Transcriptional regulation of Profilin during wound closure in *Drosophila* larvae

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Summary

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.

Key words: Wound healing, Cell migration, *Drosophila*

Introduction

Drosophila has emerged as a powerful model for studying embryonic and postembryonic wound healing (Galko and Krasnow, 2004; Jacinto et al., 2001; Mace et al., 2005; Wang et al., 2009; Wood et al., 2002). Epidermal tissues must sense the presence of the wound and respond to damage signals by migrating across the wound gap to reestablish continuity of the epithelial sheet. Several signaling pathways are required for wound closure in the fly, including the Jun N-terminal Kinase (JNK) pathway (Galko and Krasnow, 2004; Rämet et al., 2002) and two receptor tyrosine kinases (RTK). The first is Stitcher, a Ret family RTK, (Wang et al., 2009) that is upstream of extracellular signal-related kinase (ERK) activation and Grainyhead transcriptional activity (Mace et al., 2005). The second is the Platelet-Derived Growth Factor/Vascular Endothelial Growth Factor (PDGF/VEGF)-like RTK Pvr that is involved in a variety of cell migration events (Cho et al., 2002; Duchek et al., 2001; Ishimaru et al., 2004). In larvae, activation of Pvr by the soluble ligand Pvf1 has been implicated in the wound-induced actin polymerization that presumably drives closure (Wu et al., 2009) and knockdown of several known actin regulators (SCAR, Rac1, Arp14D) leads to wound closure defects (Lesch et al., 2010). Pvr acts in parallel to the JNK signaling pathway (Wu et al., 2009) that regulates the ability of the epidermal cells to shut off cuticle synthesis, or dedifferentiate, an apparent prerequisite for efficient migration. Actin mobilizes properly to the leading edge of wounded cells deficient for JNK; by contrast, knockdown of Pvr prevents actin from mobilizing to the leading edge.

The current model for how the actin cytoskeleton regulates cell migration (Pollard and Borisy, 2003), is largely based on studies of cells migrating in 2-D cultures *in vitro* and the biochemical activities and kinetics of various actin regulatory factors. The model postulates that pro-migratory extracellular stimuli prompt sequential activation of Rho-like GTPases, WASP/SCAR complexes, and ultimately, the Arp2/3 complex that nucleates new actin filaments against the cell membrane to push it forward for migration. After nucleation of new filaments, the Pollard/Borisy model proposes further steps that provide for recycling of ADP-bound actin monomers that are dissociated or severed from the pointed end of actin filaments by ADF-cofilin and other factors (Carlier et al., 1997; Rosenblatt et al., 1997). Profilin, an actin-binding protein (Kaiser et al., 1999), is thought to play a major role in recycling because it can bind to ADP-actin and facilitate the exchange to ATP-actin (Mockrin and Korn, 1980), thus facilitating the growth of the actin filament at the barbed end (Vinson et al., 1998). Profilin can also bind to Formin proteins and can stimulate their rapid processive addition of actin monomers to the pointed ends of actin filaments (Kovar et al., 2006; Romero et al., 2004).

Human cells deficient in Profilin have cell adhesion and migration defects suggesting that recycling and/or Formin-mediated filament growth is important for cells growing and moving in culture (Ding et al., 2006). However, whether there is a prominent role for either Profilin-mediated process during cell migration events *in vivo* remains an open question.

In *Drosophila*, the *chickadee* gene encodes a Profilin ortholog. *chickadee* mutants have defects in a variety of actin-dependent processes including nurse cell dumping (Cooley et al., 1992), spermatogenesis (Castrillon et al., 1993), and oogenesis and bristle formation (Verheyen and Cooley, 1994). Additionally, Profilin is required for cell migration events such as epithelial dorsal closure (DC) (Jasper et al., 2001) and axon guidance (Kim et al., 2001); it is also required for lamellipodial ruffling in S2 cells (Rogers et al., 2003). During DC, an epithelial sheet migrates across the underlying amnioserosa to establish tissue continuity by means of a contractile actin cable and actin-based processes (Jacinto et al., 2000; Kiehart et al., 2000; Millard and Martin, 2008). DC serves as a model for wound closure in the *Drosophila* embryo (Wood et al., 2002), and many genes whose mutants have defects in DC also show a defect in wound closure. About 30% of *chickadee* mutants fail to complete DC and they have fewer filopodia in the leading edge cells (Jasper et al., 2001). Profilin is also an important contributor to vertebrate development: Profilin1 is required for cell survival in early mouse development (Witke et al., 2001); it is required for glial cell adhesion in developing mouse brains (Kullmann et al., 2011); it contributes to epiboly and convergent extension in zebrafish development (Lai et al., 2008); and in *Xenopus*, XProfilin 1 regulates blastopore closure while XProfilin 2 is involved in convergent extension migrations (Khadka et al., 2009).

Here, we examine *in vivo* activities of Profilin in a larval wound healing context, where highly differentiated cells migrate long distances through extension of actin-based cellular processes. We show that Profilin is required for larval epidermal wound closure and for accumulation of leading edge actin. Profilin is transcriptionally regulated by the JNK, but not by the Pvr, signaling pathway. This regulation is via the DFos and the DJun transcription factors. Our results suggest that Profilin-mediated events are important during migration of dedifferentiated cells *in vivo*.

Results

chickadee is required for larval wound closure

Drosophila larval epidermal pinch wounds typically close by 24 hours after wounding (Fig. 1A) (Galko and Krasnow, 2004; Lesch et al., 2010; Wu et al., 2009). Closure occurs primarily through directed epidermal cell migration into the wound gap. In an ongoing RNAi-based screen for genes that affect wound closure, we found that epidermal-specific knockdown of *chickadee* (*chic*) resulted in a failure of wound closure. We tested three RNAi lines, two of which target the same sequence and one of which targets a non-overlapping portion of the gene (supplementary material Fig. S1). Two non-overlapping lines ($chic^{IR(R4)}$ and $chic^{IR(kk)}$) exhibited 60–80% open wounds at 24 hours (Fig. 1C,E,H) and showed effective on-target knockdown of Profilin in the larval epidermis (supplementary material Fig. S2C,D). The third line, $chic^{IR(R3)}$, did not efficiently knockdown expression of the protein (supplementary material Fig. S2B) and showed closed wounds (Fig. 1D,H). Although it shows no wound closure defect when expressed by itself, this line combined with $chic^{IR(R4)}$ resulted in a 100% penetrant defect in wound closure (Fig. 1B) suggesting that it does target Profilin to some degree.

