Epithelial repair is a two-stage process driven first by dying cells and then by their neighbours

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ABSTRACT
Epithelial cells maintain an essential barrier despite continuously undergoing mitosis and apoptosis. Biological and biophysical mechanisms have evolved to remove dying cells while maintaining that barrier. Cell extrusion is thought to be driven by a multicellular filamentous actin ring formed by neighbouring cells, the contraction of which provides the mechanical force for extrusion, with little or no contribution from the dying cell. Here, we use live confocal imaging, providing time-resolved three-dimensional observations of actomyosin dynamics, to reveal new mechanical roles for dying cells in their own extrusion from monolayers. Based on our observations, the clearance of dying cells can be subdivided into two stages. The first, previously unidentified, stage is driven by the dying cell, which exerts tension on its neighbours through the action of a cortical contractile F-actin and myosin ring at the cell apex. The second stage, consistent with previous studies, is driven by a multicellular F-actin ring in the neighbouring cells that moves from the apical to the basal plane to extrude the dying cell. Crucially, these data reinstate the dying cell as an active physical participant in cell extrusion.

KEY WORDS: Actin dynamics, Cell death, Myosin, Apoptosis, Epithelia, Extrusion, RhoGTPase

INTRODUCTION
An essential role of epithelial tissues is to form a tight barrier that prevents the passage of cells, macromolecules and solutes across the epithelium (Marchiando et al., 2010). In both developing and mature tissues, under both physiological and pathophysiological conditions, death of epithelial cells occurs continuously, and therefore a variety of mechanisms have evolved to remove dead or dying cells while preserving the integrity of the barrier. In intestinal epithelia, the division and differentiation of stem cells gives rise to a constant flux of cells from the crypts to the villus tips, where individual cells are extruded (Madara, 1990; Potten and Loeffler, 1990). In inner-ear sensory epithelia, cell divisions are relatively rare, but, after a damaging event, non-sensory supporting cells cleave the apical surfaces of dying hair cells, releasing the apical parts into the lumen (Bird et al., 2010). In cultured epithelial monolayers, dying cells can be extruded from the epithelium either apically (Rosenblatt et al., 2001) or basally (Slattum et al., 2009), and the latter has been suggested to be a possible cause of cancer progression (Marshall et al., 2011). Recently, cell extrusion has also been shown to play a fundamental role in tissue homeostasis (Eisenhoffer and Rosenblatt, 2013) – in both developing and mature tissues, overcrowding is relieved by extruding extra-numerary cells (Eisenhoffer et al., 2012; Marinari et al., 2012).

The molecular mechanisms underlying cell extrusion have been the focus of much attention. A seminal study reported that cell extrusion is driven by a multicellular filamentous actin (F-actin) ring that forms within the cells that surround the dying cell (Rosenblatt et al., 2001). Following the induction of cell death by UV exposure, the dying cell signals through sphingosine 1-phosphate (Gu et al., 2011). Immunostaining studies and live-imaging studies of wound healing after single-cell ablation within monolayers have revealed that this signal leads to the formation of a multicellular F-actin ring that originates at the monolayer apex (Gu and Rosenblatt, 2012; Rosenblatt et al., 2001; Tamada et al., 2007). Subsequently, myosin is recruited to the intercellular junction of the surrounding cells in a process that is dependent upon p115-RhoGEF, rho-kinase and GTPases of the rho family (Rosenblatt et al., 2001; Slattum et al., 2009; Tamada et al., 2007). The multicellular ring descends basally and, once it reaches the base of the monolayer, lamellipodial protrusions originating from the neighbouring cells join below the dying cell, reforming intercellular junctions that extrude the dead cell (Tamada et al., 2007). Based on these studies, the current consensus is that the dying cell provides a biochemical signal for its neighbours but plays no mechanical role in its own extrusion (Andrade and Rosenblatt, 2011; Cai and Sheetz, 2009; Gu and Rosenblatt, 2012). However, detailed time-resolved observations of actomyosin dynamics during cell extrusion are lacking, making a thorough understanding of the biological and biophysical mechanisms of extrusion impossible.

Here, using live three-dimensional (3D) imaging, we examine the F-actin and myosin dynamics that underlie cell extrusion from cultured monolayers over the entire duration of the process. In contrast to previous studies, we identify distinct roles for both the dying cell and its neighbours. We show that the removal of dying cells is a multistep process involving distinct stages of actomyosin activity. The initial stage, which involves an
RESULTS
A multistep process leads to cell extrusion in UV-treated monolayers

Previous studies have relied on the exposure of epithelial monolayers to UV to induce cell death (Gu et al., 2011; Marshall et al., 2011; Rosenblatt et al., 2001; Slattum et al., 2009). Although this is a robust experimental protocol, all of the cells are exposed to the stimulus and eventually die. This makes the long-term observation of single-cell extrusion events challenging because neighbouring cells often die within tens of minutes of one another. To overcome these limitations, we placed a custom-made mask in the epifluorescence light path of a microscope to expose individual cells within confluent Madin-Darby canine kidney II (MDCK) monolayers to UV, thus selectively inducing cell death. We combined this technique with 3D live imaging to follow the cellular processes that lead to cell extrusion.

