Regulation of TGFβ-activated-kinase 1 (TAK1) in Nuclear Factor-κB and Tumour Necrosis Factor/Eiger Signalling in *Drosophila melanogaster.*

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A dissertation submitted for the degree of Doctor of Philosophy (DPhil) in Biochemistry at the Department of Biochemistry, University of Oxford.

Trinity 2011

University College

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ABSTRACT

Drosophila TGFbeta-Activating-Kinase 1 (dTAK1) is an essential component of both the Immune Deficiency (IMD) innate immune and TNF/Eiger apoptotic cascades. The IMD and JNK pathways bifurcate at the level of dTAK1. Hence, elucidating the regulatory mechanism of dTAK1 is pertinent to understanding the regulation of both innate immunity and apoptosis.

In this study, Trabid was identified as a novel negative regulator of the *Drosophila* IMD pathway. Trabid interacted with dTAK1 and decreased K63-linked ubiquitination, thereby reducing immune signalling. Three tandem Npl4 Zinc Fingers (NZF) and C518 were required for Trabid activity. Lysines 142 & 156 were identified as the K63 Ub acceptor sites of dTAK1, required for K63-linked ubiquitination and signalling. Also, results show Lys 156 functioned as the K48 Ub acceptor site. Further, the ZF domain of TAK1-associated Binding Protein 2 (dTAB2) was important in modulating dTAK1 K63-linked ubiquitination and thereby the immune signal. These results indicate an elaborate and multi-tiered mechanism for regulating dTAK1 activity and modulating the immune signal.

Further, Ariadne-2 (Ari-2) was identified as a novel component of the *Drosophila* TNF/Eiger pathway which functioned at the level of dTAK1. Results indicate that Ari-2 is essential for normal development and longevity. It enhances the apoptotic signal when concomitantly over-expressed with Eiger. Further, Ari-2 interacts with dTAK1, dTAB2 and dTRAF2 which are all implicated in TNF/Eiger signalling. Thus, evidence supports the hypothesis that Ari-2 functions as an adaptor, involved in assembling a distinct signalling complex which transduces the apoptotic signal without activating immunity (245 words).

Acknowledgements

First and foremost, my deep gratitude and sincere thanks goes out to my supervisor Dr. Petros Ligoxygakis. I am deeply indebted to him for accepting me as a graduate student in his laboratory and for the wonderful support he provided throughout this period. His constant advice, guidance and help have been invaluable for my project. I very much enjoyed the little chats we had in the past year. Most of all, I am grateful for the fact that he is my friend.

My gratitude is also extended to Dr. Anthony Brown, my day to day collaborator and all-round 'guru,' for guiding me through most of my 1st year. His ever-reliable protocols and succinct methodology helped me immensely in my work. I miss him around the laboratory.

No amount of words can express the faith and help given to me by my family these past few years. It was their unwavering commitment and belief that held me up all these years.

I am also grateful to Marcus Glittenberg, Davide Roncarati, Lihui Wang and Magda Atilano, not only for their guidance but, also for extending the hand of friendship. I am also very grateful to Davide for proof reading my thesis. Their advice has been invaluable to me.

Dedication

This report is dedicated to my parents who believed in me at all times and all the hard work in getting to this point of time.

"It matters not how strait the gate,

How charged with punishments the scroll,

I am the master of my fate:

I am the captain of my soul."

- William Ernest Henley.

(Invictus)

(1849–1903)

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List of Abbreviations:

Commonly used terms:

AMP – anti microbial peptides

DAP-type PGN - meso-diaminopimelic acid type peptidoglycan

DD – death domain

DED – death effecter domain

DUB – deubiquitinating enzyme

GNBP - Gram-negative binding protein

IκB – Inhibitor of NF-κB

IKK – IκB kinase

IMD – Immune deficiency

Lys-type PGN – Lysine type peptidoglycan

NFκB – Nuclear Factor kappa B

PBS – phosphate buffered saline

PGN – peptidoglycan

PGRP – peptidoglycan recognition protein

PRR – pattern recognition receptor

RING – Really Interesting New Gene

SPE - Spätzle processing enzyme

TNF – tumour necrosis factor

TNFR – tumour necrosis factor receptor

Ub – ubiquitin

Commonly used gene names:

ari-2 - ariadne 2 (CG5709)

CYLD – cylindromatosis (CG5603)

egr – eiger (CG12919)

puc – puckered (CG7850)

Tab2 - TAK1-associated binding protein 2 (CG7417)

Tak1 - TGF-β activated kinase 1(CG18492)

Traf2 - TNF-receptor-associated factor 2 (CG10961)

trbd – trabid (CG9448)

Chapter 1: Introduction

1.1 Innate and Adaptive Immunity

Renowned Immunologist and Nobel Laureate Sir Frank Macfarlane Burnet defined immunity as “the capacity to recognise the intrusion of material foreign to the body and to mobilise cells and cell products to help remove that particular sort of foreign material with greater speed and effectiveness” (Soper, 1998). Immunity can broadly be divided as adaptive (acquired) and innate depending on the intensity and timing of the responses when exposed repeatedly to the same inducing agent/antigen (Male, 2006).

Adaptive immunity is characterised both by its specificity towards an inducing antigen and memory - where it can mount a faster and greater response in subsequent infection by the same pathogen (Male, 2006). However, this is present only in jawed vertebrates, jawless fishes and is a relative newcomer in evolution (Janeway and Medzhitov, 2002). It should be noted that evolution is a gradual process and therefore, it is highly likely that other organisms may possess rudimentary adaptive immune systems or parts of an adaptive process. One possibility that adaptive immunity has been identified in higher chordates may be because investigations may have focussed on them and have not yet investigates other possible candidates.

In contrast, innate immunity is universal, evolutionarily ancient and is the dominant system prevalent in invertebrates (Janeway and Medzhitov, 2002). Male *et al*., 2006, describes innate immunity as “simple though remarkably sophisticated systems present in all animals that are the first line of defence against pathogens and

allow a rapid response to invasion." It is an evolutionarily conserved defence mechanism that confers the host with immediate and multi-faceted defence against microbial pathogens. These include physical barriers, cellular responses and systemic humoral responses (Lemaitre and Hoffmann, 2007). Although the innate defences are closely interlinked with adaptive responses in higher chordates, all other species rely solely on innate immunity to combat pathogens. Thus, innate immunity is considered as the prototypical immune defence and is well conserved among species from plants to fruit flies to mammals (Beck, 1996).

1.2 The Systemic Innate Immune Response in *Drosophila*:

The hallmark of *Drosophila* immune defence, upon microbial challenge, is the synthesis and secretion of several families of antimicrobial peptides (AMPs) in the fat body. This constitutes the "systemic immune response" and is common to most holometabolous insects (Boman et al., 1972; Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). The fat body is a major immune-responsive tissue that is equivalent to the mammalian liver and acquires its immune competence at the onset of the first larval stage. Its large size and central position in the open circulatory system of the insect body cavity enable it to then release these immune peptides into the haemolymph or insect "blood", where they accumulate to reach effective concentrations (Bulet et al., 1999; Hoffmann and Reichhart, 2002; Lemaitre and Hoffmann, 2007).

1.2.1 Immune effectors – antimicrobial peptides (AMPs):

Most prominent and well characterised amongst the various immune effectors synthesised by the fat body in response to microbial challenge are the antimicrobial peptides (AMPs). There are some 20 AMPs grouped into 7 classes discovered thus far in *Drosophila*. These can be divided into 3 families based on their biological targets -

Gram-positive bacteria (Defensin), Gram-negative bacteria (Cecropins, Drosocin, Attacins, Diptericin), or fungi (Drosomycin, Metchnikowin) (Åsling et al., 1995; Bulet et al., 1993; Dimarcq et al., 1994; Fehlbaum et al., 1994; Levashina et al., 1995; Wicker et al., 1990). AMPs are cationic and typically small. Some are induced in surface membranes whilst others are constitutively expressed in specific tissue in the salivary glands and the reproductive tract (Imler, 2005).

Antimicrobial peptide family	No. of genes in the genome	Main biological activities	Effective conc. in the blood
Diptericin	2	Anti bacterial, Gram-negative	0.5 µM
Attacin	4	Anti bacterial, Gram-negative	nd
Drosocin	1	Anti bacterial, Gram-negative	40 µM
Cecropin	4	Anti bacterial, Gram-negative	20 µM
Defensin	1	Anti bacterial, Gram-positive	1 µM
Drosomycin	7	Antifungal	100 µM
Metchnikowin	1	Antifungal	10 µM

Figure 1 - 1: *Drosophila* antimicrobial peptides. Depicting the name of the AMP family, number of its genes in the genome, main biological activity at physiological concentrations and estimated concentration in the insect blood/hemolymph after bacterial infection. *nd: not determined*. Adapted from: Lemaitre, B. and J. Hoffmann (2007). "The Host Defense of *Drosophila melanogaster*."

1.2.2 Regulation of AMP expression:

The expression of immune-inducible polypeptides, including AMPs, is primarily regulated at level of transcription. It is now known that the promoters of genes encoding AMPs contain DNA motifs that were mandatory for immune inducibility, including NF-κB binding sites, GATA binding sites and the R1 motif (Hoffmann and Reichhart, 2002; Lemaitre and Hoffmann, 2007). Similar to their mammalian counterparts, the nuclear factor-κB (NF-κB) transactivators play a prominent role in activating and regulating AMP gene expression by interacting with the κB binding motifs (Engström et al., 1993; Ferrandon et al., 2007). Three NF-κB proteins are encoded in the *Drosophila* genome including Relish, Dorsal and Dif. All 3 proteins have a highly conserved N-terminal REL Homology Domain (RHD) which is implicated in DNA binding, dimerisation, interaction with IκB proteins and nuclear translocation. Using gel shift assays and cell culture, it has been shown that these 3 proteins bind to κB motifs and transactivate some AMP genes. Under non-infectious conditions, expression of AMP genes is suppressed by Inhibitors of NF-κB (IκB) proteins. These bind to ankyrin repeats found in NF-κB, blocking their nuclear translocation and resultant transcriptional activity (Belvin and Anderson, 1996; Lemaitre and Hoffmann, 2007).

During microbial infection, IκB proteins and/or ankyrin repeats are phosphorylated and cleaved. There is ample evidence that polyubiquitination (formation of ubiquitin chains) also plays a prominent role. The 'liberated' RHD containing N-terminus of NF-κB proteins then translocate to the nucleus, where they regulate the expression of AMP genes (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007).

Infections by different classes of pathogens result in activation of specific NF-κB transactivators, leading to expression of different subsets of AMP genes. Thus,

specific immune responses are elicited by three broad categories of microbial pathogens, namely Gram-positive, Gram-negative bacteria and fungi. Thus far, two distinct pathways (i.e. Toll and Imd) have been identified that mediate these responses (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007).

1.2.3 Detecting and recognising different classes of pathogens:

The *Drosophila* innate immune system can detect and recognise different classes of microorganisms. Microbial detection is facilitated by direct contact between host proteins called "pattern recognition receptors" (PRRs) and microbial ligands (peptidoglycans). Thus far, 2 distinct classes of PRRs have been identified - peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) (Kang et al., 1998; Lee et al., 1996; Lemaitre et al., 1997; Yoshida et al., 1996)

Bacterial invaders are discriminated by sensing different forms of peptidoglycan (PGN) of the bacterial cell wall by peptidoglycan recognition proteins (PGRPs) (Ferrandon et al., 2007). Peptidoglycan is a primary constituent of the bacterial cell wall comprising long glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues that are cross-linked to each other by short peptide bridges (Mengin-Lecreulx and Lemaitre, 2005). In Gram-negative bacteria (and a subclass of Gram-positive bacteria including *Bacillus* species), the third amino acid of the peptidic stem is a meso-diaminopimelic acid (DAP) residue while Gram-positive bacteria have a lysine (Lys) residue in this position. The working hypothesis is that the PGRPs of *Drosophila* can distinguish between DAP-type and Lys-type PGN (Leulier et al., 2003; Stenbak et al., 2004).

In addition to DAP-type and Lys-type PGN, there are numerous other types of PGN in bacteria. The PGN of Gram-positive bacteria can differ widely, with at least 8 different types based on the 3rd amino acid of the peptidic stem. Also, some species, including halophilic bacteria such as *Halobacterium halobium* and *Micrococcus morrhuae* lack peptidoglycan (Schleifer and Kandler, 1972).

So far, 13 different PGRPs have been identified in *Drosophila*. They are highly conserved and share the 160 amino acid PGRP domain, which is evolutionarily related to the bacteriophage type II amidases. These proteins can be further differentiated as catalytic PGRPs and recognition PGRPs (Werner et al., 2000). Catalytic PGRPs (PGRP-SC1, LB, SB1 and possibly SB2, SC2) have retained this enzymatic activity and modulate the immune response by removing peptides from the glycan chains of PGN of bacterial cell walls (through zinc-dependent amidase activity), thereby reducing or eliminating the biological activity of PGN. Some modulate the immune signal by scavenging PGN. On the other hand, recognition PGRPs (including PGRP-SA, SD, LA, LC, LD, LE, and LF) function as pattern recognition receptors only as they lack zinc-binding residues required for amidase activity (Mellroth et al., 2003; Werner et al., 2000).

1.3 The Toll Pathway

The Toll pathway in *Drosophila* is activated in response to microbial challenge by Gram-positive bacteria, fungi and yeast. Evolutionarily conserved, this pathway also plays a key role in several developmental processes including the formation of the dorso-ventral axis of the *Drosophila* embryo (Anderson et al., 1985; Morisato and Anderson, 1994). Homologous to the mammalian Interleukin 1 Receptor/Toll-Like Receptor (IL-1R/TLR) cascades, its canonical components include Spätzle (an

extracellular cytokine which is structurally similar to the nerve growth factor; NGF), Toll (the transmembrane receptor), Tube and MyD88 (adaptors), Pelle (a serine/threonine kinase), Cactus (the *Drosophila* homolog of IκB), and Dorsal and Dif (transcription activators) (Belvin and Anderson, 1996; Tauszig-Delamasure et al., 2002). Except for Cactus and Dorsal, deletion or inactivation of any of the other components results in a similar immune-deficient phenotype characterized by the non-expression of several immune genes and a marked susceptibility to fungal and Gram-positive bacterial infection (Lemaitre et al., 1996; Rutschmann et al., 2002).

Induction triggers cleavage and activation of the extracellular cytokine Spätzle, which then binds and activates the transmembrane receptor Toll. This leads to the recruitment of three intracellular death domain (DD) containing proteins, MyD88, Tube, and Pelle, resulting in the formation of the Toll-induced signalling complex (TISC) which, in turn, sets off an intracellular signalling cascade. This culminates in degradation of the *Drosophila* IκB homolog Cactus, leading to the activation and nuclear translocation of NF-κB-like transcription factors Dorsal (in embryos), Dorsal and Dif (in larvae) and Dif (in adults) and the resultant expression of *Drosomycin* and several other AMP genes (Leulier et al., 2006; Royet et al., 2005) (Fig. 1-2).

1.3.1 The current model for Toll signalling in adult *Drosophila melanogaster*:

1. The transmembrane receptor Toll in *Drosophila* is not activated directly by binding with microbial ligands (like the mammalian TLRs) but by the binding of a cleaved form of cytokine Spätzle. Spätzle is synthesized as an inactive pro-protein dimer where the pro-domain is natively unstructured to prevent its interaction with Toll. Spätzle is processed to a functional form by the serine protease Spätzle processing enzyme (SPE) in response to infection (Hu et al., 2004; Jang et al., 2006; Weber et al., 2003).

2. The link between SPE and receptors that recognize fungal, bacterial or viral pathogens is yet to be clearly defined. One model suggests that distinct proteolytic cascades are activated by Gram-positive and fungal infections which results in the processing and activation of SPE. According to this hypothesis these cascades are initiated by pattern recognition receptors peptidoglycan-recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) – soluble receptors present in the haemolymph (Ferrandon et al., 2007; Kambris et al., 2006).

3. PGRP-SA, PGRP-SD, GNBP1 recognise Gram-positive bacteria through Lys-type peptidoglycan (PGN), while GNBP3 detects β-glucans in yeast. Further, fungal proteases secreted by entomopathogenic fungi activate CLIP-domain containing serine proteases like Persephone (Michel et al., 2001; Wang et al., 2008; Wang et al., 2006).

4. All three cascades result in the activation of Spätzle Processing Enzyme (SPE) which, in turn, cleaves and processes Spätzle pro-protein into its mature form. Mature Spätzle (the C-terminal 106-amino-acid fragment; Spätzle C106) binds with high affinity to the extracellular domain of Toll in the ratio of one Spätzle dimer to two Toll monomers causing conformational changes in Toll and thereby initiating immune signalling (Ferrandon et al., 2007; Jang et al., 2006; Weber et al., 2003).

5. This leads to the formation of a multivalent complex centred on the intracellular TIR domain of the receptor Toll. Three intracellular death domain (DD) containing proteins, the highly conserved cytoplasmic adaptor MyD88 (myeloid

differentiation primary-response gene 88), Tube, and Pelle (a serine/threonine kinase) are recruited leading to the formation of the Toll-induced signalling complex (TISC). Complex formation is facilitated by the bipartite death domain of Tube, which contains two opposite surfaces involved in the interaction with the death domains of MyD88 and Pelle. Complex formation leads to the initiation of kinase activity of Pelle (Ferrandon et al., 2007; Galindo et al., 1995; Hecht and Anderson, 1993; Tauszig-Delamasure et al., 2002; Towb, 2009).

6. Activation of Toll immune signalling leads to the phosphorylation and degradation of the NF-κB inhibitor Cactus. Polyubiquitination is also thought to play a key role. Cactus binds to and maintains an inhibition on NF-κB transcription factors Dif and/or Dorsal. The mechanism of Cactus degradation is yet known. One model hypothesises that activated Pelle may phosphorylate Cactus. Degradation of Cactus relieves the inhibition and leads to the graded release and nuclear translocation of Dif and/or Dorsal, where they bind to NF-κB response elements (κB-RE) and regulate expression of AMP genes including *Drosomycin* (Belvin et al., 1995; Edwards et al., 1997; Fernandez et al., 2001; Groszhans et al., 1994; Yang and Steward, 1997).

7. There is evidence that Dif and Dorsal play redundant roles in AMP gene expression at the larval stage, while Dif is sufficient to mediate Toll activation in adults (Manfruelli et al., 1999).

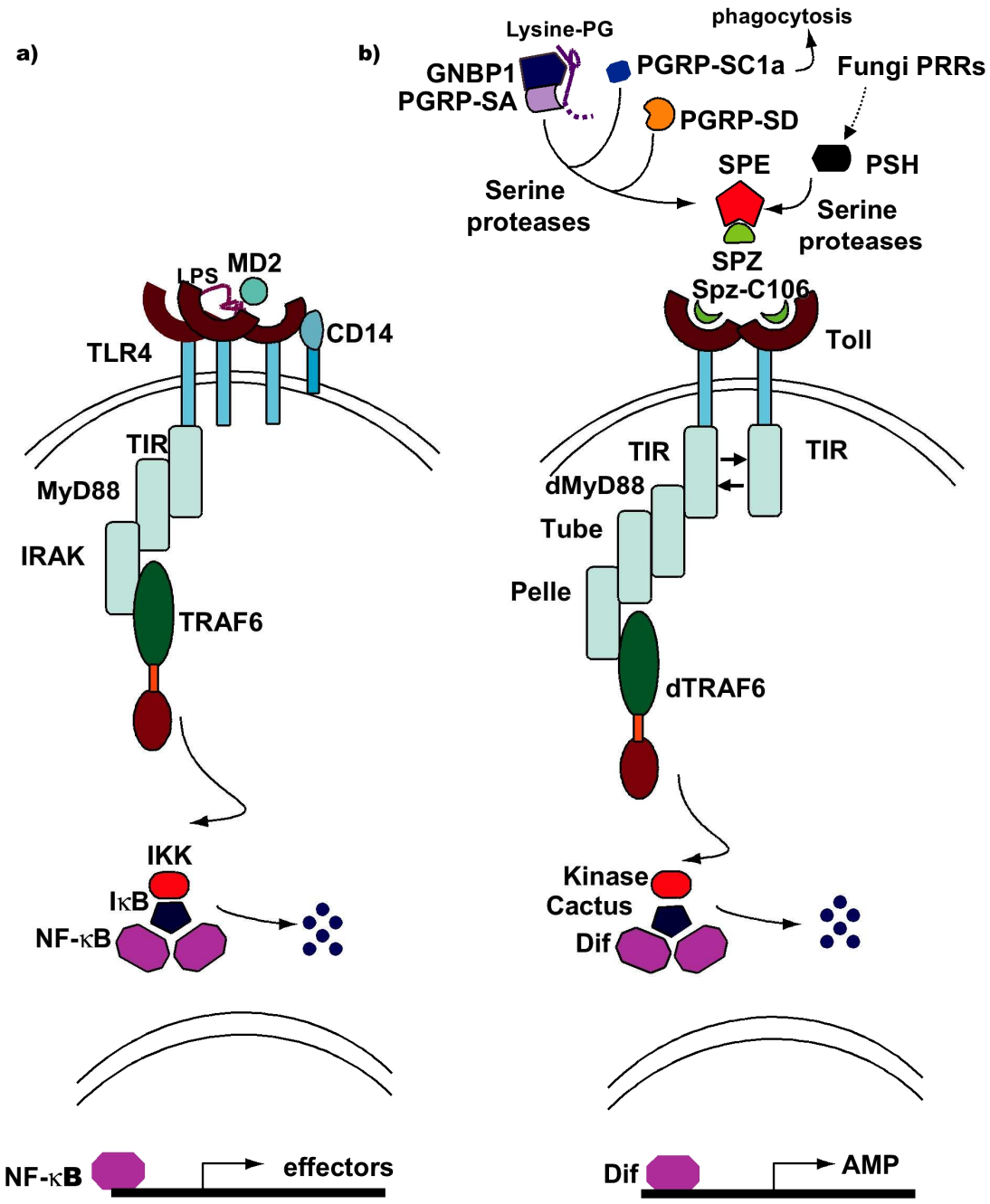


Figure 1 - 2: **(a) Mammalian TLR4 and (b) *Drosophila* Toll pathways**. (A) In the mammalian Toll-like receptor 4 (TLR4) pathway, pattern recognition receptors MD2 and CD14 present LPS to TLR4 resulting in the interaction between the cytoplasmic TIR domains of TLR4 and MyD88. Then, MyD88 recruits IL-1 receptor-associated kinase (IRAK). In turn, IRAK interacts with the adaptor TNFR-associated factor 6(TRAF6), leading to the activation of the IKK complex. IKK phosphorylates the inhibitor IκB, thereby targeting it for degradation. NF-κB translocates into the nucleus of cells and transcribes related gene expression (Wang et al., 2006). (B) The *Drosophila* Toll pathway is detailed in the text.

1.4 The IMD Pathway

The *Drosophila* IMD (Immune Deficiency) pathway is triggered in response to Gram-negative bacterial infection. Closely resembling the mammalian Tumour Necrosis Factor Receptor 1 (TNFR1) cascade, activation of IMD signalling leads to the expression of several AMP genes including *diptericin* (Lemaitre et al., 1995; Levashina et al., 1998). The canonical components of this pathway include IMD (a Death Domain containing adaptor), PGRP-LC and PGRP-LE (pattern recognition receptors), TAK1 (the Mitogen-Activated Protein 3 kinase - MAP3K), TAB2 (an adaptor) dIAP2 (a member of Inhibitor of Apoptosis proteins), IKKβ/ird5 and IKKγ/Kenny (the *Drosophila* equivalent of the mammalian IKK signalosome), dFADD (an adaptor) DREDD (apical caspase) and Relish (the NF-κB transcription factor) (Georgel et al., 2001; Lemaitre et al., 1995; Leulier, 2000; Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2000; Stoven, 2000; Vidal et al., 2001). Deletion/mutation of any of these components results in a similar phenotype – a high susceptibility to Gram-negative bacterial infection. As yet, there is no evidence linking this pathway to any developmental role (Hetru and Hoffmann, 2009; Lemaitre and Hoffmann, 2007).

Moreover, the Imd pathway bifurcates at the level of dTAK1 and also transiently activates the c-Jun amino-terminal kinase (JNK) signalling cascade during infection (Hoffmann and Reichhart, 2002) . Interestingly, dTAK1 is involved in activation of both innate immunity and apoptosis and is a nexus between the two (Silverman et al., 2003; Vidal et al., 2001).

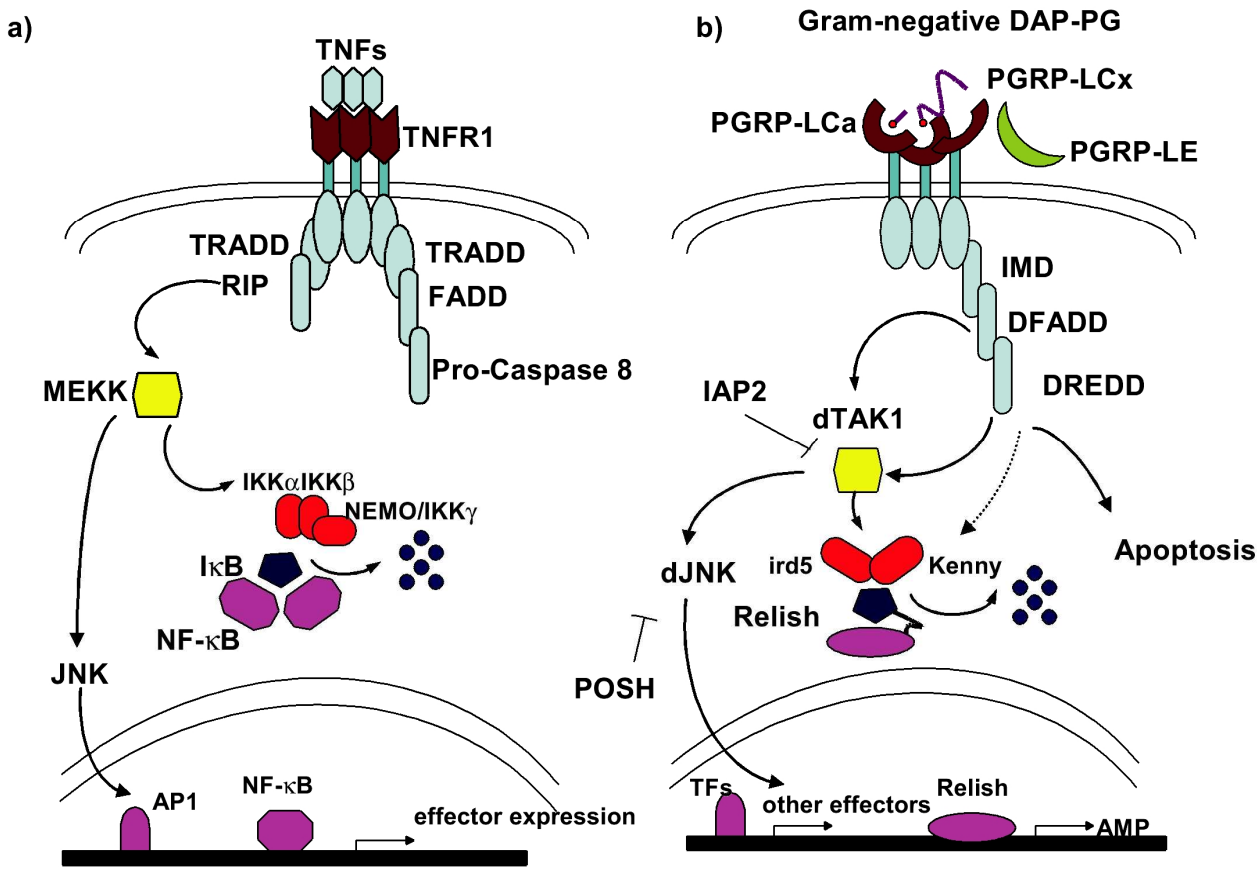


Figure 1 - 3: **(a) Mammalian TNFR-1 and (b) *Drosophila* IMD pathways**. (A) Upon stimulation, receptor TNFR1 recruits TNFR-associated death domain (TRADD) which, in turn, assembles a signalling complex comprising Fas-associated death-domain protien (FADD) and receptor interacting protein (RIP). Thereafter, FADD initiates an apoptotic cascade via pro-caspase 8, whilst RIP activates the kinase MEKK leading to phosphorylation of IκB by IKKα, IKKβ and IKKγ/NEMO complex. Phosphorylated IκB is targeted for degradation, resulting in the translocation of NF-κB into nucleus for gene expression. The mammalian Jun N-terminal kinase (JNK) pathway can be linked with the NF-κB pathway through MEKK. JNK phosphorylates and activates Jun, which is part of the AP1 transcription factor (Schutze et al., 2008). (B) The *Drosophila* IMD pathway is detailed in the text.

1.4.1 Current model for Imd signalling in adult *Drosophila melanogaster*

1. DAP-type PGN of Gram-negative bacteria is recognised in the cell surface by peptidoglycan-recognition protein-LC (PGRP-LC), a transmembrane type 11 receptor and the cytosolic PGRP-LE (a cleaved, secreted receptor in the haemolymph). Both receptors sense the presence of Gram-negative bacteria by binding directly to and being activated by monomeric or polymeric DAP-type PGNs or shorter peptidoglycan end fragments such as tracheal cytotoxin (TCT) (Choe et al., 2002; Gottar et al., 2002; Kaneko et al., 2006; Ramet et al., 2002).

2. Peptidoglycan is found in a single layer in the periplasmic space beneath the outer membrane and lipopolysaccharide (LPS) layer in Gram-negative bacteria. Tracheal cytotoxin (TCT) was identified as the minimal PGN motif capable of efficient induction of the Imd pathway. This muropeptide is located at the end of the PGN strand and is released from PGN during cell growth and division (Kaneko et al., 2004; Stenbak et al., 2004).

3. **Modulating signal intensity:** It is hypothesised that during infection, bacteria release short PGN fragments (like TCT) as a result of cell-wall remodelling during growth and division. These PGN fragments are detected, resulting in activation of the IMD pathway. Consequently, invading microorganisms are attacked by AMPs and lysozymes (effectors of the humoral immune response), leading to the release of large fragments of polymeric DAP-type PGN that were initially hidden under the LPS outer coat. These fragments can then be sensed directly by an array of membrane-bound PGRP-LC receptors (Brennan et al., 2007; Ferrandon et al., 2007). In a parallel mechanism, catalytic amidases like

PGRP-LB specifically degrade DAP-type PGN and downregulate the IMD pathway. This negative feedback mechanism provides a tight control over immune activation and modulates the level of the host immune response to the presence of infectious microorganisms (Zaidman-Rémy et al., 2006).

4. Alternative splicing of PGRP-LC can generate 3 isoforms (LCa, LCx & LCy) which possess identical N-terminal intracellular and transmembrane regions but, different extracellular PGRP domains in the C-terminus (Werner et al., 2000). For polymeric PGN recognition, at least 2 molecules of PGRP-LCx in close proximity are required while both PGRP-LCx & PGRP-LCa are necessary for monomeric PGN detection, where PGRP-LCa acts as an adaptor (Kaneko et al., 2004; Mellroth et al., 2005)

5. Binding of PGN elements to the receptors cause them to cluster or multimerise, triggering the immune signal (Lim et al., 2006). Following multimerization, death domain (DD) containing IMD (homologous to the mammalian Receptor Interacting Protein - RIP) is recruited (Georgel et al., 2001). IMD binds to the cytoplasmic RHIM-like domain of PGRPs probably through a yet unidentified mediator (Choe et al., 2005).

6. IMD then recruits *Drosophila* FAS-associated death domain (dFADD) and death-related ced-3/Nedd2-like protein (DREDD) to form a tripartite complex. It interacts with dFADD through death domains (DDs), while dFADD binds with the apical caspase DREDD via death effector domains (DEDs) (Leulier et al., 2002).

7. IMD is then cleaved in a DREDD dependent manner exposing an IAP-binding motif. IMD then binds with dIAP2 via this motif, leading to K63-linked polyubiquitination and activation of IMD (Paquette et al., 2010).

8. Once activated, IMD, in turn, activates two cascades. One process leads to the phosphorylation of the NF-κB-like transcription factor Relish through activation of dTAK1 and the IKK complex. The other process results in the cleavage of Relish by DREDD (Silverman et al., 2000; Stoven, 2000) Relish is very similar to the mammalian REL family members p105 and p100 in that it contains an N-terminal DNA-binding REL domain and C-terminal ankyrin repeats that are characteristic of the IκB family. These ankyrin repeats inhibit its activation and translocation. Relish differs from Dif and Dorsal (the NF-κB transactivators of Toll) in this regard (Stoven et al., 2003).

9. In the first process, activation of IMD results in the activation of *Drosophila* transforming growth factor-β (TGFβ)-activated kinase 1 (dTAK1) by an unknown mechanism. This Mitogen-Activated Protein 3 kinase (MAP3K) forms a dimer with the regulatory subunit *Drosophila* Tak1-binding protein 2 (dTAB2) (Geuking et al., 2005; Vidal et al., 2001). In mammals, TAK1 is can be directly activated by the E3 ubiquitin ligase, Tumour Necrosis Factor Receptor-associated factor 6 (TRAF6). TAK1 also requires TAB2 for activation (Besse et al., 2007). In *Drosophila*, the corresponding E3 ligase has not yet been identified (Lemaitre and Hoffmann, 2007).

10. Activated dTAK1 then phosphorylates and activates the *Drosophila* IκB kinase (IKK) signalling complex. The *Drosophila* IKK complex consists of the catalytic subunit Ird5 (immune-response deficient 5; the fly homologue of mammalian IKKβ) and the regulatory subunit Kenny (the fly homologue of mammalian IKKγ) (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2003).

11. The activated *Drosophila* IKK complex then phosphorylates Relish, effectively tagging it for subsequent cleavage (Lu et al., 2001; Rutschmann et al., 2000).

12. There is evidence to indicate that activation of dTAK1 and IKK involve K63-linked polyubiquitination, possibly as a scaffold for the assembly of active complexes. There is also evidence that ubiquitin conjugating E2 enzyme Bendless (homologous to mammalian ubiquitin-conjugating enzyme 13 - Ubc13) and *Drosophila* ubiquitin-conjugating enzyme E2 variant 1 (dUev1A) are essential components of IMD signalling and are probably involved in polyubiquitin chain formation. Collectively, these highlight the remarkable similarities that exist between the mammalian and insect innate immune systems (Chen, 2005; Zhou et al., 2005).

13. Activated dTAK1 (in complex with dTAB2) also transiently activates the c-Jun-N-terminal kinase (JNK) pathway. The dTAK1-dTAB2 dimeric complex is also central in the TNF/Eiger-JNK apoptosis cascade. Thus, dTAK1 is the nexus that interlinks both immunity and apoptosis (Geuking et al., 2005; Silverman et al., 2003).

14. Since dTAK1 is central to activating these cascades, it is reasonable to assume that dTAK1 activity will be carefully regulated and several processes may be involved. It is known that the JNK signal is rapidly terminated after immune activation of dTAK1 and both Relish and POSH (Plenty of SH3; an E3 ubiquitin ligase) are involved (Park et al., 2004; Tsuda et al., 2005).

15. In the second process, the activated tripartite complex of IMD, dFADD and DREDD is thought to endoproteolytically cleave phosphorylated Relish. The C-terminal ankyrin repeats of Relish remain in the cytoplasm, while the REL domain containing N-terminus migrates to the nucleus and regulates gene expression of a battery of AMP genes including *diptericin* (Stoven et al., 2003).

1.4.2 Negative regulation of the IMD pathway:

Although numerous investigations have been conducted into the activation of the IMD pathway, much less is known about mechanisms that either negatively regulate activated proteins after infection or repress antimicrobial genes in the absence of infection. Several negative regulators have been discovered in the recent past.

The RING finger protein Dnr1 was discovered as an inhibitor of DREDD in cell culture. It was also shown that immune stimulation stabilises Dnr1 in a DREDD-dependent manner indicating that Dnr1 may be a negative feedback loop where immune stimulation, via DREDD, promotes accumulation of a DREDD inhibitor (Foley and O'Farrell, 2004).

Caspar, the *Drosophila* homolog of the mammalian Fas-associating factor 1 (FAF1) was found to negatively regulate IMD signalling by blocking the nuclear translocation of Relish. It was further shown that the DREDD-dependent cleavage of Relish was the target of Caspar-mediated IMD suppression. Further, Caspar was highly specific for IMD signalling and did not affect the Toll pathway. (Kim et al., 2006).

Khush *et. al.* (2002) showed for the first time that the *Drosophila* IMD pathway is repressed by the ubiquitin-proteasome system. It was shown that loss-of-function mutations in the *SkpA* gene (the *Drosophila* homolog of human Skp1 protein) resulted in constitutive expression of *Diptericin*. Skp1 proteins function as subunits of SCF-E3 ubiquitin ligases that target substrates to the 26S proteasome. It was further shown that mutations affecting either the *Drosophila* SCF components, Slimb and dCullin1, or the proteasome also induce *Diptericin* expression. In cultured cells, inhibition of SkpA and Slimb via RNAi increases levels of both the full-length Relish protein and the processed Rel-homology domain, thereby implicating Relish as the target for ubiquitin-mediated proteasomal degradation (Khush et al., 2002).

More recently, Pirk was shown to be a negative regulator of IMD signalling. Pirk bound to both IMD and the cytoplasmic tail of PGRP-LC. It was further shown the RNAi-mediated down-regulation of Pirk resulted in hyperactivation of IMD signalling upon infection whilst Pirk overexpression drastically reduced the IMD signal (Kleino et al., 2008).

1.5 The JNK Pathway

The c-Jun-N-terminal kinase (JNK) pathway has been implicated in a variety of processes including cellular proliferation, tissue morphogenesis, apoptosis, inflammation and immune responses in both mammals and insects. It is composed of a group of Mitogen Activated Protein kinases (MAPK or stress activated protein kinases) that are generally activated by exposure to cytokines and environmental stresses (Ip and Davis, 1998; Stronach and Perrimon, 2002). The core components in *Drosophila* include JNKK Hemipterous (Hep), the JNK Basket (Bsk), and the transcription factors Jun and Fos (Geuking et al., 2005).

In mammals, there are 6 families of kinases that function as the JNKKK and involved in upstream regulation of this pathway. The scenario is much more simplified in *Drosophila* where there are only single homologues of each of the mammalian families, making it an ideal model to study this cascade (Stronach and Perrimon, 2002). During Gram-negative bacterial infection, dTAK1 transiently activates the JNK pathway although it is rapidly terminated before the sustained expression of AMP genes. There is evidence that the JNK pathway negatively regulates IMD signalling in response to infection (Kim et al., 2005; Park et al., 2004). The JNK pathway also appears to play a role in AMP gene expression but, its precise role is, as yet, unclear (Kallio et al., 2005).

The JNK pathway is also potently activated by the *Drosophila* Tumour Necrosis Factor homologue Eiger and this leads to apoptosis. In apoptotic signalling, components of the core JNK cassette were utilised. Eiger-induced cell death requires the *Drosophila* apoptosome comprising of Dronc (a caspase-9 homologue) and Dark (the Apaf-1 homologue) (Igaki et al., 2002; Moreno et al., 2002).

1.6 TNF/Eiger Apoptosis Cascade

The Tumour Necrosis Factor (TNF) family of proteins and their receptors function to transducer signals that regulate fundamental process in mammalian development and adult life. These vital processes include apoptosis, immunity, cell survival, proliferation and differentiation. Aberrations in TNF signalling are associated with numerous diseases including autoimmune conditions and cancers (Locksley et al., 2001).

Studying the mechanisms by which TNF exerts its function is complicated by the presence of numerous ligands and receptors which function through at least three intracellular signalling pathways (Locksley et al., 2001). In this light, *Drosophila* has the distinct advantage of possessing only one TNF ligand (Eiger) and one receptor (Wengen) and can be used as a simple genetically tractable model to identify all evolutionary conserved regulators and transduction components of TNF signalling (Igaki et al., 2002; Kauppila et al., 2003; Moreno et al., 2002).

Strongly resembling a subset of mammalian TNFs, Eiger is a potent activator of apoptosis. Here, Eiger uses the core cassette of the JNK signalling pathway, which in turn activates the *Drosophila* apoptotic machinery, culminating in caspase-mediated cell death through DARK and DRONC (Moreno et al., 2002). The picture of how the Eiger apoptotic signal reaches the JNK cassette has recently began to emerge. *Drosophila* TNF-associated factor-2 (dTRAF2) links the ligand-receptor complex (Eiger-Wengen) to the JNKK Hemipterous (Hep), the first component of the core cascade (Glise et al., 1995; Xue et al., 2007). The working hypothesis is that the JNKKKK, Misshapen (Msn) activates the *Drosophila* TAK1 through a receptor-adaptor complex including dTRAF2 and dTAB2. With the help of its adaptor dTAB2, dTAK1 is the JNKKK that activates

the JNKK Hemipterous, which, in turn, activate the JNK Basket and ultimately the transcription factors Jun and Fos (Geuking et al., 2005; Riesgo-Escovar et al., 1996).

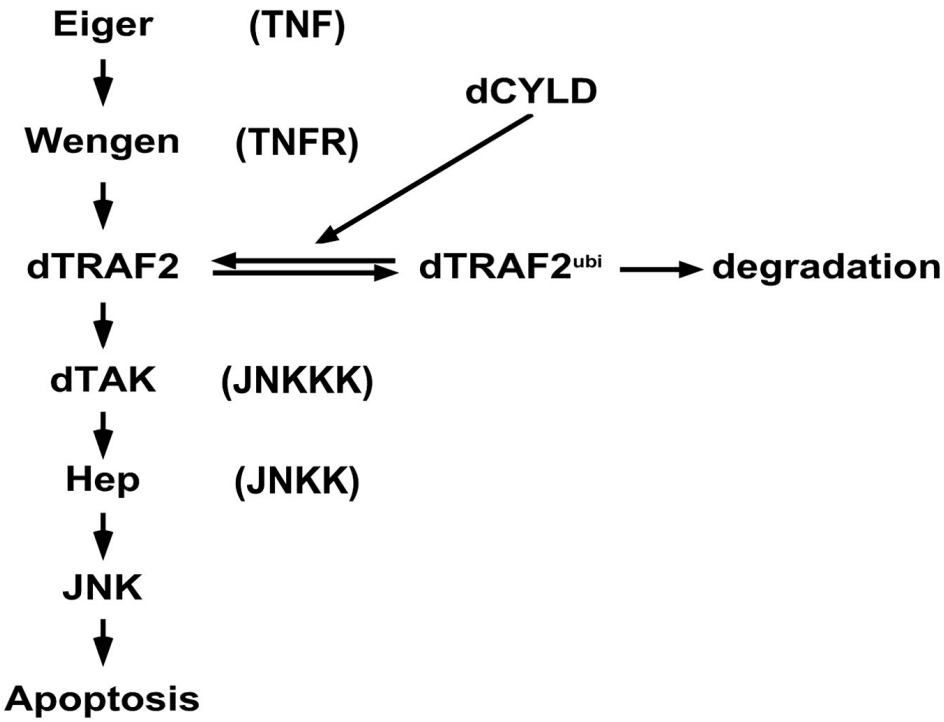


Figure 1 - 4: **A model for TNF/Eiger apoptotic signalling in *Drosophila melanogaster*.** Proposed by Xue, L., T. Igaki, et al. (2007). "Tumor Suppressor CYLD Regulates JNK-Induced Cell Death in *Drosophila*." Developmental Cell **13**(3): 446-454.

1.6.1 Current model for TNF/Eiger apoptotic signalling in adult *Drosophila melanogaster*:

1. Eiger (the *Drosophila* TNF homologue) is a potent activator of apoptosis and is named in memory of the numerous mountaineers that have been killed by the Eiger Nordwand, the "wall of death" (Moreno et al., 2002). Eiger is a type II membrane protein consisting of a short N-terminal cytoplasmic domain, a single transmembrane domain and a long C-terminal extracellular domain which comprises the TNF homology domain (THD). This domain shows significant sequence homology (20-25% identity) to other members of the TNF family. Further, similar to other members of the TNF family, Eiger can be cleaved from the cell surface and secreted into the medium (Kauppila et al., 2003; Moreno et al., 2002). Eiger has 2 isoforms called Eiger-long (Eiger-L) and Eiger-short (Eiger-s). These isoforms differ by five residues with Eiger-L predominating in abundance (in tissue) by 5 to 10-fold (Kauppila et al., 2003).

2. Wengen (named after a village at the foot of Mt. Eiger) was identified as the *Drosophila* TNF receptor (Kanda et al., 2002; Kauppila et al., 2003). The extracellular domain of Wengen contains a cystein-rich pseudorepeat which shows significant homology to other TNFR family members. It was initially reported as a type 3 membrane protein but, there is evidence it could be a type 1 membrane protein (Kanda et al., 2002; Kauppila et al., 2003). A model for Eiger induced apoptotic signalling was proposed by Geuking *et. al*., in 2005. This followed similar *albeit* less detailed models proposed by Igaki *et. al*. (2002) and Moreno *et al*. (2002).

3. In mammals, ligand-induced apoptosis is modulated by caspase-8. However, deletion of caspase-8 homologue DREDD in *Drosophila* had little effect on Eiger-induced apoptosis (Moreno et al., 2002). Here, Eiger transduced the signal through the core JNK cassette. There is evidence that Eiger induces a strong transcriptional activation of the pro-apoptotic genes *hid* and to a lesser extent, *reaper* (Moreno et al., 2002).

4. In the absence of apoptotic stimuli, inhibitor of apoptosis (IAP) proteins like *Drosophila* IAP1 (dIAP1) inhibit caspase activity by binding to them. During apoptotic signalling RHG domain containing pro-apoptotic proteins like Hid, Reaper, Grim bind to dIAP1, leading to its ubiquitin-mediated degradation and consequent release of caspases. These caspases then modulate apoptosis (Xu et al., 2009). Current research shows that TNF/Eiger-JNK signalling activates DRONC by degrading the IAPs via a transcriptional upregulation of *hid* (Moreno et al., 2002).

5. Apoptotic signalling is potently initiated by Eiger. Eiger is differentially glycosylated and can be cleaved and released into the medium. It is thought that Eiger binds to the TNFR domain (cystein-rich psuedorepeat) of Wengen through its TNF homology domain (Kanda et al., 2002; Kauppila et al., 2003).

