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Protein Kinase C: A key enzyme in mediating interferon-α signalling and a new player in its activity against Hepatitis C infection.

A thesis submitted for the degree of Doctor of Philosophy (PhD)

by

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at

University of Dublin, Trinity College

2013

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DECLARATION

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Summary

The hepatitis C virus (HCV) is a major cause of chronic liver cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). The World Health Organisation estimates that there are 170 million individuals infected globally, some ~3% of the worlds' population. While acute infection is usually asymptomatic and can be spontaneously cleared, approximately 50-80% of patients progress to chronic infection. One fifth of chronically infected patients will then develop liver cirrhosis within 20 years and amongst those with cirrhosis, 1-5% develop hepatocellular carcinoma (Cerny and Chisari, 1999; Rosen HR, 2011). Pegylated interferon-α (Peg-IFNα) combined with ribavirin has long been the standard treatment for infection. Interferons are central to the regulation of the antiviral immune response. HCV is able to utilise host factors in order to enter cells, replicate and spread. HCV is also effective in evading the host defence and establishing chronic infection. It is able to manipulate different cellular mechanisms involved in the immune response in order to escape antiviral activity. Correspondingly, IFNα therapy is often unsuccessful at clearing infection from certain HCV genotypes. In hepatocytes, classical PKC activity is required for IFNα induced STAT activation, ISG expression and HCV RNA degradation. In a number of other cell types STAT signalling activity requires activation of various PKC isotypes, including PKC δ and ε. These studies demonstrate the importance of the PKC family in IFNα mediated STAT activation and clearance of HCV. However the complete range of PKCs present in hepatocytes has not been conclusively described.

In this study we assemble a profile of the PKC isoforms that are expressed in the Huh7 hepatocarcinoma cell line. We also show that IFNα is able to induce the redistribution of PKCs α, δ and most notably ζ. We further establish that in contrast to other cell types PKC δ and ε are not required to phosphorylate STAT1 or STAT3 at serine or tyrosine residues following IFNα stimulation. We instead show that PKCε is specifically required for the IFNα induced nuclear translocation of STAT1 but not STAT3. However this inhibition of STAT1 nuclear translocation did not result in inhibition of the ISGs PKR, 2'5'-OAS or MxA following IFNα stimulation. We also showed that PKC δ is not required for the nuclear transport of these STAT proteins.

We also investigated the role of the atypical PKCζ as a host factor required by HCV to enter cells. Atypical PKCs direct the establishment of epithelia-specific junctional structures. These structures are composed of proteins such as claudin-1 and occludin, which are also host factors required for HCV cell entry. We found that IFNα is able to direct a dynamic remodelling of cell:cell contacts through the translocalisation of PKCζ from the plasma membrane. PKCζ localisation at the plasma membrane promotes its colocalisation with AJ and TJ proteins that are vital for cell:cell contact and barrier formation in Huh7 cells. When PKCζ activity is inhibited these proteins are no longer localised at the plasma membrane. This results in a reduction of claudin-1 and occludin available to HCV particles and correspondingly infection is reduced. This reveals that PKCζ is a vital host cell factor required by HCV to enter and infect liver cells and identifies a novel antiviral pathway employed by IFNα.

This body of work demonstrates the range of functions undertaken by the PKC family in hepatocytes and their close relationship with IFNα and STAT signalling. We have also uncovered an unexpected antiviral mechanism employed by IFNα that has identified a new host factor required for HCV to enter hepatocytes, which may lead to novel therapeutic targets.

To my parents and Karina.

Thank you.

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There are many people that I must express my heartfelt thanks to for helping me accomplish this thesis. It was a long and winding road but the time, thought and care that was given by so many people made this such an enjoyable process. The list is so long that if I have accidentally omitted a name please except my apologies.

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Cormac

Associated publications

Publications

"PKCβ Is Necessary For IFNα Induced Stat 3 Activation" Sarah Mullins, Cormac McKenna, Aoife Kelly, Elizabeth Ryan, Nigel Stephenson, Cliona O'Farrelly, Aideen Long. Manuscript in preparation.

PKCζ is a a new player in its activity against Hepatitis C infection. Cormac McKenna, Aoife Kelly, Nigel Stephenson, Cliona O'Farrelly, Aideen Long.
Manuscript in preparation.

Poster Presentations

"The expression of PKC isoforms and their translocation by IFNα" Cormac Mckenna, Aideen Long.
"Inflammation From Cell To Clinic", Ulster Immunology Group/ British Society for Immunology. Belfast, UK, 8/9 June 2009

"The expression of PKC isoforms and their translocation by IFNα" Cormac Mckenna, Aideen Long.
"Institute of Molecular Medicine Annual Meeting", Institute of Molecular Medicine Dublin, Ireland, 13/14 November 2009.

"The role of PKC isoforms in IFNα signalling" Cormac Mckenna, Aideen Long.
"Dangerous Liaisons – Pathogens and the Immune System", Ulster Immunology Group/ Irish Society for Immunology. Belfast, UK, 2/3 September 2010.

"The role of PKC isoforms in IFNα signalling" Cormac Mckenna, Aideen Long.
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"Falk Workshop – Liver and Immunity" Falk Foundation, Regensburg, Germany, 27/28 January 2011.

IFNα controls cell:cell contact via PKCζ Cormac Mckenna, Aideen Long.
"Irish Society for Immunology Annual Meeting", Irish Society for Immunology, Galway, Ireland, 1/2 September 2011.

IFNα controls cell:cell contact via PKCζ Cormac Mckenna, Aideen Long.
"Tercentenary Symposium" Trinity Biomedical Sciences Institute, Dublin, Ireland, 4th November 2011.

IFNα controls cell:cell contact via PKCζ Cormac Mckenna, Aideen Long.
"Irish Society for Immunology Annual Meeting", Irish Society for Immunology, Dublin, Ireland, 20/21 September 2012.

"Inflammation and Immunity", Institute of Molecular Medicine, Dublin, Ireland, 9th November 2012.

Abbreviations

2′,5′-oligoadenylate synthetase	(2'-5' OAS)
activation-loop	(A-loop)
alanine aminotransferase	(ALT)
androgen receptor-coactivator 160 kDa	(ARA160)
aPKC-specific interacting protein	(ASIP)
apolipoprotein B	(ApoB)
apolipoprotein E	(ApoE)
atypical PKC	(aPKC)
binder of ADP-ribosylation factor-like 2	(BART)
brahma-related gene 1	(BRG1)
C-type lectin like receptor	(NKG2A)
cAMP-responsive-element-binding-protein binding protein	(CBP)
classical PKC	(cPKC)
coiled-coil domain	(CC)
cytoplasmic lipid droplets	(cLD)
dendritic cells	(DC)
diacylglycerol	(DAG)
diacylglycerol acyltransterase-1	(DGAT1)
disrupter of telomere silencing 1 like	(DOT1L)
DNA-binding domain	(DBD)
ephrin receptor A2	(EphA2)
epidermal growth factor receptor	(EGFR)
extracellular signal-regulated kinases	(ERK)
fibronectin type III	(FNIII)
FKBP 12-rapamycin-associated protein	(FRAP)
fluorescence resonance energy transfer	(FRET)
four-point-one, ezrin, radixin, moesin	(FERM)
general control non-depressible 5	(GCN5)
genome-wide association studies	(GWAS)
glycosaminoglycan	(GAG)
guanine tri-phosphatase	(GTPase)
HCV cell culture system	(HCVcc)
hepatitis c virus	(HCV)
hepatocyte LDL receptor	(LDL-R)
highly sulfated heparan sulfate	(HS)
hydrophobic-motif	(HM)
interferon	(IFN)
interferon alpha receptor	(IFNAR)
interferon γ-activated site element	(GAS)
insulin receptor substrate	(IRS)
interferon regulatory factors	(IRF)
interferon sensitivity-determining region	(ISDR)
interferon stimulated gene	(ISG)
interferon stimulated gene factor 3	(ISGF3)
interferon stimulated response elements	(ISRE)
interferon /ribavirin resistance-determining region	(IRRDR)

interleukin	(IL)
internal ribosome entry site	(IRES)
intracellular domain	(ICD)
JAK homology regions	(JH)
janus kinase	(JAK1)
killer cell immunoglobulin-like receptors	(KIR)
large extracellular loop	(LEL)
leukocyte function-associated molecule-1	(LFA-1)
macrophage inflammatory protein	(MIP)
mammalian target of rapamycin	(mTOR)
messenger ribonucleic acid	(mRNA)
microRNAs	(miRNAs)
minichromosome maintenance deficient 5	(MCM5)
mitochondrial antiviral signaling	(MAVS)
mitogen activated protein kinase	(MAPK)
myxovirus resistance protein 1	(MxA)
N-terminal domain	(NT)
natural killer	(NK)
niemann-pick C1-like 1	(NCP1L1)
noncoding region	(NCR)
nonstructural proteins	(NS)
novel PKC	(nPKC)
nuclear localisation signal	(NLS)
oncostatin M receptor	(OSM-R)
open reading frame	(ORF)
partitioning defective gene-6	(PAR-6)
pathogen-associated molecular patterns	(PAMP)
pathogen-recognition receptors	(PRRs)
pegylated interferon-α	(Peg-IFNα)
peripheral blood mononuclear cells	(PBMC)
phosphatase and tensin homologue	(PTEN)
phosphatidylserine-binding	(PB1)
phosphoinositide 3-kinase	(PI3K)
phospholipase C	(PLC)
phosphotidylserine	(PS)
programmed death-1	(PD-1)
prostate apoptosis response-4	(Par-4)
protein inhibitors of activated STATs	(PIAS)
protein kinase C	(PKC)
protein kinase R	(PKR)
protein phosphatase 2A	(PP2A)
protein tyrosine phosphatases	(PTP)
receptors for activated C-kinases	(RACKs)
receptors for inactive C-kinases	(RICKs)
ribonucleic acid	(RNA)
RIG-I-like receptors	(RLR)
RING-box2	(RBX2)
RNA dependant RNA polymerase	(RdRp)
scavenger receptor class B type I	(SR-BI)

serine	(Ser)
signal transducers and activators of transcription	(STAT)
single nucleotide polymorphisms	(SNPs)
src homology 2	(SH2)
STAT3-binding elements	(SBEs)
substrates that interact with C-kinases	(STICKs)
suppressors of cytokine signalling	(SOCS)
sustained virological response	(SVR)
TATA element modulatory factor	(TMF)
threonine	(Thr)
tight junctions	(TJs)
TNF receptor-associated factor 3	(TRAF3)
toll-like receptor	(TLR)
transcriptional activation domain	(TAD)
transforming growth factor β	(TGFβ)
tumour necrosis factor	(TNF)
tumour necrosis factor-related apoptosis-inducing ligand	(TRAIL)
turn-motif	(TM)
tyrosine	(Tyr)
tyrosine kinase 2	(Tyk2)
unphosphorylated STAT	(U-STAT)
variable hinge domain	(V3)
very low-density lipoprotein	(VLDL)
zona occludin	(ZO)

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Chapter I

General Introduction

CHAPTER I

1.1 HEPATITIS C VIRUS

1.1.1 Hepatitis C Virus Prevalance and Course of Infection

The hepatitis C virus (HCV) belongs to the *Hepaciviris* genus in the *Flaviviridae* family. Human yellow fever, animal pestiviruses, dengue and tick-borne encephalitis also belong to the *Flaviviridae* family (Thiel *et al* 2005). Hepatitis C was previously known as "non A, non B hepatitis" until it was identified in 1989 (Choo *et al*, 1989). It is a major cause of chronic liver cirrhosis and hepatocellular carcinoma (Lauer and Walker, 2001). The World Health Organisation estimates that there are 170 million individuals infected globally, some ~3% of the worlds' population. While acute infection is usually asymptomatic and can be spontaneously cleared, approximately 50-80% of patients progress to chronic infection. One fifth of chronically infected patients will then develop liver cirrhosis within 20 years and amongst those with cirrhosis, 1-5% develop hepatocellular carcinoma (Cerny and Chisari, 1999, Rosen HR, 2011).

Pegylated interferon-α (Peg-IFNα) combined with ribavirin for a period of between 24 or 48 weeks has long been the standard treatment for HCV infection. Interferons are central to the regulation of the antiviral immune response, however, patients infected with HCV genotypes 1 and 4 have particularly poor virus clearance rates post treatment. It is not yet known why there is such variety in response to IFNα/ribavirin treatment. Peg-IFNα treatment causes a variety of undesired side effects such as neutropenia, anaemia and depression. Recently the approval of HCV direct-acting antiviral agents Boceprevir and Telaprevir has increased sustained viral clearance rates for genotype 1 (Fox and Jacobson, 2012). There is currently no vaccine and further developments in uncovering complementary therapeutic targets are necessary to improve patient outcome (Manns *et al*, 2001, Chung RT, 2012).

Progress in developing better therapies for HCV infection has been hampered until recently by the lack of a good quality cell culture system and a suitable small animal model. Advances in hepatitis C protein generation (Bartosh *et al*, 2003), the generation of

the HCV cell culture system (HCVcc) and HCV-JFH1 cell culture systems (Lindenbach *et al*, 2005, Wakita T *et al.*, 2005) have opened the posibility of studying HCV lifecycle in-depth. HCVcc is a full-length recombinant HCV genome that replicates and produces virus particles that are infectious in cell culture. A genetically humanized mouse model for HCV infection has now been developed which will allow for investigation of viral pathogenesis and immunity *in vivo* for the first time (Dorner *et al.*, 2011). These tools have helped uncover crucial aspects involved in processes such as viral entry into the cell, virion production, and cell-cell spread of HCV (Dhillon S *et al.*, 2010, Witteveldt J. *et al*, 2009, Bellecave P *et al* 2008).

1.1.2 HCV Structure and Genome Organisation

The hepatitis C virus is a positive-sense RNA that is 9.6kb long. There are seven major genotypes, which differ in their nucleotide sequence by 30-35% (Kuiken and Simmonds, 2009). There are several further genotype quasispecies that can differ by 20-25% due to the lack of proof-reading function of the viral RNA dependant RNA polymerase (RdRp) (Gottwein & Bukh, 2008). The genome contains a highly conserved 5' noncoding region (5' NCR) with an internal ribosome entry site (IRES) required for cap-independent translation of the viral RNA. An open reading frame (ORF) encodes one large polyprotein that is then processed into various structural and nonstructural proteins. The 3' NCR contains a highly conserved RNA element which is probably required for cap-independent translation of the viral RNA (Rijnbrand and Lemon 2000, Friebe and Bartenschlager, 2002).

The ORF encodes a 3,000 amino acid polyprotein precursor that is then processed by cellular and viral proteinases into structural and nonstructural proteins. The structural proteins are the core protein and the envelope glycoproteins E1 and E2, while the nonstructural proteins (NS) are p7 and NS2-NS5B (**Figure 1.1**).

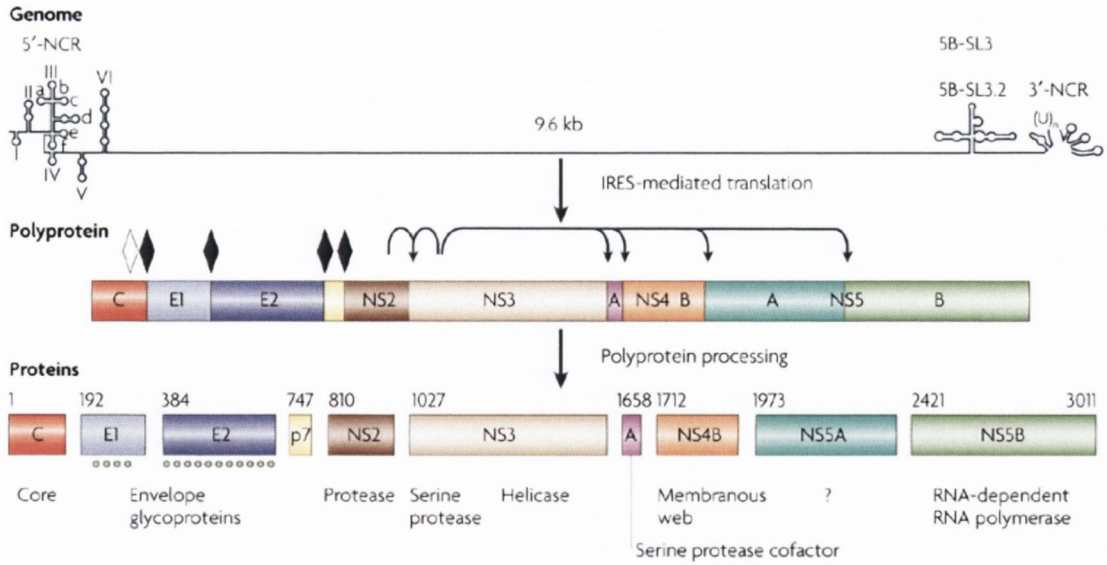


Figure 1.1 Genetic organisation and polyprotein processing of hepatitis C virus (HCV). HCV is a 9.6-kb positive-strand RNA genome shown here with simplified RNA secondary structures in the 5'- and 3'-non-coding regions (NCRs) and the core gene. The polyprotein precursor that is processed into the mature structural and non-structural proteins is shown with diamonds and arrows indicating cleavage sites. Dots in E1 and E2 indicate the glycosylation of the envelope proteins. Polyprotein processing, takes place co- and post-translationally (Adapted from Moradpour, D *et al.,* 2007).

Hepatitis C has two envelope glycoproteins, E1 and E2, which are formed by cleavage from the polyprotein. They form a noncovalent complex with transmembrane domains that promote endoplasmic reticulum retention. (Deleersnyder V *et al* 1997, Op De Beeck *et al* 2001, Dubuisson J *et al* 2002). The E2 protein also contains hypervariable regions, which are involved in receptor binding and entry to the cell (Bartosch B 2003, Callens N 2005, Roccasecca R 2003). The p7 polypeptide is 63 amino acids long and contains two transmembrane domains. p7 is required for productive infection *in vivo* but not RNA replication *in vitro* (Lin C *et al* 1994, Mizushima *et al* 1994, Dubuisson J *et al* 2002, Sakai A *et al* 2003, Steinmann *et al* 2007).

There are five non-structural proteins encoded in the hepatitis C polyprotein. Once translated these can form complexes such as the NS2-3 protease (Lorenz *et al*, 2006) and

the NS3-4A complex, which has protease and helicase activity (Wolk *et al*, 2000). NS4B hydrolyses GTP and affects HCV RNA replication (Einav *et al* 2004). NS5A is conserved amongst other flaviviruses (Reed KE *et al* 1998) and is essential for genome replication (Brass V *et al* 2002, Penin F *et al* 2004, Huang L *et al* 2005, Appel N *et al* 2006, Seeger C 2005). NS5B is also essential for RNA replication by acting as a catalyst for RNA polymerase (Ivashkina N *et al* 2002, Moradpour D *et al* 2004, Schmidt-Mende J *et al* 2001).

The core protein forms the nucleocapsid. The mature protein is 21-kDa, between 173 – 179 amino acids long and dimeric alpha-helical (Santolini *et al* 1994, McLaughlan *et al* 2002). It has been detected on the membranes of the ER and on the surface of lipid droplets of infected cells. The HCV core region also contains an alternative reading frame at a +1 ribosomal frameshift. This can encode a protein up to 160 amino acids long named ARFP or chimeric proteins (Branch AD *et al* 2005). Their functions still have to be uncovered, however, antibodies specific to this protein are produced in HCV infected patients (Bain C *et al* 2004).

1.1.3 HCV Entry to Hepatocytes

The HCV primarily targets hepatocytes, the major cell type of the liver. Its RNA has also been detected in peripheral blood mononuclear cells such as monocytes, DCs, T and B cells and brain tissue (Caussin-Schwemling *et al* 2001, Goutagny *et al* 2003, Rodriguez-Inigo *et al* 2000, Castillo *et al* 2005, Sung *et al* 2003, Kondo *et al* 2007, Radkowski *et al* 2002, Fishman *et al* 2008). The virion uses its E1 and E2 glycoproteins to bind to the host cell. Each virion can contain multiple copies of the core protein and genomic RNA (**Figure 1.2**). The major hepatic receptors for the E2 glycoprotein are CD81 and scavenger receptor class B type I (SR-BI). The tight-junction proteins Claudin-1 and Occludin are other HCV co-receptors required for HCV entry (Evans MJ *et al* 2007, Ploss A *et al*, 2009, Liu S *et al*, 2009).

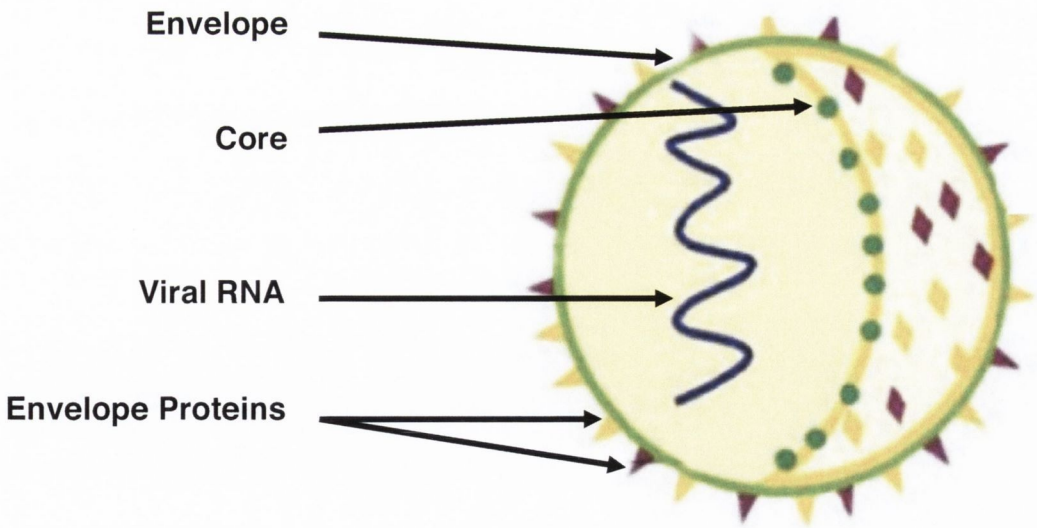


Figure 1.2 Structure of Hepatitis C Virus. The envelope glycoproteins E1 and E2 are required for cellular entry. They surround the nucleocapsid which is composed of core proteins and contains the viral RNA (Adapted from Parvaiz *et al.*, 2011).

HCV entry into hepatocytes is complex and involves multiple steps. These steps can be divided into three sections: initial cellular contact, binding to the cell surface and cellular internalisation. However, our understanding of the mechanisms of HCV entry is still poor and requires further examination.

HCV associates with the very low-density lipoprotein (VLDL) apolipoprotein B (ApoB) and ApoE in the blood (Andre *et al* 2002, Nielsen *et al* 2008). The initial contact HCV has with hepatocytes is indirect. HCV bound ApoB and ApoE interact with the hepatocyte LDL receptor (LDL-R) and highly sulfated heparan sulfate (HS) glycosaminoglycan (GAG) at the basolateral surfaces to initiate contact (Agnello *et al* 1999, Barth *et al* 2006, Barth *et al* 2003, Koutsoudakis *et al* 2006). This has been demonstrated both in primary human hepatocytes and the CD81 deficient HepG2 cell line, where LDL-R mediates HCV contact by binding HCV-ApoB and ApoE (Agnello *et al* 1999, Wunschmann *et al* 2000, Molina *et al* 2007).

Following initial contact with hepatocytes, HCV binds to the cell through surface receptors Scavenger receptor class B type I (SR-B1) and CD81. SR-B1 is an 81kDa glycoprotein

with a large extracellular loop. It is highly expressed in hepatocytes (Krieger, 2001) and can bind a variety of lipoproteins including HDL and LDL. The extracellular loop of SR-BI has been shown to interact with the HCV E2 protein (Scarselli *et al.,* 2002). It is also possible that HCV is able to associate with HDL to further enhance its binding to SR-BI (Bartosch *et al.,* 2005, Voisset *et al.,* 2005, Maillard *et al.,* 2006, Dreux *et al.,* 2009).

CD81 is a 25kDa tetraspanin that has a small extracellular loop and a large extracellular loop (LEL). It was the first protein identified to interact with a soluble HCV E2 protein and has been shown to be required for viral entry (Lindenbach *et al.*, 2005, McKeating *et al.*, 2004, Lavillette *et al.*, 2005). The LEL plays a central role in binding the E2 protein (Owsianka *et al.*, 2006, Dhillon *et al.*, 2010) while soluble CD81 LEL can inhibit HCVcc infection (Zhang *et al.*, 2004). Fluorescence resonance energy transfer (FRET) analysis has shown that CD81 associates with members of the claudin family, namely claudins 1, 6 and 9, that support HCV entry (Krieger *et al.*, 2010, Harris *et al.*, 2008, Zheng *et al.*, 2007, Meertens *et al.*, 2008).

Claudin 1 is a tetraspanin protein with four transmembrane domains, two extracellular loops and cytoplasmic N- and C- terminals. Their C-terminal domain can interact with a range of other proteins including PDZ domain containing proteins such as the Zona Occludin (ZO) family. It is highly expressed in hepatocytes and located at both tight junctions (TJs) and basolateral surfaces (Furuse *et al.*, 1998, Reynolds *et al.*, 2008). Claudin 1 forms complexes with CD81 that are required for HCV infection but has not been shown to interact directly with HCV. It is therefore postulated that Claudin 1 is required for hepatocyte entry following viral binding (Evans *et al.*, 2007, Krieger *et al.*, 2010). Claudin family members Claudin-6 & 9 have also been implicated in HCV cell entry (Zheng *et al.*, 2007). HCV uses these proteins to enter the host cell by clathrin-mediated endocytosis through an endosomal, low pH compartment (Blanchard *et al.,* 2006, Koutsoudakis *et al* .,2006, Tscherne *et al.*, 2006).

Occludin is a 65kDa integral plasma-membrane protein located at tight junctions. Similar to Claudin 1 it has four transmembrane domains, two extracellular loops and cytoplasmic N- and C- terminals. Although Occludin does not interact with HCV directly it probably

facilitates its cellular internalization following viral binding (Ploss *et al.*, 2009, Liu *et al.*, 2009, Benedicto *et al.*, 2009). Its importance to HCV cellular entry is demonstrated by mouse cells that become permissive for HCV following the expression of human occludin and human CD81 (Ploss *et al.*, 2009). HCV is a leading cause of liver transplantation and organ transplant patients are administered corticosteroids. It has been shown that when hepatocytes are treated with glucocorticosteroids, occludin expression is increased, leading to a rise in HCV entry (Ciesek *et al.*, 2010). Unfortunately this increases HCV recurrence following liver transplantation. Conversely, following HCV infection occludin is down-regulated to prevent super-infection (Liu *et al.*, 2009) (**Figure 1.3**).

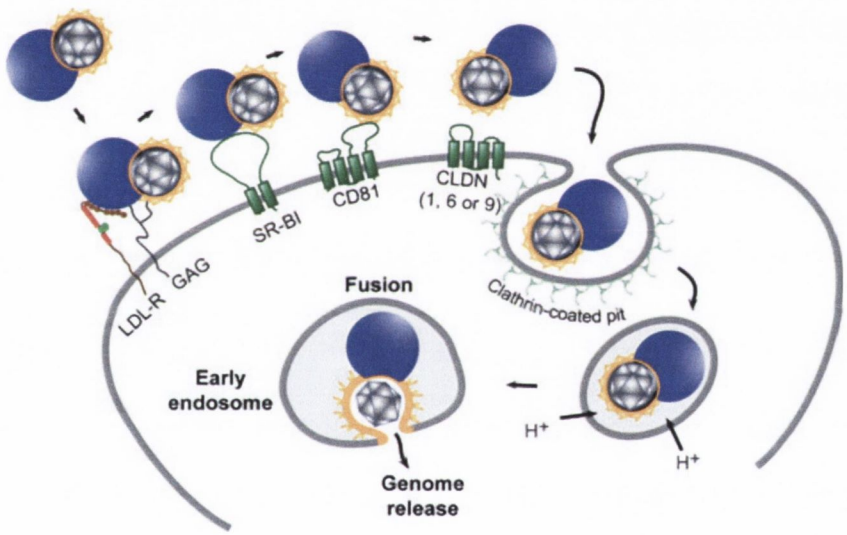


Figure 1.3 Model of HCV entry into the hepatocyte. HCV particles bind low-density lipoprotein receptors (LDL-R), glycosaminoglycan (GAG), SR-B1 and the glycoprotein CD81. The tight junction proteins Claudin 1,6 and 9 and Occludin are required for viral entry to the hepatocyte via clathrin mediated endocytosis (Adapted from Dubulsson *et al.*, 2008).

Furthermore, Niemann-Pick C1-like 1 (NCP1L1), Epidermal Growth Factor Receptor (EGFR) and Ephrin Receptor A2 (EphA2) have recently been shown to be required for HCV infection. NCP1L1 is a cholesterol uptake receptor that is responsible for cellular cholesterol absorption, *In vitro* experiments using a HCV cell culture system showed that HCV infection could be impaired by the NPC1L1 antagonist ezetimibe at a post-binding step (Sainz Jr *et al.*, 2012). EGFR and EphA2 are receptor tyrosine kinases that were found to be required for HCV following a functional RNAi kinase screen. They mediate CD81:claudin-1 co-receptor associations and viral glycoprotein-dependent membrane fusion. The stimulation of EphA2 and EGFR, with ephrin and EGF respectively, promotes HCV cell entry while blocking their kinase activity by pharmacological inhibitors and impaired infection in cell culture and a human liver chimeric mouse model *in vivo* (Lupberger J *et al.*, 2011). Some cell types are still not susceptible to HCV infection even when expressing CD81, SR-BI and Claudin-1, indicating there are still more HCV entry factors yet to be uncovered (Pileri *et al.,* 1998, Agnello *et al.,* 1999, Scarselli *et al.,* 2002, Evans *et al.,* 2007, Benedicto I *et al.*, 2009, Ploss A *et al.*, 2009).

VEGF expression in HepG2-CD81 cells is increased following HCV infection and activates the VEGF receptor 2 pathway. This reorganizes occludin, reduces the polarity of hepatocytes and results in increased HCV entry. Hepatic VEGF signalling can be inhibited by the receptor kinase inhibitor sorafenib, which then leads to the promotion of cell polarization and inhibition of HCV entry (Mee *et al.*, 2010). This correlates with reports that VEGF reduces occludin expression in HCC (Schmitt *et al.*, 2004).

Once internalised, HCV can also infect neighbouring cells in a cell-cell manner, possibly via an endocytotic-like pathway (Marsh and Helenius, 2006). This provides the virus with the advantage of avoiding neutralizing antibodies in the extracellular environment (Timpe *et al.*,2008). Similar host factors are required for cell-cell spread (Schwarz *et al.*, 2009, Timpe *et al.*,2008), although CD81 independent spread has also been described (Timpe *et al.*,2008, Witteveldt *et al.*, 2009).

1.1.4 HCV Lifecycle

HCV, similar to other positive-strand RNA viruses, synthesises a negative strand RNA that is used as a template to generate copies of the genomic positive-strand RNA. (Aizaki H *et al.,* 2004, Ali KD *et al.,* 2002, Lai VC *et al.,* 2003, Miyanari Y *et al.,* 2003, Hardy RW *et al.,* 2003, Shi ST *et al.,* 2003). Once internalized, the acidic pH of the endosomes triggers HCV envelope glycoproteins to fuse with host membranes and deliver the HCV RNA into the host cytosol for replication (Blanchard *et al.*, 2006, Smith and Helenius, 2004, Tscherne *et al.*, 2006, Haid *et al.*, 2009, Rice, 2011). The structural proteins are processed by host peptidase at the ER membrane. HCV then uses the lipoprotein pathway to assemble and secrete viral particles (**Figure 1.4**). ApoE is required for HCV assembly (Chang *et al.*, 2007, Beng *et al.*, 2010, Jian and Luo, 2009, Merz *et al.*, 2011) and HCV proteins are located within cytoplasmic lipid droplets (cLD) (McLauchlan, J. *et al.*, 2002). The importance of LDs is highlighted by diacylglycerol acyltransterase-1 (DGAT1), which is required for LD production. Inhibition of DGAT1 has been shown to decrease HCV particle production (Herker,E. *et al.*, 2010).

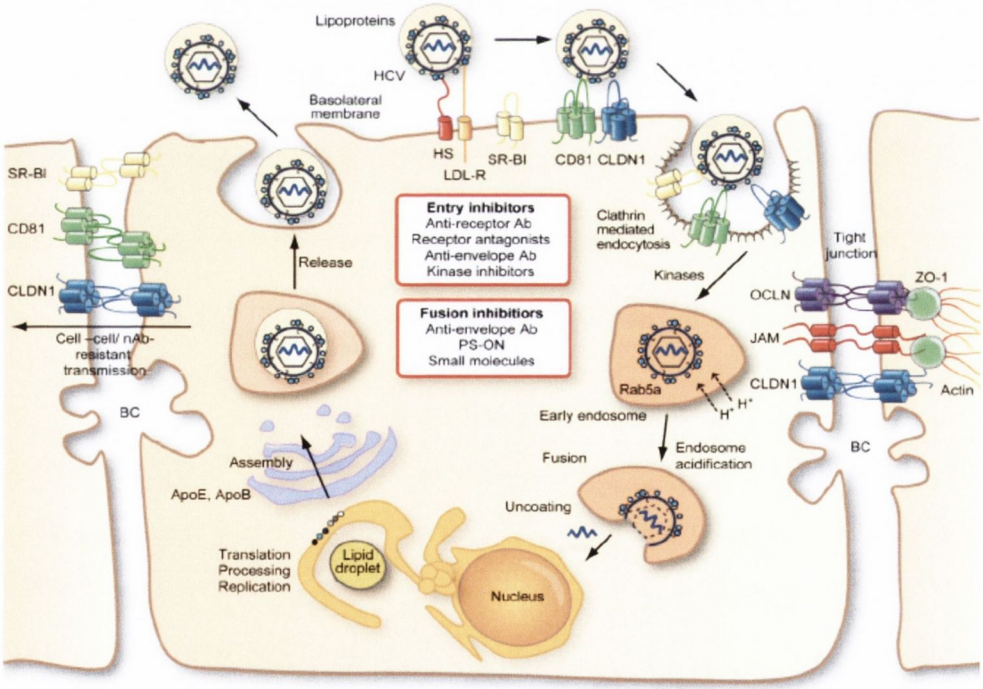


Figure 1.4 HCV lifecycle. HCV binds cell surface receptors and is internalised by clathrin endocytosis. HCV RNA is released to the cytosol for replication and translation. Viral particles are then assembled and released from the hepatocyte. (Zeisel *et al.*, 2011).

Another host factor involved in the HCV lifecycle is the role of microRNAs (miRNAs). miRNAs are non-coding RNAs that are 20-22 nucleotides long. They control gene expression by targeting mRNAs for transcriptional repression or cleavage (Bartel, 2004, Meister *et al.*, 2004, Meister and Tuschl, 2004). In the liver miR-122 comprises 70% of the miRNAs expressed. miR-122 is required for HCV replication and its depletion is associated with inhibition of HCV replication and viral production (Jopling *et al.*, 2005). miR-122 enhances the association of ribosomes with viral RNA for HCV RNA translation (Henke *et al.*, 2008) and may inhibit RIG-I recognition of HCV RNA by masking the 5'triphosphate end of the HCV genome (Jopling *et al.*, 2008, Machlin *et al.*, 2011).

1.1.5 Immunology against HCV

HCV infection is at first asymptomatic and not diagnosed until alanine aminotransferase (ALT) levels increase between 2-3 months after initial infection. Most of our current understanding of the early period of infection comes from the chimpanzee model where HCV viral titers rapidly rise within days of infection. Type I IFN is produced, slowing HCV replication and eventually viral levels plateau (Dahari H *et al.*, 2005, Bigger *et al.*, 2001, Su *et al.*, 2002).

Both innate and adaptive arms of the immune response are induced by HCV infection. HCV clearance is associated with a strong HCV-specific $CD4^+$ T cell response along with IFNγ and IL-2 production (Lechner F *et al.*, 2000, Kaplan DE *et al.*, 2007). IFNβ production and secretion by infected hepatocytes is thought to be the initial antiviral reaction. Secretion of IFNβ prompts adjacent cells into an antiviral state leading to the induction of ISGs (Guo JT *et al.*, 2004). It has been shown that infected individuals who develop chronic HCV infection have no or weak $CD4^+$ T cell responses. Loss of an originally vigorous $CD4^+$ T cell response is associated with a recovery in the prevalence of HCV (Gerlach JT *et al.*, 1999., Nascimbeni M *et al.*, 2003). Furthermore *in vivo* exhaustion of $CD4^+$ T cells from HCV-recovered chimpanzees nullifies protective CD8+ T cell immunity upon rechallenge, leading to viral persistence (Grakoui *et al.*, 2003).

$CD8^+$ T cells are found in acutely infected patients, regardless of virological outcome. These cells have diminished IFNγ production, cytotoxicity and proliferation, while high

levels of programmed death-1 (PD-1) leads to their apoptosis (Urbani S *et al.*, 2002, Kasprowicz V *et al.*, 2008). However, these cells recover their normal characteristics and memory $CD8^+$ T cells are produced once the HCV titer decreases following a successful $CD4^+$ T cell response (Urbani S *et al.*, 2006).

Dendritic cells (DC) are the primary antigen presenting cell and their activity is critical for the production of a vigorous polyclonal T-cell response (Steinman, 1991). Conventional DCs (cDCs) are required for antigen transport to the lymph nodes for optimal T cell priming. cDC maturation and IL-12 secretion may be impaired in HCV infection, resulting in insufficient T cell priming and delayed HCV-specific T cell responses (Auffermann-Gretzinger, *et al.*, 2001, Dolganiuc *et al.*, 2003). The TLR3 pathway in DCs is required for a vigorous T-cell response (Gautier *et al.*, 2005). However, DCs taken from HCV infected patients could not secrete IFNα following *in vitro* activation of the TLR3 pathway by poly(I:C) stimulation. This indicates that DC activity in these HCV patients is directed to IL-10 production that suppresses T-cell activity (Ryan *et al.*, 2011). IL-10 inhibits IFNα production (Duramad, *et al.*, 2003), promotes apoptosis of pDCs (Dolganiuc, *et al.*, 2006), and down regulates effector T cell responses. HCVcc models of infection showed that JFH-1- or H77- infected DCs also secreted higher levels of IL-10 and had diminished capability to activate T-cell responses (Saito *et al.*, 2008).

Plasmacytoid DCs (pDC) are located in inflamed tissues and are the main producers of IFN (Cella *et al.*, 1999, Colonna *et al.*, 2002, Diebold *et al.*, 2003). HCV core and NS3 can activate monocytes via TLR2 to produce TNFα, thereby inhibiting production of IFNα and stimulating apoptosis of DCs (Dolganiuc A *et al.*, 2006, Decalf J *et al.*, 2007). However, the effects of chronic HCV infection on DC function are likely to vary due to differences in the duration of infection, genotype of the virus or genetic background of the host, as some studies report no deficiencies in DC function (Piccioli *et al.*, 2005). Inhibition of the ability of DCs to drive Th1 responses was reinstated *in vitro* by the addition of the anti-viral agent, ribavirin (Miyatake *et al.*, 2007), suggesting that the virus directly affects DC function.

Natural Killer (NK) cells are found in the liver, making up 30%–50% of intrahepatic lymphocytes (Doherty and O'Farrelly 2000) and quickly induce cytotoxicity and release

cytokines. Their interaction with dendritic cells (DCs) increases NK cell activation while also promoting the maturation of DCs (Moretta *et al.*, 2006). NK cells induce cytotoxicity by direct release of perforin and granzymes or through tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated killing. They are able to produce cytokines such as IFNγ and TNFα that can inhibit viral replication and prime the adaptive response.

A balance between activatory and inhibitory receptors controls NK cell activation. Activating receptors include C-type lectin-like receptors, natural cytotoxicity receptors and CD16. The killer cell immunoglobulin-like receptors (KIR) and the C-type lectin like receptor (NKG2A) comprise the main inhibitory receptors (Moretta *et al.*, 2006). The level of NK reaction to HCV may depend on the KIR/HLA genotypes present in the individual. Patients with a stong response to infection may have a lower threshold for NK cell activation. This is due to protective KIR/HLA compound genotypes that lead to faster NK cell degranulation and IFNγ release (Ahlenstiel *et al.*, 2008, Amadei *et al.*, 2010).

During acute hepatitis C, it was demonstrated that peak NK cell activity either preceded or coincided with peak T-cell responses while degranulation of NK cells correlated with the level of HCV-specific T-cell responses, implying an indirect role for NK cells in priming adaptive immunity (Pelletier *et al.*, 2010). However, NK cells from chronic HCV-positive patients, but not from healthy controls, overexpress inhibitory receptors and produce cytokines *in vitro* such as TGF-β and IL-10 that diminish the adaptive immune reaction by restricting antigen presentation (Jinushi, M., *et al.* (2004). NK cells and their role in HCV infection is reviewed in more detail by Cheet and Khakoo (2011).

The common factor between hepatocytes, dendritic cells, NK cells and T-cells is the production of interferons in the response against HCV. Effective interferon production and function is a requirement of a successful antiviral response. In order to promote an antiviral response, patients infected with HCV are prescribed a synthetic (PegIFN) and Ribavirin. To better understand how to treat HCV positive patients and improve future treatment regimes and response rates, the interferon production and signalling pathways must be further defined.

1.2 THE INTERFERON FAMILY

Interferons are a family of structurally related cytokines that are present exclusively in vertebrates. They exert a range of effects including antiviral properties, antitumour activity and immunomodulation. Their discovery over 50 years ago as proteins that interfered with viral replication made them the first cytokine to be described (Isaacs and Lindenmann, 1957). In the late 1970's they were purified from human leukocytes (Rubinstein M, et al., 1978) and soon after their cDNA was cloned from fibroblasts and leukocytes (Taniguchi *et al.*, 1980, Nagata *et al.*, 1980, Vilcek J., 2006). IFNs are glycoproteins and are grouped into three classes (types I-III) based on their amino acid sequences. Each type of interferon binds a specific receptor dimer that then activates a signalling cascade, ultimately leading to the expression of hundreds of genes (Chelbi-Alix and Wietzerbin, 2007).

1.2.1 Type I IFNs

The best described group of IFNs is the type I family, that consists of IFNα, IFNβ, IFNω and the tissue specific IFNs κ and ε (Hardy *et al.*, 2004). Their genes lack introns and are clustered at the short arm of chromosome 9 at 9p21 in humans (Weissmann and Weber, 1986, Trent, 1982, Diaz, 1994). Their secondary structure shows a cluster of 5 α-helices held together by two disulphide bonds (**Figure 1.5**) (Mitsui, & Senda, 1997). Type I IFNs are expressed at constitutively low levels in normal individuals. This priming creates a positive feedback loop that enables a rapid increase in IFNα production and response to infection when cells are challenged (Taniguchi A and Takaoka A, 2001, Pestka *et al.*, 2004). Once produced, type I IFNs bind the IFN alpha receptor (IFNAR). This then activates the Jak/STAT signal pathway. This pathway leads to the induction of expression of hundreds of ISGs and activation of Natural Killer (NK) cells, macrophages and dendritic cells (DC) (Mitani *et al.*, 2001, Durbin *et al.*, 1996). Moreover type I IFNs are required for $CD8^+$ T cell activation, linking the innate response to adaptive immunity (Hida *et al.*, 2000).

IFNα is produced in most cell types and is the most abundant IFN released following viral or microbial infection (García-Sastre and Biron 2006). There are 13 isotypes of IFNα in humans. IFNαs are composed of 166 amino acids, except for IFNα-2, which has 165 due to a deletion at residue 44 when fully processed. Like all type I IFNs they are composed of five α-helices and have two disulphide bonds. IFNαs have a Mw range of between 17.5-27kDa and share an amino acid homology of between 75-99%. Most IFNαs are not N-glycosylated, unlike IFNβ (Bekisz *et al.*, 2004).



Figure 1.5 Structure of IFNα. The three-dimentional high resolution structure of recombinant human interferon alpha-2a (Roferon-A) determined by heteronuclear nmr spectroscopy in solution, showing a cluster of 5 α-helices as the dominant feature (Klaus *et al.,* 1997).

1.2.2 Type III IFN

The type-III family of IFN includes three members: IFN -λ1, - λ2 and - λ3, also known as

IL-29, IL-28A and IL-28B respectively. These signal through a distinct heterodimeric receptor complex composed of the ligand-binding chain, IFN- λR1 and the accessory chain, IL-10R2. Expression of IFN-λ receptors is largely restricted to cells of epithelial origin such as keratinocytes, bronchial epithelial cells and hepatocytes (Kotenko *et al.*, 2003, Sheppard *et al.*, 2003). Following ligand binding, the receptor-associated Janus tyrosine kinases, Jak1 and Tyk2, mediate trans-phosphorylation of the receptor chains to provide docking sites for STAT1 and STAT2 and the ISGF3. After it is fully assembled, ISGF3 translocates to the nucleus where it binds to IFN-stimulated response elements (ISREs) in the promoters of various ISGs. Comparative cDNA microarray analyses by several groups have shown that the repertoire of genes that are induced by IFN-I is essentially the same as that induced by IFNα (Doyle *et al.*, 2006, Marcello *et al.*, 2006). SNPs in the IL28B gene region as associated with response to cotreatment with pegylated IFNα plus ribavirin among patients with chronic HCV infection and subsequent studies have shown that IL28B genotype is associated with spontaneous HCV clearance as well (Thomas *et al.*, 2009, Rauch *et al.*, 2010). Comprehensive information on the type-III family of IFNs can be found reviewed elsewhere (Kotenko SV 2011).

1.2.3 Type I IFN Production

Type I IFN production can be induced in almost every cell type by stimuli such as viruses, bacteria or bacterial lipopolysaccharide, through a range of signalling pathways. Pathogen-associated molecular patterns (PAMP) initiate these signalling pathways by activating pathogen-recognition receptors (PRRs). For IFN production, these PRRs are either from the endosomal Toll-like receptor (TLR) family or the cytosolic RIG-I-like receptors (RLR) receptors.

Regardless of the pathway used to initiate signalling for type I IFN production, common signalling proteins are activated before type I IFN production can begin. These include TNF receptor-associated factor 3 (TRAF3) and the transcription factors IFN regulatory factors (IRF) IRF3 and IRF7 (Hacker *et al.*, 2006, Schafer *et al.*, 1998, Thompson and Locarnini 2007). Once produced, type I IFNs are secreted and can induce autocrine or paracrine signalling via the IFNAR.

1.2.4 Endosomal TLRs

The mammalian TLR family is a group of 13 receptors, with 10 of these expressed in humans. TLRs are the main sensors for RNA viruses, but can also detect DNA viruses, bacteria and fungi (Alexopoulou *et al.*, 2001, Diebold *et al.*, 2004). TLRs responsible for detecting viral infections (TLRs 3, 7-9) are mainly located in the endosomal compartments, while TLRs involved in bacterial and fungal detection (TLR 1, 2, 4-6 and 11) are located on the cell surface (Diebold 2008).

TLRs are transmembrane receptors and signal downstream through the MyD88 or TRIF adaptor proteins (Akira and Takeda 2004). TLR3 was the first receptor identified to induce IFN production in response to dsRNA, including replicating HCV-RNA (Alexopoulou *et al.*, 2001). It is localised in the endosomes of most cells but at the cell surface of fibroblasts. Once dsRNA is detected, TLR3 dimerizes (Leonard *et al.*, 2008, Wang *et al.*, 2010). This activates the TLR adaptor molecule 1 (TRIF) at its TIR domain. TRIF can activate either the IRF3 or NF-κB pathways (**Figure 1.6**).

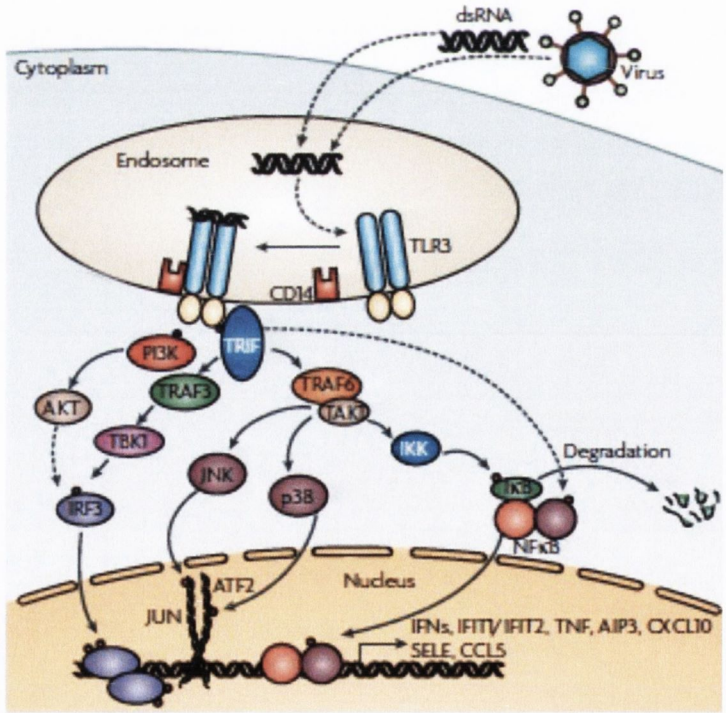


Figure 1.6 TLR3 induction of IFN production. Once activated by dsRNA, TLR3 can induce IFN production via the IRF3 transcription factor or NFκB (Borden *et al.*, 2007).

1.2.5 Cytosolic Receptors

The cytosolic RIG-I-like receptors (RLR) family are PRRs that detect intracellular viruses. Two family members, Retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) have been the focus of research in this family since its discovery in 2004 (Yoneyama *et al.*, 2004, Andrejeva *et al.*, 2004). Both of these PRRs are universally expressed ATP-dependent DExD/H RNA helicases.

RIG-I and MDA5 are composed of a caspase recruitment domain (CARD) region at their N-terminal, a central DExD/H helicase and a regulatory domain (RD) at their C-terminal (**Figure 1.7**). The RD is responsible for recognising dsRNA and regulating RLR activity. RIG-I is auto-inhibited through its RD interacting with its CARD and helicase domains. Once activated, RIG-I alters conformation, exposing the CARD domain (Saito *et al.*, 2007, Takahasi *et al.*, 2008). In contrast, MDA-5 is negatively regulated through binding of dihydroxyacetone kinase (DAK) at its RD until it detects infection (Diao *et al.*, 2007).

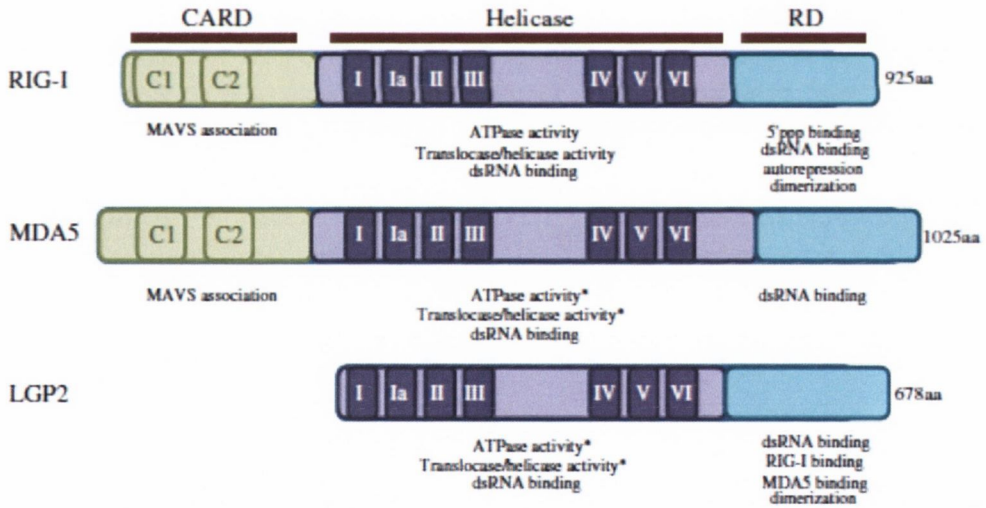


Figure 1.7 The Structure of cytosolic RIG-I-like receptors. The N-termini of RIG-I and MDA5 contain CARD domains that interact with adaptor proteins. The central domain contains the DExD/H RNA helicases. The C-domains contain RNA receptor domains and are also responsible for auto-regulation of RLR activity. The C terminus of LGP2 can also bind RIG-I and MDA5 to regulate their activity (Baum and García-Sastre 2010).

The central region of RIG-I and MDA5 is the DExD/H helicase. DExD/H helicases are ATP dependent and this region has ATPase activity that is required for IFN production (Kang *et al.*, 2002, Schmidt *et al.*, 2009, Takahasi *et al.*, 2008).

The N-terminus has two CARDs required for interaction with MAVS (also called IPS-1, VISA or Cardif) and signalling cascade activation. This signalling cascade further activates IRF3, NFκB and IFNβ transcription factors to induce IFN production (**Figure 1.8**) (Kawai *et al.*, 2005, Meylan *et al.*, 2005, Seth *et al.*, 2005, Xu *et al.*, 2005).

A final member of the RLR family is Laboratory of Genetic and Physiology 2 (LPG2). This protein also contains a DExD/H RNA helicase domain but has no CARD domains, preventing if from directing antiviral activity. LPG2 may instead be a RLR signalling regulator, inhibiting RIG-I signalling through an undetermined mechanism and promoting MDA5 activity (Rothenfusser *et al.*, 2005, Yoneyama *et al.*, 2005).

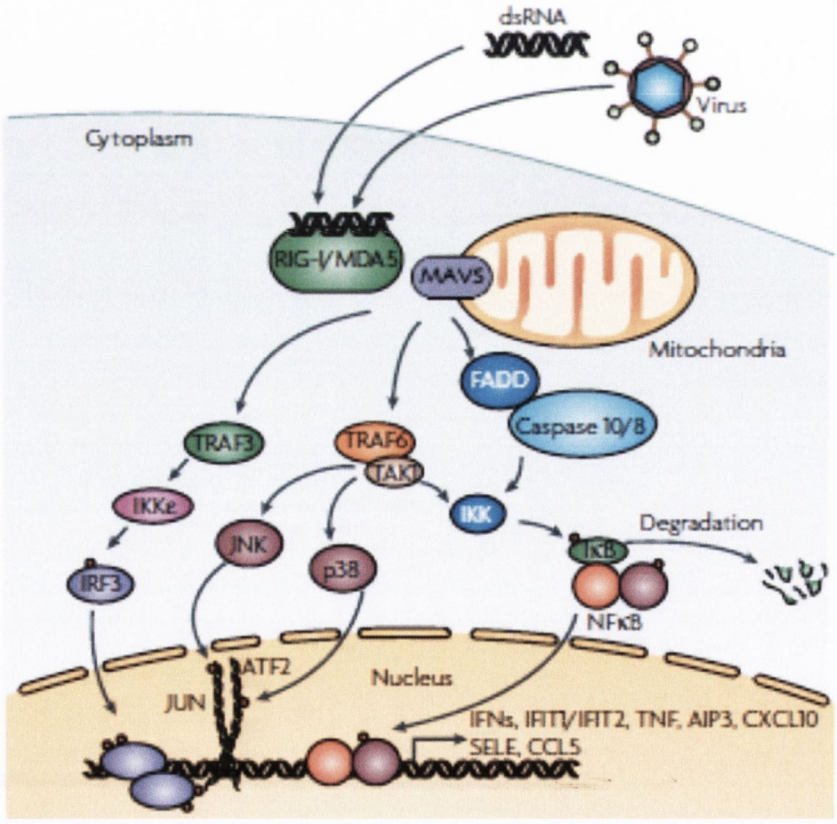


Figure 1.8 RLR induction of IFN production. RLRs activated by dsRNA can induce IFN production via the MAVS adaptor protein that then leads to the activation the IRF3 transcription factor or NFκB (Borden *et al.*, 2007).

1.3 INTERFERON-ALPHA SIGNALLING

1.3.1 IFN Signalling

Following their production, IFNs initiate signalling cascades by binding to two surface receptor subunits that each have a single transmembrane domain. The receptor subunits then dimerise, resulting in the phosphorylation of a JAK tyrosine kinase constitutively bound at its cytoplasmic terminal. The JAK proteins in turn activate pathways such as the STAT, mitogen activated protein kinase (MAPK), PI3K and Crkl pathways.

1.3.2 Type I IFN Receptors

Type I IFNs bind to the widely expressed IFNAR heterodimer. This receptor is composed of two separate subunits, IFNAR-1 and IFNAR-2. Both subunits belong to the class II helical cytokine receptor family and feature conserved structural fibronectin type III (FNIII) regions in their extracellular ligand binding domains (Bazan, 1990).

IFNAR-1 has 530 amino acid residues in its mature form, with 409 residues in the extracellular domain, 21 residues in the transmembrane domain and 100 residues in its cytoplasmic domain. It is 135kDa and is noncovalently associated with tyrosine kinase 2 (Tyk2) at its cytoplasmic terminus. It is considered to be the signalling subunit as it has a 1000 fold lower binding affinity to type I IFNs than IFNAR-2 (Uze *et al.*, 2007). IFNAR-2 has 487 residues. Exon skipping, alternative splicing and alterations in polyadenylation sites give rise to three isoforms of IFNAR-2 (Lutfalla *et al.*, 1995). IFNAR-2 noncovalently associates with Janus Kinase (JAK) 1 and has strong type I IFN binding affinity. IFNAR-2 also associates with STAT2 before induction and has a weak association with STAT1 (Stancato *et al.*, 1996, Precious *et al.*, 2005a, Tang *et al.*, Domanski and Colamonici 1996, Novick *et al.*, 1994, Uze, 1995, Pfeffer *et al.*, 1997).

IFNAR-1 and IFNAR-2 dimerize and undergo conformational change. This leads to the autophosphorylation of their associated JAK proteins. IFNα also prompts the transcriptional co-factor CBP to bind IFNAR-2 and catalyse its acetylation. Acylation allows the formation of a docking site for IFN regulatory factor 9 (IRF-9), which is a protein that is required for DNA binding. Receptor bound IRF-9, STAT1 and STAT2 are then acylated,

which may assist ISGF3 complex formation (Tang *et al.*, 2007). Activated IFNAR associates with tyrosine phosphatases to regulate the activity of Tyk2 and JAK1 in IFN signaling (Platanias, 2003).

IFNAR subunits can translocate to the nucleus following stimulation. IFNAR-I contains a nuclear localization sequence in its extracellular domain and has been shown to translocate to the nucleus following IFNβ stimulation in murine L cells overexpressing human IFNAR-1 (Subramaniam and Johnson, 2004). IFNAR-2 can be proteolytically cleaved in response to IFNα and epidermal growth factor. Once cleaved, the intracellular domain (ICD) can translocate into the nucleus and repress transcriptional activity relative to the amount of ICD present in the nucleus (Saleh *et al.*, 2004).

Following IFNα binding IFNAR, the JAK/STAT signalling pathway is activated. This pathway can then be divided into three sections; JAK phosphorylation, STAT dimerisation and nuclear translocation and finally gene transcription.

1.3.3 JAKs

Although JAK was originally the acronym for Just Another Kinase this later changed to be for Janus kinases in recognition of the Roman god Janus who had two opposing faces, suggestive of the organisation of the JAK kinase and pseudokinase regions (Wilks *et al.*, 1991). There are four types of Janus kinases; JAK1, JAK 2 and Tyk2 are expressed ubiquitously (Wilks 1989, Wilks *et al.*, 1991, Harpur *et al.*, 1992, Firmbach-Kraft *et al.*, 1990) however, JAK3 is principally limited to hematopoietic cells (Witthuhn *et al.*, 1994, Rane and Reddy 1994, Cance and Liu 1995). These kinases are involved in signal transduction from a range of cytokines besides IFNα, such as IFNγ, IFNλ, IL-6 and others that are too broad to be discussed here. This implicates JAKs in a range of activities such as inflammation, growth, metabolism and gene expression (reviewed by Kiu and Nicholson, 2012). The importance of JAKs is demonstrated in humans who have mutations in JAK3 or TYK2. These lead to primary immunodeficiencies and increased susceptibility to infections (Minegishi *et al.*, 2006, Frucht *et al.*, 2001).

JAKs are composed seven JAK homology regions (JH) within four domains. At the N-terminus a four-point-one, ezrin, radixin, moesin (FERM) domain (Yamaoka *et al.*, 2004) is situated next to a src homology 2 (SH2)-like domain (Bernards 1991). This is followed by a pseudokinase domain and finally a kinase domain at the C-terminus (**Figure 1.9**).

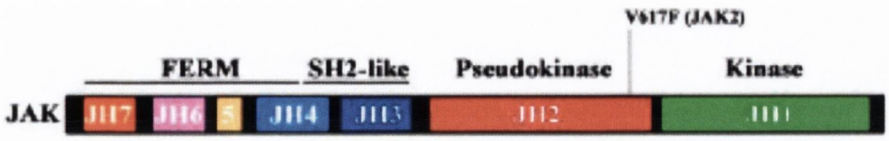


Figure 1.9 Domain organisation of the JAK protein. At the C-terminus, the JH1 region is the kinase domain while JH2 is the pseudokinase. The SH2-like domain extends over JH3 and JH4 while the FERM domain is located from JK4 to JH7 at the N-terminus (Kiu H & Nicholson SE, 2012).

The pseudokinase is involved in regulating the activity of the JH1 kinase domain. When phosphorylated, the pseudokinase domain inhibits the kinase activity of the JH1 kinase, maintaining JAKs basal inactive state (Feener *et al.*, 2004, Argetsinger *et al.*, 2004, Ishida-Takahashi *et al.*, 2006). Deletion or mutation of the pseudokinase domain gives rise to increased JAK2 and JAK3 kinase activity (Saharinen *et al.*, 2000 and 2003, Saharinen and Silvennoinen 2002).

The SH2-like domain does not retain some residues normally present in SH2 domains and appears to perform a structural role. The SH2-like domain has been shown to be required for JAK1 binding to the oncostatin M receptor (OSM-R) (Radtke *et al.*, 2005) and for TYK2 to preserve IFNAR1 surface expression (Ragimbeau *et al.*, 2003). Unlike regular SH2 domains, binding to phosphotyrosine is not necessary for its activity (Kolhuber *et al.*, 1997). The N-terminal FERM domain is involved in binding the JAK protein to the cytoplasmic domain of its associated receptor (Girault *et al.*, 1998, Cacalano *et al.*, 1999, Haan *et al.*, 2001).