Live imaging of extrusion within confluent monolayers of MDCK cells expressing the actin marker lifeact–GFP revealed that the removal of dying cells involved multiple phases of F-actin dynamics occurring in different focal planes (Fig. 1; supplementary material Movie 1). During the initial phase, a rosette formed at the monolayer apex, cleaving the dying cell and enclosing most of its body within the epithelium. This phase involved F-actin activity at the monolayer apex. In the apical plane, the intercellular junctions around the dying cell closed inwards over the course of 15–30 min (Fig. 1A). Simultaneously, the surrounding cells spread towards the centre of the dying cell, enclosing it and forming a multicellular rosette that was visible in the apical plane. This process led to the bulging of the apex of the dying cell at the surface of the monolayer before ‘scission’, which occurred as a result of junctional closure. The remainder of the cell body was enclosed within the monolayer. This scission was observed in all cell death events (48/48 cells, 12 experiments). At the onset of rosette formation, strong F-actin enrichment in the shape of a ring was visible at the apical contacts between the healthy cells and the dying cell (Fig. 1A). The closure of this apical ring occurred horizontally in a single z-plane with a steady speed of 0.2 μm.min⁻¹±0.1 (±s.d., 18 cells, four experiments; Fig. 1B; supplementary material Fig. S1A; Movie 2).

In the second phase, the apical F-actin ring moved basally around the dying cell over a period of 30–40 min, leading to the extrusion of the dying cell from the monolayer (Fig. 1C). As soon as the F-actin ring reached the basal plane, the aspect of the F-actin-enriched zone changed from ring-like to lamellipodial (supplementary material Fig. S1B). Over the next 60–90 min, the neighbouring cells crawled underneath the dying cell, in a process resembling the later stages in cell-ablation experiments (Tamada et al., 2007) (Fig. 5). This extrusion stage was observed in all death events that occurred in cells that were surrounded by healthy neighbours (17/17 cells, eight experiments). However, in cases in which one or several of the surrounding cells also died,
the extrusion of the central dying cell was not observed. These data suggest that the neighbouring cells were necessary for the process and acted collectively to bring about extrusion. Overall, the F-actin activity that was observed during extrusion in our experiments supports the notion of the multicellular F-actin-ring mechanism that is proposed to drive the extrusion of dying cells (Rosenblatt et al., 2001) and wound healing of epithelia at both the single-cell (Tamada et al., 2007) and tissue level (Martin and Lewis, 1992). Hence, our observations revealed that the extrusion of dying cells is a multistep process taking ~2.5 h to complete from the initial movement of the cell-junctions (Fig. 1D). As only part of this process had been described previously, we therefore investigated the actomyosin dynamics underlying extrusion.

Cell membranes do not become permeabilised until extrusion is complete

To gain insight into the relative timing of cell death and extrusion, we determined when membrane permeabilisation occurred by including propidium iodide (PI, a membrane-impermeant nucleic acid probe) in the imaging medium. We also determined when phosphatidylserine appeared on the outer leaflet of the plasma membrane by including Alexa-Fluor-633-tagged annexin-V [an early signal of death (Fadok et al., 1998)]. During apical contraction and rosette formation, no PI or annexin-V labelling were observed in dying cells (45/45 cells from four experiments and 8/8 cells from three experiments, respectively; Fig. 2A). By contrast, cells that had been fully extruded had both strong annexin and PI labelling (10/10 cells, three experiments; Fig. 2B), in agreement with Rosenblatt and colleagues (Rosenblatt et al., 2001). These data indicated that the integrity of the dying cells remained uncompromised during the initial apical-contraction stage and that membrane permeabilisation only occurred once extrusion was complete. This sequence of events contrasts with single-cell laser-wounding experiments, where permeabilisation precedes the formation of the F-actin ring (Tamada et al., 2007).

An F-actin ring is present in dying cells but not in surrounding cells during apical contraction and enclosure

To distinguish F-actin activity in dying cells from that in surrounding cells, we used monolayers that were formed by mixing MDCK cells that stably expressed either mRFP–actin or lifeAct–GFP (Fig. 3A). By live imaging in locations where dying cells expressed a fluorophore that was different from that expressed by their neighbours, it was possible to identify definitively whether the F-actin enrichment that was observed during the initial apical contraction was localised in the dying or surrounding cells. During rosette formation, scission and enclosure, a uniform F-actin enrichment was observed at the cortex of the dying cell in the apical plane, consistent with previous work (Rosenblatt et al., 2001) (Fig. 3B; supplementary material Movie 3, 11/11 cells, four experiments). This F-actin ring contracted inwards over 15–30 min, concurrent with junctional closure (Fig. 3B; supplementary material Movie 3), apparently driving self-scission and occurring considerably faster than reported previously (Rosenblatt et al., 2001). Simultaneously, less-organised F-actin activity was observed in the basal plane of the dying cell (Fig. 3B; supplementary material Movie 4), perhaps reflecting the loss of focal adhesions. Crucially, there was little evidence of F-actin enrichment in the surrounding cells in any plane during this initial phase, in contrast to previous descriptions (Rosenblatt et al., 2001) (Fig. 3C; supplementary material Movie 5, 9/9 cells, four experiments). At the apical surface, neighbouring cells moved inwards synchronously with the apical contraction of the dying cell (Fig. 3D, t=0–35 min; supplementary material Movies 6,7). The absence of an F-actin ring in the surrounding cells suggested that, at this stage, they were pulled inwards by the contractile ring in the dying cell, perhaps through the tight junctions that remained intact during cell removal (Fig. 4A). Immunostaining of monolayers that were fixed 2 h after UV exposure revealed the presence of strong cortical F-actin enrichment inside the tight junctions between the dying cell and its neighbours (Fig. 4A). Taken together, the data from fixed specimens, live-imaging data and the lack of membrane permeabilisation during this initial phase suggest that an F-actin ring within the dying cell drives rosette formation by pulling neighbouring cells inwards.

