cdc37 is essential for JNK pathway activation and wound closure in *Drosophila*

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ABSTRACT **Wound closure in the *Drosophila* larval epidermis mainly involves nonproliferative, endocyling epithelial cells. Consequently, it is largely mediated by cell growth and migration. We discovered that both cell growth and migration in *Drosophila* require the cochaperone-encoding gene *cdc37*. Larvae lacking *cdc37* in the epidermis failed to close wounds, and the cells of the epidermis failed to change cell shape and polarize. Likewise, wound-induced cell growth was significantly reduced, and correlated with a reduction in the size of the cell nucleus. The c-Jun N-terminal kinase (JNK) pathway, which is essential for wound closure, was not typically activated in injured *cdc37* knockdown larvae. In addition, JNK, Hep, Mkk4, and Tak1 protein levels were reduced, consistent with previous reports showing that Cdc37 is important for the stability of various client kinases. Protein levels of the integrin β subunit and its wound-induced protein expression were also reduced, reflecting the disruption of JNK activation, which is crucial for expression of integrin β during wound closure. These results are consistent with a role of Cdc37 in maintaining the stability of the JNK pathway kinases, thus mediating cell growth and migration during *Drosophila* wound healing.**

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INTRODUCTION

The healing of a mammalian skin wound is complex and involves various cellular processes, including blood clotting, inflammation, epithelial cell proliferation and migration, and matrix synthesis and remodeling, which span multiple tissues (Martin, 1997; Gurtner *et al.*, 2008; Shaw and Martin, 2009). In contrast, wound healing in the *Drosophila* larval epidermis is simple: the epidermis consists mainly of a single, nonproliferative cell layer that underlies the protective cuticle. Thus, wound closure involves primarily cuticle regeneration and cell growth and migration, but not proliferation.

Many signaling pathways are required for wound closure in the *Drosophila* epidermis (reviewed in Tsai *et al.*, 2018). Of these, c-Jun N-terminal kinase (JNK), which is required for a broad range of wound healing processes, is the most crucial. Without JNK, cells cannot properly polarize, change shape, orient toward the wound center, or migrate to close the wound (Ramet *et al.*, 2002; Galko and Krasnow, 2004; Kwon *et al.*, 2010; Park *et al.*, 2018). Conversely, some proteins acting upstream of JNK appear to be redundant in a pathway that includes both canonical and noncanonical factors in regard to the embryonic dorsal closure process (Lesch *et al.*, 2010; Rios-Barrera and Riesgo-Escovar, 2013). Specifically, both JNK and the AP-1 transcription factors DJun (Jra) and DFos (Kay) are absolutely required for wound closure, and larvae that are lacking any one of these factors cannot repair open wounds. In contrast, the Jun/stress-activated protein (SAP) 2 kinases Hep and Mkk4 are partially redundant, as are the Jun/SAP3 kinases Slpr and Tak1 (Lesch *et al.*, 2010). Although the involvement of the JNK/SAPK pathway in wound healing is well known both in insects and mammals (Angel *et al.*, 2001; Li *et al.*, 2003), the mechanisms underlying the regulation of this pathway are not well understood.

Protein kinases are often associated with the molecular chaperone Hsp90, which helps these client proteins take on their active conformation (Workman *et al.*, 2007; Taipale *et al.*, 2010). Hsp90 interacts with at least 20 other factors, called cochaperones, which either modulate the activity of Hsp90 or affect the specificity of Hsp90 client proteins (Taipale *et al.*, 2010). Cdc37 is one such cochaperone that is known to maintain the function and stability of client kinases (Pearl, 2005; Caplan *et al.*, 2007; Karnitz and Felts, 2007; Taipale *et al.*, 2010), and many kinases are regulated by

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Abbreviations used: JNK, c-Jun N-terminal kinase; SAP, stress-activated protein.

