# Requirement for Pak3 in Rac1-induced organization of actin and myosin during *Drosophila* larval wound healing

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ABSTRACT

Rho-family small GTPases regulate epithelial cell sheet migration by organizing actin and myosin during wound healing. Here, we report that Pak3, but not Pak1, is a downstream target protein for Rac1 in wound closure of the *Drosophila* larval epidermis. Pak3-deficient larvae failed to close a wound hole and this defect was not rescued by Pak1 expression, indicating differential functions of the two proteins. Pak3 localized to the wound margin, which selectively required Rac1. Pak3-deficient larvae showed severe defects in actin-myosin organization at the wound margin and in submarginal cells, which was reminiscent of the phenotypes of Rac1-deficient larvae. These results suggest that Pak3 specifically mediates Rac1 signaling in organizing actin and myosin during *Drosophila* epidermal wound healing.

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## 1. Introduction

An epithelial wound hole is sealed by collective migration of a neighboring cell sheet [1–3]. This process involves several cellular elements, including supracellular actin cables and filopodial/lamellipodial projections around the wound margin, and cell shape changes in the marginal and submarginal cells [4–7]. Studies in various wound-healing models indicate that the combined action of these elements in the marginal and submarginal cells collectively mediate wound closure [1,8].

Rho-family small GTPases mediate cell migration in morphogenesis and wound healing [9]. Each of the family members has a distinct role in wound healing. In *Drosophila* embryos, Cdc42 mediates filopodial projection into the wound leading edge (LE) and is important for suturing of the wound hole, while Rho1 mediates actin cable formation, which is critical during the initial stage of wound contraction [10]. In *Drosophila* larvae, Rac1, Cdc42, and Rho1 are essential for wound closure and organization of F-actin at the wound LE and for proper cell polarization [7,11]. These GTPases mediate wound signaling through c-Jun N-terminal kinase (JNK) [11], another essential component in cell sheet migration during morphogenesis and wound healing in *Drosophila* [6,12–14]. The

intermediate downstream targets for the GTPases involved in *Drosophila* wound healing are not known.

P21-activated kinases (Paks) are a family of serine/threonine kinases that were originally identified as a target for activated Rac1 and Cdc42 [15]. Paks regulate cytoskeletal organization during development or in diseased states in both flies and mammals [16–22]. All Pak members have a conserved kinase domain in the C-terminal half and a p21-binding domain (PBD) in the N-terminus [23]. Depending on structural differences, the Pak proteins are classified into two subgroups, group 1 and group 2. Group 1 Paks have an unique autoinhibitory domain (AID), which partially overlaps with the PBD and lies on the C-terminal side of this domain. The AID blocks the catalytic site of the kinase domain and is unlocked by binding of small GTPases to the PBD domain [23]. Involvement of Pak proteins in wound healing, however, has not yet been reported in vivo.

In the *Drosophila melanogaster* genome, three genes encode Pak proteins: *Pak1* and *Pak3* are group 1 Paks and *Mbt* is a group 2 molecule (for clarity, we will refer to *Pak* as *Pak1* to distinguish this gene from the generic name). The in vivo functions of *Drosophila* Pak1 include axon guidance of photoreceptors and olfactory neurons, synaptic organization in the neuromuscular junction, and embryonic morphogenesis [17,24–26]. For photoreceptor axon guidance, Pak1 is activated by membrane recruitment through the adaptor molecule Dock, which is associated with a guidance receptor, and also by guanine nucleotide exchange factor (GEF)-activated

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Rac1 and Cdc42 [24,27]. The in vivo function of *Drosophila* Pak3 has not been well documented; however, Pak3 acts together with Pak1 in embryonic morphogenesis, including head involution, germ-band extension, and dorsal closure [28]. Pak3 binds strongly to Rac1 and weakly to Rac2 in HEK293 cells [29]. Pak3 also binds selectively to an activated form of Cdc42, albeit weakly, in HEK293 cells.

Here, we report that *Drosophila* Pak3 is a downstream target protein for Rac1 in wound healing of *Drosophila* larval epidermis. Pak1 is not required for this process, and the functions of the two Pak proteins are clearly distinct, as observed in the mutant or RNAi knockdown phenotypes in photoreceptor connectivity and wound healing. Mechanistically, Pak3 is essential for organization of F-actin and myosin at the wound LE and in the submarginal cells.