6. The next process leading to the phosphorylation and activation of dTAK1 is yet to be fully elucidated. It is known that the JNKKKK Misshapen (a Ste20 kinase; Msn) is involved in Eiger signalling and is active upstream of dTAK1 (Geuking et al., 2005; Moreno et al., 2002). Geuking *et. al*., in 2005 proposed that

following binding of Eiger and Wengen, a signalling complex composed of *Drosophila* TNF receptor associated factor 1 (dTRAF1), Msn, *Drosophila* TAK1and *Drosophila* TAB2 forms at the cytoplasmic domain of Wengen which allows the phosphorylation and activation of dTAK1 by Msn.

7. However, it was recently shown that dTRAF2 stability was paramount for Eiger signalling. Mutations in the de-ubiquitinating enzyme dCYLD lead to increased degradation of dTRAF2 and disrupted JNK activation. Also, Eiger-induced apoptosis was completely suppressed in the absence of dTRAF2 while removal of dTRAF1 had little or no effect (Xue et al., 2007). Further, dTRAF2 exhibited a stronger physical interaction with both Wengen and dTAB2 than dTRAF1 (Geuking et al., 2005).

8. Initiation of apoptosis leads to the phosphorylation and activation of dTAK1 possibly by Msn, which is facilitated by dTAB2 (Geuking et al., 2005). Subsequently, dTAK1 activates the core JNKK-JNK module of Hemipterous (Hep) and Basket (Bsk). This leads to the activation of the *Drosophila* apoptosome consisting of DRONC and DARK and subsequent cell death (Geuking et al., 2005)

1.7 Ubiquitination-dependent regulation of NF-κB signalling

Ubiquitination has been implicated in NF-κB immune signalling in both *Drosophila* and mammals (Chen, 2005; Zhou et al., 2005). Moreover, both K63- and K48-linked polyubiquitination have been identified in signal transduction and termination in the *Drosophila* IMD pathway (Paquette et al., 2010; Tsuda et al., 2005). Ubiquitin is a highly conserved 76 amino acid residue present in most tissue in

eukaryotes. Ubiquitination is the covalent ligation of a single residue (mono-ubiquitination) or several residues in the form of a chain (poly-ubiquitination) to a substrate protein (Hershko and Ciechanover, 1998). The ubiquitin system plays an important role in the regulation of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis. It is implicated in immunity, development and apoptosis. Aberrations in the ubiquitination system lead to diseases including cancers and neurodegenerative disorders (Hershko and Ciechanover, 1998; Kirisako et al., 2006).

Conjugation of ubiquitin to substrates (either mono- or polyubiquitination) plays a crucial role in cellular processes. In formation of polyubiquitin chains, ubiquitin residues are linked through isopeptide bonds formed between an internal Lys and the C-terminal Gly (Kirisako et al., 2006). Polyubiquitin chains are formed when ubiquitin monomers attach to other ubiquitin monomers through one of the seven Lys residues. The most studied of these are the K48-linked and K63-lined chains. K48-linked polyubiquitin chains target a protein for proteasomal degradation while polyubiquitin chains liked by K63 function in signal transduction, DNA repair and gene transcription, amongst others, through a degradation-independent mechanism. There is also evidence of polyubiquitination via other Lysine residues (other than K48 and K63) and also of branching (Chen, 2005; Kim et al., 2007; Kirisako et al., 2006). Structural studies have shown that K48-and K63-linked polyubiquitin chains have different configurations thus, providing a molecular basis for their differing functions (Chen, 2005).

One of the key functions of the ubiquitin system is regulating or “recycling” proteins – ubiquitin binds to proteins and tags them for degradation via the 26S proteosome system (Kirisako et al., 2006). The discovery of the protein-regulating

system led to Aaron Ciechanover, Avram Hershko and Irwin Rose being awarded the Nobel Prize for Chemistry in 2004 (Kirisako et al., 2006; Nobel-Prize, 2004).

Ubiquitination occurs in 3 sequential steps catalysed by 3different classes of enzymes - E1, E2 and E3 (Hershko and Ciechanover, 1998).

1. **Ubiquitin activation**. Ubiquitin-activating enzyme (E1) activates the C-terminal Gly of ubiquitin in an ATP-dependent process encompassing 2 steps. Firstly, ATP is hydrolysed (releasing PP_i) and an ubiquitin-adenylate intermediate is produced. Secondly, ubiquitin binds to the active cysteine of E1 in a thiol ester linkage and AMP is released. The adenylation of an additional ubiquitin occurs in concert (Hershko and Ciechanover, 1998).

2. **Transfer of ubiquitin to an E2 enzyme**. Activated ubiquitin is then transferred from E1 to the active site cysteine of an ubiquitin-conjugating enzyme (Ubc; E2) to form a thiol ester. A large number of E2 enzymes have been identified and it has been hypothesised that E2 enzymes may recognise protein substrates directly or in association with E3 enzymes (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998).

3. **Linking ubiquitin to a substrate**. An isopeptide bond is formed between the ε-amino groups of a Lys of the substrate protein and the C-terminal glycine of ubiquitin in a reaction mediated by E3 ubiquitin-protein ligases (Hershko and Ciechanover, 1998; Kirisako et al., 2006). E3 ligases will have one of two domains:

(A) Really Interesting New Gene (RING) domain: Here, ubiquitin is transferred directly from the E2 enzymes to the substrate, catalysed by the E3 enzymes.

(B) Homologous to the E6-AP Carboxyl Terminus (HECT) domain: in this, a covalent E3-ubiquitin intermediate is formed. Thereafter, ubiquitin is transferred to the substrate (Hershko and Ciechanover, 1998).

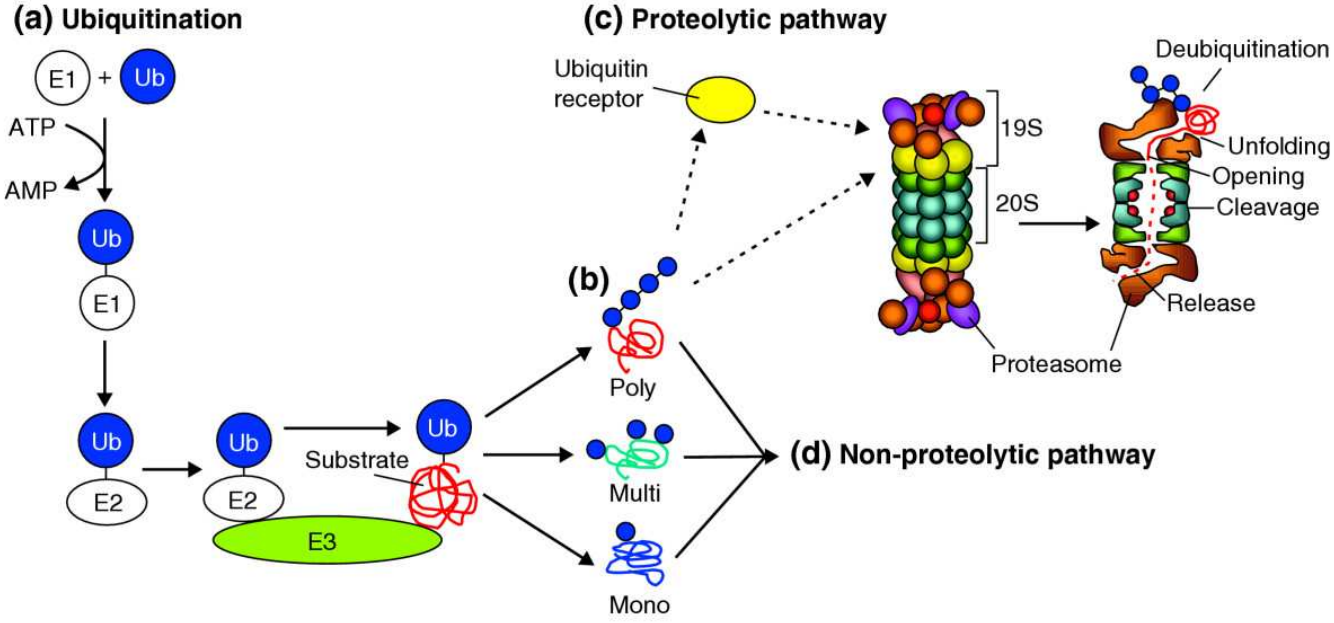


Figure 1 - 5: The Ubiquitin proteasome system showing (a) process of ubiquitination (b) attachment of ubiquitin to a substrate (c) degradation via proteolytic cleavage and (d) non-proteolytic pathway. Processes are explained in the text. Adapted from: (Kaiser and Huang, 2005).

1.8 Regulation of Innate Immune Signalling by deubiquitination

The NF-κB transcription factors regulate genes involved in inflammation and immunity. Aberrations in its regulation, especially sustained/chronic activation, lead to numerous autoimmune diseases and cancers. Therefore, activation and duration of NF-κB signalling is transient and tightly controlled (Sriskantharajah and Ley, 2010).

Under normal conditions, NF-κB is maintained in an inactive state by Inhibitors of NF-κB (IκB) proteins. Stimulation by proinflammatory cytokines lead to the degradation of IκB proteins and thereby lift the inhibition of NF-κB elements, which then migrate to the nucleus and promote the expression of inflammatory genes (Sriskantharajah and Ley, 2010). At least 3 steps of this pathway is regulated by ubiquitination including the activation of the IκB kinase (IKK), degradation of IκB (inhibitor of NF-κB) and the processing of NF-κB precursors (Chen, 2005).

1.8.1 Deubiquitinating enzymes (DUBs):

Ubiquitination, like phosphorylation, is a reversible covalent modification (Chen, 2005). Attached ubiquitin can be removed by deubiquitinating enzymes (DUBs). These are cysteine proteases that precisely cleave proteins at the Ub–protein bond (Balakirev et al., 2003). Deubiquitination is highly regulated and has been shown to be involved in numerous processes including cell cycle regulation, gene expression, DNA repair, microbial pathogenesis, kinase activation and proteasome-and lysosome-dependent protein degradation. Also, mutations in DUBs have been linked to cancers and neurological disorders (Reyes-Turcu et al., 2009). DUBs in humans are classed into 5 families. Four families are cysteine proteases including ubiquitin C-terminal hydrolase (UCH), ubiquitin-specific protease (USP/UBP), the ovarian tumour domain (OTU), and

Josephin domain (MJD). The other, JAB1/MPN/Mov34 metalloenzyme (JAMM) domain is a zinc-dependent metalloprotease family (Reyes-Turcu et al., 2009).

Recently, two deubiquitinating enzymes, CYLD and A20 have been implicated in negatively regulating the NF-κB pathway in humans (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003; Wertz et al., 2004).

Tumour suppressor protein CYLD exhibits DUB activity and functions in multiple signalling pathways by removing K48 and/or K63-linked polyubiquitin chains from its target. Mutations in the *CYLD* gene are associated with familial cylindromatosis (Brooke-Spiegler syndrome). CYLD has been shown to inhibit IKK activation in humans by cleaving Lys 63-linked polyubiquitin chains on several proteins, including TRAF2, TRAF6 and NEMO. Mutations within its DUB domains lead to the sustained activation of NF-κB, thereby contributing to the pathogenesis of the tumours (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). In *Drosophila*, CYLD was shown to function in the TNF/Eiger apoptotic signalling by deubiquitinating (and thereby stabilising) dTRAF2 (Xue et al., 2007).

The cytoplasmic protein A20 has emerged as a negative regulator of NF-κB signalling and a key element in limiting the duration of its signal through its dual ubiquitin editing functions on the adaptor RIP1 (Heyninck and Beyaert, 2005). A20 has an N-terminal OTU domain and 7 zinc-finger domains in its C-terminus. A20 cleaves K63-linked polyubiquitin chains on RIP1 through its OTU domain and re-assembles K48-linked chains through the E3 ligase activity of its zinc-finger structures, thereby tagging RIP1 for proteosomal degradation. Evidence suggests that A20 also inhibits the polyubiquitination and activation of human TRAF6 in the Toll-like receptor 4 and

interleukin-1 receptor (TLR4/IL-1R) pathways. Studies have also implicated A20 in TNF mediated cell death (Heyninck and Beyaert, 2005; Shembade et al., 2010).

1.9 Investigating *Drosophila* Tak1 regulation of immunity and apoptosis

1.9.1 *Drosophila* TAK1 the "nexus" between immunity and apoptosis

Stimulation of the IMD pathway through Gram-negative bacterial infection results in an immune response. The JNK pathway is also transiently activated and is possibly involved in regulating the immune response. However, apoptosis is not triggered (Park et al., 2004). Similarly, induction of TNF/Eiger-induced apoptosis through the core JNK cassette results in cell death but does not affect immunity (Xue et al., 2007).

Further, *Drosophila* TAK1 is an essential component of both the IMD and JNK pathways. Moreover, both pathways bifurcate at the level of and dTAK1. (Lemaitre and Hoffmann, 2007). *Drosophila* TAK1 plays a crucial role in activating IMD mediated innate immunity and mutations in dTAK1 resulted in the abrogation of the immune signal (Vidal et al., 2001). Similarly, *Drosophila* TAK1 is also an essential component of TNF/Eiger-JNK apoptotic signalling (Geuking et al., 2005).

Therefore, dTAK1 is both a principal component of and nexus between 2 pathways which elicit two very different responses (i.e. immunity and apoptosis). Hence, the question of how dTAK1 activity is regulated is very pertinent to understanding the precise regulation of both IMD and TNF/Eiger pathway activation during Gram-negative bacterial infection and also the regulation between immunity and cell death.

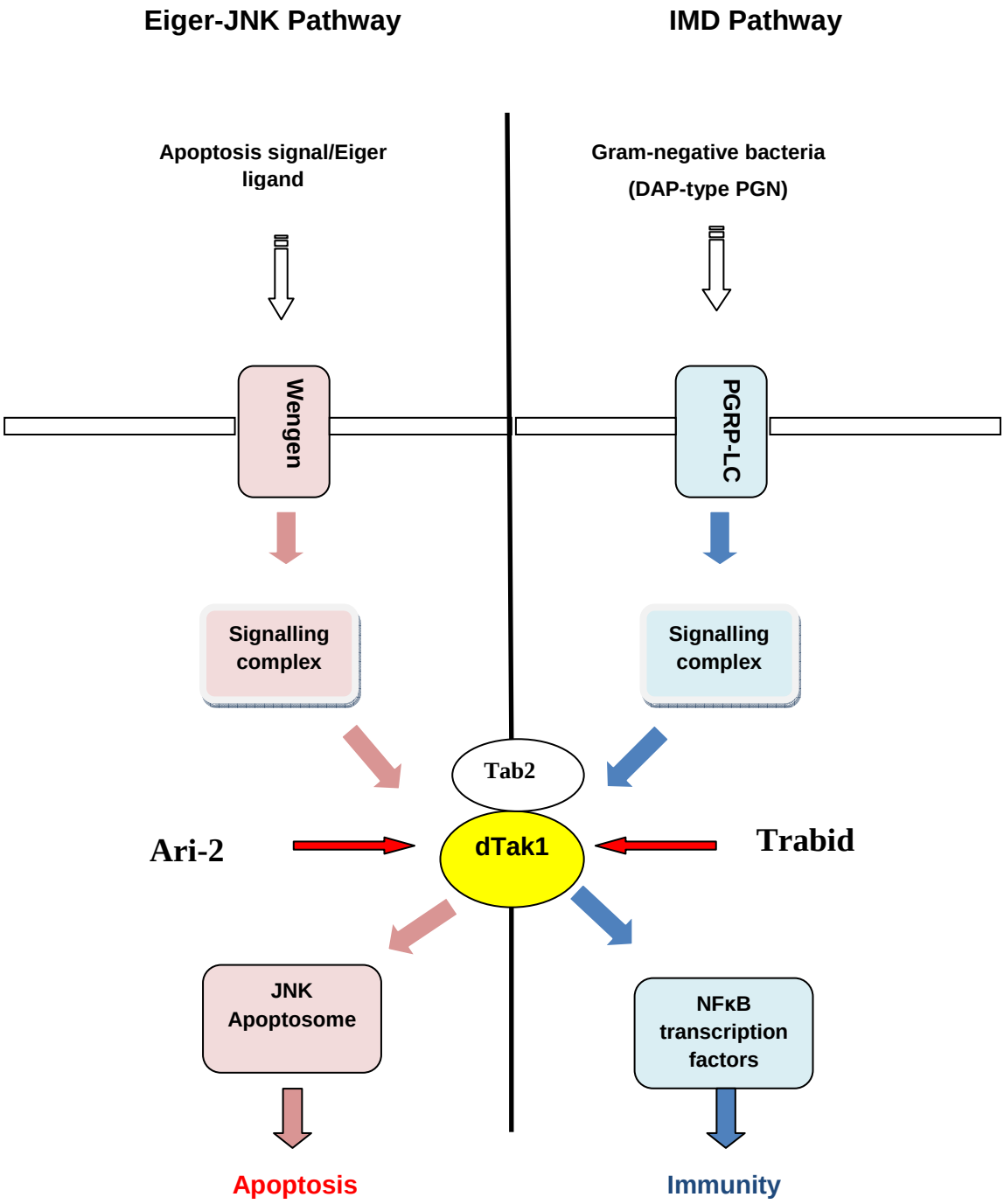


Figure 1 - 6: ***Drosophila*** **TAK1 - the 'nexus' between innate immunity and cell death**: Simplified schematic diagram depicting the central role of dTAK1 in mediating both immunity and apoptosis through the IMD and JNK pathways in response to different stimuli, with the relative positions of Trabid and Ari-2.

1.9.2 Aims and Objectives of this investigation:

The aims of this investigation were to elucidate the regulation of dTAK1 in (1) the IMD innate immune pathway and (2) the TNF/Eiger apoptotic cascade using *Drosophila melanogaster* as the model organism. A variety of biochemical, molecular biological and genetic techniques were utilised, as described in Chapter 2.

1.9.3 Summary of results of this investigation:

In this study, I report the discovery of *Drosophila* Trabid (Trbd; CG9448) as a novel component of the IMD pathway and a negative-regulator of dTAK1. Trabid alters K63-linked polyubiquitination in dTAK1 through its OTU and NZF domains attenuating the IMD innate immune signal. Evidence is presented to show that both Lysines 142 and 156 are essential for dTAK1 function in the IMD pathway and that Lys 142 is the probable K63 polyubiquitin acceptor site. Also, evidence indicates that Lysine 156 is involved in modulating both K63 and K48 polyubiquitin chains, possibly in a partially redundant role as an Ubiquitin acceptor site. Moreover, the C-terminal ZnF domain of dTAB2 plays a role in stabilising and modulating the dTAK1 signal. Taken together, my findings indicate an elaborate and multi-tiered process regulating dTAK1 function during Gram-negative bacterial infection.

I further report the characterisation of Ariadne-2 (Ari-2; CG5709), a novel component of the TNF/Eiger pathway that plays a role in stabilising dTRAF-2. Ari-2 contains a novel TRIAD (two RING and a DRIL) domain and its human ortholog Triad1 functions to stabilise growth factor independence 1 (Gfi1) during haematopoiesis. Results show that loss of Ari-2 compromised JNK dependent development and adult lifespan. Moreover, it blocked Eiger-induced cell death when the

latter was over-expressed in the adult eye. Conversely, Ari-2 enhanced signalling when concomitantly expressed with Eiger in the *Drosophila* eye. My results further show that Ari-2 functions as an adaptor for the stabilisation and assembly of a larger signalling complex comprising dTRAF2, dTAB2 and dTAK1 in the TNF/Eiger apoptotic cascade. Its human homologue Traid1 also functions to stabilise Growth factor independence 1 (Gfi1) and prevent its ubiquitination in granulocytes in a similar manner (Marteijn et al., 2007). Co-immunoprecipitation assays show that Ari-2 interacts with dTRAF2, dTAK1 and dTAB2 (with the N-terminal half being sufficient for this interaction). Deletion of the ZnF, coiled-coil and the 2 RING domains abolished the function of Ari-2 in apoptosis *in vivo*, thereby suggesting that these domains may be involved in binding with the components of the putative complex transducing the apoptotic signal. My results show that dTRAF2 is stabilised through K63-linked polyubiquitination in the presence of dTAB2 and dTAK1. In this manner, adaptors like Ari-2 specificity to such complexes (like dTRAF2-dTAB2-dTAK1) and elicit a specific response. The specificity of the complex would determine which downstream cascade is activated and would explain how dTAK1 (like its human homolog) can activate different pathways, upon stimulation by different cytokines. Finally model is proposed for *Drosophila* TNF/Eiger apoptotic signalling in which Ari-2 functions as an adaptor to form a signalling complex and stabilises dTRAF2 at the level of dTAK1.

Chapter 2: Materials and Methods

2.1 *Drosophila melanogaster* – the Model Organism

The use of *Drosophila melanogaster* as a model organism harks back to the beginning of the 20th century when T.E. Morgan, renowned Geneticist and Nobel Laureate, used it to investigate the chromosomal theory of inheritance. Since then, it has gained enormous popularity as one of the favourite model organisms of geneticists (St Johnston, 2002).

Drosophila melanogaster has distinct advantages as a model organism. It is inexpensive and easy to grow; it has a very short generation time of 10 days at 25°C and produces many progeny with each female laying around 1000 eggs (St Johnston, 2002). As a model to study genetics, it has even greater advantages. It has only 4 chromosomes which can be visualised in the giant polytene chromosomes of the larval salivary glands. Also, there is no meiotic recombination in males. Further, the exoskeleton has many external features (bristles, wing veins, compound eyes etc.) which can be affected by mutations, and the resultant mutant phenotypes observed by stereomicroscopy (St Johnston, 2002).

Recent advances have only served to increase the attractiveness of *Drosophila melanogaster* as a model. The development of the *GAL4-UAS* system has meant that any cloned gene can be selectively activated to varying degrees in a very wide variety of cell and tissue specific manner (Brand and Perrimon, 1993). New discoveries have opened new vistas where *Drosophila* has become interesting in other fields. The sequencing of the *Drosophila* genome in 2000 has added to this interest. Although

Drosophila melanogaster has only ~ 15,000 genes, a high number have clear human homologues (*E*-value $<10^{-50}$) (Adams et al., 2000; St Johnston, 2002). A large number of development processes appear to be conserved between flies and vertebrates.

Certain aspects of the immune system too, show strong conservation, including the innate immune system. Surprisingly, of the 287 known human disease genes, 178 (~62%) appear to be conserved in *Drosophila*. Strikingly, even some of the non-homologous disease genes (like Parkinson's), when expressed in *Drosophila melanogaster*, can produce symptoms similar to those in humans (Fortini et al., 2000; St Johnston, 2002).

Moreover, innate immunity can be studied at the level of the whole organism in *Drosophila* where resistance to infection by mutant strains can be monitored. Sensitivity to infection corresponds with decreased induction of the innate immune pathways which can be monitored by expression levels of AMP genes a few hours after infection (Ferrandon et al., 2007).

In addition, large-scale genetic screens for mutations that affect a given process can be carried out in *Drosophila melanogaster*. This strategy provides an unbiased way to identify genes that function in a particular process. Also, the mutants themselves are a very useful tool to elucidate the function of that particular gene (St Johnston, 2002).

Therefore, *Drosophila melanogaster* is an ideal to use in this study as a model to investigate both innate immunity and apoptosis *in vivo*.

2.2 *Drosophila* Schneider's Line 2 (S2) – Cell Culture

Drosophila Schneider's line 2 or simply 'S2' is a cell-line established from primary cultures of late stage (20-24hrs) *Drosophila* embryos as a spontaneously immortalized, nonclonal cell line. S2 cells are the most widely used *Drosophila* cell line (Rogers and Rogers, 2008; Schneider, 1972).

Drosophila S2 cells have many advantages that make them ideal as a model. Cells are easy to grow and maintain. They divide rapidly (a cell cycle of about 20hrs), can be grown at room temperature (20–25 °C) without CO_2, and do not require specialized equipment for their culture apart from a sterile hood for passage. They form a loose, semi-adherent monolayer in tissue culture flasks and in suspension in shake flasks (Invitrogen, 2002; Rogers and Rogers, 2008).

Moreover, the ease of introducing transgenes into cells enables them to be used for preliminary functional characterisation of genes before generation of fly transformants. Further, S2 cells provide an ideal platform for loss of function studies using double-stranded RNA (dsRNA) since the cells are highly susceptible to gene inhibition using RNA interference (RNAi) and have very little redundancy when compared with mammals. S2 cells are also well suited to high-resolution light microscopic assays (Dahmann et al., 2008; Rogers and Rogers, 2008). Moreover, RNA interference is much cheaper and easier in insect cells and this has led to genome-wide RNAi screens using *Drosophila* S2 cells (Dahmann et al., 2008)

S2 cells show several properties that indicate a hemocyte-derived lineage (macrophages responsible for phagocytosis of invading bacteria and play a role in

immune responses). In particular they exhibit Toll and Imd-mediated induction of AMPs and have the capacity to robustly phagocytose microorganisms. Several studies have been conducted on phagocytosis of *Escherichia coli*, *Mycobacterium fortuitum*, *Listeria monocytogenes* and *Candida albicans* in S2 cells using RNAi. Thus, S2 cells are excellent models for the study of intracellular infections and apoptosis (Ferrandon et al., 2007; Stroschein-Stevenson et al., 2005).

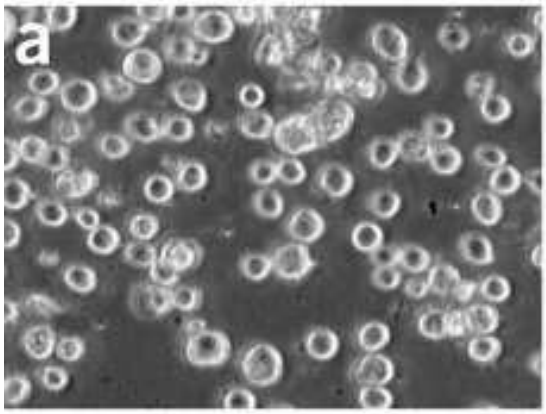


Figure 2 – 1: *Drosophila* S2 cells photographed through phase-contrast microscopy. Cells are typically 5–10µm in diameter. Adapted from: Rogers, S. L. and G. C. Rogers (2008). "Culture of *Drosophila* S2 cells and their use for RNAi-mediated loss-of-function studies and immunofluorescence microscopy." Nat. Protocols **3**(4): 606-611.

2.3 Cell Culture and Protein Biochemistry

2.3.1 General Cell Maintenance:

Drosophila S2 cells (Invitrogen) were maintained at 25°C in Schneider's *Drosophila* Medium (BioWhittaker/Lonza), supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS) and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin sulfate – all Invitrogen). Cells were maintained in $25cm^2$ treated tissue culture flasks designed for adherent cell culture (Greiner Bio One) and passaged or subcultured to a final density of 2 - 4 x 10^6 cells/ml every 3 to 4 days. All cell culture manipulations were done under aseptic conditions in a Microflow Peroxide Class 2 Advanced Biological Safety Cabinet (Astec Microflow).

2.3.2 Transient Transfection of Cells:

Introduction: Transfection is the process of introducing nucleic material into eukaryotic cells by nonviral methods. Nuclear material includes DNA, RNA, short interfering RNA (siRNA) and oligonucleotides. Various physical, lipid and chemical methods are utilised. It is a technique widely utilised in a variety of fields including microbiology and genetics. Transfection techniques, when coupled with assay-based reporter technology, enables and enhances the study of protein:protein interactions, mRNA processing, translation and recombination events, mammalian promoter and enhancer sequences, *trans*-acting proteins such as transcription factors etc. (Encyclopædia-Britannica-Online, 2011; Promega, 2011; Tuan et al., 1997)

There are 2 types of transfection – transient and stable. In transient transfection, the introduced nucleic material is not permanently incorporated into the genome of the transfected cell and so its effects will last for only a short period. By contrast, stable transfection results in the introduced nuclear material being permanently incorporated

into the genome of the cell and so, produces effects that are long lasting (Encyclopædia-Britannica-Online, 2011; Promega, 2011).

Principle: Effectene Transfection Reagent is non-liposomal lipid formulation designed to transfer DNA into eukaryotic cells with high transfection efficiencies. To achieve this, it utilises the DNA-condensation ability of Enhancer and Buffer EC. In the first step, DNA is condensed by interaction with the Enhancer in a defined buffer system (Buffer EC). In the second step, Effectene Reagent is added to the condensed DNA to produce condensed Effectene–DNA complexes (micelle structures). These structures show no size or batch variation thus ensuring excellent reproducibility of results. The Effectene–DNA complexes are then mixed with medium and directly added to the cells (QIAGEN, 2002)

Protocol: Cells were transiently transfected using Effectene Transfection Reagent (QIAGEN) according to the manufacturer's instructions. Briefly, 5ml of cells (at a concentration of $7.5x10^5$cells/ml) were subcultured into 60mm treated tissue culture dishes (Greiner Bio One) and incubated for 24 hrs to reach a confluence of ~ 90%. For each expression vector, 2ug of transfection grade plasmid DNA (in TE Buffer, pH 8.0) was used. Each expression vector was diluted in Buffer EC (DNA condensation buffer) to a total volume of 150µl in sterile 1.5ml microcentrifuge tubes (Greiner Bio One). Next, Enhancer was added in a ratio of 8µl for 1µg of DNA, the mixture gently mixed by tapping and incubated for 5 mins. Effectene reagent was then added in a ratio of 10µl for 1µg of DNA, mixed by gentle pippetting and incubated for 10 mins (to allow for transfection complex formation). Thereafter, fresh growth medium was added to the transfection complexes (to a final volume of 1ml) and gently mixed. While complex formation occurred, growth medium was gentle aspirated from cell containing tissue

culture dishes and cells washed with 4ml of tissue culture grade 1x PBS pH 7.4 (phosphate buffered saline – Sigma-Aldrich). Thereafter, 3ml of fresh growth medium was added to the cells. The transfection mixture (1ml) was then added drop-wise onto the cells, gently swirled to ensure uniform distribution and incubated at 25°C for 12 hrs. Empty pAc5.1/HA-His vectors were used to ensure equal amounts of DNA were delivered in each transfection. Subsequently, the transfection mixture was removed by gentle aspiration, cells washed once by 4ml of 1xPBS and incubated for a further 48 hours with 5ml of fresh growth medium supplemented with heat inactivated-FBS and antibiotics as detailed above.

2.3.3 Co-immunoprecipitation (Co-IP)

Introduction: This is a common technique used to identify physiologically relevant protein:protein interactions in the cytoplasm of cells. It is often used in conjunction with SDS PAGE and Western Blotting procedures (Clark, 2008).

Principle: Immunoprecipitation involves the precipitation of a protein (or other biological molecule - antigen) from a mixture of other proteins and biological molecules in a cell or tissue homogenate using an antibody specific to the target protein (antigen). By extension, if another protein is bound to the target protein (antigen), this second protein too will be precipitated from the mixture together with the primary target and this is the basis for co-immunoprecipitation assays. The antibody is incubated in the mixture, allowing it to form an immune complex with the target protein. The immune complex is then 'captured' or 'precipitated' on a solid support containing immobilized Protein A or Protein G (antibodies bind with high affinity to Protein A or G). Other non-complexed proteins are washed away. The bound antigen-antibody complex is eluted from the immobilized beads and further analysed usually by SDS PAGE and Western

Blotting for identification (Clegg, 1998; Thermo-Fisher, 2010). More frequently, small tags are engineered into target proteins which are then expressed in culture cells. This enables the use of antibodies specific to the tag, instead of the protein (Clark, 2008).

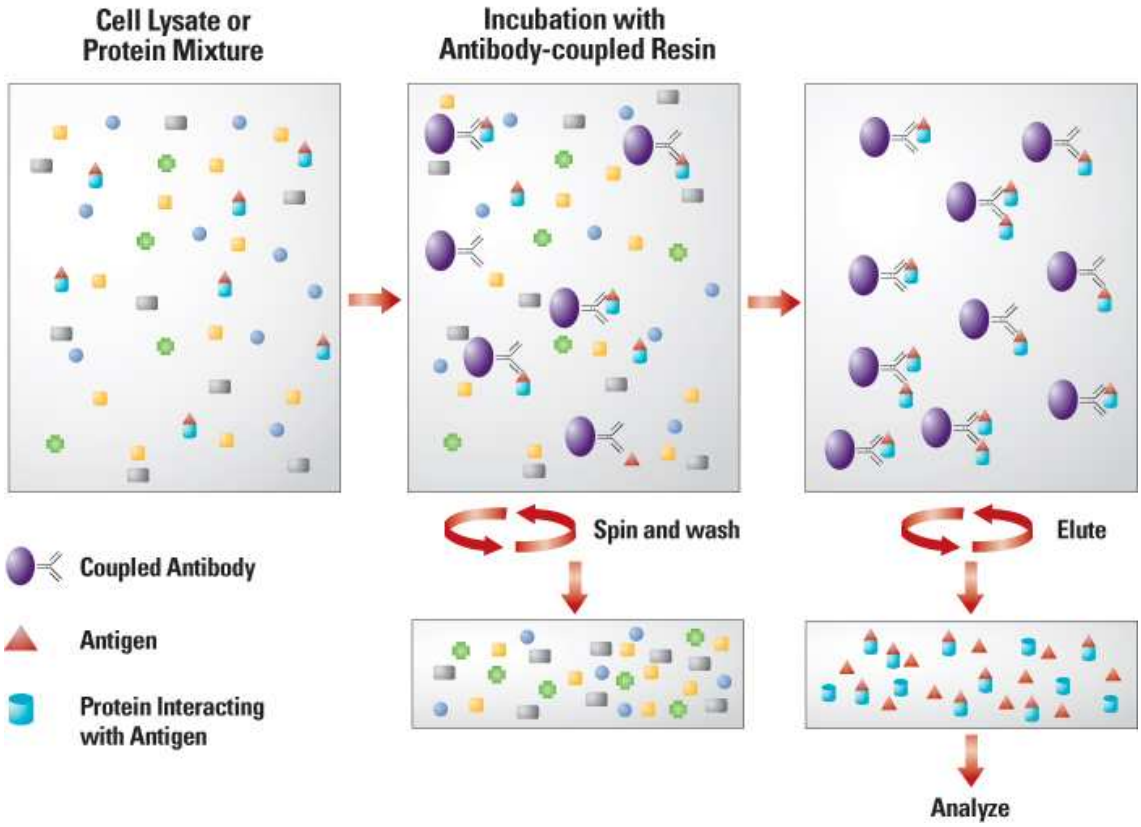


Figure 2 - 2: **Schematic summary of a standard co-immunoprecipitation assay.** Cell lysate contains a mixture of proteins. Addition of the antibody coupled resin results in the binding of the target protein (antigen) to the antibody. Secondary proteins bound to the target are also 'captured.' Washing of the antibody beads remove other proteins. The protein-protein complexes bound to the antibody are then eluted and analysed. Adapted from "Protein Interaction Technical Handbook" (Thermo-Fisher, 2010)

Co-immunoprecipitation is often used to confirm the interaction between 2 proteins from yeast-2-hybrid assays. In such instances, the 2 proteins in question can be tagged with 2 different epitopes and simultaneously expressed in cultured cells. One of the targets can then be precipitated (using an antibody specific for its tag), resolved by SDS PAGE and the presence of the other protein can be probed with different antibody for its tag using the Western blotting technique (Clark, 2008).

Protocol: *Drosophila* S2 cells were transiently transfected with the relevant expression vectors (as above). S2 cells were harvested 48 hrs post-transfection. S2 cells were removed from tissue culture dishes with the aid of cell scrapers (Greiner Bio One), collected in 15ml centrifuge tubes (Greiner Bio One), centrifuged at 1400rpm for 5 mins in an Allegra 6 centrifuge (Beckman Coulter) and supernatant decanted. Cells were resuspended in 1ml 1xPBS pH 7.4 (Sigma Aldrich), transferred to 1.5ml sterile microcentrifuge tubes (Greiner Bio One), centrifuged at 3,000rpm for 5 mins and supernatant decanted carefully.

S2 cells were lysed in RIPA buffer ((1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 0.01M Tris-Cl, pH-8, 0.14M NaCl; Sigma-Aldrich) supplemented with Complete Mini Protease Inhibitor Cocktail tablets (1 tablet in 10ml of RIPA buffer; Roche Applied Science) and Benzonase Nuclease (Sigma-Aldrich). Cell lysates were incubated at 37°C for 10 mins for the protease inhibitors to act and centrifuged at 13,000rpm for 10mins to pellet the cell debris. An aliquot of ~ 50µl was withdrawn from each transfection (for cell lysates samples), mixed with 12µl of 5x SDS sample buffer and incubated at 95°C for 5 mins in 1.5ml microcentrifuge tubes. Another aliquot of 2µl (from each transfection) was retained to measure total protein.

The remaining cell lysates were supplemented with RIPA buffer to bring the total volume to 300µl and incubated in SigmaPrep spin columns with 50µl of Anti-V5 or Anti-HA Agarose Affinity Gel (Sigma-Aldrich) for 2 hours at 4°C rotating in a Stuart SB2 Laboratory Tube Rotator (Bibby Scientific). Previously, Affinity Gel beads were washed 5 times with 1xPBS (600µl each wash - to remove traces of ethanol and sodium azide) in SigmaPrep spin columns. In between washes, the Affinity Gel beads were centrifuged at 10,000rpm for 1 min to remove the supernatant.

Alternatively, Anti-HA antibody (Sigma-Aldrich) was diluted 1:100 in cell lysates and incubated in 0.5ml microcentrifuge tubes (Greiner Bio One) at 4°C for 3 hrs rotating. The cell lysate- antibody mixture was then added to Protein A-Sepharose 4B Fast Flow beads (Sigma-Aldrich) and incubated at 4°C for a further 1.5 hrs rotating.

Affinity Gel beads and Protein A-Sepharose 4B Fast Flow beads (Sigma-Aldrich) were pre-blocked (in RIPA buffer supplemented with 0.2% BSA -New England Biolabs) at 4°C for 2 hrs rotating, before addition of cell lysates.

Affinity Gel beads or antibody-Protein A complexes were then washed with 600 µl CoIP wash buffer 900 (50 mM Tris-HCl [pH 8.0], 900 mM NaCl, 5 mM EDTA [pH 8.0], 0.5% Igepal CA-6030) four times for 10 minutes each rotating at room temperature, followed by a final wash with 600 µl CoIP wash buffer 150 (50mM Tris-HCl [pH 8.0], 150mM NaCl, 5mM EDTA [pH 8.0], 0.5% Igepal CA-6030). In between washes, the Affinity Gel beads or antibody-Protein A complexes were centrifuged at 10,000rpm for 1 min to remove the supernatant.

Immunoprecipitates were then eluted in 1x SDS sample buffer. Briefly, ~50µl of 1xSDS sample buffer was added to Affinity Gel beads or antibody-Protein A complexes

in each spin column, incubated at 95°C for 5 mins and centrifuged at 13,000rpm for 1 min to elute target and bound proteins. All centrifugations were performed in a Biofuge Pico benchtop microcentrifuge (Heraeus).

2.3.4 Protein Quantification – Bradford Protein Assay

Introduction: The Bradford Assay is a spectroscopy-based rapid and sensitive method for measuring protein concentration. It is the preferred method of protein quantification in many laboratories (Walker, 2002).

Principle: This is a colorimetric assay based on the binding of the dye Coomassie Brilliant Blue G250 to protein and the resultant shift in absorbance. The unbound cationic form has an absorbance maximum at 470nm (brownish-red) and this changes to 595nm (blue) when bound to protein. The increase in absorbance at 595nm is proportional to the amount of bound dye and consequently, the concentration of protein in the sample. The absorbance at 595nm is measured and protein concentration determined by comparison to a standard curve of proteins with known concentrations (Bradford, 1976; Walker, 2002).

Protocol: Briefly, a series of standard concentrations 0 to 20µg/ml of BSA (Bovine Serum Albumin; New England Biolabs) were prepared, 1ml of Coomassie (Plus) Bradford Protein Assay Reagent (Pierce) added, incubated for 5 mins and absorbance at 595nm measured. The data was used to generate a standard curve. Thereafter, 2µl aliquots of cell lysate from different transfections were dissolved in 18µl of sterile milliQ H_2O, incubated with 1 ml of Coomassie (Plus) Bradford Protein Assay Reagent for 5 mins and absorbance measured at 595nm in UV transparent semi-micro cuvets

(Fisher). The resulting values were extrapolated to the standard curve to determine the concentration.

2.3.5 Separation and resolution of proteins – SDS PAGE

Introduction: SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is one of the most widely used methods for protein analysis. It has numerous applications including determination of protein mass (and estimation of quantity), monitoring of protein purity and, in conjunction with Western Blotting, identification of proteins in complexes (Corley, 2005).

Principle: Electrophoresis involves the migration of charged particles under the influence of an electrical field where particles move towards the electrode bearing the opposite charge. This technique can be modified to resolve macromolecules on the basis of size, shape, conformation and charge (Creighton, 1999b; Westermeier and Gronau, 2005).

In SDS PAGE, proteins are first denatured and forced to adopt a linear configuration. SDS binds to proteins in a ratio of approximately 1.4 g SDS per 1.0 g protein, giving the proteins an overall negative charge, irrespective of their native charge. This gives an approximately uniform mass : charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. Therefore, protein mobility is proportional to the inverse of $\log_{10}$ molecular weight of the protein. Consequently, larger proteins migrate more slowly while smaller proteins travel faster and move further into the gel (Corley, 2005; Westermeier and Gronau, 2005)

Most SDS PAGE gels have a narrow “stacking gel” above the main “resolving gel.” This stacking gel has both a lower pH and polyacrylamide percentage than the resolving gel. Proteins migrate through the stacking gel and aggregate at the interface with the resolving gel. This ensures that all proteins enter the resolving gel at the same time, thereby improving reproducibility and resolution (Corley, 2005)

Protocol: Gel casting stands and frames (Atto Corporation) were assembled according to the manufacturer’s instructions. The resolving gel solution was poured in upto 1cm below the comb teeth level, overlaid with isopropanol and allowed to polymerise for ~45 mins. The isopropanol was then carefully removed, the 4% stacking gel solution poured over the resolving gel, gel combs correctly positioned and the gel solution allowed to polymerise for a further 30 mins. The combs were then carefully removed and wells thoroughly rinsed with Running Buffer. Alternatively, 10% or 12% pre-cast Precise Protein Gels (Thermo Scientific) were used.

Hand-cast gels were resolved in an AE-6450 electrophoresis chamber (Atto Corporation) according to the manufacturer’s instructions. Briefly, hand-cast gels were assembled vertically in the electrophoresis chamber and tightly secured with tension plates. The chamber was filled with 1x Tris-Glycine-SDS Running Buffer (25mM Tris, 192mM glycine and 0.1% SDS; pH 8.3). Equal amounts of total protein (150 – 250 µg) were loaded into the wells and resolved at 150V for 2 hrs using a Consort E132 electrophoresis power supply (Sigma Aldrich).

Alternatively, pre-cast gels were assembled vertically in a Novex Xcell SureLock Mini-Cell electrophoresis chamber (Invitrogen) according to the manufacturer's instructions. Adaptor plates were used to secure the pre-cast gels in the electrophoresis chamber. When using Precise Protein gels, BupH Tris-HEPES-SDS Running Buffer (0.1M Tris, 0.1M HEPES, 3mM SDS; pH 8.0; Thermo Scientific) was used. Pre-cast gels were resolved at 120V for 45 mins as per the manufacturer's instructions.

2.3.6 Detection of proteins - Western Blotting:

Introduction/Principle: Western blotting is a core technique in molecular and cell biology for the detection and characterisation of specific proteins form cultured cells and tissue homogenates. It encompasses 3 steps:

1. Separation of proteins by SDS PAGE
2. Quantitative transfer of proteins from gel to nitrocellulose (other filter membrane) by capillary action or electroblotting.
3. Detection of proteins with specific primary antibodies. Usually a secondary antibody (peroxidise-linked) is used, which binds to the primary antibody-antigen complex. Complexes are detected by visualising with a chemiluminescent agent. Alternatively, radio-labelled isotopes can be used.

The technique has several application including determination of protein mass and quantity (Corley, 2005; Hames and Higgins, 1995; Walker, 2002)

Protocol – (A) Electroblotting: Proteins were resolved by SDS PAGE as above. Immobilon P PVDF membranes (Millipore) were cut into 9cm x 7cm rectangles and activated (by incubating in 100% methanol for 15 sec) as per manufacturer's instructions. The Mini Trans-Blot Cell (Biorad) was used for electroblotting. Briefly, the gels and PVDF membranes were assembled in the gel holder cassette according to the manufacturer's instructions and inserted into the electrode module. The chamber was filled with 1x Tris-Glycine Transfer Buffer (25mM Tris, 192mM glycine in 20% methanol; pH 8.0) and PVDF membranes were electroblotted at 100V, 350mA for 1 hr with an E455 Microcomputer Electrophoresis Power Supply (Consort).

(B) Antigen-Antibody Complex Formation: PVDF blots were blocked in 50mg/ml Marvel skimmed milk (Cadbury) dissolved in PBST (1xPBS supplemented with 0.1% Tween 20) either overnight or 1 hrs with rocking at 4°C. Alternatively, 1% BSA (Fraction V; Sigma-Aldrich) in PBST was used as the blocking solution when Anti-ubiquitin antibodies (Biomol) were used. Blots were probed with the following primary antibodies in a skimmed milk-PBST binding solution: mouse Anti-V5 (1:5000, Invitrogen), mouse Anti-c-Myc peroxidase (1µg.ml^{-1}.Roche Applied Science; Clone 9E10), mouse Anti-Actin (1:1000, Pierce), mouse Anti-Actin (1µg/ml, Abcam) or rat Anti-HA antibodies (200 ng.ml^{-1}, Roche Applied Science; Clone 3F10).

Alternatively, blots were probed with Anti- mono and polyubiquitin (FK2H; HRP conjugate; 1:1000; Biomol) or Anti-polyubiquitin K63-linkage- specific (Clone HWA4C4; 1:500; Biomol) in a 5% BSA in PBST binding solution. Blots were incubated at room temperature for 2 hrs with rocking in a Model STR9 3D Rocking Platform (Stuart Scientific). Thereafter, blots were washed thrice with PBST (incubated for 5mins with rocking between washes) followed by a final wash with PBST for 15

mins. Blots were then incubated (as appropriate) with the following secondary antibodies conjugated with HRP (horse radish peroxidise): Anti-mouse (1:20,000; Sigma-Aldrich), Anti-rat (1:40,000; Sigma-Aldrich) or Anti-rabbit (1:80,000; Sigma-Aldrich). As before, blots were incubated at room temperature for 2 hrs with rocking. Blots were washed thrice with PBST (incubated for 5mins rocking between washes) followed by a final wash with PBST for 15 mins. A 3D Rocking Platform STR9 (Stuart Scientific) was used for all incubations with rocking.

