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TNF SIGNALING DURING TISSUE DAMAGE-INDUCED NOCICEPTIVE SENSITIZATION IN DROSOPHILA

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by

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A

DISSERTATION

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DOCTOR OF PHILOSOPHY

by

Juyeon Jo, M.S.
Houston, Texas
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DEDICATION

This thesis is dedicated to my parents,

Gukbong Jo, Sukjeong Oh, Sandek Suh, and An-Ja Lee

who have always loved and supported me

And to my husband,

Ji Ho Suh,

a man who I have fortunate to have,

And my lovely son,

Jason K. Suh

TNF SIGNALING DURING TISSUE DAMAGE-INDUCED

NOCICEPTIVE SENSITIZATION IN *DROSOPHILA*

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Tumor necrosis factor (TNF) signaling is required for inflammatory nociceptive sensitization in both *Drosophila* and vertebrates. In *Drosophila* larval model of nociceptive sensitization, UV irradiation in results in epidermal apoptosis and thermal allodynia. TNF/Eiger is produced from dying epidermal cells and acts its receptor in nociceptive sensory neurons to induce thermal allodynia. Inhibition of TNF signaling results in attenuation of nociceptive sensitization whereas epidermal apoptosis still occurs in the absence of TNF. Major gaps in this model are the precise relationship between apoptotic cell death and production of TNF/Eiger, downstream signaling mediators for TNFR/Wengen, and target genes that alter nociceptive behaviors. Here we show that apoptotic cell death and thermal allodynia are genetically and procedurally independent of each other while initiator caspase Dronc is required for both. An apoptotic function of Dronc activates downstream effector caspase leading to execution of epidermal cell death whereas a non-apoptotic function of Dronc induces activation of TNF signaling. Behavioral analyses with overexpression of full-length or processed soluble TNF/Eiger suggest that Dronc-mediated processing/secretion of TNF/Eiger is important for nociceptive sensitization. It is also supported by the fact that Dronc is required for thermal allodynia when TNF/Eiger is ectopically expressed in nociceptive sensory neurons that normally do not produce TNF/Eiger for nociceptive sensitization. We found that Traf3, Traf6, a p38 kinase, and the transcription factor nuclear factor kappa B mediates TNF signaling for nociceptive sensitization. Finally, downstream target genes of TNF signaling are revealed by sensory neuron specific microarray analysis and behavioral validation. A conserved epigenetic factor, Enhaner of zeste (E(z)) is required for thermal allodynia as a downstream target gene of TNF/Eiger signal transduction. Our findings suggest that an initator caspase is involved in TNF processing/secretion during nociceptive sensitization and that TNF pathway activation leads to transcription of genes required for sensory neurons to sensitize. These findings have implications for both the evolution of inflammatory caspase function following tissue damage signals and the action of TNF during sensitization in vertebrates.

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Chapter 1: Introduction

1.1 Nociceptive sensitization

The ability to differentiate noxious stimuli from innocuous stimuli is pivotal for the survival of an organism. Nociception is an evolutionarily conserved sensory process for detecting harmful stimuli and is an essential mechanism to avoid potential tissue injury. Specialized peripheral sensory neurons called nociceptors detect noxious stimuli such as extreme temperatures, harsh mechanical stimuli, and pungent chemicals (1). Sensory perception of noxious stimuli by nociceptors results in two different outputs. First, action potentials initiated by nociceptors are relayed to the central nervous system and are processed by the brain, which generates cognitive awareness of sensation. Second, nociceptive signals trigger motor neurons and induce appropriate withdrawal behavior(s) to minimize contact with damaging stimuli (2-4). Molecular, pharmacological, and genetic analyses revealed multiple ion channels as molecular transducers of noxious stimuli in nociceptors. For example, Vanilloid Transient Receptor Potential 1 (TRPV1) mediates thermal nociception as well as a response to capsaicin (5) whereas Melastatin TRP 8 (TRPM8) is a cold sensitive channel (6). PIEZO and degenerin/epithelial Na (+) (DEG/ENaC) channels are essential for mechanical nociception (7, 8) and Ankyrin TRP1 (TRPA1) responds to allyl isothiocyanate (from wasabi) and allicin (from garlic) (9).

Once noxious stimuli are perceived by molecular transducers, a variety of voltage-gated sodium, potassium, and calcium channels are activated (2). Voltage-gated sodium channels are important for generating and propagating action potentials to convey nociceptive signals to the central nervous system. Loss-of-function studies demonstrated that Nav1.7 and Nav1.8 sodium channels play a key role in mechanical and thermal hypersensitivity (4, 10). Voltage-gated calcium channels are critical for transducing alterations in membrane potential into intracellular calcium transient, thus playing a key role in neurotransmitter release and gene expression (2, 11).

One important characteristic of sensory neurons is plasticity. Receptive properties of nociceptors can be modulated by various forms of tissue damage, nerve injury, inflammation, or bacterial infection (12-15). Alteration of sensitivity in nociceptors is called "nociceptive sensitization" and this adaptive modulation protects the injured tissue from further damage and enhances repair processes. Nociceptive sensitization elicits behavioral changes such as pain response to normally innocuous stimuli (allodynia) or exaggerated pain response to noxious stimuli (hyperalgesia). In addition, spontaneous pain may persist even in the absence of stimuli (16). Tissue damage-induced nociceptive sensitization is also called inflammatory pain because it mainly arises from the production and release of inflammatory mediators such as prostaglandine E_2 (PGE_2) (17), bradykinin (18), adenosine triphosphate (ATP) (19),

protons (20), nerve growth factor (NGF) (21), histamine (22), and cytokines such as tumor

necrosis factor alpha (TNF-α) and interleukins (23, 24). These inflammatory mediators are

produced from non-neuronal cells as well as from primary sensory neuron terminals (2).

Intensive investigations of molecular mechanisms underlying the action of inflammatory

mediators revealed that most of these mediators directly activate various receptors on

nociceptors and then activate downstream signaling cascades of second messengers and

kinases (13, 25). As a result, up-regulated expression of pro-nociceptive genes such as

cytokines, post-translational modification and cellular redistribution of ion channels

collectively contributes to the induction and/or maintenance of nociceptive sensitization (26-

29). However, it still has not been examined thoroughly how these signaling cascades are

regulated, what downstream molecular targets of each signaling cascade are, and how

potential interplay/convergence of multiple signaling occurs to induce nociceptive

sensitization.

1.2 *Drosophila* model for nociceptive biology and tissue injury-induced nociceptive

sensitization

    Drosophila is an advantageous model for elucidating basic principles of neuroscience

because sophisticated genetic manipulations are available (30). The use of *Drosophila* has

contributed to important discoveries of genes affecting learning and memory, circadian rhythms, sexual behavior, cell fate specification, olfaction, aging, and neurodegeneration in the last four decades (30-32). These works have revealed that the cellular and neural network characteristics including neuronal architecture and synaptic transmission are well conserved in the *Drosophila* nervous system. Moreover, approximately 65% of human disease genes are conserved in *Drosophila*, thereby making this organism attractive as a model for human disease (32).

Drosophila has also emerged as a valuable model for studying nociceptive biology (33). *Drosophila* larvae exhibit a distinctive aversive behavior: corkscrew-like rolling when they are exposed to high temperature above 38 °C or to harsh mechanical stimuli (34-36). This aversive withdrawal behavior is mediated by nociceptive sensory neurons called class IV multiple dendritic (MD) neurons, which exhibit complex dendritic branching (34, 35, 37, 38). Nociceptive sensory neurons in *Drosophila* are relatively simple but structurally and functionally similar to mammalian nociceptors (33). In *Drosophila* larvae, class IV MD neurons are clustered with other MD neurons, external sensory organs, and internal chordotonal neurons in each segment in a repeated fashion (39). Cell bodies of class IV MD neurons are located underneath the epidermis and above the larval body wall muscle (40, 41). Each neuron in each segment has an axonal projection to the ventral nerve cord, which is

functionally analogous to the spinal cord in vertebrate (42, 43). Dendrites of class IV MD

neurons grow along the basal surface of the epidermis because interactions between neuronal

integrins and epidermal laminins prevent dendritic growth into the epidermis (41). The naked

dendritic nerve endings of each cell does not overlap with their sister branches or/and another

class IV MD neurons in the same segment but provide a complete coverage within the

segment for instant sensory detection (38). Inhibition of class IV MD neurons by expression

of tetanus toxin abrogates noxious thermal and mechanical stimuli-induced rolling behavior

(35). In contrast, optogenetic activation of class IV MD neurons produces rolling responses

in the absence of noxious stimuli, suggesting that these neurons are polymodal and are

required for nociception (34). Importantly, TRP channels and DEG/ENaC channels play an

essential role in *Drosophila* nociception similar to vertebrates (35, 44, 45). For example,

Painless TRP channel is required for both thermal and mechanical nociception (34, 35).

Drosophila TRPA1 homolog detects noxious chemicals such as allyl isothiocyanate (46) and

thermal stimuli (44). PPK1 (45), PPK26 (47) DEG/ENaC channels and Piezo (48) are

responsible for mechanical pain detection. These suggest a strong conservation of the

molecular basis for nociception in flies.

Although anatomically and functionally, *Drosophila* nociception highly resembles

vertebrate nociceptive biology, it was unknown if *Drosophila* nociceptors exhibit complex

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features such as sensitization in response to injury. Babcock et al. (36) examined if *Drosophila* larvae develop hypersensitivity following tissue injury and succeeded in modeling tissue damage-induced nociceptive sensitization in fly larvae. In this model, a sub-lethal dose of UVC irradiation results in apoptotic epidermal cell death while the underlying nociceptive sensory neurons remain intact. UV irradiated larvae develop thermal hypersensitivity displaying both allodynia and hyperalgesia. Several conserved signaling pathways have been uncovered using the fly model of nociceptive sensitization. These include TNF (36), Hedgehog (Hh) (49), and Tachykinin (Substance P in vertebrate) (50) signaling pathways. In addition, the initiator caspase Dronc was identified as a contributor from epidermal tissue for nociceptive sensitization (36). The fact that the peak response of hyperalgesia is earlier than that of allodynia and hyperalgesia resolves before allodynia peaks suggests that these two forms of hypersensitivity are regulated by distinct mechanisms. Indeed, Babcock and colleagues found that the initiator caspase Dronc, TNF, and Tachykinin signaling are only required for allodynia whereas Hh signaling is required for both allodynia and hyperalgesia (36, 49). These observations demonstrated that the fundamental mechanisms and signaling pathways of nociceptive sensitization could be elucidated using the power of *Drosophila* genetics.

1.3 The TNF Signaling Pathway in Nociceptive sensitization

TNF was initially described as a substance that caused regression of tumors and the name tumor necrosis factor alpha (TNFα) was given in 1975 (51). Since then, numerous groups identified TNF-related ligands and receptors contributing to the emergence of the TNF superfamily (52, 53). Members of the TNF superfamily are involved in both development/homeostasis and various diseases, playing important roles in immune and inflammatory responses, development of bone, angiogenesis, and cell death (53). Members of the TNF superfamily contain TNF homology domain (THD), which binds to the cysteine-rich domain of the TNF receptor (TNFR) (54). Most TNF-related ligands are membrane-bound proteins and distinct proteases process them into their soluble forms. For example, a TNFα converting enzyme (TACE) protease acts on TNFα (55, 56) whereas another metalloprotease, matrilysin processes Fas ligand, a TNF-related ligand (57). Although membrane-bound TNF can trigger downstream signaling, the mechanisms are not well understood (58). The binding of TNF to TNFR recruits adaptor proteins such as TNFR-associated death domain proteins (TRADD) or TNFR-associated factors (TRAFs) to mediate the downstream signal transduction (59, 60). This event ultimately activates nuclear factor kappa B (NF-κB) (61). Mechanisms of NF-κB activation are various and depend on the particular cell type. The assembly of downstream mediators of TNFR recruits the IκB kinase

resulting in phosphorylation and subsequent degradation of NF-κB inhibitor (62). Free NF-

κB translocates to the nucleus and activates expression of target genes (63). In addition to

NF-kB activation, caspases and mitogen-activated protein kinases (MAPKs) can be targeted

by TNF signaling mediating various cellular responses including proliferation, cell death, and

inflammation (52, 64).

As a pro-inflammatory cytokine, TNF has been associated with both inflammatory

and neuropathic pain (23). Intra-plantar injections of TNF in rats causes thermal

hypersensitivity (65) while application of TNF to the sciatic nerve induces bursting activity

in primary nociceptive afferent fibers (66). Increased expression of TNF and TNFR were

detected in a model of chronic constriction injury (CCI) as well as in human patients with

painful neuropathy (67, 68). Several studies observed downstream signaling events that are

induced by the action of TNF in the context of nociceptive sensitization. For example, p38

MAP kinase has been implicated in TNFα-induced mechanical hypersensitivity on cultured

DRG neurons (69). However, the underlying mechanisms and nociceptive sensory neuron-

specific functions of downstream players of the TNF signaling pathway in nociceptive

biology remain poorly understood. In addition, subsequent target genes subjected to

transcriptional regulation by NF-κB transcription factor during nociceptive sensitization are

unknown. A recent study focused on the loss-of-function of TNF/TNFR in primary

nociceptive sensory neuron shed light on how TNF signaling contributes to proper development and to the function of nociceptors (70) while the exact action of TNF signaling for nociceptive sensitization under tissue injury conditions still remain to be tested.

In *Drosophila*, the TNF ligand, Eiger and two TNF receptor homologs, Wengen and Grindelwald, were identified through genetic screens and bioinformatic analyses (71-73). Like its function in vertebrate system, *Drosophila* TNF signaling is associated with cell death in multiple tissues including the adult eye (71, 72, 74, 75). In addition, TNF signaling plays important roles in degeneration of neuromuscular junction, non-autonomous proliferation of imaginal epithelium, and immune responses to microbes (76-78).

Importantly, TNF/Eiger and its receptor TNFR/Wengen are involved in tissue-damaged-induced thermal allodynia caused by UV irradiation (36). In this model, TNF/Eiger is produced by apoptotic epidermal cells. Eiger acts through its receptor, Wengen, which functions in class IV MD neurons to mediate development of thermal allodynia (Figure 1.1). This study established a phylogenetically conserved role for TNF signaling in nociceptive sensitization as TNF/TNFR mediate various types of sensitization in vertebrates (23, 68). Although TNF/Eiger is produced from dying epidermal cells in UV-induced sensitization model, whole animal mutants lacking TNF/Eiger still show severe morphological changes and active caspase-3 staining in the larval epidermis following UV irradiation, suggesting
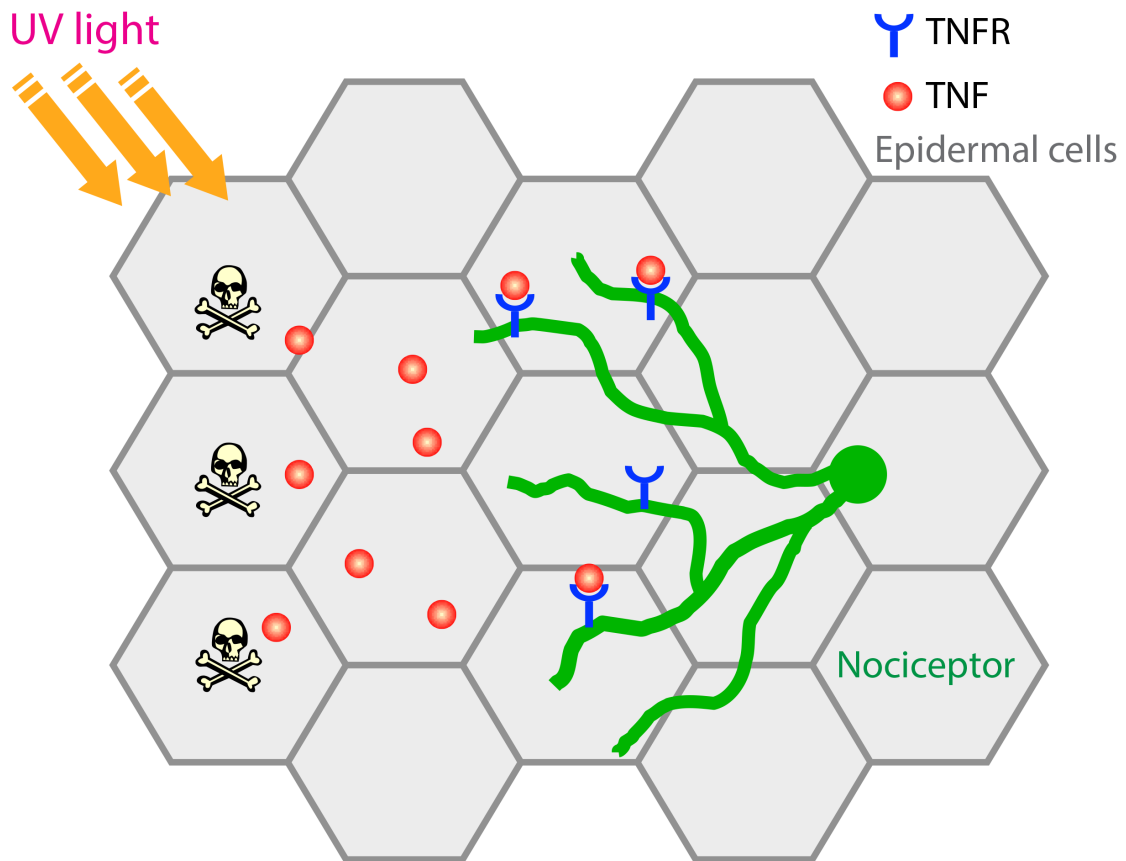
Figure 1.1 A previous model for TNF-mediated thermal allodynia in *Drosophila* larvae

UV irradiation causes apoptotic cell death in epidermis and the production or activation of

TNF/Eiger. Subsequently, the interaction of TNF/Eiger and its receptor TNFR/Wengen in

nociceptive sensory neurons induces nociceptive sensitization.

Figure is Adapted from *Babcock et al. Current biology (2009)* (36)

that TNF signaling is not required for the larval epidermal cell death. Hence, the exact relationship between apoptotic cell death and production of TNF/Eiger in the epidermis remains unclear. In addition, TNF/Eiger originates from epidermal cells and its receptor acts in the nociceptive sensory neuron, there are substantial questions regarding how this transmembrane ligand interacts/binds to its receptor to mediate sensitization. Moreover, it was not resolved what the downstream mediators for TNF/TNFR signal transduction are in class IV MD neurons to modulate its activity during nociceptive sensitization.

1.4 Apoptotic and Non-apoptotic functions of Caspases

UV irradiation leads to nociceptive sensitization in vertebrate and fly (36, 79). In vertebrates, inflammatory cytokines including interleukins and TNFα are produced by irradiated keratinocytes contributing to nociceptive sensitization (80). Acute exposure to UV also induces oxidative stress (81), DNA damage (82), inflammation (83), and apoptosis (84). UV-induced apoptotic cell death is a consequence of stimulations of several pathways including activation of p53 following DNA damages, production of reactive oxygen species (ROS), and activation of death receptors. These ultimately lead to cytochrome C release from mitochondria and activation of apoptotic machinery. The exact relationship between UV-induced apoptotic cell death and production of inflammatory mediators remains unclear.

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Apoptotic cell death is executed by a cascade of cysteine proteases called caspases (85). Caspases are categorized as either apoptotic or inflammatory caspases (86). Apoptotic caspases are highly conserved in multicellular organisms playing an essential role in regulation and execution of cell death during the development and homeostasis (87). Apoptotic caspases are classified into two large groups, the initiator caspase (Caspase-2, 8, 9, 10) and the effector caspase (Caspase-3, 6, 7) (87). The initiator caspase forms an apoptosome complex with apoptotic protease activating factor 1 (Apaf-1) to activate downstream effector caspases, subsequently leading to the deconstruction of the cellular machinery (88). In contrast to apoptotic caspases, inflammatory caspases function in inflammation and in processing of inflammatory cytokines. This group of caspases includes caspase-1, 2, 4, 5, 11, and 12. Among them, caspase-1, which is initially identified as interleukin (IL)-1β processing enzyme (89), is most well characterized. Inflammatory caspases are activated by a complex called inflammasome that senses internal and external danger signals such as bacteria, toxins, and substances released from damaged cells (86). The inflammasome also promotes the repair of injured sites (90), suggesting an essential role of inflammasome mediated-caspase-1 activation in response to cellular stress and in host defense.

