

Characterisation of the role of human Evolutionary Conserved Signalling Intermediate in Toll (ECSIT) in Mitogen Activated Protein Kinase (MAPK) signalling.

by
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Ollscoil na hÉireann Má Nuad

A thesis presented to
The National University of Ireland, Maynooth
in partial fulfilment of the thesis requirement for the
degree of
Masters of Philosophy
Institute of Immunology
National University of Ireland
Maynooth
September 2012
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Supervisor: Prof. Paul Moynagh



Funded by:

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Declaration

I, Anne Kirwan, declare that this thesis is my own work and has not been submitted in any form for another qualification at any university or Institute of Education. Information derived from the work of others has been acknowledged and cited in this text.

Signed: _____ Date: _____

Acknowledgements

‘Success is a journey, not a destination.’

This thesis would not have been possible without the guidance and the help of several individuals. First and foremost, I would like to express my sincere gratitude to my supervisor Professor Paul Moynagh, for giving me this opportunity and for his continuous support and motivation. My thanks also to all members of the Molecular Immunology Laboratory and the Department of Biology at NUIM.

In particular I would like to thank R.J. for his time and patience in helping me with experiments and for keeping me sane while in the lab. Also special thanks to Pam and Kate for providing me with endless cups of tea to feed my tea addiction. What I take most from my time at NUIM is the friends I have made, I especially want to thank all the girls I have played with on the NUIM camogie team who have made my experience at NUIM truly unforgettable.

Last but not the least I would like to thank my family. To Niamh for being the most caring person I know. To John for your knowledge of all things great and small. Your endless wisdom never ceases to amaze me. To my Mam for everything you have done for me, often with little thanks. To my Dad, my best friend and personal advisor. It is fair to say I base all my life decisions on your opinion and advice. I am eternally grateful for path you have helped me lead. To Aisling, for not only being my sister but also my very best and closest friend.

Abstract

Evolutionarily conserved signalling intermediate in Toll pathways (ECSIT) was identified originally as a TNF receptor associated factor 6 (TRAF6) interacting partner. The murine homolog mECSIT has been shown to be involved in NFκB, MAPK, BMP and mitochondrial signalling. To date there is no work published on the human homolog, hECSIT. In this thesis, I present data indicating that hECSIT is involved in NFκB, BMP and MAPK signalling. There is a striking difference in the role of hECSIT and mECSIT in the activation of inflammatory transcription factors NFκB, ELK-1 and AP-1; with mECSIT augmenting their activation and hECSIT having an inhibitory role. In addition I demonstrate that hECSIT specifically targets the p42/44 branch of MAPK signalling. Suppression of endogenous hECSIT results in increased basal and proinflammatory induced phosphorylation of p42/44 but not JNK or p38. Thus, hECSIT signalling represents a novel means of regulating p42/44 and its downstream targets.

1. Introduction

1.1 The Immune System

Multicellular organisms are constantly faced with the challenge of microbial invasion. The ability to sense and eradicate microbes is essential for survival. This is mediated by the immune system, a highly complex and adaptable system. The immune system of vertebrates is broadly divided into two distinct elements, the innate and the adaptive immune systems (Moresco *et al.*, 2011).

The innate immune system is evolutionary ancient. It is the first line of defence against microbe infection and is critical to their detection. It is characterised by germline encoded receptors which recognise conserved motifs of pathogens to mount an immediate immune response . The cells of the innate immune system, monocytes, macrophages, neutrophils, mast cells and dendritic cells (DCs), induce inflammation, phagocytose pathogens, as well as modulating the adaptive immune response . The adaptive immune response is only present in vertebrates, it is activated in a delayed fashion and is influenced by prior exposure to an antigen. In comparison with the germline encoded innate receptors, the receptors of the adaptive immune system are somatically generated by DNA recombination within variable regions and are highly specific even at molecular level. This results in a specific clonal cell response against a pathogen .

1.2 Pattern Recognition Receptors

The receptors of the innate immune system are termed Pattern Recognition Receptors (PRRs). Three main categories of PRRs have been discovered, the membrane bound Toll-like receptors (TLRs), cytoplasmic NOD like receptors (NLRs) and Rig-I-like receptors (RLRs) (Creagh and O' Neill, 2006; Kawai and Akira, 2009; Takeuchi and Akira, 2010). They are activated upon recognition of their ligands, which are broadly classed as pathogen associated molecular patterns (PAMPs) and danger associate molecular patterns (DAMPs) . PAMPs are molecular motives that are unique to pathogens, discriminating them from host proteins. They are highly conserved and essential for survival of the microbe, e.g lipopolysaccharide (LPS) of gram negative bacteria or double stranded RNA (dsRNA) from viruses. DAMPs are endogenous molecules released by the host in response to pathogenic infection, necrosis, injury or certain pathological conditions (Kawai and Akira, 2009; Matzinger, 2002). They include mitochondrial DNA (mtDNA), extracellular ATP, high mobility group box 1 protein (HMGB-1), uric acid and heat shock proteins (Piccinini and Midwood, 2010; West *et al.*, 2011).

Recognition of PAMPs or DAMPs by PRRs triggers cascades of protein signalling leading to changes in gene expression. These cascades are highly dependent on phosphorylation and ubiquitination as a means to alter protein activity . In terms of ubiquitination, proteins are mainly subjected to Lysine-48 linked polyubiquitination (K48 pUB) or Lysine-63 linked polyubiquitination (K63 pUB). In general K48 pUB is recognised by the 26s proteasome leading to protein degradation, while K63 pUB is not generally

associated with degradation and instead is more often used to form protein scaffolds (Bhoj and Chen, 2009; Chau *et al.*, 1989; [Chen and Sun, 2009](#); Deng *et al.*, 2000; [Pickart, 2001](#); Wang *et al.*, 2001). The signalling cascades converge on transcription factors such as nuclear factor kappa β (NF κ B), activating protein-1 (AP-1) and interferon regulatory factors (IRFs) which can then regulate gene expression. Depending on the PRR involved, signalling can lead to the induction of proinflammatory cytokines and chemokines, antiviral proteins, activation of the complement system and the recruitment of phagocytic cells. Integration of signals from multiple PRRs, metabolic and environmental sensors determines the ultimate outcome of signalling.

1.2.1 Toll-Like Receptors

TLRs are the most widely studied class of PRR. They were discovered in the 1990's after it was found that the *Drosophila* protein dToll, which was known to be involved in embryonic development, played a role in *Drosophila's* anti-fungal defence (Lemaitre *et al.*, 1996). The human homologue TLR4 was subsequently identified and was found to respond to LPS, lead to the activation of the proinflammatory transcription factor NF κ B and the induction of proinflammatory cytokines (Hoshino *et al.*, 1999; Medzhitov *et al.*, 1997; Poltorak *et al.*, 1998). Since the seminal work on TLR4, 10 TLRs have been discovered in humans and 13 in mice. The nature of the PAMPs detected by TLRs is diverse encompassing proteins, lipids, lipoproteins and nucleic acids derived from viruses, bacteria, fungi and protozoa ([Akira *et al.*, 2006](#); Kawai and Akira, 2010).

Structurally TLRs are members of the type-1 integral membrane protein family. They are characterised by extracellular leucine rich

repeats (LRRs) involved in ligand detection, a single transmembrane domain and a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain. The TIR domain of TLRs lacks enzymatic activity and must engage adaptor proteins to propagate signalling. (; . Five intracellular adaptor proteins, myeloid differentiation primary-response protein 88 (MyD88), MyD88 adaptor-like (MAL) , TIR-domain containing adaptor protein inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and HEAT-Armadillo motifs (SARM) , have been shown to act as adaptor proteins for TLRs and can orchestrate signalling to multiple proteins . The diversity of TLR signalling is in part due to differential engagement of these adaptor molecules (Kawai and Akira, 2010; [Yamamoto *et al.*, 2004](#)).

TLR signalling results in the activation of transcription factors such as NF κ B, AP-1 and IRF3/IRF7 which induce the expression of inflammatory cytokines, chemokines, antiviral proteins, antimicrobial products and co-stimulatory molecules. These induce local and systematic inflammation but furthermore are necessary for the activation and modulation of the adaptive immune system . Components of the TLR pathways form networks with each other and other signalling pathways to modulate the nature, magnitude and duration of innate immune responses (Kawai and Akira, 2010).

TLRs can be found on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or on internal membranes, such as endoplasmic reticulum, endosome, lysosome or endolysosome (TLR3, TLR7. TLR8, and TLR9). Cellular location is important for the detection of ligands and distinguishing between host and non-host molecules. Additionally it can dictate the type of proteins activated and the resulting response .

1.2.1.1 Cell Surface Receptors

TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the cell surface. They recognise molecular components on the surface of the pathogens.

-TLR1, TLR2 and TLR6

These TLRs are all located on the plasma membrane. TLR2 deficient mice fail to respond appropriately to several gram-positive bacteria cell wall components and are highly susceptible to *Staphylococcus aureus* infection (Takeuchi *et al.*, 2000). TLR2 can respond to wide range of ligands, due to its ability to form heterodimers with TLR1 or TLR6. As homodimers they respond to lipoteichoic acid of gram-positive and gram-negative bacteria, Mycobacteria cell wall component lipoarrabinomannan, and fungal membrane component zymosan (Kataka *et al.*, 2002; Massari *et al.*, 2002; Wetzler *et al.*, 2003). TLR1 and TLR6 homodimers bind triacetylated and diacetylated lipoproteins of bacterial membranes respectively (Takeuchi *et al.*, 2002). It follows that TLR1/2 heterodimers can recognise triacetylated lipoproteins, in addition to synthetic lipoprotein PAM3CSK4 (Takeuchi *et al.*, 2002; Wetzler *et al.*, 2003). TLR2-TLR6 heterodimers recognise diacetylated lipoproteins including mycoplasma lipoproteins (MALPs) (Takeuchi *et al.*, 2001).

-TLR4

TLR4 signalling is important for antibacterial defences. It is expressed on numerous cells including monocytes, macrophages, DC, mast cells and intestinal epithelial cells (Sallusto and Lanzavecchia, 2002). Importantly it can have cell surface or endosomal location, resulting in the activation of two different

signalling pathways (Kagan *et al.*, 2008). Its most widely studied ligand is LPS, a glycolipid component of gram-negative bacteria (Miyake *et al.*, 2004), but it can also respond to LIPID A analogs, taxol, respiratory syncynal virus, fibronectin and heat shock proteins (Lien *et al.*, 2000; Perera *et al.*, 2001). Trace amounts of LPS can be recognised by the body to produce proinflammatory cytokines, TNF α , IL-1 β and IL-6. LPS recognition by TLR4 requires LPS to be bound by LPS binding protein (LBP) (Schumam *et al.*, 1994), the LBP-LPS complex is recognised by CD14, which in turn transfers LPS to TLR4 and its associated protein MD-2 (Schromm *et al.*, 2001).

-TLR5

TLR5 recognises flagellin, a protein required for gram negative bacteria mobility (Hayashi *et al.*, 2001; Mizel *et al.*, 2003). It is expressed on monocytes, DCs, T cells, natural killer cells and epithelium cells. It is expressed on basolateral side of intestinal epithelial cells to detect only bacteria that have crossed the epithelium (Akira, 2005). Residues 386-407 of the extracellular domain of TLR5 bind flagellin directly, as mutation of these residues prevents flagellin recognition (Smith *et al.*, 2003).

-IL-1R.

IL-1R and its associate receptor IL-1RAcP should be mentioned in the same context as TLRs as the IL-1R and IL-1rAcP contain an intracellular TIR domain. They are cell surface membrane proteins and signal in a very similar fashion to cell surface TLRs through the

adaptor protein MyD88, resulting in the activation of NFκB and AP-1 (Medzhitov, 2001).

1.2.1.2 Endosomal Receptors.

TLR 3, TLR7, TLR8 and TLR9 are important for host detection of viruses. They signal from the endosomal compartments after trafficking from the ER (Leifer *et al.*, 2004). Nucleic acid sensing is often the only means of detecting viruses, however there is no distinction between host and viral nucleic acids. Compartmentalisation of these receptors to the endosome, is a strategy to prevent them from responding to host nucleic acids which are excluded from the endosome (Diebold *et al.*, 2006). Aberrant response to host DNA may be the basis of some autoimmune diseases (Leadbetter *et al.*, 2002). TLR7 and TLR9 signalling is further restricted by a requirement for their cleavage for efficient signalling. This requires acidification of the endosome, a necessary step in endosome maturation (Hacker *et al.*, 1996).

-TLR3

TLR3 recognises viral PAMPs such as dsRNA and synthetic molecule PolyI:C and leads to the activation of IRF3 and NFκB to produce type I interferons (IFNs) critical for the antiviral response (Alexopoulou *et al.*, 2001). Its restriction to the endosomes is compensated by cytoplasmic RLR receptors, which also recognise viral PAMPs in the cytoplasm (Yoneyuma *et al.*, 2004).

-TLR7 and TLR8.

TLR7 and TLR8 are highly homologous and are both expressed on the X chromosome (Wang *et al.*, 2006). They are expressed in endosomal and phagosomal compartments of monocytes and

neutrophils. They recognise ssRNA from the influenza virus, Sendai virus and azoquinidine compound R-846 which is structurally related to nucleic acid to induce type 1 interferons. TLR7 can respond to a subclass of antiviral compounds including imidazoquinoline and loxoribine (Demaria *et al.*, 2010; Jurk *et al.*, 2002).

-TLR9

TLR9 recognises DNA that is unmethylated and contains 2' CpG. The latter is characteristic of bacteria genomic DNA but not host DNA that in contrast is highly methylated and contains few CpG elements. These differences allow TLR9 to distinguish between host and microbial DNA (Hemmi *et al.*, 2000). TLR9 can cooperate with TLR5 and TLR8 (Merlo *et al.*, 2007), while it can antagonise TLR7 signalling when associated with it (Wang *et al.*, 2006). In plasmacytoid DCs, TLR9 can recognise virus derived CpG DNA to induce an anti-viral response (Krug *et al.*, 2004; Lund *et al.*, 2003; Takeshita *et al.*, 2001).

-TLR10/11

TLR10 is an orphan receptor; its absence in mice has prevented determination of its ligand. It is expressed in the spleen, lymph nodes, thymus and may form heterodimers with TLR1 and TLR2 to recognise lipoproteins (Chuang and Ulevitch, 2001). TLR11 is non-functional in humans, in mice loss of TLR11 leaves them susceptible to uro-pathogenic *E. coli* infection, the nature of the ligand is unknown but is thought to be proteinaceous in nature as treatment with proteinase K can prevent ligand recognition (Zhang *et al.*, 2004).

1.2.2 Nod-Like Receptors

The discovery of TLRs in the 1990s marked a transient leap in our understanding of innate immune signalling. A similar landmark finding occurred in the following decade with the discovery of two novel families of cytoplasmic PRRs, the RLRs and the NLRs . These receptors can sense microbial and viral signals in the cytoplasm and induce pro-inflammatory and anti-viral responses via the transcription factors NF κ B and IRF3/IRF7 and MAPK signalling .

NLRs consist of a large family of cytoplasmic receptors that detect the presence of PAMPs and endogenous DAMP molecules in the cytoplasm . They are characterised by three distinct domains- the ligand sensing LRR domain, the oligomerisation NACHT domain and a variable N-terminal effector domain, important for protein-protein interaction and initiation of signalling. Members of the NLR family are divided into four subfamilies based on the N terminal effector domain- NLRA containing an activation domain (AD), NLRB containing a baculovirus inhibitor of apoptosis repeat (BIR) domain , NLRC containing a caspase activation and recruitment domain (CARD) domain and NLRP which contain a pyrin (PYD) domain ; Ting *et al.*, 2008).

The NLRC subfamily includes the nucleotide oligomerization domain (NOD) receptors- NOD1 and NOD2, that recognise peptidoglycan constituents mesodiaminopimelic acid (meso-DAP) and muramyl dipeptide (MDP) respectively (Windheim *et al.*, 2007) to induce NF κ B and IRF transcription factors . Mutations in NOD2 have been linked to Crohn's disease (Ogura *et al.*, 2001). Consistently, macrophages lacking either NOD1 or NOD2 fail to produce cytokines following stimulation .

The NLRPs are a large NLR subgroup containing a PYD domain. They form part of multiprotein complex known as the inflammasome which functions to activate caspase 1 and/or caspase 5 ; Martinon and Tschopp, 2004). Three inflammasome complexes have so far been identified NLRP1, NLRP3 and NLRC4. The activation of caspase 1 is required for the processing of members of IL-1 family- IL-1 β , IL-18, IL-33 which are formed through TLR signalling, into a mature active form . In this manner, TLR and NLR crosstalk is vital for an appropriate immune response, as they complement and synergise each others actions. Activation of the inflammasome leads to the recruitment of ASC, which contains both CARD and PYD domains. ASC can in turn recruit Caspase 1 through CARD domain interactions (Cassel and Sutterwala, 2009; Tschopp and Schroder, 2010).

NLRP3 can be activated in response to MDP, bacterial RNA and DNA viruses. Other elements such as gout related uric acid crystals, UV-B irradiation, and asbestos may activate NLRP3 through stimulation by reactive oxygen species (ROS) . Activated NLRP3 can form a complex with apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and CARDINAL - also known as CARD8 and tumour upregulated CARD containing antagonist of caspase 9 (TUCAN) - to recruit Caspase 1 . Activation of NLRP3 is enhanced by extracellular ATP. This ATP can be sourced from bacteria or from dying cells and is recognised by the P2X7 receptor which acts on the pannexin-1 pore . The exact mechanism of how P2X7 dependent activation of pannexin-1 enhances NLRP3 activation is unknown. It is postulated P2X7 engagement promotes pannexin-1 dependent K⁺ efflux, promoting PAMP delivery into the cytoplasm resulting in NLRP3 activation ; Nakahra *et al.*, 2010). Mitochondrial signalling is important in NLRP3 functioning. Several mitochondrial derived DAMPs have been implicated in NLRP activation such as mitochondrial DNA mtDNA, ROS mROS and ATP (Lyer *et al.*, 2009; Tschopp and Schroder, 2010; [Zhou *et al.*, 2011](#)).