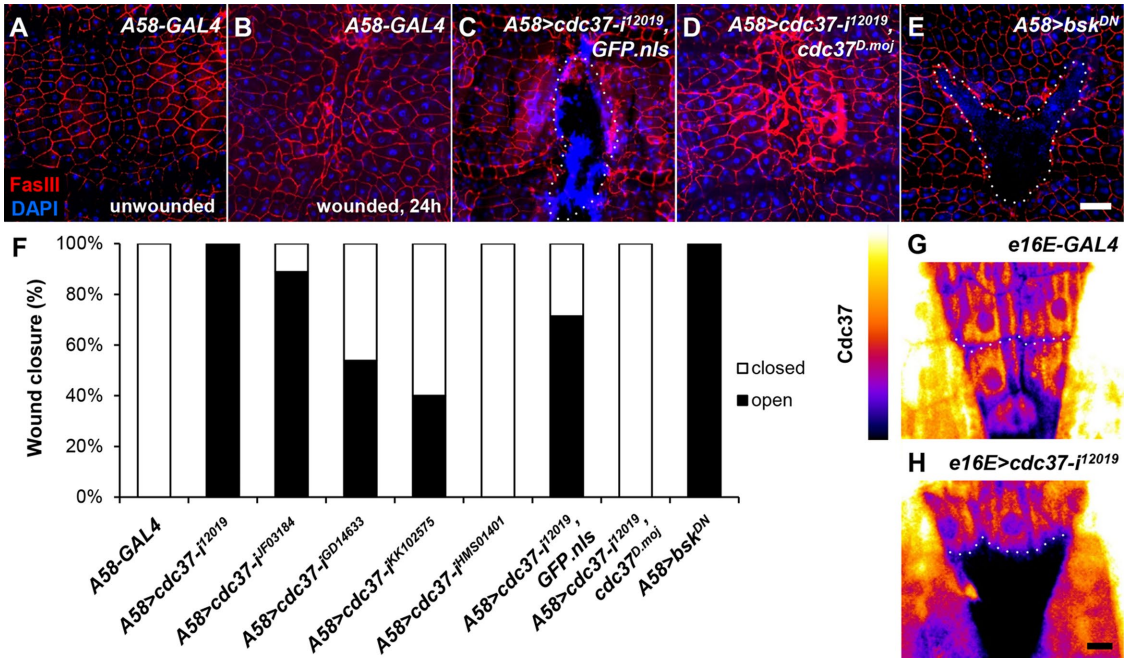

FIGURE 1: *cdc37*-knockdown larvae display defects in wound closure. (A–E) The epidermis was examined in late third instar larvae of the indicated genotypes, either before wounding (A) or 24 h after injury to the dorsal epidermis (B–E). Cell boundaries were stained red with anti-FasIII antibody, and the nuclei were stained blue with DAPI. The dotted line indicates the wound hole. (A, B) *A58-GAL4*-only control. (C) *A58-GAL4*, *UAS-cdc37-RNAi*^{12019}, *UAS-GFP.nls* (*A58>cdc37-i*^{12019}, *GFP.nls*, hereafter). The strong blue staining patterns show hemocytes attached to the wound site. (D) *A58>cdc37-i*^{12019}, *cdc37*$^{D.moj}$. (E) *A58>bsk*^{DN}. (F) Quantification of the wound closure phenotype. For each genotype, 13 or more larvae were examined. Scale bar: 100 μm (A–E). (G, H) RNAi knockdown of *cdc37* was confirmed in the larval epidermis by immunohistochemistry using anti-Cdc37 antibody, as shown in the heat map. *cdc37-RNAi* was driven using *e16E-GAL4* in the posterior half of each segment, leaving the anterior half as an internal control. The dotted line indicates the anterior–posterior compartment boundary. Anterior is up. (G) *e16E*-only control. (H) *e16E>cdc37-i*^{12019}. Scale bar: 25 μm (G, H).

Cdc37, but the relationship between Cdc37 and the JNK signaling pathway is not clear.

Cdc37 was originally identified as a yeast cell-cycle regulator that was later found to interact with Hsp90 and v-Src (reviewed in Karnitz and Felts, 2007). Hsp90 and Cdc37 are both structurally and functionally conserved in metazoans (Caplan *et al.*, 2007). In *Drosophila*, *cdc37* was initially isolated from a mutagenesis screen based on its involvement in eye development (Simon *et al.*, 1991), and was later found to be essential for Sevenless receptor tyrosine kinase signaling (Cutforth and Rubin, 1994). Null mutations in *cdc37* are recessively lethal, indicating it is required for cell viability (Cutforth and Rubin, 1994; Lange *et al.*, 2002). Cdc37 inhibits Hh and Wnt signaling pathways in both flies and mammalian cells (Swarup *et al.*, 2015) and mediates chromosome segregation and cytokinesis by modulating the function of Aurora B kinase (Lange *et al.*, 2002).

In this study, we isolated *cdc37* based on its RNA interference (RNAi) knockdown phenotype in larval epidermal wound closure in *Drosophila*, and found that *cdc37* is required not only for reepithelialization but also for cells to change shape, polarize, and grow during epidermal wound closure, and all of these phenotypes are shared by larvae lacking *JNK*. Molecularly, *cdc37* is required for maintaining the protein levels of JNK pathway components.

RESULTS

cdc37 knockdown disrupts epidermal wound closure

We isolated *cdc37* from an RNAi-based genetic screen of essential genes using an epidermal wound closure assay and the larval epidermis-specific *A58-GAL4* driver. Whereas wild-type third instar larvae closed a large wound generated by the abrasion of ~30 epidermal cells within 12–24 h (Figure 1, A and B, and Supplemental Figure S1, A–E; Galko and Krasnow, 2004; Kwon *et al.*, 2010), *cdc37*-knockdown larvae were unable to close an introduced wound even after 30 h (Figure 1, C and F, and Supplemental Figure S1, F–J). In wild-type larvae, cells located one to three rows distal to the wound margin generally undergo the most dramatic change in cell shape due to cell migration (Supplemental Figure S1, B and C; Kwon *et al.*, 2010), but in the *A58-GAL4 UAS-cdc37 RNAi* (hereafter, *A58>cdc37-i*) larvae, these cells displayed a very strong open-wound phenotype. They often retained a pentagonal or hexagonal cell shape even 24–30 h after injury (Figure 1C and Supplemental Figure S1, G–J), which is similar to unwounded epidermis or wounded epidermis expressing a dominant negative *Drosophila JNK bsk* (*bsk*^{DN}) construct (Figure 1, A and E).

We confirmed the open-wound phenotype using three different approaches. First, we tested five different *cdc37* RNAi strains. Although phenotypic strength varied, four of the five strains with the *A58-GAL4* driver displayed open wounds (Figure 1F and Supplemental Figure S2, A–D). Second, we generated a *UAS* transgenic line for *Drosophila mojavensis cdc37* (*UAS-cdc37*$^{D.moj}$) and overexpressed it in the *UAS-cdc37-i* background, which displayed the strongest phenotype, for a phenotypic rescue without interference from *Drosophila melanogaster cdc37* RNAi (Kondo *et al.*, 2009). This experiment assumed the functional conservation of *cdc37* between the two species. The sequence identity and similarity of the Cdc37 protein between the two species were 80.6% and 87.9%, respectively. Interestingly, *A58>cdc37-i; cdc37*$^{D.moj}$ larvae exhibited