(C) Detection of Protein Bands: Protein bands were detected by chemiluminescence. Blots were incubated with Pierce ECL Western Blotting Reagent (Thermo Scientific) according to the manufacturer's instructions. Briefly, blots were air-dried to remove excess wash buffer and incubated for 1 min in a working solution of Detection Reagents 1 & 2 (mixed in equal parts) for 1 min at room temperature. Approximately, 0.125ml of working solution was used per $1cm^2$ of blot surface. Excess solution was removed from blots and blots were placed in clear Saran wrap film and covered to form a protective layer. Covered blots were carefully pressed to remove bubbles or air pockets present between the membrane and plastic layer. The covered blots were then placed in a film cassette and exposed with Medical general purpose X-ray film (Kodak) in a dark room. Exposure time was varied as necessary and the film developed in Compact X4 Imaging System (Xograph).

2.4 Molecular Biology

2.4.1 Gene constructs used in this study:

(A) Full-length constructs:

Drosophila tak1, tab2, traf2, trabid, ari-2, puckered and eiger were amplified by PCR from *D. melanogaster* complementary cDNA obtained from the *Drosophila* Genomics Resource Centre (DGRC; Indiana, U.S.A.) as indicated below, and cloned into pAc5.1/V5-His, pAc5.1/HA-His or pUAST vectors. Primer sequences are detailed in Appendix 2.

Table 2-1 : Details of genes used in this study

Gene	cDNA clone	GenBank Accession No.
tak1(CG18492)	LD42274	AY051953
tab2(CG7417)	*LD40663*	BT010287
traf2(CG10961)	GH01161	AY047501
trabid (CG9448)	GH21672	AY058438
ari-2 (CG5709)	GH07166	AY119482
puc (CG7850)	SD08157	AY061616
eiger (12919)	LP03784	BT021371

EGFP: Full-length EGFP-HA was amplified by PCR using pEGFP-N1 (Clonetech) as template and cloned into pAc5.1/HA (c) (Invitrogen). Primer sequences are detailed in Appendix 2.

Ubiquitin: Full-length ubiquitin was PCR amplified from pUAS–HA–ubiquitin (a gift from Prof. Toshiro Aigaki, Tokyo Metropolitan University) with an elongated Fwd

primer containing the cMyc-epitope sequence and with a reverse primer containing a STOP codon (Appendix 2). The PCR product was then cloned into pAc 5.1/HA.

dIAP1: Full-length dIAP1 was PCR amplified from pGEX–dIAP1 (a gift from Prof. Pascal Meier, Institute of Cancer Research) and cloned into pAc5.1/HA (c) (Invitrogen). Primer sequences are detailed in Appendix 2.

(B) Ari-2 domain deletion constructs:

Due to overlapping domains, four possible representations of the Ari-2 protein have been predicted (see Appendix 4). The most probable representation was determined using SMART (Simple Modular Architecture Research Tool), a programme which allows the identification and annotation of genetically mobile domains and the analysis of domain architectures (www. smart.embl-heidelberg.de) as previously described (Schultz et al., 1998). This representation was used for domain deletions.

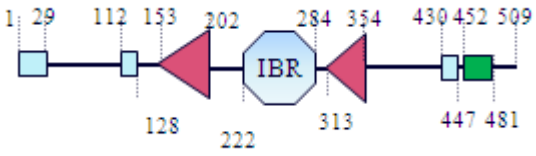


Figure 2 - 3: **Schematic diagram of the most probable representation of Ari-2.** The various motifs are: low complexity regions (blue squares), RING domains (red triangles), In Between RING domain (blue hexagon) and coiled-coil motif (green rectangle). There is also a putative C2HC zinc-finger domain within the IBR motif (http://smart.embl-heidelberg.de/).

The following domain deletion mutants of Ari-2 were made using the fusion PCR method (primer sequences are detailed in Appendix 2)

- Ari-$2^{\Delta R1}$ → Deletion of the N-terminal RING domain (153 – 202aa)
- Ari-$2^{\Delta R2}$ → Deletion of the C-terminal RING domain (313 – 358aa)
- Ari-$2^{\Delta R1R2}$ → Deletion of both RING domains (153 – 202 & 313 – 358aa)
- Ari-$2^{\Delta ZnF}$ → Deletion of the ZnF domain (270-286aa)
- Ari-$2^{\Delta CC}$ → Deletion of the C-terminal coiled-coil domain (452 – 481aa)

(C) Single and multi-site point mutation constructs:

Single and multi-site point mutation constructs were made using QuikChange Lightning Site-Directed & Multi Site-Directed Mutagenesis Kits (Agilent). Primer sequences are detailed in Appendix 2. The following point mutant constructs were made:

- ***Drosophila* Trabid**: dTrabid^{C518S} & dTrabid$^{C518S+3xNZFDel}$ (first Cys residue of the 3 NZF domains, C13, C94 and C238, were mutated to Ala in addition to C518S).
- ***Drosophila* TAK1**: dTAK1^{K134R} dTAK1^{K142R} dTAK1^{K156R} dTAK1^{K189R} & dTAK1^{K194R}
- ***Drosophila* TAB2:** dTAB2$^{\Delta ZnF}$ (mutating the first 2 Cys residues in the C-terminal ZnF motif - C769A and C772A)
- **Ubiquitin**: cMyc-Ub^{K48} (mutation of all Lys sites to Arg except K48) & cMyc-Ub^{K63} ((mutation of all Lys sites to Arg except K63). Further, cMyc-Ub^{K48R} & cMyc-Ub^{K63R} were made previously.

Transformation and processing of cDNA into desired expression vectors:

Complementary cDNA containing FTA Elute discs were transformed into competent cells according to the supplier's instructions. Briefly, discs were washed for 1 or 2 seconds with 50µl TE buffer (to remove a chemical present on the disc that would inhibit transformation) in sterile 1.5ml microcentrifuge tubes and TE buffer quickly withdrawn. Thereafter, aliquots of 50µl thawed Library Efficiency DH5α Competent Cells (Invitrogen) were mixed with the discs and incubated on ice for 30 mins. The cell-disc mixes were vortex for 1 second halfway through the incubation and just before heat shock. The cell-disc mixes were heat shocked for 2 mins at 37°C and the cells (without the discs) transferred to 1ml of Luria Bertani (LB) broth, incubated at 37°C for 1 hr with shaking. Thereafter, 200µl of each transformation was spread on pre-warmed LB agar plates containing 100µg/ml carbenicillin and incubated overnight at 37°C.

Purified expression vectors were obtained by DNA miniprep and were used as templates for PCR amplification. The protein coding sequences (CDS) were amplified with primers (flanked with appropriate restriction sites) using PfuUltra 11 Fusion Hot Start DNA polymerase (Agilent) in a Mastercycler© pro thermal cycler (Eppendorf). Generated DNA constructs were verified through standard sequencing by Source Bioscience Lifesciences. Primer sequences for cloning and standard PCR reaction mix are detailed in Appendix 2.

2.4.2 Mutagenesis:

Point mutations in DNA constructs were made using QuikChange Lightning Site-Directed Mutagenesis kits and QuikChange Lightning Multi Site-Directed Mutagenesis kits (Agilent) according to the manufacturer's instructions. Mutagenesis primers were designed using the QuikChange Primer Design Application found in the

Agilent website (www.genomics.agilent.com). A Mastercycler© pro thermal cycler (Eppendorf) was used for PCR amplification. Primer sequences, standard mutagenesis PCR reaction mixes and thermal cycling parameters for single-site and multi-site reactions are detailed in Appendix 2.

After amplification the parental (non-mutated) dsDNA strands were digested by addition of 2µl Dpn 1 enzyme (for single site mutagenesis or 1µl for multi-site mutagenesis) and incubating at 37ºC for 5 mins. Thereafter, the mutated plasmids were transformed into XL10-Gold Ultra competent Cells as detailed below.

2.4.3 Fusion PCR:

Introduction: This method enables the rapid creation of reporter gene fusions on a large scale. It was initially developed to create *gfp* fusion constructs ready to be injected into *Caenorhabditis elegans* in one day without the need for subcloning (Hobert, 2002).

Principle: Here, 2 separate PCR products (amplicon # 1 & 2) are generated from both the full length gene of interest and the *gfp* reporter to be fused (Fig 2-2). The 3' end of Primer B (1st PCR) has a 24 nucleotide overhang which is complementary to the *gfp* gene, thereby ensuring that the *gfp* is fused 'in-frame' to the gene of interest. In the 3rd PCR, amplicon # 1 & 2 are used as the template to generate amplicon #3 – the fused gene (Hobert, 2002).

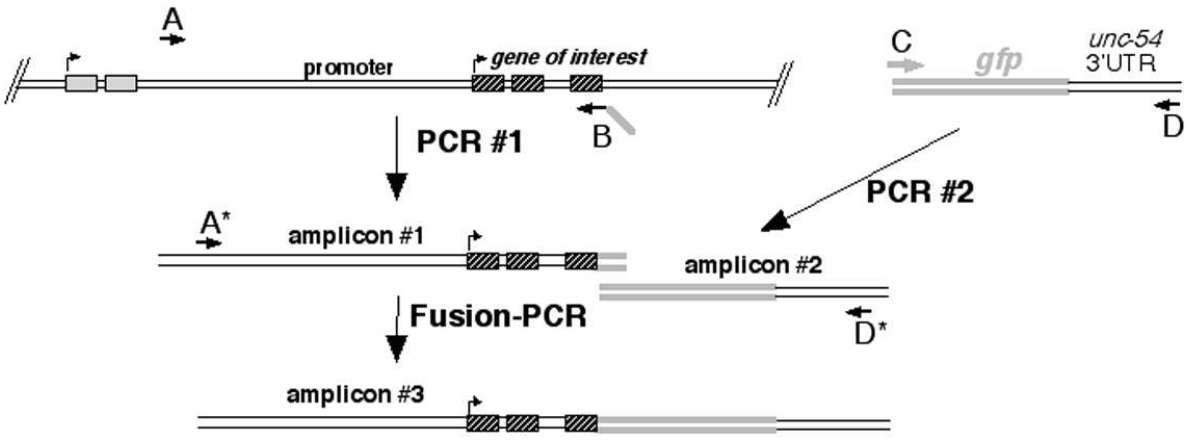


Figure 2 - 4: **Outline - PCR fusion approach to generate GFP reporters.** See text for details. Primers used: A = 5' upstream, 20-25 nucleotides; A* = nested to A (3-10bp downstream of A); B = 20–24 nucleotides of the end of the gene to fuse + 24 nucleotides of GFP vector pPD95.75; C = polylinker beginning of pPD95.75; D= at the end of *unc-54* 3′ UTR and D* = closely nested to D. Adapted from Hobert, O. (2002). "PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic *C. elegans*." Biotechniques **32**(4): 728-730.

Modified protocol for generation of domain deletions: This principle can be modified to generate gene constructs with either complete or partial deletion of protein domains. Using standard methods, a domain deletion requires 3 independent cloning procedures (Sambrook and Russell, 2001). Briefly, the 2 DNA regions flanking the domain have to be amplified by PCR and cloned separately (in-frame) into a suitable cloning vector (eg: pBluescript). Then, the 'fused' deletion construct is PCR amplified and cloned into the desired expression vector. Also, there will be several nucleotides added between the 2 flanking regions.

The modified method can generate a domain deletion gene construct without any additional nucleotides in only 3 separate PCR amplifications. Moreover, only one

cloning step is necessary - the final cloning into the desired expression vector. Thus, a deletion mutant can be generated in a single day. The protocol is as follows:

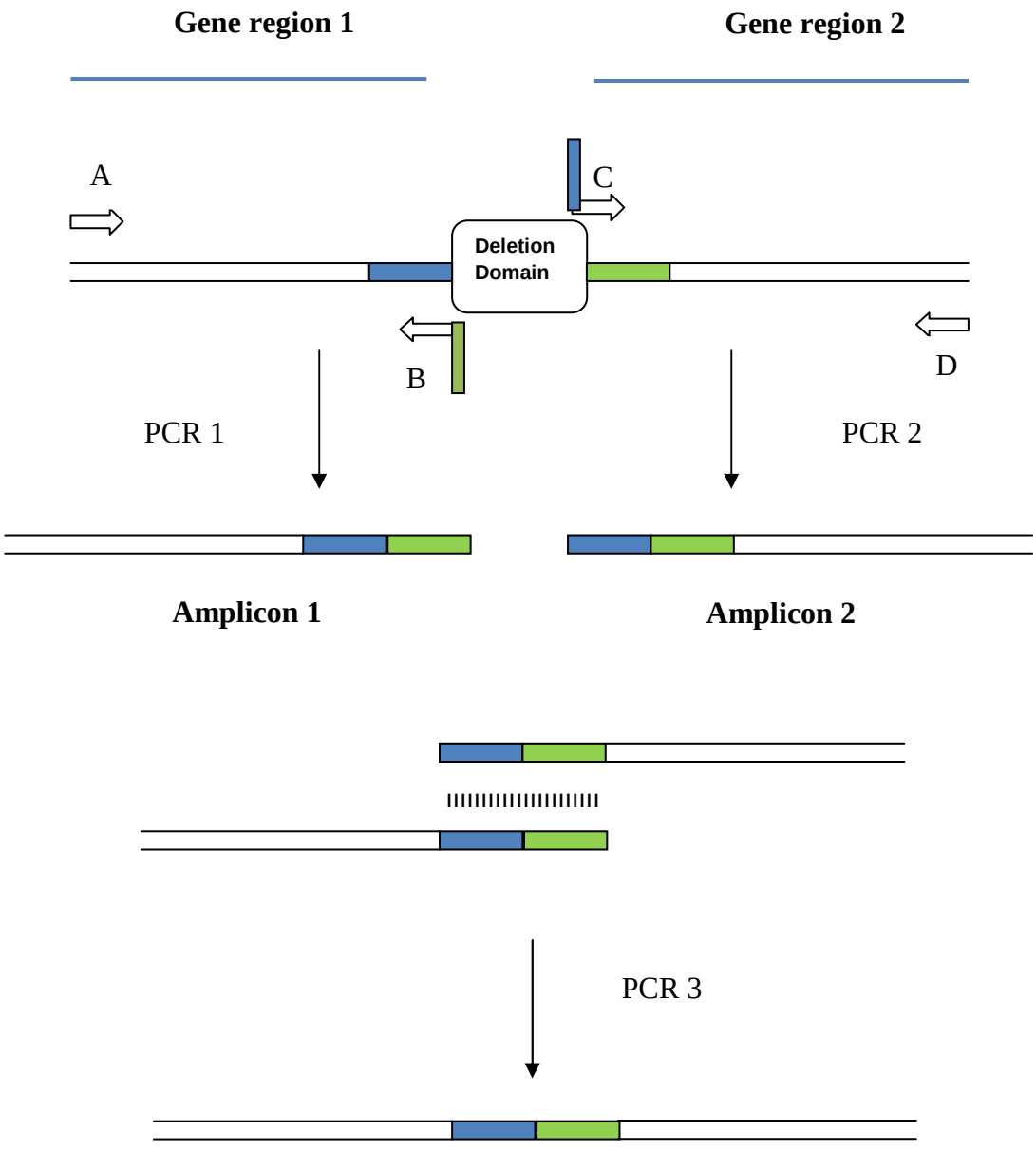


Figure 2 - 5: **Outline – Modified fusion PCR approach to generate domain deletion constructs.** The gene regions flanking the domain to be deleted are amplified by PCR with primers containing complementary overhangs (see text). The 2 PCR products are then used as the template for the 3rd PCR reaction to generate the fusion construct (see text for further details)

1. Two separate PCR amplifications (PCR 1 & 2) were performed to amplify the DNA regions (Gene region 1 & 2) on either side of the domain to be deleted (Fig. 2-3). Primer B contained an overhang of 20-24 nucleotides (at its 3' end) that was complementary to the 5' end of Gene region 2. Primer C contained a similar overhang at its 5' end that was complementary to the 3' end of Gene region 1. As a result, Amplicon 1 contained a 3' overhang which was complementary to the 5' end of Amplicon 2. Similarly, the 5' end of Amplicon 2 contained an overhang complementary to the 3' end of Amplicon 1. The overhangs would ensure that both Gene regions were 'in-frame' and lead to annealing of the 2 Gene regions during the final reaction.
2. Amplicons 1 & 2 were gel purified and concentrations determined.
3. **Fusion PCR**: 100-150ng each of Amplicons 1 & 2 was used for the template in a final reaction volume of 100µl.
4. The resultant fusion construct was gel purified, restriction digested and cloned into the appropriate expression vector.

2.4.4 Size Fractionation of DNA – Agarose Gel Electrophoresis:

Introduction/Principle: Agarose gel electrophoresis is a standard method in molecular biology to resolve and separate nucleic acids of different sizes and conformations. The polysaccharide agarose (obtained from red algae) is used as the matrix. The matrix consists of pores and migration through the matrix is dependent upon size and confirmation of the nucleic acid (Corley, 2005; QIAGEN, 2000). Shorter nucleic acid fragments migrate faster and further through the matrix pores than larger fragments in a phenomenon known as "sieving." The size of these pores is dependent on agarose concentration – higher concentrations resulting in smaller pore sizes. This phenomenon

is used to enhance fragment resolution. Low concentration gels are typically used to resolve large fragments while higher agarose concentrations are useful to separate smaller fragments (Corley, 2005; QIAGEN, 2000). The migration of DNA through the electric field is dependent upon the phosphates on the DNA backbone. Therefore, the charge to mass ratio of all DNA fragments is identical irrespective of the length. Consequently, the migration of DNA fragments is proportional to the inverse of $\log_{10}$ molecular weight of the fragment. The sizes of the unknown fragments are calculated by comparison to commercially available "ladders" which contain fragments of known sizes (Corley, 2005).

Protocol: A 1% Agarose solution was made by dissolving 1g of Agarose LE (Ambion) in 100ml of 1x Tris-Borate-EDTA buffer (90mM Tris, 90mM Boric acid and 2mM EDTA; pH 8.3) by warming the solution in a Micro Chef ST44 microwave (Proline). Ethidium bromide (0.5µg/ml) was then added and the gel poured into a UV transparent gel tray, the gel comb inserted and allowed to set. Once set, the gel was placed in a Sub Cell GT Cell (Biorad), and immersed in 1x TBE buffer. DNA samples were then loaded onto the wells and samples were resolved at 100V for ~ 2hrs. Thereafter, DNA bands were examined under UV light in a MultiImage 11 Light Cabinet (Alpha Innotech Corp.)

2.4.5 Gel Extraction, Restriction Digestion and Ligation:

Gel Extraction: Relevant DNA bands were excised from agarose gels with a sterile scalpel and transferred into 1.5ml centrifugation tubes. DNA was extracted from gel plugs using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. Briefly, 3 volumes of Buffer QG was added to 1 volume of gel and incubated at 50°C for 10 mins, vortexing briefly until the gel slices were

completely dissolved. The pH of samples were checked by examining the colour and 10µl 3M sodium acetate (pH 5.0) was added if necessary. If appropriate , one gel volume of isopropanol was added and mixed to each sample. Samples were applied to QIAquick spin columns, centrifuged for 1 min and the flowthrough discarded. The membrane bound DNA was washed with 0.75ml of Buffer PE and centrifuged for 1 min. The DNA was incubated in Buffer PE for 3 to 5 mins to remove excess salt. Columns were centrifuged again for 1 min to discard excess buffer and placed in new 1.5ml microcentrifuge tubes. Bound DNA was eluted by adding 30µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the centre of the QIAquick membrane, incubating for 1 min and centrifuging for 1 min. All centrifugations were performed at 13,000rpm (~ 17,900 x *g*) in a Biofuge Pico benchtop microcentrifuge (Heraeus).

Restriction Digestion: Purified DNA fragments were restriction digested with relevant restriction enzymes (New England Biolabs) according to the manufacturer's instructions. A typical restriction digestion reaction is detailed in Appendix 2. Reactions were incubated at the appropriate temperature (usually 37°C) for ~ 3 hrs.

PCR Clean up: The digested DNA was purified using a QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Briefly, 1 volume of each restriction digestion was mixed with 5 volumes of Buffer PB. The pHs of the samples were checked by examining the colour and 10µl 3M sodium acetate (pH 5.0) added to each, if necessary. Samples were applied to QIAquick columns, centrifuged for 1 min at 13,000rpm and the flowthrough discarded. The membrane bound DNA was washed with 0.75ml of Buffer PE and centrifuged for 1 min. The DNA was incubated in Buffer PE for 3 to 5 mins to remove excess salt. Columns were centrifuged again for 1 min to discard excess buffer and placed in new 1.5ml microcentrifuge tubes. Bound DNA was

eluted by adding 30µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the centre of the QIAquick membranes, incubating for 1 min and centrifuging for 1 min. All centrifugations were performed at 13,000rpm (~ 17,900 x *g*) in a Biofuge Pico benchtop microcentrifuge (Heraeus).

2.4.6 Quantification of DNA and RNA – Spectrophotometry

Introduction/Principle: Sprctrophotometry is a widely used technique to quantify substances in solution including nucleic acids, proteins and other metobolites. A spectrophotometer is used to measure the amount of light that a sample absorbs. The instrument passes a beam of light (a stream of photons), of known intensity (I_0) and a particular wavelength, through a sample. The molecules in the sample absorb a certain amount of the light, thereby reducing the intensity of the light beam. The intensity of the resulting output beam (I) is then detected(Creighton, 1999a; Gore, 2000).

The basis for spectrophotometry is the Lambert-Beer Law. This Law is based on the physical phenomena that certain compounds containing chromophores will absorb UV light at specific wavelengths. Here, absorbance (A) is equal to the log of the ratio of incident (I_0) and transmitted (I) light and can be expressed mathematically as follows:

$$\text{Absorbance (A)} = -\log_{10} \frac{I_0}{I}$$

Further, absorbance (A) is proportional to concentration (c) and there is a linear relationship between them. Therefore, $\mathbf{A = \varepsilon_\lambda . c . l}$ [where l = path length of the cuvette (1cm); ε_λ = extinction coefficient at λ wavelength (in $ml.g^{-1}.cm^{-1}$)]. Concentration can be determined from this equation, once absorbance is known (Gore, 2000).

Protocol: **N**ucleic acids (both DNA and RNA) were quantified using a NanoDrop ND-1000 (Thermo Scientific) according to the manufacturer's instructions. The sample volume used was 2µl.

2.4.7 Cloning of DNA constructs into expression vectors:

Purified restriction digested DNA fragments were then cloned into appropriate expression vectors using T4 DNA ligase (New England Biolabs) according to the manufacturer's instructions. Previously, expression vectors had been restriction digested, resolved by agarose gel electrophoresis, purified by gel extraction and quantified. A construct to expression vector concentration of 3:1 was used as a rule of thumb. Ligation reactions were made in 10µl volumes and were incubated overnight at 16°C. A typical ligation reaction is detailed in Appendix 2.

Expression Vectors: Expression vectors pAc5.1/V5-His, pAc5.1/cMyc-His (both Invitrogen) or pAc5.1/HA-His (modified in our laboratory) were used as expression vectors. These plasmids have an Actin binding site in the promoter which allows constitutive expression of cloned proteins in S2 cells. Vector pAc5.1/HA –His was ly previoumodified in our laboratory by replacing the V5 epitope of plasmid pAc5.1/V5-His (Invitrogen) with an HA epitope. This was achieved by ligating the following annealed phosphorylated oligomers into *Bst*BI-*Age*I-digested pAc5.1/V5-His:-

- HA-F: 5'-CGAAAGATCTGCATACCCATACGACGTCCCAGACTACGCTCGTA-3'
- HA-R: 5'-CCGGTACGAGCGTAGTCTGGGACGTCGTATGGGTATGCAGATCTTT-3'

2.4.8 Bacterial Transformation and Cloning:

Expression vectors containing cloned genes were transformed into Subcloning Efficiency DH5α Chemically Competent Cells (Invitrogen) according to the manufacturer's instructions. Briefly, competent cells were thawed on ice and 50µl aliquots (for each transformation) were transferred into pre-chilled 14-ml BD Falcon polypropylene round bottom tubes (BD Biosciences) on ice. Ligation reaction mixtures (10µl) were added to cells, mixed gently with the pipette tip and incubated for 30 mins on ice. Cells were heat shocked for 20s in a 42°C water bath and placed on ice for 2 mins. Pre-warmed S.O.C. medium (950µl; Invitrogen) was added into each tube and incubated at 37°C for 1 hr with shaking at 225rpm. Thereafter, 200µl of each transformation was spread on pre-warmed LB agar plates containing 100µg/ml carbenicillin (for selection of positive transformants) and incubated at 37°C overnight. Alternatively, the entire transformation mixture was plated. In this case, the mixture was centrifuged at 3,000rpm for 5 mins and 800µl of supernatant/medium was discarded. The cell pellet was then resuspended in the remaining medium and plated as above.

Single-site and multi-site mutants: Transformation of point mutants made using QuickChange mutagenesis kits was carried out according to the manufacturer's instructions. Briefly, 45µl aliquots of XL10-Gold Ultracompetent cells were thawed on ice in pre-chilled 14-ml BD Falcon polypropylene round bottom tubes. 2µl of β-Mercaptoethanol was added to each aliquot in order to increase transformation efficiency, gently swirled to mix and incubated on ice for 2 mins. Thereafter, 2µl of mutagenesis reaction was added to each aliquot, swirled gently to mix and incubated on ice for 30 mins. Cells were heat shocked for 30s in a 42°C water bath and incubated on ice for 2 mins. Thereafter, 500µl of pre-warmed NZY^+ broth was added into each tube and incubated at 37°C for 1 hr with shaking at 225rpm. Afterwards, 250µl of each

transformation was spread on pre-warmed LB agar plates containing 100µg/ml carbenicillin (for selection of positive transformants) and incubated at 37°C overnight.

2.4.9 Selection of Positive Transformants:

Colonies were selected from LB agar plates incubated overnight were used to prepared cultures for DNA minipreps. Briefly, single colonies were cultured in 5ml of LB broth supplemented with 100µg/ml carbenicillin and incubated at 37°C for 12 - 16 hrs with shaking at 250rpm.

Expression vectors were purified and concentrated using a QIAprep Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Briefly, bacterial cells containing expression vectors were harvested in 15ml centrifugation tubes (Greiner Bio One) by centrifugation at 5,400 x *g* for 10 mins at 4°C in an Allegra 6 centrifuge (Beckman Coulter). Cell pellets were resuspended in 250 µl Buffer P1 each and transferred into 1.5ml microcentrifuge tubes. Cells were lysed by adding 250µl Buffer P2, mixing thoroughly by gently inverting the tube 6 times and incubating for 5 mins at room temperature (RT). The lysis reaction was stopped by adding 350µl of Buffer N3 and mixing gently by inverting the tubes 6 times. Reaction mixtures were centrifuged for 10 mins, the supernatants applied to QIAprep spin columns, centrifuged for 1 min and flow through discarded. Membrane bound DNA was washed by 0.75ml of Buffer PE. The DNA was incubated in Buffer PE for 3 to 5 mins to remove excess salt before centrifugation for 1 min. Column were centrifuged again for 1 min to discard excess buffer and placed in new 1.5ml microcentrifuge tubes. The DNA was eluted by adding 50 to 100µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the centre of the QIAprep membranes, incubating for 1 min and centrifuging for 1 min. All centrifugations were

performed at 13,000rpm (~ 17,900 x *g*) in a Biofuge Pico benchtop microcentrifuge (Heraeus).

2.4.10 Glycerol Stocks of Bacteria:

Bacterial cultures were maintained on a long-term basis as glycerol stocks as previously described (Sambrook and Russell, 2001). Briefly, 150µl of 100% glycerol were aliquoted into 2ml screw-cap storage vials and sterilised by autoclaving. Thereafter, 850µl of logarithmic-phase bacterial culture were added into the pre-sterilised glycerol, mixed thoroughly by vortexing, flash-frozen in an ethanol bath and stored at -80°C (QIAGEN, 1998).

2.4.11 Confirmation of Positive Transformants:

The presence of relevant DNA constructs in expression vectors were initially confirmed by restriction digestion followed by verification of the DNA sequence by standard sequencing (Source Bioscience Lifesciences, Oxford).

2.4.12 Transfection grade expression vectors for cell culture:

Transfection grade expression vectors for cell culture were made using QIAGEN plasmid Midi Kit (QIAGEN) according to the manufacturer's instructions. Briefly, ingle colonies from overnight incubated LB agar plates (100µg/ml carbenicillin) were used to inoculate 5ml each of LB broth starter culture supplemented with 100µg/ml carbenicillin. Cultures were incubated at 37°C for 6 to 8 hrs with shaking at 250rpm. Starter cultures were diluted 1:1000 in 100ml of LB broth supplemented with 100µg/ml carbenicillin and incubated at 37°C for 16 hrs with shaking at 250rpm in a G24 Environmental Incubator Shaker (New Brunswick Scientific). Bacterial cells were harvested by centrifuging at 6,000 x *g* for 15 mins at 4°C. Cells were resuspended in

4ml of Buffer P1 (supplemented with RNase 1 and lyse blue) and transferred into polypropylene ultra-centrifugation tubes (Beckman Coulter). Cells were lysed with 4ml of Buffer P2, mixing thoroughly by inverting 6 times and incubated for 5 mins at room temperature (RT). Lysis reactions were stopped by adding 4ml of pre-chilled Buffer P3, thoroughly mixing by inverting 6 times and incubating on ice for 15 mins to precipitate SDS. Tubes were then centrifuged at 20,000 x *g* for 30 mins at 4°C. Resultant supernatants were centrifuged again at 20,000 x *g* for 15 mins at 4°C and applied to QIAGEN-tip 100 columns. These columns were previously equilibrated by application of 4ml of Buffer QBT and allowing them to empty by gravity flow. Membrane bound DNA in QIAGEN-tips were washed twice with 10ml of Buffer QC. Membrane bound DNA was eluted by applying 5ml of Buffer QF. DNA was precipitated by adding 3.5ml (0.7x volumes) of isopropanol, gently mixing and centrifuging at 20,000 x *g* for 30 mins at 4°C. Supernatants were carefully decanted. DNA pellets were washed with 2ml of 70% ethanol at RT, centrifuged at 20,000 x *g* for 10 mins at 4°C and supernatants decanted. DNA pellets were then air dried for 10 mins and gently resuspended in endotoxin-free TE buffer, pH 8.0 (QIAGEN). All centrifugations were performed in a J2-21 High Speed centrifuge (Beckman Coulter).

2.5 *Drosophila* Fly Strains

2.5.1 General Maintenance

Drosophila melanogaster flies were maintained at 25°C on standard fly food (see Appendix 1). They were transferred into new vials or “flipped” every 2 weeks. Stock strains were maintained at 18°C and “flipped” every 3 weeks. The recipe for fly food is detailed in Appendix 1

2.5.2 GAL4 – UAS System:

Introduction: This is a biochemical method used to study ectopic gene expression *in vivo*. Using this system, a cloned gene can be activated selectively in a wide variety of tissue and cell specific manner (Brand and Perrimon, 1993; Duffy, 2002).

Principle: The system consists of 2 parts - *GAL4* (a yeast transcriptional activator) and *UAS* (**U**pstream **A**ctivator **S**equence; a short section of the promoter, to which Gal4 binds and activates transcription).

The *GAL4* gene is inserted randomly into the *Drosophila* genome generating "enhancer trap" lines that express *GAL4* under the control of nearby genomic enhancers. A large collection of *GAL4* lines have now been produce that express *GAL4* in a massive variety of cell and tissue specific patterns (Brand and Perrimon, 1993; St Johnston, 2002). The target gene (gene X) is under the control of a promoter UAS which contains GAL4 binding sites and is thus under GAL4 control. The expression of gene X is driven by crossing the appropriate GAL4 enhancer-trap line to flies that carry the UAS–gene X transgene (St Johnston, 2002).

Protocol: DNA constructs containing the coding sequences of Ari-2 (wt) and Ari-2 mutants (Ari-$2^{\Delta R1R2}$, Ari-$2^{\Delta CC}$ and Ari-$2^{\Delta ZnF}$) were generated as described above using fusion PCR techniques. Full length and domain deletion constructs were then cloned into fly transformation vector pUAST (Brand and Perrimon, 1993). Primers used were:

- UASAri2-F: 5'-CCCGGTACCCAAAATGGACTCGGACATCGAAATG-3'
- UASAri2-R: 5'CCCTCTAGACTATGCGTCCACTGGGAAGA-3'.

Fly transformation was performed by Rainbow Transgenic Flies (Newbury Park, CA, USA).

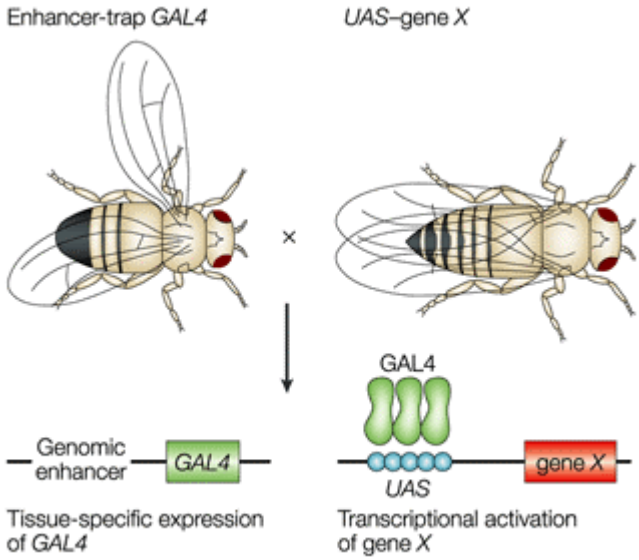


Figure 2 - 6: **Directed gene expression in *Drosophila* - GAL4/UAS system.** The genomic enhancer is silent until crossed to the GAL4 driver. When males with the GAL4 are mated with females carrying the UAS-gene X, the progeny of the cross will have gene X expressed in tissues under the control of the GAL4 genomic enhancer (Duffy, 2002). Adapted from: St Johnston, D. (2002). "The art and design of genetic screens: Drosophila melanogaster." Nat Rev Genet **3**(3): 176-188.

2.5.3 ***Drosophila stocks* and procedures:**

UAS-egr, *GMR-GAL4*, *UAS-TAK1-RNAi*, *UAS-bsk* and *heat-shock-GAL4* (*hs-GAL4*) have been described previously ((Hay et al., 1997; Leulier et al., 2002; Moreno et al., 2002; Riesgo-Escovar et al., 1996). Null mutant trbd flies (generated by a deletion the *trbd* locus, CG9448, by homologous recombination) were a kind gift from Mariann Bienz (Tran et al., 2008). *Wn*, is an isogenised wild type strain, which was the strain used as a background to generate the rel^{E20} mutant as previously described (Hedengren

et al., 1999). For the *UAS-bsk/hs-GAL4* experiments we heat-shocked synchronised cultures (25^{o}C) 3 days after puparium formation for thirty minutes at 37^{o}C. Subsequently, the eclosion rates were measured. To determine adult lifespan, cohorts of 20 wild-type male or 20 *ari-2* mutant male flies (trans-homozygotes) were placed in standard food vials at 25°C or 30°C and their survival was determined daily. Death was diagnosed by a lack of a sit-up response. JNK was activated by heat shock of flies at 37°C for 90 min or by depriving flies of food and water for 24h (dry starvation) or by exposure to filter papers soaked in 15 mM paraquat in 5% sucrose (Methyl-viologen, Sigma-Aldrich) for 90 min.

2.5.4 Quantitative PCR (qPCR):

Introduction/Principle: This technique is widely used to monitor expression of a target gene. Here, the target gene is simultaneously amplified and quantified in a single step. Several different fluorescent chemistries are used and they correlate PCR product concentration to fluorescence intensity (Wong, 2005). SYBR Green is one such widely used intercalating dye that fluoresces upon binding to double-stranded DNA. With amplification of the target gene, increasing amounts of SYBR Green binds to the product and emits a strong fluorescent signal that is easily detected (Van Guilder, 2008). The start of amplification is known as the linear phase and fluorescence emission is at background levels. The base fluorescence is calculated at this point. When amplification enters exponential/log phase, fluorescence rises above background levels. An arbitrary threshold is placed on the exponential phase and as fluorescence increases (with increasing product), this threshold is crossed. The PCR cycle in which the threshold was crossed is known as the threshold cycle (C_T). The greater the amount of the target gene in the reaction, the faster the amplification-based fluorescence will increase resulting in

a lower C_T value. Therefore, the numerical value of the C_T is inversely related to the amount of target gene in the reaction (Schmittgen and Livak, 2008; Wong, 2005).

Protocol:

RNA Extraction: Total RNA either from aliquots of S2 cells (a monolayer from a 60mm tissue culture dish or ~10^7 cells) or 20 - 30 flies was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, flies were crushed with 0.5ml of TRIzol reagent in 1.5ml microcentrifuge tubes using polypropylene pellet pestles (Sigma-Aldrich). Cell samples were lysed by gentle pippetting. Thereafter, 200µl of chloroform (Sigma Aldrich) was added to each sample, vortexed for 30s and centrifuged at 16,000rpm for 10 mins at 4°C. The RNA in the supernatant was precipitated by addition of 0.7 volumes of isopropanol (Sigma Aldrich). Samples were then centrifuged at 16,000rpm for 30 mins at 4°C, the supernatant decanted, 500µl of 70% ethanol added and centrifuged again at 16,000rpm for 10 mins at 4°C. The supertanant was discarded, RNA pellets air-dried on ice for 10 mins, resuspended in RNase free water (Agilent) and quantified.

DNA Digestion & RNA Purification: DNA in the samples was digested with RNase-free DNase I (10U/µl; Roche Applied Science) according to the manufacturer's instructions. 5µg of total RNA was used from each sample in 50µl reaction volumes. The reactions were incubated at 37°C for 30mins. Samples were then purified in 2 separate extraction with phenol:chloroform:isoamyl alcohol 25:24:1 in TE pH8.0 (Sigma Aldrich) and then chloroform (Sigma Aldrich). Briefly, 50µl of RNase free water was added to the samples to increase the volume to 100µl. Thereafter, 100µl of phenol:chloroform (24:1; Sigma-Aldrich) was added to each sample, vortexed to mix thoroughly and centrifuged at 16,000rpm for 10 mins at 4°C. The RNA containing

upper phases were extracted (~100µl), mixed with 100µl (1x volume) of chloroform, vortexed briefly and centrifuged at16,000 rpm for 5 mins at 4°C. Upper phases containing RNA were collected, mixed with 2x volumes of 100% ethanol and 0.1x volumes 3M sodium acetate (pH 5.0). Samples were then centrifuged at 16,000rpm for 30 mins at 4°C, supernatants decanted, 500µl of 70% ethanol added and centrifuged again at 16,000rpm for 10 mins at 4°C. The supernatants were discarded, RNA pellets air-dried on ice for 10 mins, resuspended in RNase-free water and quantified. All centrifugation were performed in Microcentrifuge 5417R (Eppendorf).

cDNA Synthesis: Complementary DNA (cDNA) was synthesised from RNA using the AffinityScript Multi Temperature cDNA Synthesis Kit (Agilent) according to the manufacturer's instructions (first-strand cDNA synthesis protocol). Briefly, 1µg of RNA (from each sample) was mixed with 3µl of random primers (0.1µg/µl) and RNase-free water to a total volume of 15.7µl in thin-walled PCR tubes (VWR), incubated at 65°C for 5 mins and cooled at room temperature for 10 mins (to allow the primers to anneal). The following components were then added (in order):

- 2.0 µl of 10× AffinityScript RT Buffer
- 0.8 µl of dNTP mix (25 mM each dNTP)
- 0.5 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)
- 1 µl of AffinityScript Multiple Temperature RT

Reaction components were gently mixed and cDNA synthesised using the following thermal cycling procedure in a Mastercycler Pro thermal cycler (Eppendorf):

- 25°C → 10 mins
- 42°C → 60 mins
- 70°C → 15 mins

Quantitative PCR: Quantitative PCR reactions were performed using the Brilliant 11 SYBR Green qPCR Master Mix Kit (Agilent) according to the manufacturer's instructions in a Rotor-Gene Q Real-Time PCR instrument (Corbet/QIAGEN). Briefly, reactions components (see below) were assembled in qPCR strip tubes (QIAGEN) in final volumes of 25µl. Reactions were performed in triplicate. The cycle threshold (C_t) value was determined and gene expression levels of target genes calculated, relative to the internal *Drosophila* reference gene *Rp49*, using the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008). A typical reaction was as follows:

5.5 µl of nuclease free PCR grade water

12.5 µl of 2× Brilliant II SYBR Green qPCR master mix

2.5µl of forward primer (400 nM final concentration)

2.5µl of reverse primer (400 nM final concentration)

2.0 µl of cDNA

25.0 µl → final volume

The following thermal cycling conditions were used:

Cycle	Duration	Temperature
1	10 mins	95°C
40	30 sec	95°C
	60 sec	60°C

The following primers were used for qPCR:

Primer	Sequence
Diptericin - F	5’ – GCTGCGCAATCGCTTCTACT – 3’
Diptericin - R	5’- TGGTGGAGTGGGCTTCATG – 3’
Drosomycin - F	5’ – CGTGAGAACCTTTTCCAATATGATG – 3’
Drosomycin - R	5’ – TCCCAGCACCACCAGCAT – 3’
puckered-F	5’ – TCTGGCATGTGACGAAGTG – 3’
puckered-R	5’ – ATAGATGCGGGAAAACGGG – 3’
RP49-F	5’ – GACGCTTCAAGGGACAGTATCTG – 3’
RP49-R	5’ – AAACGCGGTTCTGCATGAG – 3’

2.5.5 Reverse Transcription PCR (RT-PCR)

Introduction/Principle: This is a biochemical technique used to measure the amount of RNA in a sample. In this method, mRNA is first concerted into complementary DNA (cDNA) using the enzyme reverse transcriptase. The cDNA is then amplified by conventional PCR and analysed by agarose gel electrophoresis (Wilson and Walker, 2000).

Protocol: Total RNAs were extracted from either eight male or five female flies using TRIzol reagent(Invitrogen) as described above. Total RNA was treated with DNAse as described above. Complementary DNA (cDNA) was prepared using RETROscript First-Strand Synthesis Kit (Ambion) according to the manufacturer’s instructions. Briefly, 1µg of RNA (from each sample) was mixed with 2µl of random decamers

(50µM) and RNase-free water to a total volume of 12.0µl in thin-walled PCR tubes (VWR), incubated at 70°C for 3 mins and placed on ice. The following components were then added:

2.0 µl 10× RT Buffer

4.0 µl dNTP mix (2.5 mM each dNTP)

1.0 µl RNase Inhibitor (10 U/µl)

1.0 µl MMLV-RT (reverse transcriptase)

20.0 µl → final volume

Reaction components were gently mixed and cDNA synthesised using the following thermal cycling procedure in a Mastercycler Pro thermal cycler (Eppendorf):

- 44°C → 60 mins
- 92°C → 10 mins

PCR: PCR reactions were performed in a Mastercycler Pro thermal cycler (Eppendorf) in final volumes of 50µl. A typical reaction was as follows:

2.5 µl of cDNA from RT reaction

5.0 µl of 10× PCR buffer

2.5 µl of dNTP mix

1.25µl of forward primer (5 µM final concentration)

1.25µl of reverse primer (5 µM final concentration)

1.0 µl of Super Taq DNA polymerase

36.5 µl of nuclease-free water

50.0 µl → final volume

The following primers were used for RT-PCR:

Primer	Sequence
ari-2 – F	5’-TACAACACAGGCGAGGACTG-3’
ari-2 – R	5’-TGTGATCTGCAGGATGGTGT-3’
puckered-F	5’-CATCATCAACGGCAATGAAC-3’
puckered-R	5’-TTGGGACTTTGGCAGGTAAC-3’
RP49 – F	5’-TCCTACCAGCTTCAAGATGAC-3’
RP49 - R	5’- CACGTTGTGCACCAGGAACT-3’

The following thermal cycling conditions were used:

Segment	Cycles	Duration	Temperature
1	1	4 mins	95°C
2	30	30 sec	94°C
		30 sec	55°C
		60 sec/kb	72°C
3	1	5 mins	72°C

The PCR products were then analysed using agarose gel electrophoresis as described above.

Chapter 3: Negative Regulation of *Drosophila* TAK1 in IMD Innate Immune Signalling – Results

3.1 Ubiquitin mediated activation of TAK1

Innate immunity is highly conserved from insects to mammals to humans. In mammals, TAK1 is critical for activating several signalling cascades including tumour necrosis factor alpha (TNFα), interleukin-1β (IL-1β)-induced IκB kinase (IKK)/nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK)/activator protein 1 (AP-1) pathways (Lemaitre and Hoffmann, 2007; Tran et al., 2008). In humans, TRAF6 (an E3 ubiquitin ligase) controls the activation of TAK1 in IL-1 signalling. In this model, TAK1 is ubiquitinated by K63-linked ubiquitin chains, leading to phosphorylation and activation. TAB2 mediates TAK1 activation by linking TAK1 to TRAF6. Subsequently, activated TAK1 phosphorylates and activates IKK for NF-κB activation (Deng et al., 2000; Fan et al., 2010; Jiang et al., 2002; Takaesu et al., 2000b).

In *Drosophila*, dTAK1 has been similarly implicated in activating several pathways including NFκB and JNK cascades (Silverman et al., 2003). Due to the conserved nature of innate immune pathways between insects and mammals, it was reasonable to assume that *Drosophila* TAK1 may be regulated in a similar way to hTAK1.

3.2 Lys 142 in dTAK1 is essential for immune signalling

It was recently shown that activation of TNFα and IL-1β cascades result in the K63-linked polyubiquitination of hTAK1 at Lys 158 (within the kinase domain) and this was critical for downstream activation of above pathways (Fan et al., 2010). Therefore, we attempted to find the corresponding Lys acceptor site in *Drosophila* TAK1.

Fan *et al.*,(2010) had determined that the Lys acceptor site was located in the N-terminal half of hTAK1. Therefore, we aligned the sequences of human TAK1 (1 – 230 amino acids) and dTAK1 to determine the corresponding Lys acceptor site in *Drosophila* (Fig 3-1A). Based on the conservation of the particular sequence of residues in the region containing Lys 158 in hTAK1, Lys 142 was determined as the most probable candidate in dTAK1. The mutant dTAK1^{K142R} was constructed, with the substitute arginine maintaining the positive charge but not serving as an acceptor site for Ub modification.

