	ENSG00000006695, ENSG00000133028, ENSG00000014919	GOTERM_BP_FAT	GO:0008535 respiratory chain complex IV assembly	3	0.928793	0.007982	21.31513	0.979548
89	ENSG00000006695, ENSG00000133028, ENSG00000145431, ENSG00000110717, ENSG00000171368, ENSG00000167552, ENSG00000014919	GOTERM_BP_FAT	GO:0043623 cellular protein complex assembly	7	2.167183	0.065603	2.456064	0.970647
90	ENSG00000006695, ENSG00000133028, ENSG00000162144, ENSG00000167283, ENSG00000110717, ENSG00000131495, ENSG00000120053, ENSG00000014919	GOTERM_BP_FAT	GO:0006091 generation of precursor metabolites and energy	8	2.47678	0.309545	1.452788	0.987084
91								
92		Annotation Cluster 7	Enrichment Score: 1.2598047353013444					
93	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
94	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000183605, ENSG00000213588, ENSG00000180537, ENSG00000100722, ENSG00000010539, ENSG0000	GOTERM_MF_FAT	GO:0046914 transition metal ion binding	62	19.19505	0.006175	1.363345	0.728527
95	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000183605, ENSG00000213588, ENSG00000180537, ENSG00000100722, ENSG00000010539, ENSG0000	SP_PIR_KEYWORDS	metal-binding	65	20.12384	0.008338	1.352684	0.278586
96	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000186187, ENSG00000213588, ENSG00000180537, ENSG00000187147, ENSG00000172059, ENSG0000	SP_PIR_KEYWORDS	zinc-finger	40	12.3839	0.018422	1.44002	0.40985
97	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000186187, ENSG00000213588, ENSG00000180537, ENSG00000187147, ENSG00000142303, ENSG0000	SP_PIR_KEYWORDS	zinc	43	13.31269	0.131972	1.214939	0.770517
98	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000186187, ENSG00000112541, ENSG00000213588, ENSG00000180537, ENSG00000187147, ENSG0000	GOTERM_MF_FAT	GO:0008270 zinc ion binding	45	13.93189	0.140998	1.192482	0.930474
99	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000183605, ENSG00000103024, ENSG00000213588, ENSG00000180537, ENSG00000100722, ENSG0000	GOTERM_MF_FAT	GO:0046872 metal ion binding	76	23.52941	0.141487	1.124223	0.923388
100	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000183605, ENSG00000103024, ENSG00000213588, ENSG00000180537, ENSG00000100722, ENSG0000	GOTERM_MF_FAT	GO:0043169 cation binding	76	23.52941	0.16393	1.113731	0.938686
101	ENSG00000146587, ENSG00000175895, ENSG00000071243, ENSG00000183605, ENSG00000103024, ENSG00000213588, ENSG00000180537, ENSG00000100722, ENSG0000	GOTERM_MF_FAT	GO:0043167 ion binding	76	23.52941	0.20392	1.097449	0.945494
102								
103		Annotation Cluster 8	Enrichment Score: 1.1848092573949434					
104	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
105	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000106799, ENSG0000	GOTERM_MF_FAT	GO:0030554 adenyl nucleotide binding	37	11.45511	0.021524	1.436843	0.833927
106	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000106799, ENSG0000	GOTERM_MF_FAT	GO:0001883 purine nucleoside binding	37	11.45511	0.026417	1.415304	0.847188
107	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000106799, ENSG0000	GOTERM_MF_FAT	GO:0032553 adenyl ribonucleotide binding	35	10.83591	0.027073	1.43181	0.808087
108	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000106799, ENSG0000	GOTERM_MF_FAT	GO:0001882 nucleoside binding	37	11.45511	0.028936	1.405646	0.786734
109	ENSG00000213341, ENSG00000127334, ENSG00000134318, ENSG00000103024, ENSG00000149554, ENSG00000184304, ENSG00000082146, ENSG00000105221, ENSG0000	UP_SEQ_FEATURE	binding site:ATP	16	4.95356	0.031787	1.814217	0.937074
110	ENSG00000103024, ENSG00000132153, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000106799, ENSG00000172113, ENSG0000	GOTERM_MF_FAT	GO:0005524 ATP binding	34	10.52632	0.035861	1.403735	0.818828
111	ENSG00000132153, ENSG00000103024, ENSG00000164896, ENSG00000089737, ENSG00000185163, ENSG00000106799, ENSG00000172113, ENSG00000094804, ENSG0000	SP_PIR_KEYWORDS	atp-binding	31	9.597523	0.037833	1.445939	0.528605
112	ENSG00000120948, ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000119705, ENSG00000130811, ENSG00000164896, ENSG00000060650, ENSG0000	GOTERM_MF_FAT	GO:0000166 nucleotide binding	47	14.55108	0.052078	1.282096	0.870869
113	ENSG00000213341, ENSG00000182263, ENSG00000132153, ENSG00000127586, ENSG00000149554, ENSG00000184304, ENSG00000089737, ENSG00000185163, ENSG0000	UP_SEQ_FEATURE	nucleotide phosphate-binding region:ATP	23	7.120743	0.065142	1.469336	0.998833
114	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000167552, ENSG0000	GOTERM_MF_FAT	GO:0017076 purine nucleotide binding	40	12.3839	0.078786	1.277176	0.915224
115	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000167552, ENSG0000	GOTERM_MF_FAT	GO:0032555 purine ribonucleotide binding	38	11.76471	0.094774	1.267506	0.915063
116	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000132466, ENSG00000185163, ENSG00000167552, ENSG0000	GOTERM_MF_FAT	GO:0032553 ribonucleotide binding	38	11.76471	0.094774	1.267506	0.915063
117	ENSG00000103024, ENSG00000132153, ENSG00000112541, ENSG00000164896, ENSG00000089737, ENSG00000185163, ENSG00000167552, ENSG00000106799, ENSG0000	SP_PIR_KEYWORDS	nucleotide-binding	35	10.83591	0.102445	1.283933	0.740461
118	ENSG00000213341, ENSG00000103024, ENSG00000149554, ENSG00000184304, ENSG00000164896, ENSG00000106799, ENSG00000172113, ENSG00000068745, ENSG0000	SP_PIR_KEYWORDS	kinase	16	4.95356	0.147726	1.438346	0.78955
119	ENSG00000213341, ENSG00000135828, ENSG00000149554, ENSG00000184304, ENSG00000164896, ENSG00000145348, ENSG00000106799, ENSG00000134318, ENSG0000	GOTERM_MF_FAT	GO:0004672 protein kinase activity	14	4.334365	0.187879	1.414799	0.940763
120	ENSG00000213341, ENSG00000135828, ENSG00000134318, ENSG00000127334, ENSG00000149554, ENSG00000184304, ENSG00000082146, ENSG00000105221, ENSG0000	INTERPRO	IPR017441:Protein kinase, ATP binding site	11	3.405573	0.199174	1.497194	0.999287

