JNK-mediated Slit-Robo signaling facilitates epithelial wound repair by extruding dying cells

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Multicellular organisms repair injured epithelium by evolutionarily conserved biological processes including activation of c-Jun N-terminal kinase (JNK) signaling. Here, we show in Drosophila imaginal epithelium that physical injury leads to the emergence of dying cells, which are extruded from the wounded tissue by JNK-induced Slit-Roundabout2 (Robo2) repulsive signaling. Reducing Slit-Robo2 signaling in the wounded tissue suppresses extrusion of dying cells and generates aberrant cells with highly upregulated growth factors Wingless (Wg) and Decapentaplegic (Dpp). The inappropriately elevated Wg and Dpp impairs wound repair, as halving one of these growth factor genes cancelled wound healing defects caused by Slit-Robo2 downregulation. Our data suggest that JNK-mediated Slit-Robo2 signaling contributes to epithelial wound repair by promoting extrusion of dying cells from the wounded tissue, which facilitates transient and appropriate induction of growth factors for proper wound healing.

Wound repair is an evolutionarily conserved process that maintains tissue homeostasis upon injury1–3. It has been reported that JNK signaling acts as an essential regulator of wound repair in Drosophila epithelial tissue4–7, planarians body8,9, and zebrafish tail fin10. Genetic studies in Drosophila have shown that JNK signaling contributes to (1) actin remodeling to close wound edges6,11, (2) reconstruction of lost tissue parts by activating growth promoters such as Yorkie (Yki, a YAP homolog)12,13, Wg (a Wnt homolog)14, Dpp (a TGF-β/BMP family member)15 and Myc14, (3) facilitating cell reprogramming via reducing the activity of polycomb-dependent silencing16, and (4) induction of developmental delay by upregulating Drosophila insulin-like peptide 8 (Dilp8) to prolong the developmental period for recovery17. Particularly, JNK-dependent induction of Wg promotes regenerative growth of Drosophila wing imaginal discs after genetic ablation of the tissue14. In addition, JNK-mediated upregulation of Wg and Dpp plays critical roles in compensatory proliferation of imaginal cells after induction of massive cell death15,18,19. JNK signaling also induces apoptosis20,21, which is required for regeneration of planarian body2 or wound repair in Drosophila epithelial tissue22–24. Together, JNK regulates multiple steps of repair process from beginning to end.

Dying cells emerged in the epithelial tissue are extruded basally or apically by a coordinated mechanism25. For instance, overcrowding of cells within a limited space triggers extrusion of living or dying cells from Madin-Darby canine kidney (MDCK) epithelial monolayer26, developing zebrafish tail fin26, and Drosophila notum27. In Drosophila embryonic development, extrusion of apoptotic cells from amnioserosa promotes dorsal closure28,29, the process that shares common JNK-dependent events with epithelial wound repair, which include actin remodeling, cell migration, and epithelial zipping30,31. Similarly, JNK-dependent cell extrusion is required for tumor-suppressive cell competition, the process in which oncogenic polarity-deficient cells such as scribble (scrib) or discs large (dlg) mutant cells are actively eliminated from epithelia when surrounded by wild-type cells32–37. Importantly, extrusion of polarity-deficient cells by cell competition is driven by JNK-mediated activation of Slit-Robo2 axonal repulsive signaling that downregulates E-cadherin, as the ligand Slit, its receptor Robo2, and the downstream effector Enabled (Ena)/Vasp are all induced by JNK signaling35. During Drosophila

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embryonic development, the N-terminus of Slit produced from midline glial cells binds to the immunoglobulin (Ig) motif of Robo2 expressed in commissural axons, thereby regulating midline crossing of commissural axons via cell-cell repulsion \(^{38-42}\) and the system is well conserved throughout evolution \(^{43}\). Interestingly, it has been shown that extrusion of dying cells by Semaphorin-PlexinA axonal repulsive signaling is required for wound repair in \textit{Drosophila} and zebrafish epithelia \(^{44}\), although the role of cell extrusion in wound repair and the upstream trigger for cell-extrusion signaling remain unknown.

Here, we found in \textit{Drosophila} epithelium that physical injury induces JNK activation, which promotes extrusion of dying cells via Slit-Robo2 signaling. The Slit-Robo2-mediated cell extrusion facilitates epithelial wound repair by preventing excessive expression of growth factors Wg and Dpp upon injury.

