

PROBING THE MECHANISMS UNDERLYING THE REGULATORY EFFECTS OF CACTIN IN INNATE IMMUNE SIGNALLING PATHWAYS

by

Siobhán Gargan

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Ollscoil na hÉireann Mā Nuad

Biology Department

NUI Maynooth

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Supervisor: Prof. Paul Moynagh

Head of Department: Prof. Kay Ohlendieck

DECLARATION

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Abstract

Toll-like Receptors (TLR)s are an essential class of Pattern Recognition Receptors (PRR)s used by the innate immune system to recognise conserved microbial motifs which are characteristic of invading pathogens. Activation of TLRs initiates signalling cascades resulting in the activation of transcription factors such as NF- κ B and members of the interferon regulatory factor (IRF) family which regulate gene expression and mediate an immune response to the pathogen. Dysregulation of TLR-signalling can result in inflammatory and autoimmune diseases and for this reason they are subject to tight regulation. This study demonstrates Cactin to be a novel negative regulator of TLR-signalling pathways. Overexpression of Cactin inhibits activation of NF- κ B whilst suppressed expression of Cactin leads to stronger and more prolonged activation of NF- κ B in response to LPS. The expression of Cactin is regulated by NF- κ B, thus representing a novel negative feedback mechanism for suppression of the inflammatory response. Cactin protein is stabilised due to phosphorylation in cells treated with the pro-inflammatory cytokine IL-1 β . Similarly, the stabilisation of Cactin was observed in response to the TLR ligands LPS and Poly(I:C). Cactin also negatively regulates the activation of IRF3 and IRF7 and suppression of endogenous Cactin augments Poly(I:C)-mediated IFN β induction. Cactin localises to the nucleus by virtue of two nuclear localisation sequences (NLS)s and its nuclear expression is essential for manifesting its inhibitory effects. Within the nucleus Cactin inhibits the DNA-binding activity of the NF- κ B subunit p65. Suppression of endogenous Cactin leads to increased binding of IRF3 to the IFN β promoter, though Cactin had no effect on IRF3 phosphorylation or translocation. This indicates that Cactin also targets IRF3 at the level of DNA binding. Furthermore, Cactin negatively regulates IRF7-mediated IFN α induction and affects the protein stability of IRF7. Notably, these inhibitory effects are transcription factor specific as Cactin positively regulates the activation of AP-1 subunit c-Jun, demonstrating that Cactin can differentially regulate transcription factor activation. A number of Cactin-interacting proteins were also identified in this study. Interestingly Cactin interacts with a nuclear member of the I κ B family, I κ BL. Cactin and I κ BL show very similar nuclear expression patterns and exhibit similar negative regulatory effects on NF- κ B, IRF3 and IRF7. However, these two proteins function independently to inhibit the

activation of these transcription factors. In summary this study highlights Cactin as a novel regulator of TLR signalling pathways by regulating the ability of specific transcription factors to bind to their cognate DNA sequences and induce transcription.

Abbreviations

Amp	Ampicillin
AP-1	Activating protein-1
APC	Antigen presenting cell
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ASC-1	Activating signal cointegrator 1
ASCC1	Activating signal cointegrator 1 complex subunit 1
ATF-2	Activating transcription factor-2
bp	Base Pair
BSA	Bovine serum albumin
BMDM	Bone marrow derived macrophages
C	Carboxy
Cactin	Cactus interacting protein
cAMP	Cyclic adenosine 3', 5'-monophosphate
CARD	Caspase recruiting domain
CBP	CREB-binding protein
CD	Cluster of Differentiation
cDNA	complementary DNA
CpG DNA	2'-deoxyribo cytidine-phosphate-guanosine DNA
ChIP	Chromatin immunoprecipitation
CIP	Calf Intestinal Phosphatase
cm	centimetre
CMV	Cytomegalovirus
CNS	Central nervous system
CREB	cAMP-responsive element binding protein
CT	Control
DBD	DNA binding domain
DC	Dendritic cell
DD	Death domain
DEPC	Diethylpyrocarbonate

DIF	Dorsal-related immunity factor
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleid triphosphates
DREDD	Death related ced-3/Nedd2-like protein
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
dCactin	<i>Drosophila</i> Cactin
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal regulated kinase
EV	Empty vector
FADD	Fas-associated death domain
FBS	Foetal Bovine Serum
GNBP	β -glucan recognition protein
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
h	Hour(s)
HAT	Histone acetylase
HDAC	Histone deacetylase
HEK	Human Embryonic Kidney
HMG	High-mobility-group protein
HPRT	Hypoxanthinephosphoribosyltransferase
HSP	Heat shock protein
IAD	IRF association domain
IAP	Inhibitor of apoptosis protein
IAV	Influenza A virus
IB	Immunoblot
ICAM	Intercellular adhesion molecule 1
ID	Intermediate domain
IFN	Interferon

Ig	Immunoglobulin
IκB	Inhibitor of κB
IκBL	IκB-like
IKK	IκB kinase
IKKi	Inducible IKK
IL	Interleukin
IL-1R	IL-1 Receptor
IL-1RAcP	IL-1R accessory protein
Imd	Immune deficiency
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IPI	International protein index
IPS-1	IFNβ promoter stimulator 1
IR	Infrared
IRAK	IL-1R associated kinase
IRF	Interferon regulatory factor
ISGF3	IFN-stimulatory gene factor 3
ISRE	IFN-stimulated response element
JNK	c-Jun N-terminal kinase
K	Lysine
Kb	Kilobase
kD	KiloDalton
KIR	Kinase inhibitory region
LB	Luria Bertoni
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
Mal	MyD88-adaptor like
MAPK	Mitogen activated protein kinase
MAVS	Mitochondria anti-viral signalling protein
MBL	Mannose-binding lectin
MCMV	Murine cytomegavirus
MD-1/2	Myeloid differentiation protein-1/2

MDA5	Melanoma differentiation-associated gene 5
mDC	Myeloid dendritic cell
MEKK1	Mitogen activated protein kinase kinase kinase 1
MHC	Major histocompatibility
min	minute
ml	milli litre
mM	milli molar
MMLV RT	Moloney murine leukemia virus reverse transcriptase
MMTV	Mouse mammary tumour virus
MMR	Macrophage mannose receptor
mRNA	Messenger RNA
MS	Mass Spectrometry
MyD88	Myeloid differentiation factor 88
NAP1	NF-κB-activating kinase (NAK)-associated protein1
NC	Non-specific competitor
NES	Nuclear export signal
NF-κB	Nuclear Factor-κB
ng	nano grammes
NIK	NF-κB interacting kinase
NK	Natural killer
NLR	Nod-like receptor
NLS	Nuclear localisation sequence
nM	nanomolar
NRE	Negative regulatory element
NRF	NF-κB repressor factor
NTP	Nucleotide triphosphate
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PAK1IP1	PAK1 interacting protein 1
Pam-Cys	Pam3CysSerLys4

PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
pCAF	p300/CBP associated factor
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PI3K	Phosphatidylinositol 3-kinase
PIC	Pre-initiation complex
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl Fluoride
Poly(I:C)	Polyinosinic:polycytidylic acid
PRD	Positive regulatory domain
PRD1-BF1	PRD1 binding factor 1
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RANTES	Regulated upon activation, normal T cell expressed and secreted
RFP	Red fluorescent protein
RH	Rel homology
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor interacting protein
RISC	RNA-induced silencing protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP105	Radioprotective 105
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
s	second(s)
SARM	Sterile α and HEAT-Armadillo motifs
SC	Specific competitor

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
SEM	Standard error of the mean
SH domain	<i>Src</i> homology domain
shRNA	short hairpin RNA
SIGGIR	Single immunoglobulin IL-1 related protein
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
SR	Super repressor
SRC	Steroid receptor-co-activator
SRF	Serum response factor
SSc	Systemic sclerosis
ss Protein A	Salmon sperm DNA/protein A agarose
ssRNA	Single stranded RNA
ST2	Suppressor of tumourogenicity 2
STAT	Signal transducer and activator of transcription
sTLR	soluble TLR
TAB	TAK1-binding protein
TAE	Tris-acetate-EDTA
TAK	TGF- β -activated protein kinase 1
TANK	TRAF-family-member associated NF- κ B activator
Taq	<i>Thermophilus aquaticus</i>
TBE	Tris-Borate-EDTA
TBK1	TANK-binding protein 1
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Tween 20
TE	Tris-EDTA
TEMED	N, N, N', N' – Tetramethylethylene-diamine
TGF- β	Transforming growth factor- β
$T_{H1/2}$	Helper type 1/2 T cell
TIR	Toll/IL-1R
TLR	Toll-like receptor

TNF α	Tumour necrosis factor α
Tollip	Toll Interacting protein
TRAF	TNF receptor associated factor
TRAILR	TNF-related apoptosis-inducing ligand receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN- β
Ubc	Ubiquitin conjugating enzyme
Uev1A	Ubiquitin-conjugating enzyme E2 variant isoform
μ g	micro grammes
μ l	micro litre
μ M	micro molar
UV	Ultraviolet
VCAM	Vascular cellular adhesion molecule 1
WCL	Whole cell lysate
WNV	West Nile Virus
WT	Wild Type

Chapter 1

General Introduction

1.1 The Mammalian Immune System

The mammalian immune system is divided into two arms of defence against invading pathogens. The adaptive immune system is the late phase of response while the innate immune system represents the first line of defence against infectious agents. The adaptive immune system, or acquired immune system, is associated with immunological memory and pathogen specific responses. It is mediated by lymphocytes, T cells and B cells, which recognise a vast selection of pathogens via cell surface receptors. Each lymphocyte has a unique antigen receptor generated by somatic gene rearrangement which occurs during lymphocyte development. The result of this gene rearrangement is a huge repertoire of antigen specific receptors which can recognise virtually any pathogen (Janeway et al., 2005). Lymphocytes circulate through the lymphatic system as small inactive cells which have no effector function. Recognition of their specific antigen promotes lymphocyte proliferation and differentiation to effector cells which mount an immune response to the invading pathogen. However, antigen recognition alone is not sufficient for lymphocyte activation. Co-stimulatory molecules, provided by antigen presenting cells (APC)s of the innate immune system, must also be present to stimulate lymphocyte differentiation (Medzhitov and Janeway, 1997). In fact the presentation of antigens to lymphocytes in the absence of co-stimulatory molecules causes their inactivation. Cells of the innate immune system also release cytokines which regulate the effector responses employed by the adaptive immune system (Janeway et al., 2005).

Following activation, B cells differentiate into plasma cells which secrete antibodies that recognize and bind extracellular pathogens. Antibodies utilise three effector mechanisms for effective removal of invading pathogens. They bind to pathogens preventing them from entering cells, a process known as neutralization. Opsonization is the coating of pathogens with antibody to promote phagocytosis by innate immune cells. Antibodies can also activate the complement system which is a component of the innate immune system. It is a series of plasma proteins that assemble on the surface of pathogens and create pores which can destroy bacteria directly. More often, however, coating of pathogens with complement promotes their removal by phagocytosis. T cells are responsible for cell-mediated immune response to intracellular pathogens. Cytotoxic T cells destroy virus infected cells which present viral derived peptides to T cells in cell surface major histocompatibility (MHC)

complex class I molecules. Helper T cells guide other cells of the immune system. T_{H1} cells recognise intracellular vesicle derived peptides displayed in MHC class II molecules on the cell surface of macrophages and subsequently promotes fusion of pathogen containing vesicles with lysosomes containing bactericidal agents. T_{H2} cells also recognise vesicle derived peptides presented in MHC class II molecules and promote B cell activation (Janeway et al., 2005). Adaptive immunity occurs in the late phase of infection, typically 4 days or more after infection. This is due to the need for specific T and B cells to undergo clonal expansion and differentiation into effector cells. Conversely the innate immune response is rapidly induced and prevents the spread of infection during this time.

The innate immune system is the first line of defence against invading pathogens. In fact, many infections are cleared efficiently by cells of the innate immune system without requiring the induction of an adaptive immune response. Furthermore, the innate immune system is essential for the initiation and direction of adaptive immunity (Medzhitov and Janeway, 1997). Mechanisms of innate immunity include the epithelial surfaces of the body which act as an effective physical barrier against most microorganisms. Pathogens which pass these barriers are immediately faced with tissue macrophages which engulf the invading pathogen by phagocytosis. Furthermore, recognition of invading pathogens by macrophages leads to the initiation of an inflammatory response. The release of pro-inflammatory cytokines increases the permeability of blood vessels while chemokines attract immune cells to the site of infection. Neutrophils are the first cells recruited which help to phagocytose and remove the invading pathogen. These are followed by monocytes, the precursor of macrophages, which differentiate into additional macrophages at the site of infection. These phagocytes also produce toxic agents such as nitric oxide (NO) and antimicrobial peptides such as defensins and cationic proteins. Other cells recruited to the site of infection include granulocytes, such as mast cells, which release granules containing the small inflammatory molecule histamine and the cytokine TNF- α , and innate natural killer (NK) cells which kill virus infected cells. In addition to leukocytes, plasma proteins also accumulate at the site of infection including components of the complement system which assemble on the surface of pathogens and promote their removal by phagocytosis. The inflammatory response also promotes clotting of the blood in microvessels in the site of infection, serving to prevent the spread of infection. Recognition of invading pathogens also leads to the

expression of costimulatory molecules, CD80 and CD86, on the cell surface of macrophages and dendritic cells (DC)s. DCs travel to nearby lymph nodes and present pathogen-derived peptides to lymphocytes in MHC class II molecules, along with co-stimulatory molecules, to activate an adaptive immune response to the invading pathogen (Iwasaki and Medzhitov, 2004).

1.2 Pathogen Recognition Receptors (PRRs)

The innate immune system lacks the antigen specificity of adaptive immunity. It does, however, have the ability to discriminate between self and non-self via the use of a number of germline encoded receptors called pattern recognition receptors (PRR)s. These receptors recognise repeating patterns of molecular structure found on invading pathogens, which are not present in host cells (Janeway, 1989). For example, the DNA of bacteria contains unmethylated repeats of the dinucleotide CpG which can be detected by the innate immune system. These patterns are collectively known as pathogen associated molecular patterns (PAMPs). PAMPs are conserved structures shared by large groups of pathogens (Medzhitov and Janeway, 1997). Lipopolysaccharide (LPS), for example, is found in the cell walls of all gram negative bacteria. Double stranded (ds)RNA which is used by almost all viruses at some stage of their life cycle is also detected by PRRs. PAMPs are also essential for microbial viability, making them perfect targets for the non-specific immune system. Bacteria, viruses, fungi and protazoa all contain PAMPs and can activate an innate immune response. Some PRRs recognise PAMPs directly while others recognise products generated by PAMP recognition. There are three types of PRRs, secreted proteins, transmembrane receptors and intracellular receptors (Medzhitov and Janeway, 1997).

1.2.1 Secreted PRRs

Secreted PRRs, including collectins, ficolins and pentraxins, bind to microbes and activate the complement system. For example, Mannose-binding lectin (MBL) is a member of the collectin family which recognises a particular orientation of sugar residues on the surface of many microorganisms and initiates the complement cascade (Janeway et al., 2005).

Table 1.1 PAMP detection by TLRs

Species	PAMPs	TLRs
Bacteria, mycobacteria	LPS Lipoproteins Lipoarabinomannan PGN LTA Flagellin CpG DNA RNA	TLR4 TLR2/1, TLR2/6 TLR2/1, TLR2/6 TLR2/1, TLR2/6 TLR2/1, TLR2/6 TLR5 TLR9 TLR7
Viruses	DNA RNA Envelope protein Structural protein	TLR9 TLR3, TLR7, TLR8 TLR4 TLR2, TLR4
Fungus	zymosan, β -glucan Mannan DNA RNA	TLR2, TLR6 TLR2, TLR4 TLR9 TLR7
Parasites	tGPI-mutin (<i>Trypanosoma</i>) glycoinositolphospholipids (<i>Trypanosoma</i>) DNA hemozoin (<i>Plasmodium</i>) profilin-like molecule (<i>Toxoplasma gondii</i>)	TLR2 TLR4 TLR9 TLR9 TLR11
Host	HSP 60, 70 Fibrinogen	TLR4 TLR4

1.2.2 Transmembrane PRRs

Transmembrane PRRs include the C-type lectins, such as Dectin-1 which recognises β -glucans in fungal cell walls, and macrophage mannose receptor (MMR) (Janeway and Medzhitov, 2002). PAMP recognition by these receptors triggers cellular processes such as phagocytosis and production of reactive oxygen species (ROS). Another class of transmembrane PRRs which have been extensively studied are toll-like receptors (TLRs) and they recognise a wide range of PAMPS (Table 1.1). The expression of TLRs is cell-type specific (Iwasaki and Medzhitov, 2010). They are primarily expressed on innate immune cells such as macrophages, DCs, neutrophils and NK cells, but can be expressed on lymphocytes and also on some non-immune cells such as epithelial cells (Iwaski and Medzhitov, 2004). Cell surface TLRs recognise PAMPs on the surface of pathogens. TLR4 recognises LPS on the surface of gram-negative bacteria (Chow et al., 1999), TLR5 recognises bacterial flagellin (Hayashi et al., 2001) and TLR2 forms heterodimers with TLR1 or TLR6 to recognise a diverse range of bacterial lipopeptides (Farhat et al., 2008). Other TLRs are located on the membranes of intracellular endosomes and detect microbial nucleic acids. TLR3 recognises viral dsRNA (Alexopoulou et al., 2001), TLR7 recognises viral ssRNA (Diebold et al., 2004) and TLR9 can detect bacterial DNA by the presence of CpG repeats (Bauer et al., 2001). TLRs also recognise PAMPs derived from fungi and parasitic-protazoa (Kumar et al., 2009). PAMP recognition by TLRs initiates a signalling cascade which results in the activation of transcription factors, such as nuclear factor (NF)- κ B and members of the interferon regulatory factor (IRF) family of transcription factors (Iwaski and Medzhitov, 2004). These transcription factors up-regulate the expression of genes encoding pro-inflammatory cytokines, such as interleukin (IL)-1 β and IL-6, pro-inflammatory chemokines, such as IL-8 (also known as CXCL8), antiviral interferons (IFN)s and the co-stimulatory molecules CD80 and CD86 (Medzhitov et al., 1997).

1.2.3 Cytoplasmic PRRs

Cytoplasmic PRRs include retinoic acid-inducible gene I (RIG-I)-like receptors (RLR)s, such as RIG-I and melanoma differentiation factor 5 (MDA5). RLRs are expressed in most cell types and detect the presence of viral RNA through

their helicase domains. Activation of RLRs by viral RNA leads to the recruitment of mitochondrial antiviral signalling protein (MAVS) also called IFN β promoter stimulator (IPS)-1 which leads to downstream activation of the transcription factors NF- κ B and IRF3 (Iwasaki and Medzhitov, 2010). Another family of cytoplasmic PRRs are NOD-like receptors (NLR)s which can detect stress signals and microbial products. They have C-terminal leucine rich repeats (LRR)s responsible for PAMP recognition, a central nucleotide –binding oligomerization domain (NOD) for self oligomerization and an N-terminal effector domain. Some NLRs, such as NOD1, activate NF- κ B. Other NLRs, such as NALP1 and NALP3, form protein complexes with apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1, known as inflammasomes. The inflammasome mediates the cleavage of pro-IL-1 β and pro-IL-18 by caspase-1 to active IL-1 β and IL-18 (Creagh and O'Neill, 2006). TLR signalling leads to the production of pro-IL-1 β while NLRs are required for the cleavage of pro-IL-1 β to its active form. Given that IL-1 β is a key mediator of inflammation, it is clear that co-operation of these two PRRs is necessary for effective activation of the inflammatory response.

1.3 Toll-like Receptors (TLR)s

To date, 10 functional TLRs have been identified in humans while 12 have been identified in mice (Kawai and Akira, 2011). TLRs are type 1 transmembrane receptors with N-terminal extracellular LRRs, which are responsible for PAMP recognition, and a C-terminal intracellular signalling domain. The intracellular domain of TLRs is similar to that of the IL-1 Receptor (IL-1R) family and is consequently termed the Toll/IL-1R (TIR) domain. In fact, members of the IL-1R family use many of the same signalling intermediates as TLRs (Martin and Wesche, 2002). Unlike TLRs, however, IL-1R family members have extracellular Immunoglobulin (Ig)-like domains which mediate ligand recognition. Following PAMP recognition, TLRs form homo- or heterodimers which allows for the recruitment of various TIR domain containing adaptor proteins to the receptor. This initiates a signalling cascade which results in the downstream activation of transcription factors which regulate gene expression. NF- κ B and AP-1 regulate the expression of pro-inflammatory cytokines and chemokines while IRF3 and IRF7 regulate the expression of Type I IFNs. Myeloid differentiation factor 88 (MyD88) is

the universal adaptor protein recruited by all TLRs, with the exception of TLR3, and also by members of the IL-1R family (Medzhitov et al., 1998). Recruitment of MyD88 by all receptors results in downstream activation of NF- κ B and AP-1. In plasmacytoid DCs (pDCs) the recruitment of MyD88 by endosomal TLR7 and TLR9 can also lead to the activation of IRF7 (Kawai et al., 2004, Honda et al., 2004). TLR3 and TLR4 both recruit TIR domain –containing adaptor-inducing IFN- β (TRIF) leading to subsequent activation of IRFs and NF- κ B (Jiang et al., 2004). TLR2 and TLR4 require an additional adaptor protein, MyD88 adaptor like (Mal) which acts as a bridge between MyD88 and the receptor (Yamamoto et al., 2002). Similarly, TLR4 utilises TRIF-related adaptor molecule (TRAM) as a bridging adaptor for TRIF (Oshiumi et al., 2003).

1.3.1 TLR4

TLR4 was the first mammalian TLR discovered (Medzhitov et al., 1997). It detects LPS which is one of the best studied immunostimulatory components of bacteria. TLR4 is expressed on the cell surface of innate immune cells such as monocytes/macrophages, myeloid (m)DCs and mast cells. TLR4 is also expressed on the surface of B lymphocytes (Gerondakis et al., 2007). Furthermore, TLR4 can be expressed by non-immune cells. For example, TLR4 can be found in the Golgi apparatus of intestinal epithelial cells where it recognises internalised LPS (Hornef et al., 2003).

LPS is an essential structural component of the cell walls of gram negative bacteria. LPS is sometimes called endotoxin and consists of a core oligosaccharide, an O side chain and a highly conserved Lipid A part which serves as the PAMP that activates TLR4. Cellular recognition of LPS, however, requires several different molecules including LPS-binding protein (LBP), cluster of differentiation (CD)14, myeloid differentiation protein (MD)-2 and TLR4. LBP is an acute-phase protein which is present in the bloodstream and binds LPS (Wright et al., 1989). LBP then transfers LPS to cell surface receptor CD14 (Wright et al., 1990). CD14 is a 55 kDa glycoprotein with multiple LRRs (Setoguchi et al., 1989). It lacks a characteristic transmembrane region but instead is anchored in the cell membrane by a phosphatidylinositol linkage (Haziot et al., 1988). CD14 is expressed primarily on myeloid cells including macrophages, DCs and neutrophils. The CD14/LPS complex

then interacts with TLR4/MD-2 complexes. MD-2 is a small soluble protein that binds non-covalently to TLR4 and is essential for TLR4 recognition of LPS (Shimazu et al., 1999). The interaction of the CD14/LPS complex with TLR4/MD-2 complexes causes receptor oligomerization and initiates downstream signalling (Alexander and Rietschel, 2001).

TLR4 is unique among TLRs in that it activates two distinct signalling pathways. The TIR domain-containing adaptor proteins recruited to the receptor dictates the signalling pathway which ensues. TLR4 is also unique as it utilises all four TIR domain-containing adaptor proteins. Two distinct signalling pathways are activated by TLR4 and classified as the MyD88-dependent and MyD88-independent signalling pathways. It was originally thought that these signalling pathways were initiated simultaneously following ligand recognition. However it now appears that these pathways are initiated sequentially (Kagan et al., 2008). Recognition of LPS by TLR4 initiates MyD88-dependent signalling and activation of NF- κ B and AP-1. MyD88 is recruited to the receptor at the plasma membrane with the help of bridging adaptor Mal (Yamamoto et al., 2002). The LPS-TLR4 complex is then internalised into early endosomal structures in a process dependent on clathrin and the GTPase dynamin (Husebye et al., 2006). Once the LPS-TLR4 complex is endocytosed, MyD88-dependent signalling is abolished. TRIF can now be recruited to the receptor with the help of bridging adaptor TRAM (Oshiumi et al., 2003). Recruitment of TRIF leads to downstream activation of members of the IRF family of transcription factors and the induction of Type I IFNs. Interestingly, all pathways leading to the induction of Type I IFNs are initiated intracellularly. It was thought that TLR4 was an exception to this rule. It appears, however, that TLR4 signalling through TRIF is dependent on endocytosis of the receptor (Kagan et al., 2008). It is possible that the localization of TRAF3, a key signalling intermediate in activation of Type I IFNs, dictates that these signalling pathways must be initiated from intracellular locations. Therefore, internalisation of TLR4 brings it into close proximity to TRAF3. Once TRIF is recruited to the receptor it can interact with TRAF3 leading to downstream activation of IRFs and the induction of Type I IFNs (Kagan et al., 2008). The LPS-TLR4 complex is then trafficked to late endosomes or lysosomes for degradation and loading of associated antigens into MHC Class II molecules for presentation to helper T cells (Husebye et al., 2006).

In addition to LPS, TLR4 has also been implicated in the recognition of a diverse range of other PAMPs. TLR4 and CD14 recognise fusion (F)-protein of respiratory syncytial virus (RSV) (Kurt-Jones et al., 2000). Furthermore, TLR4 recognises the envelope protein from mouse mammary tumor virus (MMTV) (Rassa et al., 2002). TLR4 has also been implicated in the recognition of fungal pathogens. TLR4 recognises some strains of *Candida Albicans* (Netea et al., 2010) and pertussis toxin isolated from *Bordetella pertussis* (Kerfoot et al., 2004). *Candida Albicans* and *Saccharomyces Cerevisiae* derived mannan has also been shown to induce TNF- α through activation of TLR4 (Tada et al., 2002). In addition, TLR4 can also initiate an immune response to endogenous molecules. Heat-shock protein (HSP)60 serves as a danger signal for the innate immune system and activates TLR4 (Ohashi et al., 2000). Hyaluronic acid is one of the major components of the extracellular matrix and undergoes rapid degradation at sites of inflammation. Fragments of hyaluronic acid have been shown to activate TLR4 on DCs (Termeer et al., 2002). Similarly, anti-microbial β -defensin 2 released during inflammation activates TLR4 on DCs (Biragyn et al., 2002). Activation of TLR4 induces the expression of co-stimulatory molecules and maturation of DCs into mature APCs which activate cells of the adaptive immune system.

1.3.2 TLR3

TLR3 is expressed on the endosomes of immune cells such as macrophages, NK cells and mDCs and recognises viral nucleic acids (Kumar et al., 2009). TLR3 is not, however, expressed by pDCs which are specialized at producing high levels of Type I IFNs in response to viral infection via signalling by TLR7 and TLR9. This differential expression on DCs suggests a role for TLR3 in the activation of adaptive immunity by stimulating the maturation of mDCs into APCs, rather than primary production of IFNs. TLR3 is also expressed by non-immune cells including fibroblasts (Matsumoto et al., 2003), intestinal epithelial cells (Cario and Podolsky, 2000) and airway epithelial cells (Sha et al., 2004). In fibroblasts and epithelial cells TLR3 can be found on the cell surface as well as intracellularly (Matsumoto et al., 2003). While cell surface TLR3 may have a role in ligand recognition, in all cases TLR3 signalling is initiated from intracellular endosomes (Matsumoto and Seya, 2008). Notably, in human embryonic kidney (HEK) 293 cells engineered to express

TLR3, the receptor was found to localise to intracellular vesicles where signalling was initiated (Funami et al., 2004). TLR3 is expressed by neurons and induces inflammatory and antiviral responses in the brain (Lafon et al., 2006). The expression of TLR3 is up-regulated by viral infection due to the presence of an IFN-response element (ISRE) in the *TLR3* promoter region (Tanabe et al., 2003).

TLR3 recognises viral dsRNA which is the genetic information of some viruses (Alexopoulou et al., 2001). dsRNA is also produced by most other viruses at some point in their replication process. TLR3 also recognises the stable synthetic dsRNA polyriboinosinic : polyribocytidylic acid (Poly(I:C)) which is frequently used to mimic viral infection in cells. Furthermore, endogenous RNA released from necrotic cells can also serve as a ligand for TLR3 activation (Kariko et al., 2004). TLR3 has been implicated in the response to West Nile Virus (WNV). However, some reports suggested TLR3 response to WNV infection facilitates disruption of the blood-brain barrier and lethal encephalitis and so contributes to disease pathogenesis rather than protection (Wang et al., 2004). Conversely, other reports suggest a protective role of TLR3 in WNV infection as TLR3 signalling reduced the spread of viral infection the brain and CNS (Daffis et al., 2008). TLR3 has also been implicated in anti-viral responses to RSV (Rudd et al., 2006), influenza A virus (IAV) (Goffic et al., 2006), phlebovirus (Gowen et al., 2006) and murine cytomegarovirus (MCMV) (Tabeta et al., 2004). TLR3 is unique among TLRs as it does not recruit the TIR-domain containing adaptor MyD88 for signal transduction. Instead TLR3 signals through TRIF leading to downstream activation of IRFs, NF- κ B and AP-1 and the induction of Type I IFNs, pro-inflammatory cytokines and co-stimulatory molecules.

1.4 TLR signalling pathways and activation of transcription factors

MyD88 is the universal adaptor recruited by all TLRs, with the exception of TLR3, and for this reason the signalling cascades activated by TLRs are often classified as MyD88-dependent and MyD88-independent pathways (Figure 1.1). The MyD88-dependent pathway is also utilised by the widely expressed IL-1R.

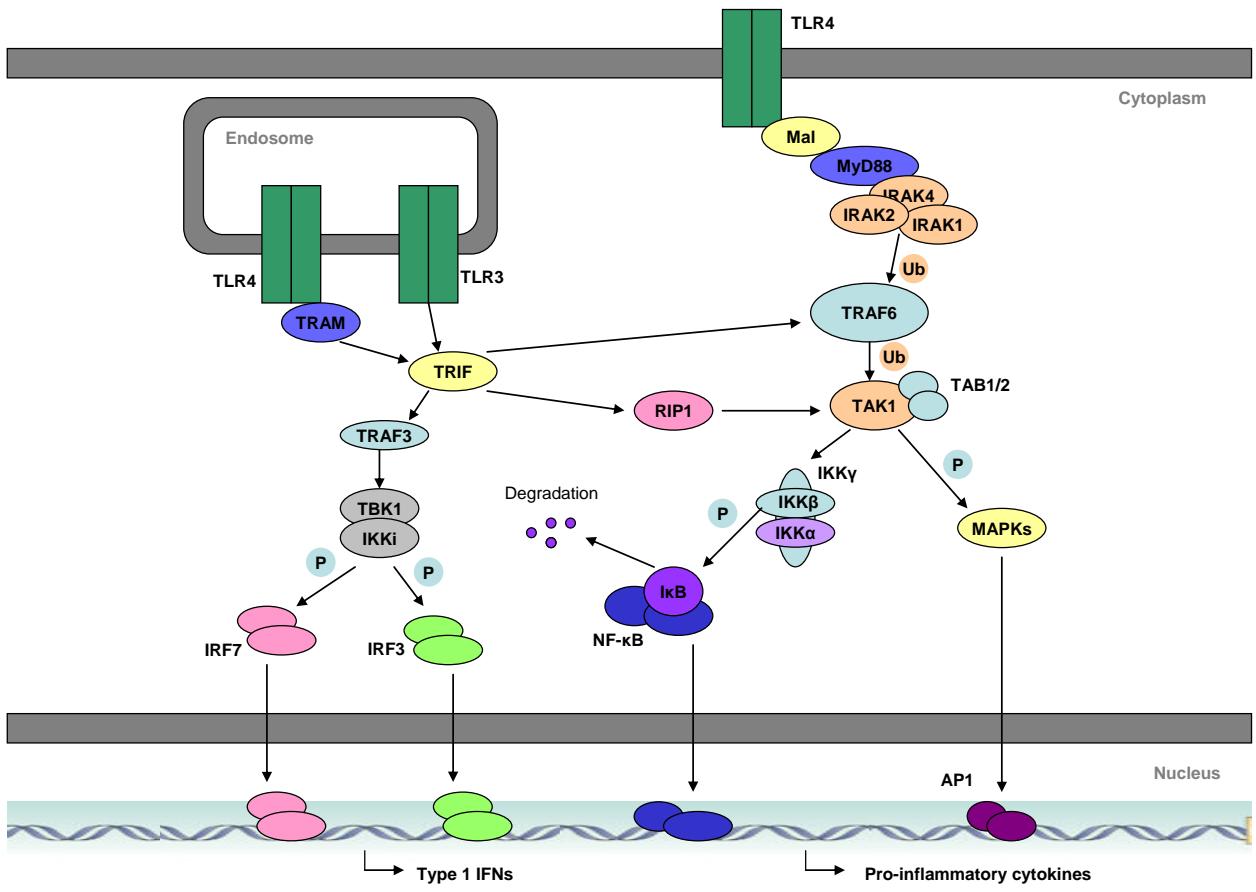


Figure 1.1 TLR3- and TLR4-mediated signalling pathways

Upon activation TLR4 recruits MyD88 to the receptor with the help of bridging adaptor Mal. This leads to the recruitment and activation of IRAKs which in turn activates TRAF6. TRAF6 ubiquitinates and activates TAK1 which in turn activates the IKK complex. The active IKK complex phosphorylates the I κ B proteins marking them as targets for proteasomal degradation. The NF- κ B dimer then translocates to the nucleus and binds to the promoter regions of its target genes. TAK1 also activates MAPKs leading to activation of AP-1 which co-operates with NF- κ B to regulate the expression of pro-inflammatory cytokines. Endocytosed TLR4 then recruits TRIF with the help of bridging adaptor TRAM. TLR3 also signals through TRIF. TRIF associates with TRAF3 which recruits the TBK1/IKKi kinase complex leading to phosphorylation of IRF3 and IRF7 promoting their dimerisation and translocation to the nucleus where they regulate the expression of Type 1 IFNs. TRIF also signals through RIP1 or TRAF6 to activate TAK1 leading to downstream activation of NF- κ B and AP-1.

1.4.1 The MyD88-dependent pathway

Upon binding of the appropriate ligand, TLRs dimerise. This causes a conformational change that creates a platform for the recruitment of TIR-domaining adaptor proteins to the receptor. MyD88 is recruited to the receptor with or without the aid of the bridging adaptor Mal. In addition to its TIR domain, MyD88 has an N-terminal death domain (DD). Upon recruitment, MyD88 associates with members of the IL-1R associated kinase (IRAK) family of protein kinases, through homotypic association of their DDs (Wesche et al., 1997). IRAK4 is thought to be recruited upstream of other IRAKs (Lye et al., 2004). IRAK4 phosphorylates critical residues in the N-terminal of IRAK1 causing its activation (Li et al. 2002). IRAK1 is then hyperphosphorylated by itself and other kinases triggering its dissociation from MyD88. IRAK2 also becomes phosphorylated and dissociates from the complex. Once dissociated, IRAK1 and IRAK2 interact with the downstream adaptor, tumour necrosis factor (TNF)-receptor associated-factor 6 (TRAF6) (Flannery and Bowie, 2010). TRAF6 is a RING domain containing E3 ubiquitin ligase which, along with E2 ubiquitin conjugating enzyme (Ubc)13 and ubiquitin-conjugating enzyme E2 variant 1 isoform A (Uev1A), catalyses the formation of polyubiquitin chains through lysine (K)63 of ubiquitin (Deng et al., 2000). Unlike K48-linked ubiquitination, this modification is not associated with proteosomal degradation. Instead, K63-linked polyubiquitination has been associated with various processes such as DNA repair, vesical trafficking and protein kinase activation (Sun and Cheng, 2004). IRAK2 plays a central role in the activation of TRAF6 (Keating et al., 2007). Interaction of IRAK2 and TRAF6 triggers the polyubiquitination of TRAF6, a process involving autoubiquitination by TRAF6 (Keating et al., 2007).

Activated TRAF6 then associates with transforming growth factor- β (TGF- β) activating kinase (TAK1) and its regulators, the TAK1 binding proteins, TAB1 and TAB2. TAK1 is an ubiquitin-dependant kinase, which is activated by K63 ubiquitination using the E3 ligase activity of TRAF6 (Wang et al., 2001). The inhibitory (I)- κ B Kinase (IKK) complex is made up of two protein kinases, IKK α and IKK β , and a scaffolding protein IKK γ which is essential for regulation and activation of the complex (Rothwarf et al., 1998). Ubiquitinated TAK1 phosphorylates IKK β and activates the IKK complex (Shim et al., 2005). In the unstimulated cell NF- κ B is found in association with I κ B proteins. The activated IKK complex phosphorylates

these I κ B proteins marking them as targets for K48-linked ubiquitination and subsequent degradation in the 26S proteosome (Hayden and Gosh, 2004). NF- κ B is then free to bind to κ B sites in the promoter regions of its target genes, including genes encoding pro-inflammatory cytokines and chemokines.

Mitogen activating protein kinases (MAPK)s are a group of intracellular signal transducing enzymes (Barr and Bogoyevitch, 2001). They are serine/threonine kinases that are activated sequentially in response to a stimulus, such as stress or microbial infection. The MAPK is phosphorylated and activated by a MAPK kinase (MAPKK or MAP2K) which is phosphorylated and activated by a MAPKK kinase ((MAPKKK or MAP3K). TAK1 is a MAP3K which can phosphorylate and activate MKK3 and MKK6 (Moriguchi et al., 1996). These MAP2Ks in turn phosphorylate and activate MAPKs JNK and p38. MAPK activation leads to subsequent activation of the transcription factor activator protein (AP)1. AP1 co-operates with NF- κ B to regulate gene expression.

1.4.2 The MyD88-independent pathway

NF- κ B and AP-1 can also be activated by a MyD88-independent pathway, involving the TIR domain containing adaptor protein TRIF. TRAM acts as a bridging adaptor for TLR4 and TRIF (Oshiumi et al., 2003), while TRIF interacts directly with TLR3. Following recruitment TRIF interacts with receptor interacting protein 1 (RIP1) (Meylan et al., 2004). RIP1 interacts with the C-terminal domain of TRIF via a RIP homotypic interaction motif. Furthermore, the N-terminal region of TRIF contains three characteristic TRAF6 binding motifs and TRAF6 has been implicated in TRIF-mediated NF- κ B activation in some cell types (Gauzzi et al., 2010). Activation of RIP1 or TRAF6 leads to downstream activation of TAK1. As in MyD88-dependent signalling TAK1 then activates the IKK complex and MAPKs. The activated IKK complex phosphorylates I κ B proteins marking them as targets for ubiquitination and proteosomal degradation which releases NF- κ B. MAPK activation leads to subsequent activation of the transcription factor AP-1. NF- κ B and AP-1 regulate the expression of genes encoding proinflammatory cytokines and chemokines.

MyD88-independent signalling also leads to the activation of members of the IRF family of transcription factors, IRF3 and IRF7. TRIF is first recruited to the

receptor which leads to the recruitment of TRAF3 (Matsumoto and Seya, 2008). This leads to subsequent recruitment and activation of tank binding kinase 1 (TBK1) and inducible IKK (IKKi). NF- κ B-activating kinase (NAK)-associated protein 1 (NAP1) also plays a role in the recruitment of these kinases (Sasai et al., 2005). TBK1 and IKKi phosphorylate key serine/threonine residues in the C-terminal region of IRF3 and IRF7 (Sharma et al., 2003). Phosphorylation of IRFs promotes their dimerisation and nuclear translocation and allows for interaction with the transcriptional co-activators cAMP responsive element binding protein (CBP)/p300 (Gauzzi et al., 2010). CBP/p300 acetylates IRF3 homodimers, which causes a conformational change that unmasks its DNA binding domain (Lin et al., 1999). IRF3 and IRF7 bind to the promoter regions and upregulate the expression of genes encoding Type I IFNs.

1.4.3 NF- κ B

NF- κ B is a key regulator of inflammation and cell survival. It was discovered in 1986 and has been extensively studied since this time (Sen and Baltimore, 1986). There are five mammalian NF- κ B/Rel proteins to date, p65 (RelA), NF- κ B1 (p50 and precursor p105), NF- κ B2 (p52 and precursor p100), c-Rel and RelB (Albert and Baldwin, 1996), each containing a conserved 300 amino acid N-terminal domain. This domain is termed the rel homology (RH) domain and is responsible for nuclear translocation, DNA-binding and dimerisation of Rel/NF- κ B family members (Ghosh et. al, 1998). RelB, c-Rel and p65 also possess a C-terminal transactivation domain. Rel subunits form homo- or heterodimers and the various dimeric combinations formed target slightly different DNA sequences allowing for distinct transcriptional activity of different NF- κ B dimers (Kunsch et al., 1992). The predominant form of NF- κ B is a p65-p50 heterodimer. Due to the lack of a C-terminal transactivation domain p50 and p52 homodimeric complexes cannot activate transcription but instead act as transcriptional repressors (Hayden and Ghosh, 2004). NF- κ B regulates the expression of a range of proteins including proinflammatory cytokines, such as IL-1 β , IL-8, IL-6 and TNF α , adhesion molecules, such as intracellular adhesion molecule (ICAM)-1 and vascular cellular adhesion molecule (VCAM)-1, and inducible enzymes such as inducible nitric oxide synthase (iNOS) which mediate the innate immune response to the invading pathogen (Pahl, 1999). Furthermore, NF- κ B regulates genes encoding co-stimulatory molecules, MHC and cytokines required for

activation and regulation of the adaptive immune system (Moynagh, 2005). NF- κ B can also promote cell survival by inducing the expression of anti-apoptotic proteins (Li and Verma, 2002).

In the unstimulated cell, NF- κ B is mainly cytoplasmic and is found in association with members of the I κ B family. I κ B proteins bind to the NF- κ B dimer through multiple ankyrin repeats and inhibit their DNA-binding activity (Whiteside and Israel, 1997). Cytoplasmic I κ B proteins, I κ B α , I κ B β and I κ B ϵ , were originally thought to sequester NF- κ B in the cytoplasm by masking its nuclear localisation sequences (NLS)s. I κ B α , however, only blocks one NLS of the NF- κ B dimer and the complexes maintain the ability to translocate to the nucleus (Malek et al., 2001). I κ B α encodes an N-terminal nuclear export signal (NES) which is responsible for shuttling the I κ B α -NF- κ B complexes out of the nucleus (Huang et al., 2000). Similarly, I κ B ϵ -NF- κ B complexes have been reported to shuttle between the nucleus and the cytoplasm (Lee and Hanink, 2002). I κ B β , in contrast, masks both NLSs of the NF- κ B dimer and thus sequesters the transcription factor in the cytoplasm (Malek et al., 2001). The N-terminal region of I κ B proteins is phosphorylated on key serine residues by the IKK complex (Yamamoto and Gaynor, 2004). Phosphorylated I κ Bs are targets for the ubiquitin ligase machinery of the cell and are polyubiquitinated and subsequently degraded by the 26S proteosome. The p65-p50 heterodimer is primarily bound by I κ B α which is rapidly degraded in response to NF- κ B activating stimuli. Active NF- κ B promotes the expression of I κ B α which is an important negative feedback regulatory mechanism which ensures termination of the NF- κ B response (Klement et al., 1996). I κ B β and I κ B ϵ are also degraded and resynthesized in response to NF- κ B signalling but with delayed kinetics when compared with I κ B α (Hoffmann et al., 2002, Kearns et al., 2006).

The NF- κ B precursor proteins p100 and p105 have C-terminal ankyrin repeats in addition to their RH domains and serve as I κ B proteins. p100 and p105 are processed, resulting in the removal of their C-terminal ankyrin repeats. p105 is constitutively processed to p50 resulting the presence of both p50- and p105-containing dimers, while p100 processing to p52 is stimulus dependent (Oeckinghaus and Ghosh, 2009). p100 preferentially binds RelB and following stimulus dependent ubiquitination and processing to p52, the RelB-p52 complex induces genes essential for B-cell development and lymphoid organogenesis (Moynagh, 2005). A number of ankyrin repeat containing proteins found in the nucleus and termed nuclear I κ Bs

regulate NF-κB transactivation. However, the nuclear IκBs characterised to date, including Bcl-3, IκB ζ and IκBNS, interact with NF-κB dimers and have been shown to both positively and negatively regulate the expression of NF-κB inducible genes (Yamamoto and Takeda, 2008).

1.4.4 The Interferon Regulatory Factors (IRFs)

The IRF family consist of nine members (IRF1-9) which all possess an N-terminal DNA binding domain (DBD) and a C-terminal IRF association domain (IAD) (Savitsky et al., 2010). They regulate the expression of Type I IFNs which are a family of cytokines involved in antiviral defence, immune activation and cell growth and regulation (Goodbourn et al., 2000). Type I IFNs prevent viral replication and limit the spread of infection. Secreted IFNs bind to common cell surface interferon receptors (IFNAR)s on surrounding infected and uninfected cells. This causes the activation of endoribonucleases that degrade viral RNA. Furthermore, PKR kinase is activated which phosphorylates the eukaryotic protein synthesis initiation factor eIF-2, preventing the translation of viral RNA. Type 1 IFN production also promotes the activation of cytotoxic T cells, by increasing the cell surface expression of MHC class I molecules on surrounding infected cells, and innate NK cells which kill virus infected cells (Janeway et al., 2005).

Type I IFNs are encoded by one IFN β gene and several IFN α genes. Of the nine family members, IRF3 and IRF7 are the most important in terms of regulation of Type I IFNs in response to viral infection (Taniguchi et al., 2001). IRF3 and IRF7 are activated by TLR-signalling pathways and also by the cytoplasmic viral sensing RLRs, RIG-I and MDA5 (Kawai et al., 2005). When activated they bind to IFN-stimulatory response elements (ISRE) found in the promoters of Type I IFNs and other genes involved in immunity and oncogenesis (Honda and Taniguchi, 2006). IRF3 is expressed at high constitutive levels in most cells and is responsible for the initial induction of IFN β in response to infection (Taniguchi et al., 2001). TBK1- or IKK α -mediated phosphorylation of IRF3 promotes its dimerisation and nuclear translocation (Sharma et al., 2003). IRF3 then co-operates in a multiprotein transcription enhancer called the enhanceosome to activate the transcription of IFN β (Panne, 2008). The enhanceosome requires the transcription factors IRF3 or IRF7, NF-κB and ATF-2/c-Jun which bind to positive regulatory domains (PRD)s in the

IFN β promoter. Furthermore, architectural protein HGM1 is required for the recruitment of histone acetyl transferases CBP/p300 to acetylate lysine residues of histone, leading to subsequent chromatin remodelling which is essential for efficient transcription of IFN β (Noppert et al., 2007).

IFN β is produced and binds to the cell surface IFN receptor termed IFNAR. Stimulation of the IFNAR results in the activation of Jak/STAT pathway (Stark et al., 1998). The IFNAR is made up of two major subunits, IFNAR1 and IFNAR2 (Mogensen et al., 1999). In the unstimulated cell IFNAR is found in association with the Janus tyrosine kinase Tyk2 (Colamonici et al., 1994), while the IFNAR2 is associated with Jak1 and signal transducer and activator of transcription (STAT)2 (Li et al., 1997). Upon IFN β binding, IFNAR1 and IFNAR2 interact facilitating transphosphorylation and activation of Tyk2 and Jak1 (Novick et al., 1994). Activated Tyk1 then phosphorylates IFNAR1 creating a new binding site for STAT2 (Yan et al., 1996). STAT2 is phosphorylated and subsequently recruits STAT1 which in turn is phosphorylated (Shaui et al., 1993). The activated STAT1/STAT2 heterodimer translocates to the nucleus where it interacts with IRF9 to form the transcription factor complex IFN-stimulated gene factor 3 (ISGF3) (Tanaguchi et al., 2001). IRF7, which is expressed at low levels in most cells is strongly induced by ISGF3 (Genin et al., 2003). IRF7 is also phosphorylated in its C-terminal region, in a manner similar to IRF3, which promotes its dimerisation and translocation to the nucleus. IRF7 is responsible for IFN α and IFN β production in the late phase of the IFN response. The positive feedback mechanism described above ensures the efficient induction of IFNs in response to viral infection. However IRF7 and IRF3 are subject to ubiquitin-dependent proteosomal degradation (Yu and Hayward, 2010) which ensures that the production of Type I IFNs only occurs for a short time, while the viral infection persists.

1.4.5 Activating Protein (AP)-1

AP-1 is a dimeric transcription factor composed of subunits from Fos, Jun, activating transcription factor (ATF) and the Maf subfamilies and it binds to TPA-response or cAMP-response elements in its target genes (Shaulian and Karin, 2002). AP-1 regulates a wide range of cellular processes including cell proliferation, cell survival and differentiation. TLR-signalling leads to the activation of MAPKs c-Jun

N-terminal kinase (JNK), p38 and extracellular signal regulated kinase (ERK) which phosphorylate and activate the subunits of the AP-1 transcription factor. Among the AP-1 subunits c-Jun, which is phosphorylated and activated by JNK, is considered the most important in terms of regulation of pro-inflammatory cytokines (Kawai and Akira, 2006). In response to some stimuli phosphorylated JNKs can also translocate to the nucleus where they phosphorylate c-Jun thereby increasing its transcriptional activity (Robinson et al., 2001). The various dimeric combinations of different AP-1 subunits govern the genes they regulate (Johnson and Lapadat, 2002). Furthermore the activation of differential subunits can be stimulus dependent. For example CpG stimulation of TLR9 induces JNK and p38 activation leading to subsequent phosphorylation of c-Jun and ATF2 and the upregulation of TNF- α and IL-12 (Hacker et al., 1998) whereas Pam3Cys stimulation of TLR2 enhances c-Fos activation and the increased expression of IL-10 (Dillon et al., 2004).

1.5 Negative Regulators of TLR-signalling

TLRs are essential for the detection of invading pathogens and the activation of innate and adaptive immune responses. However dysregulation of these pathways can be detrimental to the host and TLRs have been implicated in a wide range of autoimmune and inflammatory diseases. Most significantly, an uncontrolled inflammatory response to LPS during bacterial infection can lead to systemic inflammation and septic shock resulting in multiple organ failure and death. TLR4 is central to the progression of sepsis and TLR4-deficient mice are hyporesponsive to LPS and are thus protected from septic shock (Hoshino et al., 1999). Furthermore, MyD88-deficiency and IRAK4-deficiency also confer some resistance to sepsis in response to LPS (Weighardt et al., 2002, Suzuki et al., 2002). LPS antagonists, such as synthetic E5564, prevent the activation of TLR4 by LPS and thus represent possible novel treatments for septic shock (Mullarkey et al., 2003).

TLRs have also been implicated in other diseases. MyD88-deficient mice are less prone to the development of atherosclerosis (Michelsen et al., 2004). Furthermore, TLR4 is highly expressed in endothelial cells of human atherosclerotic lesions but poorly expressed in normal human arteries (Edfelt et al., 2002). TLR3 has also been implicated in the pathogenesis of some diseases. TLR3-deficient mice are more resistant to severe encephalitis caused by lethal West Nile virus (WNV) (Wang

et al., 2004). In this case the activation of TLR3 leads to the production of pro-inflammatory cytokines and disruption of the blood-brain barrier. TLRs also play a central role in the development of chronic inflammatory disorders such as asthma (Eisenbarth et al., 2002) and rheumatoid arthritis (RA) (Pierer et al., 2004). The recognition of self nucleic acids by TLR7 and TLR9 has also been implicated in the development of experimental lupus (Pawar et al., 2007). In fact most autoimmune and inflammatory diseases are likely to involve TLRs at some stage of disease progression, either by the initiation of inflammation or via the activation of an adaptive immune response.

As prolonged activation of TLR responses is detrimental to the host TLR signalling is tightly regulated at various stages (Figure 1.2). Some regulators of TLRs are induced by TLR ligands or cytokines as a regulatory mechanism to ensure that the response to the invading pathogen is transient. Meanwhile, other inhibitors are constitutively expressed and ensure that an immune response is not activated in the absence of a stimulus.

