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Yorkie regulates epidermal wound healing in *Drosophila* larvae independently of cell proliferation and apoptosis

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Abstract

Yorkie (Yki), the transcriptional co-activator of the Hippo signaling pathway, has well-characterized roles in balancing apoptosis and cell division during organ growth control. Yki is also required in diverse tissue regenerative contexts. In most cases this requirement reflects its well-characterized roles in balancing apoptosis and cell division. Whether Yki has repair functions outside of the control of cell proliferation, death, and growth is not clear. Here we show that Yki and Scalloped (Sd) are required for epidermal wound closure in the *Drosophila* larval epidermis. Using a GFP-tagged Yki transgene we show that Yki transiently translocates to some epidermal nuclei upon wounding. Genetic analysis strongly suggests that Yki interacts with the known wound healing pathway, Jun N-terminal kinase (JNK), but not with Platelet Derived Growth Factor/Vascular-Endothelial Growth Factor receptor (Pvr). Yki likely acts downstream of or parallel to JNK signaling and does not appear to regulate either proliferation or apoptosis in the larval epidermis during wound repair. Analysis of actin structures after wounding suggests that Yki and Sd promote wound closure through actin regulation. In sum, we found that Yki regulates an epithelial tissue repair process independently of its previously documented roles in balancing proliferation and apoptosis.

Keywords

Drosophila; Wound healing; Yorkie; JNK; Actin; Tissue repair; Scalloped

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.05.006.

1. Introduction

To cope with inevitable injury, organisms possess efficient wound healing mechanisms that maintain tissue integrity and guard against infection. However, the detailed genetic basis of wound healing is still poorly defined. Epidermal wound healing in *Drosophila* larvae serves as a powerful system to uncover the genes that are required for wound closure (Galko and Krasnow, 2004; Kwon et al., 2010; Lesch et al., 2010; Stevens and Page-McCaw, 2012). In this model, wounds made in the monolayer barrier epidermal sheet close through directed cell migration (Galko and Krasnow, 2004; Lesch et al., 2010). The receptor tyrosine kinase (RTK) Pvr, which shares homology to vertebrate platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) receptors (Cho et al., 2002; Duchek et al., 2001; Heino et al., 2001) and its ligand, Pvf1, are required for larval wound closure (Wu et al., 2009), as are other genes (Stevens and Page-McCaw, 2012; Kwon et al., 2010). Pvr regulates cellular protrusions at the wound edge (Wu et al., 2009) and also controls the directed migration of border cells (Duchek et al., 2001; Prasad and Montell, 2007) and embryonic hemocytes (Wood et al., 2006).

In addition to the Pvr pathway, the JNK pathway is also essential for larval epidermal wound healing (Galko and Krasnow, 2004; Lesch et al., 2010). Loss of function of JNK upstream kinases, Slpr and Hep, or downstream transcriptional factors, D-fos and D-jun, all cause wound closure defects in *Drosophila* (Lesch et al., 2010). Moreover, the JNK pathway regulates an actin regulator, Profilin, at the transcriptional level during healing (Brock et al., 2012). Pvr and JNK act in parallel during epidermal wound healing (Wu et al., 2009). However, whether the Pvr or JNK pathways interact with other signaling pathways during wound healing is still unclear.

Yorkie (Yki) is a transcriptional co-activator that functions as a downstream effector of the Hippo pathway (Halder et al., 2012; Huang et al., 2005; Oh and Irvine, 2010; Pan, 2007; Yu and Guan, 2013). The core of the Hippo pathway is a kinase cascade of the Hippo (MST2 in vertebrates) and Warts kinases (LATS1/2 in vertebrates) that phosphorylate Yki thereby inhibiting its nuclear localization and ability to interact with the TEAD family of transcription factors (Halder and Johnson, 2011; Pan, 2010; Yu and Guan, 2013). During normal development, the Hippo pathway regulates organ size by controlling cell proliferation (Lin and Pearson, 2014) and apoptosis (Huang et al., 2005; Udan et al., 2003). The Hippo pathway also regulates *Drosophila* intestinal and imaginal disc regeneration by promoting proliferation (Grusche et al., 2011; Karpowicz et al., 2010; Ren et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010; Sun and Irvine, 2011) and adult skin regeneration by inhibiting cell-cell fusion and promoting ploidy (Losick et al., 2013). However, the signals that regulate the activity of Yki during normal development and regeneration are not well understood (Staley and Irvine, 2012).

In this study, we found that Yki and its TEAD binding partner Scalloped (Sd) are required for wound closure (WC) in the larval epidermis. We also observed that a GFP-tagged Yki fusion protein translocates to the nucleus in some wound edge epidermal cells upon wounding. Yki's requirement for wound closure is intriguing since the larval epidermis is an endoreduplicated tissue that is likely incapable of cell division. We found that neither

blocking nor ectopically promoting cell division in the epidermis could rescue *UAS-yki^{RNAi}*-induced wound closure. Likewise, we found no role for apoptosis in wound healing, and loss of Yki did not have apparent effects on apoptosis in this context. Thus, unlike other regenerative contexts, Yki does not control the balance of mitosis or apoptosis in the healing larval epidermis, but rather seems to regulate actin polymerization in the migrating wound-edge epidermal cells.

2. Materials and methods

2.1. Drosophila stocks and genetics

Pvr^{c02859} is a hypomorphic allele (Cho et al., 2002; Wu et al., 2009). *Pvr^{c02195}* (Cho et al., 2002) is referred to as *Pvr^{null#1}*. *Pvr^{MI04181}* (Venken et al., 2011), referred to as *Pvr^{null#2}*, is a null allele based on its known molecular lesion and WC phenotype. *Pvfl^{EPI624}*, here referred to as *Pvfl^{null}*, is a null allele (Cho et al., 2002; Wu et al., 2009). *yki^{B5}*, here referred to as *yki^{null}*, is a null allele (Huang et al., 2005). *Hep^{r75}*, here referred to as *Hep^{null}*, is a null allele (Glise et al., 1995).

The GAL4/UAS system was used to drive tissue-specific gene expression of transgenes under UAS control (Brand and Perrimon, 1993). For the embryonic and larval epidermis, *e22c-Gal4* was used (Lawrence et al., 1995); for the larval epidermis, *A58-Gal4* was used (Galko and Krasnow, 2004). For live imaging of larvae, we used *e22c-Gal4, UAS-src-GFP, UAS-DsRed2-Nuc* or *A58-Gal4, UAS-src-GFP, UAS-DsRed2Nuc* (Lesch et al., 2010). *e22c-Gal4, UAS-src-GFP, UAS-DsRed2Nuc; tubP-gal80^{ts}* was used where temporal control of the Gal4/UAS system was needed (McGuire et al., 2003). *UAS-RNAi* lines employed were: *UAS-yki^{RNAi}* (N-terminal+ C-terminal, N+C) (*yk#3*), *UAS-yki^{RNAi}* (N-terminal) (*yk#4*), *UAS-yki^{RNAi}* (C-terminal) (*yk#5*), and *P{Diap1-GFP.HREx8}* (Zhang et al., 2008), which were gifts from Dr. Jin Jiang. *Ajuba^{RNAi#4}* was a gift from Dr. Gregory D. Longmore (Das Thakur et al., 2010). *P{UAS-Mer⁺.myc}* (LaJeunesse et al., 1998). *P{UAS-ex. B}* (Boedigheimer et al., 1997). *Bantam sensor, P{Tub-EGFP.ban}* (Brennecke et al., 2003).

UAS-RNAi lines from Vienna *Drosophila* Research Center (VDRC) were: *GD7185* (#38442/*Ajuba^{RNAi#2}* and #38443/*Ajuba^{RNAi#3}*), *KK109756* (*yki^{RNAi#1}*), *GD11187* (*yki^{RNAi#2}*), *GD1570* (*hpo^{RNAi#1}*), *KK101055* (*wts^{RNAi#1}*), *GD1563* (*wts^{RNAi#3}*), *KK100140* (*mats^{RNAi}*), *GD16019* (*sav^{RNAi#1}*), *KK107562* (*sav^{RNAi#3}*), *KK107857* (*hipk^{RNAi}*), *GD14350* (*ds^{RNAi#1}*), *GD2646* (*ds^{RNAi#2}*), *GD8808* (*α-cat^{RNAi}*), *GD4047* (*I(2)g^{RNAi}*), *GD14463* (*crb^{RNAi#1}*), *GD14463* (*crb^{RNAi#2}*), *KK101128* (*scrib^{RNAi#1}*), *GD11663* (*scrib^{RNAi#2}*), *KK111409* (*kibra^{RNAi}*), *GD12284* (*Drice^{RNAi#1}*), *KK108877* (*sd^{RNAi#2}*), *GD10696* (*Zyxin^{RNAi#2}*), and *GD14749* (*cycE^{RNAi}*).

