A role for Myosin in triggering and executing amnioserosa cell delaminations during dorsal closure.

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ABSTRACT

The remodeling of epithelial tissues is a critical process in morphogenesis, often involving the apoptotic removal of individual cells while preserving tissue integrity. In *Drosophila*, the amnioserosa—a highly dynamic extra-embryonic tissue—undergoes extensive remodeling, culminating in its complete elimination at the end of dorsal closure. While apoptotic cell delaminations in the amnioserosa have been proposed to contribute to dorsal closure, the cellular mechanisms underlying this process remain poorly understood. In this study, we have investigated actomyosin dynamics during cell delaminations and analyzed the consequences of perturbing non-muscle Myosin activity globally in the entire tissue as well as locally in groups of cells. We found that Myosin plays an essential role in both triggering and executing cell delaminations, with high Myosin contractility promoting cell extrusion even in the absence of caspase activity. Additionally, our results suggest that cell delaminations are governed by both cell-autonomous Myosin dynamics and mechanical cues from the tissue environment. Together, these findings provide new insights into the regulation of epithelial cell removal and the complex interplay between apoptotic and mechanical signals during tissue remodeling.

INTRODUCTION

The remodeling of epithelial tissues is a fundamental process in morphogenesis, giving rise to a diverse array of structures, including sheets, tubes, and cysts, which are essential for organ formation and function. A key aspect of epithelial remodeling is the apoptotic removal of individual cells from the epithelium while maintaining overall tissue integrity. Apoptotic cell elimination has been observed in diverse contexts contributing both to tissue morphogenesis and to the maintenance of tissue homeostasis[1–3]. In some instances, entire epithelial tissues are removed and replaced, as occurs during embryogenesis in the extra-embryonic tissues of insects[4] and during metamorphosis[5].

The amnioserosa is the single extra-embryonic tissue present in Drosophila[6]. It is specified during early embryonic development by the dorso-ventral patterning system and plays essential functions during two vital morphogenetic movements, germband retraction and dorsal closure[7]. Amnioserosa cells undergo dramatic cell shape changes during embryogenesis. During germband elongation, these cells elongate and transition from a columnar to a squamous morphology through a process known as rotary cell elongation[8]. As development progresses into germband retraction, amnioserosa cells shorten, become isodiametric, and exert a pulling force that contributes to germband retraction[9,10]. Finally, during dorsal closure, amnioserosa cells exhibit dynamic fluctuations in apical surface area, driven by periodic contractions of the actomyosin cytoskeleton, and progressively reduce their apical surface[11–14]. Around 10% of the cells of the tissue basally delaminate before closure is completed[15–18]. These cellular processes, along with a decrease in volume, generate a morphogenetic force that drives the movement of the lateral epidermal sheets towards the dorsal midline. Ultimately, the entire amnioserosa tissue is internalized and degenerates by apoptosis[19–21].

Amnioserosa cell delaminations are apoptotic, and although they are not essential for closure, enhancing or supressing apoptosis increases or reduces cell delaminations respectively, leading to either an acceleration or a slowing down of closure[16]. This led to the suggestion that apoptotic cell delaminations in the amnioserosa provides an apoptotic force that contributes to closure. Additionally, inhibiting apoptosis in the amnioserosa also

prevents cell volume decrease in the bulk of the tissue[22], showing that the apoptotic pathway controls both individual delaminations and the remodeling of the entire tissue.

The cellular mechanisms underlying the elimination of cells from an epithelium have been studied in several tissues, revealing the involvement of both cell autonomous and non-autonomous mechanisms. In the apoptotic cell, either an actomyosin ring[23–26] or medioapical actomyosin contractions[27–29] can contribute to autonomous-cell constriction, while neighbouring cells form a supracellular actomyosin cable that aids in the expulsion of the cell from the tissue[23,24,26]. In the amnioserosa, high levels of reactive oxygen species induce caspase activity and cell delaminations through the reorganization of non-muscle Myosin distribution[18]. During the early stages of dorsal closure, delaminating cells transition abruptly from a pulsatile behaviour to a rapidly, unpulsed, contracting behaviour[30]. This delamination transiently affects the oscillating behaviour of the neighbouring cells, and changes in Myosin dynamics have been observed in both the delaminating cell and its neighbours[30]. However, the precise cellular mechanisms underlying cell delamination remain unclear. Additionally, mechanical stresses have been proposed as potential triggers for both individual cell delamination of cells and the degeneration of the entire amnioserosa, but evidence for this has been lacking. In this work, we have investigated the cellular mechanisms driving cell delaminations in the amnioserosa and analyzed the consequences of perturbing non-muscle Myosin activity globally in the entire tissue as well as locally in groups of cells. Our results argue for an active role for Myosin in both triggering and executing cell delaminations and show that cells with high contractility can undergo delamination independently of caspase activity.

RESULTS

1) Actomyosin dynamics during amnioserosa cell delaminations

Cell delaminations in the amnioserosa can be observed from the end of germband retraction until the completion of dorsal closure. Although their number varies among embryos, they exhibit a consistent spatial pattern, occurring mostly in the anterior half of the amnioserosa and at the posterior canthi[15,17,18] (see below).

In epithelial tissues, cell delaminations are usually mediated by T2 transitions, in which multiple vertices converge to form a single vertex, generating a rosette-like structure. In the amnioserosa, while some cells delaminate through T2 transitions (Figure 1A-A´´), we also observe delaminating cells that do not form rosettes. Instead, these cells leave a new junction once they are delaminated (Figure 1B-B´´). This type of delamination occurs in anisotropic cells where the longer junctions, usually dorso-ventrally oriented, approach and fuse, forming a new junction between new neighbours. We found that cells forming rosettes are more isotropic at the onset of delamination than cells leaving a junction (Supplementary Information, Figure 1SA), suggesting that cell shape determines the geometry of the delamination process. Both types of cell delamination events are prevented upon expression of an apoptosis inhibitor such as p35 or Drosphila inhibitor of apoptosis 1, Diap1[15,16,18].