It has been established previously that overexpression of dTAK1 activates IMD signalling while co-overexpression of dTAK1 and dTAB2 results in increased immune signalling (Delaney et al., 2006; Vidal et al., 2001). This assay was used to determine whether dTAK1^{K142R} was functional in IMD immune signalling. Expression vectors encoding dTAK1, dTAK1^{K142R} and dTAB2 were transiently transfected in combinations shown into *Drosophila* S2 cells. Expression of *diptericin* (an established transcriptional target of IMD signalling) was assayed 48 hrs post-transfection with quantitative PCR (qPCR). Results show that the K142R mutant failed to activate IMD immune signalling (Fig 3-1B). Activation of the JNK pathway was examined (with *puckered* as the transcription target) using these samples. Results show that although the JNK pathway was only slightly activated, the K142R mutant failed to upregulate it beyond basal levels (Fig. S1 (A)– Appendix 3). Therefore, it was concluded that Lys 142 is essential for the immune activity of dTAK1 and may also be required for JNK activation.

(A)

```
hTak1,     18 MIEAPSQVLNFEEIDYKEIEVEEVVGRGAFGVVCKAKWRAKDVAIKQIESESERKAFIVE
dTak1,      1 MATASLDALQAAYVDFSEITLREKVGHGSYGVVCKAVWRDKLVAVKEFFASAEQKDIEKE
                *  *    *     *  **   * ** *  ****** ** * ** *      * *    *

hTak1,     78 LRQLSRVNHPNIVKLYG--ACLNPVCLVMEYAEGGSLYNVLHGAEPLPYYTAAHAMSWCL
dTak1,     61 VKQLSRVKHPNIIALHGISSYQQATYLIMEFAEGGSLHNFLHG-KVKPAYSLAHAMSWAR
                ***** ****  * *         * ** ****** * ***    * *  ******

hTak1,    136 QCSQGVAYLHSMQPKALIHRDLKPPNLLLVAGGTVLKICDFGTACDIQTHMTNNKGSAAW
dTak1,    120 QCAEGLAYLHAMTPKPLIHRDVKPLNLLLTNKGRNLKICDFGTVADKSTMMTNNRGSAAW
              **  * **** * ** ***** ** ****   *  ********  *  * **** *****

hTak1,    196 MAPEVFEGSNYSEKCDVFSWGIILWEVITRRKPFD
dTak1,    180 MAPEVFEGSKYTEKCDIFSWAIVLWEVLSRKQPFK
              ********* * **** *** * ****  *  **
```

(B) Ability of dTAK1, dTAK1^{K142R} & dTAK1^{K156R} to activate the IMD Pathway

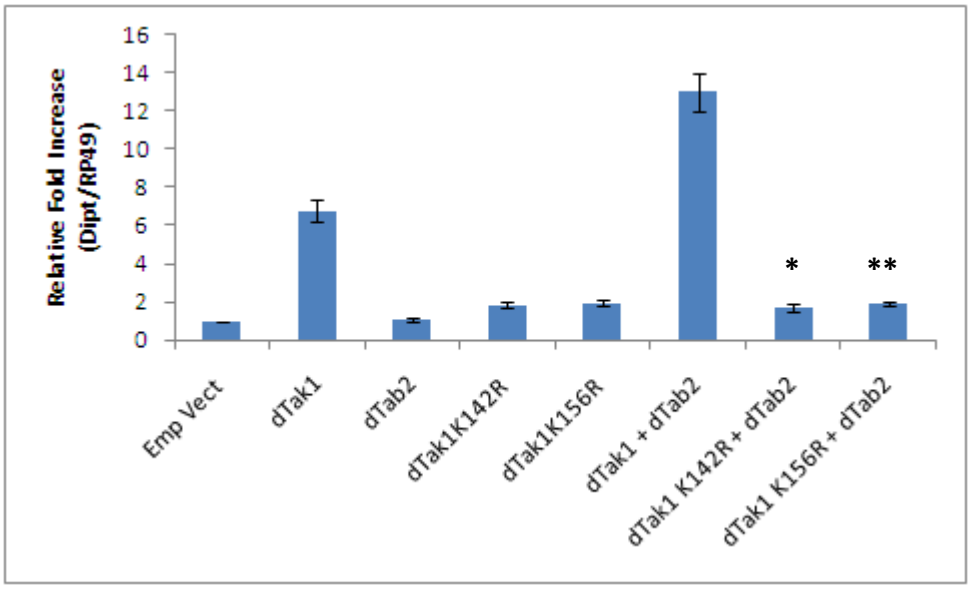


Figure 3-1: Lys 142 and 156 of *Drosophila* TAK1 are essential for immune signalling. (A) Sequence alignment of 1 to 230 amino acids of human (hTAK1) and *Drosophila* TAK1 (dTAK1). Lys 142 (red) and 156 (blue) are shown by arrows and the kinase activation loop is underlined. (B) *Drosophila* TAK1^{K142R} & dTAK1^{K156R} mutant failed to activate the IMD Pathway. *Drosophila* TAK1 (wt) or dTAK1^{K142R} or dTAK1^{K156R} mutant was expressed along with dTAB2 in combinations shown in S2 cells and *diptericin* expression was assayed 48hrs post-transfection. *Error bars represent SEM of 3 separate experiments. Results were normalized to the empty vector. Results from the K156R mutant are from 2 experiments.* * & ** indicate significance value of the results as determined by Student's t-Test (t= 7.1629, $p <$ 0.025 & t=14.8567, $p < 0.05$ respectively).

3.3 Ubiquitination of dTAK1 is altered in the K142R mutant

In humans, ubiquitination regulates TAK1 activity (Reiley et al., 2007). Activation of TNFα and IL-1β pathways induce K63-linked TAK1 polyubiquitination (Fan et al., 2010). Evidence indicates that ubiquitination also plays a role in *Drosophila* TAK1 regulation (Tsuda et al., 2005). It was sought to determine whether there was a change in the ubiquitination profile of dTAK1 and dTAK1^{K142R} mutant. It was previously shown that co-overexpression of human TAK1 and TAB1 in cell culture results in TAK1 polyubiquitination (Fan et al., 2010).

This assay was modified for *Drosophila* S2 cells. Expression vectors encoding for C-terminally V5-tagged dTAK1 or dTAK1^{K142R} were co-transfected with dTAB2-HA and cMyc – Ub into S2 cells. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5 antibody, resolved on 10% SDS PAGE and immunoblotted with anti-c-Myc antibody (Fig. 3-2). In contrast to human TAK1, where mutation of the Lys 158 Ub acceptor site resulted in non-ubiquitination, there was increased ubiquitination in dTAK1$^{K142R.}$ Examination of the cell lysates showed there was greater degradation of dTAK1^{K142R} when compared with *wild type* dTAK1.

There is a major band of slightly over 80 kDa present. This is possibly monoubiquitinated dTAK1 although it has not been experimentally established. The presence of a major band with the combined molecular weight of TAK1 + 1 Ub monomer corresponds to an observation in humans. Fan *et. al.,* (2010) found that overexpression of TAK1 and stimulation of either the IL-1β or TNFα pathways resulted in a major band that corresponded to the molecular weight of monoubiquitinated TAK1 (when immunoblotted with anti-Ub) and that this band stabilised over time even though larger polyubiquitinated entities disappeared. It is, therefore, possible that

monoubiquitinated dTAK1 plays an important role in regulating NF-κB immunity. However, it has not yet been experimentally established. Interestingly, overexpression of dTAK1 and its subsequent immunoprecipitation (included as a marker) also resulted in a band with a molecular weight of slightly over 80 kDa. The *in vivo* ubiquitination assays and overexpression of dTAK1 for the marker were performed separately and resolved in different gels. The alignment (between the ubiquitinated forms and the marker) is based on the migration of a protein ladder. In some of the marker blots, the major band is only present while in others (Figs. 3-2 & 3-4) there are smaller bands below the predominant band (which may represent unmodified dTAK1). Therefore, it appears that unmodified dTAK1 is post-translationally modified into a major band of higher molecular weight. The reason for this shift has not been experimentally established. There are several possibilities for this shift.

Based on size comparison, it is probable that this band represents monoubiquitinated dTAK1. However, this has not been experimentally established. A review of literature revealed that in *Drosophila*, investigations into ubiquitination of TAK1 have been very few. Tsuda *et al*., (2005) studied dTAK1 polyubiquitination in the context of POSH, where the assumption was that co-overexpression of dTAK1 with POSH results in dTAK1 polyubiquitination and degradation. However, there are no indications that they had witnessed a similar band shift in dTAK1 in the lysate. A further review of literature did not indicate a specific band shift observed in human TAK1. It was shown in humans, that overexpression of TAK1 and stimulation of IL-1β or TNFα pathways resulted in a major band that corresponds to the molecular weight of monoubiquitinated TAK1 and this band stabilised over time even though larger polyubiquitinated entities disappeared (Fan et al., 2010). Thus, results appear to suggest that monoubiquitinated TAK1 is important (perhaps even greater than longer polyubiquitinated forms, since the band persists longer) in both TNFα and IL-1β

induced NF-κB signalling in humans. However, as before, there is no indication of whether investigators witnessed a band shift in TAK1 in the cell lysate. It was shown later that overexpression of hTAK1 in HEK293 cells with K63-specific Ub resulted in a major band corresponding to the molecular weight of monoubiquitinated TAK1 and the size of this band was similar to the amount of hTAK1 in the lysates (Fan et al., 2011). Although no indication of a band shift is mentioned, results point to the possibility that overexpression of TAK1 with K63-specific UB may result in a significant amount of TAK1 being modified and resulting in a band shift. Also, when TAK1 was overexpressed with TAB1 or TAB3 in HEK293 cells, 2 distinct bands (very close together) are seen in TAK1 immunoprecipitates (Besse et al., 2007). Therefore, although not specifically mentioned or discussed, it appears that the band shift phenomenon has been previously encountered in humans.

It has been previously established that overexpression of dTAK1 alone was sufficient for IMD activation (Silverman et al., 2003). Further, examination of the cell lysates of the dTAK1 Ub assay (Fig. 3-2) show that almost all dTAK1 is monoubiquitinated. Taken together, one possible explanation is that overexpression of dTAK1 leads to its monoubiquitination (by endogenous ubiquitin) probably through activation of the IMD cascade. An *in vitro* ubiquitination analysis is required to establish whether ubiquitination is involved in the band shift.

Alternatively, it is possible that the band shift is due to another form of co-translational or post-translational modification of dTAK1, including glycosylation or phosphorylation. Since dTAK1 is a serine/threonine/tyrosine kinase that requires phosphorylation for its activation, both possibilities are very likely. A similar band shift phenomenon was observed with TAB1 in $CD4^+$ T cells. It was shown here that human TAB1 may be present in a glycosylated and partially phosphorylated form (72 kDa) in

the membrane and cytosol fractions of naïve $CD4^+$ T cell lysates, whereas native TAB1 protein (56 kDa) increased in anergic $CD4^+$ T cells lysates. Interestingly, deglyosylation by treatment with a peptide:*N*-glycanase (PNGase) shifted the molecular weight of TAB1 from 72 to 56 kDa (Ohkusu-Tsukada et al., 2010).

3.4 Lys 142 is the probable K63 Ub acceptor site in dTAK1

Thereafter, the linkage type (whether K48 or K63-linked) of these polyubiquitination chains on dTAK1 & $dTAK1^{K142R}$ were analysed. N-terminally cMyc-tagged Ub^{K48} or Ub^{K63} (ubiquitin mutants having only a single Lys residue at position 48 or 63 respectively) were generated and were used in determining the type of polyubiquitin chain. Expression vectors encoding dTAK1-V5 or $dTAK1^{K142R}$-V5 and dTAB2-HA together with cMyc-Ub^{K48} or cMyc-Ub^{K63} were co-overexpressed as indicated in S2 cells. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5 antibody, resolved on 10% SDS PAGE and immunoblotted with anti-c-Myc antibody. Separate dTAK1 size markers were used because dTAK1-V5 is not visible when blotted with anti-cMyc.

As expected, $dTAK1^{K142R}$ showed much greater K48-linked polyubiquitination (indicative of proteasomal degradation) and minimal K63-linked polyubiquitination (Fig. 3-3). Elimination of Lys 142 had severely compromised the ability of dTAK1 to form K63-linked polyubiquitination chains, although it had retained the ability to form K48-linked chains. Therefore, in contrast to human TAK1, there appears to be 2 separate Ub acceptor sites for K48 and K63-linked polyubiquitination chains in *Drosophila* TAK1 with Lys 142 being the probable K63 Ub acceptor site.

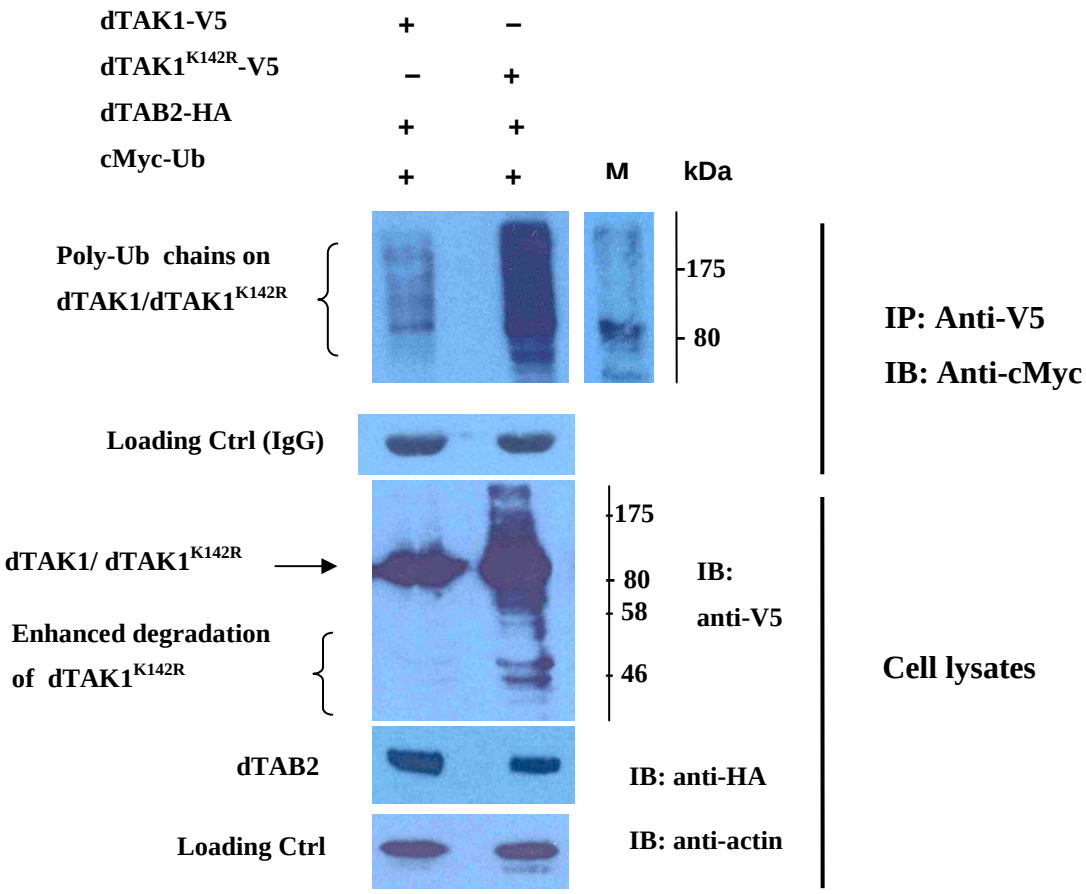


Figure 3-2: Ubiquitination profile is altered in the dTAK1^{K142R} mutant. Mutant dTAK^{K142R} shows enhanced ubiquitination. Expression vectors encoding dTAK1-V5 or dTAK1^{K142R} –V5 were transfected along with dTAB2-HA and cMyc-Ub in combinations shown in S2 cells. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5 antibody, resolved on 10% SDS PAGE, and immunoblotted with anti-cMyc antibody. *M – size marker for dTAK1 as indicated by arrow (dTAK1-V5 expressed in S2 cells, immunoprecipitated by anti-V5 and immunoblotted by anti-V5). Protein size markers are depicted adjacent to the top and middle panels with values given in kDa.*

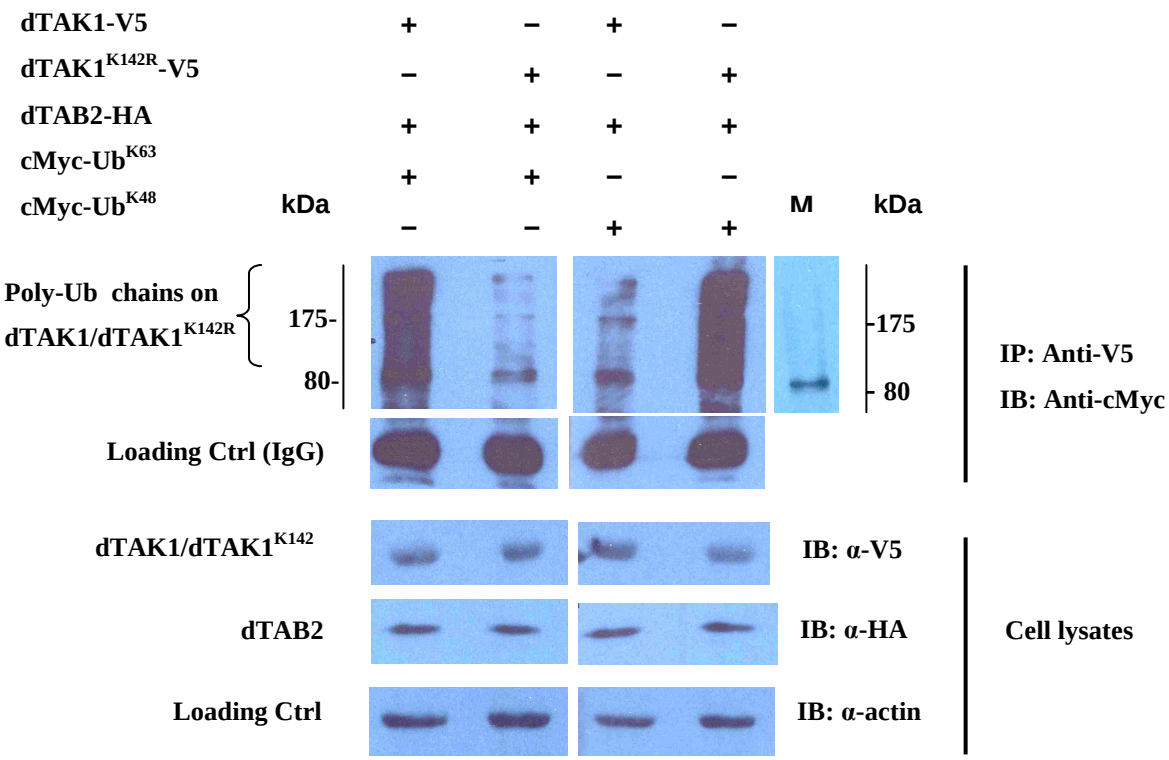


Figure 3-3: K48 ubiquitination is enhanced in the dTAK1^{K142R} mutant. Mutant dTAK1^{K142R} shows enhanced K48-linked ubiquitination and very little K63-linked ubiquitination. Expression vectors encoding dTAK1-V5 or dTAK1^{K142R} –V5 were transfected along with dTAB2-HA and cMyc-Ub^{K63} or cMyc-Ub^{K48} in combinations shown in S2 cells. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-cMyc antibody. *M – size marker for dTAK1 as indicated by arrow (dTAK1-V5 expressed in S2 cells, immunoprecipitated by anti-V5 and immunoblotted by anti-V5). Protein size markers are depicted adjacent to the top panels with values given in kDa.*

3.5 Lys 156 is the likely K48 acceptor site

It was then sought to determine the K48 Ub acceptor site. It was plausible to assume that the 2 putative Ub acceptor sites (K63 & K48) would be in close proximity to both each other and to the kinase activation domain. Hence, Lysines 134, 156, 189 and 194 were selected as likely candidates. New mutation constructs dTAK1^{K134R}-V5, dTAK1^{K156R}-V5, dTAK1^{K189R}-V5 & dTAK1^{K194R}-V5 were made mutating the Lys to Arg at these sites. To determine activity in the IMD pathway, a quantitative PCR assay was performed. Results showed that dTAK1^{K156R} showed anomalous expression (greatly decreased) of *diptericin* when compared to dTAK1 (Fig. 3-1B). K48 and K63 specific Ub assays were performed in S2 cells to determine whether Ub profiles of the new mutant constructs differed from wild type dTAK1. Results indicate that polyubiquitination was reduced in dTAK1^{K156R} (Fig. 3-4A). K48-specific Ub assays showed that K48-linked polyubiquitination was significantly diminished in dTAK1^{K156R}. Also, K63-specific Ub assays showed that K63-linked polyubiquitination was reduced in dTAK1^{K156R} (Fig. 3.4B).

3.6 Lys 156 is involved in IMD immune signalling:

To determine whether Lys 156 was involved in IMD signalling, cell culture based qPCR experiments were performed. Expression vectors encoding dTAK1 or dTAK1^{K156R} were transiently transfected together with dTAB2 into *Drosophila* S2 cells. Expression of *Diptericin* (an established transcriptional target of IMD signalling) was assayed 48 hrs post-transfection with quantitative PCR (qPCR). Results show that the K156R mutant failed to activate IMD immune signalling (Fig.3-1B).

A time-course expression analysis of dTAK1 and mutants dTAK1^{K142R} & dTAK1^{K156R} was performed after treatment with 26S proteasomal inhibitor MG132 (75µM for 8 hrs) at concentrations that are known to block proteasome activity as previously described (Stoven, 2000). Results showed that full-length dTAK1, and mutants dTAK1^{K142R} & dTAK1^{K156R} were similarly expressed (Fig. S1(B) – Appendix 3).

Taken together, results indicate that Lys 156 functions in a similar manner to Lys 158 in hTAK1, where both K48 and K63-linked polyubiquitination was abrogated upon its mutation. However, Lys 156 in dTAK1 does not completely abrogate either K63 or K48-linked polyubiquitination, although it significantly reduces both. Also, in 2 qPCR assays, the K156R mutant failed to significantly upregulate the IMD immune signal, although it was not completely abrogated. Therefore, it is very likely that Lys 142 and 156 function in a partially redundant manner to mediate K63-linked polyubiquitination of dTAK1 and modulate the IMD innate immune signal.

It cannot be ruled out that both Lys 142 and 156 are not Ub acceptor sites and the differences in ubiquitination and immune signalling observed following mutations at these 2 sites were artefacts. One possible explanation is that the kinase domain may have been disrupted following these mutations. A similar phenotype (deficient in both ubiquitination and immune signalling) may be observed if any ATP binding motif was disrupted. However, considering that the Ub acceptor site in hTAK1 is also within the kinase domain and the ATP binding motif has been identified at the very start of the protein, it is far more probable that both Lys 142 and 156 are Ub acceptor sit

(A) – Fig 3.4

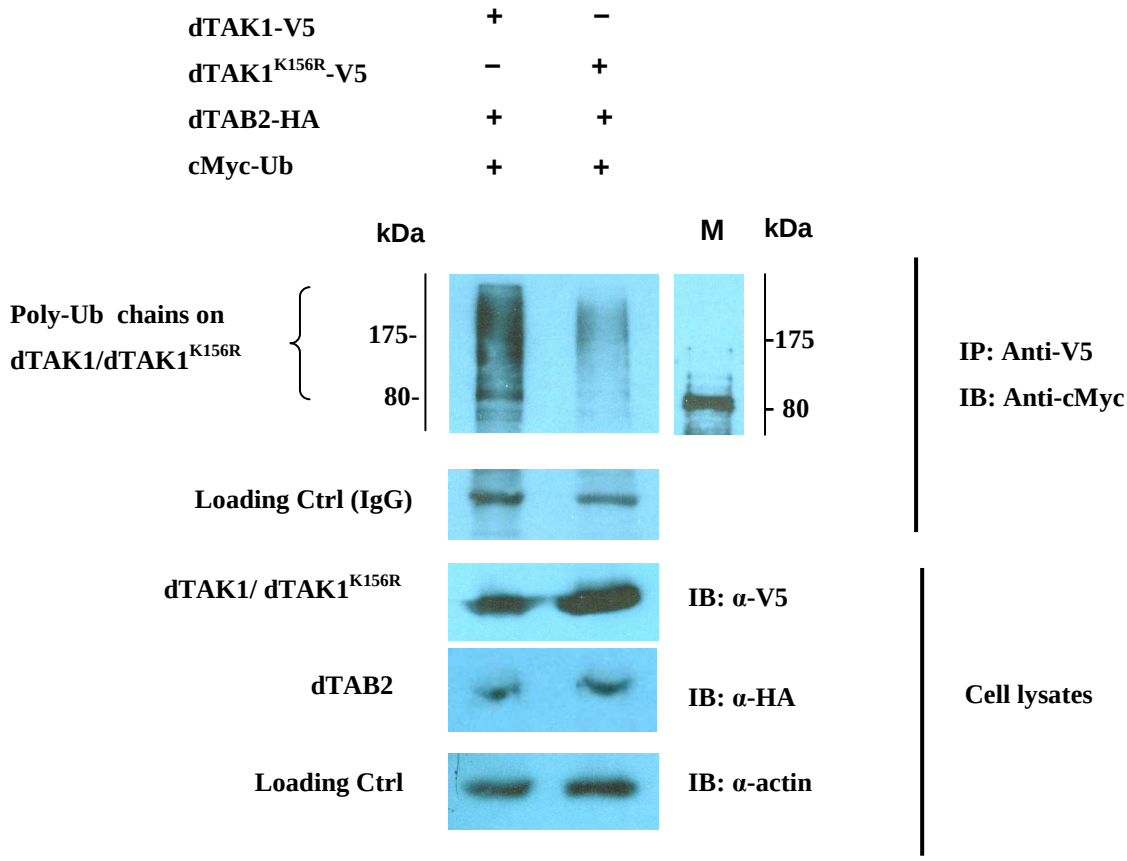


Figure 3-4: Decreased ubiquitination in the dTAK1^{K156R} mutant. (A) Mutant dTAK1^{K156R} shows decreased polyubiquitination when compared with dTAK1. *M – size marker for dTAK1 as indicated by arrow (dTAK1-V5 expressed in S2 cells, immunoprecipitated by anti-V5 and immunoblotted by anti-V5). Protein size markers are depicted adjacent to the top panels with values given in kDa.*

(B) – Fig 3.4

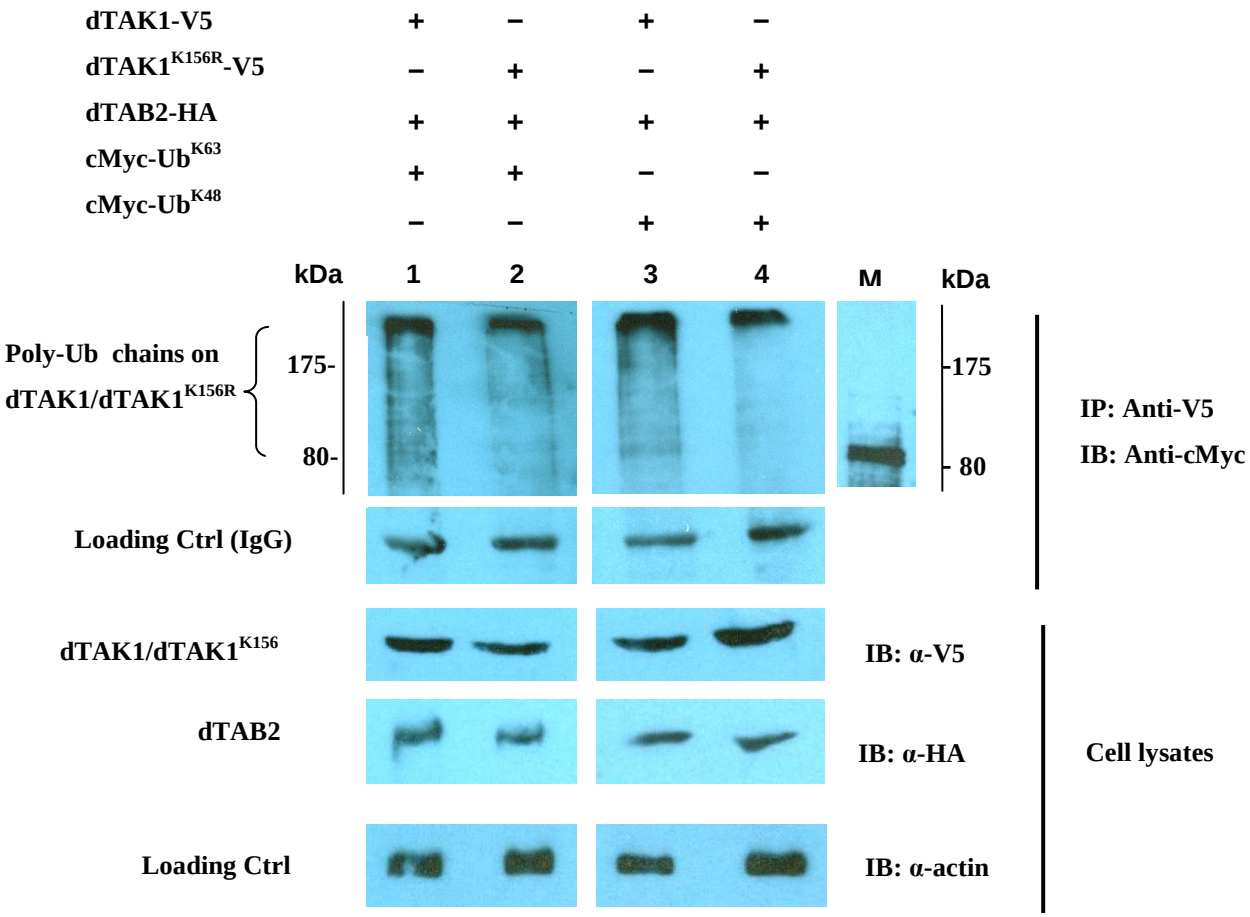


Figure 3-4: Decreased ubiquitination in the dTAK1^{K156R} mutant. (B) Both K48 and K63-linked polyubiquitination is decreased in the dTAK1^{K156R} mutant. Expression vectors encoding dTAK1-V5 or dTAK1^{K156R} –V5 were transfected along with dTAB2-HA and cMyc-Ub, cMyc-Ub^{K63} or cMyc-Ub^{K48} in combinations shown in S2 cells. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-cMyc antibody. *M – size marker for dTAK1 as indicated by arrow (dTAK1-V5 expressed in S2 cells, immunoprecipitated by anti-V5 and immunoblotted by anti-V5). Protein size marker (NEB) is depicted adjacent to the top panels with values in kDa.*

3.7 Trabid – a novel negative regulator of the IMD pathway

3.7.1 Role of de-ubiquitinating proteins in attenuating NF-κB signalling:

Since K63-ubiquitination plays a critical role in activating dTAK1, it follows that de-ubiquitination is required for inactivating dTAK1 and terminating the IMD immune signal. Recently, the deubiquitinating enzyme CYLD has recently been shown to physically interact with TAK1 in humans and inhibiting its ubiquitination and catalytic activity (Reiley et al., 2007).

In mammals, the zinc finger protein A20 (also known as TNFAIP3) plays an essential role in limiting the strength and duration of NF-κB signalling (Shembade et al., 2010). A20 proteins have been identified as negative regulators of NF-κB transcription factors in both TNF and IL-1 signalling through its de-ubiquitinating (DUB) and ubiquitin editing functions. In this model, K63-linked chains are cleaved by the N-terminal OTU domain and re-arranged to form K48-linked Ub chains through the E3 ligase activity of the C-terminal tandem Zinc-finger domains, thereby tagging the protein for proteosomal degradation (Heyninck and Beyaert, 1999; Wertz et al., 2004).

3.8 *Drosophila* Trabid and Rabex-5 interact with dTAK1:

Recently, Trabid was discovered as a positive regulator of both the mammalian and *Drosophila* Wnt pathway with a remarkable preference for binding to, and cleaving, K63-linked ubiquitin chains. Indeed, Trabid is the only representative of the A20 OTU family in flies (Tran et al., 2008). Further, *Drosophila* Rabex-5 was shown to restrict Ras signalling in the Ras pathway leading to the proper establishment of organism size, wing vein pattern, and eye versus antennal fate. It has been shown that excess Ras

signalling disrupts developmental patterning and causes developmental disorders, leading to cancers in mature tissues (Yan et al., 2010).

To determine whether Trabid and Rabex 5 also functioned in *Drosophila* IMD signalling, co-immunoprecipitation assays were performed to see whether these 2 enzymes interacted with dTAK1. Expression vectors encoding dTrabid-V5 or dRabex-5-V5 were transiently transfected along with dTAK1-HA, immunoprecipitated with anti-V5 antibody, resolved with 10% SDS PAGE and immunoblotted with anti-HA antibody. Results show that both dTrabid and Rabex-5 bound strongly with dTAK1 (Fig. 3-5A).

3.9 *Drosophila* Trabid negatively regulates the IMD immune signal in a dose-dependent manner:

Thereafter, the *in vivo* effects on IMD immune signalling of a *trbd* deletion generated by homologous recombination were tested (Tran et al., 2008). Homozygous and heterozygous *trbd* deletion flies, together with *white*$^{-}$ flies (as the background used for genetic transformation of the initial *trbd* targeting construct) and *relish*^{E20} mutants (negative control) were injected with Gram-negative bacterium *Escherichia coli* and *dipt* levels assayed after 6 hrs using qPCR. Results showed that removal of 1 copy of *trbd* resulted in a two-fold upregulation of *dipt* compared to wild type and removal of both alleles led to a 4-fold increase. A Student's t-test showed that the results were statistically significant (Fig 3-5B).

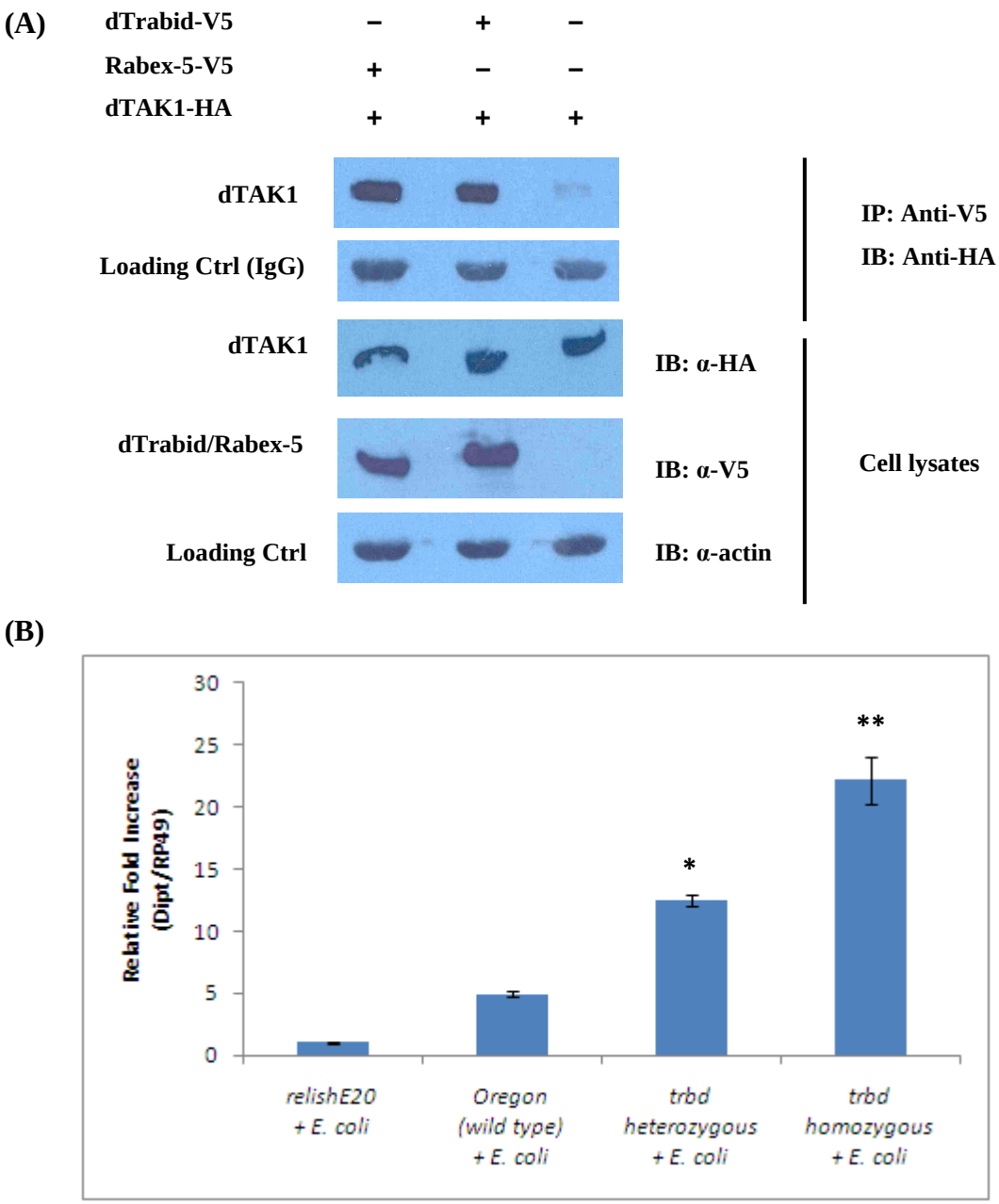


Figure 3-5: Both *Drosophila* Trabid and Rabex-5 binds to dTAK1 and dTrabid also negatively regulates the IMD signal. (A) Both *Drosophila* Trabid and Rabex-5 binds strongly with dTAK1. *Drosophila* TAK1-HA was co-transfected with or without *either* dTrabid-V5 *or* Rabex-5-V5 in S2 cells (in combinations shown), immunoprecipitated with anti-V5, resolved on 10% SDS PAGE and immunoblotted with anti-HA. (B) *Drosophila* Trabid negatively regulates the IMD signal in a dose-dependent manner. *Oregon 25174* (wt), *Relish*^{E20} (negative ctrl), *trbd* heterozygous and *trbd* homozygous mutant flies, were injected with *E. coli* and *diptericin* levels assayed after 6 hrs using qPCR. *Error bars represent the SEM of 3 separate experiments.* * & ** indicate significance value of the results as determined by Student's t-Test (t= 32.2880, $p < 0.025$ & t=8.2532, $p < 0.025$ respectively).

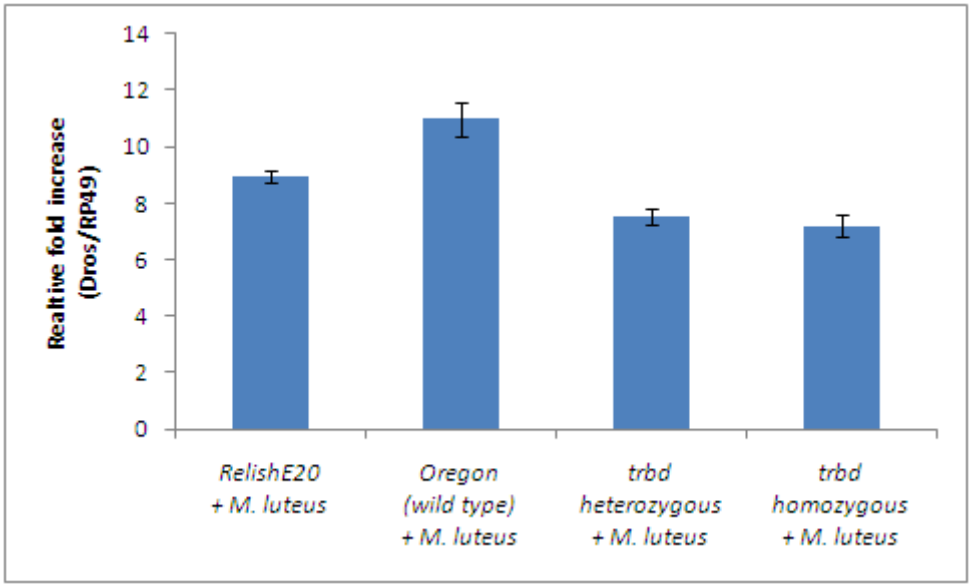
3.10 *Drosophila* Trabid does not function in either Toll signalling or survival

To determine whether dTrabid function was limited only to the IMD pathway or whether it affected innate immunity generally, similar cohorts of flies as above were injected with the Gram-positive bacterium *Micrococcus luteus* and assayed for *drosomycin* expression (target of the Toll cascade) after 24 hrs. All flies showed similar up-regulation of *drosomycin* (Fig. 3-6A).

Changes in immune signalling could potentially affect the survival of the organism. Therefore, experiments were carried out to determine whether Trabid had an impact on survival. Cohorts of 15♂ + 15♀ of *Oregon 25174* (wt), *relish*^{E20} (negative ctrl), *trbd* heterozygous and *trbd* homozygous mutant flies were injected with *E. coli* and survival assayed every 24 hrs for 5 days. Results showed that removing one or both genes of *trbd* had no effect on survival, when compared to wild type (Fig. 3-6B).

Taken together, it can be concluded that dTrabid functions solely in IMD signalling at the level of dTAK1 where it negatively regulated the immune signal in a dose-dependent manner.

(A)



(B)

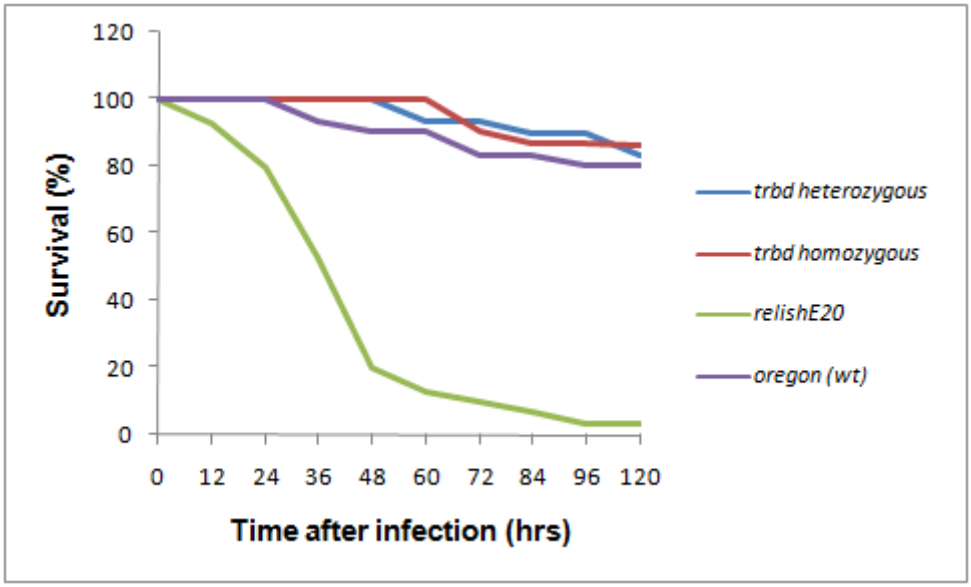


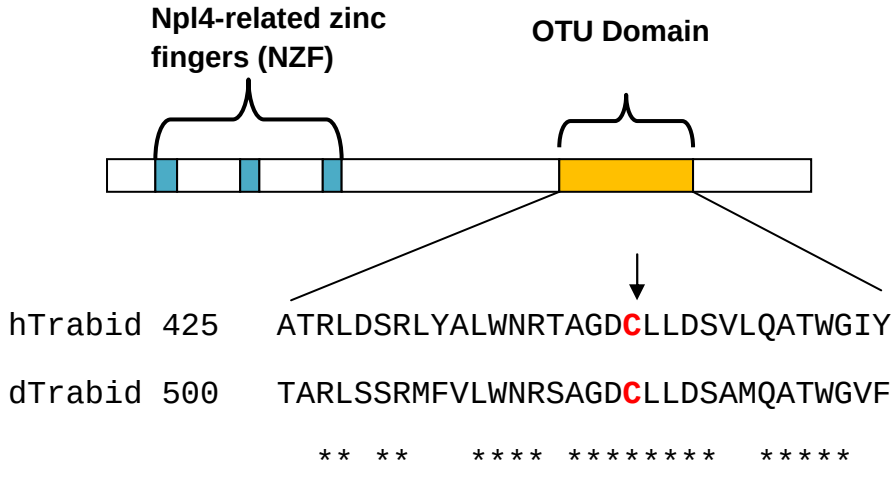
Figure 3-6: *Drosophila* Trabid (A) does not regulate Toll-dependent immunity and (B) has no impact on fly survival. (A) *Drosophila* Trabid does not regulate Toll signalling. *Oregon 25174* (wt), *Relish*^{E20} (negative ctrl), *trbd* heterozygous and *trbd* homozygous mutant flies, were injected with *M. luteus* and *drosomycin* levels assayed after 24 hrs using qPCR. *Error bars represent the SEM of 3 independent experiments.* (B) *Drosophila* Trabid does not play a role in survival. Cohorts of 15♂ + 15♀ of *Oregon 25174* (wt), *Relish*^{E20} (negative ctrl), *trbd* heterozygous and homozygous mutant flies were injected with *E. coli* and survival assayed every 12 hrs for 5 days.

3.11 C518 and the 3 NZF domains are required for dTrabid activity

Trabid (like A20) belongs to the OTU (ovarian tumour) family of deubiquitinating enzymes (DUBs). The OTU is a conserved cysteine protease domain that possesses DUB activity (Balakirev et al., 2003). Trabid possesses three N-terminal NZF (Npl4 zinc finger) domains and a C-terminal OTU domain, which shows DUB activity with a preference for K63-linked ubiquitin (Tran et al., 2008). Further, it was shown that two or more NZF domains are required for binding to K63-linked Ub chains (because of the 'outspread' conformation of K63-linked polyubiquitin). Also, C443 was shown to be the catalytic residue in the OTU domain in humans (Tran et al., 2008).