UAS-RNAi lines from the TRiP Bloomington collection were: *JF02470* (*hpo^{RNAi#2}*), *JF02471* (*wts^{RNAi#2}*), *JF02840* (*sav^{RNAi#2}*), *JF03120* (*ex^{RNAi}*), *JF02841* (*mer^{RNAi}*), *JF03270* (*zyx^{RNAi#1}*), *JF02744* (*hth^{RNAi}*), *JF02856* (*tsh^{RNAi}*), *HMS00563* (*wbp2^{RNAi}*), *GLV21013* (*mad^{RNAi#1}*), *JF01263* (*mad^{RNAi#2}*), *JF01264* (*mad^{RNAi#3}*), *JF02514* (*sd^{RNAi#3}*), *JF02843* (*fj^{RNAi}*), *GL00199* (*aurB^{RNAi#1}*), *JF03107* (*aurB^{RNAi#2}*), *GL00262*

(*cdc2^{RNAi}#1*), HMS00752 (*Diap1^{RNAi}*), HMS02335 (*Ajuba^{RNAi}#1*), JF01355 (*Luciferase^{RNAi}*), and JF03004 (*cdc2^{RNAi}#2*).

UAS-RNAi lines from NIG-Fly (<http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp>) were: 8544R-3 and 8544R-2 (*sd^{RNAi}#1* and #4, respectively), 5680R-1 and 5680R-2 (*JNK^{RNAi}*), 31196R-4 (*14-3-3^{RNAi}*), 7788R-2 (*Drice^{RNAi}#2*), 8624R-1 (*melted^{RNAi}*), 8091R-1 (*Dronc^{RNAi}*), 9553R-3 and 9553R-4 (*profilin^{RNAi}*). Other transgenic lines from Bloomington Stock Center: #11173, *puc-lacZ*. #9674, *w¹¹¹⁸*; *P{UAS-Myc. Z}132*. #64196, *w**; *P{UAS-Ras85D.V12}2*. #44248, *P{lac^W}ex697*. #28819, *w**; *P{UAS-yki. V5.O}attP2*. #44258, *w**; *P{UAS-wts. MYC}3/TM6B, Tb^l*. #44254, *y^l w**; *P{UAS-dMST.FLAG}3/TM2*. #5072, *w**; *P{UAS-p35. H}BH1*. #44253, *y^l w**; *P{lacW}fj^{pl}*. #12093, *y^l w**; *P{lacW}Diap1j5C8/TM3, Sb^l*. #28815, *y^l w**; *P{UAS-yki. GFP}4-12-1* (Oh and Irvine, 2008); #35545, *y^l w**; *P{UAS-Lifeact-Ruby}VIE-19A*.

2.2. Wounding assays

Pinch and puncture wounding of the larvae was carried out according to our detailed protocol (Burra et al., 2013). In cases where early expression of a UAS transgene was lethal (*UAS-Myc* and *UAS-ras^{V12}*), larvae bearing *tub-gal80^s*, the Gal4 driver and toxic UAS transgene were raised for six days at 18 °C to begin development, shifted to 30 °C for two days to reach mid-third-instar, and then allowed to recover at 25 °C following pinch wounding. Pinch wounds were scored as “open” if the initial wound gap remained after 24 h, and as “closed” if a continuous epidermal sheet was observed at the wound site. To calculate the percentage of larvae with open wounds, approximately 30 larvae per genotype were pinched and scored for open wounds under a stereo microscope (Leica MZ16FA).

2.3. Whole mount immunostaining and cell labeling

The third instar larval epidermis was dissected and processed as detailed previously (Burra et al., 2013). To highlight wound morphology, a mouse monoclonal antibody against Fasciclin III was used (1:50; Developmental Studies Hybridoma Bank). Rat anti-Sd was a gift from Kirsten Guss (Guss et al., 2013) and was used at 1:20. Mouse anti-GFP monoclonal antibody (Life Technologies) was used at 1: 500. Mouse anti-β-galactosidase monoclonal antibody (Promega #Z3781) was used at 1:1000. Anti-activated Caspase 3 (Cell Signaling) was used at 1:150. Mouse anti-*Drosophila* Profilin (Developmental Studies Hybridoma Bank) was used undiluted. Rabbit anti-DsRed (Clontech) was used at 1:1000. Rabbit anti-Lethal (2) giant larvae (l(2)gl) was used at 1:50 (Santa Cruz, sc-98260). Alexa 546 conjugated Phalloidin (Invitrogen) was used at 1:200. A TUNEL staining kit (Roche Diagnostics) was also used to detect DNA double-stranded breaks.

2.4. Imaging and analysis

An Olympus FV1000 Confocal microscope and Fluoview software were used to obtain images of the dissected epidermal whole mounts. Leica MZ16FA stereomicroscope with Planapo 1.6× objective and appropriate filters was used for live imaging of epidermal wounds. ImageJ software was used for image processing. For statistics, Student's T-test was used.

2.5. Quantitation of Yki nuclear translocation

Epidermal cells in unwounded or in wounded larvae at different times post-wounding were counted as containing nuclear Yki if the nuclear Yki-GFP signal was obviously greater than the cytoplasmic signal in the same cell. n =4 for each genotype.

2.6. Quantitation of wound-edge F-actin intensity

Wound-edge F-actin was labeled with *UAS-Lifeact-Ruby*. The fluorescent signal around wounds of different genotypes six hours following wounding was measured using the following protocol: In ImageJ, the wound edge was first selected based on the F-actin signals using the “wand”. Next the wound edge area was designated from the wound edge using the “make band” tool (width =6 μm) and the average fluorescent intensity was measured within the selection. The wound edge intensities were first normalized to the adjacent unwounded segments. The normalized intensities of the *UAS-yki^{RNAi#2}* and *UAS-sd^{RNAi#3}* groups were then normalized to the *UAS-Luc^{RNAi}* control. n = 7 for each genotype.

2.7. Quantitation of F-actin mean intensity and Profilin protein levels in the unwounded larval epidermis

For F-actin, whole-mount epidermis expressing different transgenes was stained with Alexa546-conjugated phalloidin. Mean phalloidin signal of one middle segment (usually abdominal segment 2 or 3) of larvae expressing *UAS-yki^{RNAi}*, *UAS-sd^{RNAi}* and *UAS-Profilin^{RNAi}* were normalized to *UAS-Luc^{RNAi}* control. n = 4 for each genotype. For measuring the mean Profilin protein level, whole-mount epidermis expressing different transgenes was stained with anti-*Drosophila* Profilin. Mean Profilin levels were measured as for F-actin mean intensity above. n = 4 for each genotype.

3. Results

3.1. Yorkie and Scalloped are required for epidermal wound closure

We have been performing a tissue-specific in vivo RNAi screen for genes required in the larval epidermis for WC (Lesch et al., 2010). While surveying the function of *Drosophila* transcriptional regulators in this context we used an established pinch wounding assay (Galko and Krasnow, 2004; Wu et al., 2009) to test whether expression of RNAi transgenes targeting *yki* in the larval epidermis affects WC. Wounds in control larvae were invariably closed 24 h after wounding (Fig. 1A,F). In contrast, wounds made in *Pvfl1^{null}* mutant larvae remained open after 24 h (Fig. 1B,F and Wu et al., 2009). Similarly, expression of multiple *UAS-yki^{RNAi}* transgenes resulted in fully or partially penetrant WC defects (Fig. 1C,F). Open wounds were observed with two different *UAS-yki^{RNAi}* transgenes that target non-overlapping regions of the *yki* gene (Fig. 1F and Fig. S1A). Overexpression of Yki in the larval epidermis via *UAS-Yki* completely rescued the *UAS-yki^{RNAi}*-induced WC defects, indicating that these effects are due to loss of Yki function in the larval epidermis and not to off-target effects (Fig. 1D,F). Expression of *UAS-Yki^{RNAi}* or Yki overexpression did not affect normal epidermal architecture. Other UAS transgenes co-expressed with *UAS-Yki^{RNAi}* (see Fig. 4E,H) did not rescue, indicating the rescue observed here is not solely a function of titration of the Gal4/UAS system by the presence of multiple UAS transgenes.

