The regulatory mechanisms of Eiger-dependent cell death signaling in *Drosophila melanogaster*

Ambika Srinivasan

Department of Neurology and Neurosurgery
Montréal Neurological Institute
McGill University
Montréal, Quebec, Canada

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In loving memory of Paati and Thatha -

the youngest 90-year-olds who have influenced my life.

***

Arise, awake, learn and stop not till the goal is reached,

for that path is sharp as a razor's edge, impassable,

and hard to go by, say the wise.

Katha Upanishad – 1.3.14 (circa 1000-400 B.C.)

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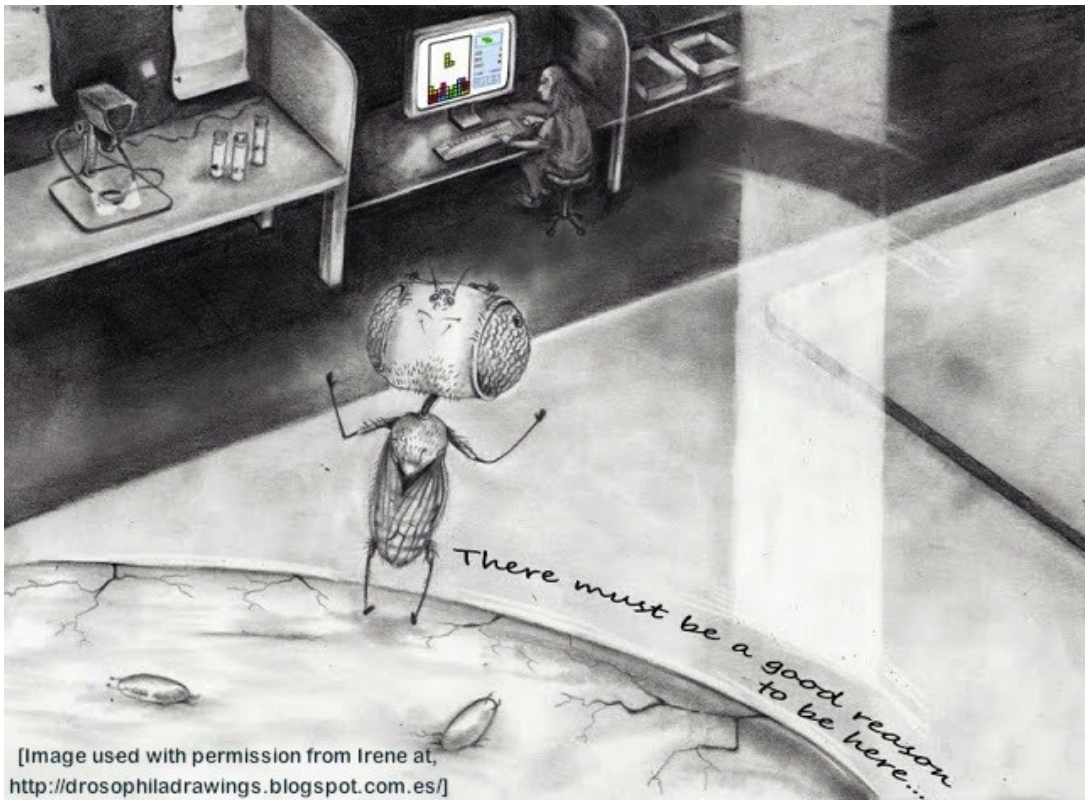

[Image used with permission from Irene at, http://drosophiladrawings.blogspot.com.es/]

…..mustn't there ?

ABSTRACT

The mammalian tumor necrosis factor receptor (TNFR) super family participate in a wide array of complex biological functions. There is accumulating evidence showing the importance of one particular TNFR, p75NTR in the activation of apoptosis *in vivo*, during development and upon injury. The caspase-9 dependent c-Jun Kinase (JNK) activation, remains the key module for p75NTR-dependent apoptosis as opposed to the caspase 8 driven mechanism adopted by other TNFR family members. However, the molecular events that lead to p75NTR-dependent JNK activation have been less clear. Phylogenetic analysis suggest a much more ancient origin of p75NTR relative to other family members, which permits the study of its signaling cascades in invertebrate model systems. The *Drosophila melanogaster* TNFR receptor, Wengen contains a cysteine-rich repeat that closely resembles similar domains in p75NTR. Wengen participates in a number of physiological events by interaction with the fly TNF, Eiger. Eiger-Wengen participate in apoptotic signaling, that is entirely dependent on JNK activation, similar to p75NTR.

Thus, structural and functional similarities between p75NTR and Wengen prompted us to explore conserved apoptotic signaling mechanisms that engage in JNK activation. The lack of genetic redundancy and the ease of genetic manipulation makes *Drosophila melanogaster* a useful model system for the identification of conserved genetic modifiers that regulate the primordial TNFR signaling cascade.
We utilize the GAL4-UAS system for the ectopic expression of fly TNF, Eiger in the eye-imaginal disc. Overexpression of Eiger in the fly eye induces a massive degree of apoptosis, resulting in the loss of a majority of photoreceptor cells, generating a 'small eye' phenotype. We use this system as a screening tool to look for regulatory proteins downstream of Eiger and upstream of the JNK signaling network.

In the first part of this dissertation we address the essential role of TNFR adaptor, dTRAF2 in Eiger-dependent signal transduction. This discovery is imperative to understand the modulatory role of dTRAF2, which we identify to be critically dependent on its expression levels. In the second part of this dissertation we address the necessity for K^{63} polyubiquitination in Eiger- dependent signal

transduction. We identify the K^{63}-conjugating enzyme, Bendless as an indispensible player that selectively activates the cell death-signaling cascade upon Eiger expression. The ubiquitinated targets downstream of the fly TNF signaling pathway remain unknown. We utilize transgenic flies that express a biotinylated form of ubiquitin, which when coupled with ectopic Eiger, allow us to identify several novel ubiquitin-conjugated proteins that lie downstream of the ligand-receptor pair. The final chapter of this dissertation recapitulates some aspects of TNF-regulated pro-degenerative events in a Drosophila model of Alzheimer's disease. We identify a multi-modal system of Eiger-signaling proteins that co-ordinate the activation of cell death through novel genetic interactions. The Eiger pro-degenerative pathway could have major implications in understanding the role of TNFR signaling in the pathology of neurodegenerative diseases. In summary, we address the role of critical proteins in fly TNF-TNFR mediated apoptosis, which would be useful to understand conserved signaling mechanisms of p75NTR dependent cell death.

RÉSUMÉ

Les membres de la famille des TNFR (tumor necrosis factor receptor) effectuent diverses fonctions biologiques chez les mammifères. En particulier, p75NTR, un des membres de cette famille, est un principal inducteur de l'apoptose cellulaire *in vivo* au cours du développement ainsi que suite à diverses lésions. Contrairement aux autres membres de cette famille qui utilisent principalement la protéine caspase 8 dans leur mécanisme d'apoptose, p75NTR active caspase 9 et JNK par des mécanismes jusqu'à présent inconnus. Une analyse phylogénétique de la famille des TNFR, démontre que l'expression de p75NTR est survenue bien avant les autres membres de la famille des TNFRs, ce qui permet l'étude de ce recepteur dans un système simple tel que les invertébrés. En effet, l'unique TNFR exprimé chez les drosophiles, nommé Wengen, possède une séquence peptidique repetée riche en cystéine, semblable à celle de p75NTR. Wengen et son ligand Eiger (TNF chez la drosophile) possèdent différents rôles physiologiques. Wengen-Eiger induisent l'apoptose cellulaire en activant JNK, identiquement à p75NTR. Ainsi, les similarités structurale et fonctionnelle entre p75NTR et Wengen, nous permettent d'étudier les mécanismes d'activation de JNK par p75NTR par l'utilisation d'un système simple et facile à manipuler génétiquement, tel que la Drosophile.

Pour ce fait, nous avons utilisé le système GAL4-UAS pour l'expression de Eiger dans les disques imaginaux des yeux de la Drosophile. La surexpression de Eiger dans les yeux de la drosophile induit l'apoptose massive résultant par la perte de la majorité des photorécepteurs, ce qui permet l'apparition du phénotype du "petit oeil" (small eye phenotype). Nous avons bénéficié de ce système pour étudier les voies de signalisation par lesquels Eiger induit la mort cellulaire par le biais de JNK.

Dans la première partie de cet essai, nous adressons le rôle important de dTRAF2, un adapteur de TNFR, dans la voie de signalisation de Eiger, induisant la mort cellulaire. Nous démontrons que dTRAF2 a un effet modulateur dans ce processus ce qui depend largement de son taux d'expression. Dans un deuxième temps, nous montrons l'importance de l'ubiquitination liée par K63 dans les signaux de transduction de Eiger. Nous avons identifié Bendless, l'enzyme de conjugaison de K63, comme un majeur activateur de la mort cellulaire suite à la surexpression de

Eiger. Les cibles de cette ubiquitination ne sont pas connues. Pour ce fait nous avons utilisé des mouches transgéniques qui expriment une forme d'ubiquitine liée à la biotine. Cette protéine, une fois couplée à Eiger nous permettra d'identifier de nouvelles protéines conjuguées à l'ubiquitine comme composants importants dans la voie de signalisation de Wengen-Eiger.

Le dernier chapitre de cette thèse relate quelques aspects du rôle de TNF dans le processus de dégénéréscence chez la Drosophile, imitant ainsi la maladie D'Alzheimer. Nous identifions un système multimodal par lequel Eiger active la mort cellulaire par le biais de nouvelles interactions génétiques. L'étude approfondie de la voie de signalisation par laquelle Eiger induit les processus de dégénéréscence est essentielle pour comprendre le rôle de TNFR dans les maladies neurodégénératives. En conclusion, ce travail adresse le rôle critique de TNF-TNFR dans l'induction de l'apoptose cellulaire chez la drosophile, ce qui sera utile dans la compréhension des mécanismes utilisés par p75NTR dans la mort cellulaire.

LIST OF FIGURES

ABBREVIATIONS

BirA	Biotin ligase
GMR	Glass Multimer Reporter
UAS	Upstream Activation Sequence
TNF	Tumor Necrosis Factor
TNFR	TNF-receptor
p75NTR	p75 neurotrophin receptor
nLC-ESI-MS/MS	Nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry
TSC	Total spectral counts
FADD	Fas-associated death domain
TRADD	TNFR-associated death domain
DISC	Death-inducing signaling complex
NT	Neurotrophin
UBC/E2	Ubiquitin-conjugating enzyme
E1	Ubiquitin-activating enzyme
TLR	Toll-like receptor
ROS	Reactive oxygen species
DAbl	Drosophila Abl tyrosine kinase network
CNS	Central nervous system
UBC	Ubiquitin-conjugating catalytic domain
1XPBS	1X phosphate buffered saline
RING	Really Interesting New Gene
TRAF	TNF receptor-associated factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Trk	Tyrosine-kinase receptor
CRD	Cysteine rich repeat domain
ICD	Intracellular domain
BDNF	Brain-derived neurotropic factor
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
DD	Death domain
MAGE	Melanoma associated antigen
APP	Amyloid precursor protein
APPL	Amyloid precursor protein like
APLP1 or 2	Amyloid precursor like protein 1 or 2
Aβ	Amyloid beta peptide
NMJ	Neuromuscular junction
PCD	Programmed cell death
BMP	Bone morphogenic protein
K^{48}	Lysine48 polyubiquitin chains
K^{63}	Lysine63 polyubiquitin chains
TLR	Toll-like receptor
IL-1	Interleukin-1
IKK	IκB kinase
UPS	Ubiquitin–Proteasome System
BIR	Baculovirus IAP repeat
MAPK	Mitogen-activated kinases
PAR-1	Partitioning defective-1
MARK	Microtubule affinity-regulating kinase

aβ42(2X)	Two tandem copies of human amyloid-*β(1-42*)
eiger^{myc}	Epitope tagged weaker isoform of Eiger
AD	Alzheimer's disease
NFT	Neurofibrillary tangles
JNK	c-Jun N-terminal kinase
BioID	Proximity-dependent biotinylation
NF-κB	Nuclear factor – kappa B

CONTRIBUTION OF AUTHORS

CHAPTER 2

I generated all the data presented in this chapter. The technique for imaging of the Drosophila adult eyes was developed by Dr.Vincent Soubannier. Susan Linn and Sarah Tse assisted me in the photography of the images taken. I wrote the first draft of the manuscript, which was corrected and edited by Dr.Philip Barker.

Contributions to figures :

Figures 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6 - Ambika Srinivasan

CHAPTER 3

I performed all the experiments presented in this chapter, except the Mass spectrometry results (Figure 3.2b). I obtained the Ubiquitin-BirA transgenes from Dr.Ugo Mayor at CICbioGUNE, Spain. Dr.Wenjing Ruan assisted me in the dissection of the eye-brain complexes that was used for Mass spectrometry. The Mass spectrometry and preliminary data analysis was performed by Tharan Srikumar in Dr.Brian Raught's laboratory. The technique for imaging of the Drosophila adult eyes was developed by Dr.Vincent Soubannier and Sarah Tse assisted me in the photography of the images taken. I wrote the first draft of the manuscript, which was corrected and edited by Dr.Philip Barker.

Contributions to figures :

Figures 3.1, 3.2a, 3.3 - Ambika Srinivasan

Figure 3.2b - Tharan Srikumar

CHAPTER 4

I generated all the data presented in this chapter. The technique for imaging of the Drosophila adult eyes was developed by Dr.Vincent Soubannier and Sarah Tse assisted me in the photography of the images taken. Dr.Wenjing Ruan generated the UAS-*eiger*^{myc} transgenic fly. The identification of Sidekick as an Eiger-signaling receptor, was by Dr.Wenjing Ruan and Susan Linn. I wrote the first draft of the manuscript, which was corrected and edited by Dr.Philip Barker.

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Figures 4.1,4.2,4.3,4.4,4.5,4.6,4.7,4.8 - Ambika Srinivasan

CHAPTER 1 – LITERATURE REVIEW

1. p75NTR and apoptosis

1.1. p75NTR, a member of the TNFR superfamily

During development of the peripheral and central nervous system, neurons are generated in excess. However, upon target tissue innervation, majority of the neurons die, but a subset of them manage to survive. A diffusible factor secreted by the innervated tissue, ensures the suppression of cell death and promotes neuronal survival (Hamburger and Levi-Montalcini, 1949). This discovery led to the formulation of the 'neurotrophic hypothesis' – the neurons that receive trophic factor support from the innervated target tissue survive, and those that fail to receive the 'survival signal', die (Oppenheim, 1991; Purves et al., 1988). This diffusible factor was later identified and characterized as the nerve growth factor (NGF) (Cohen et al., 1954; Levi-Montalcini and Cohen, 1956). Soon after the identification of this trophic factor, the search for the trophic receptor(s) began. A series of pioneering discoveries led to the isolation of two structurally distinct receptors that had the ability to interact with NGF – Tyrosine-kinase receptors (Trks) and p75 neurotrophin receptor (p75NTR) and eventually other ligands of the neurotrophin family [Brain-derived neurotrophic factor(BDNF), Neurotrophin-3 and 4 (NT-3 and NT-4)] were discovered, reviewed in (Kaplan and Miller, 2000).

The p75NTR was the first cloned member of the Tumor necrosis factor receptor (TNFR) superfamily (Chao et al., 1986; Radeke et al., 1987), but has several distinct features that separate it from the rest of the family. The characteristic structural features of TNFR superfamily members, include extracellular cysteine-rich repeat domains (CRD) and an intracellular death domain (DD). The intracellular death domain (ICD) of p75NTR is unique as it does not self-associate or interacts with death-domain containing proteins such as Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD). Further, the death domain per se, of p75NTR has not been attributed to its apoptotic functionality, unlike those of other TNFR members - such as Fas (Baker and Reddy, 1998; Kong et al., 1999; Liepinsh et al., 1997). The TNFR defining feature of p75NTR lies in the four cysteine-rich domains (CRDs) present in the extra-cellular region of the receptor, with the third CRD involved in neurotrophin interactions (Baldwin and Shooter, 1995;

Shamovsky et al., 1999; Yan and Chao, 1991). Structural and biochemical analysis have revealed that p75NTR interact with NTs in a 2:2 ratio, another unique feature of this receptor that separates it from its trimeric TNFR family members (Feng et al., 2010; Gong et al., 2008; Grob et al., 1985). The $p75^{NTR}$ dimers are held in position through disulphide linkages via a highly conserved cysteine residue (C257) present in the trans-membrane domain (Vilar et al., 2009). This event allows the ICD of the receptor to separate and engage in a number of different signaling events, particularly - apoptosis. The $p75^{NTR}$-ICD consists of the juxtamembrane domain, a Type II death domain and a C-terminal PDZ binding domain. Since the $p75^{NTR}$-ICD is catalytically inactive to participate in signaling by itself, its signaling abilities are contributed by its many interactors, reviewed in (Roux and Barker, 2002).

1.1.1 Mechanisms of p75NTR-induced cell death

NGF exerts its cellular effects by interacting with p75NTR and/or Trk family of receptors (TrkA, TrkB and TrkC). The requirement of NGF to signal through two receptors was justified by several studies that showed a pro-survival role for Trk receptor signaling and a pro-apoptotic role for p75NTR. However, in contrast to this simplistic view of neurotrophin signal transduction, a much more complex interplay between NGF activated p75NTR and Trk signaling networks began to emerge. Signaling pathways downstream of both p75NTR and Trks were found to either augment or inhibit one another. The ability of p75NTR to participate in either cell death or cell survival was influenced by the expression levels and type of Trk receptors (TrkA, TrkB or TrkC), availability of specific NT ligands (NGF, BDNF, NT-3 or NT-4), stage of neuronal developmental and the cellular type (Barrett and Bartlett, 1994; Kaplan and Miller, 2000; Yoon et al., 1998). Hence, p75NTR exerts a pro-survival signaling effect in the presence of Trk receptors, and activates apoptotic signaling, in the absence of Trk receptor activity. For example, p75NTR signals NGF-dependent apoptosis in cells lacking TrkA (oligodendrocytes) or BDNF-dependent apoptosis in cells lacking TrkB (sympathetic neurons), (Bamji et al., 1998; Casaccia-Bonnefil et al., 1996; Yoon et al., 1998). But recent evidence points to additional autonomous mechanisms of cell death activation through p75NTR, that is independent of Trk receptor inhibition. Cell death activation through p75NTR has

also been known to occur in TrkA expressing cells, through non-preferred TrkA ligands, such as BDNF or proNGF (Bamji et al., 1998; Lee et al., 2001a).

The expression levels of p75NTR peak during embryonic development and gradually decline in the adult nervous system. The pro-apoptotic function of p75NTR is further highlighted by injury-induced up-regulation of the receptor, which otherwise has restricted expression and function in the adult CNS. The ability of p75NTR to induce cell death has now been studied in a wide variety of neuronal and non-neuronal cells, in the context of both developmental and injury-induced apoptosis. Some of the cell types that undergo p75NTR –dependent apoptosis include motor neurons, hippocampal neurons, sympathetic neurons, retinal ganglion cells, Schwann cells, oligodendrocytes, sensory neurons, cholinergic neurons, cortical neurons and several cell lines, reviewed in (Roux and Barker, 2002).

Mammalian cells have the cellular machinery to undergo cell death through two distinct cellular modes, the intrinsic and the extrinsic pathway. The extrinsic mode of cell death is activated by TNF ligand interaction with death domain containing TNFRs. This engages the intracellular domain of the receptors with DD-containing proteins such as FADD or TRADD. FADD association promotes the recruitment of pro-caspase 8, which undergoes cleavage and activates downstream effector caspases such as caspase 3, 6 and 7. Effector caspases ultimately cause fragmentation of various cellular substrates, including DNA and mark the final stage of cell death. The intrinsic mode of cell death is activated in response to cellular perturbations, which lead to mitochondrial dysfunction. Cellular stress such as DNA damage or growth factors withdrawal can cause the mitochondria to function as sink holes for the accumulation of pro-apoptotic 'BH3 domain only' proteins, Bak and Bax. The oligomerization of Bak causes the formation of a pore in the mitochondria that releases cytochrome c and DIABLO/Smac (inhibitors of IAP family of proteins). Cytochrome c release promotes the organization of an active apoptosome that includes APAF-1 and Caspase 9, which in turn promotes activation of effector caspases, to complete the final stage of the cell death program, reviewed in (Dempsey et al., 2003).

One of the major signaling networks that participate in p75NTR-dependent apoptotic signaling is the c-Jun N-terminal kinase (JNK) pathway. NGF-withdrawal results in JNK activation, which is imperative for Caspase 9 activation, culminating in cell death (Aloyz et al., 1998; Bhakar et al., 2003; Deshmukh and Johnson, 1998;

Deshmukh et al., 1996; Gu et al., 1999; Martinou et al., 1999; Putcha et al., 1999). Hence, unlike the extrinsic cell death cascade activated by TNFR family members, p75NTR activates cell death through the intrinsic cell death mode (Figure 1.1). A major link that connects JNK signaling to the intrinsic neuronal cell death program is transcriptional and translational control of 'BH3 domain only' proteins (Bhakar et al., 2003; Donovan et al., 2002; Harris and Johnson, 2001; Lei and Davis, 2003; Putcha et al., 2003; Whitfield et al., 2001). The identification of a growing number of cellular contexts that undergo p75NTR dependent apoptosis, led the search for interactors of the p75-ICD, (Roux and Barker, 2002).Though the basic signaling players of p75NTR-dependent cell death are being identified, the mechanism of cell death mechanisms that engage them remain elusive.

In the next two sections I will focus attention over two crucial p75NTR interactions, that pose interesting mechanisms of JNK-dependent apoptotic signaling that will be relevant for chapters 2 and 4 – MAGE proteins and the amyloid precursor protein.

1.1.2 Role of some relevant, pro-apoptotic p75NTR interactors

1.1.2.1 NRAGE, a JNK mediator of p75NTR

The melanoma associated antigen **(**MAGE) family of proteins were initially identified as cancer-testis antigens from tumor cells and eventually also discovered as essential proteins for growth and development in the normal adult tissues. Transcripts derived from the MAGE proteins were discovered as antigens presented to the major histocompatibility antigen complex, in melanoma cells which defined the name of the family (van der Bruggen et al., 1991). The MAGE family now encodes over 60 members classified into two major groups, the Type I and the Type II, based on their chromosomal locations and gene expression patterns (Barker and Salehi, 2002). The type I group of MAGE proteins contain three sub-families, MAGE-A, MAGE-B and MAGE-C, that encode single exon coding MAGE genes. They were identified as gene clusters located on the X-chromosome, with specific expression patters in the male germ cells, placenta and serve as antigens for T-lymphocytes in tumor cells (Chomez et al., 2001). Due to their tumor specific expression, this class of MAGE proteins have served as excellent targets for cancer immunotherapy (Meek and Marcar, 2012).

The Type II group of MAGE proteins includes MAGE-D1- class of proteins that show ubiquitous expression in several cell types. These members are encoded by multiple exons with more diverse chromosomal location in the genome. Unlike the Type I family, the Type II members have more diverse roles in cellular physiology, from controlling cell cycle progression, cell survival signaling to apoptotic signaling. The defining feature of MAGE proteins is the presence of a highly conserved MAGE homology domain (MHD), with little homology between the family members outside of the domain.

MAGE proteins are very well conserved during evolution and have been identified in Zebra fish, Drosophila and Arabidopsis, but are absent in *C.elegans* and yeast. These ancient MAGE representatives were found to be phyllogenetically similar to the Type II sub-family of MAGE proteins, supporting the conserved origin of these proteins (Barker and Salehi, 2002; Chomez et al., 2001).

A MAGE-D family member, MAGE-D1/NRAGE was identified as an interactor of p75NTR from a yeast two hybrid screen. The expression pattern of NRAGE was deduced by *in situ* hybridization in E14 rats. Co-localization of NRAGE and p75NTR expression was observed in the mantle zone of the medulla oblongata, where p75NTR dependent developmental apoptosis is known to occur. Their co-expression is also observed in the trigeminal ganglia, dorsal root ganglia and facial motor neurons in the developing nervous system and extends to the basal forebrain and hippocampal neurons in the adult (Salehi et al., 2000).

1.1.2.1.1 NRAGE and p75NTR dependent apoptosis

One of the mechanisms by which NRAGE participates in p75NTR- dependent apoptosis is by the inhibition of TrkA survival signaling, thus ‘freeing’ p75NTR to participate in cell death signaling. This was shown to occur in the presence of NGF, in sympatho-adrenal cells (Salehi et al., 2000). Ectopic expression of NRAGE in PC12 cells promoted potent induction of JNK activation which resulted in transcription-dependent cytochrome c release, caspase 9 activation and cell death (Salehi et al., 2002). The *nrage-/-* mouse had pronounced phenotypes that confirmed the role of NRAGE as a pro-apoptotic adaptor protein on the p75NTR-JNK cell death pathway. Sympathetic neurons lacking NRAGE do not undergo p75NTR-dependent developmental cell death in response to BDNF. Additionally, the *nrage-/-* mice also present defects in hair follicle catagen, similar to the phenotype in *p75ntr*-/- mice.

NRAGE was also shown to be responsible for Motor neuron developmental defects, independent of p75NTR involvement (Bertrand et al., 2008a).

One of the Trk–independent mechanisms of cell death induction opted by p75NTR, is by coupling with the Sortilin receptor. Unlike NGF-dependent apoptosis, p75-NTR and Sortilin engagement is triggered by pro-NTs (Nykjaer et al., 2004). Exogenous application of proNGF in retinal ganglion cells triggered the massive activation of apoptosis, which was found to be dependent on p75NTR, Sortilin and NRAGE (Lebrun-Julien et al., 2010). A pro-apoptotic collaboration between p75NTR and NRAGE was also shown to occur in non-neuronal cells, such as keratinocytes. Apoptosis triggered by BDNF, NT-4, pro-NGF and non-NT ligand - β-amyloid, in the transit-amplifying keratinocytes of the epidermis was induced by p75NTR-NRAGE association (Truzzi et al., 2011). Hence, there is a lot of *in vivo* evidence that suggests a critical role for NRAGE in p75NTR dependent apoptotic signaling. However, the mechanism of how NRAGE and p75NTR modulate JNK-mediated cell death is still unknown.

A recent study aimed at understanding the physiological function of MAGE proteins, identified them as *bona fide* interactors of Really Interesting New Gene (RING) domain containing E3 ubiquitin ligases (Doyle et al., 2010). NRAGE has also been shown to interact with RING domain proteins, such as XIAP (Jordan et al., 2001), Praja (Sasaki et al., 2002; Teuber et al., 2013). Structural and biochemical evidence generated for several MAGE-RING E3 ligases indicate a regulatory role of MAGE proteins in ubiquitination events. Specific interaction of MAGE proteins with RING ubiquitin E3 ligases, were found to enhance the transfer of ubiquitin to relevant substrates. This effect has been shown for MAGE-RING partners – MAGE-G1 and NSE1 and MAGE-C2 and TRIM28 (Doyle et al., 2010). There are several pieces of evidence that support the role of MAGE proteins in ubiquitin-dependent degradation of signaling proteins. NRAGE is shown to be essential for ubiquitin-dependent degradation of anti-apoptotic factor CHE-1 in response to β-amyloid-induced toxicity in cortical neurons (Di Certo et al., 2007). Necdin, a MAGE protein, has also been associated with the proteasomal degradation of HIF-1, during hypoxia (Moon et al., 2005). MAGE-B18 promotes the proteasomal degradation of p53 inhibitor, NUMB in a RING dependent manner (Colaluca et al., 2008; Nie et al., 2002). Hence, there is accumulating evidence indicative of NRAGE-mediated ubiquitination, which could

potentially serve as an important regulatory mechanism for p75NTR-JNK dependent cell death.

1.1.2.2 Amyloid precursor protein, a pro-degenerative p75NTR partner

The association of amyloid precursor protein (APP) with amyloid plaques – a hallmark pathological feature of Alzheimer's disease, propelled the need for extensive research in the field of APP biology (Glenner and Wong, 1984; Kang et al., 1987; Masters et al., 1985). APP, APP-like protein 1 and 2 (APLP1 and APLP2) constitute the mammalian representatives of the amyloid protein family (Sprecher et al., 1993), with conserved counterparts in Drosophila (Amyloid precursor protein-like, APPL) (Martin-Morris and White, 1990) and *C.elegans* (APL-1) (Daigle and Li, 1993). Alzheimer's disease pathology include the accumulation of amyloid plaques, which constitute insoluble amyloid beta peptide (Aβ) fibrils and neurofibrillary tangles made up of hyper-phosphorylated Tau deposits, reviewed in (De Strooper and Annaert, 2000). APP is processed through two different pathways, the non-amyloidogenic and the amyloidogenic pathway. The non-amyloidogenic pathway is responsible for the cleavage of APP by α-secretase followed by γ-secretase. The amyloidogenic pathway cleavage of APP occurs through β-secretase followed by PSI and γ-secretase. Both these modes of cleavage generate a C-terminal intracellular domain (AICD). However, it is the N-terminal fragment produced by both these pathways that determines the cytotoxicity of the APP protein. The non-amyloidogenic pathway generates a small P3 fragment that is not associated with pathology, however the Aβ/$A\beta_{1-42}$ fragment generated by the amyloidogenic pathway is the precursor for amyloid plaques in Alzheimer's disease (Thinakaran and Koo, 2008).

Some aspects of normal, non-pathogenic APP physiology have been deduced from knockout mice. *APP-/-* mice have less neuronal viability, defective neurite outgrowth, gliosis (Perez et al., 1997), minor behavioral and motor locomotary defects (Muller et al., 1994). However the APP-/-, *APLP1-/-* and *APLP2-/-* triple knockout mice show pronounced neurite outgrowth defects in cortical neurons (Herms et al., 2004) and APP-/- APLP2-/- double knock-out mice show synaptic pruning defects in the neuromuscular junction (Wang et al., 2005). APP has also been implicated as a receptor for kinesin-1, to mediate axonal transport (Kamal et

al., 2000). These studies constitute just the tip of the iceberg to understand APP biology. Several interacting partners for APP have been identified that contribute towards the cellular function of APP, towards neuronal survival, axonal transport, axogenesis and synaptic pruning in the nervous system (De Strooper and Annaert, 2000; Reinhard et al., 2005).

1.1.2.2.1 Amyloidogenic pathway and p75NTR signaling

The pathogenic Aβ peptide and the full length APP have been shown to interact with several cell surface receptors, one of which is p75NTR. p75NTR-dependent cell death has been linked to Alzheimer's pathology due to the expression levels and strong association of this pro-apoptotic receptor with the Aβ peptide, in the adult nervous system. The most vulnerable region to be inflicted by Aβ pathology is the basal forebrain, where cholinergic neurons express p75NTR (Gimenez-Llort et al., 2007; Schliebs, 2005; Whitehouse et al., 1981; Woolf et al., 1989; Yan and Feng, 2004). Further p75NTR extracellular domain has been shown to have direct interaction with the Aβ peptide and enhance Aβ-induced toxicity (Rabizadeh et al., 1994; Yaar et al., 1997). The up-regulation of proNGF and Sortilin expression by Aβ also favors a proNGF-Sortilin-p75NTR apoptotic mechanism in Alzheimer's disease (Fahnestock et al., 2001; Pedraza et al., 2005; Saadipour et al., 2013). The co-expression and interaction of p75NTR and APP has also been shown to promote amyloidogenic processing of APP that generates the toxic Aβ peptide, leading to cell death (Fombonne et al., 2009). p75NTR expression is up regulated in response to Aβ expression and the reverse is also been shown in a number of studies (Chakravarthy et al., 2010; Ito et al., 2012; Wang et al., 2011).

There is compelling evidence showing direct Aβ-mediated p75NTR cell death mechanisms, *in vitro* and *in vivo* (Costantini et al., 2005; Coulson, 2006; Sotthibundhu et al., 2009). p75NTR has been implicated in Aβ-induced cell death that proceeds through the activation of several signaling pathways, strong evidence showing the involvement of the JNK pathway (Costantini et al., 2005; Tsukamoto et al., 2003; Yaar et al., 2002) and the Nuclear factor – kappa B (NF-κB) cascade (Costantini et al., 2005; Kuner et al., 1998). Studies also show the efficacy of drugs that mimic p75NTR ligands, to revert the neurotoxicity of the Aβ peptide, which could further our understanding of p75NTR involvement in degenerative events (Knowles

et al., 2013; Massa et al., 2006; Yang et al., 2008). Hence, the collaborative pro-apoptotic effect of Aβ-p75NTR appears to be due to multifactorial effects, where p75NTR promotes Aβ toxicity both by inhibition of its pro-survival trophic mechanisms and by the autonomous activation of its pro-apoptotic networks.

1.2 Origin and evolution of the TNFR superfamily

1.2.1 The invertebrate p75NTR homologs, an evolutionary perspective

Phylogenetic analysis provides evidence that gene duplication events coincided with the emergence of the adaptive immune system (in Gnathostomata), and this contributed to the diversity of the vertebrate TNFR superfamily as it is today. This explains the extensive involvement of several vertebrate TNFR super family members in immune signaling pathways (Collette et al., 2003). But what are the origins of the TNFR superfamily members that are less involved in immunity and contribute instead to signaling networks that shape the development of the nervous system? Until recently it was believed that, the TNFR superfamily member, p75NTR was a more recent acquisition of vertebrate evolution. This has been challenged by the accumulation of genetic information of several ancient organisms by whole genome sequencing initiatives, which have led to the identification of p75NTR homologs in several invertebrates. NTs and not surprisingly, their cognate receptors appear to have had early origins in all organisms with a centralized nervous system, which is believed to have occurred with the emergence of Bilateria (Zhu et al., 2008). However, the most ancient member of the TNFR superfamily did not originate in deuterostomes, but even before the protosome-deutersome split. TNFR members such as Troy share sequence similarity with TNFR members EDAR and EDAR2R, all of which are implicated in the development of ectodermal cells, a functional feature shared by p75NTR. Hence sequence and functional similarity suggests p75NTR, EDAR, EDAR2R and Troy represent ancient TNFR receptors (Bothwell, 2006).

Evidence of the existence of ancient p75NTR homologs in metazoans – (Additionally represented in a phylogenetic tree in Figure 1.2)

1. Radiata (Cnidaria) - Two TNFRs homologs were identified in sea anemone (*Nematostella vectensis*), one of which was found to share sequence similarity with the CRD and DD sequences of vertebrate p75NTR. This

represents the only known representative of an ancient p75NTR homologue before the emergence of Bilateria (Robertson et al., 2006).

2. Bilateria; Protostomes
    a. *Lophotrochozoa* – The genome sequencing of the mollusk, *Lottia gigantean* and Zhikong scallop, *Chlamys farreri* led to the identification of p75NTR homologs with conserved CRDs (Wilson, 2009). The sea scallop TNFR was also found to contain the DD sequence of p75NTR homologs identified in sea urchin and sea squirt (Li et al., 2009). The annelidian ancestor of p75NTR was identified in the genome of a polychaete worm (*Capitella sp.I*), with significant homology to the vertebrate p75NTR CRDs and trans-membrane region (Wilson, 2009).
    b. *Ecdysozoa* – There have been no identification of p75NTR representatives in nematodes, such as round worm *(Caenorhabditis elegans).* The fruit fly (*Drosophila melanogaster*) genome has been shown to carry a single p75NTR homolog, Wengen. The amino acid sequence similarity of Wengen however, does not extend beyond the CRD of mammalian p75NTR and EDAR (Kanda et al., 2002). But, recently a death domain like sequence for Wengen was defined, the functional significance of which still needs experimental validation (Keller et al., 2011). An additional p75NTR representative in Arthropoda was identified in a crustacean, *Daphnia pulex* that was found to possess a high degree of sequence similarity with the CRD, trans-membrane and DD of vertebrate p75NTR. Several ESTs in Daphnia have been identified to be highly similar to the fruit-fly TNF and TNFR, suggesting a close conservation of TNF-TNFR networks in Arthropoda (Wilson, 2009).
3. Bilateria; Deuterostomes
    a. Echinodermata – Seven ancestral deuterostomian TNFRs were identified in sea urchin (*Strongylocentrotus purpuratus*) by sequence homology, four of which were found to contain CRD and/or DD sequences resembling p75NTR, EDAR and Troy receptors (Bothwell, 2006; Robertson et al., 2006).

b. Hemichordata - A single p75NTR homolog was also identified in the acorn worm (*Saccoglossus kowalevskii)* from EST sequences, that resemble p75NTR CRD, TM and the DD sequences (Bothwell, 2006).
c. Cephalochordata - Two TNFR receptors with conserved CRDs were identified in lancelet (*Branchiostoma belcheri*) that bear resemblance to the CRDs of mammalian EDAR2, LT-βR, CD40, p75NTR, and have been associated with immune related functions (Yuan et al., 2007).
d. Urochordata – A BLAST search revealed two TNFRs in two species of sea squirt (*Ciona intestinalis and Ciona savignii*), one of which resembled Troy and EDAR2 (Bothwell, 2006; Robertson et al., 2006)

Hence, the phylogenetic comparisons imply the emergence of death domain containing TNFRs before bilateria (in sea anemone), which expanded during the course of deuterostomian evolution. Sequence similarity comparisons also suggest that the ancient TNFRs did not have a functional role in the extrinsic apoptotic signaling pathways as none of the ancient receptors bear homology to Fas, DR4/5 or TNFR1 (the classic death receptors). Though p75NTR has a death domain, it is known to participate in cell death through the intrinsic cell death (JNK-caspase 9) pathway unlike the extrinsic apoptotic network (FADD- caspase 8) activated by the classic death receptors of the mammalian TNFR superfamily. Further, it appears that the classic death domain functionality of the mammalian TNFRs may represent a 'recent' acquisition from invertebrate Toll receptors (Moreno et al., 2002), while the ancestral 'JNK-only' signaling function of p75NTR homologs were retained from Cnidaria to vertebrata.

Pioneering work in field of *C.elegans* biology by Sydney Brenner, John E. Sulston, and H. Robert Horvitz lay the foundation for understanding conserved mechanisms of apoptotic signaling in invertebrates. *ced-3, ced-4, ced-9* and *egl-1* are the four major genes that form the 'intrinsic-mode of apoptosis' toolkit in the round worm. Egl-1 and Ced-9 are members of the Bcl-2 family, Ced-4 is the nematode counterpart of the Apaf-1 protein and Ced-3 is an effector caspase (Caspase 3, 6 or 7) homolog in worms (Horvitz, 1999). Though there are no TNFR receptors in C.elegans, the strong presence of p75NTR in other invertebrates, coupled with its role in intrinsic cell death mechanisms in vertebrates validates the ancient origins of this receptor. The emergence of p75NTR homologs in primitive invertebrates is solely based on sequence similarity, with no experimental evidence

proving the conservation of function in these receptors. There appears to be more variability and diversity in the ligands and receptors of TNF signaling networks, but less variability in comparison, among its cytoplasmic signaling components (Robertson et al., 2006). Drosophila has a well-conserved network of proteins that exclusively signal cell death through the ancient 'JNK-only' cascade (Igaki et al., 2002; Moreno et al., 2002). Additionally, the presence of a single p75NTR homolog in the fruit fly, Wengen makes it an ideal genetic model system to decipher conserved signaling components of its apoptotic network.

1.3 A fly's eye- view of apoptosis

1.3.1 Eiger, the invertebrate TNF ligand

The first invertebrate TNF ligand, Eiger was identified in fruit flies. Two independent groups discovered the fly TNF in a mis-expression screen (using the GAL4/UAS system) aimed at uncovering cell death-inducing genes, based on their ability to induce massive apoptosis in the compound eye of *Drosophila melanogaster* (Igaki et al., 2002; Moreno et al., 2002). The screen was conducted using P-element based vectors (GS), containing UAS enhancer sequences. A set 5000 GS lines were crossed to an eye-specific promoter, Glass multimer reporter (GMR) that carried the GAL4 transcription factor (GMR-GAL4). Thus, the GAL4 dependent expression of the UAS enhancer triggered the over-expression of all the genes from the 5000 lines that carried the P-element insertion in the genome. Six GS lines generated a reduction in eye-size and were called the Regg strains (for reduced eye generator with GMR). The sequence comparison of this newly identified gene from the Regg strains was found to contain a TNF-homology domain of the mammalian TNF family of ligands, with maximum homology to EDA-A2 ligand (28%). Eiger, which stands for EDA-like cell death trigger is a Type II trans membrane protein with a short N-terminal cytoplasmic region and an extracellular C-terminal TNF homology domain. One of two isoforms of Eiger, called Eiger-s is made up of 409 amino acids with Eiger-L constituting the longer isoform, has an extension of six amino acids (GESLLS) outside TNF homology domain. RT-PCR analysis revealed comparable levels of expression of both isoforms, with Eiger-L predominating by just 5-10 fold in all the stages of development.