Both apoptotic and inflammatory caspases has been implicated in acute or chronic sensitization in several studies. For example, Caspase-6 regulates TNFα secretion from microglia in an inflammatory pain model (91). The administrations the specific inhibitors targeting caspase-3, 8, or 9 reduce both chemotherapy drug (vincristine)-induced and TNFα–induced mechanical hyperalgesia (92). Caspase-1 also has been investigated in nociceptive sensitization because of its function in processing pro-inflammatory cytokine IL-1β. Knockout mice lacking caspase-1 show reduced inflammatory mechanical hypersensitivity (93). The pretreatment of caspase-1-specific inhibitor attenuates incision-induced mechanical allodynia and thermal hyperalgesia (94). These studies suggest that both apoptotic and inflammatory caspases play an important role in nociceptive sensitization. However, exact role of caspases in nociceptive sensitization still remains undefined. Particularly, function of both categories of caspases has not been examined directly in UV-induced nociceptive sensitization although these caspases are activated by irradiation (95, 96).

In *Drosophila*, core apoptotic caspases are well conserved while the role of cytochrome C release remains contradictory (97, 98). Death regulator Nedd2-like caspase (Dronc) functions as the initiator caspase in most tissues for the execution of apoptosis (99, 100). In the absence of an apoptosis-inducing signal, the inhibitor of apoptosis protein (DIAP) prevents activation

of Dronc (101). This inhibition is released by expression of pro-apoptotic genes such as *reaper, hid, grim,* and *sickle* (102, 103). Dronc interacts with Death-associated apaf-1 related killer (Dark) to form the apoptosome (104-106) and this complex activates caspase-3-like effector caspases, Death related ICE-like caspase (Drice) (107-109) and Death caspase-1 (Dcp-1) (110) that cleave the cellular substrates that lead to downstream apoptosis (Figure 1.2) (111). *Drosophila* caspases also have been implicated in non-apoptotic processes such as innate immunity (112), sperm individualization (113), compensatory proliferation (114), and dendritic pruning (115).

In our *Drosophila* model of tissue damage-induced nociceptive sensitization, Dronc is required for both UV-induced epidermal apoptosis and thermal allodynia (36). This study directly showed that function of apoptotic initiator caspase in damaged tissue is essential for the development of nociceptive sensitization. However, the molecular mechanism of how Dronc contributes to nociceptive sensitization still needs to be elucidated. In addition, it remains unclear if upstream apoptotic genes and downstream effector caspases are required for nociceptive sensitization.
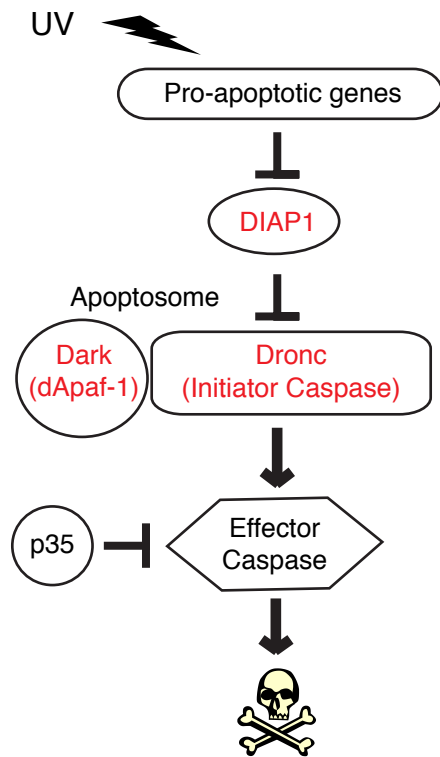
Figure 1.2 Diagram for canonical apoptotic cell death pathway in *Drosophila*

A schematic showing the signaling cascade of the apoptotic cell death pathway. An apoptosome consists of initiator caspase, Dronc and *Drosophila* Apaf-1, Dark. The apoptosome is inhibited by DIAP1. Expression of pro-apoptotic genes by stress signals (eg. UV) causes the release of DIAP1 inhibition and the resulting free apoptosome activates downstream effector caspases, thus leading to cell death. p35 is an inhibitor of effector caspases.

In this thesis, I will explore contributions of Dronc and entire cell death pathway to nociceptive sensitization. In addition, I will elucidate some aspects of TNF ligand regulation in epidermal cells as well as downstream signaling mediators and transcriptional target genes in nociceptive sensory neurons using the *Drosophila* model.

Chapter 2: Materials and Methods

2.1 Fly strains

w^{1118} (116) was used as a control for mutants. Null alleles were used for behavioral tests: $eiger^1$ and $eiger^3$ served to inhibit TNF signaling (72). $dronc^{I29}$ (117), $dark^{G8}$, $dark^{S7}$, $dark^{H16}$ (118), $Drice^{\Delta1}$ (119), $strica^4$ (120), skl^{e1}, skl^{e3} (121), and $decay^{\Delta K2}$ (122) are mutant alleles for cell death signaling pathway and caspases. $traf6^{Ex1}$ (123), $p38a^1$ (124), dl^1 (125), rel^{E20} (126) and Dif^1 (127) were used to test downstream mediators of TNF signaling. Hypomorphic alleles were used for behavioral tests: $Drice^{17}$ (128), hid^1 (129), $dredd^{EP1412}$ (130), are mutant alleles for cell death signaling pathway and caspases. $damm^{f02209}$ (131) and $dcp\text{-}1^{prev}$ (132) are undefined mutant alleles but predicted to be loss of function due to a deletion and a frameshift respectively. These are mutant alleles for cell death signaling pathway and caspases. $decay^{\Delta K3}$ (122) was used as control for $decay^{\Delta K2}$ allele. The *Gal4/UAS* system (133) was used to express transgenes in tissue specific manner. *A58 Gal4* (134), *e22C Gal4* (135) , and *pannier Gal4* (136) were used to drive larval epidermal tissue specific expression of *UAS-transgene*. *ppk1.9 Gal4* (137) was used to drive expression of *UAS-transgene* in class IV MD nociceptive sensory neurons. UAS-transgenes used: *UAS-regg^1* (72), *UAS-eiger60* (soluble TNF) (138), *UAS-Dronc* (139), *UAS-hid* (140)*, UAS-grim* (141)*,* and *UAS-reaper* (142) , *UAS-DIAP1* (140), *UAS-p35* (143), *UAS-ptc^{DN}* (144), *UAS-DTKR-GFP* (145), UAS-eGFP (Bloomington), *UAS-miRHG* (146). *UAS-Dronc$^{RNAi(8091R1)}$* (NIG)

UAS-Eiger^{RNAi} (72), *UAS-Wengen*^{RNAi} (71). *UAS-RNAi* lines from Vienna Drosophila

Research Center (VDRC) (147): 21830 (*grim*), 12045 (*reaper*), 8269 (*hid*), 34836 (*traf3*),

16125 (*traf6*), 52277 (*p38*), 45998 (*dorsal*), 107072 (*E(z)*). *JF02826* targeting *E(z)* and *UAS-*

GFP^{RNAi} (stock number: 9930) were from Bloomington stock center. *ppk-CD4-tdTom* was

used to label nociceptive sensory neurons (148). To temporally induce overexpression of

transgenes, *tub-gal80*^{ts} (149) was used in combination with Gal4/UAS.

Table 2.1 Genotypes for each figure panel

Figure	genotype
Figure 3.1A	w^{1118};;A58 Gal4/UAS-Grim
	w^{1118};UAS-Reaper/+;A58 Gal4/+
Figure 3.1B	w^{1118};tubGal80^{ts}/+;pnr-Gal4,UAS-eGFP/+
	w^{1118},UAS-Hid/+;tubGal80^{ts}/+;pnr-Gal4,UAS-eGFP/+
Figure 3.1C	w^{1118};e22C Gal4/+;
	w^{1118};e22C Gal4/+; UAS-Hid^{RNAi}(v8269)/+
	w^{1118};e22C Gal4/ UAS-RHG^{miRNA};
Figure 3.2 A-B, 3.5 A-B	w^{1118};
Figure 3.2 C	w^{1118};;hid^{1}
Figure 3.2 D	w^{1118};; hid^{1}/Df(3L)ED225, P(3'RS5+3.3')ED225
Figure 3.2 E	w^{1118};;sickle^{1}/ sickle^{3}
Figure 3.2 F	w^{1118};;dronc^{I29}
Figure 3.2 G	w^{1118};dark^{G8}/dark^{H16}
Figure 3.2 H	w^{1118};dark^{G8}/dark^{S7}
Figure 3.2 I	w^{1118};dark^{S7}/dark^{H16}
Figure 3.2 J	w^{1118}, dredd^{EP1412};
Figure 3.2 K	w^{1118};strica^{4}

Figure 3.2 L	$w^{1118};;drice^{\Delta 1}$
Figure 3.2 M	$w^{1118};dcp\text{-}1^{prev}$
Figure 3.2 N	$w^{1118};;decay^{\Delta K2}$
Figure 3.2 O	$w^{1118};damm^{f02209}$
Figure 3.3A	$w^{1118};;A58\ Gal4/+$
	$w^{1118};UAS\text{-}RHG^{miRNA};A58\ Gal4/+$
	$w^{1118};UAS\text{-}Grim^{RNAi}(v21830)/+;A58\ Gal4/UAS\text{-}Reaper^{RNAi}(v12045)\ ,UAS\text{-}Hid^{RNAi}(v8269)$
	$w^{1118};UAS\text{-}DIAP1.H/+;\ A58\ Gal4/+$
	$w^{1118};UAS\text{-}Dronc^{RNAi}\ (8081R1)/+;\ A58\ Gal4/+$
Figure 3.3B	$w^{1118};$
	$w^{1118};;hid^1$
	$w^{1118};;sickle^1/\ sickle^3$
	$w^{1118};strica^4$
	$w^{1118},\ dredd^{EP1412};$
	$w^{1118};;decay^{\Delta K3}$
	$w^{1118};;decay^{\Delta K2}$
	$w^{1118};damm^{f02209}$
Figure 3.3C	$w^{1118};$
	$w^{1118};;dronc^{I29}$
	$w^{1118};dark^{G8}/dark^{H16}$
	$w^{1118};dark^{G8}/dark^{S7}$
	$w^{1118};dark^{S7}/dark^{H16}$
	$w^{1118};;drice^{\Delta 1}$
	$w^{1118};dcp\text{-}1^{prev}$
	$w^{1118};dcp\text{-}1^{prev};drice^{\Delta 1}$
	$w^{1118};eiger^1/+$
	$w^{1118};eiger^3/+$
	$w^{1118};eiger^1/eiger^3$
Figure 3.3D, 3.4A-B	$w^{1118};;A58\ Gal4/+$
	$w^{1118};UAS\text{-}DIAP1.H/+;\ A58\ Gal4/+$
	$w^{1118};UAS\text{-}Dronc^{RNAi}\ (8081R1)/+;\ A58\ Gal4/+$
	$w^{1118};e22C\ Gal4/+$
	$w^{1118};e22C\ Gal4/UAS\text{-}p35$
	$w^{1118};e22C\ Gal4/\ UAS\text{-}Dronc^{RNAi}\ (8081R1)$

Figure 3.4C-D	w^{1118};
	w^{1118};;$dronc^{I29}$
	w^{1118};$dark^{G8}/dark^{H16}$
	w^{1118};$dark^{G8}/dark^{S7}$
	w^{1118};$dark^{S7}/dark^{H16}$
	w^{1118};;$drice^{17}$
Figure 3.4E	w^{1118};*UAS-p35/+*
	w^{1118};*UAS-DIAP1.H/+*
	w^{1118};*UAS-Dronc^{RNAi} (8081R1)/+*
Figure 3.5A-B	w^{1118};;*A58 Gal4/+*
Figure 3.5C	w^{1118};*UAS-Dronc^{RNAi} (8081R1)/+; A58 Gal4/+*
	w^{1118};*UAS-Eiger^{RNAi} /+; A58 Gal4/+*
Figure 3.6A, C-D	w^{1118};;*ppk1.9 Gal4/+*
	w^{1118};;*ppk1.9 Gal4/ UAS-Traf3^{RNAi}(v34836)*
	w^{1118};*UAS-Traf6^{RNAi}(v16125)/+;ppk1.9 Gal4/+*
	w^{1118};;*ppk1.9 Gal4/UAS-p38a^{RNAi}(v52277)*
	w^{1118};;*ppk1.9 Gal4/UAS-dl^{RNAi}(v45998)*
	w^{1118};*UAS-Dronc^{RNAi}(8081R1)/+;ppk1.9 Gal4/+*
Figure 3.6B	w^{1118};;*UAS-Traf3^{RNAi}(v34836)/+*
	w^{1118};*UAS-Traf6^{RNAi}(v16125)/+*
	w^{1118};;*UAS-p38a^{RNAi}(v52277)/+*
	w^{1118};;*UAS-Dorsal^{RNAi}(v45998)/+*
Figure 3.7A	w^{1118};
	w^{1118}, $traf6^{EX1}$;
	w^{1118};;$p38a^{1}$
	w^{1118};dl^{1}
	w^{1118};;$relish^{E20}$
	w^{1118};Dif^{1}
Figure 3.7B-C	w^{1118};
	w^{1118}, $traf6^{EX1}$;
	w^{1118};;$p38a^{1}$
	w^{1118};$dorsal^{1}$
	w^{1118};;$relish^{E20}$
Figure 3.8A	w^{1118};$regg^{1}$/+;*ppk1.9 Gal4/+*
	w^{1118}; $regg^{1}$/UAS-Wengen^{RNAi}/+;*ppk1.9 Gal4/+*

	w^{1118}; regg^1/+;;ppk1.9 Gal4/ UAS-Traf3^{RNAi}(v34836)
	w^{1118}; regg^1/UAS-Traf6^{RNAi}(v16125)/+;ppk1.9 Gal4/+
	w^{1118}; regg^1/+;;ppk1.9 Gal4/UAS-p38a^{RNAi}(v52277)
	w^{1118}; regg^1/+;;ppk1.9 Gal4/UAS-Dorsal^{RNAi}(v45998)
	w^{1118}; regg^1/UAS-Dronc^{RNAi}(8081R1)/+;ppk1.9 Gal4/+
Figure 3.8B	w^{1118};UAS-Ptc^{DN}/+;ppk1.9 Gal4/+
	w^{1118};UAS-Ptc^{DN}/UAS-Dronc^{RNAi} (8081R1);ppk1.9 Gal4/+
	w^{1118};UAS-DTKR-GFP/+;ppk1.9 Gal4/+
	w^{1118};UAS- DTKR-GFP/UAS-Dronc^{RNAi} (8081R1); ppk1.9 Gal4/+
Figure 3.9B	w^{1118};tubGal80^{ts}/+;A58 Gal4/+
	w^{1118};tubGal80^{ts}/+;A58 Gal4/UAS-Dronc
	w^{1118};tubGal80^{ts}/regg^1;A58 Gal4/+
	w^{1118};tubGal80^{ts}/UAS-Eiger 60;A58 Gal4/+
Figure 3.10	w^{1118};tubGal80^{ts}/+;A58 Gal4/+
	w^{1118};tubGal80^{ts}/+;A58 Gal4/UAS-Dronc
	w^{1118};tubGal80^{ts}/UAS-Eiger^{RNAi};A58 Gal4/UAS-Dronc
	w^{1118};tubGal80^{ts}/UAS-p35;A58 Gal4/UAS-Dronc
	w^{1118};tubGal80^{ts}/UAS-Eiger^{RNAi};A58 Gal4/+
	w^{1118};tubGal80^{ts}/UAS-p35;A58 Gal4/+
Figure 3.11A	w^{1118};;A58 Gal4/+
	w^{1118};UAS-Eiger/+;A58 Gal4/+
Figure 3.11B	w^{1118};Eiger-Gal4/UAS-GFP
Figure 3.12	w^{1118};e22C Gal4/UAS-GFP^{RNAi}(9331)
	w^{1118};e22C Gal4/UAS-TACE^{RNAi}(v2733)
	w^{1118};e22C Gal4/UAS-TACE^{RNAi}(v106335)
Figure 3.14	w^{1118};tubGal80^{ts}/+;A58 Gal4/+
	w^{1118};tubGal80^{ts}/regg^1;A58 Gal4/+
	w^{1118};tubGal80^{ts}/regg^1, UAS-Dronc^{RNAi}(8081R1) ;A58 Gal4/+
	w^{1118};tubGal80^{ts}/UAS-Eiger60;A58 Gal4/+
	w^{1118};tubGal80^{ts}/UAS-Eiger60, UAS-Dronc^{RNAi}(8081R1) ;A58 Gal4/+
Figure 3.15	w^{1118};;ppk1.9 Gal4/UAS-mcD8GFP
	w^{1118};regg^1/+;ppk1.9 Gal4/UAS-mcD8GFP

Figure 3.16A	w^{1118};UAS-GFP^{RNAi}(9331)/+;ppk1.9 Gal4/+
	w^{1118};UAS-E(z)^{RNAi}(v107072)/+;ppk1.9 Gal4/+
	w^{1118};;ppk1.9 Gal4/UAS-Garnet^{RNAi}(v31390)
	w^{1118};UAS-Allatostatin receptorA1^{RNAi}(v101395)/+ ;ppk1.9 Gal4/+
	w^{1118};;ppk1.9 Gal4/UAS-Trissin receptor^{RNAi}(v7886)
	w^{1118};UAS-Methuselah-like 8^{RNAi}(v100246)/+ ;ppk1.9 Gal4/+
Figure 3.16B	w^{1118};UAS-GFP^{RNAi}(9331)/regg1;ppk1.9 Gal4/+
	w^{1118};UAS-E(z)^{RNAi}(v107072)/regg1;ppk1.9 Gal4/+
	w^{1118};regg1/+;ppk1.9 Gal4/UAS-Garnet^{RNAi}(v31390)
	w^{1118};UAS-Allatostatin receptorA1^{RNAi}(v101395)/regg1 ;ppk1.9 Gal4/+
	w^{1118};regg1/+;ppk1.9 Gal4/UAS-Trissin receptor^{RNAi}(v7886)
	w^{1118};UAS-Methuselah-like 8^{RNAi}(v100246)/regg1 ;ppk1.9 Gal4/+
Figure 3.17B, 3.18A-B	w^{1118};UAS-GFP^{RNAi}(9331);ppk1.9 Gal4/+
	w^{1118};UAS-E(z)^{RNAi}(v107072);ppk1.9 Gal4/+
	w^{1118};UAS-E(z)^{RNAi}(BL27993);ppk1.9 Gal4/+
Figure 3.17C	w^{1118};UAS-GFP^{RNAi}(9331)/regg^1;ppk1.9 Gal4/+
	w^{1118};UAS-E(z)^{RNAi}(v107072)/regg^1;ppk1.9 Gal4/+
	w^{1118};UAS-E(z)^{RNAi}(BL27993)/regg^1;ppk1.9 Gal4/+

2.2 UV-induced tissue injury

Crosses for experiments were kept on 25°C. Early third instar larvae were selected for

UV treatment 4~5 days after copulation. Larvae were anesthetized with ethyl ether for three

minute, washed in water briefly, and aligned on a slide glass with double-sided tape in a

position of dorsal side up. Then the irradiated larvae were recovered in regular fly food for

24 hours before nociception behavioral tests. For consistent emission of UV light, a UV crosslinker (Spectronics Corporation) was pre-warmed for 100 seconds in a time-mode and right after warming up was over, larvae were placed in the UV crosslinker and irradiated in an energy-mode with 20 mJ/cm^2 setting. A UV photometer (Spectronics Corporation) was used at the same time to measure the actual administrated amount of UV each time. Comparison between the UV crosslinker setting and the actual reading with a UV photometer is shown in Table 2.2.

