8-2012

Transcriptional Regulation Of Profilin Is Required For Drosophila Larval Wound Closure

Amanda Brock

TRANSCRIPTIONAL REGULATION OF PROFILIN IS REQUIRED FOR

DROSOPHILA LARVAL WOUND CLOSURE

by

Amanda R. Brock, B.A.

APPROVED

Michael J. Galko, PhD, Supervisory Professor

_____
William Mattox, PhD

_____
Stephanie Watowich, PhD

_____
Anna Marie Sokac, PhD

_____
Vicki Huff, PhD

Approved:

_____
Dr. Michelle Barton or Dr. Michael Blackburn
Dean, The University of Texas
Graduate School of Biomedical Sciences

TRANSCRIPTIONAL REGULATION OF PROFILIN IS REQUIRED FOR

DROSOPHILA LARVAL WOUND CLOSURE

A DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
In Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Amanda R. Brock, B.A.

Houston, Texas

August, 2012

DEDICATION

This thesis is dedicated to my parents,
Thomas and Patricia Brock,
who have always loved and supported me

And to my grandmother,
Lula Brock,
who has always been a role model
of strength and independence

Acknowledgements

I would like to thank my advisor, Dr. Michael Galko, who has been tremendously supportive and encouraging throughout my graduate education. I have been fortunate to have been trained by someone with his abilities, enthusiasm, and kind nature.

I would also like to thank all the members of my committees: Drs. William Mattox, Greg May, Mary Dickinson, Stephanie Watowich, Georg Halder, Hamed Jafar-Nejad, Anna Marie Sokac, and Vicki Huff for their advice and support over the years.

I would like to thank my current and former lab members: Yan Wang, Yujane Wu, Christine Lesch, Christoph Scherfer, Juyeon Jo, Violet Han, Aimee Anderson, Sirisha Burra, Seol-Hee Im, Abanti Chattopadhyay, and Heather Turner for their scientific discussions, laboratory expertise, and friendship.

I would like to thank Dr. Renate Renkawitz-Pohl and Susanne Berger for the Lifeact-GFP reagent; Lynn Cooley, Elizabeth Chen, Mark Krasnow, Mireille Schäfer, Bloomington, FlyTrap, NIG-Fly, the Vienna Drosophila RNAi Center, and DSHB for fly stocks and monoclonal supernatants; Kenn Dunner for TEM assistance; Rong Dong and Ping Dang for fly husbandry assistance; Hank Adams and Jodie Polan for microscopy assistance; and Pat Lopez and Elisabeth Lindheim for administrative support.

Finally, I would like to thank my family and friends who have supported me throughout this endeavor: my parents, Thomas and Patricia Brock, and my grandmother, Lula Brock, have always loved and supported me; my sister, Amy Green, has always encouraged me to succeed in science; my niece, Emily Green, whose cuteness has cheered me up while writing this thesis; and my friend, Samuel Schnuer, who has always been emotionally supportive.

TRANSCRIPTIONAL REGULATION OF PROFILIN IS REQUIRED FOR

DROSOPHILA LARVAL WOUND CLOSURE

Publication No. _____

Amanda R. Brock, B.A.

Supervisory Professor: Michael J. Galko, Ph.D.

Injury is an inevitable part of life, making wound healing essential for survival. In postembryonic skin, wound closure requires that epidermal cells recognize the presence of a gap and change their behavior to migrate across it. In *Drosophila* larvae, wound closure requires two signaling pathways (the Jun N-terminal kinase (JNK) pathway and the Pvr receptor tyrosine kinase signaling pathway) and regulation of the actin cytoskeleton. In this and other systems, it remains unclear how the signaling pathways that initiate wound closure connect to the actin regulators that help execute wound-induced cell migrations. Here we show that *chickadee*, which encodes the *Drosophila* Profilin, a protein important for actin filament recycling and cell migration during development, is required for the physiological process of larval epidermal wound closure. After injury, *chickadee* is transcriptionally upregulated in cells proximal to the wound. We found that JNK, but not Pvr, mediates the increase in *chic* transcription through the Jun and Fos transcription factors. Finally, we show that *chic* deficient larvae fail to form a robust actin cable along the wound edge and also fail to form normal filopodial and lamellipodial extensions into the wound gap. Our results thus connect a factor that regulates actin monomer recycling to the JNK signaling pathway during wound closure. They also reveal a physiological function for an important developmental regulator of actin and begin to tease out the logic of how the wound repair response is organized.

Table of Contents

List of Illustrations

List of Tables

Abbreviations

EGFR …………………………………………. Epidermal Growth Factor Receptor

ERK………………………………………… Extracellular signal Related Kinases

FGF…………………………………………….. Fibroblast Growth Factor

GFP…………………………………………….Green Fluorescent Protein

JNK……………………………………………... Jun N-terminal Kinase

KGF……………………………………………..Keratinocyte Growth Factor

NIG………………………………………… National Institute of Genetics- Japan

PDGF………………………………………… Platelet Derived Growth Factor

Pvr…………………………………………PDGF/VEGF Receptor – like

RTK………………………………………… Receptor Tyrosine Kinase

TEM………………………………………… Transmission Electron Micrograph

UAS …………………………………………..Upstream Activation Sequence

VDRC………………………………………... Vienna Drosophila RNAi Center

VEGF………………………………………… Vascular Endothelial Growth Factor

Chapter One: Introduction

Wound healing is an important area of study in the realm of biomedical science. First, there are the obvious medical implications, both in healing after trauma or surgery, or in people with impaired healing, such as in elderly or diabetic patients. Secondly, it offers a wealth of interesting biological concepts that are relevant to several types of human disease. The study of wound healing can encompass diverse topics as inflammation, immunity, pain, cell proliferation, dedifferentiation, and cell migration. This thesis will primarily focus on a genetic study of the latter two topics in an invertebrate model system, *Drosophila melanogaster*, however we will first discuss the state of vertebrate wound healing research.

1.1 Vertebrate Wound Healing Models

Mouse wound healing models are complicated by the complexity of the mammalian skin, which is made up of multiple layers. The outermost layer is the epidermis. The epidermis is, itself, layered: the outermost layer is the stratum corneum, made up of anuclear corneocytes, which are derived from keratinocytes, and a lipid matrix that prevents water loss and acts as a barrier; the next layer is the granular layer, made up of keratinocytes that have keratohyalin granules of profillagrin present; next is the spinous layer, where the keratinocytes have a concentric pattern of keratin filaments; and the basal layer, where the keratinocytes proliferate, then migrate to the other layers (Hwa et al., 2011). A basal lamina separates the epidermis from the dermis, a connective tissue containing nerves, blood vessels, extracellular matrix and fibroblasts (Shaw and Martin, 2009b).

Wound healing proceeds in four stages: hemostasis, inflammation, reepithelialization, and tissue remodeling. During hemostasis, the organism stops the

blood loss that occurs following injury. This is accomplished by platelets and a fibrin clot that plugs the gap caused by tissue damage and serves as a matrix for cells to migrate upon. These platelets also secrete growth factors that stimulate the healing process (Shaw and Martin, 2009b), particularly PDGF (Platelet-Derived Growth Factor), which was one of the first factors discovered that induces epidermal cell migration (Heldin and Westermark, 1999) after wounding. Inflammation is the stage where neutrophils and macrophages are recruited to fight infection, neutrophils by phagocytosis and release of reactive oxygen species and macrophages by clearing microbes and debris by phagocytosis (Martin and Leibovich, 2005). Macrophages also release cytokines and growth factors thought to aid in healing (Martin and Leibovich, 2005). However, inflammation is not required for healing to occur (Martin et al., 2003). Reepithelialization is where the epidermal cells crawl across the provisional matrix of wound debris and the fibrin clot to reestablish tissue continuity. In the final stage, tissue remodeling, the provisional wound matrix is absorbed, resulting in a normal, undamaged tissue.

In the last twenty years, much has been discovered about the activation of non-motile cells into migration and proliferation during wound healing. For the keratinocytes to migrate requires them to dissociate from the basal lamina and express new integrins to establish links with the debris and extracellular matrix upon which it crawls (Martin, 1997). This requires the rearrangement of the actin cytoskeleton and new adhesion junctions to form for the keratinocytes to crawl across the gap and reestablish tissue continuity (Martin, 1997; Mitchison and Cramer, 1996). During the migration, these cells also gain proliferative ability, which aids in replenishing the cells that were lost during injury (Garlick and Taichman, 1994). The leading edge cells also release proteases into

the clot, to break it down and to create space for the keratinocytes to migrate (Grondahl-Hansen et al., 1988). During this phase, blood vessels are formed in the new tissue (Gurtner et al., 2008). Once reepithelialization has completed, the keratinocytes resume secretion of the basal lamina (Gipson et al., 1988).

More recently, many genes have been shown to be required for reepithelialization and wound healing. One of the most important is Keratinocyte Growth Factor (KGF), which is required for the proliferation of keratinocytes at the leading edge of a wound (Werner et al., 1994). In the migrating keratinocytes, c-Met, a receptor tyrosine kinase that binds to Hgf (hepatocyte growth factor), is required for efficient and timely migration (Chmielowiec et al., 2007; Gurtner et al., 2008). Multiple members of the FGF family are involved in cell migration, particularly FGFR2-IIIb, as well as members of the EGFR family, though these are likely functionally redundant (Gurtner et al., 2008; Werner and Grose, 2003). Furthermore, studies have been done looking not only at the transcriptome of murine wounds, which found many classes of genes to be upregulated after wounding, including cytoskeleton regulators and transcription factors, among others (Roy et al., 2008), but also the epigenetic changes that regulate gene expression during healing, which found a loss of polycomb group protein's epigenetic silencing (Shaw and Martin, 2009a).

Mouse models have many advantages for studying wound healing: they have a similar stratified epidermis and dermis to humans, allowing for discoveries about murine wound healing to have direct medical implications. Additionally, mice undergo all of the same stages of wound healing, making them the best model organism in which to study specific processes, such as angiogenesis. However, work done in mice is complicated by

the long generation time and difficulty of genetic manipulation. For wound healing

events that have an invertebrate analog, it is easier to study that event in the more

genetically tractable model organism, *Drosophila melanogaster*.

1.2 Drosophila Wound Healing models

One of the major advantages to using *Drosophila* as a model organism for wound

healing studies is the sophisticated genetic tool box that allows us to easily manipulate

genotypes, both spatially and temporally, in order to answer complex questions in an

organism with less genomic complexity. Additionally, the quick generation time allows

for ease in experimentation. An additional benefit in larval wound healing studies is the

simple, non-proliferative, endoreduplicated epidermis, which is an epithelial monolayer

that secretes the external cuticle and is separated from the body cavity by a basal lamina.

In this section, I will summarize the current state of wound healing research in

Drosophila.

1.2.1 Dorsal Closure

Dorsal closure is the developmental process by which the epidermal epithelial

sheet effects a coordinated migration over the amnioserosa, a second epithelial sheet that

covers a hole left by germ-band retraction during embryonic development, to create an

intact epithelial sheet that surrounds the embryo (Martin and Parkhurst, 2004). It is

thought to serve as a good model for wound closure because the epidermal cells healing a

wound are solving the same problem: how to effectively migrate to reestablish tissue

continuity (Wood et al., 2002). This movement is regulated by the JNK signaling

pathway (Ciapponi and Bohmann, 2002; Martin-Blanco et al., 1998; Reed et al., 2001;

Riesgo-Escovar et al., 1996). Dorsal closure begins after germ-band retraction during the

end stages of embryonic development. It is thought that the mechanical forces of germ-band retraction and amnioserosa contraction activate the JNK signaling pathway in the epithelial sheet (Jacinto et al., 2002). JNK signaling is activated in the leading edge cells of the epithelial sheet, but it goes down in the aminoserosa cells that are contracting (Jacinto et al., 2002; Reed et al., 2001; Stronach and Perrimon, 2001). After the contraction of the amnioserosa, a continuous actin cable forms at the leading edge of the migrating epidermal sheet (Jacinto et al., 2000). In the penultimate stage of dorsal closure, the epithelial sheets zipper together via the filopodia meeting from either side, pulling the hole shut, and matching the segmental patterns (Jacinto et al., 2000; Millard and Martin, 2008). In the termination stage, the migration stops and the leading edge cells begin to form adherens junctions with each other to create an intact epithelial sheet (Jacinto et al., 2002).

As mentioned, the JNK signaling pathway regulates the epithelial movements of dorsal closure. Interestingly, JNK signaling is upstream of *dpp*, which is secreted by the leading edge cells and whose receptor, *thickveins*, is required for cell elongation (Glise and Noselli, 1997; Riesgo-Escovar and Hafen, 1997b). JNK also regulates *puckered*, which functions in a negative feedback loop to reduce JNK signaling towards the end of dorsal closure (Martin-Blanco et al., 1998).

The dorsal closure model has also given a wealth of information about the actin cytoskeleton and how it acts to effect this coordinated migration. As mentioned, there is a contractile actin cable that forms along the leading edge. There have been a number of studies to determine the importance of the various forces present during dorsal closure: the contractility of the cable itself, downward forces by the amnioserosa, and the relative

importance of the crawling lamellipodia versus the contractile cable.   When the cable is ablated via laser, it is shown that the cable is required for tension within the epithelial sheet, but also that the cable alone does not provide the force for entire process (Kiehart et al., 2000). Interestingly, the amnioserosa is also required, but not sufficient, to provide the forces for dorsal closure to occur (Kiehart et al., 2000). The filopodia and lamellipodia are required for the zippering phase and correct segment alignment (Millard and Martin, 2008), but they are only required for the end phases and act to coordinate forces of the cable during earlier phases (Hutson et al., 2003). Thus, all of the forces play an important role during dorsal closure and may also be required for wound closure.

While genetic analysis of dorsal closure has revealed a number of genes that are also required for would closure, it does have some drawbacks. For one, it is a developmentally programmed event; these cells are not migrating while also coping with tissue damage or potential infection. The gap that the epidermal sheet is closing during dorsal closure is smaller than in larval wound closure. Additionally, these embryonic cells are not yet fully differentiated, so they do not necessarily act in the same way as post-embryonic, sedentary cells that have to partially dedifferentiate to become migratory.

1.2.2 Embryonic models of wound closure

Several of the wound closure models in *Drosophila* are in the embryo. It has been shown that embryonic wounds close in a similar manner to dorsal closure, in that they form a continuous actin cable at the leading edge (Wood et al., 2002) and require filopodial matching to achieve proper segment alignment in the end stages of closure (Millard and Martin, 2008). The embryo has proved useful for genetic screening (Campos et al., 2010), uncovering epithelial closure genes, though many of the genes

found were already known. Additionally, the ease of fluorescent labeling has made the embryo a good model for inflammation, or the blood cell immune response to wounding, where the cells migrate to open wounds to prevent blood loss, engulf debris and potentially infectious microbes, and potentially stimulate reepithelialization by secretion of cytokines (Wood et al., 2006). This model can also be used to track infection in real time (Vlisidou et al., 2009).

The embryo has also been used as a model to study transcriptional activation following wounding. The *grainyhead* transcription factor is required for wound healing and functions to regulate repair of the damaged cuticle barrier (Mace et al., 2005). *grainyhead* is regulated by the upstream receptor tyrosine kinase (RTK), *stitcher*, a member of the Ret-family, which activates extracellular signal-related kinase (ERK) (Wang et al., 2009). Interestingly, *grainyhead* transcriptionally activates *stitcher*, leading to a positive feedback loop that can increase the response to tissue damage as needed (Wang et al., 2009). Additionally, four genes are known to be activated following an embryonic wound, *ddc, ple, msn,* and *kkv,* which are regulated by *grainyhead* or DJun or DFos (Pearson et al., 2009), the transcription factors downstream of the JNK signaling pathway. The blood cells also induce expression of GADD45, a DNA-damage response gene, and other related genes following wounding (Stramer et al., 2008).

An important question raised by both embryonic wound repair and dorsal closure studies is the relative contribution of the actin cable and the actin based processes. Studies of the forces involved in dorsal closure suggest that the contractile force of the cable is one of the larger effectors of closure (Rodriguez-Diaz et al., 2008), while the processes are required at the end phases for zippering and proper patterning (Millard and

Martin, 2008). However, there is also a hypothesis that the cable merely acts as a platform for large amounts of process extension (Martin and Lewis, 1992). This has been tested by experiments that interrupt the actin cable by laser depletion (Rodriguez-Diaz et al., 2008), though in that system, secondary cables form and wound closure continues, so this does not truly resolve the issue of the purpose of the cable. This would best be tested if there were a method of eliminating the contractility of the cable and determining if wound closure continued.

1.2.3 Larval Wound Healing

The larval stage offers many advantages as a wound healing model. The larval epidermis is a specialized epithelial tissue that is endoreduplicated and non-migratory, while the larvae are still small and easily manageable. The major disadvantage to this system is the difficulty of live imaging, as the larvae are difficult to render immobile. The *UAS-RNAi* transgene libraries are effective in larvae and there are fewer issues of maternal effect to complicate genetic experiments. Galko and Krasnow established the *Drosophila* larvae as a viable wound healing model (Galko and Krasnow, 2004). While the hemostatic and inflammatory phases of wound healing do occur (Babcock et al., 2008; Galko and Krasnow, 2004), in this system it is simple to focus on the reepithelialization phase.   Unlike in vertebrates, the epidermal cells do not proliferate in response to the wound, allowing us to focus on cell migration. The unwounded larval epidermis is made up of hexagonal and pentagonal epithelial cells (Fig. 1.1A). Following wounding, there is a large gap in the epidermal sheet filled with cell debris (Fig. 1.1B). By four hours after wounding, the cells in the leading edge have begun elongating towards the wound (Fig. 1.1C) and by eight hours, this has started in cell rows further

away from the wound (Fig. 1.1D). By twenty four hours, the tissue has reepithelialized

(Fig. 1.1E) and is an intact epidermis, though the area where the wound had been is easily

seen, and is marked with large, oddly shaped cells and several syncytia (Lesch et al.,

2010).

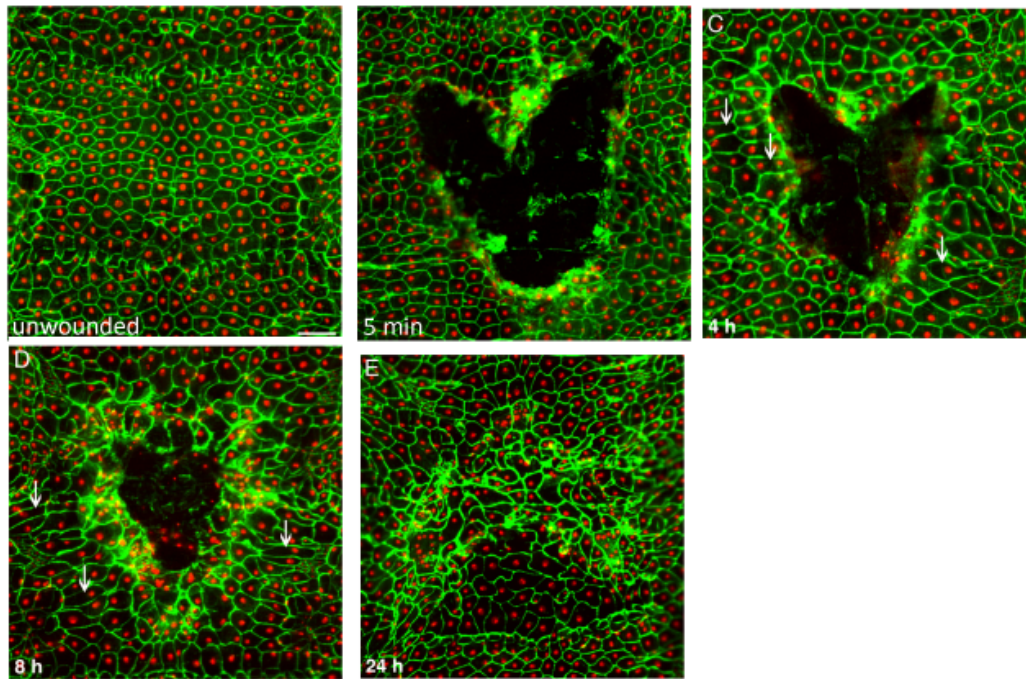**Fig. 1.1 Reepithelialization in the *Drosophila* larval epidermis**

Dissected larval epidermal wholemounts of genotype *w;e22c-Gal4, UAS-dsred2Nuc/+* and stained with FasIII. A) unwounded; B) 5 minutes after wounding; C) 4 hours after wounding. Arrows identify elongated cells. D) 8 hours after wounding. E) 24 hours after wounding.
Figure is taken from (Lesch et al., 2010) and reprinted with permission.