Extrusion involves F-actin activity in the neighbouring cells

During the later phase of cell extrusion, F-actin enrichment appeared in the surrounding cells at the monolayer apex, forming a multicellular ring encircling the dying cell (Fig. 3D, 45 min; supplementary material Fig. S1B). This multicellular ring moved basally, following the contour of the dying cell (Fig. 3D, 40–60 min; supplementary material Movies 6,7). During this time, cortical F-actin enrichment persisted at the apex of the dying cell (supplementary material Movies 1,6). Once the multicellular F-actin ring reached the basal plane, F-actin-rich protrusions...
extended beneath the dying cell, consistent with the lamellipodial crawling described in wound-healing experiments (Martin and Lewis, 1992; Tamada et al., 2007). This sequence occurred in all extrusions that were imaged to completion (6/6 cells, four experiments). In monolayer samples that were fixed 2.5 h after UV exposure, multicellular F-actin rings were clearly visible outside the tight junctions between dying cells and their neighbours (Fig. 4B). Overall, F-actin dynamics during the later extrusion phase were consistent with the multicellular F-actin rings described in single-cell laser-ablation experiments (Tamada et al., 2007) and in cell extrusion in UV-treated monolayers (Gu et al., 2011; Marshall et al., 2011; Rosenblatt et al., 2001; Slattum et al., 2009).

The dynamics of extrusion reflect those observed in single-cell wound-healing experiments
In our experiments, ~75 min separated the initial movement of intercellular junctions at the monolayer apex from the arrival of...
the multicellular F-actin ring in the basal plane – far longer than reported (20 min) in single-cell wound-healing experiments (Tamada et al., 2007). We hypothesized that the slower overall dynamics were due to the occurrence of an initial phase involving apical contraction and enclosure that was not observed during single-cell wound-healing experiments – a phase that we included in our study. To test this, we observed F-actin dynamics during single-cell wound healing under conditions identical to our UV experiments. Following laser ablation, the cell membrane was immediately permeabilised and a multicellular F-actin ring was formed around the ablated cell. This ring then descended towards the basal plane while contracting over the course of 30 min (16 cells, four experiments; Fig. 5). This behaviour was in all aspects identical to that described in a previous report (Tamada et al., 2007), and its duration was very similar to that of the extrusion phase in our UV-induced cell-death experiments. Based on its duration and characteristic F-actin dynamics, we propose that the multicellular extruding F-actin ring reported in single-cell wound healing (Tamada et al., 2007) is equivalent to the multicellular F-actin ring we observe during the extrusion of dying cells in UV-exposed monolayers.

**Active myosin colocalises with the F-actin ring and myosin activity is required during apical contraction and enclosure**

Having identified this new earlier event in cell extrusion, we investigated a potential role for myosin contraction in generating the mechanical forces underlying this phase. First, we imaged UV-exposed MDCK-cell monolayers stably expressing GFP-tagged myosin regulatory light chain (MRLC). Prior to any evidence of cell death, MRLC–GFP fluorescence was cytoplasmic, with a slight enrichment at the basal plane in focal adhesions (0 min, Fig. 6A; supplementary material Movie 8). Simultaneous with the onset of apical contraction, myosin relocalised within the dying cell to form a distinct ring in the same plane as the F-actin ring (5–10 min, Fig. 6A; supplementary material Movie 8). As the rosette formation progressed, myosin fluorescence increased, correlating with the F-actin enrichment noted in lifeact–GFP cells (16/16 cells, four experiments;
To determine whether the increase in myosin fluorescence intensity resulted from additional myosin recruitment or the concentration of already-bound protein over a smaller area, we tracked the apical junctions surrounding the dying cell during rosette formation and measured the total junctional MRLC fluorescence intensity. Between the onset of junctional movement and the completion of apical contraction, the intensity of MRLC–GFP increased 5-fold (±s.d., six cells, three experiments), indicating that the observed increase in myosin fluorescence represented protein recruitment. Furthermore, immunostaining of MDCK monolayers that were fixed 2 h after UV exposure revealed enrichment in phosphorylated myosin light chain (pMLC) at the cortex of dying cells (Fig. 6B).

