Remodeling of adhesion and modulation of mechanical tensile forces during apoptosis in *Drosophila* epithelium

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**ABSTRACT**

Apoptosis is a mechanism of eliminating damaged or unnecessary cells during development and tissue homeostasis. During apoptosis within a tissue, the adhesions between dying and neighboring non-dying cells need to be remodeled so that the apoptotic cell is expelled. In parallel, contraction of actomyosin cables formed in apoptotic and neighboring cells drives cell extrusion. To date, the coordination between the dynamics of cell adhesion and the progressive changes in tissue tension around an apoptotic cell is not fully understood. Live imaging of histoblast expansion, which is a coordinated tissue replacement process during *Drosophila* metamorphosis, shows remodeling of adherens junctions (AJs) between apoptotic and non-dying cells, with a reduction in the levels of AJ components, including E-cadherin. Concurrently, surrounding tissue tension is transiently released. Contraction of a supra-cellular actomyosin cable, which forms in neighboring cells, brings neighboring cells together and further reshapes tissue tension toward the completion of extrusion. We propose a model in which modulation of tissue tension represents a mechanism of apoptotic cell extrusion.

**KEY WORDS:** Apoptosis, Cell adhesion, *Drosophila*, Force, Tissue mechanics

**INTRODUCTION**

Throughout development and adult life, epithelia undergo constant growth and turnover. As a cell population changes, epithelial architecture needs to be maintained and this is achieved through tight intercellular adhesions supported by adherens junctions (AJs) (Harris and Tepass, 2010; Takeichi, 2014; Lecuit and Yap, 2015). Apoptosis, or programmed cell death, is the most common mechanism of eliminating damaged or unnecessary cells during embryonic development and tissue homeostasis (Jacobson et al., 1997). When cells within a tissue undergo apoptosis, the adhesions between the dying and neighboring non-dying cells need to be remodeled. This ensures the apoptotic cell can be expelled from a tissue without compromising tissue cohesion. Loss of adhesions in the apoptotic process has been studied, with a focus on the disruption of cell-cell junctions associated with caspase activation. Members of the caspase family serve as the key executors of apoptosis (Riedl and Shi, 2004; Miura, 2012). For instance, AJ components, such as E-cadherin (Shotgun, E-cad) and β-catenin (β-cat) are known substrates of caspase 3 in mammalian cells (Bannerman et al., 1998; Brancolini et al., 1997). In *Drosophila*, a reduction in the levels of E-cad from AJs is associated with cleavage of the β-cat homolog Armadillo by the *Drosophila* effector caspase DrICE (Kessler and Muller, 2009).

Along with AJ remodeling, the process of cell extrusion is also crucial in the expulsion of dying cells from a tissue. In tissue culture, apoptotic cells autonomously develop an actomyosin cable, and the neighboring non-apoptotic cells also form a supra-cellular actomyosin purge-string (Rosenblatt et al., 2001; Kuipers et al., 2014). These cables exert contractile force and contribute to extrusion (Rosenblatt et al., 2001; Kuipers et al., 2014). Recently, it was shown that E-cad is crucial for the transmission of contractile force to neighboring cells (Lubkov and Bar-Sagi, 2014), and for the recruitment of coronin 1B, which aligns the actomyosin cable (Michael et al., 2016) during apoptotic cell extrusion. Together, these findings highlight an intriguing interplay between AJ dynamics and the cytoskeleton, which leads to progressive changes in tissue tension around the apoptotic cell. The mechanisms resulting from this interplay remain to be fully understood.