Set in UV crosslinker	Measured in UV radiometer
5 mJ/cm^2	2-3 mJ/cm^2
8 mJ/cm^2	4-5 mJ/cm^2
12 mJ/cm^2	6-7 mJ/cm^2
17 mJ/cm^2	8-10 mJ/cm^2
20 mJ/cm^2	11-14 mJ/cm^2

Table 2.2 Comparison between set and measured UV doses

2.3 Nociception behavior assay and statistics

Thermal stimuli were applied with a custom-designed heat probe as described previously (36). The heat probe was set to a desired temperature and stabilized for 30 min using auto-tune mode in advance of experiment. Larvae were washed in water briefly to remove trace of

food and placed on a black plastic pad under a Leica MZ6 light microscope. Larvae were stimulated on their mid-dorsal side (3rd or 4th segment from the head) for a maximum of 20 seconds or until initiation of withdrawal behavior. Pain behavior is defined as a 360° rolling along anterior-posterior (AP) body axis within 20 seconds of a physical contact with the heat probe. The behavior latency was measured from the initial contact to the time when the rolling, withdrawal responses occured. Each larva was tested only once to avoid a potential habituation effect. Thermal allodynia was tested at 38 °C, 24 hours after UV treatment while baseline nociception was tested at both 45 and 48 °C in the absence of injury. The behavioral responses were categorized into three groups: fast (latency is up to 5 seconds), slow (latency is between 6 to 20 seconds), no response (no response within 20 seconds). Chi-square test was used to measure statistical significance in categorical data.

2.4 Tissue specific induction of transgenes

Temperature-sensitive Gal80 (*Gal80*^{ts}) was used for the temporal and regional gene expression by repression of Gal4 transcriptional activity (149). Expression of *Gal80*^{ts} is controlled by the tubulin promoter (tub *Gal80*^{ts}) and its activity is determined by the temperature. To repress Gal4, crosses were kept at the permissive temperature (18 °C) for

about 9 days and early 3rd instar larvae were exposed to heat-shock in 32 °C water bath for 24 hour to repress *Gal80^{ts}* and induce *Gal4* expression.

2.5 Immunostaining and imaging

For the analysis of epidermal tissue damage, larvae were dissected in phosphate buffered saline (PBS) using fine dissection scissors, dissection forceps, and micro dissection pins (Fine Science Tools). Larvae were washed with water to remove food traces and were placed on a sylgard plate (Dow Corning Corporation) containing PBS. Then anterior and posterior regions of larvae were pinned down in a position of ventral side up. An incision was made along the AP body axis, and each side was stretched and pinned down making a hexagonal shape. Undesired internal tissues were removed and whole-mount epidermal tissue was fixed in 3.7% formaldehyde for one hour at room temperature. After fixation, tissue was washed with PBS and treated with a blocking solution (1% Heat-inactivated normal goat serum, 0.3% Triton X-100 in PBS) for one hour at room temperature. After blocking, samples were incubated in primary antibody solutions at room at 4 °C for overnight. Tissue samples were washed 3 times in PBST (0.3% Triton X-100 in PBS) and secondary antibody was added. Tissue samples were incubated at 4 °C for overnight. Again samples were washed three times in PBST and mounted on a glass slide with Vectashield mounting media

(Vector Laboratories). For TUNEL staining, after secondary antibody treatment and washing, tissue samples were incubated in buffer (100 mM sodium citrate, 0.1% Triton X-100) for 30 minutes at 65 °C. Tissue samples were then washed 3 times in washing buffer (50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 0.5% NP-40, 1% BSA) and incubated in TUNEL dilution buffer for 10 minutes at room temperature. Tissue samples were incubated in labeling solution for 30 minutes at 37 °C and enzyme solution was added and incubated further for 3 hours. Finally samples were washed 3 times with washing buffer and mounted with vectashield mounting media. To label epidermal membranes, anti-Fasciclin III (Developmental studies Hybridoma Bank, 1:50) was used. Anti-Eiger antibody was used to label fly TNF (1:200) (72). Anti-active casapse-3 antibody (Cell signaling, 1:150) and Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) staining kit (Roche) were used for labeling apoptotic cells. Trypan blue solution was used to label pinch wound-induced necrosis in the epidermal cells. Anti-GFP antibody (life technologies, 1:500) was used to visualize induction of *Eiger Gal4* expression. Secondary antibodies: alexa488-conjugated anti-mouse (life technologies, 1:1000), Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 1:1000). Images were obtained with an Olympus Fv1000 confocal microscope and processed equally using Image J and Photoshop.

2.6 Purification of sensory neurons and Microarray analysis

Class IV MD neurons were collected from control larvae (*ppk 1.9 Gal4, UAS-mCD8 GFP*) and from larvae with nociceptive sensory neuron specific-activation of TNF signaling (*ppk1.9 Gal4, UAS-mCD8 GFP, UAS-TNF*). One hundred 3rd instar larvae were selected, washed with PBS, 70% ethanol, nuclease-free H_2O, RNase-AWAY (Sigma), and nuclease-free H_2O. Larvae were chopped into 3~4 pieces on sylgard coated petri dish and transferred to ice-cold PBS in a microcentrifuge tube. Loosely adherent tissues such as fat body, gut, imaginal discs were removed by pipetting and vortexing. To dissociate into a single cell suspension, pre-cleared larval tissue was ground with a pestle (Kontes Glass Tissue Grinder) and filtered by size using 30 μm cell strainer (Miltenyi Biotec). Magnetic beads (Dynabeads M-280 streptavidin, Invitrogen) coated with biotin-conjugated anti-mCD8 antibody (Invitrogen) solution was added to the cell suspension and incubated for one hour on ice. Cells expressing mCD8GFP bind the magnetic beads and then they were isolated with a magnetic field and following washing steps with ice-cold PBS. After checking the purity and the yield of isolation under a fluorescent microscope, mRNA isolation, amplification, labeling, hybridization, and microarray analyses were performed by Miltenyi Biotec. 250 ng of each of the sample cDNAs were used as template for Cy3 (control) and Cy5 (TNF/Eiger overexpression) labeling. Labeled cDNAs were combined and hybridized to an Agilent

whole *Drosophila* genome oligo microarray (4x44K V2) and analyses were conducted in

quadruplicate. Microarray expression and bioinformatics analyses were performed as

previously described (150). Normalized Cy5/Cy3 fold changes (Cy5/Cy3-log10 ratios) were

used to investigate differentially expressed genes with a threshold fold change >2 and a p-

value <0.01 for genes that are up-regulated in TNF signaling-activated sensory nociceptive

neurons relative to controls.

2.7 S2 cell culture

S2 cells were maintained in Schneider's medium (Gibco) supplemented with 10%

fetal bovine serum (Gibco) and 1% penicillin/streptomysin at room temperature. Cells were

transfected with Nextfect (Bioo scientific) or lipofectin (invitrogen) transfecting reagent.

pMT vector was used to overexpress TNF/Eiger (74). 24 hours after transfection, 50 uM of

$CuSO_4$ was added to induce expression. 48 hours after transfection, medium was removed for

UV irradiation and fresh medium was added after UV treatment.

2.8 Immunoprecipitation and immunoblotting

S2 cells and media were separated by centrifugation. Cells were lysed in RIPA buffer

(25 mM Tris/HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS)

containing protease inhibitor cocktail (Roche). Proteins in medium were concentrated using

Vivaspin15R (Sartorius) and protease inhibitor was added. Lysate and concentrated medium

were mixed with anti-Flag M2 affinity gel (Sigma) and incubated at 4°C overnight. The flag

gel beads were collected by a centrifugation and washed with the RIPA buffer. The flag gel

beads were boiled with 2X sample buffer for 5 min and supernatants were prepared for SDS-

PAGE gel running. Immunoblotting was performed using anti-Flag antibody conjugated with

HRP (Cell signaling). Signals were detected with ECL reagents (Amersham)

Chapter 3: Results

3.1 A non-apoptotic function of the apoptosome is required for thermal allodynia

A previous study from our lab found that RNAi-mediated knockdown of initiator caspase Dronc in epidermal tissue prevents both UV-induced apoptotic epidermal cell death and thermal allodynia, suggesting that the signaling cascade by caspases might play an important role during thermal allodynia as well as epidermal cell death in *Drosophila* larvae (36). My initial hypothesis derived from this work was that epidermal cell death is required for thermal allodynia because the prevention of epidermal cell death by knockdown of Dronc substantially reduced withdrawal responses of irradiated larvae. However, it was not entirely clear if epidermal cell death is essential for the development of thermal allodynia. Except for Dronc, other components of the cell death pathway had not been tested for either UV-induced apoptotic epidermal cell death or thermal allodynia.

To test if upstream factors prior to the initiator caspase Dronc in the cell death pathway are involved in UV-induced apoptosis in the larval epidermis, I first examined overexpression of pro-apoptotic genes including *hid, grim,* and *reaper* (Figure 3.1). Epidermal overexpression of Hid caused lethality in the early L1 stage of larval development whereas overexpression of Grim or Reaper did not induce apoptotic cell death in the larval epidermis (Figure 3.1A). To overcome lethality, temporal and local overexpression of Hid was induced using *pannier Gal4* under the control of *tubGal80^{ts}*. Controlled expression of
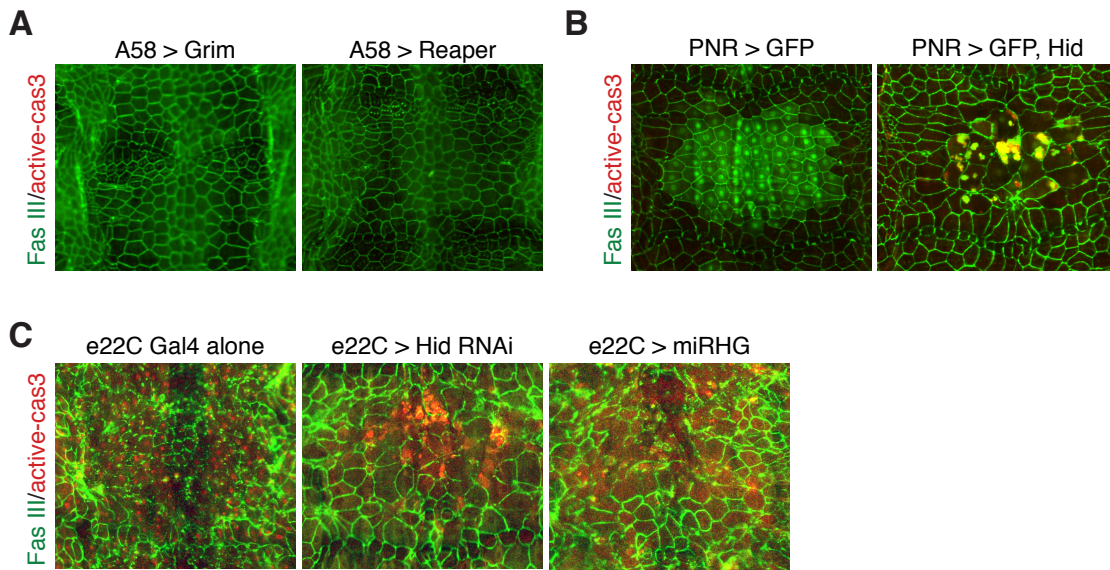
Figure 3.1 Gain and Loss-of-function of pro-apoptotic genes for epidermal cell death in

***Drosophila* larvae**

(A-C) Anti-Fasciclin-3 antibody (green) and anti-active caspase 3 antibody (red) were used

to label epidermal cell membrane and apoptosis, respectively. (A) Larval epidermal staining

shows no morphological changes with overexpression of Grim and Reaper. (B) Epidermal

morphology when overexpression of Hid is induced by *Pannier Gal4* under control of

temperature sensitive *Gal80*. (C) UV-induced epidermal apoptosis 24 hours after UV

treatment.

* Pannel A is done by Felona Gunawan.

Hid resulted in morphological disruption and strong active caspase-3staining within the

pannier Gal4-expressing patch in the larval epidermis following heat-shock treatment at 32

°C for 24 hours whereas non-pannier expressing epidermal cells were intact (Figure 3.1B).

This data indicates that Hid is capable of inducing apoptosis in the larval epidermis. To test

knockdown of Hid or all three pro-apoptotic genes in UV-induced epidermal apoptosis, a

UAS-RNAi transgene targeting Hid or *UAS-microRNA* transgene targeting Hid, Grim, and

Reaper were expressed using epidermal specific Gal4 driver, *e22C Gal4* (Figure 3.1C).

Inhibition of pro-apoptotic genes in the epidermis did not block UV-induced apoptosis,

suggesting that other signaling molecules might be involved in UV-induced epidermal

apoptosis to activate the initiator caspase Dronc.

Next, we tested UV-induced epidermal damage in the loss-of-function mutants of genes in

the canonical cell death pathway and mutants of other caspases identified in the fly genome

(Figure 3.2). The irradiated larval epidermis of control animals exhibited disrupted epidermal

cell morphology and strong TUNEL labeling in their dorsal epidermis relative to non-

irradiated controls (Figure 3.2 A and B). Consistent with tissue-specific knockdown

experiments (Figure 3.1), larvae homozygous for hid^1 or hid^1 over ED225 deficiency

spanning the *hid, grim, reaper,* and *sickle* locus fail to block UV-induced epidermal

apoptosis (Figure 3.2 C and D). *sickle* is another pro-apoptotic gene whose gene locus is adjacent to *hid, grim,* and *reaper* (103). Transheterozygous combinations of *sickle[1]* and *sickle[3]* alleles exhibited normal apoptotic cell death 24 hours after UV irradiation (Figure 3.2E). As expected from the previous tissue-specific knockdown experiments (36), homozygous null mutants for *dronc* did not exhibit morphologic hallmarks of apoptosis (Figure 3.2F). Three different transheterozygous combinations of *dark[G8], dark[H16]*, and *dark[S7]* also failed to undergo UV-induced epidermal apoptosis (Figure 3.2 G-I), suggesting the full apoptosome, defined as the complex of the initiator caspase Dronc, and the Apaf-1 adaptor protein Dark (Figure 1.2), are required for UV-induced apoptosis in the larval epidermis. In addition to Dronc, there are two more genes classified as an initiator caspase in *Drosophila* (*dredd* and *strica*) (141, 151), I tested if these initiator caspases are also involved in UV-induced epidermal cell death. Homozygous mutants for *strica* and *dredd* displayed severe morphological changes and TUNEL labeling (Figure 3.2J and K), suggesting they are not required UV-induced epidermal cell death. Finally, we tested mutants for four different effector caspases for epidermal cell death in irradiated larvae. Lack of effector caspase Drice prevented both morphological disruption and TUNEL staining in the epidermis (Figure 3.2L). Homozygous mutants for *Dcp-1, Decay,* and *Damm* failed to block UV-induced
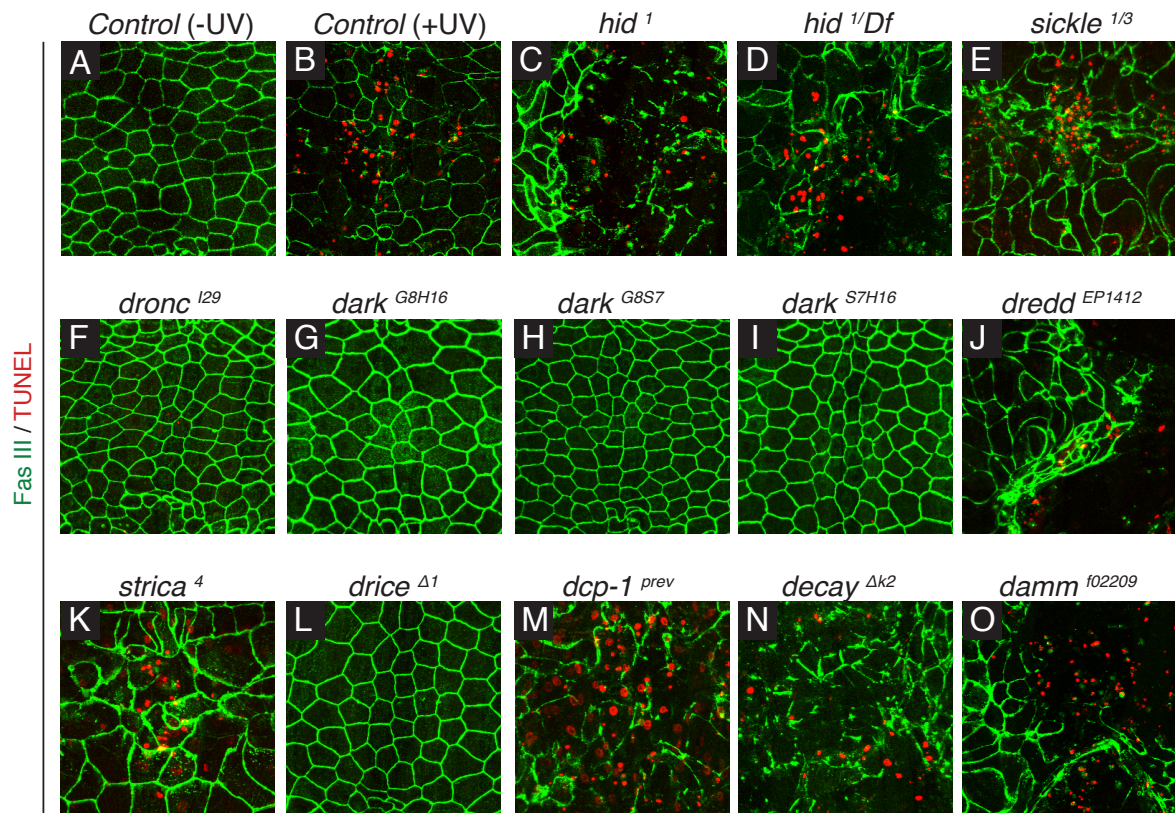
Figure 3.2 Dronc and Dark, and effector caspase Drice are required for UV-induced epidermal apoptosis in *Drosophila* larvae

24 hours after UV treatment, control or mutant larvae with the indicated allele were stained with anti-Fasciclin-3 antibody (green) and TUNEL staining (red) to label epidermal cell membrane and apoptosis, respectively.

apoptosis (Figure 3.2 M-O). Taken together, in the larval epidermis, *dronc* and *Drice* are the main initiator and effector caspase respectively.