We performed two further experiments to rule out an off-target effect of the $chic^{IR}$ lines. The first was a rescue experiment. We tested if overexpression of a *chic* cDNA through *UAS-chic* would attenuate the wound closure phenotype of the $chic^{IR(R3,R4)}$ double RNAi line. Overexpression of the cDNA alone does not interfere with wound closure (Fig. 1F). Co-expression with the RNAi transgenes reduced the percent of open wound from 100% to

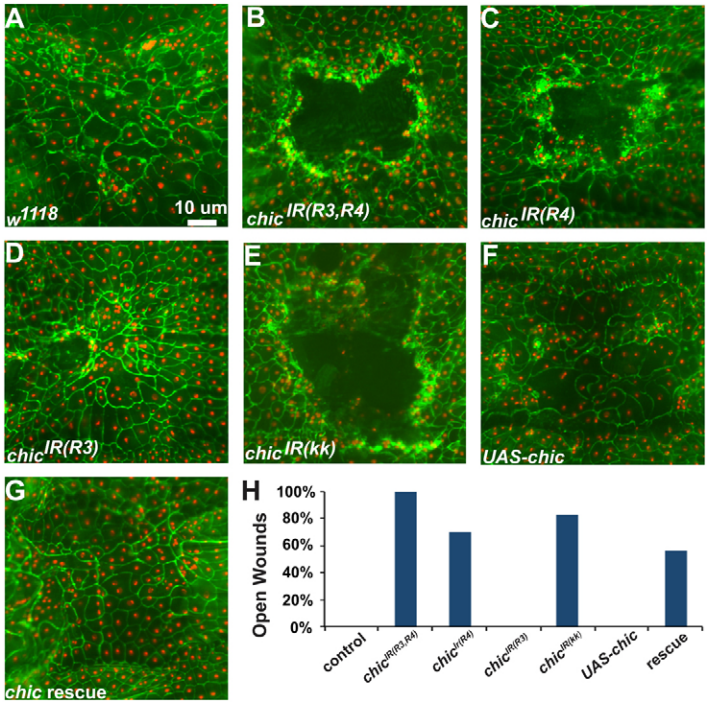


Fig. 1. $chic^{RNAi}$ results in a failure of wound closure. (**A-G**) Dissected larval epidermal wholemounts stained for anti-Fasciclin III (green). Genotype is *w; e22c-gal4, UAS-dsred2nuc* (to label epidermal nuclei, red) plus the indicated mutations or UAS transgenes. All panels show wounded larvae 24 hours after wounding. (A) w^{1118}, control. Note the presence of large, atypically shaped cells, some of which are multinucleate, at the closed wound (compare with unwounded epidermis in Fig. 2A). (B) *UAS-*$chic^{IR(R3,R4)}$. Note the open wound gap. (C) *UAS-*$chic^{IR(R4)}$. Note the open wound gap. (D) *UAS-*$chic^{IR(R3)}$. Note the closed wound. (E) *UAS-*$chic^{IR(kk)}$. Note the open wound gap. (F) *UAS-chickadee*. Overexpression of *chickadee* 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-*$chic^{IR3R4)}$, *UAS-chickadee*. $n \geq 30$. Scale bar: 10 μm.

~60% (Fig. 1F,G,H). The lack of full rescue can likely be explained by RNAi knockdown that is sufficiently potent to clear at least some of the extra expression of the *chickadee* cDNA. Second, larvae bearing a $chic^{01320}$ allele, a reported hypomorph, showed 30% open wounds (all available null alleles are larval lethal) (supplementary material Fig. S3). Interestingly, there was still Profilin protein present in *chic* hypomorphic larvae, but its expression pattern was primarily nuclear (supplementary material Fig. S2C) as opposed to the diffuse perinuclear staining seen in the control (supplementary material Fig. S2A). The combination of rescue and mutant analysis strongly suggests that the $chic^{IR}$ wound closure phenotypes are not due to off-target effects and that *chickadee* is a bona fide wound closure gene.

Epidermal Profilin is upregulated following wounding

Next we wanted to determine whether the levels of Profilin protein are regulated by wounding. A clear view of Profilin levels in the larval epidermis is difficult to obtain due to high levels of expression in the underlying body wall muscles. To circumvent this problem we examined epidermal Profilin levels in larvae that had Profilin knocked down in skeletal muscles (see Fig. 2 and Materials and Methods for genotype details). Profilin was localized in the cytoplasm and in the perinuclear area in the unwounded larval epidermis (Fig. 2A; supplementary material Fig. S2A). Following wounding, protein levels increased (Fig. 2B) especially in cells immediately proximal to the wound. For instance, leading edge cells (Fig. 2B′) stained more brightly than cells two to three cell rows away from the wound (Fig. 2B″). By 24 hours after wounding, Profilin levels remained high, but variable, in the irregularly shaped epidermal cells that mark the former wound site (Fig. 2C,C″). Interestingly, there was a very sharp boundary of Profilin levels between what we presume to be the original wound gap and the surrounding intact epidermis (Fig. 2C′). The tight boundary of Profilin staining suggests that migratory epidermal cells may need to upregulate and redistribute Profilin in order to effectively migrate into the wound gap. This upregulation of Profilin led us to ask if overexpression of Profilin would have a positive effect on wound closure. To test this, we measured the wound sizes of control larvae and larvae expressing a *UAS-chic* in the epidermis at a timepoint midway through closure (8 hours) and found that larvae overexpressing Profilin had smaller wound sizes on average (supplementary material Fig. S4), indicating that excess Profilin accelerates wound closure.

Profilin is transcriptionally upregulated after wounding

The increase in Profilin protein levels after wounding prompted us to look at the transcriptional regulation of Profilin. We used the $chic^{01320}$ *lacZ* insertion line as a reporter of transcriptional activity at the *chic* locus. β-Galactosidase activity was negligible in the unwounded epidermis (Fig. 3A). However, six hours after wounding, there was high reporter activity in leading edge cells and progressively lower expression up to 3–5 cell rows further back (Fig. 3B). By 24 hours after wounding, when reepithelialization is normally complete, reporter activity was still observed in cells that likely occupy the original wound area (Fig. 3C). This indicates that *chic* transcription increases in response to wounding primarily in the cells that are required to migrate or elongate. Further, this suggests that new transcription may no longer be necessary after healing.