1.2.3 RIG-1 Receptors

The second class of cytoplasmic PPRs are the RLRs which are comprised of two members, ribonucleic acid (RNA) helicases retinoic acid inducible gene 1 (RIG-1) and melanoma differentiation associated gene 5 (MDA5) . They are RNA helicases and can detect RNA derived from viruses such as paramyxovirus and influenza A to orchestrate antiviral responses through NFκB and IRF transcription factors ; Satoh *et al.*, 2010; [Yoneyama and Fujita, 2007](#)).

RIG-I contains tandem CARD domains at its N-terminus, a central DExD/H helicase domain and a C terminal repressor domain (RD) which binds RNA. Viral recognition, through RNA binding, induces conformational changes in RIG-1, promoting self association and interaction with downstream CARD domain containing proteins. MDA5 also contains tandem CARD domains and DExD/H domains, but it is unknown if its C terminus acts as a RD domain ([Kato *et al.*, 2011](#)).

Engagement of RLRs results in the activation of NFκB and IRF transcription factors as well as MAPK leading to induction of pro-inflammatory cytokines and type 1 IFN. RLR signalling is closely associated with mitochondria . A major target for RLR activation is the adaptor protein mitochondrial antiviral signalling adaptor protein (MAVS) also known as IPS-1, VISA and Cardif, which has been shown to localise to the mitochondria. MAVS contains an N terminal CARD-domain which interacts with the CARD domain of RLRs . MAVS-deficient mice have deficient activation of IRF3 and NFκB and induction of type 1 interferons and inflammatory cytokines by RIG-1 and MDA receptors (Kumar *et al.*, 2006). MAVS can activate NFκB to produce proinflammatory proteins by targeting complexes containing inhibitor of κB kinase (IKK) proteins and also

receptor interacting protein 1 (RIP-1). Complexes containing IKK related kinases TANK-binding kinase 1 (TBK1) and IKKi and also adaptor proteins TANK and NEMO, can signal through IRF transcription factors to produce an antiviral type 1 interferon response (Kawai *et al.*, 2005; Meylen *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). Recently it has been shown that MAVS degradation post activation is required for IRF activation by RIG-1. This is dependent on the E3 ligase TRIM25, which ubiquitinates MAVS resulting in its degradation. This releases NEMO and TBK1 into the cytosol to activate IRF proteins .

1.3 TLR Signalling.

TLR signalling is dependent on the selective use of adaptor molecules . MyD88 and TRIF are the main TLR adapters and orchestrate two distinct pathways, the MyD88-dependent and TRIF-dependent pathways (Kawai and Akira, 2008). MyD88 is used by the IL-1R and all TLRs aside from TLR3, which signals solely through the adaptor TRIF (Adachi *et al.*, 1998; Beutler *et al.*, 2005; Takeda, 2003). MyD88 can directly interact with the TIR domains of TLRs, with the exception of TLR2 and TLR4 which bind MyD88 through the adaptor protein MAL . The primary outcome of the MyD88 pathway is the activation of NFκB. TLR4 uniquely can signal via the MyD88-dependent and the TRIF-dependent pathways . The location of the TLR4 receptor governs which pathway is activated. When TLR4 is present on the cell surface it signals through the MyD88-dependent pathway. TLR4 signalling through the TRIF-dependent pathway is proposed to involve the endocytosis of TLR4 to the endosome where it engages TRIF via the adaptor TRAM . Signalling by TLR3 and TLR4 through TRIF leads to the activation of the IRF transcription factors producing an antiviral response . The

adaptor protein SARM can negatively regulate the MyD88-dependent and TRIF- dependent pathways . The duration and magnitude of TLR responses must be tightly regulated to ensure the efficient removal of a threat while minimising damage to host cells. TLRs have evolved a system of highly intricate feedback loops, which induce prompt amplification of an inflammatory response but importantly will in a delayed manner terminate the response.

1.3.1 MyD88 Dependent Pathway:

The MyD88 dependent pathway primarily leads to the activation of the transcription factor NFκB and MAPKs, essential for mounting of an immune response . NFκB activation leads to the expression of a plethora of genes to induce an inflammatory response , while MAPKs are critical for the activation of transcription factors and stabilisation of numerous mRNAs generated in an NFκB dependent manner (; Tseng *et al.*, 2010). The primary regulation of NFκB comes from their interaction with inhibitory IκB proteins, which prevents their nuclear localisation . Signalling through the MyD88-dependent pathway overcomes this negative interaction allowing NFκB to translocate to the nucleus to induce gene expression (Figure 1.1) .

Crystal structures of TLRs propose that ligand binding induces dimerisation and/or oligomerisation of the receptors, or conformational changes in preformed dimers ([Kang and Lee, 2011](#)). This results in rearrangement of the TIR domains to facilitate interaction with the C-terminus TIR domain of MyD88. Binding of MyD88 to the receptor exposes its N-terminus Death Domain (DD), which can interact with the DD of members of the Interleukin-1 receptor-associated kinase (IRAK) proteins to form a signalling

complex known as the Myddosome (Burns *et al.*, 2003; Medzhitov *et al.*, 1998; Muzio *et al.*, 1997; Suzuki *et al.*, 2002; .

TLR2 and TLR4 signalling requires the adaptor protein MAL to couple MyD88 to the receptor (. Beyond this role, MAL signalling has profound effects on TLR signalling. It is involved in induction of the transcription factor CREB and can negatively regulate TRIF-dependent signalling ([Mellett *et al.*, 2011](#);). MAL activity requires cleavage of its C-terminus by Caspase 1 which is regulated by inflammasome complexes (Martinon *et al.*, 2002). Inhibition of Caspase 1 prevents LPS activation of NFκB . IRAK proteins and suppressor of cytokine signalling 1 (SOCS-1) can phosphorylate and target MAL for degradation, possibly disassembling the Myddosome preventing further signalling (Dunne *et al.*, 2010; Mansell *et al.*, 2006).

Members of the IRAK family are essential for TLR activation of NFκB . Four IRAK family members are known, IRAK1, IRAK2, IRAK4 and IRAKM . IRAKM is proposed to be a negative regulator of TLR signalling. Knockout of IRAKM results in increased production of proinflammatory cytokines in response to a variety of TLR ligands . IRAK4 is the predominant member of the family, IRAK4 *-/-* mice display impaired NFκB and MAPK activation for all TLRs except TLR3 . The formation of the Myddosome is proposed to allow IRAK4 to autophosphorylate itself and subsequently phosphorylate and activate IRAK1 and IRAK2 which can then recruit the E3 ligase tumour necrosis factor receptor (TNFR)-associated factor-6 (TRAF-6) to the Myddosome .

IRAK1 was initially proposed to be the kinase involved in the activation of TRAF6 . However IRAK1 *-/-* mice produced unexpected results, displaying impaired but not abrogated NFκB and MAPK signalling . IRAK2 is now proposed to be responsible for TRAF6

activation . It was for a long time considered a pseudokinase, however it has now been shown to activate TRAF6 even in IRAK1 deficient cells . Interestingly IRAK2^{-/-} display normal early NFκB activation but impaired late and sustained activation and also impaired induction of NFκB dependent cytokines. Double mutants of IRAK1 and IRAK2 exhibit profound defects in NFκB and MAPK signalling. From these studies a model is now proposed where IRAK1 is thought to be initially recruited to the Myddosome and activated by IRAK4, while IRAK2 is recruited at later stage to sustain the signalling .

Phosphorylation of TRAF6, induces its oligomerisation and its E3 ligase activity . It can work in conjunction with E2 enzymes, Ubc13, UBCH5 or UBCH7, to form K63 pUB chains on itself and also on target proteins . K63 pUB chains can act as molecular scaffolds, recruiting signalling proteins via ubiquitin binding domains (UBD) which are specific to K63 pUB chains .

The K63 pUB chains of TRAF6 recruit the TAK1 complex to TRAF6 . The TAK1 complex is composed of transforming growth factor-β (TGF-β)-activated kinase-1 (TAK1) and associated TGF-β binding proteins (TAB) proteins, TAB1, TAB2 and TAB3 . The UBDs of TAB1/TAB2 facilitate the recruitment of the TAK1 complex to TRAF6. Recruitment of the TAK1 complex stimulates conformational changes in TAK1 allowing it to phosphorylate and activate itself ; Deng *et al.*, 2000; Gohda *et al.*, 2004; Hacker *et al.*, 2006; [Jiang *et al.*, 2002](#); Shibuya *et al.*, 1996; Sun *et al.*, 2004; Takaesu *et al.*, 2000). TAK1 is also ubiquitinated by TRAF6 (Hamidi *et al.*, 2011). TAK1 can phosphorylate IKK proteins and MAPK kinases (MKK), resulting in NFκB, p38 and JNK activation ; [Shirakabe *et al.*, 1997](#); Wang *et al.*, 2001). Modification of other members of TAK1 complex can regulate TAK1 activity. Recently IL-1β signalling and osmotic stress were shown to lead to the O-GlcNaylation of TAB-1 which was

required for full TAK1 and NF κ B activity and production of IL-6 and TNF α . In vitro studies have shown that the TRAF6 mediated activation of TAK1 was dependent on unanchored K63 pUB chains generated by TRAF6. These are proposed to be bound by TAB2 to activate TAK1. The importance of the unanchored chains in vivo is still undetermined.

However, TAK1 activity is redundant for NF κ B activation. In the absence of TAK1, JNK activation in response to TLR, TNFR and IL-1R signalling is completely abolished. IKK signalling, while severely reduced, was not completely abrogated. TAK1 independent IKK activation is also evident in TAK-1 deficient B cells, as NF κ B activation is comparable to WT upon BCR ligation (Sato *et al.*, 2005). Furthermore TAB1 deficient mouse embryonic fibroblasts (MEFs) show unimpaired activation of MAPK and NF κ B in response to IL-1 β , LPS and TNF α , despite the lack of TAK1 activity; Mendoza *et al.*, 2008). MEKK3 is a candidate for this TAK1 independent activation. MEKK3 is critical to TLR8 pathway. TLR8 signalling to NF κ B and JNK is completely abolished in MEK3K deficient MEFs but unimpaired in TAK1 deficient MEFs. In IL-1 β signalling, the activation of NF κ B is initially mediated through TAK1 but TRAF6 can subsequently activate NF κ B through MEKK3, independent of TAK1 activity. Activation of both is required for sufficient production of cytokines. In macrophages, TLR4 signalling requires MEKK3 for the induction of IL-6 and GMCSF, but not TNF α or IL-1 β . The importance of TAK1, MEKK3 or another kinase in the activation of NF κ B and MAPKs may be ligand and cell type specific ; ;

The IKK complex is also recruited to the K63 pUB chains of TRAF6, where it is activated by TAK1 or another TRAF6 dependent kinase. Three canonical IKKs have been found, IKK α , IKK β and IKK γ (NEMO), as well as IKK-related proteins, TBK1 and IKKi. The IKK complex, composed of IKK α , IKK β and NEMO, binds the K63 pUB

chains of TRAF6 via the UBD of NEMO ; . This is postulated to bring IKK α and IKK β into close proximity to TAK1, facilitating TAK1 to directly phosphorylate and activate the IKK complex.

Members of the IKK complex work in conjunction to phosphorylate the I κ B proteins IKK α functions primarily in the noncanonical pathway of NF κ B activation . IKK β is the major kinase responsible for phosphorylation of I κ B. IKK β deficient mice exhibit severe NF κ B defects . The regulatory element NEMO is critical to NF κ B activation and is modified with K63 ubiquitin chains upon TLR signalling . Mice depleted of NEMO no longer activate the IKK complex ; (Schmidt-Supprian *et al.*, 2000) Interestingly, it was found that NEMO is modified with and can also bind free linear pUB chains, to a 100-fold higher degree than K63 pUB chains . These chains are generated by the E3 ligase LUBAC. In MEFs deficient for HOIL1, a component of LUBAC, IL-1 β signalling is impaired but not fully abrogated . It is proposed that formation of linear chains is not necessary for initial IKK activation but may enhance its activity. Linear chains conjugated to NEMO proteins, may be bound by other NEMO proteins, potentially bringing IKK β members into close proximity allowing them to transphosphorylate each other .

NF κ B activation is regulated by I κ B proteins . There are two main members of the I κ B protein family, I κ B α and I κ B β , but there are also atypical I κ B proteins BCL3, I κ BL and I κ BNS . Their activity is mediated through their multiple ankyrin repeats which can bind to NF κ B dimers and interfere with nuclear localisation sequence (NLS) of NF κ B. Different I κ Bs have varying affinities for NF κ B dimers. I κ B α binds RelA-p50 with highest affinity, while I κ B β binds RelA homodimers. I κ B α can mask the NLS of RelA but not the NLS of p50, however it has nuclear export signal (NES) and prevents NF κ B activity by shuttling dimers out of the nucleus, preventing gene expression . IKK activation results in the phosphorylation of I κ B on

Ser32 and Ser36, resulting in K48 linked pUB on Lys 19 by the Skp1/cullin/F-box β -transducin repeat-containing protein (SCF β TRCP) and subsequent degradation by 26s proteasome . The NF κ B subunits can then translocate to the nucleus to promote gene transcription, they mediate the transcription of genes with a κ B element in their promoter including TNF α , IL-1 β , IL-6 and IL-10. Importantly NF κ B signalling also induces the transcription of NF κ B inhibitory proteins, such as A20 and I κ B .

Posttranscriptional regulation of gene expression is also important in TLR signalling. The stability of mRNA can be targeted as means to regulate protein levels, as can translational efficiency. Many cytokine mRNA transcripts contain 3' untranslated regions known as AU-rich elements (3' UTR AREs), which can target mRNA for decay. Proteins can bind to these elements and influence the stability of the transcript. RNases, such as MCP1P and ZC3H12a, can target the rate of mRNA decay. MAPK play large part in this posttranscriptional regulation .

Figure 1.1 TLR4-MyD88 signalling. Upon TLR engagement, Myd88 is activated resulting in activation of IRAK proteins, which in turn activate the E3 ubiquitin ligase TRAF6. TRAF6 activates TAK1, phosphorylates IKK β , activating the IKK complex to phosphorylate I κ B leading to I κ B degradation and release of NF κ B. TAK1 also activates the MAPKs, resulting in activation of AP1. NF κ B and AP-1 transcription factors induce transcription of proinflammatory cytokines, such as TNF- α and IL-1. Taken and modified from Zhou *et al.*, 2011.

1.3.2 TRIF-Dependent Pathway

The MyD88-independent or TRIF-dependent signalling can be activated by TLR4 and TLR3 stimulation . It was discovered from studies on MAL and MyD88 deficient cells which displayed delayed

activation of NF κ B following TLR3 and TLR4 engagement . While TLR3 can interact directly with TRIF, TLR4 requires the adaptor TRAM . TRIF signalling by TLR3/TLR4 leads to the activation of NF κ B and IRF transcription factors to induce type 1 interferons and cytokines such as TNF α , IL-6 and IL-12 .

TRIF dependent activation of NF κ B involves the recruitment of RIP1. TRIF contains a C terminus RIP homotypic interaction motif (RHIM) domain which can interact with RIP1. The RIP1-TRIF interaction is essential for TLR3 and TLR4 induction of NF κ B , It is proposed that upon TLR3 stimulation, RIP1 interacts with TRIF and is polyubiquitinated which recruits TRAF6 and TAK1 to TLR3. IRAK2 has been shown to directly bind to TLR3, and is involved in its activation of NF κ B but not IRFs. The mechanism underlying this is unknown .

TRAF proteins are central to TRIF dependent IFN production . Compared to WT mice, TRAF3^{-/-} mice have lower IFN and IL-10 levels following TLR3/4/9 stimulation, while they display higher proinflammatory cytokines . The activation of type 1 interferons is dependent on the activation of IRF transcription factors, IRF3/7. IFN α is mainly dependent on IRF7, while IFN β is dependent on IRF3, NF κ B and c-jun/ATF2 . TLR4-TRIF signalling induces only IFN β , while TLR3 signalling results in IFN α/β . Induction of IRF3 requires the N terminus of TRIF, which can associate with TRAF3 and the adaptor protein NAK-associated protein 1 (NAP1) . TRIF signalling is proposed to induce autoubiquitination of TRAF3 with K63 pUB chains, facilitating interaction with and activation of IKK related kinases, TBK1 and IKKi, which can in turn phosphorylate IRF3 on its C terminus transactivation domain ; [Saha et al., 2006](#). Phosphorylated IRF proteins can form homodimers, translocate to the nucleus and induce gene expression . In addition the MAPK JNK has been shown to phosphorylate IRF3 on its N terminus. Use of the specific JNK inhibitor SP600125 severely impaired IRF3 mediated gene

expression in response to LPS and PolyI:C, . Thus phosphorylation of IRF3 by TBK1 and JNK, on its C and N terminus respectively, may be required for full IRF3 activity. MyD88 signalling has been shown to activate the IKK related kinases without activating IRF3, indication an additional factor other than IKK related kinases activation is required for IRF3 induction. TRAF3 targeting to the endosome is proposed to be a factor . Futhermore MyD88 and MAL signalling have been shown to block TRIF dependent activation of JNK, which may in turn prevent IRF3 activation . TRIF also contains TRAF6 and TRAF2 binding sites. Simultaneous mutation of both these sites abrogated TRIF signalling to both NFκB and IRF3. Following from these results it was found that TRAF2 and TRAF6 bind to TRIF and induce its polyubiquination with K63 chains, this leads to activation of IFN-inducible pathway .

It appears TRAF3 can have positive and negative affects on TLR signalling, that may be dependent on its cellular location. TRAF3 positively regulates TLR4 and TLR3 induction of IFNs. It also positively regulates TLR9 mediated activation of IRF7 and interferons (Hacker *et al.*, 2006; Oganesyanyan *et al.*, 2006). These signalling events all occur at the endosome. In contrast, signalling by TLR4-MyD88, CD40 and TNFR1 signalling all occur proximal to the membrane and are negatively regulated by TRAF3, which inhibits the release of MAPKKK from signalling complex to activate MAPK (Hacker *et al.*, 2006; Matsuzawa *et al.*, 2009; Tseng *et al.*, 2010). It seems TRAF3s role is dependent on the individual receptors, receptor location and also the adaptors they engage (Hacker *et al.*, 2011).