Cell delaminations are driven by either a cortical actomyosin ring or pulses of contractile medioapical actomyosin[24,26–29]. To investigate the mechanisms driving cell delaminations in the amnioserosa, we visualized non-muscle Myosin (MyoII) and actin distribution in delaminating cells from live embryos carrying the ECad:mTomato (ECad:mT) and the sqh:GFP knock-in alleles, or ECad:mT and the actin reporter utrophin fused to GFP (utr:GFP) (Figure 1C-I”). Since MyoII subcellular localization and dynamics changes as dorsal closure progresses, characterized by increasing junctional MyoII and stabilisation of the medioapical pool[12,13], we examined cell delaminations at both early and late stages. During early stages, as the delaminating cell begins to apically constrict, we observed a stabilisation of medioapical MyoII, with no accumulation of junctional Myosin nor actin (Figure 1C, F). Early delaminating cells are pulsatile, and the onset of apical constriction coincides with the stabilisation of a medial MyoII pulse, giving rise to a non-pulsatile delaminating cell, as previously observed[30]. As the apical cell area reduces, cell junctions show an inward curvature (Figure 1C, C´, arrow), suggesting the existence of a pulling force from the medial part of the delaminating cell. Interestingly, we observed the presence of MyoII and actin puncta that start to form basally as the onset of the delamination process (Figure 1D´, D”, G, G´, arrows). As the apical cell area reduces, actin puncta can be seen to re-localize apically at the level of ECad (Figure 1F´, arrow) to form a continuous junctional belt. During the last steps of the process, an actomyosin junctional ring is observed subapically (Figure 1D”, E”,

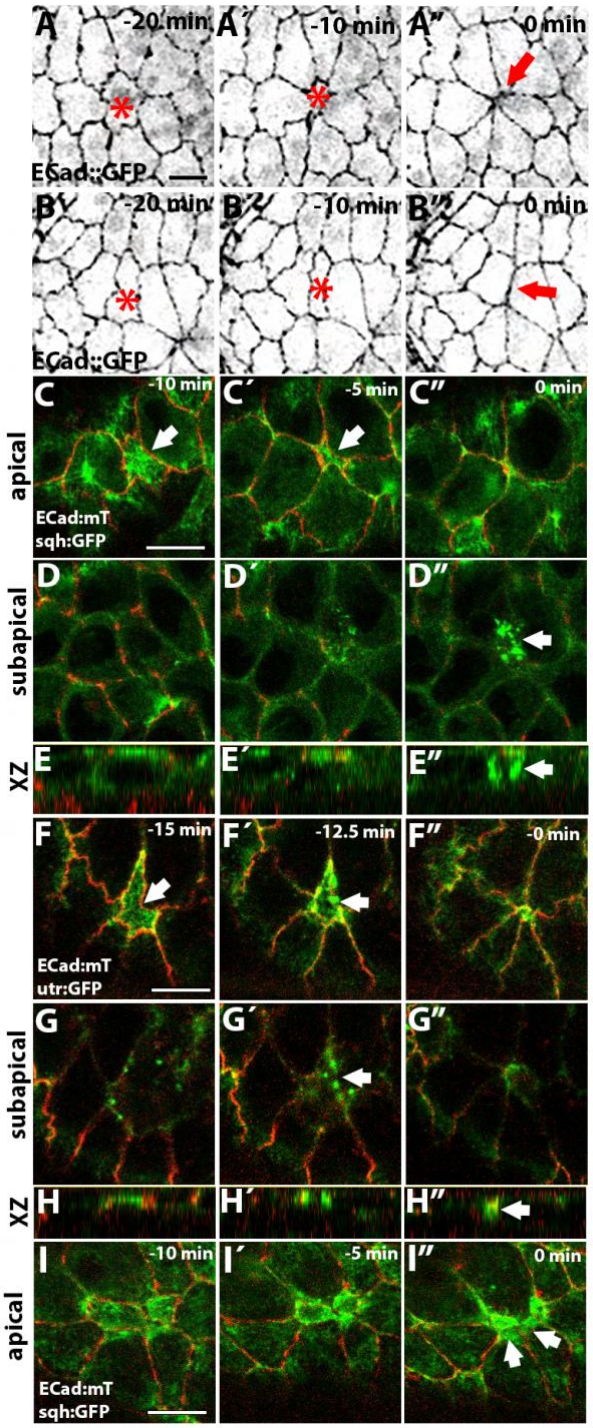

Figure 1. Actomyosin dynamics during amnioserosa cell delamination. (A,B) Still images (maximum intensity projections) from a time-lapse movie (Movie 1) of a dorsal closure Drosophila embryo carrying the knock-in allele ECad:GFP at different time points prior to the delamination of the cell (asterisk) from the plane of the epithelium, showing the formation of a rosette-like structure (A) or the formation of a new junction (B, arrow). (C-E) Still images (individual Z planes) from a time-lapse movie of an early dorsal closure embryo carrying the knock-in alleles ECad:mT and sqh:GFP (C-E, Movie 2) or the knock-in allele ECad:mT and the reporter utrophin:GFP (F-H, Movie 3) showing an apical (C, F) and subapical (D, G) planes, as well as an orthogonal view (E, H)) at indicated times prior to the delamination. Notice the stabilisation of medioapical actomyosin during the apical-contraction phase (C, C´,F, arrows), the appearance of actin puncta (F´,G´, arrows) and the formation of a subapical actomysoin ring during the final phase (D", E", arrows). (I-H) Still images (maximum intensity projections) from a time-lapse movie of a late dorsal closure embryo carrying the knock-in alleles ECad:mT and sqh:GFP. Notice the accumulation of Myosin in neighbouring cells (I", arrows), which coincides with a decrease of ECad:mT at cell junctions.

During late dorsal closure, delaminating cells also show a stabilisation of medioapical MyoII that spans the whole apical cortex as the cell area reduces (Figure 1I). An actomyosin ring is also observed as the delamination process proceeds (Figure 1I´, I”). Such cytoskeletal dynamics has been observed in both isotropic and anisotropic cell delaminations (data not shown).

In contrast to what has been observed in other epithelia, we did not observe the formation of a supracellular actomyosin ring in neighbouring cells. However, in some late delaminating cells, MyoII accumulated in neighbouring cells at the junctions with the delaminating cell (Figure 1I”, arrows) coincident with a decrease in ECad levels (Supplementary Information, Figure 1SB). We propose that this could be a mechanism to maintain junction integrity during the delamination process[31] but would not be required to expulse the cell from the tissue. Altogether, these observations indicate that amnioserosa cells delaminate through a mechanism mediated by medioapical Myosin, with the formation of an actomyosin ring in the last steps of the delamination process.

The Rho family of GTPases is a central regulator of F-actin, MyoII activity and contractility[32]. Both Rho-dependent and independent mechanisms for apoptotic cell elimination have been reported[33]. In the amnioserosa, using a Rho live sensor consisting of the Rho1 GTP-binding domain of anillin to GFP (aniRBD:GFP)[34], we have observed an increase in medioapical Rho activity as soon as the apical cell area of a delaminating cell begins to decrease (Figure 2B). Thus, it is possible that apoptotic signals induce medioapical Rho activity, which in turn regulates the cytoskeletal changes described above that participate in the execution of the delamination process. Interestingly, during late stages of dorsal closure, all amnioserosa cells show medioapical accumulation of the Rho sensor (Figure 1A, A´). Since medioapical Rho activity is observed in apoptotic delaminating cells, and caspase activity has been detected throughout the amnioserosa tissue during late dorsal closure stages (Figure 5A-B”)[22,35,36], we asked whether this late-stage medioapical Rho accumulation depends on caspase activity. The ectopic expression of Diap1 in the amnioserosa, while preventing cell delamination, does not prevent the medioapical accumulation of the Rho sensor in the bulk of the tissue during late dorsal closure (Figure 2C). This result suggests that Rho activity at the

medioapical cortex of amnioserosa cells is regulated differently in delaminating cells versus the bulk of the tissue, where it appears to be independent of apoptotic signals.