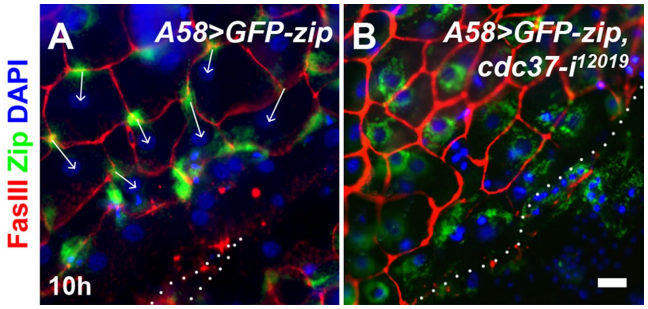

FIGURE 2: *cdc37* is required for cell polarization during wound healing. Localization of the GFP-Zip fusion protein on the rear side of cells was analyzed 10 h after injury. GFP-Zip is shown in green. Cell boundaries were stained red with anti-FasIII antibody, and the cell nuclei were visualized by DAPI staining in blue. The dotted line indicates the wound margin. (A) *A58-GFP-zip*, control. (B) *A58>GFP-zip, cdc37-i*^{12019}. The arrows indicate the directionality of the cells, based on the localization of GFP-Zip and the position of the nucleus. Scale bar: 25 μm.

complete rescue of the open-wound phenotype, whereas the control line, which had the *UAS* copy number balanced by the addition of *UAS-GFP.nls* showed only a marginal rescue, presumably due to weaker *cdc37* knockdown (Figure 1, D and F). Third, we performed immunohistochemistry experiments in knockdown larvae using anti-Cdc37 antibody to examine Cdc37 expression in the epidermis. The fluorescence intensities of Cdc37 immunostaining correlated well with the phenotypic strengths of the individual RNAi lines (Figure 1, G and H, and Supplemental Figure S2, E–H). Thus, we conclude that Cdc37 is essential for epidermal wound closure in *D. melanogaster*, and that this function has not diverged between *D. melanogaster* and *D. mojavensis*.

cdc37 is required for cell polarization during wound healing

During *Drosophila* wound healing, cells located near the wound margin polarize toward the wound center, a process that can be monitored by the localization of a GFP-Zip fusion protein (Franke *et al.*, 2005; Baek *et al.*, 2010; Kwon *et al.*, 2010). In wild-type larvae, GFP-Zip proteins localize primarily to the perinuclear region but translocate to the rear side (opposite to the wound) of the cells upon wounding, as was clearly visible 4–10 h after injury in many of the cells in the first to third rows distal to the wound margin (Figure 2A; Kwon *et al.*, 2010). In *cdc37*-knockdown larvae, GFP-Zip proteins were detected mainly around the perinuclear region and the localization was similar to that of unwounded tissues, as if the cells had not received wound signals (Figure 2B).

cdc37 is required for JNK pathway activation

The strong open-wound phenotype observed in the *cdc37* knockdown larvae prompted us to evaluate whether the JNK pathway was properly activated in these larvae using the JNK pathway reporter *msn-lacZ* (Galko and Krasnow, 2004). In wild-type larvae, wound-induced *msn-lacZ* expression was visible up to five to six cell diameters away from the wound margin (Figure 3A), but in *cdc37* knockdown larvae, *msn-lacZ* expression disappeared almost completely, similar to *bsk*^{DN}-expressing larvae (Figure 3, B and D). We wanted to confirm these results using another JNK pathway reporter *puc-lacZ,* and obtained essentially the same result (Supplemental Figure S3; Galko and Krasnow, 2004). The defects in *msn-lacZ*

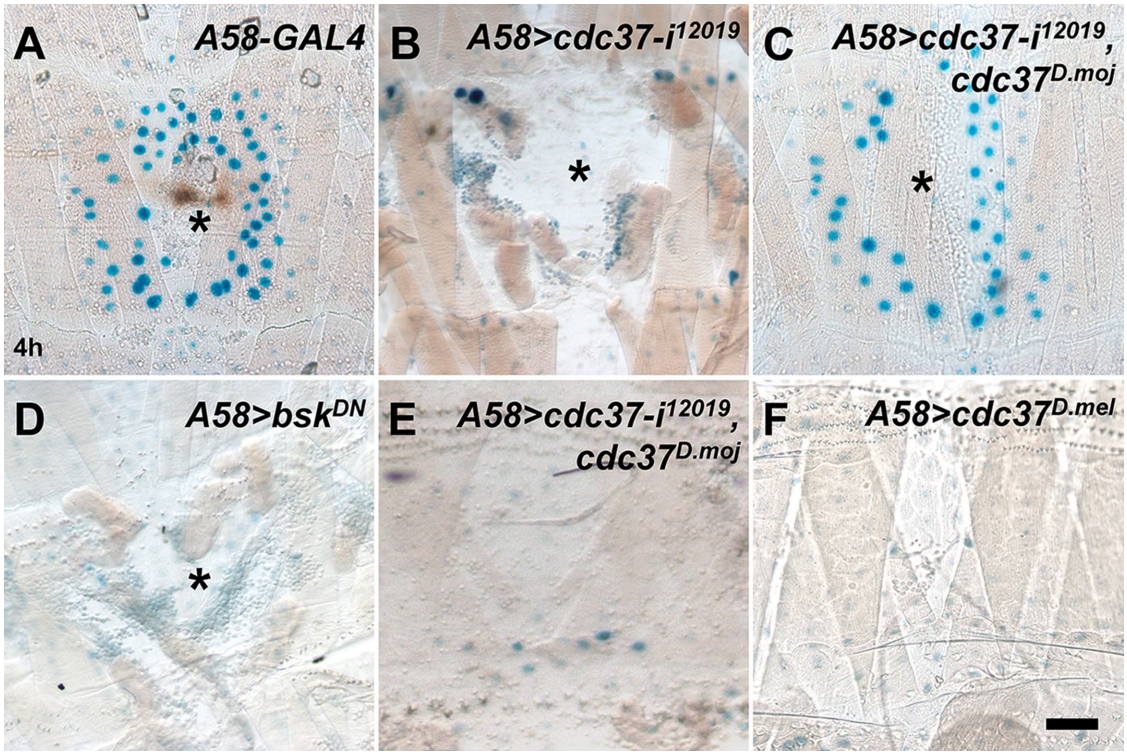

FIGURE 3: Activation of the JNK pathway is disrupted in *cdc37* knockdown larvae. Activation of the JNK pathway in larvae was monitored using the *msn-lacZ* reporter at 4 h after injury (A–D) or without wounding (E, F). β-Galactosidase expression was visualized with X-gal staining in blue. The asterisk indicates the wound hole. (A) *A58*-only control. (B) *A58>cdc37-i*^{12019}. (C) A58>*cdc37-i*^{12019}, *cdc37*$^{D.moj}$. (D) *A58>bsk*^{DN}. (E) *A58>cdc37-i*^{12019}, *cdc37*$^{D.moj}$. (F) *A58>cdc37*$^{D.mel}$. Scale bar: 100 μm.