1.2.4 Imaginal Disc Wound Healing

The imaginal discs are epithelial sacs that develop in the larval stage and give rise to adult structures during metamorphosis. It has long been established that imaginal discs have regenerative capacity after tissue damage (Hadorn et al., 1968). This is primarily executed through proliferation at the wound edge (Bosch et al., 2008). Interestingly, this proliferation is also regulated by the JNK signaling pathway (Bergantinos et al., 2010; Bosch et al., 2008; Bosch et al., 2005; Mattila et al., 2005). More recently, models to study this regeneration have been developed: a successful method of genetically ablating wing imaginal disc tissue allows for genetic screening for genes important for the proliferative response and the reestablishment of correct patterning (Smith-Bolton et al., 2009). Interestingly, the induction of tissue damage also results in a developmental delay, giving the disc time to regenerate before pupariation (Halme et al., 2010), providing a model for a whole animal response to damage. This fly wound healing model has several advantages and disadvantages: it effectively models proliferation during healing, which is an important aspect to vertebrate healing, and it effectively models developmental patterning issues that can arise with wound healing. However, it does not serve as an effective model for questions of cell migration.

1.2.5 Thorax Closure

During metamorphosis, a similar process to dorsal closure occurs where the dorsal parts of the wing imaginal disc migrate together to form the thorax (Zeitlinger and Bohmann, 1999). This process, called thorax closure, is also regulated by the JNK signaling pathway (Agnes et al., 1999; Martin-Blanco et al., 2000; Noselli, 1998). Interestingly, it has been shown that the JNK pathway is regulated by the Pvr pathway

during thorax closure (Ishimaru et al., 2004), while during larval wound closure, they act in parallel (Wu et al., 2009). This model shares some of the disadvantages of dorsal closure as a model, in that it is studying an undifferentiated tissue that is undergoing a developmentally programmed event in the absence of real tissue damage.

1.3 The JNK Signaling Pathway

The JNK signaling pathway regulates dorsal closure (Riesgo-Escovar et al., 1996), thorax closure (Agnes et al., 1999; Martin-Blanco et al., 2000; Noselli, 1998), imaginal disc regeneration (Bergantinos et al., 2010) and larval wound closure (Galko and Krasnow, 2004; Lesch et al., 2010). The canonical pathway was defined in studies of dorsal closure: the Jun4K, *misshapen* (Treisman et al., 1997) phosphorylates the Jun3K, *slipper* (Stronach and Perrimon, 2002), which phosphorylates the Jun2K, *hemipterous* (Glise et al., 1995), which in turn phosphorylates *bsk*, the *Drosophila* Jun-N-terminal Kinase (Riesgo-Escovar et al., 1996). The two transcription factors, DJun and DFos, are phosphorylated by activated JNK (Riesgo-Escovar and Hafen, 1997a). While these have all been shown to play an important role in dorsal closure, the genes in the pathway do not all give the same phenotype during larval wound closure, indicating an alteration in the pathway.

Lesch, et. al, thoroughly studied the wound closure phenotype in larval tissues knocking down genes in the JNK pathway and found that DJun, DFos, and JNK all exhibit a severe wound closure defect when expression is knocked down (Lesch et al., 2010). However, the next kinase up the pathway, *hemipterous*, exhibits a milder wound closure defect when knocked down, where a smaller percentage have open wounds and the wounds that are still open appear to be smaller, as though they had partially closed

(Lesch et al., 2010). *slipper* has an even milder defect, where the majority of wounds are closed with *e22c-gal4* or *A58-Gal4* , two drivers that express in the larval epidermis (Lesch et al., 2010). *misshapen* has the most interesting phenotype of all; when it is missing from the larval epidermal sheet, the wounds close, but instead of forming a disorganized sheet, the cells have fused into a giant syncytial cell at the former wound site, which suggests *msn* is not upstream of the JNK pathway in this process (Lesch et al., 2010) (Fig. 1.2). The fact that the upstream kinases of the canonical JNK signaling pathway do not share a phenotype with JNK or its downstream transcription factors indicates that JNK may be regulated by other factors during larval wound closure. Interestingly, the JNK signaling pathway also regulates the non-muscle myosin localization during wound healing, resulting in the cell shape changes that are seen following wounding(Kwon et al., 2010).

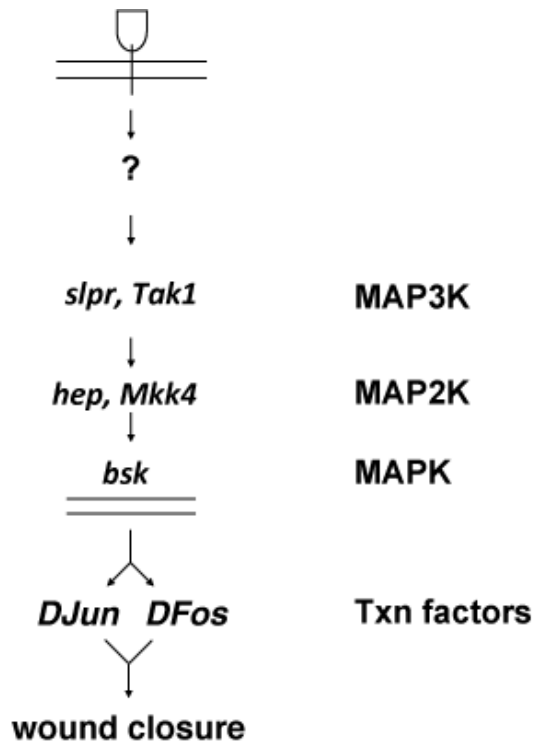**Fig. 1. 2 The JNK signaling pathway during wound closure**

This demonstrates the kinase cascade of the JNK signaling pathway as found in the work of (Lesch et al., 2010). Diagram created by Christine Lesch and used with permission.

1.4 The Pvr Signaling Pathway

The other main signaling pathway shown to be important for *Drosophila* larval wound closure is the Pvr pathway. Pvr is the *Drosophila* homolog of the PDGF/VEGF receptor and was first discovered to play a role in cell migration due to its role in directing border cell migration in the oocyte (Duchek et al., 2001; Fulga and Rorth, 2002). It has also been shown to play a role in the polarity of the wing-disc epithelium and that this role involves the polarization of the actin cytoskeleton (Rosin et al., 2004). Additionally, Pvr is important for blood cell migration (Cho et al., 2002) and blood cell survival (Bruckner et al., 2004), and the Pvr ligand, Pvf1 (McDonald et al., 2003), is important for guided migration of the border cells while additional ligands, Pvf2 and Pvf3, are required for hemocyte migration (Wood et al., 2006). Pvr plays an important role in axon guidance (Learte et al., 2008), thorax closure (Ishimaru et al., 2004), and the dorsal closure of the male terminalia (Macias et al., 2004).

Importantly, Pvr and Pvf1 are required for *Drosophila* larval epidermal wound closure (Wu et al., 2009). The current model for their function in larval wound healing is that the epidermis produces Pvf1 and shuttles it through the basal lamina into the hemolymph-filled body cavity where it circulates. In normal tissues with an intact basal lamina, the receptor on the epidermal cells does not come into contact with the Pvf1 ligand. However, in wounded tissues, the basal lamina is broken, exposing receptors on the leading edge of cells to the Pvf1 containing hemolymph, thus activating the epidermal cell and providing direction for migration (Wu et al., 2009). Currently, very few of the downstream targets of the Pvr signaling pathway are known, but it has been shown that *mbc*, *ced-12*, and *rac* facilitate Pvr's activation of JNK in thorax closure (Ishimaru et al.,

2004). One hypothesis that results from this model is that Pvr activates the actin

cytoskeleton to relocalize to the leading edge of the cell for migration, since the few

factors known to be downstream of Pvr in other systems are actin regulators.

1.5 Cell Migration

Currently, a model for how the actin cytoskeleton effects proper cell migration

comes from Pollard and Borisy (Pollard and Borisy, 2003). The majority of this model is

based on *in vitro* cell culture work or biochemical and kinetic assays of known actin

regulators. Briefly, the model suggests that an external stimulus activates a membrane

receptor that relays the signal through several downstream factors, including Rho-like

GTPases, the WASP/SCAR complex, and the Arp2/3 complex, which results in the new

nucleation of actin filaments at the leading edge of the cell membrane for migration (Fig.

1.3). The growth of filaments against the membrane leads to the formation of filopodia

and lamellipodia, actin-based structures that extend in the direction of migration and

anchor to the substrate in order to pull the cell forward. Migrating cells continuously

make new filopodia and lamellipodia to propel in the direction of a migration signal. The

Rho-GTPases are required for this process in the larval epidermal wound healing

migration (Baek et al., 2010; Lesch et al., 2010).

As actin filaments grow against the membrane, ADP-actin monomers are

dissociated from the pointed end by cofilin and additional factors (Carlier et al., 1997;

Rosenblatt et al., 1997). These monomers are recycled by Profilin, an actin binding

protein (Kaiser et al., 1999) that facilitates the exchange of ADP to ATP actin (Mockrin

and Korn, 1980) and aids in the growth of the filament against the membrane at the

barbed end (Vinson et al., 1998). This growth at the barbed end may be facilitated by

binding to formin proteins that processively bind to the end of the actin filament and

facilitate rapid growth when bound to Profilin (Kovar et al., 2006; Romero et al., 2004).

In human cell culture, cells lacking Profilin have defects in cell migration and adhesion

(Ding et al., 2006), suggesting that Profilin may play an important role in wound closure.
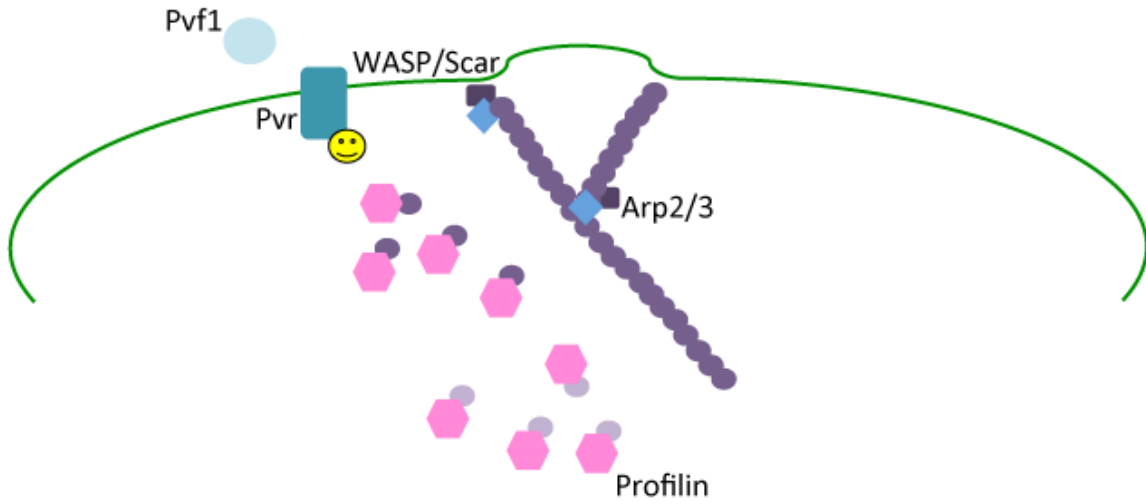
Fig. 1.3 Current model of cell migration

This diagram depicts our current model of how actin filaments grow against the membrane to effect cell migration during larval wound healing. The light blue circle represents Pvf1, a ligand that activates Pvr, represented here by a turquoise rectangle, with happy face to indicate activation. Activated Pvr activates downstream proteins, such as WASP/Scar (purple rectangle), that initiate filament growth at the membrane. Arp2/3 facilitates branching of the filaments (blue square). ATP-actin monomers are represented by the darker purple circles, while ADP-actin is in lavender. Profilin is represented by the pink hexagon.

1.6 Profilin

As mentioned, Profilin binds actin monomers that have dissociated from actin filaments and recycles them to their active state and facilitates the growth of the filament. While this biochemical role is important, Profilin can also bind to other actin regulatory proteins, including VASP (Reinhard et al., 1995), a focal adhesion protein that is thought to encourage actin polymerization by antagonizing capping proteins at the leading edge, and the formin proteins, which also encourage polymerization at the leading edge (Romero et al., 2004). A model of cell migration, the *L. monocytogenes* infection, showed that Profilin is absolutely required for polymerization, which is also required for the bacteria to replicate (Theriot et al., 1994). Profilin protein has also been shown to bind to a variety of signaling molecules, including Rac/Rho signals and membrane trafficking signaling pathways (Witke, 2004), indicating it may be important for interpreting signals to activate the cytoskeleton.

Profilin has been shown to be important for vertebrate development. Whole animal knockouts in mice (Witke et al., 2001) and *Drosophila* (Cooley et al., 1992) are embryonic lethal. Proflin1 is necessary for glial cell adhesion in developing mouse brains (Kullmann et al., 2011) as well as for epiboly and convergent extension migrations in both zebrafish (Lai et al., 2008) and *Xenopus* development (Khadka et al., 2009). *Xenopus* has two Profilins and XProfilin 1 is required for blastopore closure, while XProfilin 2 is required for the above convergent extension (Khadka et al., 2009).

The *Drosophila* Profilin gene, *chickadee*, was first discovered in a screen for defects in the process of nurse cell dumping (Cooley et al., 1992), though it is also important for spermatogenesis (Castrillon et al., 1993), oogenesis and bristle formation

(Verheyen and Cooley, 1994), axon guidance (Kim et al., 2001), and lamellipodial ruffling in S2 cells (Rogers et al., 2003). Interestingly, *chic* mutants also exhibit a dorsal closure defect: approximately 30% fail to complete dorsal closure and these have fewer leading edge filopodia than are seen in wild type cells (Jasper et al., 2001). All of this suggests that Profilin is highly likely to be required for wound closure.

In this thesis, we will characterize Profilin's wound closure defect and determine that it is regulated by the JNK signaling pathway during wound closure, and not the Pvr pathway. The data presented will suggest that fully differentiated cells may require additional transcription of actin regulators in order to become fully migratory.

Chapter 2: Materials and Methods

2.1 Fly Stocks

Fly stocks were reared at room temperature (22°C) on standard cornmeal media. Experimental crosses were raised in a 25°C incubator unless otherwise noted. The Gal4/UAS system was used to drive tissue-specific expression of a variety of transgenes, including GFP tags or *UAS- RNAi* lines (Brand and Perrimon, 1993).

Table 2.1 *Drosophila* Gal4 Lines

Name	Expression Pattern	Source
w;;A58-Gal4	Larval epidermis	(Galko and Krasnow, 2004)
w;e22c-Gal4	Late embryonic and larval epidermis	(Lawrence et al., 1995)
w;;Pnr-Gal4	Dorsal epidermal patches	(Calleja et al., 1996)
w;dmef-Gal4	Larval body wall muscles	(Zars et al., 2000)
w;;Pxn-Gal4	Hemocytes	(Stramer et al., 2005)

Table 2. Transgenes: RNAi lines and mutants

Gene	Type	Genotype	Source
chickadee	RNAi line	*w;;UAS-9553R3*	NIG
chickadee	RNAi line	*w;;UAS-9553R4*	NIG
chickadee	RNAi line	*w;UAS-9553-kk*	VDRC
chickadee	P-element insertion w/lacZ	*w;chic^{01320}*	Bloomington (Cooley et al., 1992)
Fasciclin III	GFP trap	*w;FasIIIGFP*	(Quinones-Coello et al., 2007)

chickadee	UAS	*w;UAS-chickadee*	(Geisbrecht and Montell, 2004)
hemipterous	constitutively activated UAS construct	*w; UAS- hep^{CA}*	(Adachi-Yamada et al., 1999)
Pvr	constitutively activated UAS construct	*w; UAS-λPvr*	(Duchek et al., 2001)
Pvr	RNAi line	*w; 8222R-3 (Pvr^{IR})*	NIG
n/a	actin binding label	*w;UAS-lifeactGFP*	Gift from Susanne Berger and Renate Renkawitz-Pohl
n/a	fluorescent label	*w; UAS-GFP*	(Halfon et al., 2002)
n/a	nuclear label	*w; UAS-dsrednuc21*	(Lesch et al., 2010)
basket	cDNA	*w;UAS-bsk^B*	(Boutros et al., 1998)
basket	RNAi line	*w;5680-R1*	NIG
basket	RNAi line	*w;5680-R2*	NIG
Jra (DJun)	RNAi line	*w;2275-R2*	NIG
kayak (DFos)	RNAi line	*w;15509-R2*	NIG
tubulin-Gal80^{ts}	conditionally inhibits Gal4	*tubulin-Gal80^{ts}*	(McGuire et al., 2003)

For the RNAi lines, documentation of the efficacy of those targeting *chickadee* will be shown in chapter 3. Efficient knockdown of Pvr by the RNAi line was shown in Wu, et. al (Wu et al., 2009). The *JNK^IR* line has been shown to phenocopy the dominant negative line and block *msn-lacZ* induction (Lesch et al., 2010). We show in chapter 3 of this thesis that *UAS-bsk^B* can rescue the RNAi phenotype.

2.2 Wounding Assay

The wounding assay has been described previously (Babcock et al., 2008; Galko and Krasnow, 2004; Lesch et al., 2010; Wu et al., 2009). To summarize, the larvae of the correct genotype are briefly anesthetized with ether, pinched on the dorsal side with blunted forceps (Fine Science Tools), and placed on warm food to be returned to the incubator and examined at a later timepoint. In experiments where wound closure was quantified, we defined an open wound as one where there is a clearly visible gap in the epithelial sheet without cells; we defined a closed wound as one where there is clear re-epithelialization of the epidermal sheet and there is a disorganized patch of cells where the wound had been.

2.3 Immunofluoresence

Larval epidermal wholemount dissections and immunofluorescence staining were done as described previously (Galko and Krasnow, 2004). Briefly, the larvae are pinned down in a sylgard dissection dish with the dorsal side down, then filleted open and the internal organs are removed, leaving a stretched epidermal tissue that can be fixed in 3.7% Formaldehyde, washed with 1X PBS, blocked with 1X PHT, and stained. The monoclonal antibodies chi 1J α-*chic* (undiluted) and 7G10 Fasciclin III (1:50 dilution) were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed

under the auspices of the NICHD and maintained by the University of Iowa, Dept. of Biological Sciences, Iowa City, IA. The polyclonal Rabbit α-GFP (1:500) antibody was from Invitrogen. Secondary antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA) were goat anti-mouse Cy3 (1:200) and goat anti-mouse FITC (1:200). All antibodies were diluted in 1X PHT (Phosphate Buffered Saline, 1% Heat Inactivated Normal Goat Serum (HINGS), 0.3% Triton-X). Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

2.4 Imaging

Images were obtained on either a Leica MZ16FA stereomicroscope using a PlanApo 1.6x objective with a Leica DFC300 FX color camera and Image Pro AMS v5.1 software (Media Cybernetics, Bethesda, MD, USA); an Olympus FV1000 laser confocal microscope using a 20X/0.85 NA oil or 60X/1.42 NA oil objective with Fluoview software; or a Leica DM5500 upright microscope using a 20X objective, with a Leica camera, and the extended depth of field algorithm through ImagePro Plus (v. 6.0) software with Z-slices of 1μm or 0.5μm. All images were collected at room temperature. For confocal images, Z stacks of 1μm (20X) or 0.5 μm (60X) depth per slice were collected. Green and red channels were adjusted uniformly across the entire image for optimal visualization of image features using Adobe PhotoShop. For quantification of fluorescent images, we defined a point at the approximate center of the wound. Next, we drew 32 equally spaced lines radiating through the center and intersecting the wound edge. Along each line we measured the fluorescence intensity in ImageJ from the wound edge to the first cell border and calculated the range. If the line passed through a nucleus, which was generally brighter than the surrounding cytoplasm, we deviated the line to

pass to the side of the nucleus. We performed statistical analysis using GraphPad Prism
software.