Next, we tested whether other Hippo pathway components regulate larval epidermal wound closure. Yki is commonly negatively regulated by the upstream Hippo kinase cascade (Huang et al., 2005). If Hippo/Warts regulates Yki during WC, then overexpression of these genes should phenocopy the WC defect of *UAS-yki^{RNAi}*. Overexpression of Hippo (dMst) via both the *e22c-Gal4* and *A58-Gal4* epidermal drivers resulted in lethality (Fig. 1G,H). By contrast, overexpression of Warts via the *A58-Gal4* driver was viable and caused 40% open wounds (Fig. 1H). The other upstream pathway component whose epidermal overexpression resulted in open wounds was Expanded (Fig. 1G,H). Expanded can inhibit Yki through direct binding to Yki as well as through activation of Warts (Badouel et al., 2009). Expression of *UAS-RNAi* transgenes targeting several known Yki downstream genes, such as *Diap1* and *Cyclin E*, did not affect WC (Fig. 1I). We also expressed *UAS-RNAi* transgenes targeting the factors that commonly act upstream of Yki to test if they are also required for WC. Since overexpression of Yki did not block WC (Fig. 1F), and many of the upstream components of the pathway negatively regulate Yki, expression of *UAS-RNAi* transgenes targeting these factors was not necessarily expected to affect WC. Indeed, expression of *UAS-RNAi* transgenes targeting *warts*, *hippo*, *mats*, *salvador*, *merlin*, *expanded*, and other negative regulators of Yki did not affect WC (Fig. 1K). Taken together, our results suggest that Yki is required in the larval epidermis for WC and is likely at least partially regulated by the Warts kinase in the larval epidermis.

Yki cannot bind DNA by itself (Oh and Irvine, 2010). Instead, Yki complexes with a variety of different transcription factors to regulate gene expression (Goulev et al., 2008; Oh and Irvine, 2011; Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008, 2011; Zhao et al., 2008). We surveyed many of these factors for their potential roles in WC, and found only one binding partner, Scalloped (Sd), whose knockdown resulted in WC defects. Epidermal expression of two non-overlapping *UAS-sd^{RNAi}* transgenes (Fig. S1B, *UAS-sd^{RNAi#3}* and *UAS-sd^{RNAi#4}*) resulted in WC defects (Fig. 1J). Expression of either of these *UAS-sd^{RNAi}* transgenes that blocked WC showed a significant reduction of Sd protein (compare Fig. S1E,F to Fig. S1C). A third *UAS-sd^{RNAi}* transgene that failed to block WC (*UAS-sd^{RNAi#1}*, Fig. 1J) showed little or no knockdown of Sd (Fig. S1D). These results suggest that Yki may preferentially partner with Sd during larval WC.

3.2. Yorkie transiently translocates to the nucleus in some wound-edge epidermal cells

Yki translocates to the nucleus upon activation (Dong et al., 2007; Zhao et al., 2007). To examine the localization of Yki following wounding, we expressed a GFP-tagged version of Yki, *UAS-yki-GFP*, in the larval epidermis. Overexpression of *UAS-yki-GFP* did not cause a WC defect (Fig. S2B,D). The transgene was functional as it completely rescued the *UAS-Yki^{RNAi}*-induced open wound phenotype (Fig. S2C,D). *UAS-Yki-GFP* also rescued the WC defect of *UAS-wts* (Fig. S2D), suggesting that the Wts WC defect is related to Yki function, as suggested by previous studies (Dong et al., 2007; Oh and Irvine, 2008; Zhao et al., 2007). Next, we examined the cellular localization of Yki-GFP in the larval epidermis. In unwounded larvae, Yki-GFP was predominately cytoplasmic (Fig. 2A). Ten minutes after wounding, Yki-GFP signal in the first few rows around the wound showed an overall reduction (brackets in Fig. 2B,B'). However, certain cells around the wound edge showed predominant nuclear localization (Fig. 2B,B',E). The number of cells that exhibited nuclear

Yki-GFP signal declined quickly thereafter (Fig. 2C–D',E). Taken together, these results indicate that at least some wound-edge cells transiently translocate Yki-GFP to the nucleus upon wounding.

3.3. Yorkie interacts genetically with the Jun N-terminal Kinase (JNK) pathway during wound closure

Both the JNK and Pvr pathways are essential for *Drosophila* larval WC (Galko and Krasnow, 2004; Lesch et al., 2010; Wu et al., 2009). We asked if *Yki* interacts genetically with either of these pathways during WC. For genetic interactions with *Pvr*, we used a *Pvr^{null}* allele and a *yki^{null}* allele that did not show a WC defect as heterozygotes (Fig. S3A). All of these null alleles are lethal when homozygous. Combining heterozygosity for the *Pvr^{null}* alleles with the *Yki^{null}* allele result in normal WC (Fig. S3A) as might be expected if the pathways were independent. Likewise, overexpression of Yki did not rescue the *Pvr^{hypo/hypo}* WC defect (Fig. S3B). These data indicate that the Pvr pathway and Yki do not genetically interact in the context of larval epidermal WC.

Next, we analyzed genetic interactions between *Yki* and the JNK pathway. We used a null allele of the JNK upstream kinase, *hemipterous* (*hep^{null}*) (Glise et al., 1995), which yields only 2.8% open wounds when heterozygous (Fig. 3A). Combining heterozygosity for the *hep^{null}* and *yki^{null}* alleles enhanced wound closure defects by almost three fold (9.2%), hinting at a bona fide interaction between *hep* and *yki* (Fig. 3A). We then examined *hep^{null}* hemizygous males, which yielded 24.4% open wounds. When combined with *yki^{null}*, the occurrence of open wounds was dramatically increased (to 62.7%, Fig. 3B). The percentage of open wounds in *hep^{null}/Y; yki^{null}/+* animals was greater than that expected from the sum of open wounds from *yki^{null}* and *hep^{null}/Y*, strongly suggesting synergy between *Yki* and *hep*.

Given the observed genetic interaction, Yki could act either upstream or downstream of the JNK pathway during WC. To ask if *Yki* acts upstream of JNK during wound closure we used *puc-lacZ*, an effective reporter of JNK activity during WC (Brock et al., 2012; Galko and Krasnow, 2004; Rämetsch et al., 2002). In control larvae, *puc-lacZ* is strongly induced in the cells adjacent to the wound six hours after wounding (Fig. 3C). Expression of *UAS-yki^{RNAi}* (Fig. 3D and Fig. S3C,D) or knockdown of *sd* (Fig. 3E and Fig. S3E) failed to alter the expression of *puc-lacZ* at the wound edge. The converse analysis (examining activation of a potential Yki reporter upon JNK knockdown) was not possible given that multiple such reporters (*fj-lacZ*, *exlacZ*, *Diap1-lacZ*, *Diap1-GFP* and *Bantam* sensor) were not activated in a wound-specific manner (Fig. S4). Our results suggest that Yki and Sd are not required for wound-induced JNK activation and thus are not likely to act upstream of JNK activation during WC.

3.4. Yorkie does not regulate wound closure by balancing cell proliferation and apoptosis

Next, we investigated the cellular mechanism by which Yki regulates wound closure in the *Drosophila* epidermis. In the context of organ growth control Yki regulates the balance between cell proliferation and survival (Huang et al., 2005). The cells in the larval epidermis do not appear capable of proliferation as they are endoreduplicated (Wang et al., 2015) and

there is no phosphohistone 3 staining after wounding (Lesch et al., 2010; Wang et al., 2015). However, to completely rule out the potential contribution of proliferation or programmed cell death to WC, we first sought to ectopically “force” the cell cycle in the epidermis by expressing *UAS-Myc* (Johnston et al., 1999) or an activated version of Ras, *UAS-Ras^{VI2}* (Karim and Rubin, 1998). Expression of these transgenes did not adversely affect WC (Fig. 4A,C,E). Next, we tested if *UAS-Myc* or *UAS-Ras^{VI2}* could rescue the WC defect caused by expression of *UAS-yki^{RNAi}*. Neither cell-cycle activating transgene was able to rescue *UAS-yki^{RNAi}*-induced WC defects (Fig. 4B,D,E).