To determine whether myosin contraction was necessary to drive rosette formation during the apical-contraction phase, we imaged live UV-exposed monolayers that stably expressed lifeact–Ruby in the presence of the myosin-II ATPase inhibitor blebbistatin. At low blebbistatin concentrations (10–25 μM), rosette formation began normally but, when the ring had contracted to 50–75% of the initial cell diameter, its contraction stopped and the dying cell lost its junctional actin ring and detached from the substrate, leaving an epithelial ‘hole’ (16/16 cells, four experiments; Fig. 6C; supplementary material Movie 9). Simultaneously with loss of the junctional actin ring, the intercellular junctions surrounding the cell relaxed to their initial positions. Live imaging with PI confirmed that membrane permeabilisation occurred along with the ‘tearing apart’ of the dying cell. In addition, the observed partial apical closure took 30 min±12 (±s.d., n=16), approximately twofold slower than controls, and a duration normally sufficient for complete rosette formation in control conditions. With 100 μM blebbistatin, we could not detect any evidence of F-actin-ring formation or junctional closure. Instead, the dying cell either disintegrated or appeared to be torn apart by its neighbours in the absence of any contraction, and this process was accompanied by membrane permeabilisation (22/22 cells, three experiments; Fig. 6D). These data showed that myosin contraction was necessary for successful rosette formation.

Rho-mediated contractility in the dying cell drives the apical-contraction phase of cell removal

Next, we sought to determine which of the dying or the surrounding cells provided the motile force for the progression of the initial apical-contraction phase. Previous work has shown that, during the process that we have identified as the later extrusion phase, active RhoA is necessary in the neighbouring cells but not in the dying cell (Rosenblatt et al., 2001). Immunostaining of monolayers that were fixed 2 h after UV exposure revealed that, during the apical-contraction phase, dying cells displayed strong RhoA–GTP staining (Fig. 7A). This suggested that increases in RhoA activity drive the observed increase in myosin contractility (Fig. 6A,B).

We therefore investigated the role of RhoA activity during the apical-contraction phase. To distinguish between activity in the dying cell and its neighbours, we used mosaics of lifeact–GFP MDCK cells and MDCK cells in which RhoA activity was inhibited by doxycycline-induced expression of the catalytic domain of p50-RhoGAP (also known as ARHGAP1) tagged with mCherry (Fig. 7B,C). First, we verified that high RhoGAP expression correlated with reduced RhoA–GTP labelling in MDCK cells, showing that RhoGAP expression was able to reduce RhoA–GTP levels (supplementary material Fig. S2). A similar effect was noted with pMLC staining in MDCK cells expressing p50-RhoGAP (supplementary material Fig. S3). Next, we examined how RhoGAP expression affected the two phases of cell clearance. In MDCK-mosaic monolayers, dying wild-type...
lifeact–GFP cells could still drive rosette formation and undergo self-scission even when the majority of their neighbours were RhoGAP positive (5/5 cells from three experiments; Fig. 7B; supplementary material Movie 10). Furthermore, the rosette morphology was unperturbed at junctions with RhoGAP-positive cells. By contrast, in 50% of cases, we found that dying RhoGAP-positive cells with a majority of lifeact–GFP neighbours could not complete rosette formation or scission, a phenotype that was also observed following treatment with low concentrations of blebbistatin (6/12 cells, five experiments; Fig. 6C; Fig. 7C; supplementary material Movie 11). These data suggest that in our experiments the induction of RhoGAP expression only led to a partial, rather than a total, reduction in myosin activity in dying cells. The remaining cells were able to drive normal rosette formation, perhaps by caspase-mediated rho-kinase activation (Coleman et al., 2001; Sebbagh et al., 2001) or perhaps owing to an insufficient reduction in RhoA activity by p50-RhoGAP expression (supplementary material Fig. S2).
together, these experiments indicated that RhoA-mediated myosin contractility in the dying cell was necessary to drive the initial apical-contraction phase of cell extrusion and that, during this phase, the contribution of neighbouring cells was minimal.

**Numerical simulations suggest that the apical-contraction phase can be driven by the dying cell alone**

Our experimental observations indicated that, during the apical-contraction phase, the dying cell provided the mechanical force for rosette formation through an approximately fivefold increase in myosin localisation to its apical cortex that, we assumed, resulted in a proportional increase in contractility. To verify this hypothesis and to explore alternative biophysical mechanisms leading to rosette formation, we developed a simple numerical simulation of the monolayer apex using a vertex model (Farhadifar et al., 2007; Marinari et al., 2012). Based on uniform cortical-localisation of myosin throughout apical contraction, we assumed that all cortical tension could be represented by a single contractility term ($\gamma$) acting over the cell perimeter. In our model, cells possessed an area elastic modulus ($K$) arising from limited cell-volume compressibility, a contractility ($\gamma$) arising from cortical myosin activity and an intercellular adhesion ($L$) acting at cell–cell junctions. The model was carefully calibrated to represent the mechanical equilibrium in monolayer epithelia growing on glass (see Materials and Methods). We performed numerical experiments in which, at time $t=0$, we changed the physical properties of either the dying cell ($L_D$, $\gamma_D$, $K_D$), its immediate neighbours ($L_N$, $\gamma_N$, $K_N$) or both the dying cell and its neighbours. From these, we determined the combinations of physical changes that could, in principle, drive rosette formation (defined here as a $\geq 80\%$ reduction in the apical area of the dying cell).