Cell surface activation of the IKK related proteins by IL-1β and TNFα can negatively regulate the activation of IKK proteins. IKK-related kinases are activated by IKK proteins in vitro in response to IL-1β, LPS and TNFα. In addition LPS and IL-1β can activate IKK-related

proteins independently of IKKs, involving an as yet unknown protein or may involve autophosphorylation of IKK related proteins (Clark *et al.*, 2009) The IKK related proteins can inhibit the activation of IKK proteins, limiting their activation and thus their downstream targets such as NF κ B (Clark *et al.*, 2011). It is proposed that TANK promotes IKK related protein interaction with NEMO and TRAF6, to inhibit their activity and thus inhibit IKK proteins (Clark *et al.*, 2011).

1.3.4 NF κ B

NF κ B is a master regulator of inflammation. It is elicited by viral and bacterial infection, inflammatory cytokines and engagement of antigen receptors. Environmental and physical factors can also induce NF κ B. NF κ B induces transcription of a plethora of genes to promote proinflammatory and prosurvival responses . In mammals there are five members of the transcription factor NF κ B family: RelA (p65), RelB and c-Rel and precursor proteins p105 (NF κ B1) and p100 (NF κ B2), which are processed into p50 and p65 respectively by degradation of C' I κ B-like ankrin repeats. They are characterised by a N terminus RHD domain which functions in dimerisation, nuclear translocation, DNA binding and interaction with inhibitory I κ B proteins. They act as hetero and homo dimers, binding to κ B sites in the promoter region of target genes to influence their gene expression. RelB primarily binds p100/p52, while RelA and c-Rel form dimers with p50. Some dimer combinations can have inhibitory effects on gene expression. The κ B sites are found in numerous proinflammatory genes and thus NF κ B activation can have profound effects on the cell . Targets genes include ones encoding cytokines; IL-1 β , IL-6, IL-10, TNfa, chemokines; MIP-1a/b, cell adhesion molecules; I-CAM, V-CAM, regulators of apoptosis; ASC, Bcl-xl, Bcl-2, BiM, c-FLIP. Others notables include MHC molecules required for antigen presentation by APC, iNOS and MMPs. Critically, κ B

elements are present in promoters of genes for proteins such as A20 and I κ B, which negatively regulate NF κ Bs activity to control inflammatory responses

1.3.5 IRF Transcription Factors

The antiviral interferon response is primarily regulated by the transcription factors IRF3 and IRF7 . The phosphorylated form of IRFs can dimerise and translocate to the nucleus to induce type 1 IFNs and expression of IFN-inducible genes. IRF3 proteins are constitutively and ubiquitously expressed across many cell types. Upon phosphorylation and dimerisation they can translocate to the nucleus and interact with CBP/p300 proteins to induced expression of target genes. All IRF target genes contain an interferon-sensitive response element (ISRE) domain in their promoter. IRF3 primarily leads to the induction of IFN- β , while IRF7 predominately induces IFN α IRF7. IRF7 expression is low in most cells compared with levels of IRF3 proteins. However IRF7 is an IFN-inducible gene and forms an autocrine positive feedback loop which leads to the expression of IRF7 through IFNR ; Sato *et al.*, 2000). An unexpected finding in IRAK1-/- mice was that in pDCs TLR7 and TLR9 induced IFN α was completely abolished, proposing a role for IRAK1 in IFN α production. Furthermore *in vitro* IRAK1 has been shown to directly phosphorylate IRF7. This pathway produces significant amounts of IFN α to protect from viral infection (Di *et al.*, 2009; Uematsu *et al.*, 2005).

1.3.6 Negative Regulation TLR signalling.

TLR signalling must be appropriately attenuated to prevent tissue damage. Negative regulators operate at all levels of TLR signalling

and include splice variants of adaptor proteins, ubiquitin ligases, deubiquitinases, transcriptional regulators and micro RNAs . Soluble forms of TLR receptors can compete with receptors for ligand binding to dampen immune responses . The TIR-TIR interaction between receptors and adaptors is disrupted by proteins such as MyD88s ([Burns *et al.*, 2003](#), ST2 and SIGGR . The negative adaptor SARM can interfere with both TRIF and MyD88 pathways, while MyD88 and MAL have been shown to regulate TRIF signalling to IRF proteins . Downstream of the receptor SOCS-2 and SHP-1 suppress IRAK signalling, while SOCS-2 additionally targets MAL for degradation . ATF3 prevents the TLR induction of IL-6 and IL-12p40 by regulating transcription factor activation . NFκB importantly leads to the induction of several inhibitory proteins, to form negative feedback loops such as IκB and A20 .

A20 is a potent negative regulator of NFκB signalling . It is a dual function enzyme, as it can act as deubiquinating enzyme and E3 ligase. Much of the knowledge on A20 has come from A20 knockout mice which display systemic inflammation and premature lethality. In TNFα signalling, RIP-1 is an important A20 target, while in IL-1R and TLR signalling, TRAF6 and IRAK1 are targets . It interacts with several proteins to form A20 ubiquitin editing complex composed of TAX1BP1, ITCH and RNF11. A20 also has non catalytic roles; as it can prevent the interaction of E2 and E3 enzymes to prevent ubiquitination . A20 binding to polyubiquitin chains on NEMO can block TAK-1 activation of IKK. This interaction may be promoted by ABIN1 . Under *In vitro* conditions unanchored K63 pUB chains can also promote the A20-NEMO interaction . Interestingly IKK proteins can regulate the assembly of the A20 editing complex. IKKα can phosphorylate TAX1BP1 to promote the assembly of A20 complex and its interaction with TRAF2/6 whilst IKKβ phosphorylation of A20 increases its inhibitory activity .

1.9 MAPK

Engagement of TLRs not only leads to the activation of NF κ B but also to MAPK proteins. The MAPKs can in turn activate numerous proteins and transcription factors to regulate the immune response. They are involved in all phases of immune response from initiation of innate and adaptive responses and regulating cell death of immune cells(Cargnello and Roux, 2011;.

MAPK signalling operates in a three tiered mechanism where MAPK kinase kinases (MAPKKK) phosphorylate and activate MAPK kinases (MAPKK) which then phosphorylate MAPK. MAPK require dual phosphorylation on conserved tripeptide motifs (Thr-X-Tyr) to be activated and are distinguished into three main families dependent on their specific residues within this region; Extracellular signal regulated protein kinases (ERK) Thr-Glu-Tyr (also known as p42/44), p38 Thr-Pro-Tyr and c-JUN NH₂-terminal kinases (JNK) Thr-Gly-Tyr. Recently a fourth family have been recognised, the ERK5 family. Dual-specificity phosphatases (DUSPs) regulate MAPK by reversing this dual phosphorylation, and hence have important role in immune regulation ; Marshal, C.J., 1994).

A diverse repertoire of extracellular cues induce the activity of MAPK, however different stimuli influence the different families with varying degrees. For the ERK family the main extracellular cues are growth promoting mitogenic stimuli. JNK and p38, which are also classed the stress activated kinases (SAPK), are mainly activated by stresses such as osmotic shock, hypoxia, heat shock and UV radiation and by proinflammatory signals including LPS, IL-1 β , TGF- β and TNF- α MAPK mainly regulate inflammatory responses by directly or indirectly targeting transcription factor activity, regulating mRNA stability and also protein maturation ([Cargnello and Roux 2011](#); Fan

et al., 2005; [Guhaniyogi and Brewer 2001](#); MAPK signalling integrates into several different networks to regulate cell death, proliferation and differentiation, orchestrating a dynamic flexible signalling system, that can tailor cellular responses appropriately in response to multiple signals

1.9.1 JNK

The JNK family of MAPK are involved in the regulation of cell proliferation and apoptosis. There are three known isoforms, JNK 1-3 of which JNK1 and JNK2 are widely expressed, while JNK3 expression is restricted to neuronal tissue, testis and cardiac myocytes. Several forms of stress have been shown to activate JNK signalling including heat shock, UV irradiation, cytokines, DNA damaging agents and oxidative stress . While numerous MAPKKK have been implicated in JNK activation, such as Mekk1-4, MLK 1-3, TAK1 and ASK1, JNK activation converges at the level of MAPKK specifically on two MAPKK- MKK4 and MKK7 ([Bogoyevitch *et al.*, 2010](#); Cargnello and Roux, 2011; Derijard *et al.*, 1995; Lin *et al.*, 1995; Tournier *et al.*, 1999; Weston and Davis, 2002). Tak1 is essential for JNK activation in response to inflammatory signals such as IL-1 β , TNF α , TGF- β and TLR signalling .

JNK mediates its effects primarily through the activation of transcription factors, it has been shown to target p53, ATF2, ELK-1, stat3 and c-jun ([Bogoyevitch *et al.*, 2010](#); [Gupta *et al.*, 1995](#); [Raman *et al.*, 2007](#); [Whitmarch *et al.*, 1995](#)). A major target of JNK signalling is the AP-1 complex . AP-1 is a dimeric protein complex composed of homo and heterodimers of fos, jun and ATF-2 family members, exerting its action by binding TRE elements in the promoter of target genes. The composition of the AP-1 complex determines its targets and its action. AP-1 is essential for cell differentiation and

proliferation and regulates many inflammatory genes including genes encoding cytokines, chemokines, and MCP-1. c-jun is the best characterised member of the AP-1 complex. It can form homodimers or heterodimers with c-fos. JNK can bind c-jun directly, phosphorylating and activating it (Alder *et al.*, 1992; Hibi *et al.*, 1993; Pulverer *et al.*, 1991). Many of the genes regulated by c-jun proteins are involved in cell proliferation and differentiation. Mice depleted of c-jun are embryonic lethal while fibroblasts from c-jun $-/-$ mice have defective cell cycle progression resulting from decreased expression of cyclin D and increased expression of p53 and p21.

JNK signalling is indispensable for induction of the intrinsic and extrinsic apoptotic pathways. The conflicting critical role of JNK in cell survival and cell death may be due to the differential outcome of transient and sustained JNK expression, as well as cell type and stimulus specific activity. Transient JNK expression is associated with cell proliferation and survival while sustained activation of JNK is associated with apoptosis (Javelaud and Besancon, 2001; Kamata *et al.*, 2005; Tang *et al.*, 2001).

JNK signalling is implicated in the intrinsic apoptosis pathway. Primary fibroblasts isolated from JNK1/2 $-/-$ and MKK4/7 $-/-$ mice displayed severe defects in apoptosis mediated by UV irradiation, methyl methanesulfate and anisomycin. This resistance correlates with a failure to release cytochrome C from the mitochondria. However these cells were still vulnerable to Fas mediated apoptosis. JNK can target transcription factors such as AP-1, ATF2, RXR α , RAR α and p53 to modulate the expression of pro and anti apoptotic genes. In addition it has been implicated in the regulation of Bax, Bak and Bid proteins which are central to mitochondria mediated cytochrome C release (Deng *et al.*, 2003; Lei *et al.*, 2002; Tournier *et al.*, 2001; Tsuruta *et al.*, 2004).

TNF α signalling can lead to the induction of NF κ B and thus proinflammatory and prosurvival response. But it can also lead to the DISC formation and the induction of caspase cascade, resulting in apoptosis. NF κ B induction is the default signalling pathway and must be overcome before apoptosis can proceed (De Smaele *et al.*, 2001; Tang *et al.*, 2001). JNK antagonises the prosurvival effects of NF κ B by targetting the NF κ B dependent protein c-FLIP which functions to prevent the interaction between caspase 8 and FADD and thus prevent DISC formation. Activation of JNK inactivates c-FLIP indirectly through the phosphorylation of the E3 ligase ITCH. Phosphorylated ITCH can ubiquitinate cFLIP targeting it for degradation, allowing apoptosis to proceed. ITCH deficient mice are resistant to TNF α induced acute liver failure (Chang *et al.*, 2006; Deng *et al.*, 2003).

MyD88 has been shown to negatively regulate TLR3 induced IFN- β production. It can inhibit the IKK β kinase preventing phosphorylation of IRF3. In human corneal epithelial cells, loss of MyD88 exacerbated TLR3 induced inflammatory responses in a JNK dependent manner. Mal has also been shown to inhibit TLR3 induced JNK. Mal deficiency boosted IL-6 production in response to PolyI:C.

1.9.2 P38

There are four isoforms of p38; p38 α , p38 β , p38 γ , p38 δ , each encoded by a separate gene. Upstream signalling of p38 converges on MKK3 and MKK6 which are specific to the p38 family (Cuadrado and Nebrda, 2010).

p38 kinases play important roles in cytokine signalling. They have a dual function acting as downstream mediators in cytokine signalling pathways and also are involved in the post translational production of certain cytokines. p38 upregulates cytokine production by directly phosphorylating transcription factors and indirectly through downstream kinases. They also regulate mRNA stability and translation through phosphorylation of 3' UTR adenylate/uridylate-rich elements(ARE) in target proteins (Schieven, 2005). p38 activation of MK2 is essential for the induction of TNF α . Mice deficient in MK2 have increased stress resistance and could survive LPS induced shock. It was shown that in response to LPS that TNF α levels in vivo in serum and in vitro in splenocytes were reduced when MK2 was knocked out. However the mRNA levels of the TNF α were not affected. The effect of MK2 knockout is exerted at the posttranscriptional level. 3' UTR ARE regions of mRNA transcripts are targeted by p38-activated MK2 (Kotlyarov *et al.*, 1999). p38 inhibitors in WT mice could block the production of TNF α , however these inhibitors had no affect on TNF α mutants with defective ARE regions. The p38-MK2-ARE action is targeted and inhibited by Il-10, preventing TNF α production (Kontoyiannis *et al.*, 2001). Other ARE-containing mRNAs targeted by p38 pathway include COX-2, IL-1 β , IL-6, IL-8, MIP-1, CXCL-10, c-fos and VEGF (Khabar, 2010).

p38 signalling to CREB is important for the regulation of CREB responsive genes such as TNF α , IL-2, IL-6, IL-10 and COX-2 (Avni *et al.*, 2010; Eliopoulos *et al.*, 2002; O' Donnell and Taffet, 2002; Platzer *et al.*, 1999; Shaywitz and Greenberg, 1999; Roach *et al.*, 2005; Wiggen *et al.*, 2002). LPS signalling has been shown to induce phosphorylation of CREB in p38 manner. Interesting this is dependent on MAL signalling, while the E3 ligase Pellino and TRAF6 are also involved. Phosphorylated p38 in turn activates MSK1/2

which can phosphorylate CREB (Caivono and Cohen, 2000; Eliopoulos *et al.*, 2002; Mellet *et al.*, 2011).

1.9.3 ERK

The canonical pathway of ERK activation is through the Ras-Raf-ERK pathway which is activated in response to growth factors (Kolch, 2005). However cytokine and TLR receptors activate MAPKs primarily via the MAP3K TPL-2 (Symons and Ley, 2006). ERK activation in response to LPS and IL-1 β leads to the phosphorylation of ELK-1 and C/EBP transcription factors. C/EBP proteins are important for the upregulation of several proinflammatory mediators, G-CSF, IL-6/8/1, TNF α and iNOS. ELK-1 phosphorylation leads to expression of c-fos which as part of AP-1 complex leads to upregulation of cytokines and MMP proteins (Cargnello and Roux, 2011). ELK-1 may also have anti-inflammatory roles as ELK-1 signalling has been shown to contribute to the NF κ B induction of MCPIP, a protein that negatively regulate the stability of several inflammatory transcripts such as IL-1 β and IL-12p40 (Kasza *et al.*, 2011).

In an immunological sense it seems the majority of ERK activation is through the IKK-TPL2 axis (Figure 1.2) (Gantke *et al.*, 2012). Knockout of the MAPKKK TPL2 showed that TPL2 is essential for the LPS activation of MEK1/2 and ERK1/2 but is dispensable for p38 and JNK activation. TPL2 knockout mice are resistant to LPS induced endotoxic shock (Dumitru *et al.*, 2000). A Yeast 2 hybrid screen identified p105 as a TPL2 interacting partner (Belich *et al.*, 1999). While only a small pool of p105 is associated with TPL2, the majority of cellular TPL2 is coupled to p105 ((Dumitru *et al.*, 2000; Lang *et al.*, 2004; Yang *et al.*, 2004). TPL2 does not regulate the function of p105 as TPL2-deficient cells, when treated with LPS, retain the

ability to activate p105. However, P105 was found to exert strong control of TPL2, regulating its stability and activity. In p105-deficient macrophages, TPL2 levels are near depleted and there is no LPS induced activation of ERK (Beinke *et al.*, 2005; Waterfield *et al.*, 2003). The interaction between TPL2 and p105 occurs at two points, the first between the C terminus of TPL2 and the ankyrin repeat of p105, and the second between the kinase domain of TPL2 and the death domain of p105. This second interaction blocks the activity of TPL2s kinase domain and prevents MEK1/2 activation (Beinke *et al.*, 2005). In macrophages this interaction is regulated by the IKK proteins. LPS stimulation leads to the phosphorylation of S927 and S932 in the PEST region of p105 by IKK. SCF β trcp can then target p105 with K48 pUB chains and induce its partial degradation to the p50 subunit. This proteolysis of p105 is essential for LPS activation of TPL2, as it releases TPL2 and removes inhibitory effects of p105 (Beinke *et al.*, 2004; Lange *et al.*, 2003; Salmeron *et al.*, 2001; Waterfield *et al.*, 2004). ABIN2 is also complexed with TPL2 and p105 however its role in the complex is unclear. ABIN2^{-/-} mice have comparable NF κ B activation compare to WT. However the TPL2 activation of ERK is reduced. ABIN2, like p105, is not thought to directly regulate the catalytic activity of TPL2 but controls its stability (Papoutsopoulou *et al.*, 2006).

Independent of p105 and ABIN2, TPL2 is regulated by LPS induced phosphorylation on several residues (Dumitru *et al.*, 2000; Yang *et al.*, 2004). The phosphorylation state of TPL2 has been shown to be dependent on the nutrient content of the environment. In the absence of the amino acid arginine, TPL2 associates with PP2A and is dephosphorylated, preventing MEK1/2 activation.