2.5 LacZ Staining

LacZ staining was performed as previously described (Galko and Krasnow, 2004; Lesch
et al., 2010; Wu et al., 2009). Briefly, larvae bearing the *chic-lacZ* (*chic^{01320}*) allele were
wounded, dissected at a later timepoint, fixed for 20 minutes in cold 2% glutaraldehyde
in 1X PBS, washed with 1X PBS, and stained with 5-bromo-4-chloro-3-indolyl-D-
galactopyranoside (X-gal) for two hours at 37°C. Samples were mounted in 70%
glycerol and imaged at room temperature.   Images were taken on a Leica DM5500
upright microscope using a 10X/0.40NA objective and DIC optics, Jenoptik ProgRes C14
plus camera, and the extended depth of field algorithm through ImagePro Plus (v. 7.0)
software with Z-slices of 0.5 μm.

2.6 TEM

TEM was performed as previously described (Babcock et al., 2008; Galko and Krasnow,
2004; Wu et al., 2009). Briefly, larvae were dissected in EM fixative (3%
gluataraldehyde, 2% paraformaldehyde, and 2.5% DMSO in 0.2 M sodium phosphate
buffer (pH 7.2) and incubated for 1hr in 1% Osmium tetroxide. They were then stained
overnight in 0.5% uranyl acetate, dehydrated in sequential ethanol concentrations and
embedded in SPURR resin (Electron Microscopy Sciences, Hatfield, PA, USA). Images
were taken using a JEOL JEM 1010 transmission electron microscope with AMT
(Advanced Microscopy Techniques, Woburn, MA, USA) software.

Chapter 3: Results

3.1 Profilin is required for wound closure

Previously, others in my lab undertook an RNAi-based genetic screen to find genes that are required for wound closure (Lesch et al., 2010). This is a medium-throughput genetic screen that first involved the creation of a reporter line, which is a larval, epidermal Gal4 line (*e22c-Gal4)* that drives UAS-transgene expression of a membrane-GFP and a nuclear red label, allowing the larval epidermis to be imaged live. This reporter line is then crossed to another UAS-transgene, one that leads to expression of a gene-specific sequence of mRNA that once expressed will become double-stranded and trigger the RNAi machinery to knockdown the message of both the *UAS-RNAi* transgene and the endogenous message, resulting in specific gene knockdown. Using this method, it is possible to knock down message in both a tissue- and temporal-specific manner. For the genetic screen, the *e22c-Gal4* reporter line was crossed to one of a number of *UAS-RNAi* transgenes, larvae were wounded at the third instar stage, and examined as live larvae 24 hours later for wound closure defects. The screen was effective, identifying multiple members of the JNK signaling pathway and known actin regulators as required for wound healing (Lesch et al., 2010). The screen led to a framework for analyzing wound closure phenotypes based on the morphology of the cells near the leading edge of the wound (Lesch et al., 2010). One of the genes found in the initial screen was *chickadee* (*chic*), the *Drosophila* Profilin.

After the identification of Profilin as a potential wound closure gene, we had to confirm the phenotype. First, we tested the wound closure phenotype of a control (genotype *w;e22c-Gal4, UAS-DsredNuc2/+*) and found that 100% of tested larvae (n>20) exhibited closed wounds (Fig. 3.1A, H). We compared that to a line containing two

copies of the *UAS-RNAi* transgene targeting *chic* (genotype *w;e22c-Gal4, UAS-DsredNuc2/+;UAS-9553R3, UAS-9553R4/+*) and found that 100% of the tested larvae exhibited open wounds (Fig. 3.1B, H). We had combined the lines since we thought that increasing the RNAi dosage would lead to more effective knockdown of the protein. We next tested each *UAS-RNAi* transgene in the prior experiment to look for a wound closure defect with a lower dosage of RNAi; we found that larvae expressing the *UAS-9553R3* line had closed wounds (Fig. 3.1 D,H) and those expressing the *UAS-9553R4* line had 70% open wounds (Fig. 3.1 C,H). Both RNAi lines come from NIG-Japan and target the same sequence. This indicates that the R3 line does not knockdown the message very efficiently, however, the enhanced phenotype when it is present implies there is an additive effect of the RNAi line. This does suggest that, in general, the limiting step of the RNAi may be the quantity of double stranded RNAi present and stronger knockdown can be achieved by adding more. We next tested an RNAi line that targets a different region of the *chic* gene (Fig. 3.2) to ensure the phenotype is due to on-target knockdown of *chic*; this line (genotype: *w;e22c-Gal4, UAS-DsredNuc2/UAS-chic^{kk}*) gave 80% open wounds (Fig. 3.1E, H). To further confirm that there were no off-target effects of the RNAi lines, we next did a rescue experiment, where we co-expressed a *UAS-chic* cDNA with the RNAi double line. First, we expressed the cDNA on its own (genotype: *w; e22c-Gal4, UAS-DsredNuc2/UAS-chic*), which gave closed wounds, as expected (Fig. 3.1F, H). We then did the rescue experiment, with all of the relevant transcripts, and got 60% open wounds, which is a partial rescue (Fig. 3.1 G, H). Since the RNAi line also targets the message being overexpressed, it was not necessarily unexpected that full rescue could not be achieved.
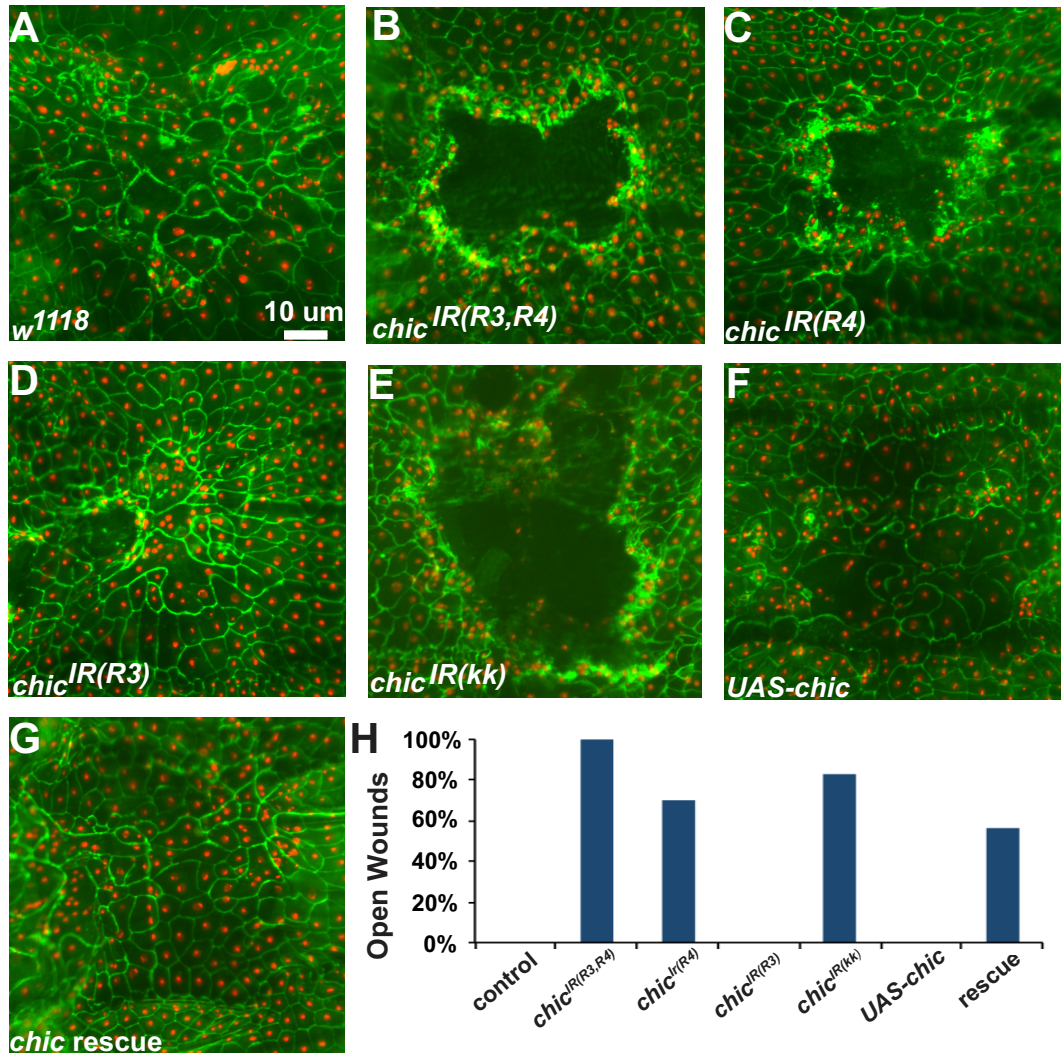
A **w^{1118}** 10 um
B **chic$^{IR(R3,R4)}$**
C **chic$^{IR(R4)}$**
D **chic$^{IR(R3)}$**
E **chic$^{IR(kk)}$**
F **UAS-chic**
G **chic rescue**
H

Fig. 3.1 *chic^{IR}* results in a failure of wound closure

(A-G) Dissected larval epidermal wholemounts stained for anti-Fasciclin III (green). Genotype is *w; e22c-gal4, UAS-dsred2nuc* (to label epidermal nuclei, red) plus the indicated mutations or UAS transgenes. All panels show wounded larvae 24 hours post-wounding. A)*w^{1118}*, control. Note the presence of large, atypically-shaped cells some of which are multinucleate at the closed wound (compare to unwounded epidermis in Figure 2A). (B) *UAS-chic^{IR(R3,R4)}*. Note the open wound gap. (C) *UAS-chic^{IR(R4)}* . Note the open wound gap. (D) *UAS-chic^{IR(R4)}* Note the closed wound. (E) *UAS-chic^{IR(kk)}*. Note the open wound gap. (F) *UAS-chickadee*. Overexpression of *chickadee* by UAS does not interfere with wound closure. (G) *UAS-chic^{IR(R3,R4)}*, *UAS-chickadee*. A closed wound is shown. (H) Percentage of larvae with an open wounds versus genotype. Rescue refers to *UAS-chic^{IR(R3,R4)}*, *UAS-chickadee*. n ≥ 30. Scale bar in (A) represents 10 μm and is the same for all panels.

Fig. 3.2 RNAi lines target non-overlapping DNA sequences.
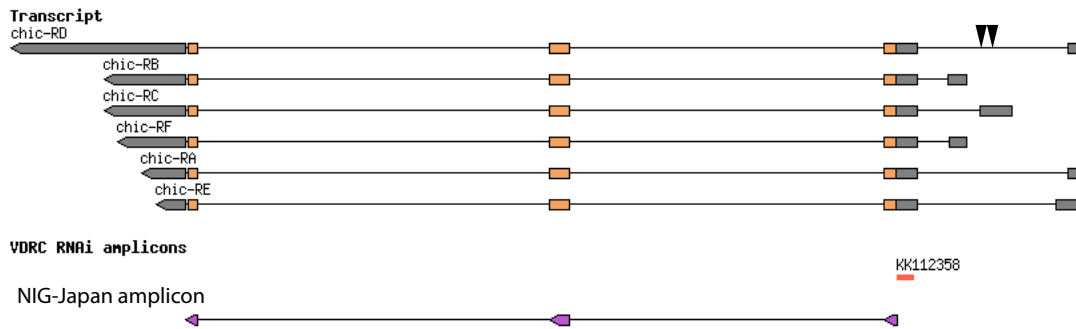
The coding region of chic is shown above. $chic^{IR(kk)}$ is from the VDRC and targets a short sequence in the 5' regulatory region. $chic^{IR(R3)}$ and $chic^{IR(R4)}$ are from NIG-Japan and target the entire coding region. The targeted regions are separated by two base pairs. The two arrowheads show the location of the AP-1 transcription factor binding sites.

33

3.2 Confirmation of RNAi lines

As a confirmation of the phenotype seen using RNAi lines, we next examined a hypomorphic allele to see if it had a similar wound closure defect. If so, it would indicate that the RNAi phenotype is the result of on-target knockdown. Ideally, a null allele would be used, however all available null alleles of *chic* are not viable at the larval stage. We tested the homozygous hypomorphic allele *chic*^{01320} and found that it had a mild wound closure defect, where it was open 20% of the time (Fig. 3.3 A,C). To further confirm that the RNAi lines were knocking down the intended target, we performed immunohistochemistry with an *α-chic* antibody. Due to intense staining of the larval body wall muscles by the *chic* antibody, we imaged the small patch of epidermal cells that are not covered by muscle tissue. In control larvae, Profilin is distributed evenly within the larval epidermal cells (Fig. 3.4A). The RNAi line (R3) that does not have a wound closure defect when expressed alone has no protein knockdown (Fig. 3.4B), while the R4 line (Fig. 3.4 D) and the double (R3,R4) do (Fig. 3.4 E). Additionally, the *UAS-chic*^{kk} line also knocks down Profilin protein efficiently (Fig. 3.4F). Interestingly, the hypomorph that shows a mild wound closure defect still shows Profilin protein present in the larval epidermis, but instead of even distribution in the cytoplasm, it appears to be localized to the nucleus (Fig. 3.4 C). All of these experiments together suggest that the phenotype seen using RNAi lines is not due to off-target effects of the RNAi, indicating that Profilin is required for epidermal wound closure.
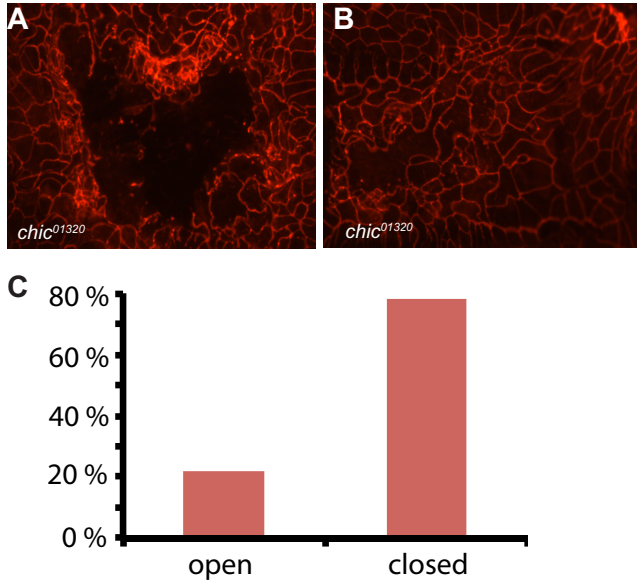
Figure 3.3 *chic^{01320}*, a hypomorph, shows a mild wound closure defect

Panels A-B show homozygous *chic^{01320}* larval wholemounts, with epidermal membranes stained with α-Fasciclin III   (A) open wound. (B) closed wound. (D) Quanitification of percent open wounds, n > 30.
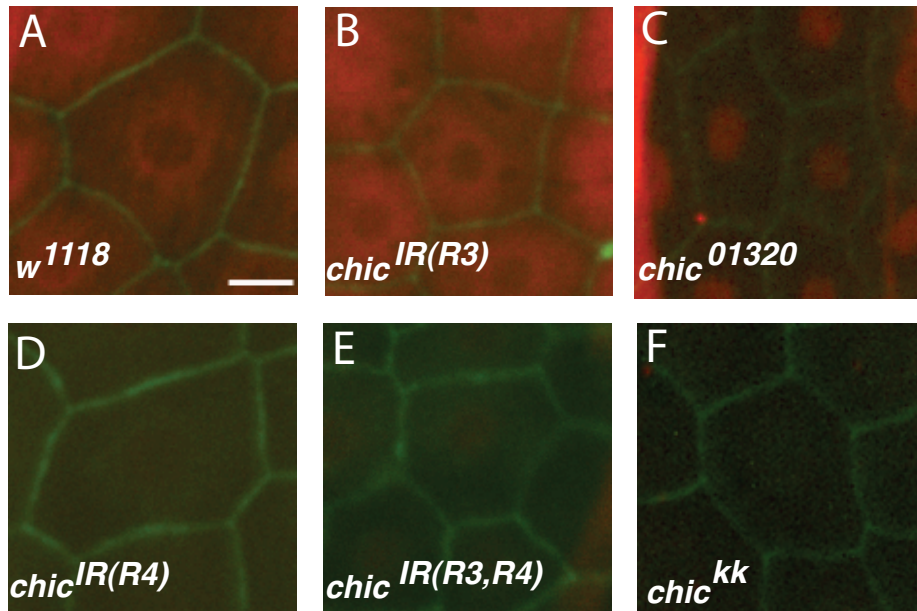
Fig. 3.4 *chic* **levels are reduced in RNAi lines**

All panels are dissected larval epidermal wholemounts stained with α-chic antibody (red), epidermal membranes labeled with FasIIIGFP (green). Genotypes of (A,B,D,E, and F) are *w;e22c-Gal4, FasIIIGFP* crossed to the indicated transgene. (C) is the homozygote of *w; chic^{01320} , FasIIIGFP*. (A) *w^{1118}* (B) *chic$^{IR(R3)}$* (C) *chic^{01320}* (D) *chic$^{IR(R4)}$* (E) *chic$^{IR(R3,R4)}$*. (F) *chic$^{IR(kk)}$*.

3.3 Profilin protein levels after wounding

After confirming that Profilin is required for wound closure, we assessed the localization and levels of the protein in a healing larval epidermis. This experiment was complicated by the fact that the *α-chic* antibody stains brightly in the larval body wall muscles that lay directly atop the epidermis and we needed to look at a larger area of the epidermal tissue than is visible between muscle fibers. There are two alternative ways to ameliorate this problem: 1) we could demuscle the larval epidermis, which is a painstaking process that is difficult to achieve without damaging the underlying tissue; or 2) we could eliminate *chickadee* in the muscles. We achieved the latter by crossing the *UAS-chic$^{IR(R3,R4)}$* double line to a *w;dmef-Gal4*, which drives expression in the larval body wall muscles, thus eliminating Profilin in the muscles, and thus eliminating staining. It was possible that eliminating *chic* in the larval body wall muscles could impact the larval epidermis, however, the wounds healed by 24 hours as in wild type, nor was there evidence of damage to the epidermal tissue in the unwounded epidermis. In the unwounded epidermis, Profilin is evenly distributed in the cells (Fig. 3.5A). By four hours after wounding, the Profilin levels have increased in the cells around the wound edge (Fig. 3.5B, B'), but not in those several cell rows away from the wound (Fig. 3.5B"). The blood cells that have accumulated in the wound debris are also expressing Profilin. By 24 hours, the wound has closed and there is a distinctive patch of high, but variable, Profilin levels in the cells that have migrated to close the wound gap (Fig.3.5, C,C',C").
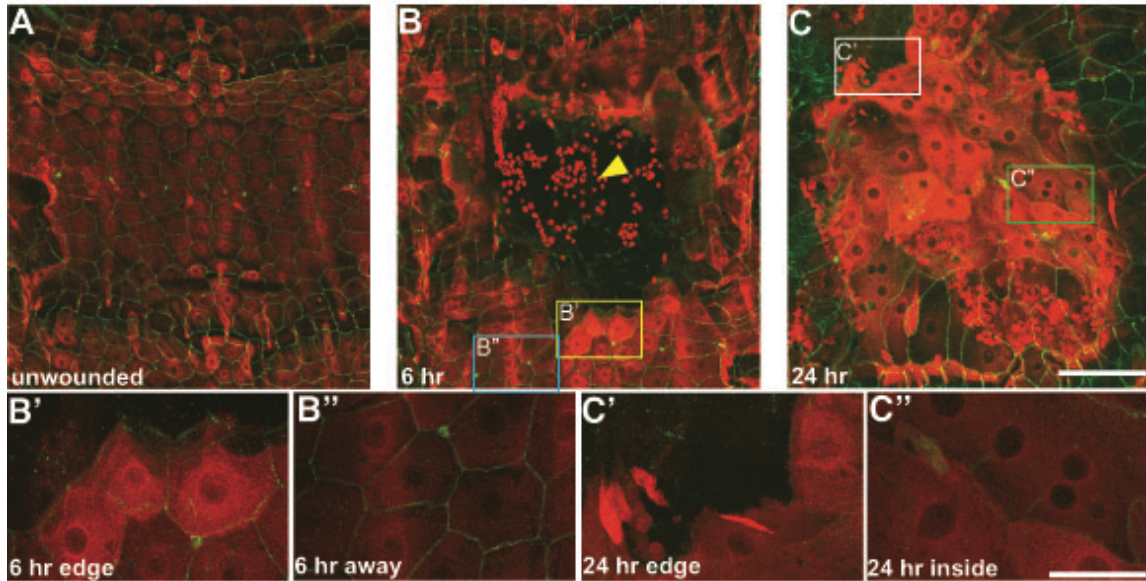
Fig. 3.5 Profilin relocalizes and levels increase following wounding

(A-C') Dissected larval epidermal wholemounts of genotype: *w;dmef2-gal4, FasIII-GFP/+;UAS-chic^{IR(R3,R4)}/+*.   In these larvae the muscle Gal4 driver *dmef2-Gal4* drives expression of *chic^{IR(R3,R4)}* in order to knock down muscle Profilin expression that would obscure a clear view of the epidermal Profilin. Epidermal membranes are labeled with *FasIII-GFP* (green); Profilin is labeled by α-chic antibody (red). (A) Unwounded larval epidermis. Note low levels of cytoplasmic Profilin staining. (B) Six hours after wounding. Note that in cells adjacent to the wound edge, Profilin levels have increased (B') compared to in cells several cell rows away from the wound (B"). Note that the Profilin antibody also labels blood cells (arrow) in the middle of the wound. (C) 24 hours after wounding. Profilin is still high in cells that have migrated to close the wound gap as indicated by the sharp boundary of the former wound area (C'). Levels are still high within the former wound (C"). Scale bar in C represents 100 mm and refers to panels A,B, and C. Scale bar in C' represents 50 mm and refers to B', B", C', and C". Note: Due to the extreme disparity in fluorescence levels between control and wounded samples red-channel levels were adjusted to different optimal levels for these samples.