## 2. Materials and methods

### 2.1. Fly strains

The following stocks were used in this study: *UAS-Pak3 RNAi* (14895R-1), *UAS-Rac1 RNAi* (2248R-2), *UAS-Rok RNAi* (9774R-2), and *UAS-MLCK RNAi* (18255R-3) from National Institute of Genetics, Japan; *UAS-Pak3 RNAi* (39843, 39844, and 44607) and *UAS-Rok RNAi* (3793) from Vienna *Drosophila* RNAi Center; *en-GAL4, GMR-GAL4, UAS-Dicer2, UAS-mCD8-GFP, UAS-Rac1^{DN}* (*UAS-Rac1^{N17}*), *UAS-Cdc42^{DN}* (*UAS-Cdc42^{N17}*), UAS-Rho1^{DN} (*UAS-Rho1^{N19}*), UAS-bsk^{DN}, Pak1^6, Pak1^{11}, Rac2^Δ, Mtl^Δ, *UAS-LIMK1 RNAi, UAS-LIMK1 KI, UAS-cofilin*, and *msn-LacZ* from the Bloomington Stock Center; Pak3^{76A}, Pak3^{27A}, *UAS-Pak1-GFP*, and *UAS-Pak3-GFP* [28]; *UAS-Pak1* [30]; *A58-GAL4* [6]; and *UAS-GFP-Zip* [31]. For the Pak3 RNAi strains, 14895R-1 contains an RNAi construct that targets the region of 36th – 533rd of the Pak3 ORF, while each of 39843, 39844, and 44607 contains the same RNAi construct that targets the region of 1295th – 1601th (http://www.shigen.nig.ac.jp; http://stockcenter.vdrc.at).

### 2.2. Wounding, dissection, and immunostaining

Third instar larvae were wounded by pinching the dorsal larval integument with forceps (Fine Science Tools, Cat. No. 11295-00), which abraded about 30–40 epidermal cells. The larvae were then returned to cornmeal-agar food paste during the incubation time for recovery. Dissection of the larval epidermis was performed in phosphate-buffered saline (PBS) using a pair of forceps and six acupuncture pins (purchased from a local store) on a silicone pad as described previously [7]. Samples were fixed in 4% paraformaldehyde (PFA) for 15–30 min at room temperature, and the muscle fibers underneath the epidermis were carefully removed for a clear image of epidermal staining for phalloidin and Pak3. The epidermal filet was washed in PBS three times each for 5 min and incubated with a primary antibody diluted in 0.1 M sodium phosphate buffer (pH 7.2) plus 0.3% Triton-X-100 overnight at 4 °C. Samples were washed in PBS three times each for 5 min and incubated with a secondary antibody alone or together with phalloidin-FITC for 2 h at room temperature. After washing away the excess secondary antibody or phalloidin-FITC with PBS, samples were mounted in 80% glycerol or Vectorshield (Vector Laboratories, Inc., Burlingame, CA) and subjected to fluorescent microscopy (Olympus BX40). Dissection and staining of the larval brain was performed as previously described [32]. The following antibodies and reagents were used in this study: rabbit anti-Pak1 (1:50, provided by N. Harden), mouse anti-Pak3 (1:250, also provided by N. Harden), mouse anti-Fasciclin III and mouse 24B10 (1:50, Developmental Studies Hybridoma Bank), goat anti-mouse IgG-Cy3 (1:200, Jackson Immuno Research), phalloidin-FITC (1:50 dilution of 400 μM stock, Sigma–Aldrich), and DAPI (1:500, Molecular Probes).

### 2.3. Quantification of GFP-Zip localization

For quantification of GFP-Zip localization, we overlaid on each of wound LE cells a symbol of compass (a circle with "×" mark in the middle) and analyzed the directionality. The compass was set to point toward the wound center or point perpendicular to the tangent line of the wound LE. We analyzed in a given microscopic field all LE cells, but excluded submarginal cells, because judging whether any particular submarginal cell was located within or outside the first three rows from the wound margin was often highly ambiguous due to cell intercalation. Any LE cell that was ambiguous in directionality due to cell–cell fusion, tissue damage, or other reasons, was also carefully excluded from the analysis.