Sequence alignment of human Trabid and *Drosophila* Trabid showed that C518 was the most probable corresponding catalytic residue in *Drosophila* (Fig 3-7A). To test the functions of both C518 and the 3 NZF domains, 2 mutant constructs, dTrabid^{C518S} and dTrabid$^{C518S+3xNZFDel}$ (where the first Cys residue of the 3 NZF domains, C13, C94 and C238, were mutated to Ala in together with the C518S mutation) were made. To test the functional importance these mutations in IMD signalling, dTAK1 and dTAB2 were co-transfected along with dTrabid^{C518S} and dTrabid$^{C518S+3xNZFDel}$ in S2 cells and relative *dipt* expression assayed using qPCR. Results showed that over-expression of dTrabid, significantly decreased the IMD signal although it did not completely abrogate it. The C518S mutation relieved the negative effect substantially and the C518S + 3xNZFDel mutations completely reversing the effect (Fig 3-7B). To determine whether dTrabid had a role in the JNK pathway, the above samples were used to assay the expression levels of *puckered* (a target of the JNK cascade) using qPCR. Results revealed that the JNK pathway was not affected, indicating that dTrabid functioned solely in the IMD pathway (Fig. S2 – Appendix 3).

(A)



(B)

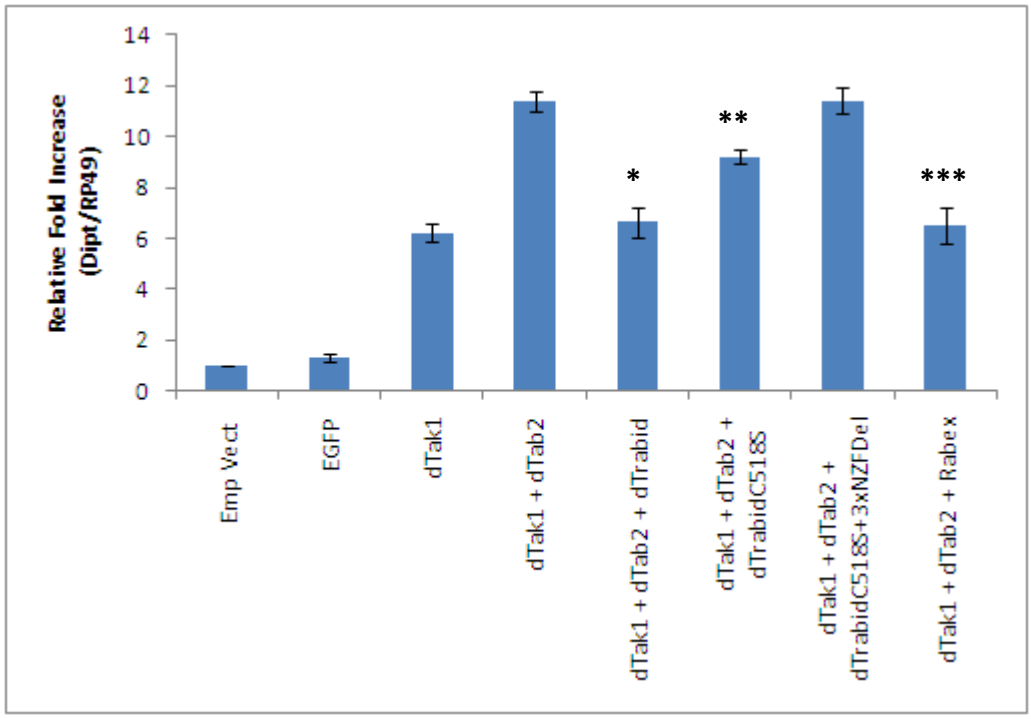


Figure 3-7: Cys 518 is the probable catalytic cysteine in *Drosophila* Trabid

(A) Schematic representation of dTrabid and sequence alignment of human and *Drosophila* Trabid in the OTU domain. The putative catalytic residue is in red & is indicated by an arrow. (B) Point mutations in C518 and the 3 NZF domains significantly affect dTrabid function in IMD signalling. Expression vectors containing dTrabid^{C518S}, dTrabid$^{C518S+3xNZFDel}$, dTAK1 and dTAB2 were co-transfected in S2 cells in the combinations shown and *diptericin* expression was assayed by qPCR 48 hrs post-transfection. *Results were normalised to an empty vector (Emp Vec) and the error bars represent SEM of 3 independent experiments*. *, ** & *** indicate significance value of the results as determined by Student's t-Test (t= 9.8004, $p < 0.025$, t=10.5654, $p < 0.01$ & t=4.4876, $p < 0.05$ respectively).

Trabid exerts its deubiquitinating activity by preferentially cleaving K63-linked polyubiquitin chains (Tran et al., 2008). Both the OTU domain and the 3 NZF motifs are essential for its function in humans. To determine the relative contribution of the catalytic C518 and the 3 NZF motifs for deubiquitination in *Drosophila*, a DUB assay was performed in S2 cells. Expression vectors encoding C-terminally HA-tagged dTrabid, dTrabid^{C518S} or dTrabid$^{C518S+3xNZFDel}$ were transiently transfected with dTAK1-V5, dTAB2-HA and cMyc-Ub^{K63}/cMyc-Ub^{K48} in S2 cells in the combinations indicated. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5 antibody, resolved on 10% SDS PAGE and immunoblotted with anti-cMyc peroxidise antibody. In agreement with the qPCR data, dTrabid substantially reduced K63-linked ubiquitination of dTAK1 when co-transfected with dTAB2, although not completely abrogated (lane 3). Further, ubiquitination was moderately affected by dTrabid^{C518S} (lane 4) and not at all by dTrabid$^{C518S+3xNZFDel}$ (lane 5) (Fig 3-8). We also similarly tested the effects of dTrabid, dTrabid^{C518S} and dTrabid$^{C518S+3xNZFDel}$ on K48-linked ubiquitination of dTAK1. Results show that dTAK1 K48-polyubiquitination was not affected by either dTrabid or the mutants (Fig. 3-9).

It is likely that dTrabid may function in a negative feedback mechanism in the same manner as A20. In agreement with this hypothesis, my results show that in *trbd* deletion mutants, the IMD innate immune signal was greatly enhanced. This is similar to the phenotype obtained with *A20*-deficient mice a previously described (Lee et al., 2000). However, dTrabid also needs to be upregulated upon IMD induction and this is yet to be established.

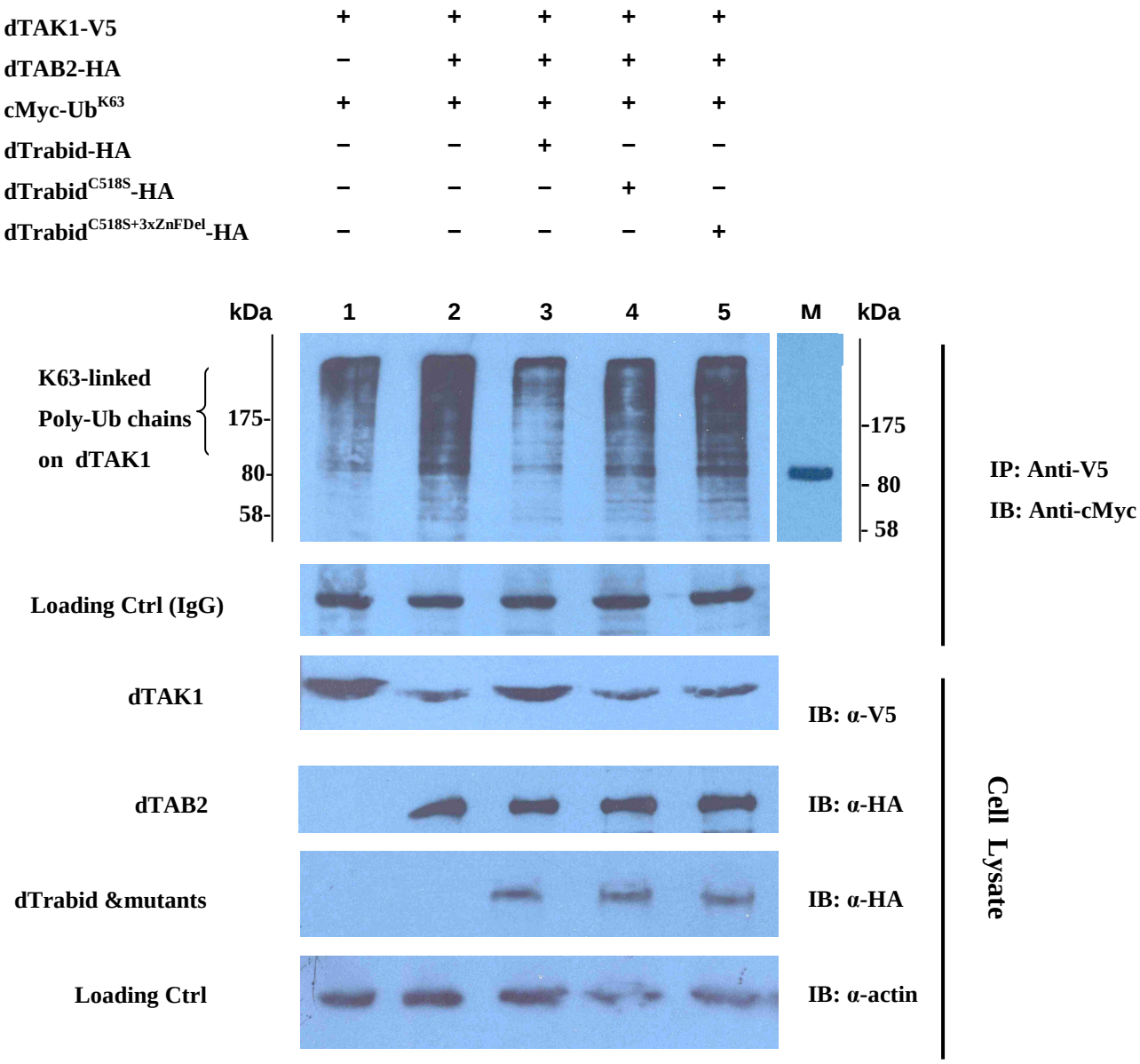


Figure 3-8: *Drosophila* Trabid significantly decreases K63-linked polyubiquitination in dTAK1. Expression vectors containing C-terminally HA-tagged dTrabid, dTrabid^{C518S} or dTrabid$^{C518S+3xNZFDel}$, along with dTAK1-V5, dTAB2-HA and cMyc-Ub^{K63} were co-transfected in S2 cells in the combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE and immunoblotted with anti-cMyc peroxidise. *M – size marker for dTAK1 as indicated by arrow (dTAK1-V5 expressed in S2 cells and immunoblotted by anti-V5). Protein size markers are depicted adjacent to top panels with values given in kDa.*

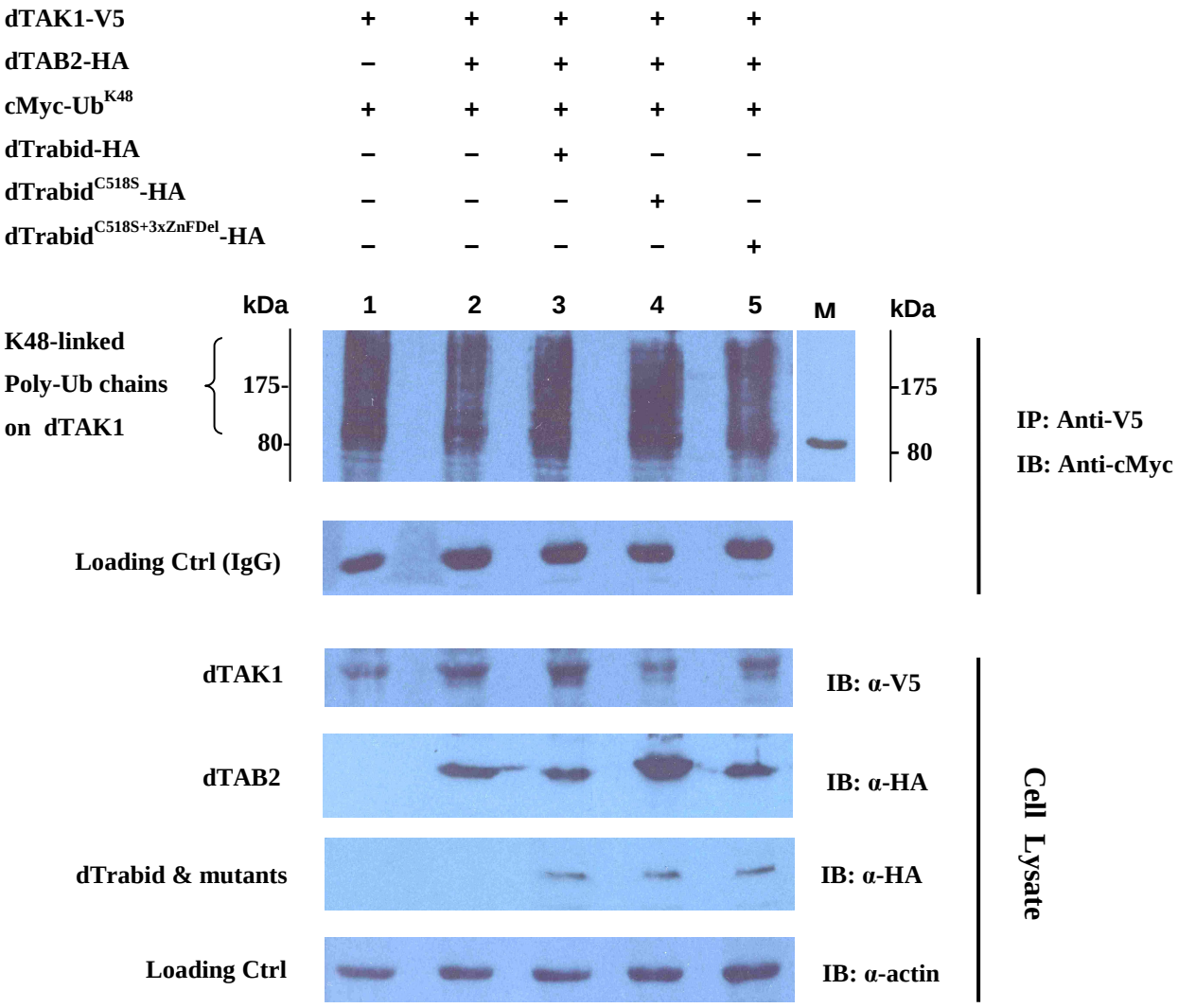


Figure 3-9: *Drosophila* Trabid does not affect K48-linked polyubiquitination in dTAK1. Expression vectors containing C-terminally HA-tagged dTrabid, dTrabid^{C518S} or dTrabid$^{C518S+3xNZFDel}$, along with dTAK1-V5, dTAB2-HA and cMyc-Ub^{K48} were co-transfected in S2 cells in the combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE and immunoblotted with anti-cMyc peroxidise. *M – size marker for dTAK1 as indicated by arrow (dTAK1-V5 expressed in S2 cells and immunoblotted by anti-V5). Protein size markers are depicted adjacent to top panels with values given in kDa.*

3.11.1 *Drosophila* Trabid – summary

Taken together, results indicate that dTrabid negatively regulates the IMD signal in a dose-dependent manner. Results show that the activity of dTrabid was limited to the IMD pathway and did not function in either the Toll or JNK cascades. It functions by de-ubiquitinating K63-linked polyubiquitin chains of dTAK1. Both C518 and the 3 NZF domains were required to fully execute this function. Also, dTrabid did not affect K48-linked ubiquitination. Results also show that deletion of the catalytic residue alone was not sufficient to abrogate its function, leaving the possibility that the Npl4-related zinc finger motifs may also play an active role in de-ubiquitination. Mutations in both the catalytic residue and the NZFs rendered the protein functionally inert. Both qPCR data (Fig 3-6B) and K63-specific ubiquitination assays (Fig. 3-7) indicate that while dTrabid significantly reduces the IMD signal, it doesn't completely abrogate it, leaving the possibility that it may function in a partially redundant manner with another regulatory component. There is evidence to indicate that Rabex-5 may be that component, although further work is required to establish this.

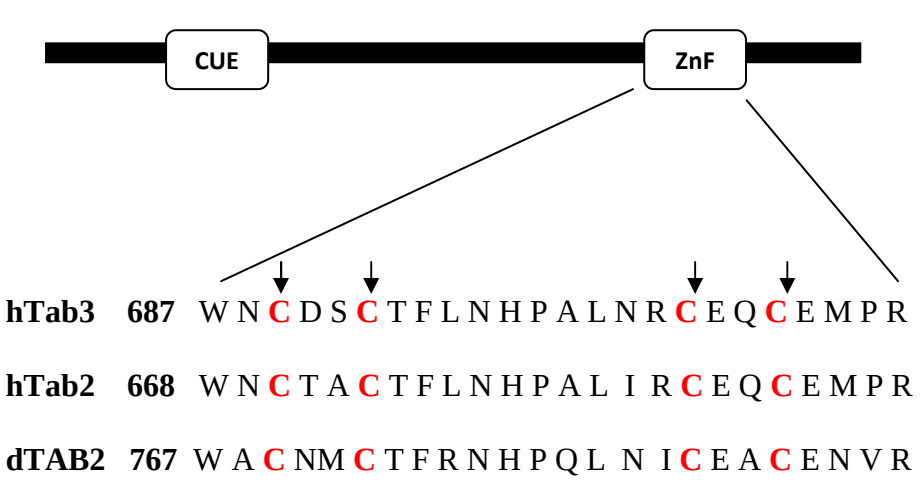
3.12 *Drosophila* TAB2 ZnF domain is involved in regulating IMD signalling

Drosophila TAK1-associated Binding Protein 2 (dTAB2; CG7417) is an adaptor protein which has been implicated in protein complex formation. Homologous to human TAB2 and TAB3 and consisting of a CUE, coiled-coil and ZnF domains, *Drosophila* TAB2 is the binding partner of dTAK1 (Geuking et al., 2005; Hetru and Hoffmann, 2009; Kleino et al., 2005; Zhuang et al., 2006).

In mammals, TAB2 and TAB3 function as adaptors which link TRAF proteins (TRAF2 and TRAF6) to TAK1, facilitating complex formation and activation of TAK1 in IL-1and TNF-induced NF-κB activation (Besse et al., 2007; Ishitani et al., 2003; Takaesu et al., 2000a) . Both TAB2 and TAB3 contain a highly conserved C-terminal novel zinc finger domain, which binds preferentially to K63-linked polyubiquitin chains. Mutation of this domain abolishes the ability of TAB2 and TAB3 to bind to polyubiquitin chains, as well as their ability to activate TAK1. (Besse et al., 2007; Kanayama et al., 2004).

It was sought to determine the functional significance of the corresponding C-terminal ZnF domain of *Drosophila* TAB2 in IMD immune signalling. *Drosophila* TAB2 was previously found to interact with dTAK1 and essential for the immune activation of JNK and NF-κB (Zhuang et al., 2006). A *Drosophila* TAB2 mutant ($dTAB2^{ZnFDel}$) was made mutating the first 2 Cys residues in the C-terminal ZnF motif to Ala (C769A and C772A) as previously performed with human TAB2 (Fig. 3-10A) (Kanayama et al., 2004). Thereafter, effect of this mutation on IMD immune signalling was assayed in cell culture using qPCR. *Drosophila* TAK1 was transfected either alone or together with dTAB2 or $dTAB2^{ZnFDel}$ into S2 cells (as indicated below) and *dipt* expression assayed 48 hrs post-transfection using qPCR. Interestingly, signalling was increased by 2-fold in the presence of the mutant $dTAB2^{ZnFDel}$ when compared with full-length dTAB2 (Fig 3-10B).

(A)



(B)

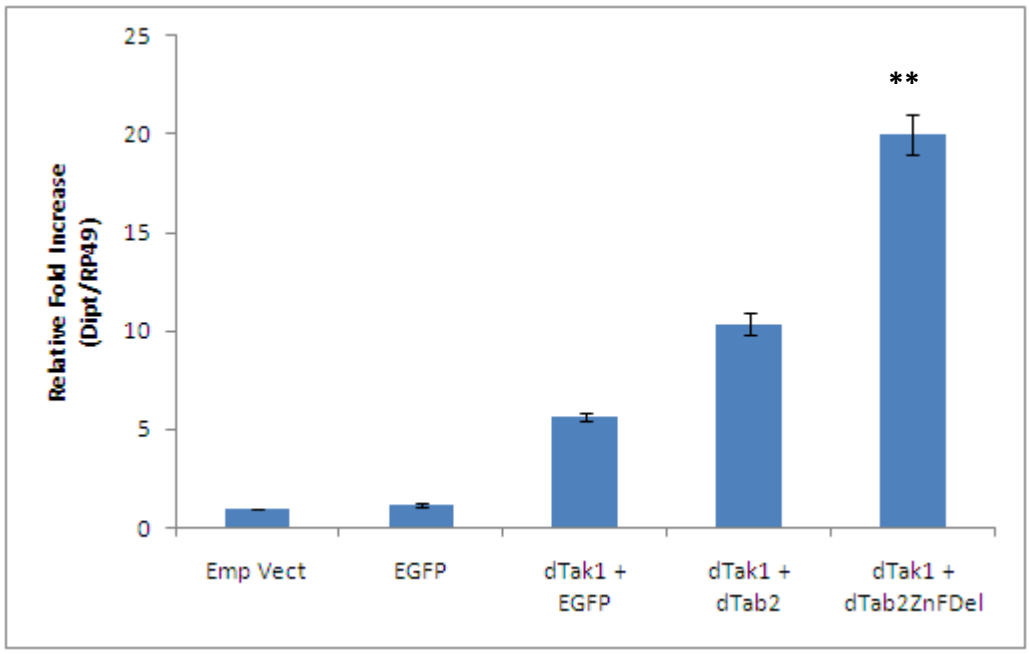


Figure 3-10: *Drosophila* TAB2 ZnF domain regulates IMD signalling (A) Sequence alignment of the C-terminal ZnF domain of hTAB2, hTAB3 and dTAB2. Conserved Cys residues are in red and indicated by arrows. (B) IMD signalling is significantly increased in dTAB2^{ZnFDel} mutant. S2 cells were co-transfected with expression vectors in combinations shown and *Diptericin* expression was assayed 48hrs post-transfection by qPCR. *Results were normalised to Emp Vec and the error bars represent the SEM of 3 independent experiments.* ** indicates significance value of the result as determined by Student's t-Test (t= 6.2838, $p < 0.025$)

Increased *Dipt* induction may be the result of greater dTAK1 activation. Therefore, the level of K63-linked ubiquitination of dTAK1 was examined in the presence of either dTAB2 or dTAB2^{ZnFDel}. Expression vectors encoding dTAK1-HA was co-transfected with cMyc-Ub^{K63} and dTAB2-V5 or dTAB2^{ZnFDel}-V5 into S2 cells. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-HA and immunoblotted with anti-cMyc. Results showed that, K63-linked ubiquitination of dTAK1 was increased in the presence of dTAB2^{ZnFDel} (Fig. 3-11A). Analysis of the cell lysate showed a greater amount of dTAK1 present with the mutant, although equal amounts of total protein (200µg per lane) were used. Taken together, results indicate that the mutation in the dTAB2 ZnF domain stabilised dTAK1 by enhancing its K63-linked ubiquitination. This led to an increase in IMD immune signalling through increased *Diptericin* induction. Therefore, it appears that the interaction of dTAK1 with the dTAB2 C-terminal ZnF domain pacifies dTAK1 activity and thereby, the IMD signal.

To test whether dTrabid binds with dTAB2, co-immunoprecipitation assays were performed. These showed that dTrabid bound to dTAB2^{ZnFDel} two-fold stronger than to dTAB2 (Fig. 3-11B). Of note, dTAB2^{ZnFDel} up-regulated *Dipt* expression by 2-fold over dTAB2. This indicated that the tighter the dTrabid bound to dTAB2 the higher the signalling capacity of dTAK1.

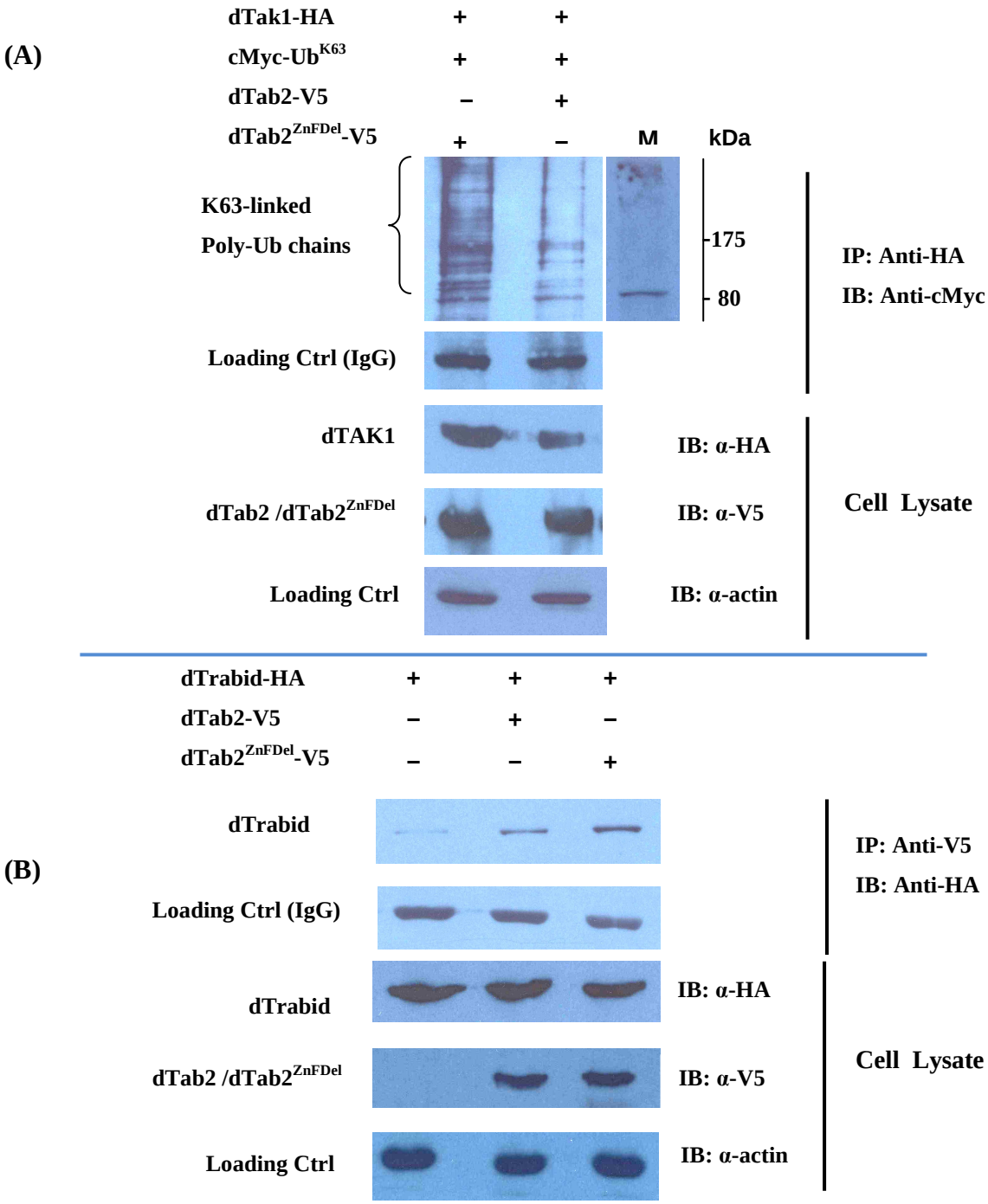


Figure 3-11: *Drosophila* TAB2 is involved in the regulation of dTAK1. (A) Mutation of dTAB2 ZnF stabilises dTAK1 and increases its K63-linked ubiquitination. *Drosophila* TAK1-HA was co-transfected with cMyc-Ub^{K63} and either dTAB2-V5 (wt) or dTAB2^{ZnFDel}–V5 into S2 cells. Cells were lysed 48hrs post-transfection, immunoprecipitated with anti-HA, resolved on 10% SDS PAGE and immunoblotted with anti-cMyc antibody. (B) *Drosophila* Trabid binds to dTAB2^{ZnFDel} but not to dTAB2 (wt). S2 cells were transfected with dTrabid-HA alone or with either dTAB2^{ZnFDel} –V5 or dTAB2-V5. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE and immunoblotted with anti-HA. *M – size marker for dTAK1. Protein size markers are depicted adjacent to M with values in kDa.*

Chapter 4: Negative Regulation of *Drosophila* TAK1 in IMD Innate Immune Signalling - Discussion

4.1 TAK1 - activation of several pathways by a single kinase

During growth and differentiation, organisms receive numerous external stimuli, whose signals of are then transduced from the cell surface to the nucleus via specific sets of intracellular signalling molecules. One such set are the Mitogen-activated protein kinases (MAPK) and they play crucial roles in cell proliferation, differentiation, and development (Nishida and Gotoh, 1993; Treisman, 1996). Activation of MAPK is by a sequential cascade of protein kinases. It is activated by dual phosphorylation through catalysis by MAPK kinase (MAPKK), which is itself phosphorylated and activated by a MAPKK kinase (MAPKKK). Prominent MAPK regulated cascades include the c-Jun amino-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway, the MAPK/extracellular signal-regulated kinase (ERK) pathway, and the p38 MAPK cascade (Davis, 1994; Marshall, 1995; Nishida and Gotoh, 1993; Waskiewicz, 1995).

TAK1 is a member of the MAPKKK superfamily. It is triggered by tumour growth factor-β (TGF-β) and therefore, can function in cascades triggered by the superfamily of TGF-β ligands as indicated in transient transfection assays using cultured cells. TAK1 has an N-terminal serine/threonine kinase domain and a C-terminal conserved domain (Takatsu et al., 2000; Vidal et al., 2001; Yamaguchi et al., 1995).

Recent evidence implicates TAK1 in various signalling pathways, including IL-1, IL-18, TNF, and receptor activator of NF-κB ligand (RANKL) (Irie et al., 2000; Ninomiya-Tsuji et al., 1999; Sakurai et al., 1999; Wald et al., 2001). It is hypothesised that upstream, including members of the TNF receptor-associated factor (TRAF) family

of adaptor proteins play a crucial role in providing specificity of downstream signalling through formation of specific complexes depending on the particular stimulation. Depending on the specificity of the complex formed, particular signalling cascades will be activated (Besse et al., 2007; Cao et al., 1996; Naito et al., 1999; Yeh et al., 1997). Therefore, the regulation of TAK1 assumes great importance in light of its activity in different pathways.

4.2 Activation of TAK1 in NF-κB signalling- the Mammalian Model:

In humans, TRAF6 has been identified as the E3 ubiquitin ligase which controls the activation of TAK1 in IL-1 signalling. In this model, TRAF6 itself is first activated by auto-ubiquitination via K63-linked polyubiquitin. The intact RING domain of TRAF6 and dimeric ubiquitin E2- conjugating enzyme complex (Ubc13/Uev1A) were found to be essential. Activated TRAF6 would subsequently polyubiquitinate TAK1 again through K63-linkages. Ubiquitinated TAK1 is then phosphorylated and activated by an unknown mechanism. Thereafter, activated TAK1 phosphorylates and activates IKK for NF-κB activation (Fan et al., 2010; Lamothe et al., 2007). Considering the conserved nature of innate immunity between insects and mammals, it is highly plausible that *Drosophila* TAK1 is activated in a similar manner. Although numerous attempts have been made, the E3 ubiquitin ligase in *Drosophila* which activates TAK1 is yet unknown (Ferrandon et al., 2007; Paquette et al., 2010).

4.3 Negative regulation of *Drosophila* TAK1:

Although many investigations have been conducted on activation of *Drosophila* IMD signalling, much less is known about its negative regulation, especially at the level of dTAK1 (Khush et al., 2002). This aspect is particularly important since IMD signalling shows an “acute phase profile.” The immune signal increases rapidly,

peaking ~6hrs post-infection and then gradually declining to reach basal levels by 24 hrs (Lemaitre et al., 1997). There appear to be no specific studies conducted on whether sustained activation of IMD results in harmful effects in *Drosophila*. My results show that IMD hyperactive *trbd* mutant flies have the same survival patterns as wild type. However, the long-term energy costs of maintaining heightened immunity may impair certain aspects of growth and development. In mammals, NF-κB regulates genes involved in both inflammation and immunity and sustained or chronic signalling has been implicated in several autoimmune diseases and cancers in humans. Therefore, under normal conditions, immune signalling is both transient and tightly controlled (Sriskantharajah and Ley, 2010).

4.4 Lys 142 in dTAK1 is essential for immune signalling

Ubiquitination plays a key role in TAK1 activation and therefore, its ubiquitin acceptor site is critical for TAK1 activity. It was previously shown that Lysine 158 was the Ub acceptor site in humans (Fan et al., 2010). Alignment of the N-terminal sequences of human and *Drosophila* TAK1 led to the determination that Lys 142 in *Drosophila* was the corresponding Ub-acceptor site. My results show that Lys 142 in *Drosophila* is essential for immune signalling and that the dTAK1^{K142R} mutant failed to activate the IMD innate immune pathway. Further, the ubiquitination profile of dTAK1^{K142R} was significantly altered – K63-linked ubiquitination was diminished whilst K48 ubiquitination was greatly enhanced. Moreover, results show greater degradation of dTAK1^{K142R} in the cell lysate, possibly indicative of K48-polyubiquitination linked proteasomal degradation. Based on my evidence, it was reasonable to conclude that *Drosophila* TAK1 has 2 different Ub acceptor sites for K63 and K48-linked chains and that Lys 142 was the probable K63 acceptor site.

4.5 Lys 156 is also an Ub acceptor site:

Investigations to find the possible K48 Ub acceptor site revealed K156 as a candidate. Ubiquitination assays showed that dTAK1^{K156R} mutant showed significantly reduced ubiquitination than wild-type dTAK1. Further analysis, with K48 and K63-

specific Ub assays showed that K48 polyubiquitination was greatly reduced in the dTAK1^{K156R} mutant. Also, K63 polyubiquitination was reduced. Therefore, Lys 156 appears to function in a manner similar to K158 of hTAK1, which is the acceptor site for both K63- and K48-linked polyubiquitin. Preliminary qPCR results show that the IMD signal is severely reduced in the dTAK1^{K156R} mutant and is similar to that of dTAK1^{K142R}. In both dTAK1^{K142R} and dTAK1^{K156R} mutants, the IMD immune signal, although greatly reduced, is not completely abrogated. More work, including quantitative qPCR & Ub assays with double mutant construct of K142R and K156R will indicate the redundancy aspect of these 2 sites.

Taken together, results suggest that Lys 142 and Lys 156 function in a partially redundant manner. Both K142 and K156 function as K63 Ub acceptor sites, whilst K156 also functions as the K48 Ub acceptor site.

Possible Monoubiquitination in dTAK1:

In ubiquitination assays of dTAK1 in S2 cells, a major band is seen at ~ 84 kDa (representing the molecular weight of monoubiquitinated dTAK1) and is visible when blotted with the antibody specific for Ub. Therefore, this band is likely to be the monoubiquitinated form of dTAK1. As described above, a similar band is observed in humans (Fan et al., 2011; Fan et al., 2010). Hence, it appears that a significant amount of total ubiquitinated dTAK1 is monoubiquitinated. Like K63 polyubiquitination,

monoubiquitination is also implicated in degradation-independent functions including protein kinase activation, DNA repair, membrane trafficking and chromatin remodelling (Liu and Chen, 2011). Different types of ubiquitin modifications (including monoubiquitination as well as polyubiquitination by K6, K63 or linear ubiquitin chains) have been reported on NEMO, the regulatory subunit of the IKK complex in NF-κB signalling. Investigations of different NF-κB signalling pathways revealed several potential ubiquitination sites on NEMO, including K285, K277, K309 and K399 (Abbott et al., 2004; Abbott et al., 2007; Hinz et al., 2010; Huang et al., 2003; Tokunaga et al., 2009; Zhou et al., 2004). In genotoxic stress induces NF-κB activation (where DNA damage signals originate in the nucleus and IKK activation occurs in the cytoplasm) NEMO is monoubiquitinated and this eventually results in activation of the TAK1 kinase complex. TAK1 then phosphorylates IKK, resulting in NF-κB activation (Wu et al., 2010). Moreover, it was recently shown that site-specific monoubiquitination of subunit IKKβ of the IKK complex regulates its phosphorylation and persistent activation (Carter et al., 2005).

Like NEMO, TAK1 is deferentially activated during NF-κB signalling. During IMD innate immune signalling *Drosophila* TAK1 also transiently activates the JNK pathway, in addition to downstream activation of the IKK complex. However, JNK activation is short-lived and is quickly terminated by a Relish-dependent process (Park et al., 2004). Tsuda *et. al.*, (2005) reported that POSH (an E3 ubiquitin ligase) was involved in terminating the JNK signal by ubiquitinating dTAK1 and tagging it for degradation.

One possible explanation is that, upon activation of IMD signalling, dTAK1 is involved in regulating several aspects of its signalling (including JNK and IMD) and is differentially ubiquitinated for these different processes. It is also possible that (like

NEMO) dTAK1 has several Ub acceptor sites which differentially ubiquitinate. Monoubiquitination itself may be sufficient for activation of dTAK1 kinase activity. This would explain the presence of a significant amount of possibly monoubiquitinated dTAK1 when the IMD pathway is activated in cell culture.

Further, overexpression of dTAK1 alone is sufficient to activate the IMD pathway, as seen by qPCR assays. *Drosophila* TAK1 immunoprecipitates (even when expressed alone) show ladder-like bands, indicative of polyubiquitination. Therefore, we can assume that dTAK1 ubiquitin modification occurs even when overexpressed alone (with the aid of endogenous components). It was shown that chronic phosphorylation of IKKβ at Ser-177/Ser-181 leads to monoubiquitin attachment at nearby Lys-163, which in turn modulates the phosphorylation status of IKKβ during chronic inflammation (Carter et al., 2005). Similarly, constitutive over-expression of dTAK1 may lead to its chronic activation and monoubiquitination. This may explain why singly overexpressed dTAK1 appears to have the same migration as a possibly monoubiquitinated form.

Alternatively, dTAK1 might be co-translationally or post-translationally modified which may explain the band shift in the marker. Further experiments are required to firmly establish this.

4.6 Role of Lys 142 in JNK Signalling

In *Drosophila*, the JNK pathway is transiently activated during IMD innate immune signalling. *Drosophila* TAK1 is a central component of the JNK cascade and is essential for its activation (Geuking et al., 2005; Silverman et al., 2003). My quantitative PCR (qPCR) data show that the JNK pathway is not strongly activated during IMD innate immune signalling, although a slight upregulation is evident (Fig. S1

(A) - Appendix 3). This is most probably due to the fact that the JNK pathway is purposely "turned off" at the onset on IMD signalling. Park *et al.*, 2005 showed that Relish plays a crucial role in limiting the duration of JNK activation and output in response to Gram-negative infections. Here, Relish activation results in proteasomal degradation of dTAK1, leading to a rapid termination of JNK signalling. It was also shown that the RING finger containing adaptor POSH, too, played a crucial role in limiting the JNK signal in innate immune signalling (Tsuda et al., 2005).Similarly, in mammals, the IKK-NF-κB module negatively regulates JNK activation (Tang et al., 2001).

My results show that, even at a low level of activation, the mutant dTAK1^{K142R} failed to activate JNK signalling above basal levels. Similarly, it was shown that Lys 158 in human TAK1 was required for TNFα and IL-1β induced IKK/NF-κB and JNK/AP-1 activation (Fan et al., 2010). Hence, it is very likely that Lys 142 is essential for JNK activation and signalling. However, further work is required to establish this firmly.

Puckered is an established transcriptional target of JNK signalling in *Drosophila*. Puckered is a dual-specificity MAP kinase phosphatase that antagonizes the JNK signal cascade through a feedback loop. It has been implicated in several processes including regulating JNK during dorsal closure, antagonising JNK-induced apoptosis and interaction with the 5′–3′ exoribonuclease *pacman*. It was recently shown that Puckered also affects Wingless (Wg) signalling where it functioned as a potential regulator of Armadillo (Grima et al., 2008; Martin-Blanco et al., 1998; McEwen and Peifer, 2005; Tuskey, 2001). Therefore, caution should be exercised when *puc* is solely used to monitor JNK activity. An alternative method would be to assay for JNK phosphorylation through specific anti-phospho-JNK antibodies or another phosphorylation assay.

Examination of the domain architecture of *Drosophila* TAK1 using the SMART programme (www.smart.embl-heidelberg.de) revealed that it contains a series of 12 to 13 serine/threonine/tyrosine kinase domains in tandem in the N-terminus. Both Lys 142 and 156 are within the dTAK1 kinase domain. Therefore, it is possible that changes in ubiquitination in the dTAK1 mutants are due to the disruption of one or more of these domains which render it inactive. It is not possible to investigate this matter further because the domain architecture has not been precisely mapped. Alternatively, disruption of an ATP binding motif could render the protein inactive. Although these explanations cannot be completely ruled out, they remain unlikely. In human TAK1, the Ub acceptor site K158, too, is within the kinase domain. In both human and *Drosophila* TAK1, the kinase activation loop is strongly conserved and the Lysines are not within this critical structure. Moreover, Lys 63 was identified as part of the ATP binding motif in human TAK1 by Fan *et. al*., (2010). A similar conserved residue can be identified in *Drosophila* TAK1 (Lys 45) by alignment analysis with hTAK1. Further, disruption of the ATP binding motif would render the protein incapable of any ubiquitination. Also, the expression profiles of both mutants (with MG132 treatment) were similar to wild type dTAK1 in the soluble fraction. Taken together, it is unlikely that changes in ubiquitination in the dTAK1 mutants are due to disruption of either the kinase domain or the ATP binding motif. It is far more likely that both Lys 142 and 156 are Ub acceptor sites.

4.7 Negative Regulation of NF-κB Signalling – the DUBs:

De-ubiquitinating enzymes (DUBs) have been implicated in regulating NF-κB signalling in mammals. A20 is a potent inhibitor of NF-κB signalling in the tumour necrosis factor receptor (TNFR) and Toll-like receptor (TLR) pathways, through the cooperative activity of its 2 ubiquitin-editing domains (Wertz et al., 2004). The N-

terminal OTU domain de-ubiquitinates K63-linked chains from receptor interacting protein 1(RIP1). Then, its C-terminal seven tandem C_2/C_2 zinc fingers polyubiquitinates it with K48-linked chains, thereby tagging RIP1 for proteosomal degradation (Wertz et al., 2004). A20 was previously shown to also bind to TRAF6 and interfere with IL-1-induced NF-κB activation (Heyninck and Beyaert, 1999). It was recently established that A20 inhibited the E3 ligase activities of TRAF6, TRAF2, and cIAP1 by antagonizing interactions with the E2 ubiquitin conjugating enzymes Ubc13 and UbcH5c. Here, A20 interacted with the E2 enzymes (together with the regulatory molecule TAX1BP1) and triggered their ubiquitination and proteosomal degradation (Shembade et al., 2010).

The tumour suppressor CYLD has also been shown to negatively regulate NF-κB signalling by deubiquitination in both humans and *Drosophila*. In human cells, CYLD interacted with NEMO and inhibited the activation of NF-κB by deubiquitinating signalling molecules of TNFR-associated pathways including CD40, XEDAR and EDAR (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). Similarly, *Drosophila* CYLD interacted with Kenny and negatively regulated NF-κB signalling through deubiquitination (Tsichritzis et al., 2007).

Also, the *Drosophila* ubiquitin-specific protease 36 (dUSP36) was recently described as a negative regulator of the IMD pathway. This ubiquitin protease interacted with IMD and prevented the accumulation of K63-polyubiquitinated IMD while promoting IMD degradation, thereby preventing the nonspecific activation of innate immune signalling (Thevenon et al., 2009).

Trabid, a member of the OTU family (as A20), was recently discovered as a regulator of the Wnt pathway of both mammals and *Drosophila* with DUB activity.

Here, it showed remarkable preference to binding and cleaving K63-linked polyubiquitin chains (Tran et al., 2008).

Recently, *Drosophila* Rabex-5 was shown to restrict the Ras signalling pathway in order to establish organism size, wing vein pattern, and eye versus antennal fate. It was further shown that Rabex-5-mediated Ras ubiquitination promoting Ras endosomal localization and leading to the suppression of ERK activation (Xu et al., 2010; Yan et al., 2010).

4.8 *Drosophila* Trabid negatively regulates IMD innate immune signalling

Co-immunoprecipitation assays show that both *Drosophila* Trabid and Rabex-5 interacts with dTAK1. Both interactions appear to be strong, leading to the assumption that these are direct bindings and not transient interactions. Therefore, it was probable that both dTrabid and Rabex-5 functioned at the level of dTAK1 and would be strong candidates for DUBs in the IMD pathway.

Examination of the *in vivo* effects of of *trbd* deletion flies (both homozygous and heterozygous) on IMD immune signalling clearly showed that dTrabid negatively regulated the immune signal in a dose-dependent manner. Results showed that removal of 1 copy of *trbd* resulted in a two-fold upregulation of *dipt* compared to wild type and removal of both alleles led to a 4-fold increase.

Importantly, injection of *trbd* deletion flies (both homozygous and heterozygous) with Gram-positive bacterium *M. luteus* and assaying for Toll upregulation with qPCR showed that dTrabid did not function in the Toll cascade. Also, co-overexpression of dTrabid and its mutants in cell culture had no effect on JNK

signalling (although the JNK pathway was not significantly upregulated). Further, survival experiments with *trbd* deletion flies showed that removal one or both genes of *trbd* had no effect on survival, when compared to wild type. Therefore, the results clearly indicate that *Drosophila* Trabid functions solely in IMD signalling at the level of dTAK1 where it negatively regulated the immune signal in a dose-dependent manner.

4.9 The role of the OTU and NZF domains in dTrabid DUB activity

In humans, the deubiquitination function of Trabid is exercised through the catalytic residue C443 in the OTU domain, with a preference for K63-linked ubiquitin. Deletion of this residue eliminated the DUB activity of Trabid. Further, the N-terminal three tandem Npl4-related zinc finger (NZF) motifs, each bear the T10 F/Y11 Φ22 signature of ubiquitin-binding NZF motifs which are predicted to be NZF surface residues that participate directly in ubiquitin binding. It was also reported that at least 2 of these motifs were required to bind K63-linked polyubiquitin which has a much more spread configuration when compared with K48-linked chains (Tran et al., 2008).

My results show that C518 is the corresponding catalytic residue in *Drosophila* Trabid. However, mutation of this residue resulted in a significant reduction (~50%) of DUB activity (as seen in both qPCR data and K63-specific Ub assays) but not its complete elimination. Mutations of the 3 NZF domains together with C518 in dTrabid$^{C518S+3xNZFDel}$ rendered it inert. The elimination of DUB function in dTrabid$^{C518S+3xNZFDel}$ could be explained in terms of the inability of the NZF motifs to bind K63-linked polyubiquitin, the first step, prior to its cleavage by the OTU domain.