3.5 Profilin overexpression accelerates wound closure

Because Profilin levels increase surrounding a wound, we questioned if overexpression of Profilin would accelerate wound closure. To do this, we expressed the *UAS-chic* in the epidermis and measured the area of the wounds at a time midway through closure in tissues expressing excess *chic* and in those expressing endogenous levels (Fig. 3.6 A,B). We determined that, on average, when the epidermal cells have excess *chic*, the wound area is smaller, indicating that additional Profilin protein can increase the speed of the cell migration required for closure (Fig. 3.6 C), assuming that wounds created with the same forceps had an equivalent average initial size between phenotypes. This further indicates the important role that Profilin plays in larval epidermal wound closure.
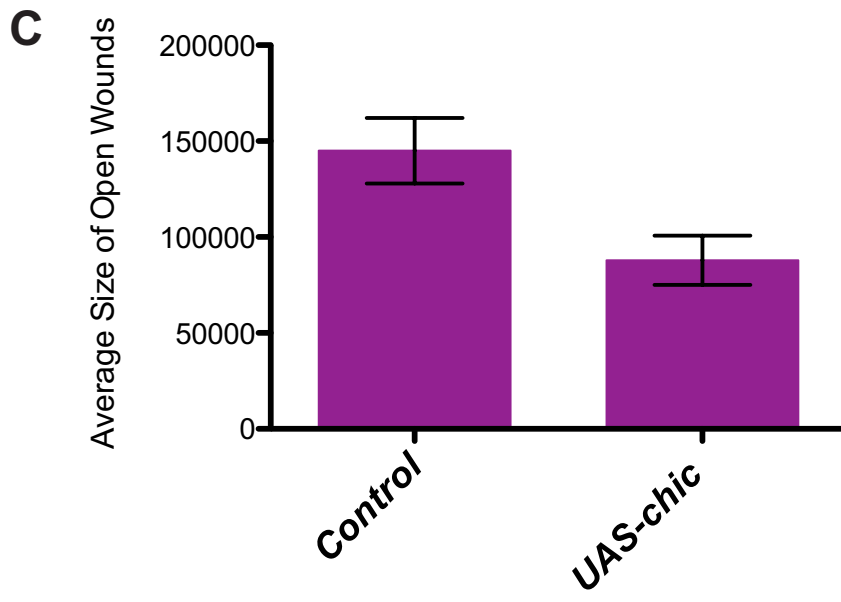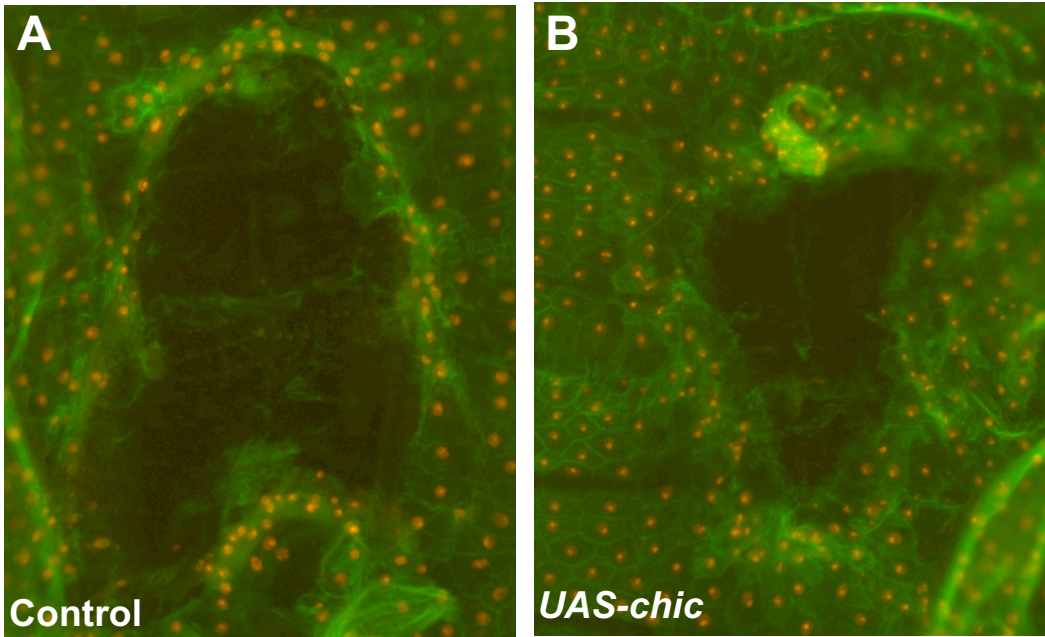
Fig. 3.6 Overexpression of *chic* results in smaller wound gaps than wild type midway through closure

(A-B) Dissected larval epidermal wholemounts of genotype *w;e22c-gal4, UAS-src-GFP, UAS-dsrednuc* plus *w^{1118}* (A) or *w;UAS-chic* (B). (C) Quantification of the area in square pixels of wound gaps eight hours following wounding. Area is significantly different between the two groups ($p < .01$, Student's T-test). n $\geq$ 15.

3.6 Profilin is transcriptionally induced after wounding

The increase in *chic* protein levels led us to ask whether or not the additional protein levels were due to an increase in transcription or translation. We attempted to resolve this in multiple ways; one method was to perform *in situ* hybridizations to detect message levels. However, there is not currently an existing protocol for that in the larval epidermis and we were unsuccessful in creating one. We were able to use the *chic* hypomorph as a lacZ reporter, as it contains a P-element insertion that includes a lacZ construct downstream of the promoter region, thus allowing for lacZ expression to be controlled by *chic*'s regulatory mechanisms.

We used the lacZ expression to do a time course experiment. In the unwounded larval epidermis, there was no expression of the lacZ, indicating that Profilin is not transcribed in the normal third instar epidermis, or at least, is transcribed at low levels undetectable by the assay (Fig. 3.7 A). However, six hours after wounding, the lacZ expression went up in the cells at the wound edge and for 2-4 cell rows back around the wound, indicating that the wound activates a local increase in *chic* transcription (Fig3.7 B). By 24 hours after wounding, when the wound has closed, there was still low level expression of the lacZ (Fig. 3.7C), which is likely an artifact of the lacZ expression, as it is a protein with a long half-life and may linger in the epidermal cells after the cessation of transcription. While it is formally possible that the lacZ expression is translationally regulated, it is unlikely that is the case; for translational regulation to be the explanation of the data, the wound would be required to induce translation of lacZ and it is unlikely that there is a mechanism for inhibiting lacZ translation.

A — Control, unwounded
B — Control, 6hr
C — Control, 24 hr
D — *Pvr^{IR}*, unwounded
E — *Pvr^{IR}*, 6hr
F — *Pvr^{IR}*, 24hr
G — *JNK^{IR}*, unwounded
H — *JNK^{IR}*, 6 hr
I — *JNK^{IR}*, 24 hr
J — Control
K — *UAS-λPvr*
L — *UAS-hep^{CA}*
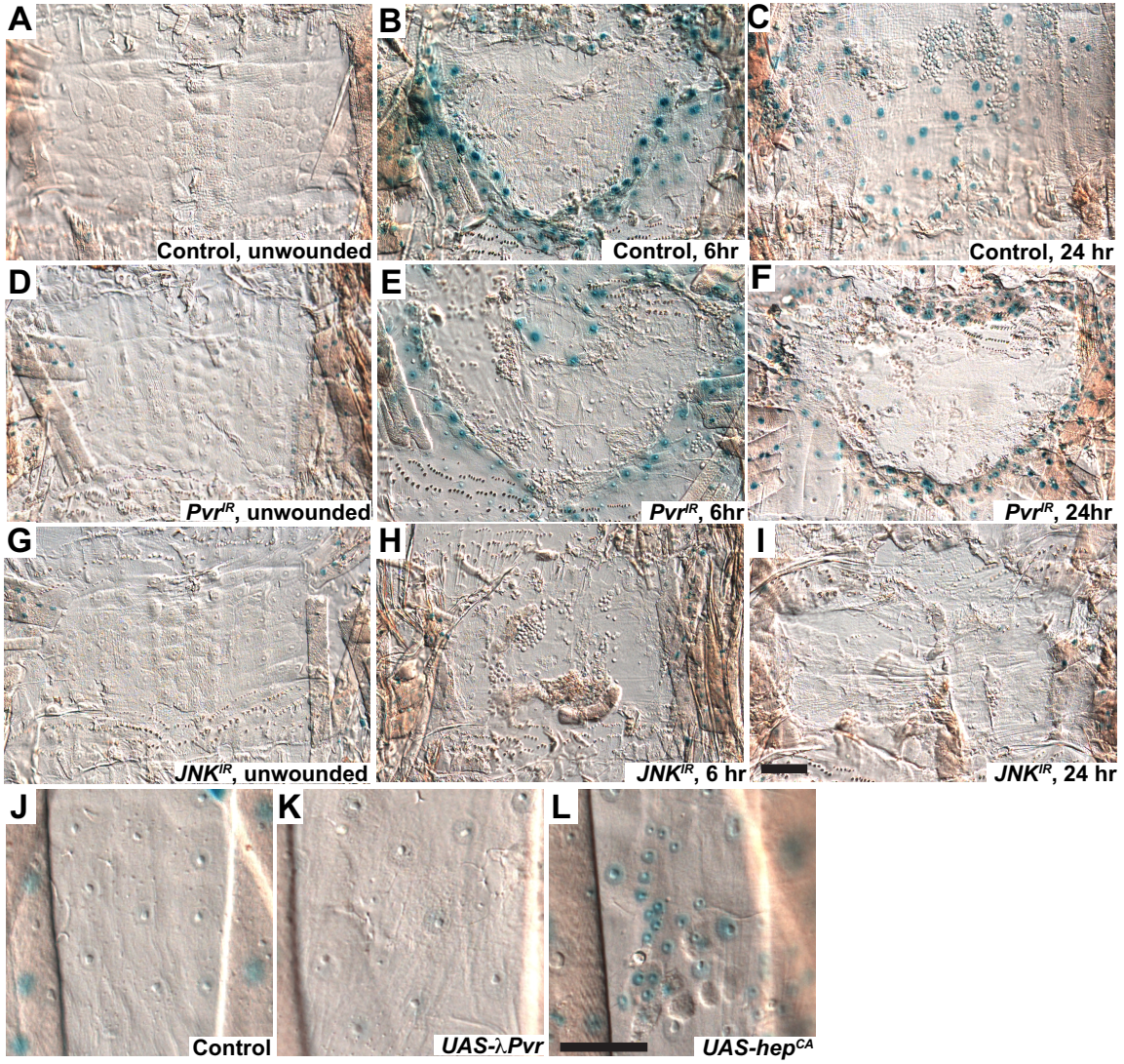
Fig. 3.7 *chic* transcription is regulated by the JNK but not the Pvr signaling

pathway

(A-I) Dissected epidermal wholemounts of larvae heterozygous for *w;e22c-Gal4, chic^{01320}* and the indicated mutants or transgenes. All are stained with X-Gal (see methods) to highlight β-Galactosidase activity in *lac-Z*-expressing nuclei (blue). (A, B, C) *w^{1118}*, (D, E, F) *UAS-Pvr^{IR}*, or (G,H, I) *UAS-JNK^{IR}*. (A,D,G) are unwounded, (B,E,H) are 6 hours after wounding and (C,F,I) are 24 hours after wounding. Note the increase in *lacZ* expression in wild type (B) following wounding. This increase is absent in cells lacking *JNK* expression (H).   (J-L) Dissected epidermal wholemounts of larvae of genotype: w;*tubgal80^{ts60}/chic^{01320}; pnr-Gal4, UAS-GFP* (J) plus *UAS-λPvr* (K), or *UAS-hep^{CA}* (L). Note the induction of *lacZ* in larvae with constitutively activated JNK expression (L). Scale bar in (I) represents 100 μm and refers to panels (A-I). Scale bar in (L) represents 50 μm and refers to panels (J-L). Assistance demuscling by Yan Wang.

3.7 *chic* expression is regulated by JNK, not Pvr

Since we determined that *chic* transcription increased after wounding, we were interested if either of the two signaling pathways that are known to be required for wound closure were regulating it. The two known signaling pathways are the JNK and Pvr pathways. Currently, the model suggests that JNK regulates the partial dedifferentiation response seen in cells at the wound edge that allowed them to stop making cuticle and detach from it, while Pvr was thought to regulate cell migration. To further confirm the JNK^{IR} line's efficacy, we did a rescue experiment to show that *bsk* cDNA can rescue the RNAi wound closure defect (Fig. 3.8), which suggests the RNAi line does not have off target effects that are resulting in the phenotype. We tested whether *chic* was regulated by either pathway by combining the lacZ reporter with JNK or Pvr RNAi lines. We hypothesized that *chic* would be regulated by Pvr, even though it had been shown to function downstream of JNK during dorsal closure (Jasper et al., 2001). We first looked at *chic-lacZ* in the absence of Pvr and found that there was no induction in the unwounded larvae, as in wild type (Fig. 3.7D). The lacZ expression was also induced six hours after wounding, indicating that *chic* transcriptional expression does not require Pvr (Fig. 3.7E). At 24 hours after wounding, the lacZ is still present, but the wound is open (as expected in an epidermis lacking Pvr) (Fig. 3.7F). It is worth noting here that this assay does not exclude the possibility that Pvr regulates *chic* protein after it is translated. We next tested whether *chic-lacZ* was induced without JNK signaling present. In the unwounded epidermis lacking *bsk*, there was no *chic-lacZ* induction, as expected (Fig. 3.7G). However, six hours after wounding, there was still no *chic-lacZ* induction, indicating that wound-induced *chic* transcription is regulated by JNK signaling (Fig. 3.7H). At 24 hours

after wounding, there was still no induction of *lacZ* expression (Fig, 3.7I), and the wound remained open, as expected.

To further confirm that *chic-lacZ* induction is regulated by JNK and not Pvr, we did a gain of function experiment where we hyperactivated the JNK pathway by expressing *UAS-hep^{CA}*, a line that has a phosphorylation mimic and is thus constitutively active; or the Pvr pathway by expressing *UAS-λPvr*, a line that is constitutively dimerized, to see if either was sufficient to induce *chic-lacZ* in the absence of wounding. Since both hyperactivated lines are lethal when expressed pan-epidermally, we used the line *tubgal80^{ts}; Pnr-Gal4, UAS-GFP*, which does not express at room temperature, but allowed us to limit the expression to when the larvae are at the permissive temperature of 37°C. When this line is crossed to *w^{1118}* or *UAS-λPvr*, there is no induction of *chic-lacZ*, as expected (Fig.3.7 J,K). However, when crossed to the *UAS-hep^{CA}*, there was induction of *chic-lacZ* expression, giving further evidence that *chic* is regulated by the JNK signaling pathway.
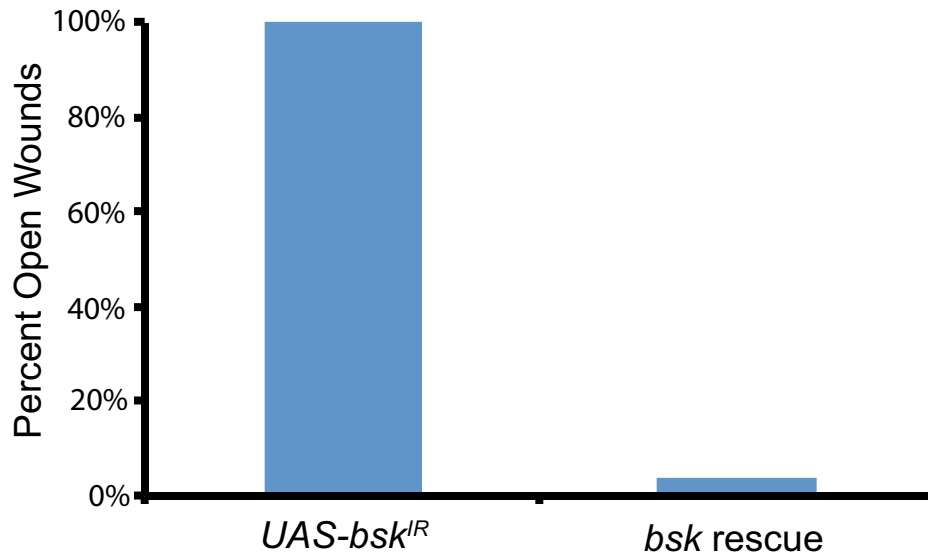
**Figure 3.8 *UAS-bsk^B* rescues the RNAi wound closure phenotype of *UAS-bsk^IR*

Percentage of larvae that exhibit an open wound phenotype after 24 hours. The left column shows that 100% of larvae of genotype *w; e22c-gal4, UAS-src-GFP, UAS-DsRed2-Nuc/+; 5680R2/+* exhibit open wounds, while the right column shows that 3.7% of larvae of genotype *w;e22c-gal4, UAS-src-GFP, UAS- DsRed2-Nuc/UAS-bsk^B; 5680R2* have open wounds. n ≥ 20.

3.8 *chic* is regulated by the Jun and Fos transcription factors

Because *chic* is regulated by the JNK signaling pathway, we investigated whether the two transcription factors that are downstream of the canonical JNK signaling pathway, DJun and DFos, were required for *chic-lacZ* induction. Interestingly, recently it has become known that in wound healing contexts, Jun and Fos may not always act together as a heterodimer, as they are commonly thought to. For example, Fos is required to activate *msn-lacZ* after wounding, while Jun is not (Lesch et al., 2010), though this experiment was tested with *DJun^{IR}*, which may not be completely effective, and Fos is required for *ddc* induction (Pearson et al., 2009). We performed the same test as above with each transcription factor and found that they were both required for the wound-induced expression of *chic-lacZ* (Fig. 3.9A,B). The control for this experiment is in Fig. 3.7B, as the experiments were performed concurrently. It is likely that they act as a heterodimer to effect *chic* induction, since only Jun has been shown to act as a homodimer. We are uncertain if Jun and Fos directly affect chic transcription or whether they regulate an upstream regulator of chic.