318

#	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
123			Annotation Cluster 9	Enrichment Score: 1.1297027188409117				
124	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
125	ENSG00000006695, ENSG00000185664, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0046148 pigment biosynthetic process	4	1.23839	0.03054	5.829778	0.969003
126	ENSG00000006695, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0006783 heme biosynthetic process	3	0.928793	0.034965	10.03065	0.952558
127	ENSG00000006695, ENSG00000185664, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0042440 pigment metabolic process	4	1.23839	0.043962	5.052474	0.955711
128	ENSG00000006695, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0006779 porphyrin biosynthetic process	3	0.928793	0.047208	8.52605	0.959258
129	ENSG00000006695, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0033014 tetrapyrrole biosynthetic process	3	0.928793	0.047208	8.52605	0.959258
130	ENSG00000006695, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0042168 heme metabolic process	3	0.928793	0.056109	7.750955	0.965369
131	ENSG00000006695, ENSG00000014919, ENSG00000188690	KEGG_PATHWAY	hsa00860:Porphyrin and chlorophyll metabolism	3	0.928793	0.088433	5.926573	0.967477
132	ENSG00000006695, ENSG00000105835, ENSG00000014919, ENSG00000188690, ENSG00000023909	GOTERM_BP_FAT	GO:0051188 cofactor biosynthetic process	5	1.547988	0.090662	2.929914	0.984902
133	ENSG00000006695, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0033013 tetrapyrrole metabolic process	3	0.928793	0.091141	5.880035	0.983302
134	ENSG00000006695, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0006778 porphyrin metabolic process	3	0.928793	0.091141	5.880035	0.983302
135	ENSG00000006695, ENSG00000105835, ENSG00000197448, ENSG00000014919, ENSG00000188690, ENSG00000023909	GOTERM_BP_FAT	GO:0051186 cofactor metabolic process	6	1.857585	0.256691	1.748933	0.984904
136	ENSG00000006695, ENSG00000014919, ENSG00000188690	GOTERM_BP_FAT	GO:0018130 heterocycle biosynthetic process	3	0.928793	0.276725	2.890187	0.985729
137								
138			Annotation Cluster 10	Enrichment Score: 1.093631290268119				
139	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
140	ENSG00000087263, ENSG00000189046, ENSG00000178467	INTERPRO	IPR005123:Oxoglutarate and iron-dependent oxygenase	3	0.928793	0.029911	10.92871	0.999639
141	ENSG00000087263, ENSG00000189046, ENSG00000178467	UP_SEQ_FEATURE	domain:Fe2OG dioxygenase	3	0.928793	0.044962	8.779513	0.997354
142	ENSG00000087263, ENSG00000189046, ENSG00000132510, ENSG00000178467	GOTERM_MF_FAT	GO:0016702 oxidoreductase activity, acting on single donors	4	1.23839	0.092157	3.711549	0.921447
143	ENSG00000087263, ENSG00000189046, ENSG00000132510, ENSG00000178467	GOTERM_MF_FAT	GO:0016701 oxidoreductase activity, acting on single donors	4	1.23839	0.095377	3.656153	0.904099
144	ENSG00000087263, ENSG00000189046, ENSG00000178467	SP_PIR_KEYWORDS	dioxygenase	3	0.928793	0.287868	2.811312	0.909942
145								
146			Annotation Cluster 11	Enrichment Score: 1.0526031513223535				
147	Genes	Category	Term	Count	%	PValue	Fold Enrich	Benjamini
148	ENSG00000103024, ENSG00000112541, ENSG00000106799, ENSG00000136159, ENSG00000172113, ENSG00000127334, ENSG00000064655, ENSG00000169446, ENSG00000...	GOTERM_MF_FAT	GO:0000287 magnesium ion binding	13	4.024768	0.06554	1.761344	0.90728
149	ENSG00000127334, ENSG00000167799, ENSG00000119414, ENSG00000106799, ENSG00000154237, ENSG00000136159	SP_PIR_KEYWORDS	manganese	6	1.857585	0.099765	2.441403	0.744948
150	ENSG00000127334, ENSG00000167799, ENSG00000119414, ENSG00000106799, ENSG00000154237, ENSG00000136159	GOTERM_MF_FAT	GO:0030145 manganese ion binding	6	1.857585	0.106343	2.385996	0.895023

