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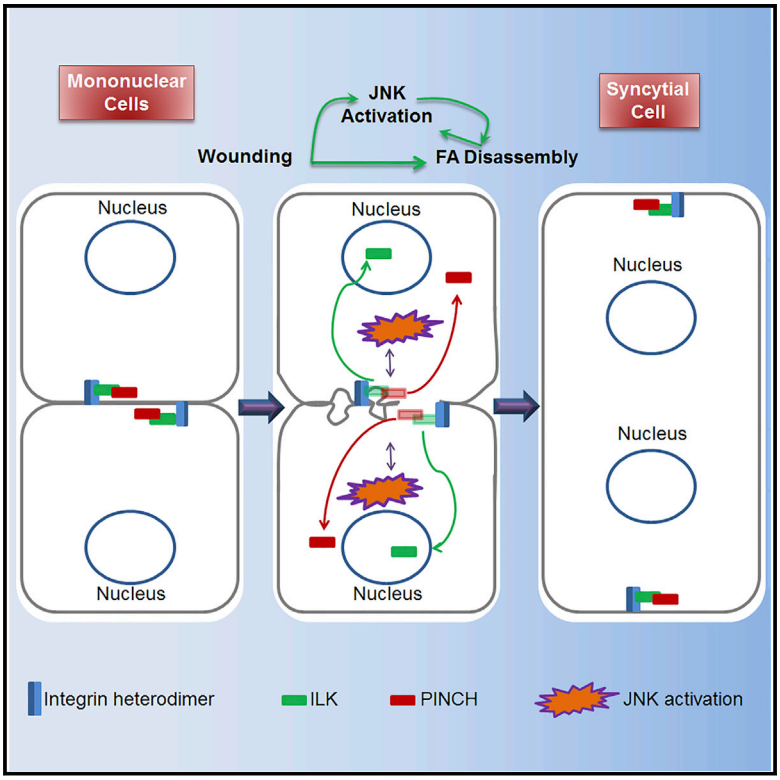
Integrin Adhesions Suppress Syncytium Formation in the *Drosophila* Larval Epidermis

Graphical Abstract



Authors

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In Brief

Wang et al. show that integrin adhesions suppress epidermal cell-cell fusion. The mechanism of epidermal syncytium formation is independent of mitosis and apoptosis and instead involves disassembly of integrin adhesion complexes and positive feedback with Jun N-terminal kinase signaling, thereby revealing a new role for integrin signaling.

Highlights

- Integrin FA complex loss leads to epidermal syncytium formation
- Wounding or integrin adhesion loss causes a unique form of cell-cell fusion
- JNK signaling correlates with and can drive epidermal syncytium formation
- JNK-induced integrin FA complex disassembly likely promotes wound-induced fusion

Wang et al., 2015, Current Biology *25*, 2215–2227
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http://dx.doi.org/10.1016/j.cub.2015.07.031

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Integrin Adhesions Suppress Syncytium Formation in the *Drosophila* Larval Epidermis

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SUMMARY

Integrins are critical for barrier epithelial architecture. Integrin loss in vertebrate skin leads to blistering and wound healing defects. However, how integrins and associated proteins maintain the regular morphology of epithelia is not well understood. We found that targeted knockdown of the integrin focal adhesion (FA) complex components β-integrin, PINCH, and integrin-linked kinase (ILK) caused formation of multinucleate epidermal cells within the *Drosophila* larval epidermis. This phenotype was specific to the integrin FA complex and not due to secondary effects on polarity or junctional structures. The multinucleate cells resembled the syncytia caused by physical wounding. Live imaging of wound-induced syncytium formation in the pupal epidermis suggested direct membrane breakdown leading to cell-cell fusion and consequent mixing of cytoplasmic contents. Activation of Jun N-terminal kinase (JNK) signaling, which occurs upon wounding, also correlated with syncytium formation induced by PINCH knockdown. Further, ectopic JNK activation directly caused epidermal syncytium formation. No mode of syncytium formation, including that induced by wounding, genetic loss of FA proteins, or local JNK hyperactivation, involved misregulation of mitosis or apoptosis. Finally, the mechanism of epidermal syncytium formation following JNK hyperactivation and wounding appeared to be direct disassembly of FA complexes. In conclusion, the loss-of-function phenotype of integrin FA components in the larval epidermis resembles a wound. Integrin FA loss in mouse and human skin also causes a wound-like appearance. Our results reveal a novel and unexpected role for proper integrin-based adhesion in suppressing larval epidermal cell-cell fusion—a role that may be conserved in other epithelia.

INTRODUCTION

Highly conserved integrin adhesion components regulate tissue morphology via cell/matrix adhesion signaling [1] and mechanotransduction [2]. Lack of integrin α6β4 in mice causes skin blistering reminiscent of epidermolysis bullosa [3, 4] where hemidesmosome failure leads to mechanical skin disruption. *Drosophila* embryos mutant for β-integrin (*myospheroid*), integrin-linked kinase (*ILK*), or the LIM-domain containing focal adhesion (FA) adaptor PINCH (*steamer duck*), exhibit defective skeletal muscle cell attachment to tendon cells [5–7]. Mutant clones of FA complex genes in the wing epithelium cause blistering from defective adhesion between two apposed epithelia [5, 7, 8]. In the embryonic epidermis, *myospheroid* is required for proper dorsal closure (DC) [9], a wound-healing-like morphogenetic movement. Whether FA components play a role in maintenance of barrier epidermal morphology is not known.

Polarized epithelial cells, like the barrier epidermal monolayer in *Drosophila* larvae [10], are usually mononuclear and tightly adherent. After DC is completed, larval epidermal cells secrete apical cuticle [11] to accompany the basal lamina assembled during embryogenesis [12]. In the epidermal plane, these endoreduplicated cells [13] pack into a highly regular sheet [10]. If wounded, they change shape to traverse the wound gap and phagocytose debris [10, 14]. Indeed, following wounding some larval and adult epidermal cells even form syncytia centered at the wound [10, 15]. Although their role in healing is not clear, these syncytia can contain over a dozen nuclei.

Formation of multinucleate cells occurs within certain epithelia. In the placental syncytial trophoblast layer [16], the vertebrate lens epithelium [17] and the *C. elegans* hypodermis [18] fusion presumably confers functional advantages. Even the adult *Drosophila* epidermis undergoes an age-dependent multinucleation whose functional significance is unclear [19]. Our molecular understanding of epithelial cell-cell fusion comes from *C. elegans* [18] where fusion is developmentally programmed. Here, fusogenic genes whose loss of function suppresses fusion [20, 21] and whose ectopic expression induces fusion [21, 22] have been identified. By contrast, negative regulation of fusion is less well understood. A *C. elegans* vacuolar ATPase (vATPase) suppresses developmental syncytium

formation [23]. Physiologically induced epithelial cell-cell fusion, and its regulation, has not been studied in any context.

Jun N-terminal Kinase (JNK) signaling can respond to cell stresses via cell death, cell proliferation, and/or morphogenetic changes/migration. In the *Drosophila* embryonic epidermis JNK signaling is required for DC [24, 25], where it controls both actin dynamics and integrin expression [26]. In the larval epidermis, JNK signaling is dispensable for normal morphology although it is required for wound closure [10]. During wound healing, JNK regulates epidermal dedifferentiation [27] and expression of actin regulators [28]. Connections between integrin expression/function and JNK signaling in unwounded larval epidermis have not been examined.

We demonstrate here a role for the integrin FA complex in suppressing epithelial syncytium formation. Knockdown of the FA adaptor PINCH in the larval epidermis resulted in multinucleate epidermal cells even without physical wounding. Temporal and local knockdowns showed that β-integrin and ILK share this fusion-suppression property. We connected the syncytia observed upon wounding or genetic loss of FA components to the local JNK hyperactivation that occurred following either event. This hyperactivation could in turn disassemble FA complexes and drive syncytium formation, independently of effects on mitosis or apoptosis. Our results suggest that epithelial cells that do not normally fuse during homeostasis have fusion-suppressive mechanisms. Last, our results suggest that one of these mechanisms monitors proper integrin-based adhesion between neighboring epidermal cells.

