The Role of Autophagy and Lipids in Mouse Norovirus Replication

Submitted by

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Statement of Authorship

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Tanya O'Donnell, 16th September 2016

Statement of Contributions by others to the thesis

A/Prof Jason Mackenzie (University of Melbourne) made significant contributions to the overall project design as well as experimental design and analysis, drafting of manuscripts and editing of this thesis overall.

Published works incorporated into the thesis

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List of Abbreviations

A	
ADP	Adenosine diphosphate
Arf1	ADP-ribosylation factor 1
Atg	Autophagy gene
ATP	Adenosine triphosphate
B	
BafA	Bafilomycin A1
BFA	Brefeldin A
BSA	Bovine serum albumin
C	
CBV3	Coxsackie B Virus 3
cDNA	Complementary DNA
CIDE	Clathrin-independent endocytosis
CME	Clathrin-mediated endocytosis
CoV	Coronavirus
CV	Calicivirus
D	
DAPI	4', 6-diamidino-2-phenylindole
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DENV	Dengue Virus
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	double-stranded RNA
E	
EDTA	Ethylenediamine tetraacetic acid
eIF2α	Eukaryotic initiation factor 2α
EM	Electron microscopy
EBV	Epstein Barr Virus
ER	Endoplasmic reticulum
F	
FCS/FBS	Fetal calf serum/Fetal bovine serum
FCV	Feline Calicivirus
FMDV	Foot and mouth disease virus
G	
GBF1	Golgi-specific brefeldin A-resistant guanine nucleotide exchange factor 1
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H	
HAV	Hepatitis A Virus
HCV	Hepatitis C Virus
HEV1	T-00127-HEV1
His	Hexahistidine
h.p.i.	hours post infection
h.p.t	hours post transfection
HSV-1	Herpes Simplex Virus 1
HuNoV	Human norovirus
I	
IAV	Influenza A Virus
IEC	Intestinal epithelial cells
IF	Immunofluorescence
IFN	Interferon
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ITZ	Itraconazole
K	
KO	Knock-out
L	
LC3	Microtubule-associated protein light chain 3
Lov	Lovastatin
M	
MAVS	Mitochondrial antiviral signalling protein
M-βCD	Methyl-β-cyclodextrin
MCS	Membrane contact site
MEF	Mouse embryonic fibroblasts
MDA-5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility complex
MNV	Mouse norovirus

m.o.i	Multiplicity of infection
mRNA	Messenger RNA
MTOC	Microtubule organizing center
MyD88	Myeloid differentiation factor 88

N

NFκB	Nuclear factor κ B
NoV	Norovirus
NS	Non-structural
NTP	Nucleotide triphosphate
NTPase	Nucleoside triphosphatase
NV	Norwalk virus

O

ORF	Open reading frame
OSBP	Oxysterol binding protein

P

PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
pfu	plaque forming units
PI4K	Phosphatidylinositol 4-kinase
PI4P	Phosphatidylinositol 4-phosphate
PKR	Protein kinase R
PRR	Pathogen recognition receptor
pSTAT	phosphorylated STAT
PV	Poliovirus

Q

qRT-PCR	Quantitative reverse-transcription PCR

R

RAG2	Recombination-activator gene-2
Rapa	Rapamycin
RC	Replication complex
RdRp	RNA dependent RNA polymerase
RHDV	Rabbit haemorrhagic disease virus
RIG-I	Retinoic acid inducible gene
RO	Replication organelle
RNA	Ribonucleic acid

S

SaV	Sapovirus
SFV	Semliki Forest virus
sgRNA	Subgenomic RNA
siRNA	Small interfering RNA
ssRNA	single-stranded RNA
STAT	Signal transducer and activator of transcription

T

TBS	Tris-buffered saline
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α

U

UTR	Untranslated region

V

VAMP	vesicle-associated membrane protein
VAP-A	VAMP-associated protein A
VLP	Virus-like particle

W

WHO	World Health Organisation
WNV	West Nile Virus

#

3MA	3-Methyladenine
25HC	25-hydroxycholestero

Summary

Human Norovirus (HuNoV) belongs to the *Caliciviridae* family and is the foremost cause of non-bacterial gastroenteritis cases worldwide. HuNoV is prevalent in developed and developing countries and is responsible for significant numbers of morbidity and mortalities each year. Despite the presence of Norovirus (NoV) in communities there is no vaccine or antiviral treatments yet available to ease this burden. This is attributed to the lack of suitable culture systems or animal models available for HuNoV. In 2003 however, a novel mouse Norovirus (MNV) was identified from immunodeficient laboratory mice which has since been used as a model for HuNoV and has provided much insight into mechanisms of NoV replication and pathogenesis.

We investigated the impact of autophagy on MNV replication and found that viral infection induces this cellular process but manipulates it in such a way that inhibits the final maturation and degradation of autophagosomes. Through chemical modulation we found that autophagy appears to be an antiviral response as inhibiting the process increased viral replication. We also show that PI4P and PI4KIIIα are required for MNV replication and MNV most likely utilises its non-structural proteins to recruit these lipids and host factors to sites of replication. Finally, this study investigated whether cholesterol and the PI4P/cholesterol counter flux was required for MNV replication. We showed that cholesterol does not appear to play a role in MNV replication as inhibition of cholesterol synthesis or OSBP had no detrimental effect on replication.

CHAPTER 1:

INTRODUCTION

1.1 The *Caliciviridae*

The family *Caliciviridae* consists of RNA viruses that have been recognised as important pathogens in humans and animals (1, 2). Belonging to the *Caliciviridae* family are genera; *Norovirus, Nebovirus, Lagovirus, Vesivirus* and *Sapovirus* (3). These family members are small (27-35 nm), icosahedral, non-enveloped viruses which possess a single-stranded positive sense RNA genome. Caliciviruses were initially classified as members of the related *Picornaviridae* family due to the presence of sequence homology, but were later reclassified as a separate virus family due to their distinct physical and chemical properties (4-6). The *Caliciviridae* possess a ~7.5 kb genome with a distinguishingly characteristic arrangement of the open reading frames (ORFs); positioned at the 5' end is a polyprotein encoding the non-structural proteins whereas the structural proteins are positioned at the 3' end of the genome (7). Expression of the calicivirus structural genes is mediated via the production of a subgenomic RNA (sgRNA) during replication. The translation of all of these viral RNA species occurs in a CAP and internal ribosome entry site (IRES)-independent manner.

The genus *Lagovirus* has a fairly narrow host range, infecting animals only of the Lagomorpha order (rabbit, hares and pikas) such as Rabbit Haemorrhagic Disease Virus (RHDV) and European Brown Hare Syndrome Virus (6). *Vesivirus* has a broader host range, mainly affecting fauna but can also infect humans. Feline Calicivirus (FCV) and Vesicular exanthema of swine virus are examples belonging to this genus which is usually associated with vesicular lesions, stomatitis and respiratory complications in humans (8, 9). *Nebovirus* is a newly characterized genus of virus that is associated with gastrointestinal disorders in cattle (10, 11).

Unlike Neboviruses, Lagoviruses and Vesiviruses, Sapoviruses and Noroviruses (NoV) are more commonly associated with causing disease in humans. Sapoviruses (SaV) can cause acute gastroenteritis in humans and swine. SaV infections are less common and milder than Norovirus infections and appear to cause disease primarily in children under 5 years of age, however they have been occasionally reported to be the cause of outbreaks in hospitals and health care facilities affecting patients from the very young to the elderly (12-16). Noroviruses (NoVs) are primarily associated with large outbreaks of gastroenteritis in humans; however there are species within this genera that infect mice, pigs, cattle, dogs and primates (17, 18). Fortunately the incidence of transmission of caliciviruses from animals to humans is relatively low, but there have been suggestions that humans may contract zoonotic diseases if exposed (19).

1.2 *Norovirus*

The Norovirus genus consists of a diverse range of viruses that infect a range of mammalian species. So far five genogroups (GI-GV) have been described (Fig 1). GI consists of NoVs that infect humans; GII consists of viruses that infect humans or swine and GIII is associated with causing disease in cattle (17, 20, 21). NoVs that infect humans are primarily those belonging to GI or GII but some have more recently been identified within GIV together with diseases affecting canines and lions (17, 22). Finally, GV strains of NoV are only found in mice (23).

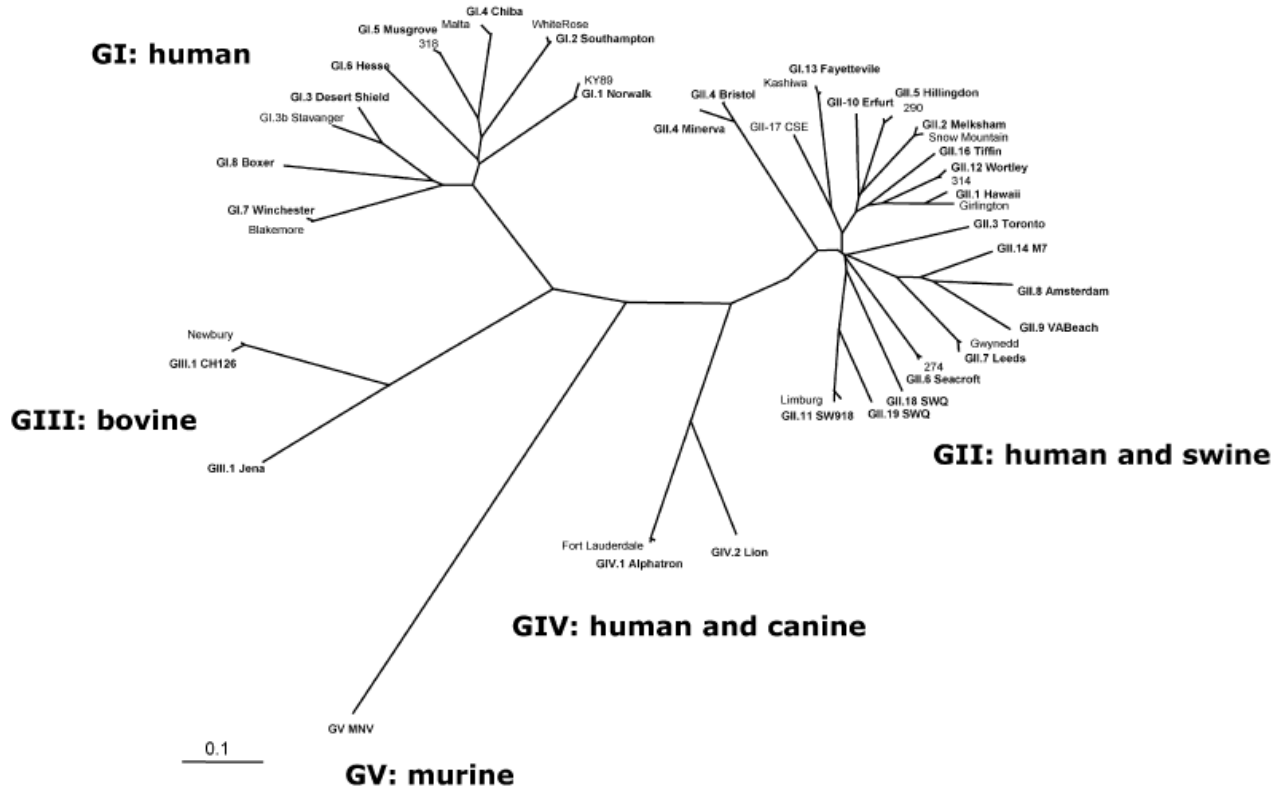

Figure 1. Phylogenetic classification of Noroviruses.
NoVs can be divided into five genogroups (GI-GV) and 32 genotypes based on sequence comparison of the capsid protein (VP1). HuNoVs primarily cluster within the GI and GII but have more recently been found within the GIV. Swine NoVs, bovine NoVs, canine NoVs, and MNV cluster within the GII, GIII, GIV, and GV respectively. Taken from Patel *et al.,* 2009.

1.3 Human Norovirus

Originally termed Norwalk virus (NV), Human Norovirus (HuNoV) was initially identified in an outbreak of acute gastroenteritis in a school in Norwalk, Ohio, in 1968 (24). HuNoV is responsible for the majority of cases of nonbacterial gastroenteritis worldwide and usually occurs in crowded locations such as schools, hospitals, restaurants, cruise ships and nursing homes (25-27). Transmission of the virus is via the faecal-oral route through ingestion of contaminated food or water (28, 29). Typical symptoms of illness include fever, nausea, vomiting and diarrhoea. Onset of illness is usually 24-48 hours after exposure while

symptoms last 1-3 days on average, although those who are immunocompromised may suffer from longer or more severe symptoms. It is estimated that 218,000 deaths and 1.1 million hospitalisations occur in developed countries each year due to HuNoV (30). While the associated illness of HuNoV is relatively short, patients have been noted to have virus detected in their stool for up to 28 days after suffering from the illness. HuNoV is highly infectious as only ~20 viral particles are sufficient to cause infection (31). Further contributing to its pathogenesis is its strong resistance to environmental degradation in various water types (32).

Outbreaks occur all year round and do not discriminate between age groups. GII strains are the most common isolates identified from NoV outbreaks followed by GI, with GIV stains being the least common cause of outbreaks. Of the GII isolates, the GII.4 strains have been shown to be the most common cause of outbreaks of NoV and are the strains associated with global outbreaks of NoV (17). Why GII.4 variants are more prolific than other GII strains is not yet fully understood, but it has been suggested that the higher mutation and evolution rate of GII.4 strains contributes to the increased viral fitness of GII.4 compared to less prolific GII strains (33).

Advances in elucidating the mechanisms of NoV replication strategies and pathogenesis have been hindered by the inability to culture HuNoV in the laboratory (23, 34). As a gastrointestinal disease it is hypothesised that intestinal epithelial cells (IEC) of the gut would be target cells for HuNoV, but extensive efforts to cultivate the virus in epithelial cells have so far been unsuccessful (35-37). In 2014 however, Jones *et al*. (38) used an unfiltered stool sample containing the GII.4 Sydney HuNoV strain to productively infect human B (BJAB) cells in culture. Upon further investigation, they discovered that HuNoV interacts with enteric bacteria which serve as cofactors for facilitating attachment and infection into B

cells (38, 39). Thus it is now suggested that NoVs are transcytosed across the epithelium to where they can access their target immune cells (40, 41).

While this cell culture system for HuNoV in B cells is still only in initial stages of development, research undertaken using the of Mouse NoV (MNV) discovered in 2003 has since proven to be a valuable model for the study of HuNoV infection due to their structural and biological similarities (34, 42-45).

1.4 Mouse Norovirus (MNV)

MNV was first isolated and characterised in 2003 when mice, lacking signal transducer activator of transcription 1 (STAT1) and Recombination-activator gene-2 (RAG-2) (STAT1/RAG2-/- mice), succumbed to an unknown, lethal viral infection (46). Sequence and phylogenetic analyses of infected samples identified the presence of a novel virus belonging to a unique cluster within the Norovirus genus of the *Caliciviridae* family. Further analysis revealed that mice deficient in STAT1 or both type I and type II IFN receptors were highly susceptible to developing a lethal infection compared to wild type mice, suggesting that STAT1-mediated innate immunity is crucial for mice to survive an otherwise lethal infection (46, 47). Subsequently, a tropism for MNV was identified to be restricted to murine mononuclear cells such as macrophages and dendritic cells (DCs) which provided the first cell culture system and animal model that could be used to potentially understand cellular processes during HuNoV infection (48). MNV can infect mice *in vivo* resulting in the classical HuNoV symptoms and tissue pathology which must be monitored in animal facilities and laboratories, as studies of other pathogens in mice may be affected (23, 49, 50).

1.5 MNV genome and non-structural protein function

All NoVs exhibit the same viral particle size (28-35 nm) and possess similar sized genomes and genome organisation. NoVs are non-enveloped, positive-sense RNA viruses of 7.2-7.5 kb in length (51). As seen in all *Caliciviridae*, the NoV genome is protein linked at the 5' end to a viral encoded protein NS5 (VPg) (52) and polyadenylated at the 3' end (53).

In particular, MNV possesses a genome consisting of 4 open reading frames (ORFs) (Fig.2). ORF1 encodes for the 6 non-structural proteins (NS1-2, NS3, NS4, NS5, NS6 [viral protease], NS7 [viral polymerase]) which are translated as a single polypeptide and is subsequently co- and post-translationally cleaved by a viral encoded protease (NS6) to produce the mature viral proteins and polyprotein subspecies (54-56). The major and minor structural proteins, VP1 and VP2 are encoded by ORF2 and ORF3 respectively which together form the virion capsid (3, 57-59). ORF4 encodes for a virulence factor (VF1) that contributes to pathogenesis by delaying the expression of innate immunity genes (55).

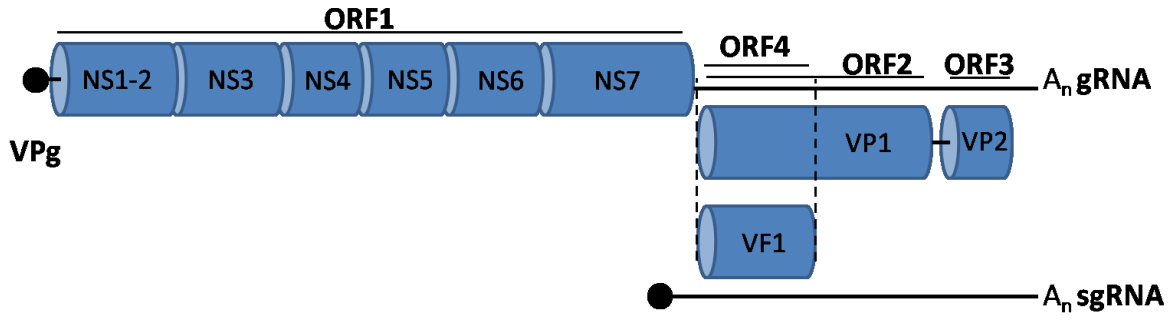

Figure 2. Mouse Norovirus (MNV) genome.
ORF1 encodes the 6 non-structural proteins, ORF2 encodes the major capsid protein VP1, ORF3 encodes minor capsid protein VP2 and ORF4 encodes VF1.

The MNV non-structural (NS) proteins have been determined to play an important role in viral replication (60-62) and likened to the non-structural proteins encoded by the *Picornaviridae* family which have been observed to aid in the assembly of viral replication complexes (60, 63, 64). The replication complex (RC) is a cellularly-derived structure created to provide an environment where efficient viral replication can occur, but is also a means to provide protection from the immune system (65, 66). Some of the NS proteins (NS1-2 and NS4) are observed to associate with the Golgi complex, endoplasmic reticulum (ER) and endosomes, and it is believed these proteins promote the intracellular membrane recruitment used to create vesicle clusters where genome replication can occur (Fig. 3)(60).

NS1-2 is believed to function as a membrane-associated protein in the Golgi apparatus and play a role in inhibiting intracellular trafficking (67). HuNoV NS1-2 was observed to inhibit surface localisation of vesicular stomatitis virus G protein which is suggested to have been mediated via an interaction between NS1-2 and vesicle-associated membrane protein (VAMP)-associated protein A (VAP-A) (67). VAP-A is a protein that plays a role in regulating vesicle trafficking between the ER and Golgi apparatus (68). To further support these findings, transient expression of MNV NS1-2 in Vero cells was observed to co-localise with calnexin in membranes of the ER (69).

Unfortunately, little is currently known about the activity of NS3 during NoV replication. When Norwalk virus (NV) was first sequenced, NS3 was identified as a "2C-like" protein named after the picornavirus 2C protein due to their vast sequence homology (64, 70). The Southampton virus (a GI NoV) NS3 has been observed to exhibit nucleoside-triphosphatase (NTPase) activity similar to the picornavirus 2C (71). Interestingly, when MNV NS3 is transfected individually into Vero cells some co-localisation with the mitochondria is observed and distinct vesicular structures are also formed (69). However in MNV-infected cells, NS3 does not form these distinct vesicular structures nor does dsRNA

co-localise with the mitochondria (69). In light of these findings, the research community is encouraged to further elucidate the function of MNV NS3 to determine the degree to which it mimics picornavirus 2C and transfected MNV NS3 associates with the mitochondria.

Like NS3, little is known about the function of NoV NS4. While it is located in a homologous location to the picornavirus 3A protein on the NoV genome, these proteins do not share similar sequence homology such as NS3 and 2C do. Picornavirus 3A associates with the ER and affects ER-to-Golgi trafficking which can lead to inhibiting of intracellular traffic which is used as an immune evasion strategy. The presence of conserved hydrophobic regions within NS4 suggests that it may be a membrane-associated protein (72). In MNV-infected cells, components of the Golgi apparatus and endosomes localise with the MNV RC (73). Likewise, transiently expressed MNV NS4 associated with markers for the Golgi apparatus and endosomes, and induced changes in Golgi apparatus morphology, implying NS4 plays a role in recruiting these membranes or parts thereof, to the RC (69).

The MNV (together with all caliciviruses) genome is covalently-linked at the 5' end to a VPg (Viral Protein genome-linked; NS5) protein. NS5 has been shown to be extremely important for various calicivirus replication and translation. The VPg protein within both FCV and Noroviruses has been shown to interact with translation factor eIF4E which is required for translation of VPg-linked viral mRNA (74, 75). Earlier studies have demonstrated that FCV virions were not infectious after digestion of VPg with proteinase K, supporting the importance of NS5 in cap-independent translation of RNA (75). Furthermore, it has been shown RHDV and NoV NS5 is uridylylated by the viral-encoded polymerase before it can act as a primer for replication (76).

Not surprisingly, NS6 has often been referred to as a "3C-like" cysteine protease due to the similarities shared between NS6 and the 3C proteases encoded by the *Picornaviridae*

(64, 70, 77, 78). Like other viral RNA proteases, NS6 cleaves the NoV ORF1 polyprotein both in *cis* and in *trans* to generate polyprotein subspecies and mature non-structural proteins required for replication (79). Interestingly, a recent report has identified and characterised a second active form of the protease, NS6-7, which was shown to exhibit different cleavage efficiencies of the ORF1 polyprotein compared to NS6 alone (80). Having two active forms of protease with different cleavage efficiencies most likely allows for additional control of post-translation mechanisms during replication. In addition, another recent study has demonstrated transiently expressed MNV NS6 in Vero 1008C cells strongly associated with the mitochondria, while during infection no such association between mitochondria and the MNV RC has been observed (69). This observation suggests MNV NS6 may be playing a role that extends past post-translational processing. Numerous studies have reported interactions between viral proteases and mitochondria of host cells where proteases are able to modulate the host immune response and apoptosis (81). For example, hepatitis A virus (HAV) co-localises with the mitochondria and is able to cleave mitochondrial antiviral signalling protein (MAVS) which disrupts activation of interferon regulatory factor 3 (IRF3) through the melanoma differentiation associated protein 5 (MDA-5) pathway (82). Likewise, picornavirus 3C has been reported to cleave retinoic acid-inducible gene I (RIG-I) during infection as a means of attenuating the host immune response (82, 83). Identifying the role of MNV NS6 during replication will undoubtedly provide insight into whether this protein can cleave cellular proteins in order to manipulate the host immune response.

Replication of all positive-strand RNA viral genomes is dependent on an encoded RNA-dependant RNA polymerase (RdRp). NS7 catalyses RNA transcription but also catalyses the nucleotidylylation of NS5 (as mentioned above) which is important in both RNA transcription and translation. The crystal structure of HuNoV RdRp, NS7, was first described in 2004 whereas the crystal structure of MNV NS7 was described in 2011 (84, 85).

As exhibited within RHDV, HCV and picornaviruses, the structure of NV NS7 possesses conserved fingers, palm and thumb domains which is typical of viral RdRps (86-88). A recent study compared 3 HuNoV-RdRps (GII.b, GII.4 and GII.7), a MNV-RdRp and 2 SaV-RdRps and found the majority of the physiological characteristics of the polymerases were all similar, implying that the use of MNV as a model for human caliciviruses is quite plausible and may be clinically useful when designing antivirals or vaccines (89).

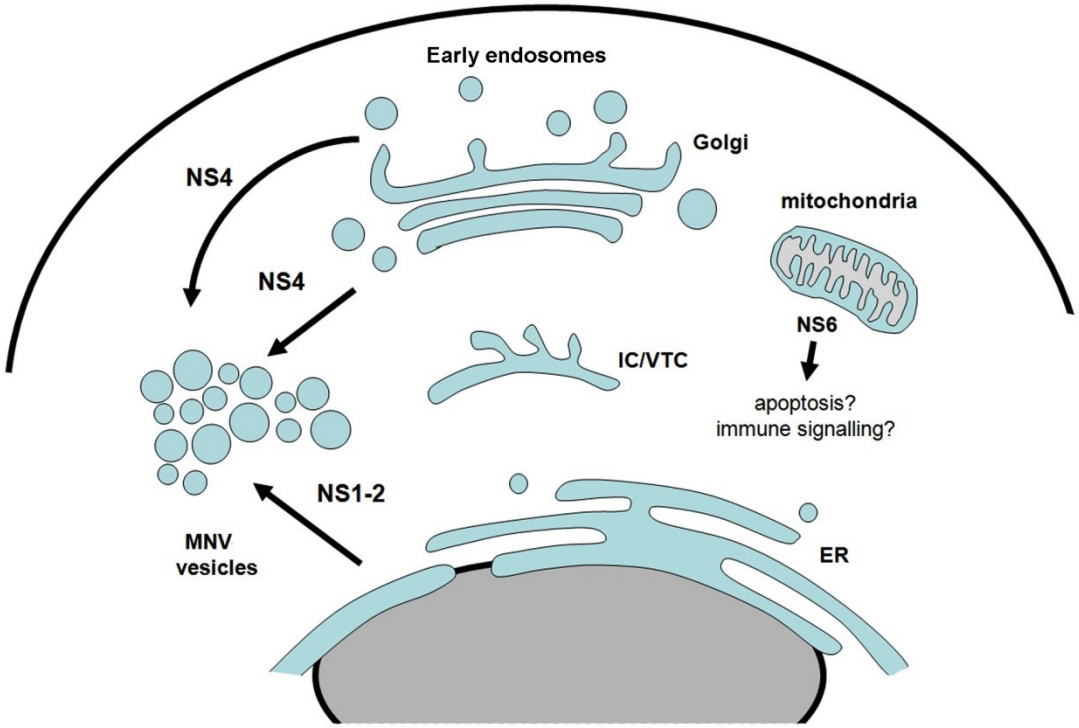

Figure 3. Model of intracellular membrane recruitment by MNV ORF1 proteins during MNV replication.
This model proposes that NS1-2 localises to the ER while NS4 localises to the Golgi apparatus and endosomes in order to recruit these membranes to the MNV RC adjacent to the nucleus. It is suggested that NS6 may localise to the mitochondria-associated proteins to modulate apoptosis or the host immune system (adapted from Hyde 2011 Thesis).

1.6 MNV Replication

1.6.1 *Entry*

Many viruses require endocytosis to gain entry and establish infection within host cells. Endocytic pathways are broadly described as either clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIDE) pathways. Viruses can exploit one of these pathways or a combination of the two (90). Recent studies of MNV replication have shown that MNV infection of RAW264.7 cells is clathrin- and caveolae-independent (91). The same study confirmed MNV infection does not require either a low pH within acidic endosomes nor flotillin-1 (91). Interestingly, MNV does require cholesterol and dynamin-II to gain entry into cells (91, 92). Many CIDE pathways originate from lipid rafts and thus require cholesterol. Methyl-β-cyclodextrin (MβCD) depletes cholesterol from the plasma membrane and disrupts lipid rafts and endocytic pathways. When cells were treated with MβCD prior to infection, MNV infection was significantly decreased. When MβCD was added post infection however, no significant change in MNV infection was seen (91, 92) indicating cholesterol plays a significant role in MNV entry into cells.

In terms of HuNoV, the capsid has been reported to bind to histo-blood group antigens (HBGAs) which are carbohydrates located on the cell surface of red blood cells. Many studies suggest that HBGAs are receptors for viral attachment and HuNoVs interact with these receptors in a strain-specific manner (93-97).

Many viruses including FCV use sialic acid (SA) as a receptor that is crucial for entry (98). It has recently been identified that ganglioside-linked terminal sialic acid also functions as attachment receptors for MNV (99). Gangliosides are glycosphingolipids that can contain sialic acid moieties. When SA-binding lectins were used as competition for MNV binding, ganglioside GD1a was depleted or sialidase treatment was added to cells prior to infection,

MNV replication was significantly decreased (99). Interestingly, we know that MNV has a specific tropism for murine macrophages, whereas murine intestinal cells (among many other cell types) also have SA and gangliosides present on their cell surface yet MNV does not, or cannot, infect these cells (100, 101). This suggests that there has to be alternate molecules that act as entry receptors which facilitate internalization of MNV on these macrophages, which in turn contribute to the restricted host tropism.

Two recent studies suggest that the interaction of norovirus with cellular receptors, and therefore the cell-entry steps, is what dictates the specific host cell tropism exhibited by noroviruses (102, 103). CD300lf is a cell-surface molecule belonging to a family of proteins that possess lipid-binding properties (104). The recent studies have shown that CD300lf is essential for MNV infection as it mediates viral binding and thus is a functional receptor for MNV (102, 103). MNV infection was significantly inhibited in CD300lf knock-out cells. Likewise, MNV infection was significantly impaired when antibody against murine CD300lf was introduced to cells before MNV infection, demonstrating that when CD300lf binding sites were occupied by antibodies, MNV could not bind and infect cells (102, 103). Interestingly, when human HeLa cells were transfected with mouse CD300lf they became susceptible to MNV infection (103). In light of these findings, future studies of MNV in human cell lines may aid in uncovering novel mechanisms of replication and pathogenesis of noroviruses across different species.

1.6.2 *Genome replication*

Viral genome replication relies on the viral proteins being translated by the host cell translation complex. As mentioned previously, MNV genomic RNA serves as a template for the initial translation of the NS proteins, whereas the structural proteins VP1 and VP2 or

VF1, are translated from subgenomic RNA (55, 105). The 5' and 3' untranslated regions (UTRs) flanking the ORFs interact with host proteins involved in regulation of translation and are essential for efficient genomic RNA translation (106) (107).

Once the host cell translation complex has been recruited, the NoV ORF1 is translated as a polyprotein, which is cleaved into the mature NS proteins by the viral protease (NS6) (108). The proteases of HuNoV and MNV share about 60% of their sequence identities and also share the conserved residues H30 and C139, which have been proven to be essential for the protease activity (109). The efficiency of protease cleavage differs at the individual ORF1 cleavage sites, revealing that cleavage at the NS2-3 and NS3-4 junction occurs at a higher rate than at the NS4-5, NS5-6 and NS6-7 junctions (110) (111).

Like any ssRNA virus, MNV genome replication is dependent on generating a negative sense intermediate, which serves as a template for the positive sense genomic RNA (Fig. 4). The positive and negative strands of the genomic and subgenomic RNA are synthesized by the viral RdRp (NS7), which is initiated by *de novo* or VPg-dependent replication (112, 113).

Replication begins with the generation of the negative sense template which is supported by the interaction of the RdRp with the S-domain of VP1 (114). The positive sense genomic RNA replication is initiated via VPg, which serves as a protein primer at the 3' end of the negative sense template (112). The RdRp covalently binds VPg to the initial nucleotide of the genomic and subgenomic RNA via a tyrosine residue that is conserved in HuNoV and MNV (Y^{27} and Y^{26}, respectively) (113). In addition to the genomic RNA, a subgenomic RNA containing the ORFs 2 and 3 is generated by the RdRp (115). The subgenomic RNA is identical with the last 2.4 kb of the genomic RNA and is also linked to VPg and poly-adenylated. Two mechanisms by which the subgenomic RNA replication is initiated have been proposed: premature termination and internal initiation (105). The latter has been

supported by findings of conserved secondary structures upstream of the VP1 sequence? (106). These structures were recently identified as the promoter for the subgenomic RNA (116) which consists of a highly conserved stem loop structure and is located 6 nucleotides upstream of VP1 in the coding region of NS7 (116). This promoter, as well as a short template sequence, are preferentially recognized by the RdRp of HuNoV and MNV, and ensure stable binding of the RdRp (117). It should be noted that the promoter sequence at both the 5' end of the genome and preceding the subgenomic RNA are the same; GTGA.

Following translation and processing of the NS proteins, these proteins then initiate the biogenesis of the viral replication complex in the cytoplasm of the host cell. The most comprehensive analysis of the MNV RC so far has demonstrated that the RC contains each of the NS proteins, together with VP1 (69, 118). This complex can be observed as punctate foci, localizing close to the microtubule organizing centre (MTOC) (119). Interestingly, VP1 has been shown to co-localise with acetylated tubulin, which suggests a potential role of the cytoskeleton in the positioning of the RC within the cell (119). The RC is comprised of host cell membranes and proteins that are redistributed and modified during infection (44, 73). These membranes are derived from the endoplasmic reticulum, Golgi apparatus and endosomes of host cells through a mechanism that is currently unknown (69, 73, 120).

1.6.3 *Assembly and Exit*

Unfortunately, the processes driving viral assembly, encapsidation and exit of MNV are still generally unknown. In terms of assembly, NV VP1 is able to self-assemble into virus-like particles (VLPs) which are indistinguishable from native virions (121) suggesting a similar route is employed during virus replication. VP2 is not required for VLP assembly but is required for the production of infectious virions as it provides stability for VP1 (72, 121).

Studies of FCV have shown a possible interaction between VP1 and VPg-linked viral RNA, which would suggest a mechanism for specific encapsidation of viral RNA (122) but it is unknown whether MNV utilizes the same mechanism.

Although much is still unknown about MNV exit from host cells, research shown that apoptosis plays an important role in the MNV life cycle which may serve as an exit strategy. While not yet thoroughly described, apoptosis is induced during MNV replication and survivin (a pro-survival factor) is down-regulated (123, 124). VF1 is able to delay apoptosis which may serve to provide MNV with more time for replication before exit (125). Importantly, when apoptosis is inhibited, MNV production was significantly reduced, suggesting the requirement of apoptosis in the viral life cycle (124).

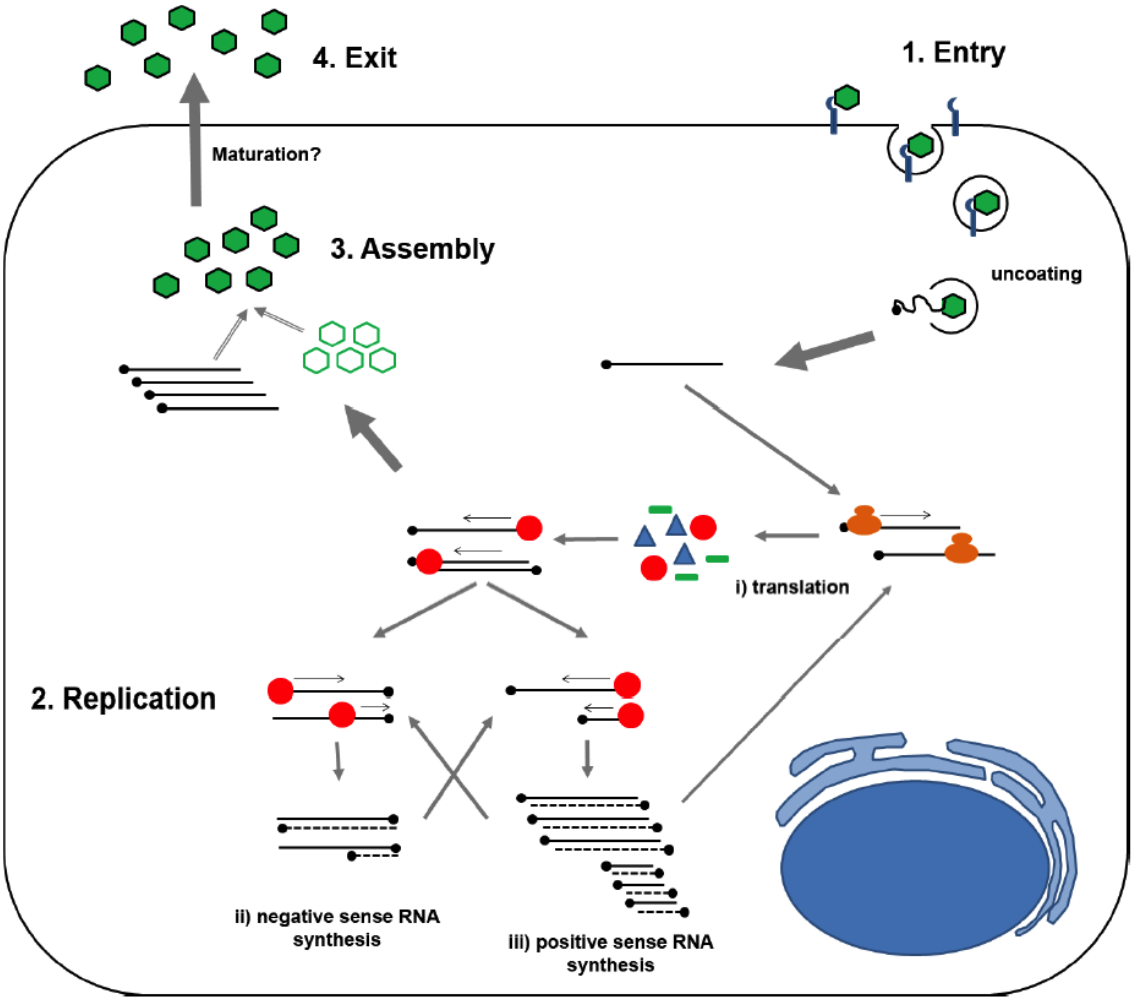

Figure 4. The replication cycle of Norovirus.
Receptor-mediated entry (1) is followed by uncoating in the cytoplasm. The positive-sense RNA genome of MNV then acts as a template and is translated by host cell machinery which generates the non-structural proteins (2i). Negative-sense RNA is transcribed from the positive sense template by the RdRp (2ii). Anti-sense gRNA is then used to generate new positive-sense gRNA and sgRNA which is then translated into viral non-structural and structural proteins (2iii). Subsequently, full-length gRNA is assembled into virions and trafficked to the periphery of the cell (3) where the viral progeny are released via a means that most likely involves apoptosis (4). Taken from Hyde 2011 Thesis.

1.6.4 *Intracellular membrane manipulation*

As demonstrated by arguably all RNA viruses, infection with MNV induces extensive reorganization of intracellular membranes which the virus uses to construct its RC (27). The evidence for and discovery of MNV prompted research into the MNV replication cycle and mechanisms. MNV has been observed to induce the formation of vesicle clusters in which the virus replicates (Fig. 5). Studies by Hyde *et al.* (2009), have established that these vesicle clusters contain components of the endocytic and secretory pathway, however, the exact origins, composition, and mechanisms of how these RCs form still remain unknown (1). Interestingly, there are parallels observed between the presentation of MNV-induced vesicle clusters and the proliferation of membranes induced by autophagy. The induction of cellular-derived vesicle clusters via viral infection produces cellular features that resemble the autophagic membranes of autophagosomes when observed using an electron microscope. Since RNA viruses require cellular membranes for RC assembly, the manipulation of autophagy is valuable for viruses as the process presents readily formed membranes which the viruses can recruit for replication (Fig. 5).