8.10 Scripts

8.10.1 EdgeR

```
#source("http://bioconductor.org/biocLite.R")
#biocLite("edgeR")
library(edgeR)
library(limma)
library(gtools)

###################################################################
#########################################
## SET THE WORKING DIRECTORY

setwd('/home/svztg/Desktop/David_haig_Rabbit_vs_Cattle/RABBIT/Edge
R_DE')


directory <- getwd()
filenames <- list.files(directory, pattern="*.counts")

for (file in filenames)

  {

  x <- read.delim(file,row.names="GENE")
  group <- factor(c(2,2,1,1))

  all_results_table = paste(file,"_all_Results_edgeR.xls")
  FDR.lt0.05_table = paste(file,"_FDR_p0.05_edgeR.xls")
  PValue.lt0.05_table = paste(file,"_PValue_p0.05_edgeR.xls")
  mds.plot.name = paste(file,".MDS.plot.pdf")
  smear.plot.name = paste(file,".smear.plot.pdf")
  volcano.plot.name = paste(file,".volcano.plot.pdf")


###################################################################
#########################################

  y <- DGEList(counts=x,group=group)
  z <- DGEList(counts=x,group=group)
  ## Since the smallest group size is TWO, we keep genes that
achieve at least one count per million (cpm) in at least TWO
samples:\n";
  keep <- rowSums(cpm(y)>1) >= 1
  y <- y[keep,]
  # Re-compute the library sizes
  y$samples$lib.size <- colSums(y$counts)
  z$samples$lib.size <- colSums(z$counts)
  # Compute effective library sizes using TMM normalization:\n";
  y <- calcNormFactors(y)
  z <- calcNormFactors(z)
  y$samples
  #  Output plot as a pdf
  pdf(mds.plot.name)
  plotMDS(y)
  dev.off()
```

```
y <- estimateCommonDisp(y)
z <- estimateCommonDisp(z)
y <- estimateTagwiseDisp(y)
z <- estimateTagwiseDisp(z)
#plotBCV(y)
et <- exactTest(y)
etz <- exactTest(z)
top <- topTags(et)

pdf(smear.plot.name)
plotSmear(y, de.tags=top, main="Differential expression using
the common dispersion")
dev.off()

## Check the individual cpm values for the top genes:
cpm(y)[rownames(top), ]
## The total number of DE genes at 5% FDR is given by:
summary(de <- decideTestsDGE(et))
## Plot the log-fold-changes, highlighting the DE genes:
detags <- rownames(y)[as.logical(de)]

## Get LogFC values
LogFCvalues <- etz$table$logFC
## Get the Genenames
RownamesforLogFC <- rownames(etz)

## Convert LogFC to FC
FC <- logratio2foldchange(LogFCvalues, base=2)
## Convert FC list to dataframe with Genenames
FClist <-  data.frame( RownamesforLogFC, FC )


# volcano plot
pdf(volcano.plot.name)
plot(et$table$logFC, -log10(et$table$PValue), pch=19, cex=0.2,
main  ="Volcano Plot", xlab = "LogFC", ylab = "-log10 pvalue")
dev.off()

## Generate an EdgeR results table
EdgeRResults <- topTags( et , n = nrow( et$table ) )$table
## Generate an counts table
gene.counts <- z$counts
## Merge tables
most.results <- merge(gene.counts, EdgeRResults, by=0, all=TRUE)
combined.results <- merge(most.results, FClist,  by=1, all=TRUE)

## Identify if a gene is up or down regulated
#combined.results.UP.G2 <- subset(combined.results, logFC > 0)
# combined.results.DOWN.G2 <- subset(combined.results, logFC <=
0)
# combined.results.UP.G2[, "G2vsG1"] <- "UP"
#combined.results.DOWN.G2[, "G2vsG1"] <- "DOWN"

## Add Up or Down to combined results
#combined.results <-
rbind(combined.results.UP.G2,combined.results.DOWN.G2)
```

321

```
## Filter combined.results to identify significant genes
significant.results0.05FDR <- subset(combined.results, FDR <=
0.05)
significant.results0.05 <- subset(combined.results, PValue <=
0.05)
## Write tables
write.table(combined.results,
file=all_results_table,row.names=FALSE,col.names=TRUE,quote=FALSE)
write.table(significant.results0.05, file=
PValue.lt0.05_table,row.names=FALSE,col.names=TRUE,quote=FALSE)
write.table(significant.results0.05FDR,
file=FDR.lt0.05_table,row.names=FALSE,col.names=TRUE,quote=FALSE)

}
```

