Modulation of Glucocorticoid Action in vivo: Role of Lipid Rafts and Clocks

A thesis submitted to The University of Manchester for the Degree of Ph.D in Medicine in the Faculty of Biology, Medicine and Health

2016

Giorgio Caratti

Division of Diabetes, Endocrinology and Gastroenterology,

School of Medical Sciences

Table of Contents:

Table of Contents; Figures

Table of Contents; Tables

Abstract: Glucocorticoids (Gcs) are a commonly used drug to target the glucocorticoid receptor (GR). The GR has a myriad of cellular and physiological effects, however, Gcs are clinically used for the treatment of inflammatory conditions due to the potent anti-inflammatory actions of GR. The anti-inflammatory effects come with serious side effects e.g. metabolic disease. I examine the role of lipid rafts in modulating the anti-inflammatory actions of Gcs, and the role of circadian rhythms in the control of Gc side effects.

I tested the role of caveolin-1 (Cav1), a constituent of membrane lipid rafts, and its role in Gc suppression of inflammation. Gene expression analysis of mouse lung tissue showed that genetic depletion of Cav1 (CAV1KO) results in increased transactivation of Gc target genes. The increased Gc action, however, does not result in an increased effect on suppression of inflammation in a model of innate immunity: aerosolised lipopolysaccharide (LPS) induced lung inflammation or in a model of adaptive immunity: Ovalbumin. CAV1KO mice were protected from LPS induced inflammation, despite increased cytokine production. This suggests a differential response to LPS in lung parenchyma and alveolar macrophages dependent on Cav1. CAV1KO results in a pro-inflammatory phenotype in macrophages, and the opposite in parenchymal tissue. These data suggest that while Cav1 is an upstream regulator of Gc response, it does not have a strong enough effect to alter the ability of GR to repress inflammation *in vivo*.

Gc treatment results in a strong metabolic phenotype, with aberrant energy metabolism, insulin resistance and hepatosteatosis, I investigated how this side effect interacts with circadian rhythms, another key determinant of energy metabolism. Using transcriptomics of whole lung and liver taken during the day or the night, I demonstrate that the metabolic actions of Gc in the liver can be temporally separated, whilst maintaining consistent anti-inflammatory actions in both liver and lung. This temporal gene regulation by Gc is controlled by REV-ERBα, a rhythmically expressed, orphan nuclear receptor, part of the core clock machinery, via a direct interaction with GR at key regulatory DNA loci. Genetic deletion of REV-ERBα protects mice from the hepatosteotosis associated with Gc treatment.

Taken together, these data suggest that Gcs are regulated upstream of the receptor by the core consitutent of membrane lipid rafts; Cav1, which modulates the Gc response *in vivo*. Also, that the GR action can be controlled by dosing at different times of day, separating the detrimental metabolic effects of Gcs from the beneficial anti-inflammatory effects. This is enabled through a direct interaction between GR and REV-ERBα at key gene regulatory sites

Declaration:

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright:

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the "Copyright") and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the "Intellectual Property") and any reproductions of copyright works in the thesis, for example graphs and tables ("Reproductions"), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property University IP Policy (see http://documents.manchester.ac.uk/display.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library's regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University's policy on Presentation of Theses

Main Supervisor: Prof. David Ray **Co-supervisor: Dr. Laura Matthews**

Advisor: Prof. Kathryn Else

List of Abbreviations

11β-HSD – 11β-Hydroxysteroid Dehydrogenase

aa – Amino Acid

Ac – Acetyl

ACTH – Adrenocorticotropic Hormone

ADP – Adenosine Diphosphate

AF – Activation Function

ALI – Acute Lung Injury

Alum – Aluminium Hydroxide

AMP – Adenosine Monophosphate

AMPK – AMP Activated Protein Kinase

AP-1 – Activator Protein-1

ApoE – Apolipoprotein E

AR – Androgen Receptor

ARAF1 – A-Raf Proto-Oncogene

ATP – Adenosine Triphosphate

BAL – Bronchioalveolar Lavage

BALF – Bronchioalveolar Lavage Fluid

Bcat2 – Branched Chain Amino Transferase 2

BMAL – Basic Helix/Loop/Helix Pas Protein

BSA – Bovine Serum Albumin

cAMP – Cyclic Adenosine Monophosphate

CAV – Caveolin

CBP – CREB-binding Protein

CDK – Cyclin Dependent Kinase

CFA – Complete Freund's Adjuvant

CLOCK – Circadian Locomotor Output Cycles Kaput

CRP – C-Reactive Protein

Cry – Cryptochrome

CXCL – C-X-C Chemokine Ligand

CXCR – C-X-C Chemokine Receptor

cGR – Cytoplasmic Glucocorticoid Receptor

ChIP – Chromatin Immunoprecipitation

ChIP-seq – Chromatin Immunoprecipitation Followed By Next Generation Sequencing

COPD – Chronic obstructive pulmonary disease

COUP-TFII – Chicken Ovalbumin Upstream Promoter-Transcription Factor II

COX – Cyclooxygenase

CREB – cAMP Responsive Element

CRH – Corticotrophin Releasing Hormone

CRM1 – Chromosomal Maintenance 1

CSD – Caveolin Scaffolding Domain

DBD – DNA Binding Domain

Dex - Dexamethasone

DMEM – Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acid

DNase – Deoxyribonuclease

DSC – Differential Scanning Calorimetry

DUSP – Dual Specificity Phosphatase

DYRK3 – Dual Specificity Tyrosine Phosphorylation Regulated Kinase 3

ECM – Extracellular Matrix

ELISA – Enzyme Linked Immunosorbent Assay

eNOS – Endothelial Nitric Oxide Synthase

ER – Estrogen Receptor

ERK – Extracellular Signal-Regulated Kinase

FCS – Foetal Calf Serum

FITC – Fluorescein Isothiocyanate

FKBP – FK506 binding protein

FMLP – *N*-Formylmethionyl-leucyl-phenylalanine

FRAP – Fluorescence Recovery After Photobleaching

FRET – Förster resonance energy transfer

G6PC – Glucocse-6 Phosphatase Catalytic Subunit

Gc – Glucocorticoid

GDP – Guanosine Diphosphate

GFP – Green Fluorescent Protein

GILZ – Glucocorticoid Induced Leucine Zipper

Glut – Glucose Transporter

GMP – Guanosine Monophosphate

GPCR – G-protein Coupled Receptor

GPI – Glycosylphosphatidylinositol

GR – Glucocorticoid Receptor

GRdim – Glucocorticoid Receptor Dimerisation Mutant

GRE – Glucocorticoid Response Element

GRIP-1 – Glucocorticoid Receptor Interacting Protein-1

GTP – Guanosine Triphosphate

H3 – Histone 3

HADH – Hydroxyacyl-CoA Dehydrogenase

HAT – Histone Acetyl Transferase

HDAC – Histone Deacetylase

HNF – Hepatocyte Nuclear Factor

HOP – HSP70/HSP90 Organising Protein

HSP – Heat Shock Protein

IκBα – Nuclear Factor of kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, alpha

ICAM – Intracellular Adhesion Molecule

IF – Immunofluorescence

IL – Interleukin

iNOS – Inducible Nitric Oxide Synthase

IP – Immunoprecipitation

IPF – Idiopathic Pulmonary Fibrosis

INF – Interferon

IRAK – Interleukin-1 Receptor-associated Kinase

IRF – Interferon Regulatory Factor

IRS – Insulin Receptor Substrate

Jak – Janus Kinase

JNK – c-Jun N-Terminal Kinase

KD – Knockdown

KLF – Kruppel-like Factor

KO – Knockout

LBD – Ligand Binding Domain

Lkb1 – Liver Kinase b1

LPS – Lipopolysaccharide

LXR – Liver X Receptor

MAPK – Mitogen Activated Protein Kinase

MED14 – Mediator of RNA polymerase II transcription subunit 14

mGR – Membrane Glucocorticoid Receptor

MMTV – Mouse Mammary Tumour Virus

MNAR – Modulator of non-genomic action of the estrogen receptor

MR – Mineralocorticoid Receptor

MyD88 – Myeloid Differentiation Primary Response 88

me – Methyl

mRNA – messenger Ribonucleic acid

NAD+ – Nicotinamide Adenine Dinucleotide (Oxidised)

NADH – Nicotinamide Adenine Dinucleotide (Reduced)

NADP+ – Nicotinamide Adenine Dinucleotide Phosphate (Oxidised)

NADPH – Nicotinamide Adenine Dinucleotide Phosphate (Reduced)

NCOR – Nuclear Corepressor

NES – Nuclear Export Sequence

NFκB – Nuclear Factor κB

nGRE – Negative Glucocorticoid Response Element

NLS – Nuclear Localisation Sequence

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

NR – Nuclear Receptor

OD – Oligomerisation Domain

PARP – Poly (ADP-ribose) Polymerase

PEPCK – Phosphoenolpyruvate Carboxykinase

Per – Period

PFK1 – Phosphofructokinase 1

PGE_2 – Prostaglandin E_2

PI3K – Phosphoinositide 3-Kinase

PKA – Protein Kinase A

PKB – Protein Kinase B

PKC – Protein Kinase C

PLA – Proximity Ligation Assay

PPAR – Peroxisome proliferator-activated receptor

PR – Progesterone Receptor

REDD – Regulated in Development and DNA Damage Response

RelA – Avian Reticuloendotheliosis Viral Oncogene Homolog A

RER – Rough Endoplasmic Reticulum

RNA – Ribonucleic acid

RNA-seq – RNA Sequencing

ROR – RAR-Related Orphan Receptor

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute

SCN – Suprachiasmatic Nucleus

SGK – Serum/Glucocorticoid Regulated Kinase

siRNA – Small Interfering RNA

SIRT – Sirtuin

Smad – Mothers Against Decapentaplegic Homolog

SMRT – Silencing Mediator for Retinoid or Thyroid-Hormone Receptors

STAT – Signal Transducer and Activator of Transcription

SWI/SNF – SWItch/Sucrose Non-Fermentable

TAK1 – Transforming Growth Factor Beta Regulated Kinase

TBP – TATA Binding Protein

TF – Transcription Factor

TGF-β – Transforming Growth Factor-β

Th1 – Type 1 T Helper Cell

Th2 – Type 2 T Helper Cell

TIF2 – Transcription Initiation Factor 2

TLR – Toll-like Receptor

TMAO – Trimethylamine *N*-Oxide

TNFα – Tumour Necrosis Factor α

TR – Thyroid Hormone Receptor

TRAF – TNF Receptor Associated Factors

tRNA – Transfer RNA

VDR – Vitamin D Receptor

VEGF – Vasculature Endothelial Growth Factor

VEGFR – Vasculature Endothelial Growth Factor Receptor

WT – Wild Type

Amino Acids

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asp	asparagine
D	Asp	aspartic acid	P	Pro	proline
E	Glu	glutamic acid	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	Lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

Chapter 1: Introduction

1.1 Glucocorticoid Structure

Glucocorticoids (Gcs) are small molecule ligands of the glucocorticoid receptor (GR), both natural and synthetic. The name is derived from glucose (by virtue of their effect on glucose metabolism) and cortex (the location of their synthesis within the adrenal). The endogenous active human Gc is cortisol (SPRAGUE *et al.*, 1950), while in rodents it is corticosterone. Corticosterone differs from cortisol by the absence of the hydroxyl group on carbon 17 of the D-ring, making it more hydrophobic (Fig.1.1). The ligands have similar effects in their respective organisms. Due to the extended carbon rings, they are extremely lipophilic molecules (Ponec *et al.*, 1986), and with this in mind the classical view of steroidal action was developed. It is assumed that due to the hydrophobic nature of Gcs and other steroids, they can easily diffuse into cells by simply crossing the plasma membrane, a process that large, ionic or hydrophilic molecules cannot.

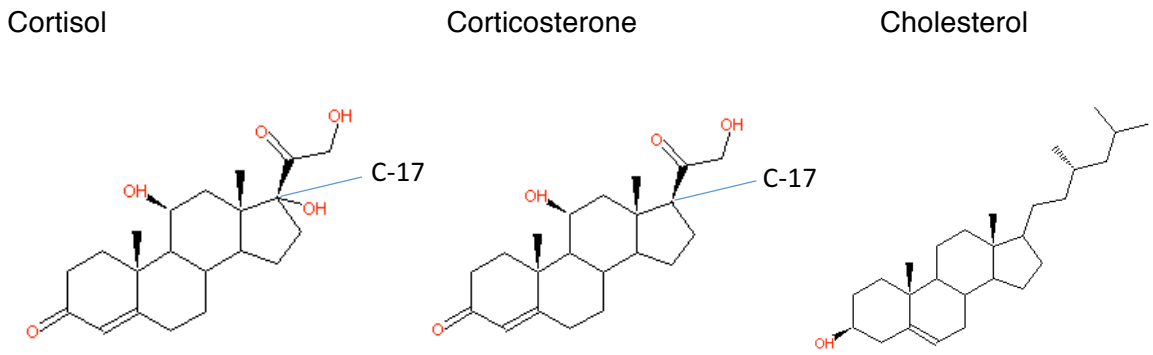

Fig.1.1 Structural comparison of cortisol (left) and corticosterone (centre) and cholesterol (right). The only difference in the hormones is the hydroxyl group on C-17. The four ring structure is common to both the endogenous glucocorticoids and cholesterol. However, it is clear that the Gcs have more hydrophilic moieties, which are involved in hydrogen bonding within the receptor, and association with proteins while in the blood serum, such as albumin and cortisol binding globulin. Images made via Java Molecular Editor applet (http://www.changbioscience.com/mis/chemdraw.html).

1.1.2 Glucocorticoid Synthesis and Release

Endogenous Gcs, cortisol and corticosterone are synthesized from cholesterol. The synthetic pathway shows the chemical relationship of all steroidal hormones. Cholesterol is initially converted into pregnenolone, which is the rate limiting step within the synthetic pathway; under control by adrenocorticotropic hormone (ACTH) (Simpson and Waterman, 1988). Pregnenolone synthesis is the initial step in the mineralocorticoid pathway as well as the Gc pathway, and by multiple redox reactions, makes its way to cortisol. Gcs are synthesised in the zona fasciculata of the adrenal cortex in response to stimulation by ACTH (Clark, 2016). ACTH is negatively regulated by Gcs, causing a negative feed-back loop by which Gc concentration inhibits further Gc synthesis (Herman *et al.*, 2016). Corticotrophin releasing hormone (CRH) is the neurotransmitter in the hypothalamus that is secreted as part of the stress response (Taylor and Fishman, 1988). This causes ACTH to be secreted from the pituitary gland directly into the blood, and thus reaching the adrenal glands. The feedback

cycle is represented in Fig.1.2. Gc are secreted in a circadian pattern too, with the highest levels in the morning, just before waking, and the lowest levels of Gcs just before sleep in humans. In mice, the endogenous Gc, corticosterone, peaks in the evening, at the onset of the nocturnal animal's active phase (Challet, 2015). However, Gcs are always found at low levels within the blood. The production of Gcs is inhibited by opioid peptides that cause a reduction in CRH levels (Briski and Vogel, 1995).

1.1.3 Glucocorticoid Physiology

Gcs have a wide range of physiological roles. They include carbohydrate and lipid metabolic control, anti-inflammatory responses and stress. The GR is constitutively expressed in almost all cell types; which reflects the nature of Gcs to control such a large variety of cellular processes. Fig.1.2 shows a generalised representation of these physiological effects. The stress induction of Gcs occurs in addition to the usual circadian pulses involved in Gc production.

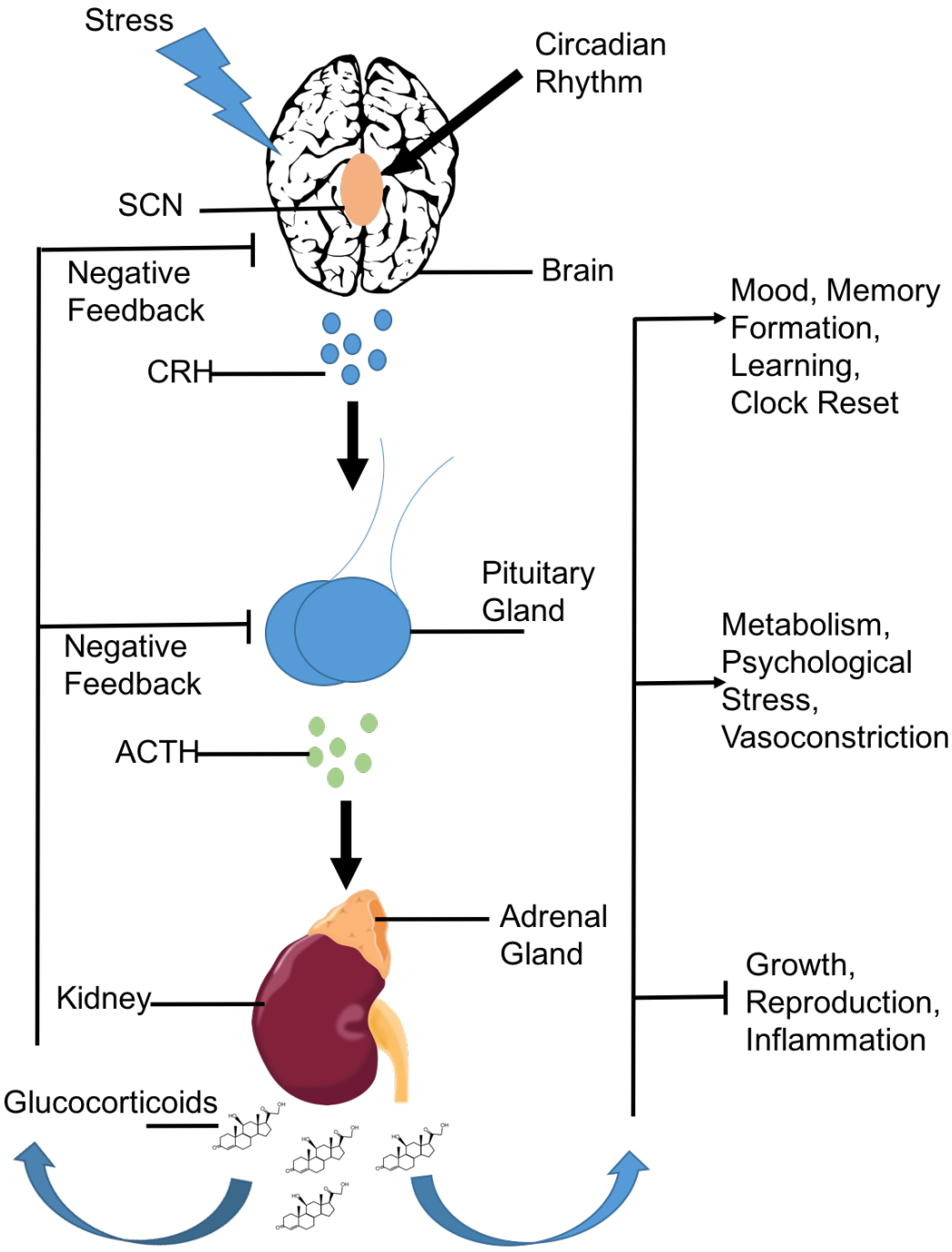

Fig.1.2 Representation of negative feedback control of Gc synthesis and release, with generalised physiological role of Gc. Gc are produced in and released from the adrenal gland into the blood. From there they act on multiple tissues, with major effects in the brain: affecting diurnal rhythms and memory, but also initiating the prevention of further Gc release. The metabolic effects occur in the liver, muscle, adipose and bone, all of which are important Gc targets, inducing regulation of gluconeogenesis and increased glycolysis in all tissues. Adapted from (Chung, Son and Kim, 2011).

1.2 Glucocorticoid Receptor Structure

The Gc receptor belongs to the Nuclear Receptor (NR) superfamily which includes the estrogen receptor (ER), mineralocorticoid receptor (MR), progestin receptor (PR), androgen receptor (AR), peroxisome proliferator activated receptor (PPARs), vitamin D receptor (VDR) and thyroid hormone receptor (TR) among others (Nrnc, 1999). Nuclear receptors have a generic, modular structure, which GR conforms to (seen in Fig.1.3): The N-terminal activation function-1 (AF-1) domain involved association with transcription factors and cofactors. The central DNA Binding Domain (DBD), which has the most evolutionary conservation (Kumar and Thompson, 1999). Lastly, the C-terminal Ligand Binding Domain (LBD) which is key for mediating protein-protein interactions and dimer formation, as well as binding ligands (Hollenberg *et al.*, 1985; Kumar and Thompson, 1999; Robinson-Rechavi, Garcia and Laudet, 2003). Ligand binding causes a conformational change in the conserved 12-helix bundle which is associated with activation of the receptor (Kauppi *et al.*, 2003). This mechanism is common to the NR family.

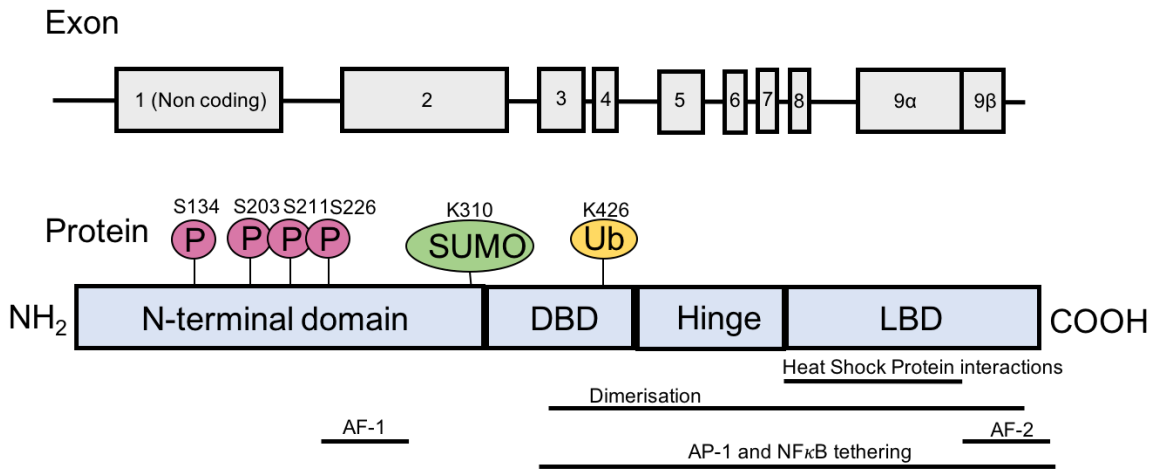

Fig.1.3 The structure of the Gc receptor. GR consists of 9 exons, of which exon 1 is non-coding, and exon 9 is alternatively spliced to form the active GRα or dominant negative GRβ. The receptor consists of the N-terminal domain, the DNA-binding domain, hinge region and ligand binding domain. Key residues that are post-transcriptionally modified have been highlighted. (Adapted from McMaster and Ray, 2008)

1.2.1 Activation Function-1 Domain

AF-1 has been shown to have the largest effect on transcriptional control (Giguere *et al.*, 1986; Kumar *et al.*, 2001; Goulding, 2004) but in solution it appears to have a dynamic secondary structure. Mutation of hydrophobic resides in the AF-1 core peptide limited the transactivation potential (Dahlman-Wright *et al.*, 1995). Kumar et al. demonstrated a hydrophobic collapse by applying trimethylamine *N*-oxide (TMAO), a naturally occurring stabilising osmolyte, to drive the formation of native fold AF-1. This lead to the observation that forcing folding with enhanced binding to transcription factors such as TATA Box Binding Protein (TBP) and CREB Binding Protein (CBP). Also that upon forming protein-protein interactions, TMAO removal did not disturb AF-1 structure, suggesting that binding stabilises or induces the AF-1 active conformation (Kumar *et al.*, 2001). Binding TBP infers that AF-1 is involved in regulating the transcriptional machinery, which is key for transactivation (Ford *et al.*, 1997). Modulator of non-genomic action of the estrogen receptor (MNAR) has been shown to elicit its strong transrepression effect through AF-1; which is an example

of how the context in which GR binds DNA leads to differential actions of GR specific to AF-1 compared to AF-2 which MNAR potentiates the effect of (Kayahara *et al.*, 2008).

1.2.2 DNA Binding Domain

As GR is a modular protein, the DBD (DNA binding domain) is capable of recognising and binding GREs (Gc response elements) without the full length receptor (Green *et al.*, 1988; Umesono and Evans, 1989). The DBD contains two zinc ions, bound by two sets of 4 cysteine residues in a tetrahedral arrangement, termed zinc fingers. The zinc finger arrangement is conserved in the NR superfamily and is common to many DNA binding proteins (Freedman *et al.*, 1988; Luisi *et al.*, 1991). The crystal structure of the DBD bound to DNA has been determined. It was found that the DBD forms a globular fold with two main modules, each being nucleated by the zinc ion. These are held together in the tertiary structure via aromatic residues, forming an hydrophobic core. Mutation to hydrophilic residues in this core causes the GR to lose its transcriptional activity, likely due to losing specific DNA contacts (Luisi *et al.*, 1991). There has been insight into the allosteric nature of DNA when binding the DBD. Luisi et al. noted this potential of DNA to initiate recognition between DBDs, as without DNA it lacks the capability to dimerise (Freedman *et al.*, 1988; Hard *et al.*, 1990). The nuclear localisation sequence-1 (NL-1) overlaps with the DBD, like in many DNA binding receptors (LaCasse and Lefebvre, 1995). The NL-1 works via the α-importin β pathway (Savory *et al.*, 1999). The classical view is that upon ligand binding, 90 kDa heat shock protein (HSP90) dissociates, thus revealing NL-1 and allowing the GR to translocate to the nucleus (Picard and Yamamoto, 1987; Pratt and Toft, 1997). However, with GR over-expression experiments, it was determined that the unliganded GR could be translocated to the nucleus. Coupled with leptomycin B treatment to inhibit nuclear export via the CRM1 pathway it was shown that GR wild type was transported to the nucleus (c.f. a GR mutant without NL-1 which was not) without ligand, suggesting that NL-1 is constitutively active, and the cytoplasmic localisation is due to shuttling, not HSP90 inhibiting the NLS (nuclear localisation sequence) via sterics. This opens up the potential for a cytoplasmic retention signal that

could be modified upon ligand binding to cause translocation, or control via variations in shuttling speeds (Savory *et al.*, 1999; Matthews *et al.*, 2011).

1.2.3 Ligand Binding Domain

Unlike the N-terminal domain, the crystal structure of the GR LBD (ligand binding domain) has been defined (Bledsoe *et al.*, 2002). Other NR receptors have a 3 layer helical sandwich which has a hydrophobic pocket for ligand binding (Bourguet *et al.*, 1995; Brzozowski *et al.*, 1997). Similarly, GR forms a 3 layer sandwich which again, creates a space for ligand binding, at the bottom of which the Activation Function-2 (AF-2) domain helix is in the "agonist bound conformation", where it packs against helices 3, 4 and 10 as part of the domain structure. The AF-2 then leads into a β-sheet which is critical for stabilisation of the AF-2 domain, and loss of which causes receptor inactivation (Zhang, Liang and Danielsen, 1996; Bledsoe *et al.*, 2002). Common to other NRs, AF-2 is important in ligand recognition and in the GR, DEX makes direct contact with AF-2 thus stabilising the activated conformation. The A ring of the ligand is oriented to a conserved arginine reside in helix-5 and the D ring towards the AF-2 helix, common to all the NR family ligand binding. Differences in the orientation of helices 6 and 7 of GR compared to other NRs can explain selectivity for Gc ligands due to the larger substituent on C17 of the D ring. Location of different amino acid residues also contribute to selectivity in GR. The polar atoms on Gcs are located differently to the ligands of the other NRs. Cortisol has a ketone on carbon-3 which accepts hydrogen bonds from Q570 and R611, compared to estrogen which has a hydroxyl which would unfavourably donate hydrogen bonding (Bledsoe *et al.*, 2002).

The ligand binding domain is also important for binding of co-activators which associate via LXXLL motifs, of which the 3 different LXXLL motifs determine specificity of the co-activator for the NR (Ding *et al.*, 1998). Specificity in the GR for co-activators such as TIF2 (transcription initiation factor 2) is mediated by two charge clamps formed by AF-2 helix and helix 3, compared to only one in ER (Bledsoe *et al.*, 2002).

The LBD contains a second NLS, termed NL-2 (Picard and Yamamoto, 1987). These alternate NLSs, which do not occur in the DBD of proteins, are likely to act under very specific conditions. Unlike the NL-1, NL-2 does not use the α-importin β pathway, as shown by GR mutants lacking NL-1. It is also a weaker signal, shown by slower transport of GR (Freedman and Yamamoto, 2004). The NL-2 appears to be hormone dependent, the antagonist RU486 (which causes translocation, but not transactivation of WT GR) did not achieve translocation in mutant GR lacking NL-1. There was also an obvious difference in kinetics of translocation via the NL-2, in that there was a 30 minute lag phase which was not apparent in NL-1. This suggests that NL-2 requires an extra protein association before translocation can occur (Savory *et al.*, 1999). The LBD, while not necessarily conserved at the amino acid level, has large amounts of conservation in folding and secondary structure between the NR superfamily, thus allowing specificity of ligand by differences at the primary level, but retaining the affinity for the steroid class of ligand by creation of a hydrophobic binding pocket (Brzozowski *et al.*, 1997; Kumar and Thompson, 1999).

1.2.4 Dimer Formation

Bledsoe et al. determined that the LBD could indeed form a dimer in their crystal structure analysis. This confirms the evolutionary differences between the GR and ER due to difference in the dimer interface where GR is unable to form a coiled coil structure upon dimerisation. Mutation of the LBD dimer interface had differential effects: P625A completely removed dimerisation and transactivation and transrepression. I628A lost transactivation; however, it retained transrepression but required a 20-fold increase in DEX concentration for full activation (Bledsoe *et al.*, 2002).

The second (carboxy-terminal) zinc finger domain has a major role in dimerisation of the DBD. This section has been shown to lose DNA binding cooperativity when mutated *in vitro* (Dahlman-Wright *et al.*, 1991) and that it is flexible as a monomer and thus unlikely to be involved in protein folding (Hard *et al.*, 1990). It provides a surface for intermolecular contacts via the residues between two of the cysteines involved in coordinating the zinc ion (C476-C482), called the “D-box” or “D-loop.” The

D-box is maintained in a β-turn by the coordination of the zinc ion in the R-configuration. Such interactions include R479 to D481 salt bridge and backbone hydrogen bonding between carbonyl and NH groups. The same zinc finger has a β-strand which causes the exposure of two hydrophobic residues to the solvent I487 and L475. This indicates a dimerisation surface. Binding to DNA appears to stabilise the binding conformation therefore explaining the cooperativity seen in GR dimerisation at GREs (Dahlman-Wright *et al.*, 1990; Hard *et al.*, 1990) as well as DNA binding aiding in the entropy penalty involved in binding and dimerising caused by conformation restriction (Luisi *et al.*, 1991).

1.2.5 Isoforms of the Glucocorticoid Receptor

The human GR gene includes 9 exons and 8 introns which account for some of the various isoforms of GR (Hollenberg *et al.*, 1985; Lewis-Tuffin and Cidlowski, 2006). However, the first exon of GR is non-protein coding, and is occurs in 5 isoforms: 1A1, 1A2, 1A3, 1B and 1C. These upstream, non-coding exons affect cellular expression specificity, and post-translational modification (Brzozowski *et al.*, 1997; Breslin, Geng and Vedeckis, 2001; Zhou and Cidlowski, 2005).

Exon 9 is responsible for the canonical GRα and another isoform; GRβ where the last 50 amino acids of the GRα C-terminus are replaced with new 15 amino acids resulting in a truncation of the receptor in the LBD (Hollenberg *et al.*, 1985). This alternate isoform GRβ is not found in mice (Otto, Reichardt and Schutz, 1997), and does not have ligand binding capability, and therefore was assumed to not affect gene transcription (Hollenberg *et al.*, 1985; Giguere *et al.*, 1986; Oakley *et al.*, 1997). However, microarray analysis revealed that GRβ does have its own transcriptional activity, although it does so mainly through indirect means; by interaction with transcription factors or co-factors. As the DBD is unchanged in GRβ it has been suggested that there could be interaction with GRβ specific response elements, but there is no strong data to support this so far (Kino *et al.*, 2009). GRβ is expressed in most tissues, but in contrast to the GRα is found mostly in the nucleus (de *et al.*, 1996; Oakley *et al.*, 1997). The best characterised effect of GRβ is its dominant negative effect on the activity of GRα, whereby it may form a heterodimer with GRα and therefore inhibits the effects of Gcs. This suggests

that GRβ is involved in regulation of Gc sensitivity of cells (Bamberger *et al.*, 1995; de *et al.*, 1996). The low levels at which GRβ is expressed in native contexts may not be enough to contribute to the dominant negative effect seen in over-expression transfection assays (Zhou and Cidlowski, 2005).

GRγ, another alternate transcript, was also found. It is constitutively expressed at around 4-9% of the total GR (Rivers *et al.*, 1999). GRγ has a single additional amino acid, arginine 453, inserted between exon 3 and 4, located between the two zinc fingers, which results in a 50% loss in transcriptional activity (Ray *et al.*, 1996) but despite this, GRγ has similar DNA binding affinity compared to GRα suggesting that it may not be differences in GRγ-DNA binding that cause its alternate effects (Meijsing *et al.*, 2009). GRγ has also been implicated to have dominant negative effects on the transrepressive activity of GRα, like GRβ (Taniguchi *et al.*, 2010), but also that GRγ has transcriptional effects on some GRγ specific genes (Meijsing *et al.*, 2009).

GR-P and GR-A are two further isoforms that have been characterised. They miss exon 8 and 9, or 5, 6 and 7 respectively (Moalli *et al.*, 1993). GR-P transfection has been shown to increase GRα activity in the presence of Gcs (de Lange *et al.*, 2001).

Finally, hGR-A (94 kDa) and hGR-B (91 kDa) are GR isoforms generated by leaky ribosomal scanning. GR-A is mainly found in cancer cells, although both GR-P and GR-A are better associated with Gc resistance (Zhou and Cidlowski, 2005). GR-B has been shown to be around two times stronger at transactivation compared to GR-A, although their transrepressive abilities are comparable. GR-B has been found endogenously in some cell lines, indicating that the ribosomal leakage can occur naturally (Zhou and Cidlowski, 2005).

1.3.1 Chaperones and Localisation of the Glucocorticoid Receptor

In the inactive state, GR is complexed by the chaperones HSP90, HSP70 (70kDa heat shock protein), HSP40 (40kDa heat shock protein), HOP (HSP70/HSP90 organising protein), p23 (prostaglandin E synthase 3) and immunophilins such as FKBP51 and FKBP52. HSP90 is extremely important in

developing the high affinity binding of the GR for Gcs. Without HSP90, GR has very low affinity for Gcs. The chaperone complex contains two HSP90 molecules bound to the LBD of one GR, with no specificity for either of the HSP90 isoforms (α or β) (Pratt and Toft, 1997). HSP90 is a target for histone deacetylase 6 (HDAC6), and the role of HSP90 as a GR chaperone is dependent on deacetylation by HDAC6. HDAC6 KD (knock down) caused a 6 fold decrease in the GR binding of DEX and a hyperacetylation of HSP90, suggesting that the chaperone mediated maturation of GR by HSP90 is lost, or at least reduced by an increase in acetylation after KD. A luciferase reporter gene assay also had reduced activation by DEX in HDAC6 KD cells. This hyperacetylation caused by the KD also limited the association of the co-chaperone p23 with HSP90 indicating that acetylation not only causes HSP90 to disassociate from GR, but also from the cochaperones involved in maintaining the overall complex. The association and chaperone activity of HSP90 to GR is an ATP-dependent process, with the acetylation causing a loss in affinity of HSP90 for ATP. DEX also appears to induce the acetylation of HSP90, but HDAC6 can remove these acetyl groups at a relatively efficient rate (Kovacs *et al.*, 2005). HSP70 is likely to be the first chaperone that recognizes new GR. It binds the LBD of GR, aided by HSP40 in an ATP dependent manner (Vandevyver *et al.*, 2013).

The classical localisation of the GR states that GR resides in the cytoplasm in complex with various chaperone proteins required for activation. Upon ligand binding and activation, the GR dissociates from the chaperones and translocates to the nucleus to elicit its transcriptional effects. This was backed up from evidence that heat shock and chemical shock causes translocation of the unliganded GR, suggesting that dissociation of the HSP90 allows the GR to translocate, and that ligand binding is the physiological method for this dissociation (Sanchez *et al.*, 1990). However, there is more and more data emerging to suggest that the GR is not static until ligand binding but is rather dynamic, moving in and out of the nucleus by the NLS-1. Similarly the chaperones may not all dissociate upon ligand binding, but move to the nucleus as a GR complex, and aid in the formation of the transactivation complex with GR to initiate gene transcription. HSP90 has been specifically noted as a requirement for GR mobility, with loss of ATP (therefore preventing HSP90 association with GR)

causing reduced GR translocation activity. The nuclear import, while not directly reliant on ATP is energy dependent, working via a GTP-GDP exchange. Therefore simply depleting ATP within a cell may have inhibited transport via reduction in GTP production and thus exchange, rather than affecting HSP90 association. Similarly, the HSP90 inhibitor geldanamycin prevented rapid GR translocation to the nucleus, to the point where the speed of translocation could be attributed to diffusion (Elbi *et al.*, 2004).

1.3.2 Nuclear Import and Export of the Glucocorticoid Receptor

The role of HSP90 in mediating rapid movement to the nucleus for entry via the nuclear pore complex can be attributed to contact with the cytoskeleton. GR does not directly interact with the cytoskeleton for rapid translocation upon ligand binding, but rather, the chaperone complex does. The immunophilin FKBP51, which is part of the chaperone complex of the resting GR, binding HSP90, is swapped out for the immunophilin FKBP52 upon ligand binding. FKBP52 mediates the interaction with the cytoskeleton that enables the rapid translocation of the GR. FKBP52 interacts with dynamitin, which in turn associates with dynein as part of the microtubule-dependent motor complex (Vandevyver *et al.*, 2013).

The GR nuclear export sequence (NES) is located between the two zinc fingers in the DBD. The export mediator calreticulin binds this sequence, in the presence of Ca^{2+}. Release of calreticulin from the endoplasmic reticulum results in a fast nuclear export. However, there is another, slower mechanism of GR export from the nucleus which is dependent on exportin-1. The two mechanisms are very similar; both require Ran-GTP and are ATP dependent. However, the exportin-1 pathway occurs when ligand is removed, rather than a stimulated release like calreticulin (Vandevyver *et al.*, 2013).

1.3.3 Post-translational Modifications of the Glucocorticoid Receptor

The GR is a phosphoprotein with S246 and T171 being constitutively phosphorylated (Popovic *et al.*, 2010). Upon ligand binding it goes through further phosphorylations by S/T kinases, resulting in a hyper-phosphorylated GR, with DNA binding capability (Ismaili and Garabedian, 2004). Due to the nature of ligand binding affecting phosphorylation, it was postulated that these modifications to the GR may affect its transcriptional activity. Both MAPKs and CDKs (cyclin dependent kinases) are involved in the post-translational modification of GR *in vivo*. From early experimentation, it was observed that in MAPKs down regulate the transcriptional activity of GR, while CDKs up-regulate, and are necessary for a full response in a yeast model. The CDKs A-CDK2 and E-CDK2 were both shown to phosphorylate S224 and S232 of the mouse GR (which are S203 and S211 in humans), and importantly that S232 phosphorylation is dependent upon S224 phosphorylation (Krstic *et al.*, 1997). The same CDKs are responsible for phosphorylation of their human counter-parts too. Interestingly, in the human GR, S203 has higher levels of basal phosphorylation than S211, which is logical considering that S211 requires S203 for its own phosphorylation. Ligand binding alters this phosphorylation rate by 4-fold and 10-fold, for S203 and S211 respectively (Wang, Frederick and Garabedian, 2002). The S211 phosphorylated GR has preferential nuclear localisation. The phosphorylation rate of S211 is correlated to the strength of the agonist (and is not phosphorylated by antagonists), with full agonists such as DEX and prednisolone generating high levels of phosphorylation, and partial agonists; RU486 generating less phosphorylation. The logical assumption is that the kinase associated with S211 interacts with the LBD; as different agonists induce different conformational changes in the LBD depending on their profile. The S203 phosphorylation is more labile than the S211, and it is likely that once both serines are phosphorylated, the S203 phosphorylation is lost, leaving behind the S211, which marks the transcriptionally active receptor that translocates to the nucleus (Wang, Frederick and Garabedian, 2002).

A good example of the negative regulatory effect that MAPKs have on GR transcriptional activity is c-Jun N-terminal Kinase (JNK) (Rogatsky, Logan and Garabedian, 1998), which controls a nuclear export pathway for the GR. JNK is a stress activated MAPK, responding to UV radiation, osmotic shock and proinflammatory cytokines such as TNF-α or IL-1 so clearly opposes the anti-inflammatory effects that GR mediates. JNK phosphorylates GR at S226, which, in the absence of DEX causes an increased, rapid nuclear export of GR and therefore limits the transcriptional effects of GR. The time difference between normal, slow nuclear export of GR which takes around 12 hours and the rapid JNK initiated export that occurs in less than 8 hours suggests that a different export pathway could be involved instead of the classical exportin 1/CRMI (Itoh *et al.*, 2002).

The MAPK p38 is required for apoptosis in cells treated with DEX. It phosphorylates S211, and when inhibited, DEX is unable to elicit an apoptotic signal. Therefore p38 is driving the activity of liganded GR (Miller *et al.*, 2005). On the other hand, CDK5 phosphorylation of GR at S203 and S211 has been shown to decrease the transcriptional activity of GR at the MMTV and SGK promoters, despite phosphorylating S211, the same target as p38. This is mediated by phosphorylation inhibiting the interaction of GR with cofactors, therefore limiting its transcriptional potential. These differences between other regulatory phosphorylation events can be explained by a cell type and/or promoter specific control of GR by phosphorylation (Kino *et al.*, 2007).

The GR is acetylated in a ligand dependent manner at the hinge region within the DBD. This occurs at the residues 492-495, in a lysine rich area. This acetylation has a negative effect on the ability for ligand stimulated GR to transrepress the activity of p65, but is removed by HDAC2. When HDAC2 was over-expressed in macrophages derived from chronic obstructive pulmonary disease (COPD) patients, there was a loss of the Gc resistance associated with the disease (Ito *et al.*, 2005).

1.4 Cellular Mechanisms of the Glucocorticoid Receptor

1.4.1 Genomic Actions: Transactivation

The GR is a ligand activated transcription factor, which upon nuclear translocation binds GREs in the target gene promoter to initiate or boost gene expression. The GR recruits the SWI/SNF chromatin remodelling complex upon binding positive GREs of which the Brm subunit has a modulatory effect on GR occupancy (Archer and Fryer, 1998). The final result is the binding and formation of the pre-initiation complex. DUSP1 (dual specificity phosphatase 1) and GILZ (Gc induced leucine zipper) are examples of genes involved in the anti-inflammatory actions of the GR transactivation mechanism. They are both activated by the classical mechanism of GR binding a GRE, and promoting transcription which can be seen in diagrammatical representation in Fig.1.4. DUSP1 functions as an anti-inflammatory gene by removing S/T/Y phosphorylations, specifically from kinases within the MAPK pathways. DUSP1 activity focuses on the dephosphorylation of JNK and p38, but has also been implicated in dephosphorylation events on ERK1/2 (Raingeaud *et al.*, 1995; Slack *et al.*, 2001). Importantly, it turns these pathways off, which prevents pro-inflammatory signalling from the MAPK pathways which promote the transcriptional activities of, for example NFκB by causing dissociation from IκB and activation. The DUSP1 phosphatase activity has also been shown to be important in macrophages. When DUSP1 KO macrophages are treated with Gcs, they have a weaker anti-inflammatory response, but DUSP1 knockout mice are overall, responsive to Gcs (Maier *et al.*, 2007). The macrophages, as well as mice also have very high levels of MAPK activation, which results in their death from endotoxic shock (Hammer *et al.*, 2006, 2010).

GILZ has its strongest effects in T cells; where in mice over-expressing GILZ the T cells show a skewing towards the Th2 response. When over-expressed in T cells, it limits the production of pro-inflammatory cytokines such as IL-2 and also protects against activation induced apoptosis. GILZ is a leucine zipper protein; however it does not bind DNA, but rather sequesters NFκB and AP-1, limiting their ability to induce pro-inflammatory genes (Clark, 2007). In a similar fashion, GR causes the up-regulation IκBα, which under conditions with no inflammatory stimulus binds and inhibits NFκB by interaction with the Rel-homology domain on the RelA subunit (Beck *et al.*, 2009).

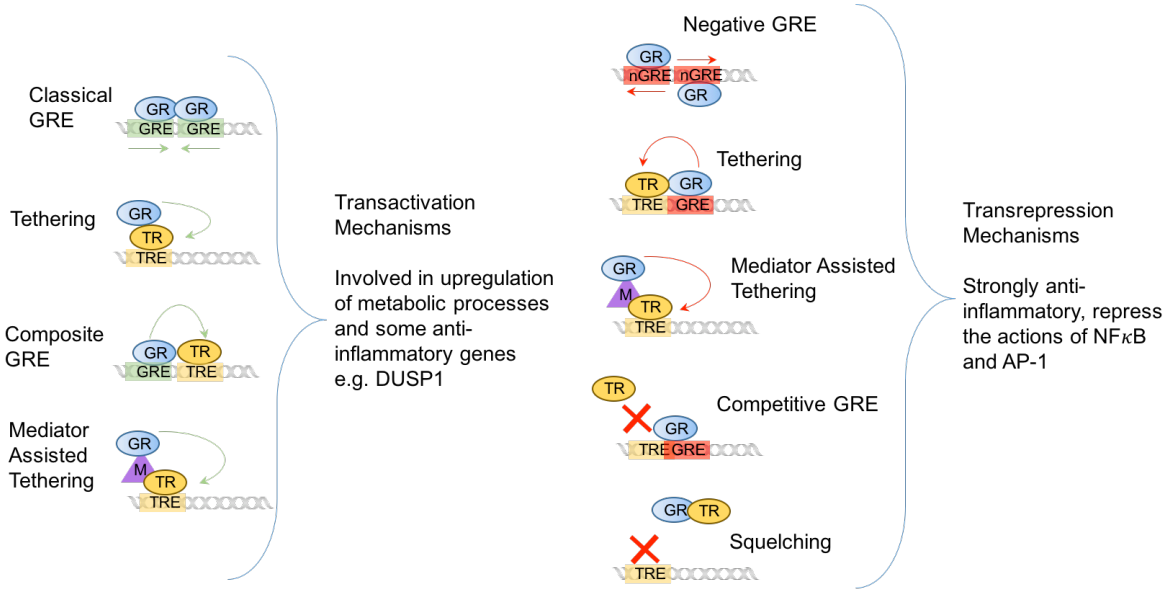

Fig.1.4 Different mechanisms of GR genomic action. The left hand side is the transactivation mechanisms, involved in up-regulating genes. The right is the transrepression which are important in down-regulating genes. The two actions of GR share similar mechanisms highlighting how important epigenetic control is in determining the action of GR. Adapted from (Ratman et al., 2012).

In a similar way, some GREs have been found to be composed of composite binding sites that can accommodate both the activated GR and another transcription factor, see Fig.3. At these sites, GR and the other transcription factor aid in each other's binding, thus promoting (and in some cases repressing) transcription of the gene. The Notch4 promoter has a composite binding motif, which contains elements of both the GR and AP-1 binding motifs (Wu and Bresnick, 2007). In endothelial cells it has been shown that while both transcription factors can bind independently, AP-1 stabilises the GR binding, which results in transactivation of the Notch4 gene. This also occurs at the phosphoenolpyruvate carboxykinase gene, where both CREB and GR bind in order to promote transcription (Ratman *et al.*, 2013).

However, using genome wide techniques to find GR binding sites in the DNA, found that most GR binding occurs at intragenic locations distant from the start of gene transcription (Reddy *et al.*, 2009; Biddie *et al.*, 2011; Grøntved *et al.*, 2013). The same has been noted in other NRs. One proposed explanation for how GR could affect transcription despite being so far away from the promoter regions is via chromosomal folding. This is where the 3D structure of DNA is arranged in such a way that the GR would be close to the start of transcription in space, but not necessarily close on the primary DNA sequence. There are examples of enhancers being located within coding exons of nearby genes that physically interact with each other. Not only does this demonstrate that folding of the DNA for transcriptional control does occur, but it would allow for tissue-specific control via DNA methylation or chromatin accessibility of the enhancer located within the coding area (Birnbaum *et al.*, 2012). GR gene transactivation results in a recruitment of histone acetyl-transferases, and thus opening of the local area allowing transcriptional machinery to enter, as well as other transcription factors (Uhlenhaut *et al.*, 2013). Linked to this, two independent studies found that the distance of the GRE seems to affect the role of GR in transcription. With those GREs that are relatively close to the promoter being involved in gene transactivation, while those that are distant, and potentially require chromatin folding for their activity are involved in transrepression. The different mechanism involved is likely tethering, which is generally attributed to the transrepressive effects of GR. It involves the direct interaction of GR with another transcription factor, resulting in either the promotion or inhibition of that transcription factor's transactivation ability. One example of tethering and transactivation is the GR and chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII). They physically interact, resulting in a Gc mediated promotion of COUP-TFII transactivation (De Martino *et al.*, 2004).

Transactivation can be mediated by post-translational modifications of the GR. An increase in the relative phosphorylation of S211 to S226 can increase the transactivation potential of GR. With a S226A mutant resulting in more GR localised to the nucleus and stronger transactivation. The mechanism by which phosphorylation at S211 increases the transactivation potential is via altering

the local structure of the GR into an α-helix, thus promoting an interaction with the cofactor MED14 (Mediator of RNA polymerase II transcription subunit 14). It was also put forward that weak GR activation, by low hormone concentrations results in the phosphorylation and MED14 dependent mechanism. High GR occupancy, resulting from high hormone concentrations appears to be independent of MED14 and phosphorylation (Chen *et al.*, 2008).

Determining which GR binding sites are transactivating or transrepressive is difficult. Surprisingly, analysis of the GR cistrome in macrophages has shown that many canonical positive GREs also have NFκB and AP-1 binding sites. However, these were found to be irrelevant to whether the gene was a transactivation or transrepression target. This suggests that it is not possible to deduce if a gene is activated or repressed by GR by only looking at the binding motifs of *cis*-regulator elements. Similarly, around 60% of transactivated and transrepressed genes recruited the GRIP-1 coregulator upon DEX treatment, meaning that is useless as a marker of activation or repression too (Uhlenhaut *et al.*, 2013).

1.4.2 Genomic Actions: Transrepression

The number of genes controlled by GR transactivation is smaller than those controlled by transrepression. The original way of determining if a gene was involved in transactivation or transrepression was via the GRdim mutant. This is a mutation in the D-loop of the GR, which is thought to prevent dimerisation and therefore distinguish between those genes involved in transactivation, which require a GR dimer (and so the GRdim is unable to activate), and those only involved in transrepression which do not require dimerization (Heck *et al.*, 1994; Reichardt *et al.*, 1998). There is discussion on this, with some evidence appearing that indicates that the GRdim is not completely unresponsive at certain genes and is still capable of dimerization, although far less so than wtGR. There is even evidence that at specific promoters the GRdim is able to form multimers, irrelevant of the DBD interface (Ratman *et al.*, 2013). A vast array of inflammatory mediators have NFκB or AP-1 binding sites in their promoters. These include, but not limited to: TNF-α, IL-6, IL-8, IL-

12 and interferon-β (De Bosscher, Vanden Berghe and Haegeman, 2003). Control of NFκB and AP-1 is therefore extremely important in the anti-inflammatory activities of the GR. This control is mediated through different mechanisms, which will be discussed in more detail.