The primary focus of most research on JAKs is their role in STAT activation following IFNα treatment. TYK2 and JAK1 are the only JAKs required for IFNα signalling and are bound

to the IFNAR1 and IFNAR2 respectively. Once IFNα binds to the IFNAR, TYK2 and JAK1 are rapidly autophosphorylated, leading to their activation (Schindler *et al.*, 1992, Fu XY 1992). Once activated by IFNα, JAKs then phosphorylate members of the STAT family.

1.3.4 STATs

There are seven STAT proteins: STAT1-4, 5a, 5b and 6. They are between 750 and 850 amino acids long and share a conserved structure. STAT proteins have a N-terminal domain (NT), a coiled-coil domain (CC), a DNA-binding domain (DBD), a linker region, a SH2 domain and a C-terminal transcriptional activation domain (TAD) (**Figure 1.10**). In primary human hepatocytes STATS 1,2, 3 and 5 are activated by IFNα (Radaeva *et al.*, 2002).



Figure 1.10 Domain organisation of the STAT family.(Kiu H & Nicholson SE, 2012).

The NT domain is involved in binding unphosphotylated STATs as anti-parallel homodimers (Lackmann *et al.*, 1998, Ndubuisi *et al.*, 1999, Braunstein *et al.*, 2003, Ota *et al.*, 2004, Mao *et al.*, 2005). The NT is also involved in DNA binding at tandem GAS elements (Mertens *et al.*, 2006, Vinkemeier *et al.*, 1998). The CC and DBD domains are also involved in binding to GAS elements.

The SH2 domain mediates receptor binding and dimerization following activation. This domain also provides targeted direction for the signaling cascade (Heim *et al.*, 1995).

The TAD at the C-terminal is the site of alternative splicing of gene transcripts that yields the truncated isoforms STAT1, 3, 4, 5a and 5b. Truncated isoforms lack functionality at their TAD but can still bind specific promoter sites of target genes. This creates competition with full length STATs at DNA binding sites and regulates transcriptional activation. Truncated STATs can also be involved in regulation of transcriptional activity by

forming complexes with other proteins to initiate ISG transcription (Vinkemeier *et al.*, 1996, Henriksen *et al.*, 2002, Maritano *et al.*, 2004, Yoo *et al.*, 2002, Hoey *et al.*, 2003).

1.3.5 IFNα mediates STAT Dimerisation and Nuclear Translocation

Unphosphorylated STATs constantly transfer between the cytoplasm and nucleus. STATs use their SH2 domain to interact with IFNAR phosphotyrosine residues following IFNα stimulation. JAKs then phosphorylate STATs, which then reorganize into active, parallel dimers by mutual SH2 and phosphotyrosine contacts. Once activated, STATs can uncouple from the receptor and translocate to the nucleus either as dimers or as part of a complex (Hahm *et al.*, 2005, Perry *et al.*, 2011) (**Figure 1.11**). However, as STAT complexes are larger than 50kDa they are therefore too large to passively translocate through the nuclear pore complex into the nucleus (Gorlich D and Kutay U 1999, Macara IG 2001) and require nuclear chaperones to enter and exit the nucleus (Onischenko and Weis 2011, Meyer and Vinkemeier 2004).

Phosphorylation of STAT1 and STAT2 results in the IFNAR2 recruiting cAMP-responsive-element-binding-protein binding protein (CBP). CBP then catalyzes IFNAR2 acetylation. This creates a docking site for interferon regulatory factor 9 (IRF9) that then binds and is itself then acetylated along with receptor bound STAT1 and STAT2 (Tang *et al.*, 2007). STAT1 and STAT2 then dimerise and recruit IRF9 to form the IFN stimulated gene factor 3 (ISGF3) complex.

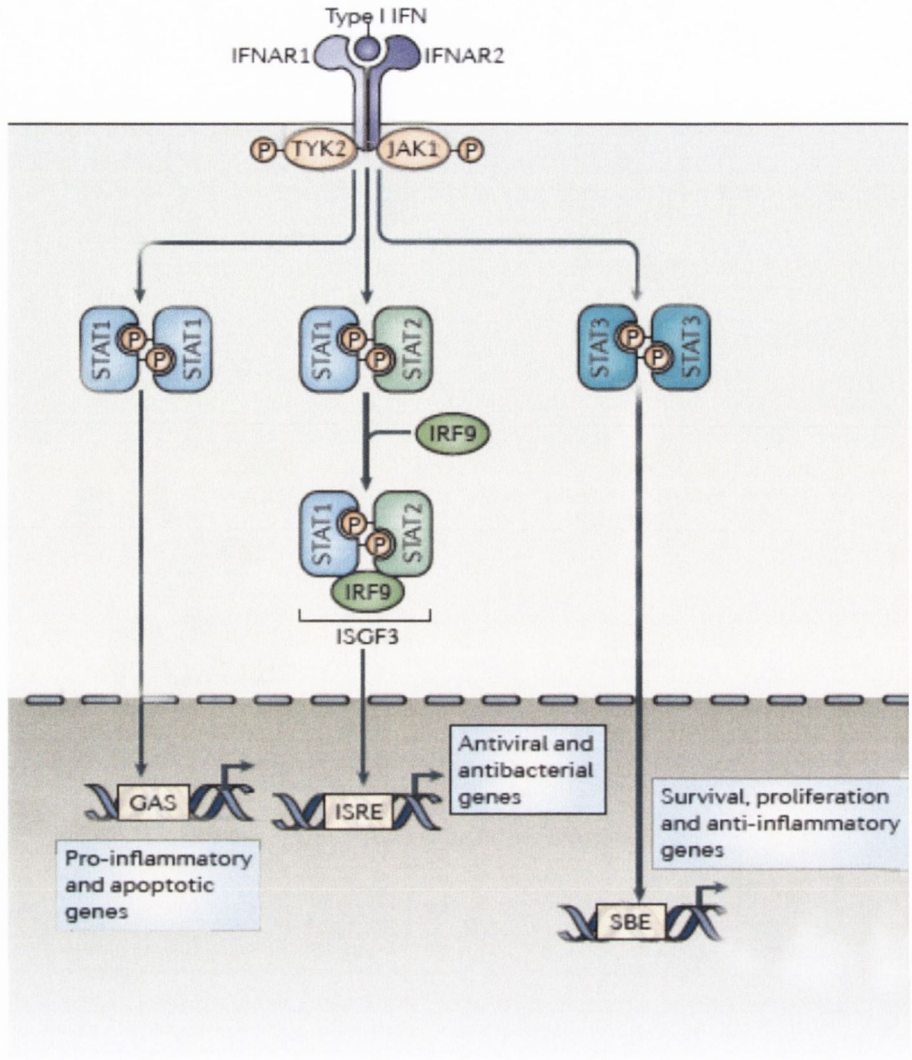


Figure 1.11 STAT Complexes and their binding elements in ISG promoters. Figure adapted from González-Navajas *et al.*, 2012.

Once STAT1 and STAT2 phosphorylate and dimerise, their nuclear localisation signal (NLS) is unmasked and binds importin α5. Importin α5 is a chaperone for the STAT dimer and actively transports the dimer into the nucleus (Sekimoto *et al.*, 1997, Melen *et al.*, 2001, Fagerlund *et al.*, 2002). Unphosphorylated STAT (U-STAT) 1 and 2 can also transport into the nucleus by directly binding nucleoporins on the nuclear envelopes or by binding other proteins that contain NLS. However, the nuclear localisation levels of U-STAT1 or 2 are lower than that of phosphorylated STAT1 or 2 (Banninger and Reich 2004, Marg *et al.*, 2004). Following ISGF3 transport into the nucleus it binds the IFN stimulated

response elements (ISREs). These elements are located in the promoters of various ISGs and initiate gene transcription following ISGF3 binding (Matikainen *et al.*, 1999, David M 2002). ISGF3 can further induce IRF9 expression, creating a positive feedback loop important in IFNα signalling (Bluyssen *et al.*, 1996).

Activated STAT1 and STAT3 can form homodimers or heterodimers with each other. In contrast, STAT2 does not form homodimers but can heterodimerise with STAT1 or STAT3. These dimers bind the IFNγ-activated site element (GAS) to induce transcription of ISGs with these elements in their promoters (Darnell *et al.*, 1994, Darnell 1997, Sato *et al.*, 1997). STAT3 homodimers can also bind STAT3-binding elements (SBEs) (Heinrich *et al.*, 1998). STAT3 is transported into the nucleus by importin α3 or occasionally importin α6, depending on cell type. Tyrosine phosphorylation is not required for STAT3 nuclear transport as its NLS is constitutively available in its CC domain for importin binding. Once imported, STAT3 recruitment of binder of ADP-ribosylation factor-like 2 (BART) is required for nuclear retention (Muromoto R *et al.*, 2008). This results in a prominent nuclear presence of STAT3 before and after tyrosine phosphorylation (Kohler *et al.*, 1997, Liu *et al.*, 2005).

1.3.6 STAT Induces ISG Transcription

Following nuclear import, STAT dimers or complexes bind ISRE or GAS elements to induce ISG transcription. Some ISGs contain only ISREs or GAS elements in their promoters while others contain both elements. This gives rise to the possibility that different combinations of these elements are required for individual gene transcription activation. All STATs can bind a palindromic element with a central $TTCN_{2\text{-}4}GAA$ consensus sequence (Horvath *et al.*, 1995). The length of the inner nucleotide sequence determines which STAT binds, with STATs 1, 3 and 4 favouring 3 nucleotides and STAT6 favouring 4 nucleotides (Ehret *et al.*, 2001).

Moreover, phosphorylation of STATs at serine 727 (Ser727) in the TAD amplifies STAT induced gene transcription allowing for full ISG expression (Boulton *et al.*, 1995, Kovarik *et al.*, 1998 and 1999, Goh *et al.*, 1999, Visconti *et al.*, 2000). Mice with serine replaced with

alanine at this position show reduced ISG expression from macrophages and increased mortality following *Listeris monocytogenes* infection (Varinou *et al.*, 2003).

Nuclear co-activators also play an important role in STAT mediated ISG transcription. Following STAT phosphorylation at Ser727, CBP, p300 and minichromosome maintenance deficient 5 (MCM5) can be recruited to STATs (Zhang JJ *et al.*, 1996, Bhattacharya S *et al.*, 1996, Zhang JJ *et al.*, (1998); DaFonseca *et al.*, 2001). CBP and p300 can increase transcription by chromatin remodeling through their histone acetyltransferase activity (Hebbes *et al.*, 1988). STAT1 can also interact with the methyltransferase Disrupter of Telomere Silencing 1 Like (DOT1L) that is localized at the *IRF1* gene *in vivo*. IFNγ mediated STAT1 DNA binding and IRF1 expression is reduced 2-fold when DOT1L is diminished in cell culture (Shah and Henriksen). Transcription can also be increased by STAT2 associating with chromatin-remodelling factor brahma-related gene 1 (BRG1) and transcriptional co-activator general control non-depressible 5 (GCN5) (Paulson *et al.*, 2002, Huang *et al.*, 2002).

Following ISG transcription, STAT complexes detach from DNA and return to an antiparallel conformation. The phosphotyrosine residues at the C-termini are then exposed to phosphatase activity. Following DNA detachment and dephosphorylation, STATs are then ready to be exported from the nucleus (Zhong *et al.*, 2005, Mertens *et al.*, 2006). This is achieved by STAT nuclear export signal recruiting the nuclear export factor, chromosome region maintenance 1 (Haspel *et al.*, 1996, Begitt *et al.*, 2000, McBride *et al.*, 2000, Mowen and David 2000, Zeng *et al.*, 2002, Bhattacharya and Schindler 2003, Meyer *et al.*, 2003, Banninger and Reich 2004, Marg *et al.*, 2004).

1.3.7 Regulation of JAK/STAT signalling

Due to the variety and complexity of JAK/STAT signalling it is no surprise that there are a number of mechanisms capable of regulating this signalling cascade. There are three main categories for regulation: direct inhibition by other proteins, dephosphorylation and receptor internalisation.

1.3.7.1 Suppressors of cytokine signalling and protein inhibitors of activated STATs

Direct inhibition of JAK/STAT activity is well characterised, with most focus on the suppressors of cytokine signalling (SOCS) and protein inhibitors of activated STATs (PIAS) families. The SOCS family has eight members, SOCS1-7 and cytokine-inducible SH2-containing protein (CIS). Expression of these proteins is induced by cytokine signalling and creates a negative feedback loop by blocking JAK and STAT phosphorylation and activation (Starr *et al.*, 1997, Hilton *et al.*, 1998). These proteins have a conserved C-terminus SOCS box motif, a central SH2 domain and a variable NT region. The SOCS box interacts with elongin B and C to recruit Cullin5 and RING-box2 (RBX2) to form an E3 ubiquitin ligase complex (Babon *et al.*, 2009). This leads to the ubiquitination and degradation of SOCS interacting proteins (Kamuraet *et al.*, 1998, Zhang *et al.*, 1999 and 2001). TATA Element Modulatory Factor (TMF) / androgen receptor-coactivator 160 kDa (ARA160) (TMF/ARA160), a Golgi resident protein, can interact with STAT3 but not STAT1 following their ubiquitination and direct their proteasomal degradation (Perry *et al.*, 2004). Furthermore, SOCS 1 and 3 can bind to IFN receptors and inhibit JAK activity via a kinase inhibitory region (Sasaki *et al.*, 1999, Nicholson *et al.*, 1999, Endo *et al.*, 1999, Fenner *et al.*, 2006, Qing *et al.*, 2005). SOCS proteins can also compete with STAT for receptor binding (Matsumoto *et al.*, 1997, Ram and Waxman 1999 and 2000, Greenhalgh *et al.*, 2002). Loss of SOCS1, 2 or 3 leads to excessive STAT activity (Croker *et al.*, 2003 and 2004, Kimura *et al.*, 2004).

The PIAS family are E3 small ubiquitin-like modifier (SUMO) ligases and is composed of four members, PIAS1, PIASx, PIAS3 and PIASy, with other isoforms formed by alternative splicing. They were originally identified when overexpression of PIAS1 and 3 inhibited STAT1 and 3 DNA binding following cytokine stimulation (Chung *et al.*, 1997, Liu *et al.*, 1998). Inhibition can be achieved by the zinc binding motif of PIAS1 binding to the N-terminal of STAT1 (Liao *et al.*, 2000). PIAS x and y inhibit STAT1 and 4 via a non-DNA binding mechanism (Liu *et al.*, 2001, Arora *et al.*, 2003).

1.3.7.2 STAT Dephosphorylation, Degradation & Receptor Internalisation

Tyrosine phosphatases including SH2 domain-containing phosphatases (SHP) 1 and 2 and protein tyrosine phosphatases (PTP) 1B can target JAK/STAT signalling at the receptor complex. SHP1 interacts with IFNAR to inhibit JAK1 phosphorylation. SHP2 can also inhibit JAK1 but can also target STAT1 and STAT5a for dephosphorylation. (David *et al.*, 1995, Klingmuller *et al.*, 1995, Chen *et al.*, 2005, Wuet *et al.*, 2002). Importantly for IFNα signaling, PTP1B can dephosphorylate both JAK1 and TYK2 (Myers *et al.*, 2001). The phosphatase and tensin homologue (PTEN) is a pro-apoptopic tyrosine phosphatase. In tissue samples of human laryngeal papillomas, STAT3 tyrosine phosphorylation was shown to be directly inverse to the presence of PTEN. Analogous to this, HeLa cells showed a reduction in STAT3 phosphorylation in a dose-dependent manner following PTEN cDNA transfection (Sun and Steinberg, 2002).

Following IFNα treatment it has been demonstrated that IFNAR2 undergoes proteolytic cleavage. This results in transmembrane stub and an intracellular domain (ICD) being produced. The ICD then translocates to the nucleus where it represses gene transcription by recruiting histone deacetylase (Saleh *et al.*, 2004).

1.3.8 Non-JAK/STAT IFN Signaling Pathways

Various signaling cascades are initiated once IFN binds its receptor. Classically this is the JAK/STAT pathway, however, other pathways such as the MAPK, PI3K and Crkl pathways can also become activated (Platanias, 2005).

Mitogen activated protein kinases (MAPKs) are serine-threonine kinases that are broadly expressed in mammalian cells (Schaeffer and Weber 1999, Platanias 2003). The MAPK pathways p38 and extracellular signal-regulated kinases (ERK) are activated by type I IFN (David *et al.*, 1995, Uddin S *et al.*, 1999). p38 is required for gene transcription of ISGs with ISRE or GAS elements independently of the JAK/STAT pathway (Uddin *et al.*, 2000).

Type I IFN can activate phosphoinositide 3-kinase (PI3K) and induce gene transcription through the insulin receptor substrate (IRS) signaling pathway. PI3K initiates ISG

translation by activating FKBP 12-rapamycin-associated protein/mammalian target of rapamycin (FRAP/mTOR), which then phosphorylates 4E-BP1, an mRNA translation repressor. Phosphorylated 4E-BP1 dissociates from the mRNA translation initiator eIF-4E, ceasing for its repression (Lekmine *et al.*, 2003). This demonstrates that the IFN activated PI3K pathway is complementary to JAK/STAT signalling as it allows for mRNA translation of JAK/STAT induced ISGs.

Type I and II IFNs can activate CrkL, an adaptor protein that can activate the small guanine tri-phosphatase (GTPase) Rap1. This can interfere with the Ras pathway and induce growth suppressive effects (Ahmad *et al.*, 1997, Alsayed *et al.*, 2000). Type I IFN can also induce CrkL to act as a nuclear adaptor for STAT5 by forming a CrkL:STAT5 complex that can then bind to ISG promoters (Fish *et al.*, 1999, Grumbach *et al.*, 2001).

1.3.9 Interferon Stimulated Genes

Following activation of the JAK/STAT pathway, a select group of genes are induced depending on the type of IFN that initiated the signalling cascade. Types I and III IFNs stimulate ISGs by binding to the ISRE elements while type II IFNs bind the GAS element to induce gene expression.

The small number of ISGs that were first reported 25 years ago (Knight and Korant 1979, Larner *et al.*, 1984) has now grown to a group containing hundreds of genes (Der *et al.*, 1998, de Veer *et al.*, 2001). Although each type of IFN induces a unique set of ISGs there is some commonality between each group (Der *et al.*, 1998). Individual ISGs can be broadly classified into having antiviral or anti-proliferative functions (van Boxel-Dezaire *et al.*, 2006, Schoggins *et al.*, 2011, Friedman, 2008). When investigating the JAK/STAT pathway, the ISGs most often examined are protein kinase R (PKR), 2′,5′-oligoadenylate synthetase (2'-5' OAS) and Myxovirus resistance protein 1 (MxA). Polymorphisms in these genes have been identified as influencing the severity of HCV infection (Knapp *et al.*, 2003).

PKR is expressed as an inactive monomer at a low basal level and is induced by type I

and III IFNs (Ank *et al.*, 2006, Gelev *et al.*, 2006, Nanduri *et al.*, 2000). PKR is a dsRNA-dependent serine/threonine kinase that recognises dsRNA through its two RNA binding motifs (RBM) at its N-terminal. For maximum activation dsRNA should be longer than 30 base pairs so both RBMs can be occupied (Nallagatla *et al.*, 2007). PKR phosphorylates eukaryotic translation initiation factor 2α (eIF2α) upon activation. Phosphorylated eIF2α then binds the limiting guanine nucleotide exchange factor EIF2β. This inhibits recycling of GDP, preventing the translation of most cellular and viral mRNAs, therefore blocking further viral replication (Roberts *et al.*, 1976, Zilberstein *et al.*, 1978). PKR can also degrade IκB, which then allows for the activation of NFκB, and therefore induce IFNβ production (Kumar *et al.*, 1994). Due to its antiviral properties many viruses undermine PKR activity to avoid host defences (Gale & Katze, 1998).

2'-5' OAS gene expression is highly induced by IFNα, while its enzymatic property can be activated by dsRNAs or HCV. Active 2'-5' OAS activates the ribonuclease L (RNaseL) to dimerises and form an endoribonuclease that can cleave viral and host ssRNA (Kerr & Brown 1978, Zhou *et al.*, 1993, Dong & Silverman, 1995, Wreschneret *et al.*, 1981, Floyd-Smith *et al.*, 1981). Inducing the cleavage of host mRNA and rRNA also impairs host machinery required for viral replication and can also lead to apoptosis. Cleaved RNA can then be used to activate RIG-I and MDA-5, leading to induction of IFNβ production (Castelli *et al.*, 1997, Castelli *et al.*, 1998, Malathi *et al.*, 2007).

MxA is a cytoplasmic large GTPase from the dynamin superfamily and has a molecular mass of 75kDa. It is composed of 3 domains, an N-terminal GTPase domain, a middle interacting domain for oligomerisation and a C-terminal GTPase effector domain for target recognition (Zürcher *et al.*, 1992, Johannes *et al.*, 1997). Once expressed, MxA self assembles into highly ordered helical oligomers that are constitutively active (Kochs *et al.*, 2002, Accola *et al.*, 2002). MxA binds viral nucleocapsids to interfere with the intracellular trafficking and function of viral polymerases (Kochs and Haller 1999). This can impede replication of several RNA viruses (Accola *et al.*, 2002, Haller *et al.*, 2007), highlighted by the fact that a polymorphism in MxA is associated with an increased sensitivity to HCV and HBV infection (Hijikata *et al.*, 2000, Suzuki *et al.*, 2004).

1.4 HCV PERSISTENCE

1.4.1 Factors for Non-Response to Treatment

The usual outcome of HCV infection is persistence, which occurs in approximately 80% of infected individuals. A sustained virological response (SVR) is defined as undetectable HCV RNA in serum after 24 weeks of post-treatment cessation follow-up (Asselah et al., 2009). A SVR marks the abolition of HCV infection and a histological improvement (Maylin et al., 2008). There are several factors that influence SVR rates and viral persistence in patients infected with HCV. These can be broadly classed into two groups: viral factors and host factors and are reviewed by Thompson 2012 and Asselah T et al., 2010.

1.4.1.1 Host Factors

Several host factors are important in influencing SVR rates. African ethnicity, male gender, older age, higher weight and steatosis are associated with poor SVR rates (Asselah *et al.*, 2006, Moucari *et al.*, 2008), as is insulin resistance, excessive alcohol intake and co-infection with HIV or hepatitis B virus (Alberti 2009, Romero-Gomez *et al.*, 2005, Khattab *et al.*, 2009).

The role ethnicity plays in predicting rates of SVR is due to genetic variations that are being resolved by human disease genome-wide association studies (GWAS). Single nucleotide polymorphisms (SNPs) of the *IL28B* gene on chromosome 19 coding for IFNλ3 strongly predict HCV treatment outcomes. Those with variants associated with positive outcomes have a SVR twice that of those with variants associated with negative outcomes (Ge *et al.*, 2009, Tanaka *et al.*, 2009, *et al.*, Suppiah; 2009, Rauch *et al.*, 2010). The frequencies of these responses can be grouped into ethnicity with the best responses in Asians > Caucasians > Hispanics > African populations (Ge *et al.*, 2009, Thomas *et al.*, 2009). Positive variants are also more common in healthy controls compared to individuals chronically infected with HCV, consistent with genetic selection. Furthermore, those with positive variants are twice as likely to spontaneously clear HCV (Rauch *et al.*, 2010, Thomas *et al.*, 2009, Grebely *et al.*, 2010, Tillmann *et al.*, 2010). Despite these associations, the mechanism by which *IL28B* variation influences viral decline and SVR

remains unclear.

Additionally, HCV patients that do not respond to IFN therapy show higher expression of protein phosphatase 2A (PP2A). This inhibits JAK/STAT signalling by increasing protein inhibitor of activated STAT1 (PIAS1) binding to STAT1 (Heim MH *et al.*, 1999, Duong *et al.*, 2004). It has also been noted that some patients infected with HCV show increased production of cytokines and levels of inhibitory receptors that can reduce the immune response. For example, TGFβ and IL-10 expression levels are increased in NK cells of patients with chronic HCV (Jinushi *et al.*, 2004).

1.4.1.2 Virological Factors

HCV has various methods of counteracting the immune response. Patients with high viral load before treatment (>2,000,000 copies/ml) do not respond as well as those with a lower load (Jensen *et al.*, 2006, Zeuzem *et al.*, 2006, von Wagner *et al.*, 2005, Berg *et al.*, 2003, Martinot-Peignoux *et al.*, 1998, Martinot-Peignoux *et al.*, 1995, Marcellin *et al.*, 1995). Furthermore, HCV quasispecies may occur in patients due to error prone replication by the virus, giving rise to variants that are able to escape treatment. Mutations in two regions of the NS5A, the IFN sensitivity-determining region (ISDR) and the IFN/ribavirin resistance-determining region (IRRDR) are associated with higher SVR rates. (Zeuzem *et al.*, 1997, Enomoto *et al.*, 1996, El-Shamy *et al.*, 2008). The NS5A of non-responding patients contains an ISDR that interacts with PKR. ISDR binding with PKR prevents PKR dimerisation and its ability to phosphorylate targets such as eIF2α and IκB (Gale *et al.*, 1998).

HCV can hamper the host antiviral response by interrupting the RIG-I and TLR3 pathways, therefore preventing IFN β expression. The HCV NS3/4A can target blocking of type I IFN production by interruption of RIG-I antiviral signalling. NS3/4A does this by the dissociation of the mitochondrial antiviral signaling (MAVS) adaptor protein from the mitochondrial membrane. This prevents the activation of the IFNα/β transcription factor IRF-3. MAVS dissociation also prevents its oligomerisation and activation of NF-κB (Baril *et al.*, 2009). NS3-4A is also able to target TLR3 induction of type I IFN by cleaving the TLR3 signaling

adaptor protein TRIF (Foy *et al.*, 2003, Meylan *et al.*, 2005, Sen & Sarkar 2005, Li K *et al.*, 2005, Li XD *et al.*, 2005). In HCV-replicon cells, NS5A and NS5B can suppress the expression of IRF-7 but not IRF-3, at a transcriptional and protein level. This leads to an inhibition of IRF-7 mediated IFNα promoter activation and IFNα expression. IFNα production can be restored in cells that have been cured of HCV by IFNα treatment (Zhang T *et al.*, 2005).

HCV can also disrupt the JAK/STAT signalling cascade by inducing the activation of the SOCS3, in order to prevent ISG transcription (reviewed by Asselah T *et al.*, 2010). HCV core protein induces expression of mRNA for the JAK-STAT inhibitors SOCS3 and SOCS7 in hepatocytes and also inhibits the induction and function of ISGF3 in various cell types (Basu A *et al.*, 2001, Bode *et al.*, 2003, Pazienza *et al.*, 2010).

Disruption of ISG function is another target for HCV immune evasion. E2 is believed to act as a competitive inhibitor to PKR and eIF2α activation through an identical phosphorylation site. This then prevents the suppression of protein production in the cell, including viral proteins (Taylor DR *et al.*, 1999, Garaigorta & Chisari, 2009). PKR is also targeted by the IRES of HCV genomic RNA. The genomic RNA competes with dsRNA to bind the dsRNA-binding domain of PKR and prevent its autophosphorylation and activation (Vyas J *et al.*, 2003). HCV core protein can also suppress synthesis of IRF-1. The loss of IRF-1 in turn then represses the expression of various ISGs such as IL-15, IL-12 and low-molecular-mass polypeptide 2 (Ciccaglione *et al.*, 2007).

1.4.2 HCV targets PKCβ function.

Another method used by HCV to avoid the effects of the immune system is inhibition of T-cell migration and cytokine secretion. One mechanism of T-cell polarity, migration and secretion of various cytokines requires Protein Kinase C (PKC) β interaction with the centrosome (Volkov *et al.*, 2001, Murphy *et al.*, 2000). In peripheral blood mononuclear cells (PBMC) leukocyte function-associated molecule-1 (LFA-1) signal induces the polarity, migration and cytokine secretion via PKCβ activity. However, PBMCs pre-treated with HCV-E2 protein show PKCβ translocation from the cytosol to lipid rafts within the cell membrane. This translocation prevents PKCβ from interacting with the centrosome in LFA-1 activated cells and thereby inhibits cell polarity, migration and secretion of macrophage inflammatory protein (MIP)-1α and MIP-1β. However, this HCV-E2 induced inhibition of polarity and migration can be prevented by pre-treatment with a peptide known to block HCV-E2 binding to CD81 (Volkov *et al.*, 2006).

IL-2 secretion by $CD4^+$ T-cells is an indicator of future SVR, while patients who are chronically infected with HCV show significantly lower levels of secreted IL-2 compared to other inflammatory liver diseases (Semmo *et al.*, 2005, Gerlach *et al.*, 1999). IL-2 secretion from anti-CD3 stimulated PBMCs was inhibited when PBMCs were pre-incubated with serum from HCV infected patients or with cell culture grown HCV particles. Furthermore, HCV-E2 was also shown to be capable of blocking the secretion of IL-2 in PMA treated cells from the HuT 78 T-cell line and from anti-CD3/anti-CD28– stimulated PBMCs. However, production of IL-2 mRNA is not significantly affected in these cells. This inhibition of secretion but not transcription was also the case for IFNγ. Further investigation revealed that the HCV-E2 induced translocation of PKCβ from the cytosol to lipid rafts within the cell membrane in HuT 78 cells, disabled cellular secretory mechanisms and prevented IL-2 secretion. (Petrovic *et al.*, 2011). This suggests that HCV specifically targets cytokine release from T-cells by inhibiting the activity of PKCβ.

As HCV can target the activity of PKCβ in T-cells, our group has investigated the possibility that it may do the same in hepatocytes. PKCβ is a member of the classical PKC subfamily that has been shown to be required for IFNα induced JAK/STAT signalling and HCV replicon clearance in hepatocytes (Fimia *et al.*, 2004). Unpublished results from our

laboratory show that anti-CD81 treatment and HCVcc infection is able to translocate PKCβ to lipid rafts in hepatocytes. Our laboratory has also demonstrated that PKCβ is specifically required for IFNα induced STAT3 tyrosine phosphorylation and that hepatocytes pre-treated with anti-CD81 or HCV-E2 have reduced IFNα induced STAT tyrosine phosphorylation (Mullins *et al.*, unpublished). These results indicate that HCV is able to target PKCβ in hepatocytes in order to evade the IFNα mediated antiviral response. However, the role of other PKC isoforms in IFNα stimulated JAK/STAT signalling and HCV immune escape has yet to be fully investigated.

1.5 PROTEIN KINASE C

1.5.1 PKC Introduction and Structure

The PKC family of enzymes are serine/threonine kinases. In all, ten PKC isotypes have now been described (Steinberg, 2008, Newton, 2010). PKC expression is ubiquitous, although isotypes expression can vary between tissues. Based on their structure, PKCs are divided into three subclasses: classical, novel and atypical (**Figure 1.12**). The classical (cPKC) are PKC α, β_I, β_{II} and γ. They are sensitive to the phospholipid diacylglycerol (DAG) and phosphotidylserine (PS) via their C1 domain and calcium via their C2 domain. The novel (nPKC) isoforms are PKC δ , ε, η and θ. These are also sensitive to DAG and PS but have no calcium interacting residues in their C2 domain. The atypical (aPKC) PKCs ζ and ι/λ have altered C1 domains and are regulated by PS at its N-terminal phosphatidylserine-binding (PB1) domain. They do not require DAG or calcium for activity.

The structure of PKCs can be divided into an N-terminal regulatory domain and a C-terminal catalytic kinase domain, separated by a variable hinge region. Although the overall PKC structure is similar between isotypes, differences between subclasses occur at the regulatory domain.

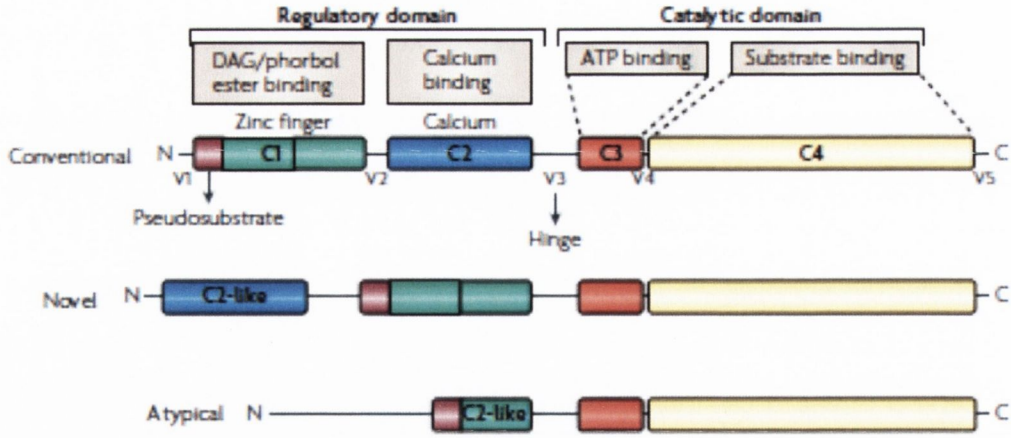


Figure 1.12 Structure of PKC subclasses. PKC has 4 conserved domains (C1-4). C1 contains DAG and phorbol ester binding sites. C2 can recognize acidic lipids and can bind calcium in cPKCs. The variable hinge domain (V3) separates C2 from C3, which has an ATP binding site while C4 binds substrates. (Mackay and Twelves, 2009).

The regulatory N-terminal contains conserved C1, C2 and PB1 domains responsible for binding lipid second messengers or other activating proteins and for tethering the protein to its subcellular location. The N-terminal also contains a pseudosubstrate sequence, where an alanine has replaced the serine/threonine residue. The pseudosubstrate sequence occupies the substrate-binding region of the C-terminus when the PKC is in its inactive state. This represses kinase activity until it is removed following PKC activation (Blumberg 1991, House and Kemp 1987).

The C-terminal contains the ATP-binding C3 domain and the C4 domain that is required for substrate binding. These domains cooperate to transfer phosphate from the ATP to the serine/threonine residue of its target substrate (Schmitz-Peiffer C and Biden TJ, 2008).

Between the N and C terminals lies a variable hinge domain (V3). Activation of PKCs leads to the loss of inhibition through the release of the pseudosubstrate sequence that normally binds the active site. PKC changes its structure by unfolding to an open conformation, with the hinge region its apex. Phosphorylation at a TFCGT motif in their activation loop is also required for optimal catalytic productivity of all PKCs (Newton, 1995,

Nishizuka, 1992, Schenk & Snaar-Jagalska, 1999, Parekh *et al.*, 2000). Following activation it is possible that the hinge region can be cleaved by calpain or caspases to create a constitutively active kinase once it is phosphorylated at its activation-loop (Smith and Smith, 2002).

1.5.2 PKC Function

PKCs are involved in a wide range of cellular activities. However, elucidating the specific roles of each isotype can prove difficult due to functional redundancy between PKC isotypes. It is also possible that the same PKC isoform performs opposite roles in different cell types. For example expression of catalytically active PKCα constructs progress the cell cycle by inducing the expression of cyclin D1 (Soh and Weinstein, 2003). However, in intestinal epithelial cells PKCα signalling resulted down-regulation of cyclin D and an increase in expression of $p21^{waf1/cip1}$ and $p27^{kip1}$, withdrawing the cells from the cell cycle and into G0 phase (Frey *et al.*, 2000). Atypical PKCs such as PKCζ are key regulators of polarity and cell contact maturation (Suzuki and Ohno, 2006). aPKCs regulate cell polarity through an evolutionarily conserved system by forming complexes with PAR 3 (or its mammalian homologue aPKC-specific interacting protein (ASIP) depending on the cell type examined) and PAR-6 which in turn regulate each others activity (Macara, 2004, Nagai-Tamai *et al.*, 2002, Ohno, 2001).

PKC localisation can be influenced through their interactions with scaffold and adaptor proteins. These proteins can anchor the PKCs to distinctive regions within the cell. Substrates that interact with C-kinases (STICKs) are phospholipid binding proteins that require kinase:substrate interaction for optimal action, whereas receptors for inactive C-kinases (RICKs) can function before activation of PKC and control substrate access and localisation. Receptors for activated C-kinases (RACKs) bind following PKC activation and control substrate access (Mochly-Rosen and Gordon, 1998, Jaken and Parker, 2000).

1.5.3 Regulation of PKC Activity

For full PKC activation, priming phosphorylation at three sites in the C-terminus must occur (Cazaubon *et al.*, 1994, Orr and Newton, 1994, Tsutakawa *et al.*, 1995, Newton *et al.*, 1995). These sites are the activation-loop (A-loop), the turn-motif (TM) and the hydrophobic-motif (HM). These three sites are conserved between all PKCs, except for aPKCs, which do not have a HM residue. Following ATP binding pocket occupation, phosphorylation is constitutive (Cameron *et al.*, 2009) and allows the PKC to form a stable, closed conformation with the pseudosubstrate blocking access to the substrate-binding site until it is activated (Gallegos and Newton, 2008).

Activation of cPKC or nPKCs generally involves PKC translocation to membranes to allow access to PS and allosteric activation by DAG or phorbol esters. DAG can be produced following activation of various receptors such as G-protein-coupled receptors, receptor tyrosine kinases and non-receptor protein tyrosine kinases. These can induce activation of phospholipase C (PLC). PLC hydrolyses the membrane-localised inositol phospholipid PtdIns(4,5)P_2 into Ins(1,4,5)P_3 and DAG, which then activates PKC (Inoue *et al.*, 1977, Kishimoto *et al.*, 1980). For cPKCs the initial step in membrane recruitment is calcium dependent, however, no such corresponding mechanism had been described for nPKCs (Newton 1995, Nishizuka 1992). For aPKCs PS can be sufficient for their activation as they do not require calcium or DAG (Chauhan *et al.*, 1990). aPKCs can also be activated by a range of other proteins and lipids such as PI3K (Naranatt *et al.*, 2003, Montiel *et al.*, 2006), Diacylglycerol kinase α (Kai *et al.*, 2009) and ceramide (Muller *et al.*, 1995, Wang *et al.*, 2009).

Once DAG is removed, cPKCs and nPKCs are deactivated and return to the cytosol. DAG can be removed by down-regulation or degradation of receptors and by conversion of DAG to phosphatidic acid by DAG kinase (Merida *et al.*, 2008). Cellular PKC levels can also be reduced. This is often initiated by the dephosphorylation of the PKC at various sites, decreasing its structural stability (Lee *et al.*, 1997, Hansra *et al.*, 1999). aPKC activity can be directly inhibited by binding prostate apoptosis response-4 (Par-4) or partitioning defective gene-6 (PAR-6) (Kotani *et al.*, 2000, Joberty *et al.*, 2000) which blocks their interaction with activating signals.

1.5.4 The role of PKC isoforms in liver disease

In HCC tissue, the transcription of PKCβ and PKCθ is statistically reduced compared to near-by non-cancerous tissue (Lu *et al.*, 2010). This was measured by real-time quantitative PCR (RT-qPCR) and confirmed by immunohistochemical staining. The reduced protein expression of PKCθ correlated with the grade of cancer cells while $PKC\beta_{II}$ reduction was associated with HCC that originated due to Hepatitis B virus (HBV) infection but not HCV infection. These results are in contrast to findings deposited in the Human Protein Atlas database, which show no presence of PKC β, θ, ε or ι and demonstrate that this database is not as yet categorical.

PKCs have been implicated in other liver diseases such as progressive familial intrahepatic cholestasis 1 (PFIC1) (Chen *et al.*, 2010). PFIC1 is a cholestatic liver disease caused by a reduction in canicular excretion of bile salts due to mutations in the ATPase Class I Type 8B Member 1 gene (FIC1). FIC1 silencing in primary human hepatocytes leads to a reduction in bile salt export pump (BSEP) promoter activity and protein. Reduction of BSEP expression leads to clinically observed cholestasis. Activation of the bile acid binding nuclear receptor Farneosid X Receptor (FXR) is required for expression of BSEP. In primary human hepatocytes, PKCζ has been shown to be required for FXR mediated BSEP expression following FXR ligand stimulation. PKCζ silencing or inhibition reduced BSEP promoter activity, FXR protein levels and redistributed remaining FXR from the nucleus to the cytoplasm. Conversely over-expression of PKCζ showed an increase in BSEP promoter activity. This demonstrates the role PKCζ can play cholestatic liver disease.

1.6 THESIS HYPOTHESES

HCV is successful in establishing chronic infection and evading the host defence. It is able to utilise host factors in order to enter cells, replicate and spread. It can also manipulate different cellular mechanisms involved in the immune response in order to escape antiviral activity. Consequently, the host immune response and Peg- IFNα therapy is often unsuccessful at clearing infection from certain HCV genotypes.

IFNα activation of the JAK/STAT signalling pathway is a central component of clinical treatment for HCV infection. In hepatocytes, cPKC activity is required for IFNα induced STAT activation, ISG expression and HCV RNA degradation (Fimia *et al.*, 2004). In a number of other cell types STAT signalling activity requires activation of various PKC isotypes, including PKC δ and ε, (Uddin *et al.*, 2002, Venkatesan *et al.*, 2006). Furthermore, it has been shown in T-lymphocytes that the HCV-E2 protein can stimulate the translocation of PKCβ to lipid rafts thus preventing LFA-1 induced T-cell migration and cytokine release (Volkov *et al.*, 2006). Using the HCVcc system it has been shown that HCV specifically prevents the release of IL-2, while HCV-E2 has been shown to prevent PKCβ activity, thereby inhibiting IL-2 secretion from Hut78 cells (Petrovic *et al.*, 2011). These studies demonstrate the importance of the PKC family in IFNα mediated STAT activation and that HCV can target PKC activity in order to evade the host response. However, the complete range of PKCs present in hepatocytes has not been conclusively described and the role of PKCs δ and ε in hepatic IFNα induced STAT activation has yet to be determined (**Figure 1.13**).

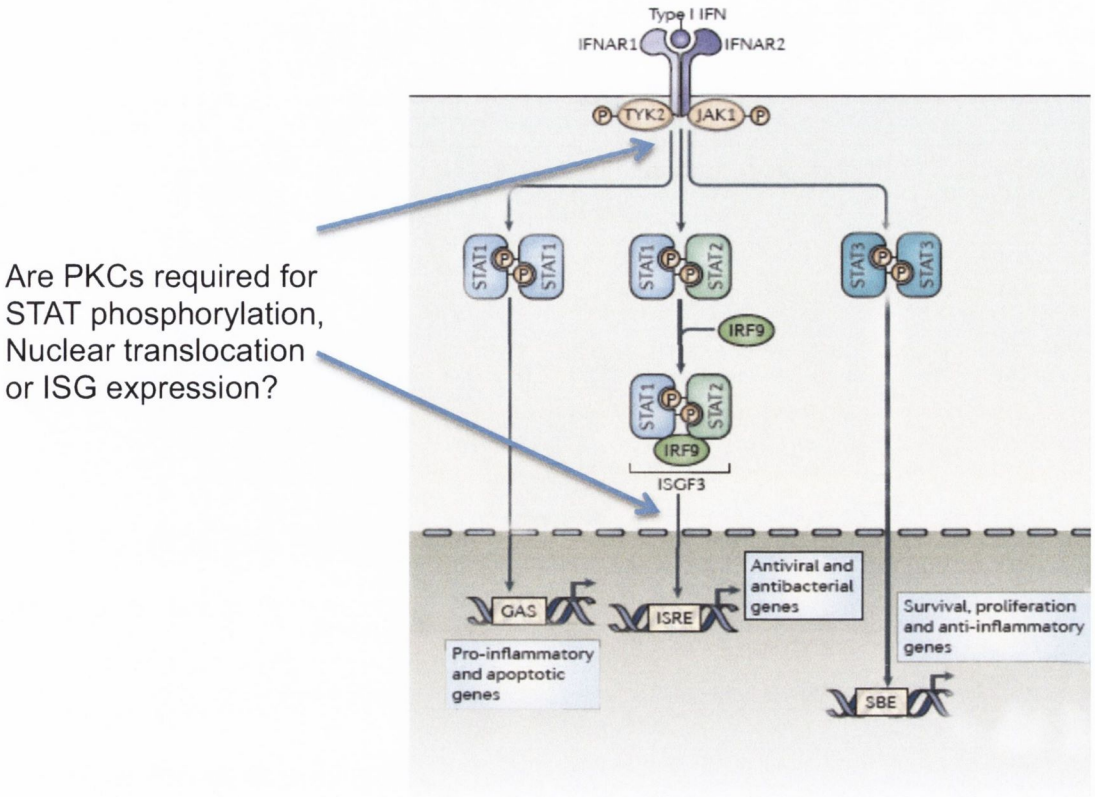


Figure 1.13. The current model of IFNα mediated Jak/STAT signalling in hepatocytes. IFNα binds its receptor and Jak kinases phosphorylate STAT proteins, which then dimerise and translocate to the nucleus. ISG expression is then induced. The requirement for novel PKC isotypes in this process must be determined. The PKC isotypes expressed in Huh7 hepatocytes must first be identified though. Figure adapted from González-Navajas *et al.*, 2012.

Atypical PKCs have been shown to direct the establishment of epithelia-specific junctional structures and cell:cell contact (Suzuki *et al.*, 2001, Nunbhakdi-Craig *et al.*, 2002). These structures are composed of proteins such as claudin-1 and occludin, which are also host factors required for HCV cell entry and replication. This gives rise to the possibility that atypical PKCs are required for hepatocyte cell:cell contact and may be required for HCV replication in hepatocytes and that regulating their activity could inhibit HCV infection by blocking its entry to hepatocytes (**Figure 1.14**).

Are aPKCs required for cell:cell contact and viral replication in hepatocytes?

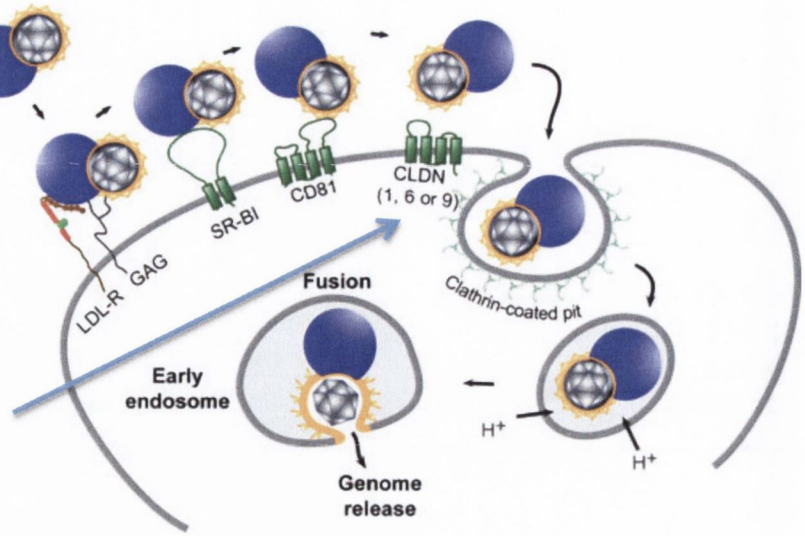


Figure 1.14. The current model of HCV entry to hepatocytes. After initial contact, HCV requires CD81 and Claudin1 to bind hepatocytes. Occludin is then required for viral internalisation. aPKCs direct Claudin and Occludin localisation and may also be required by HCV to enter hepatocytes and replicate. Figure adapted from Dubulsson *et al.*, 2008.

As it has been shown that HCV can specifically target PKC function in T-cells and that PKCs are involved in IFNα signalling in other cell types, it is important to fully describe the PKC isotypes present in Huh7 hepatocytes. PKC's role in IFNα signalling in Huh7 cells must then be investigated. The role of PKCs in cell:cell contact and HCV replication should also be investigated as aPKCs direct the localisation of proteins involved in cell polarity and HCV entry to hepatocytes.

Therefore my hypotheses are:

- That the full range and subcellular locations of PKC isotypes present in the Huh7 hepatocellular carcinoma cell line is so far inconclusive.
- That IFNα is able to translocalise PKC isotypes in hepatocytes.
- That novel PKC isotypes are required for IFNα signalling through the Jak/STAT signalling pathway and for ISG expression.
- That the atypical PKCζ is required for the membranous localisation of adherens and tight junction proteins and for Huh7 cell:cell contact.
- That PKCζ activity is required for HCVcc replication in Huh7.5 cells.

Chapter II

Materials And Methods

CHAPTER II

2. MATERIALS AND METHODS

2.1 REAGENTS

Dulbecco's modified Eagles's medium (DMEM), foetal calf serum (FCS), (Gibco). Zeocin, Non-essential amino acids (NEAA), Trypsin, Ethidium bromide, Phalloidin-TRITC, Höechst 33342, RNase ZAP, Opti-MEM I Reduced Serum Medium, Lipofectamine 2000, Lipofectamine RNAiMAX (Invitrogen). EDTA, Trypan Blue Solution, Phosphate-buffered saline (PBS), Penicillin, Streptomycin, Tween 20, Paraformaldehyde, Triton X-100, Bovine serum albumin (BSA), Sodium hydroxide (NaOH), Acetic Acid, Sodium Azide, Protein A Sepharase beads, FITC Dextran , HEPES, Phorbol 12-myristate 13-acetate (PMA), Dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Ethanol, Methanol, Isopropanol, Acetone, Chloroform (Merck) Fluorescent mounting medium (DAKO, Denmark). BCA Protein Assay Kit (Pierce, Rockford, IL). MycoAlert Mycoplasma Detection Kit (Lonza). DharmaFECT D2, ONTARGETplus SMARTpoolsiRNAs (Dharmacon, Thermo Scientific, NY). Roferon-A IFNα 2a (Hoffmann LaRoche). Rottlerin, Staurosporine, Okadaic acid, Myristolated PKCζ pseudosubstrate (Calbiochem). C16 Ceramide, C16 Dihydroceramide (Santa Cruz)

2.2 CELL CULTURE

The human hepatocellular carcinoma cell line Huh7 (a kind gift from Dr Ralf Bartenschlager, University of Heidelberg, Germany) and their derivative Huh7.5 (Apath, NY) were used in this study.

2.2.1 Source of the cell line and maintenance of cell cultures

Huh7 cells are adherent cells derived from a tumour in the liver of a 57-year-old Japanese patient with well-differentiated hepatocellular carcinoma. They have an epithelial-like morphology and low TLR3 expression. Huh7.5 cells contain an additional point mutation in *RIG-I* that inhibits its activity.

2.2.2 Culture

Huh7 cells were cultured in DMEM containing 10% foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 U/ml), L-glutamine and zeocin. Huh7 cells were grown in complete medium at 37°C in 5% CO_2 in a humidified incubator and were routinely maintained in sterile T-75 cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) in 10-15 ml of medium.

Huh7.5 cells were cultured in DMEM containing 10% foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 U/ml), L-glutamine and Non-essential amino acids (NEAA). Huh7.5 cells were grown in complete medium at 37°C in 5% CO_2 in a humidified incubator and were routinely maintained in sterile T-75 cm^2 cell culture flasks in 10-15 ml of medium.

Visual examination of both cell lines was undertaken using a phase contrast microscope (Nikon, Japan) and cells were maintained between 15-75% confluence. Huh7 and Huh7.5 cells were passaged when cell confluence reached 75% by removing the medium and washing the cells three times with sterile phosphate buffered saline (PBS). Cells were detached from the flask by incubation with 3ml of 5% trypsin for 4 minutes at 37°C in a humidified incubator. 3ml of fresh medium was then added to inactivate the trypsin and cells were centrifuged at 1,400 rpm for 4 minutes. Supernatant was then removed and cells were resuspended in 5ml of complete medium. 1ml of resuspended cells was then diluted in a fresh T-75 cm^2 flask containing 9ml complete medium. All reagents were warmed to 37°C before use and stored at 4°C, except PBS, which was stored at room temperature.

2.2.3 Cryopreservation

Huh7 cells were cryopreserved in freshly prepaired freezing media containing 90% FCS and 10% dimethyl sulfoxide (DMSO) while Huh7.5 cells were cryopreserved in Recovery Cell Culture Freezing Media (Invitrogen, NY). Cells were resuspended at a concentration of $1x10^6$ cells/ml in ice cold freezing media and transferred into sterile cryovials (Nunclon, NY). Cryovials were then transferred into a freezing container ("Mr Frosty", Nalgene, Nalgene Europe Ltd., UK) and stored at -80°C. The freezing container allows for a repeatable -1°C/min cooling rate. This allows for the freezing process to be measured enough to allow the cells to dehydrate but rapid enough to avert excessive dehydration

damage. For long-term storage, the cryovials were transferred to a liquid nitrogen tank, thereby storing the cells at -195.8°C.

Cells were thawed by transferring the cryovials from the -80°C freezer or liquid nitrogen to a 37°C water bath with an occasional gentle flick during thawing. When cells had thawed, the cryovials were transferred to a biosafety cabinet and gently diluted immediately into a sterile tube containing 4ml of warmed complete culture medium. Cells were centrifuged at 1,400 rpm for 4 minutes and the supernatant was then removed. The cell pellet was resuspended gently in 10ml of warmed complete medium and transferred to a T-75 cm^2 flask and incubated at 37°C in 5% CO_2 in a humidified incubator.

2.2.4 Cell enumeration and viability

Cells were counted using a Neubauer haemocytometer and trypan blue to assess the cell yield and viability. Cells were washed, trypsinised, centrifuged and resuspended in 5ml of complete culture medium as described for cell culturing. Once resuspended in medium, 10μl of the cell suspension was diluted in 90μl of 0.4% trypan blue in a 0.5ml microcentrifuge tube, giving a 1/10 dilution. The dilution was mixed carefully and 10μl was loaded underneath a coverslip on to each side of a Neubauer haemocytometer. Live cells appear white and non-viable cells appear blue when examined with a light microscope. Cells were counted on eight large squares (1mm x 1mm) and calculated as per manufacturers instructions.

2.3 SDS-PAGE AND PROTEIN CO-IMMUNOPRECIPITATION

2.3.1 Protein Extraction

Supernatant was removed from Huh7 or Huh7.5 cells. Cells were then incubated with ice-cold Cell Lysis Buffer (components of cell lysis buffer are provided in Appendix 1) for 5 minutes on ice. Cells were then scraped and transferred to 1.5ml tubes. Cells were further incubated on ice for 25 minutes and agitated every 5 minutes using a Vortex shaker. Unlysed cells, nuclei and detergent-insoluble materials were removed by centrifugation at 14,000x*g* for 5 minutes at 4°C in a bench-top centrifuge. Cell-free lysate supernatant was transferred to a fresh tube and stored on ice.

2.3.2 Calculation of Protein Concentration of Lysates

The protein concentration of cell lysates was calculated using a Bradford Coomassie assay with BSA standards (Bradford, 1976).

2.3.3 Acetone Precipitation of Cell Lysates

If cell lysate concentration was below 0.5 μg/μl, protein was concentrated by acetone. Cell lysates were resuspended in a 5x volume of ice-cold acetone and agitated every 5 minutes for 30 minutes at -20°C. Protein was pelleted by centrifugation at 14,000 x*g* for 5 minutes at 4°C. Supernatant was aspirated and protein allowed to air dry for 1 minute. Protein was then resuspended at 0.5 or 1 μg/μl in 1x SDS sample buffer (Appendix 1).

2.3.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE protocol developed for the Atto gel electrophoresis system (ATTO Corporation, Japan) was used to separate protein according to their molecular weight. A polyacrylamide gel (components listed in Appendix 1) was poured between two glass plates and allowed to set. A stacking gel (Appendix 1) was overlaid with a comb to form wells. When the gel was set, 200 ml of SDS-PAGE Running buffer was poured into the gel electrophoresis rig. Protein extract and molecular weight markers were then loaded into the wells. Protein was then separated by passing an electric charge through the gels at 200V and 50 mAmps until the protein dye-front reached the bottom of the gel.

2.3.5 Immunoblot Analysis

Following gel electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membrane by Western blotting using the semi-dry transfer technique as described by the manufacturer's instructions (ATTO Corporation, Japan). Five sheets of Whatmann 3 mm filter paper were soaked in transfer buffer (Appendix 1) and placed on the anode of the Western blotting apparatus. PVDF membrane (0.45μm) was activated by immersion in methanol for 1 minute. The membrane was then washed in transfer buffer and placed upon the Whatmann filter paper. The PVDF membrane was kept moist by soaking it with transfer buffer. The gel was then placed on top of the PVDF membrane and any air bubbles were carefully removed. A further 5 sheets of Whatmann 3 mm filter paper were soaked in transfer buffer and then placed on top of the gel to form a gel sandwich. The Western blotting apparatus cathode was then lowered carefully onto the gel sandwich and

any excess transfer buffer was removed by paper towels to prevent the apparatus short-circuiting. Western blotting was performed for 1 hour at 100 mAmps per gel and 200 V. Unstained molecular weight markers were then removed from the membrane and stained with Coomassie stain to identify protein standard bands.

Following Western blotting, non-protein bound sites on the PVDF membrane were blocked by a buffer containing 5% w/v milk powder in TBST, containing 1x TBS with 0.1% v/v Tween-20 for 30 minutes at room temperature with consistent gentle agitation. The PVDF membrane was washed for 5 minutes with TBST three times at room temperature. The PVDF membrane was then incubated with specific antibody to the protein of interest diluted in 5% BSA/TBS containing 0.05% sodium azide overnight at 4°C with gentle agitation. The PVDF membrane was then washed for 5 minutes with TBST three times at room temperature to remove any unbound antibody. The PVDF membrane was then incubated with a HRP- conjugated anti-mouse or anti-rabbit antibody, diluted at 1:2000, in 5% w/v milk powder in TBS for 1 hour at room temperature with consistent gentle agitation. The PVDF membrane was again washed for 5 minutes with TBST three times at room temperature to remove any unbound antibody.

Specific protein bands were visualised by Enhanced Chemiluminescence (ECL) by incubating the PVDF membrane in HRP development solution for 1 minute as previously described (Haan and Behrmann, 2007), in which 4-iodophenolboronic acid is used instead of p-Coumaric acid (Appendix 1). The PVDF membranes were then wrapped in cling film and taped on a film cassette. X-OMAT S film (Kodak, NY) was then exposed to the PVDF membrane in a darkroom for various times ranging from 1 second to 10 minutes. The film was developed using an automated film developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany) which submerges the film in developing solution for 1 minute, fixation solution for 1 minute and rinsed with distilled water and allowed air dry.

2.3.6 Stripping of Western Blot Membranes

Where indicated, PVDF membrane that had been exposed to film was stripped of its primary and secondary antibodies and re-probed with different antibodies. This was carried out by incubating the PVDF membrane with Western blot stripping buffer (Appendix 1) at 50°C for 30 minutes. The membrane was removed from the stripping

solution and washed 5 times for 5 minutes in TBST. The membrane was then blocked, probed with primary and secondary antibodies and illuminated by ECL as described above.

2.3.7 Densitomerty

Densitometric analysis of Western blots was performed by using a Image Station 440CF and 1D Image Analysis software (Kodak, NY). The relative values of the samples were determined by normalising each sample to its control sample of each experiment.

2.4 RNA EXTRACTION

2.4.1 RNA Extraction

The transcription of the interferon-stimulated genes (ISG) protein kinase R (PKR), 2'-5'-oligoadenylate synthetase (2'-5'-OAS) and Myxovirus resistance protein A (MxA) were assessed in cells treated with or without IFNα, in order to assess the functional requirements of PKC δ or ε for IFNα signalling in hepatocytes. Transcriptional activity of individual genes was measured by Real Time PCR following RNA extraction from samples.

TRIzol Reagent (Ambion, Ca) was used to isolate total RNA from cell samples. TRIzol Reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization.

IFNα was added (or not) to the Huh7 cells for 6 hours. The spent medium was then removed and 1 ml of TRIzol Reagent lysis buffer was added to the monolayer. After 5 minutes this was pipetted with sterile tips into sterile tubes, ensuring that the maximum yield was obtained from each well. After homogenizing the sample with TRIzol Reagent cell pellet can be stored at -80 °C if desired. 200μl chloroform was added, and the homogenate allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and proteins). The aqueous layer was then transferred to a fresh tube and RNA precipitated with 500μl isopropanol. RNA was then pelleted and washed with 1 ml of 75% ethanol. The RNA was pelleted again and allowed to air dry for 5 minutes before resuspension in 30μl RNase-free water.

2.4.2 Determination of RNA Yield

To assess the concentration and purity of RNA yields the absorbance of samples was measured at 230nm, 260nm and 280nm. 1μl of each sample was analysed using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Scientific, DE). An $A_{260/280}$ >1.8 and an $A_{260/230}$ ~1.2 signified good quality RNA.

2.4.3 cDNA Synthesis

Complementary DNA (cDNA) was synthesised by reverse transcription using the Omniscript Reverse Transcriptase (Qiagen Ltd., UK) kit according to the protocol supplied. Briefly, a master mix containing buffer, 1μM oligo-dT primers, 0.5 mM Deoxyribonucleotide triphosphate (dNTP) and 1 μl Omniscript Reverse Transcriptase was prepared on ice. 1μg of RNA was then added and incubated at 37°C for one hour. Reverse Transcriptase was then deactivated by heating to 95°C for 5 minutes. Samples were collected at the bottom of the tube by brief centrifugation and stored at -20°C.

2.5 IMMUNOFLUORESCENCE ANALYSIS

2.5.1 Intracellular Immunofluorescent Staining

Cells were seeded at a density of 5,000 cells per well for 96 well plates (Thermo Scientific, CA) and 96 well glass bottom plates (0.17mm; PAA, UK) or 10,000 cells per well for eight cell chamber slides (Nunc, NY) or glass bottom 96 well plates (Nunc, NY). Cells were allowed to adhere to the well surface by overnight incubation at 37°C in 5% CO_2 in a humidified incubator. Cells were then serum starved for 4 hours to arrest the cell cycle and then treated as indicated. Cells were then fixed in either 4% paraformaldehyde for 30 minutes at room temperature, ice-cold acetone or methanol for 5 minutes at -20°C or ice-cold 1:1 acetone:methanol at -20°C for 20 minutes. Fixative was then removed and cells were washed with PBS.

In order to allow antibodies access inside the cell membrane, cells were permeabilised by incubation with 0.1% Triton X-100 for 5 minutes at room temperature. Cells were then washed with PBS three times. In order to prevent non-specific antibody binding, cells were

blocked with 3% BSA in PBS for 30 minutes at room temperature and washed three times in PBS.