A *chic-lacZ, DFos^{IR}*, 6hr
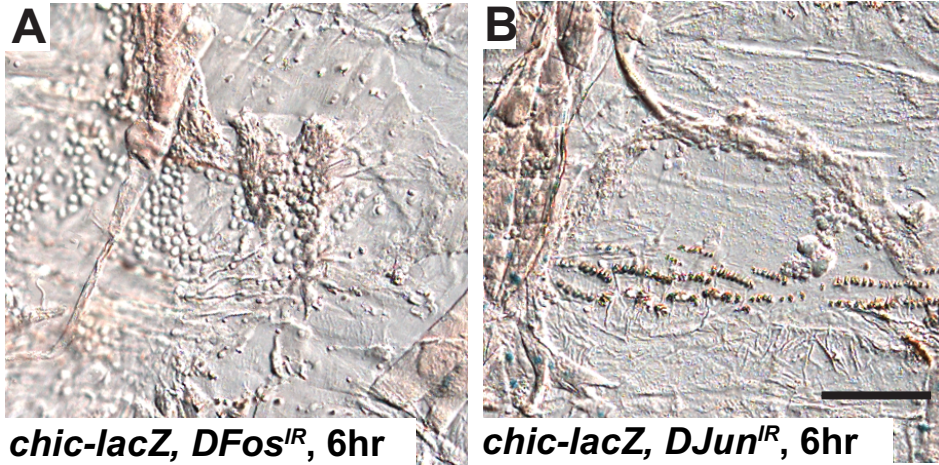
B *chic-lacZ, DJun^{IR}*, 6hr

Figure 3. 9 *chic* **is regulated by DJun and DFos**

Dissected larval epidermal wholemounts of genotype *w;e22c-Gal4, chic-lacZ* plus *UAS-DFos^{IR}* (A) or *UAS-DJun^{IR}* (B) 6 hrs after wounding.   The positive control is in Fig.

3.7A. Note the lack of lacZ induction. Assistance demuscling by Yan Wang.

3.9 AP-1 sites are located in the upstream region of *chic*

We next investigated if it was formally possible that Jun and Fos (known as AP-1) could directly bind to the *chic* promoter region to promote transcription. We looked at the gene sequence and found that the consensus sequence for AP-1 binding (TGANTCA) was located twice upstream of the *chic* translational start codon (Fig.3.2, arrowheads). Interestingly, depending on the predicted message variant of *chic*, it was either present in the promoter region, the 5'UTR, or the first intron, indicating that it is possible that wound-induced transcription of chic is regulated by Jun/Fos with a specific regulatory region that may be different from what regulates the basal levels of Profilin.

3.10 Profilin is required to localize actin to the leading edge of wounds

Since Profilin is a known actin regulator, we suspected it played a role in wound closure by regulating the actin cytoskeleton during cell migration. It was important to find a reliable way to examine the actin cytoskeleton to see if a lack of Profilin was affecting the actin-based structures (i.e. cable, filopodia, lamellipodia) at the leading edge of the wound. Typical reagents, such as phalloidin or an *α-actin* antibody are not ideal, given that they stain brightly in the larval body wall muscles, complicating our ability to clearly determine what is happening in the larval epidermis. We searched for a label that we could express via the Gal4/UAS system, allowing us to eliminate the problem of the larval body wall muscles.

Beacuse the *UAS-actin-GFP* overexpresses actin and thus could potentially interfere with the system being tested, we turned to a new reagent, Lifeact-GFP. Lifeact is a short 17-amino acid peptide that binds to actin filaments without interfering with actin polymerization or actin binding proteins (Riedl et al., 2008). One of the benefits of

this construct is that it does not overexpress actin. We were fortunate in that another lab

had made a *UAS-lifeact-GFP* transgenic fly already and were willing to share with us

(See Ch.2). We used this line crossed to *e22c-gal4* to examine the actin structures that

are present at the leading edge of the wound. In the unwounded epidermis, the actin is

evenly distributed within the cells (Fig.3.10A). Four hours after wounding, actin has

localized to the leading edge of the cells at the wound gap, forming short, discontinuous

actin cables and frequent extensions into the wound, including filopodia and lamellipodia

(Fig. 3.10D, D', D"). At 24 hours after wounding, the wound has closed, as expected,

and the actin has returned to a more even distribution, with remnants of actin cable or

stress fibers (Fig. 3.10F).

We next used this tool to examine the actin distribution and structures in

epidermal tissue with reduced Profilin. We crossed the *e22c-Gal4, UAS-Lifeact-GFP*

line to the *chic^{IR}* line. In the unwounded epidermis, the actin is evenly distributed, as in

the control experiment (Fig. 3.10 B). Four hours after wounding, there is no

relocalization of actin to the wound edge (Fig. 3.10 E, E'). There is no appreciable actin

cable, and while there are a few processes, they are short and rare (Fig. 3.10 E"). By 24

hours after wounding, the wound was still open and there were still no actin-based

structures at the wound edge (Fig. 3.10 G). Interestingly, there appeared to be multiple

vesicular structures in cells at the wound edge, as well as some dysregulation of FasIII,

indicating that there may be some defect in membrane regulation at the wound edge when

Profilin is absent or an increase in endocytosis, which is not typical of open wounds (Fig.

3.10 G'). We quantified the range of fluorescence intensity from the leading edge to the

other side of the cell to quantify the difference between the control and cells lacking

Profilin protein and found that there was a significant difference (Fig. 3.10C).

A — *w[1118]*, unwounded

B — *chic[RNAi]*, unwounded

C — Fluorescence intensity; Control, *chic[RNAi]*; *

D — *w[1118]*, 4 hr

D' — *w[1118]*, 4 hr

D'' — *w[1118]*, 4 hr

E — *chic[RNAi]*, 4 hr

E' — *chic[RNAi]*, 4 hr

E'' — *chic[RNAi]*, 4 hr

F — *w[1118]*, 24 hr

G — *chic[RNAi]*, 24 hr
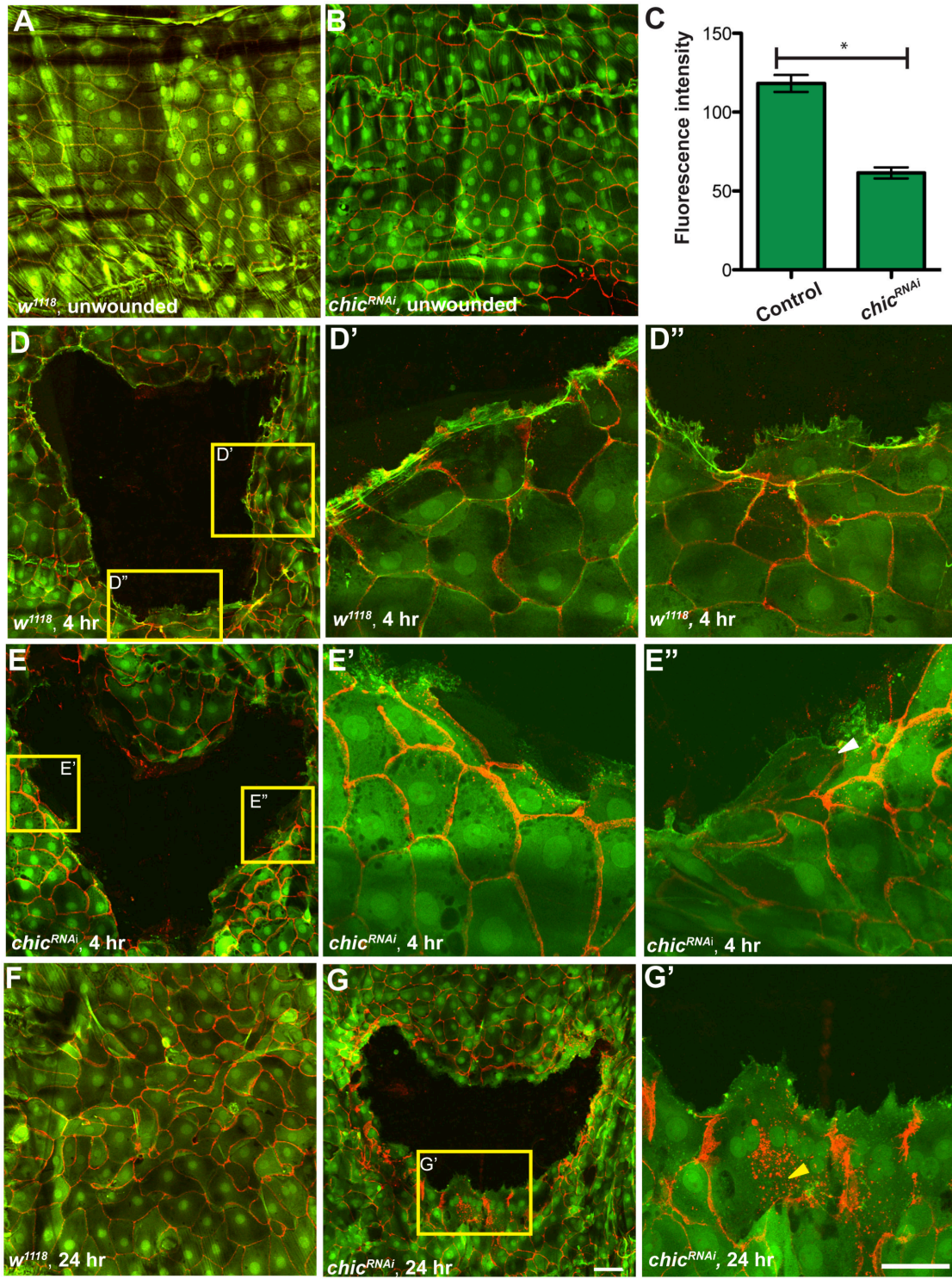
G' — *chic[RNAi]*, 24 hr

53

Fig. 3.10 Actin localizes to the wound edge and forms processes in wild type but not

in larvae lacking Profilin expression

(A-G) Dissected epidermal wholemounts of larvae heterozygous for *w;e22c-gal4, UAS-lifeact-GFP6.0* and the indicated mutations or transgenes. Epidermal membranes are labeled with FasIII (red). (A, D, F) w^{1118} (control). (B, E, G) $chic^{IR(R3,R4)}$. (A, B) Unwounded. (D, E) 4 after wounding. (F,G) 24 hours after wounding. Yellow boxes indicate the areas shown at higher magnification in (D',D", E', E'', and G').   Note the discontinuous actin cable in (D) and the presence of actin-based extensions in (D') and (D"). Note that the wound is closed after 24 hours (F).   Note the lack of actin cable in (E, E', E",G, and G'). The white arrowhead in E" indicates a small actin-based process. The yellow arrowhead in F' represents presumptive cytoplasmic vesicles. Note that wounds are open at 24 hours (F and F'). Graph in (C) shows the range of the fluorescence intensity between the leading edge and the interior of the cell in both control and $chic^{IR(R3,R4)}$ at four hours following wounding. Cable intensity is significantly different between groups ($p \leq 0.01$, Student's t-test). Error bars represent the Standard Error of the Mean. For the control group n= 96 measurements, for $chic^{IR}$, n=64. Scale bars in G and G' both represent 50 μm and refer to lower magnification and higher magnification panels, respectively.

3.11 Pvr regulates actin polymerization at the wound edge

We tested the localization of actin at the wound edge in epidermal tissues lacking JNK and Pvr using the *UAS-actin-GFP* label. Interestingly, 8 hours after wounding larvae lacking epidermal JNK, there appeared a robust actin cable and processes at the wound edge (Fig. 3.11B), though it is worth noting that these cells still have a migration defect, as the wounds remain open after 24 hours. In larval epidermal tissues lacking Pvr, there was no relocalization of actin to the wound edge and a dearth of actin-based structures, including cable or processes, indicating that Pvr regulates polymerization of actin at the wound edge in some fashion (Fig. 3.11C, D).

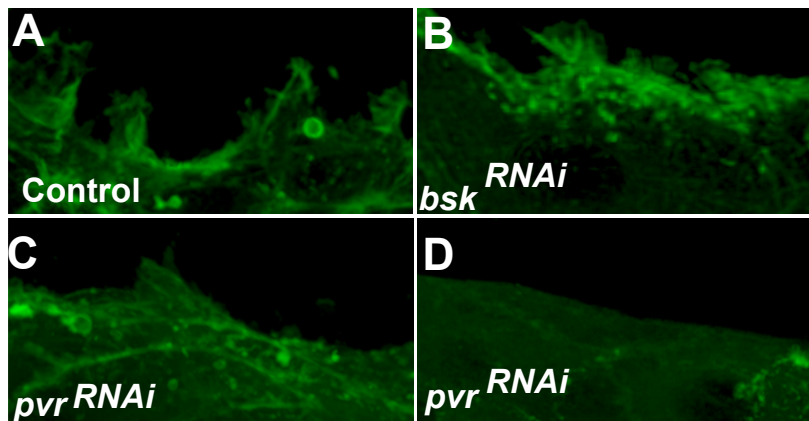**Figure 3.11 Pvr is required for actin accumulation at the wound edge, while JNK is not**

Dissected larval wholemounts done 8 hours following wounding. Genotype: A) *w; UAS-actin-GFP/+;A58-Gal4;* B) *w; UAS-actin-GFP/+;A58-Gal4/UAS-bsk^RNAi^;* C, D) *w;UAS-actin-GFP/UAS-Pvr^RNAi^;A58-Gal4*. Stained with α-GFP to enhance signal. Previously published in (Wu et al., 2009). Used with permission from the publisher.

3.12 Fos has a mild defect in actin polymerization at the wound edge

Since Jun and Fos were shown to regulate *chic* transcription, we assessed the phenotype of actin at the wound edge in epidermal tissues lacking Jun or Fos, even though the actin phenotype was normal in tissues lacking JNK, the known upstream kinase that activates them. In the unwounded epidermis, the actin in tissues lacking Jun or Fos was evenly distributed as in wild-type (Fig. 3.12 A,D). Four hours after wounding, tissues with reduced Jun had normal localization of actin to the wound edge and normal looking actin-based processes extending into the gap (Fig. 3.12E). However, in tissues lacking Fos, there was a minimal amount of localization of actin to the wound edge, less than in wild type, and there appeared to be a dearth of process extension into the wound gap (Fig. 3.12B). Interestingly, both experimental groups failed to effectively close the wound after 24 hours (Fig. 3.12C,F). We again quantified the difference in the range of fluorescence intensity for the two compared to wild type and found that tissues lacking DJun had a larger range than did wild-type, while DFos exhibited a range that was significantly lower than wild type.
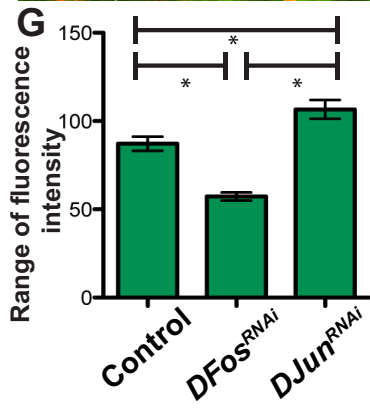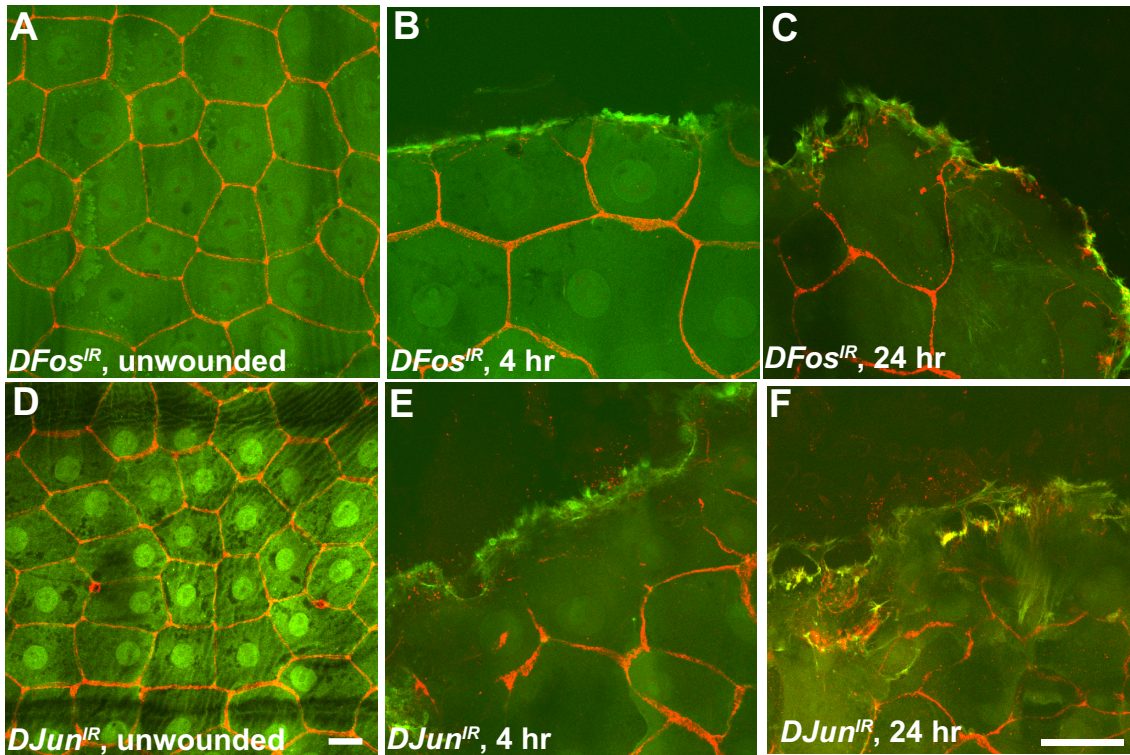
A. *DFos^{IR}*, unwounded
B. *DFos^{IR}*, 4 hr
C. *DFos^{IR}*, 24 hr
D. *DJun^{IR}*, unwounded
E. *DJun^{IR}*, 4 hr
F. *DJun^{IR}*, 24 hr

G. Range of fluorescence intensity — Control, *DFos^{RNAi}*, *DJun^{RNAi}*

Fig. 3.12 DFos is required for regular accumulation of actin at the wound edge

(A-F) Dissected larval epidermal wholemounts of genotype *w;e22c-Gal4, UAS-lifeactGFP* (to label epidermal actin in green) plus *UAS-DFos^{IR}* (A-C) or *UAS-DJun^{IR}* (D-F) and stained for Fasciclin III (red). (A, D) unwounded; (B, E) 4 hrs after wounding. Note that at four hours, *DFos^{IR}*-expressing larvae (B) have a dim actin cable, but do not appear to have processes, while *DJun^{IR}*-expressing larvae (E) resemble wild type (see Fig. 3.10). (C, F) 24 hours after wounding (G) Quantification of ranges of fluorescence intensity of the actin cable at four hours after wounding in the indicated genotypes. Cable intensity is significantly different between each group ($p \leq 0.01$, Student's t-test). Error bars represent Standard Error of the Mean. For control, n= 145, for *DFos^{IR}*, n=146, and for *DJun^{IR}*, n=100.

3.13 Actin at the leading edge of tissues lacking other important wound closure genes

Since we had developed a new assay for examining the actin-based structures during wound closure, we applied it to other genes known to be required for wound closure and to play a role in the regulation of actin. These genes included *Arp14D*, *G-protein γ1*, and *mbc (Lesch et al., 2010)*.

Arp14D is a member of the Arp2/3 complex, which is a protein complex that facilitates the branching of actin filaments off of a parent filament at the leading edge of a cell during migration. In unwounded larval epidermal tissues lacking *Arp14D*, the actin is evenly distributed, as in wild type (Fig. 3.13A). However, by four and eight hours after wounding, these cells exhibit longer than average filopodia that extend into the wound gap (Fig. 3.13B,C). At 24 hours after wounding, the wound remains open, but there are still long, actin-based structures at the wound edge (Fig. 3.13D). We hypothesize that the filopodia are extra-long due to the absence of branching, and that the branching is required for structural stability during migration. Since the tissues lacking *Arp14D* do not close, it would appear that the extra long filopodia are insufficient for healing.
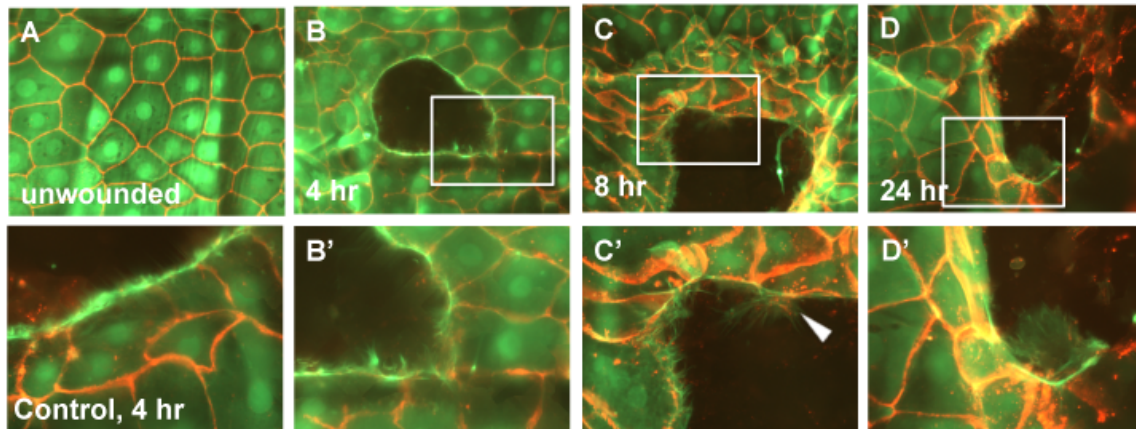
Figure 3.13 Lack of Arp14D results in long filopodia at the leading edge

Dissected larval epidermal wholemounts of genotype: *w;UAS-Lifeact-GFP;9901R-2*. A) unwounded; B, B') 4 hours after wounding; C,C') 8 hours after wounding. Arrowhead identifies long filopodia; D,D') 24 hours after wounding. Boxes in B,C,D represent the magnified area in B', C', and D'.