RESULTS

PINCH Knockdown Leads to Syncytium Formation in the Larval Epidermis

To identify genes important for epithelial organization, we knocked down proteins expressed in the larval epidermis using *RNAi* transgenes. Several were FA proteins hypothesized as important for normal tissue architecture. β-integrin, ILK, and PINCH all localized to larval epidermal cell membranes (Figures S1A–S1C). Embryonic epidermal β-integrin or ILK knockdown was lethal. However, larval-specific epidermal knockdown led to undetectable protein (Figures S1D and S1E) leaving Fasciclin III unaffected (Figures S1G and S1H). PINCH knockdown at either stage was complete (Figure S1F; data not shown), but only early PINCH knockdown caused the sporadic appearance of large oddly shaped cells (Figure S1I). None of the FA knockdowns effectively blocked wound closure (Figure S1J) compared to other genes [14, 27, 28]. Also, none of the knockdowns affected attachment of the underlying basal lamina (Figures S1K–S1Z).

Were the large cells present upon PINCH knockdown multinucleate? In controls, occasional binucleate epidermal cells were seen near the square-shaped tendon cells that attach body-wall muscles at segmental borders (Figures 1A and 1C). All other cells were mononucleate (Figure 1A). By contrast, PINCH knockdown resulted in oddly shaped cells containing up to 20 nuclei whose presence was biased toward but not solely restricted to segmental borders (Figures 1B and 1E). Quantitation revealed that the syncytia per segment nearly doubled (Figure 1F) and that large syncytia (see Experimental Procedures) were never present in controls but always present upon PINCH knockdown (Figure 1G). Thus, PINCH knockdown increases syncytia and nuclei per syncytium.

PINCH knockdown larvae also lost clear segmental borders (Figure 1B) and had detached muscle fibers apparently from misaligned tendon cells (Figure 1D; compare to control in Figure 1C). In controls, each segmental border was defined by a row of tendon cells [29] whose nuclei were not labeled by an epidermal-specific marker (Figure 1A). *PINCH^{RNAi}*-expressing larvae lacked such clearly defined rows (Figure 1B), which likely accounted for muscle fiber release from the muscle attachment site (MAS) (Figure 1D) and their locomotion defects (Movie S1). These tendon cell/muscle defects and the spatial bias of syncytia for segmental boundaries suggest that these tissues contribute to the syncytium phenotype.

We ruled out RNAi off-target effects through genetic rescue. Epidermal expression of *PINCH* did not lead to large syncytia (Figure 1G). Co-expression with *PINCH^{RNAi}* greatly reduced the proportion of larvae with large syncytia (Figure 1G). Such robust rescue was not observed with irrelevant transgenes (data not shown). In summary, the PINCH knockdown phenotypes suggest that PINCH plays a role in suppressing epidermal syncytium formation and stabilizing muscle attachment during larval homeostasis.

The PINCH Epidermal Syncytium Phenotype Requires Early RNAi Expression in Adjacent Epidermal and Tendon Cells

The multiple tissues (epidermis, tendon cells, muscles) affected upon epidermal expression of *PINCH^{RNAi}* were surprising given the reported epidermal specificity of *e22c-Gal4* [27]. Lineage tracing using a Flp-out Gal4 cassette (see Experimental Procedures) determined that *e22c-Gal4* and *pannier-Gal4*, but not *A58-Gal4*, exhibit early tendon cell expression that is later lost (Figures S2A–S2F).

To test whether early expression of *PINCH^{RNAi}* in tendon cells contributed to epidermal syncytium formation, we performed conditional knockdowns (diagrammed in Figure 2A). At L3, *pannier-Gal4* is restricted to a patch of dorsal epidermal cells within each segment (Figure 3A). Earlier, it drives expression in all dorsal epidermal cells and tendon cells (Figures S2A and S2B). Full Gal4 repression throughout early development and beyond in control and RNAi-bearing larvae blocked syncytia, dsRed2Nuc expression, and muscle detachment (Figures 2B, 2C, 2H, and 2I). By contrast, a 1-day pulse of RNAi expression during embryogenesis resulted in syncytia centered at segmental boundaries (Figure 2J) that were absent in parallel controls (Figure 2D). Accompanying their appearance, muscle fibers detached from the MASs connected to these tendon cells (Figure 2K). Following a 2-day pulse (see controls in Figures 2F and 2G), these syncytia gained dsRed2Nuc expression, confirming their multinuclearity. Further, they spread beyond the segmental boundaries (Figure 2L). Muscle detachment worsened (Figure 2M).

One possibility for how syncytia arise is developmental mis-specification of tendon cells. However, larvae expressing *PINCH^{RNAi}* still stained for a tendon cell marker [30] in these misaligned cells (Figures 2N–2Q). Further, knocking down PINCH using a tendon cell-specific Gal4 driver did not cause local syncytia (Figures S2P–2T). Importantly, restricting *PINCH^{RNAi}* expression to the late larval epidermis (see schematic

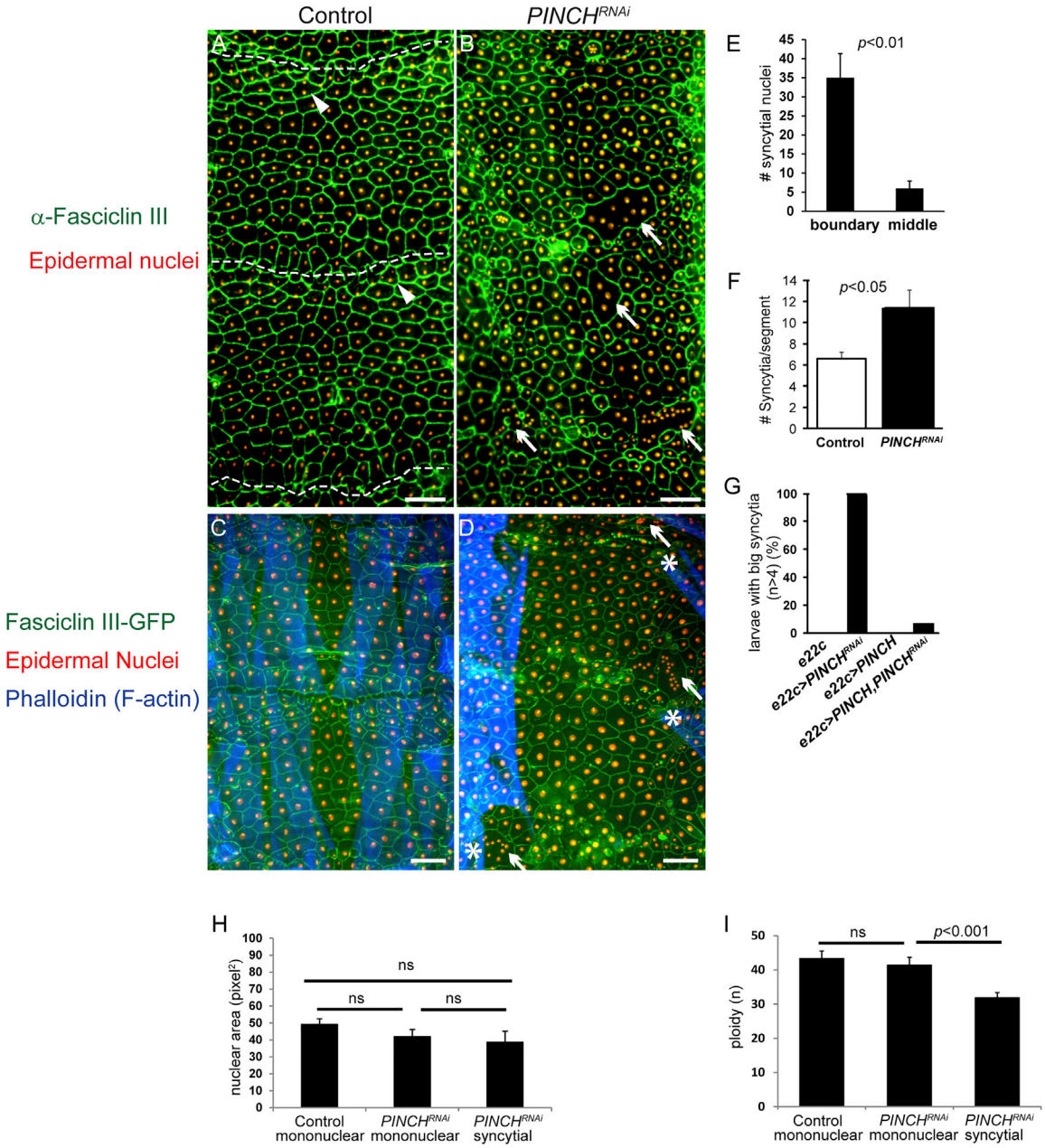

Figure 1. Epidermal *PINCH* Knockdown Leads to Excessive Syncytia in the Unwounded Larval Epidermis

(A–D) Whole mounts (*e22c-Gal4*, *UAS-dsRed2-Nuc*) immunostained with anti-Fasciclin-III (green) and Phalloidin (blue, C and D). Nuclei, red. (A and C) Control. Arrowheads, syncytia; dashed lines, tendon cells. (B and D) *UAS-PINCH^{RNAi}*. Arrows, syncytia. Asterisks, detached muscles. Scale bars, 100 μm.

