ORIGINAL PAPER

Apoptotic regulation of epithelial cellular extrusion

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Abstract Cellular extrusion is a mechanism that removes dying cells from epithelial tissues to prevent compromising their barrier function. Extrusion occurs in all observed epithelia in vivo and can be modeled in vitro by inducing apoptosis in cultured epithelial monolayers. We established that actin and myosin form a ring that contracts in the surrounding cells that drives cellular extrusion. It is not clear, however, if all apoptotic pathways lead to extrusion and how apoptosis and extrusion are molecularly linked. Here, we find that both intrinsic and extrinsic apoptotic pathways activate cellular extrusion. The contraction force that drives cellular extrusion requires caspase activity. Further, necrosis does not trigger the cellular extrusion response, but instead necrotic cells are removed from epithelia by a passive, stochastic movement of epithelial cells.

Introduction

During development and throughout adulthood, cells forming epithelial tissues undergo extensive turnover via

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cell division and death [1]. Aside from homeostatic death, epithelia are frequently exposed to toxins, inflammatory cytokines, pathogens or other forms of stress that lead to increased apoptosis or necrosis [2, 3] that could damage the epithelium. This damage could compromise the protective barrier that epithelia provide the organs and tissues they encase. Poor epithelial barriers could lead to malformations in developing embryos or edema, tissue damage, chronic inflammation and infections in adults. However, even when large numbers of epithelia cells become apoptotic, the barrier is still maintained using a process termed 'cellular extrusion'. During extrusion, an apoptotic cell signals its neighbors to form an actin and myosin contractile ring that simultaneously squeezes the dying cell out of the monolayer replacing the space the apoptotic cell leaves, thereby preventing any gaps from forming within the epithelium [4].

Several studies have linked cellular extrusion and apoptosis in vivo and in vitro [4–9]. Two models proposed that apoptosis could drive cellular extrusion, yet neither model has been examined experimentally. In one model, Mills et al. [10] suggested that extrusion results from the contractile forces that drive apoptotic cell blebbing. In the other, Peralta-Soler et al. [6] suggested the apoptotic cell would signal reorganization of actin filaments and cell junctions at the interface between the dying cell and its neighbors to orchestrate cell extrusion. Later, Rosenblatt et al. [4], through a cell addition assay, demonstrated that early apoptotic cells induce the formation of actin cables on cells in a monolayer, suggesting that the apoptotic cell signals the neighboring cells to induce the actomyosin ring to form and contract. This was an important step for understanding how apoptotic cells might activate extrusion, yet several questions remain. How does apoptosis trigger extrusion and which apoptotic signals are important for activating extrusion? Is extrusion activated in response to

both intrinsic and extrinsic apoptotic stimuli? Is apoptosis required to initiate extrusion or can extrusion occur independently of apoptosis? Can other forms of cell death such necrosis activate cell extrusion?

Here, we find that extrusion can be triggered by either intrinsic or extrinsic apoptotic stimuli. By investigating different steps in these apoptotic pathways, we found that completion of extrusion requires caspase activation. Although necrotic cells resulting from caspase inhibition do not extrude, they are removed from epithelia by stochastic movement of epithelial cells.

Materials and methods

Cell culture

MDCK II cells (gift from K. Matlin, University of Chicago, Chicago, IL) were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Invitrogen 11965-092) with 5% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Invitrogen) at 5% CO_2, 37°C. 16-HBE-14o (gift from D. Gruenert, California Pacific Medical Center, San Francisco, CA) were cultured in Minimum Essential Media (MEM) low glucose (Invitrogen 11095-080) with 5% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin in a flask coated with a solution of human fibronectin type I (BD Biosciences), bovine collagen I (Purecol; Inamed biomaterials), and BSA (Invitrogen) at 5% CO_2, 37°C.

Induction of cell death

To induce apoptosis, MDCK monolayers or HBE bilayers were treated with 120 mJ/cm^2 short-wave (UV-C) light, using a Spectrolinker (Spectroline) and incubated for 2 h after irradiation or by treating with 500 μM etoposide (Sigma-Aldrich) for 5 h or 100 ng/ml superKiller TRAIL (Enzo Life Sciences) along with 100 ng/ml Cyclohexamide (Calbiochem) for 5 h. In some experiments, MDCK cells were pre-treated with 50 μM z-VAD-fmk (Promega) or 0.1% DMSO (Sigma) before inducing apoptosis.

Cell staining

Cells were fixed with 4% formaldehyde in PBS for 20 min, permeabilized for 5 min with 0.5% Triton in PBS, rinsed three times with 0.1% Triton in PBS, and blocked with AbDil (PBS with 0.1% Triton X-100 and 2% BSA) for 20 min before incubating with primary antibodies. Cells were then incubated with the following primary antibodies (diluted in AbDil) for 1 h: 1:100 mouse monoclonal anti-Bax clone 6A7 (Sigma), 1:200 rabbit monoclonal anti

active caspase-3 (BD pharmigen), 1:100 rabbit polyclonal anti cytochrome c (Santa Cruz), mouse monoclonal anti cytochrome c (Abcam), 1:50 monoclonal mouse anti HMGB1 (Sigma) and 1:100 polyclonal rabbit anti AIF (Cell Signaling). After washing coverslips three times in 0.1% Triton X-100, coverslips were incubated in secondary antibodies (all diluted 1:100 in AbDil): Alexa Fluor® 488 goat anti-mouse, Alexa Fluor® 568 goat anti-mouse, Alexa Fluor® 488 goat anti-rabbit, Alexa Fluor® 568 goat anti-rabbit and Alexa Fluor® 647 goat anti-rabbit (all from Molecular probes, Invitrogen). Along with secondary antibodies, we incubated the cells with 1 μg/ml Hoescht 33342 (Sigma-Aldrich) and 0.25 μg/ml Alexa Fluor® 568 phalloidin or 0.25 μg/ml Alexa Fluor® 647 phalloidin (Molecular Probes, Invitrogen). After incubation with secondary antibody for 45 min, the coverslips were rinsed once with 0.1% Triton in PBS and then mounted on a micro slide (Gold Seal Products) using ProLong Gold antifade reagent (Invitrogen).