Immunofluorescent staining was accomplished by diluting a protein specific primary antibody in 5% BSA/PBS according to the manufacturer's recommendations. The primary antibody dilution was then added to cells and incubated for 1 hour at room temperature. Unbound antibody was then removed and cells were washed three times with PBS. A fluorescently-labelled secondary antibody recognising the primary antibody diluted 1:400 and Hoechst, a nuclear stain that binds chelated DNA, diluted 1:2000 in 5%BSA/PBS were then added to cells for 1 hour at room temperature. Experiments using the Philloidin stain for actin had a 1:500 dilution of Philloidin included with the secondary antibody/Hoechst mix. Unbound antibody was removed and cells washed in PBS a further three times. For chamber slides, the plastic chambers were removed and a glass coverslip mounted using DAKO fluorescent mounting medium. For 96 well plates, two drops of DAKO fluorescent mounting medium were added per well. Negative controls with only secondary antibody and Hoechst were included to ensure that visualised patterns of protein localisation were the result of primary and secondary binding and not the result of non-specific secondary antibody binding.

2.5.2 Confocal Microscopy Analysis

Cells were imaged using a Zeiss confocal workstation attached to a Zeiss LSM510 laser scanning microscope (Carl Zeiss, Thornwood, NY) using10X, 20X, 40X objectives and a 63X oil immersion objective with the Diode, Argon and DPSS lasers at the excitation wavelengths 405, 488 and 561 nm respectively. Images were obtained, processed and analysed using the Zeiss LSM Data Server software version 4.2.0.121, available for download on the Zeiss website. Representative images are shown in the results sections.

2.6 SMALL INTERFERING RNA (siRNA)

2.6.1 siRNA Technology Overview - RNA interference

RNA interference (RNAi) is a naturally occurring mechanism of sequence specific gene silencing in plants and vertebrates (Fire *et al.*, 1998). It allows the characterization of gene function by silencing gene expression in a method known as gene 'knockdown'. During the 1990s a number of post-transcriptional gene silencing (PTGS) events were detected in plants, fungi, animals and ciliates, pioneering the model of RNA silencing pathway (Wang *et al.*, 2003; Baulcombe 2000; Romano and Macino 1992). RNA that restricts the expression of a particular gene is known as RNA interference. The RNAi pathway is a process of order-specific, post-transcriptional gene silencing. It is triggered by the presence of double-stranded RNA (dsRNA) in the cell's cytoplasm and controlled by the RNA-induced silencing complex (RISC). dsRNA can either be artificially manufactured as small inferring RNA (siRNA) which is then directly transfected into cells or can be produced inside the cell by providing vectors that express short-hairpin RNA (shRNA) precursors of siRNAs (Romano *et al.*, 2006). The mechanism of converting shRNA into functional siRNA comprises cellular RNAi machinery that innately processes genome encoded microRNAs (miRNA) (Hutvagner and Zamore, 2002). miRNAs are 22–24 nucleotides in length and down-regulate gene expression by binding messenger RNAs (mRNAs) thereby blocking their translation into proteins (John *et al.*, 2006).

The RNAi pathway has a distinct progression. (**Figure 2.1**) Initially, specific dsRNA is delivered into the cell's cytoplasm. Then Dicer, a RNaseIII-like enzyme with endoribonuclease activity recognizes the dsRNA and cleaves it into small interfering (siRNA) which is 21-bp to 23-bp long, contain 5′ phosphate groups and two nucleotide 3′ over-hangs. Dicer usually contains a helicase/ATPase domain, a Piwi-Argonaute- Zwille (PAZ domain), two RNaseIII-like domains and a dsRNA-binding domain. The distance between the PAZ and the RNaseIII domains determines the size of produced siRNAs. Dicer then presents the siRNA to an Argonaut-containing RNA-induced silencing complex (RISC). Following activation by ATP, RISC then unwinds the double-stranded siRNAs, retaining one strand to act as a co-factor. Complexed with RISC this single-strand RNA recognizes its complementary mRNAs in cell's cytoplasm. The RISC complex causes a single-site cleavage of the target mRNA through endonuclease activity at its Argonaut

subunit. The resulting fragments of target mRNA is thus destabilized and degraded through natural endogenous mechanism. Activated RISC can degrade thousands of target mRNA molecules.

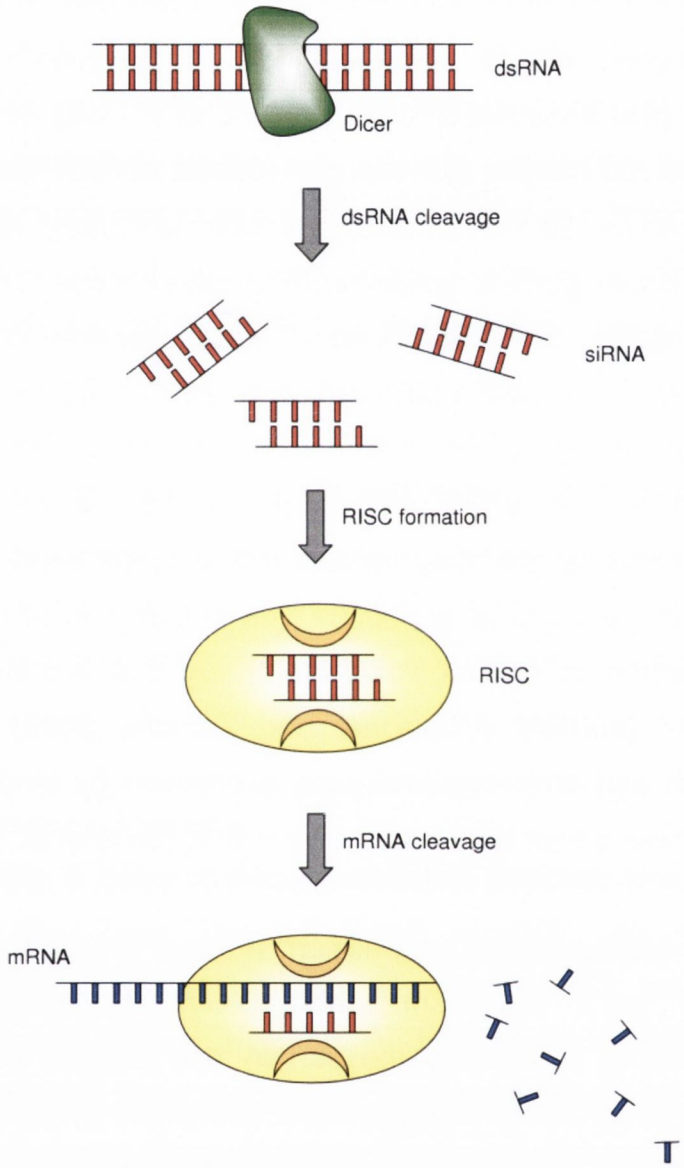


Figure 2.1 The process of RNA interference. Double-stranded siRNA is cleaved by Dicer into siRNA. RISC then processes the siRNA and binds and cleaves its target mRNA, preventing its translation (Mocellin and Provenzano, 2004).

2.6.2 Conduction of siRNA Experiments

Transfection of siRNA was undertaken by wet-reverse transfection using lipid transfection reagents (**Figure 2.2**). Reverse transfection is a method of siRNA transfection whereby the cells are transfected while in suspension, rather than the traditional (forward) method of plating cells the night before transfection. The transfection process was similar to manufacturer's instructions following optimisation experiments. The siRNA (Dharmacon; Lafayette, CO) is combined with a lipid-based transfection reagent. A control sample is also included with scrambled control siRNA instead of PKC isoform target siRNA. 200,000 Huh7 cells are then added and the mixture divided between a 12-well tissue culture plate, another 12-well plate and a 96-well plate and incubated at 37°C for 24 hours in antibiotic free media. Cells were seeded at a density of 100,000 cells per 12-well plate and 20,000 cells per 96-well plate. After 24 hours the media was replaced with fresh media and incubated for a further 48 hours. Cells seeded in one 12-well plate were lysed by NP40 and used to examine transfection efficiency. PKC isoform expression was detected in these cells by Western blot in samples transfected with PKC specific siRNA, scrambled control siRNA or non-siRNA transfected cells. PKC isoform expression was measured by densitometry and expression levels compared to actin. The ratio of PKC expression compared to actin was then compared between cells with different transfections to determine transfection efficiency and PKC protein knockdown. The cells seeded in the other 12-well plate or the 96-well plate were lysed to extract RNA or fixed for immunofluorescence staining respectively for further analysis.

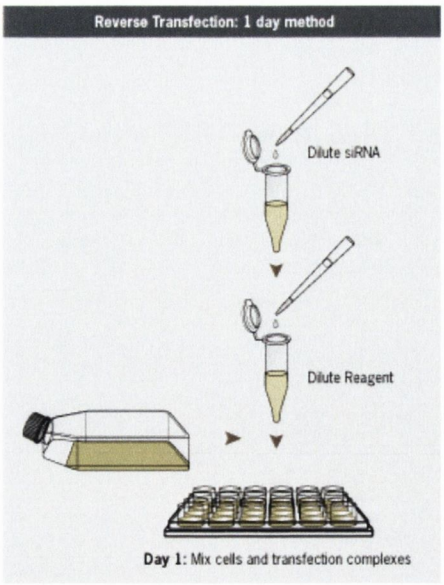


Figure 2.2 Wet reverse transfection. The wet reverse transfection method involves combining the siRNA and transfection reagent mix before transfecting the cells while still in suspension, prior to plating (Ambion® TechNotes 12).

2.6.3 PKCδ RNAi

To begin the transfection, the PKC-δ-specific siRNA mixture PRKCD was prepared, using Dharmacon ON-TARGET *plus* SMARTpool™, which comprises a combination of four PKC-isotype-specific oligonucleotides. As a control, non-targeting si control, Dharmacon ON-TARGET *plus*®, was used. The quantities outlined below are per 200,000 Huh7 cells. To make a 100 nM final siRNA concentration from a 20 μM stock, 10 μL of siRNA was added to 90 μL 1x reaction buffer (diluted with RNase-free H_20 from a 5x stock). 100 μL serum and antibiotic free DMEM was then added to this, the resulting solution left to incubate at room temperature for exactly 10 minutes. At the same time, the transfection reagent solution was prepared. Previous optimisation experiments from our group revealed DharmaFECT® 2 to be a favorable reagent when compared to different transfection reagents and was therefore selected for the remainder of the PKCδ siRNA experiments. 2 μL of DharmaFECT 2 was added to 198 μL of serum-free DMEM and also allowed to incubate at room temperature for 10 minutes.

Meanwhile, Huh7 cells were trypsinised and counted. Next, the 200 μL siRNA mix was added to the 200 μL of DharmaFECT 2 mix, and allowed to incubate at room temperature for 20 minutes. 200,000 cells were then added to the mixture and gently pipetted up and

down. The volume was then made up to a total volume of 2 ml with DMEM and a final concentration of 5% FCS. The transfected cells were then seeded in either a 12 well plate or a 96 well plate. After 24 hours of incubation at 37°C, the medium was discarded and replaced with fresh DMEM containing 5% FCS. After a further incubation of 48 hours, the knockdown is complete.

2.6.4 PKCε RNAi

RNAi duplex-Lipofectamine™ RNAiMAX (Invitrogen) proved to be a more efficient transfection reagent than DharmaFECT 2 for PKCε siRNA PRKCE experiments and was therefore selected for the remainder of the PKCε siRNA experiments.

For each batch of cells to be transfected, RNAi duplex-Lipofectamine™ RNAiMAX complexes were prepared as follows:

PKCε-specific siRNA duplex (Dharmacon ON-TARGET *plus* SMARTpool™), which comprises a combination of four PKC-isotype-specific oligonucleotides was diluted to the desired concentration of 30nM in 100 μl Opti-MEM® I Medium (Invitrogen) without serum in a 2ml tube and mixed gently. A control siRNA mix was also made. For a 30nM concentration, 3ul of 20uM stock siRNA was diluted.

Lipofectamine™ RNAiMAX was mixed gently before use and then 1μl was added to each tube containing the diluted siRNA duplex. This was mixed gently and incubated for 20 minutes at room temperature. Meanwhile, Huh7 cells were trypsinised, counted and resuspended in complete growth medium without antibiotics so that 1.9ml contained 200,000 Huh7 cells. 1.9ml of cells were then added to each tube containing siRNA duplex - Lipofectamine™ RNAiMAX complexes. The transfected cells were then seeded in either a 12 well plate or a 96 well plate. After 24 hours of incubation at 37°C, the medium was discarded and replaced with fresh DMEM containing 5% FCS. After a further incubation of 48 hours, the knockdown is complete.

2.7 QUANTATIVE REAL TIME PCR (qRT-PCR)

qRT-PCR was performed in a 96-well optical plate to quantify the expression of mRNA for PKR, 2'-5'-OAS and MxA. The PCR reaction was carried out using the SYBR Green PCR Master Mix kit (Applied Biosystems, CA), template cDNA and a mix of primers for the target genes (**Table 2.3**) and the housekeeping genes GAPDH or PPIA (**Table 2.1**), which were used as endogenous controls. 80ng cDNA (2µl) was added to the 96-well plate followed by the addition of 6µl of RNase free H_2O, 4.5µl of primer mix and the RT-PCR mix (12.5 µl of SYBR Green Master mix). The mix was then briefly centrifuged in order to collect all reagents at the bottom of the well. The reaction was performed in an Agilent Mx3005P real time thermocycler (Agilent Technologies, CA) using the following cycling program:

First step 50 °C 3 min
Second step 95 °C 10 min
Third step 60 °C 1 min
Fourth step 72 °C 30 sec

Steps 3 and 4 were repeated for 40 cycles. Fold inductions were calculated by MxPro software using the comparative CT method (Livak and Schnittgen 2001) as described in the Agilent Mx3005P manual.

For accurate gene quantification, real-time PCR data was normalised to a fixed reference using geNorm. geNorm is a system for selecting the best candidate reference gene for a given experimental scenario. To perform geNorm analysis the expression of 6 or 12 reference (house-keeping) genes is measured in a representative set of their own samples. The geNorm software ranks the reference (house-keeping) genes in order of stability of expression. The output graphs and report identify the best reference (house-keeping) genes for that particular experiment.

2.7.1 DNA VISUALISATION

Real time PCR products were occasionally visualised by UV light following electrophoresis in a 1.5% agarose gel containing 0.035% ethidium bromide to ensure there was no contamination in the product.

PKR F	TCTCAGCAGATACATCAGAGATAAATTCT
PKR R	AGTATACTTTGTTTCTTTCATGTCAGGAA
OAS2 F	GAAGCCCTACGAAGAATGTCAGA
OAS2 R	TCGGAGTTGCCTCTTAAGACTGT
MxA F	GGTGGTGGTCCCCAGTAATG
MxA R	ACCACGTCCACAACCTTGTCT

Table 2.3 ISG primer sequences

B-Actin F	AGATGACCCAGATCATGTTTGAGA
B-Actin R	CGTCACCGGAGTCCATCAC
GAP-DH F	CCACCCATGGCAAATTCC
GAP-DH R	CAGCATCGCCCCACTTG
PPIA F	GCAAATGCTGGACCCAACAC
PPIA R	GCCATTCCTGGACCCAAAG
RPS9 F	TGACCGGGTGGTTTGCTTA
RPS9 R	ATACTCGCCGATCAGCTTCAG
RPS15 F	CGGACCAAAGCGATCTCTTC
RPS15 R	CGCACTGTACAGCTGCATCA

Table 2.1 Reference (house-keeping) genes primer sequences

2.8 HIGH CONTENT ANALYSIS

2.8.1 High Content Analysis Technology Overview

High Content Analysis (HCA) uses an automated microscope to facilitate rapid, parallel biological research and drug discovery. HCA allows for the evaluation of multiple biochemical and morphological parameters in cellular systems in discovery research. By combining cell images with automated image analysis algorithms, a deep knowledge of multiple biochemical or morphological pathways at the single cell level can be obtained.

To undertake HCA, cells were seeded on 96 well plates and stained as previously described in section 2.5. Cells were then scanned using the automated microscope IN Cell Analyser 1000 HCA platform (GE Healthcare, Buckinghamshire, UK) shown in **Figure 2.3**. This platform allows for high-throughput and high-content subcellular imaging and analysis. Image data was then analysed using the IN Cell Investigator software (GE Healthcare, Buckinghamshire, UK). This software allows the measurement of a range of cellular data based on a number of protocols. Results shown here primarily measure subcellular translocation within user defined nuclear and cytoplasmic compartments.

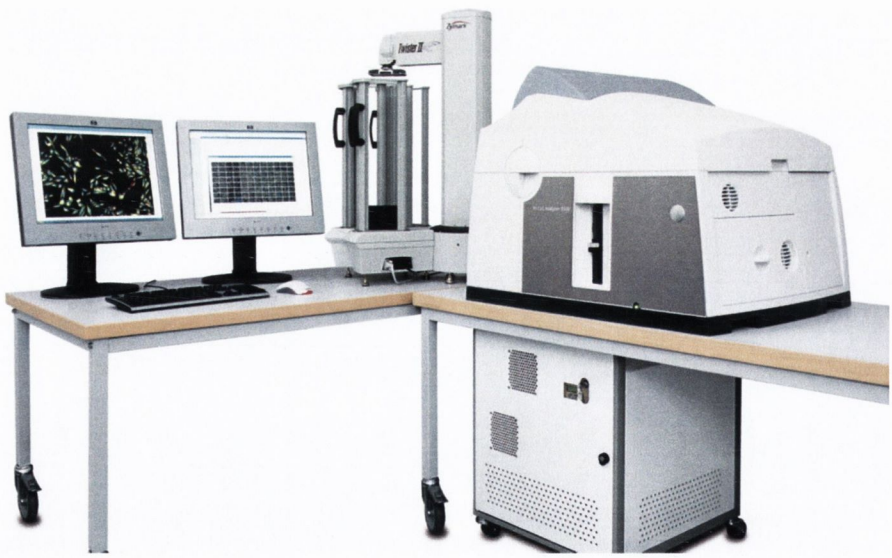


Figure 2.3 InCell Analyser (www.gelifesciences.com).

2.8.2 Analysis Parameters

Following RNAi and IFNα stimulation cells were fixed and stained for DNA and STAT1 or STAT3 as described in section 2.5. Cells were then imaged and staining intensities measured. 5-10 fields of view were taken for each sample, counting between 500 and 2000 cells. Net staining intensities were calculated by subtracting the cell background value from the nuclear or cell area intensity values. To measure nuclear translocation, the net cellular nuclear intensity was divided by the net cellular cell intensity. To analyse nuclear area, the area of DNA staining was measured in each cell and averaged for each sample.

2.9 MEASUREMENT OF CELL BARRIER PERMIABLITY

2.9.1 Measurement of dextran permeability.

Paracellular permeability was quantified by measuring the transepithelial flux of a 70-kDa fluorescein isothiocyanate (FITC)-labeled dextran molecule (Sigma-Aldrich, UK). Huh-7.5 cell monolayers were grown on 0.4-μm-poresize Transwell polycarbonate membranes (6.5mm diameter) for 6 days by seeding 20,000 cells per well. Cells were then serum starved for 2 hours before treatment with 100U/ml IFNα or 10mM PKCζ pseudosubstrate in duplicate. Following treatment all media was removed and 1μg/200μl PBS of FITC-labeled dextran was added to the apical side of the monolayer while PBS was added to the basal well. Cells were then protected from light and incubated for 2 hours at 37°C. A 100-μl aliquot of the medium was removed from the basolateral chamber to a 96-well plate and FITC-dextran fluorescence was measured by measuring the fluorescenin at 485nm 535nm 0.1s on a Victor2 (Wallac) fluorescence reader.

2.10 HCV CELL CULTURE (HCVcc) SYSTEM

2.10.1 System Overview

The cell culture derived infectious HCV system (HCVcc) has allowed for the study of virus entry to cells and its replication lifecycle (Lindenbach *et al.*, 2005). The system is comprised of 2 components: 1) cells that are permissive to *de novo* infection and allow effective replication of the full virus life cycle and release of progeny virions; and 2) a virus genome that has robust and efficient replication in tissue culture. Here the hepatoma cell line Huh7.5, made permissive by the fact that it has low TLR expression and a point mutation in RIG-I, is used while the J6/JFH and H77/JFH full-length chimeric genomes were used for replication. These chimeras use the replicative properties of the NS3-NS5B gene regions of a genotype 2a viral isolate from a Japanese fulminant hepatitis patient (JFH-1) and the core-NS2 gene regions from the infectious J6 (genotype 2a) or H77 (genotype 1a) virus strains.

2.10.2 Production Of HCV J6/JFH And H77/JFH RNA.

2.10.2.1 Transformation of *E.coli* competent cells with J6/JFH and H77/JFH circular plasmid DNA.

5μl J6/JFH HCV genotype plasmid DNA and 5μl H77/JFH HCV genotype plasmid DNA constructs were kind gifts from Dr. Charles Rice of the Rockefeller University, NY. In order to amplify DNA stocks, *E. coli* cells were transformed with the plasmid DNA constructs. 10μl MAX Efficiency DH5α competent *E.coli* cells (**Figure 2.4**) (Invitrogen) were incubated with 5μl J6/JFH plasmid DNA or 5μl H77/JFH plasmid DNA on ice for 30 minutes. The φ80*lac*ZΔM15 marker provides α-complementation of the β-galactosidase gene from pUC or similar vectors and can be used for blue/white screening of colonies on bacterial plates containing Bluo-gal or X-gal.Cells were then heat-shocked for 45 seconds at 42°C on a heating block and returned to ice for 2 minutes. 900μl if LB medium was then added to each sample. Cells were then incubated at 37°C for 1 hour @ 225 rpm in a shaking incubator in order to allow them to recover.

F⁻ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1*end*A1 *hsd*R17 (r_k-, m_k+) *pho*A *sup*E44 λ⁻ *thi*⁻1 *gyr*A96 *rel*A1

Figure 2.4 MAX Efficiency DH5α Competent Cells Genotype.

To culture the transformed *E.coli* cells, 200μl or 700μl of transformed *E.coli* cells was spread on 2 fresh Luria-Bertani agar plates containing 100μg/ml ampicillin antibiotic (LB-amp) and incubated overnight at 37°C. Six separate blue colonies were selected from each HCV genotype and picked from the LB plates with sterile pipette tips. Colonies were amplified by inoculating 10ml of LB media (containing 100μg/ml ampicillin antibiotic) at 37°C overnight @ 225 rpm. The absorbance of each culture was measured to confirm that bacteria had reached the required growth density. An A600 of 2.0-4.0 ensured that bacteria had reached the proper growth density for harvesting and plasmid DNA isolation. Glycerol stocks of each sample were prepared for long-term storage. Stocks were prepared by mixing 500μl of bacteria to 500μl of glycerol and freezing at -80°C.

2.10.2.2 Plasmid prep of Transformed *E.coli* cells using PureYield™ Plasmid Miniprep System

Once transformed *E.coli* cells have been selected and cultured overnight, plasmid DNA must then be isolated to confirm that *E.coli* contain the correct plasmid DNA sequence for use in future experiments. Plasmid DNA isolation can be achieved by using a silica membrane column. Protein, RNA and endotoxin contaminants are then removed from purified plasmid DNA by an endotoxin removal wash. This was achieved by using the PureYield™ Plasmid Miniprep System (Promega, Madison, WI). All procedures were carried out by following the manufacturer's recommendations. The Cell Lysis Buffer was checked to ensure that the solution had not precipitated, if precipitation occurred, solution was resuspended by incubating the buffer at 37°C for 30 minutes and mixing by inversion.

In each case 3ml of bacterial culture of each sample was processed. As the plasmid size is >10kb, the Elution Buffer was warmed to 50°C prior to elution. 1.5 ml of bacterial culture was centrifuged for 30 seconds at maximum speed in a microcentrifuge and supernatant discarded. The remaining 1.5 ml of bacterial culture was then centrifuged to the same tube

as before. The cell pellet was resuspended completely in 600μl of TE buffer. 100μl of Cell Lysis Buffer was added to the cells and mixed by inverting the tube 6 times. Complete lysis is indicated by the solution changing from opaque to clear blue. Within 2 minutes, 350μl of cold (4°C) neutralization solution was added, and mixed thoroughly by inverting the tube in order to prevent excessive lysis, which results in denatured plasmid DNA. The sample turns yellow when neutralization is complete and a yellow precipitate forms. The sample was inverted an additional 3 times to ensure complete neutralization.

Samples were centrifuged at maximum speed in a microcentrifuge for 3 minutes. Taking care not disturb the cell debris pellet, supernatant was transferred to a PureYield Mini-column. The mini-column was placed into a collection tube and centrifuged at maximum speed in a microcentrifuge for 15 seconds. The flow-through was then discarded, and the mini-column was returned into the same collection tube. 200μl of Endotoxin Removal Wash was then added to the mini-column and centrifuged at maximum speed in a microcentrifuge for 15 seconds. 400μl of Column Wash Solution was then added to the mini-column and centrifuged at maximum speed in a microcentrifuge for 30 seconds. The column was then incubated at room temperature for 5–10 minutes. The mini-column was transferred to a clean 1.5ml microcentrifuge tube, 50μl of Elution Buffer added directly to the mini-column matrix and allowed to stand for 1 minute at room temperature. The mini-column was centrifuged at maximum speed in a microcentrifuge for 15 seconds to elute the plasmid DNA. The microcentrifuge tube was capped and eluted plasmid DNA stored at –20°C.

The absorbance of all samples was then examined with a Nanodrop (Thermo Scientific) to determine the concentration and purity of the eluted DNA. A 260/280 nm absorbance ratio is used to assess the protein or phenol contamination of DNA. A ratio of ~1.8 indicates contamination free DNA. A 260/230 ratio is used to assess EDTA, carbohydrates or phenol contamination with a ~2 - 2.2 ratio indicating nucleic acid purity. 1000ng/10μl of each DNA sample was then sequenced by Source Biosciences to ensure that the correct plasmid DNA had been amplified by *E. coli*.

2.10.2.3 Plasmid prep of Transformed *E.coli* cells using PureYield™ Plasmid Maxiprep System

In order to produce larger quantities of purified plasmid DNA, a PureYield™ Plasmid Maxiprep System (Promega, Madison, WI) was used to isolate DNA with a silica-membrane column. This system also incorporates endotoxin removal to remove protein, RNA and other endotoxin contaminants from the plasmid DNA. Endotoxin removal was verified by Nanodrop. DNA was isolated as per manufacturers recommendations.

150μl of J6/JFH or H77/JFH plasmid that were previously selected for the highest sequence similarity to their predicted sequences were incubated with 150ml of LB-amp overnight at 37°C at 225 rpm in a shaking incubator. A glycerol stock was then prepared and stored at -80°C for each sample as described in section 2.10.2.1. The remaining cell culture was centrifuged at 4°C at 6,000 rpm for 10 minutes in a fixed rotor. The supernatant was then removed and the pellet resuspended in 12ml of cell resuspension solution. The cells were then lysed by adding 12ml of cell lysis solution, gently inverting the cells 3-5 times and incubating for 3 minutes at room temperature. Successful lysis is indicated by flocculent solution with small to medium sized clumps while a coagulated appearance indicates poor lysis. Cell lysis is neutralized by mixing 12mls of neutralization solution to the lysed cells and mixed by inverting 10-15 times. Lysate was then centrifuged at 4°C for 20 minutes at 12,000 rpm in a fixed-angle rotor centrifuge.

Cell debris was pelleted and approximately half the lysate supernatant poured onto a clearing column attached on top of a binding column and connected to a vacuum manifold. Vacuum was applied and lysate passed through both columns. The remaining lysate was poured onto the clearing column and the vacuum removed once it has passed through both columns. The clearing column was removed and 5mls of endotoxin removal wash added to the binding column. Vacuum was applied and the wash was allowed to pass through the filter. 20mls of column wash was then added and allowed to pass through the filter. The filter membrane was dried by applying a vacuum for 5 minutes.

The binding column was removed from the vacuum manifold and any excess ethanol removed from the column tip with a paper towel. A sterile 1.5 microcentrifuge tube was placed in the base of an Eluator™ Vacuum Elution Device (Promega, Madison, WI). The

device assembled and the binding column inserted into it. The assembled device and column was placed on to a vacuum manifold and 500μl nuclease free water added. a vacuum was applied at maximum pressure for 1 minute until all the water passed through the column. The microcentrifuge tube was removed and briefly centrifuged to collect all the DNA at the bottom of the tube. The DNA was immediately quantified and examined for protein, phenol, EDTA or carbohydrate contamination by examining absorbance ratios using a Nanodrop as described in section 2.10.2.2. DNA plasmids were then visually examined by electrophoresis on a 1.5% agarose gel. DNA was stored at -20°C.

2.10.2.4 DNA visualisation

DNA plasmid integrity was examined by electrophoresis on a 1.5% agarose gel containing ethidium bromide (Appendix 1) to ensure that the plasmid was the correct size and that DNA denaturing did not occur. A 1.5% agarose gel containing ethidium bromide was prepaired by dissolving 0.5g agarose powder in 1x TAE buffer. The solution was heated for 1 minute in a microwave to dissolve the powder and 2.5μl of ethidium bromide was added in order to visualise DNA following electrophoresis. The mixture was poured into a mould containing a comb to form wells. The gel was allowed solidify for 30 minutes at room temperature. When formed, the agarose gel was transferred to an electrophoresis rig. 3μg of DNA in a volume of 6μl was mixed with 2μl of DNA loading buffer and then loaded per well for each sample. 5μl of a DNA standard ladder (Hyperladder 1, Bioline,) was also loaded on the gel. Samples were electrophoresed for 15 minutes at 100 volts. DNA was then visualised by UV light using Wizard SV Gel & PCR Clean Up System.

2.10.2.5 DNA Linearization

In order to obtain linear DNA that was capable of producing HCV genotype RNA in an *in vitro* translation system, the circular plasmid DNA had to be linearized. Purified circular plasmid DNA was digested with the Xba1 restriction enzyme (New England Biolabs) in order to cleave the circular DNA at its Xba1 site and resulting in linearized DNA. 100 units of Xba1 (10μl), 10μg of DNA in a volume of 20μl and 5μl of 10x NE buffer 4 were mixed and made to a final volume of 50μl with BSA on ice and then incubated at 37°C overnight. The Xba1 was the final component to be added to this mix and added at 10 units/μg DNA to be digested. The DNA digestion reaction was stopped by heat inactivating the enzyme at 65°C for 20 minutes.

2.10.2.6 DNA Purification

In order to purify double-stranded DNA fragments from enzymatic reactions such as linearization, a MinElute Reaction Cleanup Kit (Qiagen) was used according to the manufacturers instructions. This kit uses a column with a silica membrane that can absorb DNA in high concentrations of salt and allow contaminants pass through. Optimised buffers allow for removal of contaminants and DNA recovery. Connection of the columns to a vacuum manifold enables larger volumes of DNA to be purified. The DNA-binding capacity of the MinElute columns is 5 μg.

As the maximum volume of enzymatic reaction that can be processed per MinElute column is 100μl, the reaction was split in 5 and 300μl of Buffer ERC was added to each aliquot of the split reaction. Buffer ERC was added to allow the efficient binding of double stranded DNA to the silica membrane and the removal of enzymes, salts and oligomers to the enzymatic reaction and mix. MinElute columns were placed onto a vacuum manifold and unused positions closed. DNA was bound to the silica membrane by loading the samples to the MinElute column and applying a vacuum. The vacuum was turned off once the sample had passed through the column.

750μl of the ethanol-containing Buffer PE was added to wash away salts from the MinElute column membrane and a vacuum applied. The MinElute columns were then transferred to a microcentrifuge tube and centrifuged at 10,000 x*g* in order to remove any residual ethanol from Buffer PE. The MinElute columns were then transferred into clean 1.5ml microcentrifuge tubes. DNA was eluted by adding 10 μl water to the center of the membrane, letting the column stand for 1 min, and then centrifuging the microcentrifuge for 1 min. DNA was stored at –20°C as DNA may degrade in the absence of a buffering agent.

DNA linearization was confirmed by visualising the DNA by agarose electrophoresis and quantified and examined for contaminants by using a Nanodrop as previously described in section 2.10.2.3.

2.10.2.7 J6/JFH and H77/JFH RNA Transcription

The J6/JFH and H77/JFH template DNA sequences each contain a T7 RNA polymerase promoter. An AmpliScribe T7 high yield transcription reaction kit (Cambio, UK) was used with linearized template DNA as described above in order to transcribe J6/JFH and H77/JFH RNA.

The AmpliScribe T7 reaction was combined and mixed with the appropriate volume of each reaction component in the order given in the manufacturers procedure (**Figure 2.5**). 2μl of AmpliScribe Enzyme Solution was added per μg of DNA. The reaction was mixed and incubated at 37°C for 2.5 hours. RNA was quantified and examined for contaminants by using a Nanodrop as previously described in section 2.4.2. A 260/280 absorbance ratio of ~2 is accepted to show pure RNA, with lower ratios indicating contamination. RNA was stored at -80°C.

x μl RNase-Free water
1 μg linearized template DNA with appropriate promoter
2 μl 10X AmpliScribe T7 Reaction Buffer
1.5 μl 100 mM ATP
1.5 μl 100 mM CTP
1.5 μl 100 mM GTP
1.5 μl 100 mM UTP
2 μl 100 mM DTT
0.5 μl RiboGuard RNase Inhibitor
2 μl AmpliScribe T7 Enzyme Solution
20 μl Total reaction volume

Figure 2.5 AmpliScribe T7 In vitro Transcription Mix

2.10.2.8 RNA Purification

In order to remove the AmpliScribe T7 enzyme and isolate freshly transcribed RNA, an RNeasy Mini Kit (Qiagen) was used as per manufacturers instructions. 100 μg RNA was used per reaction. The sample volume was adjusted to 100 μl with RNase-free water. 350μl Buffer RLT containing a highly denaturing guanidine-thiocyanate, was then added and mixed well to lyse and homogenise the samples.

250µl ethanol (96–100%) was then added to the diluted RNA, and mixed well by pipetting, in order to provide appropriate spin column binding conditions. The samples were transfered to an RNeasy Mini spin column placed in a 2 ml collection tube. The lid was closed and centrifuged for 15 s at 8000 x*g*. Under these conditions mRNA binds to the membrane and contaminants are washed away. This procedure also excludes 5.8S and 5S rRNA and tRNA. After centrifugation the RNeasy spin column was carefully removed from the collection tube so that the column did not contact the flow-through. The collection tube was then completely emptied.

500µl Buffer RPE was then added to the RNeasy spin column. The lid was closed gently, and the column centrifuged for 2 min at 8000 x*g* to wash the spin column membrane. The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

The RNeasy spin column was placed in a new 2 ml collection tube. The lid was closed and centrifuged at full speed for 1 min to eliminate any possible carryover of Buffer RPE. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30 µl RNase-free water was added directly to the spin column membrane. The lid was closed gently and centrifuged for 1 min at 8000 x g to elute the RNA.

RNA integrity and correct nucleotide length was confirmed by visualising the RNA by agarose electrophoresis with a RNA standard ladder as previously described for DNA visualisation in section 2.7.1. RNA was quantified and examined for contaminants by using a Nanodrop as previously described in section 2.4.2. RNA was diluted with RNase free water to 1µg/10µl, aliquoted to 10µl volumes and stored at -80°C.

2.10.3 Generation Of HCVcc Viral Particles

To generate HCVcc viral particles, *in vitro* transcribed viral RNA was transfected into Huh7.5 cells to produce viral particles. For RNA transfection Huh7.5 cells were washed 3 times with sterile PBS and trypsinised for 3 minutes at 37°C. Trypsin was then deactivated by an equal volume of DMEM containing 10% FCS and 0.1 mM non-essential amino acids (NEAA) with no antibiotics. Cells were pelleted by centrifugation (1,400 rpm for 4 minutes)

and resuspended with DMEM containing 10% FCS and 0.1 mM NEAA with no antibiotics. 100,000 cells in 2ml were seeded per well into 2 wells of a 6 well plate and cultured overnight at 37°C. Cells were then transferred to a Category 3 Safety laboratory before transfecting with virus-encoding RNA in order to produce HCVcc viral particles. 1μg of HCV RNA was incubated with 2μl of Lipofectamine 2000 (Invitrogen) in 2ml of DMEM containing 10% FCS and 0.1 mM NEAA with no antibiotics at room temperature for 20 minutes. A mock negative control was also prepared using sterile water in place of RNA. Supernatant was removed from the Huh7.5 cells and replaced with the RNA/lipofectamine mix and incubated for 5 hours at 37°C in 5% CO_2 in a humidified incubator. Following a 5 hour transfection period, supernatant was removed and replaced with DMEM containing 10% FCS and 0.1 mM NEAA with no antibiotics and incubated for a further 72 hours.

To harvest HCVcc viral particles, supernatant was collected and replaced with fresh medium at 72, 96, 120 and 144 hour time points following transfection and stored at 4°C. Media was clarified by centrifugation at 3,000x*g* for 10 minutes and then sterile-filtered (0.22μm cellulose acetate, Corning Inc, Corning, NY). For short term use (1 month) HCVcc was protected from light and stored at 4°C. For longer term storage HCVcc viral particles were stored at -80°.

2.10.4 Infection Of Huh7.5 Cells

2.10.4.1 HCVcc Infection

Huh7.5 cells were seeded in a 96 well plate at 7,500 cells per well or a 6 well plate at 100,000 cells per well. Following overnight attachment cells were then serum starved for 2 hours and treated as indicated. Cells were then infected with HCVcc at a multiplicity of infection (MOI) of 1 or 0.5, where the MOI is the ratio of viral particles to cells. Cells were infected for 48 or 72 hours before being fixed or lysed.

2.10.4.2 Virus Titration Of HCVcc

In order to establish how much virus is present in the stock a viral titration must be undertaken and the tissue culture infectious dose 50 ($TCID_{50}$/ml) established (Reed and Muench, 1938). The $TCID_{50}$/ml is a statistical calculation where the virus titer

determination is based on where the first negative well(s) is observed. It is therefore required to have a statistically significant number of replicates for each dilution. Each well is infected with HCVcc and then examined to check if any cells are positive for HCV staining. An Excel spreadsheet is then set up with the calculations shown in **Table 2.2** and used to determine the $TCID_{50}$/ml (Lindenbach, 2005).

Volume 0.1ml [a]

Dilution [b]	1.00E+00	1.00E+01	1.00E+02	1.00E+03	1.00E+04	1.00E+05
Total Wells [c]	6	6	6	6	6	6
Positive Wells [d]						
Negative Wells [e]						
Cumulative Positive [f]						
Cumulative Negative [g]						
% Infected [h]						
Prop Dist [i]						
$TCID_{50}$ [j]						
$TCID_{50}$/ml [k]						

Table 2.2 Sample Spreadsheet for $TCID_{50}$/ml Determination

a The volume of the infectious virus inoculum in the experiment.

b Reflects the 1:10 serial dilution of the virus for the experiment.

c The total number of wells used for infection of that dilution.

d The number of positive wells for the dilution determined experimentally.

e The number of negative wells for the dilution determined experimentally.

f The sum of positive wells in *d* starting at that column and including the subsequent values.

g The sum of negative wells in *e* starting at that column and including the previous values.

h The percentage of cumulative positive for that column *d*/total number of wells *c*

i =IF(E10=100,IF(F10<51,(E10-50)/(E10-F10),0),IF(E10=0,0,(E10-50)/(E10-F10))).

j =IF(G11>0,10??(LOG(G4)-G11),"). Note " is a script for Excel to repeat.

k =IF(G11>0,1/D3*1/G12,"). Here the dilution factor *a* is taken into account to determine the final $TCID_{50}$/ml.

To establish the $TCID_{50}$/ml 7,500 Huh7.5 cells per well were seeded into 38 wells of a 96 well plate and allowed to attach overnight in a 37°C incubator with 5% CO_2. Viral samples were serially diluted 10-fold in complete growth medium and used to infect the seeded cells. Cells were infected with 100μl of neat to 10^{-5} dilutions with 6 replicate per dilution. 1 well was also used to confirm that the mock negative control did not contain any viral particles by seeding a neat sample of the mock negative control. Another well was infected with neat HCVcc and used as a negative control for 9E10 antibody staining. Cells were incubated for 48 hours and then fixed and stained for HCV NS5A as previously described. The results were then used to calculate the $TCID_{50}$/ml using the spreadsheet described in Table 2.1.

2.10.4.3 Immunofluorescent Staining

Infected cells had supernatant removed and were fixed at -20°C for 20 minutes with a glacial 1:1 acetone:methanol mix. Cells were permeabilized with 0.1% Triton-X 100 for 5 minutes and blocked for 30 minutes with 3% BSA in PBS. Cells were then washed 3 times with PBS. HCV NS5A protein was then detected by overnight incubation with a 1:2000 dilution of 9E10 primary antibody (Apath, NY). Following a further 3 PBS washes, bound 9E10 antibody was detected by 1 hour incubation with anti-mouse FITC-conjugated secondary antibody and washed 3 times with PBS.

2.10.4.4 Viral Concentration

Virus was concentrated by addition of one-fourth volume sterile-filtered 40% (w/v) polyethylene glycol-8000 in PBS (final 8% (w/v)) and overnight incubation at 4°C. Virus precipitates were collected by centrifugation at 8,000x*g* for 15 minutes and resuspended in DMEM containing 10% FCS and 0.1 mM NEAA with no antibiotics.

2.10.5 MTT Metabolism Assay

The purpose of an MTT assay is to acertain cell viability of populations of cells. In normal, healthy cells, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is reduced by mitochondrial reductases into formazan. When MTT is reduced to formazan, it undergoes a colour changes from yellow to purple. These formazan crystals can then be dissolved in an acidic solution and the absorbance can be quantified using a spectrophotometer.

Following treatment of cells media was removed from cells. 100μl MTT, at a concentration of 1mg/ml in sterile PBS, was then added to each sample. The plate was then wrapped in tin foil and incubated for 2 hours at 37°C. MTT was then removed and 100 μl DMSO per sample added and incubated at 37°C for 10 minutes. The absorbance was then read on a plate reader at 595nm.

Chapter III

The expression of PKC isotypes in the Huh7 hepatocyte cell line and their role in IFNα-stimulated STAT activity.

CHAPTER III

3.1 INTRODUCTION

IFNα activates the JAK/STAT pathway in order to produce an antiviral response. The JAK family have been identified as the kinases required for phosphorylation of STAT proteins following IFNα stimulation (González-Navajas *et al.*, 2012). Other cofactors such as PKCs are also necessary for STAT phosphorylation in a range of cell types (Yanase N *et al.*, 2012, Gartsbein *et al.*, 2005). In spite of this, PKC expression in hepatocytes has been poorly described and their role in STAT phosphorylation following IFNα stimulation requires further investigation.

3.1.1 PKC expression in hepatocytes.

PKC isotype expression and function in normal hepatocytes has not been studied in detail. The Human Protein Atlas is a database of protein expression in various human tissue and cell lines. PKC protein expression data has been deposited there for most PKC isoforms and is summarised in **Table 3.1.**

PKC	α	β	γ*	δ	ε	η	θ	ζ	ι
Hepatocyte Expression	+	-	+	+	-	-/+	N/A	++	-
Main Location	C	-	C	C PM	-	C PM	N/A	C	-
Additional Locations	PM	-	PM		-	-	N/A	PM	-

Table 3.1: PKC protein expression in normal hepatocyte tissue. Protein expression is detected by immunohistochemical staining. Expression levels: - Negative, + Weak, ++ Moderate, +++ Strong, N/A Not Available. Protein localisation: C Cytoplasm, PM Plasma Membrane, N Nucleus. N/A Not Available. *Although PKCγ shows weak staining in normal liver cells this is likely to be due to antibody cross-reactivity with a different PKC isoform as PKCγ RNA is not transcribed in normal liver cells.

3.1.2 Expression of PKC isoforms in the Huh7 cell line.

Primary human hepatocytes are difficult to grow in cell culture. Human hepatocellular carcinoma cell lines have been developed to allow for *in vitro* examination. The Huh7 cell line and its derivatives are the main cell line used for studies on HCV. PKC δ, ε, ζ and a truncated version of θ have been detected in the Huh7 cell line. In 2007 Takai *et al* used cell lysate fractionation to show that PKCδ is present in the nucleus and cytosol of untreated Huh7 cells, while Nakashima *et al.*, (2010) detected PKC δ and ε, PKCζ has also been identified in Huh7 cells and found to reduce hepatic steatosis (Gupta *et al.*, 2010). PKCθ is expressed in Huh7 cells as a truncated splice variant that lacks part of its substrate binding region in its catalytic domain in comparison to a normally expressed PKCθ gene and protein in Jurkat T cells (Masuoka *et al.*, 2009). The expected molecular weight for PKCθ of 79kDa is detected by Western blot in Jurkat cells, however, in Huh7 whole cell extract PKCθ was detected as a single band at 55kDa. Furthermore, Northern blot analysis demonstrated a 2.8kb band in Jurkat cells but a 1.9kb band in Huh7 cells.

3.1.3 PKCs in IFNα mediated STAT activation.

Classical PKC activity is required for IFNα stimulated tyrosine phosphorylation of STAT1 and STAT3 in Huh7 cells. Investigations in this cell line showed that pharmacological inhibition of cPKCs decreased IFNα induced STAT phosphorylation and ISG expression (Fimia *et al.*, 2004). My group have identified PKCβ as a specific cPKC required for STAT3 tyrosine phosphorylation (unpublished). The involvement of other PKCs with the STAT pathway has been documented, in particular the role of PKCδ in STAT serine phosphorylation. PKCδ phosphorylates STAT1 and/or STAT3 in response to a variety of stimuli such as IFNα, IFNγ, IL-6 and insulin in a range of cells, including the HepG2 cell line (Uddin *et al.*, 2002, Deb *et al.*, 2003; Jain *et al.*, 1999, Gartsbein *et al.*, 2005). To date the roles of other PKC isotypes in IFNα signalling in hepatocytes has not been investigated.

3.2 HYPOTHESIS & OBJECTIVES

It is clear that PKC is involved in IFNα signalling in hepatocytes and that HCV can specifically target PKC activity in T cells. The role of PKCs in the IFNα signalling in hepatocytes needs to be further explored, however, PKC isotypes present in the Huh7 hepatocyte cell line have not been well characterized and need to be investigated. It is important to fully uncover IFNα signalling mechanisms in hepatocytes as HCV infection may target these pathways in order to evade host defences.

I hypothesise that not all PKC isoforms expressed in Huh7 cells have been uncovered so far and that PKCs alter localisation following IFNα treatment. I further hypothesise that novel or atypical PKC activity is required for IFNα induced STAT1 and STAT3 phosphorylation in Huh7 hepatocytes.

Therefore the objectives of this chapter are:

- To fully characterize PKC expression and localization in Huh7 hepatocytes.
- To determine if IFNα alters individual PKC isoform localization in Huh7 hepatocytes.
- To examine the requirement for PKC activity in IFNα induced STAT1 and STAT3 phosphorylation in Huh7 hepatocytes.

3.3 RESULTS

3.3.1 The expression and subcellular localisation of PKC isoforms in Huh7 cells.

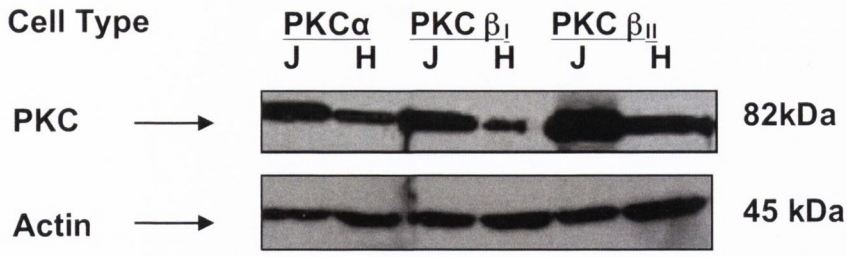
PKCs are a ubiquitous family of kinases that are required for a wide variety of cellular functions. It has been demonstrated that classical PKCs are required for IFNα induced tyrosine phosphorylation of STAT1 and 3 and an effective response against HCV *in vitro* (Fimia *et al.*, 2004). My group recently determined that PKCβ is the classical PKC specifically required for IFNα induced tyrosine phosphorylation of STAT1 and 3 (Mullins S. unpublished). Other PKC isoforms are required for STAT phosphorylation in various cell types (Yanase *et al.*, 2012). The full range of PKCs present in the Huh7 liver cell line and their response to IFNα has not been characterised. I therefore investigated the effect of IFNα on individual PKC isoform localisation in Huh7 cells and examined the requirement of PKC expression for IFNα induced STAT 1 and 3 phosphorylation.

I first established which PKCs are present in the Huh7 cell line. Huh7 cells were grown to 70-80% confluence and lysed with NP40 lysis buffer containing protease inhibitors. SDS-PAGE was performed with 10μg lysate per well. Jurkat T cell lysate was used as a positive control for PKC isoform expression except for PKCι where the lung carcinoma cell line A549 was used. PKC isoforms were then detected by Western blot by using specific antibodies for individual PKC isoforms as described in chapter 2. As shown in **Figure 3.1** the PKCs present in Huh7 cells are α, β_I, β_{II}, δ, ε, θ, ζ and ι. The novel PKC η was not detected by Western Blot in Huh7 cell lysate. Of note PKCθ was detected at a lower than expected molecular weight of 55kDa. Jurkat T cell lysate was used as a positive control for all PKC isoforms except for PKCι, where the lung carcinoma cell line A549 was used.

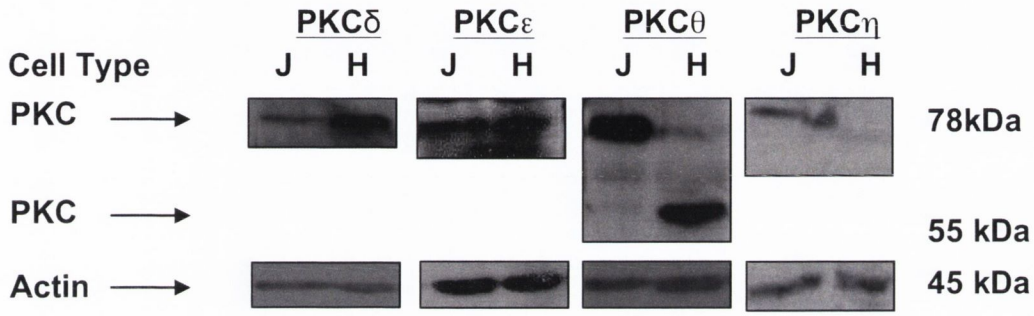
To further characterise the PKC isoforms present in Huh7 cells, I performed immunofluorescent staining of fixed Huh7 cells using antibodies specific for individual PKC isoforms as depicted in chapter 2. These cells were then examined by confocal laser scanning microscopy. As displayed in **Figure 3.2,** I elucidated the localisation of PKC α, β_I, β_{II}, δ, ε and ζ in resting cells. PKCα localised in the perinuclear area with punctate staining. PKCβ is expressed as two isoforms, β_I and β_{II}, and both were examined using specific antibodies for each. PKCβ_I showed predominantly nuclear localisation with low levels of

cytoplasmic staining while PKCβ_{II} displayed a markedly different staining pattern in comparison, being distributed exclusively in the cytoplasm. The novel PKC, PKC δ, also showed predominantly nuclear localisation with low levels of cytoplasmic staining but the other novel PKC, PKCε, was distributed within punctate compartments in the cytoplasm. PKCζ was located in the cell membrane with lower levels of cytoplasmic staining also observed. PKCs θ and ι could not be visualised by confocal immunofluorescent staining using the antibodies available within my laboratory. It is possible that the epitope for these proteins is not available to the antibody in fixed whole cells or were within compartments that were not permeablised, therefore preventing the antibody binding.

A



B



C

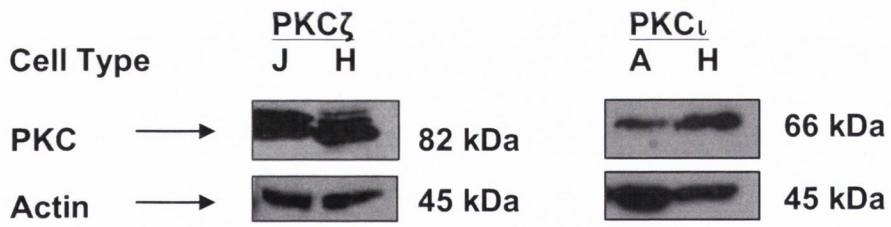


Figure 3.1: Huh7 hepatocytes express classical, novel and atypical PKCs. A) Western blot demonstrates the presence of the classical PKC isoforms α, β_I and β_{II}. B) Western blot demonstrates the presence of the novel PKC isoforms δ and ε at their expected molecular weights. PKCθ is present in Huh7 cells at 55 kDa, which is lower than in Jurkat T cells. PKCη is expressed in Jurkat T cells but not in Huh7 cells. C) Western blot demonstrates that the atypical PKCζ is expressed at 82 kDa in Huh7 cells while PKCι has a molecular weight of 66 kDa. Actin is shown as a positive control for the presence of protein. The figure is presented as a representative of three independent experiments with similar results.

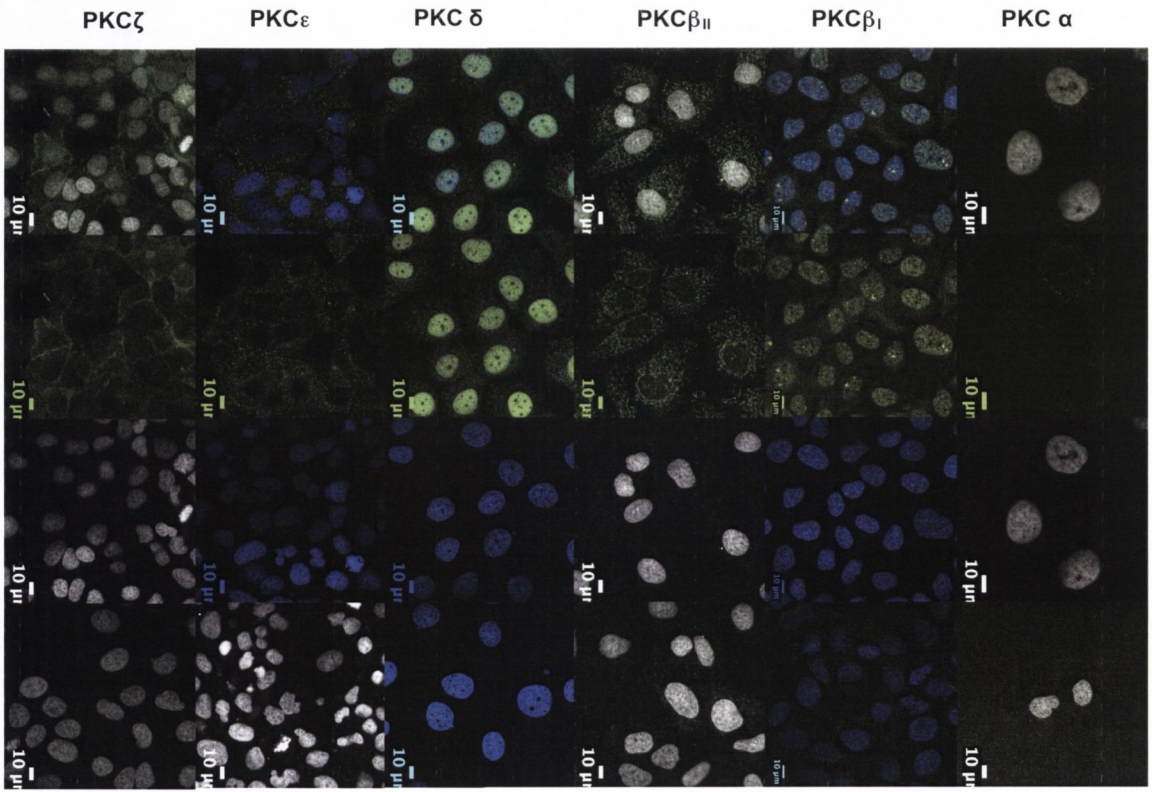


Figure 3.2 PKC isoforms are located at different subcellular locations throughout Huh7 cells. Huh7 cells were grown to 70 80% confluence and serum starved for 4 hours. Cells were fixed in 4% paraformaldehyde for 30 minutes. Cells were permiabilized and incubated with antibodies for specific PKC isoforms and the DNA stain Hoechst as described in Chapter 2. Negative controls where no primary antibody for PKC isoforms was added are shown. Images were taken using a confocal laser-scanning microscope using a 63x lens and are shown as representative samples of three independent experiments showing similar results

3.3.1.1 IFNα induces the subcellular redistribution PKC α, δ and ζ in Huh7 cells.

An alteration in sub-cellular localisation is associated with a change in PKC activity. In order to examine if IFNα is able to alter PKC localisation I stimulated resting Huh7 cells with 1000U/ml of IFNα for time points of up to two hours. Cells were then fixed and PKC localisation was compared between resting and stimulated cells using immunofluorescent confocal microscopy. Cells were also treated with 10ng/ml PMA for 30 minutes, which activates classical and novel PKCs. Orthogonal slices of cells with all treatments can be viewed for each PKC isoform in **Appendix III**. As can be seen in **Figure 3.3** PKCα is translocated from the perinuclear area into punctate compartments throughout the cytoplasm following two hours of IFNα treatment. This translocation was in contrast to PMA treatment that showed PKCα localisation remaining in the perinuclear area. PKCβI is predominantly expressed in the nucleus of resting cells but also shows a lower level of cytoplasmic staining. Following two hours of IFNα treatment PKCβI is still mainly localised in the nucleus however, its levels in the cytoplasm are increased (**Figure 3.4**). PKCβII was not translocated in response to IFNα or PMA and remained in the cytoplasm for all treatments (**Figure 3.5**). PKCδ colocalisated with the nucleus following two hours of IFNα treatment with lower cytoplasmic levels compared to resting cells or cells treated with PMA (**Figure 3.6**). PKCε maintained its punctate staining following 30 minutes of IFNα or PMA treatment. There was a marked reduction in this punctate staining pattern after one hour of IFNα treatment and a decrease in immunofluorescent staining signal following two hours IFNα treatment (**Figure 3.7**). The atypical PKCζ showed a dramatic alteration in localisation following IFNα treatment (**Figure 3.8**). Following 30 minutes of IFNα treatment, PKCζ withdrew from the membrane to the cytoplasm and could not be observed in the membrane after one hour IFNα treatment. Furthermore gaps could be identified between cells revealing that cell:cell contacts were not maintained following 2 hours IFNα treatment.

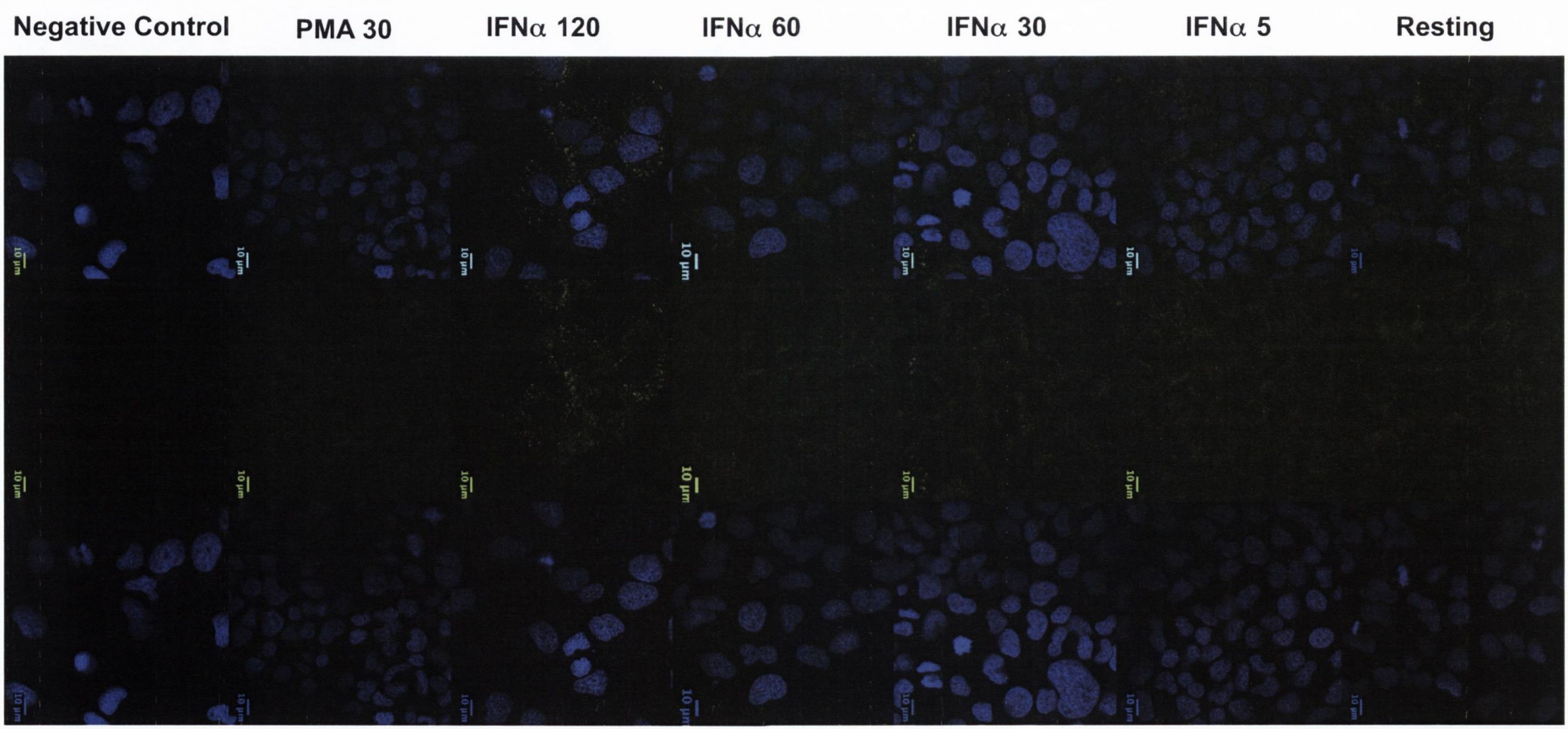


Figure 3.3 PKCα translocates from the perinuclear area to punctate compartments in Huh7 cells following IFNα stimulation. Huh7 cells were serum starved for 4 hours and then treated with 1000U/ml IFNα for time points as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCα and the DNA stain Hoechst labeling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Cells were treated for 30 minutes with 10ng/ml PMA as a positive control for PKC activation. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining.