Changes in the adhesion of the dying cell to its neighbours ($L_D$) alone could not drive rosette formation (Fig. 8A,B). Changes in the elasticity of the dying cell ($K_D$) alone could only drive rosette formation if $K_D$ was reduced to zero at the onset.
of rosette formation (Fig. 8A,B, $K_D=0$ and 0.5$K$). In our experiments, the preservation of membrane integrity (Fig. 2A) and the presence of a well-defined F-actin cytoskeleton in the dying cells (Fig. 3B) suggest that their elasticity is not reduced to zero. Furthermore, previous studies that have measured cell elasticity during cell death report either increases in elasticity (Kim et al., 2012) or decreases (at most 75%) (Kim et al., 2012; Wang and Pelling, 2010), suggesting that the formation of rosettes through a reduction of $K_D$ to zero does not occur during apoptosis. By contrast, a fivefold increase in the contractility ($\gamma_D$) of the dying cell, comparable to the results that we obtained by using live-imaging of myosin, was sufficient to generate a rosette (Fig. 8B, $\gamma_D=5\gamma$). Interestingly, a partial decrease in the elasticity of the dying cell ($K_D$) coupled with a complete loss of adhesion to its neighbours ($\Lambda_D$) could also drive rosette formation (Fig. 8B, $\Lambda_D=0$, $K_D=0.3$). Although a decrease in elastic modulus ($K_D$) during cell death has been reported in some conditions (Wang and Pelling, 2010), the complete loss of adhesion between the dying cell and its neighbours ($\Lambda_D$) is not supported by our experimental observations (Figs 3,4) or by previous work showing that the barrier function of monolayers is maintained during extrusion (Rosenblatt et al., 2001). Changes in the neighbouring cells alone or in combination with changes in the dying cell could, in theory, also give rise to rosette formation. Indeed, rosettes could be obtained through moderate increases in the adhesion of neighbouring cells to one another ($\Lambda_N$), either with or without decreases in the adhesion between the surrounding cells and the dying cell ($\Lambda_D$) (Fig. 8B, $\Lambda_N=4\Lambda$ and $\Lambda_N=3\Lambda$ with $\Lambda_D=0$). Although an increase in the elasticity ($K_N$) of neighbouring cells has been suggested to drive the extrusion of transformed cells from epithelia, even a 100-fold increase in $K_N$ – far greater than that measured experimentally (Hogan et al., 2009) – was insufficient to drive rosette formation in our model, perhaps signifying that the extrusion of transformed cells occurs through different mechanisms compared with those of dying cells (Fig. 8B, $K_N=100K$).

**DISCUSSION**

In this study, we captured, with high spatio-temporal accuracy, the cellular and cytoskeletal changes leading to the extrusion of dying cells, and, crucially, we revealed new mechanical roles for dying cells in their own extrusion. Indeed, the clearance of dying cells could be subdivided into two stages based on the location of actomyosin activity (Fig. 8C). The first, previously unidentified, stage was driven by the dying cell, which exerted tension on its neighbours through a cortical contractile F-actin ring, resulting in the formation of a cellular rosette at the epithelial surface. The second stage was driven by a multicellular F-actin ring in the...
neighbouring cells, a ring that moved apico-basally to extrude the dying cell, consistent with previous studies (Rosenblatt et al., 2001; Tamada et al., 2007).

The phase of apical contraction and enclosure involved the formation of apical rosettes through the assembly of a contractile cortical actomyosin ring downstream of RhoA in the dying cell. Live imaging of mosaics of cells that expressed F-actin markers tagged with different fluorophores, together with immunostaining data, provided definitive localisation of the contractile F-actin ring within the dying cell during this initial phase. The apical contraction of the dying cell was accompanied by a fivefold enrichment in total myosin and a clear increase in pMLC. This coincided with the scission of the dying cell, with the cell apex being shed and the remaining cell body being enclosed within the monolayer. The closure of the apical ring required myosin contractility downstream of RhoA in the dying cell because myosin inhibition and the expression of the catalytic domain of p50-RhoGAP in the dying cell both resulted in a failure of rosette formation. These results contrast with the extrusion observed in crowding-induced delamination in the Drosophila melanogaster notum, where rosette formation around the delaminating cell is driven by increased myosin contractility in the surrounding cells (Marinari et al., 2012). The present data suggest an ‘altruistic’ role for the dying cell during cell extrusion (Andrade and Rosenblatt, 2011; Mills et al., 1999). Cortical F-actin enrichment in the dying cell has been observed previously in MDCK monolayers, but the authors concluded that it did not participate in extrusion based on the lack of effect of C3-toxin (an inhibitor of RhoA, RhoC and cdc42) injected into the dying cell (Rosenblatt et al., 2001). However, in that study, dying cells were selected for injection morphologically by using phase-contrast microscopy and thus, ipso facto, were likely to have already completed the initial apical-contraction phase.

Why is it necessary to have an initial phase of apoptotic-cell extrusion that is driven by the increased contractility of the dying cell if the later extrusion phase can proceed in its absence, as is observed in single-cell wound-healing experiments (Tamada et al., 2007)? One simple explanation is that the initial phase is an altruistic act by the dying cell to trigger its own effective extrusion, while ensuring the preservation of the barrier function of the monolayer. The initial phase might harness the increase in contractility that is concomitant with cell death to bring intercellular junctions into position to seal the barrier and cleave part of the cell to facilitate extrusion, before triggering the assembly of a contractile multicellular F-actin ring in its neighbours (Eisenhoffer et al., 2012; Gu et al., 2011). Another intriguing possibility is that contractility and rosette formation during the initial phase provides an essential mechanical signal to the surrounding cells by transiently increasing junctional tension and local cellular density. This could perhaps activate molecular mechanisms similar to those involved in cell extrusion in response to overcrowding in epithelia (Eisenhoffer et al., 2012). Future work will be necessary to determine precisely how each phase contributes to overall extrusion, what signals coordinate the transition from the initial apical-contraction phase to the extrusion phase and whether basal extrusion is the result of a defective extrusion by the surrounding cells.