We then decided to block the cell cycle, independently or together with *UAS-yki^{RNAi}*, and test for effects on WC. We previously identified *UAS-RNAi* transgenes targeting *aurB* (*aurora B*) and *cdc2* (*cyclin dependent kinase-2*) that inhibit the G2 > M cell cycle transition and block potential mitotic progression in the larval epidermis without affecting endoreduplication or normal epithelial architecture (Wang et al., 2015). We expressed these transgenes in the larval epidermis and assessed WC following pinch wounding. Neither transgene perturbed epidermal WC (Fig. 4F–H). Moreover, combining these RNAi transgenes with *UAS-yki^{RNAi}* expression in the epidermis did not alter the *yki^{RNAi}*-induced WC defects (Fig. 4H). Taken together, these results suggest that regulation of mitotic progression is not involved in larval epidermal WC, either by Yki or other factors.

Does Yki promote WC by blocking epidermal apoptosis? Interestingly, we saw a differential induction of two common markers of apoptotic activity following wounding. TUNEL staining was observed immediately post-wounding (Fig. 5A) in leading edge cells and scattered cells in the wound gap. This staining persisted throughout (Fig. 5B) and past WC (Fig. 5C). We suspected based on its immediate appearance (Fig. 5A) that the observed TUNEL reactivity was more likely to be due to DNA fragmentation related to physical cell damage than to rapid activation of apoptosis. Consistent with this idea, the TUNEL reactivity was still observed at an early time point upon expression of *UAS-p35* or *UAS-Dronc^{RNAi}* (Fig. 5G,H), both potent inhibitors of apoptosis that efficiently block UV-induced apoptosis (Fig S5B–D). Activated Caspase-3 staining, a direct readout of apoptotic caspase activation by contrast, was never observed following wounding (Fig. 5D–F). These results suggest that epidermal wounding does induce DNA double strand breaks but does not activate the actual machinery of apoptosis.

Expression of *UAS-dronc^{RNAi}* alone, *UAS-p35* alone, or combination of either transgene with *UAS-yki^{RNAi}* did not block wound-induced TUNEL reactivity (Fig. 5G–I) or affect the absence of caspase activation (Fig. 5J–L). Expression of *UAS-Dronc^{RNAi}* or *UAS-p35* alone also did not affect WC (Fig. 5M,N,P) or alter the *UAS-yki^{RNAi}* mediated WC defect (Fig. 5O,P). These results, in combination with the proliferation results above, suggest that Yki mediates cellular functions other than regulating cell proliferation and apoptosis during WC.

3.5. Yorkie is required for formation of an effective actin cable during wound closure

If Yki does not regulate mitosis or apoptosis, what is its function during WC? We previously showed that an actin-based cable is formed at the wound-edge (Wu et al., 2009; Brock et al., 2012). We therefore examined F-actin intensity using a *UAS-lifeact-Ruby* transgene co-expressed in the larval epidermis with *UAS-Luc^{RNAi}* (control) or *UAS-yki^{RNAi}* (Fig. 6A). In

control larvae, actin formed a characteristic discontinuous cable around the wound six hours post wounding (Fig. 6B). The *UAS-lifect-Ruby* label in the epidermis was reduced at the wound edge when co-expressed with *UAS-yki^{RNAi}*, (Fig. 6C,E). A similar reduction in actin cable intensity was observed upon coexpression of *UAS-sd^{RNAi}* with *UAS-lifect-Ruby* (Fig. 6D,E).

Drosophila profilin, encoded by the *chickadee (chic)* gene, is required to form the actin-based cable around the wound-edge (Brock et al., 2012). Therefore, we tested if Yki and/or Sd regulate Profilin levels. Expression of *UAS-yki^{RNAi}* or *UAS-sd^{RNAi}* in the epidermis did not affect Profilin levels (Fig. S6A'-C',E), whereas *UAS-profilin^{RNAi}* did, even in unwounded larvae (Fig. S6A',D',E). Next, we tested if Yki regulates the overall F-actin level in the unwounded larvae using an alternative F-actin marker, phalloidin. *UAS-yki^{RNAi}* and *UAS-sd^{RNAi}*-expressing larvae showed similar F-actin distributions and F-actin mean intensity as the *UAS-Luc^{RNAi}* control (Fig. S6A''-C'',F), suggesting that Yki and Sd do not regulate F-actin levels in the absence of wounding. In sum, these results suggest that Yki and Sd are required for formation of a fully functional actin cable at the wound edge, and likely regulate WC primarily through control of cell migration.

4. Discussion

4.1. Proliferation, apoptosis, and Yorkie/YAP function in regenerative contexts and larval wound healing

Xenopus YAP is required for limb bud regeneration where it regulates proliferation and apoptosis in the regenerative blastema (Hayashi et al., 2014). Yki also regulates cell proliferation in *Drosophila* imaginal discs (Grusche et al., 2011; Sun and Irvine, 2011) although assessing regulation of apoptosis in this model is challenging as apoptosis is the trigger for genetically-induced regeneration. In the *Drosophila* gut Yki regulates cell proliferation following damage-induced regeneration (Karpowicz et al., 2010; Ren et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010). A similar role has been found in the mouse heart (Heallen et al., 2013; Xin et al., 2013) and in the Zebrafish blastema during fin regeneration (Mateus et al., 2015). In the skin, YAP is required for basal stem cell proliferation in the mouse epidermis and thus for epithelial maintenance (Lee et al., 2014; Schlegelmilch et al., 2011).

Our results indicate that cell proliferation and apoptosis do not play important roles during *Drosophila* larval wound healing, either by Yki or other factors. Consistent with this, well-established Yki/Sd reporters (*Diap1-LacZ* and *ex-LacZ*) that are typically activated upon suppression of apoptosis or cell-cycle activation during organ growth control are not activated or repressed upon wounding. Our results suggest that there is substantial tissue specificity to Yki functions during repair processes. They further suggest that there may be distinct target genes that are activated and/or repressed in non-proliferative tissues during repair. In adult *Drosophila*, Yorkie regulates wound-induced polyploidization but is not directly required for wound closure (Losick et al., 2013) again suggesting stage-specific differences in action even within lineage-related tissues. Taken together, our results suggest that Yki/Sd signaling must regulate some other aspect of epidermal cell behavior during epithelial wound healing.

4.2. Yki/YAP regulates actin polymerization

Our results show that Yki likely functions during WC by regulating actin polymerization. The actin cytoskeleton has been linked to Hippo signaling (Matsui and Lai, 2013). For example, F-actin regulates Hippo pathway and cell proliferation in the *Drosophila* wing imaginal disc (Fernández et al., 2011; Sansores-Garcia et al., 2011). In mammalian cell culture, YAP/TAZ activity is also regulated by tension of the actin cytoskeleton (Dupont et al., 2011). These effects may be bidirectional in some cases; the Hippo pathway also regulates the actin cytoskeleton in *Drosophila* imaginal discs (Fernández et al., 2011; Lin et al., 2014). Other studies suggest that overexpression of Yorkie can directly influence actin levels at wing hair initiation sites (Fang and Adler, 2010). In cultured mammalian hepatocytes, YAP regulates the intensity of the peripheral actin cytoskeleton (Bai et al., 2016). These studies suggest that a role for Yki in regulating actin during damage-induced cell motility is certainly plausible.

We found that Yki and Sd are required for larval WC and for efficient actin polymerization at the wound edge. During border cell migration, a developmentally programmed process, Yki gain-of-function led to enhanced migration (Lucas et al., 2013). In this context, the effects of Yki or Sd loss-of-function are not yet clear (Lin et al., 2014; Lucas et al., 2013). However, both of these studies argue that the upstream Hippo kinases may regulate actin independently of Yki. In the larval epidermis, overexpression of Wts caused a weaker WC defect than Yki loss-of-function, suggesting that Yki may have independent effects on actin depending on the cell type examined.

How does Yki/YAP regulate the actin cytoskeleton? In a mouse heart regeneration study, loss of *salvador (sav)* leads to transcriptional activation of several actin-related genes (Morikawa et al., 2015), suggesting that Yki may regulate actin regulators at the transcriptional level. Indeed, activation of migration-related genes by YAP/TEAD factors has also been observed in metastatic contexts (Liu et al., 2016). If this mechanism is conserved during larval WC, the specific Yki/Sd target genes that might be related to wound-induced migration have yet to be elucidated. These may include Yki/Sd targets that are induced in other contexts or genes whose regulation are important for larval WC (Brock et al., 2012). In breast cancer cells, lysophosphatidic acid (LPA)/GPCR/YAP signaling regulates cell migration (Yu et al., 2012). However, the identity of a putative LPA-like receptor in *Drosophila* is not entirely clear. The detailed mechanism(s) of how Yki regulates actin dynamics during larval WC will be an interesting topic for further investigation.