The JNK and Pvr signaling pathways act in parallel to promote larval epidermal wound closure (Galko and Krasnow, 2004; Wu et al., 2009). The wound-induced transcriptional upregulation of Profilin suggests that Profilin may be downstream of one or both of these pathways. To test which pathway regulates *chic* in the context of larval wound closure, we analyzed *chic-lacZ* reporter activity in larvae expressing *UAS-RNAi* transgenes targeting JNK or Pvr. Knockdown of JNK did not affect the baseline level of

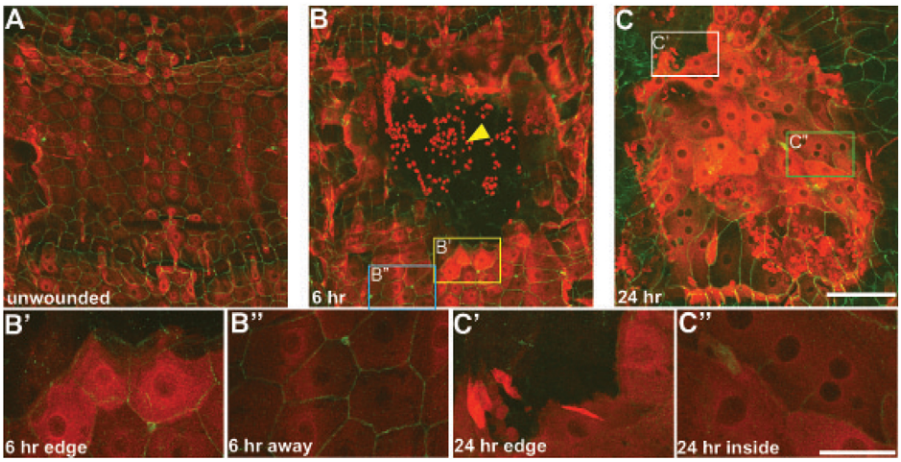


Fig. 2. Profilin relocalizes and its levels increase following wounding. (**A–C″**) Dissected larval epidermal wholemounts of genotype: *w;dmef2-gal4, FasIII-GFP/+;UAS-*$chic^{IR(R3,R4)}$*/+*. In these larvae, the muscle Gal4 driver *dmef2-Gal4* drives expression of $chic^{IR(R3,R4)}$ in order to knock 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 perinuclear Profilin staining. (B) Larvae at 6 hours after wounding. Note that in cells adjacent to the wound edge, Profilin levels have significantly increased (B′) compared with cells located several cell rows away from the wound (B″). Note that the Profilin antibody also labels blood cells (yellow arrowhead) in the middle of the wound. (C) Larvae at 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″). 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. Scale bars: 100 μm (A,B,C); 50 μm (B′,B″,C′,C″).

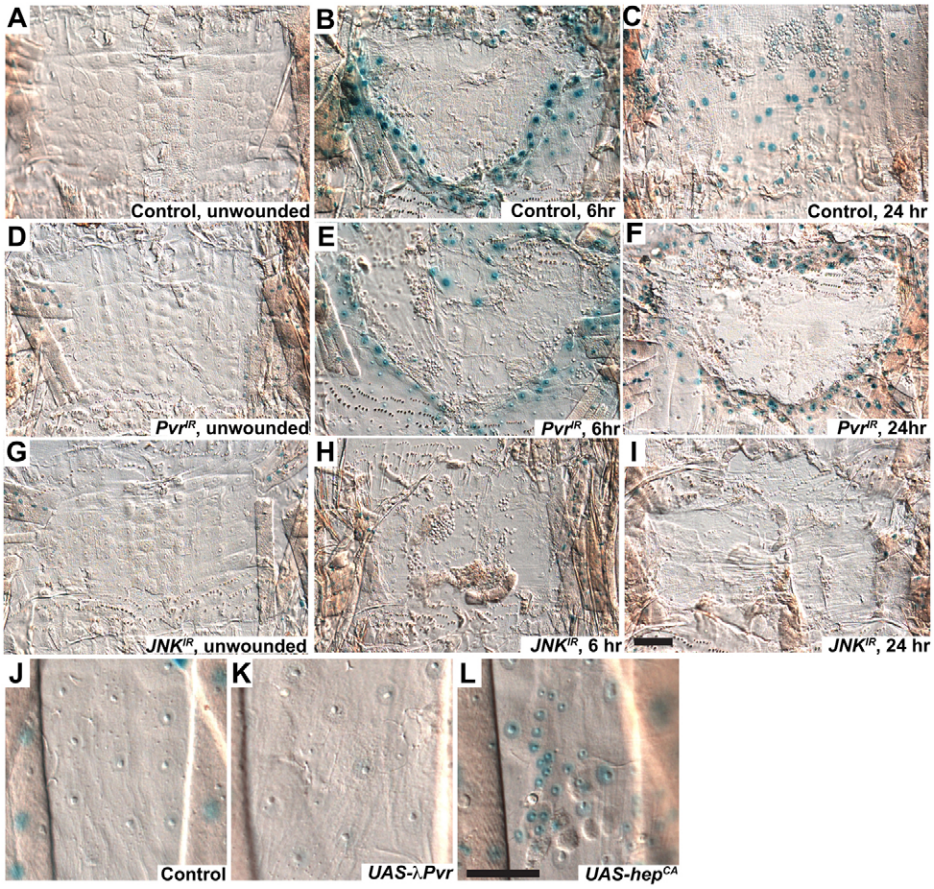


Fig. 3. *chic* transcription is regulated by the JNK but not by the Pvr signaling pathway. (**A–I**) Dissected epidermal wholemounts of larvae heterozygous for *w;e22c-Gal4, chic*^{01320} and the indicated mutants or transgenes. All are stained with X-Gal (see Materials and Methods) to highlight β-Galactosidase activity in *lacZ*-expressing nuclei (blue). (A–C) *w*^{1118}, (D–F) *UAS-Pvr*^{RNAi} or (G–I) *UAS-JNK*^{RNAi}. (A,D,G) Unwounded, (B,E,H) 6 hours after wounding and (C,F,I) 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*^{ts}*/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 bars: 100 μm (A–I); 50 μm (J–L).

chic-lacZ expression (Fig. 3G) and eliminated *chic-lacZ* induction six and 24 hours after wounding (Fig. 3H,I), when induction is strong in the controls (Fig. 3B). This indicates that *chic* transcription in the context of wounding is regulated by JNK signaling. By contrast, larvae expressing *UAS-Pvr*^{IR} showed equivalent *chic-lacZ* induction levels as controls at all time points tested (Fig. 3D–F), indicating that *chic* transcription is not regulated by Pvr. Note that Pvr mutants fail to close (Fig. 3F).