Results and Discussion

Slit-Robo2 signaling acts downstream of JNK in wound repair. To dissect the mechanism of epithelial wound repair in \textit{Drosophila}, we physically injured the wing imaginal disc, the larval epithelial primordia of adult wing. The right wing disc was injured with a tungsten needle by aseptic \textit{in situ} wounding in living larvae without further damaging the animal (hereafter denoted as “wounded” disc), with the left wing disc remained undamaged as an internal control (hereafter denoted as “intact” disc) (Supplementary Fig. 1). Wounded wing discs were repaired during animal development and form essentially normal adult wings (Fig. 1a,a′, quantified in Fig. 1g). Blocking JNK signaling by knocking down \textit{Drosophila} \textit{JNK basket (bsk)} significantly impaired wound repair (Fig. 1b,b′, quantified in Fig. 1g), indicating that JNK signaling is essential for wound repair as reported previously \(^{22-24}\). In analyzing downstream effectors of JNK signaling, we found that Slit-Robo2 signaling, the cell-extrusion signaling activated by JNK during tumor-suppressive cell competition \(^{45}\), is required for wound repair. Downregulation of Slit or Robo2 by heterogeneous deletion of these genes or by RNAi expression in the wing pouch significantly impaired wound repair (Fig. 1c′–f′, quantified in Fig. 1g) without affecting wing development in intact discs (Fig. 1c–f). Consistently, JNK activity and \textit{slit-lacZ} expression were elevated around the wound at 6hrs after wounding, as visualized by the anti-Mmp1 \(^{46}\) and \textit{slit-lacZ} reporter (Fig. 1h,i). The upregulations of Mmp1 and \textit{slit-lacZ} expression were significantly suppressed by \textit{bsk-RNAi} (Fig. 1j–k′, quantified in Supplementary Fig. 2). In addition, blocking Slit-Robo2 signaling did not exacerbate repair defect caused by \textit{bsk-RNAi} (Supplementary Fig. 3a,b, quantified in Supplementary Fig. 3c), suggesting that JNK and Slit-Robo2 participate in wound healing process in the same pathway. These data suggest that Slit-Robo2 signaling acts downstream of JNK in wound repair. The wound-repair defect caused by blocking JNK was severer than blocking Slit-Robo2 (Fig. 1g), likely because JNK has multiple functions in wound repair.

Slit-Robo2 signaling promotes extrusion of dying cells from the wounded tissue. Our finding that Slit-Robo2 signaling plays a role in epithelial wound repair suggests that JNK-mediated cell extrusion is required for this process. We thus analyzed spatial locations of dying cells in the wounded tissue by immunostaining for the cleaved form of the effector caspase Dcp1 (c-Dcp1). In wild-type background, the number of dying cells in the wing pouch significantly increased at 6hrs after wounding (Supplementary Fig. 4a,b, quantified in Supplementary Fig. 4i). Importantly, the number of dying cells within the disc was 4-fold higher at the earlier time point (Fig. 2g, 3hrs, quantified in Fig. 2f), compare to Fig. 2b, 6hrs, quantified in Fig. 2e), suggesting that dying cells are extruded from the tissue over time. Supporting this notion, the analysis of extruding/extruded dying cells in the wounded discs by classifying their locations into three classes (“in disc”, “apically extruding”, and “basally extruding”; Fig. 2a) revealed that the ratio of dying cells within the disc over basally/apically extruding cells was significantly reduced over time (compare Fig. 2k (3 hrs, wild-type), Fig. 2f (6hrs, wild-type), and Supplementary Fig. 5c,d, quantified in Supplementary Fig. 5l) after wounding, while the tendency was not observed at 3hrs after wounding likely because extrusion has not proceeded sufficiently at this time point even in the wild-type tissue (Supplementary Fig. 5a,b, quantified in Supplementary Fig. 5j).

Conversely, overexpression of \textit{slit} or \textit{robo} 2 in the wing pouch significantly decreased the number and ratio of dying cells in the disc at 3hrs (Fig. 2h,i, quantified in 2j,k) and 6hrs (Supplementary Fig. 5c,d, quantified in Supplementary Fig. 5k) after wounding, while the tendency was not observed at 9hrs after wounding likely because most cells have already been extruded by this time point even in the wild-type tissue (Supplementary Fig. 5h,i, quantified in Supplementary Fig. 5l). Together, these data indicate that JNK-induced Slit-Robo2 signaling promotes extrusion of dying cells from the injured tissue, which is essential for wound repair. Notably, blocking JNK signaling in the damaged wing pouch abolished apoptosis (as visualized by c-Dcp1 staining) but not necrosis (as visualized by Propidium Iodide (PI) staining) (Supplementary Fig. 4), indicating that apoptosis induction is also JNK-dependent as reported previously \(^{22-24}\).