Microscopy

Fluorescence micrographs cells were obtained using a Leica DM 6000B microscope captured with a Micromax charge-coupled device camera (Roper Scientific). IP Lab Software was used to control the camera and to process images. All images were processed further using Photoshop (Adobe) and Illustrator (Adobe) software. Movies were made with an Olympus IX81 spinning disk microscope with a Weather Station incubation chamber, prior motorized stage, ZDC-laser focus, and motorized objectives for Z-sectioning. Slidebook TM 5.0 software (3i intelligent Imaging Innovations) was used to control the camera and to process images. Images were later processed using ImageReady software (Adobe).

Immunoblot analysis

Antibodies used for immunoblot analysis included polyclonal rabbit anti-Bax (Santa Cruz), monoclonal rabbit anti-Bak (Abcam), monoclonal mouse anti-Bcl-2 (Abcam), polyclonal rabbit anti-GFP (Invitrogen) and Monoclonal mouse anti-alpha tubulin (Sigma) diluted to 1:10,000 in PBST (0.05% Tween 20 in PBS) with 5% milk.

DNA constructs

Human Bcl-2 alpha isoform ORF (Open Biosystems) was PCR cloned and recombined into the donor vector pDONR221 (Invitrogen) following company's instructions. The entry vector, pDONR-Bcl2-221, was then recombined with the retroviral vector pMIG [11] modified to function as a destination vector (Gift from Dr. Alana Welm [12]).

The expression vector pMIG-Bcl2 and the empty pMIG vector were used to produce retroviral particles. We synthesized Bax and Bak shRNA oligos: GCUCUGAGCAGA UCAUGAA and CCCAUUCACUACAGGUGAA, respectively [13] and cloned them into the lentiviral vector pLL5.0 (Gift from Dr. James Bear [14]). The generated vectors pLL5.0 Bax shRNA and pLL5.0 Bak shRNA along with the control vector pLL5.0 N.S. shRNA (Also a gift from Dr. James Bear) were used to make lentiviral particles. The gene fusion rat HMGB1-EGFP construct (Gift from Dr. Marco Bianchi [15]) was PCR cloned into the retroviral vector pMSCVhyg (Clontech).

Statistical analysis

The statistical analysis of data collected from three independent experiments was performed using the parametric unpaired two-tailed *t* test. In each graph, *P* values are shown and error bars are Standard Error of the Mean (SEM), unless indicated differently.

Results

Both intrinsic and extrinsic apoptotic stimuli elicit cellular extrusion in epithelial monolayers

To determine if different apoptotic pathways could trigger cell extrusion and to establish where in the apoptotic pathway extrusion is activated, we investigated whether a variety of intrinsic and extrinsic apoptotic stimuli activate extrusion in Madin-Darby Canine Kidney (MDCK) cell monolayers. Although previous studies have found that both extrinsic and intrinsic pathways can activate extrusion, these studies were done in different cell types and with only one type of stimulus [4, 6, 7]. We treated MDCK monolayers with either etoposide, a topoisomerase II inhibitor that causes double-stranded DNA breaks and activates the intrinsic death pathway, or TRAIL (Tumor Necrosis Factor (TNF)-related apoptosis-inducing ligand) to activate the extrinsic pathway. We then assayed for the number of cells undergoing apoptotic cellular extrusion by counting the number of cells with a contractile actin ring that stained positively for the apoptosis marker, active caspase-3 (Fig. 1a–c). Both treatments increased the percentage of cells undergoing apoptotic cellular extrusion compared to controls (Fig. 1b, c). To determine the treatment at which apoptotic cellular extrusion is maximized, we used increasing doses of apoptotic stimuli. Doses higher than 1 mM etoposide or 500 μg/ml TRAIL destroyed the monolayer integrity, which impaired the ability of cells to extrude (Figs. S1, S2 in Supplementary Material). Therefore, compared to apoptosis studies in single cells,

apoptotic cell extrusion can only be assessed when between 2 and 8% of the monolayer undergoes apoptosis. Because of this, all of our extrusion assays are conducted within this range of percentage of apoptotic cell extrusion.

Additionally, treatment with camptothecin, a topoisomerase I inhibitor, or Fas ligand (Fas-L), which activate the intrinsic or extrinsic pathways of cell death respectively, produced similar results to those of etoposide and TRAIL (data not shown). The same treatments in another epithelial cell line, human bronchial (HBE), gave similar results (Figs. 2e, 3d) and all cells undergoing apoptosis also undergo extrusion. Together, these results suggest that multiple types of epithelia can trigger extrusion in response to a variety of intrinsic or extrinsic apoptotic stimuli. These results also suggest that the extrusion pathway should initiate downstream of where the extrinsic and intrinsic apoptosis pathways converge.