### 2.4. Real-time PCR

Quantitative real-time PCR was performed according to the following methods. Total RNA was isolated from approximately 15–30 larvae at the 3rd instar stage using Trizol (Invitrogen, Carlsbad, CA). The cDNA was then synthesized from 2 μg of RNA with M-MLV reverse transcriptase (Promega, Madison, WI). Target cDNAs were quantified by real-time PCR (ABI Prism 7300). PCR reactions contained 2× SYBR premix Ex Taq (Takara, #RR041A) and were analyzed by the comparative Ct method. All results were normalized to the level of Rp49 mRNA in each sample, and three experiments for each condition were averaged. The following primer sets were used in this study: LIMK1, 5′-GCCGATCCGGATTTTATGC-3′ (forward) and 5′-GCGAAACTCCTGCTGATTGAG-3′ (reverse); Rok, 5′-GAGGCACTGC TCGTGGAAAC-3′ (forward) and 5′-TGCCCGCG TCTTTTGC-3′ (reverse); and MLCK, 5′-CAGTGCTGCACGGGAGAAA-3′ (forward) and 5′-TCACACAAGAGCGGCTTGAC-3′ (reverse).

## 3. Results

### 3.1. Pak3 is essential for wound closure

Since Rho-family small GTPases are involved in *Drosophila* epidermal wound healing [10,11], we examined whether Pak1 is required for the same process as a potential downstream target. Pak1^6/Pak1^{11} larvae displayed normal epidermal wound closure, examined 14 h (n = 14; data not shown) and 24 h (n = 24; Fig. 1) after wounding. The Pak1^6 allele yields a protein fragment that truncates 84% of the protein, and the Pak1^{11} allele is protein null [17,25]. The extant mutant alleles of Pak3 (Pak3^{27A} and Pak3^{76A}) [28], the other group 1 Pak in *D. melanogaster*, were lethal around the first and second instar larval stages either in homo- or heteroallelic combinations (data not shown). This lethality occurred too early to permit our regular wound closure assay. We, therefore, knocked down Pak3 activity with *UAS-Pak3 RNAi* using the larval epidermis-specific A58-GAL4 driver and found that the larvae showed a strong defect in wound closure. Upon epidermal wounding by pinching the cuticle and abrading about 30 epidermal cells during the 3rd instar larval stage, the Pak3-depleted larvae failed to seal the wound hole even after 24 h, whereas wild-type larvae typically sealed a wound hole of similar size within approximately 14 h (Fig. 1; data not shown). We confirmed this phenotype using several different RNAi strains (Fig. 1; see Section 2). The knockdown efficiency of each strain was examined
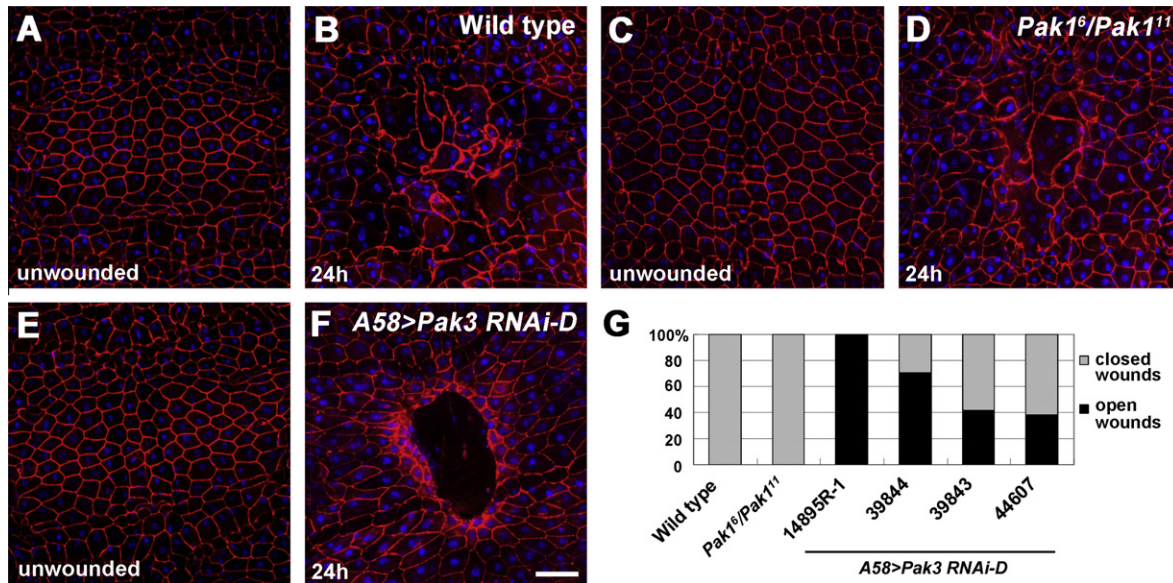
**Fig. 1.** Pak3, but not Pak1, is required for larval wound closure. Examination of larval epidermal wound closure 24 h after wounding. Cell boundaries were stained with the septate junction marker FasIII (red), and nuclei were stained with DAPI (blue). (A) Unwounded wild type. (B) Wounded wild type. (C) Unwounded $Pak1^6/Pak1^{11}$. (D) Wounded $Pak1^6/Pak1^{11}$. (E) Unwounded larvae with *UAS-Pak3 RNAi* (14895R-1) driven by the larval epidermis-specific *A58-GAL4* in the presence of *UAS-Dicer2* (hereafter, *A58>Pak3 RNAi-D* or simply *A58>Pak3i-D*). (F) Wounded *A58>Pak3 RNAi-D* (14895R-1). Bar: 100 μm (A-F). (G) Quantification of the wound closure defect. $n = 28$ (wild type), 24 ($Pak1^6/Pak1^{11}$), 26 (*A58>Pak3 RNAi-D*, 14895R-1), 17 (*A58>Pak3 RNAi-D*, 39844), 12 (*A58>Pak3 RNAi-D*, 39843), and 26 (*A58>Pak3 RNAi-D*, 44607).
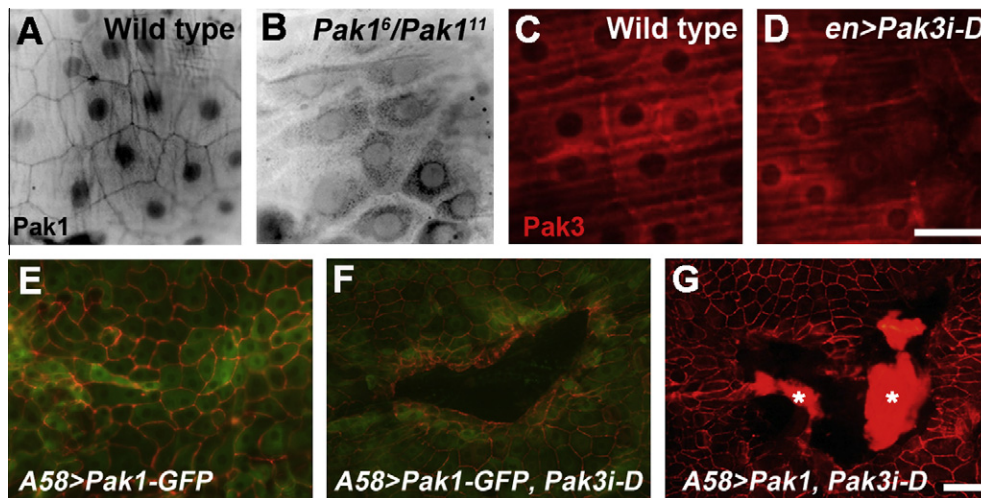