The ubiquitin assays (using both K63 and K48-linked polyubiquitin) clearly show that dTrabid functions by deubiquitinating K63-linked polyubiquitin on dTAK1. It

does not affect K48-linked polyubiquitination of dTAK1. Thus, the functional mechanism of *Drosophila* Trabid appears to be very similar to that of its human ortholog.

However, this is markedly different from the activity of A20, the most studied member of the OTU family of DUBs in humans. A20 acts at the level of TRAF2/TRAF6 (the E3 ubiquitin ligase) and prevents autoubiquitination of the E3 by binding to and polyubiquitinating the E2 enzymes with K48-linked polyubiquitin, thereby tagging them for proteosomal degradation (Shembade et al., 2010). Definite comparisons cannot be drawn since both proteins were not comparatively analysed using the same experimental techniques and so, the possibility does exist that the differences are due to the different investigative approaches.

4.10 *Drosophila* Trabid partially redundant with Rabex-5?

Co-overexpression of dTrabid together with dTAK1 and dTAB2 in cell culture did not completely abrogate the immune signal (assayed by qPCR), although it was significantly reduced by ~50% (Fig. 3-7B). Intriguingly, co-overexpression of Rabex-5 together with dTAK1 and dTAB2 in S2 cells resulted in an almost identical reduction in the IMD immune signal. Moreover, in the K63-specific ubiquitin assay, co-overexpression of dTrabid together with dTAK1 and dTAB2 in cell culture did not completely eliminate K63-linked ubiquitination of dTAK1. In agreement with the qPCR data, it significantly reduced polyubiquitination by ~50% (Fig.3-8). These results raises the very interesting possibility that dTrabid functions in a partially redundant manner with another DUB. Tran *et al.* (2008) also suggested that dTrabid may be redundant with another gene when they found that *trbd*-null mutant flies were viable and fertile.

My results suggest that Rabex-5 is a likely candidate for this role. Rabex-5 was first identified as a Rab5 guanine nucleotide exchange factor (GEF) with a highly conserved C-terminal VPS9 domain. Importantly, it also has an N-terminal zinc finger (ZnF) domain that belongs to the A20 ZnF family (that is involved in K48-linked ubiquitination) and has shown E3 ubiquitin ligase activity. It was shown to be involved in ubiquitin-mediated negative regulation of the Ras pathway (Xu et al., 2010; Yan et al., 2010). Results show that Rabex-5 interacts with dTAK1 and that it significantly decreases the IMD immune signal when co-overexpressed with dTAK1 and dTAB2 in S2 cells. Deletion allele homozygotes and heterozygotes of *Rabex-5* and even flies undergoing strong, constitutive *Rabex-5* RNAi died as giant larvae or giant pupae, often containing melanotic tumors (Yan et al., 2010). Therefore, obtaining *in vivo* evidence has proved to be problematic. However, further work (including *in vivo* evidence) is required to establish the role of Rabex-5 in IMD signalling.

As discussed previously, A20 is a negative regulator of NF-κB signalling. It functions through a negative feedback mechanism, where activation of NF-κB is required to initiate A20 transcription. Also, *A20* deficient mice showed NF-κB response and severe inflammation (Coornaert et al., 2009; Lee et al., 2000). Trabid was original discovered as a novel A20-like protein showing sequence similarity together with Cezanne (Evans et al., 2001). Cezanne was shown to inhibit NF-κB through modulation of the ubiquitination state of two of its positive regulators, RIP-1 and TRAF6 (Evans et al., 2003). Trabid was also shown to bind to and cleave K63 polyubiquitin chains in Wnt signalling (Tran et al., 2008). Given the sequence and functional similarities between A20 and Trabid, it is likely that Trabid, too, functions in a negative feedback mechanism. Like with *A20* deficient mice, *trbd* deletion flies showed enhanced activation of IMD signalling. In order to function as a negative feedback regulator,

Trabid expression must be upregulated upon Relish activation in *Drosophila*. This is yet to be established.

4.11 *Drosophila* TAB2 ZnF domain is involved in regulating IMD signalling

Drosophila TAB2 (CG7417) is an adaptor with a CUE, a coiled-coil, and a zinc-finger domain. Homologous to human TAB2/TAB3, it is the binding partner of dTAK1. This binding and the formation of the dimeric complex is essential to activate dTAK1 (Geuking et al., 2005; Kleino et al., 2005; Zhuang et al., 2006).

In humans, activation of TAK1 necessitates a complex consisting of TAK1 and two specific TAK1-binding proteins, TAB1 and TAB2. Here, TAB1 functions as an activator of TAK1, whilst TAB2 functions as an adaptor that links TAK1 to the RING domain containing E3 ligase TRAF6 in the IL-1 signalling pathway. Upon IL-1 stimulation, TAB2 facilitates the interaction between TAK1 and TRAF6. TAB3 has a redundant role with TAB2 in IL-1 signalling (Ishitani et al., 2003; Zhuang et al., 2006). Both TAB2 and TAB3 contain a highly conserved C-terminal novel zinc finger (NZF) domain, which binds preferentially to K63-linked polyubiquitin chains. Once stimulated, TRAF6 interacts with a dimeric Ub-conjugating enzyme (E2) complex consisting of Ubc13 and Uev1/Mms2 which leads to its autoubiquitination by formation of a K63-linked polyubiquitin chain. Then, TAB2 or TAB3 will bind to this K63-linked polyubiquitin chain of TRAF6 through this NZF domain. Binding of TAB2/3 to polyubiquitinated TRAF2 or TRAF6 leads to the activation of TAK1 and IKK in a proteasome-dependent manner (Takaesu et al., 2000a; Wang et al., 2001; Zhuang et al., 2006). Mutations to this domain abolish the ability of TAB2 and TAB3 to bind to

polyubiquitin chains and thereby, their ability to activate TAK1 and IKK (Besse et al., 2007; Kanayama et al., 2004).

Drosophila TAB2 is the only homolog of the human TAB proteins discovered thus far in flies. It shows little sequence similarly to the human TABs and was designated a *Drosophila* TAB homolog on the basis of conserved domain architecture - the CUE, coiled-coil, and zinc-finger domains are found together only in human TAB2 and TAB3 and in homologs of these proteins in other organisms (Geuking et al., 2005; Zhuang et al., 2006).

Contrary to the mammalian model, my results show that mutations in the C-terminal ZnF domain in dTAB2 (C769A and C772A) results in a significant upregulation of IMD immune signalling (Fig. 3-10B). Interestingly, the IMD signal increases 2-fold (~ 100%) when dTAK1 and dTAB2^{ZnFDel} are co-overexpressed in S2 cells, when compared with full-length dTAK1 and dTAB2 co-overexpression. In agreement with this, there is more K63-linked polyubiquitination in dTAK1 (again ~100% increase) in the presence of dTAB2^{ZnFDel} when compared with the full-length dTAK1 and dTAB2 scenario (Fig. 3-11A). Moreover, there is more dTAK1 (again ~ 100%) in the cell lysates when co-overexpressed with dTAB2^{ZnFDel} when compared to the full-length dTAK1 and dTAB2 co-overexpression. Strikingly, when dTAB2^{ZnFDel} is co-overexpressed with dTAK1, the IMD immune signal, dTAK1 K63-linked polyubiquitination and dTAK1 concentration in cell lysates all show ~100% increase when compared with full-length dTAB2 + dTAK1 overexpression. Very interestingly, this is almost identical to the proportional reduction of the IMD immune signal seen when either dTrabid or Rabex-5 was co-overexpressed (i.e. resulted in the reduction of the IMD immune signal by ~ 50%).

Moreover, co-immunoprecipitation assays showed dTrabid bound to dTAB2^{ZnFDel} two-fold stronger than to dTAB2 (Fig. 3-11B). This indicated that the tighter the dTrabid bound to dTAB2 the higher the signalling capacity of dTAK1.

These results are contrary to the human model where binding of polyubiquitin chains by the NZF domain of TAB2/TAB3 is necessary for activating TAK1 and IKK and mutation of the NZF domain abolishes the ability of TAB2 and TAB3 to activate TAK1 and IKK (Kanayama et al., 2004).

Taken together, results indicated that the mutation in the dTAB2 ZnF domain stabilised dTAK1 by enhancing its K63-linked ubiquitination. This led to an increase in *dipt* induction. Therefore, it appears that the interaction of dTAK1 with the dTAB2 ZnF domain pacifies dTAK1 activity and thereby, the IMD immune signal. Hence, it is improbable that this ZnF domain of dTAB2 is involved in binding to the E3 ligase which activates dTAK1. It was far more likely that this domain is involved in binding a negative regulator of dTAK1 and its mutation has resulted in releasing dTAK1 from this inhibition. Further work is necessary to explore this possibility.

4.12 Summary:

Taken together, results show a multi-tiered and complex regulation of dTAK1 signalling in the IMD cascade. *Drosophila* TAK1 activity was enhanced by K63-linked ubiquitination at Lys 142 and 156. Trabid negatively regulated this activity by reducing K63-linked ubiquitination, through its DUB function. In addition, the dTAK1-dTAB2 interaction through the latter's ZnF moderated dTAK1 activity. There are two alternative scenarios to explain this last result. *Either* tightening of the dTAB2-Trabid interaction interfered with (or altered) the dTAK1-Trabid interaction thus reducing the

Trabid influence *or* dTAB2 through its ZnF recruited another protein. The latter scenario would predict the presence of a protein that would act in concert with Trabid sharing some of its characteristics (e.g DUB activity). This would explain why loss of *trbd in vivo* resulted in a moderate increase in *Dipt* induction following *E. coli* infection. More work will be needed to distinguish between these two possibilities.

Somewhat surprisingly, belying the conserved nature of the innate immune pathways, there seems to be a marked departure from the human parallel on several levels. *Drosophila* TAK1 has 2 K63 Ub acceptor sites and one K48 Ub binding site. The key ZnF domain of *Drosophila* TAB2 functions very differently than its corresponding domains in human TABs. The DUB negative regulator acts directly on dTAK1 rather than on the E3 ligase as is the case in humans. Therefore, it appears that *Drosophila* TAK1 is regulated differently in comparison to humans. However, definite comparisons between dTAK1 and hTAK1 cannot be drawn since they were not comparatively analysed and there remains the possibility that the differences are due to the different investigative approaches adopted to study TAK1 in *Drosophila* and humans. Nevertheless, the observed deviations are interesting and merit further investigation.

Chapter 5 : Regulation of *Drosophila* TAK1 in TNF/Eiger Signalling - Results

The Tumor Necrosis Factor (TNF) family of proteins acts as signal transducers to regulate fundamental process in mammalian development and adult life including apoptosis, immunity, cell survival, proliferation and differentiation. Aberrations in TNF signalling are associated with numerous diseases including autoimmune conditions and cancer (Locksley et al., 2001). However, studying the mechanisms by which TNF exerts its functions is complicated by the presence of numerous ligands and receptors as well as at least three intracellular signalling pathways (Locksley et al., 2001).

Drosophila melanogaster, as a model organism, has the distinct advantage of possessing only one TNF ligand (Eiger) and one receptor (Wengen). Therefore, *Drosophila* can be used as a simple genetically tractable model to identify all evolutionary conserved regulators and transduction components of TNF signalling (Igaki et al., 2002, Moreno et al., 2002, Kauppila et al., 2003).

5:1 Ari-2 is a novel component of the *Drosophila* TNF/Eiger pathway

Ariadne-2 (Ari-2; CG5709) was discovered as a novel component of the TNF/Eiger apoptotic cascade in a yeast 2-hybrid screen by Dr. Anthony Brown in our laboratory. This was later confirmed in a screen involving the *Drosophila* eye used specifically for the isolation of novel genes of this pathway as previously described (Moreno et al., 2002, Geuking et al., 2005). The screen relies on the ability of Eiger to potently activate the JNK pathway and trigger apoptosis. Overexpression of Eiger in the *Drosophila* eye triggers massive apoptosis and results in a small eye phenotype.

Mutations or a reduction in copy number of genes encoding TNF/Eiger components will rescue this phenotype (Geuking et al., 2005).

Apoptosis inducer *eiger* was expressed specifically in the *Drosophila* eye either alone or in combination with *UAS-ari-2* under the control of the glass multiple reporter (GMR) promoter. Further, *eiger* was also expressed either alone or with in combination with one copy of *ari-2* in the *Drosophila* eye as above. Results show that overexpression of *eiger* causes ablation in the *Drosophila* eye previously described (Moreno et al., 2002, Geuking et al., 2005). When *eiger* was co-overexpressed with *ari-2*, this phenotype was greatly enhanced (Fig. 5-1). Therefore, *eiger* and *ari-2* appear to function synergistically in apoptosis. However, when 1 copy of *ari-2* was removed, there was a 'rescue' of the small eye phenotype, indicating that *ari-2* functioned in a dose-dependent manner.

An *ari-2* hypomorph was generated in our laboratory with Dr. Anthony Brown by combining two transposable element insertions identified in public stock centres - $ari\text{-}2^{07768}$ (554bp; a P-element insertion allele) and $ari\text{-}2^{EY00574}$ (590bp; a *gipsy* element insertion) upstream of the transcriptional start site (Fig. S3A – Appendix 4). The heteroallelic combination ($ari\text{-}2^{07768}/ari\text{-}2^{EY005}$ henceforth referred to as $ari\text{-}2^{ins}$ (or 12341/20092) showed very low levels of the *ari-2* message when the mutant was analyzed by reverse transcription PCR for *ari-2* mRNA (Fig. S3B – Appendix 4).

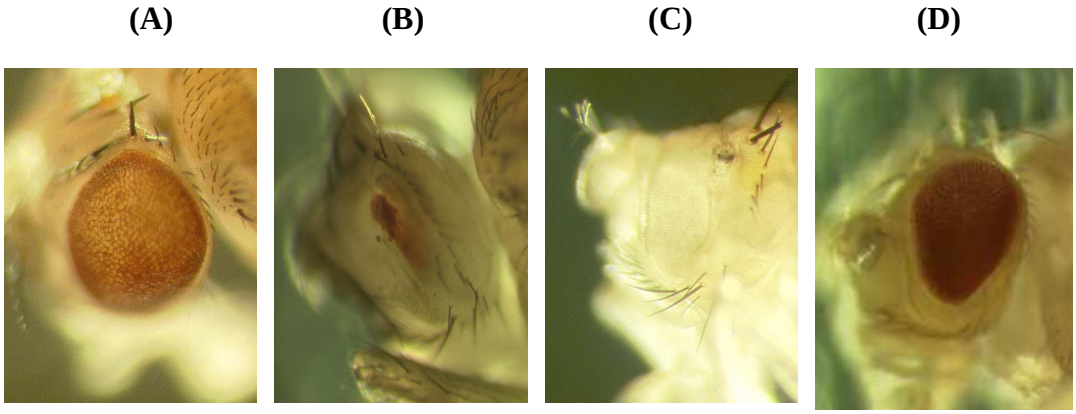


Figure 5-1: Ari-2 is a novel component of the *Drosophila* TNF/Eiger apoptotic pathway. (A) *GMR-Gal4* only eye phenotype (control). (B) Targeted overexpression of *eiger* (*GMR-Gal4/CyO;UAS-egr/+*) in the *Drosophila* eye results in a small eye phenotype. (C) This phenotype is synergistically enhanced when *ari-2* (homozygous) is co-overexpressed with *egr* (*UAS-ari2/GMR-Gal4;UAS-egr/+*). (D) Removal one copy of *ari-2* (*GMRGAL4.UASegr/20092*) greatly suppresses the eye apoptotic phenotype induced by over-expressing *eiger*.

5:2 Ari-2 is required for development

Previously, Ariadne-1 (Ari-1; CG5659) had been discovered as a novel *Drosophila* RING-finger protein required for neural development. The authors also identified a*ri-2* as a closely related gene (Aguilera et al., 2000). Therefore, *ari-2* was investigated for a possible role in development.

Firstly, *ari-2* expression patterns in different developmental stages were examined using reverse transcription PCR. Total RNA from flies of different developmental stages was extracted, DNA digested with DNase 1, cDNA synthesised from mRNA, PCR performed with 2.5µl of cDNA reaction mixture and resolved with agarose gel electrophoresis. Results show that *ari-2* was expressed by wild type flies in all developmental stages as well as in both male and female adults. Expression in *ari-2^{ins}* mutant flies was severely compromised after the 3rd larval instar (Fig 5-2A).

Additionally, the development of *ari-2* expressing wild-type flies and mutant *ari-2^{ins}* flies were examined. Successful development was determined (together with Dr. Anthony Brown) by scoring the number of embryos to hatch, the number of larvae to pupate and the number of pupae to successfully eclose. Results show that while the hatching rates of *ari-2^{ins}* embryos were comparable to wild type, the percentage of larvae entering pupariation and pupae eclosing to adults was severely reduced in comparison to wild type (Fig 5-2B). Very importantly, parallel experiments in our laboratory by Dr. Anthony Brown show that this phenomenon was reversed if the JNK *bsk* was overexpressed (through a *UAS-bsk* transgene) from 3rd larval instar onwards. Here, the transgene was expressed through a heat-shock *GAL4* driver with a single half hour heat shock at the beginning of 3rd instar. Additionally, parallel experiments in our laboratory showed that repeated balanced crosses with the two insertion alleles gave homozygous heteroallelic progeny in ratios diverting significantly from the Mendelian norm. Taken together, the above results indicated that *ari-2* is essential for development from at least late larval stage onwards.

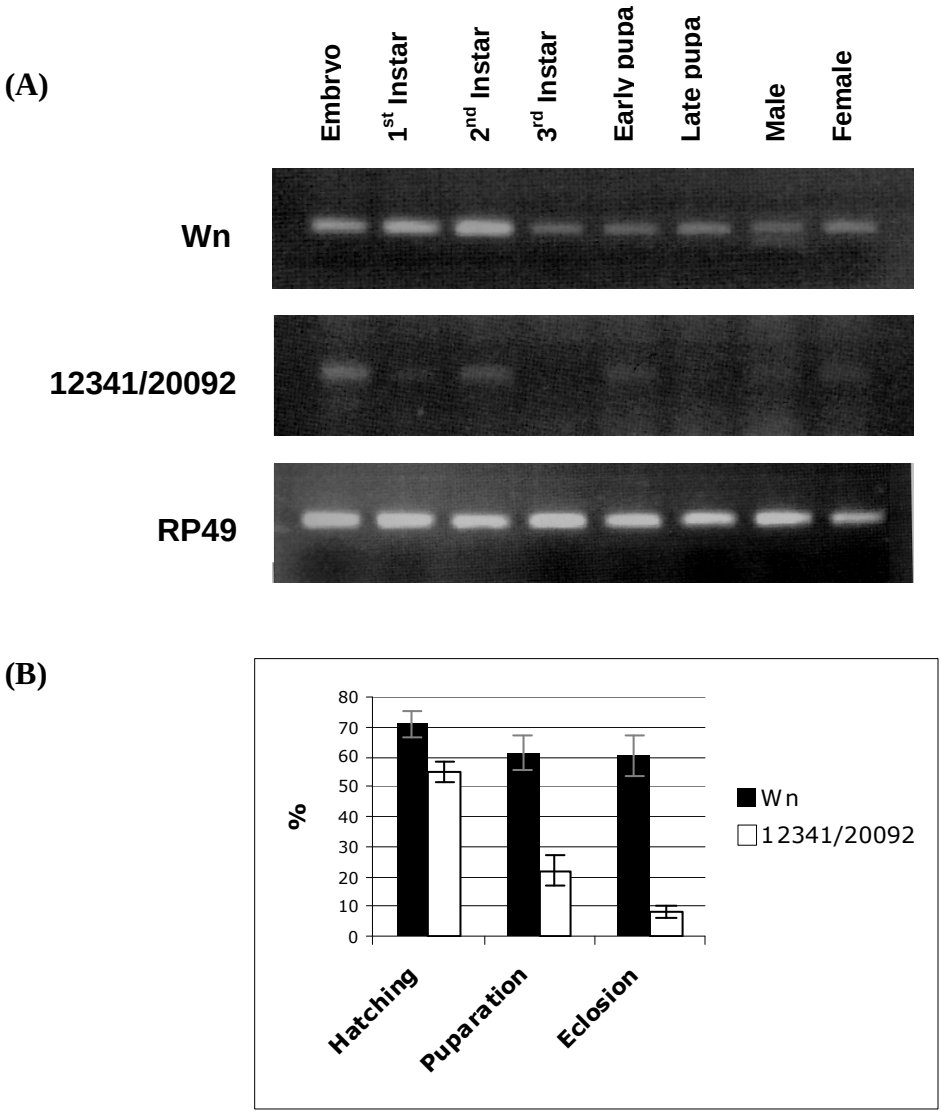


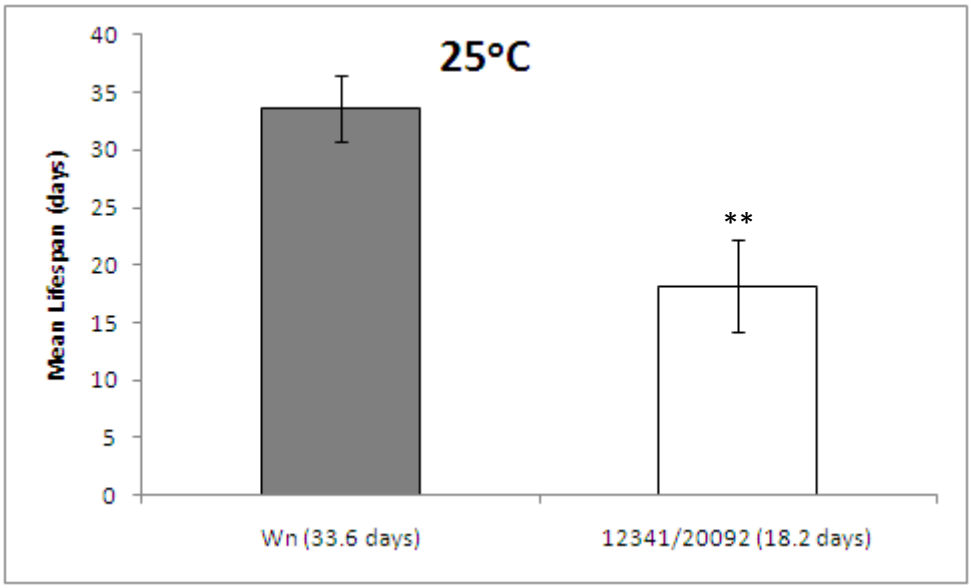
Figure 5-2: Ari-2 is required for development. (A) Expression of *ari-2* in different developmental stages. Expression of *ari-2* and of *ari-2*^{ins} mutant (12341/20092) during development was detected by reverse transcription PCR (RT-PCR) from RNA isolated from wild-type (Wn; upper panel) and mutant (12341/20092; middle panel) *Drosophila* at the indicated developmental stages. RP49 was used as a loading control for the amount of cDNA used in the RT-PCR reaction (lower panel). **(B)** Ari-2 is required for early development**.** Development of wild-type and *ari-2*^{ins} mutant flies were determined by scoring the number of embryos to hatch, the number of larvae to pupate and the number of pupae to successfully eclose. Developmental rates at each stage were determined by comparison to the initial number of embryos. *Error bars show the SEM from 3 independent experiments of 100 embryos.*

5:3 Ari-2 is required for normal adult life span and mounting a stress response

It was previously shown that mutations in *ariadne-1* resulted in reduced life-span and development abnormalities like motor impairments, and short and thin bristles (Aguilera et al., 2000). Therefore, the possible requirement of *ari-2* for life span was evaluated. Cohorts of wild-type or *ari-2*^{ins} mutant (12341/20092) flies were placed in standard food vials at 25°C and their survival determined daily. Food was changed every 3 to 4 days. The experiment was also replicated at 30°C. Results show that the lifespan of *ari-2*^{ins} mutant (12341/20092) flies was reduced. At 25^{o}C *ari-2*^{ins} mutants (12341/20092) lived significantly less than wild type (with a mean life span of 18.2 days as opposed to 33.6 days for their wild type counterparts (Fig 5-3A). Significantly, 85% of mutant flies were dead in 25 days as opposed to only 20% of their wild type counterparts (Fig 5-3B). The effect at a higher temperature was evaluated together with Dr. Anthony Brown. At 30^{o}C this phenotype was more pronounced with a mean life span of 13.4 days for *ari-2*^{ins} flies compared to 18.4 days for wild type. Further, 80% of mutant flies were dead in 17 days whilst only 40% of wild type adults died within the same period (Fig. S5 -Appendix 4).

In organisms ranging from *Drosophila* and *C. elegans* to mice and humans shortened adult lifespan may result from compromised responses to various stress conditions such as high temperature, starvation, immune challenge or exposure to free radicals (Stronach, 1999, Wang et al., 2003, Wadgaonkar et al., 2004). Several conserved signalling pathways related to the mitogen activated protein kinase (MAPK) pathway counter such stimuli, with JNK being a prominent example among them (Kyriakis and Avruch, 2001). Therefore, the possibility of *ari-2* functioning within or through the JNK pathway was examined together with Dr. Anthony Brown.

(A)



(B)

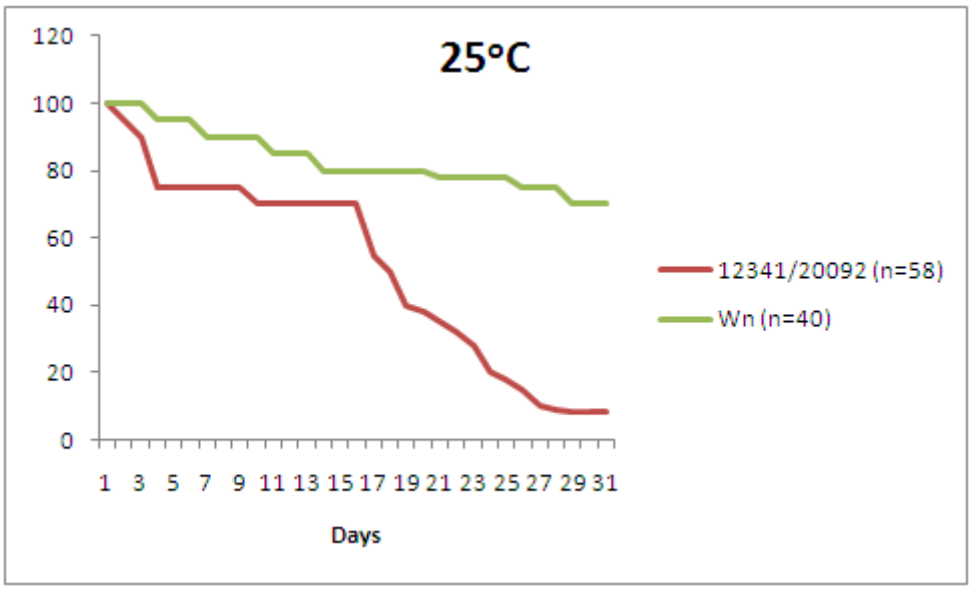


Figure 5-3: Ari-2 mutant flies have a reduced adult lifespan. (A) Mean adult lifespan of *ari-2* mutant flies (12341/20092; white bars; 18.2 days) at 25°C compared to wild-type flies (Wn; grey bars; 33.6 days). *Error bars show SEM of 3 independent experiments.* **(B).** Survival graph of above *ari-2* wild-type and mutant flies at 25ºC. Cohorts of 20 wild-type male (Wn; green line) or 20 Ari-2 mutant male (12341/20092; red line) flies were placed in standard food vials at 25°C and their survival determined daily. Death was diagnosed by a lack of a sit-up response. Experiments depict mean values from at least 2 independent experiments. ** indicates significance value of the result as determined by Student's t-Test (*P*=<0.01).

Survival of wild type and mutant (12341/20092) flies were evaluated under heat shock/high temperature conditions (37^{o}C for 4 hrs). This was compared to *UASdTAK1-RNAi* flies devoid of a JNK response.

Results show that survival of wild-type flies was approx. 50% after heat shock of 37°C for 4 hrs. In comparison, *ari-2*^{ins} mutant flies exhibited a mean survival of 30%, similar to JNK deficient *UASdTAK1-RNAi* flies. However, survival was greatly facilitated (and even improved in comparison to wild-type flies) if during the treatment an *ari-2 UAS*-transgene was expressed. Addition of a *UAS-dTAK1-RNAi* construct together with the *ari-2* UAS-transgene resulted in survival reduced to levels comparable to *ari-2*^{ins} mutants or JNK deficient flies (Fig S6 - Appendix 4).

Thereafter, the ability of wild type and *ari-2*^{ins} mutant (12341/20092) flies to activate the JNK pathway under stress conditions was evaluated using reverse transcription PCR. JNK pathway activation was assayed through the expression of *puckered* (*puc*), a prototypical target of JNK signalling in *Drosophila*. Cohorts of wild type and mutant male flies were either unchallenged or subjected to stress conditions including O_2 generation (by administration of paraquat) and heat shock (37°C for 90 mins). Total RNA from flies of different developmental stages was extracted, DNA digested with DNase 1, cDNA synthesised from mRNA, PCR performed with 2.5µl of cDNA and resolved with agarose gel electrophoresis. Results show that *ari-2*^{ins} mutant (12341/20092) flies were unable to activate the JNK pathway following heat shock and free radical generation. However, this phenotype was rescued when *ari-2* mutant flies challenged by heat shock at 37^{o}C expressed a *UAS-ari-2* transgene through *actin-GAL4* (Fig 5-4).

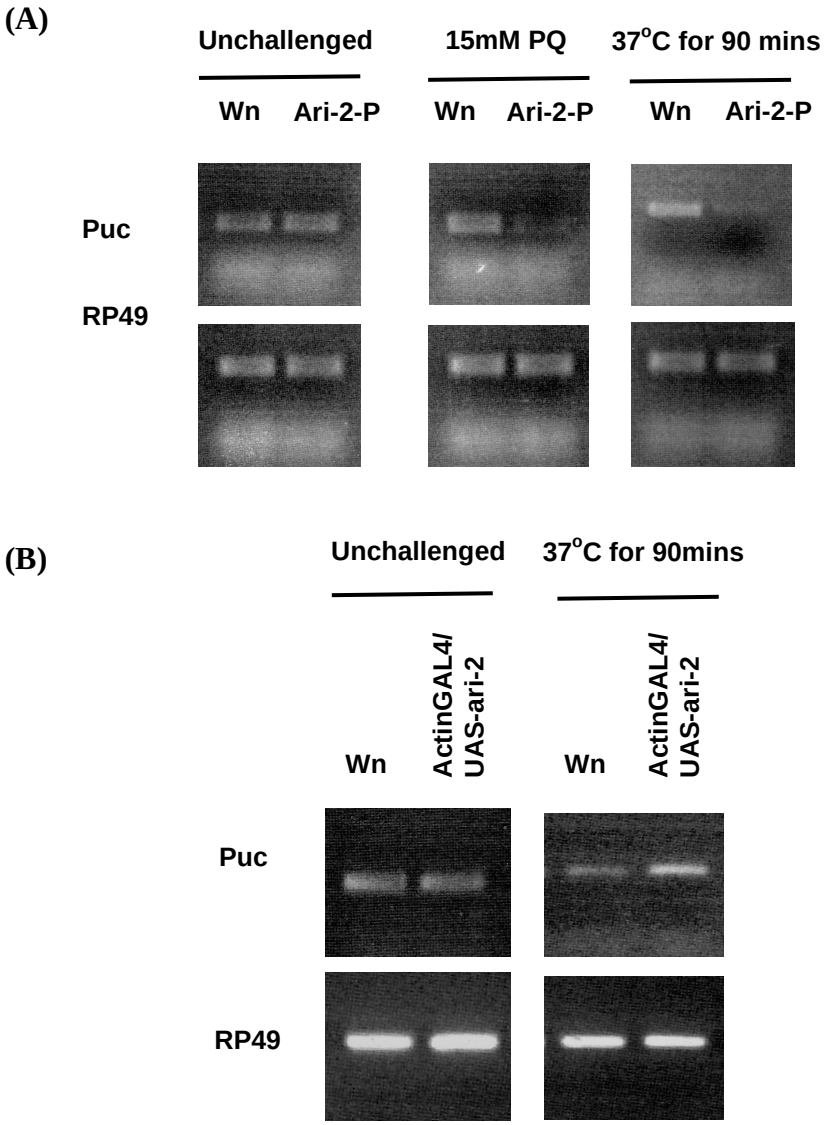


Figure 5-4: Ari-2 mutant flies are sensitive to cellular stress and fail to mount a JNK response. (A) JNK activity in *ari-2*^{ins} mutant (Ari-2-P) and wild-type (Wn) flies were compared by reverse transcriptase PCR from RNA isolated from unchallenged male flies (left), male flies exposed to a 15mM paraquat (PQ) solution (centre), or flies subject to 90 min heat shock at 37°C (right). Top panels show *puckered* (*puc*) expression as a molecular readout for JNK activity. RP49 was used as a loading control (lower panels). **(B)** *actin-GAL4/UAS-ari-2* flies exhibited increased *puc* transcript levels with respect to wild-type (Wn) flies after heat shock at 37°C for 90 min (top-right panel) when compared to unchallenged *actin-GAL4/UAS-ari-2* flies and wild type (Wn) flies (top-left panel). RP49 (lower panels) was used as a loading control.

Taken together the above results indicate that *ari-2* is required for development and a normal adult life span. Further, *ari-2* is required for mounting a stress response. Moreover, results suggest the involvement of *ari-2* in the JNK pathway upstream of (or at the same level with) *dTAK1*.

5:4 Ari-2 enhances TNF/Eiger-induced JNK activation and cell death

Ari-2 was initially discovered as a novel component of the TNF/Eiger apoptotic cascade using a screen based on the ability of Eiger to potently activate the JNK pathway and trigger apoptosis. Overexpression of Eiger in the *Drosophila* eye triggers massive apoptosis and results in a small eye phenotype (Geuking et al., 2005). The role of Ari-2 in modifying this cell death phenotype was then evaluated.

As shown above, overexpression of Eiger specifically in the *Drosophila* eye (under the control of the *GMR* promoter) resulted in a small eye phenotype. Co-overexpression of both Eiger and Ari-2 resulted in a synergistic enhancement of this phenotype and negated photoreceptor formation (Fig. 5-5A). However, this small eye phenotype was suppressed when Puckered was co-overexpressed together with both Eiger and Ari-2 (Fig. 5-5B). Also, the small eye phenotype was moderately suppressed when the JNK pathway was inhibited by using *UAS-RNAi* for dTAK1 together with Eiger and Ari-2 (Fig. 5-5C).

Taken together, these results firmly implicate Ari-2 in the TNF/Eiger-JNK cascade and place it upstream of/at the same level of dTAK1.

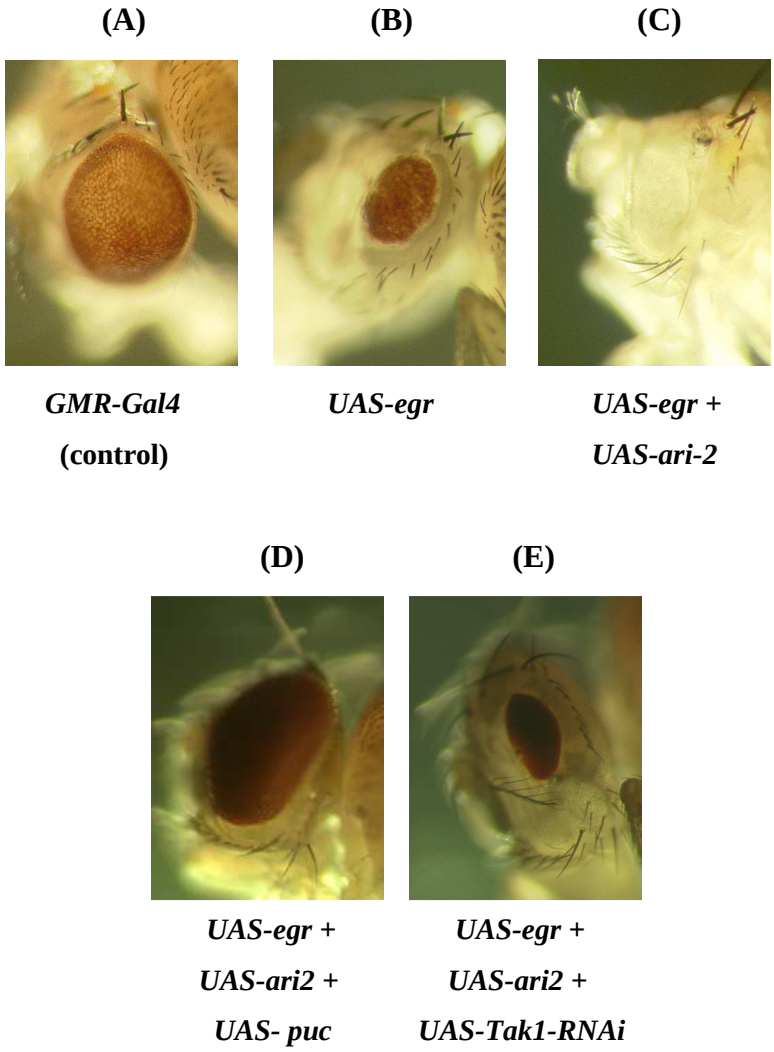


Figure 5-5: Epistatic analysis to determine relative position of Ari-2 within the JNK signalling pathway. (A) *GMR-Gal4* only eye phenotype (control). (B) Targeted overexpression of *eiger* (*GMR-Gal4/CyO;UAS-egr/+*) in the *Drosophila* eye results in a small eye phenotype. (C) This phenotype is synergistically enhanced when *ari-2* is co-overexpressed with *egr* (*UAS-ari2/GMR-Gal4;UAS-egr/+*). (D) Co-overexpression of *puckered* together with *eiger* and *ari-2* (*UAS-ari2/GMR-Gal4.UAS-egr;UAS-puc-* both on the 2nd chromosome) greatly suppressed the apoptotic eye phenotype. (E) Inhibition of the JNK pathway by co-expressing a dTAK1-dsRNA construct together with *eiger* and *ari-2* (*UAS-ari2/GMR-Gal4;UAS-egr/UAS-Tak1-RNAi*), rescues the apoptotic eye phenotype.

5:5 Ari-2 interacts with dTAK1 and dTAB2

Drosophila TAK1 has previously been shown to be a principle component of the TNF/Eiger cascade (Geuking et al., 2005). In mammals, TAK1 ubiquitination and activation is essential in both the TNF and IL-1 signalling (Besse et al., 2007). Further, the epistatic analysis placed Ari-2 upstream of/at the level of dTAK1. Therefore, Ari-2 was tested for interaction with dTAK1. Expression vectors encoding Ari-2-HA were transiently transfected in S2 cells with or without dTAK1-V5. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5 antibody, resolved on 10% SDS PAGE, and immunoblotted with anti-HA. Results show that Ari-2 interacts with dTAK1 (Fig. 5-6A).

Drosophila TAB2 was previously identified an essential component of the TNF/Eiger cascade and it was shown that the triple complex of dTRAF2-dTAB2-dTAK1 can form (Geuking et al., 2005). It was shown that dTAB2 interacts with dTAK1 and is essential for dTAK1 activation in both the JNK and NF-κB signalling pathways (Zhuang et al., 2006). A co-immunoprecipitation assay was performed to determine whether Ari-2 interacted with dTAB2. Expression vectors encoding Ari-2-HA were transiently transfected in S2 cells with or without dTAB2-V5. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA. Results show that Ari-2 interacts with dTAB2 (Fig. 5-6B).

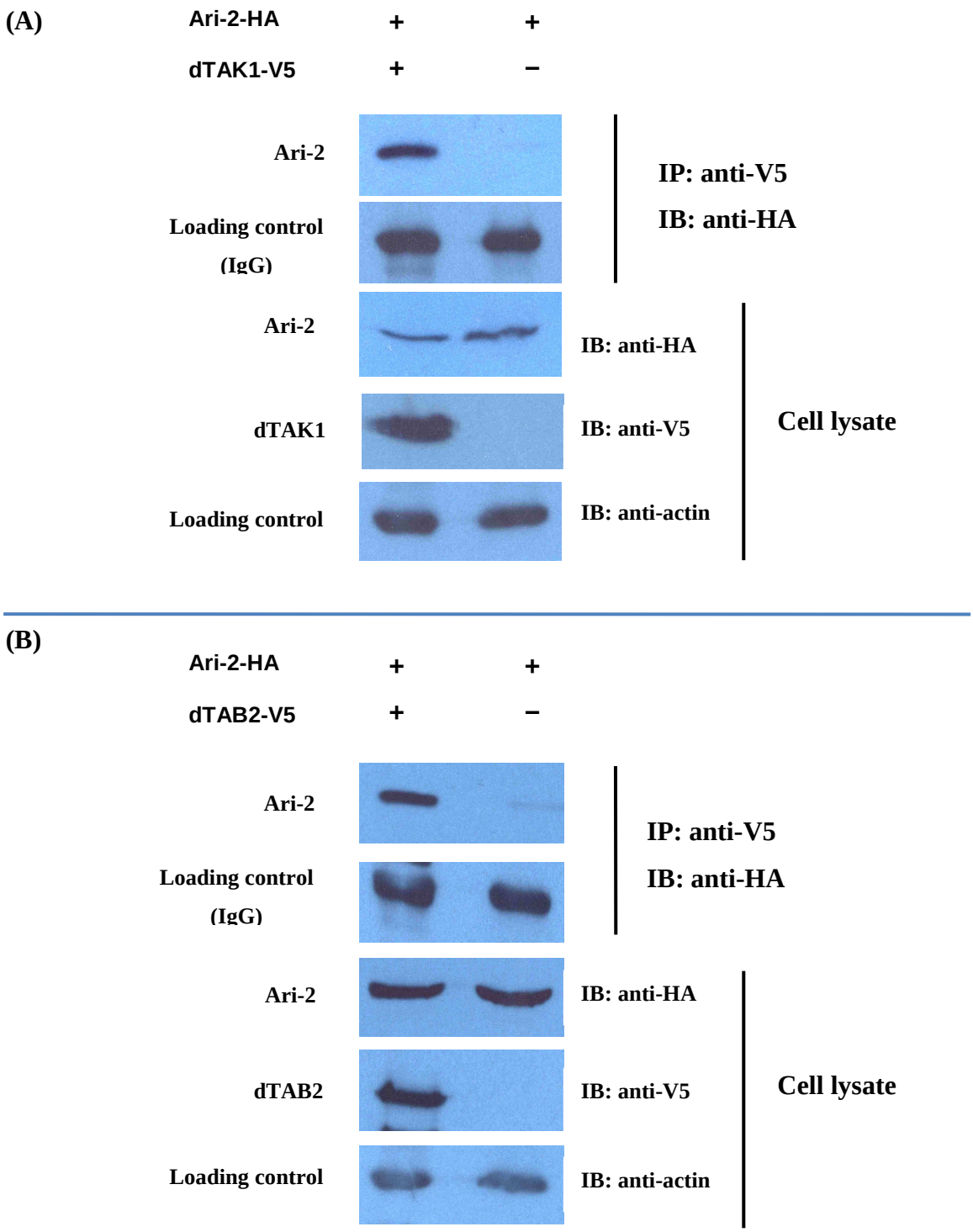


Figure 5-6: Ari-2 binds with (A) dTAK1 and (B) dTAB2. Expression vectors encoding Ari-2-HA were transiently transfected in S2 cells with or without *either* dTAK1-V5 *or* dTAB2-V5 in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA antibody.

It was previously established that the C-terminal half of dTAB2 (dTAB2-C; aa 451–831) was sufficient to bind with dTAK1 and the interaction was narrowed to the coiled-coil domain. The N-terminal half (dTAB2-N; aa 1–450), which includes the CUE domain, did not bind to dTAK1 (Geuking et al., 2005). To establish the location of the Ari-2-dTAB2 interaction in dTAB2, 2 new mutant constructs, dTAB2-N-V5 (1 to 450aa of dTAB2) and dTAB2-C-V5 (451 to 831aa) were made. Expression vectors encoding Ari-2-HA were transiently transfected in S2 cells with or without *either* dTAB2-N-V5 *or* dTAB2-C-V5. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA. Results show that Ari-2 interacts with only with the N-terminal half of dTAB2 (Fig. 5-7A).

5:6 Ari-2 interacts with dTRAF2

Drosophila TRAF2 was previously described as an essential component of the TNF/Eiger apoptotic cascade (Xue et al., 2007). It was shown that ubiquitination-mediated proteosomal degradation of dTRAF2 disrupted JNK activation and that the stability of dTRAF2 was paramount to apoptotic signalling (Xue et al., 2007). Further, dTRAF2 was found to function upstream of/at the level of dTAK1 (Geuking et al., 2005). A co-immunoprecipitation assay was performed to determine whether Ari-2 interacted with dTRAF2. Expression vectors encoding Ari-2-HA were transiently transfected in S2 cells in the presence or absence of dTRAF2-V5. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA. Results show that Ari-2 binds with dTRAF2 (Fig. 5-7B).