Gγ1 is a heterotrimeric G-protein that was found to affect actin polymerization in a screen of S2 cells(Kiger et al., 2003) and was also found to be required for wound closure in our lab's pilot genetic screen (Lesch et al., 2010). At four and eight hours after wounding, there is a distinctive actin cable, but few extensions into the wound gap (Fig. 3.14 B,C). By 24 hours after wounding in tissues lacking *Gγ1*, there is a very interesting phenotype where the wound begins to round up, as though it is being pulled by a contractile "purse string," and there is a distinct actin cable that appears to be continuous, as well as high levels of actin in the rest of the cell (Fig.3.14 D, D'). While the actin cable is contractile in dorsal closure and in embryonic wound healing, typically we do not see a continuous cable in larval wounds, nor do we see significant shape changes at the wound periphery. It appears as though *Gγ1* acts as some sort of switch that prevents normal closure from being overly dependent on the contractile actin cable and allows for wound closure to equally depend on the extensions at the leading edge. One possibility is that lack of *Gγ1* changes the tension within the epidermal sheet, leading to a different cytoskeletal arrangement for effective wound healing or that it affects the localization or levels of myosin.
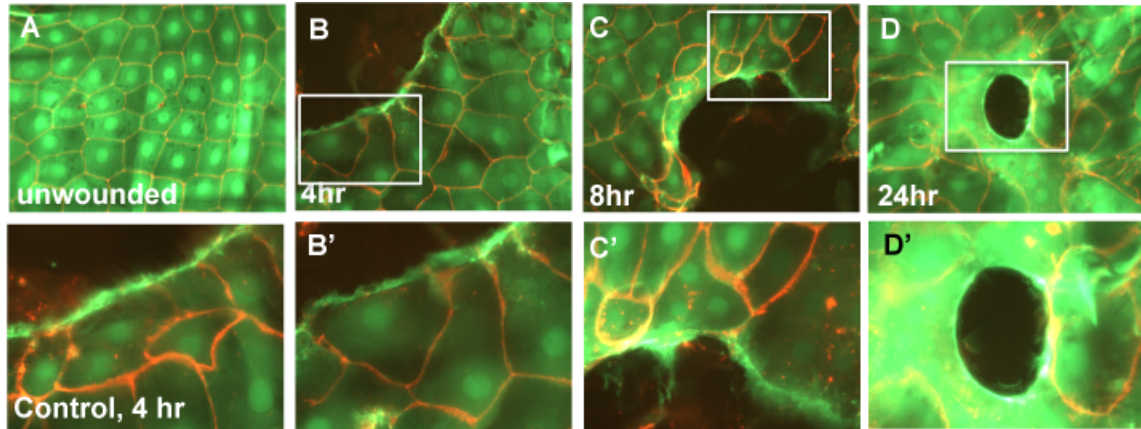
Figure 3.14 Lack of Gγ1 results in weak filopodia and rounded wounds

Dissected larval epidermal wholemounts of genotype: *w;UAS-Lifeact-GFP;8261R-1*. A) unwounded; B, B') 4 hours after wounding; C,C') 8 hours after wounding.; D,D') 24 hours after wounding. Boxes in B,C,D represent the magnified area in B', C', and D'.

myoblast city (mbc) causes a mild defect in the accumulation of actin at the leading edge during dorsal closure and is also required for larval epidermal wound closure (Lesch et al., 2010). Interestingly, we see a similar phenotype when looking at the actin in wounded epidermal tissue lacking *mbc*. Four hours after wounding, there is a limited actin-cable at the wound edge (Fig. 3.15 B) and limited processes that remain present by eight hours after wounding (Fig.3.15 C). However, by 24 hrs after wounding, the cable has diminished, though the wound is still open (Fig. 3.15D). This indicates that *mbc* may not be important for the initial formation of the actin cable, but may be required for its maintenance. It further indicates that genes discovered to regulate actin during dorsal closure may be important larval wound closure genes. Additionally, while the cable may not be required for local or purse-string contractility, this suggests it is necessary for a large concentration of actin to be maintained at the leading edge for efficient wound closure.
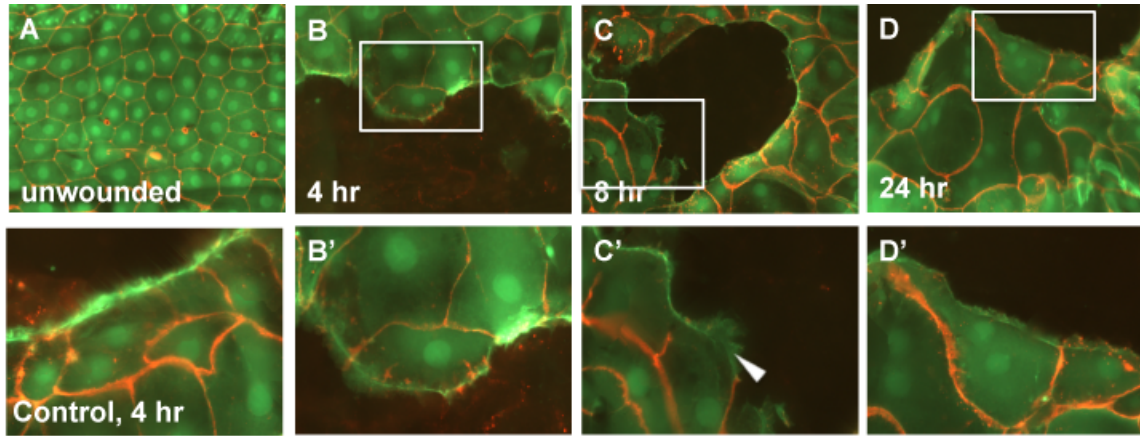
Figure 3.15 Lack of *mbc* results in a weak, transient cable

Dissected larval epidermal wholemounts of genotype: *w;UAS-Lifeact-GFP;10371R-1*. A) unwounded; B, B') 4 hours after wounding; C,C') 8 hours after wounding.; D,D') 24 hours after wounding. Boxes in B,C,D represent the magnified area in B', C', and D'.

3.14 Morphology of the leading edge of cells at the wound gap

In addition to looking at actin, we also wanted to use Transmission Electron Microscopy (TEM) to assess the morphology of the leading edge or protrusions into the wound gap. In the control epidermis, long cellular extensions into the wound gap are present by four hours after wounding (Fig. 3.16A). It appears as though these cells detach from the cuticle and extend over the cell debris to close the wound gap and reestablish tissue continuity. Previously, our lab described the morphology of wound edge cells in tissues lacking JNK or PVR (Wu et al., 2009); in tissues lacking JNK, the leading-edge cell is unable to stop producing cuticle and detach from it, such that it does not extend into the wound gap, but curls up around the debris at the edge. In tissues lacking Pvr, there is movement of cytoplasm to the wound edge, so the leading edge appears swollen, but it does not form any processes into the wound gap (Wu et al., 2009). These phenotypes led to the conclusion that JNK regulates a partial dedifferentiation that allows the cell to stop making cuticle, detach, and migrate into the wound gap, while Pvr regulates both the direction and the cytoskeletal rearrangements required for migration.

We looked at larval epidermal tissues lacking Profilin and found that at both four and 24 hours after wounding (Fig. 3.16 B,D), the leading-edge cell did not produce any long, thin processes, or look in any way as though they were preparing or able to migrate into the gap. Interestingly, the cells had neither thinned, nor shuttled cytoplasm in preparation for thinning, indicating that Profilin is required for this function.
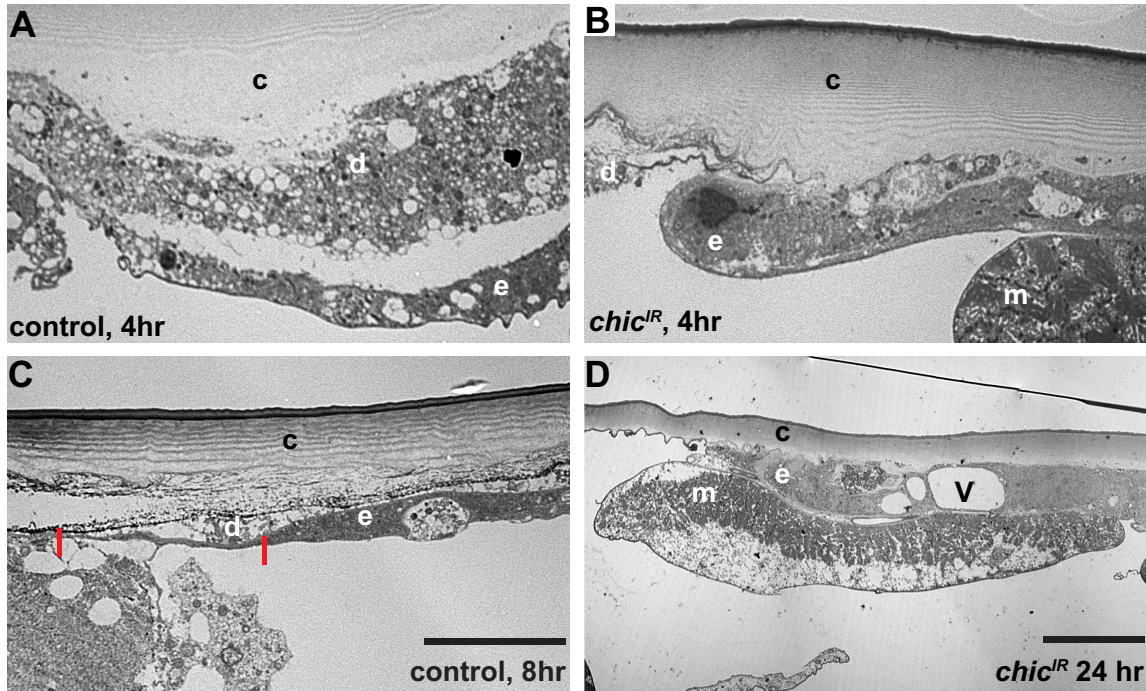
Figure 3.16 *chic* is required for cells to extend processes into the wound gap

(A-D) Transmission Electron Micrographs (TEM) of transverse sections of wounded and dissected L3 larvae of genotypes: *w; UAS-nlacZ/+; UAS-chic^{IR(R3,R4)}* (A); *w; e22c-Gal4/UAS-nlacZ; UAS-chic^{IR(R3,R4)}* (B,D); and *w;pxn-Gal4, UAS-nlacZ/+* (C). In all images, the wound gap is to the left and the cells are migrating in that direction. c = cuticle, m = muscle, e = epidermal cell, d = cell debris. (A,B) four hours after wounding. Note that the control extends a long thin process over the necrotic cellular debris (A) while cells lacking Profilin form a rounded and blunt wound edge (B). (C) Control 8 hours after wounding. The cell extends a normal process that is 14.4 μm long (between red tick marks) and 0.4 μm thick. (D) 24 hours after wounding. The wound has still not closed and the rounded cell has not appreciably extended into the wound gap. v, large cytoplasmic vesicles. Scale bars in C and D represent 10 μm (D refers to A and B as well). Preparation of samples by myself, Yan Wang, and Violet Han, microscopy assistance from Kenn Dunner, Jr.

66

We also looked at larval epidermal tissues lacking Jun or Fos. Interestingly, tissues lacking Fos looked similar to JNK, in that the cells were unable to halt cuticle secretion or detach from the cuticle to migrate into the gap. Tissues lacking Jun looked more similar to Pvr, where there was likely shuttling of the cytoplasm and a swelling at the leading edge, but no extensions into the wound gap or migrations.
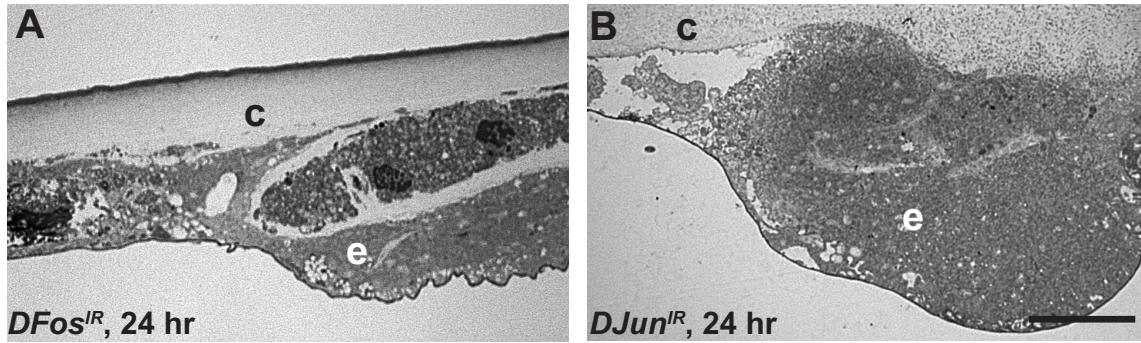
Figure 3.17 DJun and DFos are required for cells to properly extend into the wound gap

(A,B) TEM of transverse sections of wounded and dissected larvae of genotype *w;A58-Gal4, UAS-nlacZ/UAS-DFos^{IR}* (A) or *w; A58-Gal4, UAS-nlacZ / UAS-DJun^{IR}* (B). 24 hours after wounding. e = epidermal cells, c = cuticle. Scale bar in (B) represents 10 μm and refers to (A,B). Experiment performed by Michael Galko and Violet Han with microscopy assistance from Kenn Dunner, Jr.

Chapter 4: Discussion

In the previous chapter, we have shown that Profilin is definitively required for wound closure and that, during normal closure, both protein levels and transcriptional levels increase. We found that the increase in transcription was regulated by the JNK signaling pathway, and not the Pvr pathway, and more specifically, by the downstream transcription factors, Jun and Fos. Additionally, we showed that Profilin is required to localize actin to the leading edge of a wound and to form the actin-based processes that are required for cell migration. We also examined the localization of actin in tissues lacking other important wound closure genes.

4.1 Model for regulation of Profilin during wound closure

The observation that Profilin transcription is regulated by JNK signaling was surprising in light of the ability of larval epidermal tissues lacking JNK signaling to localize actin to the leading edge of the cell and form filopodia and lamellipodia that extended into the wound gap area. Since Profilin is required for this relocalization, it seemed curious that tissues unable to increase transcription of the required protein would be able to properly localize actin. However, we propose a model where the basal level of Profilin that is present in the unwounded tissue or in tissues lacking JNK signaling is sufficient for localization of actin to the leading edge, but the additional Profilin that is transcribed and translated after wounding is required for the rapid filament growth and retraction that is the hallmark of effective migration (Fig. 4.1). This explains the discrepancy between the actin localization in JNK^{IR} experiments and those in Profilin. It also logically fits with the biochemical function of Profilin; namely, to exchange ADP to ATP on actin monomers and facilitate the growth of the actin filament, possibly through formin-mediated elongation. It is logical that a fully-differentiated, non-motile cell

would need an increase in Profilin to recycle actin monomers and recruit active monomers for filament growth for efficient migration. It also fits in light of variation of regulatory regions for the different *chic* messages; since all of the isoforms are the same in coding sequence and vary only in the 5' or 3' UTR, it seems possible that the wound-induced Profilin could come from different upstream regulation than the basal level that is present in unwounded cells.

A

JNK ?

Jun/Fos

chic

Dedifferentiation

Pvf1

Pvr

Actin Nucleation

Recycling/Filament Growth

B Wild Type
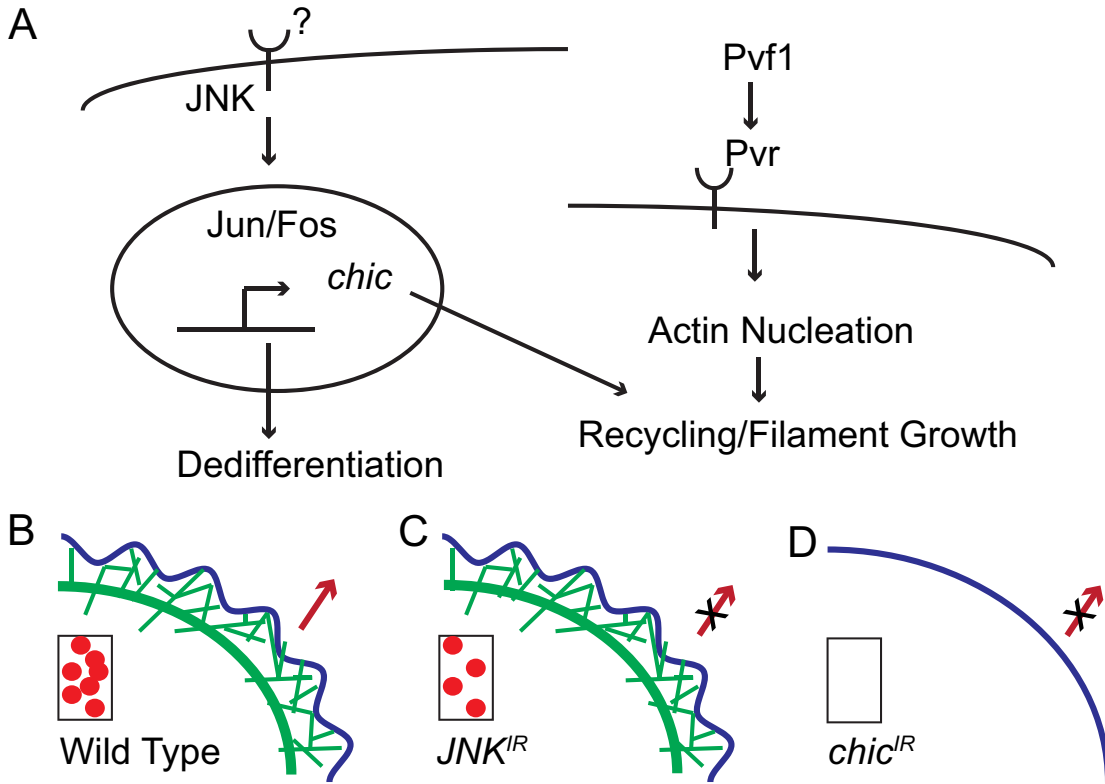
C JNK^{IR}

D $chic^{IR}$

E

Genotype	Basal Profilin	Wound-Induced Profilin	Leading Edge Actin Structures	Cell Migration
Wild Type	+	+	+	+
JNK^{IR}	+	-	+	-
$chic^{IR}$	-	-	-	-

Fig. 4.1 Model of JNK and Pvr Signaling Pathways during wound closure

A) JNK signaling is shown on the left, with an as yet unidentified extracellular ligand activating the intracellular JNK, which can in turn activate Jun and Fos. These transcription factors play a role in tissue dedifferentiation required for wound closure. Pvr signaling (shown on the right) activates actin nucleation. Here, we demonstrate that the JNK pathway converges on regulation of actin dynamics by activating transcription of the actin recycling factor *chickadee*, which is also required for wound closure. (B-D) Visual representation of the actin cytoskeleton (green) at the leading edge of the wound. (B) wild type; (C) *JNK^{IR}*; (D) *chic^{IR}*. Blue line, plasma membrane. Red arrow = direction of migration. In all three cells, the box in the lower left indicates Profilin (red dots) levels. (E) Table summarizing the Profilin expression and actin-based characteristics of the above genotypes.