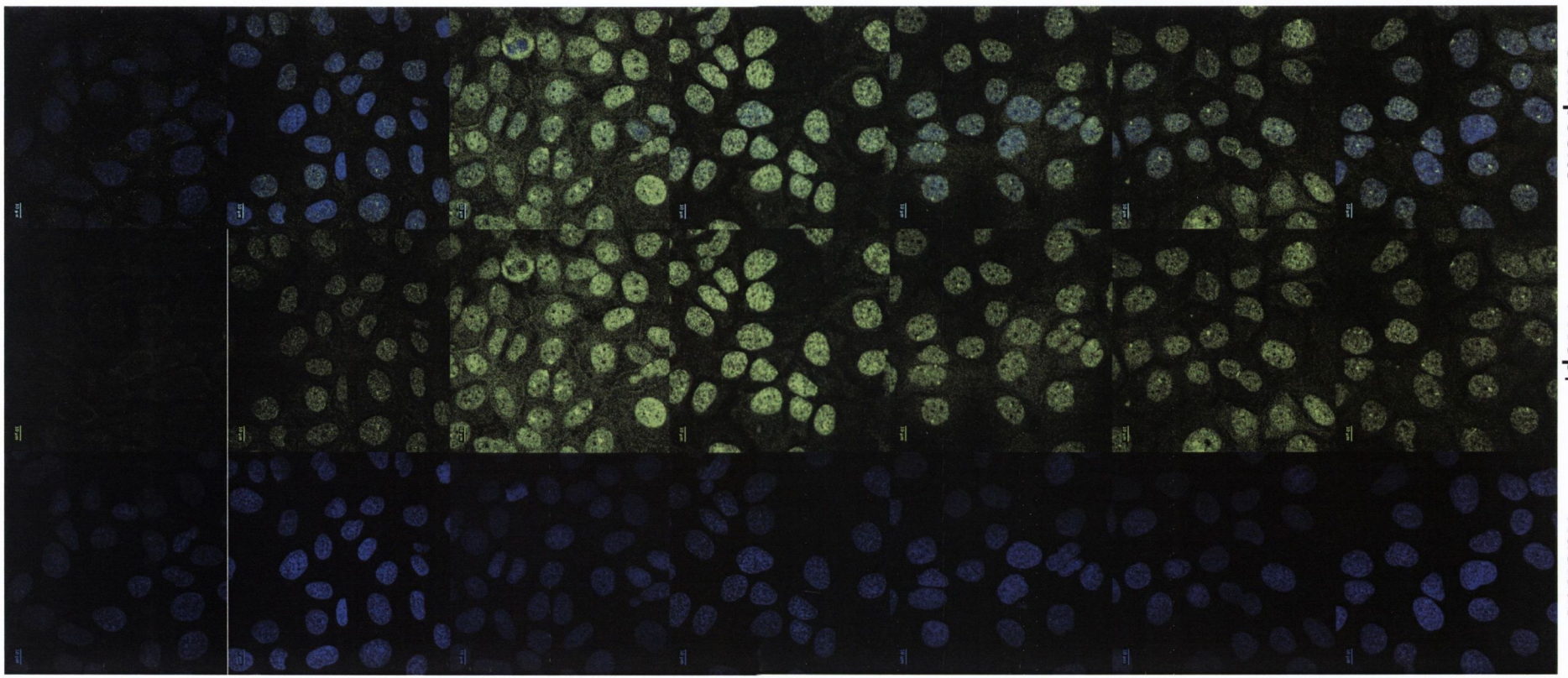


Figure 3.4 PKCβ_I is located in the nucleus of Huh7 cells and shows increased cytoplasmic staining following IFNα stimulation. Huh7 cells were serum starved for 4 hours and then treated with 1000U/ml IFNα for time pointsas indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCβ_I and the DNA stain Hoechst labeling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Cells were treated for 30 minutes with 10ng/ml PMA as a positive control for PKC activation. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining.

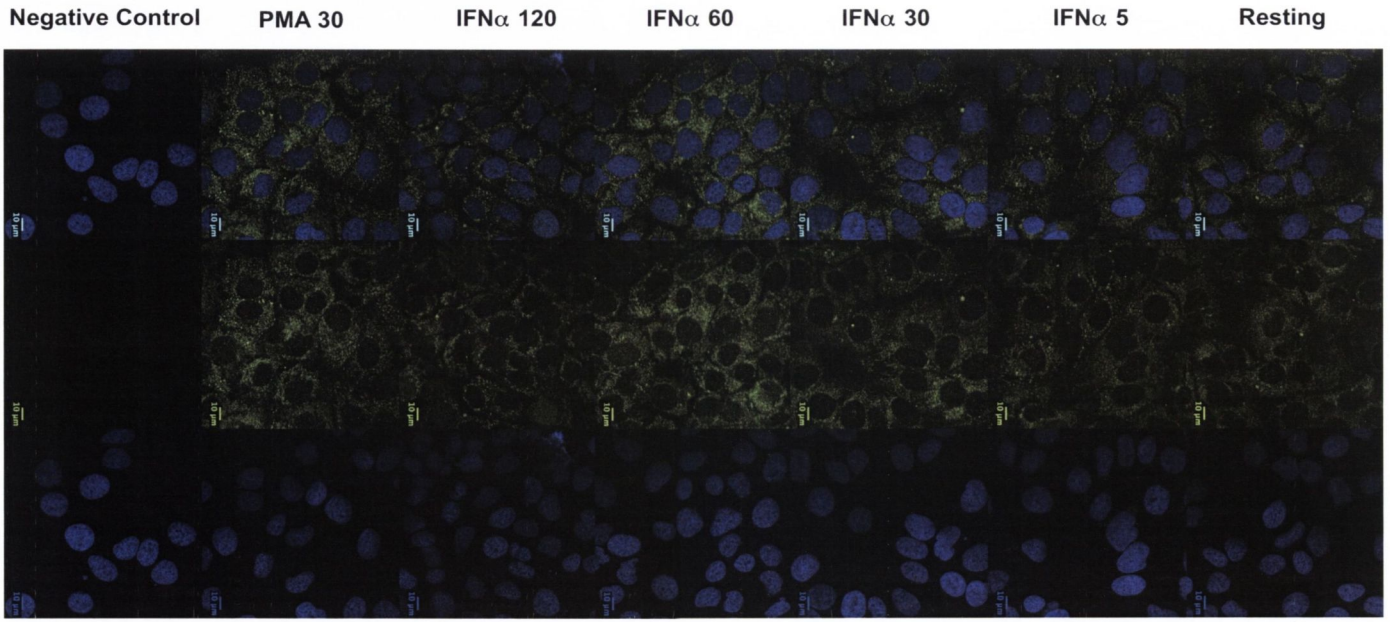


Figure 3.5 PKCβII is located in the cytoplasm of Huh7 cells is not translocated following IFNα stimulation. Huh7 cells were serum starved for 4 hours and then treated with 1000U/ml IFNα for time points as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCβII and the DNA stain Hoechst labeling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Cells were treated for 30 minutes with 10ng/ml PMA as a positive control for PKC activation. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining.

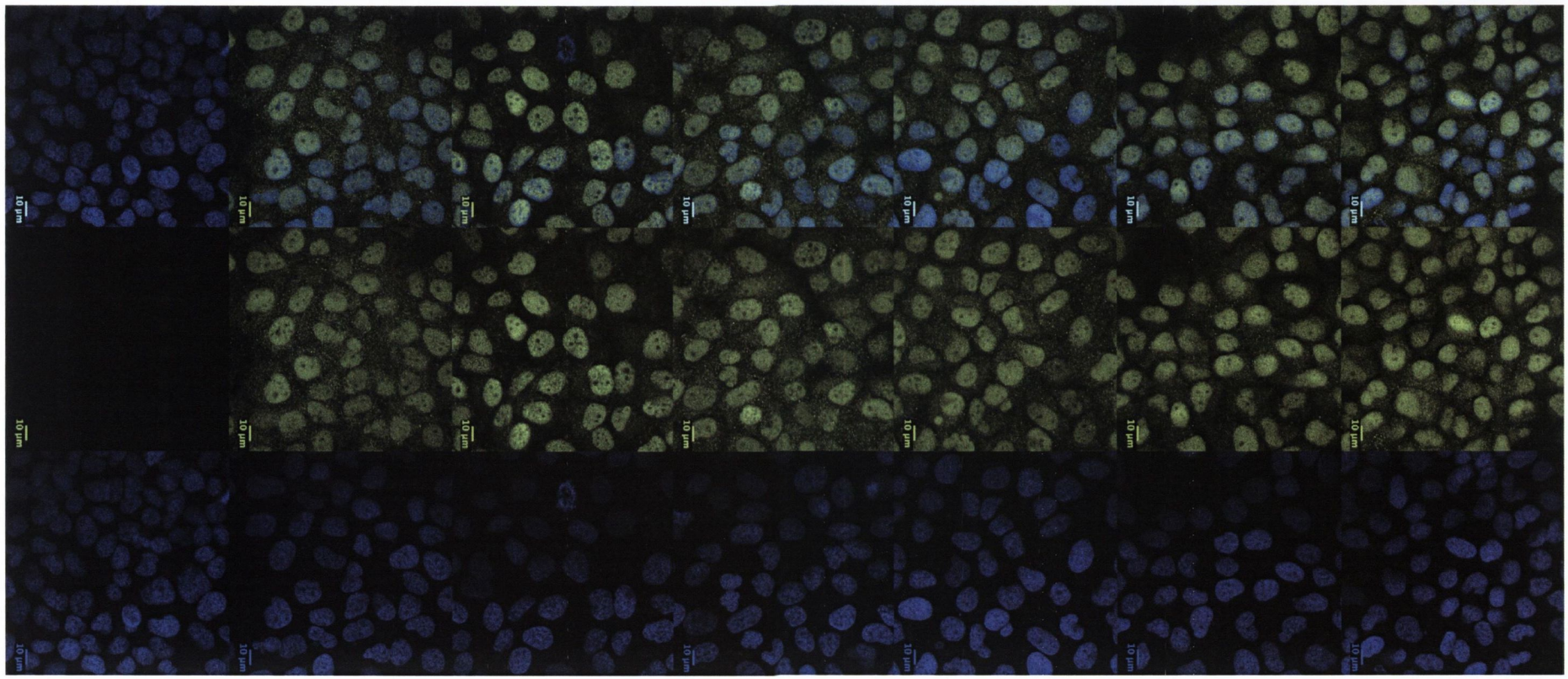


Figure 3.6 PKCδ is located in the nucleus and cytoplasm of Huh7 cells and is localized in the nucleus exclusively following IFNα stimulation. Huh7 cells were serum starved for 4 hours and then treated with 1000U/ml IFNα for time points as indicated Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCδ and the DNA stain Hoechst labeling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Cells were treated for 30 minutes with 10ng/ml PMA as a positive control for PKC activation. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining.

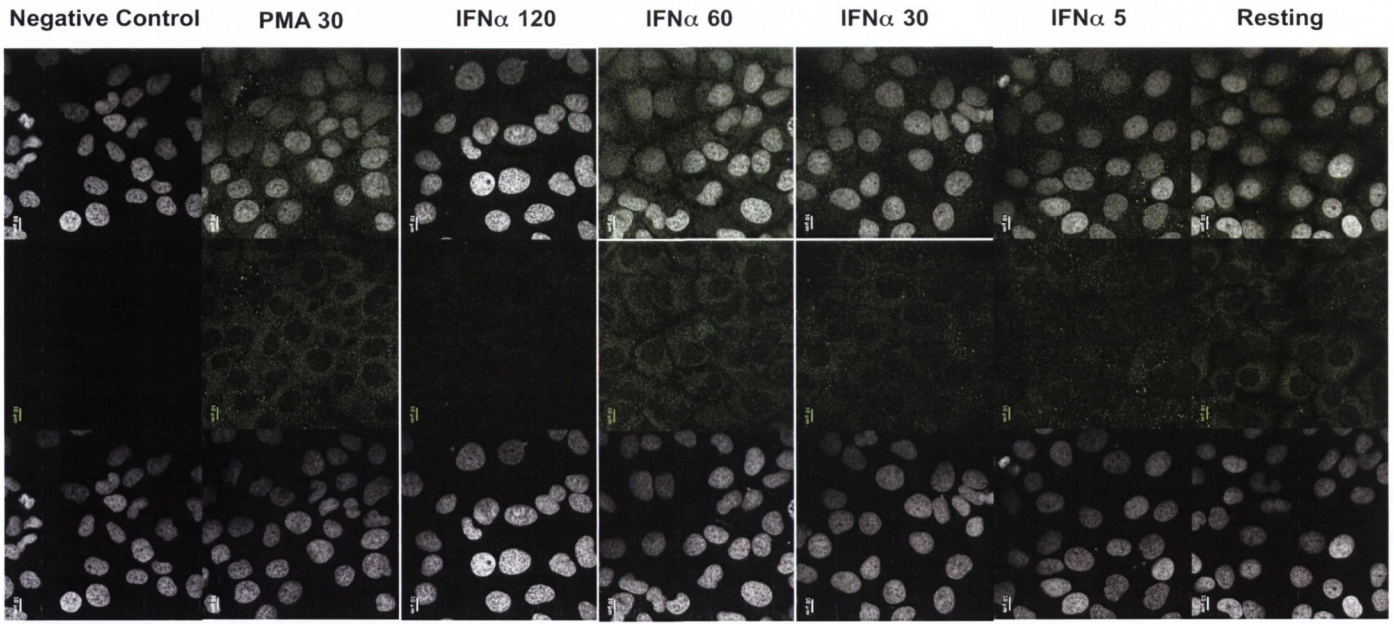


Figure 3.7 PKCε is located in the cytoplasm and within punctate compartments which are reduced following IFNα stimulation of Huh7 cells. Huh7 cells were serum starved for 4 hours and then treated with 1000U/ml IFNα for time points as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCε and the DNA stain Hoechst labeling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Cells were treated for 30 minutes with 10ng/ml PMA as a positive control for PKC activation. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining.

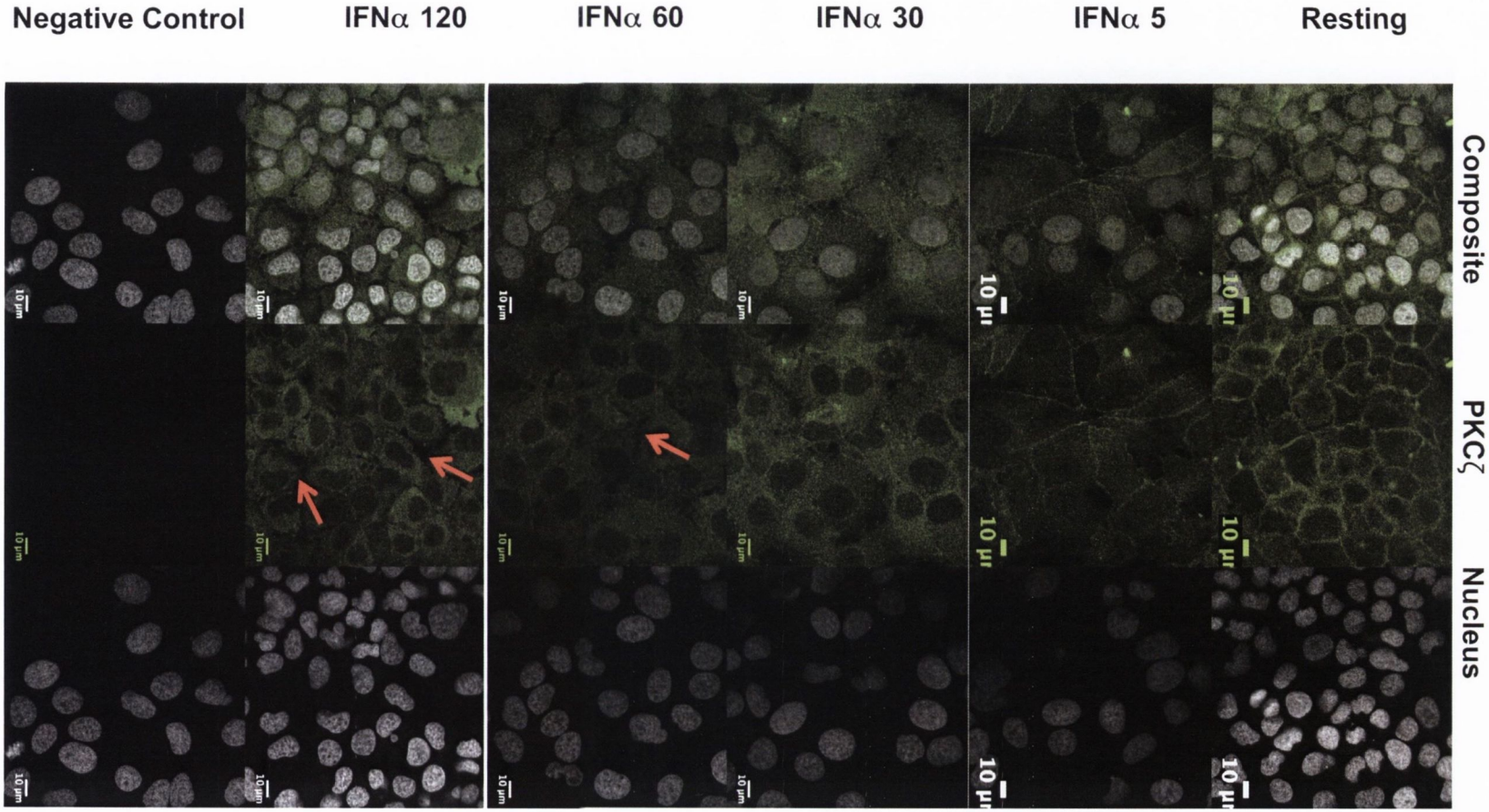


Figure 3.8 PKCζ is located at the membrane of resting Huh7 cells and is translocated to the cytoplasm following IFNα stimulation. Huh7 cells were serum starved for 4 hours and then treated with 1000U/ml IFNα for time points as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCζ and the DNA stain Hoechst labeling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Following 1 hour IFNα treatment cell:cell contacts were diminished and following 2 hour IFNα treatment gaps formed between cells as indicated by red arrows. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining

3.3.2 IFNα induces tyrosine 701/705 phosphorylation at different rates on STAT 1 and STAT3 but serine 727 is constitutively phosphorylated in Huh7 cells.

IFNα is known to induce the phosphorylation of STAT1 and 3 at tyrosine 701/705 respectively in Huh7 cells and of serine 727 in other cell types. I established the time frame required for IFNα to induce tyrosine phosphorylation of STAT 1 and 3 and examined if IFNα induced serine 727 phosphorylation in these cells. As seen in **Figure 3.9 A** STAT 1 is phosphorylated at tyrosine 701 following 5 minutes of IFNα stimulation while STAT3 1 is phosphorylated at tyrosine 705 following 30 minutes of IFNα stimulation. Both STAT proteins showed robust tyrosine phosphorylation after one hour IFNα treatment. In contrast **Figure 3.9 B** demonstrates that STAT 1 and 3 are constitutively phosphorylated at serine 727 and IFNα treatment does not increase the level of phosphorylation at this site.

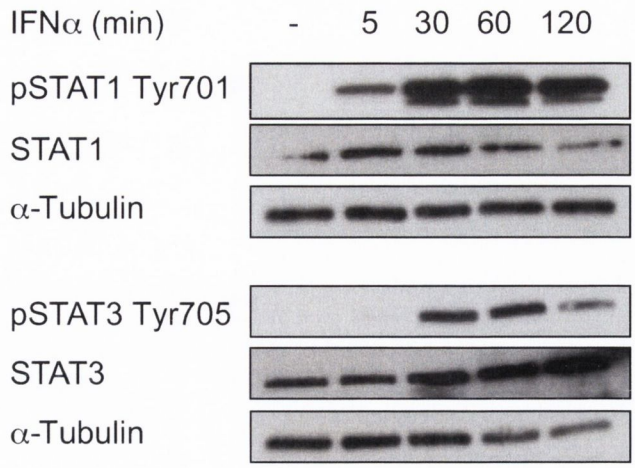
3.3.2.1 IFNα induced STAT tyrosine phosphorylation is prevented by Rottlerin but not by PKCζ inhibition in Huh7 cells.

Knowing that IFNα can alter PKC localisation and phosphorylate tyrosine sites in STAT 1 and 3 via classical PKCs I next examined the role of novel and atypical PKC activity for STAT phosphorylation. I pretreated cells with pharmacological inhibitors of novel and atypical PKC before IFNα stimulation for 30 minutes. I then extracted protein from these cells and performed SDS-PAGE and Western blot using phospho-specific STAT antibodies.

To inhibit novel PKC activity cells were pre-treated with 1 or 10μM Rottlerin for 30 minutes before being stimulated with IFNα for one hour. **Figure 3.10** demonstrates that 10μM Rottlerin prevented IFNα induction of tyrosine phosphorylation of STAT 1 and 3. Pre-treatment with DMSO was also included as a control for Rottlerin and showed a small decrease in IFNα induced STAT1 tyrosine 701 phosphorylation compared to cells stimulated with IFNα alone. The role of PKCζ in IFNα induced tyrosine phosphorylation of STAT 1 and 3 was also investigated. A myristoylated PKCζ pseudosubstrate inhibitor was used to block PKCζ activity prior to IFNα stimulation. It can be seen in **Figure 3.11** that PKCζ activity is not required for IFNα induced phosphorylation of STAT 1 and 3 at tyrosine

701 and 705 respectively. Taken together, these results demonstrate that novel PKCs are required for IFNα induced phosphorylation of STAT 1 and 3 at tyrosine 701/705 but not for serine 727 phosphorylation. Furthermore PKCζ is not required for IFNα induced phosphorylation of STAT 1 and 3 at tyrosine 701/705.

A



B

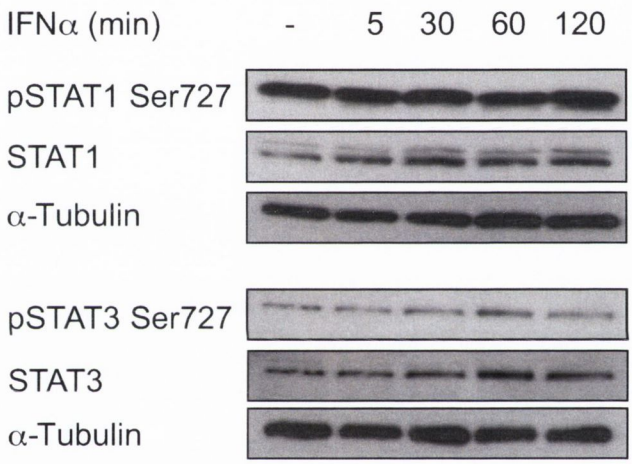


Figure 3.9 IFNα phosphorylates STAT1 at tyrosine 701 and STAT3 at tyrosine 705 in Huh7 cells. STAT1 and STAT3 are constitutively phosphorylated at serine 727 in Huh7 cells. Huh7 cells were grown to 70-80% confluence and serum starved for 4 hours. Cells were then treated with 1000U/ml IFNα for the times indicated. 10μg Huh7 lysate was loaded per well and STAT isoforms were identified by Western blot using phospho- and isoform specific antibodies. A) The time required by IFNα to induce tyrosine phosphorylation of STAT 1 and 3. B) STAT1 and STAT3 are constitutively phosphorylated at serine 727 and that IFNα treatment does not lead to an increase in phosphorylation levels of this residue. The figure is presented as a representative of three independent experiments with similar results.

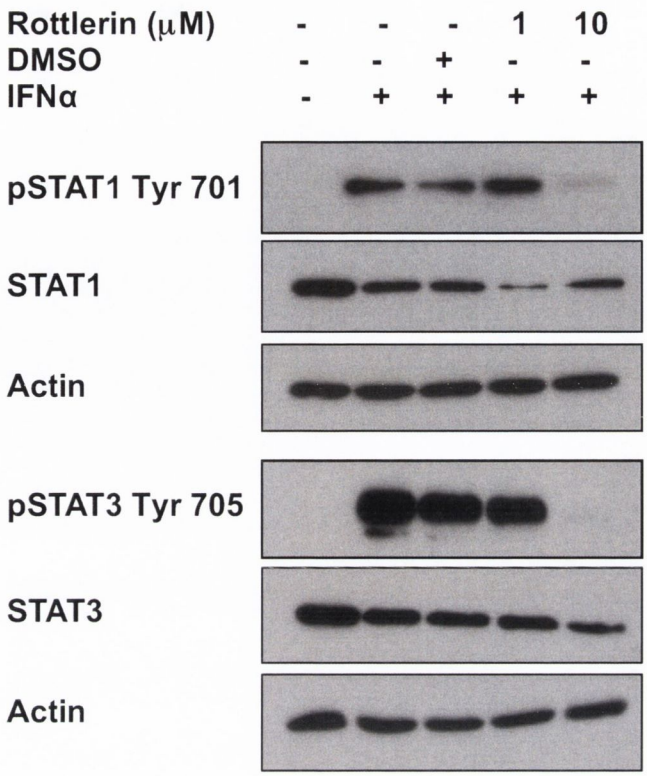


Figure 3.10 Rottlerin inhibits IFNα induction of tyrosine phosphorylation at STAT1 and STAT3. Huh7 cells were grown to 70-80% confluence and serum starved for 4 hours. Cells were then treated with Rottlerin at 1 or 10 μM for 30 minutes before 1000U/ml IFNα for the times indicated. 10μg Huh7 lysate was loaded per well and STAT isoforms were identified by Western blot using phospho- and isoform specific antibodies. 10μM Rottlerin pre-treatment is able to prevent the IFNα induction of tyrosine phosphorylation of STAT 1 and 3. The figure is presented as a representative of three independent experiments with similar results.

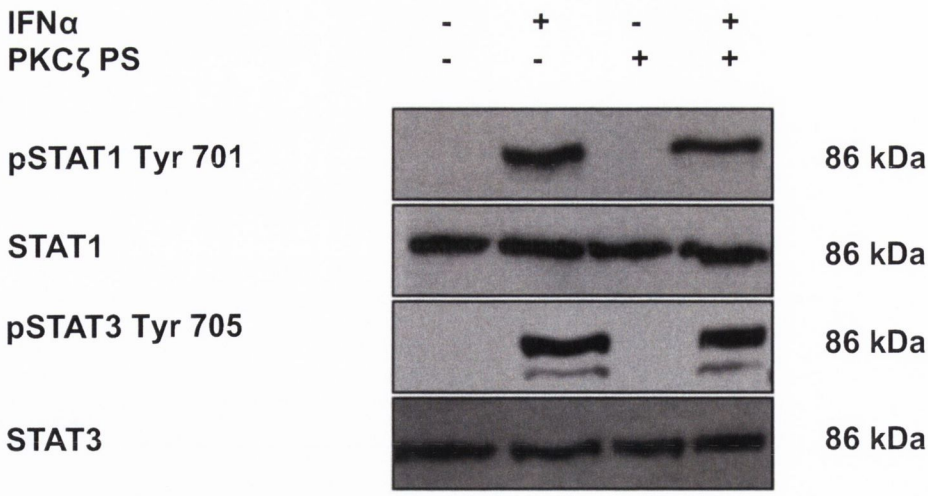


Figure 3.11 PKCζ activity is not required for IFNα induced tyrosine phosphorylation of STAT1 or STAT3. Huh7 cells were grown to 70-80% confluence and serum starved for 4 hours. Cells were then treated with 10 μM of myristoylated PKCζ pseudosubstrate inhibitor before 1000U/ml IFNα for the times indicated. 10μg Huh7 lysate was loaded per well and STAT isoforms were identified by Western blot using phospho- and isoform specific antibodies. The figure is presented as a representative of three independent experiments with similar results.

3.3.3 Ceramide cannot prevent the IFNα induced redistribution of PKCζ.

Our findings demonstrated that IFNα can redistribute PKCζ from the membrane and disrupt cell:cell contacts. Previously, a dominant-negative PKCζ model showed that disruption of cell:cell contacts could be rescued by treating cells with C16, which specifically bound to PKCζ (Wang *et al.*, 2009). I therefore decided to investigate if pre-treating cells with ceramide 16 (C16) could prevent IFNα redistribution of PKCζ or cell:cell contact disruption. Cells were treated with 10 or 25μM of C16 for either 15 minutes (**Figure 3.12 A, B**) or 60 minutes (**Figure 3.12 C, D)** prior to two hour IFNα stimulation. Cells were also treated with dihydroceramide 16 (DHC) as a negative control for C16 activity (Stiban *et al.*, 2006). As C16 and DHC were resuspended in ethanol or chloroform respectively, a comparable volume of these chemicals to the amount used for C16 and DHC treatments were incubated with cells as a vehicle control and did not induce any effects (**Figure 3.12 E**). Neither C16, DHC, ethanol nor chloroform treatment on their own did not alter the localization of PKCζ or prevent the IFNα induced redistribution of PKCζ or disruption of cell:cell contacts.

3.3.4 Ceramide does not induce tyrosine phosphorylation of STAT 1 or 3.

As ceramide can activate PKCζ (Sánchez AM *et al.*, 2008) and I observed that IFNα could alter the localization of the serine/threonine kinase PKCζ and induce STAT 1 and 3 tyrosine phosphorylation, I queried if C16 could also induce STAT 1 and 3 tyrosine phosphorylation. As can be seen in **Figure 3.13** neither C16 nor DHC could stimulate tyrosine phosphorylation of STAT 1 or 3 or prevent their phosphorylation by IFNα. This indicates that PKCζ is not involved in tyrosine phosphorylation of STAT1 at 701 or STAT3 at 705 and taken together with previous results from this chapter demonstrate that while IFNα can alter PKCζ localization PKCζ is not involved in IFNα induced STAT 1 or 3 tyrosine phosphorylation.

In this chapter I have shown that PKC isoforms are situated in a range of localisations in Huh7 cells and that PKC isoforms α, δ and ζ translocate following IFNα stimulation. Two hours treatment of this cytokine also diminishes cell:cell contact. I also demonstrate that

novel but not atypical PKCs may be required by IFNα to induce tyrosine phosphorylation of STAT 1 and 3 but serine STAT 1 and 3 phosphorylation is constitutive and not altered by IFNα stimulation or PKC inhibition. I also preclude the involvement of ceramide C16 in the IFNα induced translocation of PKCζ or phosphorylation tyrosine STAT 1 or 3.

Figure 3.12 A 10μM Ceramide pre-treatment for 15 minutes cannot prevent the IFNα induced redistribution of PKC ζ. Huh7 cells were serum starved for 4 hours and then pre-treated with 10μM ceramide C16 or DHC for 15 minutes before 1000U/ml IFNα for 2 hours as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCζ and the DNA stain Hoechst, labelling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Following 2 hour IFNα treatment gaps appeared between cells as indicated by red arrows. Neither ceramide nor DHC pre-treatment altered the effect of IFNα on cell:cell contact. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining.

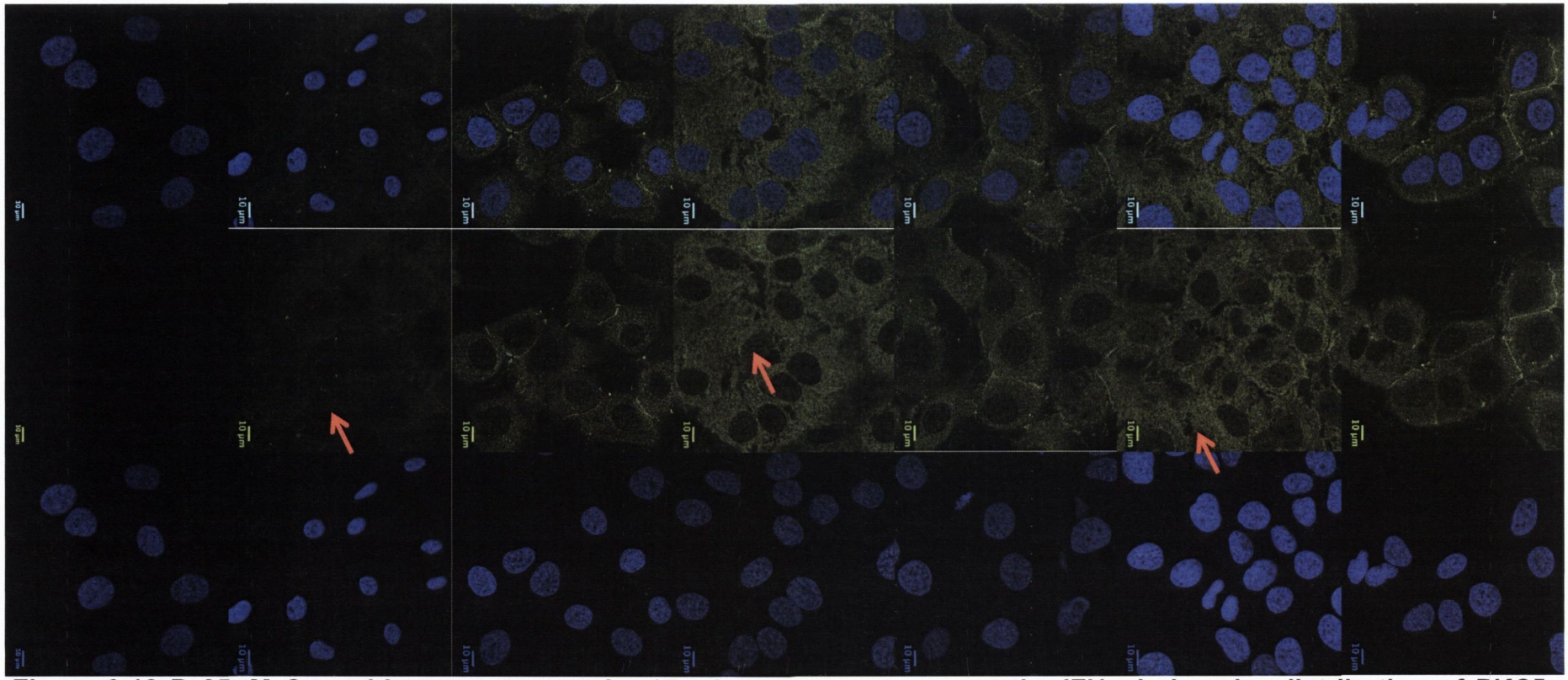


Figure 3.12 B 25μM Ceramide pre-treatment for 15 minutes cannot prevent the IFNα induced redistribution of PKCζ. Huh7 cells were serum starved for 4 hours and then pre-treated with 25μM ceramide C16 or DHC for 15 minutes before 1000U/ml IFNα for 2 hours as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCζ and the DNA stain Hoechst, labelling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Following 2 hour IFNα treatment gaps appeared between cells as indicated by red arrows. Neither ceramide nor DHC pre-treatment altered the effect of IFNα on cell:cell contact. A negative control where no PKC primary

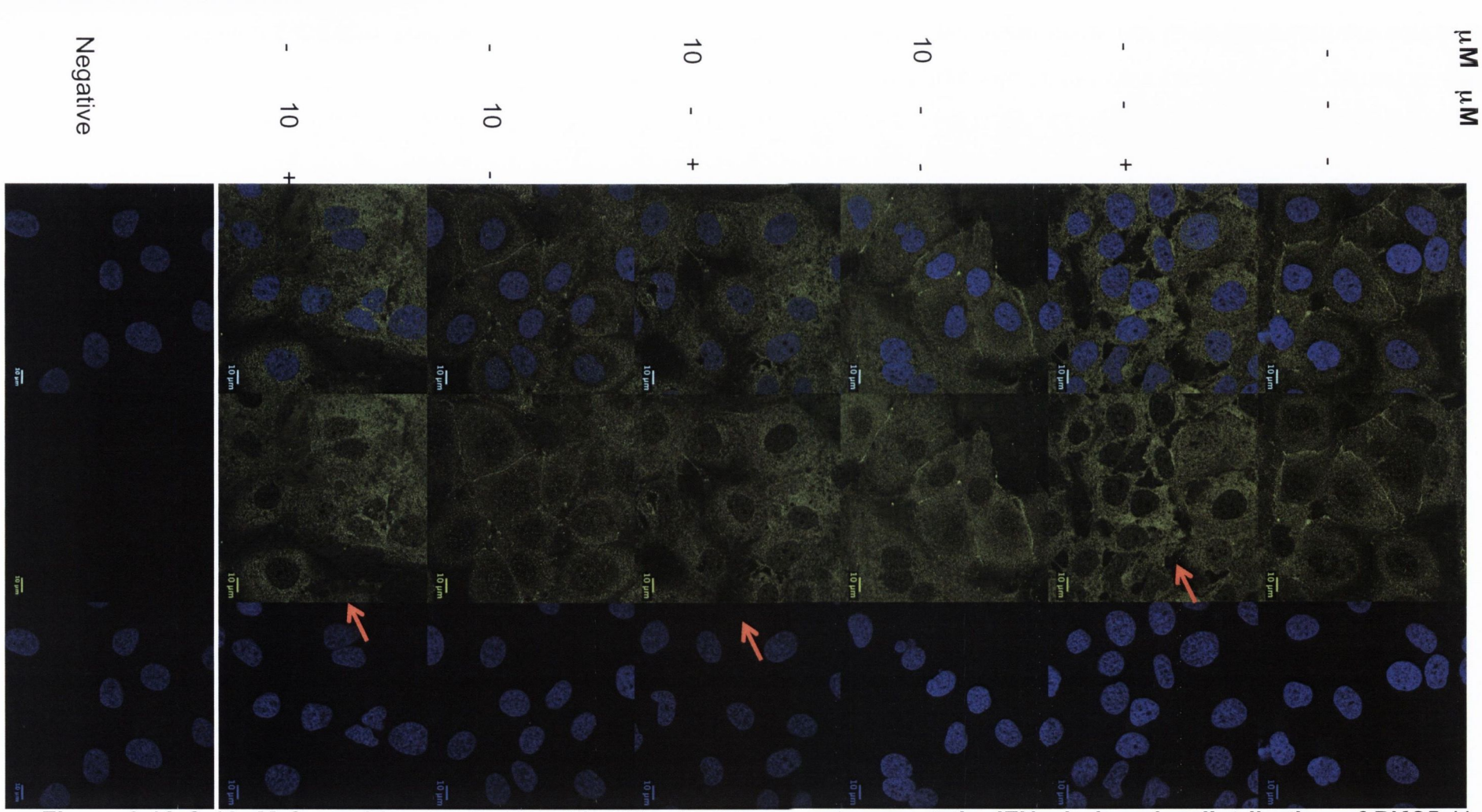


Figure 3.12 C 10μM Ceramide pre-treatment for 60 minutes cannot prevent the IFNα induced redistribution of PKCζ. Huh7 cells were serum starved for 4 hours and then pre-treated with 10μM ceramide C16 or DHC for 60 minutes before 1000U/ml IFNα for 2 hours as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCζ and the DNA stain Hoechst, labelling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Following 2 hour IFNα treatment gaps appeared between cells as indicated by red arrows. Neither ceramide nor DHC pre-treatment altered the effect of IFNα on cell:cell contact. A negative control where no PKC primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining.

Figure 3.12 D 25μM Ceramide pre-treatment for 60 minutes cannot prevent the IFNα induced redistribution of PKCζ. Huh7 cells were serum starved for 4 hours and then pre-treated with 25μM ceramide C16 or DHC for 60 minutes before 1000U/ml IFNα for 2 hours as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCζ and the DNA stain Hoechst, labelling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Following 2 hour IFNα treatment gaps appeared between cells as indicated by red arrows. Neither ceramide nor DHC pre-treatment altered the effect

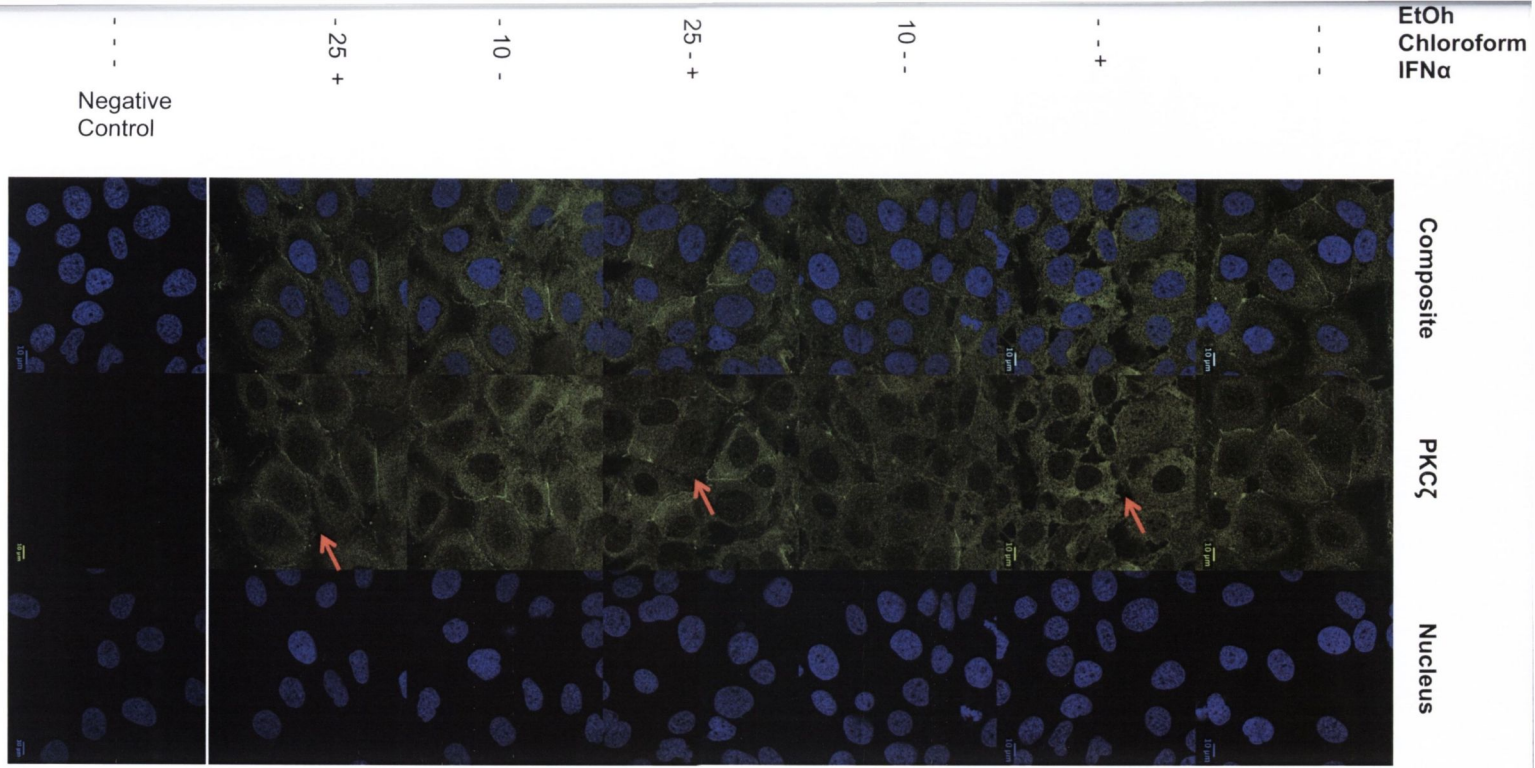
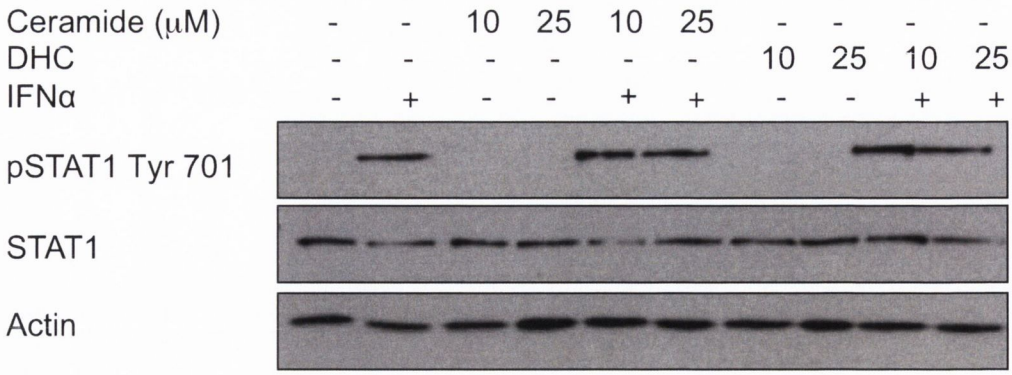


Figure 3.12 E 60 minutes of ethanol or chloroform treatment does not induce translocation of PKCζ or cell:cell contact disruption. Huh7 cells were serum starved for 4 hours and then pre-treated with ethanol or chloroform, at similar volumes to those used for ceramide C16 and DHC experiments as a vehicle control, 60 minutes before 1000U/ml IFNα for 2 hours as indicated. Cells were then fixed in 4% PFA and incubated with a primary antibody specific for PKCζ and the DNA stain Hoechst, labelling the nucleus. Cells were examined by immunofluorescent laser scanning confocal microscope at 63x magnification. Following 2 hour IFNα treatment gaps appeared between cells as indicated by red arrows. Neither ethanol nor chloroform pre-treatment altered the

A



B

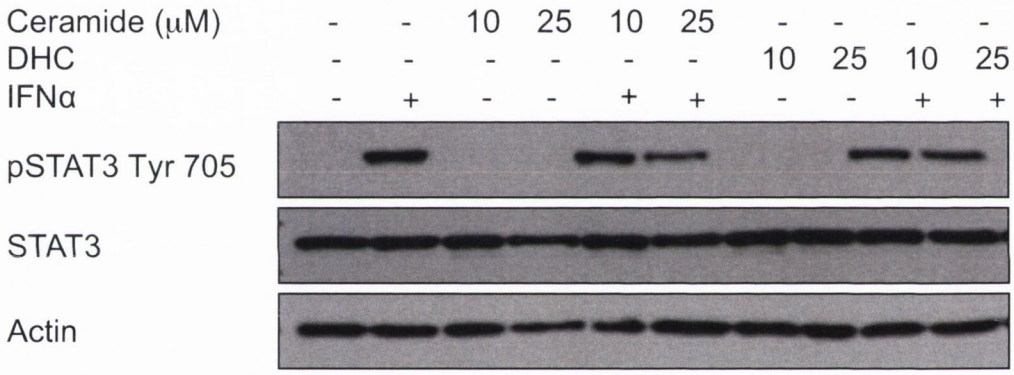


Figure 3.13 Ceramide does not induce STAT 1 or 3 tyrosine phosphorylation or prevent IFNα induced tyrosine phosphorylation of STAT 1 or 3. Huh7 cells were grown to 70-80% confluence and serum starved for 4 hours. Cells were then treated with ceramide (C16) or dihydroceramide (DHC) at 10μM or 25μM for 60 minutes. Control cells were not pre-treated with C16 or DHC. Cells were then stimulated with 1000U/ml IFNα for 30 minutes as indicated.10μg Huh7 lysate was loaded per well and STAT isoforms were identified by Western blot using phospho- and isoform specific antibodies. 10μM or 25μM ceramide (C16) or DHC is not able to prevent the IFNα induction of tyrosine phosphorylation of STAT 1 and 3. The figure is presented as a representative of three independent experiments with similar results.

3.4 DISCUSSION

IFNα is the central component of HCV treatment regimens. However, despite the recent development of direct acting antiviral medications, viral clearance rates can still be less than 50% of the patients infected with certain genotypes (Zeuzem S., 2008). This is because HCV has developed various methods of evading the immune system, including interrupting signalling of both endogenously produced and exogenously administered IFNα (Miyagi *et al.*, 2012). An effective immune response to HCV infection requires a robust IFNα response that can signal through the Jak/STAT pathway and initiate the transcription of antiviral ISGs. IFNα binds to its cell membrane receptors to induce a signalling cascade that phosphorylates STAT proteins. Once phosphorylated, STATs then dimerise and translocates to the nucleus, where they operate as transcription factors. Their gene products induce an antiviral state within the cell and also target the virus directly.

STATs have been shown to require PKCs to enable their activity in a range of cell types (Lacreusette *et al.*, 2009; Jain *et al.*, 1999; Kwak *et al.*, 2009) PKCs are a family of ten serine/threonine kinases that are universally expressed. In hepatocytes it has been demonstrated that classical PKC inhibition prevents IFNα induced tyrosine phosphorylation of STAT1 and STAT3 (Fimia *et al.*, 2004). However, a full characterisation of the specific PKCs present in hepatocytes remains to be undertaken. Although the Jak/STAT pathway has been widely studied in a variety of cell types, further characterisation is required to fully understand which PKCs are expressed and are required for IFNα signalling in hepatocytes.

I hypothesised that not all PKC isoforms had been identified in the Huh7 hepatocarcinoma cell line. In this study I assemble a profile of the PKC isoforms that are expressed in Huh7 cells. This was achieved by SDS electrophoresis followed by Western blotting using specific antibodies. The subcellular localisation of each PKC isotype was then identified by confocal immunofluorescent staining. PKCs have predicted molecular weights in between 79 & 84 kDa, except for PKCθ which was detected at 55 kDa, and have been located in a range of subcellular compartments.

The Huh7 cell line was established from hepatoma tissue that was surgically removed from a 57-year-old Japanese male with well-differentiated hepatocellular carcinoma (Nakabayashi *et al.*, 1982). Huh7 cells have very low expression of the dsRNA sensor TLR-3 (Li *et al.*, 2005). Its sub clone, Huh7.5, has a single point mutation in its RIG-I, leading to higher permissiveness for HCV RNA replication compared to other hepatocyte cell lines (Sumpter *et al.*, 2005).

The classical PKCs α, β_I and β_{II} were all detected at their expected molecular weights. When their subcellular localisation was investigated, PKCα displayed perinuclear localisation surrounding the nucleus, which could be identified by staining DNA with the stain Hoechst, a fluorescent bis-benzimidedye compound that binds the minor groove of double-stranded DNA. PKCβ is expressed as two isotypes. Protein kinase $C\beta_I$ and $PKC\beta_{II}$ are generated by alternative splicing from a single gene, but they differ at their C-terminal 50 (β_I) or 52 (β_{II}) residues. (Newton, 1995). My studies show that these isotypes are located in vastly different locations. $PKC\beta_I$ displayed a nuclear localisation, colocalising with Hoechst while $PKC\beta_{II}$ was dispersed throughout the cytoplasm. My group has recently discovered that PKCβ is required for IFNα induced tyrosine phosphorylation of STAT1 and 3. As STAT phosphorylation occurs in the cytoplasm, I can now infer that $PKC\beta_{II}$ is the isoform responsible for mediating this phosphorylation. However, other kinases must be involved in this process as PKCs are unable to directly phosphorylate tyrosine residues. The nuclear localisation of $PKC\beta_I$ is unusual but not unique and suggests that constitutively activated. In T-cells $PKC\beta_I$ can be translocated to the nucleus following PMA stimulation (Zhu P *et al.*, 2010). $PKC\beta_I$ then phosphorylates NF90. Phosphorylated NF90 is exported from the nucleus and stabilises IL-2 mRNA, allowing for its expression and involving nuclear $PKC\beta_I$ in gene expression. Nuclear expression of $PKC\beta_I$ is correlated with gene expression and tumour cell proliferation in prostate carcinoma (Metzger E *et al.*, 2010). $PKC\beta_I$ promotes gene expression by phosphorylating histone H3. Following phosphorylation H3 is unable to be regulated by lysine-specific demethylase 1 demethylation, leading to uncontrolled gene expression. As the Huh7 cell line is derived from a hepatocarcinoma it is possible that such a transcriptional imbalance occurs here also.

The expression of PKC isoforms has also been investigated in the HepG2 cell line. HepG2 cells are hepatocytes that can polarise in cell culture and form basic canaliculi between cells (Decaens *et al.*, 2008). In HepG2 cells PKCα has been shown to be involved in vascular endothelial growth factor (VEGF) induced tight junction (TJ) disruption (Schmitt *et al.*, 2004). Following VEGF stimulation, PKCα is rapidly translocated to the plasma membrane from the cytoplasm while occludin is dispersed from TJs, leading to a decrease in the formation of canaliculi. Furthermore, this phenotype can be prevented by the pre-treatment with the PKC inhibitor Gö6850 or induced by PMA. PKCα has also been implicated in both cell cycle arrest (Chiang *et al.*, 2010) and cell cycle progression and proliferation (Wu 2006) in HepG2 cells. TPA can activate PKCα, which then induces the expression of MiR-101, inducing cell arrest. MiR-101 is a microRNA that targets the degradation of components of the PRC2 complex. PRC2 enhances methylation of histone 3 lysine 27 and its degradation promotes apoptosis in hepatocytes. However, TPA induced activation of PKCα does not alter the expression of MiR-122, a microRNA associated with HCV infection (Varambally *et al.*, 2008). In contrast, the estrogen 17β-Estradiol which is associated with liver development activates PKCα and induces cell cycle progression (Marino *et al.*, 2002).

Of the novel PKCs, δ and ε were both expressed at their predicted molecular weight. Corresponding to a previous report by Takai *et al.*, 2007 that used cell lysate fractionation to examine the subcellular localisation of PKCδ, I observed this isotype predominantly in the nucleus with cytoplasmic staining also recognisable. Takai demonstrated that PKCδ is involved in regulation of phosphorylation of heat shock protein (HSP) 27. Treating Huh7 cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activated PKCδ, which then signaled through the p38 MAPK to induce phosphorylation of HSP27 and its anti-tumor properties. In HCC patients, phosphorylation of HSP27 inversely correlated with tumour stage. Studies have demonstrated that PKCδ is cleaved by caspase-3 to generate a constitutively activated catalytic fragment, which amplifies apoptosis cascades in nucleus and mitochondria. In smooth muscle cells PKCδ null mice of were resistant to apoptosis induced by UV, TNFα, or hydrogen peroxide (Leitges *et al.*, 2001), whereas embryonic fibroblasts (MEFs) cells were resistant to UV radiation-induced apoptosis (LaGory *et al.*, 2010). Loss of PKCδ also protected salivary glands against c-irradiation-induced apoptosis *in vivo* (Humphries *et al.*, 2006). Further demonstrating the role of PKCδ in regulating cell

death, PKCδ activation induced caspase-3-dependent apoptosis in three hepatocellular carcinoma (HCC) cell lines: Huh7, Hep3B and PLC5 (Hung *et al.*, 2008). In these cells PKCδ was activated by FTY720, a synthetic sphingosine immunosuppressant. In contrast, TPA activated PKCδ and PKCε also block Huh7 transforming growth factor-β1 (TGF-β1) induced apoptosis (Nakashima *et al.*, 2010). TGF-β1 increases the expression of programmed cell death 4 (PDCD4) during apoptosis. However, TPA activated PKCδ and PKCε induced the proteasomal degradation of PDCD4 and inhibited cell death. However, PKCδ also exerts its anti-apoptotic and pro-survival roles in some cases. TNFα has been shown to target PKCδ to the nucleus, where it was required for NFκB activity and cell survival (Lu *et al.*, 2009). Therefore, the varied role of PKCδ in apoptosis appears to be stimulus and cell type dependent. In the context of Huh7 cells it is possible that PKCδ is involved in a pathway to maintain the cell survival of this carcinoma line, while being separated from locations that it would otherwise become activated and induce apoptosis.

PKCε showed a punctate pattern in the cytoplasm and did not translocate following IFNα stimulation. The defined staining pattern of PKCε suggests that it is bound to a scaffold or chaperone. PKCε has been shown to interact with scaffolds and chaperones such as the Trif-related adapter molecule (TRAM) as part of the TLR-4 signalling pathway in mice (McGettrick *et al.*, 2006), the 14-3-3 scaffolds during mitosis (Saurin *et al.*, 2008) and Receptors for Activated C-Kinase2 (RACK2) to direct localisation of PKCε to locations such as the Golgi apparatus and myofilaments (Csukai *et al.*, 1997, Huang *et al.*, 2004). These provide initial leads for future studies of PKCε in Huh7 cells. PKCθ was not analysed further due to its unusual cleaved form being unlikely to represent a physiologically representative protein. In agreement with its corresponding Western blot experiments; no staining pattern was detected for PKCη, confirming that this isotype is not present in Huh7 cells. When examined by immunofluorescent staining PKCζ was shown to be located at the cell membrane. This is consistent with the evolutionarily conserved role of aPKCs in directing polarity and cell:cell contacts (Suzuki *et al.*, 2001).

PKCθ was detected at a lower than predicted molecular weight of 55 kDa corresponding to a previous finding by Masuoketa *et al.*, 2009 who had reported that PKCθ is expressed in Huh7 cells as a cleaved protein that is missing its regulatory domain due to a transcription

of a splice variation that is lacking exons at its 3' terminal. Truncated PKCθ exerted a pro-apoptotic influence in Huh7 cells in response to the lipid palmitate, potentially via an increase in the expression of DAG. Palmitate induced phosphorylation of PKCθ within its autoinhibitory domain at threonine 538. Phosphorylation at this site induces a conformational change in PKCs and allows for their binding of substrates (Liu *et al.*, 2002). PKCθ then either directly or indirectly promoted phosphorylation the anti-apoptotic MCL-1 protein at Ser159. Once phosphorylated, MCL-1 underwent proteasomal degradation by ubiquitination. As MCL-1 is a member of the anti-apoptotic Bcl-2 family, its degradation fosters palmitate-induced apoptosis. Transcription of truncated PKCθ protein appears to be unique to Huh7 cells was not analysed further due to its unusual cleaved form being unlikely to represent a physiologically representative protein in non-Huh7 liver cells. PKCη was not detected in Huh7 cells despite it being weakly detected in immunohistological stains of primary hepatocytes deposited in the Human Protein Atlas database.

Classical PKC activity has previously been shown to be required for STAT tyrosine phosphorylation by IFNα in Huh7 cells (Fimia *et al.*, 2004). Other PKC isotypes are required for phosphorylation of STAT proteins in a range of other cell types in response to a variety of stimulants (Park *et al.*, 2002; Bhattacharjee *et al.*, 2006; Litherland *et al.*, 2010). PKC activation often corresponds to subcellular translocation to the plasma membrane (Oliva *et al.*, 2005), while STAT phosphorylation results in nuclear translocation from the cytoplasm. I hypothesised that IFNα treatment would alter PKC localisation to the membrane or nucleus in Huh7 cells, suggesting PKC activation or interaction with activated STAT proteins respectively. Therefore PKC isotype localisation was investigated following IFNα stimulation at various time points.

PKC α, δ and ζ all showed a change in localisation following two hours IFNα treatment. PKCα is located in the perinuclear area of resting cells however, following 2 hours IFNα treatment this isotype translocates and displays a punctate pattern in the cytoplasm. PKC δ is activated by IFNα and interacts with STAT1 to induce its serine phosphorylation in the U-266 myeloma cell line and Molt-4 acute lymphoblastic leukemia cell line (Uddin *et al.*, 2002). I have shown that PKC δ displays a nuclear and cytoplasmic localisation in Huh7 cells but shows a decrease in cytoplasmic staining with a more predominant nuclear

localisation following 2 hours IFNα treatment. This may indicate that in Huh7 cells IFNα also induces PKCδ to complex with STAT proteins that then translocate to the nucleus following STAT phosphorylation.

In resting Huh7 cells I found that PKCζ was predominantly localized in areas of cell:cell contact and to a lesser extent in the cytoplasm. PKCζ showed a dramatic redistribution when I treated Huh7 cells with IFNα. At 1 hour following IFNα treatment PKCζ withdraws from the cell membrane, while at 2 hours following IFNα treatment cell:cell contacts are disrupted with gaps forming between the cells. As a membranous localization can indicate PKC activation this may indicate that PKCζ is constitutively activated but 2 hours IFNα exposure leads to an inhibition of its activation and translocation to the cytoplasm. This effect on PKCζ might be directly mediated by the IFNAR or could be through an intermediary protein however, I have not determined this process as yet. As aPKCs such as PKCζ are involved in cell polarity and cell:cell adhesion, the removal of PKCζ from the membrane may be the reason why I observe the abolition of cell:cell contacts. This interesting finding is further investigated in Chapter 5.

PKCζ is required to reduce hepatic steatosis in Huh7 cells (Gupta *et al.*, 2010). Hepatic insulin resistance is believed to lead to hepatic triglyceride storage and the development of steatosis. Patients with type 2 diabetes take Extendin-4 to augment their insulin production (Drucker 2001). Exendin-4 is also an agonist of the glucagon-like Peptide-1 receptor (GLP-1R) and has been shown to reduce hepatic steatosis in *ob/ob* mice (Ding *et al.*, 2006). Huh7 cells treated with Exendin-4 showed increased phosphorylation of PKCζ. This lead to a reduction of hepatic triglyceride storage (steatosis) without the requirement of further insulin treatment. Furthermore RNAi of GLP-1R was shown to prevent Extendin-4 induced phosphorylation of PKCζ and prevented the reduction in triglyceride storage.

PKCζ was located in the cytoplasm HepG2 cells (Ducher *et al.*, 1995). Following PMA stimulation PKCζ was present exclusively in the membrane. PKCζ translocation following PMA suggests that its activation may be indirectly induced, as PKCζ is not directly activated by phorbol esters. PKCζ can also be activated by pitavastatin in HepG2 cells (Arii *et al.*, 2010). Pitavastatin is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor that enhances human serum paraoxonase (PON1) gene promoter activity. Once

activated by pitavastatin, PKCζ phosphorylates the Sp1 transcription factor which increases its binding to the PON1 promoter and subsequent gene transcription.

PKC expression in the Chang liver cell line has also been investigated. Chang liver cells are a cell line that was originally derived from normal liver tissue and subsequently established via HeLa cell contamination (Kang *et al.*, 2004). Chang liver cells treated with PMA showed the activation of PKCs in a time dependent manner from between 5-30 minutes (PKC α, β_I, θ and ζ) to 12 hours for PKCε, while PKCδ was constitutively active. PKC activation led to the phosphorylation and subsequent degradation of insulin receptor substrate 1, triggering insulin resistance and induction of the secretion of the atherogenic LDL ApoB (Jin *et al.*, 2008). Furthermore, ApoB production can be inhibited at the translational, but not transcriptional, level by classical PKC inhibition in HepG2 cells demonstrating that PKCs have an important role in lipoprotein production (Sidiropoulos *et al.*, 2007).

Results in my group previously demonstrated that the classical PKC β, but not α, is required for IFNα induced STAT3 tyrosine phosphorylation. However, there can be functional redundancy between PKC isotypes. As I have demonstrated that IFNα is capable of translocating PKCδ and PKCζ, I hypothesised that novel or atypical PKCs are required for IFNα induced STAT phosphorylation. I first established the time-points that IFNα phosphorylates STAT1 and STAT3 at tyrosine and serine residues. I then used pharmacological inhibitors of novel and atypical PKC subtypes and examined their effects on IFNα induced STAT phosphorylation.