Both intrinsic and extrinsic apoptotic pathways target the mitochondrial pathway during apoptotic cellular extrusion

The extrinsic apoptotic pathway can directly activate executor caspases such as caspase 3 through the initiator caspases 8 and 10, skipping the mitochondrial pathway [16–18]. However, the extrinsic pathway does have the ability to induce mitochondrial outer membrane permeabilization (MOMP) for release of mitochondrial apoptotic factors such as cytochrome c and Smac/Diablo that will facilitate the activation of caspases and enhance the execution of apoptosis. This process occurs through the proteolytic activation of the BH3-only protein Bid by caspase 8, which in turn activates the multidomain pro-apoptotic protein Bax. Activated Bax translocates to the mitochondria where it oligomerizes to form pores and permeabilize the mitochondrial outer membrane [16, 19]. Therefore, since both intrinsic and extrinsic apoptotic pathways have the ability to target the mitochondria during apoptosis, it is possible that the mitochondria, through the release of a sequestered factor, could play a role in the activation of the extrusion machinery.

To test this possibility, we investigated if extrinsic and intrinsic stimuli target the mitochondria during apoptotic cell extrusion by immunostaining MDCK monolayers for cytochrome c and the activated form of Bax after inducing apoptosis with either etoposide or TRAIL (Fig. 1d, e, Fig. S3 in Supplementary Material). All apoptotic extruding cells, triggered by either etoposide or TRAIL treatment, show strong staining for the active form of Bax (as represented in Fig. 1d, Fig. S3a in Supplementary Material). Cytochrome c staining is also diffuse in all apoptotic extruding cells analyzed, characteristic of cytoplasmic cytochrome c release after mitochondrial permeabilization,

Fig. 1 Both intrinsic and extrinsic apoptotic stimuli elicit cellular extrusion in epithelial monolayers. MDCK monolayers treated with DMSO, etoposide, cycloheximide (CHX) or TRAIL plus CHX were fixed and stained for DNA, actin and active caspase-3 (**a**). *Insets* show a magnified view of the contraction ring, the apoptotic extruding cell and the neighbor cells distributed in a rosette pattern, characteristic of extrusion. Apoptotic extruding cells (**a**) were quantified (**b**, **c**) from three independent experiments. Cells treated with TRAIL or CHX were stained for DNA, actin, active caspase-3, and active Bax (**d**) or cytochrome c (**e**). Cartoons in (**d**) illustrate the microscope focal planes; the *bottom* view focuses on the contraction ring of the extruding cell and the *top* view focuses on the cell extruded out of the layer. *Insets* in (**e**) show the cytochrome c distribution of a live neighbor cell (*bottom* view) and of an extruding cell (top view). Scale bars, 10 µm. The number of cells counted for statistical analysis in (**b**) and (**c**) was as followed: DMSO ($n = 1046$), Etoposide ($n = 1449$), +CHX/−TRAIL ($n = 1425$), and +CHX/+TRAIL ($n = 1306$)
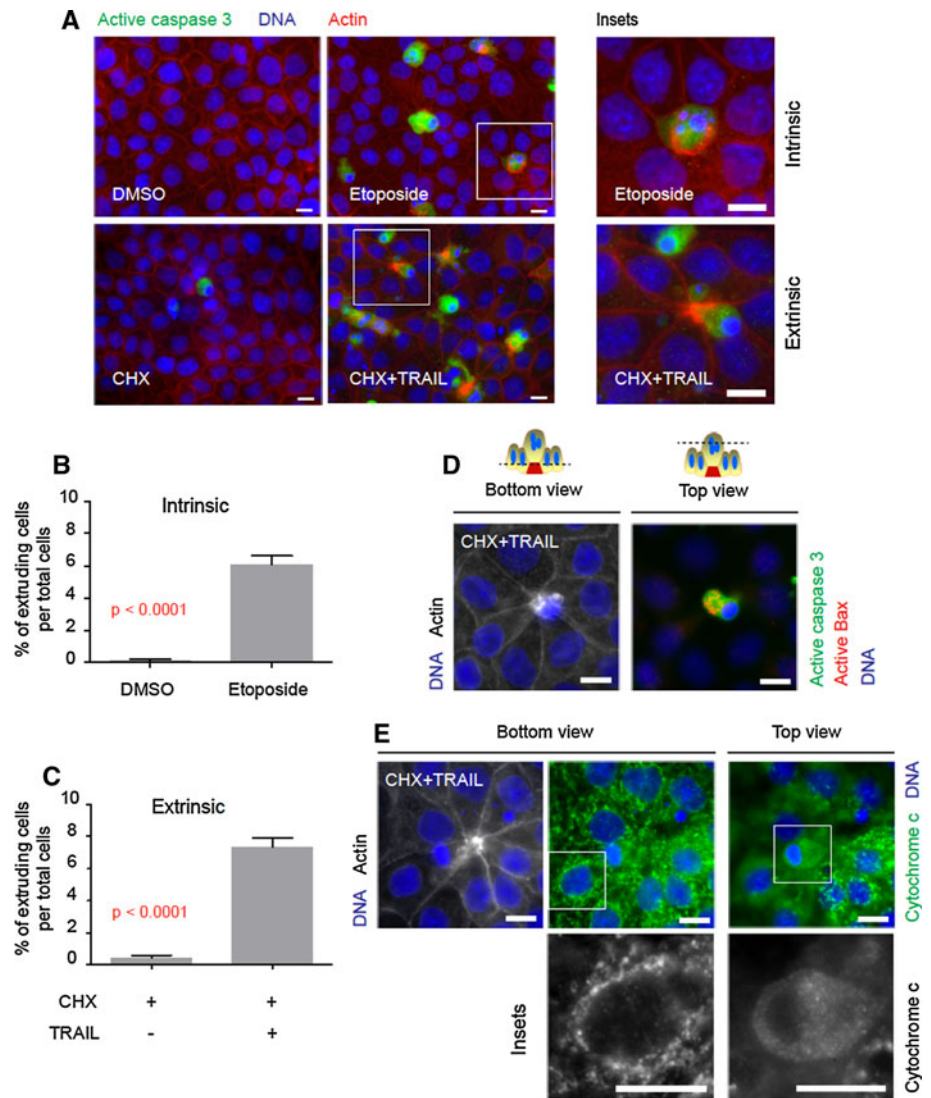
when compared to the punctate, mitochondrial staining seen in live cells (seen in bottom view of Fig 1e, Fig. S3b in Supplementary Material). These results show that both the extrinsic and intrinsic pathways activate mitochondrial permeabilization during apoptotic cell extrusion and suggested that mitochondria might sequester factors required for activation of the extrusion pathway during apoptosis.