1.5.1 Soluble decoy TLRs

One regulatory mechanism employed by TLRs is the release of soluble or decoy TLRs which prevent PAMP recognition and thus attenuate the TLR response to microbial ligands. There is a single copy of the *TLR4* gene in mammals but several mRNA products have been detected (Qureshi et al., 1999). One of these isoforms in mice encodes a soluble form of TLR4 (Iwami et al., 2000). This soluble TLR4 was found to attenuate LPS mediated NF-κB activation and TNF-α production in a mouse macrophage cell line. Furthermore, it was found to be upregulated following LPS stimulation suggesting that it acts as a regulatory feedback mechanism to inhibit an excessive response to LPS (Iwami et al., 2000).

A similar mechanism might also exist in humans. A recombinant sTLR4 was found to inhibit LPS mediated NF-κB activation and IL-8 production in human embryonic kidney (HEK) 293 cells (Hyakushima et al., 2004). Furthermore, sTLR4 was found to interact with MD-2, which is essential for TLR4 recognition of LPS. These soluble TLR4s represent a potential therapy for suppressing the immune response to LPS and the prevention of septic shock (Hyakushima et al., 2004).

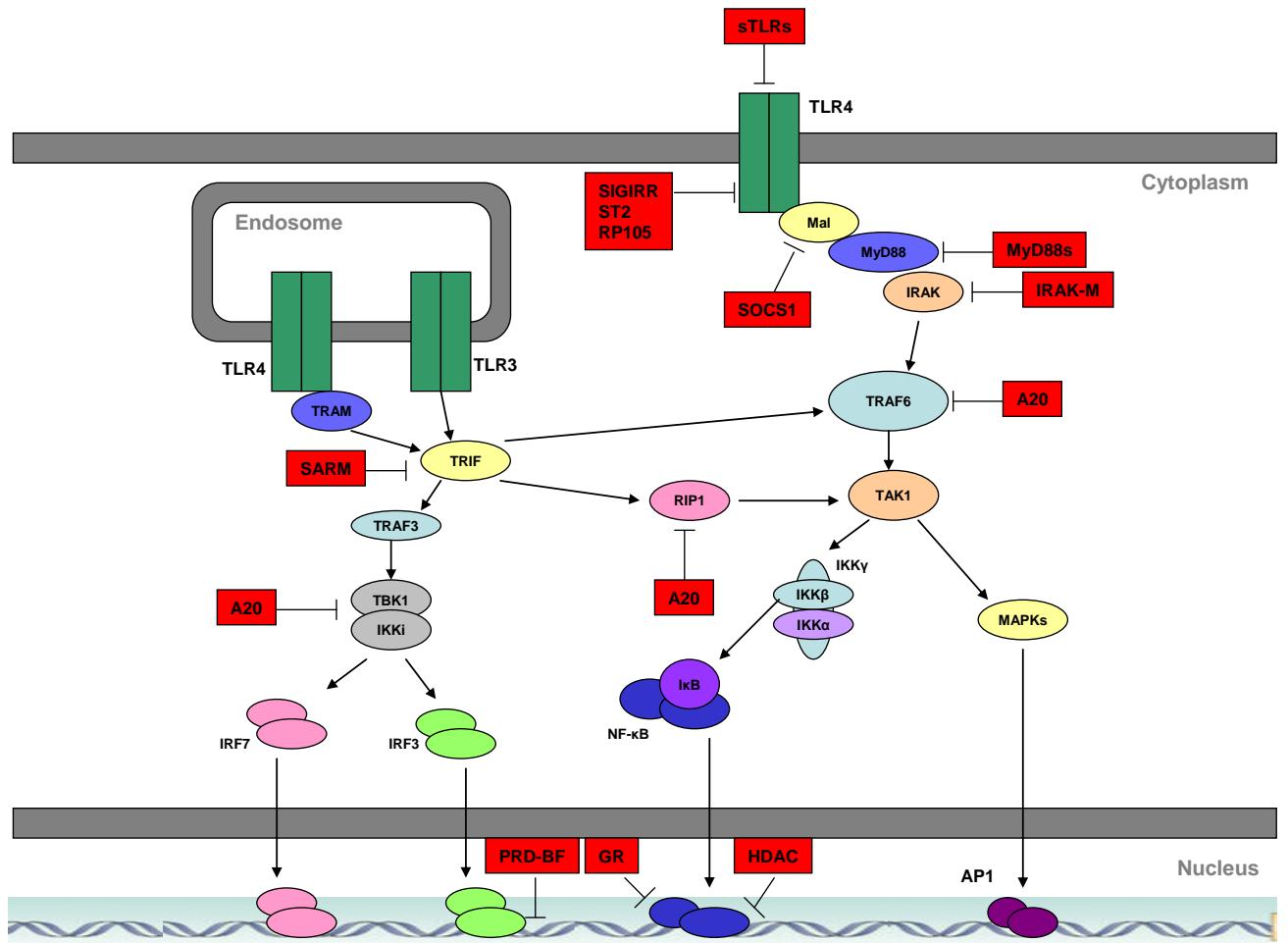


Figure 1.2 Negative regulators of TLR signalling

Soluble TLRs prevent ligand binding and activation of TLR signalling pathways. TIR domain containing transmembrane receptors, SIGIRR, ST2 and RP105, block MyD88-dependent signalling to NF- κ B. A range of intracellular regulators target multiple levels of the signalling cascade. Finally, transcription factor transactivation is regulated by nuclear regulators of TLR signalling.

Six soluble isoforms of TLR2 are produced due to post-translational modification in an intracellular compartment, creating an intracellular pool of sTLR2 (LeBourder et al., 2003). This suggests that sTLR2 might serve as an important first-line regulatory mechanism preventing an uncontrolled response to bacterial infection. These soluble TLR2s are naturally present in blood and breast milk and are released by monocytes in response to bacterial infection. sTLR2 attenuated TNF- α and IL-8 production in response to a synthetic triacylated bacterial lipopeptide, Pam3Cys, in a human monocytic cell line. This inhibition is likely due to its association with the co-receptor CD14 (LeBouder et al., 2003).

1.5.2 Transmembrane negative regulators

Some negative regulators are membrane bound and prevent downstream signalling from TLRs. ST2 is present in two forms, ST2L and sST2, due to mRNA splicing (Berger et al., 1994). ST2L is a type 1 transmembrane receptor with extracellular Ig-like domains and an intracellular TIR domain. ST2L is preferentially expressed on $T_{H}2$ cells and has been implicated in $T_{H}2$ cell effector functions (Lohning et al., 1998). IL-33 is a member of the IL-1 family which favors $T_{H}2$ responses and is recognised by a receptor complex involving ST2L and IL-1R accessory protein (IL-1RAcP) (Schmitz et al., 2005). ST2L can drive IL-33 mediated NF- κ B and MAPK activation in $T_{H}2$ cells in vitro (Schmitz et al., 2005). However, ST2L has also been implicated as a negative regulator of NF- κ B activation. Overexpression of ST2L failed to activate NF- κ B in murine thymoma EL4 cells (Brint et al., 2002). Furthermore, ST2L can interact with MyD88 and Mal preventing their interaction with downstream regulators of the signalling pathway (Brint et al., 2004). Thus, ST2L acts as a negative regulator of MyD88-dependent but not MyD88 independent pathways and ST2L-deficient mice show enhanced production of pro-inflammatory cytokines in response to IL-1 and LPS but not the TLR3 ligand Poly(I:C) (Brint et al., 2004). However, ST2L-deficient mice were no more susceptible to septic shock than wild-type mice. This is because ST2L is not constitutively expressed on the cell surface. The expression of ST2L was detected on the cell surface of macrophages following 4 h stimulation with LPS (Brint et al., 2004). So this appears to be an autoregulatory mechanism to prevent the prolonged activation of NF- κ B in response to microbial infection.

The soluble form of ST2, sST2, is also a negative regulator of inflammation which is upregulated by proinflammatory cytokines and LPS (Kumar et al., 1997, Saccani et al., 1998). sST2 competes with ST2L for IL-33 binding and negatively regulates cytokine production by T_H2 cells (Hayakawa et al., 2007). sST2 is present in normal human serum but its levels are elevated in autoimmune diseases such as SLE (Kuroiwa et al., 2001) and asthma (Oshikawa et al., 2001). sST2 binds to its receptor on bone marrow derived macrophages (BMDMs). This receptor is upregulated by LPS stimulation. Administration of sST2 *in vivo* attenuated LPS-mediated IL-6, IL-12 and TNF- α production and reduced the toxic effects of LPS (Sweet et al., 2001). Furthermore, sST2 downregulated the mRNA expression of TLR4 and TLR1 in LPS stimulated macrophages (Sweet et al., 2001).

Single-immunoglobulin interleukin-1 receptor –related (SIGIRR) is another TIR domain containing receptor which negatively regulates TLR signalling. SIGIRR is an orphan receptor with a single extracellular Ig-like domain and an intracellular TIR domain (Thomassen et al., 1999). The extracellular domain of SIGIRR interferes with the interaction of the IL-1R with IL-1RAcP and inhibits downstream signalling to NF- κ B (Qin et al., 2005). Furthermore the TIR domain of SIGIRR interacts with MyD88, IRAK1 and TRAF6 (Qin et al., 2005) thus preventing their recruitment by TLRs and the IL-1R. By this mechanism SIGIRR inhibits MyD88-dependent NF- κ B activation. Primary kidney cells from SIGIRR-deficient mice showed enhanced NF- κ B activation in response to IL-1 and LPS but not TNF α (Wald et al., 2003). This is not unexpected as the TNF receptor TNFR does not utilise MyD88. SIGIRR is highly expressed in the gut (Liew et al., 2005). This suggests that it may have a role in the suppression of intestinal inflammation in response to commensal bacteria. The expression of SIGIRR is down-regulated following LPS stimulation (Polentarutti et al., 2003) to allow for an immune response to invading pathogen.

TNF-related apoptosis-inducing ligand receptor (TRAILR) is a member of the TNF superfamily and is the receptor for TRAIL, another cell surface transmembrane protein. TRAIL-R induces apoptosis in transformed cancer cells but not non-transformed cells (Wu et al., 1999). TRAILR also has a role in the regulation of TLR-signalling. Cells from TRAILR-deficient mice showed enhanced cytokine production in response to stimulation by TLR2, TLR3 and TLR4 ligands but not the TLR9 ligand CpG DNA (Diehl et al., 2004). TRAILR has no effect on early TLR signalling events and, although its mechanism of action is unknown, it appears to affect the stabilisation

of I κ B α leading to decreased nuclear translocation of NF- κ B at later time points (Diehl et al., 2004).

Finally RP105 is a TLR4 homologue which has a similar extracellular and transmembrane domain but lacks an intracellular signalling domain. It has a similar expression pattern to TLR4 and its cell surface expression is dependent on the co-expression of MD-2 homologue MD-1 (Divanovic et al., 2005). RP105 knockout mice show enhanced production of pro-inflammatory cytokines in response to LPS. RP105 interacts directly with the TLR4-MD2 receptor complex and inhibits the complex binding to LPS (Divanovic et al., 2005).

1.5.3 Intracellular negative regulators

Intracellular regulators of TLR-signalling target various stages in the signalling pathway to inhibit the activation of transcription factors in response to TLR ligands. One such regulator is a splice variant of MyD88, termed MyD88s. This protein, like full length MyD88, has a C-terminal TIR domain and an N-terminal DD but lacks the small intermediate domain (ID) (Janssens et al., 2002). MyD88s is preferentially expressed in the spleen and is up-regulated in monocytes in response to LPS stimulation (Janssens et al., 2002). MyD88s recruits IRAK1 but not IRAK4 to IL-1R/TLRs (Burns et al., 2003). This prevents IRAK4-mediated IRAK1 phosphorylation and prevents downstream signalling events from IRAK1. MyD88s negatively regulates IL-1- and LPS- but not TNF-mediated NF- κ B activation (Janssen et al., 2002). Interestingly, however, the overexpression of MyD88s activates AP-1 (Janssens et al., 2003). This suggests that these signalling pathways may diverge at the level of MyD88 and suggests the importance of mRNA splicing in the regulation of TLR responses. A chemically modified antisense oligonucleotide (ASO) has been shown to decrease MyD88 expression and increase MyD88s expression thus attenuating MyD88-dependent signalling to NF- κ B (Vickers et al., 2006). This might prove an interesting potential therapy for inflammatory diseases.

Sterile α - and armadillo -motif containing protein (SARM) is a TIR domain containing adaptor protein. SARM has a C-terminal TIR domain and two α -motif (SAM) domains and heat-armadillo motifs in its N-terminal region (O'Neill et al., 2003). SARM interacts with TRIF and inhibits TRIF-mediated NF- κ B, IRF3 and IRF7 activation (Carty et al., 2006). Both the TIR domains and SAM domains are

required for this inhibitory effect. The protein expression of SARM is enhanced following LPS stimulation (Carty et al., 2006). In addition, SARM negatively regulates both TRIF- and MyD88-dependent AP-1 activation (Peng et al., 2010). Interestingly, however, SARM-deficiency in mice had no effect on the cytokine production of macrophages treated with TLR ligands (Kim et al., 2007). Murine SARM was found to be highly expressed in the brain where it co-localised with JNK3 and it appears to have role in the stress response in neurons (Kim et al., 2007). It is possible, however, that SARM has differential functions in mice and humans.

The TIR domain containing adaptor protein MyD88 is required for signalling from all TLRs with the exception of TLR3. Interestingly, however, MyD88 can negatively regulate TLR3 mediated IFN β induction (Siednienko et al., 2011). MyD88 inhibits IKK α - but not TBK1-mediated IRF3 activation and inhibits subsequent IFN β induction (Siednienko et al., 2011). Similarly, an inhibitory role for TIR domain containing adaptor Mal in TLR3 signalling has also been proposed (Kenny et al., 2009). Mal can interact directly with IRF7, but not IRF3, and inhibit IFN β induction by preventing IRF7 from binding to a positive regulatory domain in the IFN β promoter (Siednienko et al., 2010).

IRAK-M is a member of the IRAK family, but unlike other family members it is a negative regulator of TLR-signalling. IRAK-M was originally thought to be expressed preferentially by cells of monocytic lineage but IRAK-M expression has also been detected in other cells such as epithelial cells (Harada et al., 2006). IRAK-M prevents the dissociation of IRAK from MyD88 and the downstream formation of IRAK-TRAF6 complexes (Kobayashi et al., 2002). IRAK-M-deficient mice show enhanced cytokine production and inflammatory response to bacterial infection compared with wild type mice (Kobayashi et al., 2002). LPS induces the expression of IRAK-M and LPS-tolerance was reduced in IRAK-M-deficient mice (Kobayashi et al., 2002). IRAK-M appears to have an important regulatory role in sepsis and increased IRAK-M expression has been observed in monocytes isolated from patients with sepsis (Escoll et al., 2003). Though the mechanism of action is unknown IRAK-M appears to regulate chromatin remodelling which downregulates the expression of pro-inflammatory mediators during sepsis (Lyn-kew et al., 2010).

Splice variants of IRAK2 and IRAK1 can also act as negative regulators of TLR signalling. Overexpression of IRAK2a and IRAK2d, which both lack DDs, inhibits LPS-mediated NF- κ B activation (Hardy et al., 2004). Furthermore, a splice

variant of IRAK1, IRAK1c, negatively regulates NF- κ B activation in response to LPS and CpG (Rao et al., 2005). IRAK1c lacks kinase activity and is not phosphorylated by IRAK4 but still interacts with IRAK2, MyD88 and TRAF6 and prevents downstream signalling to NF- κ B. IRAK1c is highly expressed in the brain, while IRAK1 is not, suggesting that IRAK1c may be crucial for maintaining a non-inflammatory state in the brain (Su et al., 2007).

Toll interacting protein (Tollip) was first identified in a yeast 2 hybrid screen using the cytoplasmic tail of IL-1AcP as bait and was found to inhibit IL-1 β -mediated NF- κ B and JNK activation (Burns et al., 2000). Tollip also associates with TLR2 and TLR4 and its overexpression inhibits MyD88-dependent signalling to NF- κ B (Zhang and Ghosh, 2002). When IRAK1 is recruited to the receptor complex it is in association with Tollip. Hyperphosphorylation of IRAK1 following its recruitment leads to its dissociation from Tollip and interaction with downstream signalling protein TRAF6 (Takeda and Akira, 2004). IRAK1 also phosphorylates Tollip which may facilitate its dissociation from IRAK1 (Zhang and Ghosh, 2002). IRAK1 and Tollip are both targets for the ubiquitin ligase machinery of the cell and following ubiquitination they are subsequently degraded in the proteosome and their dissociation may facilitate this process (Zhang and Ghosh, 2002). The N-terminal of Tollip has a C2 domain which mediates its interaction with products of phosphatidylinositol-3 kinase (PI3K), phosphatidylinositol-3-phosphate and phosphatidylinositol-3,4,5-phosphate, and this interaction appears to be important for its negative regulatory role in TLR-signalling (Li et al., 2004). Tollip is also required for efficient degradation of IL-1R by trafficking it to late endosomes (Brissoni et al., 2006). Interestingly, however, Tollip-deficient mice showed no enhancement of IL-1 β - or LPS-mediated NF- κ B or MAPK activation (Didierlaurant et al., 2006). Furthermore, the production of cytokines IL-6 and TNF α by these mice was reduced compared to wildtype mice following stimulation with IL-1 β and low doses of LPS (Didierlaurant et al., 2006). So it appears the situation is more complex than originally thought and Tollip may regulate the extent of cytokine production following TLR and IL-1R activation.

The suppression of cytokine signalling (SOCS) family consists of eight family members which all possess a central Src homology 2 (SH2) domain which binds to phosphotyrosine motifs, a variable N-terminal domain and a conserved C-terminal domain known as the SOCS box (Piessevaux et al., 2008). The SOCS box mediates the formation of complexes that function as E3 ubiquitin ligases, through association

with other proteins such as Elongin C and Cullin-2 (Piessevaux et al., 2008). SOCS1 and SOCS3 possess an additional kinase inhibitory region (KIR) in their N-terminus (Dalpke et al., 2008). SOCS proteins are induced by cytokines and inhibit Jak/STAT signalling pathways that are employed by cytokine receptors. SOCS are also induced by TLR4 and TLR9 ligands LPS and CpG DNA (Dalpke et al., 2008). SOCS1 interacts with phosphorylated Mal, leading to its ubiquitination and subsequent degradation and inhibition of TLR2 and TLR4 signalling to NF- κ B (Mansell et al., 2006). SOCS1 has also been implicated in the ubiquitination and proteosomal degradation of p65-containing NF- κ B dimers (Ryo et al., 2003). Furthermore, overexpression of SOCS1 inhibits the phosphorylation of STAT1 which is involved in Type-1 IFN signalling (Baetz et al., 2004). SOCS1, therefore, indirectly down-regulates TLR-mediated IFN α/β production.

The zinc finger protein A20 was identified as the product of a gene that is rapidly induced following TNF- α stimulation (OPIPARI et al., 1990). The gene coding for A20 was found to be up-regulated by NF- κ B (Krikos et al., 1992). A20-deficient mice show increased production of inflammatory cytokines in response to TNF α and LPS (Boone et al., 2004). A20 also appears to confer some resistance to septic shock (Boone et al., 2004). Furthermore, A20 is phosphorylated and activated by the IKK complex subunit IKK β indicating a negative feedback loop to terminate NF- κ B signalling (Hutti et al., 2007). The N-terminal region of A20 contains a de-ubiquitinating domain while the C-terminus acts as an ubiquitin ligase (Wertz et al., 2004). A20 cleaves K63 linked polyubiquitin chains from TRAF6 thus inhibiting the downstream activation of TAK1 and subsequent NF- κ B activation (Boone et al., 2004). Similarly, A20 cleaves K63 linked ubiquitin chains from RIP1. A20 then catalyses K48-linked polyubiquitination which leads to subsequent proteosomal degradation of RIP1 (Wertz et al., 2004). Furthermore A20 interacts with TBK1 and IKK-i and inhibits phosphorylation and activation of IRF3 (Saitoh et al., 2005).

These negative regulators, along with others, regulate the signalling pathways leading to transcription factor translocation to the nucleus. However, these transcription factors can also be regulated at a nuclear level.

1.5.4 Nuclear regulators of TLR-signalling

The transcriptional activity of NF- κ B is regulated by transcriptional co-activators and transcriptional co-repressors. The transcriptional activators include p300/CBP, p106 proteins (steroid receptor-co-activator- (SRC)-1-3) and p300/CBP-associated factor (p/CAF). These co-activators are also important for the transactivation of nuclear receptors. p300/CBP and p106 proteins both have intrinsic histone acetylase (HAT) activity which is necessary for histone acetylation-dependent chromatin remodelling to allow for gene transcription (Rosenfeld and Glass, 2001). p106 proteins, however, display less HAT activity in the absence of p300/CBP. The NF- κ B subunit p65 is phosphorylated by Protein Kinase A (PKA) which facilitates its interaction with p300/CBP (Zhong et al., 1998). Furthermore, p65 is acetylated by p300/CBP and p/CAF which is essential for full transcriptional activity of NF- κ B and also decreases the binding ability of I κ B α (Chen and Greene, 2002). NF- κ B co-repressors include Histone Deacetylase (HDAC)-1, HDAC2 and HDAC-3. The HDACs de-acetylate p65 which represses its DNA binding activity and increases its affinity for I κ B α . (Ashburner et al., 2001, Chen et al., 2001). HDACs also de-acetylate histone proteins and return them to their previous basal state which represses gene transcription (Barns et al., 2005).

NF- κ B repressor factor (NRF) is a nuclear protein which binds to negative regulatory elements (NRE)s in the IFN β -promoter and mediates constitutive silencing of IFN β (Nourbakhsh and Hauser, 1999). The activation of the IFN β transcription enhanceosome overcomes this repression, although NRF remains bound to DNA (Nourbakhsh et al., 2000). NRF interacts directly with NF- κ B and is therefore known as an active gene repressor. The promoter region for iNOS also has a NRE and NRF inhibits both basal and cytokine induced iNOS gene expression (Feng et al., 2002). Similarly, the promoter region for IL-8 encodes a NRE. Suppression of endogenous NRF leads to spontaneous transcription of IL-8 in the absence of a stimulus (Nourbakhsh et al., 2001). Conversely, mutation of the NRE prevents IL-1 β -mediated IL-8 expression (Nourbakhsh et al., 2001). This suggests a dual role for NRF where it inhibits basal IL-8 production but co-operates in IL-8 transcription following treatment with a pro-inflammatory stimulus. Furthermore, disruption of the NF- κ B/NRF interaction, with a synthetic peptide that targets the Rel binding central

domain of NRF, decreased the expression of IL-8 in response to bacterial infection (Bartels et al., 2007).

A range of other proteins also regulate transcription factors in the nucleus. Glycogen synthase kinase (GSK)3- β affects the DNA-binding activity of p65 and is required for efficient expression of some NF- κ B regulated genes (Steinbrecher et al., 2005). Prior to gene transcription the pre-initiation complex (PIC) assembles on the promoter region with RNA Polymerase II, which is subsequently phosphorylated. The glucocorticoid receptor (GR) inhibits NF- κ B regulated gene transcription by interfering with the phosphorylation of RNA Pol II (Nissen and Yamamoto, 2000). PRDI-BF1 is a viral inducible protein that binds to PRD1 of the IFN β -promoter and represses IFN β expression (Keller and Maniatis, 1991). PRD1-BF1 recruits histone H3 methyltransferase G9a to the promoter which mediates chromatin remodelling and gene silencing (Gyory et al., 2004). Furthermore, nuclear I κ B proteins regulate the transactivation of NF- κ B (Yamamoto and Takeda, 2008).

1.6 *Drosophila* Toll

The Toll transmembrane receptor was first identified in *Drosophila Melanogaster* as a protein essential for dorso-ventral patterning in embryo development (Hashimoto et al., 1988). It was subsequently shown to play a critical role in the immune response to *Aspergillus fumigatus* in *Drosophila* (Lemaitre et al., 1996). This led to the discovery of the human Toll receptor, now known as TLR4 (Medzhitov et al., 1997). There are three NF- κ B/Rel proteins in *Drosophila*, dorsal, dorsal-related immunity factor (DIF) and Relish which regulate the expression of genes encoding immune-response peptides and proteins (Hetru and Hoffmann, 2009). Dorsal and DIF are homologous to c-Rel, p65 and RelB and are found in association with inhibitory Cactus, which is most similar to I κ B α (Huguet et al., 1997). Dif is important in immunity while dorsal plays an essential role in development (Hultmark, 2003). Relish has an N-terminal RH domain and C-terminal ankyrin repeats and is homologous to mammalian p100 and p105 precursor proteins (Hultmark, 2003). Two signalling pathways are responsible for the activation of these Rel/NF- κ B transcription factors, the toll pathway and the immune deficiency (Imd) pathway (Figure 1.3). The Toll pathway is generally activated in response to gram positive bacteria and fungal pathogens while the Imd pathway is activated in response to gram negative bacterial

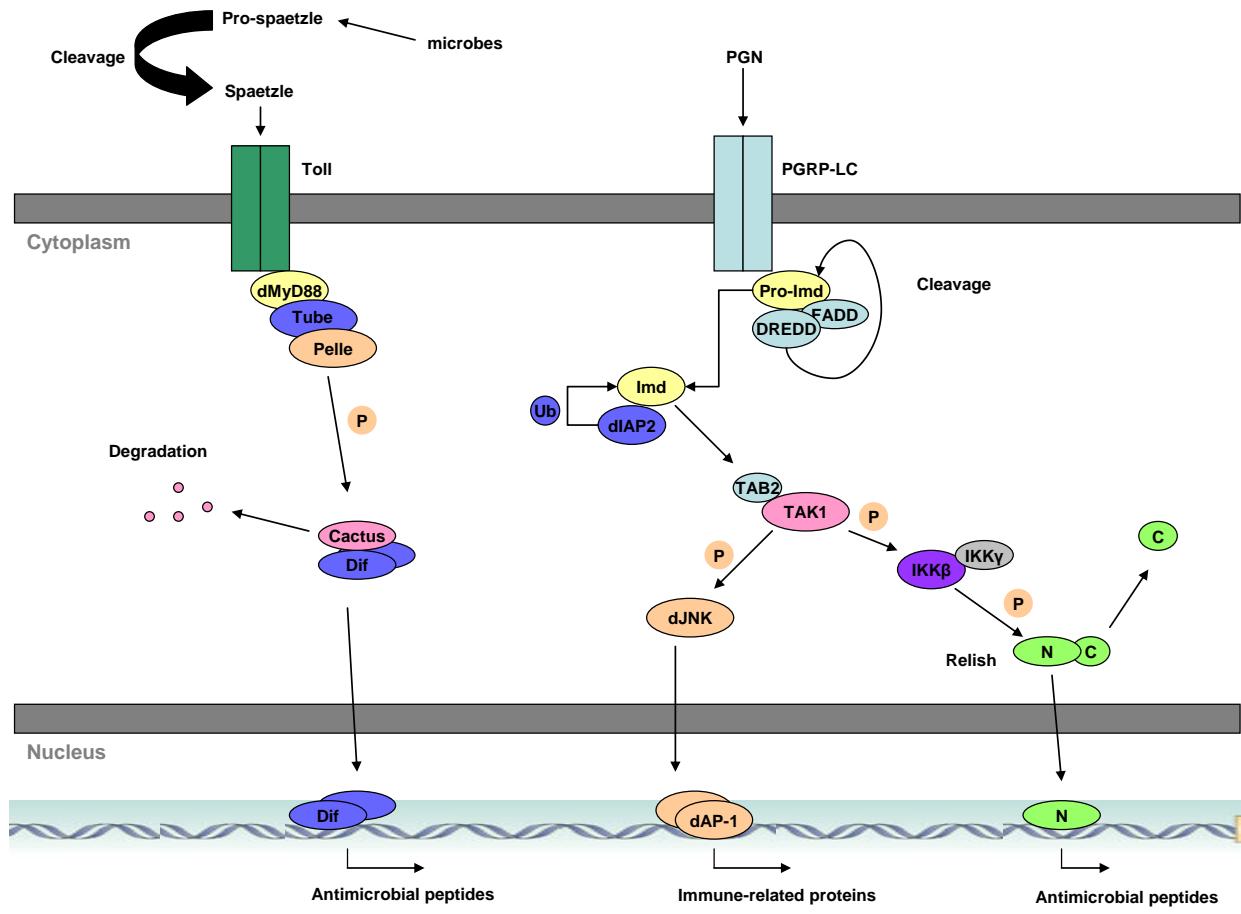


Figure 1.3 *Drosophila* Toll and Imd pathways

The presence of gram positive bacteria or fungal pathogens initiates a proteolytic cascade resulting in the cleavage of pro-Spaetzle. Cleaved Spaetzle binds and activates Toll leading to the recruitment of the dMyD88/Tube complex. This recruits Pelle which is subsequently activated. This triggers downstream phosphorylation of Cactus by an unknown kinase and its ubiquitin-dependent proteasomal degradation. Free Dif then translocates to the nucleus and up-regulates the expression of antimicrobial peptides. PGN binds directly to the PGRP-LC leading to the recruitment of Imd. This triggers the recruitment of FADD and DREDD which cleave Imd revealing a IAP association domain. Interaction of Imd with diAP2 catalyses the K63-linked polyubiquitination of Imd. This leads to downstream activation of TAK1 which phosphorylates dJNK and the dIKK complex. The dIKK complex phosphorylates Relish which is subsequently cleaved to release its C-terminal ankyrin repeats. The N-terminal region of Relish translocates to the nucleus and up-regulates the expression of anti-microbial peptides. dJNK activates dAP-1 which can regulate the transcription of immune-related genes.

infection (Hetru and Hoffmann, 2009). There are striking similarities in these signal transduction pathways and mammalian TLR-signalling pathways and they contain many homologous signalling intermediates. For this reason, *Drosophila* has proved to be a useful model organism for studying innate immune signalling pathways.

1.6.1 The Toll signalling pathway

The Toll signalling pathway is activated by peptidoglycan (PGN) found in the cell walls of gram positive bacteria and fungal β -(1,3)-glucan (Michel et al., 2001). These ligands are not recognised directly by the Toll receptor but instead are sensed by secreted peptidoglycan recognition proteins (PGRPs) and β -glucan recognition protein (GNBP) which initiate proteolytic cascades resulting in the cleavage of the cytokine Spaetzle. Cleaved Spaetzle binds to the Toll receptor which triggers Toll dimerization, initiating the intracellular signalling cascade (Weber et al., 2003). Like TLRs, Toll receptors have extracellular LRRs and an intracellular TIR domain. Dimerization of Toll creates a platform for the recruitment of intracellular signalling adaptors. *Drosophila* MyD88 (dMyD88) is recruited to the receptor via interaction with its TIR domain (Horng and Medzhitov, 2001). When dMyD88 is recruited to the receptor it is in association with Tube. Tube and MyD88 interact through homotypic associations of their DDs. Tube then recruits Pelle to the complex, again through association of DDs (Sun et al., 2002). Pelle is the *Drosophila* orthologue of IRAK and is a protein kinase. Recruitment of Pelle to the receptor complex triggers its activation. This leads to subsequent phosphorylation of Cactus by an unknown kinase leading to its ubiquitination and proteosomal degradation (Belvin and Anderson, 1995). The freed transcription factor, Dif, then translocates to the nucleus and regulates gene expression (Bergmann et al., 1996). Dif activates the transcription of genes encoding antimicrobial peptides such as Defensin, Drosomycin, Cecropin and Metchnikowin (Aggarwal and Silverman, 2008).

1.6.2 The Imd signalling pathway

The Imd signalling pathway is triggered in response to gram negative bacteria and results in the activation of Relish. Gram negative bacterial PGN binds directly to a transmembrane receptor PGRP-LC which has an extracellular PGN recognition

domain and intracellular signalling domain (Kaneko et al., 2005). Upon ligand binding PGRP-LC recruits the death domain containing adaptor protein, Imd, to the receptor (Georgel et al., 2001). This protein is similar to mammalian RIP1. Imd then recruits *Drosophila* Fas associating protein with a DD (dFADD) (Naitza et al., 2002), which in turn recruits the *Drosophila* Caspase-8 homologue, Death related ced-3/Nedd2-like protein DREDD (Leulier et al., 2000). This triggers dFADD- and DREDD-dependent cleavage of Imd which reveals a highly conserved IAP binding motif (IBM) which binds *Drosophila* inhibitor of apoptosis protein 2 (diAP2) (Paquette et al., 2010). Cleaved Imd is then rapidly K63-ubiquitinated. diAP is a RING finger protein which most likely functions as an E3 ligase in this process. Notably, Ubc13 and Uev1A are also likely to be involved (Paquette et al., 2010). This then leads to subsequent activation of TAK1. TAK1 is found in association with the homologue of mammalian TAB2 which binds to K63-linked polyubiquitin chains (Kleino et al., 2005). TAK1 activation triggers downstream activation of relish and JNK (Zhuang et al., 2006). Activation of dJNK leads to subsequent activation of dAP1. Relish activation requires the IKK complex which is made up of *Drosophila* homologues of IKK β and IKK γ (Silverman et al., 2000, Rutschmann et al., 2000). Activated IKK phosphorylates Relish on key serine residues. This results in the proteosomal-independent processing of Relish into an active transcription factor. This requires cleavage of Relish after which the N-terminal region translocates to the nucleus and regulates gene expression. This process is likely to require DREDD (Hetru and Hoffman, 2009). Relish up-regulates the expression of genes encoding antimicrobial peptides such as Diptericins, Cecropins, Drosocin and Attacins (Hoffmann and Reichhart 2002).

1.7 Cactin

In 2000 Lin et al. used Cactus as bait in a yeast two hybrid screen and identified a novel *Cactus*-interacting protein termed Cactin. They overexpressed dCactin in a sensitised Cactus background. These *Cactus*^{A2} heterozygous flies display a weak ventralizing phenotype. Interestingly, dCactin enhanced this weak ventralizing phenotype and increased embryonic lethality (Lin et al., 2000). This suggested that dCactin may affect the stability of Cactus, leading to increased degradation of Cactus and increased nuclear translocation of Dorsal (Lin et al., 2000).

Cactus is also found in association with Dif which is an important component of innate immune signalling in *Drosophila*. Due to the similarities observed between the *Drosophila* Toll signalling and TLR signalling cascades it was proposed that the human homologue of Cactin may have a similar role in TLR-signalling pathways and may target I κ B α . For this reason the human homologue of Cactin was cloned in our laboratory. Interestingly, it appears that the function of Cactin is different than that of its *Drosophila* counterpart. While dCactin appears to positively regulate the nuclear targeting of Dorsal, early studies in our laboratory indicated that hCactin negatively regulates the activation of NF- κ B and IRFs. Given the importance of negative regulators in tempering these pathways to prevent hyperinflammatory responses, this thesis explored the modulatory potential of hCactin in innate immune signalling pathways.

1.8 Aims of the project

The overall objectives of this study were to:

- Confirm that Cactin is a negative regulator of NF- κ B, IRF3 and IRF7 and characterise the underlying mechanism(s)
- Determine if Cactin is regulated by TLR-ligands or pro-inflammatory stimuli
- Determine the role of Cactin in MAPK activation
- Identify Cactin-interacting proteins in an attempt to further clarify the molecular mechanism(s) underlying the regulatory effects of Cactin.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents

Reagent	Supplier
Agar	Sigma
Agarose	Promega
Agarose, low melting point	Sigma
Alexa488	Invitrogen
Alexa594	Invitrogen
Ampicillin	Sigma
Antibodies (monoclonal) against human-	
β-Actin	Sigma
FLAG M2	Sigma
HA.11	Covance
Myc-Tag (9B11)	Cell Signaling
Myc-Tag (71D10)	Cell Signaling
Antibodies (polyclonal) against human-	
ASCC1	Sigma
C23 (Nucleolin)	Santa Cruz
ERK1/2	Cell Signaling
GFP	Santa Cruz
Phospho-ERK1/2	Cell Signaling
JNK	Cell Signaling
Phospho-JNK	Cell Signaling
IRF3	Cell Signaling
Phospho-IRF-3	Cell Signaling
IκBa	Santa Cruz
Phospho-serine	Abcam
p38	Cell signaling
phospho-p38	Cell signaling
p65 (C-20)	Santa Cruz
p65	Cell Signaling

phospho-p65	Cell Signaling
TBK1	Cell Signaling
Phospho-TBK1	BD
APS	Sigma
ASCC1 cDNA clone in CMV expression vector	OriGene
Blasticidin S	InVivo
Boric Acid	Sigma
Bovine serum albumin	Sigma
Bradford reagent dye	Bio-Rad
Bromophenol blue	Sigma
Chamber slides	Nunc
Chloroform	Sigma
Chloroquine	Sigma
CIP	New England Biolabs
Coelenterazine	Insight Biotech.
DEPC-treated water	Ambion
DMEM	Invitrogen
DMSO	Sigma
DNA ladder (1Kb) & Loading dye (6X)	Promega
dNTPs	Promega
DTT	Sigma
<i>E.coli</i> - TOP 10 competent cells	Invitrogen
EDTA	Sigma
EMSA buffer kit	Licor Biosciences
Ethanol	Sigma
Ethidium bromide	Sigma
FBS	Invitrogen
Glacial acetic acid	Merck
Glycerol	Sigma
Glycine	Sigma
Goat Serum	Vector Laboratories
GoTaq Flexi DNA Polymerase	Promega
High speed plasmid midi kit	Qiagen
HEPES	Sigma

Hoescht stain	Sigma
Human HEK 293 cell line	InVivo
Hydrochloric acid	Merck
Hygrogold	InVivo
Igepal	Sigma
IL-1 β (human)	Cell signaling
IRDye 800CW Goat Anti-Rabbit	Licor Biosciences
IRDye 680 Donkey Anti-Mouse	Licor Biosciences
Isopropanol	Sigma
Kanamycin	Sigma
L-glutamine	Invitrogen
Lipofectamine 2000	Invitrogen
Lipopolysaccharide	Alexis
Luciferase substrate	Promega
Lymphoprep	Axis-Shield
Magnesium Chloride	Sigma
2–6– β -Mercaptoethanol	Sigma
Methanol	BDH
Microlon 96-well plates	Greiner
MMLV Reverse Transcriptase	Promega
Morpholinos	Gene Tools
Nitrocellulose	Schleicher&Schuell
NF- κ B IRDye Labelled Oligonucleotides	Licor Biosciences
OptiMEM	Invitrogen
PAK1IP1-HA	Genescrypt
Paraformaldehyde	Sigma
Para-p-phenylenediamine	Sigma
PBS	Oxoid
pcDNA 3.1/Zeo	Invitrogen
Penicillin / Streptomycin / Glutamine	Invitrogen
PMSF	Sigma
Poly(I:C)	InVivo
Polybrene	Sigma

Poly-L-Lysine	Sigma
Ponceau	Sigma
Prestained molecular weight marker	Fermentas
Protease inhibitor mixture	Roche
Protease inhibitor mixture (EDTA free)	Roche
Protein A/G-agarose	Santa Cruz
Proteinase K	Sigma
Protogel	National Diagnostics
Puromycin	Sigma
QIAquick gel extraction kit	Qiagen
Random primers	Invitrogen
Reverse Transcription System	Promega
RNase Zap	Ambion
RPMI	Invitrogen
SB203580	Cell Signaling
SDS	Sigma
shRNA	Sigma
siRNA	Invitrogen
Scrambled siRNA	Invitrogen
Skim milk powder	Marvel
Sodium chloride (NaCl)	Sigma
Sodium Deoxycholate ($C_{24}H_{39}O_4Na$)	Sigma
Sodium hydroxide (NaOH)	Sigma
Sodium orthovanadate (Na_3VO_4)	Sigma
Sulphuric acid	Sigma
SensiMix SYBR Low-ROX-kit	Bioline
Synthetic oligonucleotides	MWG Biotech
TEMED	Sigma
Tissue culture ware	Greiner
TK Renilla	Promega
Tri reagent	Sigma
Tris-base	Sigma
Tris-HCl	Sigma
Triton-X	Sigma

Trypsin/EDTA	Invitrogen
Tryptone	DIFCO
Tween-20	Sigma
Vectashield	Vector Laboratories
Whatmann paper	AGB

2.1.2 Gifts

Cell lines:

- HEK293 cells and HEK293 stably expressing TLR3 or TLR4 - Prof. Douglas T. Golenbock (The University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA).
- THP-1 cell line – Prof. Catherine Godson (School of Medicine and Medical Science, Conway Institute, UCD, Dublin, Ireland).
- U373 cell line – Dr. Sinead Miggin (Biology Department, NUI Maynooth, Ireland).

Constructs:

- Flag-tagged pFLAG-CMV2-TRIF. IRF-3/7 Gal4 reporter constructs, IFN- β -luciferase reporter constructs – Dr. Kate Fitzgerald (The University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA)
- Flag-tagged MyD88 – Dr M. Muzio (Mario Negri Institute, Milan, Italy)
- IRF3-Flag, – Dr. Marion Butler (National University of Ireland, Maynooth)
- pFR-luciferase Gal4 reporter construct – Dr. Andrew Bowie (Trinity College Dublin, Ireland)
- NF- κ B-luciferase reporter construct – Prof. Luke O'Neill (Trinity College Dublin, Ireland)
- GFP-tagged I κ BL – Dr. Ross McManus (Trinity College Dublin, Ireland)
- PRDI-III-, PRDII- and PRDIV-luciferase reporter constructs - Dr. Sinead Miggin (Biology department, NUI Maynooth, Ireland)

2.2 Methods

2.2.1 Mammalian cell culture

2.2.1.1 HEK 293 and U373 adherent cell lines

The human embryonic kidney (HEK) 293 cells that stably express the TLR3 and TLR4 receptors and U373 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10 % (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a 37°C humidified atmosphere with 5 % CO₂. Cells were passaged every 2 to 3 days using 1 % (w/v) Trypsin/ethylenediaminetetraacetic acid (EDTA) solution in phosphate-buffered saline (PBS). G418 (500 µg/ml), blasticidin (100 µg/ml) and/or hygrogold (10 µg/ml) was used to select for the stably transfected TLR cell lines. HEK293 T (for SV40 large T antigen) cells were also used. This cell line was cultured in the same conditions as above in the absence of a selective agent.

2.2.1.2 THP-1 suspension cells

THP-1 suspension cells were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 2 mM L-Glutamine, 10 % (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were maintained at 37°C in a humidified atmosphere with 5 % CO₂. Cells were passaged every 3-4 days.

2.2.2 Transient transfection of mammalian HEK293 cells

2.2.2.1 Transfection of cells for luciferase reporter assay

HEK293-TLR3 and -TLR4 cells were seeded at 1.5 x 10⁵ cells/ml (200 µl DMEM/well) in 96-well plates and allowed to adhere for 24 h to approximately 60 %

confluence. Cells were transfected using Lipofectamine 2000. For each well to be transfected, 25 µl of OptiMEM (Invitrogen) was mixed with the DNA. DNA mixes were made up for the appropriate luciferase construct as outlined in section 2.2.4. Lipofectamine 2000 (0.4 µl) was diluted in OptiMEM (25 µl) per sample and the reaction was mixed gently and left at room temperature for 5 min. After incubation, the Lipofectamine/OptiMEM solution was added to the DNA/OptiMEM mix (total volume 50 µl per well to be transfected) and the combined reaction was mixed gently and incubated at room temperature for 20 min. The transfection mixture was then added to each well and mixed gently by tapping the side of the plate. Each experimental condition was represented in triplicate. Cell lysates were generated and used to measure luciferase activity.

2.2.2.2 Transfection of cells for Western blot analysis

HEK293 T, TLR3 or TLR4 cells were seeded at 1.5×10^5 cells/ml in 6-well plates (3 ml DMEM/well). Cells were grown for 24 h to approximately 60 % confluence. For each well of a 6-well plate to be transfected, DNA (2 µg) was diluted in OptiMEM (250 µl) (Invitrogen) and mixed gently. Lipofectamine 2000 (4 µl) was then diluted in OptiMEM (250 µl) and incubated at room temperature. After 5 min incubation, the diluted DNA was combined with the diluted Lipofectamine 2000, mixed gently, and incubated at room temperature for 20 min. Medium (1 ml) was removed from each well before adding the DNA-Lipofectamine complexes (500 µl). For co-immunoprecipitation experiments, constructs encoding potential interacting partners were transfected at a ratio 1:1. After 24 h, cell lysates were generated for co-immunoprecipitation studies (section 2.2.7). Lysates were analysed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (section 2.2.8)

2.2.2.3 Transfection of cells for ChIP analysis

HEK293 TLR4 cells were seeded at 3.5×10^5 cells/ml in 10 cm petri dishes (10 ml DMEM/dish). Cells were grown for 24 h to approximately 60 % confluence. For

each dish to be transfected, DNA (8 µg) was diluted in OptiMEM (1 ml) (Invitrogen) and mixed gently. Lipofectamine 2000 (8 µl) was then diluted in OptiMEM (1 ml) and incubated at room temperature. After 5 min incubation, the diluted DNA was combined with the diluted Lipofectamine 2000, mixed gently, and incubated at room temperature for 20 min. Medium (2 ml) was removed from each dish before adding the DNA-Lipofectamine complexes (2 ml). After 24 h, cells were crosslinked and lysates were generated for ChIP analysis (section 2.2.11.1)

2.2.3 Propagation of DNA

2.2.3.1 Rapid transformation of competent *E.coli* cells

Commercially available TOP 10 competent *E.coli* bacterial cells were used for propagation of plasmids of interest. Aliquots (1 µl) of plasmids (100 ng DNA) were added to TOP10 cells (5 µl). DNA was mixed gently with cells by pipetting gently up and down and then incubated on ice for 30 min. The plasmids were allowed to enter the bacterial cells by heat shocking the mixture at 42 °C for 45 seconds. The cells become permeable to allow easy entry of the plasmid. Samples were subsequently cooled on ice for 2 min making the cells once again impermeable. The transformed cells were then incubated in 1 ml Luria Bertoni (LB) broth (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 85 mM NaCl) at 37 °C on a shaker at 220 rpm for 1 h. Bacterial cells were centrifuged at 9000 rpm for 3 min and 850 µl supernatant was removed. The pellet was resuspended in the remaining LB broth and plated out onto LB agar plates (LB broth with 1.5 % (w/v) agar) containing ampicillin or kanamycin (both 50 µg/ml) depending on the antibiotic resistance of the plasmid of interest. Plates were inverted and incubated overnight at 37 °C. Plates were then stored at 4 °C.

2.2.3.2 Large scale preparation of DNA from *E. coli*

A starter culture of LB broth (2 ml) containing the relevant antibiotic (50 µg/ml) was inoculated with a single transformed *E.coli* colony and incubated at 37°C

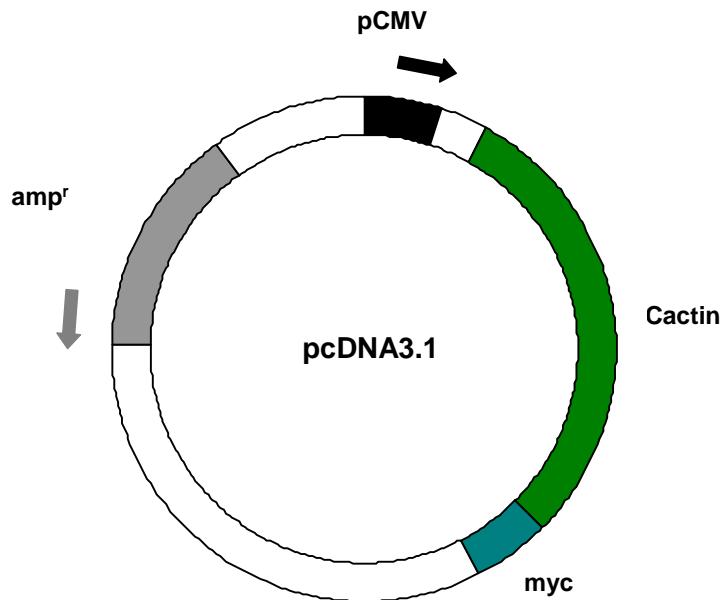


Figure 2.1 Cactin construct

The Cactin-c-myc construct used for transient expression of Cactin in HEK293 cells. The expression vector pcDNA3.1 encodes an ampicillin resistance gene used for selection of transformed bacterial cells.

with shaking at 220 rpm for 6-8 h. This was then added to a larger volume of LB broth (100 ml) containing the relevant antibiotic and incubated at 37°C overnight shaking at 220 rpm. Large plasmid preparations were made using the Qiagen high speed plasmid midi kit. The bacterial cells were centrifuged at 3000 rpm for 40 min and the supernatant was discarded and the plasmid DNA was extracted as outlined in the manufacturer's handbook. DNA was quantified using a cary spectrophotometer. After diluting the DNA appropriately in Tris-EDTA (TE) buffer, pH 8.0, (10 mM Tris-HCl, 1 mM EDTA) the absorbance of the solution was measured at 260 nm and 280 nm. All samples used had an optical density (OD)₂₆₀/OD₂₈₀ ratio in the range of 1.7 to 1.9. Ratios below 1.7 or above 1.9 indicated RNA or protein contamination, respectively. The concentration was calculated using the formula:

$$\mu\text{g/ml DNA} = 50 \mu\text{g/ml}/\text{OD}_{260} \times (\text{OD}_{260} \text{ measured}) \times (\text{dilution factor}).$$

2.2.4 Luciferase assays

HEK293 TLR3 and TLR4 cells were seeded at 1.5×10^5 cells/ml in 96-well plates (200 µl DMEM/well) and grown for 24 h. All transfections were performed using Lipofectamine 2000 transfection reagents (as described in section 2.2.2.1). Details of the constructs transfected are given below. 24 h post-transfection, the medium was removed from the cells and reporter lysis buffer (100 µl, Promega) was added to each well using a multi-channel pipette. The plate was then wrapped in aluminium foil and placed on a rocker for 30 min at room temperature before being placed at -80°C for a minimum of 1 h. After thawing at room temperature, aliquots (40 µl) of each were assayed for firefly luciferase activity using firefly luciferase substrate (40 µl, Promega), while *Renilla* luciferase activity was assayed using coelenterazine (0.1 µg/ml in 40 µl PBS). Luminescence was monitored with a Glomax microplate luminometer (Promega).

2.2.4.1 NF-κB assay

To measure activation of the NF-κB pathway, cells were transfected with NF-κB-regulated firefly luciferase reporter plasmid (80 ng), constitutively expressed *Renilla*-luciferase reporter construct phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total amount of DNA was equalised using pcDNA3.1.

2.2.4.2 IFN assays

The activation of the IFN- β promoter was assessed by transfecting the cells with an IFN- β promoter-regulated luciferase construct (80 ng), IFN α promoter-regulated luciferase construct (80 ng), PRDI-III-regulated luciferase construct (80 ng), PRDII-regulated luciferase construct (80 ng) or PRDIV-regulated luciferase construct (80 ng) and phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total DNA concentration was equalised using pcDNA3.1.

2.2.4.3 IRF3 assay

To measure the activation of IRF3, cells were transfected with pFR-Luc (60 ng), the *trans*-activator plasmid pFA-IRF3 (IRF3 fused downstream of the yeast Gal4 DNA binding domain, 30 ng), phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total DNA concentration was equalised using pcDNA3.1.

2.2.4.4 IRF7 assay

To measure the activation of IRF7, cells were transfected with pFR-Luc (60 ng), the *trans*-activator plasmid pFA-IRF7 (IRF7 fused downstream of the yeast Gal4 DNA binding domain, 25 ng), phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total DNA concentration was equalised using the pcDNA3.1.

2.2.4.5 c-Jun assay

To measure the activation of c-Jun, cells were transfected with pFR-Luc (60 ng), the *trans*-activator plasmid pFA-Jun (activation domain of c-Jun fused downstream of the yeast Gal4 DNA binding domain, 30 ng), phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total DNA concentration was equalised using the pcDNA3.1.