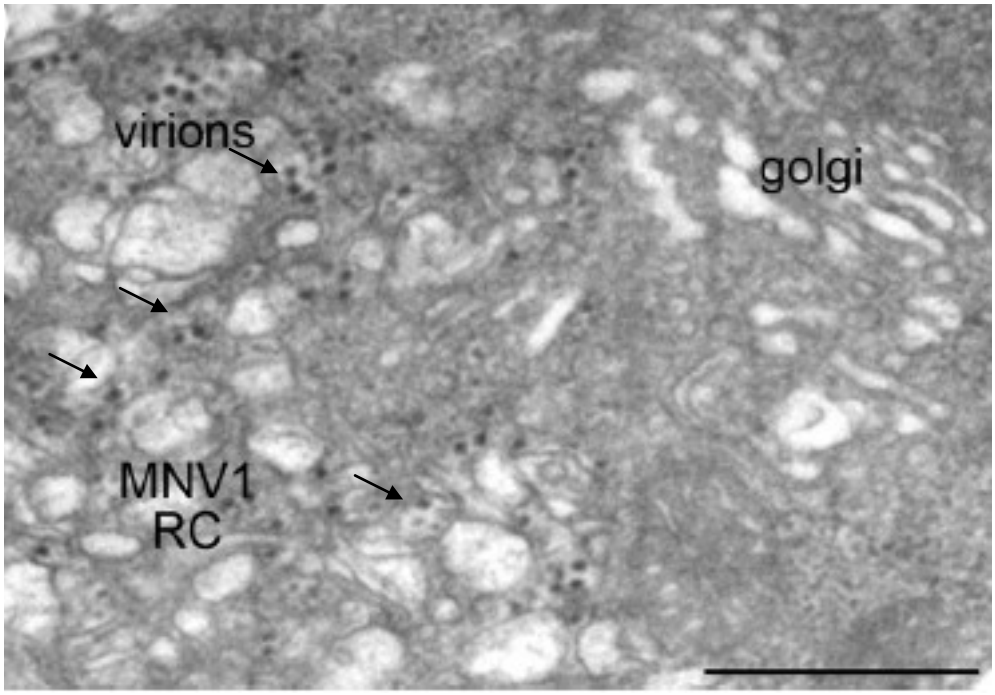

Figure 5. MNV induces intracellular membrane arrangements to establish the replication complex.
Resin-embedded sections of MNV-infected RAW264.7 cells demonstrating the creation of the MNV RCs with associated virions. Arrows indicate cellular-derived vesicle clusters where viral replication takes place. Taken from Hyde *et al.,* 2009.

1.7 Autophagy

Autophagy is a cellular mechanism by which the cell is able to degrade, recycle and manage intracellular contents. Importantly, it can also be used as a survival mechanism under starvation conditions or during threat from pathogens such as bacteria and viruses (126, 127).

Autophagy involves the creation of an autophagosome, a double membraned vesicle which is facilitated by the presence of microtubule-associated protein light chain 3 (LC3) and Autophagy (Atg) proteins (128). There are over 30 Atg proteins involved in the process of autophagy. LC3 and Atg proteins assist in both the creation of the autophagosome and its elongation, as cytoplasmic contents are sequestered (Fig. 6) (128). Once the autophagosome is matured it fuses with lysosomes and the contents are degraded or recycled (129). One of the major complexes required for autophagy consists of Atg16L1 bound to an Atg5-Atg12 conjugate that is responsible for the elongation and closure of the autophagosome (130, 131).

A recent study has confirmed that mice lacking both IFNα/β and IFNγ receptors are unable to prevent an MNV infection from becoming lethal however when only one receptor is lacking, the mice are resistant to the potentially lethal infection (132). In addition, they showed that Atg5 expression was essential for the control of MNV infection in mice with compromised IFNα/β signalling and also Atg5 was critical for the IFNγ-mediated control of MNV infection in macrophages (132). Interestingly, while the degradative role of autophagy appeared to have no effects on the control of MNV replication by IFNγ, the conjugation of Atg5-Atg12 is required together with the binding of Atg16L1 to this conjugate for IFNγ-mediated suppression of MNV (132).

Autophagy also plays an important role in the adaptive immune system as it promotes the major histocompatibility complex (MHC) class II presentation of cytosolic antigens (133-135). Whilst autophagy is primarily regarded as a defence mechanism against invading

bacteria or viruses, the pathway can be manipulated by internal pathogens to promote their own proliferation (136, 137). There are many suspected reasons for which viruses use autophagy to facilitate replication; the double membraned autophagosomes provide a scaffold for RNA viruses on which to construct their RC, a means to eschew immune detection or may also provide a mechanism for extracellular delivery without lysing the cell (137, 138).

1.7.1 *Microtubule-associated protein light chain 3 (LC3)*

LC3 is a mammalian homologue of Atg8 and was originally identified as one of three light protein chains associated with MAP1A and MAP1B and was thought to be involved with the regulation of microtubule assembly and disassembly (139). It was then discovered that LC3 has an autophagy-specific role and became a widely used marker to indicate the occurrence of autophagy in cells (139-141).

Once autophagy has been induced, LC3 conjugates to phosphatidylethanolamine (PE) and is recruited to autophagosomal membranes (128). LC3 will stay associated with the autophagosomes throughout the process of autophagy and will eventually be degraded or recycled back into the cell (142). The unconjugated form of LC3 (LC3-I) and the conjugated form (LC3-II) can be clearly distinguished from one another via immunofluorescence or blotting (128, 140). LC3-I is distributed ubiquitously throughout mammalian cells whereas the presence of LC3-II is indicative of autophagosomes and appears as large punctate regions (142).

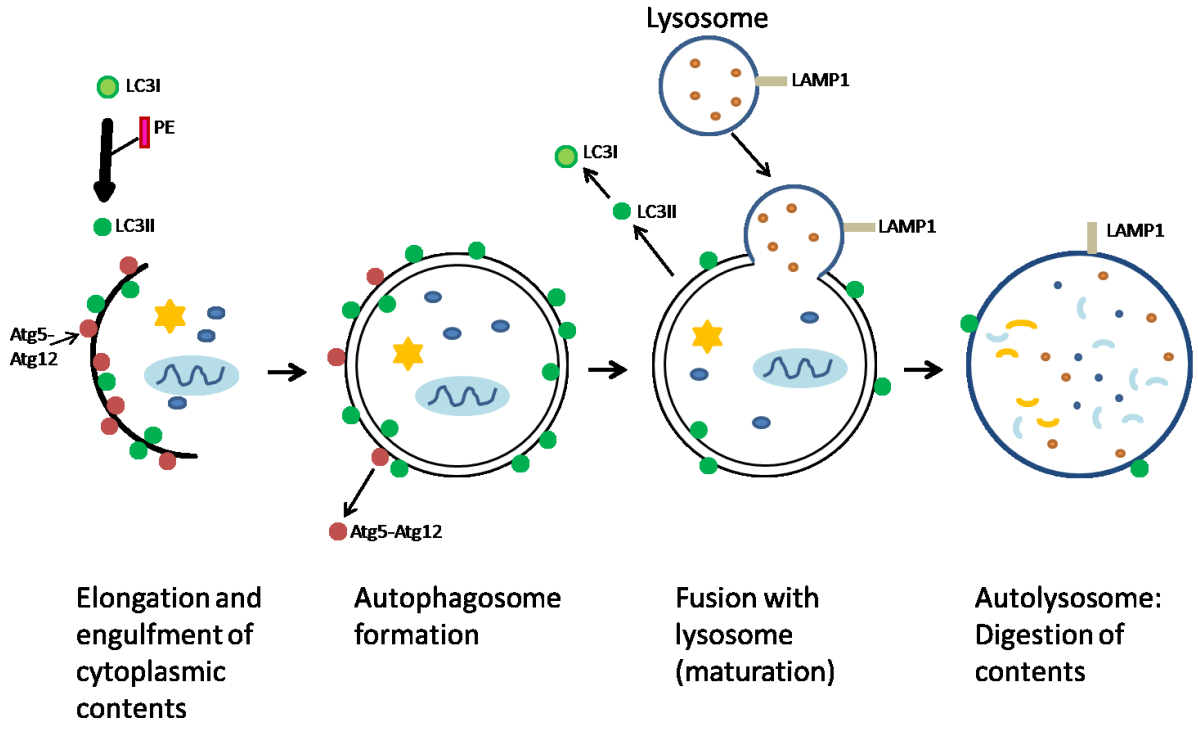

Figure 6. The role of LC3 in the autophagy pathway.
When autophagy is induced LC3 and the Atg5-Atg12 complex are recruited and facilitate the formation and maturation of the autophagosome. LC3 remains associated with the autophagosome throughout the duration of autophagy where it can be recycled back into the cell after the contents of the autophagosome are degraded.

1.8 Autophagy and viruses

Many viruses are able to manipulate the process of autophagy to benefit their own means of replication. Virus survival depends on the innate ability of the virus to avoid cellular anti-viral defences and to effectively manipulate cellular processes for their own proliferation (136, 138).

In particular, many studies have been conducted on picornaviruses and how they are able to manipulate autophagy (138, 143). Poliovirus (PV) and Foot-and-Mouth Disease Virus (FMDV) both induce rearrangements of intracellular membranes into vesicular structures where replication of their genome takes place (144, 145). It has also been recently discovered

that these viruses use mechanisms of autophagy to aid and facilitate replication (146, 147). PV is able to manipulate the cell in such a way that it induces the formation of autophagic membranes but inhibits their final maturation and ability to fuse with lysosomes potentially to prevent nascent virions being degraded (138, 146-148). PV RCs are located on these reorganized intracellular membranes that resemble autophagosomes and are created by the non-structural proteins (146, 149-151). Likewise, FMDV non-structural proteins 2B, 2C and 3A co-localise with the autophagosome marker LC3 which indicates that replication is occurring at the site of autophagy. FMDV is able to trigger autophagy which in turn enhances viral replication by generating a proliferation of membranes which FMDV uses for creating RCs (144, 146). While it is not yet known how FMDV is able to induce the activation of the autophagic machinery it has been shown that FMDV replication is dependent on autophagy (146). FMDV infection is also able to reduce the number of MHC class I molecules on the surface of epithelial cells and thus will limit or decrease the presentation of viral peptides and delay detection by the host (152, 153).

Infection with HCV also induces the process of autophagy and it has been shown that the autophagic machinery is imperative for initial HCV RNA translation/replication (154). In the absence (inhibition or knock-down) of autophagy, HCV could not establish a productive infection. Interestingly, autophagy was not required for HCV entry or exit and neither were autophagosome membranes recruited for RC assembly, which suggests HCV exploits this pathway via an undefined mechanism during early phases of its life cycle (154).

It is well established that DENV infection induces autophagy and this process is required for efficient DENV replication (155-157). Like HCV, DENV proteins do not associate with autophagosomes, but interestingly, DENV-induced autophagy regulates lipid metabolism (155). DENV infection led to the autophagy-dependent depletion of lipid droplets and triglycerides. The decrease in triglycerides was subsequently associated with increased β-

oxidation and generation of ATP, factors which are known to enhance DENV replication (155). These studies provide us with yet another mechanism by which viruses are able to utilize autophagy.

As mentioned previously, most of the positive strand RNA viruses are in fact very similar in terms of both replication and the induction of membranous vesicle formation to create RCs (158, 159). In particular, many comparisons between the C*aliciviridae* and *Picornaviridae* can be made. The similarities between caliciviruses and picornaviruses include their method of replication which occurs in cellular-derived membrane vesicles induced by viral infection, however little is known about the composition and mechanisms by which these RCs are formed (1, 160, 161). Also shared are similar mechanisms for releasing their non-structural proteins from the polyproteins by cleavage via specific viral proteases (162, 163). Due to the similarities between the two families of viruses, it is a reasonable assumption that norovirus may also use or manipulate autophagy for its own benefit using similar methods.

1.8.1 *Autophagy as an antiviral response*

The process by which autophagosomes engulf viruses or viral components leading to their destruction is known as xenophagy (Fig. 7a) (164). This process is regulated by dsRNA-dependent protein kinase (PKR) and eukaryotic initiation factor 2α (eIF2α) phosphorylation (165, 166). Activation of PKR leads to phosphorylation of the α subunit of eIF2α which leads to the shutdown of host and viral protein synthesis and viral replication. Many viruses have evolved strategies to antagonise PKR function and avoid the translational shutdown. It has been revealed that xenophagy plays a role in degrading herpes simplex virus type 1

(HSV-1), however HSV-1 encodes a neurovirulence protein, ICP34.5, that is able to dephosphorylate eIF2α and negate the activity of PKR and inhibit autophagy (165).

There are two highly important mammalian innate immune pathways that function to combat RNA viruses. One of these pathways involves cytoplasmic helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5). These enzymes are structurally similar but recognise different virus-derived immunostimulatory RNA structures (isRNA), 5'-triphosphorylated RNA or dsRNA respectively (167, 168). When activated, these sensors facilitate a signalling pathway that eventually leads to production of type I interferons (169).

When wildtype mouse embryonic fibroblasts (MEFS) and Atg5 knockout (KO) MEFS were treated with Poly(I:C) the Atg5 KO MEFS produced significantly higher levels of type I IFN mRNA (170) suggesting that the mechanism of autophagy suppresses IFN production. When examined further it was established that the Atg5-Atg12 conjugate interacts with RIG-I and MDA5 which suppresses the production of type I IFNs (170). This finding presents another explanation of why viruses such as vesicular stomatitis virus (VSV) induce autophagy during infection; not only do autophagosomes readily provide membranes to recruit, but autophagy contributes to viral replication by inhibiting the innate immune response.

The second innate immune pathway acting to combat RNA virus infections involves Toll-like receptors (TLRs). Plasmacytoid dendritic cells (pDCs) and macrophages have TLR7 and TLR9 in the endosomes which recognise ssRNA and dsDNA respectively (171, 172). When triggered, activation of these TLRs leads to expression of type I IFNs (173, 174). Though the mechanism is not yet fully understood, autophagosomes can deliver cytoplasmic viral nucleic acids to endosomal TLRs. TLRs bind to the ligands and activate interferon

regulatory factor 7 (IRF7), which then leads to type I IFN-dependent immune responses (Fig. 7b) (175).

The other important arc of autophagy as an antiviral defence is antigen presentation. Autophagy plays a role in the adaptive immune response as this process can promote major histocompatibility complex (MHC) class II presentation of cytosolic antigens (Fig. 7c) (176). It has been shown that autophagy is a constitutively active pathway in all MHC class II positive cells including dendritic, epithelial and B cells (177). Moreover, autophagosomes fuse with MHC class II-loading compartments (MIICs) and the targeting of antigens to autophagosomes led to increased MHC class II presentation (177). An example of this is the MHC class II processing of the nuclear antigen 1 (EBNA1) of the Epstein-Barr virus (EBV). EBNA1 was shown to associate with lysosomes and autophagosomes after inhibition of lysosomal acidification and the inhibition of autophagy significantly down-regulated the MHC class II processing of EBNA1 (177).

While the exact mechanisms of autophagy acting as an antiviral response still remain unclear, there is no doubt that this response plays a vital role in innate and adaptive defences against invading pathogens.

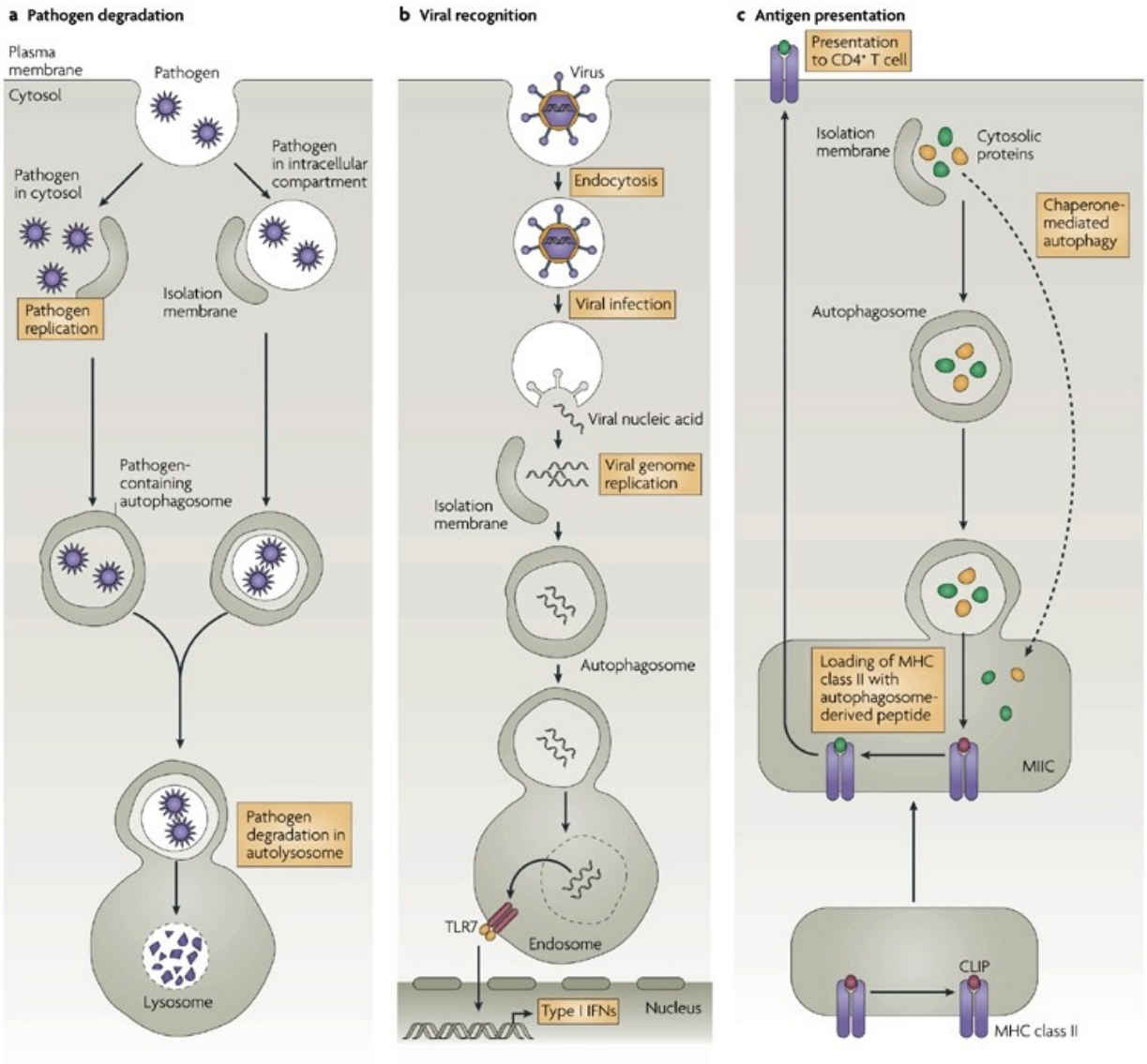

Figure 7. The process of autophagy acting as an antiviral response.
a) Intracellular pathogens are engulfed by autophagosomes which then fuses with lysosomes and is degraded in the autolysosomes. b) autophagy transfers viral nucleic acids from the cytoplasm to endosomes containing TLR7 which signals the induction of type I interferon production (innate immunity) c) viral antigens are engulfed by autophagosomes that fuse to MIICs and are loaded onto MHC class II molecules for presentation (adaptive immunity). (Taken from Levine and Deretic 2007).

1.8.2 *Non-canonical means of inducing autophagy*

Recent studies have demonstrated that autophagy can be induced in a PI3K-independent manner which differs from canonical autophagy. Unfortunately the mechanisms are not yet known of how the process of autophagy is induced independently of PI3K. Hepatitis C Virus (HCV) is a member of the *Flaviviridae* family and is also a positive-stranded RNA virus. Infection with HCV induces the accumulation of autophagosomes in those host cells and it is thought that the cellular process enhances viral replication (178). Interestingly, HCV is able to manipulate the cell and induce autophagy in a PI3K-independent manner. This has been demonstrated by the presence of LC3-II in infected cells when treated with 3-Methyladenine and also with siRNA directed against Beclin-1 (178).

Pattern recognition receptors (PRRs) such as TLRs play an important role in the activation of autophagy upon the recognition of pathogen molecules (179). TLRs are a class of proteins that play a very important role in the innate immune system. In particular, TLRs 3, 7 and 8 are generally confined to endosomes, and recognize viral nucleic acid, particularly single stranded RNA oligonucleotides containing guanosine- and uridine-rich sequences (180, 181). Lee *et al* have shown that the process of autophagy delivers Pathogen-associated molecular patterns (PAMPs) to the endosomal and lysosomal TLRs (175). Conversely, a more recent study by Delgado *et al* demonstrated that PAMPs may in fact stimulate autophagy through TLR ligand recognition (181). They have shown that TLR7 is required specifically for TLR ligand-induced autophagy but not required for starvation-induced autophagy (181).

This area of research is part of the broader research aim to aid in elucidating how NoV interacts with and manipulates the host cell. Like any virus, the more known about its

pathogenesis, the more opportunities may arise to create vaccines, improve treatment methods and prevent the illnesses they inflict.

1.9 RNA viruses require intracellular membranes

All RNA viruses induce membrane structures inside their host, whether it be animal, plant or insect cells. Replication of positive-sense RNA viruses is directly associated with these unique membrane structures that form around the replication complex and provide a microenvironment where RNA synthesis can occur. These membrane-bound RCs generally consist of small vesicles or platforms that accumulate near the perinuclear region (182-190). These vesicles provide a stable environment for viral replication but also provide a means of hiding replicating RNA from the host's immune response (191). These membranes can be recruited from many different organelles within the cell. In fact, it appears that each family of viruses has their own method to manipulate and recruit host cells membranes to form their RC. The RCs of picornaviruses, flaviviruses, arteriviruses and bromoviruses are known to be largely associated with the endoplasmic reticulum, whereas togavirus replication is associated with endosomes and lysosomes (188, 192-194). Tombusviruses require chloroplasts and peroxisomes whereas nodaviruses use mitochondria for sites of replication (195, 196).

As obligate intracellular parasites it's obvious that manipulating host membranes is crucial for viral proliferation. Investigation into which particular lipids viruses are recruiting to sites of replication will provide insight into mechanisms of pathogenesis and also hopefully aid in the treatment of these viruses.

1.10 Cellular lipids known to be recruited by viral replication

Lipids are a sundry group of naturally occurring compounds made up of fatty acids and their derivatives (197). Lipids are one of the most profuse types of cellular molecules that exhibit vast amounts of biochemical and physiological cellular functions. They are the main constituent of the plasma membrane, ER, Golgi, endosomes and lysosomes; though the lipid composition of these structures vary amongst tissue types (198, 199). Varying cell types together with the location of the cells determines the lipid compositions and cellular membrane structure. These variations in lipid composition may determine how viruses target their host cells and provide another example of viruses adapting to manipulate their host (200).

It was once thought that the primary role of lipids were limited to membrane morphogenesis; but advances in lipidomics has illuminated the role of lipids in various cellular functions such as energy production, structural changes of membranes, protein modification and stability, signalling platforms (such as lipid rafts) and inflammation (199, 201).

Viral replication is a complex process which requires and regulates many host factors including lipid metabolism and redistribution (202, 203). At any stage during the viral life cycle, cellular lipids can play a very important role. For instance, many viruses such as PV, echovirus I (EV1) and Semliki Forest virus (SFV) depend on cholesterol-rich regions in the cellular membrane to gain entry into host cells (204-206). Fatty acids, lipid droplets, free cholesterol and phosphatidylinositol-4-phosphate (PI4P) have all been shown to play a key role in various viral life cycles and replication (156, 207, 208). Additionally, enveloped viruses require association with lipids for viral exit. Alphaviruses Semliki Forest virus and

Sindbis virus are both dependent on cholesterol for exit and budding from host cells (209, 210).

1.10.1 *Phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4-kinases (PI4Ks)*

Phosphoinositides are an important class of lipids that are involved in cellular lipid metabolism, transport and lipid-mediated signal transduction. Of particular interest to this study is phosphatidylinositol 4-phosphate (PI4P) which is harboured in the membranes of the Golgi complex, plasma membrane and to a lesser extent the ER (211, 212). PI4P regulates forward trafficking from the Golgi to cell periphery (213, 214). In humans, there are two classes of phosphatidylinositol 4-kinases (PI4Ks) responsible for the production of PI4P. Type II PI4Ks (PI4KIIα and PI4KIIβ) are responsible for the majority of PI4K activity in the plasma membrane whereas Type III PI4Ks (PI4KIIIα and PI4KIIIβ) are responsible for the generation of PI4P in the Golgi apparatus (215-217). This study will focus on PI4KIIIα and PI4KIIIβ. PI4KIIIα resides primarily in the ER of mammalian cells whereas PI4KIIIβ is the resident kinase of the Golgi apparatus (218, 219).

Recently, it has been determined that many viruses utilize or manipulate PI4P and its production within the host cell. For example, PI4KIIIβ has been determined to play a crucial role in the replication of multiple picornaviruses. Replication of both Coxsackie Virus B3 (CVB3) and poliovirus (PV) from the *Enterovirus* genus crucially relies on PI4KIIIβ activity (220, 221). The 3A protein recruits the kinase to sites of replication to promote PI4P production (220). The enriched regions of PI4P likely attract the RdRp (called $3D^{pol}$), as this binds to PI4P and facilitates replication (220). Additionally, Rhinovirus has also been shown to depend on PI4KIIIβ activity and its generation of PI4P-rich regions to support replication (222).

Like other members of the *Picornaviridae,* Aichi virus (AiV) of the genus *Kobuvirus,* recruits PI4KIIIβ to sites of viral replication (223). Unlike the *Enteroviruses* however, AiV non-structural proteins (2B, 2BC, 2C, 3A and 3AB) bind to the Golgi protein acyl-coenzyme A binding domain containing 3 (ACBD3) and PI4KIIIβ which then forms a complex and promotes the generation of PI4P at viral replication sites (223). Due to the similarity of picornaviruses and caliciviruses we hypothesized that MNV would also require PI4P for the generation of the replication complex.

The Arf family of GTPases are required for protein trafficking through the Golgi apparatus and regulating membrane dynamics. Arf1 is activated and becomes membrane-bound by a guanine exchange factor (GEF) known as GBF1, where GBF1 catalyses the GDP/GTP exchange on Arf1 which then in turn recruits coat proteins or lipid modifying enzymes such as PI4KIIIβ (224-227). The 3A protein of CVB3 has been reported to interfere with GBF1, inhibiting the activation of Arf1 which induces a general inhibition of secretory pathway transport, i.e., ER-to-Golgi transport has been blocked (228). The reasoning behind this inhibition of transport may be to prevent the secretion of cytokines and delivery of viral antigens to MHC molecules.

On the other hand, some viruses actively recruit Arf1 and GBF1 to sites of viral replication. Activated Arf1 is responsible for the delivery of PI4Kβ to the Golgi membrane where the production of PI4P is increased (229). Enriched pools of PI4P are required for HCV replication, and it has been shown that Arf1, GBF1 and PI4KIIIβ all co-localise during infection, suggesting that HCV requires all these factors for the generation of PI4P and efficient replication (229). Further studies revealed that the NS5A protein of HCV recruits PI4KIIIα (predominately found in the ER) to the viral replication complex and NS5A

stimulates PI4Kα activity to enhance PI4P accumulation which is essential for HCV replication (208, 230).

Considering the interaction picornaviruses have with PI4Ks, it will be interesting to discover whether MNV also utilises these kinases and lipids for the biogenesis of the RC.

1.10.2 *Cholesterol*

Mammalian cellular membranes are composed of a lipid bilayer containing phospholipids and cholesterol. Membrane fatty acids, phospholipids and cholesterol content can be modified in mammalian cells which disturbs membrane fluidity and affects a variety of cellular functions (231). Cholesterol biosynthesis is regulated within the ER which involves membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs) (232). SREBPs play a role in activating genes that up-regulate cholesterol synthesis and uptake, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and fatty acid synthase (233). Cellular cholesterol is also regulated by the intake of extracellular cholesterol (234) and *de novo* synthesis cholesterol (235).

De novo synthesis of cholesterol is regulated by various enzymes including 3-hydroxy-3methyl- glutaryl-coenzyme A (HMG-CoA) reductase and acyl-CoA:cholesterol acyltransferase (ACAT) (236). HMG-CoA and ACAT are critical in cholesterol synthesis, as HMG-CoA acts as a rate-limiting enzyme and is modulated by complex regulatory controls, while ACAT converts excess intracellular free cholesterol to cholesteryl ester in cells (236). Statins are HMG-CoA reductase inhibitors which reduce plasma cholesterol levels by up-regulating low-density lipoprotein receptors (LDLR) and promoting uptake of LDL bound cholesterol to cells.

Enteroviruses use clathrin-mediated endocytosis (CME) to gain entry into cells but also exploit this process to enrich intracellular free cholesterol pools and traffic cholesterol to sites of replication (237). Confocal microscopy has demonstrated that all replication organelles (ROs) of CVB3, PV rhinovirus and echovirus are enriched with free cholesterol. The cholesterol found in these ROs is required for efficient enterovirus replication as cholesterol regulates the 3CD polymerase (237). Fascinatingly, the 3A protein recruits recycling endosomes through Rab11 and targets cholesterol back into the RO instead of it cycling back to the PM (237).

Several authors have established that Flaviviruses such as WNV and DENV both depend on cholesterol to mediate viral replication (238-240). When cellular cholesterol levels were depleted by inhibiting HMGCR activity together with the release of cholesterol from late endosomes and lysosomes, DENV virions were trapped in late endosome/lysosome compartments (241) and replication was reduced, however the formation of RCs was not altered, indicating that the depletion of cellular cholesterol effects DENV replication at a different stage of the replication life cycle (239). In contrast, Mackenzie *et al.,* (2007) showed that WNV redistributes cholesterol to viral replication complexes by up-regulating cholesterol biosynthesis. Reduction of HMGCR, manipulation of cholesterol level and altering cellular geranylgeranylated protein concentrations drastically reduced viral replication, probably through disruption of membrane biogenesis (238). Semliki Forest virus has a very specific lipid requirement for attachment to the target membrane. Fusion of SFV and host membranes requires cholesterol, particularly, one cholesterol molecule per two phospholipids (242).

As mentioned previously (1.6.1), cholesterol plays an important role in entry of MNV into target cells however it is not known to what extent cholesterol plays a role in MNV replication. One study has shown that down-regulation of cholesterol biosynthesis with

statins significantly increased NV RNA levels in replicon-bearing cells (243). In addition to this, the study found treatment of statins lead to increased expression of LDLR in replicon-bearing cells. Furthermore, the study demonstrated that ACAT inhibitors significantly reduced levels of NV proteins together with mRNA levels of LDLR suggesting that cholesterol does play some role in NoV replication (243). This study will hopefully elucidate whether cholesterol plays a role during MNV replication.

1.11 Viral recruitment of host factors VAP-A and OSBP

The ability of viral proteins to interact with host factors and lipids is an important focus of research and proffers potential targets for drug developments. The lipid flux between adjacent membranes has been identified to play an important role in the replication of many viruses. Oxysterol binding protein (OSBP) is a lipid binding/transfer protein with an affinity for oxysterols, cholesterol and PI4P, and is involved in regulating lipid transport and sterol homeostasis (244). Thus it has a high affinity for various oxysterol regulators of cholesterol synthesis (245). Vesicle-associated membrane protein (VAMP)-associated protein-A (VAP-A) is a type II integral membrane protein. Early studies suggested that VAP-A plays a role in both transporting proteins between the Golgi and ER, and in the process of vesicle transport and fusion (244, 246). VAP-A can bind to vesicle-SNARES (vSNARES) which mediate vesicle trafficking (246). Further investigation revealed the tight interaction of VAP-A and OSBP confirming its role in Golgi to ER transport (247).

Logically, those viruses utilizing the host secretory pathway and requiring the PI4Ks would also associate with OSBP and use the PI4P/cholesterol exchange to assist or facilitate replication. The secretory pathway provides a means of transporting cargo between specific organelle compartments via a series of vesicle budding and fusion events (248). The inherent

nature of this pathway is the reason numerous RNA viruses utilize these organelles. Hence, cellular secretory-trafficking machinery is reorganised into viral replication organelles containing lipid-rich environments that are crucial for viral replication.

1.11.1 *PI4P and Cholesterol exchange*

Membrane contact sites (MCSs) are regions where two organelles are closely juxtaposed. MCS are important domains where the efficient exchange of lipids and signals, such as cholesterol and calcium, takes place between organelles (249, 250). A revolutionary study in 2011 demonstrated a yeast OSBP related protein (ORP) Osh4p, promoted the rapid exchange of sterols for PI4P between the ER and *trans*-Golgi membranes, i.e., sterol release is followed by PI4P extraction in the Golgi and PI4P release is followed by sterol extraction in the ER (250, 251).

A subsequent study in mammalian cells confirmed this and further characterized this transfer mechanism (247). OSBP is tethered between the ER and Golgi membranes via VAP-A on the ER and PI4P on the Golgi (247, 252) (Fig 8). This membrane tethering by OSBP is vital in the exchange of lipids at these MCSs. PI4P synthesised at the Golgi is transported by OSBP to the ER where it is then hydrolysed by Sac1. Sac1 is an ER-resident PI4P-phosphatase which converts PI4P into phosphatidylinositol (PI) (253) (Fig 8). This hydrolysis by Sac1 in the ER supplies OSBP with the energy to function in a cyclic manner; as forward transfer of sterol (from the ER to Golgi) by OSBP is coupled to backward transport of PI4P (Golgi to ER) (250, 252). This particular MCS and exchange of lipids has recently become a focus of numerous studies to identify whether this region is manipulated by viral replication.

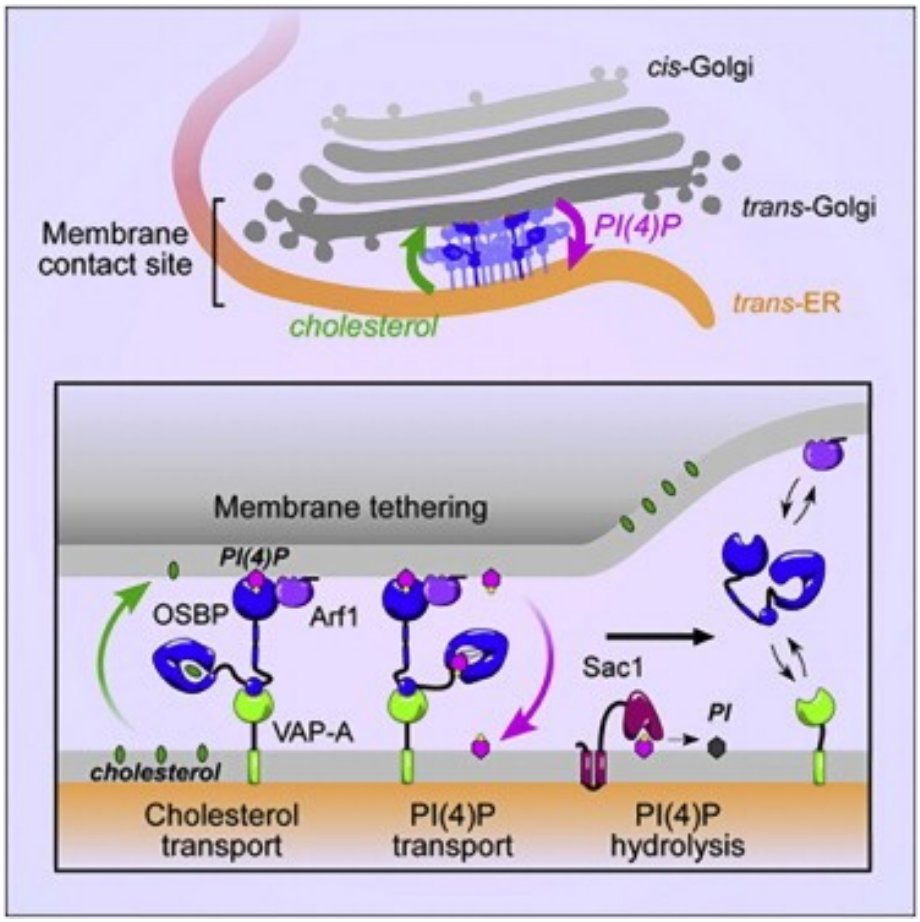

Figure 8. PI4P and cholesterol exchange at the Golgi and endoplasmic reticulum membrane contact site.
OSBP is tethered to the ER via VAP-A and to the Golgi apparatus via PI4P. Arf1 also acts as a membrane anchor. PI4P is transferred from the Golgi to ER while cholesterol is transferred from the ER to Golgi. Sac1 dephosphorylates PI4P which provides the energy for OSBP to modulate the lipid exchange. Taken from Mesmin *et al* 2013.

As mentioned previously, transfected NV p48 (NS1-2) targets intracellular vesicles and then binds to VAP-A, which may form a stable complex (67). Binding of p48 to VAP-A demonstrated disruption of intracellular protein trafficking of the VSV G protein to the cell surface (67). Although not yet seen during infection, it is hypothesised that p48 recruits VAP-A to the replication complex and the concurrent inhibition of intracellular protein trafficking is a means of combating the host's immune responses (interferon production or antigen presentation) as this has been reported during PV infection (254).

Human rhinoviruses (HRV) require Golgi membranes and PI4KIIIβ for replication (255). It has since been demonstrated that cholesterol plays a crucial role in supporting HRV replication. Concomitantly, blocking or silencing OSBP with 25-hydroxycholesterol or siRNA significantly decreased HRV infection and replication, suggesting this virus relies heavily on the cycling of PI4P and cholesterol between the ER and Golgi (222). Additionally, OSW-1 is a natural compound extracted from the plant *Ornithogalum saundersiae* that has been used as an anti-cancer activity. Recent studies have shown that OSW-1 has a high affinity target for OSBP (256). When enterovirus-infected cells were treated with OSW-1 significant decreases in replication were observed (257). It has been established that the PI4P-rich environments of ROs elicits the recruitment of OSBP which then tethers the RO to the ER generating unique MCS to create a PI4P/cholesterol counterflux (Fig. 9)(258, 259).

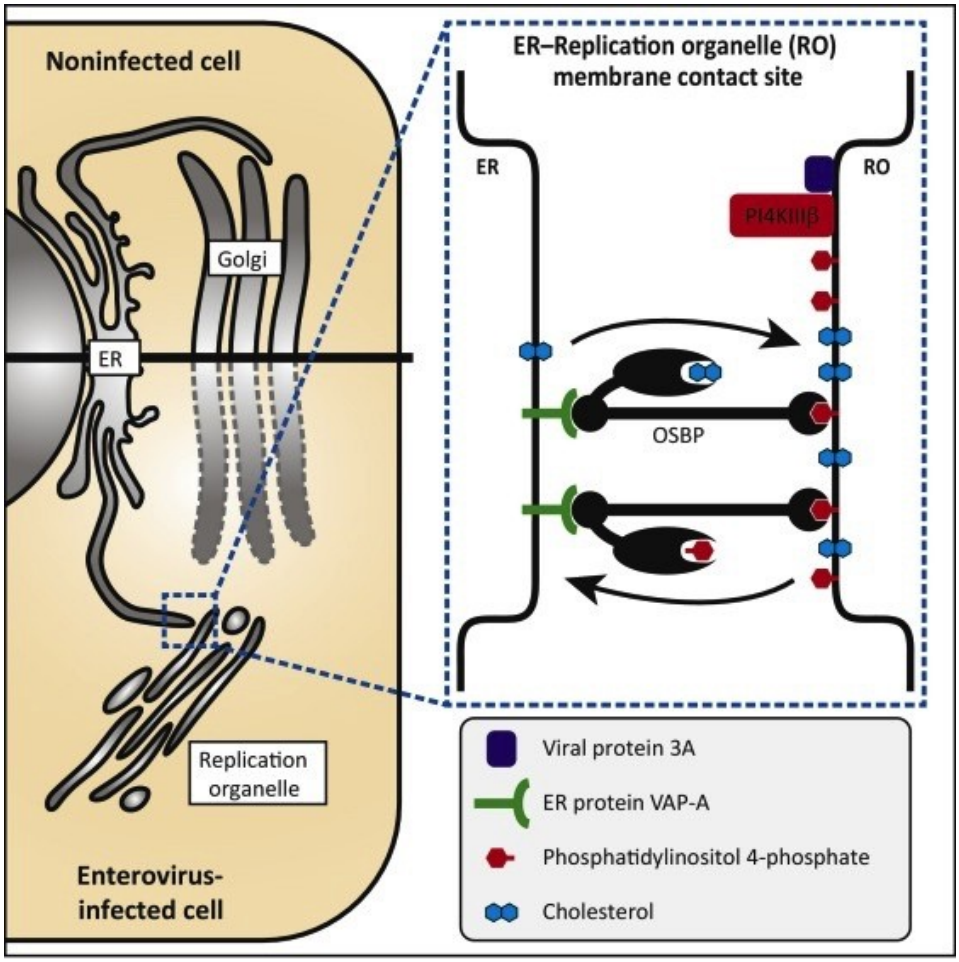

Figure 9. Enteroviruses create novel membrane contact sites between replication organelles and the ER to facilitate lipid exchange.
Enterovirus replication organelles are rich in PI4P which elicits the recruitment of OSBP and tethers the RO to the ER, generating unique MCS to create PI4P/cholesterol counterflux. Taken from Strating *et al* 2015.