8.10.2 Ballgown

```
## ----loadmethods, echo=FALSE, message=FALSE, warning=FALSE------
---------
## source("http://bioconductor.org/biocLite.R")
## biocLite("ballgown")

library(edgeR)
library(limma)
library(ballgown)
library(gtools)


## ----Variables------------------------------------------------
---------

group = factor(c(1,1,2,2))
data_directory
='/home/svztg/Desktop/David_haig_Rabbit_vs_Cattle/COW/assemblies'

## ----makebg, message=FALSE-------------------------------------
------

setwd(data_directory)
directory <- getwd()
bg = ballgown(dataDir=data_directory, samplePattern='assembly',
meas='all')


## ----getexpr---------------------------------------------------
---------

gene_expression = texpr(bg, 'FPKM')
transcript_values = texpr(bg, 'all')
transcript_names = transcriptNames(bg)


## ----pData-----------------------------------------------------
---------

pData(bg) = data.frame(id=sampleNames(bg), group)
phenotype_results = pData(bg)


## ----getStats--------------------------------------------------
---------

stats_results = stattest(bg, feature='transcript',
gexpr=gene_expression, getFC=TRUE, meas='FPKM', covariate='group')
stats_results <- merge(transcript_names, stats_results, by=0,
all=TRUE)
stats_results = subset(stats_results,
select=c("x","fc","pval","qval"))
colnames(stats_results)[1] <- "t_name"
colnames(stats_results)[2] <- "Ratio"
Log_FC <- log2(stats_results$Ratio)
FC <- logratio2foldchange(Log_FC, base=2)
stats_results <- cbind(stats_results, Log_FC, FC)
stats_results = subset(stats_results,
select=c("t_name","Ratio","FC","Log_FC","pval","qval"))
stats_results <- merge(stats_results, transcript_values)
colnames(stats_results)[1] <- "Transcript_Name"
```

```
stats_results <- stats_results[ order(stats_results$pval) ,]

## ----writeTable-------------------------------------------------
--------------

#transcript_table = paste("transcript_table", sep="" )
#write.table(transcript_values,
file=transcript_table,row.names=FALSE,col.names=TRUE,quote=FALSE,s
ep="\t")

phenotype_results_table = paste("phenotype_results_table", sep =
"" )
write.table(phenotype_results,
file=phenotype_results_table,row.names=FALSE,col.names=TRUE,quote=
FALSE,sep="\t")

stats_results_table = paste("stats_results_table", sep="" )
write.table(stats_results,
file=stats_results_table,row.names=FALSE,col.names=TRUE,quote=FALS
E,sep="\t")


## ----sessioninfo, results='markup'-----------------------------
---------
#sessionInfo()
```

8.10.3 Hisat

```
#!/usr/bin/bash

#HISAT and StringTie wrapper script v0.0.1
#Tom Giles
#09.12.14
#thomas.giles@nottingham.ac.uk

###############################################################
# FUNCTIONS:
###############################################################

help_readme() {

# Help Section

    echo "

HISAT and StringTie wrapper script v1.0.1 by Tom Giles (21.10.15)
(thomas.giles@nottingham.ac.uk) `basename $0`

This pipeline aligns reads to a genome sequence using Hisat v2
(Kim D, Langmead B and Salzberg SL. HISAT: a fast spliced aligner
with low memory requirements. Nature Methods 2015). Technical
replicates from different lanes are merged using samtools (Heng
Li, Et Al, The Sequence Alignment/Map format and SAMtools.
Bioinformatics 2009) and then assembled to identify transcipt
level expression using stringtie (Pertea M, Et Al. StringTie
enables improved reconstruction of a transcriptome from RNA-seq
reads Nature Biotechnology 2015).

Usage:

Hisat_Wrapper.sh <genome_directory> <read_directory>

The genome_directory should contain the following 2 files:

    # GENOME_SEQUENCE=     genome_name.fa
    # GENOME_ANNOTATIONS   =     genome_name.gtf

if you wish to use the SNP aware indexer the genome folder should
also contain:

    # GENOME_SNPS     =     genome_name.vcf

The read_directory should contain trimmed.gz reads in pairs:

You will be prompted to enter the number of CPUS's to use, the
amount of RAM and the unique read suffixes

The number of cores and amount of RAM should be specified as whole
numbers (eg 20 = 20gb)

The read suffixes should be unique:

    # *_R1_001.fq.gz
    # *_R2_001.fq.gz
```

325

for optimum compatibility we recomend that the reads are formated
as follows:

```
    # SAMPLE_REPLICATE_LANE_READSUFFIX
```

If the reads are not formated in this manner it is likely that the
script will crash whilst merging of the sorted bamfiles.

This script assumes that the reads were generated across multiple
lane (eg: L001, L002).

If the reads were not generated across multiple lanes then the
script will crash whilst merging of the sorted bamfiles. In this
instance you may need to re-write the Merge_bam_files function or
rename your reads so that the technical replicates are defined
after the second underscore in the readname.