2.2.5 siRNA studies

Pre-designed siRNA targeting Cactin was purchased from Invitrogen (sense sequence: 5' – UCUUGAAGUGCUCUGCCUCCUUCUC -3') and a corresponding scrambled siRNA was designed (sense sequence 5' – GAGAAGGAGGCAGAGCACUUCAAGA - 3') and purchased from Invitrogen.

2.2.5.1 Transfection of siRNA

HEK293 TLR3 cells were seeded at 1.5×10^5 cells/ml in 6-well plates (3 ml DMEM/well). Cells were grown for 24 h to approximately 60 % confluence. The appropriate amount of Cactin siRNA or scrambled siRNA (for a final concentration of 25 nM/well) was diluted in OptiMEM (250 μ l). Lipofectamine 2000 (4 μ l) was diluted in OptiMEM (250 μ l) per sample, and the reaction was mixed gently and left at room temperature for 5 min. After the incubation, the Lipofectamine/OptiMEM solution was added to the siRNA mix (total volume 500 μ l per well to be transfected) and the combined reaction was mixed gently and incubated at room temperature for 20 min. 500 μ l sample was added to each well and mixed gently by rocking the plate back and forth. Cells were incubated for 48 h prior to generation of extracts for Western immunoblotting (2.2.8) or extraction of RNA for quantitative real-time PCR (2.2.9).

2.2.6 shRNA studies

Cactin-specific shRNA 5' - AGGTCA GATT CAGAGGAAGAG -3',

I κ BL-specific shRNA

5'CCGGGCCTCCCATGAAACCCAGGAAC TCGAGT CCTGGGTT CATGGGA GGCTTTTG – 3' and a control shRNA (CT) were purchased from Sigma. The shRNA CT is a non-targeting shRNA vector that will activate the RNA-induced silencing complex (RISC) and the RNAi pathway, but it does not target any human or mouse genes. The short-hairpin sequence contains 5 base pair mismatches to any known human or mouse gene. (See Fig. 2.2 for the map of the plasmid).

2.2.6.1 Transfection of shRNA for luciferase assays

HEK293 TLR3 or TLR4 cells were seeded at 1.5×10^5 cells/ml in 96-well plates (200 μ l DMEM/well). Cells were grown for 24 h to approximately 60 % confluence. DNA mixes were made up for the appropriate luciferase construct as outlined in section 2.2.4. Cactin-specific shRNA (100 ng), I κ BL-specific shRNA (100 ng) or shRNA CT (100 ng) was added to the DNA mix which was subsequently diluted in OptiMEM (25 μ l) and mixed gently. Lipofectamine 2000 (0.4 μ l) was diluted in OptiMEM (25 μ l) per sample, and the reaction mixed gently and left at room temperature for 5 min. After the incubation, the Lipofectamine/OptiMEM solution was added to the DNA mix (total volume 50 μ l per well to be transfected) and the combined reaction mixed gently and incubated at room temperature for 20 min. Sample (50 μ l) was added to each well and mixed gently by tapping the side of the plate. Cells were harvested 48 h post-transfection.

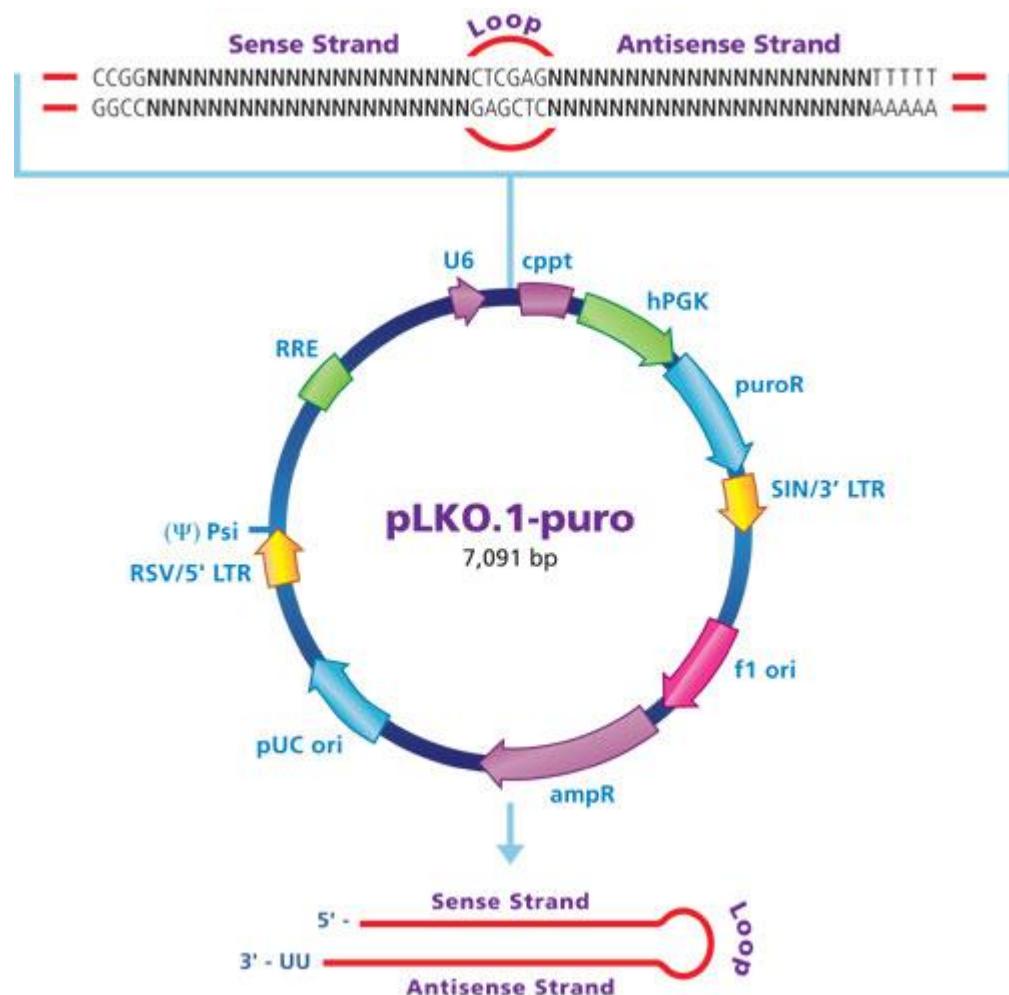


Figure 2.2 shRNA Lentiviral Vector map.

The pLKO.1-puro lentiviral plasmid vector contains the ampicillin and puromycin antibiotic resistance genes for selection of inserts in bacterial or mammalian cells respectively.

2.2.6.2 Transfection of shRNA for expression analysis or Western immunoblotting

HEK293 TLR4 cells were seeded at 1.5×10^5 cells/ml in 6-well plates (3 ml DMEM/well). Cells were grown for 24 h to approximately 60 % confluence. Cactin-specific shRNA (1 μ g), I κ BL-specific shRNA (1 μ g) and shRNA CT (1 μ g) was diluted in OptiMEM (250 μ l). Lipofectamine 2000 (4 μ l) was diluted in OptiMEM (250 μ l) per sample, and the reaction mixed gently and left at room temperature for 5 min. After the incubation, the Lipofectamine/OptiMEM solution was added to the DNA mix (total volume 500 μ l per well to be transfected) and the combined reaction mixed gently and incubated at room temperature for 20 min. During this incubation 1 ml of DMEM was removed from each well. 500 μ l sample was added to each well and mixed gently by rocking the plate back and forth. Cells were incubated for 48 h prior to generation of extracts for Western immunoblotting (2.2.8) or extraction of RNA for quantitative real-time PCR (2.2.9).

2.2.7 Co-immunoprecipitations (CoIPs)

HEK293 TLR4 cells were transfected with Lipofectamine 2000 as previously described in section 2.2.2.2 with equal amounts (2 μ g) of expression constructs encoding the potential interaction partners. Cell extracts were generated on ice or at 4°C as follows. Cells were first washed with pre-chilled 1X PBS (1 ml) then lysed with pre-chilled CoIP lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5 % (v/v) igepal and 50 mM NaF, with 1 mM Na₃VO₄, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor mixture (leupeptin (25 μ g/ml), aprotinin (25 μ g/ml), benzamidine (1 mM), trypsin inhibitor (10 μ g/ml)) for 30 min on a rocker at 4°C. Lysates were scraped into pre-chilled 1.5 ml eppendorf tubes and centrifuged at 12,000 g for 10 min at 4°C. Supernatants were removed to fresh tubes (a sample retained for whole cell lysate analysis) and incubated for 30 min with mouse or rabbit immunoglobulin (Ig) G (1 μ g) (depending on the primary antibody) and Protein A/G agarose beads (10 μ l) on a rotator at 4 °C.

Samples were centrifuged at 1000 g for 5 min at 4°C to pellet beads with non-specifically bound protein and supernatants were removed to fresh pre-chilled tubes. Samples were incubated overnight with primary antibody (1 µg). The following day Protein A/G agarose beads (20 µl) were added to each sample and they were again incubated at 4°C overnight. The subsequent day samples were centrifuged at 16,000 g for 1 min. The supernatant was removed and the beads were washed with CoIP lysis buffer (500 µl) and subject to re-centrifugation. This step was repeated four times. 40 µl of 1X sample buffer (0.125 M Tris-HCl, pH 6.8, containing 20 % (w/v) glycerol, 4 % (w/v) SDS, 1.4 M β-mercaptoethanol and 0.0025 % (w/v) bromophenol blue) was added to the beads for 30 min at RT. Samples were centrifuged at 16,000 g for 2 min and the supernatant was subsequently boiled at 100°C for 10 min and analyzed using SDS polyacrylamide gel electrophoresis and western blotting (section 2.2.8).

2.2.8 Western blot analysis

2.2.8.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli (Laemmli 1970), as modified by Studier (Studier 1973). Samples and appropriate prestained (26.6-180 kDa) protein markers were loaded into separate wells. Electrophoresis was performed at 80 V through a 5 % SDS polyacrylamide stacking gel and then through a 10 % SDS polyacrylamide resolving gel at 110 V for 1-1.5 h, depending on the size of the proteins being electrophoresed.

2.2.8.2 Immunoblotting

Following separation by electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membranes in a wet transfer unit at 150 V for 1.5 h using Whatmann and nitrocellulose pre-soaked in cold transfer buffer (25 mM Tris Base, 0.2 M glycine and 20 % (v/v) methanol) for 10 min. Following transfer, non-

specific binding was blocked by incubating the nitrocellulose membranes at room temperature for 1 h (or overnight) in TBS (20 mM Tris-HCl pH 7.5, containing 0.05 % (v/v) Tween 20 and 0.5 M NaCl) containing 5 % (w/v) skimmed milk powder. The membranes were then washed 3 times for 5 min each in TBS prior to incubation at 4°C overnight with the primary antibodies diluted in TBS containing 5 % (w/v) skimmed milk powder or BSA. The membranes were subsequently subjected to 5 x 10 min washes in TBS prior to incubation with secondary antibody (1:5000 dilution) specific for the primary antibody in question (anti-rabbit or anti-mouse) in TBS containing 5 % (w/v) skimmed milk powder for 1.5 h in the dark at room temperature. The membranes were then washed a further 5 times for 10 min each in TBS in the dark. The immunoreactive bands were detected using Odyssey Infrared Imaging System from Licor Bioscience, according to the instructions of the manufacturer.

1° antibody	Dilution	2° antibody*
ASCC1	1:500	mouse
β-actin	1:5000	mouse
c-Myc	1:1000	mouse
Erk1/2	1:1000	rabbit
Phospho Erk1/2	1:1000	rabbit
Flag	1:1000	mouse
GFP	1:500	rabbit
HA	1:1000	mouse
IκBα	1:500	rabbit
JNK	1:1000	rabbit
Phospho-JNK	1:1000	rabbit
IRF-3	1:1000	rabbit
p38	1:1000	rabbit
Phospho-p38	1:1000	rabbit
Phospho-IRF-3	1:1000	rabbit
p65	1:1000	rabbit
Phospho-p65	1:1000	rabbit
Phospho-Serine	1:200	rabbit

Nucleolin (C23)	1:500	rabbit
TBK1	1:1000	rabbit
Phospho-TBK1	1:250	rabbit

* All secondary antibodies were used at a dilution of 1:5000.

2.2.9 Isolation of RNA and cDNA synthesis

2.2.9.1 Isolation of total RNA from HEK293, THP-1 and U373 cells

In order to minimise RNA degradation, a number of precautions were taken throughout the following procedures. All water and salt based solutions were treated with diethylpyrocarbonate (DEPC) (0.2 % v/v). All plasticware was certified RNase-free. Before commencing, equipment was wiped with “RNase Zap”, an RNase decontamination solution. Gloves were worn at all times and regularly changed. Total RNA was isolated using the Tri Reagent (Sigma) as per manufacturer’s instructions. The amount of isolated RNA was quantified by measuring the absorbance at wavelengths of 260 nm and 280 nm on a spectrophotometer, where an absorbance of 1 unit at 260 nm is ~ 40 µg/ml. Pure RNA preparations have an OD₂₆₀/OD₂₈₀ ratio of 1.6-1.8. Extracted RNA was stored at -80°C.

2.2.9.2 Synthesis of first strand cDNA from messenger RNA (mRNA)

1 µg of RNA was placed in nuclease-free microcentrifuge tubes and incubated for 10 min at 70°C. The mixture was then chilled on ice and centrifuged briefly. The following components were then added:

Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT)	0.25 µl
dNTPs (10 mM)	2 µl
5X MMLV Buffer	4 µl
RNasin (40 U/µl)	0.5 µl
Random Primers (0.5 µg/µl)	1 µl
Nuclease-Free Water	to 20 µl

The reaction mixture was incubated for 10 min at RT allowing the primers to anneal to the RNA. Next, the reaction was incubated at 42°C for 1 h. MMLV RT was then deactivated by heating to 95°C for 5 min followed by cooling to 0-5°C for 5 min. Generated cDNA was stored at 4°C for short-term storage or -20°C for long-term storage.

2.2.10 Quantitative Real Time PCR

Cells were seeded (1.5×10^5 cells/ml; 3 ml) into 6-well plates and grown for 24 h to approximately 60 % confluency. In some cases, cells were transfected, as previously described in section 2.2.5.1 and 2.2.6.2 and/or stimulated with Poly(I:C) (25 µg/ml) or LPS (100 ng/ml) for various time periods. Cells were washed with 1X PBS (1 ml) and RNA was extracted, as described in section 2.2.9.1. cDNA was then generated as described in section 2.2.9.2. Samples were assayed by quantitative real time PCR for levels of IFNβ, Cactin or IκBL cDNA using SensiMix SYBR Low-ROX kit (Bioline). PCR was conducted with the CFB-322001G Opticon thermal cycler (Bio-Rad Laboratories). Reactions were performed using pre-validated primers (Eurofins MWG Operon). The sequence of primers used were as follows:

Cactin	Forward	5' TACACCAACACCGACAACCC 3'
	Reverse	5' TTCAGCTCCTTCTCCTCCAG 3'
IFN β	Forward	5' GACCAACAAGTGTCTCCTCCAAA 3'
	Reverse	5' CTCCTCAGGGATGTCAAAGTTCA 3'
I κ BL	Forward	5' GCCTACACCGATTCTTCC 3'
	Reverse	5' CCTCCTCTTCAGCAGAATCC 3'
HPRT	Forward	5' GGTGAAAAGGACCCCACGAA 3'
	Reverse	5' GGCGATGTCAATAGGACTCCAGAT 3'

Accumulation of gene-specific PCR products was measured continuously by means of fluorescence detection over 40 cycles. Samples were initially heated for 10 min at 95°C. This was followed by 10 seconds at 95°C, 10 seconds at 59°C and 1 min at 72°C for 40 cycles. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method.

2.2.11 Chromatin ImmunoPrecipitation (ChIP)

2.2.11.1 X-ChIP

HEK293 TLR4 cells were seeded at 3.5×10^5 cells/ml in 10 cm petri dishes (10 ml DMEM/dish) and grown to approximately 60 % confluency. They were then transfected with or without myc-tagged hCactin and HA-tagged p65 as previously described in section 2.2.2.3. 24 h post-transfection cells were cross-linked by adding 37.5 % (v/v) formaldehyde/10 % (v/v) methanol stock directly to the culture medium to a final concentration of 1 %. After 10 min incubation at 37°C, medium was removed and the cells washed three times in ice-cold PBS. Cells were collected in PBS and centrifuged at 8000 x g at 4°C for 5 min. Cells were lysed for 5 min in 500 μ l L1 buffer (50 mM Tris-HCl pH 8.0, containing 0.2 mM EDTA, 0.1 % (w/v) igepal and 10 % (w/v) glycerol). Nuclei were pelleted by centrifugation at 8000 x g for 5 min at 4°C. The supernatant constituting the cytoplasmic fraction was removed and the nuclei were resuspended in 500 μ l buffer L2 (50 mM Tris pH 8.0, containing 5 mM EDTA, 1 % (w/v) SDS). After 10 min incubation on ice, chromatin was sheared by

sonication. Four 12 s pulses were applied per sample using a Sanyo Soniprep 150 at half of the total power. Samples were incubated on ice for a minimum of 1 min between pulses. Lysis buffer was periodically monitored for SDS precipitation. SDS was redissolved by warming the tube by hand. Following sonication, nuclear debris was removed by centrifugation at 8000 x g at 4°C. An aliquot was retained as an input sample to normalize PCR reactions and analyze shearing efficiency. The remaining sample was divided into two aliquots, which were diluted ten-fold in dilution buffer (50 mM Tris pH 8.0, containing 0.5 % (w/v) igepal, 0.2 M NaCl and 5 mM EDTA). These samples were precleared for 3 h by rotation at 4°C with 40 µl of a 50 % suspension of salmon sperm-saturated protein A-agarose (ssProtein A). Samples were then centrifuged for 2 min at 1000 x g to pellet ssProtein A. The supernatant was removed to a fresh tube and incubated overnight with rocking at 4°C with 2 µg anti-p65 antibody or rabbit IgG antibody. Immuno-complexes were collected by incubation with 20 µl ssProtein A for 1 h at 4°C, followed by centrifugation at 1000 x g. The supernatant containing unbound DNA was removed. A series of washes was then performed. Each wash involved resuspension of the immuno-complexes in wash buffer and 5 min incubation with rocking at 4°C. Immuno-complexes were washed consecutively with low salt wash buffer (20 mM Tris pH 8.0, 0.2 mM EDTA, 0.1 % (w/v) SDS, 1 % (w/v) igepal, 0.15 M NaCl), high salt wash buffer (20 mM Tris pH 8.0, 0.2 mM EDTA, 0.1 % (w/v) SDS, 1 % (w/v) igepal, 0.5 M NaCl), 0.5 M LiCl and twice with TE buffer. After each wash, immune-complexes were pelleted by centrifugation at 1000 x g for 2 min at 4°C.

2.2.11.2 PCR analysis of immunoprecipitated DNA

DNA was eluted from immune-complexes by incubation for 15 min with extraction buffer (1X TE with 2 % (w/v) SDS). During the incubation, tubes were gently agitated at regular intervals to maintain immune-complexes in suspension. Reversion of protein-DNA cross-linking was achieved by incubating immunoprecipitated and input samples overnight at 65°C. DNA was incubated with proteinase K at 50°C for 2 h. DNA was extracted using the Qiaquick purification kit (Qiagen) according to the manufacturer's instructions. In order to monitor chromatin

shearing, an aliquot of each input sample was electrophoresed in an agarose gel. DNA used for PCR had an average size of 650 bp.

The conditions for standard PCR were as follows:

DNA template	5 µl
Forward and reverse primers (at 100pmol)	1.25 µl
25 mM MgCl ₂	2 µl
dNTP mix	2 µl
GoTaq Flexi DNA Polymerase (5 U/µl)	0.25 µl
5X Green GoTaq Flexi Buffer	5 µl
PCR-grade water	to 25 µl

Samples were initially heated to 95°C for 2 min. This was followed by 40 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 30 s. Samples were then incubated at 72°C for 10 min and then stored at 4°C. The sequences of primers used are as follows:

IL-8 promoter 5'- GGAAGTGTGATGACTCAGGTTGC -3'

5' - GATGGTTCCTCCGGTGGTTCTTC -3'

2.2.11.3 Agarose gel electrophoresis

Agarose gels were prepared by suspending agarose (0.8-1 % (w/v)) in TAE (0.484 % (w/v) Tris base, 0.1 % (v/v) glacial acetic acid, 0.2 M EDTA). This was heated in a microwave until the agarose had completely dissolved, and was then cooled to less than 50°C. Ethidium bromide (5 µg/ml) was added and the agarose poured into the gel tray. Following solidification, agarose gels were electrophoresed in TAE (1X). Samples were mixed with loading dye (0.017 % (w/v) xylene cyanol, 0.017 % (w/v) bromophenol blue and 5 % (w/v) glycerol). Samples were run with molecular size markers, with a 100 bp ladder. Gels were run at 110 V for 30 min. Nucleic acids were visualised under UV light (254 nm) and images acquired using the Syngene G box gel documentation system (Frederick, MD, USA).

2.2.12 Immunofluorescence

HEK293 TLR3/TLR4 cells were seeded at 1.5×10^5 cells/ml in 4 well chamber slides (Lab-Tek) (800 μ l DMEM/well) or on Poly-L-lysine coated coverslips in 6 well-plates (3 ml DMEM/well) and grown for 24 h to approximately 60 % confluence. Cells were transfected using Lipofectamine 2000 with plasmids as detailed in the figure legends. Medium was removed and the cells were gently washed with 1X PBS, being careful not to disrupt the cells. The cells were then fixed by addition of 4 % (v/v) paraformaldehyde (500 μ l). After 30 min incubation at RT the paraformaldehyde was removed and the cells were washed with PBS. 500 μ l Hoescht (1:1000 diluted in PBS) was added to each well. After 20 min incubation the Hoescht was removed and the cells were washed four times with PBS before removing the chamber walls. A single drop of Vectashield hard-set mounting medium was applied to each well. Coverslips were then mounted and sealed on the sides with nail varnish, before being wrapped in tinfoil and stored at 4°C.

Alternatively, after fixing with paraformaldehyde cells were incubated with 0.2 % Triton X (500 μ l) for 20 min. Cells were then gently washed with PBS. Cells were then incubated in 10 % goat serum for 2 h at room temperature to block non-specific binding. Cells were probed overnight with anti-myc (1:500), anti-flag (1:500) or anti-ASCC1 (1:500) antibodies at 4°C. The following day cells were washed 3 times for 5 min with PBS and then probed with anti-rabbit Alexa-Floura 488 (1:250) and/or anti-mouse Alexa-Floura 594 (1:250) antibodies as deemed appropriate in 5 % goat serum with Hoescht (1:1000) for 1.5 h at room temperature. Cells were then gently washed 6 times for 10 min with PBS. The Poly-L-Lysine coated coverslips were then removed from the 6 well plates and mounted on slides with a drop of anti-fade mounting media (50 % (v/v) glycerol, 0.5X PBS, 2 μ g/ μ l para-p-phenylenediamine). Coverslips were sealed on the sides with nail varnish, before being wrapped in tinfoil and stored at 4°C.

Confocal images were captured using the 40 x or 63 x objective lens on the Olympus Fluoview FV1000 confocal laser scanning microscope equipped with the appropriate filter sets. Acquired images were analysed using the Olympus FV-10 ASW imaging software.

2.2.13 Lentiviral shRNA-Cactin infection and stable cell lines

2.2.13.1 Lentiviral production

HEK293 T cells were seeded at 2×10^5 cells/ml in 6-well plates (3 ml DMEM/well) and grown for 24 h to approximately 80 % confluency. The cells were transfected as in section 2.2.2.2. The DNA mixture contained packaging plasmid (900 ng), envelope plasmid (100 ng) and the sh-pLKO.1 vector (1 μ g). hCactin sh-RNA sequences were as follows:

hCactin shRNA 1 5'- CAGGGATACAAGTTCAACATC -3'

hCactin shRNA 2 5'- AGGTCAGATTAGAGGAAGAG -3'

A control shRNA (CT) was also used in the transfection. It is a non-targeting shRNA vector that will activate the RNA-induced silencing complex (RISC) and the RNAi pathway, but it does not target any human or mouse genes. The short-harpin sequence contains 5 base pair mismatches to any known human or mouse gene. The shRNA hCactin and shRNA control plasmids were purchased from Sigma (Fig. 2.2 for the map of the plasmid). To remove the transfection reagent, the media was changed 24 h post-transfection and replaced with fresh high serum (30 %) growth media. The cells were then incubated for 24 h. The media containing lentivirus were harvested ~ 40 h post-transfection and transferred to a polypropylene tube for storage at -20°C. The media was replaced with fresh high serum (30 %) growth media and the cells were incubated for 24 h. The virus was harvested one more time and after the final harvest the packaging cells were discarded.

2.2.13.2 Lentiviral infection

THP-1 and U373 cells were seeded at 2×10^5 cells/ml in 6-well plates (3 ml/well RPMI or DMEM, respectively). U373 cells were incubated overnight prior to infection to allow the cells to adhere. The growth media was then removed and replaced with fresh media containing polybrene (8 μ g/ml) and 600 μ l of virus. The plates were incubated at 37°C. The media was removed 24 h post-infection and replaced with fresh growth media containing puromycin (10 μ g/ml) to select for cells

transduced with shRNA. The polyclonal population of cells were cultured for 2-3 weeks and used for EMSA and Western Blot experiments.

2.2.14 Electrophoretic mobility shift assay (EMSA)

EMSA was used to detect the presence of DNA-binding proteins in nuclear extracts from lenti-viral transduced THP-1 cells, siRNA-transfected HEK293 TLR3 cells and transfected HEK293 TLR4 cells. NF-κB Infrared Dye labelled oligonucleotides were purchased from Licor Biosciences while Infrared Dye labelled oligonucleotides for IFN β were purchased from MWG.

2.2.14.1 Preparation of sub-cellular fractions

HEK293 TLR3 cells were transfected with siRNA as described in section 2.2.6.1. and treated with Poly(I:C) (25 μ g/ml). HEK293 TLR4 cells were transfected as described in section 2.2.2.2 with various expression vectors as detailed in the figure legends. Cells were washed with 1 ml of ice cold PBS. Cells were then detached with 1 ml of hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF). Alternatively THP-1 cells previously transduced with shRNA were grown in 6-well plates and stimulated with LPS (100 ng/ml). Cells were centrifuged, washed with 1 ml of ice cold PBS and resuspended in 1 ml hypotonic buffer. All cells were pelleted in hypotonic buffer by centrifugation at 21000 x g for 10 min, and then lysed for 10 min on ice in hypotonic buffer A (30 μ l) containing 0.1 % (w/v) Igepal. Lysates were centrifuged at 21000 x g for 10 min. The resulting supernatants constituted cytosolic extracts and were stored at -20°C in fresh microcentrifuge tubes. The remaining pellets were resuspended in 25 μ l of Buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25 % (w/v) glycerol and 0.5 mM PMSF) and incubated on ice for 15 min. Incubations were then centrifuged at 21000 x g for 10 min. The supernatants were then removed into new tubes and 75 μ l of Buffer D (10 mM HEPES pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20 % glycerol, 0.5 mM PMSF and 0.5 mM DTT) was added. Such samples constituted

nuclear extracts. Protein concentrations were determined by the method of Bradford and assayed for protein-DNA binding activity as outlined in the following sections.

2.2.14.2 Determining protein concentration – Method of Bradford

Protein content of BSA standards and cell extracts was measured by the method of Bradford (Bradford 1976). Standards and extracts (10 µl) were diluted in water and mixed with aliquots (190 µl) of Bradford protein reagent (Bio-Rad), diluted 1:5 in distilled water, by pipetting up and down. A blank was prepared using the same dilution of water. Absorbance was measured for each sample in a 96-well plate at 590 nm using ELx800TM microplate reader with Gen5 Data Analysis Software. Six protein standards of BSA (10, 8, 6, 4, 2 and 0 µg/µl) were made and used to construct a standard curve which was subsequently used to determine protein concentration of the cell extracts.

2.2.14.3 Incubation of nuclear extracts with NF-κB and PRDI-III Infrared Dye labelled oligonucleotides

Nuclear extracts (5μg of protein), generated as described in section 2.2.14, were incubated with the following reaction mix:

10x Binding Buffer (100 nM Tris, 500 mM KCl, 10 mM DTT; pH 7.5)	2 μl
Poly (dI-dC) 1 μg/μl in 10 mM Tris, 1 mM EDTA; pH 7.5	1 μl
25 mM DTT	2 μl
1 mM MgCl ₂	1 μl
NF-κB/PRDI-III IRDye labelled oligonucleotides	<u>1 μl</u>
	7 μl
Nuclear extracts (5 μg of protein)	...
Water	to 20 μl

NF-κB consensus oligonucleotide:

5' - AGTGAGGGGACTTCCCAGGC - 3'

3' - TCAACTCCCCTGAAAGGGTCCG - 5'

PRDI-III consensus oligonucleotide

5' – GTAAATGACATAGGAAAAGTGAAGGGAGAAGTGAAAGTGG – 3'

3' – CATTACTGTATCCTTGACTTCCCTCTTCACCTTCACC – 3'

Incubations were performed for 1 h at room temperature in the dark. Poly (dI-dC) was added to prevent non-specific binding of proteins to the oligonucleotide probe and thereby to reduce background. Following incubation, orange loading dye (2 µl) was added and the incubations were then subjected to electrophoresis for 2 h at 90 V in a non-denaturing 4 % (w/v) polyacrylamide gel. Gels were run in 0.5 x TBE buffer. Gels were then removed from glass plates and visualised using Odyssey Infrared Imaging System from Licor Biosciences.

2.2.15 Statistical Analysis

Data are expressed relative to untreated cells transfected with empty vector, and are the mean +/- standard error of the mean of triplicate determinations from three independent experiments. For statistical comparisons between two groups, paired Student's t-test (one tail) was used. A p value of < 0.05 was considered significant

Chapter 3

**The role of Cactin in NF-κB signalling: probing the
underlying mechanism of inhibition**

3.1 Introduction

The transcription factor NF- κ B is activated by a range of signalling pathways. Toll Like Receptors (TLRs) recognise conserved microbial motifs and respond by activating transcription factors such as NF- κ B. The IL-1 Receptor (IL-1R) and TNF Receptor family activate NF- κ B in response to endogenous proinflammatory stimuli. NOD1 and NOD2 are cytoplasmic receptors for microbial ligands and are another example of receptors that can trigger the activation of NF- κ B (Philpott and Girardin, 2004). NF- κ B regulates the expression of a wide variety of genes involved in both innate and adaptive immunity. These genes include proinflammatory cytokines and chemokines, adhesion molecules, stress response proteins and anti-apoptotic proteins (Li and Verma, 2002). In the unstimulated cell, NF- κ B is predominantly cytoplasmic and is found in association with members of the Inhibitory (I)- κ B family. The various signalling pathways that lead to NF- κ B activation all result in the phosphorylation of the I κ B proteins, marking them as targets for ubiquitination and subsequent degradation by the proteosome. This allows for NF- κ B to bind to motifs in the promoter regions of its target genes and thus regulate gene expression. Members of the NF- κ B, or Rel, family of transcription factors share a conserved N-terminal rel homology (RH) domain. This domain mediates dimerisation of Rel/NF- κ B family members, their nuclear translocation and binding to DNA (Ghosh et al., 1998). The Rel/NF- κ B family currently has five mammalian members; p65 (RelA), NF- κ B1 (p50 and precursor p105), NF- κ B2 (p52 and precursor p100), c-Rel and RelB (Albert and Baldwin, 1996). RelB, c-Rel and p65 also possess a C-terminal transactivation domain. Rel subunits form homo- or heterodimers and the most common combination found is a p65/p50 heterodimer. p50 and p52 homodimers cannot activate transcription directly but do, however, have the ability to repress it (Hayden and Ghosh, 2004).

Dysregulation of NF- κ B can lead to various autoimmune and inflammatory diseases and so these pathways are tightly regulated. ST2 (suppression of tumorigenicity 2) is a TIR domain containing receptor which sequesters MyD88 and Mal and thereby inhibits TLR4 and IL-1R signalling to NF- κ B (Brint et al., 2004). IRAK-M is a member of the IRAK family which is expressed in monocytes and

macrophages and negatively regulates TLR-signalling. IRAK-M prevents the dissociation of IRAK1 and IRAK4 from MyD88, thereby preventing their association with downstream regulators of the pathway (Kobayashi et al., 2002). A20 is another negative regulator of NF- κ B which functions by interfering with the ubiquitination of receptor interacting protein (RIP), an essential mediator of TNFR signalling, and TNF receptor associated factor (TRAF)6 (Wertz et al., 2004, Boone et al., 2004). Suppressor of cytokine signalling 1 (SOCS1) is a member of the cytokine-induced SOCS family which inhibits LPS mediated NF- κ B activation (Akira and Takeda, 2004).

In *Drosophila* the Toll signalling pathway triggers the activation of Rel transcription factors. Three such transcription factors have been identified in *Drosophila*; Dorsal, Dif and Relish. Like their mammalian counterparts, these transcription factors all contain a conserved RH domain which mediates dimerisation, DNA-binding and interaction with I κ B-inhibitor proteins (Govind, 1999). Following nuclear translocation Rel transcription factors bind to κ B sites in genes controlling many biological processes including early development and immune response. The components of the Toll pathway are evolutionarily conserved and most have homologues in the mammalian TLR-signalling pathway. In *Drosophila* Rel transcription factors are sequestered in the cytoplasm through their association with Cactus, the human orthologue of which is I κ B α . Cactus is phosphorylated following Toll activation, allowing for the Rel transcription factor to translocate to the nucleus and regulate gene expression. In 2000, Cactus was used as bait in a yeast-2-hybrid screen which led to the discovery of a novel *Cactus*-interacting protein termed Cactin (Lin et al., 2000). dCactin was overexpressed in females with *cactus* haplo-insufficient phenotype which displays weak ventralisation. Overexpression of dCactin enhanced this weak ventralising phenotype suggesting that dCactin may function as a positive regulator of Dorsal nuclear translocation by affecting the stability of Cactus protein (Lin et al., 2000). Due to the similarities of the insect Toll signalling pathway and mammalian TLR signalling and given that I κ B α , the human homologue of Cactus, is vital to controlling NF- κ B activation and inflammation, we were keen to investigate if human Cactin can regulate innate immune signalling. Prior to this study the human homologue of Cactin was cloned in the author's laboratory and previous studies indicated that Cactin inhibits NF- κ B activation.

3.1.1 Specific aims of chapter 3

1. Confirm that Cactin is a negative regulator of NF-κB activation.
2. Uncover a possible mechanism by which Cactin exerts its inhibitory effects on NF-κB.
3. Examine if, like other negative regulators of TLR-signalling, Cactin is regulated by proinflammatory stimuli.

3.2 Results

3.2.1 Cactin acts as a negative regulator of the NF-κB pathway

Drosophila Cactin was first discovered in a yeast-two hybrid screen using Cactus as bait (Lin et al., 2000). Cactus is the *Drosophila* orthologue of human IκBα which is a regulator of NF-κB. Thus, human Cactin was initially probed for its effects on the NF-κB pathway. In order to investigate this, HEK293 cells stably transfected with TLR3 and TLR4 were employed. These cells were transfected with increasing concentrations of an expression plasmid encoding myc-tagged Cactin and assessed for their ability to induce a co-transfected NF-κB regulated luciferase gene in response to various stimuli. Overexpression of Cactin significantly inhibited the activation of NF-κB in response to the TLR4 ligand LPS (Figure 3.1A) and the TLR3 ligand Poly(I:C) (Figure 3.1B). Overexpression of Cactin also inhibited the activation of NF-κB in response to the endogenous proinflammatory stimulus IL-1 β (Figure 3.1C). When the TIR-domain containing adaptor protein MyD88 was used to drive NF-κB activation Cactin displayed similar inhibitory effects (Figure 3.1D). These results are consistent with previous findings from the laboratory suggesting that Cactin is a negative regulator of the NF-κB pathway.

Because the above studies were performed using cells engineered to express TLRs it was necessary to demonstrate a role for Cactin in a more physiologically relevant system. It was also hoped to complement the above overexpression studies and assess the effects of suppressing endogenous expression of Cactin on the NF-κB pathway. Thus, cells that naturally express TLRs were employed. THP-1 is a human, acute monocytic, leukemia cell line that naturally express TLR4 and respond to LPS while U373 human astrocytoma cells express TLR3 and TLR4 and respond to both LPS and Poly(I:C). These cells are not easily transfected and so they were transduced with lentiviral particles containing a plasmid encoding Cactin-specific shRNAs in order to suppress Cactin expression. Cells were also transduced with a plasmid encoding a control shRNA that does not target Cactin or any other human gene. These plasmids also encode a puromycin resistance gene allowing for selection of cells that have been stably transduced with the constructs. In the absence of an available antibody for Cactin protein, quantitative realtime PCR was used to confirm knockdown of endogenous Cactin in these cells lines. THP-1 cells exhibited 80 %

knockdown of endogenous Cactin (Figure 3.2A) while Cactin expression in U373 cells was decreased by an average of 50 % (Figure 3.2B).

THP-1 cells, previously transduced with control or Cactin-specific shRNA were treated for various time points with LPS. Nuclear extracts were generated and then assayed for NF- κ B activation by electrophoretic mobility shift assay (EMSA) using an oligonucleotide encoding a consensus binding site for NF- κ B. THP-1 cells transduced with Cactin specific shRNA displayed, stronger and more prolonged activation of NF- κ B compared with those transduced with the control shRNA (Figure 3.3). The protein-DNA complexes observed contained the Rel subunits p65 and c-Rel as confirmed by supershift analysis. It is also worth noting that THP-1 and U373 cells transduced with Cactin shRNA demonstrated greatly enhanced expression of IL-8 and TNF- α , most likely due to potentiation of NF- κ B activation. These findings demonstrate a functional role for Cactin in regulating activation of NF- κ B in these cell lines.

The ability of Cactin to regulate the phosphorylation of p65 was next examined. U373 cells transduced with Cactin specific shRNA and control shRNA were treated with LPS for various time points. Samples were harvested in SDS-PAGE sample buffer and subjected to Western immunoblotting. Surprisingly, knockdown of endogenous Cactin did not lead to increased levels of phospho-p65 as detected by immunoblotting using an anti-phospho-p65 antibody (Figure 3.4). As we previously demonstrated that knockdown of endogenous Cactin led to increased activation of NF- κ B by EMSA this suggests that Cactin may be exerting its negative effects downstream of p65 phosphorylation and possibly at the nuclear level.

3.2.2 Cactin is localised in the nucleus and its nuclear localisation is important for manifesting its inhibitory effects on NF- κ B

Given the likely action of Cactin at the nuclear level the subcellular localisation of Cactin was examined by confocal microscopy. HEK293 cells were transfected with GFP-tagged Cactin. Cells were fixed and stained with hoescht to visualise the nuclei. Cactin was found to be expressed exclusively in the nucleus (Figure 3.5A). Bioinformatic analysis of Cactin using a protein family and domain database, PROSITE, predicts two bipartite nuclear localisation sequences (NLSs) in the N-terminal region. In order to assess the importance of nuclear localisation for the

inhibitory effects of Cactin, a truncated form of Cactin was cloned which lacks both nuclear localisation sequences (Figure 3.5B). The C-terminal 200 residues of Cactin are highly conserved and, in *Drosophila*, are responsible for interaction with Cactus. This conserved C-terminal region is intact in the truncation mutant. However, the conserved mid region of Cactin which contains coiled-coil domains is disrupted by the truncation. In order to confirm that truncated Cactin is expressed in the cytoplasm HEK293 cells were transfected with GFP-tagged truncated Cactin. Again, the cells were fixed and stained with hoescht to allow for visualisation of the nuclei. Truncated Cactin was found to be localised in the cytoplasm (Figure 3.5C).

The inhibitory effects of truncated Cactin were then compared with that of the full length protein. HEK293 TLR4 cells were transfected with increasing concentrations of plasmids encoding myc-tagged truncated Cactin and myc-tagged Cactin. Transfected cells were then assayed for the activation of a co-transfected NF- κ B regulated luciferase gene. In response to the TLR4 ligand LPS, full length Cactin exhibited statistically significant inhibitory effects when only 30 ng of plasmid was transfected while truncated-Cactin only exhibited inhibitory effects when higher concentrations of the plasmid were used (Figure 3.6A). Similarly, in response to the endogenous pro-inflammatory stimulus IL-1 β full length Cactin showed statistically significant inhibitory effects at all concentrations while truncated Cactin was ineffective (Figure 3.6B). Finally in cells co-transfected with MyD88, full length Cactin again proved a more powerful inhibitor of NF- κ B as it exhibited significant effects when the lowest concentration of plasmid DNA was transfected and more significant effects than truncated Cactin when higher concentrations of plasmid were used (Figure 3.6C). These results indicate that the nuclear localisation of Cactin is important for manifesting its regulatory effects on the NF- κ B pathway.

3.2.3 Cactin does not bind to a consensus NF- κ B-binding motif and does not co-immunoprecipitate with p65

Given that Cactin appears to exert its negative effects downstream of p65 phosphorylation and is expressed in the nucleus it seemed plausible that Cactin itself might bind to DNA thus preventing NF- κ B from binding to the promoter regions of the various genes it regulates. In order to investigate this possibility, HEK293 cells were transfected with plasmids encoding HA-tagged p65 and myc-tagged Cactin.

HA-tagged p65 was used as a positive control for DNA-binding. Nuclear lysates were generated and incubated with an oligonucleotide encoding a consensus binding site for NF- κ B. Overexpressed p65 translocates to the nucleus in the absence of a stimulus and so the p65-DNA complex was detected by electrophoretic mobility shift assay (EMSA) (Figure 3.7). The specificity of this band was confirmed by supershift analysis (Figure 3.7B) and by the use of a specific competitor for NF- κ B (Figure 3.7A). Nuclear lysates from cells transfected with myc-tagged Cactin were also assessed for DNA-binding activity. However, no protein-DNA complexes were detected indicating that Cactin cannot bind to the consensus NF- κ B binding site excluding the binding of Cactin to the NF- κ B motif as a possible mechanism to explain the inhibitory effects of Cactin on NF- κ B.

Next we investigated if Cactin interacts directly with the NF- κ B subunit p65, thus preventing it from binding to DNA and upregulating gene expression. In order to investigate this proposal, HEK293 cells were co-transfected with myc-tagged Cactin and HA-tagged p65. Cell lysates were generated and immunoprecipitated with an anti-myc antibody. No p65-HA was detected in immunoprecipitates although it was detected in the appropriate whole cell lysates and myc-tagged Cactin was also detected in the immunoprecipitates (Figure 3.8). This suggests that Cactin and the NF- κ B subunit p65 do not interact so again this is unlikely to be the mechanism by which Cactin negatively regulates NF- κ B. Similarly, no interaction was detected between Cactin and the NF- κ B subunits c-Rel or RelB.

3.2.4 Cactin prevents p65 from binding to DNA

Although Cactin does not bind to DNA or interact directly with p65 it was still plausible that Cactin could prevent p65 from binding to DNA by some other mechanism. In order to investigate if this is the case HEK293 TLR4 cells were co-transfected with plasmids encoding myc-tagged Cactin and HA-tagged p65. Nuclear lysates were generated and assessed for DNA binding activity. Again overexpressed p65 displayed the ability to bind DNA in the absence of a stimulus by EMSA and the specificity of this band was confirmed with the use of a specific competitor for NF- κ B. Interestingly in nuclear lysates from cells transfected with both HA-tagged p65 and myc-tagged Cactin there was significantly less p65 bound to the oligonucleotide than in lysates from cells expressing p65 alone (Figure 3.9A). HA-tagged p65 was

detected in both nuclear lysates (Figure 3.9B). This suggests that Cactin inhibits the NF- κ B subunit p65 at the level of DNA-binding.

The inhibition of p65 binding to its cognate DNA sequence by Cactin would predict that the latter could reduce the transactivation potential of p65. To test this hypothesis HEK293 TLR4 cells were transfected with increasing concentrations of a plasmid encoding myc-tagged Cactin and assessed for the regulatory effects of Cactin on p65-HA-induced expression of the co-transfected NF- κ B-regulated luciferase gene. Cactin inhibited p65-induced expression of NF- κ B-regulated luciferase in a statistically significant manner ($p < 0.05$) (Figure 3.10). Finally, ChIP analysis was carried out to confirm that Cactin could block p65 binding to a target promoter in an *in vivo* setting. Again HEK293 TLR4 cells were transfected with plasmids encoding p65-HA and Cactin-myc. Nuclear lysates were generated, sonicated, and immunoprecipitated with anti-p65 or anti-IgG antibodies. PCR analysis was then performed using primers to specifically amplify a region of the IL-8 promoter that contains an NF- κ B binding site. As expected the IL-8 promoter fragment was amplified in immunoprecipitates from cells expressing p65-HA. The IL-8 promoter was not, however, amplified from immunoprecipitates from cells expressing both Cactin-myc and p65-HA, although IL-8 was detected in corresponding Input sample, indicating that Cactin can prevent p65 from binding to the IL-8 promoter (Figure 3.11).

3.2.5 There is some regulation of Cactin mRNA in response to LPS and Poly(I:C)

Given that Cactin inhibits NF- κ B activation we were interested to see if the expression of Cactin can be regulated by NF- κ B as part of autoregulatory system. Firstly, we investigated if the expression of Cactin, at the mRNA level, changes following stimulation with the TLR ligands LPS and Poly(I:C). HEK293 TLR3 cells were treated for various time points with Poly(I:C) while HEK293 TLR4 cells were treated with LPS. RNA was then extracted and converted to cDNA. Realtime PCR was carried out to quantify the amount of Cactin cDNA at each time point. There were only very small changes in levels of Cactin mRNA in response to Poly(I:C) stimulation. There were no statistically significant changes observed but a similar trend was seen each time (Figure 3.12A). In response to LPS there was a more

significant increase in Cactin mRNA following 5 h stimulation (Figure 3.12B). This suggests that there is also some transcriptional regulation of Cactin following NF-κB activation.

3.2.6 The expression of Cactin is stabilised by IL-1 β , LPS and Poly(I:C)

As only modest changes were detected in the mRNA levels of Cactin following LPS and Poly(I:C) stimulation we next investigated if Cactin is regulated at the protein level. To investigate this HEK293 TLR4 or TLR3 cells were transfected with myc-tagged Cactin and treated for various time-points with the different stimuli. IL-1 β , LPS and Poly(I:C) all triggered the stabilisation of Cactin, as visualised by Western Immunoblotting with an anti-myc antibody (Figure 3.13). This regulation of expression was also visualised by fluorescent microscopy. HEK293 TLR4 cells were transfected with GFP-tagged Cactin and stimulated with LPS. At time zero the cells were fixed and stained with hoescht to allow for visualisation of the nuclei. In non-stimulated cells Cactin is expressed in localised nuclear dots. Following LPS-stimulation GFP-Cactin appears more intense and now covers the entire nucleus, no longer in localised dots (Figure 3.14).

3.2.7 The stabilisation of Cactin in response to IL-1 β and Poly(I:C) is dependent on NF-κB activation

In order to investigate if this regulation of Cactin is dependent on NF-κB activation a plasmid encoding a mutated form of I κ B α was employed. I κ B α -Super Repressor (SR) has a mutated serine residue and cannot be phosphorylated, and therefore is not ubiquitinated or degraded, and can suppress NF-κB activation. HEK293 cells were co-transfected with a plasmid encoding myc-tagged Cactin and with or without a plasmid encoding I κ B α -SR. Cells were then treated with IL-1 β . Again the expression of Cactin was stabilised following IL-1 β stimulation. Intriguingly, in cells expressing the I κ B α -SR this stabilisation of expression was not observed (Figure 3.15). This indicates that the regulation of Cactin is dependant on NF-κB activation.

Next we investigated if the regulation of Cactin in response to Poly(I:C) stimulation is also due to NF-κB activation. HEK293 TLR3 cells were co-transfected

with a plasmid encoding myc-tagged Cactin and with or without a plasmid encoding I κ B α -SR and treated for various time-points with Poly(I:C). Stabilisation of Cactin expression was observed following Poly(I:C) stimulation. Once more this regulation was prevented by the overexpression of the NF- κ B repressor (Figure 3.16). These results indicate that Cactin is regulated by NF- κ B.

3.2.8 Cactin is not ubiquitinated in response to LPS stimulation

The stabilisation of Cactin may be due to post-translational modification following NF- κ B activation. Thus, we wanted to investigate what these modifications might be. Ubiquitination plays a very important role in TLR-signalling as many of the signalling intermediates of the pathway are activated by ubiquitination. For this reason we first wanted to examine if Cactin is ubiquitinated following LPS stimulation. To do this HEK293 TLR4 cells were co-transfected with plasmids encoding myc-tagged Cactin and HA-tagged ubiquitin. Transfected cells were then treated with LPS. Cell lysates were generated and immunoprecipitated using an anti-myc antibody and probed for the presence of HA. There was no ubiquitin-HA detected in immunoprecipitates although it was detected in the corresponding whole cell lysates (Figure 3.17). Also, Cactin-myc was detected in the immunoprecipitates indicating that the immunoprecipitation process was successful. It is worth noting that it may appear from the whole cell lysates that Cactin is having some effect on global ubiquitination, however the observed effect is possibly due to competition for the transcription machinery of the cell. The above findings indicate that Cactin is not ubiquitinated and therefore the stabilisation of expression must be due to some other form of modification and mechanism.

3.2.9 Cactin is phosphorylated following IL-1 β stimulation

Next we investigated if Cactin is phosphorylated following NF- κ B activation. HEK293 TLR4 cells were transfected with a plasmid encoding myc-tagged Cactin and treated for various time-points with IL-1 β . Cell extracts were generated, split and incubated with and without Calf Intestinal Alkaline Phosphatase (CIP) for 1 h at 37°C. CIP can be used to catalyse the hydrolysis of phosphate groups from phosphorylated serine, tyrosine and threonine residues in proteins. The extracts were then subjected

to Western blotting. Stabilisation of Cactin was observed in extracts from cells treated with IL-1 β when samples were incubated without CIP. Interestingly the same stabilisation was not observed when the same extracts had been incubated with CIP (Figure 3.18). These results suggest that the stabilisation of Cactin following IL-1 β stimulation is due to phosphorylation.

To confirm these findings another approach was taken in which HEK293 TLR4 cells were transfected with either myc-tagged Cactin or empty vector pcDNA3.1. Cell lysates were generated and interacting proteins were dissociated. The lysates were then immunoprecipitated using an anti-myc antibody. The immunoprecipitates were probed for the presence of phosphorylated serine residues which were detected in immunoprecipitates from cells expressing Cactin-myc. The strongest phosphorylation was observed 10 minutes after IL-1 β stimulation (Figure 3.19). Therefore Cactin is phosphorylated at one or more serine residues following NF- κ B activation. Immunoprecipitates were also probed for the presence of phosphorylated tyrosine and threonine residues but none were detected.

