GENETIC ANALYSIS OF NINJURIN A, A STRESS-REGULATED PROTEIN THAT INDUCES NONAPOPTOTIC CELL DEATH

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To Mom, Dad, Mat and Katie

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TABLE OF CONTENTS

Page

DEDICATION……ii
ACKNOWLEDGEMENTS.....iii

LIST OF TABLES.....v

LIST OF FIGURES.....vi

CHAPTER I: INTRODUCTION.....1

Summary 1

Introduction to Ninjurin 1

Overview of the *Drosophila* immune response.....6

Summary of cell stress responses.....13

CHAPTER II: *DROSOPHILA* NINJURIN A INDUCES NONAPOPTOTIC CELL DEATH.....19

Abstract.....19

Introduction.....20

Results.....21

Discussion.....50

Materials and Methods.....54

Acknowledgements.....60

CHAPTER III: NINJURIN A MAY BE REQUIRED FOR UV-INDUCED CELL DEATH: PRELIMINARY STUDIES 61

Introduction.....61

Results.....63

Discussion....71

Materials and Methods....72

CHAPTER IV: CONCLUDING REMARKS....74

Summary....74

Conclusions and Future Directions....80

Significance....83

WORKS CITED....86

APPENDIX I: JNK EXPRESSION IS INDUCED BY COLD SHOCK....94

APPENDIX II:FOXO EXPRESSION IS NOT DEPENDENT ON THE *NIJA*^{D3} CHROMOSOME....95

APPENDIX III: *NIJAβ* OVER-EXPRESSION CAUSES CELL DEATH IN THE EPIDERMIS....97

LIST OF TABLES

Table Page

1.1 *Ninjurin A* is up-regulated in response to stress.....2

2.1 Organismal death resulting from GMR-driven expression of a cell-death gene is not dependent on the gene acting non-autonomously.....34

LIST OF FIGURES

Figure		Page
1.1	Overview of the classical larval immune response mechanisms	12
2.1	Ninjurin A protein response to septic wounding	24
2.2	*NijA*^{D3} mutants do not express mRNA from the *NijA* genomic locus	26
2.S1	*NijA* is not required for survival to immune challenge	29
2.S2	*NijA* is not required for Toll-mediated antimicrobial peptide induction	30
2.S3	*NijA* is not required for phagocytosis of *E. coli*	31
2.S4	*NijA* is not required for resistance to starvation	32
2.3	Ninjurin A over-expression in the lymph gland causes nonapoptotic cell death	38
2.S5	NijA is not required for developmentally programmed cell death in the embryo.	40
2.4	NijA induces cell death in *Drosophila* S2 cell culture	45
2.5	NijA appears to kill in a cell-autonomous manner	46
2.6	*NijA* mutants behave *in vivo* as they do in cell culture	48
2.S6	The secreted ectodomain of *NijA* does not induce cell death	49
3.1	*Ninjurin A* may be required for UV-irradiation induced cell death	65
3.2	*Ninjurin A null* mutants are not resistant to UV-induced DNA damage	66
3.3	*Ninjurin A* is required for H2AV modification after UV-irradiation	68
3.4	Ninjurin A protein increases expression at the cell surface after UV-irradiation	70
A1	JNK expression is induced in the epidermis after cold shock.	94
A2	FOXO expression is not dependent on the *NijA*^{D3} chromosome	95

A3 Over-expression of *NijA*β is sufficient to cause a loss of cell-cell adhesion *in vivo* 97

CHAPTER I

INTRODUCTION

Summary:

Understanding of the complex connections between cellular stress, immunity, and cellular death are essential for treating several diseases. The proper function of these fundamental cellular processes is pivotal in the onset of diseases like cancer and sepsis. The Ninjurin proteins constitute a conserved family of membrane bound proteins that increase expression in response to stress or immune stimuli, which makes them ideal candidates for modulating these cellular processes. Unfortunately the current literature is confusing and lacks a true genetic null model for the function of the Ninjurin family.

Introduction to Ninjurin:

Although the function of Ninjurins is murky, the expression pattern of *Ninjurins* is not. *Ninjurins* are up-regulated after a stress stimulus in both mammalian and *Drosophila* model systems. This is not surprising, Ninjurin proteins were first discovered in rats after nerve injury, and homologous Ninjurin family members were later identified in *Drosophila* [1, 2]. Ninjurins are two-pass transmembrane proteins that are speculated to function in cell adhesion and cell death regulation [1-7]. Cellular stresses like infection, wounding, and X-ray irradiation are reported to increase *Ninjurin* expression [1, 2, 4, 5, 8-14]. Although not discussed in the text of the reports, additional expression data found in the

supplementary materials of studies that conducted microarrays suggest that *Ninjurins* increase expression in response to a broad range of cellular stresses (Table 1.1) [see supplemental data in 15, 16-18]. The conserved expression of *Ninjurins* in response to cellular stresses suggests a conserved function for this family of proteins.

Organism	Stimulus	Fold Change	Reference
Fly	Septic Injury	+12-fold	De Gregario, 2001
Fly	Septic Injury	+2-fold	Vodovar, 2005
Fly/ in vitro	LPS treatment	+3-fold	Boutros, 2002
Fly	Septic Injury	+3-fold	Boutros, 2002
Fly	Insulin (chico) mutant	+>2-fold	Clancy, 2001
Fly	Biotin Deficient Diet	+>5-fold	Smith, 2007
Mouse	Spinal Cord Injury	+6-fold	Di Giovanni, 2005
Mouse	Spinal Cord Injury	+2-fold	Jensen, 2007
Human	X-irradiation	+5-fold	Koike, 2008
Human Cell Culture	Leprosy Infection	+1.5-fold	Cardoso, 2007

Table 1.1 Ninjurin A is up-regulated in response to stress.

There are two reported Ninjurin genes in mammals, *Ninjurin 1* and *Ninjurin 2* [4]. There are three predicted *Drosophila* Ninjurin genes, *Ninjurin A*, *Ninjurin B*, and *Ninjurin C* [2]. The predicted Ninjurin family members have the same conserved topology of a two-pass transmembrane protein and a conserved sequence called the Ninjurin domain [2]. Ninjurins are relatively small proteins of approximately 16-27 kDa. The *Drosophila* NijA protein, the focus of this work, is predicted to have a 166

amino acid N-terminal extra-cellular domain (ectodomain), a 66 amino acid intracellular domain, and a 13 amino acid C-terminal extra-cellular domain (Fig 2.5 C) [2]. Despite the localization of the NijA protein to the cell membrane, there is no predicted signal sequence in the *NijA* gene [2].

The expression patterns of two Ninjurin family members, *Drosophila* NijA and the mammalian Ninjurin 1 proteins, have been examined using antibodies specific to either *Drosophila* NijA or Mammalian Ninjurin 1. NijA is primarily detected in epithelial tissues including the epidermis and trachea of *Drosophila*, and mammalian Ninjurins are detected in nervous tissue and macrophages [1, 2, 7]. In this thesis I will also report that the *Drosophila NijA* is expressed in the hemocytes (blood cells), fat body (similar to the mammalian liver), and lymph gland (a site of larval hematopoiesis).

Although there are no antibodies to the *Drosophila* Ninjurin B and Ninjurin C proteins, whole genome assays for mRNA expression have provided information about the mRNA expression of these genes. The *NijA* gene is expressed in response to immune stimuli and starvation [19]. The expression of the *NijA* and *NijC* mRNA peaks during the prepupa and metamorphosis stages [19]. The tissue with the highest *NijA* expression is the larval carcass, whereas the tissue with the highest *NijC* expression is the larval midgut [19]. *NijB* mRNA is detected at all developmental time points at very low levels, and does not change expression in response to stress stimuli [19]. There are no reported functional studies of *NijB* or *NijC*. Despite their homology, the *Drosophila Ninjurins A*, *B*, and *C*, have distinct

expression patterns that suggests these family members may not have redundant functions.

There are three predicted splice forms for the *Drosophila NijA* gene: α (245 amino acids), β (229 amino acids), and γ (241 amino acids). The differences between the splice forms are 12 amino acids, and these differences are in the N-terminal ectodomain of the protein. The α and β splice forms are predicted, whereas the *NijA γ* splice form sequence was previously determined from the sequence of a EST isolated from a *Drosophila* cDNA library [20]. There are no reported functional differences between the splice forms of *NijA*.

While there is striking trend of Ninjurin expression after cellular stress there are limited functional studies on Ninjurins, and those studies report conflicting results. The first study investigating the function of Ninjurins was done by over-expressing the mammalian *Ninjurin 1* gene in a cell culture model. *Ninjurin 1* over-expression resulted in cell aggregation. This study examined the role of Ninjurin 1 in inducing cell aggregation further by plating cells over-expressing Ninjurin 1 with wild-type cells. The results of this experiment showed that only cells over-expressing Ninjurin 1 could aggregate with cells that also over-expressed Ninjurin 1. The authors of this work concluded that Ninjurin 1 directly promotes cell adhesion through homophillic binding of the Ninjurin 1 ectodomain [1, 5]. Another study over-expressing the *Drosophila Ninjurin A* (*NijA*) in S2 cell culture observed a loss of cell adhesion, which conflicts with the previously published results [2]. Although both studies suggest that Ninjurins regulate adhesion, they indicate disparate roles for the Ninjurin proteins.

The results indicating that Ninjurin 1 is directly forming homophillic attachments between two cells is difficult to conceive when you consider the scale of extracellular space. The *Drosophila* Ninjurin A (NijA) has a 160 amino acid N-terminal extra-cellular ectodomain. This is a relatively small extracellular domain in comparison to other know transmembrane proteins that regulate adhesion by homophillic binding like cadherins, which have extracellular domains of more than 1000 amino acids [21]. Despite the reports of homophilic cell aggregation when *Ninjurin 1* is over-expressed, there is no experimental evidence demonstrating a direct interaction between Ninjurin ectodomains. The relatively small size of the Ninjurin protein ectodomain compared to known adhesion modulators raises the possibility that Ninjurins may indirectly mediate cell adhesion.

The studies reporting the over-expression of Ninjurin family members are plagued by conflicting results, and could yield novel phenotypes unrelated to the native biological function of Ninjurin caused by the robust and non-biological expression levels and patterns of the protein. Phenotypes from genetically null animals would not be prone to the same interpretation concerns as phenotypes observed from over-expression experiments, and may clarify the existing conflicts in the literature. Unfortunately there are no reported genetic nulls in any model system. There are loss-of-function studies that use a neutralizing antibody to inhibit the function of the mammalian Ninjurin 1 protein in lieu of a genetic null. Unfortunately, in the absence of a loss-of-function model the authors were unable to directly assess the ability of this antibody to inhibit Ninjurin 1 function. Studies using this untested neutralizing antibody reported that Fibroblast Like Cells (FLC)

cultured in the presence of the neutralizing Ninjurin 1 antibody were unable to non-cell autonomously promote the survival of co-cultured neurons [22]. This study suggests that Ninjurin 1 functions non-autonomously to promote cell survival. In contrast, the application of the Ninjurin 1 neutralizing antibody to developing ocular tissue of rats prevents cell death-mediated vascular regression [7]. This work suggests that Ninjurin 1 functions to promote cell death, since in the absence of Ninjurin 1 the vasculature is unable to undergo cell death. Again both studies suggest that Ninjurin 1 is regulating cell death, but suggest disparate functions for the Ninjurin 1 protein. The current literature suggests that Ninjurins regulate adhesion and/or cell death and that Ninjurins either promote or inhibit these cellular processes in a context dependent manner.

The conserved expression patterns of Ninjurin proteins from mammals to *Drosophila* suggest a conserved function for the Ninjurin family of proteins, and suggest that *Drosophila* maybe a viable model system to investigate the function of Ninjurin proteins. Because the current literature lacks a genetic null mutant to examine the function of Ninjurins after a stress stimulus we used a novel *Drosophila NijA null* mutant to examine NijA function. We focused the investigation of NijA function on the conserved *Drosophila* innate immune response, which is a time when *Ninjurins* are dramatically up-regulated in both mammals and *Drosophila*.

Overview of the Drosophila immune response:

The *Drosophila* immune system is an excellent model to study the analogous mammalian innate immune response [23]. The innate immune response is

sometimes referred to as the "fast" or "primary" immune response because in mammals it is the only immune response for the first four days after immune challenge [24]. The innate immune response is a nonspecific immune response that promiscuously attenuates pathogens. After the first four days of infection the acquired immune system responds with the production of antibodies that provide specificity and memory against pathogens to which the organism has been previously exposed. Although a recent study reports that some pathogens can illicit specificity and memory from the *Drosophila* innate immune system this response does not result in the production of antibodies [25]. *Drosophila* do not have an acquired immune response. Despite this limitation, the *Drosophila* immune response has been a tremendously powerful tool to model the mammalian innate immune response.

The *Drosophila* immune response is often segregated into the cellular and humoral immune responses, although these systems have significant cross-talk to coordinate a successful immune response. A major component of the cellular immune response are the hemocytes, which are migratory cells that respond to immune challenges and clear cellular debris (Fig 1.1). There are at least three distinct classes of hemocytes that respond to immune challenge [23, 26, 27]. The first and most abundant population is the plasmatocytes, which encompass 95% of the hemocytes in third instar larvae. Plasmatocytes are circulating surveillance cells that function to clear pathogens and cell corpses by phagocytosis in a manner similar to the mammalian macrophage [28]. The second class of hemocytes are the crystal cells, which are small round cells that undergo an antimicrobial process

called melanization (Fig 1.1). Crystal cells are less than 5% of the hemocytes found after infection, and are not detectable prior to immune challenge [26, 29]. The activation of crystal cell melanization is considered analogous to the complement system in the mammalian immune response [23]. The third class of hemocytes are the lamellocytes, which are large thin cells that encapsulate parasitic wasp eggs in an integrin dependent manner (Fig 1.1) [29-32]. One report suggests that the lamellocytes closest to the surface of the wasp egg undergo a necrotic cell death, although the immunological benefits are unknown [32]. Lamellocytes constitute fewer than 5% of the hemocytes found in the hemolymph after an immune stimulus, and are not found prior to immune challenge. The sub-populations of hemocytes are specifically differentiated in response to specific needs from the hematopoietic organ, the lymph gland, shortly after immune challenge (Fig 1.1) [26, 33, 34]. The lymph gland is found only in the larval life cycle and bursts just prior to pupariation. The proliferation and differentiation of hemocytes in the lymph gland is modulated by the JAK/STAT pathway [35, 36].

Hemocytes have at least three reported sites of differentiation. The first site of differentiation is in the head mesoderm of the developing *Drosophila* embryo. The embryonic hemocytes are not eliminated by histolysis during metamorphosis, and are persistent into adulthood [37]. The second site of hemocyte differentiation is from the cardiac mesoderm, which contributes to the formation of the primary hematopoietic organ, the lymph gland [37]. The third site of hematopoiesis is a population of hemocytes found in the extreme posterior segments of third instar larvae, and their embryonic origin is unknown [38]. When a third instar larva is

wounded, undifferentiated pro-hemocytes in the posterior of the animal and the lymph gland differentiate. The differentiated hemocytes are then released into circulation to attenuate the immune challenge. The tissue of the lymph gland has three distinct populations of cells, the self-renewing cells of the posterior signaling center, undifferentiated pro-hemocytes of the medulluary zone, and the differentiated hemocytes found in the cortical zone [39, 40]. When the larvae approaches metamorphosis the lymph gland receives developmental cues to burst and release the hemocytes into the larva [41]. The larval hemocytes, like the embryonic hemocytes, are persistent into adulthood. Although extensive research into the signaling pathways that regulate hemocyte differentiation and development are ongoing, there has been little or no research into hemocyte programmed cell death after immune challenge or programmed cell death in the lymph gland during hematopoiesis [42].

Another major immune response mechanism is the humoral response, which can regulate the production of anti-microbial peptides (AMPs) in response to an immune stimulus among other antimicrobial agents like reactive oxygen species. Mammals have a analogous system to also produce AMPs. AMPs are small peptides that are released into the hemolymph where they can attenuate infections (Fig 1.1) [23]. AMPs are produced primarily by the fat body, hemocytes, and barrier epithelia. The Imd and Toll pathways are pathogen recognition-initiated pathways that can mediate AMP production. The Imd pathway is activated when the peptidoglycan-recognition protein-LC (PGRP-LC) comes in contact with the peptidoglycans on the surface of bacteria [23, 43, 44]. Once stimulated, the transmembrane PGRP protein

binds to the transmembrane Imd protein to initiate intracellular signal transduction that results in the translocation of the transcription factor Relish to the nucleus. Once in the nucleus, Relish promotes transcription of AMPs like Diptericin [45-49]. The other pathogen recognition-initiated pathway, Toll, is initiated when the extracellular PGRP-SA initiates proteolytic cascades that result in the cleavage of the Spaetzle ligand [50-52]. Cleaved Spaetzle binds to the transmembrane Toll receptor initiating intracellular signal transduction that results in the translocation of the redundant Dif and Dorsal transcription factors to the nucleus. Dif and Dorsal can promote transcription of AMPs like Drosomycin and Drosocin [51, 53, 54]. The Dif, Dorsal, and Relish transcription factors are collectively known as the rel factors, and are homologous to the NF-kB family of mammalian transcription factors [55-59]. Recent studies suggest that stress regulation pathways like the FOXO pathway can also modulate the production of Drosomycin [60].

Two major immune-response cell types conduct the majority of Imd and Toll signaling: the hemocytes (blood cells) and fat body cells (fatty tissue similar to the mammalian liver) [23, 28, 30, 61-64]. The fat body is a very large heterogeneous tissue that produces the majority of the AMPs found in the larvae after infection [65]. Since AMPs are small peptides found predominantly in the hemolymph of the larvae, they are difficult to measure directly. There are two accepted ways to assess AMP production in a *Drosophila* larva after immune challenge. One is with the use of AMP reporters made by engineering the promoter region up-stream of the AMPs of interest to either a LacZ gene or a GFP gene [66]. After immune challenge the amount of the reporter expression is assessed. The second way to assess AMP

expression is by measuring the mRNA expression of the AMPs by qPCR or Northern blot analysis [67].

The stress of infection can induce *Ninjurin A* expression, the differentiation of pro-hemocytes, the release of hemocytes into circulation, and the production of AMPs to attenuate the infection among many other biological processes. Despite the ongoing research into the *Drosophila* immune system activation, there is limited research on the recovery of the *Drosophila* immune system after an immune challenge has been resolved.

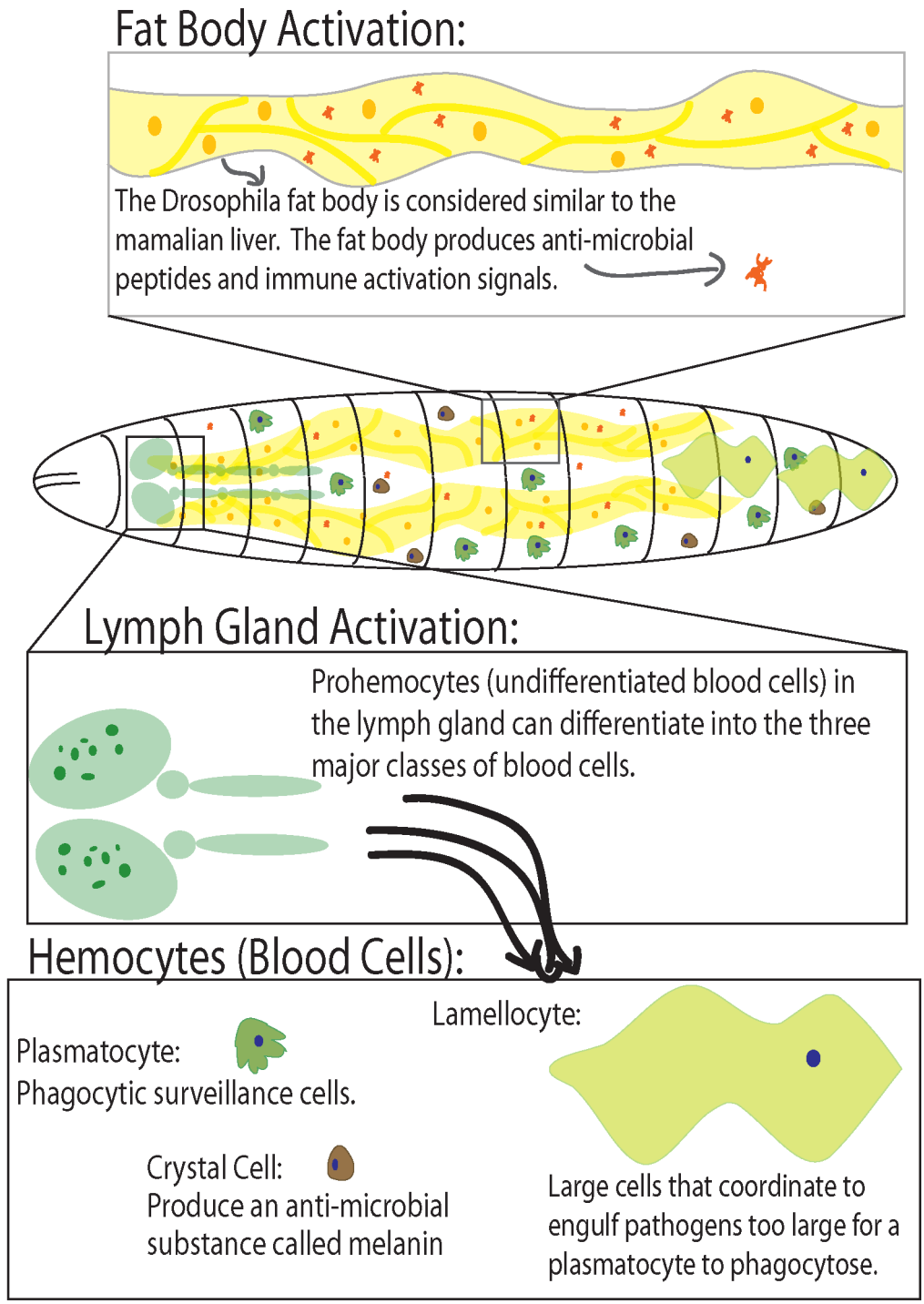


Figure 1.1 Overview of the classical larval immune response mechanisms.

Summary of cell stress responses:

Immune challenge is just one kind of cellular stress. There are many ways to disrupt the homeostasis of a cell, which can cause tissue and organismal responses. Reponses to these cell stresses are essential for organismal survival. In some cases the damage caused by cellular stress can be too severe for the cell to recover from, which results in a programmed cell death event [68]. I report in this thesis that the over-expression of *NijA* is sufficient to induce programmed cell death *in vivo*. The increase of NijA expression at the cell surface after cellular stress, and the cell death induced by the over-expression of *NijA* suggest that NijA may modulate the cell stress response. While there are many different mechanisms of cell death, the persistence of a damaged cell can be deleterious to the function of the tissue. The processes that control the survival and death of a cell are complex and biomedically important for treating autoimmune disorders and cancers [68-71].