4.3. Relationship of Yki translocation to the requirement of Yki for wound closure

Yki-GFP shows predominant nuclear localization in some wounded edge epidermal cells within ten minutes of wounding. Other studies have shown that Yap/Yki nuclear translocation can be regulated through physical and mechanical cues (Aragona et al., 2013; Dupont et al., 2011; Rauskolb et al., 2014). The Yki translocation we observed here may be due to the mechanical force of wounding or to alterations in epidermal tension following tissue damage, as the predominant rapid nuclear localization decreases quickly over the next hour. The functional consequence of this rapid translocation, in terms of Yki transcriptional regulation or other functions related to WC is not yet clear, especially given the rapid

kinetics of translocation versus the long timescale of wound closure. One possibility here is that Yki may have non-transcriptional functions during WC as previously reported during cytokinesis (Bui et al., 2016).

4.4. The Yki/Sd signaling axis in larval wound healing

Our genetic interaction experiments suggest that Yki interacts strongly with the JNK pathway. Previous work has linked the JNK pathway to the Hippo pathway. In the wing imaginal disc, JNK phosphorylates Ajuba/LIM1D, which results in Warts inhibition, Yki activation, and cell proliferation (Sun and Irvine, 2013; Codelia et al., 2013). Two observations suggest that Ajuba may not be the link between JNK and Yki during WC. First, there is no cell proliferation in the larval epidermis. Second, epidermal expression of a phenotypic *UAS-Ajuba^{RNAi}* transgene (Das Thakur et al., 2010; Rauskolb et al., 2014) does not phenocopy expression of *UAS-Yki^{RNAi}*.

Additional connections between JNK activation and Hippo signaling have been found during gut regeneration. In particular, Misshapen (*msn*), a MAP4K sterol family kinase acting upstream of JNK, phosphorylates and activates Warts leading to Yki inhibition in the intestinal stem cells (Li et al., 2014). In larval WC, expression of *UAS-msn^{RNAi}* leads to a peculiar and specific phenotype of enhancing wound-induced epithelial cell-cell fusion (Lesch et al., 2010; Wang et al., 2015). However, expression of *UAS-Yki^{RNAi}* or *UAS-Yki* did not result in this epidermal cell fusion phenotype, suggesting that *Msn > Wts* mediated Yki regulation may not occur in the larval epidermis.

Wound-induced JNK activation was not blocked by *UAS-RNAi* targeting Yki or Sd. This makes it similarly unlikely that Yki acts upstream of JNK (Ma et al., 2015) during larval WC. However, the JNK downstream transcriptional factors, Activation Protein-1 family genes (AP-1), D-Jun and D-Fos, are required for larval WC (Lesch et al., 2010). Jun physically interacts with TEAD4 in colon cancer cells (Liu et al., 2016) and could potentially interact directly with Yki to regulate transcription as observed in cancer cells (Verfaillie et al., 2015; Zanconato et al., 2015). Some of the transcriptional targets that shared AP-1 and TEAD binding sites include actin regulators (Liu et al., 2016). The *Drosophila* ortholog of at least one of these target genes, DOCK, is a known larval WC gene (Lesch et al., 2010). Additionally, JNK may directly phosphorylate and activate Yki, as mammalian JNK has been shown to phosphorylate YAP to regulate apoptosis in keratinocytes and a squamous cell carcinoma (Tomlinson et al., 2010). Finally, JNK inhibits Yki activation and wound-induced polyploidization in the *Drosophila* adult epidermis (Losick et al., 2016). Since ploidization is not directly related to cell division, this observation, together with the other instances of transcriptional co-regulation, provide potentially fertile leads for future work as to how Yki and JNK cooperate to effect wound closure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

β-Gal	β-Galactosidase
<i>crb</i>	<i>crumbs</i>
D-fos	<i>Drosophila fos</i>
D-Jun	<i>Drosophila Jun</i>
<i>ds</i>	<i>dachsous</i>
<i>ex</i>	<i>expanded</i>
<i>ff</i>	<i>four-jointed</i>
<i>hep</i>	<i>hemipterous</i>
JNK	c-Jun N-terminal kinase
<i>l(2)gl</i>	<i>lethal (2) giant larvae</i>
<i>Luc</i>	<i>luciferase</i>
MAP4k	mitogen-activated protein 4 kinase
<i>puc</i>	<i>puckered</i>
Pvr	Platelet Derived Growth Factor/Vascular-Endothelial Growth Factor receptor
PDGF	platelet-derived growth factor
RTK	receptor tyrosine kinase
<i>sd</i>	<i>scalloped</i>
<i>sav</i>	<i>salvador</i>
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
VEGF	vascular endothelial growth factor
WC	wound closure
<i>yki</i>	<i>yorkie</i>

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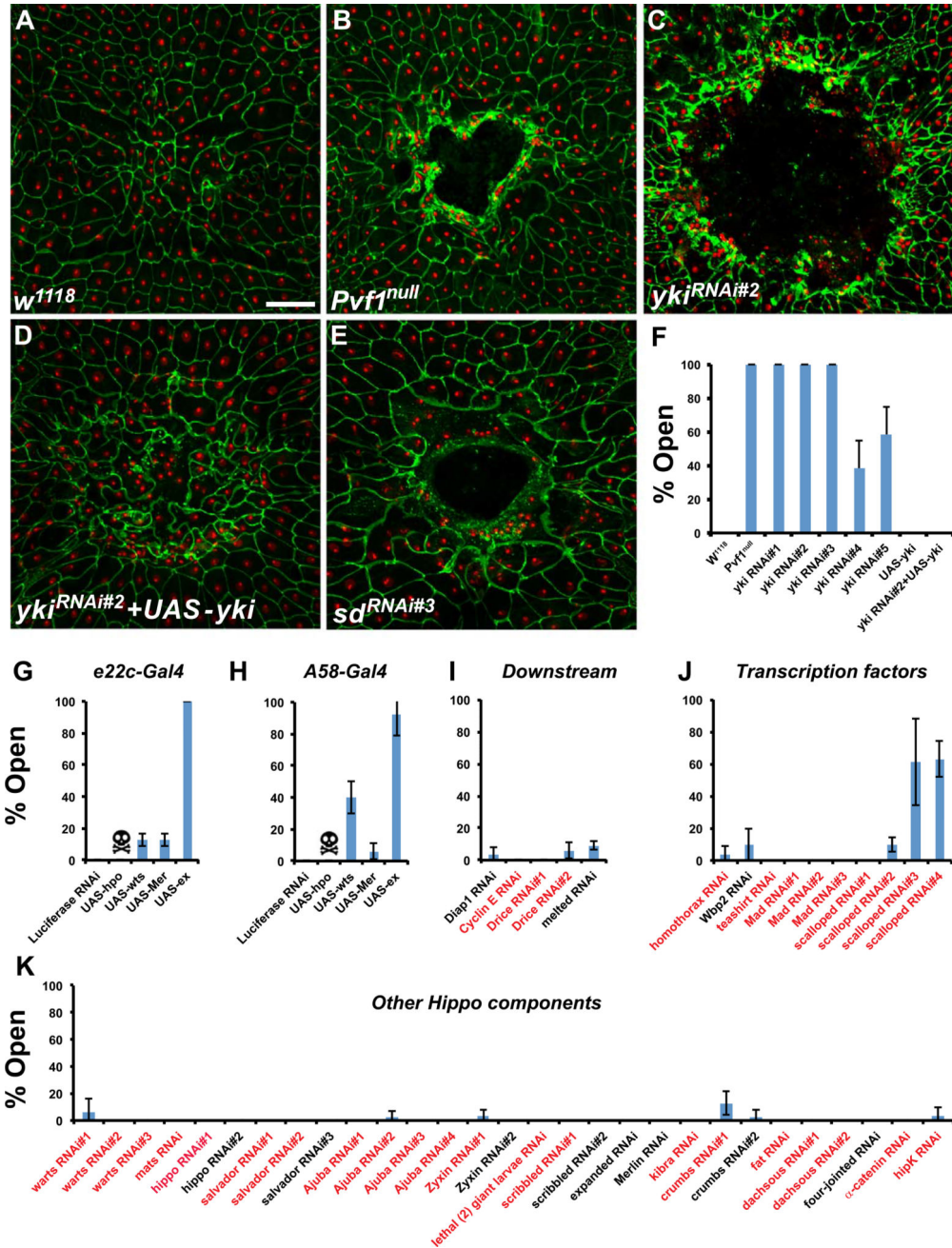