Dependencies (The folowing tools must be installed at $/PATH):

```
    # HISAT (tested with version 2.0.0-beta)
    # samtools (tested with version 0.1.19)
    # HTSeq_count (tested with version 0.6.1)
    # StringTie (tested with version 1.0.4)
```

If not set, the CPU and RAM settings determined automatically
based on the avalible system resources

```
    "

  exit 0

}

#-----------------------------------------------------------#

Extract_Splice_sites(){

# Extract Splice sites from GFF

  START=$(date +%s)

  if [ -f $SPLICE_SITES ];

      then
      echo "Splice site file $SPLICE_SITES exists"
      else
      echo "Extracting splice sites from $GENOME_GTF"
      python $HISAT2_DIRECTORY/extract_splice_sites.py $GENOME_GTF
> $SPLICE_SITES

  fi;
  wait

  END=$(date +%s)
  TIME_SPLICE_SITES=$((END-START))
```

```
   echo "Time to extract Splice sites from GFF: $TIME_SPLICE_SITES
seconds"

}

#-----------------------------------------------------------#

Extract_Exons(){

# Extract Exons from GFF

   START=$(date +%s)

   if [ -f $EXONS ];

       then
       echo "Exon file $EXONS exists"
       else
       echo "Extracting exons sites from $GENOME_GTF"
       python $HISAT2_DIRECTORY/extract_exons.py $GENOME_GTF >
$EXONS

   fi;
   wait

   END=$(date +%s)
   TIME_EXONS=$((END-START))
   echo "Time to extract Exons from GFF: $TIME_EXONS  seconds"

}

#-----------------------------------------------------------#

Extract_SNPs(){

# Extract Exons from VCF

   START=$(date +%s)

   if [ -f $SNPS ];

   then
       echo "Exon file $SNPS exists"
       else
       echo "Extracting SNPs from $GENOME_VCF"

   cat $GENOME_VCF | awk -F $'\t' 'BEGIN {OFS = FS}{
               if (index($8, "deletion")) { print
$3,"deletion"$1,$2,length($5);}
               else if (index($8, "SNV")) { print
$3,"single"$1,$2,$5;}
               else if (index($8,"insertion")) { print
$3,"insertion"$1,$2,$5;}
         }' | tr '[,]' '[\t]' | awk -F $'\t' 'BEGIN {OFS = FS}{
               if ($6=="") { print $1,$2,$3,$4,"$5;}
               else { for (i=4; i<=NF; i++) print
$1,$2,$3,$4,"$i;
       }' >  $SNPS
```

327

```
  fi;
  wait

  END=$(date +%s)
  TIME_EXONS=$((END-START))
  echo "Time to extract SNPs from GFF: $TIME_SNPs  seconds"

}

#------------------------------------------------------------#

Index_reference_genome_no_SNPs(){

# Build the reference genome without the SNP aware indexer

  START=$(date +%s)

  if [ -f $INDEX_NAME.1.ht2 ] ;

     then
     echo "GENOME_SEQUENCE $INDEX_NAME Exists. If you need to re-
index the genome please remove current version in
$GENOME_DIRECTORY"
     else
     echo "Indexing $GENOME_SEQUENCE with splice site aware
algorithm";
     hisat2-build --verbose -p $NPROC --ss $SPLICE_SITES --exon
$EXONS  $GENOME_SEQUENCE $INDEX_NAME


  fi;
  wait

  END=$(date +%s)
  TIME_INDEXING=$((END-START))
  echo "Time to index the reference sequence: $TIME_INDEXING
seconds"

}

#------------------------------------------------------------#

Index_reference_genome_with_SNPs(){

# Build the reference genome with the SNP aware indexer

  START=$(date +%s)

  if [ -f $INDEX_NAME.1.ht2 ] ;

     then
     echo "GENOME_SEQUENCE $INDEX_NAME Exists. If you need to re-
index please remove current version"
     else
     echo "Indexing $GENOME_SEQUENCE with splice site aware
algorithm";
```

328

```
        hisat2-build --verbose -p $NPROC --snp $SNP --ss
$SPLICE_SITES --exon $EXONS  $GENOME_SEQUENCE $INDEX_NAME


  fi;
  wait

  END=$(date +%s)
  TIME_INDEXING=$((END-START))
  echo "Time to index the reference sequence: $TIME_INDEXING
seconds"

}

#------------------------------------------------------------#

Run_alignment(){

#Run hisat on all read pairs

  START=$(date +%s)

  for fq_pair_1 in $WORKING_DIRECTORY/*$READ1_SUFFIX ;

      do

      filename=${fq_pair_1%$READ1_SUFFIX}
      fq_pair_2=${filename}$READ2_SUFFIX

      samfile=$filename.$SAMFILE
      mapping_statistics=$filename.$MAPPING_STATISTICS
      novel_splice_sites=$filename.$NOVEL_SPLICE_SITES
      unaligned_singletons=$filename.$UNMAPPED_SINGLETONS
      unaligned_pairs=$filename.$UNMAPPED_PAIRS

      if [ -f $samfile ] ;
           then
           echo "$samfile Exists"
           else
           echo "now aligning $fq_pair_1 and $fq_pair_2"
           hisat2 -x $INDEX_NAME -p $NPROC --known-splicesite-
infile $SPLICE_SITES --novel-splicesite-outfile
$novel_splice_sites -1 $fq_pair_1 -2 $fq_pair_2 -S $samfile --met-
file $mapping_statistics --un-gz $unaligned_singletons --un-conc-
gz $unaligned_pairs;
      fi
  done;
  wait

  END=$(date +%s)
  TIME_ALLGINING=$((END-START))
  echo "Time to run hisat: $TIME_ALLGINING seconds"

}

#------------------------------------------------------------#

Convert_SAM_to_BAM(){
```