**Fig. 2.** Differential functions of Pak1 and Pak3. (A–D) Analysis of protein expression of Pak1 (A and B) and Pak3 (C and D) in the larval epidermis. Pak1 and Pak3 were stained using anti-Pak1 and anti-Pak3 antibodies, respectively. (A) Wild type. (B) $Pak1^6/Pak1^{11}$. (C) Wild type. (D) *en>Pak3 RNAi-D*. The right half of the panel is the Engrailed-expression domain (also see Supplementary Fig. 1). (E) Normal wound closure in *A58>Pak1-GFP* was examined 24 h after wounding. (F) Defective wound closure in *A58>Pak1-GFP, Pak3 RNAi-D* was examined 24 h after wounding. (G) Defective wound closure in *A58>Pak1, Pak3 RNAi-D* was examined 24 h after wounding. Asterisks indicate tissue debris stuck in the wound hole. Cell boundaries were stained with FasIII (red; E–G) and Pak1-GFP was in green (E and F). Bar: 50 μm (A–D) and 100 μm (E–G).

by immunohistochemistry using anti-Pak3 antibody (Supplementary Fig. 1; also see Fig. 2C and D). Since the strain 14895R-1 induced the highest knockdown efficiency, we selected this strain for further studies.

### 3.2. Distinct in vivo functions of Pak1 and Pak3

We further investigated the differential roles of Pak1 and Pak3. During photoreceptor axon pathfinding, Pak1 is essential for the formation of a regular topographic array of axon terminals in the larval brain that reflects the positions of the photoreceptor cell bodies in the eye imaginal disc [24]. Whereas $Pak1^6/Pak1^{11}$ larvae failed to establish this spatial array as described previously [24],

Pak3 RNAi larvae exhibited a grossly normal projection pattern, indicating that Pak1 and Pak3 have different in vivo functions (Supplementary Fig. 2A–C).

The differential roles of Pak1 and Pak3 may stem from different protein interaction networks or perhaps be simply attributable to different tissue-specific expression patterns of the two proteins that have otherwise similar biochemical functions. The latter possibility was taken into account since Pak1 and Pak3 showed high structural similarity (35.4% identity and 47.9% similarity overall; 63.4% identity and 79.9% similarity in the kinase domain) [29].

We first examined expression patterns of Pak1 and Pak3. In the larval brain, only Pak1 was expressed. Pak1 was localized to the photoreceptor axons as previously shown [24], whereas Pak3
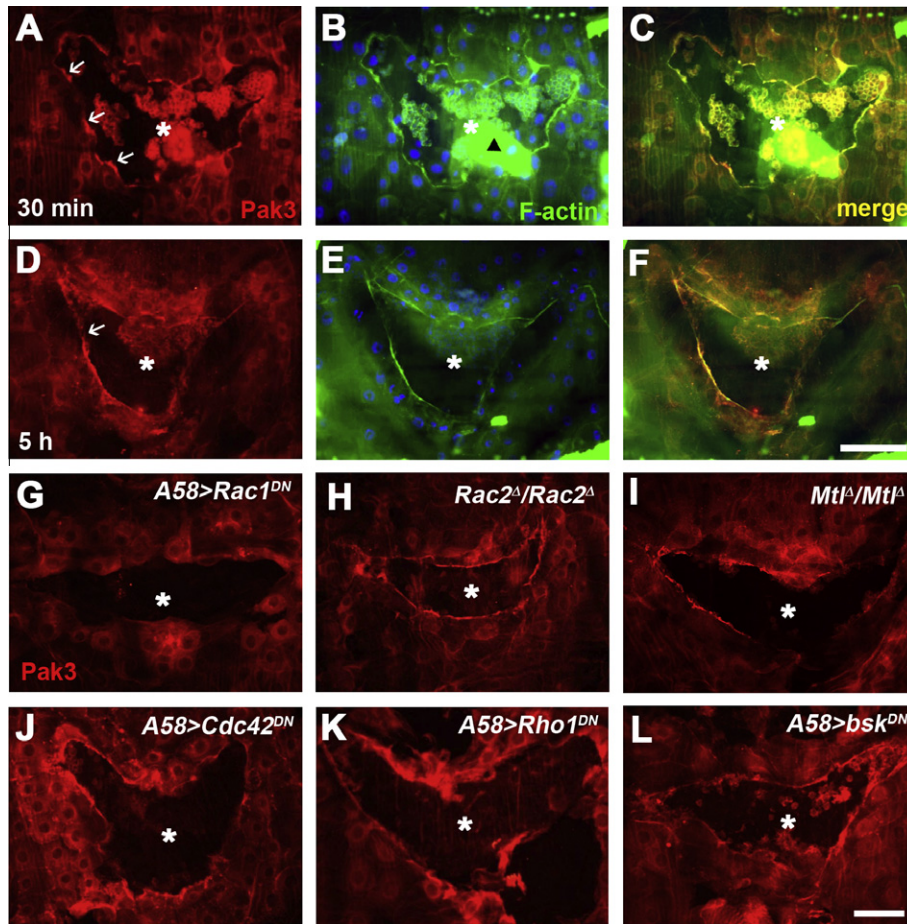
**Fig. 3.** Pak3 localization to the wound leading edge requires Rac1. (A–F) Pak3 localization was examined 30 min (A–C) or 5 h (D-F) after wounding. (A and D) Anti-Pak3 staining is shown in red. Arrows indicate Pak3 localization to actin-based protrusions. Hemocyte aggregates were observed in the wound hole. (B and E) Phalloidin staining for F-actin is shown in green. The black triangle indicates muscle remnants after dissection. (C and F) Merged pictures. (G–L) Pak3 localization was examined 5 h after wounding in larvae with the indicated genotype. (G) $A58>Rac1^{DN}$. (H) $Rac2^{\Delta}/Rac2^{\Delta}$. (I) $Mtl^{\Delta}/Mtl^{\Delta}$. (J) $A58>Cdc42^{DN}$. (K) $A58>Rho1^{DN}$. (L) $A58>bsk^{DN}$. Pak3 was stained with anti-Pak3 (red). Asterisks indicate wound holes. The lower right parts of the tissues in (I and K) were damaged during muscle dissection. Bar: 100 μm.