DNA damage by ultraviolet-irradiation (UV-irradiation) is a robust cellular stress that is associated with skin cancers, and severe DNA damage from UV-irradiation can promote programmed cell death. Recently, a method of UV-irradiation on *Drosophila* third instar larva has been established to assess the nociceptive response [72]. This stress involves exposing *Drosophila* larvae to UV-irradiation using an UV Stratalinker to induce DNA damage and programmed cell death in the dorsal epidermis of larvae [72, 73]. Other researchers have used UV-irradiation to induce programmed cell death in the *Drosophila* eye to investigate cell death in the non-essential eye tissue [73].

Although there are few studies using UV-induced cell death in *Drosophila*, UV-induced DNA damage is a well-established area of research. The sun produces UV-irradiation wavelengths in the UVA (400-315 nm), UVB (315-280 nm), and UVC (280-100 nm) range, although the ozone blocks the UVC wavelengths, which prevents UVC radiation from reaching the earth's surface [74]. UVA and UVB-irradiation result in DNA damage by causing thymidine dimers in the DNA strands and reactive oxygen species, which cause double-stranded breaks in the DNA helix. In contrast, UVC irradiation causes only the formation of thymidine dimers, but not double-stranded breaks from reactive oxygen species [74]. The formation of thymidine dimers in the DNA are recognized and repaired by the DNA repair machinery. If the repair process is unsuccessful the cell is programmed to undergo cell death. Using a UV Stratalinker, the dose of UV-irradiation can be titrated to cause sufficient damage to force the exposed cells to undergo programmed cell death [72].

Prior to cell death there are established biological hallmarks of a cell detecting DNA damage and initiating programmed cell death. DNA damage recognition involves modification of the H2AV histones, the homologs of the mammalian H2AX histones, to γH2AV at the sites of DNA damage [75]. Some cell death programs are mediated by the activation of caspase proteins that promote the formation of the apoptosome that ultimately results in DNA and organelle degradation [76]. During the process of DNA degradation the chromatin condenses to form a small dense pyknotic nucleus. Most cells undergoing programmed cell death loose adhesion to surrounding cells and the extracellular matrix. Ultimately

the cell membrane of the cell undergoing programmed cell death becomes permeable.

Although there are many biological processes that result in cell death (anoikis, NETosis, etc), there are three major mechanisms that control cell death, apoptosis, autophagy, and necroptosis. Apoptosis is the classic programmed cell death that is dependent on caspases, does not initiate inflammation, and involves organized DNA degradation/fragmentation. Apoptosis is considered an organized cell death, and the cell corpse is cleared by either neighbor cells or a macrophage [77]. Autophagy, a process in which the cell digests its own components, can regulate a variety of cellular processes including viral clearance and signal transduction regulating metabolic processes [78, 79]. Autophagy can promote both cell survival and cell death in response to stress [80]. The mechanism of autophagy-dependent cell death utilizes components of the apoptotic machinery [81]. Autophagic cell death is dependent on the Autophagy Related (Atg) family of proteins that are required for autophagosome formation, does not always require caspase activation, and can sometimes have DNA degradation. The unique hallmarks distinguishing the autophagic and apoptotic cell death pathways are still being defined. Previous reports suggest that autophagic and apoptotic cell death pathways have redundant components and compensate in the absence of the other, which indicates that there are intricate control mechanisms regulating programmed cell death [82, 83].

The most recently described cell death mechanism is necroptosis, and is distinct from autophagy and apoptosis by the inflammatory response it elicits.

Necrotic cell death was not previously considered a type of programmed cell death, and instead it was considered a biophysical response to cell shearing or cell swelling. Necrotic cell death was recently reported as dependent on the post-translational modification of the Receptor Interacting (RIP) proteins in mammalian cell culture, which suggests that necrotic cell death is the result of a programmed cell death pathway [84]. Necroptosis involves the bursting of a cell, which results in inflammation. When apoptosis is inhibited then the necroptosis pathway is initiated to compensate suggesting that these pathways inhibit one another [85]. There is an extensive redundancy between the apoptotic signal transduction pathways and those for necroptosis. There has been limited research on necroptosis in *Drosophila*, and it is unclear if *Drosophila* cells have a homologous pathway to initiate necroptosis, although it can be induced genetically [86, 87]. Some studies suggest that the *Drosophila* Imd protein is analogous to the mammalian RIP family of proteins required for necroptotic cell death in mammals; however, the Imd proteins do not have the kinase function of the mammalian RIP proteins, and there are no reports investigating the role of Imd in necroptotic cell death [23].

Necrotic cell death has been observed in *Drosophila* as a cellular response to immune challenge [32]. The crystal cells and a few lamellocytes undergo an antimicrobial process known as melanization [32]. In crystal cells melanization is stimulated by inflammatory signals. Crystal cells then burst to release the contents of the cell, which could further contribute to inflammation [27]. The lamellocytes, as previously discussed, are required to encapsulate wasp eggs [32]. As the lamellocytes encapsulate the wasp egg the cells closest to the egg die by necrosis as

new cells are layered on top [32]. Although it is unclear why this would be beneficial, it is possible that lamellocyte necrosis is increasing inflammation by releasing cytoplasmic contents when the lamellocyte ruptures. It is unknown if these necrotic cell deaths in the *Drosophila* immune system are the result of necroptosis programmed cell death.

In conclusion, Ninjurin proteins across species are up-regulated after a stress stimulus at a time when cell death is regulated. There are several mechanisms for cell death; however, they function redundantly and compensate for the absence of one another. The *Drosophila* and mammalian Ninjurins are highly up-regulated in response to the cellular stress of infection. The *Drosophila* immune response is analogous to the mammalian innate immune system, and *Drosophila* does not have the secondary acquired immune response found in mammals. There has been extensive research into the activation of the *Drosophila* immune system, but limited studies on the recovery of the immune system during immune challenge resolution.

In this document, I will report that *NijA* is up-regulated in adult flies after immune challenge, and increases expression at the cell surface of the larval immune response tissues, the fat body and hemocytes. Activation of the Toll immune response pathway is sufficient to induce an increase in *NijA* expression in larvae. *NijA* null mutants are viable, morphologically wild type, and do not have immune response defects. *NijA* over-expression in differentiated hemocytes induces a non-apoptotic cell death, and preliminary results may suggest that *NijA* null mutants are unable to induce cell death after UV-irradiation to the same extent as wild-type larvae. The investigation of *NijA*-regulated cell death contributes to the

understanding of stress-induced cell death regulation. The Ninjurin family of proteins increases expression at the cell surface in response to stress stimuli making it a good candidate for modulating stress response.

CHAPTER II

Drosophila Ninjurin A induces nonapoptotic cell death

This chapter is published and was authored by the following people [6]:

Sarah Broderick, Xiaoxi Wang, Nicholas Simms, Andrea Page-McCaw

Abstract:

Ninjurins are conserved transmembrane proteins that are upregulated across species in response to injury and stress. Their biological functions are not understood, in part because there have been few *in vivo* studies of their function. We analyzed the expression and function of one of three *Drosophila* Ninjurins, NijA. We found that NijA protein is redistributed to the cell surface in larval immune tissues after septic injury and is upregulated by the Toll pathway. We generated a null mutant of *NijA*, which displayed no detectable phenotype. In ectopic expression studies, NijA induced cell death, as evidenced by cell loss and acridine orange staining. These dying cells did not display hallmarks of apoptotic cells including TUNEL staining and inhibition by p35, indicating that NijA induced nonapoptotic cell death. In cell culture, NijA also induced cell death, which appeared to be cell autonomous. These *in vivo* studies identify a new role for the Ninjurin family in inducing nonapoptotic cell death.

Introduction:

Ninjurins are a conserved family of transmembrane proteins first identified by upregulation in injured rat nerves [1]. There are two Ninjurin family members in mammals, Ninjurin1 and Ninjurin2 [4], and three in *Drosophila*, Ninjurin A, B, and C [2]. Ninjurins are small proteins of ~16-27 kDa, with an N-terminal ectodomain and two predicted transmembrane domains near the C-terminal end. In humans, mice, and *Drosophila*, Ninjurin transcripts are upregulated upon injury, infection, or stress suggesting that not just their structure but also their function is conserved [1, 10, 12-14, 88, 89] [see supplemental data in 15, 18, 90].

Although little is known about the functions of Ninjurins, many studies have implicated them as adhesion molecules, either directly through homophilic binding on the cell surface [see for example 1, 3, 5, 91] or by regulating adhesion via their ectodomain [2]; yet these adhesion studies have been limited to cell culture models. *In vivo*, some data suggest that Ninjurin1 may promote hyaloid vasculature regression in mouse embryos, as neutralizing antibodies against Ninjurin1 delay this regression, although the relationship between Ninjurin1 and cell death *in vivo* is unclear from these studies [91]. To our knowledge, no Ninjurin mutants or knock-outs have been reported in any organism.

In this study, we show that Ninjurin A (NijA) protein responds to septic injury in a developmentally regulated manner, as whole-animal levels increase in adults but not in larvae. Rather, in larvae the protein distribution is altered in immune tissues after injury, and NijA protein levels can be elevated via the Tl immune signaling pathway, suggesting that NijA may function in the immune

system. We generated several deletion mutants of *NijA* including a molecular null allele but no phenotype was observed in these animals. In a gain-of-function approach, however, we found that *NijA* induced cell death at a level comparable to the known apoptotic gene *hid*, yet the NijA-induced death does not have the hallmarks of apoptosis. From cell culture studies, we conclude that NijA is likely to induce cell death in a cell-autonomous manner, rather than as a nonautonomous signaling molecule.

Results:

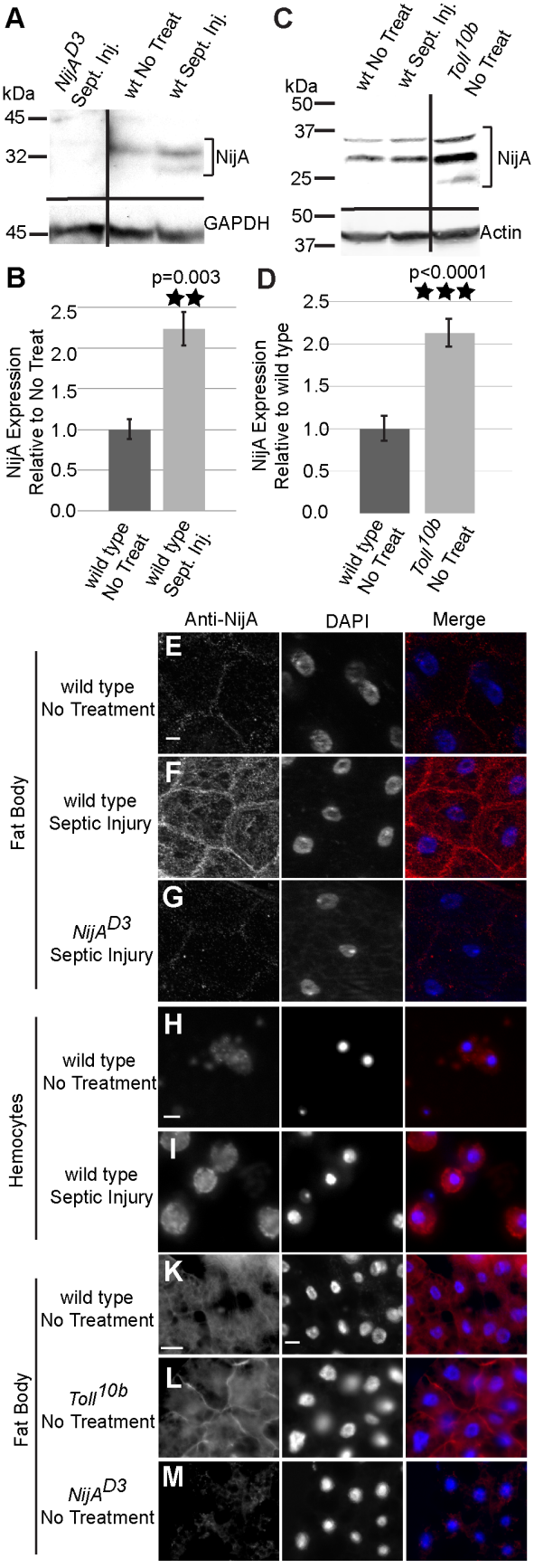
NijA distribution is regulated in larval immune tissues

Ninjurin A (NijA) is one of three *Drosophila* Ninjurin family members, and genome-wide analyses have indicated that its transcript is upregulated between 3-12 fold upon septic injury in adults or immune challenge in cultured cells [13, 15, 90]. Using a polyclonal antibody we made to the N-terminal peptide of NijA [2], we determined by western blotting that the protein levels in whole adults increase 2h after septic injury by about two-fold, verifying the microarray studies (Fig. 2.1A,B). In contrast, in larvae treated with septic injuries, we did not observe an increase in NijA protein in lysates from whole animals in each of six replicates (Fig. 2.1C, left lanes). Because western blots of whole larvae might obscure changes in tissue-specific expression or protein localization, we compared NijA protein by immunohistochemistry in tissues from untreated larvae or larvae 2h after septic wounding. We examined three candidate larval tissues that respond to septic wounding: fat body, hemocytes (immune cells of the blood), and epidermal wound

sites. There was no change in NijA at the site of injury at the wound site (data not shown). Fat bodies are known to be heterogeneous across the tissue [65], so we reduced the variability by examining only the cells surrounding the testis; in this area NijA protein distribution was clearly altered after septic wounding in 4/4 fat bodies compared to 6 unwounded (Fig. 2.1E-G; p=0.0048, Fisher's exact test). Blood cells were examined *ex vivo*, and NijA staining was altered in 9/9 samples of blood cells after septic wounding compared to 9 unwounded (Fig. 2.1H,I; $p=4.1x10^{-5}$, Fisher's exact test). In both fat and blood cells, there was a marked increase in NijA localized to the cell surface, easily observed in unpermeabilized tissue since our antibody recognizes an extracellular epitope of the NijA transmembrane protein [2]. Thus NijA protein responds to septic injury in both adults and larvae.

Since fat body and blood cells are both *Drosophila* immune organs [23], we asked whether the immune regulator Tl was capable of regulating NijA [51]. We found that whole larvae with the constitutively active Tl^{10b} mutation have higher levels of NijA protein, even in the absence of injury (Fig. 2.1C, D). Anti-NijA immunostaining of the fat body indicated that NijA levels were increased in this tissue in 9/9 Tl^{10b} mutants compared to wild type (Fig. 2.1K-M; $p=4.1x10^{-5}$, Fisher's exact test), and this Tl-mediated upregulation appears to increase NijA levels at the cell surface. The sufficiency of Tl to upregulate *NijA* in larvae is consistent with the microarray findings of De Gregorio *et al* that *spz* flies, which cannot activate the Tl pathway, also cannot upregulate *NijA*; in contrast, larvae mutant for the Imd pathway were able to upregulate *NijA* like wild type [90]. The regulation of *NijA* by the *Tl* pathway, combined with its relocalization after septic injury in the immune

tissues of the blood and fat body, suggest that NijA functions in the immune system of larvae.


A
NijA^D3 Sept. Inj.
wt No Treat
wt Sept. Inj.
kDa
45
32
NijA
45
GAPDH
C
wt No Treat
wt Sept. Inj.
Toll^10b No Treat
kDa
50
37
25
NijA
50
37
Actin
B
p=0.003
NijA Expression Relative to No Treat
2.5
2.0
1.5
1.0
0.5
0.0
wild type No Treat
wild type Sept. Inj.
D
p<0.0001
NijA Expression Relative to wild type
2.5
2.0
1.5
1.0
0.5
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wild type No Treat
Toll^10b No Treat
Anti-NijA
DAPI
Merge
Fat Body
wild type No Treatment
E
wild type Septic Injury
F
NijA^D3 Septic Injury
G
Hemocytes
wild type No Treatment
H
wild type Septic Injury
I
Fat Body
wild type No Treatment
K
Toll^10b No Treatment
L
NijA^D3 No Treatment
M

Figure 2.1. Ninjurin A protein response to septic wounding.
(**A)** Western blot of whole adult male lysates probed with anti-NijA. NijA increases expression two hours after infection in adults. *NijA*^{D3} null lysates demonstrate antibody specificity. Black lines indicate regions of the blot that were omitted for clarity. **(B)** Graph representing three replicates of the western blot pictured in (A). NijA levels increase significantly in adults after septic injury (p=0.003). **(C)** Western blot of whole male larval lysates probed with anti-NijA. NijA levels do not change 2h after septic injury in third instar larvae; in contrast larval *Toll*^{10b} gain-of-function mutant larvae have increased levels of NijA protein. **(D)** Graph representing five replicates of the western blot pictured in (C). NijA levels increase significantly in constitutively activate *Toll*^{10b} mutant larvae (p<0.0001). **(E-M)** Anti-NijA (red) and DAPI (blue) labeling nuclei. All scale bars are 10μm. **(E-G)** Anti-NijA stained non-permeabilized fat bodies of male third instar larvae show an increase in NijA at the cell surface 2h after septic injury (compare E and F). **(G)** *NijA*^{D3} larvae demonstrate the NijA antibody specificity. **(H,I)** Anti-NijA stained non-permeabilized hemocytes of third instar larvae *ex-vivo* show an increase in NijA at the cell surface 2h after septic injury. **(K-M)** Anti-NijA stained permeabilized fat bodies of male third instar larvae show increased NijA expression in gain-of-function *Toll*^{10b} mutants. Error bars in (B,D) represent standard error of the mean.

NijA is not required for viability

To understand the functional requirements for *NijA*, we made a deletion mutant by excising a *P* element at the genomic locus. Three imprecise excisions were generated that removed part of the *NijA* coding sequence: *D3, E1,* and *F9* (Fig. 2.2A). The *D3* allele removed the 5' UTR and most of the coding region including the last internal methionine, suggesting that *D3* may be a null allele. To determine whether there was internal translation of the 3' remnant of the gene in the *D3* allele, we performed quantitative PCR on the fourth exon, present in the *D3* allele, comparing its transcription level to the third exon, deleted from the *D3* allele and acting as a negative control. We found no transcription of either the third or fourth exon, confirming that the *D3* allele is a null (Fig. 2.2B). $NijA^{D3}$ homozygous mutants were viable and fertile with no obvious developmental abnormalities (data not shown). Thus *NijA* is not required for viability.

To examine the role of *NijA* in the immune system, we tested viability of $NijA^{D3}$ homozygous mutants after wounding or infection with gram positive or negative bacteria, but found no differences in survival or melanization (Fig. 2.S1 and data not shown). The ability of $NijA^{D3}$ mutants to mount an antimicrobial peptide response after septic injury was examined by measuring Drosomycin (Drs) or Drosocin (Dro), both targets of the Tl pathway [51]. Both antimicrobial peptides were elevated in the $NijA^{D3}$ mutant in a manner not significantly different from wild type (Fig. 2.S2A,B). To further assess a potential role for *NijA* in Tl signaling, we performed an epistasis test to ask whether *NijA* is required for the upregulation of a Tl pathway target when *Tl* is genetically activated by mutation in the fat body (by

driving *Tl*^{10b} with the *c564-GAL4* driver). Examining the levels of Drs, we found that Drs elevation was similar after septic wounding of wild type or genetic activation of *Tl*^{10b} in the fat body, and these levels were not affected by the *NijA*^{D3} mutation in *Tl*^{10b} *NijA* double mutants (Fig. 2.S2C). To examine the cellular immune response we assayed phagocytosis and found that the capacity of hemocytes or S2 cells to phagocytose labeled *E. coli* did not depend on *NijA,* although hemocyte phagocytosis was sensitive to a dominant locus on the *NijA*^{D3} chromosome (Fig. 2.S3). Similarly, a dominant locus on the *NijA*^{D3} chromosome obscured our ability to assess a homozygous mutant phenotype in response to starvation (Fig. 2.S4). Thus we were unable to identify a function for *NijA* using loss-of-function approaches.

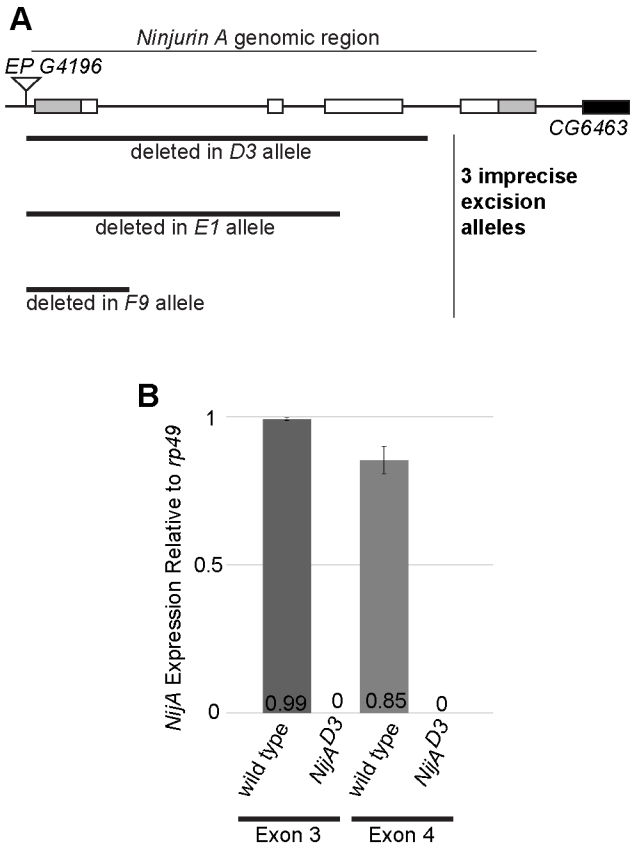
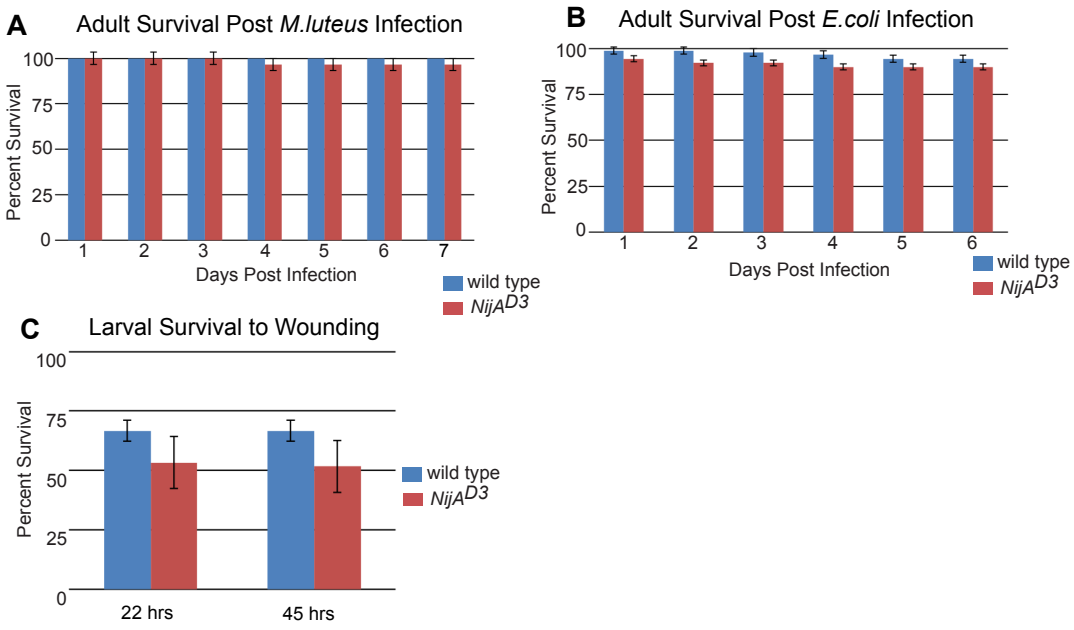


Figure 2.2. *NijA^{D3}* mutants do not express mRNA from the *NijA* genomic locus. **(A)** Schematic of the *NijA* locus showing all four exons. Gray indicates untranslated regions and white indicates open reading frame. Three excision alleles (*D3, E1,* and *F9*) were generated from imprecise excisions of *EP G4196*. **(B)** qPCR data from primers specific to exon 3 (a negative control, as it is deleted in the *D3* allele) or exon 4 of *NijA*. The *NijA^{D3}* mutant did not produce any detectable mRNA from exon 4 of the *NijA* locus, even though exon 4 remains in the genome, indicating that the *D3* allele is a null. Error bars represent standard error of the mean.



