After wounding, a G-protein coupled receptor restores tension to epithelial cells in a dynamic inward-traveling wave

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Abstract

The maintenance of epithelial barrier function is due in part to cellular tension, with cells pulling on their neighbors to maintain epithelial integrity. Wounding interrupts cellular tension and wound-induced changes in tension may serve as an early signal to initiate epithelial repair. To characterize how wounds alter cellular tension, we used a laser-recoil assay to map cortical tension around wounds in the epithelial monolayer of the *Drosophila* pupal notum. Within a minute of wounding, there was widespread loss of cortical tension along both radial and tangential directions. This tension loss was similar to levels observed with Rok inactivation. Tension was subsequently restored as an inward traveling wave that reached the wound margin about 10 minutes after wounding. Restoring tension required the GPCR Mthl10 and the IP3 receptor, indicating the importance of this calcium signaling pathway known to be activated by cellular damage. The wave of tension restoration correlated with an inward-moving contractile wave that has been previously reported; however, the contractile wave itself was not affected by Mthl10 knockdown. These results indicate that cells may transiently increase tension and contract in the absence of Mthl10 signaling, but that pathway is critical for fully resetting baseline epithelial tension after it is disrupted by wounding.

Introduction

In response to epithelial damage, an organism must have robust mechanisms for wound repair or else risk exposure to dangers such as infection. Many aspects of the epithelium are damaged by wounding: some cells are lost or die, other cells are damaged but recover, and yet other cells lose cortical tension (O'Connor *et al.*, 2021a). In healthy epithelia, tension is maintained by cells pulling on each other through cellular junctions tethered to the actin cytoskeleton. Tension forces are usually balanced, so cells do not move, but temporary adjustments in tension allow epithelial cells to alter their positions in response to the internal or external environment while still maintaining their sheet-like organization (Fernandez-Gonzalez *et al.*, 2009; Zulueta-Coarasa and Fernandez-Gonzalez, 2018). By removing cells, wounds impair the ability of pairs of cells to maintain balanced tension. Thus, one aspect of epithelial wounding is a loss of cellular tension, and one aspect of repair is restoring that tension.

A wound-induced change in tension has been considered as a possible early wound signal, sensed by surrounding epithelial cells, triggering wound-repair behaviors (Xu and Chisholm, 2011; Antunes *et al.*, 2013; Enyedi and Niethammer, 2015; Cao *et al.*, 2017; Franco *et al.*, 2019); however, the extent of this potential signal around wounds has not yet been explored. For example, how far from the wound do changes in tension extend? Do tension levels change in a gradient from the wound? Further, specific mechanisms to restore cellular tension after wounding have not been identified. Here we answer these questions in the particular case of the *Drosophila* pupal notum.

The pupal notum epidermis is an epithelial monolayer that offers an excellent system to probe the dynamics of wound repair. This tissue can be imaged *in vivo* continuously – before, during, and after laser ablation, throughout the entire process of wound repair – and pupae can survive wounding and imaging to eclose and walk away (Shannon *et al.*, 2017). Using this system and harnessing the power of fly genetics, our laboratories previously identified a wound-induced signaling pathway that acts through the cell surface receptor Mthl10, a G-protein coupled receptor (GPCR) coupled to $G_{\alpha q}$. The wound-induced activation of Mthl10 is transduced downstream by $G_{\alpha q}$ and PLCβ, generating IP_3 to release Ca^{2+} from

internal stores through the IP_3 receptor (IP3R; O'Connor *et al.*, 2021b). Although Mthl10 improves viability after wounding, how its activation affects cells and promotes wound repair is not known. Two potential clues are that the second messenger downstream of Mthl10, i.e., Ca^{2+}, has a profound impact on the actin cytoskeleton (Wales *et al.*, 2016), and it has been shown that increasing Ca^{2+} alone is sufficient to induce cellular contraction (Kong *et al.*, 2019).

In this study, we investigated wound-induced changes in cellular tension. Using laser-induced recoil to infer cellular tensions quantitatively, (Ma *et al.*, 2009) we mapped changes in tension at different locations and time points after wounding. We found that that within one minute, tension was lost around the wound in a widespread and uniform manner, in both the radial and tangential directions. Tension was restored over the next ~10 minutes in a spatiotemporal pattern, from outward in, as a wound-induced contractile wave advanced toward the wound. Restoring tension required signaling by Mthl10 and its downstream release of calcium via the IP3R. Even when wounds were administered using a method that did not generate an evident contractile wave, *mthl10* was still required for the restoration of tension, suggesting that Mthl10 may restore tension around many types of epithelial wounds.

Results

Laser Recoil Measures Changes in Tension across the *Drosophila* Notum

An epithelial sheet consists of cells that are connected with each other under tension. Breaking those connections by creating a wound in the tissue would be expected to lead to changes in tension. To probe cortical tension around a wound, we used a laser-induced recoil assay. This assay is performed by severing an apical epithelial cell border with laser micro-ablation and measuring the retraction velocity of the corresponding tricellular junctions (TCJs) at the ends of the severed border; higher recoil velocities correspond to higher cortical tension, while lower recoil velocities correspond to lower cortical tension (Fig. 1D-D′, Movie 1). We measured tension at specific sites, using the border of the *pnr* domain as a landmark (Fig. 1B), and we measured tension in two axes, severing cell borders that were aligned with the anterior-posterior (AP) and the mediolateral (ML) axes of the tissue (Fig. 1C). To establish the assay and set a baseline, we measured tension in unwounded control samples to map epithelial tension across the notum. We found that tension was fairly constant at all locations tested for both ML and AP epithelial cell borders, with no evidence of location-specific or *pnr*-specific effects (Fig. 1E). This finding indicates that epithelial tension in the unwounded notum is reasonably constant.

Because there are no *pnr*-domain-specific effects in control pupae, we can drive genetic manipulations within the *pnr* domain using *pnr-Gal4*, with the neighboring domain acting as an internal control. It was unclear, however, whether a loss of tension generated in the *pnr* domain would equilibrate across the notum to the control domain or whether it would remain in the *pnr* domain. To determine if we could detect different levels of tension across the notum, we mapped tension in unwounded samples with *Rok RNAi* expressed in just the *pnr* domain. Rok, or Rho kinase, modulates stress and contractility by phosphorylating cytoskeletal proteins including myosin II, and inhibiting or knocking down *Rok* reduces cortical tension (Mizuno *et al.*, 1999). Interestingly, there was a decrease in tension at locations in the *pnr* domain with *Rok RNAi* compared to the control domain (Fig. 1F). This trend was clear for both ML and AP borders. Tension in the control domain was relatively constant and similar to previous levels; tension

in the *pnr>Rok RNAi* domain was also relatively constant, but at a decreased value. These results demonstrate that we can detect domain-specific changes in tension.

Wounding Reduces Tension Locally

Next we measured tension around wounds. Wounds were generated using the same laser used for measuring recoil velocity but at 4-fold higher power. Previous work by our group found that this type of laser ablation generates specific and reproducible types of cellular damage at different distances from the center of the wound (O'Connor *et al.*, 2021a). One of the most far-reaching types of damage is the loss of nuclear membrane integrity, visible in the *pnr* domain by the loss of mCherry.NLS from the nuclei, extending out ~70 µm from the center of the wound. We used this nuclear membrane damage as the landmark for the most proximal testing location, measuring tension just beyond this visible region of damage, and at two more fixed distances distal to this location on the *pnr* side. We mirrored these three locations on the other side of the *pnr* border, testing tension at the same three radii in the control domain. For these locations, ML borders were radial to the wound and AP borders were tangential to the wound. A few minutes after wounding control samples, tension was reduced in a gradient in both ML and AP cell borders (Fig. 1G), with the lowest tension found closest to the wound. This lowest tension was similar to the level observed with *Rok RNAi*. The tension for the furthest locations, ~210 µm from the wound, were similar to those of unwounded samples. As with unwounded samples, tension in the control domain was comparable to tension in the *pnr* domain. Thus, a few minutes after wounding, tension in apical epithelial cell borders increased as the distance from the wound increased.

Tension is Restored over Time

After generating a spatial map of tension around wounds, we generated a temporal map, investigating if post-wounding tension at a certain location changed over time. Our previous measurements were taken between 1 to 10 minutes after wounding. To create our temporal map, we measured tension at ~70 µm at 1-2 minutes and 15-18 minutes after wounding using mediolateral borders.

We observed a dramatic restoration of tension to pre-wound levels at the later times, indicating that tension is dynamically regulated around wounds (Fig. 2A).