Like other members of the mammalian TNF superfamily, Eiger was also found to undergo cleavage (between amino acids 145 and 146) and released the secreted form of the TNF ligand, when expressed in S2 cells (Kauppila et al., 2003; Moreno et al., 2002). Though the enzyme responsible for its cleavage is not known, it was found to harbor a canonical furin cleavage site, that generated the secreted form of the protein in HEK293T cells (Narasimamurthy et al., 2009). Sequence analysis and biochemical tests revealed the presence of three N-glycosylation sites at amino acids 226, 339, and 406 (Kauppila et al., 2003). *In situ* hybridization studies revealed the expression of Eiger in all stages of development, with predominant expression in the nervous system (Shklover et al., 2015) that included the brain and imaginal disc complex. Neuronal expression was specifically observed in the terminally differentiated neurons and proliferating cells at the morphogenic furrow of the imaginal discs (Igaki et al., 2002). Injury induced expression of Eiger has been reported in the larval epidermis (Babcock et al., 2009), peripheral glial cells at the neuromuscular junction (NMJ) (Keller et al., 2011), hemocytes (Parisi et al., 2014) and the fat body (Mabery and Schneider, 2010) of the innate immune system. Mutagenesis studies discovered the requirement of both the TNF homology domain and the membrane-proximal stalk region (amino acids 60-145) for the cell death inducing potential of Eiger (Narasimamurthy et al., 2009).

1.3.2 Wengen, the invertebrate TNFR homolog

Soon after the discovery of the fly TNF ligand, the fly TNFR - Wengen was identified. Wengen was discovered in a dominant modifier screen, which was performed by genetic crosses between deficiency lines of over 70% of all the genes in the fly genome, and the GMR-GAL4>UAS-*eiger* transgene. One of Eiger cell death suppressors identified, was the deficiency line *Df(1)E128/+.* One of the genes that was lost in this deficiency line was found to contain the CRD, characteristic of the mammalian TNFR superfamily members (Kanda et al., 2002). Wengen, named after a village on the foothills of Mt.Eiger, is made up of 343 amino acids that constitute the N-terminal extracellular domain, trans-membrane stalk and C-terminal cytoplasmic domain. Wengen was found to contain a single CRD in the N-terminus with maximum homology to the corresponding domains in mammalian EDA2R and p75NTR (Bothwell, 2006; Kanda et al., 2002). However, Wengen carries a unique

cytoplasmic domain not observed in any TNFR superfamily members (Kanda et al., 2002). A putative death domain-like sequence in the intracellular region of Wengen was proposed, based on homology to death domain interacting proteins Myd88 and RIP2, but functional significance supporting this claim remain inconclusive (Keller et al., 2011).

The initial discovery of Wengen classified it as a Type III receptor with no identifiable signal peptide sequence, but was re-designated a Type I receptor with a putative signal peptide sequence in the N-terminus of the protein (Kauppila et al., 2003). Further, Wengen TNFR homology domain could physically interact with the TNF homology domain of Eiger, as shown by co-immunoprecipitation experiments (Kanda et al., 2002). RT-PCR and *in situ* hybridization studies revealed expression patterns of Wengen to span all developmental stages, with predominant expression of the protein in the nervous system, that includes the larval brain-imaginal disc complex, photoreceptor axons in the adult eye (Ruan et al., 2013) and Motor neurons of the NMJ (Keller et al., 2011).

1.3.3 Mechanisms of Eiger-induced cell death signaling

The GAL4-UAS system of gene expression is being widely used as a versatile tool for targeted gene expression. The GAL4 transcription factor was first identified in yeast as a regulator of Galactose inducible genes, with specific binding sites in DNA, known as the upstream activation sequence (UAS) (Giniger et al., 1985; Laughon et al., 1984). GAL4 was thus found to be capable of transcribing genes placed under UAS control in Drosophila. A seminal paper published by Brand and Perrimon pioneered the usage of the GAL4-UAS system in Drosophila genetics (Brand and Perrimon, 1993). Tissue specific expression of the GAL4-UAS system can be regulated, by the incorporation of specific promoters for the GAL4 gene. The GMR promoter is routinely utilized for transgene expression in all the post-mitotic cells posterior to the morphogenic furrow, of Drosophila third-instar larva eye-imaginal disc (Ellis et al., 1993; Freeman, 1996). The GMR-GAL4>UAS system of targeted gene expression was used (as described in sections 1.3.1 and 1.3.2) to identify the leading components of the Fly TNF-TNFR ligand-receptor system. The ectopic of Eiger in the eye-imaginal disc, induced massive activation of cell death, leading to loss of majority of the photoreceptor cells (Igaki et al., 2002). This phenotype was

defined as the 'small-eye' phenotype of Eiger over-expression. Some of the key signaling components uncovered using modifier screens using GMR-GAL4-UAS>*eiger* are described below, such as the identification of -

Kinases – Ectopic Eiger triggered massive expression of puckered, a dual-specific phosphatase whose expression is induced by JNK activation. Puckered was known to act as a negative regulator of JNK signaling via de-phosphorylation of the fly JNK, Basket (Martin-Blanco et al., 1998). Genetic epistasis experiments led to the identification of several signaling components of the JNK apoptotic signaling pathway, such as the Drosophila homologs of MAP4K- Misshapen, MAP3K- dTAK1, MAP2K- Hemipterus, MAPK- Basket/JNK, downstream of Eiger (Igaki et al., 2002; Moreno et al., 2002).

Adaptor proteins - The connectivity of the MAPK cascade to Eiger signaling was strengthened by the discovery of adaptor proteins such as the TNF receptor-associated factors (TRAFs), that have been known to play important roles in bridging the gap between ligand-receptor and downstream signaling components in mammalian signaling networks (Dempsey et al., 2003). Two TRAF homologs dTRAF1 and dTRAF2 have been linked to the Eiger-JNK pathway (Geuking et al., 2005; Moreno et al., 2002; Xue et al., 2007). The Chapter 2 of this thesis defines the indispensible and non-redundant role for dTRAF2 in the Eiger signaling pathway. Subsequently, the fly homolog of the TAK1 interactor, dTAB2 was also identified as a regulator of the Eiger 'small-eye' phenotype (Geuking et al., 2005).

Ubiquitin regulators - Several regulatory components of the ubiquitin system were also identified as key players of the Eiger cell death-inducing network. dCYLD, a deubiquitinating enzyme was identified as a regulator of the JNK pathway via the modulation of dTRAF2 expression levels (Xue et al., 2007). Regulation of a signaling pathway through ubiquitin modifications requires three major components, an ubiquitin activator – E1, an ubiquitin conjugating enzyme – E2, and an ubiquitin ligase- E3. Chapter 2 of this thesis addresses the importance of dTRAF2 as an a potential RING-E3 on this pathway and Chapter 3 of this thesis validates and addresses the importance of several E2s that have now been added to the regulatory network of the Eiger signaling pathway. Several additional RING domain E3 proteins have also been identified on the Eiger signaling pathway such as NOPO (Ma et al., 2012) and POSH (Zhang et al., 2010), but the mechanism of how these ubiquitin regulators function requires further study and validation.

Caspases and caspase regulators - Eiger-induced cell death was unaltered by the loss of the caspase 8 homolog, Dredd. However, Eiger 'small-eye' phenotype was reverted by the expression of a pan-caspase inhibitor, p53. An earlier study utilized a dominant negative form of caspase 9, Dronc and observed a strong reversal of the 'small-eye' Eiger phenotype, which placed caspase 9 on the Eiger signaling pathway (Moreno et al., 2002). A more recent study generated and utilized a null allele of Dronc to show a dispensable role for caspase 9 on the fly JNK signaling network (Kanda et al., 2011). However, the fly homolog of mammalian caspase 9 activator, Apaf-1 (called dARK in the fly) has been shown to operate on the Eiger pathway (Moreno et al., 2002). Additionally, the ectopic-expression of the anti-apoptotic, caspase inhibitor – dIAP1 (IAP stands for Inhibitor of apoptosis) suppressed Eiger-induced cell death (Moreno et al., 2002). dIAP1 has been shown to inhibit apoptosis by the inhibition of DRONC (Wilson et al., 2002), in model of programmed cell death, activated by the ectopic expression of IAP inhibitors - Hid, Grim and Reaper (Bergmann et al., 1998; Yoo et al., 2002). Though there has been no known role for Eiger in programmed cell death (PCD) (Shklover et al., 2015), *in situ* hybridization studies revealed a massive up-regulation of *hid* mRNA levels, in response to the ectopic expression of Eiger (Moreno et al., 2002).

Oxidative stress modulators - A deficiency screen discovered the involvement of energy production enzymes as suppressors of the 'small-eye' phenotype of Eiger over-expression. Eiger was found to generate cytotoxic reactive oxygen species (ROS) downstream of JNK, via mitochondrial associated metabolic enzymes, highlighting a novel oxidative stress-driven mechanism of apoptosis (Kanda et al., 2011).

Hence, Eiger-induced apoptosis is executed via the conserved JNK signaling pathway, similar to mammalian p75NTR (described in section 1.1.1), where as the extrinsic cell death components that include the FADD-caspase 8 network, are dedicated to innate immunity pathways via the Toll receptor in Drosophila (Hu and Yang, 2000; Leulier et al., 2000). Eiger participates in cell death through a number of interesting partners, with multiple modes of apoptosis induction. Hence, it is imperative to understand the regulatory mechanisms of proteins identified on the Eiger pathway, to better appreciate the conserved physiological functions of this primordial TNF-TNFR signaling network.

1.3.4 Physiological functions of Eiger-dependent signaling

The architecture of the Eiger apoptotic-signaling pathway has been largely deduced through screens relying on its ectopic expression in the eye or wing imaginal discs. The role of endogenous Eiger and its pro-death signaling components has been elusive until recently. Genetic studies have yet again proved powerful in determining the physiological role of Eiger *in vivo*. Some of the recently characterized functions of the Eiger signaling network are listed below –

Eiger as an intrinsic tumor suppressor - In 1967 a gene named *lgl (lethal giant larvae)* was identified which when mutated had the ability to generate unregulated invasive tumors that eventually killed the fly larva. This was the first *in vivo* example of a tumor suppressor gene in any metazoan (Gateff, 1978), which was followed by the discovery of two additional genes with similar tumor suppressive properties, *scrib (scribbled)* (Bilder et al., 2000) and *dlg (disc large*) (Stewart et al., 1972). The fruit fly has a modest lifespan of around 30 days, which is a very short span of time to accumulate mutations to promote cancer progression. Though flies can develop cancers, they rarely do so in the wild, hence cancer-causing mutations have been discovered largely through experimental interventions via genetic screens. The genes *lgl, dlg or scrib* do not directly affect cell growth as would be expected for genes involved in cancer, but rather its polarity. The loss of polarity (by the mutation of one of three genes) in normal cells contributed to the formation of disorganized clumps of tumors, reminiscent of many features of human cancerous cells (Hanahan and Weinberg, 2000).

However, normal epithelial tissues possess a protective mechanism to prevent neoplastic growth, when polarity genes are mutated in a selective population of cells. Thus epithelial cells that are deficient in cell polarity (*lgl, scrib or dlg* mutations) are cleared by neighboring wild type cells. The signaling mechanism responsible for this clearance was discovered to be an intrinsic property of the Eiger-Wengen-JNK signaling network. The mechanism for the elimination of neoplastic clones required Eiger-JNK signaling both in the surrounding wild-type cells, for the activation of a PVR-ELMO/Mbc-mediated engulfment pathway (Ohsawa et al., 2011), and in the polarity deficient cells for Rab5 dependent endocytic activation of cell death (Igaki et al., 2009). An alternate mechanism was also proposed that involved a systemic immune response that involved Eiger-JNK signaling, for the

activation of the PVR-proliferation pathway in the polarity deficient cells. PVR signaling was shown to induce the proliferation of hemocytes, which led to the activation of Toll receptor signaling in the larval fat body. The non-cell autonomous generation of Eiger by both the hemocytes and the fat body caused the final elimination of the polarity deficient clones (Parisi et al., 2014). These studies established the role for Eiger-JNK cell death signaling in cell-competition for epithelial structure maintenance.

Eiger as a tumor promoter, a paradoxical role - One of the consequences of assuming the state of a mis-polarized cell, is the susceptibility for oncogenic transformation. As described in the section above, wild type cells have an inherent sense of 'duty' to eliminate such neoplastic transformants. In rare situations, these polarity deficient clones acquire additional mutations (mutations that cause them to be constitutively active) in genes such as *ras* or *notch*, which renders them resistant to elimination by their neighbors (Brumby and Richardson, 2003). It was soon discovered that the combination of cell polarity loss (*scrib/lgl/dlg*$^{-/-}$) and the constitutive activation of *ras* (*ras*^{V12}) promoted tumor growth and metastasis by the active participation of the Eiger-Wengen-JNK signaling network (Cordero et al., 2010; Igaki et al., 2006; Ma et al., 2014; Ma et al., 2013a; Ma et al., 2013b). Elegant studies by the usage of genetic mosaics revealed the key that turned Eiger from a tumor suppressor to a tumor promoter, was the deactivation of Hippo signaling cascade. Mitochondrial derived ROS and *ras*^{V12} in the mutant cells were found to collaborate to induce neoplastic growth in the wild type neighboring cells, by activation of the JNK pathway. Additionally, the source of Eiger for JNK activation was found to be tumor associated hemocytes (Cordero et al., 2010). The tumor promoting JNK signaling effects was executed by the inactivation of the Hippo tumor suppressor signaling pathway, whose deregulation caused the over-proliferation of wild type cells via up regulation of genes, *unpaired* (*interleukin-6* homologue) and *wingless* (a *wnt* homologue) (Ohsawa et al., 2012). The tumor–promoting role of Eiger-JNK signaling, has been shown to be mediated by the flyTNFR, Wengen (Igaki et al., 2006). However, an additional TNFR receptor was uncovered very recently, named Grindelwald that has been shown to participate in the *ras*^{V12}*scrib/lgl/dlg*$^{-/-}$ tumor-inducing effect through direct interaction with Eiger. The importance of this

new TNFR member in Eiger-signaling events needs to be further clarified. Chapter 4 of this thesis reflects upon the potential for Eiger to signal through multiple receptors.

Role of Eiger in dorsal patterning - The bone morphogenic protein (BMP) signaling pathway has been known to play an essential role in the dorsal specification of tissues during development (Irish and Gelbart, 1987). The expression of Eiger in the dorsal side of the embryo is regulated by BMP signaling, both at the transcription and translational levels. Eiger-JNK signaling was identified as part of a positive feedback loop for the maintenance of BMP signaling in the dorsal side of the pregastrula embryo of the fruit fly (Gavin-Smyth et al., 2013).

Role of Eiger in innate immune signaling – The pro-apoptotic role of Eiger was deduced from over-expression genetic studies. However the *eiger*^{1} mutants present no dramatic phenotypes. It was only when the *eiger*^{1} mutants were challenged with pathogens, a role in host defense emerged. *eiger*^{1} mutants were shown to be defective in the phagocytic clearance of pathogenic bacteria like *S.aureus* (Schneider et al., 2007) and *V.cholerae* (Berkey et al., 2009), but was found to delay host lethality in response *S.* typhimurium (Brandt et al., 2004). Eiger and its JNK signaling network were also identified as important mediators of melanization, as part of the natural host defense response in the larva (Bidla et al., 2007). As highlighted in the section above, an increase in tumor burden was found to induce a systemic immune response (Toll dependent) from the circulating tumor associated hemocytes and the fat body, which through the production of Eiger, was found to induce tumor-cell death (Parisi et al., 2014). Hence the role of Eiger in immune signaling parallels the inflammatory responses of mammalian TNF ligands.

Eiger in nociceptive sensitization – Eiger and Wengen were identified to participate in a non–cell autonomous mechanism of UV-induced nociceptive sensitization. Eiger, produced from UV-injured epidermal cells induced thermal sensitization by binding Wengen in dendritic sensory neurons (Babcock et al., 2009). The signaling pathway downstream of Wengen has not been elucidated but hints to a potential collaboration between Wengen and the TRPV1 channel homolog, Painless. TRPV1 and Painless have been associated in mammals and Drosophila

respectively, with nociceptive responses to noxious stimuli like heat (Caterina et al., 1997; Tracey et al., 2003).

Eiger in injury-induced degeneration – Analysis of the central nervous system in *eiger*^{1} mutants confirmed the lack of involvement of Eiger-JNK signaling in developmental apoptosis, despite high levels of neuronal and glial cell expression (Shklover et al., 2015). However, *eiger* was transcriptionally regulated by p53 in response to UV-induced DNA damage. The cell death inducers involved in PCD – *hid, grim* and *reaper* are also known targets of p53 in response to ionizing radiations (Brodsky et al., 2004). The ectopic expression of Hid, when used to create a damage induced- model of neuronal death, required Eiger expression independent of JNK activation. Additionally, Eiger was also found to be essential for the transcriptional up-regulation of *hid* in response to X-ray - induced neuronal injury in the Drosophila larva (Shklover et al., 2015). A glial derived pro-degenerative model at the NMJ was shown to depend on Eiger and Wengen. Eiger produced by glia in response to neuronal damage, activated JNK-independent synaptic loss via Wengen, expressed in the Motor neurons. This pro-degenerative mechanism was shown to require the initiator caspase homolog - Dronc, effector caspase homolog - Dcp-1, Apaf-1 homolog- dARK and Bcl-2 homolog – Debcl (Keller et al., 2011).

Eiger signaling in glial cell proliferation – Extensive pruning of axons has been known to occur during fruit fly metamorphosis, through the glia-dependent activity of the engulfment receptor, Draper (Awasaki et al., 2006). TNF-α has been known to participate in the glial response to neuronal injury in the mammalian central nervous system (Rolls et al., 2009). Additionally, the ectopic expression of genes involved in PCD such as *hid*, *reaper* and *grim,* induce cell death accompanied by proliferation of surrounding cells through JNK, Dpp and Wnt signaling pathways (Ryoo et al., 2004), a process termed as ‘compensatory proliferation’. During the eclosion of the adult fly, neurons in the ventral nerve cord undergo extensive *reaper* and *grim*-induced PCD in response to the Ecdysone hormone (Robinow et al., 1993).The essential role for an Eiger-dependent, but Wengen–independent signaling mechanism was shown in the adult fruit fly brain during Ecdysone-mediated PCD and in response to injury in antenna neurons. The ability of Eiger to induce glial cell proliferation at the site of neuronal injury was ablated in *eiger*^{1} mutants, which was rescued upon glial specific

expression of Eiger (Kato et al., 2009). The downstream signaling components of this pathway remain unknown. Glial proliferation was also shown to be induced by an alternate mechanism, involving Eiger-Wengen-NF-κB signaling pathway, in response to stab injury in the larval ventral nerve cord (Kato et al., 2011). An over-view of the Eiger-Wengen signaling pathway, with some of its physiological functions is illustrated in Figure 1.3.

1.4 Ubiquitination - an important post-translational modification of cellular proteins.

One of the major and widely studied post-translational modifications of cellular signaling components is the covalent attachment of a 76 amino acid protein, ubiquitin. The attachment of ubiquitin to a substrate requires the sequential activity of three enzymes – An ubiquitin activating enzyme (E1), which activates the Glycine residue of an ubiquitin monomer in an ATP-dependent manner, and transfers it to an ubiquitin-conjugating enzyme (E2). The ubiquitin carrier E2 interacts with a specific ubiquitin ligase enzyme (E3) and can either contribute to the auto-ubiquitination of the E3 itself or a substrate protein, the specificity of which is determined by the E3. The E2-ubiquitin thioester is transferred to the substrate by the formation of an isopeptide bond between the C-terminus of the ubiquitin monomer and the ε-amino group of a specific Lysine residue on the substrate. Ubiquitin transfer can be successively repeated by the addition of more ubiquitin to the previously conjugated ubiquitin, through one of seven Lysine (K6, K11, K27, K29, K33, K48, K63) residues present in ubiquitin. This gives rise to a diverse array of ubiquitin modifications, ranging from mono-ubiquitination, multi-monoubiquitination, homotypic or heterotypic polyubiquitination, that directly influences the fate of the substrate. Recently reviewed in (Komander and Rape, 2012).

The two main poly-ubiquitin linkages that are being extensively studied in the regulation of signaling events are Lysine48 (K^{48}) and Lysine63 (K^{63}) poly-ubiquitin linkages. The other 'atypical' ubiquitin linkages, involving K^{6}, K^{11}, K^{27}, K^{29} and K^{33} have also been recently gaining attention in signaling contexts, such as DNA repair, cell cycle regulation and NF-κB activation (Kulathu and Komander, 2012). K^{48} poly-ubiquitin chains were first identified in proteins associated with the proteasomal complex in yeast (Bachmair and Varshavsky, 1989), and has been attributed since

as a signal for protein degradation. A few years after the discovery of K^{48} linkages, proteins involved in DNA repair mechanisms were found to associate with non-degradative K^{63} linkages (Spence et al., 1995). The involvement of both forms of ubiquitin linkages have been studied extensively over the years, that have shaped our understanding of several signaling cascades, one in particular – TNFR signaling.

1.4.1 Lessons from mammalian TNFR signaling, what do we know about K^{48} and K^{63} ubiquitin-dependent regulation?

The first association between apoptotic signaling and ubiquitin was made in studies focused on programmed cell death in the intersegmental muscles of the Tobacco hawk moth (Schwartz et al., 1990). This highlighted the importance of ubiquitin in apoptosis. However, the induction of cell death as a direct consequence of proteasome inhibition was one of the first clues that suggested a modulatory role for ubiquitin in apoptotic signaling pathways (Drexler, 1997). This modulatory role of ubiquitin was first studied in the context of TNFR-dependent NF-κB signaling. Stimulation of cells with TNF-α leads to the phosphorylation of the NF-κB inhibitor – IκB, in an IKK complex (IκB kinase)-dependent manner. This serves as a signal for the K^{48} polyubiquitin– dependent proteasomal degradation of IκB, by the E2, Ubc4/5 and the SCF ubiquitin E3 complex (Chen et al., 1995; Chen et al., 1996; Yaron et al., 1998). The same study also revealed the requirement of a non-degradative poly-ubiquitination event, for the activation of the IKK complex (Chen et al., 1996), which was later identified as K^{63} poly-ubiquitination. Studies in Toll-like receptor (TLR) signaling and Interleukin-1 (IL-1) signaling led to the identification of TRAF6, the E3 and Ubc13-Uev1a complex, the E2 involved in K^{63} poly-ubiquitination (Deng et al., 2000). The activator of the IKK complex was later discovered to be the TAK1-TAB2/3 complex (Ishitani et al., 2003; Wang et al., 2001a). The key to the activation of the TAK1-TAB2/3 complex itself, was identified as K^{63} auto-ubiquitination via Lys^{124} of TRAF6, in response to IL-1 (Ea et al., 2004; Lamothe et al., 2007). Thus the K^{63} poly-ubiquitin linkage provided the function of a signaling scaffold, for the recruitment and activation of both TAK1 and IKK.

A similar mechanism was discovered for ubiquitin regulation in TNFR signaling. Signaling via TNF-α is initiated by its binding to and trimerization of the TNFR1 receptor. The receptor trimer can interact with either death-domain protein

FADD to signal caspase 8-dependent cell death, or TRADD-RIP1-TRAF2 to signal NF-κB or JNK signaling (Hsu et al., 1996). The activation of TAK1 and IKK was dependent on K^{63}-polyubiquitination at the $Lysine^{377}$ of RIP1 (Ea et al., 2006). Analogous to the scaffolding function provided by TRAF6 K^{63} linkages in IL-1 signaling, RIP1 poly-ubiquitin chains serve as adaptors to recruit the TAK1-TAB2/3 and IKK complexes. The inhibitor of apoptosis proteins, cIAP1/2 and Ubc13-Uev1a were identified as the E3 and E2 respectively, for the K^{63}-polyubiquitination of RIP1. Additionally, the de-ubiquitination of RIP1 favored its participation in FADD-procaspase8 apoptotic signaling (Bertrand et al., 2008b; Festjens et al., 2007).

TNFR signaling is also equipped with negative regulators that keep check on unrestrained signal transduction. Dual-purpose regulator A20, was identified as a negative regulator of NF-κB signaling, by its ability to de-ubiquitinate the K^{63} linkages on RIP1 and subject it to K^{48} dependent degradation (Wertz et al., 2004b). CYLD was also identified as a negative regulator of NF-κB signaling, by participation in the de-ubiquitination of NEMO (component of IKK complex), TRAF2 and TRAF6 (Kovalenko et al., 2003; Trompouki et al., 2003). The ubiquitin-proteasomal system also exerts a regulatory effect on TNFR dependent cell death pathways by controlling the levels of the main executioners of apoptosis – the caspases. The caspases are kept under check by their inhibitors, the IAP family of proteins, by direct interaction via the baculovirus IAP repeat (BIR) domains and inhibition by their RING E3 ubiquitin ligase activity. The role of mammalian IAPs – XIAP, cIAP1 and cIAP2 have been shown to inactivate caspase 3 and 7 by UbcH5 subfamily (E2) mediated K^{48} poly-ubiquitination, reviewed in (Vaux and Silke, 2005). To permit cell death to proceed in response to appropriate cell death stimuli, the IAPs are also auto-regulated to prevent degradation of active caspases. This was first shown by the ability of XIAP and cIAP1 to promote RING-dependent auto-ubiquitination and degradation, by the release of IAP antagonists (Smac/Diablo) from the mitochondria (Du et al., 2000; Verhagen et al., 2000; Yang et al., 2000).

TNFR receptors also participate in apoptotic signaling pathways through JNK activation, depending on the nature of the stimulus, cell type and duration of ligand activation. JNK dependent cell death proceeds via a conserved apoptotic mechanism involving the Bcl-2 gene family that induces apoptosis by the activation of caspase 9 (Varfolomeev and Ashkenazi, 2004). The role of ubiquitin dependent regulation of JNK signalling has not been extensively studied. However, ubiquitin

dependent regulation of the JNK transcription factor *c-jun* has been reported (Fuchs et al., 1997). The SCF-complex has been shown to interact with the phosphorylated form of *c-jun* and target it for degradation in PC12 cells (Nateri et al., 2004). The E3 ligase hDET1 was also identified as a regulator of *c-jun* levels in non-neuronal cells (Wertz et al., 2004a). Thus, degradative and scaffolding functions of poly-ubiquitin linkages govern the hierarchy, duration and outcome of cellular signaling events.

1.4.2 Ubiquitin conjugating enzymes (UBC/E2s)

The E2s are important decision makers that control the transfer of ubiquitin linkages onto target proteins. The amino acid sequence of E2s contains a conserved (35-40%), catalytic UBC domain that mediates its critical ubiquitin conjugating functions. The critical cysteine in the UBC domain is essential for the thioester bond formation with an active ubiquitin monomer. Studies in bacteria led to the identification of an ubiquitin-like posttranslational event involving a ~7KDa protein called Pup. The conjugation of the prokaryotic-ubiquitin required the Proteasome-associated factor A (PafA), which though lacked the eukaryotic UBC domain, served a dual purpose role of an E2 and an E3 for substrate linkage modification (Iyer et al., 2008). Thus, substrate ubiquitin conjugation has been widely accepted as a eukaryotic invention. A recent study concluded the presence of 13 genes in *Saccharomyces cerevisiae*, 25 genes in *Drosophila melanogaster* and 22 genes in *Caenorhabditis elegans*, to form the primordial set of E2s in metazoan evolution (Michelle et al., 2009).

All the known E2s have been categorized into four families – Class I, include E2s made up exclusively of the UBC domain, Class II – possess C-terminal extensions in addition to the UBC domain, Class III – possess N-terminal extensions upstream of the UBC domain and Class IV constitute a combination of N and C terminal extensions flanking the UBC domain. The human genome consists of 2 ubiquitin activating enzymes or E1s, 38 E2s and >1000 ubiquitin ligases or E3s. The main function of E2s is determining the specificity of ubiquitin linkages between lysine residues of ubiquitin monomers. The favorable orientation of an ubiquitin monomer at the active site of the UBC domain, governs the nature of the linkage formed. For example, E2s - Ubc4 and Ubc5 favor K^{48}-polyubiquitination and Ubc13-Uev1a complex favors K^{63} ubiquitin catalysis, reviewed in (van Wijk and Timmers, 2010).

1.4.3 K^{63} polyubiquitination, a dedicated function for Ubc13

K^{63} polyubiquitination has emerged as an essential modification that governs non-degradative function of signaling proteins. In mammalian TNFR signaling contexts, the only E2 that is responsible for catalysis of this nature is Ubc13. Ubc13 regulates the K^{63} poly-ubiquitination by interacting with mammalian homologs of yeast Mms2, that lack the catalytical cysteine in the UBC domain. Human Ubc13-Mms2 complex was shown to participate in the assembly of K^{63} poly-ubiquitin chains in a RAD6-dependent DNA repair response pathway (Hofmann and Pickart, 1999). The structure of human Ubc13-Mms2 complex provided evidence for the preference of this heteromeric complex to orient ubiquitin in a conformation, that specifies the exclusive formation of K^{63} –linked ubiquitin chains (McKenna et al., 2001; Moraes et al., 2001). Ubc13 interaction with an additional Mms2 homolog – Uev1a was subsequently purified as the complex responsible for TRAF6-dependent activation of IκB kinase, via K^{63} poly-ubiquitin linkages (Deng et al., 2000). TRAF6 was found to undergo auto-ubiquitination by selective interaction with Ubc13-Uev1A and generate K^{63} linkages, which served as a scaffold for downstream activation of kinases such as TAK1 and IKK, in the IL-1/LPS induced NF-κB pathway (Lamothe et al., 2007; Wang et al., 2001a). The same was also observed in TNF-α mediated NF-κB signaling where cIAP1/2 were both found to associate with Ubc13-Uev1a and catalyze K^{63} poly-ubiquitination of key substrate RIP1, for TAK1 and IKK activation (Bertrand et al., 2008b; Ea et al., 2006; Li et al., 2006).

The discovery of Ubc13 for the non-proteolytic poly-ubiquitination in response to IL-1 and TNF-α provided proof for the modulatory role of K^{63} poly-ubiquitin linkages in NF-κB signaling pathway. This mechanism is a highly conserved process as K^{63} poly-ubiquitin dependent modulation of TAK1 and IKK is also observed in innate immune signaling in Drosophila. Ubc13 homolog- Bendless and Uev1a homolog – dUev1a are both essential components downstream of the IMD pathway, in response to Gram-negative bacterial infection (Zhou et al., 2005a). Bendless has also been implicated as critical regulator of Drosophila TNF-JNK signaling (Ma et al., 2014)(unpublished data, Chapter 3), though the specific targets and E3s on this pathway remain unknown. Ubc13-Uev1A complex has also been implicated in regulating signaling events by modulating the sub-cellular localization of proteins.

TNF-α dependent K^{63} poly-ubiquitination of TRAF2 was shown to require Ubc13-Uev1A activity. The ubiquitin linkage was necessary to target the modified TRAF2 proteins into insoluble fractions, for activation of the JNK pathway (Habelhah et al., 2004; Shi and Kehrl, 2003). Hence, K^{63} poly-ubiquitination is the intrinsic function of Ubc13, in association with UBC variants, Uev1a or Mms2.

1.5 TNFR associated factors (TRAFs), important regulatory proteins in TNFR signaling

The TRAF proteins are defined by the presence of a characteristic domain made up of anti-parallel β-sheets, in the C-terminus of the protein, called the MATH/TRAF-C domain. Several MATH/TRAF-C domain-containing proteins have been identified in the genome that includes proteins such as meprin proteases, USP7, TRIM37 and the TRAFs. The meprins and USP7 proteins are involved in proteolytic processing events and several TRIM37 and TRAF members are E3 ubiquitin ligases. Hence, a common theme among MATH/TRAF-C domain containing proteins is their participation in regulatory events such as ubiquitination, which appears to be well conserved in the eukaryotic phylogenetic tree (Zapata et al., 2007).The mammalian TRAF families of proteins possess the conserved MATH/TRAF-C domain, preceded by a coiled-coil domain that constitutes the TRAF-N domain of the protein. The six mammalian representatives of the TRAF family carry additional protein motifs and domains that account for their functional variability. TRAFs 2,3,4,5 and 6 carry an N-terminal RING domain followed by a variable number of Zinc-coordinating motifs (Chung et al., 2002; Rothe et al., 1994) (Domain structure illustrated in Figure 1.4).

The TRAF family of proteins participate in signal transduction pathways, by interacting either directly or indirectly with TNFRI and TNFRII family of receptors. The TNFRI receptors are mainly associated with the induction of apoptosis through their death domain interaction with the death-inducing signaling complex (DISC). However, they can also participate in inflammatory responses and transduce signals through the JNK or NF-κB pathway, via a complex of TRADD, TRAF1, TRAF2 and RIP1. The TNFRI receptors thus lack a direct TRAF-binding motif and communicate with the TRAF proteins, through the DD containing protein – TRADD. In contrast, TNFRII class of receptors encode distinct TRAF binding motifs in their intracellular

domains, that allow their direct communication (Dempsey et al., 2003) (illustrated in Figure 1.5).

The generic TRAF binding sequence - PXQX(T/S) was defined based on studies in TNFRII receptors such as CD40, CD27 and LMP1 (Arch et al., 1998) . TRAF6 interaction appears to be unique based on the elucidation of its distinct binding site - QXPXEX from RANK and CD40 receptors (Darnay et al., 1999; Ishida et al., 1996a). By engaging in specific interactions with upstream receptors and down-stream kinases, the TRAFs aid in the sequential activation of signaling cascades, which accounts for their convergent function as signaling scaffolds. The divergent functions of TRAF proteins is accounted for, by the presence of the RING domain, associated most commonly with ubiquitin ligase activity (Pickart, 2001). Hence, TRAF members can also participate in ubiquitin-dependent regulatory events in addition to serving as interaction platforms in apoptotic and inflammatory signaling pathways.

1.5.1 Role of mammalian TRAF1,2,3 and 5 in TNFR signaling

Rothe and colleagues identified the founding members of the mammalian TRAF family, TRAF1 and TRAF2 as interacting partners of the TNFRII receptor complex (Rothe et al., 1994). The main difference between the two TRAFs is the absence of the N-terminal RING domain and Zn-finger motifs in TRAF1. The main function of TRAF1 and TRAF2 in TNF-α signaling, is suppression of caspase8-dependent apoptosis and activation of the NF-κB pathway. This occurs by their interaction with and engagement of TRADD, RIP1, cIAP1 and 2 with TNFR I (Park et al., 2000; Wang et al., 1998). Activation of the IKK complex was shown to require RIP1 K^{63}-ubiquitination, which was postulated to be a consequence of TRAF2-RING E3 ligase activity (Lee et al., 2004). TRAF2 association with K^{63} specific E2 – Ubc13-Uev1a further supported this result (Habelhah et al., 2004). Recent structural analysis of the TRAF2 RING domain shows the structural inability of TRAF2-Ubc13 interaction, which dispels the role of TRAF2 as an E3 ubiquitin ligase activity (Yin et al., 2009a). Hence, the role of TRAF2 in NF-κB signaling is the recruitment of other essential E3 ligases for RIP1, such as cIAP1 and cIAP2 (Bertrand et al., 2008b).

The exact role of TRAF1 in NF-κB signaling is not well defined, but several theories deduced from *traf1*-/- mice phenotypes, point to the role of TRAF1 in

modulating the availability of soluble TRAF2 for anti-apoptotic NF-κB activation, in response to CD40 signaling (Arron et al., 2002). TRAF5 structure closely resembles that of TRAF2 and also participates in parallel signal transduction pathways. The loss of TRAF2 sensitized cells to apoptosis and impaired JNK activation, but did not inhibit NF-κB signaling (Lee et al., 1997; Yeh et al., 1997). Phenotypes of TRAF2, TRAF5 double knockout mice hinted to a compensatory effect of TRAF2 by TRAF5, in NF-κB signaling (Tada et al., 2001).

TRAF5 was identified by two independent yeast two hybrid screens for TNFR receptors, CD40 (Ishida et al., 1996b) and LTβR interactors (Nakano et al., 1996). Unlike the ubiquitous expression of TRAF2 in several tissues, TRAF5 has a more tissue specific expression pattern, with major contributions towards CD40 and CD27 lymphocyte signaling (Ishida et al., 1996b; Nakano et al., 1999). TRAF3 was identified as an interactor of TNFR receptor proteins CD40 and LMP-1 (Hu et al., 1994). Unlike the role of other TRAF members in activation of NF-κB and JNK signaling, TRAF3 has been shown to negatively regulate non-canonical NF-κB signaling, by modulating the levels of NF-κB-interacting kinase, NIK (Liao et al., 2004).

1.5.2 TRAF4 and TRAF6 – the primordial TRAF proteins.

The search for homologs of TRAF proteins in Drosophila led to the identification of the TRAF4 homolog – dTRAF1 and TRAF6 homolog – dTRAF2. (Grech et al., 2000; Liu et al., 1999). TRAF4, unlike other TRAF proteins was not isolated by its interaction with any TNFR receptor. It was identified as a protein over-expressed in breast cancer cells (Tomasetto et al., 1995). TRAF4 has not been placed downstream of the classic TRAF-signaling pathways of TNFR family members. Analysis of *traf4-/-* mice does not present any immunological defects, unlike other TRAF knockout mice phenotypes (Cherfils-Vicini et al., 2008). It does not interact with TNFR superfamily members other than a weak interaction with LTβR and strong interaction with the intracellular domain of p75NTR (unpublished data),(Krajewska et al., 1998; Ye et al., 1999). The functional significance of these TNFR interactions remain unknown.

A pro-migratory role for TRAF4 has been suggested by its localization at tight junctions in epithelial and endothelial through a Rac1 and phosphoinositide

dependent mechanism (Kedinger et al., 2008; Rousseau et al., 2013; Wu et al., 2005). It has also been shown to contribute towards cancer cell metastasis through TGF-β signaling and Wnt/β-catenin signaling (Wang et al., 2014; Zhang et al., 2013). The expression profile of TRAF4 suggests a specific enrichment in the nervous system (Masson et al., 1998), that correlates with myelination defects and neural tube closure defects observed in the *traf4-/-* mice (Blaise et al., 2012; Regnier et al., 2002). However, the TRAF4 homolog in Drosophila has been shown to participate in both TRAF-specific pathways, such as NF-κB and JNK signaling mechanisms. Genetic and cell-culture experiments have shown the involvement of dTRAF1 in innate immune signaling by the interaction and activation of IRAK homolog, Pelle (Zapata et al., 2000). dTRAF1 has been implicated in JNK-dependent dorsal closure events, and dorsal-ventral patterning defects are also observed in dTRAF1-/- flies (Cha et al., 2003; Liu et al., 1999; Mathew et al., 2009). A regulatory role for dTRAF1 has been suggested in both Reaper- (Kuranaga et al., 2002) and FlyTNF- (Geuking et al., 2005; Moreno et al., 2002) induced cell death (addressed in Chapter 2 of this thesis). Despite such high degree of similarity between TRAF4 and dTRAF1, the main difference that stands out is the absence of the RING domain in dTRAF1 (Liu et al., 1999), which could explain striking differences in cellular functions of the homologs.

Sequence comparison revealed dTRAF2/TRAF6 to be the most divergent TRAF family member (Grech et al., 2000). The functional characterization of TRAF6 also supported the unique nature of this family member, due it its involvement in signaling pathways outside the TNFR superfamily, such downstream of the TLR and IL-1 receptor immune signaling mechanisms (Cao et al., 1996; Lomaga et al., 1999). TRAF6 was identified in a yeast two-hybrid screen that utilized the TNFR receptor CD40 as the bait (Ishida et al., 1996a) and has also been characterized as an essential adaptor for RANK signaling in osteoclasts (Armstrong et al., 2002). PI3K-Akt activation has been shown to occur through a TRAF6-dependent signaling mechanism, downstream of the TNFR receptor, TRANCE (Wong et al., 1999). TRAF6 has also been shown to participate in NF-κB signaling by interacting with p75NTR (Khursigara et al., 1999). TRAF6, is the only TRAF member to have known E3 ubiquitin ligase activity, through its interaction with Ubc13-Uev1a. Together they catalyze K^{63}-linked poly-ubiquitin chains, which aid the activation of downstream kinases such as TAK1 and the IKK complex protein, for NF-κB signaling (Deng et al.,

2000; Lamothe et al., 2007; Wang et al., 2001a). The involvement of TRAF6 in JNK-dependent signaling is evidenced by neural tube closure defects and impaired developmental apoptosis in *traf6-/-* mice (Lomaga et al., 2000). Further, TRAF6 has also been shown to activate JNK-dependent cell death in response to Lipopolysaccharide treatment (Hull et al., 2002).