We elected to study tissue replacement during histoblast expansion in *Drosophila*. At the onset of pupal development, the quiescent abdominal histoblasts (precursors of the adult epidermal cells) are grouped in nests embedded amongst larval epidermal cells (LECs) (Fig. 1A; Movie 1). As metamorphosis progresses, histoblasts exhibit rapid proliferation and active migration, leading to growth and expansion of the nests (Ninov et al., 2007, 2010). Concurrently, pre-existing LECs undergo caspase-3-mediated apoptosis (Nakajima et al., 2011). The majority of the apoptotic cells in *Drosophila*, including LECs, extrude basally. This is observed in, for example, embryonic tissue (Kiehart et al., 2000; Toyama et al., 2008; Meghana et al., 2011; Mulyiil et al., 2011; Sokolow et al., 2012; Monier et al., 2015), larval tissue (Ninov et al., 2007), imaginal discs (Shen and Dahmann, 2005; Manjón et al., 2007; Monier et al., 2015) and adult tissue during pupa (Kuranga et al., 2011; Marinari et al., 2012). Intriguingly, this is in contrast to apical cell extrusion in most vertebrates (Rosenblatt et al., 2001; Eisenhofer et al., 2012; Yamaguchi et al., 2011). Around 80% of LECs undergo apoptosis in proximity to histoblast nests (hereafter referred to as ‘boundary’ LECs) and ~20% of LECs die away from the nests (hereafter referred to as ‘non-boundary’ LECs) (Ninov et al., 2007; Bischoff and Cseresnyes, 2009; Nakajima et al., 2011). Although proliferation of histoblasts is known to contribute to histoblast expansion (Ninov et al., 2007; Ninov et al., 2010), it was not clear whether the mechanics of LEC apoptosis also contributes to the expansion (Ninov et al., 2007; Bischoff and Cseresnyes, 2009).

In this study, we reveal that during the extrusion of apoptotic LECs, AJs between apoptotic and neighboring cells become remodeled, and that this is associated with a reduction in the levels of AJ components. Furthermore, this correlates with a transient release of tissue tension. Towards the end of the extrusion process, neighboring cells are...
brought together by the formation and contraction of supra-cellular actomyosin cables within the neighboring cells. This further restores tissue tension. We conclude that adhesion remodeling and changes in tissue tension are mechanically coordinated, and that this represents a mechanism of apoptotic cell extrusion. Furthermore, we reveal that the extrusion process of death-committed LECs is able to drive histoblast expansion mechanically.

RESULTS
Apoptotic cell extrusion coincides with neighboring cell deformation
To explore the effects of extrusion of apoptotic LECs on histoblast expansion, we collected confocal images of the surface of wild-type pupae expressing a DE-cad::GFP (Drosophila E-cadherin GFP). We found that boundary LECs undergo apical constriction (Fig. 1B, filled circle; Movie 2) and basal extrusion (Movie 3). Concurrently, histoblast cells surrounding apoptotic LECs exhibited biased cell shape deformations (Fig. 1B). Histoblasts that directly contact apoptotic LECs become progressively elongated (Fig. 1B, yellow dotted outlines). The next-to-nearest-neighbor histoblasts also elongated (Fig. 1B, white and magenta dotted outlines). With time, the space originally occupied by the apoptotic LECs was covered by neighboring cells. To quantify histoblast deformation upon boundary LEC extrusion, we monitored cell elongation by measuring the anisotropy of cell shape (Materials and Methods; Fig. 1C; Movie 4). Analyzing the anisotropy revealed that histoblast elongation correlated with LEC extrusion (Fig. 1C), and the degree of elongation decayed as a function of the distance from the extruding cell (Fig. S1A). These observations indicated that the mechanical impact of apoptotic cell extrusion was not restricted to the nearest-neighbor histoblast, but propagated to the next-to-nearest-neighbor cells. The elongated histoblasts (Fig. 1B) underwent either cell division or cell-cell junction remodeling after cell extrusion had completed, and did not maintain their elongated shape (Fig. S2). These rearrangements, however, had no impact on tissue expansion, with the edge of the histoblasts remaining in place after cell extrusion was complete (Fig. S2). This suggests that the mechanical impact of boundary LEC apoptosis on tissue expansion is preserved even after the completion of LEC extrusion.