Supporting Information S1. *NijA* is not required for survival to immune challenge. Survival of adult males injected with either *M. luteus* **(A)** or *E. coli* **(B)**. *NijA* was not required in adults for survival to *M. luteus* or *E. coli*. **(C)** Survival of third instar larvae wounded with a non-sterile fine needle. *NijA* was not required in larvae to survive wounding, as $NijA^{D3}$ mutants were not significantly different from wild type in their ability to survive wounding (Student's T test). Error bars represent standard error of the mean.

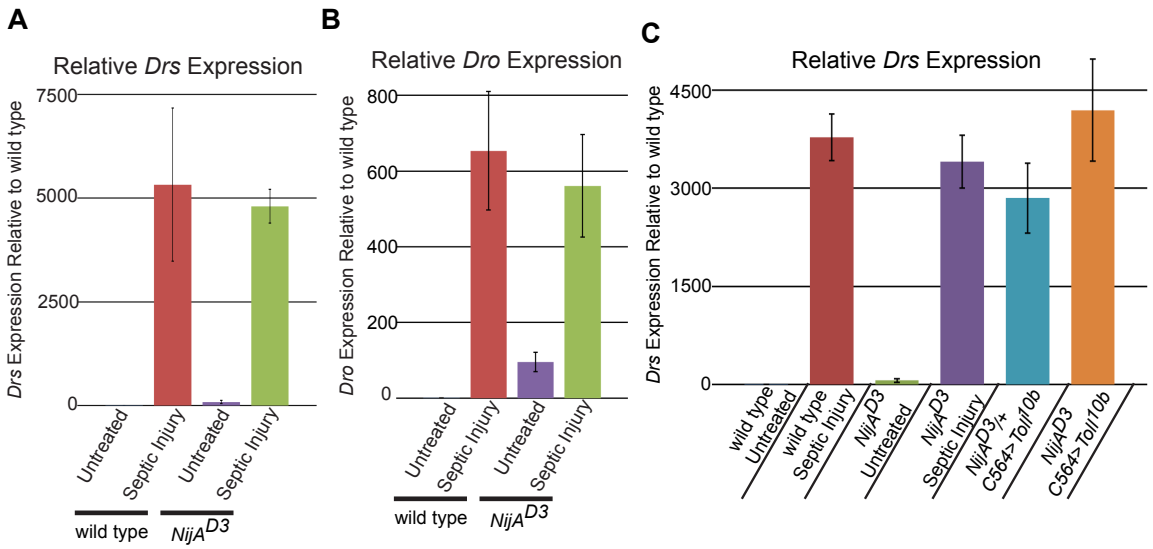
Methods:

Adult Infection:

Adult males were collected from a healthy bottle 24h after clearing, aged two days in a 25°C incubator, and injected with 69nl of a log-phase growth culture of either *M. luteus* or *E. coli* using a Nanoject apparatus (Drummond) into the lateral side of the abdomen just below the halteres. 10 animals were placed in a vial containing 10ml of standard molasses food and allowed to recover at 25°C in a humidified incubator. An adult was scored as dead if it was not standing up, and vials were scored every 24h. All adults survived the first 5h. For *M. luteus* three replicates were performed with 20 animals each. For *E. coli* three replicates were performed of 30 animals each.

Larval Wounding:

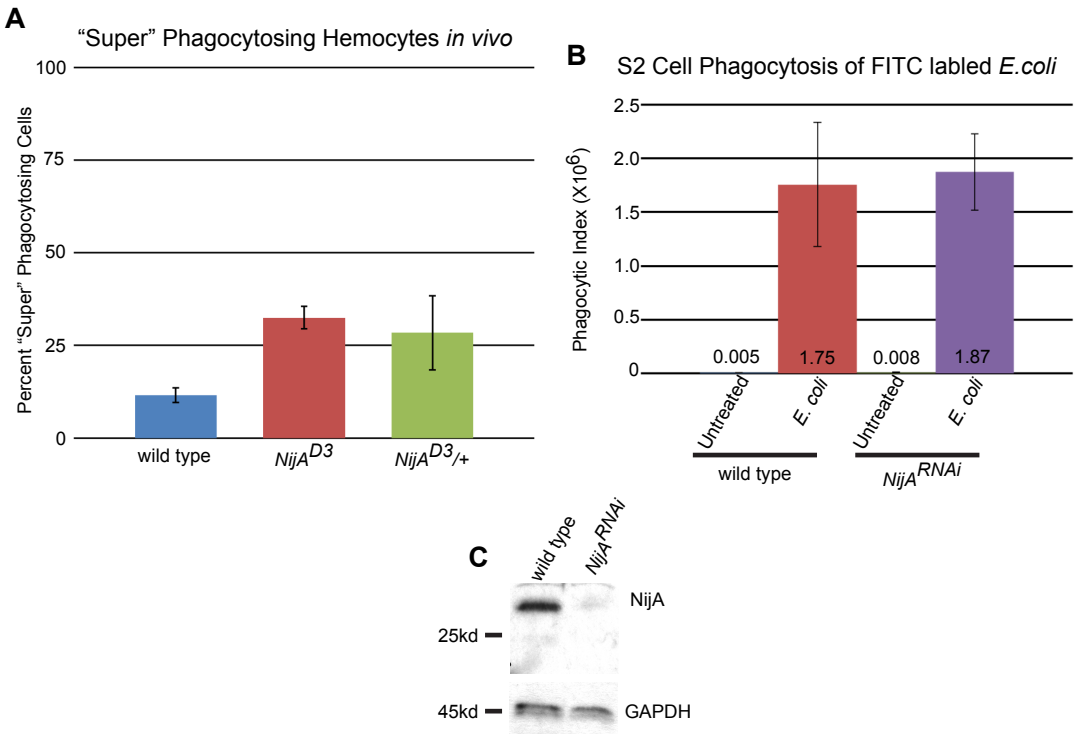
Third instar larvae were collected from the food of a healthy bottle and impaled with a fine needle (Fine Science Tools) in the posterior third of the animal near the lateral side to avoid puncturing the gut or damaging the dorsal vessel. 20 larvae were allowed to recover on a grape juice plate with wet yeast in a humidified 25°C incubator. Larvae were scored as dead if they did not respond to gentle prodding with a probe and if the dorsal vessel did not beat. Three replicates were performed of 20 animals each.



Supporting Information S2. *NijA* is not required for Toll-mediated antimicrobial peptide induction.
(A-B) qPCR analysis of relative *Drosomycin* (*Drs*, A) expression or *Drosocin* (*Dro*, B) expression in male third instar larvae after septic injury with *M. luteus*. $NijA^{D3}$ homozygotes were able to respond to immune challenge by upregulating both antimicrobial peptides similarly to wild type. **(C)** qPCR analysis of relative *Drosomycin* expression after septic injury, or in $C564{>}Toll^{10b}$ larvae where *Tl* is genetically activated in the fat body. $NijA^{D3}$ homozygous mutants were able to respond to *Tl* gain-of-function in the fat body by increasing *Drosomycin* to levels similar to heterozygous sibling controls. The slight increases in *Drs* and *Dro* observed in $NijA^{D3}$untreated larvae in all three panels are not statistically significant.

Methods:
Larvae were pierced with a fine needle (Fine Science Tools) dipped in a log-phase growth culture of *M. luteus* in LB. qPCR was performed as described in Materials and Methods, except that 2µl of the cDNA pools were primed with validated primers sets for *rp49* (R2<0.99), *Drosocin* (R2<0.98), and *Drosomycin* (R2<0.99), as previously described by [1]. All values are reported relative to untreated wild-type samples. Each sample was run in triplicate, and a minimum of three independent biological replicates was performed per condition.

1. Leulier F, Lhocine N, Lemaitre B, Meier P (2006) The Drosophila inhibitor of apoptosis protein DIAP2 functions in innate immunity and is essential to resist gram-negative bacterial infection. Mol Cell Biol 26: 7821-7831.



Supporting Information S3. *NijA* is not required for phagocytosis of *E. coli*.
(A) Heat-killed fluorescently labeled *E. coli* particles were injected into third instar larvae, and after 30 min hemocytes were scored *ex vivo* for number of particles engulfed per cell. Cells engulfing five or more particles were considered "super" phagocytosing cells. Both $NijA^{D3}$ homozygotes and heterozygotes had significantly more super-phagocytosing cells than wild type, indicating that the effect was likely caused by a dominant locus on the $NijA^{D3}$ chromosome. **(B)** Drosophila S2 cells were incubated with fluorescently labeled *E. coli* and scored using flow cytometry. S2 cells treated with a *NijA-RNAi* construct were able to phagocytose at the same efficiency as wild-type cells. **(C)** Western blot of S2 cell lysates probed with anti-NijA demonstrating a strong reduction in NijA protein in *NijA-RNAi* treated S2 cells. This western was repeated twice. Error bars represent standard error of the mean.

Methods.

In vivo phagocytosis:

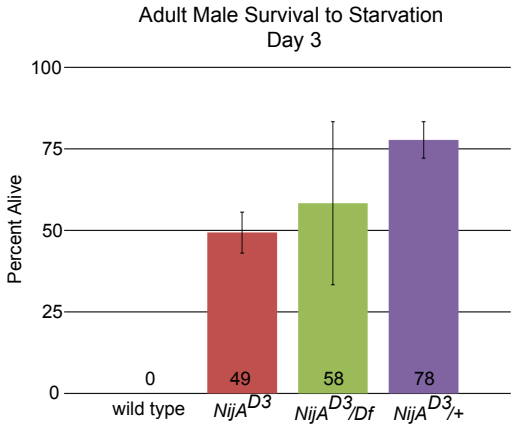
Wandering 3rd instar larvae from healthy bottles were septically wounded with a fine needle (Fine Science Tools) dipped in a concentrated mix of *E. coli* and *M. luteus*. The larvae were allowed to recover on "drinking plates" at 25°C in a humidified incubator for 2h. (Drinking plates are grape juice plates with wet yeast, scored with a probe in one quarter of the plate, and the scored areas filled with distilled water.) The larvae were then injected in the lateral side using a Nanoject apparatus (Drummond) with 69nl of $1.0x10^6$ heat-killed FITC labeled *E. coli* particles (Bioparticles, Molecular Probes/Invitrogen) suspended in phosphate buffered saline (PBS). Larvae were allowed to recover on "drinking plates" for 30 min at 25°C in a humidified incubator. The larval body was torn open in the posterior end with forceps and hemolymph was collected. Hemolymph from five animals was pooled in 10µl of 0.4% Trypan Blue in PBS to quench fluorescence of the extracellular particles, and all 10µl loaded on a hemocytometer for scoring. Hemocytes were viewed on the hemocytometer with a Zeiss Imager M2 microscope with a 20X objective, and the number of fluorescent particles per cell was scored. Five or more particles per cell were considered "super" phagocytosers. Each sample was tested in three independent pools of five animals for each genotype.

Flow Cytometry Measurements of Phagocytosis:

$2.0x10^5$ Drosophila S2 cells were plated in 200µl complete media in each well of a 24-well plate. Selected wells were treated with 6µg of dsRNA against *NijA* for 30mins. All wells were then supplemented with 400µl of complete media and cultured at 25°C for 48hrs. The phagocytosis assay was conducted as previously described [1]. Briefly, to each well was added 2µl of a $1.0x10^6$ particle/µl solution of heat-killed FITC labeled *E. coli* particles (Bioparticles, Molecular Probes/Invitrogen) in PBS. Plates were placed on ice for 30 min then transferred to room temperature for 15 min. Cells were suspended with vigorous pipetting and mixed 1:1 with 0.4% Trypan Blue to quench extracellular fluorescence. Cells were analyzed on a FACSaria flow cytometry machine (BD Biosciences) for 10,000 events per well, and the phagocytic index (phagocytosis events multiplied by mean fluorescence of phagocytosing cells) was calculated as previously described by Kocks et. al. [2]. Six wells were run on two different days for each condition.

1. Ramet M, Manfruelli P, Pearson A, Mathey-Prevot B, Ezekowitz RA (2002) Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. Nature 416: 644-648.

2. Kocks C, Cho JH, Nehme N, Ulvila J, Pearson AM, et al. (2005) Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell 123: 335-346.



Supporting Information S4. *NijA* is not required for resistance to starvation.
Although *NijA*^{D3} homozygotes are significantly more resistant to starvation than wild type, the resistance is likely to be caused by a dominant locus on the *NijA*^{D3} chromosome because the *NijA*$^{D3/+}$ heterozygote also displays increased resistance to starvation. It appears that *NijA* is not required for resistance to starvation, as the *NijA* homozygote does not have increased resistance compared to the heterozygote.

Methods:
Adult males were collected from a bottle cleared 24h prior to collection. Ten males were placed in vials containing two Kimwipes with 1.5ml sterile water for starvation conditions. Ten males were placed in vials containing 10ml of standard molasses food for fed conditions. Adult males were scored as dead when they were no longer standing upright. All animals were alive on day two and all animals were dead by day four in the starvation vials. Three independent replicates were scored concurrently. Error bars represent standard error of the mean.

Ectopic expression of *NijA* induces nonapoptotic cell death

Because NijA is upregulated on septic injury, we focused on analyzing its function by ectopically upregulating *NijA* using the *GAL4/UAS* system for a gain-of-function approach. Under the ubiquitously expressed *tubulin* and *actin* drivers, we found that embryos died with morphological abnormalities around the time of cellularization (data not shown), which is also when zygotic transcription begins [92, 93]. Lethality was also observed when we expressed *NijA* with *A58-GAL4* in the larval epidermis [94] or in most blood cells with *He-Gal4* [95] (data not shown). Attempts to control the onset of lethality by using the conditional $GAL80^{ts}$ inhibitor [96] or the inducible *GeneSwitch-GAL4* driver [97] both failed because even under restrictive conditions (18° or absence of RU486, respectively) leaky expression of *NijA* still caused lethality (data not shown). We expressed *NijA* in a tissue not required for viability, the eye, with *GMR-GAL4* and *ey-GAL4*; to our surprise, *NijA* expression with each driver was pupal lethal (Table 2.1 and data not shown). Expression of *NijA* with *hml-GAL4*, expressed in differentiated blood cells of the larval lymph gland and in circulation [98, 99], was not lethal and allowed us to ask about the cellular consequences of NijA overexpression.

cross	# *GMR>UAS* progeny observed	Expected Mendelian # if viable	χ^2 value
GMR-GAL4/CyO x UAS-NijA/TM3	3/338 (0.89%)	85.5/338 (25%)	104.8 (p<0.001)
GMR-GAL4/CyO x UAS-hid/CyO	3/175 (1.7%)	58.3/175 (33%)	78.7 (p<0.001)

Table 2.1: Organismal death resulting from GMR-driven expression of a cell-death gene is not dependent on the gene acting non-autonomously.

Hml expressing cells, visualized by *Hml>GFP*, can be observed under the cuticle of whole third instar larvae (Fig. 2.3A,B); however, when *NijA* is co-expressed, the cells appear to be absent (Fig. 2.3C). To compare the effects of *NijA* to a known apoptosis inducer, we drove the expression of *hid* with *hml-GAL4*, and found a similar loss of GFP-labeled cells (Fig. 2.3D). The similarity of these results suggested that *NijA* induces cell death in differentiated blood cells. We confirmed by quantitative Western blotting that GFP was absent from animals co-expressing *GFP* and either *NijA* or *hid* (Fig. 2.3E,F). This lack of GFP indicated that larvae were missing nearly all *Hml*-expressing cells, both in circulation and in the lymph gland, the site of hematopoiesis in larvae.

We examined dissected lymph glands expressing *NijA*. Although 7/7 control (*hml>GFP*) lymph glands did not stain with acridine orange, which can only permeate dead cells, in contrast all lymph glands expressing either *NijA* or the apoptosis inducer *hid* stained brightly with acridine orange (7 of each genotype, compare Fig. 2.3G-I'). The dying cells appeared to be restricted to differentiated cells that express *hml*, as undifferentiated hemocytes expressing *hemese* but not *hml* were still present in lymph glands expressing *hml>NijA* or *hml>hid* (Fig. 2.2J-L). We were able to detect little to no GFP signal with an anti-GFP antibody in the lymph glands of *hml>NijA,GFP* or *hml>hid,GFP* animals (Fig. 2.3J'-L'), consistent with the dramatic decrease in GFP expression in whole animals by western blot analysis (Fig. 2.3E-F). Interestingly, *NijA* was not required for developmentally regulated apoptosis, however, as apoptosis in the stage 10 embryonic head region occurs

normally in a *NijA*^{D3} mutant with no zygotic or maternal *NijA* (Fig. 2.S5). Thus *NijA* induces cell death when ectopically expressed.

To examine whether NijA induces cell death via apoptosis, we examined lymph glands by TUNEL staining, a nuclear label for apoptotic cells. We compared lymph glands expressing *GFP* and *NijA* to glands expressing only the *GFP* marker as a negative control, and to glands expressing *GFP* and the apoptosis-inducer *hid* as a positive control. We counted the number of TUNEL positive nuclei in primary lymph gland lobes: fewer than 30 nuclei was considered background, and more than 30 (often uncountable) was considered apoptotic. By these criteria, we found that 10/11 *hid*-expressing lymph glands were apoptotic, whereas 22/23 GFP lymph glands were TUNEL-negative and 9/13 *NijA*-expressing lymph glands were TUNEL-negative (Fig. 2.3J''-L''). The penetrance of apoptosis in *NijA* lymph glands is significantly different from *hid* lymph glands by Fisher's exact test (p=0.004). Some of the unexpected variability in our *NijA* overexpressing lymph glands may be an artifact of our cut-offs, as 3 of the 4 *NijA* expressing lymph glands scored as "apoptotic" qualitatively appeared to have fewer TUNEL positive cells than the *hid* expressing glands; another possibility is that there is significant cross-talk between cell death pathways (see Discussion).

As a second independent assay to assess whether *NijA* induces apoptotic or nonapoptotic cell death, we co-expressed *NijA* with *p35*, an apoptotic inhibitor that blocks cell death when co-expressed with *reaper* or *hid* [100, 101]. Examining GFP-labeled hemocytes in live animals, we found that p35 inhibited Hid-induced cell death as expected, but importantly p35 did not inhibit NijA-induced cell death (Fig.

2.3M-P). Our data indicate that *NijA* induces a nonapoptotic form of cell death, as dying cells do not label with TUNEL and cell death is not inhibited by p35.

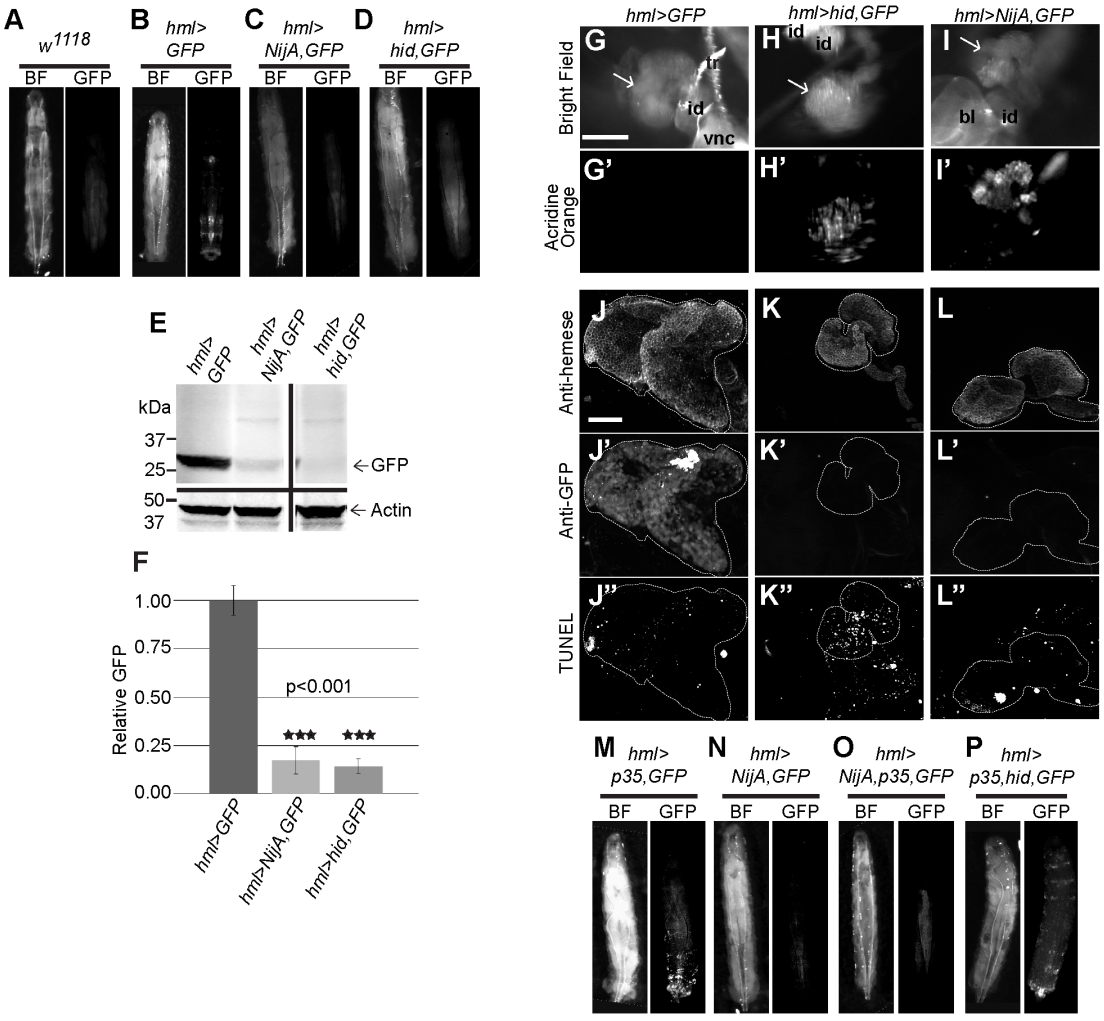
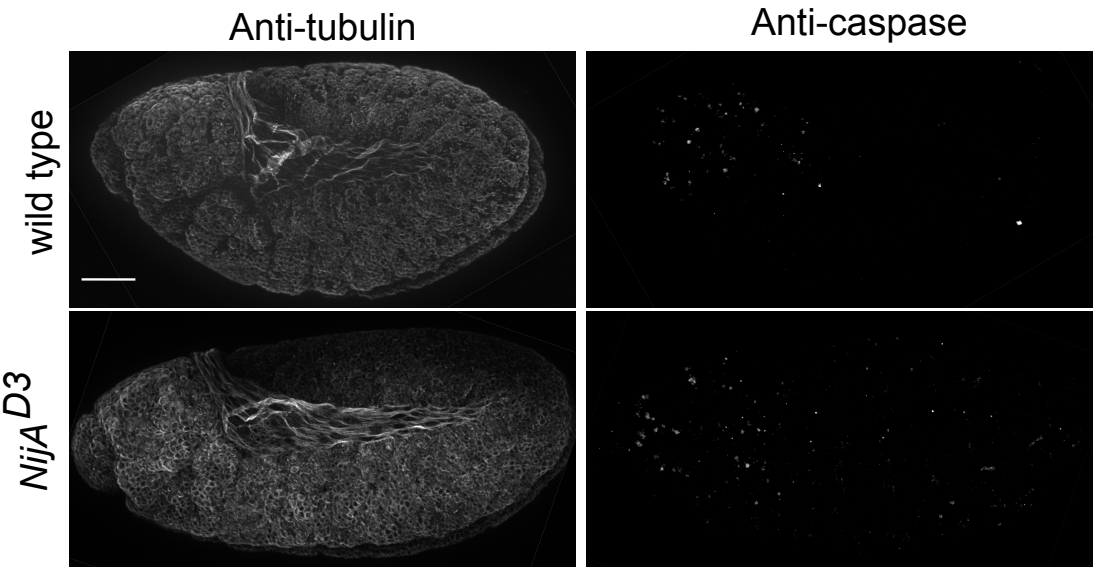