The Drosophila TRAF6 homolog, dTRAF2 has also been shown to participate in parallel signaling pathways. dTRAF2 has been shown to be essential for the activation of NF-κB signaling in innate immunity, by the interaction with IRAK homolog, Pelle (Shen et al., 2001). A similar IRAK-TRAF6 signaling module is seen in mammalian IL-1 signaling (Jiang et al., 2002). Thus, the TRAF-related pathway that has been explored in detail is the NF-κB pathway. In contrast, the mechanism of TRAF-dependent JNK signaling has remained elusive. Components of the JNK-signaling pathway are being currently explored in a flyTNF model of cell death signaling. The TNFR member in Drosophila, Wengen has been shown to participate in JNK-dependent cell death, downstream of fly TNF, Eiger (Igaki et al., 2002; Kanda et al., 2002). Both dTRAF1 and 2 have also been shown to be essential for JNK-dependent cell death signaling (Geuking et al., 2005; Moreno et al., 2002; Xue et al., 2007). However, data from Chapter 2 aims to clarify the role of dTRAF2 and not dTRAF1 in fly TNF-apoptotic signaling.

Based on the TRAF-C domain homology, dTRAF1 and dTRAF2 are the closest descendants of a single primordial TRAF gene found in *C.elegans* (Grech et al., 2000; Wajant et al., 1998). TRAF4 and TRAF6 may thus represent the most ancient TRAFs of the family, with recent additions of TRAF 1, 2, 3, and 5 to the mammalian TRAF family. The growing complexity of the mammalian TNFR signaling network could have contributed to an expansion of TRAF function in vertebrates. The two main signaling pathways that are activated by TRAF proteins are NF-κB and JNK signaling. The components of the Nf-kB signaling network and a TNF signaling network are not conserved in *C.elegans* (Rubin et al., 2000). However, strong conservation of JNK and apoptotic signaling components in *C.elegans* (Hay et al., 2004; Kawasaki et al., 1999), suggest the engagement of TRAFs in an ancient JNK signaling network. Thus, studying TRAF signaling pathways in Drosophila could provide useful insight into conserved signaling mechanisms of mammalian TRAF-related pathways.

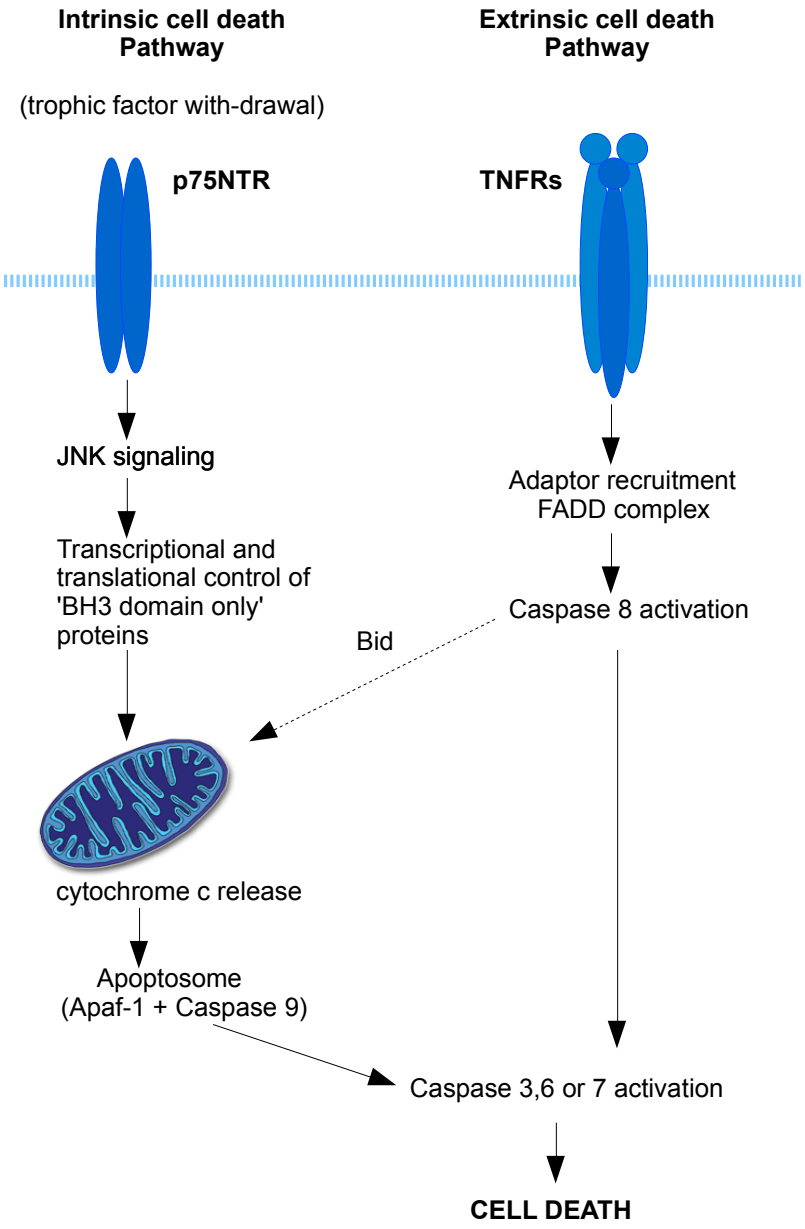

Figure 1.1 The extrinsic and intrinsic cell death pathways induced by TNFRs
The extrinsic mode of cell death is initiated by death receptors in the TNFR superfamily, followed by the recruitment of the FADD complex that initiates cell death by caspase 8 activation. The intrinsic pathway is activated by internal cellular stress signals (such as growth factor withdrawal in case of p75NTR-dependent cell death), which through JNK signaling modulates the release of apoptogenic factors from the mitochondria, such as cytochrome c. The cytochrome c release activates the formation of the apoptosome complex, resulting in caspase 9 driven cell death activation.

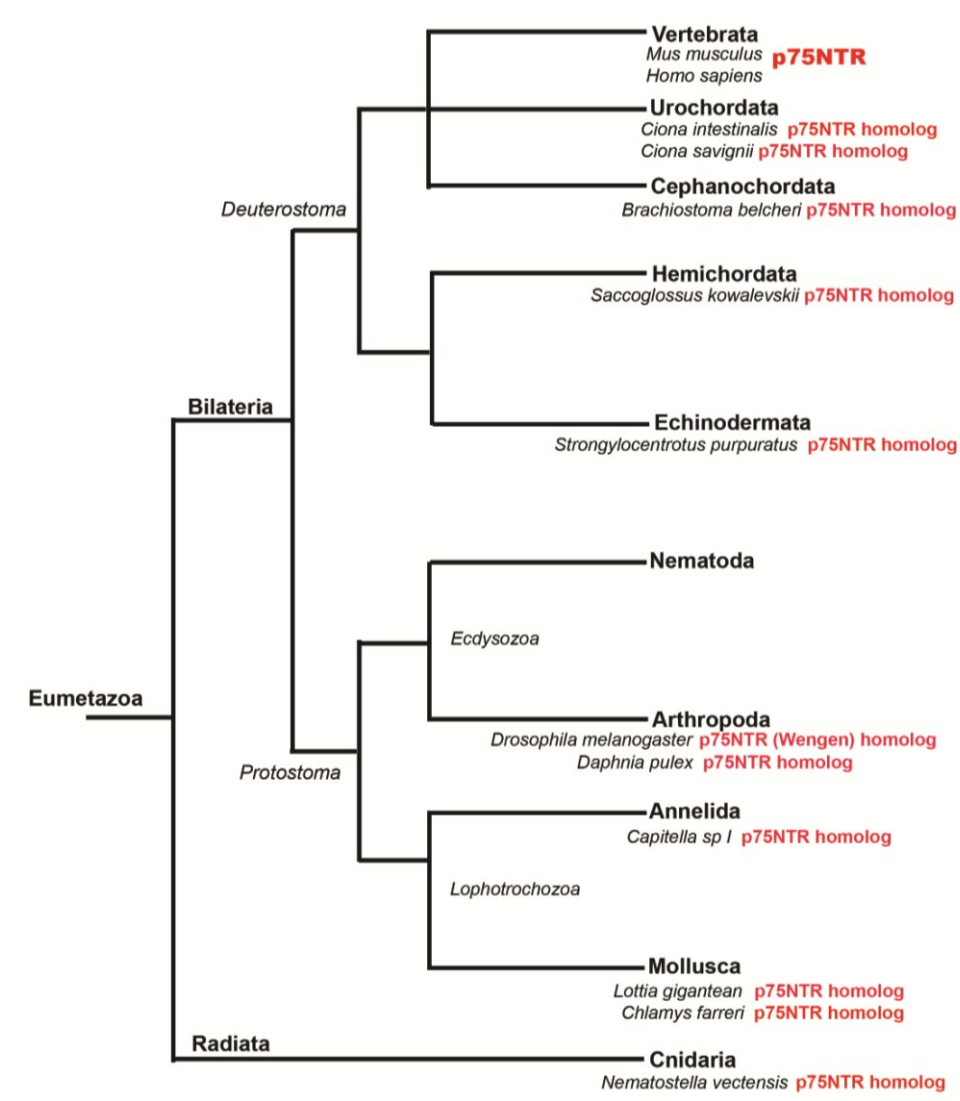

Figure 1.2 Illustration of phylogenetic distribution of p75NTR homologs during metazoan evolution.

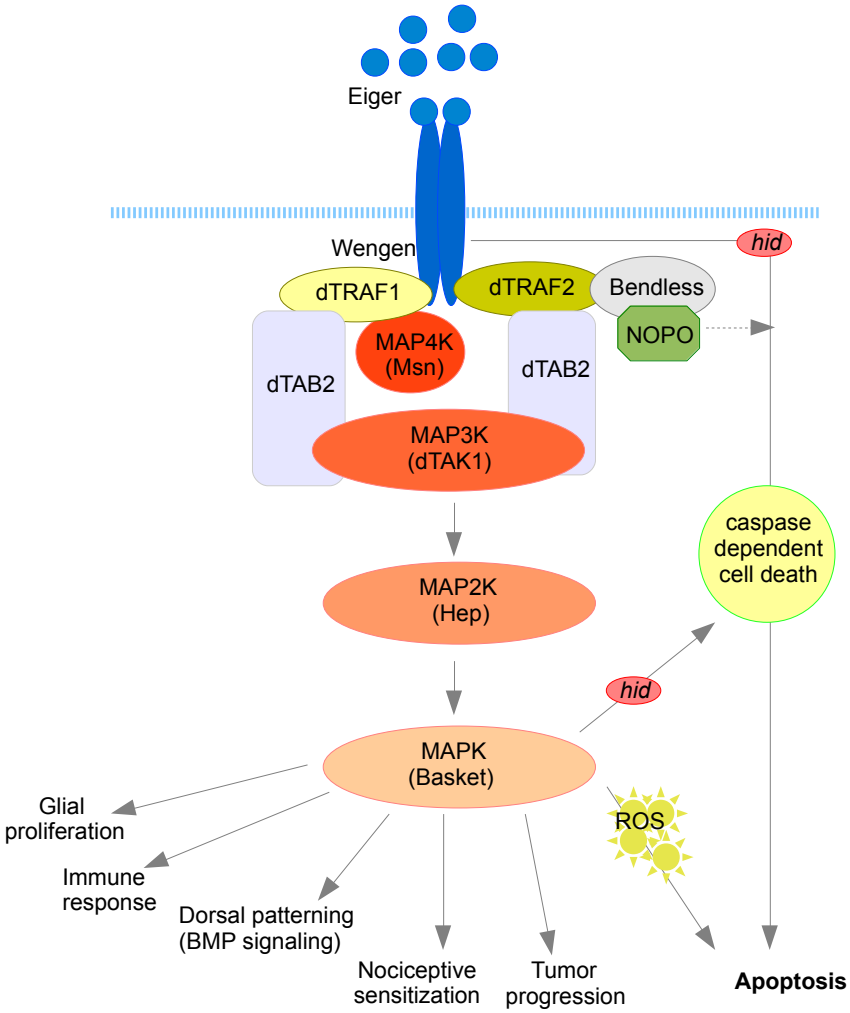

Figure 1.3 Fly TNF-TNFR signaling.
Eiger interaction Wengen results in the recruitment of adaptor proteins – dTRAF1 or dTRAF2, that initiate the hierarchical phosphorylation and subsequent activation of the MAPK cascade. The interaction of RING domain containing protein dTRAF2 with ubiquitin conjugating enzyme – Bendless, is suggestive of ubiquitin-dependent dTRAF2 modulation of the MAPK cascade. Bendless has also been shown to interact with other RING domain containing proteins, such as NOPO, which engages in a JNK-independent but caspase-dependent cell death mechanism. TRAF engagement of the receptor, initiates the MAPK cascade by the phosphorylation of MAP4K, Misshapen (Msn), which in turn phosphorylates and activates the MAP3K – dTAK1. dTAK1 recruitment to the signaling cascade is mediated by the adaptor protein dTAB2. An active dTAK1 kinase prolongs the signaling cascade by phosphorylation of the MAP2K – Hemipterous(Hep). Hep activates the terminal kinase in the cascade, the MAPK/JNK homolog – Basket. Basket allows the phosphorylation and nuclear translocation of transcription factors, which control gene expression for engagement in a variety of cellular outcomes. Eiger-Wengen signaling has also been shown to be essential for caspase-dependent and independent modes of cell death activation, depending on the cellular context. A detailed description of all the cellular outcomes controlled by Eiger-Wengen signaling has been described in section 1.3.4

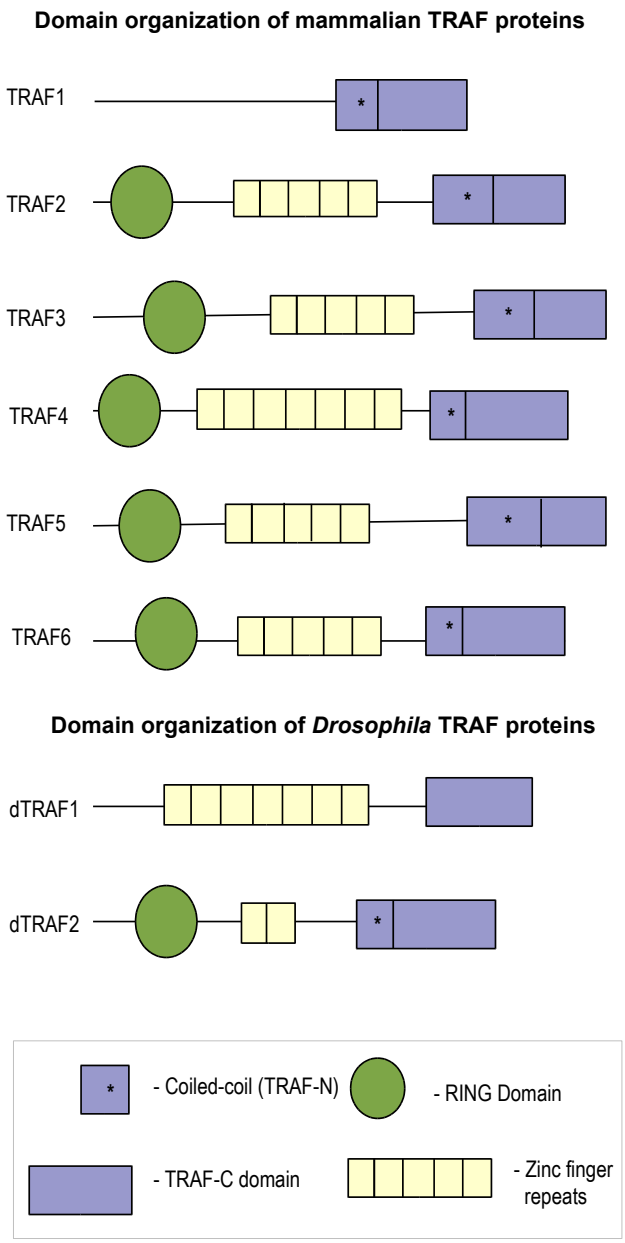

Figure 1.4 Domain organizations of mammalian and Drosophila TRAF family members.
All TRAF family members, with the exception of mammalian TRAF1 and Drosophila dTRAF1 carry a RING domain in the N-terminus of the protein, followed by variable number of Zinc finger repeats. The C-terminal is made up of the coiled-coil domain and the TRAF-C domain, which is essential for TRAF interaction with TNFR and its signaling components. The phylogenetic analysis of TRAF proteins is based on the TRAF-C sequence homology. The Drosophila TRAF proteins – dTRAF1 and dTRAF2 are most homologous to mammalian TRAF4 and TRAF6 respectively.
Adapted from (Grech et al., 2000).

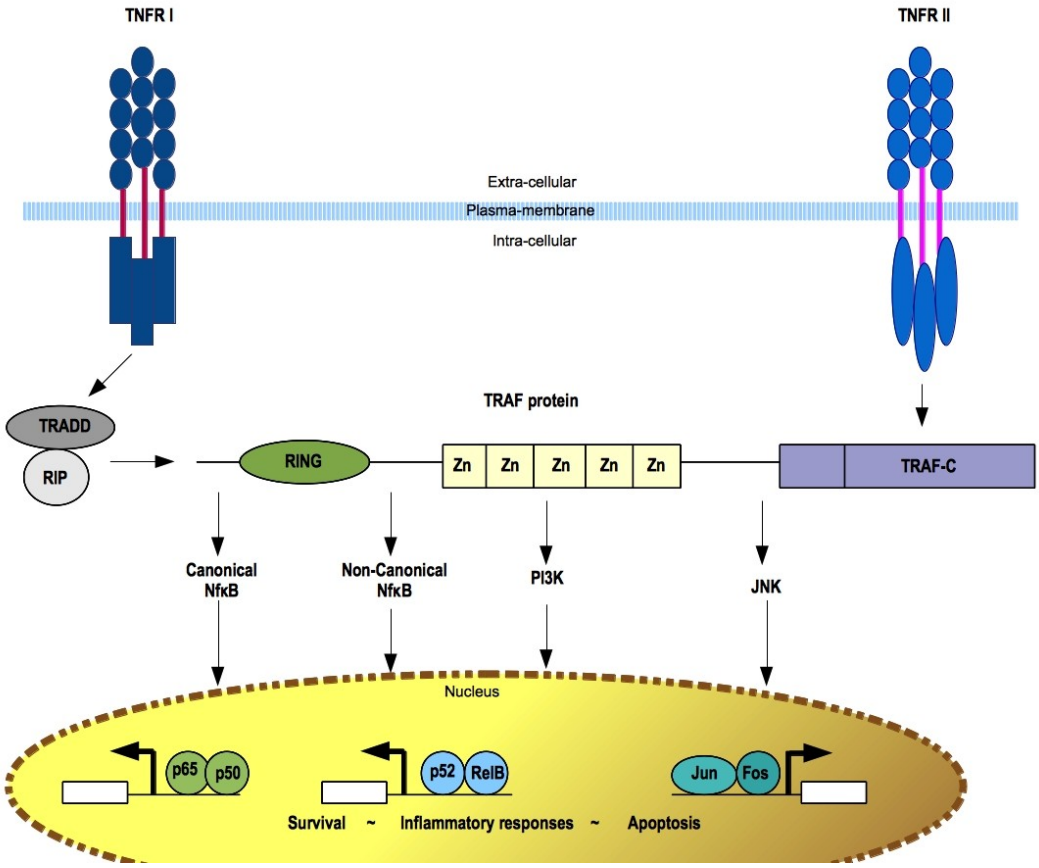

Figure 1.5 TRAF-signal transduction downstream of TNFR I and TNFR II family members.
TRAF proteins engage in TNFR signal transduction by indirect interaction with TNFR I receptors, through TRADD-RIP complex or via direct TNFR II receptor interaction. The TRAF-C domain directly interacts with the TNFR II intracellular domain. The common TRAF related pathways include NF-κB and JNK signaling with additional engagement in pathways, such as the PI3K cascade. The main signaling outcomes of TRAF signaling include cell survival, cell death and inflammatory responses. Adapted from (Dempsey et al., 2003)

RATIONALE AND OBJECTIVES

Rationale

The focus of my project is to understand some of the fundamental signalling proteins that mediate JNK dependent apoptosis in a *Drosophila melanogaster* model system. Drosophila is amendable to genetic manipulations, which can provide invaluable insights *in vivo*, into conserved regulatory events that govern cell death signalling in vertebrates. Mammalian TNFR signalling proteins engage in physiological events, that over-lap both immune regulation and apoptosis. However, in Drosophila the segregation of Toll receptor- dependent immune signalling pathways from the TNFR-regulated apoptotic network allows careful study of conserved components of TNFR signalling. The fly TNF, Eiger is involved in a conserved signalling network, for the elimination of injured neurons during development or injury. Further, the pro-inflammatory role of Eiger is also utilized for the elimination of pre-cancerous lesions in the epithelia. Extensive genetic screens have shaped up the identity of the signalling network utilized by Eiger, for its pro-apoptotic functions. My doctoral thesis attempts to define some of the underlying conserved mechanisms of TNFR signalling, that will provide new dimensions into understanding the basic cellular principles of vertebrate development and homeostasis.

Objectives

The three main objectives of this thesis are -

1. To determine the role of conserved TNFR adaptor, dTRAF2 in Eiger-dependent apoptotic signalling. – Chapter 2

2. To establish an *in vivo* genetic and biochemical tool to assess the importance of ubiquitin-regulated control over the flyTNF signalling network – Chapter 3

3. To assess the modulatory role of Eiger in pro-degenerative signalling events and identify novel Eiger-regulated signalling partners, that are implicated in the progression of Alzheimer's disease – Chapter 4

PREFACE TO CHAPTER 2

The mammalian TRAF proteins are one of the main adaptors that connect TNFR receptors to downstream signalling pathways. There are two TRAF homologs in the fly, namely dTRAF1 and dTRAF2. The dTRAF2 protein is homologous to the mammalian TRAF6 protein, which represents the most ancient and functionally divergent member of the TRAF family. TRAF6 plays a dual-purpose role of serving as a signalling scaffold and an E3-ubiquitin ligase enzyme, in mammalian TNFR and TLR signalling contexts. The RING domain that is responsible for the E3 ubiquitin ligase activity of TRAF6, is well conserved in the fly counterpart, however, the role of dTRAF2 has not been explored in invertebrate TNFR signalling pathways. We provide genetic evidence for the critical role of dTRAF2 and dispel the role of dTRAF1, in Eiger-mediated JNK signalling. By careful modulation of protein expression levels, we show the critical dependence dTRAF2 function, in Eiger-dependent apoptotic signalling. Thus by understanding the primordial role of dTRAF2, we will gain to understand the molecular basis of non-redundant TRAF6 signal transduction.

CHAPTER 2 - dTRAF2, is an essential scaffolding protein that regulates FlyTNF-mediated apoptotic signaling

Ambika Srinivasan[1], Sarah Tse[1], Susan Linn[1] and Dr. Philip Barker[1]
[1]Montreal Neurological Institute, McGill University, Canada

[1]**Corresponding author -** email: phil.barker@mcgill.ca

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2. ABSTRACT

The cellular events that govern the viability of any eukaryotic cell, such as cell death, survival, proliferation, growth regulation and differentiation require carefully orchestrated cascades that are activated, regulated and mediated by a large repertoire of signaling proteins. With the rise in complexity of cellular organization, the intricacies of any given signaling network and proteins associated with it have expanded over the course of evolution. However, understanding the role of signaling proteins in phylogenetic ancient model systems may shed light on the basic cellular principles and the resulting variations that have evolved from it. In our current study, we utilize *Drosophila melanogaster* to understand conserved signaling mechanisms of TNF-mediated apoptotic signaling. We observe an indispensible and non-redundant role for a TNF adaptor protein - dTRAF2, as the crucial effector of TNF-induced cell death. Using the power of Drosophila genetics, we show that one of the unexpected mechanisms of cell death regulation utilized by dTRAF2, is the auto-regulation of its expression levels.

2.1. INTRODUCTION

The tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamily consist of a group of secreted or membrane-bound ligands and receptors, that regulate cellular processes such as cell survival, apoptosis, and immunity. TNFRs interact with a family of intracellular adapter molecules to promote cell survival by the activation of downstream protein kinases, which in turn modulate transcription factors belonging to the Nuclear factor – kappa B (NF-κB) and AP-1 family. These transcription factors can activate relevant genes to exert the biological effect of the ligand-receptor pair (Locksley et al., 2001). A family of intracellular proteins identified on the basis of their ability to bind to TNFRII (Rothe et al., 1994) and subsequently CD40 (Hu et al., 1994) were termed TNF receptor-associated factors, TRAFs. To date, six proteins of this family have been identified in mammals (TRAF1-TRAF6) (Chung et al., 2002). Mammalian TRAFs activate both c-Jun N-terminal kinase (JNK) and NF-κB signaling pathways downstream of TNFR receptors. There has been a large body of research dedicated to understand the role of each TRAF member in TNFR signaling. The studies include cell-based analysis and extensive work done in mouse genetics, has made it easier to understand the *in vivo* function of each individual TRAF member (Lomaga et al., 1999; Nakano et al., 1996; Nakano et al., 1999; Nishitoh et al., 1998; Regnier et al., 2002; Rothe et al., 1995; Xu et al., 2002). However, sequence similarity between each member makes it difficult to account for redundancy of TRAF function.

Fortunately, *Drosophila melanogaster* has only two TRAF homologs, dTRAF1 and dTRAF2 that are homologous to TRAF4 and TRAF6 respectively (Grech et al., 2000; Liu et al., 1999). The fly TRAFs, dTRAF1 and dTRAF2 do have conserved roles in signaling pathways attributed to their mammalian counterparts. dTRAF1 has been shown to activate JNK signaling upstream of Mishappen and is required for dorsal closure during embryogenesis (Cha et al., 2003; Liu et al., 1999) and for Pelle -dependent innate immune signaling (Zapata et al., 2000). dTRAF1 has been known to act as a critical effector of JNK-dependent cell death, in response to the ectopic expression of pro-apoptotic protein, Reaper (Kuranaga et al., 2002). dTRAF2 has also been implicated in coordinating Toll receptor signaling through NF-κB activation through the interaction with Pelle (Cha et al., 2003; Kopp et al., 1999;

Shen et al., 2001), but its role in JNK-dependent cell death has been poorly defined. Relative to the mammalian signaling pathway, the receptor-activated cell death pathway, is simplified in *Drosophila melanogaster*, owing to the presence of just a single TNF ligand, Eiger (Igaki et al., 2002; Moreno et al., 2002) and two TNFRs, Wengen (Kanda et al., 2002) and Grindelwald (Andersen et al., 2015). Genetic epistasis studies have uncovered several conserved apoptotic proteins downstream of Eiger. It is certain that Eiger induces cell death that is JNK dependent (Igaki et al., 2002; Moreno et al., 2002), but the upstream mechanisms that drive JNK activation are the focus of ongoing research. Like all TNF-TNFR signaling, the Eiger-Wengen cascade requires adaptor proteins to participate in signaling. dTRAF1 was reported to be the critical adaptor for cell death signaling through Eiger (Geuking et al., 2005; Moreno et al., 2002). However conflicting results (Xue et al., 2007) showed the importance of dTRAF2 and not dTRAF1, downstream of Eiger. Hence, in the current study we sought to clarify the role for both dTRAF proteins in Eiger signaling and define the need for dTRAF2 in Eiger-dependent cell death signaling.

The TRAF family members share a conserved domain, termed the Really Interesting New Gene (RING). The RING domain is structured by a series of Cysteine and Histidine residues arranged in a cross-braced manner. This structural feature defines their function as E3 ubiquitin ligases, which allow them to interact with specific ubiquitin conjugating enzymes (E2s) (Joazeiro and Weissman, 2000; Lovering et al., 1993). Experimental evidence from a range of studies suggests that all TRAF members can function as adaptors, but not all are E3 ubiquitin ligases. TRAF6 is a *bona fide* E3 ubiquitin ligase, which interacts with Ubc13-Uev1A complex, for participation in TLR/IL-1R signaling (Bradley and Pober, 2001; Cao et al., 1996). Several studies have shown the importance of TRAF2 in the catalysis of K^{63} – linked polyubiquitination, downstream of TNF signaling, by interacting with a heteromeric E2-complex, Ubc13-Uev1a (Lee et al., 2004; Shi and Kehrl, 2003; Wertz et al., 2004b). However, recent structural evidence has challenged the notion that TRAF2 has intrinsic E3 ligase activity (Yin et al., 2009a). Distinct amino acid differences in the RING domain and the N-terminus region of the TRAF structure prevent TRAF2, but not TRAF6, from interacting with the K^{63} –conjugating E2, Ubc13 (Yin et al., 2009a). In Drosophila, the TRAF6 homolog dTRAF2 interacts and signals with the Ubc13 homolog, Bendless, (Ma et al., 2014), but it's potential for E3

ubiquitin ligase activity has not been addressed. The ease of genetic manipulation and high degree of homology of mammalian TNFR signaling components, makes *Drosophila* melanogaster an ideal tool to address the mechanism of TNF-induced cell death and in the current study we address the importance and physiological function of dTRAF2 in Eiger signaling.

2.2. MATERIALS AND METHODS

Drosophila stocks and genetic crosses –

Fly line	*Genotype*	*Collection*
UAS-*tsg*-RNAi	w[1118];P{GD6488}v45355/TM3	VDRC
UAS-*dtraf1*-RNAi	w[1118]; P{GD10100}v21213/TM3	VDRC
UAS-*dtraf2*-RNAi	w[1118];P{GD7146}v1612 6/TM3	VDRC
UAS-*relish*-RNAi	w[1118]; P{GD1199}v49413	VDRC
dtraf2 ^{ex1}	dtraf2 ex1/FM7c no Bar sn	Dr.Jongkyeog Chung (Cha et al., 2003)
dtraf2 BAC clone (genomic transgene)	w[1118]; Dp(1;3)DC186,PBac{y[+mDint2] w[+mC]=DC186}VK00033	BDSC
rel^{e20}	Rel[E20] – BM55714	BDSC
UAS-*dakt1*	y[1] w[1118]; P{w[+mC]=UAS-Akt1.Exel}2	BDSC
akt1^{04226}	ry[506]P{ry[+t7.2]=PZ}Akt1[04226]/TM3, ry[RK] Sb[1] Ser[1]	BDSC
UAS-*eiger*	yw; UAS-eiger/CYO(T655 D449)	Dr.Konrad Basler (Moreno et al., 2002)
GMR-GAL4	w[1118]; P{GMR-GAL4.w[-]}2/CyO	BDSC

VDRC=Vienna Drosophila RNAi Center

BDSC =Bloomington Drosophila Stock Center

All stocks were raised in standard Drosophila media (recipe taken from BDSC) and all crosses were carried out at 25°C, unless specified.

Generation of dtraf2 transgenic flies - To generate UAS-dTRAF2^{wildtype}, UAS-dTRAF2$^{C104A/H121A}$, *tubulinα1*>dTRAF2^{wildtype} and *tubulinα1*>dTRAF2$^{C104A/H121A}$ transgenic flies, dTRAF2 cDNA (GH01161) was obtained from the Berkeley Drosophila Genome Project. Two critical E2 binding sites in the RING domain of Drosophila dTRAF2 (C104 and H121) were mutated to alanine using the Quick-change multi site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). The fidelity of point mutations were verified by DNA sequencing. Full-length dTRAF2 ^{wildtype} and dTRAF2$^{C104A/H121A}$ were amplified by PCR-based cloning techniques and sub-cloned into pUAST vector to generate UAS-transgenes and sub-cloned into pOP118 for the *tubulinα1* promoter driven transgenes. The pOP118 vector was a

kind gift from Dr. Konrad Basler, University of Zürich (Geuking et al., 2005). The transgenic flies were generated by Best Gene Inc.

Imaging - Adult fly eyes were photographed using with a Canon EOS 1000D DSLR (rebel XS) camera mounted on a Zeiss Axioskop 40 microscope with a10× objective (0.25 = N.A). Several images of the adult fly eye were taken at different focal planes, for extended depth. The focus stacking of each set of images was done using Helicon Focus software (HeliconSoft) to generate the final image. The quantification of eye area was done using Fiji image processing software. Data presented as bar graphs were created using GraphPad Prism 4. One-way ANOVA or student t-test was used to calculate statistical significance as indicated in the relevant figure legends, using GraphPad Prism 4.

Antibody production- The dTRAF2 mouse polyclonal antibody was directed against a glutathione-S-transferase (GST)-fusion protein containing the full-length dTRAF2. The GST-dTRAF2 construct was sub-cloned into pGEX-4T-1 (GE). The dTRAF2-pGEX-4T-1 was used to express glutathione-S-transferase (GST)-dTRAF2 in E. coli strain BL21. GST-dTRAF2 fusion protein was purified from bacterial lysates using a standard GST-fusion protein purification protocol (GE), and injected into mice to generate anti-dTRAF2 sera.

Western blotting – To test the dTRAF2 expression levels four fly heads/genotype were suspended in 100µl of 2×Laemmli sample buffer. The samples were homogenized and boiled for 5 minutes prior to their separation by SDS-PAGE. After the samples were separated by 10% SDS-PAGE, the proteins were transferred onto nitrocellulose membranes at 100V for 90 minutes. The membranes were rinsed in 1×TBST and blocked in 1× TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2% Tween 20) supplemented with 5% (w/v) dried skim milk. Primary and secondary antibody incubations were performed in 1×TBST containing 2.5% (w/v) dried skim milk blocking solution. Incubation with primary antibodies (mouse polyclonal anti-dTRAF2 sera 1:4000, home-grown antibody; mouse monoclonal anti-actin antibody, Fischer) was performed overnight at 4°C. Poly-HRP secondary antibody (used between 1:2000 – 1:5000 dilutions, Jackson ImmunoResearch) incubations were performed for 1 hour at room temperature. Membranes were washed repeatedly in 1× TBST

after each incubation. Immunoreactive bands were detected using enhanced chemiluminescence solution kit (Perkin-Elmer Life Sciences, Norwalk, CT, USA), as per the manufacturer's instructions.

2.3. RESULTS

dTRAF2 and not dTRAF1 is the important adaptor for Eiger-mediated cell death signaling – We have utilized the Glass multimer reporter (GMR) promoter to drive transgene expression in all post-mitotic cells posterior to the morphogenic furrow, of Drosophila third-instar larva eye-imaginal disc (Ellis et al., 1993; Freeman, 1996). Using this promoter to drive ectopic of Eiger in the eye-imaginal disc induces potent activation of cell death via JNK activation, leading to loss of most photoreceptor cells in the eye (Igaki et al., 2002). Knockdown of dTRAF1 expression using a transgenic RNAi lines failed to suppress Eiger-induced cell death (Figure 2.1a-E). However, using an RNAi line to knockdown dTRAF2 expression completely inhibited Eiger-induced cell death (Figure 2.1a-F). We validated this dTRAF2 modifier effect, by using a null allele of dTRAF2, *Dfdtraf*^{ex1} (Cha et al., 2003). The 'small-eye' phenotype of Eiger over-expression is greatly suppressed in a dTRAF2 heterozygous female *Dfdtraf2*^{ex1}/+, and completely suppressed in a dTRAF2 hemizygous male, *Dfdtraf*^{ex1}/Y (Figure 2.1a-G,H). Further, the suppression of the 'small-eye' phenotype of Eiger over-expression in a dTRAF2 null fly (*Dfdtraf2* ^{ex1}), was reverted by introducing a genomic rescue transgene of dTRAF2 (Figure 2.1b, compare F and G, J and K). A minimum of one copy of endogenous dTRAF2 was required for the induction of cell death, in response to Eiger. Hence, dTRAF2 and not dTRAF1, is the critical adaptor protein that links Eiger to down-stream signaling players.

The ectopic expression of dTRAF2 inhibits Eiger signaling – Given the sequence homology between mammalian TRAF6 and dTRAF2, we wanted to address the potential E3 ubiquitin ligase activity of dTRAF2, specifically in Eiger signaling. In order to address this, we generated transgenic flies that over-express UAS-*dtraf2*$^{wild\ type}$ and dTRAF RING mutants, UAS-*dtraf2*^{C104A} and UAS-*dtraf*^{H121A}. Mutation of the corresponding cysteine or histidine residues of TRAF6 renders the RING domain null for E3 ubiquitin ligase activity. The TRAF6 RING mutants (C70 and H87) loose their ability to interact with Ubc13-Uev1a and are inefficient for K^{63}-linked polyubiquitination (Deng et al., 2000; Wooff et al., 2004). Given the requirement for dTRAF2 in the induction of cell death, we expected to see a potentiation of the Eiger cell death phenotype upon dTRAF2 over-expression.

However, to the contrary, the over-expression of UAS-*dtraf2*^{wildtype} failed to potentiate Eiger-mediated cell death (Figure 2.2a, compare E with F), but rather inhibited it. We observe the same effect by the ectopic expression of the RING mutants, UAS-*dtraf2*^{C104A} and UAS-*dtraf*^{H121A} (Figure 2.2a, compare E with G and H). Further, we found that the co-expression of UAS-*dtraf2*^{wildtype} (or UAS-*dtraf2*^{C104A}/UAS-*dtraf*^{H121A}) with GMR-GAL4>UAS-*eiger*, generated a unique 'bulging eye' phenotype with a mild disruption of the eye lattice structure (Figure 2.2b-B).

Over-expression of dTRAF2 does not inhibit pro-apoptotic signaling by activating pro-survival pathways - Activation of pro-survival cascades, such as the NF-κB pathway, can reduce JNK-dependent cell death (De Smaele et al., 2001; Tang et al., 2001). In mammals, this serves as a means to regulate inflammation-induced apoptosis, in response to mammalian TNF signaling. This bi-model control of signaling is also known to occur in Drosophila innate immune signaling contexts. In response to gram-negative bacterial infection, NF-κB prevents JNK activation by sequestering an apical kinase in the pathway, dTAK1 (Park et al., 2004). Over-expression of UAS-*dtraf2* has been previously shown to induce the activation and nuclear translocation of Drosophila NF-κB factors, Dif and Relish (Cha et al., 2003). We therefore asked if activation of the NF-κB signaling pathway accounted for the inhibition of Eiger-induced cell death observed upon dTRAF2 overexpression. Figure 2.3 shows that the inhibition of Eiger-induced cell death by UAS-*dtraf2* over-expression, is not prevented upon Relish knockdown, using UAS-*relish*-RNAi (compare D and L) or in the presence of a Relish loss of function allele, *rel*^{e20} (compare D and H), indicating activation of NF-κB does not contribute to the survival induced by dTRAF2 overexpression.

dTRAF2 and Akt signaling cross-talk- TRAF6 controls Akt activation by inducing it's membrane recruitment in response to mammalian IGF-1 (Yang et al., 2009) and in response to TRANCE activation (Wong et al., 1999). Drosophila Akt1 (dAkt1) has been shown to be important for the regulation of cell growth, downstream of the Drosophila insulin receptor signaling. Interestingly, the ectopic expression of dAkt1 was also shown to generate a 'bulging eye' phenotype (Verdu et al., 1999) similar to what we observed upon dTRAF2 overexpression. This led us to assess the role of dAkt1 in the inhibition of dTRAF2 mediated Eiger-induced cell

death. Loss of dAkt1 expression using a dAkt1 loss of function allele, *akt1*^{04226}, failed to revert the UAS-*dtraf2* blockade of Eiger- cell death phenotype (Figure 2.4.1, compare D and H). However, loss of dAkt1 did inhibit the 'bulging eye' phenotype of dTRAF2 and Eiger co-expression, (Figure 2.4.2, compare C and F) whereas over-expression of UAS-*dAkt1* enhanced the 'bulging eye' phenotype observed with dTRAF2 and Eiger co-expression (Figure 2.4.2, compare C and I). We conclude that the block in Eiger-induced cell death induced by UAS-*dtraf2* overexpression is not due to NF-κB or Akt pathway activation. However, the Eiger and dTRAF2 bulging eye phenotype does require dAkt1 participation.