Next, we tested if the canonical cell death pathway and other caspases are required to induce thermal allodynia (Figure 3.3). Epidermal expression of *UAS-RNAi* or *UAS-microRNA* targeting pro-apoptotic genes did not alter thermal allodynia (Figure 3.3A). Homozygous or heterozygous combinations of mutant alleles for *hid*, *sickle*, *dredd*, and *strica,* also showed normal development of thermal allodynia, suggesting that these are not required for either UV-induced nociceptive sensitization (Figure 3.3B). Larvae that are homozygous mutant for *dronc* or carrying three different transheterozygous allelic combinations for *dark* showed attenuated withdrawal behavior responses in comparison to control, suggesting that formation of the apoptosome is required for both processes, apoptotic cell death and thermal allodynia following UV irradiation (Figure 3.3C). By contrast, when we examined null mutants for all effector caspases including Drice, which very effectively blocks epidermal cell death, they all still showed a strong degree of thermal allodynia (Figure 3.3B-C). This was surprising because *Drice* is a well-known downstream target of Dronc in the context of cell death (98). Although *Dcp-1* single mutants were not able to block UV-induced apoptosis in the epidermis, we examined the possibility that *Drice* and *Dcp-1* might
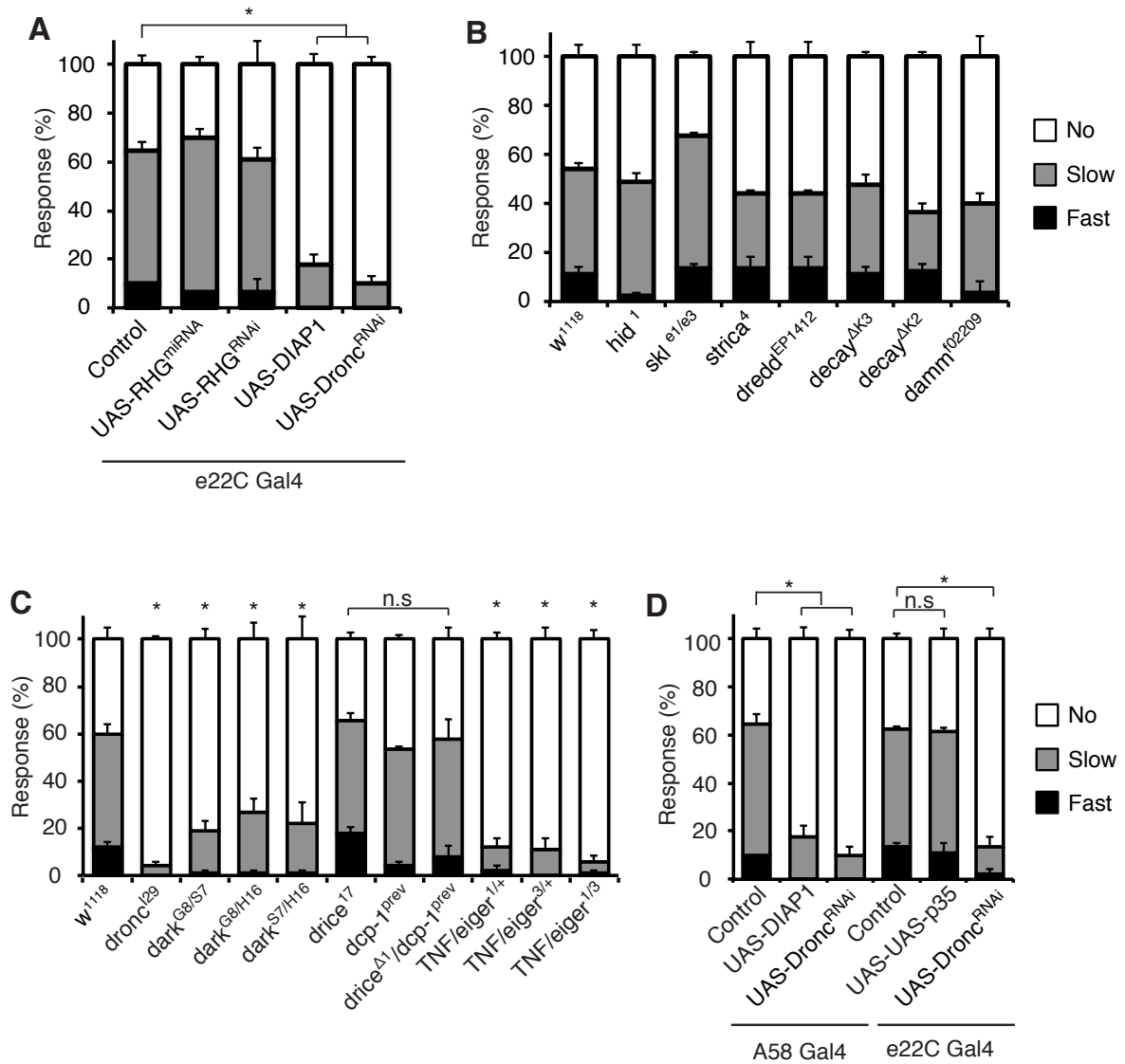
Figure 3.3 The apoptosome but not effector caspase is required for UV-induced thermal allodynia

(A-D) Quantification of UV-induced thermal allodynia at 38 °C, 24 hour post UV irradiation.

Larval behavior was categorized as "no withdrawal" (white), "slow withdrawal" (gray, response between 6 and 20 s), or "fast withdrawal" (black, response ≤ 5 s) in this and other

figures. n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicates p value less than 0.05,

n.s indicates no significance. (A) Measurement of aversive withdrawal behavior when pro-

apoptotic genes are inhibited in larval epidermal cells (e22C Gal4). UAS-DIAP1 and UAS-

DroncRNAi were used as a positive control to inhibit initiator caspase Dronc. (B-C)

Measurement of aversive withdrawal behavior of larvae homozygous or transheterozygous

for indicated mutant alleles. w^{1118} was used as a control.(D) Measurement of aversive

withdrawal behavior when initiator or effector caspases were inhibited in larval epidermis

(A58 or e22C Gal4).

cooperate together to induce thermal allodynia. However, *Drice* and *dcp-1* double mutants still developed thermal allodynia, suggesting that Dronc might have different downstream mediators for thermal allodynia (Figure 3.3C).

The results presented above predict that inhibiting the apoptosome should block both cell death and thermal allodynia whereas inhibiting effector caspases should block only cell death. To test this hypothesis we expressed transgenes that target either Dronc (*UAS-DIAP1*) or Drice/Dcp-1 (*UAS-p35*) activity using the epidermal specific Gal4 driver, *e22C-Gal4*. Overexpression of either transgene blocked cell death but only DIAP1 inhibited development of thermal allodynia (Figure 3.3D), suggesting that only Dronc, but not Drice and Dcp-1, is required to induce thermal allodynia.

To examine if lack of apoptosome or effector caspase function interferes with baseline nociception in the absence of tissue injury, we measured withdrawal behavior to a normally noxious stimulus of 45 °C and 48 ºC (Figure 3.4). Larvae homozygous mutant for *dronc* displayed a mild defect in baseline nociception, raising a possibility that Dronc or the apoptosome is required for the normal locomotion or for the proper development of Class IV MD neurons. However, larvae transheterozygous for *dark* showed normal baseline nociception, suggesting reduced thermal allodynia in *dark* mutants is not due to the baseline

A 45 °C, No UV

B 48 °C, No UV

C 45 °C, No UV

D 48 °C, No UV

E 38 °C, 24hr post UV
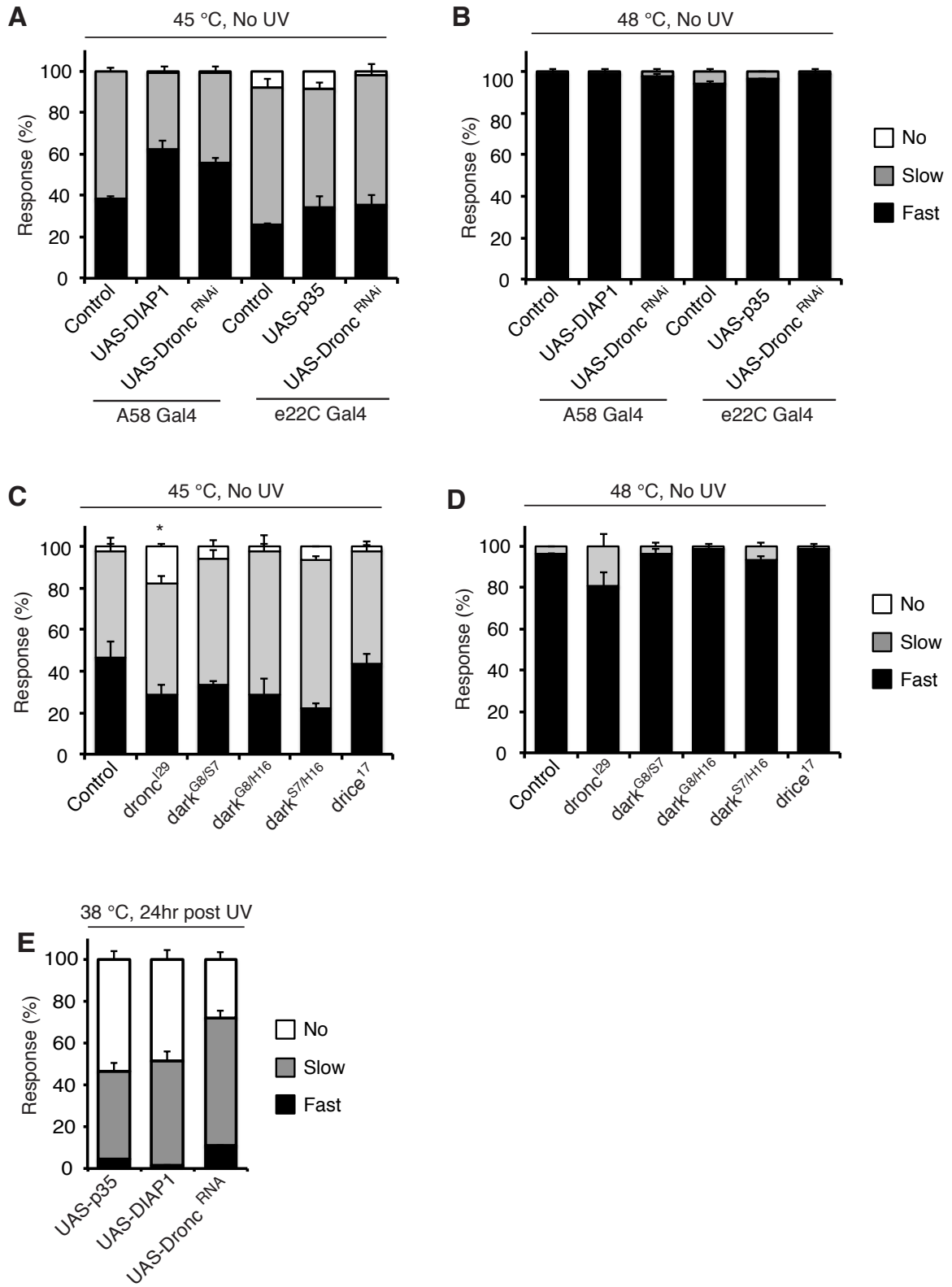
Figure 3.4 Baseline thermal nociception in cell death pathway genes and UV-induced thermal allodynia of UAS-alone control.

(A-D) Quantification of thermal withdrawal behavior in the absence of UV irradiation. (A-B) Measurement of aversive withdrawal behavior of larvae expressing UAS-indicated transgene under control of epidermal specific driver, *A58* or *e22C Gal4* at 45 °C (A) or 48 °C (B). Gal4 only was used as control. (C-D) Measurement of aversive withdrawal behavior of mutant larvae lacking indicated component of cell death pathway at 45 °C (C) or 48 °C (D). w^{1118} is used as control. (E) Quantification of UV-induced thermal allodynia of *UAS-transgene* targeting indicated gene alone control at 38 °C, 24 hour post UV irradiation. n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicates p value less than 0.05, n.s indicates no statistical significance.

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defect (Figure 3.4C and D). Moreover, there were no significant differences in baseline nociception between control and larvae overexpressing *UAS-Diap1* or *UAS-p35* in the epidermis. (Figure 3.4A and B), supporting the idea that alterations of nociceptive sensitization in larvae defective for the apoptosome are not caused by the baseline nociception defect. Overall, these results demonstrate that Dronc has a non-apoptotic function in promoting thermal allodynia and this non-apoptotic function is exerted independently of the downstream effector caspases that Dronc usually cooperates with in apoptotic contexts. Interestingly, while confirming previous results that TNF/Eiger is required for thermal allodynia, we noticed that this mutant is in fact haploinsufficient for thermal allodynia (Figure 3.3C). Taken together, our findings indicate that apoptosis and thermal allodynia are genetically separable, meaning that there exist genotypes in which each process occurs independently of the other (Table 3.1).

3.2 UV-induced thermal allodynia can be evoked below the threshold for UV-induced apoptosis

As an alternative test of the question whether epidermal cell death is necessary to induce thermal allodynia, we examined if larvae exhibit nociceptive sensitization when treated with low doses of UV that do not cause overt apoptosis. To determine the lowest dose

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	Required for Epidermal Cell death?	Required for Allodynia?
Other caspases (Dcp-1, Decay, Strica)	NO	NO
Apoptosome (Dronc, dApaf-1, DIAP1)	YES	YES
Effector caspase defect (DrIce, p35)	YES	NO
TNF signaling	NO	YES

Table 3.1 Genetic separation between apoptotic cell death and allodynia.

Comparison of UV-induced cell death and thermal allodynia in larvae lacking TNF signaling,

apoptosome, effector caspases, or other caspases. NO indicates that it is not required. YES

indicaites that it is required for the process.

of UV that causes apoptosis, we examined TUNEL labeling in the larval epidermis exposed to decreasing amounts of UV. We found that UV doses higher than 12 mJ/cm^2 caused both morphological disruption and TUNEL staining in the epidermis, whereas doses less than 8 mJ/cm^2 do not (Figure 3.5A). As expected from a previous work, thermal allodynia developed in a dose-dependent manner at UV doses where overt apoptosis was observed (Figure 3.5B). Interestingly, an attenuated but still substantial thermal allodynia response was observed even at lower UV doses (8 mJ/cm^2) that did not provoke overt apoptotic cell death (Figure 3.5B). Although the magnitude of thermal allodynia is significantly reduced with UV doses of less than 12 mJ/cm^2 in comparison with thermal allodynia induced by normally employed dose (20 mJ/ cm^2), Dronc and TNF/Eiger were still required for these low UV doses-induced thermal allodynia (Figure 3.5C). These data first suggest that there is no distinct signaling mechanism that induces thermal allodynia when apoptosis is absent. Second, the data suggest that the signal(s) that activate nociceptive sensitization can be produced even in the absence of overt cell death and that Dronc can promote these signal(s) independently of engagement of downstream apoptotic activation.

3.3 Canonical TNF/Eiger signaling factors are required for TNFR/Wengen signal transduction within nociceptive sensory neurons during UV-induced thermal allodynia

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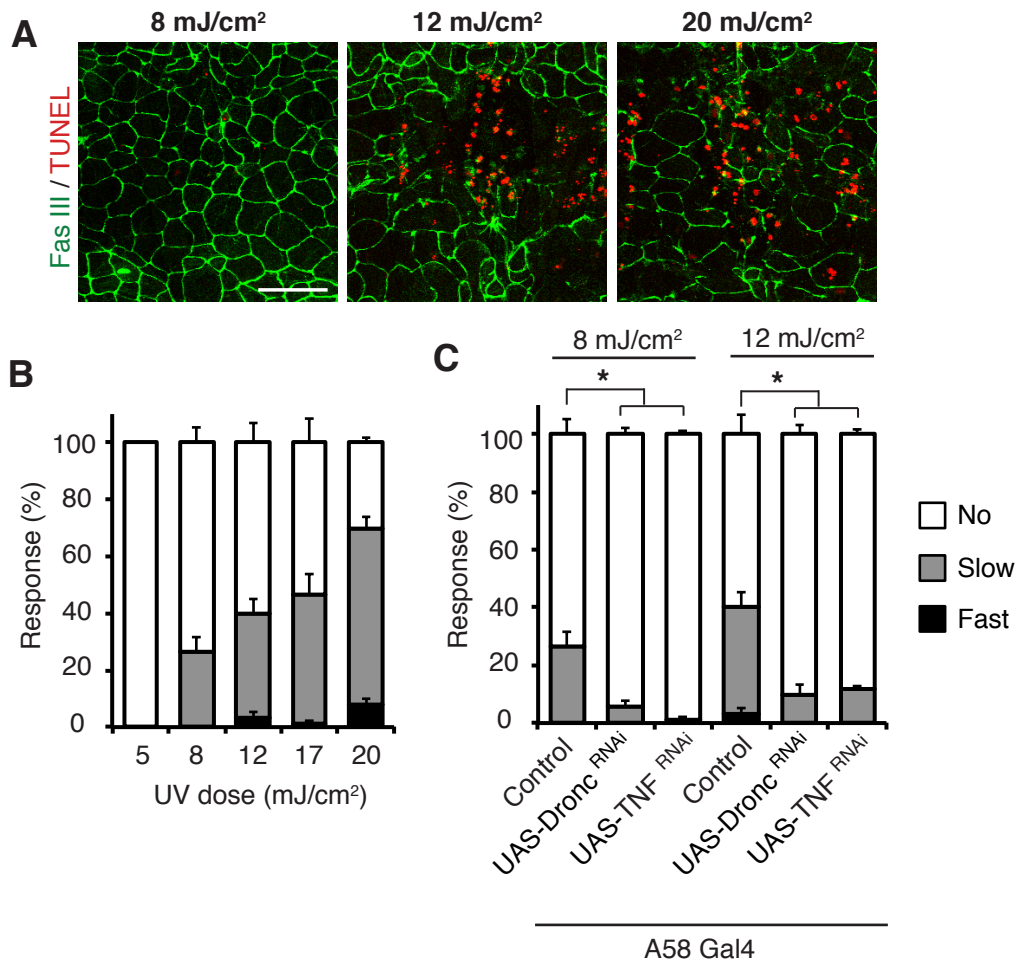
Figure 3.5 Procedural separation of thermal allodynia from epidermal cell death.

(A) Epidermal morphology changes and TUNEL staining of A58 Gal4/+ larvae with

different doses of UV, 24 hour post-irradiation Anti-Fasciclin-3 antibody (membranes, green)

and TUNEL labeling (apoptotic cells, Red) were used. (B) Dose response of UV-induced

thermal allodynia of A58 Gal4/+ larvae at various doses of UV, 24 hour post irradiation. (C)

UV-induced thermal allodynia when Dronc (initiator caspase) or Eiger (TNF) was inhibited,

at 38 °C, 24 hour post UV irradiation *UAS-RNAi* lines are indicated. Gal4 only was used as

control. n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicates p value less than 0.05,

n.s indicates no statistical significance.

Our lab previously found that TNF/Eiger and its receptor TNFR/Wengen are required for UV-induced allodynia (36). However, that study did not resolve howthe signal from the epidermis is transduced in sensory neurons. To identify downstream signaling mediators of TNFR/Wengen during thermal allodynia, we focused on candidate downstream targets implicated in TNF/Eiger signaling pathway during immune and inflammatory responses (64, 152). These included TNF receptor-associated factors (TRAFs), MAP kinases, and NF-κB transcription factors. We first tested nociceptive sensory neuron-specific expression of *UAS-RNAi* transgenes targeting TRAF3 and TRAF6, p38a kinase and the NF-κB-like transcription factor Dorsal for UV-induced thermal allodynia. Expression of *UAS-RNAi* transgenes targeting all of these factors decreased thermal allodynia relative to the Gal4 driver alone and UAS-alone controls (Figure 3.6A and B). Importantly, sensory neuron-specific expression of *UAS-RNAi* transgene targeting Dronc did not interfere with thermal allodynia demonstrating that Dronc functions specifically in the epidermis during development of UV-induced thermal allodynia. Baseline nociception was normal with expression of *UAS-RNAi* transgenes targeting TRAF3, TRAF6, p38a kinase, and Dorsal in class IV MD neurons when tested at normally noxious temperature 45 °C and 48 ºC in the absence of UV treatment (Figure 3.6C and D). To rule out off-target effects of RNAi, we examined null mutants for *traf6* and *p38a/c* and found they also show significant decreases in withdrawal behavior in comparison

Figure 3.6 Canonical downstream mediators of TNF signaling are required for UV-induced thermal allodynia

(A-B) Quantification of UV-induced thermal allodynia, at 38 °C, 24 hour post UV

irradiation. (A) *UAS-RNAi* transgene targeting indicated genes were expressed in class IV

MD neuron with *ppk1.9 Gal4*. Gal4 only was used as control. (B) *UAS-RNAi* transgene alone

control. (C-D) Measurement of baseline thermal nociception of indicated genotype at 45 °C

(C) and 48 °C (D) in the absence of UV irradiation. n = 3 sets of 30 larvae. Error bars

represent S.E.M. * indicates p value less than 0.05, n.s indicates no statistical significance.

with control larvae (Figure 3.7A). Since there are three NF-κB homologs in *Drosophila*, we tested mutants of all of theirs: *Dorsal, Relish*, and *Dif*. Null mutants for *dorsal* and *relish* showed reduced thermal allodynia but larvae homozygous for *Dif* exhibited normal thermal allodynia (Figure 3.7A). Baseline nociception was measured in homozygous mutants for *traf6, p38a/c, dorsal,* and *relish* at 45 °C and 48 ºC in the absence of UV irradiation to test if decreased sensitization is due to the developmental defect (Figure 3.7B and C). All mutants for canonical downstream mediators displayed normal baseline nociception, suggesting TNF signaling is not required for a proper development of class IV nociceptive sensory neuron in *Drosophila*.