Given these results, we realized the observed tension gradient around wounds had a temporal component, so we investigated if tension at distal locations would be reduced immediately (< 1 minute) after wounding. We measured tension in ML borders at ~135 µm from the wound about 30 seconds after wounding and compared it to tension at the same distance and orientation 5-6 minutes after wounding. Excitingly, at the 30 second time point, we observed an initial loss of tension at ~135 µm to levels similar to *Rok RNAi* (Fig. 2B). Although our previous measurements over a wider and slower time range had missed this initial decrease, tension was restored to pre-wound levels at this location by 5-6 minutes. Thus, cortical tension was restored in a temporal gradient – first distally, then proximally.

Tension is Restored as a Contractile Wave Moves toward the Wound

A previous study reported a mysterious phenomenon after laser wounding: a symmetrical wave around the wound that starts distally ~2 minutes after wounding and travels inward to the wound margin (Antunes *et al.*, 2013). The wave consists of successive synchronous cell contractions, beginning with an outer ring of cells contracting and expanding synchronously over ~ 5 minutes; as they expand, their proximal neighbors begin contracting. This pattern propagates inward across 4-5 rows of cells until the contracting ring reaches the cells at the leading edge of the wound ~12-14 minutes after wounding (Fig. 3A, Movie 2). Each cellular contraction is preceded by the temporary accumulation of actin and myosin at the apical cell surface (Antunes *et al.*, 2013; Fig. 3B-C, Movie 3). Given the involvement of actin and myosin in the contractile wave, we wondered if the cellular contractions might affect tension around the wound.

Previous measurements at ~70 µm indicate reduced tension levels at 2 minutes after wounding and restored tension levels after 15 minutes, with these two times bracketing the expected passage of the propagating contractile wave by this location (Fig. 3D). To directly test the role of the wave, we imaged the wave by tracking cell contraction around a wound and measured tension at ~70 µm before and after

the directly observed contractile wave passed through that location (Fig. 3E-E″, Movie 4). To ensure that the first measurement did not interfere with the second, measurements were acquired on the right and left side of the wound at ~70 µm. We found that the tension at ~70 µm was low at levels similar to *Rok RNAi* before the wave passed through and was then restored to near pre-wound levels shortly after the wave passed through the location (Fig. 3F). Thus, we conclude that tension is restored as the contractile wave passes through.

Mthl10 is Required to Restore Tension

Tension is restored remarkably quickly around the wound: at 135 µm from the wound, tension is restored by 5-6 minutes post-wound (Fig. 2B). Indeed, looking closely at the data in Fig. 1G, we found that by three minutes post-wound, tension was already restored for all mediolateral borders measured at 110 µm, with recoil velocities reaching an average of 41 nm/s (n=5). What kind of signal can act this fast? Our labs previously discovered that the GPCR Methuselah-like 10 (Mthl10) is activated in epithelial cells surrounding a wound between 45-75 seconds after wounding (Shannon *et al.*, 2017; O'Connor *et al.*, 2021b). The GPCR triggers the $G_{\alpha q}$-signaling pathway, resulting in the release of calcium via the IP_3 Receptor (IP3R) in the endoplasmic reticulum.

To investigate whether Mthl10 signaling modulates tension, we measured tension in both unwounded and wounded samples with *mthl10 RNAi* expressed in the *pnr* domain. Similar to previous unwounded control samples, unwounded tension was relatively homogeneous across both control and knockdown regions for both ML and AP cell borders (Fig. 4A). In the wounded samples, Mthl10 was required for restoring tension at all distances measured (Fig. 4B). These results were confirmed independently with a second *UAS-mthl10 RNAi #2* line (Fig. S1A). To test if Mthl10 signaling restores tension through IP3R, we knocked down *IP3R* and found that it was required to restore tension, indicating the release of calcium from the ER restores tension around wounds (Fig. 4C). We also tested an additional far distal location in the *pnr > IP3R* domain at ~270 µm from the wound and found unwounded levels of tension, indicating that wounding reduces tension in a limited region around the wound (Fig. S1B).

Surprisingly, knockdown of *mthl10* did not disturb the contractile wave (Fig. 4D-G; Movies 5 and 6). The wave was visible as it traveled across cell borders toward the wound, even in the absence of Mthl10, and the pattern of MyoII-GFP levels moving toward the wound was also similar between control and knockdown regions around the wound. To see if the rate of travel was altered by *mthl10* knockdown, we analyzed the location of the wave on the two sides of the wound (control and *pnr*) in both control samples (+ and +) and in samples in which *mthl10* was knocked down in the *pnr* domain (+ and *mthl10 RNAi*). We found that in both cases, the wave was at similar locations on the two sides of the wound, indicating that Mthl10 did not significantly alter the wave's rate of travel. Finally, when we knocked down *mthl10*, tension remained low over the first 15 minutes at ~70 µm from the wound (Fig. 4H) in contrast with the control data from Figure 2A. Thus, the contractile wave cannot restore tension in the absence of Mthl10.

Scanned Wounds Also Require Mthl10 to Restore Wound-Induced Tension Loss

The laser wounds we analyzed thus far generate several different types of cellular damage arrayed in a regular pattern around the center of the wound, including a large region where the plasma membrane is damaged and then repaired (O'Connor *et al.*, 2021a). To ask if these damaged cells contribute to the loss of tension around wounds, we measured tension around a different type of wound. To eliminate wound-induced plasma membrane damage, we used a scanning ablation technique in which low-energy laser pulses are scanned repeatedly across a circular area to lyse all cells; this process takes about 2 minutes. The scanned area matched the area of cell death in the wounds that we created previously. These wounds showed no evidence of a contractile wave (Fig. 5A,B; Movies 7 and 8). Within a few minutes after scanning ablation, we did not see any changes in tension across the notum in control samples (Fig. 5C); however, the longer duration of the scanning ablation may have allowed the restoration of tension to proceed even as the wound was being created. To test this possibility, we generated scanned wounds in pupae with *mthl10* knocked down in the *pnr* domain and found that tension levels in the *pnr* domain were reduced to levels similar to the *Rok* knockdown (Fig. 5D). Thus, we conclude that Mthl10 is required to

restore tension around epithelial wounds even in the absence of plasma membrane damage, suggesting that epithelia generally lose tension around wounds, and then tension is restored by Mthl10 signaling.

Discussion

In this study, we used a laser-induced recoil assay to measure changes in cortical tension after epithelial wounding in the *Drosophila* notum. Although before wounding, epithelial cells have relatively constant cortical tension, we find that wounds trigger loss of tension across a discrete area. Within this area, tension is reduced uniformly, to similar levels as when *Rok* is knocked down, and to similar extents along orthogonal axes. Tension is then restored over 2-10 minutes – first at locations distal to the wound and proceeding proximally. This restoration of tension requires calcium signaling through the IP_3 Receptor downstream of the wound-responsive G-protein-coupled receptor Methuselah-like 10 (Mthl10). Our labs previously found that *mthl10* knockdown throughout the notum decreased pupal survival by ~30% (O'Connor *et al.*, 2021b). The newly discovered tension restoration function of Mthl10 signaling may contribute to the importance of Mthl10 for wound recovery.

The term "tension" is used to represent multiple different phenomena in mechanobiology. For the laser-recoil assays used here, the measured tension is a combination of membrane tension, cell-cell adhesive zippering, and cortical actomyosin activity. Variability in the measurements arises from both the stochastic nature of laser ablation and cell-to-cell variability in the epithelium: each measured point averages tension over a local region of four cells (see Fig. 1D′). The local nature of these measurements is illustrated by the experiments with Rok knockdowns. When the knock down is conducted in a limited region (*pnr* domain), measured tension is lower throughout that domain, and yet tension is normal as close as 70 µm (~7 cell diameters) outside this region. Although mechanical effects can act over long distances in an epithelium (Alisafaei *et al.*, 2021), these experiments show that local regions can maintain significantly higher or lower tension.

Before wounding, tensions in control epithelia were relatively uniform and isotropic, i.e., having no preferred direction. Our naive expectation was that making a circular wound would yield a pattern of tension changes matching those around a hole in a viscoelastic sheet. This pattern would have two orthogonal

gradients: tensions in radial directions would be reduced around the wound and then gradually rise toward the uniform far-field tension; those in tangential directions would be stronger circling the wound and gradually fall toward the far-field limit (Timoshenko and Goodier, 1951; Vishay Micro-Measurements, 2007). Instead, the measurement showed that tension was reduced to a similar extent in both radial and tangential directions. Although our initial experiments appeared to uncover a spatial gradient of tension loss (e.g., Fig. 1G), further investigation revealed that this result was actually a spatiotemporal gradient of tension restoration. When tension levels were analyzed in the *Mthl10* knockdowns, which were unable to restore tension, we found that wounding induced a loss of tension uniformly out to at least 210 µm from the wound center (although not as far as 270 µm; Fig S1B). Measurements in control pupae as fast as 30 s after wounding confirmed this uniform loss out to at least 135 µm, and its distal to proximal restoration over the next ~10 minutes.