To examine the kinematics of extruding LECs further, we analyzed the temporal progression of cell shape changes. Boundary LECs exhibited an apical constriction in the course of cell extrusion (Fig. 1D). The time at which the cell area transitioned, \( \tau_{\text{area}} \), was defined as the time when the cell started to constrict with higher speed (Materials and Methods; Fig. S3). Intriguingly, the boundary LECs exhibited characteristic kinematics before and after \( \tau_{\text{area}} \). The deformation in the shape of an extruding LEC was local and anisotropic before \( \tau_{\text{area}} \). Here, the major deformation was at the cell boundary adjacent to the growing histoblasts (Fig. 1E, arrow), whereas the cell boundaries next to adjacent LECs showed little or no deformation (Fig. 1E, arrowheads). We speculate that the local deformation, before \( \tau_{\text{area}} \), was due to compressive stress exerted by proliferating histoblasts, which caused LECs to be passively deformed. By contrast, the contraction of the dying LEC was more isotropic after \( \tau_{\text{area}} \) (Fig. 1E'), which might have been due to the contraction of actomyosin rings formed upon apoptosis. The different kinematics of apoptotic LECs before and after \( \tau_{\text{area}} \) were further supported by quantitative measurements of the cell shape (Fig. S4). Moreover, quantification of a non-extruding LEC that was next to an LEC undergoing extrusion revealed that although it was static before \( \tau_{\text{area}} \), it exhibited local deformations in response to the extrusion of its neighboring cell (Fig. S5A). In this case, the major deformation after \( \tau_{\text{area}} \) was at the cell boundary adjacent to the extruding LEC. The cell boundaries away from the cell undergoing extrusion showed little or no deformation (Fig. S5B,B'). Together, we found that the effect of boundary LEC extrusion was restricted to the nearest-neighbor LECs, but propagated into several layers of histoblasts.

We also noticed that apoptosis of non-boundary LECs was not strongly associated with histoblast deformation (Fig. S6). Hereafter, we focus on the apoptotic process of boundary LECs, which contributes mechanically to histoblast expansion.

Caspase-3 activation precedes apical constriction in apoptotic cells
To clarify the time at which apical constriction commenced, with respect to apoptotic signaling, we monitored caspase activity in
LECs by imaging pupae incorporating a genetically encoded fluorescence resonance energy transfer (FRET)-based caspase-3-like DEVDase (hereafter referred to as caspase-3) sensor (SCAT3) (Takemoto et al., 2003; Nakajima et al., 2011; Levayer et al., 2016). This sensor displayed a reduced FRET ratio as caspase-3 activation increased (Fig. 2A; Materials and Methods). We used the bipartite GAL4-UAS system (Brand and Perrimon, 1993) to express SCAT3 in the pupal epithelium, including LECs, by tsh-GAL4. To monitor the apical constriction of apoptotic LECs in parallel with caspase-3 activity, we imaged DE-cad::Tomato (Huang et al., 2009) together with SCAT3 (Fig. 2B). Caspase-3 activity progressively increased with time (Fig. 2A; Movie 5; Fig. 2C, orange line). The initiation of caspase-3 activation was defined as $\tau_{\text{Cas3}}$, the time when the cell started to show higher caspase-3 activity (Fig. S3). The time delay between $\tau_{\text{area}}$ and $\tau_{\text{Cas3}}$ indicated that the initiation of caspase-3 activation preceded the onset of apical constriction (Fig. 2D). Qualitatively, our data are in agreement with recent findings from apoptotic extrusion in Drosophila notum (Levayer et al., 2016).

Remodeling of AJs during apoptotic cell extrusion

To understand how AJs and the associated cytoskeleton were remodeled in the course of LEC apoptosis, we examined the distribution of E-cad (ubi-DE-cad::GFP) and Myosin II [non-muscle Myosin II regulatory light chain (Spaghetti squash, Sqh); MyoII::mCherry]. Stills from time-lapse movies show that the level of E-cad at the interfaces between apoptotic LECs and neighboring cells, including both histoblasts and non-apoptotic LECs, was reduced (Fig. 3A,A’, arrowheads; Movie 6). By contrast, there was no change in the level of DE-cad::GFP at cell junctions of neighboring cells, away from the interface (Fig. 3A’, double arrows). Concomitant to the reduction of E-Cad, MyoII distribution around the periphery of apoptotic LECs showed two myosin cable-like structures (Fig. 3B, arrows and double arrows). Merged images of E-cad and MyoII (Fig. 3C) indicated that the supra-cellular cable was in the neighboring cells (referred to as the ‘outer’ cable; Fig. 3B,D, double arrows) whereas the other cable was in the apoptotic cell (referred to as the ‘inner’ cable; Fig. 3B,D, arrows). The distribution of actin resembled that of MyoII (Fig. S7). We also noticed that E-cad accumulated between the two neighboring cells (Fig. 3C’, arrows). These loci could link the actomyosin cables, thereby forming a supra-cellular actomyosin purse-string-like structure. Importantly, upon completion of apical constriction, de novo AJs were formed between the non-apoptotic neighboring cells (Fig. 3A, t=45 min; Fig. S8).