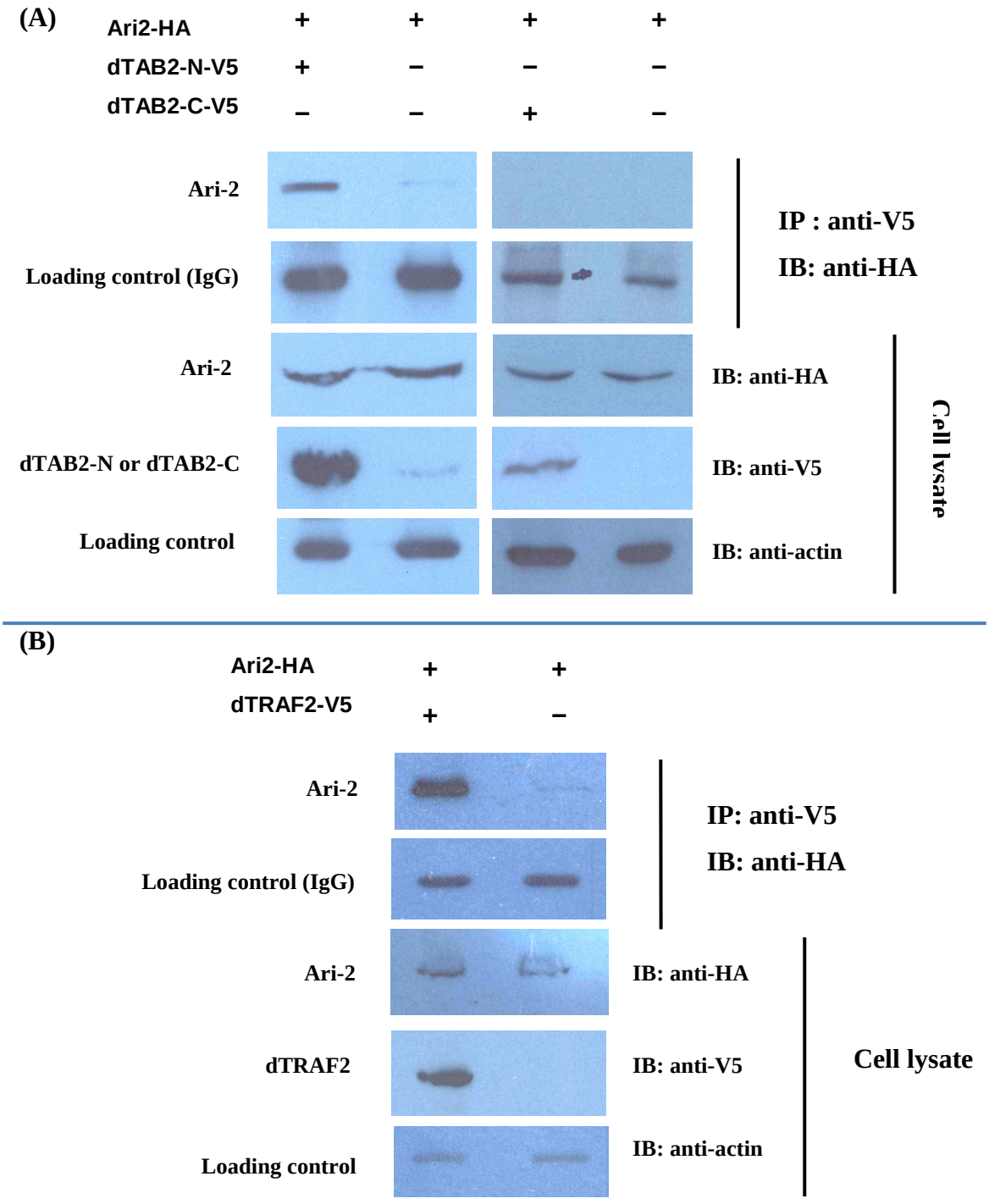


Figure 5-7: Ari-2 binds with (A) the N-terminus of dTAB2 and not with the C-terminus & (B) with dTRAF2. Expression vectors encoding Ari-2-HA were transiently transfected in S2 cells with or without dTAB2-N-V5, dTAB2-C-V5 or dTRAF2-V5 in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA antibody.

5:7 Analysis of the discrete functions of the N- and C-terminal RING domains of Ari-2

It was previously shown that Traid1 (human homolog of Ari-2) bound to and stabilised Growth factor independence 1 (Gfi1) by preventing its ubiquitination and proteosomal degradation and that the C-terminal RING domain (R2) mediated this interaction (Marteijn et al., 2007). It was also shown that the N- and C-terminal RING domains of Traid1 differentially bound ubiquitin-conjugating enzymes, UbcH7 and Ubc13 respectively and exhibited dual ubiquitin ligase activity (i.e. formed both K48- and K63-linked polyubiquitin) in myelopoiesis (Marteijn et al., 2009). To determine whether the 2 RING domains of Ari-2 similarly performed discrete roles, two mutant constructs, Ari-$2^{\Delta R1}$ and Ari-$2^{\Delta R2}$, were made by deleting the the N-terminal (R1; 153 – 202aa) and C-terminal (R2; 313 – 358aa) RING domains, respectively, as described in Chapter 2.

To determine whether deletion of either RING domain affected the interactions of Ari-2 with dTAK1, dTAB2 and dTRAF2 (and thereby determine the binding domains), co-immunoprecipitation assays were performed. Expression vectors encoding Ari-$2^{\Delta R1}$-HA or Ari-$2^{\Delta R2}$-HA were transiently transfected in the presence or absence of dTAK1-V5, dTrab2-N-V5 or dTRAF2-V5 in combinations detailed below. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA antibody.

Results show that both Ari-$2^{\Delta R1}$ and Ari-$2^{\Delta R2}$ interacted with dTAK1 (Fig. 5-8), dTAB2-N (Fig. 5-9) and dTRAF2 (Fig. 5-10) in the same manner as full-length Ari-2.

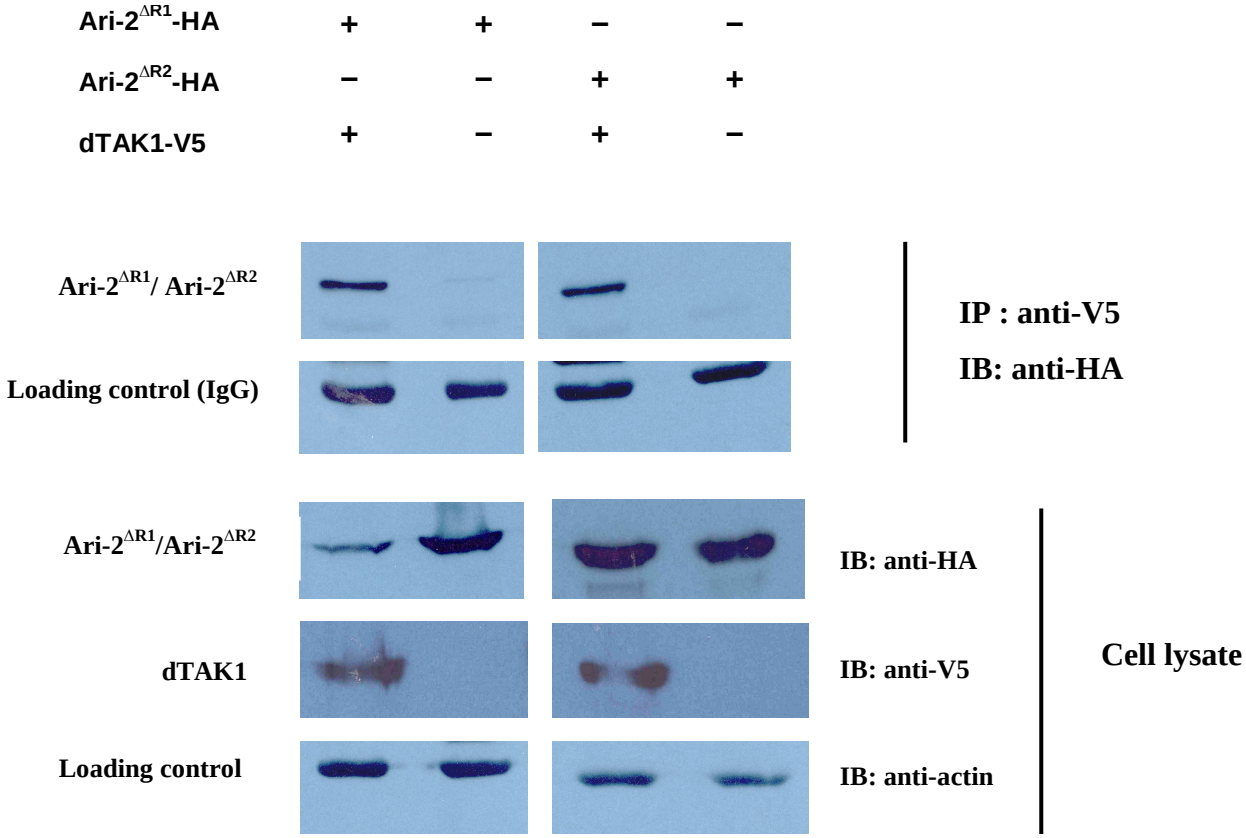


Figure 5-8: Ari-2$^{\Delta R1}$ and Ari-2$^{\Delta R2}$ mutants bind with dTAK1. Expression vectors encoding Ari-2$^{\Delta R1}$-HA or Ari-2$^{\Delta R2}$-HA were transiently transfected in S2 cells in the presence or absence of dTAK1-V5 in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA antibody.

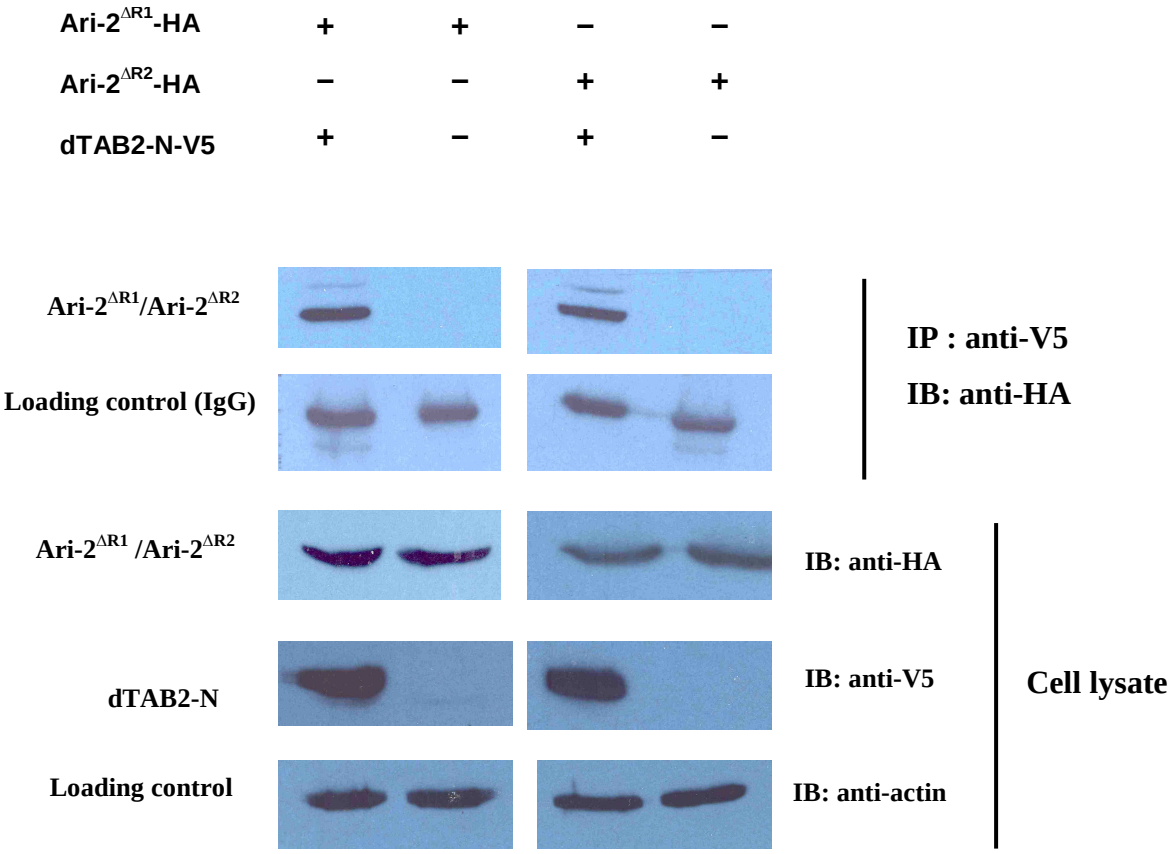


Figure 5-9: Ari-2$^{\Delta R1}$ and Ari-2$^{\Delta R2}$ mutants bind with dTAB2-N. Expression vectors encoding Ari-2$^{\Delta R1}$-HA or Ari-2$^{\Delta R2}$-HA were transiently transfected in S2 cells with or without dTAB2-N-V5 in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA antibody.

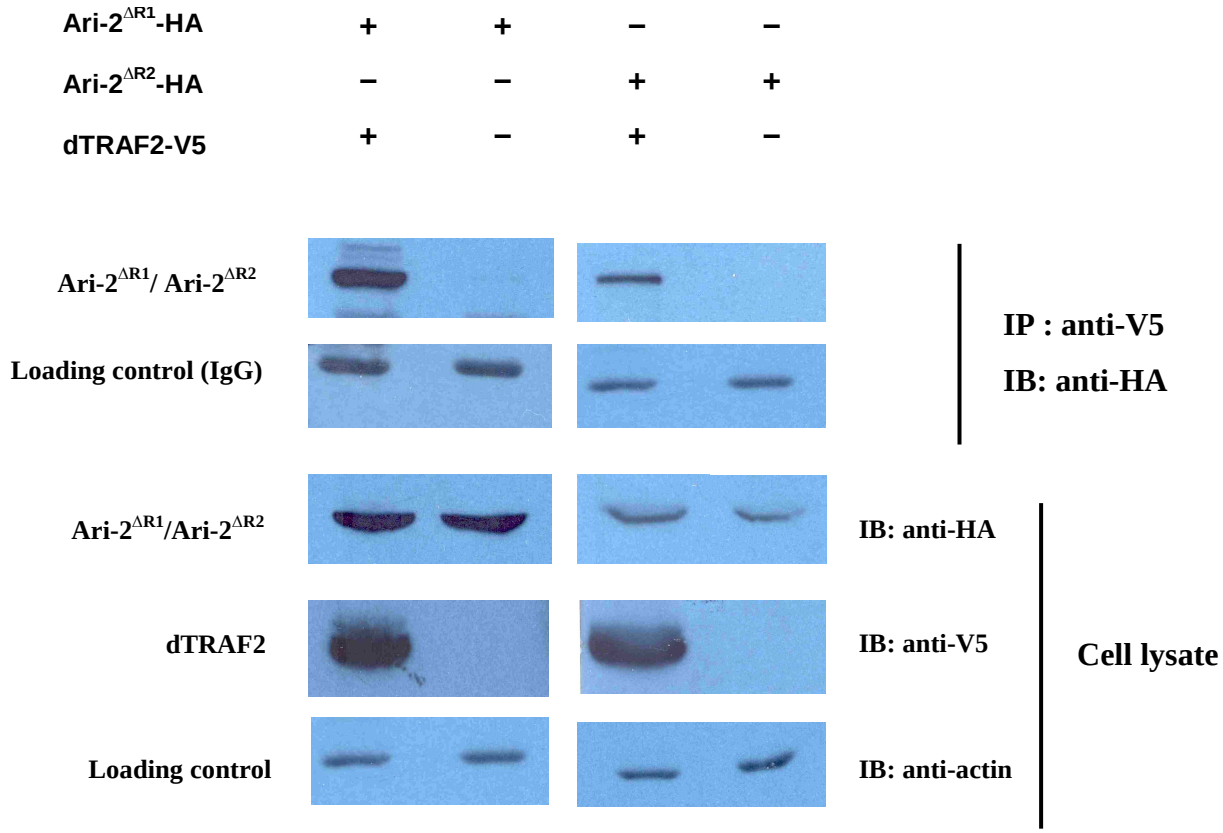


Figure 5-10: Ari-2$^{\Delta R1}$ and Ari-2$^{\Delta R2}$ mutants bind with dTRAF2. Expression vectors encoding Ari-2$^{\Delta R1}$-HA & Ari-2$^{\Delta R2}$-HA were transiently transfected in S2 cells with or without dTRAF2-V5 in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE, and immunoblotted with anti-HA antibody.

5:8 Analysis of the discrete functions of the Zinc-finger, coiled-coil and 2 RING domains of Ari-2

As seen above, deletion of either the N-terminal (R1) or C-terminal (R2) RING domains of Ari-2 did not inhibit its interactions with dTRAF2, dTAB2-N and dTAK1. Both Ari-$2^{\Delta R1}$ and Ari-$2^{\Delta R2}$ interacted with proteins in the same manner as full-length Ari-2. Therefore, the interactions with dTRAF2, dTAB2 and dTAK1 may be modulated by the C_6HC In Between RING (IBR) domain or the zinc-finger (ZnF) or coiled-coil (CC) domains of Ari-2. To determine whether this was the case, 3 new mutant constructs, Ari-$2^{\Delta R1R2}$ (deletion of both R1 and R2; 153-202aa & 313 – 358aa), Ari-$2^{\Delta ZnF}$ (deletion of the ZnF domain; 270 – 286aa) and Ari-$2^{\Delta cc}$ (deletion of the coiled-coil domain; 452 – 481aa) were made as described in Chapter 2. To observe the effects of these 3 mutations on the function of Ari-2 in TNF/Eiger signalling, the mutant constructs were cloned into the fly transformation vector *pUAST* as previously described (Brand and Perrimon, 1993). Fly transformation was performed by Rainbow Transgenic Flies (Newbury Park, CA, USA). The Ari-2 mutants were co-overexpressed with Eiger in the *Drosophila* eye, as before, under the control of *GMR-Gal4*.

Results show that, in contrast to the full length construct, the Ari-$2^{\Delta R1R2}$, Ari-$2^{\Delta ZnF}$ and Ari-$2^{\Delta CC}$ mutants failed to synergistically activate apoptosis, when co-overexpressed with Eiger and the *Drosophila* eye reverted back to a size similar to the control (Fig 5-11).

Taken together, results show that the N- and C-terminal RING domains (in tandem), zinc-finger domain and coiled-coil domain are essential for Ari-2 function in TNF/Eiger induced apoptosis.

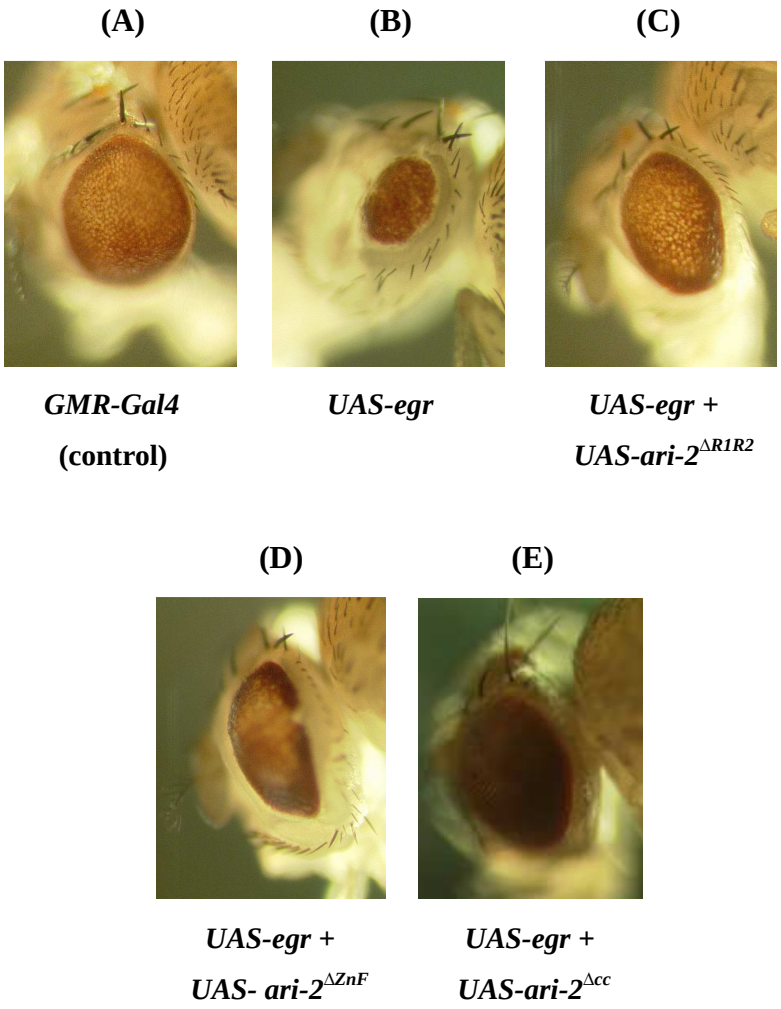


Figure 5-11: Functional analysis of the zinc-finger, coiled-coil and 2 RING domains (N- and C-termini) of Ari-2 in apoptosis. (A) *GMR-Gal4* only eye phenotype (control). (B) Targeted overexpression of *eiger* (*GMR-Gal4/CyO;UAS-egr/+*) in the *Drosophila* eye results in a small eye phenotype. (C) Ari-$2^{\Delta R1R2}$ is non-functional in TNF/Eiger-induced apoptosis. Co-expression of *ari-*$2^{\Delta R1R2}$ with Eiger (*UAS-ari-*$2^{\Delta R1R2}$*/GMRGal4;UAS-egr/+*) failed to induce the apoptotic phenotype observed when *eiger* is expressed alone. (D) Ari-$2^{\Delta ZnF}$ is also non-functional in TNF/Eiger-induced apoptosis. Co-expression of *ari-*$2^{\Delta ZnF}$ with *eiger* (*UAS-ari-*$2^{\Delta ZnF}$*/GMR-Gal4;UAS-egr/+*) failed to induce apoptosis observed when *eiger* is expressed alone (E) Ari-$2^{\Delta CC}$ is also non-functional in TNF/Eiger-induced apoptosis. Co-expression of *ari-*$2^{\Delta CC}$ with *eiger* (*UAS-ari-*$2^{\Delta CC}$*/GMR-Gal4;UAS-egr/+*) failed to induce the apoptotic phenotype observed when *eiger* is expressed alone.

5:9 Role of dTRAF2 in formation of signalling complexes

In humans, TAK1 has the capacity to be activated by various cytokines and is known to activate multiple pathways including IL-1, IL-18, TNF, and receptor activator of NF-κB ligand (RANKL) (Ninomiya-Tsuji et al., 1999, Irie et al., 2000, Sakurai et al., 1999, Wald et al., 2001). Here, upstream adaptor molecules (like the TRAF family of adaptors) are critical in providing the selectivity of initiating distinct signalling events upon receptor activation (Besse et al., 2007). It is thought that, upon stimulation by a specific cytokine, a particular adaptor will sequester TAK1 into a distinct complex and the specificity of this complex determines which downstream signalling cascade is activated. This enables in single kinase like TAK1 to activate multiple pathways (Besse et al., 2007). It has the been shown that TNF receptor-associated factor (TRAF) family of adaptor proteins are involved in coupling activated TNF and IL-1 receptors to NF-κB and MAPKs pathways (Cao et al., 1996, Yeh et al., 1997, Naito et al., 1999, Silverman and Maniatis, 2001). Further, Xue et al., in 2007 found that the stability of *Drosophila* TRAF2 was essential for TNF/Eiger-induced apoptotic signalling. Hence, the role of *Drosophila* TRAF2 as an adaptor was explored.

5.9.1 *Drosophila* TRAF2 is probably capable of auto-ubiquitination:

In humans, the TRAF6 RING finger domain functions as an ubiquitin E3 ligase and is capable of autoubiquitination via K63-linked polyubiquitin chains. It is believed that these non-degradative K63-linked polyubiquitin chains serve as docking sites for formation of signaling complexes, and that K63-linked autoubiquitination of TRAF6 is essential for formation and activation of a complex involving TAK1 and its adapters, TAB1 and TAB2 in IL-1 and TNF signaling (Walsh et al., 2008). It is also known that the intact RING domain is required for TRAF6 signalling and mutation of the first Cys residue of the RING domain (C70) abolished TRAF6 auto-ubiquitination (Lamothe et

al., 2007). Of the 2 TRAF proteins identified in *Drosophila* (i.e. dTRAF1 and dTRAF2), only dTRAF2 contained a RING domain and is the ortholog of TRAF6 (Liu et al., 1999, Cha et al., 2003). To determine whether auto-ubiquitination occurred in dTRAF2, two RING mutant constructs, dTRAF2^{C104A} (mutation of the first Cys residue in the RING domain) and dTRAF2^{C124A} (mutation of the 4th Cys residue in the RING domain) were constructed as described in Chapter 2. Expression vectors encoding for cMyc-Ub were transiently transfected with dTRAF2-V5 or dTRAF2^{C104A}-V5 or dTRAF2^{C124A}-V5 in S2 cells. Cells were lysed 48 hrs post-transfection and immunoblotted with anti-cMyc antibody. Results show that dTRAF2 exhibit strong ubiquitination (when co-overexpressed only with Ub) and this requires an intact RING domain. Mutation of either C104 or C124 (which disrupted the RING) abolished its ubiquitination capability (Fig. 5-12). In order to determine conclusively whether dTRAF2 is capable of self-catalysis, an *in vitro* ubiquitination analysis is required.

5.9.2 *Drosophila* TRAF2 interacts with dTAB2 and E2 enzymes

Lamothe et al.,(2007) showed that the K63 specific E2 conjugating enzyme dimer Ubc13/Uev1A was required for human TRAF6 auto-ubiquitination, in addition to the intact RING domain. It was also reported that human TAB2 facilitates TRAF6 ubiquitination and thereby mediates IL-1 signalling (Kishida et al., 2005). To determine whether the *Drosophila* E2 homologues of Ubc13 (*bendless*) and Uev1A interacted with dTRAF2, co-immunoprecipitation assays were performed. Further, the possible binding of dTAB2 with dTRAF2 was also examined. Expression vectors encoding for dTRAF2-V5 or dTRAF2-HA were transiently transfected in S2 cells with dUbc13-cMyc and dUev1A-cMyc or dTAB2-V5 in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-cMyc (for E2 enzymes) or anti-V5 (for dTAB2) and immunoblotted with anti-HA antibody. Results show that dTRAF2 binds

with both dUbc13 and dTAB2 in a similar manner to its human homologue TRAF6 (Fig. 5-13).

5.9.3 *Drosophila* TRAF2 is stabilized in the dTRAF2-dTAB2-dTAK1 complex

It was previously reported that dTAB2 binds dTAK1 to dTRAF2 and thereby forming a tripartite complex in *Drosophila* TNF/Eiger signalling (Geuking et al., 2005). Further, Xue *et al.* (2007) found that the stability of *Drosophila* TRAF2 was essential for TNF/Eiger apoptotic signalling. K63-linked ubiquitination is known to stabilise proteins and aid in complex formation (Chen, 2005). Therefore, the K63-specific ubiquitination status of dTRAF2 when in complex with dTAK1 and dTAB2 was analysed in S2 cells using a ubiquitin construct which has a Lys at position 63 only (cMyc-Ub^{K63}). Expression vectors encoding for dTRAF2-V5 were transiently transfected in S2 cells with or without dTAK1-HA and dTAB2-HA in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5, resolved on 10% SDS PAGE and immunoblotted with anti-V5 antibody. *Drosophila* TRAF2 showed significantly enhanced K63-linked ubiquitination when co-overexpressed with both dTAK1 and dTAB2. The cell lysate showed significantly increased dTRAF2 levels (when co-overexpressed with dTAB2 and dTAK1). Both dTRAF2 ubiquitination and cell lysate levels reduced progressively as dTAK1 and dTAB2 were withdrawn. Lowest levels of ubiquitination and cell lysate levels were observed when only dTRAF2 and Ub were co-overexpressed (Fig. 5-14). Therefore, results clearly indicate that dTRAF2 is stabilised when in complex with dTAK1 and dTAB2 through enhanced K63-linked ubiquitination.

Taken together, results show that dTRAF2 is capable of probably capable of auto-ubiquitination (an *in vitro* ubiquitination analysis is required for verification) and

that this requires an intact RING domain. Further, dTRAF2 binds with both the adaptor dTAB2 and K63 specific E2 ubiquitin conjugating enzyme dUbc13. Moreover, dTRAF2 is stabilised in the presence of dTAB2 and dTAK1 through K63-linked polyubiquitination.

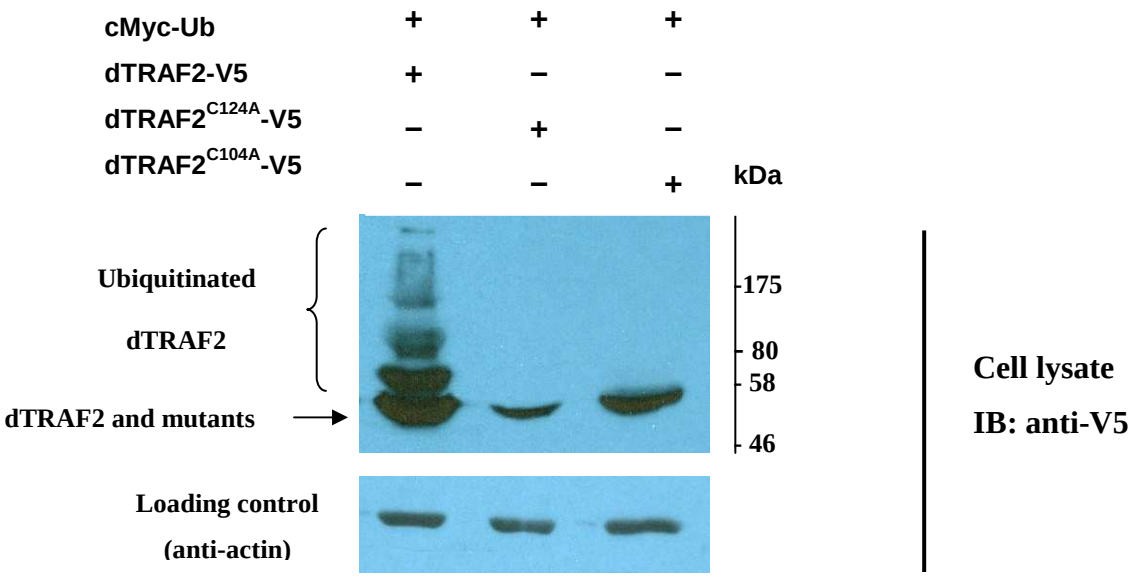


Figure 5-12: *Drosophila* TRAF2 can auto-ubiquitinate and requires an intact RING domain for this. Expression vectors encoding dTRAF2-V5, dTRAF2^{C124A}-V5 or dTRAF2^{C104A}-V5 were transiently transfected in S2 cells together with cMyc-Ub in combinations shown. Cells were lysed 48 hrs post-transfection and immunoblotted with anti-V5 antibody. *Protein size markers are depicted adjacent to the top panel with values given in kDa.*

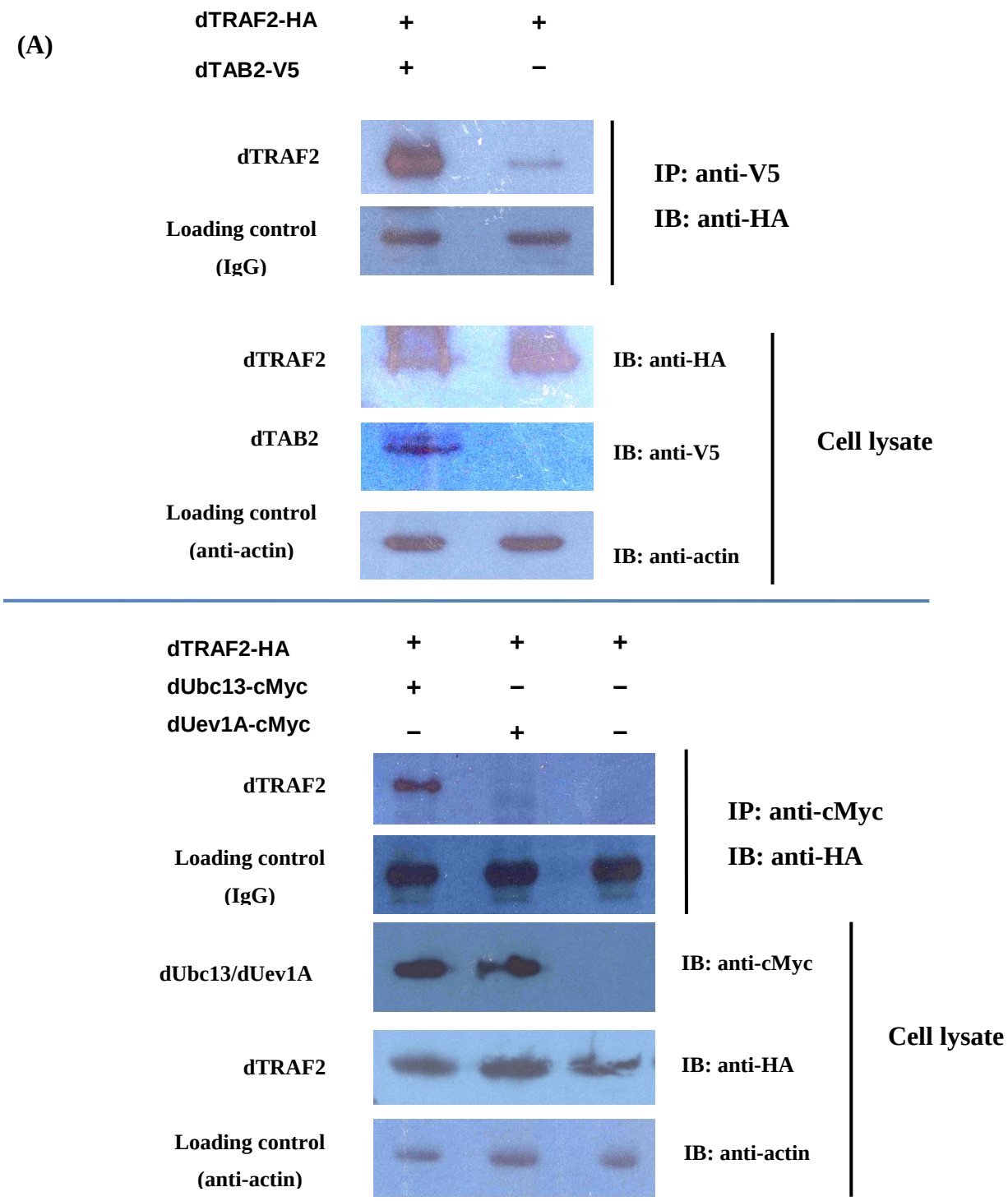


Figure 5-13: *Drosophila* TRAF2 binds with (A) dTAB2 & (B) with E2 enzyme dUbc13. Expression vectors encoding dTRAF2-HA were transiently transfected in S2 cells with or without dTAB2 -V5 or dUbc13-cMyc or dUev1A-cMyc in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5 or anti-cMyc antibody(as indicated), resolved on 10% SDS PAGE, and immunoblotted with anti-HA.

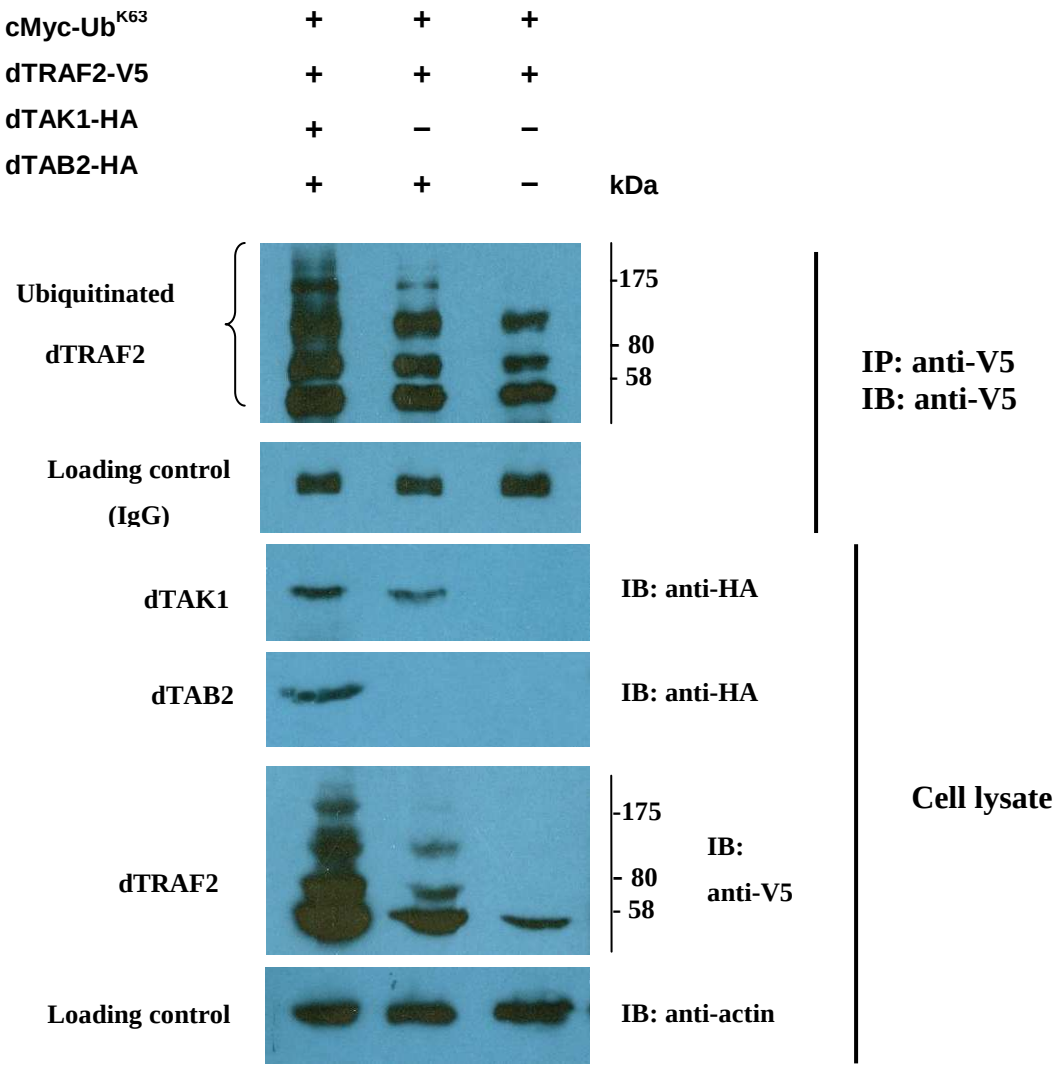


Figure 5-14: *Drosophila* TRAF2 is stabilised when in complex with dTAK1 and dTAB2. Expression vectors encoding dTRAF2-V5 and cMyc-Ub^{K63} were transiently transfected in S2 cells with or without dTAB2–HA and dTAK1-HA in combinations shown. Cells were lysed 48 hrs post-transfection, immunoprecipitated with anti-V5 antibody, resolved on 10% SDS PAGE, and immunoblotted with anti-V5 antibody. *Protein size markers are depicted adjacent to the top panel and panel 5 from top (showing cell lysates of dTRAF2) with values in kDa.*

Chapter 6 Regulation of *Drosophila* TAK1 in TNF/Eiger Signalling - Discussion

6:1 Ariadne-2: A Novel Component of the TNF/Eiger pathway

Ariadne-2 (Ari-2; CG5709) was first discovered as a novel component of the TNF/Eiger pathway in a yeast 2-hybrid assay by Dr. Anthony Brown of our laboratory. Ariadne-2 belongs to a new class of proteins which contain a cysteine-rich C_6HC motif (termed DRIL – Double RING finger linked). This motif was defined as the fourth family member of the zinc-binding RING, LIM, and LAP/PHD fingers (Van Der Reijden et al., 1999). In most instances, this motif is flanked by 2 RING domains. These C_3HC_4 RING domains show deviation from the traditional RING motif. In the N-terminal RING domain (R1), the spacing between C7 and C8 is 4 instead of 2 amino acids whilst in the C-terminal RING (R2) there are 1 – 4 amino acids instead of 2 between C5 and C6. Also, the histidine residue conservation in R2 varies and there is a preference for basic residues after C6 instead of the consensus hydrophobic components. Further, the 2 RING and DRIL motifs form a large tripartite structure termed TRIAD (two RING fingers and DRIL) and the strong conservation of these 3 sub-domains indicate that they may be functionally linked. Triad1 is the human homolog of Ari-2 (Van Der Reijden et al., 1999).

6:2 Role of Ari-2 in JNK-dependent development & stress response

Results indicate that Ari-2 is required in JNK-dependent development and stress response. Results show that Ari-2 expresses in developmental all stages and in both sexes. It is essential for development from at least late larval stage onwards and development of *ari-2*^{ins} mutants was compromised. Further, *ari-2*^{ins} mutants were unable to activate JNK signalling in response to heat shock, oxidative stress and dry

starvation. This phenomenon was reversed when the JNK Bsk was overexpressed via a transgene. These results firmly implicate Ari-2 in the JNK pathway. Further, epistatic analyses and co-immunoprecipitation assays place Ari-2 upstream of/at the level of dTAK1.

Aguilera *et al*., in 2000, had reported the discovery of a closely related gene, *ariadne-1* as a novel *Drosophila* RING-finger protein, which was required for neural development. They showed that both Ari-1 and Ari-2 (together with the corresponding murine and human homologues) belonged to a new conserved protein family RBR (Ring Between Ring) or TRIAD. Also, *ari-1* was found to be expressed in most tissue and all developmental stages with increased levels in the adult nervous system and female gonads. Null *ari-1* mutants show 24% - 29% lethality in pupal stage (Aguilera et al., 2000). Additionally, these mutants exhibited motor task and neural connectivity defects. Also, deletions in the N-terminal RING domain had a deleterious effect in oogenesis. Interestingly, it was shown that ari-1 and ari-2 were non-redundant and functionally distinct (Aguilera et al., 2000).

6:3 The Role of Ari-2 in the TNF/Eiger apoptotic cascade:

Eiger is a potent activator of apoptosis and its targeted expression in the *Drosophila* eye results in ablation leading to a small sized eye. Co-overexpression of a dominant modifier together with Eiger results in enhanced ablation (Geuking et al., 2005, Moreno et al., 2002). Results show that overexpression of Ari-2 with Eiger resulted in enhancement of the small eye phenotype. Further, removal of 1 copy of *ari-2* significantly suppressed the eye apoptotic phenotype induced by over-expressing Eiger, effectively rescuing this phenotype. Co-overexpression of Puckered together with Ari-2 and Eiger suppresses apoptosis and reverts the eye back almost to normal size. Similar

results were observed (albeit a less dramatic rescue) when *UAS-dTAK1-RNAi* was expressed along with Ari-2 and Eiger. The somewhat subdued suppression may be due to the fact that RNAi 'blockage' is not complete and a sizeable amount of dTAK1 will be present. Taken together, these results firmly implicate Ari-2 as a dominant modifier of the TNF/Eiger apoptotic cascade which functions through the core JNK cassette.

6:4 The Zinc-finger, coiled-coil and both RING domains (in tandem) of Ari-2 are required for TNF/Eiger apoptotic signalling

In Triad1, the N- and C-terminal RING motifs play discrete roles by differentially binding to ubiquitin-conjugating enzymes, UbcH7 and Ubc13 respectively and exhibiting dual ubiquitin ligase activity (i.e. formed both K48- and K63-linked polyubiquitin) in myelopoiesis. Interestingly, deletion of either the N-terminal (R1) or C-terminal (R2) RING domains of Ari-2 did not inhibit its interactions with dTRAF2, dTAB2 and dTAK1. Both Ari-$2^{\Delta R1}$ and Ari-$2^{\Delta R2}$ interacted with these proteins in the same manner as full-length Ari-2, including not binding with dTAB2-C. The Co-IP system immunoprecipitating with anti-V5 and subsequently immunoblotting with either anti-HA or anti-cMyc has been well optimized and yield the best results. These results have been confirmed with the reciprocal assays (i.e. immunoprecipitating with anti- HA or anti-cMyc and immunoblotting with anti anti-V5). The optimized results are shown. Since the physiological interactions were maintained, both mutants were assumed to be functional. Thus, through the process of elimination, it is likely that interactions with dTRAF2, dTAB2 and dTAK1 were modulated by the intact In Between RING (IBR), Zinc-finger or coiled-coil domains of Ari-2.

Further, results show that Ari-$2^{\Delta R1R2}$ (deletion of both R1 and R2), Ari-$2^{\Delta ZnF}$ (deletion of the ZnF domain) and Ari-$2^{\Delta CC}$ (deletion of the coiled-coil domain) mutants

failed to synergistically activate apoptosis, together with Eiger in the *Drosophila* eye. This clearly indicates that the ZnF, the coiled-coil domain and 2 RING motifs in tandem were essential for Ari-2 activity in apoptosis. Therefore, it is very likely that the IBR, ZnF and coiled-coil domains mediate interactions with dTRAF2, dTAB2 and dTAK1.

It is unlikely that the 2 RING domains (R1 and R2) function in a redundant manner. Although both R1 and R2 show a C_3HC_4 motif, the spacing between Cys and His residues are very different and R2 shows a more pronounced deviation from consensus. Alternatively, it is possible that the intact TRIAD domain (consisting of R1, IBR and R2) is required for signalling and deletion of any part may render it inert. It was previously postulated that due to the conserved nature of the TRIAD domain that the entire and intact tripartite structure would be required for signalling in Triad proteins (Van Der Reijden et al., 1999).

6:5 A novel cell culture based platform to activate Ari-2 and study its function under active apoptosis conditions

One of the key elements in trying to elucidate the mechanism of Ari-2 function is to examine the ubiquitination states of Ari-2 (both K48 and K63-linked) in the presence or absence of key components of the Eiger cascade, namely dTAK1 and dTRAF2. My observations indicate that Ari-2 did not ubiquitinate when the Eiger pathway was inactive. Therefore, it is highly likely that Ari-2 is activated only when the Eiger pathway is stimulated. Since Eiger is a potent activator of apoptosis, its overexpression in cell culture will result in cell death. Therefore, to address this difficulty, the need arose for an experimental platform where the TNF/Eiger apoptosis cascade could be activated without the resultant apoptosis. Theoretically, this could be achieved in a S2 cell culture system by abrogating downstream signalling by

overexpressing *Drosophila* IAP1. This is proposed as a possible solution.

Drosophila Inhibitor of Apoptosis 1 (IAP1) is involved in modulating programmed cell death (Xu et al., 2009). Apoptosis is executed by Cys-proteases termed "caspases." *Drosophila* IAP1 binds to caspases and inhibit their activity (Xu et al., 2009). In JNK signalling, *Drosophila* IAP1 functions below the level of *puckered.*

The proposed novel cell culture platform would be a stable S2 cell line expressing *Drosophila* IAP1. Initial qPCR tests have shown that the TNF/Eiger cascade can be activated by addition of media in which Eiger had been previously overexpressed. It was previously shown that when Eiger is overexpressed in cell culture, its mature form is cleaved and released into the medium and this is sufficient to activate apoptosis (Kauppila et al., 2003, Igaki et al., 2002). Activation of apoptosis and changes in apoptotic signalling can be monitored by qPCR using *puckered* as the transcriptional target since dIAP1 functions downstream of this. This is especially useful since all of the components in this study (i.e. Ari-2, dTAK1, dTAB2 and dTRAF2) are upstream of *puckered*. This system provides an ideal platform to study Ari-2 and its functions in an activated scenario. The system, however, has not been completed as yet, is untested and has not been used in this study.

6:6 Drosophila TRAF2 – the "switch" between apoptosis and innate immunity?