In line with previous studies I demonstrated that IFNα induces phosphorylation at tyrosine 701/705 in STAT1/3 within 30 minutes of treatment. When I examined serine 727 phosphorylation of STAT1/3 I observed that this residue is constitutively phosphorylated and that IFNα does not induce an increase in its phosphorylation. I found that protein levels of total STAT1 detected seemed to decrease following two hours of IFNα treatment. This may be due to STAT tyrosine phosphorylation and translocation to the nucleus following IFNα treatment. This would lead to a decrease in cytoplasmic STAT while nuclear STAT would not be detected as the lysis buffer used to lyse these cells was NP40, which does not solubilize the nuclear fraction. Serine phosphorylation of STAT proteins is

associated with maximal transcriptional activity (Yokogami *et al.*, 1999; Lufei *et al.*, 2007) however, STAT proteins constantly shuttle in and out of the nucleus in un-activated cells. Not withstanding this it was unexpected that IFNα did not induce an increase in phosphorylation at this residue. However, this finding may be due to the oncogenic properties of this cell line as constitutive STAT serine phosphorylation is associated with tumour cell survival (Darnell JE Jr., 2002).

When cells were pretreated with the novel PKC inhibitor Rottlerin before IFNα activation tyrosine phosphorylation at serine 701/705 was dramatically inhibited compared to cells that did not have Rottlerin pretreatment. Others have reported that the novel PKC δ is required for STAT phosphorylation in response to IFNα and IL-6 in other cell lines (Uddin *et al.*, 2002; Novotny-Diermayr *et al.*, 2002). Again I found that the levels of total STAT proteins appear decrease following STAT tyrosine phosphorylation. This is probably due to the use of NP40 lysis buffer as described in the preceding paragraph. These results indicate that PKCδ is coordinated by IFNα and may be involved in the Jak/STAT pathway. However, the specificity of Rottlerin is controversial and cannot be relied upon (Soltoff, 2007; Tapia *et al.*, 2006). RNA interference (RNAi) that targets PKC isotypes δ and ε is required in order to conclusively establish if these PKC isotypes are involved in STAT activation. This has been further studied and is detailed in Chapter 4.

The requirement for atypical PKC activity in IFNα induced STAT activation was also examined. A PKCζ pseudosubstrate (PKCζPS) inhibitor was used to occupy the substrate-binding region of PKCζ and prevent its activation with its activators. When 701/705 tyrosine STAT1/3 phosphorylation was examined, PKCζPS pretreatment did not prevent IFNα inducing the phosphorylation of these residues. However, my immunofluorescent staining results demonstrated that IFNα was able to translocate PKCζ from the membrane and interrupt cell:cell contact. The requirement for PKCζ activity and localization in cell:cell contact has been further studied in Chapter 5

I also hypothesised that activation of PKCζ could induce STAT1/3 tyrosine 701/705 phosphorylation without the need for IFNα treatment. I pretreated cells with ceramide, a sphingolipid found at the plasma membrane and known to bind to the carboxyl-terminal of PKCζ and induce its activation (Wang G *et al.*, 2005; Fox TE *et al.*, 2007). As a control to

deactivate ceramide activity, I pretreated some cells with dihydroceramide (DHC). DHC is similar to ceramide except that it lacks a *trans* double bond at the 4, 5 position. It is an intermediate in the *de novo* ceramide synthesis pathway and is believed to be biologically inactive in that it cannot cause cells to undergo apoptosis (Bielawska *et al.*, 1993; Sugiki *et al.*, 2000). Dihydroceramide also does not form channels in planar phospholipid membranes or in liposomes (Siskind and Colombini 2000). It can also be inhibitory against ceramide activity, interfering with ceramide channel formation both in mitochondria and in liposomes (Stiban *et al.*, 2006). My results showed that ceramide C16 could not induce STAT tyrosine phosphorylation while DHC pretreatment did not prevent IFNα induced STAT tyrosine phosphorylation.

Ceramide has been shown to preserve cell:cell adhesion in epithelial cells that were induced have their epithelium integrity disrupted (Wang G *et al.*, 2009). Ceramide pretreatment did not alter PKCζ localization or prevent its translocalization by IFNα. This demonstrates that although IFNα can control PKCζ localization, PKCζ is not involved in the Jak/STAT pathway and that ceramide cannot inhibit IFNα induced translocation of PKCζ.

These results show for the first time the complete range of PKC isoforms expressed in the Huh7 cell line. I identify that these isoforms display a range of localisations, suggesting that different isoforms are involved in distinct roles in Huh7 cells and propose PKCβ_{II} as the specific isoform required for IFNα induced STAT 1 and 3 tyrosine phosphorylation. I have also shown that another kinases or kinases are required to tyrosine phosphorylate these as PKCs can not directly phosphorylate tyrosine residues and that Rottlerin, an inhibitor that has not previously been reported to inhibit PKCβ, was able to prevent IFNα induced STAT 1 and 3 tyrosine phosphorylation. I also show that IFNα is able to translocate PKC α, δ and ζ. The translocation of PKCζ away from the membrane corresponded with the appearance of gaps between the cells, demonstrating a new role for IFNα in regulating cell:cell contact and that this is not via ceramide signalling to PKCζ.

Chapter IV

The role of PKCs δ and ε in IFNα mediated STAT activation, nuclear translocation and ISG expression.

CHAPTER IV

4.1 INTRODUCTION

The involvement of PKCs with the STAT pathway has been previously documented, in particular the role of PKCδ in STAT serine phosphorylation. In Chapter III I demonstrated that the novel PKC inhibitor Rottlerin inhibits the IFNα induced tyrosine phosphorylation of STAT 1 and STAT 3. I further investigated these findings in Huh7 cells as PKCδ is required to phosphorylate STAT1 and/or STAT3 in response to a variety of stimuli such as IFNα, INFγ, IL-6 and insulin in a range of cells, including the HepG2 cell line (Uddin *et al.,* 2002, Deb *et al.,* 2003; Jain *et al.,* 1999, Gartsbein *et al.,* 2005). IFNα has been used to treat various cancers and the Jak/STAT pathway has been examined in many types of cancer (Uddin *et al.,* 2002). In myeloma U-266 and Molt-4 cells, once IFNα binds its receptor, PKCδ becomes phosphorylated and its kinase domain becomes active. PKCδ then interacts with STAT1 and phosphorylates it at serine 727. U-266 cells treated with IFNα show that STAT1 and PKCδ co-immunoprecipitate. It was also shown that luciferase activity was diminished in the dominant-negative PKCδ cells in comparison to cells over expressing a dominant-negative PKCε mutant. Conversely, in HepG2 cells, IL-6 activates PKCδ to directly bind STAT3 and phosphorylate it at Ser727. This phosphorylation leads to the inhibition of STAT3 DNA binding and transcriptional activity. This group also reported that STAT3 co-immunoprecipitated with PKCε in an IL-6 independent manner (Jain *et al.,* 1999).

Relationships between STAT proteins and other PKCs have also been described, although not with the same frequency as for PKCδ PKCε expression levels are correlated with the aggressiveness of prostate cancer while activation of STAT3 has also been shown to be involved in the development of prostate cancer (Wu *et al.,* 2002; Huang HF *et al.,* 2005). PKCε has also been shown to be required for serine 727 STAT1/3 phosphorylation in a mouse model of ischemic preconditioning (PC) (Ping *et al.,* 1999, Shen *et al.,* 2004

PKC has other roles in STAT signalling besides STAT phosphorylation. In myoblast cells, the hormone angiotensin II activates PKC, leading to the phosphorylation of the transcription factor GATA-4 (Wang, *et al.,* 2005). Following phosphorylation, GATA-4 then binds DNA and recruits STAT1 for optimal induction of gene transcription. GATA-4 DNA binding can be inhibited by the broad range inhibitor BIM, leading to a decrease in gene expression. This demonstrates the importance of PKC activity in STAT signaling, even though STAT1 phosphorylation was not induced by PKC in this case.

IFN α, β and γ phosphorylate PKC θ in human T-cells (Srivastava *et al.,* 2004). PKCθ phosphorylation is required for IFN α and γ induced gene transcription at GAS sites. This was demonstrated in Molt-4 cells by siRNA and dominant negative mutant over expression studies of PKCθ showing that loss of active PKCθ inhibited GAS driven transcription. Interestingly knockdown of PKCθ does not alter STAT1 tyrosine 701 phosphorylation or DNA binding, however, the authors did not examine phosphorylation serine 727 or other members of the STAT family in this report.

4.2 HYPOTHESIS & OBJECTIVES

IFNα stimulated JAK/STAT signalling in hepatocytes is a vital mechanism in the host response to, and clinical treatment of, HCV infection. PKCs δ and ε clearly play an important role in JAK/STAT signalling in a variety of cell types.

Following my observation that Rottlerin inhibits the IFNα induced phosphorylation of STAT1 and STAT3 I hypothesise that, as with other cell types, the expression of PKCs δ or ε is required for the IFNα mediated phosphorylation STAT 1 and 3 and for their subsequent nuclear translocation. I also hypothesise that the expression of PKCs δ or ε is required for ISG expression in hepatocytes either by preventing STAT phosphorylation and nuclear translocation or by disrupting transcription machinery as demonstrated in myoblast cells and T-cells.

Therefore, the objectives of this chapter are:

- To determine if PKC δ or ε is required for IFNα stimulated STAT 1 or STAT 3 phosphorylation in Huh7 hepatocytes.
- To determine if PKC δ or ε is required for IFNα stimulated nuclear translocation of STAT 1 or STAT 3 in Huh7 hepatocytes.
- To determine if PKC δ or ε is required for ISG transcription in Huh7 hepatocytes.

4.3 RESULTS

4.3.1 The novel PKCs δ and ε are not required for IFNα induced phosphorylation of STAT 1 or 3.

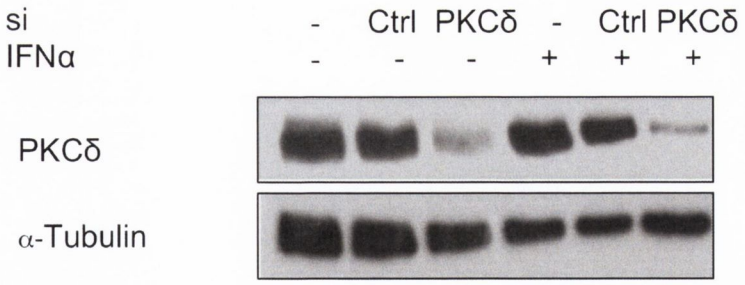
Novel PKCs are required for IFNα signalling and STAT activation in many cell types. I previously confirmed the expression of the novel PKCs δ and ε by Western blot and immunofluorescent staining and observed that the novel PKC inhibitor Rottlerin could inhibit IFNα induced tyrosine phosphorylation of STAT 1 and 3. I therefore assessed if these specific PKC isoforms were required for IFNα signalling in Huh7 cells by knocking down either PKC δ or ε and then analysing cellular responses to IFNα.

I first established protocols that allowed for the efficient knock down of PKC δ and ε by siRNA (**Chapter 2**). Wet reverse transfection of 100nm PRKCD siRNA with D2 DharmaFECT transfection reagent for 72 hours was optimum to achieve knock down of PKCδ (**Figure 4.1 A**) while wet reverse transfection of 30nm PRKCE siRNA with RNAiMAX transfection reagent for 72 hours was optimum to achieve knock down of PKCε (**Figure 4.1 B**). Wet reverse transfection allowed the knockdown reaction to be split into multiple wells, allowing for the confirmation of knockdown by Western blot while using a sample from the same reaction to be used for different experiments. The percentage of knockdown for each individual experiment is shown in **Figure 4.2**. A control with random scrambled siRNA transfected instead of PKC specific siRNA was transfected in parallel to show that the effects I found were not due to off target effects.

Having established protocols that allowed for knockdown of either PKC δ or ε I then investigated the requirement for these isoforms for phosphorylation of tyrosine residues in STAT 1 and STAT3 by IFNα. PKC δ or ε was knocked down and cells were then either stimulated or not with IFNα for 30 minutes. As can be seen in **Figure 4.3** and **Figure 4.4**, neither PKCδ nor PKCε are required by IFNα to induce tyrosine phosphorylation of STAT 1 or 3 in Huh7 cells. Phosphorylation of serine 727 is constitutive on both STAT 1 and 3 in Huh7 cells. I examined if PKC δ or ε are required for phosphorylation at this site. **Figure 4.5** and **Figure 4.6** show that neither PKCδ nor PKCε are required for STAT 1 or 3 serine 727 phosphorylation in resting or IFNα stimulated Huh7 cells.

These results demonstrate that neither PKCδ nor PKCε are required by IFNα to induce phosphorylation of STAT1 or STAT3 at tyrosine 701 or 705 respectively. These kinases are also not required for the constitutive phosphorylation of serine 727 of STAT1 or STAT3.

A



B

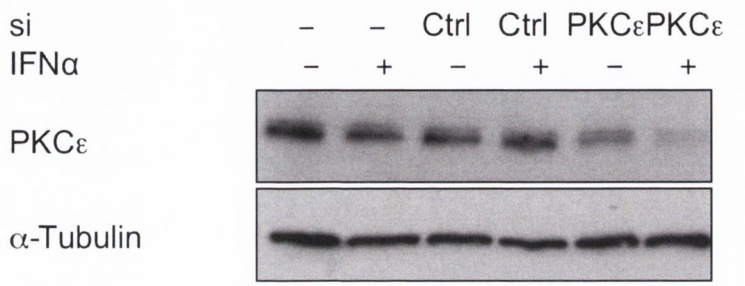
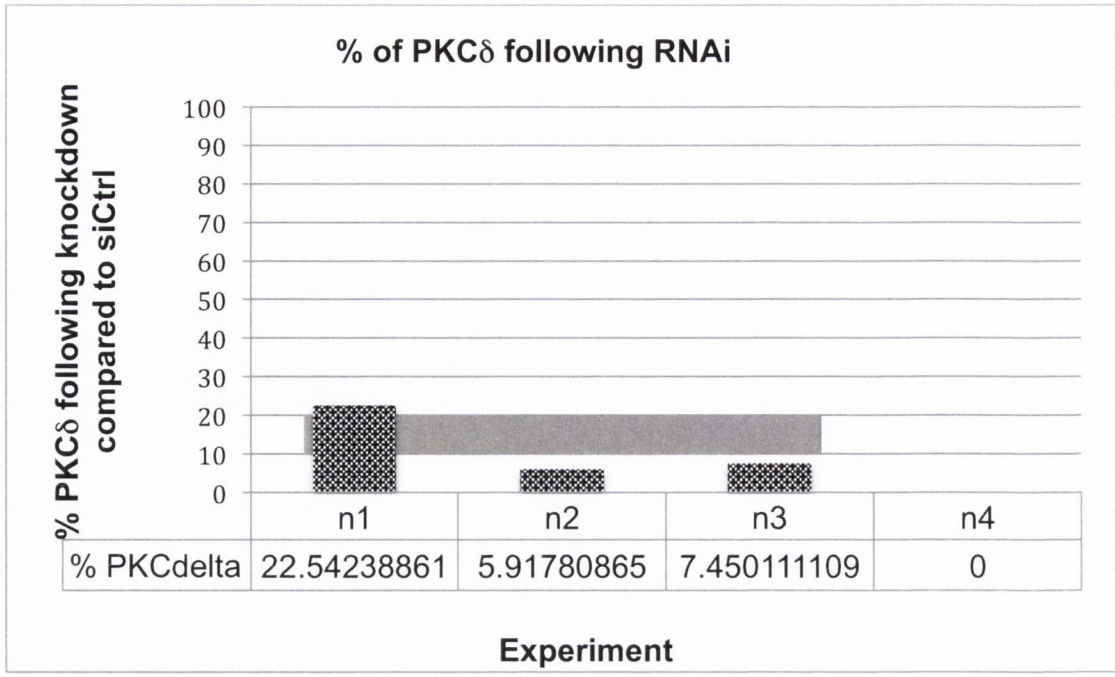


Figure 4.1 RNAi knockdown of PKC δ and ε in Huh7 cells. Huh7 cells were transfected with a mixture of four siRNAs designed to target the PRKCD or PRKCE genes. A scrambled siRNA was included as a control for transfection. siRNA was transfected by wet reverse transfection using D2 DharmaFECT or RNAiMAX transfection reagent as described in Chapter 2. SDS-PAGE was performed with 10μg of lysate per well and PKC knockdown was verified by Western Blot using antibodies specific for PKCδ (Figure 4.1 A) or PKCε (Figure 4.1 B). α-Tubulin was used as a loading control. The figure shown is representative of four independent experiments.

A



B

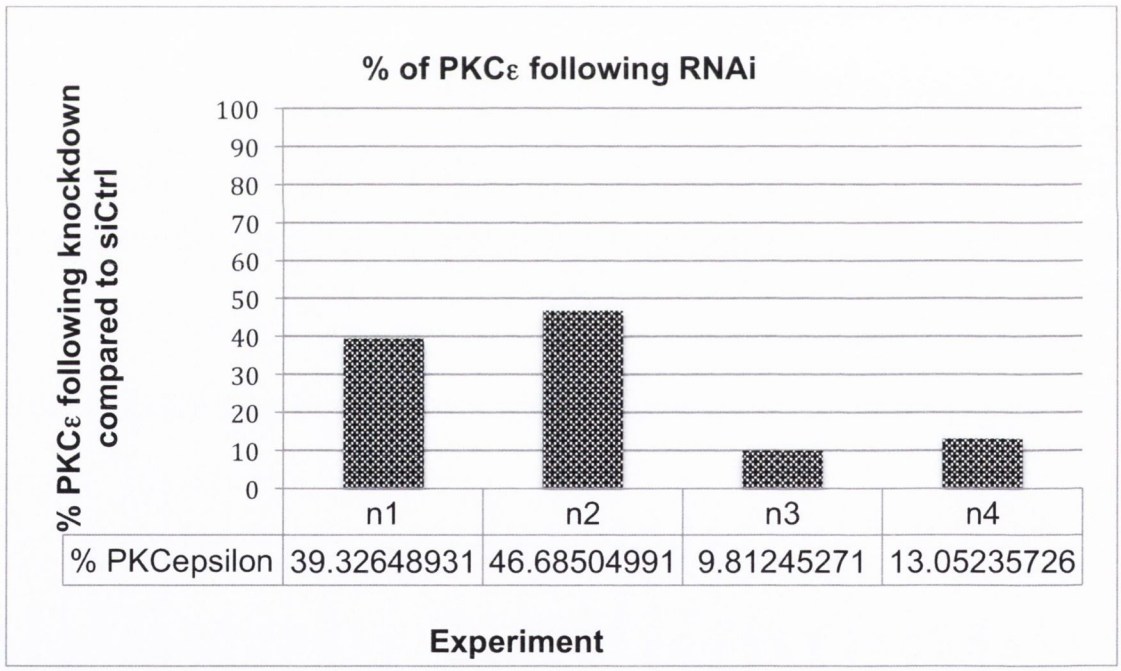
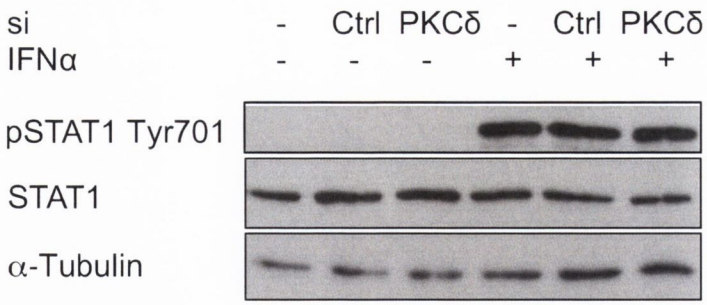


Figure 4.2 Percentage of PKC δ and ε protein remaining following RNAi in four individual experiments. Protein concentration was normalized and PKC δ or ε detected by Western blot. Densitometry was undertaken to compare the amount of PKC present in cells transfected with PKC siRNA compared to cells transfected with control siRNA. The percentage of PKC δ (Figure 4.2 A) and PKC ε (Figure 4.2 B) present in knocked-down cells compared to control cells is shown.

A



B

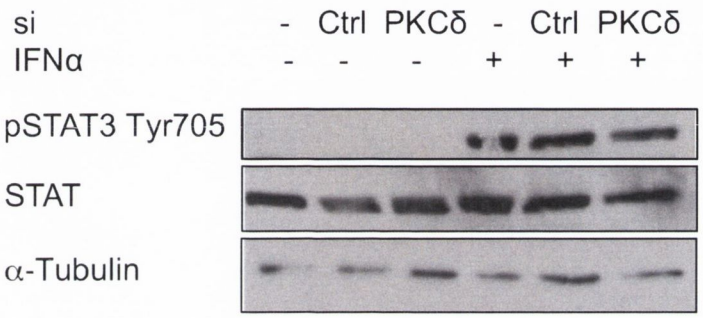
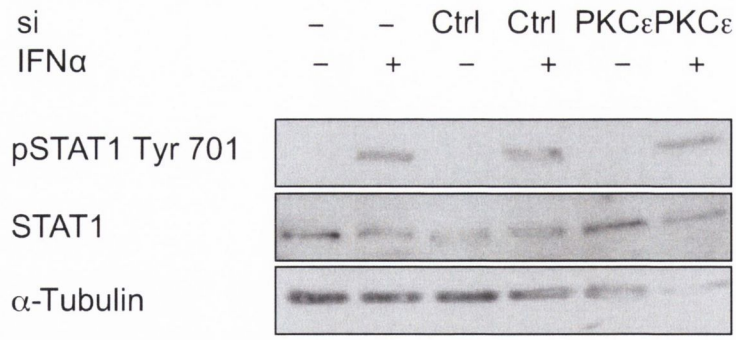


Figure 4.3 IFNα induced tyrosine phosphorylation of STAT1 and STAT3 does not require PKCδ in Huh7 cells. Huh7 cells with PKCδ knocked down were stimulated with IFNα to induce phosphorylation of STAT1 at Tyr 701 and STAT3 at Tyr705. SDS-PAGE was performed with 10μg of lysate per well. Total and phospho STAT1 (Figure 4.3 A) and STAT3 (Figure 4.3 B) proteins were identified by Western Blot using specific antibodies. α-Tubulin was used as a loading control. The figure shown is representative of four independent experiments.

A



B

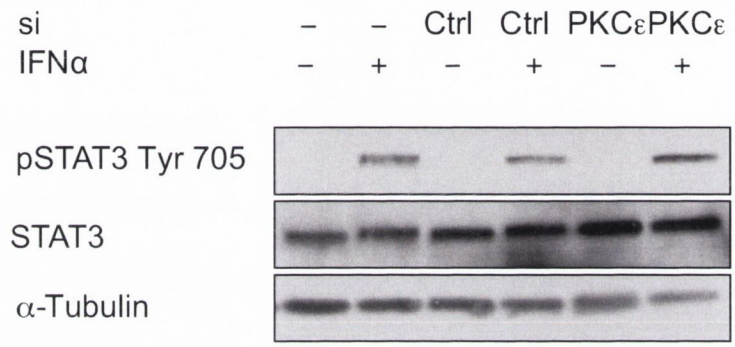
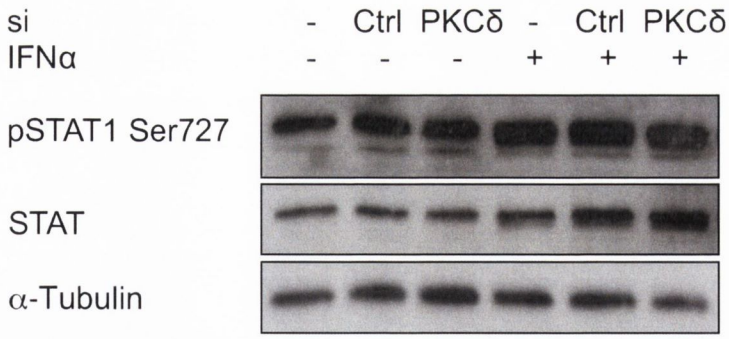


Figure 4.4 IFNα induced tyrosine phosphorylation of STAT1 and STAT3 does not require PKCε in Huh7 cells. Huh7 cells with PKCε knocked down were stimulated with IFNα to induce phosphorylation of STAT1 at Tyr 701 and STAT3 at Tyr705. SDS-PAGE was performed with 10μg of lysate per well. Total and phospho STAT1 (Figure 4.4 A) and STAT3 (Figure 4.4 B) proteins were identified by Western Blot using specific antibodies. α-Tubulin was used as a loading control. The figure shown is representative of four independent experiments.

A



B

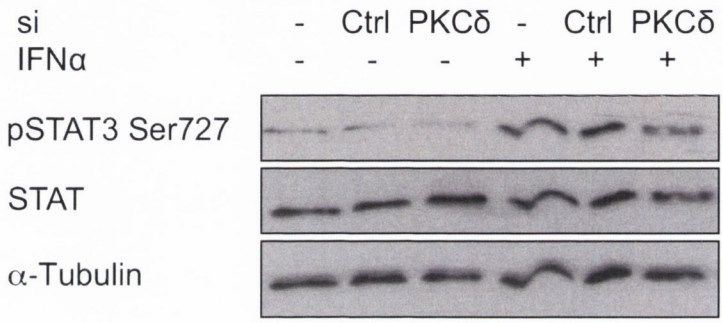
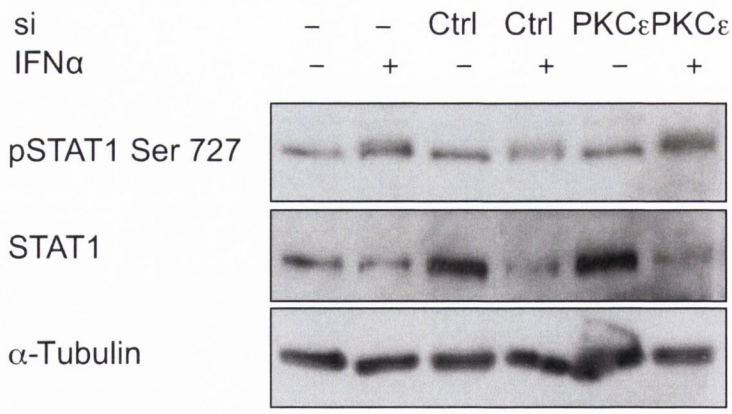


Figure 4.5 Serine 727 phosphorylation of STAT1 and STAT3 does not require PKCδ in Huh7 cells. Huh7 cells with PKCδ knocked down were stimulated with IFNα for 30 minutes. SDS-PAGE was performed with 10μg of lysate per well. Total and phospho STAT1 (Figure 4.5 A) and STAT3 (Figure 4.5 B) proteins were identified by Western Blot using specific antibodies. α-Tubulin was used as a loading control. The figure shown is representative of four independent experiments.

A



B

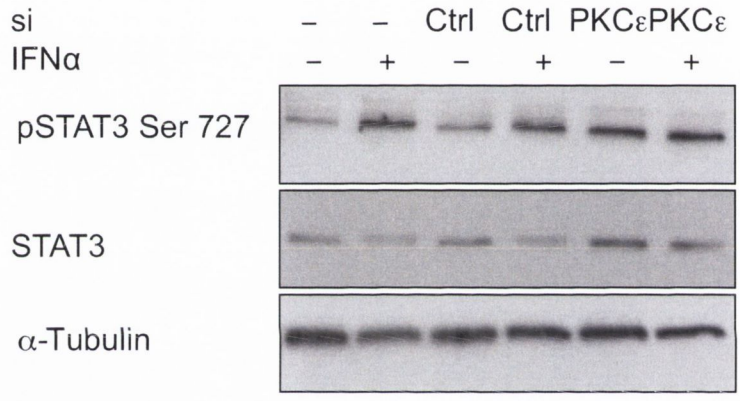


Figure 4.6 Serine 727 phosphorylation of STAT1 and STAT3 does not require PKCε in Huh7 cells. Huh7 cells with PKCε knocked down were stimulated with IFNα for 30 minutes. SDS-PAGE was performed with 10μg of lysate per well. Total and phospho serine 727 STAT1 (Figure 4.6 A) and STAT3 (Figure 4.6 B) proteins were identified by Western Blot using specific antibodies. α-Tubulin was used as a loading control. The figure shown is representative of four independent experiments.

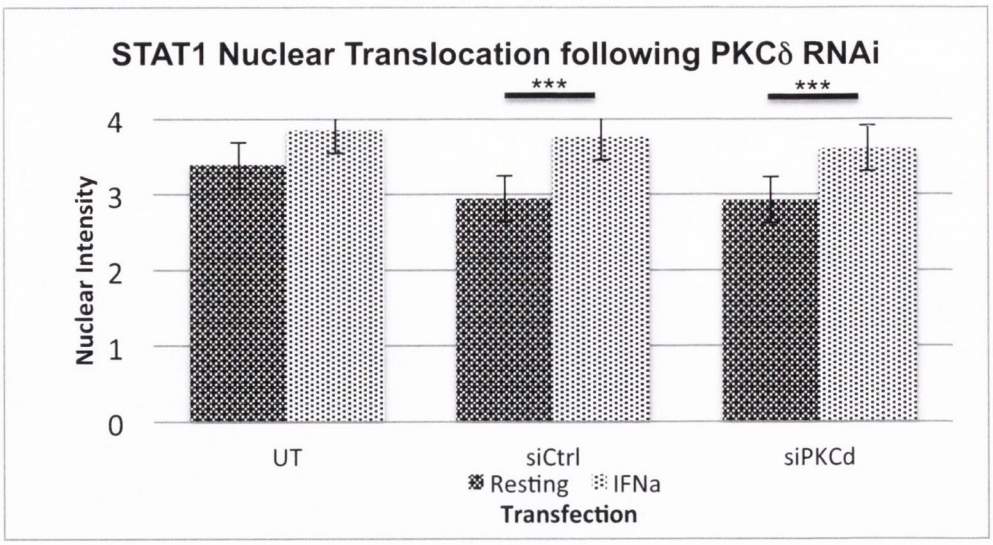
4.3.2 IFNα induced STAT 1/3 translocation and changes in nuclear area following novel PKC knockdown.

IFNα stimulation induces the translocation of STAT proteins from the cytoplasm into the nucleus. In order to assess if PKCδ or PKCε are required for nuclear translocation of STAT1 or STAT3 to occur following IFNα treatment I developed an assay using the InCell high content analyser as described in chapter 2. Following RNAi for either PKC isoform, cells were stimulated or not with IFNα for one hour before being fixed and stained for DNA and either STAT 1 or 3. The intensity of STAT staining within the nuclear area was then measured using the InCell software and compared before and after IFNα stimulation. As shown in **Figure 4.7** the intensity of STAT 1 and 3 in the nucleus is significantly higher following IFNα treatment in control cells however, knockdown of PKCδ does not prevent STAT 1 or 3 nuclear translocation following IFNα treatment.

When PKCε was knocked down it was observed that nuclear translocation of STAT1 by IFNα was prevented and was significantly decreased in comparison to IFNα treated control cells. In contrast, STAT3 maintained its ability to translocate into the nucleus following IFNα stimulation in cells with PKCε knocked down (**Figure 4.8**). As an increase in nuclear area can indicate transcriptional activity I assessed if the size of nuclei in cells with PKC δ or ε knocked down was increased following IFNα treatment. I show that nuclear size was the same between cells transfected with control scrambled siRNA before or after IFNα treatment. Nuclear size increased following IFNα treatment in cells with PKC δ knocked down but not with PKC ε knocked down (**Figures 4.9** and **4.10**).

These results demonstrate that PKCε is required for IFNα to induce the nuclear translocation of STAT1 but not STAT3, revealing a distinct pathway of STAT activation by IFNα within Huh7 cells. It was further demonstrated that PKCδ is not required for IFNα induced nuclear translocation of either STAT protein. However, PKCδ may be required for IFNα induced transcriptional activity as nuclear area increases following PKCδ knockdown, suggesting an increase in transcriptional activity.

A



B

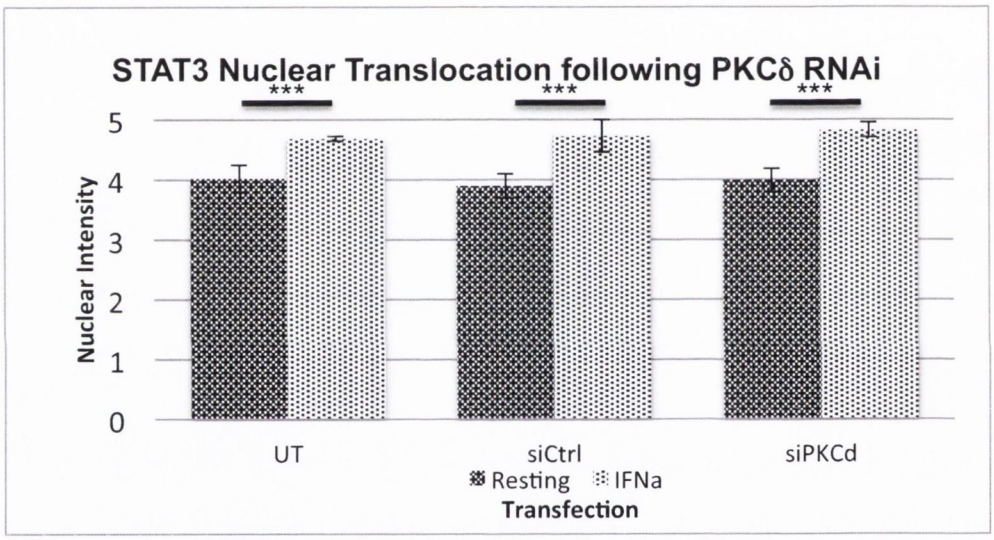
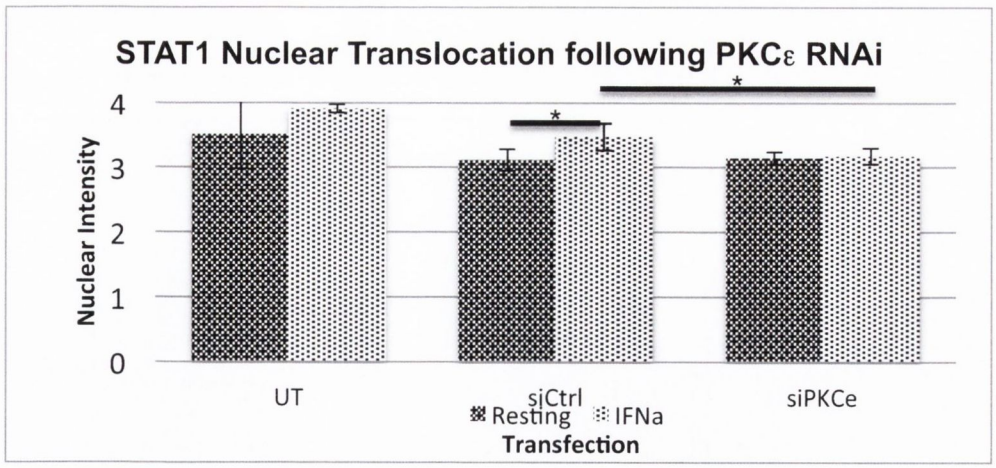


Figure 4.7 Nuclear translocation of STAT 1 or 3 by IFNα does not require PKCδ. Huh7 cells were either not transfected (UT), transfected with scrambled control siRNA (siCtrl) or PKCδ specific siRNA (siPKCd) for 72 hours as previously described. Cells were then serum starved and further treated or not with IFNα for one hour. Cells were then fixed and stained for total STAT 1 or 3 and DNA. Nuclear translocation was determined with an InCell analyzer by measuring the net nuclear intensity of STAT1 (Figure 4.9 A) or STAT3 (Figure 4.9 B) staining before and after IFNα treatment. Results shown are an average of four independent experiments. Standard T Test p values are shown to indicate statistical significance (* = $p<0.05$, *** = $p<0.01$). Error bars are of Standard Error of the Mean.

A



B

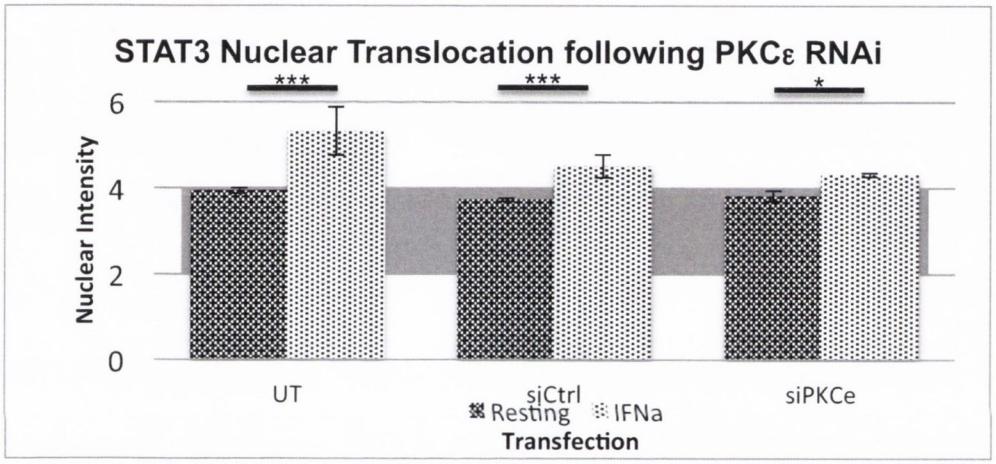


Figure 4.8 PKCε is required for IFNα induced STAT1 but not for STAT3nuclear translocation. Huh7 cells were either not transfected (UT), transfected with scrambled control siRNA (siCtrl) or PKCε specific siRNA (siPKCe) for 72 hours as previously described. Cells were then serum starved and further treated or not with IFNα for one hour. Cells were then fixed and stained for total STAT 1 or 3 and DNA. Nuclear translocation was determined with an InCell analyzer by measuring the net nuclear intensity of STAT1 (Figure 4.9 A) or STAT3 (Figure 4.9 B) staining before and after IFNα treatment. Results shown for STAT1 are an average of three independent experiments. Results shown for STAT3 are an average of two independent experiments. Standard T Test p values are shown to indicate statistical significance (* = $p<0.05$, *** = $p<0.01$). Error bars are of Standard Error of the Mean.

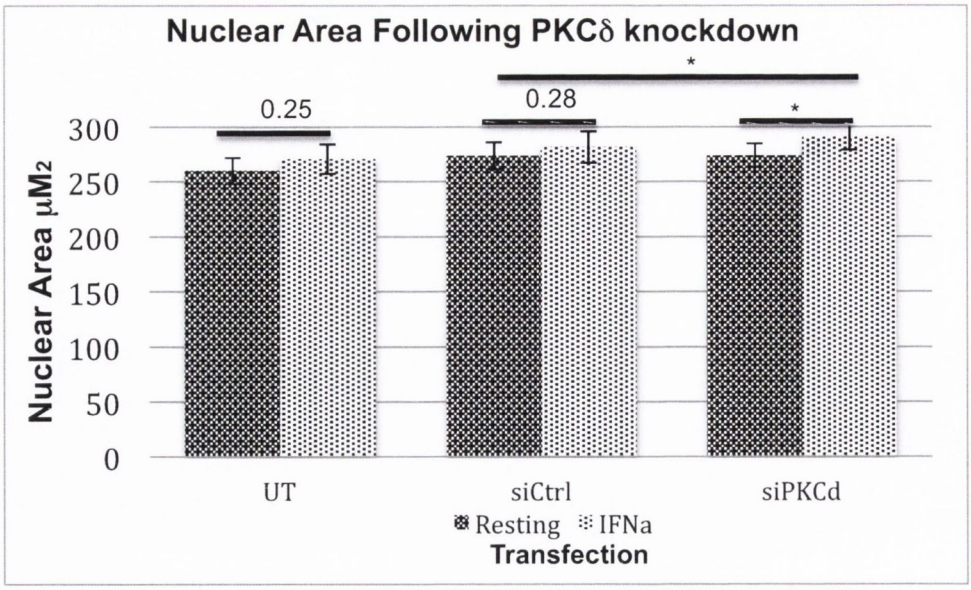


Figure 4.9 Nuclear area increases following IFNα stimulation in cells with PKCδ knockdown. Huh7 cells were either not transfected (UT), transfected with scrambled control siRNA (siCtrl) or PKCδ specific siRNA (siPKCd) for 72 hours as previously described. Cells were then serum starved and further treated or not with IFNα for one hour. Cells were then fixed and stained for DNA with Hoechst. Nuclear area was then measured by an InCell analyzer. Results shown are an average of four independent experiments. Standard T Test p values are shown to indicate statistical significance. Error bars are of Standard Error of the Mean.

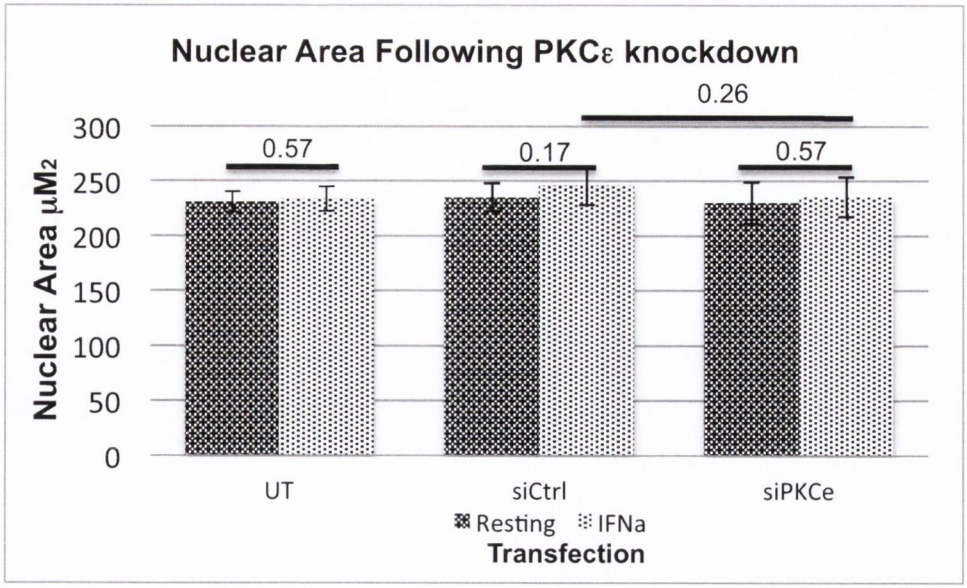


Figure 4.10 Nuclear area does not increase following IFNα stimulation in cells lacking PKCε expression. Huh7 cells were either not transfected (UT), transfected with scrambled control siRNA (siCtrl) or PKCε specific siRNA (siPKCe) for 72 hours. Cells were then serum starved and further treated or not with IFNα for one hour. Cells were then fixed and stained for DNA with Hoechst. Nuclear area was then measured by an InCell analyzer. Results shown are an average of three independent experiments. Standard T Test p values are shown to indicate statistical significance. Error bars are of Standard Error of the Mean.

4.3.3 IFNα stimulated ISG expression following novel PKC knockdown.

PKCs are required for IFNα induced transcription in T cells (Srivastava, *et al.*, 2004). Having shown that PKCε is required for IFNα stimulated STAT1 translocation to the nucleus and that PKCδ is localised in the nucleus I evaluated the requirement of PKC δ and ε for IFNα stimulated gene (ISG) transcription. I examined the induction of the ISGs protein kinase R (PKR), 2′-5′ oligoadenylate synthetase (2'5'-OAS) and myxovirus resistance protein 1 (MxA) by IFNα following knockdown of PKC δ or ε using quantitative real-time polymerase chain reaction (RT-PCR). The ratio of ISG expression in all samples was first compared to a stable housekeeper gene as determined by the GeNorm algorithm. ISG induction of transcription was then normalised to resting samples and the ratio of IFNα induction of ISGs then was compared to this. **Figure 4.11** shows that PKCδ knockdown appears to decrease the IFNα stimulated induction of expression of each ISG. However, a statistically significant difference is not observed. This is likely due to conflicting results from individual experiments (**Appendix II**). The total level of ISG mRNA expressed is also lower in cells with PKCδ knocked down compared to siRNA control cells for both resting and IFNα stimulated samples (**Figure 4.12**). When PKCε is knocked down the induction and total expression of PKR and 2'5'-OAS is not changed significantly (**Figure 4.13 A & B** and **Figure 4.14 A & B**). The total mRNA levels and IFNα induction of MxA appears to be decreased but a statistically significant difference is not observed (**Figure 4.13 C and Figure 4.14 C)**. Again this is likely due to conflicting results from individual experiments (**Appendix II**). This data suggests that the novel PKC isoforms δ and ε are not required by IFNα to induce ISG transcription in Huh7 cells. However, this is a tentative conclusion as there was a significant difference in the results of individual experiments.

Although the pharmacological novel PKC inhibitor Rottlerin prevented IFNα inducing tyrosine phosphorylation of STAT1 and STAT3 I show that this is not via PKCδ or PKCε, demonstrating that another pathway must be required for IFNα to induce this phosphorylation. I also show that PKCδ and PKCε are not required for serine 727 phosphorylation of STAT1 and STAT3. However, I reveal that PKCε is required by IFNα to facilitate the nuclear translocation of STAT1 specifically and that this pathway may be required for MxA expression. Taken together I show that PKCε is required for IFNα

signalling through STAT1 but not STAT3 and that IFNα does not signal though PKCδ in Huh7 cells.

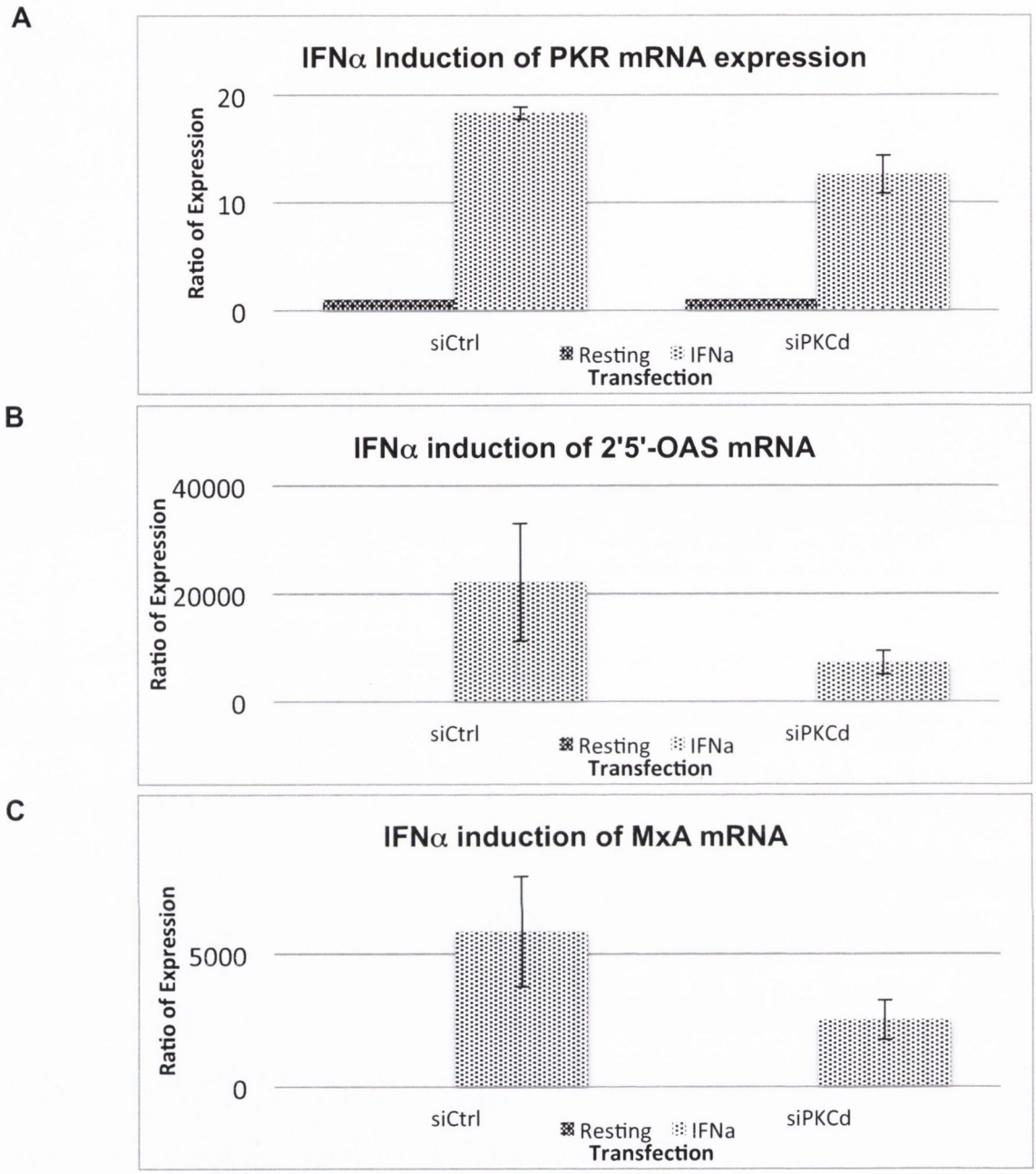
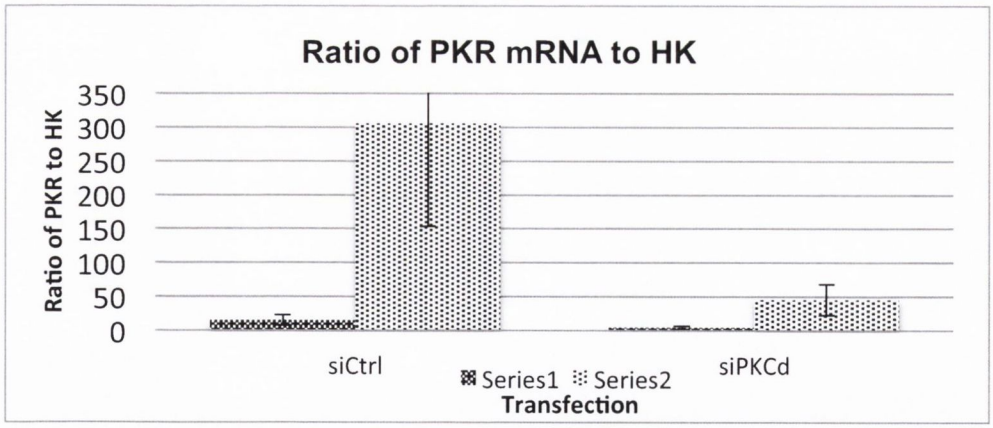
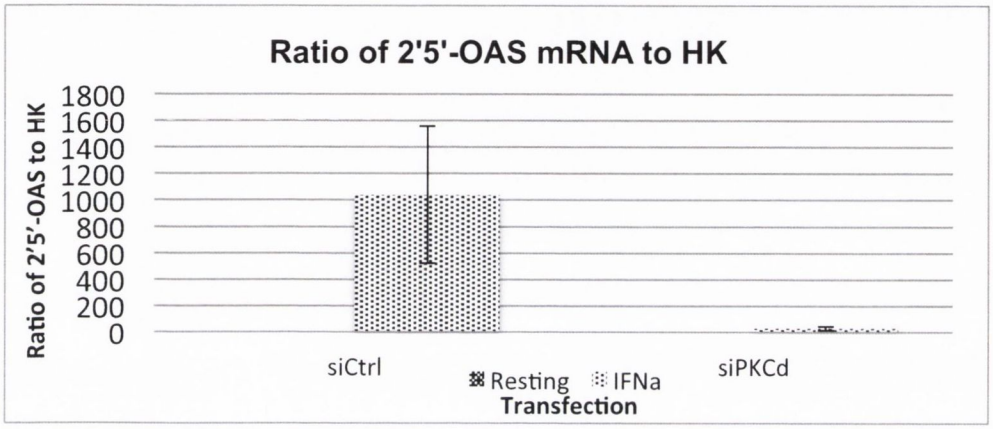


Figure 4.11 PKCδ is not required for IFNα induction of ISG transcription. ISG expression was measured by RT-PCR following PKCδ knockdown and IFNα stimulation. IFNα induction of ISGs was determined by normalising the expression of ISGs to resting samples and calculating fold increase of ISGs following 6 hours of IFNα stimulation. The ratio of PKR (A). 2'5'-OAS (B) and MxA (C) induced by IFNα appeared to decrease following PKCδ knockdown however, there is no statistically significant decrease. Results shown are an average of four independent experiments. Error bars are of Standard Error of the Mean.

A



B



C

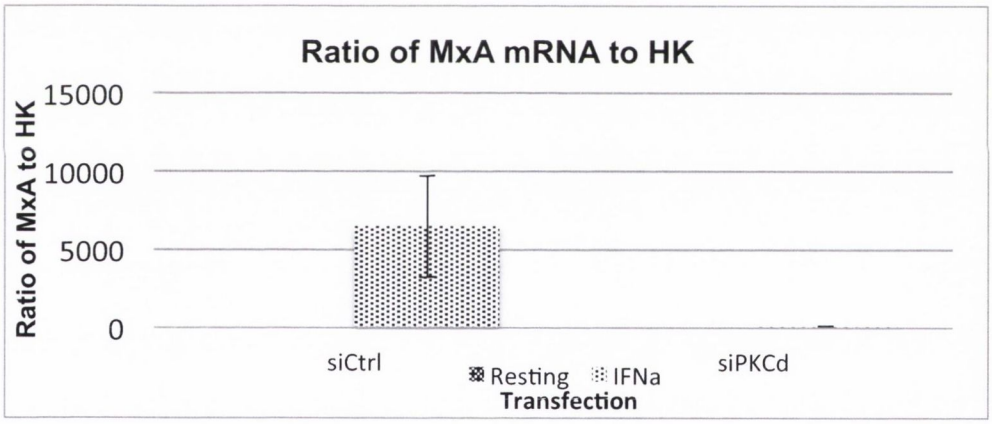


Figure 4.12 PKCδ is not required for ISG transcription. ISG expression was measured by RT-PCR following PKCδ knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene GAPDH. The levels of PKR (A). 2'5'-OAS (B) and MxA (C) expressed appeared to decrease following PKCδ knockdown, however, there is no statistically significant decrease. Results shown are an average of four independent experiments. Error bars are of Standard Error of the Mean.

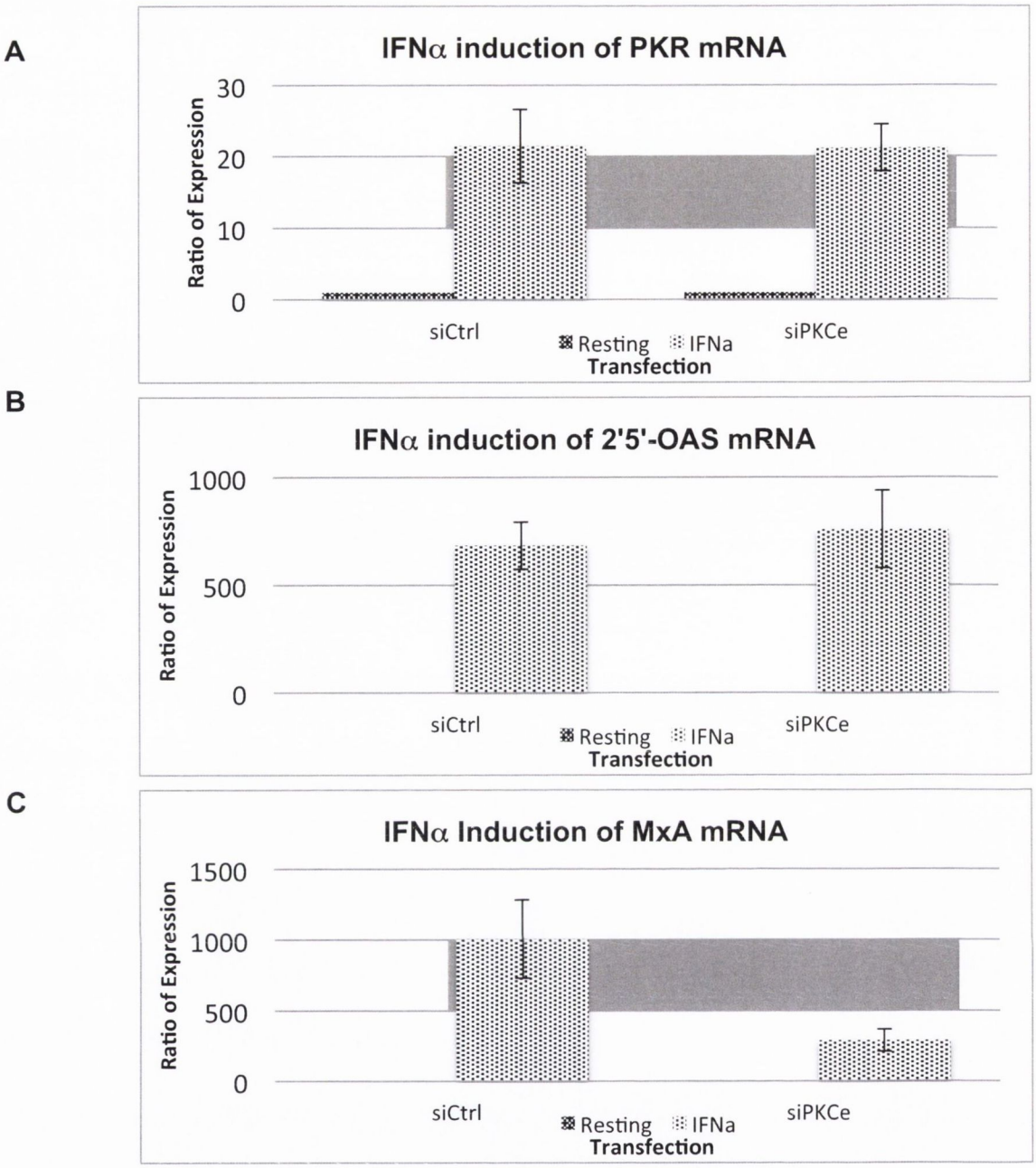
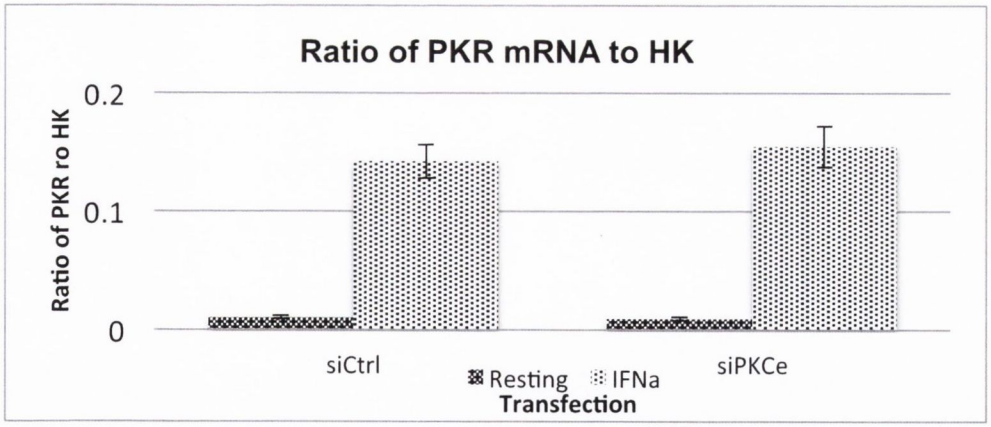
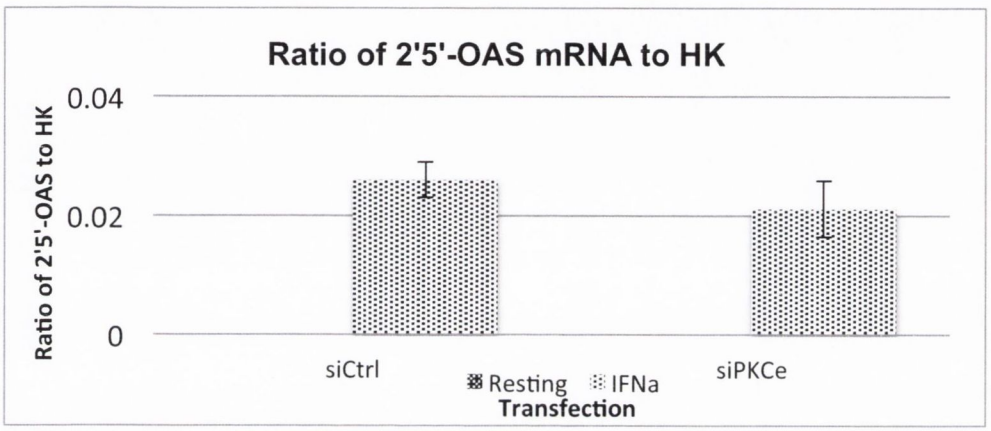


Figure 4.13 PKCε is not required for IFNα induction of ISG transcription. ISG expression was measured by RT-PCR following PKCε knockdown and IFNα stimulation. IFNα induction of ISGs was determined by normalising the expression of ISGs to resting samples and calculating fold increase of ISGs following 6 hours of IFNα stimulation. Following PKCε knockdown the ratio of PKR (A) and 2'5'-OAS (B) induced by IFNα is unchanged while the ratio of MxA (C) induced by IFNα appeared to decrease, however, there is no statistically significant decrease. Results shown are an average of four independent experiments. Error bars are of Standard Error of the Mean.

A



B



C

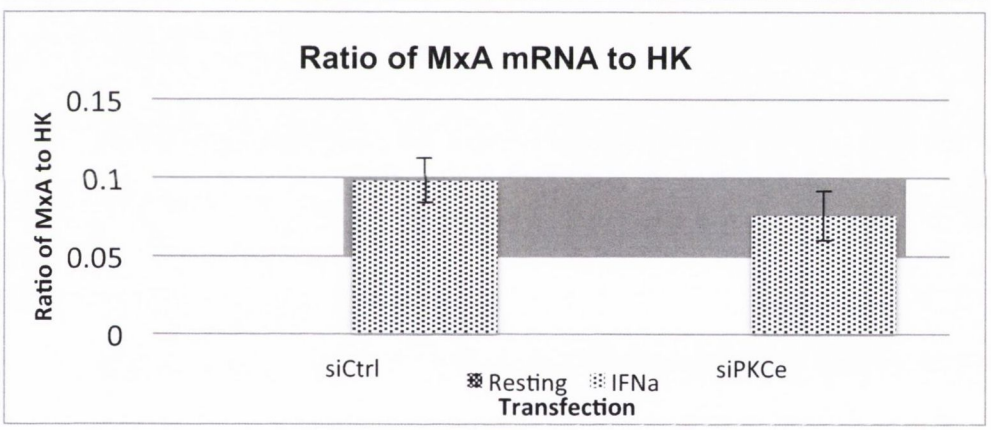


Figure 4.14 PKCε is not required for ISG transcription. ISG expression was measured by RT-PCR following PKCε knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene PPIA. The levels of PKR (A). 2'5'-OAS (B) and MxA (C) expressed appeared to decrease following PKCε knockdown, however, there is no statistically significant decrease. Results shown are an average of four independent experiments. Error bars are of Standard Error of the Mean.