To characterize further the progressive reduction of E-cad levels at the interfaces between apoptotic LECs and their neighboring cells, we imaged a GFP knock-in fly line that replaced the endogenous DE-cad (DE-cad::GFPKl) (Huang et al., 2009). The level of E-cad at the interface between apoptotic LECs and neighboring cells progressively decreased with time (Fig. 3E) and the initiation of the decrease in the level of E-cad ($\tau_{\text{EcadKl}}$) followed the onset of apical constriction ($\tau_{\text{area}}$) (Fig. 3E,F). Furthermore, we found that E-cad levels in pupae with two copies of E-cad [E-cad::GFPKl; $\tau_{\text{EcadKl_red}}$ 38.3$\pm$3.4 min (mean$\pm$s.e.m.) after $\tau_{\text{area}}$, n=6; Fig. 3F] or with four copies of E-cad [ubi-E-cad::GFP; $\tau_{\text{Ecad_red}}$ 36.4$\pm$2.9 min after $\tau_{\text{areas}}$, n=9; Fig. S9A] reduced at similar times, indicating that the strong reduction in E-cad levels during apoptosis is insensitive to the expression level of E-Cad.

The other major components of AJs, $\alpha$- and $\beta$-catenin, behaved similarly to E-cad (Fig. S9B,C). Flies expressing a $\beta$-cat homolog fusion construct, Armadillo::YFP, or a Dc-catenin::RFP fusion marker both showed a reduction of these components (Fig. S9B,C; Movies 7, 8) at the same time that the level of E-cad decreased (Fig. S9A; $\tau_{\text{cat_red}}$ and $\tau_{\text{cat_red}}$). To test the possibility that the reduction in the levels of E-cad from AJs is associated with cleavage of $\beta$-cat at AJs by caspase-3 (Kessler and Muller, 2009), we used a fly line that replaced endogenous DE-cad with a GFP-tagged E-cad-$\alpha$-cat fusion protein (DE-cad-$\alpha$-cat::GFP) (Morais-de-Sá and Sunken, 2013). Similar to E-cad, the level of this fusion protein was reduced during extrusion (Fig. S9A,D). This suggests that it is the cleavage of $\beta$-cat from a cytoplasmic pool of the protein, rather than the $\beta$-cat linking E-cad with $\alpha$-cat in AJs, that plays a role in the reduction of E-cad. Moreover, we cannot rule out the possibility that the reduction in the levels of E-cad is due to the cleavage of the cytoplasmic region of E-cad by caspase-3 (Bannerman et al., 1998).

The observation that two myosin cables form when E-cad is reduced, suggests a loosening of E-cad-dependent cell-cell adhesion. To follow the kinematics of cell-cell contacts during apoptotic cell extrusion, we visualized the overall plasma membrane using the PH domain of PLCy (Phosphoinositide phospholipase C, $\gamma$ form) fused to GFP (PH::GFP) together with MyoII::mCherry. The plasma membranes of apoptotic and neighboring cells sometimes, but not always, detached at the apical section of the cell once the separation of the two myosin cables became apparent (Fig. 3G; Fig. S10). In this case, the myosin cables of each cell were approximately at the edge of plasma membranes (Fig. 3G’). By contrast, the two plasma membranes of apoptotic and neighboring cells could maintain their
contacts even when the two myosin cables became apparent (Fig. S11).

To gain insight into the dynamics of cell-cell contacts at the basolateral section of the cell, we analyzed the behavior of septate junctions (SJs). These are positioned basal to AJs in Drosophila in an opposite orientation relative to that of tight junctions, the functional equivalent of SJs in vertebrates (Oda and Takeichi, 2011). We imaged pupae expressing GFP-tagged Neuroglian, a cell surface trans-membrane protein essential for SJ function (Nrg::GFP). In contrast to AJs, SJs were stable throughout the apical constriction (Fig. S12; Movie 9). This suggests that the permeability barrier function is preserved at SJs even when AJs are disengaged during apoptosis of LECs.