The main outcome of TPL2 knockout is a drastic decrease in LPS induced TNF α production in macrophages. It has since been revealed that the TPL2-ERK axis can target TNF α at transcriptional,

posttranscriptional and protein levels. TPL2/ERK can target the transcription of TNF α mRNA as well as its translation through phosphorylation of its 3' UTR ARE regions (Dumitru *et al.*, 2000). TNF α is produced in a pre-TNF form which is a type II transmembrane protein and must be cleaved by the enzyme TACE to form the soluble form. Inhibition of ERK in macrophages significantly decreases the levels of soluble TNF α . This is explained by the phosphorylation of TACE on thr735 by LPS induced ERK (Rousseau *et al.*, 2008). TPL2 deficiency also resulted in reduced IL-10 and IL-1 β mRNA levels, while IL-12 and IFN- β mRNA levels were increased. The regulation of IL-12 by TPL-2 is a combination of direct regulation of IL-12 and indirect regulation through IL-10 regulation. Conversely the regulation of IFN- β was completely independent of IL-10, but involves the induction expression of the transcription factor c-fos (Kaiser *et al.*, 2009). Loss of TPL2 in addition affects macrophage production of PGE₂. TPL2 is required for ERK stimulation of MSK1, which can activate CREB to regulate COX-2 transcription. COX-2 then regulates PGE₂ production (Eliopoulos *et al.*, 2002). Despite the pro- and anti-inflammatory effects, TPL2 is considered to be a proinflammatory protein as it is required for clearance of *Listeria monocytogenes* bacteria (Mielke *et al.*, 2009).

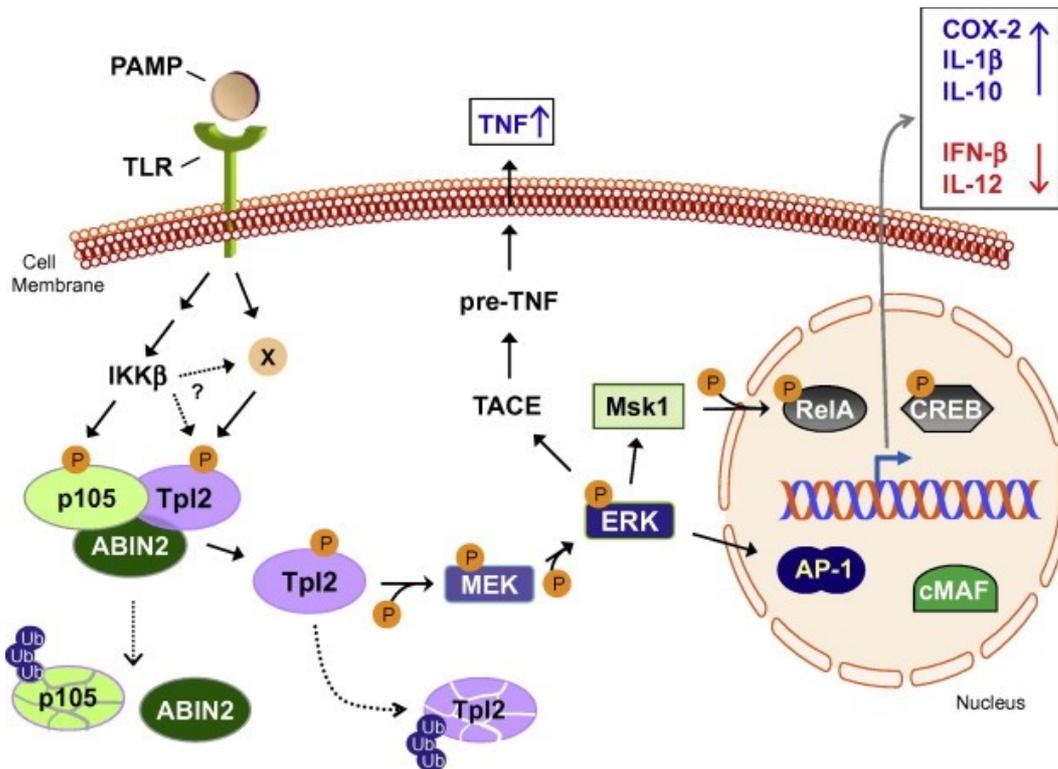


Figure 1.2: Tpl-2 Signalling pathway. Under Basal conditions Tpl2 associates with p105 and ABIN2. TLR4 signalling results in IKKβ activation, which can phosphorylate p105 leading to its processing to p50 and release of Tpl2. Tpl2 can then target the p42/44 pathway by activating MEK. P42/44 subsequently can activate and induce transcription factors with both pro- and anti-inflammatory activity. It also plays a role in the post transcriptional activation of TNF-α by targeting the enzyme TACE. Taken and modified from Vougioukalaki *et al.*, 2011.

1.10 ECSIT

Evolutionary Conserved signalling intermediate in toll (ECSIT) protein was first discovered in 1999 by Kopp *et al.*, In a bid to understand the signalling mechanisms linking TRAF6 to IKK activation they performed a yeast two hybrid screen of TRAF6 against mouse liver library. ECSIT was discovered as 1.2kDa cDNA transcript whose encoded protein interacted with TRAF6. Further northern blot analysis revealed 3 alternatively spliced forms of the protein, named ECSIT 1/2/3. ECSIT displayed no homology to any

known proteins and had no known protein domains. However the protein was highly conserved across *Drosophila* and *C. elegans* and thus was termed ECSIT. The initial functional characterisation of ECSIT found that ECSIT specifically interacted with TRAF6 and not TRAF2 or TRAF5 and its overexpression increased NF κ B in a dose dependent manner in the presence of TLR4, IRAK1 and TRAF6. A dominant negative version of ECSIT blocked NF κ B activation in presence of IRAK1 but not RIP1 indicating ECSIT was specific for TLR4 signalling and not TNFR signalling. Furthermore ECSIT was found to interact with MEKK1 and lead to increased MEKK1 processing into the proposed active 80kDa isoform. Based on these finding it was postulated that ECSIT formed the critical link between TRAF6 and MEKK1 leading to NF κ B and AP-1 activation. However this theory is now widely disputed as MEKK1 knockout mice don't display defects in NF κ B signalling (Zhang *et al.*, 2003).

ECSIT knockout mice display embryonic lethality and die on day E7.5. Interestingly analysis of these embryos revealed a phenotype that mimicked that of BMPR1a knockout mice (Xiao *et al.*, 2003). BmpR1a is the receptor for BMP4 a member of TGF β superfamily. It signals via SMAD proteins and is critical during embryonic development with roles in cell proliferation, survival and formation of the mesoderm (Winnier *et al.*, 1995). ECSIT null mutants displayed decreased cell proliferation, altered epiblast patterning and impaired mesoderm. ECSIT was found to constitutively interact with SMAD4 and to interact with SMAD1 in a BMP inducible manner. A SMAD4/1/ECSIT complex was found to bind the promoter of Tlx2, a BMP inducible gene leading to TLx2 expression. ECSIT ShRNA was used to knockdown ECSIT levels and was found to abolish Tlx2 activity and LPS induced NF κ B activation (Xiao *et al.*, 2003).

Since these initial discoveries ECSIT has slowly become a prime focus in innate signalling, with several groups publishing on ECSIT in

recent years. ECSIT localises to the mitochondria via a N-terminal target sequence. It can interact with the chaperone protein NDUFUFA1 in 500 and 850kDA complexes and is involved in the assembly and function of Complex1 in the mitochondrial respiratory chain (Vogel *et al.*, 2007). The relevance of ECSITs mitochondrial localisation in the context of innate immune signalling was established by the work of West *et al.*, 2012. Bacterial killing in phagocytes is mediated by the generation of ROS in phagocytes. This has always been considered due to production of ROS by NADPH oxidase. However recent studies looking at mouse macrophage killing of bacteria has implicated mROS to be important, requiring signalling from bacterial sensing TLRs. West *et al.*, 2012 demonstrated that TRAF6 and ECSIT are required for macrophage killing of intracellular bacteria. Upon TLR 1, 2 and 4 signalling in macrophages, mitochondria are recruited to the phagosome, while TRAF6 translocates to the mitochondria. TRAF6 can bind to and ubiquitinate ECSIT resulting in an enrichment of ECSIT to the mitochondrial periphery and a concomitant increase in both mitochondrial and cellular production of ROS. Macrophages deficient in TRAF6 and ECSIT fail to clear infection. Thus proving in a physiological context the importance of mECSIT in TLR signalling.

ECSIT role is not confined to TLR signalling and has recently been shown to extend to RLR signalling (Kondo *et al.*, 2012). As with the original discovery of ECSIT by Kopp *et al.*, ECSITs role in RLR was eluded from a yeast two hybrid screen. In this case TRIM59, a newly discovered ER-localised TRIM protein, was used as bait. The authors investigated the role of ECSIT and TRIM59 in RLR signalling as the common mitochondrial location of MAVS and ECSIT and their overlapping signalling intermediates raised the possibility that they interacted. Indeed this was confirmed by coimmunoprecipitation and confocal studies which showed that ECSIT interacts with MAVS and enhances MAVS mediated IFN- β promoter activation. TRIM59

was found to negatively affect the MAVS induced transcriptional activation in the NF κ B responsive element and IFN- β and IRSE promoters. It was postulated that TRIM59 may negatively affect MAVS signalling via ECSIT. However TRIM59 fails to ubiquitinate ECSIT. Although not conclusive these results implicate ECSIT in RLR signalling. TRIM59 knockout mice once generated will further establish ECSITs role.

1.12 Aims of the project.

The main objectives of this project were:

- Determine the functional role of hECSIT in MAPK signalling and its ability to modulate activation of the transcription factors ELK-1 and Ap-1.
- To screen for ECSIT interacting partners by an unbiased proteomic approach.
- To identify a possible mechanism of action underlying the regulatory effects of hECSIT in MAPK signalling.

2. Materials and Methods.

2.1 Materials

2.1.1 Reagents

Supplier

| | | |
|--|----------------|---------|
| Agar | | Sigma |
| Agarose | | Promega |
| Agarose, low melting point | | Sigma |
| Ampicillin | | Sigma |
| Antibodies (monoclonal) against human- | | |
| | β -Actin | |
| | Sigma | |
| | FLAG M2 | Sigma |
| | HA-Tag (6E2) | Cell |
| Signalling | | |
| | MYC-Tag (9B11) | Cell |
| Signalling | | |

Antibodies (polyclonal) against human-

ECSIT/SITPEC

MyBioSource.com

Ampholytes

Amersham Bioscience

Acetic Acid

Sigma

Acetonitrile

Amersham Bioscience

Ammonium Bicarbonate

Sigma

APS

Sigma

Bovine serum albumin

Sigma

Bradford reagent dye

Bio-Rad

Bromophenol blue

Sigma

Chaps

Sigma

Chloroform

Sigma

Coelenterazine

Insight

Biotech.

Coomassie

Blue

Sigma

Cover fluid

Amersham

Bioscience

DEPC-treated water

Ambion

DMEM

Invitrogen

DMSO

Sigma

dNTPs

Promega

DTT

Sigma

E. coli - TOP 10 competent cells

Invitrogen

EDTA

Sigma

| | | | |
|-----------------------------|-------|--------|-------------|
| Ethanol | | | |
| Sigma | | | |
| FBS | | | Invitrogen |
| Formalin | | | Sigma |
| Formic Acid | | | Fluka |
| Glycerol | | | Sigma |
| Glycine | | | Sigma |
| HEPES | | | Sigma |
| High speed plasmid midi kit | | | Qiagen |
| Hydrochloric acid | | | Merck |
| Igepal | | | |
| Sigma | | | |
| IL-1 β (human) | | | RandD |
| Systems | | | |
| Iodoacetamide | | | Sigma |
| IPG strips | | | Amersham |
| Bioscience | | | |
| IRDye | 800CW | Goat | Anti-Rabbit |
| Licor Biosciences | | | |
| IRDye | 680 | Donkey | Anti-Mouse |
| Licor Biosciences | | | |
| Isopropanol | | | Sigma |
| L-glutamine | | | Invitrogen |
| Lipofectamine 2000 | | | |
| Invitrogen | | | |
| Lipopolysaccharide | | | Alexis |
| Luciferase substrate | | | Promega |
| Magnesium Chloride | | | Sigma |
| β -Mercaptoethanol | | | Sigma |
| Methanol | | | BDH |
| Microlon 96-well plates | | | Greiner |
| Nitrocellulose | | | |
| Schleicher and Schuell | | | |

| | |
|---|----------------------|
| OptiMEM | |
| Invitrogen | |
| PBS | Oxoid |
| pcDNA 3.1/Zeo | Invitrogen |
| Penicillin / StreptoMYCin / Glutamine | |
| Invitrogen | |
| PMSF | Sigma |
| Poly (I:C) | Sigma |
| Polybrene | |
| Sigma | |
| Ponceau | Sigma |
| Potassium ferricyanide | Sigma |
| Prestained molecular weight marker (See Blue Plus) | |
| Invitrogen | |
| Protein A/G-agarose | Santa |
| Cruz | |
| Protogel | National Diagnostics |
| Puromycin | |
| Sigma | |
| RNase Zap | Ambion |
| RPMI | Invitrogen |
| SDS | Sigma |
| siRNA hECSIT specific | |
| Ambion | |
| siRNA Lamin a/c | Ambion |
| Skim milk powder | Sigma |
| Silver nitrate | Sigma |
| Sodium chloride (NaCl) | Sigma |
| Sodium hydroxide (NaOH) | Sigma |
| Sodium orthovanadate (Na ₃ VO ₄) | Sigma |
| Sodium nitrate | Sigma |
| Sodium Phosphate | Sigma |

| | |
|----------------------------|----------|
| Sodium Potassium Carbonate | Sigma |
| Sodium thiosulphate | Sigma |
| Sulphuric acid | Sigma |
| TEMED | Sigma |
| TMB | Sigma |
| Tissue culture ware | Greiner |
| TK Renilla | Promega |
| TNF- α | RandD |
| Systems | |
| Thiourea | |
| Sigma | |
| Trifluoroacetic acid | Sigma |
| Tris-base | Sigma |
| Tris-HCl | Sigma |
| Triton-X | Sigma |
| Trypsin/EDTA | |
| Invitrogen | |
| Tween-20 | Sigma |
| Ultrapure | Protogel |
| National Diagnostics | |
| Urea | Sigma |
| Whatmann paper | |
| AGB | |

Cell Lines given as gifts:

- HEK293 and HEK293 stably expressing TLR4 - Prof. Douglas T. Golenbock (The University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA).
- A549 cells- Dr Shirley O' Dea (Epithelial Immunobiology Laboratory, NUI Maynooth, Co. Kildare).

- U373 cells- Dr Sinead Miggen (Immune signalling Laboratory, NUI Maynooth, Co. Kildare).

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Adherent Cell Lines

Human embryonic kidney (HEK) 293 cells, HEK 293 cells that stably express TLR4 receptor (HEK 293 TLR4 cells), human U373 astrocytoma cells stably transfected with CD14 (U373-CD14) and carcinomic human alveolar basal epithelial A549 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% (v/v) foetal bovine serum (FBS), 100 µg/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). The neoMYCin analog G418 (500 µg/ml) was used to select for the stably transfected TLR cell lines and maintenance of CD14 expression.

2.2.2 Propagation of DNA

2.2.2.1 Rapid transformation of competent *E. coli* cells

TOP10 chemically competent *E. coli* cells were used for propagation of plasmids. Plasmid (100-400ng) was added to 5 µl of TOP10 cells. DNA and the cells were mixed gently with a pipette and incubated

on ice for 30 min. The plasmids were allowed to enter the bacterial cells by heat shocking the mixture at 42°C for 60 seconds. The cell membrane becomes permeable to allow easy entry of the plasmid and cooling on ice for 2 min reverses the membrane to an impermeable state. 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 2-4hr. Transformed cells were pelleted by centrifuging at 1000rpm for 2min at 4 °C and then resuspended in 100µl of LB. An aliquot (50µl) of mix was plated out on LB agar plates (LB broth with 1.5% (w/v) agar) containing 100 µg/ml ampicillin. Plates were inverted and incubated overnight at 37 °C. Plates were then stored at 4 °C for up to four weeks.

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

A starter culture of LB broth (2 ml) containing ampicillin (50 µg/ml) was inoculated with a single transformed *E. coli* colony and incubated at 37°C 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 by spectrophotometry (Nanodrop; Thermo Scientific).

2.2.3 Luciferase assay.

2.2.3.1 Transfection of HEK293 cells for luciferase reporter assay

HEK 293 TLR4 cells were seeded at 1.8×10^5 cells/ml (200 μ l DMEM/well) in 96-well plates and allowed to adhere for 24 h to approximately 70% confluency. Cells were transfected using Lipofectamine 2000. For each well to be transfected, 25 μ l of OptiMEM (Invitrogen) was mixed with the DNA. DNA mixes were made up for the appropriate luciferase construct as outlined in section 2.2.3.2. Lipofectamine 2000 (0.4 μ l) was diluted in OptiMEM (25 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 DNA/OptiMEM mix (total volume 50 μ l 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 sample was transfected in triplicate. 24 h after transfection the supernatants were removed, cell lysates were generated and used to measure luciferase activity.

2.2.3.2 Luciferase Assays

24 h post-transfection (as described in section 2.2.3.1), the medium was removed from the cells and reporter lysis buffer (100 μ l, 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 μ l) of each were assayed for firefly luciferase activity using firefly luciferase substrate (40 μ l, Promega), while Renilla luciferase activity was assayed using coelenterazine (0.1 $\mu\text{g/ml}$ in PBS). Luminescence was monitored with a Glomax microplate luminometer (Promega).

2.2.3.2.1 NF- κ B Assay

To measure activation of the NFκB pathway, cells were transfected with NFκB -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 maintained at 200 or 250 ng using pcDNA3.1.

2.2.3.2.2 AP-1 Assay

To measure the activation of Ap-1, cells were transfected AP-1-regulated firefly luciferase (80ng), phRL-TK (20ng) and varying amounts of expression constructs (detailed in figure legends). The total DNA concentration was maintained at 230 or 250 ng using the appropriate empty vector.

2.2.3.2.3 ELK-1 Assay

To measure the activation of ELK-1, cells were transfected with pFR-Luc (60 ng), the trans-activator plasmid pFA-ELK-1 (ELK-1 fused downstream of the yeast Gal4 DNA binding domain, (3ng), phRL-TK (20 ng) and varying amounts of expression constructs (detailed in figure legends). The total DNA concentration was maintained at 230 ng using the appropriate empty vector.