Unsurprisingly, HCV NS5A recruits OSBP to sites of replication; in fact OSBP is required to establish the crucial integrity of the membranous webs of HCV replication sites (260). It has been established that PI4KIIIα and PI4P are responsible for recruitment of OSBP to these membranous webs and inhibition of OSBP significantly decreased HCV replication (260). Furthermore, cholesterol plays a significant part in HCV replication as RCs

consist of lipid rafts (261) and it is now known that the recruitment of cholesterol to RCs is dependent on OSBP and PI4KIIIα (260).

Evidently, reasons for viral recruitment of the secretory and endocytic pathways may be two fold; firstly, in order to generate a replication complex/organelle and secondly, to dampen the host's immunity. Lipids are abundant in these organelle membranes so commandeering these readily available platforms for biogenesis of RCs is ubiquitous among RNA viruses. Recruitment of key aspects of the secretory pathway may also be used as a pro-viral strategy. By inhibiting cellular protein secretion this can inhibit the production of interferons and hinder the transfer of antigens to MHC presentation molecules (228, 262).

1.12 Thesis aims

Positive-strand RNA viruses are highly dependent on intracellular membranes for replication and commonly manipulate and remodel these membranes in order to form a replication complex. Little is known about MNV replication and the formation of the replication complex. We hypothesise that because MNV recruits intracellular membranes from the secretory and endocytic pathway, it is likely that MNV is using or manipulating cellular phospholipids and cholesterol. A common feature of picornaviruses is the ability to induce and manipulate the process of autophagy to benefit viral replication. Due to similarities between picornaviruses and caliciviruses, and also the presence of double-membraned vesicles in resin EM images, we hypothesised that MNV may induce the process of autophagy during infection.

Specifically, the aims of this thesis are:

1. Determine if MNV infection induces the cellular process of autophagy
2. Investigate the role of autophagy during MNV replication
3. Identify if MNV utilises the phosphoinositide PI4P to establish a membrane platform for efficient virus replication
4. Identify the individual viral protein(s) that enable recruitment of host factors to the sites of viral replication
5. Investigate whether MNV utilises or manipulates the PI4P-cholesterol counter flow to promote replication.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Cell lines and passaging

RAW264.7 murine macrophages, Wild type bone derived macrophage cells (BDMCs), TLR7-/-, TLR9-/- and MYD88/TRIF-/- (double knockout) BDMCs were provided by Dr Ashley Mansell (Hudson Institute, Melbourne) and grown and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% v/v foetal bovine serum (FBS), 200 mM Glutamax™ (Glx; Life Technologies) and 50 U/mL/50 µg/mL penicillin/streptomycin respectively (P/S; Life Technologies). Vero C1008 (African green monkey epithelial) and 293T (Human embryonic kidney) cell lines were grown and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 6% v/v foetal bovine serum (FBS), 200 mM Glutamax™ (Glx; Life Technologies) and 50 U/mL/50 µg/mL penicillin/streptomycin respectively (P/S; Life Technologies). Adherent macrophage cell lines were passaged using repetitive syringing to detach cells from the culture dish and then pelleted at 1500 rpm for 3 minutes. Supernatant was discarded and cells then resuspended and re-seeded in appropriate growth medium. Veros were passaged using 0.25% Trypsin to detach cells, then resuspended and re-seeded in appropriate growth medium. Semi-adherent 293Ts were detached from a flask by repetitive pipetting and then grown as above. All cells were incubated at 37ºC in 5% CO_2.

2.2 Antibodies

Numerous MNV-specific antibodies were used: MNV-specific rabbit polyclonal antibodies were generously provided by Herbert Virgin (Washington University School of Medicine, St Louis, MO). Rabbit polyclonal sera raised against MNV NS6 and NS7 were generated and purchased from Invitrogen. Guinea pig anti-NS1-2, -NS4, -NS5, -NS6, -NS7 antibodies were kindly provided by Stanislav Sosnovtsev (National Institute of Health, Bethesda, USA) and have been described previously (72). Rabbit anti-MNV-1 (VP1) was generously provided by

Christiane Wobus (Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, USA). Mouse monoclonal anti-double-stranded RNA (anti-dsRNA; clone J2) was purchased from English & Scientific Consulting Bt. (Hungary). For the detection of hexahistidine (His)-tagged viral proteins; rabbit anti-6xHis (Abcam) or mouse anti-His (Merck-Calbiochem) were used.

Antibodies to cellular markers used were purchased from the following companies: rabbit anti-actin and rabbit anti-LC3B (Sigma); rabbit anti-Sac1 and rabbit anti-SQSTM1 (Santa Cruz Biotechnology). Mouse anti-PI4P was purchased from Sapphire, rabbit anti-PI4KB was purchased from Merck-Calbiochem, rabbit anti-PI4K was sought from Cell Signalling Technology and mouse β-tubulin was from Invitrogen. Rabbit anti-LC3 was purchased from MBL, and mouse anti-LAMP1 was purchased from BD Pharmingen (USA).

Goat anti-rabbit IgG (H+L) HRP (Life Technologies) was used for detection of primary antibodies in ECL western blot analysis. Anti-rabbit-, anti-guinea pig- and anti-mouse-specific IgG-Alexa Fluor 488, 594 and 647 were used to detect primary antibodies in Immunofluorescence (IF) and western blot analysis (Molecular Probes; Invitrogen). Saponin and filipin were purchased from Sigma and used in IFA.

2.3 Drugs and reagents

Table 1. Reagents used to treat cells.

Drug	Solvent	Concentration used	Company
Rapamycin	DMSO	10 μM	Merck
3-Methyladenine	DMEM	5 μM	Sigma
Bafilomycin A1	DMSO	10 μM	Sigma
PIK93	DMSO	10 μM or 30 μM	Merck
T-00127-HEV1	DMSO	10 μM	Glixx Laboratories
Tyrphostin AG1478	DMSO	20 μM	Sigma
GW5074	DMSO	5 μM	Sigma
Itraconazole	DMSO	10 μM	Sigma
Lovastatin	100% Ethanol	10 μM	Sigma
25-Hydroxycholesterol	DMSO	5 μM	Sigma

All drug stock solutions were stored at -20°C. All chemicals were tested for cytotoxicity by measuring lactate dehydrogenase activity using a cell cytotoxicity kit (Promega) as recommended by the manufacturers.

2.4 Virus infections

2.4.1 ***Propagation***

MNV (strain CW1) was kindly provided by Christiane Wobus (Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, USA) and was propagated and maintained in RAW 264.7 cells supplemented with DMEM containing 2% FBS for 24 hours after which time tissue culture supernatant was collected and clarified by ultra-centrifugation at 23,100 rpm in a Beckman Optima XL-90 Ultracentrifuge (Beckman Coulter), where subsequently the pellet was resuspended in 3 mL DMEM and aliquoted and stored at -80 ºC until required.

2.4.2 ***Infection***

RAW264.7 cells were infected with tertiary stocks of MNV at a multiplicity of infection of 5 for 1-24 hours post infection as specified.

2.4.3 ***Plaque assay***

Plaque assays were performed in 12 well plates by seeding 2.5 x 10^5 RAW 264.7 cells in 1 ml complete DMEM (as for cell and virus stock) the previous day. Cell monolayers were infected with 220 µl of ten-fold dilutions (10^{-1} to 10^{-8} with 10^{-1} and 10^{-3} discarded) of virus in DMEM for 60 minutes. Plates were rocked every 10 min to prevent the cell monolayer from drying out. 2 ml of overlay containing 70% DMEM, 2.5% v/v FCS, 0.9 M NaHCO3, 200 IU/ml Penicillin, 200 µg/ml Streptomycin, 1 M HEPES (Sigma), 2 mM Glutamax and 0.35%

w/v low-melting-point agarose were added per well and subsequently incubated for 30 min at 4 ºC to allow the overlay to solidify and then returned to 37 ºC for 48 hours. Cells were then fixed by adding 1 ml of 10% buffered formalin solution (Sigma) directly to the overlay for 1 hour at room temperature. The overlay was removed and cells stained with 1% Toluidine Blue (in MQH_2O) for 30 minutes. The stain was removed and wells rinsed with water and plaques counted. Plaque assays were performed in duplicate and the viral titre calculated as an average.

2.5 Transformation of DNA

Purified plasmid DNA was transformed into chemically competent *Escherichia coli* JM109 cells. 100 µl of competent cells were thawed on ice before 1 µl of purified plasmid DNA was added and incubated on ice for 10 minutes. The cell/plasmid DNA samples were heat shocked at 42 ºC for 90 seconds and returned to ice for a further 2 minutes. 900 µl of Luria Bertani (LB) media was added and placed in an environmental shaker for 20 minutes at 37 ºC. Following incubation the mixture was spread onto an LB plate containing 100 µg/ml Kanamycin (Sigma) and incubated at 37 ºC overnight. A single colony was subsequently cultured overnight in 200 ml LB containing 100 µg/ml kanamycin.

2.6 Plasmids

GFP-LC3 was generously provided by Karla Kirkegaard, Department of Microbiology and Immunology, Stanford University School of Medicine, California, USA. ARF1-GFP and GBF1-venus were kindly provided by George A. Belov, Department of Veterinary Medicine, The University of Maryland, USA. OSBP-FLAG and VAP-A-mCherry plasmids were kindly provided by Michael Beard, Molecular and Cellular Biology, The University of Adelaide,

Australia. pcDNA3.1 expression vectors containing the individual MNV ORF1 proteins were constructed as previously described (263).

2.6.1 ***Plasmid purification***

Following transformation, plasmids were amplified using a Hi-Speed Midiprep kit as per manufacturer's instructions (QIAGEN).

2.7 Transfection

2.7.1 ***Lipofectamine 2000***

Cells were seeded in complete DMEM and grown to 70% confluence overnight. Approximately 750 ng of DNA was incubated with 1.5 µL Lipofectamine 2000 (Life Technologies) in 100 µL DMEM (for a 24-well plate) at RT for 15 minutes and then added drop-wise to cells already in 200 µL DMEM. 4 hours post transfection media was removed and replaced with DMEM containing 2% FCS and 1% Glx and incubated for 12-24 hours at 37°C.

2.7.2 ***FuGENE6***

Cells were seeded in complete DMEM and grown to 70% confluence overnight. 1.5 µL of FuGENE6 (Promega) was incubated in 25 µL Opti-MEM (Life Technologies; per well) for 5 minutes. Approximately 700 ng of DNA was then added to the FuGENE6/media mix and incubated for a further 15 minutes. Transfection mix was then added on top of cells in 300 µL DMEM/10%FCS/1%Glx and maintained at 37°C for 12-24 hours.

2.8 Immunofluorescence

2.8.1 ***Cell fixation***

Cells were aspirated once in PBS to remove debris dead cells. Cells were then fixed in 4% w/v paraformaldehyde (PFA)/PBS at RT for 10 minutes and then permeabilised in 0.1% Triton X-100 for 10 minutes at RT. Following two PBS rinses, treatment with 0.2 M glycine was performed for 7 minutes at RT to reduce background fluorescence.

Alternatively, when filipin was used to observe cholesterol, cells were permeabilised with Saponin throughout fixation and labelling.

2.8.2 ***Immunofluorescent labelling***

After aspiration with PBS, cells were incubated with primary antibodies (see section 2.2) in 1% w/v BSA/PBS for 1 hour at RT. After 3 washes with 0.1% w/v BSA/PBS cells were incubated with species-specific secondary antibodies conjugated to either Alexa Fluor 488, 594 or 647 (Invitrogen) for 45 minutes at RT. Cells were washed a further 2 times in PBS then nuclei were counterstained with 4 µg/mL 4, 6-diamidino-2-phenylindole (DAPI; Sigma). Cells were rinsed with PBS then MilliQ water and mounted on coverslides using Ultramount mounting media (Fronine). Cells were analysed using Zeiss LSM700 or Zeiss LSM710 Confocal Microscopes and figures assembled using Adobe Photoshop™. Co-localisation was determined by evaluation of Pearson's coefficients in the JaCOP plugin in ImageJ. If $Rr > 0.5$ co-localisation is deemed to be occurring.

2.9 Western Blotting

Infected or transfected cells were aspirated with PBS to remove dead cells and debris before lysis with SDS lysis buffer (0.1% SDS, 0.5 mM EDTA, 10 mM Tris pH8.0, 150 mM NaCl) containing protease inhibitors (Protease inhibitor cocktail III, Astral Scientific). SDS

loading buffer (Bio-Rad) was added to lysates and boiled at 95°C for 5 minutes before separation on a 10-15% Tris-glycine polyacrylamide gel. Separated proteins were subsequently transferred to a PVDF membrane (Bio-Rad) in Western Transfer Buffer (25 mM Tris-HCl, 0.2 M glycine, 20% methanol) at 100V for 70 minutes. Non-specific binding sites were blocked for 2 hours in 5% BSA in PBS-0.05% Tween (PBS-T; Sigma). Primary antibodies were incubated with the membrane in 5% BSA/PBS-T overnight at 4 ºC. The following day the membrane was washed 4 times in PBS-T and incubated with either secondary antibodies conjugated to either Alexa Fluor 488 or 647 in PBS-T (Fluorescence imaging) or incubated with goat-anti-rabbit HRP in PSB-T (ECL imaging) for 2 hours at room temperature. For fluorescence analysis the membrane was then washed with twice with PBS-T and twice again with PBS and subsequently visualized on the Bio-Rad Pharos FX Plus system. For ECL analysis the membrane was washed 6 times in PBS-T after the HRP incubation and incubated for a maximum of 5 minutes in 1 mL of Pierce ECL Plus Western Blotting Substrate and Reagent (Thermofisher). Blots were then analysed on the MF-ChemiBIS (DNR).

2.10 Lipid-protein interactions: PIP strips

293T cells were transfected with pcDNA3.1 vectors expressing MNV NS4 or NS7 with Lipofectamine 2000 and maintained in complete DMEM. 24 hpt protein lysates were collected. PIP Strips (Echelon Biosciences) were washed in 3% Fatty acid free (FAF) BSA/PBS for 2 hours at RT. Approximately 500 ng of NS4, NS7 or HCV G2a RdRp (JFH1) in 3% FAF BSA/PBS was incubated on each PIP Strip overnight at 4°C. PIP Strips were then washed 3 times in 3% FAF BSA/PBS. Strips were then incubated with primary antibodies in 3% FAF BSA/PBS for 2 hours at RT followed by 3 subsequent washes and secondary

antibody incubation in 3% FAF BSA/PBS for 2 hours at RT. Strips were washed twice in PBS and visualized on the Bio-Rad Pharos FX Plus system. HCV G2a RdRp (JFH1) was used as a positive control and was kindly provided by Peter White, School of Biotechnology and Biomolecular Sciences, University of NSW, Australia.

2.11 Knock down by siRNA

Cells were treated with 40 pmol siRNA once and then again 24 hours later. 12 hours after the second treatment cells were infected with MNV at an moi of 5. 12 hpi cells were fixed with 4% PFA for IFA, TCF collected and protein/RNA lysates were harvested. Mouse PI4Kα was purchased from Dharmacon (L-066305-00-0005, SMARTpool). siRNA was transfected into cells using Lipofectamine RNAiMAX Transfection Reagent as per manufacturer's instructions (Invitrogen, CA). Negative control siRNA was purchased from Origene and used at the same concentration.

2.12 RNA extraction

Cells for RNA extraction were rinsed in PBS and then lysed with Trizol (Life Technologies). Lysates were pipetted repeatedly to ensure cellular breakdown and then stored at -80°C until RNA extraction. Lysates were thawed at RT for 20 min to ensure complete dissociation of RNA-nucleoprotein complexes. Chloroform was added and vigorously mixed, then centrifuged at 12,000 rcf for 15 min at 4°C. The aqueous phase was collected and RNA precipitated with isopropanol and 20 μg glycogen (Sigma) at RT for 10 min, then pelleted at 12,000 rcf, 4°C for 10 min. The RNA pellet was washed in 70% v/v ethanol/DEPC MilliQ H_20, then re-dissolved in DEPC MilliQ H_20 at 55°C for 15 min.

2.13 cDNA synthesis and qPCR

Total RNA concentration was quantified using a Nanodrop and 1 µg total RNA was treated with RQ1 DNase (Promega) at 37 ºC for 45 minutes. cDNA was reverse-transcribed from treated RNA with MNV primers, described previously (264) and an internal control (GAPDH) using Superscript III (Invitrogen) at 50 ºC for 50 minutes. Samples were heat-inactivated at 70 ºC and reactions were diluted in DEPC water. cDNA levels were quantified by qPCR with Sybr GreenER (Bio-Rad) using the following cycling conditions (50 ºC for 8 minutes, 95 ºC for 2 minutes, 40 cycles of 15 seconds at 95 ºC, 1 minute annealing/extension at 60 ºC followed by final extension of 10 minutes) and analysed with ICycler software (Bio-Rad). Fold induction of RNA was compared to the housekeeping gene (GADPH) and error bars indicate triplicate experiments.

Table 2. qPCR primers and sequences.

Primer	Sequence
RT MNV NS3 F	GTTCTCTGGGATGATTTCGG
RT MNV NS3 R	CAATTGAGTGTCACTGGGCA
RT GAPDH F	ACAGTCCATGCCATCACTGCC
RT GAPDH R	GCCTGCTTCACCACCTTCTTG

CHAPTER 3:

MOUSE NOROVIRUS INFECTION PROMOTES AUTOPHAGY INDUCTION TO FACILITATE REPLICATION BUT PREVENTS FINAL AUTOPHAGOSOME MATURATION

3.1 Introduction

Autophagy is a cellular mechanism where the cell is able to degrade, recycle and manage intracellular contents. This process can be used as a survival mechanism when cells are under starvation, or during threat from pathogens such as bacteria and viruses (126, 127). Autophagy involves the creation of an autophagosome, a double membraned vesicle which is facilitated by autophagy (Atg) proteins (128). LC3, Atg and SQSTM1 proteins assist in both the creation of the autophagosome and its elongation, which will then sequester and engulf cytoplasmic contents (128). Once the autophagosome is matured it fuses with lysosomes and the contents are degraded or recycled (129).

Many viruses are able to manipulate and exploit autophagy to enhance replication (265-268). PV induces autophagy, which creates a plethora of intracellular membranes (autophagosomes) that the virus can then use to construct the viral RC (269). In the case of CVB3, viral infection induces the process of autophagy however the virus stalls the final fusion of autophagosomes and lysosomes, potentially to prevent degradation of nascent virions (270). During the course of this study it was reported that the autophagy proteins Atg5-Atg12 and Atg16L localise to the MNV RC upon treatment of cells with IFN-γ and prevent MNV translation and replication (129). The authors observed that this antiviral effect was ineffective in cells deficient in Atg5 indicating that autophagy may be an antiviral cellular response to infection (129).

In this study we show that infection of murine macrophage cells with MNV induces the process of autophagy however MNV does not appear to utilise the autophagosomal membranes to construct a RC. We observed that although there was induction and accumulation of LC3-positive puncta, representing autophagosomes, these puncta showed minimal co-localisation with the MNV RdRp NS7. Additionally, we observed that MNV

appears to block the fusion of autophagosomes with lysosomes as there was sustained levels of LC3II during infection, accumulation of the host protein SQSTM1 (p62) that should normally be degraded during autophagy induction and active recruitment of the lysosomal protein Lamp1 to the site of MNV replication, away from lysosomes. Thus we identified that MNV inhibits subsequent degradation and autophagosome maturation from occurring. We observed that chemical inhibition of the autophagy pathway (via 3-methyladamine) resulted in a significant increase in MNV genome replication, protein production and infectious virus production suggesting that autophagy is an antiviral response to MNV infection. In addition, we observed that MNV-induced autophagy stills occurs in the presence of 3-methyladamine suggesting that induction may occur via a non-canonical pathway.

The current question though is whether MNV actively stimulates the early induction of autophagy for some unknown purpose, or whether MNV allows autophagy induction but controls downstream events during this process. Further studies aim to investigate this question and additionally identify what viral factors (*i.e.* viral RNA, viral proteins, and viral replication) trigger the induction of autophagy. Another question of great importance is whether the inhibition of the final autophagosome maturation stage prevents antigen cross-presentation via this pathway which could be a crucial mechanism employed by MNV during the infection process.

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Mouse Norovirus infection promotes autophagy induction to facilitate replication but prevents final autophagosome maturation

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ABSTRACT

Autophagy is a cellular process used to eliminate intracellular pathogens. Many viruses however are able to manipulate this cellular process for their own advantage. Here we demonstrate that Mouse Norovirus (MNV) infection induces autophagy but does not appear to utilise the autophagosomal membrane for establishment and formation of the viral replication complex. We have observed that MNV infection results in lipidation and recruitment of LC3 to the autophagosome membrane but prevents subsequent fusion of the autophagosomes with lysosomes, as SQSTM1 (an autophagy receptor) accumulates and Lysosome-Associated Membrane Protein1 is sequestered to the MNV replication complex (RC) rather than to autophagosomes. We have additionally observed that chemical modulation of autophagy differentially affects MNV replication. From this study we can conclude that MNV infection induces autophagy, however suppresses the final maturation step of this response, indicating that autophagy induction contributes to MNV replication independently of RC biogenesis.

1. Introduction

Human Noroviruses (HuNoV) belong to the family *Caliciviridae* and cause the majority of cases of non-bacterial gastroenteritis worldwide. Until 2014, lack of ability to culture HuNoV in cells has hindered research (Akira et al., 2006) of its replication strategies and pathogenesis, but recent studies have demonstrated the ability of GII.4 Sydney strain of HuNoV to infect B cells in culture with enteric bacteria acting as cofactors for infection (Jones et al., 2015).

While the cell culture system is still in initial stages of development, the discovery of Mouse Norovirus (MNV) has proven to be a valuable model for the study of HuNoV infection due to their similarities (Hardy, 2005; Karst et al., 2003; Wobus et al., 2004, 2006). MNV is a non-enveloped, positive-sense RNA virus, and possesses a genome consisting of 4 open reading frames (ORFs), the first of which encodes for the 6 nonstructural proteins (NS1–2, NS3, NS4, NS5, NS6 [viral protease], NS7 [viral polymerase]) (McFadden et al., 2011; Sosnovtsev et al., 2006). The major and minor structural proteins, VP1 andVP2 are encoded by ORF2 and ORF3, respectively (Asanaka et al., 2005) and ORF4 encodes for a virulence factor (VF1) that contributes to pathogenesis (McFadden et al., 2011).

As demonstrated by arguably all RNA viruses, infection with MNV induces extensive reorganisation of intracellular membranes for which the virus uses to construct its replication complexes (RC) (Hyde et al., 2009; Mackenzie, 2005). The nonstructural proteins encoded in the MNV genome are presumably responsible for recruiting these membranes from the endocytic and secretory pathway (Hyde and Mackenzie, 2010; Hyde et al., 2009).

Some parallels can be drawn between the induced reorganisation of cellular membranes due to MNV infection and those membranes created by the process of autophagy. Autophagy is the cellular mechanism by which cells are able to manage starvation, recycle and degrade proteins and organelles, and also eliminate invading pathogens (Kirkegaard et al., 2004). In addition, autophagy plays a vital part in the host immune response to infection. The process by which pathogens such as bacteria or viruses are captured by autophagosomes and are degraded is known as xenophagy (Espert et al., 2007). Most importantly, when virions or viral proteins are captured by the autophagosome, its fusion with

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the endosomal/lysosomal pathway induces Toll-like receptor (TLR) recognition and delivers the viral proteins to the major histocompatibility complex (MHC) class II antigen presentation pathway (Espert et al., 2007; Kirkegaard et al., 2004).

Many viruses, such as those belonging to the *Picornaviridae* family, are able to manipulate and exploit autophagy to enhance replication (Kudchodkar and Levine, 2009; Lee and Iwasaki, 2008; Shoji-Kawata and Levine, 2009; Wileman, 2006). For example, it has been shown that Poliovirus (PV) induces autophagy, by which this process creates a plethora of intracellular membranes (autophagosomes) that the virus can then use and exploit by commandeering these membranes on which to construct RC (Taylor and Kirkegaard, 2008). Likewise, other viruses known to manipulate the autophagic pathway are; Dengue virus, Foot-and-Mouth disease virus (FMDV) and Coxsackievirus B3 (CVB3) (Jackson et al., 2005; Klein and Jackson, 2011; Lee et al., 2008). In the case of CVB3, viral infection induces the process of autophagy however the virus is able to stall the final fusion of autophagosomes and lysosomes, potentially to prevent degradation of nascent virions (Wong et al., 2008).

In this study we show that infection of murine macrophage cells with MNV induces the process of autophagy however MNV does not appear to utilise the autophagosomal membranes to construct a RC. Additionally, we have also observed that MNV appears to block the fusion of autophagosomes with lysosomes and thus inhibits degradation from occurring. Our results suggest that MNV requires autophagy for replication, but does not use autophagosome membranes for biogenesis of the RC. However, the final maturation of the autophagosome pathway must be inhibitory to MNV and as such the virus disables the final process of degradation as a method of self-preservation.

2. Materials and methods

2.1. Cells and virus

RAW264.7 murine macrophage were grown and maintained in Dulbecco modified Eagle medium (DMEM; Life Technologies) and supplemented with 10% v/v foetal calf serum (FCS; Thermofisher), 2 mM Glutamax (Gibco) and penicillin (100 U/ml) – streptomycin (100 μg/ml)(Gibco), termed DMEM complete. Vero C1008 (African green monkey kidney cells) and 293 T (human embryonic kidney) cells were maintained in DMEM supplemented with 6% v/v foetal calf serum, 2 mM Glutamax and penicillin (100 U/ml) – streptomycin (100 μg/ml). MNV (strain CW1) was propagated and maintained in RAW264.7 cells supplemented with DMEM for 24 h. After this time tissue culture supernatant was collected and clarified by ultra-centrifugation at 23,100 rpm in a Beckman Optima XL-90 Ultracentrifuge (Beckman Coulter), where subsequently the pellet was resuspended in 3 mL DMEM and aliquoted and stored at −80 °C until required. The viral titre was determined by plaque assay (as indicated below). Cells in this study are referred to as MNV-infected or mock-infected, where mock-infected pertains to cells being treated with neat DMEM only instead of DMEM containing virus,

2.2. Reagents

(1) **Antibodies.** Anti-rabbit-, anti-guinea pig- and anti-mouse-specific Ig G-Alexa Fluor 488, 594 and 647 were purchased from Molecular Probes (Invitrogen). MNV-specific guinea pig polyclonal antibodies were kindly provided by Kim Green (National Institute of Health, USA) and have been described previously (Sosnovtsev et al., 2006). MNV-specific rabbit polyclonal antibodies were generously provided by Herbert Virgin (Washington University School of Medicine, St Louis, MO). Rabbit polyclonal sera raised against MNV NS6 and NS7 were generated and purchased from Invitrogen. Anti-actin and anti-SQSTM1 were purchased from Sigma, anti-LC3 was purchased from MBL and Sigma, anti-β-tubulin was purchased from Invitrogen, and anti- Lysosome-Associated Membrane Protein1 (LAMP1) was purchased from BD Pharmingen. Goat anti-rabbit Ig G HRP was purchased from Life Technologies.

(2) **Chemicals.** Autophagy inhibitor 3-Methyladenine (Sigma) was added to infected cells at a concentration of 5 mM and Autophagy inducer Rapamycin (Merck) was added to infected cells at a concentration of 1 μM. Bafilomycin A1 was purchased from Sigma and used at a concentration of 1 μM. All chemicals were tested for cytotoxicity by measuring lactate dehydrogenase activity using a cell cytotoxicity kit (Promega) as recommended by the manufacturers. All assays were performed in triplicate and no cytotoxic effects were observed for the concentrations used within this study.

2.3. Plaque assay

Plaque assays were performed in 12 well plates by seeding 2.5×10^5 RAW 264.7 cells in 1 ml complete DMEM (as for cell passaging) the previous day. Cell monolayers were infected with 220 μl of ten-fold dilutions (10^{-1}–10^{-8} with 10^{-1} and 10^{-3} discarded) of virus in DMEM for 60 min. Plates were rocked every 10 min to prevent the cell monolayer from drying out. 1 ml of overlay containing 70% DMEM, 2.5% v/v FCS, 0.9 M NaHCO3, 200 IU/ml Penicillin, 200 μg/ml Streptomycin, 1 M HEPES (Sigma), 2 mM Glutamax and 0.35% w/v low-melting-point agarose was added per well and subsequently incubated for 30 min at 4 °C to allow the overlay to solidify and then returned to 37 °C for 48 h. Cells were then fixed by adding 1 ml of 10% buffered formalin (Sigma) directly to the overlay for 1 h at room temperature. The overlay was removed and cells stained with 1% Toludine Blue (Sigma) (in MQH_2O) for 30 min. The stain was removed and wells rinsed with water and plaques counted. Plaque assays were performed in duplicate and the viral titre calculated as an average.

2.4. Transformation of DNA

Purified plasmid DNA was transformed into chemically competent *Escherichia coli* JM109 cells. 100 μl of competent cells were thawed on ice before 1 μl of purified plasmid DNA was added and incubated on ice for 10 min. The cell/plasmid DNA samples were heat shocked at 42 °C for 90 s and returned to ice for a further 2 min. 900 μl of Luria Bertani (LB) media was added and placed in an environmental shaker for 20 min at 37 °C. Following incubation the mixture was spread onto an LB plate containing 100 μg/ml Kanamycin (Sigma) and incubated at 37 °C overnight. A single colony was subsequently cultured overnight in 200 ml LB containing 100 μg/ml Kanamycin.

2.5. Transfection

1.25×10^5 RAW264.7 or 293T cells (per transfection) were seeded and left to proliferate overnight. Cells were then transfected with Lipofectamine 2000 (as per manufacturer's instructions; Life Technologies) and DNA plasmids. 4 h posttransfection (h.p.t.) media was removed and replaced with 2% FCS in DMEM. 24 h.p.t. cells were infected with MNV (where applicable) and fixed with 4 % paraformaldehyde (PFA; Sigma)/PBS 12 h post-infection (h.p.i) or 24 h.p.t.

2.6. Resin thin sections for electron microscopy

Cells were fixed with 3% w/v glutaraldehyde in 0.1 M cacodylate buffer for 2 h at room temperature. Cells were then washed several times in 0.1 M cacodylate buffer followed by fixation with 1% OsO4 in 0.1 M cacodylate buffer for 1 h. After washing the cells in 0.1 M cacodylate buffer, specimens were dehydrated in graded acetones for 10–20 min each. Subsequently, samples were infiltrated with EPON resin and polymerised in moulds for 2 days at 60 °C. 50 nm thin sections were cut on a Leica UC7 ultramicrotome using a Diatome diamond knife and collected on formvar and carbon-coated copper mesh grids. Before viewing in TF30 transmission electron microscope cells were post-stained with 2% aqueous uranyl acetate (UA) and Reynold's lead citrate.

2.7. Immunofluorescence

1.25×10^5 RAW264.7 cells seeded onto coverslips, allowed to propagate over night and subsequently infected with MNV at an M.O.I. of 5 and at the appropriate time points were fixed with 4% paraformaldehyde (PFA) for 10 min. Cells were then permeabilized with 4% PFA/0.1% Triton-X-100 (Sigma) for a further 10 min. The PFA was removed, cells washed 2 times with PBS and treated with 0.2 M glycine for 10 min and finally washed with PBS.

Primary antibodies were incubated with blocking buffer (PBS containing 1% BSA) for 1 h at room temperature, washed 3 times with PBS containing 0.1% BSA before being incubated with species specific secondary antibodies in blocking buffer. Cells were washed in PBS, stained for 5 min with 4 μg/mL 4′, 6-diamidino-2-phenylindole (DAPI) to counterstain the nucleus, washed with PBS and Milli-Q H_2O and mounted with Ultramount (Fronine) onto coverslides. Cells were analysed on a Leica TCS SP2 Confocal Microscope or Zeiss confocal microscope (LSM 700 and LSM 710) and figures were assembled using Adobe Photoshop™. Co-localisation was determined by Pearson's coefficient using the ImageJ JACoP plugin software on images collected from triplicate experiments and expressed as $Rr \pm SD$. In each case, we provide the *Rr* for the presented image but include combined quantification within the text. A co-efficient value exceeding 0.500 is considered as co-localisation.

2.8. Western blotting

RAW264.7 cells were infected with MNV at a M.O.I. of 5 and incubated for desired time periods before lysis with SDS lysis buffer (0.1% SDS, 0.5 mM EDTA, 10 mM Tris pH 8.0, 150 mM NaCl) containing protease inhibitors (Protease inhibitor cocktail III, Astral Scientific). Alternatively, transfected 293T cells were incubated for 24 h before lysis with SDS lysis buffer. SDS loading buffer was added to lysates, before separation on a 10–15% Tris-glycine polyacrylamide gel. Separated proteins were subsequently transferred to a PVDF membrane (Bio-Rad) in Western Transfer Buffer (25 mM Tris-HCl, 0.2 M glycine, 20% methanol) and non-specific binding sites were blocked for 2 h in 5% BSA in PBS-0.05% Tween (PBS-T; Sigma). Primary antibodies were incubated with the membrane in 5% BSA/PBS-T overnight at 4 °C. The following day the membrane was washed 4 times in PBS-T and incubated with either secondary antibodies conjugated to either Alexa Fluor 488 or 647 in PBS-T (Fluorescence imaging) or incubated with goat-anti-rabbit HRP in PSB-T (ECL imaging) for 2 h at room temperature. For fluorescence analysis the membrane was then washed with twice with PBS-T and twice again with PBS and subsequently visualised on the Bio-Rad Pharos FX Plus system. For ECL analysis the membrane was washed 6 times in PBS-T after the HRP incubation and incubated for a maximum of 5 min in 1 mL of Pierce ECL Plus Western Blotting Substrate and reagent (Thermofisher). Blots were then analysed on the MF-ChemiBIS (DNR).

2.9. RNA extractions and qPCR analyses

1×10^6 RAW264.7 cells were seeded into 6-well plates and the next day were infected with MNV at an MOI of 5. 1 hpi Rapamycin or 3-MA was added. 18 hpi cells were rinsed in PBS then lysed in 1 ml Trizol Reagent (Invitrogen). RNA extractions were performed as directed by the manufacturer (Invitrogen). Total RNA concentration was quantified using a Qubit fluorometer (Invitrogen) and 1 μg total RNA was treated with RQ1 DNase (Promega) at 37 °C for 45 min. cDNA was reverse-transcribed from treated RNA with MNV primers, described previously (Hyde et al., 2012) and an internal control (GAPDH) using Superscript III (Invitrogen) at 50 °C for 50 min. Samples were heat-inactivated at 70 °C and reactions were diluted in DEPC water. cDNA levels were quantified by qPCR with Sybr GreenER (Bio-Rad) using the following cycling conditions (50 °C for 8 min, 95 °C for 2 min, 40 cycles of 15 s at 95 °C, 1 min annealing/extension at 60 °C followed by final extension of 10 min) and analysed with ICycler software (Bio-Rad). Fold induction of RNA was compared to the housekeeping gene (GADPH) and error bars indicate triplicate experiments.

3. Results

3.1. MNV infection induces autophagosome formation

Previously we have characterized the MNV replication complex (RC) at the ultrastructure level (Hyde et al., 2009). Upon closer inspection of these micrographs we identified an increasing number of double membrane vesicles within the cytoplasm of infected cells of the course of the infection (Fig. 1A(b–d)). As the presence of double membrane vesicles is a hallmark and indicator of autophagy induction we aimed to investigate the possible MNV-mediated induction of autophagy and the contribution autophagy has on MNV replication.

Cellular changes in localisation of LC3 are a common method for detecting the induction of autophagy. Normally, LC3-I is diffusely distributed throughout cells, however when autophagy is induced LC3 is recruited to autophagosomal membranes and becomes lipidated to form LC3-II, which is redistributed to distinct punctuate regions (Kabeya et al., 2000; Kuma et al., 2007). To investigate if autophagy was induced in MNV-infected cells, RAW264.7 cells were transfected with a recombinant plasmid expressing GFP-LC3 and subsequently infected with MNV. At 12 h post-infection (h.p.i.) the cells were fixed and assessed for the localisation of GFP-LC3 and the viral protein NS7 (Fig. 1B). In uninfected cells LC3 was observed to be dispersed throughout the cell, with very few discrete foci observed (Fig. 1B(a–c)). Conversely, distinct changes in LC3 distribution and localisation were observed in MNV-infected cells, where LC3 accumulated in large punctuate regions (Fig. 1B(d–f)), with a significant increase in the number of GFP-LC3 in MNV-infected cells (Fig. 1C). This change in distribution of LC3 indicated that MNV infection potentially induces the formation of autophagosome membranes. Interestingly, we observed minimal colocalisation between LC3 and the viral polymerase NS7, indicating MNV does not appear to utilise or actively recruit the autophagosomal membranes for replication.

3.2. Accumulation of autophagosomes occurs over time during MNV infection

Autophagosomes are transient vesicles and will generally deliver their cargo to lysosomes within minutes (Klionsky, 2005).

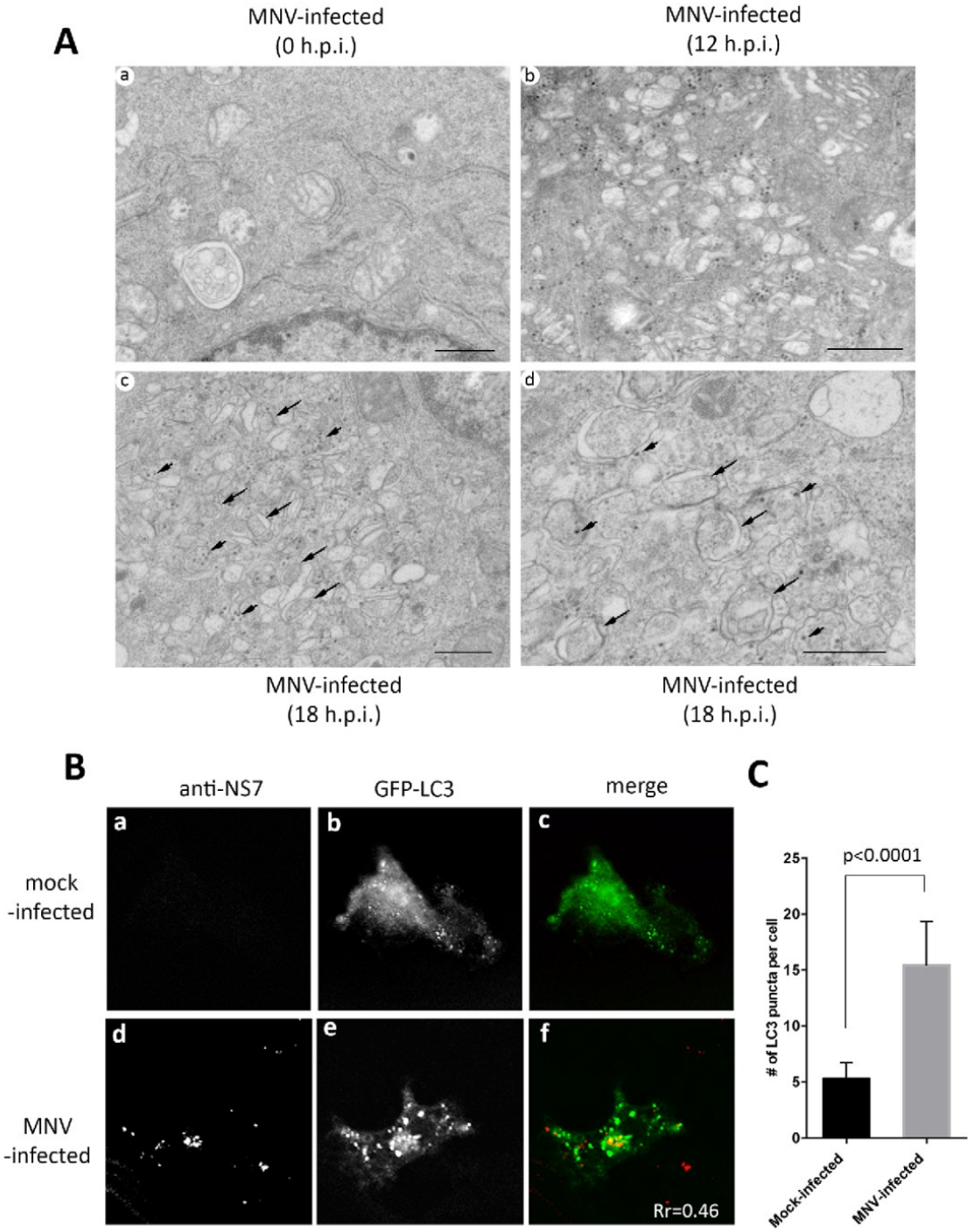

Fig. 1. MNV infection induces autophagy. (A) RAW264.7 cells were infected with MNV and fixed for resin embedding at 0, 12 and 18 h.p.i. Numerous double membrane vesicles can be observed in the cytoplasm of cells infected with MNV at 12 (panel b) and 18 h.p.i. (panels c and d) with none observed at 0 h.p.i. (panel a). The double membrane vesicles are identified with arrows, and MNV virions with arrowheads. Magnification bars represent 200 nm. (B) RAW264.7 cells were transfected with GFP-LC3 (green), and 24 h later infected with MNV at an M.O.I. of 5, fixed at 12 h.p.i. and labelled with NS7 (red). MNV was observed to induce alterations and changes in morphology of LC3 indicating the induction of autophagy. $Rr = 0.46 \pm 0.16$. Pearson's coefficient (colocalisation) was determined by the JaCOP plugin software in ImageJ. (C) Quantification of the number of GFP-LC3 puncta in mock-infected cells compared to MNV-infected at 12 hpi, Error bars indicate number of foci per cell ± SD and significance was determined by students *t*-test.