Either overexpression of Bcl-2 or knockdown of Bax and Bak block cell extrusion via the intrinsic but not extrinsic apoptotic pathway

To determine the potential role of mitochondria in the activation of cellular extrusion, we blocked its permeabilization, and evaluated if cellular extrusion was blocked when the intrinsic pathway of apoptosis was stimulated. To block MOMP, and therefore the release of mitochondrial factors during apoptosis, we use two approaches:

overexpression of the anti-apoptotic protein Bcl-2 or knockdown of the pro-apoptotic genes Bax and Bak. To do this, we transduced MDCK cells with a vector containing a Bcl-2-IRES-EGFP cassette or sequentially transduced HBE cells with vectors containing a Bax shRNA and a Bak shRNA sequence (Fig. 2a, b). For the respective controls, we transduced MDCK cells with an empty vector (IRES-EGFP) or HBE cells with non-specific shRNA sequence (Fig. 2a, b). Epithelial monolayers overexpressing Bcl-2 or with Bax/Bak knockdown were treated with UV-C irradiation or etoposide and scored for their abilities to undergo apoptosis and extrusion. In addition to apoptosis, overexpression of Bcl-2 or downregulation of both Bax and Bak inhibited cell extrusion (Fig. 2c–e, Fig S4 in Supplementary Material), suggesting that mitochondria permeabilization is required for the activation of the extrusion pathway. We obtained similar results when we over-expressed Bcl-2 in HBE cells (data not shown). Therefore, the signal that
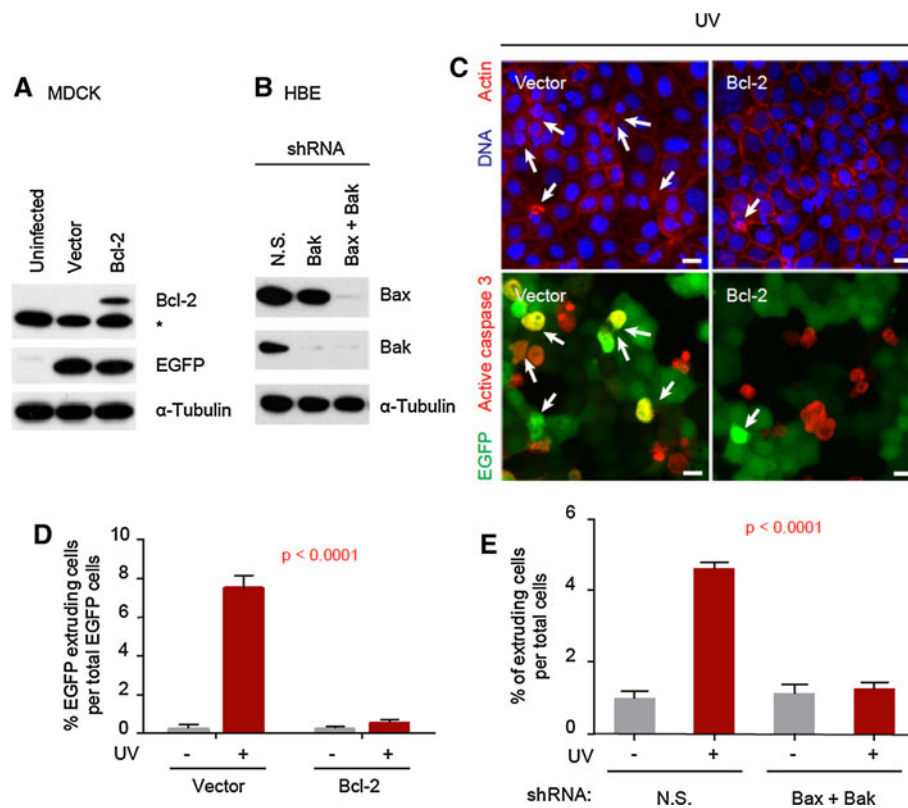
Fig. 2 Over-expression of Bcl-2 or knockdown of Bax and Bak blocks cell extrusion induced by intrinsic apoptosis stimuli. Immunoblot analysis of Bcl-2 in MDCK (**a**) or Bax and Bak in HBE (**b**) cells transduced with the indicated constructs. In (**a**), *asterisk* denotes non-specific immunogen; and in (**b**), N.S. stand for non-specific shRNA. Extrusion in MDCK monolayers with over-expression of Bcl-2 block cell death and extrusion, where *arrows* indicate apoptotic extruding EGFP-positive cells (**c**). The EGFP-negative cells in the vector control and Bcl-2 monolayers (**c**) are internal control cells. Note that internal control cells are extruding in both Vector and Bcl-2 monolayers. *Scale bars*, 10 μm. The number of apoptotic extruding cells was quantified (**d**, **e**) from three independent experiments. The *P* values in (**d**) and (**e**) correspond to *t* test analyses of the experiments indicated with *red bars*. The number of cells counted for statistical analysis in (**d**) and (**e**) were as followed: Vector −UV = 790, Vector +UV = 619, Bcl2 −UV = 1319, Bcl2 +UV = 1050, NS −UV = 1907, NS +UV = 2078, Bax/Bak −UV = 2047, and Bax/Bak +UV = 2356