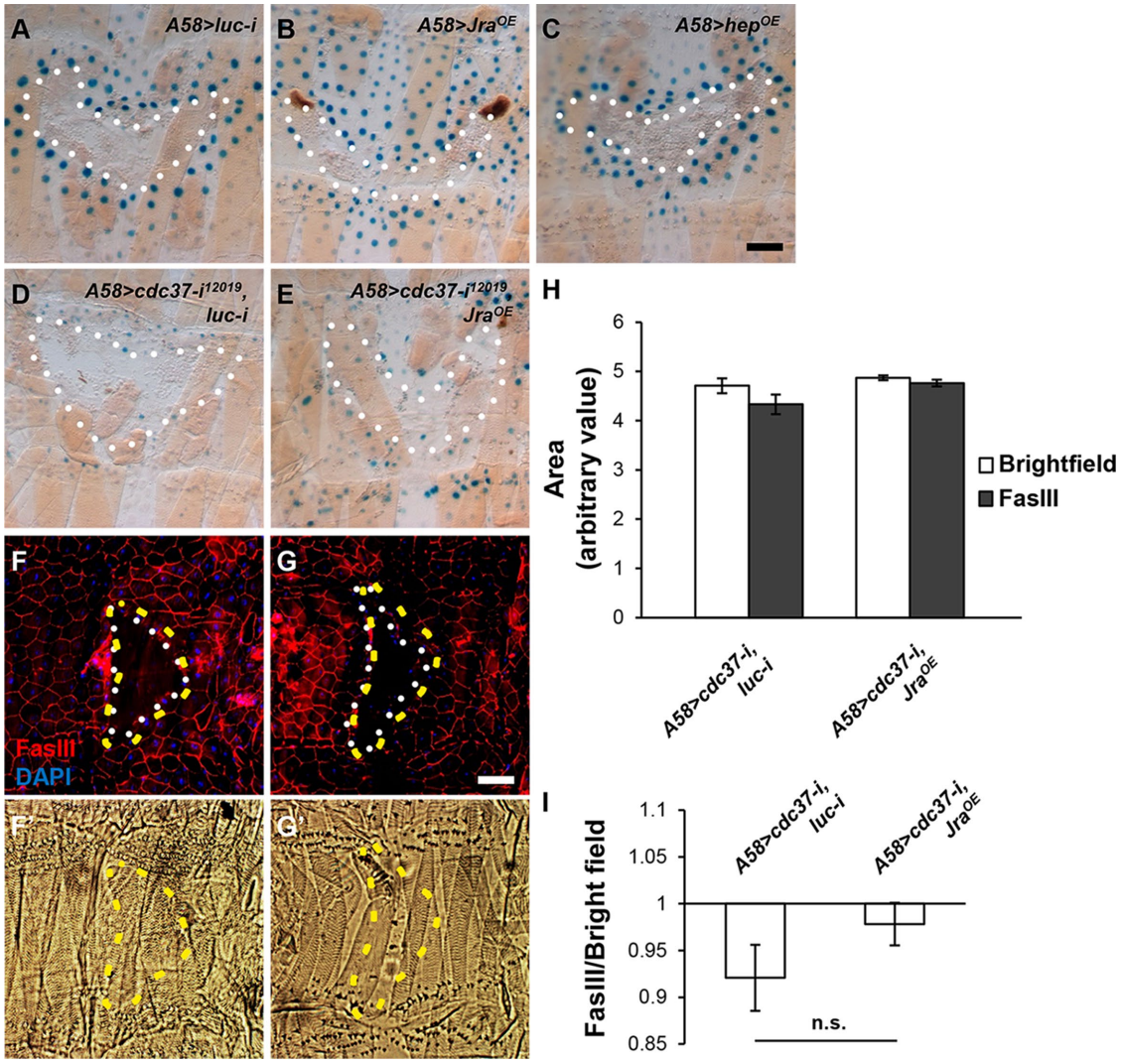

FIGURE 4: Overexpression of *Jra* does not rescue the wound healing defects displayed by *cdc37*-knockdown larvae. (A–E) Examination of JNK pathway activation using the *msn-lacZ* reporter in the larvae of the indicated genotypes, 4 h after injury. (A) *A58>luc-i* (control). (B) *A58>Jra*. (C) *A58>hep*. (D) *A58>cdc37-i, luc-i* (control). (E) *A58>cdc37-i, Jra*. β-Galactosidase expression was visualized with X-gal staining in blue. The white-dotted line indicates the wound margin. (F–G′) Wound closure analysis in the larvae of the indicated genotypes, 30 h after injury. (F, F′) *A58>cdc37-i, luc-i* (control). (G, G′) *A58>cdc37-i, Jra*. (F, G) Cell boundaries were stained red with anti-FasIII antibody, and the nuclei were stained blue with DAPI. The white-dotted line indicates the epidermal wound margin, 30 h after injury, and the yellow-dashed line indicates the original wound margin, left as impressions on the cuticle. (F′, G′) Bright-field micrographs of the cuticle layer. Scale bar: 100 μm. (H) Quantification of the size of the wound hole measured in the bright-field micrograph (original) or in the fluorescent micrograph (recovered). (I) Ratios of the area of the epidermal wound hole (white-dotted) to the area of the cuticle impression (yellow-dotted), which may indicate wound recovery. The difference is not significant. The error bars represent SEM.