4.4 DISCUSSION

In Chapter III I observed that the nPKCs δ and ε are expressed in Huh7 cells. These PKC isotypes are required for STAT 1 and 3 phosphorylation in cell types such as Daudi B lymphoma cells (Yanase *et al.,* 2012), human peripheral blood monocytes (Bhattacharjee, *et al.,* 2006) and human prostate cancer cell lines (Aziz *et al.,* 2007). PKCδ interacts with STAT1 and phosphorylates it at serine 727 in myeloma cells cell lines. This was demonstrated by Western blot with specific anti-phospho PKCδ antibodies and by kinase assays using PKCδ immunoprecipitates treated with or without IFNα to examine phosphorylation of exogenous histone H1. Immunoprecipitated STAT1 from lysates of unstimulated cells that are then mixed with recombinant active PKCδ show strong serine 727 phosphorylation following addition of active PKCδ (Uddin *et al.,* 2002). Similarly, in experiments using cells derived from the IFNα sensitive chronic myelogenous leukemia (CML) cell line KT-1, IFNα was shown to activate PKCδ and that this activation was required for STAT1 serine 727 phosphorylation (Kaur *et al.,* 2005). After using rottlerin Stat1 serine 727 phosphorylation was blocked, while ISG15 gene expression was also blocked. Chromatin immunoprecipitation (ChIP) assays showed that this inhibition of gene expression was independent of ISRE promoter binding at ISG15 indicating that PKCδ is not required for ISGF3 complex formation or its binding to promoter elements but is necessary for optimal ISG expression. Furthermore, by examining colony formation of bone marrow or PBMCs from CML patients it was shown that rottlerin subdued the anti-leukemic properties of IFNα.

I therefore examined the requirement of PKC δ and ε in IFNα signalling via STAT 1 and 3 in Huh7 cells. In Chapter III I pretreated cells with Rottlerin, which was identified in 1994 by Gschwendt *et al.,* as an inhibitor of PKCδ. I found that this pretreatment lead to the inhibition of IFNα induced phosphorylation of tyrosine 701/705 in STAT1/3. This suggests that PKCδ is required for optimal IFNα induced activation of the Jak/STAT pathway. However, the specificity of Rottlerin has been called into question (Soltoff SP, 2007). Rottlerin leads to reduced cellular ATP levels (Soltoff SP, 2001) by uncoupling mitochondrial respiration from oxidative phosphorylation which may then have an indirect inhibition on PKCδ activation. Davies S.P. *et al., 2000* analysed the activity of a range of kinases following Rottlerin treatment. They found that Rottlerin had virtually no effect on

PKCδ but in fact inhibited p38-regulated/activated protein kinase (PRAK) and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2).

In order to make definitive conclusions regarding PKC function, RNAi is required to be undertaken. I used panels of siRNAs to separately knock down the expression of PKC δ and ε. I then treated these cells with IFNα before lysing them and examining the phosphorylation status of STAT 1 and 3 or their ISG expression. I also fixed cells and examined the ability of IFNα to translocate STAT proteins to the nucleus in cells where these PKCs had been knocked-down. In order to check the transfection efficiency of PKC protein knockdown in each experiment I lysed the cells and then used SDS-electrophoresis and Western blotting to detect PKC δ, ε and α-tubulin protein levels. Protein levels were then measured by densitometry. I then normalized the values by comparing the amount of α-tubulin protein present in each sample. I then calculated the relative levels of PKC δ/ε in PKC δ/ε siRNA samples compared to siRNA control samples for each experiment (**Figure 4.2**).

Despite previous reports showing that PKC δ and ε are required for STAT phosphorylation at tyrosine and serine sites in other cell types, I found that these PKC isotypes were not required for IFNα induced STAT tyrosine or serine phosphorylation in Huh7 cells. Chen *et al.,* 2013 recently investigated IFNα signalling in Huh7 cells the context of hepatitis B virus (HBV) infection. In conflict with my results Chen *et al.,* deduced that PKCδ is required for optimal IFNα signalling through the Jak/STAT pathway and that HBV targets PKCδ activity to evade the IFNα response. When they treated Huh7 cells with IFNα they observed an increase in serine 727 phosphorylation at STAT 1 and 3 and that STAT 1 co-immunoprecipitated with PKCδ following IFNα treatment. When they treated cells with Rottlerin they reported a decrease in phosphorylation at serine 727 but not tyrosine 701/705 of STAT 1 and 3 following IFNα treatment. Ectopic expression of HBV polymerase (Pol) suppressed IFNα induced STAT serine 727 phosphorylation. Furthermore they demonstrated that Pol interacted with PKCδ and perturbed its association with STAT1. They also observed that Pol was able to interfere with nuclear transport of the STAT1/2 dimer by competitively competing for the region that binds importin-α5. However, these conclusions are undermined as they did not examine the ability of IFNα to phosphorylate STAT in cells with RNAi of PKCδ. Furthermore, their

studies did not examine the possibility that PKCε may be required for STAT phosphorylation.

Following tyrosine phosphorylation, STAT proteins dimerise and translocate from the cytoplasm to the nucleus and initiate gene transcription. Retaining a balance between nuclear and cytoplasmic levels of a particular protein can be necessary for regulating cell fate. Proteins over ~40 kDa must be actively transported into the nucleus (Ma J *et al.,* 2012). Transport of these proteins into the nucleus is via nuclear pore complexes and is mediated by the importin superfamily. Therefore nucleocytoplasmic shuttling is a highly coordinated process and is often coupled with other levels of regulation such as protein phosphorylation. In most cases, phosphorylation enhances nuclear import of cargo proteins, although abrogation of nuclear import has also been reported (Nardozzi *et al.,* 2010).

Even though I have shown that PKC δ and ε are not required for IFNα phosphorylation of STAT 1 and 3 proteins, I hypothesized that PKCs may still be required for phosphorylation of adaptor proteins such as 14-3-3 that facilitate the nuclear transport of STAT 1 and 3 following IFNα stimulation. STAT1 phosphorylation induces its homo-dimerization, forming a dimer-specific nuclear localization sequence (NLS) that is recognized by importin-α (McBride *et al.,* 2002). Furthermore STAT3 has been shown to interact with 14-3-3ζ, a scaffold protein that regulates STAT3 activity, in U266 multiple myeloma cells (Zhang *et al.,* 2012). 14-3-3ζ binds STAT3 at its phosphorylated serine 727 site. Depletion of 14-3-3ζ prevented nuclear translocation of STAT3 and reduced the ability of STAT3 to bind DNA. 14-3-3 also associates with c-Abl (Yoshida and Miki, 2005) where phosphorylation of 14-3-3 leads to its dissociation from c-Abl, exposing the NLS of the latter and inducing its nuclear import. 14-3-3 proteins also regulate PKCs (Van Der Hoeven *et al.,* 2009; Van Der Hoeven *et al.,* 2000; Wheeler-Jones *et al.,* 1996; Saurin *et al.,* 2008). In U266 cells Zheng *et al.* showed that 14-3-3ζ knock-down resulted in a significant decrease in the kinase activity of PKCs while its overexpression increased PKC kinase activity. I therefore used RNAi to separately knockdown the expression of PKC δ and ε in Huh7 cells. Cells were then stimulated with IFNα to induce the translocation of STAT proteins from the cytoplasm to the nucleus. Nuclear translocation was then examined by high content analysis of immunofluorescent stained cells. My results demonstrated that nuclear import of STAT 1

and 3 following IFNα were not significantly inhibited in cells with PKC δ knocked down. However, I showed that PKCε expression was required for the IFNα induced nuclear translocation of STAT1 but not STAT3 indicating that nuclear translocation of different STAT proteins may be regulated by different pathways following IFNα stimulation.

In 293T cells PKCδ was located in the cytoplasm, however, following activation it alters conformation to expose a NLS that is conserved in PKCs. This then allows for the interaction with importin-α and the complex is translocated to the nucleus. As I have demonstrated that PKCδ is constitutively located in the nucleus it is possible that PKCδ is constitutively activated in Huh7 cells. This is in contrast to 293T cells where nuclear translocation of PKCδ induces apoptosis (Adwan *et al.,* 2011; DeVries TA *et al.,* 2002). Failure of nuclear localized PKCδ to induce apoptosis in Huh7 cells may be one of the reasons why this cell line is a carcinoma.

Kinase activity of PKCs is also required for nuclear export of some proteins (Filatova *et al.,* 2009). Sub-confluent porcine kidney cells show nuclear localization of porcine RS1 (pRS1) while confluent cells have decreased nuclear levels of pRS1. PKC activation was found to result in the export of RS1 protein from the nucleus in sub-confluent cells where as PKC inhibition did not alter its nuclear localization. Confluent cells however, showed serine phosphorylation of pRS1 at a PKC-dependent site that is conserved between pigs and humans. However, pRS1 was not exported from the nucleus when cells reached confluence when this phosphorylation site was mutated to alanine. I did not observe an increase in nuclear localisation of STAT 1 and 3 in unstimulated cells that had PKC δ or ε knocked-down. Therefore my results rule out the requirement of PKC δ or ε for nuclear export of STAT 1 and 3.

Given that PKCε was able to inhibit IFNα induced STAT1 nuclear translocation and in Chapter III I observed that PKC δ is expressed in the nucleus, I investigated the requirement for PKCs in transcriptional activity and specifically ISG transcription following IFNα stimulation. I first examined if PKC δ or ε are required for transcriptional activity. Gene transcription is associated with an increase in nuclear area. I therefore investigated if siRNA knock-down of PKC δ or ε would prevent an increase in nuclear area following IFNα stimulation. I found that IFNα stimulation leads to a slight increase in nuclear area.

However, nPKC knockdown does not prevent this increase in nuclear size, suggesting that transcriptional activity is still present in these cells.

I then examined if PKC δ or ε are required for IFNα induced transcription of ISGs. The novel PKC θ is required for IFNα dependent gene transcription in T cells (Srivastava *et al.,* 2004). Knock-down of PKCθ blocked the IFNα induced transcription of ISGs but not the tyrosine phosphorylation of STAT1 or the formation of STAT1:1 or STAT1:3 dimers. PKCs have also been shown to phosphorylate the transcription factor GATA-4 and enhance its DNA binding in response to angiotensin II, a cardioregulatory hormone (Wang *et al.,* 2005). GATA-4 is then able to recruit STAT proteins to induce maximal transcription at the atrial natriuretic factor promoter. Promoter regions for other genes that contain STAT-binding sites were examined and also found to be synergistically activated by GATA-4 and STAT1. This raises the possibility that other transcription factors activated by PKCs are required for maximal expression of genes that require STAT dimers for their transcription, such as ISGs. I therefore examined if PKCs were required for maximal transcriptional expression of ISGs following IFNα stimulation. I utilized real-time PCR to assess the transcription of three ISGs, PKC, 2'-5'-OAS and MxA, in Huh7 cells that were stimulated with IFNα for 6 hours following siRNA knock-down of PKC δ or ε. However, I did not find consistent changes in ISG expression following IFNα stimulation in nPKC RNAi cells between each experiment (**Appendix II**). RT-PCR experiments were repeated four times however, none of the experiments showed a consistent trend of ISG expression either increasing or decreasing between replicates. It is not clear why there was such a difference between experiments, as all controls appeared to be satisfactory. There also appears to be no correlation between the rates of PKC isoform knockdown and ISG expression. It was surprising that RNAi of PKCε did not result in a decrease in ISG expression as nuclear localisation of STAT1 is decreased in these cells. However, it is still possible that PKCε is required for the expression of another subgroup of ISGs that I did not examine. Another possibility is that PKCε is required for STAT mediated transcription stimulated by other cytokines such as IFNγ or IL-6 but not IFNα (see Mohr A. *et al.,* 2012 for review).

Taken together, these results rule out the requirement of PKC δ and ε for IFNα induced tyrosine or serine phosphorylation of STAT1 and STAT3, which is in contrast to other cell types. I instead show that another kinase is required following my earlier observations that

Rottlerin inhibits IFNα induced tyrosine phosphorylation of STAT1 and STAT3. I uncover that PKCε expression is required for IFNα induced nuclear translocation of STAT1 but not STAT3, uncovering a new pathway of STAT regulation. However, nPKC are not required for the IFNα induced transcription of PKR, 2'5'-OAS or MxA.

Chapter V

The role of PKC ζ in Huh7 cell:cell contact

CHAPTER V

5.1 INTRODUCTION

The liver is comprised of 60% hepatocytes that generate 90% of the total hepatic proteins and control most of the major liver functions. Hepatocytes are epithelial cells and as such are polarised, forming ordered structures and boundaries between the internal components of the liver and the external environment. Unlike simply polarised cells, hepatocyte polarity is complex (**Fig. 5.1**) and organised to have several apical and basolateral poles (Decaens *et al.,* 2008). Polarised cells are separated into apical and basolateral domains. These domains are separated by adheren junctions (AJ) and tight junctions (TJ) and are required for cell barrier function. Polarity is directed by conserved polarity complexes such as aPKC:Par3:Par6 that recruit junction proteins including Claudin-1 and Occludin to cell junctions. In hepatocytes, the apical poles form a continuous network of bile canaliculi (BC). The BC is exposed to the external environment where bile is secreted (Easter *et al.,* 1983). The basal pole is termed the sinusoidal and is in contact with the blood and can secrete proteins into circulation and uptake recycled biliary salts (Lee and Luk, 2010). The border between apical and basolateral poles are formed by closely associated AJs and TJs (Nelson 2003; Mellman and Nelson, 2008).

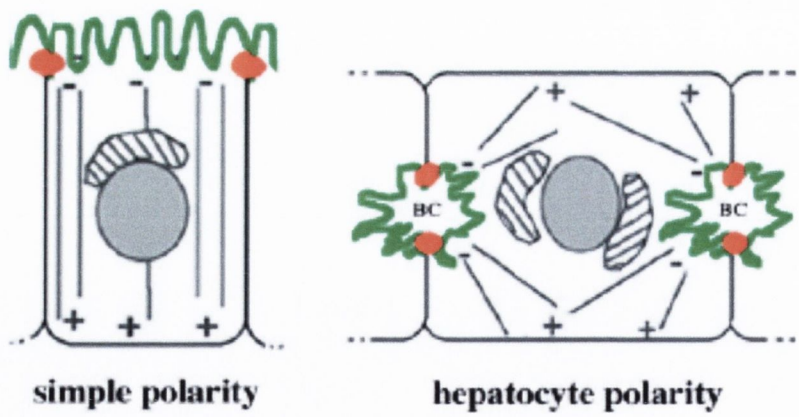


Figure 5.1. The polarity of simple epithelial cells and hepatocytes. The apical pole (green) is separated from the basolateral pole by tight junctions (red). The Golgi apparatus (dashed area) always faces the apical pole. Microtubules are orientated with minus-end at the apical pole. Bile canaliculi form between the apical poles of adjacent hepatocytes (Decaens *et al.,* 2008).

5.1.2 Adherins Junctions

AJs can be persistently produced and disassembled as required due to evolving changes in cell packing resulting from cell division, cell death, delamination and tissue organisation (Fujita *et al.,* 2002; Pilot *et al.,* 2006; Cavey *et al.,* 2008; de Beco *et al.,* 2009). AJs can also regulate cell polarity by acting as a reference point for the cell's apical-basal axis. E-Cadherin and β-Catenin are the main components of AJs in epithelial cells. They are composed of a single transmembrane domain and long extracellular and cytoplasmic domains. The cytoplasmic domain of cadherins associate with other proteins involved in cell polarity and vesicle transport and with catenins that link cell:cell adhesion to the actin-myosin network (Drees *et al.,* 2005). In order to allow for AJ turnover E-cadherin can be actively internalized and recycled back to the plasma membrane by clathrin-mediated endocytosis (Le *et al.,* 1999; Bryant and Stow, 2004). β-Catenin can transduce physical signals detected by E-cadherin at cell:cell junctions to allow for relief of membrane tension by remodelling the actin network. β-Catenin can free α-catenin to interact with actin and other actin-binding proteins such as ZO-1, vinculin and α-actinin to mediate actin cytoskeleton remodelling (Kobielak and Fuchs, 2004, Yonemura *et al.,* 2010).

5.1.3 Tight Junctions

Once initial cell:cell contact and AJs have been established, robust paracellular barrier formation can be generated by formation of TJs. TJs are coordinated complexes of transmembrane proteins that play an important role in determining cell polarity of hepatocytes. They mediate cell-cell adhesion and can also interact with the actin cytoskeleton to rearrange the cell structure (for review see Gonzalez-Mariscalet *et al.,* 2003; Schneeberger and Lynch, 2004). Three protein families are centrally involved in TJ formation and function; they are the Claudin, Occludin and Zona Occludin (ZO) families.

The Claudin family are 20-27kDa proteins with four transmembrane domains with two extracellular loops. Both the N- and C-terminal tails reside in the cytoplasmic side of the plasma membrane (Morita *et al.,* 1999; Koval 2006). They have been identified as the major transmembrane component of TJs (Tsukita and Furuse, 2002). The interaction of their C-terminal with occludin and ZO proteins stabilizes the TJ and maintains their role in perneability regulation (Umeda *et al.,* 2006; Itoh *et al.,* 1999; Muller *et al.,* 2003 Mrsny *et*

al., 2008). Claudins can interact with other claudin proteins laterally within the membrane (heteromeric interactions) or by head-to-head binding between adjacent cells (heterotypic interactions). Phosphorylation of claudin can both increase and decrease TJ assembly (D'Souza *et al.,* 2005, Ikari *et al.,* 2006) and paracellular transport (Turner *et al.,* 1999, Haorah *et al.,* 2005, Banan *et al.,* 2005).

Occludin is a 65 kDa tetraspan protein, with two extra cellular domains and a cytoplasmic C-terminal that is capable of binding ZO proteins (Tsukita and Furuse, 1999, Feldman *et al.,* 2005). Occludin is believed to have a role in regulating barrier function as cells transfected with a truncated occludin construct show increased paracellular flux of uncharged molecules (Balda *et al.,* 1996).

ZOs are scaffold proteins that cluster protein:protein interactions at the TJs. They anchor claudins and occludin to the cytoskeleton and can be phosphorylated by PKC and PKA (Avila-Flores *et al.,* 2001; Tsukita and Furuse, 2002; Piontek *et al.,* 2008).

5.1.4 The Role of the PAR Complex in Polarity

In Chapter III I demonstrated that the aPKC ζ is expressed in Huh7 cells and is localised at the cell membrane. I also demonstrated that IFNα disrupted cell:cell contacts of Huh7 cells and that PKCζ was translocalised away from the membrane (**Figure 3.8**). The aPKC:Par3:Par6 complex is necessary for anterior-posterior polarity in *Caenorhabditis elegans* and apical-basal polarity of epithelial cells in *Drosophila melanogaster* (Cox *et al.,* 2001, Drubin and Nelson, 1996). This complex has been conserved in mammalian epithelial cells and is located at the apical end where TJs are formed. Their importance can be demonstrated by kinase dead aPKC mutants, which prevent contact stimulated formation of junctions and reduce cell polarity (Suzuki *et al.,* 2001, Yamanaka *et al.,* 2001). It is possible to restrict the localization of aPKC:Par-3:Par-6 by Lethal giant larvae (Lgl), which localizes at basolateral membrane and confines aPKC:Par-3:Par-6 to the apical membrane by antagonizing aPKC (Hutterer *et al.,* 2004, Chalmers *et al.,* 2005).

5.2 HYPOTHESIS & OBJECTIVES

I hyopthesise that PKCζ activity is required for normal Huh7 cell:cell contact. This is because in Chapter III I demonstrated that PKCζ is localised at the plasma membrane of cells and is highly expressed at cell:cell contacts. Following IFNα stimulation PKCζ translocates away from the plasma membrane and cell:cell contacts degrade. It is well established that aPKC is required for normal cell:cell contact by directing the localisation of TJ proteins such as Claudin-1, Occludin and ZO-1. Due to its cell membrane localisation I hypothesise that PKCζ colocalises with AJ and TJ proteins and when this colocalisation is disrupted, by IFNα or PKCζ inhibition, cell:cell contact is disrupted.

Therefore the objectives of this chapter are:

- To examine the localisation of PKCζ in resting and long term IFNα stimulated Huh7 cells.
- To examine the localisation of PKCζ and cell:cell contacts in Huh7 cells following PKCζ inhibition.
- To examine the relationship between PKCζ and the TJ proteins Claudin-1, Occludin and ZO-1 in resting, IFNα stimulated and PKCζ inhibited Huh7 cells.
- To examine the relationship between PKCζ and the AJ proteins E-cadherin and β-catenin in resting, IFNα stimulated and PKCζ inhibited Huh7 cells.

5.3 RESULTS

5.3.1 PKC ζ Membrane localisation is required to maintain cell:cell contact

In Chapter III I observed that IFNα induced the translocalisation of the atypical PKC ζ from the membrane to the cytoplasm and disrupted cell:cell contact. Atypical PKCs play an important role in directing the localisation of proteins involved in maintaining cell:cell contacts (Iden S *et al.,* 2012). I set out to examine if PKCζ activity was required to maintain cell:cell contacts of Huh7 cells and if PKCζ activity was also required to direct the localisation of cell junction proteins.

As I had previously witnessed that IFNα inhibited normal cell:cell contact after 2 hours of treatment I first confirmed that cell:cell contact could reform following longer term treatment that would be typical of medicinal administration of IFNα. **Figure 5.1** shows that cell:cell contacts appear normal when Huh7 cells are treated for 24 hours with IFNα or allowed 22 hours to recover following 2 hours IFNα treatment. Notably PKCζ in these cells is found in its normal membrane localisation. I can also show that IFNα induced phosphorylation of STAT1 tyrosine 701 after 24 hours is reduced compared 2 hours of IFNα suggesting that the effects of IFNα are not long lasting (**Figure 5.2**). To investigate if PKCζ activity is required to maintain cell:cell contacts in Huh7 cells I treated cells with a cell permeable myristoylated PKCζ pseudosubstrate inhibitor (PKCζPS) that specifically binds the pseudosubstrate region of PKCζ which then prevents its substrate binding. When cells were treated with this inhibitor at 10μM for one hour I observed a decrease in the membrane localisation of PKCζ and a decrease in cell:cell contact similar to that seen by 2 hours IFNα treatment (**Figure 5.3**). I also pre-treated cells with PKCζPS for one hour before 2 hours IFNα stimulation to examine if the decrease in PKCζ translocalisation and cell:cell contact could be additive. Pre-treated cells appeared to have a similar phenotype to cells treated with PKCζPS alone suggesting that IFNα does not utilise another pathway to further translocate PKCζ or disrupt cell:cell contact. When cells were treated with 50μM of PKCζPS for one hour with or without further IFNα stimulation I found gross changes in cell morphology and large spaces forming between cells (**Figure 5.4)**. It was therefore decided to continue further PKCζPS experiments at a concentration of 10μM. To further confirm the requirement for PKCζ in maintenance of cell:cell contacts, I assessed the

ability of Huh7 monolayers to form barriers between apical and basal spaces before and after PKCζPS or IFNα treatment. I grew cells in transwell inserts for 5 days before treating cells with IFNα or PKCζPS. I then placed 5μg/ml of a 70kDa Dextran polymer conjugated to fluorescein isothiocyanate (FITC-Dextran) in the apical chamber and measured the amount of FITC-Dextran that had passed to the basal chamber following 2 hours incubation. The higher the value of the column the higher the paracellular barrier formed between cells. Results in **Figure 5.5** show that cells are able to form a paracellular barrier that prevents FITC-Dextran from passing from the apical to basal side. Inserts with no cells (Blank) or with cells treated overnight with 2μM staurosporine, which is known to induce apoptosis, were included as negative controls showing no paracellular barrier. It can also be seen that barrier permeability is higher in cells treated with PKCζPS than cells treated with IFNα. There is also a slight increase in barrier permeability in cells treated with IFNα for 2 hours compared to cells treated with IFNα for 24 hours, however, this is not statistically significant. Taken together these results indicate that PKCζ membrane localisation and substrate binding activity is imperative for normal cell:cell contact formation in Huh7 cells.

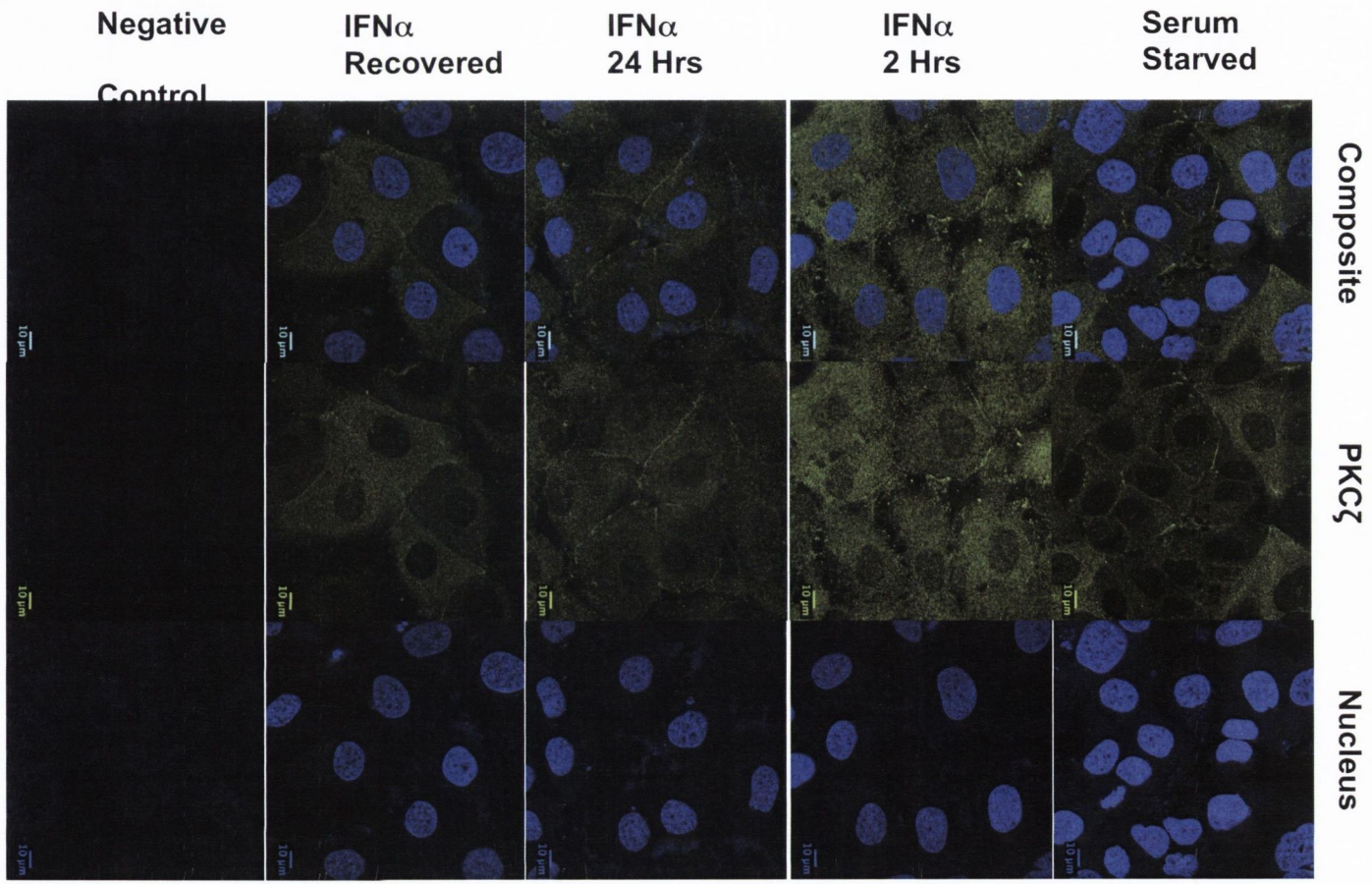


Figure 5.1 Huh7 cell:cell contacts recover following prolonged IFNα treatment. Huh7 cells were serum starved for 4 hours and then treated with 1000U/ml IFNα for the times indicated. IFNα recovered cells were treated with IFNα for 2 hours before their medium was replaced with DMEM. Cells were fixed in 4% PFA and incubated with primary antibody specific for PKCζ and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

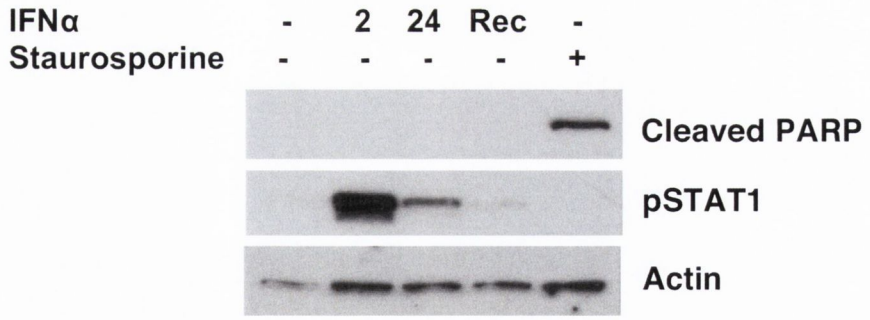


Figure 5.2 24 Hours IFNα treatment does not induce apoptosis. Huh7 cells were grown to 70-80% confluence and treated with IFNα for the time points indicated. IFNα recovered cells were treated with IFNα for 2 hours before their medium was replaced with DMEM. Cells treated with 1μM staurosporine for 24 hours are shown as a positive control for the expression of cleaved PARP. Cells were then lysed with NP40 lysis buffer containing protease inhibitors. SDS-PAGE was performed with 10μg Huh7 lysate per well and cleaved PARP was detected by Western blot. The figure is representative of three independent experiments.

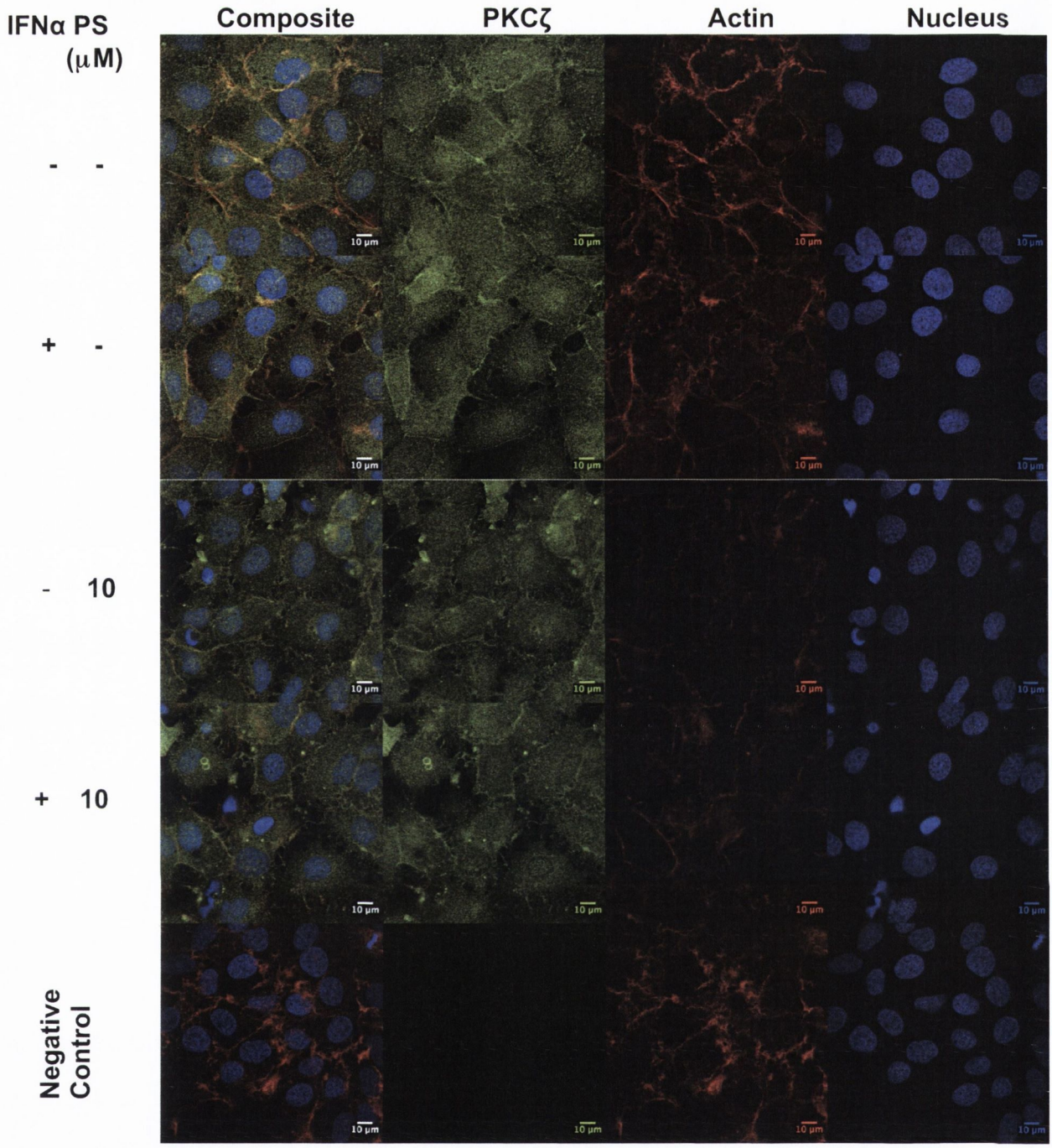


Figure 5.3 10μM PKCζ pseudosubstrate inhibition disrupts cell:cell contacts. Huh7 cells were serum starved for 4 hours, then pre-treated with 10μM PKCζ pseudosubstrate inhibitor for 1 hour and then treated with 1000U/ml IFNα for the times indicated. IFNα recovered cells were treated with IFNα for 2 hours before their medium was replaced with DMEM. Cells were fixed in 4% PFA and incubated with primary antibody specific for PKCζ the actin stain Phillodin and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

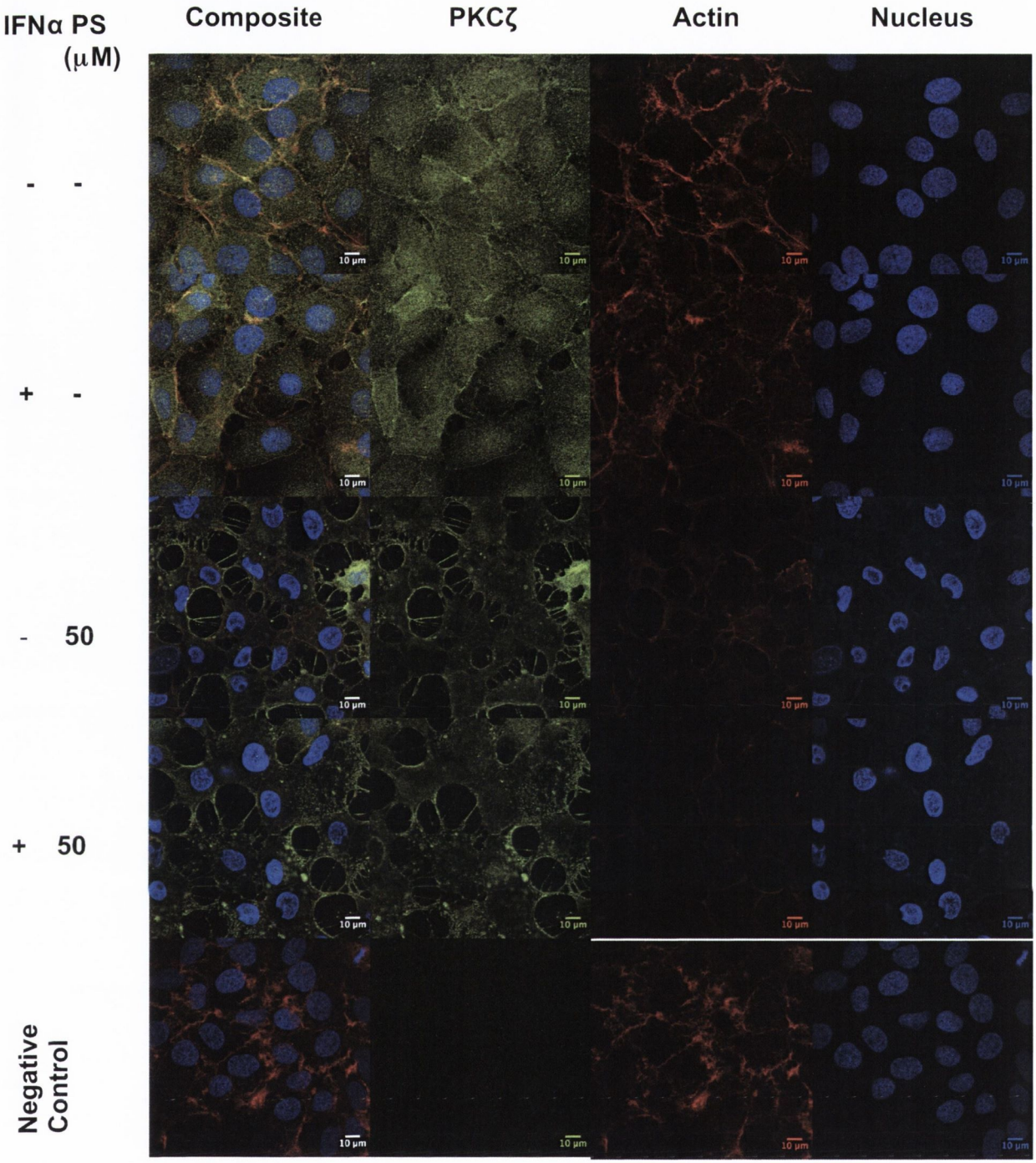


Figure 5.4 50μM PKCζ pseudosubstrate inhibition disrupts cell:cell contacts. Huh7 cells were serum starved for 4 hours and then pre-treated with 50μM PKCζ pseudosubstrate inhibitor for 1 hour and then treated with 1000U/ml IFNα for the times indicated. IFNα recovered cells were treated with IFNα for 2 hours before their medium was replaced with DMEM. Cells were fixed in 4% PFA and incubated with primary antibody specific for PKCζ the actin stain Phalloidin and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

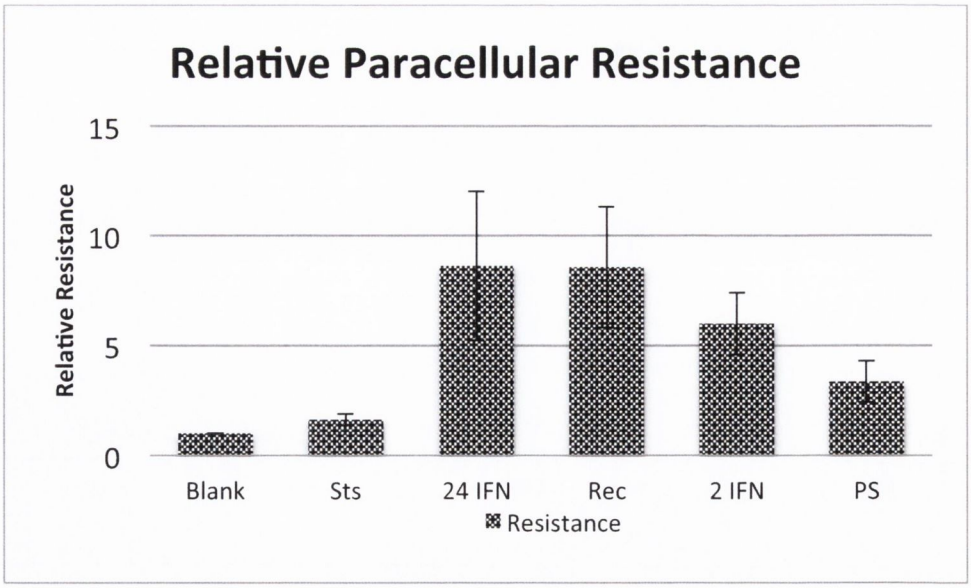


Figure 5.5 FITC-Dextran Paracellular Permeability Assay. Huh7.5 cells were seeded on a transwell insert and grown for 5 days to form a monolayer. Cells were then serum starved for 2 hours and treated with IFNα for the time points indicated. IFNα recovered cells were treated with IFNα for 2 hours before their medium was replaced with DMEM. Cells treated with 1μM staurosporine for 24 hours are shown as a positive control for paracellular permeability. FITC-dextran was added to the apical layer of cells for 2 hours. Samples of media were then taken from the basal well and FITC-Dextran detected by fluorescence emission at 535nm. A blank well with no cells seeded was also included as a control to show transwell membrane permeability. All treatments were performed in duplicate. Readings were then normalised to the amount of FITC-Dextran that passed through the blank wells as shown by the fold decrease in FITC-Dextran measured compared to the blank control. The graph is representative of the average of three independent experiments.

5.3.2 PKC ζ activity is required for E-Cadherin stability and directs the localisation of Adhesion Junction proteins

I next examined if PKCζ activity was required to direct proteins involved in cell:cell adhesion and if IFNα treatment interrupted any interaction between PKCζ and these proteins. I utilised confocal immunofluorescent microscopy to examine if PKCζ colocalised with the adherens junction (AJ) proteins β-Catenin and E-Cadherin in resting and IFNα stimulated cells and if PKCζPS inhibition would alter AJ localisation. **Figure 5.6** illustrates that PKCζ and β-Catenin colocalise at the membrane of resting Huh7 cells. It can also be seen that when IFNα disrupted cell:cell contact after 2 hours that colocalisation at the membrane is lost at areas where cells are not in contact with each other, however, the reduction of β-Catenin at the membrane appears to be less than that of PKCζ suggesting that translocation of PKCζ from the membrane occurs before or at a faster rate than β-Catenin. Cells treated with PKCζPS show a similar phenotype, demonstrating that PKCζ activity is required to preserve β-Catenin localisation at the membrane. I also examined if PKCζ colocalised with E-Cadherin in a similar manner. **Figure 5.7** shows results comparable to those for β-Catenin, however, it can be seen that when cells were treated with PKCζPS all colocalisation with PKCζ and E-Cadherin is lost. Orthogonal views of Z-stack images of PKCζ colocalisation with β-Catenin and E-Cadherin can be found in **Appendix III.** When protein expression of these AJs was examined by Western blot I found that PKCζ inhibition lead to a cleaved fragment of E-Cadherin being detected while expression of β-Catenin is unaffected (**Figure 5.8**). These results show that PKCζ activity is required for the localisation of AJ proteins at the cell membrane and protein stability of E-Cadherin.

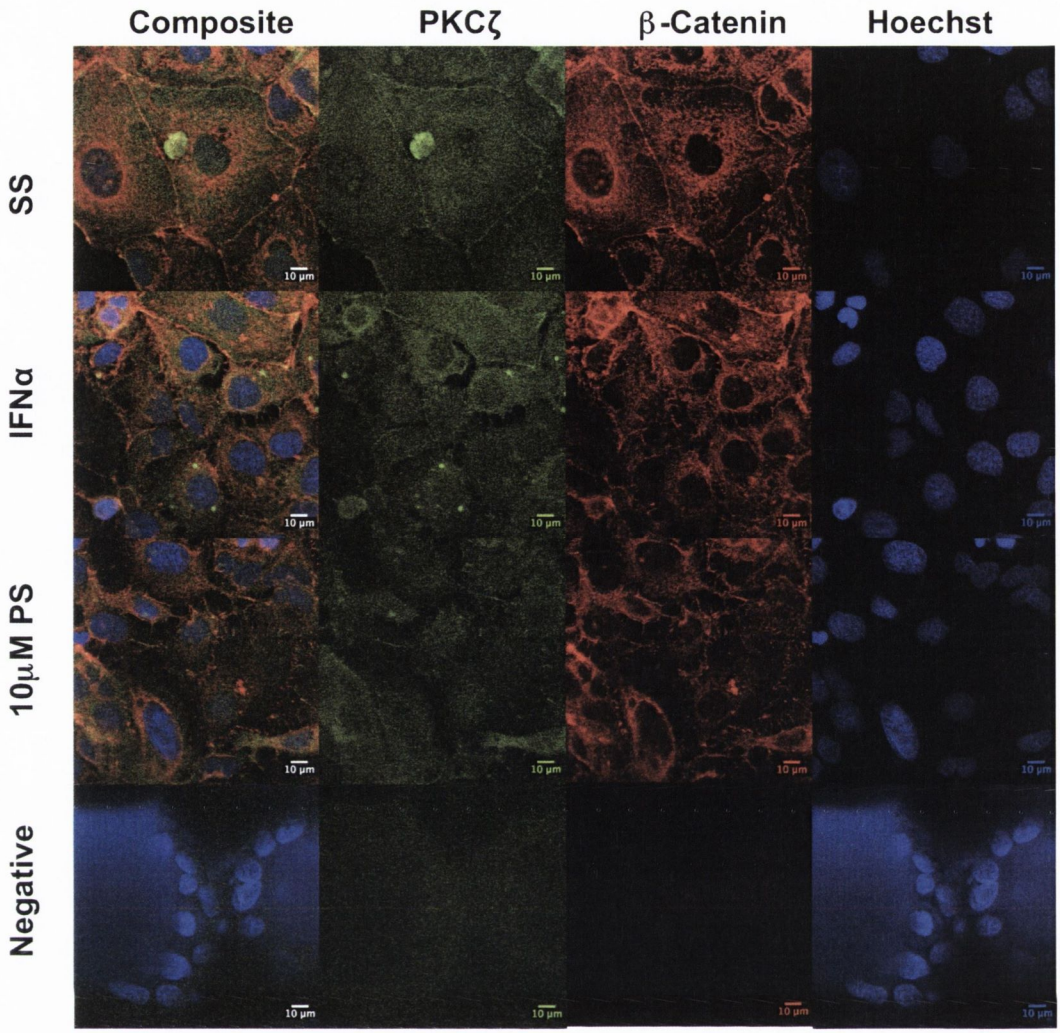


Figure 5.6 PKCζ Colocalisation with Beta-Catenin following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours and then treated with either 10μM PKCζ pseudosubstrate inhibitor for 1 hour or with 1000U/ml IFNα for two hours. Cells were fixed in acetone and incubated with primary antibody specific for PKCζ and beta-Catenin and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

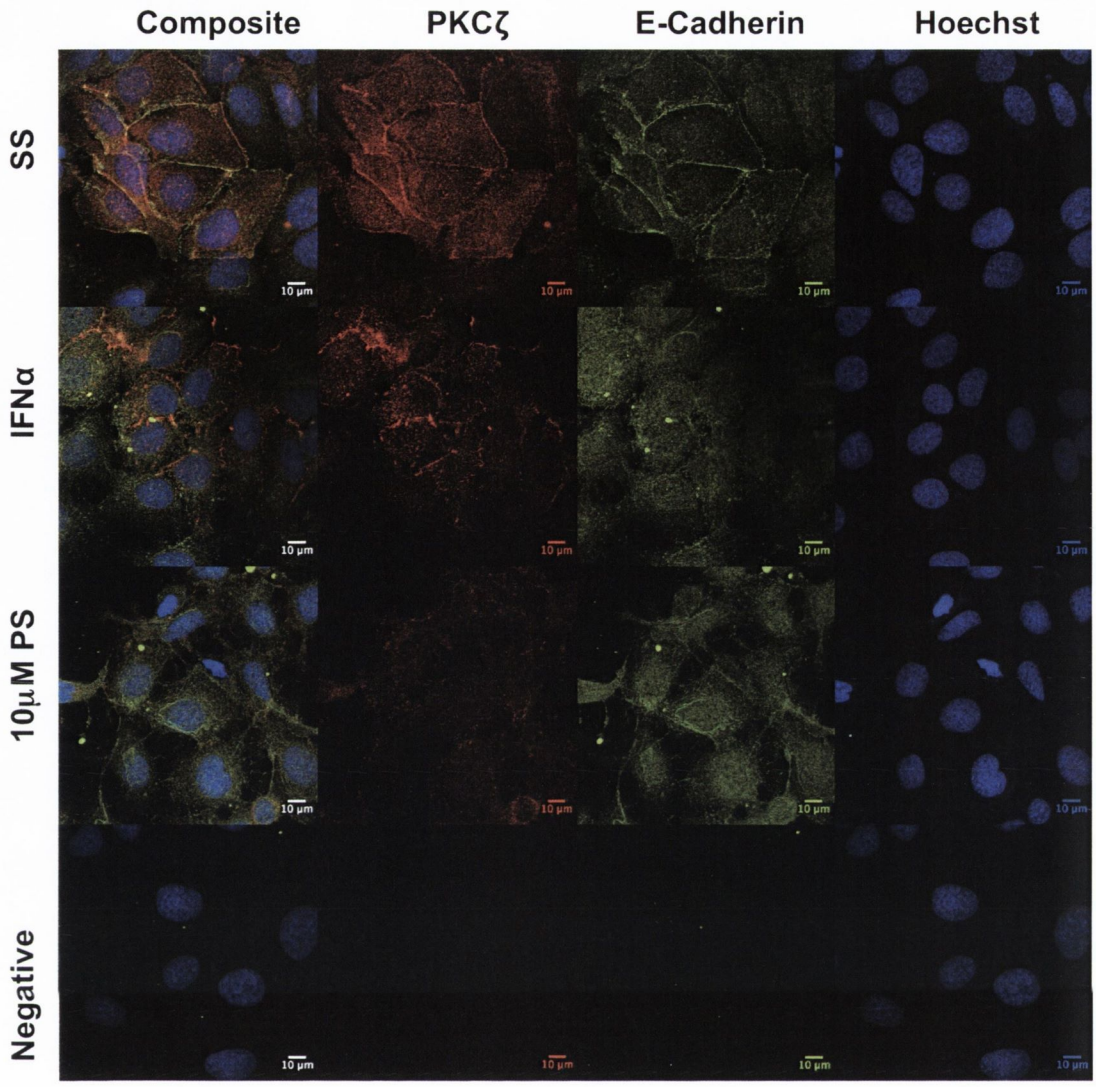


Figure 5.7 PKCζ Colocalisation with E-Cadherin following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours and then treated with either 10μM PKCζ pseudosubstrate inhibitor for 1 hour or with 1000U/ml IFNα for two hours. Cells were fixed in acetone and incubated with primary antibody specific for PKCζ and E-Cadherin and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

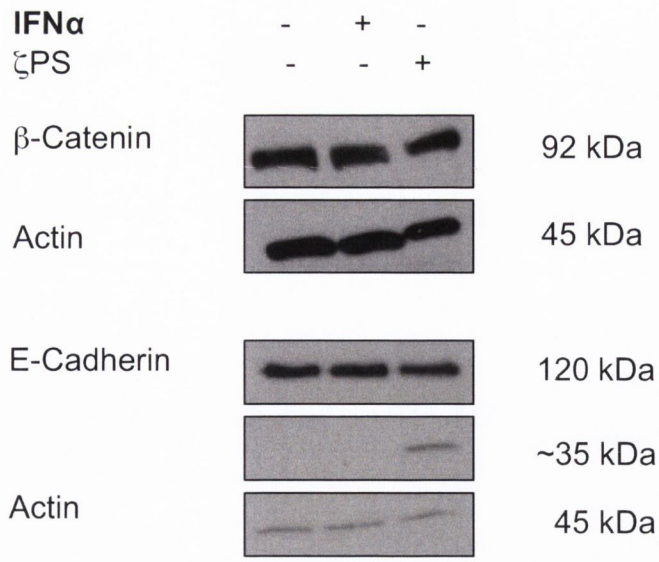


Figure 5.8 E-Cadherin is cleaved to form a ~35 kDa fragment following 10mM PKCζ pseudosubstrate inhibition. β-Catenin protein expression is not altered by IFNα or 10mM PKCζ pseudosubstrate inhibition. Huh7 cells were serum starved for 4 hours and treated with 1000U/ml IFNα for 2 hours or 10mM PKCζ pseudosubstrate. SDS-PAGE was performed and E-Cadherin and β-Catenin detected by Western blot using specific antibodies. Actin is shown as a loading control. Following PKCζ pseudosubstrate treatment E-Cadherin showed a band at ~35 kDa as well as it's predicted 120 kDa molecular weight. Results are representative of three independent experiments.

5.3.3 PKC ζ activity directs the localisation of Tight Junction Proteins

To see if PKCζ activity is also required to direct the localisation of tight junction (TJ) proteins in Huh7 cells I then examined the expression of claudin-1, occludin and ZO-1 following IFNα stimulation and PKCζPS inhibition. I found that these proteins were all localised at the cell membrane as would be expected. Claudin-1 colocalised with PKCζ in resting cells (**Figure 5.9**) but did not following IFNα treatment where there was still some membrane localisation of claudin-1 but not for PKCζ. Results for PKCζPS inhibition showed similar findings, highlighting the importance of PKCζ activity in Claudin-1 localisation. When I similarly examined the localisation of the TJ proteins occludin and ZO-1 with PKCζ I found results highly comparable with those for claudin-1. Occludin colocalised with PKCζ in resting cells (**Figure 5.10**) but did not following IFNα treatment where there was a reduction of membrane localisation and colocalisation of occludin and PKCζ. Results with PKCζPS inhibition showed similar findings, highlighting the importance of PKCζ activity in occludin localisation. ZO-1 colocalised with PKCζ in resting cells (**Figure 5.11**) but did not following IFNα treatment where there was a reduction of membrane localisation and colocalisation of ZO-1 and PKCζ. Results with PKCζPS inhibition showed similar findings, highlighting the importance of PKCζ activity in ZO-1localisation. Orthogonal views of Z-stack images of PKCζ colocalisation with TJ proteins. can be found in **Appendix III.** I then examined TJ protein expression by Western blot (**Figure 5.12**). I found that PKCζPS treatment but not IFNα induced cleavage of occludin into fragments of ~40 and 55 kDa. I did not detect cleavage of Claudin-1, while technical limitations prevented the detection of ZO-1 by Western blot. I can conclude that PKCζ activity is required for the localisation of TJ proteins at the cell membrane and protein stability of occludin.

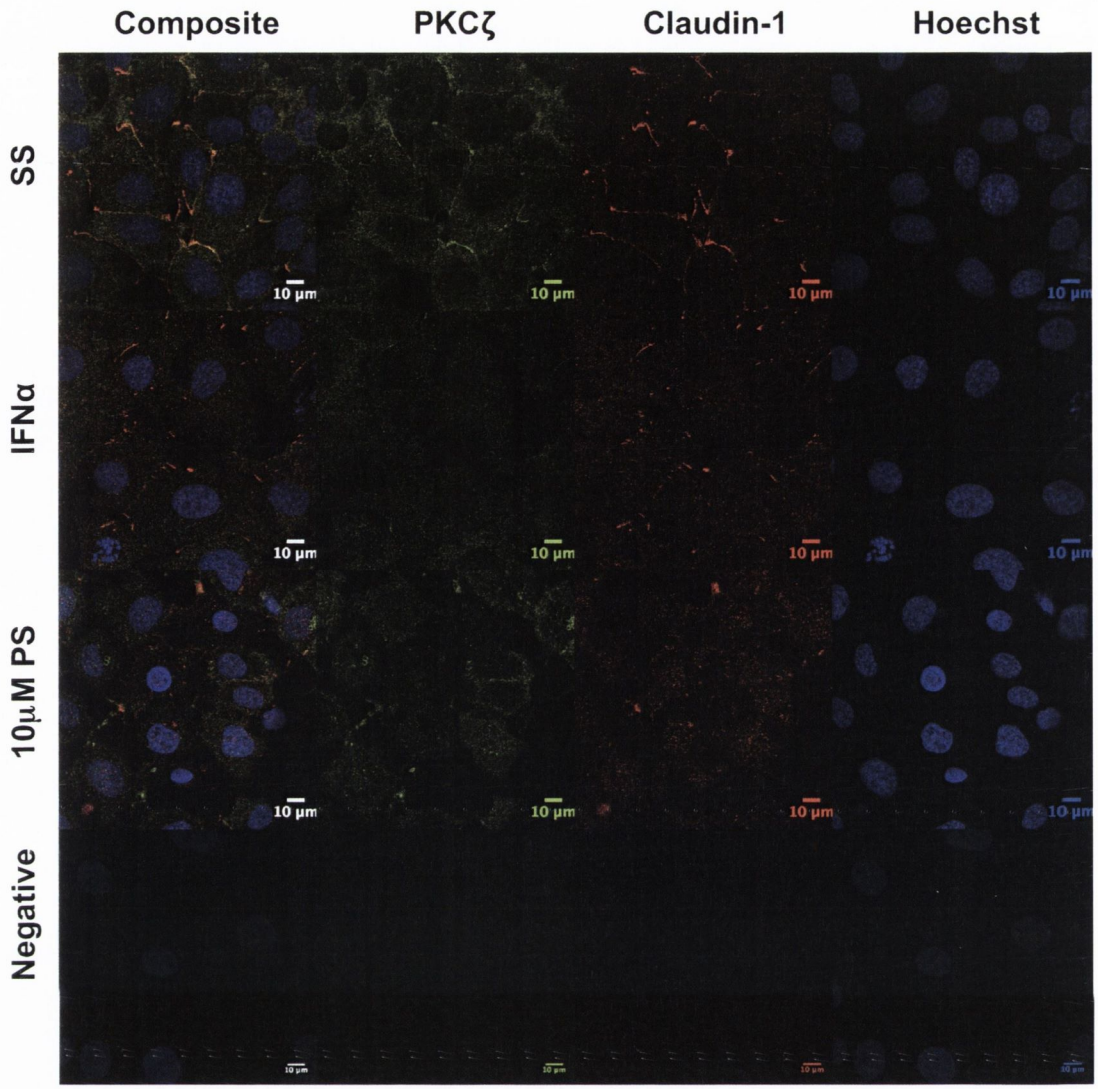


Figure 5.9 PKCζ Colocalisation with Claudin-1 following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours and then treated with either 10μM PKCζ pseudosubstrate inhibitor for 1 hour or with 1000U/ml IFNα for two hours. Cells were fixed in acetone and incubated with primary antibody specific for PKCζ and claudin-1 and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

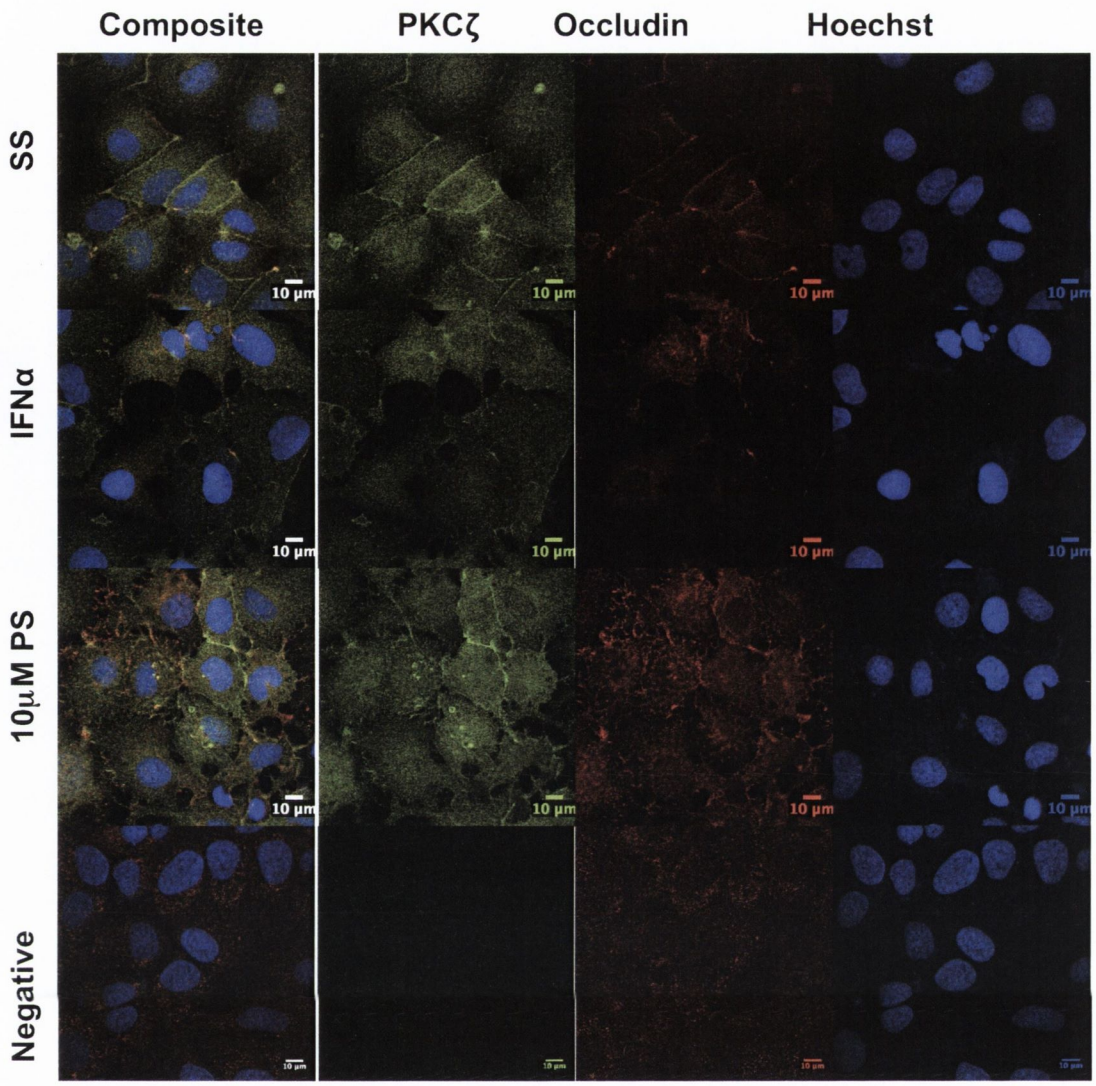


Figure 5.10 PKCζ Colocalisation with Occludin following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours and then treated with either 10μM PKCζ pseudosubstrate inhibitor for 1 hour or with 1000U/ml IFNα for two hours. Cells were fixed in acetone and incubated with primary antibody specific for PKCζ and occludin and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

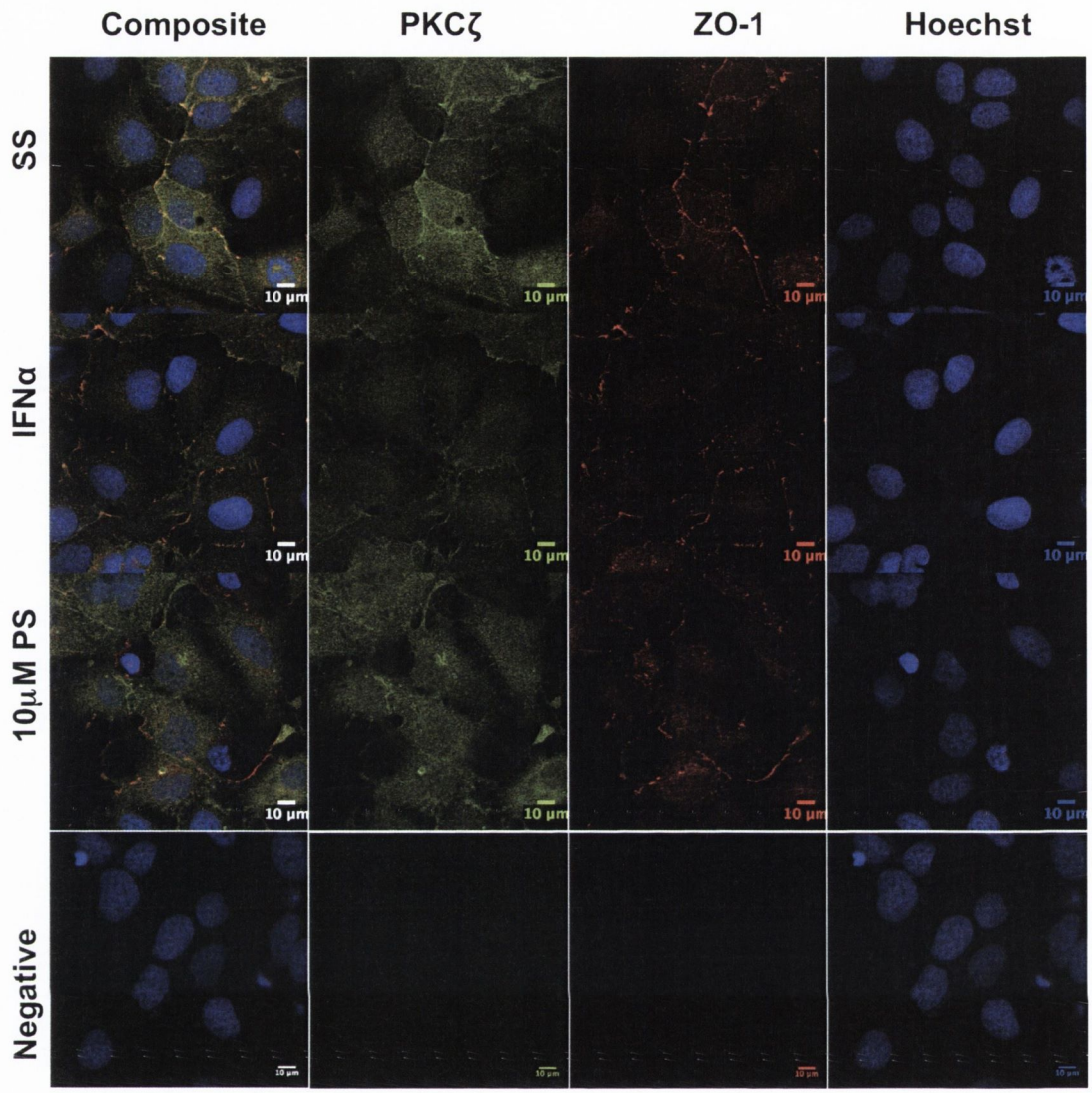


Figure 5.11 PKCζ Colocalisation with ZO-1 following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours and then treated with either 10μM PKCζ pseudosubstrate inhibitor for 1 hour or with 1000U/ml IFNα for two hours. Cells were fixed in acetone and incubated with primary antibody specific for PKCζ and ZO-1 and the DNA stain Hoechst. Cells were examined by a laser-scanning confocal microscope with a 63x objective. A negative control where no PKC antibody was used was included to exclude the possibility of non-specific antibody staining. Results are representative of three independent experiments.