1.4.3 Negative Glucocorticoid Response Elements

Analogous to the GREs, which mediate transactivation, there are negative Gc response elements (nGREs), where GR binding directly to the promoter region results in a repression of gene transcription. See Fig.1.4. The nGREs, identified by ChIP-seq (chromatin immunoprecipitation-sequencing) were found to be similar in DNA motif structure to the positive GREs and proximal to the NFκB and AP-1 binding sites (Uhlenhaut *et al.*, 2013). These nGREs work by binding the nuclear corepressor (NcoR), silencing mediator for retinoid or thyroid-hormone receptors (SMRT) and HDACs. Unlike the tethering mechanism, the nGREs contain specific GR binding sites on the DNA, and are stopped by the GRdim mutation. The repression mediated by nGREs is also very sensitive to the antagonist RU486 which the tethering mechanism is relatively resistant to. RU486 promotes nuclear translocation, but prevents further gene repression via the nGRE binding mechanism. This suggests that some structural changes must be involved to allow nGRE binding, which is not relevant in tethering (Surjit *et al.*, 2011). As mentioned previously, composite transcription factor binding elements exist. The AP-1/GR composite site is very common, and can be part of the transrepressive activity of GR too as seen in Fig.3. (Ratman *et al.*, 2013).

The mechanism of GR binding nGREs is different to binding classical positive GREs. Rather than two monomers binding at one site, in a cooperative fashion, there are two binding sites of differential affinity. The nGRE binding results in the GR monomers being oriented away from each other. From previous experimentation, mainly using the GRdim mutation, it's known that GR monomers (or at least non-canonical dimers) are involved with transrepression, compared to dimerisation being involved in transactivation. Therefore the nGRE may be acting as an allosteric regulator, preventing dimerisation, and thus ensuring that GR represses the gene in question. Interestingly, unlike at

positive GREs, the binding of the first GR monomer at an nGRE, results in negative cooperativity. This is caused by the change in DNA conformation upon the high affinity binding of the first monomer. The DNA constricts at the major groove and expands at the minor groove, which is the opposite of what occurs at positive GREs. It was postulated that GR nGRE binding is in competition with GR dimerisation. This hypothesis was supported by two GR mutants: the GRdim and a GR double mutant R460D D462R. Both limit GR dimerisation, and potentiated nGRE action, suggesting that monomeric GR is needed for nGRE binding (Hudson, Youn and Ortlund, 2013). Binding of the proinflammatory transcription factors NFκB (p65/RelA) and AP-1 (in this case c-Jun) at GREs meant that there was a higher chance of transcriptional change occurring, compared to if GR was solely bound to the DNA. However, this was also true of positive GREs. Therefore it was hypothesised that the nature of the GRE is not as important as previously thought. This was backed up by mutation of canonical GREs resulting in loss of transrepression, showing DNA binding is a key part of the transrepression mechanism. The GR binding recruits HDACs at repressed genes to limit the binding of other proteins, and effectively silence the area. However, there was no effect on NFκB and AP-1 binding, meaning that GR must repress them specifically by preventing their activation of genes, rather than stopping their direct binding to the DNA (Uhlenhaut *et al.*, 2013).

1.4.4 Protein-Protein Interactions: Tethering and Squelching

The transrepressive mechanisms that involve direct protein-protein interactions between GR and other transcription factors are very important to the control of inflammation. They are also involved in the transactivation mechanisms of GR. GR and NFκB or GR and AP-1 interactions are the most well documented, and interestingly the transrepressive actions are reciprocated: GR is also inhibited by these transcription factors upon association. The difference between tethering and squelching is the location of the repressed transcription factor (see Fig.1.4.). Squelching (Wright *et al.*, 1991) is not an uncommon way for GR to mediate transrepression, it was first noted when GR repression of AP-1 resulted in decreased binding of both c-Jun and GR (Yang-Yen *et al.*, 1990; Brogan *et al.*, 1999). T-

bet, a Th1 specific transcription factor, is repressed in a similar mechanism to NFκB. Both can be bound to prevent their DNA binding, which is mediated by the first zinc finger of the DBD on GR (Ratman *et al.*, 2013). GR binding and sequestering the opposing transcription factor, without any DNA binding involved is squelching. If the GR binds already DNA bound NFκB or AP-1, this is known as tethering (Nissen and Yamamoto, 2000; De Bosscher, Vanden Berghe and Haegeman, 2003). This mechanism was proposed after ChIP-seq found that GR or the repressed transcription factor remained bound to the DNA during the repression, meaning that the mechanism of squelching was insufficient to explain all the mechanisms of protein-protein interaction based repression. Both mechanisms result in the prevention of the transcription factor from initiating mRNA production. Using pull down experiments with GR, there is evidence that both c-Jun and c-Fos subunits of AP-1 are bound by GR. RelA is also bound by GR. Further experimentation found that although the DBD of GR was enough to allow interaction, shown by pull down, it was not able to repress the transcriptional control elicited by NFκB. A chimeric protein on the other hand, which included the transcriptional repressive domain from Mad1 and the DBD from GR was able to repress NFκB (De Bosscher, Vanden Berghe and Haegeman, 2003). At the collegenase-1 promoter, GR tethering is suggested to be the major mechanism of repression, where it binds AP-1 to prevent up-regulation of the gene. There is further evidence though that a mediator may be required to elicit this tethering. Trip6 (Thyroid receptor-interacting protein 6) is an example of one such mediator. When looking at AP-1 and GR occupancy, KD of Trip6 resulted in a large decrease of GR bound, but no reduction of AP-1, and a loss of transrepression; suggesting that it provides an intermediate connection between the two (Kassel *et al.*, 2004).

These protein-protein interactions are likely to be more important than direct DNA binding as only 25-30% of GR and NFκB have GRE or NFκB response elements (Rao *et al.*, 2011). This data is seemingly in direct contradiction to Uhlenhaut et al. where DNA binding was implicated to be the most important mechanism (Uhlenhaut *et al.*, 2013). However, both studies found the importance of large DNA binding sites, which include AP-1, NFκB and GR binding motifs in mediating the

transrepression of GR. The fact that many genes repressed by Gcs are dependent on inflammatory stimulus is also a common idea to both studies. DNA bound RelA recruits GR to the gene, thus only resulting in repression when the inflammatory stimulus and Gcs are around. Therefore showing that the Gc mediated repression of these inflammatory genes is not pre-emptive, but reactionary (Rao *et al.*, 2011; Uhlenhaut *et al.*, 2013). Although, activation of GR or RelA can result in the increase of the other's binding to DNA, but not necessarily at the same location. The explanation Rao et al. put forward was that each factor could be involved in chromatin remodelling, thus exposing an area for the other to bind (Rao *et al.*, 2011).

1.4.5 Tissue Specificity of Genomic Actions

The mechanism of cell type specific GR binding is controlled by the epigenetic state of that cell. DNA methylation or histone acetylation controls access of GR to DNA sites, and thereby dictates the transcriptomic response. John et al found that 71% of GR binding sites were located at places within the genome that were already accessible and sensitive to DNase I; i.e. sites which are constitutively accessible due to high histone acetylation. A further 9% were in locations that had moderate DNase I sensitivity, again suggesting that they are easily accessible. Only around 11% of these accessible sites were the same between a mouse mammary and mouse pituitary cell line, highlighting the tissue specificity. There were some sites found that required other transcription factors to bind and open the chromatin before the GR could also bind. However, these sites were in the minority compared to those that were already DNase I sensitive (John *et al.*, 2011). In fact, most transcription factor binding DNA motifs are heavily methylated, meaning that in general, transcription factors are unable to bind the majority of their target, however the transcription factor binding sites that are active within the tissue appear to be hypo-methylated, thus limiting incorrect binding of transcription factors in a tissue (Choy *et al.*, 2010). This is changed by cell type, with those motifs that are required for transcriptional control containing little to no methylation (Ratman *et al.*, 2013). When comparing the GR cistrome of macrophages, adipocytes, pituitary and mammary cells, the overlap of binding

sites were 1% between all 4 cell types. If compared in pairs, the overlap was still below 15%. Logically, the metabolic binding sites were less occupied, and the inflammatory binding sites were more occupied in macrophages compared to the other cell types (Uhlenhaut *et al.*, 2013).

1.4.6 Non-Genomic Actions

The idea that Gcs may work by additional, non-transcriptional mechanisms originated from the observation that Gcs can elicit rapid responses, which are incompatible with the slow speed of transcriptional control. These rapid actions also seem to evoke a different response from the GR itself as confirmed by classical antagonists such as mifepristone, which are unable to inhibit these actions. The range of rapid actions in Gcs is wide and found in multiple tissues, it is even conserved throughout evolution. In male roughskin newts physiological levels of Gcs have been shown to decrease their reproductive behaviour, showing that these rapid effects have both behavioural and physiological actions (Lewis and Rose, 2003). Similarly, the cortisol increase after exercise, and thus the acute, rapid actions of Gcs has been shown to aid in memory formation and learning whereas the stress response, working via the transcriptional pathway has been shown to inhibit learning and memory formation. The HPA feedback inhibition via Gcs is highly dependent on the non-genomic effects, with around 50% of the negative feedback by Gcs on the ACTH production being mediated by the rapid actions, therefore providing a fast negative regulation on its own release. Importantly this action of Gcs in the brain is reliant on the canonical GR. Knockdown (KD) of the GR in the brain inhibits these responses and thus suggests that the non-genomic actions are working via the same receptor as the genomic responses (Tasker, Di and Malcher-Lopes, 2005). Actin polymerisation has been shown to increase in response to rapid Gc effects too (Falkenstein, Norman and Wehling, 2000).

PKC and PKA have been implicated in the rapid Gc effects, with PKC inhibitors preventing the inhibition of voltage gated calcium channels in the hippocampus by Gcs. Via this PKC pathway, Gcs are able to initiate the phosphorylation of MAPKs p38, JNK and ERK1/2 in hippocampal cell lines. As these signalling cascades end in the nucleus, this may be an alternative way that Gcs affect

transcription. However, the ERK1/2 pathways are activated much slower by a stress response (1-2 hrs), and are sensitive to knockout of the GR. This suggests that they are controlled by the usual transcriptional mechanisms. However the same study also found that the downstream effectors were activated quickly, in around 30mins, suggesting a rapid Gc control of these (Tasker, Di and Malcher-Lopes, 2005).

1.4.7 The Membrane Glucocorticoid Receptor

The body of work that looked at rapid Gc actions within the brain has found a link between the rapid Gc actions and GPCRs. This has led to the hypothesis of membrane bound Gc receptors (mGR). Radiolabelled Gcs in the membrane fraction of roughskin newt brains was found to be inhibited by GTP-γ-S and enhanced by magnesium which are good signs that the signalling pathway is working via a GPCR. Similarly, the rapid ACTH suppression caused by Gcs is inhibited by pertussis toxin, a classical GPCR inhibitor which prevents the GTP/GDP exchange of the G_i α-subunit (Tasker, Di and Malcher-Lopes, 2005). It was expected that the mGR is encoded by a separate gene than the cytosolic GR (cGR). However, many antibodies directed against cGR, detect the same epitopes on mGR, suggesting strong similarity. Initially, over-expression of the cGR (specifically GRα) gene was shown to not increase the amount of mGR at the surface, which many took to indicate that two separate genes must encode for the cGR and mGR (Bartholome *et al.*, 2004). More recent evidence found that the two proteins are encoded for by the same gene. Using RNA interference, where different siRNA molecules aimed at the 9 different exons were developed and found that mGR was not decreased more than cGR, whereas KD of the whole gene did result in decreased mGR and cGR. These data indicate that not only do the two proteins come from the same gene, but are unlikely to be different splice variants. Considering that the mGR is also dependent on Golgi transport for targeting to the membrane, it is likely to be modified in some way to aid in membrane association (Strehl *et al.*, 2011). More evidence suggests that the exocytotic pathway is key came from experimentation that showed immunostimulation by LPS caused up-regulation of the mGR, and could be stopped by

inhibition of the exocytotic pathway in monocytes (Bartholome *et al.*, 2004). Interestingly, caveolae also begin formation at the golgi. This raises the question as to whether GR and CAV1 association begins, preformed at the golgi, or whether association is by random interaction events at the plasma membrane. The BSA-DEX model was explored further; using a GILZ reporter gene assay, it was possible to show that BSA conjugated cortisol does not activate the transactivation mechanisms of the GR. The mGR was also shown to be highly important in the anti-inflammatory actions of Gc treatment. mGR stimulation by BSA-cortisol reduced prostaglandin E synthase p23, an important subunit for the translocation potential of the GR-chaperone complex, but also a key enzyme in the generation of prostaglandin E_2 (PGE_2) which are pro-inflammatory signalling molecules (the PGE_2 pathway is a target for over-the-counter anti-inflammatory drugs such as aspirin). In addition to the anti-inflammatory actions, the mGR was shown to mediate metabolic pathways such as glycolysis and gluconeogenesis as well as amino-acyl tRNA synthesis by non-genomic actions (Vernocchi *et al.*, 2013).

When Gcs are administered to immune cells, they are able to drastically and immediately reduce the oxygen consumption of activated lymphocytes. Mostly by altering the Na^+,K^+-ATPase activity but only *in vitro* and using relatively high concentrations of Gc (double the concentration of therapeutic Gc doses). It was postulated that these effects could be mediated by the mGR, but also that the high concentrations could cause non-specific binding of Gcs in the membrane, thus altering the physical environment and inhibiting the ion pumps (Schmidt, Holsboer and Spengler, 2001).

The ER isoforms ERα and ERβ both associate with membranes. Thus the highly related GR may also behave like this, meaning the classical cytosolic receptor may be involved in some of the rapid effects rather than a specific Gc liganded GPCR. Antibody detection has shown that the cGR is localised at the membrane. This is further backed up by fluorescence studies which also show the localisation of cGR to the membrane. However, the mGR is dependent on G proteins indicating a different method of action. Association with the membrane could cause it to behave differently leaving idea that it is the same as the cytosolic receptor open (Tasker, Di and Malcher-Lopes, 2005).

The control of the MAPK pathway by the mGR (membrane Glucocorticoid Receptor), specifically p38, is supported by data using BSA-conjugated DEX (BSA-DEX). This molecule is unable to cross the membrane due to the large protein attachment, and therefore only activates the mGR. In CD14+ monocytes, this mGR specific ligand caused differential activation of signalling cascades, and an increase in p38 MAPK signalling at a significantly higher rate than simple DEX treatment or LPS stimulation (Strehl *et al.*, 2011).

Membrane associated GR is also involved in transcriptional activities. Those GRs located within lipid raft microdomains have been shown to be dependent on this association for transcriptional control; disrupting the rafts inhibits the GR. The GR agonist DEX also stimulates increased association to detergent resistant membranes (Jain *et al.*, 2005).

1.5 Glucocorticoids and Physiology

1.5.1 Lung Development

The link between Gcs and lung development has been known since the late 1960's, when it was observed that antenatal Gc treatment reduced respiratory distress syndrome in prematurely born infants (Ballard and Ballard, 1972). It became common practice for physicians to administer Gcs to mothers who have a chance of premature birth. This is in order to enhance lung development of the child. Part of this developmental control that Gcs mediate is the synthesis of surfactants, produced by the epithelial type II cells and the Clara cells. Gcs stimulate the epithelial type II cells to produce the phospholipid surfactants by increasing fatty acid synthase expression (Garbrecht *et al.*, 2006). They also stimulate the increased expression of Clara cells' protein surfactants (surfactant protein-B, C and D) and aid in lengthening the mRNA (messenger ribonucleic acid) half-life of these genes (Ballard *et al.*, 1996).

Gcs stimulate multiple effects of lung structural development; these include alveolar wall thinning to aid in gas exchange, cell proliferation and differentiation (epithelial cell type II). Gcs also control a variety of messengers: TGF-β (Transforming Growth Factor-β) is an important example (Wen *et al.*, 2003). TGF-β inhibits lung branching via the Smad2, Smad3 pathway, although its necessity for lung morphogenesis is unknown, *tgfb1* knockout (KO) mice do not show any obvious signs of structural defects. Vasculature Endothelial Growth Factor (VEGF), which is also controlled by Gcs, has an important part in the development of the endothelial-epithelial exchange within the foetal lung (Cardoso and Lü, 2006). KO of GR gene results in mice that show respiratory distress and die after a few hours following birth. This is partly due to lack of lung inflation (Cole *et al.*, 1995). This can be explained by Gcs controlling the change of osmotic pressure via stimulating the synthesis of epithelial Na^+ channels and thus causing fluid absorption rather than expulsion from the epithelia to lumen (Garbrecht *et al.*, 2006). Indeed, within the GR KO mice, the sodium transporter expression was decreased. The GR KO mice also lacked development of late bronchioles and alveoli indicating control of the final step in lung development.

1.5.2 Glucocorticoids and Metabolism

The effect of Gcs on fat and glucose metabolism is well known, however their actions are entirely tissue specific, and contribute to the role of increasing glucose concentration in the blood (Britton and Silvette, 1931), whilst sparing glucose uptake in adipose tissue (Di Dalmazi *et al.*, 2012) and skeletal muscle (Kuo, Harris and Wang, 2013). Glucocorticoids also directly regulate the transcription of multiple genes involved in the gluconeogenic pathway. Phosphoenolpyruvate caboxykinase (PEPCK), glucose-6-phosphatase (G6PC) and phosphofructokinase (PFK1) are all key genes required for gluconeogenesis, and are regulated by Gc. Gc also induce the degradation of protein in skeletal muscle and lipolysis in adipose tissue, resulting in more precursors (amino acids and glycerol) for use in gluconeogenesis (Exton *et al.*, 1972; Kuo, Harris and Wang, 2013). Gc cause increases in glycogenolysis and liver fatty acid uptake, which can result in liver steatosis (discussed later). This

gives Gc an important role in fasting. The stress of fasting causes increased levels of circulating Gc, and therefore results in increased levels of gluconeogenesis, and thus available glucose for the central nervous system. On the other hand, Gc cause a switch from glucose utilisation in the liver, muscle and adipose tissue, to use of fatty acids as energy. Therefore Gc have the opposing action of insulin signaling, and interestingly, cooperate with growth hormone for their actions via the transcription factor STAT5 (Mueller *et al.*, 2012). Gc effects are therefore associated with insulin resistance (discussed later).

The shift from glycolysis and utilization of glucose to beta-oxidation of lipids as a source of energy can be seen not only through the above mechanisms of preventing glucose uptake, but also in how Gc directly regulate mitochondrial dynamics, mitochondrial genes, and nuclear encoded mitochondrial genes, shown in multiple species (Weber *et al.*, 2002; Hernández-Alvarez *et al.*, 2013; Li *et al.*, 2013).

Gc act on adipose tissue through two key enzymes: hormone sensitive lipase and acetyl-CoA carboxylase, which causes and increase in free fatty acids and a decrease in fatty acid synthesis respectively, therefore acting on both the lipolytic and lipogenic pathway (Divertie, Jensen and Miles, 1991; Slavin, Ong and Kern, 1994; Gathercole *et al.*, 2011). Paradoxically, Gc also cause an increase in adipocyte accumulation, by acting directly on preadipocytes and stimulating differentiation (Campbell *et al.*, 2010). However, *in vitro* models of adipose catabolism do not mirror what is seen in the clinic or in chronically treated animal models. People with high levels of endogenous Gc, or who are chronically treated with synthetic Gc display an increase in fat mass. Linked to this, patients with Cushing's Syndrome, and animals on chronic Gc treatment have been shown to choose high calorie foods over lower calorie alternatives, likely through increased production of Neurotransmitter-Y (Peckett, Wright and Riddell, 2011).

The role of Gc in causing muscle atrophy is widely accepted, and dates back to the first clinical uses of Gc in the 1950's (Hoberman, 1950). The effect of Gc in muscle depends entirely on fibre type, with fast twitch fibres being the most susceptible to Gc induced atrophy, through a mechanism involving regulation of KLF15 and REDD1, up regulating protein degradation and amino acid metabolism through Bcat2. The lack of Gc induced atrophy in slow twitch fibres is likely due to a reduced expression of GR in those particular cells (Shimizu *et al.*, 2011). Gc also have similar effects in muscle as in adipose, with a decrease in glucose uptake, by suppression of the glucose transporter Glut4, and an increase in glycogenolysis similar to that seen in liver.

1.5.3 Immunity and Inflammation Homeostasis

While inflammation is important for the resolution of infection or tissue damage, the process must be turned off, in order to protect from the deleterious effects of prolonged inflammation. GR can interact with and repress the activities of Nuclear Factor-κB (NFκB), Activating Protein-1 (AP-1), and cAMP Responsive Element Binding protein (CREB), a coactivator, thus limiting the associations that CREB can form with proinflammatory transcription factors (Goulding, 2004). Gcs also stimulate the production of anti-inflammatory proteins such as interleukin-10 (IL-10) and interleukin-1 receptor antagonist, important in the resolution of inflammation (Mozo, Suárez and Gutiérrez, 2004).

Gcs have major effects on all immune cells; in neutrophils they cause the inhibition of movement, whether that is within blood vessels, or extravasation. This movement inhibition is mediated by reducing the expression of endothelial adhesion molecules (ICAM-1, -2 and -3) and neutrophil adhesion molecules (integrins β2). However, they also increase the concentration of neutrophils within the blood stream, but induce apoptosis in this population by increasing annexin-1 expression. This is consistent with the idea that Gcs are inflammatory homeostatic agents, rather than simple inflammatory repressors (Baschant and Tuckermann, 2010; Busillo and Cidlowski, 2011). Dendritic cells are also targets of Gc inflammatory homeostasis, although their monocyte precursors are not.

Gcs induce apoptosis in dendritic cells whilst simultaneously reducing MHCII and cytokine expression.

Inhibition of cyclooxygenase (COX), to prevent prostaglandin release, and therefore limit inflammation, is a large part of how Gcs exert their anti-inflammatory effects. This occurs strongly within both T cells and macrophages. The suppressive effects elicited by Gcs on macrophages have been shown to work through non-genomic mechanisms, as well as the classical genomic repression, via the MAPK (mitogen activated protein kinase) pathway using GR KO (knock-out) macrophages (Bhattacharyya *et al.*, 2007). It appears that Gcs are important in suppressing the adaptive immune response, but supporting the innate response. This support can be shown in Gcs abilities to increase macrophage survival when presented with lipopolysaccharide (LPS) whilst simultaneously repressing activity. T cells, a critical part of the adaptive immune system at show Gc induced apoptosis, and a marked Gc-induced inhibitory effect on expression of cytokine and chemokine production. Interestingly, unlike most cell types, T cells have an increase in GR expression when exposed to Gcs. This aids them in reaching the threshold protein concentration of GR needed for Gc induced apoptosis, essentially a positive feedback loop (Necela and Cidlowski, 2004). There is also inhibition of cytokine production from Th1 and Th2 cells. Physiological concentrations of Gcs can cause immunity to become more dependent on Th2 cells, however at pharmacological concentrations, there is repression of T-cells in general (Baschant and Tuckermann, 2010).

1.5.4 Clinical Applications of Glucocorticoids

Since the 1940's, Gcs have been used to treat inflammatory and auto-immune diseases due to their strong anti-inflammatory effects. They are currently used to treat asthma, allergies, autoimmune diseases (including lupus and organ transplants), rheumatoid arthritis and sepsis. Gcs have divided opinion since their introduction to the clinic. Gcs such as prednisolone are used to treat asthma; it inhibits Th2 cell production of proinflammatory cytokines and vasodilators. Gcs also reduced the recruitment and activation of eosinophils within the airways, thus eliciting the protective effect

against asthma (Barnes, 1995). Using Gcs in asthma treatment is not entirely efficient. There is evidence that patients become resistant to the low dosage in a short amount of time. This means that the side effects caused by Gc medication do not always outweigh the benefits in asthma, as the concentration must be increased to retain the beneficial effects (Keatings *et al.*, 1997).

1.6 Lipid Rafts and Caveolae

Lipid rafts are small areas with altered fluidity and chemical composition of the plasma membrane. The idea developed with the identification of Triton X-100 (non-ionic detergent) resistant membranes. It was found that sphingomyelin was more difficult to solubilise with surfactants at 20°C, compared to 40°C after the phase shift (Robson and Dennis, 1979). This data was supported and enhanced when looking at detergent resistance in membranes when including cholesterol. It was noted that for resistance to detergents to occur, the lipid composition of the bilayer must have unsaturated acyl chains. Such resistant membranes were found to be in the liquid ordered state, whereby the cholesterol interdigitates with the lipid, resulting in wider membranes, due to an increase in trans-conformation of the acyl-chain, and therefore increased length of the fatty acid tails. These detergent resistant membranes have characteristics of liquid phase membranes (lateral diffusion), but show a lower temperature phase transition from liquid to gel. Therefore, sphingolipids, in conjunction with cholesterol stretch their carbon chain to the full length via these trans-conformations. This is made possible by full (or close to full) saturation, thus limiting the number of kinks in the chain formed by double bonds, and by favourable Van de Waal forces between the acyl chains and cholesterol. Cholesterol; a rather rigid molecule, especially in comparison to the acyl chains, stabilises this full extension. Due to their full extension, the lipids within this area form a membrane domain that is thicker than its surroundings (Davies *et al.*, 1990). However, the conditions used to produce such detergent resistance membranes are distinctly non-

physiological, and they may simply be an artefact of the process. Recently, the use of atomic force microscopy, which importantly is done at (close to) physiological temperatures, has enabled visualisation of discrete lipid domains. Consistent with the liquid ordered phase theory, removal of cholesterol disrupted these membrane domains. The study also showed that the inner-leaflet is extremely different to the outer-leaflet in protein architecture (Cai *et al.*, 2012). While this does directly show that membrane domains do exist, the actual lipid constituents are not characterised, and the fact that the inner-leaflet is covered with protein may be one reason for detergent resistance that has not entirely been investigated. The size of lipid rafts is another controversial issue. The atomic force microscopy measurements found the raft domains to be in the range of 100-300nm in size, but Förster resonance energy transfer (FRET) and differential scanning calorimetry (DSC) measurements have sizes in the range of 2-7nm radius (Petruzielo *et al.*, 2013). Nuclear magnetic resonance studies have put the rafts in the size order of 45-75nm (Bunge *et al.*, 2008). This large variance in results, across 2 orders of magnitude, is clearly an issue, and may be due to the different probes used, or the different raft types viewed.

The rafts contain different lipids to the surrounding membrane. They are enriched in sphingolipids and cholesterol, generally in a 3:1 stoichiometric ratio in the plasma membrane (Brown, 1998). This separation of lipids into different domains is in part due to the high affinity of cholesterol for sphingolipids in comparison to other lipids (Tsamaloukas, Szadkowska and Heerklotz, 2006). However, the exact reason for affinity is not quite known, and there is debate over the differing effects of chain length and head-group on the affinity (Nyholm *et al.*, 2010; Lönnfors *et al.*, 2011). It is most likely to be a compound effect of the head and tail groups to explain the affinity for sphingolipids over other plasma membrane lipids (Sankaram and Thompson, 1990).

Lipid rafts are hypothesised to be domains which aid in signalling by localising signalling molecules. This could be integral membrane receptors, GPI-anchored proteins or lipophilic signalling molecules. Some of the raft sizes alone are too small for this to be of any real significance, but there are two

explanatory hypotheses. One is that the membrane domains coalesce, forming larger domains for signalling. This has been demonstrated in non-cholesterol containing rafts (Rajendran *et al.*, 2003), but the action can be inferred to those containing cholesterol. Another is that activated receptors move into membrane domains, forming clusters. Both of these explain how signalling cascades can be amplified; by concentrating all the relevant parts within space thus aiding in the kinetics of the subsequent reactions and associations that are initiated. Whether the protein is stimulated by ligand and then translocates to the raft domain, or if translocation is required for ligand induced activation isn't known, but the environment is highly dynamic and thus ligand induced conformational changes are likely to alter lipid affinity, just as lipid environment is likely to alter ligand affinity by allowing energetically favourable conformational changes. Coalescing of membranes and activation dependent protein association are not mutually exclusive. It is likely that both of these may occur, and caveolae are an example of how this might happen.

Caveolae are membrane domains characterised by their physical appearance: small flask shaped invaginations directed towards the cytoplasm. Caveolae are constitutively found in almost all cells, but are most numerous in adipoyctes. They are a specific membrane domain involved in endocytosis and signalling, coated by the caveolin proteins on the inner-leaflet of the plasma membrane. For endocytotic activity, caveolae are not independent. The budding caveolae require dynamin to break from the membrane and enter the cell, similar to other protein coated pits. Caveolae are involved with the internalisation of GPI-linked proteins and external ligands or proteins. Upon endocytosis the caveolae fuse with caveosomes. They have a similar function to endosomes, but are distinct by the fact that they are mediated by caveolin proteins, have neutral pH and do not contain any endosomal markers (Pelkmans and Helenius, 2002). While caveolin proteins are the main protein components for caveolae, other proteins are responsible for cooperative association with caveolin and promote formation of caveolae: cavins. Cavin KO mice develop the same phenotype as caveolin KO mice (discussed later) and have no caveolae, thus linking the caveolae loss to the phenotype rather than

the loss of cavin or caveolin (Liu *et al.*, 2008). Cavins only associate with caveolin at the membrane. This association is aided by phosphatidylserine and cholesterol in the inner leaflet of the plasma membrane, which are commonly found in caveolae rafts. Cavins have multiple low affinity interactions with phosphatidylserine and thus it is likely that cavin and caveolin associate with raft domains to form the curvature of caveolae in a cooperative manner (Hill *et al.*, 2008).

Caveolae are trafficked via cytoskeletal attachment. Microtubules are the main attachment which allows movement of the caveolae to and from the plasma membrane, and loss of caveolae at the cell surface was caused via KO of microtubule stabilisers; β1 integrin and integrin-linked kinase. On the other hand, caveolae seem to be localised along actin stress fibres, visualised via electron microscopy and electron tomography. This potentially allows polymerisation of the fibers to organise caveolae in response to mechanical stress. This may be in part of a potential role of caveolae in sensing mechanical changes in the membrane (Parton and Del Pozo, 2013).

1.6.1 Caveolin Structure and Oligomerisation

Caveolae are dependent on cholesterol for their function, depletion of sterols in the membrane causes complete loss of caveolae. This suggests that formation of caveolae is dependent on a cholesterol binding molecule. Caveolae were initially characterised as “non-clathrin coated pits” as a way to differentiate them from other endocytotic pathways. It was clear that another protein was in use as clathrin-coated pits could be removed by high salt concentrations, whereas the caveolae remained intact, suggesting that the protein coat at least acts like an integral membrane protein. Conversely, addition of filipin and other sterol binding agents caused loss of caveolae, but not of clathrin-coated pits. They were determined to contain a separate protein, termed caveolin via antibody detection (Rothberg *et al.*, 1992).

The primary structure of caveolin-1 allows identification of the putative helical intramembrane domain. It is located between amino acids 105-125, where there is a stretch of hydrophobic amino acids (see Fig.1.5). Caveolin-1 (CAV1) differs from caveolin-2 (CAV2) and caveolin-3 (CAV3) by being

16 amino acids longer than CAV2 and 37 amino acids longer than CAV3 at the C-terminus (Fig.1.5). CAV1 can also be found in two isoforms, the α-isoform, the β-isoform which is truncated at the N-terminal compared to the α-isoform. This truncation results in amino acids 1-31 missing and thus a key target for kinases; tyrosine 14 (Fig.1.5) *(http://www.uniprot.org/uniprot/Q03135)*.

The role of CAV1 as not only a protein that is involved in endocytosis, but also in signal transduction came initially from sources of structural information.

CAV1 can form high order oligomers, causing the small 22kDa monomer to reach complexes of around 400kDa of both homooligomers and heterooligomers, which causes ordering of the lipid membrane to concentrate sphingolipids in the same area as the caveolin allowing detergent resistant membranes to form as caveolae *in vivo.* The rafts are indeed the cause of insolubility, not the CAV1 oligomers. The interaction with itself, including heterooligomerisation with other caveolins has been determined to be mediated by 41 amino acids on the N-terminal side (Monier *et al.*, 1995) of the membrane inserted region (CAV1 61-101) (Fig.1.5), termed the oligomerisation domain (OD) (Sargiacomo *et al.*, 1995). This stretch of amino acids also contains a smaller section of 20, termed the caveolin scaffolding domain (CSD), 82-101 (Fig.1.5) which specifically mediates interactions with other proteins. The terminal domain (TD) at the C-terminal of CAV1, amino acids 168-178, mediates oligomer:oligomers interactions, allowing the formation of high order structures that would be impossible if only the OD was involved in their generation. These domains can be seen in Fig.1.5 which shows their interaction with the membrane and oligomerisation.

A

```
        10         20         30         40         50
MSGGKYVDSE GHLYTVPIRE QGNIYKPNNK AMADELSEKQ VYDAHTKEID
        60         70         80         90        100
LVNRDPKHLN DDVVKIDFED VIAEPEGTHS FDGIWKASFT TFTVTKYWFY
       110        120        130        140        150
RLLSALFGIP MALIWGIYFA ILSFLHIWAV VPCIKSFLIE IQCISRVYSI
       160        170
YVHTVCDPLF EAVGKIFSNV RINLQKEI
```

B

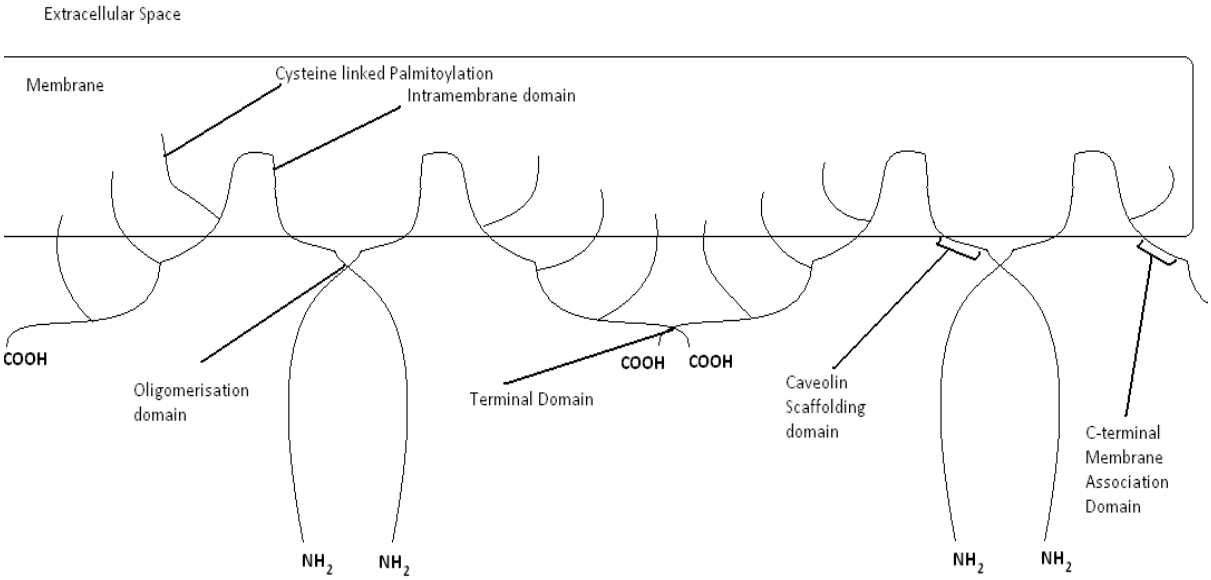

*Fig.1.5 (**A**) Human caveolin-1 primary sequence. Green is the CAV-β isoform truncation. Red is the OD, with the CSD contained within the black box. Blue is the hypothetical intramembrane domain. The yellow section represents the size difference between CAV3 and CAV1, while the black box is the TD. Orange and yellow combined represent the size difference between CAV2 and CAV1. N.B. The sequences of CAV2 and CAV3 are not the same as CAV1, therefore the highlighted sections are merely a guide to size. Adapted from (*http://www.uniprot.org/uniprot/Q03135*). (**B**) Caveolin membrane association and oligomerisation. Adapted from* (Schlegel and Lisanti, 2001).

1.6.2 Caveolin Membrane Association

The binding of caveolin to cholesterol is very stable, and occurs at around a 1:1.4 stoichiometric ratio. This odd ratio may have been due to interaction with cholesterol and the detergent used to remove caveolin (Murata *et al.*, 1995). CAV1 binds cholesterol via amino acids 94-101 which are located in the CSD. There is evidence that a CSD peptide can attach to the membrane without the intramembrane domain (Schlegel and Lisanti, 2001), which may be due to its cholesterol binding properties. The intramembrane domain contains strong α-helical structure in a hairpin formation, broken in the centre by proline 110 to form the loop, loss of this proline causes CAV1 to take on an transmembrane helical conformation (Aoki *et al.*, 2010). However, the CSD forms a β-sheet which runs parallel to the membrane, causing the whole 82-134 run to form a "wedge" shape, which may be important for causing membrane curvature in caveolae (Hoop *et al.*, 2012). The membrane association is aided by three cysteine residues 133, 143 and 156 (*http://www.uniprot.org/uniprot/Q03135*) which have post-translational modifications with palmitoyl groups. These are not needed for membrane attachment, but certainly aid in it.

1.6.3 Caveolin-protein Interactions

CAV1 interactions with other proteins are mediated by the CSD. Using phage display libraries, it was found that only the CSD binds strongly to peptides, of which contain large hydrophobic residues in two consensus sequences: (ϕ*X*ϕ*XXXX*ϕ) or (ϕ*XXXX*ϕ*XX*ϕ), which can also be found in a composite form. Some *in silico* evidence exists to explain how the CSD binds the consensus sequences. CAV1 is commonly phosphorylated in Y14, which is thought to aid the CSD association with other proteins. The phosphorylation disrupts a network between Y14, H12 and E20, forcing E20 to move away from the newly dense negative charge, and R19 to move towards the phosphate. This causes the N-term to become more compact, thus providing space for the CSD to rearrange to promote interactions with other proteins (Shajahan *et al.*, 2012). The sequences that bind CAV1, were not the same as those that bind CAV2, indicating the ability of the two isoforms to co-localise different molecules.

CAV1 and CAV3 do have similar binding sequence properties. This can be explained by their different expression patterns, which will be discussed later.

1.6.4 Caveolin Localisation

Around 90% of CAV1 is located in the plasma membrane and if expressed in knockout cells, caveolae will spontaneously form. Caveolae do not form on the plasma membrane surface, but instead, precursors form in the trans-Golgi (see Fig.1.6). These move as small vesicles, which then fuse to the plasma membrane generating caveolae. This movement is mediated by CAV1 and CAV2 association at the trans-Golgi where the precursors originate from (Pelkmans and Zerial, 2005). Once at the plasma membrane, association with the cytoskeleton prevents much lateral diffusion. As mentioned previously, over expression of CAV1 can cause aberrant localisation. This is thought to be due to a non-physiological ratio of caveolin to cavins. When cavin expression is reduced (or CAV1 is over expressed) caveolae lose their characteristic morphology, and CAV1 is lost to the lysosomal degradation pathway (Hill *et al.*, 2008).

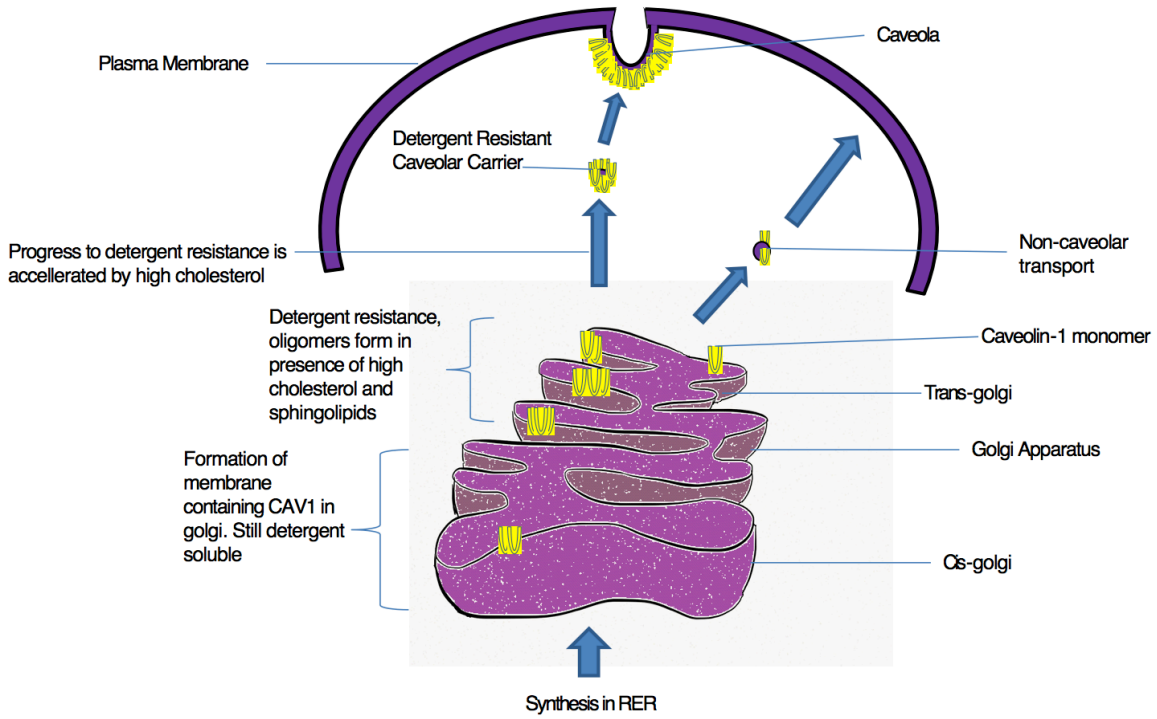

Fig.1.6 Mechanism of CAV1 localisation to the plasma membrane. After synthesis in the RER, CAV1 monomers start to form oligomers in the golgi apparatus. During progression from the cis to the trans-golgi, which contains more cholesterol and sphingolipids, these oligomers grow larger until they bud as vesicles and are targeted to the membrane. Some CAV1 monomers or small oligomers do not go via this method, but are still targeted to the plasma membrane (Parton and Simons, 2007).

1.6.5 Caveolin Physiology: Endocytosis

Due to the caveolae morphology, the most obvious physiological role of caveolin is in endocytosis. However, caveolae seem to be rather stable structures that will remain on the surface of the cell, and have very little capacity to enter the cell via endocytosis. Using GFP-labelling and FRAP (fluorescence recovery after photobleaching), it was shown that the labelled caveolin doesn't move from caveolae. This indicates that they are relatively stable and not involved in a rapid endocytosis. The same study also showed that this stability can be removed by cholesterol depletion (therefore loss of caveolin) and by inhibition of actin polymerisation (therefore loss of cytoskeletal attachment) (Thomsen *et al.*, 2002). Contrary to the idea of stationary caveolae, experimentation using GFP-linked CAV1 and live cell imaging, viewing fluorescence intensity, it was possible to see that

individual “quanta” of caveolae would move to the membrane, and then remain in the same location upon docking, rather than diffusion of fluorescence intensity. This was further backed up by pH quenching of FITC (Fluorescein isothiocyanate) conjugated Cholera toxin. In order to show that the caveolae were indeed docking and fusing with the membrane, the extracellular space was acidified, to quench the FITC signal. When a caveola containing FITC-Cholera toxin fused with the plasma membrane, the fluorescence signal disappeared due to exposure to low pH. Interestingly, there was a delay of quenching by 1 second, suggesting that the caveolae vesicles dock, then fuse. When vesicles move caveosomes which are neutral pH, reversal of the quenching could be seen indicating that the caveolae not only traffic back and forth, but also may be picking up the FITC-Cholera toxin and transporting it with them (Pelkmans and Zerial, 2005).

Linked to endocytosis, CAV1 function has been linked to transcytosis, specifically in endothelia. CAV1 KO mice have a compensatory mechanism for transcytosis in endothelia, leading to the hyperpermeable microvasculature. Rather than using CAV1 as an endocytotic mediator to achieve transcytosis, there was a drastic increase in paracytosis, which is an important part of the inflammatory process. This was shown by substantially faster radio-labelled BSA clearance from the blood. The endothelia viewed by EM showed morphologically smaller tight junctions, and in lung endothelia, there was even some detachment from the basement membrane. Both of these differences between the WT and the CAV1 KO mice can explain the hyperpermeable phenotype. Endothelial nitric oxide synthase (eNOS) inhibitors managed to reduce this hyperpermeability of the microvasculature to similar levels as the WT mice, whilst also not altering the WT mice permeability, suggesting that nitric oxide (NO) is involved in the mechanism (Schubert *et al.*, 2002). CAV1 negatively controls the TNF-α stimulation of tight barrier permeabilisation, thus implicating CAV1 in an anti-inflammatory role. This is done via inhibition of Rac1, a G-protein α-subunit (Shao *et al.*, 2013).

1.6.6 Caveolin Physiology: Endothelial Nitric Oxide Synthase

eNOS is indeed localised within caveolae, where CAV1 (and even the CSD alone) acts as a negative regulator for eNOS by holding eNOS in an inactive conformation. This inhibition is abolished by Ca^{2+}-calmodulin. The interaction of CAV1 and eNOS is modulated by phosphorylation of both CAV1 and eNOS. Src phosphorylates CAV1 on tyrosine-14 and therefore using phosphomimicking mutants (Y14D), it was possible to show that CAV1 phosphorylation is an important step in mediating eNOS inhibition (Chen *et al.*, 2012). The CAV1 association with eNOS is important in an inflammatory context too. As mentioned above, eNOS produced NO is involved with vasculature permeability, vasodilation, preventing apoptosis of endothelia, and thrombosis. Apolipoprotein E (apoE) expressed on macrophages can be internalised by endothelia. ApoE then competes for the CSD with eNOS, thus displacing it and preventing the inhibition of eNOS. Therefore, macrophages potentiate the inflammatory signalling at their site of action by interfering with CAV1, causing eNOS activation. Cell fractionation gave evidence that in fact ApoE may be causing CAV1 to lose its membrane attachment as it was found in the high density fraction, although this is not conclusive (Yue *et al.*, 2012). Contrary to this, C-reactive protein (CRP) which is stimulated by macrophage interleukin-6 release during acute phase infection has an inhibitory effect on eNOS. This is mediated via CRP binding FCγRIIB receptor, which causes activation of a phosphatase pathway, resulting in reduction of eNOS phosphorylation, thus activation and gene expression. The FCγRIIB receptor is localised to caveolae in B-cells, thus allowing for signal amplification due to colocalisation with eNOS (Tanigaki *et al.*, 2009).

Interestingly, in CAV1 KO mice, stromal cells, such as fibroblasts, exhibit signs of hypoxia. This is partly due to constitutive activation of nitric oxide synthase, resulting in more NO within the cell, and also due to an increase in reactive oxygen species (ROS) production causing inhibition of mitochondrial oxidative phosphorylation enzymes. The inhibition occurs via nitrosylation of tyrosine residues of both Complex I and Complex IV by free NO, inactivating them. The reduction in viable mitochondria drives the cells to use glycolysis as their main means of energy production, and

explains why glycolysis inhibition is lethal to both CAV1 KO stromal cell lines, and whole mice. Furthermore, the KO results in hypoxia-inducible factor (HIF) and NFκB activation, thus increasing glycolysis related enzyme expression as a compensatory factor for losing active mitochondria as well as inflammatory response. ROS cause DNA damage, which causes the induction of Poly (ADP-ribose) polymerase (PARP). PARP acts as cofactor for NFκB, to promote inflammation. It also is a cofactor for other Gc modulated transcription factors such as AP-1. This activation of HIF and NFκB, coupled with production of ROS and NO is linked to the fibrotic phenotype found in both the CAV1 KO mice, and pulmonary fibrosis (Pavlides *et al.*, 2010). In WT mice, oxidative stress up-regulates CAV1 via the MAPK pathway. The up-regulation is instrumental in causing senescence in cells (Dasari *et al.*, 2006).

1.6.7 Caveolin Physiology: The Lung

CAV1 is important in lung development. In mouse models it is found at E10 and onwards in the lung, but only in developing blood vessels, not in epithelia. In adult lung, CAV1 is found in epithelial and endothelial type I cells. This change in expression is only seen postnatally. By specifically detecting CAV1α and CAV1β, it's been established that CAV1α expression and new vasculogeneis occur concomitantly. This seems contradictory to CAV1's role in the anti-proliferative and tumour suppressor pathways, as the new endothelia must reproduce heavily during development. Although, in CAV1 KO mice, the epithelia in alveolar walls proliferate more, resulting in thicker walls, suggesting that CAV1 restricts proliferation here, but less so in endothelia. The expression profile of CAV1 in development highlights how cell type specific production of CAV1 is important in CAV1 physiology (Ramirez *et al.*, 2002).

Systemic LPS challenge in CAV1 KO mice, results in a decrease in sequestration of neutrophils to the lungs suggesting that CAV1 KO have decreased response to LPS challenge. The protective effect against inflammation was ascribed to eNOS. With constitutive activation of eNOS due to no CAV1 repression, the increased NO signalling can cause a negative feedback on NFκB. This was backed up by non-selective NOS inhibitors bringing the inflammatory response to the same level as the WT

mice (Garrean *et al.*, 2006). Although a systemic LPS model comes with inherent limitations, when a systemic infection of Salmonella was analysed in CAV1 KO mice, the opposite was noted. An increase in the inflammatory effects, increase in cytokine production and neutrophilia. Despite the increase in the immune and inflammatory response, the CAV1 KO mice were found to be very susceptible to infection, with a higher rate of mortality compared to WT mice (Medina *et al.*, 2006). Using exogenous CAV1 KO neutrophils, Hu et al. demonstrated that they had a weaker response to fMLP, resulting in less movement and less ROS production. The decrease in observed ROS production in neutrophils was proposed to be a direct result of losing CAV1. The authors propose that without CAV1 to act as a scaffold for the NADPH oxidase subunits, the subunits are not localised to the plasma membrane and thus not activated. These data both implicate that CAV1 is involved in pro-inflammatory signalling, and that disruption of lipid rafts limits TNF-α signalling is consistent with this (Hu *et al.*, 2008). However, there also seems to be some contradictory evidence. For example, administering the CSD has been shown to inhibit microvascular permeability and leukocyte adhesion *in vivo* indicating that CAV1 has an anti-inflammatory effect (Bucci *et al.*, 2000). In CAV1 siRNA KD macrophages, LPS stimulation also increased the cytokine production and inflammatory response. Importantly, the macrophage cell line was derived from alveolar macrophages (Wang, Kim, *et al.*, 2006). Another example is that CAV1 KD enhances ROS production in bovine endothelial cells (Gonzalez *et al.*, 2004) and mouse fibroblasts (Grande-García *et al.*, 2007) seemingly inconsistent with the results in neutrophils. Toll-like Receptor 4 (TLR-4), which is involved in LPS detection in macrophages has been shown to localise to caveolae, and directly interacts with CAV1 via a CSD binding motif thus down-regulating TLR-4 (Wang *et al.*, 2009). Whether these are examples of cell type specific actions of CAV1 or the differing methodologies generated different effects is unknown.

1.6.8 Caveolin Physiology: Fibrosis

Idopathic pulmonary fibrosis (IPF) is a chronic, degenerative disease whereby the lungs become filled with scar tissue, ultimately leading to death. It has very little in the way of treatment, other than

lung transplantation, and the mortality rate of those diagnosed with IPF is extremely high (Schwartz *et al.*, 1994). It is common after infection, resulting in excessive differentiation, proliferation and activation of fibroblasts. The differentiated form; myofibroblasts are a diagnostic marker of IPF, although the signalling events that cause differentiation to the ECM secreting myofibroblasts are not fully understood. Damaged alveolar epithelial cells release cytokines such as TNF-α and TGF-β which can attract fibroblasts to the area of damage where they proceed to differentiate and produce ECM proteins. There is also evidence that the alveolar type II epithelial cells may also differentiate into myofibroblasts directly at the site of damage (Moore and Herzog, 2013). TGF-β is a potent mediator of alveolar epithelial-mesenchymal transition, i.e. the loss of epithelial cell characteristics and localisation to form cells resident in connective tissue. TGF-β1 can be activated by cytoskeletal changes caused by epithelial damage, and importantly, TGF-β1 signalling has been shown to localise to caveolae.