induction were rescued to near wild-type levels by the simultaneous overexpression of *cdc37*$^{D.moj}$(Figure 3C). We noticed, however, that *msn-lacZ* reporter induction was no stronger or broader in the wounded, rescued *cdc37* knockdown larvae than in wounded wild-type controls, and that *msn-lacZ* expression was not induced in unwounded larvae (Figure 3E). These results indicate that *cdc37*$^{D.moj}$ expression does not generate a gain-of-function phenotype. To investigate this finding further, we generated a *UAS* transgenic line of *D. melanogaster cdc37* (*UAS-cdc37*$^{D.mel}$) and expressed *cdc37*$^{D.mel}$ in the epidermis. Overexpression of *cdc37*$^{D.mel}$ alone neither induced *msn-lacZ* expression in unwounded larvae nor increased the induction of *msn-lacZ* expression in wounded larvae (Figure 3F; unpublished data). Together, these results indicate that *cdc37* is necessary, but not sufficient, for activation of the JNK pathway during wound healing, suggesting that *cdc37* functions as a permissive factor in JNK signaling.

We next examined whether forced activation of the JNK pathway could rescue the wound healing defects of *cdc37* knockdown larvae. First of all, overexpression of the constitutively active form of *Hep* (*Hep*^{CA}) degenerated epidermal tissues, and thus could not be used for wound analysis, while overexpression of *Hep* or *Jra* did not interfere with normal wound closure, examined at 24 h after injury. Overexpression of *Jra* enhanced *msn-lacZ* induction upon injury, whereas overexpression of *Hep* did not (Figure 4, A–C), indicating

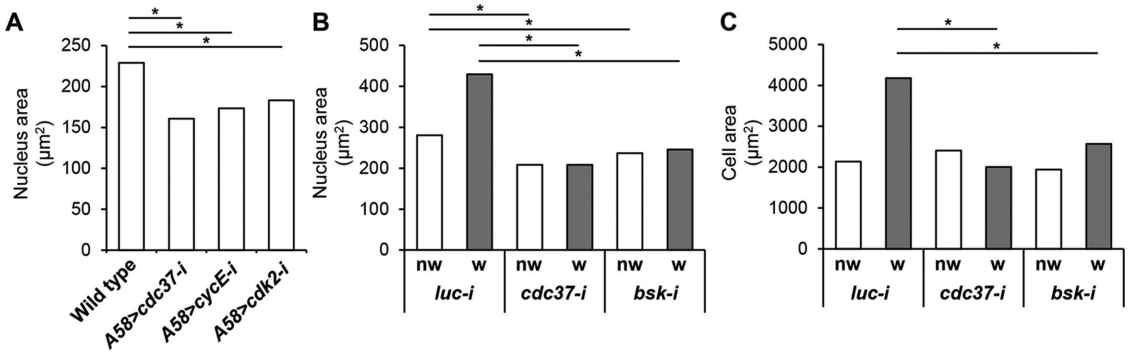

FIGURE 5: *cdc37* knockdown disrupts the wound-induced growth of cells and the nuclei. Nuclear width was assessed based on DAPI fluorescence. Nuclei were ranked by size, and those in the top 15% were chosen for additional analysis. (A) The average width of a nucleus that was within the top 15% in unwounded epidermis. (B) The average width of a nucleus that was within the top 15% selected from nonwounded (nw) and wounded (w) segments of the epidermis 7 h after injury. (C) The average width of a cell that was chosen for the analysis indicated in B. In B and C, *luc-i* was used as a control, and all the RNAi constructs were driven using *A58-GAL4*. At least eight larval epidermal samples were analyzed for each genotype. *, $p < 0.01$; Wilcoxon rank-sum test.

that the latter did not enhance the JNK pathway activation. Thus, we used *Jra* for further analysis. Overexpression of *Jra* in the background of *cdc37* knockdown did not induce *msn-lacZ* upon injury (Figure 4, D and E), indicating that *Jra* still requires *cdc37* for *msn-lacZ* expression. We also examined wound closure in these larvae at 30 h after injury. Wound recovery rates did not differ significantly between *cdc37* knockdown larvae and *Jra*-overexpressing, *cdc37*-knockdown larvae (Figure 4, F–I). These results indicate that *cdc37* is absolutely required for JNK pathway activation.

cdc37 knockdown disrupts the wound-induced growth of cells and nuclei

Wounding induces endoreplication, and consequently cell growth, in the *Drosophila* adult epidermis (Losick *et al.*, 2013; Zielke *et al.*, 2013). We noted that the epidermal cells in *cdc37*-knockdown larvae remained small even 24 h after injury. Thus, we wanted to determine whether *cdc37* is required for endoreplication. In unwounded epidermis, *cdc37* knockdown via *A58-GAL4* significantly reduced nuclear width ($p < 0.01$; similar to that observed following knockdown of *cycE* or *cdk2*, the genes that encode the cyclin and cyclin-dependent kinase required for S phase entry, respectively), consistent with the argument that *cdc37* is required for the developmental endocycle of epidermal cells (Figure 5A). Knockdown of *cycE* or *cdk2* did not noticeably interfere with wound closure or wound-induced *msn-lacZ* expression (Supplemental Figure S4, A–D), indicating that other factors may compensate for retarded cell growth, including cell migration, cell–cell fusion (Losick *et al.*, 2013; Lee *et al.*, 2017), and possibly the thinning of epidermal tissues, which would increase the two-dimensional width.