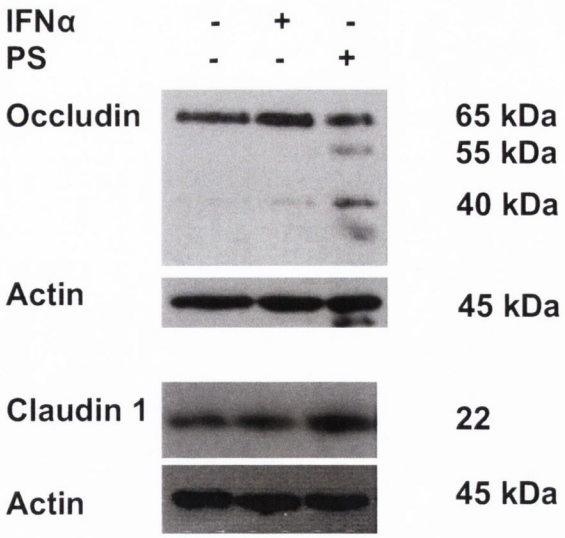


Figure 5.12 Claudin-1 & Occludin expression following IFNα treatment or PKCζ inhibition. Huh7 cells were grown to 70-80% confluence and serum starved for 4 hours and treated with 1000U/ml IFNα for 2 hours or 10mM PKCζ pseudosubstrate. SDS-PAGE was performed and Occludin and Caludin-1 detected by Western blot using specific antibodies. Actin is shown as a loading control. Following PKCζ pseudosubstrate treatment Occludin showed a band at ~34 and ~55 kDa as well as it's predicted 65 kDa molecular weight. Results are representative of three independent experiments.

5.4 DISCUSSION

Epithelial cells exhibit an apicobasal polarity in order to control their cytoskeletal organization and distribution of membrane proteins. Polarity also helps form a selective paracellular barrier that restricts the passage of solutes through the cell monolayer, maintaining distinct compartments within the organism (Gonzalez-Mariscal L *et al.,* 2003). Following initial cell:cell contact via adherens junctions (AJ), tight junctions (TJ) form ordered contact points between adjacent cells. The intercellular space is then sealed by a network of structures integrated within the plasma membrane of the involved cells (Furuse M, 2010). At the molecular level, TJs are complexes that link adjacent cells and can interact through their intracellular domains with cytoplasmic proteins providing anchorage for the actin cytoskeleton (Matter K, Balda MS. *et al.,* 2003, Aijaz S *et al.,* 2006). These adaptor proteins can also interact with one another. All these interactions regulate TJ function and play a role in the transduction of signals involved in the control of key cell functions, including cell proliferation and differentiation. This is demonstrated by IFNγ, which can increase claudin endocytosis and tight junction permeability (Chiba *et al.,* 2006).

In Chapter III I observed that IFNα treatment translocated PKCζ away from the membrane and that cell:cell contacts appeared to degrade. In this chapter I demonstrated that the Huh7 cell monolayer that was initially disrupted following 2 hours of IFNα treatment could be re-established following prolonged treatment with this cytokine. This re-establishment was associated with a return of PKCζ localisation to the cell membrane. Inhibition of PKCζ activity, by blocking its ability to bind substrates using a pseudosubstrate peptide (PKCζPS) also lead to PKCζ translocalisation from the membrane and cell:cell contact disruption in a similar fashion to that observed with IFNα. Paracellular permeability assays also demonstrated that PKCζ inhibition reduced cell barrier function in Huh7 cells. This reveals a novel mechanism of action for IFNα by controlling PKCζ localisation which then directs Huh7 cell:cell contact maintenance.

Atypical PKCs (aPKCs) such as PKCζ are key regulators of polarity and cell contact maturation (Suzuki and Ohno, 2006). aPKCs regulate cell polarity through an evolutionarily conserved system by forming complexes with PAR-3 (or its mammalian homologue aPKC-specific interacting protein (ASIP) depending on the cell type examined)

and Par-6 proteins which in turn regulate each others activity (Macara, 2004, Nagai-Tamai *et al.,* 2002, Ohno, 2001. aPKC and the scaffold protein PAR-6 interact through their N-terminal phox and Bem1p (PB1) domains (Suzuki *et al.,* 2003; Suzuki *et al.,* 2001). The scaffold protein PAR-6 works as an adaptor molecule that links Rac1/Cdc42 GTPase activity to aPKC. Cdc42 is involved in establishing cell polarity by transmitting polarity signals to the aPKC complex (Etienne- Manneville and Hall, 2001; Gotta *et al.,* 2001, Hutterer *et al.,* 2004, Yamanaka *et al.,* 2001). The aPKC kinase domain also interacts with the central conserved region of the scaffold protein, PAR-3. Phosphorylation of this region by aPKC decreases PAR-3 interaction with aPKC (Izumi *et al.,* 1998, Nagai-Tamai *et al.,* 2002).

I further investigated the ability of PKCζ to direct the localisation of proteins that form AJ and TJ in Huh7 cells and found the localisation of the AJ proteins β-catenin and E-cadherin and the TJ proteins claudin-1, occludin and ZO-1 were all translocated away from the membrane when cells were treated with IFNα or PKCζPS. PKCζPS treatment also resulted in protein cleavage of E-cadherin and occludin demonstrating a protective role of PKCζ for these proteins. This indicates that IFNα is able to alter the localisation of PKCζ in order to disrupt AJ and TJ proteins localisation and regulate cell:cell contact, either through a direct or indirect mechanism.

Possible mechanisms that IFNα could use to translocate PKCζ include signalling through the extracellular signal-regulated kinase (ERK) pathway. IFNα has previously been shown to activate the ERK pathway (David M *et al.,* 1995, Wang F *et al.,* 2004). It has also been shown in Madin–Darby canine kidney (MDCK) epithelial cells that activated ERK is able to induce the disassembly TJs (Togawa A). It is therefore possible that this pathway is also utilised by IFNα to translocate TJ proteins in Huh7 cells. In MDCK cells PKCζ forms a complex with PAR-6 and PAR-3 at TJs. However, PKCζ:PAR-6 association can be inhibited by Cdc42 binding PAR-6, leading to the disassociation of TJs. Cells treated with hepatocyte growth factor (HGF) activate ERK, which leads to Cdc42 binding PAR-6 and disassociating TJ structures, demonstrating how ERK pathway can alter polarisation.

Another mechanism that could dismantle cell:cell contact is inhibition of PKCζ by activation of a specific PKCζ inhibitor such as protein phosphatase 2A (PP2A) or prostate androgen

responsive-4 (Par-4) which are known to regulate PKCζ and lead to a reduction in tight junction stability. When I inhibited PKCζ I observed the translocalisation of JT proteins. PP2A is a serine/threonine phosphatase that has been shown to regulate PKCζ activity and TJ assembly in MDCK cells (Nunbhakdi-Craig V *et al.,* 2002). PP2A is recruited to regions of cell:cell contact and interacts with claudin-1, occludin and ZO-1 leading to their dephosphorylation and in increase in paracellular permeability. However, to date, there has not been a clear pathway identified involving both IFNα and PP2A. Likewise Par-4 is able to inhibit PKCζ enzymatic activity (Moscat J *et al.,* 2006). Par-4 interacts with aPKC at its PB1 domain and prevents PKCζ activating NF-κB (Diaz-Meco MT *et al.,* 1996; Diaz-Meco MT *et al.,* 1999) while Par-4$^{-/-}$ knockout mice show hyperphosphorylation of PKCζ (Garcia-Cao I, *et al.,* 2003).

These findings demonstrate that IFNα is able to direct a dynamic remodelling of cell:cell contacts through the translocalisation of PKCζ from the plasma membrane by a mechanism that has yet to be uncovered. PKCζ inhibition also induced cleavage of E-Cadherin and occludin proteins. PKCζ localisation at the plasma membrane promotes its colocalisation with AJ and TJ proteins that are vital for cell:cell contact and barrier formation in Huh7 cells. As TJ proteins are also involved in HCV entry to hepatocytes I examined if PKCζ activity was required for HCV replication in Huh7.5 cells. These results are described in Chapter VI.

Chapter VI

PKC ζ inhibition reduces J6/JFH HCVcc replication in Huh7.5 cells

CHAPTER VI

6.1 INTRODUCTION

HCV viral entry and replication mechanisms within hepatocytes still need to be fully understood. Until 2005, when the HCVcc system was developed, there was no efficient cell culture system to examine the HCV lifecycle. The HCVcc system created full-length chimeric genomes with the use of the core-NS2 gene regions from the infectious J6 (genotype 2a) or H77 (genotype 1a) virus strains and a genotype 2a subgenomic replicon, SGRJFH1, which efficiently replicates in cell culture without adaptive mutations (Lindenbach *et al.*, 2005). In this system Huh7.5 cells, which contain a point mutation in RIG-I as well as low TLR expression, are transfected with full-length chimeric genomic RNA. The RNA is translated and HCVcc viral particles are then produced by the cell machinery and secreted from the cell. Medium containing secreted viral particles can then be used to infect naïve cells.

I have shown in Chapter III that IFNα treatment translocates PKCζ away from the plasma membrane and in Chapter V that PKCζ is required for the membrane localization of tight junction proteins. As described in Section 1.1.3 Claudin-1 and Occludin expression at the cell surface is required by HCV to enter hepatocytes and replicate (Benedicto I *et al.,* 2012). However the role of PKCζ activity in HCV lifecycle has not been investigated thus far.

6.2 HYPOTHESIS & OBJECTIVES

I have demonstrated in Chapter III that IFNα treatment translocates PKCζ away from the plasma membrane and in Chapter V that PKCζ inhibition is also able to translocate PKCζ away from the plasma membrane and that Claudin-1 and Occludin are also translocated from the plasma membrane. I hypothesise that PKCζ translocation away from the membrane results in a withdrawal of HCV entry factors from the cell surface, thereby inhibiting the ability of HCV to enter and replicate in hepatocytes. In this chapter I have used the HCVcc system and peptide inhibition of PKCζ to examine this hypothesis. If this were to be the case then this would be a novel IFNα antiviral response and identify PKCζ activity as a new target for HCV treatment.

Therefore, the objectives of this chapter are:

- To generate J6/JFH and H77/JFH RNA.
- To generate J6/JFH HCVcc viral particles.
- To examine the role of PKCζ in HCVcc infection of Huh7.5 cells.

6.3 RESULTS

6.3.1 Production of J6/JFH and H77/JFH DNA and RNA

In order to generate HCVcc viral particles, large quantities of J6/JFH and H77/JFH DNA and RNA first had to be generated as described in section 2.10. *E.coli* competent cells transfected with the appropriate circular plasmid DNA were grown overnight and DNA then isolated. This circular DNA plasmid was then linearized in order to be able to be used for RNA synthesis. The circular plasmid DNA and linearized DNA was visualised on an agarose gel in order to confirm that they were not contaminated and were the correct base pair length (**Fig 6.1**). Once the linearized DNA was confirmed to be of the correct base pare length, RNA was transcribed by a AmpliScribe T7 kit and RNA then isolated and purified. The RNA was then visualised on an agarose gel in order to confirm that the RNA was of correct base length and of good purity (**Fig 6.2**).

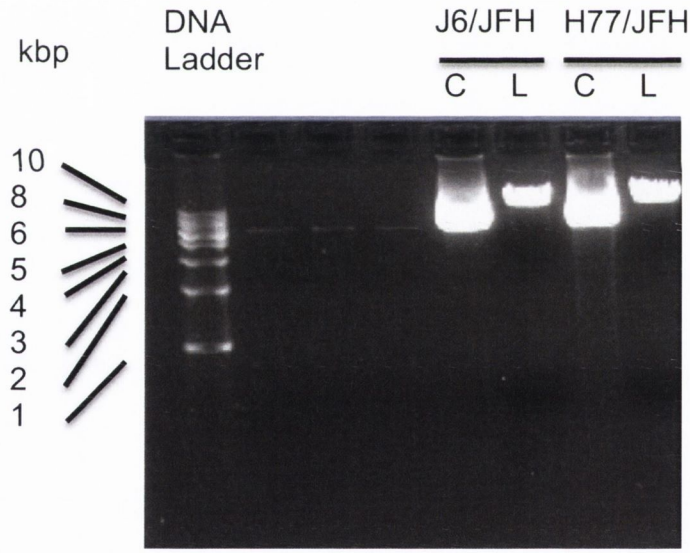


Figure 6.1 Coiled and linear HCVcc J6/JFH and H77/JFH DNA visualisation by agarose gel. Coiled (C) and linearized (L) J6/JFH and H77/JFH HCVcc DNA can be seen as a single band at 12.3kbp. Coiled DNA moves through agarose gel at a faster rate than linearized DNA and therefore appears to have a lower base pair length.

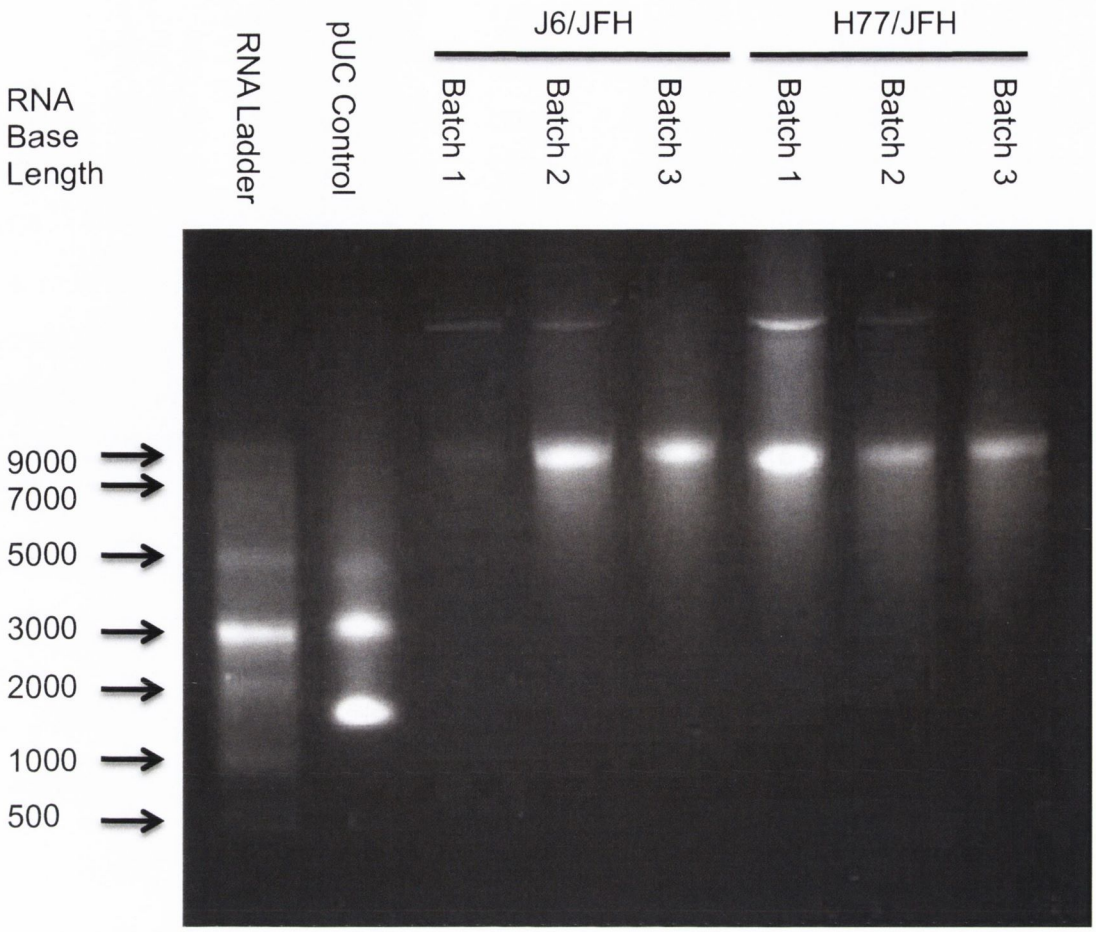


Figure 6.2 HCVcc J6/JFH and H77/JFH RNA visualisation by agarose gel. J6/JFH and H77/JFH HCVcc RNA can be seen as a single 9.6kb band. A pUC control at 3000 and 1500 bases is shown as a positive control.

6.3.2 J6/JFH HCVcc viral particles infect Huh7.5 cells

As the cell surface expression of claudin-1 and occludin are required by HCV to enter hepatocytes I investigated if inhibition of PKCζ could reduce the ability of HCV to replicate within Huh7.5 hepatocytes. I generated J6/JFH HCVcc viral particles using J6/JFH RNA transcribed as described in chapter 2. I first verified that these particles could infect and replicate in Huh7.5 cells. I incubated viral particles with Huh7.5 cells for 72 hours before visualising HCV replication by staining for the HCV non-structural protein NS5A (**Figure 6.3**). As predicted NS5A was detected in the cytoplasm of Huh7.5 cells (**Figure 6.4**). In parallel to all HCVcc experiments I also treated cells with a mock virus control that did not contain HCV in order to confirm that any results shown were caused specifically by HCVcc replication (data not shown). Using serial dilutions I was able to determine the tissue culture infectious dose 50 ($TCID_{50}$/ml) of viral particles generated. This allowed us to infect Huh7.5 cells at a standard MOI of 0.5 for future PKCζ inhibition studies. A preliminary examination of PKCζ localisation following HCVcc infection showed that PKCζ remained at the areas of cell:cell contact (**Figure 6.5**), however, this study must be repeated using confocal microscopy.

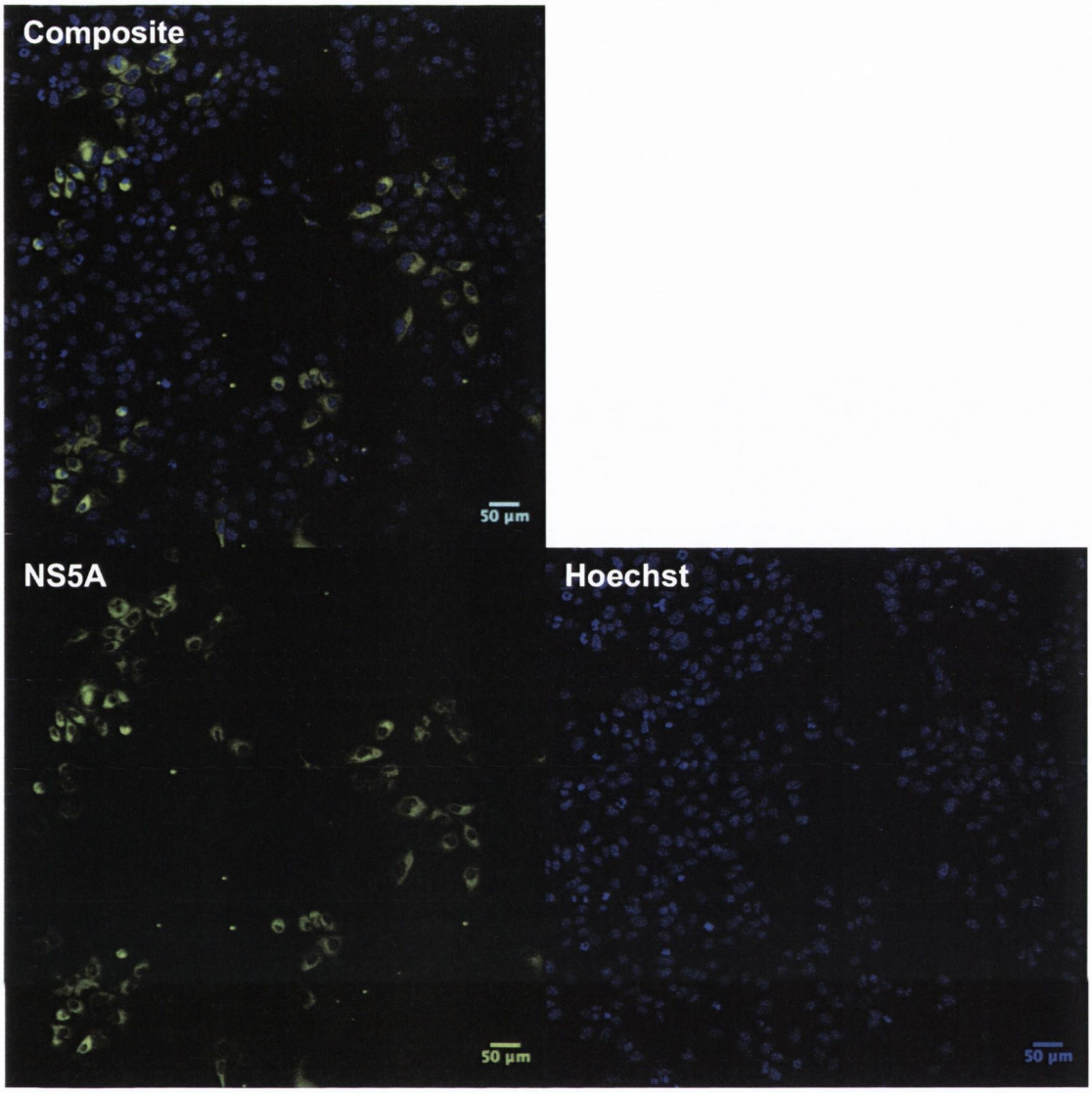


Figure 6.3 J6/JFH HCVcc infects Huh7.5 cells. Huh7.5 cells were grown to 40-50% confluence and then infected with J6/JFH for 72 hours. Inoculant was then removed and cells fixed in a 1:1 mix of acetone and methanol. Cells were then incubated with a primary antibody specific for HCV NS5A and the DNA stain Hoechst. Cells were imaged by a laser microscope using a 20x objective lens. A negative control where no PKC primary antibody was added was used to exclude the possibility of non specific secondary antibody staining. A mock infection where no viral particles were added to cells was also performed. Images are representative of three independent experiments.

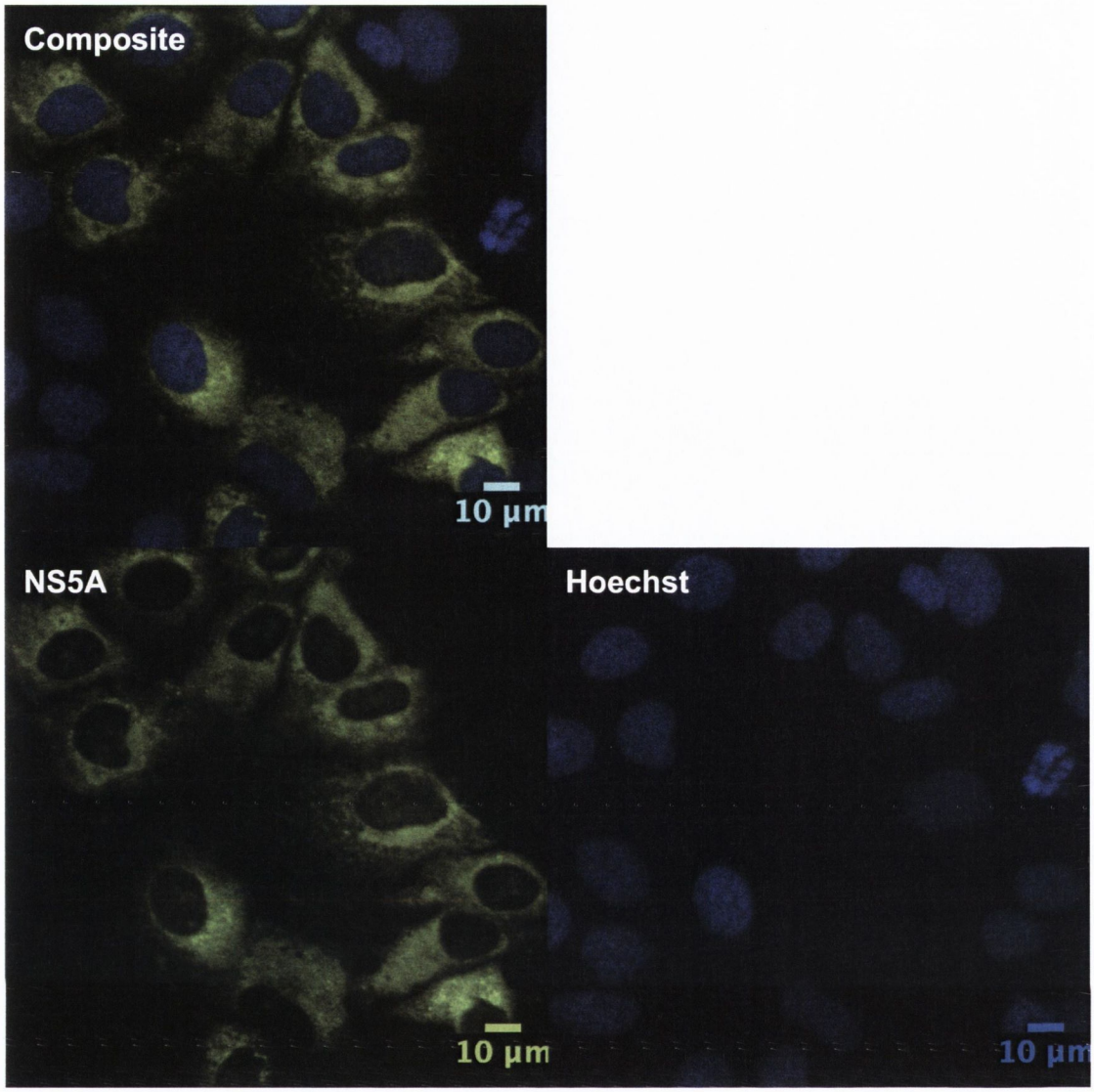


Figure 6.4 HCVcc NS5A is localised in the cytoplasm of Huh7.5 cells. Huh7.5 cells were grown to 40-50% confluence and then infected with J6/JFH for 72 hours. Inoculant was then removed and cells fixed in a 1:1 mix of acetone and methanol. Cells were then incubated with a primary antibody specific for HCV NS5A and the DNA stain Hoechst. . Cells were imaged by a laser microscope using a 20x objective lens and a digital zoom applied. A negative control where no PKC primary antibody was added was used to exclude the possibility of non specific secondary antibody staining. A mock infection where no viral particles were added to cells was also performed. Images are representative of three independent experiments.

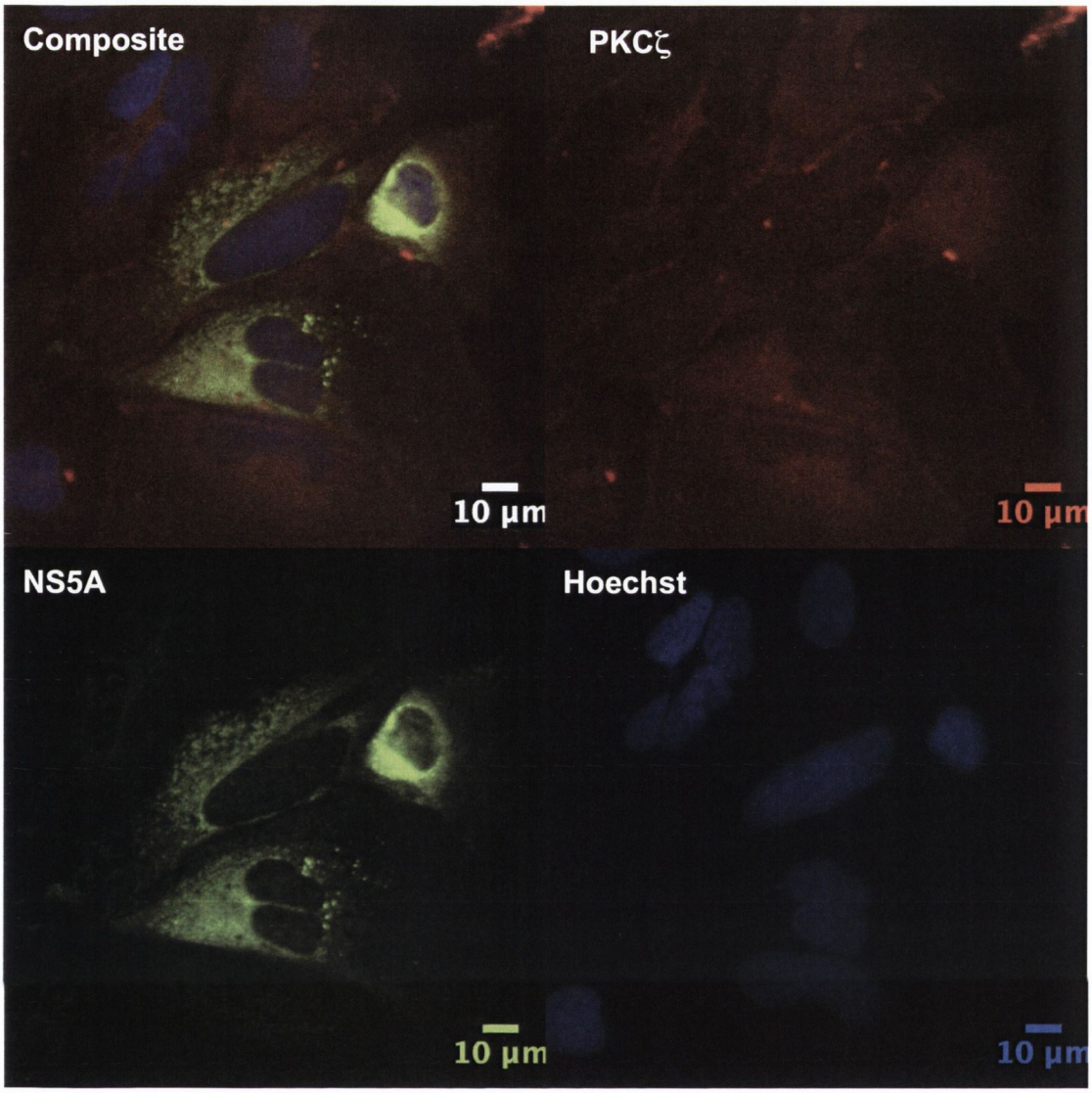


Figure 6.5 HCVcc does not translocate PKCζ. Huh7.5 cells were grown to 40-50% confluence and then infected with J6/JFH for 72 hours. Inoculant was then removed and cells fixed in a 1:1 mix of acetone and methanol. Cells were then incubated with a primary antibody specific for HCV NS5A and the DNA stain Hoechst. . Cells were imaged by a laser microscope using a 20x objective lens and a digital zoom applied. A negative control where no PKC primary antibody was added was used to exclude the possibility of non specific secondary antibody staining. A mock infection where no viral particles were added to cells was also performed. Images are representative of one experiment.

6.3.3 PKC ζ inhibition reduces HCVcc replication in Huh7.5 cells.

I examined if inhibition of PKCζ with a peptide inhibitor could prevent HCVcc replication within Huh7.5 cells. I first evaluated the metabolic activity of Huh7.5 cells that were treated with PKCζPS for one hour and then allowed 48 hours to recover without HCVcc infection in order to confirm that cells can survive following PKCζPS inhibition. **Figure 6.6** shows that metabolic activity in cells treated with 10μM of PKCζPS is significantly reduced to approximately one third at 48 hours after medium change compared to cells without PKCζ inhibition indicating a high level of cell death. Other concentrations of PKCζPS did not significantly alter metabolic activity compared to control cells although cells treated with 7.5μM of PKCζPS show a roughly 50% reduction in metabolic activity. However, cells treated with up to 5μM of PKCζPS appear to be viable. I then examined HCVcc replication in Huh7.5 cells following PKCζPS inhibition. I seeded cells overnight in a 96-well plate before treating cells with the PKCζPS for one hour at various concentrations. Medium was then removed and cells infected with HCVcc at a MOI of 0.5 for 48 hours. Cells were then fixed and stained for HCVcc NS5A and DNA. I then utilised the InCell Analyser to image cells in 7 fields of view per well. Each treatment was performed in duplicate in each experiment however, due to time constraints this experiment was only performed twice. In total up to 7000 cells were examined per treatment. The number of cells per treatment was counted by the InCell Analyser by adding the number of nuclei present in each field of view (**Figure 6.7**). I found a significant degrease in cell numbers following 10 or 5 μM of PKCζPS treatment while cell numbers following 1μM of PKCζPS were almost half those of non-inhibited cells. Cells with HCVcc replication were then counted manually to eliminate the possibility of false positives being detected by the InCell Analyser (**Figure 6.8**). I found that PKCζPS inhibition at any concentration significantly reduced the total numbers of cells with NS5A compared to cells that were not treated with the PKCζPS peptide. To see if this reduction was as due to PKCζ inhibition or as a result of a decrease in cell numbers I calculated the percentage of cells with NS5A in each treatment. I found that treatment with the PKCζPS inhibitor almost halved the percentage of cells with NS5A (**Figure 6.9**). When the percentage of cells with NS5A was normalised as a percentage of cells with NS5A in uninhibited cells I found that 1μM PKCζPS significantly reduced the rate of NS5A detected by 40% while 5μM of PKCζPS lead to a more variable reduction of about 50% (**Figure 6.10**). These results show that inhibition of PKCζ activity by using a pseudosubstrate

inhibitor at low concentrations is capable of decreasing NS5A detection in Huh7.5 hepatocytes.

In Conclusion I demonstrated that PKCζ inhibition was able to reduce HCVcc replication in Huh7.5 cells, revealing for the first time that PKCζ activity is required for HCV replication within hepatocytes.

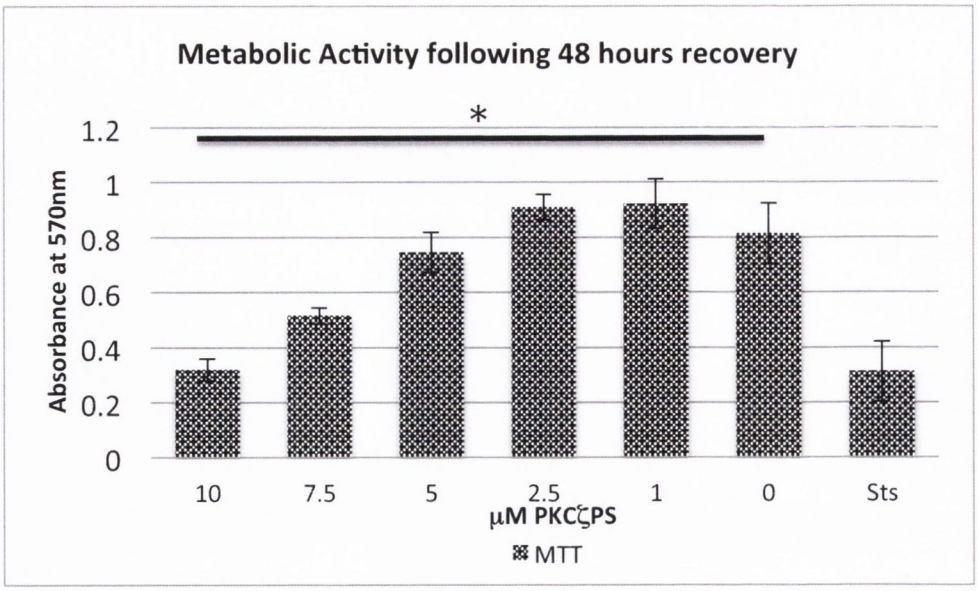


Figure 6.6 Metabolic activity of Huh7.5 cells 48 hours after 1 hour PKCζPS treatment. Huh7 cells were grown to 70-80% confluence in a 96-well plate and serum starved for 4 hours before treatment with PKCζ pseudosubstrate for 1 hour. Medium was then replaced with fresh DMEM and cells allowed to recover for 48 hours. MTT was then added to the cells at a final concentration of 1mg/ml and incubated for 2 hours. Media was then removed and DMSO added to each sample for 10 minutes. The absorbance was then read at 570nm in a absorbance plate reader. Results are representative of three independent experiments.

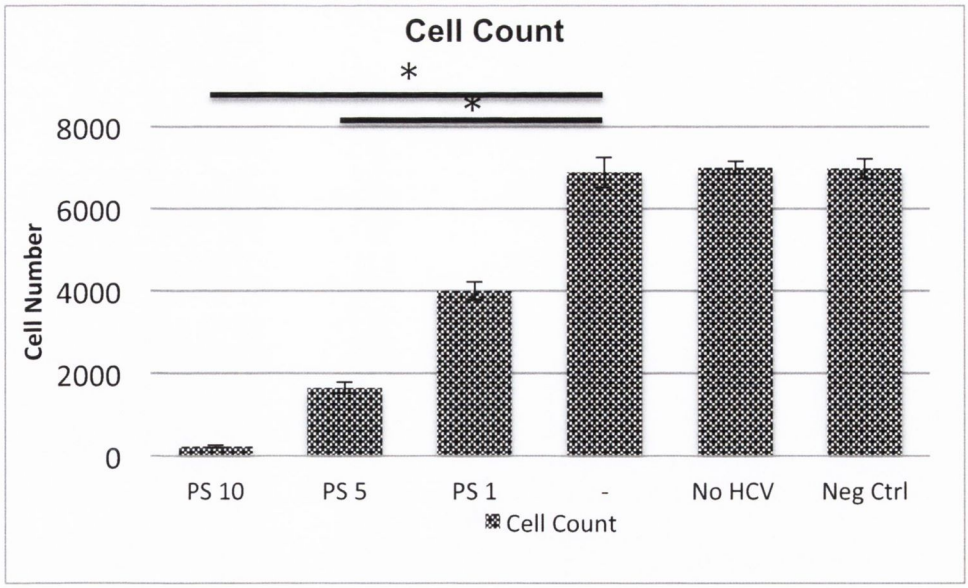


Figure 6.7 Cell number following PKCζ inhibition and HCVcc infection in Huh7.5 cells. Cells were grown to 40-50% confluence in a 96-well plate and serum starved for 4 hours before treatment with PKCζ pseudosubstrate for 1 hour. Cells were then inoculated with J6/JFH HCV viral particles at a MOI of 0.5 for 48 hours. Cells were then fixed in a 1:1 mix of acetone and methanol. Cells were then incubated with a primary antibody specific for HCV NS5A and the DNA stain Hoechst. A negative control where no NS5A primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining. Cells were imaged by an InCell Analyser 1000 and nuclei counted by InCell Analyser analysis software. A mock infection control without HCV particles was also included. Results are representative of the average of three independent experiments.

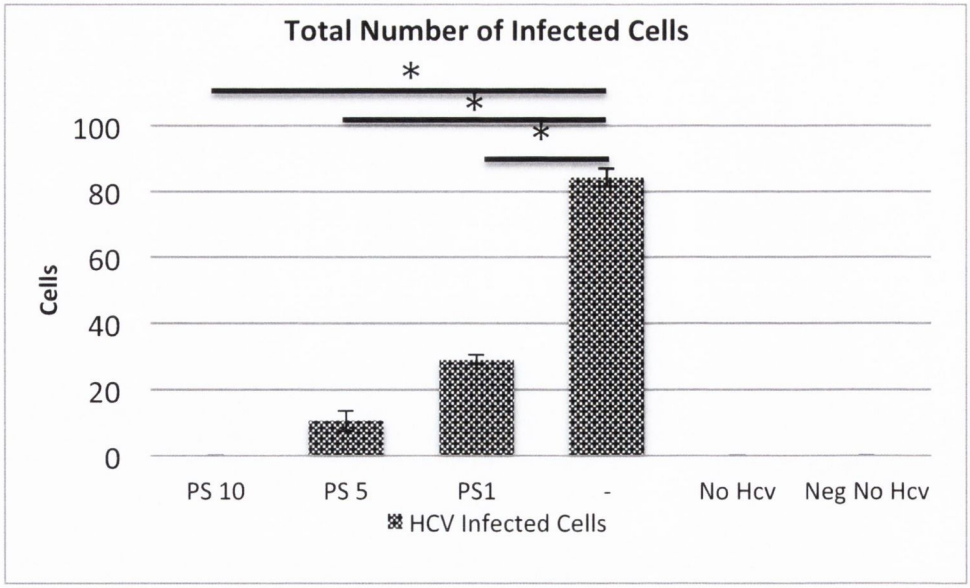


Figure 6.8 Cells infected by HCVcc following PKCζ inhibition in Huh7.5 cells. Cells were grown to 40-50% confluence in a 96-well plate and serum starved for 4 hours before treatment with PKCζ pseudosubstrate for 1 hour. Cells were then inoculated with J6/JFH HCV viral particles at a MOI of 0.5 for 48 hours. Cells were then fixed in a 1:1 mix of acetone and methanol. Cells were then incubated with a primary antibody specific for HCV NS5A and the DNA stain Hoechst. A negative control where no NS5A primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining. Cells were imaged by an InCell Analyser 1000 and nuclei counted by InCell Analyser analysis software. HCV replicatiing cells were counted manually. A mock infection control without HCV particles was also included. Results are representative of the average of three independent experiments.

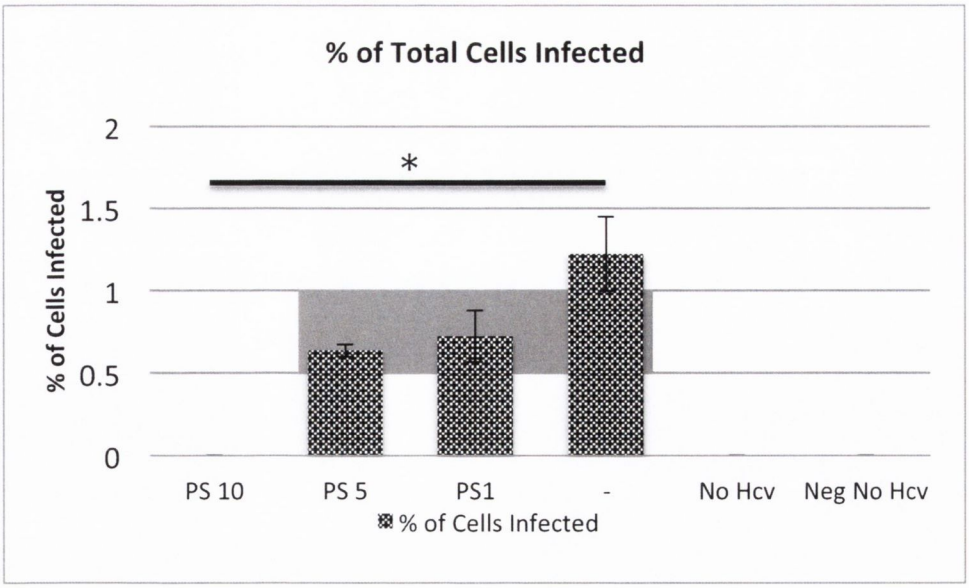


Figure 6.9 PKCζ inhibition reduces the percentage of cells infected by J6/JFH HCVcc. Cells were grown to 40-50% confluence in a 96-well plate and serum starved for 4 hours before treatment with PKCζ pseudosubstrate for 1 hour. Cells were then inoculated with J6/JFH HCV viral particles at a MOI of 0.5 for 48 hours. Cells were then fixed in a 1:1 mix of acetone and methanol. Cells were then incubated with a primary antibody specific for HCV NS5A and the DNA stain Hoechst. A negative control where no NS5A primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining. Cells were imaged by an InCell Analyser 1000 and nuclei counted by InCell Analyser analysis software. HCV replicating cells were counted manually and the percentage of cells infected is shown. A mock infection control without HCV particles was also included. Results are representative of the average of three independent experiments.

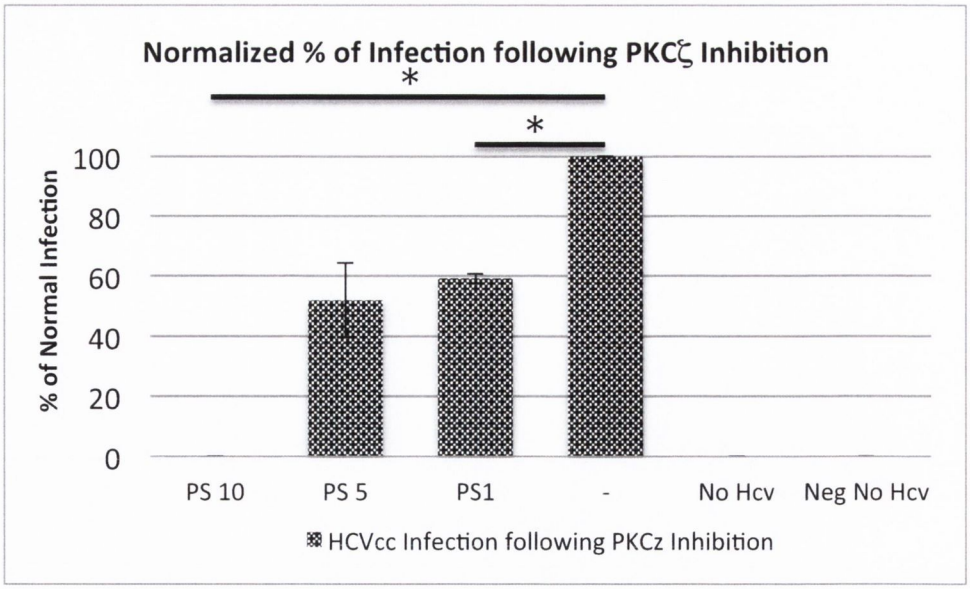


Figure 6.10 Rate of cells infected by HCVcc following PKCζ inhibition is reduced. Cells were grown to 40-50% confluence in a 96-well plate and serum starved for 4 hours before treatment with PKCζ pseudosubstrate for 1 hour. Cells were then inoculated with J6/JFH HCV viral particles at a MOI of 0.5 for 48 hours. Cells were then fixed in a 1:1 mix of acetone and methanol. Cells were then incubated with a primary antibody specific for HCV NS5A and the DNA stain Hoechst. A negative control where no NS5A primary antibody was added was used to exclude the possibility of non-specific secondary antibody staining. Cells were imaged by an InCell Analyser 1000 and nuclei counted by InCell Analyser analysis software. HCV infected cells were counted manually and relative HCV replication rates are shown. A mock infection control without HCV particles was also included. Results are representative of the average of three independent experiments.

6.4 DISCUSSION

HCV interacts with TJ proteins at the cell membrane in order to enter host cells. I have demonstrated in Chapter III that IFNα treatment translocates PKCζ away from the plasma membrane while, in Chapter V I have shown that PKCζ activity is required for the membrane localization of these TJ proteins. I therefore examined if PKCζ inhibition could prevent HCVcc replication in Huh7.5 cells. Examination of cell viability using 48 hour MTT assays showed that cells remained viable after PKCζPS treatment except at concentrations of 10μM where metabolic activity was significantly lower. I do not believe that 10μM concentrations of PKCζPS affected earlier experiments, as these cells were immediately fixed following one-hour PKCζPS treatment compared to MTT experiments where cells were maintained for 48 hours following PKCζPS treatment. Also the PKCζPS and IFNα phenotypes were similar in these previous experiments, while it was shown that longer term IFNα treatment did not induce apoptosis (**Figure 5.2**). Furthermore when IFNα induced phosphorylation of STAT proteins was examined following PKCζPS treatment it was shown that this cell signalling pathway remained functional (**Figure 3.11**). When I stained cells I found a large decrease in cell number following PKCζPS treatment. This might be explained by cells being washed away during the staining process, as they may be unable to adhere to the 96-well plate due to a withdrawal of adhesion proteins from the cell surface. Coating 96-well plates with poly-L-lysine to facilitate cell attachment may improve findings of future experiments.

Using an assay that measures HCVcc replication I found that PKCζ inhibition by PKCζPS at concentrations as low as 1μM could reduce HCVcc replication rates to roughly half the levels of uninhibited cells. In order to exclude the possibility that the rate of HCVcc replication appeared lower due to a decrease in cell numbers, due to cell death or cells being washed away, I calculated the percentage of cells present that showed HCVcc replication. I then compared these percentages between the various treatments. Even at 1μM and 5μM concentrations of PKCζPS, which showed similar metabolic activity compared to uninhibited cells, the percentage of cells showing HCVcc replication was markedly reduced compared to uninhibited cells. This demonstrates that PKCζ activity is not only required for maintenance of cell:cell contact but is also required for HCVcc infection.

Although my assays have not categorically determined if HCVcc infection is reduced by PKCζ inhibition at the cell entry to Huh7.5 stage or the viral replication stage it would appear more likely that it is at the cell entry stage. HCV entry is the first step of virus-host cell interactions that leads to infection and represents a possible target for antiviral therapy. Our previous results (Chapter V) show that when PKCζ is translocated from the membrane, claudin-1 and occludin are also removed from the membrane. It is possible that this then denies HCV access to the receptors it requires to enter hepatocytes. HCV entry is believed to occur through a coordinated pathway involving several viral and host cell factors, thus offering several novel targets for antiviral therapy. Claudin-1 has been identified as an essential HCV hepatocyte entry factor, probably during a post-binding step as no direct HCV:Claudin-1 interaction has been demonstrated (Evans MJ *et al.,* 2007, Krieger SE *et al.,* 2010). Claudin-1 associates with CD81 and the formation of Claudin-1:CD81 complexes is crucial for HCV infection. Mutations at residues 32 and 48 in the Claudin-1 extra-cellular loop 1 abolish its association with CD81 and the viral receptor activity (Harris HJ *et al.,* 2010). Occludin has been identified as another host cell factor critical for HCV entry, probably at a late post-binding event (Ploss A *et al.,* 2009; Liu S *et al.,* 2009; Benedicto I *et al.,* 2009). So far, there is no evidence of its direct interaction with HCV but occludin expression on hepatocytes as well as HCV entry is increased upon glucocorticoid treatment (Ciesek S *et al.,* 2010) while occludin expression is down-regulated upon HCV infection to prevent super-infection (Liu S *et al.,* 2009).

TJ proteins are not the only proteins that require PKCζ to direct their localisation to the membrane. In Huh7 cells PI3K has been shown to signal through PKCζ to induce translocation of sodium taurocholate co-transporting polypeptide (Ntcp) to the plasma membrane (McConkey M *et al.,* 2004). They demonstrated that cyclic AMP (cAMP) activated PI3K which then further activated PKCζ and Akt in Huh7 cells. They showed that activation of this pathway resulted in uptake of taurocholate (TC) and membrane localisation of Ntcp in hepatocytes. They also demonstrated that a dominant negative PKCζ inhibited cAMP-induced TC uptake and Ntcp translocation in HuH7-Ntcp cells showing a role of PKCζ in the internalisation of extracellular particles. The PI3K pathway is also rapidly activated by HCV and utilized to enhance infection, whereas PI3K inhibition reduces HCV infection (Liu Z *et al.,* 2012). It is therefore conceivable that PI3K activates

PKCζ that then promotes the membrane localisation of proteins such as claudin-1 and occludin and that HCV further activates this pathway to facilitate its infection of cells. Targeting of this pathway by IFNα could therefore be a strategy that blocks HCV replication by temporarily removing PKCζ-regulated entry factors from the cell surface and inhibiting HCV internalisation. However problems exist with targeting PKCζ. Firstly it is necessary to target hepatocytes specifically as these are the main reservoirs of HCV. Targeting is also important as if other epithelial cell types were to have their barrier formation compromised it could lead to systemic failures within the patient's body. It may be possible to conjugate a PKCζ inhibitor to a liver specific receptor or develop a precursor compound that is metabolised into an active form in the liver. It is important that any therapeutic must not have long lasting affects. This is because cells must be able to reform TJs in order for the liver to remain functional. Long-term reduction in TJs would affect hepatocyte polarity, which would then lead to the disruption of bile canaliculi and bile secretion. This is a benefit that IFNα has, as I have shown that TJs are able to reform following 24 hour IFNα treatment.

In conclusion I show that when PKCζ activity is inhibited HCV replication in Huh7.5 cells is reduced. This reveals that PKCζ is a vital host cell factor required by HCV to replicate in hepatocyte cells. This study also demonstrates that PKCζ is a novel target of IFNα in the context of its anti-viral effects.

Chapter VII

General Discussion

CHAPTER VII

7.1 GENERAL DISCUSSION

Hepatitis C (HCV) is a global epidemic with 130 million people chronically infected with the virus. HCV patients are at risk of developing severe and even fatal liver disease. However, detection rates of HCV are inadequate and the actual number of infections may be much higher than currently estimated. Hepatitis C is caused by hepatitis C virus (HCV), a positive sense, single-stranded RNA virus of the Flaviviridae family. HCV frequently establishes a chronic infection and displays a robust replicative fitness in combination with a high error prone polymerase that results in a tremendous genetic diversity. This diversity corresponds to a rapidly developed viral resistance (De Francesco R and Migliaccio G 2005). Recently improvements have been made to the standard of care therapy. Now a combination of pegylated interferon alpha (peg- IFNα), ribavirin and one of two drugs that interfere with the HCV NS3/4a protease have improved the management of HCV infection in countries with a developed medical infrastructure. The current treatment is not fully successful for all HCV genotypes or patient cohorts and must be administered in a frequent and burdensome dosing regimen over several months. It also causes considerable side effects, which require close medical supervision (McHutchison JG *et al.,* 2009; Jacobson IM *et al.,* 2011; Poordad F *et al.,* 2011; Kwo PY *et al.,* 2010). Interferons are not only important for medical administration but are also central to the host response to HCV. The endogenous IFN system in the chronically infected liver is constantly activated and hundreds of IFN stimulated genes (ISGs) are strongly expressed in hepatocytes via a response that can last decades. However, viral escape strategies allowing HCV to persist despite such strong activation of the hepatic IFN system are poorly understood. (MacQuillan GC *et al.,* 2003; Asselah T *et al.,* 2005; Asselah T *et al.,* 2008; Sarasin-Filipowicz M *et al.,* 2008; Chen L *et al.,* 2005).

Our group had previously found that T cell activation and interleukin 2 (IL-2) secretion is reduced in HCV infected patients compared to those with other inflammatory liver diseases. This was caused by HCV interacting with CD81 at the cell surface and targeting the translocation PKCβ into lipid rafts which prevented its function as part of the IL-2 the secretion pathway (Petrovic *et al.,* 2011). Classical PKC activity is required for IFNα to

induce tyrosine phosphorylation of STAT1 and STAT3 in Huh7 cells (Fimia *et al.,* 2004). The characterization of PKC isoforms in hepatocytes is incomplete and the roles of individual PKC isoforms in IFNα signalling in hepatocytes has not been fully explored. Therefore I identified the PKC isoforms present in Huh7 cells and detailed their subcellular localizations before and after IFNα stimulation. I further investigated if novel and atypical PCKs were also required for IFNα induced STAT phosphorylation and determined that PKCε expression is required IFNα induced STAT1 nuclear translocation. PKC δ is not active in the IFNα Jak/STAT pathway. PKCs have also been shown to regulate the expression of cell surface proteins that are used by HCV to enter hepatocytes (Suzuki and Ohno, 2006; Ploss and Dubuisson, 2012). I therefore examined the role of PKCζ in mediating HCV entry into hepatocytes and how IFNα could take advantage of this pathway to protect cells against infection.

In this study I have uncovered the PKC isoforms expressed in Huh7 cells and found some surprising results, most notably that inhibition of PKCζ activity reduces HCV replication of Huh7.5 cells. I first showed that Huh7 cells express the classical PKCs α, β_I and β_{II}, the novel PKCs δ, ε and a cleaved version of θ and the atypical PKCs ζ and ι. These PKC isoforms were localised in a variety of locations within the cell. This was to be expected as different PKC isotypes have different substrates, activity status and functions to each other within the cell. Importantly PKCζ was localised in the membrane in resting cells suggesting that it is constitutively activated, while PKC β_I and δ were both localized in the nucleus which is unusual for PKCs. When I then stimulated cells with IFNα I found that PKCζ was translocated from the membrane to the cytoplasm and that cell:cell contacts were diminished. I investigated if pretreatment with the PKCζ activator ceramide could prevent IFNα translocation of PKCζ. Interestingly ceramide has also been shown to induce association of the PKCζ inhibitor PAR-4 with PKCζ in embryonic mouse stem cells (Wang *et al.,* 2005). However, I found that ceramide had no effect on PKCζ localisation. This suggests that PKCζ localisation is not controlled by sphingolipids in Huh7 cells and that IFNα does not utilise this pathway to translocate PKCζ. I also found that IFNα was able to alter the localisation of PKCs α and δ but not β_I, β_{II} and ε. This shows that IFNα is specifically able to target certain PKC isotypes or their substrates, further demonstrating the functional variety of PKCs.This shows for the first time the full range of PKC isoforms expressed in Huh7 hepatocytes and forms a clear linkage between IFNα signalling and the

PKC family.

Interestingly for PKC isoforms, β_I and δ localization was found to be in the nucleus. The nuclear localisation of PKCβ_I that I observed may be due to PKCβ_I being constitutively activated. Nuclear expression of PKCβ_I is correlated with tumour cell proliferation (Metzger E *et al.,* 2010). This is relevant as the Huh7 cell line is derived from a hepatocarcinoma. Indeed prostate carcinoma tumour progression correlates with PKCβ_I expression. In androgen receptor dependent tumour cells PKCβ_I is localised in the nucleus and inhibits the regulation of gene expression by phosphorylating histone H3. Following phosphorylation H3 is unable to be demethylated by lysine-specific demethylase 1 leading to an increase in gene expression. Nuclear localisation PKCβ_I has also been reported in undifferentiated murine embryonic stem cells (Costa-Junior HM *et al.,* 2010). PMA stimulation again lead to an increase in nuclear localisation of PKCβ_I while differentiation of these cells lead to the export of PKCβ_I to the cytoplasm. In T-cells PKCβ_I can be translocated to the nucleus following PMA stimulation (Zhu P *et al.,* 2010). Once activated by PMA, PKCβ_I directly phosphorylates NF90. Phosphorylated NF90 is then exported from the nucleus to the cytoplasm where it then stabilises IL-2 mRNA and allows for its expression, demonstrating again the role of nuclear PKCβ_I in gene expression. These reports demonstrate that PKCβ_I activation can lead to a nuclear localisation where PKCβ_I can repress transcriptional regulation and lead to cancer progression, which may be the case for Huh7 cells.