Ectopic overexpression of TNF/Eiger in the class IV MD neurons is sufficient to cause "genetic allodynia" (36), which we define as thermal allodynia induced by genetic manipulations in the absence of tissue damage. Therefore, we examined if expression of *UAS-RNAi* transgenes targeting TRAF3, TRAF6, p38a kinase, and Dorsal can attenuate the genetic allodynia caused by TNF/Eiger overexpression. As expected, expression of *UAS-RNAi* transgenes for TNFR/Wengen reduced genetic allodynia (Figure 3.8A). Likewise, RNAi-mediated knockdown of TRAF3, TRAF6, p38, and Dorsal also attenuated TNF-induced genetic thermal allodynia, suggesting these factors mediate downstream signaling

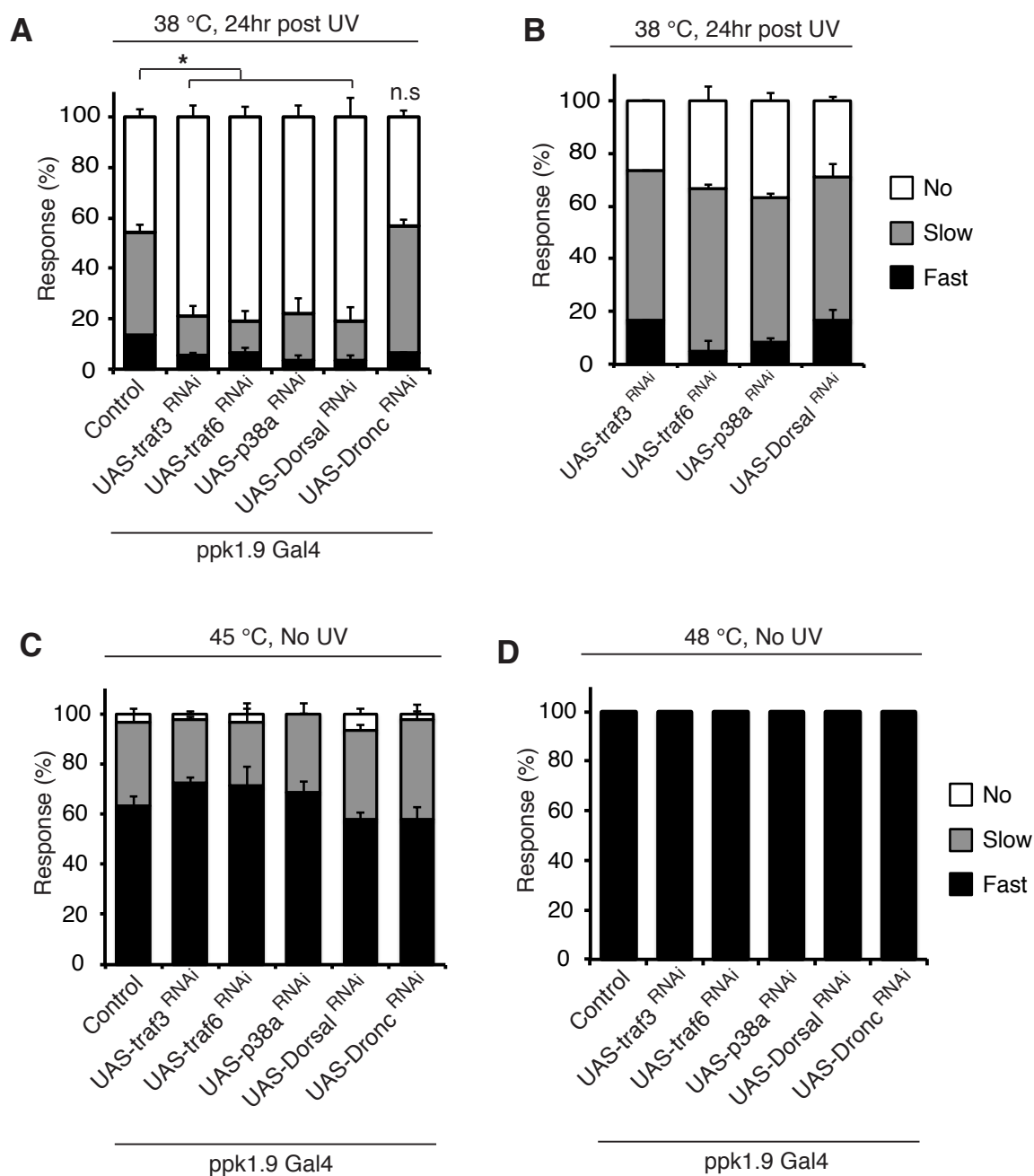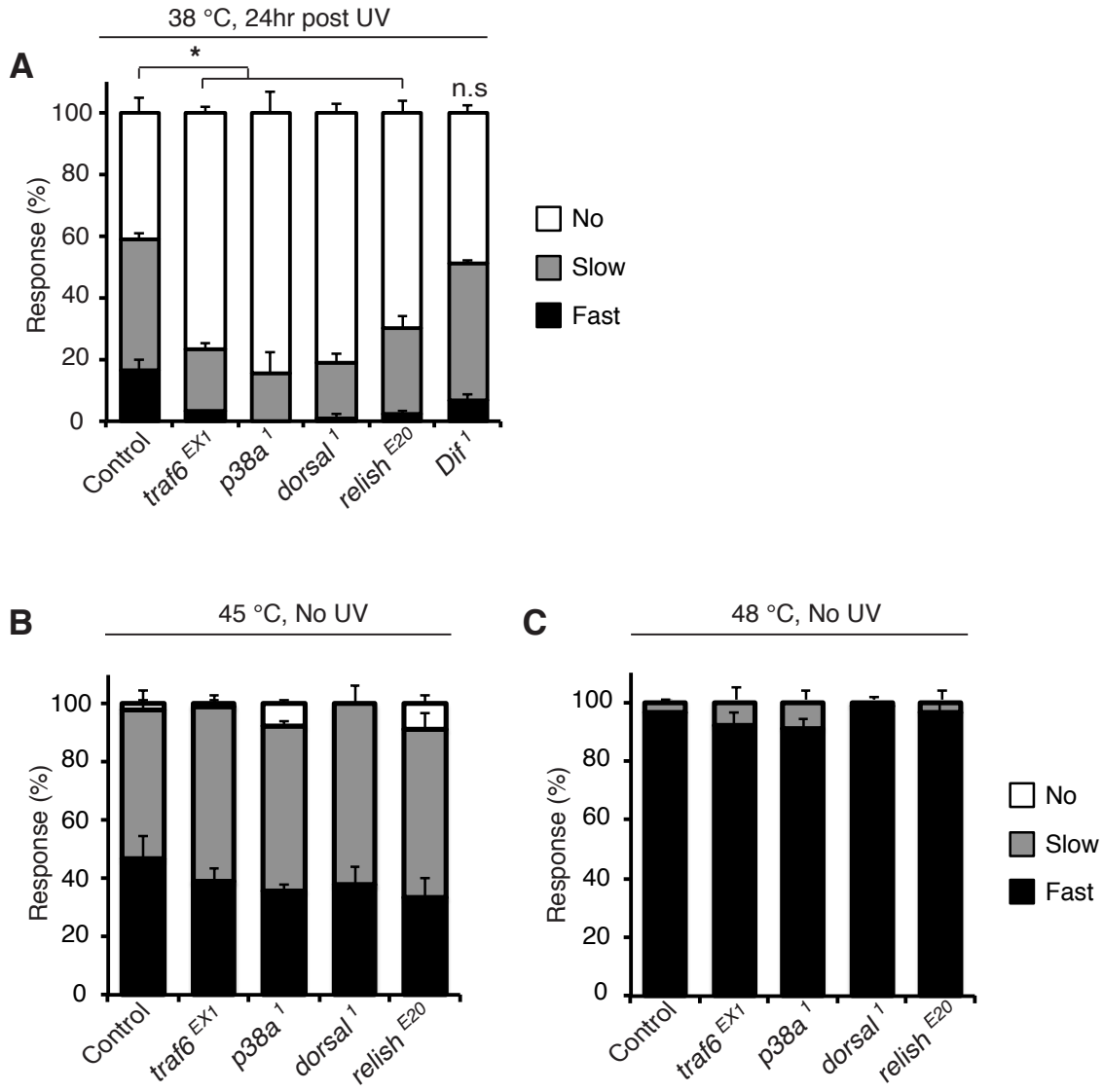**Figure 3.7 Mutants larvae for canonical downstream mediators of TNF signaling display reduced thermal allodynia.**

(A) Quantification of UV-induced thermal allodynia in homozygous mutants, at 38 °C, 24 hour post UV irradiation. w^{1118} was used as a control. (B-C) Baseline thermal nociception of larvae homozygous for indicated mutant alleles at 45°C (B) and at 48°C (C) in the absence of tissue damage.

n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicates p value less than 0.05, n.s

indicates no statistical significance.
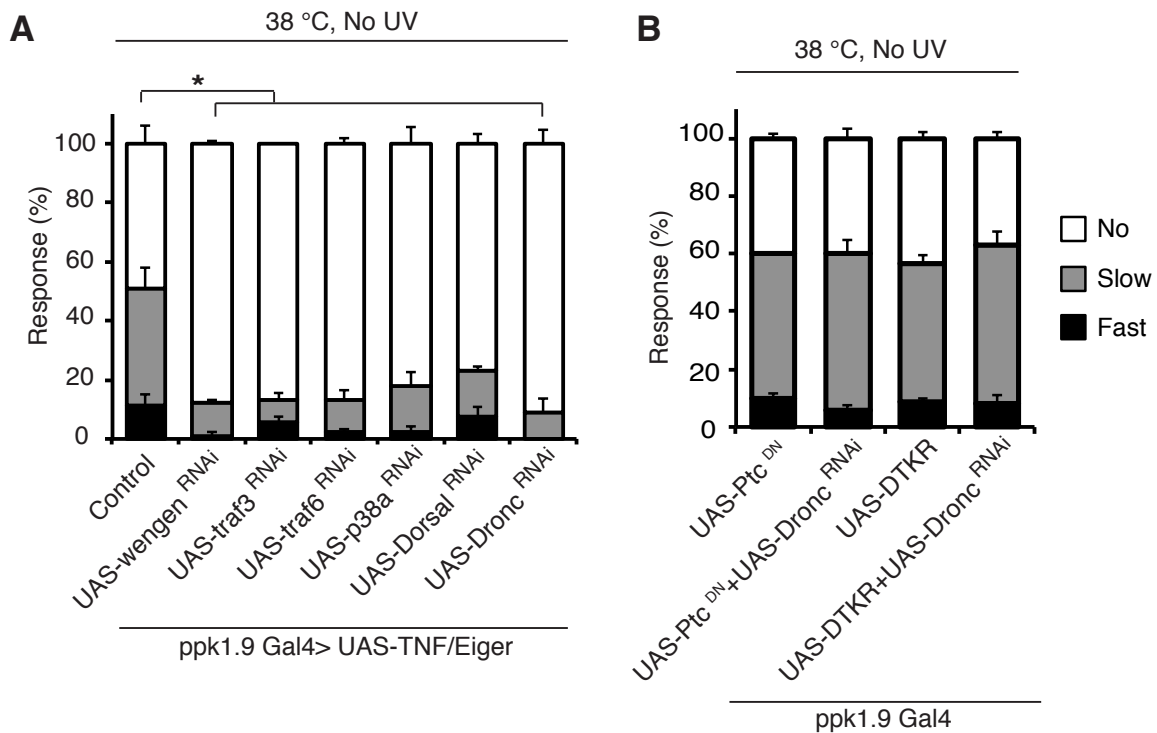
Figure 3.8 Knockdown of Traf3 and Traf6, p38a, Dorsal, and Dronc attenuates TNF-induced genetic thermal allodynia.

(A) Quantification of TNF-induced genetic thermal allodynia in the absence of tissue damage when indicated *UAS-RNAi* transgene is expressed, at 38°C. UAS-TNF/+; ppk1.9 Gal4/+ larvae were used as control. (B) Quantification of genetic thermal allodynia induced by Hedgehog or Tachykinin signaling in the absence of tissue damage, with or without *UAS-RNAi* transgene targeting Dronc, at 38°C. n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicates p value less than 0.05

for TNF/TNFR to modulate nociceptive sensitization. Interestingly, Dronc, which is not required within class IV MD neurons for UV-induced thermal allodynia (Figure 3.6A) is required for TNF-induced genetic thermal allodynia (Figure 3.8A). This requirement of Dronc in class IV sensory neurons was specific to TNF signaling, as expression of *UAS-RNAi* transgenes targeting Dronc did not block Hedgehog or Tachykinin signaling-induced genetic thermal allodynia (49, 50) (Figure 3.8B). Taken together, our results suggest that Dronc may be required for some aspect of generating functional TNF.

3.4 Dronc requires TNF/Eiger but not effector caspases to produce thermal allodynia

Because epidermal Dronc is required for UV-induced thermal allodynia, we tested if epidermal overexpression of Dronc could cause genetic thermal allodynia in the absence of UV irradiation. Because activation of Dronc causes apoptotic cell death and larval lethality, I employed the *tub-Gal80^{ts}* system to avoid developmental defects in the early larval stages (Figure 3.9A). Larvae were reared at a permissive temperature (18°C) and temporal overexpression of the relevant transgene was induced by a heat-shock at 32°C for 24 hours. There was no thermal allodynia in any genotype in the absence of heat-shock (Figure 3.10A). Conditional overexpression of Dronc by heat-shock caused strong genetic thermal allodynia
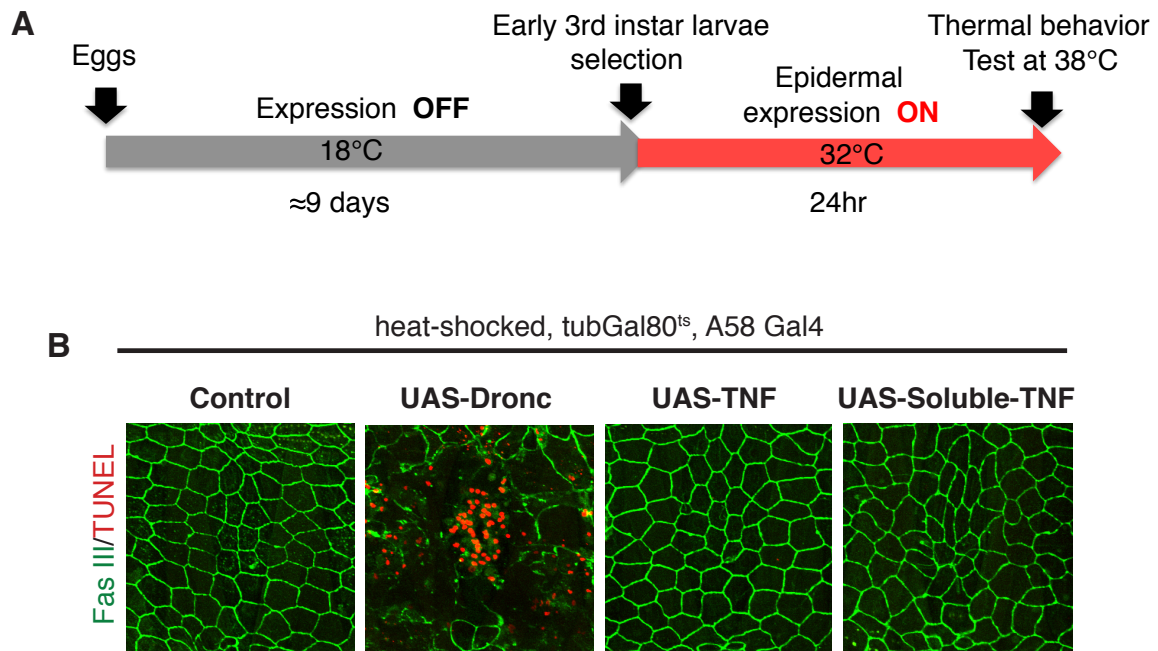
Figure 3.9 Overexpression of Dronc but not TNF causes epidermal cell death.

(A) Schematic of *UAS-transgene* activation by heat-shock. *A58-Gal4*-mediated transgene

expression was controlled by *tubGal80^{ts}*. (B) Epidermal staining of the indicated genotypes.

Anti-Fasciclin-3 antibody (membranes, green) and TUNEL labeling (apoptotic cells, red)

were used.
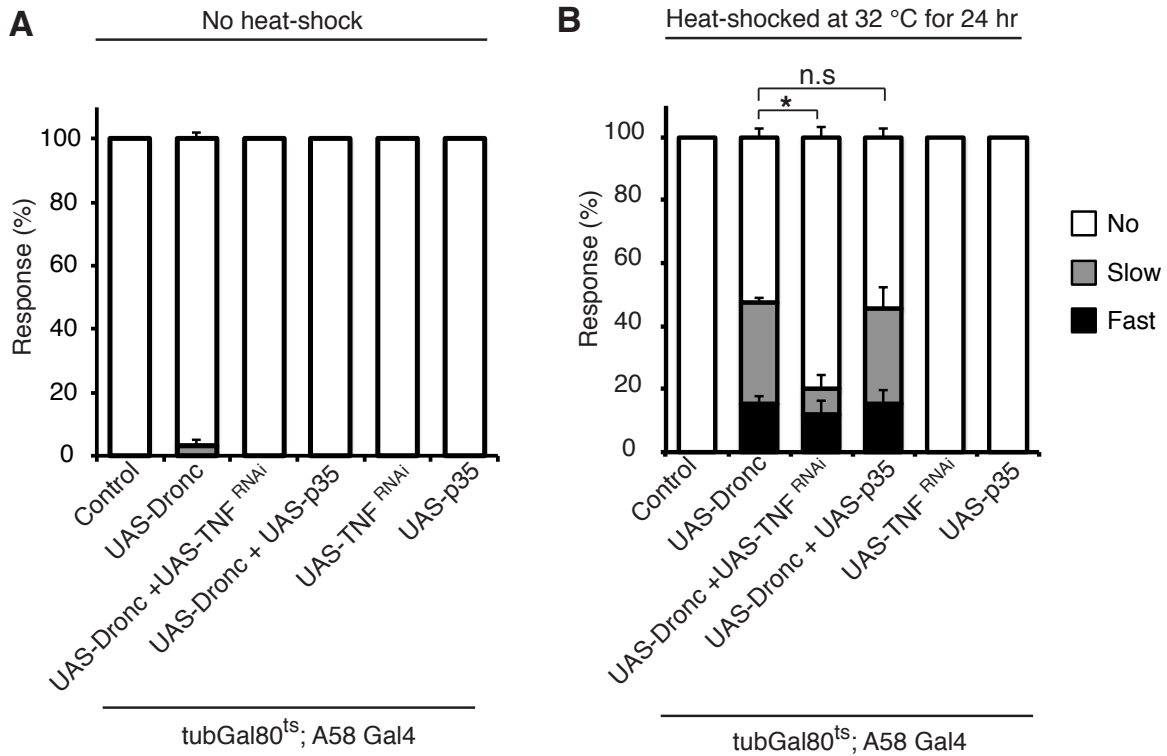
Figure 3.10 Dronc utilizes TNF signaling to induce thermal allodynia.