Next, we attempted to measure the growth of the cells and nuclei in the wounded epidermis. It is generally true that cells proximal to the wound tend to undergo the most prominent changes. However, we also noticed occasionally that cells away from the wound were enlarged greatly, while some cells of the wound margin shrank. These factors interfered with simple size measurement of wound-proximal cells. Therefore, we first measured the width of all cell nuclei in a defined region and then chose those with the largest nuclear areas (top 15%) for further analysis, by marking the cell boundaries for those cells. First, although the average size of the cell nuclei (top 15%) in wounded versus unwounded wild-type larvae increased significantly ($p > 0.01$), this increase was disrupted in both *cdc37*-knockdown larvae and bsk^{DN}-expressing larvae (Figure 5B). Second, the average size of the cells containing the largest nucleus (top 15%) also increased after wounding in wild-type larvae, but not in *cdc37*-knockdown or bsk^{DN}-expressing larvae (Figure 5C). These results suggest that *cdc37* is required for endoreplication and cell growth during wound healing, which also depends on Cdk2, and is consistent with a previous report showing that Cdk2 interacts with Cdc37 (Prince *et al.*, 2005).

JNK and integrin β subunit protein levels are reduced in *cdc37*-knockdown larvae

As a way to examine whether Cdc37 might influence protein stability, we measured global protein levels of the JNK pathway signaling components in the epidermis of *cdc37*-knockdown larvae compared with wild-type larvae by Western blotting. JNK protein levels were reduced in the epidermis of knockdown larvae, even before wound incubation (Figure 6, A and B; unpublished data). Activated JNK, assessed using an anti–phospho-JNK antibody, was also reduced, potentially reflecting a reduction in total protein levels (Figure 6, A and C), as were the levels of Hep, Mkk4, and Tak1, kinases acting upstream of JNK (Figure 6, A and D–F). These results are consistent with previous reports showing that Cdc37 increases the stability of client kinases (Caplan *et al.*, 2007; Karnitz and Felts, 2007), although our results cannot exclude the potential involvement of transcriptional or translational regulation.

Because JNK up-regulates protein levels of the integrin β subunit βPS and integrins are essential for wound closure in *Drosophila* (Lee *et al.*, 2017; Park *et al.*, 2018), we also measured βPS and talin protein levels. βPS levels were greatly reduced in the epidermis of *cdc37*-knockdown larvae compared with wild-type controls, whereas talin levels did not differ between the two groups of larvae (Figure 6, A, G, and H). These results were confirmed by immunostaining the wounded larval epidermis with anti-βPS antibody (Figure 6, I and J).

DISCUSSION

Our results indicate that *cdc37* is an essential factor in the activation of the JNK pathway during *Drosophila* wound healing. Without *cdc37*, the levels of many proteins involved in JNK pathway signaling, including JNK itself, were severely reduced, suggesting that the stability of these proteins is compromised in the absence of *cdc37*.

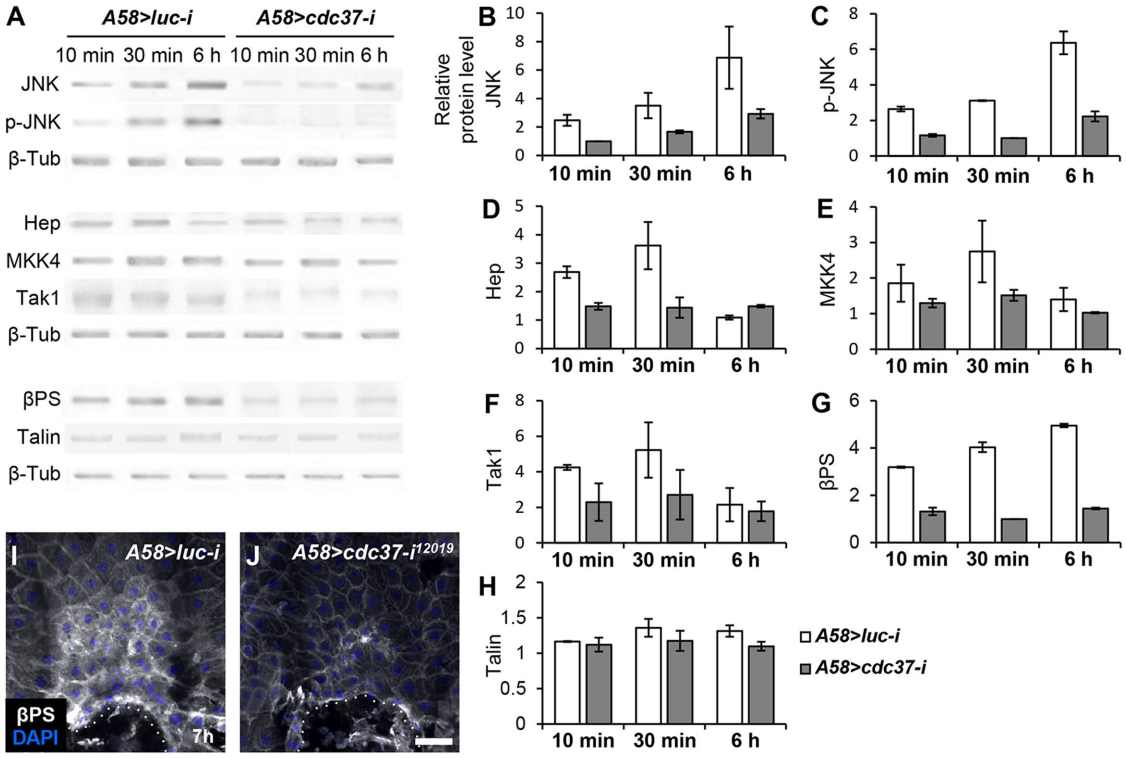

FIGURE 6: The JNK pathway kinase and βPS integrin protein levels are reduced in *cdc37*-knockdown larvae. (A) Western blotting was performed on epidermal samples from control (*luc* knockdown) and *cdc37*-knockdown late third instar larvae at the indicated times after wounding. β-Tub was used as a loading control. (B–H) Quantification of three independent Western blotting results. Error bars represent the SEM. (I, J) Protein levels of βPS integrin were analyzed by immunostaining using an anti-βPS antibody 7 h after wounding. Cell nuclei were stained blue using DAPI. The dotted line indicates the wound margin. Scale bar: 100 μm.