Therefore any accumulation of autophagosomal membranes will be a result of increased formation or decreased degradation of autophagosomes. To elucidate the temporal pattern of autophagy induction we assessed the distribution of GFP-LC3 in MNV-infected cells over the course of the infection (Fig. 2). From as early as 9 h.p.i. changes in GFP-LC3 localisation were observed indicating that the induction of autophagy occurs rapidly in the presence of MNV replication (Fig. 2A(d–o)). As the infection progressed over the 18 h course, the number of GFP-LC3 puncta also increased over time reaching maximum numbers at 18 h.p.i. (Fig. 2A(m–o)), which correlates with peak virus replication (Wobus et al., 2006). As noted previously, we observed only minimal colocalisation with GFP-LC3 and viral NS7.

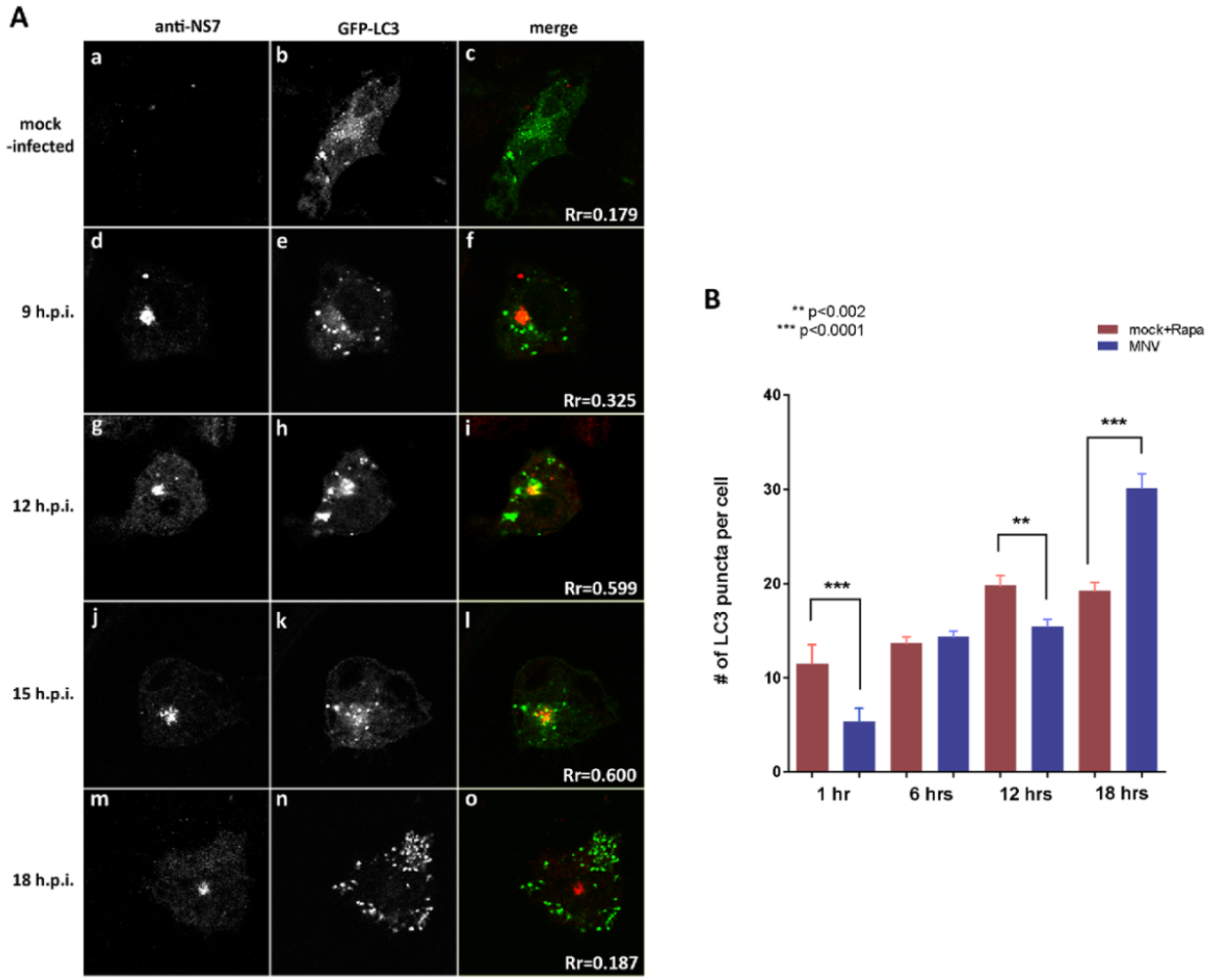

Fig. 2. MNV infection induces autophagy and causes accumulation of autophagosomes over time. (A) RAW264.7 cells were transfected with GFP-LC3 (green), and 24 h later infected with MNV at an MOI of 5, fixed at 3, 6, 9, 12, 15 and 18 h.p.i. and labelled with NS7 (red). Pearson's coefficient (colocalisation) was determined by the JaCOP plugin software in ImageJ. (B) Quantification of GFP-LC3 puncta in mock-infected cells and cells treated with Rapamycin (Rapa) compared to MNV-infected at 6, 12 and 18 h.p.i., $n=3$. Error bars indicate number of foci per cell $\pm$ SD and significance was determined by students t-test.

In comparison, we also treated RAW264.7 cells with Rapamycin to induce autophagy and assessed the number of GFP-LC3 puncta under these conditions (Fig. 2B). Over the course of 18 h after cells were treated with rapamycin, numbers of GFP-LC3 puncta rise as an indication of autophagy and finally decrease in number as a sign of autophagic degradation. Conversely, numbers of GFP-LC3 puncta continue to rise over the 18 h course of MNV infection (Fig. 2B), indicating the autophagic turnover is not occurring. When examined 12 and 18 h post-stimulation, we observed that the number of GFP-LC3 puncta was approximately half that observed during MNV infection at 18 h.p.i. (after 18 h.p.i there was extreme cell death and no time points were included after 18 h.p.i.). Due to the increase in autophagosomes induced during MNV infection we suggest that autophagosomes are not being degraded in these cells, and perhaps MNV may be intervening within this pathway to prevent fusion and degradation.

3.3. MNV infection inhibits the fusion of autophagosomes and lysosomes

During the induction of autophagy in mock cells, LC3 and LAMP1 colocalise as the autophagosome fuses with the lysosome to degrade its contents. To investigate whether the fusion of autophagosomes and lysosomes was occurring in infected cells, we determined the form of LC3 predominately accumulating in cells in the presence or absence of MNV infection. As can be observed in Fig. 3A LC3-I and LC3-II were abundant in similar ratios in uninfected cells, with an associated accumulation of LC3-II observed when turnover is prevented with the inhibitor Bafalomycin A (BafA). In stark contrast, most of the LC3-I was lipidated to LC3-II in the MNV-infected cells, supporting the observation that autophagosomes accumulate in infected cells. Quantitation of total LC3 observed via immunoblot is expressed as percentage of LC3II out of total LC3 present. 43% (43 ± 1.2, $n=3$) of total LC3 in mock-infected cells is LC3II, whereas 65% (65 ± 9.7, $n=3$) of total LC3 present in MNV-infected cells is LC3II (Fig. 3B).

Additionally, MNV-infected cells were co-labelled with antibodies against the viral protein NS6, LAMP1 and LC3 and then analysed by confocal microscopy (Fig. 3C). In uninfected cells treated with Rapamycin we observed considerable overlap between LC3 and LAMP1 (Fig. 3e–h; $Rr=0.57 \pm 0.13$). However, during infection with MNV it was observed that there was greatly reduced colocalisation between LC3 and LAMP1 in infected cells ($Rr=0.53 \pm 0.10$), whereas MNV NS6 and LAMP1 displayed a high

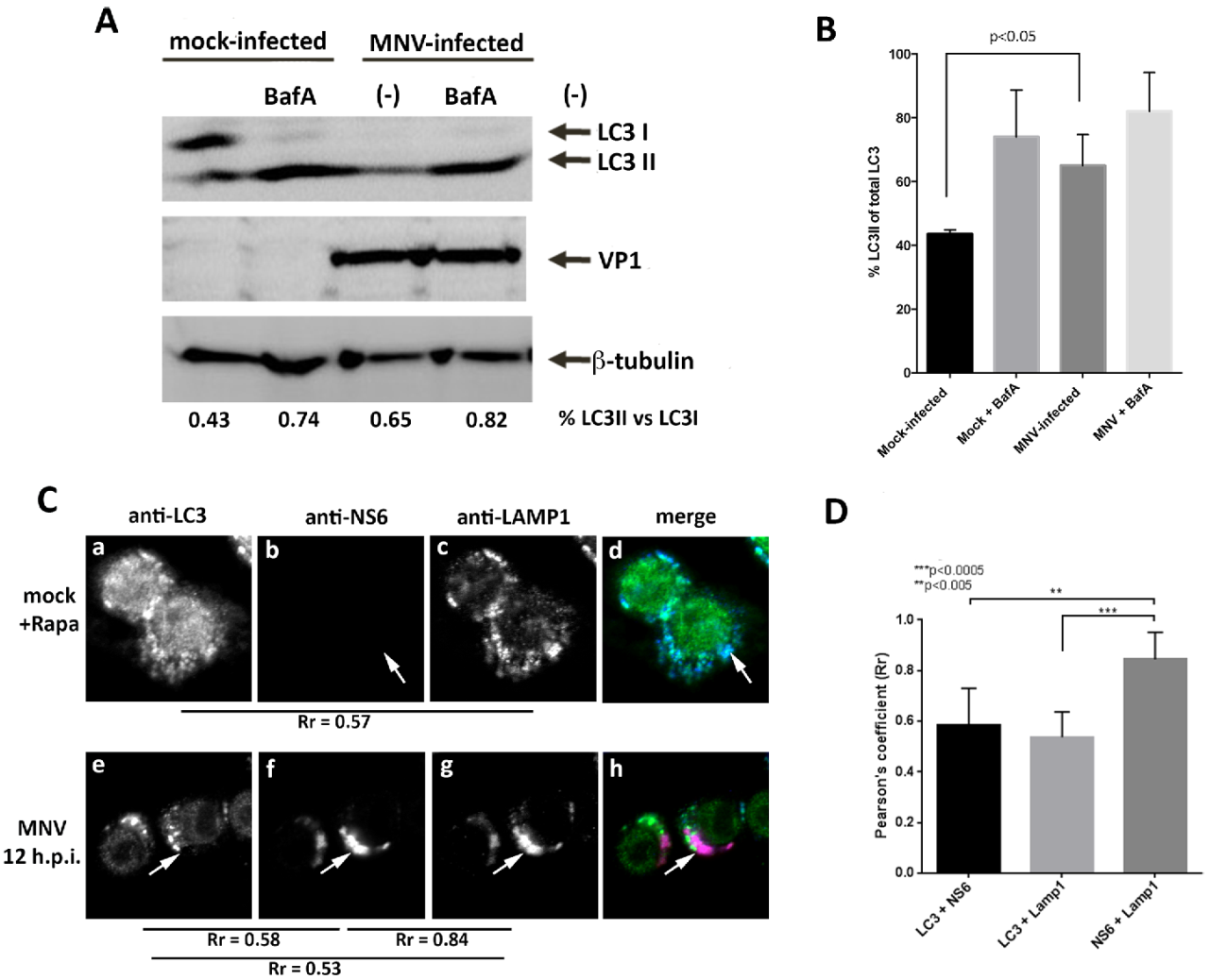

Fig. 3. MNV stalls the final degradation of autophagolysosome and colocalises with LAMP1 during infection. RAW264.7 cells were infected with MNV at an M.O.I. of 5, treated with Bafilomycin A1 (BafA) 10 h.p.i and fixed at 12 h.p.i. for IFA or protein lysates collected. (A) Protein lysates were analysed by western blot using antibodies against LC3, MNV VP1 and cellular internal control β-tubulin. BafA was used to demonstrate the accumulation of LC3II. (B) Graph showing quantitation of the % LC3II from total LC3 expressed ($n=4$). Error bars indicate intensity of protein ± SD and significance was determined by students *t*-test. (C) Fixed cells were labelled with LC3 (green), NS7 (red) and LAMP1 (blue). Rapamycin (Rapa) was added to uninfected cells to demonstrate the colocalisation between LAMP1 and LC3 when autophagy is induced. MNV infection induced autophagy but only minimal colocalisation between LC3 and LAMP1 occurred. MNV was observed to colocalise with LAMP1 suggesting that the virus may be blocking the fusion of autophagosomes and lysosomes. Pearson's coefficient (colocalisation) was determined by the JaCOP plugin software in ImageJ. LC3−NS6 Rr = 0.58 ± 0.14. LC3−Lamp1 Rr = 0.53 ± 0.10. NS6−Lamp1 Rr = 0.84 ± 0.10. (D) Graph showing quantitation of Rr values from triplicate experiments. Error bars indicate intensity of protein ± SD and significance was determined by students *t*-test.

degree of colocalisation (Fig. 3C(i–l) and D; Rr = 0.84 ± 0.10). As observed previously there was very little colocalisation between LC3 and MNV NS6 (Rr = 0.58 ± 0.14) confirming our previous observations.

These results would suggest that MNV may be inhibiting the fusion between lysosomes and autophagosomes and may even be recruiting lysosome components for replication.

3.4. *Sequestosome 1 (SQSTM1) accumulates during MNV infection*

SQSTM1 is a ubiquitin binding protein that can also bind LC3 and as such can induce autophagosomal degradation of protein aggregates (Pankiv et al., 2007). As we had observed that MNV appeared to prevent the fusion between autophagosomes and lysosomes we aimed to determine the localisation and levels of SQSTM1 during infection. In unaffected cells we observed very few SQSTM1 foci within the cytoplasm of cell (Fig. 4A(i–iv)), and slightly increased numbers of foci upon stimulation of autophagy with Rapamycin (Fig. 4A(v–viii)). In contrast, we observed a vast increase in SQSTM1 foci and foci size in cells infected with MNV but additionally observed that a small proportion of the SQSTM1 pool appeared to relocate to the MNV RC (Fig. 4A(ix–xii); Rr = 0.498). We additionally observed a vast increase in SQSTM1 protein in MNV-infected cells compared to mock-infected or Rapamycin treated cells (Fig. 4B). Quantitiation of these foci indicate that the size of these SQSTM1 foci are significantly larger in MNV-infected cells (Fig. 4C). These observations corroborate our findings in Fig. 3 and suggest that MNV interferes with the final maturation of autophagosomes as we observe accumulated LC3 and SQSTM1 suggesting MNV may be restricting the fusion of autophagosomes and lysosomes, and thus protein degradation.

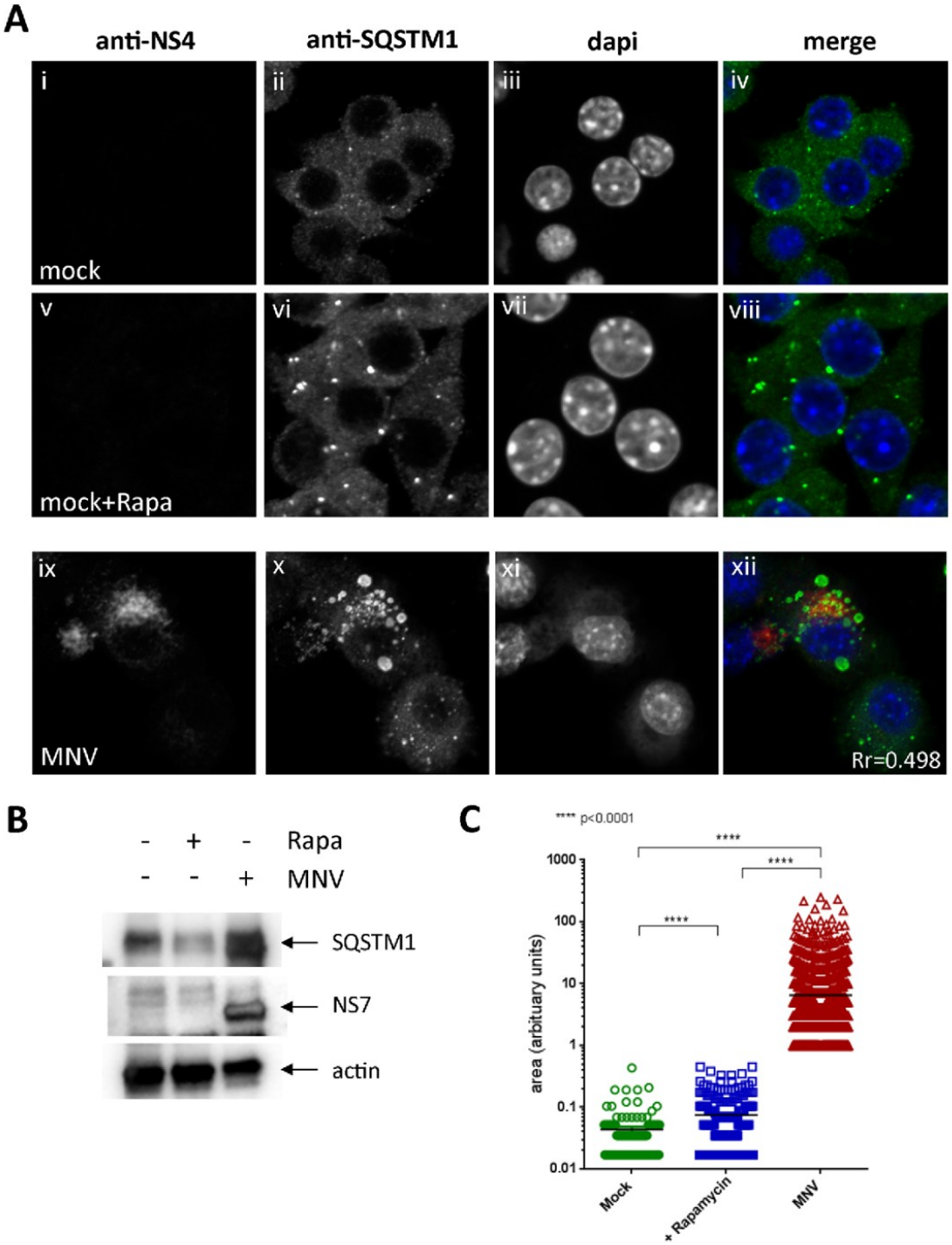

Fig. 4. SQSTM1 accumulates during MNV infection. RAW264.7 cells were infected with MNV at an M.O.I. of 5 and fixed for IFA or protein lysates collected at 12 h.p.i. Alternatively, cells were mock-infected and treated with Rapamycin (Rapa) and fixed for IFA or protein lysates collected 12 h later. (A) IFA demonstrated diffuse staining of SQSTM1 throughout mock-infected cells (A(i–iv)) and distinct foci of SQSTM1 forming in Rapamycin treated cells (A(v–viii)). In MNV-infected cells numerous, very large foci of SQSTM1 accumulate (A(ix–xii)). Pearson's coefficient (colocalisation) was determined by the JaCOP plugin software in ImageJ. If Rr > 0.5 then it is deemed to colocalise. (B) Protein lysates analysed by anti- SQSTM1 western blot. (C) Foci area was analysed by ImageJ software. $p < 0.0001$, SQSTM1 foci were enumerated for $n = 30$ cells for each sample from duplicate experiments and graphed with Prism 6. Error bars indicate the area of foci per cell ± SD and significance was determined by students *t*-test.

3.5. *Chemical modulation of autophagy influences MNV replication*

Numerous viruses are able to manipulate autophagy in such a way to benefit their replication. Picornaviruses have been robustly studied and it has been determined that compounds inducing or inhibiting autophagy affects viral replication by enhancing or decreasing it respectively (Espert et al., 2007; Kabeya et al., 2000; Kudchodkar and Levine, 2009; Wileman, 2006). Rapamycin is a widely used compound that induces the process of autophagy (Noda and Ohsumi, 1998). 3-Methyladenine (3-MA) is a compound

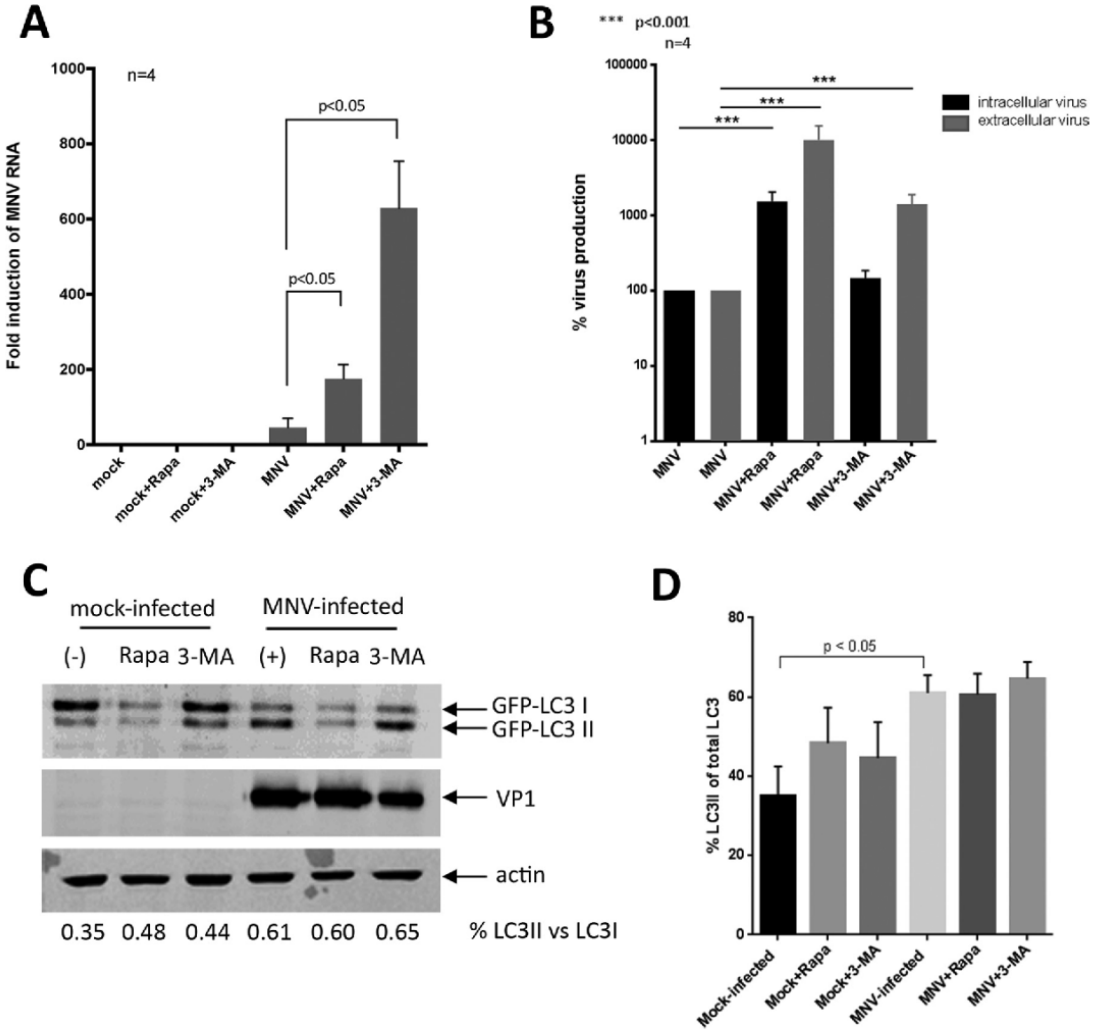

Fig. 5. The modulation of autophagy has positive impacts of MNV replication. (A) RAW264.7 cells were infected with MNV at an M.O.I. of 5, treated with Rapamycin (Rapa) or 3-Methyladenine (3-MA) at 1 h.p.i. and at 18 h.p.i. lysates were collected. RNA was then extracted and analysed via qPCR ($n=4$, * indicates $p < 0.02$). (B) Tissue culture fluid was collected at 18 h.p.i. and analyzed for the production of infectious virus by plaque assay. For virus titre analyses, intracellular virus ($n=4$) and extracellular virus ($n=4$) were measured by plaque assay for pfu/ml and expressed as % of WT MNV infection. (C) Immunoblot of cell lysates collected at 18 h.p.i were analysed for changes in viral protein and the conversion of LC3-I to LC3-II when treated with Rapa and 3-MA. (D) Graph showing quantitation of the % LC3II from total LC3 expressed ($n=4$). Error bars indicate percentage of LCII compared to LCI ± SD and significance was determined by students t-test.

that is commonly used as an autophagy inhibitor as it exercises inhibitory effects on class III PI3K activity which is required for the induction of autophagy (Wu et al., 2010).

To determine the contribution autophagy plays during MNV infection, we investigated the effects of Rapamycin- and 3-MA-treatment on MNV replication. RAW264.7 cells were infected with MNV and subsequently treated with Rapamycin or 3-MA 1 h.p.i. and viral RNA levels, protein expression and release of infectious virus was assayed at 18 h.p.i. (Fig. 5). Qualitative PCR analysis showed a significant increase in viral RNA in the presence of either 3-MA and/or Rapamycin (Fig. 5A). Whilst the amount of RNA produced in the presence of Rapamycin was higher when compared to untreated cells, there was a substantial fold-increase in the amount of MNV RNA in the presence of 3-MA. This suggests that 3-MA may influence genome amplification but not duly affect protein translation. Furthermore, it appeared that chemical treatment with 3-MA lead to a greater proportion of secreted MNV virions compared to intracellular accumulation of virions ($p < 0.001$ vs. NS, respectively), whereas Rapamycin treatment lead to an overall increase in both intracellular and extracellular virus (Fig. 5B). Analysis of intra- and extracellular viral titres also demonstrated that both 3-MA and Rapamycin treatment increased viral secretion by ~10–100 fold compared to untreated infected cells. Rapamycin treatment produced a 100-fold increase in MNV secretion when compared to untreated infected cells, which indicates that the process of autophagy is beneficial for MNV replication. However, we cannot exclude that this may reflect the influence of Rapamycin on host capped mRNA translation (Feigenblum and Schneider, 1996), a possibility we are currently exploring. Interestingly, MNV extracellular secretion was increased in the presence of 3-MA which was unexpected. However, we cannot exclude any potential off target effects induced by both Rapamycin or 3-MA, at this point in time. This data suggests that the modulation of autophagy contributes differentially to MNV infection.

When we assessed the lipidated state of GFP-LC3 in treated mock-infected and infected samples, it was observed that more abundant LC3-I was observed in mock-infected cells and mock-infected cells treated with 3-MA. In contrast, MNV-infected cells demonstrated increased conversion of LC3-I to LC3-II (Fig. 5C).

Interestingly, even in the presence of 3-MA, MNV-infected cells more abundant LC3-II protein. As 3-MA is an autophagy inhibitor, this was unexpected suggests that MNV has the capacity to induce autophagy even in the presence of 3-MA. Quantitation of total LC3 observed via immunoblot is expressed as percentage of LC3II out of total LC3 present. 35% (35 ± 13, $n=4$) of total LC3 in mock-infected cells is LC3II, whereas 61% (61 ± 7.8, $n=4$) of total LC3 present in MNV-infected cells is LC3II (Fig. 5D).

Based on these findings we would suggest that the induction of autophagy may be an important factor for MNV replication as the virus is able to induce autophagy in the presence of 3-MA, potentially demonstrating a PI3K-independent induction of autophagy. We also suggest that stabilizing or inhibiting the final maturation process of autophagy is also important for MNV replication.

4. Discussion

The replication processes of many viruses have been widely studied and it is well documented that RNA viruses induce extensive intracellular membrane rearrangements in order to assist replication (Black and Brown, 1978; Mackenzie, 2005; Miller and Krijnse-Locker, 2008). Replication complexes are required for efficient virus replication and also aid as a means to avoid immune detection, although little is known about the composition and mechanism by which noroviruis (NoV) RCs are formed (Bailey et al., 2010; Belov and Ehrenfeld, 2007; Hyde et al., 2012; Hyde and Mackenzie, 2010; Love and Sabine, 1975; Mackenzie, 2005). Many studies have also elucidated how some viruses are able to manipulate cellular processes including autophagy to induce these membrane structures (Shoji-Kawata and Levine, 2009). Whilst autophagy is regarded as a mechanism to degrade intracellular pathogens, some viruses are able to overcome these anti-viral properties and utilise the autophagic pathway to facilitate their own replication (Kudchodkar and Levine, 2009; Lee and Iwasaki, 2008). Members of the *Picornaviridae* family of viruses are a well known example of viruses that manipulate the autophagy pathway and exploit it to enhance replication (Taylor and Kirkegaard, 2008). Due to the similarities between picornaviruses and caliciviruses, in terms of genome structure and function, it was reasonable to suggest MNV infection may also induce autophagy and manipulate the process to its advantage. To this end, we have shown that autophagy is induced during infection of macrophages with MNV; which appears to be a response favoring virus infection. We have observed that: (i) MNV does not appear to utilise the autophagosome membrane for biogenesis of its RC, (ii) MNV potentially inhibits autophagosome maturation by preventing fusion with lysosomes, (iii) MNV replication is enhanced in cells where autophagy has been chemically stimulated and (iv) when autophagy has been chemically inhibited by 3-MA, MNV is still able to induce the process of autophagy.

We observed that MNV-infected cells displayed a dramatic redistribution and accumulation of LC3-positive puncta over the course of infection (Figs. 1 and 2). This was apparent as immunofluorescent signatures representative of GFP-LC3 formed very large, distinct puncta upon infection with MNV, as opposed to remaining dispersed throughout the cells as it does when cells are not undergoing autophagy. These observations suggested that at least in part, autophagy was induced during MNV infection. However, we observed minimal colocalisation between LC3 and the MNV RC, strongly suggesting that MNV does not appear to utilise the autophagic membrane for the biogenesis of the RC. Previous studies investigating Picornavirus infection have demonstrated the proliferation of GFP-LC3 puncta in cells infected with PV, FMDV and CVB3 (Klein and Jackson, 2011; O'Donnell et al., 2011; Wong et al., 2008). However, for these viruses it was observed that viral replicase proteins colocalised with LC3, indicating that the viruses were utilising the autophagic membranes for replication (Klein and Jackson, 2011).

Previous studies have shown that chemical inhibition of autophagy dramatically reduced poliovirus titre and replication (Jackson et al., 2005) presumably as a result of fewer membranes forming due to lack of autophagy. Interestingly, our findings suggest that this is not what occurs during MNV infection. When autophagy was modulated by 3-MA and Rapamycin in MNV-infected cells, viral RNA replication and secretion both increased compared to untreated infected cells. Furthermore, when 3-MA was added to MNV-infected cells and analysed by immunoblotting (Fig. 5C), there was increased LC3-II compared to mock-infected 3-MA treated cells. This was unexpected as 3-MA inhibits the process of autophagy, but lead us to propose that even in the presence of 3-MA, MNV can still induce autophagy and possibly by unblocking the activity of 3-MA on the enzyme PI3K, or by a novel unidentified mechanism. One suggestion is that MNV may induce autophagy in a PI3K-independent manner, a mechanism we are currently investigating.

Generally, autophagy is induced by events such as starvation, Rapamycin treatment or infection by viruses such as FMDV. Following this, LC3 and LAMP1 colocalise due to the fusing of the autophagosomes and lysosomes at the end of the autophagic process (Kuma et al., 2007; Levine and Deretic, 2007). When autophagosomes and lysosomes fuse, the contents of autophagosomes are degraded, recycled or in the instance of infection, antigens are presented via the MHC molecules (Dhodapkar et al., 2001; Rammensee et al., 1993). Interestingly, during MNV infection we observed minimal colocalisation of LC3 and LAMP1 during the infection process (Fig. 3B). This suggests that MNV may prevent the fusion of the lysosomes and autophagosomes and thus inhibit the degradation process. The lack of interaction between LC3 and LAMP1 correlates with the findings of increased number of autophagosomes seen in infected cells, and these results both indicate the prevention of autophagosome degradation.

Why MNV blocks this fusion and degradation of autophagosomes remains unclear. It may be a mechanism similar to that which FMDV utilises to inhibit the presentation of viral antigens by MHC molecules (Sanz-Parra et al., 1998). Similarly, CVB3 has also been reported to inhibit the fusion of autophagosomes and lysosomes (Kemball et al., 2010; Wong et al., 2008). However, CVB3 has also been reported to use autophagosomal membranes and thus there are predictions that CVB3 inhibits the fusion to protect any nascent virions in the autophagosomes from being degraded and also as method of non-lytic release (Kemball et al., 2010; Klein and Jackson, 2011). This manipulation of the autophagic pathway by MNV may be a way to prevent degradation of viral proteins and therefore antigen presentation by the cell.

To further support this data, we investigated the localisation and amount of SQSTM1 in MNV-infected cells. SQSTM1 binds directly to LC3 during autophagy and facilitates the degradation of ubiquitinated protein aggregates (Kuusisto et al., 2001) and will eventually be itself degraded by this process. We observed very large and abundant SQSTM1 foci in MNV-infected cells when compared to mock-infected or Rapamycin treated cells (Fig. 4A). Likewise, by immunoblotting we also saw a large increase in SQSTM1 protein in MNV-infected cells compared to mock-infected and Rapamycin treated cells. This correlates with our findings suggesting that MNV is able to inhibit the end degradation process of autophagy.

A recent study has shown that SQSTM1 is induced in activated macrophages, in particular by IFN-γ and TLR stimulation (Kim and Ozato, 2009). They also demonstrated that in IFN-γ/CpG- stimulated cells SQSTM1 is able to inhibit the expression of NF-κB-

dependent cytokine genes (Kim and Ozato, 2009). In addition, it was recently shown that Atg5-Atg12/Atg16L1 and Atg7 are required for IFN-γ to inhibit MNV replication (Hwang et al., 2012). These findings may suggest why MNV permits the process of autophagy to occur, but limits its final fusion and degradation stages. The induction of these large SQSTM1 puncta may be a method MNV is using to inhibit some of the antiviral cellular responses and thus the virus does not want these foci to be degraded.

We demonstrate that the process of MNV infection triggers autophagy in host cells, and appears to block the downstream degradation of autophagosomes. Why the virus disrupts the final stages of autophagy is still unclear, however we suggest that this may prevent unwanted degradation of viral products or to inhibit antigen cross-presentation. Further research into how and why MNV interrupts this fusion of lysosomes and autophagosomes may help in understanding the complex mechanisms of autophagy and also provide more insight into the pathogenesis of NoVs.

Acknowledgements

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CHAPTER 4:

EFFICIENT MOUSE NOROVIRUS REPLICATION IS DEPENDENT ON PHOSPHATIDYLINOSITOL 4-PHOSPHATE AND THE ASSOCIATED KINASE PI4KIIIα

4.1 Introduction

Arguably the replication of all (+)RNA viruses induce extensive reorganization of intracellular membranes for which these viruses utilise to construct their RC (182, 183). These unique membrane structures form around the viral replicative components and provide a microenvironment where efficient viral RNA synthesis can occur. These membrane-bound RCs generally consist of small vesicles or tubular platforms that accumulate near the perinuclear region shortly after the viral latent period (182-190). The cellular membranes that are recruited to these sites can be commandeered from many different organelles within the cell. In particular, the secretory pathway is a common target for membrane recruitment as multiple studies have demonstrated the RCs of picornaviruses, flaviviruses, arteriviruses and bromoviruses are comprised of proteins and membranes largely derived from the endoplasmic reticulum, whereas togavirus replication is occurs on membrane platforms associated with endosomes and lysosomes (188, 192-194). Previous reports from our laboratory have demonstrated that the MNV RC is generated from membranes of the endocytic and secretory pathway (263, 271). In addition, our laboratory showed that the MNV non-structural proteins, NS1-2 and NS4 localise independently to the ER and Golgi apparatus/endosomes, respectively, are thus are presumably responsible for recruiting these membranes for RC biogenesis (69).

One of the major constituents of these cellular membranes is phosphoinositides. Phosphoinositides are an important class of lipids that are involved in cellular lipid metabolism, intracellular transport and lipid-mediated signal transduction. Of particular interest is phosphatidylinositol 4-phosphate (PI4P) which is resident in membranes of the Golgi complex, plasma membrane and to a smaller extent; the ER (211, 212). In humans, there are two classes of phosphatidylinositol 4-phosphate kinases (PI4Ks) responsible for the production of PI4P. Type II PI4Ks (PI4KIIα and PI4KIIβ) are responsible for the majority of

PI4K activity in the plasma membrane whereas Type III PI4Ks (PI4KIIIα and PI4KIIIβ) are responsible for the generation of PI4P in the Golgi apparatus (215-217).

Recent studies have revealed that a number of (+)RNA viruses utilize or manipulate PI4P and its production within the host cell. For example; Hepatitis C Virus (HCV) requires the activity of PI4KIIIα and its subsequent generation of PI4P pools to facilitate efficient viral replication. During the HCV replication cycle, the viral NS5A protein actively stimulates PI4KIIIα activity and thus the accumulation of PI4P in infected cells (220, 272, 273). Similarly, PI4KIIIβ has been identified to perform a crucial role in the replication cycle of multiple picornaviruses, as the replication of both CVB3 and PV replication is dependent on PI4KIIIβ activity (220). Due to the similarity of picornaviruses and caliciviruses, we hypothesized that MNV would also require PI4P for the generation of the RC and thus efficient replication.

Protein trafficking through the Golgi apparatus and regulating membrane dynamics are governed by the Arf family of GTPases. Arf1 is activated and becomes membrane-bound by a guanine exchange factor (GEF) known as Golgi brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1). GBF1 catalyses the GDP/GTP exchange on Arf1 which then in turn recruits coat proteins or lipid modifying enzymes such as PI4KIIIβ (221-224). The 3A protein of CVB3 has been reported to interfere with GBF1, inhibiting the activation of Arf1 which induces a general inhibition of secretory pathway transport, i.e., ER-to-Golgi transport has been blocked (228). The reasoning behind this inhibition of transport may be to prevent the secretion of cytokines and delivery of viral antigens to MHC molecules.

On the other hand, some viruses actively recruit Arf1 and GBF1 to sites of viral replication. Activated Arf1 is responsible for the delivery of PI4KIIIβ to the Golgi membrane where the production of PI4P is increased (229). Enriched pools of PI4P are required for

HCV replication, and it has been shown that Arf1, GBF1 and PI4KIIIβ all co-localise during infection, suggesting that HCV requires all these factors for the generation of PI4P and efficient replication (229). Further studies revealed that the NS5A protein of HCV recruits PI4KIIIα (predominately found in the ER) to the viral replication complex and NS5A stimulates PI4Kα activity to enhance PI4P accumulation which is essential for HCV replication (208, 230).