(A-B) Quantification of withdrawal responses in larvae of indicated genotype to 38 °C thermal stimuli in the absence of UV irradiation. *tubGal80^{ts}; A58 Gal4* only was used as control. (A) Larvae carrying indicated transgene were maintained at permissive temperature, 18 °C. (B) Heat-shock induced overexpression of Dronc with or without expression of *UAS-RNAi* transgene targeting TNF/Eiger and with or without the inhibitor of effector caspases, p35. Heat-shock was given at 32 °C for 24 hours. n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicates p value less than 0.05, n.s indicates no statistical significance.

(Figure 3.10B) as well as massive apoptotic cell death in the epidermis (Figure 3.9B). *UAS-Dronc* alone control did not show thermal allodynia, suggesting heat-shock alone could not cause thermal allodynia (Figure 3.10B). Since Dronc and TNF/Eiger are required in the same tissue; epidermis for UV-induced nociceptive sensitization, we hypothesized that epidermal Dronc activates TNF/Eiger to produce thermal allodynia. Therefore, I examined if epidermal- specific inhibition of TNF/Eiger could block the genetic thermal allodynia induced by Dronc overexpression. A *UAS-RNAi* transgene targeting TNF/Eiger significantly attenuated Dronc-induced ectopic thermal allodynia (Figure 3.10B). By contrast, overexpression of the effector caspase inhibitor p35 did not alter the ectopic thermal allodynia induced by Dronc overexpression. Taken together, I conclude that a non-apoptotic Dronc-mediated regulation or induction of TNF/Eiger is critical to induce thermal allodynia in *Drosophila* larvae.

3.5 Pre-Processed TNF/Eiger does not require Dronc to induce thermal allodynia

Previously, our lab found that TNF/Eiger is required in the epidermis for UV-induced thermal allodynia. However, the relative contribution(s) of potential transcriptional, translational, processing/secretion, or other regulatory mechanisms in regulating TNF activity during UV-induced thermal allodynia remained unclear. To understand how

TNF/Eiger is regulated in the epidermis, I first measured changes in transcription and translation of TNF following UV treatment. There were no detectable alterations of protein levels (Figure 3.11A) or transcriptional reporter (Figure 3.11B) of TNF/Eiger, suggesting that the regulation is not likely at the expression level, rather at the posttranslational modification and subcellular localization level.

Previous studies in mammalian cells show that the ectodomain of TNF-α can be secreted from the membrane following proteolytic cleavage by the metalloprotease TNF- α converting enzyme (TACE). Therefore, processing/secretion of TNF could be required to activate TNF/Eiger in the epidermis following UV exposure. This hypothesis is also supported by the fact that TNF/Eiger and its receptor are required in different tissues, epidermis and class IV MD neurons respectively. Proteolytic processing of TNF/Eiger has been reported in S2 cells (74) and Tace, a homolog of mammalian TACE exists in fly genome, although it has not been validated biochemically whether Tace in *Drosophila* directly processes TNF/Eiger into soluble form. Because TNF/Eiger is required in the epidermis, tissue specific inhibition of Tace in the epidermis was examined for nociceptive sensitization after UV irradiation. Expression of *UAS-RNAi* targeting Tace in the epidermis exhibited normal development of thermal allodynia (Figure 3.12), suggesting that either
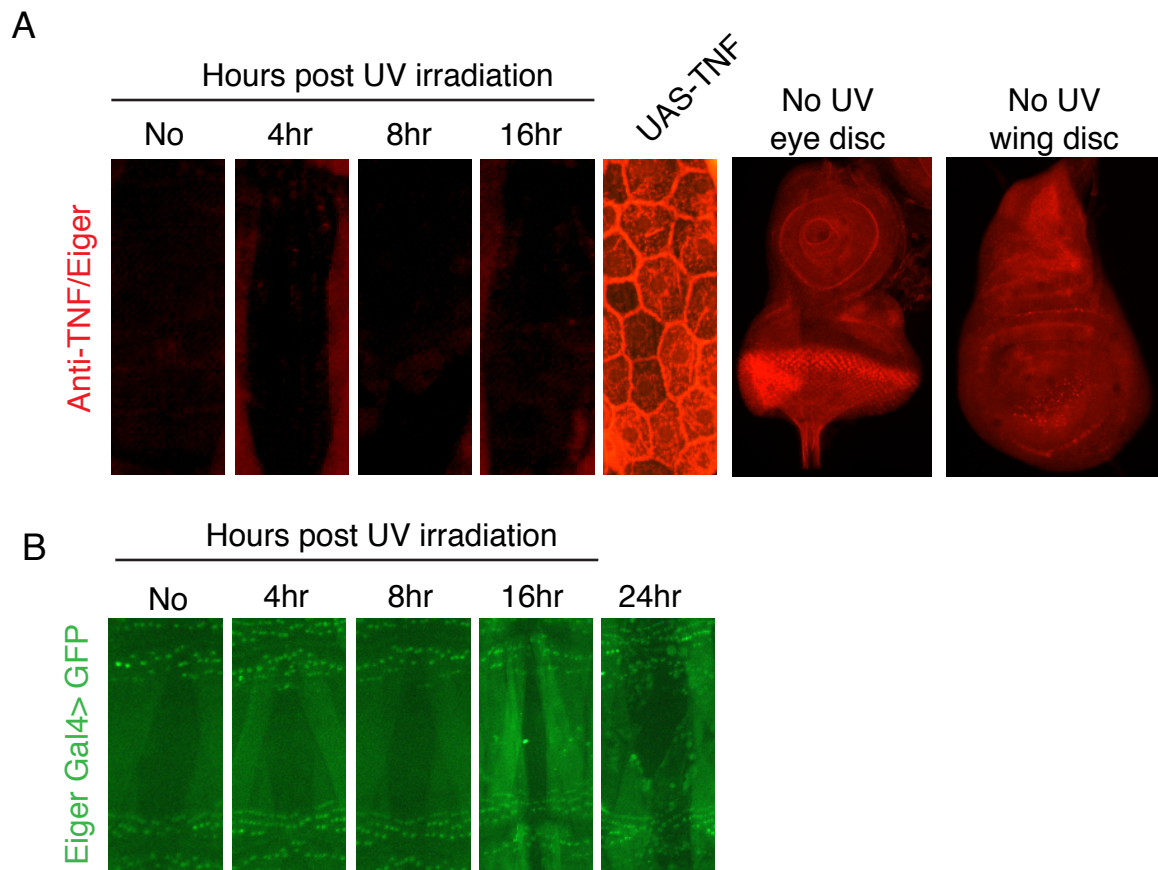
Figure 3.11 A TNF/Eiger transcriptional reporter and TNF/Eiger translation are not induced by UV irradiation.

(A) Anti-TNF/Eiger staining of the larval epidermis and eye and wing imaginal discs (red) at the indicated times after UV irradiation. *A58 Gal4* alone is used for non-UV treated or UV-treated experiments. For positive control of epidermal staining, *UAS-TNF/Eiger* was used with *A58 Gal4*. Imaginal discs were used as controls for antibody staining. (B) Epidermal whole mounts in larvae bearing *UAS-GFP* under control of *TNF/Eiger-Gal4* after UV irradiation (Anti-GFP, green).
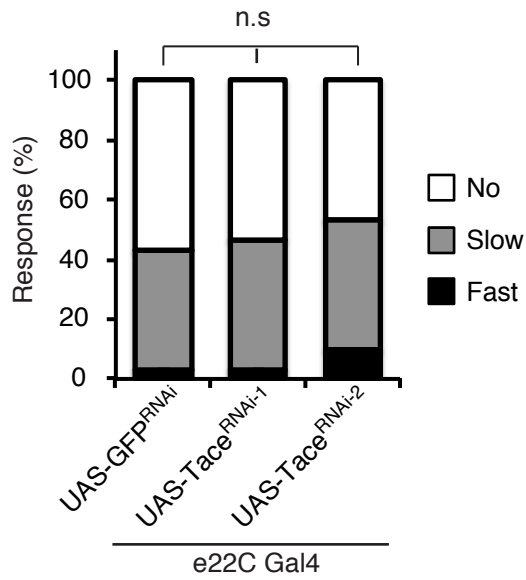
Figure 3.12 Epidermal Tace is not required for UV-induced thermal allodynia.

Quantification of UV-induced thermal allodynia in homozygous mutants, at 38 °C, 24 hour

post UV irradiation, in larvae with epidermal tissue specific expressions of two independent

UAS-RNAi transgenes targeting Tace. *UAS-RNAi* transgene targeting GFP was used as

control. n = 30 larvae. n.s indicates no statistical significance.

epidermal Tace is not required for thermal allodynia or RNAi is ineffective to knockdown

Tace.

Given that active processing of TNF/Eiger has been reported in *Drosophila* S2 cells, I

tested if UV irradiation promotes processing or secretion of TNF/Eiger in S2 cells. To find

UV dose that does not cause apoptotic cell death, active caspase-3 staining was performed 24

hours after irradiation at multiple UV doses and $5mJ/cm^2$ was selected for the next

experiment (Figure 3.13A). As expected from a previous study in S2 cells, two different sizes

of TNF/Eiger proteins were detected in cell lysate and media, meaning cell autonomous

processing of TNF/Eiger occurs in the absence of any stimuli or stresses (Figure 3.13B).

However, increased secretion of small size TNF/Eiger proteins into media was detected 30

minutes after UV irradiation in comparison with non-irradiated control. This data suggests

that UV treatment can trigger production of the active TNF/Eiger.

To test a hypothesis that processing or secretion of TNF/Eiger is induced by UV

irradiation to mediate thermal allodynia, I examined genetic allodynia induced by

overexpressing full-length or pre-processed soluble TNF/Eiger in the epidermis. Since

overexpression of soluble TNF/eiger in the epidermis was lethal, the *tub-Gal80^{ts}* system was

employed again to temporally control overexpression (Figure 3.9A). When larvae were

raised at the permissive temperature of 18 °C, there was no thermal allodynia (Figure 3.14A).
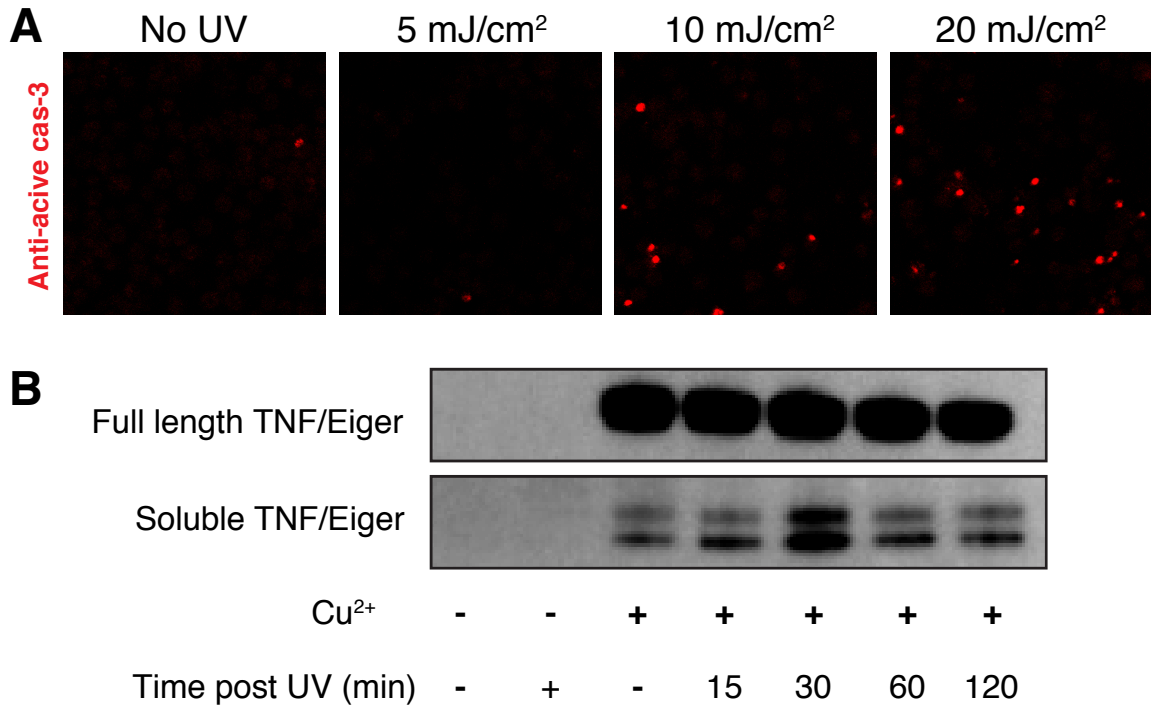
Figure 3.13 Secretion of soluble TNF/Eiger is constitutive and is enhanced by the UV irradiation in S2 cells.

(A) Apoptotic cell death was examined at different dose of UV using anti-active caspase-3 staining (red) in S2 cells. (B) Western blot analysis shows full-length TNF/Eiger from cell lysate and soluble TNF/Eiger from media with or without induction by Cu2+ or UV treatment.
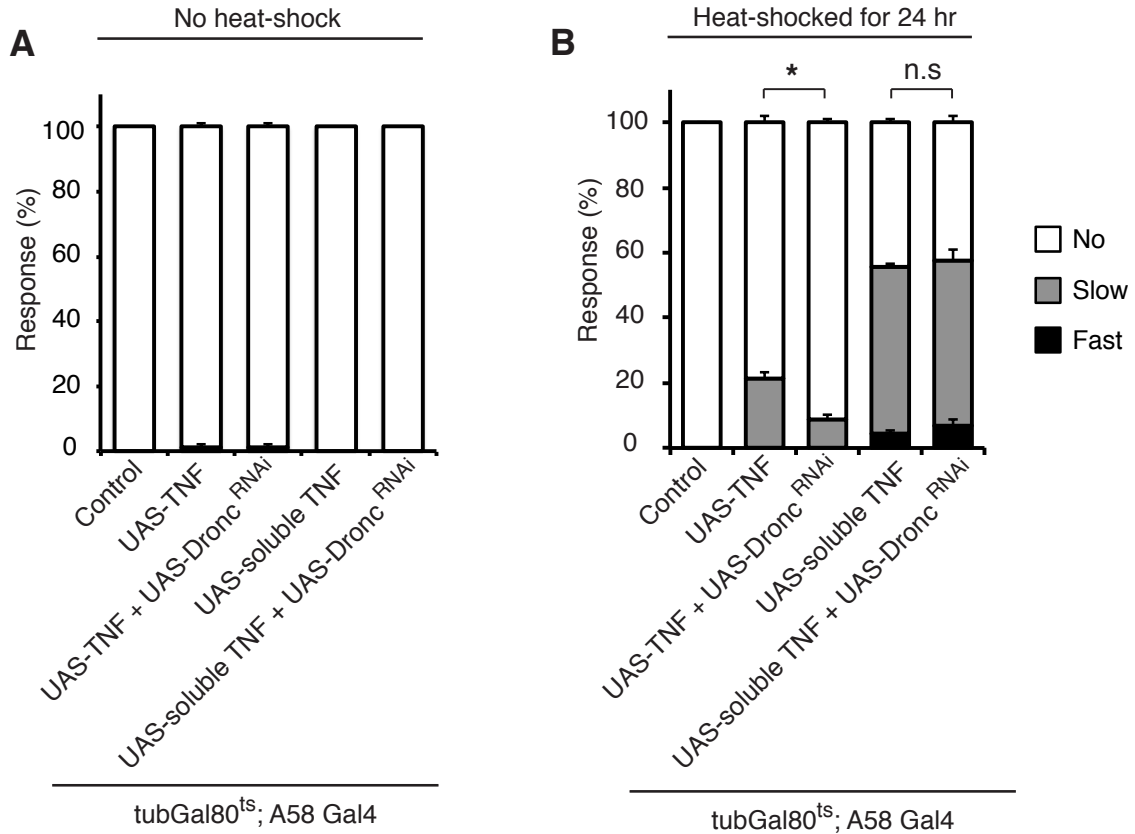
Figure 3.14 Dronc activates TNF signaling by regulating active TNF ligand production.

(A-B) Quantification of withdrawal responses in larvae of indicated genotype to 38 °C thermal stimuli in the absence of UV irradiation. (A) Larvae carrying indicated transgene were maintained at permissive temperature, 18 °C. (B) Heat-shock induced overexpression of full-length or soluble TNF/Egier with or without expression of *UAS-RNAi* transgene targeting Dronc. Heat-shock was given at 32 °C for 24 hours.

n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicated p value less than 0.05. n.s indicates no statistical significance.

In the absence of UV irradiation, I found that conditional epidermal overexpression of full-length TNF/Eiger did not cause strong genetic allodynia (Figure 3.14B). By contrast, conditional epidermal overexpression of a pre-processed soluble form of TNF/Eiger produced robust genetic thermal allodynia (Figure 3.14B). There was no apoptotic cell death in the epidermis upon overexpression of either full-length or soluble TNF/Eiger (Figure 3.9B). Consistent with the idea that Dronc might be required for some aspect of TNF processing or trafficking rather than downstream receptor signaling, I found that epidermal expression of *UAS-RNAi* transgene targeting of Dronc could not block the genetic allodynia induced by soluble TNF/Eiger (Figure 3.14B), demonstrating Dronc is not acting downstream of the TNFR.

3.6 Identification of functional downstream target genes relevant to TNF-mediated nociceptive sensitization

I showed that NF-kb transcription factor Dorsal mediates TNF signaling in class IV MD neurons during nociceptive sensitization. Requirement of Dorsal for TNF signaling-mediated thermal allodynia implies that downstream target genes could be up or down regulated at the gene expression level to induce nociceptive sensitization. To find transcriptionally regulated downstream target genes upon TNF signaling activation in

nociceptive sensory neurons, tissue specific microarray analysis was performed in collaboration with the Cox lab (Neuroscience Institute, Georgia State University). Nociceptive sensory neurons were isolated from control larvae and genetically sensitized larvae that overexpress TNF/Eiger in sensory neuron (Figure 3.15A). In comparison with control, a total of 86 genes were modulated in TNF signaling-activated class IV MD neurons: 50 genes were relatively up-regulated (>2 folds) while 36 genes were down-regulated (<2 folds). 27 genes in up-regulated group and 21 genes in down-regulated group have clear human orthologs. Table 3.2 shows the list of up-regulated genes that have human orthologs. These up-regulated genes include G protein coupled receptors (GPCRs), enzymes, transcription factors, and ion channels (Figure 3.15B). To test whether increased expression of these genes contributes to nociceptive sensitization, I examined expression of *UAS-RNAi* transgenes targeting these candidate genes for both UV-induced and TNF/Eiger-induced thermall allodynia. I found that expression of *UAS-RNAi* transgenes targeting five distinct genes decreased both UV-induced and genetic thermal allodynia (Figure 3.16), suggesting that these genes are functional downstream target genes by TNF signaling in nociceptive sensory neurons. These five genes are *garnet*, *Allatostatin receptor A1(AstA-R1)*, *Trissin receptor (TrissinR)*, *Enhancer of zeste (E(z)),*and *methuselah-like 8 (mthl8)*. *mthl8* is a *Drosophila* specific gene whereas other four genes have human ortholog. *garnet* is an
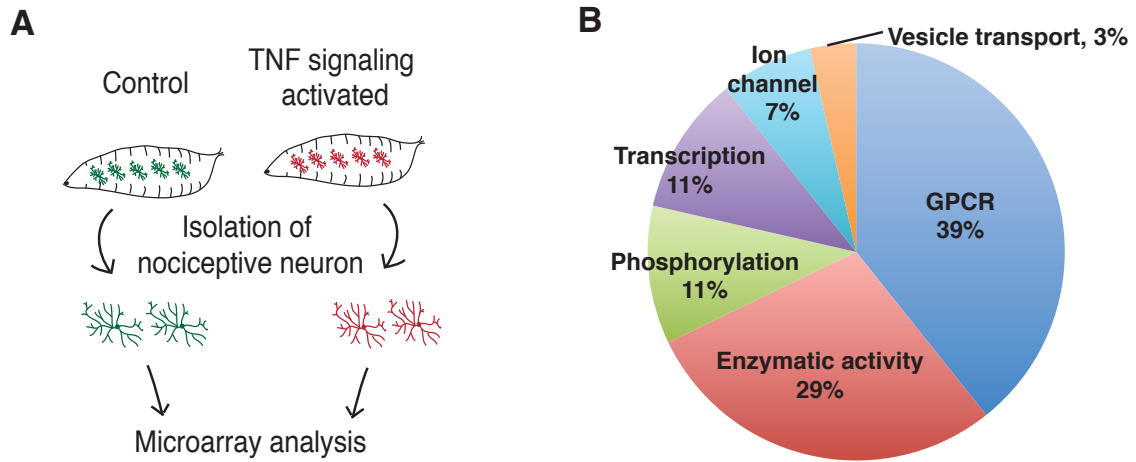
Figure 3.15 Microarray analysis reveals downstream target gene of TNF/Eiger signaling.