(E) Spatial bias of syncytial nuclei for boundaries. n = 5 segments.

(F) Syncytia per segment in (A) and (B). n ≥ 22.

(G) Big syncytia in indicated genotypes. n ≥ 10 larvae.

(H) Nuclear area in indicated groups/genotypes. Control: 550 nuclei/five larvae; *UAS-PINCH^{RNAi}* mononucleate: 851 nuclei/six larvae; *UAS-PINCH^{RNAi}* syncytial: 136 nuclei/six larvae.

(I) Ploidy in indicated groups/genotypes. Control: 129 nuclei/six larvae; *UAS-PINCH^{RNAi}* mononucleate: 95 nuclei/four larvae; *UAS-PINCH^{RNAi}* syncytial: 109 nuclei/four larvae. Stats: Student's t test, ns ($p > 0.05$).

Error bars, SEM. See also Figure S1 and Movie S1.

Figure S2G) did not cause syncytia or muscle detachment (Figures S2H–S2O). Taken together, these results suggest that early expression of *PINCH^{RNAi}* in adjacent tendon and epidermal cells initiates syncytium formation between them with concomitant muscle detachment. Subsequently, these syncytia expand into the adjacent epidermal segments.

If epidermal syncytia arise from an early defect in adjacent epidermal and tendon cells then constitutive expression of

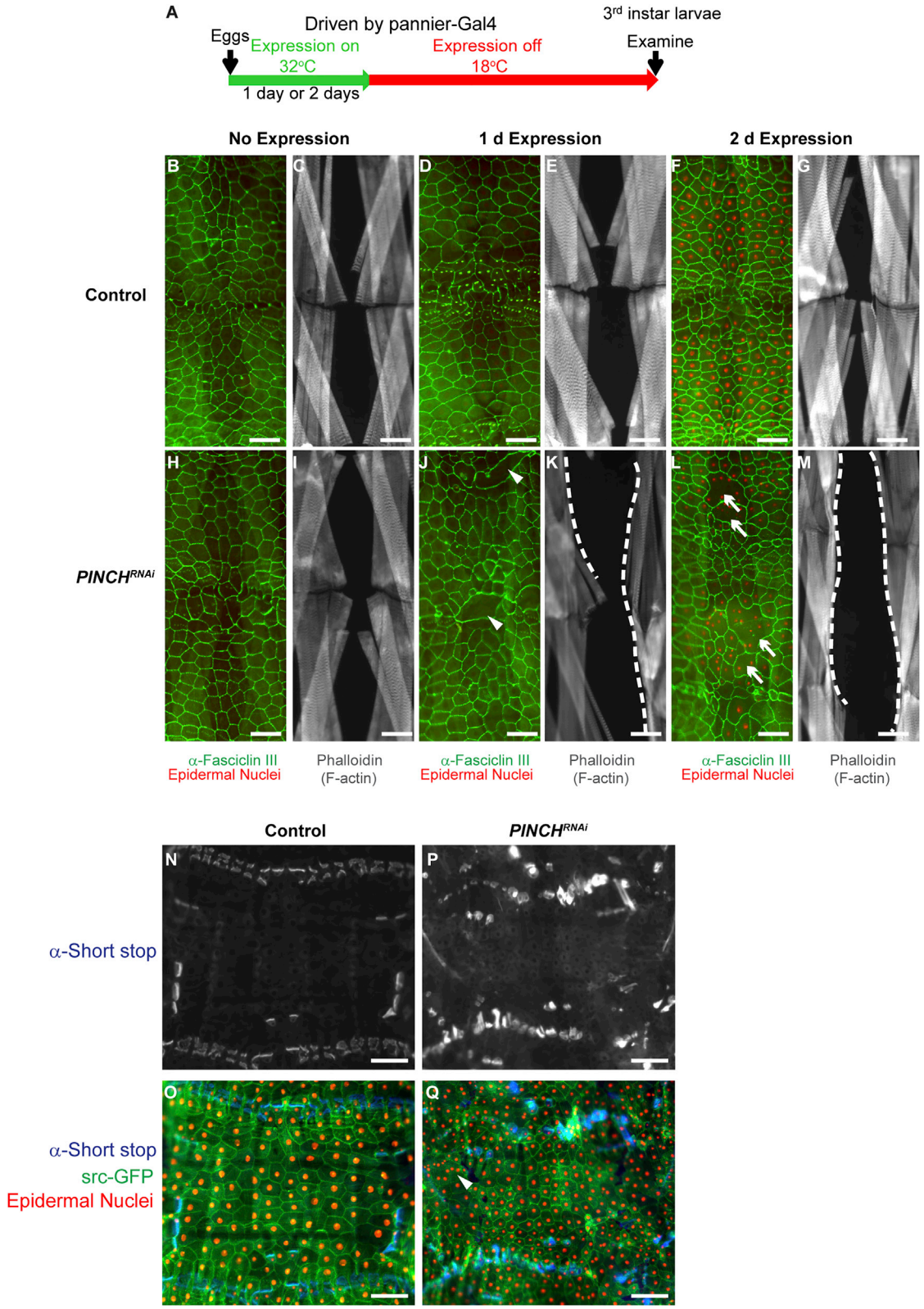

Figure 2. Early PINCH Knockdown Induces Syncytia and Muscle Detachment without Affecting Tendon Cell Differentiation

(A) Temporal transgene expression strategy schematic.

(B–M) Whole mounts of *tubulin-gal80^{ts}*, *pannier-Gal4, and UAS-dsRed2Nuc* crossed to *w^{1118}* (B–G) or to *UAS-PINCH^{RNAi}* (H–M) immunostained with anti-Fasciclin-III (B, D, F, H, J, and L; green) or phalloidin (C, E, G, I, K, and M; gray). Nuclei, red. (B and C) Control, no temperature shift (TS). (D and E) Control, 1 day

(legend continued on next page)

PINCH^{RNAi} and *eGFP* via *pannier-Gal4* (see control late expression pattern, Figure 3A) might lead to labeled intra-patch syncytia reflecting late driver expression. In addition unlabeled ''boundary'' syncytia sandwiched between the patches (reflecting early tendon cell expression) might also arise. Indeed, both types were observed (Figures 3B, S3A, and S3B). Intra-patch syncytia contained more nuclei (Figure S3C) and were more numerous (Figure S3A) than the occasional binucleate control cells. Boundary syncytia (defined in Supplemental Information) were biased for the anterior/posterior patch borders housing tendon cells (Figure S3D). Syncytia were never present in controls but *PINCH*^{RNAi}-expressing larvae contained more than one per segment (Figures 3B and S3A). DAPI labeling confirmed their multinuclearity (Figure 3B, inset).

Regional β-Integrin and ILK Knockdown Also Leads to Exuberant Syncytia

We next tested whether syncytium suppression was a general property of integrin FA proteins. RNAi transgenes targeting ILK and β-integrin were lethal with *e22c-Gal4.* Expression via *A58-Gal4*, which omits tendon cells (Figure S2F), did not cause syncytia (Figures S1G and S1H). Therefore, we tried *pannier-Gal4*, hoping that its early tendon cell expression and later spatially restricted epidermal expression might permit survival and reveal phenotypes. As with *PINCH*^{RNAi} (Figures S3E and S3F) both *ILK*^{RNAi} and *β-integrin*^{RNAi} led to undetectable target protein levels within the *pannier-Gal4* patches (Figures S3G, S3H, 4A, and 4D). Both also caused intra-patch and boundary syncytia (Figures 3C and 3D). β-integrin knockdown strongly altered epidermal morphology, with adjacent *pannier-Gal4* patches nearly merging (Figure 3D). For *PINCH* and *β-integrin*, independent *RNAi* transgenes targeting non-overlapping gene regions gave similar phenotypes (Figure 3I), ruling out RNAi off-target effects. In these cases *pannier-Gal4*-mediated knockdown also disrupted muscle attachment (cf. Figures 3F and 3H [detachment] with Figures 3E and 3G [no detachment]).