2.2.4 SDS PAGE Electrophoresis and Western Blotting

2.2.4.1 Transfection of cells for Western Blot analysis

HEK293 or U373 cells were seeded at 2×10^5 cells/ml in 6-well plates (2.5 ml DMEM/well). Cells were grown for 24 h to approximately 70% confluency. For each well of a 6-well plate to be transfected, DNA (amount depending on individual assays as outlined in relevant sections) 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. 500µl of DNA-Lipofectamine mixture was added to each well. After 24hrs cells were treated with appropriate ligands for various times. Samples were then analysed by SDS-Gel Electrophoresis and Western Blotting.

2.2.4.2 Transfection of cells with siRNA for Western Blot

Analysis.

Lamin a/c siRNA and siRNA targeting hECSIT was purchased from Ambion Inc. (target sequence : 5' GGTTCTTTGCCCAAAGACTT 3').

U373 cells were seeded at 2×10^5 cells/ml in a 6-well plates (2.5 ml DMEM/well). Cells were grown for 24 h to approximately 70% confluency. 10nM of hECSIT-specific siRNA or Lamin a/c control siRNA, which has no significant homology to any known human gene sequences, were diluted in OptiMEM (250 µl) mixed gently and incubated for 5min. Lipofectamine 2000 (4 µl) was also diluted in 250µl of OptiMEM per sample, the reaction was mixed gently and left at room temperature for 5 min. After the incubation, the Lipofectamine/OptiMEM solution was added to the OptiMEM-siRNA mix (total volume 500 µl per well to be transfected) and the combined reaction mixed gently and incubated at room temperature for further 20 min. 500 µl sample was then added to each well and mixed gently by rocking the plate back and forth. Cells were incubated for 48 h prior to treatment and harvesting.

2.2.4.3 Harvesting of Cells for Western Blotting

U373 cells or HEK293 cells seeded at a density of 2×10^5 /ml in 6-well plates. Cells were grown for 24 h to approximately 80% confluency. The next day, cells were stimulated with TLR ligands for 1 h-3 h. To prevent induction of stress activated MAPKs during harvesting, harvesting was done rapidly at room temperature. All

media was removed from 6 well plate, cells were washed with room temperature PBS and then 100-150µl of cell lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% (w/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 µg/ml), aprotinin (25 µg/ml), benzamidine (1 mM), trypsin inhibitor (10 µg/ml)). Cells were gently scraped from the plate and transferred into pre-cooled microcentrifuge tubes. Samples were centrifuged at 15000 RPM for 15mins at 4 degrees. Supernatant was transferred to new microcentrifuge tubes and was mixed 4:1 with SDS-PAGE sample buffer (0.125 Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 1.4 M β-mercaptoethanol and 0.0025% (w/v) bromophenol blue). All samples in sample buffer were boiled for 5min and either stored at -20°C or separated on SDS-PAGE gels.

2.2.4.4 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 1.5-3 h, depending on the size of the proteins being electrophoresised.

2.2.4.5 Immunoblotting

Following separation by electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membranes in a Hoefer TE 70 Semiphor semi-dry transfer unit at 110 mA for 2 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 minutes. 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 4 minutes each in TBS prior to incubation at 4°C overnight with the primary antibodies diluted in TBS containing 5% (w/v) skimmed milk powder. The membranes were subsequently subjected to 3 x 5 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 Odyssey Blocking Buffer (Licor, Bioscience) for 1 h at room temperature. The membranes were then washed a further 3 times for 4 min each in TBS. The immunoreactive bands were detected using Odyssey Infrared Imaging System from Licor Biosciences, according to the instructions of the manufacturer.

| 1 ^o Antibody | Dilution | 2 ^o Antibody* |
|-------------------------|----------|--------------------------|
| β-Actin | 1:1000 | mouse |
| MYC | 1:1000 | mouse |
| ECSIT | 1:1000 | rabbit |
| Flag | 1:500 | mouse |
| HA | 1:1000 | mouse |
| Phospho-p38 | 1:500 | rabbit |
| Phospho-p42/44 | 1:1000 | rabbit |
| Phospho-p65 | 1:1000 | rabbit |
| Phospho-JNK | 1:500 | rabbit |
| P38 | 1:1000 | rabbit |
| P42/44 | 1:1000 | rabbit |
| JNK | 1:1000 | rabbit |

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

2.2.4.6 Co-ImmunoPrecipitation (CoIPs)

HEK293 TLR4 cells were transfected with Lipofectamine 2000 as previously described in section 2.2.4.1 with equal amounts of potentially interacting proteins. Cell extracts were generated on ice. Cells were first washed with pre-chilled 1 x PBS (1 ml) then lysed with pre-chilled 500 μ l cell lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% (w/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 $^{\circ}$ C. Lysates were scraped into pre-chilled 1.5 ml microcentrifuge tubes and centrifuged at 12,000 g for 10 min at 4 $^{\circ}$ C. Supernatants were removed to fresh tubes (10% of sample was 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 $^{\circ}$ C. Samples were centrifuged at 1000 g for 5 min at 4 $^{\circ}$ C to pellet beads with non-specific protein and supernatants were removed to fresh pre-chilled tubes. Samples were incubated overnight with primary antibody (2 μ g). The following day Protein A/G agarose beads (20-30 μ l) were added to each sample and they were again incubated at 4 $^{\circ}$ C overnight. The subsequent day samples were centrifuged at 16,000 g for 1 minute and supernatant removed. The beads were washed with CoIP lysis buffer (500 μ l) and subjected to re-centrifugation. This step was repeated five times. The columns were moved to fresh tubes and 20-40 μ l of 2 x 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 columns for 30 minutes at RT. Samples were centrifuged at 16,000 g for 2 minutes, to pellet agarose beads. Supernatants were transferred to new

ependorf tubes and subsequently boiled at 100°C for 5-10 minutes and analyzed using SDS polyacrylamide gel electrophoresis and western blotting.

2.2.5 hECSIT Lentiviral shRNA infection and generation of stable cell lines

2.2.5.1 Lentiviral production

HEK293 T cells were seeded at 2×10^5 cells/ml in 6-well plates (2ml DMEM/well) and grown for 24 h to approximately 70% confluency. The cells were transfected as in section 2.2.4.1. The DNA mixture contained packaging plasmid (900 ng), envelop plasmid (100 ng) and the hECSIT-shRNA or control shRNA (1 μ g) for a total of 2 μ g DNA to be transfected. shRNA hECSIT sequence was as follows:

ShRNA hECSIT A7

5'-

CCGGGCCCTTTGAGTGTACAGCAAACCTCGAGTTTGCTGTACACTCAAAG
GGCTTTTTG -3'

A control shRNA 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 shRNA hECSIT and shRNA control plasmids were purchased from Sigma. 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 ~48 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 further 24 h. The virus was harvested one more time and after the final harvest the packaging cells were discarded.

2.2.5.2 Lentiviral infection

U373 cells were seeded at 2×10^5 cells/ml in 6-well plates (2 ml/well DMEM). The cells were left to recover overnight and then infected with 600 μ l of virus-containing conditioned media. Polybrene (8 μ g/ml) was added to improve transduction efficiency. The plates were incubated at 37°C. The media was removed 24 h post-infection and replaced with fresh growth media containing puromycin (5 μ g/ml) to select for cells transduced with shRNA. Cells were cultured for 4 weeks before suppression of hECSIT expression was determined. These stable cell lines were then used for Western Blot experiments.

2.2.6 Two-Dimensional Electrophoresis (2-DE)

2.2.6.1 Transfection and Co-IP of Samples

HEK293 T cells were seeded at 2×10^5 cells/ml in T175cm flasks (20ml/flask DMEM). This was done in five replicates to be pooled when harvested. Cells were transfected the next day with 50 μ g hEcsit or 50 μ g PcDNA3.1+ per flask. To transfect DNA, 50 μ g of DNA was added to 3mls of optiMEM, while 50 μ l of lipofectamine was added to 3mls of optiMEM. Both mixtures were left for 5 min and then gently mixed. After incubating for 20 min, this DNA-lipofectamine-optiMEM mix was carefully pipetted onto cells.

After 24 hr the media was removed, cells were washed in 5 ml of ice-cold PBS, and 2 ml of NP40 lysis buffer was added. Cells were left on rocker for 30 min at 4°C. Following this period, cells were removed from flask by gentle scraping and collected in pre-cooled microcentrifuge tubes. Cell debris was pelleted by spinning at 12000g for 10min at 4°C. The supernatant was then transferred to new microcentrifuge tubes and 4µl/ml of anti-c- MYC antibody was added to samples, and left for 24 hr. 10µl of A/G beads was then added per 1µg of antibody. The subsequent day samples were added to chilled spin columns (Cytosignal) and centrifuged at 16,000 g for 1 minute. The beads were washed with lysis buffer (as described in section 2.2.4.6) (500 µl) and subject to re-centrifugation. This step was repeated five times. The columns were moved to fresh tubes and 100µl of proteomic lysis buffer (7 M urea, 2M thiourea, 65 mM (3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane sulfonate) (CHAPS), 100 mM dithiothreitol (DTT) and 5% (v/v) ampholytes, 5 tablets/50ml of buffer Mini Complete) was added and incubated at RT for 1hr. Sample was eluted and collected in microcentrifuge tubes by a final centrifugation at 16000g for 1 min. To remove DNA, 2µl of DNAase-1 were added per 100µl of lysis buffer.

2.2.6.2 Bradford Assay

To ensure equal loading between samples protein content was quantified by Bradford assay determination. Protein content of BSA standards and cell extracts were measured by the method of Bradford (Bradford 1976). Standards and extracts (20 µl) were diluted in water and mixed with aliquots (180 µl) of Bradford protein reagent (Bio-Rad) by vortexing at room temperature. A blank was prepared using the same dilution of water: lysis buffer as the samples. Absorbance was measured for each sample in a 96-well plate at 590 nm using ELx800™ microplate reader with Gen5 Data

Analysis Software. Five serial dilutions of a BSA (500 µg/ml) protein standard were made and used to construct a standard curve which was subsequently used to determine protein concentration of the cell extracts.

2.2.6.3 In-gel rehydration.

In-gel rehydration in a reswelling tray from Amersham Biosciences/GE Healthcare (Little Chalfont, Bucks., UK) was used to load samples onto IPG strips. 300µg of sample was brought to a final volume of 450µl with rehydration buffer (7 M urea, 2 M thiourea, 65mM CHAPS, 100 mM DTT and 5% (v/v) ampholytes; containing 0.05% (w/v) bromophenol blue as tracking dye). Samples were gently pipetted into reswelling tray wells, with care taken to distribute samples evenly and without air bubbles. 24cm ph3-10 IPG strips were placed gel side down in the reswelling tray and left for 12 hr.

2.2.6.4 Isoelectric focussing (IEF)

After re-swelling, the IPGstrips were loaded gel side up in an Amersham Ettan IPGphor manifold and covered with 108 ml of cover fluid. The following protocol was used;

| Time | Voltage | Mode |
|-----------|---------|------|
| 21.30 hrs | 3500 | |
| 10m | 8000 | Grad |
| 4hr | 8000 | Step |
| 12hr | 100v | Hold |

2.2.6.5 Equilibration of IPG strips

Following isoelectric focusing, IPG strips were equilibrated twice for 20 minutes

using 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 100mM Tris-HCl, pH 8.8. The first incubation step was performed with the addition of 100mM DTT while the second incubation step was with 0.25 M iodoacetamide. The strips were then briefly washed in SDS running buffer (0.0125 M Tris, 0.96 M glycine, 0.1% (w/v) SDS) and placed on top of a 12.5% (w/v) SDS resolving gels and set using a 1% (w/v) agarose sealing gel.

2.2.6.6 Second Dimensional Electrophoresis

The gel electrophoretic separation in the second dimension was carried out by standard SDS-PAGE using an Amersham Ettan DALT-Twelve system (Doran *et al.*, 2006). Gels were electrophoresed at 1.5 W overnight until the bromophenol blue dye front had migrated off the gel.

2.2.6.7 Silver Staining

After electrophoresis, the gels were placed into fixing solution (30% ethanol, 10% acetic acid) for a minimum of 30 minutes. The gels were then rinsed in 20% ethanol twice for 10 minutes, which was followed by two 10 minute washes in milli-Q dH₂O. Sensitising solution (0.8mM sodium thiosulfate) was poured onto the gels for one minute after which the gels were once again washed in milli-Q dH₂O twice for two minutes. The staining solution (12 mM silver nitrate) was then left on the gels for 20 minutes to 2 hours. After the staining solution was removed and gels were washed in milli-Q dH₂O for 10 seconds the developing solution (3% sodium potassium carbonate, 250ml formalin, 125 ml 10% sodium thiosulfate) was added to the gel. Once the protein map was visualised the gel was placed into stopping solution (40g Tris, 2% actic acid) storing.

2.2.7 ESI LC/MS

2.2.7.1 Sample Preparation for Mass Spectrometry (MS)

The gel was first washed with deionised water (2 x 10 min) and the silver stained spots of interest were excised from the gels under water to prevent contamination and placed into siliconised 1.5 ml microcentrifuge tubes. Silver stain first had to be destained from sample. Equal amounts of 30mM potassium ferricyanide and 100mM sodium thiosulphate stocks were added together and 50µl added to each gel plug. Plugs were incubated in this solution until stain was removed and then washed 3-4 times in deionised water. 100µl of 200mM Ammonium Bicarbonate was added for 20mins at room temperature on shaker. Samples were centrifuged briefly and solution was removed. 70µl of 200mM Ammonium Bicarbonate/Acetonitrile 2:3 (v/v) was added to each tube and incubated at 37 degrees for 15 min while shaking. Again the samples were centrifuged briefly and solution was removed. 70µl of 50mM Ammonium Bicarbonate was added. After 5 min, an equal volume of acetonitrile was added. After 15 min of incubation all the liquid was removed and the gel plugs were then dehydrated in 100% acetonitrile. The acetonitrile was removed and the gel plugs were then dried down for 30 min using a Heto type vacuum centrifuge from Jouan Nordic A/S (Allerød Denmark). Individual gel plugs were then rehydrated in enough digestion buffer (1mg of trypsin in 20ml of 50mM Ammonium Bicarbonate) to cover the gel plugs. Additional digestion buffer was added if all the initial volume had been absorbed by the gel pieces. The samples were then incubated at 37°C over night. The peptides generated by tryptic digestion were recovered by removing supernatants from the digested gel plugs. Further recovery was achieved by adding 30% acetonitrile/ 0.2% trifluoroacetic acid to the gel plugs for 10 min at 37°C with gentle agitation. The resulting supernatants were added

to the initial peptide recovery following trypsin digestion. The sample volume was reduced until dry through vacuum centrifugation. Samples were resuspended in 15 ml of ultrapure ddH₂O and 0.1% formic acid for identification by ion trap LC/MS (Liquid Chromatography/ Mass Spectrometry) analysis.

2.2.7.2 Ion Trap Mass Spectrometry

The mass spectrometric analysis of peptides was carried out in the Proteomics Suite of the National University of Ireland, Maynooth with a Model 6340 Ion Trap LC/MS apparatus from Agilent Technologies (Santa Clara, CA). Excision, washing, destaining and treatment with trypsin was performed by the above optimised method. Separation of peptides was performed with a nanoflow Agilent 1200 series system, equipped with a Zorbax 300SB C18 5mm, 4mm, 40 nl 71 precolumn and a Zorbax 300SB C18 5 mm, 43mm x 75mm analytical reversed phase column using HPLC-Chip technology (Staples *et al.*, 2009). The mobile phases utilised were A: 0.1% formic acid, B: 50% acetonitrile and 0.1% formic acid. Samples (5ml) were loaded into the enrichment column at a capillary flow rate set to 4ml/min with a mix of A and B at a ratio of 19:1 (v/v). Tryptic peptides were eluted with a linear gradient of 10-90% solvent B over 15 min with a constant nano pump flow rate of 0.60ml/min. At 1 min post time of solvent A was used to remove sample carry over. The capillary voltage was set to 2000 V and the flow and temperature of the drying gas were 4ml/min and 300°C, respectively. For protein identification, database searches were carried out with Mascot MS/MS Ion search (Matrix Science, London, UK).

3. Results

3.1 Introduction

Elucidating the signalling pathways propagated from cell surface receptors presents a major research challenge. However understanding of how these signalling events lead to physiological effects is essential for discovering how these pathways are altered in pathological conditions and developing appropriate therapeutics . The innate immune system acts as the first line of defence for the body, acting to sense and eradicate invading microbes. It is mediated by cells such as monocytes, macrophages, neutrophils and dendritic cells, which act to induce local and systematic inflammation, phagocytose microbes and initiate and modulate the adaptive immune system . The immune system is a double edged sword in that it is essential for eradicating infection but also underlies inflammatory diseases and thus must be tightly regulated .

Cells of the innate immune system sense pathogens through pathogen recognition receptors (PPRs). TLRs are the most characterised class of PPRs and can produce proinflammatory and anti-viral responses. Signalling through TLRs, TNFR1 and IL-IR converge on the activation of the kinase TAK1 which phosphorylates and activates IKK proteins. IKK proteins can phosphorylate I κ B, leading to I κ B degradation and the release of the proinflammatory transcription factor NF κ B to induce changes in gene expression. TAK1 activation simultaneously leads to the activation of MAPK proteins by activating both MAPKKK and MAPKK proteins. The MAPKs are involved in the induction of gene expression, as well as playing roles in the regulation of mRNA stability. Detection of viral particles by TLRs mediates an anti-viral interferon response through the activation of IRF transcription factors, NF κ B and MAPKs . Since the discovery of TLRs in the 1990s, deciphering of TLR signalling has been the focus of many research groups. Indeed it has been

acknowledged that signalling by TLRs does not induce simple linear pathways but that they integrate into complex signalling networks that ultimately control the physiological response.

Some of the earliest work on TLRs led to the discovery of the protein ECSIT. It was discovered in 1999 by Kopp *et al.*, which identified it as a TRAF6 interaction protein through a yeast two hybrid screen. Murine ECSIT (mECSIT) was found to enhance NFκB responses and lead to MEKK1 modulation. Since then, mECSIT has been characterised as a mitochondrial localised protein, involved in mitochondrial complex assembly and mitochondrial signalling. Knockout of mECSIT results in embryonic lethality, which led to the discovery that mECSIT was involved in BMP signalling. The Moynagh Lab is concerned with the human orthologue of ECSIT, hECSIT, which was cloned by members of this lab. To date no functional data on hECSIT has been published. However extensive research in the Moynagh Lab has characterised hECSIT as a negative regulator of NFκB activation. My project focussed on the ability of hECSIT to regulate the MAPK pathways and to explore the mechanistic basis to any effects of ECSIT on the MAPK pathways.