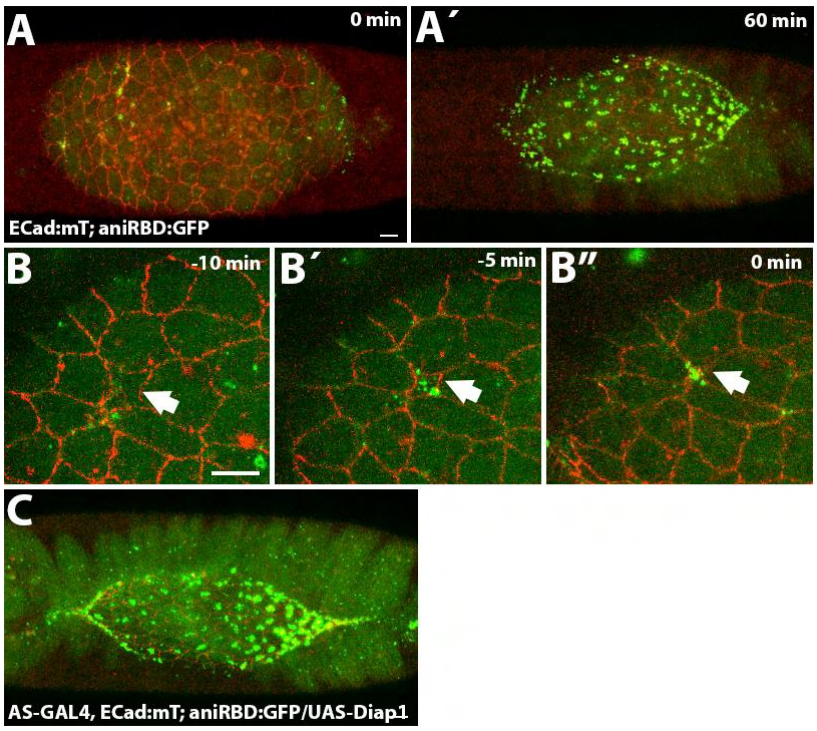

Figure 2. Rho activity in the amnioserosa. (A, A´) Still images (maximum intensity projections) from a time-lapse movie of a dorsal closure Drosophila embryo carrying the knock-in allele ECad:mT and the Rho sensor (aniRBD:GFP). During most of dorsal closure, the activity of the Rho sensor is only detected in delaminating cells (A). By the end of the process, most of the cells show medioapical accumulation of the Rho sensor (A´). (B) Magnification of the same time-lapse than in A (Movie 4), showing a delaminating cell (arrows) and the medioapical accumulation of the Rho sensor prior to cell delamination at the times indicated.

2) Perturbing Myosin levels in the whole tissue does not alter the number of cell delaminations

To investigate the requirement for Myosin in the triggering and the elimination of cells from the tissue, we aimed to perturb Myosin II activity in the whole tissue. We have previously shown that the ectopic expression of a constitutive active form of Mbs in the amnioserosa (Mbs is the Myosin binding subunit of the protein phosphatase PP1), decreases phosphorylated Myosin, decreases the rate of contraction of amnioserosa cells and slows dorsal closure[37,38]. Here, we analyzed whether cell delaminations are affected upon ectopic expression of MbsN300 (Figure 3B, D). Because dorsal closure takes longer in embryos