Fig. 1. Yki and Sd are required for larval epidermal wound closure. (A–E) Dissected epidermal whole mounts of pinch wounded larvae expressing *UAS-DsRed2Nuc* via the *e22c-Gal4* driver and the indicated mutations or transgenes. Epidermal nuclei (red); cell boundaries (immunostained with anti-Fasciclin III, green). (A) Control (B) *Pvf1^{Null}* (C) *UAS-yki^{RNAi#2}* (D) *UAS-yki^{RNAi#2}* and *UAS-yki* (E) *UAS-Sd^{RNAi#3}*. Scale bar: 100 μ m. (F–K) Percentage of open wounds in larvae expressing the indicated transgenes via the *e22c-Gal4* driver (G, I–K) or *A58-Gal4* driver (H). (F) *UAS-yki^{RNAi}* transgenes, Yki overexpression, and rescue. (G, H) Overexpression of *yki* negative regulators. (I) *UAS-RNAi* transgenes targeting *yki*

downstream genes (genes that also act upstream of *yki* are included in Fig. 1K). (J) *UAS-RNAi* transgenes targeting *yki* transcriptional partners. (K) *UAS-RNAi* transgenes targeting Hippo pathway components upstream of *yki*. 3 sets of n = 8 for each genotype. Error bars, standard deviation. Previously published *UAS-RNAi* transgenes are labeled in red.

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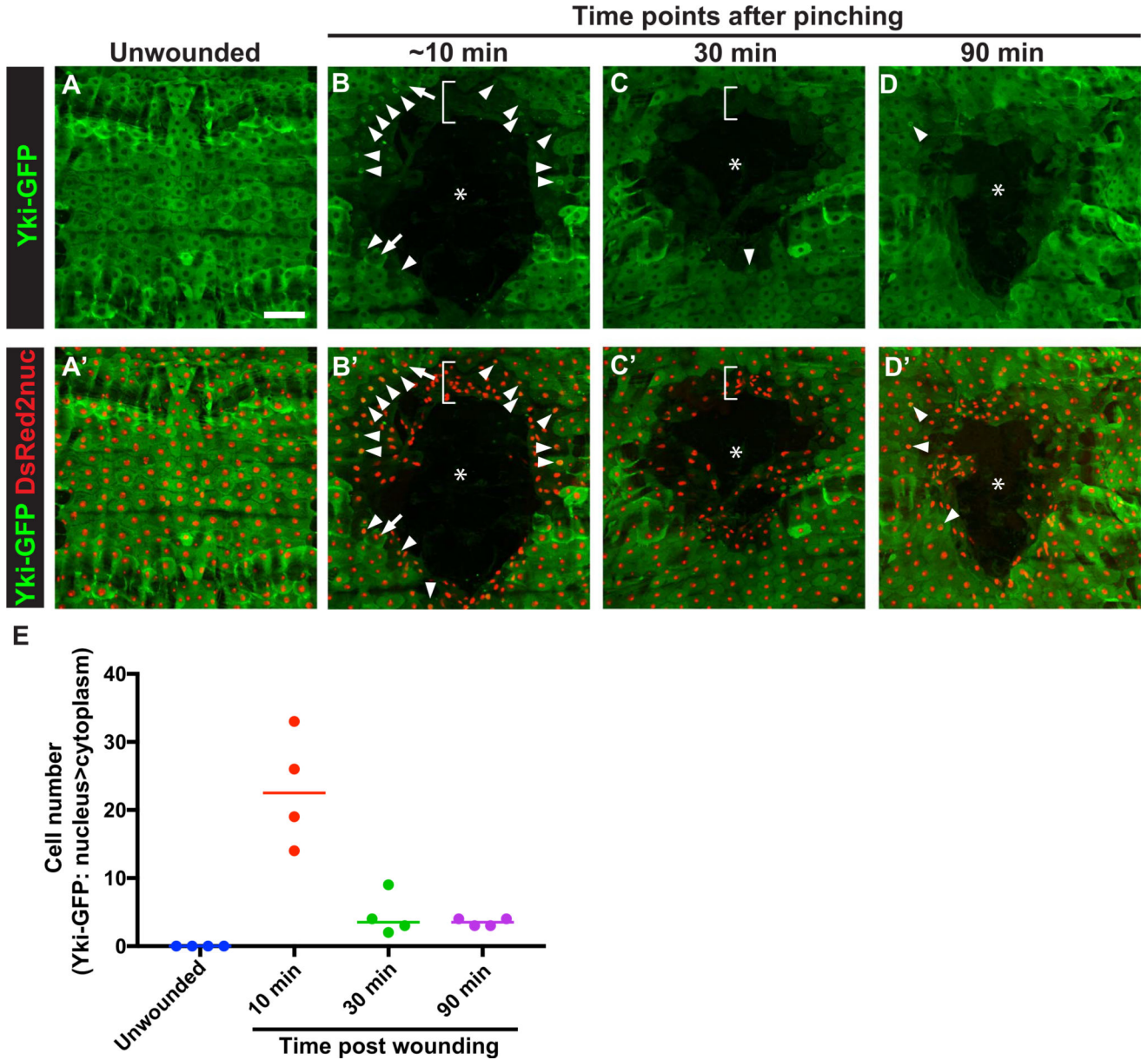


Fig. 2. Yki-GFP rapidly translocates to the nucleus in some wound-edge epidermal cells upon wounding. (A–D) Dissected epidermal whole mounts of unwounded (A) or pinch wounded (B–D) larvae expressing the indicated transgenes via *e22c-Gal4* at the indicated time points following wounding. Cell nuclei were labeled by DsRed2nuc in A'–D'. Asterisks in (B–D') indicate the center of the wounds. Arrowhead in (B–D) show nuclei with prominent Yki-GFP localization. Arrows in (B) show examples of cells with Yki-GFP cytoplasmic localization. Scale bar in (A) indicates 100 μ m is for (A–D'). (E) Quantitation of cells with predominant Yki-GFP nuclear localization at different time points after wounding. n=4 for each condition. Each dot represents one larva.