3.2 Results

3.2.1 hECSIT and mECSIT display differential effects on NFκB activation.

Kopp *et al.*, (1999) characterised mECSIT as a positive intermediate of NFκB activation; it can induce NFκB activation and enhances NFκB activation in response to IL-1β and LPS signalling. The present study compared the efficacies of the hECSIT and mECSIT in

activating NFκB as measured by induction of a NFκB-dependent reporter gene. HEK 293 TLR4 cells were transiently transfected with hECSIT or mECSIT expression constructs and assayed for their ability to induce the expression of a co-transfected NFκB-regulated firefly luciferase gene (Figure 3.1). As shown by Kopp *et al.*, (1999), mECSIT induces NFκB activation. In contrast hECSIT fails to up-regulate NFκB activation. Following IL-1β and LPS stimulation, mECSIT further enhances the activation of NF-κB. hECSIT however does not up-regulate IL-1β and LPS induced NFκB activation and displays slight inhibitory effects on the ability of IL-1 to activate NFκB.

3.2.2 Numerous forms of hECSIT can be detected by Mass Spectromerty.

Given that the human form of ECSIT fails to mimic the functional capacity of its murine equivalent, we were keen to study the human form in more detail with a view to providing some understanding of its function. With this in mind we applied a proteomics-based approach to identify ECSIT-interacting proteins that may provide clues to its function. HEK 293 T cells were transiently transfected with or without a MYC-tagged hECSIT expression construct. The following day cell lysates were generated and immunoprecipitated using an anti-MYC antibody. Western blotting was used to confirm the presence of MYC-tagged ECSIT in the immunoprecipitated samples (Figure 3.2). Immunoprecipitates were then subjected to two-dimensional gel electrophoresis and stained with silver stain solution (Figure 3.3). 8 proteins spots, unique to samples overexpressing hECSIT, were cut from the gels and identified by mass spectrometry (Table 3.1). Five of the spots were identified as hECSIT based on mass spectrometry and MASCOT analysis, indicating the presence of various modified forms of hECSIT.

Furthermore the probing of the 2-D gels with an anti-hECSIT antibody confirmed the presence of hECSIT at those spots previously identified as hECSIT by Ion-trap Mass Spectrometry (Figure 3.4). 3 other proteins were identified by mass spectrometry, they were dystroglycan, leukotriene C4 synthase and Zinc finger protein 419. Two of these proteins, dystroglycan and leukotriene C4 synthase have been characterised previously. In skeletal muscle dystroglycan forms part of large complex that functions to link the extracellular matrix to the cytoskeleton. While it is expressed in tissues other than muscle, its function in them is not fully known . Leukotriene C4 synthase is an enzyme that converts leukotriene A4 and glutathione into leukotriene C4, a step in the production of leukotrienes from arachidonic acid . Leukotrienes are critical to the inflammatory response, acting on cell surface receptors to promote bronchoconstriction, increase vascular permability and cell adherence amongst other roles . In contrast to dystroglycan and leukotriene C4 synthase, there was minimal information available on the function of ZNF419 . ZNF419 has been characterised in terms of potential domains, and contains a zinc finger domain. Zinc finger domains are present in several key TLR proteins and therefore it was decided to further probe the interaction of hECSIT and ZNF419 and the role of ZNF419 in TLR signalling ; Jaaflela *et al.*, 1996; Minoda *et al.*, 2006; Wallis *et al.*, 2003).

3.2.3 hECSIT fails to co-immunoprecipitate with ZNF419.

ZNF419 was identified by Ion-trap Mass Spectrometry as a hECSIT interacting protein. It was selected for further analysis as zinc fingers domains have been shown to be important in TLR signalling proteins. We aimed to confirm this interaction by assessing if hECSIT-MYC co-immunoprecipitates with ZNF419. We initially planned to study the interaction of hECSIT with endogenous ZNF419

but the commercially available anti-ZNF419 failed to detect ZNF419 (at its molecular weight of 59kDA) even when cells were transfected with an expression construct encoding ZNF419 (Figure 3.5). Therefore hECSIT was next examined for its ability to interact with overexpressed ZNF419 that was FLAG tagged. HEK 293 cells were transiently co-transfected with ZNF419-FLAG constructs, with or without MYC-tagged hECSIT construct. The following day cell lysates were generated and immunoprecipitated using anti-MYC antibody. Immunoprecipitates were then analysed for protein interaction by Western immunoblotting using FLAG antibody. Whilst immunoprecipitation of hECSIT was confirmed by anti-MYC blotting, ZNF419 was not detectable in the hECSIT-immunoprecipitated samples (Figure 3.6). Immunoblotting with an anti-HA antibody confirmed overexpression of ZNF419.

3.2.4 ZNF419 does not affect NF κ B activation.

Although we failed to confirm interaction of ZNF419 with hECSIT, we were keen to assess if the former could regulate pathways such as NF κ B that have been associated with ECSIT. Furthermore zinc finger containing proteins are known to regulate several aspects of TLR signalling (Jaaflela *et al.*, 1996; Minoda *et al.*, 2006) and thus we probed if ZNF419 could regulate NF κ B signalling. HEK 293 TLR4 cells were transiently transfected with or without ZNF419-FLAG expression constructs and assayed for their ability to regulate the expression of a co-transfected NF κ B-dependent reporter gene. ZNF419 failed to activate NF κ B in this assay system and had no affect on the ability of LPS, IL-1 β or TNF α to activate NF κ B (Figure 3.7).

3.2.5 Knockdown of endogenous hECSIT by lentiviral encoding shRNA constructs decreases the phosphorylation of SMAD1-5-8 while increasing the phosphorylation of p42/44 at basal levels.

Given that hECSIT fails to mimic mECSIT in activating NFκB and our proteomics-based screen did not reveal novel ECSIT-interacting proteins we next probed the potential role of hECSIT as a regulator of other pathways that are associated with mECSIT. Xiao *et al.*, (2003) reported that knockout of ECSIT in murine models resulted in embryonic lethality and a phenotype that was very similar to loss of BMP1R. Further investigation revealed mECSIT was critical to BMP signalling and was essential for mesoderm formation during embryonic development. We thus assessed the function of hECSIT in BMP4 signalling. BMP4 signals through the BMPR1 receptor and propagates signalling through SMAD proteins, SMAD 1, SMAD 5, and SMAD8 (SMAD 1-5-8) . In order to examine the role of hECSIT in the BMPR1 pathway, the endogenous expression of hECSIT was suppressed using lentiviral-deliver hECSIT specific shRNAs and examined for effects on activation of SMAD 1-5-8 following BMP4 stimulation. A549 cells are human adencarcinomic alveolar basal epithelial cells, they show increased activation of SMAD 1-5-8 in response to BMP4 stimulation and thus were chosen as the cell line for the purpose of this study. Lentivirus was initially produced in HEK 293 T cells by co-transfecting packaging and envelope expression constructs with shRNA vectors encoding hECSIT-specific shRNA or control shRNA (that doesn't target any human gene). Media containing lentivirus was collected 48h post-transfection and then used to infect A549 cells in the presence of polybrene (8µg/ml) to improve infection efficiency. Virus was removed 24h post-infection, cells were then cultured in the presence of a selective reagent puromycin (5µg/ml) to ensure stable integration of shRNA was obtained. To confirm selective knockdown of

endogenous hECSIT shRNA, cell lysates were generated and subjected to Western immunoblotting using ECSIT-specific antibodies. hECSIT shRNA clone A7 showed very strong knockdown of hECSIT protein expression while control shRNA had no effect on hECSIT expression (Figure 3.9). The A549 cell lines with stably integrated shRNA constructs were stimulated with BMP4 for various time periods from 0', 15', 45' up to 180' min. Cell extracts were then generated and probed for phosphorylation of SMAD 1-5-8 (Figure 3.9). BMP4 induced phosphorylation of SMAD 1-5-8 at 15min and peaked at 45' min in cells that had been transduced with lentivirus containing control shRNA. Knockdown of hECSIT expression with hECSIT-specific shRNA greatly reduced Smad-induced phosphorylation by BMP-4. These data suggest that hECSIT mimics the role of its murine counterpart in the BMPRI pathway. In order to assess if this role is specific for regulation of Smad phosphorylation we also probed the role of hECSIT in SMAD-independent pathways that are also triggered by BMPRI. BMP stimulation can also activate SMAD-independent pathways, of which p42/44 MAPK is a known target (Li *et al.*, 2012; Zhou *et al.*, 2007). BMP4 promoted low level phosphorylation of p42/44 in cells transduced with control shRNA. Increased phosphorylation was apparent at 15' min post BMP4 stimulation and declined to basal levels by 180' min. However suppression of hECSIT caused strong increases in the basal and BMP4-induced levels of phosphorylated p42/44 (Figure 3.8). All samples displayed equivalent levels of β -actin demonstrating that the changes in SMAD 1-5-8 and p42/44 phosphorylation were not due to non-specific changes in general protein expression.

3.2.6 hECSIT fails to interact with SMAD4.

Xiao *et al.*, (2003) showed that mECSIT could interact with SMAD4 to regulate its function. Given that the above findings indicated that hECSIT may be able to regulate Smad phosphorylation, I assessed the interaction of hECSIT and SMAD4. HEK 293 cells were transiently co-transfected with HA-tagged SMAD4 with or without MYC-tagged hECSIT construct. The following day cell lysates were generated and immunoprecipitated using anti-MYC antibody. Immunoprecipitates were then analysed for the presence of SMAD4 by Western immunoblotting using anti-HA antibody. Unlike its murine form, hECSIT shows no interaction with SMAD4 (Figure 3.9). The expression of MYC-hECSIT and HA-SMAD4 were confirmed by probing WCL with anti-MYC and anti-HA antibodies respectively. The immunoprecipitation of MYC-hECSIT was confirmed by probing co-immunoprecipitation samples with an anti-MYC antibody.

3.2.7 Knockdown of endogenous hECSIT by lentiviral encoding shRNA constructs enhances phosphorylation of p42/44.

Since suppression of hECSIT resulted in strong induction p42/44 phosphorylation in response to BMP4, we next examined if hECSIT could regulate p42/44 phosphorylation in TLR signalling pathways. A549 stable cell lines were again transduced with lentiviral particles containing hECSIT-specific shRNA and stable cell lines were generated showing suppressed expression of hECSIT. Cells were treated with LPS for various time periods, cell extracts generated and subjected to Western immunoblotting. LPS promoted increased phosphorylation of p42/44 in cells transduced with control shRNA. Suppression of hECSIT resulted in greatly enhanced phosphorylation of basal p42/44 phosphorylation that was not further augmented by LPS (Figure 3.10). This is comparable with results obtained following

BMP4 signalling (Figure 3.8). From these results it appears hECSIT plays a role in controlling basal phosphorylation of p42/44.

3.2.8 hECSIT and mECSIT display differential effects on the activation of ELK-1.

Given that hECSIT can regulate basal phosphorylation of p42/44 we next probed the downstream functional consequences of such regulation. ELK-1 is a transcription factor involved in the induction of genes such as c-fos which forms vital part of AP-1 complex. It can be activated and phosphorylated by p42/44 and also by p38 and JNK . HEK 293 TLR4 cells were co-transfected with pFA-ELK-1 trans-activator plasmid in conjunction with pFR-luciferase construct with or without hECSIT or mECSIT plasmid constructs. The following day cells were treated with IL-1 β or LPS for 6hr. Cell lysates were then generated and assayed for ELK-1 regulated luciferase activity. Similar to regulation of NF- κ B, hECSIT and mECSIT display opposing effects on activation of ELK-1 (Figure 3.11). Neither hECSIT or mECSIT induce ELK-1 activation. However mECSIT enhances ELK-1 activation following IL-1 β and LPS treatment. In contrast hECSIT displays marginal inhibitory effects on IL-1 β and LPS-induced activation of ELK-1. The inhibitory effects of hECSIT on ELK-1 are consistent with the inhibitory effects of hECSIT on upstream p42/44 .

3.2.9 hECSIT and mECSIT display differential effects on AP-1 activation.

A key consequence of ELK-1 activation is the induction of c-fos expression, an Ap-1 subunit. AP-1 is key transcription factor induced by MAPK to mediate innate immune responses. It is a dimeric protein composed primarily of c-fos and c-jun proteins . HEK 293 TLR4 cells were transiently transfected with hECSIT or mECSIT

expression constructs with or without a MyD88 expression construct and assayed for their ability to induce the expression of a co-transfected AP-1- dependent reporter gene. The following day cell lysates were generated and assayed for AP-1 regulated luciferase activity. hECSIT and mECSIT displayed opposing effects on activation of AP-1 (Figure 3.12). mECSIT enhances MyD88 induced AP-1 activation, while in contrast hECSIT displays inhibitory effects on MyD88 induced activation of AP-1.

3.2.10 Overexpression of hECSIT decreases MEKK1 induced phosphorylation of p42/44.

The luciferase assays detailed above indicate that overexpression of hECSIT has an inhibitory effect on the activation of p42/44. Furthermore Kopp *et al.*, (1999) reported that mECSIT was involved in processing of MEKK1. MEKK1 is a MAP kinase kinase kinase that is an upstream regulator of all 3 classes of MAPKs including p42/44. Thus hECSIT was investigated for its potential to regulate MEKK1 activation of the various MAPKs. HEK 293 cells were co-transfected with MEKK1-encoding construct and increasing amounts of hECSIT-encoding constructs. The following day cell lysates were generated and subjected to western immunoblotting using antibodies against the phosphorylated and total forms of p42/44, p38 and JNK. The overexpression of MEKK1 promoted increased phosphorylation of all 3 MAPKs. Intriguingly the co-expression of hECSIT strongly inhibited MEKK1-induced phosphorylation of p42/44 whilst leaving the levels of phosphorylated p38 and JNK relatively unaffected (Figure 3.13). This data indicates a strong degree of specificity for hECSIT in regulating the p42/44 pathway.

3.2.11 Suppression of endogenous hECSIT by lentiviral encoding shRNA constructs increases the phosphorylation of p42/44.

Given the strong effect of shRNA-mediated knockdown of hECSIT on phosphorylation of p42/44 in A549 cells we were eager to show that this effect was not an artefact of the A549 cell line and thus we performed similar shRNA knockdown studies in U373 cells. The latter are brain astrocytic cells that respond to LPS, IL-1 β and TNF α . U373 cells stably expressing hECSIT specific shRNA constructs were produced as described in section 3.2.5. The U373 cell lines with stably integrated shRNA constructs were stimulated with LPS for various time periods. Cell extracts were then generated and probed for the phosphorylation of p42/44, p38 and JNK. Knockdown of hECSIT with shRNA increased the basal levels of phosphorylated p42/44 and this was further augmented by LPS (Figure 3.14). In control samples, the levels of p42/44 phosphorylation decrease to near basal levels by 180' minutes, however when hECSIT levels are suppressed the levels of p42/44 phosphorylation remain elevated. The phosphorylation of p38 and pJNK are comparable between control shRNA and hECSIT shRNA. All samples displayed equivalent levels of total p42/44, p38, JNK and β -actin demonstrating that the changes in p42/44 phosphorylation were not due to non-specific changes in general protein expression.

The effect of hECSIT suppression on p42/44 phosphorylation in response to IL-1 β (Figure 3.15) and TNF α (Figure 3.16) signalling was also determined. As with LPS stimulation, the levels of p42/44 phosphorylation are greatly enhanced when hECSIT is suppressed, while there is no significant effect on the phosphorylation of p38 and JNK. β -actin levels are consistent throughout the samples demonstrating that the changes in p42/44 phosphorylation were not due to changes in protein levels.

3.2.12 Suppression of endogenous hECSIT by siRNA increases the phosphorylation of p42/44.

Given the strong effect of shRNA-mediated knockdown of hECSIT on phosphorylation of p42/44 in A549 and U373 cells we were keen to show that this effect was not an artefact of the shRNA knockdown approach, we thus complemented this approach by using siRNA-mediated knockdown of hECSIT in U373 cells. U373 cells were transfected with Lamin a/c control siRNA or hECSIT-specific siRNA. 48hrs post transfection cells were treated with LPS at various time points from 0', 5', 10', 30', 60' up to 180' mins. Cells lysates were extracted and subjected to western blotting and probed with antibodies against the phosphorylated and total forms of p42/44 and p38. In control siRNA-transfected cells, basal levels of p42/44 phosphorylation are low and peak at 60 min post LPS stimulation. Transfection of cells with hECSIT-specific siRNA caused strong suppression of hECSIT expression and this led to greatly increased basal levels of p42/44 that were further enhanced by LPS (Figure 3.17). The phosphorylation of p38 is comparable between control siRNA and hECSIT siRNA again indicating that hECSIT specifically targets the p42/44 pathway. Total levels of p42/44 and p38 remain constant across the samples, while all samples displayed equivalent levels of β -actin demonstrating that the changes in p42/44 phosphorylation were not due to non-specific changes in general protein expression.

3.2.13 hECSIT does not affect the processing of p105 to p50.

While p38, JNK and p42/44 are members of the same family of proteins, they have distinct roles and are activated differentially in

response to different ligands. They differ in the upstream kinases that can activate them, with each having distinct MAPKKK and MAPKK that can solely or preferentially activate them. Tpl2 has been shown to be critical to the activation of p42/44 but not other MAPKs (Rousseau *et al.*, 2005). As hECSIT specifically targets the regulation of phosphorylation of p42/44 but not that of p38 and JNK, I postulated that hECSIT could be acting at the level of Tpl2. Tpl2 stability depends on its interaction with p105, while partial degradation of p105 into the p50 form is necessary for Tpl2 signalling. I investigated if hECSIT regulated the levels of p105 or its processing in response to LPS signalling (Beinke *et al.*, 2003). Stable hECSIT knockdown U373 cell lines were generated as before using hECSIT specific shRNA. Cells were treated with LPS at various timepoints. Following stimulation, cell lysates were generated and subjected to western blotting. They were probed with anti-p105/p50 antibody. The levels and degradation of p105 over the indicated timecourse were comparable between control and hECSIT shRNA samples, implying hECSIT does not affect the processing of p105 (Figure 3.18). Suppression of hECSIT results in increased p42/44 phosphorylation as shown previously.