Figure 2.3. Ninjurin A over-expression in the lymph gland causes nonapoptotic cell death. (A) Wild-type w^{1118} larva demonstrating background autofluorescence. **(B)** *hml>GFP* larva with GFP-positive differentiated hemocytes along posterior body wall and in lymph gland. **(C)** *hml>NijA,GFP* larva lacked GFP-positive cells. **(D)** *hml>hid,GFP* larva, a cell-death positive control, lacked GFP-positive cells. **(E)** Western blot of whole larval lysates probed with anti-GFP. *hml>NijA,GFP* and *hml>hid,GFP* larvae were devoid of GFP. **(F)** Quantification of three western blots probed for anti-GFP as in (E). GFP is virtually absent from *hml>NijA,GFP* and *hml>hid,GFP* larvae. Error bars represent standard error of the mean. **(G-I')** Live partially dissected 3rd instar larval lymph glands (arrows) were stained with acridine orange to detect cell death. Scale bars are 200μM. tr: trachea; id: imaginal disc; vnc: ventral nerve cord; bl: brain lobe. **(G')** *hml>GFP* larval glands did not stain with acridine orange. **(H')** *hml>hid,GFP* glands, a cell-death positive control, stained with acridine orange. **(I')** *hml>Nij*A,GFP glands stained with acridine orange, demonstrating that *NijA* induced cell death. **(J-L'')** Larval lymph glands were fixed, TUNEL labeled, and antibody stained. Scale bars are 50μm. **(J-L)** Anti-Hemese staining labeled the lymph glands. **(J'-L')** Anti-GFP staining shows no GFP-positive

(hml+) hemocytes in *hml>hid,GFP* (K') or *hml>NijA,GFP* (L') larval glands. **(J''-L'')** TUNEL-labeled glands. **(J'')** Few TUNEL positive cells in *hml>GFP* negative control glands. **(K'')** Many TUNEL positive cells in *hml> hid,GFP* positive control glands. **(L'')** Few TUNEL positive-cells in the *hml>NijA,GFP* glands, indicating that *NijA* does not induce apoptosis. **(M)** Larvae expressing the apoptotic inhibitor p35 (*hml>p35,GFP)* displayed GFP-positive hemocytes similar to *hml>GFP* in (B). **(N-O)** p35 did not inhibit the NijA-induced loss of the GFP-positive cells in *hml>NijA,p35,GFP* larvae. **(P)** p35 inhibited the hid-induced loss of the GFP-positive cells in *hml>hid,p35,GFP* larvae, a positive control for p35 inhibition. In (A-D, M-P), anterior is up.



Supporting Information S5. *NijA* is not required for developmentally programmed cell death in the embryo.
Stage 10 embryos were fixed and stained with anti-tubulin to show embryo morphology and anti-cleaved-caspase 3 to label apoptotic cells. Cleaved-caspase 3 staining in the anterior of the embryo appeared similar in the *NijA*^{D3} mutant and the wild-type embryos. Anterior is on the left and dorsal is up.

Methods.
Embryos from an overnight collection of *w*^{1118} or homozygous *NijA*^{D3} mothers were dechorionated in 50% Clorox bleach, fixed at the interface of heptane and 4% formaldehyde (Ted Pella), and deviteillinized in methanol/heptane. Embryos were slowly rehydrated, blocked in 1% Bovine Serum Albumin (BSA) in 1X PBS + 0.2% Tween 20 (PBST) for 30 min at room temperature with gentle rocking, and stained overnight at 4°C with rat anti-tubulin at 1:200 (AbD Serotec, clone YL1/2) and rabbit anti-cleaved-caspase 3 at 1:50 (Cell Signaling, #9661) diluted in the blocking solution. Embryos were washed several times in PBST, and stained for 2h at RT with FITC-labeled goat anti-rat and Cy3-labeled goat anti-rabbit, each at 1:200 in blocking solution. Embryos were washed in PBST, dehydrated with methanol, and mounted in clearing solution (2:1 Benzyl Benzoate: Benzyl Alcohol). Embryos were photographed using a Zeiss Imager M2 with Apotome. Images are a projection of a Z-series to show all of the caspase-positive cells present in the embryo. All stage 10 embryos of both genotypes were caspase-positive.

NijA appears to kill cells in a cell-autonomous manner

Because the expression of NijA in the eye with *ey-GAL4* or *GMR-GAL4* was lethal to the animal, we asked whether this phenotype represented tissue nonautonomous cell death, *i.e.*, if NijA expressed in the eye disc was effectively instructing tissues outside the eye to die. Alternatively, it was possible that even the autonomous destruction of a large tissue may release toxic factors that could cause animal lethality. To determine if we could assess tissue autonomy in this assay, we expressed *hid*, which is known to be an autonomous cell-death gene [100] also under *GMR-GAL4* and found that the overexpression of *hid* caused organismal lethality with very few escapers (Table 2.1 and data not shown). Either *GMR-GAL4* is not eye-specific or the massive developmentally-inappropriate induction of cell death is sufficient to cause organismal death; in either case this assay cannot indicate the autonomy of NijA-induced cell death.

As another means to assess the autonomy of NijA-induced cell death, we turned to cell culture. We previously reported that when overexpressed in cultured *Drosophila* S2 cells, NijA inhibits cell adhesion in a nonautonomous manner within a few hours of its induction. This NijA-mediated phenotype was dependent on the activity of the protease Mmp1, and the NijA ectodomain was sufficient to release adhesion even when Mmp1 proteolysis was inhibited [2]. We revisited these experiments and found that when S2 cells express *NijA* for longer periods, the cells died as measured by trypan blue exclusion: after 24h, 20.2% of cell had died (not shown), and after 48 hours 33% of cells had died, a significant increase over the background death rate of about 9% in non-expressing cells (Fig. 2.4A).

Interestingly, like in whole flies, *NijA* was not required for apoptosis, as the apoptosis-inducer actinomycin D [102, 103] was able to induce death at similar levels in wild-type and *NijA* knock-down cells (Fig. 2.4C,D), indicating that *NijA* is not an essential component of the cell death machinery. In contrast to our previous adhesion results, NijA-induced death does not require Mmp1 (Fig. 2.4B). Similarly, we found that the NijA ectodomain is not sufficient to trigger cell death, even though we were able to detect the tagged ectodomain in cells, cell lysates, and in the culture medium (Fig. 2.S6). These Mmp1 and NijA ectodomain results suggested that NijA may induce death in a cell-autonomous manner.

To examine the autonomy of Ninjurin-induced cell death, we considered testing conditioned media, but we were concerned that dying cells may release toxic factors into the media even if the death were cell autonomous, similar to our results in whole animals. Instead, we chose to examine the relationship between NijA transfection status labeled by GFP and cell death measured by trypan blue exclusion. We co-transfected NijA and GFP expression vectors and counted the number of GFP-expressing cells remaining after 48h induction, as transfected cells are expected to take up both vectors simultaneously (Fig. 2.5). When transfected with only the *GFP* plasmid, 39% of cells expressed GFP, and 8% of cells were dead as measured by trypan blue exclusion (Fig. 2.5A), consistent with the baseline rate of cell death we measured in these cultures (Fig. 2.4). In contrast, when cells were co-transfected with *NijA* and *GFP*, only 9% of cells expressed GFP, and 37% of the cultured cells were dead as measured by trypan blue exclusion (Fig. 2.5A). Thus it appeared that *NijA*-expressing cells were much more likely to die than their non-

transfected neighbors. We continued this analysis to ask which residues were important for inducing cell death, examining four site-directed *NijA* mutants generated by alanine-scanning [104], in which charged residues in the ectodomain were replaced with alanines (Fig. 2.5A,C). We found that D140 was absolutely required, as this point mutation ablated NijA's ability to induce death; importantly the D140A mutant protein was not able to localize correctly to the cell surface (Fig. 2.5D-F), suggesting that cell-surface localization is critical for inducing death. Surprisingly, two mutants seemed to increase the potency of NijA, as D124A or the double mutant K131A, K132A were more toxic to cells than wild-type NijA, resulting in no GFP-positive cells; yet because the fraction of dead cells was similar to the sum of the baseline death rate plus the transfection rate, it appears that this overactive toxic mutant still killed in a cell-autonomous manner. The double-mutant R152A, E156A killed cells similarly to wild-type NijA (Fig. 2.5A), and the mutant protein localized at the cell surface similarly to wild-type NijA (Fig. 2.5G). In a separate experiment, we asked which domains were required to induce death (Fig. 2.5B). The N-terminal ectodomain was required, as a deletion removing it ($NijA^{\Delta N\text{-}term}$) induced only 8% death with 52% of the cells expressing GFP, similar to the GFP-alone controls. The ectodomain was also required for localization of NijA to the cell surface, as the $NijA^{\Delta N\text{-}term}$ protein (carrying a myc epitope at the new N-terminus) was not detectable in unpermeabilized cells (Fig. 2.5I). The predicted 20-amino acid intracellular domain was partially required, as a mutant replacing most of the intracellular sequence ($NijA^{\Delta intracell}$) with the myc epitope gave an intermediate level of death, with 20% dead cells and 32% GFP-expressing cells. Taken together, these

data strongly suggest that NijA induces death in a cell-autonomous manner, requiring cell surface localization to kill cells.

To determine if these mechanisms applied to NijA cell death induction *in vivo*, we transformed flies with GAL4-inducible constructs encoding the *NijA* double mutant *R152A, E156A* and the NijA ectodomain (*UAS-NijA*^{ect}). As in cell culture, the double mutant phenocopied wild-type *NijA*, whereas the ectodomain alone did not induce cell death comparable to wild-type *NijA* (Fig. 2.6). We conclude that when expressed at high levels, *NijA* kills cells by a nonapoptotic mechanism, likely in a cell autonomous manner.

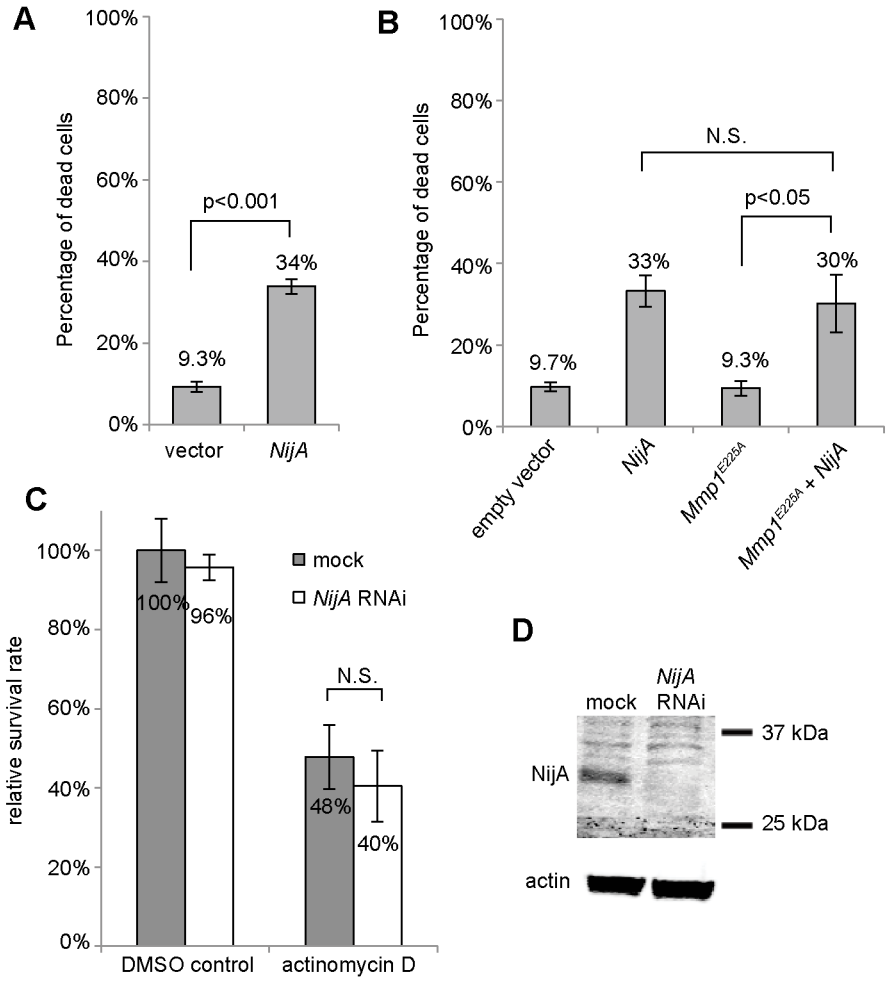


Figure 2.4. NijA induces cell death in *Drosophila* S2 cell culture.
(A) *NijA* expression kills S2 cells. Cells were transiently transfected with *pRmHa3* empty vector or *pRmHa3-NijA* and induced with copper for 48h. The percentage of dead cells was determined by dividing the number of trypan blue positive cells by that of total cells counted for each sample. Data from 8 experiments are shown. Error bars indicate S.E.M, and Student's T test was used to calculate p value. **(B)** Mmp1 activity is not required for *NijA*-induced cell death. Cells were transiently transfected and induced for 48h. $Mmp1^{E255A}$ is a dominant-negative catalytically inactive mutant of *Mmp1*. Data from 4 experiments are shown. **(C)** *NijA* is not required for actinomycin D-induced apoptosis. Cells were treated with *NijA* dsRNA or no dsRNA (mock) for 48h, then incubated with 100nM actinomycin D for 6h. Trypan blue staining was used to determine cell survival, which was normalized to the untreated (DMSO), wild-type (mock) sample. Data from 4 experiments are shown. **(D)** Western blot showing the NijA protein levels in mock and *NijA* dsRNA-treated cells. Actin was used as the loading control.

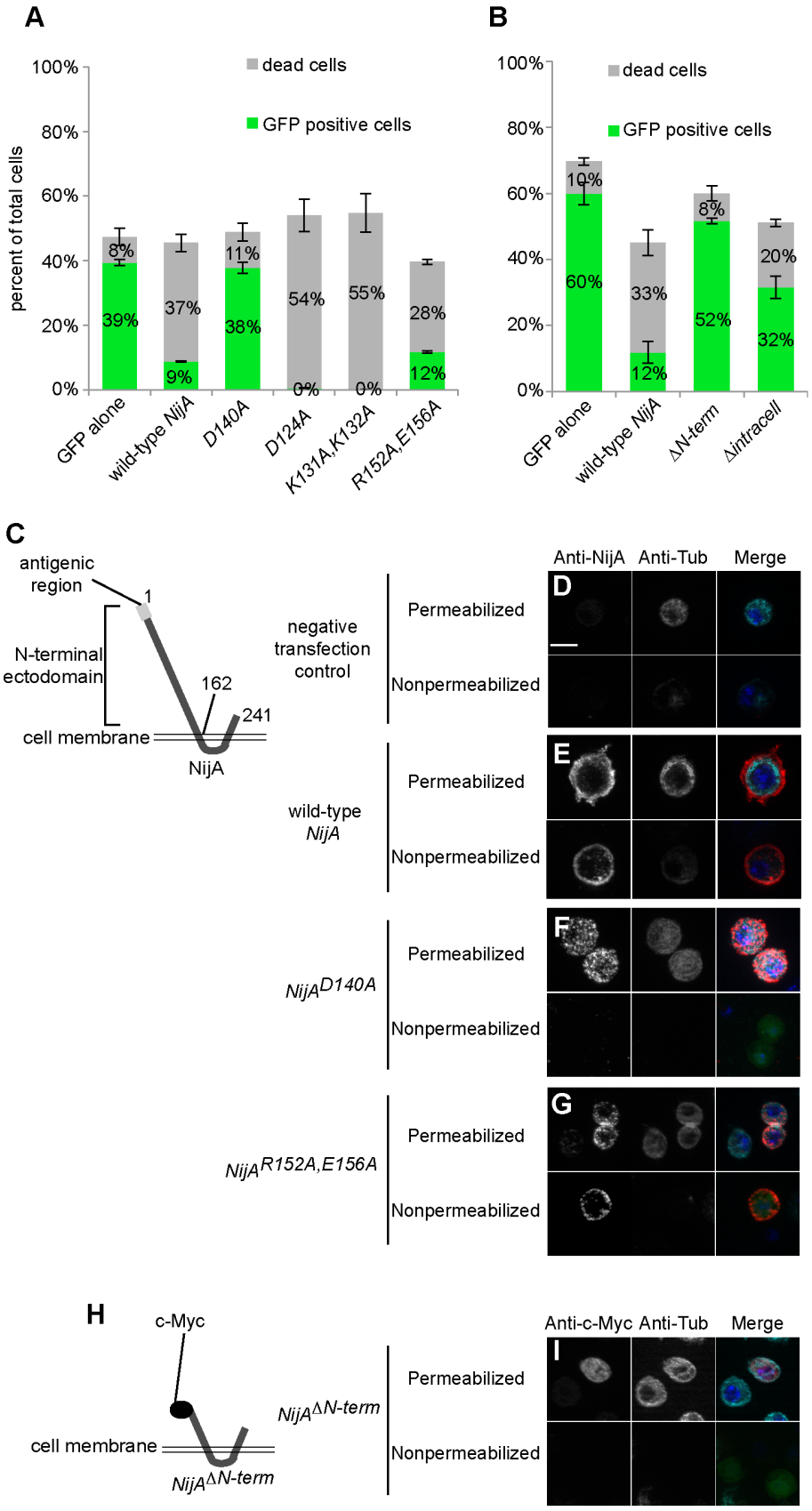

A
100%
80%
60%
40%
20%
0%
percent of total cells
dead cells
GFP positive cells
8%
39%
37%
9%
11%
38%
54%
0%
55%
0%
28%
12%
GFP alone
wild-type NijA
D140A
D124A
K131A,K132A
R152A,E156A
B
100%
80%
60%
40%
20%
0%
dead cells
GFP positive cells
10%
60%
33%
12%
8%
52%
20%
32%
GFP alone
wild-type NijA
ΔN-term
Δintracell
C
antigenic region
1
N-terminal ectodomain
162
241
cell membrane
NijA
negative transfection control
wild-type NijA
NijAD140A
NijAR152A,E156A
Permeabilized
Nonpermeabilized
Anti-NijA
Anti-Tub
Merge
D
E
F
G
H
c-Myc
cell membrane
NijAΔN-term
NijAΔN-term
Anti-c-Myc
Anti-Tub
Merge
I

Figure 2.5. NijA appears to kill in a cell-autonomous manner. (A-B) Cells were transiently co-transfected with *pRmHa3-GFP* and various mutants of *pRmHa3-NijA* as indicated under each column; mock is empty *pRmHa3* vector. 48h after induction, viability was assessed by trypan blue staining, and transfection status was assessed as GFP fluorescence. Wild-type *NijA* and most *NijA* mutants killed cells, whereas the mock control, the *D140A* mutant, and the N-terminal deletion (B) showed low levels of cell death. The sum of transfected live cells (GFP+) plus dead cells was relatively constant across samples despite the augmented or compromised capacity to kill cells, indicating that NijA kills the cell it transfects but not others. Data from 3 replicates are shown. Error bars indicate S.E.M. **(C)** Schematic showing topology of NijA (form A) protein and the extracellular region recognized by our polyclonal antibody. Amino acid residue numbers are indicated. **(D-G,I)** Immunofluorescence localization of wild-type NijA or NijA mutant forms expressed in S2 cells and stained with anti-NijA (D-G) or anti-c-Myc (I), both extracellular epitopes. For each construct, staining was performed on permeabilized cells to show NijA protein levels, and on unpermeabilized cells to show NijA cell-surface localization. Permeabilization status was verified by anti-tubulin staining. The merge image combines images for NijA (red), tubulin (cyan), DAPI (blue) and GFP fluorescence as a transfection control (green). Bar: 10 μm. **(H)** Diagram showing placement of the myc epitope for (I), necessary because the NijA antigenic region was deleted in this mutant.