What could explain this widespread, isotropic and uniform loss of tension immediately after wounding? The low-tension region extends considerably farther than the area of plasma membrane damage caused by the laser-induced cavitation bubble (~ 110 µm), indicating that tension loss is not an artifact of cavitation; further, scanned wounds with smaller cavitation bubbles still lose tension, discernible in *Mthl10* knockdowns, indicating that tension loss is independent of cavitation and plasma membrane damage. One potential explanation for the uniform widespread loss of tension is that cellular mechanics are both passively and actively nonlinear. It would be interesting if this nonlinearity around wounds was actually adaptive and limited further damage. An analogy for this possibility would be when clothing gets snagged on a thorn – pulling will rip the clothing but relaxing (i.e., reducing tension) prevents ripping.

The widespread and uniform loss of tension around wounds is fast, occurring within 30 sec (the limit of our ability to make a wound and retarget the laser to measure tension). Tension is then restored over the next ~ 10 min in a distinct spatiotemporal pattern, from outward in. Tension restoration requires the cell surface G-protein coupled receptor Mthl10, which has fast activation kinetics starting ~45 sec after wounding, and a distinct spatial domain of activation around wounds (O'Connor *et al.*, 2021b), both of which are consistent with the spatiotemporal pattern of restoring cellular tension. However, there is one important

difference: Mthl10 activation starts near the wound and moves outward, but tension restoration starts at the edge of the low-tension domain and moves inward. Although there are many possible explanations for this outward-in phenomenon, the simplest might be that cells farther from the wound are less damaged than those closer in (O'Connor *et al.*, 2021a), and so they are able to restore tension sooner. This attractive hypothesis is at present untestable: we could reduce the damage gradients by creating scanned ablation wounds, but creating these requires ~ 2 min, preventing us from measuring dynamic changes in tension on few-minute time scales. Another explanation for the outward-in restoration of tension is that cells must pull on fully-tensioned neighbors to restore their own tension, available only to the outer ring of low-tension cells.

Another phenomenon that travels distal to proximal around wounds is a contractile wave, previously reported by Antunes *et al.* (2013). Coordination between the contractile wave and the restoration of tension is summarized in Figure 6. The wave first becomes evident when a ring of cells ~100 µm from the wound simultaneously contract. As these cells then relax and expand, their wound-proximal neighbors contract, and the process repeats over about 10 minutes until the contractile wave meets the wound margin. The spatiotemporal dynamics of this wave seem to match the Mthl10-mediated restoration of tension, and indeed we found that tension is low before the wave and is restored after it passes through. Interestingly, though, the wave continues unperturbed in *mthl10* knockdown cells. Propagation of the wave requires cells to increase tension as they contract, and Antunes *et al.* (2013) noted that these contracting cells also transiently accumulate more actin and myosin at their apical surfaces, so *mthl10* knockdown cells must be able to temporarily increase cellular tension; however, they are unable to maintain this tension after the wave passes through. We thus propose that Mthl10 regulates a ratchet-like mechanism, maintaining high tension after the contractile wave temporarily restores it through cellular contraction.

Many systems that regulate cellular biomechanics have mechanical feedback, e.g., stretch-induced contractions (Odell *et al.*, 1981; Zulueta-Coarasa and Fernandez-Gonzalez, 2018), that make the systems more responsive and robust. Is there any such feedback through Mthl10-regulated calcium signaling? Mthl10 is activated after wounding by the Gbp cytokines, which exist outside the cell in latent pro-Gbp forms and are

converted to active cytokines by proteases released from the wound. A similar ligand-dependent Mthl10 activation occurs when isolated *Drosophila* wing discs are exposed to fly extract, which contains its own proteases and pro-Gbps. Interestingly, similar *in vitro* experiments demonstrate a mechanical sensitivity in extract-stimulated calcium signals (Narciso *et al.*, 2017). It is not yet clear whether mechanical cues are involved in Mthl10 signaling in vivo, but several GPCRs are mechanically gated, and it is possible that Mthl10 activation requires two triggers, Gbp ligand binding and the loss of cellular tension after wounding.

In summary, this study reports an unexpected widespread reduction in tension around epithelial wounds. This loss of tension occurs within 30 seconds, and its restoration over 2-10 minutes is regulated by a cell surface receptor activating a signal transduction cascade, resulting in the release of cytoplasmic calcium. The receptor Mthl10 does not have an ortholog in vertebrates, but it is a member of the broadly conserved $G_{\alpha q}$-coupled GPCR family. It is possible that similar but not orthologous biochemical signaling cascades may be utilized by vertebrate epithelia to restore tension after wounding.

Materials and Methods

Drosophila melanogaster genotypes

All fly genotypes used for recoil measurements had GFP-labeled E-cadherin (*Ecad-GFP*, Kyoto DGGR 109007) in apical epithelial cell borders. They also had *UAS-mCherry.NLS* (BDSC 38424) – mCherry RFP with a nuclear localization signal – driven by *pnr-Gal4* (BDSC 25758) and *tubP-Gal80^{TS}* (BDSC 7017). The *pnr* domain is an hourglass-shaped domain in the center of the notum (Fig. 1C) that expresses *pannier (pnr)*, a gene required for dorsal closure (Herranz and Morata, 2001). The Gal4-UAS system allows us to drive genetic manipulations using enhancer UAS (Upstream Activation Sequence) next to our gene of interest and having transcription activator protein Gal4 bind to UAS to activate gene transcription (Brand and Perrimon, 1993). With *pnr-Gal4*, Gal4 is only expressed in the *pnr* domain and thus genetic manipulations can be confined there, setting up a system where the area outside the *pnr* domain – called the control domain – can serve as an internal control. Gal80^{TS} is a temperature-sensitive protein that binds and inhibits Gal4 transcriptional activity at 18ºC (Zeidler *et al.*, 2004). At temperatures at or above 29ºC, Gal80^{TS} cannot bind Gal4 and can no longer repress it, activating Gal4-driven gene expression.

The control genotype was Ecad-GFP cyo; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+. For tension mapping, other genotypes had knockdowns driven by *pnr-Gal4, tubP-Gal80^{TS}*. The genotype Ecad-GFP UAS-Rok RNAi; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+ had *UAS-Rok RNAi* (VDRC 104675), Ecad-GFP UAS-mthl10 RNAi; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+ had *UAS-mthl10 RNAi* (BDSC 62315), and the genotype Ecad-GFP UAS-IP3R RNAi; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+ had *UAS-IP$_3$R RNAi* (BDSC 25937). The *mthl10 RNAi* knockdown results were verified with *UAS-mthl10 RNAi#2* (BDSC 51753) in the genotype Ecad-GFP UAS-mthl10 RNAi#2; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+.

For contractile wave tracking, cell shape changes were visualized with Ecad-GFP in the control fly genotype Ecad-GFP cyo; pnr-Gal4, UAS-mCherry.NLS, Gal80TS+ and in the *mthl10* knockdown genotype Ecad-GFP UAS-mthl10 RNAi; pnr-Gal4, tubP-Gal80TS, UAS-mCherry.NLS+. The apical

accumulation of myosin in contracting cells was visualized using the fluorescence signal of MyoII-GFP (BDSC 51564), an endogenously tagged non-muscle myosin II heavy chain. Genotypes used were control genotypes yw; MyoII-GFP cyo and MyoII-GFP+; pnr-Gal4, UAS-mCherry.NLS, Gal80TS+ and *mthl10* knockdown genotype MyoII-GFP UAS-mthl10 RNAi; pnr-Gal4, UAS-mCherry.NLS, Gal80TS+.
The complete genotypes for each *Drosophila melanogaster* line used in this study are detailed in Table S1. The complete genotype for each figure panel and movie is provided in Table S2.

***Drosophila melanogaster* husbandry**

Flies were fed standard cornmeal-molasses food supplemented with dry yeast. MyoII-GFP flies without $Gal80^{TS}$ were maintained at room temperature (around 20ºC). For control samples with $Gal80^{TS}$, new vials were maintained at room temperature to inhibit Gal4 activation during embryogenesis and then incubated at 29ºC for 2-5 days until experimentation to activate Gal4. For samples with *Rok RNAi, mthl10 RNAi,* or *IP_3R RNAi* expressed in the *pnr* domain, animals were kept at 18ºC for 3-5 days to inhibit Gal4 activation during embryogenesis and then moved to 29ºC for 3-4 days until experimentation to activate Gal4.