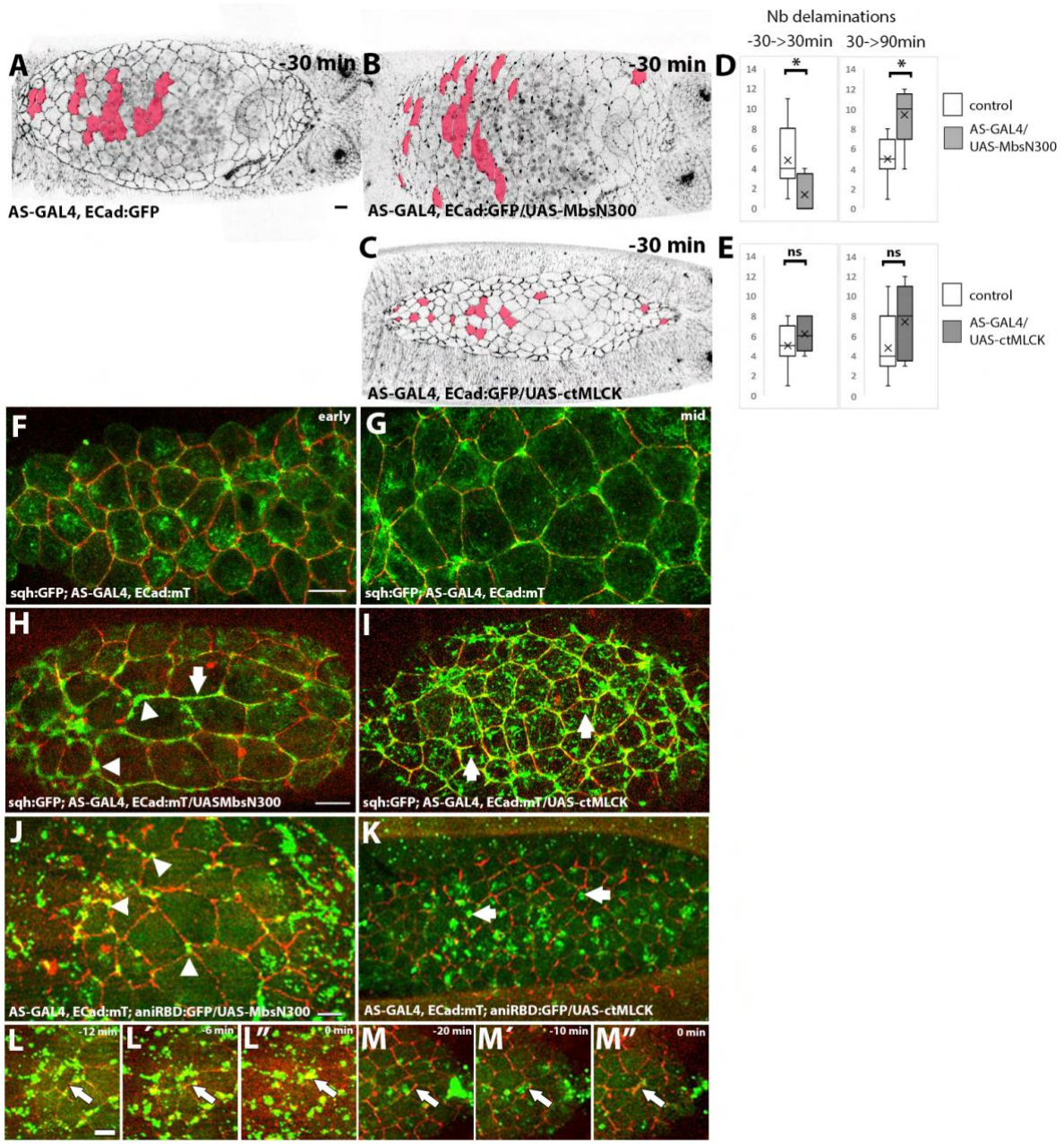

Figure 3. Effect of perturbing Myosin activity in the amnioserosa on cell delaminations and actomyosin contractility. (A-C) Still image of a control ECad:GFP embryo (A, Movie 1), an AS-GAL4, ECad:GFP/UAS-MbsN300 (B, Movie 5) and an AS-GAL4, ECad:GFP/UAS-ctMLCK embryo (C, Movie 6) at -30 min of dorsal closure (staging relative to the distance of posterior spiracles). The cells labelled in red are cells that delaminate during dorsal closure. (D) Number of delaminating cells in AS-GAL4, ECadGFP (n=11) and AS-GAL4, ECadGFP/UAS-MbsN300 (n=5) embryos, between -30 and 30 min (left panel, p = 0.039) and between 30 and 90 min (right panel, p = 0.029). Although the total number of delaminating cells is similar between the two genotypes, AS-GAL4, ECadGFP/UAS-MbsN300 embryos show a delay in the appearance of cell delaminations, with most of them occurring from 30 min of dorsal closure. (E) Number of delaminating cells in AS-GAL4, ECadGFP (n=11) and AS-GAL4, ECadGFP/UAS-ctMLCK (n=5) embryos, between -30 and 30 min (left panel) and between 30 and 90 min (right panel). No differences in the total number of delaminating cells nor a change in the timing of appearance of cell delaminations is observed in these two conditions. (F, G) Still images from time-lapse movies of embryos carrying the knock-in alleles ECad:mT and sqh:GFP at early (F) and mid dorsal closure (G). (H, I) Still images from time-lapse movies of embryos carrying the knock-in alleles ECad:mT and sqh:GFP and ectopically expressing MbsN300 (H) or ctMLCK (I). (J, K) Still images from time-lapse movies of embryos carrying the knock-in allele ECad:mT and the Rho sensor and ectopically expressing MbsN300 (J, Movie 7) or ctMLCK (K, Movie 8). Notice the accumulation of Sqh:GFP and the Rho sensor at bicellular and tricellular junctions in H, J (arrow and arrowheads respectively) and their medioapical accumulation in I, K (arrows). (L, M) Still images of a delaminating cells from an embryo ectopically expressing MbsN300 (I) or ctMLCK (J) at the times indicated prior to cell delamination.

ectopically expressing MbsN300, to compare the number of delaminations between the two genotypes, we considered the delaminations occurring during the same period of time, from -30 min to 100 min of dorsal closure, and staging the embryos according to the distance between the posterior spiracles. Surprisingly, we found that the total number of cell delaminations in MbsN300 embryos is indistinguishable from that in control embryos. However, when comparing the timing of cell delaminations, we observed a delay: the number of cell delaminations between -30 and 30 min of dorsal closure is lower in AS-GAL4, ECad:GFP/UAS-MbsN300 than in controls but is higher between 30 and 100 min of dorsal closure (Figure 3D). These results suggest an early requirement of MyoII for triggering cell delaminations, however, unexpectedly, during later stages, cell delaminations resume and become more frequent.

To explore what could be driving late delaminations in these embryos, we analyzed MyoII localization in sqh:GFP; AS-GAL4, ECad:mT/UAS-MbsN300 embryos. While MyoII localizes in medioapical foci in early dorsal closure, in older embryos, MyoII shows an aberrant distribution, accumulating at bicellular junctions and cell vertices, without forming the dense medioapical mesh observed in control embryos (Figure 3H, compare with 3F and 3G, see also[31]). Interestingly, the Rho sensor is also mislocalized, accumulating at cell junctions and cell vertices (Figure 3J). In these embryos, delaminating cells display junctional rather than medioapical Rho1 activity (Figure 3L-L”), along with strong junctional MyoII (Supplementary Information, Figure 2SA). These observations suggest that, in these embryos, cell delamination does not involve medioapical MyoII activity but rather junctional Rho1 and MyoII.

Reciprocally, we aimed to increase Myosin activity in the amnioserosa by ectopically expressing a constitutive active form of Myosin Light Chain Kinase (ctMLCK). We have previously shown that these cells are more contracted and accumulate both junctional and medioapical MyoII[12,39]. While in other tissues increasing Myosin activity perturbs cell delaminations[40], in the amnioserosa neither the number nor the timing of cell delaminations are altered (Figure 3C, E). These cells exhibit an accumulation of Myosin at cell junctions and a persistent, dynamic medioapical network (Figure 3I), which further accumulates in extruding cells (Supplementary Information, Figure 2SB). Interestingly, most of amnioserosa

cells with ectopic ctMLCK show medioapical accumulation of the Rho sensor (Figure 3K), which becomes more pronounced in delaminating cells (Figure 3M-M”). These observations, together with the junctional localization of the Rho sensor upon ectopic expression of MbsN300, show that perturbing Myosin localization impacts Rho localization. These observations are in agreement with the existence of a positive feedback between the upstream regulator Rho and MyoII[34] (see Discussion).

3) In a tissue with tension heterogeneity, cells with higher levels of MyoII are more likely to delaminate

We were intrigued by the observation that perturbing Myosin activity does not affect the overall rate of cell delaminations. While increased actomyosin contractility underlies cell ingression in several tissues[41,42], a global increase of junctional MyoII has been found to inhibit both apoptotic basal cell delamination and apoptotic apical extrusion[40,43]. To further