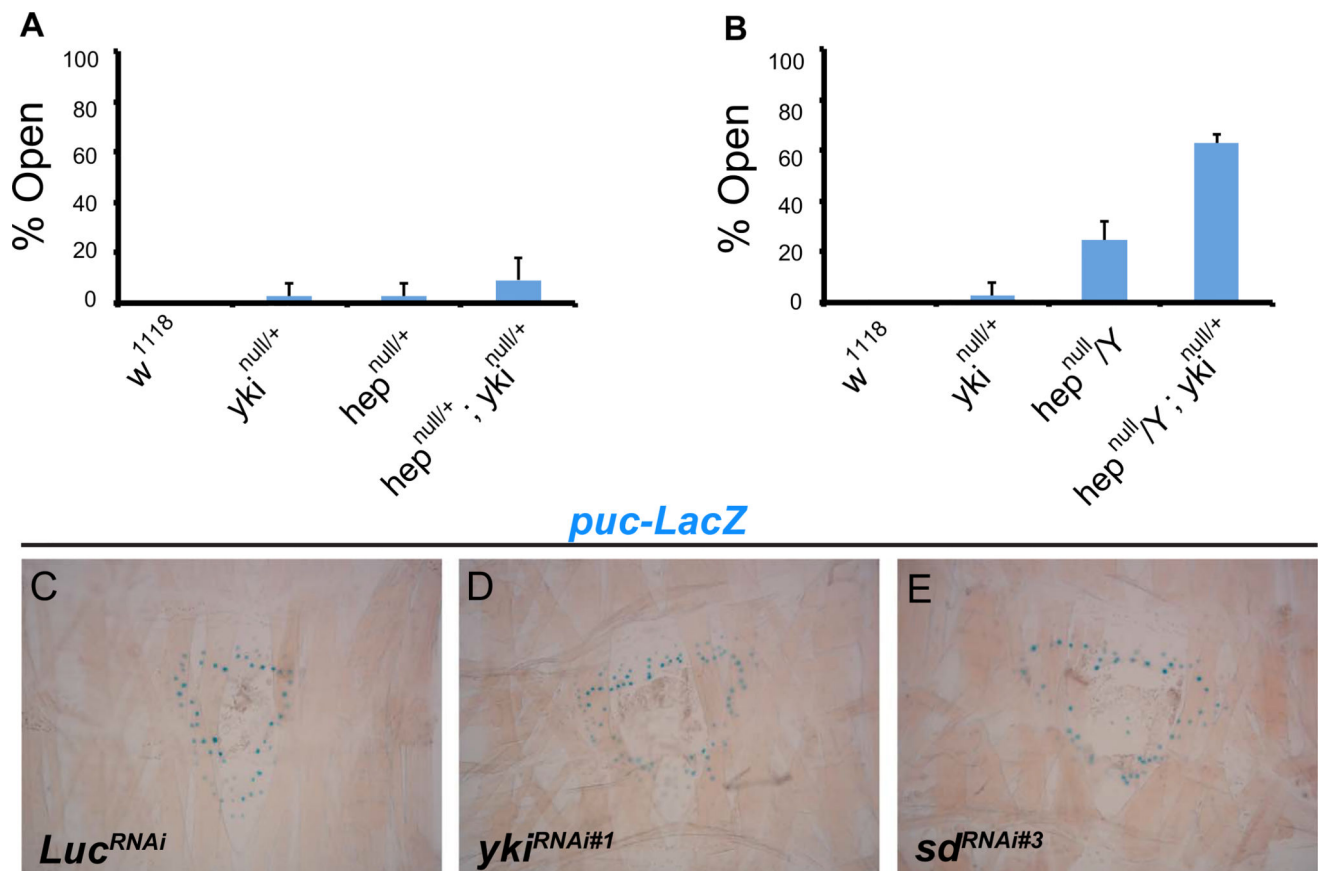


Fig. 3. *yki* genetically interacts with the JNK pathway. (A, B) Percentage of open wounds in larvae of indicated genotypes. 3 sets of n = 8 for each genotype. Error bars, standard deviation. (C–E) X-gal staining of dissected epidermal whole mounts of larvae expressing *puc-lacZ* and the indicated transgenes via *e22c-Gal4*. All wounds are 6 h post-wounding. Blue dots; *puc-lacZ* positive nuclei.

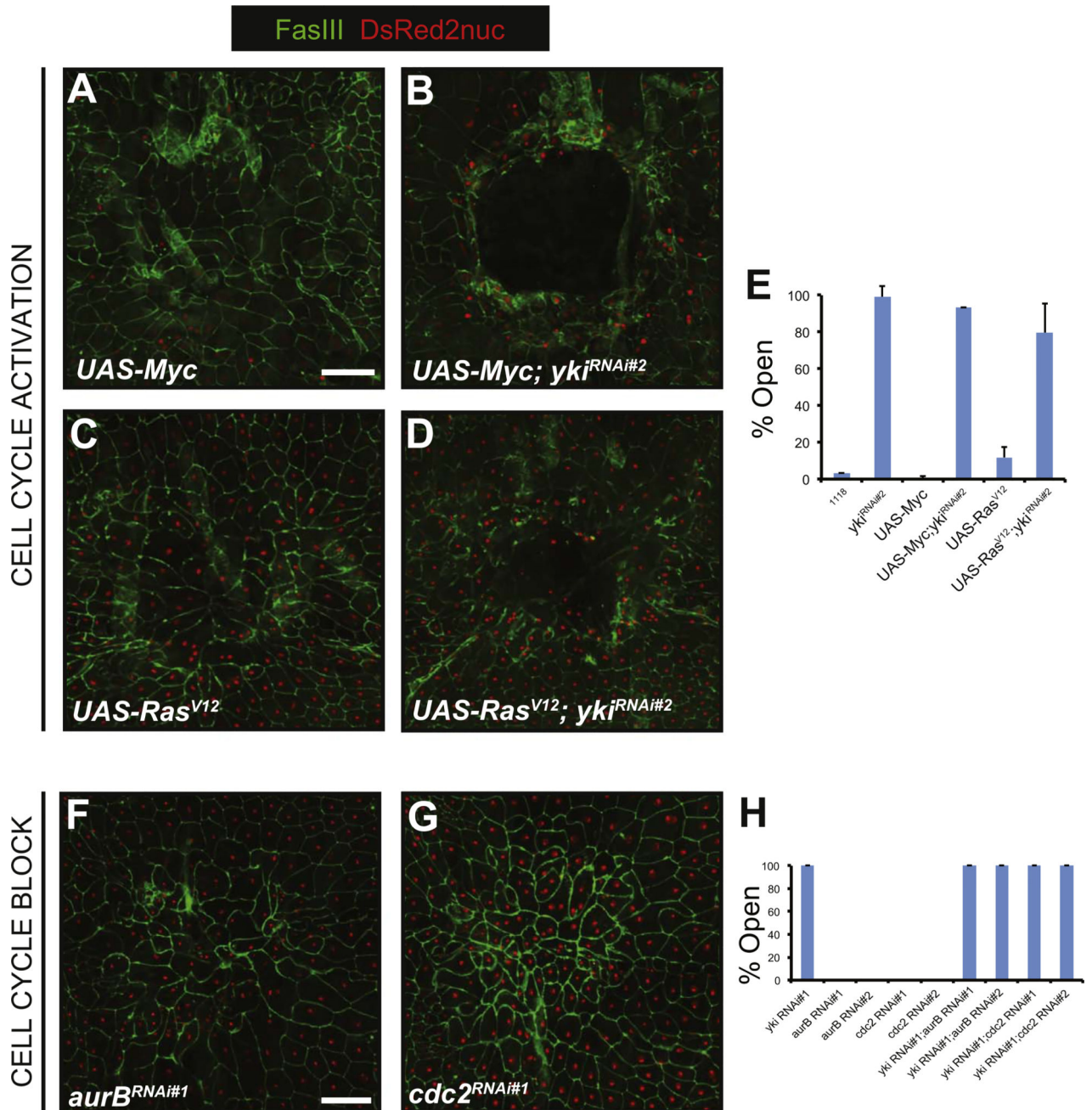


Fig. 4. Yki does not regulate cell proliferation in epidermal wound closure. (A–D) Dissected epidermal whole mounts of pinch wounded larvae expressing the indicated transgenes via the *e22c-Gal4* driver. Cell membranes (immunostained with anti-Fasciclin III), green; nuclei labeled with *UAS-DsRed2Nuc*; Scale bar: 100 μ m. (A) *UAS-Myc* (B) *UAS-Myc* + *UAS-yki^{RNAi#2}* (C) *UAS-Ras^{V12}* (D) *UAS-Ras^{V12}* + *UAS-yki^{RNAi#2}*. (E) Percentage of open wounds on expression of the indicated *UAS-RNAi* lines or transgenes. (F, G) Dissected epidermal whole mounts of pinch wounded larvae expressing the indicated transgenes via the *e22c-GAL4* driver. Cell membranes (immunostained with anti-Fasciclin III), green;

nuclei labeled with *UAS-DsRed2Nuc*; Scale bar: 100 μm . (F) *UAS-aurB^{RNAi#1}* (G) *UAS-cdc2^{RNAi#1}* (H) Percentage of open wounds on expression of indicated *UAS-RNAi* transgenes.