Pupal mounting

White prepupae were aged at 29º C for 12 to 18 hours or room temperature for 15-23 hours after puparium formation and mounted with nota pressed against a coverslip (Shannon *et al.*, 2017; O'Connor *et al.*, 2022). Pupae were placed ventral side down onto double-sided tape (Scotch brand, catalog #665) on a microscope slide. The pupae were tilted about the anterior-posterior axis to allow access to the control domain. Each anterior puparium was removed using fine-tipped forceps to expose the notum (Fig. 1A). A 35 mm x 50 mm coverslip (Fisherbrand, cat#125485R) was prepared by layering two pieces of double-sided tape along one long edge. Then, the tape with dissected pupae was gently transferred to the coverslip such that the posterior tips of the pupae rested on the layers of double-sided tape on the coverslip and the nota were between the coverslip and piece of tape. This procedure allowed for nota to be pressed up against the coverslip for microscopy and laser ablation. An oxygen-permeable membrane

(YSI, standard membrane kit, cat#1329882) was secured over the coverslip to prevent the pupae from becoming dehydrated or oxygen-deprived.

Live imaging

In vivo live imaging was performed with a Nikon Spinning Disk confocal microscope (Yokogawa CSU-X1 spinning disk head with Andor DU-897 EMCCD camera) using a 40X 1.3 NA oil-immersion objective. The camera and laser were positioned by moving ~200 μm down the midline from the head-thorax joint and then laterally to the *pnr* border. For all samples, a pre-wound z-stack scan was taken using the 488-nm laser line to capture Ecad-GFP or MyoII-GFP and the 560-nm laser line to capture mCherry-labeled *pnr* nuclei. For tension measurements, 45-second videos were taken of Ecad-GFP labeled cell borders to track tricellular junction (TCJ) movement following micro-ablation. For contractile wave tracking, z-stacks were taken every 30 seconds after wounding or every minute when making before- and after-wave measurements. When imaging live, images at previous timepoints for the current movie can be viewed as soon as they are taken, allowing the contractile wave to be monitored almost in real-time in order to measure tension before and after the wave passed through (Fig. 3E-E″, Movie 4).

Laser wounding

All laser ablations for single-shot wounding and laser-induced recoil were performed with single pulses of the 3rd harmonic (355 nm) of a Q-switched Nd:YAG laser (5 ns pulse-width, Continuum Minilite II, Santa Clara, CA). Laser pulse energy for the wound was adjusted to create a ~70-μm radius of nuclear membrane damage, with specific energy values varying from 1.5 to 2.0 μJ. For scanned wounds, a series of micro-ablations with laser pulse energy of 0.4 μJ were created in the pattern of a circle with a radius of ~40 μm, which matches the predicted radius of cell lysis for a single-shot wound with a ~70 μm radius of nuclear membrane damage. The initial wound in both single-shot and scan-wounded samples always occurred on the *pnr* border, with the center of the wound positioned by moving ~200 μm down the midline from the head-thorax joint and then moving laterally to the *pnr* border. Wounding on the *pnr* border allowed for symmetrical comparisons between *pnr* and control domain in internally controlled samples.

Locations for tension measurements

Tension was measured in mediolateral (ML) and anterior-posterior (AP) borders at different radii lateral to the *pnr* border in both the *pnr* and control domains (Fig. 1B). The closest location in unwounded samples was 70 µm from the *pnr* border. The corresponding location in wounded samples was at the edge of the region of nuclear membrane damage as indicated by a disrupted mCherry signal in the *pnr* domain; a symmetric location was used on the control side. This radius ranged from 60-90 µm but was ~70 µm in most samples. The second location was 110 µm from the *pnr* border in unwounded samples and 40 µm away from the edge of nuclear membrane damage in wounded samples. The farthest location was 210 µm from the *pnr* border in unwounded samples and 140 µm from the edge of nuclear membrane damage in wounded samples. For scan-wounded control samples, the location right outside of the scan, ~40 µm from the *pnr* border, was tested for tension in addition to the 70, 110, and 210 µm locations. After initial tension mapping, ~135 µm was added as a distance for tension testing to represent a location distal to the wound that was not as far as 210 µm. Unless otherwise specified, tension mapping was performed between 1 to 10 minutes post-wound in wounded samples and over a similar time period in unwounded samples.

Laser-induced recoil assay

Cortical tension, a combination of tension from the membrane, cytoskeleton, and cell adhesions, can be measured by laser-induced recoil. In this assay, an apical epithelial cell border labeled by Ecad-GFP is severed with a laser micro-ablation using the same laser that is used for wounding but at a lower power (Movie 1). Laser pulse energy for the laser-induced recoil micro-ablations was adjusted to ablate a single cell border, with specific energy values varying from 0.4 to 0.8 µJ. After ablating a cell border, the velocity at which the corresponding tricellular junctions (TCJs) retract from each other is measured. In this assay, higher cortical tension leads to higher recoil velocities after ablation while lower cortical tension leads to lower recoil velocities after ablation (Fig. 1D′).

Calculating recoil velocities

After ablating the cell border in laser-induced recoil, the distance between the corresponding TCJ's of the severed border was measured every 2 seconds for 30 seconds with the length measurement tool in the Nikon NIS-Elements platform (RRID:SCR_014329) or ImageJ (Schindelin *et al.*, 2012). TCJ's show up as brighter spots of Ecad-GFP fluorescence. The first frame with visible TCJ's after micro-ablation was set as t = 0 s. For each sample, the length measurements were analyzed in Mathematica (Wolfram, Champaign, IL) to calculate the average recoil velocity (in nm/s) over the first 30 seconds based on either a linear or nonlinear model – using the fit with the more negative Akaike information criterion (AIC) value to select the more appropriate regression for each sample. The nonlinear model was $x(t) = d_0 + d_1\left(1 - e^{-t/\tau}\right)$, where x is distance between TCJ's at time t after micro-ablation and d_0, d_1 and τ are fitting parameters.

Cell contraction analysis

The contractile wave can be visualized over time using Ecad-GFP, which labels apical epithelial cell borders (Movies 2, 4, and 5). To make visualizing these contractions easier, we processed post-wound Ecad-GFP movies in ImageJ using a "contraction filter." First, we took the difference images of frames that were 2 minutes apart (i.e., subtracting the image at one point of the movie from the image 2 minutes later). The difference image of a contracting cell should have a bright inner ring surrounded by a dark outer ring, and vice versa for an expanding cell. Next, a top-hat filter with a radius of 7 pixels was applied to turn the ring-like structures for individual cells in a difference image into a bright region (contracting) or dark region (expanding). For the top-hat filter, the ImageJ MorphoLibJ plug-in was used on each difference image (Legland *et al.*, 2016). Finally, a "Fire" LUT was applied to highlight the bright contractile region.

MyoII-GFP intensity analysis

To quantify MyoII-GFP intensity as the contractile wave travels inwards, we used the ImageJ plug-in Radial Profile Extended (Ferri *et al.*, 2008) to plot the average MyoII-GFP intensity profile as a function of distance from the center of the wound. For each MyoII-GFP movie, radial profile angle

analysis was applied to images every 2 minutes starting from 2 minutes post-wound and ending at 14 minutes post-wound (Fig. 3C). A custom R script (R Core Team, 2021) was then used to determine the each timepoint's peak MyoII-GFP intensity and its distance from the wound. This distance was used to represent the wave's location for calculating the average distance of the wave from the wound over time, which was graphed in Microsoft Excel (Fig. 3D).

Statistical analysis

All statistical analyses were performed with GraphPad Prism version 9.2.0, which was also used to graph the recoil velocity values. Each graph displays the value for each sample as a point, with bars representing mean and SEM. Data from the control domain are shown in black and indicated by negative distance values. Data from the *pnr* domain are shown in red and indicated by positive distance values. Statistical analyses were conducted by one-way ANOVA with multiple comparisons for recoil velocities from each genotype in Fig. 1, Fig. 2, Fig. 3A-C, Fig. 5, and Fig. S1. For unwounded control and *mthl10 RNAi* samples, along with the Fig. 2 graphs showing post-wound tension over time and Fig. S1B, the mean of each column was compared with the mean of every other column. For unwounded *Rok RNAi* and the rest of the wounded samples, means were compared between the most proximal and distal locations on each domain, and then between control and *pnr* domains for the most proximal and distal locations. For scan-wounded samples, the 40 μm location was left out of statistical tests because this location was not tested in other types of samples. For Fig. 3 and Fig. 4H, paired sample t-tests were used to compare the two groups, with each sample producing one pair of tension measurements. For Fig. 4G, an unpaired t-test was used to compare the two groups.