CAV1 inhibits TGF-β receptor phosphorylation of Smad2 and therefore prevents the signal transduction (Razani *et al.*, 2001) as well as inhibiting the nuclear translocation of Smad3, but having no effect on Smad4 or 7 (Yang *et al.*, 2013)Oxidative stress is another important factor in IPF which is also regulated by CAV1. The inflammatory response causes ROS production and (through TGF-β signalling) a down regulation of glutathione (an anti-oxidant) production thus driving the fibrosis (Margaritopoulos *et al.*, 2013). There is indeed a correlation between ECM protein deposition and TGF-β signalling, as seen in CAV1 KO mice. These mutant mice have an accelerated lung deterioration which is normally associated with age, with an increase in collagen and elastin deposition causing decreased lung compliance. Importantly, the deposition of ECM correlated with TGF-β signalling spikes, indicating that it was likely the source of the fibrosis seen in the CAV1 KO lung tissue (Le Saux *et al.*, 2008) TGF-β has also been demonstrated to decrease CAV1 expression levels in a dose dependent manner, adding even more evidence to the role of CAV1 and TGF-β signalling in fibrosis. In both humans and rats with IPF, there is a decrease found in the CAV1 expression levels and similar protein levels within the lung, including both alveolar epithelia and

myofibroblasts. Using a viral vector with the CAV1 gene, it was possible to protect mice against bleomycin induced fibrosis; with less fibrotic injury and lower levels of inflammation in the group treated with the CAV1 vector. This was reproduced *in vitro* where CAV1 overexpression elicited a protective effect against TGF-β stimulation, resulting in lower ECM deposition. The signalling pathways implicated in TGF-β signalling negatively controlled by CAV1 are the MAPKs ERK (involved in collagen production and deposition) and JNK (involved in fibronectin production and deposition), but not p38 (Wang, Zhang, *et al.*, 2006). CAV1 expression is also lower in leukocytes in IPF patients, mirroring the phenotype seen in the fibroblasts, epithelia and endothelia. This phenotype is also generated from healthy leukocytes by incubation with TGF-β or TNF-α. Exogenous addition of the CSD limits the migration of neutrophils and macrophages to the lungs in bleomycin induced IPF, showing CAV1's anti-inflammatory ability. The CSD also inhibited MAPK activation through ERK, JNK and p38 in leukocytes, limiting the fibrotic phenotype, in a similar way to the effects of the CSD in epithelial cells (Tourkina *et al.*, 2010). The fibrotic phenotype (mimicked by TGF-β and TNF-α) also results in the over-expression of CXCR4 and an increased response of monocytes to CXCL12 chemotaxis which again, is blocked by the CSD, preventing the collagen producing monocytes from migrating to the lung. This seems to be protective against both fibrosis, but also inflammation (Tourkina *et al.*, 2010).

1.6.9 Caveolin and Kinases

While caveolae are implicated in clustering of signalling, it is important to note that caveolar dynamics and structure are controlled by kinases too. Out of 6 identified, they all seem to have rather different effects on caveolin. ARAF1 has a stabilising effect on caveolin oligomers. MGc26597 and src interference resulted in larger caveolae, indicating that these tyrosine kinases play a role in limiting the association of caveolin oligomers. Loss of KIAA0999 and MAP3K2 activity results in decreased caveolae movement. This suggests that they are responsible for control of the machinery required to pinch in the caveolae for endocytosis. Another MAPK related kinase DYRK3 had the

opposite effect to the previous two serine/threonine kinases, in that when removed, causes the caveolae to pinch and dock more frequently, suggesting that DYRK3 is involved in stabilising the caveolin oligomers at the cell surface (Pelkmans and Zerial, 2005). As previously mentioned, CAV1 has been implicated in control of MAPK pathways, by inhibition of ERK, JNK and p38. Much of the data generated on this is via comparing CAV1 KO or CAV1 KD cells with WT cells treated with selective MAPK inhibitors.

The ERK1/2 MAPK pathway is important in cell differentiation and growth factor stimulation; therefore it is relevant in the context of CAV1 control over fibrotic diseases and proliferative pathways. ERK is localised within caveolae membranes and upon activation, it dissociates and initiates the p42/44 MAPK cascade. This is supported by the observation that down regulation of CAV1 results in constitutively activated ERK *in vivo*, therefore it is likely that CAV1 holds ERK in the inactive conformation by interaction with the CSD, relying on receptor activation to force dissociation from CAV1, allowing interaction with the rest of the cascade. This p42/44 pathway also has a regulatory effect on CAV1 expression, suggesting a positive feedback mechanism, whereby activation of the pathway limits CAV1 expression, resulting in stronger activation, or priming for more signal, but is normally negatively regulated by CAV1 (Engelman *et al.*, 1998).

In alveolar macrophages, CAV1 KD has been shown to increase proinflammatory cytokines TNF-α and IL-6 and decrease the anti-inflammatory cytokine IL-10 upon LPS stimulation. Over expression in the same cell type has the directly opposite effects. This is mediated by the p38 MAPK pathway, with increasing CAV1 expression, increasing p38 phosphorylation. This seems to modulate the NFκB and AP-1 signals that drive production of the regulated cytokines. CAV1 over expression causes a decrease in both NFκB and AP-1 binding. When a p38 inhibitor was included with CAV1 over expression, this inhibition of transcription factor binding was abolished thus implicating p38 and CAV1 in anti-inflammatory roles in immune cells. Interestingly, in the LPS stimulation model of alveolar macrophages, ERK and JNK phosphorylation was decreased, while the p38 was increased contrary to the accepted view that CAV1 is a negative regulatory element (Wang, Kim, *et al.*, 2006).

The p38 MAPK pathway activation during cellular stress results in phosphorylation of CAV1 on tyrosine 14. While p38 is an S/T kinase, it can activate c-Src as a downstream Y kinase, which results in the CAV1 phosphorylation on Y14. The phosphorylation event is important for recruiting signal transduction molecules such Grb2 and SOS1 again showing CAV1 as a scaffolding protein for multiple protein-protein interactions within a signalling pathway. This stress induced pathway of p38 activation leading to CAV1 phosphorylation can be decoupled by limiting cellular cholesterol, and thus losing caveolin based lipid rafts, therefore highlighting their importance in mediating the signal transduction possibly by concentrating the pathway intermediates into the small area of a caveola (Volonte *et al.*, 2001).

The internal anti-proliferative pathway, initiated by BCL2 and BCLxL on mitochondrial outer-membranes is also controlled by CAV1. Phosphorylation of Y14 by tyrosine kinases such as Src, Abl and Fyn promotes the association of CAV1 with BCL2 and BCLxL, which is not observed in the CAV1-β isoform that lacks Y14. This association promotes the phosphorylation of both BCL2 and BCLxL thus initiating the anti-proliferative pathway when stimulated with the apoptosis inducer paclitaxel. Immunoprecipitation of phosphorylated CAV1 and phosphorylated JNK gave some initial evidence that JNK is associating with CAV1 phospho-Y14, and when coupled with specific inhibition of JNK, it was possible to show that the JNK pathway is involved in phosphorylation and therefore activation of BCL2 and BCLxL, which is promoted by CAV1 phospho-Y14. This is an example of how CAV1 can scaffold signalling molecules at locations other than the plasma membrane (Shajahan *et al.*, 2012).

1.6.10 Caveolin and the Glucocorticoid Receptor

Initially, the only evidence that GR could be complexed with CAV1 came by extrapolating data from ER which was shown to be associated with CAV1 at the membrane. Later, GR in complex with its chaperone; HSP90 was found in detergent resistant membrane fractions along with CAV1 and STAT3. DEX administration increased the amount of GR found in the detergent resistant membranes, suggesting a link between activation and localisation. Whether DEX induces GR to associate with

rafts or whether ligand stimulated GR dissociates from rafts and is replaced by free cytosolic GR is unknown, however the latter is favoured (Jain *et al.*, 2005). There is some contradictory evidence; using fluorescence microscopy techniques, it was shown that CAV1 and mGR do not co-localise in caveolae (Spies *et al.*, 2006). This experiment depended on the over-expression of CAV1 with a fluorescent tag, which can be localised to different compartments due to a difference in CAV1:Cavin ratios (Parton and Del Pozo, 2013), which may explain the contradictory findings, especially as the cellular specific location of CAV1 was not established, only whether it was colocalised with mGR. Filipin III disruption of membrane rafts by cholesterol depletion (Bavari *et al.*, 2002) causes a reversible inhibition of mGR signalling assessed using a luciferase reporter gene with a GRE. Similar results were found using progesterone as a raft disruptor. DEX administration causes phosphorylation of CAV1 which allows the CSD to interact with many proteins. This suggests that both CAV1 and GR modulate each other's signalling events. Selective inhibition showed that this phosphorylation was dependent on both GR and PI3K, with the tyrosine kinase responsible for the phosphorylation likely to be c-src. Using the CSD as a dominant negative it was possible to show that there was less phosphorylation of CAV. It also inhibited Gc induced phosphorylation of PKB. The CSD was able to stop TNF-α reporter gene activation, suggesting that CAV1 phosphorylation is required for TNF-α signal transduction events. However, it did not prevent activation of a similar reporter gene containing a GRE. Similarly in a CAV1 KD model, TNF-α signalling was abrogated, but GR transactivation was unaffected using the same reporter genes. These data indicate that CAV1 is not essential for GR translocation (Matthews *et al.*, 2008).

The non-genomic pathways of Gcs are controlled by CAV1. Gcs have potent anti-proliferative effects; however, this ability is lost in CAV1 KD or KO cells. Due to the fact that CAV1 does not affect the transactivation potential of GR, the observation that loss of CAV1 stops the anti-proliferative signalling can be attributed to CAV1 control over the non-genomic effects of the GR. The transrepression was not analysed, therefore it is possible that CAV1 may affect these properties of GR too (Matthews *et al.*, 2008).

High throughput proteomic analysis has aided in the understanding of how CAV1 interacts with mGR. Using BSA conjugated Gcs it is possible to distinguish the mGR effects from the cGR effects. The BSA moiety prevents the steroid crossing the plasma membrane and therefore is only accessible by the mGR. Proteomics analysis found that mGR and CAV1 are associated. When using the 5E4 anti-GR antibody to validate the result via *in situ* proximity ligation assay (PLA) no GR was detected, however mGR was detected using the M20 antibody. This was explained by the previous observation that 5E4 recognises GR by the AF-1 domain, which is the proposed site of mGR-CAV1 association (Vernocchi *et al.*, 2013). KD of CAV1 resulted in a 70% loss in CAV1-mGR dimers and a reduction in membrane localised GR. Interestingly CAV1 can control the outcome of mGR stimulation. In cells expressing CAV1, stimulation with BSA conjugated cortisol results in down regulation of HADH and CoxVb genes, two genes that are both classically regulated by GR. However upon CAV1 KD, the cortisol-BSA stimulation resulted in an up regulation of CoxVb, and no change for HADH. When the same cortisol-BSA assays were performed in CCRF-EFM cells and Jurkat cells, which do not express CAV1 unlike the U2-0S cells used before, the GR was still able to translocate to affect transcription. This suggests that CAV1 is not needed for GR nuclear translocation, unlike the ER (Vernocchi *et al.*, 2013).

GR (and MR, but not the sex steroids) agonists have been shown to increase the expression of CAV1, and the total protein content in vascular cell lines (Igarashi *et al.*, 2013). The expression increase is reversibly stopped by GR inhibitors. There is indeed a GRE 1526bp upstream of the CAV1 promoter which is implicated in transactivation of CAV1. This increase in expression was also reproduced in lung epithelial cells, however because the whole organ lysates did not show a change in mRNA or protein levels after 48hours, it is unlikely that DEX affects CAV1 concentration in non-endothelial cells, at least according to these data.

When looking at VEGFR signalling, the DEX induced CAV1 up regulation caused attenuation of the phosphorylation events at VEGFR2, Akt, ERK1/2 and eNOS. This was abolished by using either siRNA

for CAV1, or by using a GR antagonist. The role of GR activation in inhibiting the VEGF signal is by increasing the amount of CAV1, as fractionation showed that CAV1 was localised to the membrane whether the cells were stimulated with DEX or not. This could be a physiological control of angiogenesis by Gcs. Interestingly; CAV1 up-regulation by Gcs can explain some of the anti-inflammatory effects by increasing the CAV1 mediated inhibition of eNOS. The same study also implicated the same pathway induced by DEX and modulated by CAV1 in limiting microtubule formation (Igarashi *et al.*, 2013).

1.7.1 *In vivo* Models of Inflammation Sensitive to Glucocorticoids: Aerosolised Lipopolysaccharide Challenge

TLR4 is the major receptor involved in the response to its ligand LPS. It is highly expressed in immune cells; macrophages, dendritic cells and B cells. LPS is a marker of Gram negative bacteria (Strieter *et al.*, 2002). It is also expressed on epithelial cells. Bronchial epithelial cells are the first to come into contact with aerosolised LPS. Upon TLR4 activation, MyD88 is recruited, and through a series of MAP kinases, the signal is propagated to activate NFkB and AP-1 transcription factors resulting in the induction of pro inflammatory cytokines (Akira and Takeda, 2004). Gc repress both of these transcription factors.

The inhaled LPS mimics the pathology of ALI (acute lung injury), and is a way to separate the adaptive immune response from the innate immune mechanisms thanks to it being a sterile challenge, i.e. there is no microorganism in the challenge, only the TLR4 ligand, LPS.

The challenge causes high levels of pulmonary neutrophilia, and chemokines associated with neutrophil movement in the BALF. These neutrophils can be found throughout the lung, at the bronchioles and in alveolar spaces (Roos *et al.*, 2014).

As the LPS is inhaled, it is not absorbed into the blood stream, which minimizes systemic effects. Systemic LPS can lead to complications, and cause death, however the localisation of the challenge to the lung means that less Gc release as the HPA axis will not be highly activated. The release of Gc;

corticosterone in mice, results in immune suppression and therefore will mask the effects of any exogenous Gc treatment.

TLR4 activation can propagate via two independent pathways, the MyD88 pathway, or the MyD88 independent pathway. The MyD88 pathway signals through the adaptor proteins IRAK-4, IRAK-1 and TRAF6. IRAK-4 KO macrophages and mice show defects in LPS stimulation and are resistant to sepsis (Suzuki *et al.*, 2002), while IRAK-1 KO macrophages show a less severe phenotype (Swantek *et al.*, 2000). The cascade then reaches TAK-1 (TGFb-associated kinase-1), a MAPK (MAP3K8). TAK-1's role in the inflammatory signalling is complex, KD *in vivo* results, paradoxically, in a pro-inflammatory phenotype, (Vink *et al.*, 2013) however, a full KO, results in poor inflammatory signalling, with a lack of activation of JNK and IKKB, but not IKKa (Shim *et al.*, 2005). Nevertheless, TAK-1 activation results in phosphorylation of IKK, therefore degradation of IKB, and activation of NFkB. Similarly, TAK-1 signals through MAPKs (JNK, p38) to activate AP-1(Lu, Yeh and Ohashi, 2008).

The MyD88 independent pathway is less well studied, and results in another transcription factor being recruited as well as NFkB and AP-1: IRF3 (Interferon regulatory factor). This pathway is involved more in an anti-microbial/anti-viral response than the MyD88 pathway (Bowie and Haga, 2005).

1.7.2 *In vivo* Models of Inflammation Sensitive to Glucocorticoids: Ovalbumin Sensitisation

Ovalbumin is used as a model for antigen induced asthma. The type of airway inflammation induced is dependent on the adjuvant, ovalbumin plus Alum (aluminium hydroxide) results in a Th2 type response, with increased airway eosonophilia, in comparison to the CFA (Complete Freund's Adjuvant) which consists of inactivated mycobacteria, and generates a TNF-a dependent Th1 response, with high neutrophilia, which is resistant to Gc suppression (Dejager *et al.*, 2015).

The procedure can vary, with no treatment regimen being accepted as the de facto way to induce asthma pathology. However, all procedures follow a similar pattern of intraperitoneal or

subcutaneous injection of ovalbumin with an adjuvant over 2+ weeks to induce allergy and T-cell proliferation, followed by an inhaled challenge of dissolved ovalbumin to induce airway inflammation, cellular infiltrate and hyperresponsiveness. The ovalbumin model does not depend on mast cells, as mice lacking mast cells are indistinguishable from littermate controls in terms of response to ovalbumin, despite being implicated in many allergic diseases (Takeda *et al.*, 1997). The mouse allergic response displays some similarity to human asthma, as previously mentioned - eosoniphilia, Th2 response, airway remodelling, and a reversible inflammatory response (Kumar, Herbert and Kasper, 2004).

The ovalbumin model does has its limitations; it is not a perfect model of human asthma. This is demonstrated by there being poor, or no late phase response of airway hyperresponsiveness, despite large numbers of cells found at this time point, in mice. While a late phase response is characteristic of asthma in humans (Zosky *et al.*, 2008).

CavKO mice are reported to show an increase in airway responsiveness at baseline, and after ovalbumin challenge compared to WT mice. CavKO mice also showed an increase in cellular infiltrate, increase in TNFα receptor and IL4 receptor in comparison to WT, both at baseline and when treated with ovalbumin. Interestingly, WT mice show an increase in cav1 protein, and cavin proteins, suggesting that cav1 has a protective role in airway hyperresponsivity. The authors attribute this to increased ariginase activity, resulting in decreased NO and therefore increased airway tone. They also highlight the increased collagen deposition found in CavKO mice which contributes to the observed phenotype (Aravamudan *et al.*, 2012).

1.7.3 Molecular Mechanisms of Resistance to Glucocorticoid Treatment During Inflammation

At the most basic level, Gc resistance occurs by a down-regulation of GR expression caused by administration of GR agonists. The down regulation becomes apparent rather quickly, often coming into effect within 24 hours. This physiological effect of Gcs is exacerbated by chronic administration.

A meta-analysis of many different data has suggested that the GR has around 50% expression reduction upon addition of agonist (Schaaf and Cidlowski, 2002), although, all of the data were obtained in various cell-lines, so how this affects the HPA-axis isn't defined. The mechanism for the down-regulation of GR by agonists is not well characterised. There is evidence that it is controlled at the transcriptional phase (Rosewicz *et al.*, 1988) whereby there is a direct down-regulation of GR mRNA, which is correlated to the decrease in GR protein. However, there is counter evidence to this, as mRNA half-life was unaffected by DEX treatment, indicating that there is post-transcriptional control on GR production (Dong *et al.*, 1988). Therefore, it's expected to involve more than one mechanism (Schaaf and Cidlowski, 2002). Degradation of GR via the proteosome contributes to the down-regulation of GR. Ligand binding causes hyperphosphorylation of GR, which has been implicated in the stability of GR (Webster *et al.*, 1997). This phosphorylation mediates ubiquitination of the GR at K426, located in the PEST sequence (Wallace and Cidlowski, 2001). This proteosomal ligand-dependent degradation was confirmed by the use of proteosome inhibitors and mutation of the ubiquitination site (K426A); resulting in a loss of ligand induced GR down-regulation (Wallace and Cidlowski, 2001).

The alternate isoforms GRβ (Bamberger *et al.*, 1995; Oakley *et al.*, 1997; Taniguchi *et al.*, 2010), GRγ (Ray *et al.*, 1996; Taniguchi *et al.*, 2010), GR-P and GR-A (Moalli *et al.*, 1993) (discussed in more detail eailer) have been implicated in Gc resistance, in the case of GRβ and GRγ via a dominant negative action. The mechanisms of the dominant negative effects produced by these isoforms are not well characterised. One hypothesis states it may be mediated by formation of transcriptionally inactive heterodimers between GRα and the other isoforms (Oakley *et al.*, 1997; Strickland *et al.*, 2001). Opposing hypotheses exist, such as that GRβ may compete for co-activators with GRα, thus reducing the transactivation effects (Yudt *et al.*, 2003; Taniguchi *et al.*, 2010).

NFκB is another factor involved in Gc resistance, due to it having a repressive effect on GR activity via interaction of GR with the p65 subunit found in NFκB proteins (Caldenhoven *et al.*, 1995; McKay and Cidlowski, 1998). This inhibition of the GR via the p65 subunit is mirrored by other NRs, like ER

(Galien, Evans and Garcia, 1996). Translocation, transactivation and DNA binding of NFκB is not required for the mechanism of mutual inhibition, as shown by removal of these individual domains (Doucas *et al.*, 2000), however the same research showed that it does require PKA (protein kinase A) phosphorylation of NFκB. This suggests that NFκB is unable to inhibit GR when it is not activated and held in an inactive conformation by IκB. This was backed up by further research which implicates that IκB must be removed to allow the mutual inhibition of GR and NFκB (Zhong *et al.*, 1997). However, there is evidence to suggest the opposing view; that GR, NFκB and IκB complex together in both the nucleus and cytoplasm via the p65 and p50 subunits. Importantly this was shown to occur without stimulation from Gcs or TNFα (tumor necrosis factor α), indicating that it occurs constitutively within the cell. The same study found that TNFα increases GR association with HSP90 (90kDa heat shock protein), showing a cross-talk between the pathways of inflammation and anti-inflammation (Widen, Gustafsson and Wikstrom, 2003). Therefore, problematically, inflammation, the pathology that Gcs are used to treat can cause resistance to the drug. This means that treatment requires careful consideration into dose. It must be able to both be enough to over-come the effects of resistance, but as many have recommended; the lowest dose possible to limit side effects.

1.8 Circadian Rhythms

Circadian rhythms are defined as self-sustaining oscillations that occur over 24 hour periods. These oscillations can be found throughout evolution, and in mammals are controlled by light signalling, input into the brain to the master pace maker the suprachiasmatic nucleus (SCN) located in the hypothalamus. The SCN is entrained by light, that is, it will synchronise its 24 hour oscillations with light in the environment, however, in constant darkness, it will continue to maintain a 24 hour rhythm. Surgical lesioning of the SCN causes loss of circadian behaviour (Ralph *et al.*, 1990). This ensures that the physiology of the organism is matched to the rhythmic environment. Light is not the only time information, different organs respond strongly to other signals, for example the liver can be entrained to feeding signals, causing mice to phase shift under restricted feeding regimens,

where food is only available in the usual inactive phase. However, this does not fully switch their clock, as evidenced by their plasma corticosterone levels. Corticosterone normally peaks directly before the active phase, but reverse phase fed mice display a biphasic peak, where the corticosterone peaks before feeding, but then also again before their usual active phase (Le Minh *et al.*, 2001). The clocks in tissues other than the SCN are termed *peripheral clocks*. They operate independently to the SCN, but are synchronized by it. This can be demonstrated by the fact that tissues removed from the body, are able to oscillate independently for days, with no time signal.

Circadian rhythms control many aspects of physiology, including inflammation and metabolism. Glucose homeostasis is tightly controlled, and hormones involved in it display rhythmicity. Insulin, glucagon and Gcs are all secreted rhythmically (Boden *et al.*, 1996; Ruiter *et al.*, 2003). SCN lesioned mice lose any rhythmicity in plasma glucose concentrations, demonstrating the importance of the daily rhythm in controlling these fluctuations. Interestingly, the glucose concentration was unaffected by scheduled or *ad libitum* (at will) feeding, with glucose peaking at the onset of the animal's active phase, showing that food timing does not affect plasma glucose, but daily rhythms do (La Fleur *et al.*, 1999). Lipid metabolism is also controlled in a circadian manner, with many genes being directly regulated by circadian transcription factors. Circulating lipids and cholesterol are higher in the active phase of rats, and this is likely controlled by changes in lipoproteins such as apolipoprotein B, which is higher at the end of the active phase (Pan and Hussain, 2007).

Energy production seems to be one of the key aspects of biology that circadian rhythms control. Mitochondrial oxidative function is modulated by the clock. This is through daily cycles of NAD^+ which are the ligand for the NAD^+ dependent deacetylase SIRT3. Due to the cycling levels of the co-factor, NAD^+, SIRT3 deacetylation of the mitochondrial proteins and therefore the inactivation of said proteins also cycles. In mutant mice deficient in circadian rhythms (BMAL1 knockout mice), the SIRT3 activity was reduced thanks to an overall reduction in NAD^+ suggesting that cycling mitochondrial activity is dependent on a functional clock (Peek *et al.*, 2013).

Protein translation is strongly circadian regulated. Ribosomal production changes over a 24 hour cycle, and authors speculated that translation would be decreased by roughly 20% in the inactive phase. Therefore, protein production is highest when the mammal is active and feeding, caused by an increase in total rRNA, and activation of EIF4E (eukaryotic translation initiation factor 4 E) via phosphorylation (Jouffe *et al.*, 2013). BMAL1 also associates directly with ribosomes after phosphorylation by ribosomal s6 protein kinase 1. This also stimulates protein synthesis, adding to the rhythmic control of protein synthesis directly by the circadian machinery (Lipton *et al.*, 2015).

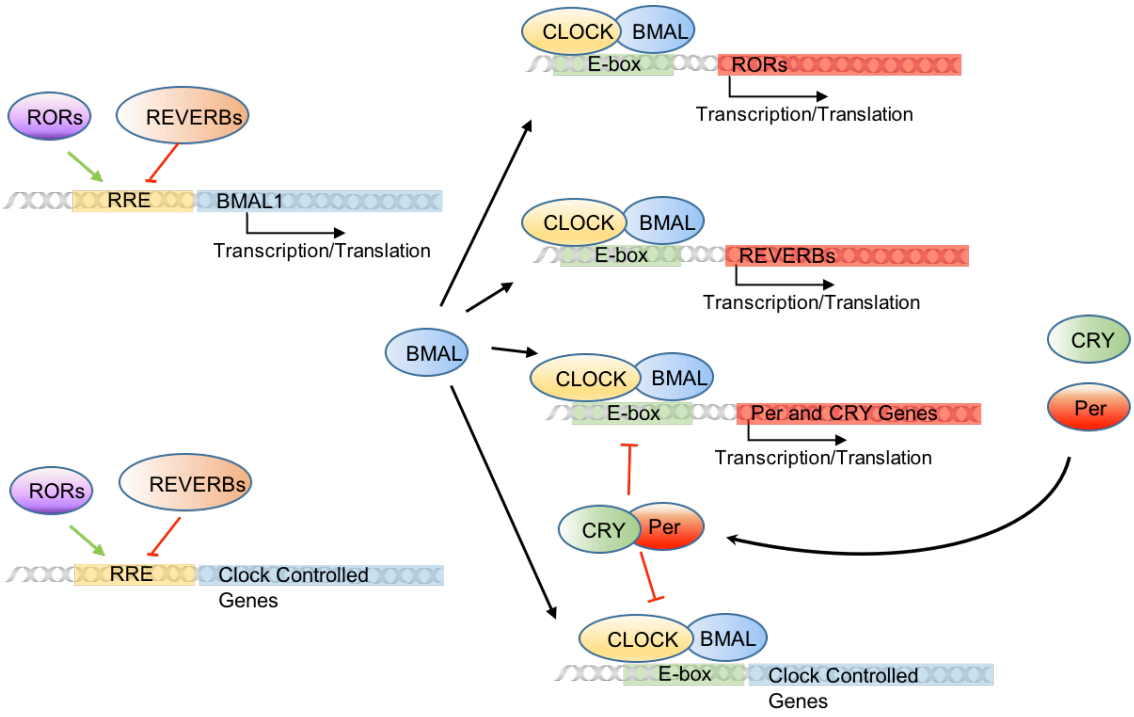

Fig.1.7. The molecular clockwork. BMAL and CLOCK cooperate to regulate rhythmic, clock controlled, genes, as well as the repressors Per and Cry, which act as a negative feedback loop to turn off the expression of BMAL, therefore reducing their own expression, allowing BMAL to be expressed again,

restarting the loop. BMAL also controls the expression of the RORs and REV-ERBs, which activate and repress genes respectively. The RORs and REV-ERBs act as a fine tuning to gene expression within the clock controlled genes, and BMAL expression. Adapted from (Ko and Takahashi, 2006).

1.8.1 The Molecular Clock.

The core clock consists of multiple transcription factors, which form a transcriptional-translational feedback loop, whereby BMAL1 (Brain and Muscle ARNT-Like 1) and CLOCK (Circadian Locomotor Cycles Kaput) activate the transcription of many genes, including the negative arm of the clock (Fig.1.7). Cry (Cryptochrome) 1 and 2 along with Per (Period) 1 and 2 are thereby translated, and inhibit the action of BMAL1 and CLOCK, thus preventing their own transcription, and starting the cycle over again. This transcriptional-translational feedback loop oscillates roughly every 24 hours (Doherty and Kay, 2010). Alongside this "core clock" consisting of BMAL1, CLOCK, Cry and Per, there is the auxiliary loop. This consists of the nuclear receptors NR1D1, NR1D2 (Rev-erbα, Rev-erbβ) as the negative arm and NR1F1 (RORα) as the positive arm. The DNA binding sites of the BMAL1 and Rev-erb RORα overlap, indicating that Rev-erb and RORα have an input into the action of the core clock loop (Cho *et al.*, 2012; Zhang *et al.*, 2015). See Fig.1.7 for a diagram of the molecular clock work and transcriptional translational feedback mechanism.

The actions and dynamics of the core clock have been well studied. An extensive study using ChIP-seq (Chromatin Immunoprecipitation followed by high throughput sequencing) demonstrated that the activation and repression of circadian controlled transcription is not only down to transcription factor binding, but daily remodeling of chromatin caused by the core clock. The histone marks H3K4me3, H3K9Ac and H3K27Ac (all activating marks showing poised or activated enhancer regions) are rhythmic, driven by the molecular clock. Similarly, RNA polymerase II is differentially recruited to chromatin depending on the time of day, driving circadian oscillations in mRNA production. This gave insight into how transcription varies by time of day: Enhancers become activated at circadian time 6-8, with peak transcription occurring at 15 hours, followed by repression from 16 to 22 hours

circadian time (Koike *et al.*, 2012). This rhythmic transcription can be found in multiple organs, and those rhythmic mRNAs are specific to each organ. The phase at which most of the circadian transcription occurs varies by tissue also, with different tissues running out of phase with one another. It was also found that many of the rhythmic genes were targets for major drugs, suggesting that the concept of chronotherapy is more important than previously thought (Zhang *et al.*, 2014).

The molecular action of the Rev-erb/RORα is different from the common competition model assumed. Recent advancements by Zhu et al demonstrated that the competition model is unlikely as the repressors Rev-erb and the activator RORα are bound to chromatin at the same time, suggesting a facilitated loading model, whereby RORα and BMAL1 open the chromatin, and allow Rev-erb to bind, thus initiating the repression. Opposing the repression model, over expression of BMAL1 and RORα increase the binding capacity of Rev-erb, which has a preference for open chromatin (Zhu *et al.*, 2015).

Rev-erbα is strongly associated with metabolic actions within the clock. Rev-erbα knockout mice gain significantly more fat mass than wild type mice when put on a high fat diet, and rely on lipids more as energy than their wild type littermates. Similarly, glucose metabolism functions are perturbed in Rev-erbα KO mice, they are hyperglycemic, but not insulin resistant. Nor do they have defects in the gluconeogenic pathway as shown by a pyruvate tolerance test (Delezie *et al.*, 2012). The effects of Rev-erbα are not limited to the liver, it is essential for mitochondrial biogenesis, and Rev-erbα knockout mice show a decreased exercise tolerance, due to a decrease in mitochondrial number (which can be recovered *in vitro* by Rev-erbα over expression). The AMPK (AMP dependent protein kinase) pathway, which is important in sensing AMP/ATP levels and mitochondrial activity/biogenesis, is also affected by loss of Rev-erbα. The mRNA levels of Lkb1 (liver kinase b1), the AMPK activator are decreased as are the activity levels of AMPK itself, suggesting this is the mechanism for decreased muscular mitochondria (Woldt *et al.*, 2013).

1.8.2 Interaction Between the Molecular Clock and the Glucocorticoid Receptor

Corticosterone the endogenous Gc in mice is rhythmic, as is the equivalent in humans (cortisol). It peaks at the onset of the active phase, in mice, at dusk. It is involved in inducing wakefulness and activity. When the food zeitgeber is altered, and access to food is restricted to the inactive phase (light phase), mice will alter their sleep/wake cycle to fit this, and move from nocturnal to diurnal, however, this only affects the circadian rhythm of peripheral clocks, such as the liver, while the dominant pace maker in the SCN remains unchanged. Adrenalecatomised mice more rapidly become accustomed to the phase switching compared to their sham operated counterparts, suggesting that Gc inhibit this phase change in the peripheral tissues by resetting the clock genes (Le Minh *et al.*, 2001). Further evidence that Gc reset clock controlled genes comes from transriptomic studies. Gc reset and therefore synchronise the circadian transcriptome, likely through direct effects on some genes, but also by regulating core clock genes such as Per1, BMAL1 and Cry1. The liver has a strongly circadian transcriptome, and Gc administration affect around 60% of rhythmic transcripts in mice. Interestingly, many of the circadian genes that Gc affect also contain HNF4α binding elements. These genes lose rhythmicity in SCN lesioned animals and HNF4α knockout animals, but maintain Gc responsiveness only in the SCN lesioned animals, suggesting that HNF4α is key for regulation of GR action *in vivo* (Reddy *et al.*, 2007). The GR directly regulates Rev-erbα, a key repressive element of the circadian clock at the transcriptional level in both rat and human primary hepatocytes. This shows a direct interaction with the circadian clock, and also how Gc may affect lipid metabolic processes in liver - by repressing a repressor, thus resulting in an increase in hepatic lipid levels (Torra *et al.*, 2000).

Using immunoprecipitation, it's been shown that the GR interacts directly with the core clock components Cry1 and Cry2, but on a physical level, rather than a transcriptional level. Further dissection of this interaction in Cry1/2 double knockout mouse embryonic stem cells shows that Gc elicit a stronger transcriptional response, with higher amounts of Gc induced transcript being produced in the double knockout cells. This suggests that the interaction between the Cry proteins

and GR is inhibitory in nature, and indeed, glucose tolerance tests show that Gc treated Cry double knockout mice have elevated fasting blood glucose and decreased glucose tolerance compared to wild type Gc treated mice (Lamia *et al.*, 2011).

Despite the removal of cryptochomes causing an increase in Gc action in the liver, GR requires an intact clock for its immunosuppressive functions in the lung. Loss of BMAL1 in bronchial epithelial cells causes an increase in CXCL5, a pro inflammatory cytokine. This loss of BMAL1, and therefore the loss of clock function in the epithelial cells is also associated with decreased GR binding to CXCL5 promoter suggesting that the intact clock in responsible for correct GR loading onto the DNA. This can also be seen in the glutamine synthetase promoter upon bronchial epithelial cell specific BMAL1 ablation, suggesting the GR loading effect is not limited to immunosuppressive function only (Gibbs *et al.*, 2014).

1.9 Side Effects Associated with Glucocorticoid Use

The use of Gcs, especially chronic use has been associated with serious side effects; including, but not limited to: osteoporosis, metabolic syndrome, cardiovascular disease, infections, osteonecrosis, cataracts and diabetes (Moghadam-Kia and Werth, 2010). These side effects are mainly dose and time dependent, but even short term use of Gcs was shown to have an effect on risk of bone fracture (De Vries *et al.*, 2007). Gc use can cause the development of exogenous Cushing's syndrome by dysregulating the HPA (Hypothalamic-Pituitary-Adrenal) axis. This leads to further problems when Gc treatment is withdrawn from patients, as the continual down-regulation of the HPA axis causes a decrease in endogenous Gc release (Hopkins and Leinung, 2005).

Due to the potent anti-inflammatory actions of Gcs, one risk of prolonged usage is infection. It has been found that limiting the Gc dose (even while maintaining a chronic usage) has a significant effect at preventing potential infections (Cutolo *et al.*, 2008). The dose dependent risk factors have been quantified (Dixon *et al.*, 2012) allowing for better treatment of patients whilst limiting the risk of

infection. The anti-inflammatory effects also cause issues in wound healing, mainly by inhibition of proinflammatory cytokines (Beer, Fassler and Werner, 2000).

1.9.1 Glucocorticoid Induced Side Effects; Osteoporosis

Osteoporosis is a common and serious risk factor with Gc treatment. Glucocorticoid induced osteoporosis (GIO) includes two phases: an early rapid phase characterized by bone loss caused by bone resorption and a second phase when bone formation is impaired. Gcs have been shown to limit the development of osteoblasts by inhibition of Krox-20 (Leclerc *et al.*, 2005) as well as causing apoptosis of osteoblasts and production of proteins that suppress their activity (Leclerc *et al.*, 2004). While not especially deadly in younger patients, it does lead to a decreased quality of life and extra pain symptoms and therefore must be taken into account when initiating Gc therapy, particularly in demographics that are already at risk of osteoporosis. There is also evidence that Gc induced transrepression affects collagen I and osteocalcin, which are important for bone mineralisation. Gcs can work indirectly too, by limiting calcium absorption in the gut, and calcium reabsorption within the kidneys too (Bultink, Baden and Lems, 2013). Transrepression appears to be the main mechanism of Gc action on bone. Studies using the GRdim (dimerisation deficient) mouse, demonstrated that the Gc induced osteoporosis was still in effect, while a full deletion of GR in osteoblasts via the Runx2 Cre-driver abrogated the side effects. Interestingly, it was found that the cytokine IL-11 played a major role in the differentiation of preosteoblasts to osteoblasts, which was inhibited by Gc (Rauch *et al.*, 2010).

Linked to osteoporosis, Gc treatment also causes irreversible osteonecrosis, resulting in 9-40% of patients receiving Gc therapy and the highest cause of all non-traumatic osteonecrosis cases. The apoptosis of osteocytes caused by Gcs leads to a build-up of dead cells in the bone due to their unavailability for phagocytotic removal (Weinstein, 2012).

1.9.2 Glucocorticoid Induced Side Effects; Insulin Resistance

As previously mentioned, Gc up regulate the gluconeogenic pathway in liver, whilst simultaneously down regulating glucose utilisation in muscle and adipose tissue. Gc also increase lipolysis, therefore resulting in increased levels of serum fatty acids and glucose. However, Gc have been shown to increase abdominal fat deposits, which are linked to insulin resistance. The effects in muscle are particularly important. 80% of glucose uptake stimulated by insulin is done by skeletal muscle, and the bulk of glycogen reserves are found in skeletal muscle (Rafacho *et al.*, 2014). Glucocorticoids drastically reduce the effect of insulin by reducing Glut4 (glucose transporter) transcription, reducing IRS1 (insulin receptor substrate) and IRS2 transcription and activity, and inhibiting the action of PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) by up regulating the transcription of the regulatory subunit; PIK3R (Saad *et al.*, 1993; Dimitriadis *et al.*, 1997). This reduces the uptake of glucose into skeletal muscle, and reduces the effect of insulin simultaneously. The same effects can be seen in the liver, but there are data to show that the effects of Gc on glucose homeostasis in the liver are dependent on the orphan nuclear receptor LXRβ (Liver X Receptor), where LXRβ is important for the Gc induced transcription of PEPCK, the rate limiting step in gluconeogenesis (Patel *et al.*, 2011). Patel et al. demonstrated increased glucose tolerance, lower levels of insulin and faster glucose clearance in LXRα/β–/– double knockouts compared to WT, while mice were treated chronically with Gc over 5 days. This highlights an interesting link between other nuclear receptors and the actions of GR. The Gc antagonist RU486 can aid with the insulin resistance in mice, again demonstrating a key role for the GR in mediating this effect (Taylor *et al.*, 2009). The effects of Gc on insulin production are counter intuitive. Chronic dosing of Gc causes an increase in insulin production, attributed to the constant hyperglycaemia caused by Gc treatment, which outweighs any effect Gc have on pancreatic β-cells (Petersons *et al.*, 2013). Gc have been shown to induce apoptosis on β-cells *in vitro*, however, when tested *in vivo*, the effect was found to be reversible after cessation of Gc treatment (Rafacho *et al.*, 2010).

1.9.3 Dissociative Agonists

Due to the previously mentioned side effects of Gcs, there has been a lot of effort put into finding agonists which retain the anti-inflammatory actions, but do not cause the unwanted detrimental effects. The logic behind such attempts is that Gcs work via transrepression, transactivation and non-genomic mechanisms. The transactivation generally has the unwanted effects, and thus if this action can be avoided, new, stronger, safer, synthetic Gcs can be sold. These so called "dissociative agonists" come in two flavours: "soft steroids" which are rapidly metabolised, and thus the mechanism to stop side effects is speedy expulsion from the system. Or have a local application (e.g. topical and inhalation) in order to limit the bioavailability and therefore side effects. These are not applicable to every use of Gcs though, especially in cases of chronic inflammation or ALI (acute lung injury). The other type of drug is the more precise definition of "dissociative agonists", which attempt to separate the anti-inflammatory effects from deleterious effects. A major issue when trying to create dissociative Gcs is that even classic Gcs have dose dependent dissociative effects; DEX (dexamethasone) needs a lower concentration for transrepression effects than for the transactivation effects (McMaster and Ray, 2008). There have been advances, where apparently fully dissociated molecules have been found, although their mechanism of action seems to augment Gc use, rather than replace it (De Bosscher *et al.*, 2005) suggesting that a new approach to how side effects of Gc therapy are combated is needed.

1.10 Aims and Hypothesis

Gc responses have been shown to be strongly affected by both cellular cues (Matthews *et al.*, 2011) and environmental cues (Nader *et al.*, 2010; Uhlenhaut *et al.*, 2013), resulting differential control of genes by Gcs. Caveolin-1 has been previously implicated in Gc responses, both in non-genomic (Matthews *et al.*, 2008) and genomic (Peffer *et al.*, 2014) mechanisms, thus understanding how Cav1-GR interaction can affect inflammation is necessary due to Gcs being used as anti-inflammatory compounds.

In terms of environmental effect on Gc signaling, Gcs potently regulate core clock genes (Torra *et al.*, 2000; So *et al.*, 2009). The circadian clock has also been shown to affect GR DNA binding and transcriptome, using knockout models (Lamia *et al.*, 2011). However, whether this changes the transcriptome of Gc responses throughout the day had not yet been explored. Therefore, the following aims, hypotheses and objectives were formulated.

Aim 1:

To assess the role of the membrane lipid raft component caveolin-1, in modulating the anti-inflammatory actions of glucocorticoids in the lung using mouse models of airway inflammation.

Hypothesis 1: Caveolin-1 is essential for Gc signalling in inflammation. Loss of caveolin-1 will result in a reduction of inflammation in response to LPS, and a pro-inflammatory response to ovalbumin. Both of these inflammatory challenges will be resistant to Gc treatment due to the loss of caveolin-1.

Objectives:

1) Characterise Gc transcriptional responses *in vivo* and *in vitro* in a murine model of Cav1KO using qRT-PCR to identify genes differentially regulated by Gcs in Cav1KO tissue.
2) Characterise the anti-inflammatory effects of Gcs in lung inflammation *in vivo* with Cav1KO mice, focusing on inflammatory cell infiltrate and cytokine production.

3) Determine whether any effect found is due to a lung specific phenotype of CAV1KO mice, or due to the interaction of CAV1 and GR in the innate immune system by comparing two different models of lung inflammation – an innate immunity LPS challenge and an adaptive immunity ovalbumin challenge.

Aim 2:

To determine the effect of circadian rhythms on glucocorticoid responses in different tissues, and determine the transcription factors involved in the effect found, in order to separate side effects of Gc treatment from beneficial anti-inflammatory effects.

Hypothesis 2: Time of day regulates Gc response, which is modulated by the core components of the molecular clock through direct DNA interactions between clock components and GR. Circadian oscillations in chromatin architecture will determine the accessibility for GR and thus Gc responsiveness.

Objectives:

1) Determine the transcriptome of C57BL/6 mice in response to Gcs during the day and the night using RNA-sequencing in lung and liver tissue.
2) Identify any genes regulated by Gcs specifically at one time of day or another, and determine a suitable animal model to test any ontological link.
3) Propose a mechanism for Gc time of day regulated genes by looking directly at core clock transcription factors through *in silico* methods. Then use knockout this target and assess the time of day Gc responses in WT and KO mice.

CHAPTER 2: Materials and Methods

2.1 Animals

Experimentation was performed on mouse strains C57BL/6J (WT) from Harlan Blackthorn, UK. All mice were acclimatised in the biological services facility for one week before any procedures were undertaken. All procedures were performed in compliance with Animals (Scientific Procedures) Act of 1986. While housed in Manchester, mice had free access to food (unless stated) and water and were multiply housed in a 12:12 light/dark cycle. Animals were randomly allocated to treatment groups and coded, then samples processed and decoded post analysis to limit any investigator bias.

2.2 Transcript analysis

2.2.1 RNA Extraction

Samples were lysed and total RNA was prepared using SV Total RNA Isolation System (Promega). RNA quality was checked using the RNA 6000 Nano Assay, RNA samples with a 260:280 nM ratio of ~2 taken forward for analysis.

2.2.2 Two-Step qRT-PCR

Total RNA was reverse transcribed to cDNA using High Capacity RNA to cDNA kit (Applied Biosystems) and subjected to qPCR using SYBR Green (KAPA Biosystems) detection in a q-PCR machine. All samples were analysed in duplicate. The mRNA expression levels were measured using appropriate primer sets (Europhins). Expression levels were calculated using the δδCT method normalising to GAPDH control.

2.3 Histological analysis

2.3.1 Processing tissue for histology

Lung: After cervical dislocation, mice were intubated and the lungs were instilled with 1ml of 4% *para*-formaldehyde. The bronchus was clamped and the lungs fixed by immersion in 4% *para*-

formaldehyde overnight. Tissue was embedded in paraffin blocks and cut into 5µm sections (Leica RM2255 Microtome).

2.3.2 Immunohistochemical Preparation

Sections were rehydrated and washed 3 times in PBS. Endogenous peroxides were quenched via incubation with 0.003% v/v hydrogen peroxide. (DAB staining only). Antigen retrieval was performed by boiling sample in 10mM citric acid pH 6 for 20 minutes, and then washed 3 times in PBS. Samples were incubated over-night at 4°C with primary antibody (GR 1:400, clone M-20, sc-1004, Santa Cruz; Caveolin-1 1:200, clone N-20, sc-894, CCSP 1:200, clone diluted in PBS (pH 7.4, 0.1% v/v Triton-X 100) with 3% goat or horse serum (Invitrogen).

2.3.3 3,3'-diaminobenzidine Staining

Following antigen retrieval samples were washed 3 times in PBS and incubated with biotinylated secondary antibody (BA-1000, Vector Laboratories) diluted in PBS (pH 7.4, 0.1% v/v Triton-X 100) for 2 hours at 4°C, followed by another 3 PBS washes. Samples were then incubated with streptavidin conjugated Horse radish peroxidase (SA-5004, Vector Laboratories) for 1 hour at 4°C. Sample were incubated with *3,3'-diaminobenzidine* (DAB, SK-4100, Vector Laboratories) dissolved in water, washed 3 times in PBS, then incubated for 5-10 minutes with DAB dissolved in water with 3% hydrogen peroxide added. After DAB staining, sections were counterstained with Toludine blue and dehydrated. Samples were then dehydrated and mounted using entallan.

2.3.4 Haematoxylin and Eosin Staining

Paraffin embedded adipose sections were dehydrated and brought to distilled water. Frozen liver sections were equilibrated to room temperature, then immersed in distilled water. All sections were then processed in parallel. Nuclei were stained with Haemotoxylin, then rinsed in tap water, then stained with Eosin (2min), rinsed with tap water, dehydrated and mounted using entallan.

2.4 Microscopy

2.4.1 Brightfield

Images were acquired on an Axio Imager.A1 (Zeiss) microscope using either a 10x Zeiss EC Plan-NEOfluar or 20x Zeiss EC Plan-NEOfluar objective. Images were collected using AxioCam MRc (Zeiss). Raw images were visualised using AxiovisionRel. 4.7 (Zeiss) and processed using Image J.

2.4.2 Deltavision

Images were acquired on a Delta Vision RT (Applied Precision, GE Healthcare) restoration microscope using either a 40X/0.85 Uplan Apo objective or a 60X/1.42 Plan Apo N objective and the Sedat Quad filter set (Chroma 86000v2, VT, USA). The images were collected using a Coolsnap HQ (Photometrics, AZ, USA) camera with a Z optical spacing of 0.5µm. Raw images were deconvolved using the Softworx software (GE Healthcare) and average intensity projections of these deconvolved images processed using Image J.

2.5 Statistical Analysis

Standard statistical tests were completed using Graph pad prism statistical software. Data is presented as group means with standard deviation or as individual data points with median (as indicated in the figure legend). Column statistics were used to determine whether data was normally distributed, and parametric or non-parametric tests used accordingly, determined by pairwise or multiple comparisons. Data were analysed by student's t-test, Mann-Whitney test, 1-way analysis of variance (ANOVA) with the Holm-Sidak post-hoc analysis, Kruskal-Wallis with Dunn's multiple comparisons test or 2-way ANOVA with the Tukey's post-hoc test. Specific tests used can be found in the figure legends along with an indicated level of support (p-value<0.05 or 0.01).

3.1 Caveolin-1 Regulates Glucocorticoid Function in Lung Parenchyma to Suppress Inflammation

3.2 Abstract**:** Glucocorticoids (Gc) are a commonly used treatment for a wide variety of inflammatory diseases and are known to inhibit inflammatory signalling through transrepression of NF-κB induced genes, and induction of multiple anti-inflammatory genes such as DUSP1 and GILZ. Caveolin-1 (cav1) is also an important modulator of inflammatory signalling, regulating the activity of NF-κB, through interacting with mitogen activated kinases. Previous work identified Cav-1 as an interting partner protein with GR, and capable of regulating antiproliferative GR function. Therefore, here we investigate the effect of Cav1 on Gc inhibition of pulmonary inflammation in two *in* vivo models. We show that mice lacking Cav1 have reduced inflammatory cellular infiltrate in response to aerosolized lipopolysaccharide (LPS), a TLR4 agonist. This model of acute lung injury showed equivalent repression of inflammation by Gc in both wild type and *cav1* knockout mice. Using a model of allergic asthma, loss of cav1 had no effect on the extent of inflammation measured by cellular infiltrate in response to an ovalbumin sensitization and challenge, and again the repression of inflammation by Gc was unaffected. Our data therefore suggests that Cav1 regulates innate inflammation, but does not affect adaptive immune responses. Despite regulating GR function in vitro, and affecting GR transactivation of genes in lung tissue there was no Cav-dependent Gc anti-inflammatory phenotype observed in two *in vivo* models.

3.3 Introduction

Glucocorticoids (Gc) are steroid hormones, widely used as anti-inflammatory agents to treat chronic and acute inflammatory diseases such as asthma and rheumatoid arthritis. Gc signal through the Gc receptor (GR); a ligand activated transcription factor, which upon activation translocates to the nucleus, where it can transactivate or transrepress genes, in a cell type specific manner. Many Gc

targets are genes involved in regulating the immune response. GR binds to proinflammatory transcription factors such as NF-κB and AP-1 to repress their activity (Payvar *et al.*, 1981, 1983; McKay and Cidlowski, 1998; Uhlenhaut *et al.*, 2013), resulting in potent immunosuppression. Gc activated targets also include important anti-inflammatory proteins, such as GILZ and DUSP1 which also contribute to the Gc dependent suppression of inflammation (Hübner *et al.*, 2015).

Caveolin-1 (cav1), a protein involved in membrane lipid raft formation, and the key constituent of caveolae, flask shaped invaginations found in the plasma membrane. Cav1 is known to be an essential regulator of endocytosis, cholesterol and lipid metabolism (Murata *et al.*, 1995; Schlegel and Lisanti, 2001; Liu, Rudick and Anderson, 2002; Razani *et al.*, 2002), but also in inflammatory signalling, specifically through regulating the activity of NF-κB. Knockout of *cav1* is associated with protection from inflammation, through reduced NF-κB activity and less efficient immune cell infiltration (Garrean *et al.*, 2006; Jiao *et al.*, 2013; Wu *et al.*, 2016). However, there is also evidence that Cav1 loss can cause increased inflammatory activation of macrophages (Zhang *et al.*, 2013), suggesting that the role of caveolin-1 in inflammation is both complex and poorly understood. Previously we have identified a functional interaction between cav1 and GR in cell cycle regulation in lung epithelial cells (Matthews *et al.*, 2008), therefore we tested how these two key mediators of inflammation interact during an *in vivo* inflammatory challenge.