The levels of dTRAF2 is a critical determinant for Eiger-mediated apoptotic signaling- Crystal structure and biochemical analysis have revealed a unique structure for TRAF6. TRAF6 monomers form mushroom shaped trimers with it's C-terminus (TRAF-C binding domain) and dimers with it's N-terminus (RING domain and Zinc fingers), leading to the formation of an intricate oligomeric lattice-like arrangement (Yin et al., 2009b). The evolutionary conservation of critical residues that contribute to TRAF6 oligomerization suggests a similar configuration for the dTRAF2 oligomeric structure (Figure 2.5a). Hence, we hypothesize that the GMR-driven levels of UAS-dTRAF2, expressed well above physiological levels of protein expression, was responsible for the disruption of this intricate lattice like arrangement, which prevented it from participation in Eiger signaling. To confirm our hypothesis, we utilized a *tubulinα1* promoter to drive the expression of dTRAF2 (*tubulinα1> dtraf2*^{wildtype}/ *tubulinα1>dtraf2*^{C104A} and *tubulinα1>dtraf*^{H121A}). In order to observe the subtle changes in the phenotype, we performed our crosses at 18°C, which reduces GAL4 activity (Duffy, 2002). In order to assess the expression level of dTRAF2 transgenic flies, we generated a polyclonal antibody against full-length dTRAF2. Figure 2.5b shows that GMR-driven levels of UAS-*dtraf2* are considerably higher than either the tubulin-driven or endogenous levels of the protein.

We were surprised to observe a significant increase in the eye size of flies that over-express the *tubulinα1>dtraf2*^{wildtype} transgene, that is lost upon mutation of critical residues in the RING domain. Hence, *tubulinα1>dtraf2*^{wildtype}, in the absence of Eiger is capable of participating in a signaling pathway that effects the over-all eye size, in a RING dependent manner (Figure 2.6a-B,C, D and 2.6b). We then tested the ability of *tubulinα1>dtraf2*^{wildtype}/ *tubulinα1>dtraf2*^{C104A}/ *tubulinα1>dtraf*^{H121A}, to

modify the cell death phenotype of Eiger, in a dTRAF2 null background. Importantly, we observed a small induction of cell death by *tubulinα1> dtraf2*^{wildtype} in heterozygous dTRAF2 null flies (*Dfdtraf2*^{ex1}/+; compare 2.6a-F and G). However, *tubulinα1>dtraf2*^{H121A} or *tubulinα1>dtraf*^{C104A} fails to induce Eiger-dependent cell death in *Dfdtraf2* ^{ex1}/+ flies (compare 2.6a-F with H and F with I, respectively). In order to better assess this subtle effect, we quantified the area of the eye, of *tubulinα1> dtraf2* transgenic flies in the presence and absence of Eiger (Figure 2.6c).

The confounding Eiger-independent effect of *tubulinα1>dtraf2*^{wildtype} towards the regulation of eye size, prevented us from being able to directly compare the eye area of the *tubulinα1>dtraf2*^{wildtype} transgene, against the two dTRAF2 RING mutants, in the ectopic Eiger - *Dfdtraf2* ^{ex1}/+ null flies. However, we observed a significant Eiger-dependent decrease in the eye-size of *Dfdtraf2*^{ex1}/+ flies upon *tubulinα1>dtraf2*^{wildtype} expression, when compared to the eye size of *tubulinα1>dtraf2*^{wildtype} transgenic flies (Figure 2.6c, compare columns 1 with 2; [t (15)=3.8422, p=0.0016]). The ectopic expression of Eiger failed to induce a decrease in eye size, upon expression of *tubulinα1>dtraf2*$^{H121A/C104A}$ (Figure 2.6c, compare columns 3 with 4 and 5 with 6). A paired-samples t-test revealed a significant increase in the eye area of transgenic flies that co-express *tubulinα1>dtraf2*^{H121A} or *tubulinα1>dtraf2*^{C104A} with Eiger in a *Dfdtraf2*^{ex1}/+ null background, compared to the eye area of *tubulinα1>dtraf2*^{H121A} [t (15)=8.422, p<0.0001] or *tubulinα1>dtraf2*^{C104A} [t (15)=7.476, p<0.0001] transgenic flies.

We conclude that dTRAF2 must be expressed at physiological levels to recover the Eiger-dependent ‘small-eye’ phenotype and that the RING domain within dTRAF2 is essential for this cascade.

2.4. DISCUSSION

TRAF6 is a unique member of the TRAF family, as it participates both in TNFR signaling cascades and in non-TNFR cascades, including those induced by the Toll-like receptor (TLR) and Interleukin-1 receptor (IL-1R) (Cao et al., 1996; Ishida et al., 1996a; Khursigara et al., 1999; Lomaga et al., 1999). An additional unique feature of TRAF6 lies in the sequence specificity of its receptor binding abilities, which is distinct from other TRAF family members (Darnay et al., 1999; Pullen et al., 1998). Sequence analysis and the unique features of TRAF6 mentioned above suggest it to be the most divergent and well conserved member of the TRAF family (Grech et al., 2000). Fly TNFR, Wengen has a cysteine rich domain that is most similar to p75NTR than other members of the TNFR superfamily (Bothwell, 2006). The *Drosophila* TRAF proteins, dTRAF1 and 2 are the only representatives of the more recent mammalian TRAF family. Our unpublished data shows that p75NTR selectively interacts with TRAF4 and TRAF6, the homologs of dTRAF1 and dTRAF2. Hence, there is a high degree of conservation of p75NTR, TRAF6 and JNK signaling components in *Drosophila melanogaster*. In this present study we have addressed the role of this ancient TRAF family member in Fly TNF-driven JNK signaling. We have utilized a well-established cell death model, driven by the mis-expression of Eiger in the eye-imaginal disc of *Drosophila melanogaster* (Igaki et al., 2002). The resulting phenotype generates a 'small-eye' and has been extensively used to identify modifiers of the Eiger-driven signaling pathway (Andersen et al., 2015; Geuking et al., 2005; Geuking et al., 2009; Igaki et al., 2002; Kanda et al., 2002; Kanda et al., 2011; Ma et al., 2012; Ma et al., 2014; Ma et al., 2013b; Moreno et al., 2002; Zhang et al., 2010).

We observe an indispensible role for dTRAF2 in Eiger-mediated cell death through JNK activation. Contrary to previous reports, we do not observe the dependency of Eiger signaling on dTRAF1 function (Geuking et al., 2005; Moreno et al., 2002). In order to understand the physiological role of dTRAF2 in JNK signaling, we created transgenes that over-express dTRAF2, driven by the GMR promoter. We found that the ectopic expression of dTRAF2, which exceeded endogenous expression levels of the protein, suppressed Eiger-mediated cell death signaling. We tested the role of dTRAF2 over-expression in anti-apoptotic signaling pathways, such as NF-κB and Akt1 signaling. We did not observe a role for both these signaling

pathways in the suppression of Eiger-dependent cell death, by dTRAF2 over-expression. By careful regulation of dTRAF2 expression levels, we show a critical dependence of the dosage of dTRAF2 on the Eiger signaling network. Lessons from mammalian TRAF signaling studies have provided clues to understand this dosage dependency of dTRAF2.

An elegant crystallographic study (Yin et al., 2009b) showed that TRAF6 exists as C-terminal trimers and N-terminal dimers in solution. The N-terminal dimerization of TRAF6, unleashes the ability of this protein to organize into higher order oligomers. Each TRAF6 trimer is formed by trimerization of its C-terminus and coiled-coiled stalk domain. However, the N-terminus of TRAF6 showed a preference for dimerization with the N-terminus of the adjacent TRAF6 trimer thus facilitating heterologous dimerization. Mutagenesis studies show that the loss of ability of TRAF6 to dimerize affected its subsequent oligomerization tendencies, which interfered with its biological activity. The main regulatory function of TRAF6 is contributed by its E3 ubiquitin ligase activity. TRAF6 catalyzes the formation of non-degradative K^{63}-linked polyubiqutin chains with Ubc13-Uev1A that aids the activation of the downstream signaling pathway. TRAF6 has been shown to participate in signaling by auto-ubiquitination (Deng et al., 2000; Lamothe et al., 2007) and promote the activation of TAK1 (Wang et al., 2001a) via K^{63}-linked polyubiquitin chains. Disruption of the N-terminal TRAF6 dimers did not inhibit the ability of TRAF6 to interact with its E2 binding partner, Ubc13-Uev1a but failed to induce K^{63}-polyubiquitination. This careful arrangement of TRAF oligomers thus serves as a point of convergence for ubiquitin conjugation that activates downstream kinases. The ability to participate in signaling after oligomerization could serve as a regulatory mechanism to prevent individual monomers from interacting with receptors in their ligand free state. This has been shown for TRAF2, which as monomers have very low affinity for TNFR (Ye and Wu, 2000) or CD40 (Pullen et al., 1999). Hence, massive over-expression of dTRAF2 could potentially hamper the oligomeric structure and impair Eiger-induced signaling, suggesting that dTRAF2 expression levels need to be tightly regulated.

We used temperature control and a weaker gene promoter (*tubulinα1*) to regulate levels of dTRAF2 expression, to ask if it is an important E3 ubiquitin ligase

on the Eiger signaling pathway. The E2 (Ubc13) interaction with TRAF6 requires residues within and preceding the RING domain of TRAF6. Hence, though the RING domain alone is not sufficient for the Ubc13 interaction, it is still very essential to ensure proper E2 alignment for catalysis. Not surprisingly, the TRAF6^{C70A} mutant is deficient for E3 ubiquitin ligase activity, since it has lost its ability to interact with E2, Ubc13 (Deng et al., 2000; Lamothe et al., 2007; Wooff et al., 2004; Yin et al., 2009b). We find that *tubulinα1*>dTRAF2^{wildtype} induces a small degree of Eiger-mediated cell death, but the *tubulinα1*>dTRAF2$^{C104A/H121A}$, failed to engage in Eiger apoptotic signaling, that suggests a RING-dependent function of dTRAF2 in Eiger-mediated cell death signaling. Interestingly we observe an Eiger-independent involvement of *tubulinα1*>dTRAF2^{wildtype} in a signaling pathway, that regulates the overall eye size, also in a RING domain-dependent manner.

We conclude that an optimum level of dTRAF2 expression is required to conduct signaling events through Eiger. Further, the RING domain of dTRAF2 maybe crucial for the auto regulation of its expression levels, in addition to the modulation of other protein targets on the Eiger signaling network. The over-expression of dTRAF2 in an SL2 insect cell, resulted in extensive modification of dTRAF2, with a ladder like appearance on a western blot, which is lost upon deletion of the RING domain (Shen et al., 2001). The significance and nature of the modification is not known, but could very well represent the auto-ubiquitination of dTRAF2. The regulation of dTRAF2 expression levels have been linked to a de-ubiquitinating enzyme - dCYLD, which controls the levels and hence the degradation of dTRAF2(Xue et al., 2007). We also observe differences in the levels of dTRAF2^{wildtype} versus dTRAF2$^{C104A/H121A}$ expression (Figure 2.5b). The dTRAF2$^{C104A/H121A}$ are expressed at a higher level in comparison to dTRAF2^{wildtype}. This could represent the need for an internal quality control mechanism that ensures the optimum levels of dTRAF2 is available (K^{48}-linked auto-ubiquitination), to participate in cell death signaling (through K^{63}-linked auto-ubiquitination). The expression of dTRAF2^{wildtype} could potentially be essential to drive a sub-optimal levels of the auto-ubiquitinated protein, required to signal through Eiger, which accounts for a lower level of protein expression compared to the dTRAF2 RING mutants. TRAF6^{C70A,} has also been shown to be defective in K^{63}-linked auto-ubiquitination, which inhibits its participation in Nf-kB and MAPK signaling. The auto-

ubiquitination site of TRAF6 has been mapped to K124, which is well conserved in dTRAF2 (Deng et al., 2000; Lamothe et al., 2007). The *tubulinα1*>dTRAF2$^{C104A/H121A}$ may fail to undergo auto-ubiquitination, which could thus reflect a failure of auto-ubiquitin catalysis in addition to the misregulation of auto-expression, hence failing to participate in Eiger-induced apoptotic signaling. Future studies need to be performed to validate the E3 ubiquitin ligase activity of dTRAF2 *in vitro* and identify protein targets that are modulated by this TRAF family member, on the Eiger signaling network.

The current study highlights the importance of dTRAF2 in Eiger-mediated cell death signaling. We show that the levels of dTRAF2 is crucial for effective regulation of the signaling network, and minor perturbation of expression levels of dTRAF2 is sufficient to impair cell death signaling, potentially through a RING-dependent mechanism downstream of the flyTNF. Thus, the importance of ubiquitin dependent regulation of TRAF proteins is a phylogenetically conserved mechanism, which could be used to understand the divergent functions of TRAF proteins in mammalian signaling contexts.

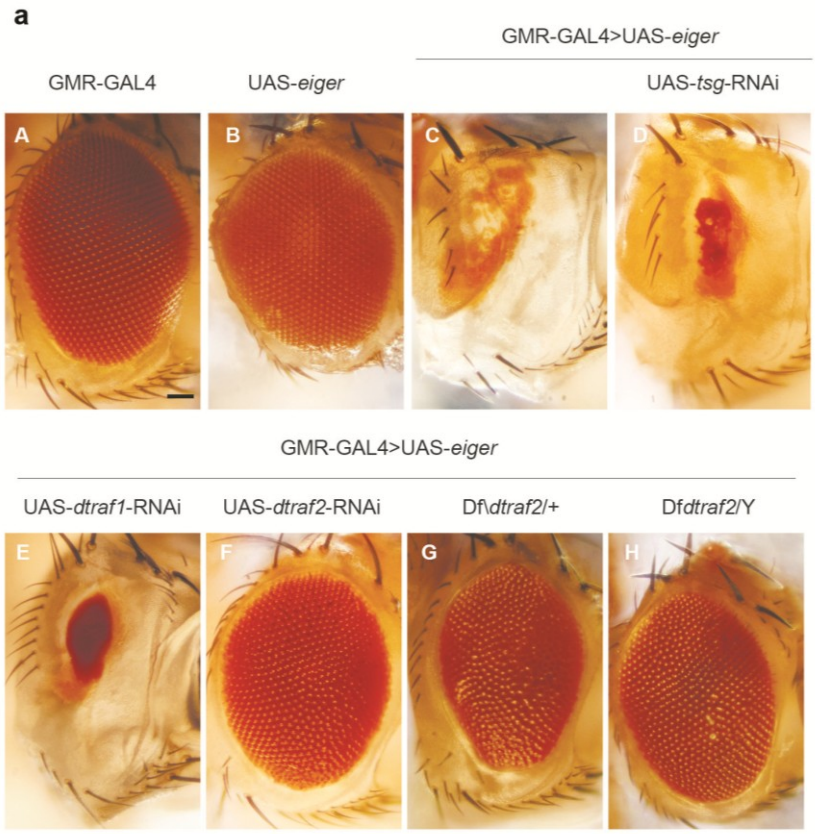

Figure 2.1.a dTRAF2 is indispensible for Eiger-induced cell death signaling

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A-H Over-expression of Eiger generates a 'small eye phenotype', that is unaffected by the loss of dTRAF1 expression, but completely suppressed by the loss of dTRAF2 expression. *Dfdtraf2*^{ex1}, is a null allele of dTRAF2. dTRAF2 is located on the X-chromosome, hence the heterozygous female fly, *Dfdtraf2*^{ex1}/+ has half the dosage of endogenous dTRAF2 expression levels and *Dfdtraf2*^{ex1}/*Y* represents the hemizygous male fly, which is a complete null for dTRAF2. The individual genotypes are listed below. UAS-*dTRAF1*-RNAi *and UAS-dTRAF2*-RNAi, express dsRNA for RNAi of dTRAF1 and dTRAF2 respectively, under UAS-control. Wild type eyes, A.GMR-GAL4/*CyO*, B. UAS-*eiger*/CyO. Over-expression of Eiger generates a 'small eye phenotype' C. GMR-GAL4,UAS-*eiger*/*CyO*. Small eye phenotype is unaffected by the knockdown of D. an unrelated gene, *tsg,* GMR-GAL4,UAS-*eiger*/+; UAS-*tsg*-RNAi/+ (used as a negative control), E. loss of dTRAF1 expression by RNAi, GMR-GAL4,UAS-*eiger*/+;UAS-*dtraf1*-RNAi/+; F. completely suppressed by dTRAF2 loss of expression using an RNAi line, GMR-GAL4,UAS-*eiger*/+;UAS-*dtraf2*-RNAi/+. The Eiger-mediated cell death phenotype is greatly suppressed by the loss of one copy of dTRAF2, as reflected in a heterozygous dTRAF2 background (dTRAF2+/-) G. *Dfdtraf2*^{ex1}/Fm7c,sn;GMR-GAL4,UAS-*eiger*/*CyO,* and completely suppressed in a hemizygous dTRAF2 background(dTRAF2-/-) H. *Dfdtraf2*^{ex1}/Y;GMR-GAL4,UAS-*eiger*/*CyO.* Scale bar - 100µm.

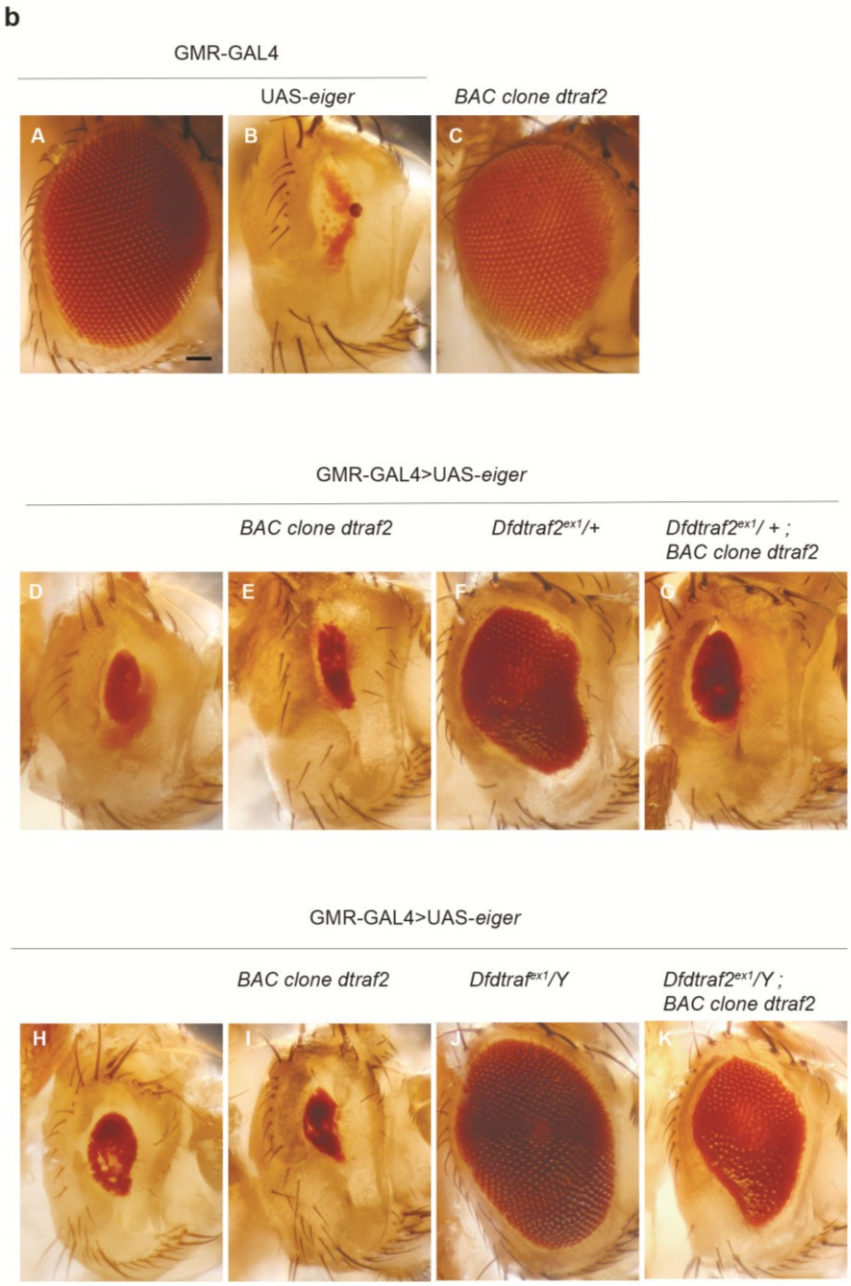

Figure 2.1.b dTRAF2 is indispensible for Eiger-induced cell death signaling

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A-K Introduction of a dTRAF2 genomic rescue transgene, strongly induces Eiger-mediated apoptosis in a dTRAF2 null background. *Dfdtraf2*^{ex1}, is a null allele of dTRAF2. dTRAF2 is located on the X-chromosome, hence the heterozygous female fly, *Dfdtraf2*^{ex1}/+ has half the dosage of endogenous dTRAF2 expression levels and *Dfdtraf2*^{ex1}*/Y* represents the hemizygous male fly, which is a complete null for dTRAF2. *PBAC(dtraf2)* is a genomic rescue dTRAF2 BAC clone that represents one endogenous copy of dTRAF2. The individual genotypes are listed below. A. Wild type eye, GMR-GAL4/*CyO*; B. 'small-eye' phenotype of Eiger over-expression, GMR-GAL4,UAS-*eiger*/*CyO*. *PBAC(dtraf2)* does not induce a cell death phenotype C. in the absence of Eiger, *PBAC(dtraf2)/+*.

The ‘small-eye’ phenotype of Eiger over-expression, in a female fly D. +/+; GMR-GAL4,UAS-*eiger*/+, is not enhanced by an additional genomic copy of dTRAF2, E. GMR-GAL4,UAS-*eiger*/+*; PBAC(dT2)/+.* However, suppression of Eiger-induced cell death observed in a heterozygous dTRAF2 (dTRAF2+/-) female fly, F. Df*dtraf2*^{ex1}/+;GMR-GAL4,UAS-*eiger*/+ is reverted by the introduction of a dTRAF2 genomic rescue transgene, G.Df*dtraf2*^{ex1}/+;GMR-GAL4,UAS-*eiger*/+; *PBAC(dT2)*/+. The ‘small-eye’ phenotype of Eiger over-expression, in a male fly, H.+/Y;GMR-GAL4,UAS-*eiger*/+, is not enhanced by the introduction of a genomic copy of dTRAF2, I. GMR-GAL4,UAS-*eiger*/+*; PBAC(dT2)/+.* The complete suppression of Eiger-induced cell death observed in a hemizygous dTRAF2 (dTRAF2-/-) male fly, J. Df*dtraf2*^{ex1}/Y;GMR-GAL4,UAS-*eiger*/+, is reverted by the introduction of a genomic copy of dTRAF2, K. Df*dtraf2*^{ex1}/Y;GMR-GAL4,UAS-*eiger*/+; *PBAC(dT2)/+.* Scale bar - 100µm.

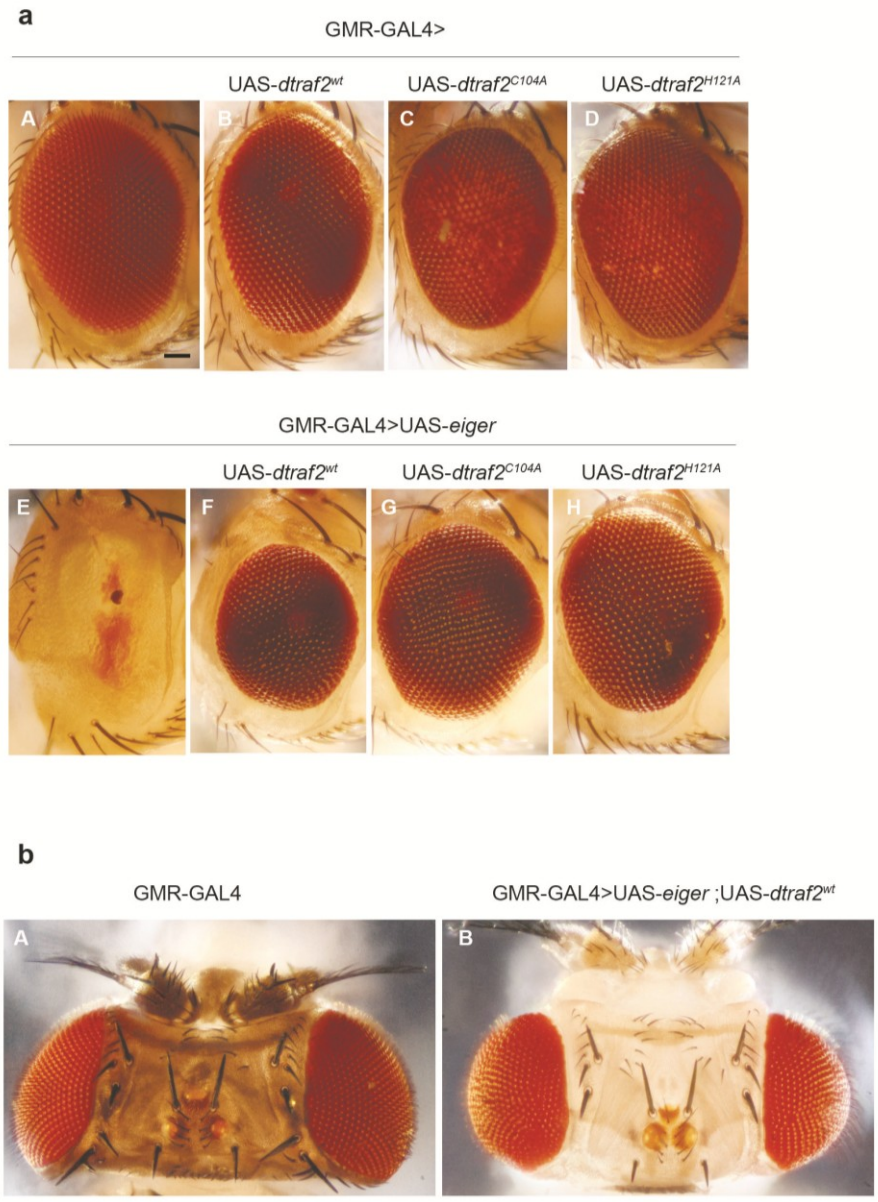

Figure 2.2 Transgenic over-expression of dTRAF2 does not potentiate Eiger-mediated cell death, but rather inhibits it.

a. Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A-H GMR-driven over-expression of UAS-*dtraf2* transgene, inhibits Eiger-induced apoptosis. The individual genotypes are listed below. A. Wild type eye, GMR-GAL4/*CyO*. Over-expression of B. wild type dTRAF2 transgene, GMR-GAL4/*CyO*;UAS-*dtraf2-wt*/*TM6B,Tb*[1], C. C104A RING mutant dTRAF2 transgene, GMR-GAL4/*CyO*;UAS-*dtraf2-C104A*/*TM6B,Tb*[1], and D. H121A RING mutant dTRAF2 transgene, GMR-GAL4/*CyO*;UAS-*dtraf2-H121A*/*TM6B,Tb*[1], does not induce cell death. The 'small-eye' phenotype of Eiger over-expression, E. GMR-GAL4,UAS-*eiger*/*CyO,* is suppressed by the co-expression of *F.* a wild type dTRAF2 transgene*,* GMR-GAL4,UAS-*eiger*/*CyO;*UAS-*dtraf2-wt*/*TM3,Sb*[1], G. a C104A RING domain mutant of dTRAF2, GMR-GAL4,UAS-*eiger*/*CyO;*UAS-*dtraf2-C104A*/*TM6B,Tb*[1], and by H. an H121A RING mutant of dTRAF2,

GMR-GAL4,UAS-*eiger*/*CyO;*UAS-*dtraf2-H121A*/*TM6B,Tb*[1]. Scale bar - 100µm.

b. Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. The transgenic over-expression of full length-wild type dTRAF2 with Eiger, generates a novel 'bulging-eye' phenotype. Genotype used in A. Wild type eye, GMR-GAL4/*CyO;TM3,Sb*[1]*/+,* and the 'bulging-eye' phenotype of Eiger and dTRAF2 co-expression, B. GMR-GAL4,UAS-*eiger*/*CyO;*UAS-*dtraf2-wt*/*TM3, Sb*[1].

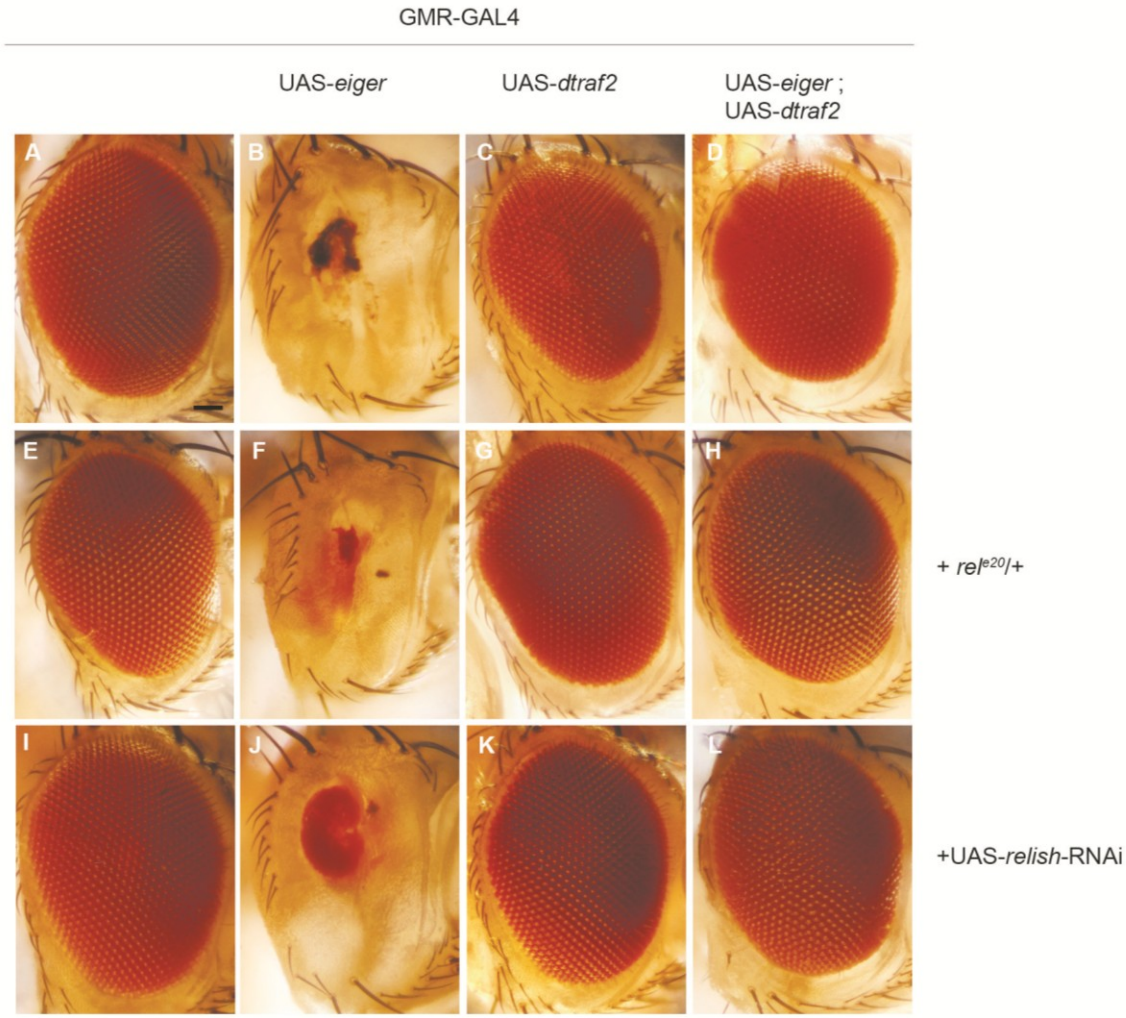

Figure 2.3 The suppression of Eiger-induced cell death by dTRAF2 over-expression, is not due to anti-apoptotic NF-κB signaling.

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A-L GMR-driven over-expression of *dtraf2* transgene, inhibits Eiger-induced apoptosis, independent of NF-κB signaling. rel^{e20} is a null allele for Drosophila NF-κB homolog, Relish. UAS-*relish*-RNAi, expresses dsRNA for RNAi of Relish under UAS-control. The individual genotypes are listed below. A. Wild type eye,GMR-GAL4/*CyO*; B. The 'small-eye' phenotype of Eiger over-expression, GMR-GAL4,UAS-*eiger*/*CyO; C.* dTRAF2 over-expression, GMR-GAL4/*CyO*;UAS-*dtraf2*/TM6B,Tb^1; D. The suppression of Eiger-induced cell death, by dTRAF2 expression, GMR-GAL4,UAS-*eiger*/*CyO;*UAS-*dtraf2*/*TM6, Tb*^{1}. The loss of one copy of Relish, rel^{e20}/+ does affect E. a wild type eye,GMR-GAL4/ rel^{e20}, F. 'small-eye' phenotype of Eiger over-expression, GMR-GAL4,UAS-*eiger*/rel^{e20}, *G.* dTRAF2 over-expression, rel^{e20}/GMR-GAL4;UAS-*dtraf2*/+, H. or the suppression of cell death by Eiger and dTRAF2 co-experession, GMR-GAL4,UAS-*eiger*/rel^{e20};UAS-*dtraf2*/+. The loss Relish expression using an RNAi line has the same effect as the Relish loss of function allele, rel^{e20} in a wild type eye, I.GMR-GAL4/UAS-*relish*-RNAi; Eiger over-expression

background, J. GMR-GAL4,UAS-*eiger*/UAS-*relish*-RNAi*;* dTRAF2 over-expression background, K. UAS-*relish*-RNAi*/*GMR-GAL4;UAS-*dtraf2*^{wildtype}/+ ; or Eiger and dTRAF2 co-expression background, L.GMR-GAL4,UAS-*eiger*/UAS-*relish*-RNAi*;*UAS-*dtraf2*/+,Scale bar - 100µm.

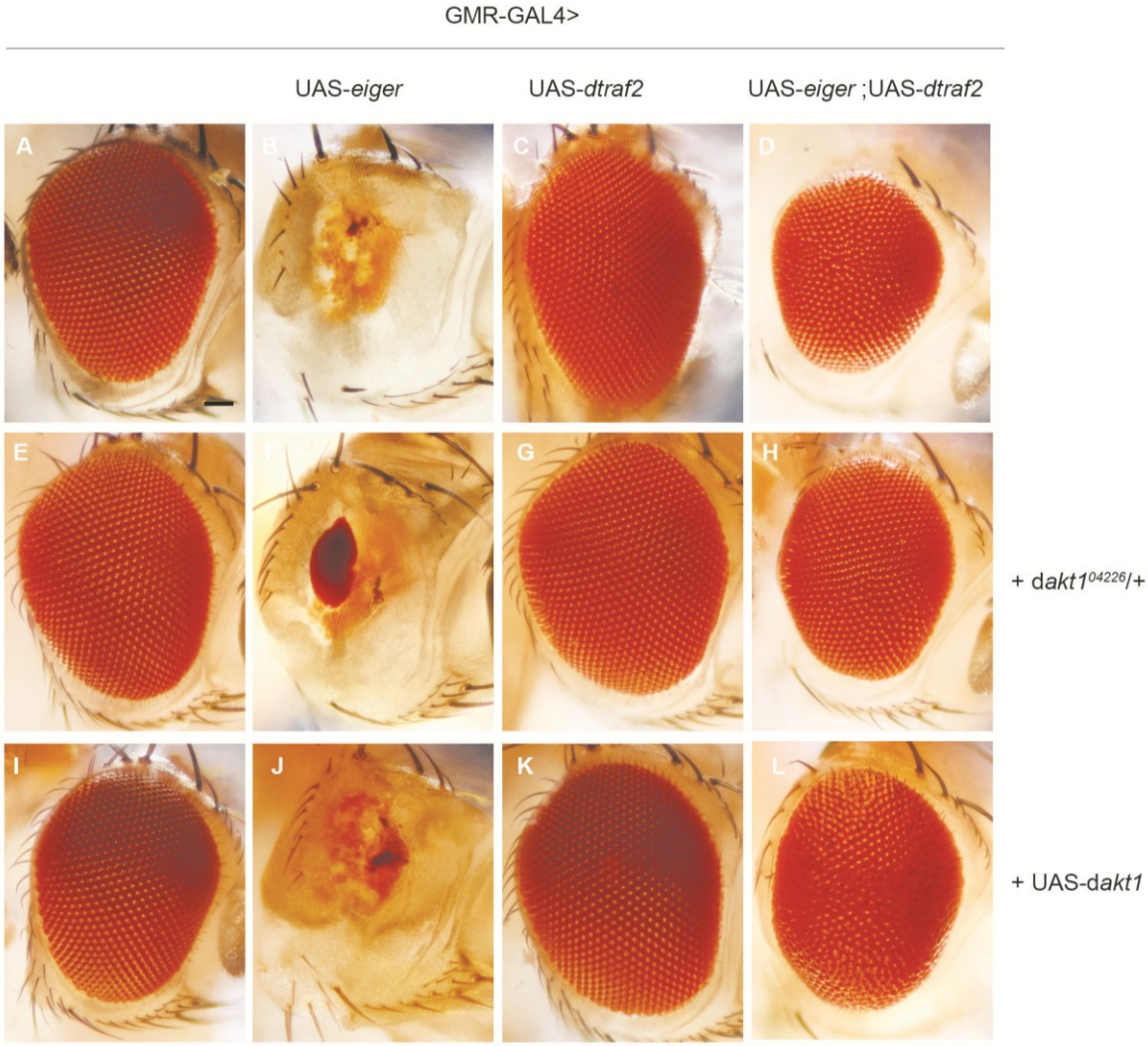

Figure 2.4.1 The suppression of Eiger-induced cell death by dTRAF2 over-expression, is not due to pro-survival dAkt1 signaling.

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A-L GMR-driven over-expression of *dtraf2* transgene, inhibits Eiger-induced apoptosis, independent of Akt1 signaling. *dakt1*^{04226} is a P-element insertion line, which represents a loss of function allele of Drosophila Akt1 homolog, dAkt1. UAS-*dakt1,* is a transgene that expresses wild-type dAkt1 under UAS control. The individual genotypes are listed below. A. Wild type eye, GMR-GAL4/+;*TM3,Sb*^{1}. B. The 'small-eye' phenotype of Eiger over-expression, GMR-GAL4,UAS-*eiger*/+;*TM3,Sb*^{1}/+. *C.* Over-expression of wild-type dTRAF2 transgene,GMR-GAL4/*CyO*;UAS-*dtraf2*/*TM3,Sb*^{1}, D. Co-expression of Eiger and dTRAF2, GMR-GAL4,UAS-*eiger*/*CyO;*UAS-*dtraf2*/*TM3, Sb*^{1}. The loss of one copy of dAkt1, *dakt1*^{04226}/+ does not affect E. wild type eye, GMR-GAL4/+;*dakt1*^{04226}/+, F. 'small-eye' phenotype of Eiger over-expression, GMR-GAL4,UAS-*eiger*/+;*dakt*^{104226}/+, *G.* dTRAF2 over-expression, GMR-GAL4/+;UAS-*dtraf2/dakt*^{104226}, H. or the suppression of cell death by Eiger and dTRAF2 co-expression, GMR-GAL4,UAS-*eiger*/+;UAS-*dtraf2*/*dakt*^{104226}. The transgenic over-expression of dAkt1 does not effect I. a wild type eye,

GMR-GAL4/UAS-*dakt1;TM6B,Tb*^{1}/+, J. the ‘small eye’ phenotype of Eiger over-expression,GMR-GAL4,UAS-*eiger*/UAS-*dakt1*, K. or dTRAF2 over-expression, UAS-*dakt1/*GMR-GAL4;UAS-*dtraf2*/+. The ectopic expression of dAkt1 does not revert the suppression of the Eiger cell death phenotype, by dTRAF2 over-expression, L. GMR-GAL4,UAS-*eiger*/UAS-*dakt1;*UAS-*dtraf2*/+,Scale bar - 100μm.