Together, our results show that the AJs between dying cells and their neighbors rearranged during apoptosis, and that this is associated with a reduction in the levels of AJ components.

**MyoII accumulates in apoptotic and neighboring cells at different times during apoptosis**

To characterize further the formation of actomyosin cables in apoptotic LECs (inner cable), or in neighboring cells (outer cable), we quantified the temporal progression of MyoII accumulation in pupae expressing GFP-tagged MyoII (MyoII::GFP), in either histoblasts by esg-GAL4, or LECs by Eip71CD-GAL4 (Fig. 4). These flies also ubiquitously expressed MyoII::mCherry. In GFP-positive histoblasts, which were considered non-dying neighboring cells, MyoII accumulated in apoptotic and neighboring cells at different times during apoptosis.
cells, MyoII::GFP was found to accumulate within the vicinity of the apoptotic boundary LECs (Fig. 4A, double arrows; Movie 10). This accumulation commenced when the space between the two MyoII cables became apparent (t=60; Fig. 4A, arrowhead and arrow) and increased with time (Fig. 4B). The time when MyoII started to accumulate, $\tau_{outer\_MyoII}$, was 35.7±3.8 min after $\tau_{area}$ ($n=9$; Fig. 4E; Materials and Methods), which was not significantly different from the timing of E-cad reduction. This indicated that the initiation of MyoII accumulation in neighboring cells coincided with remodeling of AJs. Moreover, the intensity of MyoII in neighboring LECs increased concurrently with MyoII in histoblasts (t=60 and 70; Fig. 4A, arrowheads), suggesting that the formation of actomyosin cables in the two different cell types (i.e. non-dying histoblasts and LECs) was coordinated. To facilitate the analysis of MyoII accumulation in apoptotic LECs, we quantified the intensity of the MyoII::GFP signal (Fig. 4C; Movie 11) that originated solely from the cell periphery of apoptotic LECs facing histoblasts (Fig. 4C′, arrows). In dying cells, MyoII started increasing as apical constriction commenced ($\tau_{inner\_MyoII}$; Fig. 4D,E). This observation implied that the actomyosin cable in the apoptotic cell is present before the reduction of E-cad levels.

Altogether, the analyses of MyoII distribution showed that the formation of actomyosin cables in apoptotic and neighboring cells occur at different times, implying that the two cables are involved in different stages of apoptotic cell extrusion.

Actomyosin cable formation, apical constriction, and a reduction in E-cad levels upon apoptosis are caspase-3 dependent

In apoptotic LECs, caspase-3 is activated prior to the initiation of apical constriction (Fig. 2), the reduction in E-cad levels (Fig. 3) and the accumulation of MyoII in dying and neighboring cells (Fig. 4).
To investigate the role of caspase-3 in apoptotic cell extrusion, we ectopically expressed a baculovirus caspase inhibitor, p35, in the majority of LECs by Eip71CD-GAL4. This fly also ubiquitously expressed DE-cad::GFPKl or MyoII::GFP. As previously reported (Ninov et al., 2007), tissue expansion (Fig. S13A) and the extrusion of LECs are delayed when p35 is expressed, with cells requiring more time to leave the tissue (Fig. 5A; Movie 12; Fig. 5B, pink line). The number of LECs undergoing extrusion was reduced by more than 90%; however, extrusion did not cease completely (38.7±3.3 and 3.0±1.0 cells within 5 h in wild-type and p35 pupae, respectively). During extrusion of p35-positive LECs, no significant reduction in E-cad levels (Fig. 5A; Fig. 5B, blue line) was observed. Similarly, MyoII did not further accumulate in either the extruding or neighboring cells (Fig. 5C-D; Fig. S13B; Movie 13), and the space between MyoII cables (Fig. 5C) was not observed. These results indicated that the extrusion of p35-expressing LECs is different in nature to that of caspase-positive wild-type LECs. Indeed, Ninov et al. reported that extruded p35-positive cells were viable under the epithelium, and were not engulfed by hemocytes (Ninov et al., 2007). We speculate that the extrusion of p35-positive LECs occurs when they are pushed out by expanding histoblasts (Fig. 1E). Together, our data showed that the formation of actomyosin cables, the apical constriction, and the reduction in E-cad levels observed during wild-type LEC apoptosis are caspase dependent.