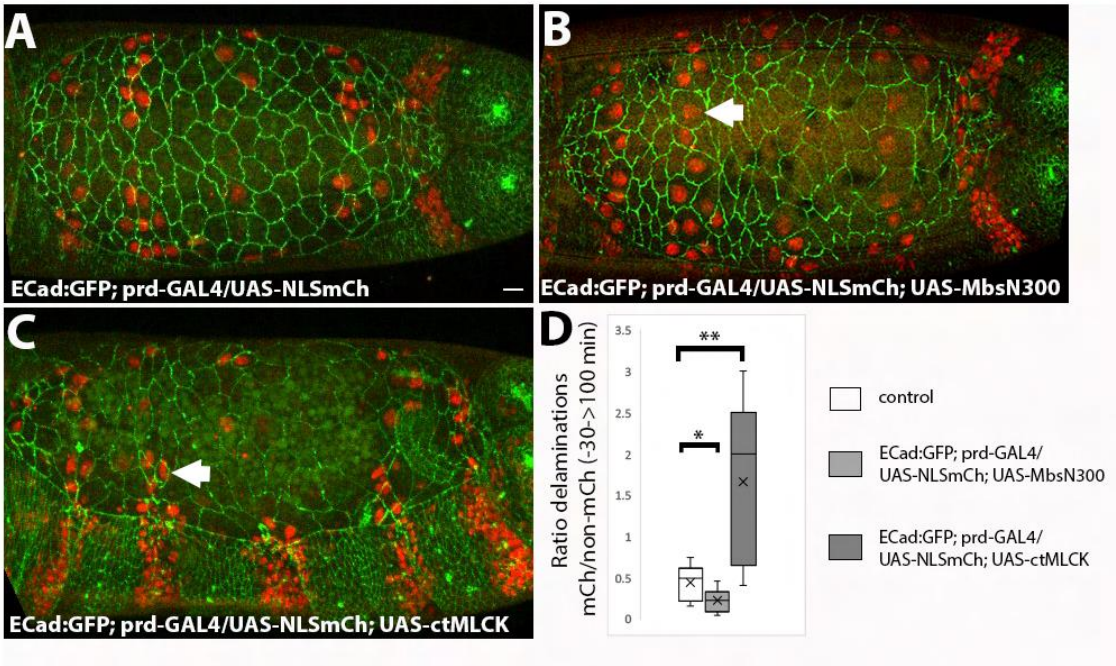

Figure 4. Effect of perturbing Myosin activity in groups of amnioserosa cells on cell delaminations. (A-C) Still images of from time-lapse movies of ECad:GFP; prd-GAL4/UAS-NLS:mCh (A, Movie 9), ECad:GFP; prd-GAL4/UAS-NLS:mCh; UAS-MbsN300 (B, Movie 10) and ECad:GFP; prd-GAL4/UAS-NLS:mCh; UAS-ctMLCK (C, Movie 11) embryos at 0 min of dorsal closure. (D) Ratio of NLS:mCherry to non-NLS:mCherry delaminating cells in ECad:GFP;prd-GAL4/UAS-NLS:mCh (n=11), ECad:GFP;prd-GAL4/UAS-NLS:mCh; UAS-MbsN300 (n=6) embryos and ECad:GFP;prd-GAL4/UAS-NLS:mCh; UAS-ctMLCK (n=5) embryos. Cells with lower levels of Myosin activity than their neighbours delaminate less than their neighbours ($p = 0.049$) while cells with higher levels of Myosin activity than their neighbours delaminate more ($p = 0.009$).

investigate this, we generated heterogeneities in MyoII levels across the tissue by selectively increasing or decreasing Myosin activity in groups of cells. For this, we ectopically expressed UAS-MbsN300 or UAS-ctMLCK using the prd-Gal4 driver, which is expressed in alternate segments in the epidermis and in the amnioserosa, along with a NLS:mCherry (NLS:mCh) reporter to identify MbsN300 or ctMLCK expressing cells. To assess whether changes in MyoII activity influence the likelihood of a cell delaminating, we calculated the ratio between the number of delaminating cells NLS-mCh positive and the number of delaminating cells NLS-mCh negative for each embryo.

In ECad:GFP, prdGAL4/UAS-NLS:mCh, UAS-MbsN300 embryos, we observe that MbsN300 expressing cells show a larger apical cell area (Figure 4B, arrow, compare with 4A), confirming that these cells have lower actomyosin contractility. In these embryos, the ratio of NLS-mCh delaminating cells (and thus expressing MbsN300) to non NLS-mCh delaminating cells (with wild type MyoII levels) is lower than in control embryos (Figure 4D). These results confirm a requirement for medioapical MyoII in cell delamination. Conversely, in prdGAL4/UAS-NLS:mCh; UAS-ctMLCK embryos, we observe that ctMLCK positive cells show a smaller apical cell area (Figure 4C, arrow, compare with 4A). In this case, the ratio of NLS:mCh delaminating cells (with higher both junctional and medioapical MyoII) to non-NLS:mCh delaminating cells (with wild type MyoII levels) is greater than in control embryos (Figure 4D). Thus, although no increase in the number of delaminations is observed when Myosin activity is increased across the whole tissue, we find that cells with higher levels of Myo are more likely to delaminate when surrounded by cells with wild type MyoII activity. Importantly, delaminations of cells with high levels of MyoII occur throughout the entire process of dorsal closure, suggesting that high MyoII levels alone do not immediately induce delamination but increase the probability of a cell to delaminate. These results, together with the observation that increasing MyoII activity across the whole tissue does not result in an increase in the number of delaminations, suggest that high tissue stiffness could disrupt cell delamination.

4) Blocking endocytosis prevents cell delaminations

In the pupal epidermis, a decrease in endocytic activity impairs or accelerates cell delaminations depending on the region of the segment where delaminations occur[5,29]. Therefore, we aimed to investigate the effect of blocking endocytic activity in the amnioserosa. We have previously shown that the ectopic expression of Rab5DN in the amnioserosa compromises apical constriction and apical membrane removal[44]. We