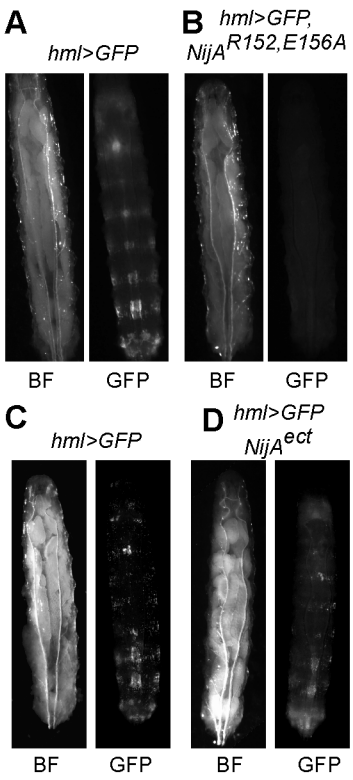
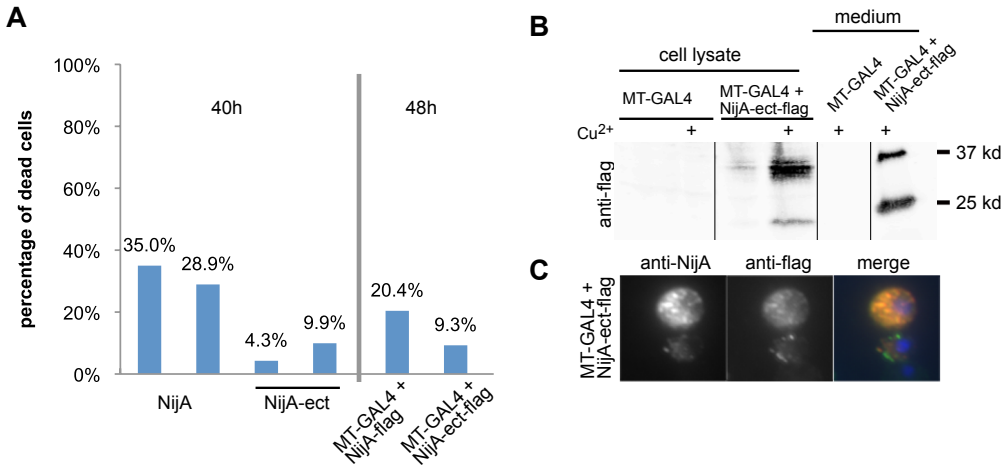


Figure 2.6. *NijA* mutants behave *in vivo* as they do in cell culture. (A) Whole third instar *hml>GFP* larvae have visible GFP expression in the hemocytes attached to the body wall in a segmental arrangement. **(B)** Third instar *hml>GFP,NijA*^{152156A} larvae were devoid of GFP-positive hemocytes, indicating that these mutants were capable of inducing cell death and that polar amino acids 152 and 156 were not required for cell death. **(C-D)** *hml>NijA*^{ect}*,GFP* larvae displayed visible GFP-positive hemocytes on the body wall (compared with *hml>GFP* larvae in C), suggesting that NijA^{ect} was not sufficient to induce cell death.



Supporting Information S6. The secreted ectodomain of *NijA* does not induce cell death.

(A) Cells were transfected with *pRmHa3-NijA* (columns 1 and 2) or *pRmHa3-NijA-ectodomain* (columns 3 and 4) and induced for 40h. To express C-terminal flag-tagged forms, cells were co-transfected with *pRmHa3-GAL4* and *UAS-NijA-flag* (column 5) or *UAS-NijA-ecto-flag* (column 6) and induced for 48 hrs. Percentage of dead cells was determined by counting trypan blue positive cells. **(B)** *NijA* ectodomain was expressed and secreted into the medium. Western blot with *anti-flag* was performed on cell lysate and medium collected from cells transfected with *pRmHa3-GAL4* and *UAS-NijA-ecto-flag* or *pRmHa3-GAL4* alone (control). **(C)** Localization of *NijA-ecto-flag* shown by immunofluorescence staining with *anti-NijA* (red) and *anti-flag* (green). Cell nuclei were stained in blue by DAPI.

Discussion:

In the present study, we found that *Drosophila* NijA is upregulated or relocalized in tissues of the immune system upon septic injury. The NijA upregulation observed in adults may result in more protein localized to the cell surface, a similar effect to the relocalization to the cell surface observed in larvae after septic injury. NijA protein levels are upregulated via the Tl pathway, an established immunoregulatory pathway, and in activated Tl mutants, there is more NijA observed at the surface of fat body cells, supporting the idea that NijA upregulation and relocalization are functionally similar immune responses. In whole-animal genetic experiments we found that the ectopic upregulation of NijA induces cell death characterized by acridine orange staining and tissue loss. However, NijA-induced cell death is not associated with DNA fragmentation, as assessed by TUNEL labeling, and is not suppressible by p35, an apoptotic inhibitor. These data indicate that NijA induces nonapoptotic cell death. Similar death phenotypes were observed both in tissues of whole larvae and in cultured S2 cells, and the cell death appears to be autonomous. In cultured cells, *NijA* mutants that cannot localize to the cell surface also cannot induce cell death, suggesting that *in vivo* the observed increase of NijA levels at the cell surface after septic injury may be critical to NijA function.

Originally identified as gene products upregulated on nerve injury, Ninjurins have been characterized as adhesion molecules [1, 3, 5, 91], anti-adhesion signals [2], and mediators of cell cycle regulation [9]. This lack of consensus likely stems from the fact that these studies have been performed in cultured cells. There is

scant information about Ninjurin function *in viv*o, in part because animal studies have focused almost exclusively on expression analysis [1-4, 91]. The only study investigating Ninjurin function *in vivo* was performed with neutralizing antibodies to Ninjurin1 during rat ocular development [91], and it found that Ninjurin antibodies slowed the regression of the hyaloid vasculature, an embryonic tissue that is removed by cell death during development.

Our *in vivo* gain-of-function data suggests that Ninjurins promote nonapoptotic cell death. In addition to apoptosis, the other main types of programmed cell death are autophagy and programmed necrosis (sometimes called necroptosis) [105]. Autophagy, a process in which the cell digests its own components, can regulate a variety of cellular processes including viral clearance and signal transduction [78, 79]. Autophagy can result in cell survival in response to stress or can lead to cell death [80]. The death outcome utilizes components of the apoptotic machinery [81]. The differences between autophagic death and apoptosis are still being elucidated, and it is likely that in some contexts they can compensate for each other if one death mechanism fails [82, 83]. The pathways leading to programmed necrosis also have considerable crosstalk with the pathways leading to apoptosis, and they are believed to inhibit each other [85]. Unfortunately, programmed necrosis has not been characterized in *Drosophila*, although it may be induced genetically [86, 87]. We speculate that the interplay between these different types of cell death may explain why we observe no cell-death phenotypes in the *NijA* null mutants. Because cell death mechanism are known to compensate for each other, it is possible that in our *NijA* null mutants, cells that would have been

killed by a NijA-dependent mechanism are now killed by another cell-death mechanism. Of note, genome-wide expression studies report that NijA is expressed during metamorphosis at levels ~12-fold higher than during any other time in the *Drosophila* life cycle [106, as reported at flybase.org], suggesting that NijA may function during cell and tissue death in development. Interestingly, several Toll family members (Toll, 18-wheeler/tollo, Toll-6, and Toll-7) are also highly expressed during metamorphosis as well [107], and our data demonstrates that Tl can upregulate NijA.

Another possibility to explain the lack of cell death or other phenotypes in our *NijA* null mutants is genetic redundancy among the three *Drosophila* Ninjurin genes, *NijA, NijB,* and *NijC*. However, expression data does not support the idea of Ninjurin family redundancy. Genome-wide expression data sets, examining developmental timing and tissue-specific expression of all genes, indicate that *NijA, NijB*, and *NijC* are not expressed in similar times or tissues in developing flies. Nevertheless, it is possible that analysis of double and triple mutants may uncover redundant Ninjurin functions. In our preliminary examination of *NijA, NijC* double mutants we have not found any obvious developmental abnormalities (X. Wang and A. Page-McCaw, unpubl.).

Our data suggest that Ninjurin may participate in an immune response that promotes cell death. Although cell death is critical in the mammalian immune system [reviewed in 85], in *Drosophila* it is unclear why immune tissues would initiate death in response to immune challenge. One possibility is that NijA is required not for the initial immune response, but rather for resolving the immune

response once a pathogen has been neutralized. In mammals, superfluous neutrophils are cleared by cell death after resolution of an immune challenge [108], and if neutrophils are not cleared excessive inflammation can result in damage to healthy tissue [109]. In *Drosophila*, there is a dramatic increase in circulating hemocytes after immune challenge [26], and it is not clear if these hemocytes persist after the challenge has been eliminated; it is possible that NijA may participate in their clearance. Another possibility is that increased NijA primes cells to die in an orderly manner if they should become injured in the course of an infection. A third possibility is based on the idea that physiological increases of NijA in response to septic injury are significantly smaller than those in our genetic overexpression system; perhaps on immune challenge, moderate levels of NijA initiate an autophagic mechanism of pathogen clearance from cells, but at physiologic levels NijA does not promote cell death at all.

Our genetic gain-of-function studies indicate that NijA induces cell death in a cell-autonomous manner. This is different from the nonautonomous apoptotic cell death observed in endothelial cells adjacent to Ninjurin1-positive macrophages that was interpreted as a result of altered adhesion [91], and it is different from the cell nonautonomous loss-of-adhesion signaling we observed in cultured cells [2]. It is possible that in different contexts Ninjurins can act either autonomously or nonautonomously, as their cell-membrane location would allow them to relay information from outside to inside the cell, or allow them to signal via their extracellular domain to other cells. Our study highlights a novel and potentially medically important role for the conserved Ninjurin family in inducing cell death.

Materials and Methods:

Drosophila genetics and imaging

Unless otherwise noted flies were raised at 25° under standard conditions. *Hml-GAL4, UAS-GFP* and *UAS-hid* were from J. Royet [98]; *C564-GAL4* and Tl^{10b} were from K.V. Anderson [54, 61]; *UAS-*Tl^{10b} was from S. Cherry [50]. The *EP* element *G4196* was generated by Genexel (Korea) and deposited at the Bloomington *Drosophila* Stock Center. The site of the insertion was determined by sequencing genomic DNA to be 51 bp upstream of the transcription start site. The insertion line was outcrossed 3 times to w^{1118} before excising the transposon. 60 excision lines were screened in pools of five by PCR amplification of a 3561 bp genomic fragment surrounding the P insertion site; from these three imprecise excisions (shown in Figure 2) were identified by gel electrophoresis of PCR products. The *UAS-NijA* line was generated by ligating the cDNA *RE5744* (Berkeley *Drosophila* Genome Project) corresponding to NijA-RA, into the *pUAST* vector at the EcoR1 and BamH1 sites. The fly transformation vectors *UAS-*$NijA^{ect}$ and *UAS-*$NijA^{R152A,E156A}$ were generated by ligating the inserts from the corresponding *RmHa3* plasmids (see below) into *pUAST*. Transformants were generated by Genetic Services Inc (Cambridge, MA). We examined two independent transformants of the *UAS-NijA* and *UAS-*$NijA^{ect}$, and in both cases we saw comparable results.

For *hml>GFP* analysis in whole larvae, third instars were selected from the food of a healthy vial, washed in sterile water to remove debris, and placed on a grape juice plate. GFP-labeled blood cells were scored in live larvae under a Zeiss

LumarV12 fluorescence stereomicroscope prior to heat-killing for imaging. A minimum of 20 animals were scored per genotype. For imaging, each larva was placed in 20µl of PBS on a cover slip, and heated for 5 sec at 95˚C to kill the larvae. Larvae were immediately imaged by bright field and epifluorescence with a Zeiss 0.8X Neolumar objective, on magnification setting 64X. Images were cropped and edited using Adobe Photoshop.

For imaging lymph glands live-stained with acridine orange, third instar larvae were selected from the food of a healthy vial and washed in sterile water to remove debris. Each larva was placed in a 50µl drop of freshly diluted $1.6x10^{-6}$ M acridine orange in *Drosophila* Ringer's solution on a Sylgard dissection plate. The dorsal cuticle was carefully torn away from posterior to the anterior to expose the internal organs. The flap of dorsal cuticle was pinned to the plate, and the fat surrounding the dorsal vessel quickly cleared away for imaging. Just prior to imaging the acridine orange solution was removed and replaced with *Drosophila* Ringer's solution. Bright field and epifluorescence images were taken on a Zeiss LumarV12 with a 1.5X Neolumar objective and processed using Adobe Photoshop.

Western blotting and antibody staining

For Western blots, lysates were made by mechanically grinding samples on ice in Laemmli buffer and heating at 95˚C for 5 min. Blots were probed with guinea pig anti-NijA at 1:1500 [2], mouse anti-GAPDH at 1:2000 (IMGENEX, #IMG3073), mouse anti-Actin at 1:2000 (Abcam, #ab6276), and rabbit anti-GFP at 1:2000 (Abcam, #ab6556). Detection was performed by either HRP-mediated

chemiluminescence or fluorescence imaging (Licor, Odyessy). Figure 1A and Supplementary Figure 3C were probed with HRP-labeled goat anti-guinea pig at 1:5000 (Santa Cruz) and HRP-labeled goat anti-mouse at 1:5000 (Jackson Immuno-research) secondary antibodies. Figures 1C, 2E, and 4D were probed with IRDye 680-labeled donkey anti-mouse, IRDye 800CW-labeled donkey anti-guinea pig, IRDye 680-labeled donkey anti-rabbit, or IRDye 800CW-labeled donkey anti-mouse, all diluted 1:5000 (Licor). Data was quantified using the ImageJ software. Blots were cropped and edited using Adobe Photoshop.

For antibody staining, samples were dissected, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20mins, and then blocked for 30mins in 1% bovine albumin serum (BSA) in PBS+0.2%Tween (PBST) for permeabilized samples or in 5% normal goat serum (NGS) in PBS for non-permeabilized samples. Primary antibodies were diluted in blocking reagent and incubated with sample overnight at 4^0C. Primary antibodies used were guinea pig anti-NijA at 1:100 [2], mouse IgG2a anti-hemese at 1:100 (from Istvan Ando [110]), and rabbit anti-GFP at 1:50 (Abcam, #ab6556). Samples were washed and labeled with secondary antibody for 2h in the dark at room temperature. Secondary antibodies used were Cy3-labeled goat anti-guinea pig, DyLight 649-labeled goat anti-mouse, and FITC-labeled goat anti-rabbit , all diluted 1:500 (Jackson ImmunoResearch). TUNEL labeling was performed according to the manufacturer's directions (Roche *In situ* cell death detection Kit TMR red). Tissues were mounted in Vectashield (Vector Lab) and imaged on a Zeiss Imager M2 with Apotome. Images

are 2D projections of Z-sections. Projections were generated by the ImageJ software, and images were cropped and edited using Adobe Photoshop.

Hemocytes were recovered for *ex vivo* staining as described [36]. In brief, the posterior end of a clean larva was bled onto a glass slide, and the hemolymph was recovered with a pulled glass needle. (To control the needle suction with a P20 pipettor, the dull end of the pulled needle was attached to the small end of a P20 tip by melting the plastic tip and sealing with nail polish.) The hemolymph was transferred to a Multitest slide (MP Biomedicals) in 10ul of PBS, and the hemocytes were allowed to settle for 10mins. Hemocytes were fixed for 7mins in 4% paraformaldehyde in PBS, rinsed briefly in PBS, and then blocked for 15mins in 1% BSA in PBST. The primary antibody was applied to the samples for 2h while the slide was in a humidified chamber. The samples were washed in several changes of PBST, and the secondary antibody was applied for 1h in a dark humidified chamber. The samples were washed several times in PBST, and once in PBS prior to mounting in Vectasheild mounting media for viewing on a Zeiss Imager M2 with a Plan Neofluor 40X oil objective.

qPCR Analysis

Total RNA was extracted from whole third instar males using the TRIzol reagent (Ambion) according to the manufacture's directions. Only males were used because of sex-specific differences in antimicrobial peptides [111]. Total RNA extracts were treated with DNase to remove contaminating DNA with the TURBO DNA-free kit (Ambion). 800ng of total DNase-treated RNA was reverse-transcribed

into cDNA pools using the iScript cDNA Synthesis Kit (BioRad) according to the manufacture's directions in an Eppendorf AG 22331 Hamburg Thermocycler. 2µl of the cDNA pools were primed with validated primers sets for $NijA^{Exon\ 3}$ (Fwd:AACTGTTGGAGGCAACGGAG, Rev:AAAGGAGAAACTGGGTCGTCTT. R^2<0.99), $NijA^{Exon\ 4}$ (Fwd:GCGTGGGCCTTATATTGATG, Rev:TGTTCGCCCGGCAGATAT, R^2<0.99), and rp49 (R^2<0.99)[112]. qPCR reactions were run using the SSO Advanced SYBR Green Super Mix (BioRAD) for SYBR green chemistry according to the manufacture's directions in a CFX96 Real-Time C1000 Thermocycler (BioRad). Resulting Ct values were analyzed in Microsoft Excel. Ct values were fit to a standard dilution curve for correction to primer efficiency and then normalized to the *rp49* housekeeping gene. Three replicates were performed for each condition.

Cell culture and transient transfection

Drosophila S2 cells were obtained from the *Drosophila* Genomics Resources Center (Bloomington, IN) and maintained at 27 ℃ in Schneider's *Drosophila* medium (Gibco) containing 10% heat inactivated Fetal Bovine Serum (Gibco, 16140) and 100 U/ml penicillin/streptomycin. For transient transfection, 3 × 10^5cells were seeded per well in 750 µl of complete medium in a 24-well plate. The next day, cells were transfected with 2 µg plasmid DNA/well (1 µg DNA/well for co-transfected GFP plasmids) using the calcium phosphate method according to manufacturer's protocols (Invitrogen). *NijA-RA* wild-type, mutant, deleted, tagged, and ectodomain constructs were each cloned into *pRmHa3* for inducible expression from the metallothionine promoter; the wild-type and ectodomain constructs were

described in [2]. The *Mmp1*^{E225A} mutant, also in *pRmHa3*, was in splice isoform 1 (*Mmp1-RD*) and encodes an inactivating mutation at the catalytic core rendering the protein a dominant negative [2, 113]. 16-24h after transfection, cells were washed once in complete medium, and copper sulfate was added to a final concentration of 0.7 mM to induce gene expression from the metallothionine promoter in *pRmHa3*. Transfection with empty *pRmHa3* was used as the vector control. For immunostaining, S2 cells were fixed in 4% formaldehyde for 20 min, washed with PBS, permeabilized in PBS + 0.1% Triton X-100 for 15 min and blocked in PBS + 0.1% Triton X-100+1% NGS + 1% BSA. Triton X-100 was removed from all washing and blocking solutions for nonpermeabilized staining. Primary antibodies used were guinea pig anti-NijA (1:500), rabbit anti-myc (1:500, Abcam), and mouse anti-β-tubulin (1:500) as permeablization control.

S2 cell death assay

At different time points after induction (24h, 40h or 48h), cells were resuspended, mixed with 0.4% trypan blue (Gibco) and applied to a hemocytometer for counting. The percentage of dead cells was calculated by dividing the number of trypan blue positive cells by that of the total cells counted. For counting GFP positive cells, different plasmids were co-transfected with *pRmHa3-GFP*. 48h after the induction, cells were resuspended and 30-50 µl were applied to single wells of a 12-well multi-test slide (MP Biomedicals), allowed to settle for 2h and then fixed and mounted in Vectashield with DAPI. Pictures were taken with a 20× Plan Aprochromat objective on a Zeiss AxioImager M2 microscope. GFP

positive/negative cells were counted from three randomly chosen fields. 600-2500 cells were counted for each sample. For cell death after 100 nM actinomycin D treatment, 5 µg/well dsRNA against NijA was added to 3×10^5 cells in 250 µl serum-free medium in a 24-well plate. After incubation for 1h, 500 µl complete medium was added and 48h later, actinomycin D was added to a final concentration of 100 nM. 6h after actinomycin D treatment, total numbers of living (trypan blue negative) cells were counted using the hemocytometer and the survival rate was determined relative to the untreated (DMSO), wild-type sample.

Acknowledgements:

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CHAPTER III

NINJURIN A MAY BE REQUIRED FOR UV-INDUCED CELL DEATH: PRELIMINARY STUDIES

Introduction:

Chapter II described a cell-death function for NijA. When *NijA* is overexpressed in *Drosophila* hemocytes they undergo nonapoptotic death. I also reported that *NijA* was up-regulated in response to cell stress events. The results from chapter II suggest that the wild-type function of NijA may be to induce cell death in response to stress. The mechanisms that regulate cell death in response to stress have great biomedical importance for the cancer and inflammation fields [68, 70, 76, 114, 115]. If a cell programmed to undergo cell death becomes a persistent cell, it can result in cancerous tissues or auto inflammatory diseases [70, 116]. Persistent cells often become persistent because of mutations in DNA. If these cells do not die through programmed cell death then they can produce more daughter cells with the same mutant DNA. Over time these progeny of a persistent cell can become unchecked and form a cancerous mass [116]. After an immune challenge has been resolved, immune response cells are eliminated through programmed cell death. If these immune response cells are persistent in the absence of an immune stimulus this can lead to tissue damage and autoimmune disorders [108].

Understanding the mechanisms that control cell death is essential for understanding the progression of cancers and autoimmune diseases.

When a cell is exposed to stress it undergoes a recognition and repair process, which ultimately results in one of two outcomes: cellular processes resume or cell death programs are initiated [68, 70, 71, 76, 78, 80, 116]. This chapter focuses on the mechanisms that regulate the cell death outcome after exposure to an UV-induced cellular stress. (UV-irradiation is a light wavelength in the 100-400 nm range.) There are three distinct wavelength ranges for UV-irradiation that have distinct biological consequences. As described in Chapter I, UVC (100-280 nm) irradiation is characterized by a type of DNA damage, thymidine dimers. Thymidine dimers are inappropriate covalent linkages between two pyrimidine bases, and results in a conformational change in the DNA that is recognized by repair enzymes [117]. Thymidine dimers are typically repaired by the nucleotide excision repair mechanism [118], although they can also be repaired through a photolyase dependent process after exposure to light wavelengths greater than 300 nm, a process known as photoreactivation [119]. The sites of DNA damage are distinguished by the phosphorylation of the H2AX histones, now γH2AX, which is a biomarker of DNA damage [120]. The H2AX proteins are homologous to the H2AV protein in *Drosophila* [121]. If the cell is unable to repair the thymidine dimer mutations caused by UV-irradiation then the cell arrests and undergoes death. UV-irradiation can induce any of the three cell death mechanisms discussed in Chapter I: apoptosis, autophagic death, and necroptosis [72-74, 121-124]. The apoptotic and autophagic cell death processes can be dependent on the Caspase family of

proteases [69, 78]. Caspase-3 is an executioner caspase that is a biomarker of late stages of cell death [70, 72, 125, 126]. Cells that contain the thymidine-dimer DNA mutations from UV-irradiation that do not die can lead to melanoma cancers in humans [127].

Recent studies have used UV-irradiation to induce cell death in the dorsal epidermis of third instar larvae to study the nociceptive response of *Drosophila* [72]. Using a modified version of this protocol, I UV-irradiated third instar larvae at a dose sufficient to induce cell death. This method allowed me to examine the role of *NijA* in regulating cell death in response to a cellular stress. All of the data in this chapter are preliminary and were generated using a single Stratalinker as a UV light source. When the UV bulbs in the Stratalinker were exchanged for new bulbs, the dose of UV-irradiation became unreliable, which stymied this investigation.

Despite these caveats, I found that *NijA null* mutants were resistant to cell death after UV-irradiation, as they had significantly fewer cleaved caspase-3 positive cells and pyknotic nulcei. The *NijA null* mutants stained positive for thymidine dimers after UV-irradiation, which indicates that *NijA null* mutants are not resistant to UV-induced DNA damage. There was a reduction in the number of γH2AV positive nuclei of *NijA null* mutants when compared to wild type. This result suggests that *NijA* is required to detect DNA damage, which is usually considered to be a cell autonomous process. These results were surprising because it was unexpected that a trans-membrane protein on the cell surface would mediate DNA damage detection in the nucleus.