initiates extrusion could either be sequestered in the mitochondria or activated downstream mitochondrial permeabilization (i.e. caspase activation).

Although our data show TRAIL targets the mitochondria during apoptotic cell extrusion, it might not require MOMP to induce apoptosis, as it can directly activate executioner caspases via caspase 8 and 10. If the extrusion-initiating signal is sequestered in the mitochondria, then blocking MOMP after initiating the extrinsic pathway should not allow cells to extrude. However, if the extrusion initiating signal is activated downstream of caspase activation, then TRAIL-dependent activation of the extrinsic death pathway should cause both apoptosis and extrusion even when MOMP is blocked. To test between these models, we treated MDCK and HBE monolayers with TRAIL and evaluated cell death and extrusion when MOMP is blocked by Bcl-2 overexpression or Bax/Bak knockdown (Fig. 3a–d). Neither overexpression of Bcl-2 nor Bax/Bak knockdown blocked apoptosis induced by

TRAIL treatment, as expected from other studies [16, 18]. Importantly, cell extrusion also occurred in these lines, favoring the hypothesis that the extrusion-initiating signal is activated downstream caspase activation.

To verify that TRAIL-induced apoptotic cell extrusion occurred independently of mitochondrial permeabilization, we immunostained TRAIL-treated monolayers for active Bax and cytochrome c (Fig. 3e). Apoptotic extruding cells from monolayers where Bcl-2 was overexpressed still showed release of cytochrome c; however, cytochrome c staining appeared punctate when Bax and Bak were knocked down (Fig. 3e, right panel insets). Although some mitochondrial permeability may occur through other mechanisms (i.e. VDAC) [20, 21], lack of complete diffuse cytochrome c staining suggested that Bax/Bak knockdown blocked most of MOMP. Correspondingly, activated Bax was absent in apoptotic extruding cell where Bax and Bak were knocked down, yet Bcl-2 overexpression still resulted in activated Bax staining (Fig. 3e, left panel insets). Failure
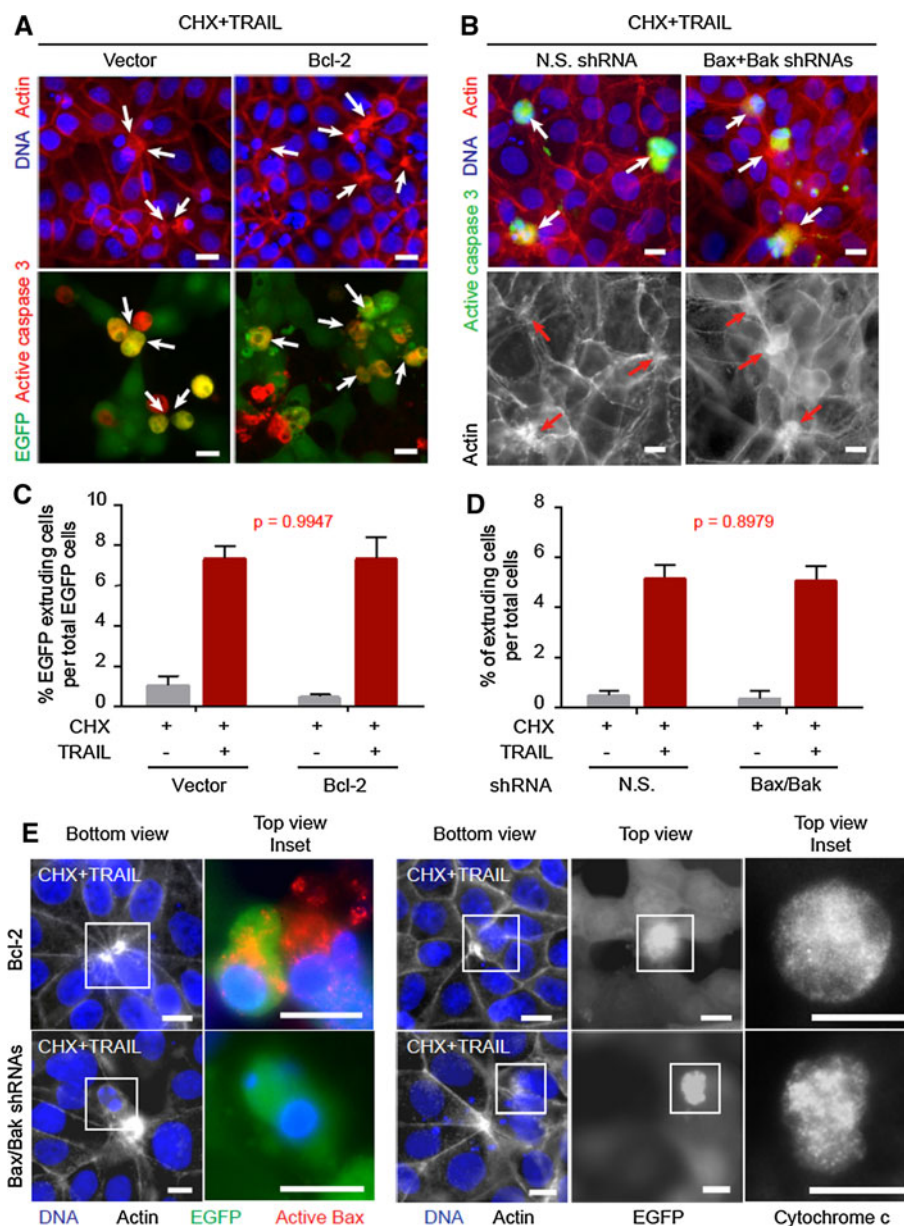
Fig. 3 Neither over-expression of Bcl-2 nor Bax/Bak knockdown blocks cell extrusion induced by extrinsic apoptosis stimuli. Extrusion in MDCK and HBE monolayers transduced with Bcl-2 or Bax/Bak shRNAs were treated with TRAIL and CHX (**a**, **b** respectively). *Arrows* in (**a**) and (**b**) show apoptotic extruding cells. In (**a**), the *arrows* in *upper panels* point to closed actin rings with condensed and fragmented DNA and those in *lower panels* show EGFP-positive cells with caspase-3 activity. The EGFP-negative cells in (**a**) are internal control cells. The number of apoptotic extruding cells was quantified (**c**, **d**) from three independent experiments. The *P* values in (c) and (**d**) correspond to *t* test analyses of the experiments indicated with *red bars*. Monolayers treated with TRAIL and CHX were stained for