We also tested whether epidermal syncytia could result from perturbations to adhesion/polarity. We targeted Adherens Junctions (AJ), E-Cadherin (ECAD); Septate Junctions (SJ), Fasciclin III (Fas III), and Neuroglian (Nrg); the apico-basal polarity complex (AP), Lethal Giant Larvae (Lgl), and Discs Large (Dlg); and the basal polarity complex (BP), Bazooka (Baz). Each protein had robust antibodies that allowed protein knockdown verification upon epidermal expression of gene-specific RNAi transgenes: Fasciclin III (Figures S3I–-S3K); Lgl (Figures S3N–-S3P); Bazooka (Figures S3S–S3U); and ECAD (Figures S3X–S3AA). In no case did knockdown of these adhesion/polarity components exceed 40% syncytia (most lines 0%–20%) (Figure 3I), while FA protein knockdowns were mostly in excess of 60% (most lines 80%–90%). Knockdowns of Fasciclin III (Figures S3L and S3M), Lgl (Figures S3Q and S3R), Bazooka (Figures S3V and S3W), and E-Cadherin (Figures S3AB–S3AD) revealed normal epidermal morphologies despite the absence of the targeted proteins. In summary, suppression of epidermal syncytium formation is a general property of certain proteins that contribute to integrin-mediated adhesion.

Syncytium Formation after β-Integrin Knockdown or Wounding Involves Cytoplasmic Content-Mixing and Membrane Breakdown

Multinucleate cells can arise by nuclear division without cytokinesis or by membrane breakdown and subsequent cytoplasmic content mixing. Which mechanism occurs in the PINCH-deficient epidermis? Consistent with the epidermis being post-mitotic and endoreduplicated, anti-phospho-Histone H3 staining revealed no nuclear mitosis in both control (data not shown) and syncytia within the *PINCH*^{RNAi}-expressing tissue (Figures S5A and S5B). In addition, nuclear sizes were equivalent within cells in controls, and mononuclear or syncytial cells of *PINCH*^{RNAi}-expressing larvae (Figure 1H), and ploidy did not reduce greater than 2-fold (Figure 1I) arguing against reductive nuclear division.

To examine cytoplasmic mixing we observed the edges of *pannier-Gal4* patches expressing *eGFP* and *β-integrin*^{RNAi}. Here, either GFP or cytoplasmic β-integrin might traffic from cell to cell. In controls, β-integrin was primarily membrane-localized, with some protein cytoplasmic (Figures S1A and 4A). In the center of *β-integrin*^{RNAi}-expressing patches, β-integrin was absent (Figure 4D). At the margins, however, some individual patch-localized cells expressing *eGFP/β-integrin*^{RNAi} still contained cytoplasmic β-integrin whereas some non-patch cells acquired GFP (Figures 4D–4F and S4). Such cells could have acquired β-integrin via cell-cell fusion with their immediate neighbors outside the patch.

More conclusive evidence for cell-cell fusion required live imaging. We used laser wounding of the pupal epithelium because pupae are less motile and contractile than larvae. The unwounded pupal epithelium also contains highly regular mononucleate cells (Figure 4G). Live imaging of pupae expressing E-Cadherin-GFP revealed some cell membranes near the wound disintegrating so that larger presumably multinucleate cells formed within 1 hr of wounding (Figures 4H–4K and Movie S2) and persisted for up to 2.5 hr (Figures 4L and 4M). No mitotic figures were observed in these cells. Taken together, our results indicate that syncytium formation following wounding derives from a unique form of cell-cell fusion precipitated by locally destabilized adhesion.

Blocking Cell Division Rules Out a Mitotic Contribution to Wound-Induced and Loss of PINCH-Induced Epidermal Syncytium Formation

We took a parallel genetic approach to test a role for mitosis. Because of epidermal endoreduplication, we identified inhibitors of G2>M to block mitosis without affecting S phase. First, we tested whether *RNAi*-mediated knockdown of G2>M promoters in eye imaginal discs could ablate tissue growth. *RNAi*

expression. (F and G) Control, 2 days expression. (H and I) *UAS-PINCH*^{RNAi}, no TS. (J and K) *UAS-PINCH*^{RNAi}, 1 day expression. Arrowheads, syncytia near segmental borders. Dashed lines, muscle detachment. (L and M) *UAS-PINCH*^{RNAi}, 2 days expression. Arrows, syncytia. Dashed lines, detached muscles. Scale bars, 100 μm.

(N–Q) Whole mounts (*e22c-Gal4*, *UAS-src-GFP, UAS-dsRed2-Nuc*) immunostained with anti-Short stop (gray, N and P; blue, O and Q). (N and O) Control. (P and Q) *UAS-PINCH*^{RNAi}. Membranes, green; nuclei, red; arrowhead, large syncytium.

Scale bars, 100 μm. See also Figure S2.

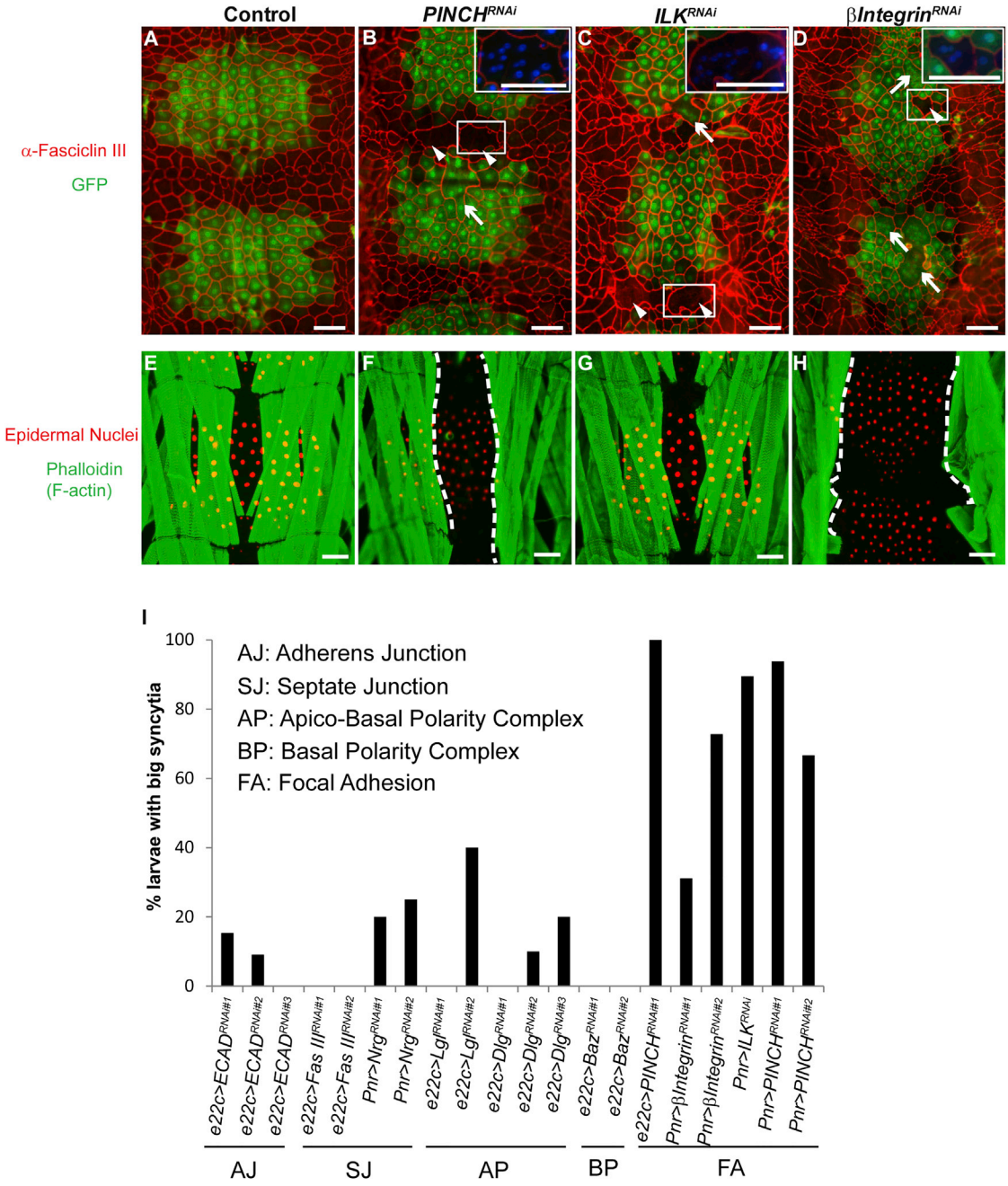

Figure 3. Other FA Protein Knockdowns Also Cause Syncytia and Muscle Detachment

(A–H) Whole mounts (*pannier-Gal4*, *UAS-eGFP*, green) immunostained with anti-Fasciclin-III (red) (A–D) and DAPI (blue) (insets in B–D) or expressing *UAS-DsRed2Nuc* (red) (E-H) stained with Phalloidin (green) (E–H) and expressing the indicated transgenes. (A and E) Control. (B and F) *UAS-PINCH^{RNAi}*, (C and G) *UAS-ILK^{RNAi}*, (D and H) *UAS-β-integrin^{RNAi}*. Arrows, intra-patch syncytia; arrowheads, boundary syncytia; insets, DAPI-stained nuclei in boundary syncytia; Dashed lines, muscle detachment. Scale bars, 100 μm.