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FIGURES AND FIGURE LEGENDS

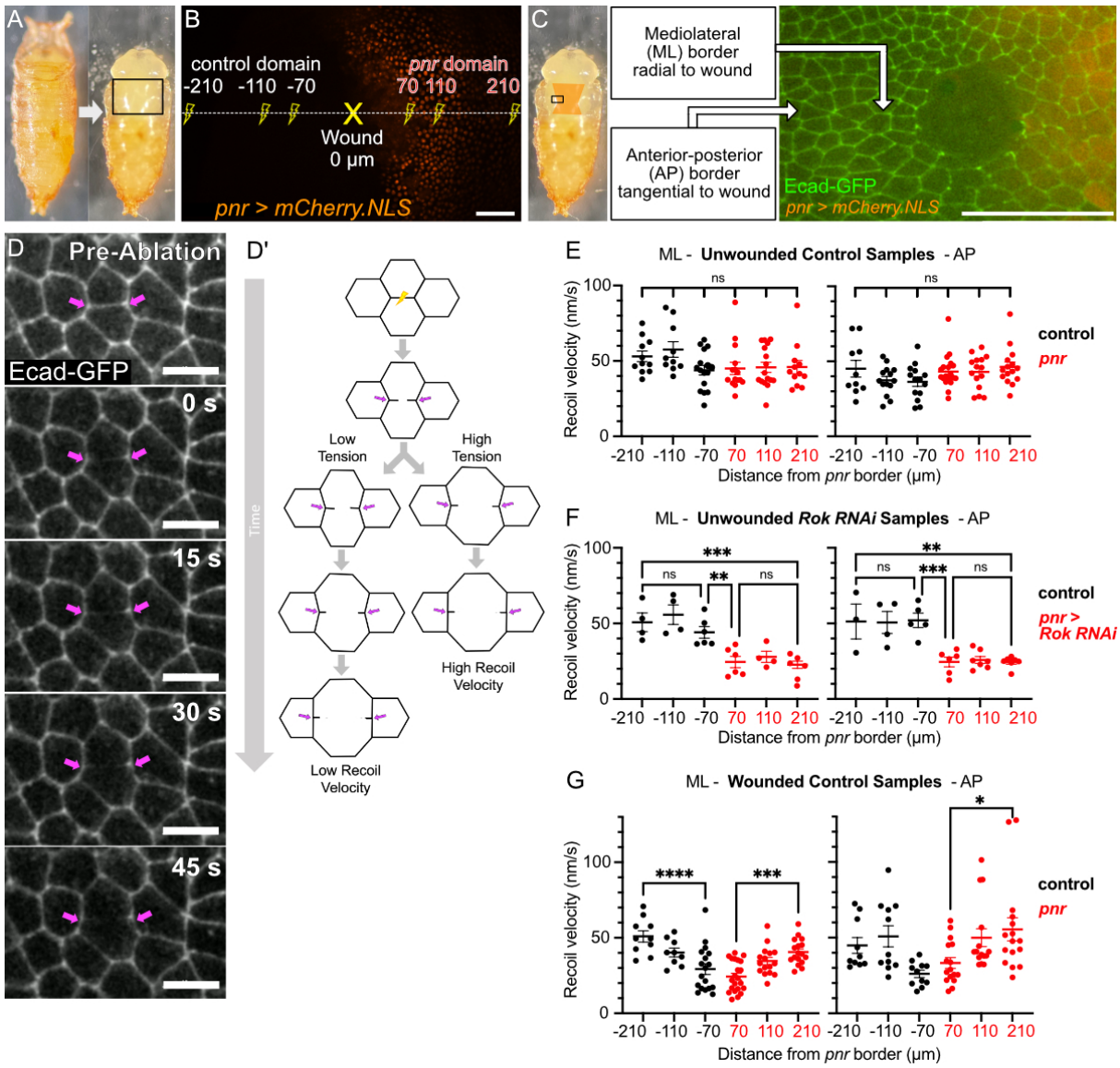

Figure 1. Tension is lost around wounds.
(A) *Drosophila melanogaster* pupa (left) with pupal case removed (right) to expose notum (outlined by black rectangle). **(B)** Map of distances where tension was measured. Wound is designated as 0 µm (yellow X). Tension was measured at ~70, 110, and 210 µm on either side of the wound (yellow lightning bolts). Map is overlaid on an image of the notum with *pnr* domain labeled by red nuclei. Closest distance (~70 µm) corresponds to the edge of nuclear membrane damage visible in wounded samples.
(C) Two types of borders measured: mediolateral (ML) borders, which are radial to the wound at this location, and anterior-posterior (AP) borders, which are tangential to the wound at this location.
(D) Example of laser-induced recoil for a mediolateral cell border. Tricellular junctions of ablated cell border indicated by magenta arrows. See also: Movie 1. **(D′)** Diagram illustrating how laser-induced recoil reports cortical tension: low tension corresponds to low recoil velocity; high tension corresponds to high recoil velocity.

(E) In unwounded control samples, tension was fairly constant at all locations tested for both ML and AP borders.
(F) In unwounded *pnr* > *Rok RNAi* samples, significantly lower tension was detected in both ML and AP borders. Reduced tension was confined to the domain expressing *Rok RNAi***.**
(G) In wounded control samples, tension was reduced in a gradient for both ML and AP borders, with lower tension closer to the wound. Measurements taken 1-10 minutes post-wound. Graph bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with multiple comparisons. Scale bars = 50 µm (B and C), or 10 µm (D).

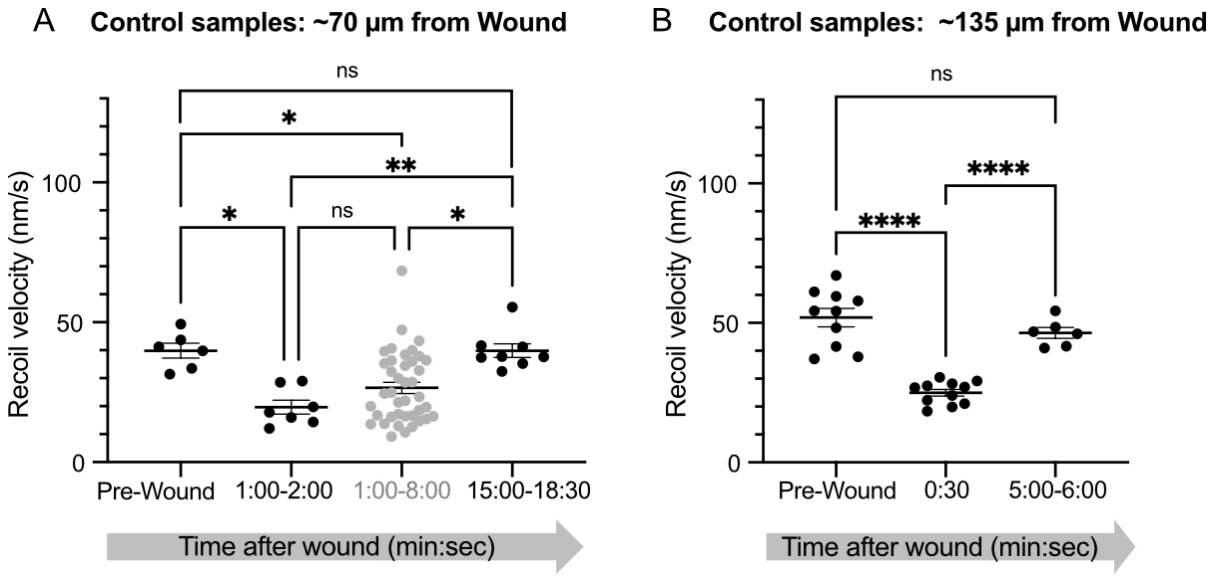

Figure 2. Tension is restored over time.
(A) At ~70 µm from the wound, wounding reduces cortical tension by half, evident 1-2 min after wounding. Tension is restored to pre-wound levels by 15-18 minutes after wounding. Data collected 1-8 min after wounding shows a mix of tension levels (light gray, from Fig. 1G) **(B)** At ~135 µm from the wound, wounding reduces cortical tension by half, evident 30 sec after wounding. Tension is restored to pre-wound levels by 5-6 minutes after wounding. All measurements conducted on ML borders. Graph bars represent mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001 by one-way ANOVA with multiple comparisons.