DNA, actin, and either active Bax or cytochrome c (**e**). *Bottom* views show actin rings of extruding cells while *insets* of *top* views show the apoptotic extruding cell features. The second of the Bcl-2 panels shows two adjacent extruding cells positive for active Bax: one EGFP-positive and the other EGFP-negative (internal control). *Scale bars*, 10 μm. The number of cells counted for statistical analysis in (**c**) and (**d**) were as followed: Vector +CHX/−TRAIL = 905, Vector +CHX/+ TRAIL = 681, Bcl2 +CHX/−TRAIL = 1288, Bcl2 +CHX/+TRAIL = 1146, NS +CHX/−TRAIL = 924, NS +CHX/ + TRAIL = 850, Bax/Bak +CHX/−TRAIL = 1157, and Bax/Bak +CHX/+ TRAIL = 1004

of Bcl-2 overexpression to block TRAIL-induced Bax activation and cytochrome c release suggests that other anti-apoptotic Bcl-2 family members, rather than Bcl-2, could efficiently inhibit Bax activation and cytochrome c

release triggered by TRAIL (i.e. Bcl-x$_L$, Mcl-1, Bfl-1 and Bcl-w) [22–24]. However, because knockdown of Bak/Bax blocked both Bax activation and significant cytochrome c release but still allowed apoptotic extrusion to occur; we

reasoned that the caspase cascade may be important for cellular extrusion.

Epithelial cellular extrusion requires caspase activity for the proper formation and contraction of the actomyosin ring

To test the role of caspase activity for activating apoptotic cell extrusion, we inhibited caspase activation using the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl-ketone (z-VAD-fmk) after UV irradiation or etoposide-treatment. As expected, we observed an almost complete inhibition of apoptotic cell death, as evaluated by caspase activation and DNA morphology (Fig. 4a, c). Since either pharmacological or genetic inhibition of caspase following apoptotic stimuli could lead to other cell death mechanisms such as necrosis [25–29] or caspase-independent cell death [30–33], we evaluated the High Motility Group Box 1 (HMGB1) marker localization to explore the viability of the z-VAD-fmk–treated UV-irradiated cells. In living cells, HMGB1 is localized in the nucleus of interphase cells. During apoptosis, HMGB1 remains in the fragmented nucleus or is released into the cytoplasm. During necrosis or other forms of non-apoptotic cell death, HMGB1 is released extracellularly and its staining is lost [15, 34, 35].