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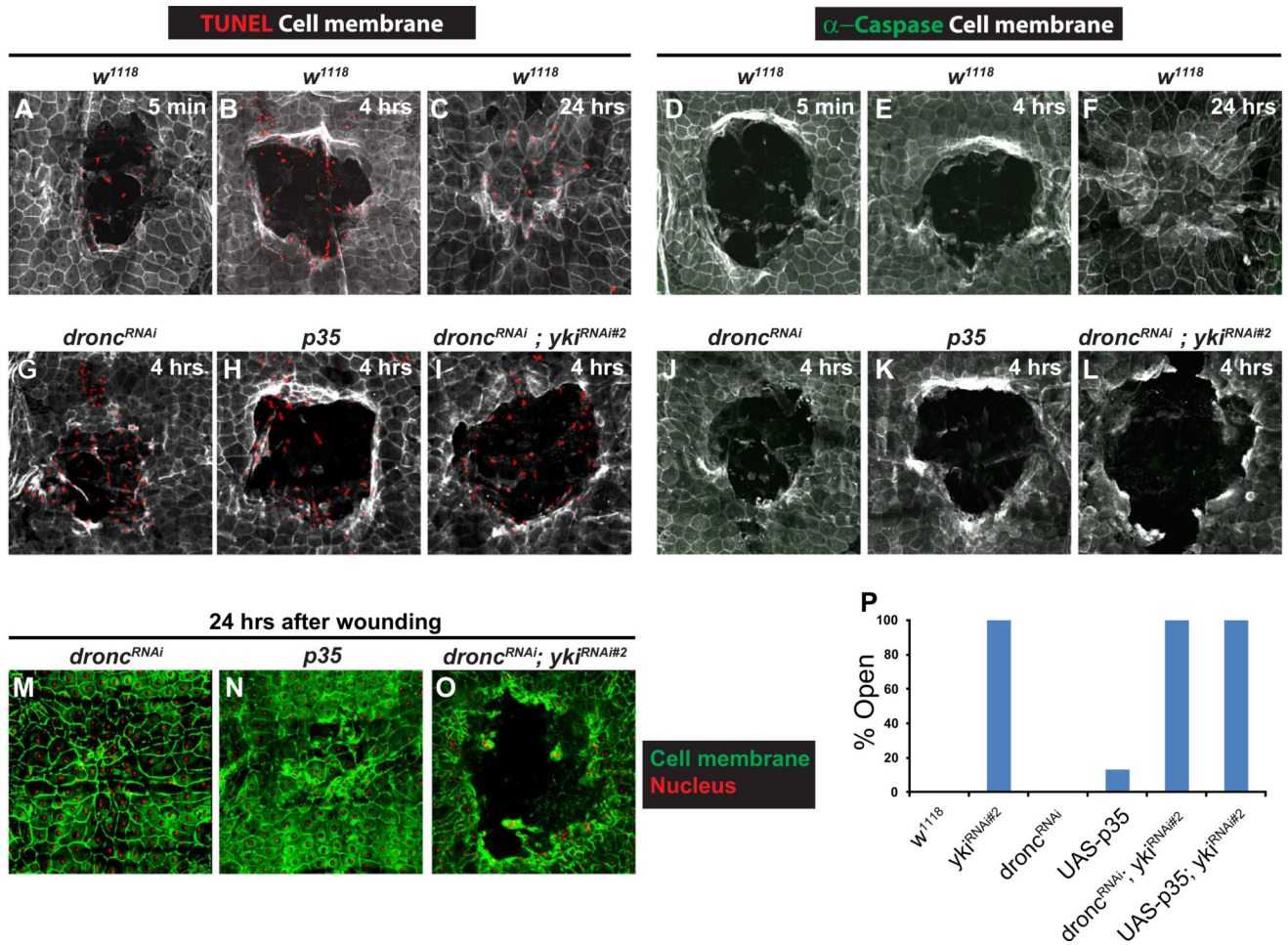
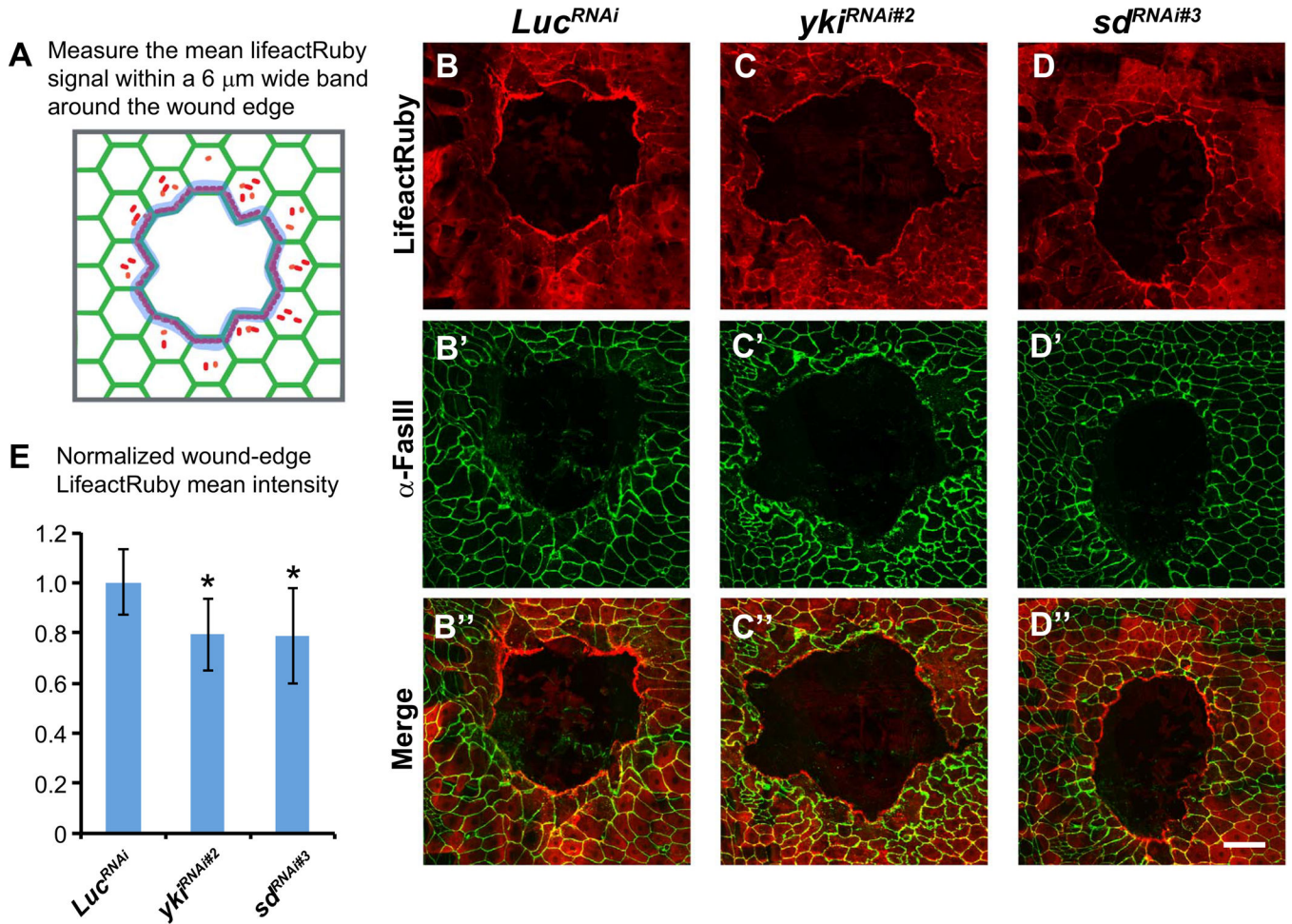


Fig. 5.

Yki does not regulate apoptosis during epidermal wound closure. (A–C, G–I) Dissected epidermal whole mounts of pinch wounded larvae of the indicated genotypes and at the indicated time points post wounding stained with anti-Fasciclin III antibody (white) and TUNEL (red). (A–C) *w¹¹¹⁸* control. (A) 5 mins. (B) 4 h. (C) 24 h. (G) *UAS-dronc^{RNAi}*, 4 h. (H) *UAS-p35*, 4 h. (I) *UAS-dronc^{RNAi} + UAS-yki^{RNAi#2}*, 4 h. (D–F, J–L) Dissected epidermal whole mounts of pinch wounded larvae of the indicated genotypes and at the indicated time points post wounding immunostained with anti-Fasciclin III antibody (white) and anti-cleaved-Caspase 3 antibody (green). (D–F) *w¹¹¹⁸* control. (D) 5 mins. (E) 4 h. (F) 24 h. (J) *UAS-dronc^{RNAi}*, 4 h. (K) *UAS-p35*, 4 h. (L) *UAS-dronc^{RNAi} + UAS-yki^{RNAi#2}*, 4 h. (m–O) Dissected epidermal whole mounts of larvae expressing *UAS-dsRed2Nuc* (red) and the indicated transgenes 24 h after pinch wounding, stained with anti-Fasciclin III antibody (green). (M) *UAS-dronc^{RNAi}*, 24 h. (N) *UAS-p35*, 24 h. (O) *UAS-dronc^{RNAi} + UAS-yki^{RNAi#2}*, 24 h. (P) Percentage of open wounds on expression of the indicated *UAS-RNAi* line or transgenes.

**Fig. 6.**

Yki regulates wound-edge F-actin levels during epidermal wound closure. (A) Illustration of wound-edge F-actin measurement. (B–D) Dissected epidermal whole mounts of wounded larvae expressing the indicated transgenes and *UAS-lifeact-Ruby* (F-actin, red, B–D) via the *e22c-Gal4* six hours post wounding. (B'–D') Green, anti-Fascin III. (B''–D'') Merged images. Scale bar: 100 μm . (E) Average F-actin intensities around the wound sites of larvae expressing the indicated transgenes. Error bars indicate standard deviation. Student's *t*-test was used to test for significance. *, $P < 0.05$.