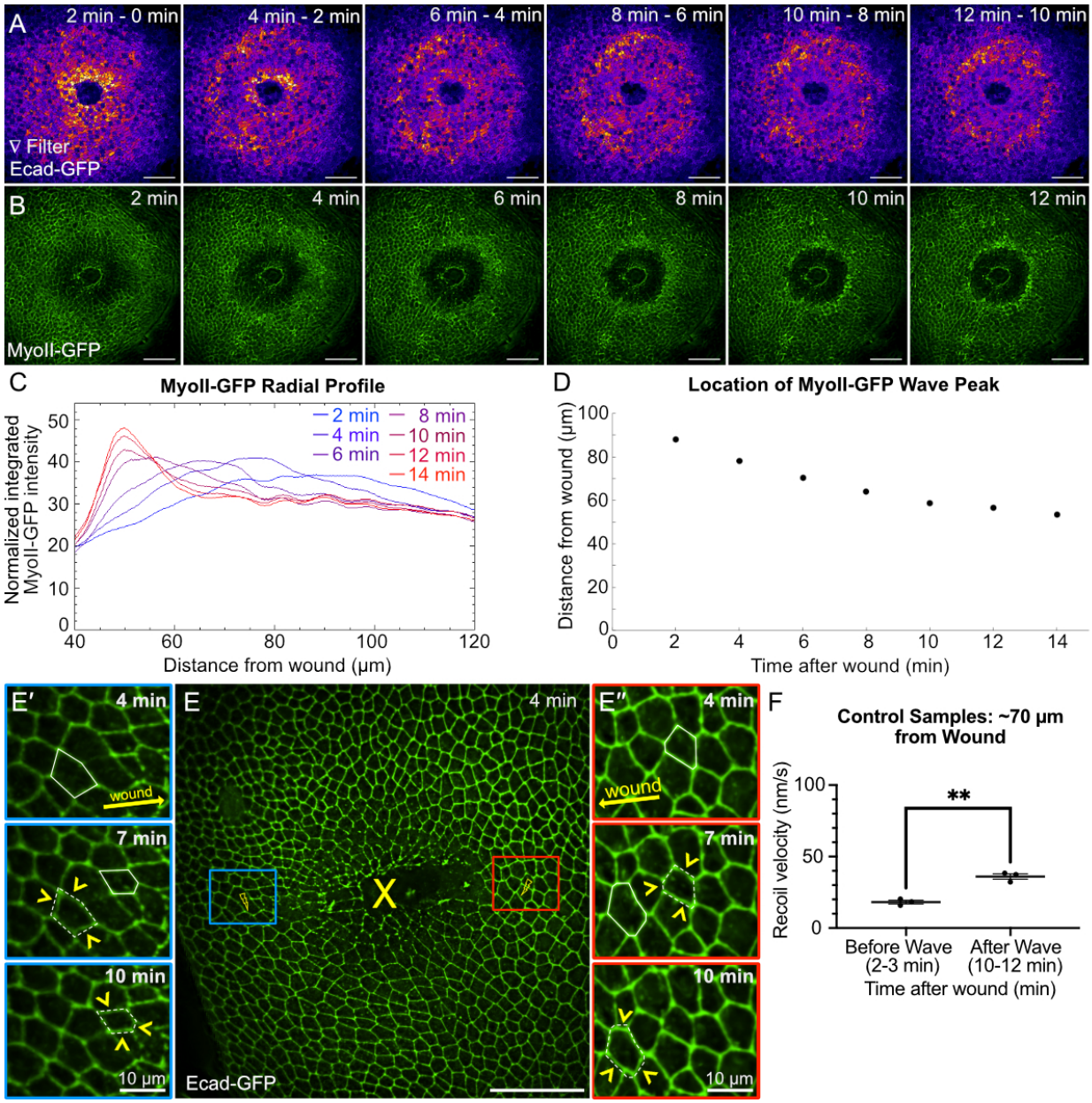

Figure 3. Tension is restored as the contractile wave moves toward the wound.
(A) A wave of cell contraction travels inward over 12 minutes after wounding. These contractions can be visualized in still frames by applying a top-hat filter on the Ecad-GFP difference images of successive frames (n = 5). See also Movie 2.
(B) MyoII-GFP accumulates apically just before each cell contraction. (n = 7). See also Movie 3.
(C) Peaks of radial MyoII-GFP intensity move closer to the wound over time, tracking progression of the contractile wave.
(D) The average location of the wave over time, as determined from data in panel C (n = 7).
(E-E″) Using Ecad-GFP to monitor the contractile wave, tension was measured before (blue) and after (red) the contractile wave passed through a cell ~70 μm from the wound. Yellow arrowheads in E′ and E″ show contracting cells. See also Movie 4.
(F) Tension at ~70 μm from the wound is restored as the wave passes through. Graph bars represent mean ± SEM. **p<0.01 by paired sample t-test. Scale bars = 50 μm (A, B, and E), or 10 μm (E′ and E″).

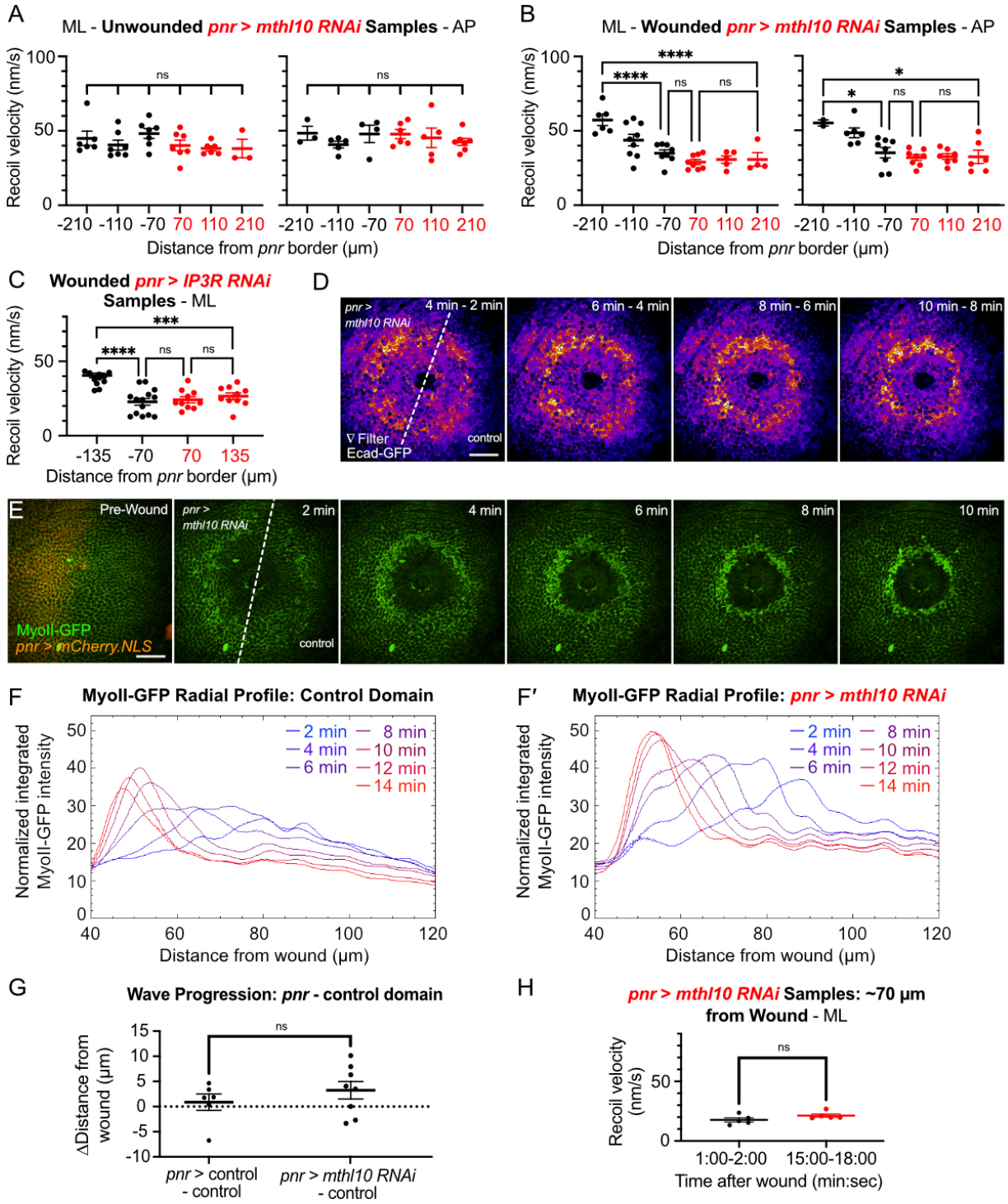

Figure 4. The restoration of tension after wounding requires the GPCR Mthl10.
(A) In unwounded flies, knockdown of *mthl10* within the *pnr* domain had no effect on cortical tension for both ML and AP borders.
(B) The wound-induced loss of tension persists after wounding in the *pnr* domain where *mthl10* was knocked down, indicating that Mthl10 is required for the restoration of tension.

(C) *IP3R* is downstream of Mthl10 and is also required for the restoration of tension after wounding, indicating that Mthl10 signaling restores tension through IP3R. Measurements in B-C taken 1-10 minutes post-wound.
(D-G) The contractile wave does not require *mthl10.* Wave symmetry was not disturbed when *mthl10* was knocked down in the *pnr* domain, as visualized with a top-hat filter applied to Ecad-GFP difference images of successive frames (D; n = 4; see also Movie 5) or with MyoII-GFP (E; n = 7, see also Movie 6). Radial profile analysis (F and F′) showed no significant difference between control and *pnr* domains in *pnr* > *mthl10 RNAi* samples compared to control samples (G).
(H) The contractile wave exists without *mthl10*, but it requires *mthl10* to restore tension after wounding. Tension is not restored at ~70 µm even 15 minutes post-wound in *pnr* > *mthl10RNAi* samples. Graph bars represent mean ± SEM. *p<0.05, ***p<0.001, ****p<0.0001 by one-way ANOVA with multiple comparisons (A, B, C), unpaired t-test (G), or paired sample t-test (H). Scale bars = 50 µm.