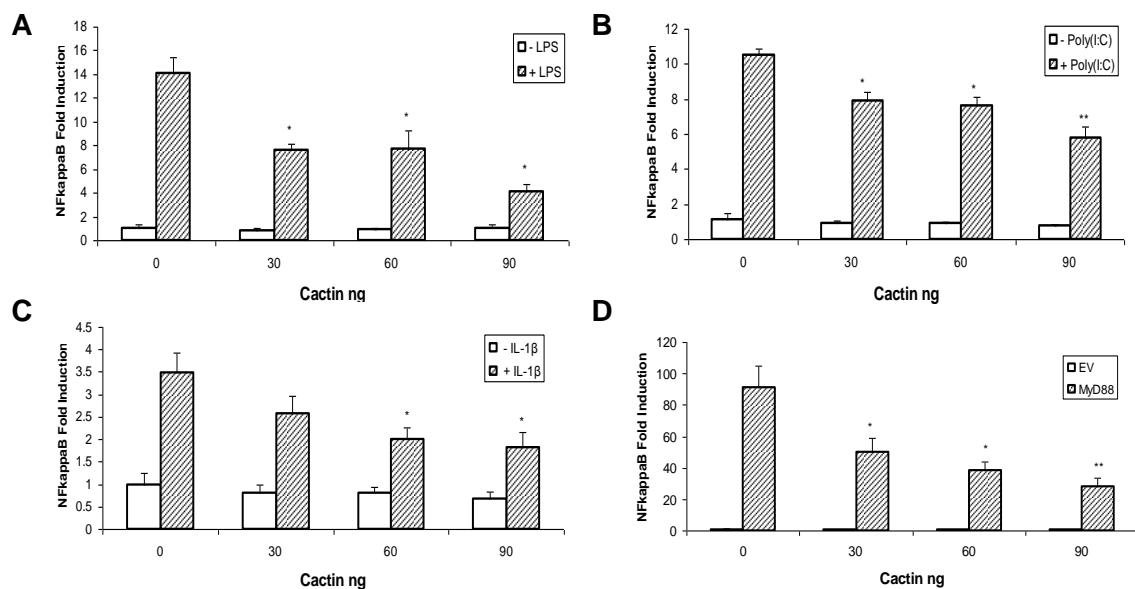


Figure 3.1 Cactin inhibits LPS, Poly(I:C), IL-1 β and MyD88-mediated activation of NF- κ B

HEK293 cells stably transfected with TLR4 or TLR3, were cotransfected with plasmids encoding NF- κ B-regulated firefly luciferase (80 ng) and constitutively expressed TK Renilla (20 ng) and with or without plasmids encoding myc-tagged Cactin (30, 60 or 90 ng) or MyD88 (50 ng). Empty vector (EV) pcDNA3.1 was used to normalise the amount of total DNA transfected. Transfected cells were left overnight and then treated with (A) LPS (100 ng/ml), (B) Poly(I:C) (25 μ g/ml) or (C) IL-1 β (10 ng/ml) for 24 h. Cell lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data are presented as the mean +/- S.E.M of triplicate determinations from three independent experiments. * p<0.05, ** p<0.01 paired t-test.

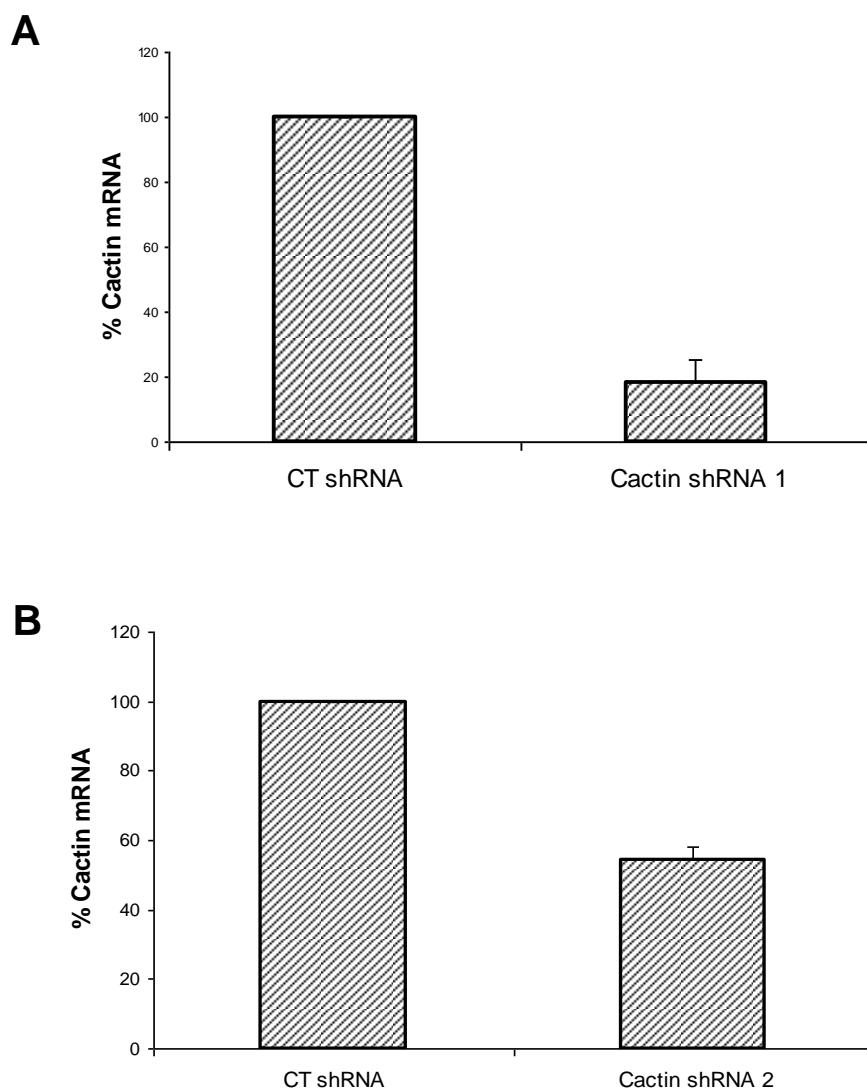


Figure 3.2 Knockdown of endogenous Cactin by lentiviral vector-encoded shRNA in THP-1 and U373 cell lines

(A) THP-1 and (B) U373 cells stably transduced with Control or Cactin specific-shRNA were cultured in the presence of puromycin (10 µg/ml). Total RNA was extracted and cDNA was generated using RT-PCR. Samples were subsequently assayed by quantitative real-time PCR for levels of Cactin. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments.

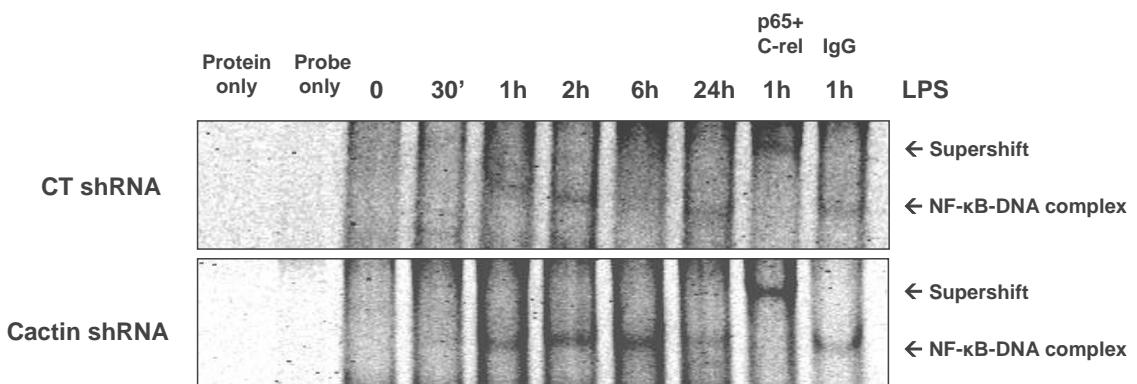


Figure 3.3 Cactin-specific shRNA augments LPS-induced activation of NF-κB in THP-1 cells

THP-1 cells stably transduced with Control and Cactin-specific shRNA were grown in 6-well plates for 24 h. Cells were then treated with LPS (100 ng/ml) for the indicated time periods. Nuclear extracts were generated and assayed for binding to a fluorescently labelled oligonucleotide encoding a consensus NF-κB-binding motif by EMSA. Nuclear extracts from cells stimulated for 1 h with LPS were pre-incubated with anti-p65 and c-rel or non-specific IgG antibody before assaying NF-κB-DNA binding activity. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are indicative of two independent experiments.

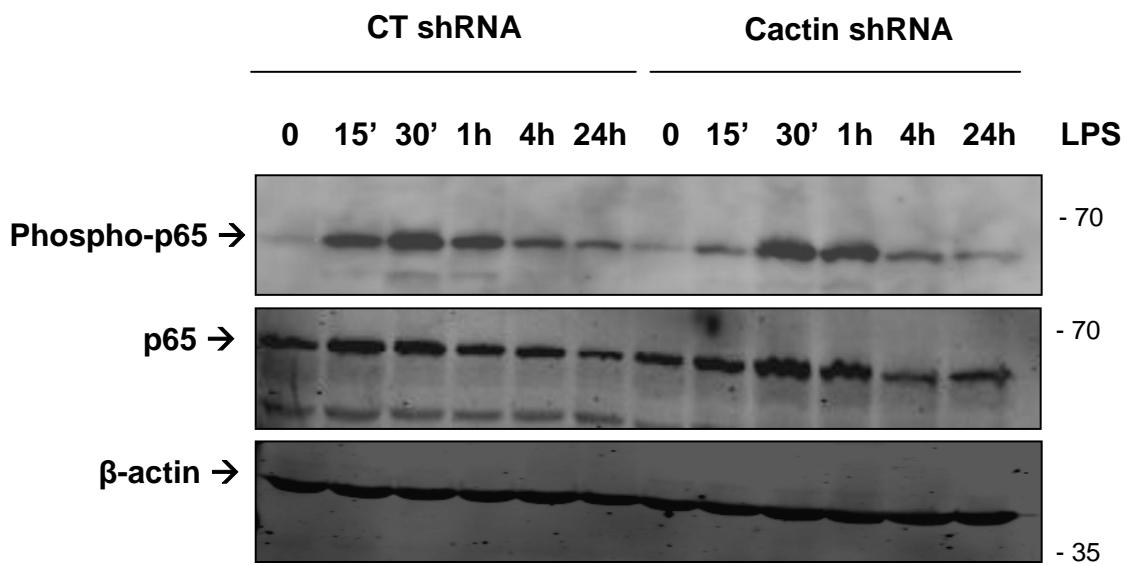


Figure 3.4 Knockdown of endogenous Cactin does not affect phosphorylation of p65 in U373 cells

U373 cells stably transduced with Control or Cactin-specific shRNA were grown in 6-well plates for 24 h. Cells were then stimulated with LPS (100 ng/ml) for the indicated time periods. Cells were harvested in SDS-PAGE sample buffer and lysates were resolved by SDS-PAGE. Western blotting was carried out probing with anti-phospho-p65, anti-p65 and anti-β-actin antibodies. Blots shown are representative of two independent experiments.

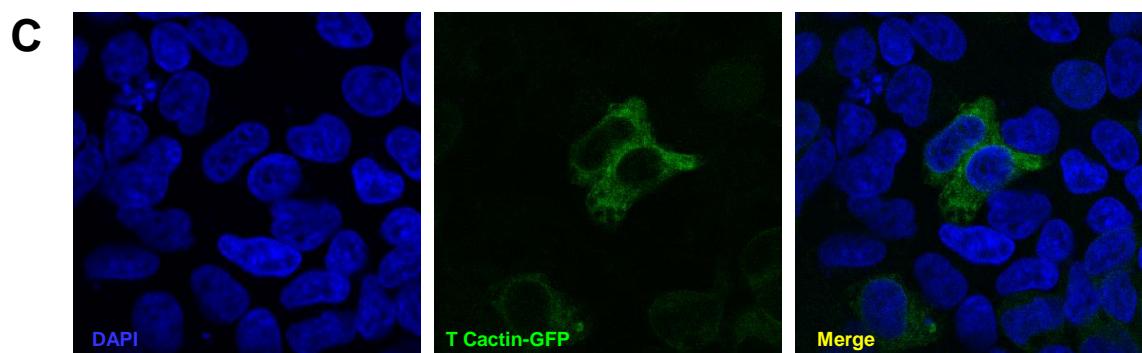
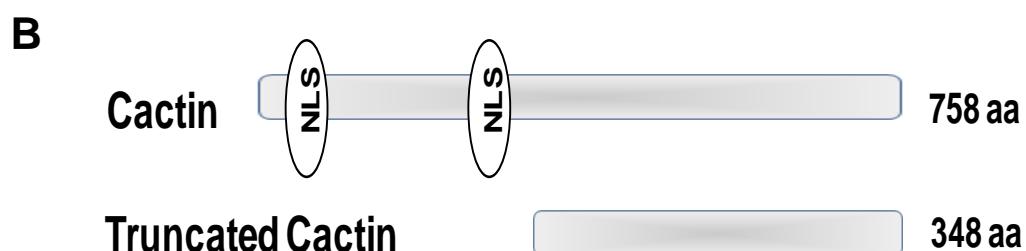
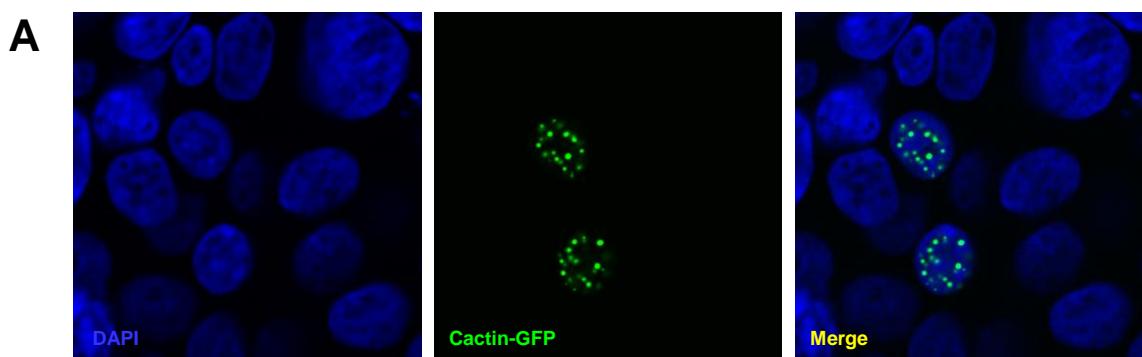


Figure 3.5 Cactin is localised in the nucleus while the truncated Cactin mutant is expressed in the cytoplasm

HEK293 TLR4 cells were transfected with (A) GFP-tagged Cactin or (C) GFP-tagged truncated Cactin (800 ng). Cells were fixed 24 h post-transfection, stained with hoescht, and mounted in anti-fade medium. Confocal images were captured using Olympus FluoView FV1000 confocal laser scanning microscope equipment. Data analysis was performed using the Olympus FV-10 ASW imaging software. (B) Schematic representation of full length and truncated Cactin with location of nuclear localisation sequences (NLS)s depicted.

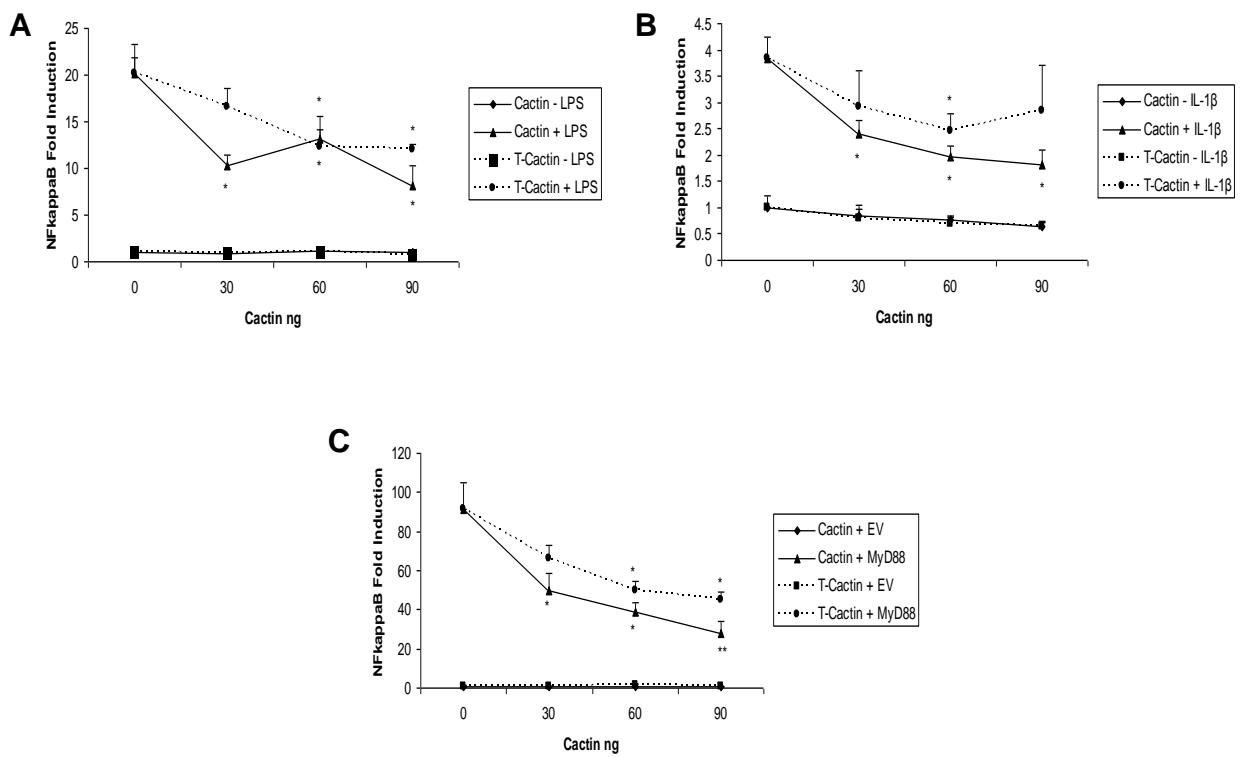


Figure 3.6 Full length Cactin is a more powerful inhibitor of LPS, IL-1 β and MyD88-mediated activation NF- κ B than truncated Cactin

HEK293 cells stably transfected with TLR4 were co-transfected with plasmids encoding NF- κ B regulated firefly luciferase (80 ng) and constitutively expressed TK Renilla (20 ng) and with or without plasmids encoding Cactin-myc (30, 60 or 90 ng), truncated Cactin-myc (30, 60 or 90 ng) or MyD88 (50 ng). Empty vector (EV) pcDNA3.1 was used to normalise the amount of total DNA transfected. Transfected cells were left overnight and then treated with (A) LPS (100 ng/ml) or (B) IL-1 β (10 ng/ml) for 24 h. Cell lysates were assayed for firefly and *Renilla* luciferase activity. Data are presented as the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p<0.05, **p<0.01; ligand stimulated cells transfected with EV compared with ligand stimulated cells transfected with Cactin/T-Cactin.

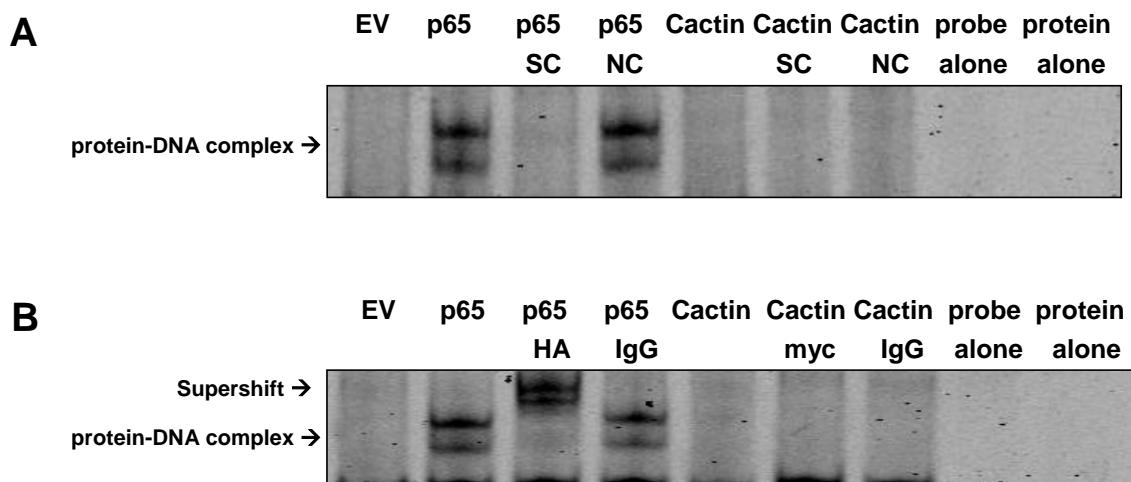


Figure 3.7 Cactin does not bind to a consensus NF-κB-binding motif

HEK293 cells were transfected with empty vector (EV) pcDNA3.1 (1 µg), p65-HA (1 µg) or Cactin-myc (1 µg). Nuclear lysates were generated and assayed for binding to a fluorescently labelled oligonucleotide containing a consensus NF-κB-binding motif by EMSA. **(A)** Nuclear extracts from cells transfected with p65-HA and Cactin-myc were preincubated with an unlabelled oligonucleotide encoding the same binding motif, specific competitor (SC), or an oligonucleotide encoding an unrelated DNA sequence, non-specific competitor (NC), before assaying for DNA binding activity. **(B)** Nuclear extracts from cells transfected with p65-HA and Cactin-myc were preincubated with anti-HA, anti-myc or non-specific IgG antibodies before assaying for DNA binding activity. Results are representative of two independent experiments.

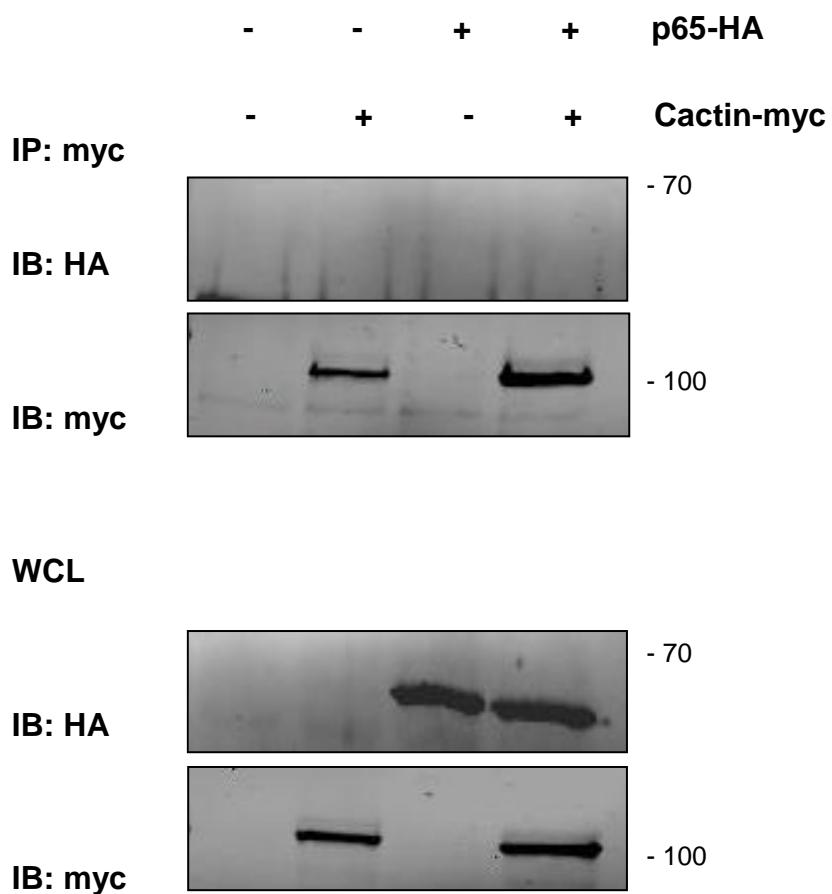


Figure 3.8 Cactin does not interact with p65

HEK293 TLR4 cells were transfected with or without myc-tagged Cactin (2 µg) and HA-tagged p65 (2 µg). pcDNA3.1 was used to normalise the amount of total DNA transfected. Cell extracts were generated 24 h post transfection and were immunoprecipitated with an anti-myc antibody. Immunoprecipitates and whole cell lysates (WCL) were subjected to Western immunoblotting using anti-HA and anti-myc antibodies. Immunoreactivity was visualised using the Odyssey infrared imaging system. Blots shown are representative of two independent experiments.

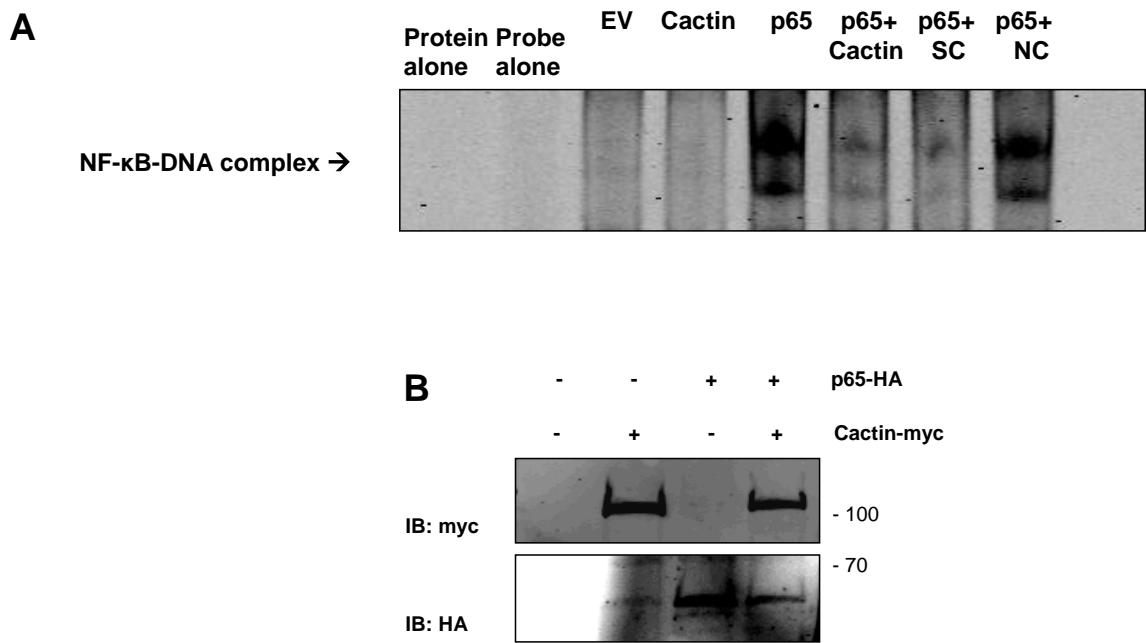


Figure 3.9 Cactin inhibits p65 binding to a consensus NF-κB binding site

HEK293 TLR4 cells were transfected with plasmids encoding myc-tagged Cactin (1 µg) and HA-tagged p65 (1 µg). Empty vector (EV) pcDNA3.1 was used to normalise the amount of total DNA transfected. 24 h post transfection nuclear extracts were generated and (A) assayed for binding to a fluorescently labelled oligonucleotide encoding a consensus NF-κB-binding motif by EMSA. Nuclear extracts from cells transfected with p65-HA were preincubated with an unlabelled oligonucleotide encoding the same binding motif, specific competitor (SC), or an oligonucleotide encoding an unrelated DNA sequence, non-specific competitor (NC), before assaying for DNA binding activity. (B) Nuclear lysates were also subjected to Western blotting using anti-myc and anti-HA antibodies. Results shown are representative of two independent experiments.

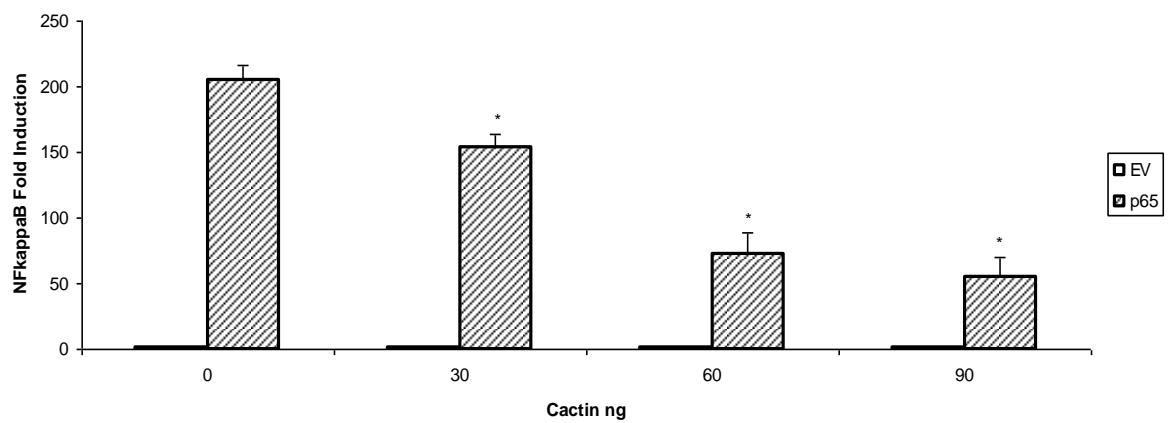


Figure 3.10 Cactin inhibits p65-mediated activation of NF-κB

HEK293 TLR4 cells were co-transfected with plasmids encoding NF-κB-regulated firefly luciferase (80 ng) and constitutively expressed TK Renilla (20 ng) and with or without plasmids encoding Cactin-myc (30, 60 and 90 ng) and p65-HA (50 ng). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection cell lysates were generated. Lysates were assayed for firefly and *Renilla* luciferase activity. The data presented are mean +/- S.E.M of triplicate determinations from a representative of three independent experiments. * p < 0.05, paired t-test.

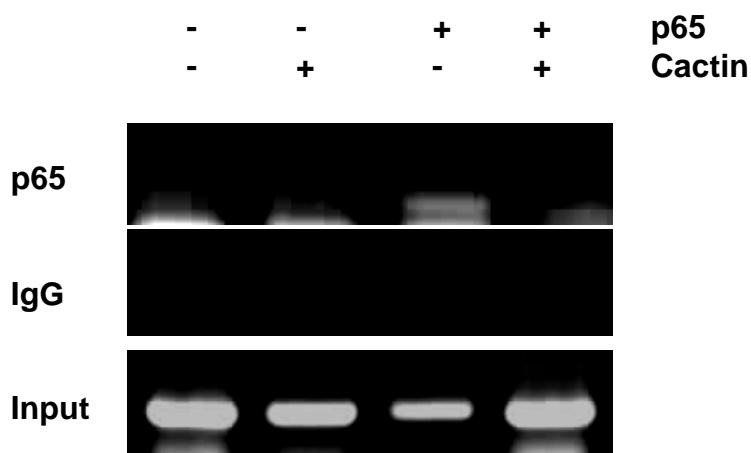


Figure 3.11 Cactin inhibits binding of p65 to the IL-8 promoter

HEK293 TLR4 cells were transfected with HA-tagged p65 (8 µg) and myc-tagged Cactin (8 µg). Empty vector pcDNA3.1 was used to normalise the amount of total DNA transfected. Sonicated nuclear lysates were immunoprecipitated with anti-p65 or non-immune IgG antibodies. Immunoprecipitated DNA was analysed for the presence of specific IL-8 promoter fragments by PCR. Input DNA was removed from the sonicated nuclear lysates before immunoprecipitation and was also analysed for the presence of IL-8 promoter. Results are representative of two independent experiments.

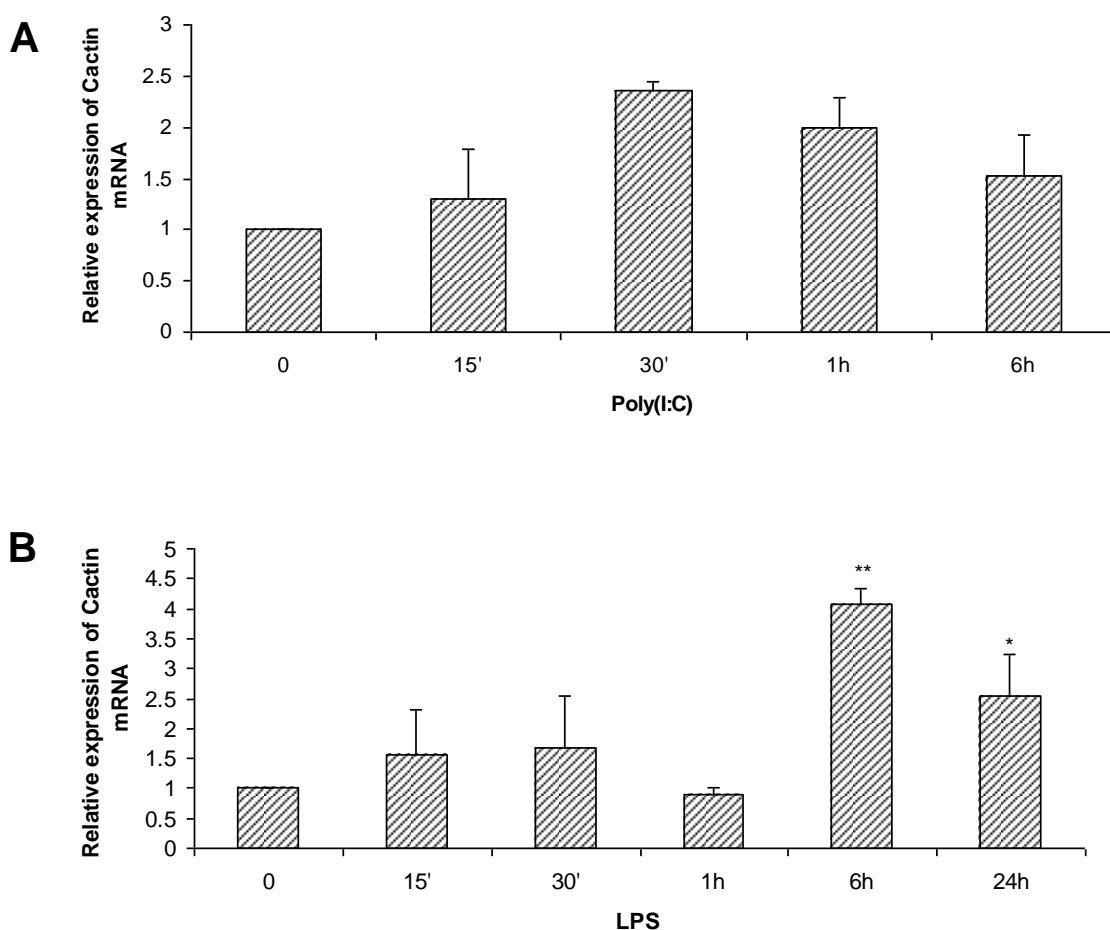


Figure 3.12 Regulation of Cactin mRNA expression by LPS and Poly(I:C) stimulation

(A) HEK293 TLR3 were treated with Poly(I:C) (25 µg/ml) while (B) HEK293 TLR4 cells were treated with LPS (100 ng/ml) for the indicated time periods. Total RNA was extracted and cDNA was generated using RT-PCR. Samples were subsequently assayed by quantitative real-time PCR for levels of Cactin. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. * p<0.05, **p<0.01 paired t-test.

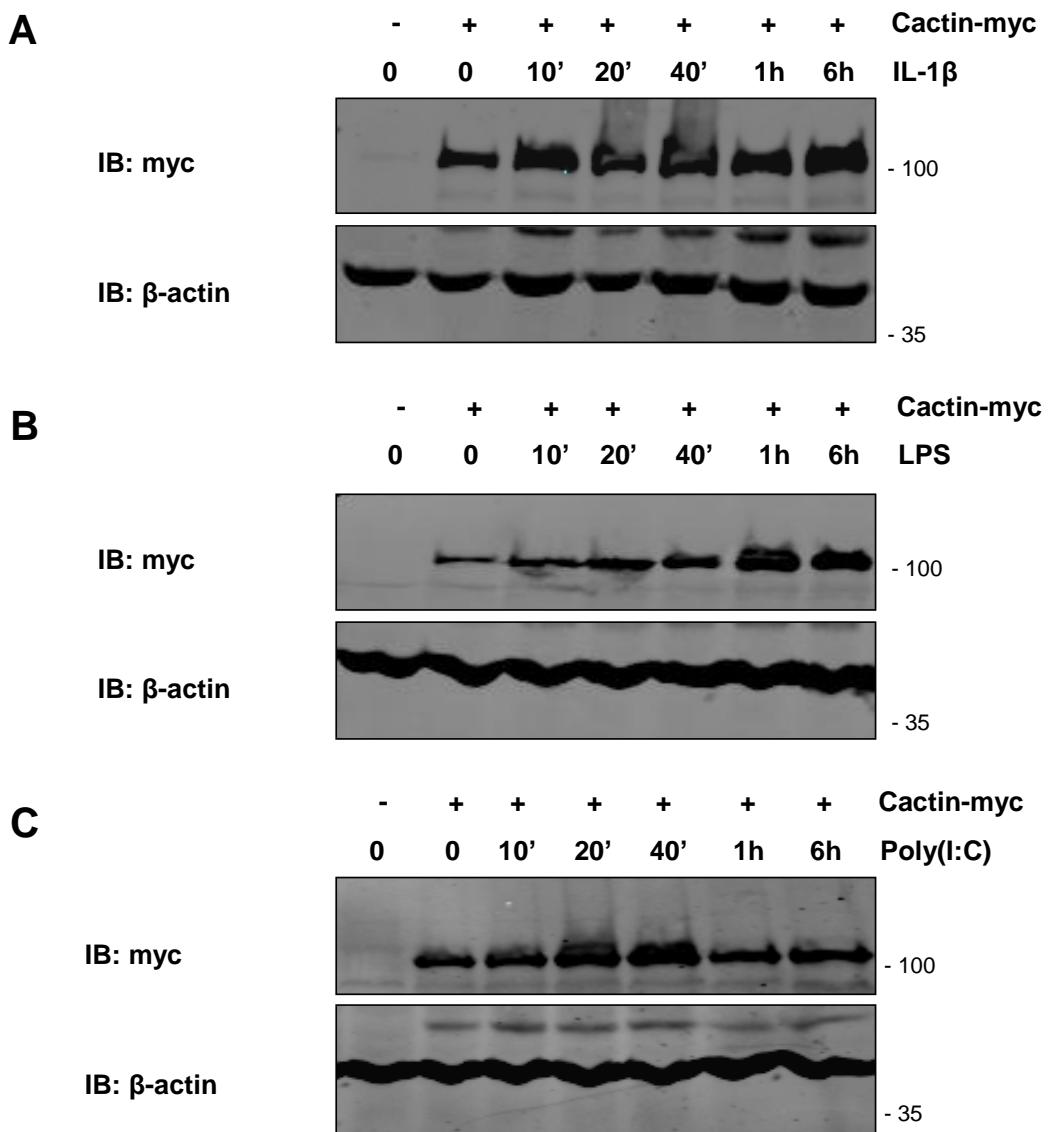


Figure 3.13 IL-1 β , LPS and Poly(I:C) regulate protein expression levels of Cactin
 HEK293 cells stably transfected with TLR4 or TLR3 were transfected with a plasmid encoding myc-tagged Cactin (2 μ g) or empty vector pcDNA3.1 (2 μ g). The cells were stimulated with (A) IL-1 β (10 ng/ml), (B) LPS (100 ng/ml) or (C) Poly(I:C) (25 μ g/ml) for the indicated time periods. Cell lysates were generated and subjected to Western blotting using anti-myc and anti- β -actin antibodies. Results are indicative of three independent experiments.

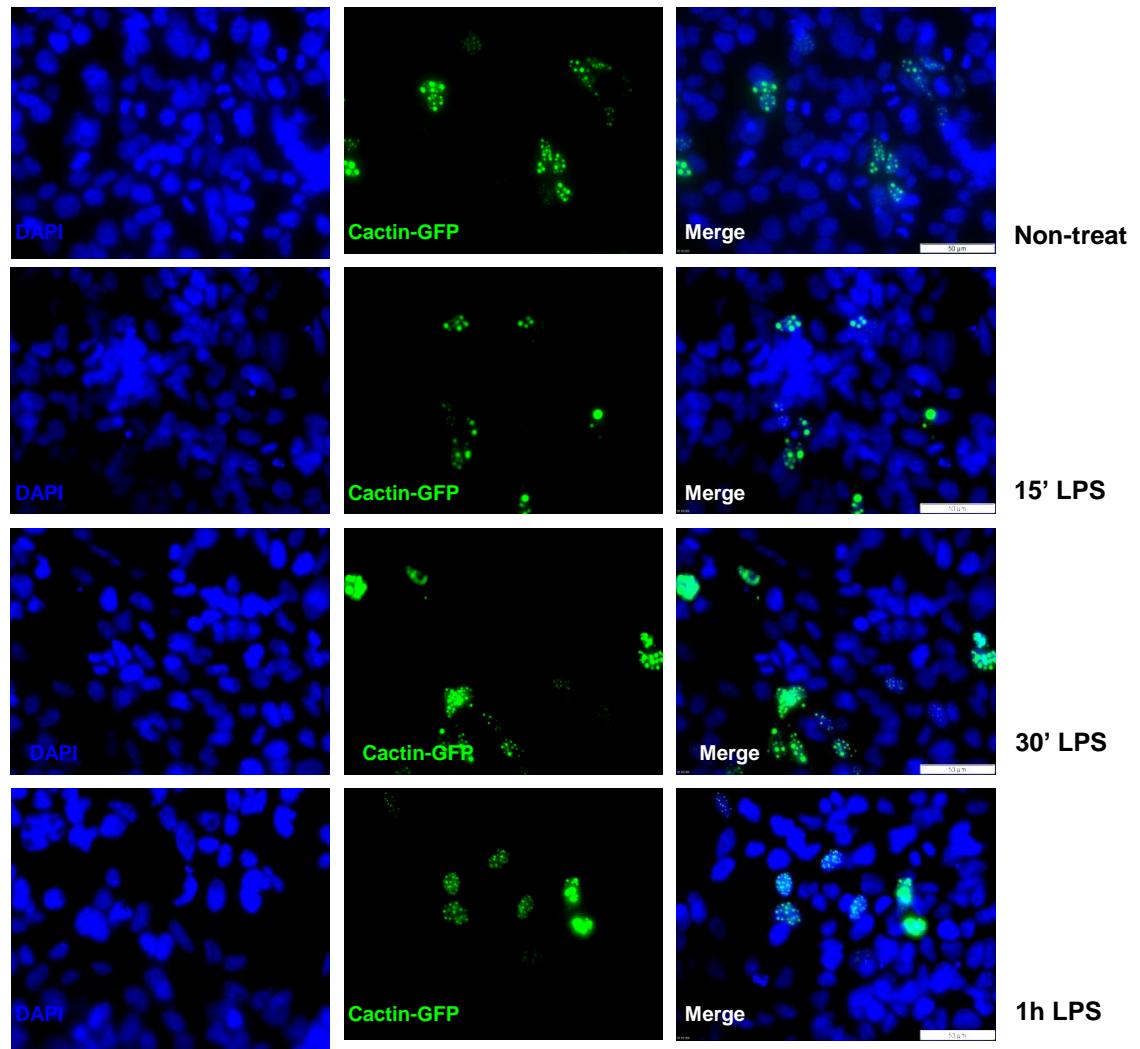


Figure 3.14 The expression pattern of Cactin is regulated by LPS

HEK293 TLR4 cells were transfected with GFP-tagged Cactin (1 μ g). Transfected cells were left overnight and then treated for the indicated time-points with LPS (100 ng/ml). Cells were then fixed, stained with hoescht, and mounted in anti-fade media. Slides were visualised using fluorescent microscopy. Results shown are a representative of three independent experiments.

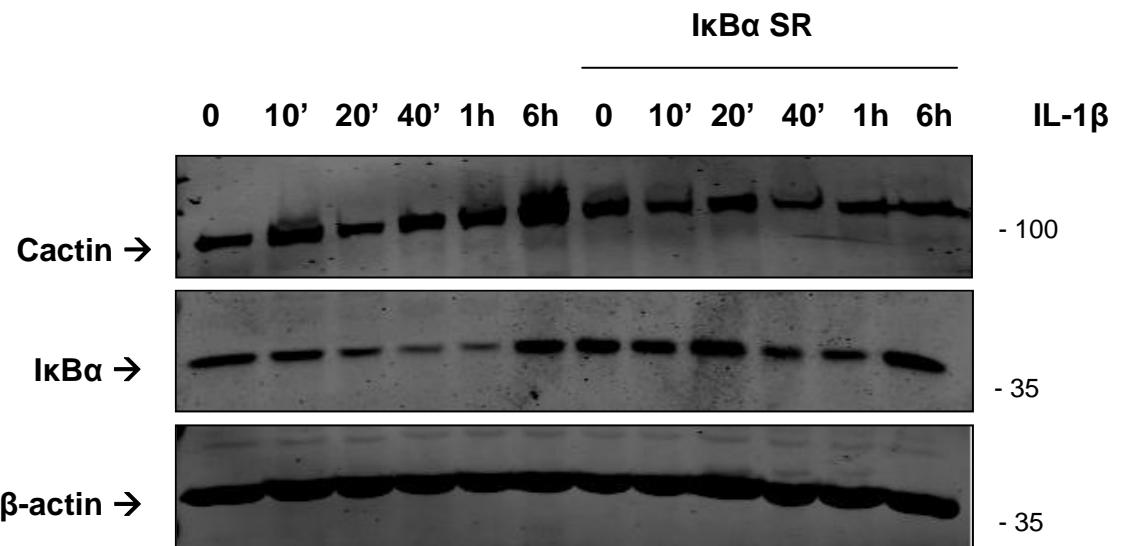


Figure 3.15 Regulation of Cactin in response to IL-1 β is dependent on NF- κ B activation

HEK293 TLR4 cells were transfected with myc-tagged cactin (2 μ g) and with or without I κ B α Super Repressor (SR) (2 μ g). pcDNA3.1 was used to normalise the amount of total DNA transfected. Transfected cells were left overnight and then treated with IL-1 β (10 ng/ml) for the indicated time periods. Cell lysates were generated and subjected to Western blotting using anti-myc, anti-I κ B α and anti- β -actin antibodies. Blots shown are representative of three independent experiments.

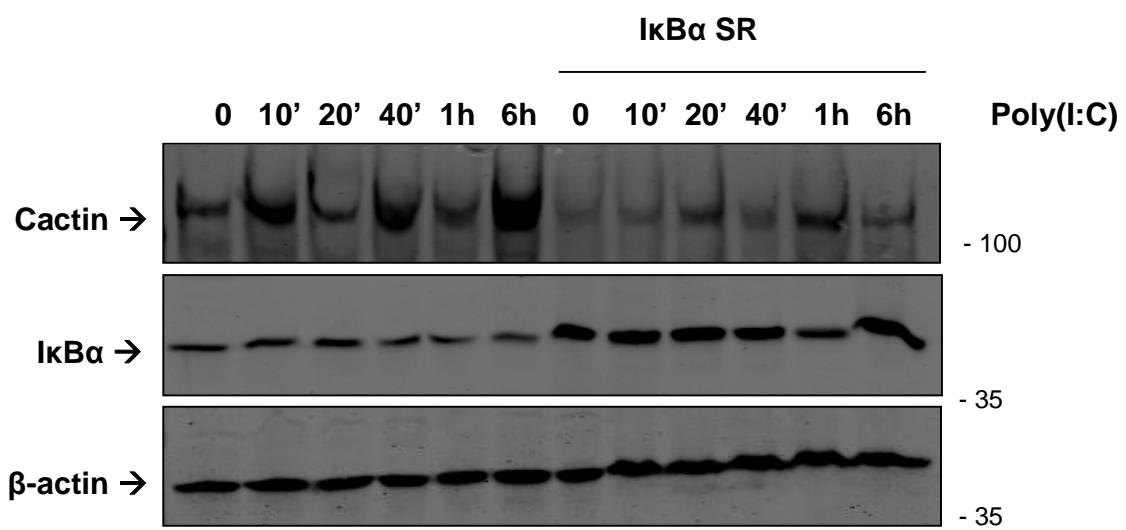


Figure 3.16 Regulation of Cactin in response to Poly(I:C) is dependent on NF- κ B activation

HEK293 TLR3 cells were transfected with myc-tagged Cactin (2 μ g) and with or without I κ B α -Super Repressor (SR) (2 μ g). pcDNA was used to normalise the amount of total DNA transfected. Transfected cells were left overnight and then treated with or without Poly(I:C) (25 μ g/ml) for the indicated time periods. 24 h post transfection cell lysates were generated and subjected to Western blotting using anti-myc, anti-I κ B α and anti- β -actin antibodies. Results shown are indicative of three independent experiments.

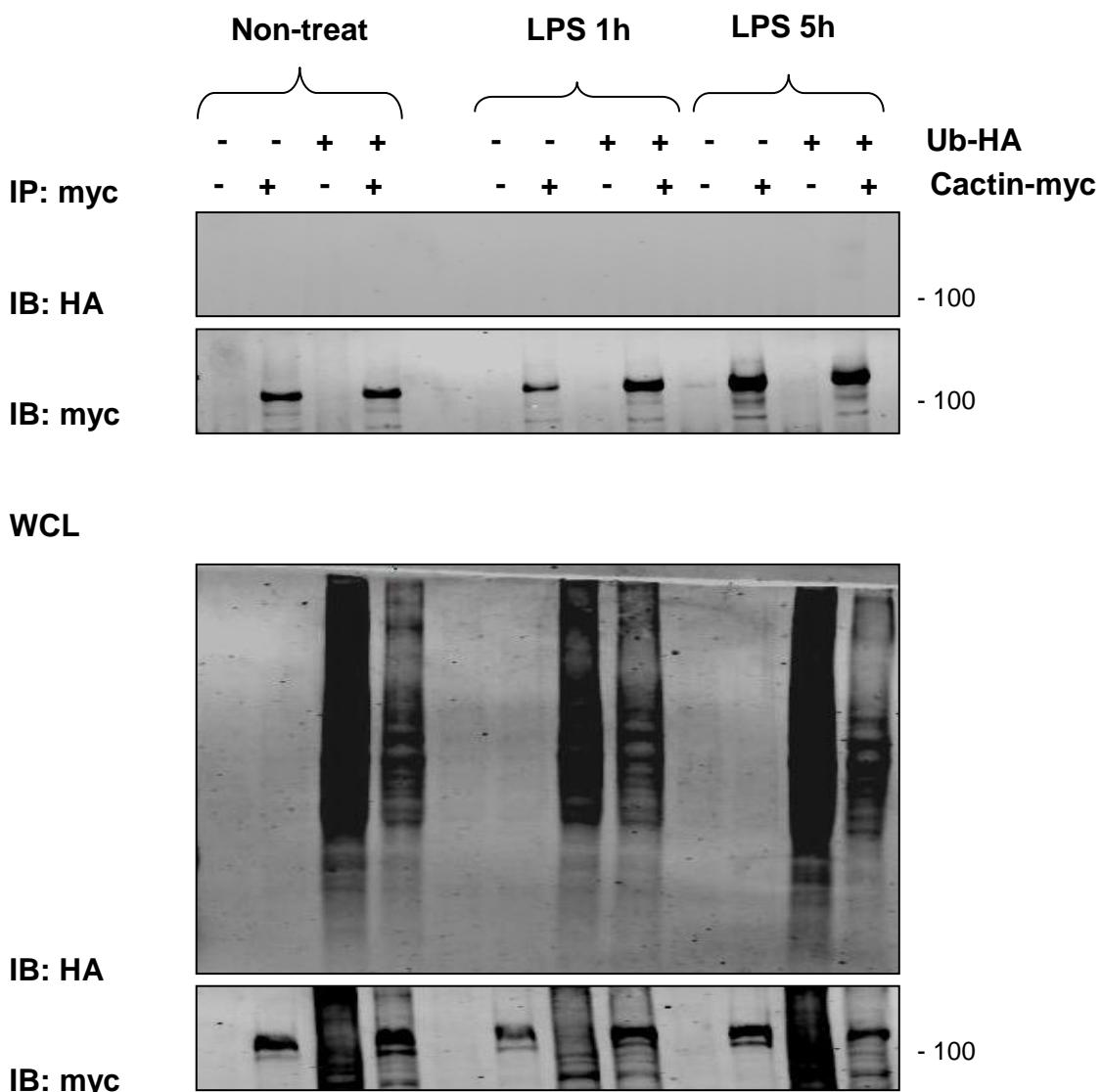


Figure 3.17 Cactin is not ubiquitinated following LPS stimulation

HEK293 TLR4 cells were co-transfected with or without myc-tagged Cactin (1 μ g) and HA-tagged ubiquitin (3 μ g). pcDNA3.1 was used to normalise the total amount of DNA transfected. Transfected cells were left overnight and then treated with or without LPS (100 ng/ml) for the indicated time periods. Lysates were generated and immunoprecipitated with an anti-myc antibody. Immunoprecipitates and whole cell lysates were subjected to Western blotting using anti-HA and anti-myc antibodies. Blots shown are representative of three independent experiments.