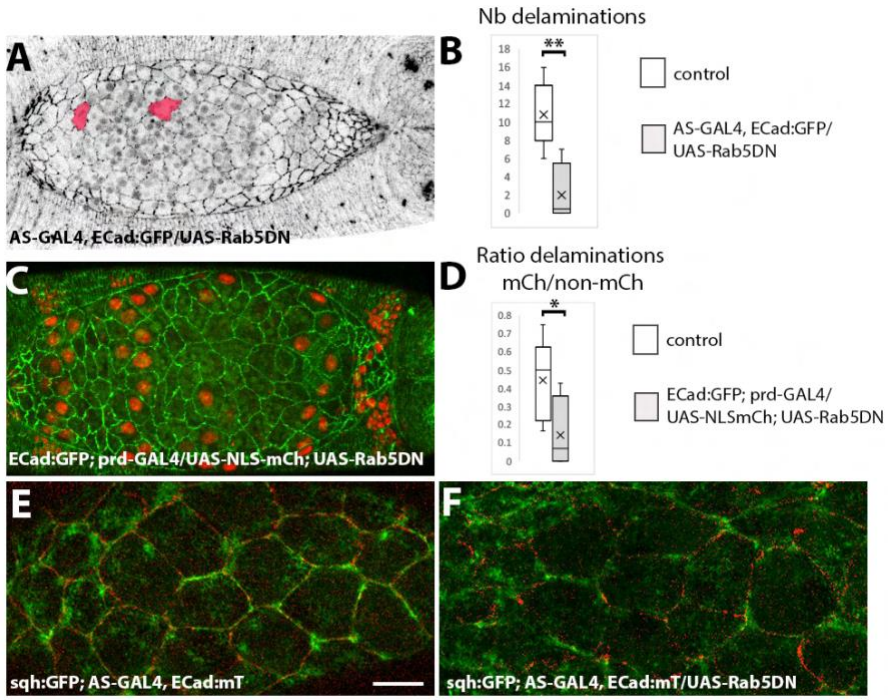

Figure 5. Blocking endocytosis prevents cell delaminations. (A) Still image from a time-lapse movie of an AS-GAL4, ECad:GFP/UAS-Rab5DN embryo at -30 min of dorsal closure (Movie 12). (B) Number of delaminating cells between -30 min and 100 min in control (n=7) vs AS-GAL4, ECad:GFP/UAS-Rab5DN (n=3) embryos ($p < 0.001$). (C) Still image from a time-lapse movie (MIP) of an ECad:GFP; prd-GAL4/UAS-Rab5DN embryo at 0 min of dorsal closure (Movie 13). (D) Ratio of NLS:mCherry to non-NLS:mCherry delaminating cells in ECad:GFP;prd-GAL4/UAS-NLSmCh (n=11) and ECad:GFP;prd-GAL4/UAS-NLSmCh; UAS-Rab5DN (n=4) embryos ($p = 0.03$). (E, F) Still images from a time lapse movie from sqh:GFP; AS-GAL4, ECad:mT (E) and sqh:GFP; AS-GAL4, ECad:mT/UAS-Rab5DN (F) embryos.

observed a decrease in the number of cell delaminations with only few delaminations that occurring during late stages of dorsal closure (Figure 5A, B). The expression of Rab5DN in a mosaic manner using the prd-GAL4 driver shows that Rab5DN-expressing cells have a greater apical cell area than their neighbours (Figure 5C), confirming that blocking endocytosis affects apical contraction. In these embryos, cells expressing Rab5DN are less likely to delaminate than control cells (Figure 5D). We examined the subcellular localization

of MyoII and found that although MyoII pulses are present, there is a decrease in junctional MyoII (Figure 5E, F). In fact, it has been shown that Rab5DN expressing amnioserosa cells show lower junctional tension, suggesting that endocytosis is important to maintain junctional tension[31]. These results show that endocytic activity is required for amnioserosa cell delaminations, which could be related, at least in part, to a decrease in tissue tension.

5) Interaction between contractility and caspase activity

Our results show that cells with increased MyoII activity due to the ectopic expression of ctMLCK are more likely to delaminate when they are surrounded by cells with control levels of MyoII (Figure 4C, D). This observation raised the question of whether increased Myosin activity affects caspase activity. To investigate this possibility, we explored the relationship between Myosin and caspase activity. The apoptotic sensor Apoliner has been widely used in several systems to monitor capsase activity[45]. In this sensor, the fluorescent proteins mRFP and eGFP, are linked by a caspase-specific cleavage site from Diap1. Caspase activation leads to the cleavage of Apoliner and to the translocation of the eGFP from the cytoplasm to the nucleus. In the amnioserosa, during early stages of dorsal closure, the sensor is not active, except in the most posterior cells (Figure 6A). As dorsal closure progresses, the sensor is activated first in anterior cells and then in the whole tissue before closure is complete (Figure 6B, C and see also[22,35]). The activation of the sensor is completely blocked in embryos ectopically expressing Apoliner and Diap1 even after the amnioserosa has been internalized underneath the epidermis (Supplementary Information, Figure 3S). Thus, Apoliner can detect low levels of caspase activity, that start to build up in the amnioserosa as dorsal closure progresses, but this activity does not inevitably lead to the cell delamination since most of the cells remain in the bulk of the tissue. We then turned to a different sensor, GC3Ai, which has been shown that can be detected in apoptotic cells throughout the whole process of programmed cell death[46]. In this sensor, the C and N termini of the GFP have been linked by a fragment containing a DEVD caspase cleavage site that must be cleaved for the GFP to fluoresce. In embryos ectopically expressing GC3Ai in the amnioserosa, sensor activity is only observed in delaminating cells (Figure 6C). The activity of the GC3Ai sensor in delaminating cells is detected as the cell starts to apically constrict until after it has ingressed

and start to undergo fragmentation, a process that takes up to 40 minutes (Figure 6C´-C´´´´). Thus, the GC3ai sensor detects apoptotic levels of caspase activity.