In *Drosophila*, JNK mediates diverse wound healing responses, including gene expression, cell shape change and polarization, reepithelialization, and cell fusion (Ramet *et al.*, 2002; Galko and Krasnow, 2004; Bosch *et al.*, 2005; Mattila *et al.*, 2005; Campos *et al.*, 2010; Kwon *et al.*, 2010; Lesch *et al.*, 2010; Brock *et al.*, 2012; Losick *et al.*, 2016; Lee *et al.*, 2017; Park *et al.*, 2018). The present study suggests that the JNK pathway also mediates wound-induced endoreplication and cell growth. However, prior reports have indicated that JNK suppresses wound-induced endoreplication in adult stages (Losick *et al.*, 2016), which is a discrepancy that requires further investigation.

Larvae lacking *cdc37* displayed disrupted activation of the JNK pathway and displayed phenotypes similar to those of larvae lacking active JNK. Thus, we conclude that most of the *cdc37*-knockdown phenotypes we analyzed were likely caused by the disruption of JNK activation during wound healing. It should be noted, however, that cell nucleus size and JNK protein levels were also reduced in the unwounded epidermis of *cdc37* knockdown larvae, indicating that loss of cdc37 expression also causes developmental defects. This was not unexpected, given that *cdc37* null mutations are cell lethal (Lange *et al.*, 2002). Considering that *A58-GAL4* is only active after early larval stages (Galko and Krasnow, 2004), and that endoreplicating cells are resistant to apoptosis (Mehrotra *et al.*, 2008; Hassel *et al.*, 2014; Zhang *et al.*, 2014), the wound healing defects in *cdc37*-knockdown larvae may have been uncovered luckily due to cell stress caused by wounding in the apoptosis-resistant epidermal cells.

Cdc37 is best known as a cochaperone that confers client kinase specificity to Hsp90 (Karnitz and Felts, 2007; Taipale *et al.*, 2010). The client kinases requiring the Hsp90-Cdc37 complex for activity and stability are diverse and include Cdk2, Cdk4, Src, Aurora B, Raf1, and RIP3 (Stepanova *et al.*, 1996; Lange *et al.*, 2002; Prince *et al.*, 2005; Li *et al.*, 2015; and reviewed in Hunter and Poon, 1997). However, Cdc37 may also function as an independent molecular chaperone alone, similar to Hsp90 (Kimura *et al.*, 1997). Our investigation into the possible involvement of *Hsp90* (also known as *Hsp*83 in *Drosophila*) in wound healing using multiple *Hsp90* RNAi lines did not yield any definitive answer. We also assessed whether *aurora B*, *cdc2*, or *ckII* were involved in wound healing, as these factors reportedly interact with *cdc37* in various contexts (Cutforth and Rubin, 1994; Kimura *et al.*, 1997; Lange *et al.*, 2002; Miyata and Nishida, 2004). Larvae deficient of each of these factors closed wounds normally (Supplemental Figure S4, E–G). Finally, we did not observe any noticeable changes in the protein level or localization of Cdc37 during wound healing (Supplemental Figure S5). Thus, the requirement of *cdc37* for JNK activation is a novel finding. Nonetheless, defining the detailed molecular mechanisms underlying Cdc37 functions requires further investigation.

MATERIALS AND METHODS

Fly stocks

The following stocks were obtained from the Bloomington Stock Center: *Oregon R*, w^{1118}, *msn-lacZ* (msn^{06946}), *puc-lacZ*, *UAS-Jra*, *UAS-hep*, *e16E-GAL4*, *hs-GAL4*, *UAS-cdc37-RNAi* (JF03184, GD14633, HMS01401), *UAS-*bsk^{DN}, *UAS-luciferase RNAi*, *UAS-GFP. nls*, *UAS-Dcr-2*, *UAS-cdk2-RNAi* (HMS00174; Sopko *et al.*, 2014), *UAS-cycE-RNAi* (HMS00060), and *UAS-ckIIα*^{DN} (Lin *et al.*, 2002). The following stocks were obtained from the Vienna *Drosophila* RNAi

Center in Austria: *UAS-cdc37-RNAi* (KK102575), *UAS-aurora B-RNAi* (GD11982, KK112558; Bell *et al.*, 2015), and *UAS-cdc2-RNAi* (106130). *UAS-cdc37-RNAi* (12019R-2) was obtained from the National Institute of Genetics in Japan. *D. mojavensis* was obtained from The National *Drosophila* Species Stock Center.

Generating *cdc37*$^{D.mel}$ and *cdc37*$^{D.moj}$ transgenic flies

Total RNA was isolated from mid- to late-third instar larvae of *D. melanogaster* or *D. mojavensis*. The corresponding cDNAs were synthesized and the *cdc37* open reading frame (ORF) was amplified from the resulting cDNA pools using the following primer sets: 5′-AGCGGCCGCATGGTGGACTACAGCAAG-3′ (forward) and 5′-AGACTCGAGTCAGTCAACGTCCTCGGT-3′ (reverse) for *D. melanogaster* and 5′-AGCGGCCGCATGGTTGACTACAGCAAG-3′ (forward) and 5′-AGACTCGAGTCAGTCGAGGTCATTGGT-3′ (reverse) for *D. mojavensis*. These ORFs were subcloned into a pUAST vector at the *Not*I-*Xho*I sites and injected into *w*^{1118} embryos to produce transgenic flies.

Generating anti-Cdc37 antibody

Total RNA was isolated from third instar larvae and cDNAs were synthesized. A fragment of the *cdc37* gene corresponding to amino acids 29–183 was amplified from the cDNA pool by PCR using the following primer sets: 5′-AAGGATCCTTCCGCTGGCGGCACCAG-3′ (forward) and 5′-AACTCGAGTTATTACGTCTCCTCGCCCACCAA-3′ (reverse) for N-terminal His tagging and 5′-AACCATGGTTTTCCGCTGGCGGCACCAG-3′ (forward) and 5′-AAGCGGCCGCCGTCTCCTCGCCCACCAA-3′ (reverse) for C-terminal His tagging. The resulting fragments were subcloned into the vector pET28a for expression in the *Escherichia coli* (BL21). His-tagged proteins were purified using a Ni-bead column. Polyclonal antibodies against the purified Cdc37 peptide were raised in rabbits by Young In Frontier (Seoul, Korea). In this study, we primarily used antiserum obtained using the C-terminal His-tagged fusion (Supplemental Figure S5, A and B).