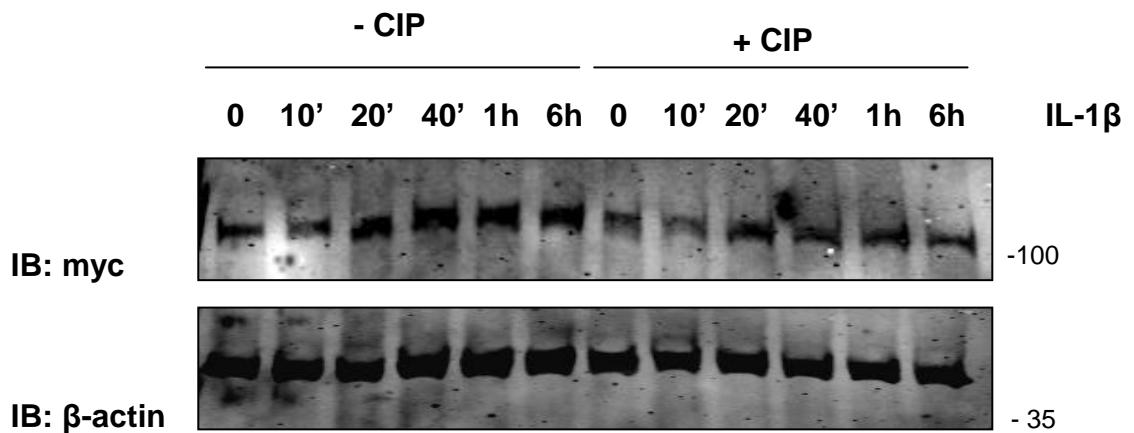


Figure 3.18 Cactin is phosphorylated following IL-1 β stimulation

HEK293 TLR4 cells were transfected with Cactin-myc (2 μ g). The cells were left overnight and then treated with IL-1 β for the indicated time periods. Lysates were generated 24 h post transfection. Cell lysates were split and then incubated with or without CIP at 37°C for 1 h. Lysates were then resolved by SDS-PAGE and subjected to Western blotting using anti-myc and anti- β -actin antibodies. Immunoreactivity was visualised using the Odyssey infrared imaging system. Blots shown are representative of three independent experiments.

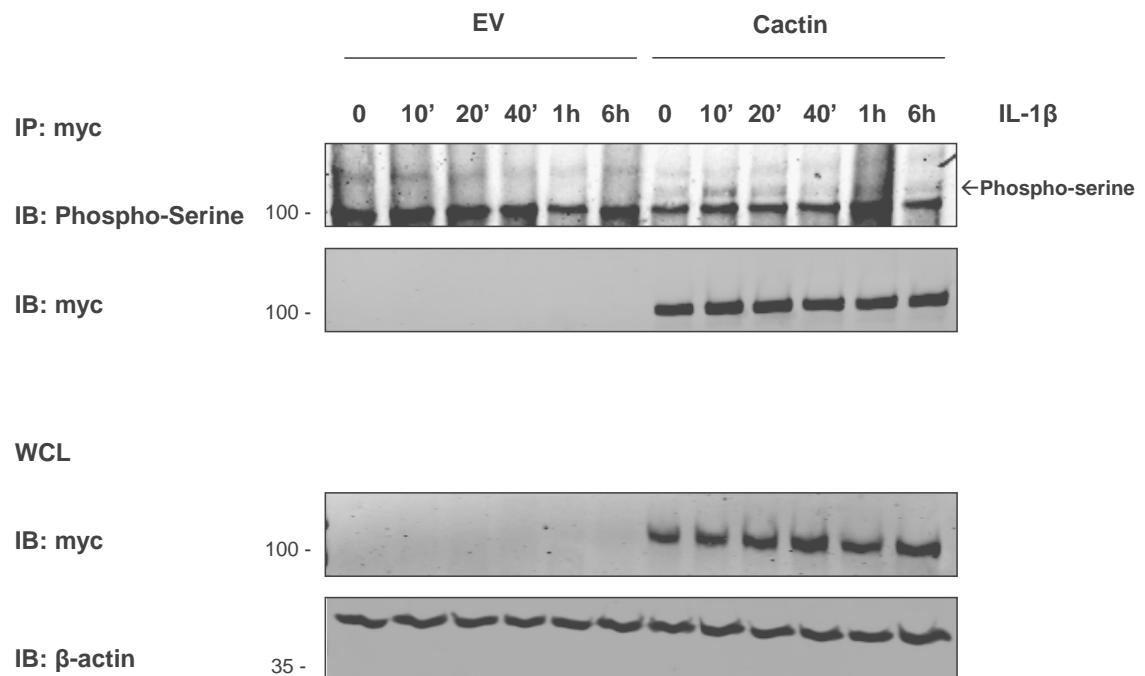


Figure 3.19 Cactin is serine phosphorylated following IL-1 β stimulation

HEK293 TLR4 cells are transfected with myc-tagged Cactin (2 μ g) or empty vector (EV) pcDNA3.1 (2 μ g). Transfected cells were left overnight and then treated with IL-1 β (10 ng/ml) for the indicated time periods. Cell lysates were generated and incubated at 95°C for 5 min with 0.5% SDS to dissociate interacting proteins. Lysates were then immunoprecipitated with an anti-myc antibody. Immunoprecipitates and whole cell lysates were then subjected to Western immunoblotting with anti-phospho-serine, anti-myc and anti- β -actin antibodies. Blots shown are representative of two independent experiments.

3.3 Discussion

Given that *Drosophila* Cactin appears to positively regulate the translocation of the Rel transcription factor dorsal to the nucleus it may appear surprising that the human orthologue acts as a negative regulator of NF-κB activation. However, homology does not always correspond to conserved function (Jensen, 2001). Cactin inhibits the activation of NF-κB in response to a variety of stimuli including the TLR4 and TLR3 ligands LPS and Poly(I:C) and the pro-inflammatory cytokine IL-1β. The effects of Cactin are also independent of cell-type as the inhibitory effects of Cactin were observed in HEK, THP-1 and U373 cell lines. It is worth noting that suppression of endogenous Cactin in THP-1 cells led to more prolonged activation of NF-κB in response to LPS. This suggests that Cactin may function as an endogenous control system, designed to prevent prolonged activation of NF-κB in response to inflammatory stimuli. Constitutive activation of NF-κB is associated with a number of diseases including rheumatoid arthritis, multiple sclerosis and asthma (Li and Verma, 2002), highlighting the importance of negative regulators such as Cactin in controlling innate immune signalling.

Cactin was first observed in 1999 as an antigen in patients with renal cell carcinoma and was termed NY-REN-24 (Scanlon et al., 1999). Cactin is also differentially expressed in patients with multiple myeloma (Davies et al. 2003). Furthermore, the gene for Cactin maps closely to a microsatellite marker, D19S216, a site which is defective in uterine cancers (Lee et al., 1998). This is not entirely unexpected given that Cactin is a regulator of NF-κB which upregulates pro-survival genes and has been implicated in many Cancers. Interestingly dysregulation of Rel transcription factors leads to the production of melanotic tumours in *Drosophila* and unpublished findings from Prof. Ruth Stewards laboratory indicate that larvae from transgenic flies expressing a *dCactin*-RNAi construct develop melanotic tumours (<http://waksman.rutgers.edu/Waks/Steward/steward.html>). Such studies in conjunction with the regulatory effects of Cactin on the NF-κB pathway suggests that Cactin may play a regulatory role in tumourogenesis and cancer.

dCactin interacts with Cactus, the homologue of human IκBα and modulates Rel transcription factor activation (Lin et al., 2000). In contrast, human Cactin appears to function downstream of IκBα. Phosphorylation and degradation of the IκBs is not sufficient for maximal NF-κB response (Oeckinghaus and Ghosh, 2009).

The subunits of NF-κB are also subject to post-translational modifications including phosphorylation, acetylation and ubiquitination (Perkins, 2006). This is another point at which the activation of NF-κB can be controlled. Interestingly, Cactin did not affect the phosphorylation of p65 indicating that Cactin targets the pathway downstream of this NF-κB subunit modification. Cactin is localised to the nucleus and the nuclear localisation appears to be important for manifesting its inhibitory effects. These findings led us to ask the question, can Cactin target NF-κB DNA-binding? Overexpressed p65 translocates to the nucleus where it binds κB sites in target genes in the absence of a proinflammatory stimulus. This allowed us to investigate if Cactin can inhibit p65 at the level of DNA-binding. EMSA was initially employed to show that Cactin can inhibit p65 binding to a consensus DNA-binding motif. A more physiologically relevant model system, using ChIP analysis, was also used to show that Cactin can inhibit p65 binding to the IL-8 promoter.

Many inhibitors of NF-κB are regulated by proinflammatory stimuli and thus establish a self-regulatory control system. Sterile α and HEAT-armadillo motifs (SARM) is a TIR domain containing adaptor which negatively regulates TLR signalling. The expression of SARM is strongly enhanced by LPS stimulation (Carty et al., 2006). The negative regulator A20 also increases in expression in response to LPS and TNF (Opiari et al., 1990). Another example is SOCS1 protein which is induced by cytokine stimulation (Akira and Takira, 2004). Cactin is induced by LPS stimulation. Higher levels of Cactin mRNA were detected in cells treated for 5 h with LPS, quantified by real-time PCR. Furthermore, Cactin appears to be stabilised by IL-1β, LPS and Poly(I:C) stimulation due to post-translational modification. Cactin appears to be serine phosphorylated in response to pro-inflammatory signals and this seems to be associated with increase protein stability. Furthermore this stabilisation is dependent on NF-κB activation. Again this is not unusual as NF-κB upregulates the transcription of many genes, including genes encoding its own inhibitors, such as IκBa. This again demonstrates that Cactin may function as an endogenous hauling mechanism for NF-κB activation. NF-κB might upregulate the expression of the gene encoding the protein kinase responsible for Cactin phosphorylation. Alternatively NF-κB activation might induce a phosphatase inhibitor leading to increased phosphorylation of Cactin.

This chapter demonstrates that Cactin is a negative regulator of NF-κB by inhibiting its DNA-binding activity. Cactin is also a powerful negative regulator of

IRF3 and IRF7. It was interesting to determine if Cactin inhibits the activation of these transcription factors by a similar mechanism and this forms the content of the next chapter.

Chapter 4

**Exploring the role of Cactin in IRF3 and IRF7
regulation and characterising the effect of Cactin on
TLR-mediated MAPK activation**

4.1 Introduction

In addition to NF- κ B, TLR-signalling also regulates members of the Interferon Regulatory Factor (IRF) family of transcription factors. The IRF family consist of nine members which all possess an N-terminal DNA binding domain (DBD) and a C-terminal IRF association domain (IAD) (Savitsky et al., 2010). IRFs regulate the expression of Type 1 interferons (IFNs) which are multifunctional secreted proteins involved in antiviral defence, immune activation and cell growth and regulation (Goodbourn et al., 2000). Type I IFNs are encoded by one IFN β gene and several IFN α genes. The IRF family members IRF3 and IRF7 have been extensively studied in the context of Type I IFN regulation in response to viral infection (Taniguchi et al., 2001). IRF3 is expressed at high constitutive levels in most cells and is expressed predominantly in the cytoplasm. Viral or bacterial infection leads to specific phosphorylation of a serine/threonine cluster in the C-terminal region of IRF3 which promotes dimerisation, nuclear translocation and association with co-activators CBP and p300 (Yoneyama et al., 1998). IRF3 can then bind to the IFN β promoter with the co-operation of other transcription factors such as NF- κ B and induce the early phase of IFN β production. This leads to the subsequent activation of the transcription factor complex IFN-stimulated gene factor 3 (ISGF3), made up of signal transducer and activator of transcription (STAT)1, STAT2 and IRF9 (Taniguchi et al., 2001). IRF7 is expressed at much lower levels in most cells but is strongly induced by ISGF3 (Genin et al., 2003). IRF7 is also phosphorylated in its C-terminal region which promotes dimerisation, with itself or with IRF3, and translocation to the nucleus. IRF3 and IRF7 then cooperate to amplify IFN α and IFN β production in the late phase of the IFN response.

TLR3 is located on the membrane of endosomes and phagosomes and recognises viral double stranded dsRNA derived from dsRNA viruses or as replication intermediates from ssRNA viruses (Akira et al., 2006). Upon recognition of dsRNA TLR3 recruits the adaptor protein TRIF to the receptor. TRIF can then associate with TRAF3 which activates inducible IKK (IKK-i) and TRAF family member-associated NF- κ B activator (TANK)-binding kinase 1 (TBK1). Both kinases have been shown to directly phosphorylate and activate IRF3 and IRF7 (Sharma et al., 2003). TLR3

stimulation also leads to TRIF-dependent NF- κ B activation through activation of receptor interacting protein (RIP)1 (Meylan et al., 2004). In conjunction with MyD88-mediated NF- κ B activation, TLR4 also utilises the adaptor protein TRIF to activate IRF3 and IRF7 in response to bacterial LPS (Fitzgerald et al., 2003). Once activated, IRF3 and IRF7 bind to regulatory elements in the IFN β promoter. The IFN β promoter contains four overlapping positive regulatory domains (PRDs). PRD1 and PRDIII are closely related to the IFN-stimulated response element (ISRE), found in genes transduced by Type I IFNs, and are bound by members of the IRF family, including IRF3 and IRF7. PRDII is bound and activated by NF- κ B and PRDIV is regulated by ATF-2 homodimers or ATF-2/c-Jun heterodimers. These transcription factors interact with each other and also with CBP/p300 and high-mobility-group protein (HMG) to form a multiprotein transcription-promoting complex called an enhanceasome (Maniatis et al., 1998).

TLR-signalling can also lead to the activation of Mitogen-activated protein kinases (MAPKs). MAPKs are evolutionary conserved enzymes which transduce extracellular signals into cellular responses. MAPKs are controlled by complex signalling cascades and regulate a wide range of cellular processes including cell proliferation, apoptosis, differentiation, cell migration and gene expression. There are three groups of mammalian MAPKs, extracellular signal-related kinases (ERK)s, p38 proteins and Jun amino-terminal kinases (JNK)s (English et al., 1999). MAPK activation involves a three-tiered signalling cascade involving a MAPK, a MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase (MAPKKK or MEKK). ERK1 and ERK2 have been linked to cell proliferation but also have roles in other processes. JNK1/2 and 3 are activated in response to environmental stresses and are implicated in apoptosis, cell transformation and cytokine biosynthesis (English et al., 1999). JNK phosphorylates and activates c-Jun which, along with the ATF2 or c-Fos, forms the transcription factor AP-1. AP-1 plays a role in the regulation of inflammation (Krishnan et al., 2007). p38 MAPKs can also be activated by stress or bacterial infection and have roles in processes such as cytokine biosynthesis, muscle differentiation and B cell proliferation (English et al., 1999). p38 is also involved in the activation of subunits of AP-1 (Krishnan et al., 2007)

4.1.1 Specific aims of chapter 4

1. Given that previous studies from the lab indicated that Cactin is a negative regulator of IRF3 and IRF7 this chapter aimed to dissect the underlying mechanism(s) involved.
2. Determine if this inhibition has a functional effect on the production of type 1 IFNs.
3. Investigate if Cactin regulates MAPK activation.

4.2 Results

4.2.1 Cactin inhibits IRF3 and IRF7 activation

In response to viral and bacterial infection IRF3 and IRF7 regulate the expression of Type I IFNs. Type I IFNs encode powerful antiviral molecules and also have an important role in bridging innate and adaptive immunity by upregulating co-stimulatory molecules on antigen-presenting cells (Moynagh, 2005). TLR3 recognises dsRNA and activates IRF3 and IRF7, while TLR4 activates IRFs in response to bacterial LPS. In order to investigate if Cactin regulates the activation of IRF3 and IRF7 in response to viral and bacterial infection, HEK293 cells stably transfected with TLR3 and TLR4 were used. Cells were transfected with plasmids encoding pFA-IRF3 or pFA-IRF7 in conjunction with a pFR luciferase reporter plasmid and increasing concentrations of a plasmid encoding myc-tagged Cactin. Cactin significantly inhibited the activation of IRF3 and IRF7 in response to the synthetic double dsRNA Poly(I:C) in HEK293 TLR3 cells (Figure 4.1A, Figure 4.1B). This inhibitory effect is not stimulus specific as Cactin also significantly impaired the activation of IRF3 and IRF7 in response to LPS stimulation in HEK293 TLR4 cells (Figure 4.1C, Figure 4.1D).

Because Cactin exhibited such potent inhibitory effects on IRF3 and IRF7 it was important to exclude the possibility that Cactin was preventing gene expression from the pFA plasmids. HEK293 TLR3 cells were thus co-transfected with the pFA-IRF3 plasmid and a myc-tagged Cactin plasmid. Cells were also treated in the presence or absence of Poly(I:C). The co-expression of Cactin failed to affect the expression levels of pFA-IRF3 (Figure 4.2). This indicates that Cactin regulates the activation of IRF3 rather than preventing the expression of IRF3 from the the plasmid. Because the above studies were carried out in cells engineered to express TLRs it was necessary to assess if Cactin exhibited the same inhibitory effect in cells naturally expressing TLRs. U373 astocytoma cells naturally express TLR3 and respond to Poly(I:C). U373 cells previously transduced with control and Cactin-specific shRNA were treated with Poly(I:C) and assessed for phosphorylation of TBK1 as an index of TLR3 signalling since TBK1 is the kinase responsible to IRF3 and IRF7 phosphorylation. Interestingly U373 cells transduced with Cactin shRNA showed increased basal phophorylation of TBK1 compared with cells expressing the control

shRNA (Figure 4.3). This suggests that the pathway may be constitutively active in these cells. Interestingly, suppression of Cactin had no effect on Poly(I:C) induced phosphorylation of TBK1. It is worth noting that suppression of Cactin in U373 cells led to increased expression of the IRF responsive gene RANTES, thus confirming the inhibitory effects of Cactin in these cells (Atzei et al., 2010).

4.2.2 Cactin inhibits IFN β gene expression

Since IRFs regulate the expression of Type I IFNs, the effects of Cactin on IFN β gene expression was next assessed. HEK293 TLR3 cells were transfected with plasmids encoding myc-tagged Cactin or empty vector (EV) pcDNA3.1 and stimulated with Poly(I:C). Real-time PCR was performed to quantify the amount of IFN β mRNA transcribed. Over-expression of Cactin significantly inhibited IFN β induction following 6 h stimulation with Poly(I:C) (Figure 4.4). In order to complement the above findings that used overexpression approaches, the effects of Cactin suppression on Poly(I:C) induction of IFN β was next assessed. HEK293 cells were thus transfected with Cactin specific siRNA. Real-time PCR confirmed that there is ~ 55% knockdown of endogenous Cactin in cells transfected with Cactin-specific siRNA compared with cells transfected with a scrambled control (Figure 4.5). Suppression of endogenous Cactin in HEK293 TLR3 cells augmented the induction of IFN β mRNA following stimulation with Poly(I:C) (Figure 4.6). This confirms the earlier findings indicating a functional role for cactin in regulation of IRFs and induction of IFN β .

Given the inhibitory effects of Cactin on IRFs and IFN β expression Cactin was next probed for its regulation of the IFN β promoter. HEK293 TLR3 cells were transfected with a reporter plasmid encoding a luciferase gene regulated by the IFN β -promoter in the absence or presence of a plasmid encoding Cactin-myc. Cactin significantly inhibited Poly(I:C) mediated IFN β activation (Figure 4.7A). The IFN β promoter contains four overlapping PRD domains. PRDI and PRDIII are bound and regulated by IRFs, while PRDII is activated by NF- κ B and PRDIV is bound by ATF-2/c-Jun. In order to investigate which domain of the IFN β promoter are subject to regulation by Cactin, HEK293 TLR3 cells were transfected with PRDI-III-Luc, PRDII-Luc and PRDIV-Luc reporter plasmids in the presence or absence of Cactin-myc. Interestingly, Cactin failed to inhibit Poly(I:C)-induced activation of PRDII.

Indeed there was increased Poly(I:C) activation of PRDII in cells transfected with Cactin compared with cells transfected with Empty vector (Figure 4.7B). Cactin did, however, inhibit Poly(I:C) mediated activation of PRDI-III and to a lesser extent PRDIV (Figure 4.7B). These results indicate that Cactin negatively regulates IFN β activation by targeting IRFs and inhibiting IRF-mediated activation of the IFN β promoter.

4.2.3 Cactin does not inhibit IRF3 phosphorylation or prevent the translocation of IRF3 or IRF7 to the nucleus.

Because the activation of the IRF pathway requires phosphorylation of IRF3 which subsequently dimerises and translocates to the nucleus, Cactin was next assessed for its ability to regulate IRF3 phosphorylation. HEK293 TLR3 cells were transfected with Cactin-specific siRNA and a scrambled siRNA control. Interestingly suppression of Cactin did not augment Poly(I:C) mediated IRF3 phosphorylation as detected by Western blotting using an anti-phospho-IRF3 antibody (Figure 4.8). In fact, there was decreased phosphorylated IRF3 detected in cells transfected with Cactin specific siRNA. This indicates that Cactin may exert its inhibitory effects downstream of IRF3 phosphorylation and so it was next investigated if Cactin inhibits IRF3 translocation to the nucleus. HEK293 TLR3 cells were thus co-transfected with plasmids encoding IRF3-flag and Cactin-myc. Following 30 min stimulation with Poly(I:C), cells were fixed and stained with anti-flag and anti-myc antibodies. Cells were also stained with hoescht to visualise the nuclei. IRF3-flag was detected in the nucleus of Poly(I:C) stimulated cells as visualised by confocal microscopy (Figure 4.9). Interestingly, IRF3-flag was also detected in the nucleus of Poly(I:C) stimulated cells expressing Cactin-myc (Figure 4.9). This suggests that Cactin does not inhibit the translocation of IRF3 to the nucleus. Similarly, when cells were transfected with plasmids encoding IRF7-flag and Cactin-myc, IRF7-flag was detected in the nucleus of Poly(I:C) stimulated cells and this was still apparent in cells expressing Cactin-myc (Figure 4.10). The above findings indicate that Cactin is functioning downstream of phosphorylation and translocation of IRF3 and IRF7 to the nucleus and so this prompted us to investigate if Cactin manifests its inhibitory effects on IRFs within the nucleus.

4.2.4 The nuclear localisation of Cactin is essential for manifesting its inhibitory effects on IRF3 and IRF7

In order to determine if the nuclear localisation of Cactin is important for its negative regulation of IRFs a truncated-Cactin mutant was employed. Truncated-Cactin lacks a NLS and is expressed exclusively in the cytoplasm (see Figure 3.5 in previous chapter). HEK293 TLR3 or TLR4 cells were transfected with increasing concentrations of plasmids encoding Cactin-myc or truncated-Cactin-myc and pFA-IRF3 or pFA-IRF7 and pFR-regulated firefly luciferase. Truncated Cactin had modest inhibitory effects on Poly(I:C), LPS, and TRIF-mediated IRF3 and IRF7 activation but the inhibitory effects were greatly reduced when compared with full-length Cactin (Figure 4.11). This suggests that the regulatory effects of Cactin on IRFs are heavily dependent on its nuclear localisation.

4.2.5 Cactin does not co-immunoprecipitate with IRF3 or IRF7.

As Cactin appears to manifest its inhibitory effects in the nucleus we then investigated if Cactin interacts directly with IRF3 and IRF7 and affects their ability to bind DNA. HEK293 TLR3 cells were co-transfected with plasmids encoding IRF3-flag and Cactin-myc. Cells were then treated with Poly(I:C) to stimulate IRF3 phosphorylation and translocation to the nucleus to allow for possible interaction with Cactin. Lysates were generated and immunoprecipitated with an anti-myc antibody. IRF3-flag was not detected in immunoprecipitates from cells expressing Cactin-myc and IRF3-flag, although IRF3-flag was detected in the corresponding whole cell lysate (Figure 4.12). HEK293 TLR3 cells were also transfected with plasmids encoding IRF7-flag and Cactin-myc. Again, cells were stimulated with Poly(I:C) to stimulate IRF7 phosphorylation and translocation to the nucleus. Lysates were immunoprecipitated, using an anti-flag antibody. Cactin-myc failed to co-immunoprecipitate with IRF7-flag although it was detected in the whole cell lysate (Figure 4.13). This suggests that Cactin does not interact directly with IRF3 or IRF7 hinting at an indirect mechanism of action.

4.2.6 Suppression of Cactin leads to increased binding of IRF3 to PRDI-III of the IFN β promoter

Nuclear lysates from HEK293 TLR3 cells transfected with Cactin-specific siRNA and a scrambled siRNA control were subjected to Western immunoblotting using an anti-IRF3 antibody. Suppression of Cactin did not augment Poly(I:C) mediated nuclear translocation of IRF3 to the nucleus. However, higher levels of IRF3 were detected in nuclear lysates from cells transfected with Cactin-specific siRNA, compared to cells transfected with a scrambled control, following 24 h stimulation with Poly(I:C), presumably due to increased binding of IRF3 to its target promoter (Figure 4.14). Nucleolin/C23 was used as a loading control for nuclear lysates. In order to determine if Cactin prevents IRF3 from binding to the IFN β promoter, HEK293 TLR3 cells were transfected with siRNA specific for Cactin and a scrambled siRNA control. Nuclear lysates were generated and incubated with an oligonucleotide encoding PRDI-III of the IFN β promoter. Interestingly, there was stronger and more prolonged activation of PRDI-III following Poly(I:C) stimulation in cells suppressing endogenous Cactin (Figure 4.15). Supershift analysis indicates the presence of IRF3 in the protein-DNA complexes. The above findings indicate that Cactin inhibits IRF3 at the level of DNA-binding and knockdown of Cactin leads to more prolonged binding of IRF3 to the IFN β promoter.

4.2.7 Cactin inhibits IRF7-induced IFN α expression and affects the stability of IRF7 protein

Given the regulatory effects of Cactin on IRF3, Cactin was next assessed for its regulation of IRF7 activity. HEK293 TLR3 cells were transfected with a plasmid encoding a luciferase reporter gene regulated by the IFN α -1 promoter and with or without plasmids encoding Cactin-myc and IRF7-flag. Cactin significantly inhibited IRF7-induced activation of IFN α -luc (Figure 4.16). These findings suggest that Cactin targets IRF7 in addition to IRF3. IRF7 is subject to post-translational modifications including phosphorylation and ubiquitination following Poly(I:C) stimulation (Ning et al., 2011) which regulates its expression. To examine the effect of Cactin on IRF7 protein expression, HEK293 TLR3 cells were transfected with a plasmid encoding flag tagged IRF7 and with or without Cactin-specific siRNA or a

plasmid encoding myc-tagged Cactin. Interestingly, suppression of Cactin led to stabilization of IRF7-flag in unstimulated cells (Figure 4.17A). Furthermore, over-expression of Cactin inhibited poly(I:C) mediated stabilisation of IRF7-flag (Figure 4.17B). These results further confirm that Cactin negatively regulates IRF7.

4.2.8 Cactin positively regulates the activation of the MAPKs JNK, p38 and ERK1/2

As part of the above studies, interrogating the effects of Cactin on IFN β expression, it was noted that Cactin, whilst showing strong regulatory potential on IRFs and the PRDI-III region of the IFN β promoter, it also showed some modest regulation of the PRDIV domain. Given that the latter is driven by AP-1 complexes that are regulated by MAPK signalling the susceptibility of the MAPK pathways to Cactin was next probed. JNK phosphorylates and activates the AP-1 subunit c-jun and so to investigate if Cactin regulates JNK signalling HEK293 cells were transfected with plasmids encoding pFA-Jun and pFR-regulated firefly luciferase. MEKK1 was also over-expressed in these cells as a positive control to drive the activation of c-Jun. Intriguingly over-expression of Cactin enhanced MEKK1 activation of c-Jun (Figure 4.18). To examine if Cactin regulates JNK activation in a physiologically relevant system U373 cells previously transduced with Cactin-specific shRNA or a non-specific shRNA control were employed. Samples were generated from Poly(I:C) stimulated cells and subjected to Western immunoblotting. There were decreased levels of phospho-JNK detected in cells suppressing endogenous Cactin (Figure 4.19). Furthermore, there were also decreased levels of phospho-p38 and phospho-ERK1/2 detected in cells transduced with Cactin-specific shRNA compared with cells transduced with a control shRNA (Figure 4.19). These data indicate that Cactin can act as a positive regulator of JNK, p38 and ERK1/2 activation in response to TLR3 stimulation. We next examined if such a role for Cactin in regulating MAPK activation extends to the TLR4 pathway.

U373 cells transduced with Cactin-specific and control shRNA were treated with LPS for various time-points and the levels of phospho-ERK1/2, p38 and JNK were again visualised by Western Immunoblotting. Interestingly, similar levels of phosphorylated ERK1/2, p38 and JNK were detected in cells suppressing endogenous Cactin (Figure 4.20). This suggests that the effects of Cactin on these MAPKs may be

stimulus specific. These results were confirmed using densitometry analysis (Figure 4.21).

4.2.9 p38 positively regulates IRF3 and IRF7 activation

As Cactin was displaying such strong regulatory effects on Poly(I:C) induced p38 phosphorylation it seemed necessary to investigate if phosphorylation of p38 is required for Cactin to manifest its inhibitory effects. To investigate this, HEK293 TLR3 cells were transfected with pFA-IRF3 and pFR-regulated luciferase constructs. Cells were also transfected with a plasmid encoding a dominant negative mutant of p38 (Δ p38). Interestingly overexpression of Δ p38 inhibited Poly(I:C) mediated IRF3 activation (Figure 4.22A). Furthermore, pre-treatment of cells with the p38 inhibitor SB 203580 inhibited Poly(I:C)-mediated IRF3 activation (Figure 4.22B). Similarly, overexpression of Δ p38 inhibited Poly(I:C) induced activation of IRF7 as did treatment with SB 203580 (Figure 4.23). As phosphorylation of p38 promotes the activation of IRF3 and IRF7, this indicates that phosphorylation of p38 is not important for Cactin's inhibitory effects on IRFs.

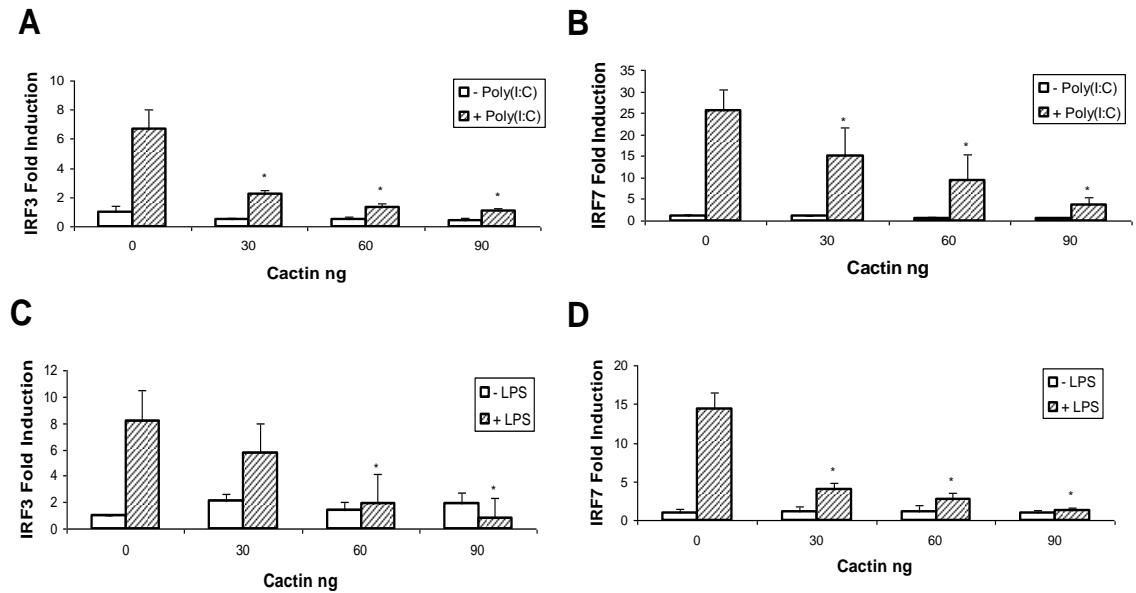


Figure 4.1 Cactin inhibits Poly(I:C) and LPS-mediated activation of IRF3 and IRF7

HEK293 TLR3 or TLR4 cells were cotransfected with plasmids encoding (**A, C**) pFA-IRF3 (30 ng) or (**B, D**) pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla luciferase (20 ng) in the absence or presence of a plasmid encoding myc-tagged Cactin (30, 60 or 90 ng). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post-transfection cells were treated overnight with (**A, B**) Poly(I:C) (25 µg/ml) or (**C, D**) LPS (100 ng/ml). Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Results shown are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, paired t-test, ligand stimulated cells transfected with Cactin compared with ligand stimulated cells transfected with EV.

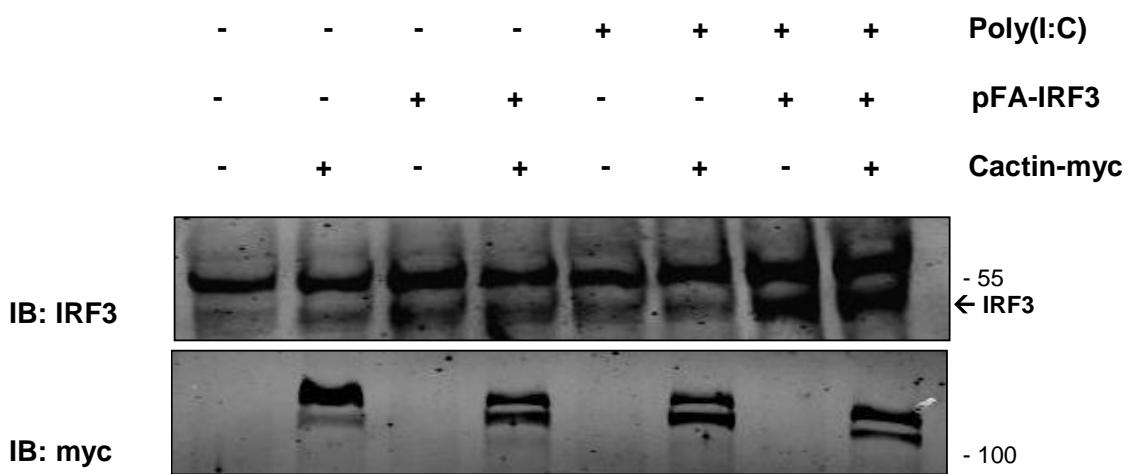


Figure 4.2 Cactin does not affect expression levels of pFA-IRF3

HEK293 TLR3 cells were transfected with plasmids encoding pFA-IRF3 (2 µg) and Cactin-myc (2 µg). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection cells were treated with or without Poly(I:C) (25 µg/ml) for 1 h. Lysates were generated and subjected to Western Immunoblotting using anti-IRF3 and anti-myc antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of two independent experiments.

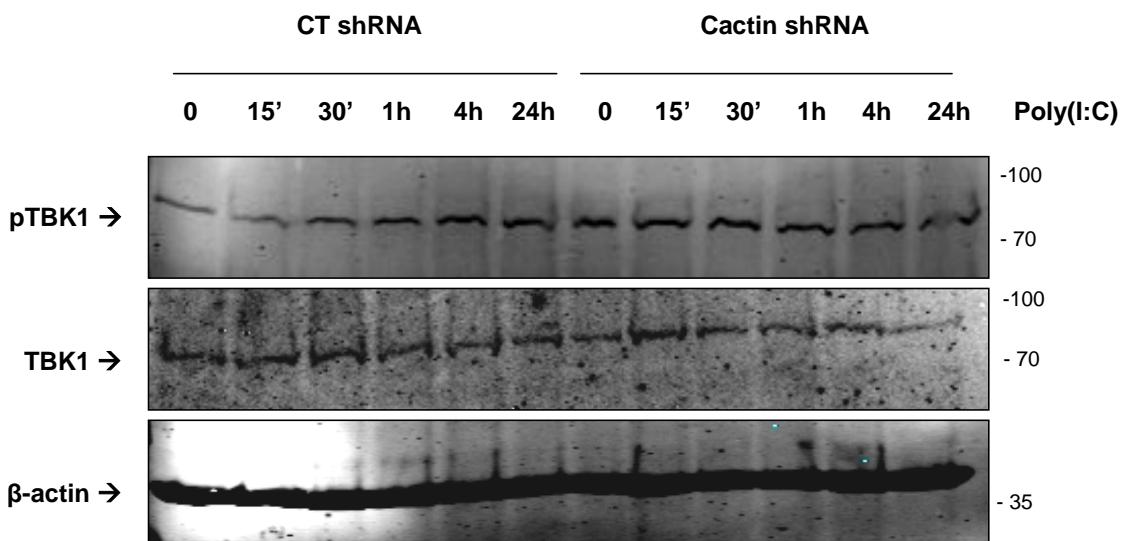


Figure 4.3 Effect of Cactin knockdown on phosphorylation of TBK1 in U373 cells

U373 cells stably transduced with Control shRNA and Cactin specific shRNA were treated with Poly(I:C) (10 µg/ml) for the indicated time periods. Cells were lysed in SDS-PAGE sample buffer and samples subjected to Western blotting using anti-phospho-TBK1, anti-TBK1 and anti-β-actin antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are indicative of three independent experiments.

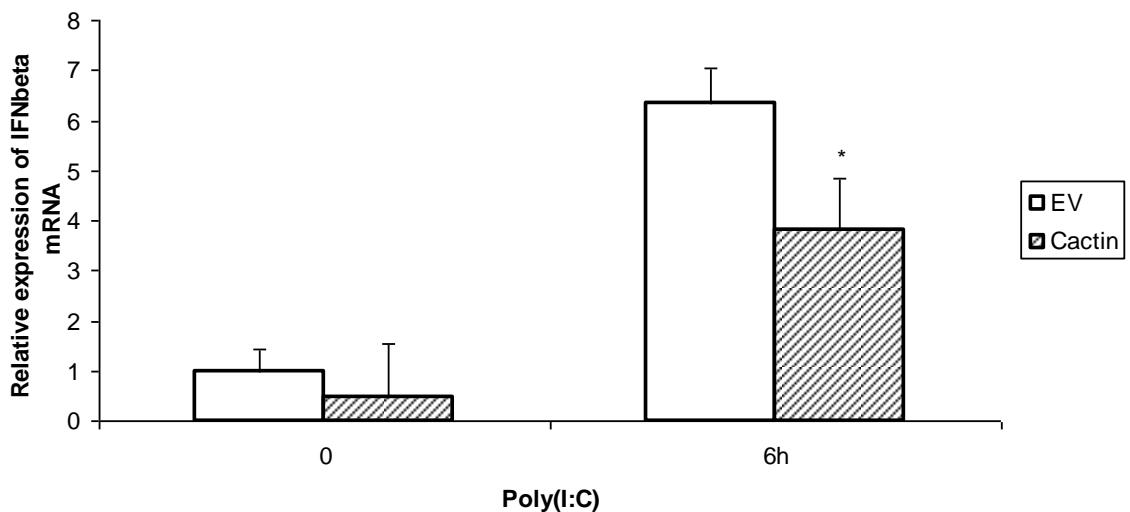


Figure 4.4 Overexpression of Cactin inhibits Poly(I:C) induction of IFN β

HEK293 TLR3 cells were transfected with myc-tagged Cactin (2 μ g) or Empty Vector (EV) pcDNA3.1 (2 μ g). 24 hours post-transfection cells were treated with Poly(I:C) (25 μ g/ml) for the indicated time periods. Total RNA was extracted and cDNA was generated using RT-PCR. Samples were subsequently assayed by quantitative real-time PCR for levels of IFN β . Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test, Poly(I:C) stimulated cells expressing Cactin compared with ligand stimulating cells transfected with EV.

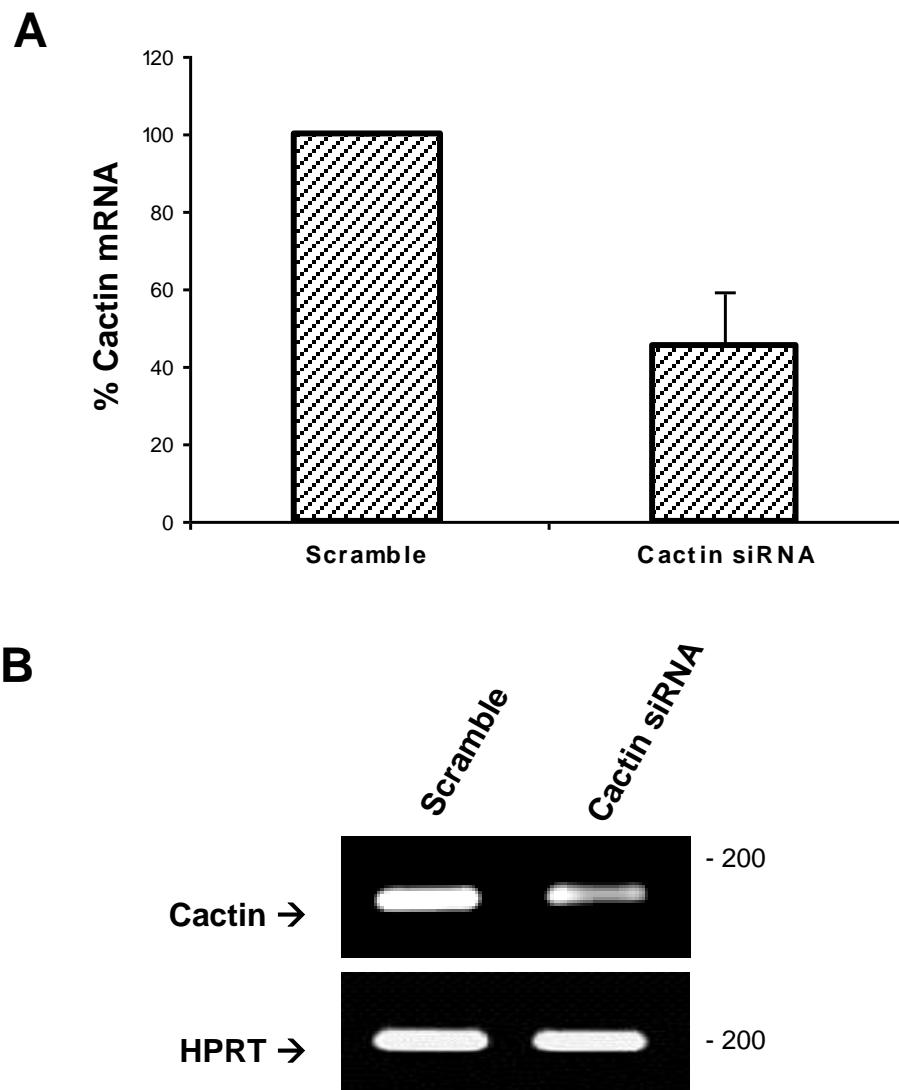


Figure 4.5 Cactin-specific siRNA suppresses endogenous Cactin expression in HEK293 cells

HEK293 TLR3 cells were transfected with Cactin specific siRNA or scrambled siRNA (25 nM). Total RNA was extracted and cDNA was generated using RT-PCR. Samples were subsequently assayed by quantitative real-time PCR for levels of Cactin. **(A)** Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. **(B)** Real-time products were separated by gel electrophoresis. Results shown are representative of three independent experiments.

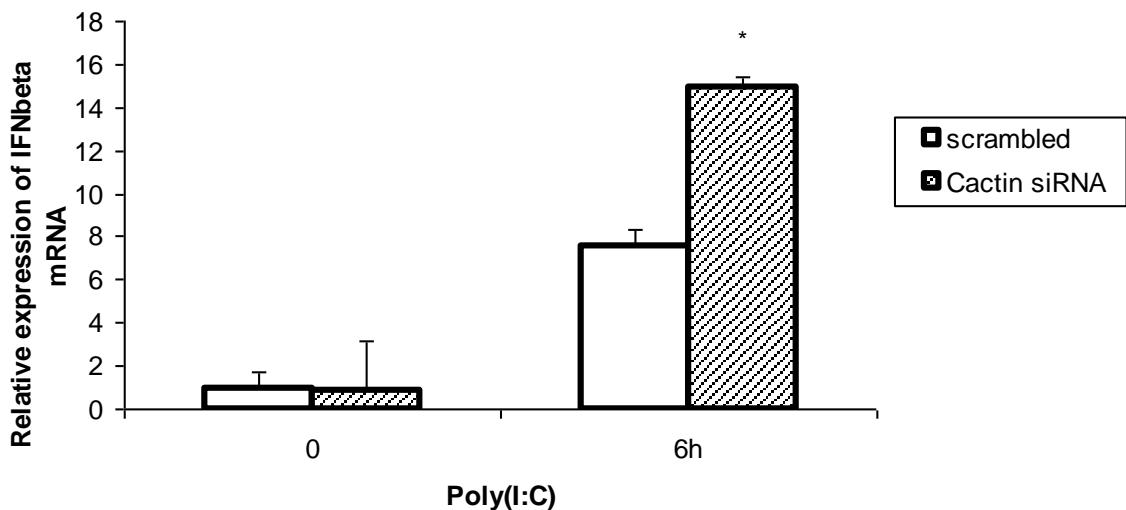


Figure 4.6 Suppression of endogenous Cactin augments Poly(I:C) induced IFN β
 HEK293 TLR3 cells were transfected with Cactin specific and scrambled siRNA (25 nM). Cells were treated for the indicated time-points with Poly(I:C) (25 μ g/ml). Total RNA was extracted 48 h post-transfection and cDNA was generated by RT-PCR. Samples were then assayed by real-time PCR for levels of IFN β . Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. * p < 0.05, paired t-test, Poly(I:C) stimulated cells transfected with Cactin siRNA compared with cells transfected with scrambled siRNA control.

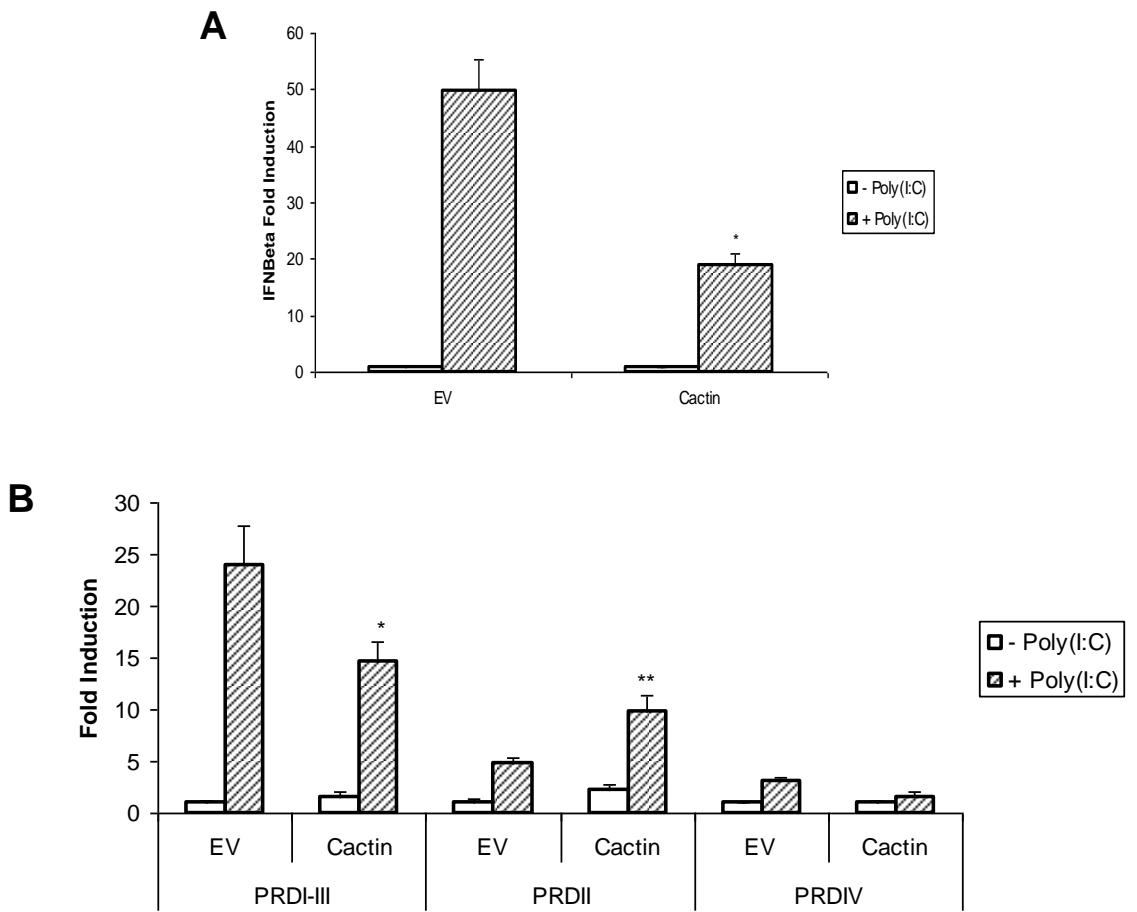


Figure 4.7 Cactin inhibits Poly(I:C)-mediated IFN β activation and inhibits the activation of PRDI-III and PRDIV but not PRDII of the IFN β promoter

HEK293 TLR3 cells were co-transfected with plasmids encoding (A) IFN β luciferase reporter plasmid (80 ng) or (B) PRDI-III, PRDII and PRDIV luciferase reporter plasmids (80 ng) and constitutively expressed TK Renilla (20 ng) and were transfected with plasmids encoding Cactin-myc (90 ng) or Empty Vector (EV) pcDNA3.1 (90 ng). Transfected cells were left overnight and then treated with Poly(I:C) (25 μ g/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data presented and the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, ** p < 0.01, paired t-test, Poly(I:C) stimulated cells expressing Cactin compared with Poly(I:C) stimulated cells transfected with EV.

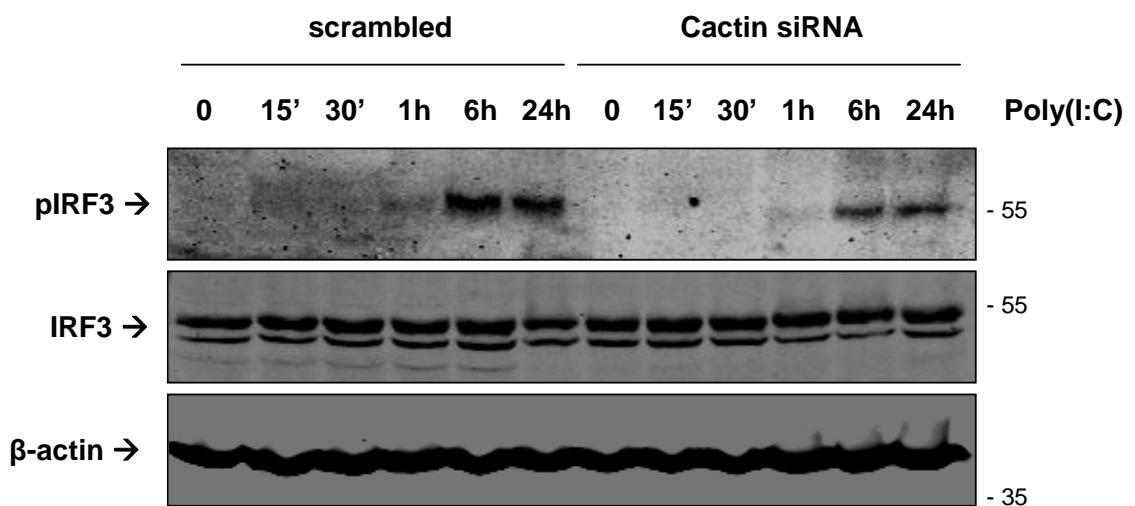


Figure 4.8 Effect of Cactin knockdown on IRF3 phosphorylation

HEK293 TLR3 cells were transfected with Cactin specific and scrambled siRNA (25 nM). 24 h post-transfection cells were treated with the indicated time periods with Poly(I:C) (25 µg/ml). Lysates were generated and subjected to Western Immunoblotting using anti-phospho-IRF3, anti-IRF3 and anti-β-actin antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Blots shown are a representative of three independent experiments.