To further test whether JNK or Pvr regulates *chic* expression, we examined whether hyperactivation of JNK or Pvr signaling would induce *chic* transcription in the unwounded larval epidermis. Since pan-epidermal expression of *UAS-hep*^{CA} or *UAS-λPvr* is lethal, we used a conditional strategy to drive these activating transgenes. Briefly, we combined the *pnr-Gal4* driver, which expresses in dorsal epidermal patches, with a ubiquitously-expressed temperature-sensitive Gal80 (*tubulin-Gal80*^{ts}) capable of inhibiting Gal4 at lower temperatures (18°C) but not at higher ones (32°C). Conditional expression of *UAS-hep*^{CA}, activated the JNK pathway and was sufficient to induce *chic-lacZ* activity (Fig. 3L) relative to control (Fig. 3J) in unwounded larvae. By contrast, hyperactivation of Pvr via conditional expression of *UAS-λPvr* was not able to induce *chic-lacZ* activity (Fig. 3K). Together, these data reveal that the JNK, but not the Pvr, pathway induces *chic* transcription after wounding.

Profilin is required to form actin-based structures at the wound edge

Since Profilin is a known actin regulator and actin accumulates at the leading edge of larval epidermal wounds (Kwon et al., 2010; Wu et al., 2009), we examined the formation of these actin-based structures in controls and in larvae lacking epidermal Profilin. To label actin without overexpressing it, we used Lifeact-GFP, which is a small polypeptide that binds to actin filaments without interfering with polymerization or interaction with actin-binding proteins (Riedl et al., 2008). Using a *UAS-lifeact-GFP* transgene and the epidermal specific *e22c-Gal4* driver, we labeled actin filaments in the larval epidermis. Actin was present at similar levels and was evenly distributed in the cytoplasm of unwounded epidermal cells in both control and *UAS-chic*$^{IR(R3,R4)}$-expressing larvae (Fig. 4A,B). Actin accumulated along segments of the wound edge in control larvae by 4 hours (Fig. 4D) and appeared to form a discontinuous cable. Additionally, filopodia and lamellipodia extended into the wound gap (Fig. 4D′,D″). The discontinuous actin cable was maintained at 8 hours after wounding (data not shown). By 24 hours (Fig. 4F), closed wounds showed a high concentration of cytoplasmic actin with sporadic linear concentrations of actin that may represent cable remnants.

To test whether larval epidermal cells lacking Profilin would be able to form the cable and the actin-based processes, we used the *e22c-Gal4* driver to drive both *UAS-lifeactGFP* and *UAS-chic*$^{IR(R3,R4)}$ (Fig. 4E–G). Actin did not accumulate at the wound edge by 4 hours after wounding (Fig. 4E). Further, there were only rare, thin and short processes into the wound gap (Fig. 4E′,E″). Wounds remained open at 24 hours in these larvae (Fig. 4G) and the cells at the wound edge appeared disorganized, with broader FasIII staining along membrane boundaries and on what appear to be cytoplasmic vesicles (Fig. 4G′). We quantified the range of fluorescence intensity of the GFP signal from the leading edge of