329

```
#Convert all .sam files to sorted .bam files in batches of NPROC

  START=$(date +%s)

  for samfile in $WORKING_DIRECTORY/*.$SAMFILE;

      do

      bamfile="${samfile%.sam}.alignment"

      if [ -f $bamfile.bam ];
            then
            echo "$bamfile.bam Exists"
            else
            echo "Now sorting $samfile."
            samtools view -bS $samfile | samtools sort  - $bamfile
&
            PROC=$((PROC+6))

            if [ "$PROC" -ge "$NPROC" ];
                  then
                  wait
                  PROC=0
            fi
      fi
  done;
  wait

  END=$(date +%s)
  TIME_SORTING=$((END-START))
  echo "Time to convert .sam files to sorted .bam files:
$TIME_SORTING seconds"

}

#----------------------------------------------------------#

Merge_bam_files(){

#merge the sorted .bam files

  START=$(date +%s)

  grouped_sample_ID=$(find "$WORKING_DIRECTORY" -name
"*.alignment.bam" -exec echo "{}" \;)
  echo "$grouped_sample_ID" >
$WORKING_DIRECTORY/list_of_alignments.tmp
  sample_IDs=$(cat $WORKING_DIRECTORY/list_of_alignments.tmp | rev
| cut -d"/" -f1 | rev | cut -d"_" -f1-2 | sort | uniq)

  for sample in $sample_IDs;
      do
      list=$(find  "$WORKING_DIRECTORY" -name
"$sample*alignment.bam");

      echo "merging sorted bam files"
      echo " $list"
```

330

```
        samtools merge
$WORKING_DIRECTORY/"$sample".merged_alignment.bam $list
  done;

  wait

  END=$(date +%s)
  TIME_MERGING=$((END-START))
  echo "Time to merge .bam files: $TIME_MERGING seconds"

}

#-----------------------------------------------------------#

Generate_assembly(){

#Run stringtie on sorted .bam files

  START=$(date +%s)

  for bamfile in $WORKING_DIRECTORY/*merged_alignment.bam;

      do
      results_summary="${bamfile%.merged_alignment.bam}_assembly/r
esults_summary.txt"
      results_folder="${bamfile%.merged_alignment.bam}_assembly"

      if [ -f $results_summary ];
      then
            echo "$results_summary Exists"
            else
            echo "Now running StringTie on $bamfile."
            mkdir $results_folder
            stringtie $bamfile -G $GENOME_GTF -p $NPROC -b
$results_folder > $results_summary
      fi
  done;
  wait

  END=$(date +%s)
  TIME_ASSEMBLING=$((END-START))
  echo "Time to run StringTie: $TIME_ASSEMBLING seconds"

}

#-----------------------------------------------------------#

Generate_counts(){

#Run HTSeq on sorted .bam files

  START=$(date +%s)

  for bamfile in $WORKING_DIRECTORY/*merged_alignment.bam;

      do
      results_counts="${bamfile%.bam}.counts"
```

331

```
        if [ -f $results_counts ];
        then
                echo "$results_summary Exists"
                else
                echo "Now running HTSeq on $bamfile."
                samtools view $bamfile | htseq-count - $GENOME_GTF >
$results_counts  &

                PROC=$((PROC+2))

                if [ "$PROC" -ge "$NPROC" ];
                        then
                        wait
                        PROC=0
                fi
        fi
  done;
  wait

  END=$(date +%s)
  TIME_COUNTING=$((END-START))
  echo "Time to count alignments with HTSeq counts: $TIME_COUNTING
seconds"

}

#--------------------------------------------------------------#

Generate_statistics(){

#Calculate total runtime, the number of reads and Mapping
Statistics

  START=$(date +%s)

  echo "Generating statistics"

  for fq_pair_1 in $WORKING_DIRECTORY/*$READ1_SUFFIX;

        do

        filename=${fq_pair_1%$READ1_SUFFIX}
        fq_pair_2=${filename}$READ2_SUFFIX
        samfile=$filename.$SAMFILE
        bamfile=$filename.alignment.$BAMFILE
        sample_name=${filename##*/}


            number_of_R1_input_reads=$(zcat "$fq_pair_1" | echo $((`wc
-l`/4)))
        number_of_R2_input_reads=$(zcat "$fq_pair_2" | echo $((`wc -
l`/4)))
        flagstat=$(samtools flagstat $bamfile)

        echo "Filename = $sample_name
number of reads = $number_of_R1_input_reads +
$number_of_R2_input_read
Samtools flagstats =
```

```
$flagstat

            "
  done > "$WORKING_DIRECTORY/Run_statistics.tsv"

  END=$(date +%s)
  TIME_GENERATE_STATISTICS=$((END-START))

}

#-------------------------------------------------------------#

Move_files(){

#Make new directories and move files accordingly

 if [ ! -d "$WORKING_DIRECTORY/reads" ]; then
     mkdir $WORKING_DIRECTORY/reads
 fi;

 if [ ! -d "$WORKING_DIRECTORY/reads/trimmed" ]; then
     mkdir $WORKING_DIRECTORY/reads/trimmed
     mv $WORKING_DIRECTORY/*$READ1_SUFFIX
$WORKING_DIRECTORY/reads/trimmed
     mv $WORKING_DIRECTORY/*$READ2_SUFFIX
$WORKING_DIRECTORY/reads/trimmed
 fi;

 if [ ! -d "$WORKING_DIRECTORY/reads/unmapped_singletons" ]; then
     mkdir $WORKING_DIRECTORY/reads/unmapped_singletons
     mv $WORKING_DIRECTORY/*$UNMAPPED_SINGLETONS
$WORKING_DIRECTORY/reads/unmapped_singletons
 fi;

 if [ ! -d "$WORKING_DIRECTORY/reads/unmapped_pairs" ]; then
     mkdir $WORKING_DIRECTORY/reads/unmapped_pairs
     mv $WORKING_DIRECTORY/*.unmapped.pairs.fq.*.gz
$WORKING_DIRECTORY/reads/unmapped_pairs
 fi;

 if [ ! -d "$WORKING_DIRECTORY/alignments" ]; then
     mkdir $WORKING_DIRECTORY/alignments
 fi;

 if [ ! -d "$WORKING_DIRECTORY/alignments/merged_alignment" ];
then
     mkdir $WORKING_DIRECTORY/alignments/merged_alignment
     mv $WORKING_DIRECTORY/*.merged_alignment.bam
$WORKING_DIRECTORY/alignments/merged_alignment
 fi;

 if [ ! -d "$WORKING_DIRECTORY/alignments/samfiles" ]; then
     mkdir $WORKING_DIRECTORY/alignments/samfiles
     mv $WORKING_DIRECTORY/*$SAMFILE
$WORKING_DIRECTORY/alignments/samfiles
 fi;

 if [ ! -d "$WORKING_DIRECTORY/alignments/sorted_bamfiles" ]; then
```