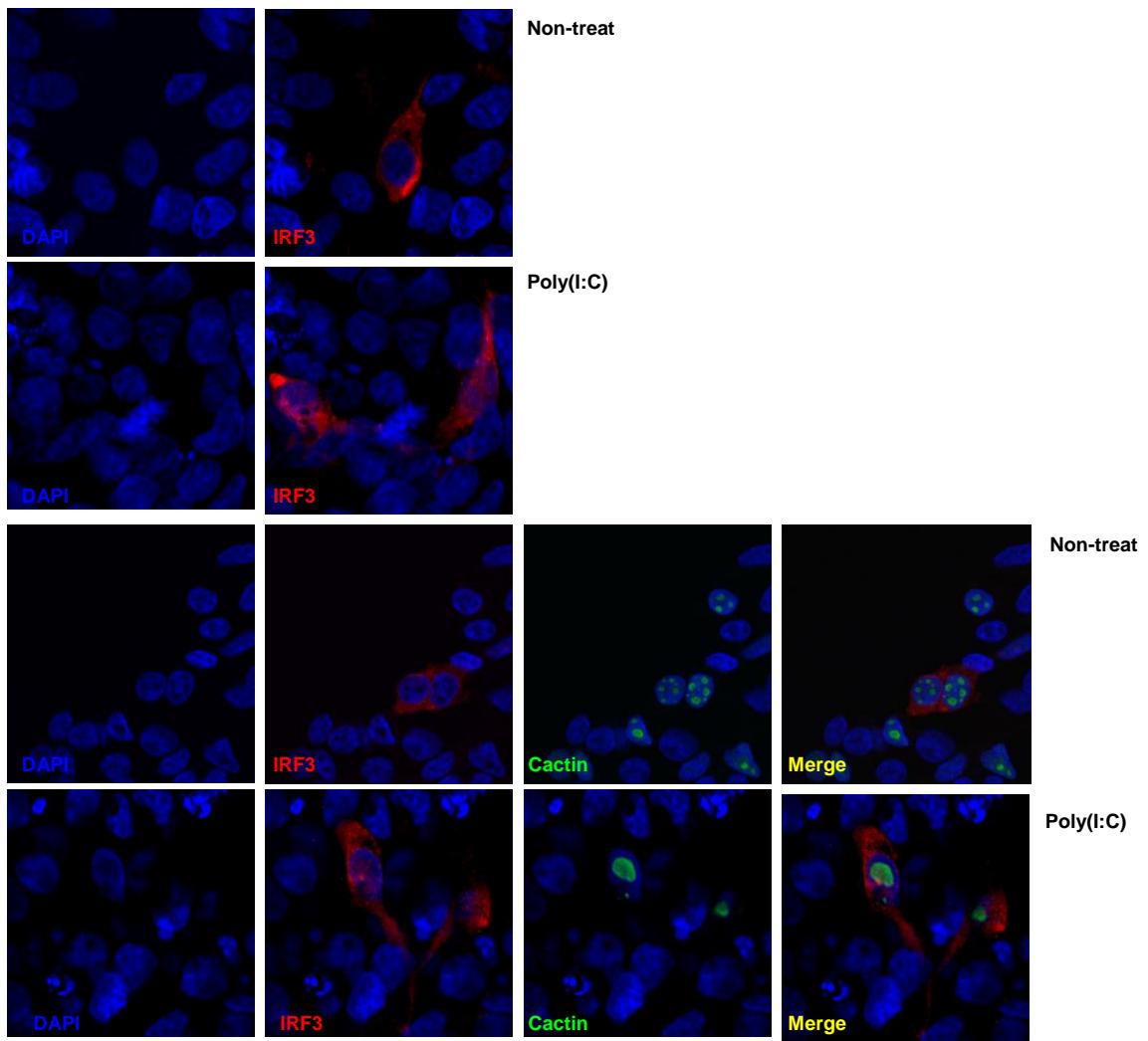


Figure 4.9 Overexpression of Cactin does not prevent the translocation of IRF3 to the nucleus

HEK293 TLR3 cells were co-transfected with plasmids encoding Flag-tagged IRF3 (2 µg) and myc-tagged Cactin (2 µg). pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection cells were treated with or without Poly(I:C) (25 µg/ml) for 30 min. Cells were fixed, stained with hoescht, anti-flag and anti-myc antibodies and mounted in anti-fade medium. Confocal images were captured using the Olympus Fluoview FV1000 confocal laser scanning microscope. Data analysis was performed using the Olympus FV-10 ASW imaging software. Results shown are representative of two independent experiments.

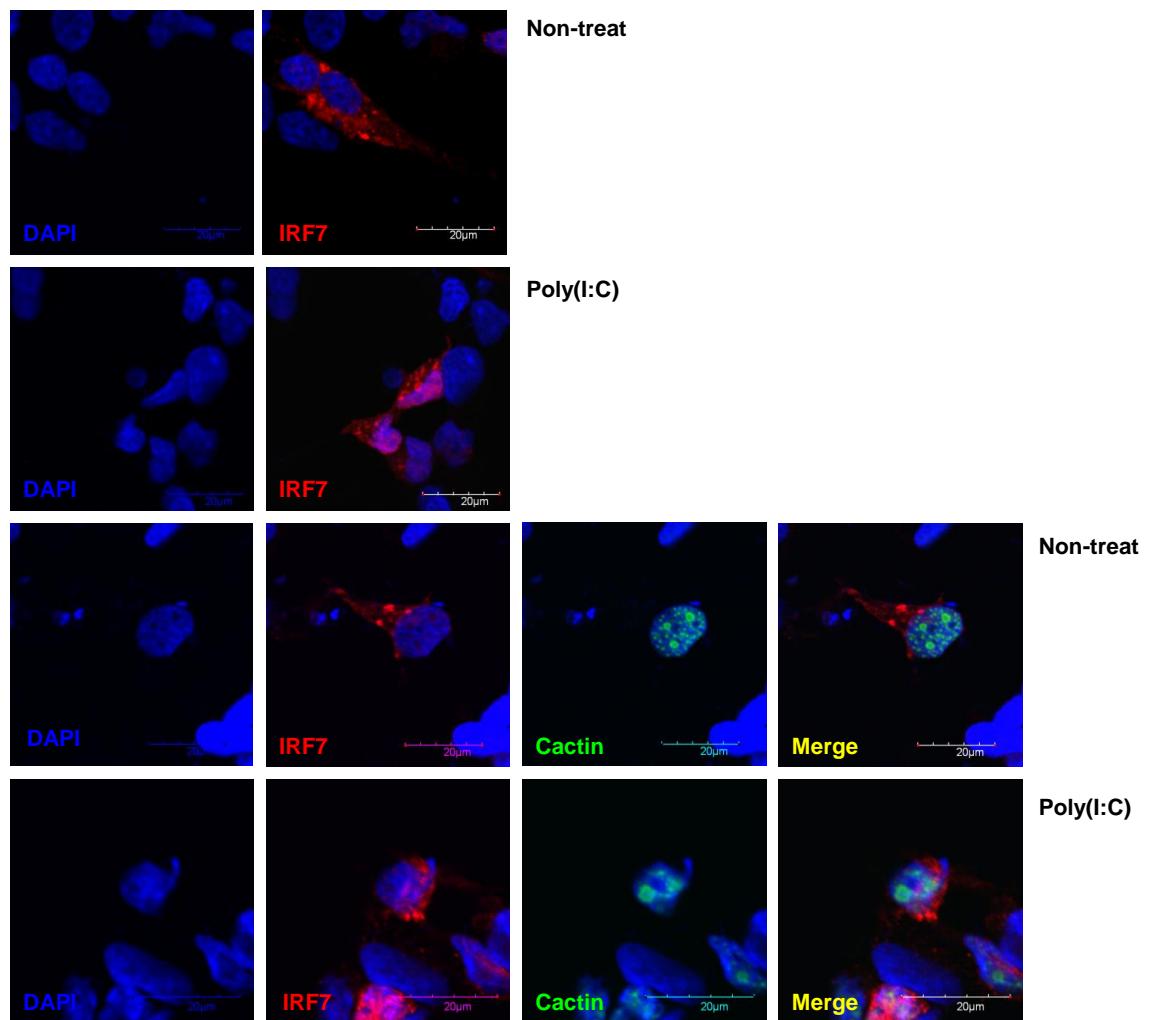


Figure 4.10 Overexpression of Cactin does not prevent nuclear translocation of IRF7

HEK293 TLR3 cells were co-transfected with flag-tagged IRF7 (2 µg) and myc-tagged Cactin (2 µg). 24 h post-transfection cells were treated with Poly(I:C) (25 µg/ml) for 30 min. Cells were fixed, stained with anti-flag and anti-myc antibodies, stained with hoescht and mounted in anti-fade medium. Confocal images were captured with the Olympus FluoView FV1000 confocal laser scanning microscope and data analysis was performed using the Olympus FV-10 ASW imaging software.

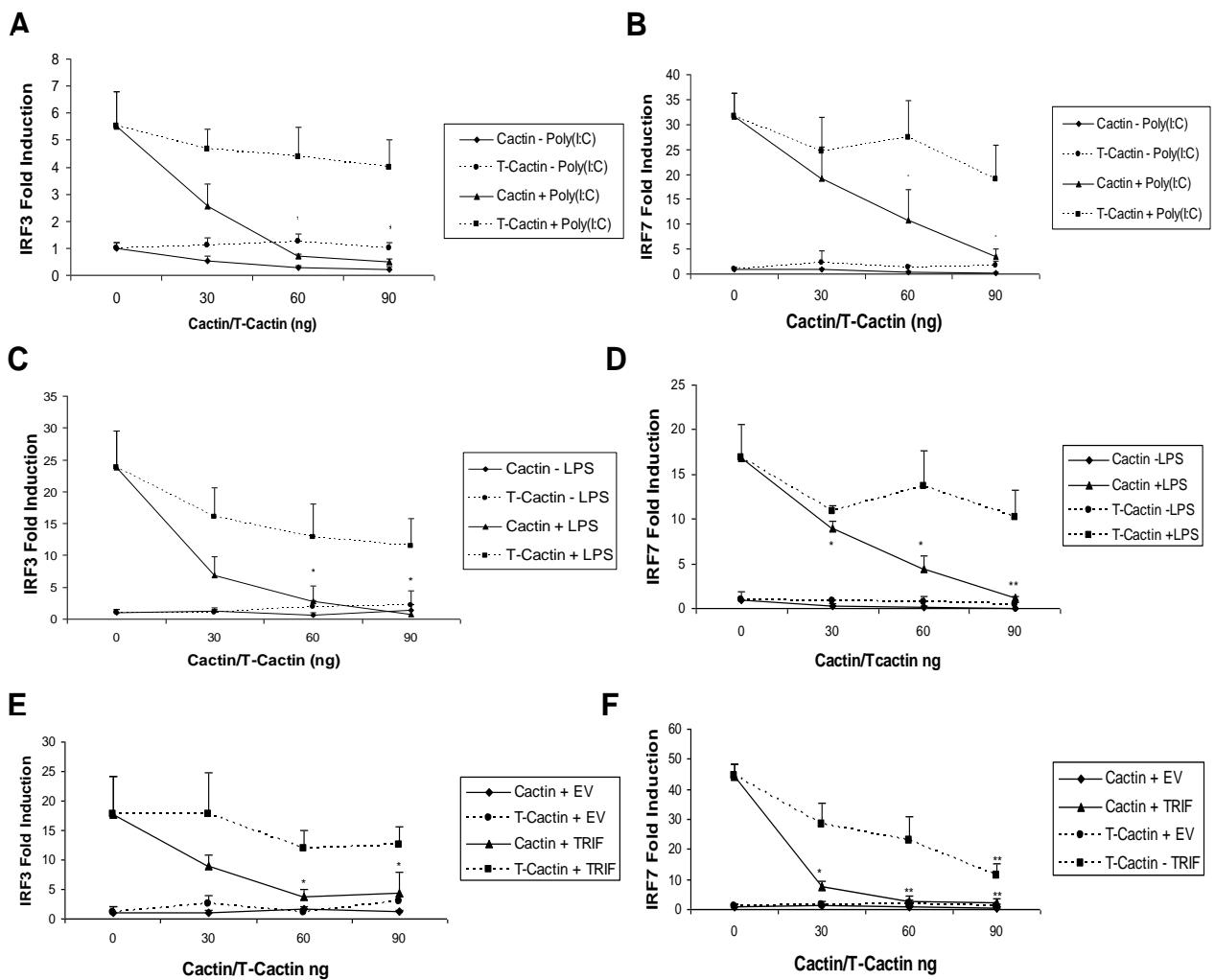


Figure 4.11 Nuclear localisation of Cactin is important for manifesting its inhibitory effects on IRF3 and IRF7

HEK293 TLR3 or TLR4 cells were co-transfected with (A, C, E) pFA-IRF3 (30 ng) or (B, D, F) pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla (20 ng) and transfected with or without plasmids encoding myc-tagged Cactin (30, 60 or 90 ng), myc-tagged truncated Cactin (30, 60 or 90 ng) and TRIF (50 ng). Empty vector (EV) pcDNA3.1 was used to normalise the amount of DNA transfected. Cells were treated with and without (A, B) Poly(I:C) (25 µg/ml) or (C, D) LPS (100 ng/ml) overnight. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data are the mean +/- S.E.M. for triplicate determinations from three independent experiments. * p < 0.05, ** p < 0.01, paired t-test, ligand stimulated or TRIF expressing cells transfected with Cactin or T-Cactin compared with ligand stimulated or TRIF expressing cells transfected with EV.

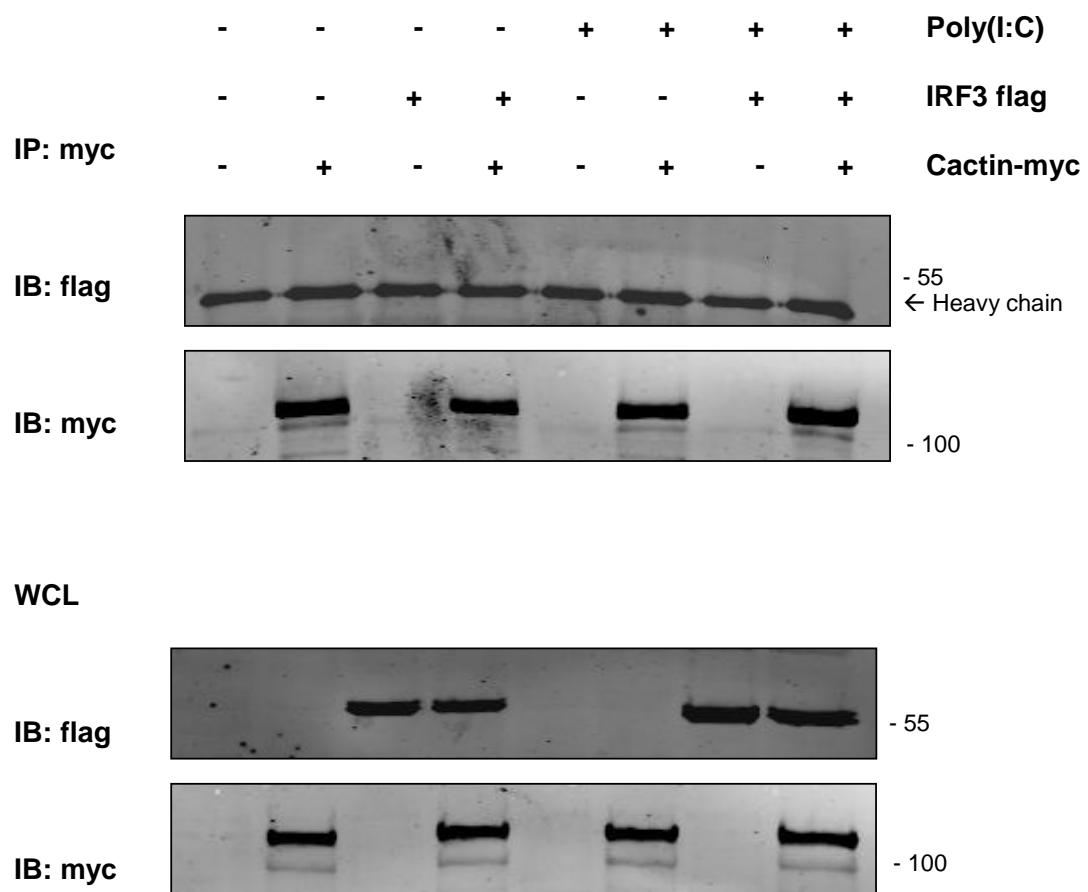


Figure 4.12 IRF3 does not co-immunoprecipitate with Cactin

HEK293 TLR3 cells were co-transfected with flag-tagged IRF3 (2 µg) and myc-tagged Cactin (2 µg). Empty vector pcDNA 3.1 was used to normalise the total amount of DNA transfected. Transfected cells were left overnight and then treated for 30 min with Poly(I:C) (25 µg/ml). Lysates were generated and immunoprecipitated with an anti-myc antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western immunoblotting with anti-flag and anti-myc antibodies. Immunoreactivity was visualised with the Odyssey Infrared imaging system. Results shown are representative of three independent experiments.

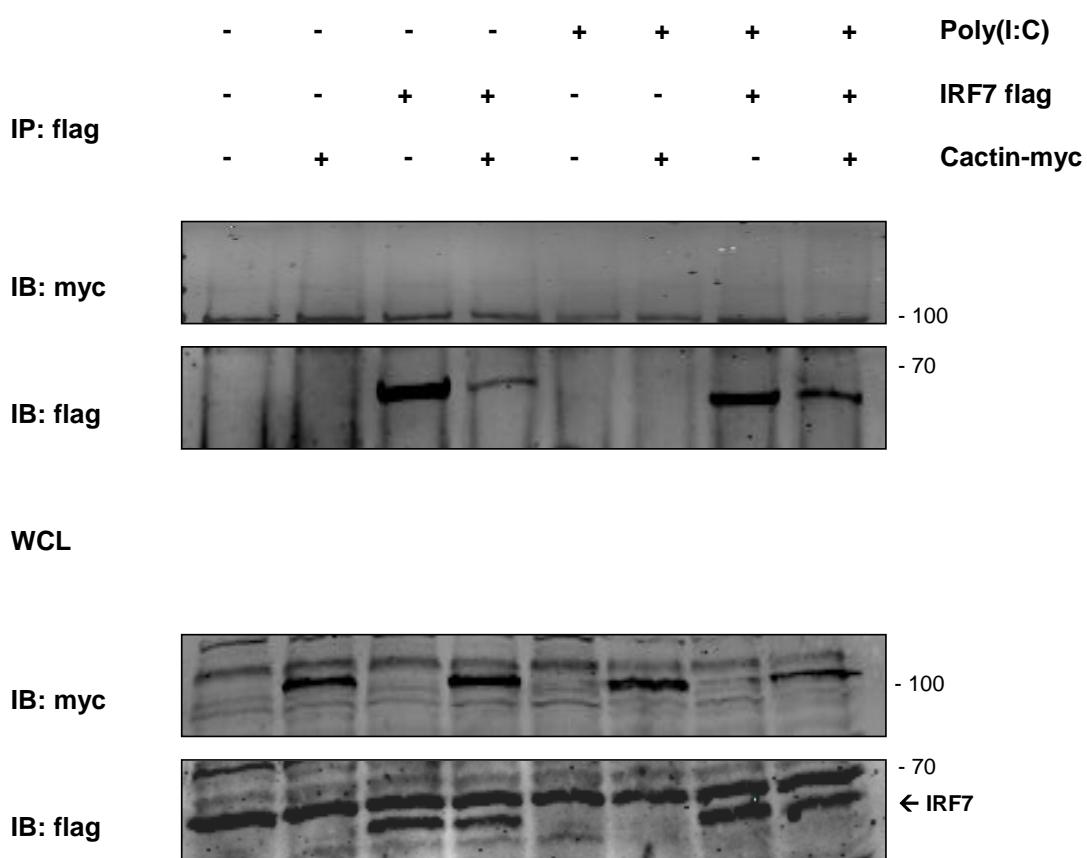


Figure 4.13 Cactin does not co-immunoprecipitate with IRF7

HEK293 TLR3 cells were co-transfected with myc-tagged Cactin (2 µg) and flag-tagged IRF7 (2 µg). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. Transfected cells were left overnight and then treated with Poly(I:C) (25 µg/ml) for 30 mins. Lysates were generated and then immunoprecipitated with an anti-flag antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western Immunoblotting using anti-myc and anti-flag antibodies. Immunoreactivity was visualised with the Odyssey Infrared imaging system. Blots shown are indicative of three independent experiments.

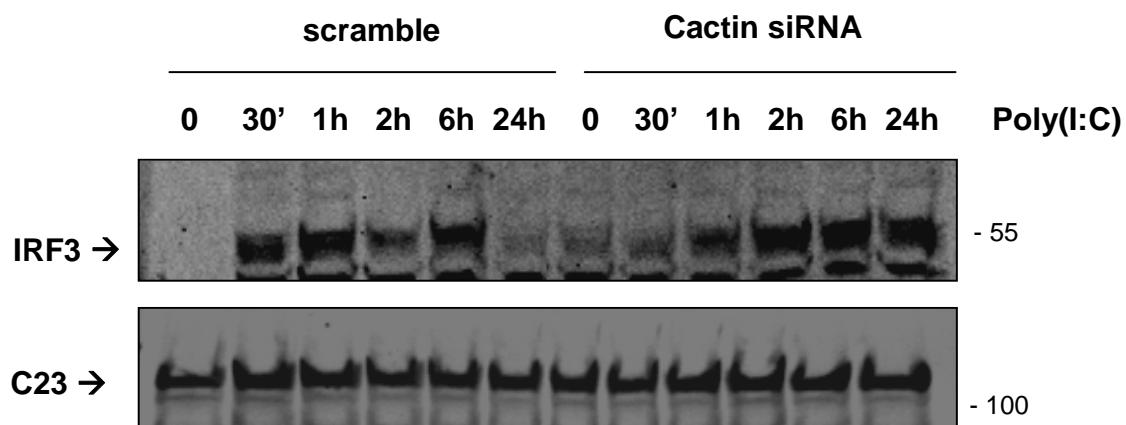


Figure 4.14 Suppression of endogenous Cactin leads to increased nuclear levels of IRF3 in response to Poly(I:C)

HEK293 TLR3 cells were transfected with Cactin specific and scrambled siRNA (25 nM). 24 h post-transfection cells were treated for the indicated time-periods with Poly(I:C) (25 µg/ml). Nuclear lysates were generated and subjected to Western immunoblotting using anti-IRF3 and anti-Nucleolin antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Blots shown are indicative of two independent experiments.

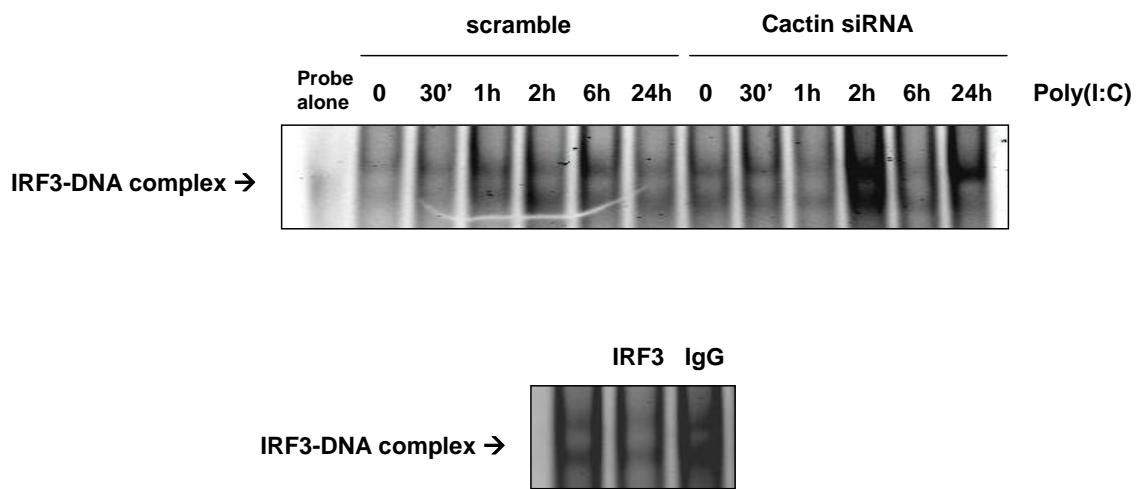


Figure 4.15 Suppression of endogenous Cactin expression augments IRF3 binding to the IFN β promoter

HEK293 TLR3 cells were transfected with Cactin specific and scrambled siRNA (25 nM). 24 h post-transfection cells were treated with Poly(I:C) (25 μ g/ml) for the indicated time periods. Nuclear lysates were generated and assayed for binding to an oligonucleotide encoding PRDI-III of the IFN β promoter by EMSA. Lysates from cells treated with Poly(I:C) for 2 h and transfected with Cactin specific siRNA were incubated with an anti-IRF3 antibody or non specific IgG before assaying for DNA-binding activity. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of three independent experiments.

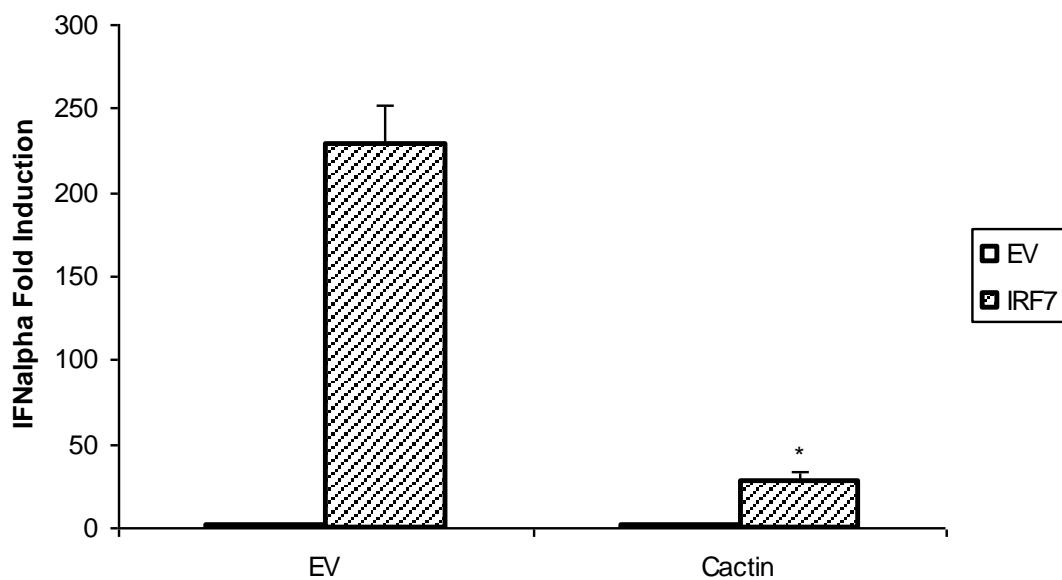


Figure 4.16 Cactin inhibits IRF7-induced activation of the IFN α -1 promoter

HEK293 TLR3 cells were co-transfected with plasmids encoding an IFN α -1 firefly luciferase reporter plasmid (80 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without plasmids encoding myc-tagged Cactin (90 ng) and flag tagged IRF7 (50 ng). Empty Vector (EV) pcDNA3.1 was used to normalise the total amount of DNA transfected. Lysates were generated 24 h post transfection and assayed for firefly and *Renilla* luciferase activity. Data shown are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, paired t-test, IRF7 transfected cells expressing Cactin compared with IRF7 expressing cells transfected with EV.

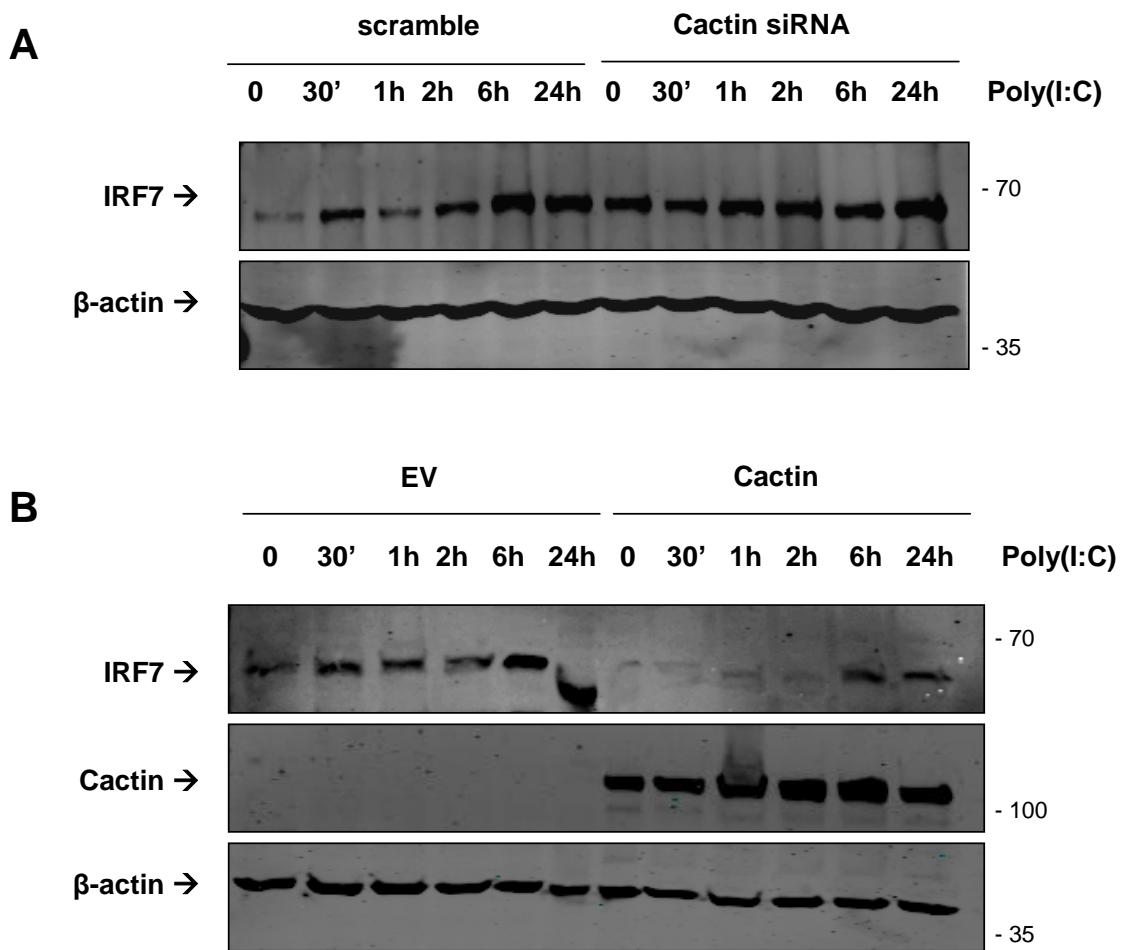


Figure 4.17 Cactin regulates protein levels of IRF7

(A) HEK293 TLR3 cells were co-transfected with Cactin specific and scrambled siRNA (25 nM) and a plasmid encoding flag-tagged IRF7 (2 µg). (B) HEK293 TLR3 cells were co-transfected with a plasmid encoding flag-tagged IRF7 (2 µg) and with or without a plasmid encoding myc-tagged Cactin (2 µg). Empty Vector (EV) pcDNA3.1 was used to normalise the total amount of DNA transfected. Transfected cells were left overnight and then treated for the indicated time points with Poly(I:C) (25 µg/ml). Lysates were generated 48 h post-transfection and subjected to Western immunoblotting using anti-flag, anti-β-actin and anti-myc antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Blots shown are representative of three independent experiments.

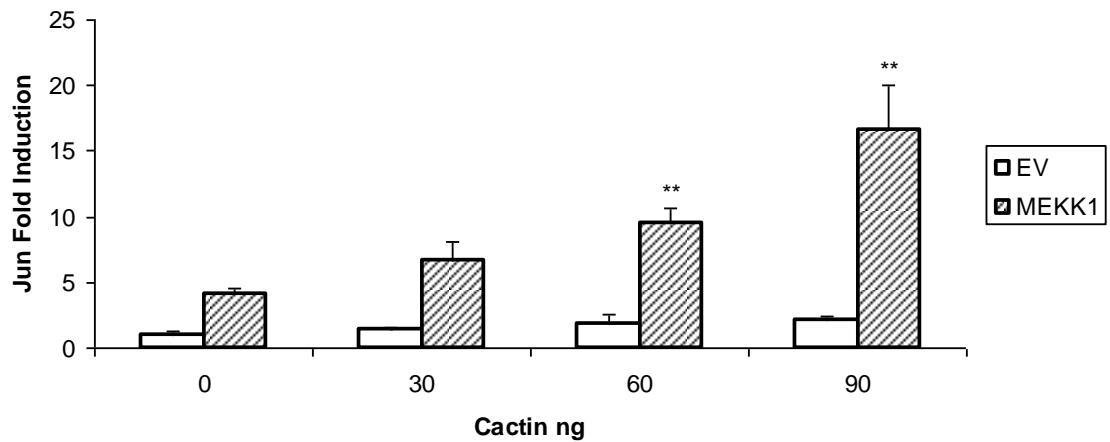


Figure 4.18 Cactin positively regulates c-Jun activation

HEK293 TLR4 cells were co-transfected with plasmids encoding pFA-jun (30 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without plasmids encoding myc-tagged Cactin (30, 60 and 90 ng) and MEKK1 (20 ng). Empty Vector (EV) pcDNA3.1 was used to normalise the total amount of DNA transfected. Lysates were generated 24 h post transfection and assayed for firefly luciferase activity and normalised for transfection efficiency using *Renilla* luciferase activity. Data are presented as the mean +/- S.E.M. of triplicate determinations from three independent experiments. ** p < 0.01, paired t-test, MEKK1 expressing cells transfected with Cactin compared with MEKK1 expressing cells transfected with EV.

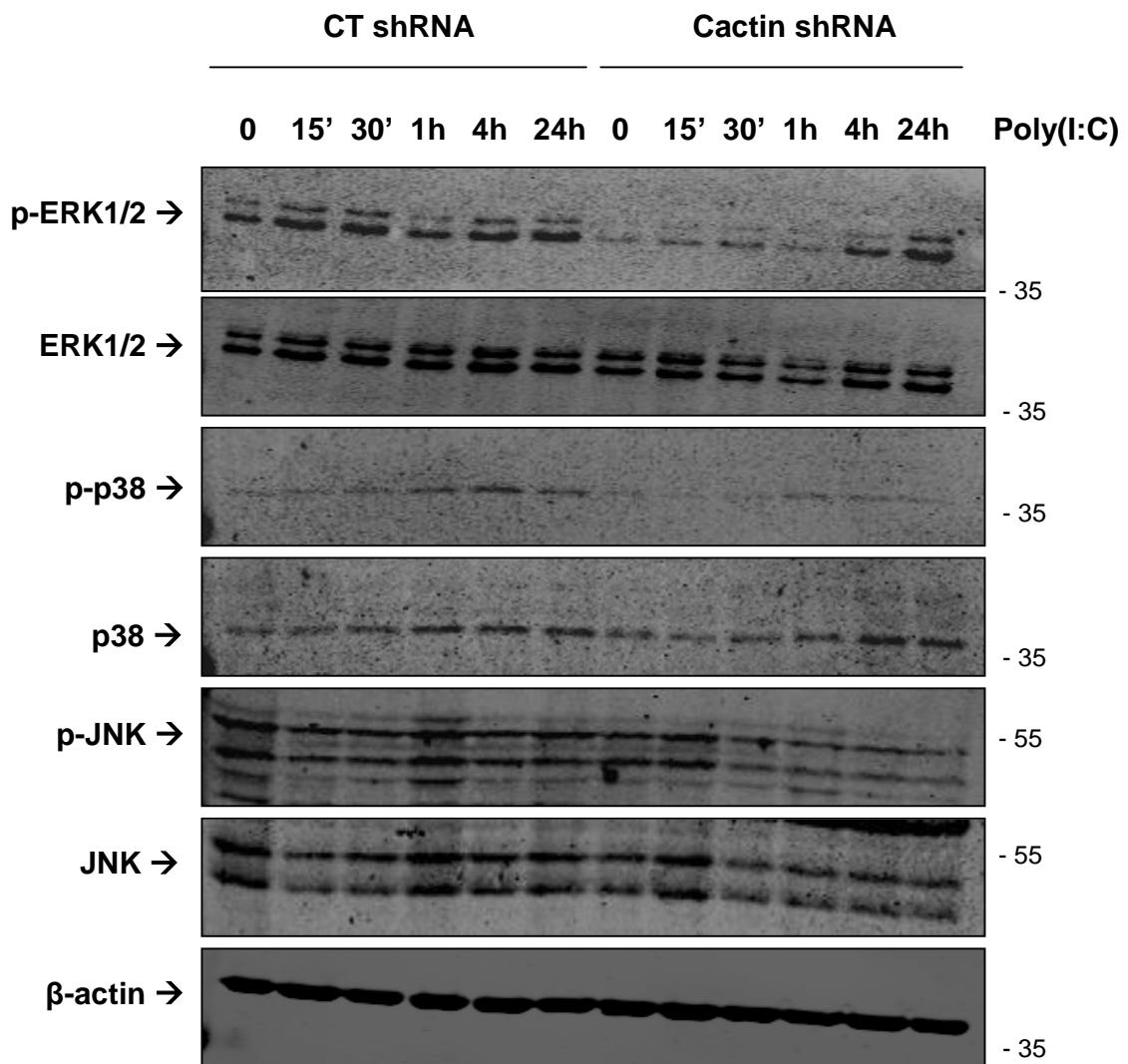


Figure 4.19 Knockdown of Cactin inhibits TLR3-induced MAPK signalling in U373 cells

U373 cells previously transduced with control and Cactin specific shRNA were treated for the indicated time periods with Poly(I:C) (10 µg/ml). Cells were harvested in SDS-PAGE sample buffer and subjected to Western immunoblotting using anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK and anti-β-actin antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of two independent experiments.

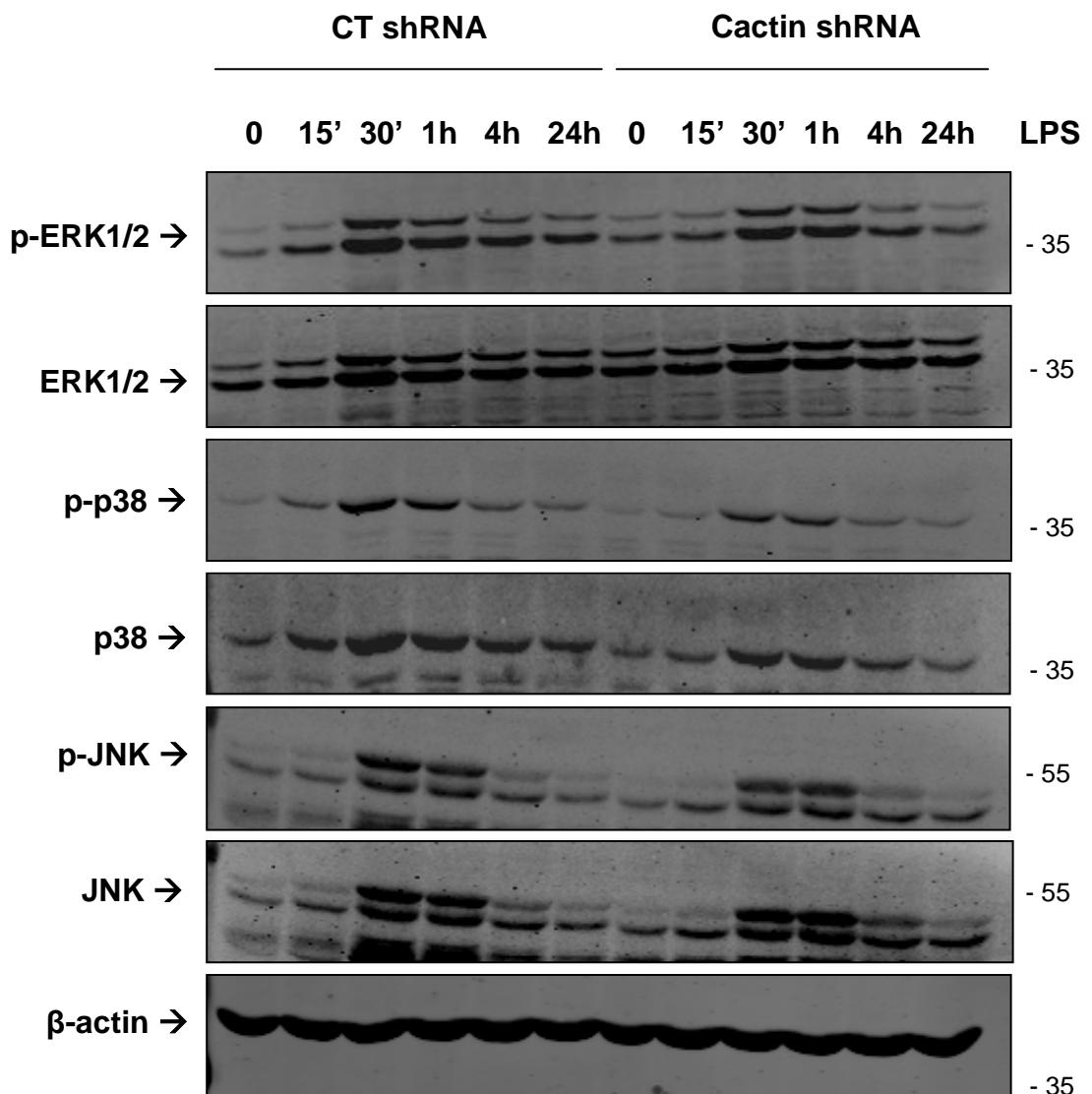


Figure 4.20 Suppression of endogenous Cactin fails to affect LPS mediated phosphorylation of ERK1/2, p38 and JNK in U373 cells.

U373 cells previously transduced with control and Cactin specific shRNA were treated for the indicated time periods with LPS (100 ng/ml). Cells were harvested in SDS-PAGE sample buffer and subjected to Western immunoblotting using anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK and anti- β -actin antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of two independent experiments.

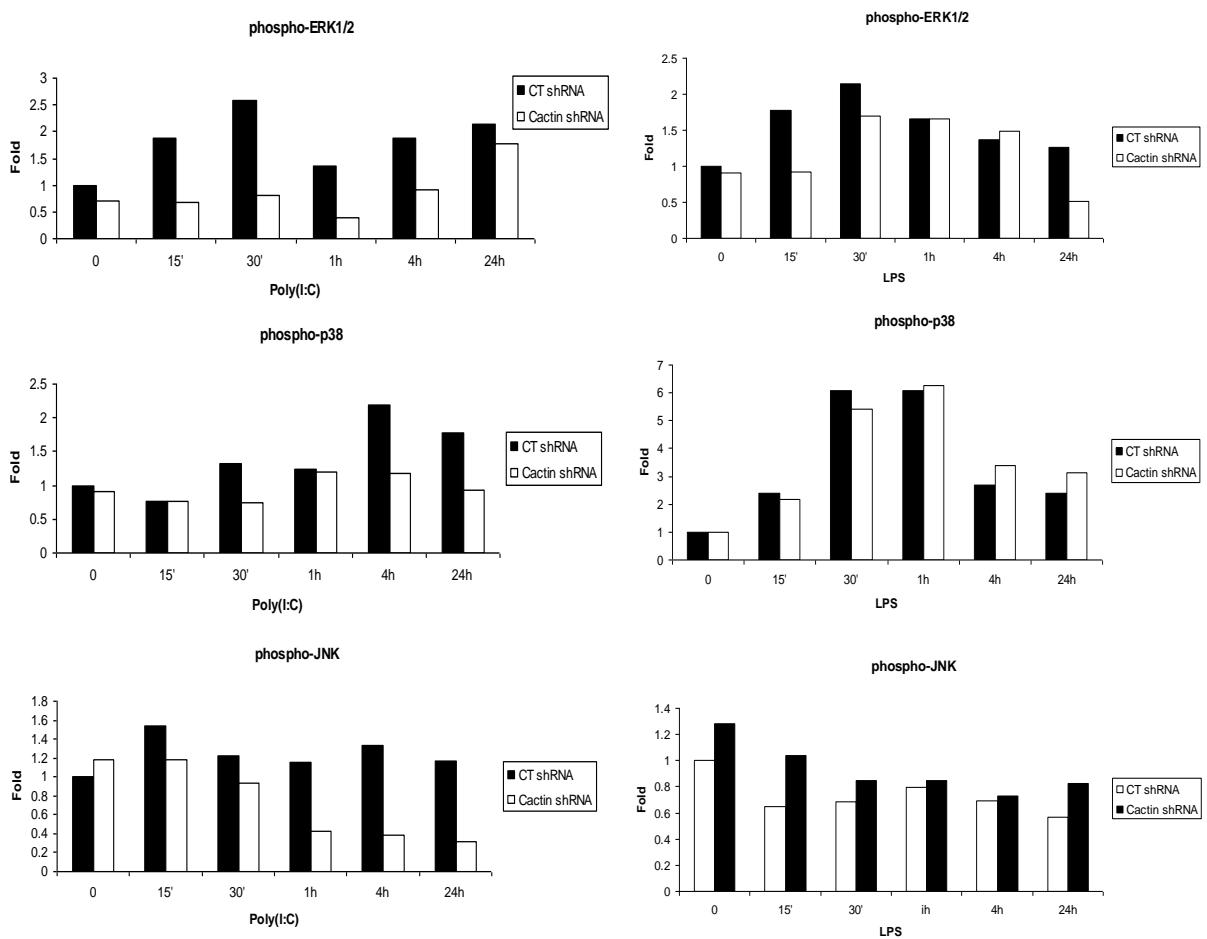


Figure 4.21 Densitometry analysis of western blots seen in Figure 4.19 and Figure 4.20.

Levels of phospho-ERK1/2, phospho-p38 and phospho-JNK were determined by densitometry and normalised to the amount of total ERK1/2, p38 or JNK and relative to the amount detected in non-treated samples. Densitometry was performed using ImageJ software.

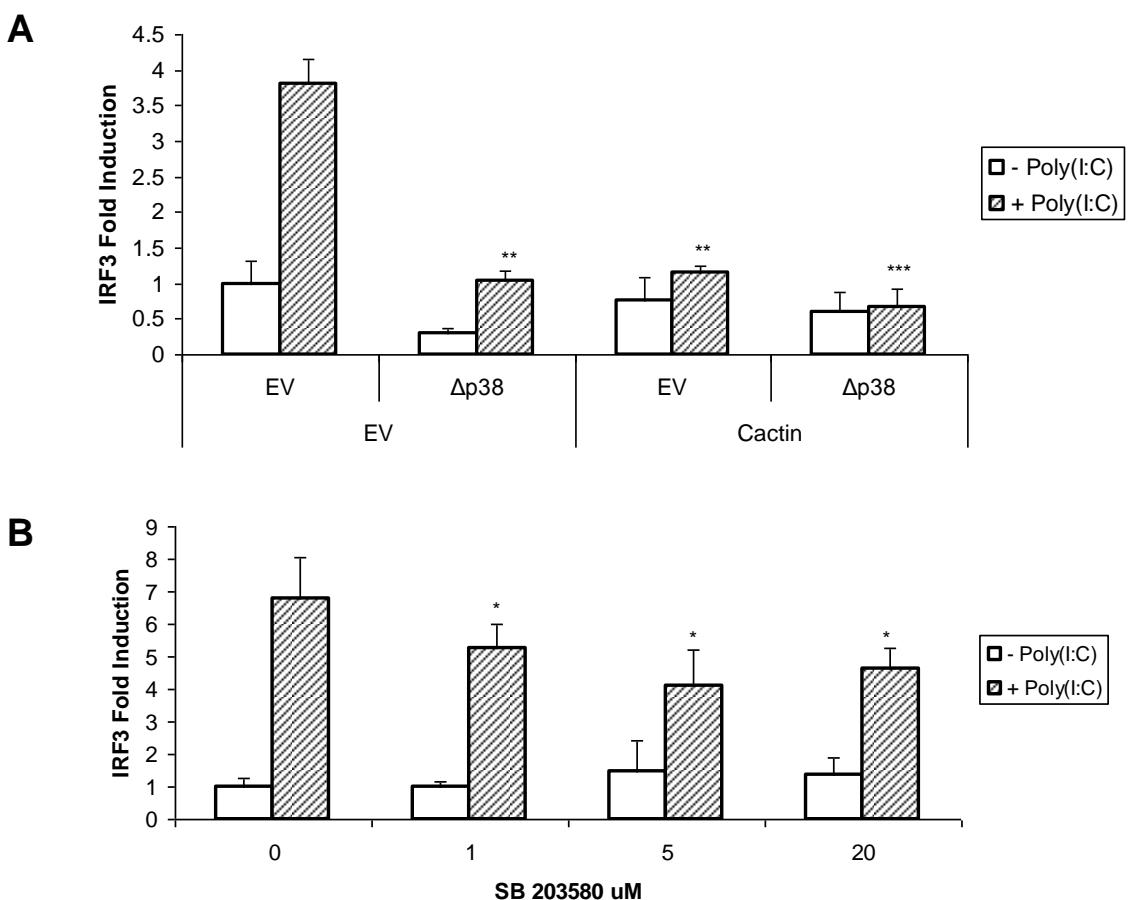


Figure 4.22 p38 positively regulates IRF3 activation

(A) HEK293 TLR3 cells were co-transfected with plasmids encoding pFA-IRF3 (30 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla (20 ng) and with or without plasmids encoding myc-tagged Cactin (90 ng) and a dominant negative mutant of p38 ($\Delta p38$) (50 ng). Transfected cells were left overnight and then treated with Poly(I:C) (25 $\mu\text{g/ml}$) for 24 h. (B) HEK293 TLR3 cells were co-transfected with plasmids encoding pFA-IRF3 (30 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla (20 ng). Cells were pre-treated with various concentrations of SB 203580 prior to stimulation with Poly(I:C) (25 $\mu\text{g/ml}$) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data presented are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, paired t-test, (A) Ligand stimulated cells transfected with EV compared with ligand stimulated cells transfected with Cactin and/or $\Delta p38$ and (B) Ligand stimulated cells pretreated with SB 203580 compared with ligand stimulated cells pre-treated with DMSO.

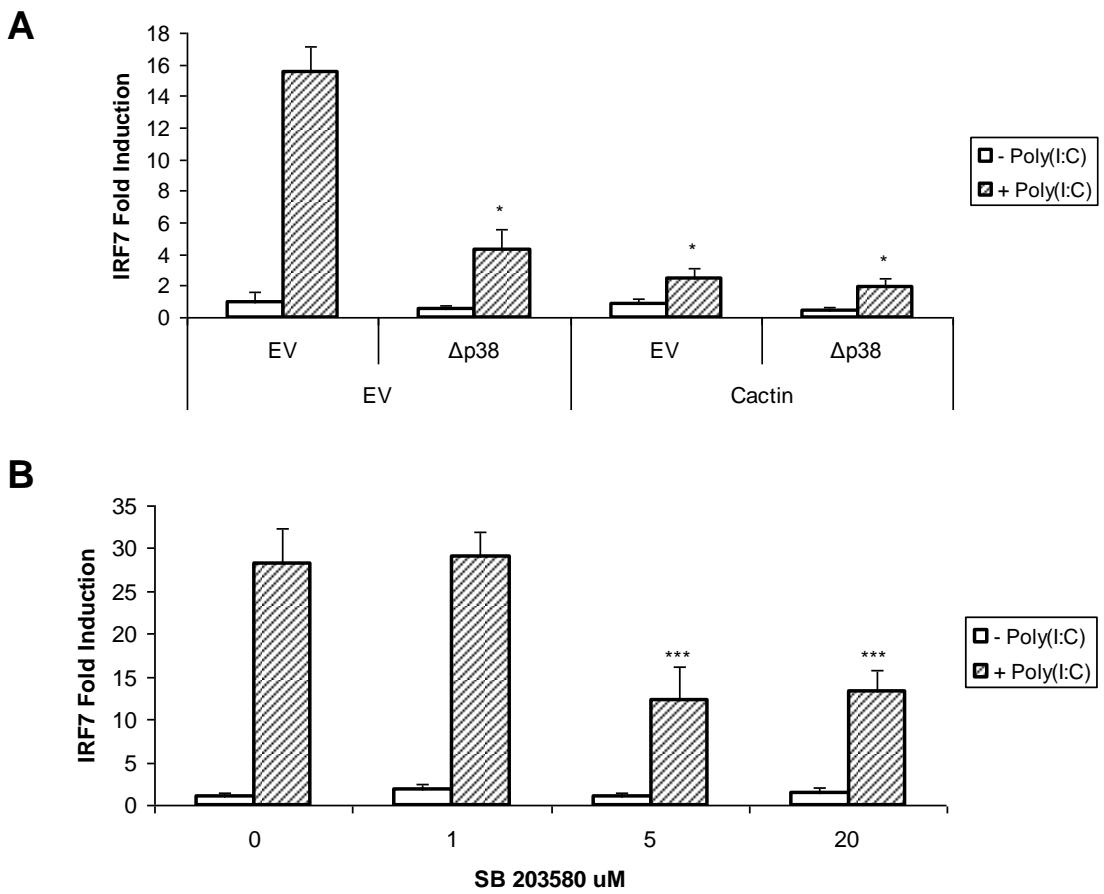


Figure 4.23 p38 positively regulates IRF7 activation

(A) HEK293 TLR3 cells were co-transfected with plasmids encoding pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla (20 ng) and with or without plasmids encoding myc-tagged Cactin (90 ng) and a dominant negative mutant of p38 (Δ p38) (50 ng). Transfected cells were then treated with Poly(I:C) (25 μ g/ml) for 24 h. (B) HEK293 TLR3 cells were co-transfected with plasmids encoding pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla (20 ng). Cells were pretreated with various concentrations of SB 203580 for 1 h prior to stimulation with Poly(I:C) (25 μ g/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data presented are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * $p < 0.05$, *** $P < 0.001$, paired t-test, (A) Ligand stimulated cells transfected with EV compared with ligand stimulated cells transfected with Cactin and/or Δ p38 and (B) Ligand stimulated cells pretreated with SB 203580 compared with ligand stimulated cells pre-treated with DMSO.