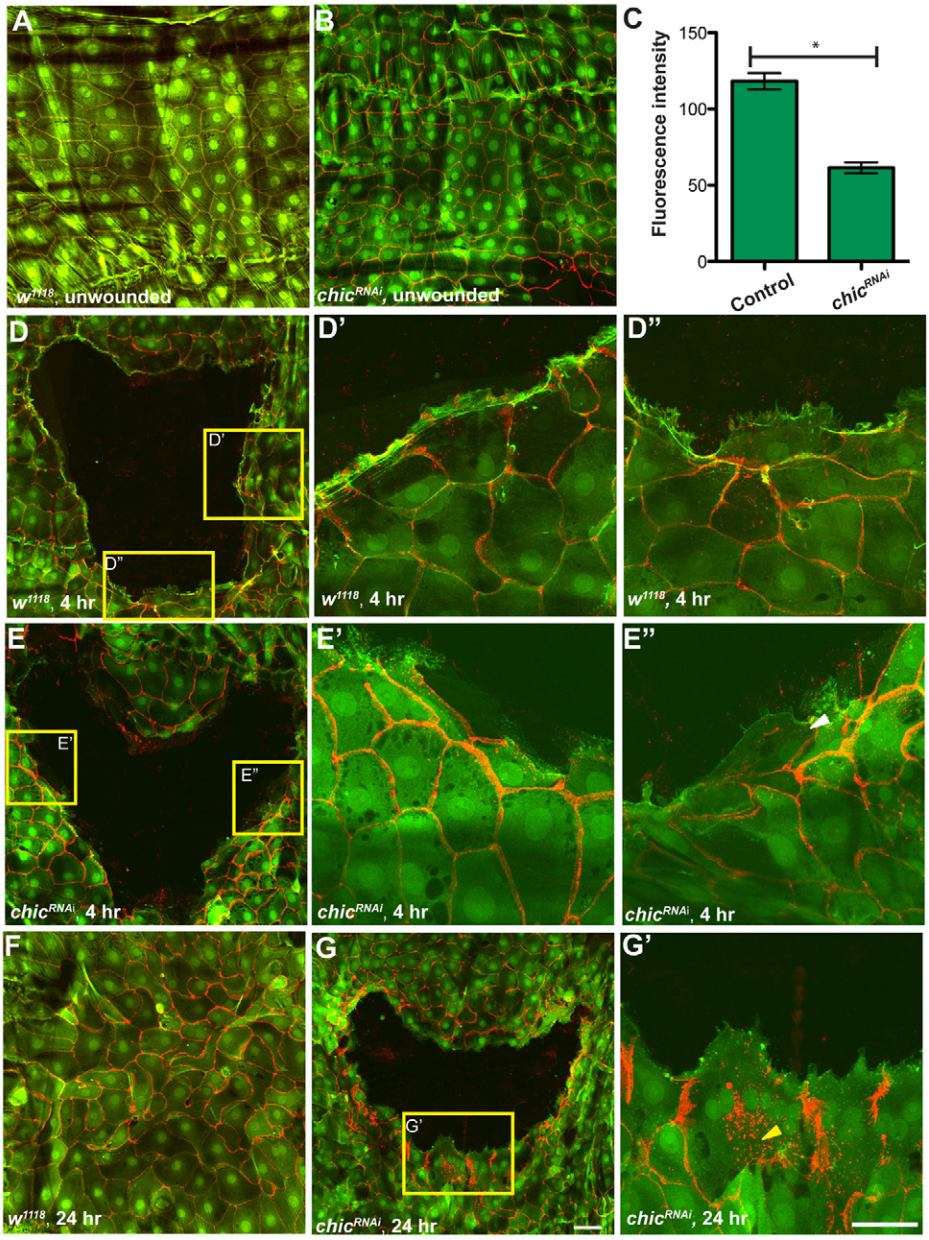


Fig. 4. Actin localizes to the wound edge and forms processes in wild type but not in larvae lacking Profilin expression. (**A,B,D-G**) Dissected epidermal wholemounts of larvae heterozygous for *w;e22c-gal4, UAS-lifeact-GFP6.0* and the indicated mutations or transgenes. Epidermal membranes are labeled with FasIII (red). (A,D,F) w^{1118} (control). (B,E–E″,G,G′) $chic^{IR(R3,R4)}$. (A,B) Unwounded, (D,E′′) 4 hours after wounding and (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′, G and G′. The white arrowhead in E″ indicates a small actin-based process. The yellow arrowhead in G′ represents presumptive cytoplasmic vesicles. Note that wounds are open at 24 hours (G,G′). (**C**) Graph 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 4 hours following wounding. Cable intensity is significantly different between groups (*$P \leq 0.01$, Student's *t*-test). Error bars represent the s.e.m. For the control group $n=96$ measurements; for $chic^{IR}$, $n=64$. Scale bars: 50 μm.

the cell to the interior (Fig. 4C) and showed that there is a significant difference between the ranges of the control and tissues lacking Profilin. This data suggests that Profilin is necessary for the organization of both an actin cable and actin-based processes at the wound edge during closure.

Additionally, we examined the wound edge morphology by Transmission Electron Microscopy (TEM) in controls and in larvae lacking epidermal Profilin. As seen previously (Wu et al., 2009), at 4 and 8 hours after wounding, leading edge cells extend long and thin cellular projections over residual debris and into the wound gap (Fig. 5A,C). In epidermal tissues lacking Profilin, leading edge cells lacked the prominent extensions into the debris field that are seen in controls (Fig. 5B,D). Instead, the cells exhibited a rounded morphology, similar to, but less severe than, that seen in TEM of cells lacking Pvr (Wu et al., 2009). This data suggests that the actin defects shown above are manifested at the ultrastructural level as a defect in cell process extension into the wound gap.

DFos and DJun regulate *chic* expression after wounding

Recent evidence suggests that DJun and DFos, the transcription factors downstream of the JNK pathway, can act independently in *Drosophila* embryonic and larval epidermal wound healing (Lesch et al., 2010; Pearson et al., 2009). Jun is not required for the activation of a *Dopa decarboxylase* (*ddc*)-derived wound reporter in embryonic wound healing, while a specific isoform of DFos is required (Pearson et al., 2009). Further, the JNK reporter *msn-lacZ* is activated by DFos after wounding, but not by DJun (Lesch et al., 2010). We tested if the knockdown of these two transcription factors would have an actin phenotype more closely resembling that observed in controls or upon epidermal expression of $UAS\text{-}JNK^{IR}$ (intact cable and processes) (Wu et al., 2009) or that of $UAS\text{-}chic^{IR}$ (lack of cable and processes). We previously showed that expression of $UAS\text{-}DFos^{IR}$ and $UAS\text{-}DJra^{IR}$ transgenes resulted in potent, on-target protein knockdown (Lesch et al., 2010). Knockdown of DJun did not

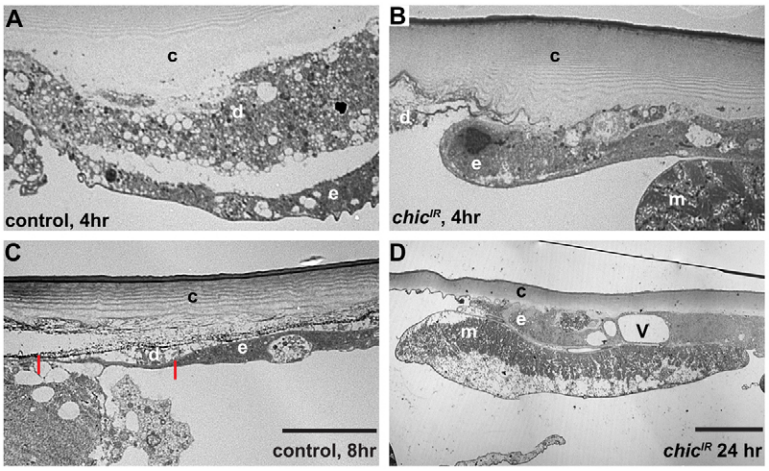


Fig. 5. *chic* is required for cells to extend processes into the wound gap. (**A–D**) TEM images of transverse sections of wounded and dissected L3 larvae of genotypes: *w; UAS-nlacZ/+; UAS-chic$^{IR(R3,R4)}$* (A); *w; e22c-Gal4/UAS-nlacZ; UAS-chic$^{IR(R3,R4)}$* (B,D); and *w;pxn-Gal4, UAS-nlacZ/+* (C). In all images, the wound gap is to the left and the cells are migrating in that direction. (A,B) Larvae at 4 hours after wounding. Note that the control extends a long thin process over the necrotic cellular debris (A), whereas cells lacking Profilin form a rounded and blunt wound edge (B). (C) Larvae at 8 hours after wounding. The cell extends a normal process that is 14.4 μm long (between the red marks) and 0.4 μm thick. (D) Larvae at 24 hours after wounding. The wound has still not closed and the rounded cell has not appreciably extended into the wound gap. c, cuticle; m, muscle; e, epidermal cell; d, cell debris; V, large cytoplasmic vesicle. Scale bars: 10 μm.

affect the distribution or levels of actin in the unwounded larval epidermis (compare Fig. 6D with Fig. 4A), while knockdown of DFos appeared to cause a reduction in nuclear-localized actin (compare Fig. 6A with Fig. 4A). Interestingly, when DFos was knocked down by epidermal expression of *UAS-DFos^{IR}*, the resulting actin phenotype in the wounded epidermis was intermediate between these two extremes. Leading edge cells were able to form a weak actin cable but did not extend actin-based processes by 4 hours after wounding (Fig. 6B and see Fig. 6G for quantification of cable intensity). By contrast, when DJun was knocked down in the larval epidermis, the actin phenotype upon wounding was comparable to *UAS-JNK^{IR}* and controls at 4 hours after wounding (Fig. 5E). Despite normal-seeming actin processes and a cable that was more robust than