The other PKC isoform that is found in the nucleus of Huh7 cells is PKCδ although cytoplasmic localization of this protein is also observed. This is interesting because PKCδ is normally associated with pro-apoptotic functions and nuclear translocation of PKCδ is regarded as amplifying the apoptotic process (reviewed by Zhao M *et al.,* 2012). However, TNFα has been shown to target PKCδ to the nucleus, where it was required for NFκB activity and cell survival (Lu *et al.,* 2009). It is possible therefore that PKCδ is cooperating with a factor such as NFκB in Huh7 cells to maintain cell survival, while being removed from locations where it would otherwise become activated and induce apoptosis. When cells were treated with IFNα it could be seen that cytoplasmic levels of PKCδ decreased but its nuclear localization remained. It is possible that this decrease in cytoplasmic

localisation is due to PKCδ binding with a STAT protein which then translocates into the nucleus following IFNα mediated signalling. Indeed it has been shown that PKCδ:STAT1 co-immunoprecipitate (Chen *et al.,* 2013).

I also examined the role of PKCs in IFNα signalling. Results in our group have recently shown that PKCβ is the classical PKC required by IFNα to induce STAT 1 and 3 tyrosine phosphorylation (Mullins S *et al.,* unpublished). The nuclear localization of β_I and the cytoplasmic localisation of β_{II} therefore suggest that the β_{II} isoform is responsible for inducing tyrosine phosphorylation of these STATs as unphosphorylated STAT proteins are localised in the cytoplasm before translocating to the nucleus following tyrosine phosphorylation. As PKCs are serine/threonine kinases so this phosphorylation must be mediated through another kinase. It is therefore probable that IFNα induces PKCβ_{II} to activate another kinase that then directly tyrosine phosphorylates STAT 1 and 3.

Interestingly, when I pretreated cells with the novel PKC pharmacological inhibitor Rottlerin before IFNα stimulation I found that tyrosine phosphorylation of STAT1 and STAT3 was markedly inhibited, while an inhibitor for the atypical PKCζ did not inhibit tyrosine phosphorylation. PKC δ has been implicated in cytokine-induced phosphorylation of STATs in other cell types (Uddin *et al.,* 2002, Deb *et al.,* 2003; Jain *et al.,* 1999, Gartsbein *et al.,* 2005). However, when I then followed up these findings by knocking down the expression of either PKC δ or ε by RNAi I found no such inhibition of IFNα stimulated tyrosine phosphorylation of STAT1 and STAT3 or of serine phosphorylation, which I previously showed to be constitutive in Huh7 cells. This therefore implies that Rottlerin inhibits another kinase or kinases that then phosphorylates STAT 1 and 3. While I was unable to confirm the kinase(s) inhibited in this case, previous papers have examined the specificity of Rottlerin and suggested that Rottlerin also inhibits p38-regulated/ activated protein kinase (PRAK) and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) (Davies *et al.,* 2000) as well as c-Jun N-terminal kinase 1α1 (JNKα1), mitogen- and stress-activated protein kinase 1 (MSK-1), cAMP-dependent protein kinase (PKA), 3-phosphoinositide-dependent protein kinase-1 (PDK-1), protein kinase B a (Akt), and glycogen synthase kinase 3b (GSK3b) (Soltoff SP, 2007). PKCδ has recently been suggested to be required for serine but not tyrosine phosphorylation of STAT1 in Huh7 cells (Chen *et al.,* 2013). Our results did not agree with this as I found serine 727 to be

phosphorylated at STAT1 in resting cells and that this was not increased following IFNα treatment. Also I did not find inhibition of STAT serine phosphorylation following PKCδ RNAi with or without further IFNα stimulation. Chen *et al.,* did not use RNAi and instead relied upon Rottlerin inhibition. However, they did show PKCδ:STAT1 interaction following co-immunoprecipitation which could be blocked by the hepatitis B virus (HBV) directly binding to PKCδ. This illustrates a difference between HCV and HBV, as there was no colocalisation between PKCδ and HCV NS5A when I examined infected Huh7.5 cells

My thesis has advanced the "current" model of IFNα signalling (**Figure 1.13**) to reveal that PKCε is required for IFNα induced STAT nuclear translocation (**Figure 7.1**). This identifies PKCε as a new component in IFNα signalling in Huh7 hepatocytes. RNAi of PKCε prevented the IFNα induced nuclear translocation of STAT1 but not of STAT3. I further examined the expression of 3 ISGs known to be strongly induced following IFNα treatment and widely used to examine IFNα signalling in published literature. I expected to see a reduction of ISG expression when PKCε was knocked down due to the reduction of nuclear localized STAT, however, I could not show a consistent pattern of ISG induction by IFNα following RNAi. That these genes were still able to be expressed despite the reduction of nuclear STAT1 suggests that there may be functional redundancy for ISG expression between the different STAT proteins. However, the selective requirement of STAT1 for PKCε to facilitate its nuclear translocation may also show a novel mechanism of regulation for ISG induction. Microarray studies have shown that hundreds of ISGs, with a range of functions, are expressed following IFN treatment (see Schoggins and Rice, 2011, for review). ISGs are induced to vastly different levels during viral infection or IFN treatment, and expression levels are often dependent on time, dose, and cell type. It is therefore still possible that PKCε is required for the expression of a subgroup of ISGs that I did not examine. Another possibility is that PKCε is required for STAT mediated transcription stimulated by other cytokines such as IFNγ or IL-6 but not IFNα. Besides STAT induced gene transcription, PKCε interaction with STAT1 may also be required for a non-signalling function as other STAT proteins have also been shown to interact with microtubule stabilization and the mitochondrial respiratory chain (see Mohr A. *et al.,* 2012 for review).

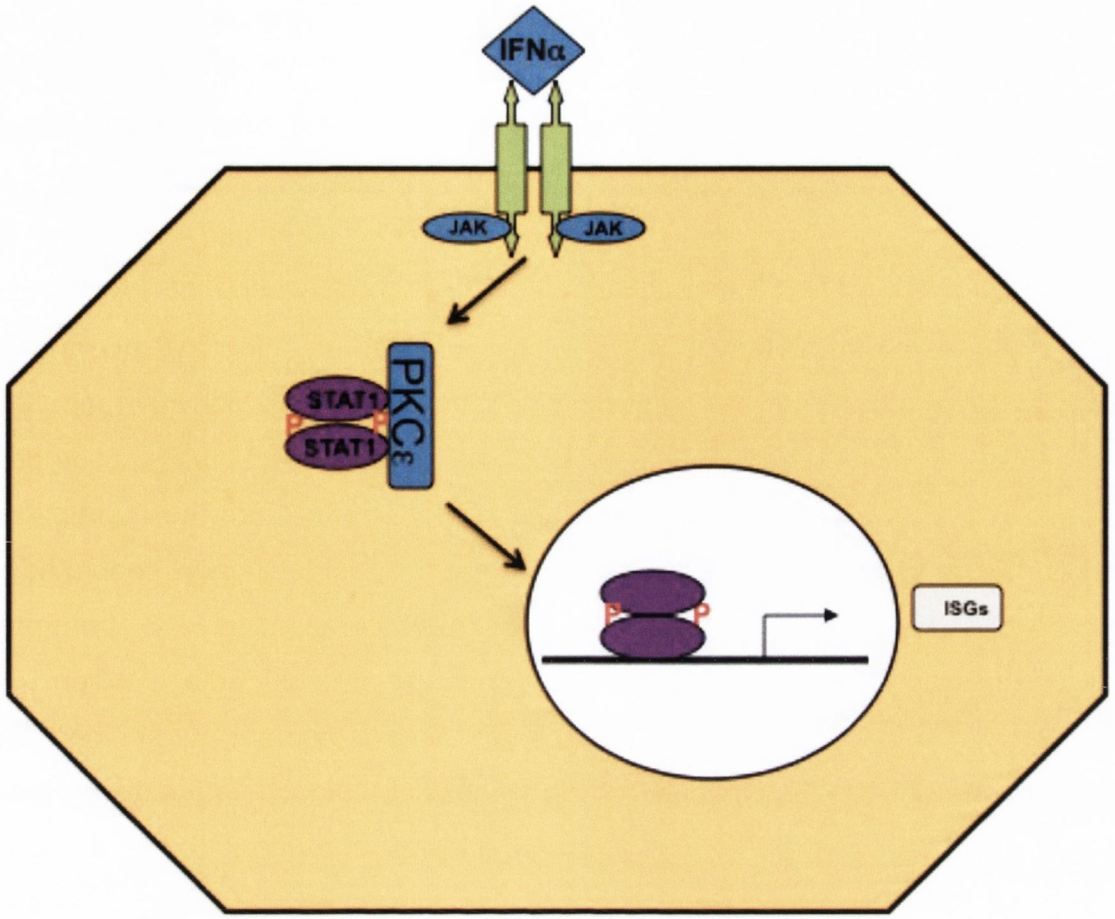


Figure 7.1. Advanced model of IFNα signalling through the Jak/STAT pathway and PKCε. My thesis has advanced the "current" Jak/STAT pathway in Huh7 cells by showing that PKCε is required by IFNα to translocate STAT1 into the nucleus from the cytoplasm but that this does not affect the transcription of ISGs.

To summarize our studies into the role of the PKC family in IFNα signalling through STAT 1 and 3, I propose PKCβ_{II} to be required for indirect tyrosine phosphorylation of STAT1 and STAT3 but rule out the requirement for PKCs δ, ε and ζ in this process. However, PKCε but not δ is required to translocate STAT1 but not STAT3 from the cytoplasm to the nucleus following IFNα stimulation. This may represent a novel mechanism of regulation within the STAT family. However, the inhibition of STAT1 translocation did not result in inhibition of expression of the ISGs, PKR, 2'5'-OAS or MxA, indicating that PKCε translocation of STAT1 may instead be required for expression of other genes or for transcription of genes induced by a different cytokine.

The second half of this thesis investigated the role PKCζ plays in maintaining cell:cell

contacts in Huh7 cells and how PKCζ is required for HCV replication in Huh7.5 cells. I demonstrated that inhibition of PKCζ by a pseudosubstrate inhibitor was able to withdraw PKCζ from the membrane and disrupt cell:cell contacts in Huh7 cells. For the first time I have shown that PKCζ activity is required for HCVcc replication in Huh7.5 cells. Targeting PKCζ function is a novel antiviral mechanism utilised by IFNα and this kinase may be considered for a future HCV therapeutic (**Figure 7.2**). Inhibition of PKCζ resulted a phenotype similar to that observed following treatment of the cells for 2 hours with IFNα. As PKCζ is known to be a key regulator of cell polarity (Gonzalez-Mariscal L *et al.,* 2003), I examined the effect of IFNα and PKCζ inhibition on adherens and tight junction proteins. I found that IFNα was able to translocate these proteins away from the membrane which resulted in a loss of cell:cell contact. I propose that this is specifically modulated through translocation of PKCζ away from the membrane, thereby preventing PKCζ from promoting the membrane localisation of other proteins involved in cell:cell contact. When I inhibited PKCζ activity I found identical results to those for IFNα treatment, further demonstrating the requirement of PKCζ in maintaining Huh7 cell:cell contact. Interestingly, prolonged treatment with IFNα showed that PKCζ was able to return to the cell membrane and cell contacts reformed. This was further demonstrated by paracellular assays. These results are interesting because for the first time it implicates IFNα as a regulator of cell:cll contact and polarity. aPKCs are accepted as key regulators of polarity and cell contact through their association with the evolutionarily conserved aPKC:PAR-6:PAR-3 complex. This complex is dynamically regulated by proteins such as PP2A and Par-4. The relationship between IFNα, PP2A and Par-4 and the aPKC:PAR-6:PAR-3 complex was beyond the scope of this study but could provide a novel pathway for cell polarity regulation.

Finally, I investigated the requirement of PKCζ activity in HCV infection of Huh7.5 cells. HCV enters hepatocytes through a stepwise process that requires membrane localised claudin-1 and occludin. Having already demonstrated that IFNα can translocate PKCζ away from the cell membrane and that inhibiting PKCζ activity removes claudin-1 and occludin from the membrane, I finally set out to determine if PKCζ inhibition can prevent HCV infection. By using the HCVcc infectious system I demonstrated that PKCζ pseudosubstrate pretreatment at concentrations as low as 1μM could reduce HCV infection rates to nearly half the level of that seen in cells not treated with PKCζPS. For the first time this identifies PKCζ as a host factor that is required by HCV to enter and infect

hepatocytes. I have shown that if PKCζ localisation can be targeted, the viral entry proteins that HCV require are removed and HCV can no longer infect cells. This also uncovers a novel antiviral mechanism deployed by IFNα. The discovery of this mechanism now opens the possibility of novel targets to prevent HCV infection. The role of the aPKC:PAR complex has not been investigated yet in the context of HCV infection but must now be considered a key regulator of HCV infection. The role of endogenous PKCζ inhibitors in preventing HCV entry should also be examined. An important benefit of targeting host factors required for cell entry is that any new therapies developed should be effective across all HCV genotypes, thereby avoiding the problems associated with IFNα and direct acting antiviral therapies where certain genotypes that do not respond well to treatment.

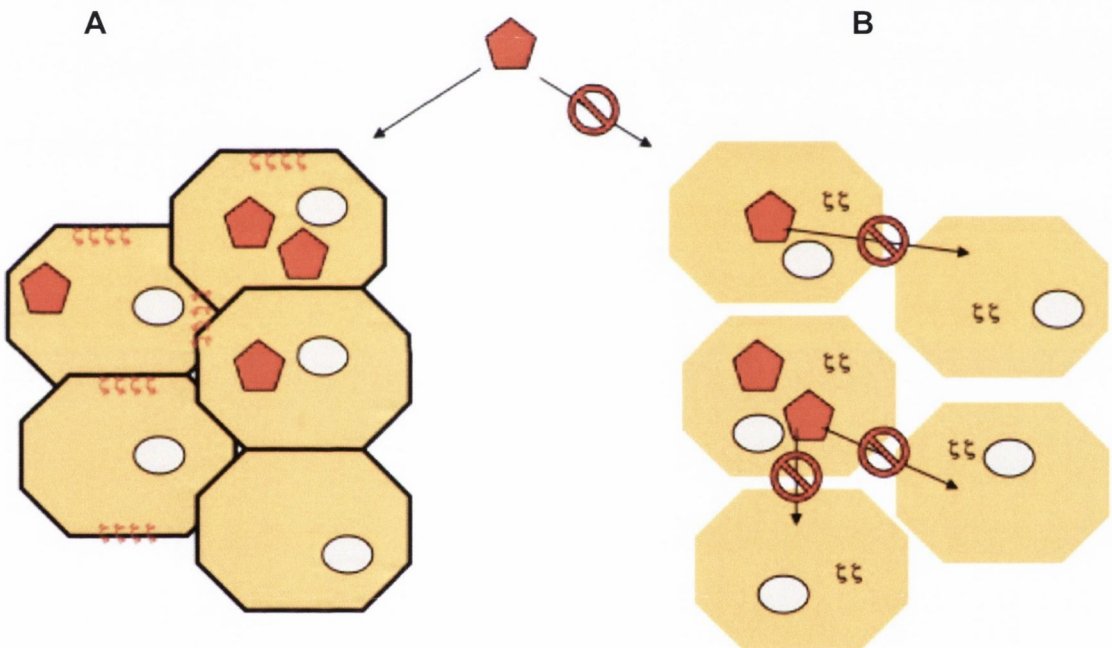


Figure 7.2. Advanced model of cell:cell contact in Huh7 cells and HCVcc replication in Huh7.5 cells. My thesis has advanced the "current" understanding of cell:cell contact of Huh7 cells and HCVcc replication in Huh7.5 cells by showing that PKCζ activity is required for both activities. This is the first time that PKCζ activity has been shown to be required for HCVcc replication. "A" shows that when PKCζ is expressed at the cell membrane cell:cell contact is maintained and HCV particles are able to enter, replicate and spread within hepatocytes. "B" shows that when PKCζ is removed from the membrane that cell:cell contacts degrade and HCV can not enter hepatocytes.

Overall I have helped resolve the IFNα signalling pathway in Huh7 cells by characterising the PKC isoforms present and outlining their requirement in the processes of STAT phosphorylation and nuclear translocation. I have also discovered a novel antiviral mechanism employed by IFNα and identified PKCζ as a key protein required for HCV infection.

7.2 FUTURE WORK

The work described in this thesis forms the basis for future research that will increase our understanding of how IFNα mediates inflammatory signals in the liver and how new agents that will protect us against HCV infection can be developed.

The finding that IFNα can alter PKCζ localisation and that PKCζ is required for HCV infection must be further investigated. I have not identified the pathway that IFNα uses to interact with PKCζ. This must be uncovered as it is possible that permanent inhibition of PKCζ could be fatal for cells. When I utilised MTT assays to measure metabolic activity following PKCζ inhibition I found that there was no significant decrease in metabolic activity when using lower levels of a PKCζ inhibitor suggesting cells remained viable. However, when I stained cells for HCV protein expression I found a large decrease in cell numbers following PKCζ inhibition. Therefore the mechanisms IFNα utilises to transiently alter the membrane localisation of PKCζ in order to allow enough cell membrane expression of PKCζ and AJ/TJ proteins for cell adhesion must further defined. These mechanisms may be direct, with PKCζ possibly binding the IFNAR or Jak proteins or indirect by IFNα signalling through scaffold proteins that interact with PKCζ or PAR proteins. The aPKC:PAR complex is dynamically regulated and it is possible that IFNα promotes the activity of a regulator of this complex such as PP2A or PAR-4. Uncovering the precise mechanisms involved by IFNα to regulate PKCζ should yield promising targets to develop into clinical therapies. Further confocal immunofluorescent staining and coimmunoprecipitation/proteomic studies should prove valuable in determining the proteins involved. The role of the other atypical PKC isoform ι must also be investigated to determine if there is any functional redundancy between the aPKCs for regulating cell:cell contact. HCV cell:cell spread is a distinct mechanism utilised by the virus to infect cells and evade the immune response. The role that PKCζ plays in this method of infection should also be examined as TJ proteins are known to be involved in this process.

Furthermore these findings should be validated in other cell lines. While the Huh7.5 cell line is well suited for studying HCV entry and lifecycle, it is not the optimal choice for studying cell polarity as these cells do not form complex polarity or bile canalicular structures typical of fully polarized hepatocytes. Using primary hepatocytes is also

problematic as they tend to rapidly de-differentiate. One possible option would be to use matrigel to allow Huh7.5 cells grow in a 3D environment and develop bile canalicular structures (Molina-Jimenez F *et al.,* 2012), while another option would be to use HepG2-CD81 cells developed by the McKeating group (Mee CJ *et al.,* 2009) that are able to polarize and support HCV infection, allbeit at a lower level than Huh7.5 cells. Ultimately the recently developed humanized mouse model for HCV infection could be employed if future findings are promising (Dorner M. *et al.,* 2011).

The role of PKC isoforms in STAT signalling should also be further investigated. I have identified PKCε as being required for nuclear translocation of STAT1, however, I was unable to determine the functional consequences of this and did not fully explore the biochemical relationship between these proteins due to time constraints. A starting point for future work for this would be to investigate if these proteins physically interact, either directly or via an adaptor protein such as an importin. The role PKCε plays in associating STAT1 with its importin protein should be investigated based on our findings that PKCε is required for nuclear translocation of STAT1. ISG expression following PKCε knockdown could also be looked at again. STAT induced gene expression is complex and the canonical pathway of STAT dimerisation and binding either ISRE or GAS elements alone is too simplistic and not reflected in reality. Thus the three ISGs examined in this thesis may not be affected by the loss of PKCε while other ISGs may be.

Finally, having characterised the PKCs present in Huh7 hepatocytes, the role these kinases play in activating STAT proteins other than STAT 1 and 3 should be completed. Of particular interest would be STAT2, which is also activated by IFNα. The role of PKCs in STAT activation induced by other cytokines should be investigated. IFNγ also signals through the STAT pathway and has been shown to exert antiviral properties against HCV. Understanding how these pathways are activated will help us to better understand the host defence and develop better therapeutic strategies and may also provide biomarkers to indicate clinical outcome following HCV infection.

HCV is a global burden on human wellbeing and healthcare providers, which also exerts a costly economic liability. It is vital therefore to elucidate different aspects of HCV infection and its evasion of host defenses and medical therapy. Current anti-HCV therapy has

limited success, is long lasting and toxic and must urgently be improved to better treat this disease. The work in this thesis should contribute to a better understanding of the molecular mechanisms underlying the host response to infection, the mechanism of action of the medication used to treat it and how the virus itself utilizes host factors to enter hepatocytes. These will contribute to the development of more effective therapeutic and preventative approaches in the future. Any new therapeutic will require that compounds used will be specifically targeted to hepatocytes. Peg-IFNα is an unspecific, broad acting compound that causes many unwanted side effects. Using a hepatocyte specific PKCζ inhibitor could allow for a Peg-IFNα free regimen, which is highly desired amongst patients and medical professionals. A hepatocyte specific PKCζ inhibitor is also required as inhibiting PKCζ in other cells could cause major unwanted side effects such as disruption of epithelial tissue integrity. Another important feature of a new therapeutic is that it's PKCζ inhibition is transient and allows for PKCζ activity and localisation to return to normal and for cells to recover. This is important because when I inhibited PKCζ activity during HCVcc infection studies I observed that cells detached and were washed away during the staining process. This indicates that cells without long term PKCζ activity are no longer able to retain their adherence. If this situation were to occur in a patients liver this would most likely lead to fatal liver failure. This is obviously not the case with Peg-IFNα as cells are able to recover, as seen by my 24-hour IFNα cell barrier function assays.

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Appendix I

Activators/Inhibitors

Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich)

Roferon-A IFNα 2a (Hoffmann LaRoche)

Myristolated PKCζ pseudosubstrate (Calbiochem)

Okadaic acid (Calbiochem)

Staurosporine (Calbiochem)

Rottlerin (Calbiochem)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)

C16 Ceramide (Santa Cruz)

C16 Dihydroceramide (Santa Cruz)

Lysis Buffers

NP40 buffer

20mM Tris-Hydrochloric acid (HCl) pH 7.4

Trizma (Sigma-Aldrich)

150mM Sodium chloride (NaCl) (Sigma-Aldrich)

Glycerol

5mM NaF (Sigma-Aldrich)

5mM Sodium Pyrophosphate (NaPP) (Sigma-Aldrich)

5mM EDTA (Sigma-Aldrich)

5mM EGTA (Sigma-Aldrich)

1% IGEPAL/NP40 (Sigma-Aldrich)

20mM Leupeptin (Sigma-Aldrich)

1mM Sodium Orthovanidate (Na_3VO_4) (Sigma-Aldrich)

1mM PMSF (Sigma-Aldrich)

Sodium Dodecyl Sulphate Buffer

40mM Tris-HCL pH 7.4

120 NaCl (Sigma-Aldrich)

0.5% Triton X-100

0.3% Sodium Dodecyl Sulphate

RIPA Buffer

0.1% w/v SDS

1% w/vSoduim Deoxycholate

20mM Tris-Hydrochloric acid (HCl) pH 7.4

Trizma (Sigma-Aldrich)

150mM Sodium chloride (NaCl) (Sigma-Aldrich)

Glycerol

5mM NaF (Sigma-Aldrich)

5mM Sodium Pyrophosphate (NaPP) (Sigma-Aldrich)

5mM EDTA (Sigma-Aldrich)

5mM EGTA (Sigma-Aldrich)

1% IGEPAL/NP40 (Sigma-Aldrich)

20mM Leupeptin (Sigma-Aldrich)

1mM Sodium Orthovanidate (Na_3VO_4) (Sigma-Aldrich)

1mM PMSF (Sigma-Aldrich)

SDS PAGE

30% Acrylamide (Sigma-Aldrich)

1.5 M Tris-HCl (pH 8.8) (Sigma-Aldrich)

Sodium Dodecyl Sulphate (SDS) (Sigma-Aldrich)

Ammonium persulphate (APS) (Sigma-Aldrich)

TEMED (Sigma-Aldrich)

1 M Tris-HCl (pH 6.8) (Sigma-Aldrich)

Isobutanol (Sigma-Aldrich)

Glycine (Sigma-Aldrich)

Molecular Weight Markers (Sigma-Aldrich)

Bromophenol blue (Sigma-Aldrich)

DL-Dithiothreitol (DTT)

polyvinylidene difluoride (Pierce)

Chromatography paper (Whatman)

Coomassie stain and destain solutions

Coomassie brilliant blue G-250 (Bio-Rad)

Milk powder (Marvel)

HRP Development Solution

100nM Tris-HCL (pH 8.5)

H_2O_2 (BDH labs)

120ul of 250 mM luminol (Sigma-Aldrich)

53.2 ul of 90 mM p-Courmaric acid (Sigma-Aldrich)

Light Sensitive Film (Kodak, Rochester, NY).

Stock solutions required

NP40 (Sigma catalogue 56741)

Store at room temperature

500 mM Tris-HCl (pH 7.4) (Sigma catalogue T-1503)

Dissolve 6.055g Trizma in 80 ml dH20

Adjust pH to 7.4 with HCl and add dH20 to 100 ml

Store at room temperature

1 M NaCl (Sigma catalogue S-7653)

Dissolve 5.844g NaCl in 100 ml dH20

Store at room temperature

100 mM NaF (Sigma catalogue S-7920)

Dissolve 0.4199g NaF in 100 mls dH20

Store at room temperature

100 mM Sodium Pyrophosphate decahydrate (Sigma catalogue 223168)

Dissolve 4.46g NaPP in 100 mls dH20

Store at room temperature

100 mM EDTA (Sigma catalogue 100933T)

Dissolve 3.72g EDTA in 80 ml dH20

Adjust pH to 8.0 with NaOH and add dH20 to 100 ml

Store at room temperature

100 mM EGTA (Sigma catalogue E0396)

Dissolve 3.8g EGTA in 80 ml dH20

Adjust pH to 8.0 with NaOH and add dH20 to 100 ml

Store at room temperature

100 mM PMSF (Sigma catalogue P7626)

Dissolve 0.174g PMSF in 10 ml ethanol

Aliquot into 0.5 ml tubes and store at -20 degrees.

1 mg/ml leupeptin (Sigma catalogue L8511)

Dissolve leupeptin to 1 mg/ml in dH20

Aliquot into 0.5 ml tubes and store at -20 degrees.

100 mM Na3VO4 (sodium orthovanadate) (Sigma catalogue S-6508)

Dissolve 1.839g of sodium orthovanadate in 100 ml dH20.

Adjust the pH to 10.0 using NaOH or HCl.

Boil the solution until it turns colourless (approximately 10 minutes). Cool to room temperature.

Aliquot and store at -20 degrees.

Pierce® BCA Protein Assay

Reagent A – sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1 M sodium hydroxide Reagent B – 4% cupric sulphate Albumin standards – bovine serum albumin at 2.0 mg/ml in 0.9% saline, 0.05% sodium Azide

10% Acrylamide Resolving gel

Component Volume

dH20 7.9 ml

30 % Acrylamide 6.7 ml

1.5 M Tris-HCl (pH 8.8) 5.0 ml

10 % w/v SDS 200 µl

10 % w/v APS 200 µl

TEMED 8 µl

Stacking gel

Component Volume

dH20 3.4 ml

30 % Acrylamide 0.83 ml

1.0 M Tris-HCl (pH 6.8) 0.63 ml

10 % w/v SDS 50 µl

10 % w/v APS 50 µl

TEMED 5 µl

30 % acrylamide (Sigma catalogue A3699)

Store at 4°C

10 % w/v ammonium persulphate (10 % APS) (Sigma catalogue A3678)

Dissolve 0.1g APS in 1 ml dH20

Store at 4 °C

1.5 M Tris-HCl (pH 8.8) (Sigma catalogue T-1503)

Dissolve 90.825g Trizma in 400 ml dH20

Adjust pH to 8.8 with HCl and add dH20 to 500 ml

Store at room temperature

1.0 M Tris-HCl (pH 6.8) (Sigma catalogue T-1503)

Dissolve 60.55g Trizma in 400 ml dH20

Adjust pH to 6.8 with HCl and add dH20 to 500 ml

Store at room temperature

10 % w/v SDS (Sodium Dodecyl Sulphate) (Sigma catalogue L-5750)

Dissolve 10g of SDS in 100 ml dH20

Coomassie stain solution

2.5 g Coomassie brilliant blue G-250 (Bio-Rad; catalogue number 161-0406)

450 ml dH20

450 ml Methanol

100 ml glacial acetic acid

Store at room temperature

Coomassie destaining solution

450 ml dH20

450 ml Methanol

100 ml glacial acetic acid

Store at room temperature

Western blot blocking buffer

5 % w/v milk powder (Marvel) in Western blot wash buffer (TBS-T)

Western blot wash buffer

1X TBS with 0.1% v/v Tween-20 (TBS-T)

10X TBS:

24.2g Trizma base (Sigma catalogue T-1503)

80g NaCl (Sigma catalogue S-7653)

900 ml dH20

pH solution to 7.6 with HCl

Add dH20 to 1L.

Store at room temperature and dilute to 1X with dH20 before use

HRP development solution

24 mls of 100 mM Tris-HCl (pH 8.5)

14.4 µl of 30 % H2O2 (BDH labs catalogue number 285194F)

120 µl of 250 mM luminol (Sigma catalogue 09253, dissolved in DMSO)

532 µl of 4-iodophenylboronic acid (4-IPBA)

8% PFA preparation

Weigh out 8g PFA. Add ~ 80ml PBS and add a few drops of concentrated NaOH.

Add a magnetic stirrer and heat/mix solution to 60°C in the fume hood. Keep the lid of the container loose. Once dissolved, allow the PFA to cool to RT.

pH the solution to 7.0 bring solution up to 100ml with PBS.

Aliquot and store at -20°C.

10 X PBS (Phosphate Buffered Saline)

80 g NaCl

2.0 g KCl

2.4 g KH2PO4

14.4 g Na2HPO4

900 ml of distilled water

Dissolve completely and pH the solution to 7.4 by adding conc. HCl

1.5% agarose gel

A 1.5% agarose gel containing ethidium bromide was prepared by dissolving 0.5g agarose powder in 1x TAE buffer. The solution was heated for 1 minute in a microwave to dissolve the powder and 2.5μl of ethidium bromide was added in order to visualise RNA following electrophoresis. The mixture was poured into a mould containing a comb to form wells. The gel was allowed solidify for 30 minutes at room temperature.

ANTIBODIES

Antibody	Source	Catalogue #	Manufacturer	Clone
PKC α	α-mouse	sc-8393	Santa Cruz	H-7
PKC β_I	α-mouse	sc-8049	Santa Cruz	E-3
PKC β_{II}	α-rabbit	sc-210	Santa Cruz	C-18
PKC δ	α-rabbit	sc-937	Santa Cruz	C-20
PKC ε	α-rabbit	sc-214	Santa Cruz	C-15
PKC θ	α-rabbit	sc-212	Santa Cruz	C-18
PKC η	α-rabbit	sc-215	Santa Cruz	C-15
PKC ζ	α-rabbit	sc-216	Santa Cruz	C-20
PKC ι	α-mouse	sc-17837	Santa Cruz	H-12
Actin	α-rabbit	A2066	Sigma	
STAT1	α-mouse	sc-464	Santa Cruz	C-136
STAT3	α-mouse	sc-81385	Santa Cruz	STAAD22A
pSTAT1Y701	α-rabbit	#9167S	Cell Signalling	58D6
pSTAT3Y705	α-rabbit	#9145S	Cell Signalling	D3A7
pSTAT3S727	α-rabbit	#9134S	Cell Signalling	
pSTAT1S727	α-rabbit	07-714	UpState	
Claudin-1	α-mouse	37-4900	Invitrogen	2H10D10
Occludin	α- rabbit	71-1500	Invitrogen	
ZO-1	α- rabbit	61-7300	Invitrogen	
E-Cadherin	α-mouse	610181	TransductionBD	36/E-Cadherin
E-Cadherin	α- rabbit	sc-7870	Santa Cruz	H-108

Table A2.1: Primary antibodies used for Western Blot and immunfluorescence experiments.

Antibody	Conjugated	Catalogue #	Manufacturer
2° HRP	α-rabbit	#7074	Cell Signalling
2° HRP	α-mouse	#7076	Cell Signalling
2° Alexa Fluor 488	α-rabbit	A11008	Invitrogen
2° Alexa Fluor 488	α-mouse	A11001	Invitrogen
2° Alexa Fluor 568	α-rabbit	A11036	Invitrogen
2° Alexa Fluor 568	α-mouse	A11031	Invitrogen

Table A2.2: Secondary antibodies used for Western Blot and immunfluorescence experiments.

Company details

Ambion® Ambion® Applied Biosystems, Austen, TX, USA.

BDH BDH, VWR International Ltd., England.

BD Pharmingen BD Biosciences, CA, USA.

Bio-Rad Bio-Rad Laboratories GmbH, Mu□nchen, Germany.

Calbiochem EMD Chemicals, NJ, USA.

Cell Signaling Cell Signaling Technology, Danvers, MA, USA.

Chivers Chivers Ireland Ltd., Dublin 5, Ireland.

Dako Dako A/S, Denmark.

Dharmacon® Thermo Fisher Scientific, Lafayette, CO, USA.

Gibco Gibco BRL, Grand Island, NY, USA.

Macherey-Nagel Macherey-Nagel, GmbH & Co. KG, Du□ran, Germany.

Merck Merck KGaA, Darmstadt, Germany.

Molecular Probes Invitrogen Corporation, California, USA.

Pall Pall Gelman Laboratories, Ann Arbor, MI, USA.

Peprotech Peprotech, Inc., Rocky Hill, NJ, USA.

R&D Systems R&D Systems, MN, USA.

Roche Hoffmann-La Roche, Basel, Switzerland.

Santa Cruz Biotechnology Santa Cruz Biotechnology Inc., CA, USA.

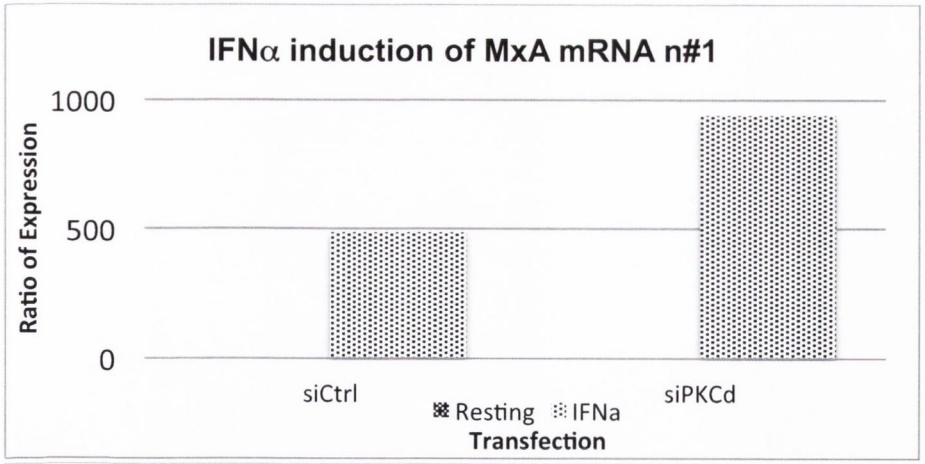
Sigma Sigma Chemicals Company, St Louis, MO, USA.

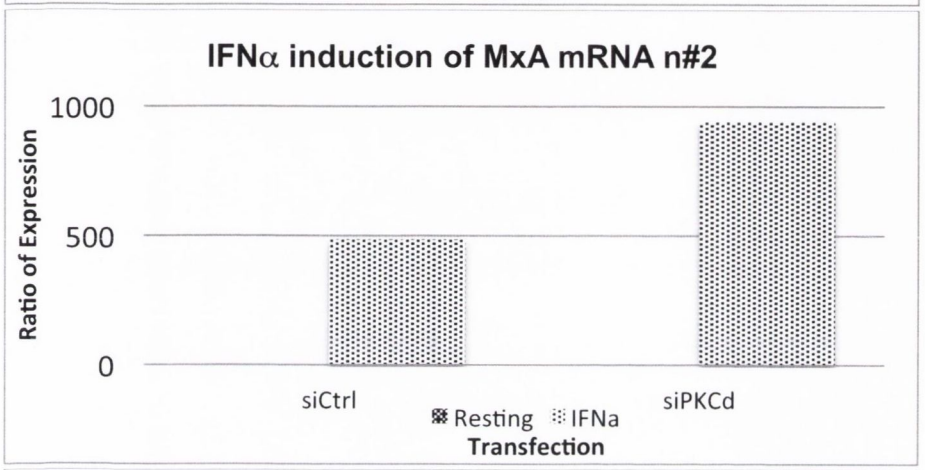
Appendix II

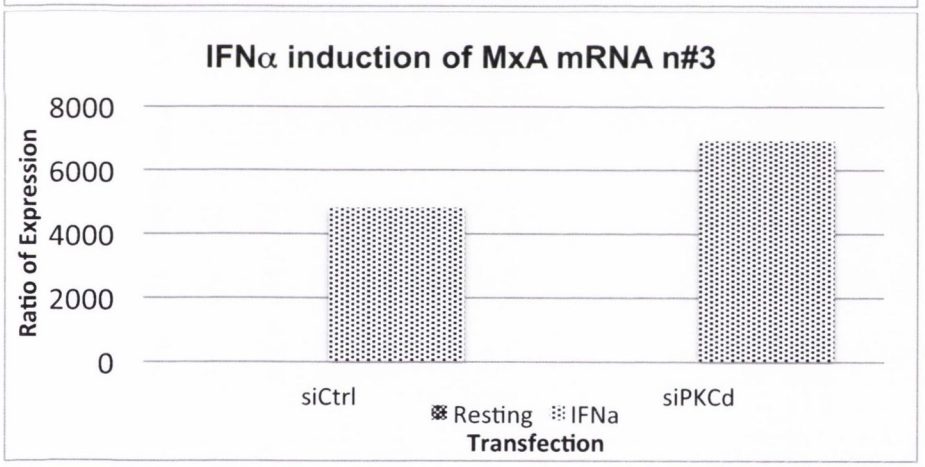
Results of individual qRT-PCR experiments examining PKR, 2'5' OAS and MxA expression in Huh7 cells with RNAi for either PKC δ or ε following six hour IFNα stimulation.

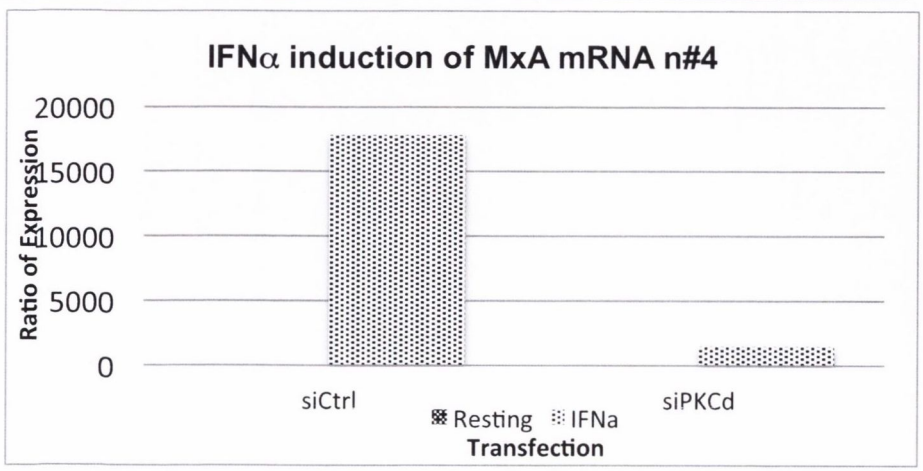
Appendix 4.1 IFNα induction of PKR following PKCδ RNAi. PKR expression was measured by RT-PCR following PKCδ knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene GAPDH. Results of individual experiments (n#) are shown here. Series1 are resting cells, Series2 are IFNα stimulated cells.

Appendix 4.2 IFNα induction of 2'5'-OAS following PKCδ RNAi. 2'5'-OAS expression was measured by RT-PCR following PKCδ knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene GAPDH. Results of individual experiments (n#) are shown here. Transfection 1 is scrambled control siRNA and transfection 2 is PKCδ siRNA.









Appendix 4.3 IFNα induction of MxA following PKCδ RNAi. MxA expression was measured by RT-PCR following PKCδ knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene GAPDH. Results of individual experiments (n#) are shown here.

Appendix 4.4 IFNα induction of PKR following PKCε RNAi. PKR expression was measured by RT-PCR following PKCε knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene PPIA. Results of individual experiments (n#) are shown here.

Appendix 4.5 IFNα induction of 2'5'-OAS following PKCε RNAi. 2'5'-OAS expression was measured by RT-PCR following PKCε knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene PPIA. Results of individual experiments (n#) are shown here.

Appendix 4.6 IFNα induction of MxA following PKCε RNAi. MxA expression was measured by RT-PCR following PKCε knockdown and IFNα stimulation. The levels of ISGs expressed were determined by normalising the expression of ISGs in comparison to expression of the housekeeper gene PPIA. Results of individual experiments (n#) are shown here.

Appendix III

Composite orthogonal views of PKCζ colocalisation with Adherens Junction proteins and Tight Junction proteins.

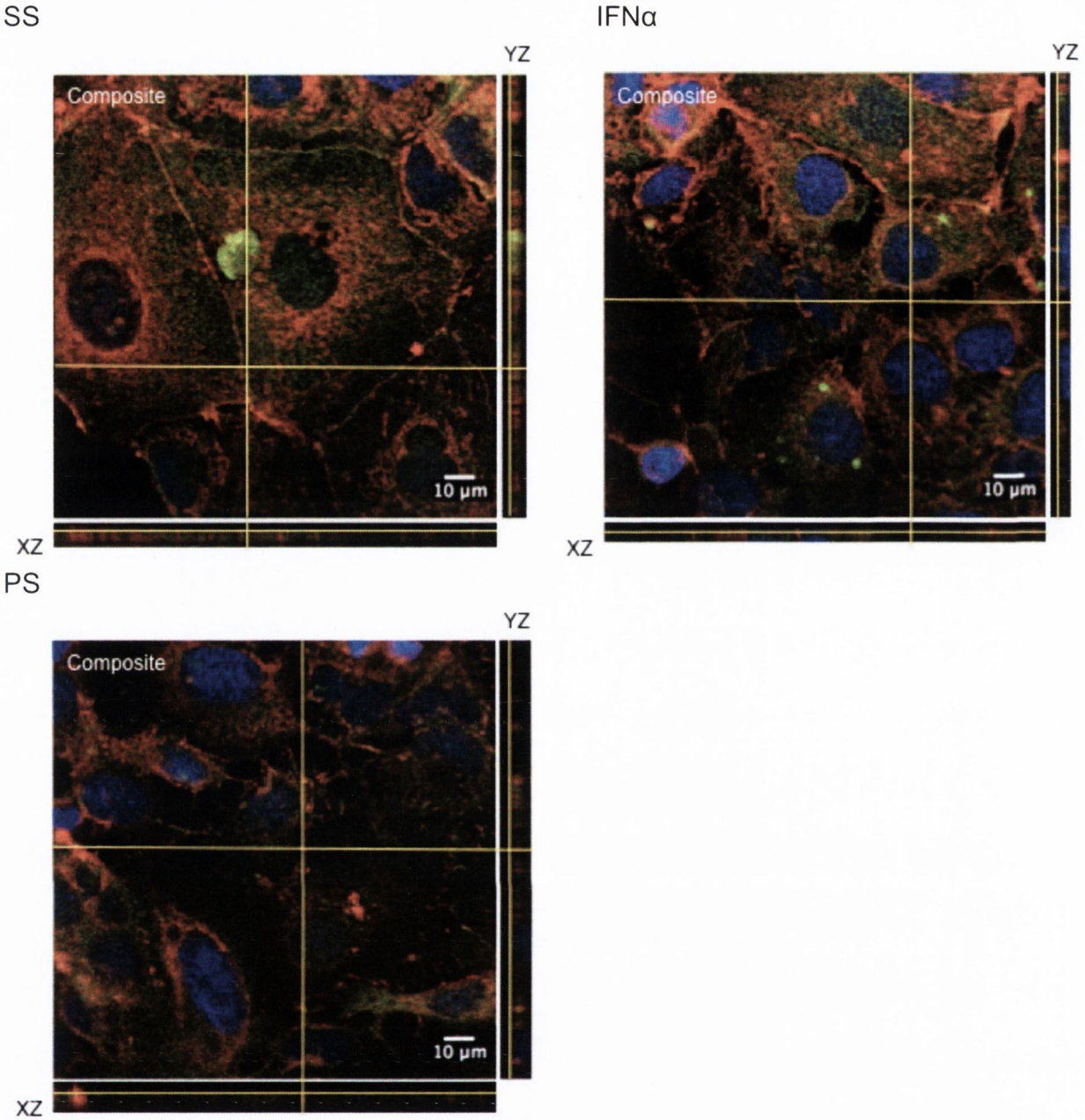


Figure A3.1 Composite orthogonal views of PKCζ colocalisation with Beta-Catenin following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours (SS) and then treated with either 10μM PKCζ pseudosubstrate (PS) inhibitor for 1 hour or with 1000U/ml IFNα for two hours (IFNα). Cells were fixed in acetone and incubated with primary antibody specific for PKCζ (green) and beta-Catenin (red) and the DNA stain Hoechst (blue). Yellow signal shows areas of PKCζ and beta-Catenin colocalisation. Cells were examined by a laser-scanning confocal microscope with a 63x objective.

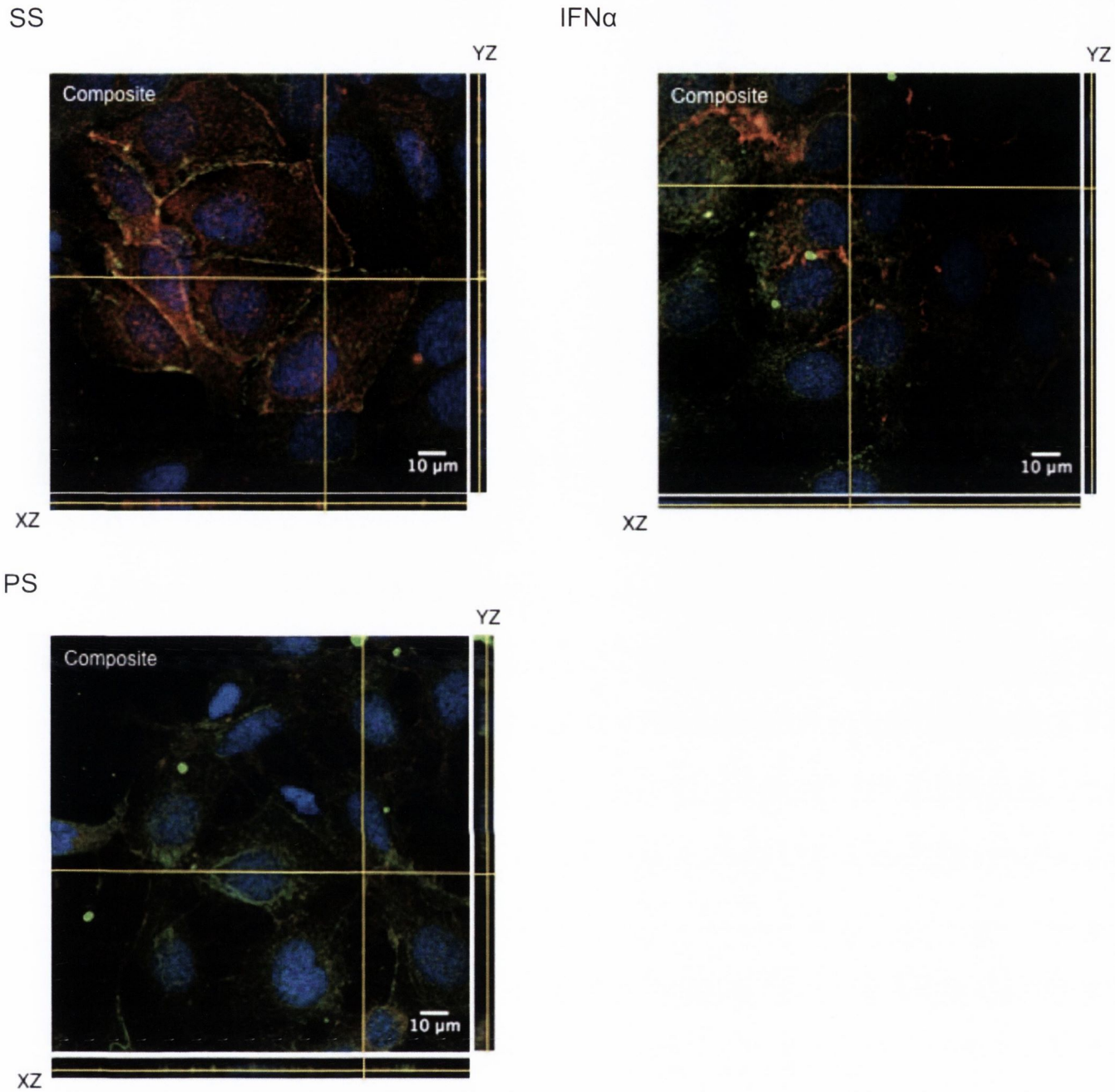


Figure A3.2 Composite orthogonal views of PKCζ colocalisation with E-Cadherin following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours (SS) and then treated with either 10μM PKCζ pseudosubstrate (PS) inhibitor for 1 hour or with 1000U/ml IFNα for two hours (IFNα). Cells were fixed in acetone and incubated with primary antibody specific for PKCζ (green) and E-Cadherin (red) and the DNA stain Hoechst (blue). Yellow signal shows areas of PKCζ and E-Cadherin colocalisation. Cells were examined by a laser-scanning confocal microscope with a 63x objective.

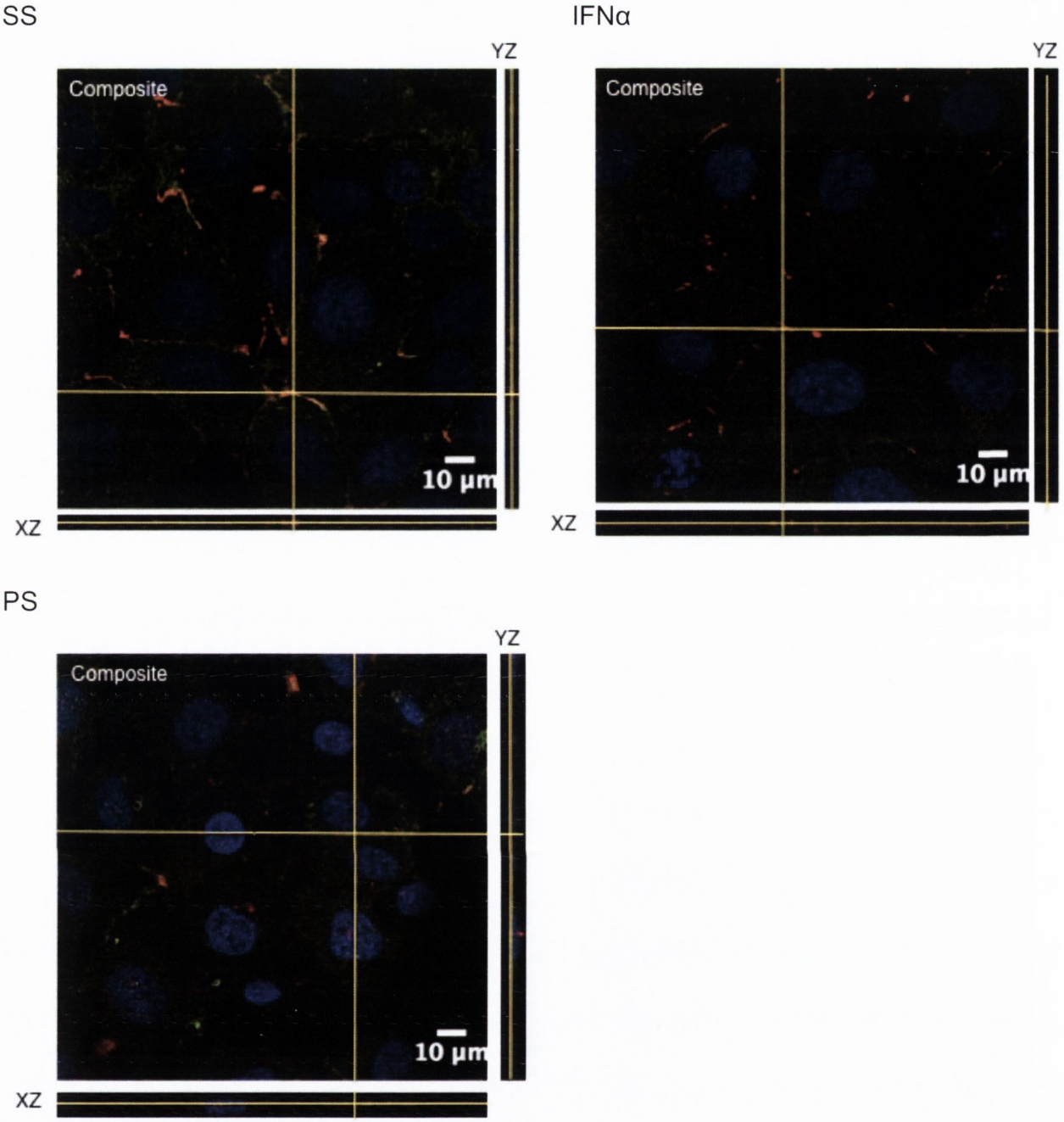


Figure A3.3 Composite orthogonal views of PKCζ colocalisation with Claudin-1 following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours (SS) and then treated with either 10μM PKCζ pseudosubstrate (PS) inhibitor for 1 hour or with 1000U/ml IFNα for two hours (IFNα). Cells were fixed in acetone and incubated with primary antibody specific for PKCζ (green) and Claudin-1 (red) and the DNA stain Hoechst (blue). Yellow signal shows areas of PKCζ and Claudin-1 colocalisation. Cells were examined by a laser-scanning confocal microscope with a 63x objective.

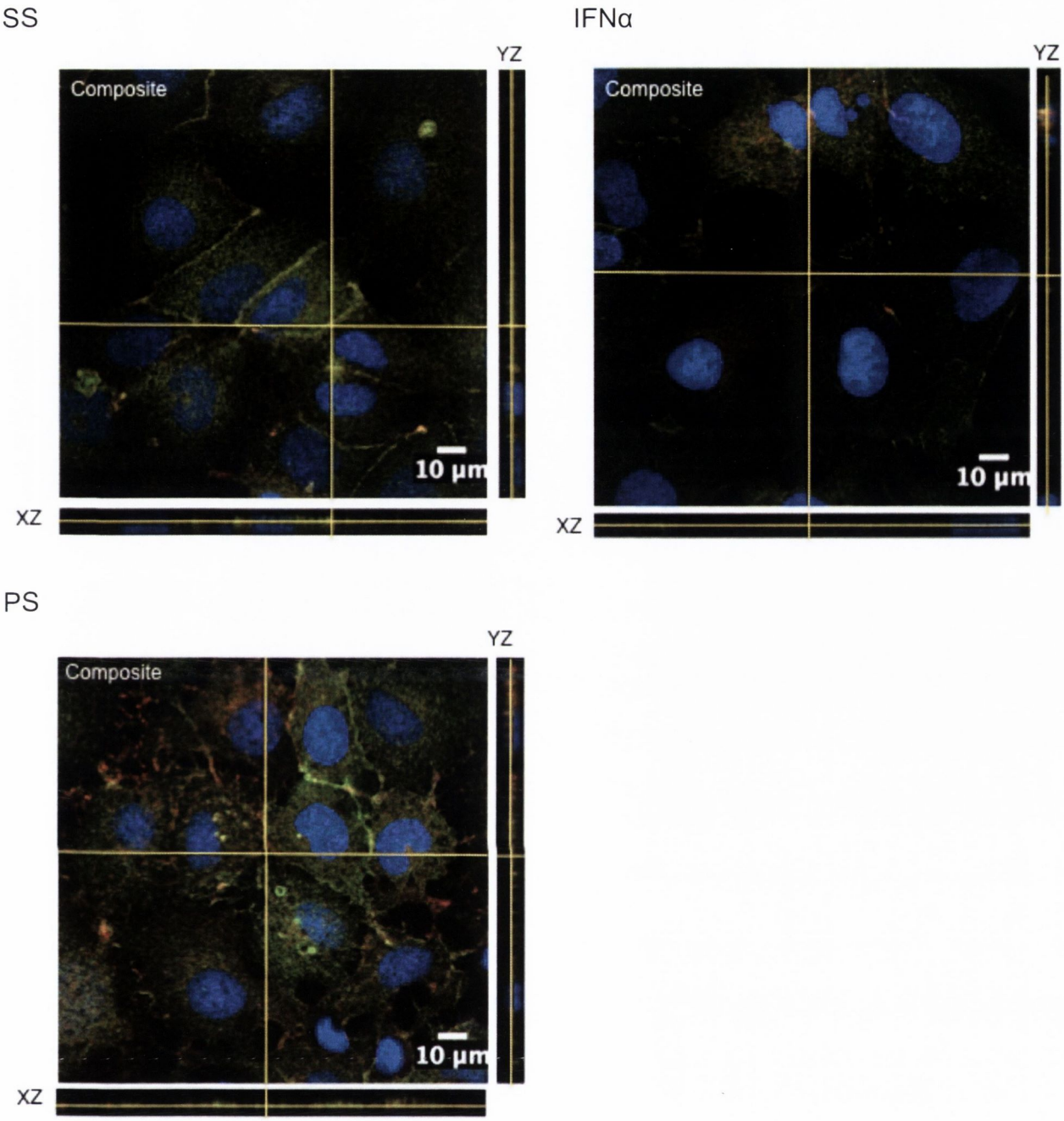


Figure A3.4 Composite orthogonal views of PKCζ colocalisation with Occludin following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours (SS) and then treated with either 10μM PKCζ pseudosubstrate (PS) inhibitor for 1 hour or with 1000U/ml IFNα for two hours (IFNα). Cells were fixed in acetone and incubated with primary antibody specific for PKCζ (green) and Occludin (red) and the DNA stain Hoechst (blue). Yellow signal shows areas of PKCζ and Occludin colocalisation. Cells were examined by a laser-scanning confocal microscope with a 63x objective.

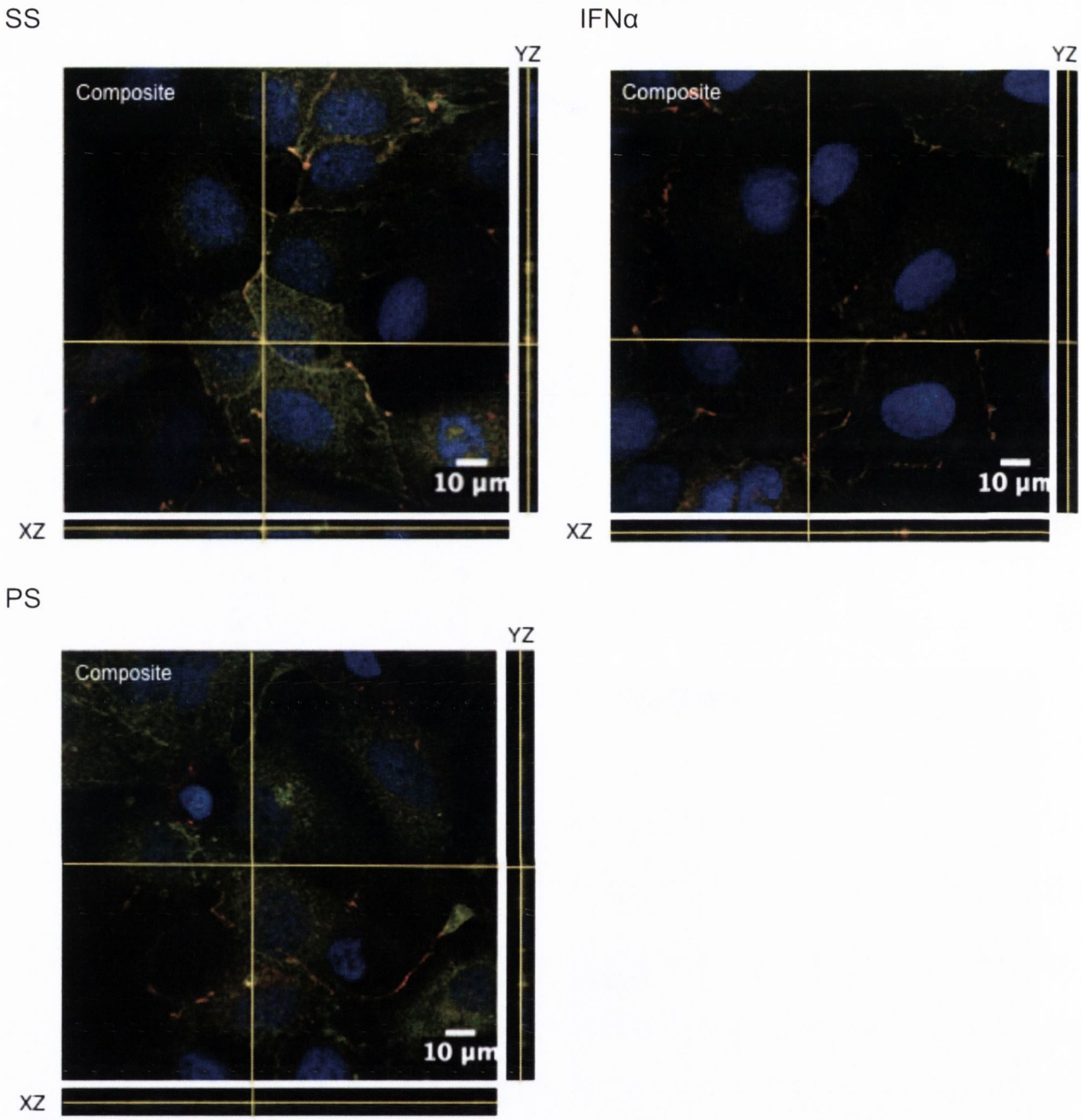


Figure A3.5 Composite orthogonal views of PKCζ colocalisation with ZO-1 following PKCζ inhibition or IFNα treatment. Huh7 cells were serum starved for 4 hours (SS) and then treated with either 10μM PKCζ pseudosubstrate (PS) inhibitor for 1 hour or with 1000U/ml IFNα for two hours (IFNα). Cells were fixed in acetone and incubated with primary antibody specific for PKCζ (green) and ZO-1 (red) and the DNA stain Hoechst (blue). Yellow signal shows areas of PKCζ and ZO-1 colocalisation. Cells were examined by a laser-scanning confocal microscope with a 63x objective.