Using two models of pulmonary inflammation, LPS induced acute lung injury, and ovalbumin sensitization to mimic allergic asthma we examined at the effect of loss of Cav1 on the suppression of inflammation by Gcs in order to identify new signalling pathways involved in immunosuppression and aid in the identification of therapeutic targets upstream of the GR.

The number of animals required for the LPS challenge (Fig. 3.2, C and Fig. 3.4) was determined from (Gibbs *et al.*, 2014). The number of animals required for the Ovalbumin challenge (Fig. 3.6) was calculated using G*Power statistical power analysis software (University of Dusseldorf) via F-test (ANOVA, Fixed Effects) with the final calculated total sample size as 39 animals, for an effect size of 0.9, and a confidence interval of 0.95. Fewer animals however, were used due to the poor breeding

and low yield of homozygous CAV1KO mice.

3.4 Methods

3.4.1 Animal Work

Experimentation was performed on mouse strains C57BL6 (WT) from Harlan Blackthorn, UK and B6.Cg-*Cav1*^{tm1Mls}/J (CAV1KO) from Jackson Laboratories, Maine, USA, courtesy of Professor Michael Lisanti (University of Manchester). Mice were aged between 10 and 24 weeks. All procedures were conducted in compliance with Animals (Scientific Procedures) Act of 1986. Mice had free access to food and water and were housed in a 12 hour light/dark cycle.

3.4.2 Alveolar Macrophages Isolation

Following cervical dislocation of C57BL6 and CAV1KO mice, lungs from naïve mice, were lavaged using 1ml RPMI-1640 instilled and removed via tracheal cannula 3 times. The media was then centrifuged at 1500 rpm for 5 minutes and the pellet was washed twice and resuspended in RPMI-1640. After 2 hours incubation, non-adherant cells were washed off using RPMI-1640 and cultured in RPMI-1640 supplemented with 10% v/v FBS or charcoal dextran stripped fetal calf serum (sFCS; Invitrogen) and 1% v/v penicillin and streptomycin (Invitrogen) treated the next day.

3.4.3 Explant Culture

Lungs were collected and washed twice with serum free DMEM. Whole lung was diced and transferred to DMEM containing sFCS and incubated in a humidified atmosphere of 5% carbon dioxide at 37°C overnight. Samples were then treated with dexamethasone 100nM (Sigma Aldrich) or vehicle (Dimethylsulfoxide, Sigma Aldrich) for 2 hours before rinsing with PBS and extraction of total RNA.

3.4.4 Aerosolised LPS challenge

Age matched C57BL6 (Harlan Blackthorn, UK) and CAV1KO mice were pre-treated with dexamethasone (1mg/kg, intraperitoneal), at ZT2, for 1h then placed into a Perspex chamber and exposed to aerosolised lipopolysaccharide (0127:B8; 2mg/ml) or vehicle (saline) for 20 min at ZT3. The animals were returned back to their cages for 5h before sacrifice (pentobarbital, intraperitoneal).

3.4.5 Ovalbumin Challenge

Age matched C57BL6 (Harlan Blackthorn, UK) and CAV1KO mice were sensitised to ovalbumin using an adjuvant (10ug ovalbumin, 2mg Aluminium Hydroxide per mouse, Sigma, UK) injected intraperitoneal on days 0 and 14. Mice were then given intraperitoneal dexamethasone (Sigma, UK) or vehicle (Cyclodextrin, Sigma, UK) 3 hours before intranasal dosing of ovalbumin (1mg/ml, 50ul) on days 24, 25 and 26. 24 hours later, mice were sacrificed via pentobarbital, intraperitoneal.

3.4.6 Collection of BAL and processing of protein and cell content

After inflammatory challenge, the lungs were lavaged using 1ml BAL fluid (10mM EDTA and 1% BSA) instilled and removed via a tracheal cannula. The left lobe of the lung was collected for protein analysis and the right for RNA analysis. The lavage fluid was centrifuged and the supernatant utilised for cytokine/chemokine analysis using the Bioplex Suspension Array System (BioRad) or ELISA (R&D Systems). The cell pellet was re-suspended in fresh BAL fluid to allow quantification of total cell numbers using a Casy Cell Counter (Schärfe System, Germany), and cytospins were stained with Leishman's eosin-methylene blue (VWR) to enable quantification of macrophages and neutrophils. Images were captured using a Leica DM2000 microscope and Leica DFC296 camera and macrophages and neutrophils were counted from 5 individual fields to determine the relative percentage of each. By combining total cell counts and relative cell counts, total numbers of macrophages and neutrophils were calculated.

3.4.7 Flow Cytometry

Resident cells were isolated from naive lung at CT0 and CT12 via lavage with BAL fluid. Fc receptors were blocked (1:100 anti-CD16/32, eBioscience #14-0161) before application of the following antibodies in 30 µl FACS buffer (PBS, 1% BSA and 0.1% sodium azide): CD11b-PerCP-Cy5.5 (1:200, clone **M1/70, #45-0112**), CD11c-APC (1:400, **clone N418, #17-0114**), Ly6G-FITC (1:100, clone **RB6-8C5, #11-5931**)(all purchased from eBioscience), Siglec-F-Alexafluor700 (1:100, clone **1RMN44N**). After washing, cells were resuspended in 50µl FACS buffer and fixed by addition of an equal volume of 3.6% formaldehyde for 20 min. Cells were resuspended in FACS buffer, and analysis was carried out on a BD LSR II flow cytometer. Neutrophils were identified as $CD11b^{+}Ly6G^{+}$. Alveolar macrophages were identified as $CD11c^{+}CD11b^{lo}Ly6G^{-}$. Eosoniphils were identified as $Siglec\text{-}F^{+}CD11b^{lo}$.

3.4.8 *Immunoblot Analysis*

Lung tissue was collected at ZT12 and protein prepared in FastPrep-24 lysing matrix tubes (MP Biomedicals) then lysed using Radio-Immunoprecipitation Assay (RIPA) buffer (50 mM TricCl pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease (Calbiochem, San Diego, CA, USA) and phosphatase inhibitors (Sigma-Aldrich Corp.). 15 µg protein was run on an SDS 8–12% Tris-Glycine gel (Novex, Life Technologies) and then transferred onto a 0.2-µm nitrocellulose membrane (BioRad) overnight, blocked with 1% milk and probed for GR (1:1000, clone M-20, sc-1004, Santa Cruz), Cav1 (1:1000, clone N20, Santa Cruz), Cavin (1:1000 O-24, Santa Cruz), actin (1:1000, SP-124, Sigma Aldrich), TFIIB (1:1000, C-18 Santa Cruz). Primary antibodies were detected using donkey anti-rabbit-HRP linked secondary antibody (VWR). Immunoreactivity was visualized using enhanced chemiluminescence (GE Healthcare).

3.4.9 Immunofluorescence

Following the histological preparation, samples were incubated in permeabilisation buffer (TBS, 0.1% v/v Triton-X 100) overnight at 4°C. Sections were then incubated overnight at 4°C with primary antibody (Cav1 1:200, clone N20, Santa Cruz) diluted in PBS (pH 7.4, 0.1% v/v Triton-X 100) with goat or horse serum. Sections were washed 3 times in PBS and incubated with fluorophore conjugated secondary diluted in PBS (pH 7.4, 0.1% v/v Triton-X 100) for 2 hours at 4°C, followed by another 3 PBS washes and incubated over night at 4°C in another primary (CCSP 1:500, 07-623, Merck Millipore) diluted in PBS (pH 7.4, 0.1% v/v Triton-X 100) with goat or horse serum. After washing 3 times in PBS, samples were incubated with an alternate fluorophore secondary antibody diluted in PBS (pH 7.4, 0.1% v/v Triton-X 100) for 2 hours at 4°C. After 3 washes in PBS, samples were mounted using Vectamount AQ (Vector Laboriatories, Peterborough, UK) containing DAPI.

3.4.10 Trichrome Staining

Sections were deparaffinised in xylene and rehydrated using ethanol and distilled water. Samples were re-fixed in Bouin's solution for 1 hour at 56°C and rinsed with tap water. Samples were then stained in Weigert's iron hematoxylin for 10 minutes, rinsed in tap water for a further 10 minutes and washed in distilled water. Following this, samples were stained in Biebrich scarlet-acid fuishin for 10 minutes, washed in distilled water then differentiated in phosphomolybdic-phsphotungstic acid solution for 10 minutes. Sections were then immersed in aniline blue for 5 minutes, rinsed in distilled water and differentiated in 1% acetic acid for 5 minutes. Sections were mounted in entellan.

3.5 Results

Caveolin-1 knockout results in Gc sensitivity

Caveolin-1 is important in lung development (Drab *et al.*, 2001) and lung fibrosis (Wang, Zhang, *et al.*, 2006). Adult CAV1KO mice display the characteristic hypercellularity and increased alveolar septum thickness are previously described (Fig.3.1A), while aged CAV1KO mice (40+ weeks) display a clear increase in collagen deposition around the bronchioles when stained using Masson's Trichrome

(Fig.3.1B). As Gcs are important in both lung development (Cole *et al.*, 1995) and in the treatment of fibrosis (Flaherty *et al.*, 2001; Dik *et al.*, 2003) we analysed the capacity for Gc transactivation in lung explants from WT and CAV1KO mice (Fig.3.1C, top) and *in vivo* transactivation in lungs isolated from WT and CAV1KO mice (Fig.3.1C, bottom) Gc treatment *in vitro* and *in* vivo, transactivated well characterised Gc target genes, GILZ, DUSP1 and FKBP5 in WT and CAV1KO lung tissue. The response to Gc was significantly greater in the CAV1KO lungs, suggesting increased sensitivity to Gc. We identified no difference in either the expression or subcellular distribution of the glucocorticoid receptor in lung sections (Fig.3.1D, left), or lung tissue extracts (Fig.31D, right), suggesting that the increased Gc response seen in the CAV1KO lung is not simply due to increased levels of GR protein, or greater nuclear occupancy.

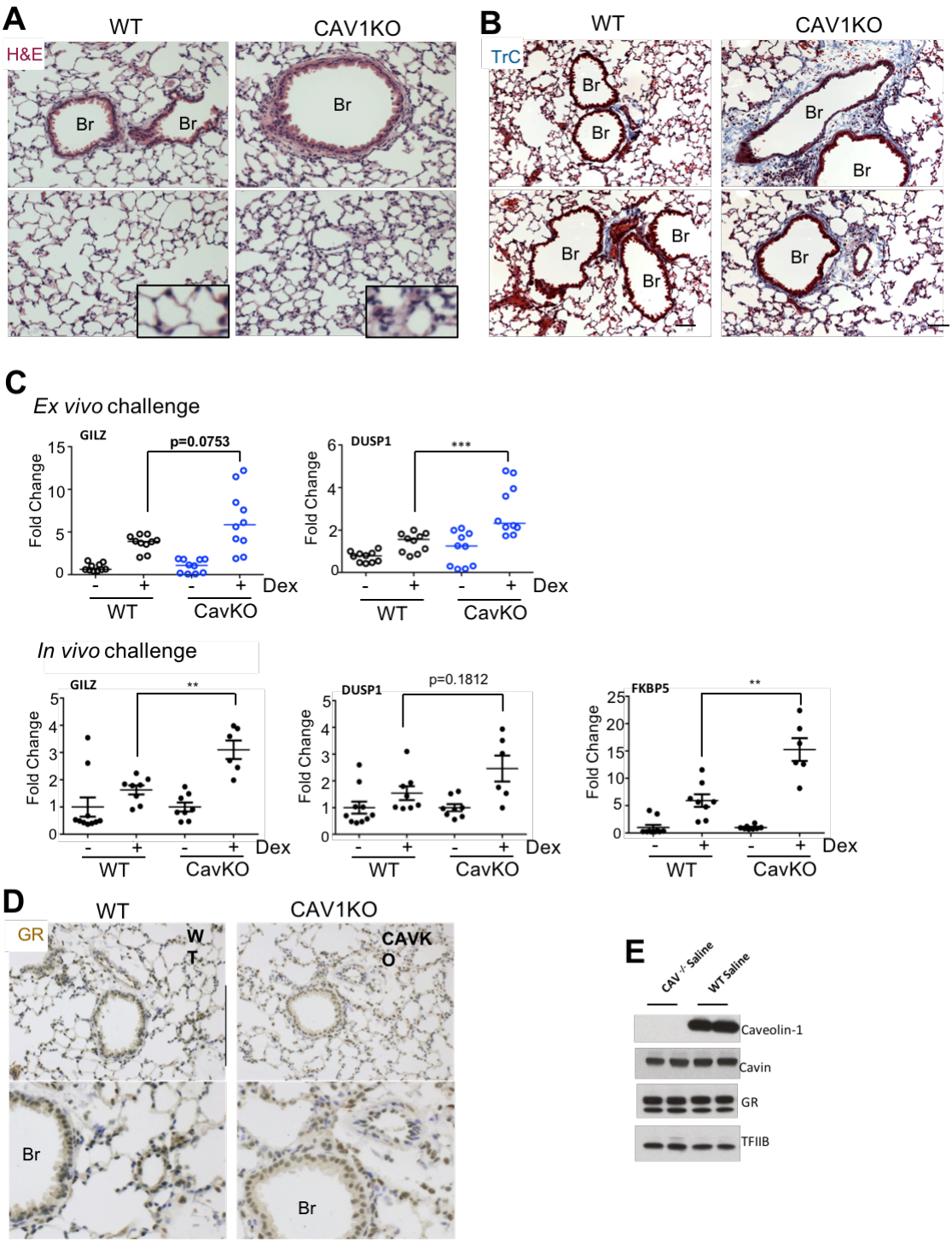

Figure 3.1 Caveolin-1 Knockout Affects Gc Sensitivity. Lungs from C57BL/6 mice and B6.Cg-*Cav1*^{tm1Mls}/J mice were fixed and embedded in paraffin. Sections were stained by H&E (A) and Masson's Trichrome (B). Lungs taken from C57BL/6 mice and B6.Cg-*Cav1*^{tm1Mls}/J mice, were lavaged to remove immune cells, then cultured *ex vivo* with DMSO vehicle or Dex (100nM). qRT-PCR was used to determine Gc response in the tissue (C, top). C57BL/6 mice and B6.Cg-*Cav1*^{tm1Mls}/J mice were

treated with cyclodextrin vehicle or dexamethasone (1mg/kg), lungs harvested and analysed by qRT-PCR for Gc responsive genes (C, bottom). Lungs from WT and CAVKO mice were also fixed with 4% PFA and analysed by immunohistochemistry for GR localisation (brown) (D) or lysed and protein content analysed via western blot for Cav1, Cavin, GR and TFIIB loading control (E). Data analysed by 1-way ANOVA, mean and standard error displayed unless otherwise mentioned. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Caveolin-1 facilitates the pulmonary innate immune response

To assess the role of Cav1 in a modulating Gc transrepression, we first used a model of innate immunity. WT and CAV1KO mice were pre-treated with vehicle (cyclodextrin) or dexamethasone (1mg/kg, I.P.) for 1 hour before exposure to aerosolised LPS (1mg/ml), or saline for 20 minutes, then sacrificed 5 hours later (Fig.3.2A). The expression levels of caveolin-1 and GR in lungs were unaffected by either LPS treatment or dex treatment, and the CAV1KO mice lacked caveolin-1 protein (Fig.3.2B).

Quantification of immune cells number present in BAL fluid shows an increased in immune cell infiltration in lungs of both WT and CAV1KO mice post LPS challenge. CAV1KO mice however, have a reduced inflammatory response to LPS, with fewer immune cells found in the BAL fluid compared to the WT. The overall the response to dexamethasone treatment, reduced immune cell infiltration was was comparable between genotypes (Fig.3.2C, left). Analysis of immune cell subtypes revealed significantly less neutrophilia in the CAV1KO mice post LPS challenge (Fig.3.2C, centre), with a similar suppression of neutrophil infiltrate by dexamethasone in both the WT and CAV1KO mice. Macrophage numbers were unaffected by treatment or genotype (Fig.3.2C, right).

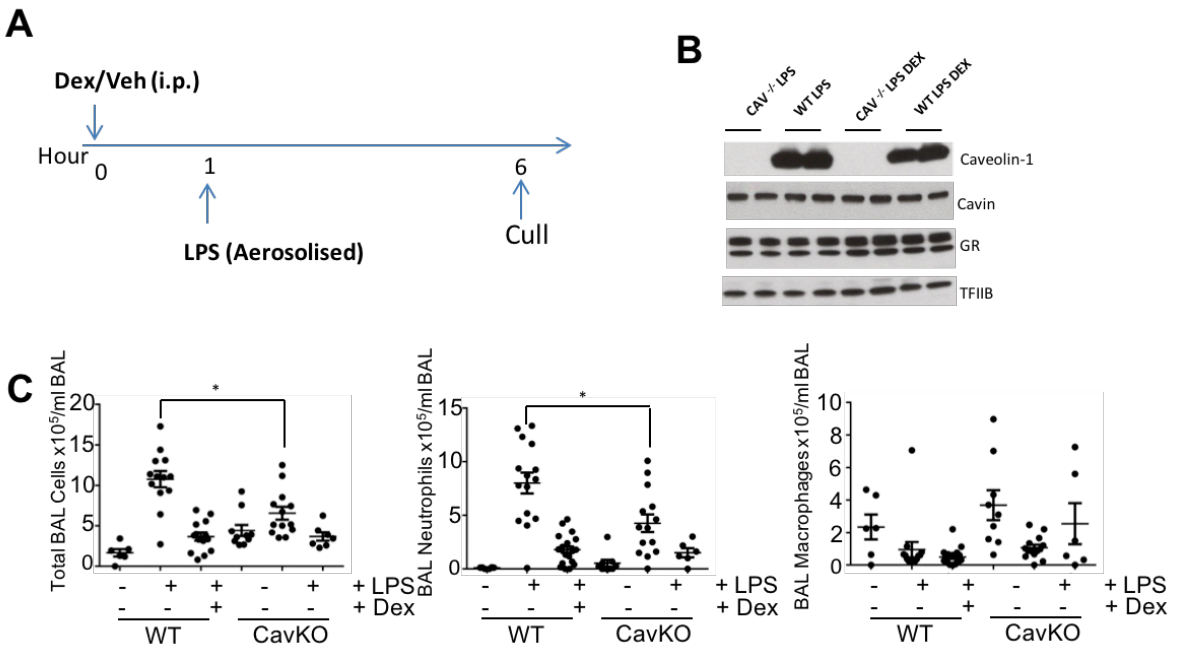

Figure 3.2. Innate Immunity is Regulated by Caveolin-1 Knockout WT and CAVKO mice were treated with saline or dexamethasone (1mg/kg) for 1 hour before being exposed to aerosolised saline or LPS (1mg/ml) for 20 minutes before being culled 5 hours later (A). Animals were then sacrificed and lung cell infiltrate was removed by bronchoalveolar lavage prior to lysis of lungs for protein extration. Protein was analysed by western blot for Cav1, Cavin, GR and TFIIB loading control (B), cells from BAL fluid were counted and stained for cell specific markers (Ly6G and CD11b) or counted by cytospin (C), data is combination of two independent experiments. Data analysed by 1-way ANOVA, mean and standard error displayed unless otherwise mentioned. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Caveolin-1 is absent from club cells

As club cells are important mediators of LPS induced lung inflammation (Elizur *et al.*, 2007) we performed dual immunofluorescence staining of WT lungs for Cav1 (red) and club cell secretory protein (CCSP) (green). There was no overlap in the localisation of the two proteins (Fig.3.3A), suggesting that club cells do not express Caveolin, and therefore the reduced inflammatory response in CAV1KO is unlikely to be mediated by club cells. The same result can be seen using

immunohistochemistry in mouse lungs (Fig.3.3B, left), which we then further confirmed in human lung tissue (Fig.3.3B, right), and suggesting that the lack of Cav1 in club cells is not a murine specific phenomenon.

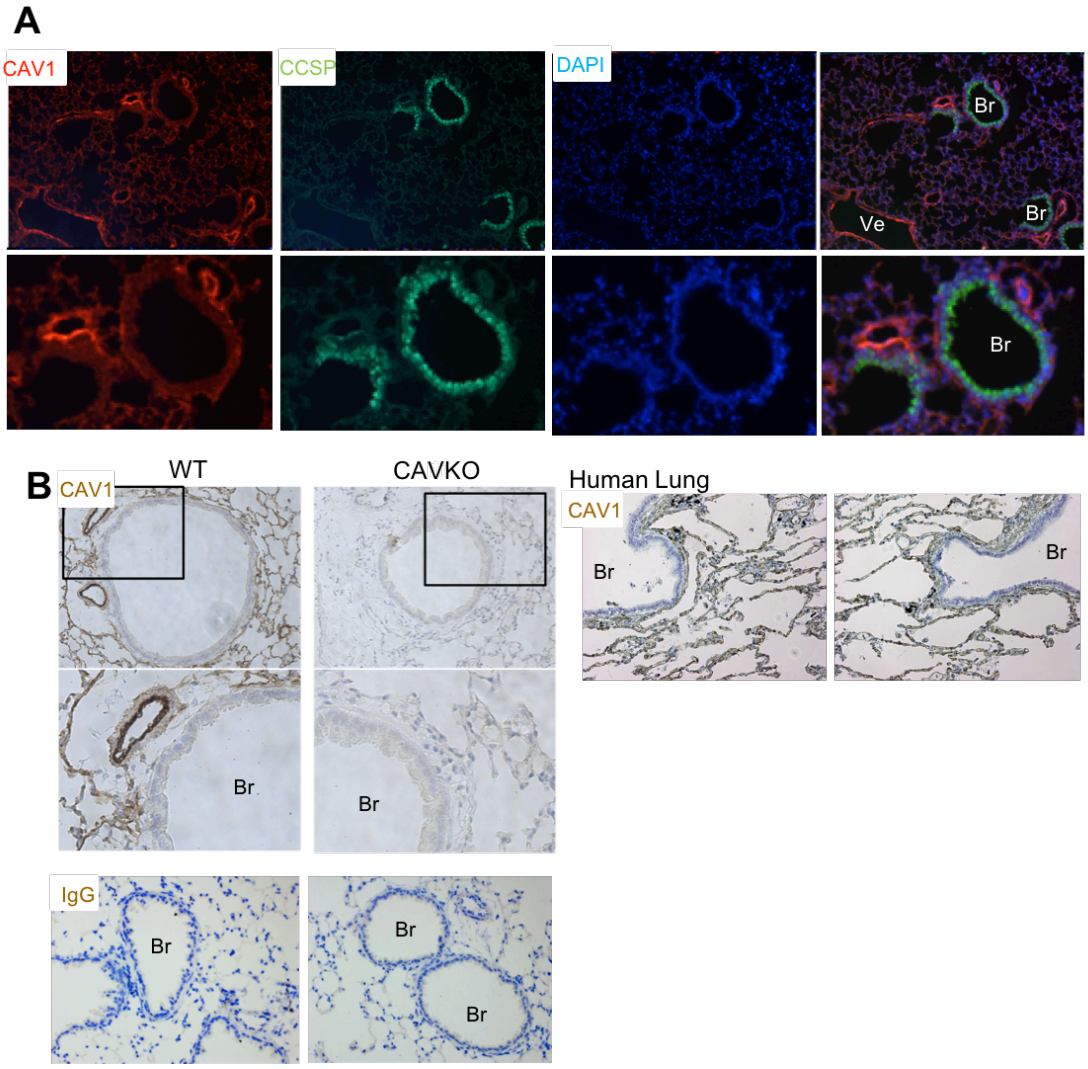

Figure 3.3. Caveolin-1 is Not Expressed in Bronchial Epithelial Cells Lungs from C57BL/6 mice and B6.Cg-*Cav1*^{tm1Mls}/J mice were fixed and embedded in paraffin. Sections were costained for caveolin-1 (red), clara cell secretory protein (green) and nuclei were stained with DAPI (blue). The merge shows cellular localisation of each protein (A).

Mouse lungs were also stained for caveolin-1 shown in brown (B, left). Human lung sections were also stained for caveolin-1 in brown (B, right).

Loss of Caveolin-1 affects inflammation in a cytokine specific manner

Despite a significant total reduction in cellular infiltrate in the BAL fluid, cytokines found in the BAL are upregulated by loss of caveolin-1, upon LPS stimulation (Fig.3.4). For example, CCL3, CCL4 and IL-12p70/p40 for example, were all increased in the CAV1KO mice following LPS treatment compared to WT. We therefore grouped the cytokines according to the effect of CAV1KO with LPS and whether the cytokines were sensitive to Gcs in both WT and CAV1KO (Fig.3.4A), sensitive in just WT (Fig.3. B), or non-responsive to Gcs (Fig3.4C).

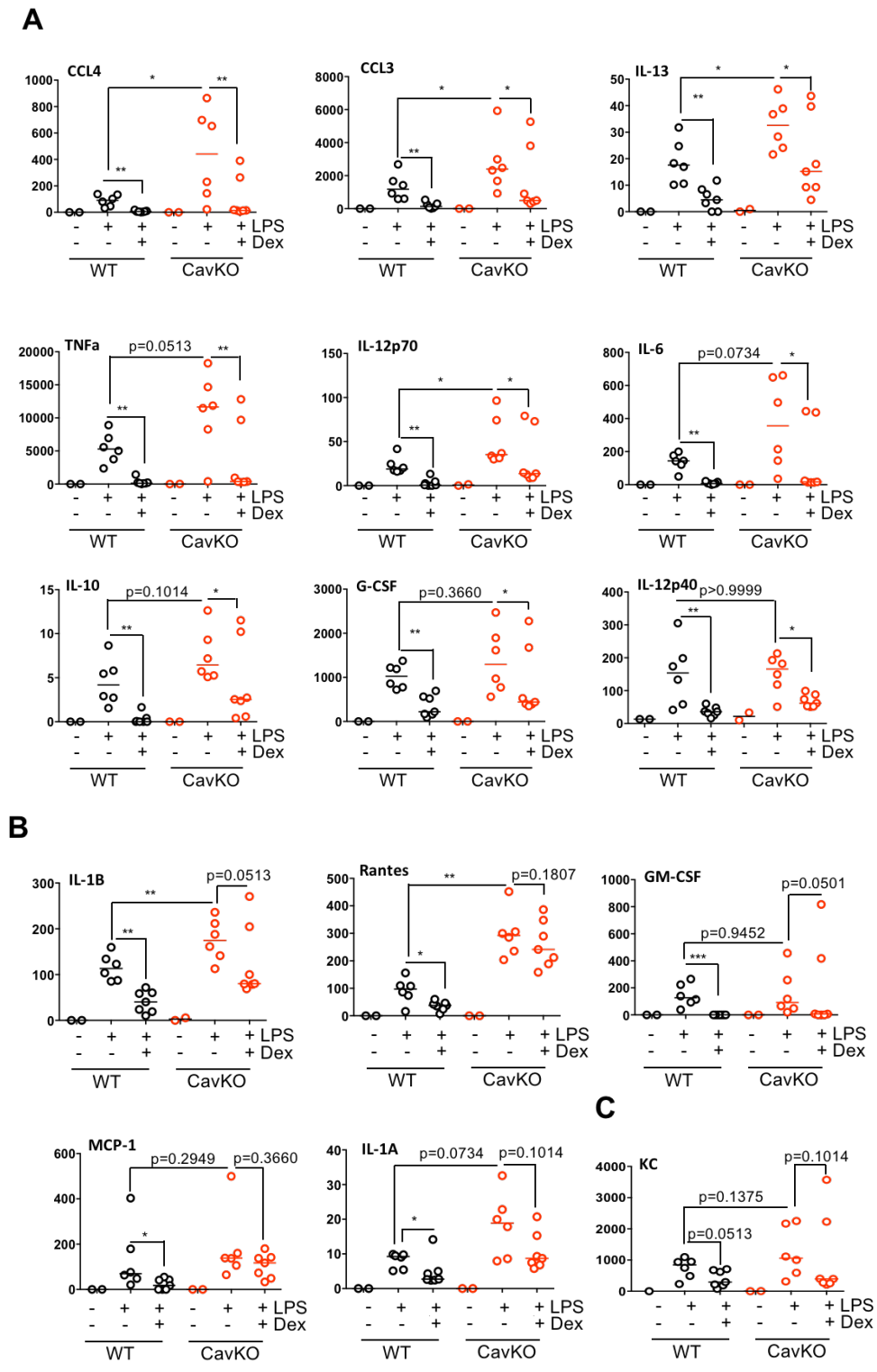

Figure 3.4 Caveolin-1 Knockout Causes Differential Expression of Cytokines: WT and CAVKO mice were treated with saline or dexamethasone (1mg/kg) for 1 hour before being exposed to aerosolised saline or LPS (1mg/ml) for 20 mins. Animals were then sacrificed 5 hours later, and BAL fluid was extracted. Cytokine content of BAL fluid was assessed by multiplex. Cytokines were grouped into 3

groups: significantly different between WT and CAVKO after LPS, and responsive to Dex in both genotypes (A), not significantly different between WT and CAVKO after LPS, and responsive to Dex (B) and unresponsive to Dex (C). Data analysed by 1-way ANOVA, median displayed unless otherwise mentioned. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Caveolin-1 regulates inflammation in a cell type specific manner

The cytokines grouped as Gc responsive in both genotypes, and showing significantly higher response in CAV1KO mice are regulated in macrophages, therefore we isolated RNA from "immune cell depleted" lung (Fig3.5A) (i.e. lung which has been lavaged) and also from purified alveolar macrophages (Fig3.5B) recovered from the BAL. Samples were treated with LPS, or LPS+Dex and the expression of CCL3 and CCL4 were examined by Q-RT PCR. The whole lung had significantly less CCL3 and CCL4 upon CAV1KO, however the CAV1KO alveolar macrophages had significantly more CCL3 and CCL4 expression. This result conforms with the literature (Wang, Kim, *et al.*, 2006), that caveolin-1 knockout in macrophages induces a pro-inflammatory phenotype, and may also explain the increased cytokines found in the BAL.

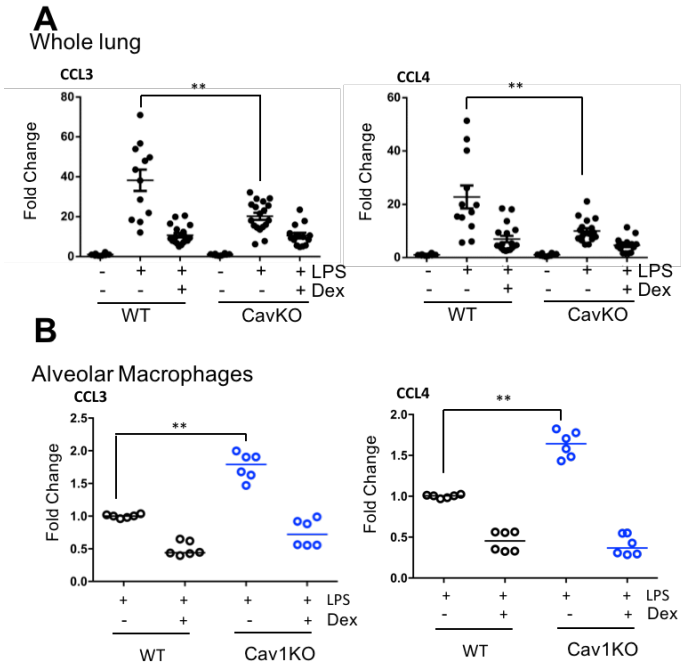

Figure 3.5. Caveolin-1 is Pro-Inflammatory in Lung Parenchyma and Anti-Inflammatory in Alveolar Macrophages: Lungs from WT and CAVKO mice were extracted and cultured *ex vivo* and treated

with vehicle (DMSO) or dex (100nM) for 1 hours before vehicle (saline) or LPS (100ng/ml) treatment for 5 hours and qRT-PCR analysis of CCL3 and CCL4 expression was performed (A). Alveolar macrophages were harvested from WT and CAVKO mice and treated with vehicle (DMSO) or dex (100nM) for 1 hour then LPS (100ng/ml) for 5 hours. RNA was extracted and qRT-PCR analysis of CCL3 and CCL4 expression was performed (B). Data analysed by 1-way ANOVA, mean and standard error displayed displayed for whole lung, median displayed for alveolar macrophages. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Caveolin-1 does not regulate T cell mediated immunity in the lung

To investigate the role of cav1 in controlling the anti-inflammatory effects of steroids during allergic asthma, C57BL/6 (WT), or Caveolin-$1^{-/-}$ (CAV1KO) mice were sensitized with I.P. ovalbumin (10μg), with an aluminium hydroxide adjuvant (2mg) (hereby referred to as OVA sensitisation) once a week for 2 weeks before three intranasal (I.N.) challenges of ovalbumin (50μg) on 3 consecutive days in the third week. Mice were given I.P. 1mg/kg dexamethasone (dex) or vehicle (1mg/kg cyclodextrin) 3 hours before each dose of I.N. ovalbumin. (Fig.3.6A).

Cell numbers in the bronchioalveolar lavage (BAL) fluid were quantified and cell type was determined by FACS. There was an increased in the total number of immune cells in both genotypes when challenged with ovalbumin, and these are significantly reduced upon dex treatment (Fig.3.6B, left). Eosinophils (Fig.3.6B, centre), neutrophils (Fig.3.6B right) and alveolar macrophages (Fig.3.6B bottom) were all increased upon ovalbumin challenge, and similarly reduced by dex. The majority of cellular infiltrate was made up of eosinophils.

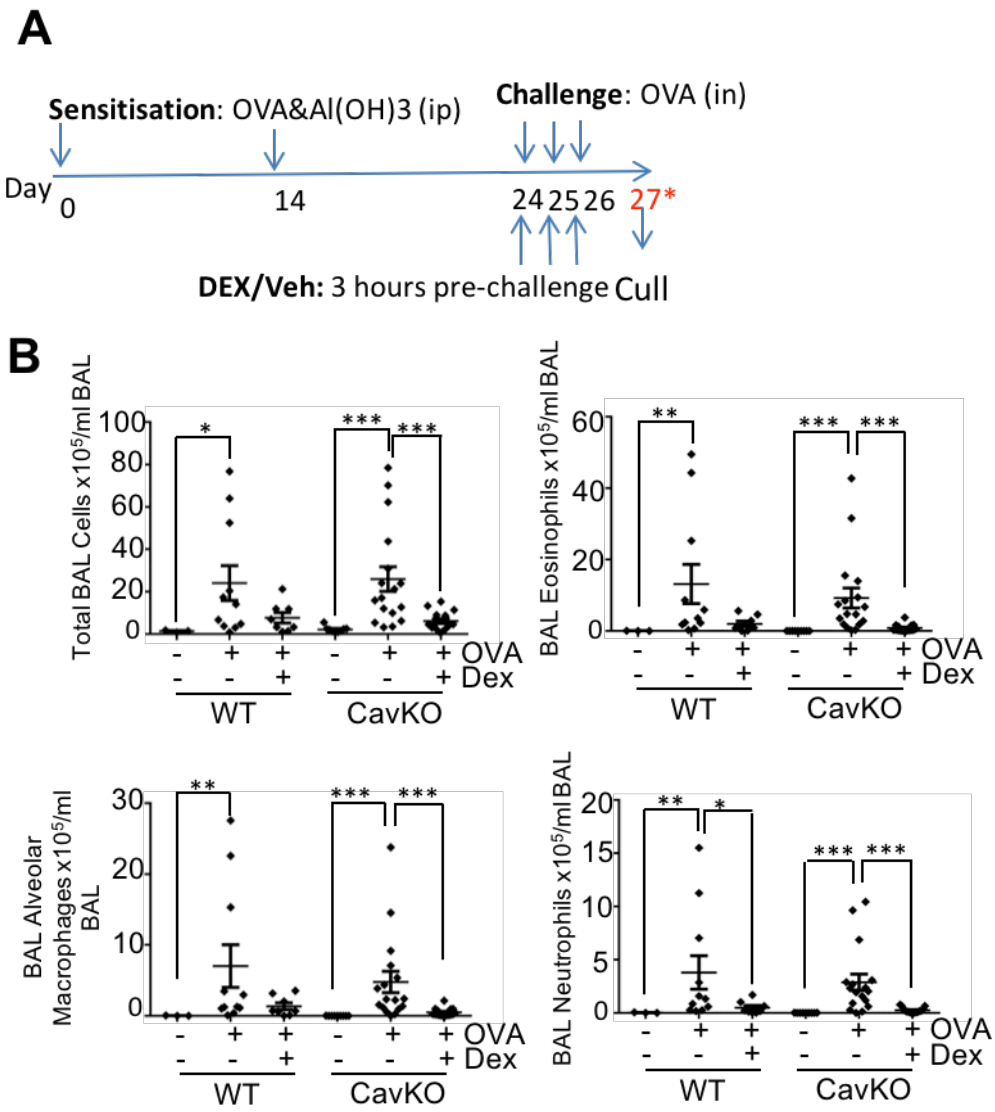

Figure 3.6. Caveolin-1 Knockout Does Not Affect Adaptive Immunity In the Lung: WT and CAVKO mice were treated with OVA and alum adjuvant for I.P. once a week for 2 weeks before treatment with cyclodextrin vehicle or dexamethasone (1mg/kg) for 3 hours before being exposed to I.N. OVA every 24 hours for 3 days, and culled 24 hours later (A). Animals were then sacrificed and lung cell infiltrate was removed by bronchoalveolar lavage, counted and stained for cell specific markers (Ly6G, Siglec-F and CD11b) (B). Data analysed by 1-way ANOVA, mean and standard error displayed displayed unless otherwise mentioned. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

3.6 Discussion

Here we show that loss of CAV1 results in an increase in Gc action, specifically transactivation, both *ex vivo* and *in vivo* in lung tissue. This is in contrast to data previously published from our lab (Matthews 2008), where no effect was seen on the genomic actions of GR, and also contrasting to data published on neural stem cell cultures (Peffer 2014) which showed a reduction in Gc action. As CAV1 expression is linked to various pulmonary inflammatory diseases such as asthma and COPD (Hackett 2013), it could potentially be used as a biomarker to predict if patients are likely to be Gc sensitive or resistant.

Contrary to previously published data, there was not an increase in the total number of cells infiltrating into the lung after OVA challenge (Aravamudan *et al.*, 2012). The total number of cells in the CAV1KO mice (mean $2.59x10^6$), however, is higher than that Aravamudan et al. published, as are the total cells in WT (mean $2.40x10^6$). This result may be due to the different conditions in animal units between research groups. It is widely accepted that different housing conditions can affect immune responses, and that housing mice from different conditions together can affect the immunity of both (Beura *et al.*, 2016). We did not yet assess the airway remodeling in the OVA challenged mice, and due to the importance of CAV1 in airway smooth muscle cells (Gosens *et al.*, 2011), and their role in airway remodeling in asthma (Bara *et al.*, 2010), it is important to determine the effect of CAV1KO in this context.

We also determine the effect of CAV1KO in localized lung inflammation, using the aerosolised LPS challenge. The local inflammation limits the activation of the hypothalamic-pituitary-adrenal axis, therefore resulting in less endogenous Gc release upon inflammation, which would confound the effect of dex treatment. Surprisingly, despite the increase in Gc transactivation seen in the lung explant culture, there was no more reduction in inflammation. Gc reduced the total cells, neutrophilia and cytokine production to the same level in the WT and the CAV1KO. This may be a limitation of the inflammatory model; in that it is extremely sensitive to Gc treatment. To establish whether the increased transactivation affects the inflammatory status in CAV1KO, above that of WT,

it would be necessary to titrate the Gc dosage back. As transactivation is the key determinant of the anti-inflammatory effect of GR (Vandevyver *et al.*, 2013), it is likely that the CAV1KO mice would require a lower Gc dose to sufficiently repress the inflammatory response, at least in the LPS model. Importantly, these data do not determine as to whether the seemingly protective CAV1KO is due to increased transactivation under basal conditions. Increased sensitivity to corticosterone may be another potential mechanism for the decreased inflammatory cell infiltrate seen in the CAV1KO. This should be assessed before moving forward. This is an unlikely explanation though, as chronic exposure to Gc results in increased inflammatory markers in response to allergen, and increase in systemic cytokine production. Furthermore, it results in an insensitivity to the anti-inflammatory actions of Gcs (Bailey *et al.*, 2009; Cheng, Jope and Beurel, 2015).

The reduced immune cell infiltrate in CAV1KO mice under LPS conditions conforms with the literature (Garrean *et al.*, 2006; Wu *et al.*, 2016), as does the increase in cytokine production (Wang, Kim, *et al.*, 2006). Macrophages are a key source of the cytokine production in the lung (Pribul *et al.*, 2008), therefore, despite the lung parenchyma displaying a reduction in inflammatory activation in the CAV1KO mice, the pro-inflammatory macrophages are able to compensate with increased cytokine production. This, however, does not result in an increase in cell number due to the effect of CAV1KO on cell motility. CAV1KO macrophages are indeed proinflammatory when assessed *ex vivo* via LPS stimulation, but also in response to *in vivo* serum cytokine analysis (Medina *et al.*, 2006), however, this is coupled by a decreased potential for movement to the site of damage, especially the lung (Garrean *et al.*, 2006). The mechanism of this is proposed to be linked to a lack of polarization in the mobile cells (Grande-García *et al.*, 2007), but also a loss of the necessary markers for leukocyte motility such as ICAM-1 (Garrean *et al.*, 2006) and VCAM-1 (Wu *et al.*, 2016) in the endothelia.

The lack of CAV1 in Club cells, but the importance of CAV1KO in causing a pro-inflammatory phenotype in macrophages sheds light on the role of each of these cells in the aerosolized LPS model. Loss of Club cells results in a proinflammatory phenotype, however, some of this increase in

inflammation and cytokines originates from macrophages of club cell depleted mice (Snyder *et al.*, 2010). Macrophages play a key role in inflammation, producing key cytokines for the induction of the innate immune response (Fujiwara and Kobayashi, 2005). Somewhat paradoxically, loss of macrophages results in an increase of proinflammatory cytokines during lung inflammation (Elder *et al.*, 2005) implying a repressive role of the alveolar macrophages. This can also be seen in resident lung macrophages that reduce the capacity for inflammation in alveolar epithelial cells through inhibitory calcium signaling (Westphalen *et al.*, 2014). These data suggest that the macrophage is more important than the Club cell in determining inflammation in response to both ovalbumin and LPS, with the role of the "time keeper" being the responsibility of the Club cell.

3.7 Supplementary Data

Table 3.S1 PCR Primers

Gene	Primers
β-Actin	F - AGG TCA TCA CTA TTG GCA ACG A
	R - CAC TTC ATG ATG GAA TTG AAT GTA GTT
CCL3	F - GCT CTC TGC AAC CAG TTC TCT
	R - TCG CTT GGT TAG GAA GAT GAC A
CCL4	F - CCA GCC AGC TGT GGT ATT C
	R - CAC CTA ATA CAA TAA CAC GGC ACA
TNFa	F - TGT TGT AGC AAA CCC TCA AGC
	R - TGT AGG CCC CAG TGA GTT CT
IL-6	F - CAA TGA GGA GAC TTG CCT GGT G
	R - GGT TGG GTC AGG GGT GGT TA
FKBP5	F - CGG AAA GGC GAG GGA TAC TC
	R - CGT GTA CTT GCC TCC CTT GA

CHAPTER 4 RESULTS: The Molecular Clock and Glucocorticoid Action

4.1 REV-ERBα Confers Circadian Control to Glucocorticoid Action

4.2 Abstract

The glucocorticoid receptor (GR) is a major drug target in inflammatory disease. However, chronic glucocorticoid (Gc) treatment leads to disordered energy metabolism, including increased weight gain, adiposity and hepatosteatosis; all programmes regulated by the circadian clock. Here we demonstrate that while anti-inflammatory actions are maintained irrespective of dosing time, the liver was significantly more sensitive during the day. Temporal segregation of Gc action was underpinned by physical interaction of GR with circadian transcription factor REV-ERBα, and cooperative binding of the two nuclear receptors on chromatin. Deletion of *Reverbα* inverted circadian liver Gc sensitivity. REV-ERBα dependent Gc-responses segregated metabolic actions, with daytime responsive genes regulating carbohydrate metabolism, and night-time responsive genes controlling lipid metabolism. Importantly, *Reverbα* null mice were protected from the increased adiposity and hepatosteatosis induced by chronic Gc administration in wild-type mice. This reveals a new mechanism by which the circadian clock acts through REV-ERBα to direct GR action on energy metabolism.

4.3 Introduction

Glucocorticoids (Gcs, cortisol in humans, corticosterone in rodents) are critical regulators of energy metabolism and immunity. Synthetic Gc are the most potent anti-inflammatory agents known, and are widely used therapeutically. However, long term use is accompanied by severe side effects, notably fat accumulation, hyperglycaemia, and hepatosteatosis (Schäcke, Döcke and Asadullah, 2002). Many aspects of metabolism and immunity are regulated by circadian mechanisms, and close coupling between cellular clock machinery and the glucocorticoid receptor (GR) has been

established. Specifically, interaction between the circadian factor cryptochrome (CRY) and GR influences Gc signalling to carbohydrate metabolism (Lamia *et al.*, 2011). Given the potential importance of dosing time of Gc action, we investigated circadian control of Gc responses. Number of animals for RNA-seq analysis was determined using ENCODE guidelines. For chronic dex dosing of WT and REV-ERBαKO mice, group sizes were decided for primary outputs (GTT, ITT, hepatic steatosis), based on (Patel *et al.*, 2011).

4.4 Results

We first defined the time-dependency of acute Gc responses using the synthetic Gc dexamethasone (dex; 1mg/kg) in a non-metabolic (lung) and metabolic (liver) tissue. Secretion of endogenous Gc follows a circadian pattern, with peak serum concentrations before the active phase (night in rodents). Dex administration was therefore timed to the middle of the day (ZT6, 1pm) or the middle of the night (ZT18, 1am), when endogenous corticosterone concentrations are similar (**Fig. 4.1A**). In both tissues, GR expression and nuclear localisation did not vary between times (**Fig. 4.1B, Fig. 4.S1A**). In total, 2419 Gc-regulated genes were identified. Of these, the majority were tissue-specific (627 genes in lung, 1665 genes in liver). Only 127 genes were regulated by Gc in both tissues, and were associated with anti-inflammatory Gc effects (**Fig. 4.1C**).

In lung, a similar total number of genes were responsive to Gc at the two times of dex administration, but of these, only 43% of genes were regulated at both time points, indicating remodelling of the Gc response (**Fig. 4.1D**). Gene ontology analyses revealed anti-inflammatory pathways as highly Gc-regulated at both times (**Extended data Table 4.1, Extended data Table 4.2**), and transactivated and transrepressed genes were equally represented (**Fig. 4.1E**). Time of day effects were confirmed by qRT-PCR for EFNA1, a night-specific Gc target, and Wt1, a day-specific Gc target (**Fig. 4.1F,G**).

Timing of administration had a major effect on Gc sensitivity in the liver, with 1709 genes responsive to daytime Gc administration, and only 211 genes regulated at night (**Fig. 4.1H**). Again, similar proportions of transactivated and transrepressed genes were observed at both times. The time-dependent switch in Gc sensitivity was validated by qRT-PCR for DIO1 and Aldh1b1 (**Fig. 4.1I,J**). The mapped GR cistrome annotated to (85%) of the Gc-regulated genes (**Fig. 4.S1B**). Pathway analyses revealed 'daytime-specific' Gc targets in the liver to be regulators of energy metabolism (**Fig. 4.S2, Table 4.3, Table 4.4**). Highlighting this time-dependent impact of Gc administration on cellular energy metabolism and oxidative phosphorylation there was a profound Gc-induced loss of mitochondrial mass only in daytime-treated animals (**Fig. 4.S3A, B**), with a concomitant rise in hepatic AMP concentration, and reduction in NAD^+ (**Fig. 4.S3C-E**).

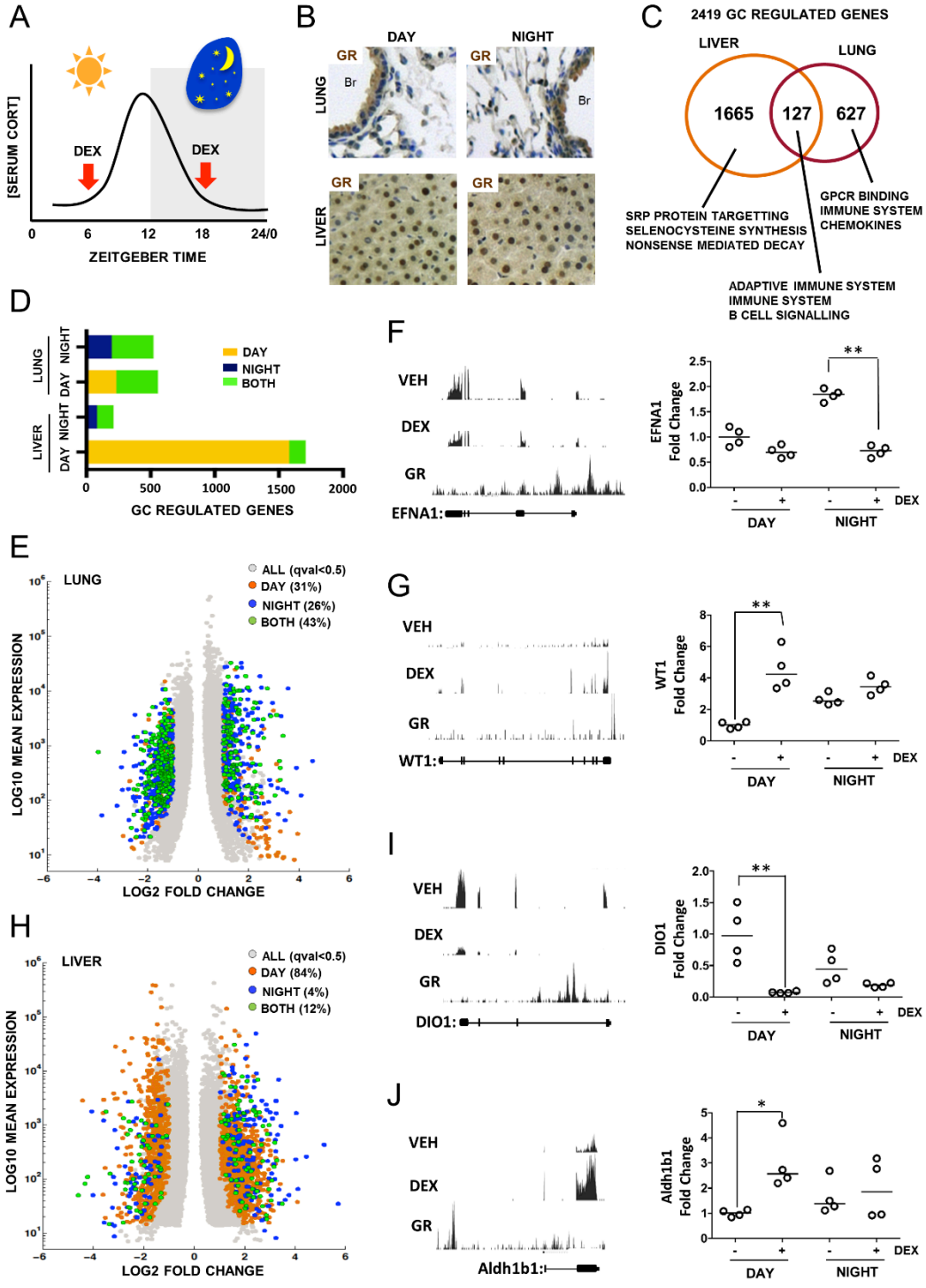

Figure 4.1 Gc sensitivity in liver is regulated by time of day. C57BL/6 mice were given vehicle or 1mg/kg I.P. dexamethasone at ZT6 (1pm, day) or ZT18 (1am, night), culled 2 hours later and lung and liver analysed by RNA-seq (**A**). GR immunohistochemistry in lung and liver at both times. GR expression is shown in brown, nuclei are blue. Br, bronchioles (**B**). Venn depicting all Gc regulated genes identified by DE-seq (N=2). Lung and liver specific targets are indicated, with gene ontology

terms for each group listed below (**C**). Time of day dependent Gc targets for each tissue are also shown (**D**). Base mean expression vs log2 fold-change plots for Gc regulated genes in lung show direction of regulation (**E**) and two time specific exemplars were validated by QPCR (**F, G**). Base mean expression vs log2 fold-change plots for Gc regulated genes in liver show direction of regulation (**H**) and two time specific exemplars were validated by QPCR (**I, J**). Individual data points are shown with median. Statistical analysis by Kruskal-Wallis test with a Dunn's multiple comparisons correction, where * $p < 0.05$, ** $p < 0.01$. RNA-seq analysis and mapping of GR ChIP-seq peaks (E, H, F, G, I) performed by M.I. All animal treatments, sample collection, IHC, ontological analysis and Q-PCR (B, C, D, E, F, G, H, I, J) performed by G.C.