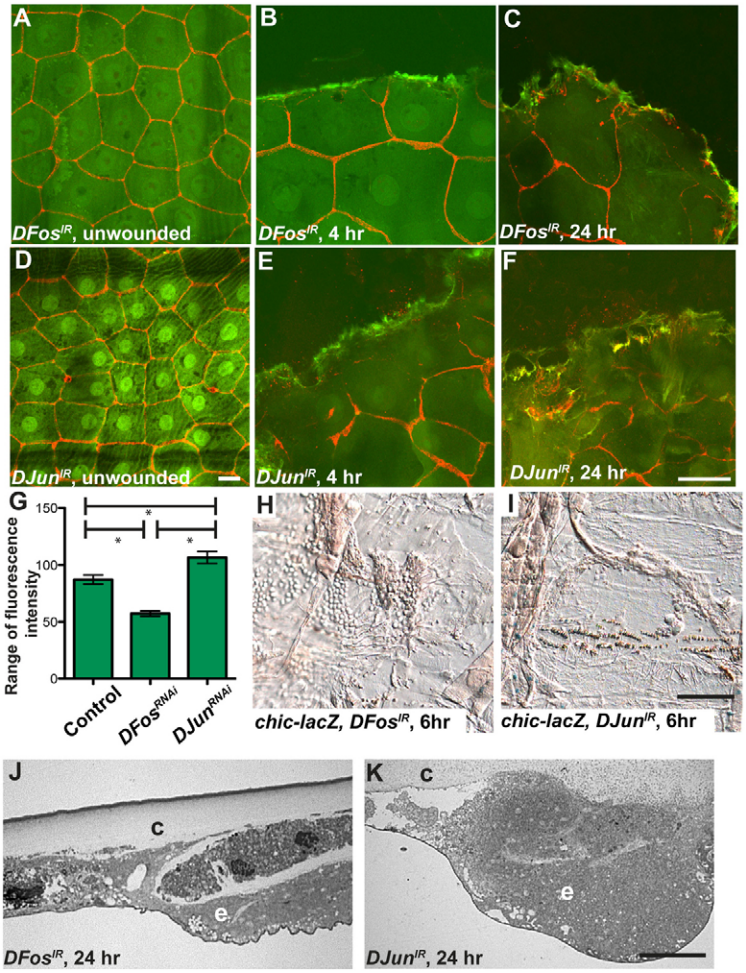


Fig. 6. DFos and DJun are required for *chic* transcription. (**A–F**) Dissected larval epidermal wholemounts of genotype *w;e22c-Gal4, UAS-lifeactGFP* (to label epidermal actin in green) plus *UAS-DFos^{IR}* (A–C) or *UAS-DJun^{IR}* (D–F) and stained for Fasciclin III (red). (A,D) Unwounded, (B,E) and 4 hours after wounding. Note that at 4 hours, the *DFos^{IR}*-expressing larva (B) has a dim actin cable, but does not appear to have processes, whereas *DJun^{IR}*-expressing larvae (E) resemble wild type. (C,F) Larvae at 24 hours after wounding. (**G**) Quantification of ranges of fluorescence intensity of the actin cable at 4 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 s.e.m. For control, $n=145$, for *DFos^{IR}*, $n=146$, and for *DJun^{IR}*, $n=100$. (**H,I**) Dissected larval epidermal wholemounts of genotype *w;e22c-Gal4, chic-lacZ* plus *UAS-DFos^{IR}* (H) or *UAS-DJun^{IR}* (I) at 6 hours after wounding. Note the lack of *lacZ* induction. (**J,K**) TEM of transverse sections of wounded and dissected larvae of genotype *w;A58-Gal4, UAS-nlacZ/UAS-DFos^{IR}* (J) or *w; A58-Gal4, UAS-nlacZ/UAS-DJun^{IR}* (K) at 24 hours after wounding. e, epidermal cells; c, cuticle. Scale bars: 50 μm (A–F); 100 μm (H,I); 10 μm (J,K).

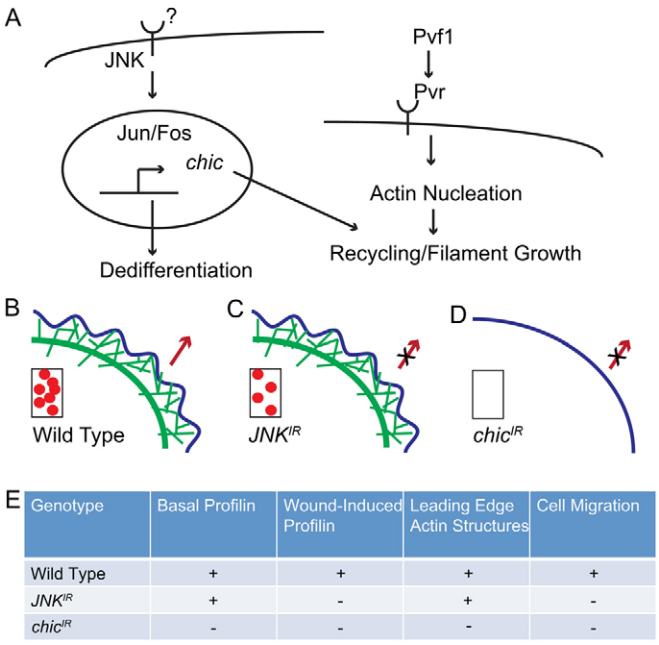
normal (Fig. 6G), by 24 hours after wounding the wounds in *UAS-DJun*^{IR} expressing larvae remained open, suggesting that the processes were insufficient to effect closure.

Because the actin and *msn-lacZ* activation data suggested different roles for *DJun* and *DFos*, we also tested whether they were both required to induce the *chic-lacZ* reporter and whether their knockdown led to similar leading edge morphologies by TEM. *chic-lacZ* was not induced in cells lacking DFos or DJun six hours after wounding (Fig. 6H,I), as compared to the control (Fig. 3B). This indicates that the wound-induced *chic* transcription is mediated by both DFos and DJun. The TEM morphologies of some cells lacking either DJun or DFos both appear similar to those that lack JNK; they continue to secrete cuticle near the wound edge (Fig. 6J,K) – a phenotype we have interpreted as a defect in dedifferentiation (Galko and Krasnow, 2004; Wu et al., 2009). However, other samples of cells lacking DJun or DFos exhibit morphologies more similar to that of cells lacking Profilin (Fig. 4E and data not shown); they have a rounded edge and do not extend into the wound gap. Interestingly, cells lacking Profilin do not appear to continue secreting cuticle near the wound edge. This suggests that while DJun and DFos regulate *chickadee* and dedifferentiation, *chickadee* itself is not required for dedifferentiation.