**Disruption of MyoII compromises apoptotic cell extrusion, without preventing reduction of E-cad levels**

To investigate further the mechanism behind the strong reduction in E-cad levels and to define the contribution of actomyosin contractility in this mechanism, we ectopically expressed sqh-RNAi (MyoII regulatory light chain RNAi) in the majority of LECs by Eip71CD-GAL4. This fly also ubiquitously expressed either DE-cad::GFPKl or MyoII::GFP (Fig. 6). The level of MyoII::GFP (Fig. 6A,B) and...
phosphorylated myosin regulatory light chain (Fig. S14) in LECs was reduced in sqh-RNAi-expressing pupae compared with wild-type pupae. This was accompanied by a strongly delayed apical constriction (Fig. 6C,D), a low level of E-cad in the LECs (Fig. S15), and less, or absence of, mechanical impact on the surrounding histoblasts (Fig. S1B), compared with wild-type pupae. We observed a strong reduction of E-cad in all sqh-RNAi-positive apoptotic cells (n=9; Fig. 6E, arrowheads; Movie 14). Furthermore, we noticed a space existed between MyoII cables (Fig. 6F; n=10 out of 19 cells) in around half of the apoptotic cells whereas in the other apoptotic cells this space was not observed (Fig. 6G; n=9 out of 19 cells). Together, these results suggest that the strong reduction of E-cad in apoptotic LECs could be independent of actomyosin cable contraction, whereas the formation of the space between actomyosin.
cables, which was normally observed during wild-type LEC apoptosis, required actomyosin contractility.

**Disruption of MyoII in neighboring cells strongly delays apoptotic cell extrusion**

To understand the role of the supra-cellular actomyosin cable in apoptotic cell extrusion, we sought to impair actomyosin contractility only in neighboring non-dying cells. To this end, we expressed sqh-RNAi in a subset of LECs using the mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 1999) (Fig. 7A-B'; Movie 15). We then examined apoptosis of wild-type LECs, which were positioned next to RNAi-positive LECs (Fig. 7B, blue dotted outlines). In this case, all but one of the neighboring cells were wild type, and the contractility of the supra-cellular actomyosin cable was therefore only partially affected. First, we qualitatively compared the apical constriction of apoptotic wild-type LECs that were next to RNAi-positive cells (hereafter referred to as LECWT/RNAi; Fig. 7B, red dotted outlines) with that of wild-type LECs fully surrounded by wild-type cells (hereafter referred to as LECWT/WT; Fig. 7B, white dotted outlines). This comparison was performed on cells in the same pupa. We found that LECs with MyoII-defective neighbors (LECWT/RNAi) exhibited a slower apical constriction, which, compared with controls, also required a longer time to complete (Fig. 7C). Quantitative analyses further supported these observations (Fig. 7D) and we reasoned that this delay was the result of the partially compromised supra-cellular actomyosin cable in neighboring cells. The contour plot of the apoptotic LECWT/RNAi shown in Fig. 7B shows that the apical constriction appears to be biased toward the RNAi-positive cell (Fig. 7E, arrow). This observation was further supported by tracking the trajectory of the centroid of the apoptotic cell shape (Fig. 7F,F'). These observations suggested that supra-cellular actomyosin cables with partially defective myosin contraction lead to eccentric deformation of apoptotic cells. Together, our data showed that the actomyosin cable that formed in neighboring cells upon apoptosis plays an important role in the extrusion process.