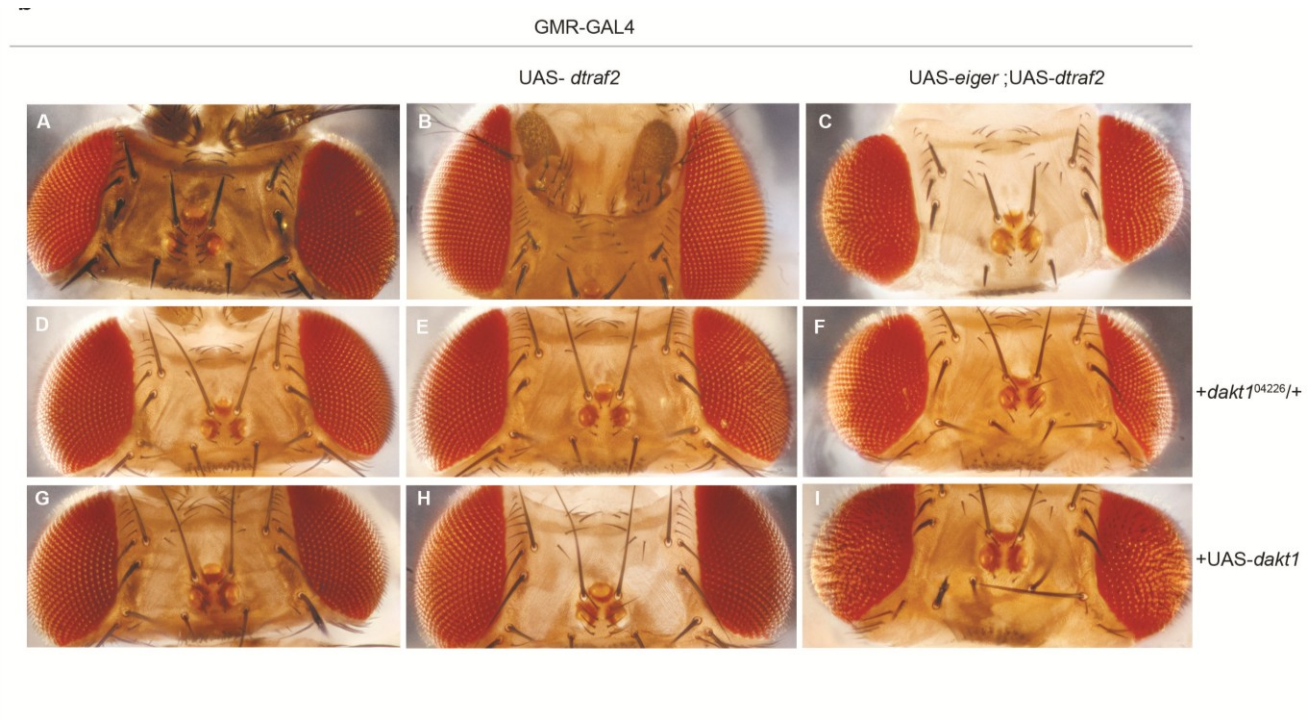

Figure 2.4.2 The Eiger - dependent 'bulging-eye' phenotype of dTRAF2 over-expression is due to dAkt1 signaling.

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A-I GMR-driven co-expression dTRAF2 and Eiger transgenes, generates a 'bulging-eye' phenotype, that is dependent of Akt1 signaling. The 'bulging eye' phenotype is suppressed by the loss of dAkt1 expression, *dakt*^{104226} and enhanced by the transgenic over-expression of wild type dAkt1. *dakt*^{104226} is a P-element insertion line, which represents a loss of function allele of Drosophila Akt1 homolog, dAkt1. UAS-*dakt1,* is a transgene that expresses wild type dAkt1 under UAS control. The individual genotypes are listed below. A. Wild type eye, GMR-GAL4/+*;TM3,Sb*^{1}/+; B. over-expression of dTRAF2, GMR-GAL4/*CyO*;UAS-*dtraf2*/TM3,Sb^{1}; C. and the 'bulging' eye' phenotype, induced by the co-expression of Eiger and dTRAF2, GMR-GAL4,UAS-*eiger*/*CyO;*UAS-*dtraf2*/TM3, Sb^{1}. The loss of one copy of dAkt1, *dakt1*^{04226}/+ does not affect D. the wild type eye, GMR-GAL4/+;*dakt*^{104226}/+; E. dTRAF2 over-expression phenotype, GMR-GAL4/+;UAS-*dtraf2/dakt*^{104226}; F. but inhibits the 'bulging-eye' phenotype of dTRAF2 and Eiger co-expression, GMR-GAL4,UAS-*eiger*/+*;*UAS-*dtraf2*/*dakt1*^{04226}. Ectopic expression of dAkt1 induces, G. a mild 'bulging-eye' phenotype GMR-GAL4/UAS-*dakt1;* H. that is unaffected by dTRAF2 over-expression,GMR-GAL4/UAS-*dakt1*;UAS-*dtraf2*/+; I. but potentiated by the co-expression of Eiger and dTRAF2, w^{1118}; GMR-GAL4,UAS-*eiger*/UAS-*dakt1/+;*UAS-*dtraf2*/+. Scale bar - 100μm.

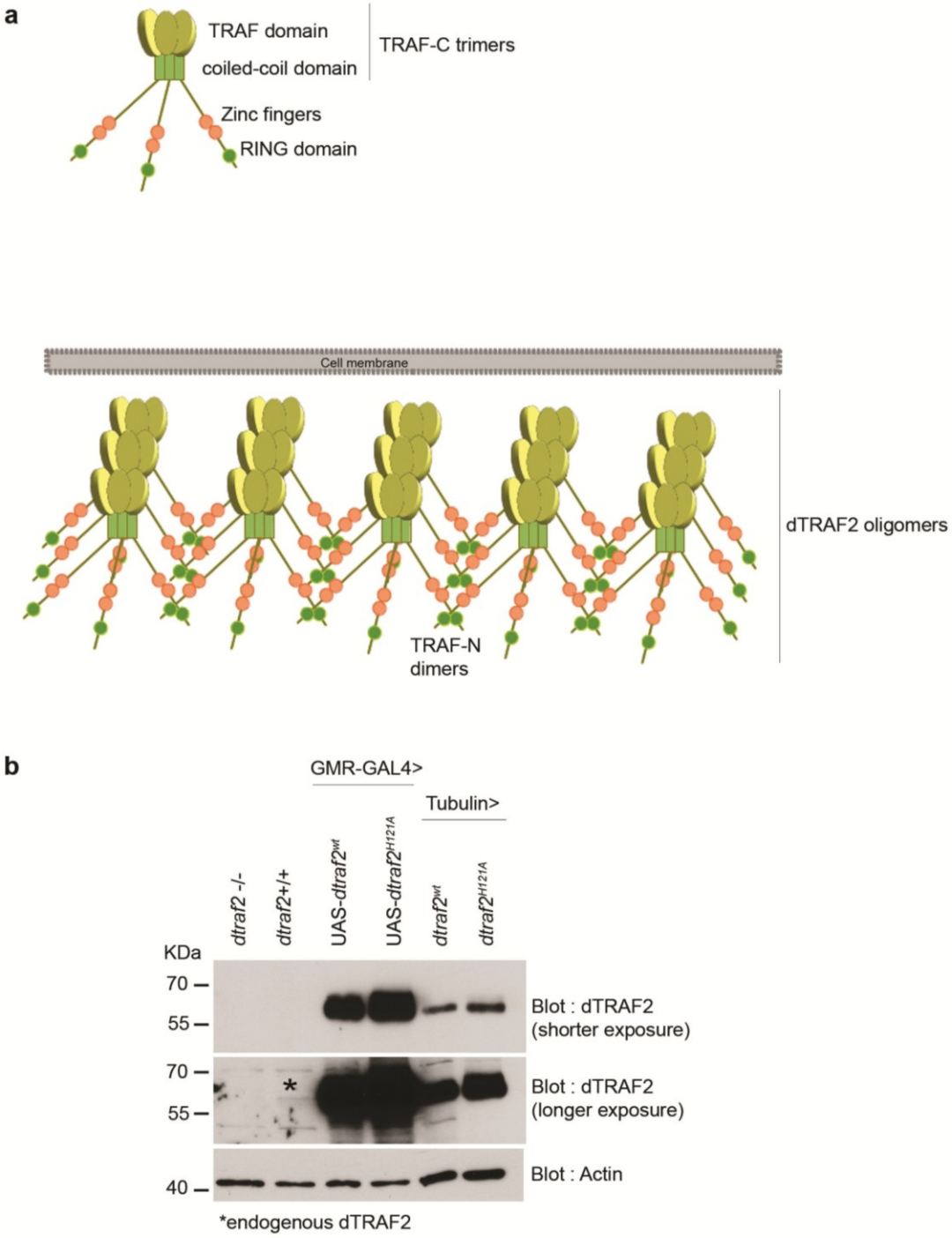

Figure 2.5 The expression levels of dTRAF2 is a critical determinant for Eiger-mediated cell death signaling

a. Illustration depicting the structure of dTRAF2, adapted from the oligomeric structure of mammalian homolog, TRAF6 (Wang et al., 2010; Yin et al., 2009b). The TRAF-C domains of individual TRAF monomers trimerize forming a mushroom-shaped head, which represents the receptor-binding structure. The TRAF-N, which constitutes the Zinc-finger and RING domains undergo heterologous dimerization, which upon oligomerization leads to the formation of an intricate lattice like scaffolding network.

b. Comparison of dTRAF2 expression levels in wild type, GMR-driven transgenic dTRAF2 levels and *tubulinα1* promoter driven transgenic dTRAF2 levels, from adult fly-heads. The levels of dTRAF2 in decreasing order of expression, GMR-driven>*tubulinα1*-driven>endogenous dTRAF2. $dTRAF2^{H121A}$ RING mutant is expressed at a higher level than $dTRAF2^{wildtype}$, under GMR or *tubulinα1* promoter control. Four fly heads/genotype were used for each sample. Western blotting of the lysates with anti-dTRAF2 sera and anti-actin antibody. Genotypes of the samples are as specified.

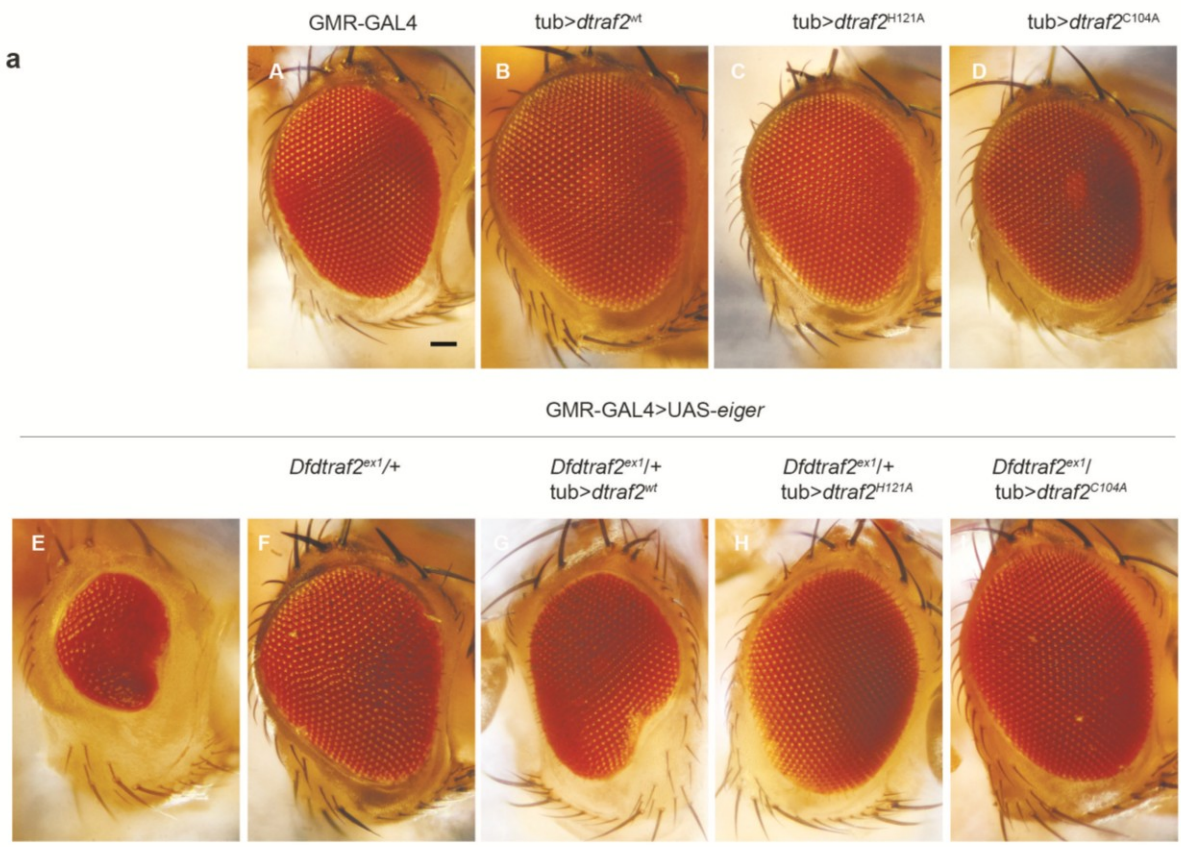

Figure 2.6 dTRAF2 induces Eiger-dependent cell death, in a RING domain domain dependent manner.

a. Light micrograph images of adult Drosophila eye phenotypes with indicated . Images taken at 10X magnification. A-I GMR-driven expression of UAS-*eiger* transgenes, generates a 'small-eye' phenotype, is suppressed in the absence of dTRAF2. The suppression of the small eye phenotype by the null allele of *dTRAF2* can be marginally reverted by introducing *dTRAF*^{wt}, driven by the *tubulinα1(tub) promoter. Dfdtraf2*^{ex1}, is a dTRAF2 null allele. *tubulinα1 promoter driven levels of RING mutants, dTRAF2*$^{C104A/H121A}$ *impair Eiger-signaling, in a heterozygous (Dfdtraf2*^{ex1}*/+) dTRAF2 background.* The individual genotypes are listed below. A. Wild type eye,GMR-GAL4/ *CyO*. The phenotype of *tubulinα1 promoter driven* B. wild type dTRAF2, *tubulinαdtraf2*^{wt}/TM3, Sb^{1}, C. H121A RING domain mutant dTRAF2, *tubulinαdtraf2*^{H121A}/TM3, Sb^{1}, D. C104A RING domain mutant dTRAF2, *tubulinαdtraf2*^{C104A}/Fm7. The 'small-eye' phenotype of Eiger over-expression in a female fly, E. GMR-GAL4,UAS-*eiger*/CyO, is suppressed by the loss of dTRAF2 expression in a dTRAF2 heterozygous female fly, F. *Dfdtraf2*^{ex1}/+;GMR-GAL4,UAS-*eiger*/+. The suppression of Eiger-induced cell death in a dTRAF2 heterozygous female fly, is partially reverted by tubulin promoter driven G. wild type dTRAF2, *Dfdtraf2*^{ex1}/+;GMR-GAL4,UAS-*eiger*/+; *tubulinαdtraf2*^{wildtype}/+, but not by tubulin promoter driven dTRAF2 RING mutants, H. dTRAF2^{H121A} - *Dfdtraf2*^{ex1}/+;GMR-GAL4,UAS-*eiger*/+; *tubulinαdtraf2*^{H121A}/+. or I. dTRAF2^{C104A} - *Dfdtraf2*^{ex1}/ *tubulinαdtraf2*^{C104A};GMR-GAL4,UAS-*eiger*/+. Scale bar - 100μm.

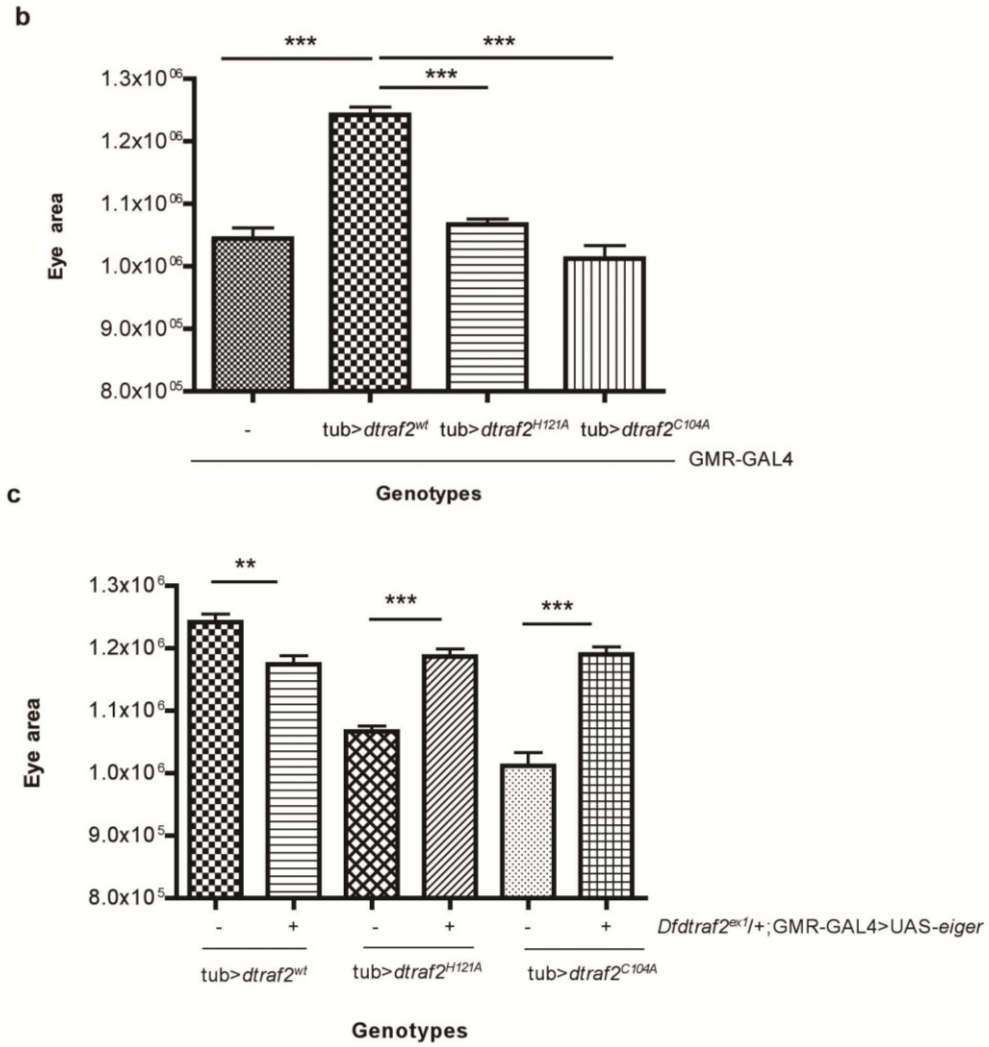

Figure 2.6 *dTRAF2 induces Eiger-dependent cell death*, in a RING dependent manner.

b. Quantification of the eye area of the indicated genotypes. *tubulinαdtraf2*^{wildtype} transgene expression (in an Eiger-independent manner) resulted in an increase of over-all eye size that is impaired in the *tubulinαdtraf2*$^{H121A/C104A}$ mutant transgenic flies. Eye area is compared to wild-type eye, GMR-GAL4. Comparisons between groups were made using One-way ANOVA ($F_{3,60} = 44.85$, $p<0.0001$), followed by Post hoc comparisons using Tukey's multiple comparison test. n=16 per genotype, values represent mean ± SEM, ***p<0.001.

c. Quantification of the eye area of the indicated genotypes. *tubulinαdtraf2*^{wildtype} *but not tubulinαdtraf2*$^{H121A/C104A}$ induces Eiger-dependent cell death in a dTRAF2 heterozygous null fly (*Dfdtraf2*^{ex1}*/+)*. There is a significant decrease in the area of the eye upon *tubulinαdtraf2*^{wildtype} *expression, in an Eiger-Dfdtraf2*^{ex1}*/+* background, that is not observed in the *tubulinαdtraf2*$^{H121A/C104A}$ mutant transgenic flies. Comparisons were made using paired-*t* test, n= 16, values represent mean ± SEM, **p<0.01, ***p<0.0001.

PREFACE TO CHAPTER 3

TNFR signaling receptors have evolved efficient regulatory mechanisms that prevent deregulated activation of its signaling networks. Further, the hierarchical engagement of signaling proteins is ensured by distinct post-translational modifications that either suppress or enhance cellular signaling events. Ubiquitin has emerged as an effective strategy to regulate the cellular outcome and function of proteins. The ubiquitin-dependent control of the JNK signaling module has not been explored due to the lack of robust tools to isolate and identify ligand activated ubiquitin modified conjugates. In this chapter we describe the generation and validation of a robust tool, to isolate and subsequently identify proteins that are selectively ubiquitinated in response to Eiger activation, that lie upstream of the fly JNK homolog - Basket. We discover an Eiger-specific enrichment of ubiquitin-conjugated targets that include Bendless and other well-conserved K^{48}-polyubiquitin conjugating E2s. We also genetically validate the non-redundant role of K^{63}-polyubiquitin conjugating enzyme, Bendless in pro-apoptotic Eiger signaling. This study represents, to our knowledge, the first to show the importance of ubiquitin-dependent regulation of the Eiger-JNK signaling network.

CHAPTER 3- The role of ubiquitin-dependent regulation in a Drosophila model of TNF signal transduction

Ambika Srinivasan[1], Dr. Wenjing Ruan[1], Tharan Srikumar[3], Dr. Ugo Mayor[2], Dr. Brian Raught[3], and Dr. Philip Barker[1]
[1]Montreal Neurological Institute, McGill University, Canada
[2]CICbioGUNE, Spain
[3]Princess Margaret Cancer Centre, University of Toronto, Canada

[1]**Corresponding author -** email: phil.barker@mcgill.ca

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Number of words: 3533
Figures : 3

3. ABSTRACT

The regulatory events that prevent the constitutive activation of any signaling network, is key to ensure cellular viability. In this study, we have utilized *Drosophila melanogaster*, as a model system to understand ancient and conserved mechanisms of ubiquitin-dependent control over TNF (Tumor Necrosis factor) signaling. We use an avidin-tagged ubiquitin construct that can be biotinylated *in vivo, and* readily incorporated to modify substrates. Proteins that are subjected to ubiquitination in response to TNF-α signaling can be enriched and efficiently detected by virtue of the tag. Using the power of fly genetics and molecular proteomics, we identify conserved proteins that ensure hierarchical control over the TNF-α signaling network.

3.1. INTRODUCTION

The fly homolog of TNF, termed Eiger, has been shown to induce apoptosis that is dependent on signaling by Basket, the Drosophila form of c-Jun N-terminal kinase (JNK) (Igaki et al., 2002; Moreno et al., 2002). Unlike the mammalian TNF-receptor (TNFR) superfamily, the Drosophila TNFR family consists of only two receptors Wengen and Grindelwald (Andersen et al., 2015; Kanda et al., 2002). Sequence similarity indicates that Wengen is more similar to the p75 neurotrophin receptor (p75NTR), than other members of the mammalian TNFR superfamily (Bothwell, 2006). p75NTR has been regarded as a pro-apoptotic receptor and has been shown to activate cell death that is dependent on caspase-9 and JNK activation, unlike the caspase-8 dependent mode of cell death induction of most other pro-apoptotic TNFRs (Bhakar et al., 2003; Chibuk et al., 2001; Friedman, 2000; Troy et al., 2002). The molecular events that allow Eiger, Grindelwald or p75NTR to induce the activation of JNK activation remain largely unknown.

Ubiquitination of proteins has emerged as a critical post-translational modification, which regulates several cellular events. The ubiquitin-system requires three major components, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and an E3 ubiquitin-ligase that collaborate to tag substrates with a variety of ubiquitin modifications, through one of the seven unique lysine residues present in ubiquitin. A typical ubiquitination reaction is initiated by ATP-dependent activation of a ubiquitin monomer. The ubiquitin is then transferred to a specific E2, which forms an E2-Ubq thioester. The E2-Ubq thioester interacts with a specific E3, which determines the specificity of the substrate modified by the ubiquitin linkage. The nature of the ubiquitin linkage (mono, di, or polyubiquitin) plays a major role in determining the outcome of the post-translational modification (reviewed in (Komander and Rape, 2012). The proteolytic targeting that is mediated by ubiquitination was first studied in the context of the NF-κB pathway (Chen et al., 1996), in response to TNF-α. The non-proteolytic function of polyubiquitin linkages are mainly involved in signal transduction pathways, specifically those of mammalian TNF-TNFRs (Sun and Chen, 2004). The first evidence of a non-proteolytic ubiquitin modification was shown by TRAF6-dependent, Ubc13-Uev1a mediated polyubiquitination of IKK (Deng et al., 2000). TNF-dependent signaling pathways are

activated in a hierarchical manner that is initiated with ligand binding to the receptor and culminates in the engagement of proteins that exert transcriptional control over relevant genes, required to transmit the cellular outcome. The transmission of this signal from the plasma membrane to the nucleus requires a tightly coordinated signaling platform, to prevent deregulation of normal physiology. The deregulation of apoptotic signaling pathways can be traced to a growing number of diseases, which has fuelled a massive accumulation of research over the years, to understand the regulatory determinants of it's signaling pathways.

In our current study, we have undertaken a proteomic approach, using *Drosophila melanogaster* as a model system, to identify critical regulators in the Eiger-mediated JNK signaling pathway. The identification of conserved proteins in the invertebrate p75NTR signaling pathway would shed light over mechanisms of ubiquitin–driven p75NTR regulation in mammals.

3.2. MATERIALS AND METHODS

Drosophila stocks and genetic crosses –

Fly line	*genotype*	*Collection*
UAS-*tsg*-RNAi	w[1118];P{GD6488}v45355/TM3	VDRC
UAS-*uev1a*-RNAi[1]	w[1118];P{GD6650}v32267	VDRC
UAS-uev1a-RNAi[2]	P{KK110298}VIE-260B	VDRC
UAS-*bendless*-RNAi	w[1118]; P{GD1387}v9413	VDRC
UAS-*ubcD1*-RNAi	w[1118]; P{GD10600}v26011	VDRC
UAS-*ubcD2*-RNAi	w[1118]; P{GD14776}v31158	VDRC
UAS-*ubcD4*-RNAi	w[1118]; P{GD13878}v35872	VDRC
UAS-*CG7656*-RNAi	w[1118]; P{GD13193}v26881	VDRC
UAS-*CG40045*-RNAi	P{KK115994}VIE-260B	VDRC
UAS-*basket*-RNAi	w[1118];P{GD10555}v34139/CyO	VDRC
ben[1]	w[118] ben[1]/C(1)A, y[1]	BDSC
UAS-*eiger*	yw; UAS-eiger/CYO(T655 D449)	Dr.Konrad Basler (Moreno et al., 2002)
GMR-GAL4	w[1118]; P{GMR-GAL4.w[-]}2/CyO	BDSC
UAS-*birA*	y[1] w[*]; P{w[+mC]=UA-birA}3	Dr.Ugo Mayor (Franco et al., 2011)
UAS-*ubqbirA*	y[1] w[*]; P{w[+mC]=UAS-Ubi6-birA}3	Dr.Ugo Mayor (Franco et al., 2011)

VDRC=Vienna Drosophila RNAi Center

BDSC =Bloomington Drosophila Stock Center

All stocks were raised in standard Drosophila media (recipe taken from BDSC) and all crosses were carried out at 25°C.

Imaging - Adult fly eyes were photographed using with a Canon EOS 1000D DSLR (rebel XS) camera mounted on a Zeiss Axioskop 40 microscope with a10× objective (0.25 = N.A). Several images of the adult fly eye were taken at different focal planes, for extended depth. The focus stacking of each set of images was done using Helicon Focus software (HeliconSoft) to generate the final image.

Dissection of larval eye-brain complex – About 72 hours after egg laying, the Drosophila third-instar larvae stop feeding and climb onto the walls of the fly vial. The larvae are gently removed from the walls of the vial using a pair of blunt forceps and

suspended with the dorsal side up, in 1× phosphate buffered saline (1×PBS), on a Sylgard dish. The anterior end of the larvae containing the mouthparts was immobilized with a pair of forceps. Another pair of forceps was used to immobilize the posterior end of the larva and gently pull the body of the larva away from the anterior end, leaving the imaginal disc complex and the brain attached to the anterior mouthparts. Any extraneous tissues attached to the eye-brain complex was carefully cleaned and the tissue was immediately frozen at -20°C and stored at -80°C for later use.

Extract preparation and streptavidin pull downs – Twenty eye-brain complexes of the specified genotypes, were dissected and homogenized under denaturing conditions using 375 µl of Lysis buffer. The lysate was centrifuged at maximum speed on a tabletop centrifuge briefly. The resulting supernatant was mixed with 1125 µl of Binding buffer, bringing the total volume of each sample to 1500 µl. The lysates were incubated with 75 µl of streptavidin-agarose bead suspension (Thermo Scientific) for 2 hours, at room temperature. The beads were washed with 1ml Lysis buffer (once) followed by 1×PBS (thrice). The beads were re-suspended in 75 µl of 2× Laemmli sample buffer and boiled for 5 minutes prior for their separation using SDS-PAGE. The mass spectrometry analysis was performed on 200 eye-brain complexes for each genotype, using the same protocol described above. The volume of buffers used was 500µl of Lysis buffer, 1500 µl of Binding buffer. The lysates were incubated with 100 µl of streptavidin-agarose bead suspension for 2 hours, at room temperature. The beads were washed (same as above) and immediately stored at -80C. Lysis buffer composition – Buffer made in 1×PBS containing 8M Urea, 1%SDS, 50mM *N*-ethylmaleimide, supplemented with protease inhibitor cocktail (Complete Mini Protease Inhibitor Tablets, Roche Molecular Biochemicals, Basel, Switzerland). Binding buffer composition – Buffer made in 1×PBS containing 1M NaCl, 50mM *N*-ethylmaleimide, supplemented with protease inhibitor cocktail.

Mass spectrometry and analysis - The mass spectrometry and subsequent analysis was performed as previously described (Dingar et al., 2015).

Western blotting – After the samples were separated by SDS-PAGE, the proteins were transferred onto nitrocellulose membranes at 100V for 90 minutes. The

membranes were rinsed in 1×TBST and blocked in 1× TBST (10 mM Tris pH 8.0, 150 mM NaCl, 2% Tween 20) supplemented with 5% (w/v) dried skim milk. Primary and secondary antibody incubations were performed in 1× TBST containing 2.5% (w/v) dried skim milk blocking solution. Incubation with primary antibodies (mouse monoclonal anti-Biotin 1:2000,Jackson ImmunoResearch); mouse monoclonal anti-Ubiquitin [PD41] 1:2000,Covance) were performed overnight at 4°C. Poly-HRP secondary antibody (used between 1:2000 – 1:5000 dilutions, Jackson ImmunoResearch) incubations were performed for 1 hour at room temperature. Membranes were washed repeatedly in 1× TBST after each incubation. Immunoreactive bands were detected using enhanced chemiluminescence solution kit (Perkin-Elmer Life Sciences, Norwalk, CT, USA), as per the manufacturer's instructions.

3.3. RESULTS

In vivo biotinylation of ubiquitin to identify Eiger dependent ubiquitinated conjugates- We have utilized flies that express an upstream activation sequence (UAS) transgene that constitutes a polyubiquitin chain, fused with a C-terminus Biotin ligase (BirA) enzyme (Franco et al., 2011). This resulting construct (Ubq-BirA) was expressed under the control of the Glass multimer reporter (GMR) promoter, which drives transgene expression in all the post-mitotic cells posterior to the morphogenic furrow, of Drosophila third-instar larva eye-imaginal disc (Ellis et al., 1993; Freeman, 1996).

In order to isolate novel ubiquitinated conjugates downstream of Eiger, we activated signaling by the ectopic expression of Eiger (GMR-GAL4>UAS-*eiger*) and prevented apoptosis by suppressing the expression of the terminal kinase of the pathway, Basket (Figure 3.1a-E). Thus, the Ubq-BirA transgene was co-expressed with Eiger and UAS-*basket*-RNAi. We also co-expressed the Ubq-BirA transgene with GMR-GAL4>UAS-*basket*-RNAi, which represents the baseline level of proteins that are biotinylated in the absence of both JNK and Eiger expression. The BirA transgene, lacking the fused polyubiquitin was co-expressed with GMR-GAL4>UAS-*basket*-RNAi, in the presence and absence of Eiger. Both these samples served as our negative controls, to represent the non-specific biotinylation of endogenous proteins. This strategy allowed us to accumulate targets that were specifically ubiquitinated in eye-brain complexes of flies expressing Eiger (Figure 3.1b).

Biotinylated conjugates can be isolated efficiently, using streptavidin-agarose beads, owing to the high affinity of avidin for biotin and the efficient biotinylation of ubiquitin observed is our system, as shown in Figure 3.1c. GMR drives expression mainly in the eye and to examine Eiger-induced ubiquitination events, we examined proteins within the larval eye-brain complex. We performed streptavidin pull-downs to enrich for biotinylated proteins within eye-brain lysates and then performed immunoblots. We observed that Eiger expression induced a massive accumulation of biotinylated proteins (Figure 3.1c, 3.2a lysates), whereas the expression of UAS-*birA* or UAS-*basket*-RNAi with the Ubq-BirA transgene, did not increase levels of biotinylated ubiquitin conjugates (Figure 3.1c). Therefore, Eiger expression specifically induces the accumulation of ubiquitinated target proteins.

Identification of ubiquitination targets induced by Eiger -To identify specific proteins that become ubiquitinated in response to Eiger expression, we dissected 200 eye-brain complexes from GMR-GAL4>UAS-*eiger* flies crossed to UAS-*ubqbirA* and UAS-*basket*-RNAi. These were lysed, insoluble material was removed and then the cleared lysates were incubated with streptavidin-agarose beads. The streptavidin-agarose beads were washed, bound proteins were trypsinized and eluted peptides were analyzed using nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS). MS data analysis was done using XTandem search engine and the peptides were identified using (iProphet) analysis as part of the Trans-Proteomic Pipeline (TPP), as described previously (Dingar et al., 2015).

When complied across all conditions, a total of 87 proteins with a TPP probability score of > 0.7 were identified. 48 of these were present in all samples, including the control conditions used in this study [GMR-GAL4>UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA/TM6,Tb*^{1}],[GMR-GAL4>UAS-*basket*-RNAi/*Bc*,UAS-*birA/TM6,Tb*^{1}], [GMR-GAL4>UAS-eiger,UAS-*basket*-RNAi/*Bc;*UAS-*birA/TM6,Tb*^{1}], and were therefore excluded as non-specific background proteins. Of the 39 proteins that remained in this pool, we excluded 22 that had total spectral counts (TSC) less than 10, leaving a total of 17 proteins (Figure 3.2b). However, two E2 family members, UbcD2 and UbcD4, that were just below our arbitrary cut-off of TSC < 10 were included in subsequent analyses. Figure 3.2b lists the 19 proteins that emerged from the screen, along with the TSC for each, obtained from flies that did or did not express Eiger. For 17 of the 19 proteins, spectral counts were increased by Eiger expression, indicating that these proteins became ubiquitinated in the presence of Eiger, whereas 2 of the 19 proteins showed an Eiger-dependent decrease in ubiquitination. Based on available functional data in FlyBase, these were grouped into four functional categories: ubiquitination system, oxidative stress, axogenesis and miscellaneous. The largest of these groups were proteins involved in ubiquitination and our subsequent analyses focused on this group.

Critical E2s that regulate Eiger-dependent cell death signaling - Six E2s (Bendless, UbcD1/Effete, UbcD2, UbcD4, CG40045, CG7656) that showed increased ubiquitination in flies that overexpressed Eiger are listed, with putative

mammalian homologs, in Figure 3.3a. Of these, Bendless was most highly ubiquitinated in response to Eiger expression. Bendless is the Drosophila homolog of Ubc13, an E2 required for K^{63}-mediated ubiquitination that is essential for mammalian TNFR (Deng et al., 2000), Toll-like receptor (TLR) (Fukushima et al., 2007) and IL-1 (Yin et al., 2009b) signaling pathways. To establish whether Bendless is required for Eiger-induced cell death, the Eiger-dependent small-eye phenotype was assessed in flies depleted of Bendless using RNAi and in flies lacking the *bendless* allele. Figure 3.3b-F,G,H shows that flies lacking Bendless function, show no evidence of photoreceptor cell death, indicating that Bendless normally plays an indispensible role in Eiger-induced apoptosis. In mammalian systems, Ubc13 functions in concert with an essential structural component, Uev1a (Andersen et al., 2005; Deng et al., 2000) but depletion of Drosophila Uev1a (using two independent UAS-*uev1a*-RNAi lines (Figure 3.3b-D,E) did not have a significant effect on Eiger-induced cell death.

UbcD1/effete and UbcD2 are closely related E2s that share strong phylogenetic conservation with yeast UBC4/5 homologs. UbcD1/effete has been implicated in a broad array of Drosophila functions (Cipressa and Cenci, 2013) but UbcD2 is less well characterized. RNAi-mediated knockdown of UbcD1 (Figure 3.3b-M) or UbcD2 had only minor effects on Eiger-induced cell death (Figure 3.3b-L). Therefore, we conclude that these closely related E2s are likely to have compensatory effects that together normally contribute to Eiger-induced cell death signaling pathways.

UbcD4 – UbcD4 was identified as an E2 required during embryonic development and cell cycle progression (Canning et al., 2002; McPhee et al., 2013). It does not appear to be a major player in Eiger-induced cell death since RNAi-mediated knockdown of UbcD4 produced only a minor suppression of the small eye phenotype (Figure 3.3b-I).

CG40045 and CG7656 – Two E2s identified in the screen (Figure 3.3a) have not previously been characterized in Drosophila. They were closely related to one another and both showed sequence similarity to mammalian Ubc7, an E2 implicated in signaling and in proteasomal degradation of mis-folded proteins (Kostova et al., 2007). Individual knockdown of CG40045 and CG7656 did not reduce Eiger-induced cell death (Figure 3.3b-J,K), suggesting that CG40045 and CG7656 may exert overlapping activities that facilitate Eiger-induced cell death.

3.4. DISCUSSION

Here we have identified ubiquitinated conjugates that are regulated in the developing eye by the Eiger signaling cascade. The enrichment of ubiquitinated targets in response to Eiger, demonstrates that ubiquitin-based signaling plays a crucial role in transducing signals initiated by the Drosophila TNF homolog.

Several of the key targets identified in our screen were phylogenetically conserved E2s components. Ubc13, the mammalian homolog of Bendless, has been studied extensively in mammalian signaling pathways, and is the only E2, other than Rad6 (Silva et al., 2015) to catalyze non-proteolytic K^{63}-linked polyubiquitination (Hofmann and Pickart, 2001).Mammalian Ubc13 and Uev1a function as a stable complex in the catalysis of non-degradative poly-ubiquitin chains (Andersen et al., 2005; Deng et al., 2000). The fly homologs, Bendless and dUev1a have also shown to collaborate in IMD-innate immune signaling (Zhou et al., 2005b) and NOPO–induced cell death (Ma et al., 2012). Thus, our biochemical and genetic data confirms the importance of Bendless in this cascade, as recently shown by Xianjue Ma and colleagues (Ma et al., 2014). We observe a minor suppression of Eiger-induced cell death upon loss of dUev1a expression, by two independent RNAi lines. Our data does not discard the dependence of dUev1a in Eiger-induced cell death signaling, which has been validated by other groups, using a P-element *DG14805* mutant and deficiency lines, *Df(3L) Exel6104* and *Df(3L)Exel6105* (Ma et al., 2013b). The partial effect of the dUev1a RNAi lines could be attributed to the fact that RNAi does not always generate a complete null phenotype. However, we did not observe the association of Bendless with dUev1a in our screen, which raises important questions regarding the involvement of dUev1a with Bendless, in Eiger-mediated ubiquitin regulation.