(A) Schematic of the isolation procedure of nociceptive sensory neurons and microarray analysis. (B) Categories of genes that have clear human orthologs among genes that are up-regulated in nociceptive sensory neurons that TNF signaling pathway is genetically activated.

CG number	Gene	Molecular Function	Human ortholog with best score	Fold change
CG31201	Glutamate receptor IIE	Ion channel	GRIK2	2.1630475
CG3772	cryptochrome	GPCR signaling	CRY1, CRY2	4.27233
CG1147	neuropeptide F receptor	GPCR signaling	NPY5R	4.200895
CG33183	Hormone receptor-like in 46	Transcription	RORB	4.195125
CG18741	Dopamine receptor 2	GPCR signaling	ADRA1B	4.4514525
CG33344	Cardioacceleratory peptide receptor	GPCR signaling	NPSR1	2.2945625
CG34381	Trissin receptor	GPCR signaling	GPR139	3.1337875
CG31760		GPCR signaling	GPR179	2.1514775
CG7431	Tyramine receptor	GPCR signaling	ADRA1D	3.85525
CG10888	Rhodopsin 3	GPCR signaling	OPN4	2.826415
CG33513	NMDA receptor 2	Ion channel	GRIN2D	2.1088125
CG7383	eagle	Transcription	VDR	1.895905
CG9918	Pyrokinin 1 receptor	GPCR signaling	NMUR1, NMUR2	2.5064225
CG15744		GPCR signaling	ADGRA2	2.6660225
CG6899	Protein tyrosine phosphatase 4E	Phosphatase, receptor	PTPRB	1.87305
CG2872	Allatostatin Receptor	GPCR signaling	GALR2	2.910375
CG4007	Neurospecific receptor kinase	Receptor tyrosine kinase	MUSK	2.0737025
CG11783	Hormone receptor-like in 96	Transcription	NR1I2	3.283885
CG34384		enymatic activity	DGKH	4.6278075
CG11111	retinal degeneration B	enymatic activity	PITPNM2	3.2450125
CG2171	Triose phosphate isomerase	enymatic activity	TPI1	2.66855
CG6502	Enhancer of zeste	enymatic activity	EZH2	2.6339075
CG10986	garnet	protein transport	AP3D1	3.28213
CG4747		enymatic activity	GLYR1	4.1603975
CG2155	vermilion	enymatic activity	TDO2	2.4186625
CG12529	Zwischenferment	enymatic activity	G6PD	1.9283325
CG6728	ninaG	enymatic activity	CHDH	3.8052075

Table 3.2 List of genes that are up-regulated in the TNF/Eiger overexpressing class IV

MD neurons. Up-regulated genes that have human ortholog are listed.
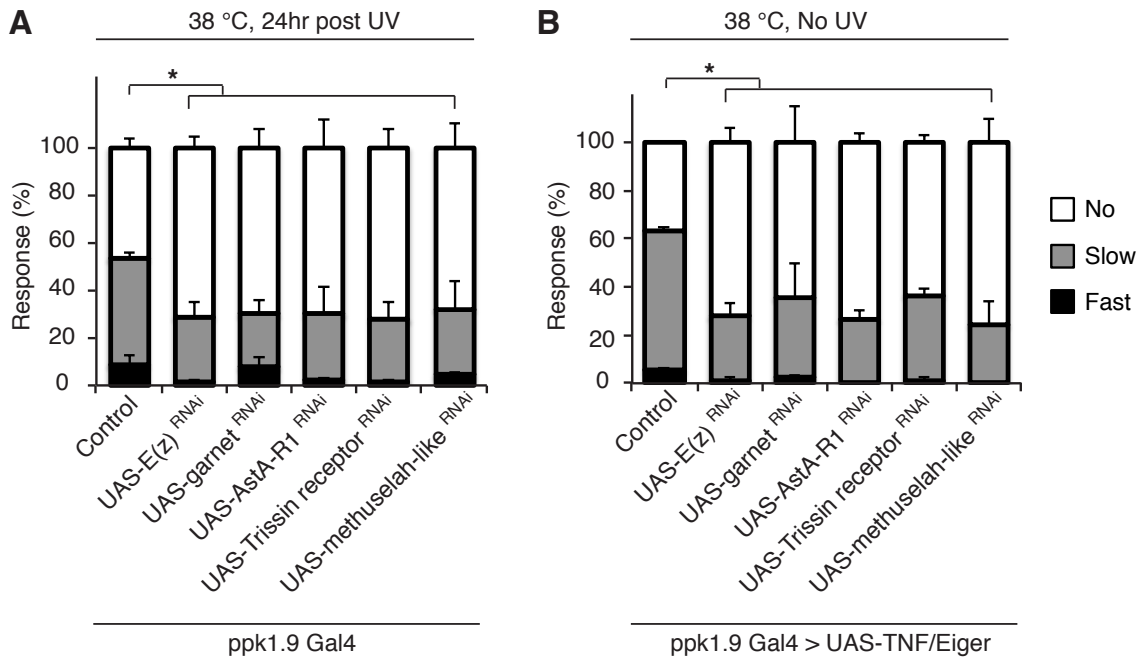
Figure 3.16. Behavioral validation of candidate genes from microarray reveals functional downstream target genes of TNF/Eiger signaling during nociceptive sensitization.

(A) Quantification of UV-induced thermal allodynia (24 hour post UV irradiation) and (B) Quantification of TNF/Eiger-induced thermal allodynia was measured at 38 °C, when indicated *UAS-RNAi* transgene were expressed in class IV nociceptive sensory neurons, using *ppk1.9 Gal4*. n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicated p value less than 0.05

adaptor protein complex 3 for clathrin-mediated endocytosis. *TrissinR*, and *AstA-R1* are GPCRs. *E(z)* is an epigenetic regulator. Among them, I further validated one particular gene of interest, *E(z)* by testing a non-overlapping *UAS-RNAi* transgenes (Figure 3.17A). Non-overlapping *UAS-RNAi* transgenes targeting different regions of *E(z)* transcript also displayed attenuated thermal allodynia in both UV-induced and TNF/Eiger-induced thermall allodynia (Figure 3.17B), demonstrating that reduction of thermal allodynia was not due to off-target effect. Baseline nociception was measured with expression of *UAS-RNAi* transgenes targeting *E(z)* and a mild but statistically significant behavioral defect was found with expression of independent *UAS-RNAi* transgenes (Figure 3.18). Taken together, I identified a sensory neuron specific target gene regulated by for TNF signaling during nociceptive sensitization.
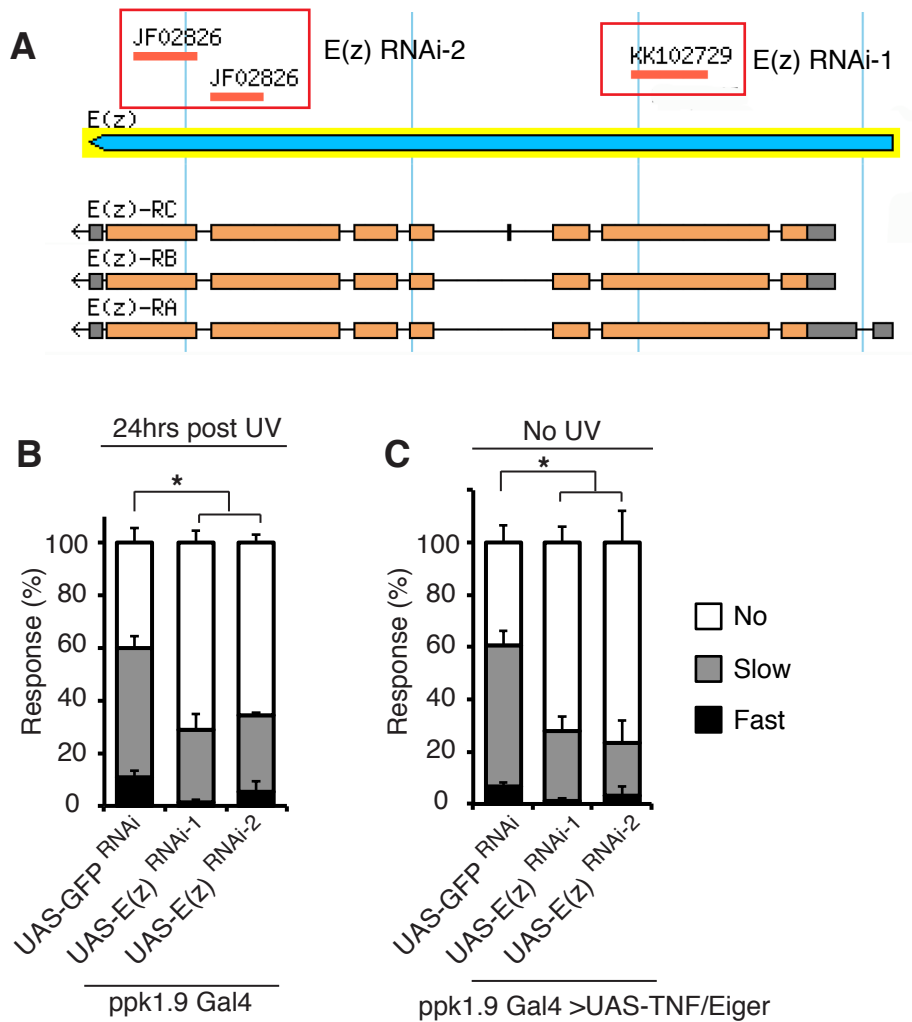
Figure 3.17. E(z) is a functional downstream target gene for TNF-mediated nociceptive sensitization.

(A) Schematic of non-overlapping RNAi lines targeting *E(z)*. (B) Quantification of UV-induced thermal allodynia of larvae with class IV nociceptive sensory neuronal expression of *UAS-RNAi* transgenes targeting *E(z)*, at 38 °C, 24 hour post UV irradiation. *UAS-GFP RNAi* was used as a control. (C) Quantification of TNF/Eiger-induced thermal allodynia of larvae

with class IV nociceptive sensory neuronal expression of *UAS-TNF/Eiger* and *UAS-GFP*

RNAi or *UAS-RNAi* transgenes targeting *E(z)*, at 38 °C in the absence of UV irradiation.n = 3

sets of 30 larvae. Error bars represent S.E.M. * indicated p value less than 0.05.
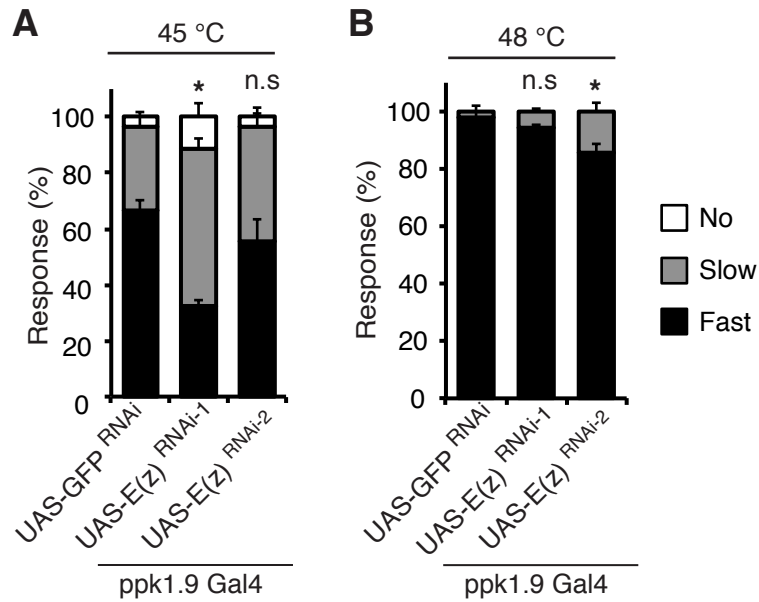
Figure 3.18. Baseline thermal nociception in larvae expressing UAS-RNAi for E(z).

(A-B) Baseline thermal nociception at 45 °C (A) and 48 °C (B) of larvae with class IV nociceptive sensory neuronal expression of *UAS-RNAi* transgenes that are indicated. n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicated p value less than 0.05. n.s indicates no statistical significance.

Chapter 4: Conclusions and Future directions

In the previous chapter, I have shown overall architecture of TNF signaling: ligand regulation, signaling mediators for receptor, a transcription factor, and target gene regulation during nociceptive sensitization in *Drosophila* larvae (Figure 3.19). Described here are important findings, a number of interesting questions raised by my study, and future directions.

4. 1 Activation of apoptosome complex in UV irradiated epidermis

A previous study in our lab identified initiator caspase Dronc for UV-induced apoptotic cell death. Here I characterized more completely the function of the apoptosis machinery for the larval epidermal cell death induced by UV treatment. In addition to Dronc, the Apaf-1 factor Dark and downstream effector caspase Drice were essential for the larval epidermal apoptosis whereas other initiator or effector caspases were not required in larval epidermal cell death. These data suggest that the full apoptosome consists of Dronc and Dark activates a specific downstream effector caspase Drice in the larval epidermal cell death. It also indicates that the larval epidermis use classical apoptotic caspases that are commonly utilized by other fly tissues. However, it still remains unclear if the larval epidermis uses classical upstream factors to activate epidermal Dronc and a subsequent cascade of caspases in the context of UV irradiation-induced cell death. In most fly tissues, the apoptosome is inhibited by DIAP1 in the absence of apoptotic signals. DIAP1 can be antagonized by one or
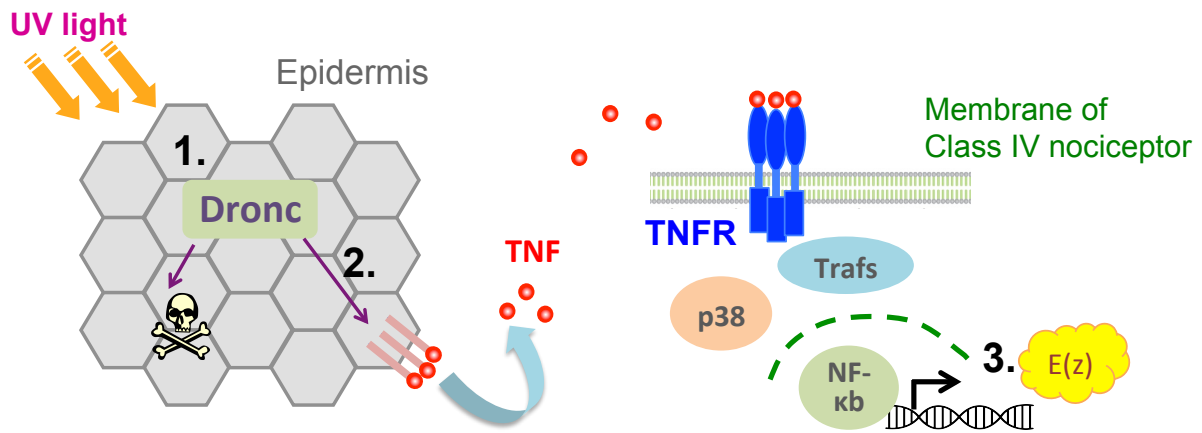
Figure 3.19. Current model of TNF signaling-mediated nociceptive sensitization in

Drosophila **larvae.**

1. UV irradiation induces the activation of initiator caspase Dronc. The apoptotic function of Dronc is required for epidermal apoptotic cell death. 2. Non-apoptotic activity of Dronc promotes the production of the active TNF/Eiger from the epidermis. TNF/Eiger binds its receptor TNFR/Wengen in class IV MD neuron, thus leading to downstream signal transduction. Traf3, Traf6, p38 MAP kinase, and NF-kb transcription factor Dorsal mediate this signal transduction, 3. which eventually increases expression level of epigenetic regulator, Enhancer of zest.

Blue arrow indicates processing or secretion of TNF. Green dashed line is nuclear membrane of nociceptive sensory neuron.

combinations of pro-apoptotic genes: *grim, reaper, hid,* and *sickle*. Epidermal overexpression of Hid caused lethality and temporal overexpression was enough to induce epidermal apoptotic cell death. By contrast, overexpression of Grim and Reaper is dispensable for apoptotic cell death. These gain-of-function experiments suggest Hid as a strong candidate gene among pro-apoptotic genes for the activation of epidermal apoptosome. However, tissue specific knockdown of *hid* or homozygous hypomorphic mutant for *hid* did not block UV-induced apoptotic cell death in the epidermis. The result of loss of function study could be due to contributions of other pro-apoptotic genes to alternate or amplify function of Hid. However, tissue specific knockdown of Hid, Grim, and Reaper with expression of either *UAS-RNAi* or *UAS-microRNA* targeting all three genes did not interfere with epidermal apoptosis after UV irradiation. It is still conceivable that tissue specific knockdown or hypomorphic allele incompletely inhibit the functional Hid, Grim, and Reaper level although *UAS-RNAi* or *UAS-microRNA* targeting all three genes are shown to work effectively in the other *Drosophila* tissues in previous studies (121, 146). Homozygous null mutant animals lacking *hid* or all pro-apoptotic genes are embryonic lethal limiting the investigation to heterozygous or hypomorphic mutants, which still show substantial apoptotic cell death in epidermis. In the future, a tissue specific conditional knockout of pro-apoptotic genes using CRISPR/Cas9-mediated conditional mutagenesis (153) would be useful to test the

requirement of pro-apoptotic genes for activation of Dronc and following epidermal cell death induced by UV irradiation in *Drosophila* larvae.

Another possible scenario is that Dronc is activated by a pro-apoptotic genes-independent mechanism for UV-induced epidermal cell death. UVC irradiation strongly induces instant DNA damage such as cyclobutane pyrimidine dimers, a covalent linkage between neighboring pyrimidine bases and can be removed by nucleotide excision repair (NER) (154). In addition to the formation of pyrimidine dimers, UVC irradiation is effective at generating reactive oxygen species (ROS) (155). All these cellular events induced by UV irradiation are triggers of apoptosis. It is not known if initiator caspase Dronc can be directly activated by DNA damage or ROS. However, it is notable that ROS directly mediates caspase-9 activation via facilitating a disulfide link between caspase-9 and Apaf-1 in mammalian cells (156). In the future it would be interesting to test if DNA damage, members of the nucleotide repair machinery or generation of ROS directly contribute to the activation of Dronc and subsequent apoptosis. To test this hypothesis, proof-of-principle experiments should be performed first to test if UVC irradiation induces pyrimidine dimers or production of ROS in larval epidermis. The formation of pyrimidine dimers could be visualized using anti-thymine dimer antibody (157). A previous study showed that UVC irradiation induces conversion of non-fluorescent H_2DCF into fluorescent 2', 7'-DCF (158) in larval epidermis

and muscle, suggesting production of ROS by UV irradiation (159). Therefore, it would be interesting to test if the UV doses that are used in my study facilitate ROS production in epidermis. A contributions of DNA damage or ROS to UV-induced cell death could be tested by epidermal knockdown of components of NER (160) or overexpression of anti-oxidant enzyme such as catalase (161). Finally, Dronc activity could be monitored using a genetic probe containing a motif that Dronc specifically recognizes and cleaves (162, 163) in the contexts that NER or production of ROS is blocked.