Slit-Robo2-mediated extrusion of dying cells prevents excessive growth factor expressions in the wounded tissue. We next examined the role of Slit-Robo2-mediated extrusion of dying cells in wound repair. It has been shown that secreted growth factors Wg and Dpp are essential for regeneration of damaged epithelium in \textit{Drosophila} \(^{14,15,18,19,47}\). Interestingly, we found that at 24hrs after wounding, aberrant dying cells with highly elevated Wg and Dpp expressions were emerged in \textit{slit} /+ or \textit{robo} 2/++ wounded wing discs (Fig. 3g,h,k,l) compared to wild-type background (Fig. 3c,d, quantified in Fig. 3m,n), while intact discs did not possess such aberrant cells (Fig. 3a,b,e,f,i,j, quantified in Fig. 3m,n). In addition, these aberrant cells frequently located nearby or within JNK-activated cells as visualized by \textit{TRE-DsRed reporter} (Supplementary Fig. 6g (63.6%, n = 11), h (100%, n = 5), k (85.7%, n = 14), l (87.5%, n = 14)) compared to wild-type background (Supplementary Fig. 6c (30.8%, n = 13), d (22.2%, n = 9)). These data indicate that impaired Slit-Robo2 signaling in the wounded tissue results in the emergence of abnormal cells with excess production of Wg and Dpp, which may disturb the wound healing process. Indeed, downregulation of Wg or Dpp by halving the \textit{wg} or \textit{dpp} gene significantly suppressed the wound healing...
defects in slit^{2}/+ or robo^{2}/+ flies (Fig. 4b,c,e,f, quantified in Fig. 4g, and Supplementary Fig. 7b,c,e,f, h,i, quantified in Supplementary Fig. 7j), while heterozygosity for wg or dpp on its own did not affect wound repair (Fig. 4a,d, quantified in Fig. 4g, and Supplementary Fig. 7a,d,g, quantified in Supplementary Fig. 7j). In addition, halving the wg gene indeed suppressed the emergence of aberrant cells with excess Wg (Supplementary Fig. 8a–f, quantified in Supplementary Fig. 8g). These results suggest that the emergence of aberrant cells with excess growth factors is

Figure 1. Slit-Robo2 signaling acts downstream of JNK in wound repair. (a–f) Control intact (a–f) and wounded (a’–f’) adult wings in each genotype taken from the same individuals. All pictures were taken at the same magnification. Scale bar, 500 µm. (g) Boxplot with dots representing wound repair (%) (see Methods) in each genotype (wild-type (n = 136), bsk-RNAi (n = 131), slit^{2}/+ (n = 139), slit-RNAi (n = 113), robo^{2}/+ (n = 154), and robo2-RNAi (n = 189)). Mann-Whitney U-test; *p < 0.05, **p < 0.01, and ***p < 0.001. (h–k”) Wing discs of wild-type (h–i”) and nub > bsk-RNAi (j–k”) flies were dissected at 6hrs after wounding. Wing pouches were labeled with GFP using the nub-gal4 driver (green). JNK activity (magenta), slit-lacZ expression (white), and nuclei (cyan) were detected by anti-Mmp1, anti-β-gal (for slit-lacZ), and DAPI, respectively. Yellow dashed lines and arrowheads indicate locations of wounds. Red arrowheads indicate ectopic slit-lacZ expressions. Asterisks indicate the position of endogenous slit-lacZ expression. Scale bars, 50 µm. See Supplementary Information for detailed genotypes.
Figure 2. Slit-Robo2 promotes extrusion of dying cells from the wounded tissue. (a) Diagram of the analysis for dying cell locations in the cross-section of the wing disc. See Methods for further details. (b–k) Images show xy and yz cross-sections of wounded wing discs of wild-type (b), slit^+/^+ (c), and robo2^+/^+ (d) larvae dissected at 6hrs after wounding; xy and yz cross-sections of wild-type (g), nub > slit (h), and nub > robo2 (i) larvae dissected at 3hrs after wounding. Dying cells were detected by anti-c-Dcp1 staining (magenta) and wing pouches were marked with Venus using the nub-gal4 driver (cyan), and F-actin was visualized with Phalloidin (green). Yellow dashed lines indicate the positions of wounds. White dashed lines indicate the positions of yz cross-section shown in the right panel. The two-direction arrow indicates apical (A) and basal (B) sides of the disc. Asterisks in right panels indicate dying cells classified as “in disc” (white), “apically extruding” (yellow), and “basally extruding” (blue). Scale bars, 50 µm. Quantification of the number of dying cells classified as “in disc” at 6hrs after wounding in each genotype (e) (wild-type (n = 12), slit^+/^+ (n = 12), and robo2^+/^+ (n = 12)); 3hrs after wounding in each genotype (j) (wild-type (n = 11), nub > slit (n = 12), and nub > robo2 (n = 12)). Welch's T-test; mean ± s.d.; *p < 0.05 and **p < 0.01. (f,k) Quantification of the ratio of dying cells classified into 3 types (as shown in A) at 6hrs after wounding in each genotype.
responsible for the repair defect. Together, our data indicate that JNK-induced Slit-Robo2 signaling contributes to wound repair by promoting extrusion of dying cells with excess growth factors (Fig. 5).