4.3 Discussion

The production of Type I IFNs is essential for mounting an efficient immune response to invading pathogens. However dysregulation of the pathways controlling Type I IFN expression can contribute to the development of autoimmune and autoinflammatory disorders such as Rheumatoid arthritis (RA) (Ivashkiv and Hu, 2003) and systemic lupus erythematosus (SLE) (Hu et al., 2008). The activation of Type I IFNs is tightly regulated at various stages to avoid the development of autoimmune disorders and also to prevent undue stress in uninfected cells. For example, the half-life of IFN-induced IRF7 is only 0.5-1 h, most likely to ensure that the production of Type I IFNs is transient (Sato et al., 2000). Peptidyl-prolyl isomerase Pin1 is a negative regulator of Type I IFNs and ubiquitinates activated IRF3, marking it as a target for proteosomal degradation (Saitoh et al., 2006). Some members of the IRF family, IRF2 and IRF8, bind to ISREs and negatively regulate gene expression (Goodbourn et al., 2000). The present studies now suggest that Cactin is a novel negative regulator of Type I IFNs.

Consistent with previous findings from the lab the data here indicates that Cactin is a powerful negative regulator of IRF3 and IRF7 activation. This inhibitory effect was found to be stimulus and cell type independent as Cactin negatively regulated the activation of IRF3 and IRF7 in response to Poly(I:C) and LPS in HEK293 cells and suppression of endogenous Cactin led to increased expression of RANTES in U373 cells. Importantly this study showed that Cactin negatively regulates IFN β induction. Over-expression of Cactin inhibited Poly(I:C) induced IFN β while suppression of endogenous Cactin enhanced IFN β expression. Interestingly, Cactin has no effect on the phosphorylation of IRF3 or translocation of IRF3 or IRF7 to the nucleus. However, suppression of Cactin leads to increased binding of IRF3 to PRDI-III of the IFN β promoter. Together these results suggest that Cactin inhibits IRFs at the level of DNA binding. This is consistent with findings from the previous chapter indicating that Cactin inhibits NF- κ B-DNA binding. Indeed nuclear localisation was shown to be critical for Cactin to manifest its inhibitory effects on IRF3 and IRF7 as a truncated-Cactin mutant failed to manifest the same inhibitory effects on LPS, Poly(I:C) and TRIF-mediated IRF3 and IRF7 activation. Interestingly, this inhibition was not achieved through direct binding of Cactin to IRF3. It is possible that Cactin interacts with other IRF3 binding proteins. For

example, association of CBP/p300 and IRF3 has been shown to be essential for IRF3-DNA binding activity (Suhara et al., 2002). Interference with this association could therefore repress the DNA binding activity of IRF3.

IRF7 is essential for the induction of IFN α genes. Notably, Cactin inhibited IRF7-induced activation of the IFN α -1 promoter. This confirms that Cactin also targets IRF7. IRF7 is subject to post-translational modifications including phosphorylation and ubiquitination following Poly(I:C) stimulation (Ning et al., 2011). K48-linked ubiquitination of IRF7 promotes its proteosomal degradation. The results here indicate that overexpressed IRF7 is degraded in the absence of Poly(I:C) in HEK293 cells. Interestingly, suppression of Cactin expression prevented this degradation. Furthermore, overexpression of Cactin inhibited the Poly(I:C) induced stabilisation of Cactin and this is also a contributory mechanism to the inhibitory effects of Cactin on IFN β expression. The regulatory effects of Cactin on IRF7 are particularly interesting due to the role of IRF7 in autoimmune diseases such as SLE, psoriasis and systemic sclerosis (SSc) (Sozzani et al., 2010). This raises the possibility of exploiting Cactin for the treatment of these autoimmune diseases. It would be interesting, in the future, to look at the expression levels of Cactin in autoimmune diseases such as SLE.

As Cactin negatively regulates the activation of a range of transcription factors including NF- κ B, IRF3 and IRF7 it was important to ensure that such effects were not non-specific, reflecting inhibitory effects on global transcription. Importantly, Cactin enhanced c-Jun-induced gene expression indicating that Cactin can differentially regulate specific transcription factors. It also suggests that Cactin is a positive regulator of JNK activation which was further confirmed when suppression of endogenous Cactin decreased Poly(I:C) mediated JNK phosphorylation. Furthermore, Cactin also positively regulates Poly(I:C) mediated phosphorylation of MAPKs p38 and ERK1/2. p38 has a role in LPS mediated IRF3 activation in U373 cells (Navarro and David, 1999). p38 is also required for CpG-DNA mediated IRF7 activation in plasmacytoid dendritic cell precursors (Takauchi et al., 2002). This current study indicates that p38 is also a positive regulator of Poly(I:C) mediated IRF3 and IRF7 activation. This appears to be somewhat of a contradiction as Cactin positively regulates the activation of p38 which should lead to increased activation of IRF3. However, Cactin inhibits IRF3 at the level of DNA binding and so this inhibition would override any upstream affects on MAPK activation. This also would also

explain how Cactin can positively regulate JNK phosphorylation but negatively regulates binding of AP-1 heterodimers to PRDIV of the IFN β promoter. The positive regulation of p38 by Cactin also explains how suppression of endogenous Cactin leads to decreased phosphorylation of IRF3 in response to Poly(I:C).

In order to explore the mechanism by which Cactin negatively regulates TLR-signalling it is important to identify novel Cactin-interacting proteins and to characterise the functional consequences of these interactions. Such studies form the content of the next chapter.

Chapter 5

**Identifying Cactin-interacting proteins and
determining the functional consequences of these
interactions**

5.1 Introduction

In order to further dissect the mechanism by which Cactin negatively regulates TLR-signalling it was necessary to investigate potential protein-interacting partners for Cactin. Prior to this current study Mass Spectrometry analysis was carried out to identify proteins that co-immunoprecipitated with Cactin and this approach identified several potential Cactin interactors (Table 5.1). This study was especially interested in validating putative interactions with proteins of relevance to pathways that are regulated by Cactin. One such interactor was Activating signal cointegrator 1 complex subunit 1 (ASCC1). ASCC1 was identified as a component of the ASC-1 complex which regulates NF- κ B, AP-1 and serum response factor (SRF) (Jung et al. 2002). The ASC-1 complex binds directly to SRF, AP-1 subunit Jun and NF- κ B subunits p50 and p65 and positively regulates transactivation. ASC-1 is also a transcription coactivator of nuclear receptors (Kim et al., 1999). ASCC1 contains an RNA-binding motif termed KH and this domain appears to be essential for its function and interaction with other components of the ASC-1 complex (Jung et al., 2002). Potential interaction of Cactin and ASCC1 seemed interesting as ASCC1 has been shown to enhance NF- κ B and AP-1 transactivation and so this study aimed to confirm this interaction by co-immunoprecipitation.

PAK1 interacting protein 1 (PAK1IP1) was another potential Cactin-interactor identified by Mass Spectrometry. The p21-activated kinases (PAKs) are a family of serine-threonine protein kinases regulated by small GTPases, CDc42 and Rac (Manser et al., 1994). There are four known isoforms, PAK1-4, which are differentially expressed in mammalian cells and regulate diverse cellular functions. PAK1 has been shown to activate the MAPK signalling cascades resulting in JNK and p38 activation (Bagrodia et al., 1995). PAK1 can also activate NF- κ B in response to various stimuli including LPS (Frost et al., 2000). PAK proteins all possess an N-terminal autoregulatory domain and C-terminal kinase domain and form inactive dimers in unstimulated cells. PAKs are activated by binding of GTPases to the N-terminal domain disrupting the dimer and resulting in an active kinase. PAK1IP1 interacts with the N-terminal autoregulatory domain of PAK1 and inhibits PAK1 mediated activation of NF- κ B and JNK (Xia et al., 2001). Given that PAK1IP1 and Cactin both

negatively regulate NF-κB activation we wanted to confirm this possible interaction and to determine if these proteins co-operate in order to inhibit NF-κB activation.

Given that *drosophila* Cactin was discovered as an interactor of Cactus, we decided to investigate if human Cactin interacts with IκBα, or other members of the IκB family. IκB family members all possess five to seven ankyrin repeat motifs which mediate interaction with the RHD of Rel/NF-κB proteins (Oeckingus and Ghosh, 2009). In the unstimulated cell, cytoplasmic IκB proteins, IκBα, IκBβ, and IκBε are found in association with NF-κB dimers. The amino terminal of cytoplasmic IκBs contains conserved serine and lysine residues which can be phosphorylated and ubiquitinated respectively, marking them as targets for K48-linked ubiquitination and subsequent proteosomal degradation (Manavalan et al., 2010). This allows for NF-κB to translocate to the nucleus and regulate gene expression. Nuclear IκBs, including IκBζ, IκBNS, IκB-like (IκBL) and Bcl-3 also possess these ankyrin repeats and some have been shown to interact directly with NF-κB dimers. IκBζ inhibits DNA binding and transactivation of p65-containing NF-κB complexes but also appears to upregulate the expression of some NF-κB-regulated genes (Oeckingus and Ghosh, 2009). Bcl-3 has also been shown to both promote and inhibit transcriptional activity (Yammamoto and Takeda, 2008). Nuclear IκBs have been shown to interact with proteins other than NF-κB subunits. For example, IκBζ can interact with and inhibit STAT3 activity (Wu et al., 2009). IκBL has been shown to interact with translation elongation factor 1 α and CTP synthase 1 and can also associate directly with mRNA, suggesting a role for IκBL in mRNA processing or in regulation of translation (Greetham et al., 2007). It is clear that nuclear IκB proteins are complex regulators of gene expression. Given that Cactin is expressed in the nucleus it was interesting to investigate if Cactin might interact with nuclear members of the IκB family thus regulating NF-κB activation. Using a combination of the unbiased proteomic-based screening (described above) with more targeted analysis of IκB proteins, the studies described in this chapter hoped to provide an increased understanding of the mechanisms underlying the regulatory effects of Cactin by identifying and validating Cactin interactors of functional relevance.

5.1.1 Specific aims of Chapter 5

1. Validating Cactin interactors detected by Mass Spectrometry analysis.
2. Investigate if Cactin interacts with IkB α or other members of the IkB family.
3. Determine the functional relevance of any interactions detected.

5.2 Results

5.2.1 Cactin-myc does not co-immunoprecipitate with PAK1IP1-HA

Proteomic-based analysis, carried out prior to this study, identified PAK1IP1 as an interactor of Cactin. HEK293 T cells were transfected with Myc-tagged hCactin and cell lysates were immunoprecipitated using anti-IgG mouse or anti-myc antibodies. The immunoprecipitates were resolved in a SDS acrylamide gel and both lanes in the gel were cut into slices and the proteins were digested with trypsin and subjected to nano-electrospray liquid chromatography mass spectrometry (Nano-LC MS/MS). We were keen to confirm this interaction by co-immunoprecipitation. To do this HEK293 TLR4 cells were co-transfected with plasmids encoding HA-tagged PAK1IP1 and myc-tagged Cactin. Lysates were generated and immunoprecipitated with an anti-HA antibody. PAK1IP1-HA was detected in immunoprecipitates from cells expressing PAK1IP1-HA indicating that the immunoprecipitation process was successful (Figure 5.1). However Cactin-myc was not detectable in immunoprecipitates from cells expressing both PAK1IP1-HA and Cactin-myc although it was detected in the corresponding whole cell lysates (Figure 5.1). This suggests that PAK1IP1 and Cactin do not interact. It is worth noting that cells were also treated with IL-1 β and Poly(I:C) to assess if any potential interaction could be stimulus dependent but again no interaction was detected.

5.2.2 ASCC1 co-immunoprecipitates with Cactin-myc

ASCC1 is another protein that had been identified as a Cactin interactor by our proteomic approach. To confirm this interaction HEK293 TLR4 cells were co-transfected with plasmids encoding ASCC1 and myc-tagged Cactin. Lysates were generated and immunoprecipitated with an anti-myc antibody. ASCC1 was detected in immunoprecipitates from cells over-expressing ASCC1 and Cactin-myc, indicating that there was some interaction between the two proteins (Figure 5.2). To further confirm this interaction by confocal microscopy HEK293 TLR4 cells were transfected with plasmids encoding Cactin-myc and ASCC1, fixed and stained with anti-ASCC1 and anti-myc antibodies. Cells were also stained with Hoescht to visualise the nuclei. ASCC1 was expressed in the cytoplasm and the nuclei of the cells while Cactin-myc

was again found exclusively in the nucleus (Figure 5.3). ASCC1 and Cactin-myc co-localised in some but not all cells which was detected by overlapping red and green staining which forms yellow/orange immunofluorescence (Figure 5.3). It is worth noting that similar results were observed when cells were treated with the pro-inflammatory stimulus IL-1 β .

In order to investigate the functional relevance of the interaction between ASCC1 and Cactin, the ability of ASCC1 to regulate NF- κ B, IRF3 and IRF7 was next examined. Over-expression of ASCC1 was found to have no effect on the activation of a co-transfected NF- κ B-regulated luciferase gene by LPS (Figure 5.4A). This was somewhat surprising as ASCC1 was identified as a component of the ASC-1 complex which enhances NF- κ B transactivation in HeLa cells (Jung et al., 2002). The ability of ASCC1 to regulate IRF3 and IRF7 was then examined. HEK293 TLR3 cells were transfected with plasmids encoding pFA-IRF3 or pFA-IRF7 and pFR-regulated luciferase in the absence or presence of ASCC1. Interestingly over-expression of ASCC1 inhibited Poly(I:C) mediated IRF3 and IRF7 activation (Figure 5.4B, C). This is interesting as Cactin is also a negative regulator of IRF3 and IRF7 activation and so these two proteins may co-operate in order to inhibit IRFs. However the magnitude of inhibition effected by ASCC1 was more modest than Cactin, especially in relation to regulation of NF- κ B and thus the functional importance of this interaction was not further considered in this thesis.

5.2.3 Cactin fails to interact with I κ B α but does interact with nuclear I κ BL

Given that *Drosophila* Cactin interacts with Cactus we investigated if Cactin interacts with the human homologue of Cactus which is I κ B α . In order to investigate this HEK293 TLR4 cells were transfected with a plasmid encoding myc-tagged Cactin and subsequently treated with the pro-inflammatory stimulus IL-1 β for various time periods to see if these proteins interact under inflammatory conditions. Lysates were generated and immunoprecipitated with an anti-myc antibody. Whilst the expression of both Cactin-myc and I κ B α was confirmed by Western blotting, I κ B α was not detected in samples of immunoprecipitated Cactin, even in samples from cells previously stimulated with IL-1 β (Figure 5.5). This is not altogether surprising as I κ B α is expressed predominantly in the cytoplasm and Cactin-myc displays a nuclear localisation. For this reason, it was then investigated if Cactin interacts with a nuclear

member of the I κ B family, I κ BL. To do this HEK293 T cells were co-transfected with plasmids encoding GFP-tagged I κ BL and myc-tagged Cactin. Lysates were generated and immunoprecipitated with an anti-myc antibody. I κ BL-GFP was detected in immunoprecipitates from cells expressing I κ BL-GFP and Cactin-myc indicating that there was interaction between the two proteins (Figure 5.6).

To further confirm this association HEK293 TLR4 cells were co-transfected with plasmids encoding GFP-tagged I κ BL and RFP-tagged Cactin. The cells were then fixed and stained with hoescht to visualise the nuclei. I κ BL-GFP and Cactin-RFP showed very similar nuclear expression patterns. Co-localisation was apparent in many cells as evidenced by the overlapping RFP and GFP formation of yellow/orange immunofluorescence (Figure 5.7). As a control HEK293 T cells were co-transfected with plasmids encoding GFP-tagged Cactin and myc-tagged I κ B α . Lysates were generated and immunoprecipitated using an anti-myc antibody. Cactin-GFP was not detected in immunoprecipitates from cells expressing both I κ B α -myc and Cactin-GFP although I κ B α -myc was detected in the immunoprecipitates and Cactin-GFP was detected in the whole cell lysates (Figure 5.8). This further confirms that Cactin does not interact with I κ B α and also that the association seen between I κ BL-GFP and Cactin-myc is more likely to be a true interaction and not an artefact of expressing GFP and myc tagged proteins.

5.2.4 Cactin and I κ BL function independently to inhibit NF- κ B activation

To investigate the functional relevance of Cactin and I κ BL association, the ability of I κ BL to regulate NF- κ B activation was first examined. To do this HEK293 TLR4 cells were transfected with or without a plasmid encoding I κ BL-GFP and assessed for their ability to activate a co-transfected NF- κ B-regulated luciferase gene in response to IL-1 β and LPS stimulation. I κ BL-GFP significantly inhibited IL-1 β and LPS mediated activation of NF- κ B (Figure 5.9). As Cactin is also a negative regulator of NF- κ B we were keen to investigate if the association of these two proteins is necessary for manifesting the inhibitory effects of both proteins on NF- κ B. In order to do this Cactin- and I κ BL-specific shRNA was used to suppress endogenous expression of these proteins. Quantitative real-time PCR was used to confirm the specific effects of these two shRNAs. Real-time PCR confirmed ~55% knockdown of endogenous I κ BL by shRNA in HEK293 TLR4 cells (Figure 5.10A,B) while there

was ~50% less Cactin in HEK293 TLR3 cells transfected with Cactin-specific shRNA compared to the non-specific shRNA control (Figure 5.10C,D).

In order to determine if I κ BL is required for Cactin's negative regulation of NF- κ B, HEK293 TLR4 cells were transfected with plasmids encoding I κ BL-specific shRNA or a non-specific shRNA control and assessed for the ability of Cactin-myc to inhibit LPS mediated activation of a co-transfected NF- κ B-regulated luciferase reporter plasmid. As expected, knockdown of I κ BL using shRNA augmented LPS activation of NF- κ B (Figure 5.11) further confirming its role as a negative regulator of NF- κ B. However, over-expression of Cactin-myc inhibited LPS-mediated NF- κ B activation in cells suppressing I κ BL to the same extent as in cells transfected with the non-specific shRNA control (Figure 5.11). This suggests that Cactin inhibits NF- κ B activation independently of its association with I κ BL. Next we examined if the regulatory effects of I κ BL on NF- κ B are dependent on its association with Cactin. To do this HEK293 TLR4 cells were co-transfected with plasmids encoding Cactin-specific shRNA or a non-specific shRNA control, I κ BL-GFP or Empty Vector (EV) pcDNA3.1 and NF- κ B-regulated luciferase. As before, suppression of endogenous Cactin augmented LPS mediated NF- κ B activation (Figure 5.13). Interestingly, there was no impairment of the ability of I κ BL to inhibit LPS mediated NF- κ B activation in cells transfected with Cactin-specific shRNA compared with cells transfected with a non-specific shRNA control (Figure 5.12). This indicates that I κ BL inhibits NF- κ B activation independent of Cactin and so the two proteins appear to function independently to regulate NF- κ B.

5.2.5 I κ BL inhibits IRF3 and IRF7 activation

Given that Cactin and I κ BL appear to function independently in terms of NF- κ B regulation we decided to investigate if the association of I κ BL and Cactin is important for the regulation of IRFs. Firstly, the effects of I κ BL on Poly(I:C) mediated IRF3 and IRF7 activation was examined. To do this HEK293 TLR3 cells were co-transfected with plasmids encoding pFA-IRF3 or pFA-IRF7 and a pFR-regulated luciferase gene and with or without a plasmid encoding I κ BL-GFP. Like Cactin, I κ BL-GFP inhibited the activation of IRF3 and IRF7 in response to Poly(I:C) (Figure 5.13).

5.2.6 I κ BL negatively regulates IFN β induction

To investigate the functional consequence of I κ BL negatively regulating IRF3 and IRF7 activation, the ability of I κ BL to regulate IFN β induction was next examined. HEK293 TLR3 cells were transfected with plasmids encoding I κ BL-specific shRNA or a non-specific shRNA control. Cells were then stimulated with Poly(I:C) before extracting RNA. Real-time PCR was performed to quantify the amount of IFN β mRNA transcribed. In keeping with regulatory effects of Cactin on IRF activation, suppression of endogenous I κ BL augmented Poly(I:C) induction of IFN β (Figure 5.14). To further confirm that I κ BL inhibits IFN β induction by targeting IRF-mediated activation of the IFN β promoter HEK293 TLR3 cells were transfected with plasmids encoding I κ BL-specific shRNA or a non-specific shRNA control and assessed for their ability to activate a co-transfected IFN β -, PRDI-III-, PRDII- or PRDIV-regulated luciferase reporter construct. Suppression of endogenous I κ BL augmented the activation of the IFN β promoter in response to Poly(I:C) (Figure 5.15). Interestingly I κ BL appears to target PRDI-III of the IFN β promoter as suppression of endogenous I κ BL augmented Poly(I:C) activation of PRDI-III-luciferase (Figure 5.15), although the figures did not reach statistical significance. Suppression of endogenous I κ BL had no affect on Poly(I:C) mediated PRDII or PRDIV activation. Given that Cactin was also found to be a powerful negative regulator of IFN β induction by targeting PRDI-III of the IFN β promoter it was interesting to determine if these proteins co-operate in order to inhibit the activation of IRFs and induction of IFN β .

5.2.7 I κ BL has no affect on Poly(I:C) -induced phosphorylation of IRF3

Phosphorylation of IRF3 is essential for its activation as it promotes dimerisation and translocation to the nucleus. In an effort to investigate the effect of I κ BL on Poly(I:C) mediated IRF3 phosphorylation, HEK293 TLR3 cells were transfected with plasmids encoding I κ BL-specific shRNA or a non-specific shRNA control. Suppression of endogenous I κ BL had no effect on Poly(I:C) mediated IRF3 phosphorylation as detected by Western immunoblotting using an anti-phospho-IRF3 antibody (Figure 5.16). This indicates that, similarly to Cactin, I κ BL manifests in

inhibitory effects downstream of IRF3 phosphorylation and most likely downstream of nuclear translocation.

5.2.8 Cactin and I_kBL function independently to inhibit IRF3 and IRF7 activation

In order to determine if Cactin's negative regulation of IRF3 and IRF7 activation is dependent on its interaction with I_kBL, HEK293 TLR3 cells were transfected with plasmids encoding pFA-IRF3 or pFA-IRF7 and pFR-regulated luciferase. Cells were also transfected with or without plasmids encoding I_kBL-specific shRNA or a non-specific shRNA control and Cactin-myc. Suppression of endogenous I_kBL augmented Poly(I:C) mediated IRF3 and IRF7 activation (Figure 5.17), further confirming the role of I_kBL as a negative regulator of IRFs. However there was no impairment of Cactin's ability to inhibit Poly(I:C) mediated IRF3 or IRF7 activation in cells suppressing endogenous I_kBL (Figure 5.17). This indicates that interaction with I_kBL is not essential for Cactin to negatively regulate IRF3 or IRF7 activation. It was still possible, however, that I_kBL inhibits IRF3 and IRF7 activation in a Cactin-dependent manner. In order to investigate this HEK293 TLR3 cells were again transfected with plasmids encoding pFA-IRF3 or pFA-IRF7 and pFR-regulated luciferase. Cells were also transfected with or without plasmids encoding Cactin-specific shRNA or a non-specific shRNA control and I_kBL-GFP. Suppression of endogenous Cactin augmented Poly(I:C) mediated IRF3 and IRF7 activation. Again there was no impairment of I_kBL's inhibitory effects in cells suppressing endogenous Cactin compared with cells transfected with a non-specific shRNA control (Figure 5.18). The above data suggests that I_kBL and Cactin function independently to negatively regulate IRF3 and IRF7 activation.

Table 5.1 List of Cactin interacting proteins identified by Mass Spectrometry and listed by IPI number.

protein name	Description	Gene Name	prot score
IPI00736681.1	predicted: chromosome 19 open reading frame 29 isoform 1.	C19orf29	1
IPI00031508.1	atp-dependent rna helicase ddx8.	DHX8	1
IPI00385143.1	microfibrillar protein 2 (fragment).	PTF1A	1
IPI00005055.3	hypothetical protein flj22965.	CXorf56	1
IPI00022276.3	mitochondrial 28s ribosomal protein s28.	MRPS28	1
IPI00006970.1	mitochondrial 28s ribosomal protein s2.	MRPS2	1
IPI00249529.2	predicted: ip63 protein isoform 1.	CEP78	1
IPI00001757.1	splice isoform 1 of rna-binding protein 8a.	RBM8A	1
IPI00061245.4	mitochondrial 28s ribosomal protein s10.	MRPS10	1
IPI00036742.5	hypothetical protein loc25962 isoform 1.	KIAA1429	1
IPI00219335.9	60s ribosomal protein l3-like.	RPL3L	1
IPI00011875.1	protein red.	IK	1
IPI00411680.6	similar to protein-l-isoaspartate.	PCMT1	1
IPI00643356.1	karp-1-binding protein.	CEP170	1
IPI00643414.1	3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondria	BCKDK	1
IPI00001690.1	cullin-7.	CUL7	1
IPI00297900.4	probable atp-dependent rna helicase ddx10.	DDX10	1
IPI00006379.1	nucleolar protein nop5.	NOP5	1
IPI00219350.1	splice isoform 2 of 28s ribosomal protein s11; mitochondrial precursor.	MRPS11	1
IPI00305833.3	smu-1 suppressor of mec-8 and unc-52 homolog.	SMU1	1
IPI00395337.1	splice isoform 1 of pre-mrna 3'-end-processing factor fip1.	FIP1L1	1
IPI00007049.1	28s ribosomal protein s18c; mitochondrial precursor.	HPSE	1
IPI00013721.2	serine/threonine-protein kinase prp4 homolog.	PRPF4B	1
IPI00045051.1	transcriptional activator protein pur-beta.	PURB	1
IPI00013180.2	protein g10 homolog.	BUD31	1
IPI00017451.1	splicing factor 3 subunit 1.	SF3A1	1
IPI00030820.1	39s ribosomal protein l47; mitochondrial precursor.	MRPL47	1
IPI00069750.1	fuse-binding protein-interacting repressor isoform a.	PUF60	1
IPI00074690.1	kiaa1839 protein (fragment).	RNPC3	1
IPI00175136.3	rna binding motif protein 15b.	RBM15B	1
IPI00290566.1	t-complex protein 1 subunit alpha.	TCP1	1
IPI00430079.1	splice isoform 2 of phd finger protein 8.	PHF8	1
IPI00514173.1	cyclophilin-33b.	PPIE	1
IPI00554737.1	serine/threonine-protein phosphatase 2a 65 kda regulatory subunit a alpha isoform.	PPP2R1A	1
IPI00022793.4	trifunctional enzyme beta subunit; mitochondrial precursor.	HADHB	1
IPI00514399.1	ribosomal protein s27.	RPS27	1
IPI00654654.1	mrps33 protein (fragment).	MRPS33	1
IPI00002902.5	nucleolar protein 9.	NOL9	1
IPI00005492.2	wd-repeat protein 5.	WDR5	1

IPI00006181.1	eukaryotic translation initiation factor 3 subunit 7.	EIF3S7	1
IPI00020729.1	insulin receptor substrate 4.	IRS4	1
IPI00022373.1	art-4 protein.	NOB1	1
IPI00023086.3	mitochondrial ribosomal protein l15.	LYPLA1	1
IPI00073779.1	28s ribosomal protein s35; mitochondrial precursor.	MRPS35	1
IPI00083708.2	hbxag transactivated protein 2.	BAT2D1	1
IPI00167009.3	cdna flj38620 fis; clone heart2007767; weakly similar to mus musculus e-map-115 protein.	RPRC1	1
IPI00300659.4	parafibromin.	CDC73	1
IPI00305068.5	u5 snrnp-associated 102 kda protein.	PRPF6	1
IPI00337736.1	splice isoform 2 of kinectin.	KTN1	1
IPI00410067.1	splice isoform 1 of zinc finger ccch type antiviral protein 1.	ZC3HAV1	1
IPI00418403.4	hypothetical protein loc51490.	C9orf114	1
IPI00427242.1	protein c14orf111.	FCF1	1
IPI00434390.1	splice isoform 2 of activating signal cointegrator 1 complex subunit 1.	ASCC1	1
IPI00549540.3	p21-activated protein kinase-interacting protein 1.	PAK1IP1	1
IPI00550037.2	28s ribosomal protein s15; mitochondrial precursor.	MRPS15	1
IPI00643426.1	ring-finger protein.	TOPORS	1
IPI00300952.3	splice isoform 1 of peptidyl-prolyl cis-trans isomerase-like 3.	PPIL3	0.99
IPI00472102.2	60 kda heat shock protein; mitochondrial precursor.	HSPD1	0.99
IPI00550227.2	Nephrocystin-3	NPHP3	0.99
IPI00032416.6	splice isoform long of jagged-2 precursor.	JAG2	0.99
IPI00456940.4	ribosomal protein l7; eukaryotic form family protein.	RPL7L1	0.98
IPI00220302.1	splice isoform 1 of wilms' tumor 1-associating protein.	WTAP	0.98
IPI00641770.1	phosphatidylinositol 4-kinase; catalytic; beta polypeptide.	PIK4CB	0.97
IPI00021435.2	26s protease regulatory subunit 7.	PSMC2	0.92
IPI00004459.1	probable dimethyladenosine transferase.	DIMT1L	0.92

HEK293 T cells were transfected with Myc-tagged hCactin and cell lysates were immunoprecipitated using anti-IgG mouse or anti-myc antibodies. The immunoprecipitates were resolved in a SDS acrylamide gel and both lanes in the gel were cut into slices and the proteins were digested with trypsin and subjected to nano-electrospray liquid chromatography mass spectrometry (Nano-LC MS/MS). Proteins were identified that were immunoprecipitated with the anti-myc antibody but not the control anti-IgG and the top Protein Prophet (PP) scoring proteins are listed above ; performed by Dr. Paola Atzei and Dr. Patricia Maguire

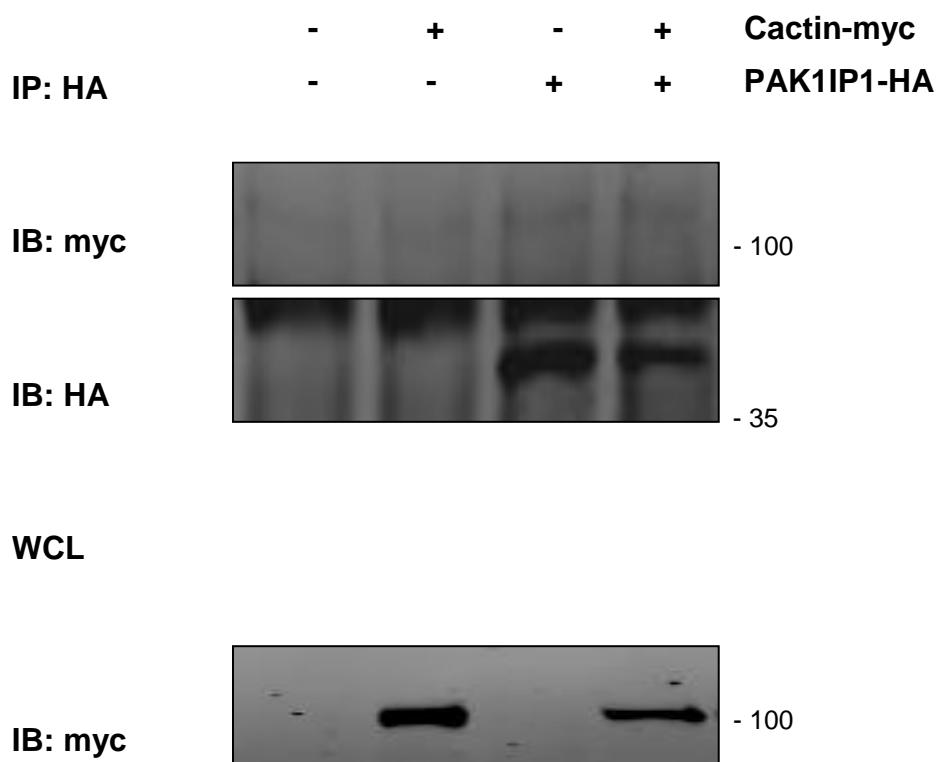


Figure 5.1 Cactin-myc does not co-immunoprecipitate with PAK1IP1-HA

HEK293 TLR4 cells were co-transfected with plasmids encoding myc-tagged Cactin (2 µg) and PAK1IP1-HA (2 µg). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection lysates were generated and immunoprecipitated with an anti-HA antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western immunoblotting using anti-myc and anti-HA antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of three independent experiments.

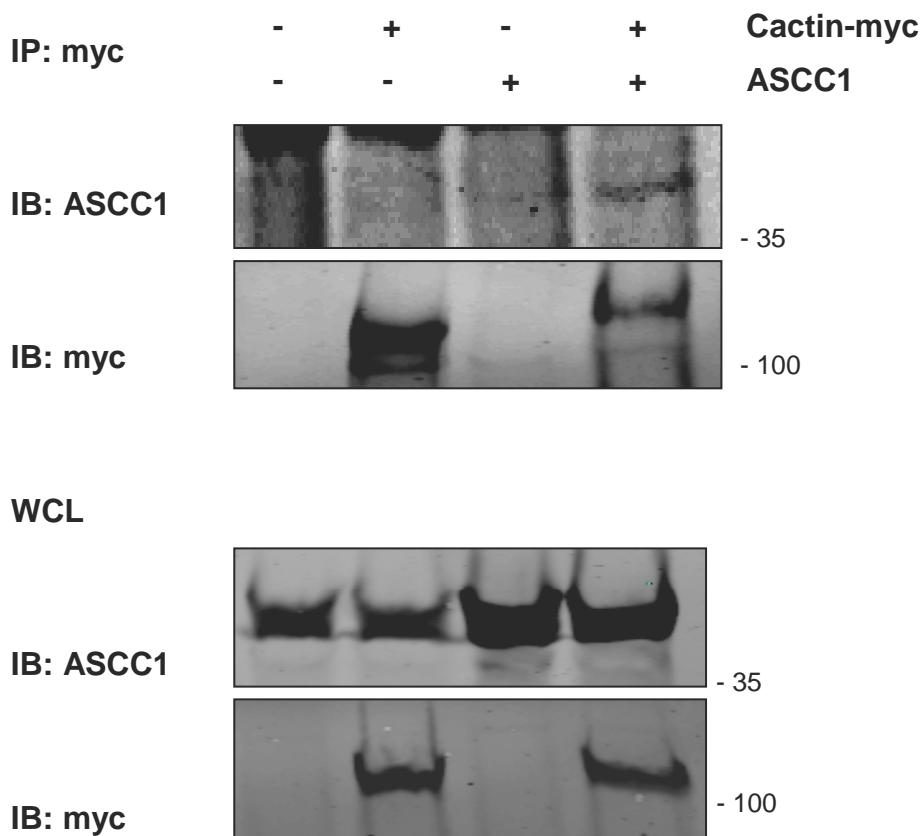


Figure 5.2 ASCC1 co-immunoprecipitates with Cactin-myc

HEK293 TLR4 cells were co-transfected with plasmids encoding myc-tagged Cactin (2 µg) and ASCC1 (2 µg). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection lysates were generated and immunoprecipitated with an anti-myc antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western immunoblotting using anti-ASCC1 and anti-myc antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are indicative of three independent experiments.

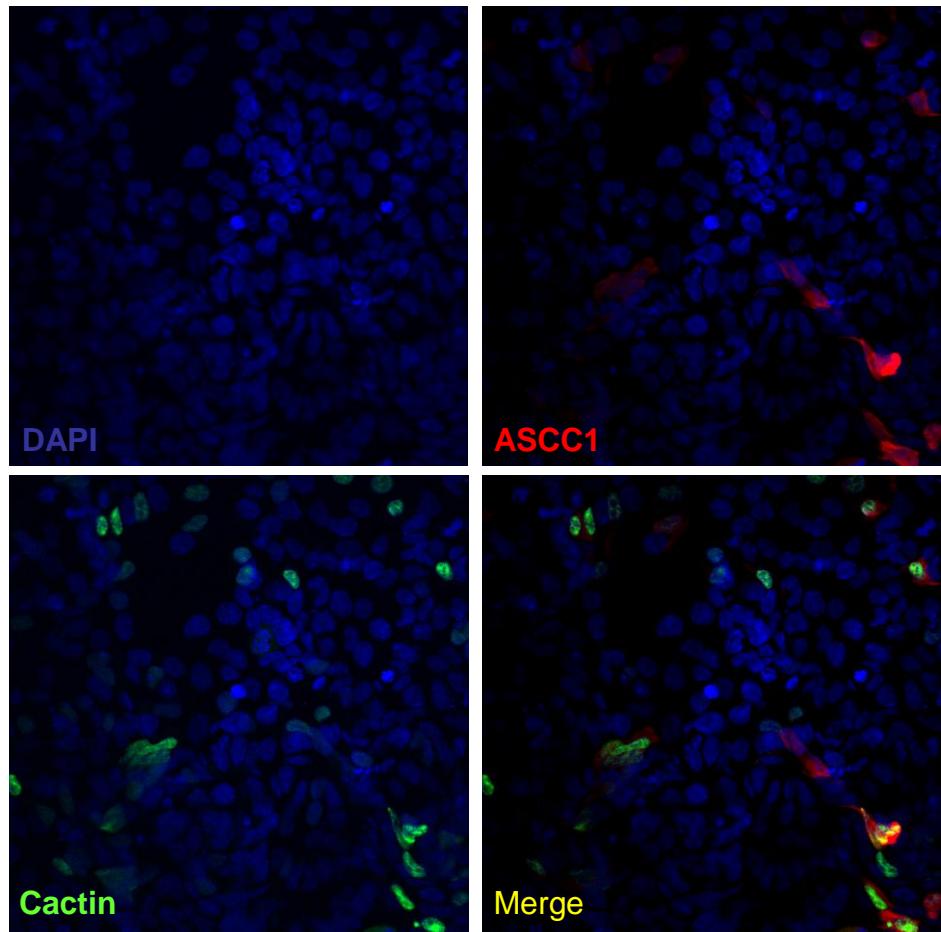


Figure 5.3 Cactin-myc and ASCC1 co-localise in some cells

HEK293 TLR4 cells were co-transfected with plasmids encoding myc-tagged Cactin (2 µg) and ASCC1 (2 µg). 24 h post-transfection cells were fixed, stained with anti-myc and anti-ASCC1 antibodies, stained with hoescht and mounted in anti-fade medium. Confocal images were captured with the Olympus FluoView FV1000 confocal laser scanning microscope and data analysis was performed using the Olympus FV-10 ASW imaging software. Images shown are representative of two independent experiments.

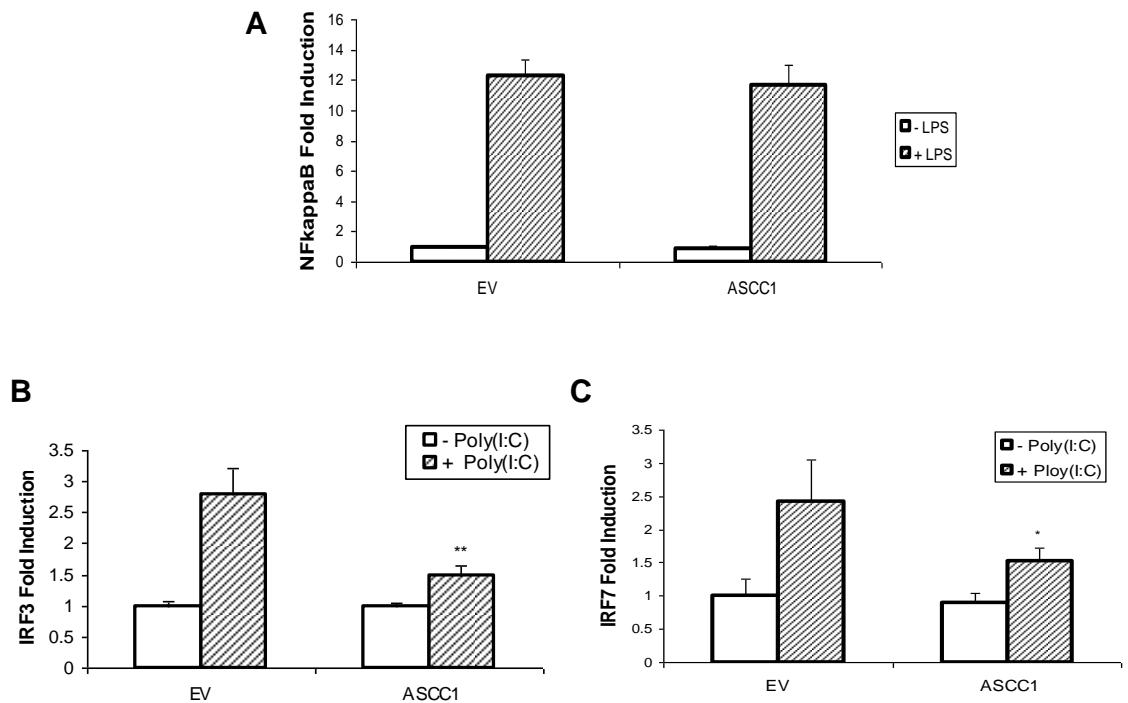


Figure 5.4 ASCC1 inhibits Poly(I:C)-induced activation of IRF3 and IRF7 but has no effect on LPS-induced activation of NF-κB

HEK293 TLR4 or TLR3 cells were transfected with plasmids encoding (A) NF-κB-regulated luciferase (80 ng) and constitutively expressed TK Renilla luciferase (20 ng), (B) pFA-IRF3 (30 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla luciferase (20 ng) or (C) pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without plasmids encoding ASCC1 (90 ng) or Empty vector (EV) pcDNA3.1 (90 ng). Transfected cells were left overnight and then treated with (A) LPS (100 ng/ml) or (B, C) Poly(I:C) (25 µg/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Results shown are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, ** p < 0.01, paired t-test, Ligand stimulated cells transfected with ASCC1 compared with ligand stimulated cells transfected with EV.

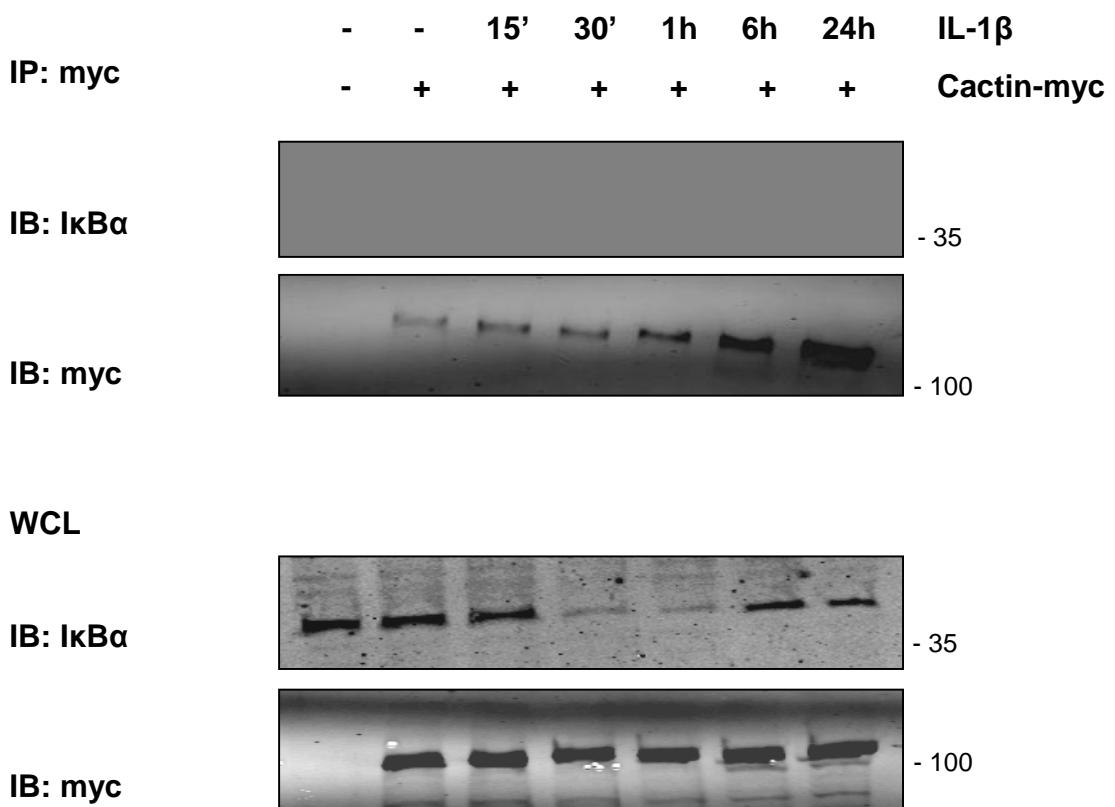


Figure 5.5 I κ B α does not co-immunoprecipitate with Cactin–myc

HEK293 TLR4 cells were transfected with a plasmid encoding myc-tagged Cactin (2 μ g) or empty vector pcDNA3.1 (2 μ g). Transfected cells were left overnight and then treated for the indicated time periods with IL-1 β (10 ng/ml). Lysates were generated and immunoprecipitated with an anti-myc antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western immunoblotting using anti-I κ B α and anti-myc antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of three independent experiments.

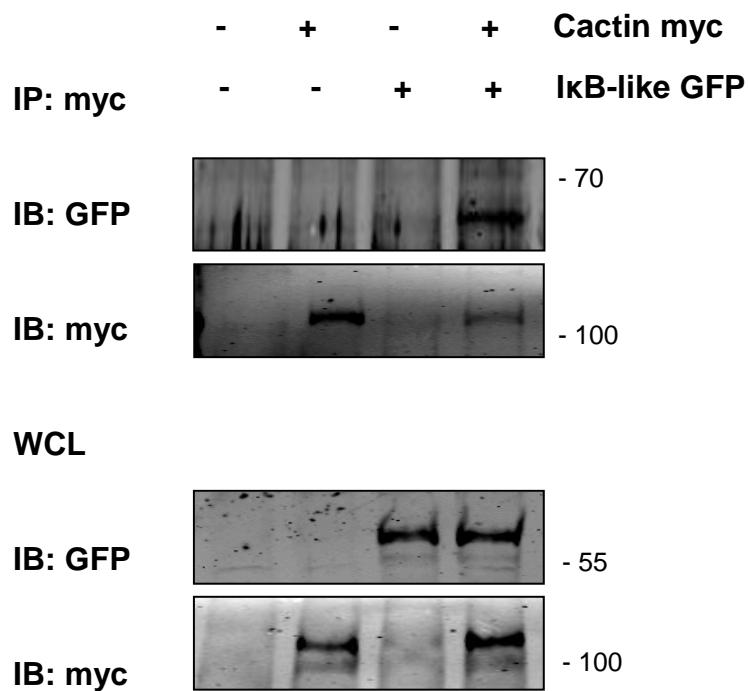


Figure 5.6 I_kBL co-immunoprecipitates with Cactin-myc

HEK293 T cells were co-transfected with plasmids encoding myc-tagged Cactin (2 µg) and GFP-tagged I_kBL (2 µg). Empty vector pcDNA 3.1 was used to normalise the total amount of DNA transfected. 24 h post-transfection lysates were generated and immunoprecipitated with an anti-myc antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western immunoblotting using anti-GFP and anti-myc antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Blots shown are representative of three independent experiments.

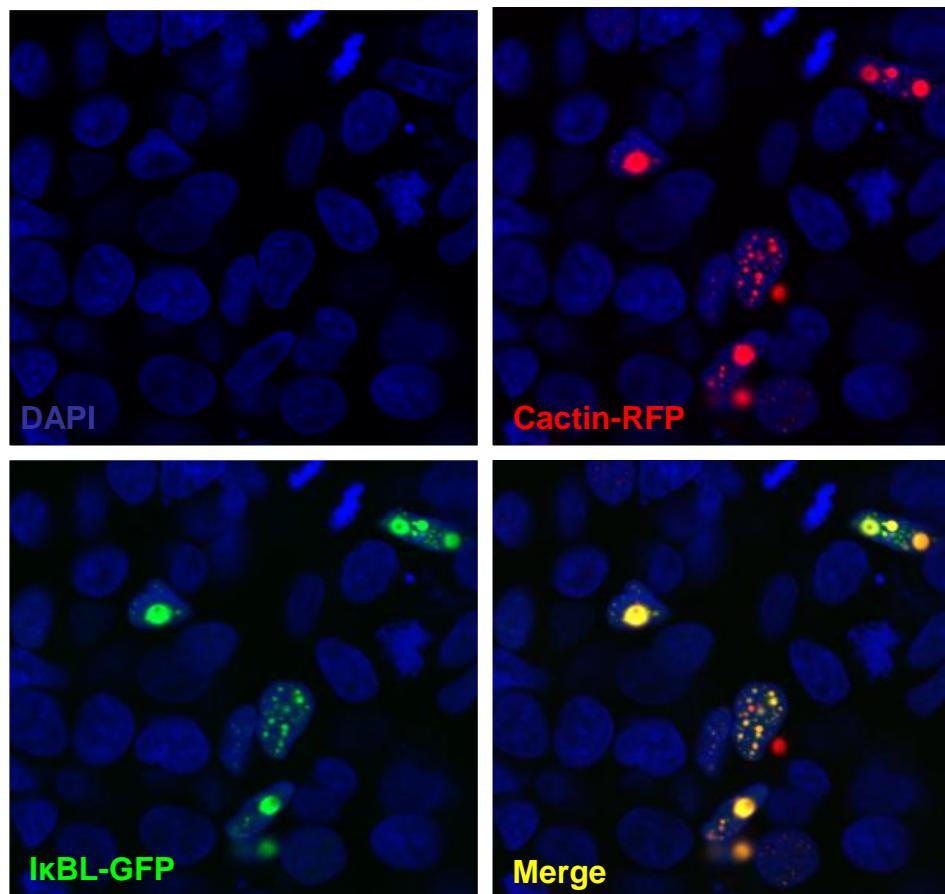


Figure 5.7 IkBL-GFP co-localises with Cactin-RFP

HEK293 TLR4 cells were transfected with GFP tagged IkBL (400 ng) and RFP-tagged Cactin (400 ng). 24 h post transfection cells were fixed and stained with hoescht. Confocal images were captured using the Olympus FluoView FV1000 confocal laser scanning microscope and data analysis was performed using the Olympus FV-10 ASW imaging software. Results shown are representative of two independent experiments.