Our computational simulations of the apical-contraction phase indicated that increases in myosin contractility (γD) in the dying cell alone were sufficient to drive rosette formation. Interestingly, an increase in the adhesion (AN) of surrounding cells to one another could also drive rosette formation, something that should manifest itself as an increase in cadherin recruitment to intercellular junctions between the surrounding cells. Further work will be necessary to determine whether and to what extent an increase in intercellular adhesion between surrounding cells also contributes to rosette formation. Finally, the physical processes underlying the extrusion phase still remain unclear, and 3D computational models explicitly modelling the apical area, basal adhesion and lateral junctions are required to gain the necessary physical insight to understand this process.

**MATERIALS AND METHODS**

**Cell culture**

Madin-Darby Canine Kidney II (MDCK) cells were maintained at 37°C under 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal calf serum and 1% penicillin and streptomycin. MDCK expression mosaics were created by mixing RFP- and GFP-expressing cell lines in a 1:10 ratio before plating.

**Generation of cell lines**

MDCK cell lines stably expressing fluorescent markers for F-actin and myosin (actin-mRFP and MRLC–GFP) were generated as described previously (Charras et al., 2006). Lifeact–GFP and lifeact–ruby (Riedl et al., 2008) were gifts from Roland Wedlich-Soldner (Max Planck Institute, Martinsried). The generation of retroviruses and cell subcloning are described previously (Harris et al., 2012). To establish MDCK cells that stably expressed the mCherry–p50RhoGAP catalytic domain in a tetracycline-inducible manner, MDCK-pTR cells (Hogan et al., 2009) were transfected with pcDNA/TO/mCherry-p50RhoGAP catalytic domain (Hogan et al., 2009), followed by selection in medium containing 5 μg.ml$^{-1}$ blasticidin (Invitrogen) and 400 μg.ml$^{-1}$ zeocin (Invitrogen). The cells were then subcloned to obtain a monoclonal cell line with an epithelial phenotype.

**Inducing cell death of individual cells by UV exposure**

Cells were exposed to UV light for 3 min in a Stratalinker1800 to induce cell death (Rosenblatt et al., 2001). For the majority of live-imaging experiments, an aluminium mask with eight holes (1.5 mm apart) was placed in the epifluorescence light path of a microscope, resulting in illumination spots of ~10 μm diameter that were 75 μm apart on the sample. UV exposure (20 min) resulted in the death of individual cells within 2 h, whereas the surrounding cells remained healthy.

**Single-cell ablation and junction cutting**

Cell ablations and intercellular junction cutting were carried out by using a Chameleon-XR Ti-sapphire laser coupled to a Zeiss LSM510Meta upright confocal microscope. Cells were ablated by scanning 890-nm laser light over a 9×9 pixel region of interest (ROI) (0.28 μm$^2$ pixels) at 100% laser power (63 mW nominal) with a dwell time of 3.2 μs.pixel$^{-1}$ for 20 iterations, using a ×40 NA 0.8 objective. To cut junctions, 4×4

| Table 1. A comparison of the results from the vertex model of tissue mechanics and experimental data |
|---------------------------------------------------------------|--------|--------|
| Length change of cut junction (%)                                  | 17±13  | 17±13  |
| Area change of two cells joined by the cut junction (%)           | 42±12  | 20±10  |
| Area change of ablated cell (%)                                    | 10±7   | 15±10  |
pixel ROIs, 20% laser power, 720 nm (12 mW) and a dwell time of 164 μs/pixel were used.

Confocal imaging
Fixed samples were imaged by using an inverted Zeiss LSM510Meta microscope, ×63 NA 1.4 oil objective or a ×20 NA 0.7 objective. For live imaging, the cells were placed in a heated chamber (37°C under 5% CO2) on a spinning-disc confocal microscope (UltraviewERS, Perkin Elmer, ×40 NA 1.3 oil objective). Z-stacks (26×0.7 μm) were recorded at 30-s intervals over 4–6 h. In some experiments, 1 μg/ml of PI was added to the imaging medium to identify permeabilised cells. For laser ablations, Z-stacks were acquired in seven planes at 1-μm separation at 30-s intervals.

Immunocytochemistry
For ZO-1 staining, monolayers were simultaneously fixed and permeabilised using a solution of 1.75% paraformaldehyde, 1% sucrose and 0.5% Triton X-100 in PBS:DMEM (1:1) at 37°C for 20 min. For pMLC and RhoA staining, the monolayers were fixed with a solution of 4% paraformaldehyde in DMEM at room temperature for 15 min and were exposed either to acetone at −20°C for 5 min (pMLC) or 0.5% Triton X-100 in PBS at 4°C for 5 min (RhoA). The samples were incubated for 10 min at 4°C in blocking solution (10 mg/ml BSA in PBS) prior to incubation with primary antibodies. The following primary and secondary antibodies were used: anti-ZO-1 (rabbit polyclonal, 2.5 μg/ml, Invitrogen), anti-pMLC (rabbit polyclonal, 1:100 dilution, Cell Signaling), anti-RhoA–GTP (mouse monoclonal, 5 μg/ml, NewEast Biosciences), goat anti-mouse-IgG Alexa-Fluor-647 and Alexa-Fluor-488 (10 μg/ml, Invitrogen) and donkey anti-rabbit-IgG Alexa-Fluor-568 (10 μg/ml, Invitrogen). Phalloidin–Alexa-Fluor-488 (Invitrogen) or Atto-647 (Sigma) were used at 33–50 nM and DAPI was used at 1 μM.