In this chapter we demonstrate that infection of RAW264.7 cells results in an increased production of PI4P. We also observed that during the biogenesis of the MNV RC, MNV actively recruits both PI4KIIIα and PI4KIIIβ to sites of replication resulting in an increased localised production of PI4P. Using chemical inhibition of PI4P production we identified PI4KIIIα to be the major kinase required for the observed PI4P production facilitating efficient virus replication. In addition to the PI4Ks and PI4P, we observed an active recruitment of the trafficking protein Arf1 and the guanine nucleotide exchange factor for Arf1, GBF1, to the sites of MNV replication Surprisingly, we observed that transient expression of the MNV non-structural proteins did not influence PI4P homeostasis or distribution and we observed minimal co-localisation of the transiently expressed MNV proteins and PI4Ks or GBF1. However, we did observe that MNV NS3 and NS6 proteins displayed significant co-localisation with Arf1, suggesting an active role for these proteins in recruiting Arf1 to promote PI4KIIIα activation and subsequent PI4P production. In addition, we observed that the MNV NS4 protein is able to bind PI4P in a similar manner to that of HCV NS5A. These studies have identified that PI4P is a major component of the MNV RC and that Arf1 and PI4KIIIα are the major cellular proteins modulated by MNV for the increased PI4P production during MNV replication.

4.2 Results

4.2.1 PI4P localises to sites of MNV replication

Recent reports have indicated that the intracellular replication of many (+)RNA viruses is dependent on the phosphoinositide PI4P (200, 221, 222, 230, 258). Our previous localisation studies of the MNV RC indicated that the membranes comprising the MNV RC were derived from the ER and endocytic pathway (73). Intriguingly, a notable feature of these membranes is that they are rich in PI4P. Thus, in light of these previous observations we aimed to determine if the MNV RC recruited PI4P to facilitate efficient viral replication.

RAW264.7 cells were mock- or infected with MNV for 12 hours, fixed and immunolabelled with anti-PI4P antibodies and antibodies to the viral replicative protein NS4 (Fig. 10). In mock-infected cells we observed that the levels of PI4P were quite low and the distribution of the lipid was fairly dispersed throughout the cytoplasm (Figs. 10a-d), although some did appear to accumulate within the perinuclear region (Figs 10e-h). In contrast, we observed a dramatic increase in the redistribution and accumulation of PI4P only in the MNV-infected cells (Figs. 10i-l). This staining pattern of PI4P strongly co-localised with that of the MNV protein NS4 (Figs 15m-p; $Rr = 0.86$). As the NS4 protein (with most if not all of the other MNV ORF1 proteins) localises to the MNV RC (182) these observations suggested that MNV recruits PI4P to the RC during replication.

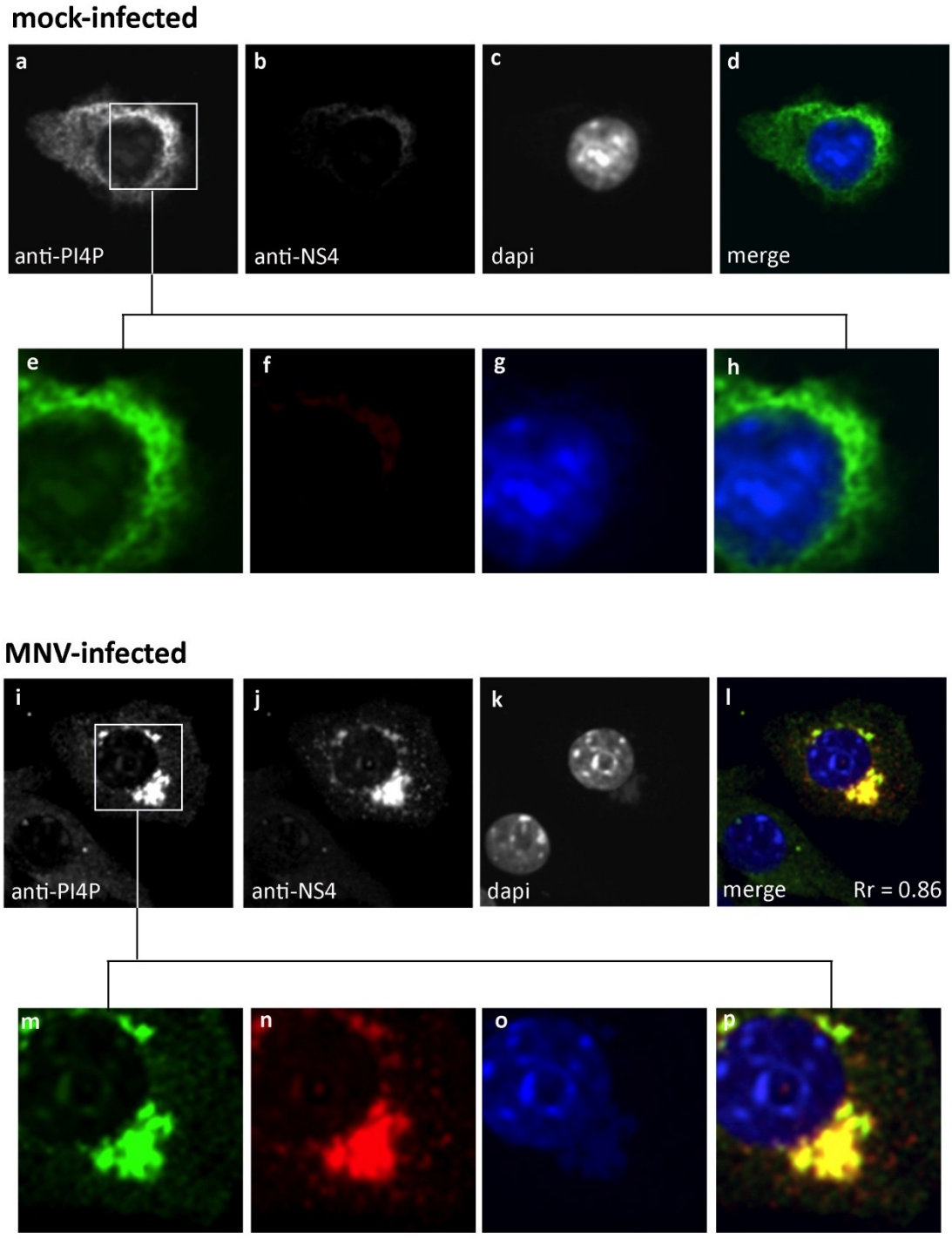


Figure 10. PI4P localises to sites of MNV replication.
RAW264.7 cells were infected with MNV at an M.O.I. of 5, fixed at 12 h.p.i. and labelled with anti-PI4P (green; panels a, e, i and m)) and anti-NS4 (red; b, f, j, and n) antibodies and the nuclei were counterstained with dapi (panels c, g, k and o). In MNV-infected cells PI4P presence was observed to dramatically increase, and to accumulate and co-localise with the MNV RC (*Rr* = 0.86). Merged panels are shown in panels d, h, l and p and co-localisation is shown as a yellow hue. Images are taken at 63x magnification. Pearson's coefficient (*Rr*; co-localisation) was determined by the JaCOP plugin software in ImageJ.

4.2.2 **PI4Ks localise to sites of MNV replication.**

The results in Figure 10 indicated that MNV recruited PI4P to the RC, and thus we aimed to investigate whether MNV recruits or utilizes PI4Ks for the production of the lipid. For these analyses, RAW264.7 cells were mock- or infected with MNV for 12 hours and subsequently fixed and stained with antibodies specific for either PI4KIIIα or PI4KIIIβ and the MNV protein NS4 (Fig. 11). In mock-infected cells we observed that PI4KIIIα was present quite diffuse within the cytoplasm (Figs. 11a-d), whereas PI4KIIIβ was also diffuse within the cytoplasm but also accumulated within the perinuclear region typical of staining of the Golgi apparatus (Figs. 11e-h). These staining patterns are consistent with the known localisation of these two proteins (208, 209).

Within the MNV-infected cells, we observed, that both PI4KIIIα and PI4KIIIβ displayed a dramatic redistributed to be confined within the perinuclear region and both displayed a high degree of co-localisation with the viral NS4 protein (Figs. 11i-p; *Rr* = 0.83 and *Rr* = 0.68 for PI4KIIIα or PI4KIIIβ respectively). Together these results and those described in section 4.2.1 indicate that MNV actively recruits the lipid kinases PI4KIIIα and PI4KIIIβ to facilitate and increase the local production of PI4P within the MNV RC during replication.

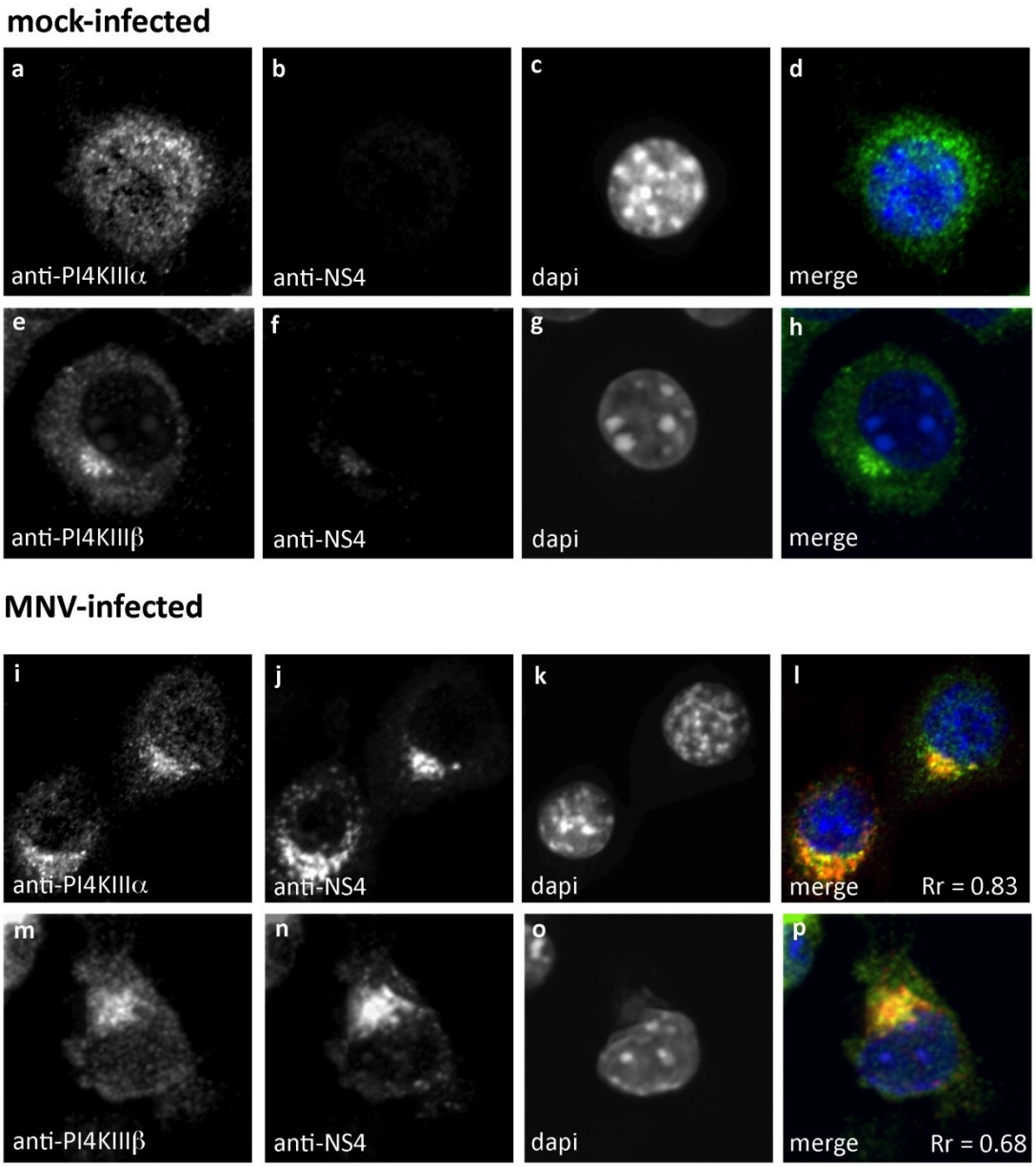


Figure 11. PI4KIIIα and PI4KIIIβ localise to the sites of MNV replication.
RAW264.7 cells were infected with MNV at an M.O.I. of 5, fixed at 12 h.p.i. and labelled with antibodies to PI4KIIIα (green; panels a and i) or PI4KIIIβ (green; e and m), the MNV NS4 protein (red; panels b, f, j and n) and the nuclei were counterstained with dapi (blue; panels c, g, k and o)). Merged panels are shown in panels d, h, l and p and co-localisation is depicted as a yellow hue. In MNV-infected cells both PI4KIIIα and PI4KIIIβ were observed to accumulate and co-localise with the MNV RC (*Rr* =0.83 and *Rr* =0.68, respectively). Images are taken at 63x magnification. Pearson's coefficient (*Rr;* co-localisation) was determined by the JaCOP plugin software in ImageJ.

4.2.3 **Inhibition of PI4KIIIβ function with the chemical inhibitor T-00127-HEV1 did not affect MNV RC formation or replication.**

The compound T-00127-HEV1 (HEV1) is termed an enviroxime-like compound and has antiviral activity against a number of viruses, particularly those of the *Picornaviridae* family (274, 275). The major target of HEV1 is PI4KIIIβ and the antiviral activity is due to the reduction in PI4P production in the presence of HEV1. As the *Caliciviridae* and *Picornaviridae* share many characteristics, most importantly a requirement for PI4P during replication, we aimed to determine of HEV1 was equally potent against MNV compared to the members of the *Picornaviridae*. For these analyses, RAW264.7 cells were infected with MNV at 5 m.o.i. and 1 hr post-infection the cells were treated with 10μM HEV1 or left untreated and MNV replication was assessed by IF analysis, plaque assay and western blot at 12 hrs p.i. (Fig. 12).

Our initial assessment of the effects of HEV1 on MNV were by IF analysis and we observed that the presence and formation of the MNV RC remained relatively unaltered in the presence of HEV1 (compare Figs. 12a-d with 12e-h). Interestingly we still observed a significant amount of PI4P produced and redistributed to the MNV RC in the presence of HEV1, as identified with anti-PI4P antibodies (Fig. 12e). These results suggest that HEV1-dependent inhibition of PI4KIIIβ did not affect the production of PI4P in MNV-infected cells.

To further assess the effects of HEV1 on MNV replication we measured viral protein production (Fig. 12B) and viral titres (Fig. 12C) during HEV1 treatment. We observed minimal impact of HEV1 on the production of MNV infectious virus and on the production of viral protein NS7 (or other MNV viral proteins; data not shown). Overall we can conclude

from these observations that PI4KIIIβ is not required for the production of PI4P in MNV-infected cells.

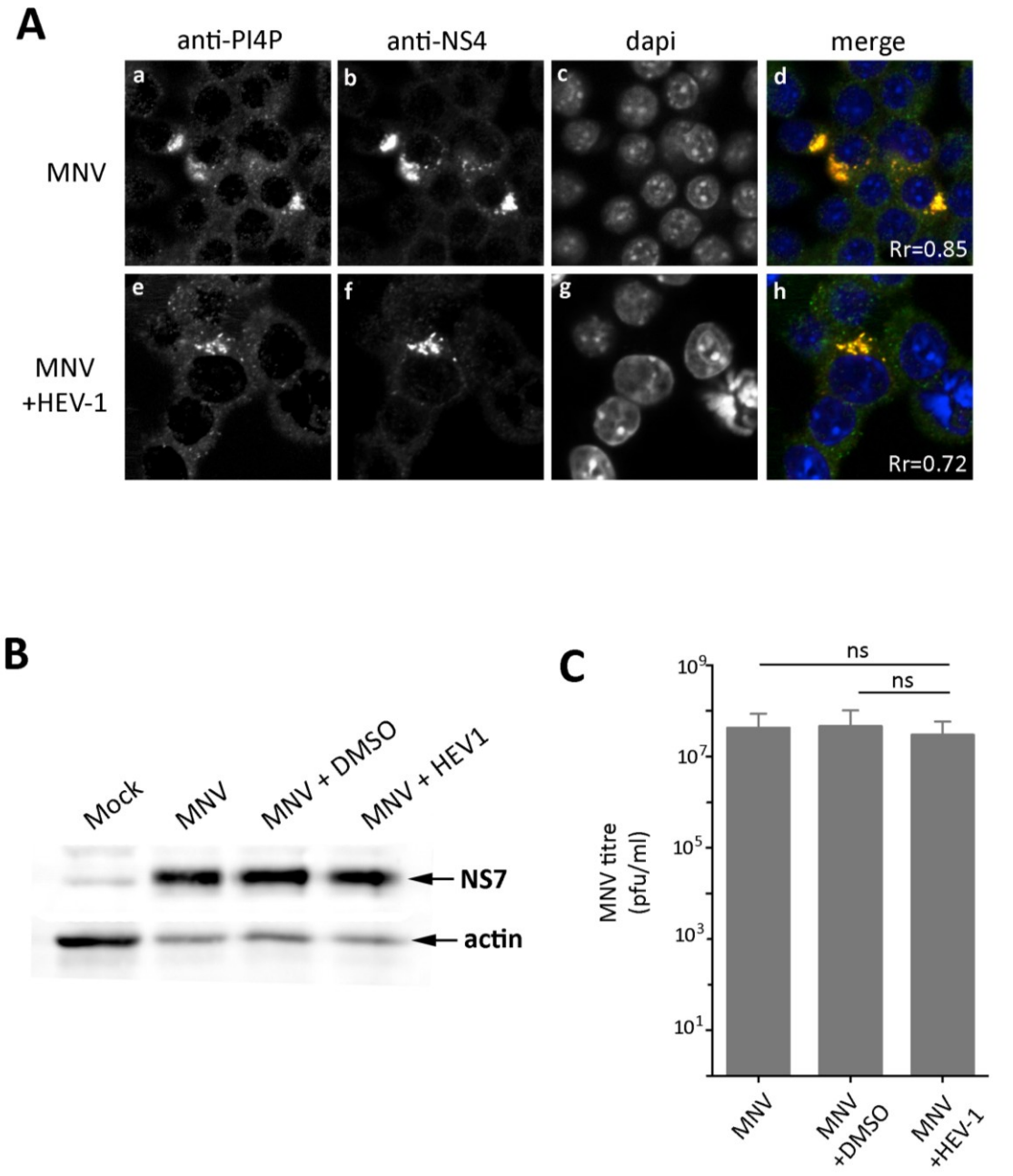


Figure 12. HEV1-mediated inhibition of PI4KIIIβ does not impair MNV replication.
(A) RAW264.7 cells were infected with MNV at an M.O.I. of 5 and at 1 h.p.i. were treated with 10μM HEV1. At 12 h.p.i. the cells were fixed and labelled with anti-PI4P (green; panels a and e) or anti-NS4 (red; panels b and f) antibodies and the nuclei were counterstained with dapi (blue; panels c and g). Merged panels are shown in panels d and h, and co-localisation is depicted as a yellow hue. In MNV-infected cells treated with HEV1, PI4P was seen to accumulate and co-localise with the MNV RC (*Rr*=0.72). Images are taken at 63x magnification. **(B-C)** At 18 h.p.i. tissue culture fluid and lysates were collected for analysis by plaque assay and western blotting, respectively. In **(B)** the production of viral protein in cell lysates was analysed by western blotting with antibodies against the viral protein NS7 and the cellular protein actin, as a loading control. In **(C),** the production of infectious extracellular virus was measured by plaque assay in the presence of the vehicle solvent (DMSO) or HEV-1 and enumerated as pfu/mL ($n = 4$). Significance was determined by students *t*-test using Graph Prism 6.

4.2.4 **The lipid kinase PI4KIIIα, but not PI4KIIIβ, contributes to the increased production of PI4P during MNV replication.**

Several recent studies have shown that the chemical compound PIK93 is a useful compound to inhibit PI4K activity (274, 276). The use of PIK93 at different concentrations affects the different kinases, such that use of PIK93 at a concentration of 10μM inhibits the activity of PI4KIIIβ whilst when used to a higher concentration of 30μM inhibits PI4KIIIβ and PI4KIIIα activity. As we had observed that HEV1 had a minimal impact on MNV replication we aimed to examine whether either 10μM or 30μM PIK93 could affect the production of PI4P during MNV replication and assess the impact of the compound on NV replication efficiency (Fig. 13). Thus, RAW264.7 cells were infected with MNV and treated with either 10μM or 30μM of PIK93 at 1 h.p.i. or treated with the vehicle solvent DMSO alone. At 12 hrs h.p.i. cellular lysates and tissue culture fluid were collected for analysis by IF, western blot, qRT-PCR and plaque assay.

Our initial IF analysis revealed that treatment of cells with PIK93 at a concentration of 10μM still resulted in a significant amount of PI4P produced and redistributed to the MNV RC, as identified with anti-PI4P antibodies (Fig. 13h). However, treatment with PIK93 at a concentration of 30μM resulted in an impairment of PI4P production and a minimal accumulation and redistribution of PI4P to the MNV RC (Fig 13l). These results confirm those in section 4.2.3 that indicate that PIK93-dependent inhibition of PI4KIIIβ does not affect the production of PI4P in MNV-infected cells but that PIK93-dependent inhibition of PI4KIIIα plays a critical role in the production of PI4P in MNV-infected cells.

In addition to the IF analysis, our western analysis showed a significant decrease in MNV NS7 protein levels in the 30μM PIK93 treated cells whilst only a slight decrease in NS7 was observed in cells treated with 10μM PIK93 (Fig. 13B). In agreement with the western blot data we also observed a significant fold decrease ($p<0.02$; $n = 3$) in the

transcription of MNV genomic RNA transcription in 30μM PIK93 treated cells, whereas only a slight, non-significant reduction was observed upon treatment with 10μM PIK93 (Fig. 13C). This difference was additionally reflected in the production and secretion of infectious MNV particles, where a 1-2 $\log_{10}$ decrease ($p<0.02$; $n = 4$) was observed in cells treated with 30μM PIK93, compared a slight non-significant decrease in the 10μM PIK93-treated cells (Fig. 13D).

Taken together these results suggest that PI4KIIIα rather than PI4KIIIβ is required for the increased production of PI4P during MNV replication and that inhibition of this enzyme activity duly affects the replication efficiency of MNV in macrophages.

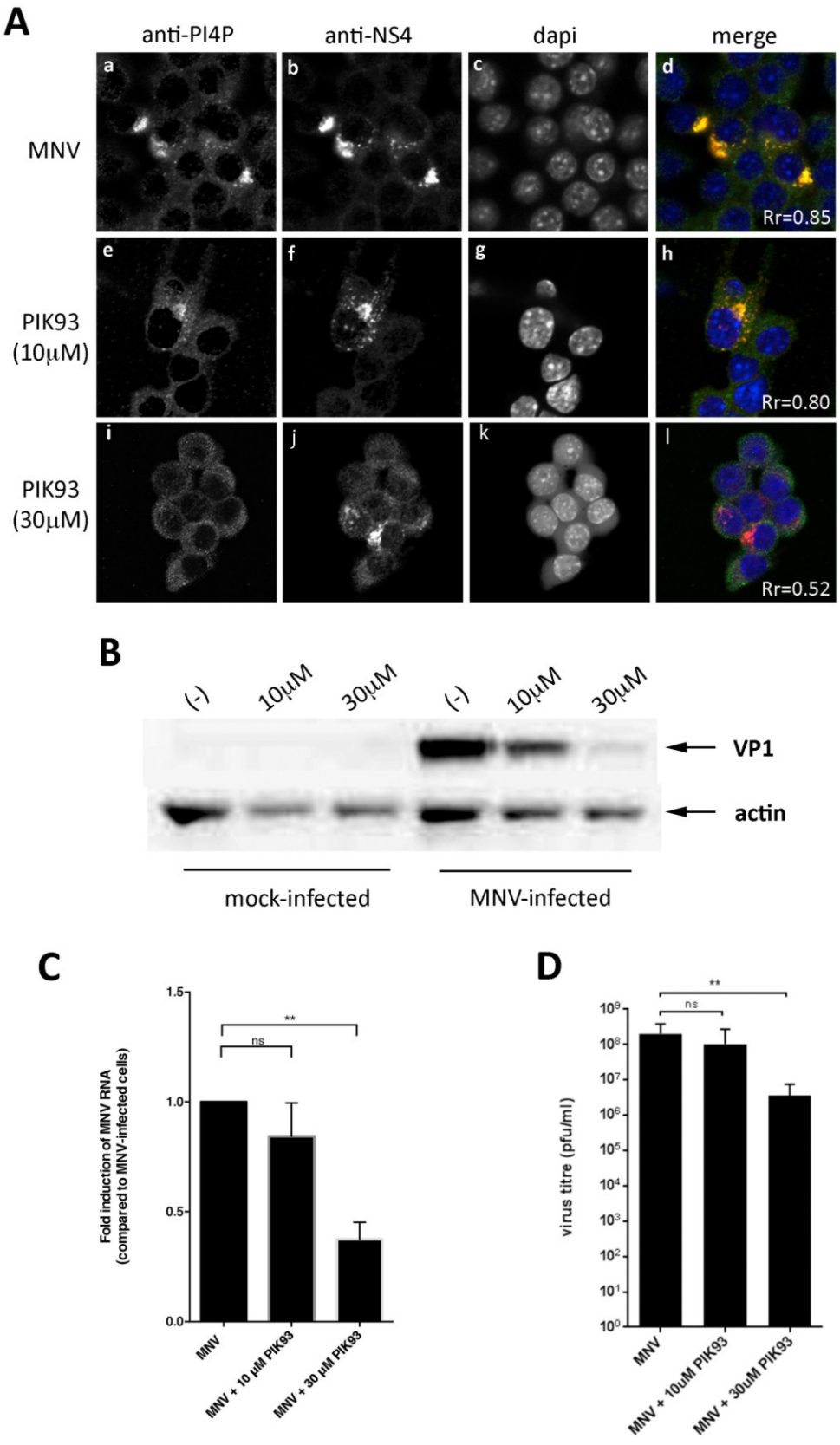


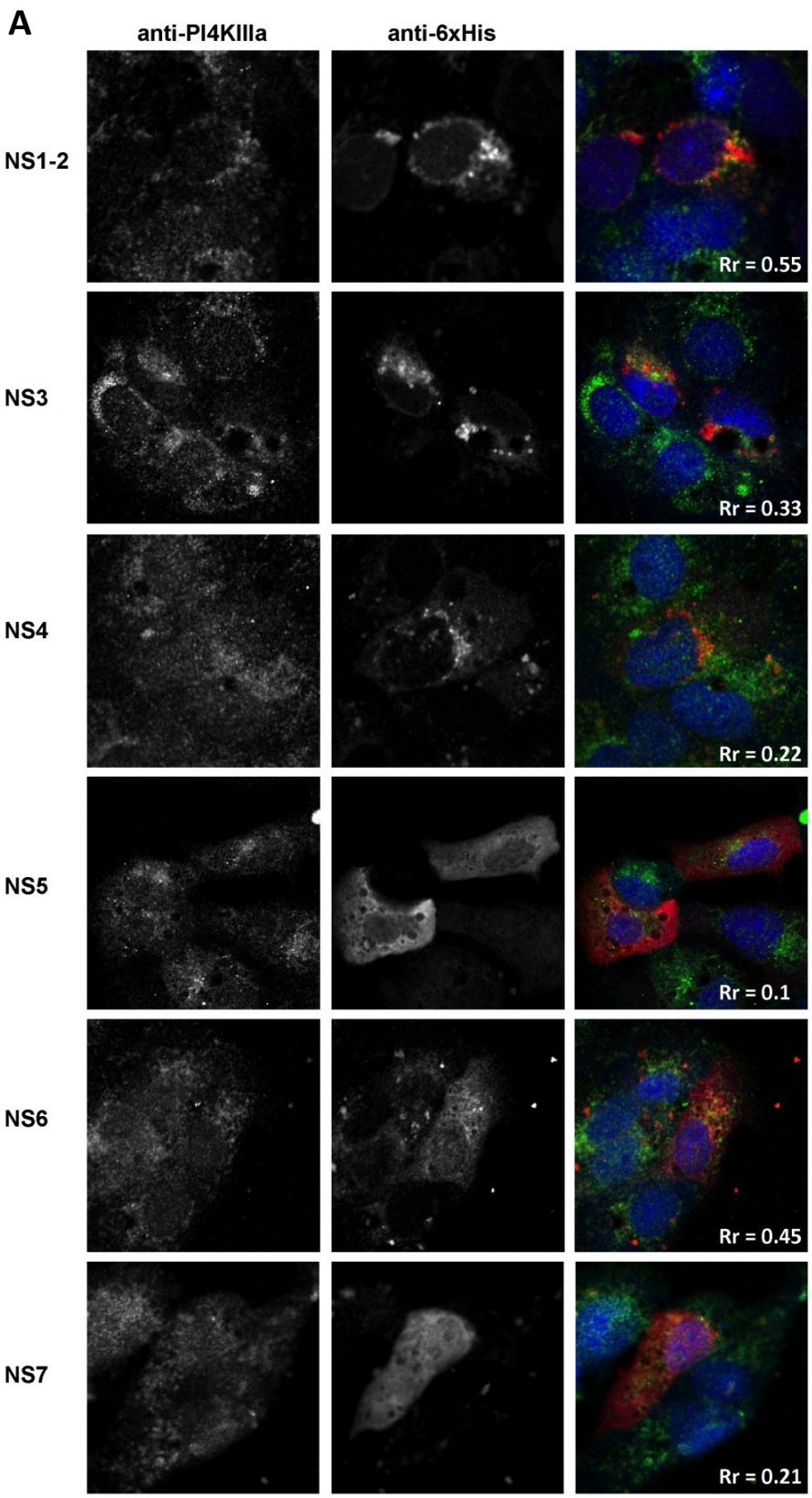
Figure 13. PI4KIIIα rather than PI4KIIIβ is required for MNV replication.
RAW264.7 cells were treated with 10 or 30 μM PIK93 and infected with MNV at an M.O.I. of 5 1 hour later. 12 h.p.i. cells were fixed and labelled with anti-PI4P (green) or anti-NS4 (red) or 18 h.p.i.

lysates and tissue culture fluid were collected. **(A)** In MNV-infected cells treated with 10 μM PIK93 PI4P was seen to accumulate and co-localise with the MNV RC (*Rr*=0.80). In MNV-infected cells treated with 30 μM PIK93 very minimal PI4P was seen to accumulate and co-localise with the MNV RC (*Rr*=0.52). Images are taken at 63x magnification. **(B)** Immunoblots of cell lysates were analysed for changes in viral protein under different PIK93 concentrations. Significance was determined by students *t*-test using Graph Prism 6. **(C)** RNA was then extracted and analysed via qPCR ($n = 3$, * indicates $p < 0.02$). **(D)** Extracellular virus ($n = 4$) was measured by plaque assay for pfu/ml and expressed as pfu/mL.

4.2.5 **MNV ORF1 proteins do not influence nor co-localise with the lipid kinases PI4KIIIα and PI4KIIIβ.**

As we observed co-localisation of PI4Ks with NS4 during MNV infection, we aimed to investigate if NS4 or other MNV non-structural proteins were recruiting PI4Ks. Transiently expressed proteins in the absence of infection allow further insight into whether replication is needed to recruit membranes within host cells or if the NS innately do this. Vero C1008 cells were transfected with each individual MNV ORF1 protein and were subsequently fixed 24 hours later and stained for PI4KIIIα or PI4KIIIβ and anti-6xHis (Fig 14).

Immunofluorescence analyses revealed that NS1-2 was the only ORF1 protein which co-localised with PI4KIIIα (*Rr* =0.55; Fig 14A) and NS4 was the only ORF1 protein that co-localised with PI4KIIIβ (*Rr* =0.59; Fig 14B). This data suggests that NS1-2 may be recruiting PI4KIIIα, while NS4 may be recruiting PI4KIIIβ to the RC in order to generate PI4P-rich regions.


A
anti-PI4KIIIa
anti-6xHis
NS1-2
Rr = 0.55
NS3
Rr = 0.33
NS4
Rr = 0.22
NS5
Rr = 0.1
NS6
Rr = 0.45
NS7
Rr = 0.21

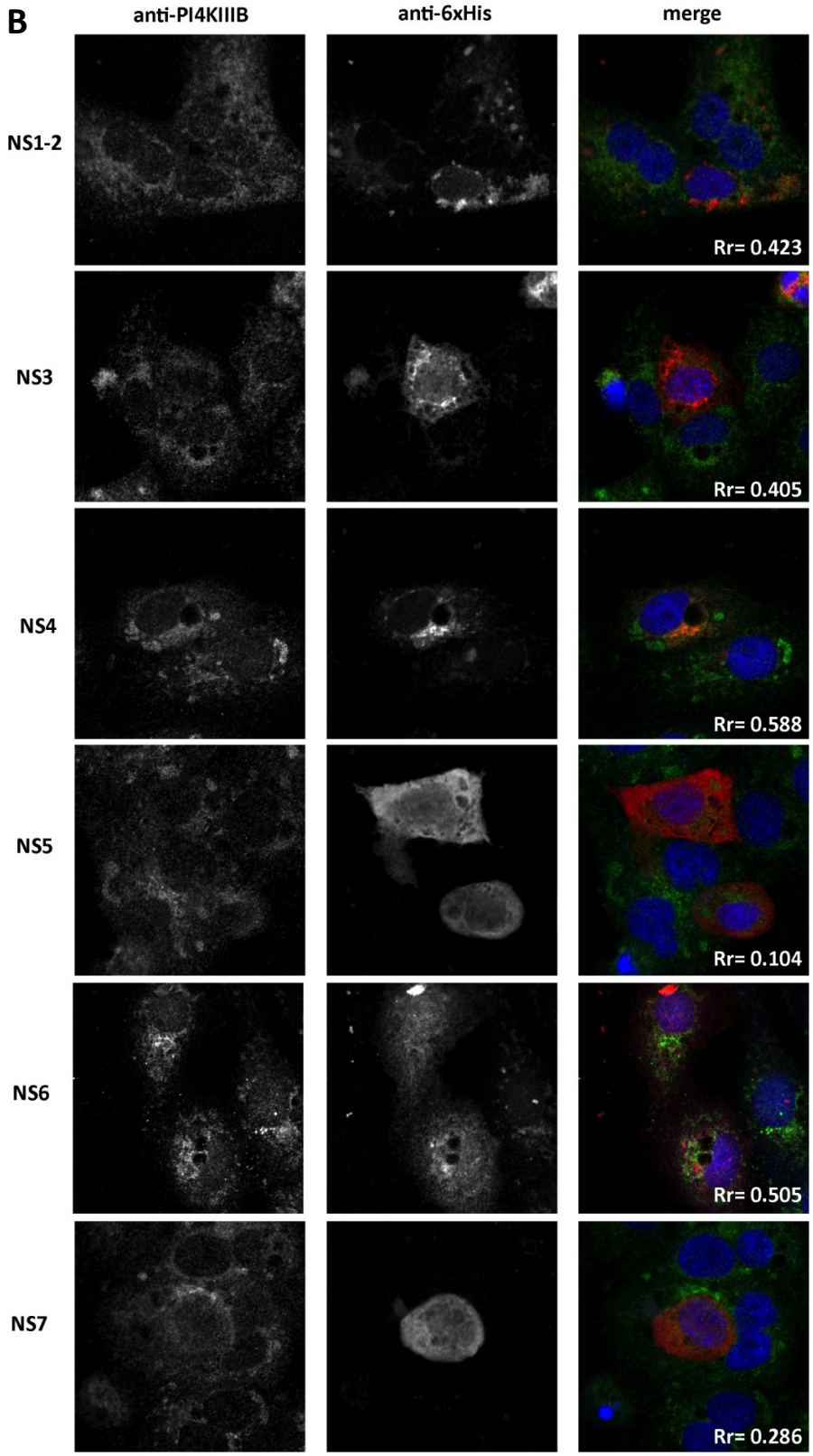


Figure 14. MNV ORF1 proteins NS1-2 and NS4 recruits PI4KIIIα and PI4KIIIβ, respectively. Vero C1008 cells were transfected with each individual MNV ORF1 proteins for 24 hours and then labelled for **(A)** anti-PI4Kα (green) and anti-6xHis (red) or **(B)** anti-PI4KIIIβ (green) and 6xHis (red). Images are taken at 63x magnification. Pearson's coefficient (co-localisation) was determined by the JaCOP plugin software in ImageJ.

4.2.6 **Transiently expressed MNV ORF1 proteins do not co-localise with PI4P.**

A recent study has shown that individually expressed PV 2C-containing putative precursors (2BC, 2BC3A and 2C3A), but not 2C or other virus proteins, activated PI4P production and accumulation (284). Our lab has shown previously that transient expression of the MNV ORF1 proteins alone do not induce RC formation, but that each NS protein is associated with the RC during replication (60).

As we had only observed minimal, if any, co-localisation of the MNV ORF1 proteins with either PI4KIIIα or PI4KIIIβ, we aimed to determine whether transient expression of the individual MNV ORF1 proteins resulted in the co-localisation and redistribution of regions rich in PI4P. To determine this, Vero C1008 cells were transfected with each individual MNV ORF1 proteins and were subsequently fixed 24 hours later and labelled for anti-PI4P and anti-6xHis antibodies (Fig. 15). We observed that none of the MNV ORF1 proteins accumulated pools of PI4P such as we observed in Figure 10, and there was no significant overlap of NS proteins and PI4P staining (Fig. 15). These findings suggest that MNV replication must occur for the virus to recruit or redistribute PI4P.

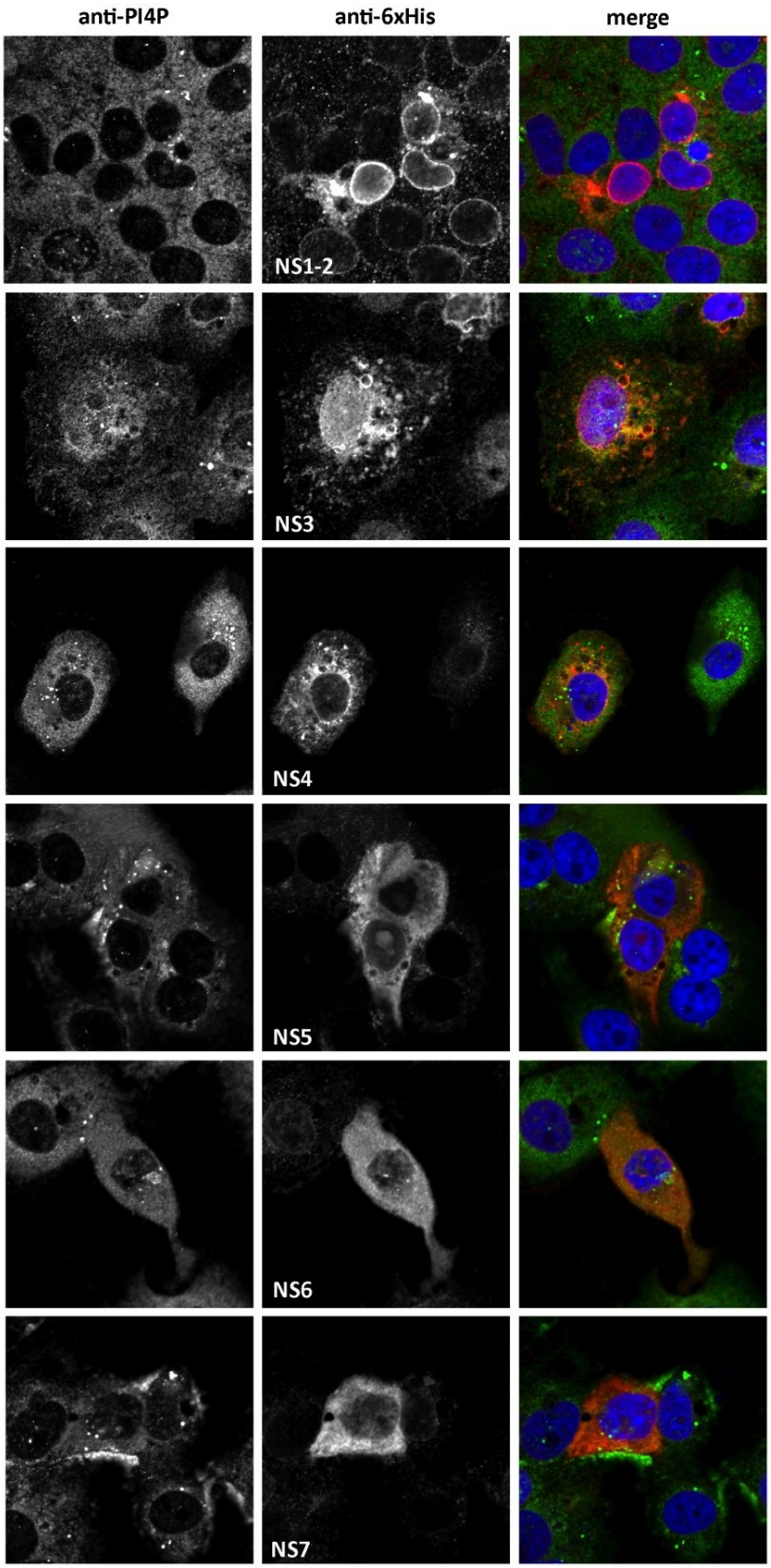


Figure 15. Individually expressed MNV ORF1 proteins do not induce pools of PI4P.
Vero C1008 cells were transfected with each individual MNV ORF1 proteins for 24 hours and then fixed and labelled for anti-PI4P (green) and anti-6xHis (red). Images are taken at 63x magnification.