(I) Big syncytia in RNAi knockdown larvae of the indicated genotypes.

See also Figure S3.

transgenes targeting *ial* (*aurora-like kinase*) and *cdc2* (*cyclin dependent kinase-2*) attenuated disc growth (Figures S5C–S5G) suggesting an efficient cell-cycle block. We then tested whether these could block the various types of experimentally induced epidermal syncytium formation.

Wounded control larvae developed a central syncytial cell surrounding the wound (Figures 5A, 5K, and 5L). Global epidermal expression of *cdc2^{RNAi}* or *ial^{RNAi}* (Figures 5B and 5C) did not alter the nuclear numbers in the central syncytium (Figure 5L). By contrast, *PINCH^{RNAi}*-expressing larvae had

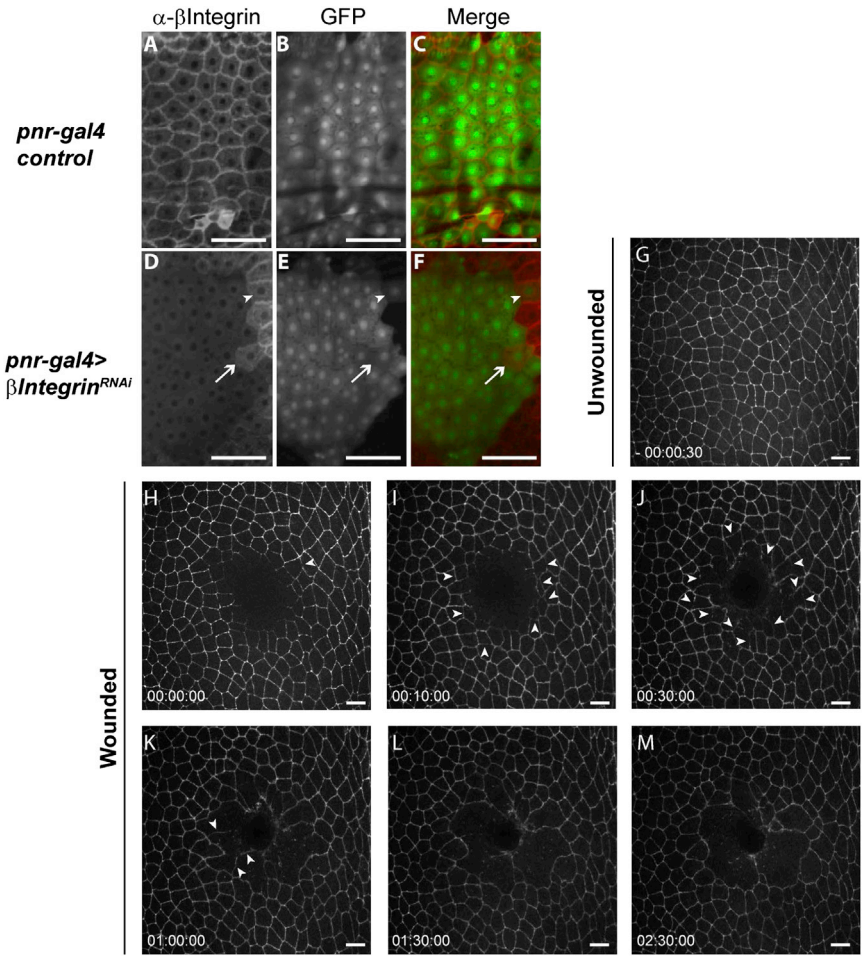

Figure 4. Epidermal Syncytia Formation on β-Integrin Knockdown or Wounding Is via Epidermal Cell-Cell Fusion

(A–F) Whole mounts stained with anti-β-integrin bearing *pannier-Gal4*, *UAS-eGFP* alone (A–C) or *UAS-β-integrin*^{RNAi} (D–F). (A and D) α-β-integrin. (B and E) *UAS-eGFP*. (C and F) Merge. Arrow, cell at patch boundary expressing *UAS-eGFP* containing cytoplasmic but not membrane β-integrin. Arrowhead, boundary cell expressing *UAS-eGFP* with both cytoplasmic and membrane β-integrin. Scale bars, 100 μm.

(G–M) Movie stills of unwounded (G) and wounded (H–M) live pupae expressing E-Cadherin-GFP. Arrowheads, disintegrating membranes.

Scale bars, 100 μm. See also Figure S4 and Movie S2.

more nuclei in their central syncytia and syncytia beyond the wound (Figures 5D and 5L). Combining *cdc2*^{RNAi} or *ial*^{RNAi} with *PINCH*^{RNAi} in the unwounded epidermis (Figures 5E–5J) did not suppress *PINCH*^{RNAi}-induced exuberant syncytium formation (Figure 5M). Neither transgene, either alone or together, caused activation of mitosis (Figures S5N–S5T). Together, these results confirm that mitotic progression does not contribute to syncytial formation following epidermal wounding or global epidermal PINCH loss.

JNK Signaling Hyperactivation Correlates with and Can Drive Epidermal Cell-Cell Fusion

The cellular similarities between wound-induced epithelial fusion and loss-of-FA-induced syncytium formation prompted a search for molecular regulators. JNK signaling activation correlates with wound-induced cell-cell fusion [10]. We therefore tested JNK activation following global epidermal PINCH knockdown. In controls, basal epidermal JNK activation, assessed with *msn-lacZ* [10], was low (Figure 6A). In the *PINCH*^{RNAi}-expressing epidermis, however, it was locally higher within or bordering syncytia (Figure 6B, arrows). In the wounded epidermis, JNK loss cannot suppress wound-induced cell-cell fusion [10]. Similarly, co-expressing *JNK*^{RNAi} did not suppress *PINCH*^{RNAi}-induced syncytia (Figure 6C). However, the spatial correlation between high JNK activity and cell-cell fusion prompted us to test whether local JNK hyperactivation without wounding could drive syncytium formation.

We conditionally expressed (see Experimental Procedures) a constitutively activated Jun kinase kinase *hemipterous* (*hep*^{CA}) in *pannier-Gal4* patches to locally activate JNK signaling. In controls, cells within and near the patches were uniform in size and shape and predominantly mononuclear (Figures 6D and 6E). Temperature-shift (TS) induced DsRed2Nuc expression but did not alter cell morphology (Figure 6E). In *hep*^{CA}-expressing larvae without TS, epidermal morphology was normal and DsRed2Nuc absent (Figure 6F). However, 16 hr after TS, nearly all patch cells lost Fasciclin III staining between adjacent nuclei and a giant syncytium comprising the entire *pannier-Gal4* expression domain formed (Figure 6G). The syncytial nuclei were slightly larger than control nuclei, arguing against nuclear division/aborted cytokinesis (Figure 6H). Further, nuclear number was not changed within the patch (60.5 ± 4.9 nuclei [controls] and 62.3 ± 9.8 [*hep*^{CA}]). In sum, these data suggest that JNK hyperactivation can directly drive epidermal syncytium formation.