UbcD1/Effete has been implicated as a key regulator of several processes ranging from morphogenesis, oogenesis/spermatogenesis, dendritic pruning, alternation of microtubule dynamics, chromatin remodeling and apoptosis, to name a few, reviewed in (Cipressa and Cenci, 2013). In the context of apoptosis, UbcD1 has been shown to be essential for the turnover of anti-apoptotic protein, DIAP1 in response to cell death inducers, Reaper or Grim (Ryoo et al., 2002; Yoo, 2005). UbcD2 has been shown to have a minor role in axon pruning of Drosophila

mushroom body neurons, during metamorphosis (Watts et al., 2003). Here, we show that UbcD1 and UbcD2 are ubiquitinated in flies overexpressing Eiger, but fail to demonstrate suppression of Eiger-mediated cell death upon loss of expression of the individual E2s by RNAi. Hence, we propose that UbcD1 and UbcD2 share overlapping functions on the Eiger signaling pathway and are currently testing the combined genetic suppression of the two E2s. Similarly, we identified two mammalian Ubc7 homologs (CG40045 and CG7656) as targets of Eiger-induced ubiquitination that when individually depleted, do not inhibit Eiger–induced killing. Future studies aimed at co-depletion of the two closely related E2s would address the importance of the two novel E2s, in the Eiger signaling pathway.

Activation of the Eiger signaling cascade also led to the identification of a ubiquitinated pool of proteins, that have not been genetically validated in our current study, and present interesting targets for future studies (Figure 3.2b). We have recovered Eiger specific enrichment of proteins that are linked to mitochondrial energy homeostasis, such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Aconitase. Over-expression of Eiger has been linked with the induction of oxidative stress, which is dependent on genes associated with mitochondrial energy homeostasis, such as GAPDH and Aconitase. Through the generation of reactive oxygen species (ROS) in the mitochondria, Eiger was shown to signal apoptosis in tumorigenic cells, thus functioning as an intrinsic tumor suppressor (Igaki et al., 2009; Kanda et al., 2011). Our study also validates the association of GADPH and Aconitase with the Eiger signaling network.

We have also isolated interesting targets, which suggest a strong mechanistic association of Eiger with the DNA damage response. We observe an Eiger-specific accumulation of proteins implicated in DNA repair mechanisms. One such protein is Rad23, a DNA damage sensor that participates in Nuclear excision repair, by regulating the turnover of the it's partner and DNA binding protein, Rad4 (Xie et al., 2004). We observe a strong accumulation of ubiquitinated Histone2A in response to Eiger over-expression. Ubiquitination of Histone2A has been associated with DNA damage repair mechanisms (Messick and Greenberg, 2009). Mono-ubiquitination of K119 residue in Histone2A is catalyzed by the PRC1 complex (Wang et al., 2004), is commonly associated with gene repression. Recent data suggests a role for PRC1-

dependent gene repression as an essential mechanism for DNA damage protection (Facchino et al., 2010; Gieni et al., 2011; Pan et al., 2011). However K^{63}-linked polyubiquitination of K13-15 of Histone2A is mediated by RNF8/RNF168 (Mattiroli et al., 2012) and has also been linked to DNA damage response of a cell. Df31, is a Histone3 binding protein that is associated with chromatin compaction (Crevel et al., 2001; Guillebault and Cotterill, 2007; Schubert et al., 2012), was also recovered in our screen. These targets suggest a strong mechanistic association of Eiger with the DNA damage response. Future studies will need to be performed to understand the mechanism of mitochondrial derived ROS, in mediating Eiger-regulated cell death that potentially involves DNA damage sensors. DNA damage response genes are often mutated in cancers and deregulation of this safety mechanism could lead to uncontrolled tumor progression, reviewed in (Khanna and Jackson, 2001). Given the well-established role of Eiger as an intrinsic tumor suppressor (Igaki et al., 2009), these protein targets could provide mechanistic insights of TNF-dependent tumor suppression in metazoans.

We have also isolated genes such as Fax and Nrt, that have been implicated in DAbl signaling. DAbl signaling is essential for the formation and maintenance of the neuronal architecture in the embryonic central nervous system (CNS) (Henkemeyer et al., 1990; Hill et al., 1995). The selective expression of Eiger in the Drosophila nervous system, (Igaki et al., 2002), could suggest a novel link between Eiger signaling and axonal growth.

A recent study highlighted the importance of MAGE proteins as regulators of ubiquitination events by serving as RING-E3 ubiquitin ligase facilitators (Doyle et al., 2010). The Type II MAGE family of proteins are being extensively studied as key proteins involved in cell cycle regulation and cell death (Barker and Salehi, 2002). Work from our lab has previously shown the importance of NRAGE (MAGE-D1), as a p75NTR interacting protein in mediating JNK dependent cell death in vivo (Bertrand et al., 2008a; Salehi et al., 2000; Salehi et al., 2002). We also observe a requirement of dMAGE, the Drosophila homolog of NRAGE in the regulation of ubiquitinated targets downstream of Eiger (data not shown). Using this robust tool, future studies aimed at the analysis of Eiger-induced global ubiquitin changes, that are sensitive to

dMAGE loss of function, could provide essential clues to the regulatory role of dMAGE in Eiger signal transduction.

We have generated an important tool, which to our knowledge is the first to permit the isolation and analysis of global protein changes that occur in response to TNF-α, treatment, upstream of JNK activation. This genetic tool can be thus be used to understand the modulatory role of ubiquitination in the regulation of several signal transduction networks, downstream of ligand activation.

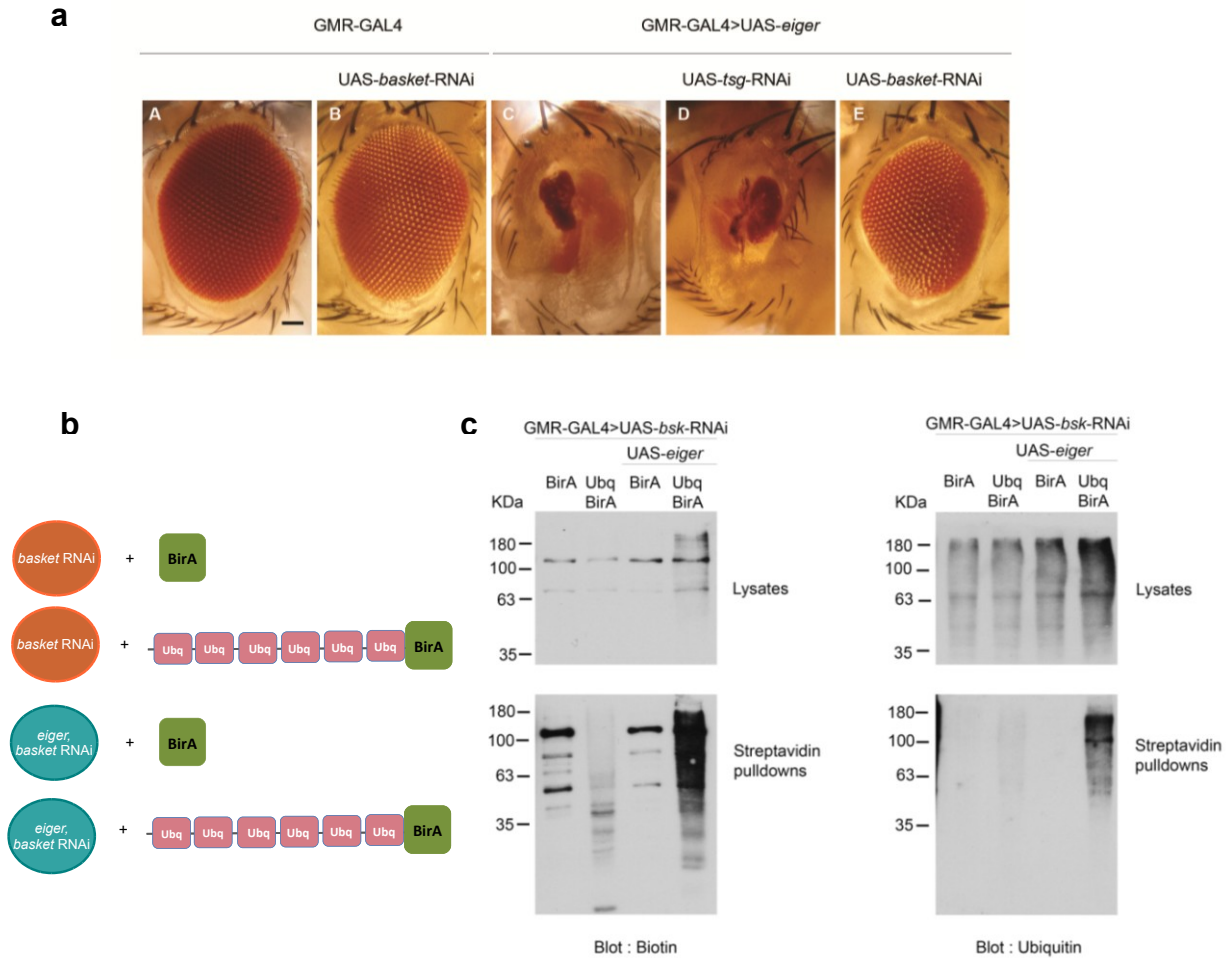


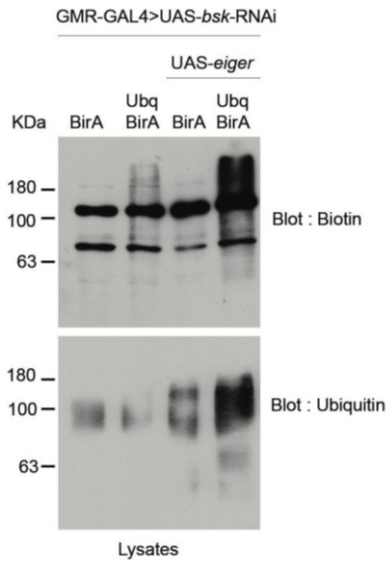
Figure 3.1 Increase in the ubiquitination of target proteins, in response to eiger over-expression.

a. Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A-E Over-expression of Eiger generates a 'small eye phenotype', that is dependent on JNK signaling. Basket(bsk), Drosophila homolog of JNK. bsk knockdown using a transgenic RNAi line, suppresses and interrupts Eiger-induced cell death. Genotypes used in A. GMR-GAL4/ *CyO*, B. GMR-GAL4,UAS-*basket*-RNAi/*CyO* , C.GMR-GAL4,UAS-*eiger*/*CyO*, D. GMR-GAL4,UAS-*eiger*/+ ; UAS-*tsg*-RNAi/+(used as a negative control), E. GMR-GAL4,UAS-*eiger*,UAS-*basket*-RNAi/+;*TM3,Sb*^{1}/+. Scale bar - 100µm

b. Scheme showing the genotypes used in the study using the in *vivo* biotinylation of ubiquitin system. UbqBirA, a poly-ubiquitin precursor with a C-terminus biotin ligase (BirA) and N-terminus biotin acceptor peptide tag. GMR driven expression of this construct allows the poly-ubiquitin to be cleaved by endogenous de-ubiquitinating enzymes, allowing individual ubiquitin monomers to be biotinylated in vivo by the BirA enzyme. Over-expression of the ubiquitin precursor with Eiger in the presence of Basket (bsk) knockdown, to isolate ubiquitinated proteins that accumulate downstream of eiger and upstream of bsk.

c. Isolation of biotin-tagged ubiquitinated targets by streptavidin pull-down. Western blotting with anti-biotin and anti-ubiquitin, showing an increase in both biotin and ubiquitin in response to Eiger over-expression. 20 eye-brain complexes were used for each pull-down. Genotype of the main experimental sample - GMR-GAL4,UAS-*eiger*,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA/TM6B,Tb*[1]. Control conditions used in this study[GMR-GAL4,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA*/*TM6B,Tb*[1]],[GMR-GAL4,UAS-*basket*-RNAi/*Bc*;UAS-*birA*/*TM6B,Tb*[1]], [GMR-GAL4,UAS-*eiger*, UAS-*basket*-RNAi/*Bc*;UAS-*birA*/*TM6B,Tb*[1]].

a



b

Gene ID	Gene Name	Annotation Symbol	Total spectral counts (TSC)				Protein name	Protein function
			bsk RNAi,ubqBirA		*egr, bsk RNAi,ubqBirA*			
			TSC1	TSC2	TSC1	TSC2		
Proteins associated with the ubiquitin system								
35998	*uba1*	CG1782	97	81	276	262	Ubiquitin activating enzyme 1	Ubiquitin activating enzyme (E1)
38456	*ubi-p63E*	CG11624	311	318	600	619		Ubiquitin precursor
32358	*ben*	CG18319	3	2	80	78	Bendless	Ubiquitin conjugating enzyme (E2)
3355079	*CG40045*	CG40045			33	30	(Uncharecterized protein)	Ubiquitin conjugating enzyme (E2)
41785	*ef/ubcD1*	CG7425			28	23	Effete	Ubiquitin conjugating enzyme (E2)
39691	*CG7656*	CG7656			15	14	(Uncharecterized protein)	Ubiquitin conjugating enzyme (E2)
34487	*ubcD2*	CG6720			9	6	Ubiquitin conjugating enzyme 2	Ubiquitin conjugating enzyme (E2)
39133	*ubcD4*	CG8284			9	7	Ubiquitin conjugating enzyme 4	Ubiquitin conjugating enzyme (E2)
Proteins involved in oxidative stress								
3772632	*his2A*	CG33832			76	69	Histone2A	Chromatin assembly & disassembly
35418	*df31*	CG2207	3		24	15	Decondensation factor 31	Chromatin binding protein
41840	*hsc70-4*	CG4264			42	55	Heat shock protein cognate 4	Chaperone protein
43785	*rad23*	CG1836			16	13	-	DNA repair component
35728	*gapdh1*	CG12055			21	13	Glyceraldehyde-3-phosphate dehydrogenase 1	Glucose metabolism
44149	*acon*	CG9244				20	Aconitase	Aconitase
Proteins involved in axogenesis								
39826	*fax*	CG4609	36	36	76	79	failed axon connections	axogenesis
39873	*Nrt*	CG9704			13	10	Neurotactin	axogenesis
37238	*betaTub56D*	CG9277	5		60	64	β-Tubulin at 56D	Cytoskeletal component
Miscellaneous								
5740505	*CG34411*	CG34411	14	24			-	G-protein coupled receptor activity
38094	*CG42553*	CG42553		28			-	Unknown protein

Figure 3.2 Proteomic screen- Identification of novel ubiquitinated conjugates, involved in Eiger-induced signal transduction.

a. Input from the streptavidin pull-downs of biotin-tagged ubiquitinated targets. 200 eye-brain complexes used for each genotype.
b. Streptavidin pull-downs from 2a, used for MS/MS. Identification of ubiquitinated conjugates in response to Eiger over-expression by Tandem mass spectrometry (MS/MS).The data is presented as total spectral counts(TSC), which represents the number of times a peptide from the indicated protein was observed in the analysis. TSC1 and TSC2 refer to technical replicates of each sample. The genotypes used for MS/MS are – GMR-GAL4,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA*/*TM6B,Tb*[1]], (control) and GMR-GAL4,UAS-*eiger*,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA/TM6B,Tb*[1] (experimental sample).

a

Gene Name	Protein name	Nature of ubiquitin linkages	Mammalian homologs
ben	Bendless	K63	Ubc13/UBE2N
eff/ubcD1	Effete	K48	UBE2D1-D2
ubcD2	Ubiquitin conjugating enzyme 2	K48	UBE2D1-D2
ubcD4	Ubiquitin conjugating enzyme 4	K48	HIP2/UBE2K
CG40045	(Uncharecterized protein)	K48	Ubc7/UBE2G2
CG7656	(Uncharecterized protein)	K48	Ubc7/UBE2G2

b

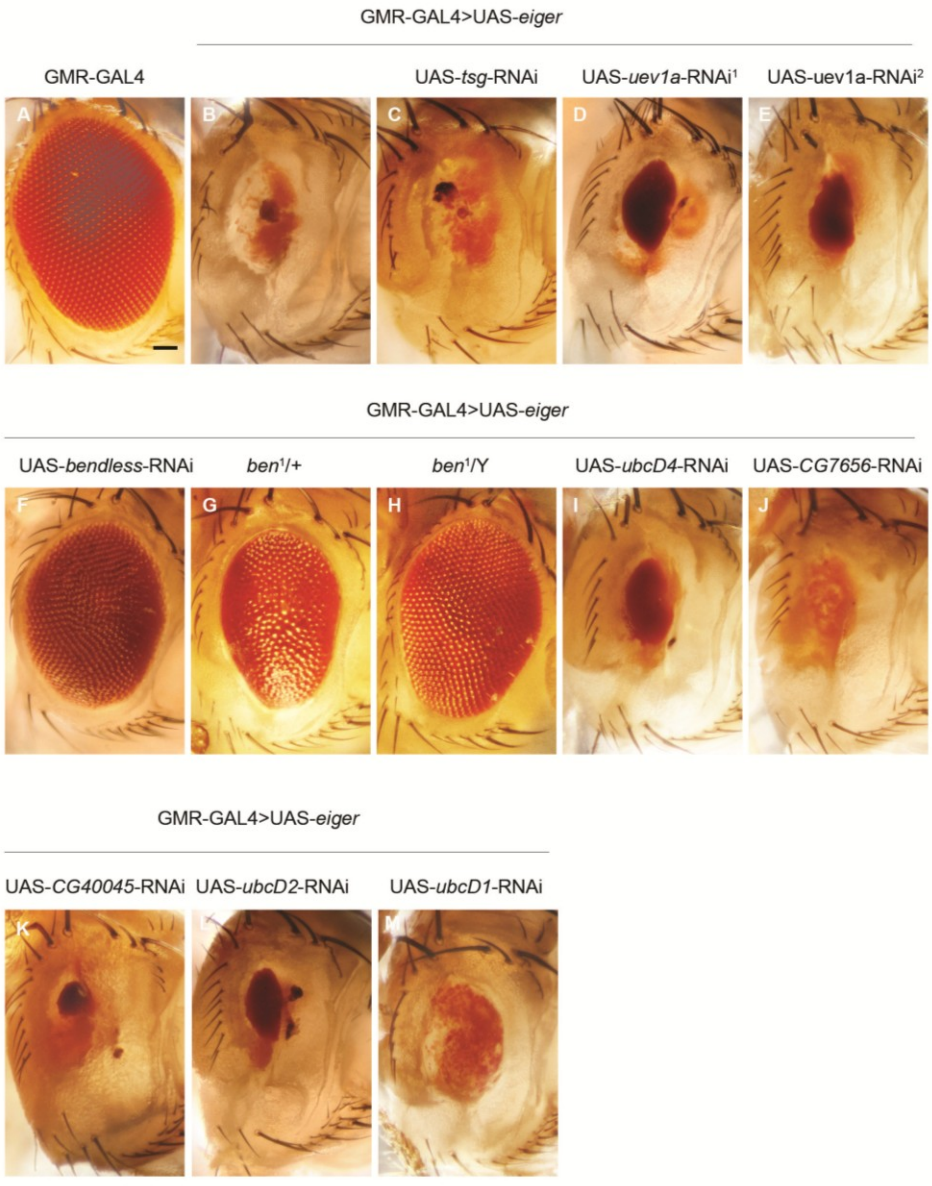


Figure 3.3 Identification of ubiquitinated E2s in Eiger - induced signaling.

a. Table with the E2s identified in the screen with their mammalian homologs and the nature of ubiquitin linkages they are associated with.

b. Bendless, an ubiquitin-conjugating enzyme (E2) required for K^{63}

ubiquitination, plays an indispensible non-redundant role in Eiger-induced cell death. Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A. Wild type eye, GMR-GAL4/ *CyO*. B. Over-expression of Eiger generates a 'small eye phenotype', GMR-GAL4,UAS-*eiger*/*CyO*. Small eye phenotype is unaffected by the knockdown of a C. unrelated transgene, GMR-GAL4,UAS-*eiger*/+ ; UAS-*tsg*-RNAi/+(negative control). The small eye phenotype is marginally suppressed by the knockdown of Uev1A using two independent RNAi lines D. GMR-GAL4,UAS-*eiger*/+;UAS-*uev1a*-RNAi^{1}/+ and E. GMR-GAL4,UAS-*eiger*/UAS-*uev1a*-RNAi^{2}. Eiger 'small eye' phenotype is completely suppressed by Bendless knockdown using an RNAi line F. GMR-GAL4,UAS-*eiger*/+; UAS-*bendless*-RNAi/+.The small eye phenotype is partially suppressed in a heterozygous female, *ben*^{1}/+ G. *ben*^{1}/+; GMR-GAL4,UAS-*eiger*/+. and completely suppressed in a hemizygous male, H.*ben*^{1}/Y G. *ben*^{1} /Y; GMR-GAL4,UAS-*eiger*/+. *ben*^{1} is a Bendless null allele.
Eiger 'small eye phenotype' is poorly suppressed by the knockdown of individual E2s identified in the screen – I. UbcD4 - UAS-*ubcD4*-RNAi/GMR-GAL4,UAS-*eiger*, J. CG7656- GMR-GAL4,UAS-*eiger*/+; UAS-*CG7656*-RNAi/+, K.CG40045 - GMR-GAL4,UAS-*eiger*/UAS- *CG40045*-RNAi, L.UbcD2 - GMR-GAL4,UAS-*eiger*/UAS-*ubcD2*-RNAi, M.UbcD1 - GMR-GAL4,UAS-*eiger*/+;UAS-*ubcD1*-RNAi/+. Scale bar - 100μm.

PREFACE TO CHAPTER 4

Aβ-induced neurotoxicity has been shown to progress through the cumulative effect of a number of different cellular pathways, the complexity of which is due to its interaction with a number of binding partners. In this current chapter, we identify two novel Eiger-dependent signaling mechanisms that contribute to the severity of degenerative processes in a Drosophila AD model system. Our study identifies PAR-1, a dTau kinase as a potent enhancer of Eiger-induced apoptosis, which progresses through a Bendless dependent, but Basket independent mechanism. Our results provide evidence for the involvement of Eiger in an Aβ-driven pathway, that is independent of its JNK signaling potential. We highlight the involvement of Eiger to signal through multiple receptors other than Wengen, in pro-apoptotic processes. Lastly, we show the modulation of Eiger pro-apoptotic signaling through activation of the dToll-1 receptor. Thus, the current chapter recapitulates a synergy between PAR-1 dependent and Aβ-induced pro-degenerative mechanisms, which are amplified by the ectopic expression of Eiger.

CHAPTER 4- Eiger, the Drosophila form of TNF, Eiger, participates in Aβ-mediated pro-degenerative signaling

4. ABSTRACT

The human amyloid-*β (1-42*)/Aβ-42 peptide has been associated with the neuropathology of Alzheimer's disease, through multiple mechanisms that are not fully understood. In this current study we have utilized the power of Drosophila genetics to identify and address important cellular players that contribute towards Aβ-induced degeneration. We identify the Drosophila Tumor Necrosis factor (TNF), Eiger as a potent enhancer of Aβ-42-induced cell death, through a TNFR-independent mechanism. Additionally, we show the ability of Eiger to transmit inflammatory signaling responses through a novel mechanism involving Drosophila Toll-1 (dToll-1) receptor activity. One of the most widely studied mechanisms of Aβ-42-induced cytotoxicity involves the hyper-phosphorylation of microtubule-associated protein Tau. The aberrant phosphorylation of Tau is mediated by several kinases and has been shown to induce microtubule destabilization, which ultimately contributes towards neuronal dysfunction and death. We identify a novel collaboration between Eiger and a physiological Tau kinase, PAR-1 that together transmit the activation of a novel cell death cascade, via a TNFR and ubiquitin-dependent mechanism. The genetic interactions uncovered in this study show a complex interplay of cellular responses engaging Eiger, dToll-1, PAR-1 and Aβ-42, which synergistically amplify cellular toxicity, in our model of retinal degeneration.

4.1. INTRODUCTION

Alzheimer's disease (AD) is a progressive disease of the nervous system that is pathologically characterized by the accumulation of amyloid plaques. The components of these plaques were identified as 40 or 42 amino acid containing peptides (Aβ- 40/Aβ-42) derived from the amyloid precursor protein (APP) (Glenner and Wong, 1984). Aberrant enzymatic activation of two proteases, γ and β secretases, have been implicated in the generation of these insoluble peptides. Mutations in full length APP and the two APP processing enzymes have been associated with the enhanced accumulation of Aβ peptides, and are also genetically linked with early onset AD (Tanzi and Bertram, 2005). Aggregated Aβ peptides can act as an initiator of AD, but a general agreement on molecular mechanisms that allow this pathogenesis to proceed has not emerged. One of the most widely studied mechanisms contributing towards disease progression is Aβ-induced Tau toxicity, which ultimately leads to neuronal dysfunction and death (Ittner and Gotz, 2011).

Tau is a microtubule-associated protein whose main physiological roles include microtubule stabilization and regulation of axonal transport. An increase in Tau phosphorylation negatively regulates its affinity for microtubule interactions (Gotz et al., 2006). The hyper-phosphorylation of Tau causes its detachment and intracellular accumulation in neurofibrillary tangles (NFT), which have been pathologically linked to several neurodegenerative diseases/tauopathies such as frontotemporal dementia, Parkinson's disease and AD (Lee et al., 2001b). Though there are no heritable mutations that directly link tauopathies and AD, several Tau haplotypes have been suggested to contribute towards AD susceptibility (Kauwe et al., 2008; Mukherjee et al., 2007). *In vitro* and *in vivo* studies point to multiple mechanisms of Tau and Aβ toxicity. The amyloid hypothesis places the Aβ peptides as the initiator of neurotoxicity, which progresses through one of several mechanisms, involving the accumulation of hyper-phosphorylated Tau (Gotz et al., 2004).

The dramatic reduction in mis-phosphorylated Tau accumulation in response to Aβ immunotherapy (Oddo et al., 2004) in transgenic mouse models of AD suggests that Tau phosphorylation is a consequence of Aβ peptide toxicity. The loss of Tau has been shown *in vivo* to reduce Aβ-induced excitotoxicity, which has been

functionally linked to the role of Tau as an important dendritic scaffold (Ittner et al., 2010; Roberson et al., 2007). The loss of Tau expression has also been associated with the rescue of axonal transport defects induced by Aβ toxicity (Vossel et al., 2010). There are also studies showing independent modes of Aβ or Tau toxicity that contribute synergistically towards neuronal dysfunction by affecting similar cellular processes. Aβ and Tau contribute towards mitochondrial respiratory chain defects, which amplify the neuronal toxicity observed in a triple transgenic mouse AD model (Rhein et al., 2009). Hence, serial and parallel modes of Aβ-Tau-induced neuronal dysfunction have been proposed as mechanisms of AD progression (Small and Duff, 2008). However, the array of cellular proteins that act downstream of Aβ-peptides remain elusive.

In this current study we have utilized *Drosophila melanogaster* as a genetic model system to address the molecular mechanisms of Aβ induced neurotoxicity. Fruit flies have been used to study AD due to conservation of a number of genes involved in APP biology, coupled with the ease to conduct unbiased-large scale genetic screens for the assessment of disease phenotypes and potential modifiers (Moloney et al., 2010). The fly homolog of γ secretase has been shown to process human APP (Fossgreen et al., 1998), followed by a more recent identification of a β secretase equivalent (Carmine-Simmen et al., 2009). Additionally, flies also carry a homolog for APP, called the APPL (amyloid precursor like) (Rosen et al., 1989). However, APPL does not harbor the same sequences as APP, required for the processing of the Aβ peptides, by the two successive cleavage events (Luo et al., 1992). Hence several Drosophila AD models have been generated using the transgenic expression of the human APP gene (Iijima-Ando and Iijima, 2010). The over-expression of human Aβ-42 in the Drosophila compound eye was shown to generate a ‘reduced and rough-eye‘ phenotype, indicative of photoreceptor cell loss. This phenotype was scored against transposon EP insertion lines, which led to the identification of several chromatin modifiers as key enhancers of the Aβ-42-induced degeneration phenotype (Cao et al., 2008). A loss of function screen also led to the identification of the dToll-1 receptor and its Nuclear factor – kappa B (NF-κB) signaling components as key enhancers of Aβ-induced retinal toxicity (Tan et al., 2008).

We utilize a transgenic fly that expresses two tandem copies of human A*β-42,* that phenocopy the APP gene duplication associated with early onset familial AD (Casas-Tinto et al., 2011). We use the retinal expression driven, GAL-UAS system to screen for potential genetic interactors in the Aβ and Tau-dependent degeneration pathway. We observe a complex interplay between components of the Fly TNFR signaling cascade and PAR1- a Tau kinase, in mediating the toxic effects of A*β-42*-induced cell death.

4.2. MATERIALS AND METHODS

Drosophila stocks and genetic crosses –

Fly line	*Genotype*	*Collection*
UAS-tsg-RNAi	w[1118];P{GD6488}v45355/TM3	VDRC
UAS-wengen-RNAi	w[1118];P{GD3427}v9152	VDRC
UAS-dtraf2-RNAi	w[1118];P{GD7146} v16126/TM3	VDRC
UAS-sidekick-RNAi	P{KK105116}VIE-260B - v106217	VDRC
UAS-grindelwald-RNAi	P{KK109939}VIE-260B – v104538	VDRC
UAS-basket-RNAi	w[1118];P{GD10555}v34139/CyO	VDRC
UAS-bendless-RNAi	w[1118]; P{GD1387}v9413	VDRC
UAS-par-1	w[*]; P{UAS-PAR1}IN1	Kyoto DGRC
UAS-toll-1	P{w[+mC]=UAS-Tl.10b}11, y[1]w[*]	BDSC
UAS-toll-1-RNAi	P{TRiP.JF01491}attP2 - #31044	BDSC
UAS-par1-RNAi	P{TRiP.GL00253}attP2 -#35342	BDSC
GMR-GAL4	w[1118]; P{GMR-GAL4.w[-]}2/CyO	BDSC
UAS-eiger	yw; UAS-eiger/*CyO* (T655 D449)	Dr.Konrad Basler (Moreno et al., 2002)
UAS-aβ42(2X)	yw; UAS-*aβ42(2X)/CyO*	Dr. Pedro Fernandez-Funez (Casas-Tinto et al., 2011)

VDRC=Vienna Drosophila RNAi Center
BDSC =Bloomington Drosophila Stock Center
Kyoto DGRC = Kyoto Drosophila Genetic Resource Center

All stocks were raised in standard Drosophila media (recipe taken from BDSC) and all crosses were carried out at either 25°C or 29°C, as indicated in the figure legends.

Generation of epitope tagged, weaker isoform of Eiger - To generate UAS-eiger^{myc} transgenic flies, *eiger* cDNA (LP03784), was obtained from the *Drosophila Genomics Resource Center.* The full-length gene sequence of Eiger-s (shorter isoform) was amplified from LP03784, with an extension of six amino acids (GESLLS) and C-terminal 2xmyc epitope tags, by PCR and sub-cloned using standard cloning procedures, into the pUAST vector. Thus the UAS-eiger^{myc} represents the epitope tagged, longer isoform of Eiger. The transgenic flies were generated by Best Gene Inc.

Imaging - Adult fly eyes were photographed using with a Canon EOS 1000D DSLR (rebel XS) camera mounted on a Zeiss Axioskop 40 microscope with a10× objective (0.25 = N.A). Several images of the adult fly eye were taken at different focal planes, for extended depth. The focus stacking of each set of images was done using Helicon Focus software (HeliconSoft) to generate the final image.

4.3. RESULTS

Aβ42-induced toxicity is exacerbated by the flyTNF, Eiger – We utilized the pan-retinal GMR-GAL4 promoter to drive the ectopic expression of two tandem copies of human amyloid-*β(1-42*) under UAS control – 'UAS-*aβ42(2X)'.* The ectopic expression of *aβ42(2X)* in the eye results in a reduction of eye size. The surface of the compound eye shows deformed ommatidia, dotted with necrotic spots, which we refer to as the 'enhanced-rough eye' phenotype (Figure 4.1-D). The ectopic expression of fly TNF, Eiger in the eye-imaginal disc, induces potent activation of cell death via c-Jun N-terminal kinase (JNK) homolog - Basket, leading to the loss of majority of photoreceptor cells, thus generating a 'small+-eye' phenotype (Igaki et al., 2002) (Figure 4.1-B). The expression of an weaker epitope-tagged isoform of Eiger ($Eiger^{myc}$) generates a less potent cell death phenotype, characterized by fused, irregular arrangement of ommatidia, which we refer to as the 'rough-eye' phenotype (Figure 4.1-C). Thus the mis-expression of *aβ42(2X)* or Eiger generates phenotypes that can be utilized to screen for genetic suppressors or enhancers of cell death. Importantly, we observe a dramatic enhancement of cell death upon co-expression of *aβ42(2X)* with both the untagged or epitope-tagged isoforms of Eiger (Figure 4.1, compare B,D with E and compare C, D with F). Thus, fly TNF potentiates the toxicity of human amyloid-*β(1-42*) *in vivo* and represents an uncharacterized signaling mechanism of cell death induction, in this model of AD.

PAR-1 enhances Eiger-induced cell death – In order to address the molecular mechanisms of Eiger in the context of *aβ42(2X)*-induced toxicity, we addressed the importance of a PAR-1, a critical regulator of synaptic pathologies in human amyloid-*β(1-42*) disease models. The ectopic-expression of PAR-1 has been shown to generate a 'rough-eye' phenotype with disrupted ommatidial arrangement (Chatterjee et al., 2009; Nishimura et al., 2004) (Figure 4.2-D). We observe a potent enhancement of the cell death phenotype upon co-expression of Eiger and PAR-1. Ectopic expression of Eiger with PAR-1 results in pre-pupal lethality (Figure 4.2-E), whereas PAR-1 potentiates cell death induced by $Eiger^{myc}$ (Figure 4.2, compare C and F). Hence PAR-1 is a novel regulator of cell death, induced by Eiger.

PAR-1 is a critical kinase downstream of Eiger- Aβ42(2X)–induced toxicity –

One of the mechanisms of neurotoxicity induced by the human amyloid-*β(1-42*), is the promotion of aberrant Tau phosphorylation (Iijima et al., 2010). Further, PAR-1 has been previously linked as a regulator of *aβ42*-induced dTau-neurotoxicity (Lee et al., 2012). Since Eiger promotes cytotoxicity in collaboration with either PAR-1 or *aβ42(2X)* (Figure 4.1 and 4.2), we addressed the participation of Eiger in the *aβ42*-PAR-1-Tau module. The ectopic expression of PAR-1 enhances *aβ42(2X)*-induced toxicity (Figure 4.3, compare C and G). Additionally, the ectopic expression of PAR-1 enhances the toxicity of Eiger over-expression (Figure 4.2-E and 4.3-F) and Eiger-*aβ42(2X)* co-expression (Figure 4.3, compare D and H), as shown by the lethality of the pre-pupa. This indicates that PAR-1 is a critical downstream kinase whose ectopic expression enhances toxicity downstream of both Eiger and *aβ42(2X)*. However, the loss of expression of PAR-1 by RNAi failed to suppress the cell death phenotype induced by the over-expression of Eiger (Figure 4.3, compare B and J) or *aβ42(2X)* (Figure 4.3, compare C and K) and did not suppress the lethality of Eiger-*aβ42(2X)* co-expression (Figure 4.3, compare D and L). This data shows a critical gain-of-function role for PAR-1 in the regulation of cytotoxicity induced by overexpression of Eiger, in the presence of aβ42(2X). Additionally, the data shows that Eiger or *aβ42(2X)* are capable of participating in alternate cell death promoting cascades, in the absence of PAR-1.

Eiger potentiates PAR-1 induced cell death through a novel ubiquitin-dependent mechanism, downstream of Fly TNFR, Wengen - Ectopic expression of Eiger, causes induction of Basket (FlyJNK homolog)-dependent apoptosis, (Figure 4.4, compare B with N) downstream of Fly TNFR, Wengen (Figure 4.4, compare B and Q)(Igaki et al., 2002; Kanda et al., 2002). Genetic epistasis studies have identified several components of the Basket-cell death module, downstream of Eiger. The fly TRAF protein, dTRAF2 (unpublished data)(Xue et al., 2007) and ubiquitin-conjugating enzyme, Bendless (unpublished data)(Ma et al., 2014) are indispensible for Eiger-induced cell death. This is supported by the complete suppression of $Eiger^{myc}$-induced 'rough-eye' phenotype, upon loss of expression of either of these two proteins (Figure 4.4, compare B with K; compare B with H). RING domain containing dTRAF2 serve a dual-purpose role of a potential E3 ubiquitin ligase and a signaling scaffold that connects Eiger-Wengen to kinase cascades and Bendless acts as an E2 enzyme necessary for non-degradative K^{63} poly-ubiquitination. Hence,

we asked if PAR-1 regulation of Eiger-cytotoxicity was mediated by the classic components of the Wengen-Basket signaling module. We observed a massive induction of cell death upon co-expression of Eiger^{myc} with PAR-1 (Figure 4.2-F, 4.4-D). Eiger^{myc}- PAR-1 induced cell death is poorly suppressed by the loss of the critical TNFR adaptor, dTRAF2 (Figure 4.4, compare D and M) or terminal kinase of the JNK pathway, Basket (Figure 4.4, compare D and P). However, the loss of Bendless completely suppressed the cell death phenotype of Eiger^{myc}-PAR-1 expression (Figure 4.4, compare D and J). The loss of Wengen expression partially, yet substantially suppressed the cell death induced by the collaboration of Eiger^{myc}-PAR-1 (Figure 4.4, compare D and S). Additionally, both Wengen and Bendless serve upstream of PAR-1 on this pathway, as the loss of expression of Wengen or Bendless fail to suppress the 'rough-eye' phenotype of PAR-1 over-expression (Figure 4.4, compare C with I and R).

Eiger does not utilize the classic components of the Basket-cascade to induce aβ42(2X)-cytotoxicity – The loss of expression of Eiger-signaling components such as dTRAF2 (Figure 4.5, compare D with J), Bendless (Figure 4.5, compare D with G) or Basket (Figure 4.5, compare D with M) failed to suppress Eiger-*aβ42(2X)* induced toxicity. Thus, Eiger utilizes an uncharacterized, novel mechanism to induce *aβ42(2X)* toxicity.

Eiger can induce cell death signaling through multiple receptors – The suppression of Eiger-induced 'small-eye' apoptotic phenotype by a TNF homology domain containing receptor, led to the identification of the fly TNFR, Wengen. However, the suppression of Eiger-induced cell death by Wengen RNAi (Figure 4.6, compare B and C) (Kanda et al., 2002), is not complete, suggesting that other Eiger-interacting receptors exist. An X-chromosome deficiency screen uncovered a novel genetic interaction between Eiger and a cell adhesion receptor, Sidekick (unpublished). Sidekick loss of function significantly rescued Eiger-induced cell death (Figure 4.6, compare B and D). Additionally, a second TNFR receptor, Grindelwald - was recently identified as an interactor and promoter of Eiger-induced Basket signaling (Figure 4.6, compare B and E)(Andersen et al., 2015). We wanted to address the importance of these three Eiger-signaling receptors in *aβ42(2X)*-induced toxicity. Surprisingly, loss of Wengen (Figure 4.6, compare G and H), Sidekick

(Figure 4.6, compare G and I) or Grindelwald (Figure 4.6, compare G and J) had no effect on Eiger-*aβ42(2X)*-induced toxicity. Hence, Eiger partners with a novel receptor to signal *aβ42(2X*)-induced cell death.

Eiger partners with the Toll-1 receptor to induce cell death – An early genetic screen isolated the Drosophila Toll-1 receptor (dToll-1) as an enhancer of *aβ42(2X)*-induced neurodegeneration (Tan et al., 2008). Hence, we asked if Eiger was capable of promoting cytotoxicity, in the absence or presence of *aβ42(2X)*, through the dToll-1 receptor. Figure 4.6 shows that the Eiger-induced 'small-eye' phenotype was dramatically suppressed by the RNAi-dependent depletion of dToll-1 expression (compare B and F). Additionally, the GMR-driven expression of the dToll-1 receptor, with either untagged (Figure 4.7, compare B and E) or epitope tagged Eiger (Figure 4.7, compare C and F), massively enhanced cell death in the compound eye. Surprisingly, loss of dToll-1 expression did not suppress, but rather enhanced Eiger-*aβ42(2X*) toxicity (Figure 4.6, compare G and K).

4.4. DISCUSSION

The compound eye of Drosophila serves as an effective model to study cell death related pathways. Each adult eye comprises ~800 ommatidia made up of photoreceptor cells and supporting cells that differentiate from the larval eye-imaginal disc (Ready et al., 1976). The main regulators of cell number in the eye are pro-apoptotic inducers Hid, Grim and Reaper, which induce caspase-dependent programmed cell death (PCD) during morphogenesis (Richardson and Kumar, 2002). However, cell death can also be mediated by the activation of the c-jun Kinase (JNK)/Basket pathway that can activate cell death by caspase-dependent and independent mechanisms (Kanda et al., 2011). FlyTNF, Eiger induces potent activation of cell death through a hierarchical Basket-dependent pathway. The retinal expression of Eiger induces apoptosis and generates a visible reduction in the eye size, caused by the loss of photoreceptor cells (Igaki et al., 2002; Moreno et al., 2002). We and other research groups have previously shown the importance of adaptor protein, dTRAF2 (Chapter 2)(Xue et al., 2007) and ubiquitin-conjugating enzyme, Bendless (Chapter 3) (Ma et al., 2014) as indispensible regulators of Eiger-dependent cell death signaling.