4.2.7 **Arf1 and GBF1 localise to sites of MNV replication.**

As we had observed that none of the MNV ORF1 proteins convincingly confer the capacity to recruit the PI4Ks or PI4P individually, we sought to investigate the role of the accessory proteins Arf1 and GBF1 in the process of forming the MNV RC. It has been previously reported that the Enterovirus protein 3A interacts with GBF1 and requires this host factor for replication, although the exact role GBF1 plays in replication remains elusive (228, 259, 277). In addition, it was observed that Arf1 and GBF1 aid in generating a PI4P-rich environment facilitating HCV replication (270). Thus in this study, we aimed to investigate whether Arf1 and GBF1 were also recruited to the sites of MNV replication to enable and facilitate the production of PI4P.

RAW264.7 cells were transfected with Arf1-GFP or GBF1-venus expression plasmids and mock- or infected with MNV 24 hours later. Cells were subsequently fixed at 12 h.p.i. and stained with antibodies specific for the MNV NS4 protein (Fig. 16). In MNV-infected cells that were also transfected we observed NS4 staining and Arf1-GFP or GBF1–venus fluorescence accumulate within the perinuclear region typical of staining of the Golgi apparatus (Figs. 16a-l). These staining patterns are consistent with the known localisation of these three proteins. Within the transfected MNV-infected cells, we observed that both Arf1 and GBF1 co-localised with the viral NS4 protein (Figs. 16a-d; Rr=0.64 and Rr=0.88 for Arf1 or GBF1 respectively). These results indicate that in addition to the PI4Ks and PI4P, these host factors are also recruited to the sites of MNV replication.

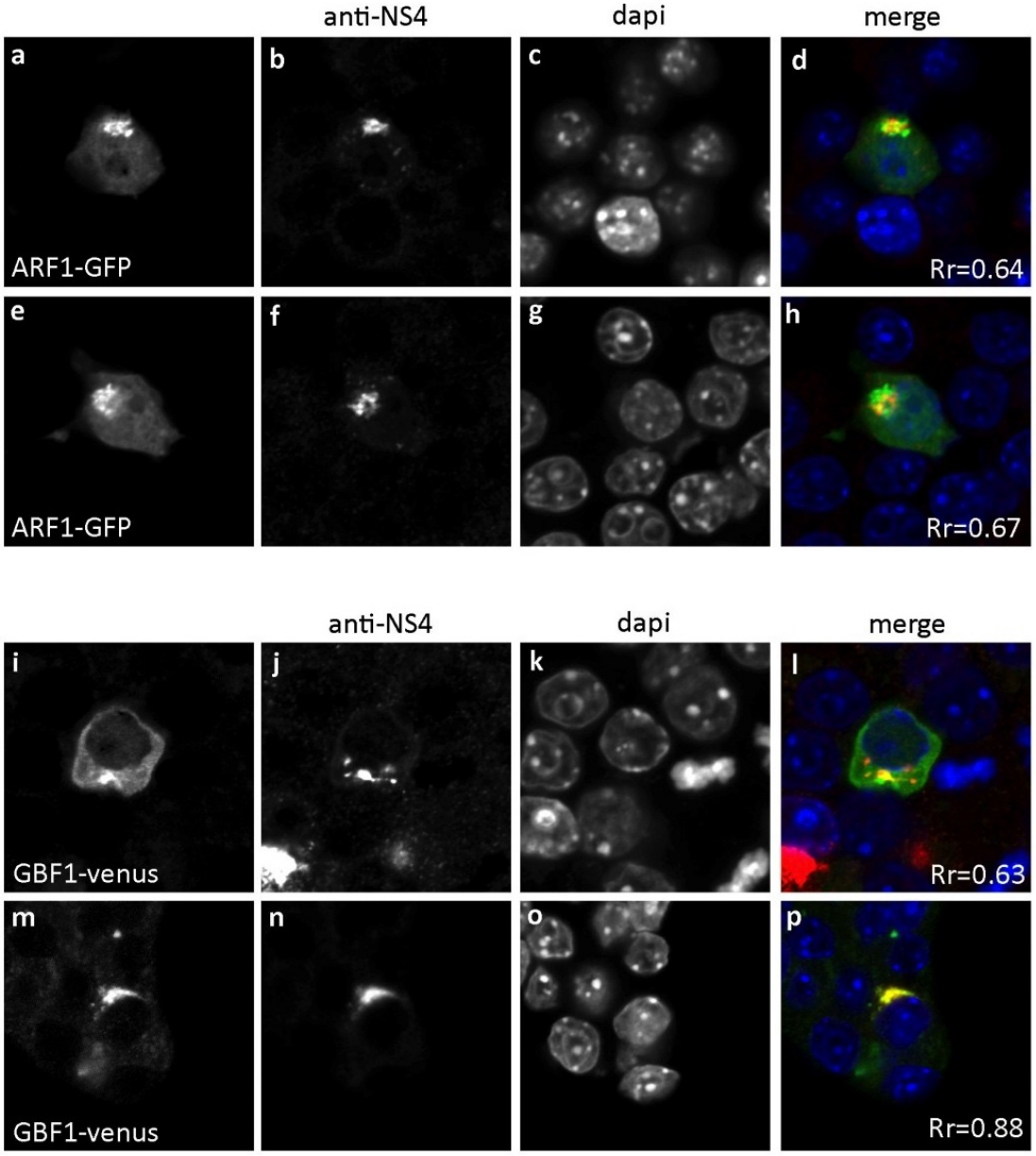


Figure 16. Arf1 and GBF1 co-localise with sites of MNV replication.
RAW264.7 cells were transfected with Arf1-GFP or GBF1-venus plasmids (green; panels a, e, i, m, respectively) and infected with MNV at an M.O.I. of 5, fixed at 12 h.p.i. and labelled with antibodies to the MNV NS4 protein (red; panels b, f, j and n) and the nuclei were counterstained with dapi (blue; panels c, g, k and o). Merged panels are shown in panels d, h, l and p and co-localisation is depicted as a yellow hue. In transfected, MNV-infected cells Arf1-GFP was observed to co-localise with the MNV RC (*Rr* =0.64 and *Rr* =0.67). GBF1-venus was observed to co-localise with the MNV RC (*Rr* =0.63 and *Rr* =0.88). Images are taken at 63x magnification. Pearson's coefficient (*Rr;* co-localisation) was determined by the JaCOP plugin software in ImageJ.

4.2.8 **Chemical inhibition of GBF1 activity with AG1478 has no effect on MNV replication.**

Brefeldin A (BFA) is a recognized inhibitor of enteroviruses. BFA inhibits the transport of cargo from the ER to the Golgi by disrupting vesicles and the integrity of the ER-Golgi intermediate compartment (ERGIC) via inhibition of GBF1 (278). Previous studies from our laboratory indicated that BFA had no significant effect on MNV viral protein production or production of infectious virus (73). Recently the compound, AG1478 was described to induce Golgi complex disassembly and specifically inhibit GBF1, which subsequently prevented Arf1 activation (279). As we had observed that both Arf1 and GBF1 co-localised with the MNV RC during infection, we aimed to determine whether AG1478 had any detrimental effect on MNV replication.

RAW264.7 cells were infected with MNV at an m.o.i of 5 and treated with AG1478 1 h.p.i. At 12 h.p.i. cells were fixed for immunofluorescence and cellular lysates and tissue culture fluids were collected for subsequent analysis. IF analysis revealed no distinct change in RC formation and PI4P was still seen to be significantly produced and redistribute and co-localise with the MNV RC (*Rr* = 0.72) (Fig 17A). Our western blot analysis showed a slight decrease in MNV VP1 protein levels in the AG1478-treated cells compared to untreated MNV-infected cells (Fig. 17B). In agreement with the western blot data we observed a slight but non-significant difference in the production and secretion of infectious MNV particles in cells treated with AG1478 compared to untreated MNV-infected cells (Fig. 17C).

Thus we conclude that although GBF1 is known to stimulate Arf1 activity, this function is not required for the ability of Arf1 to generate a PI4P-rich environment for MNV replication. These results are also in agreement with our previous results showing that MNV replication is resistant to BFA treatment.

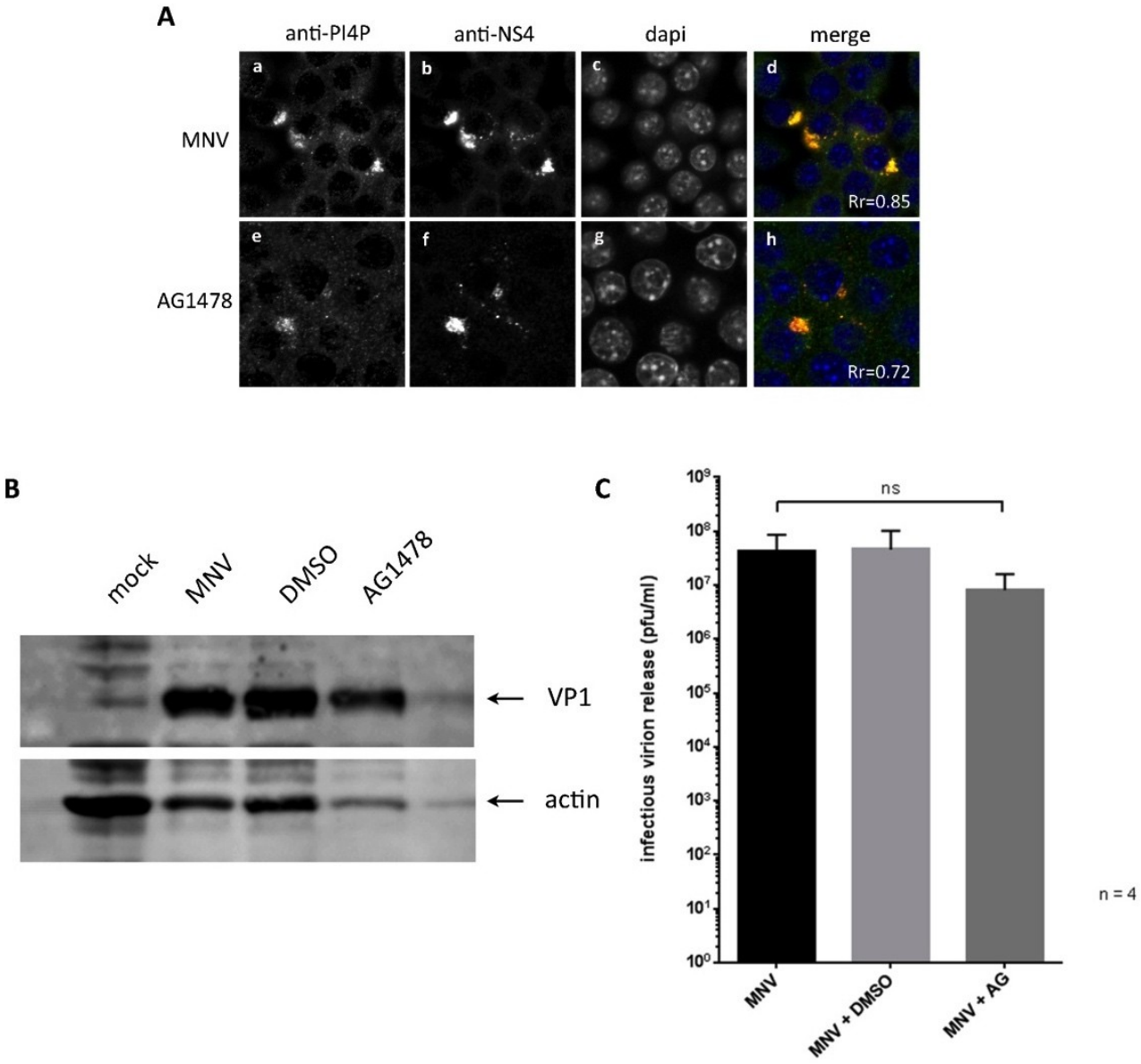


Figure 17. GBF1 inhibition by AG1478 has no effect on MNV replication.
RAW264.7 cells were treated with AG1478 and infected with MNV at an m.o.i. of 5 1 hour later. 12 h.p.i. cells were fixed and labelled with anti-PI4P (green) or anti-NS4 (red) or 18 h.p.i. lysates and tissue culture fluid were collected. **(A)** In MNV-infected cells treated with AG1478 PI4P was seen to accumulate and co-localise with the MNV RC (*Rr*=0.72). **(B)** Immunoblots of cell lysates were analysed for changes in viral protein when MNV-infected cells were treated with AG1478. **(C)** Extracellular virus (n = 4) was measured by plaque assay and expressed as pfu/mL +/-S.E.M.

4.2.9 **Individually expressed MNV non-structural proteins NS3, NS4 and NS6 co-localise with Arf1, but no individually expressed MNV non-structural proteins co-localise with GBF1.**

As we observed that Arf1 and GBF1 are recruited to the sites of MNV replication and that none of the MNV ORF1 proteins individually associated with the PI4Ks or increased PI4P production, we aimed to examine whether any MNV non-structural proteins were responsible for the recruitment of either Arf1 or GBF1. To determine this, Vero C1008 cells were co-transfected with each individual MNV ORF1 proteins and either Arf1-GFP or GBF1-venus. Cells were subsequently fixed 24 hours later and visualised for the GFP or venus expression and immune-labelled with anti-6xHis antibodies for detection of the MNV ORF1 proteins (Figs. 18 and 19). In cells co-transfected with Arf1-GFP and ORF1 proteins, we observed a significant co-localisation between Arf1-GFP with NS3 (*Rr* =0.54), NS4 (*Rr* =0.64) and NS6 (*Rr* =0.83) (Fig 18). In each case both Arf1-GFP and the MNV proteins were observed to accumulate in the perinuclear region of the cell. Interesting, the most significant co-localisation was observed between the MNV protease and Arf1-GFP

In contrast, no co-localisation was observed between GBF1-venus and the MNV ORF1 proteins (Fig 19). Additionally, the expression of the MNV ORF1 proteins did not duly affect the distribution and localisation of GBF1. Thus we can conclude that GBF1 does not play a critical role during MNV replication and neither its activity (see section 4.2.8) nor its physical association with the MNV replicative proteins is required during the MNV replication cycle.

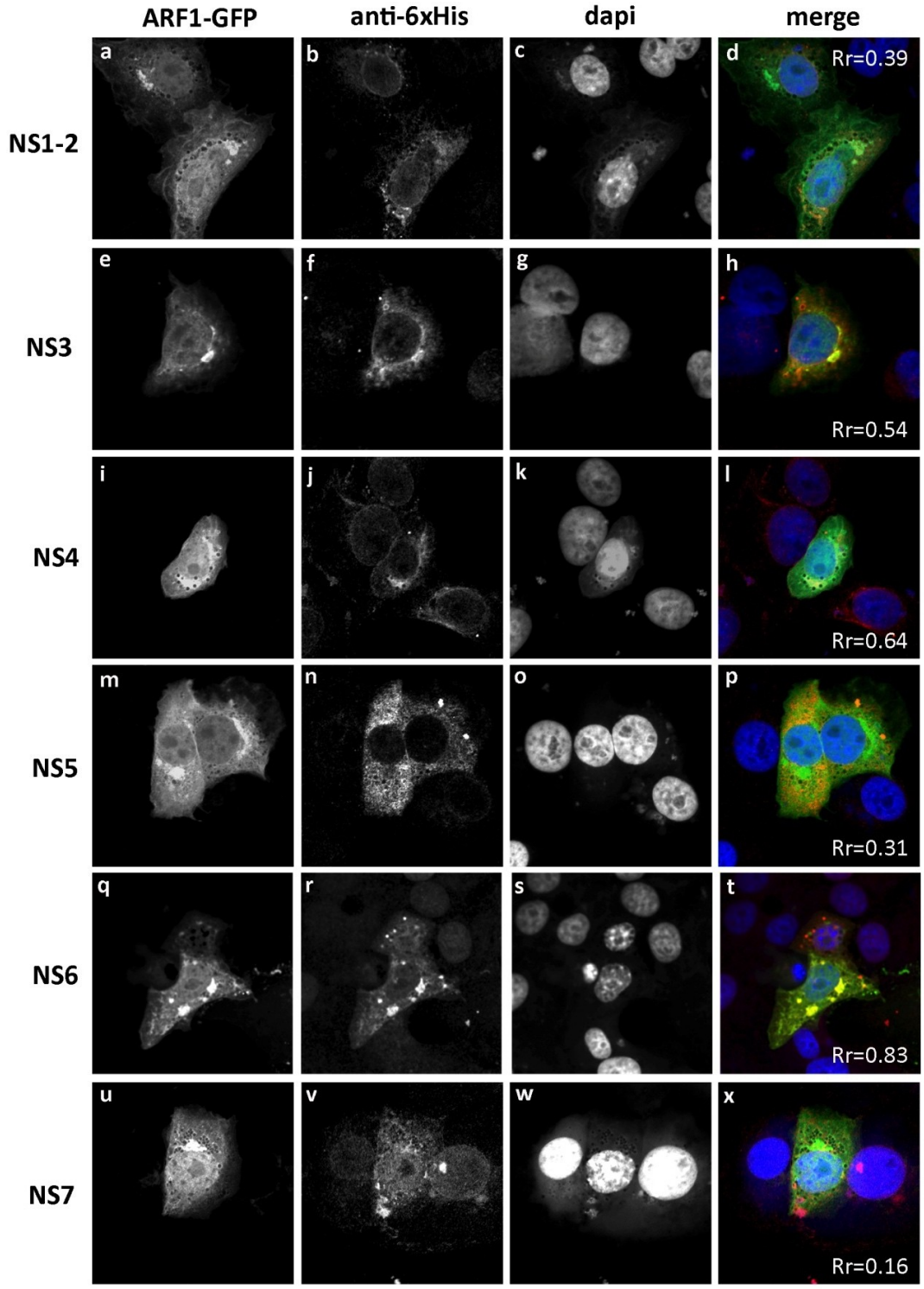


Figure 18. Arf1-GFP co-localises with transiently expressed MNV NS3, NS4 and NS6 proteins. Vero C1008 cells were co-transfected with Arf1-GFP (green; panels a, e, i, m, q and u) and MNV ORF1 proteins and fixed 24 hours later. Cells were labelled with anti-6xHis (red; panels b, f, j, n, r and v) and the nuclei were counterstained with dapi (blue; panels c, g, k, o, s and w). Merged panels are shown in panels d, h, l, p, t and x and co-localisation is depicted as a yellow hue. In co-transfected cells NS3, NS4 AND NS6 was observed to co-localise with Arf1-GFP (*Rr* =0.54, *Rr* =0.64 and *Rr* =0.83, respectively). Images are taken at 63x magnification. Pearson's coefficient (*Rr;* co-localisation) was determined by the JaCOP plugin software in ImageJ.

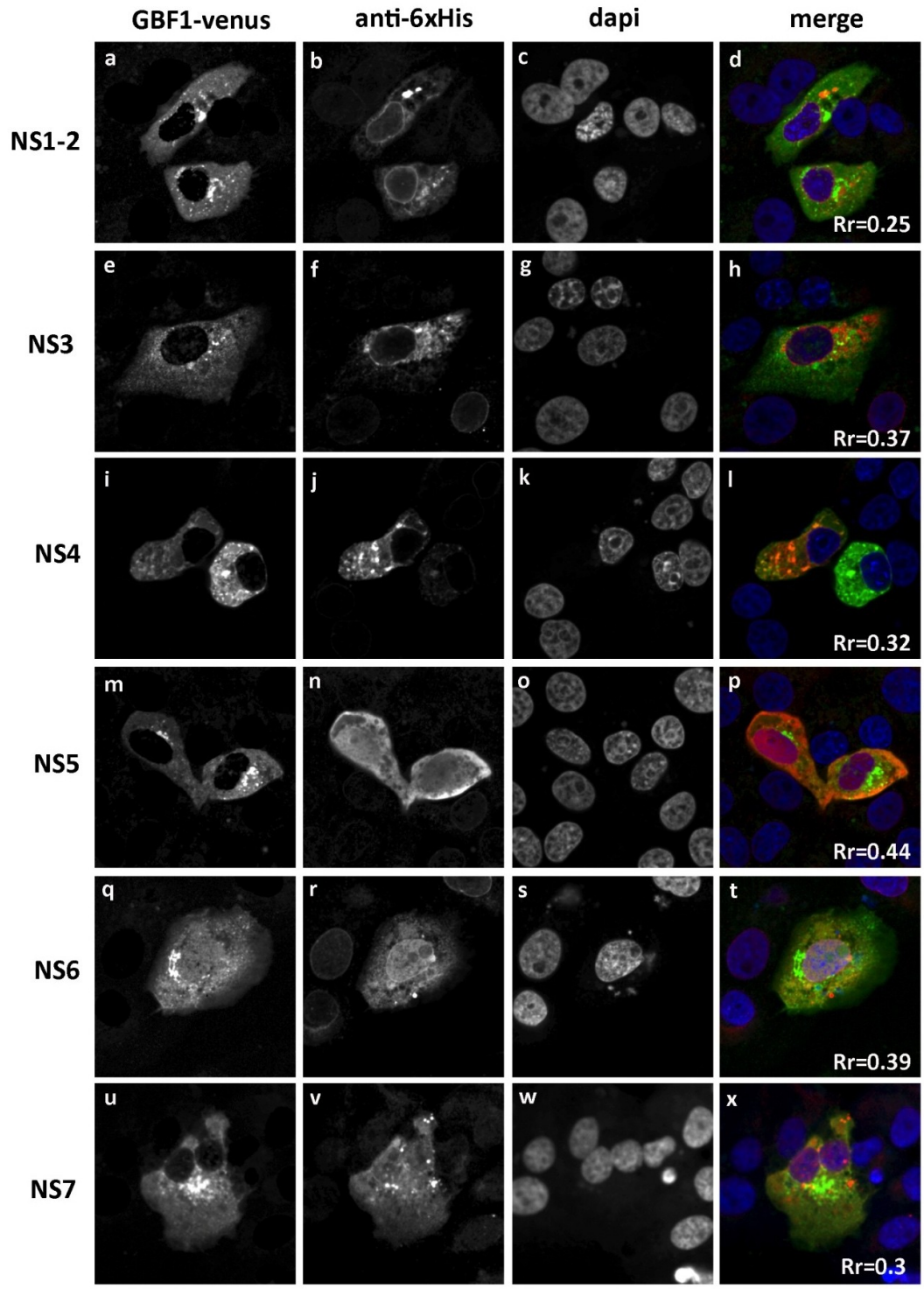


Figure 19. GBF1-venus does not co-localise with any transiently expressed MNV ORF1 proteins.
Vero C1008 cells were co-transfected with GBF1-venus (green; panels a, e, i, m, q and u) and MNV ORF1 proteins and fixed 24 hours later. Cells were labelled with anti-6xHis (red; panels b, f, j, n, r and v) and the nuclei were counterstained with dapi (blue; panels c, g, k, o, s and w). Merged panels are shown in panels d, h, l, p, t and x and co-localisation is depicted as a yellow hue. In co-transfected cells no co-localisation of GBF1-venus and MNV NS proteins was observed. Images are taken at 63x magnification. Pearson's coefficient (*Rr;* co-localisation) was determined by the JaCOP plugin software in ImageJ.

4.2.10 **The MNV NS4 protein interacts with and binds the lipid PI4P**

Lastly, we aimed to determine whether any of the MNV ORF1 proteins had the capacity to interact with and bind lipid. Previous reports have shown that the 3A protein of the *Picornaviridae,* and NS5A of HCV have an affinity for the PI4P lipid (206, 226, 227). We initially screened the NS4 and NS7 proteins as NS4 is homologous to the picornavirus 3A protein and NS7 is the viral RdRp. We hypothesised that these two proteins would most likely interact with lipids as NS4 would have an analogous function to that of the picornavirus 3A protein and that NS7 would require a lipid rich membrane for polymerase function. Thus, HEK 293T cells were transfected with recombinant expression plasmids encoding the MNV NS4 or NS7 proteins and lysed for analysis 24 hours later. Protein lysates along with purified HCV (JFH1) G2a RdRp protein (kindly provided by Peter White, UNSW and used as a positive control) were each incubated on PIP Strips overnight to allow protein-lipid binding. Following primary and secondary antibody incubations, the PIP Strips were subsequently scanned and analysed. As expected, the HCV RdRp bound to the phosphoinositides PI3P, PI4P and PI5P as described previously (208). In contrast, we observed that MNV NS4 bound to the PIP strips with a strong affinity for some phosphoinositides, including PI4P and PI5P (Fig. 20). In contrast, we did not observe any binding of MNV NS7 with any of the phosphoinositides on the phospholipid strips (Fig 20).

These results indicate that although the MNV NS4 protein does not stimulate the production or coalescence of PI4P in cells, it has an affinity for this lipid and may thus provide a scaffold for the biogenesis of the MNV RC on a PI4P-rich membrane platform.

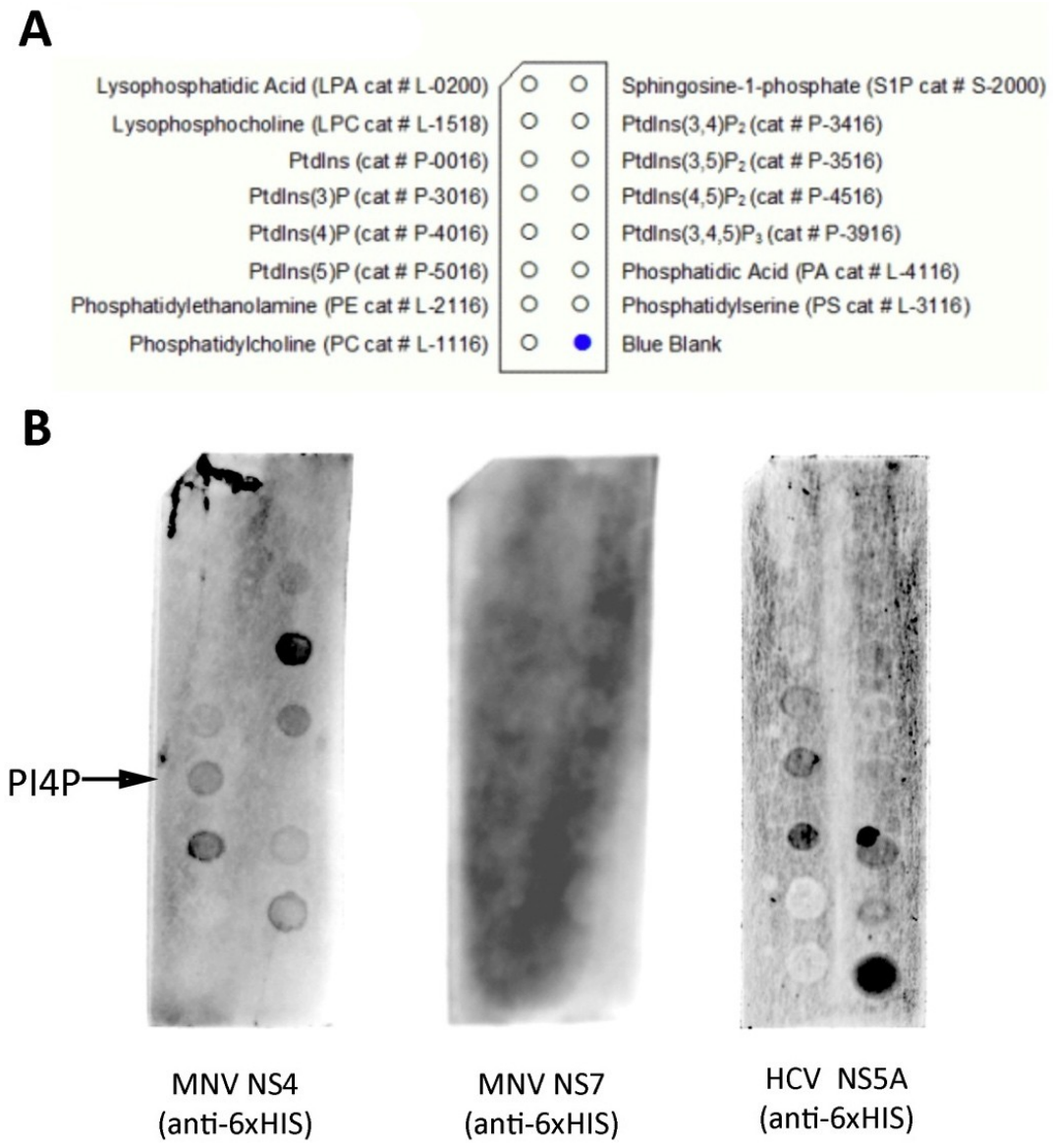


Figure 20. The MNV NS4 protein binds to host lipids including PI4P.
(A) A schematic of the PIPstrips used during this study indicating the position of the individual phospholipids and phosphoinositides. **(B)** HEK293T cells were transfected with recombinant DNA expression plasmids encoding the MNV NS4-6xHis and NS7-6xHis proteins and at 24 h.p.t. the cells were lysed and the lysate was incubated on the PIPstrip membrane before subsequent detection with anti-6xHis antibodies. Purified HCV NS5B-6xHis protein was also incubated with the PIPstrip membrane as a control.

4.3 Discussion

The replication processes of many viruses have been widely studied and it is well documented that (+)RNA viruses induce extensive intracellular membrane rearrangements in order to assist replication (183, 280, 281). These replication complexes are required for efficient virus replication but also aid as a means to avoid immune detection, although little is known about the composition and mechanism by which NoV RCs are formed (183, 282-284). As described previously (271), our laboratory has shown that MNV recruits membranes from the ER and late secretory pathway in order to form its RC (271), and additionally we observed that the membrane re-distribution is potentially mediated via the MNV NS1-2 and NS4 proteins (120). In this study we observed that the increased production of PI4P via PI4KIIIα is important for MNV replication. We observed that: (i) during infection MNV recruits PI4KIIIα, PI4KIIIβ, Arf1, GBF1 and PI4P to sites of virus replication, (ii) chemical inhibition of PI4KIIIα specifically decreases MNV replication and viral secretion, (iii) MNV NS3, NS4 and NS6 co-localised with Arf1, and (iv) no individually expressed ORF1 proteins co-localised with PI4KIIIα, PI4KIIIβ, PI4P or GBF1, although the NS4 protein was able to bind PI4P independently of the other viral proteins. These results suggest that a PI4P rich membrane platform is required for the biogenesis of the MNV RC and efficient MNV replication is equally dependent on PI4P production in a PI4KIIIα-dependent manner.

The role of PI4P in the replication of many (+)RNA viruses has been increasingly studied and have revealed that picornaviruses such as poliovirus (PV) and Coxsackie virus B3 (CVB3) heavily rely on PI4KIIIβ to generate pools of PI4P for replication. Conversely, HCV primarily requires the activity of PI4KIIIα for the establishment and maintenance of replication (220, 273, 285, 286). Due to many similarities between caliciviruses and picornaviruses, these lead us to investigate whether MNV replication also required PI4P and one or more of the associated kinases.

In our initial immunofluorescence analyses we observed that the presence of PI4P was significantly increased in MNV-infected cells and that the pools of PI4P accumulated, and strongly associated with NS4, at sites of viral replication (Fig 10). Our previous studies have revealed that MNV recruits membranes from the endocytic and secretory pathways, hence taken together with our present observations; this suggests that MNV may be recruiting PI4P from the Golgi or ER. Additionally, we observed strong to moderate co-localisation between MNV NS4 and the PI4P kinases PI4KIIIα or PI4KIIIβ during MNV-infection. We have previously reported that all non-structural proteins of MNV associate with the RC during replication (73) so we feel comfortable using a non-structural protein such as NS4 for a marker of the MNV RC. Unfortunately, we could not use the anti-dsRNA antibody for these studies as both it and the anti-PI4P and anti-PI4K antibodies are of mouse origin. From these findings we propose that MNV may be recruiting PI4Ks in order to utilize and generate pools of PI4P at sites of replication.

To interrogate the contribution of each of the PI4Ks in generating the increased PI4P pools we utilised chemical inhibition of PI4K activity with the compounds T-00127-HEV1 and PIK93. HEV1 has been identified as an enviroxime-like compound that inhibits enterovirus replication and specifically, HEV1 inhibits the activity of PI4KIIIβ resulting in decreased production of PI4P in the Golgi apparatus (274, 275, 287). In contrast, PIK93 is a small molecule inhibitor of PI4KIIIβ (288) that has been reported to inhibit replication of viruses requiring PI4P (220). Further investigation into the mechanisms of PIK93 revealed that at low concentrations PIK93 specifically inhibits PI4KIIIβ activity, whereas at higher concentrations PIK93 targets PI4KIIIα activity (289). Both of these compounds were used to the contributions of both PI4KIIIα and PI4KIIIβ during HCV replication as was initially reported that HCV required both PI4KIIIα and PI4KIIIβ for efficient replication (289). However, subsequent investigation utilising HEV1 and PIK93 at differing concentrations

established that only PI4KIIIα, and not PI4KIIIβ, is crucial for HCV replication (208, 275, 290).

In the studies described here, we observed no visual disruption to the MNV RC and distinct, accumulated pools of PI4P were observed to co-localise with the MNV RC in both HEV1- and 10μM PIK93-treated cells. The increased production and accumulation of PI4P at sites of replication in these treated cells indicates that the recruitment of PI4P to sites of MNV replication occurs independently of PI4KIIIβ. Our subsequent analyses of virus production and secretion, and our western blot analyses also established no significant difference between the HEV1- and 10μM PIK93-treated MNV-infected cells and the untreated MNV infected cells. Again, these findings suggest that production, redistribution and utilisation of PI4P during MNV replication occurs in a PI4KIIIβ-independent manner.

In stark contrast to the above results, we observed a dramatic decrease in PI4P fluorescence in MNV-infected cells treated with 30μM PIK93 and the degree of co-localisation between PI4P and NS4 were significantly diminished. Our subsequent western blot analysis demonstrated a significant decrease in MNV VP1 protein levels in the 30μM PIK93-treated cells. In agreement with the western blot data we observed a significant decrease in the transcription of MNV genomic RNA transcription, and a significant decrease (1-2 $\log_{10}$) in MNV-infected cells treated with 30 μM PIK93. These results suggest that PI4KIIIα rather than PI4KIIIβ is required for the production of PI4P during MNV replication and that inhibition of PI4KIIIα affects the replication efficiency of MNV in macrophages.

From this study, we suggest that MNV is recruiting PI4Ks to sites of replication in order to generate pools of PI4P. We have shown that inhibition of these kinases, particularly PI4KIIIα, significantly repressed MNV replication. However, it is understandable that chemical modulation of cells may not be the most accurate way to demonstrate inhibition of a

protein as there may be unknown off-target effects. We did attempt siRNA knockdown of PI4KIIIα, but unfortunately due to time constraints and the difficult and inefficient nature of transfecting RAW264.7 cells, we were not able to achieve knock-down at this point in time. However, the laboratory is endeavouring to continue with these experiments.

Numerous studies have confirmed the recruitment of PI4KIIIα to sites of HCV replication is due to the action of NS5A (230), whilst the recruitment of PI4KIIIβ to sites of enterovirus replication is due to actions of the 3A protein (220). Considering the NoV NS4 protein has been called a "3A-like" protein in the past, and the MNV NS7 is the RdRp we aimed to establish whether any of the MNV ORF1 proteins, but especially NS4 or NS7, were recruiting any of these kinases to sites of MNV replication. To assist in identifying whether any of the MNV NS proteins were recruiting the PI4Ks and accumulating PI4P, Vero C1008 cells were transfected with individual NS proteins and labelled with anti-PI4KIIIα, anti-PI4KIIIβ or anti-PI4P antibodies and examined for co-localisation of MNV proteins with the kinases or lipid. Overall, our IF analyses were not compelling in identifying any significant co-localisation, nor any significant redistribution or accumulation of either kinase or PI4P during the transfection studies. We did observe that NS1-2 demonstrated some co-localisation with PI4KIIIα (*Rr* = 0.55). PI4KIIIα is primarily found in the ER and is responsible for the generation of PI4P in these regions (219). Our previous findings have shown that it is likely NS1-2 is recruiting ER membranes to the MNV RC as transient expression of NS1-2 co-localises with calnexin (69). In addition we also observed that NS4 was the only ORF1 protein to potentially co-localise with PI4KIIIβ (*Rr* = 0.588). Previous findings have demonstrated MNV NS4 localises with markers of the Golgi apparatus (69) and PI4KIIIβ is the primary PI4K residing in the Golgi (219). Although our findings from the HEV1 and PIK93 treatments, would suggest that PI4KIIIβ is not as important for MNV replication thus the co-localisation of NS4 and PI4KIIIβ may only be coincidental.

Interestingly, we did not observe any co-localisation between ORF1 proteins and PI4P neither did we observe any of the dramatic increase or redistribution of PI4P we observed during MNV replication. This suggests that replication of MNV may be required for the production and recruitment of PI4P to occur. This may be because the presence of the MNV polyprotein may be required to recruit PI4P, or the effects of multiple NS proteins on host factors are required to induce these PI4P-rich regions.

Based on the lack of evidence for roles for the MNV ORF1 proteins in recruitment of PI4KIIIα, PI4KIIIβ and PI4P we investigated the role of additional accessory molecules in facilitating the increased PI4P induction. We investigated the involvement of Arf1 and GBF1 proteins, recently identified to play a critical role in the establishment of PI4P-rich membrane microdomains facilitating HCV replication. In agreement with the HCV reports we also observed significant redistribution and accumulation of both Arf1 and GBF1 to the MNV RC. However, our chemical inhibition studies revealed that GBF1 activity did not contribute and we have been unable to directly determine the role of Arf1 but this is part of in-going studies within the laboratory. In contrast to our investigations with PI4KIIIα, PI4KIIIβ and PI4P, we observed that transient expression of NS3, NS4 and NS6 all showed some degree of co-localisation and accumulation with Arf1, with none of the proteins associating with GBF1. Perhaps it is not surprising for NS3 or NS4 to associate with Arf1 as (i) NS3 is a putative GTPase and may be able to modulate Arf1 activity via this inherent function, and (ii) NS4 is localised to the Golgi apparatus where Arf1 also resides.

Our previous findings have demonstrated that individually expressed NS6 is extensively associated with the mitochondria, however no association with mitochondria is observed during MNV infection (69). In consideration of this, our observation of NS6 strongly co-localising with Arf1-GFP was surprising. Interestingly, a recent study by Ackema

et al. revealed that Arf1 plays a role in mitochondria homeostasis (291). Whilst Arf1 generally plays an important role in regulating transport along the secretory pathway, Ackema *et al* established the first report of Arf1 carrying out a role in mitochondria dynamics and function which in independent of its Golgi/vesicular transport functions (291). However at this time we are unable to completely elucidate the role NS6 may play in the recruitment and potential modulation of Arf1.

To advance our understanding further we aimed to determine whether MNV NS4 or NS7 would bind to PI4P, as previous reports demonstrated the binding of enterovirus 3A protein and HCV NS5A to PI4P (208, 258). We initially chose NS4 and NS7 as NS4 shares some homology to the enterovirus 3A protein and NS7 is the MNV RdRp and the enterovirus $3D^{pol}$ protein was observed to bind PI4P (206, 227). In addition, we included the HCV (JFH1) G2a NS5A protein as a positive control. As expected, HCV NS5A bound to PI3P, PI4P and PI5P as has been described previously (208). Significantly, we observed that MNV NS4 bound strongly to PI(4,5)P2 but also with PI4P and PI5P. We did not observe any binding of NS7 with to any of the lipids presence on the PIP strip. It is interesting to note that PI(4,5)P2 and PI4P are enriched on membranes of the endosomes and Golgi apparatus, respectively, organelles where NS4 also resides. Intriguingly, although we observed that NS4 could bind to PI4P it does not stimulate its production nor accumulate or recruit PI4P. There are two possible explanations to this (i) the function of NS4 is solely to associate to PI4P-rich membranes and the interactions with other viral and cellular proteins are required to establish the RC or (ii) that an additional viral protein or proteins are required to recruit and activate PI4KIIIα to produce PI4P. We speculate that the recruitment of PI4P to the MNV RC may be the result of mechanisms of multiple NS proteins working simultaneously to achieve these lipid-rich regions.