was not found at these axons (Supplementary Fig. 2D–G). In the larval epidermis, however, Pak1 and Pak3 were both expressed (Fig. 2A–D). Pak1 was mainly associated with the plasma membrane and, was also observed, rather unexpectedly, in the nucleus as well (Fig. 2A). Pak3 was distributed in the cortical region and the cytosol but was excluded from the nucleus (Fig. 2C). The specificities of the antibody staining were confirmed in the corresponding mutant or RNAi knockdown backgrounds (Fig. 2B and D). Thus, Pak1 was present in the epidermis but was unable to replace Pak3 in the absence of the latter.

Second, we examined whether Pak1 overexpression could rescue the wound closure defect of Pak3 RNAi larvae. We made sure that overexpression of Pak1-GFP fusion protein [33] did not interfere with wound closure in an otherwise wild-type background (Fig. 2E). When overexpressed in Pak3 RNAi larvae, Pak1-GFP did not rescue the wound closure defect of Pak3 RNAi larvae (Fig. 2F). In these larvae, Pak1-GFP protein was normally expressed and not knocked down by an off-target effect of Pak3 RNAi. Overexpression of Pak1 without the GFP tag still did not rescue the wound closure defect of Pak3 RNAi (Fig. 2G). These findings exclude the possibility that Pak1 and Pak3 have an overlapping biochemical function but a loss of function for Pak3 only displays wound closure defect because Pak3 is normally present in the epidermis at a higher protein level. Taken together, these results indicate that Pak3 has an unique function in epidermal wound closure, despite the sequence similarity between Pak1 and Pak3.