333

```
    mkdir $WORKING_DIRECTORY/alignments/sorted_bamfiles
    mv $WORKING_DIRECTORY/*$BAMFILE
$WORKING_DIRECTORY/alignments/sorted_bamfiles
 fi;

 if [ ! -d "$WORKING_DIRECTORY/alignments/statistics" ]; then
    mkdir $WORKING_DIRECTORY/alignments/statistics
    mv $WORKING_DIRECTORY/*$MAPPING_STATISTICS
$WORKING_DIRECTORY/alignments/statistics
 fi;

 if [ ! -d "$WORKING_DIRECTORY/alignments/novel_splice_sites" ];
then
    mkdir $WORKING_DIRECTORY/alignments/novel_splice_sites
    mv $WORKING_DIRECTORY/*$NOVEL_SPLICE_SITES
$WORKING_DIRECTORY/alignments/novel_splice_sites
 fi;

 if [ ! -d "$WORKING_DIRECTORY/assemblies" ]; then
    mkdir $WORKING_DIRECTORY/assemblies
    mv $WORKING_DIRECTORY/*assembly
$WORKING_DIRECTORY/assemblies
 fi;
if [ ! -d "$WORKING_DIRECTORY/counts" ]; then
    mkdir $WORKING_DIRECTORY/counts
    mv $WORKING_DIRECTORY/*counts $WORKING_DIRECTORY/counts
 fi;

}

#############################################################
# Either run the pipeline or exit based on input variables
#############################################################


GENOME_DIRECTORY=$1;
WORKING_DIRECTORY=$2;

if [ "$1" = -h ];
    then

    help_readme

elif [ "$1" = --help ];
    then

    help_readme

elif [  -d "$1" ] && [ -d "$2" ];
    then

    gtf=$(ls $GENOME_DIRECTORY/*.gtf | wc -l)
    fa=$(ls $GENOME_DIRECTORY/*.fa | wc -l)
    vcf=$(ls $GENOME_DIRECTORY/*.vcf | wc -l)

    if [ "$gtf" = "1" ] && [ "$fa" = "1" ];
        then
```

334

```
            echo "Run Started"
            echo "HISAT and StringTie wrapper script v1.0.1 by Tom
Giles (21.10.15) (thomas.giles@nottingham.ac.uk)"
            echo "for more information on how to run this script
please use -h or --help"
            echo ""
            echo "Genome folder = $1"
            echo "Read folder = $2"


            #----------------------------------------------------
-------#

            # Set global variables

            echo -n "Enter the number of CPU cores to use > "
            read number_of_cores
            echo -n "Enter the amount of RAM (in GB) to use > "
            read amount_of_ram
            echo -n "Enter the unique read_1 suffix > "
            read READ1_SUFFIX
            echo -n "Enter the unique read_2 suffix > "
            read READ2_SUFFIX

            #----------------------------------------------------
-------#

            # Define the status of the system

            NPROC=$(grep -c ^processor /proc/cpuinfo)

            if [ "$number_of_cores" -lt "$NPROC" ];
                then

                NPROC=$number_of_cores
                else
                echo "$number_of_cores is greater than the
avalible number of cores. \n Using $NPROC cores"
            fi

            KB_RAM=$(head -1 /proc/meminfo | awk '{ print $2 }');
            GB_RAM=$(echo "scale=2; $KB_RAM /1048576" | bc);
            GB_RAM_ROUNDED=$(echo ${GB_RAM%%.*});
            RAM=$((GB_RAM_ROUNDED-2));


            if [ "$amount_of_ram" -lt "$RAM" ];
                then

                RAM=$amount_of_ram
                else
                echo "$amount_of_ram is greater than the
avalible amount of RAM. \n Using $RAM GB of RAM"
            fi

            echo "Using $RAM GB of RAM and $NPROC cores"
```