**Tissue tension is transiently released upon E-cad reduction and rebuilt afterwards**

The appearance of two actomyosin cables associated with a reduction in E-cad level implied that tissue tension is altered during the apoptotic cell extrusion. Indeed, we found that a subset of neighboring histoblast cell boundaries, which are orthogonal to the interface between apoptotic and neighboring cells, lose their...
progression of AJ disengagement (Fig. 5). As caspase-3 is not activated in the neighboring cells, we speculate that the reduction of E-cad is a consequence of a loss of trans-interactions between E-cad in the neighboring cell and E-cad in the apoptotic cell, which undergoes caspase-3-dependent cleavage. This often, but not always, leads to plasma membrane separation (Fig. 3G), which is suggestive of a loosening of AJ-dependent adhesion. Reyes et al. reported that anillin organizes and stabilizes actomyosin contractile rings at AJs and its knockdown is associated with a reduction of E-cad and β-cat levels at AJs, leading to AJ disengagement (Reyes et al., 2014). A gradual decrease in the level of E-cad (Fig. 3E) and a gradual increase in MyoII accumulation in apoptotic cells (Fig. 5D) was observed prior to the strong reduction of E-cad levels. This leads us to hypothesize that mechanical tension exerted on the cell interface between apoptotic LECs and neighboring cells by the contraction of the actomyosin cable, which forms in the apoptotic cell, is large enough to rupture the weakened contacts between plasma membranes at AJs upon the strong reduction of E-cad levels. Interestingly, and by contrast, there are cases when AJs are not disengaged even after the level of E-cad is reduced. In these cases, the cells exhibit a separation of actomyosin cables from the membrane (Fig. S11). We speculate that the state of cell-cell contacts at AJs, i.e. whether they will disengage or remain engaged during apoptosis, is dependent on which of the following links is weaker: the link between two plasma membranes, or the link between the plasma membrane and the actomyosin cable. Both of these links would be weakened by a strong, albeit incomplete, reduction of E-cad levels. When the former is weaker than the latter, the two plasma membranes could be detached. When the former is stronger than the latter, the two plasma membranes could remain in contact, and the actomyosin cable could be detached from the plasma membrane.

In parallel with the reduction of E-cad levels and the associated release of tension (Fig. 8), a supra-cellular actomyosin cable begins to relax (Fig. 3A, arrow) when the outer actomyosin cable contracted (Fig. 3B, double arrow). Quantification of the linearity of the cell boundaries (Materials and Methods) further supported these observations (Fig. 3D). To characterize the change in tension of neighboring cells during apoptosis, we probed the junctional tension at different stages of apoptosis by laser nano-ablation (Hara et al., 2016) (Fig. 8A–C; Movie 16). The laser ablations (arrowheads) were performed at three different stages of apoptotic cell extrusion: before reduction of E-cad levels (A,A'); right after reduction (B,B'); and during neighboring actomyosin cable (double arrow in C) contraction (C,C'). Scale bar: 5 μm. (D,E) Statistical comparison of the cell boundary linearity (D) and the recoil velocities (E) at each stage of cell extrusion shown in A-C. n=17, 12 and 27 for control, after reduction, and, during cable contraction, respectively. Error bars indicate s.e.m. *P<0.05; ***P<0.001.
to form in neighboring cells (Fig. 4). These observations prompt us to speculate that the release of tissue tension triggers MyoII accumulation in neighboring cells. Subsequent contraction of this outer ring helps to re-shape tissue tension, which is transiently released when E-cad is reduced. As a consequence, the neighboring cells are stretched (Fig. 1). Upon completion of apical constriction, neighboring non-apoptotic cells form de novo AJs (Fig. S8) and the stretched cells undergo cell division and/or cell-cell contact rearrangement (Fig. S2). These processes allow a relaxation of the high tension associated with the stretching of cells (Fig. 7E). Finally, our measurements of caspase-3 activity (Fig. 2), and our observations from caspase inhibition experiments (Fig. 5), help us to conclude that the characteristics associated with apoptotic cell extrusion reported in this study are the consequences of the apoptotic process, rather than the cause.