Inhibitors
The myosin-II inhibitor blebbistatin was added to the culture medium immediately after UV exposure and was left for the duration of the experiment. For live imaging, ruby–lifeact MDCK cells were used at 568-nm excitation to circumvent the photo-inactivation and phototoxicity issues reported with blebbistatin at 488 nm (Sakamoto et al., 2005).

Image processing and analysis
XYZT image stacks were cropped into smaller regions (50 μm2) around individual cell-death events using custom-written Matlab routines. Each cell-death event was then examined in ImageJ using the View5D plug-in (Rainer Heintzmann). Quantitative analysis was performed using Matlab. To generate kymographs, the final image of actin-ring closure was superimposed onto an image taken prior to ring formation (for Fig. 1B, the image at 18 min from supplementary material Fig. S1A was superimposed onto the image at 0 min). A line was drawn through the centre of the ring and the intensity values along this line were plotted as a function of time (red line, left panel, Fig. 1B). The time over which the ring contraction took place was measured (in Fig. 1B, between 120 and 140 min) and the slopes of the movement of the junctions were used to calculate the speed of closure and time taken for closure.

Quantification of changes in myosin activity
The quantification of changes in myosin activity was performed using Matlab routines. Briefly, the perimeter of the dying cell was manually drawn in the apical-junctional plane using the MRLC–GFP time-lapse images (or brightfield images) for timepoints that were separated by 2-min intervals starting from 10 min prior to any observable movement of the cell junctions up until rosette formation. We assumed that rosette formation was complete when the area of the dying cell in the confocal plane had decreased by 90%.

To measure the temporal evolution of the myosin intensity along the cell contour, we positioned ROIs (area 4 μm2) around the cell perimeter. The mean ROI intensity at each timepoint was calculated for the z-plane of maximum myosin intensity. The background (mean intensity for an image plane below the coverslip) was subtracted for each data set. The time-series were smoothed with a running average of window-size 3.

Vertex model of tissue mechanics for MDCK monolayers growing on a substrate
To model MDCK monolayers growing on a substrate, we adapted an existing computational model of Drosophila epithelium (Marinari et al., 2012) that is based upon previous vertex models (Farhadifar et al., 2007; Käfer et al., 2007). It uses the following work function W:

\[ W = \sum_{ij} \frac{K}{2} (A_i - A_0)^2 + \sum_{i<j} 2A_i + \sum_i \frac{\gamma}{2} H_i^2 \]

The first term in the work function reflects an area constraint, where \( K_a \) is the effective bulk modulus, \( A_i \) is the current area and \( A_0 \) is the preferred area of a cell, \( x \). The second and third terms have a certain degree of redundancy. The second term represents a line tension acting along the length of a junction \( l \) connecting vertices \( i \) and \( j \), reflecting the balance of intercellular adhesion and cortical tension. The third term represents the actomyosin contractility \( \gamma \) acting around the cell perimeter \( L_\alpha \) owing to forces acting along the intracellular actin belt present at adhesions junctions. We showed uniform localisation of myosin at the cell cortex throughout apical contraction and reasoned that a tension applied to the entire perimeter of the cell was sufficient to reflect the mechanical contribution of cortical myosins, and, thus, cortical tension could be represented by \( \gamma \) alone and that \( \alpha \) was only due to intercellular adhesion.

The energy of the system is minimised by random sampling using a Monte Carlo method. The probability of a vertex move being accepted or rejected depends on the associated energy change in the work function: if the move decreases the energy of the system, we accept the move \( (\Delta W < 0, P=1) \); if the move does not change the energy of the system, it is accepted with a probability of 0.5 \( (\Delta W = 0, P=0.5) \); if the move increases the energy of the system, it is rejected \( (\Delta W > 0, P=0) \).

We undertook a thorough calibration to choose values of the parameters \( K, \alpha \) and \( \gamma \) that were representative of our experimental conditions (rather than the original Drosophila notum). This involved running simulations to find the parameter set that was best able to replicate the experimental data used for calibration.

To probe the mechanical properties of the system, we used laser ablation to cut individual intercellular junctions. By comparing the movement of the vertices of the cut junction to simulations, it was possible to calibrate the relative magnitudes of model parameters \( \alpha \) and \( \gamma \) (Farhadifar et al., 2007). Following ablation, the vertices of the cut junction moved apart from one another. Simultaneously, the perimeters of the two cells expanded a small amount. Equilibrium was reached within 40–140 s. The length change of the cut junction and the area and perimeter changes for the two cells linked by the cut junction were measured (nine cuts, three experiments): junction length increased by 1.0 μm±1.0 and cellular perimeter increased by 2.5 μm±2.5 (mean perimeter: 9 μm±4), suggesting that junction cutting affects both \( \alpha \) and \( \gamma \). If only \( \alpha \) for the cut junction was affected, the absolute increase in cell perimeter should be the same as the change in junction length. However, the perimeter of both cells increased by a length 2.5-fold greater than the increase in junction length, suggesting that \( \gamma \) was also affected. The outward movement of the vertices showed that junctions were under positive line tension. To numerically simulate junction cutting, a random junction was chosen from an equilibrium configuration of the tissue. For that junction, adhesion (\( \alpha \)) and contractility (\( \gamma \)) were set to zero for the two cells linked by the cut junction, as described previously (Farhadifar et al., 2007). The other parameters remained unchanged. The change in the ‘cut’ junction length was then calculated.