Discussion

The traditional model of the actin cytoskeleton in cell migration, based on *in vitro* cell culture and biochemical assays, provides a useful framework for the mechanics of how cell migration is regulated. However, there is need for *in vivo* studies in order to answer important questions that are not addressed by the current model: 1. Is there a role for Profilin-mediated recycling during wound-induced migration of differentiated cells *in vivo*? 2. Is there a role for transcriptional regulation of actin regulators during such migrations? This latter question emerges because the basic model generally assumes that migratory cells have an intact actin-regulatory apparatus that needs only to be activated to initiate and sustain migration. While this assumption may be correct for migrating cells in developmental contexts (Castrillon et al., 1993; Cooley et al., 1992; Jasper et al., 2001; Verheyen and Cooley, 1994) one could imagine that initially non-migratory differentiated cells may need more than their resting complement of actin regulators in order to effect long-distance migration.

Unwounded larval epidermal cells have an even distribution of actin and Profilin throughout the cytoplasm and are thought to be non-migratory. These fully differentiated epithelial cells secrete an apical cuticle and a basal lamina (Snyder et al., 1981). They respond to the physiological signal of tissue damage by partially dedifferentiating and becoming migratory. Here we show that the leading edge cells form multiple actin-based structures including a discontinuous cable, filopodia, and lamellipodia. We propose a working model (Fig. 7) where the basal levels of Profilin are sufficient to make actin-based structures, but wound-induced transcription of *chic* is required for the cells to efficiently migrate (Fig. 7B–E). The lack of actin-based structures at the wound edge in cells lacking Profilin would indicate that if Formin-mediated actin nucleation is involved in wound healing, it likely requires Profilin. An epidermal sheet lacking detectable Profilin fails to close wounds or form any actin-based structures at the wound edge whereas a sheet containing only a basal level of Profilin (i.e. one that is lacking proteins that transcriptionally regulate Profilin after wounding, such as JNK, Fos, or Jun) forms



E

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

Fig. 7. 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.

actin structures at the wound edge, but is ultimately unable to efficiently migrate and close the wound (Fig. 7E). This model is complicated by the fact that cells lacking JNK, Fos, or Jun also have defects in dedifferentiation, as these cells do not stop secreting cuticle following wounding. Thus, we cannot completely exclude the possibility that the lack of wound closure is due to defects in dedifferentiation. However, it is entirely possible that upregulation of actin-binding regulators is an important component of the dedifferentiation process, as this involves returning to a state during which these cells were competent to migrate.

Current wound closure models have identified two signaling pathways that are important for healing. One is Pvr signaling, where the secreted VEGF-like ligand Pvf1 activates the Pvr receptor (Wu et al., 2009). Currently, only a few proteins are suspected of being downstream of Pvr signaling (Duchek et al., 2001; Ishimaru et al., 2004), but Profilin is not among them. Given that epidermal cells lacking Pvr are unable to mobilize actin to the wound edge, Pvr is likely upstream of actin regulatory proteins that initiate actin polymerization at the leading edge of migrating cells (Fig. 7A). The second pathway is JNK signaling, which is required for closure but not for actin polymerization at the wound edge (Wu et al., 2009). Naively, we anticipated that wound-induced *chic* expression would be regulated by Pvr since epidermal expression of *UAS-chic*^{RNAi}

also blocks actin accumulation at the wound edge. Surprisingly, this is not the case. *chic-lacZ* expression is instead regulated by JNK signaling, as it is in the developing embryo during DC (Jasper et al., 2001). This data reveals that although the JNK signaling pathway is not required for actin nucleation at the wound edge (Wu et al., 2009) it contributes to actin dynamics through regulating expression of *chic* and perhaps other genes important for migration.

How does JNK signaling activate *chic* transcription after wounding? Although the upstream signal for the JNK signaling pathway is still unknown, the kinase cascade is well-defined (Lesch et al., 2010) and is thought to culminate with the phosphorylation of the transcription factors, DJun and DFos. These two proteins are commonly thought to act as a dimer (AP-1) to mediate transcriptional activation of target genes (Kockel et al., 2001). In the early DC studies chickadee expression was shown to depend on the JNK signaling pathway (Jasper et al., 2001). This study did not address the roles of DJun and DFos in particular, although these transcription factors are required for DC (Riesgo-Escovar and Hafen, 1997b; Riesgo-Escovar and Hafen, 1997a). In wound healing contexts, however, it appears that DFos can act without DJun to activate a *ddc*-wound reporter (Pearson et al., 2009) and a *msn-lacZ* wound reporter (Lesch et al., 2010). Here we find that both DJun and DFos are required to activate *chic*. Additionally, two consensus binding sequences for the AP-1 transcription factor (TGANTCA) are located upstream of the *chic* start codon (depending on the message isoform the sites are located in the 5′UTR, the first intron, or the promoter region – see supplementary material Fig. S1), indicating that it is at least possible that the upregulation of *chic* transcription is directly accomplished by Jun and Fos (Perkins et al., 1988). The consensus sequence is also located upstream of the human Pfn1, indicating that there is potential for this regulation to be conserved. This suggests that in the migrating cells at the wound edge, DFos can act either as a homodimer, with unidentified binding partners, or with DJun to regulate the necessary transcriptional targets.

In *Drosophila* embryonic models of wound closure both the contractile actin cable and filopodial processes are important for wound closure, but their relative contributions are still unclear (Wood et al., 2002). There has been debate over whether the cable mediates closure through contraction (Kiehart et al., 2000), through serving as a platform for extension of processes into the wound gap (Martin and Lewis, 1992), or through a combinaton of these functions. From the data shown here it seems that actin-based contraction is not a major contributor to larval wound closure. First, the actin concentrations that appear at larval wound edges are discontinuous. Second they do not appear to be locally contractile given that the cells behind prominent concentrations do not obviously taper toward the wound. This is similar to what has been observed in the embryonic *Xenopus* epithelium where actin cables form but differently shaped wounds do not round up as would be expected from cable contraction (Davidson et al., 2002). Thus it would appear that in larvae the actin concentrated at the wound edge primarily facilitates process extension into the wound gap.