To genetically test whether mitosis was involved in JNK hyperactivation-induced epidermal syncytium formation, we co-expressed G2>M inhibitors and *hep*^{CA} in *pannier-Gal4* patches. In control patches, epidermal cells were mononuclear (Figure 6I), whereas TS-induced expression of *hep*^{CA} caused syncytia encompassing most of the dorsal patch (Figure 6J). *cdc2*^{RNAi} and *ial*^{RNAi} alone did not affect epidermal morphology (Figures 6K and 6M) and did not suppress *hep*^{CA}-induced syncytia (Figures 6L and 6N). The number of syncytial nuclei was equivalent upon mitosis inhibition (Figures 6O and S5U–S5Z). Thus, mitosis is dispensable when syncytium formation is mediated by JNK hyperactivation.

Given the intimate relationship between JNK signaling and cell death in many epithelial tissues [31, 32], the possibility remained that syncytium formation might result from JNK-mediated apoptosis. To test this, we combined inhibitory transgenes of apoptosis with wounding, global expression of *PINCH*^{RNAi}, or

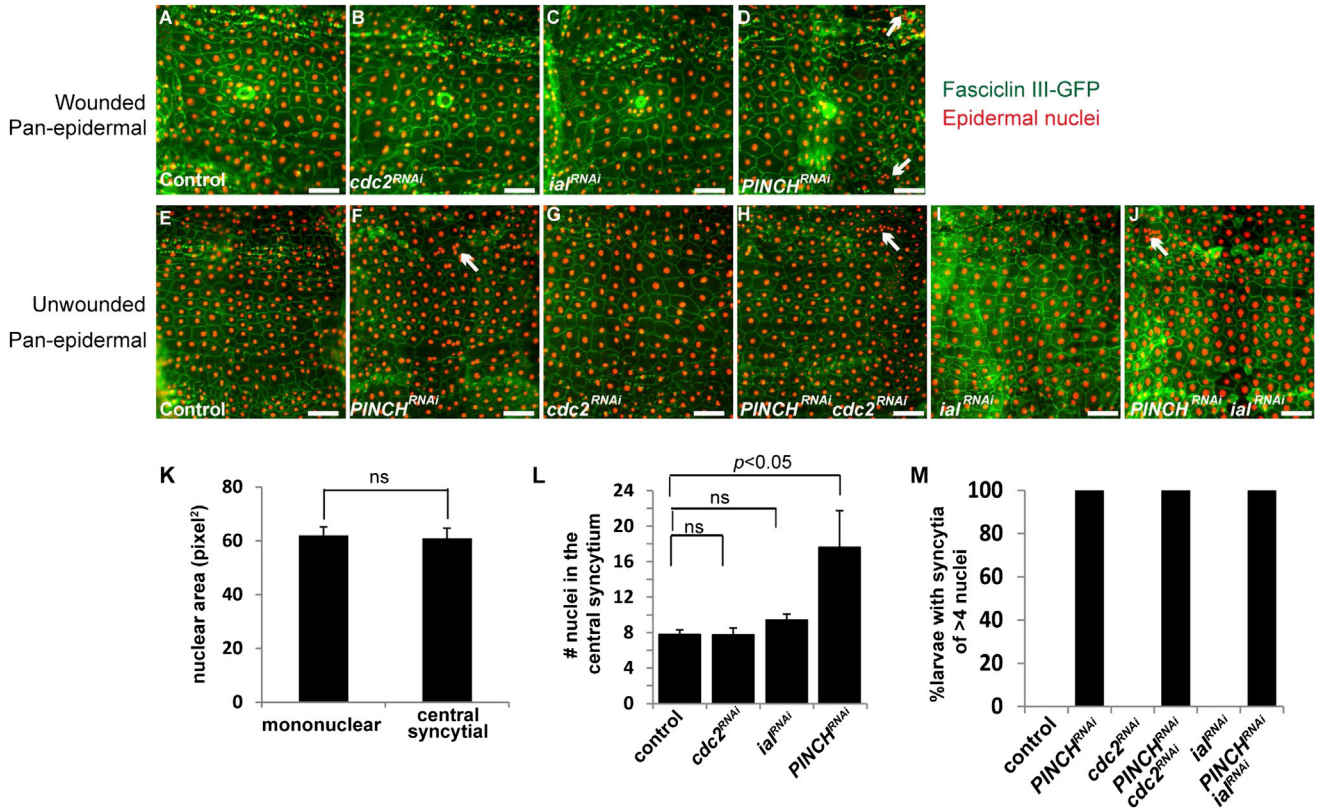

Figure 5. Misregulated Cell Division Is Not the Mechanism of Wound- and *PINCH*^{RNAi}-Induced Cell-Cell Fusion

(A–J) Whole mounts bearing *e22c-Gal4*, *UAS-DsRed2Nuc* (red), the indicated transgenes, and *Fasciclin-III-GFP* (green). (A–D) 24 hr post puncture wound. (A) Control. (B) *UAS-cdc2*$^{RNAi\#2}$. (C) *UAS-ial*$^{RNAi\#2}$. (D) *UAS-PINCH*^{RNAi}. (E–J) Unwounded. Samples co-stained with anti-phospho-Histone H3 (see Figures S5N–5S) to assess mitosis. (E) Control. (F) *UAS-PINCH*^{RNAi}. (G) *UAS-cdc2*$^{RNAi\#1}$. (H) *UAS-PINCH*^{RNAi} and *UAS-cdc2*$^{RNAi\#1}$. (I) *UAS-ial*$^{RNAi\#2}$. (J) *UAS-PINCH*^{RNAi} and *UAS-ial*$^{RNAi\#2}$. Arrows, big syncytia. Scale bars, 100 μm.

(K) Average nuclear area of cells in (A) ($n \geq 63$).

(L) Nuclei in central wound-induced syncytia in (A)–(D). $n \geq 4$ larvae. For (K) and (L), error bars, SEM ns ($p > 0.05$), Student's t test.

(M) Larvae with big syncytia in (E)–(J). $n \geq 5$.

See also Figure S5.

local expression of *hep*^{CA} to test whether blocking apoptosis altered the progression of epidermal syncytium formation in any case (Figures S6A–S6AD). As with our analysis of mitosis, we found that apoptosis is not required for syncytium formation in any of the above cases.

Wounding Relocalizes PINCH and ILK from Epidermal Cell Membranes near the Wound

If mitosis and apoptosis do not control epidermal syncytium formation, how do these multinucleate cells form? We hypothesized that wounding might destabilize FA complexes at the epidermal membranes near the wound, leading to subsequent fusion. We thus examined PINCH and ILK localization following wounding or JNK hyperactivation. Puncture wounding rapidly relocalized both proteins. In cells proximal to the wound PINCH moved from epidermal membranes (Figure 7A) to the cytoplasm as early as 4 hr post wounding and completely by 8 hr (Figure 7B), when even more distal cells lacked membrane-localized PINCH. By 24 hr, PINCH was still cytoplasmic in proximal cells but reappeared on the membranes more distally (Figure 7C).

Similar to PINCH, ILK was localized to unwounded epidermal cell borders (Figure 7D). At 4 hr, ILK in wound-proximal cells relocalized to the nucleus while in more distal cells some was retained on the membrane (Figure 7E). By 24 hr, membranes proximal to the wound, including the central syncytial border, possessed ILK while nuclear localization diminished (Figure 7F). Wounding thus provoked a striking relocalization of PINCH and ILK, indicating disassembly of functional FA complexes concomitant with syncytium formation (Figure S6AE). This disassembly was strongest in the proximal cells that contribute syncytial nuclei.