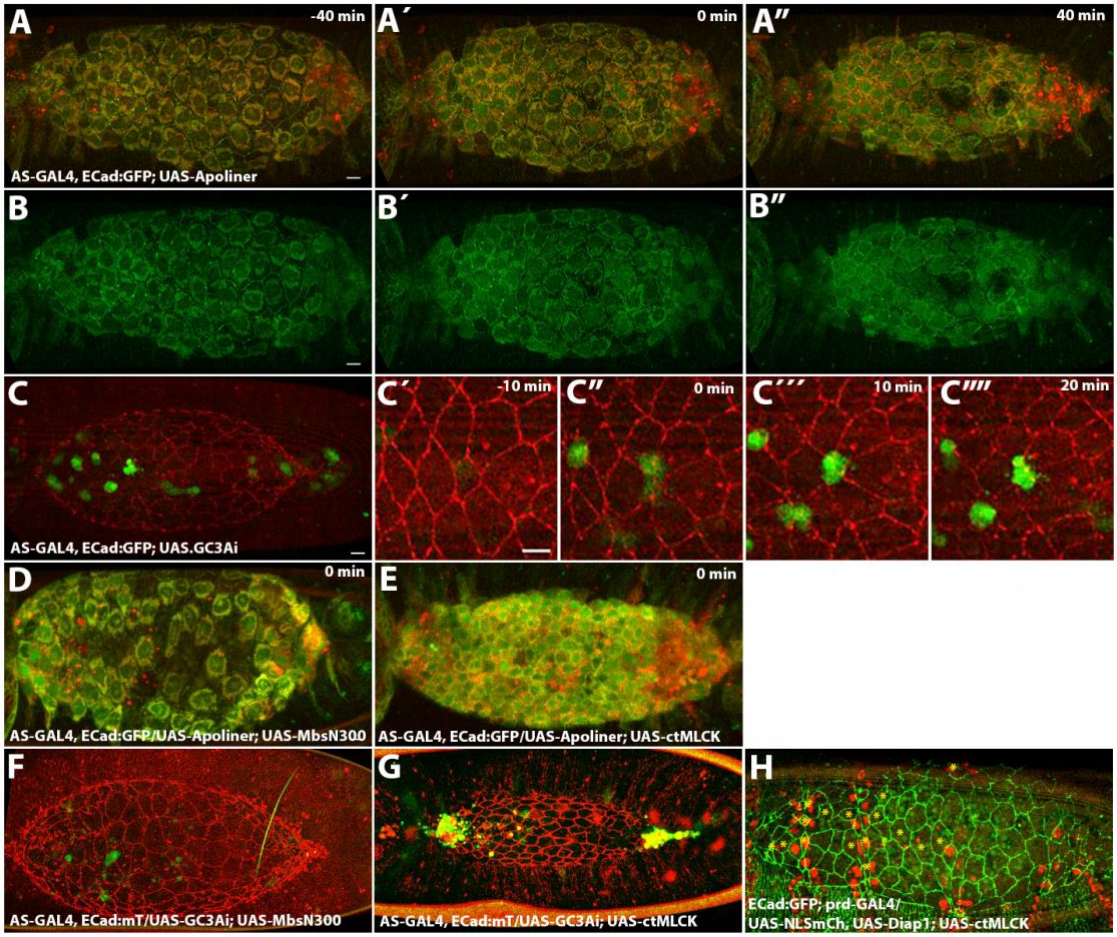

Figure 6. Caspase activity in the amnioserosa. (A-B”) Still images (MIP) from a time-lapse movie of a dorsal closure embryo ectopically expressing the caspase sensor Apoliner in the amnioserosa (Movie 14). Early during dorsal closure, only posterior cells show caspase activity (A,B). As dorsal closure progresses, most amnioserosa cells located in the anterior part of the tissue start showing caspase activity while the majority of central cells still show low levels of nuclear GFP (A´,B´). During late dorsal closure, all amnioserosa cells have exhibit caspase activity (A”,B”). (C) Still images (MIP) from a time-lapse movie of an embryo ectopically expressing the apoptotic sensor GC3Ai (Movie 15). (C´-C´´´´) Time course of GC3Ai activation during the delamination of a cell. (D) Still image of an AS-GAL4/UAS-GC3Ai; UAS-MbsN300 (Movie 16), with cells showing almost normal levels of caspase activity. (E) Still image of an AS-GAL4/UAS-Apoliner; UAS-ctMLCK (Movie 17), with cells showing higher levels of caspase activity. (F, G) Still images from a time-lapse movie of an AS-GAL4/UAS-GC3Ai; UAS-MbsN300 (F) and an AS-GAL4/UAS-GC3Ai; UAS-ctMLCK (G) embryo. Only cells that delaminate have GC3Ai signal. (H) Still image of an ECad:GFP; prd-GAL4/UAS-Diap1, UAS-NLS:mCh; UAS-ctMLCK (Movie 18). Delaminating cells are indicated with yellow asterisks.

We analyzed caspase activity with both sensors in embryos with perturbed Myosin activity. We wanted to determine whether, in AS-GAL4/UAS-MbsN300 embryos, the delay in the appearance of cell delaminations was due to an absence of caspase activity or to an

impairment of the expulsion of the cell from the tissue. If this was the case, we expected to see an activation of the apoptotic sensors in the tissue with no delamination. In AS-GAL4/UASApoliner, UAS-MbsN300 embryos that complete dorsal closure, the activation of the sensor followed a similar temporal pattern than in control embryos (Figure 6D, compare with 6A), while the activity of the GC3ai sensor was only observed in delaminating cells (Figure 6F). These observations show that apoptotic levels of caspase activity do not arise independently of cell delamination and suggest that proper levels of Myosin activity are required to trigger apoptotic cell delaminations.
Similarly, in embryos ectopically expressing ctMLCK, GC3Ai activity was only detected in cells that delaminate (Figure 6G). In contrast, in AS-GAL4/UASApoliner, UAS-ctMLCK embryos, we observed a consistent premature activation of Apoliner (Figure 6E, compare with 6A). These observations suggest that increasing MyoII activity can induce low levels of caspase activity.

These observations lead us to analyze whether the preferential delamination of cells with high MyoII levels in prd-GAL4/UAS-NLS:mCh; UASctMLCK embryos was due to an increase of caspase activity induced by MyoII. For this, we asked whether delaminations of ctMLCK cells in these embryos could be prevented by the ectopic expression of Diap1. Surprisingly, in ECadGFP; prd-GAL4/UAS-NLS:mCh, UASDiap1; UASctMLCK, NLS:mCh embryos, we observe NLS:mCh positive cells that delaminate (Figure 6H). Although we cannot rule out the possibility that Diap1 levels in this experiment are insufficient to fully prevent caspase activity, this result suggests that cell delamination can occur in response to high levels of MyoII even in the absence of caspase activity. This finding reveals that in a mechanically heterogeneous tissue, cells with high contractility can be eliminated independently of caspase activity.

DISCUSSION

In this work, we have investigated the mechanisms underlying the triggering and execution of apototic cell delaminations in the amnioserosa during dorsal closure, before the tissue completely ingresses into the embryo and degenerates. Our results show that cell delaminations involve, firstly, the stabilisation of the medioapical actomyosin cortex, and secondly, the formation of an actomyosin ring, which completes the elimination of the

apoptotic cell from the tissue. We have observed the appearance of actin puncta prior to the formation of the actomyosin ring, initially subapically and then co-localizing with ECadherin at the level of junctions. We propose that these puncta represent sites of actin nucleation for building the apoptotic actomyosin ring[47]. Although less prominent, Myosin puncta were also present, and we hypothesize they could be recruited by the nascent actin bundles. Our results also suggest that the small GTPase Rho regulates the reorganization of the actomyosin cytoskeleton to form the apoptotic actomyosin ring. Rho activity has mostly been shown to be required in neighbouring cells to the delaminating cells to form a supracellular actomyosin cable that squeezes extruding cells apically from the epithelium, but its requirement in the apoptotic cell itself is more controversial[23,33]. In the amnioserosa, all delaminating cells showed accumulation of a sensor of Rho activity in the medioapical cortex, strongly supporting the role of this small GTPase in regulating the cytoskeletal changes associated with cell delamination in the delaminating cell.

Our observations do not provide evidence for the involvement of cell neighbours in the elimination of the delaminating cells. Although it has been shown that a delaminating cell induce a deformation in its neighbours and impacts their Myosin pulses[16,30], we have not observed the formation of a supracellular actomyosin cable in neighbouring cells. An accumulation of MyoII close to cell junctions between the delaminating cell and its neighbours is only observed when ECad levels decrease significantly, suggesting that this might be a mechanism to preserve junction integrity. In fact, a mechanosensitive mechanism involving the actomyosin cytoskeleton to maintain junction integrity has been proposed[31].

Interestingly, we find that Rho subcellular localization changes upon perturbation of Myosin activity. In embryos ectopically expressing MbsN300, both MyoII and the Rho sensor accumulate at cell junctions, while in embryos ectopically expressing ctMLCK, Myo forms a dense apicomedial mesh, and the Rho sensor shows a more persistent apicomedial localization. These observations suggests that MyoII recruits Rho either to the junctional or to the medioapical region of cells, providing evidence for the existence of a positive feedback between Rho and MyoII. We have also observed oscillatory Rho activity in non-delaminating cells (unpublished observations) and as dorsal closure progresses, medioapical

Rho activity is stabilised medioapically in the whole tissue. An advection-positive feedback mechanism between Rho and its downstream effectors Rock and MyoII has been shown to underlie actomyosin pulsatility in epidermal cells during germband elongation[34]. It is possible that such a mechanism is in place in the amnioserosa, contributing to the progressive stabilization of Rho and the medioapical acomyosin cortex at the level of the whole tissue.