Eiger has been shown to signal apoptosis through the TNFR homolog, Wengen (Kanda et al., 2002). Additional receptors, Grindelwald (Andersen et al., 2015) and Sidekick (unpublished data) have also been identified from genetic screens designed to identify novel Eiger receptors. However, the JNK signaling components downstream of these receptors need to be further characterized. Grindelwald has been shown to interact with Eiger to activate cell death for the clearance of epithelial cells with aberrant polarity (Andersen et al., 2015). Sidekick, is a homotypic cell adhesion receptor, associated with the laminar organization of photoreceptor cells (Nguyen et al., 1997). The loss of expression of Sidekick, resulted in a significant rescue of Eiger-induced photoreceptor death (unpublished data). Lastly, Eiger-Wengen signaling has been shown to be responsible for stress-induced activation of cell death in motor neurons at the neuro-muscular junction (NMJ) (Keller et al., 2011). Hence, we wanted to address the involvement of Eiger-induced JNK signaling in mediating the cytotoxicity of human amyloid-*β*.

Eiger and aβ42(2X) synergistically activate retinal degeneration, through a

JNK-independent mechanism - The retinal expression of transgenic human *aβ42(2X)* causes a degenerative phenotype observed by the disruption of ommatidial arrangement coupled with visible necrotic spots on the eye surface. We utilized this phenotype to look for enhancers or suppressors of *aβ42(2X)* toxicity. We identify the fly TNF, Eiger, as a potent enhancer of *aβ42(2X*)-induced toxicity. The ectopic co-expression of Eiger and *aβ42(2X*) caused a massive induction of cell death, characterized by the complete loss of photoreceptor cells. We additionally observed a dispensable role for Eiger-signaling components – Wengen, Grindelwald, Sidekick, dTRAF2, Bendless or Basket in contributing towards *aβ42(2X*)-dependent retinal degeneration. The mis-expression of *aβ42(2X)* has been linked to the activation of cell death through caspase-dependent and JNK-dependent mechanisms (Tare et al., 2011). In accordance with this, we observed a minor rescue of the *aβ42(2X)* retinal phenotype upon loss of JNK homolog, Basket. Hence, *aβ42(2X)* sequesters Eiger away from participating in the canonical-Basket signaling cascade, and engages in cell death signaling through an unknown and novel mechanism.

Tau phosphorylation kinase, PAR-1 potentiates Eiger-Wengen dependent cell death signaling, through an ubiquitin-dependent mechanism – In vivo and *in vitro* evidence presents a strong case for a Tau-dependent mechanism in the induction of *aβ42(2X)* toxicity. Several proteins, such as GSK-3β, Cdk2 and 5, PKA, CaMKII, and MARK have been identified as Tau kinases (Lee et al. 2001), but their relative contributions have not been elucidated *in vivo.* PAR-1/MARK is involved in a number of different processes ranging from cellular proliferation, differentiation and polarity determination in humans and flies (Hurov and Piwnica-Worms, 2007). But PAR-1/MARK2 was identified as a key regulator of microtubule dynamics, by the modulation of Tau phosphorylation levels (Drewes et al., 1997). Mutation of critical PAR-1 phosphorylation sites on Tau abolished Tau-dependent toxicity, and conversely neuronal expression of PAR-1 was found to induce tau hyper-phosphorylation and degeneration (Nishimura et al., 2004). We identify a novel genetic interaction between PAR-1 and Eiger. Eiger-induced cell death by strong and weak isoforms is potentiated by the over-expression of PAR-1. The Eiger-PAR-1 toxicity was found to occur through a Wengen, Bendless-dependent, but dTRAF2, Basket-independent mechanism.

Eiger and aβ42(2X) induce degeneration through PAR-1 dependent and independent ways – Eiger-*aβ42(2X*)– induced toxicity is exacerbated by the ectopic expression of PAR1. Paradoxically, the co-expression of Eiger-*aβ42(2X)* with PAR-1 RNAi also enhances toxicity by promoting pupal lethality. Hence, the perturbations of PAR-1 levels have an impact on cytotoxicity, in the presence of Eiger and *aβ42(2X)* through a complex interplay of several mechanisms. The ectopic expression of PAR-1 was found to induce a stronger neurodegeneration phenotype than Tau over-expression. Hence, PAR-1 is capable of inducing cytotoxicity through multiple mechanisms involving several substrates, which could account for the phenotypes observed in response to PAR-1 perturbations. PAR-1 over-expression has been shown to negatively regulate the post-synaptic targeting of the PSD-95 homolog, Dlg (Zhang et al., 2007), which correlates well with synaptic PSD-95 defects seen in AD (Gylys et al., 2004). The ectopic expression of PAR-1 can thus influence synapse development by regulating both Tau and Dlg independently. However, the loss of PAR-1 also induces synaptic dysfunction, shown by an overgrowth phenotype of the post-synaptic reticular structures (Zhang et al., 2007). Thus by impacting microtubule dynamics via Tau phosphorylation, and synapse formation via Dlg, the deregulated levels of PAR-1(gain and loss of function) can account for synaptic dysfunction which often precedes amyloid plaque and NFT formation in AD (Selkoe, 2002; Tu et al., 2014).

A Drosophila Tau over-expression model highlighted the requirement of PAR-1/MARK phosphorylation as the prerequisite for activation of other kinases such as GSK-3β and Cdk5 (Nishimura et al., 2004). There has also been a recent study showing the role of GSK-3 as the inhibitory kinase of MARK2 (Timm et al., 2008). Additionally, many Tau-kinases, such as GSK-3β, Cdk5 and Erk2 have also been linked to Tau-dependent A*β* neurotoxicity (Mazanetz and Fischer, 2007). However, the mechanism of how these different kinases contribute towards Tau hyper-phosphorylation is unknown. Hence, *aβ42(2X)*- induced neurodegeneration can proceed through a number of different mechanisms, which explains the lack of suppression of the *aβ42(2X)*-'enhanced-rough eye' phenotype upon PAR-1 knockdown.

Eiger induces cell death through Toll-1 receptor activation – Our results show a dispensable role for Eiger-signaling receptors in *aβ42(2X)*-induced retinal degeneration, upon individual knockdown of receptors Wengen, Sidekick or Grindelwald. But, we cannot disregard the requirement of a full complement of all three Eiger-signaling receptors for the promotion of Eiger-*aβ42(2X)*-induced cell death. However, a deficiency screen uncovered the innate immune receptor, dToll-1 as a suppressor of *aβ42*-induced 'rough-eye' phenotype (Tan et al., 2008). This prompted us to test the involvement of dToll-1 in Eiger-*aβ42(2X)*-induced retinal degeneration. Quite surprisingly, we observe an enhancement of retinal degeneration upon dToll-1 knock down, in response to the co-expression of Eiger and *aβ42(2X)*. We do not completely disregard the idea of a Toll-dependent mechanism contributing towards Eiger- *aβ42(2X)*-induced signaling, as Drosophila has a total of 9 different Toll receptors in the genome (Tauszig et al., 2000). Hence, other members of the Toll family could compensate for the loss of dToll-1. The ectopic expression of cellular stressors (Eiger+*aβ42(2X))* combined with dToll-1 knock-down may amplify toxicity through alternate receptors and could account for the enhanced lethality of the pupae.

Due to the participation of multiple dToll receptors in innate immune activation, targeting a single receptor would be ineffective in understanding the mechanism of Eiger+*aβ42(2X*)-induced toxicity. However studying the contribution of common downstream dToll signaling components, would aid the identification of the signaling mechanism involved in degeneration. dToll-receptors participate in innate immune responses by activation of the NF-κB signaling pathway. The dToll receptor pathway is activated in response to gram-positive bacteria, fungi and yeast infections. The pathogens are recognized by peptidoglycan recognition proteins, which stimulate the cleavage of the dToll-1 ligand, Spätzle. Interaction of Spätzle with dToll-1 activates its engagement with the death-domain containing protein dMyd88, which assembles downstream kinases such as Pelle and Tube, to activate the NF-κB transcription factors Dif and Dorsal. These transcriptional factors then mediate the relevant cellular effects, such as the generation of anti-microbial peptides (Lemaitre and Hoffmann, 2007). The NF-κB components, Pelle, Tube, Dif and Dorsal were identified as suppressors of the *aβ42*- rough eye phenotype (Tan et

al., 2008), and we are currently testing the importance of these components in the Eiger-induced *aβ42(2X)* degeneration paradigm.

Interestingly, we observe a dependence of Eiger-induced cell death signaling on dToll-1 receptor activity. Knockdown of the dToll-1 receptor by RNAi, induces a strong suppression of Eiger-dependent cell death. Conversely, ectopic-expression of Toll-1 enhances Eiger-induced cell death eye-phenotype. dToll-1 receptor activity has been shown previously, to regulate the intrinsic tumor suppressor ability of Eiger-JNK signaling. Tumor cells were shown to activate a systemic immune response through Eiger-JNK signaling in hemocytes, which led to dToll-1 activity in the fat body. The dToll-1 activation was shown to trigger the elimination of tumorigenic cells through an elusive Eiger-dependent mechanism (Parisi et al., 2014). But, the involvement of dToll-1 receptor in the direct modulation of Eiger-dependent cell death has never been addressed before. This current study places dToll-1 as a major potentiator of Eiger-induced cell death. During the preparation of this manuscript, a very recent study by Lei Xu and colleagues also highlighted the importance of the dToll-1 receptor as a downstream modulator of Eiger-dependent cell death (Wu et al., 2015).

Unlike mammalian TNF-α signaling, which proceeds via multiple mechanisms involving NF-κB and JNK activation (Dempsey et al., 2003), the engagement of Fly TNF or TNFRs in NF-κB signaling pathways is poorly understood. This study opens up an undiscovered mechanism of TNF-dependent NF-κB signaling pathway involving dToll receptor engagement. This will be particularly useful to understand emerging discoveries of innate immune responses identified in fly neurodegenerative disease models. Petersen *et al*. have recently shown the importance of innate immune responses towards glial-derived degeneration, in a Drosophila model of Ataxia-telangiectasia (Petersen et al., 2012). Additionally, the NF-κB factor, Relish has been shown to contribute towards light-induced degeneration in a photoreceptor neuronal model of retinal diseases (Chinchore et al., 2012). In mammalian systems, the microglia represent the resident immune modulators of the nervous system, which engage in immune activation through Toll-dependent pathways (Olson and Miller, 2004). The deposition of amyloid aggregates has been shown to induce microglial-derived immune responses, which contribute towards neuronal loss and

cognitive decline in AD (Akiyama et al., 2000). Additionally, pro-inflammatory responses have also been implicated in the hyper-phosphorylation of Tau (Kitazawa et al., 2011). This study is thus relevant to understand conserved mechanisms of Toll-dependent inflammatory responses commonly observed in neurodegenerative diseases such as AD (Amor et al., 2010; Arroyo et al., 2011).

We propose a novel pathway (Figure 4.8) of *aβ42(2X)*-induced degeneration that is potentiated by the flyTNF, Eiger through a putative dToll-receptor dependent mechanism. We also identify a downstream kinase, PAR-1 as a novel modulator of Eiger-Wengen signaling that progresses via an ubiquitin-dependent mechanism, involving the E2, Bendless. Lastly, we identify a synergy of Eiger-modulated PAR-1 dependent and independent pathways that amplify *aβ42(2X)*-induced degeneration.

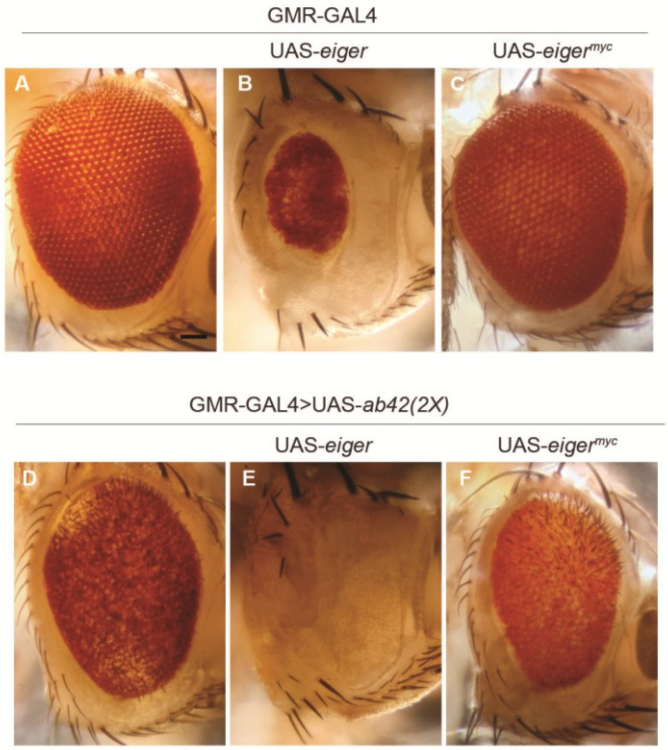


Figure 4.1. Aβ42-induced toxicity is exacerbated by the flyTNF, Eiger

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images were taken at 10X magnification. Crosses were carried out at 29°C. A-F Over-expression of Aβ42 generates an 'enhanced-rough eye' phenotype, that is potentiated by the co-expression of epitope tagged or untagged Eiger. UAS-*aβ42(2X)* transgene is made up of two tandem copies of human amyloid-*β(1-42*) under UAS control. UAS-*eiger* is an untagged isoform of Eiger and UAS-*eiger*^{myc} is an epitope tagged, weaker isoform of Eiger. The individual genotypes are listed below. Wild type compound eye, A.GMR-GAL4/*CyO*. Over-expression of Eiger generates a 'small+-eye' phenotype, B.GMR-GAL4,UAS-*eiger*/*CyO*. Over-expression of Eiger^{myc} generates a less potent cell death phenotype, which we refer to as the 'rough-eye' phenotype, C. GMR-GAL4,UAS-*eiger*^{myc}/*CyO*. Over-expression of two tandem copies of human *aβ42* peptide, induces cytotoxicity in the eye with necrotic spots, which we refer to as the 'enhanced-rough eye' phenotype, D. GMR-GAL4/UAS-*aβ42(2X).* Co-expression of untagged isoform of Eiger with UAS-*aβ42(2X)* enhances cytotoxicity, as shown by the complete ablation of the compound eye, E. GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X);TM3,Sb*^{1}/+. Co-expression of *myc* tagged isoform of Eiger with UAS-*aβ42(2X)* enhances cytotoxicity, as shown by the reduction in eye size, coupled with deformed ommatidia, F. GMR-GAL4,UAS-*eiger*^{myc}/UAS-*aβ42(2X).* Scale bar - 100μm. [Note: Since the phenotype of *aβ42(2X)* is dosage dependent, we utilized the temperature sensitivity of the GAL4-UAS system and performed these crosses at 29°C. The cytotoxicity of *aβ42(2X)* expression at 25°C induces less potent phenotypic changes than at 29°C. However, Eiger-*aβ42(2X)* co-expression induces degeneration at both temperatures.]

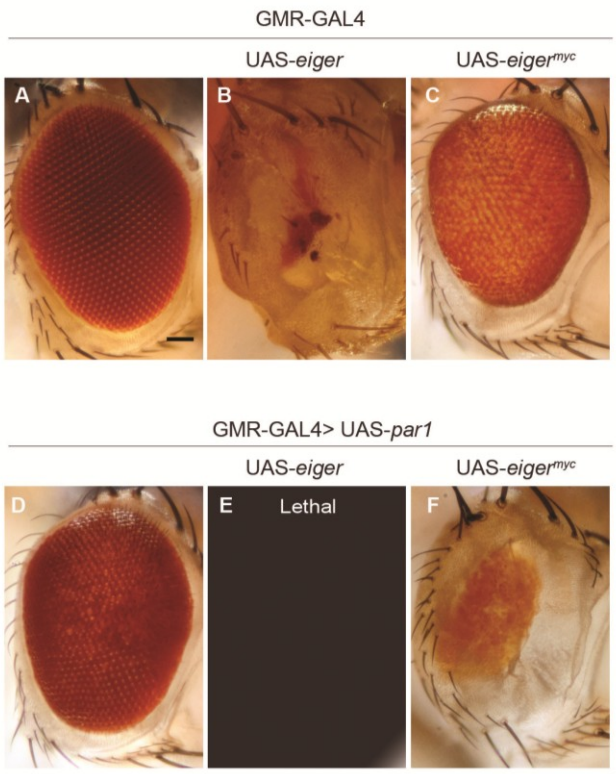


Figure 4.2 PAR-1 enhances Eiger-induced cell death

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images were taken at 10X magnification. Crosses were carried out at 25°C. A-F Over-expression of PAR-1 generates a 'rough-eye' phenotype, that is enhanced by the co-expression of epitope tagged or untagged Eiger. UAS-*par-1* transgene carries the full-length *par-1* gene. UAS-*eiger* is an untagged isoform of Eiger and UAS-*eiger*^{myc} is an epitope tagged, weaker isoform of Eiger. The individual genotypes are listed below. Wild-type compound eye, A.GMR-GAL4/*CyO*. Over-expression of Eiger generates a 'small-eye' phenotype, due to induction of apoptosis, B. GMR-GAL4,UAS-*eiger*/*CyO*. Over-expression of Eiger^{myc} generates a less potent cell death phenotype, which we refer to as the 'rough-eye' phenotype, C. GMR-GAL4,UAS-*eiger*^{myc}/*CyO;TM6B,Tb*^{1}/+. Over-expression of the *par-1* transgene, induces a mild 'rough-eye' phenotype, D. GMR-GAL4/*CyO;*UAS-*par-1*/*TM6B,Tb*^{1}. Co-expression of untagged isoform of Eiger with UAS-*par-1* enhances cytotoxicity, due to increased pupal lethality, E. GMR-GAL4,UAS-*eiger/+;*UAS-*par-1*/+. Co-expression of *myc* tagged isoform of Eiger with UAS-*par-1* enhances cytotoxicity, as shown by complete ablation of the compound eye, F. GMR-GAL4,UAS-*eiger*^{myc}*/CyO;*UAS-*par-1*/*TM3,Sb*^{1}. Scale bar - 100µm. [Note the enhanced potency of cell death induced by the UAS-*eiger* transgene, when expressed at 25°C compared to 29°C - compare Figure 2B with 1B. Hence we have adopted the nomenclature of 'small-eye' to refer to the ectopic expression of Eiger at 25°C and 'small+-eye' to refer to the ectopic expression of Eiger at 29°C]

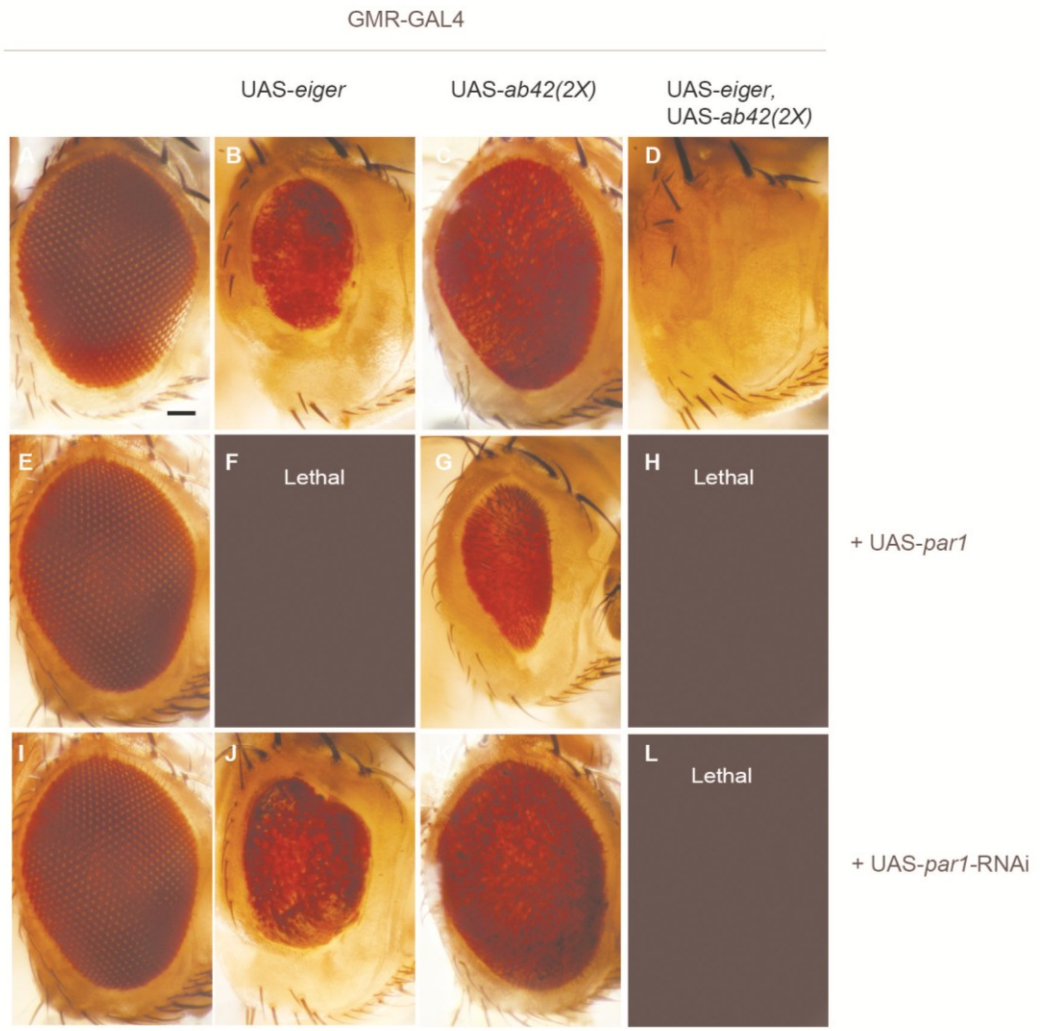


Figure 4.3 PAR-1 is a critical kinase downstream of Eiger-Aβ42(2X)–induced toxicity.

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images were taken at 10X magnification. Crosses were carried out at 29°C. A-L The gain or loss of PAR-1 expression enhances Eiger-Aβ42(2X)-induced toxicity. UAS-*par-1* transgene carries the full-length *par-1* gene. UAS-*par-1*-RNAi, expresses dsRNA for RNAi of PAR-1, under UAS-control. UAS-*eiger* is an untagged isoform of Eiger and UAS-*aβ42(2X)* transgene is made up of two tandem copies of human amyloid-*β(1-42*). The individual genotypes are listed below. Wild-type compound eye, A.GMR-GAL4/+*;TM3,Sb*^{1}/+. Over-expression of Eiger generates a 'small+-eye' phenotype, due to induction of apoptosis, B. GMR-GAL4,UAS-*eiger*/+*;TM3,Sb*^{1}/+. Over-expression of Aβ42(2X) generates 'enhanced-rough eye' phenotype, C. GMR-GAL4/UAS-*aβ42(2X);TM3,Sb*^{1}/+. Co-expression of Eiger and Aβ42(2X) induces massive cell death causing ablation of the compound eye, D. GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X);TM3,Sb*^{1}/+. The ectopic expression of PAR-1, E.GMR-GAL4/*CyO;*UAS-*par-1*/*TM3,Sb*^{1}, enhances toxicity by the induction of pupal lethality in the presence of Eiger

over-expression, F.GMR-GAL4,UAS-*eiger/+;*UAS-*par-1*/+ or Eiger-Aβ42(2X) co-expression, H. GMR-GAL4,UAS-*eiger/*UAS-*aβ42(2X);* UAS-*par-1*/+. The ectopic expression of PAR-1 enhances cell death in the presence of Aβ42(2X), as observed by the significant reduction in the eye-size, G. GMR-GAL4*/* UAS-*aβ42(2X);* UAS-*par-1*/+. The loss of PAR-1 expression by RNAi, I. GMR-GAL4/+*;*UAS-*par-1-RNAi*/+ does not suppress Eiger-induced 'small+-eye' phenotype, J. GMR-GAL4,UAS-eiger/*CyO*;UAS-*par-1-RNAi*/*TM3,Sb*^{1} or Aβ42(2X)-induced 'enhanced-rough eye' phenotype, K. GMR-GAL4/UAS-*aβ42(2X);*UAS-*par-1-RNAi*/+. The loss of PAR-1 expression however enhances toxicity of Eiger-Aβ42(2X) co-expression, by inducing pupal lethality, L. GMR-GAL4,UAS-eiger/UAS-*aβ42(2X);*UAS-*par-1-RNAi*/+. Scale bar - 100μm.

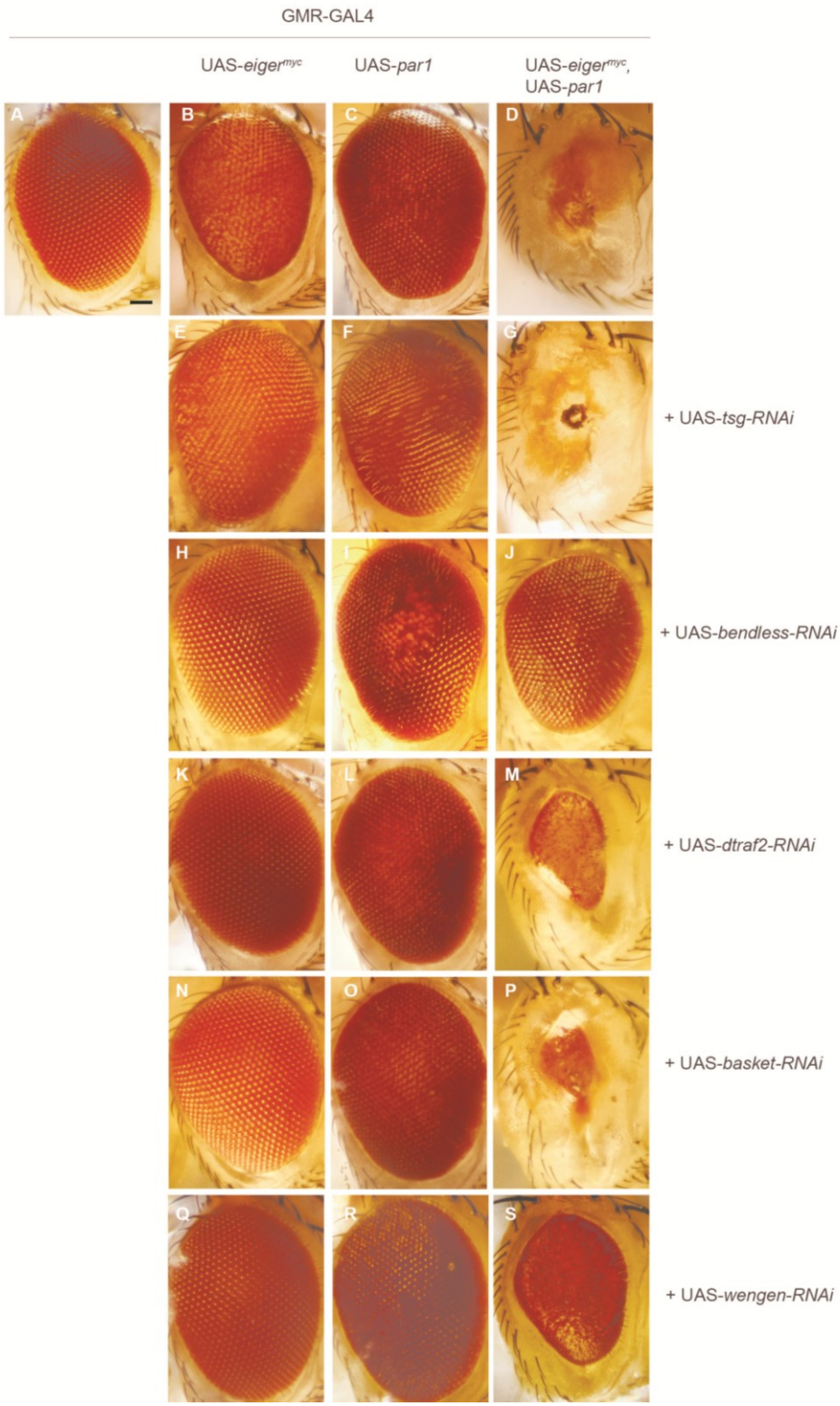


Figure 4.4 Eiger potentiates PAR-1 induced cell death through a novel ubiquitin-dependent mechanism, downstream of Fly TNFR, Wengen

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images were taken at 10X magnification. Crosses were carried out at 25°C. A-S Eiger co-ordinates PAR-1 mediated cell death, through TNFR, Wengen and ubiquitin-conjugating enzyme, Bendless. UAS-*par-1* transgene carries the full-length *par-1* gene. UAS-*eiger*^{myc} is an epitope

tagged, weaker isoform of Eiger. The individual genotypes are listed below. Wild-type compound eye, A. GMR-GAL4/*CyO*. Over-expression of Eiger^{myc} generates a 'rough-eye' phenotype, due to weak induction of apoptosis, B. GMR-GAL4,UAS-*eiger*^{myc}/*CyO,* that is unaffected by the knockdown of E. an unrelated gene, *tsg,* GMR-GAL4,UAS-*eiger*^{myc}/+;UAS-*tsg*-RNAi/+ (used as a negative control). Over-expression of PAR-1 generates a mild 'rough-eye' phenotype, C. GMR-GAL4/*CyO;*UAS-*par-1*/*TM6B,Tb*^{1} *,* that is unaffected by the knockdown of F. an unrelated gene, *tsg,* GMR-GAL4/+*;*UAS-*par-1*/UAS-*tsg*-RNAi (used as a negative control). Co-expression Eiger^{myc} and PAR-1 enhances cytotoxicity, as shown by complete ablation of the compound eye, D. GMR-GAL4,UAS-*eiger*^{myc}*/CyO;*UAS-*par-1*/*TM3,Sb*^{1}, that is unaffected by the knockdown of G. an unrelated gene, *tsg,* GMR-GAL4,UAS-*eiger*^{myc}*/+;*UAS-*par-1*/UAS-*tsg*-RNAi (used as a negative control). The activation of cell death through the ectopic expression of Eiger^{myc} is induced through a Wengen, dTRAF2, Bendless and Basket dependent mechanism. This is shown by the suppression of the Eiger^{myc} 'rough-eye' phenotype, by the loss of expression of Bendless- H. GMR-GAL4,UAS-*eiger*^{myc}/+;UAS-*bendless*-RNAi/+, dTRAF2 – K. GMR-GAL4,UAS-*eiger*^{myc}/+;UAS-*dtraf2*-RNAi/+, Basket– N. GMR-GAL4,UAS-*eiger*^{myc}/UAS-*basket*-RNAi, and Wengen Q. GMR-GAL4,UAS-*eiger*^{myc}/+;UAS-*wengen*-RNAi/+, by RNAi. The loss of expression of the Eiger signaling components, fails to suppress the mild 'rough-eye' phenotype of PAR-1 over-expression, as follows: Bendless - I.GMR-GAL4/+*;*UAS-*par-1*/UAS-*bendless*-RNAi ; dTRAF2 - L. GMR-GAL4/+*;*UAS-*par-1*/UAS-*dtraf2*-RNAi ; Basket - O. GMR-GAL4/UAS-*basket-RNAi;*UAS-*par-1*/+ and Wengen - R. GMR-GAL4/+*;*UAS-*par-1*/UAS-*wengen*-RNAi. However, the cell death induced by the co-expression of Eiger^{myc} and PAR-1 is predominantly suppressed by the loss of expression of Bendless, J.GMR-GAL4,UAS-*eiger*^{myc}*/+;*UAS-*par-1*/UAS-*bendless*-RNAi and Wengen, S. GMR-GAL4,UAS-*eiger*^{myc}*/+;*UAS-*par-1*/UAS-*wengen*-RNAi, but not by dTRAF2, M. GMR-GAL4,UAS-*eiger*^{myc}*/+;*UAS-*par-1*/UAS-*dtraf2*-RNAi or Basket, P. GMR-GAL4,UAS-*eiger*^{myc}*/*UAS-*basket-RNAi;*UAS-*par-1*/+ loss of expression. Scale bar - 100μm.

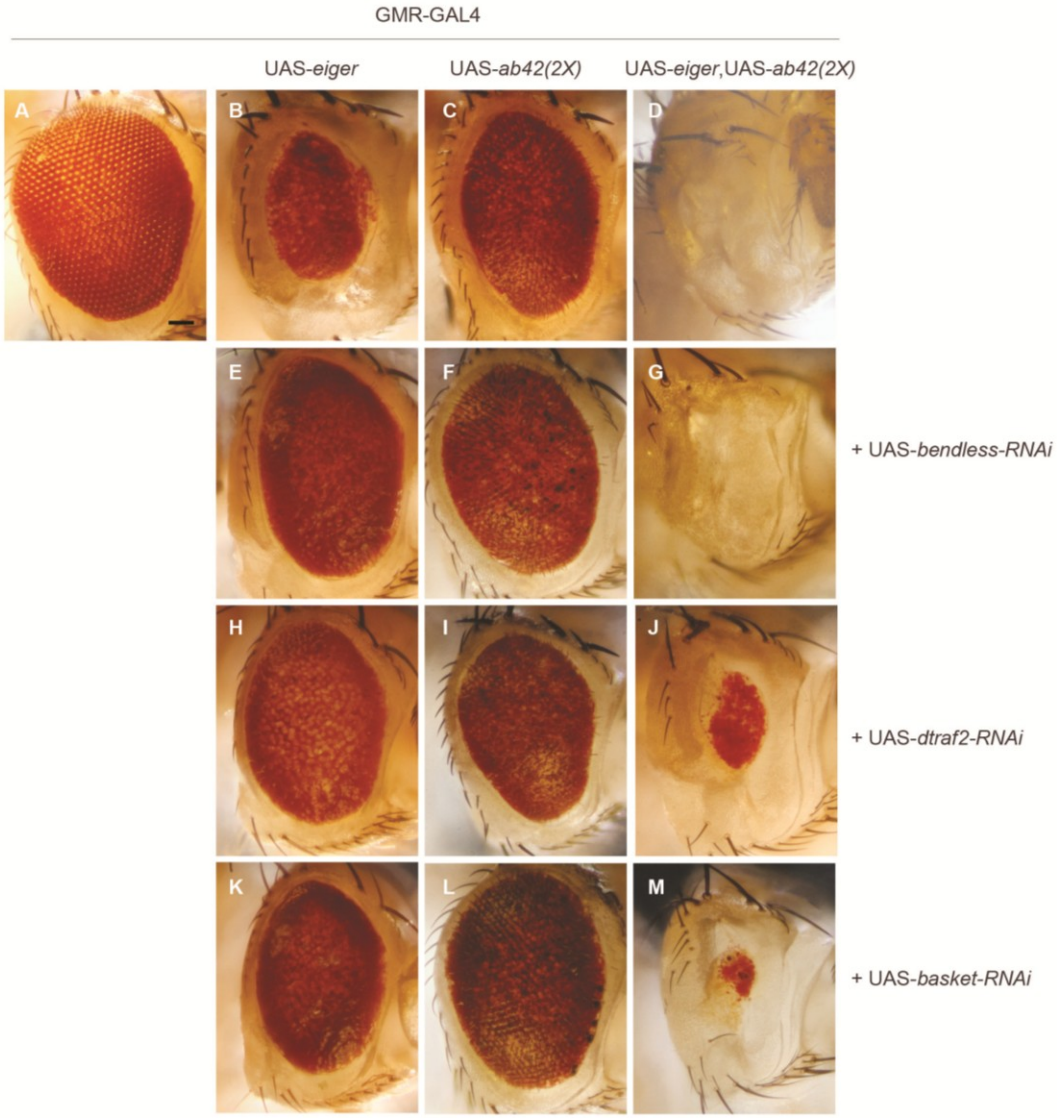


Figure 4.5 Eiger does not utilize the classic components of the Basket-cascade to induce Aβ42(2X)-cytotoxicity

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images were taken at 10X magnification. Crosses were carried out at 29°C. A-M Eiger does not signal through the canonical TNFR pathway in the presence of Aβ42(2X). UAS-*aβ42(2X)* transgene is made up of two tandem copies of human amyloid-*β(1-42*) under UAS control. UAS-*eiger* is an untagged isoform of Eiger. The individual genotypes are listed below. Wild-type compound eye, A. GMR-GAL4/*CyO*. The activation of cell death through the ectopic expression of Eiger is induced through a dTRAF2, Bendless and Basket dependent mechanism. This is shown by the suppression of the Eiger 'small+-eye' phenotype, B. GMR-GAL4,UAS-*eiger*/+;*TM3,Sb*^{1}/+, by the loss of expression of Bendless- E. GMR-GAL4,UAS-*eiger*/+;UAS-*bendless*-RNAi/+, dTRAF2 – H. GMR-GAL4,UAS-*eiger*/+;UAS-*dtraf2*-RNAi/+ or Basket– K.GMR-GAL4,UAS-*eiger*/UAS-*basket*-RNAi, by RNAi. The cytotoxicity of *aβ42(2X)* expression, induces an 'enhanced-rough eye' phenotype with necrotic spots, C. GMR-GAL4/UAS-*aβ42(2X);TM3,Sb*^{1}/+, that is unaltered by

the loss of expression of Bendless – F.GMR-GAL4/UAS-*aβ42(2X)*;UAS-*bendless*-RNAi/+, dTRAF2– I.GMR-GAL4/UAS-*aβ42(2X)*;UAS-*dtraf2*-RNAi/*TM3,Sb*^{1} or Basket- L. GMR-GAL4,UAS-basket-RNAi/UAS-*aβ42(2X)*, by RNAi. The co-expression of Eiger and UAS-*aβ42(2X)* enhances cytotoxicity, by complete ablation of the compound eye, D. GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X);TM3,Sb*^{1}*/+.* This phenotype is largely unaffected by the loss of expression of classic Eiger signaling components such as, Bendless – G.GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X)*;UAS-*bendless*-RNAi/+, dTRAF2, J. GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X)*;UAS-*dtraf2*-RNAi/*TM3,Sb*^{1}, and Basket, M. GMR-GAL4,UAS-*eiger,UAS-basket*-RNAi/UAS-*aβ42(2X);TM6B,Tb*^{1}*/+.* Scale bar - 100μm. [Note : The irregular arrangement of ommatidia in B,E,H and K in comparison to A maybe attributed to the expression of Eiger at an elevated temperature of 29°C].