The time-dependent switch in Gc sensitivity suggests regulation by a daytime activator, or night-time repressor in liver (**Fig 4.2A**). A major regulator of energy metabolism in the liver is the circadian clock. The molecular clockwork is conserved in all cell types, and is driven by rhythmic transcriptional/translational feed-forward (BMAL1, CLOCK/NPAS2) and negative feedback (Cry1/2, Per1/2 and REV-ERBα/β) loops (**Fig 4.2B**). The chromatin-loading of these core clock transcription factors shows strong daily variation (**Fig 4.2C**). To define the role of the core clock in setting time-of-day Gc sensitivity we compared our Gc-regulated genes with annotated circadian transcription factor responsive gene lists (Cho *et al.*, 2012; Koike *et al.*, 2012). The largest overlap was observed with REV-ERBα (58%) and REV-ERBβ (53%) target genes, with more restricted overlap of CRY1 (48%) and CRY2 (39%) (**Fig. 4.2D**), and other circadian clock factors (**Fig. 4.S4**). Moreover, GR and REV-ERBα were found in the same molecular complexes by co-immunoprecipitation (**Fig. 4.2E**), supporting a direct modulatory effect of REV-ERBα in shaping Gc-response.

To profile genome-wide binding patterns of GR and circadian factors, we determined the proximity of the mapped cistromes of GR to each of the circadian transcription factors in liver (**Fig. 4.2F, Fig. 4.S5A**). Putative co-binding was defined as a distance of less than 120bp between ChIP-Seq summits,

and high stringency (FE30) co-bound sites indicative of cooperative binding (leftmost peak in **Fig. 4.2F**). A surprisingly high frequency of co-binding with GR was observed for REV-ERBα and REV-ERBβ, with median inter-peak distances of only 93 and 80bp respectively (**Fig. 4.S5B**). Co-binding of GR and CRY was also revealed, consistent with the reported physical interaction between GR and CRY (Lamia *et al.*, 2011). However, sites of REV-ERBα and REV-ERBβ co-binding represented 52% and 49% of GR peaks, compared with 36% and 28% of GR summits lying in proximity with CRY1 and CRY2 summits, respectively (**Fig. 4.S5C**). There was large overlap between GR-REV-ERBα and GR-REV-ERBβ co-bound genes (**Fig. 4.S5D**).

Given the potential influence of REV-ERBα on Gc action, time-of-day dependent dex-responses were defined in livers of *Reverbα*$^{-/-}$ mice (REV-ERBαKO). In contrast to previous reports on CRY regulation of GR, *Reverbα* null mice exhibited suppression of endogenous corticosterone by dex, and normal glucose tolerance (**Fig. 4.S6**). However, absence of REV-ERBα caused a dramatic change in the temporal characteristics of Gc response, with loss of many daytime-responsive genes, and acquisition of additional Gc-targets at night (**Fig. 4.2G, Fig. 4.S7A**). Importantly, there was restricted overlap between lost daytime and acquired night Gc targets (**Fig. 4.S7B**), suggesting REV-ERBα-dependent rewiring of the Gc response, and not simply inversion of the liver clock. Across both time-points, this revealed a REV-ERBα-dependency in Gc-response for 2018 genes, of which 46% contained cobound sites for REV-ERBα and GR (**Fig. 4.2H,J**). The REV-ERBα-dependent, co-bound, day-specific Gc target genes were associated with glucose metabolism (**Fig. 4.2H,I**), while REV-ERBα-dependent, co-bound, night-specific Gc regulated genes were associated with lipid metabolic processes (**Fig. 4.2J,K**). In keeping with REV-ERBα acting as a transcriptional repressor, those genes which acquired Gc regulation in the REV-ERBαKO mice were mainly transactivated (**Fig. 4.3A, Extended data Table 4.5**). We considered CRY1 and LXR as potential mediators of the REV-ERBα effect, however, REV-ERBα deletion did not affect CRY1 or LXR targets genes (**Fig. 4.S8, Extended data Table 4.6**). Additionally, there was no effect of REV-ERBα loss on anti-inflammatory Gc effects

in liver or isolated bone marrow derived macrophages (**Fig. 4.S9**), further reinforcing the target-specific cross talk between GR and REV-ERBα.

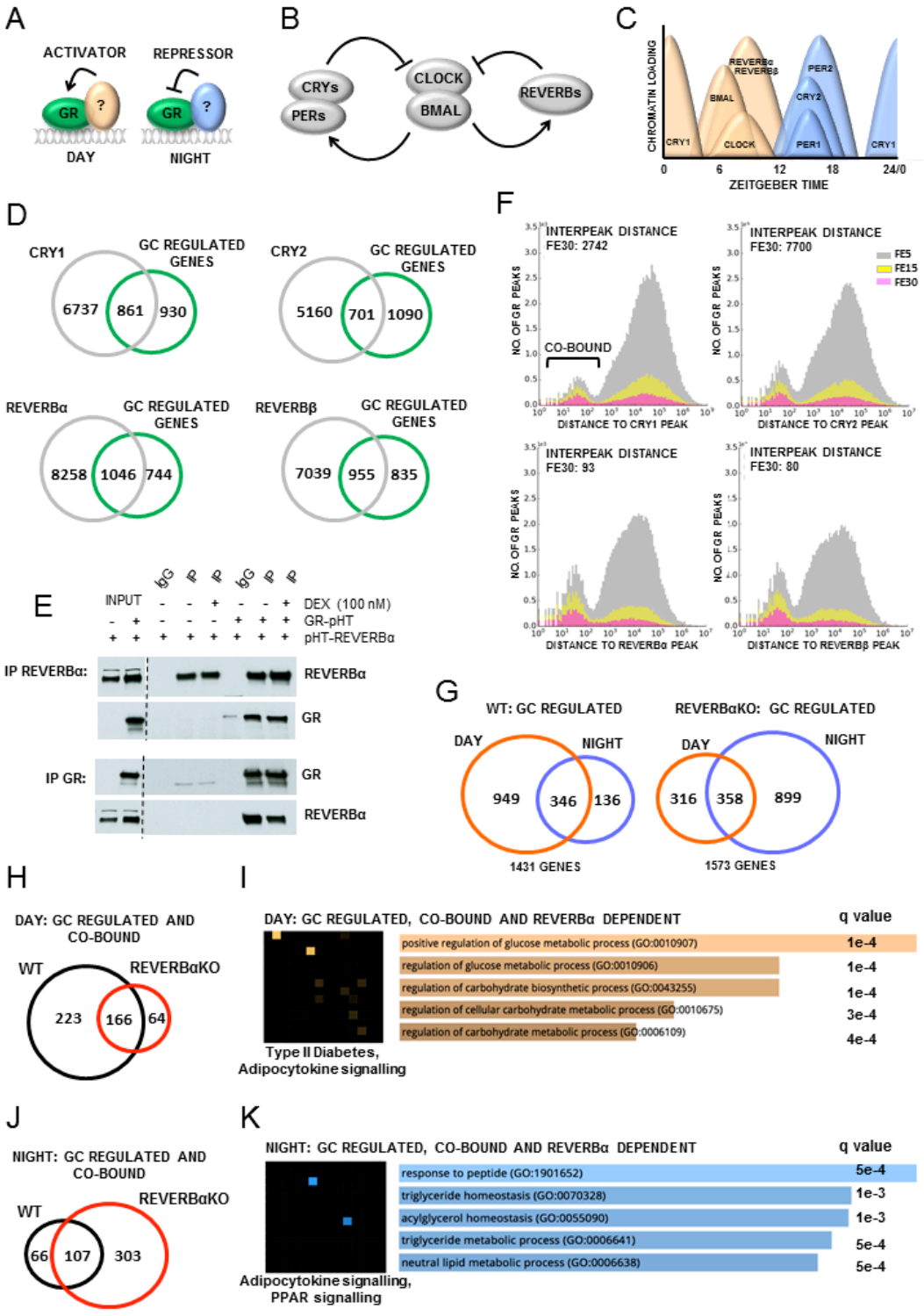

Figure 4.2. REV-ERBα regulates GR function. The time of day variation in Gc sensitivity suggests regulation of GR function by a day activator or night repressor (**A**). Components of the molecular

clockwork (**B**) function as time of day specific regulators of transcription[25] (**C**). Venns show overlap between Gc target genes, and clock transcription factor (CRY1, CRY2, REV-ERBα and REV-ERBβ) regulated genes in liver (**D**). Co-immunoprecipitation of epitope tagged REV-ERBα and GR (**E**). Co-binding analysis, histograms depict the distance between GR binding events and the nearest clock transcription factor ChIP-seq summit, using three stringencies (fold enrichment (FE) scores). Median interpeak distances for the highest stringency (FE30) is shown inset (**F**). C57BL/6 (WT) and REV-ERBα knockout (REV-ERBαKO) mice were given 1mg/kg I.P. dexamethasone at ZT6 (day) or ZT18 (night), culled 2 hours later and livers analysed by RNA-seq. (**G**). Day (**H**) and night (**J**) regulated Gc targets were further stratified for regions of co-binding from (**F**). Gene ontology of Gc regulated, co-bound, REV-ERBα dependent genes using Enrichr in the day (**I**) and at night (**K**).) I.P. (E) performed by M.P. Co-binding analysis (F) performed by M.I. RNA-seq analysis performed by P.W. (G). All animal treatments, sample collection comparison of ChIP-seq to RNA-seq and gene ontology performed by G.C. (D, G, H, I, J, K)

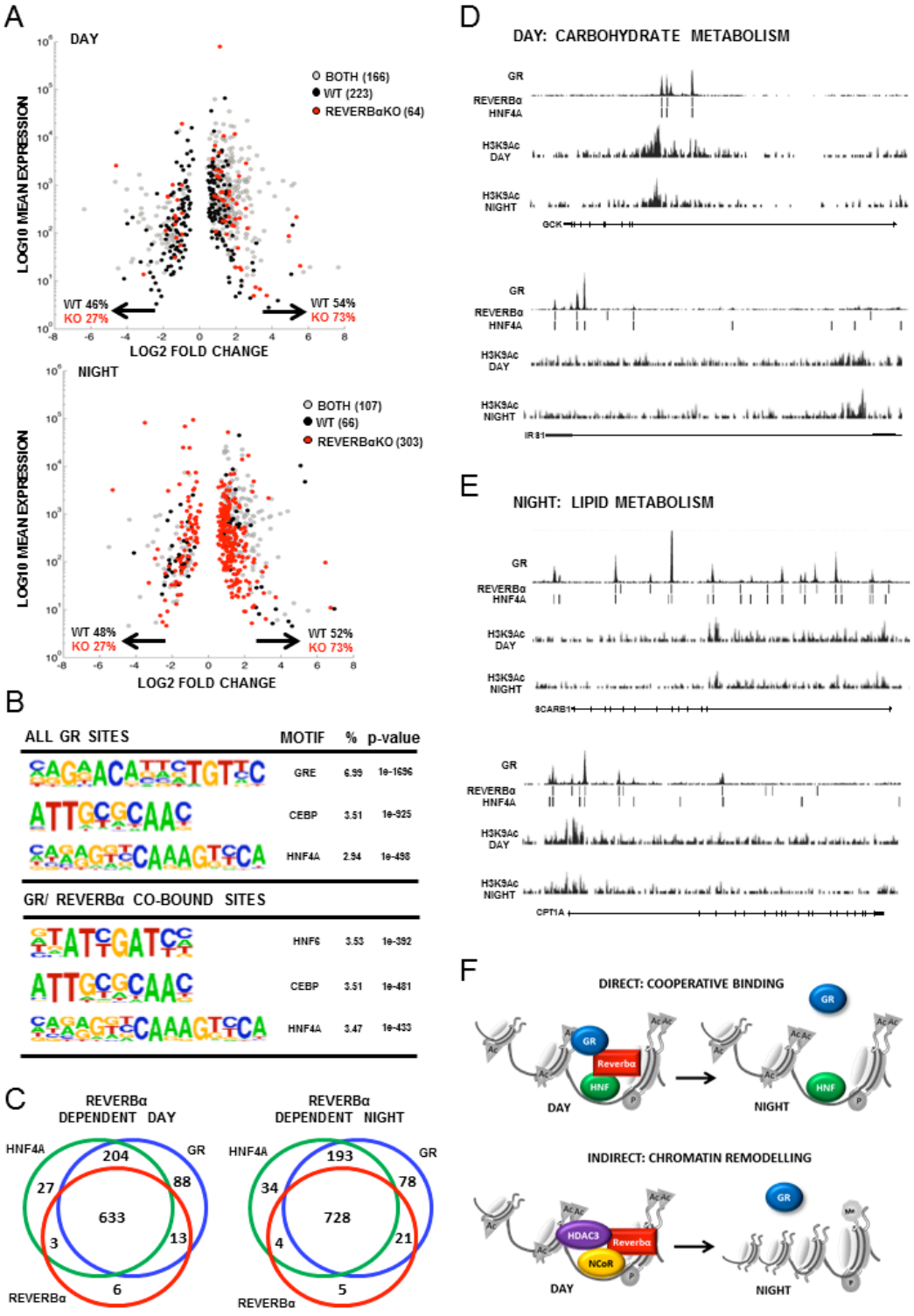

Figure 4.3. Rhythmicity of Gc sensitivity in liver is determined by REV-ERBα and HNF transcription factors. Base mean expression vs log2 fold-change plots for Gc regulated genes in day and night show direction of gene regulation (**A**). Summary of top ranked motifs (by coverage % observed/expected) under all GR peaks or GR/REV-ERBα co-bound peaks (interpeak distance <120

bp) (**B**). Overlay of Gc regulated genes with GR/ REV-ERBα/HNF ChIP-seq in the day and night (**C**). Gene tracks of WT specific (IRS1, GcK) Gc targets in the carbohydrate ontology (**D**), and REV-ERBαKO specific (SCARB1, CPT1A) Gc targets in the lipid ontology (**E**). UCSC gene tracks showing alignment of GR peaks, REV-ERBα/HNF4A summits, and H3K9Ac peaks in day/night. Schematic summarising the role of REV-ERBα in regulating liver specific, time dependent changes in Gc sensitivity (**F**). RNA-seq analysis (A) performed by P.W. Motif analysis and ChIP-seq peak mapping (B,D,E) performed by M.I. Animal treatments, sample collection and comparison of ChIP-seq peak locations (A, C) performed by G.C.

Motif analysis at sites of GR:REV-ERBα co-binding revealed enrichment for hepatocyte-specific transcription factors, HNF6 and HNF4A (**Fig. 4.3B, Fig. 4.S10,4.S11**). HNF4A is highly expressed in the liver compared to HNF6 (**Fig. 4.S12**), and there was clear overlap seen between binding sites of HNF4A, GR and REV-ERBα at REV-ERBα-dependent Gc-regulated genes (**Fig. 4.3C**). For example, Gc-regulated carbohydrate (IRS1, GcK) and fatty acid (SCARB1, CPT1A) metabolic genes show striking alignment of GR, REV-ERBα, and HNF4A binding (**Fig. 4.3D,E)**, identifying these as sites of functional cross-talk. Consistent with our findings, REV-ERBα was recently shown capable of binding DNA by tethering to HNF4A in a mechanism that does not require the REV-ERBα DNA binding domain (Zhang *et al.*, 2015) and HNF4A has been suggested as a GR pioneer factor (Reddy *et al.*, 2007). REV-ERBα repressive action involves recruitment of NCOR and HDAC3 (Zhang *et al.*, 2015), and indeed, many co-bound enhancers show time of day changes in Histone H3K9Ac (**Fig. 4.3D,E)**, a mark regulated by HDAC3. Thus, our data indicate that time-dependent Gc effects are a product of (i) the daily rhythm of REV-ERBα expression with attendant HDAC3 recruitment and (ii) cell lineage-determining transcription factors (eg HNF4A; **Fig. 4.3F**).

Long-term Gc treatment drives abnormal carbohydrate, and lipid metabolism and we therefore investigated the impact of GR:REV-ERBα cross-talk on the metabolic consequences of chronic Gc

treatment. Mice were treated with dex at ZT6 every 48 hours for 8 weeks. There was marked thymic atrophy in both WT and REV-ERBαKO animals (**Fig. 4.4A**), further supporting a lack of cross-talk between GR and REV-ERBα in immune regulation (Gibbs *et al.*, 2012, 2014). Dex-treated WT mice gained weight, while REV-ERBαKO mice did not (**Fig. 4.4B, Fig. 4.S13A**). Gc treatment did not affect glucose tolerance, insulin levels, or liver glycogen in either genotype, but caused a significant increase in fasting glucose only in WT animals (**Fig. 4.S13B-E**).

Importantly, while WT mice accumulated significant fat mass on long-term Gc treatment, REV-ERBα null mice were protected from Gc-related adiposity (**Fig. 4.4C,D, Fig. 4.S14A**). Dex treatment also increased adipocyte size, and heterogeneity only in WT mice (**Fig. 4.4E-G, Fig. 4.S14B**). The catabolic actions of Gc, with accumulation of adipose tissue, are characteristic of the changes seen in people treated with Gc, and these changes impose a major limitation to therapeutic use of synthetic Gc. Moreover, Gc actions in the liver inhibit beta-oxidation of fatty acids, ketogenesis, and promote synthesis of triglycerides resulting in hepatosteatosis. REV-ERBα deletion provided a striking protection from Gc-induced hepatic triglyceride accumulation and lipid droplet formation, hallmarks of hepatosteatosis (**Fig. 4.4H,I, Fig. 4.S15**), with no effect on serum free fatty acids or triglycerides (**Fig. 4.4J,K**).

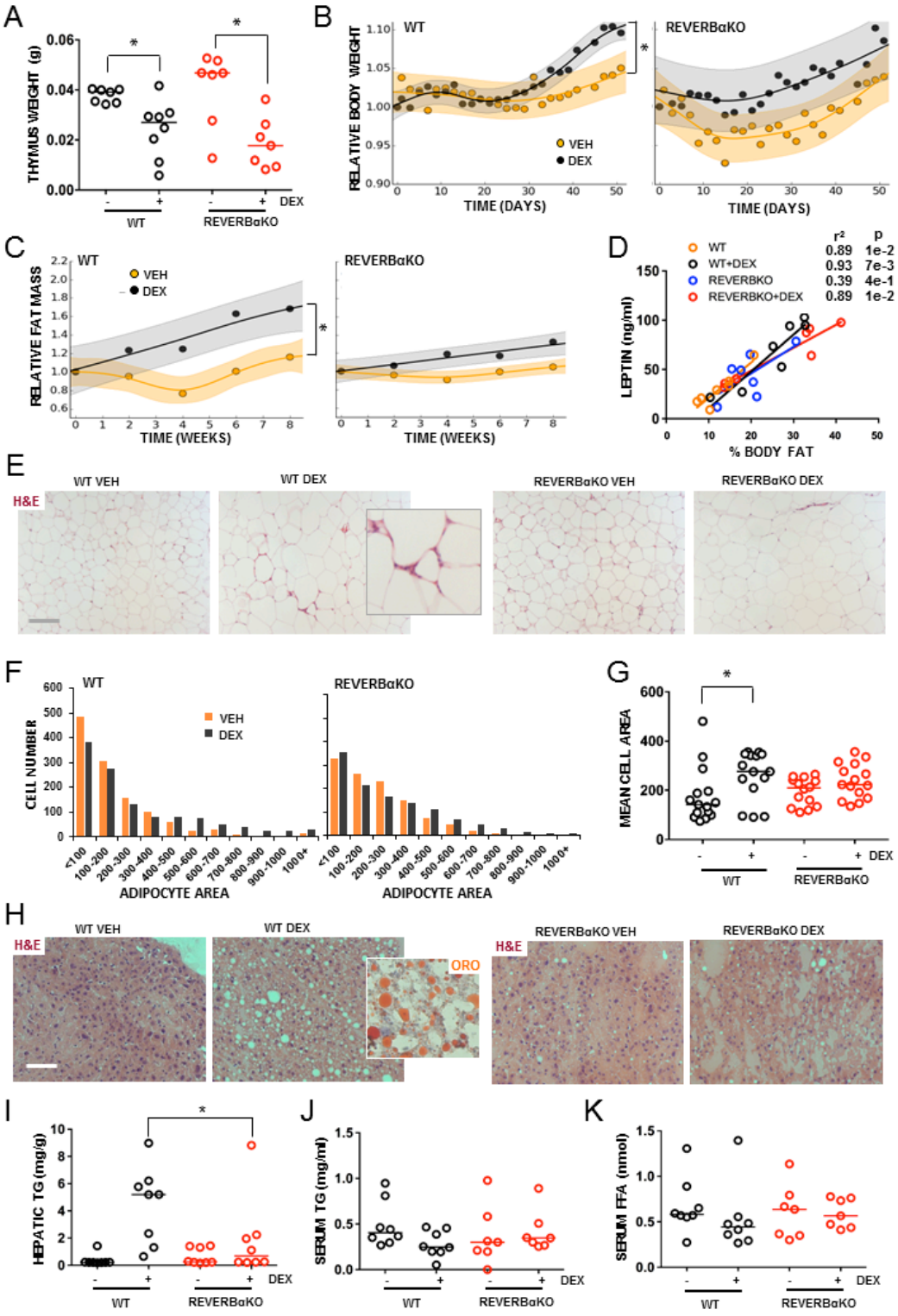

Figure 4.4. REV-ERBα mediates Gc dependent triglyceride accumulation. C57BL/6 (WT) and REV-ERBαKO mice were given 1mg/kg I.P. dexamethasone or vehicle at ZT6 every 48hrs for 8 weeks. Thymus weight was measured at cull (**A**), and body weight tracked throughout the study (**B**). Body fat percentage was measured every 14 days by Echo MRI (**C**). Serum leptin was plotted against body

fat percentage, with r^2 and p values shown inset (**D**). H&E of visceral adipose (**E**) and quantification of adipocyte size (**F,G**). H&E of liver, collected at cull with oil red O stain shown inset (**H**). Liver triglycerides (**I**), serum triglycerides (**J**) and free fatty acids (**K**) were also analysed. Graphs show either the mean for each group, or individual animals with median. In (**A**) and (**F**), the solid lines and filled regions show the model output (mean and credible regions) while the circles are the data points (mean over replicates). Statistical analysis via two-way ANOVA repeated measures (body weight and fat mass), Mann-Whitney test (Thymus weight) or Kruskal-Wallis test with a Dunn's multiple comparison correction (hepatic triglycerides) * $p < 0.05$, **$p < 0.01$. Mathematical modelling of weight and fat mass (B, C) performed by M.I. H&E (E, H) performed by R.V. Oil Red-O (H) performed by L.M. Leptin, Triglyceride and FFA (D, I, J, K) performed by A.T. All sample collection, weighing of Thymus and animals, echo-MRI, analysis of all data (A, B, C, D, E, F, G, H, I, J, K) performed by G.C. Animal treatments performed by G.C with assistance from D.B.

4.5 Discussion

Therapeutic use of Gc in people remains common, but is plagued by major off-target effects, notably disordered lipid metabolism that can lead to metabolic syndrome, a major risk for cardiovascular disease. Gc use is a major risk factor for hepatosteatosis, a state leading to disruption of liver function, with resulting inflammation, fibrosis and organ failure. There is renewed interest in timing of Gc therapy, with night-time release of prednisolone offering a small additional therapeutic effect (Buttgereit *et al.*, 2008). We now demonstrate that timed administration of Gc can minimise off-target Gc effects, and thereby increase therapeutic index, by utilising the underlying circadian rhythm of liver Gc sensitivity. The time of day variation in Gc response is mediated by the core clock transcription factor REV-ERBα. Our data suggests that timing of Gc administration to coincide with the nadir of REV-ERBα expression (midday in humans), or using REV-ERB ligands may restrict the spectrum of Gc activities. More generally embedding clock logic in therapeutic use of Gc can be a powerful tool in targeting specific physiological and pathological programmes.

Acknowledgements: We gratefully acknowledge Andy Hayes and Stacey Holden (University of Manchester Genomics facility), Peter Walker and Natalie Partington (University of Manchester Histology facility), Rupasri Mandal (The Metabolomics Innovation Centre, University of Alberta), and Bruce Wisely and Paul Holmes (Protein Cellular and Structural Sciences, GlaxoSmithKline) for technical assistance. We acknowledge GlaxoSmithKline for backcrossing and breeding of the REV-ERBα null mice. We acknowledge MRC, Wellcome Trust, and GlaxoSmithKline for funding. Gc was supported by a MRC CASE studentship. LCM was supported by a University of Manchester Stepping Stones fellowship.

Author contributions: Gc, LCM and DWR conceived the project. Gc completed all *in vivo* studies, processed and analysed tissue samples, primary data and performed statistical analysis. MI analyzed RNA-seq data, ChIP-seq data, performed co-binding and motif analysis and modelled time series data. PW analysed RNA-seq data. RMV, NB, MP, ZZ, LMI, MB, TMP and DAB assisted with *in vivo* studies. Gc, AJT and JLW completed biochemical assays. MP completed co-immunoprecipitation studies. ZZ performed *ex vivo* LPS challenge in macrophages. DRS and DAD generated the REV-ERBα monoclonal antibody. ASIL, DAB, MR, LCM and DWR supervised the project. Gc, DAB, MR, LCM and DWR wrote the paper. Gc and LCM prepared the figures. All authors edited the manuscript.

4.6 Materials and Methods

4.6.1 Materials

Mouse GR antibody (Cat# sc-1004) was purchased from Santa Cruz biotechnology, human GR antibody (Cat# 24050-1-AP) was purchased from ProteinTech. GSK6F05 anti-REV-ERBα was generated in collaboration with GlaxoSmithKline. Dexamethasone, methyl-cyclodextrin, dextrose and standard chemicals were purchased from Sigma Aldrich.

4.6.2 Cell Lines

Mycoplasma free HEK293 cells were purchased from ATCC, and maintained in Dulbecco's modified eagle's medium (DMEM 4500mg glucose/L, 110mg sodium pyruvate/L and L-glutamine, Sigma Aldrich) with 10% v/v heat inactivated bovine serum (FBS, Invitrogen) in a humidified atmosphere of 5% carbon dioxide at 37°C.

Primary bone marrow derived macrophages were purified and cultured in growth media (DMEM containing 4500mg/l glucose, 110mg/l sodium pyruvate, L-glutamine and 10% heat inactivated bovine serum) supplemented with 0.1mg/ml M-CSF in a humidified atmosphere of 5% carbon dioxide at 37°C.

4.6.3 Animals

Experimentation was performed on mouse strains C57BL/6J (WT) from Harlan Blackthorn, UK and global REV-ERBα knockout mice and littermate controls imported from GlaxoSmithKline, Stevenage UK. There, the REV-ERBα knockout mice were re-derived (from the original colony held by Ueli Schibler, Geneva) using in-vitro fertilisation procedures with resulting heterozygous REV-ERBα animals subsequently backcrossed to a C57BL/6J background to over 98%, as confirmed by MAXBax. Homozygous REV-ERBα knockout and wildtype littermate controls were generated through Heterozygous x Heterozygous matings. Animals housed at GlaxoSmithKline were multiply housed in autoclaved Techniplast GM500 IVC cages containing IPS Lignocel BK8/15 bedding with Datesand Paper Shaving nesting material, within a Techniplast SmartFlow ventilation system. Animals are maintained at an ambient temperature of 20.5ºC to 23.5ºC and relative humidity of 39% to 61%, maintained on a 7am to 7pm light–dark cycle, with free access to food (Labdiet Irradiated 5LF2 Maintenance Diet) and animal grade drinking water. All mice (8-22 weeks old, male and female) were acclimatised in the biological services facility for one week before any procedures were undertaken. All procedures were performed in compliance with Animals (Scientific Procedures) Act of 1986. While housed in Manchester, mice had free access to food (unless stated) and water and

were multiply housed in a 12:12 light/dark cycle. Animals were randomly allocated to treatment groups and coded, then samples processed and decoded post analysis to limit any investigator bias. To give sufficient power to identify differences between treatment groups for RNA-seq studies, group sizes of 2 (lung and liver) or 5 (WT and REV-ERBα) were used, for *in vivo* biology group sizes of 8 were used, and for metabolic profiling, groups of 10 were used.

4.6.4 Acute Gc treatment

Mice were treated at 6 hours ZT (6 hours after lights on, 1:30pm) or at 18 hours ZT (6 hours after lights off, 1:30am) with dexamethasone (1 mg/kg intraperitoneal) or vehicle (methylcyclodextrin 1mg/kg intraperitoneal) for either 2 or 4 hours before sacrifice by cervical dislocation.

4.6.5 Chronic Gc treatment

Mice (N=8) were treated at 6 hours ZT (6 hours after lights on) with dexamethasone (1mg/kg intraperitoneal) or vehicle (saline intraperitoneal) every 48 hours for 8 weeks before sacrifice by cervical dislocation. Two mice (one REV-ERBαKO vehicle, one REV-ERBαKO dex) were sacrificed prior to the end of the study for ill health. With the exception of hepatic triglycerides, an early, and robust Gc response, all other samples were excluded from subsequent analysis.

4.6.6 Glucose Tolerance Tests

Mice were fasted over-night (12 hours) and injected at ZT6 with 2g/kg dextrose (glucose tolerance test, GTT). Blood glucose was measured over 3 hours (GTT) (Aviva Accucheck). GTT was performed prior to chronic treatment (week -1) N=16 biological replicates, during treatment (week 7) WT N=8 biological replicates, REV-ERBαKO N=7 biological replicates.

4.6.7 Body weight and adiposity

Mice were weighed every 48 hours between ZT3 and ZT5 and placed in EchoMRI 900 (Echo Medical Systems) every two weeks between ZT6 and ZT9 for a total read time of between 140s to 160s. An average of 3 readings were taken. Percentage body fat was calculated from values determined by the Echo MRI. WT N=8 biological replicates, REV-ERBαKO N=7 biological replicates. Raw data on body weight (26 measurements) and body fat (5 measurements) were normalized to the first reading for each animal, as shown in Fig. S13A and 14A, respectively.

4.6.8 Ex vivo LPS challenge

Bones from global REV-ERBα knockout mice and littermate controls (males, aged 12-16 weeks) were collected and processed independently (N=3). Primary cells were cultured in growth media supplemented with 0.1mg/ml M-CSF for 7 days prior to experimentation. On day 8, cell culture medium was replaced (without M-CSF) and cells treated with vehicle or 100nM dex for 1hour, and then 100ng/ml LPS for a further 4hours. Cells were lysed and processed for qRT-PCR.

4.6.9 RNA-seq and qRT-PCR

Lung, liver and macrophages were lysed and total RNA prepared using SV Total RNA Isolation System (Promega). RNA quality was checked using the RNA 6000 Nano Assay, RNA samples with a 260:280 nM ratio of ~2 taken forward for analysis. Quality and integrity of total RNA samples were assessed using a 2100 Bioanalyzer or a 2200 TapeStation (Agilent Technologies) according to the manufacturer's instructions.

RNA sequencing (RNA-seq) libraries were generated using the TruSeq® Stranded mRNA assay (Illumina, Inc.) according to the manufacturer's protocol. Briefly, total RNA (0.1-4ug) was used as input material from which polyadenylated mRNA was purified using poly-T, oligo-attached, magnetic beads. The mRNA was then fragmented using divalent cations under elevated temperature and then reverse transcribed into first strand cDNA using random primers. Second strand cDNA was then synthesised using DNA Polymerase I and RNase H. Following a single 'A' base addition, adapters were

ligated to the cDNA fragments, and the products then purified and enriched by PCR to create the final cDNA library. Adapter indices were used to multiplex libraries, which were pooled prior to cluster generation using a cBot instrument. The loaded flow-cell was then paired-end sequenced (101 + 101 cycles, plus indices) on an Illumina HiSeq2500 instrument. For comparison of time of day Gc responses in lung and liver N=2 biological replicates were sequenced, for comparison time of day responses in liver between WT and REV-ERBαKO mice N=5 biological replicates were sequenced. Demultiplexing of the output data (allowing one mismatch) and BCL-to-Fastq conversion was performed with CASAVA 1.8.3.

FastQ files containing paired-end reads were quality checked with FastQC tool, followed by Trimmomatic (Bolger, Lohse and Usadel, 2014) in order to trim the low-quality and adapter sequences from the reads. Filtered reads were aligned to GRCm38.71 (mm10) assembly of the mouse genome using Tophat-2.0.11 (Trapnell, Pachter and Salzberg, 2009), reporting best score matches for every read. Mapped reads were counted to genes, for Ensembl annotation GRCm38.71, using HTSeq-count (v0.5.4p5, (Anders, Pyl and Huber, 2015)), with default quality score, and with options stranded=reverse, *and* intersection_nonempty.

DESeq2 (Love, Huber and Anders, 2014) was used to perform normalization and pairwise comparisons (flat design). Differentially expressed (DE) genes were reported for *qval <= 0.05* and fold change of 2 (Fig. 1) or *qval <= 0.1* (Fig. 3), and were taken forward for further downstream analysis. High stringency q values were used for the small sample group (n=2), and lower stringency q values used for the larger group size (n=5). Online bioinformatics tools, webgestalt (http://bioinfo.vanderbilt.edu/webgestalt/) and enrichr (http://amp.pharm.mssm.edu/Enrichr/) were used for enrichment analysis of the DE genes.

4.6.10 Mitochondrial Genome Quantification

Livers and lungs were homogenised and DNA extracted using TRIzol (Invitrogen) and mitochondrial DNA (ND1) was quantified in using qRT-PCR (Applied Biosystems). Expression levels were calculated

using the δδCT method normalising to the nuclear genome (GAPDH). Primer sequences are described in Extended data Table7. N=10 for lung, 10 vehicle liver ZT6, 9 dex liver ZT6 and vehicle liver ZT18 and 7 dex liver ZT18.

4.6.11 ChIP-seq Analysis

GR ChIP-seq and matched control data from GEO: GSE46047 (Grøntved *et al.*, 2013) was downloaded and FastQ files were mapped to mm10 genome using Bowtie1 (Langmead *et al.*, 2009) with default options except '−m1' option, keeping only uniquely mapped reads. Peaks were called using MACS 2.1.0 (*17*) with default parameters, except *–nolambda* option. To associate identified peaks to mm10 annotated genes, HOMER (*18*) (annotatePeaks.pl) was used, with default options.

For co-binding analysis, ChIP-seq datasets (BED files for mm9 assembly) for core clock transcription factors were downloaded from GEO: GSE3986 and GSE34020 (Cho *et al.*, 2012; Koike *et al.*, 2012) and lifted to mm10 assembly using UCSC liftover tool (*https://genome.ucsc.edu/cgi-bin/hgLiftOver*). For each of the clock TFs, histograms were drawn for summit to summit distances between each GR peak to its nearest clock TF peak as described previously (Amin *et al.*, 2015).

4.6.12 Motif Analysis

Regions from the GR BED file with a REV-ERBα peak within 120bp were extracted and analysed further for enriched motifs. HOMER (findMotifsGenome.pl) was used to find enriched de novo and known motifs in these co-bound regions, as well as, locations of top motifs. Motifs were built using options *-len 8,12,15*, and *-size 200*, while finding locations of the selected motifs, option *-size given* was used. Output motifs were ranked based on %Ratio (observed/expected frequencies). Duplicate motifs, motifs with %Ratio<1.5 or total coverage <5% were removed.

4.6.13 Histology Paraffin Embedded Tissues

Lungs were immediately infused with 1ml of 4% *para*-formaldehyde then submerged in 4% *para*-formaldehyde overnight. Livers and visceral adipose were submerged in 4% *para*-formaldehyde overnight. Tissues were embedded into paraffin blocks and cut into 5µm sections (Leica RM2255 Microtome). For *frozen tissues*, livers were embedded in optimal cutting temperature compound (OCT) and frozen in liquid nitrogen. 5µm sections were cut (Leica CM3050 Cryostat).

4.6.14 Oil Red O Stain

Frozen liver sections were equilibrated to room temperature, and air dried for 5 minutes, then incubated in water for 10 minutes. Sections were stained with Oil Red O for 10 minutes, transferred to 70% isopropanol (2 minutes) then rinsed in tap water (2 minutes) and counterstained with Mayers Haemotoxilin (1 minute). After blueing under running tap water, sections mounted in glycerin.

4.6.15 Image Analysis

All images were acquired on an Axio Imager.A1 (Zeiss) microscope using either a 10x Zeiss EC Plan-NEOfluar or 20x Zeiss EC Plan-NEOfluar objective. Images were collected using AxioCam MRc (Zeiss). Raw images were visualised using AxiovisionRel. 4.7 (Zeiss), processed and quantified using Image J (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Adipocytes_Tool).

4.6.16 Metabolic profiling

Quantification of ATP, ADP, AMP, NAD, NADP, NADPH from flash frozen liver was performed by the the Metabolomics Innovation Centre (TMIC) at the University of Alberta, Canada.

4.6.17 Immunoprecipitation and immunoblotting

HEK293 cells were transfected with 1µg halo-tagged REV-ERBα and/or 1µg halo-tagged GR using PEI (3:1 v/w ratio) and left overnight. Cells were transferred to media containing charcoal stripped FBS

(Invitrogen) 4 hours before treatment with dexamethasone (100nM) or DMSO for 1 hour. Cells were lysed (150 mM NaCl, 20 mM Tris-HCl, 10% glycerol, 1% TritonX-100, 1mM PMSF, 10 mM NEM, PhosSTOP, Complete EDTA-free protease inhibitor cocktail) on ice and cell debris cleared by centrifugation (13k xg, 5 min).

1 µg of anti-REV-ERBα antibody (mouse monoclonal GSK6F05, or 1 µg of mouse IgG) or 1 µg of anti GR antibody (rabbit polyclonal, Proteintech, or 1 µg of rabbit IgG) was incubated with protein lysates for 1 hour on a rotating wheel at 4°C. Antibody complexes were captured by addition of beads (Mouse - Sure beads Protein G magnetic beads, BioRad; rabbit - MagResyn Protein A, Biosciences) for 45 minutes at 4°C. Beads were washed three times with lysis buffer, then boiled for 10 minutes in SDS loading dye. Beads were cleared using magnetic separator and supernatants electrophoresed on Mini Protean TGX Precast Gels 4-15% (BioRad). Resolved proteins were transferred to a 0.2 µm pore size Protran nitrocellulose membrane then rinsed with Tris-HCl pH 7.6 buffered saline (TBS) solution supplemented with 0.1% Tween-20 (TBST). Membranes were blocked (5% skim milk powder in TBST) for 1 hour at room temperature and then incubated with mouse monoclonal GSK6F05 anti-REV-ERBα or rabbit polyclonal anti-GR (ProteinTech) antibodies overnight at 4°C. Membranes were washed 3 × 10 minutes with TBST and secondary HRP-linked antibodies (GE Healthcare) were incubated for 1 hour. After 3 × 10 minute TBST washes immunoreactive bands were detected using Supersignal West Dura (ThermoScientific) and chemiluminescence visualised on Kodak BioMax MR or XAR Film. Experiments were repeated on four separate occasions, and full scans of images from Fig 4.2E are shown in Fig. 4.S16.

4.6.18 Corticosterone measurements

Blood was left to clot for 30 min, centrifuged for 10 minutes at 1000xg and serum collected. ELISAs were performed according to the manufacturer's instructions (Corticosterone ELISA kit, Cat# ADI-900-097, ENZO life sciences). Samples were diluted 1:40, and quantified in duplicate and run alongside a standard curve within the range 32-27,000pg/ml. N=5 biological replicates).

4.6.19 Insulin and Leptin measurements

Blood was left to clot for 30 min, centrifuged for 10 minutes at 1000xg and serum collected. Serum was diluted 1:2 and samples analysed according to the manufacturer's instructions (MILLIPLEX MAP Mouse Bone Magnetic Bead Panel, Cat# MBNMAG, Millipore) using the Bioplex 200 system (BioRad) alongside standards curves ranging from 37-150,000pg/ml (insulin) and 10-40,000pg/ml (leptin). WT N=8, REV-ERBαKO N=7 biological replicates.

4.6.20 Liver Glycogen measurements

Liver homogenates (10mg/100µl) were boiled for 5 min and cleared by centrifugation at 13kxg for 10 min. Assays were performed according to the manufacturer's instructions (Glycogen Assay Kit Cat# MAK016, Sigma Aldrich). Single measurements of 0.5 and 0.1 µl sample/well were run alongside a 0.2-2µg glycogen standard curve. WT N=8, REV-ERBαKO N=7 biological replicates.

4.6.21 Triglyceride and Free Fatty Acid measurements

Liver homogenates (10mg/100µl) and serum was isolated, and triglycerides and free fatty acids assays were performed according to the manufacturers' instructions (Serum Triglyceride Determination Kit Cat# TR0100, Free Fatty Acid Quantitation Kit Cat# MAK044, Sigma Aldrich). Serum samples (undiluted) and liver homogenates (diluted 1:2) were run in duplicate alongside a standard curve of glycerol (0.037-2.5mg/ml range, TG assay) or palmitic acid (0.2-1nmole range, FFA assay). Triglycerides isolated from liver were normalised to total protein content of the tissue using data from a Bradford assay run in parallel. All samples were measured using the Glomax Multi Detection System (Promega). WT N=8, REV-ERBαKO N=8 biological replicates.

4.6.22 Statistical Analysis

To model the underlying trend in the body weight and body fat time-series data, a Gaussian Processes based model (Rasmussen, C.E. and Williams, 2006) using the GPy software package ('GPy:

A Gaussian process framework in python [https://github.com/SheffieldML/GPy]', no date) was built to fit a non-linear curve to the means over the replicates. The model output with mean and credible region (corresponding to two standard deviations of the posterior distribution) is shown in Fig4.4A and 4.4F.

4.7 Supplementary Data

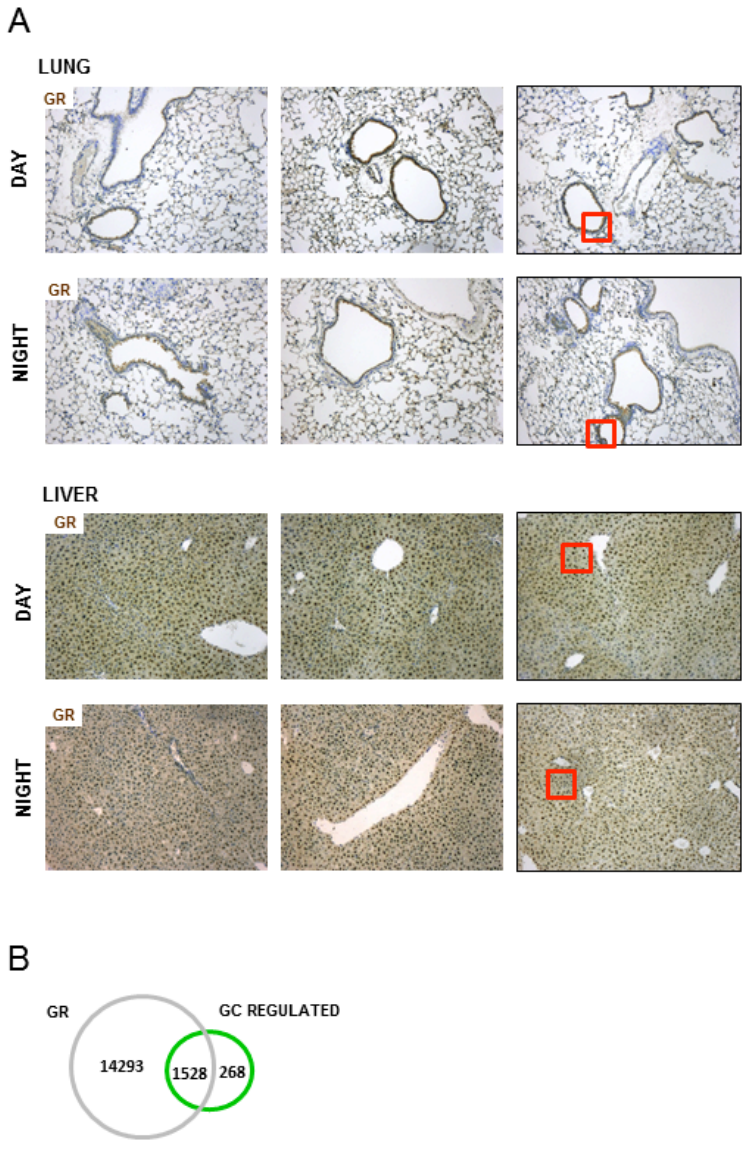

Figure 4.S1. GR expression does not alter throughout the day in lung or liver. Lung and liver were analysed by immunohistochemistry for GR expression (brown) and nuclei counterstained with toluidine blue. Three examples are shown, and higher magnification regions shown in Fig 1

highlighted with red boxes. 10x magnification (**A**). Gc regulated genes in the liver were compared to ChIP-seq annotated genes bound by GR in mouse liver (**B**). All performed by G.C.

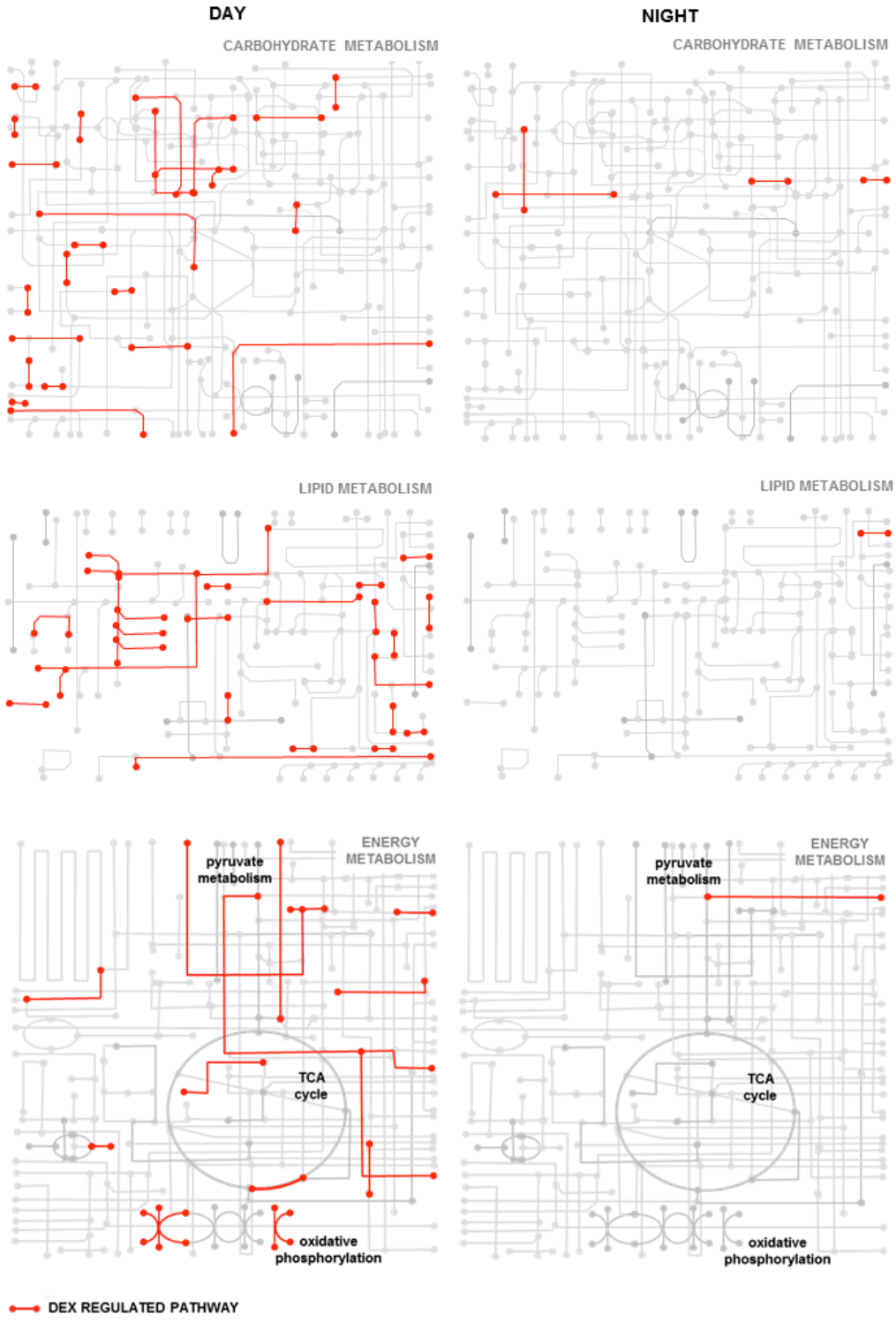

Figure 4.S2. Day specific Gc regulated genes in liver control key metabolic pathways. Schematic summarizing KEGG Pathway analysis of Gc regulated genes in liver. Gc regulated pathways are shown in red. Ontology analysis performed by G.C.