What triggers cell delaminations? The apoptotic removal of epithelial cells from a tissue can be regulated by both biochemical and mechanical signals[1,48]. Mechanical compression, local topological defects and the geometry of cell packing have all been shown to promote cell elimination from a tissue[49–51]. In the amnioserosa, it has been suggested that mechanical signals are involved in the triggering of cell delaminations[15,18,36]. Here, we have observed that decreasing MyoII activity in the entire tissue delays the appearance of cell delaminations. Since low MyoII activity is associated with low tissue tension[37], our results argue for a role for mechanics in triggering of cell delaminations. However, increasing tissue tension through increasing Myo levels in the whole tissue does not increase cell delaminations. A global increase in junctional Myosin has also been shown to block cell delamination in the fly notum[40], and to disrupt apoptotic extrusion in epithelial monolayers. In the latter, increasing Myosin in apoptotic cells or in the surrounding cells disrupted extrusion[43]. This contrasts with what we observe in the amnioserosa, where cells with higher MyoII than their neighbours are more likely to delaminate. Altogether, our results suggest that high tissue tension or a stiff mechanical environment may exert an inhibitory effect on the ability to delaminate.

It is important to notice that cells with increased MyoII do not delaminate instantaneously but are more likely to do so over the entire process of dorsal closure. This suggests that other signals contribute to the decision of whether a cell will delaminate. In the fly notum, it has been found that the apical size of a cell, compared to other cells within the tissue and relative to the immediate neighbours, is a predictor of apoptotic cell delamination[52]. Interestingly, we have found here that cells with lower levels of Myosin and showing a greater apical cell area are less likely to delaminate, while cells with higher Myo levels and with a smaller apical cell area, are more likely to delaminate. Our results suggest that MyoII

activity is a key determinant of apical cell size that could be mediating the relation between cell size and apoptotic fate.

The fact that cells with higher MyoII levels than their neighbours can delaminate when caspase activity is prevented by the ectopic expression of Diap1 is intriguing. As mentioned above, we cannot rule out that the levels of Diap1 are not sufficient to fully prevent caspase activity due to a dilution of the GAL4 transcriptional activator. Nonetheless, it is worth pointing out that live basal cell delamination plays a crucial role in tumour cell invasion[53]. Interestingly, RasV12-transformed cells in epithelial monolayers are typically extruded apically through a mechanism called "epithelial defence against cancer"[54]. However, some cells evade this defence mechanism and instead delaminate basally, a process suggested to contribute to malignancy. The mechanisms underlying the shift in the direction of cell elimination from epithelia remain unclear, but one possible factor is Rho hyperactivation, which increases actomyosin contractility[53]. Our results provide further evidence for a role for actomyosin contractility in driving live basal cell delamination.

The amnioserosa is a tissue in which the delamination of cells from the tissue occurs alongside the subsequent complete elimination of the tissue. This phenomenon also takes place in the larval epidermis, which is replaced by histoblasts during metamorphosis[55], and is likely to occur in other insect extra-embryonic tissues[4]. Both in the amnioserosa and in the larval epidermis, all the cells show low, non-apoptotic, levels of caspase activity well before the tissue is eliminated. There is growing evidence that caspases have non-lethal functions and can influence a diverse array of cellular processes such as signalling, proliferation, differentiation and migration[56]. In the amnioserosa, a role for caspases in controlling cell volume decrease has been found[22], and it is possible that low levels of caspases play a role in the remodelling of the cytoskeleton not directly linked to cell death.

An important question, however, is how global tissue levels of caspases arise. We have found here that increasing Myo activity favours caspase activation. In the larval epidermis, it has also been proposed that MyoII potentiates caspase activity[5]. An interesting possibility is that actomyosin contractility influences caspase activity and thus the fate of amnioserosa cells. A relationship between mechanical cues and cell fate has been known for several

years[57]. The Hippo pathway is involved in transforming mechanical stimuli into biochemical signalling pathways both in vertebrates and in Drosophila[58–60]. Notably, in wing imaginal disc cells, it has been found that medial actomyosin flows promote the medial accumulation of Kibra and the formation of a Hippo complex, which activates the Hippo pathway to repress the pro-growth transcriptional effector Yorkie[61]. The role of the Hippo pathway in the amnioserosa has not been explored yet but would be an interesting avenue for future research.

MATERIALS AND METHODS

Fly stocks and genetics:

The following stocks were used in this work:

ECad:GFP and ECad:mT knock-in alleles [62]; sqh:GFP [2] ; aniRBD:GFP [34]; AS-GAL4 (332.3 GAL4 line, RRID:BDSC_5398); prd-GAL4 (RRID:BDSC_1947); UAS-MbsN300 [63]; UAS-ctMLCK [64]; UAS-Apoliner [45]; UAS-GC3Ai [46]; UAS-NLSmCherry (RRID:BDSC_38425); UAS-Diap1 (II); UAS-Rab5DN (RRID:BDSC_42704).

Stocks built during this work:

AS-GAL4, ECad:GFP and AS-GAL4, ECad:mT; sqh:GFP

AS-GAL4, ECad:mT; aniRBD:GFP

sqh:GFP; AS-GAL4, ECad:mT

UAS-NLS:mCherry; UAS-MbsN300

UAS-NLS:mCherry; UAS-ctMLCK

UAS-Apoliner; UAS-MbsN300

UAS-Apoliner; UAS-ctMLCK

UAS-GC3Ai; UAS-MbsN300

UAS-GC3Ai; UAS-ctMLCK

ECad:GFP; prdGAL4

UAS-Diap1; UAS-MbsN300

UAS-Diap1; UAS-ctMLCK

UAS-Diap1, UAS-NLS:mCherry; UAS-ctMLCK

Live-Imaging: Stage 12-13 Drosophila embryos were dechorionated, mounted in coverslips with the dorsal side glued to the glass and covered with Voltalef oil 10S (Attachem). The AS

was imaged at 25-28ºC. using an inverted LSM 710 Meta laser scanning microscope or a Leica SP8 with a 40X or a 63X oil immersion Plan-Fluor (NA=1.3) objective. GFP and mTomato cytoskeletal reporters were simultaneously imaged with an argon laser and a 651 diode laser. For whole AS imaging, 15-30 z sections 1-1.5µm apart were collected every 2 minutes. For cytoskeletal dynamics imaging, 10 z sections 1µm apart were collected every 30 seconds.

Image analysis and statistics: Images were processed and assembled in Fiji (version 2.16.0) and Adobe Photoshop (version 23.5.0). Images were projected via the maximum intensity projection to cover the entire tissue or a single cell in case it was located in a curved region of the tissue. The identification of delaminating cells was done manually on the maximum intensity projections. Statistical analysis of the significance of differences in phenotype across genotypes was performed using the Wilcoxon-Mann-Whitney test using the R package.

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Author contributions statement: NG conceived the project, performed experiments, analysed data and wrote the paper, YF, JG and GS performed experiments, JR analysed data.

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