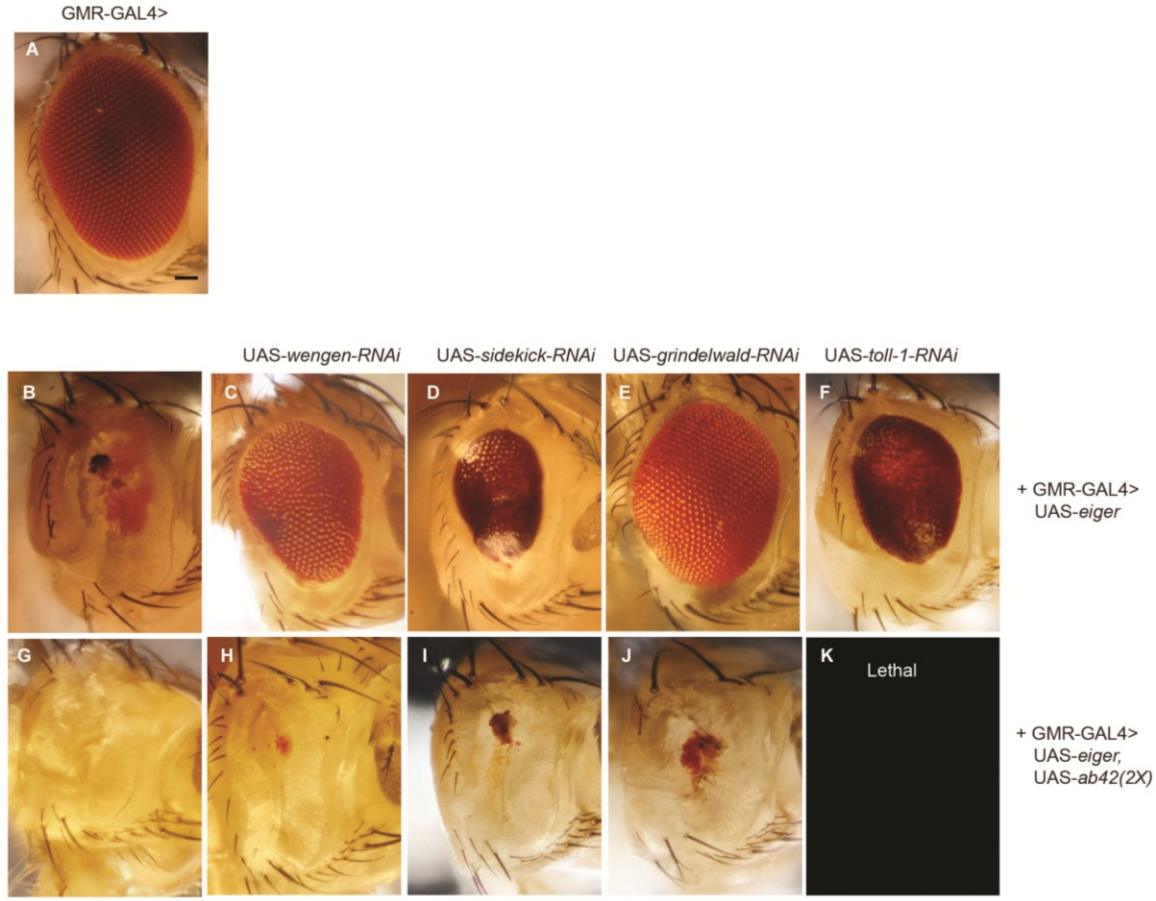


Figure 4.6 Eiger can induce cell death signaling through multiple receptors.
Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images were taken at 10X magnification. Crosses were carried out at 25°C. A-F Eiger activates cell death signaling through multiple receptors. G-K Eiger-*aβ42(2X)* toxicity is not mediated through Eiger-signaling receptors. The UAS- *aβ42(2X*) transgene is made up of two tandem copies of human amyloid-*β(1-42*) under UAS control. UAS-*eiger* is an untagged isoform of Eiger. The individual genotypes are listed below. Wild-type compound eye, A. GMR-GAL4/*CyO*. Eiger-induced 'small-eye' phenotype, B.GMR-GAL4,UAS-*eiger*/*CyO,* is suppressed by the loss of expression of flyTNFR, Wengen - C. GMR-GAL4,UAS-*eiger*/+;UAS-*wengen*-RNAi/+, Sidekick-D. GMR-GAL4,UAS-*eiger,*UAS-*sidekick*-RNAi/*CyO*, Grindelwald–E.GMR-GAL4,UAS-*eiger*/UAS-*grindelwald*-RNAi and the Toll-1 receptor – F.GMR-GAL4,UAS-*eiger*/+;UAS-*toll-1*-RNAi/+. Eiger-*aβ42(2X)* co-expression induced toxicity, G.GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X);TM3,Sb*^{1}/+ is not suppressed by the loss of expression of - Wengen H. GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X)*;UAS-*wengen*-RNAi/+ , Sidekick I. GMR-GAL4,UAS-*eiger,*UAS-*sidekick-RNAi*/UAS-*aβ42(2X*);*TM3,Sb*^{1}/+ or Grindelwald J. GMR-GAL4,UAS-*eiger,*UAS-*grindelwald-RNAi*/UAS-*aβ42(2X*);*TM3,Sb*^{1}/+. However, loss of expression of Toll-1 receptor enhances the toxicity of Eiger-*aβ42(2X)* co-expression, K. GMR-GAL4,UAS-*eiger*/UAS-*aβ42(2X)*;UAS-*toll-1*-RNAi/+. Scale bar - 100μm.

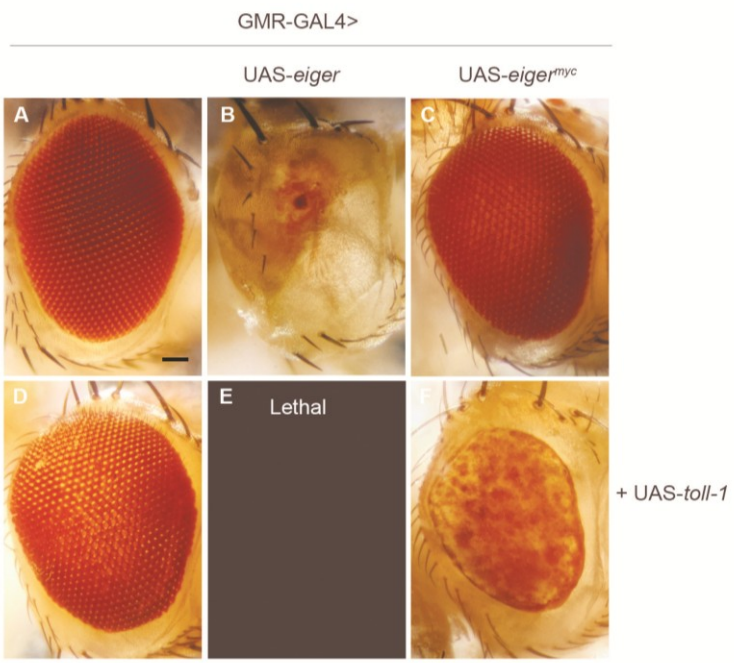


Figure 4.7 Eiger partners with the Toll-1 receptor to induce cell death signaling.

Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images were taken at 10X magnification. Crosses were carried out at 25°C. A-F Over-expression of Eiger generates a 'small-eye' phenotype that is enhanced by the co-expression of the Toll-1 receptor. UAS-*toll-1* transgene carries encodes the full length Toll-1 receptor. UAS-*eiger* is an untagged isoform of Eiger and UAS-*eiger*^{myc} is an epitope tagged, weaker isoform of Eiger. The individual genotypes are listed below. Wild-type compound eye, A.GMR-GAL4/+*;TM3,Sb*^{1}/+. Over-expression of Eiger generates a 'small-eye' phenotype, due to induction of apoptosis, B. GMR-GAL4,UAS-*eiger*/+*;TM3,Sb*^{1}/+. Over-expression of Eiger^{myc} generates a less potent 'rough-eye' phenotype, C. GMR-GAL4,UAS-*eiger*^{myc}/*CyO*. Over-expression of the Toll-1, does not present any phenotypic changes in the eye, D. UAS-toll-1/+;GMR-GAL4/+. Co-expression of untagged isoform of Eiger with UAS-*toll-1* enhances cytotoxicity, due to increased pupal lethality, E. UAS-toll-1/+;GMR-GAL4,UAS-*eiger*/+. Co-expression of *myc* tagged isoform of Eiger with UAS-*toll-1* enhances cytotoxicity, as shown by the reduction and disruption of the compound eye, F. UAS-toll-1/+;GMR-GAL4,UAS-*eiger*^{myc}/+. Scale bar - 100µm.

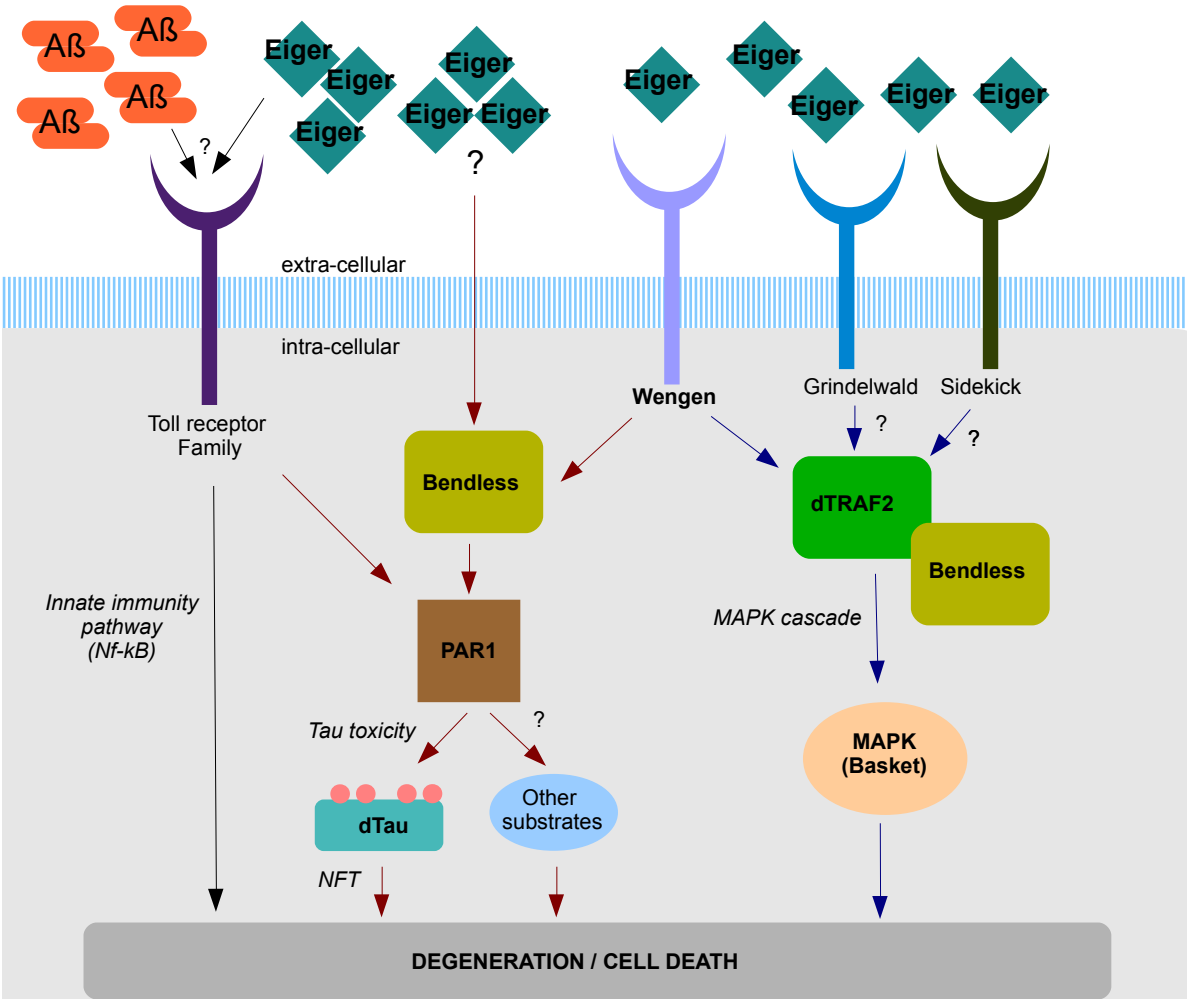


Figure 4.8 Model depicting multiple modes of Eiger-dependent pro-degenerative signaling.

FlyTNF, Eiger activates cell death through the hierarchical activation of the JNK/Basket signaling pathway (blue arrows), via TNFR Wengen, adaptor protein dTRAF2 and ubiquitin conjugating enzyme(E2), Bendless. Genetic screens have revealed the presence of additional receptors, Grindelwald and Sidekick as effectors of Eiger-dependent cell death signaling. Grindelwald has been shown to interact with dTRAF2, but the down-stream mechanism of the two newly identified receptors are currently unknown. This current study highlights two alternate cascades of Eiger-dependent cell death signaling. We identify a PAR-1 mediated Eiger signaling module that is partially dependent on TNFR, Wengen and completely dependent upon E2, Bendless (red arrows). PAR-1 regulated degeneration has been shown to occur via multiple mechanisms, but predominantly through the formation of neurofibrillary tangles (NFT), induced by the destabilization and hyper-phosphorylation of dTau. Additionally, the progression of *aβ42*-mediated toxicity has been shown to occur via a PAR-1-Tau signaling module. Our results show a strong induction of *aβ42*-mediated cell death, in the presence of Eiger. We identify a novel genetic interaction between Eiger and a dToll-dependent signaling cascade (black arrows), which could synergistically amplify *aβ42(2X)*-induced degeneration, through PAR-1 dependent and independent pathways.

CHAPTER 5 – GENERAL DISCUSSION OF THE THESIS

5. Major findings

The work in this thesis establishes the role for important regulators of JNK-dependent cell death signaling, in a Drosophila model of TNF-α signaling. In our current study, we have utilized a well-established genetic model system, driven by the ectopic expression of fly TNF, Eiger in the eye-imaginal disc of *Drosophila melanogaster*. We genetically validate the importance of the TRAF6 homolog, dTRAF2 in Eiger-mediated JNK signaling and show the critical dependence of dTRAF2 dosage in cell death signal transduction. In order to gain insight into regulatory mechanisms that modulate the Eiger-JNK signaling cascade, we use an *in vivo* ubiquitin tagging system to isolate and identify novel Eiger-induced ubiquitinated conjugates. We genetically validate the importance of an essential ubiquitin-conjugating enzyme, Bendless that is up regulated in response to Eiger activation, highlighting the importance of ubiquitin-dependent regulation over the JNK signaling network. We also generate a model system, for Aβ-induced degeneration that requires the participation of Eiger in a novel signaling pathway, independent of its JNK signaling potential. Thus, we use the power of Drosophila genetics to dissect the signaling potential of fly TNF, Eiger in multiple pro-apoptotic signaling contexts, to understand the basic similarities and phylogenetic differences of mammalian TNF signaling mechanisms.

5.1. Understanding p75-mediated JNK signaling through *Drosophila melanogaster*

p75NTR engages in apoptotic signaling both during development and injury, in a variety of cell types (Roux and Barker, 2002). p75NTR is a unique member of the TNFR superfamily, due to its preference to signal apoptosis through the intrinsic cell death pathway, as opposed to the cell-surface regulated death receptor pathway. p75NTR engages effector caspases in cell death, through mitochondrial control as opposed to the FADD-caspase 8 driven mechanism utilized by majority of the TNFRs (Gu et al., 1999; Troy et al., 2002; Wang et al., 2001b). There is lot scientific evidence that shows the importance of JNK activation for the transcriptional and translational control of the mitochondrial-caspase 9-intrinsic pathway (Bhakar et al., 2003; Donovan et al., 2002; Harris and Johnson, 2001; Lei and Davis, 2003;

Putcha et al., 2003; Whitfield et al., 2001). The p75NTR signaling networks are governed by their dependence over other co-receptors and multiple ligands, making it difficult to dissect its molecular details in *vivo* (Barker, 2004). Majority of the cellular assays utilized to study p75NTR-dependent cell death, focus on over-expression paradigms, that have led to the identification of several p75NTR interacting proteins upstream of the JNK signaling pathway (Roux and Barker, 2002). However, the mechanism of how these essential interactors regulate the JNK signaling network requires extensive study.

Evolutionary origin of diverse members of the TNFR superfamily coincides with the evolution of adaptive immunity in jawed vertebrates (Collette et al., 2003). But sequence analysis and functional similarities suggest the origin of receptors EDAR, EDAR2R, Troy and p75NTR, to be an earlier event in TNFR evolution. Invertebrate TNFR receptors show no sequence resemblance to the classic death receptors (e.g. Fas/TNFRI/DR4/5) of the vertebrate TNFR superfamily. BLAST sequence analysis revealed a close similarity between the CRD of p75NTR and fruit-fly p75NTR homolog, Wengen (Bothwell, 2006). Wengen like p75NTR, has been shown to engage in JNK-dependent cell death signaling (Kanda et al., 2002). Further, the fruit-fly FADD-caspase 8 signaling cassette is dedicated to innate immune signaling functions (Lemaitre and Hoffmann, 2007) and thus has not been shown to engage in apoptotic signaling events, downstream of the FlyTNF pathway (Moreno et al., 2002). Thus, the vertebrate TNFR extrinsic cell death cascade may appear to be a ‘recent’ evolutionary acquisition from the invertebrate Toll receptors, with the more ancient invertebrate TNFRs dedicated to engage in the primordial JNK-dependent route, to signal cell death processes. Thus, understanding the mechanism of invertebrate TNF-TNFR mediated JNK signaling events in Drosophila, will highlight conserved cellular regulatory principles that govern p75NTR-dependent cell death in vertebrates.

5.1.1. Eiger-induced cell death signaling is fine tuned by the levels of adaptor protein, dTRAF2

The discovery of Drosophila TNF homolog - Eiger (Igaki et al., 2002; Moreno et al., 2002) and TNFR homolog - Wengen (Kanda et al., 2002) propelled the study

of invertebrate TNFR signaling pathways. The ectopic expression of Eiger in the eye-imaginal disc has been shown to induce massive apoptosis, causing ablation of the compound eye. Further, this mode of cell death induction was identified to engage in JNK activation, rather than in FADD-caspase8-mediated cell signaling (Moreno et al., 2002). The intrinsic cell death pathway in Drosophila is different from mammalian systems, by the dispensable need for mitochondrial involvement. The release of IAP antagonists such as Smac/Diablo from the mitochondria trigger the release of Caspase 9 from IAP control, thus promoting the formation of the apoptosome (Apaf-1 + Caspase 9). However, the fruit-fly homologs of IAP antagonists – Hid, Grim and Reaper, are not controlled by mitochondrial release but rather require JNK-dependent transcriptional events to mediate Caspase 9/Dronc activation (Kornbluth and White, 2005). Thus, there maybe evolutionary variations in the mechanisms of cell death induction downstream of JNK signaling, but the proteins engaged in the signaling cascade upstream are very well conserved.

Several components of the Drosophila JNK signaling network were identified by extensive genetic screens (Andersen et al., 2015; Geuking et al., 2005; Geuking et al., 2009; Igaki et al., 2002; Kanda et al., 2002; Kanda et al., 2011; Ma et al., 2012; Ma et al., 2014; Ma et al., 2013b; Moreno et al., 2002; Zhang et al., 2010). TNF-TNFR ligand-receptors pairs communicate with downstream signaling players through intermediate adaptor proteins. The classic adaptor proteins of the mammalian TNFR superfamily, the TRAF proteins serve as scaffolds that bring JNK or Nf-κB signaling networks to specific cellular compartments and exert regulation over them (Dempsey et al., 2003). The RING domain in TRAF proteins, provides an added potential to the TRAF members to engage in E3-Ubiquitin ligase activity (Fang et al., 2003). The role of fruit-fly TRAF homologs – dTRAF1 and dTRAF2 have not been clearly established in Eiger-Wengen signaling. Chapter 2 of this thesis highlights the indispensible role for dTRAF2 in Eiger-mediated signaling and dispels the need for dTRAF1. We show that a careful regulation of dTRAF2 expression levels is imperative to govern the extent of cell death activation, in response to ectopic Eiger expression. By careful promoter-regulated titration of dTRAF2 expression, we show the failure of participation of dTRAF2 in Eiger-induced cell death, when its expression levels exceed endogenous levels of the protein. Co-

expression of Eiger and dTRAF2 cause their engagement in the growth-regulatory network of Akt1 signaling, thus intercepting the JNK apoptotic signaling module.

Our preliminary data suggests the importance of the dTRAF2 RING domain in cell death signaling, which we show by mutating critical Zn^{+2} coordinating residues (C104A and H121A) in the RING domain of the protein. Optimal expression levels of the $dTRAF2^{wild\text{-}type}$ but not $dTRAF2^{C104A/H121A}$, is essential for Eiger-induced cell death signaling. Our work thus highlights the critical importance of the scaffold protein - dTRAF2, which through a RING-dependent manner governs the extent of Eiger-dependent cell death induction.

5.1.2. Eiger mediates cell death signaling, through ubiquitin-dependent regulation

The covalent modification of cellular proteins with monomers or polymers of ubiquitin, have a direct impact on their turnover and cellular function. Ubiquitin-conjugating enzymes/E2s control the nature of the lysine residue utilized to tag cellular proteins, which affect the fate of corresponding substrates. The specificity of the substrate is under the control of the E3-ubiquitin ligases/E3s that interact with both the substrate and a specific E2 in order to facilitate ubiquitin transfer (Pickart, 2001). The ubiquitin-dependent regulation of signaling proteins, have been extensively studied with regard to TNF-dependent Nf-κB signaling. Polyubiquitin liked via K^{48} regulates the turnover of proteins and tags them for proteasomal degradation. The proteasomal degradation of IκB, by K^{48}- linked polyubiquitin occurs via the E2, Ubc4/5 and the SCF ubiquitin E3 complex, which facilitate the activation of TNF-α dependent Nf-κB signaling (Chen et al., 1995; Chen et al., 1996; Yaron et al., 1998). Studies in IL-1 and TLR signaling have highlighted the importance of K^{63}-linked chains, which engage in protein-protein interactions and serve a scaffolding function in signaling pathways.

TRAF6 was first identified as an E3 for the activation of the TAK1-TAB2/3 complex, by auto-ubiquitination via K^{63}-specific E2, Ubc13. Ubc13 forms a heteromeric complex with Uev1A and is essential for K^{63}- linked non-degradative ubiquitin modifications (Deng et al., 2000). Ubc13 also engages in polyubiqutination

of RIP1 in response to TNF-α, for activation of the Nf-κB signaling network (Bertrand et al., 2008b; Ea et al., 2006; Li et al., 2006). The role of Ubc13 in ubiquitin-regulation is also well conserved in Drosophila. The Ubc13 homolog, Bendless has been shown to participate in K^{63} poly-ubiquitin dependent modulation of TAK1 and IKK, in the innate immune signaling pathways. Ubc13 homolog- Bendless and Uev1a homolog – dUev1a are both essential components downstream of the IMD pathway, in response to Gram-negative bacterial infection (Zhou et al., 2005b). The chapter 3 of this thesis highlights the indispensible role for K^{63}-polyubiqutinating E2, Bendless in Eiger-dependent JNK signaling. We show a complete suppression of Eiger-induced cell death in response to Bendless loss of function or expression. However, the ubiquitinated targets that lie on the Eiger signaling pathway have not been identified. To address this, we express a modified form of ubiquitin that undergoes metabolic tagging with Biotin, *in vivo.* The co-expression of this form of ubiquitin with Eiger enabled us to isolate a pool of ubiquitinated proteins that are selectively enriched in response to Eiger. We utilize MS/MS to identify these isolated targets and validate the enrichment of proteins belonging to the ubiquitin regulatory system. We observe the conjugation of Bendless with ubiquitin, in addition to four other E2s with conserved roles in K^{48}-linked ubiquitin modifications.

We also show an enrichment of DNA damage response genes, in response to ectopic Eiger, suggesting an enhanced oxidative stress response. We have also discovered Eiger-specific ubiquitin-conjugated proteins, such as GAPDH and Aconitase, which are associated with the mitochondrial energy homeostasis network. Eiger has been previously shown to activate ROS production, downstream of JNK signaling, by destabilization of metabolic enzymes in the mitochondria. The generation of ROS, was shown to activate a caspase-independent mode of cell death, employed to eradicate tumorigenic cells with aberrant polarity in the epithelia (Kanda et al., 2011). The induction of oxidative stress was shown to enhance the susceptibility of tumor cells for Eiger-dependent elimination. Thus, our study hints to a potential relationship between oxidative stress-induced DNA damage and the induction of Eiger-induced apoptosis.

5.1.3. Eiger participates in pro-degenerative signaling, through novel JNK-independent genetic interactions.

There are very few proteins other than p75NTR, that are notorious for their participation in a bewildering list of complex cellular functions that directly impact both cellular physiology and disease pathology. One such protein is APP, and understanding its biology remains a major scientific challenge. Our knowledge of APP has stemmed from extensive studies done in animal models and the identification of its many protein-binding partners (Reinhard et al., 2005). The identification of pathological roles of APP, has been attributed to the detection of aberrant cleavage products of the full-length protein. The toxic-by product of APP cleavage – the Aβ peptide have been identified in large aggregates in AD and has since been associated as the major causative agent of cytotoxicity. A multitude of mechanisms have been proposed to understand the biological pathways adopted by Aβ in neuropathology (O'Brien and Wong, 2011).

Aβ is known to engage in the signaling pathways of several receptors, one of which involves p75NTR, whose expression and engagement has been shown to promote Aβ-induced toxicity (Xia et al., 2014). Aβ-p75NTR dependent collaboration has been shown to occur through JNK and Nf-κB dependent mechanisms (Costantini et al., 2005; Kuner et al., 1998; Tsukamoto et al., 2003; Yaar et al., 2002). But the molecular details of the signaling networks that communicate the neurotoxic effect of the Aβ peptide remain elusive.

In our current study (Chapter 4), we have utilized an Alzheimer's disease model in *Drosophila melanogaster*, to address the importance of the fly TNF-TNFR signaling network in pro-degenerative signaling. Our work defines a novel role for Eiger, as a potent activator of Aβ-induced toxicity. The ectopic expression of Aβ engages Eiger in a novel signaling pathway and thus intercepts its participation in the JNK signaling module. Our data suggests the involvement of Eiger in a dToll receptor pathway, which contributes towards Aβ-induced degenerative mechanisms. We also identify the dTau kinase, PAR-1 as an essential modulator of Eiger-induced degenerative signaling. Co-expression of PAR-1 with Eiger triggers a massive induction of cell death, which is partially dependent on the p75NTR homolog,

Wengen, completely dependent on K^{63}-specific E2, Bendless and independent of Basket/JNK control. Our study links over-lapping signaling networks of Eiger-dependent pro-degenerative cellular pathways that contribute towards Aβ-induced toxicity.

5.2. Conclusions and future directions

Our study highlights the importance of dTRAF2 in the fine-tuning of the Eiger signaling pathway (Chapter 2). The TRAFs in mammalian signaling pathways have been shown to engage in interactions with TNFRs, in order to activate downstream kinases. Though we show an indispensible need for dTRAF2 in the Eiger signaling pathway, we fail to observe a direct physical interaction between dTRAF2 and Wengen (data not shown in this thesis). Further, the ectopic expression of Eiger triggers massive apoptosis, but the transgenic expression of full-length Wengen or its intracellular domain, is not sufficient to engage in cell death activation (data not shown in this thesis). Thus, under physiological conditions, the over-expression of Wengen is not sufficient to activate JNK signaling, potentially due to the limited availability of Eiger. This could also be due to the stringent regulation of dTRAF2 recruitment by crucial signaling modulators, to prevent deregulated activation of cell death. We and other groups have shown the ability of Eiger to mediate apoptotic signaling through multiple receptors (Chapter 4), in addition to Wengen. Thus, Wengen may require the collaboration of other co-receptors to engage in dTRAF2 interaction and down-stream Basket/JNK signaling.

The system is more complex than previously imagined and future studies aimed at the identification of the Eiger-activated receptor complex and the proteins there in, will be imperative to understand the conserved constituents of the JNK signaling network. The generation of an epitope tagged form of Eiger based on a proximity-dependent biotinylation *(BioID)* strategy would permit the identification of the protein complex at the ligand-receptor vicinity. Our study places dTRAF2 as a critical determinant downstream of the ligand-receptor complex, which exerts control over its signaling network by auto-regulation of its expression levels. Further, our data points to the critical importance of the RING domain in Eiger signaling. Future studies aimed at further validation of dTRAF2 E3 ubiquitin ligase activity and

identification of the substrates regulated by the RING-dependent activity of dTRAF2, would further our knowledge of TRAF-regulated signaling strategies.

We have created an essential tool to isolate and identify proteins that are conjugated by ubiquitin, in response to Eiger signal transduction (Chapter 3). Our results highlight the enrichment of ubiquitinated proteins in the Eiger signaling pathway. We provide genetic and biochemical validation for the requirement of E2, Bendless on the Eiger signaling pathway. Our results show the prerequisite for non-degradative K^{63}-linked ubiquitination in the JNK signaling network. Additionally, we also identify an Eiger specific enrichment of ubiquitin-conjugated E2s, which have not been previously studied with regard to both mammalian and fly TNFR signaling. We were unable to observe a suppression of Eiger-mediated cell death upon loss of expression of the individual ubiquitin-conjugated E2s (UbcD1, UbcD2, CG40045 and CG7656), identified in the screen. We attribute this result to the redundancy of the closely related E2s (UbcD1 and UbcD2 ; CG7656 and CG40045), and we are currently performing double knockdowns of the homologous E2 pairs, to validate their importance on the Eiger cell death signaling network. Future studies aimed at further validation using specific loss of function or null alleles of the novel Eiger-specific E2s identified, followed by a search for specific substrates that are regulated by these novel Eiger-specific E2s will add to our knowledge of the pro-apoptotic TNFR signaling cascade.

The ubiquitin-tagging system can also be utilized to understand the modulatory role of dMAGE in ubiquitin-dependent regulation of novel substrates. A MAGE-D family member, MAGE-D1/NRAGE was identified as an interactor of p75NTR from a yeast two hybrid screen (Salehi et al., 2000). There is a lot of *in vivo* evidence that suggests a critical role for NRAGE in p75NTR dependent apoptotic signaling, shown in PC12 cells, sympathetic neurons, retinal ganglia and non-neuronal cells like keratinocytes (Bertrand et al., 2008a; Lebrun-Julien et al., 2010; Salehi et al., 2002; Truzzi et al., 2011). But the mechanism of NRAGE involvement in p75-dependent cell death remains unknown. Our preliminary results address the importance of the NRAGE homolog, dMAGE in Eiger-mediated JNK signaling. We observe a minor but not a complete suppression of Eiger-induced apoptosis, upon loss of dMAGE expression using $dMAGE^{RNAi}$ or a dMAGE loss of function allele

(dMAGE$^{XL+/-}$) (Figure 5.1a-D,E). This hints to a modulatory role for dMAGE in Eiger-JNK signaling.

The MAGE protein family members have been shown to serve as enhancers for RING-dependent ubiquitination events, by acting as *bona fide* interactors of RING domain containing E3-Ubiquitin ligases (Doyle et al., 2010). Several RING-domain containing proteins have also been implicated in Eiger-dependent JNK activation, such as dTRAF2 (unpublished data), NOPO (Ma et al., 2012) and POSH (Zhang et al., 2010). We observe a substantial reduction of Eiger-induced ubiquitin-bound conjugates upon loss of dMAGE expression (Figure 5.1b). Thus our study introduces the *in vivo* ubiquitin tagging system, as an effective read-out to assess changes in global ubiquitination targets that are selectively affected by dMAGE knockdown, in response to Eiger signal transduction. Genetic screening can also be conducted for the identification of other unknown RING domain containing proteins, which are potentially modulated by dMAGE in the Eiger-JNK pathway.

We have also uncovered interesting ubiquitin-associated participants that can be studied to understand the pro-inflammatory role of Eiger, through a ROS-driven mechanism of TNF signaling. This will have a major impact specifically with regard to our understanding of tumor cell death susceptibility, in response to TNF-up regulation and ROS generation. Our knowledge of oxidative stress-driven cell death signaling mechanisms will have implications in the development of more effective chemotherapeutics in the field of cancer biology.

Our results also show the importance of Eiger in the pathology of an Aβ-disease model in Drosophila (Chapter 4). We identify two novel Eiger-mediated mechanisms that contribute to the severity of degenerative processes in an AD model system. We identify PAR-1 an enhancer of Eiger-induced cell death. PAR-1 plays an essential role in the regulation of microtubule dynamics, via phosphorylation of dTau (Drewes et al., 1997; Nishimura et al., 2004). Deregulation of dTau phosphorylation by the aberrant activation of PAR-1 has been associated with the formation NFT in AD and tauopathies (Iijima-Ando and Iijima, 2010). Further, PAR-1 has been shown *in vivo,* to contribute towards dTau hyper-phosphorylation,

downstream of Aβ-induced toxicity at the NMJ (Lee et al., 2012). The ectopic expression of Eiger contributes to the severity of PAR-1 over-expression, which is dependent on Bendless and Wengen, but independent of Basket/JNK activation.

Our study highlights the upstream activators of PAR-1-modulated degeneration. Future studies aimed at identifying the down-stream modulators of the Eiger–Wengen-Bendless-PAR-1 signaling module will provide mechanistic insight into the contributions of the dTau kinase in AD pathology. The transgenic expression of dTau using the pan-retinal promoter GMR-GAL4, has been shown to generate a degenerative eye phenotype (Nishimura et al., 2004). Eiger and its signaling components can be assessed as potential modifiers of the dTau over-expression phenotype. This can also be accompanied by assessment of the phosphorylation status of Tau at AD related sites, in response to ectopic Eiger expression.

Additionally, we also identify a PAR-1 independent mechanism of Eiger-induced Aβ toxicity (Chapter 4). There is accumulating evidence that suggests a major contribution of the innate immune response in neurodegenerative disease progression. The microglia, which are the macrophages of the nervous system, engage in immune activation through mammalian Toll-like receptors (TLRs). Aβ aggregation has been shown to promote microglia-derived chronic innate immune responses, which contribute towards disease progression in AD (Akiyama et al., 2000). This has been validated by the efficacy of non-steroidal anti-inflammatory drugs/NSAIDS in reducing the severity of the disease (McGeer et al., 1990), accompanied by neuro-immune modulatory effects studied in AD model systems (Morgan et al., 2005). Aβ-induced microglia activation has been shown to induce the up-regulation of pro-inflammatory cytokines like TNF-α and ROS, in an Nf-κB dependent manner, which collectively contributes towards AD disease progression (Combs et al., 2001). The up-regulation of TNF-α has also been observed in AD patient samples (Bruunsgaard et al., 1999; Fillit et al., 1991), which correlates well with our results that show a potentiation of Aβ toxicity, in response to ectopic flyTNF - Eiger.

Paradoxical reports show the involvement of mammalian TLR2, TLR4, TLR6 and TLR9 in neuro-protective effects, by the promotion of Aβ uptake and clearance (Chen et al., 2006; Doi et al., 2009; Song et al., 2011; Tahara et al., 2006). Thus a tight regulation of TLR signaling ensures the pro or anti-degenerative responses of microglia, where transient activation of TLRs facilitates a protective response against Aβ, but aberrant, prolonged activation promotes neurotoxicity (Akiyama et al., 2000).

Our study recapitulates the innate immune response in a Drosophila model of AD. We observe an Eiger-dependent potentiation of Aβ-induced toxicity, through a putative dToll dependent mechanism. Due to the engagement of multiple dToll receptors in innate immune activation, loss of expression of dToll-1 alone did not prevent degeneration induced by Eiger and Aβ co-expression, which maybe attributed to the redundancy of other eight dToll family members. In mammalian TLR signaling, with the exception of TLR3, all TLRs utilize MyD88 as the main adaptor for the activation of downstream Nf-κB signal transduction. Further, the loss of MyD88 has been associated with a decrease in AD severity (Lim et al., 2011). Thus, future studies aimed at addressing the downstream signaling players such as dMyD88, Pelle and Tube, in the dToll signaling cascades will prove to be more informative in delineating the pathway that contributes towards AD progression. Further, since the TLRs/dTolls represent the first line of defense against pathogens, the therapeutic intervention of its signaling components in AD requires careful consideration and further study. Our results thus provide a mechanistic over-view of PAR-1 dependent and independent mechanisms of Aβ-induced degeneration, which is potentiated by Eiger expression.

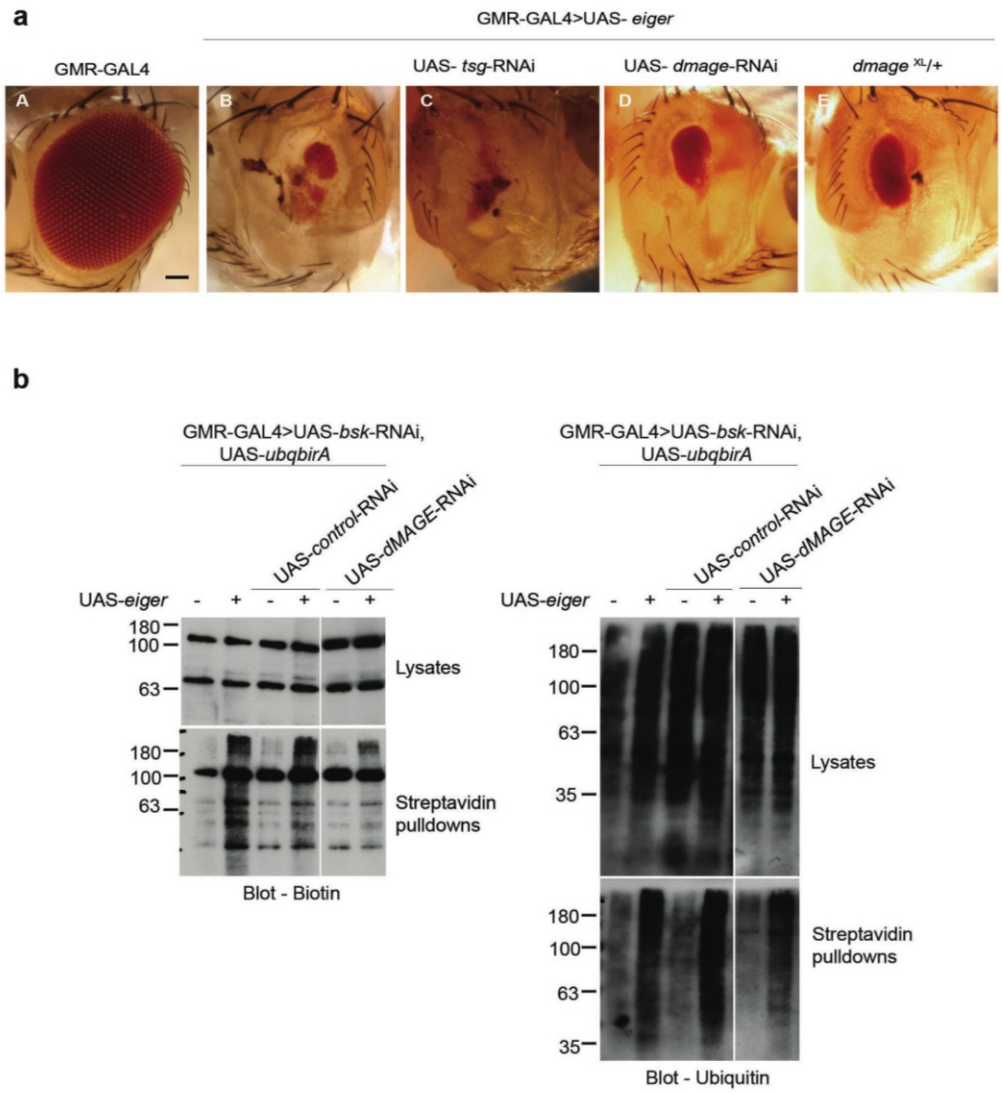


Figure 5.1 dMAGE, an E3 Ubiquitin Ligase facilitator on the Eiger pathway

a. Light micrograph images of adult Drosophila eye phenotypes with indicated genotypes. Images taken at 10X magnification. A. Wild type eye, GMR-GAL4/ *CyO*. B. Over-expression of Eiger generates a 'small eye phenotype', GMR-GAL4,UAS-*eiger*/*CyO*. Small eye phenotype is unaffected by the knockdown of a C. unrelated transgene- GMR-GAL4,UAS-*eiger*/+;UAS-*tsg*-RNAi/+(negative control). The small eye phenotype is marginally suppressed by the knockdown of dMAGE using an RNAi line D. GMR-GAL4,UAS-*eiger*/+;UAS-*dmage*-RNAi/+ and dMAGE loss of function allele, dmage^{XL}/+. E. GMR-GAL4,UAS-*eiger*/+;dmage^{XL}/+. Scale bar - 100µm

b. Loss of dMAGE expression decreases the accumulation of ubiquitinated target proteins, in response to Eiger-over expression. Isolation of Biotin-tagged ubiquitinated targets by streptavidin pull-down. Western blotting with anti-biotin and anti-ubiquitin. The increase in both biotin and ubiquitin levels in response to Eiger over-expression, decreases upon knockdown of dMAGE expression. 20 eye-brain complexes were used for each pull-down. Genotypes used for pull-downs are, [GMR-GAL4,UAS-*eiger*,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA/TM6B,*Tb^{1}],[GMR-GAL4,UAS-*eiger*,UAS-*basket*-

RNAi/*Bc*;UAS-*ubqbirA*/UAS-*tsg*-RNAi], [GMR-GAL4,UAS-*eiger*, UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA*/UAS-*dmage*-RNAi], [GMR-GAL4,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA/TM6B,*Tb[1]] [GMR-GAL4,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA*/UAS-*tsg*-RNAi], [GMR-GAL4,UAS-*basket*-RNAi/*Bc*;UAS-*ubqbirA*/UAS-*dmage*-RNAi].

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