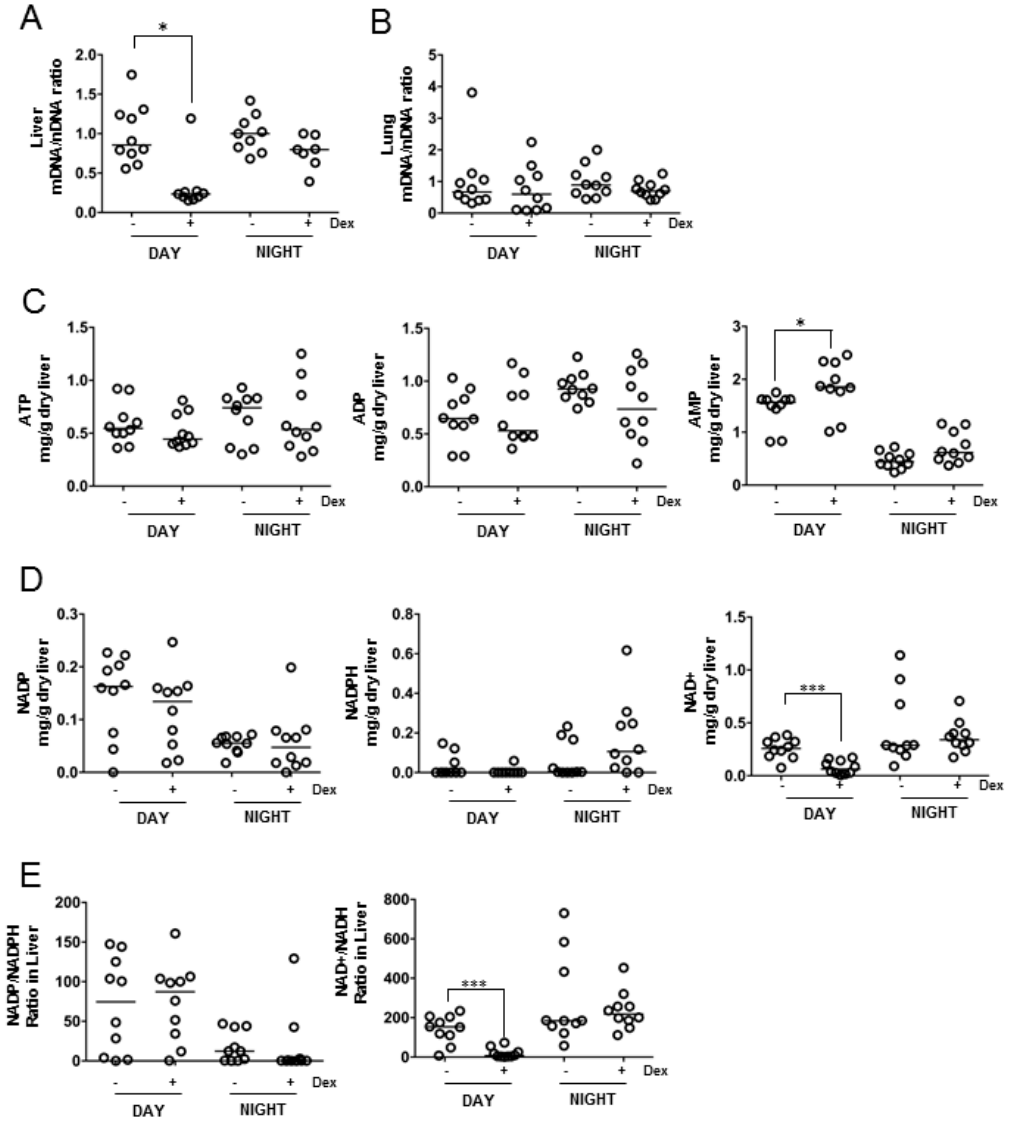

Figure 4.S3. Gc reduces mitochondrial number in the liver only in the day. C57BL/6 mice were treated with 1mg/kg I.P. dexamethasone at either ZT6 or ZT18 and culled 4 hours later. Relative mitochondrial DNA in liver (**A**) and lung (**B**), were normalised to vehicle. AMP, ADP, ATP (**C**) and NADP, NADPH, NAD+ and NADH (**D**) were measured via HPLC in dry mouse liver. Ratios of NADP to NADPH and NAD+ to NAD H (**E**) were calculated. Data shown as median. Statistical analysis of Gc treatment via one-way ANOVA with a Holm-Sidak multiple comparisons correction (mitochondrial

quantification AMP, ADP and ATP) or one-way ANOVA, followed by a t-test NADP, NADPH, NAD+ NADP/NADPH ratio and NAD+/NADH ratio), $p < 0.05$ * $p < 0.001$ **, $p<0.0001$ ***. QPCR (A) performed by A.T. Metabolite concentrations (B) performed by The Metabolomics Innovation Centre, Alberta, Canada. All animal treatments, sample generation and analysis by G.C.

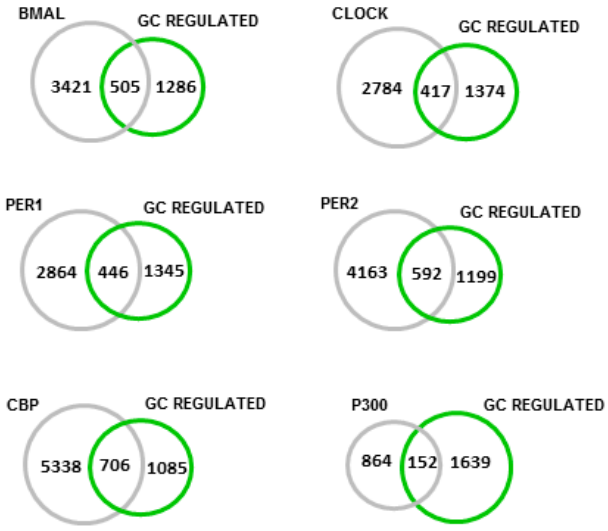

Figure 4.S4. Gc regulated genes show limited overlap with core clock transcription factors. Venns showing overlap between genes regulated by Gc and core clock transcription factors. Performed by G.C.

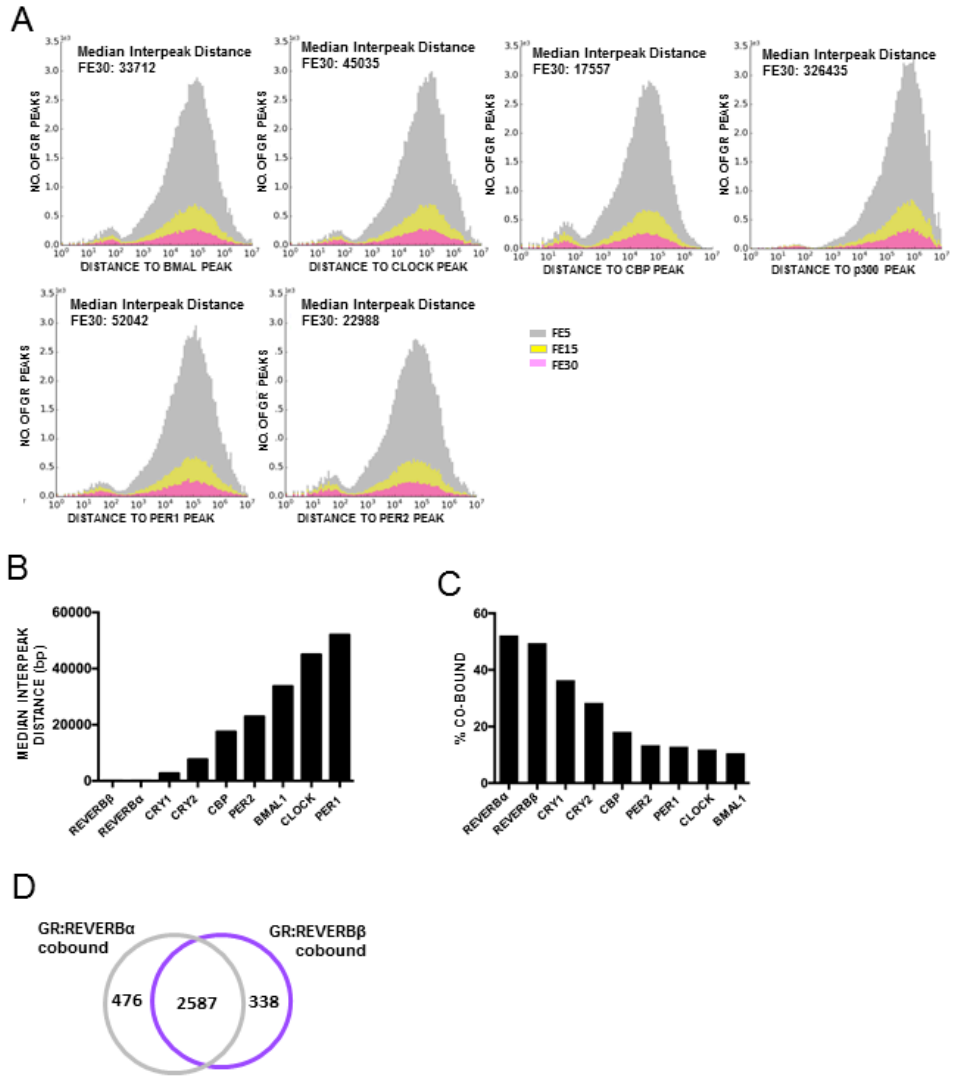

Figure 4.S5. GR binding shows minimal overlap with binding of some core clock transcription factors. ChIP-seq data for GR and core clock transcription factors was compared to determine proximity of binding. Histograms depict the number of GR binding peaks against distance from closest transcription factor summit using three stringencies (fold enrichment (FE) scores). Median interpeak distances for the highest stringency (FE30) are inset (**A**), and plotted in order of ranking (**B**). The percentage of GR co-binding with each transcription factor is also plotted (**C**). Genes co-bound by GR and REV-ERBα or REV-ERBβ (**D**). Cobinding analysis and mapping of ChIP-seq peaks (A, B, C, D) performed by M.I. Downstream analysis (B, C, D) performed by G.C.

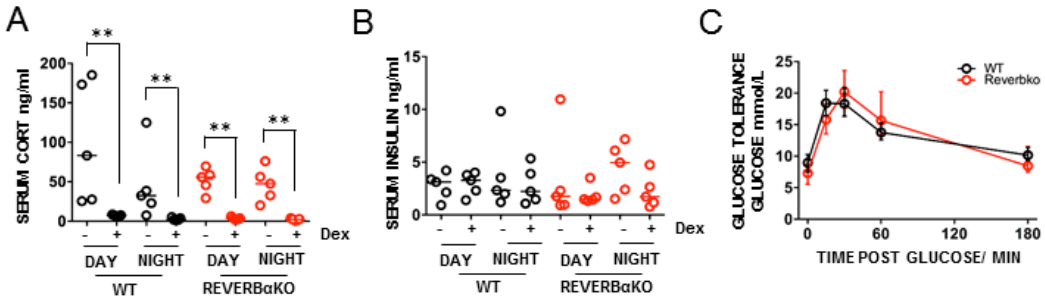

Figure 4.S6. REV-ERBα knockout have normal Gc sensitivity, serum insulin and glucose tolerance. REV-ERBα knockout and littermate controls were treated with dex at either ZT6 or ZT18 and culled 2 hours later. Serum was harvested and corticosterone (**A**) and insulin (**B**) analysed by ELISA. REV-ERBα knockout and littermate controls were fasted overnight and injected I.P. with 2g/kg glucose at ZT6 and blood glucose was measured over 180 minutes (N=12) (**C**). Statistical analysis via Mann-Whitney (serum Cort), Kruskal-Wallis with a Dunn's multiple comparisons correction (serum insulin) and two-way ANOVA repeated measures with a Tukey's multiple comparisons correction (GTT) $p < 0.01$ **. All performed by G.C.

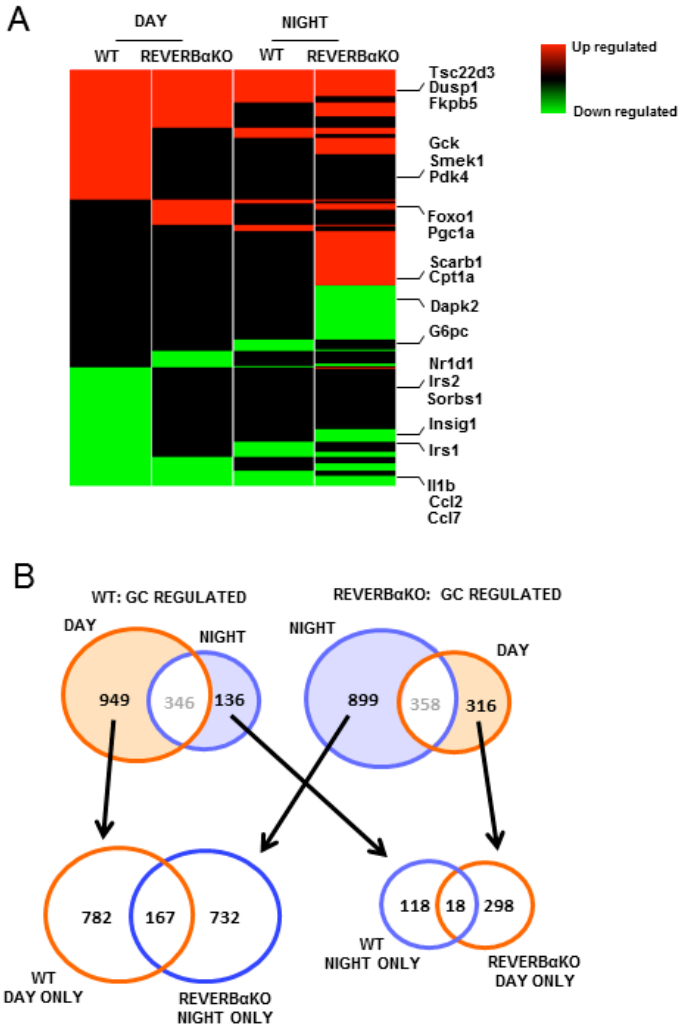

Figure 4.S7. REV-ERBα modulates the Gc response. REV-ERBαKO mice and littermate controls were treated with dex at either ZT6 or ZT18 and culled 2 hours later. Livers were harvested and analysed by RNA-seq (N=5). Heat map of genes differentially regulated by dex treatment (**A**). Day and night specific genes in WT and REV-ERBαKO were compared (**B**). RNA-seq analysis and heat map (A) generated by P.W. Identification of ChIP-seq binding sites (B) performed by M.I. Downstream analysis of regulated genes compared to ChIP-seq binding (B) performed by G.C.

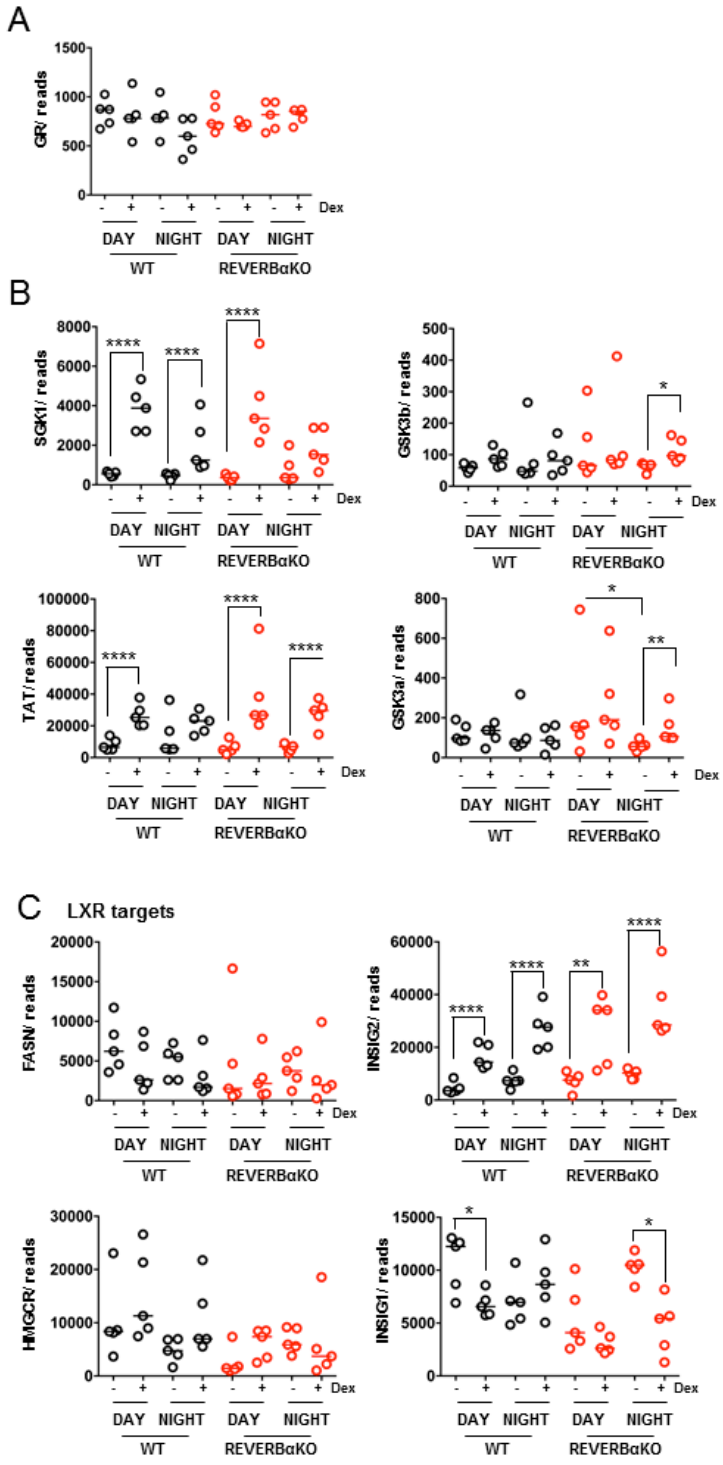

Figure 4.S8. The effect of REV-ERBα on Gc action is not mediated indirectly through activation of CRY1 or LXR. Graphs show RNA-seq reads for GR (**A**), and reported CRY1 (**B**) and LXR (**C**) target genes. Individual samples (N=5) are plotted with the median for each group. q < 0.05*, q < 0.01**, q

< 0.001***, q < 0.0001****. DEseq pairwise analysis, Fisher's Exact test adapted for negative binomial distribution (RNA-seq). Analysis of RNA-seq performed by P.W. Graphs generated by G.C.

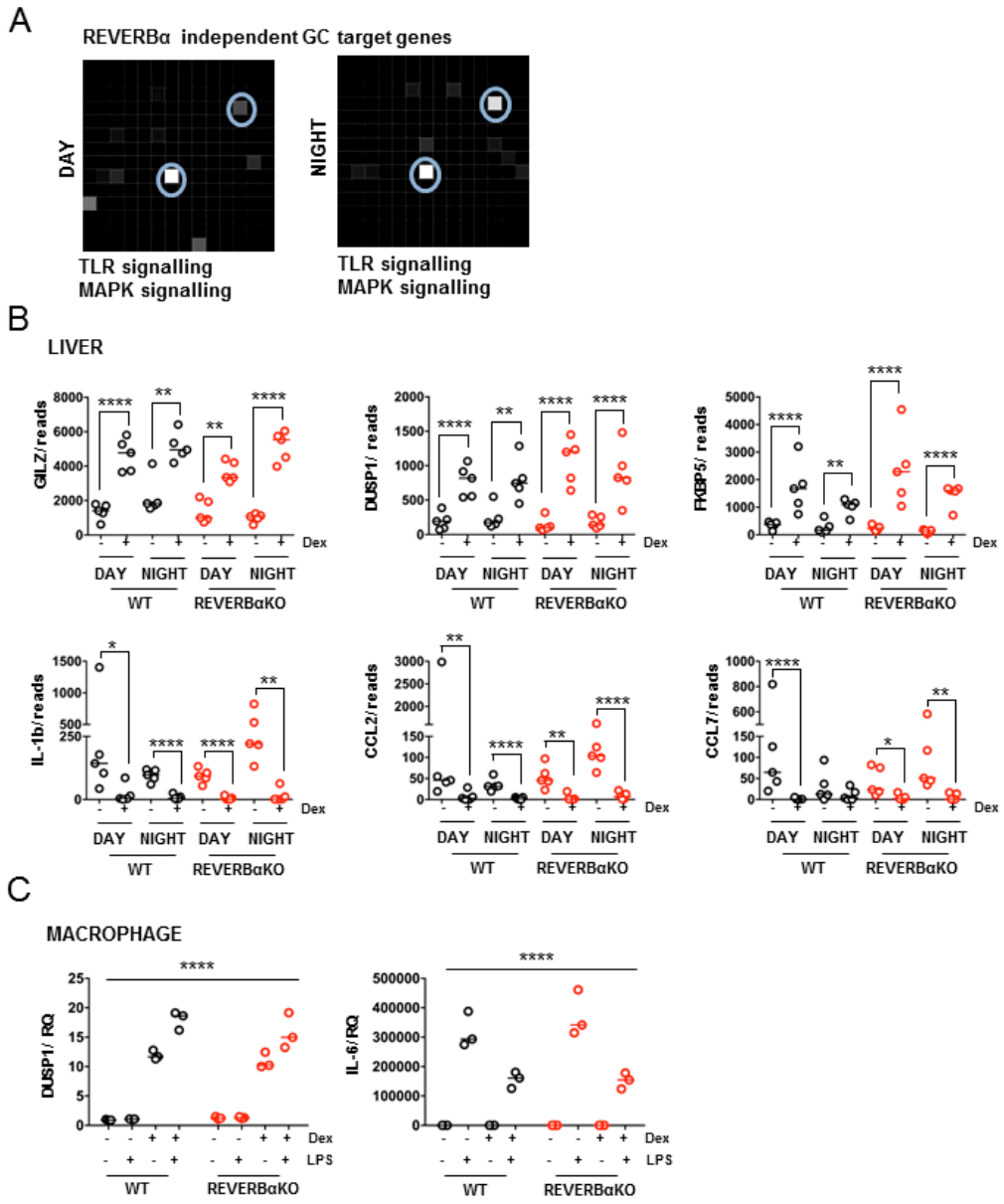

Figure 4.S9. REV-ERBα does not regulate anti-inflammatory Gc effects. Enrichr canvases for REV-ERBα independent Gc targets with the two highest ranking terms listed underneath (**A**). Graphs show RNA-seq reads for anti-inflammatory Gc target genes from liver RNA-seq. Individual samples (N=5) are plotted with the median for each group (**B**). Bone marrow derived macrophages were isolated from REV-ERBαKO and WT littermate control mice, treated with vehicle or 100nM dex for 1 hour, then with 100ng/ml LPS for 4 hours. Gc regulation was determined by q-RTPCR for DUSP1 and

IL-6; no genotypic differences were observed (**C**). q < 0.05*, q < 0.01**, q < 0.001***, q < 0.0001**** Two-way ANOVA (macrophage) DEseq pairwise analysis, Fisher's Exact test adapted for negative binomial distribution (RNA-seq). Gene ontology analysis (A) performed by G.C. RNA-seq analysed (B) by P.W. Macrophage isolation, treatment and QPCR performed by R.V. and Z.Z.

Figure 4.S10. Analysis of all GR binding sites shows enrichment of GREs. Motif analysis for all GR binding sites. Observed/Expected ratios, and p-values are indicated for each motif. Motif analysis performed by M.I.

Figure 4.S11. GR-REV-ERBα co-bound regions are enriched for HNF motifs. Motif analysis for all GR-REV-ERBα co-bound sites (where binding occurs within 120bp). Observed/Expected ratios, and p-values are indicated for each motif. Motif analysis performed by M.I.

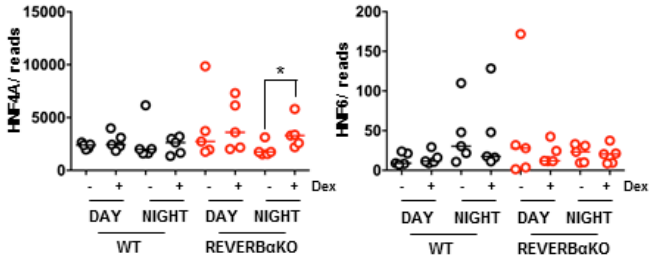

Figure 4.S12. Gc target genes are enriched for HNF binding sites. Graphs show RNA-seq reads for HNF4A and HNF6. Individual samples (N=5) are plotted with the median for each group. RNA-seq analysis performed by P.W.

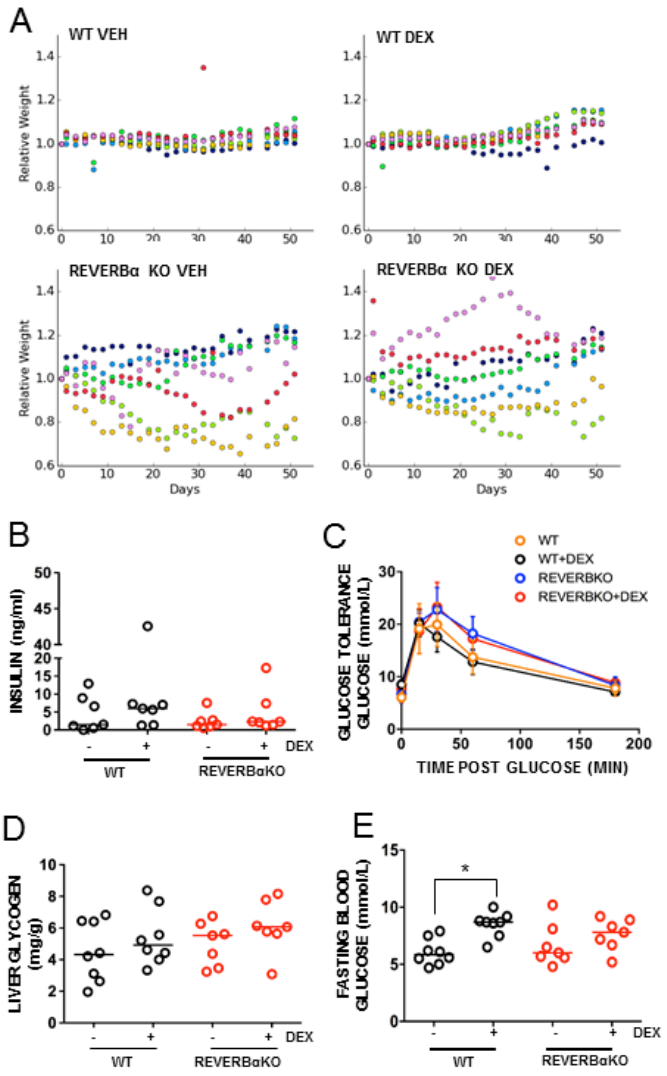

Figure 4.S13. Loss of REV-ERBα protects from Gc induced weight gain. REV-ERBα knockout and littermate controls were treated with dex or vehicle at ZT6 every 48 hours for 8 weeks. Weight was tracked every 48 hours and change relative to initial weight plotted for each animal in the group. The group means are shown in Fig4B (**A**). Serum insulin (**B**), glucose tolerance (**C**), liver glycogen (**D**) and fasting blood glucose (**E**) were quantified (n=8 WT, 7 KO). Statistical analysis via Kruskal-Wallis with a Dunn's multiple comparison correction (insulin, liver glycogen and fasting glucose) or two-way ANOVA repeated measures with a Tukey's multiple comparison correction (GTT). $p < 0.05$*. Modelling of weight (A) performed by M.I. Insulin and glycogen assays (B, D) performed by A.T. GTT (C) performed by G.C. with assistance from N.B., A.T., R.V. and M.B. Fasting glucose (E), all treatments and sample collection performed by G.C.

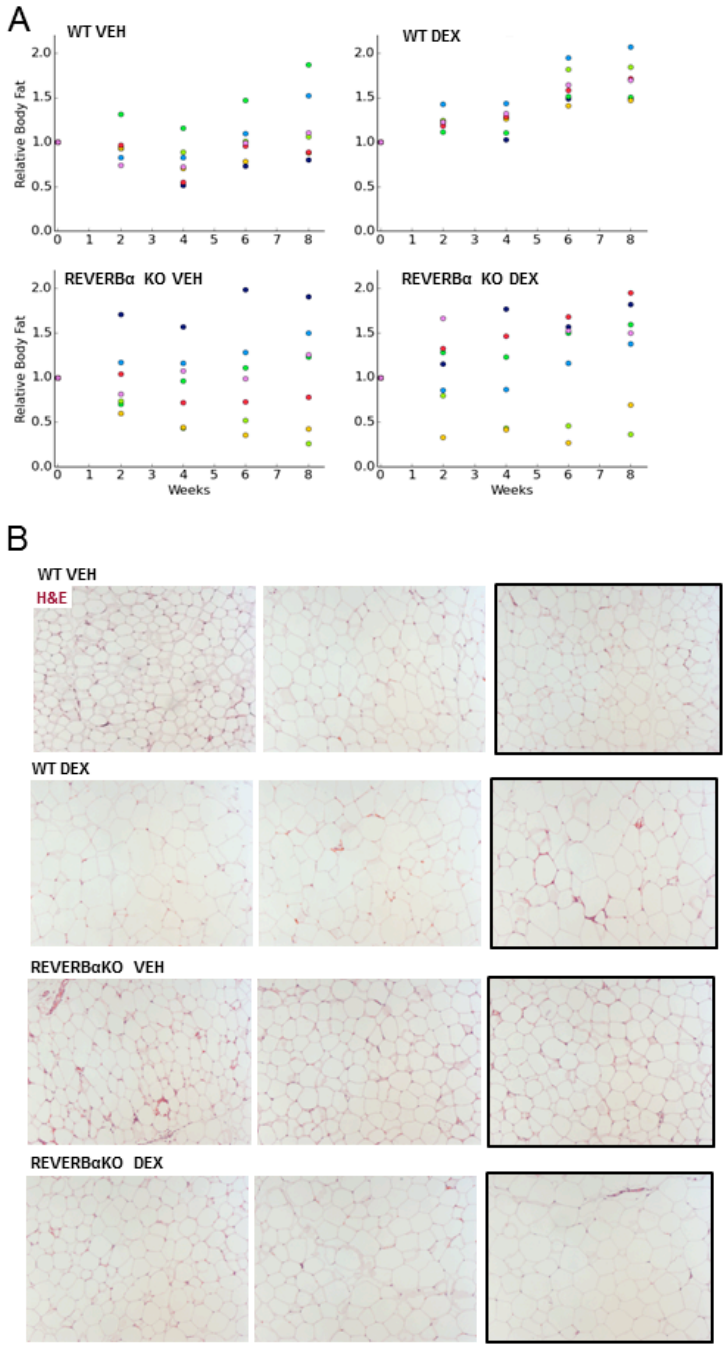

Figure 4.S14. Dex treatment increases adiposity, adipocyte size and heterogeneity in WT but not REV-ERBαKO mice. REV-ERBα knockout and littermate controls were treated with dex or vehicle at ZT6 every 48 hours for 8 weeks. Fat mass was tracked every 14 days and relative change in fat mass for each animal plotted. Means across each group are shown in Fig4C (**A**). Visceral adipose was collected at cull and analysed by H&E. Representative images from three animals per group are shown (**B**). Highlighted images are included in Fig4E. Modelling of relative fat mass (A) performed by

M.I. H&E (B) performed by R.V. All treatments, echo-MRI analysis and sample collections performed by G.C.

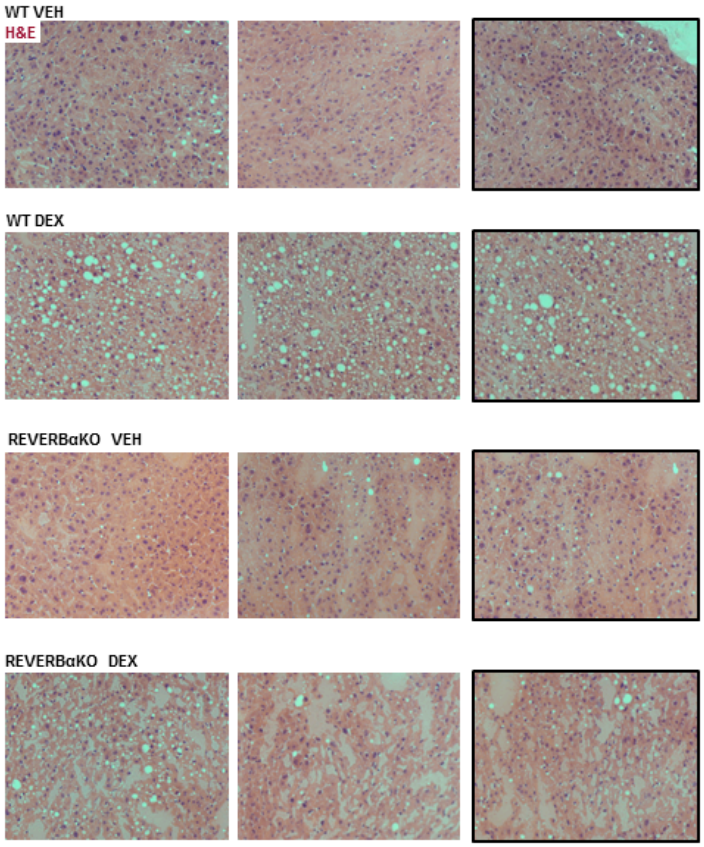

Figure 4.S15. Loss of REV-ERBα protects from Gc induced hepatosteatosis. REV-ERBα knockout and littermate controls were treated with dex or vehicle at ZT6 every 48 hours for 8 weeks. Liver was collected at cull and analysed by H&E. Representative images from three animals per group are shown. Highlighted images are included in Fig4H. H&E performed by R.V. All treatments and sample collection performed by G.C.

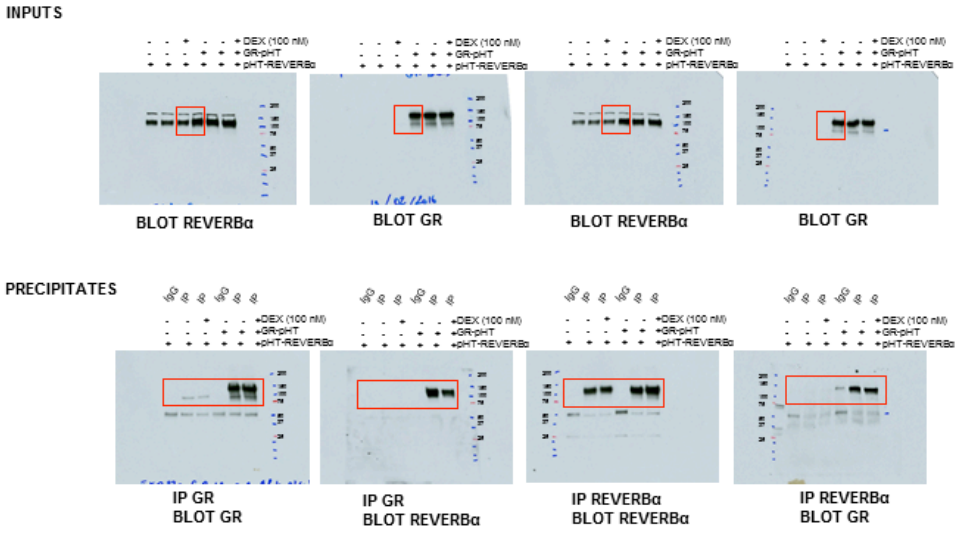

Figure 4.S16. GR interacts with REV-ERBα in vitro. Expanded gel images of GR/ REV-ERBα co-immunoprecipitation studies. Full gel scans are shown with cropped images shown in Fig2E highlighted by red boxes. Molecular weight markers are indicated. Perfomed by M.P.

PathwayName	#Gene	Statistics
Cytokine-cytokine receptor interaction	25	C=245;O=25;E=2.27;R=11.02;rawP=1.25e-18;adjP=1.54e-16
B cell receptor signaling pathway	14	C=76;O=14;E=0.70;R=19.89;rawP=1.18e-14;adjP=7.26e-13
Hematopoietic cell lineage	13	C=82;O=13;E=0.76;R=17.11;rawP=7.98e-13;adjP=3.27e-11
Metabolic pathways	40	C=1184;O=40;E=10.97;R=3.65;rawP=3.63e-12;adjP=1.12e-10
Natural killer cell mediated cytotoxicity	13	C=125;O=13;E=1.16;R=11.23;rawP=1.91e-10;adjP=4.70e-09
Chemokine signaling pathway	14	C=185;O=14;E=1.71;R=8.17;rawP=2.60e-09;adjP=4.00e-08
Complement and coagulation cascades	10	C=76;O=10;E=0.70;R=14.20;rawP=2.36e-09;adjP=4.00e-08
Pathways in cancer	18	C=325;O=18;E=3.01;R=5.98;rawP=2.20e-09;adjP=4.00e-08
Leishmaniasis	9	C=64;O=9;E=0.59;R=15.18;rawP=8.23e-09;adjP=1.12e-07
Malaria	8	C=46;O=8;E=0.43;R=18.77;rawP=9.86e-09;adjP=1.21e-07
Cell adhesion molecules (CAMs)	11	C=149;O=11;E=1.38;R=7.97;rawP=1.70e-07;adjP=1.75e-06
Osteoclast differentiation	10	C=118;O=10;E=1.09;R=9.15;rawP=1.71e-07;adjP=1.75e-06
MAPK signaling pathway	14	C=268;O=14;E=2.48;R=5.64;rawP=2.72e-07;adjP=2.57e-06
Neuroactive ligand-receptor interaction	14	C=277;O=14;E=2.57;R=5.46;rawP=4.05e-07;adjP=3.32e-06
Staphylococcus aureus infection	7	C=50;O=7;E=0.46;R=15.11;rawP=3.98e-07;adjP=3.32e-06
T cell receptor signaling pathway	9	C=110;O=9;E=1.02;R=8.83;rawP=9.53e-07;adjP=7.21e-06
NOD-like receptor signaling pathway	7	C=57;O=7;E=0.53;R=13.26;rawP=9.97e-07;adjP=7.21e-06
Jak-STAT signaling pathway	10	C=153;O=10;E=1.42;R=7.06;rawP=1.88e-06;adjP=1.28e-05
Toxoplasmosis	9	C=127;O=9;E=1.18;R=7.65;rawP=3.17e-06;adjP=2.05e-05
p53 signaling pathway	7	C=70;O=7;E=0.65;R=10.79;rawP=4.08e-06;adjP=2.51e-05

PathwayName	#Gene	Statistics
Cytokine-cytokine receptor interaction	27	C=245;O=27;E=2.14;R=12.60;rawP=1.54e-21;adjP=1.60e-19
Hematopoietic cell lineage	16	C=82;O=16;E=0.72;R=22.31;rawP=2.27e-17;adjP=1.18e-15
Primary immunodeficiency	11	C=34;O=11;E=0.30;R=37.00;rawP=4.90e-15;adjP=1.70e-13
Natural killer cell mediated cytotoxicity	16	C=125;O=16;E=1.09;R=14.64;rawP=2.40e-14;adjP=6.24e-13
T cell receptor signaling pathway	15	C=110;O=15;E=0.96;R=15.59;rawP=5.99e-14;adjP=1.25e-12
B cell receptor signaling pathway	12	C=76;O=12;E=0.66;R=18.06;rawP=3.28e-12;adjP=5.69e-11
Chemokine signaling pathway	16	C=185;O=16;E=1.62;R=9.89;rawP=1.10e-11;adjP=1.63e-10
Osteoclast differentiation	13	C=118;O=13;E=1.03;R=12.60;rawP=4.54e-11;adjP=5.25e-10
Graft-versus-host disease	10	C=54;O=10;E=0.47;R=21.18;rawP=4.06e-11;adjP=5.25e-10
Chagas disease (American trypanosomiasis)	12	C=100;O=12;E=0.87;R=13.72;rawP=9.20e-11;adjP=9.57e-10
Leishmaniasis	10	C=64;O=10;E=0.56;R=17.87;rawP=2.38e-10;adjP=2.25e-09
Type I diabetes mellitus	9	C=59;O=9;E=0.52;R=17.44;rawP=2.38e-09;adjP=2.06e-08
MAPK signaling pathway	15	C=268;O=15;E=2.34;R=6.40;rawP=1.96e-08;adjP=1.57e-07
Toll-like receptor signaling pathway	10	C=101;O=10;E=0.88;R=11.32;rawP=2.26e-08;adjP=1.68e-07
Cell adhesion molecules (CAMs)	11	C=149;O=11;E=1.30;R=8.44;rawP=9.58e-08;adjP=6.64e-07
p53 signaling pathway	8	C=70;O=8;E=0.61;R=13.07;rawP=1.90e-07;adjP=1.23e-06
Toxoplasmosis	10	C=127;O=10;E=1.11;R=9.00;rawP=2.01e-07;adjP=1.23e-06
African trypanosomiasis	6	C=32;O=6;E=0.28;R=21.44;rawP=3.24e-07;adjP=1.87e-06
Rheumatoid arthritis	8	C=81;O=8;E=0.71;R=11.29;rawP=5.95e-07;adjP=3.26e-06
Apoptosis	8	C=86;O=8;E=0.75;R=10.64;rawP=9.45e-07;adjP=4.91e-06

Table 4.34 KEGG Pathway analysis of night Gc targets in Liver

PathwayName	#Gene	Statistics
Endocytosis	37	C=220;O=37;E=6.17;R=6.00;rawP=2.43e-18;adjP=2.88e-16
Metabolic pathways	92	C=1184;O=92;E=33.21;R=2.77;rawP=3.05e-18;adjP=2.88e-16
Pathways in cancer	40	C=325;O=40;E=9.12;R=4.39;rawP=6.90e-15;adjP=4.35e-13
Ribosome	24	C=119;O=24;E=3.34;R=7.19;rawP=3.23e-14;adjP=1.53e-12
MAPK signaling pathway	35	C=268;O=35;E=7.52;R=4.66;rawP=5.59e-14;adjP=2.11e-12
Focal adhesion	27	C=200;O=27;E=5.61;R=4.81;rawP=1.87e-11;adjP=5.89e-10
Vascular smooth muscle contraction	19	C=123;O=19;E=3.45;R=5.51;rawP=1.78e-09;adjP=4.81e-08
Leishmaniasis	14	C=64;O=14;E=1.80;R=7.80;rawP=2.28e-09;adjP=5.39e-08
Regulation of actin cytoskeleton	25	C=216;O=25;E=6.06;R=4.13;rawP=2.73e-09;adjP=5.73e-08
Axon guidance	19	C=131;O=19;E=3.67;R=5.17;rawP=5.24e-09;adjP=9.90e-08
Chemokine signaling pathway	22	C=185;O=22;E=5.19;R=4.24;rawP=1.46e-08;adjP=2.51e-07
Adipocytokine signaling pathway	13	C=68;O=13;E=1.91;R=6.82;rawP=4.74e-08;adjP=7.47e-07
Wnt signaling pathway	19	C=154;O=19;E=4.32;R=4.40;rawP=7.62e-08;adjP=1.11e-06
Toxoplasmosis	17	C=127;O=17;E=3.56;R=4.77;rawP=1.10e-07;adjP=1.48e-06
Amoebiasis	16	C=116;O=16;E=3.25;R=4.92;rawP=1.70e-07;adjP=2.14e-06
Osteoclast differentiation	16	C=118;O=16;E=3.31;R=4.83;rawP=2.16e-07;adjP=2.55e-06
Insulin signaling pathway	17	C=137;O=17;E=3.84;R=4.42;rawP=3.35e-07;adjP=3.72e-06
Starch and sucrose metabolism	10	C=45;O=10;E=1.26;R=7.92;rawP=3.82e-07;adjP=4.01e-06
NOD-like receptor signaling pathway	11	C=57;O=11;E=1.60;R=6.88;rawP=4.61e-07;adjP=4.59e-06
Bile secretion	12	C=71;O=12;E=1.99;R=6.03;rawP=6.32e-07;adjP=5.69e-06

PathwayName	#Gene	Statistics
Cysteine and methionine metabolism	3	C=39;O=3;E=0.05;R=56.34;rawP=2.16e-05;adjP=0.0001
Metabolic pathways	8	C=1184;O=8;E=1.62;R=4.95;rawP=0.0002;adjP=0.0005
Protein processing in endoplasmic reticulum	3	C=169;O=3;E=0.23;R=13.00;rawP=0.0016;adjP=0.0027
Steroid hormone biosynthesis	2	C=55;O=2;E=0.08;R=26.63;rawP=0.0026;adjP=0.0032

Table 4.S6 REV-ERBα dependent Gc Targets: Carbohydrate Metabolism

GENE	DAY	NIGHT
INSIG1	WT ↓	KO ↓
SCARB1	-	KO ↑
CPT1A	-	KO ↑
DGAT2	-	KO ↑
G6PC	-	WT ↓
HNF4A	-	KO ↑
SESN2	-	KO ↑
MIA2	-	KO ↑
ACSL1	KO ↑	KO ↑
CYP2E1	-	KO ↑
LPIN1	WT ↑, KO ↑	KO ↑
LPIN2	KO ↑	KO ↑

GENE	DAY	NIGHT
IRS1	WT ↓	WT ↓
PPP13RG	WT ↓	-
SMEK1	WT ↑	-
PPP1R3B	WT ↑	-
IRS2	WT ↓	-
SORBS1	WT ↓	-
PDK4	WT ↑	-
NR1D1	WT ↓	-
GCK	WT ↑	-
FOXO1	KO ↑	KO ↑
ACER2	WT ↑	-
PGC1A	KO ↑	KO ↑

GENE	PRIMERS
β-Actin	F-AGGTCATCACTATTGGCAACGA
	R-CACTTCATGATGGAATTGAATGTAGTT
EFNA1	F-GTGGAGAAGCCTGTGGGAAC
	R-GTGTGTATCGCTCCATGGCT
Wt1	F-CACGGCACAGGGTATGAGAG
	R-GTTGGGGCCACTCCAGATAC
Aldh1b1	F-AGCCTCTGTTCAAGTTCAAG
	R-CCTTAAAGCCTCCGAATGG
DIO1	F-GTGGTGGACACAATGCAGAAC
	R-ACGATTGGGTCTATAAGTGGC
GAPDH (Genomic)	F-CAAGAAACAGGGGAGCTGAG
	R-TTGGGTTGTACATCCAAGCA
Mt ND1 (Genomic)	F-GGATCCGAGCATCTTATCCA
	R-GGTGGTACTCCCGCTGTAAA
IL-6	F-GCTACCAAACTGGATATAATCAGGA
	R-CCAGGTAGCTATGGTACTCCAGAA
DUSP1	F-GTGCCTGACAGTGCAGAATC
	R-CACTGCCCAGGTACAGGAAG

CHAPTER 5 Discussion:

Gc are essential regulators of inflammation, and as such are in wide-spread clinical use in the treatment of a range of inflammatory diseases. However, their clinical application is hindered by the development of local Gc resistance at site of inflammation, and also the development of severe side effects. New strategies to overcome resistance and offer improved safety profiles are needed. Two novel modulators of GR action, CAV1 and REV-ERBα are directly related to Gc efficacy in inflammation, and Gc side effects. The first is the membrane lipid raft protein, CAV1, which regulates inflammatory responses in the lung – a key Gc target tissue. The second is REV-ERBα, a nuclear receptor, which regulates lipid metabolism in the liver – which is dysregulated by long term Gc treatment.

CAV1, an integral membrane protein coordinates the complex interactions between the kinase and adaptor protein signalling pathways underlying inflammatory signalling. Loss of CAV1, selectively drives pro-inflammatory innate responses in alveolar macrophages, resulting in increased cytokine expression and increased cytokines found in the BAL. Paradoxically, CAV1KO limits immune cell infiltration into the lung. The disconnect between increased chemotactic signal, and the reduction in immune cell recruitment to the airways implies a defect in cell migration (Garrean *et al.*, 2006; Wu *et al.*, 2016) and trafficking to the site of inflammation. Both ICAM1 and VCAM1 are necessary for leukocyte motility during LPS induced inflammation, and CAV1KO causes a reduction in the levels of both (Garrean *et al.*, 2006; Wu *et al.*, 2016). Interestingly, ICAM1 and ELAM1 (the leukocyte expressed cognate receptor for ICAM1) are both Gc targets, and are down regulated by dex treatment, causing a reduction in immune cell infiltrate (Cronstein *et al.*, 1992). This reduction in ICAM1 and VCAM1 is a potential mechanism for the decreased immune cell infiltration seen in the CAV1KO mice, despite the increase of cytokine production. CAV1 expression in the lung increases in the first few hours of a rat model of ALI (Qiaoli *et al.*, 2016). This may explain the increase in immune

cell infiltrate into the lungs during injury, as the increase in CAV1 would potentiate the chemotactic signals, by allowing more efficient trafficking of cells.

The regulatory role of CAV1 appears to lie upstream of GR, which means that the apparent increase in Gc sensitivity in lung is not necessarily through a direct interaction with GR itself. As the overall impact of CAV-1 on the physiological actions of Gc in-vivo was quite limited I chose not to pursue this line of investigation further, as even if I could find a new mechanism of interaction between CAV-1 and the GR the resulting impact on lung immunity was likely to be small, and the translational potential of the work would be predicted to be low. I therefore elected to move my project on to analyse another promising candidate for regulation of the GR, namely the circadian clock.

There are a number of issues to consider when studying the circadian clock, and overall targeting the core circadian clockwork is unattractive as it is likely to result in quite a severe phenotype. For example, shift-work, or global circadian clock disruption in animal studies leads to aberrant energy metabolism, and cancer (Peek *et al.*, 2013; Kettner *et al.*, 2016). REV-ERBα is a transcriptional repressor, a key component of the molecular clockwork, and is an important regulator of inflammation and metabolism. REV-ERBα is an orphan nuclear receptor to which a number of synthetic drug-like ligands have been designed. Importantly global loss of REV-ERBα results in only a minor circadian phenotype, with a slight lengthening of the circadian period seen under constant darkness (Cho *et al.*, 2012). Loss of REV-ERBα causes differential regulation of Gc target genes, resulting in a subset of lipid metabolic genes to be unaffected by Gc treatment. This is associated with a decrease in Gc sensitivity shown by loss of the highly prevalent phenotype of hepatic steatosis associated with chronic Gc treatment. REV-ERBα directs GR to specific binding sites on the DNA, either by closing areas of chromatin through recruitment of HDAC3, or by interacting with lineage determining factors and directing GR to key genomic loci with multiple nuclear receptors binding.

Both CAV1 (Bucci *et al.*, 2000; Garrean *et al.*, 2006; Wang, Kim, *et al.*, 2006; Wang *et al.*, 2009; Tourkina *et al.*, 2010) and REV-ERBα (Migita, Morser and Kawai, 2004; Barish *et al.*, 2005; Gibbs *et al.*, 2012; Eichenfield *et al.*, 2016) are known to be important regulators of inflammation, neither appear to have a direct involvement on Gc regulation of anti-inflammatory actions. CAV1 loss does not result in increased repression of inflammation, despite increased transactivation capacity in lung. This may have been due to a limitation of the global knockout system, as loss of CAV1 results in a decrease in ICAM-1 induction during inflammation and decreased PBMC (Garrean *et al.*, 2006). This reduction in immune cell infliltration capacity affects the core readout of inflammation. Using a cell specific knockout may lead to different results, and in particular provide a model to dissect out the role of CAV1 in airway macrophages. Similarly, chronic treatment of WT and REV-ERBα knockout mice with Gc reduced thymus weight to a similar extent in both genotypes. Treatment *ex vivo* of bone marrow derived macrophages revealed no differences in the immunosuppressive actions of Gc with loss of REV-ERBα, and in acute treatment studies Gc had the same effect on inflammatory mediator genes in liver with or without the presence of REV-ERBα.

These data predict that in people treated with Gc that exposure during the day when REV-ERBα is low will not adversely impact the anti-inflammatory profile of the therapeutic Gc, but will minimise the adverse effects on liver function; by comparison with exposure to Gc during the night when the liver is far more sensitive to the actions of Gc. This is relevant now as there are moves to target Gc exposure overnight in an attempt to target the early morning symptoms seen in diseases such as rheumatoid arthritis. In this way standard prednisolone is re-formulated with a delayed release coating. The tablets (LODOTRA) are ingested at night but release active compound while the patient is asleep, overnight (Buttgereit *et al.*, 2008). These studies predict that such release kinetics are likely to amplify the adverse effect profile of the prednisolone, and this should be analysed in treated patients.

These results provide two distinct mechanisms of regulating Gc sensitivity involving cross-talk between GR and other signalling molecules including the membrane protein CAV1, and the nuclear receptor REV-ERBα. CAV1 has been previously reported to both be involved in the repression of inflammation, and also the promotion of inflammation. These differences though seem to be affected by cell type, or interactions between cell types. The anti-inflammatory actions are seen in macrophages (Wang, Kim, *et al.*, 2006), while the pro-inflammatory actions are seen systemically (Garrean *et al.*, 2006). Both of these observations also fit with these data, where the acute lung inflammation in response to nebulised LPS is reduced in the CAV1KO mice, but cytokine production is increased, and similarly I see increased pro-inflammatory cytokine production in *ex-vivo* activated CAV-1 null macrophages. As CAV1 likely acts upstream of the GR, and is involved in regulating multiple kinase signalling cascades, it is unlikely that CAV1 expression could be used as a marker for Gc sensitivity, or indeed a useful drug target for the improvement of Gc efficacy in lung inflammation. A prediction made on the basis of my data is that reduced CAV1 expression should result in increased Gc mediated transactivation. The complexities of cell-type specific CAV-1 actions in lung inflammation will need further study in the context of Gc treatment.

REV-ERBα is also associated with a pro-inflammatory phenotype in macrophages (Gibbs *et al.*, 2012) however, genetic ablation of REV-ERBα results in increased systemic inflammation (Sato *et al.*, 2013). Unpublished data from our lab also shows a pro-inflammatory phenotype in REV-ERBα knockout mice using an ovalbumin model and aerosolised LPS model, highlighting its role as a suppressor of inflammation in multiple different contexts. This may be because REV-ERBα is a constitutive repressor, and directly associates with the DNA to affect transcription.