One possible scenario (depicted in Figure 21) is that (1) NS3 and/or NS6 bind and activate Arf1, (2) the activation of Arf1 equally stimulates the activity of PI4KIIIα to produce PI4P, (3) NS4 redistributes and accumulates to these sites due to its high affinity for PI4P, (4) NS4 then recruits the viral RdRp and replications ensues. Part of the future aims will be to investigate these specific protein-protein and protein-lipid interactions.

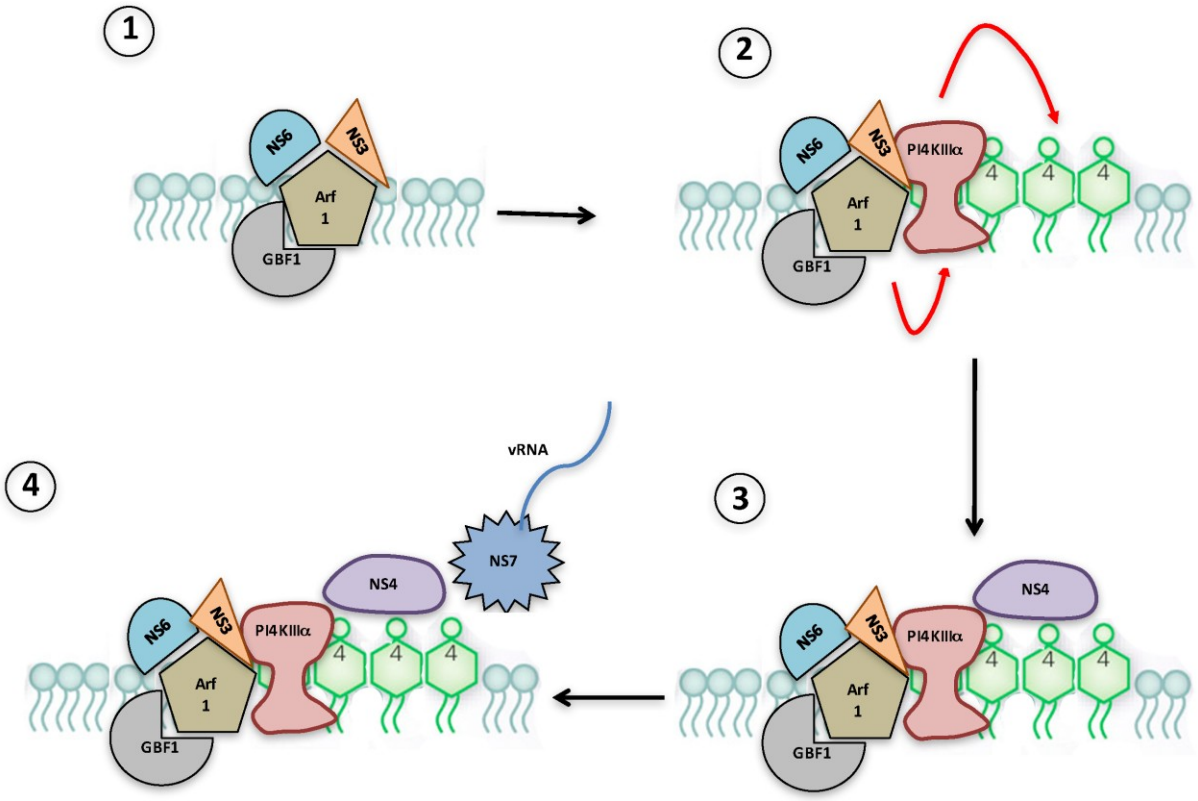


Figure 21. Model of the production and utilization of PI4P during the MNV replication cycle.
A schematic representation of our hypothesis of the increased PI4P production and its utilisation as a membrane platform for viral RNA replication during intracellular replication of MNV. We speculate that the MNV NS3 and/or NS6 proteins stimulate Arf1 to activate PI4KIIIa to increase the local concentration of PI4P. This increase leads to NS4 recruitment, due to its inherent capacity to bind PI4P, and NS4 recruits NS7 (the viral RdRp) to promote replication.

CHAPTER 5:

MNV INTERACTION AT THE PI4P-CHOLESTEROL SHUTTLING INTERFACE

5.1 Introduction

The secretory pathway provides a means of transporting cargo, including pro-inflammatory and antiviral mediators, between specific organelle compartments via a series of vesicle budding and fusion events (248). The inherent nature and the unique lipid composition of particular membrane sites within this pathway is the reason numerous (+)RNA viruses utilize these organelles to derive a membrane platform required for replication. Hence, cellular secretory-trafficking machinery is reorganised into viral replication organelles containing lipid-rich environments that are crucial for viral replication.

Membrane contact sites (MCSs) are domains where two organelles are closely positioned together where the efficient exchange of lipids and signals can take place between these organelles (249, 250). Of particular interest to this study is the MCS where the ER and Golgi apparatus are juxtaposed. This MCS has been the subject of many recent studies concerning the flux of two key lipids, PI4P and cholesterol, between these two organelles and whether this is beneficial for viral replication. There are a number of key host factors associated with regulation of the lipid exchange between this MCS that include oxysterol-binding protein (OSBP), vesicle-associated membrane protein (VAMP)-associated protein-A (VAP-A) and Sac1.

OSBP is a lipid binding/transfer protein with an affinity for oxysterols, cholesterol and PI4P and is involved in regulating lipid transport and sterol homeostasis (244). VAP-A is a type II integral membrane protein that plays a role in transporting proteins between the Golgi and ER and the process of vesicle transport and fusion (244, 246). OSBP is tethered between the ER and Golgi membranes via VAP-A on the ER and PI4P on the Golgi (247, 252). This membrane tethering by OSBP is vital in the exchange of lipids at these MCSs. In contrast, Sac1 is an ER resident lipid phosphatase that hydrolyses PI4P in the ER to

phosphatidylinositol to be transported back to the Golgi apparatus to be recycled to generate further PI4P pools, via PI4Ks. Previously it was observed that multiple enteroviruses and HCV actively recruit OSBP to sites of viral replication in order to encourage the exchange of PI4P and cholesterol between the Golgi and ER (257, 260, 274). In addition to the role of PI4P, there is increasing evidence that the host sterol cholesterol contributes significantly to the establishment of the RC of multiple viruses and is required for efficient viral replication. Previously, our laboratory was one of the first to show that direct recruitment of cholesterol to the WNV_{KUN} RC and the critical requirement for continual intracellular cholesterol synthesis to facilitate and maintain WNV_{KUN} replication (235). Recently, the dependency of cholesterol has been extended to many members of the *Picornaviridae* and to HCV. Although the exact function for cholesterol in the biogenesis of the viral RC and on-going virus replication is not fully understood, it is speculated that cholesterol inclusion within the membranes of the RC provides structural integrity and decreases the membrane fluidity within these viral organelles. In addition, there are also increasing reports suggesting that cholesterol and other sterols, particularly 25-hydroxycholesterol, are mediators of immune sensing and activation (294).

In this chapter we show that VAP-A and Sac1, but not OSBP, are actively recruited to the MNV RC in MNV-infected cells. In support of this, we observed that VAP-A strongly co-localised with the individually expressed MNV NS1-2 protein. Additionally, we could not observe any significant recruitment of cholesterol to the MNV RC during infection, as detected with filipin staining, and the chemical modulation of cholesterol synthesis and the PI4P-cholesterol counter-flux had little effect on MNV replication. Thus, our observations suggest that although MNV replication is dependent on the host lipid PI4P, it does not utilize the PI4P/cholesterol counter-flux to establish the local concentration of PI4P pools, and that cholesterol equally does not play an important role during MNV replication. However, our

observations potentially indicate that MNV may disturb the cholesterol-PI4P counter-flux by modulating the activity of VAP-A and Sac1 to restrict the function of OSBP and hydrolysis of PI4P, respectively, resulting in accumulation of PI4P available for establishment of the MNV RC.

5.2 Results

5.2.1 VAP-A but not OSBP co-localises to sites of MNV replication.

Given our previous findings (see Chapter 4) that MNV replication utilizes PI4P, we aimed to determine whether the host factors OSBP and VAP-A were additionally recruited to the MNV RC during MNV replication given the central roles these two molecules play during lipid and sterol transport between the ER and the Golgi apparatus. It has been established that the PI4P-rich environments of enterovirus RCs elicits the recruitment of OSBP which then prompts the tethering of the RC to the ER, generating a MCS to create a PI4P/cholesterol counter-flux (258, 259).

To aid this objective, RAW264.7 cells were transfected with OSBP-FLAG or VAP-A-mCherry expression plasmids and subsequently infected with MNV 24 hours later. At 12 h.p.i cells were fixed and stained with anti-FLAG and anti-NS4 antibodies or visualised for mCherry (Fig 22). In cells that were transfected but mock-infected we observed OSBP-FLAG to localise primarily within the perinuclear region indicative of the Golgi apparatus (Fig. 22a-d) and consistent with its known distribution (193, 292). In contrast VAP-A-mCherry was observed to have a more diffuse distribution within the cytoplasm with some staining also observed within the perinuclear region (Figs. 22i-l). Again this distribution is consistent with the known localisation of VAP-A within both ER and Golgi apparatus membranes (193, 229).

In cells transfected with OSBP-FLAG and MNV-infected we observed no significant co-localisation of the viral protein NS4 with OSBP-FLAG (*Rr* = 0.39), although staining of NS4 and OSBP-FLAG did both appear to be in the perinuclear region but the staining of OSBP-FLAG did not appear to be dramatically altered and remained similar to its

localisation within the Golgi apparatus (Fig 22e-h). Interestingly the staining of OSBP-FLAG was interspersed with that of NS4 and was almost visualised solely where NS4 was absent. Conversely, in the VAP-A-mCherry transfected, MNV-infected cells we observed a very significant co-localisation between NS4 and VAP-A (*Rr* = 0.803) (Fig 22m-p). It also appeared that there was less staining of VAP-A-venus in the cytoplasm suggesting that MNV actively sequestered VAP-A from the cytoplasm to within the RC. These results suggest that VAP-A but not OSBP may be recruited to the MNV RC; the site of MNV replication.

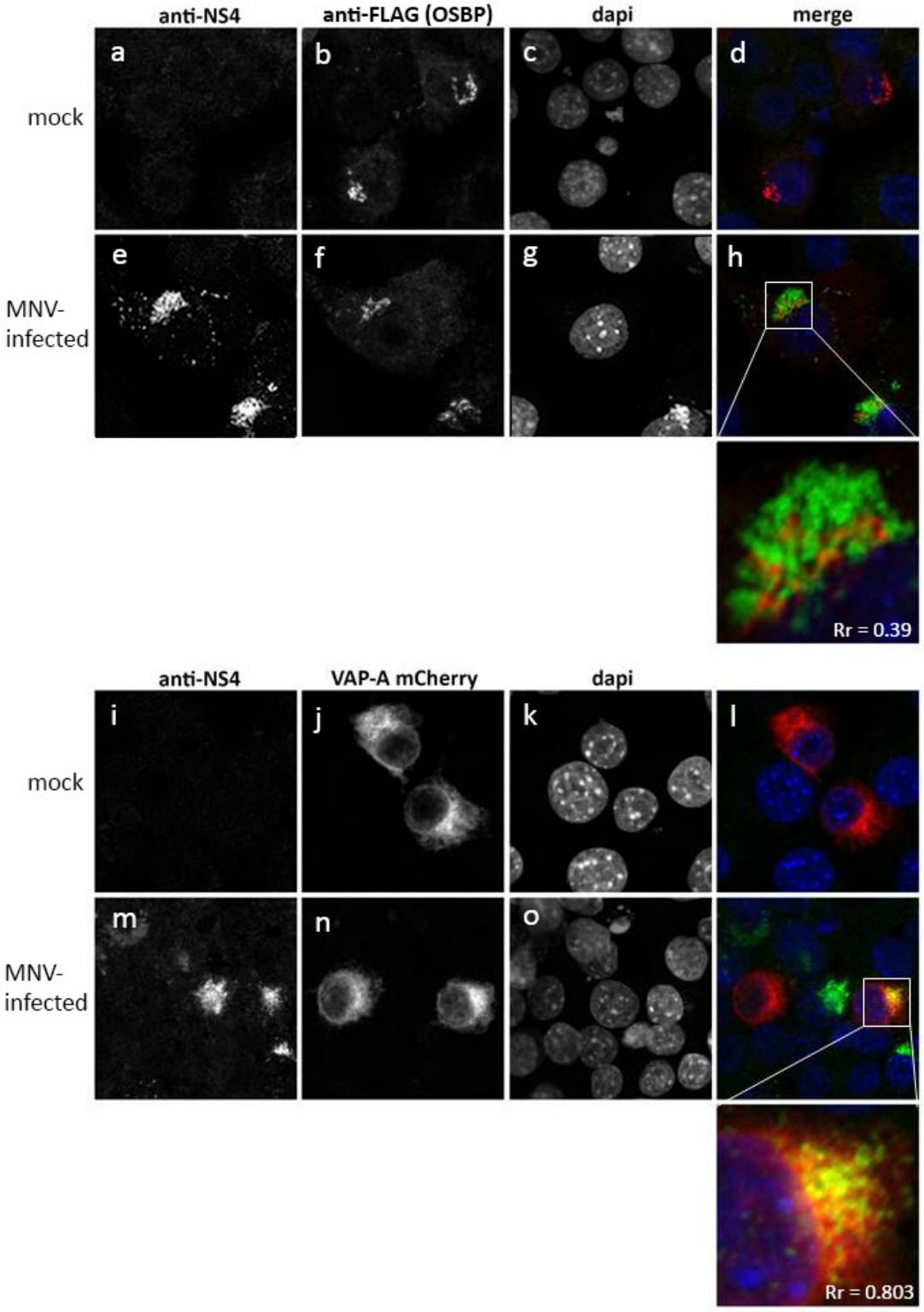


Figure 22. VAP-A but not OSBP co-localises with the MNV RC.
RAW264.7 cells were transfected with OSBP-FLAG or VAP-A-mCherry plasmids and infected with MNV at an m.o.i. of 5 24 hours later. 12 h.p.i cells were fixed and labelled with anti-NS4 (green; panels a, e, i and m), anti-FLAG (red; panels b, f, j and n) and dapi was used to counterstain the cell nuclei (blue; panels c, g, k, and o). Merged images (panels d, h, l and p) depict co-localisation as a yellow hue. Images are taken at 63x magnification. Pearson's co-efficient values (*Rr*) were calculated using the JaCOP plugin in ImageJ software, and are provided in the merged panels, with a value >0.500 corresponding to co-localisation.

5.2.2 **MNV NS protein NS4 does not co-localise with OSBP or VAP-A, but NS1-2 strongly co-localises with VAP-A.**

Previously our laboratory has shown that individual expression of MNV NS1-2 lead to an association with the ER whilst NS4 localised with components of the Golgi apparatus (69). Furthermore, HuNoV NS1-2 was previously observed to interact with VAP-A and inhibited intracellular trafficking (67). Given these findings and our previous observation we aimed to determine whether OSBP or VAP-A were recruited by any of the MNV non-structural proteins to the RC. Vero C1008 cells were co-transfected with MNV NS1-2 or NS4 proteins and either OSBP-FLAG or VAP-A-mCherry. Cells were subsequently fixed 24 hours later and visualised for anti-6xHis and anti-FLAG antibodies or mCherry fluorescence (Fig. 23). We observed minimal or no co-localisation between NS4 and OSBP and NS1-2 and OSBP (*Rr*= 0.32 and *Rr* = 0.04 respectively) as only minimal amounts of yellow hue can be seen which is generally an indication for co-localisation. Interestingly, although we did not identify any significant association of NS1-2 with OSBP we did observe that expression of NS1-2 appeared to effect the distribution of OSBP to a more dispersed staining pattern (Figs. 24e-h). This observation suggests that NS1-2 may disrupt or interfere with Golgi apparatus morphology potentially disrupting membrane protein trafficking as previously observed for HuNoV NS1-2 (67).

Expression of NS4 and VAP-A exhibited minimal co-localisation (*Rr* = 0.26), while a very strong and significant co-localisation of VAP-A and NS1-2 was observed (*Rr* = 0.67). We observed that both NS1-2 and VAP-A localised to discrete foci with the cytoplasm with some diffuse cytoplasmic staining also observed. Both proteins did not appear to be significantly altered in their distribution and localisation but were observed within the same cytoplasmic structures.

These observations suggest that the MNV NS1-2 protein may be required for the active recruitment of VAP-A to the MNV RC. In addition, the results support our observations that MNV does not recruit or require OSBP for efficient replication. This would suggest that MNV may utilise a different function for VAP-A than the known function to promote the role of OSBP in the PI4P-cholesterol counter-flux.

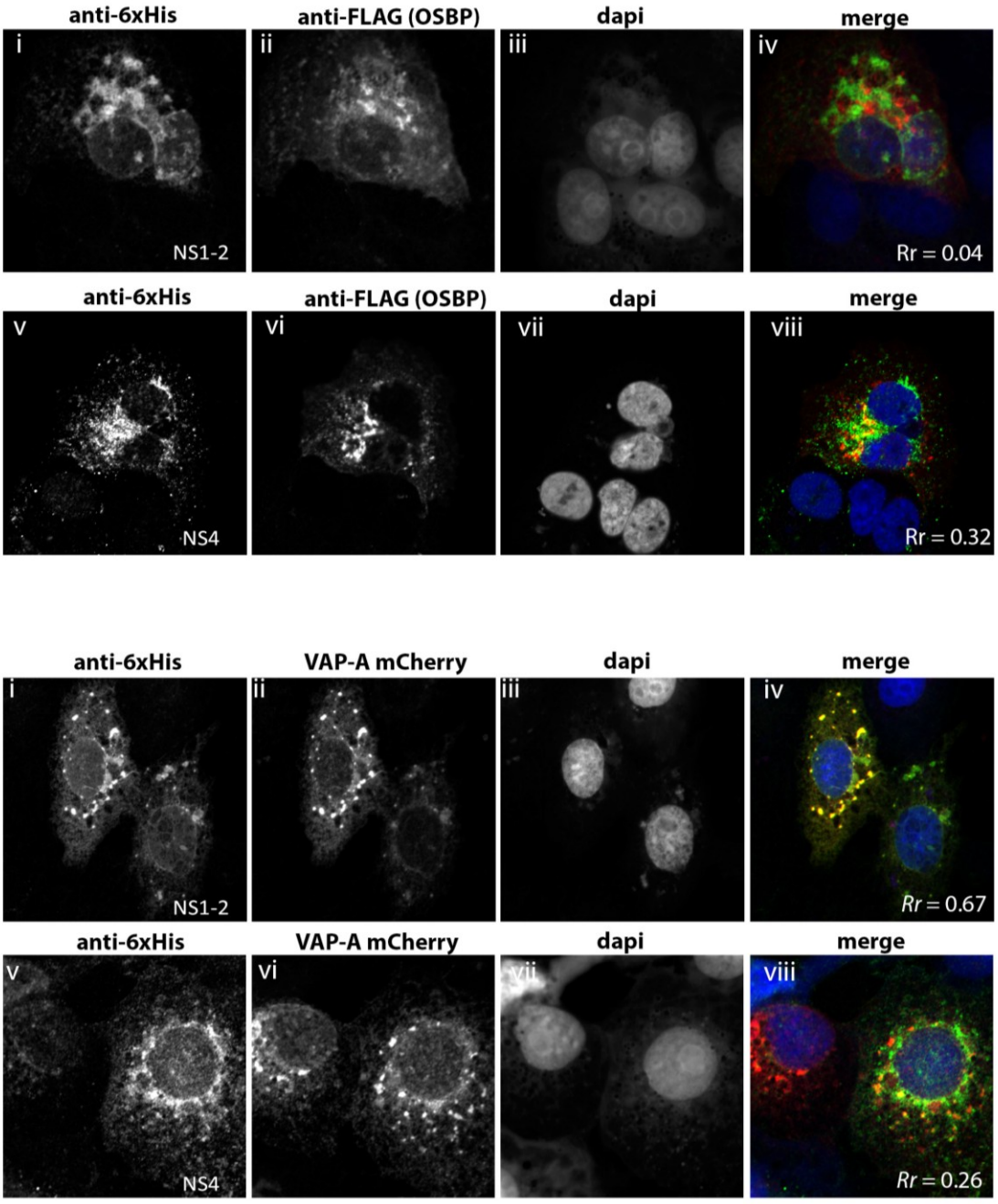


Figure 23. MNV NS1-2 strongly co-localises with VAP-A.
RAW264.7 cells were co-transfected with OSBP-FLAG or VAP-A-mCherry and MNV NS1-2 or NS4 plasmids and fixed 24 hours later. Cells were labelled with anti-6xHis (green; panels a, e, i, m and q), anti-FLAG (red; panels b, f, j, n and r) and dapi was used to counterstain the cell nuclei (blue; panels c, g, k, o and s). Merged images (panels d, h, l, p and t) depict co-localisation as a yellow hue. Images are taken at 63x magnification. Pearson's co-efficient values (*Rr*) were calculated using the JaCOP plugin in ImageJ software, and are provided in the merged panels, with a value >0.500 corresponding to co-localisation.

5.2.3 **Sac1 co-localises with sites of MNV replication.**

Sac1 is an ER-resident PI4P-phosphatase which converts PI4P into phosphatidylinositol (PI) (253). PI4P synthesised within membranes of the Golgi apparatus is transported by OSBP to the ER where it is then hydrolysed by Sac1. The hydrolysis of PI4P by Sac1 in the ER supplies OSBP with the energy to transfer sterol (*e.g.* cholesterol) from the ER to Golgi and transport PI4P from the Golgi to the ER (250, 252). Thus Sac1 plays a major role in facilitating the PI4P-cholesterol exchange at the ER-Golgi MCS.

As we observed co-localisation of PI4P, PI4KIIIα, Arf1, GBF1 and VAP-A with the MNV RC, we also wished to observe if Sac1 was recruited to the MNV RC. Thus, RAW264.7 cells were mock- or MNV-infected for 12 hours, fixed and immune-labelled with anti-Sac1 and anti-NS4 antibodies. In uninfected cells our IF analysis revealed that Sac1 was primarily associated with small discrete foci scattered throughout the cytoplasm with some diffuse cytoplasmic labelling also observed. We hypothesise that the small discrete foci most likely represent the ER-Golgi MCS (Fig 24). In contrast, we observed a dramatic redistribution and accumulation of Sac1 with NS4 in the MNV-infected cells (*Rr* = 0.83) (Fig 24). We observed an almost complete overlap of the staining patterns of NS4 and Sac1 in the infected cells and the distribution is drastically different to that observed in the neighbouring uninfected cells in the same panel.

These results would suggest that MNV recruits a select composition of proteins from the ER-Golgi MCS to the viral RC. It is also intriguing to consider why MNV would recruit a PI4P phosphatase to replication sites that require the lipid for efficient replication. One consideration could be to derive energy from the hydrolysis of the lipid or to recruit and modify the activity of Sac1 to restrict PI4P hydrolysis.

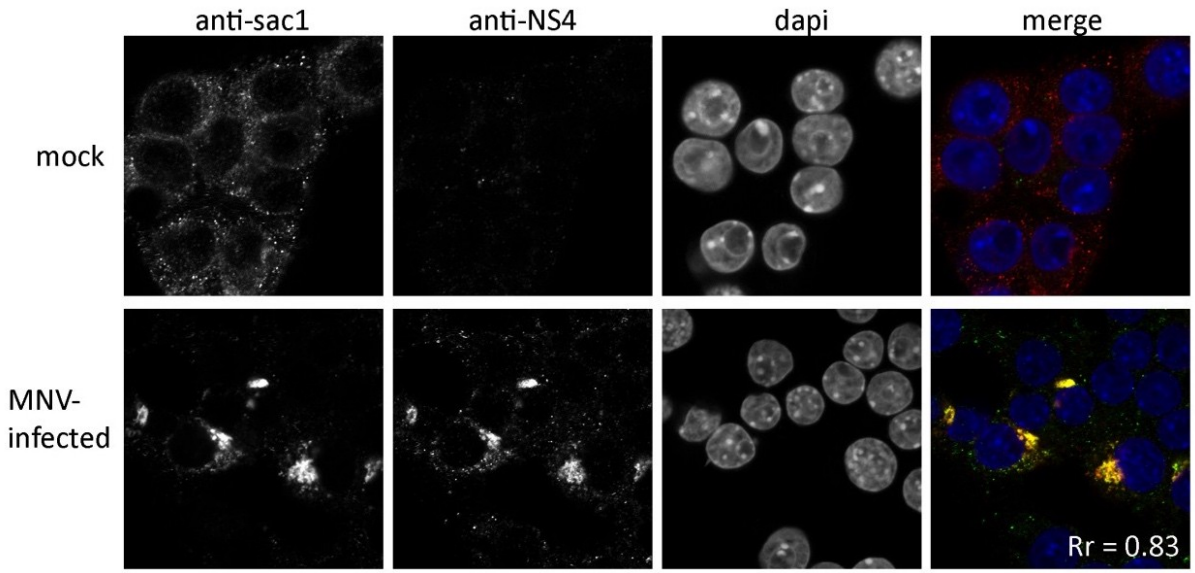


Figure 24. SAC1 co-localises with the MNV RC during MNV replication.
RAW264.7 cells were infected with MNV at an m.o.i. of 5, fixed 12 h.p.i and labelled with anti-NS4 (green), anti-SAC1 (red) and dapi was used to counterstain the cell nuclei (blue). Merged images depict co-localisation as a yellow hue. Images are taken at 63x magnification. Pearson's co-efficient values (*Rr*) were calculated using the JaCOP plugin in ImageJ software, and are provided in the merged panels, with a value >0.500 corresponding to co-localisation.

5.2.4 **Cholesterol and the cholesterol/PI4P counter-flux at the ER-*trans*-Golgi MCS do not appear to be required for MNV replication.**

Cholesterol plays an important role in the replication of many (+)RNA viruses (209, 222, 238). Several studies have demonstrated that cholesterol is required for optimal genome replication of enteroviruses (237, 257) however, the ongoing synthesis of cholesterol does not impact the replication of these viruses (222). This suggests that cholesterol is crucial for the formation of the replication organelles but not important for the later stages in the viral life cycles. The role of cholesterol during MNV infection still remains unknown. While entry of MNV is reliant on cholesterol (91), there is no current data to indicate whether this lipid is utilized in the replication complex. One study demonstrated that statins up-regulated Norwalk virus replicon replication, but inhibitors of acyl-CoA:cholesterol acyltransferase (ACAT) down-regulated Norwalk virus replicon replication (243) suggesting that cholesterol does play some form of role in replication. Given these observations, we aimed to investigate whether cholesterol is recruited to the RC and required during MNV infection.

To determine the involvement of cholesterol during the MNV replication cycle, RAW264.7 cells were mock- or MNV-infected for 12 hours, after which they were fixed and stained for filipin and with anti-dsRNA antibodies (Fig. 25). In mock-infected cells we observed that the filipin stain was evenly distributed throughout the cytoplasm with some accumulation in the perinuclear region and small discrete foci also observed. The latter two staining patterns most likely reflect the accumulation of cholesterol in the Golgi apparatus and endosomes, respectively. Our IF analyses of the MNV-infected cells revealed no significant co-localisation between the filipin staining and the anti-dsRNA antibody staining (Rr = 0.21), suggesting that cholesterol was most likely not recruited to the sites of MNV replication. In addition, we did not observe any dramatic change in the localisation or distribution of cholesterol, as determined by the filipin stain, although the filipin staining was

perhaps slightly more prominent in the cytoplasm compared to the perinuclear staining in the MNV-infected cells.

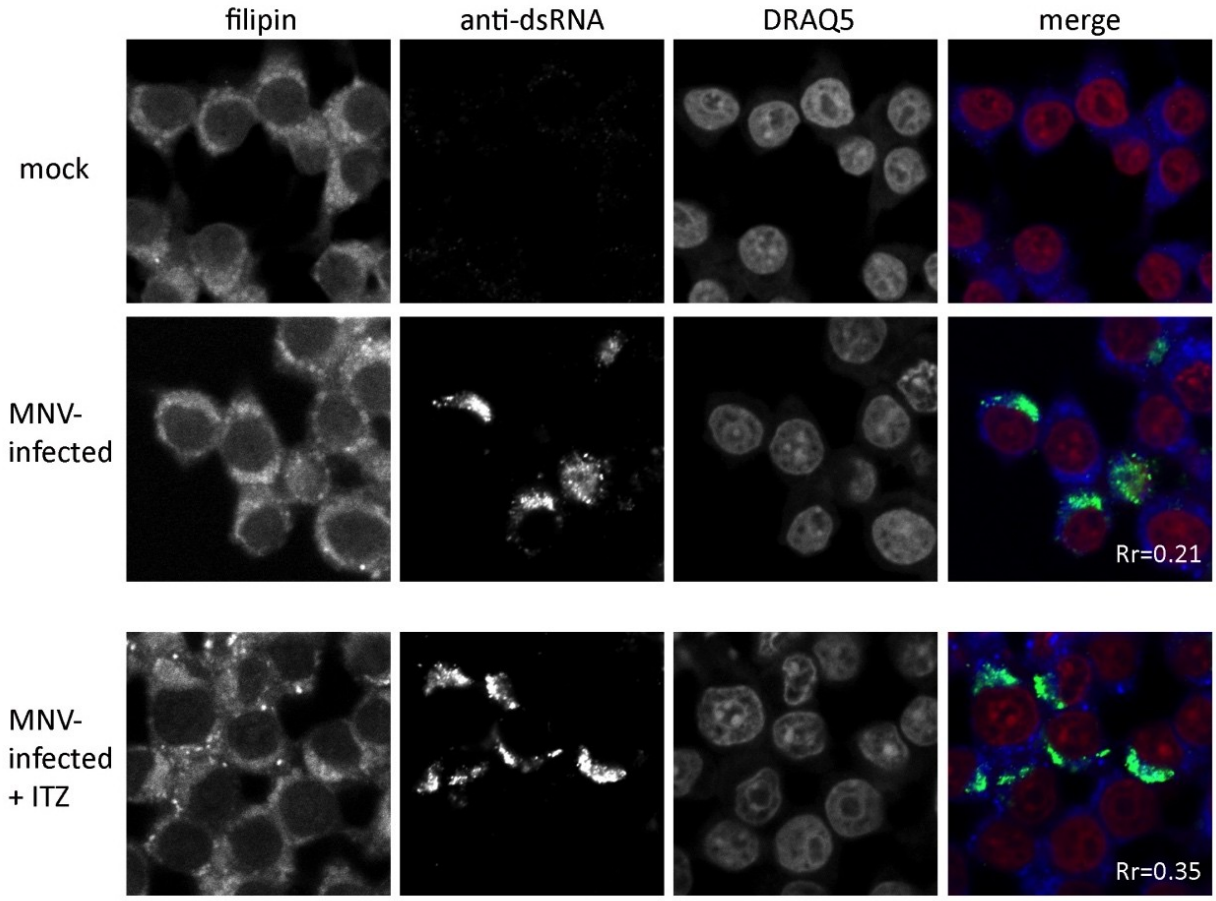


Figure 25. Filipin does not co-localise with the MNV RC.
RAW264.7 cells infected with MNV at an m.o.i. of 5, treated with ITZ 1 h.p.i. and subsequently fixed and labelled 12 h.p.i. Cells were labelled with filipin (blue), anti-dsRNA (green) and nuclei stained with DRAQ5 (red). Merged images depict co-localisation as a yellow hue. Images are taken at 63x magnification. Pearson's co-efficient values (*Rr*) were calculated using the JaCOP plugin in ImageJ software, and are provided in the merged panels, with a value >0.500 corresponding to co-localisation.

5.2.5 **Chemical modulation of cholesterol synthesis and transport does not affect MNV RC biogenesis or replication efficiency.**

Even though we had not observed a redistribution of cholesterol to the MNV RC we aimed to further examine the contribution of cholesterol to MNV replication. Thus, we treated MNV-infected cells with three inhibitors of sterol synthesis; Itraconazole (ITZ), Lovastatin and 25-hydroxycholesterol. ITZ inhibits sterol synthesis, and more significantly impairs the function of OSBP to shuttle cholesterol from the ER to the Golgi apparatus. Lovastatin inhibits HMG-CoA reductase within the mevalonate pathway, thus blocking subsequent steps of cholesterol synthesis (293). 25-hydroxycholesterol (25-HC) is an oxygenated sterol that reduces the activity of HMG-CoA reductase on a transcriptional level (294, 295). IF analyses revealed no visible difference to the MNV RC when MNV-infected cells were treated with ITZ (Fig 25).

To interrogate the modulation of cholesterol biosynthesis and transport on MNV replication efficiency, RAW264.7 cells were treated with ITZ, lovastatin, 25-HC or vehicle solvent 1 hour post MNV-infection. Subsequently, at 18 h.p.i cell lysates were collected for viral RNA or protein analyses and the tissue culture fluid was collected to determine the impact on the production of infectious virus. Interestingly, we observed no significant difference in MNV genomic RNA transcription of MNV-infected cells compared to the drug treated MNV-infected cells (Fig 26A). Nonetheless, we did observe a ~3-fold increase in viral genomes in the Lovastatin-treated cells which was greater, although not significantly so, when compared to the other treatments overall. In contrast, our western analysis showed a substantial decrease in MNV NS7 protein levels in the 25-HC treated cells, however no decrease in NS7 was observed for the other treatments (Fig. 26B). This dramatic decrease is intriguing as we saw no effect of 25-HC on viral RNA levels, suggesting that 25-HC is acting at a translational rather than transcriptional level. In concurrence with the western blot data

we observed a slight increase and a drastic decrease in the production and secretion of infectious MNV particles in cells treated with lovastatin and 25-HC, respectively, but this was not deemed to be significant (Fig. 26C).

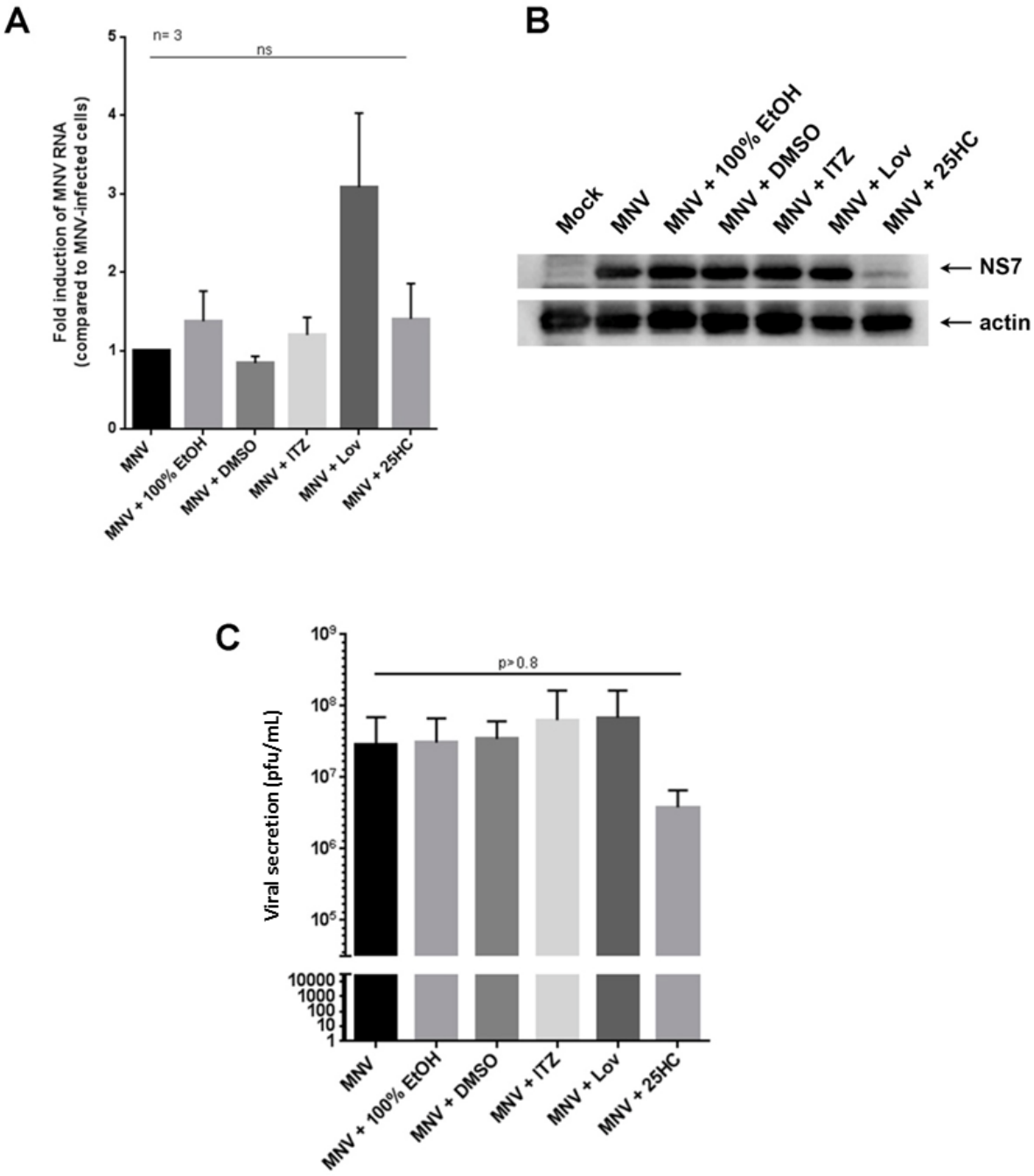


Figure 26. Inhibition of cholesterol synthesis does not affect MNV replication.
RAW264.7 cells were infected with MNV at an m.o.i. of 5 and treated with ITZ, lovastatin or 25HC 1 h.p.i. 18 h.p.i. lysates and tissue culture fluid were collected. **(A)** RNA was then extracted and analysed via qPCR (n = 3). **(B)** Immunoblots of cell lysates were analysed for changes in viral protein when MNV-infected cells were treated with ITZ, lovastatin or 25HC. **(C)** Extracellular virus (n = 4) was measured by plaque assay and expressed as pfu/mL +/-S.E.M.

5.3 Discussion

The secretory pathway is utilized by many viruses as the unique lipid composition of particular membrane sites within this pathway are recruited to form a membrane platform required for replication (248). The MCS where the ER and Golgi apparatus are juxtaposed has been the subject of many recent studies concerning the flux of two key lipids, PI4P and cholesterol, between these two organelles and whether this is beneficial for viral replication. Furthermore, interactions of viral proteins and the key host factors associated with regulation of the lipid exchange between this MCS have been the focus of numerous studies. It has been reported that multiple enteroviruses and HCV actively recruit OSBP to sites of viral replication in order to encourage the exchange of PI4P and cholesterol between the Golgi and ER (257, 260, 274). There is increasing evidence that the host sterol cholesterol contributes significantly to the establishment of the RC of multiple viruses and is required for efficient viral replication. In this study we observed that MNV does not utilize the PI4P/counter-flux between the ER and Golgi, and MNV replication does not appear to recruit cholesterol to the RC. We observed that (i) MNV actively recruits VAP-A but not OSBP to sites of MNV replication, (ii) VAP-A and MNV NS1-2 strongly co-localised, (iii) MNV actively recruits Sac1 to sites of viral replication, (iv) no significant recruitment of cholesterol to the MNV RC during infection, and (v) chemical modulation of cholesterol synthesis and the PI4P-cholesterol counter-flux had little effect on MNV replication. These results suggest that although MNV replication is dependent on the host lipid PI4P (see Chapter 4), it does not utilize the PI4P/cholesterol counter-flux to establish the local concentration of PI4P pools, and that cholesterol equally does not play an important role during MNV replication.