Wounding and dissection

Mid- to late-third instar larvae were wounded using a pair of forceps (Fine Science Tools; Cat. No. 11295-00) to pinch the outer integument to abrade ~30 epidermal cells on the dorsal side of segment A2 or A3. The larvae were then placed on cornmeal–agar media for recovery. Epidermal tissues were dissected in phosphate-buffered saline (PBS), as described in Park *et al.* (2018). Samples were fixed in 4% paraformaldehyde for 15 min. For Western blotting and coimmunoprecipitation, five different dorsal segments were wounded to maximize wound healing responses. Unless otherwise specified, at least six larvae were examined for each experiment.

Immunohistochemistry

Fixed samples were washed three times in PBS and incubated with primary antibodies diluted in PBS plus 0.5% Triton X-100 (PBST) supplemented with 1% normal goat serum for 2 h at room temperature. The following primary antibodies were used: mouse anti–Fasciclin III (1:50 dilution; Developmental Studies Hybridoma Bank [DSHB]; Cat. No. 7G10) and rat anti-Cdc37 (1:200 dilution; obtained from M. Therrien at the University of Montreal in Canada; Cutforth and Rubin, 1994). Samples were washed in PBST plus 5% normal goat serum three times for 10 min each and incubated with secondary antibodies diluted in PBST plus 1% normal goat serum overnight at 4°C. The following secondary antibodies were used: Cy3-conjugated goat anti-mouse immunoglobulin G (IgG) (1:100; Jackson ImmunoResearch; Cat. No. 75512), Alexa Fluor 488–conjugated goat anti-mouse IgG (1:200; Molecular Probes; Cat. No. A11001), and Alexa Fluor 546–conjugated goat anti-rat IgG (1:200; Molecular Probes; Cat. No. A11081). After washing in PBST three times for 10 min each, the samples were mounted in VECTASHIELD (Vector Laboratories; Cat. No. H-1000). Cell nuclei were counterstained in a 1:500 dilution of 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes; Cat. No. D1306).

β-Galactosidase staining

Mid- to late-third instar larvae were dissected in PBS and fixed in 2% glutaraldehyde for 15 min at room temperature. The samples were washed three times in PBS and incubated in 10 mM $NaPO_4$, 150 mM NaCl, 1 mM $MgCl_2$, 3.1 mM $K_4[FeII(CN)_6]$, 3.1 mM $K_3[FeIII(CN)_6]$, 0.3% Triton X-100, and 0.2% X-Gal for 30 min at 37°C.

Measurement of the cell and nucleus

Micrographs of DAPI-stained epidermal tissues were analyzed using ImageJ. Background signals were eliminated using the Threshold tool, and the widths of all of the cell nuclei in the trapezoidal area of a dorsal segment (Kwon *et al.*, 2010) were measured. Cells containing the widest nuclei (widest 15%) were selected for further analysis. The cell boundaries of these cells were marked using the Freehand Selection tool and the width of the cell was measured. At least eight animals per genotype were analyzed and a Wilcoxon rank-sum test was used to perform the statistical analysis in Figure 5.

Western blot analysis and coimmunoprecipitation

For Western blotting, epidermal filets were boiled in SDS sample buffer (250 mM Tris-HCl, pH 6.8, 0.5M dithiothreitol, 10% SDS, 0.25% bromophenol blue, and 50% glycerol) at 100°C for 5 min, subjected to 8% or 10% SDS–PAGE, and transferred to nitrocellulose membranes. The membranes were then blocked with 5% skim milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 2 h and probed with primary antibodies at 4°C overnight. The following antibodies were used: rabbit anti-JNK (1:1000 dilution; Santa Cruz Biotechnology; Cat. No. sc-571), rabbit anti-pJNK (1:1000 dilution; Promega; Cat. No. V793A), rat anti-Cdc37 (1:400 dilution; Figure 1; Supplemental Figures S2 and S5, C–F′; Cutforth and Rubin, 1994), rabbit anti-Cdc37 (1:1000 dilution; Supplemental Figure S5, A and B), rabbit anti-Hep (1:1000 dilution; Rallis *et al.*, 2010), guinea pig anti-Mkk4 (1:1000 dilution; Rallis *et al.*, 2010), rabbit anti-Tak1 (1:1000 dilution; Paquette *et al.*, 2010), rabbit anti-Slpr (1:500 dilution; Polaski *et al.*, 2006), mouse anti-βPS (1:1 dilution; DSHB; Cat. No. CF.6G11), mouse anti-talin (1:1 dilution; DSHB; Cat. No. A22A), and goat anti–β-tubulin (1:1000 dilution; Santa Cruz Biotechnology). The membranes were then washed three times with TBST and incubated with horseradish peroxidase–conjugated secondary antibodies (1:1000 dilution; anti-rabbit [Cat. No. sc-2004], anti-rat [Cat. No. sc-2006], anti-mouse [Cat. No. sc-2005], anti-goat [Cat. No. sc-2056], and anti-guinea pig [Cat. No. sc-2438], all purchased from Santa Cruz Biotechnology) in TBST plus 1% skim milk for 1 h. After washing in TBST three times, the membranes were visualized using the WEST-ZOL Plus Western blot detection system (iNtRon; Cat. No. 16024).

For coimmunoprecipitation, epidermal filets were briefly lysed in NP-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40) at 4°C and incubated with protein G–sepharose beads (Sigma Aldrich; Cat. No. P3296) plus antibodies for coupling at 4°C overnight. The beads were then washed with NP-40 buffer and used for Western blot analysis.

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