REV-ERBα is a core component of the molecular clock. While the REV-ERBs and the related orphan retinoid receptors, the RORs, were originally considered an “accessory loop” for fine tuning the main rhythm, however deletion of both REV-ERBα and REV-ERBβ results in loss of circadian behaviour in mice (Cho *et al.*, 2012), making the REV-ERBs bona fide clock genes. The ability for REV-ERBα to bind

DNA is influenced by RORs, which recognise and bind to identical DNA sequences in the genome (O'Malley Cell 2015). Early work suggested a mechanism of competition, whereby the RORs and REV-ERBs would compete to bind and this would result in either activation (bound by RORs), or repression (bound by REV-ERBs) or target genes. However, now it appears that the binding of RORs prepared the element for subsequent loading of REV-ERBα.

As loss of REV-ERBα alters the Gc induced transcriptome, it is feasible that REV-ERBα (or even the RORs) are involved in the assisted loading of the GR. Therefore, loss of REV-ERBα not only alters chromatin accessibility by reducing the amount of HDAC3 activity (resulting in Gc responsive genes gained in the REV-ERBα KO), but also means there is no longer a potential assisted loading mechanism, whereby REV-ERBα aids in the binding of GR to chromatin (resulting in Gc responsive genes lost in REV-ERBα KO mice). The role of REV-ERBα in maintaining rhythmic metabolic processes in the liver is the link between the GR and REV-ERBα. While the GR has its rhythmic activity modulated by both endogenous ligand exposure and chromatin binding site availability, it appears that REV-ERBα also aids in directing the GR to the correct binding sites at the right time of day. Interestingly, there are other nuclear receptors that also co-bind with GR such as PPARα and LXRβ both of which are also heavily involved in lipid metabolism (Siersbæk *et al.*, 2014). Dysregulation of any one of these nuclear receptors, by mutation, disease state or ligand availability could result in altered and pathophysiological lipid metabolism, such as that seen under chronic Gc treatment.

There is very little evidence that CAV1 and circadian rhythms interact, although it has been shown that CAV1 is a Per1 target gene (Stow *et al.*, 2012) and that another component of caveolae, Cavin3, regulates circadian period length through direct interaction with Per2 (Schneider *et al.*, 2012). As CAV1KO mice do not display a circadian phenotype, it is unlikely that alteration of the daily rhythm contributes to the phenotype I see under inflammation.

REV-ERBα and CAV1 are heavily involved in lipid metabolism, however I did not assess the role of CAV1 in the metabolic actions of Gcs, limiting the project to inflammation only. REV-ERBα controls lipid and carbohydrate metabolism (Cho *et al.*, 2012; Delezie *et al.*, 2012; Zhang *et al.*, 2015), as does GR, however the cooperation of GR and REV-ERBα in the liver seems to be mostly involved with regulation of lipid metabolic processes. GR function is traditionally linked to the regulation of glucose metabolism, especially in the liver, with strong effects on free serum glucose and gluconeogenesis. Upon chronic Gc treatment, the REV-ERBα dependent Gc regulation of metabolism was more strongly associated with lipids. The most obvious physiological effect seen was the protection from hepatosteotosis after Gc treatment in the REV-ERBα null mice. The genes regulated at the mRNA level included both carbohydrate and lipid metabolic processes. The lack of change seen in the glucose sensitivity, insulin resistance and only minor change in fasting glucose could be attributed to the involvement of other tissues. The REV-ERBα mice have more adipose tissue, and less muscle mass, which may buffer against the regulation of carbohydrate genes in the liver. We do see an effect of Gc on regulating the body fat of mice under chronic treatment, and the loss of REV-ERBα results in a modest protection from this, suggesting that the REV-ERBα:GR interaction may play a role in liver and adipose tissue as serum triglycerides were unaffected by REV-ERBαKO.

CAV1 is upregulated in obesity in adipose tissue, with an increased number of caveolae, however it's unclear as to whether this is causative or an effect of the obesity (Grayson *et al.*, 2013). CAV1KO mice are lean, and resistant to high fat diet (Razani *et al.*, 2002), and caveolin-1 plays a role in the transport and enclosure of lipid droplets, but also the regulation of lipid droplet transport by modulating PKA (Cohen *et al.*, 2004), another known Gc interacting kinase (Christoffels *et al.*, 1998).

The activity of the GR can be modulated in multiple different ways. The initial binding of the Gc to the GR initiates so called non-genomic effects, whereby the ligand binding to receptor causes interaction with kinases to initiate signalling cascades (Mitre-Aguilar, Cabrera-Quintero and Zentella-

Dehesa, 2015). CAV1 has been shown to affect the non-genomic actions of the GR *in vitro* (Matthews *et al.*, 2008), however my data suggests that it also affects the genomic actions *in vivo*, whereas published data only looks at this interaction *in vitro* (Peffer *et al.*, 2014). My data also shows the effect on genomic actions of GR, with CAV1 KO mice showing an increase in transactivation in whole lung. However, it was not possible to assess the non-genomic effects *in vivo* as the LPS treatment requires a long time course that far surpasses the non-genomic window of less than 1 hour.

REV-ERBα has a predominantly nuclear localisation (Chopin-Delannoy *et al.*, 2003), limiting the potential for interaction between GR in the cytoplasm and regulation of non-genomic effects. These two proteins interact with GR via disparate mechanisms, with CAV1 likely affecting the interaction between GR and other kinases by modulating their localisation and/or their activity. REV-ERBα on the other hand is involved in directing GR to appropriate binding sites on the DNA, regulating the interaction between GR and key tissue specific, lineage determining factors. CAV1 modulation of Gc sensitivity could therefore be recapitulated by altering kinase activity, and a kinase activity array could provide obvious CAV1 regulated kinases which could potentially alter GR activity on the transcriptional level.

Interestingly, both CAV1 and REV-ERBα have tissue specific effects. While there does not appear to be a cell type specific interaction between CAV1 and GR, not many tissues or cell types were examined. REV-ERBα, and its strong effect on metabolism, may be due to having a tissue specific interaction with GR in an organ heavily associated with metabolic control: the liver. Whether the interaction between REV-ERBα and GR is due to specific target genes i.e. only those that regulate lipid metabolism, or whether it is entirely due to tissue specific, lineage determining factors was not addressed. Therefore, a key question remains as to whether other Gc induced side effects can be limited in other tissues by finding further nuclear receptors that interact with GR and the appropriate GR target genes.

There has been a long and relatively fruitless search for so called "dissociative agonists." That is, agonist which repress inflammation, but do not result in the side effects associated with Gc treatment. Generally these ligands are reported to have reduced transactivation activity, and increase (or maintained) transrepressive activity through GR. Work on the various GR dimerization mutant mice, which have a dramatically reduced capacity for transactivation, with retained transrepression highlights that this approach may not be appropriate. GR^{dim} mice show increased inflammation compared to WT controls (Vandevyver *et al.*, 2012), and loss of the ability to repress inflammation through Gc treatment (Kleiman *et al.*, 2012; Vandevyver *et al.*, 2012; Vettorazzi *et al.*, 2015). The approach I have taken is to look for endogenous regulators of Gc action, which modulate the effects of Gc treatment in specific tissues, both at the cytoplasmic and genomic level. This data is proof of concept that it is possible to dissociate side effects from anti-inflammatory actions of GR via genetic ablation of specific proteins. While it may not be possible, or safe to directly target specific genes in the clinic, kinases and nuclear receptors are easily druggable targets. This allows for further potential therapeutic approaches to modulation of Gc side effects by targeting appropriate kinases or nuclear receptors that interact with the GR.

5.2 Summary

My data demonstrates two independent pathways of affecting Gc sensitivity *in vivo*. Caveolin-1 loss increases Gc transactivation capacity in classically activated Gc target genes. This likely involves a pathway whereby loss of CAV1 alters MAPK signalling cascades, resulting in an indirect mechanism of increased Gc activity through increases phosphorylation of GR co-activators. Caveolin-1 also promotes a pro-inflammatory phenotype in lung parenchyma, but an anti-inflammatory phenotype in alveolar macrophages. REV-ERBα modulates GR activity directly through interacting with GR on the genome. This alters GR binding locations, causing differential regulation of genes depending on the circadian phase. In the liver, REV-ERBα directs GR to regulate triglyceride metabolism, resulting

in hepatic steatosis, and thus loss of REV-ERBα results in mice being protected from this common Gc metabolic side effect.

5.3 Future work

To confirm the role of CAV1 in macrophage – epithelial cell cross talk, co-cultures between CAV1 depleted macrophages and WT epithelial cells (and the reverse experiment) would aid in the explanation of how there are more pro-inflammatory cytokines, but a lower level of cellular infiltrate after LPS induced lung inflammation. To further test the involvement of non-genomic Gc action and CAV1 in inflammation, use of cells or animals with impaired nuclear translocation of GR could be used. This would limit the transrepressive action of GR on the pro-inflammatory transcription factors such as AP-1 and NFκB and only allow the interaction of GR with kinases and other effector molecules in the cytoplasm.

Identifying the involvement of CAV1 in Gc regulation of metabolic processes would be an important follow up to the inflammation data. As both CAV1 and GR regulate lipid metabolism, the cross talk between the two is an interesting area of further study. Furthermore, to test the application of Gc and the role of CAV1 in inflammation, it would be important to reassess using more clinically relevant models. Streptococcus pneumonia challenge or cecal ligation and puncture are more accepted models of acute lung inflammation and sepsis. Furthermore, use of cell type specific knockout mice may give deeper insight into how CAV1 affects inflammation and Gc suppression of inflammation. The use of CX_3CR1^{Cre} mice would allow for deletion of CAV1 solely in resident, alveolar macrophages, thus would not affect the infiltration capacity of cells from the monocyte lineage.

The use of kinase activation arrays to determine the dysregulation of signalling pathways in the CAV1KO mice in a cell type specific manner. This would enable identification of which pathways result in the increase of inflammation in the macrophage, but a decrease in inflammatory signalling

in the parenchyma. Furthermore, it would highlight candidate kinases which may be responsible for the observed increase in CAV1KO dependent Gc induced transactivation.

To confirm the hypothesis that time of day treatment affects Gc induced side effects, but maintains a reduction in inflammation, use of a clinically relevant model, collagen induced arthritis, and a short half-life Gc (such as prednisolone) to treat mice either during the day, or the night to coincide with REV-ERBα peak and trough expression. This would allow assessment of clinical score of the arthritis to determine the anti-inflammatory impact, and also side effects such as hepatic steatosis or fat accumulation. Alternatively, the use of REV-ERBα antagonists could be used to mimic the time of day effect.

Comparing REV-ERBα, GR and other NRs (HNF4α (Reddy *et al.*, 2007; Lim *et al.*, 2015) HNF6 (Lim *et al.*, 2015; Zhang *et al.*, 2016), FOXA1 (Swinstead *et al.*, 2016), PPARα (Ratman *et al.*, 2016), LXRβ (Patel *et al.*, 2011) chromatin co-localisation using publically available data sets would also be an important step forward in understanding how nuclear receptor interactions control metabolic homeostasis, and helpful in the identification of nuclear receptor directed hot spots of transcription. Furthermore, it would be important to test whether ligand treatment against specific nuclear receptors alters the GR cistrome, transcriptome and metabolic or inflammatory phenotype of mice. This approach may be more easily adhered to in humans, as circadian disruption is very common in people, as we do not abide to a set 12 hour light:dark cycle (thus making timing of the Gc treatment more difficult to gauge).

Performing both GR ChIP-seq and REV-ERBα ChIP-seq at both ZT8 and ZT20 with and without dex, to determine how Gc treatment rewires the REV-ERBα cistrome. Similarly, GR ChIP-seq in REV-ERBαKO mice to determine the change in genomic location of GR without the co-binding partner. It would also be important to assess the Gc responsiveness in HNF4a and HNF6 KO mice. Similarly, to identify the genomic location and potential co-binding of GR and REV-ERB upon loss of the hepatic nuclear receptors. Finally, ChIP-re-ChIP assays to determine whether GR, REV-ERBα, HNF4α and HNF6 all

bind at the same genomic loci, followed up with an Array MAPPIT (Tavernier *et al.*, 2002; Lievens *et al.*, 2009) to determine the interaction domains between the nuclear receptors in a high-throughput manner.

Chapter 6: References

Akira, S. and Takeda, K. (2004) 'Toll-like receptor signalling', *Nature Reviews Immunology*. Nature Publishing Group, 4(7), pp. 499–511.

Amin, S., Donaldson, I. J., Zannino, D. A., Hensman, J., Rattray, M., Losa, M., Spitz, F., Ladam, F., Sagerström, C. and Bobola, N. (2015) 'Hoxa2 selectively enhances Meis binding to change a branchial arch ground state', *Dev Cell*, 32(3), pp. 265–277.

Anders, S., Pyl, P. T. and Huber, W. (2015) 'HTSeq--a Python framework to work with high-throughput sequencing data', *Bioinformatics*, 31(2), pp. 166–169.

Aoki, S., Thomas, A., Decaffmeyer, M., Brasseur, R. and Epand, R. M. (2010) 'The Role of Proline in the Membrane Re-entrant Helix of Caveolin-1 *'.

Aravamudan, B., VanOosten, S. K., Meuchel, L. W., Vohra, P., Thompson, M., Sieck, G. C., Prakash, Y. S. and Pabelick, C. M. (2012) 'Caveolin-1 knockout mice exhibit airway hyperreactivity.', *American journal of physiology. Lung cellular and molecular physiology*. American Physiological Society, 303(8), pp. L669-81.

Archer, T. K. and Fryer, C. J. (1998) 'Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex.', *Nature*, 393(6680), pp. 88–91.

Bailey, M. T., Kierstein, S., Sharma, S., Spaits, M., Kinsey, S. G., Tliba, O., Sheridan, J. F., Panettieri, R. A. and Haczku, A. (2009) 'Social Stress Enhances Allergen-Induced Airway Inflammation in Mice and Inhibits Corticosteroid Responsiveness of Cytokine Production', *The Journal of Immunology*, 182(12).

Ballard, P. L. and Ballard, R. A. (1972) 'Glucocorticoid receptors and the role of glucocorticoids in fetal lung development.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 69(9), pp. 2668–72.

Ballard, P. L., Ertsey, R., Gonzales, L. W. and Gonzales, J. (1996) 'Transcriptional regulation of human pulmonary surfactant proteins SP-B and SP-C by glucocorticoids', *Am.J.Respir.Cell Mol.Biol.*, 14(6), pp. 599–607.

Bamberger, C. M., Bamberger, A. M., de, C. M. and Chrousos, G. P. (1995) 'Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans', *J.Clin.Invest*, 95(6), pp. 2435–2441.

Bara, I., Ozier, A., Tunon de Lara, J.-M., Marthan, R. and Berger, P. (2010) 'Pathophysiology of bronchial smooth muscle remodelling in asthma', *European Respiratory Journal*, 36(5).

Barish, G. D., Downes, M., Alaynick, W. A., Yu, R. T., Ocampo, C. B., Bookout, A. L., Mangelsdorf, D. J. and Evans, R. M. (2005) 'A Nuclear Receptor Atlas: Macrophage Activation', *Molecular Endocrinology*, 19(10), pp. 2466–2477.

Barnes, P. J. (1995) 'Inhaled Glucocorticoids for Asthma', *New England Journal of Medicine*, 332(13), pp. 868–875.

Bartholome, B., Spies, C. M., Gaber, T., Schuchmann, S., Berki, T., Kunkel, D., Bienert, M., Radbruch, A., Burmester, G.-R., Lauster, R., Scheffold, A. and Buttgereit, F. (2004) 'Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis.', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 18(1), pp. 70–80.

Baschant, U. and Tuckermann, J. (2010) 'The role of the glucocorticoid receptor in inflammation and immunity', *The Journal of Steroid Biochemistry and Molecular Biology*, 120(2–3), pp. 69–75.

Bavari, S., Bosio, C. M., Wiegand, E., Ruthel, G., Will, A. B., Geisbert, T. W., Hevey, M., Schmaljohn, C., Schmaljohn, A. and Javad Aman, M. (2002) 'Lipid Raft Microdomains: A Gateway for Compartmentalized Trafficking of Ebola and Marburg Viruses ✪', *The Journal of Experimental Medicine @BULLET*, 195(5), pp. 593–602.

Beck, I. M. E., Vanden Berghe, W., Vermeulen, L., Yamamoto, K. R., Haegeman, G. and De Bosscher, K. (2009) 'Crosstalk in Inflammation: The Interplay of Glucocorticoid Receptor-Based Mechanisms and Kinases and Phosphatases', *Endocrine Reviews*, 30(7), pp. 830–882.

Beer, H. D., Fassler, R. and Werner, S. (2000) 'Glucocorticoid-regulated gene expression during cutaneous wound repair', *Vitam.Horm.*, 59, pp. 217–239.

Beura, L. K., Hamilton, S. E., Bi, K., Schenkel, J. M., Odumade, O. A., Casey, K. A., Thompson, E. A., Fraser, K. A., Rosato, P. C., Filali-Mouhim, A., Sekaly, R. P., Jenkins, M. K., Vezys, V., Haining, W. N., Jameson, S. C. and Masopust, D. (2016) 'Normalizing the environment recapitulates adult human immune traits in laboratory mice', *Nature*. Nature Research, 532(7600), pp. 512–516.

Bhattacharyya, S., Brown, D. E., Brewer, J. A., Vogt, S. K. and Muglia, L. J. (2007) 'Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase', *Blood*, 109(10), pp. 4313–4319.

Biddie, S. C., John, S., Sabo, P. J., Thurman, R. E., Johnson, T. A., Schiltz, R. L., Miranda, T. B., Sung, M.-H., Trump, S., Lightman, S. L., Vinson, C., Stamatoyannopoulos, J. A. and Hager, G. L. (2011) *Transcription Factor AP1 Potentiates Chromatin Accessibility and Glucocorticoid Receptor Binding*, *Molecular Cell*.

Birnbaum, R. Y., Clowney, E. J., Agamy, O., Kim, M. J., Zhao, J., Yamanaka, T., Pappalardo, Z., Clarke, S. L., Wenger, A. M., Nguyen, L., Gurrieri, F., Everman, D. B., Schwartz, C. E., Birk, O. S., Bejerano, G., Lomvardas, S. and Ahituv, N. (2012) 'Coding exons function as tissue-specific enhancers of nearby genes.', *Genome research*. Cold Spring Harbor Laboratory Press, 22(6), pp. 1059–68.

Bledsoe, R. K., Montana, V. G., Stanley, T. B., Delves, C. J., Apolito, C. J., McKee, D. D., Consler, T. G., Parks, D. J., Stewart, E. L., Willson, T. M., Lambert, M. H., Moore, J. T., Pearce, K. H. and Xu, H. E.

(2002) ‘Crystal Structure of the Glucocorticoid Receptor Ligand Binding Domain Reveals a Novel Mode of Receptor Dimerization and Coactivator Recognition’, *Cell*, 110(1), pp. 93–105.

Boden, G., Ruiz, J., Urbain, J. L. and Chen, X. (1996) ‘Evidence for a circadian rhythm of insulin secretion.’, *The American journal of physiology*, 271(2 Pt 1), pp. E246-52.

Bolger, A. M., Lohse, M. and Usadel, B. (2014) ‘Trimmomatic: a flexible trimmer for Illumina sequence data’, *Bioinformatics*, 30(15), pp. 2114–2120.

De Bosscher, K., Berghe, W. Vanden, Beck, I. M. E., Van Molle, W., Hennuyer, N., Hapgood, J., Libert, C., Staels, B., Louw, A. and Haegeman, G. (2005) ‘A fully dissociated compound of plant origin for inflammatory gene repression’, *Proceedings of the National Academy of Sciences of the United States of America*, 102(44), pp. 15827–15832.

De Bosscher, K., Vanden Berghe, W. and Haegeman, G. (2003) ‘The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression.’, *Endocrine reviews*, 24(4), pp. 488–522.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H. and Moras, D. (1995) ‘Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha’, *Nature*, 375(6530), pp. 377–382.

Bowie, A. G. and Haga, I. R. (2005) ‘The role of Toll-like receptors in the host response to viruses’, *Molecular Immunology*, 42(8), pp. 859–867.

Breslin, M. B., Geng, C. D. and Vedeckis, W. V (2001) ‘Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids’, *Mol.Endocrinol.*, 15(8), pp. 1381–1395.

Briski, K. P. and Vogel, K. L. (1995) ‘Role of endogenous opioid peptides in central glucocorticoid receptor (GR)-induced decreases in circulating LH in the male rat’, *Neuropeptides*, 28(3), pp. 175–181.

Britton, S. W. and Silvette, H. (1931) ‘Some Observations on the Cortico-Adrenal Hormone’, *Science*, 73(1892).

Brogan, I. J., Murray, I. A., Cerillo, G., Needham, M., White, A. and Davis, J. R. . (1999) ‘Interaction of glucocorticoid receptor isoforms with transcription factors AP-1 and NF-κB: lack of effect of

glucocorticoid receptor β', *Molecular and Cellular Endocrinology*, 157(1), pp. 95–104.

Brown, R. E. (1998) 'Sphingolipid organization in biomembranes: what physical studies of model membranes reveal', *Journal of Cell Science*, 111(1), pp. 1–9.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A. and Carlquist, M. (1997) 'Molecular basis of agonism and antagonism in the oestrogen receptor', *Nature*, 389(6652), pp. 753–758.

Bucci, M., Gratton, J. P., Rudic, R. D., Acevedo, L., Roviezzo, F., Cirino, G. and Sessa, W. C. (2000) 'In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation.', *Nature medicine*, 6(12), pp. 1362–7.

Bultink, I. E. M., Baden, M. and Lems, W. F. (2013) 'Glucocorticoid-induced osteoporosis: an update on current pharmacotherapy and future directions', *Expert Opinion on Pharmacotherapy*, 14(2), pp. 185–197.

Bunge, A., Müller, P., Stöckl, M., Herrmann, A. and Huster, D. (2008) 'Characterization of the Ternary Mixture of Sphingomyelin, POPC, and Cholesterol: Support for an Inhomogeneous Lipid Distribution at High Temperatures', *Biophysical Journal*, 94(7), pp. 2680–2690.

Busillo, J. M. and Cidlowski, J. A. (2011) 'The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore', *Trends in Endocrinology & Metabolism*, (0).

Buttgereit, F., Doering, G., Schaeffler, A., Witte, S., Sierakowski, S., Gromnica-Ihle, E., Jeka, S., Krueger, K., Szechinski, J. and Alten, R. (2008) 'Efficacy of modified-release versus standard prednisone to reduce duration of morning stiffness of the joints in rheumatoid arthritis (CAPRA-1): a double-blind, randomised controlled trial', *Lancet*, 371(9608), pp. 205–214.

Cai, M., Zhao, W., Shang, X., Jiang, J., Ji, H., Tang, Z. and Wang, H. (2012) 'Direct Evidence of Lipid Rafts by in situ Atomic Force Microscopy', *Small*, 8(8), pp. 1243–1250.

Caldenhoven, E., Liden, J., Wissink, S., de Stolpe, A., Raaijmakers, J., Koenderman, L., Okret, S., Gustafsson, J. A. and der Saag, P. T. (1995) 'Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids', *Molecular*

Endocrinology, 9(4), pp. 401–412.

Campbell, J. E., Peckett, A. J., D'souza, A. M., Hawke, T. J. and Riddell, M. C. (2010) 'Adipogenic and lipolytic effects of chronic glucocorticoid exposure', *American Journal of Physiology - Cell Physiology*, 300(1).

Cardoso, W. V and L□ü, J. (2006) 'Regulation of early lung morphogenesis: questions, facts and controversies', *Development*, 133(9), pp. 1611–1624.

Challet, E. (2015) 'Keeping circadian time with hormones', *Diabetes, Obesity and Metabolism*. Blackwell Publishing Ltd, 17(S1), pp. 76–83.

Chen, W., Dang, T., Blind, R. D., Wang, Z., Cavasotto, C. N., Hittelman, A. B., Rogatsky, I., Logan, S. K. and Garabedian, M. J. (2008) 'Glucocorticoid receptor phosphorylation differentially affects target gene expression.', *Molecular endocrinology (Baltimore, Md.)*, 22(8), pp. 1754–66.

Chen, Z., Bakhshi, F. R., Shajahan, A. N., Sharma, T., Mao, M., Trane, A., Bernatchez, P., van Nieuw Amerongen, G. P., Bonini, M. G., Skidgel, R. A., Malik, A. B. and Minshall, R. D. (2012) 'Nitric oxide-dependent Src activation and resultant caveolin-1 phosphorylation promote eNOS/caveolin-1 binding and eNOS inhibition.', *Molecular biology of the cell*, 23(7), pp. 1388–98.

Cheng, Y., Jope, R. S. and Beurel, E. (2015) 'A pre-conditioning stress accelerates increases in mouse plasma inflammatory cytokines induced by stress.', *BMC neuroscience*. BioMed Central, 16, p. 31.

Cho, H., Zhao, X., Hatori, M., Yu, R. T., Barish, G. D., Lam, M. T., Chong, L.-W. W., DiTacchio, L., Atkins, A. R., Glass, C. K., Liddle, C., Auwerx, J., Downes, M., Panda, S. and Evans, R. M. (2012) 'Regulation of circadian behaviour and metabolism by REV-ERB-α and REV-ERB-β', *Nature*, 485(7396), pp. 123–127.

Chopin-Delannoy, S., Thénot, S., Delaunay, F., Buisine, E., Begue, A., Duterque-Coquillaud, M. and Laudet, V. (2003) 'A specific and unusual nuclear localization signal in the DNA binding domain of the Rev-erb orphan receptors.', *Journal of molecular endocrinology*. BioScientifica, 30(2), pp. 197–211.

Choy, M.-K., Movassagh, M., Goh, H.-G., Bennett, M. R., Down, T. A. and Foo, R. S. Y. (2010) 'Genome-wide conserved consensus transcription factor binding motifs are hyper-methylated', *BMC Genomics*, 11(1), p. 519.

Christoffels, V. M., Grange, T., Kaestner, K. H., Cole, T. J., Darlington, G. J., Croniger, C. M. and Lamers, W. H. (1998) 'Glucocorticoid receptor, C/EBP, HNF3, and protein kinase A coordinately activate the glucocorticoid response unit of the carbamoylphosphate synthetase I gene.', *Molecular and cellular biology*, 18(11), pp. 6305–15.

Chung, S., Son, G. H. and Kim, K. (2011) 'Circadian rhythm of adrenal glucocorticoid: Its regulation and clinical implications', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1812(5), pp. 581–591.

Clark, B. J. (2016) 'ACTH Action on StAR Biology', *Frontiers in Neuroscience*. Frontiers, 10, p. 547.

Cohen, A. W., Razani, B., Schubert, W., Williams, T. M., Wang, X. B., Iyengar, P., Brasaemle, D. L., Scherer, P. E. and Lisanti, M. P. (2004) 'Role of Caveolin-1 in the Modulation of Lipolysis and Lipid Droplet Formation', *Diabetes*, 53(5).

Cole, T. J., Blendy, J. A., Monaghan, A. P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., Schutz, G. and Schütz, G. (1995) 'Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation', *Genes Dev.* Cold Spring Harbor Laboratory Press, 9(13), pp. 1608–1621.

Cronstein, B. N., Kimmel, S. C., Levin, R. I., Martiniuk, F. and Weissmann, G. (1992) 'A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1.', *Proceedings of the National Academy of Sciences of the United States of America*, 89(21), pp. 9991–5.

Cutolo, M., Seriolo, B., Pizzorni, C., Secchi, M. E., Soldano, S., Paolino, S., Montagna, P. and Sulli, A. (2008) 'Use of glucocorticoids and risk of infections', *Autoimmunity Reviews*, 8(2), pp. 153–155.

Dahlman-Wright, K., Baumann, H., McEwan, I. J., Alml"f, T., Wright, A. P., Gustafsson, J. A. and H„rd, T. (1995) 'Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor', *Proceedings of the National Academy of Sciences of the United States of America*, 92(5), pp. 1699–1703.

Dahlman-Wright, K., Siltala-Roos, H., Carlstedt-Duke, J. and Gustafsson, J. A. (1990) 'Protein-protein interactions facilitate DNA binding by the glucocorticoid receptor DNA-binding domain', *J.Biol.Chem.*, 265(23), pp. 14030–14035.

Dahlman-Wright, K., Wright, A., Gustafsson, J. A. and Carlstedt-Duke, J. (1991) 'Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids', *Journal of Biological Chemistry*, 266(5), pp. 3107–3112.

Di Dalmazi, G., Pagotto, U., Pasquali, R., Vicennati, V., Di Dalmazi, G., Pagotto, U., Pasquali, R., Vicennati, V., Di, D. G., Pagotto, U., Pasquali, R. and Vicennati, V. (2012) 'Glucocorticoids and type 2 diabetes: from physiology to pathology', *J.Nutr.Metab*. Hindawi Publishing Corporation, 2012, p. 525093.

Dasari, A., Bartholomew, J. N., Volonte, D. and Galbiati, F. (2006) 'Oxidative stress induces premature senescence by stimulating caveolin-1 gene transcription through p38 mitogen-activated protein kinase/Sp1-mediated activation of two GC-rich promoter elements.', *Cancer research*, 66(22), pp. 10805–14.

Davies, M. A., Schuster, H. F., Brauner, J. W. and Mendelsohn, R. (1990) 'Effects of cholesterol on conformational disorder in dipalmitoylphosphatidylcholine bilayers. A quantitative IR study of the depth dependence', *Biochemistry*, 29(18), pp. 4368–4373.

de, C. M., Elliot, S., Kino, T., Bamberger, C., Karl, M., Webster, E. and Chrousos, G. P. (1996) 'The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role', *Mol.Med.*, 2(5), pp. 597–607.

Dejager, L., Dendoncker, K., Eggermont, M., Souffriau, J., Van Hauwermeiren, F., Willart, M., Van Wonterghem, E., Naessens, T., Ballegeer, M., Vandevyver, S., Hammad, H., Lambrecht, B., De Bosscher, K., Grooten, J. and Libert, C. (2015) 'Neutralizing TNFα restores glucocorticoid sensitivity in a mouse model of neutrophilic airway inflammation', *Mucosal Immunology*. Nature Publishing Group, 8(6), pp. 1212–1225.

Delezie, J., Dumont, S., Dardente, H., Oudart, H., Gréchez-Cassiau, A., Klosen, P., Teboul, M.,

Delaunay, F., Pévet, P. and Challet, E. (2012) 'The nuclear receptor REV-ERBα is required for the daily balance of carbohydrate and lipid metabolism', *FASEB J*, 26(8), pp. 3321–3335.

Dik, W. A., McAnulty, R. J., Versnel, M. A., Naber, B. A. E., Zimmermann, L. J. I., Laurent, G. J. and Mutsaers, S. E. (2003) 'Short course dexamethasone treatment following injury inhibits bleomycin induced fibrosis in rats.', *Thorax*. BMJ Publishing Group Ltd and British Thoracic Society, 58(9), pp. 765–71.

Dimitriadis, G., Leighton, B., Parry-Billings, M., Sasson, S., Young, M., Krause, U., Bevan, S., Piva, T., Wegener, G. and Newsholme, E. A. (1997) 'Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle.', *The Biochemical journal*. Portland Press Ltd, (Pt 3), pp. 707–12.

Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J. and Stallcup, M. R. (1998) 'Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities', *Mol.Endocrinol.*, 12(2), pp. 302–313.

Divertie, G. D., Jensen, M. D. and Miles, J. M. (1991) 'Stimulation of Lipolysis in Humans by Physiological Hypercortisolemia', *Diabetes*, 40(10).

Dixon, W. G., Abrahamowicz, M., Beauchamp, M. E., Ray, D. W., Bernatsky, S., Suissa, S. and Sylvestre, M. P. (2012) 'Immediate and delayed impact of oral glucocorticoid therapy on risk of serious infection in older patients with rheumatoid arthritis: a nested case-control analysis', *Annals of the Rheumatic Diseases*, 71(7), pp. 1128–1133.

Doherty, C. J. and Kay, S. A. (2010) 'Circadian Control of Global Gene Expression Patterns', *Annual Review of Genetics*, 44(1), pp. 419–444.

Dong, Y., Poellinger, L., Gustafsson, J. A. and Okret, S. (1988) 'Regulation of Glucocorticoid Receptor Expression: Evidence for Transcriptional and Posttranslational Mechanisms', *Molecular Endocrinology*, 2(12), pp. 1256–1264.

Doucas, V., Shi, Y., Miyamoto, S., West, A., Verma, I. and Evans, R. M. (2000) 'Cytoplasmic catalytic

subunit of protein kinase A mediates cross-repression by NF-kB and the glucocorticoid receptor', *Proceedings of the National Academy of Sciences*, 97(22), pp. 11893–11898.

Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C., Schedl, A., Haller, H. and Kurzchalia, T. V. (2001) 'Loss of Caveolae, Vascular Dysfunction, and Pulmonary Defects in Caveolin-1 Gene-Disrupted Mice', *Science*, 293(5539).

Eichenfield, D. Z., Troutman, T. D., Link, V. M., Lam, M. T., Cho, H., Gosselin, D., Spann, N. J., Lesch, H. P., Tao, J., Muto, J., Gallo, R. L., Evans, R. M. and Glass, C. K. (2016) 'Tissue damage drives co-localization of NF-κB, Smad3, and Nrf2 to direct Rev-erb sensitive wound repair in mouse macrophages.', *eLife*. eLife Sciences Publications, Ltd, 5.

Elbi, C., Walker, D. A., Romero, G., Sullivan, W. P., Toft, D. O., Hager, G. L. and DeFranco, D. B. (2004) 'Molecular chaperones function as steroid receptor nuclear mobility factors.', *Proceedings of the National Academy of Sciences of the United States of America*, 101(9), pp. 2876–81.

Elder, A., Johnston, C., Gelein, R., Finkelstein, J., Wang, Z., Notter, R. and Oberdörster, G. (2005) 'LUNG INFLAMMATION INDUCED BY ENDOTOXIN IS ENHANCED IN RATS DEPLETED OF ALVEOLAR MACROPHAGES WITH AEROSOLIZED CLODRONATE', *Experimental Lung Research*, 31(6), pp. 527–546.

Elizur, A., Adair-Kirk, T. L., Kelley, D. G., Griffin, G. L., deMello, D. E. and Senior, R. M. (2007) 'Clara cells impact the pulmonary innate immune response to LPS', *AJP: Lung Cellular and Molecular Physiology*, 293(2), pp. L383–L392.

Engelman, J. A., Chu, C., Lin, A., Jo, H., Ikezu, T., Okamoto, T., Kohtz, D. S. and Lisanti, M. P. (1998) 'Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain.', *FEBS letters*, 428(3), pp. 205–11.

Exton, J. H., Friedmann, N., Wong, E. H., Brineaux, J. P., Corbin, J. D. and Park, C. R. (1972) 'Interaction of glucocorticoids with glucagon and epinephrine in the control of gluconeogenesis and glycogenolysis in liver and of lipolysis in adipose tissue.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 247(11), pp. 3579–88.

Falkenstein, E., Norman, A. W. and Wehling, M. (2000) 'Mannheim Classification of Nongenomically Initiated (Rapid) Steroid Action(s)', *The Journal of Clinical Endocrinology & Metabolism*. Endocrine Society, 85(5), pp. 2072–2075.

Flaherty, K. R., Toews, G. B., Lynch, J. P., Kazerooni, E. A., Gross, B. H., Strawderman, R. L., Hariharan, K., Flint, A. and Martinez, F. J. (2001) 'Steroids in idiopathic pulmonary fibrosis: a prospective assessment of adverse reactions, response to therapy, and survival.', *The American journal of medicine*, 110(4), pp. 278–82.

La Fleur, S. E., Kalsbeek, A., Wortel, J. and Buijs, R. M. (1999) 'A suprachiasmatic nucleus generated rhythm in basal glucose concentrations.', *Journal of neuroendocrinology*, 11(8), pp. 643–52.

Ford, J., McEwan, I. J., Wright, A. P. H. and Gustafsson, J. …ke (1997) 'Involvement of the Transcription Factor IID Protein Complex in Gene Activation by the N-Terminal Transactivation Domain of the Glucocorticoid Receptor in Vitro', *Molecular Endocrinology*, 11(10), pp. 1467–1475.

Freedman, L. P., Luisi, B. F., Korszun, Z. R., Basavappa, R., Sigler, P. B. and Yamamoto, K. R. (1988) 'The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain', *Nature*, 334(6182), pp. 543–546.

Freedman, N. D. and Yamamoto, K. R. (2004) 'Importin 7 and importin alpha/importin beta are nuclear import receptors for the glucocorticoid receptor.', *Molecular biology of the cell*. American Society for Cell Biology, 15(5), pp. 2276–86.

Fujiwara, N. and Kobayashi, K. (2005) 'Macrophages in inflammation.', *Current drug targets. Inflammation and allergy*, 4(3), pp. 281–6.

Galien, R., Evans, H. F. and Garcia, T. (1996) 'Involvement of CCAAT/enhancer-binding protein and nuclear factor-kappa B binding sites in interleukin-6 promoter inhibition by estrogens', *Mol.Endocrinol.*, 10(6), pp. 713–722.

Garbrecht, M. R., Schmidt, T. J., Krozowski, Z. S. and Snyder, J. M. (2006) '11Beta-hydroxysteroid dehydrogenase type 2 and the regulation of surfactant protein A by dexamethasone metabolites', *Am.J.Physiol Endocrinol.Metab*, 290(4), pp. E653–E660.

Garrean, S., Gao, X.-P., Brovkovych, V., Shimizu, J., Zhao, Y.-Y., Vogel, S. M. and Malik, A. B. (2006) 'Caveolin-1 regulates NF-kappaB activation and lung inflammatory response to sepsis induced by lipopolysaccharide.', *Journal of immunology (Baltimore, Md. : 1950)*, 177(7), pp. 4853–60.

Gathercole, L. L., Morgan, S. A., Bujalska, I. J., Hauton, D., Stewart, P. M. and Tomlinson, J. W. (2011) 'Regulation of Lipogenesis by Glucocorticoids and Insulin in Human Adipose Tissue', *PLoS ONE*. Edited by E. Buratti. Public Library of Science, 6(10), p. e26223.

Gibbs, J. E., Blaikley, J., Beesley, S., Matthews, L., Simpson, K. D., Boyce, S. H., Farrow, S. N., Else, K. J., Singh, D., Ray, D. W. and Loudon, A. S. (2012) 'The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines', *Proc Natl Acad Sci U S A*, 109(2), pp. 582–587.

Gibbs, J., Ince, L., Matthews, L., Mei, J., Bell, T., Yang, N., Saer, B., Begley, N., Poolman, T., Pariollaud, M., Farrow, S., DeMayo, F., Hussell, T., Worthen, G. S., Ray, D. and Loudon, A. (2014) 'An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action', *Nat Med*, 20(8), pp. 919–926.

Giguere, V., Hollenberg, S. M., Rosenfeld, M. G. and Evans, R. M. (1986) 'Functional domains of the human glucocorticoid receptor', *Cell*, 46(5), pp. 645–652.

Gonzalez, E., Nagiel, A., Lin, A. J., Golan, D. E. and Michel, T. (2004) 'Small interfering RNA-mediated down-regulation of caveolin-1 differentially modulates signaling pathways in endothelial cells.', *The Journal of biological chemistry*, 279(39), pp. 40659–69.

Gosens, R., Stelmack, G. L., Bos, S. T., Dueck, G., Mutawe, M. M., Schaafsma, D., Unruh, H., Gerthoffer, W. T., Zaagsma, J., Meurs, H. and Halayko, A. J. (2011) 'Caveolin-1 is required for contractile phenotype expression by airway smooth muscle cells', *Journal of Cellular and Molecular Medicine*. Blackwell Publishing Ltd, 15(11), pp. 2430–2442.

Goulding, N. J. (2004) 'The molecular complexity of glucocorticoid actions in inflammation - a four-ring circus', *Curr.Opin.Pharmacol.*, 4(6), pp. 629–636.

'GPy: A Gaussian process framework in python [https://github.com/SheffieldML/GPy]' (no date).

Grande-García, A., Echarri, A., de Rooij, J., Alderson, N. B., Waterman-Storer, C. M., Valdivielso, J. M. and del Pozo, M. A. (2007) 'Caveolin-1 regulates cell polarization and directional migration through Src kinase and Rho GTPases.', *The Journal of cell biology*. The Rockefeller University Press, 177(4), pp. 683–94.

Grayson, T. H., Chadha, P. S., Bertrand, P. P., Chen, H., Morris, M. J., Senadheera, S., Murphy, T. V. and Sandow, S. L. (2013) 'Increased caveolae density and caveolin-1 expression accompany impaired NO-mediated vasorelaxation in diet-induced obesity', 139(2).

Green, S., Kumar, V., Theulaz, I., Wahli, W. and Chambon, P. (1988) 'The N-terminal DNA-binding "zinc finger" of the oestrogen and glucocorticoid receptors determines target gene specificity', *EMBO J.*, 7(10), pp. 3037–3044.

Grøntved, L., John, S., Baek, S., Liu, Y., Buckley, J. R., Vinson, C., Aguilera, G. and Hager, G. L. (2013) 'C/EBP maintains chromatin accessibility in liver and facilitates glucocorticoid receptor recruitment to steroid response elements', *EMBO J*, 32(11), pp. 1568–1583.

Hammer, M., Echtenachter, B., Weighardt, H., Jozefowski, K., Rose-John, S., Männel, D. N., Holzmann, B. and Lang, R. (2010) 'Increased inflammation and lethality of Dusp1-/- mice in polymicrobial peritonitis models.', *Immunology*. Wiley-Blackwell, 131(3), pp. 395–404.

Hammer, M., Mages, J., Dietrich, H., Servatius, A., Howells, N., Cato, A. C. B. and Lang, R. (2006) 'Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock', *Journal of Experimental Medicine*, 203(1).

Hard, T., Dahlman, K., Carlstedt-Duke, J., Gustafsson, J. A. and Rigler, R. (1990) 'Cooperativity and specificity in the interactions between DNA and the glucocorticoid receptor DNA-binding domain', *Biochemistry*, 29(22), pp. 5358–5364.

Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P. and Cato, A. C. (1994) 'A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1.', *The EMBO journal*. European Molecular Biology Organization, 13(17), pp. 4087–95.

Herman, J. P., McKlveen, J. M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., Herman, J. P., McKlveen, J. M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J. and Myers, B. (2016) 'Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response', in *Comprehensive Physiology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., pp. 603–621.

Hernández-Alvarez, M. I., Paz, J. C., Sebastián, D., Muñoz, J. P., Liesa, M., Segalés, J., Palacín, M. and Zorzano, A. (2013) 'Glucocorticoid Modulation of Mitochondrial Function in Hepatoma Cells Requires the Mitochondrial Fission Protein Drp1', *Antioxidants & Redox Signaling*. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA , 19(4), pp. 366–378.

Hill, M. M., Bastiani, M., Luetterforst, R., Kirkham, M., Kirkham, A., Nixon, S. J., Walser, P., Abankwa, D., Oorschot, V. M. J., Martin, S., Hancock, J. F. and Parton, R. G. (2008) 'PTRF-Cavin, a Conserved Cytoplasmic Protein Required for Caveola Formation and Function', *Cell*, 132(1), pp. 113–124.

Hoberman, H. D. (1950) 'Endocrine regulation of amino acid protein metabolism during fasting.', *The Yale journal of biology and medicine*. Yale Journal of Biology and Medicine, 22(4), pp. 341–67.

Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G. and Evans, R. M. (1985) 'Primary structure and expression of a functional human glucocorticoid receptor cDNA', *Nature*, 318(6047), pp. 635–641.

Hoop, C. L., Sivanandam, V. N., Kodali, R., Srnec, M. N. and van der Wel, P. C. A. (2012) 'Structural characterization of the caveolin scaffolding domain in association with cholesterol-rich membranes.', *Biochemistry*, 51(1), pp. 90–9.

Hopkins, R. L. and Leinung, M. C. (2005) 'Exogenous Cushing's Syndrome and Glucocorticoid Withdrawal', *Endocrinology and Metabolism Clinics of North America*, 34(2), pp. 371–384.

Hu, G., Ye, R. D., Dinauer, M. C., Malik, A. B. and Minshall, R. D. (2008) 'Neutrophil caveolin-1 expression contributes to mechanism of lung inflammation and injury.', *American journal of physiology. Lung cellular and molecular physiology*, 294(2), pp. L178-86.

Hübner, S., Dejager, L., Libert, C. and Tuckermann, J. P. (2015) 'The glucocorticoid receptor in inflammatory processes: transrepression is not enough', *Biological Chemistry*, 396(11), pp. 1223–

1231.

Hudson, W. H., Youn, C. and Ortlund, E. A. (2013) 'The structural basis of direct glucocorticoid-mediated transrepression', *Nat Struct Mol Biol*, 20(1), pp. 53–58.

Igarashi, J., Hashimoto, T., Shoji, K., Yoneda, K., Tsukamoto, I., Moriue, T., Kubota, Y. and Kosaka, H. (2013) 'Dexamethasone induces caveolin-1 in vascular endothelial cells: implications for attenuated responses to VEGF', *American Journal of Physiology - Cell Physiology*, 304(8).

Ismaili, N. and Garabedian, M. J. (2004) 'Modulation of Glucocorticoid Receptor Function via Phosphorylation', *Annals of the New York Academy of Sciences*. Blackwell Publishing Ltd, 1024(1), pp. 86–101.

Ito, K., Ito, M., Elliott, W. M., Cosio, B., Caramori, G., Kon, O. M., Barczyk, A., Hayashi, S., Adcock, I. M., Hogg, J. C. and Barnes, P. J. (2005) 'Decreased Histone Deacetylase Activity in Chronic Obstructive Pulmonary Disease', *New England Journal of Medicine*. Massachusetts Medical Society , 352(19), pp. 1967–1976.

Itoh, M., Adachi, M., Yasui, H., Takekawa, M., Tanaka, H. and Imai, K. (2002) 'Nuclear export of glucocorticoid receptor is enhanced by c-Jun N-terminal kinase-mediated phosphorylation.', *Molecular endocrinology (Baltimore, Md.)*, 16(10), pp. 2382–92.

Jain, S., Li, Y., Kumar, A. and Sehgal, P. B. (2005) 'Transcriptional signaling from membrane raft-associated glucocorticoid receptor.', *Biochemical and biophysical research communications*, 336(1), pp. 3–8.

Jiao, H., Zhang, Y., Yan, Z., Wang, Z.-G., Liu, G., Minshall, R. D., Malik, A. B. and Hu, G. (2013) 'Caveolin-1 Tyr14 phosphorylation induces interaction with TLR4 in endothelial cells and mediates MyD88-dependent signaling and sepsis-induced lung inflammation.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 191(12), pp. 6191–9.

John, S., Sabo, P. J., Thurman, R. E., Sung, M.-H., Biddie, S. C., Johnson, T. A., Hager, G. L. and Stamatoyannopoulos, J. A. (2011) 'Chromatin accessibility pre-determines glucocorticoid receptor binding patterns', *Nature genetics*, 43(3), pp. 264–268.

Jouffe, C., Cretenet, G., Symul, L., Martin, E., Atger, F., Naef, F. and Gachon, F. (2013) 'The circadian clock coordinates ribosome biogenesis.', *PLoS biology*. Public Library of Science, 11(1), p. e1001455.

Kassel, O., Schneider, S., Heilbock, C., Litfin, M., Göttlicher, M. and Herrlich, P. (2004) 'A nuclear isoform of the focal adhesion LIM-domain protein Trip6 integrates activating and repressing signals at AP-1- and NF-kappaB-regulated promoters.', *Genes & development*, 18(20), pp. 2518–28.

Kauppi, B., Jakob, C., Fa¤rnegardh, M., Yang, J., Ahola, H., Alarcon, M., Calles, K., Engstrom, O., Harlan, J., Muchmore, S., Ramqvist, A. K., Thorell, S., Ohman, L., Greer, J., Gustafsson, J. A., Carlstedt-Duke, J. and Carlquist, M. (2003) 'The Three-dimensional Structures of Antagonistic and Agonistic Forms of the Glucocorticoid Receptor Ligand-binding Domain: RU-486 Induces a Transconformation That Leads To Active Antagonism', *Journal of Biological Chemistry*, 278(25), pp. 22748–22754.

Kayahara, M., Ohanian, J., Ohanian, V., Berry, A., Vadlamudi, R. and Ray, D. W. (2008) 'MNAR functionally interacts with both NH2- and COOH-terminal GR domains to modulate transactivation', *Am.J.Physiol Endocrinol.Metab*, 295(5), pp. E1047–E1055.

Keatings, V. M., Jatakanon, A., Worsdell, Y. M. and Barnes, P. J. (1997) 'Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD', *American Journal of Respiratory and Critical Care Medicine*, 155(2), pp. 542–548.

Kettner, N. M., Voicu, H., Finegold, M. J., Coarfa, C., Sreekumar, A., Putluri, N., Katchy, C. A., Lee, C., Moore, D. D. and Fu, L. (2016) 'Circadian Homeostasis of Liver Metabolism Suppresses Hepatocarcinogenesis', *Cancer Cell*, 30(6), pp. 909–924.

Kino, T., Ichijo, T., Amin, N. D., Kesavapany, S., Wang, Y., Kim, N., Rao, S., Player, A., Zheng, Y.-L., Garabedian, M. J., Kawasaki, E., Pant, H. C. and Chrousos, G. P. (2007) 'Cyclin-Dependent Kinase 5 Differentially Regulates the Transcriptional Activity of the Glucocorticoid Receptor through Phosphorylation: Clinical Implications for the Nervous System Response to Glucocorticoids and Stress', *Molecular Endocrinology*. Endocrine Society , 21(7), pp. 1552–1568.

Kino, T., Manoli, I., Kelkar, S., Wang, Y., Su, Y. A. and Chrousos, G. P. (2009) 'Glucocorticoid receptor (GR) beta has intrinsic, GRalpha-independent transcriptional activity',

Biochem.Biophys.Res.Commun., 381(4), pp. 671–675.

Kleiman, A., Hübner, S., Rodriguez Parkitna, J. M., Neumann, A., Hofer, S., Weigand, M. A., Bauer, M., Schmid, W., Schütz, G., Libert, C., Reichardt, H. M. and Tuckermann, J. P. (2012) 'Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages.', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. Federation of American Societies for Experimental Biology, 26(2), pp. 722–9.

Ko, C. H. and Takahashi, J. S. (2006) 'Molecular components of the mammalian circadian clock', *Human Molecular Genetics* , 15(suppl 2), pp. R271–R277.

Koike, N., Yoo, S. H., Huang, H. C., Kumar, V., Lee, C., Kim, T. K. and Takahashi, J. S. (2012) 'Transcriptional architecture and chromatin landscape of the core circadian clock in mammals', *Science*, 338(6105), pp. 349–354.

Kovacs, J. J., Murphy, P. J. M., Gaillard, S., Zhao, X., Wu, J.-T., Nicchitta, C. V, Yoshida, M., Toft, D. O., Pratt, W. B. and Yao, T.-P. (2005) 'HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor.', *Molecular cell*, 18(5), pp. 601–7.

Krstic, M. D., Rogatsky, I., Yamamoto, K. R. and Garabedian, M. J. (1997) 'Mitogen-activated and cyclin-dependent protein kinases selectively and differentially modulate transcriptional enhancement by the glucocorticoid receptor', *Mol Cell Biol*, 17(7), pp. 3947–3954.

Kumar, R. K., Herbert, C. and Kasper, M. (2004) 'Reversibility of airway inflammation and remodelling following cessation of antigenic challenge in a model of chronic asthma', *Clinical <html_ent glyph='@amp;' ascii='&'/> Experimental Allergy*, 34(11), pp. 1796–1802.