Intriguingly, it has been reported that an axon guidance molecule PlexinA plays an important role in cell extrusion during epithelial wound repair in Drosophila and zebrafish. In addition, Slit has been proposed to bind to PlexinA in mammals, suggesting that multiple axon guidance signaling contribute to wound healing by promoting dying cell extrusion. Our findings suggest that dying cells remained in the tissue with excess growth factors need to be removed for proper wound healing, by promoting epithelial fusion and/or facilitating transient and appropriate production of growth factors.

Methods
Fly strains. Flies were cultured with standard food in plastic vials at 25 °C. 3rd instar wandering larvae were analyzed in all the experiments. Fly strains are used as follows: *uben* (Bloomington Drosophila Stock Center [BDSC] #42699), *rn-gal4*Gal4UAS (BDSC #7405), UAS-CD8-PARP-Venus (gift from Yasushi Hiromi), UAS-bsk-RNAi (National Institute of Genetics [NIG] #5680R-2), *slit* (Drosophila Genomics and Genetic Resources [DGGR] #106948), UAS-slit-RNAi (BDSC #31468), robo2 (DGGR #106843), UAS-robo2-RNAi (BDSC #34589), slit-Gal4 (slit-lacZ, BDSC #12189), UAS-slit (gift from Tom Kidd), UAS-robo2-HA (gift from Talia Volk), gfp (BDSC #2978), robo2 (BDSC #405), dpp (DGGR #10644), dpp-kat2 (DGGR #106649), TRE-DsRed (BDSC #59012), P[PIZ]/dpp10638 (dpp-lacZ, BDSC #12379).

Physical in situ wounding. 3rd instar wandering larvae were randomly collected and anesthetized with ice-water for around 10 minutes. Then their wing discs (which were marked by fluorescent proteins GFP or Venus) were injured on ice with a sharpened 0.3 mm tungsten needle by performing aseptic in situ wounding (by pushing the wing pouch region using the needle) in living larvae without further damaging the animal. Wounding was performed under the fluorescence binocular microscope. After wounding, larvae were cultured in fresh food vials and kept at 25 °C again. Late 3rd instar larvae before wounding were wondered only when we analyzed wing discs 24 hrs after wounding. See Supplementary Fig. 1 for further information.

Histology. Larval tissues were fixed and stained using standard immunohistochemical methods with rabbit anti- cleaved-Dcp1 (1:100, Cell Signaling Technology), chicken anti-β-galactosidase (1:2500, abcam), mouse anti-Mmp1 (1:100, from cocktail of 3A6B4, 3B8D12 and 5H7B11, Developmental Studies Hybridoma Bank [DSHB]), mouse anti-Wingless (1:100, DSHB), anti-mouse Alexa 546, 647 (1:250, Molecular Probes), anti-rabbit anti-Mmp1 (1:100, from cocktail of 3A6B4, 3B8D12 and 5H7B11, Developmental Studies Hybridoma Bank [DSHB]), anti-chicken Alexa 546, 647 (1:250, Molecular Probes), anti-chicken Alexa 647 (1:250, Molecular Probes), and were mounted with DABCO (wild-type (n = 17), nub > slit (n = 13), and nub > robo2 (n = 13)). Chi-squared test; ***p < 0.001; * the absolute value of adjusted residual > 2.56. See Supplementary Information for detailed genotypes.

Measurement of wing size. The right and left wings of adult flies were mounted on slide glasses. Leica binocular stereo microscope with LEICA FIRECAM ver. 3.4 (Leica Microsystems) was used to take pictures of the wings and whole bodies of adult flies. The wing size was automatically measured by Fiji ver. 2.0.0-rc-49/1.51k (https://imagej.net/Fiji). “Wing repair (%)) was defined as injured right wing area divided by intact left wing area (%), calculated by Microsoft Excel for Mac ver. 16.16.4 (Microsoft).