FA Protein Relocalization Precedes Cell-Cell Fusion upon JNK Hyperactivation

Finally, we tested whether JNK hyperactivation without wounding also led to FA protein relocalization. We examined the levels and localization of PINCH, ILK, and β-integrin in *pannier-Gal4* patches where *hep*^{CA} expression was induced. Immediately before TS, Fasciclin III (Figures 7K and 7S), PINCH (Figure 7G), ILK-GFP (Figure 7O), and β-integrin (Figure 7W) were primarily on epidermal cell borders. From 8 to 16 hr after TS, however,

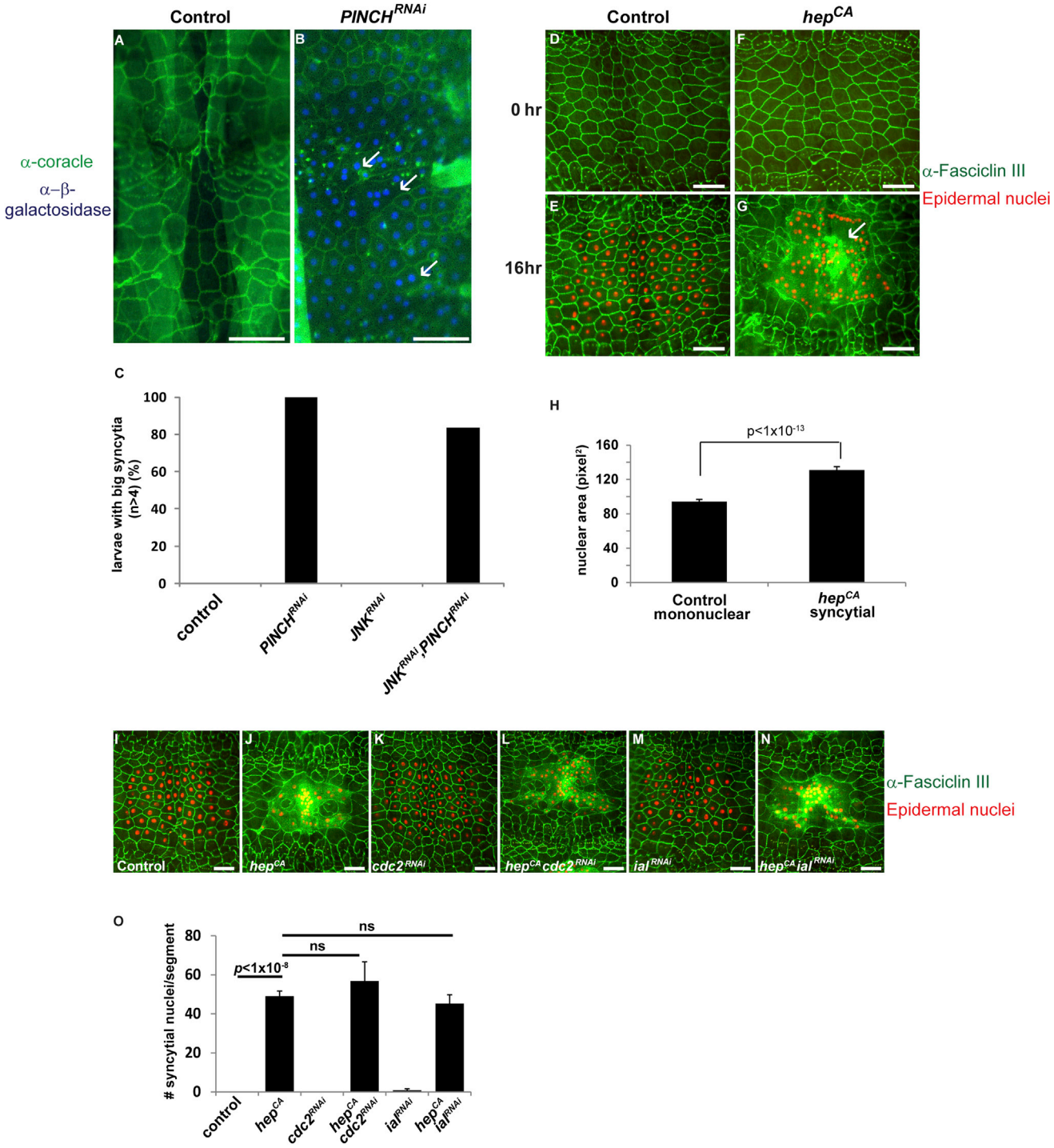

Figure 6. JNK Activation Correlates with and Can Drive Syncytium Formation in the Larval Epidermis

(A and B) Whole mounts bearing *e22c-Gal4, msn-lacZ* and the indicated transgenes immunostained with anti-coracle (green) and anti-β-Gal (blue). (A) Control. (B) *UAS-PINCH*^{RNAi}. Arrows, JNK^{+} nuclei. Scale bars, 100 μm.

(C) Big syncytia in larvae expressing transgenes via *e22c-Gal4*. n ≥ 9 larvae.

(D–G) Whole mounts bearing *tubulin-Gal80*^{ts}, *pannier-Gal4*, and *UAS-DsRed2Nuc* (red) without (D and F) or with (E and G) 16 hr TS activation of *UAS-hep*^{CA}, immunostained with anti-Fasciclin III (green). Arrow, giant syncytial cell in (G). Scale bars, 100 μm.

(H) Nuclear area in control and syncytia in (E) and (G). Control: 242 nuclei/four larvae; *UAS-hep*^{CA}: 374 nuclei/six larvae. Error bars, SEM ns ($p > 0.05$), Student's t test.

(legend continued on next page)

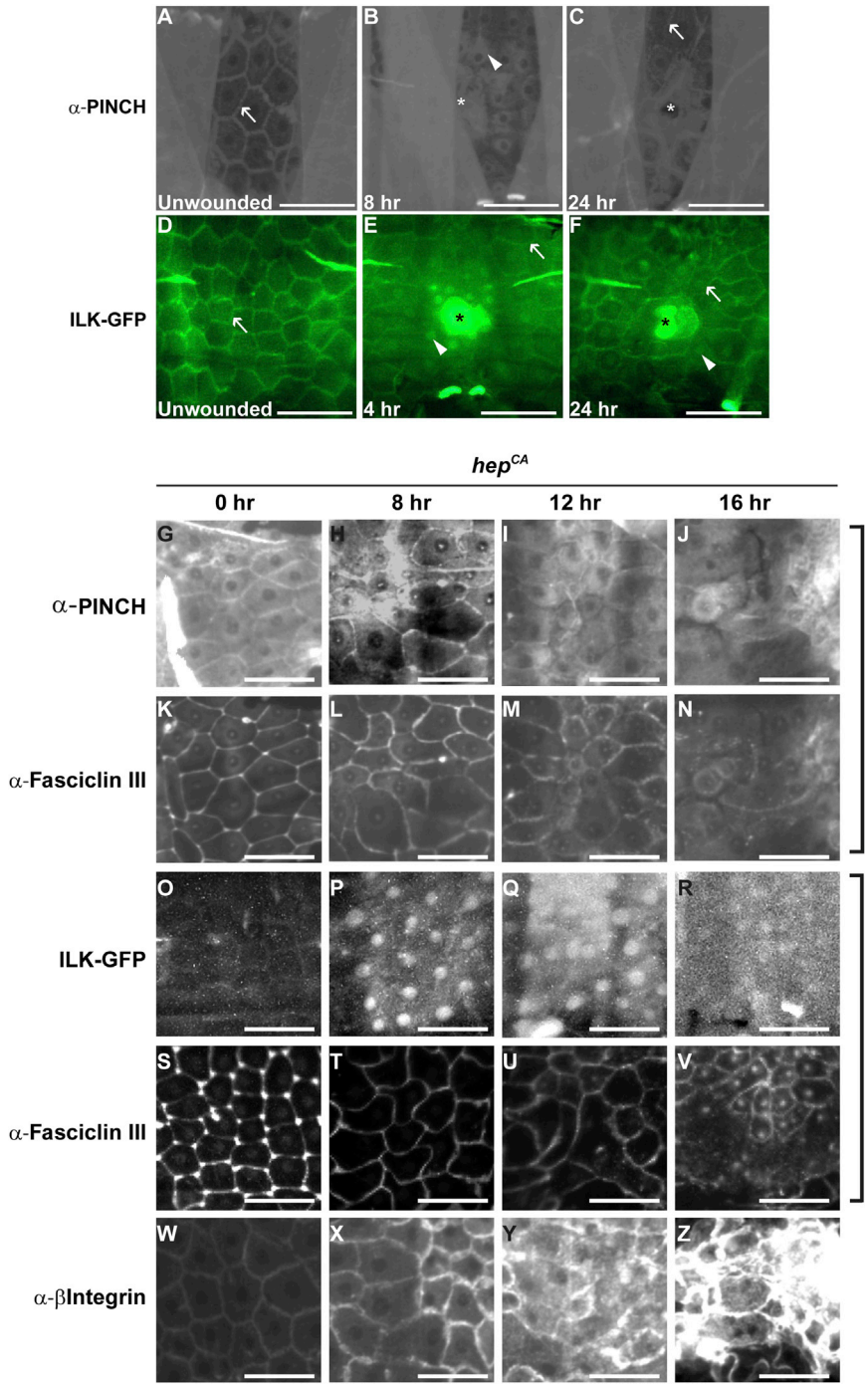

Figure 7. Disassembly of FA Complexes upon Wounding and JNK Activation

(A–F) Whole mounts of (w^{1118}) (A–C) or w;*ILK-GFP* larvae (D–F) immunostained with anti-PINCH (A–C) at indicated times after wounding. (A and D) Unwounded. (B, C, E, and F) Post-wounding. Arrows, membrane localization; arrowheads, cytoplasmic or nuclear localization; asterisks, wounds. Scale bars, 100 μm.