335

```
#~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~#

        # Automatically define the additional core variables

        GENOME_SEQUENCE=$(find $GENOME_DIRECTORY -type f -name
*.fa)
        GENOME_GTF=$(find $GENOME_DIRECTORY -type f -name
*.gtf)

        INDEX_NAME="${GENOME_SEQUENCE%.fa}"
        SPLICE_SITES="${GENOME_GTF%.gtf}.splice_sites"
        EXONS="${GENOME_GTF%.gtf}.exons"
        HISAT2_DIRECTORY=$(which hisat2 | sed
's,/*[^/]\+/*$,,')
        SAMFILE="sam"
        BAMFILE="bam"
        MAPPING_STATISTICS="mapping_statistics.txt"
        NOVEL_SPLICE_SITES="novel_splice_sites.tsv"
        UNMAPPED_SINGLETONS="unmapped.singletons.fq.gz"
        UNMAPPED_PAIRS="unmapped.pairs.fq.gz"

        if [ "$vcf" = "1" ];
            then

            $GENOME_VCF=$(find $GENOME_DIRECTORY -type f -
name *.vcf)
            $SNPS="${GENOME_VCF%.vcf}.snps"
            echo "SNP file present - will attempt to
generate a SNP sensitive genome index"
            else
            echo "SNP file not present - will not attempt to
generate a SNP sensitive genome index"
        fi


    #~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~#

        # call functions

        Extract_Splice_sites;
        Extract_Exons;

        if [ "$vcf" = "1" ];
            then

            Extract_SNPs;
        fi

        if [ "$vcf" = "1" ];
            then

            Index_reference_genome_with_SNPs;
            else

            Index_reference_genome_no_SNPs;
```

336

```
            fi
            Run_alignment;
            Convert_SAM_to_BAM;
            Merge_bam_files;
            Generate_counts;
            Generate_assembly;
            Generate_statistics;
            Move_files;



     #~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~#

            # Collect Statistics

            echo "Run Finished"
            rm $WORKING_DIRECTORY/*.tmp

            Run_date=$(date);

            TIME_SPLICE_SITES=$(echo $TIME_SPLICE_SITES | awk
'{print int($1/60)"m:"int($1%60)}"s"')
            TIME_EXONS=$(echo $TIME_EXONS | awk '{print
int($1/60)"m:"int($1%60)}"s"')
            TIME_INDEXING=$(echo $TIME_INDEXING | awk '{print
int($1/60)"m:"int($1%60)}"s"')
            TIME_ALLGINING=$(echo $TIME_ALLGINING | awk '{print
int($1/60)"m:"int($1%60)}"s"')
            TIME_SORTING=$(echo $TIME_SORTING | awk '{print
int($1/60)"m:"int($1%60)}"s"')
            TIME_MERGING=$(echo $TIME_MERGING | awk '{print
int($1/60)"m:"int($1%60)}"s"')
            TIME_COUNTS=$(echo $TIME_COUNTS | awk '{print
int($1/60)"m:"int($1%60)}"s"')
            TIME_ASSEMBLING=$(echo $TIME_ASSEMBLING | awk '{print
int($1/60)"m:"int($1%60)}"s"')
            TIME_GENERATE_STATISTICS=$(echo
$TIME_GENERATE_STATISTICS | awk '{print
int($1/60)"m:"int($1%60)}"s"')

            echo ""
            echo "PERFORMANCE STATISTICS:"
            echo "Number of CPUs = $NPROC | Amount of Ram used =
$RAM"

            if [ "$TIME_SPLICE_SITES" != "0m:0s" ];
            then
                 echo "Time to extract splice sites from GFF:
$TIME_SPLICE_SITES"
            else
                 echo "splice sites file existed";
            fi
            if [ "$TIME_EXONS" != "0m:0s" ]; then echo "Time to
extract exons from GFF: $TIME_EXONS";
            else
                 echo "Exon file already existed";
            fi
            if [ "$TIME_INDEXING" != "0m:0s" ]; then
```

337

```
                echo "Time to index the reference sequence:
$TIME_INDEXING";
            else
                echo "Genome was already indexed"
            fi
            if [ "$TIME_ALLGINING" != "0m:0s" ];
            then
                echo "Time to run HISAT: $TIME_ALLGINING";
            fi
            if [ "$TIME_SORTING" != "0m:0s" ];
            then
                echo "Time to convert .sam files to sorted .bam
files: $TIME_SORTING";
            fi
            if [ "$TIME_MERGING" != "0m:0s" ];
            then
                echo "Time to merge .bam files: $TIME_MERGING";
            fi
            if [ "$TIME_COUNTS" != "0m:0s" ];
            then
                echo "Time to count alignments with HTSeq
counts: $TIME_COUNTS";
            fi
            if [ "$TIME_ASSEMBLING" != "0m:0s" ];
            then
                echo "Time to run StringTie: $TIME_ASSEMBLING";
            fi
            if [ "$TIME_GENERATE_STATISTICS" != "0m:0s" ];
            then
                echo "Time to generate statistics:
$TIME_GENERATE_STATISTICS";
            fi
            exit 0

    else
        help_readme

    fi
else

help_readme

fi
```

338

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