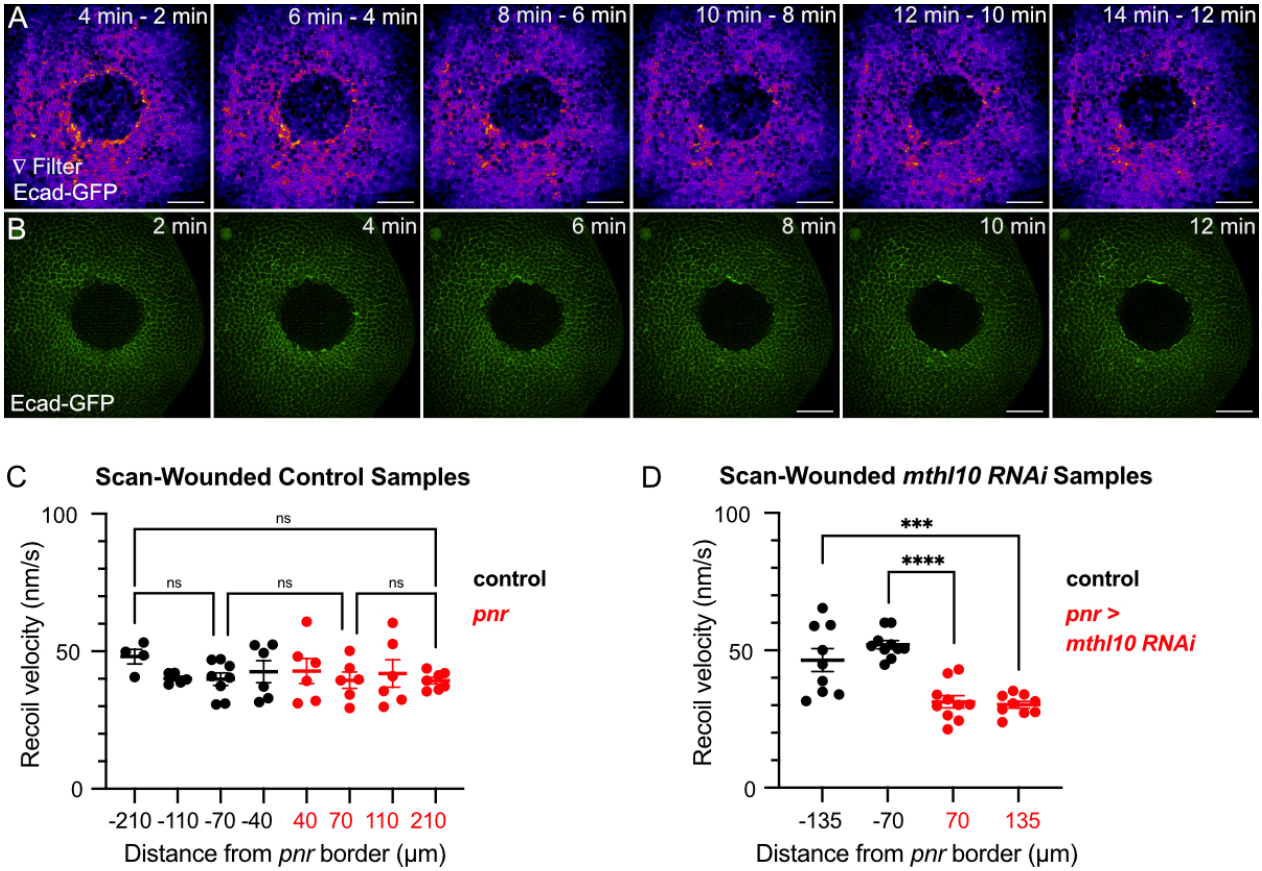

Figure 5. Mthl10 is also required for tension restoration around scanned wounds.
(A-B) In scan-wounded control samples, there is no evident wave of contraction (n = 5; see also Movie 7) or myosin accumulation (n = 5; see also Movie 8). **(C)** Within ~2-10 minutes of completing a scanned wound, post-wound tension is fairly uniform at all locations tested. **(D)** In contrast, for scan-wounded *pnr* > *mthl10 RNAi* samples, there is reduced tension in the knockdown domain, indicating that scanned wounds also require Mthl10 for tension restoration. All measurements were taken on ML cell-cell borders. Graph bars represent mean ± SEM. ***p<0.001, ****p<0.0001 by one-way ANOVA with multiple comparisons. Scale bars = 50 µm.

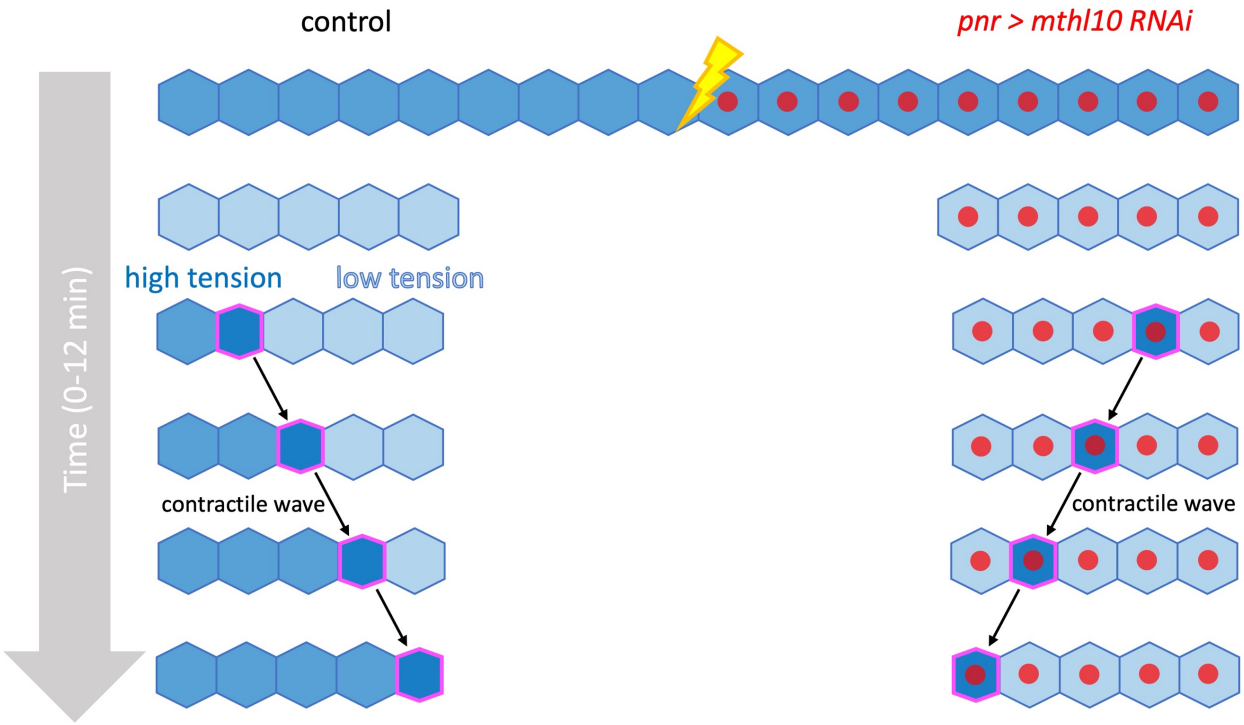

Figure 6. Schematic of how Mthl10-driven calcium signaling and the wound-induced contractile wave coordinate to restore tension around epithelial wounds.