Analysis of dying cells. Apoptotic dying cells were detected by c-Dcp1 antibody. For the analysis of spatial locations of c-Dcp1-positive dying cells, the locations were classified into 3 classes: (1) “basally extruding”, as dying cells located at the basal tip of the disc proper, (2) “apically extruding”, as dying cells located at the apical tip of the disc proper, and (3) “in disc”, as dying cells located within the disc proper (see Fig. 2a for further information). Wing discs with wounding that crosses center of the wing pouches were analyzed for special locations of c-Dcp1-positive cells. c-Dcp1-positive cells classified as “in disc” were manually counted using single xz-section images, and pock areas were manually measured with Fiji and calculated with Microsoft Excel for Mac. For the analysis of total number of c-Dcp1-positive cells in the pouch, the number of c-Dcp1-positive cells in the wing pouch and the size of the wing pouch areas were automatically counted using Z-stacked images with Fiji and calculated with Microsoft Excel for Mac. For the analysis of necrotic dying cells detected by PI staining, the number of PI-positive cells in the wing pouch were automatically counted using single xy-section images with Fiji and calculated with Microsoft Excel for Mac.
Figure 3. Defects in dying cell extrusion cause aberrant Wg and Dpp expression. (a–l) xy cross-section images of intact and wounded wing discs of wild-type (a–d’), slit2/+ (e–h’), and robo22/+ (i–l’) larvae at 24hrs after wounding. Dying cells were detected with anti-c-Dcp1 antibody (magenta). Wg and Dpp expressions were detected using anti-Wg antibody and anti-β-gal antibody (for dpp-lacZ), respectively (green). Yellow arrowheads indicate representative dying cells expressing aberrantly high levels of Wg or Dpp. Asterisks indicate endogenous signals. Scale bars, 50 µm. (m,n) Boxplot with dots representing normalized intensity of anti-Wg in c-Dcp1-positive cells (see Methods) in each genotype (wild-type (intact: n = 12, wounded: n = 11), slit2/+ (intact: n = 16, wounded: n = 17), and robo22/+ (intact: n = 15, wounded: n = 13)). (m) Boxplot with dots representing normalized intensity of anti-β-gal antibody (for dpp-lacZ) in c-Dcp1-positive cells (see Methods) at 24hrs after wounding in each genotype (wild-type (intact: n = 9, wounded: n = 11), slit2/+ (intact: n = 5, wounded: n = 6), and robo22/+ (intact: n = 12, wounded: n = 10)). (n) Mann-Whitney U-test; *p < 0.05, **p < 0.01, ***p < 0.001; n.s.; not significant. See Supplementary Information for detailed genotypes.
Figure 4. wg and dpp mutants could rescue Slit-Robo2 defect. (a–f’) Control intact (a–f) and wounded (a’–f’) adult wings in each genotype taken from the same individuals after wounding. All pictures were taken at the same magnification. Scale bar, 500 µm. (g) Boxplot with dots representing wound repair (%) (see Methods for details) in each genotype (wild-type (n = 138), wg1/+(n = 89), dpp6/+(n = 64), slit2/+(n = 139), slit2/+, wg1/+(n = 105), slit2/+, dpp6/+(n = 77), robo2/+(n = 154), robo2/+(n = 128), and robo2/+(n = 95)) Mann-Whitney U-test; *p < 0.05, **p < 0.01, ***p < 0.001; n.s.; not significant. See Supplementary Information for detailed genotypes.

Figure 5. Model for the role of JNK-Slit-Robo-mediated extrusion of dying cells in wound repair. See text for details.
Statistical analysis. All experiments were repeated at least three times. Mann-Whitney non-parametric test was used for analyzing adult wing sizes and signal intensity, Welch’s T-test was used for analyzing the number of dying cells, and chi-squared test was used for analyzing spatial locations of dying cells. Error bars in all bar graphs indicate standard deviation (s.d.). n.s.; not significant indicates p ≥ 0.05 or the absolute value of adjusted residual ≤ 2.56. All bar graphs and stacked graphs were prepared with Microsoft Excel for Mac. All boxplot graphs include the data of all individuals as dots and were prepared with R ver. 3.2.3 (https://www.r-project.org).

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C.I., S.O., K.T., M.Y., G.M. and T.I. designed experiments; C.I. performed all experiments; C.I., S.O., K.T., M.Y., G.M. and T.I. analyzed the data; C.I., S.O., K.T. and T.I. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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