Parallel experiments by Dr. Anthony Brown show that activation of the TNF/Eiger pathway does not induce innate immune signalling *in vivo*. This is further confirmed by my preliminary qPCR data in cell culture. This 'separation' between apoptosis and innate immunity exists despite the fact that dTAK1 is a central component of and a nexus between both the TNF/Eiger and IMD innate immune pathways. It has been previously shown that the TRAF family of proteins function as

upstream adaptors that sequester TAK1 into specific signalling complexes and selectively activate downstream cascades (Besse et al., 2007). TRAF1 and TRAF2 are the only representatives of the TRAF family in *Drosophila*, with TRAF2 having a RING domain (Cha et al., 2003). Moreover, the stability of dTRAF2 has been shown to be essential for Eiger-induced apoptotic signalling (Xue et al., 2007). My results show that *Drosophila* TRAF2 is possibly capable of auto-ubiquitination and the intact RING domain is essential for this. Further, dTRAF2 binds with dTAB2 (dimeric partner of dTAK1) and the E2 conjugating enzyme dUbc13. In these aspects, dTRAF2 is very similar to its human homologue TRAF6. Moreover, results clearly show that dTRAF2 is stabilised when in complex with dTAK1 and dTAB2. Both dTRAF2 K63-linked ubiquitination and its concentration in the cell lysate decreases as dTAK1 and dTAB2 are withdrawn from this mix. Moreover, preliminary qPCR data from cell culture show overexpression dTRAF2 together with dTAK1 and dTAB2, results in the dampening of the innate immune signal, although the signal is not abrogated. Overexpression of the dTRAF2^{C104A} (which is incapable of ubiquitination) 'rescues' this inhibition thereby suggesting that ubiquitination is essential for this partial dampening.

Taken together, results appear to suggest that dTRAF2 may also function to dampen innate immune signaling in *Drosophila* thereby, helping to partially "switch off" innate immune signaling and providing specificity to the apoptotic signal. More work is required to establish this.

6:7 Ari-2 functions as an adaptor in TNF/Eiger apoptotic signalling

Adapter or linker molecules have been previously described as proteins that lack enzymatic activity and contain multiple binding domains and sequence motifs to which such domains bind (Samelson, 2002). Phosphorylation alters the surface of these adaptors and allows additional enzymes or adapters to bind, leading to the formation of

multiprotein signalling complexes. These complexes are generally at the proximal end of a series of subsequent activation events, and multiple signalling complexes and pathways can be involved. Generally, these processes result in the transfer of information, that is, the transmission of an event on the exterior of the cell (receptor-ligand binding) to activation and regulation of multiple intracellular events occurring at the plasma membrane, cytosol, or nucleus (Samelson, 2002, Downward, 2001).

It has been previously shown that human TAK1 has the capacity to be activated by various cytokines and is known to activate multiple pathways including IL-1, IL-18, TNF, and receptor activator of NF-κB ligand (RANKL) (Ninomiya-Tsuji et al., 1999, Irie et al., 2000, Sakurai et al., 1999, Wald et al., 2001). A central point of interest in TNF/Eiger signalling is how TAK1 is activated (Sakurai et al., 2000, Shibuya et al., 1996). The working hypothesis is that in a number of different biological contexts TAK1 functions as the biologically active molecule in a complex of TAK1-binding proteins (TABs) and TNF-associated proteins (TRAFs). The biological specificity of the complex is likely determined by the repertoire of constituent components and the nature of the external signal rather than the intrinsic specificity of a particular kinase, i.e. TAK1 (Delaney, 2006).

Results show that Ari-2 functions as an adaptor for the stabilisation and assembly of a larger signalling complex comprising dTRAF2, dTAB2 and dTAK1 in the TNF/Eiger apoptotic cascade. Its human homologue Traid1 also functions to stabilise Growth factor independence 1 (Gfi1) and prevent its ubiquitination in granulocytes in a similar manner (Marteijn et al., 2007). Co-immunoprecipitation assays show that Ari-2 interacts with dTRAF2, dTAK1 and dTAB2 (with the N-terminal half being sufficient for this interaction). It was previously reported that dTAB2 interacted with dTAK1

through its C-terminal coiled-coil domain (Geuking et al., 2005). It was also shown that dTRAF2 was in a complex with dTAB2 and dTAK1. Moreover, dTRAF2 interacted with the TNF receptor Wengen (Geuking et al., 2005). My results show that dTRAF2 is stabilised through K63-linked polyubiquitination in the presence of dTAB2 and dTAK1. Taken together, it appears that Ari-2 forms a complex with dTRAF2, dTAB2 and dTAK1 which modulates TNF/Eiger signalling.

Deletion of the ZnF, coiled-coil and the 2 RING domains abolished the function of Ari-2 in apoptosis *in vivo*, thereby suggesting that these domains may be involved in binding with the components of the putative complex transducing the apoptotic signal. In this manner, adaptors like Ari-2 may provide specificity to such complexes (like dTRAF2-dTAB2-dTAK1) and elicit a specific response. However, this aspect requires further investigation.

Xue *et al*., (2007) showed that the stability of dTRAF2 was essential for transducing the TNF/Eiger apoptotic signal. They found that mutations in the de-ubiquitinating enzyme CYLD resulted in increased ubiquitination-mediated proteosomal degradation of dTRAF2 and the consequent breakdown in JNK mediated apoptosis. Thus, the ubiquitination status of dTRAF2 was shown to play a critical role in modulating TNF/Eiger signalling in *Drosophila*. Parallel cell culture experiments by Dr. Anthony Brown indicate that Ari-2 may stabilise dTRAF2. Taken together, results indicate that Ari-2 may play a similar role by stabilising dTRAF2.

Also, whilst both dTAK1 and dTAB2 are essential components of both the TNF/Eiger and IMD innate immune pathways, *ari-2*^{ins} mutant flies showed the same degree of sensitivity to Gram-negative bacterial infection as wild type flies. This shows

that the functions of Ari-2 are limited to the apoptotic cascade. Moreover, whilst dTRAF2 dampened innate immune signalling when overexpressed with dTAK1 and dTAB2, it did not abolish it. This suggests that dTRAF2 may play a redundant role in "switching off" innate immunity during apoptosis. Ari-2, with its relative position in the cascade (at the level of dTAK1) and its domains will be a prime candidate for the role of the redundant partner of dTRAF2. Based on the results the following model is proposed for *Drosophila* TNF/Eiger apoptotic signalling in which Ari-2 functions as an adaptor to form a signalling complex and stabilises dTRAF2 at the level of dTAK1.

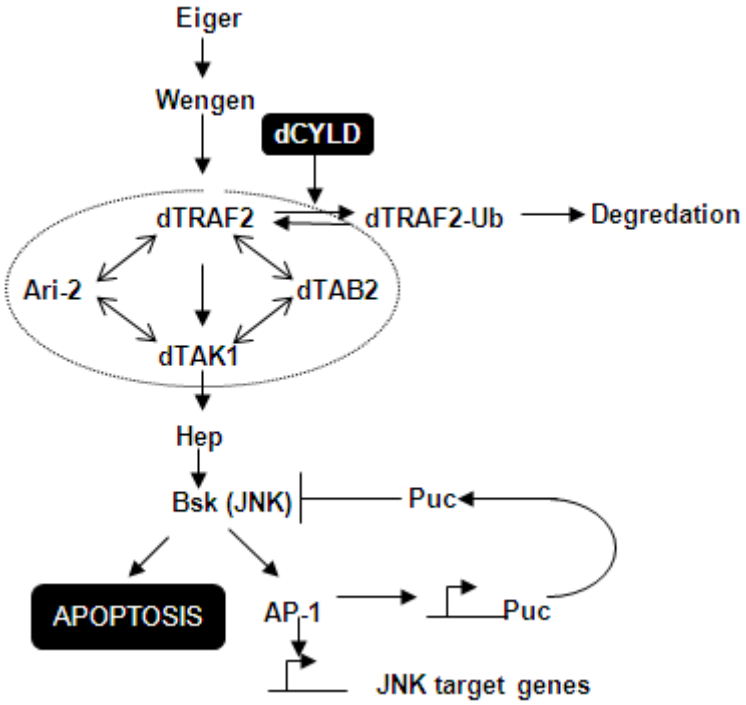


Figure 6 - 1: Proposed model for the TNF/Eiger pathway in *Drosophila melanogaster*. The apoptotic signal is transduced from the ligand-receptor complex of Eiger-Wengen to a complex composed of Ari-2, dTRAF2, dTAK1 and dTAB2. Here, Ari-2 functions as an adaptor to form the complex. The apoptotic signal is then transduced to the core JNK cassette of Hep (JNKK) and Bsk (JNK) leading to the activation of the *Drosophila* "apoptosome" consisting of DARK and DRONC.

Future Perspectives:

Negative Regulation of *Drosophila* TAK1 in IMD Innate Immune Signalling:

Quantitative PCR and Ub assays with double mutant construct of dTAK1 comprising the point mutations K142R and K156R needs to be performed to elucidate the redundant nature of these 2 sites in dTAK1 activity.

Also, in vivo data in a Rabex-5 RNAi background is necessary to establish its role in the IMD pathway. Moreover, a double knockout of dTrabid and Rabex-5 will be required to establish the proposed partial redundancy between these 2 enzymes in negatively regulating the IMD signal in *Drosophila*.

Regulation of *Drosophila* TAK1 in TNF/Eiger Signalling:

The mechanism of Ari-2 activity needs to be elucidated in a background where the TNF/Eiger pathway is activated. In this regard, the novel cell-culture based platform will be very useful. This system will enable both qPCR and Ub assays to be performed in an active TNF/Eiger background.

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Appendix 1: Supplementary Data – Standard recipes

Luria –Bertani broth (1 litre)

10 g of bacto-tryptone

5 g of bacto – yeast extract

10 g of NaCl

Dissolve in 800ml of autoclaved milliQ water, adjust to pH 7.0 and make upto 1 litre with autoclaved milliQ water. Add 15g/L of agar if making medium. Autoclaved before use.

NZY$^+$ Broth (per litre)

10 g of NZ amine (casein hydrolysate)

5 g of yeast extract

5 g of NaCl

Add 800ml of autoclaved milliQ water, adjust to pH 7.5 using NaOH and make upto 1 liter with autoclaved milliQ water. Autoclave. Add the following filer-sterilized supplements prior to use:

12.5 ml of 1 M $MgCl_2$

12.5 ml of 1 M $MgSO_4$

20 ml of 20% (w/v) glucose

Carbenicillin stock solution (100mg/ml):

Dissolved 1.0g of carbenicillin in 10ml of autoclaved milliQ water, filter-sterilesed and stored at -20ºC.

2x SDS sample buffer (Laemmli Buffer):

2.5ml of 0.5 M Tris-HCl, pH 6.8

2.0ml of glycerol (100%)

4.0ml of 10% (w/v) SDS

0.5ml of 0.1% (w/v) Bromophenol Blue

Make upto 10ml with autoclaved milliQ water. Add 2-Mercaptoethanol to final concentration of 5.0%.

10% SDS solution:

Dissolve 10g of SDS in 90ml of milliQ water with gentle stirring. Make upto 100ml with milliQ water.

TE Buffer

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

***Drosophila* fly food:**

(A) 20.0g of agar

750ml of autoclaved milliQ water

125ml of golden syrup

(B) 275ml of autoclaved milliQ water

125g of wholemeal flour

(C) 250ml of autoclaved milliQ water

12ml of nipagin (stock solution 25g dissolved in 250ml alcohol)

7ml propionic acid

Stir all A ingredients into a saucepan and bring to a boil. Mix B ingredients to a fine paste, add to the saucepan and bring it to a boil. Simmer the mixture for 5 mins. Mix C ingredients away from the flame. Add it to the saucepan, bring it to a boil and pour into sterile vials or bottles. Add live Baker's yeast to the top of the food before use.

Appendix 2: Supplementary data – primer sequences & standard reactions

Table A2-1: Primer sequences for generation of full length constructs

Construct	Primer name	Primer sequence
dTAK1	AcTAK1-F	5'- CCCGAATTCCAAAATGGCCACAGCATCGCTGGACG-3'
	AcTAK1-R	5'- CCCCTCGAGCGCATTGTGATGCGGTGGGATGAC-3'
Ari-2	AcAri2-F	5'- CCCGAATTCCAAAATGGACTCGGACATCGAAATG-3'
	AcAri2-R	5'- CCCGCGGCCGCTGCGTCCACTGGGAAGAAGTC-3'
Trabid	AcTrbd-F	5' - CCCGAATTCCAAAATGTGCGATACCAAAGACGATGCG - 3'
	AcTrbd-R	5' - CCCGCGGCCGCCTCCTCATCCGAGTCGCCATC - 3'
dTRAF2	AcTRAF6-F	5'-CCCGAATTCCAAAATGCAGCGAGTCCAGGCC-3'
	AcTRAF6-R	5'-CCCGCGGCCGCCACAATATTGATCTCGATCTTGATG-3'
dTAB2	AcTAB2-F	5'- CCCGCGGCCGCCAAAATGGCGGCTACACCACCAATG-3'
	AcTAB2-R	5'- CCCCTCGAGTGTATGCAGAGCGTACGGCTGCTG-3'
Ubiquitin	AcUb-F	5'-CCCGAATTCCAAAATGGCCGAACAAAAACTTATTTCTGAAGAA GATCTGGGTGCCGGTATGCAGATCTTCGTGAAAACCCTTAC-3'
	AcUb-F	5'-CCCGCGGCCGCTTAACCACCTCTCAGACGCAGG-3'
Eiger	AcEgr-F	5'-CCCGAATTCCAAAATGACTGCCGAGACCCTCAAGC-3'
	AcEgr-R	5'-CCCGCGGCCGCCACCTTGAAGATGCCAAAGTAGC-3'
Puckered	AcPuc-F	5'-CCCGCGGCCGCCAAAATGTGTGTGAATCGAGAAACGC-3'
	AcPuc-R	5' - CCCCTCGAGGTCCCTCGTCAAATTGCTAGCC - 3'
dIAP1	AcdIAP1-F	5' - CCCGAATTCCAAAATGGCATCTGTTGTAGCTGATCTTCC – 3'
	AcdIAP1-R	5' - CCCGCGGCCGCAGAAAAATATACGCGCATCACATCGG – 3'
Rabex-5	Rabex-5-F	5'- CCCGCGGCCGCCAAAATGTCAACGGCGGCGCGACCTC-3'
	Rabex-5-R	5'- CCCCTCGAGTTTGGGAGCATCCGCGCCGTCCTGTCC-3'
EGFP	EGFP-F	5'-CCCGAATTCCAAAATGGTGAGCAAGGGCGAGGAGC-3'
	EGFP-R	5'-CCCGCGGCCGCCTTGTACAGCTCGTCCATGC-3'

**Restriction sites are underlined.*

Table A2-2: Primer sequences for generation of dTAB2-N and dTAB2-C constructs

Construct	Primer name	Primer sequence
dTAB2-N	AcTAB2-F	5'- CCCGCGGCCGCCAAAATGGCGGCTACACCACCAATG-3'
	TAB2-N-R	5'- CCCCTCGAGCGGCTCACAGGGCGTTGCGTACAAGG-3'
dTAB2-C	TAB2-C-F	5'-CCCGCGGCCGCCAAAATGGTGACAAGTGTTCCGGCAAACC-3'
	AcTAB2-R	5'- CCCCTCGAGTGTATGCAGAGCGTACGGCTGCTG-3'

**Restriction sites are underlined.*

Standard PCR reaction

40.0 µl milliQ H_20 (sterile)

1.0 µl DNA template (100ng/µl)

5.0µl PfuUltra11 reaction buffer (10x)

1.0µl dNTP mix (25 mM each dNTP)

1.0 µl reverse primer (10µM)

1.0 µl forward primer (10µM)

1.0µl PfuUltra11 fusion HS DNA polymerase

50.0 µl → total volume

PCR Cycling parameters:

Cycle	Temperature	Duration
1	95°C	2 mins
30	95°C	20 sec
	Primer T_m - 5°C	20 sec
	72°C	15 sec/kb
1	72°C	3 mins

Table A2-3: Mutagenesis primers for fusion PCR – *Drosophila* Ari-2

Construct	Primer name	Primer sequence
Ari-2$^{\Delta R1}$	Ari-2$^{\Delta R1}$-A	5'- CCCGAATTCCAAAATGGACTCGGACATCGAAATG-3'
	Ari-2$^{\Delta R1}$-B	5'- CCTCGGGCACCCTAACATTCATCTGGCTGCGGTACTGTGG-3'
	Ari-2$^{\Delta R1}$-C	5'-CCACAGTACCGCAGCCAGATGAATGTTAGGGTGCCCGAGG-3'
	Ari-2$^{\Delta R1}$-D	5'- CCCGCGGCCGCTGCGTCCACTGGGAAGAAGTC-3'
Ari-2$^{\Delta R2}$	Ari-2$^{\Delta R2}$-A	5'- CCCGAATTCCAAAATGGACTCGGACATCGAAATG-3'
	Ari-2$^{\Delta R2}$-B	5'-GGGTTGTCCTTGTAGCGAGACTTGGGGCAGTCTTTGGTGTG-3'
	Ari-2$^{\Delta R2}$-C	5'-CCACACCAAAGACTGCCCCAAGTCTCGCTACAAGGACAACCC-3'
	Ari-2$^{\Delta R2}$-D	5'- CCCGCGGCCGCTGCGTCCACTGGGAAGAAGTC-3'
Ari-2$^{\Delta ZnF}$	Ari-2$^{\Delta ZnF}$-A	5'- CCCGAATTCCAAAATGGACTCGGACATCGAAATG-3'
	Ari-2$^{\Delta ZnF}$-B	5'-GGCGCACTTGGTCAGCCAGCCCGTGTGGCAAGCCTTGC-3'
	Ari-2$^{\Delta ZnF}$-C	5'-GCAAGGCTTGCCACACGGGCTGGCTGACCAAGTGCGCC-3'
	Ari-2$^{\Delta ZnF}$-D	5'- CCCGCGGCCGCTGCGTCCACTGGGAAGAAGTC-3'
Ari-2$^{\Delta CC}$	Ari-2$^{\Delta CC}$-A	5'- CCCGAATTCCAAAATGGACTCGGACATCGAAATG-3'
	Ari-2$^{\Delta CC}$-B	5'-CTCGAGATCACCCAGGTCACCGGCCTCCATGTAATAGG-3'
	Ari-2$^{\Delta CC}$-C	5'-CCTATTACATGGAGGCCGGTGACCTGGGTGATCTCGAG-3'
	Ari-2$^{\Delta CC}$-D	5'- CCCGCGGCCGCTGCGTCCACTGGGAAGAAGTC-3'

**Restriction sites are underlined.*

Note: Ari-2$^{\Delta R1R2}$ was made with the same primers used for making Ari-2$^{\Delta R1}$ but, using Ari-2$^{\Delta R2}$ as the template.

Table A2-4: Mutagenesis primers – *Drosophila* TAK1 single site mutations:

Construct	Primer name	Primer sequence
dTAK1 (K45R)	dTak1A137G-F	5'-CAAGCTGGTTGCCGTCAGGGAGTTCTTCGC-3'
	dTak1A137G-R	5'-GCGAAGAACTCCCTGACGGCAACCAGCTTG-3'
dTAK1 (K134R)	dTak1A401G-F	5'-ATATTTGCATGCCATGACGCCAAGACCACTAATACATCG-3'
	dTak1A401G-R	5'-CGATGTATTAGTGGTCTTGGCGTCATGGCATGCAAATAT-3'
dTAK1 (K142R)	dTak1K142R-F	5' - CACTAATACATCGCGACGTGAGGCCGCTGAACC - 3'
	dTak1K142R-R	5' - GGTTCAGCGGCCTCACGTCGCGATGTATTAGTG - 3'
dTAK1 (K156R)	dTak1A467G-F	5'-AAGGGACGCAATCTGAGGATATGCGACTTCGGC-3'
	dTak1A467G-R	5'-GCCGAAGTCGCATATCCTCAGATTGCGTCCCTT-3'
dTAK1 (K189R)	dTak1A566G-F	5'-GGTCTTCGAAGGCTCCAGGTATACGGAGAAGTGTG-3'
	dTak1A566G-R	5'-CACACTTCTCCGTATACCTGGAGCCTTCGAAGACC-3'
dTAK1 (K194R)	dTak1A578G-F	5'-GCTCCAAGTATACGGAGAGGTGTGACATTTTTAGCTG-3'
	dTak1A578G-R	5'-CAGCTAAAAATGTCACACCTCTCCGTATACTTGGAGC-3'

Table A2-5: Mutagenesis primers – *Drosophila* TAB2 single site mutations:

Construct	Primer name	Primer sequence
dTAB2$^{\Delta FP}$	dTab2FP-F	5'-GAGATGAAGCAGGAGGCAGCCACGATACCCGATGCC-3'
	dTab2FP-R	5'- GGCATCGGGTATCGTGGCTGCCTCCTGCTTCATCTC-3'
dTAB2$^{\Delta ZnF}$	dTab2C769A/C772A-F	5'-GCTGGACTCGTGGGCCGCCAACATGGCTACATTCCGCA ACCAT - 3'
	dTab2C769A/C772A-R	5' - ATGGTTGCGGAATGTAGCCATGTTGGCGGCCCACGAGTC CAGC - 3'

Table A2-6: Mutagenesis primers – *Drosophila* Trabid single site mutations:

Construct	Primer name	Primer sequence
dTrabid^{C518S}	TrbdT1552A-F	5'-CAGTGCTGGCGACAGCCTGCTGGACTC-3'
	TrbdT1552A-R	5'-GAGTCCAGCAGGCTGTCGCCAGCACTG-3'
dTrabid^{C13A}	TrbdT37G/G38C-F	5'-GACGATGCGCAGAAATGGAAAGCTGAAACTTGCACCTACG-3'
	TrbdT37G/G38C-R	5'-CGTAGGTGCAAGTTTCAGCTTTCCATTTCTGCGCATCGTC-3'
dTrabid^{C95A}	TrbdT283G/G284C-F	5'-CAGCGAAAAGTGGCCCGCCAAGGTGTGCACCTAT-3'
	TrbdT283G/G284C-R	5'-ATAGGTGCACACCTTGGCGGGCCACTTTTCGCTG-3'
dTrabid^{C238A}	TrbdT712G/G713C-F	5'-CTATGTCTCCAAATGGGCGGCTAACTCTTGCACTTACGAA-3'
	TrbdT712G/G713C-R	5'-TTCGTAAGTGCAAGAGTTAGCCGCCCATTTGGAGACATAG-3'

- Construct dTrabid^{C518S} was made with primers TrbdT1552A-F & TrbdT1552A-R with full-length dTrabid as the template using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent).
- Construct dTrabid$^{C518S+3xZnF}$ was made with primers TrbdT37G/G38C-F, TrbdT283G/G284C-F & TrbdT712G/G713C-F using full dTrabid^{C518S} as the template with a QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent).

Table A2-7: Mutagenesis primers – *Drosophila* TRAF2 single site mutations:

Construct	Primer name	Primer sequence
dTRAF2^{C104A}	dTraf2^{C104A} - F	5'-GGACTCGCGATACGAGGCCGCCATTTGCATCGAT-3'
	dTraf2^{C104A} - R	5'-ATCGATGCAAATGGCGGCCTCGTATCGCGAGTCC-3'
dTRAF2^{C124A}	dTraf2^{C124A} - F	5'-TTGACGTCGTGCGGCGCTCGATTTGCTCGCAGCTGCCTCAC-3'
	dTraf2^{C124A} - R	5'-GTGAGGCAGCTGCGAGCAAATCGAGCGCCGCACGACGTCAA-3'

Standard single-site mutagenesis reaction

40.0 µl milliQ H_20 (sterile)

1.0 µl dsDNA template (100ng/µl)

5.0µl Reaction buffer (10x)

1.0µl dNTP mix (25 mM each dNTP)

1.0 µl oligonucleotide primer # 1 (125ng)

1.0 µl oligonucleotide primer # 1 (125ng)

1.5 µl QuikSolution reagent

50.0 µl → total volume

Then added:

1.0 µl of QuikChange Lightning Enzyme

PCR Cycling parameters:

Segment	Cycle	Temperature	Duration
1	1	95°C	2 mins
2	18	95°C	20 sec
		60°C	20 sec
		68°C	30 sec/kb
3	1	68°C	5 mins

Table A2-8: Mutagenesis primers –Ubiquitin multi site mutations:

Primer name	Primer sequence
Ub-A17G-F	5'-ATGCAGATCTTCGTGAGAACCCTTACCGGCAGG-3'
Ub-A17G-R	5'-CCTGCCGGTAAGGGTTCTCACGAAGATCTGCAT-3'
Ub-A32G-F	5'-AAAACCCTTACCGGCAGGACCATCACCCTTGAG-3'
Ub-A32G-R	5'-CTCAAGGGTGATGGTCCTGCCGGTAAGGGTTTT-3'
Ub-A80G_A86G-F	5'-CACCATCGAAAATGTGAGGGCCAGGATCCAGGATAAGGAAG-3'
Ub-A80G_A86G-R	5'-CTTCCTTATCCTGGATCCTGGCCCTCACATTTTCGATGGTG-3'
Ub-A98G-F	5'-GCCAGGATCCAGGATAGGGAAGGCATTCCC-3'
Ub-A98G-R	5'-GGGAATGCCTTCCCTATCCTGGATCCTGGC-3'
Ub-A143G-F	5'-AGGCTCATCTTTGCAGGCAGGCAGCTGGAAG-3'
Ub-A143G-R	5'-CTTCCAGCTGCCTGCCTGCAAAGATGAGCCT-3'
Ub-A188G-F	5'-TCTTTCTGACTACAACATCCAGAGAGAGTCGACCCT-3'
Ub-A188G-R	5'-AGGGTCGACTCTCTCTGGATGTTGTAGTCAGAAAGA-3'

Mutant constructs cMyc-Ub^{K48} & cMyc-Ub^{K63} were made using a QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). Only the forward primer set were used. Mutagenesis was performed in 2 steps.

1. Full-length cMyc-Ub was used as the template and nucleotides 32, 80, 86 and 143 (or 188) were mutated from A to G. This generated 2 mutant constructs with either K48 (A143G) or K63 (A188G) mutated together with sites 32, 80 and 86.
2. Thereafter, sites 17 and 98 were mutated using both the K48 and K63 mutant constructs as templates.

Standard multi-site mutagenesis reaction

X µl sterile milliQ H_20 (upto a final volume of 25µl)

X µl dsDNA template (100ng)

2.5µl reaction buffer (10x)

1.0µl dNTP mix (25 mM each dNTP)

X µl oligonucleotide primer # 1 (100ng)

X µl oligonucleotide primer # 1 (100ng)

X µl oligonucleotide primer # 1 (100ng)

0.75 µl QuikSolution reagent

1.0 µl of QuikChange Lightning Enzyme

25.0 µl → total volume

PCR Cycling parameters:

Segment	Cycle	Temperature	Duration
1	1	95°C	2 mins
2	30	95°C	20 sec
		55°C	30 sec
		65°C	30 sec/kb
3	1	65°C	5 mins

Standard restriction digestion reaction

X µl sterile milliQ H_20 (upto a final volume of 100µl)

X µl dsDNA template (2.5µg)

10.0µl appropriate reaction buffer (10x)

10.0µl bovine serum albumin (10x)

2.5 µl restriction enzyme # 1

2.5 µl restriction enzyme # 2

100.0 µl → total volume

Incubated at 37ºC for 3 hrs

Typical ligation/cloning reaction

X µl sterile milliQ H_20 (upto a final volume of 10µl)

X µl restriction digested expression vector DNA (~50ng)

X µl restriction digested DNA construct (~150ng)

1.0µl reaction buffer (10x)

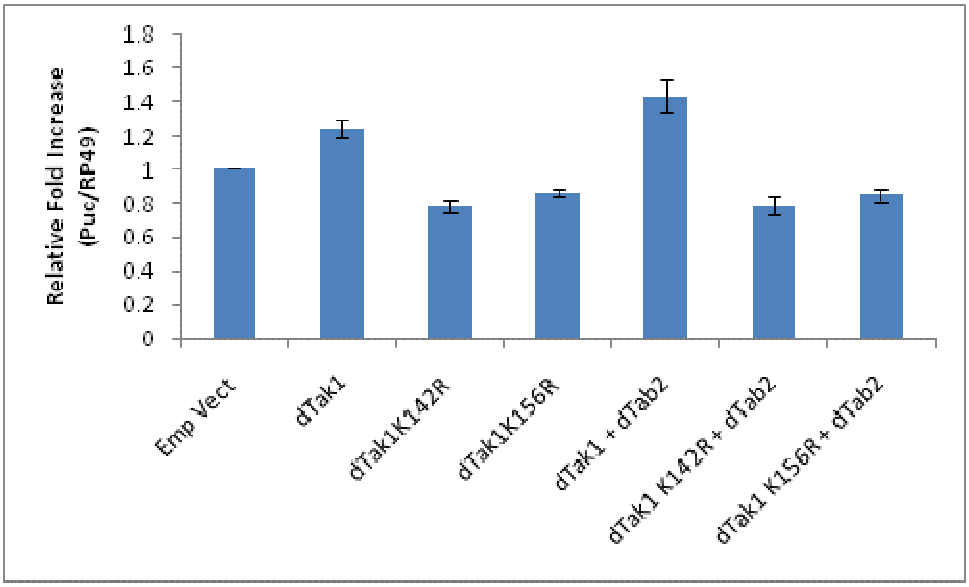
1.0µl DNA Ligase enzyme

10.0 µl → total volume

Incubated at 16ºC for 12 - 16 hrs

Appendix 3 – Supplementary Data - Chapter 3

(A)



(B)

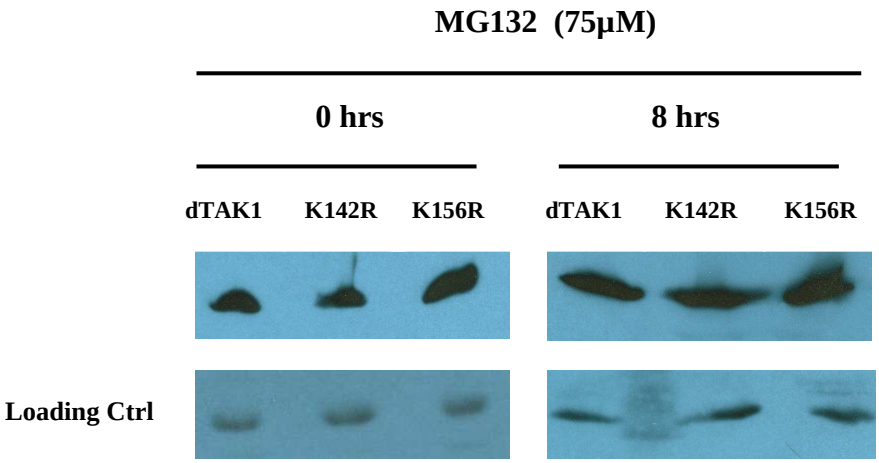
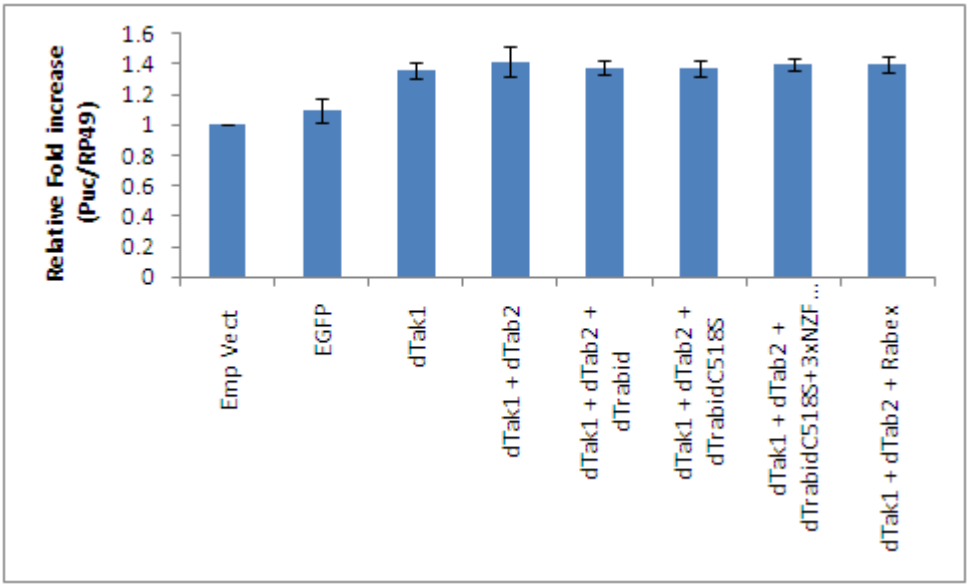


Figure S1: JNK activity & expression profiles of Lys 142 and 156 mutants of *Drosophila* TAK1. (A) *Drosophila* TAK1^{K142R} & dTAK1^{K156R} mutant failed to activate the JNK signalling above basal levels. *Drosophila* TAK1 (wt) or dTAK1^{K142R} or dTAK1^{K156R} mutant was expressed along with dTAB2 in combinations shown in S2 cells and *puckered* expression was assayed 48hrs post-transfection. *Error bars represent SEM of 3 separate experiments. Results were normalized to the empty vector.* **(B)** Both dTAK1^{K142R} & dTAK1^{K156R} show similar expression profiles to dTAK1. A time-course expression analysis after treatment with proteasomal inhibitor MG132 (75µM for 8 hrs) show the profiles of both mutants were similar to full-length dTAK1.

(A)



(B)

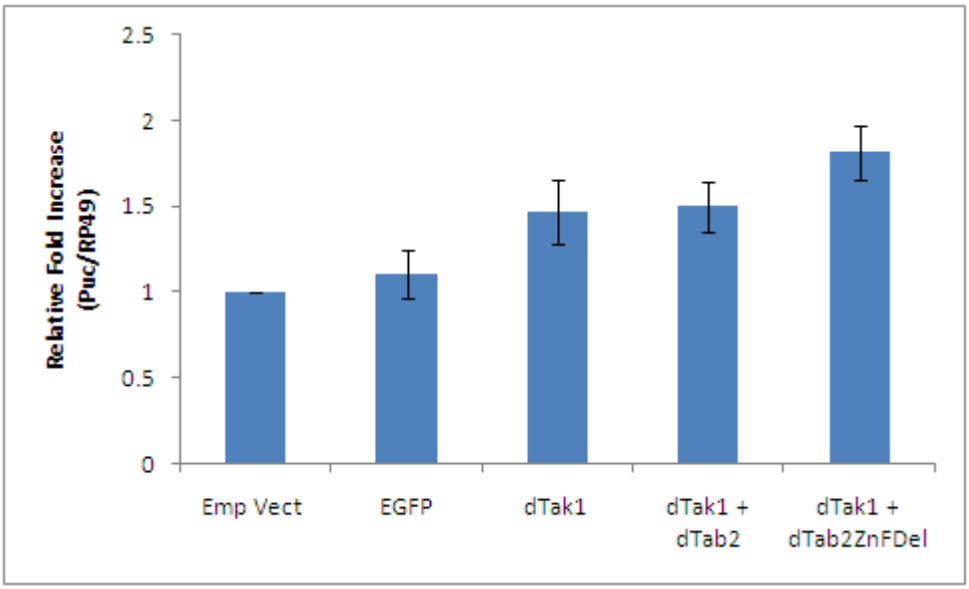
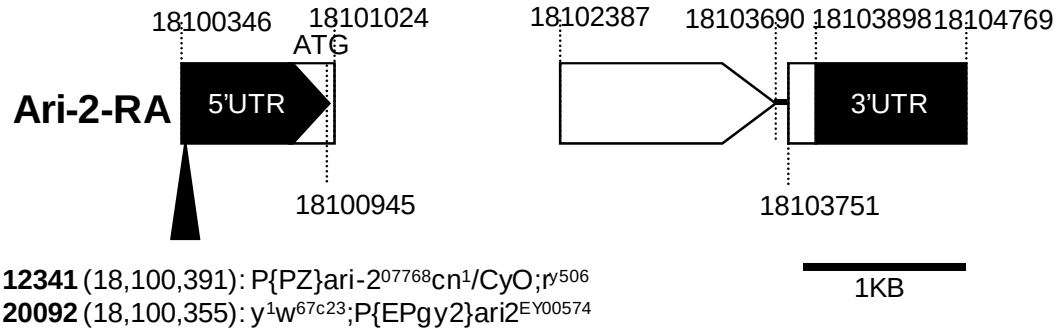


Figure S2: JNK activity in the presence of (A) dTrabid and mutants & (B) dTAB2 and dTAB2^{DelZnF} . (A) Activity of the JNK pathway was not affected by dTrabid or its mutants. S2 cells were co-transfected with expression vectors in combinations shown and *puckered* expression was assayed 48hrs post-transfection by qPCR. *Results were normalised to the empty vector (Emp Vec) and the error bars represent the SEM of 3 independent experiments.* (B) The JNK pathway is slightly activated above basal levels in the presence of *Drosophila* TAB2^{DelZnF} mutant when compared with full-length dTAB2. S2 cells were co-transfected with expression vectors in combinations shown and *puckered* expression was assayed 48hrs post-transfection by qPCR. *Results were normalised to the empty vector (Emp Vec) and the error bars represent the SEM of 3 independent experiments.*

Appendix 4 – Supplementary Data - Chapter 5

(A) Chromosome 2R:18,100,346..18,104,769



(B)

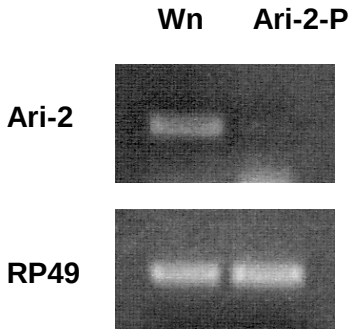


Fig. S3. Characterisation of P-element insertion lines. (A) Schematic genomic map of *Ari-2-RA*, depicting positions of insertion of the P-elements in the two mutant lines (black arrow) used to generate the transheterozygous mutant Ari-2 stock utilised in the study. **(B)** The transheterozygous 12341/20092 mutant was analyzed by reverse transcriptase PCR for *ari-2* mRNA (upper panel), and shown to be a strong hypomorph when compared with wild-type flies (Wn). *rp49* (also known as *RpL32*) was used as a loading control (lower panel).

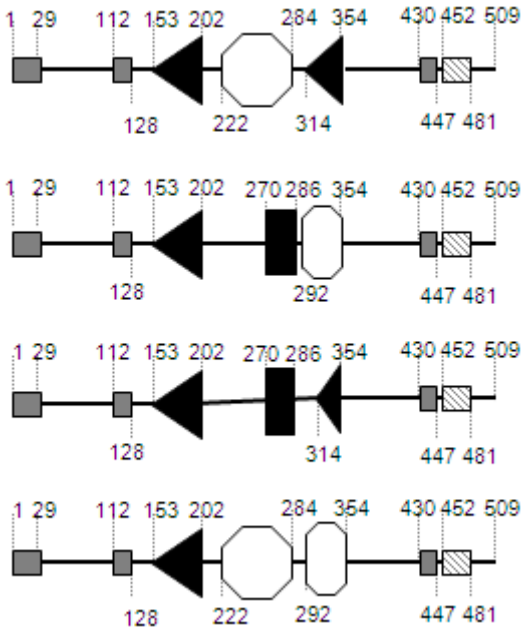
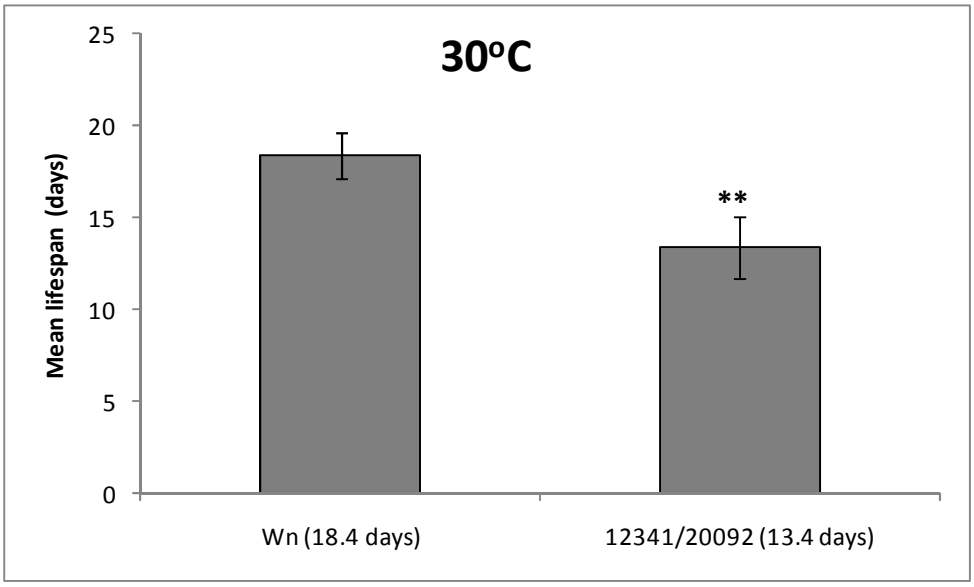


Fig. S4. Possible Representations of Ari-2. Due to overlapping domains, Ari-2-PA is predicted to form four possible representations of the Ari-2 protein (http://smart.embl-heidelberg.de/). Grey box represents a low complexity region; Black triangle represents a RING finger domain; White hexagon represents an IBR (In Between RING fingers domain); Black rectangle represents a C_2HC-type zinc finger; Hatched rectangle represents a coiled coil region.

(A)



(B)

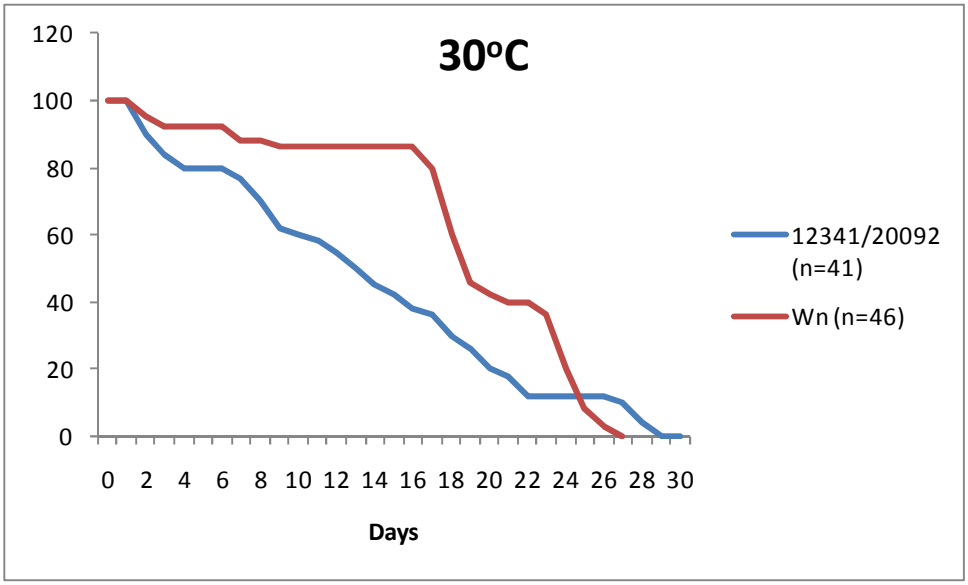


Figure S5: Ari-2 mutant flies have a reduced adult lifespan. (A) Mean adult lifespan (± s.e.) of *ari-2* mutant flies (12341/20092; white bars; 13.4 days) at 30°C compared to wild-type flies (Wn; grey bars; 18.4 days). (B) Survival graph of above *ari-2* wild-type and mutant flies at 30ºC Cohorts of 20 wild-type male (Wn; black line) or 20 Ari-2 mutant male (12341/20092; grey line) flies were placed in standard food vials at 30°C and their survival determined daily Experiments depict mean values from at least 2 independent experiments. ** indicates significance value of the result as determined by Student's t-Test (*P*=<0.01).

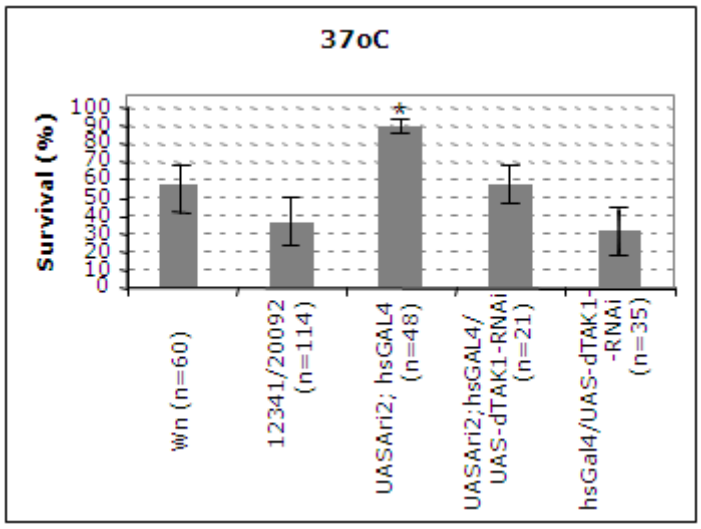


Figure S6: Ari-2 mutant flies are sensitive to cellular stress and fail to mount a JNK response. Cohorts of 20 male flies were placed in standard food vials and exposed to heat shock at 37°C for 4 hours. Survival (determined by a sit-up response) was scored after this period. Mean values ±s.e. are depicted from at least 3 independent experiments. The survival of Ari2 mutant flies (12341/20092) was compromised with respect to wild-type flies (wCam), and found to be comparable to flies devoid of a JNK response (*CyO/If;hsGal4/UASdTak1-RNAi*). Survival, however, could be extended by overexpressing *ari-2* (*UAS-ari2/CyO;hsGal4/+*). This extended lifespan phenotype could however, be restored to wild-type levels by co-expressing a dTAK1 dsRNA (*UAS-ari-2/CyO;hsGal4/UAS-dTAK1-RNAi*).

Publication List:

1. Fernando, M. D. A., and Ligoxygakis, P. (2011). “Trabid is a novel negative regulator of the *Drosophila* Immune-deficiency pathway at the level of TAK1” (in submission)

2. Fernando, M. D. A., Brown, A. E., Ligoxygakis, P. (2011). “The putative E3 Ubiquitin ligase Ariadne-2 is a novel component of the *Drosophila* Eiger/TNF-JNK pathway” (being finalized for submission)