In addition, we performed experiments in which we ablated a single cell. Following ablation, the area change was measured in the targeted
cells and compared with simulations to estimate the relative magnitudes of $k$ and $\gamma$. Laser ablation caused outward recoil of the surrounding cell junctions that relaxed over a period of $-140$ s, and the area of the ablated cell increased by $15\% \pm 10$ (12 cells, three experiments). Laser ablation damaged the target cells, and we assumed that this decreased the cellular elastic modulus owing to the permeabilisation and perturbation of the cell (Fig. 5). The outward junctional movement suggested that the targeted cell lost its pull on neighbouring cells, and therefore that both $\gamma$ and $\Lambda$ were affected by ablation. Because some cellular remnants were still present following ablation, we assumed that cellular elasticity ($K$) and adhesion energy ($\Lambda$) were not reduced to zero. In our simulation of single-cell ablation, a random cell was chosen from an equilibrium configuration of the tissue. For that cell, the effective bulk modulus ($K$) and the adhesion ($\Lambda$) were set to half of their original values and the contractility ($\gamma$) was set to zero. The change in the area of the ablated cell was then calculated.

To calibrate the model, simulations were run to find the parameter set that was best able to replicate the experimental data that were gathered from analysis of cell packing, single-junction cuts and single-cell ablations. The aim was to find a configuration representative of the principal forces acting across the monolayer that could then be used as the equilibrium starting point from which to simulate rosette formation during cell death. Our goal was to generate an equilibrium configuration with mechanical properties that could replicate junction cutting and cell ablation experiments and that could attained configurations that approximated the observed MDCK-cell packing.

Simulations were run in which we varied $\Lambda$, $\gamma$, $K$ and the amount of compression applied to the monolayer (Farhadifar et al., 2007). For each parameter set, the starting configuration was a network of 30 hexagonal cells of perimeter length $L$ = 6. This was allowed to ‘grow’ for a specified number of cell divisions or time-steps. The resulting configuration was then used to simulate junction cutting and single-cell ablation. The simulations were compared with experimental data using four criteria: (1) $\Delta A_{\text{cut}}$, the percentage length change in the cut junction, (2) $\Delta A_{\text{area}}$, the percentage area change in the cells linked by the cut junction, (3) $\Delta A_{\text{stable}}$, the percentage area change in the ablated cell, (4) $\Delta P$, the sum of squared deviations for the probability of n-sided cells (Gibson et al., 2006): $\Delta P = \sum_{n=1}^{N} (\pi^{\text{new}} - \pi^{\text{old}})^2$. The parameter set that was best able to replicate our experimental calibration data was $\delta = 0$ and $\zeta = -1.4$, after one round of cell division with the preferred area $A_0$ of daughter cells equal to that of mother cells. Numerical results are compared to experimental data in Table 1. Given that the model is a simplified description of an adherent MDCK monolayer, we would not expect it to perfectly mimic experiments. In particular, the simplifying assumptions made in generating the numerical model and in the boundary conditions that were chosen for junction cutting and ablation experiments might contribute to differences between the results of numerical simulations and experiments. However, with the optimal parameter set, our model was able to replicate all of the qualitative results of the calibration experiments as well as most of the quantitative results.

**In-silico extrusion experiments**

To investigate the respective role of the dying cell and its neighbours in the process of rosette formation, our code allowed us to separately specify the physical parameters for the dying cell ($K_D$, $\Lambda_D$, $\gamma_D$) and those of its immediate neighbours ($K_N$, $\Lambda_N$ and $\gamma_N$). Using initial conditions in which monolayers had reached mechanical equilibrium, we performed numerical experiments in which, at time $t=0$, we changed the physical properties of either the dying cell (to test mechanisms driven by a single dying cell, parameterised by $\Lambda_D$, $\gamma_D$, $K_D$), or its immediate neighbours (to test mechanisms in which neighbours participated, parameterised by $\Lambda_N$, $\gamma_N$, $K_N$) or both the dying cell and its immediate neighbours. The code was then run until it reached mechanical equilibrium and the area change of the dying cell was determined. Based on experimental observations, we defined rosette formation as resulting in a greater than 80% reduction in the apical area of the dying cell.

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**Competing interests**

The authors declare no competing interests.

**Author contributions**

J.E.G., G.C. and D.K. conceived and designed the experiments. D.K. performed the experiments and analysed the data, with some contributions from J.E.G. and G.C. J.E.G., G.C. and D.K. interpreted the data. L.P. contributed image analysis routines. T.D., A.M. and D.K. designed the simulation. M.K., Y.F. and G.C. generated reagents. D.K., G.C. and J.E.G. wrote the manuscript.

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**Supplementary material**

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.138289/-/DC1

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