Kumar, R., Lee, J. C., Bolen, D. W. and Thompson, E. B. (2001) 'The Conformation of the Glucocorticoid Receptor AF1/tau1 Domain Induced by Osmolyte Binds Co-regulatory Proteins', *Journal of Biological Chemistry*, 276(21), pp. 18146–18152.

Kumar, R. and Thompson, E. B. (1999) 'The structure of the nuclear hormone receptors', *Steroids*, 64(5), pp. 310–319.

Kuo, T., Harris, C. A. and Wang, J.-C. (2013) 'Metabolic functions of glucocorticoid receptor in skeletal

muscle', *Molecular and Cellular Endocrinology*, 380(1), pp. 79–88.

LaCasse, E. C. and Lefebvre, Y. A. (1995) 'Nuclear localization signals overlap DNA- or RNA-binding domains in nucleic acid-binding proteins', *Nucleic Acids Research*, 23(10), pp. 1647–1656.

Lamia, K. A., Papp, S. J., Yu, R. T., Barish, G. D., Uhlenhaut, N. H., Jonker, J. W., Downes, M. and Evans, R. M. (2011) 'Cryptochromes mediate rhythmic repression of the glucocorticoid receptor', *Nature*, 480(7378), pp. 552–556.

de Lange, P., Segeren, C. M., Koper, J. W., Wiemer, E., Sonneveld, P., Brinkmann, A. O., White, A., Brogan, I. J., de Jong, F. H. and Lamberts, S. W. (2001) 'Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells', *Cancer Res.*, 61(10), pp. 3937–3941.

Langmead, B., Trapnell, C., Pop, M. and Salzberg, S. L. (2009) 'Ultrafast and memory-efficient alignment of short DNA sequences to the human genome', *Genome Biol*, 10(3), p. R25.

Leclerc, N., Luppen, C. A., Ho, V. V, Nagpal, S., Hacia, J. G., Smith, E. and Frenkel, B. (2004) 'Gene expression profiling of glucocorticoid-inhibited osteoblasts', *Journal of Molecular Endocrinology*, 33(1), pp. 175–193.

Leclerc, N., Noh, T., Khokhar, A., Smith, E. and Frenkel, B. (2005) 'Glucocorticoids inhibit osteocalcin transcription in osteoblasts by suppressing Egr2/Krox20-binding enhancer', *Arthritis Rheum.*, 52(3), pp. 929–939.

Lewis-Tuffin, L. J. and Cidlowski, J. A. (2006) 'The physiology of human glucocorticoid receptor beta (hGRbeta) and glucocorticoid resistance', *Ann.N.Y.Acad.Sci.*, 1069, pp. 1–9.

Lewis, C. M. and Rose, J. D. (2003) 'Rapid corticosterone-induced impairment of amplectic clasping occurs in the spinal cord of roughskin newts (taricha granulosa)', *Hormones and Behavior*, 43(1), pp. 93–98.

Li, R., Zou, H., Jia, Y. and Zhao, R. (2013) 'Glucocorticoid receptor is involved in the breed-dependent transcriptional regulation of mtDNA- and nuclear-encoded mitochondria genes in the liver of newborn piglets', *BMC Veterinary Research*. BioMed Central, 9(1), p. 87.

Lievens, S., Vanderroost, N., Van der Heyden, J., Gesellchen, V., Vidal, M. and Tavernier, J. (2009) 'Array MAPPIT: High-Throughput Interactome Analysis in Mammalian Cells', *Journal of Proteome Research*. American Chemical Society, 8(2), pp. 877–886.

Lim, H. W., Uhlenhaut, N. H., Rauch, A., Weiner, J., Hübner, S., Hübner, N., Won, K. J., Lazar, M. A., Tuckermann, J. and Steger, D. J. (2015) 'Genomic redistribution of GR monomers and dimers mediates transcriptional response to exogenous glucocorticoid in vivo', *Genome Res*, 25(6), pp. 836–844.

Lipton, J. O., Yuan, E. D., Boyle, L. M., Ebrahimi-Fakhari, D., Kwiatkowski, E., Nathan, A., Güttler, T., Davis, F., Asara, J. M. and Sahin, M. (2015) 'The Circadian Protein BMAL1 Regulates Translation in Response to S6K1-Mediated Phosphorylation.', *Cell*. Elsevier, 161(5), pp. 1138–51.

Liu, L., Brown, D., McKee, M., LeBrasseur, N. K., Yang, D., Albrecht, K. H., Ravid, K. and Pilch, P. F. (2008) 'Deletion of Cavin/PTRF Causes Global Loss of Caveolae, Dyslipidemia, and Glucose Intolerance', *Cell metabolism*, 8(4), pp. 310–317.

Liu, P., Rudick, M. and Anderson, R. G. W. (2002) 'Multiple functions of caveolin-1.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 277(44), pp. 41295–8.

Lönnfors, M., Doux, J.-P. F., Killian, J. A., Nyholm, T.-K. M., Slotte, J. P., Lönnfors, M., Doux, J.-P. F., Killian, J. A., Nyholm, T.-K. M. and Slotte, J. P. (2011) 'Sterols Have Higher Affinity for Sphingomyelin than for Phosphatidylcholine Bilayers even at Equal Acyl-Chain Order', *Biophysical Journal*. The Biophysical Society, 100(11), pp. 2633–2641.

Love, M. I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biol*, 15(12), p. 550.

Lu, Y.-C., Yeh, W.-C. and Ohashi, P. S. (2008) 'LPS/TLR4 signal transduction pathway', *Cytokine*, 42(2), pp. 145–151.

Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R. and Sigler, P. B. (1991) 'Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA', *Nature*,

352(6335), pp. 497–505.

Maier, J. V., Brema, S., Tuckermann, J., Herzer, U., Klein, M., Stassen, M., Moorthy, A. and Cato, A. C. B. (2007) 'Dual Specificity Phosphatase 1 Knockout Mice Show Enhanced Susceptibility to Anaphylaxis but Are Sensitive to Glucocorticoids', *Molecular Endocrinology*, 21(11), pp. 2663–2671.

Margaritopoulos, G. A., Tsitoura, E., Tzanakis, N., Spandidos, D. A., Siafakas, N. M., Sourvinos, G. and Antoniou, K. M. (2013) 'Self-eating: friend or foe? The emerging role of autophagy in idiopathic pulmonary fibrosis.', *BioMed research international*. Hindawi Publishing Corporation, 2013, p. 420497.

De Martino, M. U., Alesci, S., Chrousos, G. P. and Kino, T. (2004) 'Interaction of the glucocorticoid receptor and the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII): implications for the actions of glucocorticoids on glucose, lipoprotein, and xenobiotic metabolism.', *Annals of the New York Academy of Sciences*, 1024, pp. 72–85.

Matthews, L., Berry, A., Ohanian, V., Ohanian, J., Garside, H. and Ray, D. (2008) 'Caveolin mediates rapid glucocorticoid effects and couples glucocorticoid action to the antiproliferative program.', *Molecular endocrinology (Baltimore, Md.)*, 22(6), pp. 1320–30.

Matthews, L., Johnson, J., Berry, A., Trebble, P., Cookson, A., Spiller, D., Rivers, C., Norman, M., White, M. and Ray, D. (2011) 'Cell Cycle Phase Regulates Glucocorticoid Receptor Function', *PLoS ONE*. Edited by J.-M. Vanacker. Public Library of Science, 6(7), p. e22289.

McKay, L. I. and Cidlowski, J. A. (1998) 'Cross-Talk between Nuclear Factor-Kappa B and the Steroid Hormone Receptors: Mechanisms of Mutual Antagonism', *Molecular Endocrinology*, 12(1), pp. 45–56.

McMaster, A. and Ray, D. W. (2008) 'Drug insight: selective agonists and antagonists of the glucocorticoid receptor', *Nat.Clin.Pract.Endocrinol.Metab*, 4(2), pp. 91–101.

Medina, F. A., de Almeida, C. J., Dew, E., Li, J., Bonuccelli, G., Williams, T. M., Cohen, A. W., Pestell, R. G., Frank, P. G., Tanowitz, H. B. and Lisanti, M. P. (2006) 'Caveolin-1-deficient mice show defects in innate immunity and inflammatory immune response during Salmonella enterica serovar

Typhimurium infection.', *Infection and immunity*, 74(12), pp. 6665–74.

Meijsing, S. H., Pufall, M. A., So, A. Y., Bates, D. L., Chen, L. and Yamamoto, K. R. (2009) 'DNA binding site sequence directs glucocorticoid receptor structure and activity', *Science*, 324(5925), pp. 407–410.

Migita, H., Morser, J. and Kawai, K. (2004) 'Rev-erbα upregulates NF-κB-responsive genes in vascular smooth muscle cells', *FEBS Letters*, 561(1–3), pp. 69–74.

Miller, A. L., Webb, M. S., Copik, A. J., Wang, Y., Johnson, B. H., Kumar, R. and Thompson, E. B. (2005) 'p38 Mitogen-activated protein kinase (MAPK) is a key mediator in glucocorticoid-induced apoptosis of lymphoid cells: correlation between p38 MAPK activation and site-specific phosphorylation of the human glucocorticoid receptor at serine 211.', *Molecular endocrinology (Baltimore, Md.)*, 19(6), pp. 1569–83.

Le Minh, N., Damiola, F., Tronche, F., Schütz, G. and Schibler, U. (2001) 'Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators', *Embo j*. European Molecular Biology Organization, 20(24), pp. 7128–7136.

Mitre-Aguilar, I. B., Cabrera-Quintero, A. J. and Zentella-Dehesa, A. (2015) 'Genomic and non-genomic effects of glucocorticoids: implications for breast cancer.', *International journal of clinical and experimental pathology*. e-Century Publishing Corporation, 8(1), pp. 1–10.

Moalli, P. A., Pillay, S., Krett, N. L. and Rosen, S. T. (1993) 'Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells', *Cancer Res.*, 53(17), pp. 3877–3879.

Moghadam-Kia, S. and Werth, V. P. (2010) 'Prevention and treatment of systemic glucocorticoid side effects.', *International journal of dermatology*. NIH Public Access, 49(3), pp. 239–48.

Monier, S., Parton, R. G., Vogel, F., Behlke, J., Henske, A. and Kurzchalia, T. V (1995) 'VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro.', *Molecular biology of the cell*, 6(7), pp. 911–27.

Moore, M. W. and Herzog, E. L. (2013) 'Regulation and Relevance of Myofibroblast Responses in

Idiopathic Pulmonary Fibrosis.', *Current pathobiology reports*. NIH Public Access, 1(3), pp. 199–208.

Mozo, L., Suárez, A. and Gutiérrez, C. (2004) 'Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes.', *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*, 34(3), pp. 406–12.

Mueller, K. M., Themanns, M., Friedbichler, K., Kornfeld, J.-W., Esterbauer, H., Tuckermann, J. P. and Moriggl, R. (2012) 'Hepatic growth hormone and glucocorticoid receptor signaling in body growth, steatosis and metabolic liver cancer development', *Molecular and Cellular Endocrinology*, 361(1), pp. 1–11.

Murata, M., Peränen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V and Simons, K. (1995) 'VIP21/caveolin is a cholesterol-binding protein.', *Proceedings of the National Academy of Sciences of the United States of America*, 92(22), pp. 10339–43.

Nader, N., Ng, S. S., Lambrou, G. I., Pervanidou, P., Wang, Y., Chrousos, G. P. and Kino, T. (2010) 'AMPK regulates metabolic actions of glucocorticoids by phosphorylating the glucocorticoid receptor through p38 MAPK', *Mol Endocrinol*, 24(9), pp. 1748–1764.

Necela, B. M. and Cidlowski, J. A. (2004) 'Mechanisms of Glucocorticoid Receptor Action in Noninflammatory and Inflammatory Cells', *Proceedings of the American Thoracic Society*, 1(3), pp. 239–246.

Nissen, R. M. and Yamamoto, K. R. (2000) 'The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain.', *Genes & development*. Cold Spring Harbor Laboratory Press, 14(18), pp. 2314–29.

Nrnc (1999) 'A Unified Nomenclature System for the Nuclear Receptor Superfamily', *Cell*, 97(2), pp. 161–163.

Nyholm, T. K. M., Grandell, P. M., Westerlund, B. and Slotte, J. P. (2010) 'Sterol affinity for bilayer membranes is affected by their ceramide content and the ceramide chain length', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1798(5), pp. 1008–1013.

Oakley, R. H., Webster, J. C., Sar, M., Parker, C. R. and Cidlowski, J. A. (1997) 'Expression and

Subcellular Distribution of the Beta-Isoform of the Human Glucocorticoid Receptor', *Endocrinology*, 138(11), pp. 5028–5038.

Otto, C., Reichardt, H. M. and Schutz, G. (1997) 'Absence of glucocorticoid receptor-beta in mice', *J.Biol.Chem.*, 272(42), pp. 26665–26668.

Pan, X. and Hussain, M. M. (2007) 'Diurnal regulation of microsomal triglyceride transfer protein and plasma lipid levels.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 282(34), pp. 24707–19.

Parton, R. G. and Del Pozo, M. A. (2013) 'Caveolae as plasma membrane sensors, protectors and organizers', *Nat Rev Mol Cell Biol*, 14(2), pp. 98–112.

Parton, R. G. and Simons, K. (2007) 'The multiple faces of caveolae', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 8(3), pp. 185–194.

Patel, R., Patel, M., Tsai, R., Lin, V., Bookout, A. L., Zhang, Y., Magomedova, L., Li, T., Chan, J. F., Budd, C., Mangelsdorf, D. J. and Cummins, C. L. (2011) 'LXRβ is required for glucocorticoid-induced hyperglycemia and hepatosteatosis in mice.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 121(1), pp. 431–41.

Pavlides, S., Tsirigos, A., Vera, I., Flomenberg, N., Frank, P. G., Casimiro, M. C., Wang, C., Fortina, P., Addya, S., Pestell, R. G., Martinez-Outschoorn, U. E., Sotgia, F. and Lisanti, M. P. (2010) 'Loss of stromal caveolin-1 leads to oxidative stress, mimics hypoxia and drives inflammation in the tumor microenvironment, conferring the "reverse Warburg effect": a transcriptional informatics analysis with validation.', *Cell cycle (Georgetown, Tex.)*, 9(11), pp. 2201–19.

Payvar, F., DeFranco, D., Firestone, G. L., Edgar, B., Wrange, O., Okret, S., Gustafsson, J. A. and Yamamoto, K. R. (1983) 'Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region', *Cell*, 35(2 Pt 1), pp. 381–392.

Payvar, F., Wrange, O., Carlstedt-Duke, J., Okret, S., Gustafsson, J. A. and Yamamoto, K. R. (1981) 'Purified glucocorticoid receptors bind selectively in vitro to a cloned DNA fragment whose transcription is regulated by glucocorticoids in vivo.', *Proceedings of the National Academy of*

Sciences of the United States of America. National Academy of Sciences, 78(11), pp. 6628–32.

Peckett, A. J., Wright, D. C. and Riddell, M. C. (2011) 'The effects of glucocorticoids on adipose tissue lipid metabolism.', *Metabolism: clinical and experimental*. Elsevier, 60(11), pp. 1500–10.

Peek, C. B., Affinati, A. H., Ramsey, K. M., Kuo, H. Y., Yu, W., Sena, L. A., Ilkayeva, O., Marcheva, B., Kobayashi, Y., Omura, C., Levine, D. C., Bacsik, D. J., Gius, D., Newgard, C. B., Goetzman, E., Chandel, N. S., Denu, J. M., Mrksich, M. and Bass, J. (2013) 'Circadian clock NAD+ cycle drives mitochondrial oxidative metabolism in mice', *Science*, 342(6158), p. 1243417.

Peffer, M. E., Chandran, U. R., Luthra, S., Volonte, D., Galbiati, F., Garabedian, M. J., Monaghan, A. P. and DeFranco, D. B. (2014) 'Caveolin-1 regulates genomic action of the glucocorticoid receptor in neural stem cells.', *Molecular and cellular biology*. American Society for Microbiology, 34(14), pp. 2611–23.

Pelkmans, L. and Helenius, A. (2002) 'Endocytosis Via Caveolae', *Traffic*, 3(5), pp. 311–320.

Pelkmans, L. and Zerial, M. (2005) 'Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae.', *Nature*, 436(7047), pp. 128–33.

Petersons, C. J., Mangelsdorf, B. L., Jenkins, A. B., Poljak, A., Smith, M. D., Greenfield, J. R., Thompson, C. H. and Burt, M. G. (2013) 'Effects of Low-Dose Prednisolone on Hepatic and Peripheral Insulin Sensitivity, Insulin Secretion, and Abdominal Adiposity in Patients With Inflammatory Rheumatologic Disease', *Diabetes Care*, 36(9).

Petruzielo, R. S., Heberle, F. A., Drazba, P., Katsaras, J. and Feigenson, G. W. (2013) 'Phase behavior and domain size in sphingomyelin-containing lipid bilayers', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1828(0), pp. 1302–13.

Picard, D. and Yamamoto, K. R. (1987) 'Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor', *EMBO J.*, 6(11), pp. 3333–3340.

Ponec, M., Kempenaar, J., Shroot, B. and Caron, J.-C. (1986) 'Glucocorticoids: Binding Affinity and Lipophilicity', *Journal of Pharmaceutical Sciences*, 75(10), pp. 973–975.

Popovic, N., Ruzdijic, S., Kanazir, D. T., Niciforovic, A., Adzic, M., Paraskevopoulou, E., Pantelidou, C.,

Radojcic, M., Demonacos, C. and Krstic-Demonacos, M. (2010) 'Site-specific and dose-dependent effects of glucocorticoid receptor phosphorylation in yeast Saccharomyces cerevisiae.', *Steroids*. Elsevier, 75(6), pp. 457–65.

Pratt, W. B. and Toft, D. O. (1997) 'Steroid Receptor Interactions with Heat Shock Protein and Immunophilin Chaperones', *Endocrine Reviews*, 18(3), pp. 306–360.

Pribul, P. K., Harker, J., Wang, B., Wang, H., Tregoning, J. S., Schwarze, J. and Openshaw, P. J. M. (2008) 'Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development.', *Journal of virology*. American Society for Microbiology, 82(9), pp. 4441–8.

Qiaoli, S., Yi, S., Jie, Z. and Deyun, C. (2016) 'KLF2 and caveolin-1 as early indicators of acute lung injury induced by paraquat', *European Review for Medical and Pharmacological Sciences*, 20(1), pp. 138–145.

Rafacho, A., Ortsäter, H., Nadal, A. and Quesada, I. (2014) 'Glucocorticoid treatment and endocrine pancreas function: implications for glucose homeostasis, insulin resistance and diabetes.', *The Journal of endocrinology*. BioScientifica, 223(3), pp. R49-62.

Rafacho, A., Quallio, S., Ribeiro, D. L., Taboga, S. R., Paula, F. M. M., Boschero, A. C. and Bosqueiro, J. R. (2010) 'The adaptive compensations in endocrine pancreas from glucocorticoid-treated rats are reversible after the interruption of treatment', *Acta Physiologica*. Blackwell Publishing Ltd, 200(3), pp. 223–235.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J. and Davis, R. J. (1995) 'Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine.', *The Journal of biological chemistry*, 270(13), pp. 7420–6.

Rajendran, L., Masilamani, M., Solomon, S., Tikkanen, R., Stuermer, C. A. O., Plattner, H. and Illges, H. (2003) 'Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells.', *Proceedings of the National Academy of Sciences of the United*

States of America. National Academy of Sciences, 100(14), pp. 8241–6.

Ralph, M., Foster, R., Davis, F. and Menaker, M. (1990) 'Transplanted suprachiasmatic nucleus determines circadian period', *Science*, 247(4945).

Ramirez, M. I., Pollack, L., Millien, G., Cao, Y. X., Hinds, A. and Williams, M. C. (2002) 'The alpha-isoform of caveolin-1 is a marker of vasculogenesis in early lung development.', *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 50(1), pp. 33–42.

Rao, N. A., McCalman, M. T., Moulos, P., Francoijs, K. J., Chatziioannou, A., Kolisis, F. N., Alexis, M. N., Mitsiou, D. J. and Stunnenberg, H. G. (2011) 'Coactivation of GR and NFKB alters the repertoire of their binding sites and target genes', *Genome Res*, 21(9), pp. 1404–1416.

Rasmussen, C.E. and Williams, C. K. . (2006) *Gaussian processes for machine learning*. MIT Press.

Ratman, D., Vanden Berghe, W., Dejager, L., Libert, C., Tavernier, J., Beck, I. M. and De Bosscher, K. (2013) 'How glucocorticoid receptors modulate the activity of other transcription factors: A scope beyond tethering', *Molecular and Cellular Endocrinology*, 380(1–2), pp. 41–54.

Ratman, D., Mylka, V., Bougarne, N., Pawlak, M., Caron, S., Hennuyer, N., Paumelle, R., De Cauwer, L., Thommis, J., Rider, M. H., Libert, C., Lievens, S., Tavernier, J., Staels, B. and De Bosscher, K. (2016) 'Chromatin recruitment of activated AMPK drives fasting response genes co-controlled by GR and PPAR␣', *Nucleic Acids Research*.

Rauch, A., Seitz, S., Baschant, U., Schilling, A. F., Illing, A., Stride, B., Kirilov, M., Mandic, V., Takacz, A., Schmidt-Ullrich, R., Ostermay, S., Schinke, T., Spanbroek, R., Zaiss, M. M., Angel, P. E., Lerner, U. H., David, J.-P., Reichardt, H. M., Amling, M., Schütz, G. and Tuckermann, J. P. (2010) 'Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor.', *Cell metabolism*. Elsevier, 11(6), pp. 517–31.

Ray, D. W., Davis, J. R., White, A. and Clark, A. J. (1996) 'Glucocorticoid receptor structure and function in glucocorticoid-resistant small cell lung carcinoma cells', *Cancer Res.*, 56(14), pp. 3276–3280.

Razani, B., Combs, T. P., Wang, X. B., Frank, P. G., Park, D. S., Russell, R. G., Li, M., Tang, B., Jelicks, L.

A., Scherer, P. E. and Lisanti, M. P. (2002) 'Caveolin-1-deficient mice are lean, resistant to diet-induced obesity, and show hypertriglyceridemia with adipocyte abnormalities.', *The Journal of biological chemistry*, 277(10), pp. 8635–47.

Razani, B., Zhang, X. L., Bitzer, M., von Gersdorff, G., Bottinger, E. P. and Lisanti, M. P. (2001) 'Caveolin-1 Regulates Transforming Growth Factor (TGF)-beta /SMAD Signaling through an Interaction with the TGF-beta Type I Receptor', *Journal of Biological Chemistry*, 276(9), pp. 6727–6738.

Reddy, A. B., Maywood, E. S., Karp, N. A., King, V. M., Inoue, Y., Gonzalez, F. J., Lilley, K. S., Kyriacou, C. P. and Hastings, M. H. (2007) 'Glucocorticoid signaling synchronizes the liver circadian transcriptome', *Hepatology*, 45(6), pp. 1478–1488.

Reddy, T. E., Pauli, F., Sprouse, R. O., Neff, N. F., Newberry, K. M., Garabedian, M. J. and Myers, R. M. (2009) 'Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation', *Genome Research*. Cold Spring Harbor Laboratory Press, 19(12), pp. 2163–2171.

Reichardt, H. M., Kaestner, K. H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P. and Schütz, G. (1998) 'DNA Binding of the Glucocorticoid Receptor Is Not Essential for Survival', *Cell*, 93(4), pp. 531–541.

Rivers, C., Levy, A., Hancock, J., Lightman, S. and Norman, M. (1999) 'Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing', *J.Clin.Endocrinol.Metab*, 84(11), pp. 4283–4286.

Robinson-Rechavi, M., Garcia, H. E. and Laudet, V. (2003) 'The nuclear receptor superfamily', *Journal of Cell Science*, 116(4), pp. 585–586.

Robson, R. J. and Dennis, E. A. (1979) 'Mixed micelles of sphingomyelin and phosphatidylcholine with nonionic surfactants. Effect of temperature and surfactant polydispersity', *Biochim.Biophys.Acta*, 573(3), pp. 489–500.

Rogatsky, I., Logan, S. K. and Garabedian, M. J. (1998) 'Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase.', *Proceedings of the National Academy of*

Sciences of the United States of America. National Academy of Sciences, 95(5), pp. 2050–5.

Roos, A. B., Berg, T., Ahlgren, K. M., Grunewald, J. and Nord, M. (2014) 'A Method for Generating Pulmonary Neutrophilia Using Aerosolized Lipopolysaccharide', *Journal of Visualized Experiments*, (94), pp. e51470–e51470.

Rosewicz, S., McDonald, A. R., Maddux, B. A., Goldfine, I. D., Miesfeld, R. L. and Logsdon, C. D. (1988) 'Mechanism of glucocorticoid receptor down-regulation by glucocorticoids', *Journal of Biological Chemistry*, 263(6), pp. 2581–2584.

Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y.-S., Glenney, J. R. and Anderson, R. G. W. (1992) 'Caveolin, a protein component of caveolae membrane coats', *Cell*, 68(4), pp. 673–682.

Ruiter, M., La Fleur, S. E., van Heijningen, C., van der Vliet, J., Kalsbeek, A. and Buijs, R. M. (2003) 'The Daily Rhythm in Plasma Glucagon Concentrations in the Rat Is Modulated by the Biological Clock and by Feeding Behavior', *Diabetes*, 52(7).

Saad, M. J., Folli, F., Kahn, J. A. and Kahn, C. R. (1993) 'Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 92(4), pp. 2065–72.

Sanchez, E. R., Hirst, M., Scherrer, L. C., Tang, H. Y., Welsh, M. J., Harmon, J. M., Simons, S. S., Ringold, G. M. and Pratt, W. B. (1990) 'Hormone-free mouse glucocorticoid receptors overexpressed in Chinese hamster ovary cells are localized to the nucleus and are associated with both hsp70 and hsp90.', *The Journal of biological chemistry*, 265(33), pp. 20123–30.

Sankaram, M. B. and Thompson, T. E. (1990) 'Modulation of phospholipid acyl chain order by cholesterol. A solid-state deuterium nuclear magnetic resonance study', *Biochemistry*, 29(47), pp. 10676–10684.

Sargiacomo, M., Scherer, P. E., Tang, Z., Kübler, E., Song, K. S., Sanders, M. C. and Lisanti, M. P. (1995) 'Oligomeric structure of caveolin: implications for caveolae membrane organization.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy

of Sciences, 92(20), pp. 9407–11.

Sato, S., Sakurai, T., Ogasawara, J., Takahashi, M., Izawa, T., Imaizumi, K., Taniguchi, N., Ohno, H. and Kizaki, T. (2013) 'A Circadian Clock Gene, Rev-erbα, Modulates the Inflammatory Function of Macrophages through the Negative Regulation of Ccl2 Expression', *The Journal of Immunology*, 192(1).

Le Saux, O., Teeters, K., Miyasato, S., Choi, J., Nakamatsu, G., Richardson, J. A., Starcher, B., Davis, E. C., Tam, E. K. and Jourdan-Le Saux, C. (2008) 'The role of caveolin-1 in pulmonary matrix remodeling and mechanical properties', *AJP: Lung Cellular and Molecular Physiology*, 295(6), pp. L1007–L1017.

Savory, J. G. A., Hsu, B., Laquian, I. R., Giffin, W., Reich, T., Hach,, R. J. G. and Lefebvre, Y. A. (1999) 'Discrimination between NL1- and NL2-Mediated Nuclear Localization of the Glucocorticoid Receptor', *Molecular and Cellular Biology*, 19(2), pp. 1025–1037.

Schaaf, M. J. M. and Cidlowski, J. A. (2002) 'Molecular mechanisms of glucocorticoid action and resistance', *The Journal of Steroid Biochemistry and Molecular Biology*, 83(1â€"5), pp. 37–48.

Schäcke, H., Döcke, W. D. and Asadullah, K. (2002) 'Mechanisms involved in the side effects of glucocorticoids', *Pharmacol Ther*, 96(1), pp. 23–43.

Schlegel, A. and Lisanti, M. P. (2001) 'The caveolin triad: caveolae biogenesis, cholesterol trafficking, and signal transduction.', *Cytokine & growth factor reviews*, 12(1), pp. 41–51.

Schmidt, P., Holsboer, F. and Spengler, D. (2001) 'Beta(2)-adrenergic receptors potentiate glucocorticoid receptor transactivation via G protein beta gamma-subunits and the phosphoinositide 3-kinase pathway.', *Molecular endocrinology (Baltimore, Md.)*, 15(4), pp. 553–64.

Schneider, K., Köcher, T., Andersin, T., Kurzchalia, T., Schibler, U. and Gatfield, D. (2012) 'CAVIN-3 regulates circadian period length and PER:CRY protein abundance and interactions', *EMBO reports*. John Wiley & Sons, Ltd, 13(12), pp. 1138–1144.

Schubert, A.-L., Schubert, W., Spray, D. C. and Lisanti, M. P. (2002) 'Connexin family members target to lipid raft domains and interact with caveolin-1.', *Biochemistry*, 41(18), pp. 5754–64.

Schwartz, D. A., Helmers, R. A., Galvin, J. R., Van Fossen, D. S., Frees, K. L., Dayton, C. S., Burmeister,

L. F. and Hunninghake, G. W. (1994) 'Determinants of survival in idiopathic pulmonary fibrosis.', *American journal of respiratory and critical care medicine*. American Public Health Association, 149(2 Pt 1), pp. 450–4.

Shajahan, A. N., Dobbin, Z. C., Hickman, F. E., Dakshanamurthy, S. and Clarke, R. (2012) 'Tyrosine-phosphorylated caveolin-1 (Tyr-14) increases sensitivity to paclitaxel by inhibiting BCL2 and BCLxL proteins via c-Jun N-terminal kinase (JNK).', *The Journal of biological chemistry*, 287(21), pp. 17682–92.

Shao, M., Yue, Y., Sun, G.-Y., You, Q.-H., Wang, N. and Zhang, D. (2013) 'Caveolin-1 Regulates Rac1 Activation and Rat Pulmonary Microvascular Endothelial Hyperpermeability Induced by TNF-α', *PLoS ONE*. Edited by P. Mukhopadhyay. Public Library of Science, 8(1), p. e55213.

Shim, J.-H., Xiao, C., Paschal, A. E., Bailey, S. T., Rao, P., Hayden, M. S., Lee, K.-Y., Bussey, C., Steckel, M., Tanaka, N., Yamada, G., Akira, S., Matsumoto, K. and Ghosh, S. (2005) 'TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo.', *Genes & development*. Cold Spring Harbor Laboratory Press, 19(22), pp. 2668–81.

Shimizu, N., Yoshikawa, N., Ito, N., Maruyama, T., Suzuki, Y., Takeda, S., Nakae, J., Tagata, Y., Nishitani, S., Takehana, K., Sano, M., Fukuda, K., Suematsu, M., Morimoto, C. and Tanaka, H. (2011) 'Crosstalk between Glucocorticoid Receptor and Nutritional Sensor mTOR in Skeletal Muscle', *Cell Metabolism*, 13(2), pp. 170–182.

Siersbæk, R., Rabiee, A., Nielsen, R., Sidoli, S., Traynor, S., Loft, A., Poulsen, L. L. C., Rogowska-Wrzesinska, A., Jensen, O. N. and Mandrup, S. (2014) 'Transcription Factor Cooperativity in Early Adipogenic Hotspots and Super-Enhancers', *Cell Reports*, 7(5), pp. 1443–1455.

Simpson, E. R. and Waterman, M. R. (1988) 'Regulation of the Synthesis of Steroidogenic Enzymes in Adrenal Cortical Cells by ACHT', *Ann. Rev. Physioi*, 50, pp. 427–40.

Slack, D. N., Seternes, O.-M., Gabrielsen, M. and Keyse, S. M. (2001) 'Distinct Binding Determinants for ERK2/p38 and JNK MAP Kinases Mediate Catalytic Activation and Substrate Selectivity of MAP Kinase Phosphatase-1', *Journal of Biological Chemistry*, 276(19), pp. 16491–16500.

Slavin, B. G., Ong, J. M. and Kern, P. A. (1994) 'Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes.', *Journal of lipid research*, 35(9), pp. 1535–41.

Snyder, J. C., Reynolds, S. D., Hollingsworth, J. W., Li, Z., Kaminski, N. and Stripp, B. R. (2010) 'Clara Cells Attenuate the Inflammatory Response through Regulation of Macrophage Behavior', *American Journal of Respiratory Cell and Molecular Biology*, 42(2), pp. 161–171.

So, A. Y.-L., Bernal, T. U., Pillsbury, M. L., Yamamoto, K. R. and Feldman, B. J. (2009) 'Glucocorticoid regulation of the circadian clock modulates glucose homeostasis.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 106(41), pp. 17582–7.

Spies, C. M., Schaumann, D. H. S., Berki, T., Mayer, K., Jakstadt, M., Huscher, D., Wunder, C., Burmester, G.-R., Radbruch, A., Lauster, R., Scheffold, A. and Buttgereit, F. (2006) 'Membrane glucocorticoid receptors are down regulated by glucocorticoids in patients with systemic lupus erythematosus and use a caveolin-1-independent expression pathway', *Annals of the Rheumatic Diseases*, 65(9), pp. 1139–1146.

SPRAGUE, R. G., POWER, M. H., MASON, H. L., ALBERT, A., MATHIESON, D. R., HENCH, P. S., KENDALL, E. C., SLOCUMB, C. H. and POLLEY, H. F. (1950) 'Observations on the Physiologic Effects of Cortisone and ACTH in Man', *Archives of Internal Medicine*. American Medical Association, 85(2), p. 199.

Stow, L. R., Richards, J., Cheng, K.-Y., Lynch, I. J., Jeffers, L. A., Greenlee, M. M., Cain, B. D., Wingo, C. S. and Gumz, M. L. (2012) 'The Circadian Protein Period 1 Contributes to Blood Pressure Control and Coordinately Regulates Renal Sodium Transport Genes', *Hypertension*, 59(6).

Strehl, C., Gaber, T., Löwenberg, M., Hommes, D. W., Verhaar, A. P., Schellmann, S., Hahne, M., Fangradt, M., Wagegg, M., Hoff, P., Scheffold, A., Spies, C. M., Burmester, G.-R. and Buttgereit, F. (2011) 'Origin and functional activity of the membrane-bound glucocorticoid receptor.', *Arthritis and rheumatism*, 63(12), pp. 3779–88.

Strickland, I., Kisich, K., Hauk, P. J., Vottero, A., Chrousos, G. P., Klemm, D. J. and Leung, D. Y. (2001)

'High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids', *J.Exp.Med.*, 193(5), pp. 585–593.

Strieter, R. M., Belperio, J. A., Keane, M. P., Knowles, M., Boucher, R., Ganz, T., McCormack, F., Whitsett, J., Medzhitov, R., Janeway, C., Ghosh, S., May, M., Kopp, E., Medzhitov, R., Preston-Hurlburt, P., Janeway, C., Hoshino, K., Beutler, B., Poltorak, A., Yoshimura, A., Wolfs, T., Dinarello, C., Murphy, J., Robert, C., Kupper, T., Fantuzzi, G., Bazzoni, F., Beutler, B., Laichalk, L., Brieland, J., Mehrad, B., Strieter, R., Standiford, T., Keane, J., Zlotnik, A., Yoshie, O., Proost, P., Sozzani, S., Allavena, P., Vecchi, A., Mantovani, A., Gu, L., Boutten, A., Rodriguez, J., Mehrad, B., Standiford, T., Tsai, W., Moore, T., Tsai, W., Mehrad, B., Dawson, T., Beck, M., Kuziel, W., Henderson, F., Maeda, N., Huffnagle, G., Lindell, D., Standiford, T., Mancuso, P., Leshen, Z., Huffnagle, G., Mosmann, T., Cherwinski, H., Bond, M., Giedlin, M., Coffman, R., Driggers, P., Rollins, B., Cooper, A., Flynn, J., Condos, R., Rom, W., Schluger, N., Deng, J., Tateda, K., Zeng, X., Standiford, T., Standiford, T., Strieter, R., Lukacs, N., Kunkel, S., Greenberger, M. and Steinhauser, M. (2002) 'Cytokines in innate host defense in the lung.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 109(6), pp. 699–705.

Surjit, M., Ganti, K. P., Mukherji, A., Ye, T., Hua, G., Metzger, D., Li, M. and Chambon, P. (2011) 'Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor', *Cell*, 145(2), pp. 224–241.

Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W. and Yeh, W.-C. (2002) 'Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4', *Nature*. Nature Publishing Group, 416(6882), pp. 750–756.

Swantek, J. L., Tsen, M. F., Cobb, M. H. and Thomas, J. A. (2000) 'IL-1 receptor-associated kinase modulates host responsiveness to endotoxin.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 164(8), pp. 4301–6.

Swinstead, E. E., Miranda, T. B., Paakinaho, V., Baek, S., Goldstein, I., Hawkins, M., Karpova, T. S.,

Ball, D., Mazza, D., Lavis, L. D., Grimm, J. B., Morisaki, T., Grøntved, L., Presman, D. M. and Hager, G. L. (2016) 'Steroid Receptors Reprogram FoxA1 Occupancy through Dynamic Chromatin Transitions', *Cell*, 165(3), pp. 593–605.

Takeda, K., Hamelmann, E., Joetham, A., Shultz, L. D., Larsen, G. L., Irvin, C. G. and Gelfand, E. W. (1997) 'Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice.', *The Journal of experimental medicine*. The Rockefeller University Press, 186(3), pp. 449–54.

Tanigaki, K., Mineo, C., Yuhanna, I. S., Chambliss, K. L., Quon, M. J., Bonvini, E. and Shaul, P. W. (2009) 'C-reactive protein inhibits insulin activation of endothelial nitric oxide synthase via the immunoreceptor tyrosine-based inhibition motif of FcgammaRIIB and SHIP-1.', *Circulation research*. NIH Public Access, 104(11), pp. 1275–82.

Taniguchi, Y., Iwasaki, Y., Tsugita, M., Nishiyama, M., Taguchi, T., Okazaki, M., Nakayama, S., Kambayashi, M., Hashimoto, K. and Terada, Y. (2010) 'Glucocorticoid receptor-beta and receptor-gamma exert dominant negative effect on gene repression but not on gene induction', *Endocrinology*, 151(7), pp. 3204–3213.

Tasker, J. G., Di, S. and Malcher-Lopes, R. (2005) 'Rapid central corticosteroid effects: evidence for membrane glucocorticoid receptors in the brain.', *Integrative and comparative biology*. Oxford University Press, 45(4), pp. 665–71.

Tavernier, J., Eyckerman, S., Lemmens, I., Van der Heyden, J., Vandekerckhove, J. and Van Ostade, X. (2002) 'MAPPIT: a cytokine receptor-based two-hybrid method in mammalian cells1', *Clinical <html_ent glyph='@amp;' ascii='&'/> Experimental Allergy*. Blackwell Science, Ltd, 32(10), pp. 1397–1404.

Taylor, A. I., Frizzell, N., McKillop, A. M., Flatt, P. R. and Gault, V. A. (2009) 'Effect of RU486 on Hepatic and Adipocyte Gene Expression Improves Diabetes Control in Obesity-type 2 Diabetes', *Hormone and Metabolic Research*, 41(12), pp. 899–904.

Taylor, A. L. and Fishman, L. M. (1988) 'Corticotropin-Releasing Hormone', *New England Journal of*

Medicine, 319(4), pp. 213–222.

Thomsen, P., Roepstorff, K., Stahlhut, M. and van Deurs, B. (2002) 'Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking.', *Molecular biology of the cell*, 13(1), pp. 238–50.

Torra, I. P., Tsibulsky, V., Delaunay, F., Saladin, R., Laudet, V., Fruchart, J. C., Kosykh, V. and Staels, B. (2000) 'Circadian and glucocorticoid regulation of Rev-erbalpha expression in liver.', *Endocrinology*, 141(10), pp. 3799–3806.

Tourkina, E., Richard, M., Oates, J., Hofbauer, A., Bonner, M., Gooz, P., Visconti, R., Zhang, J., Znoyko, S., Hatfield, C. M., Silver, R. M. and Hoffman, S. (2010) 'Caveolin-1 regulates leucocyte behaviour in fibrotic lung disease', *Annals of the Rheumatic Diseases*, 69(6), pp. 1220–1226.

Trapnell, C., Pachter, L. and Salzberg, S. L. (2009) 'TopHat: discovering splice junctions with RNA-Seq', *Bioinformatics*, 25(9), pp. 1105–1111.

Tsamaloukas, A., Szadkowska, H. and Heerklotz, H. (2006) 'Thermodynamic Comparison of the Interactions of Cholesterol with Unsaturated Phospholipid and Sphingomyelins', *Biophysical Journal*, 90(12), pp. 4479–4487.

Uhlenhaut, N. H., Barish, G. D., Yu, R. T., Downes, M., Karunasiri, M., Liddle, C., Schwalie, P., Hübner, N. and Evans, R. M. (2013) 'Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes', *Molecular cell*, 49(1), pp. 158–171.

Umesono, K. and Evans, R. M. (1989) 'Determinants of target gene specificity for steroid/thyroid hormone receptors', *Cell*, 57(7), pp. 1139–1146.

Vandevyver, S., Dejager, L., Van Bogaert, T., Kleyman, A., Liu, Y., Tuckermann, J. and Libert, C. (2012) 'Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 122(6), pp. 2130–40.

Vandevyver, S., Dejager, L., Tuckermann, J. and Libert, C. (2013) 'New insights into the anti-inflammatory mechanisms of glucocorticoids: an emerging role for glucocorticoid-receptor-mediated transactivation.', *Endocrinology*, 154(3), pp. 993–1007.

Vernocchi, S., Battello, N., Schmitz, S., Revets, D., Billing, A. M., Turner, J. D. and Muller, C. P. (2013) 'Membrane glucocorticoid receptor activation induces proteomic changes aligning with classical glucocorticoid effects.', *Molecular & cellular proteomics : MCP*, 12(7), pp. 1764–79.

Vettorazzi, S., Bode, C., Dejager, L., Frappart, L., Shelest, E., Klaßen, C., Tasdogan, A., Reichardt, H. M., Libert, C., Schneider, M., Weih, F., Henriette Uhlenhaut, N., David, J. P., Gräler, M., Kleiman, A. and Tuckermann, J. P. (2015) 'Glucocorticoids limit acute lung inflammation in concert with inflammatory stimuli by induction of SphK1', *Nat Commun*, 6, p. 7796.

Vink, P. M., Smout, W. M., Driessen-Engels, L. J., de Bruin, A. M., Delsing, D., Krajnc-Franken, M. A., Jansen, A. J., Rovers, E. F., van Puijenbroek, A. A., Kaptein, A., Nolte, M. A., Garritsen, A. and van Eenennaam, H. (2013) 'In Vivo Knockdown of TAK1 Accelerates Bone Marrow Proliferation/Differentiation and Induces Systemic Inflammation', *PLoS ONE*. Edited by R. Johnson. Public Library of Science, 8(3), p. e57348.

Volonte, D., Galbiati, F., Pestell, R. G. and Lisanti, M. P. (2001) 'Cellular Stress Induces the Tyrosine Phosphorylation of Caveolin-1 (Tyr14) via Activation of p38 Mitogen-activated Protein Kinase and c-Src kinase: EVIDENCE FOR CAVEOLAE, THE ACTIN CYTOSKELETON, AND FOCAL ADHESIONS AS MECHANICAL SENSORS OF OSMOTIC STRESS', *Journal of Biological Chemistry*, 276(11), pp. 8094–8103.

De Vries, F., Bracke, M., Leufkens, H. G. M., Lammers, J.-W. W. J., Cooper, C. and Van Staa, T. P. (2007) 'Fracture risk with intermittent high-dose oral glucocorticoid therapy', *Arthritis & Rheumatism*. Wiley Subscription Services, Inc., A Wiley Company, 56(1), pp. 208–214.

Wallace, A. D. and Cidlowski, J. A. (2001) 'Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids', *J.Biol.Chem.*, 276(46), pp. 42714–42721.

Wang, X. M., Kim, H. P., Nakahira, K., Ryter, S. W. and Choi, A. M. K. (2009) 'The heme oxygenase-1/carbon monoxide pathway suppresses TLR4 signaling by regulating the interaction of TLR4 with caveolin-1.', *Journal of immunology (Baltimore, Md. : 1950)*, 182(6), pp. 3809–18.

Wang, X. M., Kim, H. P., Song, R. and Choi, A. M. K. (2006) 'Caveolin-1 confers antiinflammatory

effects in murine macrophages via the MKK3/p38 MAPK pathway.', *American journal of respiratory cell and molecular biology*. American Thoracic Society, 34(4), pp. 434–42.

Wang, X. M., Zhang, Y., Kim, H. P., Zhou, Z., Feghali-Bostwick, C. A., Liu, F., Ifedigbo, E., Xu, X., Oury, T. D., Kaminski, N. and Choi, A. M. K. (2006) 'Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis', *The Journal of Experimental Medicine*, 203(13), pp. 2895–2906.

Wang, Z., Frederick, J. and Garabedian, M. J. (2002) 'Deciphering the phosphorylation "code" of the glucocorticoid receptor in vivo.', *The Journal of biological chemistry*, 277(29), pp. 26573–80.

Weber, K., Brück, P., Mikes, Z., Küpper, J.-H., Klingenspor, M. and Wiesner, R. J. (2002) 'Glucocorticoid Hormone Stimulates Mitochondrial Biogenesis Specifically in Skeletal Muscle', *Endocrinology*. Endocrine Society, 143(1), pp. 177–184.

Webster, J. C., Jewell, C. M., Bodwell, J. E., Munck, A., Sar, M. and Cidlowski, J. A. (1997) 'Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein', *J.Biol.Chem.*, 272(14), pp. 9287–9293.

Weinstein, R. S. (2012) 'Glucocorticoid-Induced Osteoporosis and Osteonecrosis', *Endocrinology and Metabolism Clinics of North America*, 41(3), pp. 595–611.

Wen, F. Q., Kohyama, T., Skold, C. M., Zhu, Y. K., Liu, X., Romberger, D. J., Stoner, J. and Rennard, S. I. (2003) 'Glucocorticoids modulate TGF-beta production by human fetal lung fibroblasts', *Inflammation*, 27(1), pp. 9–19.

Westphalen, K., Gusarova, G. A., Islam, M. N., Subramanian, M., Cohen, T. S., Prince, A. S. and Bhattacharya, J. (2014) 'Sessile alveolar macrophages communicate with alveolar epithelium to modulate immunity', *Nature*, 506(7489), pp. 503–506.

Widen, C., Gustafsson, J. A. and Wikstrom, A. C. (2003) 'Cytosolic glucocorticoid receptor interaction with nuclear factor-kappa B proteins in rat liver cells', *Biochem.J.*, 373(Pt 1), pp. 211–220.

Woldt, E., Sebti, Y., Solt, L. A., Duhem, C., Lancel, S., Eeckhoute, J., Hesselink, M. K. C., Paquet, C., Delhaye, S., Shin, Y., Kamenecka, T. M., Schaart, G., Lefebvre, P., Nevière, R., Burris, T. P., Schrauwen,

P., Staels, B. and Duez, H. (2013) 'Rev-erb-α modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy.', *Nature medicine*. NIH Public Access, 19(8), pp. 1039–46.

Wright, A. P. H., McEwan, I. J., Dahlman-Wright, K. and Gustafsson, J.-Å. (1991) 'High Level Expression of the Major Transactivation Domain of the Human Glucocorticoid Receptor in Yeast Cells Inhibits Endogenous Gene Expression and Cell Growth', *Molecular Endocrinology*. Oxford University Press, 5(10), pp. 1366–1372.

Wu, H., Deng, R., Chen, X., Wong, W. C., Chen, H., Gao, L., Nie, Y., Wu, W. and Shen, J. (2016) 'Caveolin-1 Is Critical for Lymphocyte Trafficking into Central Nervous System during Experimental Autoimmune Encephalomyelitis', *Journal of Neuroscience*, 36(19), pp. 5193–5199.

Wu, J. and Bresnick, E. H. (2007) 'Glucocorticoid and growth factor synergism requirement for Notch4 chromatin domain activation.', *Molecular and cellular biology*. American Society for Microbiology (ASM), 27(6), pp. 2411–22.

Yang-Yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smeal, T., Schmidt, T. J., Drouin, J. and Karin, M. (1990) 'Transcriptional interference between c-Jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction', *Cell*, 62(6), pp. 1205–1215.

Yang, Y., Wolfram, J., Shen, J., Zhao, Y., Fang, X., Shen, H. and Ferrari, M. (2013) 'Live-cell single-molecule imaging reveals clathrin and caveolin-1 dependent docking of SMAD4 at the cell membrane.', *FEBS letters*. NIH Public Access, 587(24), pp. 3912–20.

Yudt, M. R., Jewell, C. M., Bienstock, R. J. and Cidlowski, J. A. (2003) 'Molecular origins for the dominant negative function of human glucocorticoid receptor beta', *Mol.Cell Biol.*, 23(12), pp. 4319–4330.

Yue, L., Bian, J.-T., Grizelj, I., Cavka, A., Phillips, S. A., Makino, A. and Mazzone, T. (2012) 'Apolipoprotein E enhances endothelial-NO production by modulating caveolin 1 interaction with endothelial NO synthase.', *Hypertension (Dallas, Tex. : 1979)*, 60(4), pp. 1040–6.

Zhang, P.-X., Murray, T. S., Villella, V. R., Ferrari, E., Esposito, S., D'Souza, A., Raia, V., Maiuri, L.,

Krause, D. S., Egan, M. E. and Bruscia, E. M. (2013) 'Reduced Caveolin-1 Promotes Hyperinflammation due to Abnormal Heme Oxygenase-1 Localization in Lipopolysaccharide-Challenged Macrophages with Dysfunctional Cystic Fibrosis Transmembrane Conductance Regulator', *The Journal of Immunology*, 190(10), pp. 5196–5206.

Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E. and Hogenesch, J. B. (2014) 'A circadian gene expression atlas in mammals: implications for biology and medicine.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(45), pp. 16219–24.

Zhang, S., Liang, X. and Danielsen, M. (1996) 'Role of the C terminus of the glucocorticoid receptor in hormone binding and agonist/antagonist discrimination', *Molecular Endocrinology*, 10(1), pp. 24–34.

Zhang, Y., Fang, B., Damle, M., Guan, D., Li, Z., Kim, Y. H., Gannon, M. and Lazar, M. A. (2016) 'HNF6 and Rev-erbα integrate hepatic lipid metabolism by overlapping and distinct transcriptional mechanisms.', *Genes & development*. Cold Spring Harbor Laboratory Press, 30(14), pp. 1636–44.

Zhang, Y., Fang, B., Emmett, M. J., Damle, M., Sun, Z., Feng, D., Armour, S. M., Remsberg, J. R., Jager, J., Soccio, R. E., Steger, D. J. and Lazar, M. A. (2015) 'Discrete functions of nuclear receptor Rev-erbα couple metabolism to the clock', *Science*, 348(6242), pp. 1488–1492.

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P. and Ghosh, S. (1997) 'The Transcriptional Activity of NF-kB Is Regulated by the IkB-Associated PKAc Subunit through a Cyclic AMP-Independent Mechanism', *Cell*, 89(3), pp. 413–424.

Zhou, J. and Cidlowski, J. A. (2005) 'The human glucocorticoid receptor: One gene, multiple proteins and diverse responses', *Steroids*, 70(5â€"7), pp. 407–417.

Zhu, B., Gates, L. A. A., Stashi, E., Dasgupta, S., Gonzales, N., Dean, A., Dacso, C. C. C., York, B., O'Malley, B. W., O'Malley, B. W. and O'Malley, B. W. (2015) 'Coactivator-Dependent Oscillation of Chromatin Accessibility Dictates Circadian Gene Amplitude via REV-ERB Loading', *Mol Cell*, 60(5), pp. 769–783.

Zosky, G. R., Larcombe, A. N., White, O. J., Burchell, J. T., Janosi, T. Z., Hantos, Z., Holt, P. G., Sly, P. D.

and Turner, D. J. (2008) 'Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation', *Clinical & Experimental Allergy*, 38(5), pp. 829–838.