In this study we establish a connection between a known wound-induced signaling pathway, JNK signaling, and Profilin-mediated regulation of the actin cytoskeleton. We speculate that transcriptional induction of actin-regulators may be a general feature of cell migration in differentiated cells as suggested by a recent study of cells undergoing EMT (Haynes et al., 2011). By connecting upstream signaling pathways to downstream actin dynamics, this work begins to unravel the logic of how the cellular movements required for wound closure are orchestrated.

Materials and Methods

Fly stocks

All fly stocks were reared at 25℃ on standard cornmeal media. $chic^{01320}$ (also referred to here as *chic-lacZ*) is a larval viable hypomorphic allele that bears a *lacZ*-containing P-element insertion (Cooley et al., 1992). *FasIII-GFP* (Quiñones-Coello et al., 2007) is a GFP trap insertion in the *Fas3* locus producing a Fasciclin III-GFP fusion protein that is expressed on larval epidermal cell membranes.

The Gal4/UAS system (Brand and Perrimon, 1993) was used to drive transgene expression in selected tissues. *A58-Gal4* (Galko and Krasnow, 2004) drives in the larval epidermis, *e22c-Gal4* (Lawrence et al., 1995) in the embryonic and larval epidermis, *Pnr-Gal4* (Calleja et al., 1996) in dorsal epidermal patches, *Pxn-Gal4* in the hemocytes (Stramer et al., 2005) and *dmef2-Gal4* (Zars et al., 2000) in the larval body wall muscles. *UAS-chickadee* (Geisbrecht and Montell, 2004) was used to overexpress Profilin in rescue experiments. $UAS\text{-}hep^{CA}$ (Adachi-Yamada et al., 1999) was used to induce activation of the JNK signaling pathway and *UAS-λPvr* (Duchek et al., 2001) was used to induce activation of the Pvr signaling pathway. *UAS-lifeact-GFP* was used to label filamentous actin. For the *UAS-lifeact-GFP* construct, the codons (atgggcgtggccgatctgatcaagaagttcgagagcatcagcaaggaggaa) of the lifeact peptide [MGVADLIKKFESISKEE; (Riedl et al., 2008)] were cloned in frame into the pUASt-green vector, containing an eGFP cassette (Brand and Perrimon, 1993; Jung et al., 2007) by using synthetic oligonucleotides (CTCTAGatgggcgtggccgatctgatcaagaagttcgagagcatcagcaaggaggaA; CCGGTtcctc-cttgctgatgctctcgaacttcttgatcagatcggccacgcccatCTAGAGGTAC) and restriction sites for *Kpn*I and AgeI. Transgenic flies were established as reported previously (Berger et al., 2008). *UAS-GFP* (Halfon et al., 2002) was used to select *Pnr-Gal4*-expressing larvae. *UAS-DsRed2-Nuc* labels nuclei in red (Lesch et al., 2010). $UAS\text{-}bsk^{B}$ was used to express *bsk* cDNA in a rescue experiment (Boutros et al., 1998). $tubulin\text{-}Gal80^{ts}$ (McGuire et al., 2003) was used to conditionally inhibit Gal4-mediated transgene expression.

To knock down gene expression we used transgenic lines that express inverted repeats (IR) under UAS control. All RNAi lines were from NIG-Fly (http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp) or the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007). Lines used included: *9553R-3* ($chic^{IR(R3)}$); *9553R-4* ($chic^{IR(R4)}$); a stock that combined these transgenes ($chic^{IR(R3,R4)}$); *9553 #112358* ($chic^{IR(kk)}$); *8222R-3* (Pvr^{IR}); a stock that combined two lines targeting *basket*, *5680R-1* and *5680R-2* (JNK^{IR}); *2275R-2* ($DJun^{IR}$); and *15509R-2* ($DFos^{IR}$). Epidermal expression of the RNAi lines targeting Pvr, Fos, and Jun result in efficient knockdown of the targeted proteins with no off-target effects (Lesch et al., 2010; Wu et al., 2009). The JNK^{IR} line has been shown to phenocopy the $UAS\text{-}bsk^{DN}$ line (Lesch, et. al, 2010) and block *msn-lacZ* induction (Lesch et al., 2010). Additionally, we show here that $UAS\text{-}bsk^{B}$ can fully rescue *UAS-5680R-2* (supplementary material Fig. S5).

Wounding assay

The wounding assay was performed as described previously (Babcock et al., 2008; Galko and Krasnow, 2004). Briefly, larvae were lightly anesthetized with ether, pinched on the dorsal side within segment A4, A5 or A6 with blunted laboratory forceps (Fine Science Tools), and returned to food to be examined for wound closure at a later timepoint. For quantification of wound closure, the wound was defined as open if a gap was visible in the epithelial sheet with no apparent cells and as closed if there was a re-epithelialized sheet with a patch of atypically organized cells.

Immunofluorescence and imaging

Larval epidermal wholemount dissections and immunofluorescence staining were done as described previously (Galko and Krasnow, 2004). The monoclonal antibodies chi 1J (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. 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 1× 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). Images were obtained on either a Leica MZ16FA stereomicroscope (Fig. 1) using a Planapo 1.6× objective with a Leica DFC300 FX color camera and Image Pro AMS v5.1 software (Media Cybernetics, Bethesda, MD, USA) or on an Olympus FV1000 laser confocal microscope (Figs 2,4,5) using a 20×/0.85 NA oil or 60×/1.42 NA oil objective with Fluoview software. All images were collected at room temperature. For confocal images, Z stacks of 1 μm (20×) or 0.5 μm (60×) 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 (Figs 4,6), 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.

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* allele were wounded, dissected at a later timepoint, fixed for 20 minutes in cold 2% glutaraldehyde in 1× PBS, washed with 1× PBS, and stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) for two hours at 37℃. Samples were mounted in 70% glycerol and imaged at room temperature. Images were taken on a Leica DM5500 upright microscope using a 10×/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.

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% glutaraldehyde, 2% paraformaldehyde, and 2.5% DMSO in 0.2 M sodium phosphate buffer (pH 7.2) and incubated for 1 hr 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 on a JEOL JEM 1010 transmission electron microscope with AMT (Advanced Microscopy Techniques, Woburn, MA, USA) software.

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