Results:

NijA is required for cell death after UV-induced DNA damage

Using a UV Stratalinker, I induced a cellular stress that resulted in cell death in a wild-type larva. Wild-type and $NijA^{D3}$ mutant larvae were anesthetized briefly with cold and were exposed to 20mJ/cm^2 of UVC-irradiation on the dorsal side of the larva. The epidermis was filleted away from the internal organs, fixed, and examined by antibody staining. The cells of >15 wild-type dorsal epidermises were exposed to UV-irradiation were either missing or stained positive for cleaved caspase expression, had nuclei with a pyknotic morphology, and disorganized FasIII staining (septate junction marker) (Fig 3.1 A-B). These results indicated the wild-type cells were dying by programmed cell death in response to UV-induced DNA damage. In contrast, the $NijA^{D3}$ mutant dorsal epidermis cells exposed to UV-irradiation were persistent and expressed limited cleaved caspase (12/14 animals examined) (Fig 3.1 C-D). The $NijA^{D3}$ mutants had significantly fewer cleaved caspase positive cells when compared to the wild type ($p<0.014$) (Fig 3.1 E). These results suggested that *NijA* could be required to induce cell death in response to the cell stress of UV-irradiation.

An alternative conclusion could be that the *NijA null* mutants were resistant to DNA damage, and therefore they were not initiating cell death. To determine if the $NijA^{D3}$ mutants were resistant to DNA damage, epidermal samples of UV-irradiated larvae were antibody stained for thymidine dimers. Both the $NijA^{D3}$ and the wild-type cells exposed to UV-irradiation stained positively for thymidine

dimers, which indicates that the DNA of *NijA null* mutants was damaged from the UV-irradiation (Fig 3.2 A-C).

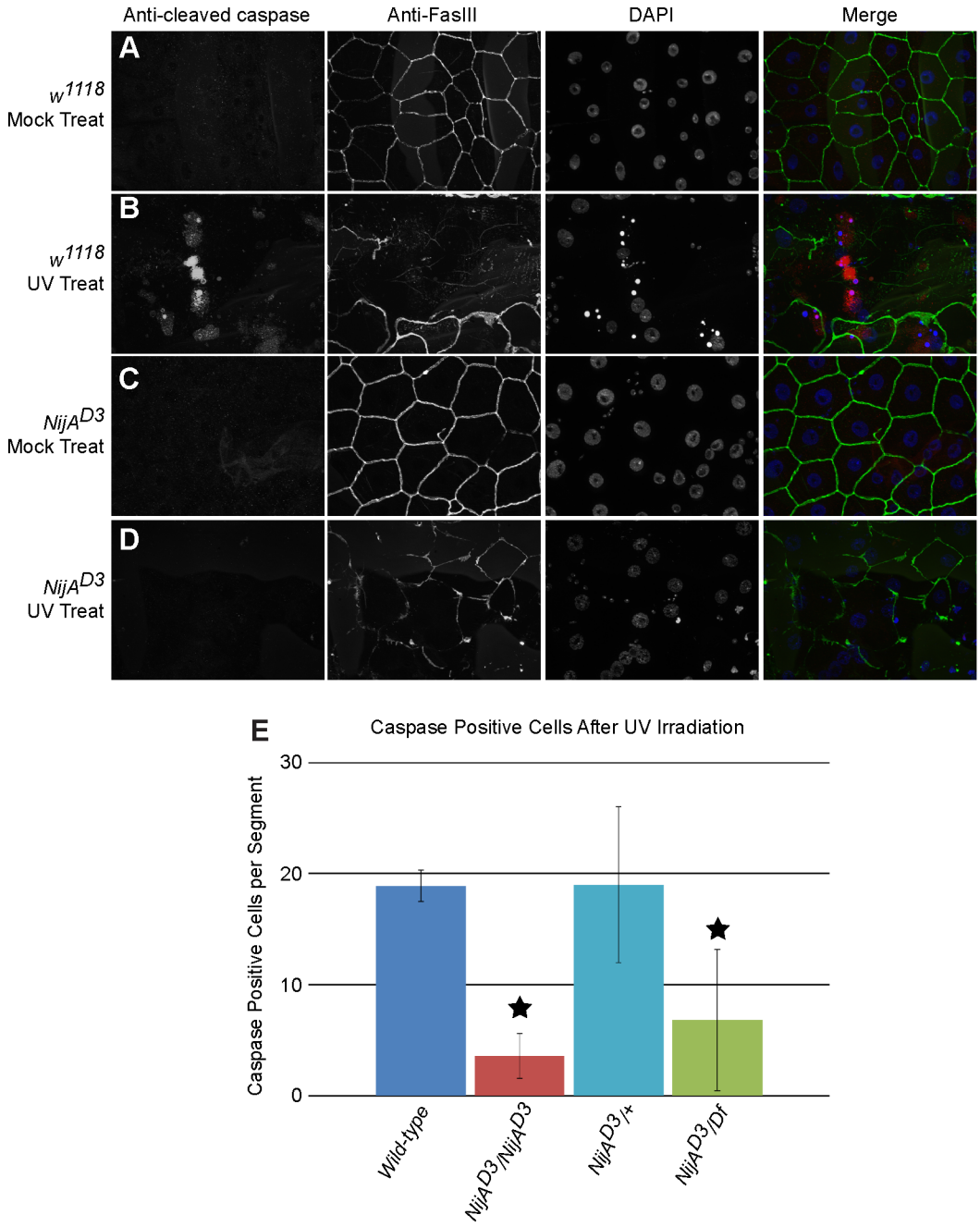

Figure 3.1 *Ninjurin A* may be required for UV-irradiation induced cell death (Preliminary Results).
(A-D) Dorsal epidermis of third instar larvae antibody stained for anti-cleaved caspese in red, anti-FasIII in green, and stained for DAPI in blue. *NijA*^{D3} larvae have fewer cleaved capsase-positive cells. **(E)** The number of cleaved caspase-positive cells per segment was quantified, and there are statistically fewer ($p<0.05$) cleaved caspase-postive cells in both the UV-irradiated *NijA*^{D3} mutants and the *NijA*^{D3}*/Df(3L)BSC377* transheterozygotes than the wild type.

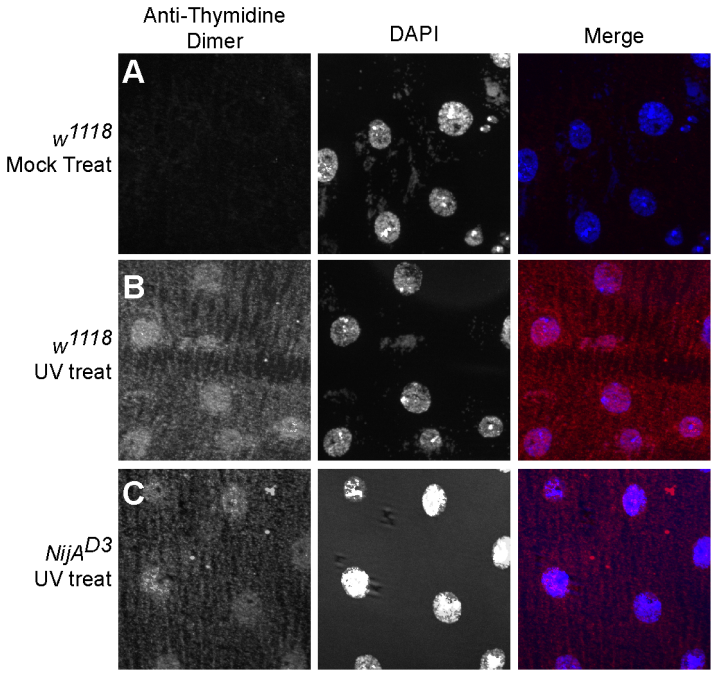

Figure 3.2 *Ninjurin A null* mutants are not resistant to UV-induced DNA damage. (Preliminary Results)
(A-C) Dorsal epidermis of either mock or UV-irradiated larvae antibody stained for thymidine dimers in red and DAPI stained in blue. The *NijA*^{D3} mutants stain positive for thymidine dimers after UV-irradiation.

Preliminary data suggests *NijA* may be required for DNA damage detection

To determine at what point the $NijA^{D3}$ larvae were unable to induce cell death I began systematically examining biomarkers for the first stages of DNA damage repair. One of the first cellular changes is phosphorylation of the H2AV histone to make γH2AV at the sites of DNA damage. I examined the dorsal epidermis of wild-type and *NijA* mutant larvae after UV-irradiation for γH2AV staining. The $NijA^{D3}$ larvae had qualitatively fewer γH2AV positive nuclei than the wild type (6/6 animals) (Fig 3.3 A-B). These results suggest that *NijA* could be required for DNA damage detection. It was counterintuitive to model how a membrane-localized protein, NijA, could be regulating DNA damage detection in the nucleus. Instead we hypothesized that NijA was non-cell autonomously sensitizing neighboring cells to DNA damage detection, a model that will be discussed in further detail in the discussion.

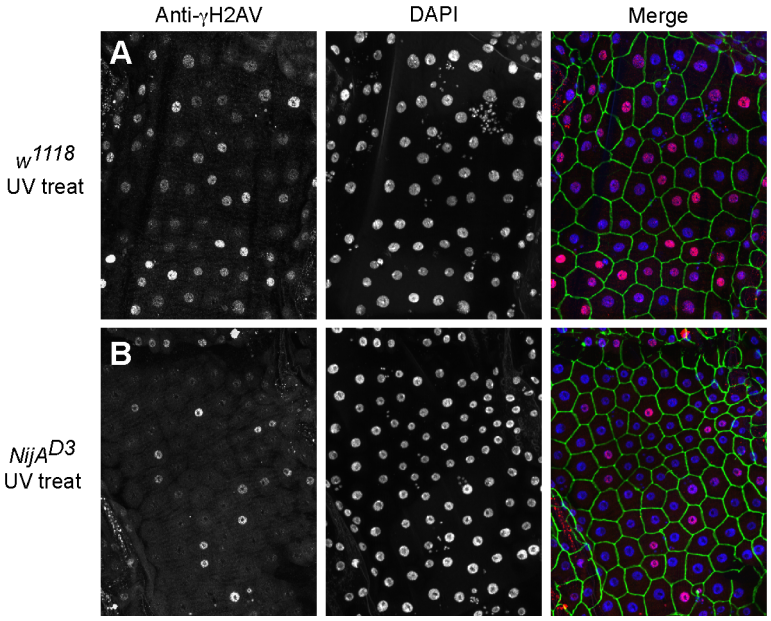

Figure 3.3 *Ninjurin A* maybe required for H2AV modification after UV-irradiation. (Preliminary Results)
(A-B) Dorsal epidermis of UV-irradiated larvae stained for anti-γH2AV in red, anti-FasIII in green, and DAPI stained in blue. There are qualitatively fewer γH2AV positive nuclei in the *NijA*^{D3} mutants.

NijA increases expression at the cell surface of the dorsal epidermis after UV-irradiation

In chapters I and II, I extensively discussed the increase in NijA at the cell surface and in whole protein lysates after the cellular stress of septic injury. I wanted to know if this was also the case after the cellular stress of UV-irradiation. The localization of NijA was examined in an unpermeabilized sample of wild-type dorsal epidermis after UV-irradiation using the anti-NijA antibody. I was able to qualitatively detect an increase in NijA at the cell surface of the dorsal epidermis of the larvae exposed to UV-irradiation in 4/6 samples. There was no change in Ninjurin expression in 2/6 samples examined (Fig 3.4 A-B).

I assessed the changes in *NijA* mRNA by qPCR to quantitatively assess the increase in NijA after UV-irradiation. I was unable to detect a significant ($p<0.05$) increase in *NijA* expression in the dorsal epidermis of wild-type larvae exposed to UV-irradiation (Fig 3.4 C). Indeed, the opposite occurred: these data suggest that there was a decrease in the cellular mRNA for *NijA* in the dorsal tissues, and an increase in cellular mRNA for *NijA* in the ventral epidermis. These results were unexpected because the general expectation is for protein levels to increase as a result of increased transcription at the gene locus. Closer inspection of these preliminary results suggest that perhaps the translation of NijA could be triggering a feedback-mediated decrease in transcription to prevent an over abundance of NijA protein from being made. This could be preliminary evidence that there are two levels of NijA expression control both at the transcriptional and translational levels.

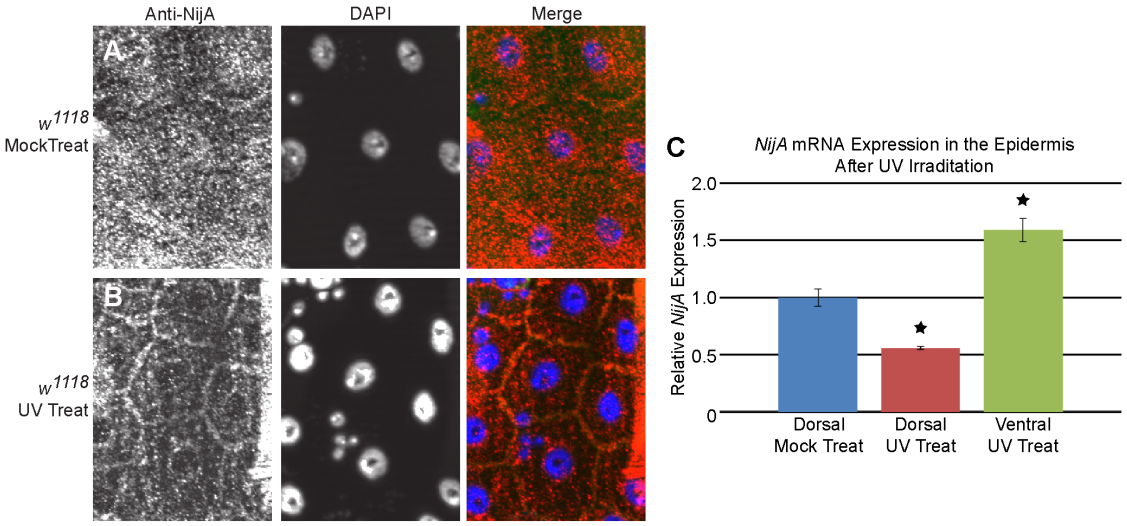

Figure 3.4 Ninjurin A protein increases expression at the cell surface after UV-irradiation. **(A-B)** Unpermeabilized dorsal epidermis of wild-type larvae after UV-irradiation antibody stained with anti-NijA in red and DAPI stained in blue. There is an increase in NijA protein detected at the cell surface after UV-irradiation. **(C)** Quantitative PCR results of *NijA* mRNA expression in the epidermis after UV-irradiation or mock treatment after normalization to rp49 expression. Surprisingly, there was a significant ($p<0.05$) decrease in the *NijA* mRNA in the irradiated dorsal epidermis after UV-irradiation, and a significant increase in *NijA* mRNA in the non-irradiated ventral epidermis after UV-irradiation.

Discussion:

The preliminary data presented in this chapter suggest that *NijA* may be required for stress-induced cell death. The *NijA null* mutants were not resistant to DNA damage; however both *NijA* homozygotes and transheterozygotes (over deficiency) failed to undergo cell death in response to UV-irradiation in a manner similar to wild type. *NijA null* mutants did not modify the H2AV histone to the same extent as wild type in response to UV-irradiation, which suggests that *NijA* is required to detect DNA damage in the nucleus. There was an increase in NijA protein expression at the cell surface in response to UV-irradiation, but this increase was not due to an increase in transcription. The increase in NijA expression at the cell surface is consistent with work reported in Chapters I and II, which suggests that NijA localization to the cell surface is important for inducing cell death. The *NijA null* phenotype, cells persisting under conditions that induce cell death in wild-type cells, is the opposite of the *NijA* over-expression phenotype, in which nonapoptotic death is induced in cells in the absence of a stress event. Although there is no rescue confirmation that *NijA* is the gene responsible for stress induced cell death, it is noteworthy that the loss of *NijA* and the over-expression of *NijA* have opposite effects.

The reduction in cleaved caspase staining in the *NijA null* mutant suggests that NijA is regulating a caspase-dependent cell death pathway. Both apoptosis and autophagic cell death processes can induce effector caspase cleavage [77, 78, 80, 124, 125, 128, 129]. Since the over-expression of *NijA* induces nonapoptotic cell death, I would hypothesize that NijA is regulating autophagic cell death in response

to cell stress. Previous reports have elucidated the pathways regulating UV-induced autophagy in mammalian cell culture [130]. It is possible that NijA is regulating this autophagic pathway to promote DNA damage sensitization after UV-irradiation.

The *NijA null* mutant also has a reduction in γH2AV positive nuclei when compared to wild type, which suggests that NijA is regulating DNA damage detection in the nucleus after UV-irradiation. The requirement for NijA, a membrane bound protein that signals non-cell autonomously in S2 cell culture, for the nuclear detection of DNA damage is surprising. The bystander effect could account for these unexpected results. In mammalian cell culture UV-irradiated cells can signal non-autonomously to unexposed neighbor cells to initiate DNA damage repair in the undamaged cell [131, 132]. The bystander effect suggests that DNA damage repair can be initiated non-cell autonomously, which could explain how a cell surface protein, like NijA, could regulate DNA damage detection. Although the bystander effect is an interesting phenomenon there are no *in vivo* biological contexts for this observation, only cell culture. Similarly, there is no evidence to suggest NijA is functioning non-autonomously in these contexts, although NijA has been reported to act non-cell autonomously in cell culture to induce loss-of-adhesion [2].

These preliminary results suggest that NijA could be required to regulate a caspase-dependent cell death by detecting DNA damage in response to UV-irradiation. These preliminary studies coupled with the results of Chapter II suggest that fine control over the amount of NijA protein is required to maintain the homeostatic balance between cellular life and death.

Materials and Methods:

UV-Irradiation:

Male third instar larvae were collected from the food of healthy bottles. Larvae were briefly anesthetized on a 4^0C cold block on pre-chilled grape juice plates for less than four minutes. An alternative anesthetization method was explored after experiments examining JNK expression indicated that the brief cold treatment was inducing the JNK stress response process (Fig. A1). Anesthetization after 2mins of ether exposure was sufficient to anesthetize the larvae, and did not induce JNK expression. Once anesthetized the larvae were oriented dorsal side up and exposed to 20mJ/cm^2 of UVC irradiation from a UV-Stratalinker, or mock treated larvae were placed for the same length of time just outside the Stratalinker box on the bench. Wet yeast was added to the plate, and the larvae were allowed to recover at 25^0C in a humidified incubator for 24hrs. The larval epidermis was dissected away from the internal organs and prepared for antibody staining as described in Chapter II.

CHAPTER IV

CONCLUDING REMARKS

Summary:

This thesis reports a cell death function for the stress-induced NijA protein. The idea of stress-induced cell death is not a new one, and it has been discovered and addressed in many different biological contexts. Stress stimuli can range from glucose starvation, which causes an accumulation of unfolded proteins in the ER triggering the unfolded protein response to the DNA damage response [68]. Stress induced cell death is a conserved and essential cellular function that promotes the homeostasis of an organism [68, 76, 128]. When cells are unable to induce cell death in response to stress the remaining persistent cells can be damaging to the surrounding tissues [78, 123, 128]. For example, when immune response cells are unable to undergo neutrophil clearance after an immune challenge has been resolved, the persistent cells can cause inflammation in the absence of a stimulus [108]. This inflammation is damaging to the surrounding tissues and could lead to the potentially lethal autoimmune disorder, sepsis [70]. Cells with aberrant cell cycle regulation are eliminated by cell death mechanisms to prevent the persistence of cells with uncontrolled proliferation, which can be deleterious to the function of a tissue [123, 133]. In extreme cases, cells with unchecked cell cycle regulation can lead to reduced organ function and even cancer [134]. Understanding the

mechanisms that regulate cell death is important to understanding the fundamental cellular process of homeostasis maintenance.

I reported that *NijA* over-expression induces a nonapoptotic cell death in Chapter II. The phrase "nonapoptotic cell death" suggests two alternative cell death mechanisms *NijA* over-expression could be inducing: necroptosis and autophagic cell death. (This is assuming that NijA over-expression is not inducing a novel cell death pathway.) Preliminary data suggest that *NijA* is required for caspase activation after UV-irradiation, and since necroptosis is not mediated by caspases these results suggest that *NijA* over-expression could induce an autophagic cell death mechanism [84]. Autophagy is a process that promotes the formation of a double-membrane vesicle that isolates cellular components, and targets the vesicle contents to the lysosome for degradation [78, 80, 82]. Autophagy not only promotes autophagic cell death but a plethora of biological processes from signal transduction to viral pathogen clearance [79]. It is possible that the cell death I observed was a result of robust *NijA* over-expression by the UAS-Gal4 system but did not reveal NijA's normal function. The UAS-Gal4 system drives the expression of *NijA* far beyond the biological levels of stress induced *NijA* expression. This robust *NijA* expression could be inducing robust autophagosome formation, which ultimately would promote a cell autonomous death when taken to non-physiological levels. In this model, the normal function of NijA is to promote autophagosomes, which in turn would promote one of the many other biological processes mediated by autophagy, such as immune clearance. In the future, the requirement for autophagosome formation in NijA- induced cell death could be analysed in an

Autophagy-specific gene (Atg) mutant background. Atg genes are required for autophagosome formation [80].

Although it is unclear if autophagosome formation is required for NijA-induced cell death there is a little data indicating that *NijA* is not required for autophagic cell death processes in development. Preliminary data suggest that *NijA* is not required for the developmentally programmed autophagic cell-death dependent regression of the gastric caeca in *Drosophila* metamorphosis (personal communication from John Cao) [78, 135]. These data could indicate that NijA is mediating an autophagy process independent of autophagic cell death. It is also interesting that *NijA* is not required for developmentally programmed cell death in the embryo. This result fits with the onset of *NijA* expression in the late stages of embryogenesis. It is unknown if *NijA* is required for the stress induced autophagy processes like viral clearance [79].

Previous reports suggest that NijA can both promote and inhibit cell death [7, 22]. It is interesting to note that autophagy can also promote cell survival as well as cell death in a context specific manner [78, 80]. This might account for the conflicting functions for the Ninjurin family in the literature. Some studies suggest that Ninjurin 1 inhibition promotes cell survival while others suggest that the inhibition of Ninjurin 1 is sufficient to induce cell death [7, 11]. The preliminary data reported in Chapter III also suggests that Ninjurins may be required to induce cell death. This functional dichotomy would fit with the literature surrounding autophagy regulation.

Current literature and this thesis often conflict on the reported function for the Ninjurin family of proteins. Previous reports investigating the role of Ninjurin 1 in cell death have relied on a neutralizing antibody thought to inhibit Ninjurin 1 protein function [1, 5, 7, 22]. Unfortunately there were no controls done to determine if the neutralizing antibody functioned as an inhibitor. Previous reports suggest that Ninjurin 1 functions by homophilic interaction [5]. It is tempting to speculate that the Ninjurin 1 neutralizing antibody may promote dimerization of the Ninjurin 1 protein by increasing the proximity of Ninjurin 1 proteins. Dimerization could promote Ninjurin 1 function instead of inhibiting function, and if so the antibody would yield similar phenotypes to the over-expression of the Ninjurin 1 protein. This confusion about the function of the Ninjurin 1 neutralizing antibody could account for some of the disparities in the published literature from my results that the over-expression of NijA promotes cell death. Without further experiments to test how the Ninjurin 1 antibody acts on Ninjurin 1, it is not possible to draw conclusions from experiments using this antibody to determine Ninjurin 1 function.