To evaluate cell death in the presence of the pan-caspase inhibitor, MDCK monolayers incubated with z-VAD-fmk and treated with either UV-C or etoposide were immuno-stained for HMGB1 in combination with DNA, active caspase-3, cytochrome C or AIF (Fig. 4a). Very few cells (1.5%) within these monolayers were apoptotic, as characterized by positive caspase-3 and HMGB1 immuno-staining, and chromatin condensation and fragmentation (Fig. 4a, c). A higher percentage (5.3%) of cells with z-VAD-fmk showed neither HMGB1 nor caspase-3 staining (Fig. 4a, c). The sum of both percentages corresponds to the percentage of cells that would normally die through apoptosis in the absence of z-VAD-fmk (Fig. 4c). Further, the mitochondria in the HMGB1 negative cells appear to be permeabilized, as cytochrome c was absent from mitochondria (Fig. 4a), confirming that these cells were indeed dying, although through a cell death mechanism different from apoptosis. To determine whether these cells were dying through caspase-independent cell death or necrosis, we first evaluated apoptosis inducing factor (AIF) localization. During caspase-independent cell death, AIF is released from the mitochondrial outer membrane space and translocates to the nucleus to mediate chromatin condensation and large-scale DNA fragmentation [30, 36, 37]. Although AIF is absent from mitochondria, it does not translocate into the nucleus, suggesting that these cells were probably dying through necrosis, rather than caspase-

independent cell death (Fig. 4a). Their DNA appearance, the absence of HMGB1 and caspase staining as well as their positive staining for propidium iodide (PI), strongly suggest that the z-VAD-fmk-treated cells were dying through necrosis (Fig. 4a, e).

When evaluating whether cells extrude from monolayers treated with z-VAD-fmk, we found that the small population of apoptotic cells with caspase-3 activity undergo cell extrusion, while the HMGB1 negative cells do not (Fig. 4d). Instead, HMGB1 negative cells show a slight accumulation of actin at the cell cortex where they contact their neighboring cells, resembling a non-continuous actin ring (Fig. 4b). Although the necrotic cells appear to form a reduced actin extruding ring, they remain uncontracted in the monolayer compared to control cells at 2 h post-irradiation (Fig. 4b, d).To determine whether these cells eventually extrude, we filmed these cells by time-lapse microscopy by treating MDCK cells stably expressing a HMGB1-EGFP protein fusion with z-VAD-fmk and UV-C (Fig. 4e). To monitor cell permeabilization, we added PI to the culture medium. As previously observed, control monolayers treated with the DMSO and UV irradiation undergo apoptotic cell extrusion one to two hours post-irradiation, as characterized by contracting, blebbing cells surrounded by their neighbor cells in a rosette pattern (Fig. 4e, asterisks). These apoptotic cells retain HMGB1 in their nuclei and are negative for PI staining throughout the extrusion process until the extruding ring has been closed at 70–80 min after extrusion initiates (Fig. 4e, Supplementary Movie 1). By contrast, when caspases are blocked, cells undergo necrosis, lose HMGB1 and become PI-permeant, while no extrusion occurs (Fig. 4b, e, Note lack of rosette pattern). In this case, necrotic cells have impaired plasma membrane integrity and should compromise the epithelium protective barrier, compared to monolayers where apoptotic cells extrude. Nevertheless, although necrotic cells are unable to trigger the extrusion machinery, they are eventually removed from the epithelium by a passive cell movement. The movement by constant cell rearrangements in the epithelium acts to shove the necrotic cells out of the way as their integrity declines (Fig. 4e, Supplementary Movies 2, 3). These movies are representative of other movies, where apoptotic extrusion occurs within 90 min, whereas necrotic cell removal can take up to 5 h (Fig. S5 in Supplementary Material). This wide variation is likely due to variance in the stochastic movements of cells.