The role of the host factor OSBP, as well as the role of cholesterol during viral replication has been increasingly studied. Many enteroviruses and HCV rely on OSBP and

the counter-flux of PI4P and cholesterol, while many flaviviruses have been shown to require cholesterol for efficient replication (238, 258, 260, 296).

In our initial immunofluorescence analyses we observed that OSBP did not co-localise with MNV NS4 in MNV-infected cells (Fig 22). In contrast, VAP-A strongly co-localised with NS4 at sites of viral replication in MNV-infected cells. Our previous studies have revealed that MNV recruits membranes from the endocytic and secretory pathways (73), hence taken together with our present observations; this suggests that MNV may be recruiting VAP-A from the ER. Further analyses of the recruitment of VAP-A and OSBP with individually expressed MNV ORF1 proteins revealed MNV NS1-2 strongly co-localised with VAP-A (Fig 23). NS1-2 did not co-localise with OSBP, nor did NS4 co-localise with OSBP or VAP-A. HuNoV NS1-2 has been previously observed to interact with VAP-A and inhibited intracellular trafficking (67). These observations suggest that the MNV NS1-2 protein may be required for the active recruitment of VAP-A to the MNV RC. In addition, these results support our observation that MNV does not recruit OSBP to sites of replication. This suggests that MNV may not be utilising the PI4P-cholesterol counter-flux and is recruiting VAP-A for an alternate function rather than promoting the mechanisms of OSBP.

Furthermore, we aimed to determine whether Sac1 plays a role in MNV replication. Because Sac1 is an ER-resident PI4P-phosphatase which converts PI4P into phosphatidylinositol (PI) and plays a major role in facilitating the PI4P-cholesterol exchange at the ER-Golgi MCS (250, 252), we wanted to interrogate whether this phosphatase is utilized or not during MNV replication. Our IF analyses revealed dramatic redistribution and accumulation of Sac1 with NS4 in the MNV-infected cells (Fig 24). This result would suggest that MNV recruits a select composition of proteins from the ER-Golgi MCS to the viral RC. It is interesting to consider why MNV would recruit a PI4P phosphatase to replication sites that require the lipid for efficient replication. One consideration could be to

derive energy from the hydrolysis of the lipid or to recruit and modify the activity of Sac1 to restrict PI4P hydrolysis as MNV requires pools of PI4P for efficient replication (see Chapter 4).

Finally, as MNV does not appear to recruit OSBP, we aimed to determine whether cholesterol plays a role in MNV replication. Cholesterol biosynthesis is regulated within the ER which involves membrane-bound transcription factors called sterol regulatory element-binding protein (232). Enteroviruses use clathrin-mediated endocytosis (CME) to gain entry into cells but also exploit this process to enrich intracellular free cholesterol pools and traffic cholesterol to sites of replication (237). The cholesterol found in these ROs is required for efficient enterovirus replication as cholesterol regulates the 3CD polymerase (237). Similarly, several studies have established that Flaviviruses such as WNV and DENV both depend on cholesterol to mediate viral replication (238-240). Whilst it has been shown cholesterol plays an important role in entry of MNV into target cells however it is not known to what extent cholesterol plays a role in MNV replication (91).

Our IF analysis revealed no co-localisation of staining between the anti-dsRNA antibody with filipin (Fig 25). In addition, we did not observe any dramatic change in the localisation or distribution of cholesterol, as determined by the filipin stain in MNV-infected cells. This suggests that MNV does not appear to recruit cholesterol to sites of replication. Itraconazole inhibits sterol synthesis, and more significantly impairs the function of OSBP to shuttle cholesterol from the ER to the Golgi apparatus (255, 293, 294). When MNV-infected cells were treated with ITZ, we observed no significant visual disruption to the MNV RC.

Based on our observations that MNV does not appear to recruit OSBP or cholesterol, MNV-infected cells were treated with ITZ, Lovastatin or 25-HC to confirm cholesterol does not play an important role in MNV replication. Lovastatin inhibits HMG-CoA reductase

within the mevalonate pathway, thus blocking subsequent steps of cholesterol synthesis (293). 25-hydroxycholesterol (25-HC) is an oxygenated sterol that reduces the activity of HMG-CoA reductase on a transcriptional level (294, 295). No significant difference in MNV RNA, NS7 protein production or viral particle secretion was observed between MNV-infected cells and treated MNV-infected cells (Fig 26). The only discrepancy observed was 25-HC treated MNV-infected cells displayed decreased NS7 protein which may be due to the role 25-HC plays in immune sensing and activation (297). 25-HC is an immunoregulatory lipid that is produced in response to activation of the innate immune system by TLRs in macrophages which then subsequently regulates the adaptive immune response (294). Therefore, decreased viral replication or protein production in 25-HC treated MNV-infected cells could be due to the cell producing an immune reaction in response to the overexpression of 25-HC rather than the inhibition of cholesterol synthesis.

In conclusion, our observations suggest that cholesterol does not appear to a play an important role in MNV replication as inhibition of cholesterol synthesis or mechanisms of OSBP also had minimal to no effect on MNV replication. Interestingly, NS1-2 strongly associated with VAP-A which presents the idea that MNV may be recruiting this protein to inhibit cellular protein secretion or to inhibit OSBP from binding to VAP-A. We propose that MNV may disturb the cholesterol-PI4P counter-flux by modulating the activity of VAP-A and Sac1 to restrict the function of OSBP and hydrolysis of PI4P, respectively, resulting in accumulation of PI4P available for establishment of the MNV RC. Thus we have proposed a model (depicted in Fig 27) that describes the overall interactions of MNV proteins with PI4P, PI4KIIIα and Arf1 (Chapter 4) together with the observations from this chapter.

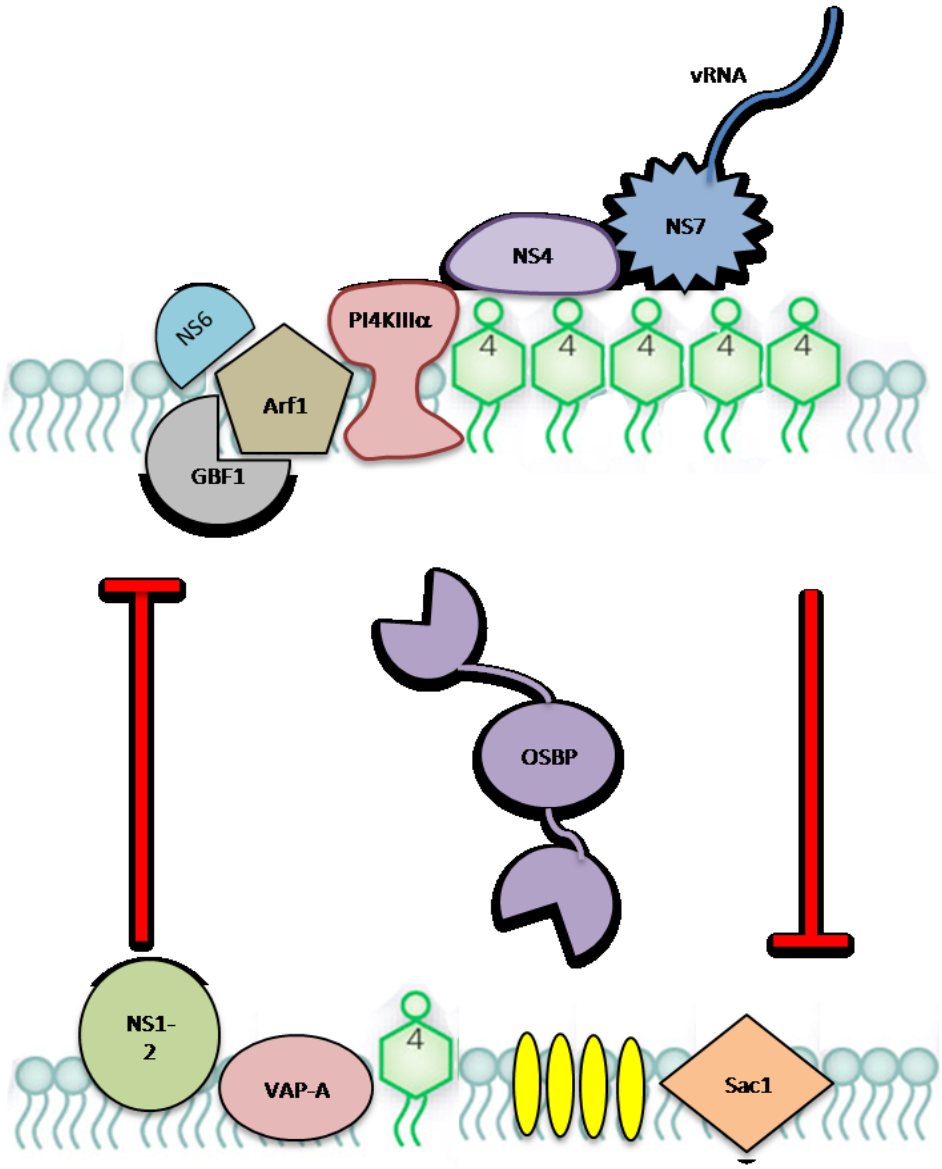


Figure 27. Model of the MNV NS proteins interacting with host factors involved in the PI4P-cholesterol shuttling interface.
A schematic representation of our hypothesis of the manipulation and utilisation of the host PI4P/cholesterol shuttling pathway during intracellular replication of MNV. We speculate that the MNV NS6 protein stimulates Arf1 to activate PI4KIIIa to increase the local concentration of PI4P while MNV NS1-2 binds to or sequesters VAP-A in order to prevent OBSP from binding to VAP-A. This prevents shuttling of PI4P into the ER and retains rich pools of PI4P within the Golgi-derived membrane scaffolds required for MNV replication.

CHAPTER 6:

DISCUSSION AND FUTURE DIRECTIONS

The emergence of HuNoVs during the previous decades and recurrent appearances in today's media highlights the significance of this human pathogen, and the need to research and understand the replication and pathogenesis of this group of viruses. Unfortunately, due to the lack of efficient tissue culture systems and small animal models, research into this pathogen has been hindered. The discovery of Mouse Norovirus in 2003 has provided the opportunity to research, and driven investigation into, the mechanisms of NoV replication and pathogenesis. In this study we have used MNV as a surrogate model to characterise previously unidentified aspects of NoV replication as described in Chapters 3-5.

6.1 Autophagy induction and its role during MNV infection

Previous observations of MNV-infected cells at the ultra-structural level have demonstrated a dramatic reorganisation of intracellular membrane architecture giving rise to juxtaposed membranes, increased accumulation of heterogeneously sized vesicular structures and the formation of double membraned vesicles within these cells (73). The induction of double membraned vesicles is a hallmark of the induction of the autophagy process, thus our previous observations, described above, prompted us to investigate whether the double membraned vesicles formed during MNV replication were autophagosomes potentially comprising and housing the MNV RC. Although little is known about the composition and mechanism by which NoV RCs are formed (120, 183, 264, 282-284), many studies have demonstrated how some other viruses are able to manipulate autophagy and utilize these membrane structures for their RCs (268).

Autophagy is generally regarded as a mechanism to degrade intracellular pathogens, however some picornaviruses manipulate the autophagy pathway and exploit it to enhance replication and subsequently release infectious viral progeny (175, 269). Due to the

similarities between picornaviruses and caliciviruses, in terms of genome structure and function, and the visual presence of double membraned vesicles in the cytoplasm of MNV-infected cells, it was reasonable to propose that MNV infection may also induce the autophagy process and manipulates this process to facilitate increased virus replication and intracellular survival.

In our initial analysis, we observed that MNV-infected cells displayed a dramatic redistribution and accumulation of LC3-positive puncta over the course of infection, indicating the formation of autophagosomes (Chapter 3, Figs. 1 and 2). The LC3-positive puncta are formed during autophagy induction when LC3 is lipidated with phosphatidylethanolamine increasing its association with the autophagosome membrane, and thus its distribution changes from a relatively dispersed cytoplasmic distribution to a more membrane associated one. From our observations, we could state with confidence that, at least in part, autophagy was induced during MNV infection. Interestingly, we observed minimal co-localisation between LC3 and the MNV RC, strongly suggesting that MNV does not appear to utilize the autophagic membrane for the biogenesis of the RC. This was unexpected as many studies have demonstrated that the replicase proteins of picornaviruses that induce autophagy co-localise with LC3, which indicates these viruses utilize autophagic membranes for replication (270, 298, 299).

We observed that MNV prevented the final stages of autophagosome maturation that resulted in the accumulation of autophagosomes over the course of the infection. During MNV infection we observed minimal co-localisation of LC3 and the lysosomal marker LAMP1, suggesting that MNV may prevent the fusion of lysosomes and autophagosomes and thus inhibit the degradation process (Chapter 3, Fig 3B). The lack of interaction between LC3 and LAMP1 correlates with the increased number of autophagosomes observed during MNV replication. To further support this data, we investigated the localization and amount of

SQSTM1 in MNV-infected cells. SQSTM1 binds directly to LC3 during autophagy and facilitates the degradation of ubiquitinated protein aggregates (300) and will eventually also be degraded by this process. We observed very large and abundant SQSTM1 foci in MNV-infected cells when compared to mock-infected cells or cells stimulated with the autophagy-inducer Rapamycin (Chapter 3, Fig 4A). Assessment of protein abundance by immunoblotting also revealed a significant increase in SQSTM1 protein in MNV-infected cells compared to mock-infected and Rapamycin-treated cells. Again, these observations support and correlate with our findings suggesting that MNV inhibits the final maturation of the autophagosome that should result in degradation and turnover of autophagy components. Why MNV would block the fusion and degradation of autophagosomes remains unclear. However, we can speculate that it may be a mechanism acting to inhibit the presentation of viral antigens by MHC molecules, as the degraded contents of autophagosomes can be presented via the MHC molecules (301, 302). Alternatively, it may be that MNV utilises the autophagy pathway for release of progeny virions and inhibits the fusion of autophagosomes to lysosomes which would reduce any degradation of virions within these structures. However, our results indicate that the most likely of these is the former as we only observed a significant increase in virus titre during chemical inhibition of the autophagy process.

Further studies are also required to determine how MNV may prevent the fusion between lysosomes and autophagosomes. Some of the major players for this role would be the cellular proteins UVRAG, Rubicon and Syntaxin 17 (303-306). Each of these proteins have previously been observed to be manipulated by other viruses (*e.g.* HCV; (307)) to impede this fusion process. It would also be worthwhile to determine if one of the MNV ORF1 proteins can individually invoke this inhibition. Recombinant expression plasmids could be used to determine the ability of the expressed MNV protein to prevent the co-localisation between LC3 and LAMP1 upon Rapamycin treatment. This type of experiment

would also reveal additional information regarding the function of the MNV proteins during replication.

When we modulated autophagy by treating MNV-infected cells with 3-MA and Rapamycin, we observed that viral RNA replication and secretion both increased compared to untreated infected cells. Surprisingly, when 3-MA was added to MNV-infected cells we also observed an increased and sustained conversion of LC3-I to LC3-II when compared to mock-infected 3-MA treated cells (Chapter 3, Fig. 5C). This was unexpected, as 3-MA is reported to inhibit the process of autophagy at a very early stage along the pathway, upstream of the lipidation of LC3. Thus in the presence of 3-MA one should observe a reduction in LC3 and an accumulation of LC3I. Our observation of increased LC3II in the presence of 3-MA leads us to propose that MNV is inducing autophagy by two possible mechanisms: (i) through unblocking the activity of 3-MA on the enzyme PI3K; or (ii) through a novel non-canonical and unidentified mechanism. A recent study has also demonstrated that HCV is able to induce autophagy in a PI3K-independent manner (178). In light of these findings, it will be interesting to learn how these viruses are inducing autophagy.

It has recently been reported that the autophagy proteins Atg5-Atg12 and Atg16L localise to the MNV RC upon treatment of cells with IFN-γ and prevent MNV translation and replication (129). The authors observed that this antiviral effect was ineffective in cells deficient in Atg5 indicating that autophagy may be an antiviral cellular response to infection (129). Considering these findings, together with our own, we speculate that MNV induces autophagy in order to sequester the non-canonical function of the Atg proteins during the antiviral response (*i.e.* if ATG12/16L are sequestered to autophagosomes, they are not available to facilitate the IFN-g-mediated function and of course do not cross-present antigen). This has been summarised in Fig 28.

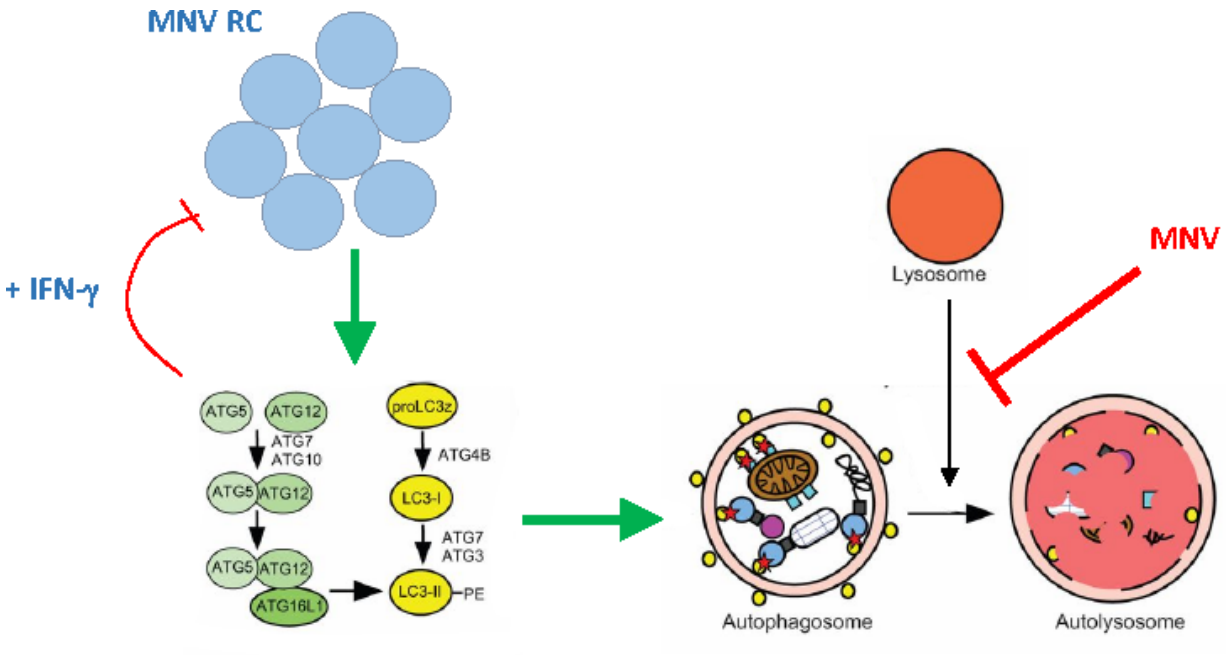


Figure 28. Model of the induction of autophagy during MNV replication.
A schematic representation of our hypothesis that MNV induces the cellular process of autophagy in order to sequester Atgs proteins away from IFN-ɣ to inhibit the antiviral response. We speculate that MNV also inhibits the antiviral response by inhibiting lysosome and autophagosome fusion to prevent antigen presentation.

Finally, it will be of great benefit to investigate the non-canonical roles of the autophagy proteins in facilitating an antiviral state in cells and the potential non-canonical induction of autophagy that is mediated by MNV. In addition, further research into how and why MNV induced autophagy (and the complex interplay as to how the autophagy process is induced), is manipulated and how this contributes to our antiviral immune response will be a vital to aid to understanding the pathogenesis of NoVs.

6.2 MNV replication requires a membrane platform rich in the host lipid PI4P

Viral replication complexes are generated on membrane platforms that have been sequestered and manipulated following the induced rearrangement and reorganisation of intracellular membranes (183, 280, 281). These complexes are required for efficient virus replication and also aid as a means to avoid immune detection. Due to the lack of an efficient tissue culture system for the laboratory cultivation of NoVs, little is known regarding the composition and mechanism by which NoV RCs are formed (183, 282-284). With the discovery of MNV and the subsequent identification of the tropism of MNV for mononuclear cells, advancements in this area have started to be reported. Our laboratory has revealed that MNV recruits membranes from both the ER and late secretory pathway in order to form its RC (271), and additionally we observed that the membrane re-distribution is potentially mediated via the MNV NS1-2 and NS4 proteins (120).

The role and contribution of the phosphoinositide PI4P to the biogenesis of viral RCs and in facilitating efficient replication of many (+)RNA viruses has been increasingly studied. These reports have revealed that enteroviruses rely heavily on the PI4P kinase PI4KIIIβ to generate pools of PI4P for replication. Conversely, HCV primarily requires the activity of another PI4P kinase, PI4KIIIα, for the establishment and maintenance of replication (220, 273, 285, 286). Due to the requirement of PI4P for efficient replication of these (+)RNA viruses, we aimed to investigate whether MNV replication had an equal dependency for PI4P during its replication cycle.

In pursuing these studies we observed a significant increase in the production of PI4P in MNV-infected cells and observed that these pools of PI4P accumulated the two PI4P lipid kinases PI4KIIIα or PI4KIIIβ, and strongly associated with NS4, at sites of MNV replication. To determine if either or both PI4Ks (PI4KIIIα or PI4KIIIβ) were required for the generation

of PI4P at the MNV replication membrane platform, chemical inhibition of these kinases was used to assess whether MNV replication was decreased under these conditions. We used the chemical HEV1, which is specific for PI4KIIIβ or PIK93 and selectively inhibits the activity of PI4KIIIα and/or PI4KIIIβ in a concentration-dependent manner. Our results showed that MNV-induced production of PI4P and efficient MNV replication still occurred in the presence of HEV1. Thus, these findings demonstrate that production, redistribution and utilisation of PI4P during MNV infection occur in a PI4KIIIβ-independent manner.

The PI4KIIIβ-independent production of PI4P induced during MNV replication was additionally supported in MNV-infected cells treated with 10μM PIK93 (which selectively inhibits PI4KIIIβ activity), whereupon increased PI4P production, its redistribution to the MNV RC and minimal impact on MNV replication were observed. In stark contrast, we observed a drastic decrease in PI4P production within cells treated with 30μM PIK93 (which selectively inhibits PI4KIIIα activity) and further that the extent of co-localisation between PI4P and NS4 within the MNV RC was significantly diminished. In addition, we demonstrated that the 30μM PIK93 treatment of MNV-infected cells resulted in a significant decrease in the transcription of MNV genomic RNA, a significant decrease in viral protein production and a significant decrease in the production and secretion of infectious progeny virions. In conjunction, these determinations strongly implicate a role for PI4KIIIα, rather than PI4KIIIβ, in the production of PI4P during MNV replication and that inhibition of PI4KIIIα activity duly affects the replication efficiency of MNV in macrophages. Again, considering the genetic similarities between picornaviruses and noroviruses, our finding that MNV requires PI4KIIIα activity rather than PI4KIIIβ activity for efficient replication was surprising.

Furthermore, in our attempts to elucidate a mechanism and role for the MNV ORF1 in the recruitment of PI4P and associated kinases, we found that none of the viral MNV proteins, co-localised with, or affected the localisation and distribution of either: PI4P; PI4KIIIα; or PI4KIIIβ when expressed individually. Conversely, numerous studies have shown that HCV NS5A protein actively stimulates PI4KIIIα activity infected cells whilst the 3A protein of picornaviruses interacts with PI4KIIIβ during replication (208, 220, 257, 272, 273, 275).

To interrogate a potential recruitment mechanism we widened our search for factors that could activate and promote PI4P production in cells. This included Arf1 and GBF1 that are two accessory proteins known to aid in the generation of PI4P-rich membrane microdomains facilitating HCV replication. Normally, GBF1 (acting as a GEF) activates Arf1 to subsequently activate PI4KIIIα to generate PI4P pools. Using recombinant expression plasmids encoding both of these proteins, we observed that both Arf1 and GBF1 were recruited and co-localised within the MNV RC. Interestingly we observed that chemical inhibition of GBF1 activity did not influence MNV-induced PI4P production nor significantly affect MNV replication. Thus an exact functional role for GBF1 within the RC is unknown. It may simply be a passenger during membrane recruitment based on its interaction with Arf1 within Golgi complex membranes. Unfortunately, a specific inhibitor for Arf1 was not available to the author during this research period, so the functional role for this protein during MNV replication has not been elucidated but will be pursued in further experiments. Interestingly, we did observe that the MNV proteins NS3, NS4 and NS6 showed significant co-localisation with Arf1, suggesting that these proteins play a role in the recruitment and/or activation of Arf1. It is intriguing that NS3 is a GTPase and thus there is the potential that NS3 may be a surrogate GEF to Arf1, a premise that warrants further investigation.

In our final assessment of the contribution of PI4P to the MNV replication cycle we aimed to determine whether the MNV NS4 or NS7 proteins had the inherent capacity to bind to PI4P. We chose NS4 and NS7 as the analogous enterovirus proteins 3A and $3D^{pol}$ have all been observed to bind to PI4P when individually expressed (208, 258). Interestingly, we observed that NS4 bound strongly to the phosphoinositides PI4P, PI(2,3)P2 and PI5P but NS7 did not bind to any of the phosphoinositides. The affinity of NS4 to these phosphoinositides is consistent with the residency of NS4 within the Golgi apparatus and endosome, and implicates NS4 with a potential tethering function to facilitate assembly of the MNV RC on membrane platforms rich in PI4P. It is intriguing that transient expression of NS4 alone did not stimulate or redistribute PI4P; however those observations strongly suggest that an additional viral protein or proteins are required to recruit and activate PI4KIIIα to produce PI4P. We speculate that the recruitment of PI4P to the MNV RC may be the result of mechanisms of multiple NS proteins working simultaneously to achieve these lipid-rich regions. Our current hypothesis is that NS3, NS4 and/or NS6 stimulate Arf1 to activate PI4KIIIα to produce PI4P (Fig 29). The increased local concentration of PI4P recruits NS4 that subsequently binds NS7 to facilitate genome replication. Many of these points still require robust interrogation to prove our hypothesis correct.

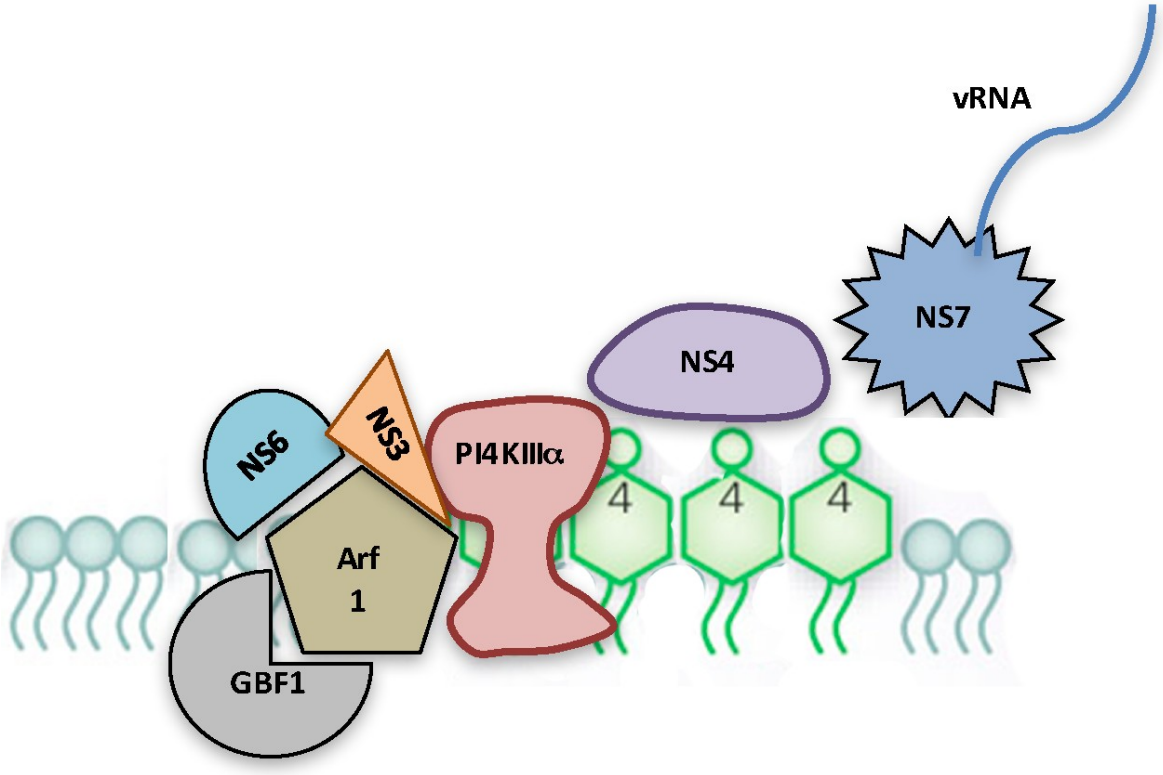


Figure 29. Our current model proposing the protein-protein and protein-lipid interactions that occur during MNV RC biogenesis and efficient MNV replication.
A schematic representation of our hypothesis of the increased PI4P production and its utilisation as a membrane platform for viral RNA replication during intracellular replication of MNV. We speculate that the MNV NS3 and/or NS6 proteins stimulate Arf1 to activate PI4KIIIa to increase the local concentration of PI4P. This increase leads to NS4 recruitment, due to its inherent capacity to bind PI4P, and NS4 recruits NS7 (the viral RdRp) to promote replication.

6.3 MNV replication and the cholesterol-PI4P shuttling interface

In addition to identifying that PI4P and its associated PI4KIIIα play an important role in MNV replication, this chapter further interrogates the role that host factors associated with the MCS between the ER and Golgi play, as well as cholesterol, in MNV replication.

The secretory pathway is utilized by many viruses as the unique lipid composition of particular membrane sites within this pathway are recruited to form the membrane platform required for replication (248). The MCS where the ER and Golgi apparatus are juxtaposed has been the subject of many recent studies examining the flux of two key lipids, PI4P and cholesterol, between these two organelles and whether this is beneficial for viral replication. Furthermore, interactions of viral proteins and the key host factors associated with regulation of the lipid exchange between this MCS have been the focus of numerous studies. It has been reported that multiple enteroviruses and HCV actively recruit OSBP to sites of viral replication in order to encourage the exchange of PI4P and cholesterol between the Golgi and ER (257, 260, 274). There is increasing evidence that the host sterol cholesterol contributes significantly to the establishment of the RC of multiple viruses and is required for efficient viral replication. The role of the host factor OSBP, as well as the role of cholesterol during viral replication has been attracting greater interest. Many enteroviruses and HCV rely on OSBP and the counter-flux of PI4P and cholesterol, while many flaviviruses have been shown to require cholesterol for efficient replication (238, 258, 260, 296).

In our initial immunofluorescence analyses we observed that OSBP did not co-localise with MNV NS4 in MNV-infected cells (Fig 22). In contrast, VAP-A strongly co-localised with NS4 at sites of viral replication in MNV-infected cells. Our previous studies have revealed that MNV recruits membranes from the endocytic and secretory pathways (73), hence in conjunction with our present observations this suggests that MNV may be recruiting

VAP-A from the ER. Further analyses of the recruitment of VAP-A and OSBP with individually expressed MNV ORF1 proteins revealed MNV NS1-2 strongly co-localised with VAP-A (Fig 23). NS1-2 did not co-localise with OSBP, nor did NS4 co-localise with OSBP or VAP-A. These observations suggest that the MNV NS1-2 protein may be required for the active recruitment of VAP-A to the MNV RC. In support of these findings, HuNoV NS1-2 has been previously observed to interact with VAP-A which resulted in inhibition of intracellular trafficking (67). Similarly, another study has shown that PV non-structural proteins 2B and 3A inhibit secretory vesicle trafficking, which has been predicted to utilize a mechanism that involves, or interferes with, VAP-A (251). As we did not observe recruitment of OSBP to sites of MNV, we speculate that MNV may not be utilising the PI4P-cholesterol counter-flux and is instead recruiting VAP-A for an alternate function rather than promoting the mechanisms of OSBP.

Given our hypotheses that MNV may not be utilizing the PI4P-cholesterol counter-flux, we aimed to determine whether Sac1 plays a role in MNV replication. Since Sac1 is an ER-resident PI4P-phosphatase which converts PI4P into phosphatidylinositol (PI) and plays a major role in facilitating the PI4P-cholesterol exchange at the ER-Golgi MCS (250, 252), we wanted to interrogate whether this phosphatase is utilized or not during MNV replication. Our IF analyses revealed dramatic redistribution and accumulation of Sac1 with NS4 in the MNV-infected cells (Fig 24). This observation together with our previous observations would suggest that MNV recruits a select composition of proteins from the ER-Golgi MCS to the viral RC. It is interesting to consider why MNV would recruit a PI4P phosphatase to replication sites that require the lipid for efficient replication. One consideration could be to derive energy from the hydrolysis of the lipid or to recruit and modify the activity of Sac1 to restrict PI4P hydrolysis as MNV requires pools of PI4P for efficient replication (see Chapter 4). One study has reported that HRV-A1A and -A16 infection is significantly inhibited in the

presence of siRNA targeting Sac1 (219). The counter flow of PI4P and cholesterol has been shown to play an important role in many enteroviruses' replication (219, 244, 254), thus inhibition of Sac1 is detrimental to viral replication as the energy derived from the PI4P hydrolysis is not supplied to OSBP and thus the flux of lipids cannot occur. Since no specific inhibitors or siRNA were available to the author during this body of work, determination as to whether Sac1 is required for MNV replication remain beyond the scope of this dissertation. It would be interesting however to determine this and we speculate that a knockdown of Sac1 may only have slight detrimental effects on MNV replication as MNV does not appear to require the PI4P/cholesterol exchange. If MNV is deriving energy from the hydrolysis of the lipid however, this will have major impacts on replication.

Finally, as MNV does not appear to recruit OSBP, we aimed to determine whether cholesterol plays a role in MNV replication. Cholesterol biosynthesis is regulated within the ER which involves membrane-bound transcription factors called sterol regulatory element-binding protein (232). Enteroviruses use clathrin-mediated endocytosis (CME) to gain entry into cells but also exploit this process to enrich intracellular free cholesterol pools and traffic cholesterol to sites of replication (237). The cholesterol found in these ROs is required for efficient enterovirus replication as cholesterol regulates the 3CD polymerase (237). Similarly, several studies have established that Flaviviruses such as WNV and DENV both depend on cholesterol to mediate viral replication (238-240). Whilst it has been shown that cholesterol plays an important role in entry of MNV into target cells, it is not known to what extent cholesterol plays a role in MNV replication (91).

Interestingly, our IF analyses revealed no co-localisation of staining associated with the anti-dsRNA antibody and filipin (Fig 26). In addition, we did not observe any dramatic change in the localisation or distribution of cholesterol, as determined by the filipin stain in MNV-infected cells. This suggests that MNV does not appear to recruit cholesterol to sites of

replication. To investigate this further, we examined the effects of modulating cholesterol shuttling and synthesis on MNV replication, by treating MNV-infected cells with various compounds.

Itraconazole (ITZ) is a drug with antifungal and anticancer activities which also inhibits sterol biosynthesis (308, 309). A recent study has demonstrated that Enterovirus and Cardiovirus replication is significantly inhibited in the presence of ITZ (258). The study revealed that OSBP is a novel target of ITZ, where ITZ binds to OSBP and disrupts its lipid shuttling properties. This inhibition of the counterflux of lipids between the Golgi and ER is responsible for the decreased viral replication (255, 293, 294). To identify whether ITZ treatment negatively affects MNV replication, MNV-infected cells were treated with ITZ and labelled with anti-dsRNA and filipin stained. We observed no significant visual disruption to the MNV RC and no visible changes to filipin localisation, which suggests that the inhibition of OSBP via ITZ causes no visual disruption of viral replication.

Furthermore, based on our observations that MNV does not appear to recruit OSBP or cholesterol, MNV-infected cells were treated with ITZ, Lovastatin or 25-HC to confirm cholesterol does not play an important role in MNV replication. Lovastatin inhibits HMG-CoA reductase within the mevalonate pathway, thus blocking subsequent steps of cholesterol synthesis (293). 25-hydroxycholesterol (25-HC) is an oxygenated sterol that reduces the activity of HMG-CoA reductase on a transcriptional level (294, 295). No significant difference in MNV RNA, NS7 protein production or viral particle secretion was observed between MNV-infected cells and ITZ-, Lov- or 25-HC treated MNV-infected cells (Fig 27). The only discrepancy observed was 25-HC treated MNV-infected cells displayed decreased NS7 protein which may be due to the role 25-HC plays in immune sensing and activation (297). A study in 2013 demonstrated that 25-HC can act as an effector of the innate-interferon response in macrophages to induce antiviral functions (310). While it has been

reported that 25-HC is a negative feedback mediator of the sterol pathway, the authors present a previously uncharacterized role for 25-HC in mediating antiviral cellular functions through recruitment of Stat1 which is directly coupled to IFN responses (301). The reduction in MNV NS7 protein and viral secretion could be explained by this mechanism rather than the decrease in protein production and secretion being attributed to inhibition of cholesterol biosynthesis.

In conclusion, our observations suggest that cholesterol does not appear to a play an important role in MNV replication as inhibition of cholesterol synthesis or mechanisms of OSBP also had minimal to no effect on MNV replication. Interestingly, NS1-2 strongly associated with VAP-A which raises the proposition that MNV may be recruiting this protein to inhibit cellular protein secretion or to inhibit OSBP from binding to VAP-A. We propose that MNV may disturb the cholesterol-PI4P counter-flux by modulating the activity of VAP-A and Sac1 to restrict the function of OSBP and hydrolysis of PI4P, respectively, resulting in accumulation of PI4P available for establishment of the MNV RC.

6.4 Future directions

The studies outlined in this thesis have provided initial insights into key events associated with MNV replication and pathogenesis. In the absence of a viable HuNoV tissue culture system, MNV provides a valuable surrogate model for the study of NoV replication. It is becoming more evident that while caliciviruses have been likened to picornaviruses in the past, emerging data together with the findings presented in this thesis illustrates significant differences between these families and the unique nature of caliciviruses.

We have shown that MNV induces autophagy and utilizes this process in a unique manner that has not been described for other viruses. In particular, further studies are required

to determine why and how MNV may prevent the fusion between lysosomes and autophagosomes. This will provide much insight as to whether this is a countermeasure against the host antiviral response or a means of dissemination from host cells.

It would also be of great benefit to investigate the mechanism of inducing autophagy via a PI3K-independent manner. Investigating the potential non-canonical induction of autophagy that is mediated by MNV is worth examining as this will uncover a significant mechanism in MNV pathogenesis. In addition, further research into how and why MNV induces the complex process of autophagy, and how the virus is able to manipulate this pathway which contributes to our antiviral immune response will be vital to help in understanding the pathogenesis of NoVs.

Previously, MNV NS4 has been likened to picornavirus 3A; however these studies have highlighted the unique nature of MNV non-structural proteins. For example, our laboratory has shown that NS1-2 and NS4 play important roles in recruiting intracellular membranes for the construction of the RC, while this thesis has demonstrated that these NS proteins obviously play multiple roles within the cell to facilitate replication during MNV infection. We have shown that PI4P and PI4KIIIα are required for efficient MNV replication. While we did not observe any co-location with MNV ORF1 proteins with either PI4P or PI4KIIIs, we did observe NS3, NS4 and NS6 co-localisation with Arf1. To continue this interrogation into whether Arf1 is recruited in order to recruit PI4K and stimulate the production of PI4P, knockdown or inhibition of Arf1 should be employed to examine the effects on MNV replication. Likewise, knockdown of PI4KIIIs is required to confirm our observations. In addition, co-immunoprecipitation experiments should be performed to examine direct interactions between viral non-structural and cellular proteins and lipids.

Finally, it is interesting to consider why MNV would recruit a PI4P phosphatase to replication sites that require the lipid for efficient replication. Investigation into the relationship of MNV and Sac1 needs to be extended to aid in identifying whether or not cholesterol is important for MNV replication. Likewise, further experiments need to be performed to clarify the relationship between NS1-2 and VAP-A. It would be beneficial to discover if MNV is able to inhibit intracellular secretion as a method of enhancing pathogenesis.

Extensive investigation into the MNV non-structural proteins is suggested in order to discover what unique and multifaceted roles these play during infection. This will significantly help understand and fill in the gaps in Norovirus pathogenesis we currently have.

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