I reported in Chapter III that *NijA* might be required for the modification of the H2AV histone near sites of DNA damage. The modification of H2AV to γH2AV has been previously used as an indicator of DNA damage [121]. These results were surprising because Ninjurin A is localized to the cell membrane. It is difficult to explain how a protein not typically detected in the nucleus could be affecting a process there. Wild-type cells exposed to UV-irradiation have variable intensities of γH2AV staining; however they all appear to have some increase γH2AV staining

when compared to unirradiated controls. There are many possible reasons for this variability. The first obvious reason is that DNA damage induction is stochastic, although the large DNA target size within each nucleus should average out the noise to give fairly similar responses from nucleus to nucleus if they were equally sensitive and received the same dose. However, nuclei may not be equally sensitive, as their asynchronous endoreduplicating cell cycles would configure the DNA differently, and replicating DNA is likely to be more exposed to UV damage than than condensed interphase DNA. Additionally, during normal DNA replication double stranded breaks can occur even in the absence of UV, and the same mechanism of γH2AV directed repair occurs during this process [121], although this level of baseline repair would be apparent in the unirradiated controls. Another source of variability could be the efficiency of the cell to repair damage. γH2AV remains phosphorylated at the site of DNA damage until repair is successful [121]. It is possible that the less intensely stained nuclei have repaired the DNA damage faster. Despite the variability of the intensity of γH2AV staining in wild type exposed to UV, preliminary data suggests that the *NijA*^{D3} mutants do not detect the DNA damage to the same extent as the wild type.

As discussed in Chapter III, the bystander effect could account for the role of a cell membrane protein in regulating DNA damage detection. The bystander effect posits that cell-cell communication occurs when cells are damaged by UV-irradiation. This cell-cell communication is sufficient to activate DNA repair in unexposed cells. An observation that supports the hypothesis that NijA is required for this cell-cell communication is the presence of few robustly positive γH2AV cells

in the *NijA null* mutants, and almost none of the lower intensity γH2AV positive cells. Perhaps a few cells can initiate damage detection cell autonomously (high intensity γH2AV staining) but most cells require a nonautonomous signal from damaged neighbor cells to initiate damage repair (low intensity γH2AV staining). It is tempting to speculate that one source of the variability seen in the wild-type samples could be from proximity to a "NijA competent" cell, which is sensitizing neighbor cells to DNA damage. An alternative hypothesis is that *NijA null* mutants are exceptionally efficient at DNA repair eliminating the less intense nuclei staining. This hypothesis is less appealing because it does not account for the cell membrane localization of NijA after UV-irradiation or the persistence of UV damaged cells 24hrs after irradiation. Further experiments using clones to assess the fate of wild-type cells neighboring *NijA null mutant* cells after UV-irradiation will need to be done to determine if this hypothesis is valid.

I reported in Chapter III that NijA increases expression at the cell surface in response to the stress of septic injury and UV-irradiation. I also reported that the activation of the Toll pathway is sufficient to increase NijA at the cell surface of the *Drosophila* fat body. The common factor among these mechanisms is the localization of NijA to the cell surface. Over-expression of *NijA* mutations in cell culture that were unable localize appropriately to the cell surface were insufficient to induce cell death. These data combined suggest that the localization of NijA to the cell surface is important for promoting cell death. While localization is important to NijA function there are at least two kinds of NijA regulation, transcription and translocation. After septic injury in adults and after Toll activation I was able to detect an increase in

transcription of NijA. In contrast, after septic injury in larvae and UV-irradiation of larvae I was only able to detect an increase in NijA expression at the cell surface suggesting a translocation independent of transcriptional changes. Although there appear to be two levels of NijA regulation, the localization of NijA to the cell surface appears to be essential for NijA function.

Conclusions and Future Directions:

In Chapter II, I discussed cases where both the $NijA^{D3}$ homozygous mutant and the $NijA^{D3}/+$ heterozygous mutants exhibited a mutant phenotype. These results indicate that there is a mutation in another gene on the same chromosome as the $NijA^{D3}$ mutation, known as a second-site mutation, causing this phenotype. The presence of at least one second-site mutation stymied my attempts to assess the role of *NijA* in regulating phagocytosis, starvation, and Foxo localization (Figs 2.5A, 2.6, and A2). Although the $NijA^{D3}$ deletion was generated from a strain that was outcrossed three times to a wild-type stock before P-element excision, three outcrosses would only be expected to replace 87.5% of the genome on average, and in the case of the $NijA^{D3}$ allele this was clearly not enough. In the *Drosophila* $NijA^{D3}$ stock, the homozygotes carrying the $NijA^{D3}$ mutation are present but they do not outnumber the balanced heterozygotes, as would be expected if the homozygotes were perfectly healthy and fertile; rather, the multiply inverted balancer chromosome is maintained, even though balancers are selected against when competing with wild-type chromosomes. The mutation on the $NijA^{D3}$ chromosome that confers this selection weakness could be either the $NijA^{D3}$ mutation or the

second-site mutation that is plaguing the mutant analysis. Using currently available tools, the only way to assess the requirement for *NijA* without using the $NijA^{D3}$ mutant would be RNAi analysis. There is a strong RNAi line I used in Chapter II that would be useful for *NijA* loss-of-function analysis in the future but a clean *NijA null* mutation is an invaluable tool. Isolating a brand new *NijA* null mutant after extensively outcrossing the starting chromosome to randomly eliminate second site mutations could eliminate the second-site mutation. This would be labor intensive but valuable considering the consistency of the results for starvation and Foxo localization suggesting that a mutation on the third chromosome is regulating the starvation stress response, which is also a process that is regulated by autophagy. Pathogen clearance is also regulated by autophagy, and I was unable to assess the requirement for NijA in this process as well because of second site mutations.

Previous research suggests that Ninjurins regulate adhesion. In mammalian cell culture over-expression of Ninjurin1 promotes homophillic cell aggregation, which suggests an increase in cell-cell adhesion [1]. In *Drosophila* cell culture over-expression of NijA reduces cell-substrate adhesion, and Xiaoxi Wang observed an adhesion reduction prior to the cell death reported in Chapter II (Fig 2.9 and 2.11) [2]. Preliminary experiments over-expressing a weak insertion of *NijA* in the larval epidermis resulted in a breakdown in the cell membrane markers FasIII (at septate junctions) and Armadillo (aka β-catenin, at adherens junctions). The dead larvae over-expressing *NijA* in the epidermis appeared deflated and were surrounded by a pool of hemolymph, as if the hemolymph had leaked out of the larvae through the epidermis. These preliminary results could indicate that the cells are loosing

adhesion prior to cell death *in vivo* as well (Fig A3). As the cells lose adhesion the pro-survival signals provided by the extracellular matrix and neighbor cells are also lost, and this process results in a cell death mechanism known as anoikis. Recently anoikis was described as a response to the cellular stress of over-crowding [71]. During metamorphosis the *Drosophila* notum, an epithelial sheet that experiences cell crowding, eliminates superfluous cells through anoikis [71]. Because NijA is up regulated during metamorphosis, NijA is implicated in regulating adhesion prior to cell death, and *NijA* over-expression is sufficient to induce cell death. It could be valuable to investigate if *NijA* regulates the anoikis response to cellular crowding.

I have reported in Chapter II that NijA is up-regulated at the cell surface of immune response cells after septic injury, and in Chapter III that NijA over-expression induces nonapoptotic cell death. It seems counter intuitive to increase the expression of a cell death inducer after septic injury in a population of cells required to respond to infection. Immune cell death after immune challenge, a process called neutrophil clearance, has been previously reported in mammalian systems. Neutrophil clearance is essential to immune response recovery. After infection mammalian systems are known to dramatically increase the number of circulating neutrophils to attenuate the immune challenge. When the immune challenge is resolved the immune system needs to recover to a pre-challenge state, which involves the programmed cell death of the superfluous neutrophils [108]. *Drosophila* also increases the number of circulating surveillance cells, the hemocytes, in response to an immune challenge, and $NijA^{D3}$ mutants are capable of increasing the number of circulating hemocytes. What is unclear is if this population

of new hemocytes is cleared through programmed cell death during the recovery from immune challenge. This would be promising area for future NijA research. The recovery of the *Drosophila* larval immune response is relatively unresearched, and this could be due to the short duration of the larval stages. Some preliminary studies on wild-type larvae would be required to determine if the recovery of the larval immune response is a feasible area for future research. Another indirect method to assess immune system recovery in adult *Drosophila* would be to examine egg-laying. It has been found that the cost of responding to immune challenge in adult *Drosophila* is a reduction in the fecundity [136]. If *NijA* is required for cell-death mediated immune system recovery, they should experience a persistent decrease in fecundity after immune challenge. Future experiments could assay fecundity of $NijA^{D3}$ *null* mutants at a time point when wild-type animals have recovered, and I hypothesize that the $NijA^{D3}$ mutants will have a persistent decrease in fecundity.

An alternative explanation for the expression of NijA in a population of cells that are responding to infection is the wild type function of NijA might not be to induce cell death. Instead NijA may be required to activate autophagic processes like pathogen clearance. NijA is increased at the cell surface of circulating hemocytes after immune challenge, and Toll over-activation is sufficient to induce NijA expression. Previous reports suggest that autophagy is required for viral pathogen clearance in a Toll-7 mediated manner, and perhaps NijA could be mediating this autophagic dependent process [79]. Future experiments on the role of NijA in viral pathogen clearance would need to be done to test this hypothesis.

Significance:

With the advent of the whole genome sequence and current bioinformatics annotation it has become starkly apparent that we understand the function of only a fraction of the predicted genes in our genomes. *Ninjurins* are among those genes with limited functional data, although there is a large body of expression data suggesting a conserved and seemingly important function across species. By examining the function of novel genes we expand the breadth of scientific knowledge when it is often tempting to limit ourselves to expanding the depth of knowledge of a well known process.

While expanding the breadth of knowledge by examining the function of the novel gene, NijA, in *Drosophila* I also added to the cancer biology and immunology fields. Preliminary data suggests that in the absence of *NijA,* cells damaged by UV-irradiation are unable to die and become persistent cells. Persistent cells are extremely dangerous to tissue function and integrity, and persistent cells can ultimately result in cancers. UV-irradiation induced cell damage is strongly correlated with an increase in skin melanomas, a type of cancer [123].

The work on the Toll pathway pioneered in the *Drosophila* immune system led to discoveries of a conserved family of proteins in mammals call the Toll-Like Receptors (TLRs). Previously it was unknown that the NijA protein is one of the genes regulated downstream of Toll activation. My work provides deeper understanding of how this process could be functioning. Regulating cell death after immune response is essential to control the deleterious effects of an over activated immune system that can lead to many autoimmune disorders. Further research into

the function of the Ninjurin family members could provide further insights into autophagic mediated processes and Toll mediated immune responses.

WORKS CITED

1. Araki, T. and J. Milbrandt, *Ninjurin, a novel adhesion molecule, is induced by nerve injury and promotes axonal growth.* Neuron, 1996. **17**(2): p. 353-61.
2. Zhang, S., et al., *An MMP liberates the Ninjurin A ectodomain to signal a loss of cell adhesion.* Genes Dev, 2006. **20**(14): p. 1899-910.
3. Ahn, B.J., et al., *Ninjurin1 is expressed in myeloid cells and mediates endothelium adhesion in the brains of EAE rats.* Biochemical and Biophysical Research Communications, 2009. **387**(2): p. 321-325.
4. Araki, T. and J. Milbrandt, *Ninjurin2, a novel homophilic adhesion molecule, is expressed in mature sensory and enteric neurons and promotes neurite outgrowth.* J Neurosci, 2000. **20**(1): p. 187-95.
5. Araki, T., et al., *Mechanism of homophilic binding mediated by ninjurin, a novel widely expressed adhesion molecule.* J Biol Chem, 1997. **272**(34): p. 21373-80.
6. Broderick, S., et al., *Drosophila Ninjurin A induces nonapoptotic cell death.* PLoS One, 2012. **7**(9): p. e44567.
7. Lee, H.J., et al., *Ninjurin1 mediates macrophage-induced programmed cell death during early ocular development.* Cell Death Differ, 2009. **16**(10): p. 1395-407.
8. Koike, M., Y. Ninomiya, and A. Koike, *Characterization of Ninjurin and TSC22 induction after X-irradiation of normal human skin cells.* J Dermatol, 2008. **35**(1): p. 6-17.
9. Toyama, T., et al., *Ninjurin1 increases p21 expression and induces cellular senescence in human hepatoma cells.* J Hepatol, 2004. **41**(4): p. 637-43.
10. Kim, J.W., et al., *Up-Regulation of ninjurin expression in human hepatocellular carcinoma associated with cirrhosis and chronic viral hepatitis.* Mol Cells, 2001. **11**(2): p. 151-7.
11. Ahn, B.J., et al., *Ninjurin1 is expressed in myeloid cells and mediates endothelium adhesion in the brains of EAE rats.* Biochem Biophys Res Commun, 2009. **387**(2): p. 321-5.
12. Buchon, N., et al., *Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation.* Cell Host Microbe, 2009. **5**(2): p. 200-11.
13. Boutros, M., H. Agaisse, and N. Perrimon, *Sequential activation of signaling pathways during innate immune responses in Drosophila.* Dev Cell, 2002. **3**(5): p. 711-22.
14. Di Giovanni, S., et al., *In vivo and in vitro characterization of novel neuronal plasticity factors identified following spinal cord injury.* J Biol Chem, 2005. **280**(3): p. 2084-91.
15. De Gregorio, E., et al., *Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays.* Proc Natl Acad Sci U S A, 2001. **98**(22): p. 12590-5.

16. Njau, F., et al., *Restriction of Chlamydia pneumoniae replication in human dendritic cell by activation of indoleamine 2,3-dioxygenase.* Microbes Infect, 2009. **11**(13): p. 1002-10.
17. Ma, S.F., et al., *Bioinformatic identification of novel early stress response genes in rodent models of lung injury.* Am J Physiol Lung Cell Mol Physiol, 2005. **289**(3): p. L468-77.
18. Miyairi, I., et al., *The p47 GTPases Iigp2 and Irgb10 regulate innate immunity and inflammation to murine Chlamydia psittaci infection.* J Immunol, 2007. **179**(3): p. 1814-24.
19. Graveley, B.R., et al., *The developmental transcriptome of Drosophila melanogaster.* Nature, 2011. **471**(7339): p. 473-9.
20. Rubin, G.M., et al., *A Drosophila complementary DNA resource.* Science, 2000. **287**(5461): p. 2222-4.
21. Iwai, Y., et al., *Axon patterning requires DN-cadherin, a novel neuronal adhesion receptor, in the Drosophila embryonic CNS.* Neuron, 1997. **19**(1): p. 77-89.
22. Jerregard, H., *Sensory neurons influence the expression of cell adhesion factors by cutaneous cells in vitro and in vivo.* J Neurocytol, 2001. **30**(4): p. 327-36.
23. Lemaitre, B. and J. Hoffmann, *The host defense of Drosophila melanogaster.* Annu Rev Immunol, 2007. **25**: p. 697-743.
24. Janeway, C.A., Jr., *How the immune system protects the host from infection.* Microbes Infect, 2001. **3**(13): p. 1167-71.
25. Pham, L.N., et al., *A specific primed immune response in Drosophila is dependent on phagocytes.* PLoS Pathog, 2007. **3**(3): p. e26.
26. Lanot, R., et al., *Postembryonic hematopoiesis in Drosophila.* Dev Biol, 2001. **230**(2): p. 243-57.
27. Williams, M.J., *Drosophila hemopoiesis and cellular immunity.* J Immunol, 2007. **178**(8): p. 4711-6.
28. Babcock, D.T., et al., *Circulating blood cells function as a surveillance system for damaged tissue in Drosophila larvae.* Proc Natl Acad Sci U S A, 2008. **105**(29): p. 10017-22.
29. Kurucz, E., et al., *Definition of Drosophila hemocyte subsets by cell-type specific antigens.* Acta Biol Hung, 2007. **58 Suppl**: p. 95-111.
30. Irving, P., et al., *New insights into Drosophila larval haemocyte functions through genome-wide analysis.* Cell Microbiol, 2005. **7**(3): p. 335-50.
31. Xavier, M.J. and M.J. Williams, *The Rho-family GTPase Rac1 regulates integrin localization in Drosophila immunosurveillance cells.* PLoS One, 2011. **6**(5): p. e19504.
32. Russo, J., et al., *Insect immunity: early events in the encapsulation process of parasitoid (Leptopilina boulardi) eggs in resistant and susceptible strains of Drosophila.* Parasitology, 1996. **112 (Pt 1)**: p. 135-42.
33. Crozatier, M. and A. Vincent, *Drosophila: a model for studying genetic and molecular aspects of haematopoiesis and associated leukaemias.* Dis Model Mech, 2011. **4**(4): p. 439-45.
34. Krzemien, J., et al., *Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre.* Nature, 2007. **446**(7133): p. 325-8.

35. Minakhina, S., W. Tan, and R. Steward, *JAK/STAT and the GATA factor Pannier control hemocyte maturation and differentiation in Drosophila.* Dev Biol, 2011. **352**(2): p. 308-16.
36. Sorrentino, R.P., J.P. Melk, and S. Govind, *Genetic analysis of contributions of dorsal group and JAK-Stat92E pathway genes to larval hemocyte concentration and the egg encapsulation response in Drosophila.* Genetics, 2004. **166**(3): p. 1343-56.
37. Holz, A., et al., *The two origins of hemocytes in Drosophila.* Development, 2003. **130**(20): p. 4955-62.
38. Markus, R., et al., *Sessile hemocytes as a hematopoietic compartment in Drosophila melanogaster.* Proc Natl Acad Sci U S A, 2009. **106**(12): p. 4805-9.
39. Evans, C.J., V. Hartenstein, and U. Banerjee, *Thicker than blood: conserved mechanisms in Drosophila and vertebrate hematopoiesis.* Dev Cell, 2003. **5**(5): p. 673-90.
40. Jung, S.H., et al., *The Drosophila lymph gland as a developmental model of hematopoiesis.* Development, 2005. **132**(11): p. 2521-33.
41. Grigorian, M., L. Mandal, and V. Hartenstein, *Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the Drosophila lymph gland.* Dev Genes Evol, 2011. **221**(3): p. 121-31.
42. Evans, C.J. and U. Banerjee, *Transcriptional regulation of hematopoiesis in Drosophila.* Blood Cells Mol Dis, 2003. **30**(2): p. 223-8.
43. Lemaitre, B., et al., *A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense.* Proc Natl Acad Sci U S A, 1995. **92**(21): p. 9465-9.
44. Gottar, M., et al., *The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein.* Nature, 2002. **416**(6881): p. 640-4.
45. Choe, K.M., et al., *Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila.* Science, 2002. **296**(5566): p. 359-62.
46. Dushay, M.S., B. Asling, and D. Hultmark, *Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of Drosophila.* Proc Natl Acad Sci U S A, 1996. **93**(19): p. 10343-7.
47. Han, Z.S. and Y.T. Ip, *Interaction and specificity of Rel-related proteins in regulating Drosophila immunity gene expression.* J Biol Chem, 1999. **274**(30): p. 21355-61.
48. Hedengren, M., et al., *Relish, a central factor in the control of humoral but not cellular immunity in Drosophila.* Mol Cell, 1999. **4**(5): p. 827-37.
49. Stoven, S., et al., *Activation of the Drosophila NF-kappaB factor Relish by rapid endoproteolytic cleavage.* EMBO Rep, 2000. **1**(4): p. 347-52.
50. Hu, X., et al., *Multimerization and interaction of Toll and Spatzle in Drosophila.* Proc Natl Acad Sci U S A, 2004. **101**(25): p. 9369-74.
51. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.

52. Ramet, M., et al., *Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli.* Nature, 2002. **416**(6881): p. 644-8.
53. Matova, N. and K.V. Anderson, *Drosophila Rel proteins are central regulators of a robust, multi-organ immune network.* J Cell Sci, 2010. **123**(Pt 4): p. 627-33.
54. Schneider, D.S., et al., *Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the Drosophila embryo.* Genes Dev, 1991. **5**(5): p. 797-807.
55. Adams, M.D., et al., *The genome sequence of Drosophila melanogaster.* Science, 2000. **287**(5461): p. 2185-95.
56. Engstrom, Y., et al., *kappa B-like motifs regulate the induction of immune genes in Drosophila.* J Mol Biol, 1993. **232**(2): p. 327-33.
57. Ip, Y.T. and M. Levine, *Molecular genetics of Drosophila immunity.* Curr Opin Genet Dev, 1994. **4**(5): p. 672-7.
58. Ip, Y.T., et al., *Dif, a dorsal-related gene that mediates an immune response in Drosophila.* Cell, 1993. **75**(4): p. 753-63.
59. Uvell, H. and Y. Engstrom, *Functional characterization of a novel promoter element required for an innate immune response in Drosophila.* Mol Cell Biol, 2003. **23**(22): p. 8272-81.
60. Becker, T., et al., *FOXO-dependent regulation of innate immune homeostasis.* Nature, 2010. **463**(7279): p. 369-73.
61. Brennan, C.A., et al., *Psidin is required in Drosophila blood cells for both phagocytic degradation and immune activation of the fat body.* Curr Biol, 2007. **17**(1): p. 67-72.
62. Rosetto, M., et al., *Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line.* Biochem Biophys Res Commun, 1995. **209**(1): p. 111-6.
63. Samakovlis, C., et al., *In vitro induction of cecropin genes--an immune response in a Drosophila blood cell line.* Biochem Biophys Res Commun, 1992. **188**(3): p. 1169-75.
64. Ekengren, S., et al., *A humoral stress response in Drosophila.* Curr Biol, 2001. **11**(9): p. 714-8.
65. Lemaitre, B., et al., *Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila.* EMBO J, 1995. **14**(3): p. 536-45.
66. Senger, K., et al., *Immunity regulatory DNAs share common organizational features in Drosophila.* Mol Cell, 2004. **13**(1): p. 19-32.
67. Zaidman-Remy, A., et al., *The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection.* Immunity, 2006. **24**(4): p. 463-73.
68. Fulda, S., et al., *Cellular stress responses: cell survival and cell death.* Int J Cell Biol, 2010. **2010**: p. 214074.
69. Domingos, P.M. and H. Steller, *Pathways regulating apoptosis during patterning and development.* Curr Opin Genet Dev, 2007. **17**(4): p. 294-9.
70. Hotchkiss, R.S. and D.W. Nicholson, *Apoptosis and caspases regulate death and inflammation in sepsis.* Nat Rev Immunol, 2006. **6**(11): p. 813-22.