However, it is also possible that the defect in dedifferentiation of JNK^{IR} is the sole reason these cells cannot migrate. Per the TEM, cells lacking JNK are unable to stop secreting new cuticle and are unable to detach from the cuticle to extend processes into the gap (Wu et al., 2009). Currently, we cannot experimentally verify whether JNK^{IR} epidermal cells would be capable of migration were they able to detach from the cuticle. One possible experiment, if live-imaging were less technically fraught, would be to quantify whether the filopodial and lamellipodial "ruffling" at the wound edge was as robust in JNK^{IR} cells as it is in wild type cells. However, we must also consider the possibility that the partial dedifferentiation that occurs in the leading edge cells includes a return to a migratory state. It is often seen in epithelial cancers that loss of epithelial identity (as defined by apical basal polarity and location) coincides with a gain of migratory and proliferative abilities. As the JNK pathway is a stress-response pathway that regulates many processes after wounding, it may be impossible to de-activate cuticle synthesis without also activating migration.

It is also conceivable that cessation of cuticle production is not a sufficient marker for dedifferentiation and that stopping cuticle secretion in order to migrate in response to tissue damage is a behavior that is within the epidermal cell's identity. The apical microvilli that secrete cuticle proteins are lost in the cellular extension over the debris (unpublished data), though this morphological change may be insufficient evidence of true de-differentiation. While the physiological evidence suggests the cells engage in new behaviors following wounding, currently there is no molecular evidence to suggest a return to a multipotent state; i.e. reverting to a state where it had the potential to become

something other than an epidermal cell. Without this evidence, it may be that

dedifferentiation is the wrong term for the cellular changes regulated by the JNK

signaling pathway.

It was surprising to discover that *chic* is regulated by both the Jun and Fos

transcription factors. While they are thought to act as a heterodimer in the canonical JNK

signaling pathway (Kockel et al., 2001), previous work has suggested that they have

separate roles in wound healing. For example, DFos can act by itself to activate a *ddc*

wound reporter (Pearson et al., 2009); additionally, DFos, but not DJun, is required for

msn-lacZ induction after wounding (Lesch et al., 2010). Since the AP-1 transcription

factor binding site (consensus sequence: TGAGTCA) is located in the promoter region of

two isoforms of *chic*, it is certainly possible that DFos and DJun together regulate a

wound induced response. In the future, it would be interesting to undertake a tissue

specific microarray to examine how many genes DJun and/or DFos regulate following

wounding, whether they do so independently or together, and to categorize how many of

them regulate cell migration or cuticle synthesis.

4.2 Pvr and cell migration

There is another known signaling pathway, apart from JNK, that is required for

Drosophila larval epidermal wound closure, namely, the Pvr pathway. As shown here,

absence of Pvr in epidermal cells results in a complete lack of actin localization at the

wound edge. Pvr has been shown in other systems to be required for cell migration, so

this is not ultimately surprising. We had expected that Pvr would regulate *chic*

transcription, which it does not. However, this does not preclude a post-transcriptional

role for Pvr signaling in regulating Profilin or other actin regulators. Currently, the

downstream signaling pathway of Pvr is largely unknown, though the targets that are known in thorax closure are known actin cytoskeleton regulators (Ishimaru et al., 2004). There is currently no evidence that the Pvr signaling pathway regulates transcription, indicating that its entire role may be activating post-translational modifications or relocalizations that induce the actin cytoskeleton rearrangements required for migration. This would also explain the need for two signaling pathways to regulate this process: it is possible that the JNK signaling pathway activates transcription all over the genome of a variety of important genes, while the Pvr signaling pathway regulates existing proteins and structures for effective healing.

One could imagine a model where the Pvr receptor, and its ligand, Pvf1, are among the early detectors of tissue damage and are then responsible for activating the actin polymerization machinery along the wound edge, and thus, priming the cell for migration. Such a model would also explain why Pvr has such a strong defect in actin accumulation at the wound edge while other actin regulators have subtler phenotypes. For example, if Pvr is an upstream activator of Arp2/3, Mbc, $G\gamma 1$, and others, it would be expected that knocking down Pvr would result in none of the downstream targets being activated, thus, the strong phenotype of no actin accumulation. However, when any single actin regulator is knocked down, Pvr would still be able to activate all of the other actin regulators, meaning specific events are left undone. This model fits with the available data; for example, Arp2/3, which is known to branch actin filaments, has a subtle phenotype where filopodia appear longer than is typical at the wound edge, indicating a defect in branching, but no larger defect in actin localization or polymerization.

4.3 Actin Cable

A role for both the actin cable and the filopodia and lamellipodia has been previously shown in *Drosophila* embryonic wound healing. In that system, wound closure is dependent on the contractile actin cable, though it is debated whether the cable is required for contractility or as a platform for the actin-based processes at the wound edge that are required for the final zippering stage and the correct developmental patterning. It appears that larval wound closure differs in that respect, since the data suggest that there is no continuous "purse string" actin cable that contracts to close the wound. It also does not appear that the cable is locally contractile, as one would imagine that resulting in a tapering of cells towards the wound edge that is not present. We also have the impression that they are locally mutually exclusive; in areas of robust lamellipodial extension, there may be less cable and vice versa (Fig. 3.10, D"). Work in cell culture systems have shown a similar phenomenon (Anon et al., 2012). Together, this indicates that, at least in larvae, the cable is functioning more as a platform for the filopodia and lamellipodia that effect migration.

There is some evidence that the shape of the wound determines whether a contractile cable can form or not; in the *Xenopus* epithelium, differently shaped wounds form cables but do not contract (Davidson et al., 2002). MCDK cells in culture have more lamellipodia along epithelial gaps with lower curvature than in highly curved areas where there is mostly contractile cable (Anon et al., 2012). Thus, the difference we see between embryonic and larval wound closure may have something to do with the method of wounding; most of the work done in embryos features wounds created by lasers, which makes for perfectly round wounds, while in larvae, we use forceps that create irregularly

shaped wounds. It's possible that larval wounds that were perfectly round would form a continuous cable. Interestingly, when Gγ1 is knocked out, the actin showed a phenotype where the wound became round and a continuous cable formed, indicating that there may be a genetically regulated process that prevents wound closure from being dependent on cable contractility. If strong lamellipodia take actin away from the cable, it would indicate that there is an advantage of strong lamellipodia over contractility during larval wound healing.

4.4 Future Directions

There are several sets of small experiments that would further clarify the work in this thesis. While we did not stain the larval epidermis for Profilin in tissues lacking JNK or Pvr due to the technical difficulty of demuscling the epidermis, this experiment would answer questions of how quickly protein levels increase following wounding in tissues with or with JNK and would answer whether Pvr signaling is required for localization changes. We could also do RT-PCR in addition to the lacZ staining performed in this thesis to quantify a precise level of increase in *chic* message levels.   Another small experiment to further analyze the work done in this thesis would be to do a time-course experiment in cells with *chic* overexpression to determine the time range of closure compared to wild type. A major hurdle to overcome which would greatly enhance this work, as well as future work in the field, is to develop a protocol for larval live-imaging. Immobilizing the larvae without killing them is exceedingly difficult; current strategies for doing so manage mere minutes before the larvae move, which is not a long enough length of time to truly analyze what the cells at the leading edge are doing.

This work has produced many interesting hypotheses that would be worth investigating in the future. First, it would be worthwhile to determine whether JNK signaling transcriptionally regulates a whole suite of actin regulatory genes or if it is phenomenon unique to Profilin. To do this, we could either look for or create lacZ reporters for other actin regulators with wound closure defects, though this is potentially laborious. Another method would be to perform microarray analysis on the cells surrounding a wound with and without JNK to determine which genes are transcriptionally upregulated following wounding. We would expect that regulation by JNK signaling is a general feature of actin regulators that must be induced to make a non-motile cell migratory. While doing this, it would also be interesting to conduct a similar experiment with Jun and Fos to see if there is a pattern to which genes they upregulate together and which genes only require Fos for transcriptional regulation following wounding.

Second, performing a similar microarray experiment with and without Pvr would test another hypothesis; namely, that the actin regulation performed by Pvr is post-translational and not transcriptional. This can also be accomplished by immunostaining of actin regulators, including Profilin, after wounding to see if the localization within the cell is different when Pvr signaling is present or not. Further, since Pvr is an RTK, it would be interesting to see if the phosphorylation status of actin regulators change following wounding. It would be particularly interesting to see if Arp2/3, Mbc, or $G\gamma1$ localization or activation changes in the absence of Pvr. Currently, a postdoc in the lab is working on a screen to find downstream regulators of Pvr by knocking genes down in the

hemocytes and testing their ability to form lamellipodia in response to Pvf1 treatment. I would hypothesize that she will find multiple actin regulators in this screen.

Both of these sets of experiments would help answer the question of why wound healing has evolved parallel pathways to regulate closure, and if they reveal a clear division of labor for activating migration for wound closure. It would be interesting to look at the same pathways in vertebrate wound healing to determine if they are parallel, and if so, if the different, but necessary, effects the pathways have are conserved.

4.5 Medical Implications

Knowledge of how cells migrate following wounding has profound medical implications. For example, drugs enhancing the JNK signaling pathway or Profilin itself could theoretically lead to increased healing speed, or enhanced healing in wounds that remain open, such as in diabetic patients.

However, there are also implications in the realm of cancer treatment. A hypothesis in the field has been that cancer cells hijack wound healing pathways during metastasis, as these cells have to achieve similar tasks: proliferate (in vertebrate wound healing), partially change identity, and migrate (Schafer and Werner, 2008). Knowing how the signaling is regulated when properly activated could lead to understanding the mechanisms when this process is dysregulated in cancers. For example, it is not inconceivable that an inhibitor of Profilin could act to prevent migration and, thus, metastasis, though it might also have a deleterious effect on healthy tissues.

4.5 Conclusions

In this thesis, we have shown that Profilin is required for larval epidermal wound closure and that it is regulated by the JNK signaling pathway. Currently, it is unknown if

new transcription of actin regulatory proteins is a general feature of wound healing or if it is unique to Profilin. I would hypothesize that it is a general feature and part of the dedifferentiation process required for effective wound closure.

Appendix

During wound closure, the larval blood cells (hemocytes) attach themselves to the wound site in a process similar to vertebrate inflammation. In vertebrate tissues, neutrophils diapedese from local blood vessels to the site of tissue damage in a directed cell migration and release reactive oxygen species and fight potential infection through phagocytosis (Martin and Leibovich, 2005) and macrophages follow later to continue phagocytosing debris and infectious microbes. This process has been studied in *Drosophila* embryos, where it was found that, despite drastically different circulatory systems, hemocytes directly migrate to wound sites (Stramer et al., 2005). As insects, *Drosophila* have an open circulatory system. In the larvae, blood cells and hemolymph are pumped from the posterior through the dorsal vessel (heart) where they are released at the anterior of the larvae, and then float through the hemolymph towards the posterior, propelled by the peristaltic motion of the larvae. Our lab found that the larval hemocytes attach to the wound site directly from circulation (Babcock et al., 2008) as opposed to directly migrating. Since the size and movement of a larvae, along with the interior lining of the epithelial epidermis, is similar to a vertebrate blood vessel lined with endothelial cells, this system offered a nice model for studying a mechanism of how blood cells may act inside the vessel. The question that arose was the molecular mechanism that led to the direct capture of blood cells from circulation. I undertook an RNAi-based screen to identify the cell surface molecule(s) on the hemocytes that recognize tissue damage and attach to fight infection at the wound site.

To do this, I used an *Nrg-GFP;Pxn-Gal4, UAS-yfp* reporter line that expresses an epidermal membrane marker and labels the blood cells with a yfp, allowing us to look at

inflammation in a live larva.   I designed an assay where I would wound the larvae and

image the live larvae six hours later in order to examine the inflammatory response. We

devised a semi-quantitative system for scoring how much blood cell coverage there was
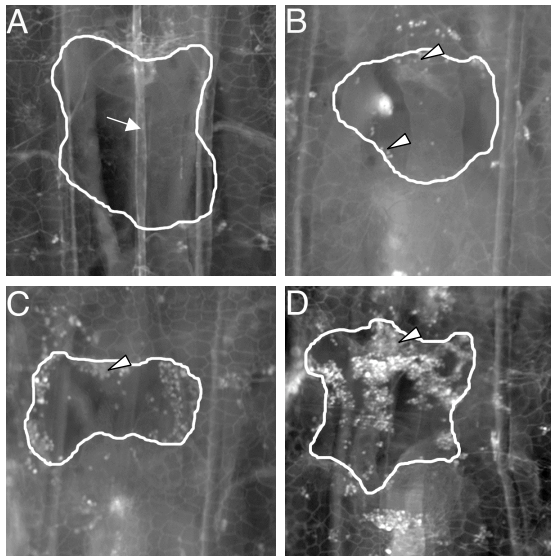
within the wound (Fig. A.1).

Figure A.1 Semiquantitative scale for live scoring of wound-induced inflammation.
(A–D) Live whole-mount preparations of *w,Nrg-GFP/+;Pxn>YFP/+* larvae. For
quantitation of wound-induced inflammation in live larvae bearing *Nrg-GFP, Pxn>YFP*,
we devised a semiquantitative scale for scoring inflammation. (A) Score = 0, no
inflammation (B) Score = 1, minimal inflammation, mostly around wound edges. (C)
Score = 2, moderate inflammation, some cells within wound gap (D) Score = 3, dense
inflammation covering much of the wound gap. Arrow, intact dorsal vessel in one larva.
Arrowheads, accumulations of blood cells. Figure and legend previously published in
(Babcock et al., 2008). Published here with permission of Dan Babcock and the
publisher.

We used this scoring system to identify genes that when knocked down by RNAi, led to a lack of inflammation (i.e. those that scored 0s and 1s). We generated a list of RNAi lines to test that seemed like likely candidates for being the membrane receptor that binds to cell debris (Table A.1).

Table A.1 Categories of RNAi lines for Inflammation Screen

Category	# of genes in the category	# of RNAi lines we possess	# screened
Tolls	9	8	8
Scavenger receptors	21	21	21
Cell adhesion molecules	158	126	51
GPCR	247	211	78
TM receptor actvity	102	14	8
Plasma membrane	364	30	13
Integral to membrane	497	14	9

membrane	770	16	6

We screened 195 RNAi lines without finding a gene that gave no inflammation. We suspected therefore that our assay was ineffective. To confirm that the reporter line we were using was efficiently knocking down protein, we tested an RNAi line of Pvr, a gene that was expressed in the blood cells and a line that we knew resulted in efficient protein knock down in epidermal tissues (FigA.2) (Wu et al., 2009).
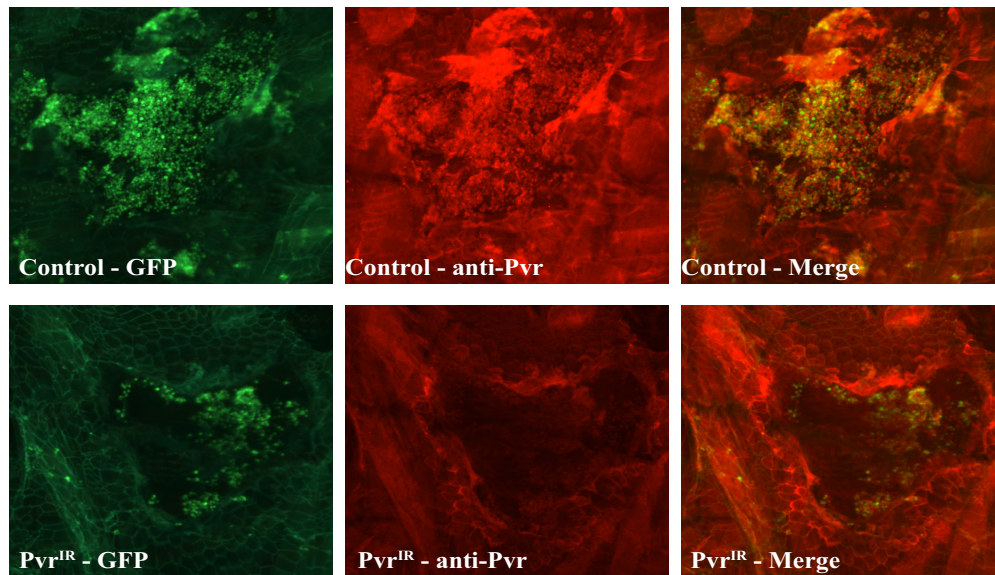
Figure A.2 Blood cells do not efficiently knock down Pvr expression

In the first row, blood cells are stained with anti-Pvr. Pvr is expressed in the blood cells. In the second row, blood cells are expressing *UAS-Pvr^IR*. However, the Pvr expression remains.

Since blood cells expressing *UAS-Pvr^IR* are still expressing moderate levels of Pvr, though it is reduced, we concluded that the Gal4 line we were using either did not turn on early enough for efficient RNAi knockdown, or that the blood cells themselves were not

efficient at the process, meaning there are no conclusions to be drawn from the screening data.

For success at this screen in the future, it may be possible to try an alternative Gal4 line, *hmlΔ-Gal4*, which is thought to have stronger expression, or to add a *UAS-dicer2* to the reporter line, as it strengthen the RNAi. This would be a worthwhile project for someone to undertake in the future, provided they identify a driver that effectively causes protein knockdown in the blood cells.

References

Adachi-Yamada, T., K. Fujimura-Kamada, Y. Nishida, and K. Matsumoto. 1999. Distortion of proximodistal information causes JNK-dependent apoptosis in Drosophila wing. *Nature*. 400:166-169.

Agnes, F., M. Suzanne, and S. Noselli. 1999. The Drosophila JNK pathway controls the morphogenesis of imaginal discs during metamorphosis. *Development*. 126:5453-5462.

Anon, E., X. Serra-Picamal, P. Hersen, N.C. Gauthier, M.P. Sheetz, X. Trepat, and B. Ladoux. 2012. Cell crawling mediates collective cell migration to close undamaged epithelial gaps. *Proceedings of the National Academy of Sciences of the United States of America*. 109:10891-10896.

Babcock, D.T., A.R. Brock, G.S. Fish, Y. Wang, L. Perrin, M.A. Krasnow, and M.J. Galko. 2008. Circulating blood cells function as a surveillance system for damaged tissue in Drosophila larvae. *Proceedings of the Natlional Academy of Sciences of the United States of America*. 105:10017-10022.

Baek, S.H., Y.C. Kwon, H. Lee, and K.M. Choe. 2010. Rho-family small GTPases are required for cell polarization and directional sensing in Drosophila wound healing. *Biochemical and Biophysical Research Communications*. 394:488-492.

Bergantinos, C., M. Corominas, and F. Serras. 2010. Cell death-induced regeneration in wing imaginal discs requires JNK signalling. *Development*. 137:1169-1179.

Bosch, M., J. Baguna, and F. Serras. 2008. Origin and proliferation of blastema cells during regeneration of Drosophila wing imaginal discs. *The International Journal of Developmental Biology*. 52:1043-1050.

Bosch, M., F. Serras, E. Martin-Blanco, and J. Baguna. 2005. JNK signaling pathway required for wound healing in regenerating Drosophila wing imaginal discs. *Developmental Biology*. 280:73-86.

Boutros, M., N. Paricio, D.I. Strutt, and M. Mlodzik. 1998. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell*. 94:109-118.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 118:401-415.

Bruckner, K., L. Kockel, P. Duchek, C.M. Luque, P. Rorth, and N. Perrimon. 2004. The PDGF/VEGF receptor controls blood cell survival in Drosophila. *Developmental Cell*. 7:73-84.

Calleja, M., E. Moreno, S. Pelaz, and G. Morata. 1996. Visualization of gene expression in living adult Drosophila. *Science*. 274:252-255.

Campos, I., J.A. Geiger, A.C. Santos, V. Carlos, and A. Jacinto. 2010. Genetic screen in Drosophila melanogaster uncovers a novel set of genes required for embryonic epithelial repair. *Genetics*. 184:129-140.

Carlier, M.F., V. Laurent, J. Santolini, R. Melki, D. Didry, G.X. Xia, Y. Hong, N.H. Chua, and D. Pantaloni. 1997. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *The Journal of Cell Biology*. 136:1307-1322.

Castrillon, D.H., P. Gonczy, S. Alexander, R. Rawson, C.G. Eberhart, S. Viswanathan, S. DiNardo, and S.A. Wasserman. 1993. Toward a molecular genetic analysis of

spermatogenesis in Drosophila melanogaster: characterization of male-sterile mutants generated by single P element mutagenesis. *Genetics*. 135:489-505.