(G–Z) Whole mounts bearing *tubulin-Gal80^{ts}*, *pannier-Gal4*, and *UAS-hep^{CA}*, immunostained with indicated antibodies (G–N and S–Z) or visualized with ILK-GFP (O–R) at indicated times post-TS. For anti-PINCH (G–J), anti-Fasciclin (K–N = PINCH double stain, S–V = ILK-GFP double stain) and anti-β-integrin (W–Z), *UAS-eGFP* marked the *pannier-Gal4* patch except in *ILK-GFP* (O–R). Scale bars, 100 μm.

See also Figure S7.

PINCH levels increased and became progressively more cytoplasmic (Figures 7H–7J). Initially, Fasciclin III remained membrane-localized (Figure 7L), but 12–16 hr after JNK activation some membranes were losing protein or had lost it altogether (Figures 7M and 7N). ILK-GFP upregulation and relocalization to nuclei were similarly dramatic (Figures 7P–7R). As with PINCH, the relocalization of ILK-GFP preceded Fasciclin III loss (Figures 7T–7V). Lastly, at 8 hr β-integrin levels were markedly increased (Figure 7X), and by 12–16 hr this increase was accompanied by loss from some membranes (Figures 7Y and 7Z). Of note, FA complex loss in *pannier-Gal4* patches did not by itself lead to loss or relocalization of other adhesion/polarity complex proteins (AJ, SJ, AP, BP; see Figure S7), indicating that FA destabilization-induced syncytium formation is probably not through secondary effects on these other proteins.

Taken together, these data indicate that the mechanism of epidermal syncytium formation following wounding or JNK hyperactivation likely involves FA complex disassembly at the epidermal membrane.

DISCUSSION

Our data suggest a novel integrin adhesion complex function—suppressing larval epidermal syncytium formation. Three events that lead to epidermal syncytium formation all act through destabilization of integrin adhesion complexes: (1) direct genetic loss early in adjacent tendon and epidermal cells; (2) physical wounding; or

(I–N) Whole mounts bearing *tubulin-Gal80^{ts}*, *pannier-Gal4,* and *UAS-DsRed2Nuc* (red) and the indicated transgenes 16 hr after TS, immunostained with anti-Fasciclin III (green) and anti-phospho-Histone H3 (see Figures S5U–S5Z). (I) Control. (J) *UAS-hep^{CA}*. (K) *UAS-cdc2$^{RNAi\#1}$*. (L) *UAS-hep^{CA}* and *UAS-cdc2$^{RNAi\#1}$*. (M) *UAS-ial$^{RNAi\#1}$*. (N) *UAS-hep^{CA}* and *UAS-ial$^{RNAi\#1}$*. Scale bars, 100 μm.

(O) Total syncytial nuclei per segment in indicated genotypes (I–N). n ≥ 4. Error bars, SEM ns ($p > 0.05$), Student's t test.

See also Figure S5 and S6.

(3) epidermal JNK hyperactivation. Our graphical abstract summarizes these interactions. Below, we discuss the features and implications of this model both for integrin function and cell-cell fusion.

Interestingly, the syncytia in PINCH-knockdown epithelia localized near tendon cells. Lineage tracing of *e22c-Gal4* and *pannier-gal4* revealed early tendon cell expression before restriction to the epidermis. Early expression of $PINCH^{RNAi}$ in tendon cells and adjacent epidermal cells initiated syncytium formation. Expression solely in epidermal cells or tendon cells did not. We suspect that tendon cells, which depend on integrins to form proper MASs [33], represent tissue weak points sensitive to FA component loss. The physical strain of larval locomotion likely not only initiates syncytia (akin to physical wounding; see below), but also aids their propagation, leading to the later syncytia where over 20 nuclei are sometimes observed. This is similar to $\alpha6\beta4$ integrin and other skin-specific FA component knockouts [34–36]. Although the actual tissue morphology differs—skin blistering in mice versus syncytial formation in *Drosophila*—in both cases, mechanical disruption of the tissue leads to a phenotype that resembles skin wounding.

Integrins are among the main cellular mechanoreceptors. The JNK signaling pathway is also stress responsive and in many cells is activated by mechanical force [37, 38]. In *Drosophila* S2 cells, Integrin and Talin loss activates JNK signaling [39]. Here, pan-epidermal PINCH loss activates JNK signaling that spatially correlates with syncytium formation. This is consistent with PINCH's role as a repressor of JNK activation [40] in DC. Relocalized PINCH/ILK presumably cannot participate in functional adhesion, and their removal from the membrane may help initiate fusion.

Our results suggest a positive feedback loop between integrins and JNK signaling. Specifically, hyperactivation of JNK, in addition to correlating with loss of membrane-localized FA components, also drives their relocalization and syncytium formation. How might JNK signaling mediate FA complex disassembly? JNK can phosphorylate at least one FA complex member, Paxillin, in vertebrate cells [41], suggesting a possible direct role. We speculate that JNK activation may cause phosphorylation of other FA proteins leading to their disassembly or relocalization from the FA complex upon mechanical perturbation of the cell.

Our results also reveal a novel suppressive role for integrins in what may be a unique form of cell-cell fusion. In sperm-egg fusion, integrins play a positive role; an oocyte-localized integrin $\alpha6/\beta1$ heterodimer binds to a sperm-localized metalloprotease disintegrin to trigger fusion [42, 43]. Positive roles for $\beta1$, $\alpha3$, and $\alpha9$ integrins in myoblast fusion in vertebrates have also been reported [44, 45]. In both fertilization and myoblast fusion, integrins are thought to appose the fusing cells closely enough to enable fusogens to act. Indeed, in myoblast and macrophage fusion, Paxillin has a stabilizing effect on fusion [46]. We show here in the *Drosophila* larval epidermis that *loss* of FA components acts similarly. Since little is known about the negative regulation of cell-cell fusion, it will be interesting to investigate potential mechanistic connections between the integrin FA complex and a vATPase complex that suppresses epithelial cell-cell fusion [23].

Finally, our experiments suggest that this distinct form of cell-cell fusion does not involve altered cytokinesis/apoptosis and occurs via direct membrane breakdown. Initiation by membrane damage or destabilization of adhesion complexes distinguishes it from the developmentally programmed cell-cell fusions in the *C. elegans* hypodermis or skeletal muscle [18, 47]. Developmentally programmed and wound-induced epithelial fusions likely exhibit further differences during actual fusion. The chaotic nature of wound-induced fusion asks, does it involve induction, localization, or activation of a putative fusogen, as has been shown for hypodermal fusion in *C. elegans* [20, 21] and myoblast fusion [48]? Wound-induced fusion may instead result from spontaneous dissolution of integrin adhesion structures following direct physical membrane disruption. This dissolution seems to involve vesiculation of adjacent cell membranes, which are then resolved into a continuous cytoplasm containing the original fusion partner nuclei. Whatever the specific fusion mechanism, it is clear from this work that the larval epidermis, and perhaps other epithelia, possesses adhesion-based fusion-suppressive mechanisms that keep the unperturbed tissue mononucleate.

EXPERIMENTAL PROCEDURES

Fly Strains and Genetics

Crosses were performed on cornmeal/dextrose medium at 25°C unless noted. w^{1118} was control genotype. See Supplemental Information for more details.

Puncture and Pinch Wound Assays

Pinch and puncture wound assays were as described [10].

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.07.031.

ACKNOWLEDGMENTS

We thank members of M.J.G.'s lab for comments; Jodie Polan for confocal assistance; Guy Tanentzapf, Andreas Wodarz, and Talila Volk, for fly stocks/antibodies; the Bloomington Drosophila Stock Center, the Vienna Drosophila RNAi Center, and the Kyoto stock center for fly strains; and the Developmental Studies Hybridoma Bank for antibodies. This work was supported by a March of Dimes Basil O'Connor Award (5-FY06-588) and NIH R01 GM083031 to M.J.G., NIH R01 GM084103 to J.L.K., and European Research Council Starting Grant (2007-StG-208631) to A.J.

Received: September 2, 2014
Revised: June 15, 2015
Accepted: July 10, 2015
Published: August 6, 2015

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