71. Marinari, E., et al., *Live-cell delamination counterbalances epithelial growth to limit tissue overcrowding.* Nature, 2012. **484**(7395): p. 542-5.
72. Babcock, D.T., C. Landry, and M.J. Galko, *Cytokine signaling mediates UV-induced nociceptive sensitization in Drosophila larvae.* Curr Biol, 2009. **19**(10): p. 799-806.
73. Luo, X., et al., *Foxo and Fos regulate the decision between cell death and survival in response to UV irradiation.* EMBO J, 2007. **26**(2): p. 380-90.
74. Besaratinia, A., et al., *Wavelength dependence of ultraviolet radiation-induced DNA damage as determined by laser irradiation suggests that cyclobutane pyrimidine dimers are the principal DNA lesions produced by terrestrial sunlight.* FASEB J, 2011. **25**(9): p. 3079-91.
75. Mehrotra, S. and K.S. McKim, *Temporal analysis of meiotic DNA double-strand break formation and repair in Drosophila females.* PLoS Genet, 2006. **2**(11): p. e200.
76. Ulukaya, E., C. Acilan, and Y. Yilmaz, *Apoptosis: why and how does it occur in biology?* Cell Biochem Funct, 2011. **29**(6): p. 468-80.
77. McCall, K. and H. Steller, *Facing death in the fly: genetic analysis of apoptosis in Drosophila.* Trends Genet, 1997. **13**(6): p. 222-6.
78. Denton, D., S. Nicolson, and S. Kumar, *Cell death by autophagy: facts and apparent artefacts.* Cell Death Differ, 2012. **19**(1): p. 87-95.
79. Nakamoto, M., et al., *Virus recognition by toll-7 activates antiviral autophagy in Drosophila.* Immunity, 2012. **36**(4): p. 658-67.
80. Baehrecke, E.H., *Autophagy: dual roles in life and death?* Nat Rev Mol Cell Biol, 2005. **6**(6): p. 505-10.
81. Yu, L., et al., *Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8.* Science, 2004. **304**(5676): p. 1500-2.
82. Ryoo, H.D. and E.H. Baehrecke, *Distinct death mechanisms in Drosophila development.* Curr Opin Cell Biol, 2010. **22**(6): p. 889-95.
83. Bass, B.P., et al., *Cell-autonomous requirement for DNaseII in nonapoptotic cell death.* Cell Death Differ, 2009. **16**(10): p. 1362-71.
84. Han, J., C.Q. Zhong, and D.W. Zhang, *Programmed necrosis: backup to and competitor with apoptosis in the immune system.* Nat Immunol, 2011. **12**(12): p. 1143-9.
85. Han, J., C.-Q. Zhong, and D.-W. Zhang, *Programmed necrosis: backup to and competitor with apoptosis in the immune system.* Nature Immunology, 2011. **12**(12): p. 1143-1149.
86. Kanda, H., et al., *Conserved metabolic energy production pathways govern Eiger/TNF-induced nonapoptotic cell death.* Proc Natl Acad Sci U S A, 2011. **108**(47): p. 18977-82.
87. McCall, K., *Genetic control of necrosis - another type of programmed cell death.* Curr Opin Cell Biol, 2010. **22**(6): p. 882-8.
88. Koike, M., Y. Ninomiya, and A. Koike, *Characterization of Ninjurin and TSC22 induction after X-irradiation of normal human skin cells.* The Journal of dermatology, 2008. **35**(1): p. 6-17.
89. Kubo, T., et al., *Analysis of genes induced in peripheral nerve after axotomy using cDNA microarrays.* J Neurochem, 2002. **82**(5): p. 1129-36.

90. De Gregorio, E., et al., *The Toll and Imd pathways are the major regulators of the immune response in Drosophila.* EMBO J, 2002. **21**(11): p. 2568-79.
91. Lee, H.J., et al., *Ninjurin1 mediates macrophage-induced programmed cell death during early ocular development.* Cell death and differentiation, 2009. **16**(10): p. 1395-1407.
92. Edgar, B.A. and G. Schubiger, *Parameters controlling transcriptional activation during early Drosophila development.* Cell, 1986. **44**(6): p. 871-7.
93. Anderson, K.V. and J.A. Lengyel, *Rates of synthesis of major classes of RNA in Drosophila embryos.* Dev Biol, 1979. **70**(1): p. 217-31.
94. Galko, M.J. and M.A. Krasnow, *Cellular and genetic analysis of wound healing in Drosophila larvae.* PLoS Biol, 2004. **2**(8): p. E239.
95. Zettervall, C.J., et al., *A directed screen for genes involved in Drosophila blood cell activation.* Proc Natl Acad Sci U S A, 2004. **101**(39): p. 14192-7.
96. McGuire, S.E., Z. Mao, and R.L. Davis, *Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila.* Sci STKE, 2004. **2004**(220): p. pl6.
97. Osterwalder, T., et al., *A conditional tissue-specific transgene expression system using inducible GAL4.* Proceedings of the National Academy of Sciences, 2001. **98**(22): p. 12596-12601.
98. Charroux, B. and J. Royet, *Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response.* Proceedings of the National Academy of Sciences, 2009. **106**(24): p. 9797-9802.
99. Goto, A., T. Kadowaki, and Y. Kitagawa, *Drosophila hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects.* Dev Biol, 2003. **264**(2): p. 582-91.
100. Grether, M.E., et al., *The head involution defective gene of Drosophila melanogaster functions in programmed cell death.* Genes Dev, 1995. **9**(14): p. 1694-708.
101. White, K., E. Tahaoglu, and H. Steller, *Cell killing by the Drosophila gene reaper.* Science, 1996. **271**(5250): p. 805-7.
102. Primrose, D.A., et al., *Interactions of DNR1 with the apoptotic machinery of Drosophila melanogaster.* Journal of Cell Science, 2007. **120**(7): p. 1189-1199.
103. Wright, C.W., et al., *The baculovirus anti-apoptotic protein Op-IAP does not inhibit Drosophila caspases or apoptosis in Drosophila S2 cells and instead sensitizes S2 cells to virus-induced apoptosis.* Virology, 2005. **335**(1): p. 61-71.
104. Wertman, K.F., D.G. Drubin, and D. Botstein, *Systematic mutational analysis of the yeast ACT1 gene.* Genetics, 1992. **132**(2): p. 337-50.
105. Kroemer, G., et al., *Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009.* Cell Death Differ, 2009. **16**(1): p. 3-11.
106. Graveley, B.R., et al., *The developmental transcriptome of Drosophila melanogaster.* Nature, 2011. **471**(7339): p. 473-479.
107. Tauszig, S., et al., *Toll-related receptors and the control of antimicrobial peptide expression in Drosophila.* Proc Natl Acad Sci U S A, 2000. **97**(19): p. 10520-5.

108. Bratton, D.L. and P.M. Henson, *Neutrophil clearance: when the party is over, clean-up begins.* Trends Immunol, 2011. **32**(8): p. 350-7.
109. Salmen, S., et al., *Mechanisms of neutrophil death in human immunodeficiency virus-infected patients: role of reactive oxygen species, caspases and map kinase pathways.* Clin Exp Immunol, 2007. **150**(3): p. 539-45.
110. Kurucz, E., et al., *Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2622-7.
111. Taylor, K. and D.A. Kimbrell, *Host immune response and differential survival of the sexes in Drosophila.* Fly (Austin), 2007. **1**(4): p. 197-204.
112. Leulier, F., et al., *The Drosophila inhibitor of apoptosis protein DIAP2 functions in innate immunity and is essential to resist gram-negative bacterial infection.* Mol Cell Biol, 2006. **26**(21): p. 7821-31.
113. Glasheen, B.M., A.T. Kabra, and A. Page-McCaw, *Distinct functions for the catalytic and hemopexin domains of a Drosophila matrix metalloproteinase.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2659-64.
114. Fernandez-Marcelo, T., et al., *Differential expression of senescence and cell death factors in non-small cell lung and colorectal tumors showing telomere attrition.* Oncology, 2012. **82**(3): p. 153-64.
115. Song, Z. and H. Steller, *Death by design: mechanism and control of apoptosis.* Trends Cell Biol, 1999. **9**(12): p. M49-52.
116. Oren, M., *Decision making by p53: life, death and cancer.* Cell Death Differ, 2003. **10**(4): p. 431-42.
117. Kemmink, J., et al., *Conformational changes in the oligonucleotide duplex d(GCGTTGCG) x d(CGCAACGC) induced by formation of a cis-syn thymine dimer. A two-dimensional NMR study.* Eur J Biochem, 1987. **162**(1): p. 37-43.
118. Yang, W., *Surviving the sun: repair and bypass of DNA UV lesions.* Protein Sci, 2011. **20**(11): p. 1781-9.
119. Weber, S., *Light-driven enzymatic catalysis of DNA repair: a review of recent biophysical studies on photolyase.* Biochim Biophys Acta, 2005. **1707**(1): p. 1-23.
120. Cleaver, J.E., *gammaH2Ax: biomarker of damage or functional participant in DNA repair "all that glitters is not gold!".* Photochem Photobiol, 2011. **87**(6): p. 1230-9.
121. Madigan, J.P., H.L. Chotkowski, and R.L. Glaser, *DNA double-strand break-induced phosphorylation of Drosophila histone variant H2Av helps prevent radiation-induced apoptosis.* Nucleic Acids Res, 2002. **30**(17): p. 3698-705.
122. Betz, A., et al., *STAT92E is a positive regulator of Drosophila inhibitor of apoptosis 1 (DIAP/1) and protects against radiation-induced apoptosis.* Proc Natl Acad Sci U S A, 2008. **105**(37): p. 13805-10.
123. Pfeifer, G.P. and A. Besaratinia, *UV wavelength-dependent DNA damage and human non-melanoma and melanoma skin cancer.* Photochem Photobiol Sci, 2012. **11**(1): p. 90-7.
124. Zhou, L. and H. Steller, *Distinct pathways mediate UV-induced apoptosis in Drosophila embryos.* Dev Cell, 2003. **4**(4): p. 599-605.

125. Shearwin-Whyatt, L.M. and S. Kumar, *Caspases in developmental cell death.* IUBMB Life, 1999. **48**(2): p. 143-50.
126. Tinsley, K.W., et al., *Caspases -2, -3, -6, and -9, but not caspase-1, are activated in sepsis-induced thymocyte apoptosis.* Shock, 2000. **13**(1): p. 1-7.
127. Vink, A.A. and L. Roza, *Biological consequences of cyclobutane pyrimidine dimers.* J Photochem Photobiol B, 2001. **65**(2-3): p. 101-4.
128. Bergmann, A. and H. Steller, *Apoptosis, stem cells, and tissue regeneration.* Sci Signal, 2010. **3**(145): p. re8.
129. Steller, H., *Regulation of apoptosis in Drosophila.* Cell Death Differ, 2008. **15**(7): p. 1132-8.
130. Liang, C., et al., *Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG.* Nat Cell Biol, 2006. **8**(7): p. 688-99.
131. Hu, B., et al., *The time and spatial effects of bystander response in mammalian cells induced by low dose radiation.* Carcinogenesis, 2006. **27**(2): p. 245-51.
132. Nagasawa, H., et al., *Involvement of membrane signaling in the bystander effect in irradiated cells.* Cancer Res, 2002. **62**(9): p. 2531-4.
133. Alexandre, J., et al., *Novel action of paclitaxel against cancer cells: bystander effect mediated by reactive oxygen species.* Cancer Res, 2007. **67**(8): p. 3512-7.
134. Jones, R.G. and C.B. Thompson, *Tumor suppressors and cell metabolism: a recipe for cancer growth.* Genes Dev, 2009. **23**(5): p. 537-48.
135. Denton, D., et al., *Larval midgut destruction in Drosophila: not dependent on caspases but suppressed by the loss of autophagy.* Autophagy, 2010. **6**(1): p. 163-5.
136. McKean, K.A., et al., *The evolutionary costs of immunological maintenance and deployment.* BMC Evol Biol, 2008. **8**: p. 76.

APPENDIX I

JNK expression is induced by cold shock.

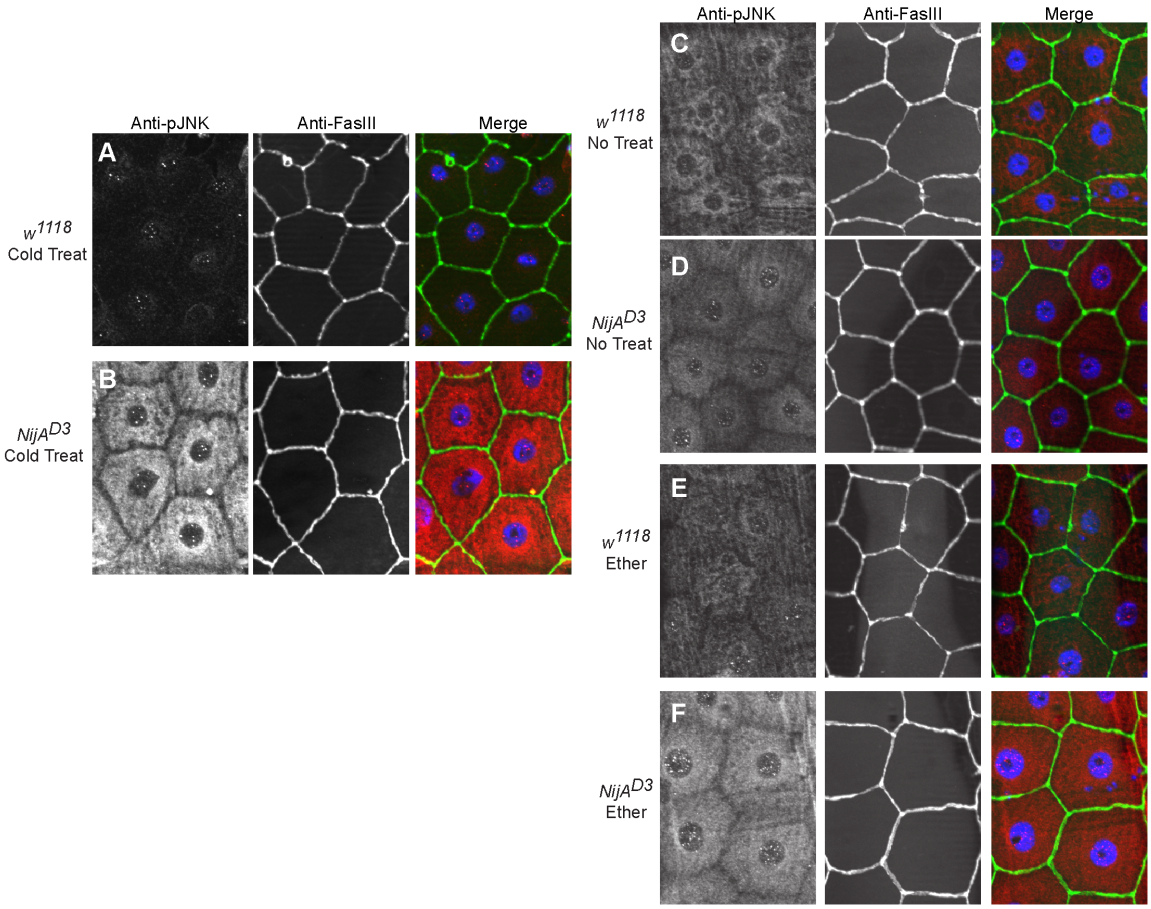

Figure A1. JNK expression is induced in the epiderims after cold shock. (A-B) JNK expression was assessed in fixed tissues 15mins after a 3min cold treatment. At first it appeared that the animals with the *NijA*^{D3} mutation had a more robust JNK expression pattern (3/3 epidermises examined). **(C-F)** Further examination shows a reduced JNK expression in the *NijA*^{D3} animals after ether treatment that is variable from the wild-type levels shown to the most robust JNK expression shown above (6 samples examined). Anti-JNK in red, Anti-Fas-III in green, DAPI staining in blue.

APPENDIX II

FOXO expression is not dependent on *NijA*.

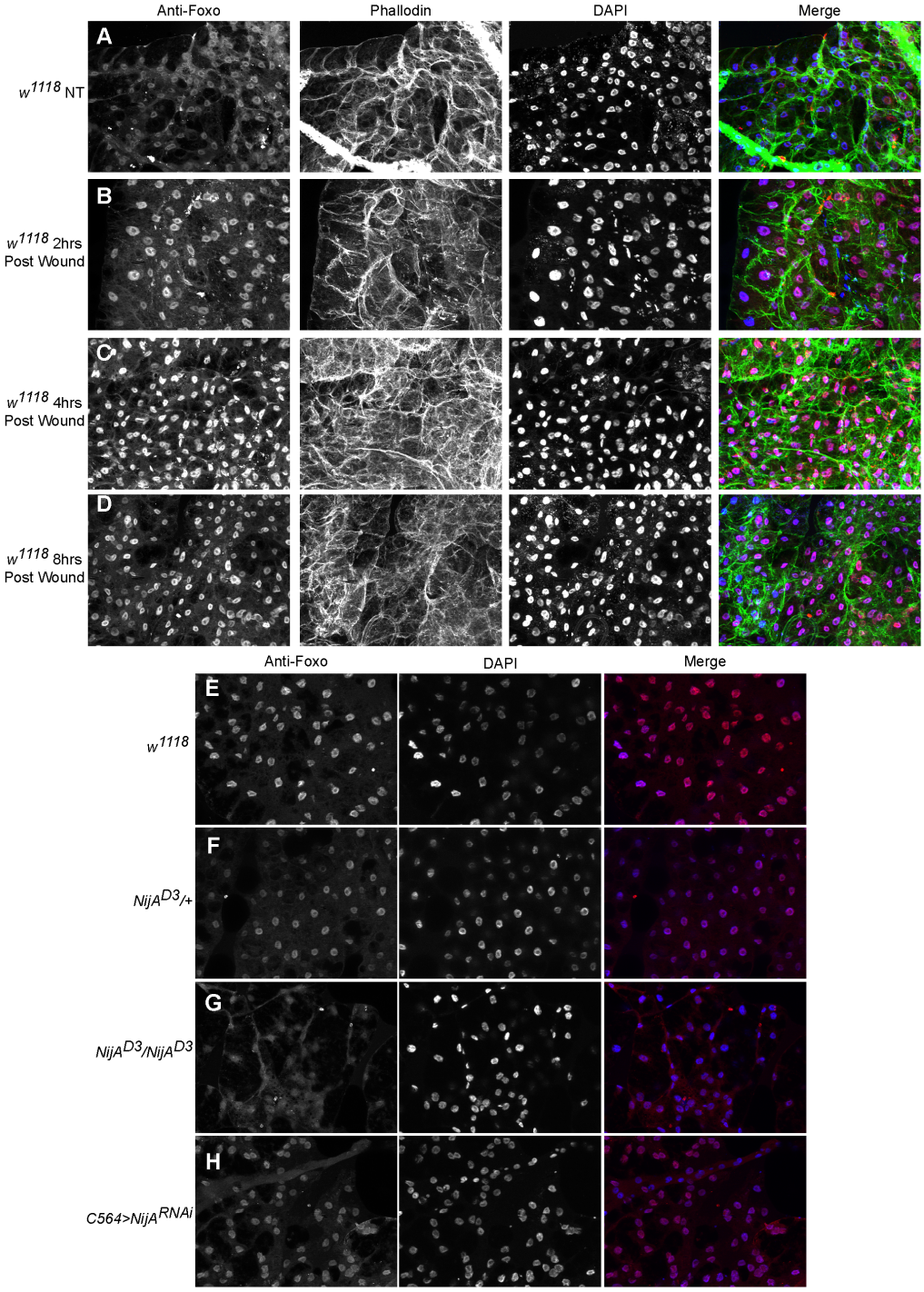

Figure A2. Foxo localization is regulated independently of *NijA* in the fat body. **(A-D)** Fat body tissue from w^{1118} larvae at various times after septic injury stained with Phalloidin (green), DAPI (blue), and antibody stained with anti-Foxo (red). Foxo has peak nuclear expression around 4hrs after septic injury in the fat body of wild-type animals. **E-H** Fat body tissue from wild-type larvae (E), $NijA^{D3}/+$ (F), $NijA^{D3}$ homozygotes (G), and $NijA^{RNAi}$ expressed in the fat body (H) stained with DAPI (blue) and antibody stained with anti-Foxo (red) 4hrs after septic injury. Foxo expression appeared reduced in the homozygous $NijA^{D3}$ mutants, although Foxo expression was not reduced in $C546>NijA^{RNAi}$ mutants. This suggests that Ninjurin A is not the gene responsible for the reduced Foxo expression in the $NijA^{D3}$ mutants.

Methods: Wandering third instar larvae were decapitated at approximately the third larval segment to remove the head fat of the larva. The fat body from the remaining body segments was carefully forced out of the remaining epidermal sac. The fat body tissue was antibody stained as described in Chapter III. All fat body images were taken in the region of the testis to eliminate the heterogeneity of the fat tissue. Anti-Foxo antibody was a kind gift of Heather Broihier, PhD (Case Western Reserve) and used at a 1:500 dilution.

APPENDIX III

NijAβ over-expression causes loss of adhesion in the epidermis.

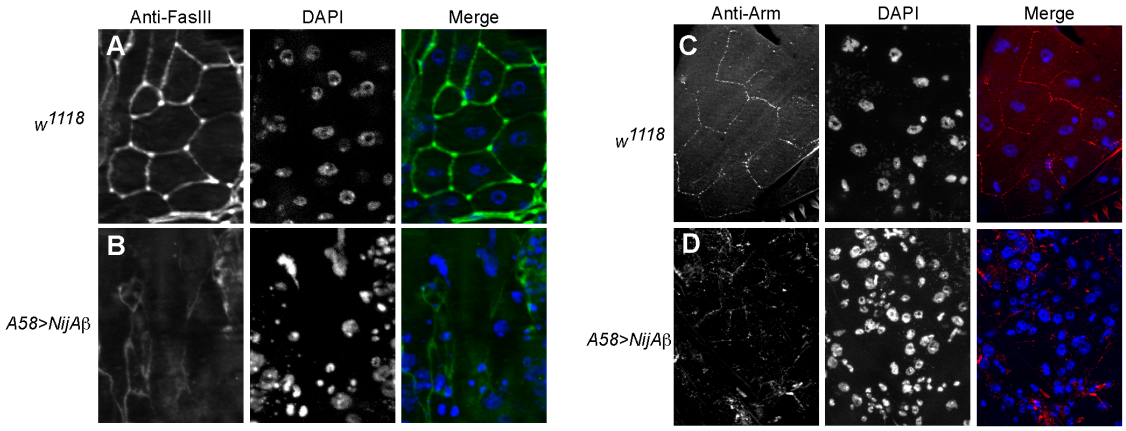

Figure A3. Over-expression of NijAβ is sufficient to cause a loss of cell-cell adhesion *in vivo*. A-B Epidermis of an early third instar larva from a w^{1118} (A) or a larva over-expressing *NijAβ* in the epidermis (B). Epidermal samples were antibody stained with anti-FasIII (green) and stained with DAPI (blue). Animals over expressing *NijAβ* in the epidermis are lacking the organized expression of FasIII at cell-cell borders. **C-D** Epidermis of an early third instar larva from a w^{1118} (C) or a larva over-expressing *NijAβ* in the epidermis (D). Epidermises were antibody stained with Anti-armadillo (red) and stained with DAPI (blue). Larvae over-expressing *NijAβ* in the epidermis are lacking armadillo staining at cell-cell borders.