SUPPLEMENTAL MATERIALS

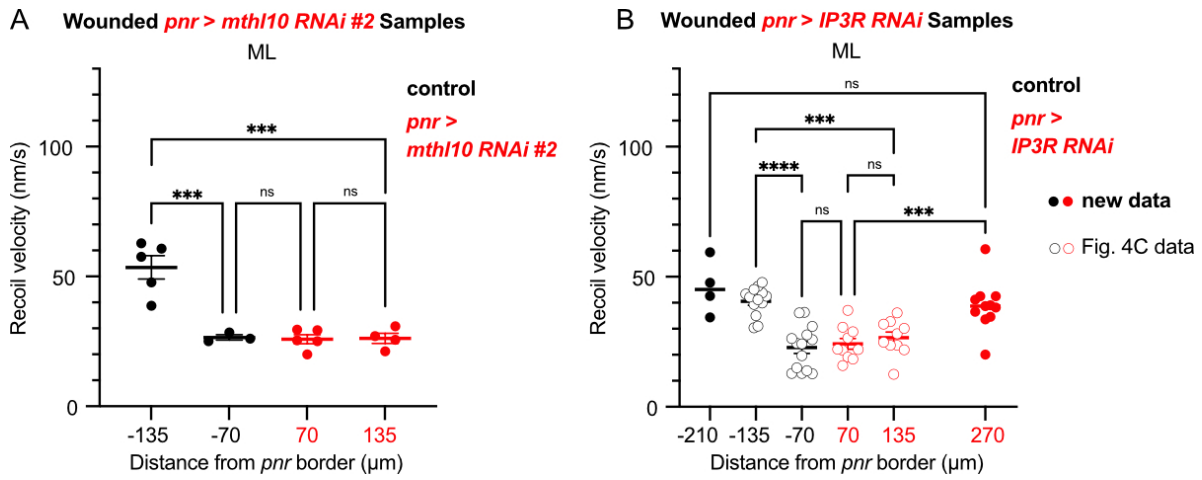

Figure S1. Additional tension mapping with *pnr > mthl10 RNAi #2* and *pnr > IP3R RNAi.*
(A) In wounded *pnr > mthl10RNAi #2* samples, tension loss persisted in the knockdown region in measurements taken 1-10 minutes post-wound, verifying the first *mthl10 RNAi* line. **(B)** Tension was reduced at ~70 µm and ~135 µm in the knockdown region for *pnr > IP3R RNAi* but not at ~270 µm, suggested that IP3R is required to restore wound-induced tension loss but this initial tension loss is confined to a limited region that does not extend to ~270 µm from the wound. These measurements were taken 1-10 minutes post-wound. Graph bars represent mean ± SEM. ***p<0.001, ****p<0.0001 by one-way ANOVA with multiple comparisons.

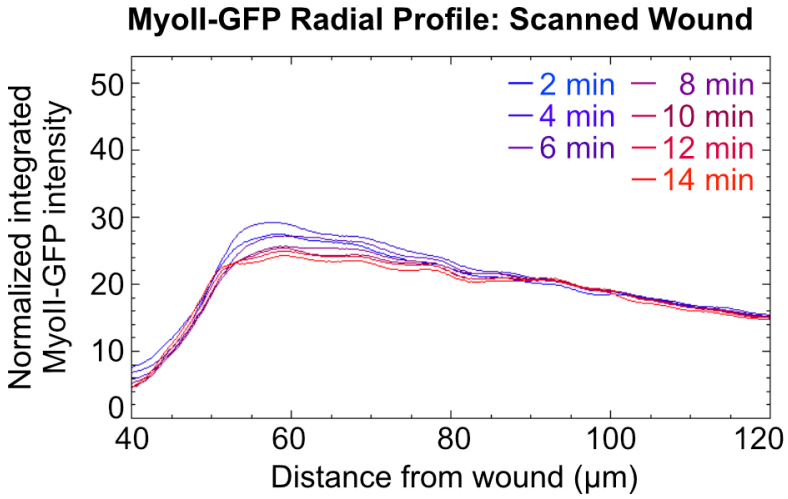

Figure S2. There is no apparent contractile wave around scanned wounds.
Radial profile analysis of the MyoII-GFP images around scanned wounds shown in Figure 5 and Movie 8.

Table S1. Complete genotypes of source flies obtained for this study

Fly lines	Complete genotype of source flies
"Ecad-GFP"	*w; {ubi-p63E-shg.GFP, w+}5* (FlyBase ID: FBtp0014096)
"MyoII-GFP"	*w[*]; P{w[+mC]=PTT-GC}zip[CC01626]/SM6a* (FlyBase ID: FBst0051564)
"pnr-Gal4"	*P{w[+mC]=UAS-Dcr-2.D}1, w[1118]; P{w[+mW.hs]=GawB}pnr[MD237]/TM3, Ser[1]* (FlyBase ID: FBst0025758)
"tubP-Gal80^{ts}"	*w[*]; P{w[+mC]=tubP-GAL80[ts]}2/TM2* (FlyBase ID: FBst0007017)
"UAS-IP3R RNAi"	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01957}attP2* (FlyBase ID: FBst0025937)
"UAS-mCherry.NLS"	*w[*]; P{w[+mC]=UAS-mCherry.NLS}3* (FlyBase ID: FBst0038424)
"UAS-mthl10 RNAi"	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23672}attP40/CyO* (FlyBase ID: FBst0062315)
"UAS-mthl10 RNAi#2"	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03304}attP2* (FlyBase ID: FBst0051753)
"UAS-Rok RNAi"	*P{KK107802}VIE-260B* (FlyBase ID: FBst0476533)

Table S2. Complete genotypes of source flies obtained for this study

Figure(s)	Full Genotype of Sample
Fig. 1A-E, G Fig. 2 Fig. 3A, E-F Fig. 5A Fig. S2A Movies 1, 2, 4, S1	Ecad-GFPcyo; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+
Fig. 1F	Ecad-GFP UAS-Rok RNAi;pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+
Fig. 3B Fig. S2B, C Movie 3, S2	yw; MyoII-GFPcyo
Fig. 3D	yw; MyoII-GFPcyo and MyoII-GFP+;pnr-Gal4, UAS-mCherry.NLS, Gal80TS+
Fig. 4A, B, D, H Fig. 5B Movie 5	Ecad-GFPUAS-mthl10 RNAi;pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+
Fig. 4C Fig. S1B	Ecad-GFP UAS-IP3R RNAi; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+
Fig. 4E-F′ Movie 6	MyoII-GFPUAS-mthl10 RNAi; pnr-Gal4, UAS-mCherry.NLS, Gal80TS+

Fig. 4G	MyoII-GFP+; pnr-Gal4, UAS-mCherry.NLS, Gal80TS+ (left) MyoII-GFPUAS-mthl10 RNAi; pnr-Gal4, UAS-mCherry.NLS, Gal80TS+ (right)
Fig. S1A	Ecad-GFPUAS-mthl10 RNAi#2; pnr-Gal4, UAS-mCherry.NLS, tubP-Gal80TS+

Movies 1-8: attached with submission

Movie 1. Laser-induced recoil example
Tricellular junctions (TCJ's) of ablated cell border retract after laser microablation. Quantifying the recoil velocity of retraction was used to estimate cortical tension. Movie shows a control sample mediolateral border being ablated and then recoiling for ~60 seconds. Scale bar = 10 µm.

Movie 2. Contractile wave visualized by cell contractions in wounded control sample
The post-wound contractile wave was visualized with Ecad-GFP labeling apical epithelial cell borders as they contract in concentric rings one at a time, moving closer to the wound over time. Movie shows a control sample from 0-15 minutes every 30 seconds after wounding. Scale bar = 50 µm.

Movie 3. Contractile wave visualized by myosin accumulation in wounded control sample
The post-wound contractile wave was also visualized with MyoII-GFP labeling non-muscle myosin II, which temporarily accumulates apically in each cell before it contracts. Movie shows a control sample from 0-15 minutes every 30 seconds after wounding. Scale bar = 50 µm.

Movie 4. Monitoring contractile wave to measure tension before and after
Tension was measured before and after the contractile wave passed through by using Ecad-GFP to monitor the wave. Movie shows a control sample from 4-10 minutes every minute after wounding, right after a before-wave tension measurement is taken and right before an after-wave tension measurement is taken. Scale bar = 50 µm.

Movie 5. The contractile wave is not disrupted in *pnr > mthl10 RNAi* samples (Ecad-GFP)
The contractile wave, visualized with Ecad-GFP, was not disrupted in *pnr > mthl10 RNAi* samples. Movie shows a *pnr > mthl10 RNAi* sample from 0-14 minutes every 30 seconds after wounding, with a pre-wound image containing the red channel (*mCherry.NLS*) to indicate where the *pnr* knockdown domain is. Scale bar = 50 µm.

Movie 6. The contractile wave is not disrupted in *pnr > mthl10 RNAi* samples (MyoII-GFP)
The contractile wave, visualized with MyoII-GFP, was not disrupted in *pnr > mthl10 RNAi* samples. Movie shows a *pnr > mthl10 RNAi* sample from 0-16 minutes every 30 seconds after wounding, with a pre-wound image containing the red channel (*mCherry.NLS*) to indicate where the *pnr* knockdown domain is. Scale bar = 50 µm.

Movie 7. No apparent contractile wave after scan-wounding (Ecad-GFP)
With Ecad-GFP labeling cell borders, there was no evidence of a contractile wave around scanned wounds. Movie shows a control sample from 2-15.5 minutes every 30 seconds after scan-wounding. Scale bar = 50 µm.

Movie 8. No apparent contractile wave after scan-wounding (MyoII-GFP)
With MyoII-GFP labeling non-muscle myosin II, there was no evidence of a contractile wave around scanned wounds. Movie shows a control sample from 2-15.5 minutes every 30 seconds after scan-wounding. Scale bar = 50 µm.

Recoil Velocity Data Spreadsheet: attached with submission

Contains information for every data point represented on recoil velocity graphs, including specific sample, time after wounding, standard error values, and regression AIC values.