**Apoptosis mechanically drives tissue expansion**

In addition to the progressive remodeling of AJs and modulation of tissue tension during apoptosis, we examined the mechanical role of apoptosis ‘apoptotic force’ in tissue morphogenesis, which has been proposed, demonstrated and discussed previously (Stenn et al., 1998; Toyama et al., 2008; Suzanne et al., 2010; Teng and Toyama, 2011; Miura, 2012; Kuranaga, 2012; Monier et al., 2015; Okuda et al., 2016; Pérez-Gario and Steller, 2015; Monier and Suzanne, 2015; Ambrosi et al., 2016). We show that the mechanical force generated by the contraction of actomyosin cables formed when LECs undergo apoptosis, especially boundary LECs, promotes tissue expansion (Fig. 1; Fig. S1), along with histoblast proliferation and migration (Ninov et al., 2007, 2010). Nonetheless, we cannot rule out the possibility that this apical contraction is in part driven by a decrease in cell volume, which can be triggered by caspase activation (Saias et al., 2015). Intriguingly, we found that apoptosis of non-boundary LECs did not affect tissue expansion (Figs S5, S6). This raised the possibility that the mechanical influence of apoptosis in neighboring tissues is dependent not only on the physical connections between cells but also on the mechanical properties of cells, including cell compliance. If a tissue is soft, for instance, the tensile forces generated by the apoptotic process could be absorbed by nearest-neighbor cells and would not propagate to cells further than a single cell away. We speculate that the apoptotic process could mechanically contribute to cell death-related morphogenesis, but only when apoptosis takes place among a tissue with optimal mechanical properties.

Here, we present a framework for understanding how cell adhesions and tissue tension are progressively modulated during apoptosis in a developing epithelium (Fig. S17). We conclude that tissue tension re-shaping, including the transient release of tension upon a reduction in the levels of AJ components, represents a mechanism of apoptotic cell extrusion. It would be important to explore how this transient modulation in mechanical tension would further influence the biochemical nature of neighboring non-apoptotic cells.

**MATERIALS AND METHODS**

**Drosophila stains, fly husbandry, sample preparation and live imaging**

Fly lines are described in supplementary Materials and Methods. Flies and crosses were raised on standard media at 25°C. Staged pupae were collected, dissected, mounted and imaged as previously described (Ninov and Martín-Blanco, 2007). Z-stack images were captured every 1.6 μm along the apical-basal axis of the tissue at 5-min intervals (except for Fig. 3D, which was at 2.5-min intervals) using a Nikon A1R MP confocal microscope with an Apo 40× WI.5. S DIC N2, N.A 1.25 objective, or a Zeiss LSM 510 Meta inverted confocal microscope with a LD C-Apo 40×, N.A 1.1 objective. All imaging was performed at room temperature.

**Image analyses and quantification**

All confocal images shown (except for those in Fig. 8, Fig. S12 and Fig. S16) are maximum projections of ~3 μm along the apical-basal axis of a cell around AJs. The images shown in Fig. 8, Fig. S12 and Fig. S16 are single confocal sections at AJs. We used ImageJ, Packing Analyzer V2.0 (Aigouy et al., 2010), and/or Matlab software (MathWorks) for image quantification and subsequent analyses. See supplementary Materials and Methods for further details of image quantification and subsequent analyses of cell shape anisotropy, caspase 3 activity, apical constriction timings, MyoII intensity, cell boundary linearity, initial recoil velocity and cell shape deformation.

**Immunohistochemistry**

Pupae were staged as described by Ninov and Martín-Blanco (2007), and dissected and fixed following protocols described by Wang and Yoder (2011). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). The primary antibody anti-pMLC (Cell Signaling, #3671) was used at 1:50. A secondary antibody was Alexa-546-conjugated donkey anti-rabbit (1:1000, LifeTechnology, A10040).

**Laser ablation**

Laser ablation of histoblasts was performed using a Leica TCS SP5 multiphoton confocal microscope with a 63×/1.4-0.6 HCX PL Apo objective. Ablation was carried out at the AJ plane with a multi-photon laser (Mai-Tai HP from Spectra Physics, CA, USA) set to 800 nm, with a laser power of 40% of the 2.8 W maximum output. Further details can be found in Founnounou et al. (2013).

Laser ablation of LECs was performed using a femtosecond laser (FP1030 from Fianium, UK) interfaced to a Nikon A1R MP confocal microscope. The laser power was set to 140 mW at the back aperture of the objective. Further details can be found in Hara et al. (2016).

**Statistics**

All the P-values were calculated using Student’s t-test with P<0.05 considered significantly different.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

X.T. and Y.T. designed the research and prepared the manuscript. X.T., R.L.B., and X.T. performed the experiments and analyses. L.Q. contributed new reagents. Y.T. oversaw the project.

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**Supplementary information**

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