3.2.14 hECSIT does not interact with TPL2

Tpl2 has been shown to form a signalling complex with p105 and ABIN2 which regulates the stability and activation of Tpl2. We hypothesised that hECSIT may exert its affect on p42/44 phosphorylation by interacting with Tpl2. I investigated this possible interaction. HEK 293 cells were transiently co-transfected with HA-tagged Tpl2 with or without MYC-tagged hECSIT construct. The following day cell lysates were generated and immunoprecipitated using anti-MYC antibody. Immunoprecipitates were then analysed

for protein interaction by Western immunoblotting using anti-HA antibody. The expression of the plasmids was confirmed by probing whole cell lysates with anti-HA antibody and anti-MYC antibody, while the immunoprecipitation of MYC-tagged hECSIT was confirmed by probing immunoprecipitates with anti-MYC antibody. hECSIT showed no interaction with Tpl2 (Figure 3.19).

3.3 Figures

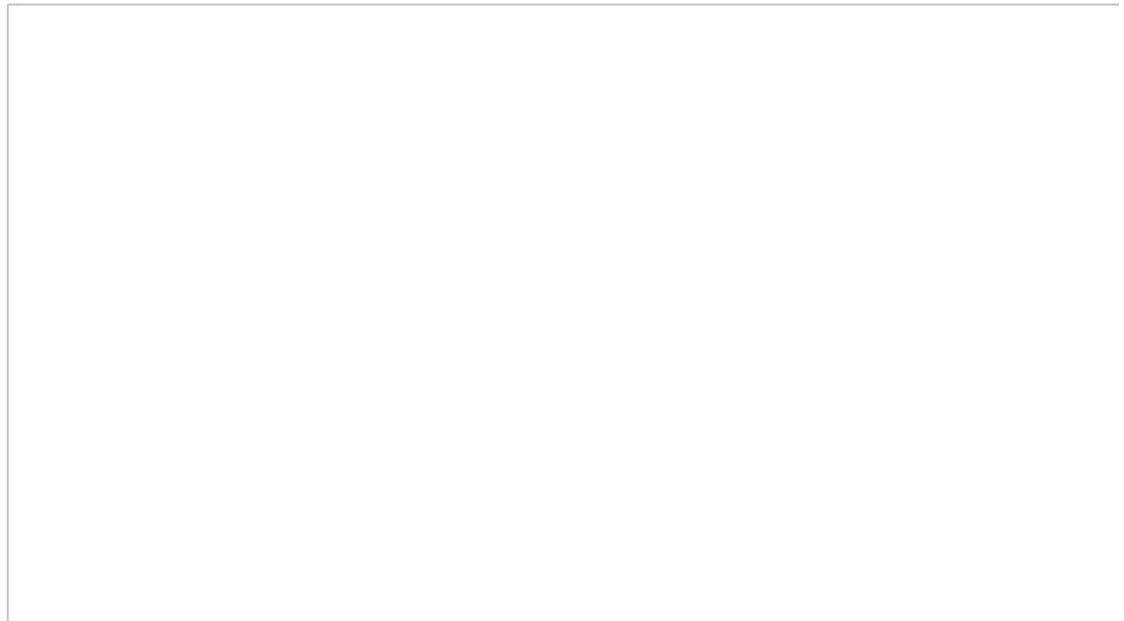


Figure 3.1 hECSIT and mECSIT display differential effects on IL-1 β and LPS-induced NF- κ B activation.

HEK293 TLR4 cells were co-transfected with NF κ B firefly luciferase reporter construct (80ng), TK Renilla (20ng) and hECSIT or mECSIT (100ng). Empty Vector (EV) pcDNA3.1 was used to normalise total amount of DNA. The following day cells were treated with either IL-1 β (10ng/ml) or LPS (100ng/ml) for 6h. Cell lysates were then generated and assayed for firefly and Renilla luciferase activity. Results represent mean +/- SD of triplicate determinations and is a representative of three independent experiments.

Figure 3.2 Confirmation of immunoprecipitation of hECSIT-MYC.

HEK 293 T cells were transfected with 1ug hECSIT-MYC or control EV PcDNA (CTRL). The following day cell lysates were harvested and subjected to immunoprecipitation with anti-MYC antibody. To confirm the specific immunoprecipitation of hECSIT-MYC, samples were subjected to SDS-PAGE separation and western blotting with anti-MYC antibody.

A



B

Figure 3.3 2-D electrophoresis of proteins co-immunoprecipitating with hECSIT

HEK 293 T cells were transfected with 1 μ g hECSIT-MYC (A) or control EV PcDNA (B). The following day cell lysates were harvested and subjected to immunoprecipitation with anti-MYC antibody. Protein concentration was measured by Bradford assay and equal amounts of coimmunoprecipitates (100 μ g) were separated in the first dimension by isoelectric focusing on pH 3-10 18mm IEF strips and in the second dimension by SDS-PAGE. Proteins were visualised by silver staining.

| Spot # | Name | Gene ID | Gene Description |
|--------|--------|---------|---|
| 1 | ECSIT | 51295 | ECSIT homolog (<i>Drosophila</i>) |
| 2 | ECSIT | 51295 | ECSIT homolog (<i>Drosophila</i>) |
| 3 | ECSIT | 51295 | ECSIT homolog (<i>Drosophila</i>) |
| 4 | ECSIT | 51295 | ECSIT homolog (<i>Drosophila</i>) |
| 5 | ECSIT | 51295 | ECSIT homolog (<i>Drosophila</i>) |
| 6 | DAG1 | 1605 | dystroglycan (dystrophin-associated glycoprotein 1) 1 |
| 7 | LTC4 | 4056 | leukotriene C4 synthase |
| 8 | ZNF419 | 79744 | Zinc Finger Protein 419 |

Table 3.1 Ion-trap Mass-spectrometry identified proteins that interact with hECSIT. Criteria thresholds were lowered to minimum criteria to achieve maximum peptide hits. Any identified proteins were then validated by molecular means to determine the results.

Figure 3.4 hECSIT undergoes various forms of modification

HEK 293 T cells were transfected with 1 μ g hECSIT-MYC. The following day cell lysates were harvested and subjected to immunoprecipitation with anti-MYC antibody. Samples were separated in the first dimension by isoelectric focusing on pH 3-10 18mm IEF strips and in the second dimension by SDS-PAGE and subsequently subjected to western blotting with anti-hECSIT antibody.

Figure 3.5 Anti-ZNF419 fails to detect overexpressed ZNF419

HEK293 cells were transfected with increasing amounts of plasmid encoding ZNF419 (0.5, 0.8, 1, 1.5 and 2 μ g). The empty vector (EV) pcDNA3.1 was used as a negative control. Cell lysates were generated the following day and subjected to SDS-PAGE separation. Western blotting was performed probing with anti-ZNF419 antibody. β -actin was used as a loading control.

Figure 3.6 ZNF419 fails to co-immunoprecipitate with hECSIT

Hek 293 T cells were transfected with 1 μ g hECSIT-MYC and/or 1 μ g ZNF419-FLAG as indicated. DNA levels were normalised with empty vector pcDNA3.1. Lysates were harvested 24 h post-transfection and immunoprecipitated with an immobilised anti-MYC antibody. Immunoprecipitates were subjected to polyacrylamide gel electrophoresis and subsequently to Western immunoblotting using an anti-FLAG antibody. They were probed with anti-MYC antibody to confirm the co-immunoprecipitation. Whole cell lysates (WCL) were also analysed by Western blotting with anti-MYC and anti-FLAG antibody to confirm expression of plasmids. Immunoprecipitation blot shown above is representative of two independent experiments.

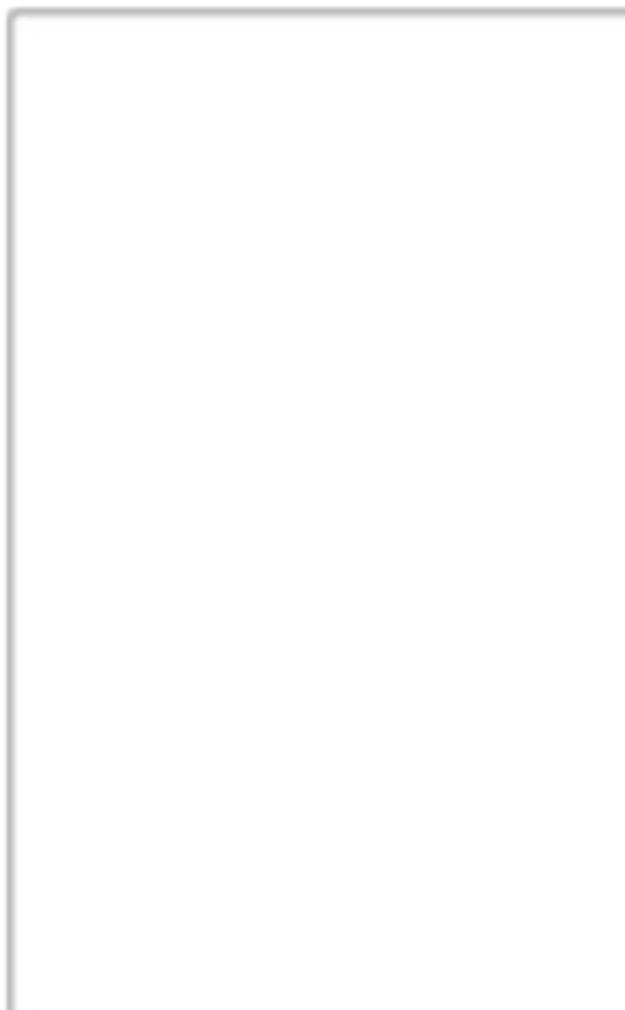


Figure 3.7 ZNF419 does not affect LPS, IL-1 β or TNF α induced activation of NF κ B.

HEK293 TLR4 cells were transfected with NF κ B-luciferase (80ng), TK renilla (20ng) with or without ZNF419-FLAG (100ng). EV PcDNA3.1 was used to equalise DNA concentration, while TK renilla was used to normalise for transfection efficiency. The following day cells were treated with LPS (100ng/ml), IL-1 β (10ng/ml) or TNF α (50ng/ml) and harvested after 6h. Cells lysates were assayed for firefly luciferase and TK Renilla luciferase. Results represent mean \pm SD of triplicate determinations and is a representative of two independent experiments.

Figure 3.8 Knockdown of endogenous hECSIT in the A549 cell line decreases the phosphorylation of receptor regulated SMAD proteins (SMAD1-5-8) and enhances the phosphorylation of p42/44 following BMP-4 stimulation.

A549 cells were transduced with hECSIT-specific lentiviral shRNA or Control shRNA. Cells were cultured in the presence of selective reagent puromycin (5µg/ml). Knockdown of endogenous hECSIT was confirmed by immunoblotting. Once achieved, the cells were then treated with BMP-4 (50ng/ml) for various time points (15, 45 and 180min). Cell lysates were then generated and subjected to SDS-PAGE and subsequently to Western immunoblotting with antibodies against phosphorylated SMAD 1-5-8 (p-SMAD),

phosphorylated P42/44 (p-P42/44) and ECSIT. β -actin was used as a loading control. These results are representative of two independent experiments.

Figure 3.9 Smad4 fails to co-immunoprecipitate with hECSIT

HEK 293 T cells were transfected with 1 μ g hECSIT-MYC and/or 1 μ g Smad4-HA as indicated. DNA levels were normalised with empty vector pcDNA3.1. Lysates were harvested 24 h post-transfection and immunoprecipitated with an immobilised anti-MYC antibody. Immunoprecipitates were subjected to polyacrylamide gel electrophoresis and subsequently to Western immunoblotting using an anti-HA antibody. They were subsequently probed with anti-MYC

antibody to confirm the co-immunoprecipitation. Cell lysates were also analysed by Western immunoblotting to confirm expression of the constructs. Immunoprecipitation blot shown above is representative of two independent experiments.

Figure 3.10 Knockdown of endogenous hECSIT in the A549 cell line results in increased phosphorylation of p42/44 following LPS stimulation.

A549 cells were transduced with hECSIT-specific lentiviral shRNA or Control shRNA. Cells were cultured in the presence of selective reagent puromycin (5µg/ml). Knockdown of endogenous hECSIT was confirmed by immunoblotting. Once achieved, the cells were then treated with LPS (100ng/ml) for various time points (20, 60, 180min). Cell lysates were then generated and subjected to

SDS-PAGE and subsequently to Western immunoblotting with antibodies against phosphorylated P42/44 (p-P42/44). hECSIT knockdown was confirmed by probing with anti-hECSIT antibody. β -actin was used as a loading control. These results are representative of two independent experiments.

Figure 3.11 Differential effects of hECSIT and mECSIT on IL-1 β and LPS induced ELK-1 activation.

HEK293 TLR4 cells were transfected with ELK-1-gal4 (30ng), the gal4 responsive promoter pFR-ELK-1 luciferase (80ng), tk renilla (20ng) and either hECSIT or mECSIT (100ng). EV PcDNA3.1 was

used to equalise DNA concentration, while tk renilla was used to normalise transfection efficiency. The following day cells were treated with or without IL-1 β (10ng/ml) or LPS (100ng/ml) and harvested after 6h. Cells lysates were assayed for firefly luciferase and TK Renilla luciferase. Results represent mean +/- SD of triplicate determinations and is a representative of two independent experiments.

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Figure 3.12 Differential effects of hECSIT and mECSIT on MYd88 induced activation of AP-1.

Hek293 T cells were cotransfected with ap-1 luciferase (80ng), tk renilla (20ng), hECSIT or mECSIT (100ng), and Myd88 (50ng). PcDNA was used to normalise DNA concentration, while TK renilla was used to normalise tranfection efficiency. Cells were harvested the following day and lysates were assayed for firefly luciferase and TK renilla. Results represent mean +/- SD of triplicate determinations and is a representative of two independent experiments.

EV MEKK1

Figure 3.13 hECSIT inhibits the MEKK1 induced phosphorylation of p42/44 but not p38 and JNK.

HEK293 cells were transfected with various amounts of plasmids encoding hECSIT (0.5, 1, and 1.5 μ g), and MEKK1 (.5 μ g). The empty vector (EV) pcDNA3.1 was used as a negative control. Cell lysates were generated the following day and subjected to SDS-PAGE. Levels of phosphorylated p38 (p-p38), p42/44 (p-p42/44) and JNK (p-JNK) and total p38, p42/44 and JNK were assessed by Western immunoblotting. β -actin was used to measure total protein levels in samples.

Figure 3.14 Knockdown of endogenous hECSIT in the U373 cell line enhances the phosphorylation of p42/44 following LPS stimulation.

U373 cells were polyclonally transduced with hECSIT-specific lentiviral shRNA or Control shRNA. Cells were cultured in the presence of selective reagent puromycin (5µg/ml). Knockdown of endogenous hECSIT was checked by immunoblotting. Once achieved, the cells were then treated with LPS (100ng/ml) for various time points (5,10, 30, 60, 180mins). Cell lysates were then generated and subjected to SDS-PAGE. Levels of phosphorylated p38 (p-p38), p42/44 (p-p42/44) and JNK (p-JNK) and total p38, p42/44 and JNK were assessed by Western β-actin was used as a loading control. These results are representative of two independent experiments.

Figure 3.15 Knockdown of endogenous hECSIT in the U373 cell line enhances phosphorylation of p42/44 following IL-1 β stimulation.

U373 cells were polyclonally transduced with hECSIT-specific lentiviral shRNA or Control shRNA. Cells were cultured in the presence of selective reagent puromycin (5 μ g/ml). Knockdown of endogenous hECSIT was checked by immunoblotting. Once achieved, the cells were then treated with IL-1 β (10ng/ml) for

various time points (5,10, 30, 60, 180mins). Cell lysates were then generated and subjected to SDS-PAGE. Levels of phosphorylated p38 (p-p38), p42/44 (p-p42/44) and JNK (p-JNK) and total p38, p42/44 and JNK were assessed by Western immunoblotting β -actin was used as a loading control. These results are representative of two independent experiments.

Figure 3.16 Knockdown of endogenous hECSIT in the U373 cell line enhances phosphorylation of p42/44 following TNF- α stimulation.

U373 cells were polyclonally transduced with hECSIT-specific lentiviral shRNA or Control shRNA. Cells were cultured in the presence of selective reagent puromycin (5 μ g/ml). Knockdown of

endogenous hECSIT was checked by immunoblotting. Once achieved, the cells were then treated with TNF- α (50ng/ml) for various time points (5,10, 30, 60, 180mins). Cell lysates were then generated and subjected to SDS-PAGE. Levels of phospho-p38, phospho-p42/44, phospho-JNK and total p42/44, were assessed by Western immunoblotting. β -actin was used as a loading control. These results are representative of two independent experiments.

Figure 3.17 Knockdown of hECSIT with siRNA augments IL-1 β induced phosphorylation of p42/44.

U373 cells were transfected with hECSIT siRNA (10nm). Cells were harvested 48hrs later in RIPA buffer. 3 hrs before harvesting cells were treated with IL-1 β at indicated times (5, 10, 30, 60 and 180 mins). Whole cell extracts were generated and subjected to SDS-PAGE. Levels of phosphorylated p38 (p-p38) and p42/44 (p-p42/44) and total p38 and p42/44 were assessed by Western immunoblotting.. β -actin was used a measure of total protein loaded. These results are representative of two independent experiments.

Figure 3.18 Knockdown of endogenous hECSIT in the U373 cell line does not affect the processing of p105 following LPS stimulation.

U373 cells were polyclonally transduced with hECSIT-specific lentiviral shRNA or Control shRNA. Cells were cultured in the presence of selective reagent puromycin (5 μ g/ml). Knockdown of endogenous hECSIT was checked by immunoblotting. Once achieved, the cells were then treated with LPS (100ng/ml) for various time points (5,10, 30, 60, 180mins). Cell lysates were then generated and subjected to SDS-PAGE and subsequently to Western immunoblotting with antibodies against p105/p50 and hECSIT. β -actin was used as a loading control. These results are representative of two independent experiments.

Figure 3.19 hECSIT does not interact with Tpl-2.