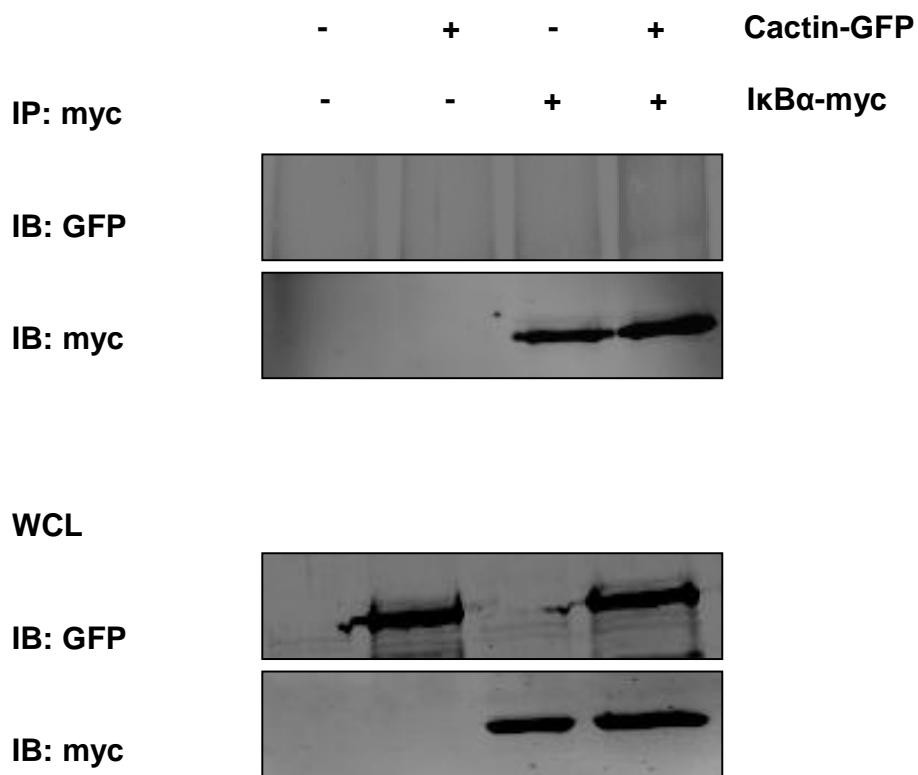


Figure 5.8 Cactin-GFP does not co-immunoprecipitate with I κ B α -myc

HEK293 T cells were co-transfected with GFP tagged Cactin (2 μ g) and myc tagged I κ B α (2 μ g). 24 h post transfection lysates were generated and immunoprecipitated with an anti myc antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western immunoblotting using anti-GFP and anti-myc antibodies. Images were captured using the Odyssey Infrared imaging system. Blots shown are indicative of three independent experiments.

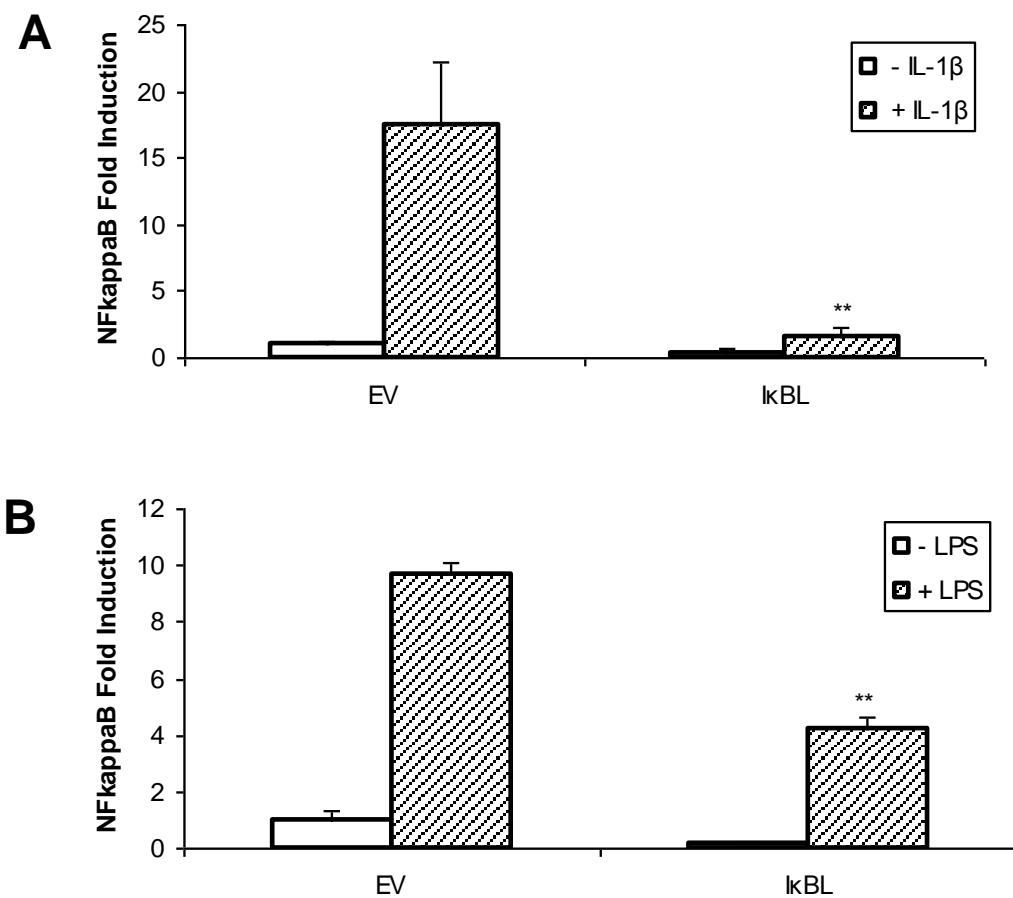


Figure 5.9 IκBL inhibits IL-1 β and LPS-induced activation of NF-κB

HEK293 TLR4 cells were transfected with plasmids encoding NF-κB –regulated firefly luciferase (80 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without a plasmid encoding GFP tagged IκBL (90 ng). Empty vector (EV) pcDNA3.1 was used to normalise the total amount of DNA transfected. Transfected cells were left overnight and then stimulated with (A) IL-1 β (10 ng/ml) or (B) LPS (100 ng/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data shown are the mean +/- S.E.M of triplicate determinations of a representative of three independent experiments. ** p < 0.01, paired t-test, Ligand stimulated cells transfected with IκBL compared with ligand stimulated cells transfected with EV.

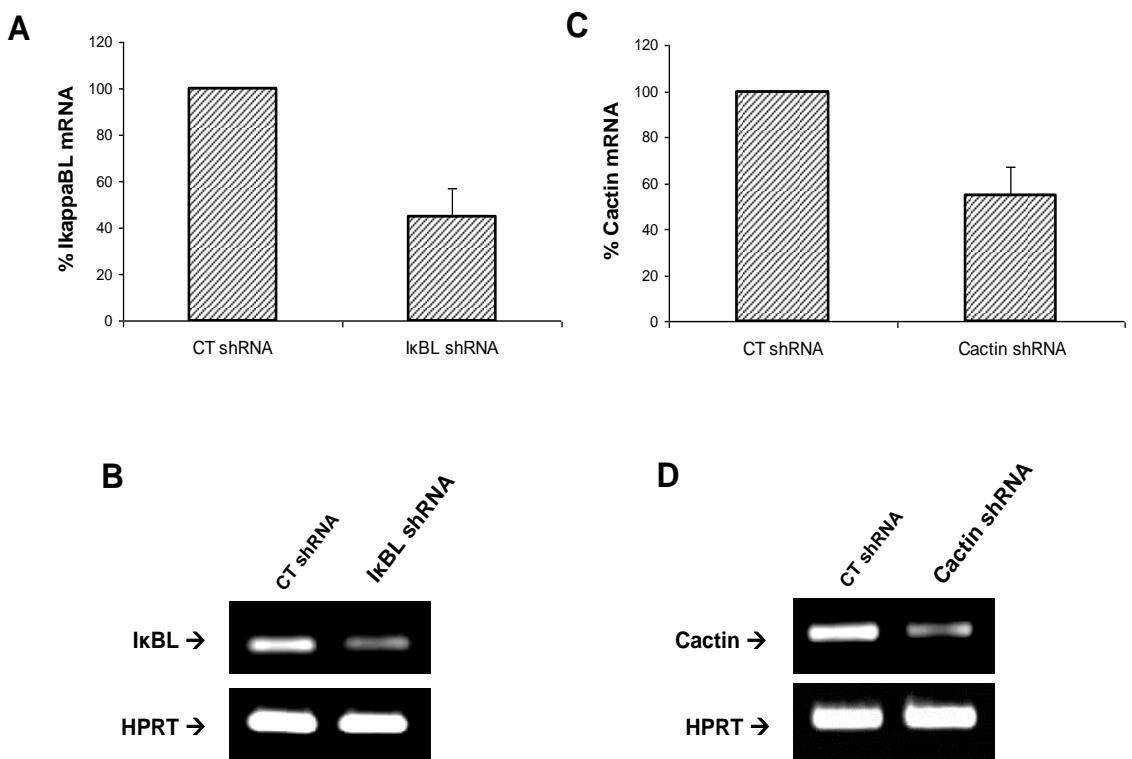
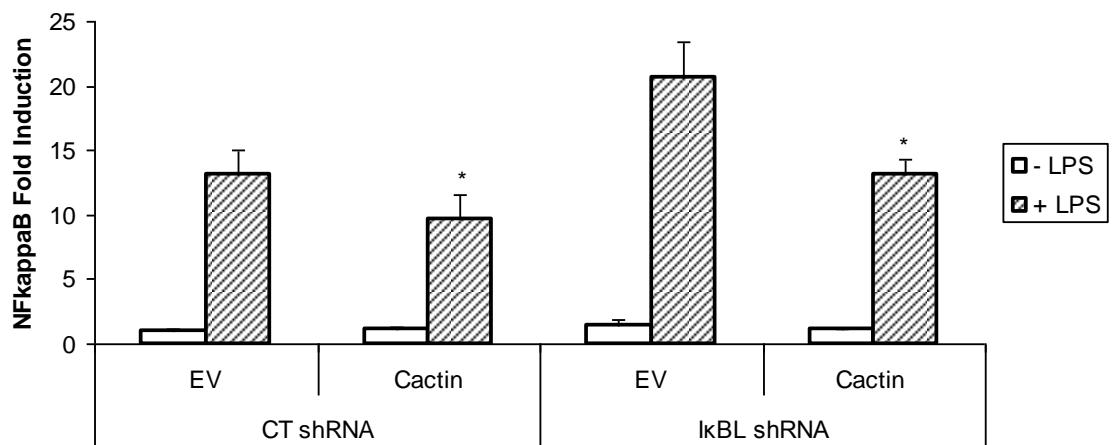


Figure 5.10 shRNA-mediated knockdown of I κ BL and Cactin

HEK293 TLR4 or TLR3 cells were transfected with plasmids encoding (**A, B**) I κ BL-specific shRNA (1 μ g), (**C, D**) Cactin-specific shRNA (1 μ g), or a non-specific shRNA control (1 μ g). Total RNA was extracted and converted to cDNA by RT-PCR. Real-time PCR was then performed to quantify the amount of (**A**) I κ BL or (**C**) Cactin. Gene expression was quantified relative to the housekeeping gene HPRT and data analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. (**B, D**) Real-time products were separated by gel electrophoresis.



5.11 Suppression of endogenous I κ BL does not affect the ability of Cactin to inhibit LPS-induced activation of NF- κ B

HEK293 TLR4 cells were transfected with plasmids encoding NF- κ B-regulated luciferase (80 ng) and constitutively expressed TK Renilla luciferase (20 ng), myc-tagged Cactin (90 ng) or Empty Vector (EV) pcDNA3.1 (90 ng) and I κ BL-specific shRNA (100 ng) or a non-specific shRNA control (100 ng). Transfected cells were left overnight and then treated with LPS (100 ng/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data presented are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, paired t-test, LPS stimulated cells transfected with Cactin compared with LPS stimulated cells transfected with EV.

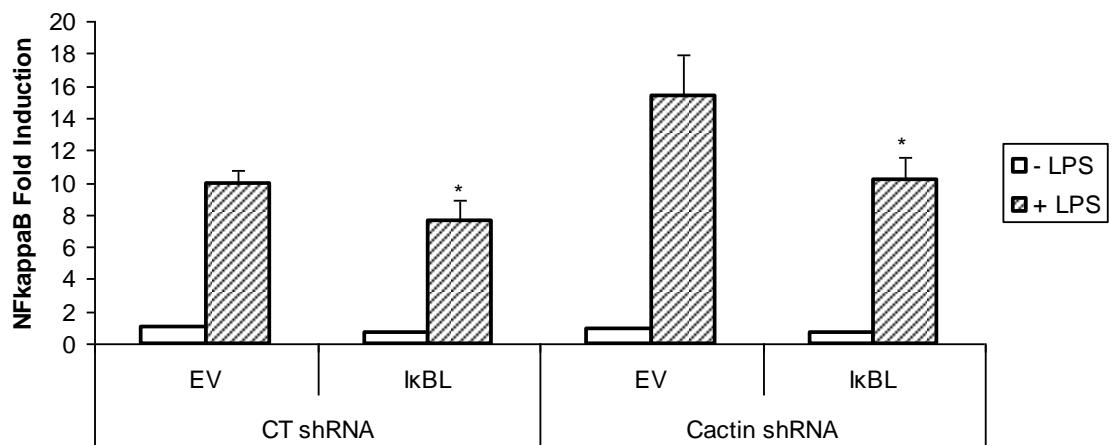


Figure 5.12 Suppression of endogenous Cactin does not affect the ability of I κ BL to inhibit LPS induced activation of NF- κ B

HEK293 TLR4 cells were transfected with plasmids encoding NF- κ B-regulated luciferase (80 ng) and constitutively expressed TK Renilla luciferase (20 ng), GFP-tagged I κ BL (90 ng) or Empty Vector (EV) pcDNA3.1 (90 ng) and Cactin-specific shRNA (100 ng) or a non-specific shRNA control (100 ng). Transfected cells were left overnight and then treated with LPS (100 ng/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data shown are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, paired t-test, LPS stimulated cells transfected with I κ BL compared with LPS stimulated cells transfected with EV.

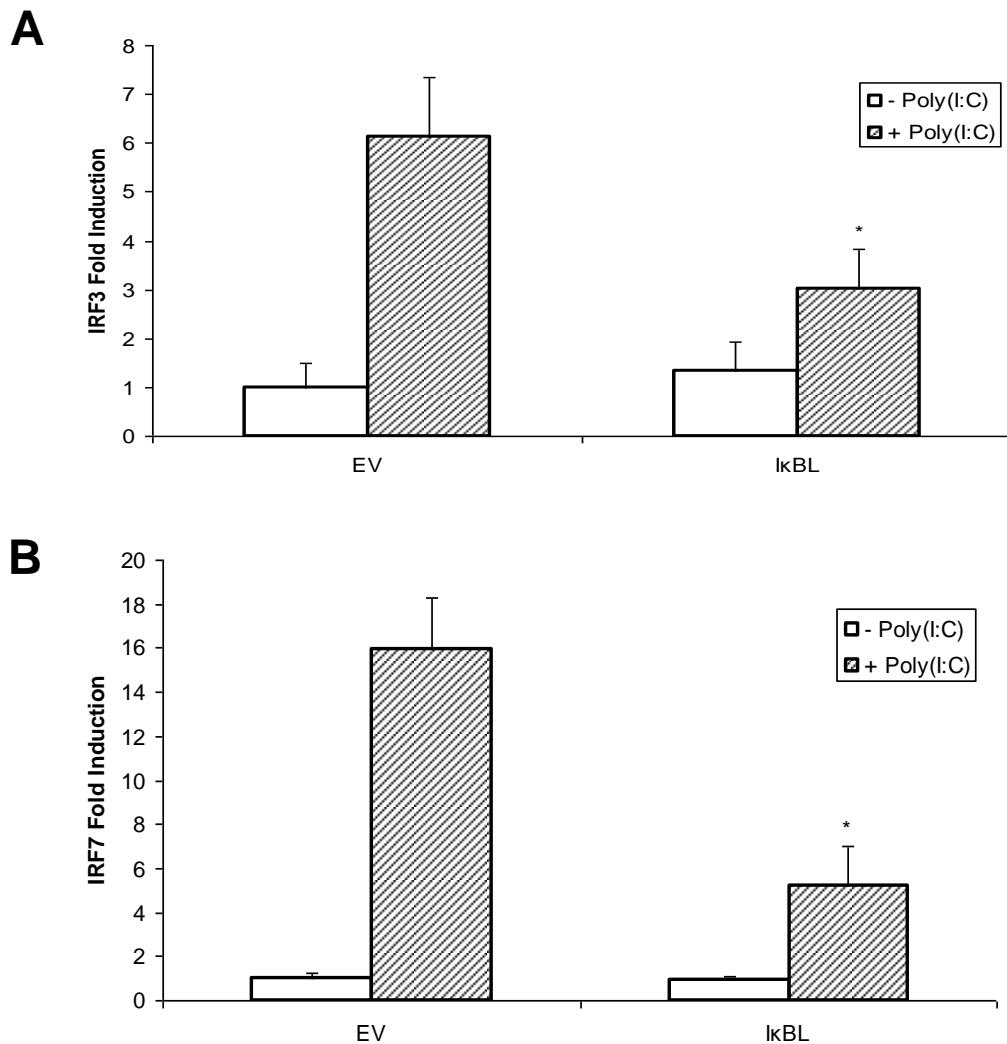


Figure 5.13 IκBL inhibits Poly(I:C) induced activation of IRF3 and IRF7

HEK293 TLR3 cells were transfected with plasmids encoding (A) pFA IRF3 (30 ng) or (B) pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without plasmids encoding GFP-tagged IκBL (90 ng) or Empty Vector (EV) pcDNA3.1 (90 ng). Transfected cells were left overnight and then treated with or without Poly(I:C) (25 µg/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data represent the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, paired t-test, Ligand stimulated cells transfected with IκBL compared with ligand stimulated cells transfected with EV.

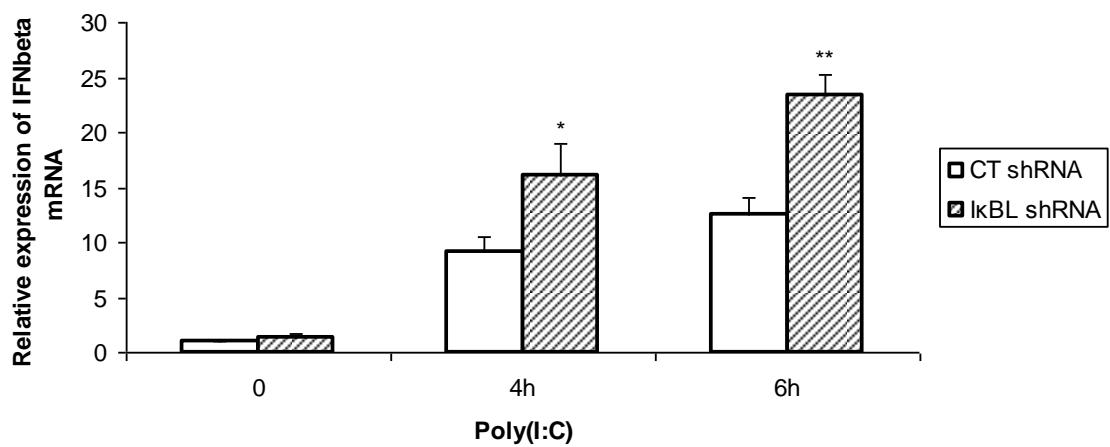


Figure 5.14 Suppression of endogenous IkBL augments Poly(I:C) induction of IFN β

HEK293 TLR3 cells were transfected with IkBL-specific shRNA (1 μ g) or a non-specific shRNA control (1 μ g). Cells were treated with Poly(I:C) (25 μ g/ml) for the indicated time-periods. Total RNA was extracted 48 h post-transfection and converted to cDNA by RT-PCR. Realtime PCR was then carried out to quantify the amount of IFN β in each sample. Gene expression was quantified relative to the housekeeping gene HPRT and data analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data presented are the mean +/- S.E. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, paired t-test, Cells transfected with IkBL-specific shRNA compared with cells transfected with a non-specific shRNA control.

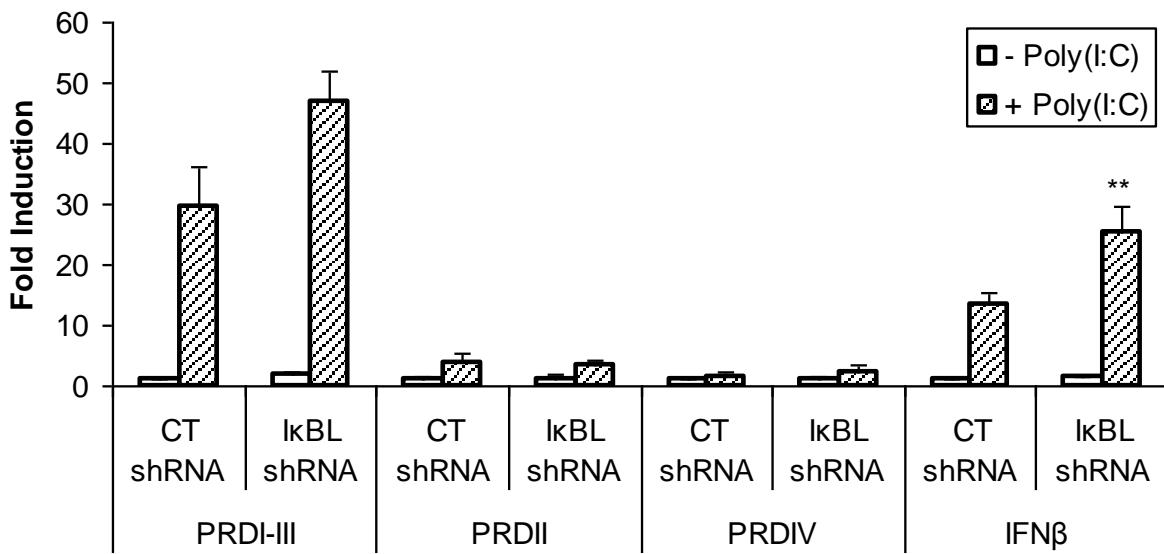


Figure 5.15 Suppression of endogenous I κ BL augments Poly(I:C)-induced activation of PRDI-III and the IFN β promoter

HEK293 TLR3 cells were transfected with plasmids encoding PRDI-III-, PRDII-, PRDIV- or IFN β -regulated firefly luciferase (80 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without plasmids encoding I κ BL-specific shRNA (100 ng) or a non-specific shRNA control (100 ng). 24 h post-transfection cells were treated with Poly(I:C) (25 μ g/ml) overnight. Lysates were generated 48 h post-transfection and assayed for firefly and *Renilla* luciferase activity. Data shown are the mean +/- S.E.M of triplicate determinations from three independent experiments. ** p < 0.01, paired t-test, Poly(I:C) stimulated cells transfected with I κ BL-specific shRNA compared with Poly(I:C) stimulated cells transfected with a non-specific shRNA control.

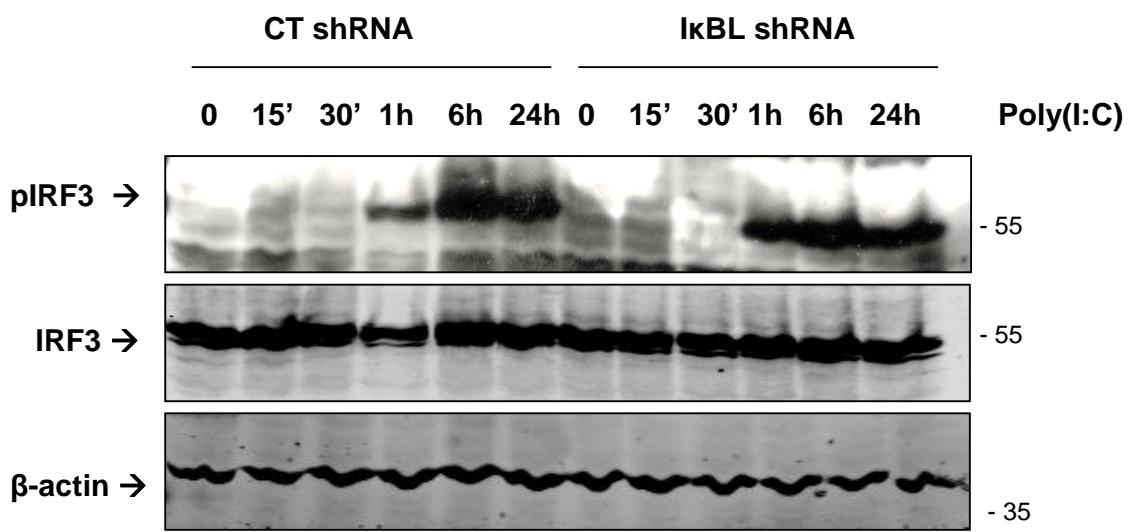


Figure 5.16 Suppression of endogenous IkBL has no effect on Poly(I:C)-induced phosphorylation of IRF3

HEK293 TLR3 cells were transfected with IkBL-specific shRNA (1 µg) or a non-specific shRNA control (1 µg). 24 h post-transfection cells were stimulated for the indicated time-periods with Poly(I:C) (25 µg/ml). Cell lysates were generated 48 h post-transfection and subjected to Western immunoblotting using anti-phospho-IRF3, anti-IRF3 and anti-β-actin antibodies. Results shown are indicative of three independent experiments.

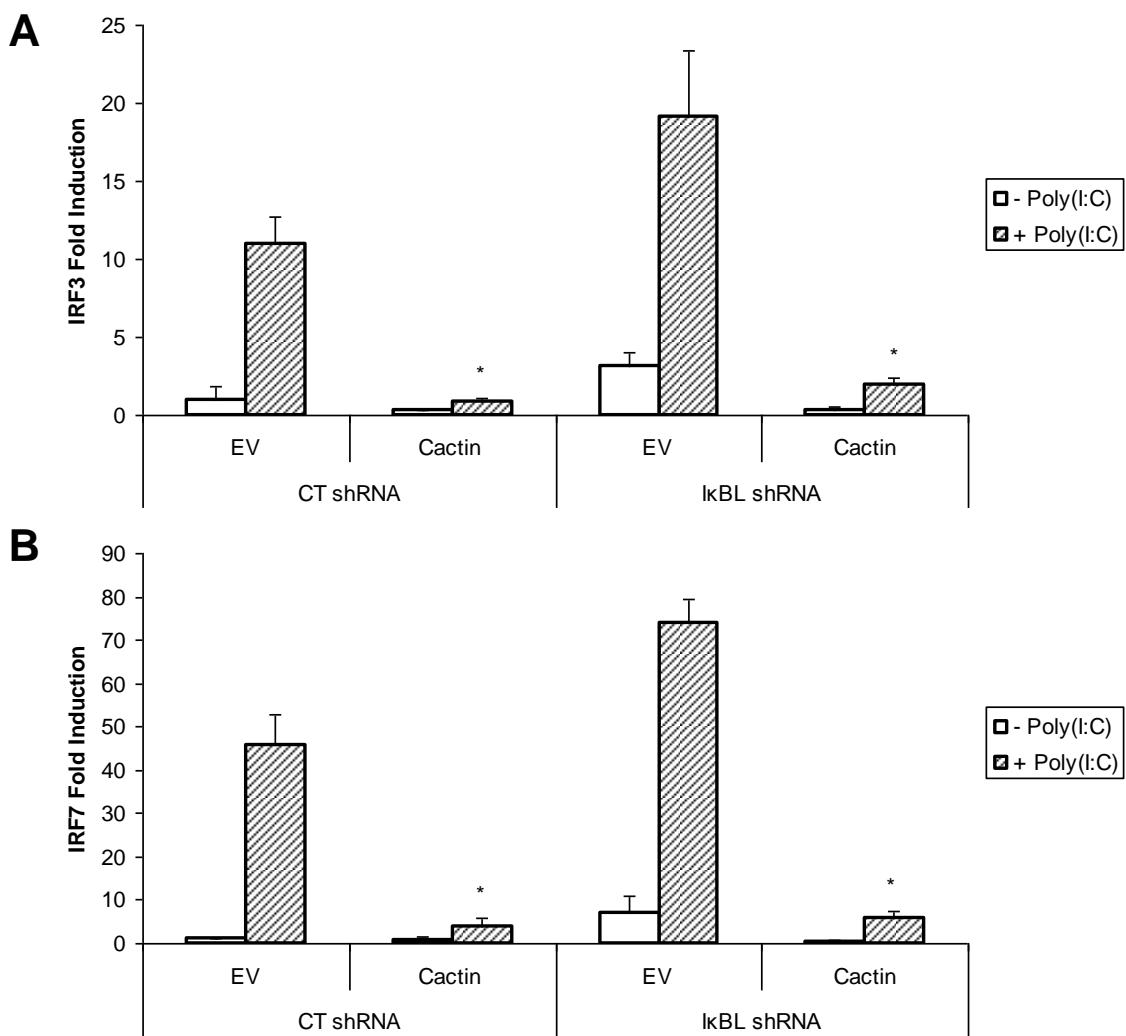


Figure 5.17 Cactin inhibits Poly(I:C) mediated IRF3 and IRF7 activation independently of I κ BL

HEK293 TLR3 cells were transfected with plasmids encoding (A) pFA-IRF3 (30 ng) or (B) pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without plasmids encoding myc-tagged Cactin (90 ng), Empty Vector (EV) pcDNA3.1 (90 ng), I κ BL-specific shRNA (100 ng) or a non-specific shRNA control (100 ng). Transfected cells were left overnight and then stimulated with Poly(I:C) (25 μ g/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data shown are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, paired t-test, Ligand stimulated cells transfected with Cactin compared with ligand stimulated cells transfected with EV.

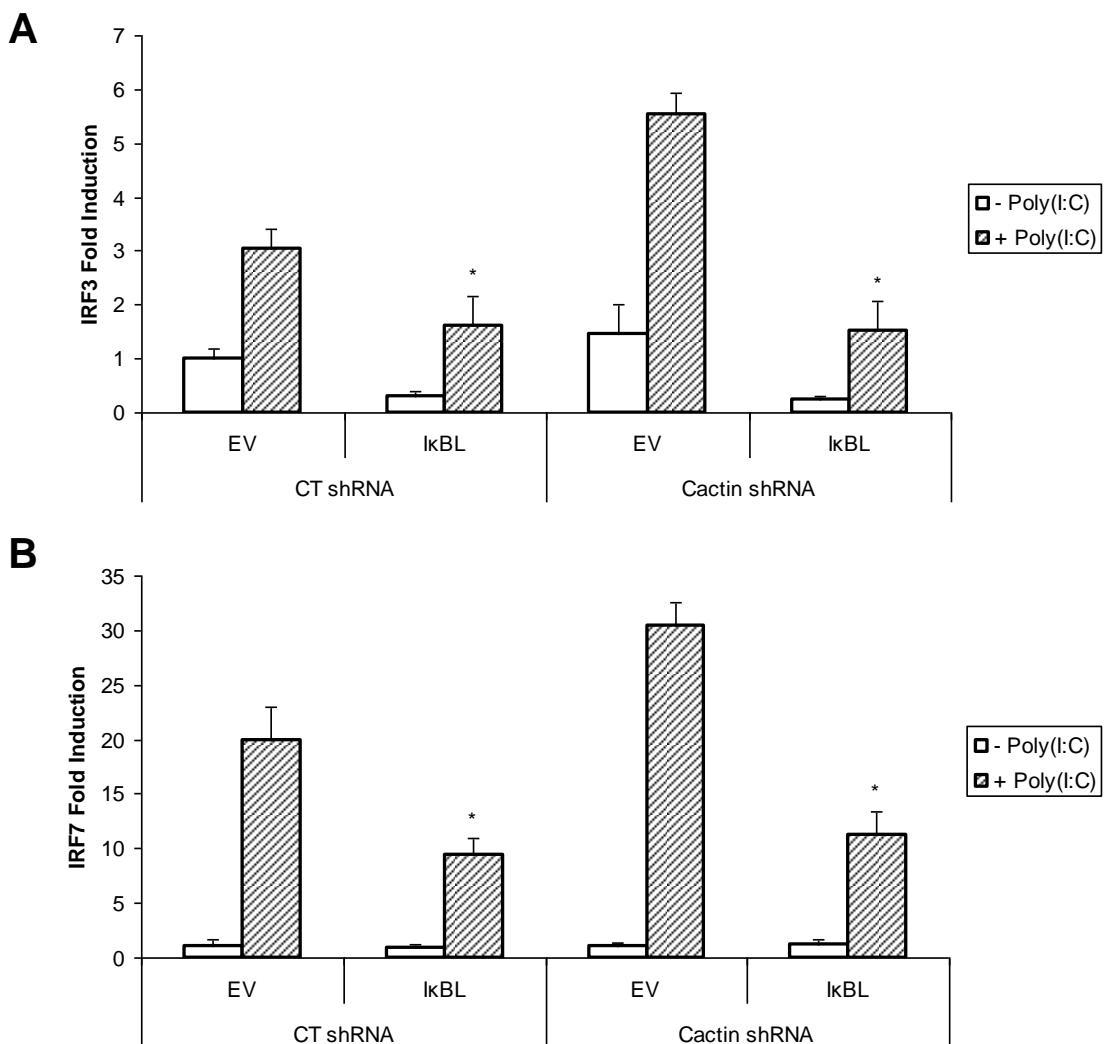


Figure 5.18 IκBL inhibits Poly(I:C) mediated IRF3 and IRF7 activation independently of Cactin.

HEK293 TLR3 cells were transfected with plasmids encoding (A) pFA-IRF3 (30 ng) or (B) pFA-IRF7 (25 ng), pFR-regulated firefly luciferase (60 ng) and constitutively expressed TK Renilla luciferase (20 ng) and with or without plasmids encoding GFP-tagged IκBL (90 ng), Empty Vector (EV) pcDNA3.1 (90 ng), Cactin-specific shRNA (100 ng) or a non-specific shRNA control (100 ng). Transfected cells were left overnight and then stimulated with Poly(I:C) (25 µg/ml) for 24 h. Lysates were generated and assayed for firefly and *Renilla* luciferase activity. Data shown are the mean +/- S.E.M. of triplicate determinations from three independent experiments. * p < 0.05, paired t-test, Ligand stimulated cells transfected with IκBL compared with ligand stimulated cells transfected with EV.

5.3 Discussion

An unbiased proteomic approach in our laboratory had identified ASCC1 as an interactor of Cactin and we were keen to validate this interaction by other techniques. Some interaction was detected by co-immunoprecipitation and co-localisation of over-expressed Cactin and ASCC1 was detected in some cells by confocal microscopy. This interaction is interesting as ASCC1 has been shown to be essential for ASC-1 transactivation of AP-1 (Jung et al., 2002) and most likely NF- κ B. As ASCC1 appears to be an important component of the ASC-1 complex it is possible that interaction with Cactin could displace ASCC1 from the complex, thus affecting the ability of ASC-1 to regulate NF- κ B transactivation. Further investigation is required, however, to determine the importance of ASCC1 and Cactin association. Mass Spectrometry analysis also identified PAK1IP1 as an interactor of Cactin. However this study failed to confirm the interaction by co-immunoprecipitation in both non-stimulated and ligand treated cells. This suggests that PAK1IP1 may not be a true interactor of Cactin. According to Mass spectrometry analysis Cactin also interacts with proteins involved in RNA processing and a number of DEAD-box (DDX) RNA helicases. This is potentially interesting as a member of the RNA helicase family, namely DDX3, has been shown to enhance TBK1 mediated IFN β induction (Schroeder et al., 2008). However, these interactions need to be further confirmed by other methods.

We also decided to exploit homology relationships in order to identify potential interactors of Cactin. We initially assessed the ability of Cactin to interact with cytoplasmic I κ B α . There was no detectable interaction of Cactin and I κ B α in non-stimulated cells or after stimulation with pro-inflammatory stimuli. This is not surprising as I κ B α is expressed in the cytoplasm while Cactin localises to the nucleus. For this reason, we next assessed the ability of Cactin to interact with a nuclear member of the I κ B family, I κ BL. Interaction between Cactin and I κ BL was detected by co-immunoprecipitation. Co-localisation was also observed by confocal microscopy and I κ BL and Cactin appear to have very similar nuclear expression patterns. Mutation in the gene coding for I κ BL has been associated with various autoimmune disorders. For example, the gene for I κ BL has been identified as a susceptibility gene for RA (Greetham et al., 2007). Furthermore I κ BL gene

polymorphism increases the risk of developing SLE and primary Sjogren's syndrome (pSS) (Castiblanco and Anaya, 2008).

A role for I κ BL in mRNA processing and regulation of translation has previously been suggested (Greetham et al., 2007). We were keen to investigate a role for I κ BL in innate immune signalling. Similarly to Cactin, I κ BL negatively regulates the activation of NF- κ B by the TLR ligand LPS and the endogenous pro-inflammatory stimulus IL-1 β . Interestingly, the association of Cactin and I κ BL does not appear to be essential for Cactin or I κ BL's negative regulation of NF- κ B. Cactin displays the same inhibitory effects when endogenous I κ BL expression is suppressed. Similarly knockdown of endogenous Cactin expression has no effect on I κ BL's inhibitory effects. These findings suggest functional redundancy for Cactin and I κ BL in terms of NF- κ B signalling. As Cactin is also a powerful inhibitor of IRFs we next assessed the ability of I κ BL to regulate IRF signalling pathways. Overexpression of I κ BL inhibited Poly(I:C) mediated IRF3 and IRF7 activation while suppression of endogenous I κ BL augmented Poly(I:C) induction of IFN β . Intriguingly, like Cactin, knockdown of endogenous I κ BL had no effect on Poly(I:C) stimulated IRF3 phosphorylation. Furthermore, both Cactin and I κ BL target PRDI-III of the IFN β promoter and inhibit IFN β expression. These results suggest that Cactin and I κ BL manifest their regulatory effects on IRFs by similar mechanism. Interestingly these two proteins appear to function independently to inhibit IRFs as knockdown of endogenous Cactin had not effect on I κ BL's ability to negatively regulate IRF3 and IRF7, while knockdown of I κ BL similarly did not affect the regulatory effects of Cactin. Again these findings suggest functional redundancy for Cactin and I κ BL in regulating IRF as well as NF- κ B signalling. It is possible that both Cactin and I κ BL function as part of a multi-protein complex which regulates transcription factors in the nucleus.

It is also worth noting that I κ BL is encoded in the MHC class III gene region of chromosome 6. Susceptibility loci to several diseases such as Graves disease, Crohn's disease and SLE have been localised to this region of the genome (Matsuzaka et al., 2001). Given that this gene cluster has been associated with autoimmune disorders a high throughput yeast two hybrid system was used to identify interaction partners for proteins encoded by the MHC class III region (Lehner et al., 2003). Interestingly, Cactin was identified as an interaction partner for two of these proteins, RD binding protein (RDBP) and leukocyte-specific transcript 1 protein (LST1)

(Lehner et al., 2003). Furthermore, the TNF gene is also located within this region. Cactin has been shown to negatively regulate TNF- α mediated NF- κ B activation and suppression of endogenous Cactin augmented LPS mediated TNF- α expression (Atzei et al., 2010). Given that Cactin targets a number of proteins encoded by the MHC class III region, either directly or functionally, it appears that Cactin functions as a key regulator of this gene cluster. This also means that Cactin may prove to be important in preventing the development of autoimmune disease as this genomic region has been associated with susceptibility to diseases such as RA and SLE. Going forward it will be of interest to explore the role of Cactin in autoimmunity.

Chapter 6

Concluding remarks

6.1 Concluding Remarks

Drosophila is used as a model organism for the study of innate immune signalling due to the striking similarities in the *Drosophila* Toll and Imd pathways and mammalian TLR signalling cascades. *Drosophila* Cactin was identified as a regulator of *Drosophila* Toll signalling that positively regulated the activation of the Rel/NF- κ B transcription factor dorsal (Lin et al., 2000). For this reason the human homologue of Cactin was cloned in our laboratory to examine its role in TLR signalling pathways but it was found to have a different function than its *Drosophila* orthologue. Cactin negatively regulates the activation of NF- κ B, IRF3 and IRF7. The aim of this current work was to probe the mechanisms underlying the negative regulatory effects of Cactin.

Cactin is expressed exclusively in the nucleus and a truncated mutant of Cactin, which lacks both of its NLSs and is expressed in the cytoplasm, showed considerably reduced inhibitory effects on NF- κ B. This indicates that the nuclear localisation of Cactin is important for its function. Suppression of endogenous Cactin resulted in increased DNA binding activity of NF- κ B in response to LPS but had no effect on LPS mediated p65 phosphorylation, indicating that Cactin might function downstream of this event. The predominant form of NF- κ B is a p65/p50 heterodimer (Li and Verma, 2002). For this reason we focussed our studies on the NF- κ B subunit p65. Overexpressed p65 dimerises and translocates to the nucleus where it binds to κ B sites its target genes. This occurs in the absence of a stimulus due to the lack of I κ B proteins to export the p65 dimers to the cytoplasm. This provided us with a tool to determine if Cactin can inhibit p65 transactivation. This study showed that Cactin prevented p65 from binding to a consensus NF- κ B-binding motif. Furthermore, Cactin prevented p65 binding to the endogenous IL-8 promoter. Intriguingly this inhibition was not achieved through direct interaction with p65 or by competing with p65 for DNA-binding activity. Notably, p65 is acetylated by p300/CBP and this modification is essential for full transcriptional activity (Chen and Greene, 2002). Conversely, deacetylation of p65 represses its DNA binding activity and increases its binding affinity for I κ B α (Ashburner et al., 2001). It is possible that Cactin recruits the histone deacetylating proteins, HDACs, responsible for p65 deacetylation. These HDACs are also responsible for chromatin remodelling and gene silencing. Alternatively Cactin might interact with CBP/p300 and prevent their association with

p65, thus preventing its acetylation and DNA binding. Cactin could also recruit other proteins that compete for binding at the NF- κ B binding site.

This study also showed that Cactin is regulated by pro-inflammatory stimuli. The regulation of Cactin is both transcriptional and post-translational in response to LPS. Cactin expression was induced following 5 h stimulation with LPS. However, more apparent than this regulation was the stabilization of Cactin protein following stimulation of cells with the endogenous pro-inflammatory stimulus IL-1 β , and the TLR ligands LPS and Poly(I:C). This stabilisation appears to be due to serine phosphorylation of Cactin. Furthermore, the stabilisation of Cactin was shown to be dependent on NF- κ B activation as repression of NF- κ B prevented its stabilisation. The kinase responsible for Cactin phosphorylation might be encoded by an NF- κ B-regulated gene. Conversely, NF- κ B might upregulate the expression of the gene encoding the activator of the responsible kinase. Many negative regulators of TLR signalling are regulated by NF- κ B activation and represent negative feedback mechanisms to ensure the termination of the inflammatory response. Prolonged activation of NF- κ B can cause damage to the host and is associated with a range of inflammatory and autoimmune diseases. This highlights the therapeutic potential of exploiting the inhibitory effects of Cactin. Also, because Cactin targets the DNA-binding activity of NF- κ B it can negatively regulate its activation by any stimulus and any signalling pathway.

Cactin is also a powerful negative regulator of IRF3 and IRF7. This study showed that Cactin inhibits the induction IFN β in response to Poly(I:C). Again we wanted to dissect the mechanism by which Cactin inhibits the activation of these transcription factors. IRF3 and IRF7 are both phosphorylated which promotes their dimerisation and translocation to the nucleus. Interestingly this study found that Cactin had no effect on Poly(I:C) mediated phosphorylation of IRF3. Nor did it affect the translocation of IRF3 or IRF7 to the nucleus. Suppression of Cactin by siRNA technology did, however, lead to increased binding of IRF3 to the IFN β promoter. This suggests that Cactin can also inhibit the DNA-binding activity of IRF3 and this is the mechanism by which it inhibits IFN β induction. Furthermore, Cactin inhibited the transactivation of IRF7 as it prevented its induction of an IFN α -regulated reporter plasmid. Again this inhibition at the level of DNA binding does not involve direct interaction of Cactin with either IRF3 or IRF7. It is possible that Cactin interacts with other IRF3 or IRF7 interacting partners to inhibit their DNA binding activity. For

example association of CBP/p300 promotes IRF3 acetylation and unmasks its DNA-binding domain (Lin et al., 1999). Furthermore this association has been shown to be indispensable for IRF3 DNA-binding activity (Suhara et al., 2002). Disruption of this interaction would inhibit the DNA binding activity of IRF3. Histone acetylation by CBP/p300 is also important for chromatin remodelling essential for the initiation of transcription. CBP/p300 interaction has also been shown to be important for IRF7-mediated IFN β induction (Yang et al., 2004). However, the association of CBP/p300 with IRF7 is weaker than that of CBP/p300 and IRF3 (Genin et al., 2009).

Interestingly, Cactin regulates the stability of IRF7 protein. The expression of IRF7 is tightly regulated both transcriptionally and post-translationally to ensure tight regulation of Type 1 IFN responses. IRF7 is ubiquitinated by TRAF6 which positively regulates its activation (Kawai et al., 2004). A mutant of IRF7 that cannot be ubiquitinated lost its transactivational activity (Ning et al., 2008). Disruption of this modification by Cactin, or by a deubiquitinase recruited by Cactin could potentially repress its DNA binding activity. IRF7 is also targeted by SUMO E3 ligase tripartite motif-containing protein (TRIM)28 which triggers its SUMOylation and transcriptional repression (Liang et al., 2011). Similarly IRF3 is SUMOylated and this negatively regulates Type 1 IFN induction (Kubota et al., 2009). Acetylation of IRF7 by pCAF at a specific site also reduces its DNA binding activity by masking its DNA-binding domain (Caillaud et al., 2002). Furthermore RAUL catalyses the K48-linked ubiquitination of IRF7 leading to its proteosome-dependent degradation (Yu and Hatward, 2010). IRF7 has a very short half-life which ensures that Type 1 IFNs are not produced in the absence of viral infection. Interestingly, RAUL suppression leads to the accumulation of IRF7 in HEK293 T cells (Yu and Hayward 2010). Similarly, in the present study, more overexpressed IRF7 was detected in cells transfected with Cactin-specific siRNA compared with cells transfected with a scrambled siRNA control. It is possible that Cactin promotes K48-linked ubiquitination and proteosomal degradation of IRF7, thereby negatively regulating the production of Type 1 IFNs. Type 1 IFNs play an important role in the pathogenesis of autoimmune diseases such as SLE and psoriasis (Baccala et al., 2007, Nestle et al., 2005). High levels of Type 1 IFNs are often observed in patients with SLE and high levels of IFN α are associated with susceptibility to the disease (Salloum and Niewold, 2011). IFN α production also plays a central role in the activation of autoreactive T cells in the progression of psoriasis (Nestle et al., 2005). In both cases, self-nucleic

acids are recognised by endosomal TLRs leading to the production of Type 1 IFNs and the activation of an adaptive immune response to autoantigens. Again this highlights the therapeutic potential of Cactin, as it can negatively regulate the production of Type 1 IFNs. In a xenograft model Nestle et al. (2005) showed that blocking Type 1 IFNs can prevent the development of psoriasis. The ability of Cactin to target IRF7 is of particular interest as it is considered the master regulator of IFN α production and so targeting IRF7 is potentially therapeutic in these disease situations.

Since Cactin displayed negative regulatory effects on a number of transcription factors it was possible that it acts as a global repressor of all transcription factor transactivation. We were able to demonstrate, however, that this is not the case. Cactin failed to inhibit the transactivation of c-jun, a subunit of the AP-1 transcription factor. Furthermore there was potentiation of MEKK1 mediated c-jun activation observed in the presence of Cactin. This prompted an investigation into the affect of Cactin on MAPK activation in response to TLR ligands. Intriguingly, Cactin positively regulated MAPK activation by Poly(I:C) but not LPS. It will be interesting in the future to further examine the effects of Cactin on MAPK signalling pathways.

In an attempt to further dissect the mechanism by which Cactin negatively regulates NF- κ B and IRF activation this study aimed to look at Cactin-interactors. Prior to the start of this project a proteomic-based analysis had been carried out to identify novel interactors of Cactin. We decided to investigate two of the putative interactors that were identified using this approach. We focussed on PAK1IP1 and ASCC1 since these proteins had previously been implicated in the regulation of NF- κ B and as described earlier Cactin proved to be an effective regulator of this transcription factor. Thus it was envisaged that such interactions may shed light on the mechanism(s) underlying the regulatory effects of Cactin. However, we failed to confirm the interaction of Cactin with PAK1IP1, by co-immunoprecipitation. The second protein, ASCC1, did co-immunoprecipitate with Cactin and co-localisation of these two proteins was detectable in some cells by confocal microscopy. ASCC1 is a subunit of a multiprotein coactivator complex called the ASC1 complex which positively regulates the transactivation of NF- κ B and AP-1 (Jung et al., 2002). Therefore, the interaction of Cactin and ASCC1 was potentially interesting. It seems plausible that Cactin's interaction with ASCC1 could displace the subunit from the ASC-1 complex thus preventing the ASC-1 complex from positively regulating NF- κ B activation. However, this project failed to reproduce the positive regulatory effects

of ASCC1 in HEK293 cells and ASCC1 showed modest inhibitory effects on the IRFs. For this reason ASCC1 and Cactin association was not further investigated in this project.

Drosophila Cactin was identified as an interactor of *Drosophila* Cactus. We therefore investigated if Cactin could interact with the human homologue of Cactus, I κ B α . It may seem unlikely that these two proteins would interact as I κ B α is expressed predominantly in the cytoplasm. However, following NF- κ B activation, I κ B α expression is upregulated and it can enter the nucleus and bind active NF- κ B dimers resulting in their export to the cytoplasm. However, no interaction of Cactin and I κ B α was detectable. Cells were also treated with IL-1 β to determine if Cactin and I κ B α interact in a stimulus-dependent manner but again no interaction was detected. This prompted us to investigate if Cactin interacts with I κ BL, a nuclear member of the I κ B family. I κ BL co-immunoprecipitated with Cactin and confocal analysis revealed that they both display very similar patterns of expression within the nucleus. Examination of the effects of I κ BL on NF- κ B and IRFs showed that I κ BL mimics the inhibitory effects of Cactin. These findings were further confirmed by the use of shRNA to suppress the endogenous expression of I κ BL. Suppression of endogenous I κ BL augmented LPS mediated NF- κ B activation and Poly(I:C) mediated IRF3 and IRF7 activation. Furthermore, suppression of endogenous I κ BL augmented Poly(I:C) induction of IFN β . Interestingly both Cactin and I κ BL inhibit the activation of IFN β and target the same positive regulatory domain, PRDI-III, of the IFN β promoter. I κ BL, like Cactin, had no effect on Poly(I:C) mediated IRF3 phosphorylation suggesting that both of these proteins carry out their inhibitory effects in the nucleus. A role for I κ BL in mRNA processing or regulation of translation has been previously been suggested (Greetham et al., 2007). Similarly, the mass spectrometry analysis suggested that Cactin may also have a role in similar processes. However shRNA-based studies revealed that Cactin and I κ BL function independently, at least in terms of regulation of NF- κ B, IRF3 and IRF7. This suggests functional redundancy for Cactin and I κ BL. It is possible that they both function as part of the same multi-protein complex which regulates the activation of transcription factors in the nucleus. I κ BL has been a subject of interest in recent years as genetic defects in the gene coding for I κ BL confers susceptibility to diseases such as RA (Greetham et al., 2007) and SLE (Castiblanco and Anaya, 2008).

Given the regulatory effects of Cactin on inflammatory signalling pathways, it will be interesting in the future to look at the role of Cactin in autoimmune diseases, initially looking at its expression levels in diseases such as SLE. We are currently at somewhat of a disadvantage due to the lack of an antibody for endogenous Cactin and therefore must rely on over-expression studies. Such antibodies will be of great use for future work on Cactin. Looking forward, it would also be of great benefit to generate Cactin-deficient mice and examine the role of Cactin on TLR signalling *in vivo*. Such models would also offer the potential to evaluate the importance of Cactin in a various models of inflammatory disease. The present studies implicate Cactin as an effective molecular regulator of TLR signalling but we await with interest an interrogation of its functional importance in physiological and pathological settings.

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