Chmielowiec, J., M. Borowiak, M. Morkel, T. Stradal, B. Munz, S. Werner, J. Wehland, C. Birchmeier, and W. Birchmeier. 2007. c-Met is essential for wound healing in the skin. *The Journal of Cell Biology*. 177:151-162.

Cho, N.K., L. Keyes, E. Johnson, J. Heller, L. Ryner, F. Karim, and M.A. Krasnow. 2002. Developmental control of blood cell migration by the Drosophila VEGF pathway. *Cell*. 108:865-876.

Ciapponi, L., and D. Bohmann. 2002. An essential function of AP-1 heterodimers in Drosophila development. *Mechanisms of Development*. 115:35-40.

Cooley, L., E. Verheyen, and K. Ayers. 1992. chickadee encodes a profilin required for intercellular cytoplasm transport during Drosophila oogenesis. *Cell*. 69:173-184.

Davidson, L.A., A.M. Ezin, and R. Keller. 2002. Embryonic wound healing by apical contraction and ingression in Xenopus laevis. *Cell Motility and the Cytoskeleton*. 53:163-176.

Ding, Z., A. Lambrechts, M. Parepally, and P. Roy. 2006. Silencing profilin-1 inhibits endothelial cell proliferation, migration and cord morphogenesis. *Journal of Cell Science*. 119:4127-4137.

Duchek, P., K. Somogyi, G. Jekely, S. Beccari, and P. Rorth. 2001. Guidance of cell migration by the Drosophila PDGF/VEGF receptor. *Cell*. 107:17-26.

Fulga, T.A., and P. Rorth. 2002. Invasive cell migration is initiated by guided growth of long cellular extensions. *Nature Cell Biology*. 4:715-719.

Galko, M.J., and M.A. Krasnow. 2004. Cellular and genetic analysis of wound healing in
Drosophila larvae. *PLoS Biology*. 2:E239.

Garlick, J.A., and L.B. Taichman. 1994. Fate of human keratinocytes during
reepithelialization in an organotypic culture model. *Laboratory Investigation; a
Journal of Technical Methods and Pathology*. 70:916-924.

Geisbrecht, E.R., and D.J. Montell. 2004. A role for Drosophila IAP1-mediated caspase
inhibition in Rac-dependent cell migration. *Cell*. 118:111-125.

Gipson, I.K., S.J. Spurr-Michaud, and A.S. Tisdale. 1988. Hemidesmosomes and
anchoring fibril collagen appear synchronously during development and wound
healing. *Developmental Biology*. 126:253-262.

Glise, B., H. Bourbon, and S. Noselli. 1995. hemipterous encodes a novel Drosophila
MAP kinase kinase, required for epithelial cell sheet movement. *Cell*. 83:451-461.

Glise, B., and S. Noselli. 1997. Coupling of Jun amino-terminal kinase and
Decapentaplegic signaling pathways in Drosophila morphogenesis. *Genes &
Development*. 11:1738-1747.

Grondahl-Hansen, J., L.R. Lund, E. Ralfkiaer, V. Ottevanger, and K. Dano. 1988.
Urokinase- and tissue-type plasminogen activators in keratinocytes during wound
reepithelialization in vivo. *The Journal of Investigative Dermatology*. 90:790-795.

Gurtner, G.C., S. Werner, Y. Barrandon, and M.T. Longaker. 2008. Wound repair and
regeneration. *Nature*. 453:314-321.

Hadorn, E., R. Hurlimann, G. Mindek, G. Schubiger, and M. Staub. 1968.
[Developmental capacity of embryonal blastema in Drosophila following

cultivation in an adult host]. *Revue Suisse de Zoologie; Annales de la Societe Zoologique Suisse et du Museum D'histoire Naturelle de Geneve*. 75:557-569.

Halfon, M.S., S. Gisselbrecht, J. Lu, B. Estrada, H. Keshishian, and A.M. Michelson. 2002. New fluorescent protein reporters for use with the Drosophila Gal4 expression system and for vital detection of balancer chromosomes. *Genesis*. 34:135-138.

Halme, A., M. Cheng, and I.K. Hariharan. 2010. Retinoids regulate a developmental checkpoint for tissue regeneration in Drosophila. *Current Biology : CB*. 20:458-463.

Heldin, C.H., and B. Westermark. 1999. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiological Reviews*. 79:1283-1316.

Hutson, M.S., Y. Tokutake, M.S. Chang, J.W. Bloor, S. Venakides, D.P. Kiehart, and G.S. Edwards. 2003. Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. *Science*. 300:145-149.

Hwa, C., E.A. Bauer, and D.E. Cohen. 2011. Skin biology. *Dermatologic Therapy*. 24:464-470.

Ishimaru, S., R. Ueda, Y. Hinohara, M. Ohtani, and H. Hanafusa. 2004. PVR plays a critical role via JNK activation in thorax closure during Drosophila metamorphosis. *The EMBO Journal*. 23:3984-3994.

Jacinto, A., W. Wood, T. Balayo, M. Turmaine, A. Martinez-Arias, and P. Martin. 2000. Dynamic actin-based epithelial adhesion and cell matching during Drosophila dorsal closure. *Current Biology : CB*. 10:1420-1426.

Jacinto, A., W. Wood, S. Woolner, C. Hiley, L. Turner, C. Wilson, A. Martinez-Arias, and P. Martin. 2002. Dynamic analysis of actin cable function during Drosophila dorsal closure. *Current Biology : CB*. 12:1245-1250.

Jasper, H., V. Benes, C. Schwager, S. Sauer, S. Clauder-Munster, W. Ansorge, and D. Bohmann. 2001. The genomic response of the Drosophila embryo to JNK signaling. *Developmental Cell*. 1:579-586.

Kaiser, D.A., V.K. Vinson, D.B. Murphy, and T.D. Pollard. 1999. Profilin is predominantly associated with monomeric actin in Acanthamoeba. *Journal of Cell Science*. 112 (Pt 21):3779-3790.

Khadka, D.K., W. Liu, and R. Habas. 2009. Non-redundant roles for Profilin2 and Profilin1 during vertebrate gastrulation. *Developmental Biology*. 332:396-406.

Kiehart, D.P., C.G. Galbraith, K.A. Edwards, W.L. Rickoll, and R.A. Montague. 2000. Multiple forces contribute to cell sheet morphogenesis for dorsal closure in Drosophila. *Journal of Cell Biology*. 149:471-490.

Kiger, A.A., B. Baum, S. Jones, M.R. Jones, A. Coulson, C. Echeverri, and N. Perrimon. 2003. A functional genomic analysis of cell morphology using RNA interference. *Journal of Biology*. 2:27.

Kim, Y.S., S. Furman, H. Sink, and M.F. VanBerkum. 2001. Calmodulin and profilin coregulate axon outgrowth in Drosophila. *Journal of Neurobiology*. 47:26-38.

Kockel, L., J.G. Homsy, and D. Bohmann. 2001. Drosophila AP-1: lessons from an invertebrate. *Oncogene*. 20:2347-2364.

Kovar, D.R., E.S. Harris, R. Mahaffy, H.N. Higgs, and T.D. Pollard. 2006. Control of the assembly of ATP- and ADP-actin by formins and profilin. *Cell*. 124:423-435.

Kullmann, J.A., A. Neumeyer, C.B. Gurniak, E. Friauf, W. Witke, and M.B. Rust. 2011. Profilin1 is required for glial cell adhesion and radial migration of cerebellar granule neurons. *EMBO Reports*. 13:75-82.

Kwon, Y.C., S.H. Baek, H. Lee, and K.M. Choe. 2010. Nonmuscle myosin II localization is regulated by JNK during Drosophila larval wound healing. *Biochemical and Biophysical Research Communications*. 393:656-661.

Lai, S.L., T.H. Chan, M.J. Lin, W.P. Huang, S.W. Lou, and S.J. Lee. 2008. Diaphanous-related formin 2 and profilin I are required for gastrulation cell movements. *PloS One*. 3:e3439.

Lawrence, P.A., R. Bodmer, and J.P. Vincent. 1995. Segmental patterning of heart precursors in Drosophila. *Development*. 121:4303-4308.

Learte, A.R., M.G. Forero, and A. Hidalgo. 2008. Gliatrophic and gliatropic roles of PVF/PVR signaling during axon guidance. *Glia*. 56:164-176.

Lesch, C., J. Jo, Y. Wu, G.S. Fish, and M.J. Galko. 2010. A targeted UAS-RNAi screen in Drosophila larvae identifies wound closure genes regulating distinct cellular processes. *Genetics*. 186:943-957.

Mace, K.A., J.C. Pearson, and W. McGinnis. 2005. An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. *Science*. 308:381-385.

Macias, A., N.M. Romero, F. Martin, L. Suarez, A.L. Rosa, and G. Morata. 2004. PVF1/PVR signaling and apoptosis promotes the rotation and dorsal closure of the Drosophila male terminalia. *The International Journal of Developmental Biology*. 48:1087-1094.

Martin, P. 1997. Wound healing--aiming for perfect skin regeneration. *Science*. 276:75-81.

Martin, P., D. D'Souza, J. Martin, R. Grose, L. Cooper, R. Maki, and S.R. McKercher. 2003. Wound healing in the PU.1 null mouse--tissue repair is not dependent on inflammatory cells. *Current Biology*. 13:1122-1128.

Martin, P., and S.J. Leibovich. 2005. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends in Cell Biology*. 15:599-607.

Martin, P., and J. Lewis. 1992. Actin cables and epidermal movement in embryonic wound healing. *Nature*. 360:179-183.

Martin, P., and S.M. Parkhurst. 2004. Parallels between tissue repair and embryo morphogenesis. *Development*. 131:3021-3034.

Martin-Blanco, E., A. Gampel, J. Ring, K. Virdee, N. Kirov, A.M. Tolkovsky, and A. Martinez-Arias. 1998. puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. *Genes & Development*. 12:557-570.

Martin-Blanco, E., J.C. Pastor-Pareja, and A. Garcia-Bellido. 2000. JNK and decapentaplegic signaling control adhesiveness and cytoskeleton dynamics during thorax closure in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America*. 97:7888-7893.

Mattila, J., L. Omelyanchuk, S. Kyttala, H. Turunen, and S. Nokkala. 2005. Role of Jun N-terminal Kinase (JNK) signaling in the wound healing and regeneration of a Drosophila melanogaster wing imaginal disc. *The International Journal of Developmental Biology*. 49:391-399.

McDonald, J.A., E.M. Pinheiro, and D.J. Montell. 2003. PVF1, a PDGF/VEGF homolog, is sufficient to guide border cells and interacts genetically with Taiman. *Development*. 130:3469-3478.

McGuire, S.E., P.T. Le, A.J. Osborn, K. Matsumoto, and R.L. Davis. 2003. Spatiotemporal rescue of memory dysfunction in Drosophila. *Science*. 302:1765-1768.

Millard, T.H., and P. Martin. 2008. Dynamic analysis of filopodial interactions during the zippering phase of Drosophila dorsal closure. *Development*. 135:621-626.

Mitchison, T.J., and L.P. Cramer. 1996. Actin-based cell motility and cell locomotion. *Cell*. 84:371-379.

Mockrin, S.C., and E.D. Korn. 1980. Acanthamoeba profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine 5'-triphosphate. *Biochemistry*. 19:5359-5362.

Noselli, S. 1998. JNK signaling and morphogenesis in Drosophila. *Trends in Genetics : TIG*. 14:33-38.

Pearson, J.C., M.T. Juarez, M. Kim, O. Drivenes, and W. McGinnis. 2009. Multiple transcription factor codes activate epidermal wound-response genes in Drosophila. *Proceedings of the National Academy of Science of the United States of America*. 106:2224-2229.

Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 112:453-465.

Quinones-Coello, A.T., L.N. Petrella, K. Ayers, A. Melillo, S. Mazzalupo, A.M. Hudson, S. Wang, C. Castiblanco, M. Buszczak, R.A. Hoskins, and L. Cooley. 2007. Exploring strategies for protein trapping in Drosophila. *Genetics*. 175:1089-1104.

Reed, B.H., R. Wilk, and H.D. Lipshitz. 2001. Downregulation of Jun kinase signaling in the amnioserosa is essential for dorsal closure of the Drosophila embryo. *Current Biology*. 11:1098-1108.

Reinhard, M., K. Giehl, K. Abel, C. Haffner, T. Jarchau, V. Hoppe, B.M. Jockusch, and U. Walter. 1995. The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. *The EMBO Journal*. 14:1583-1589.

Riedl, J., A.H. Crevenna, K. Kessenbrock, J.H. Yu, D. Neukirchen, M. Bista, F. Bradke, D. Jenne, T.A. Holak, Z. Werb, M. Sixt, and R. Wedlich-Soldner. 2008. Lifeact: a versatile marker to visualize F-actin. *Nature Methods*. 5:605-607.

Riesgo-Escovar, J.R., and E. Hafen. 1997a. Common and distinct roles of DFos and DJun during Drosophila development. *Science*. 278:669-672.

Riesgo-Escovar, J.R., and E. Hafen. 1997b. Drosophila Jun kinase regulates expression of decapentaplegic via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure. *Genes & Development*. 11:1717-1727.

Riesgo-Escovar, J.R., M. Jenni, A. Fritz, and E. Hafen. 1996. The Drosophila Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. *Genes & Development*. 10:2759-2768.

Rodriguez-Diaz, A., Y. Toyama, D.L. Abravanel, J.M. Wiemann, A.R. Wells, U.S. Tulu, G.S. Edwards, and D.P. Kiehart. 2008. Actomyosin purse strings: renewable

resources that make morphogenesis robust and resilient. *HFSP Journal*. 2:220-237.

Rogers, S.L., U. Wiedemann, N. Stuurman, and R.D. Vale. 2003. Molecular requirements for actin-based lamella formation in Drosophila S2 cells. *Journal of Cell Biology*. 162:1079-1088.

Romero, S., C. Le Clainche, D. Didry, C. Egile, D. Pantaloni, and M.F. Carlier. 2004. Formin is a processive motor that requires profilin to accelerate actin assembly and associated ATP hydrolysis. *Cell*. 119:419-429.

Rosenblatt, J., B.J. Agnew, H. Abe, J.R. Bamburg, and T.J. Mitchison. 1997. Xenopus actin depolymerizing factor/cofilin (XAC) is responsible for the turnover of actin filaments in Listeria monocytogenes tails. *The Journal of Cell Biology*. 136:1323-1332.

Rosin, D., E. Schejter, T. Volk, and B.Z. Shilo. 2004. Apical accumulation of the Drosophila PDGF/VEGF receptor ligands provides a mechanism for triggering localized actin polymerization. *Development*. 131:1939-1948.

Roy, S., S. Khanna, C. Rink, S. Biswas, and C.K. Sen. 2008. Characterization of the acute temporal changes in excisional murine cutaneous wound inflammation by screening of the wound-edge transcriptome. *Physiological Genomics*. 34:162-184.

Schafer, M., and S. Werner. 2008. Cancer as an overhealing wound: an old hypothesis revisited. *Nature Reviews Molecular Cell Biology*. 9:628-638.

Shaw, T., and P. Martin. 2009a. Epigenetic reprogramming during wound healing: loss of polycomb-mediated silencing may enable upregulation of repair genes. *EMBO Reports*. 10:881-886.

Shaw, T.J., and P. Martin. 2009b. Wound repair at a glance. *Journal of Cell Science*. 122:3209-3213.

Smith-Bolton, R.K., M.I. Worley, H. Kanda, and I.K. Hariharan. 2009. Regenerative growth in Drosophila imaginal discs is regulated by Wingless and Myc. *Developmental Cell*. 16:797-809.

Stramer, B., M. Winfield, T. Shaw, T.H. Millard, S. Woolner, and P. Martin. 2008. Gene induction following wounding of wild-type versus macrophage-deficient Drosophila embryos. *EMBO Reports*. 9:465-471.

Stramer, B., W. Wood, M.J. Galko, M.J. Redd, A. Jacinto, S.M. Parkhurst, and P. Martin. 2005. Live imaging of wound inflammation in Drosophila embryos reveals key roles for small GTPases during in vivo cell migration. *Journal of Cell Biology*. 168:567-573.

Stronach, B., and N. Perrimon. 2002. Activation of the JNK pathway during dorsal closure in Drosophila requires the mixed lineage kinase, slipper. *Genes & Development*. 16:377-387.

Stronach, B.E., and N. Perrimon. 2001. Investigation of leading edge formation at the interface of amnioserosa and dorsal ectoderm in the Drosophila embryo. *Development*. 128:2905-2913.

Theriot, J.A., J. Rosenblatt, D.A. Portnoy, P.J. Goldschmidt-Clermont, and T.J. Mitchison. 1994. Involvement of profilin in the actin-based motility of L. monocytogenes in cells and in cell-free extracts. *Cell*. 76:505-517.

Treisman, J.E., N. Ito, and G.M. Rubin. 1997. misshapen encodes a protein kinase involved in cell shape control in Drosophila. *Gene*. 186:119-125.

Verheyen, E.M., and L. Cooley. 1994. Profilin mutations disrupt multiple actin-
dependent processes during Drosophila development. *Development*. 120:717-728.

Vinson, V.K., E.M. De La Cruz, H.N. Higgs, and T.D. Pollard. 1998. Interactions of
Acanthamoeba profilin with actin and nucleotides bound to actin. *Biochemistry*.
37:10871-10880.

Vlisidou, I., A.J. Dowling, I.R. Evans, N. Waterfield, R.H. ffrench-Constant, and W.
Wood. 2009. Drosophila embryos as model systems for monitoring bacterial
infection in real time. *PLoS Pathogens*. 5:e1000518.

Wang, S., V. Tsarouhas, N. Xylourgidis, N. Sabri, K. Tiklova, N. Nautiyal, M. Gallio,
and C. Samakovlis. 2009. The tyrosine kinase Stitcher activates Grainy head and
epidermal wound healing in Drosophila. *Nature Cell Biology*. 11:890-895.

Werner, S., and R. Grose. 2003. Regulation of wound healing by growth factors and
cytokines. *Physiological Reviews*. 83:835-870.

Werner, S., H. Smola, X. Liao, M.T. Longaker, T. Krieg, P.H. Hofschneider, and L.T.
Williams. 1994. The function of KGF in morphogenesis of epithelium and
reepithelialization of wounds. *Science*. 266:819-822.

Witke, W. 2004. The role of profilin complexes in cell motility and other cellular
processes. *Trends in Cell Biology*. 14:461-469.

Witke, W., J.D. Sutherland, A. Sharpe, M. Arai, and D.J. Kwiatkowski. 2001. Profilin I is
essential for cell survival and cell division in early mouse development.
Proceedings of the National Academy of Sciences of the United States of America.
98:3832-3836.

Wood, W., C. Faria, and A. Jacinto. 2006. Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in Drosophila melanogaster. *Journal of Cell Biology*. 173:405-416.

Wood, W., A. Jacinto, R. Grose, S. Woolner, J. Gale, C. Wilson, and P. Martin. 2002. Wound healing recapitulates morphogenesis in Drosophila embryos. *Nature Cell Biology*. 4:907-912.

Wu, Y., A.R. Brock, Y. Wang, K. Fujitani, R. Ueda, and M.J. Galko. 2009. A blood-borne PDGF/VEGF-like ligand initiates wound-induced epidermal cell migration in Drosophila larvae. *Current Biology*. 19:1473-1477.

Zars, T., M. Fischer, R. Schulz, and M. Heisenberg. 2000. Localization of a short-term memory in Drosophila. *Science*. 288:672-675.

Zeitlinger, J., and D. Bohmann. 1999. Thorax closure in Drosophila: involvement of Fos and the JNK pathway. *Development*. 126:3947-3956.

Vita

Amanda R. Brock was born in Wichita, Kansas, in June, 1982. After graduating from Wichita High School East with her International Baccalaureate Diploma in Wichita, KS, she moved to Houston, TX, where she attended Rice University. She graduated from Rice with a Bachelor of Arts and a double major in Biology and History in May, 2004. In August, 2006, she entered the University of Texas Graduate School of Biomedical Sciences at the University of Texas MD Anderson Cancer Center in Houston, TX. She currently lives in Houston.

Permanent Address:

7318 E. Norfolk Dr.

Wichita, KS 67206