### 3.3. Pak3 localizes to the wound LE, and this process selectively requires Rac1

Polarized localization of Pak protein during cell migration and tissue movement has been reported previously [17,34,35]. In unwounded wild-type larval epidermal cells, Pak3 was located in the cytosol and the cortical region (Fig. 2C). Following wounding, Pak3 localized to the wound LE, which co-localized with F-actin as early as 30 min post-wounding (Fig. 3A–C). Furthermore, an increase in Pak3 protein level was observed in wound LE cells and submarginal cells 5 h after wounding (Fig. 3D–F). To determine which genetic components are required for Pak3 localization to the wound LE, we examined Pak3 staining in various genetic backgrounds 5 h after wounding. Pak3 localization was absent in larvae that overexpressed Rac1$^{DN}$, suggesting that Pak3 acts downstream of Rac1 (Fig. 3G). The Rac1 null mutant is embryonic lethal [36]; thus, we confirmed this result using a UAS-Rac1 RNAi strain (Supplementary Fig. 3). We also examined the possible involvement of the other two Rac proteins, Rac2 and Mtl, in wound healing. Null mutant larvae for either Rac2 or Mtl had no defect in wound closure or Pak3 localization (Fig. 3H and I; Supplementary Fig. 3). In larvae overexpressing either Cdc42$^{DN}$, Rho1$^{DN}$, or Bsk$^{DN}$, the LE localization of Pak3 was not as distinct as in wild type (Fig. 3J–L), and this difference may be attributable to complex defects in reepithelialization of these larvae [7,11]. Nevertheless, Pak3 was localized to the wound LE, and these findings indicate that Pak3 localization is selectively dependent on
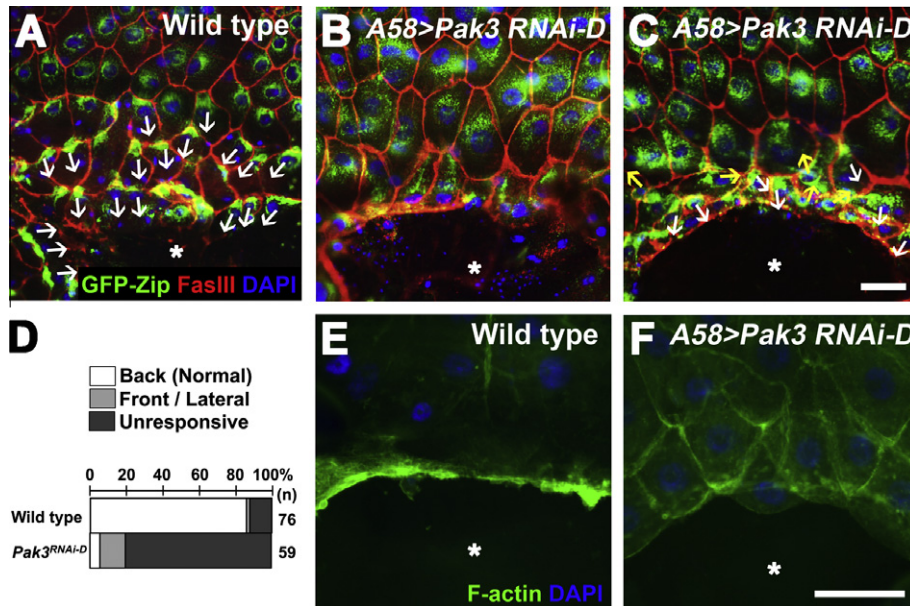
**Fig. 4.** Assembly of actin and myosin is disrupted in *Pak3* RNAi larvae during wound healing. (A–D) Cell polarization and directionality was analyzed by GFP-Zip (green) localization 8 h after wounding. FasIII staining of cell boundaries (red) and DAPI staining of nuclei (blue) are shown. Asterisks indicate wound holes. Arrows indicate directionality as determined by GFP-Zip localization in each cell. A normal directionality, which points toward the wound center, was marked in white, and the wrong directionality was marked in yellow. (A) Wild type. (B and C) Two representative micrographs for *A58>Pak3 RNAi-D*. (D) Quantification of the GFP-Zip localization. Wound LE cells were sorted into three categories depending on the GFP-Zip localization pattern (see Section 2 for details). (E and F) F-actin localization was examined 6 h after wounding. F-actin was visualized by phalloidin-FITC (green), and nuclei were stained with DAPI (blue). Asterisks indicate wound holes. (E) Wild type. (F) *A58>Pak3 RNAi-D*. Bar: 50 μm.

Rac1 but not Rac2, Mtl, Cdc42, or Rho1. We also noticed that the Pak3 induction by wounding was not disrupted in any of the genetic backgrounds we tested, indicating that Pak3 up-regulation by wounding is mediated by a separate pathway.