HEK 293 T cells were transfected with 1 μ g hECSIT-MYC and/or 1 μ g Tpl2-HA as indicated. DNA levels were normalised with empty vector pcDNA3.1. Cells lysates were harvested 24 h post-transfection and immunoprecipitated with an immobilised anti-MYC antibody. Immunoprecipitates were subjected to polyacrylamide gel electrophoresis and subsequently to Western immunoblotting using an anti-HA antibody. They were subsequently probed with anti-MYC antibody to confirm the co-immunoprecipitation. Cell lysates were also analysed by Western immunoblotting with anti-MYC antibody and anti-HA antibody to confirm expression of the constructs.

Immunoprecipitation blot shown above is representative of two independent experiments.

4. Discussion

In 1999 Kopp *et al.*, described a newly identified protein termed ECSIT. It was discovered during a yeast two hybrid screen where TRAF6 was used as the bait and it was subsequently found to enhance NF κ B activation as well as playing a role in MEKK1 processing. When null mutants of ECSIT were generated, the embryos died on day E7.5 due to defects in mesoderm formation. There were striking similarities in the phenotype of ECSIT null embryos and those that had BMPRI1 gene deletions, leading to the discovery that ECSIT was essential in BMP signal transduction (Xiao *et al.*, 2003). Since then, ECSIT has been established as a predominantly mitochondrial located protein that is involved in the assembly of mitochondrial complex 1 (Vogel *et al.*, 2007). The relevance of ECSITs mitochondrial location and its interaction with TRAF6 was deciphered by West *et al.*, (2011) when they demonstrated that TRAF6 and ECSIT are required for macrophage killing of intracellular bacteria. They showed that signalling through TLR 1, 2 and 4 in macrophages results in mitochondrial recruitment to the phagosome. TLR signalling also induces TRAF6 translocation to the mitochondria, where it can bind to and ubiquitinate ECSIT resulting in an enrichment of ECSIT to the mitochondrial periphery and a concomitant increase in both mitochondrial and cellular production of ROS, required for killing of intracellular bacteria. Macrophages deficient in TRAF6 and ECSIT fail to clear infection, as do those with deficient mitochondrial production of ROS. Such findings have established ECSIT as key protein in TLR signalling, critical to innate immune clearance of bacteria.

All previous studies have focussed on the murine form of ECSIT, mECSIT. While murine models are routinely used as *in vivo* models in immunology, the distinct difference between the human and murine immune systems means that results in murine models do not always translate directly to human models. Indeed in terms of TLR signalling, while generally there is high level of conservation across human and mice there are differences in the TLR expression with 13 TLRs in mice and 11 in humans. Murine and human TLRs can show differential recognition of ligands . It is therefore important when using mice as experimental tools to consider differences that may be present in human forms. The human form of ECSIT, hECSIT, was cloned in the Moynagh Lab, who have since been concerned with characterising its role in TLR signalling. Despite being homologs of each other, hECSIT and mECSIT display opposing roles in NFκB activation. This has been shown extensively as part as ongoing research in the Moynagh lab (unpublished) and is briefly exemplified in my work (Figure 3.1). mECSIT can induce NFκB activation and enhance LPS- and IL-1β-induced NFκB activation. This is agreement with the initial characterisation by Kopp *et al.*, (1999) that mECSIT is a positive intermediate in NFκB activation. In contrast hECSIT fails to induce NFκB and displays mild inhibitory effects on its activation upon LPS and IL-1β stimulation. It is therefore proposed as a negative regulator of NFκB activation.

In an effort to understand how hECSIT signalling differs from that of mECSIT, I set to uncover hECSIT interacting proteins. A proteomic screen of proteins that could co-immunoprecipitate with hECSIT was undertaken. This was considered as an unbiased approach of detecting interacting proteins, as it would survey all hECSIT interacting proteins within the cell and identify them by mass spectrometry. The experiment required the large scale culturing of HEK293 T cells transiently expressing hECSIT and subsequent immunoprecipitation of hECSIT and its interacting proteins. The

experimental model was restricted by low protein returns following the co-IP, even after pooling of samples, limiting the amount of protein available for two-dimensional electrophoresis (2-DE) separation (Figure 3.3). From this screen, 8 proteins spots were identified as coimmunoprecipitating with hECSIT (Table 3.1). No known ECSIT interacting partners were identified. This may be due to the low protein levels loaded or because these interactions were below the detection level of the assay. In addition, of the proteins separated by 2-DE, the majority are non-specific as they are present in control and hECSIT immunoprecipitated samples. The high proportion of non-specific proteins may be masking the presence of specific interacting proteins, preventing their detection. In a repeat of this experiment in the future, in addition to loading increased protein amounts, further washing of the co-IP samples to remove non specific proteins would be of benefit. As mECSIT has been shown to have a predominately mitochondrial location, isolation of cell lysates into mitochondrial fractions prior to performing the coimmunoprecipitation may also increase the detection of hECSIT partners.

Of the 8 proteins subjected to mass spectrometry identification, 5 were identified as hECSIT, indicating that hECSIT is present in several forms within the cell. Probing gels with anti-hECSIT antibody confirmed the hECSIT can be detected at more than one point on the gel (Figure 3.4). This is not entirely unexpected as ECSIT is proposed to have four isoforms due to alternative splicing of transcripts (http://www.ncbi.nlm.nih.gov/sites/entrez?db=geneandcmd=Retrieveanddopt=full_reportandlist_uids=51295). As post translation modification of proteins by phosphorylation and ubiquitination is so critical to TLR signalling, it could be postulated that these spots may represent post translational modified forms of hECSIT. However these questions were not addressed as part of my thesis, instead I focussed on the protein identified as ZNF419. It

was chosen as a protein of interest due to the presence of a zinc finger domain within the protein. Several proteins involved in TLR signalling contain zinc finger domains, such as ZCCHC11, Gfi1 and A20 (Jaaflela *et al.*, 1996; Minoda *et al.*, 2006; Wallis *et al.*, 2003). ZNF419 is characterised as a KRAB domain containing C2H2 zinc finger protein, which is the largest subfamily of the zinc finger family. KRAB domains are associated with transcriptional repression, indeed KRAB domain proteins have been previously shown to negative regulate innate immune signalling (Kamitani *et al.*, 2011).

As the commercial available antibody against ZNF419 failed to detect ZNF419 expression (Figure 3.5), a FLAG-tagged version of ZNF419 had to be engineered. When the interaction between hECSIT and ZNF419-FLAG was probed by conventional co-IP methods, no interaction could be detected (Figure 3.6). This questioned the physiological relevance of the identification of ZNF419 as a hECSIT-interacting protein by mass spectrometry. Increasing the stringency of criteria used for accepting protein hits from mass spectrometry such as percentage of peptide matched may prevent such false results in future screens.

Considering that zinc finger domains are present in numerous proteins relevant to TLR signalling, it was still of interest to investigate if ZNF419 could affect NF κ B activity. As shown by luciferase assay, ZNF419 does not play a role in the LPS, IL-1 β or TNF α induced activation of NF κ B (Figure 3.7) Recently, Broen *et al.*, (2011), measured the levels of ZNF419 expression in fibroblasts, keratinocytes and proximal tubular epithelial cells and found the expression to be low. Interestingly they found that IFN- γ caused an increase in ZNF419 expression in keratinocytes.

As mECSIT has been shown to be critical to BMP signalling (Xiao *et al.*, 2003), I wanted to establish if hECSIT has a role in BMP signalling and whether it would act comparably with mECSIT to promote BMP signalling or act differentially as it does in NFκB signalling. BMP signalling from the cell surface to the nucleus is propagated by the SMAD family of transcription factors which can regulate gene transcription by binding to the promoter of BMP responsive genes. BMP signalling initially activates a distinct subgroup of receptor regulated SMADs (R-SMADS), SMAD1, SMAD5 and SMAD8 (SMAD1-5-8), which once activated can interact with SMAD4 to induce gene transcription. Xiao *et al.*, (2003) discovered that mECSIT is critical to BMP signalling, it can basally interact with SMAD4 and interacts with SMAD1 in a BMP4 inducible manner to promote that transcription of BMP4 inducible genes, such as Tlx-2. My first approach to address the role of hECSIT in BMP4 signalling was to suppress endogenous hECSIT expression and investigate the effect on SMAD1-5-8 activation. When hECSIT is suppressed, the phosphorylation of SMAD1-5-8 is decreased (Figure 3.8). As mECSIT mediates its effects on BMP signalling through interaction with SMAD proteins, it was of interest if hECSIT could similarly interact with SMADs. However no interaction between hECSIT and SMAD4 could be detected (Figure 3.8). It would be of value for future studies to probe the interaction of hECSIT with other SMAD family members. This initial investigation into the role of hECSIT in BMP4 signalling indicates that similar to mECSIT, hECSIT is involved in the positive regulation of BMP4 signalling. However the mechanism by which hECSIT achieves this and the result of hECSIT regulation of SMAD1-5-8 remain unanswered. More extensive studies in the future will address if hECSIT interacts with any members of the SMAD family and how hECSIT regulates the expression of BMP-inducible genes.

While BMP4 primarily signals through SMAD proteins, it can also signal through non-canonical SMAD-independent pathways (Yang *et al.*, 2006). Several studies have shown that BMP4 can mediate signalling by targeting the MEK1/2- p42/44 pathway (Li *et al.*, 2012; Zhou *et al.*, 2007), and therefore I looked at the effect of hECSIT suppression on BMP4 induced p42/44 activity. Interestingly when hECSIT is knocked down, p42/44 exhibits drastically higher levels of phosphorylation under resting conditions compared to control cells (Figure 3.8). While control cells show induction of p42/44 activation upon BMP4 stimulation, cells with suppressed expression of hECSIT display no further induction. Similar results are found in response to TLR4 signalling (Figure 3.10), indicating that hECSIT is involved in the basal regulation of p42/44 activity. To further investigate the role of hECSIT in the regulation of MAPK activation other cells lines, such as HEK293 T, HEK293 TLR4 and U373 cells which display strong induction of MAPK proteins in response to proinflammatory signals were used.

Considering that hECSIT and mECSIT display striking differences in their regulation of the transcription factor NF κ B (Figure 3.2), it was of relevance to compare the roles of hECSIT and mECSIT in the regulation of MAPK induced transcription factors. Numerous transcription factors have been shown to be targets of MAPK signalling including ELK-1, CHOP1, CREB, ATF4, c-fos, NFAT, STAT3 and p53, with individual MAPK members showing overlapping and distinct targeting of their activation (Cargnello and Roux, 2011). To assess if ECSIT could regulate MAPK activation of transcription factors, luciferase assays monitoring the activation of ELK-1 and AP-1 were preformed. ELK-1 is a key target of p42/44, but can also be targeted by p38 and JNK. P42/44 can bind to and directly activate ELK-1 to induce immediate early genes. One such target gene encodes the protein c-fos, which can then form dimers with c-jun to form the AP-1 transcription factor . JNK is also critical to the

induction of AP-1 as it can directly bind to activate the AP-1 subunit c-jun. Heterodimeric complexes of AP-1 composed of c-fos and c-jun, can regulate numerous proinflammatory genes, including IL-1 β and TNF α . Similar to their opposing roles on NF κ B activation, hECSIT and mECSIT display differential effects on the activation of ELK-1 and AP-1. mECSIT augments the activation of ELK-1 and AP-1 (Figure 3.11) While hECSIT displays marginal inhibitory effects on ELK-1 and AP-1 activation, this is consistent with its inhibitory effects on the upstream regulator p42/44.

The initial studies on mECSIT had proposed mECSIT to be the key linker protein that couples TRAF6 to MAPK activation as mECSIT could bind both TRAF6 and MEKK1 (Kopp *et al.*, 1999). mECSIT was also indicated to play a role in MEKK1 processing. Consequently I wanted to determine if hECSIT could regulate MEKK1-induced activation of the MAPKs. While there are negligible effects on p38 and JNK activation, hECSIT shows strong inhibitory effects towards p42/44 phosphorylation (Figure 3.13). To validate the results alluded to by the overexpression studies, RNAi technology was used to suppress the expression of endogenous hECSIT. hECSIT specific siRNA and lentiviral shRNA was therefore employed. Suppression of endogenous hECSIT again specifically impacts p42/44 activation, resulting in increased basal and proinflammatory induction of p42/44 phosphorylation (Figure 3.14, 3.15, 3.16 and 3.17) The overexpression studies and the complementary siRNA and shRNA studies define hECSIT as being a negative regulator of p42/44 phosphorylation.

The discriminatory regulation of p42/44, and not the p38 or JNK, by hECSIT indicates the hECSIT functions by targeting proteins that operate solely in the p42/44 pathway. Potential candidates include MAP3K, MAP2K, DUSP and certain scaffold proteins (Kolch, 2005).

The activation of p42/44 in response to growth factors primarily is through the Ras-Raf pathway, where Raf acts as a MAPKKK to activate MEK1/2 which can then subsequently activate p42/44 (Kolch, 2000). However in terms of immune signalling, where proinflammatory stimulation leads to p42/44 activation, pathways involving other MAPKKK such as MEKK1 and Tpl2 are employed (Symon *et al.*, 2006). Tpl2 was of interest to this study as it specifically acts on p42/44 and not p38 or JNK. Under resting conditions the activity of Tpl2 is regulated by its interaction with the NF κ B subunit p105, which prevents Tpl2 from interacting with and activating MEK1/2. In response to proinflammatory signals, IKK proteins induce the processing of p105 protein to the p50 form. This partial degradation of p105, releases Tpl2 allowing it to activate the MEK1/2-p42/44 pathway (Robinson *et al.*, 2007). These signalling events have been shown to be critical to the production of LPS induced TNF α and IL-1 β (Rousseau *et al.*, 2008). I set out to investigate if hECSIT was acting at the level of Tpl2 to regulate p42/44 activity.

As the NF κ B subunit p105 has a critical role on the regulation of Tpl2 activity, I examined whether the suppression of endogenous hECSIT by hECSIT shRNA affected the levels and processing of p105 to p50. However no significant differences between the control and hECSIT shRNA samples could be witnessed (Figure 3.18). In addition to its interaction with p105, Tpl2 also interacts with ABIN-2 (Papoutsopoulou *et al.*, 2006) and the p38 isoforms p38 γ and p38 δ (Risco *et al.*, 2012). In macrophages, deletion of any of these components results in Tpl2 degradation, preventing p42/44 activation. As the interacting partners of Tpl2 are so critical to its activation of p42/44, it was proposed that hECSIT could form part of this complex to regulate Tpl2 activity. However no interaction between hECSIT and Tpl2 could be detected when co-immunoprecipitations were performed (Figure 3.19)

Although hECSIT suppression has no effect on p105 processing and does not interact with Tpl2, it is still possible that hECSIT may regulate Tpl2 activity resulting in the augmented p42/44 phosphorylation that is observed under conditions of hECSIT suppression. Future studies should probe any possible relationship between hECSIT and Tpl2 in depth starting by investigating the phosphorylation of Tpl-2 in response to proinflammatory signals when hECSIT is suppressed. GSK β 3 is another viable candidate for mediating the regulatory effects of hECSIT on the p42/44 pathway. GSK β 3 is a negative regulator of p42/44 activity and it is active in its unphosphorylated state (Wang *et al.*, 2006). Signalling through the PI3K pathway, phosphorylates and inactivates GSK β 3 to promote the activity of p42/44. It will be of great interest to assess if hECSIT suppression affects the activity of GSK β 3. The basal activity of GSK β 3 in its unphosphorylated state will be of special interest given the increased basal phosphorylation of p42/44 when hECSIT is suppressed. `

While my thesis has focussed on the Tpl2 pathway of p42/44 activation, hECSIT may be relevant to the Ras-Raf pathway. Interestingly a tandem affinity purification (TAP) screen undertaken recently by members of the GHOSH lab has shown that mECSIT can interact with rac-1 (West, A. 2011- PhD thesis). They used a C-terminally TAP-tagged ECSIT expression vector containing both streptavidin and calmodulin binding peptides. After TAP-purification of mECSIT and its binding partners, samples were separated by SDS-PAGE and identified by mass spectrometry. A possible interaction between hECSIT and Rac-1 is highly relevant to hECSITs regulation of p42/44. Rac-1 is a member of the RHO family of GTPase and has been shown to be activated in response to TLR signalling. Importantly it has been shown to phosphorylate PAK, which can in turn phosphorylate MEK1 to enhance MEK1 interaction with ERK2 (Eblen *et al.*, 2002; 2004). In a negative feedback

mechanism ERK is proposed to also phosphorylate MEK1, preventing PAK phosphorylation of MEK1. Based on hECSIT inhibitory role in p42/44 phosphorylation, it is plausible to propose that the interaction of hECSIT with Rac-1 under resting conditions may regulate Rac-1 activity. Indeed the p42/44 inhibitor RKIP has been shown to act in such a way (Trakul *et al.*, 2005). Under resting conditions it binds to Raf and MEK preventing their physical interaction and Raf-induced activation of MEK. RKIP interaction with Raf is also thought to prevent PAK induced activation of Raf. Mitogenic stimulation results in the dissociation of RKIP and Raf to allow MEK1/2 activation. Interestingly RKIP like hECSIT can also target NF κ B activation (Yeung *et al.*, 2001). Thus future investigations of the molecular and functional interactions of hECSIT and rac-1 will be of great interest in the context of signalling by mitogenic and proinflammatory stimuli.

mECSIT was identified over ten years ago, but to date there are no published reports on the human form of hECSIT. Here I show that hECSIT specifically targets the p42/44 branch of MAPK signalling. It acts as a negative regulator of p42/44 phosphorylation, and functions under resting conditions and when proinflammatory signalling pathways are induced. How hECSIT inhibits p42/44 phosphorylation remains unknown, as does the physiological consequences on p42/44 signalling when hECSIT is suppressed. In addition West *et al.*, (2011) showed that TRAF6 ubiquitination of mECSIT was required for ECSIT signalling. Whether the ubiquitination status of hECSIT, or indeed its phosphorylation status, is relevant to its regulation of p42/44 needs to also be addressed. The importance of mitochondrial signalling in innate immune signalling is gaining more appreciation. mECSIT is now known to be a predominately mitochondrially located protein, with its mitochondrial location critical to its signalling. Establishing the cellular location of hECSIT signalling and its signalling partners is

essential to understanding hECSIT signalling and may explain the opposing role of hECSIT and mECSIT on NF κ B and p42/44 activity.

5. References

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