It is particularly interesting how Dronc is activated and if identical upstream factors activate the dual functions of Dronc- apoptotic cell death and thermal allodynia. The examination of this hypothesis would provide some clues how apoptotic and nociceptive sensitization functions of Dronc are distinguished. If the same upstream factors activate both functions of Dronc, activity levels and execution efficiencies might determines outcome of Dronc activation. Florentin et al. (164) showed that the levels of the pro-caspases (not yet activated caspases) are directly proportional to apoptotic potential. The activation of Dronc might also be proportional to does of UV. Thermal allodynia might be induced even if the activity level of Dronc is low whereas apoptotic cell death only occurs when the activity level of Dronc is above certain threshold. This hypothesis could be tested with differential activation/overexpressions of Dronc by controlling lengths of heatshock time using *Gal80^ts*.

In addition, the comparison of Dronc and Drice-specific activity reporter could help to figure out how the activity level of Dronc is related to the activity of Drice for apoptotic cell death.

It is also conceivable that post-translational modifications of Dronc are different for apoptotic cell death and thermal allodynia. Yang et al. (165) showed that metabolic alterations including changes of cellular NADPH levels modulate activity of Dronc through phosphorylation, suggesting that post-translational modifications are important for the regulating activity of Dronc. Therefore, it would be interesting to test if Dronc is differentially modified, through phosphorylation or other potential conjugates, with low and high doses of UV.

4. 2 Production of active TNF ligand by a non-apoptotic function of Dronc

UV-induced nociceptive sensitization is used in various model organisms as a reliable model to understand mechanisms of inflammatory pain (166). UV-induced damages in the skin and subsequent alterations in pain are very similar in many organisms including human (83, 166, 167). Because damages in skin are obvious as epidermis mainly absorb UV light and many inflammatory cytokines are produced from epidermal tissue, epidermal damages are considered as a main reason of nociceptive sensitization. However, it was unclear whether UV-induced cell death itself is a direct cause of nociceptive sensitization. Here I

found that epidermal apoptotic cell death and thermal allodynia are completely separable. First, there are four distinct genotypes that show genetic separation between epidermal apoptotic cell death and thermall allodynia. Second, thermal allodynia can be induced by a low dose of UV that does not cause apoptotic cell death in the epidermis. Lastly, in the absence of apoptotic tissue damage, it was enough to provoke thermal allodynia by genetic manipulation such as overexpression of TNF/Eiger and knockdown of Cactus (I kappa B), suggesting that cell death is unnecessary to induce nociceptive sensitization.

Whereas cell death and nociceptive sensitization are independent, I found that the initiator caspase Dronc plays a critical role in induction of thermal allodynia through activation of TNF signaling and that this function is independent of the canonical downstream effector caspase, Drice. I conclude that a non-apoptotic function of Dronc contributes to production of active TNF/Eiger in the epidermis. This conclusion is supported by several lines of evidence. Because there is no clear transcriptional or translational up-regulation of TNF/Eiger in the epidermis after UV irradiation, post-translational modifications including processing/secretion or increased membrane trafficking could be a key step to induce TNF signaling and thermal allodynia. The fact that TNF/Eiger is a transmembrane ligand and TNF receptor/Wengen functions in a separate tissue supports the conclusion that activation of TNF/Eiger pathway requires cleavage of TNF from the

epidermal tissue to produce soluble ligand. Indeed, overexpression of full-length or soluble

TNF/Eiger from the epidermis produced substantially different degrees of thermal allodynia;

the latter is much more potent than the former. Moreover, epidermal knockdown of Dronc

attenuated full-length TNF/Eiger-induced genetic allodynia. By contrast, the function of

Dronc was dispensable for soluble TNF/Eiger-induced genetic alldoynia, indicating

processed form of TNF does not require Dronc. Lastly, I found that Dronc is not required in

class IV sensory neuron for UV-induced thermal allodynia, in which context that TNF/Eiger

is produced in the epidermal cells wherease nociceptive sensory neuronal Dronc is required

for thermal allodynia induced by ectopic expression of TNF/Eiger in class IV nociceptive

sensory neurons. These results imply that the requirement of Dronc for thermal allodynia is

dependent on whether the tissue makes active TNF/Eiger, supporting my idea that production

of active TNF requires functional Dronc. In mammals, UV irradiated keratinocytes produce

TNFα via transcriptional up-regulation (168). However, it is not well known what regulates

TNF secretion. My study suggests a potential mechanism that initiator caspase might

contribute to production of active TNF in UV-treated tissue. In addition, it would make

initiator caspase as a strong target for inflammatory pain treatment, not simply alleviating

tissue damage but as a reagent that inhibits release of inflammatory cytokine and signaling

activation for nociceptive sensitization.

One of the questions that should be addressed in the future study is a mechanism of how the initiator caspase Dronc contributes to the production of active TNF/Eiger. Because caspase is a protease, one can imagine that Dronc directly processes TNF/Eiger for thermal allodynia. However, TNF/Eiger does not have the consensus sequence that caspases recognize and cleave. The difference in subcellular localization of Dronc and TNF/Eiger is another reason to think that Dronc might not directly process TNF, although there is a precedent study that showed increased TNF secretion by secreted extracellular caspase (91). In other organisms, TNF is processed by TACE, a metalloprotease. Therefore, it is conceivable that Dronc activates a TNF/Eiger by activating its processing enzyme. However, epidermal tissue specific inhibition of *Drosophila* Tace with expression of *UAS-RNAi* transgenes failed to block nociceptive sensitization. This result might be due to ineffective knockdown although the same RNAi line was effectively used in other tissue (78). Because there is no available mutant allele for *Tace*, in the future, it would be interesting to generate and test mutants of *Tace* or other metalloproteases for thermal allodynia to find a link between Dronc and TNF/Eiger processing. Alternatively, it has been shown that TNF trafficking and secretion are regulated by exocytosis in both mammals and *Drosophila* (169). Therefore, examining exocytosis-related genes as mediators for Dronc to activate TNF secretion would be a potential future interest. This could be tested by measuring genetic

thermal allodynia together with overexpression of Dronc and inhibition of exocytosis in the epidermis. An experiment testing this hypothesis would be similar to those described in Figure 3.10.

4. 3 Canonical TNF signaling mediators

In other studies, downstream signaling mediators of the TNF/TNFR pathway, including TRAF3 and p38 MAP kinase, have been implicated in nociceptive sensitization (28, 69, 170). However, nociceptive sensory neuron specific functions of these downstream mediators were unclear. Here I have shown that nociceptive sensory neuron intrinsic functions of Traf2, Traf6, p38a kinase, and the NF-κb factor Dorsal are important to mediate TNF signaling transduction and nociceptive sensitization. Of particular interests are, in the future, to resolve exactly how these downstream components of TNF signaling cooperate or interplay with each other and other pathways to induce thermal allodynia. For example, it needs to be addressed whether p38a kinase directly activates NF-κb by phosphorylating IκB kinase or activates different downstream signaling.

Several members of the canonical TNF signaling cascade have not yet been tested in our UV-induced thermal allodynia model. While Wengen was known as a sole TNF receptor in *Drosophila*, a recent study identified one more TNF receptor, Grindelwald (73), raising the

question of whether this novel TNFR is also required for nociceptive sensitization as vertebrate TNFR 1 and 2 show similar or distinct functions depending on the contexts. In addition, extra-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) have been strongly associated with TNF signal transduction (64). Therefore, it would be interesting to test if fly orthologs of ERK and JNK have similar functions in the context of nociceptive sensitization. I found that *Drosophila* NF-κb transcription factor, Relish (171), is also required for nociceptive sensitization in our model. However, it is unclear whether Relish is required in class IV nociceptive sensory neuron and mediates TNF signaling because nociceptive sensory neuronal expression of *UAS-RNAi* targeting Relish did not block UV-induced thermal allodynia (data not shown). In the future, addressing tissue specificity of two different NF-κb factors and if they have shared target genes for mediating thermal allodynia would be interesting to investigate.

4.4 Novel downstream target genes including E(z)

When it comes to the role of TNF signaling in inflammatory pain, the traditional view has been that TNF signaling and downstream NF-kB activation induce more expression of inflammatory mediators to modulate neuronal activity. Nociceptive sensory neuron-specific microarray analysis in my study revealed that a diverse group of genes including GPCRs and

transcription factors are modulated by the activation of TNF signaling, indicating the function of TNF signaling during nociceptive sensitization is not limited to regulation of inflammatory mediators. In addition, it suggests that expansion of the gene expression profiling analysis with the activation of other signaling pathways will likely identify additional target genes regulating nociceptive sensitization. One of the limitations of the microarray analysis is that it is limited to transcriptional changes. In addition, alterations of gene expression are not specific for nociceptive sensitization although it is performed in class IV MD neurons. To increase the specificity and to narrow down candidate genes for behavioral analysis, the comparison of gene expressions could be performed in differentially sensitized class IV MD neurons by activating TNF signaling in various ways including UV irradiation and NF-kB activation. Another interesting question remains unclear is if the increased expression of these genes is due to the transcriptional activity of Dorsal. Because binding sequences for Dorsal is characterized (172), a combinational analysis of bioinformatics and biochemical experiments such as binding motif search, chromatin immunoprecipitation with an anti-Dorsal antibody (173) would help to test if certain candidate genes are targeted by Dorsal.

Among up-regulated genes in microarray analysis, interestingly, an epigenetic regulator E(z) appeared as a target gene of TNF signaling during UV-induced nociceptive

sensitization. E(z) is a member of the polycomb group (PcG) proteins and encodes a histone

methyltransferase enzyme that usually modifies H3K27 (174). A well-known function of

PcG proteins is in the maintenance of the identity of a cell by creating repressive chromatin

environment. Thus many PcG proteins are involved in development and carcinogenesis.

However, their roles in nociceptive biology are completely unknown. Therefore, in the future,

it would be important to test if *E(z)* is a target gene of TNF signaling and plays a key role in

nociceptive sensitization in vertebrate system. Interestingly, the human ortholog of *E(z)*,

EZH2 has been implicated as a target of TNF signaling in skeletal myogenesis (175),

indicating that *E(z)* can be modulated by TNF signaling in some context. In addition, the

examination of other members of polycomb group protein would be interesting to find if *E(z)*

has an independent function or has a role within a classically defined complex.

How does epigenetic regulation contribute to nociceptive sensitization? In recent

studies, epigenetic regulations including histone acetylation have been implicated in

nociceptive sensitization. For example, nociceptive sensory neuron specific conditional

knockout mouse lacking histone deacetylase HDAC4 displayed decreased thermal

sensitization while baseline nociception was normal (176). Also, HDAC4 knockout mouse

showed reduced TRPV1 expression, suggesting that the expression of ion channels for

noxious stimuli detection could be regulated by alteration of chromatin environment.

Because E(z) creates a more repressed chromatin environment, the function of E(z) during nociceptive sensitization might be inhibiting expression of negative regulators for sensitization. Identification of mechanisms and target genes regulated by E(z) will likely help to understand unexpected role of TNF signal transduction and relationship between chromatin environment and nociceptive sensitization.

Appendix

Different modes of tissue damage might employee distinct molecular mechanisms to induce nociceptive sensitization. Previous studies in the lab established pinch wound-induced nociceptive sensitization using *Drosophila* larvae. In this model, pinch wound creates a gap by removing epidermal cells as well as damaging underlying nociceptive sensory neurons. Damaged area for pinch wound is much smaller than one by UV irradiation on whole dorsal midline epidermis. In addition, epidermal and neuronal damage by pinching do not cause apoptotic cell death because active caspase-3 staining is absent (Figure A1A). Instead, pinch wounding creates necrotic cell death as the damaged area showed positive trypan blue staining (Figure A1B). Both pinch wound-induced and UV irradiation-induced tissue damages cause thermal allodynia but their onset and duration are different. Pinch wound-induced allodynia response peaks around 4 to 8 hours and decreases around 16 hours after pinching whereas UV-induced allodynia shows a maximum response at 24 hours and disappears 48 hours after irradiation (Figure A1C). To test whether these difference come from employing different signaling pathways to activate nociceptive sensory neurons, we examined signaling pathways identified in UV-induced allodynia, such as Dronc, TNF, and Hh signaling for pinch wound-induced allodynia. Consistent with our hypothesis that

different modes of damage activate distinctive signaling pathways, knockdown of three

signaling pathways did not block pinch
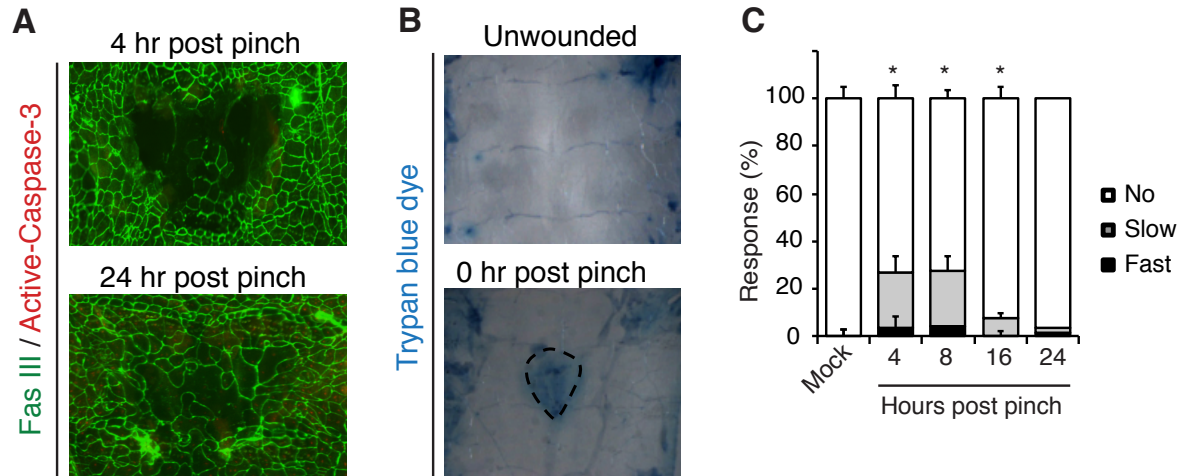
Figure A1. Pinch wound-induced allodynia differs from UV-induced allodynia in

***Drosophila*.**

(A) Epidermal morphology and apoptotic cell death after pinch wound. Anti-Fasciclin-3

antibody (membranes, green) and anti-active caspase 3 antibody (apoptotic cells, red) were

used. (B) Necrotic cell death after pinch wound. Trypan blue dye was used to label necrotic

cells (blue). (C) Quantification of pinch wound-induced thermal allodynia at different time

point after pinch-wound, at 38 °C.

n = 3 sets of 30 larvae. Error bars represent S.E.M. * indicated p value less than 0.05

wound-induced allodynia (Figure A2A and B). In addition, a signaling pathway for wound

repair is not involved in pinch wound-induced allodynia. Inhibition of JNK did not block

both initiation and cessation of pinch wound-induced thermal allodynia (Figure A2C)

although wound is not healed due to lack of JNK signaling (134). Moreover, eliminating

hemocytes did not help to reduce pinch wound-induced allodynia (Figure A2D) while pinch

wound instantly attract them into the wounded area (177), suggesting hemocytes do not

contribute to nociceptive sensitization in this model.
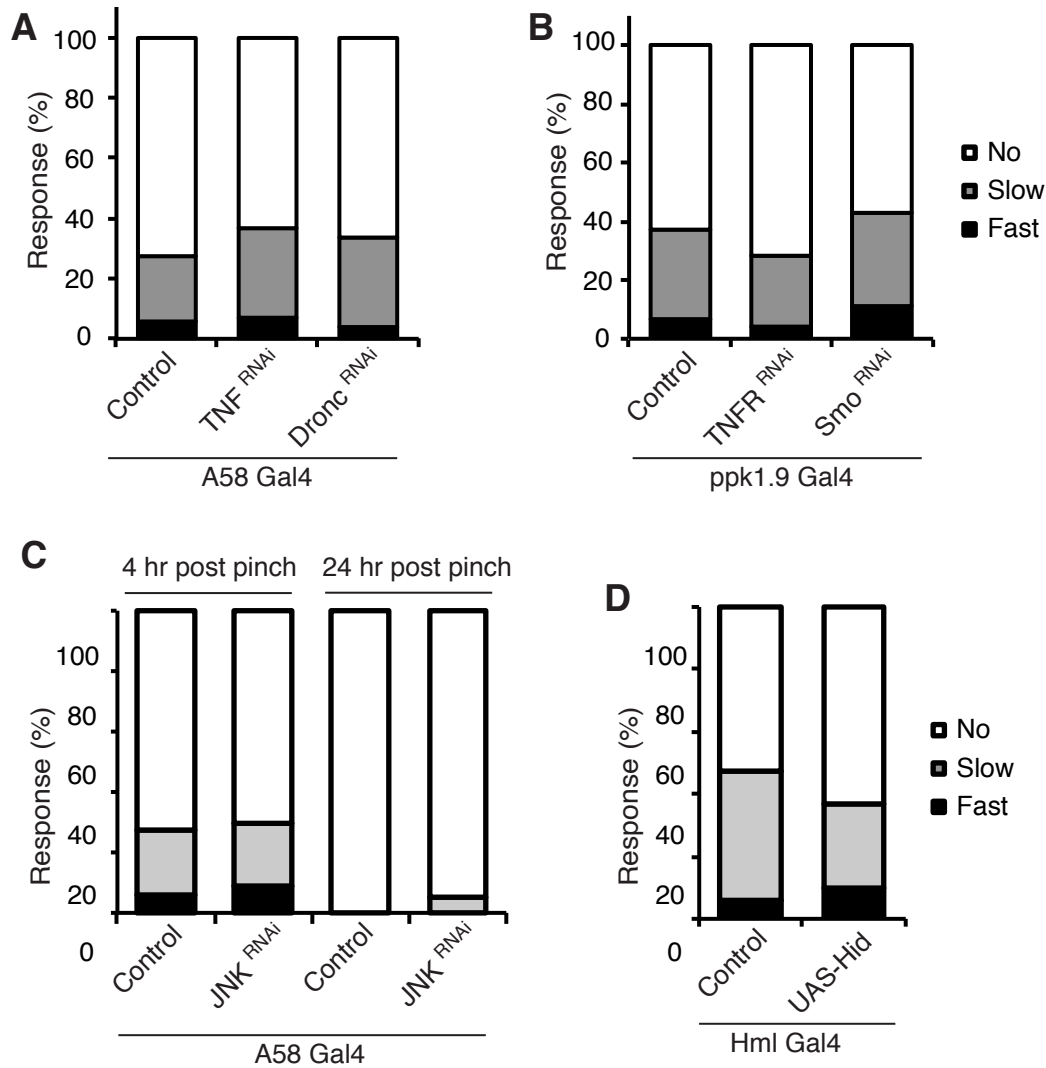
Figure A2. Distinct signaling pathways are required for pinch wound-induced allodynia in *Drosophila*.

(A-D) Quantification of pinch wound-induced thermal allodynia at 38 °C. (A-B) Epidermal or class IV MD neuronal expression of *UAS-RNAi* transgene targeting TNF/Eiger or Dronc were induced using *A58 Gal4* or *ppk1.9 Gal4*. Thermal allodynia was measured 4 hour post pinch-wound. (C) Epidermal expression of *UAS-RNAi* transgene targeting JNK was induced

using *A58 Gal4.* Thermal allodynia was measured 4 or 24 hour post pinch-wound. (D)

Hemocyte were eliminated by overexpression of pro-apoptotic gene Hid using hemocyte

specific *Hml Gal4.* Thermal allodynia was measured 4 hour post pinch-wound.

n $\geq$ 30 larvae.

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