### 3.4. Pak3 is required for assembly of actin and myosin during wound healing

To analyze the wound healing defect of Pak3 knockdown larvae mechanistically, we examined the localization pattern of the non-muscle myosin II heavy chain, which is encoded by *zipper* (*zip*), during wound healing. In wild-type larval epidermis, the GFP-Zip fusion protein (and the endogenous Zip protein as well) localizes to the rear side of the cells that are located within 2–3 rows of the wound margin (Fig. 4A) [7]. In Pak3 RNAi larvae, this localization pattern was disrupted (Fig. 4B and C). In some cases, cells did not respond at all to the wound, and this lack of response was reminiscent of larvae that overexpress the dominant-negative form of Basket (Bsk[DN]) [7]. In other cases, cells managed to polarize GFP-Zip, but the directionality was random, similar to larvae that overexpress a dominant-negative form of either Rac1, Cdc42, or Rho1 (Fig. 4B–D) [11].

We also examined F-actin localization by phalloidin staining. In wild type, F-actin was observed at the wound LE 6 h after wounding as described previously (Fig. 4E) [7]. In Pak3 RNAi larvae, no distinct LE localization of F-actin was observed 6 h after wounding (Fig. 4F). Instead, weak localization of F-actin at the cell peripheries was observed, which may also reflect the loss of wound directionality.

### 4. Discussion

Our present study demonstrates that Pak3 is required for reepithelialization in *Drosophila* larval epidermal wound healing. Based on our cellular and genetic analysis of Pak3 function combined with the previous biochemical findings [29], we propose that

Pak3 selectively mediates Rac1 signaling via recruitment to the wound LE, where the protein organizes actin and myosin assembly. To our knowledge, this study provides the first in vivo evidence indicating that Pak is required for wound healing. Numerous reports have shown the involvement of Pak proteins in wound healing in cultured cell-based studies [23,37]. No knockout phenotype regarding wound healing, however, has yet been reported in mice, which might be due to functional redundancies between the six Pak members.

The differential phenotypes of Pak1 and Pak3 are interesting, given the structural similarities between the two proteins. In fact, the two proteins have similar in vitro binding preferences with their potential upstream regulators, such as the small GTPases [27,29]. We found that Pak1 is expressed in the larval epidermis, yet Pak1 mutant larvae heal wounds normally. In the embryonic surface ectoderm, Pak1 and Pak3 are both expressed [28,38], and the two proteins show partial redundancy in embryonic morphogenesis, while concomitantly exhibiting unique non-redundant functions [28]. Therefore, Pak1 may be involved in a subtle aspect of wound healing, and in this case, a sufficiently sensitive assay should be applied to examine this function. Alternatively, Pak1 may simply lack the ability to form a functional in vivo complex with proteins involved in wound healing.

The molecular mechanism underlying Pak protein organization of the cytoskeleton has been described for Pak1 in the context of axon growth and guidance in *Drosophila*. During the axon growth of the *Drosophila* mushroom body neurons, GTP-loaded forms of Rac1 and Cdc42 activate Pak1, which then activates LIM kinase (LIMK). LIMK inhibits the actin-severing protein cofilin [16]. GTP-loaded Rho1 activates Rho kinase (Rok), which activates the myosin light chain kinase (MLCK) and also converges on LIMK. Currently, it is not clear whether Pak3 works in a similar manner during wound healing; we observed normal wound healing in larvae that were deficient in LIMK1, Rok, or MLCK or in larvae that overexpressed cofilin (Supplementary Fig. 4).

JNK is a strong downstream candidate to mediate Pak3 function as evidenced by the relationship between the GTPases and JNK in larval epidermal wound healing [11]. As stated above, the wound healing phenotypes of $Bsk^{DN}$ and $Pak3$ RNAi larvae are very similar. JNK, however, is normally activated in Pak3-deficient larvae following wounding as determined by analysis of the $msn-LacZ$ reporter (Supplementary Fig. 5). We speculate that, just as the larvae that singly overexpress either $Rac1^{DN}$, $Cdc42^{DN}$, or $Rho1^{DN}$ exhibit wound closure defect but have normal $msn-LacZ$ induction upon wounding likely as a result of redundancy among these proteins [11], Pak3 functions partially redundantly with factors acting downstream to the other Rho-family small GTPases.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.061.

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