Discussion

Although several studies both in vivo and in vitro show a connection between cellular extrusion and apoptosis [4–9],
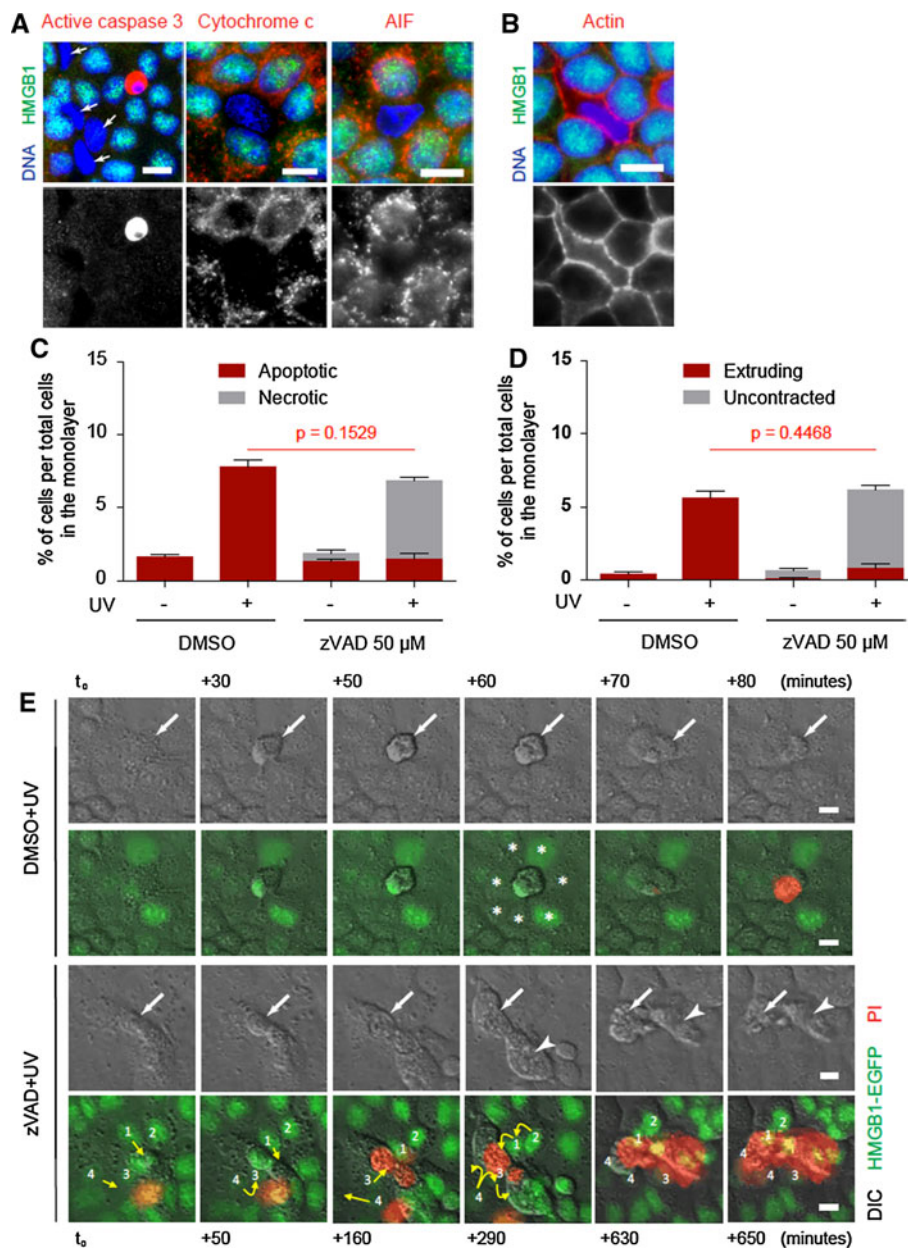
Fig. 4 Epithelial cellular extrusion requires caspase activity for proper formation and contraction of the actomyosin ring. MDCK monolayers incubated with 50 μM zVAD-fmk (caspase inhibitor) and irradiated with UV-C were immuno-stained for DNA and HMGB1 as well as active caspase-3, cytochrome c, AIF or actin (**a**, **b**). Apoptotic (with active caspase-3) and necrotic cells (negative for both active caspase-3 and HMGB1) as well as extruding and uncontracted cells were quantified in monolayers subjected to treatments indicated in (**c**) and (**d**). Results shown represent mean, where error bars are ± SEM from three independent experiments. The number of cells counted for statistical analysis in (**c**) and (**d**) were as followed: DMSO −UV = 1832, DMSO +UV = 1952, zVAD −UV = 1899, and zVAD +UV = 1875. MDCK monolayers stably expressing the fusion HMGB1-EGFP were treated with UV-C irradiation with or without zVAD (**e**) and time-lapsed every 10 min for 12 h. Propidium iodide (PI) was added to monitor permeability of the dying cells. *Arrows* in the *top panels* show an apoptotic extruding cell, where *asterisks* in the *second panel* highlight rosette pattern formed around the dying cell, which is characteristic of cell extrusion. *Arrows* in the *third panel* show a necrotic, uncontracted cell, which loses its HMGB1 (*bottom panel*) and becomes PI-positive and bursts. *Arrowheads* in the *third panel* point to another cell undergoing necrosis at a faster rate than the cell with *arrows*. In the *bottom panel*, numbers indicate four cells changing positions to illustrate the stochastic movement of cells in the epithelium while *yellow arrows* indicate the direction the cell moves. *Scale bars*, 10 μm

little was previously known about how the pathways of apoptosis and extrusion are linked. In this study, we found that several intrinsic (UV irradiation, etoposide, camptothecin and serum deprivation) and extrinsic (Fas-L and TRAIL cytokines) apoptotic stimuli induce cells to extrude from monolayers of two different cell lines
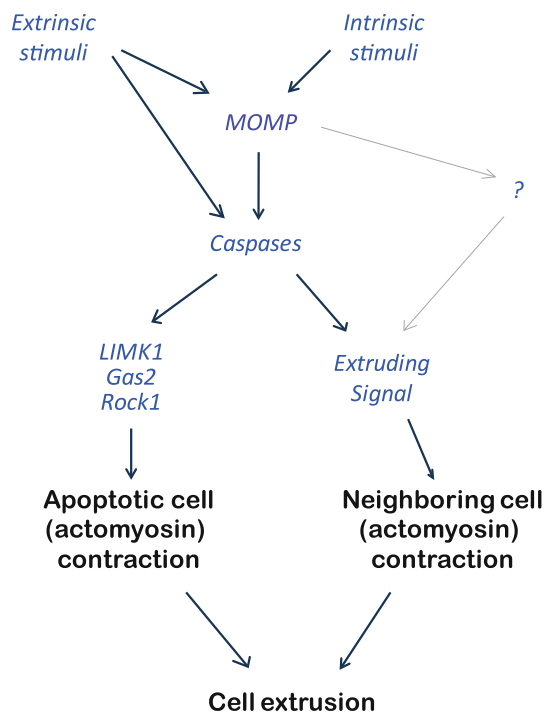
Fig. 5 A model for apoptotic regulation of cellular extrusion. This model illustrates pathways that can link apoptosis with extrusion. Here, caspases may orchestrate the removal of the apoptotic cells by activating several signals. Question mark suggests that other non-apoptotic signals may also contribute to formation of actin extruding ring, which partially forms in the presence of caspase inhibitors

(MDCK and HBE). We also found that caspase activity is required for cellular extrusion. When caspases are inhibited, cells become necrotic and are removed from epithelia by random epithelial cell movement. Taken together, our results lead to a model whereby all known apoptotic signals feed into a conserved step in the pathway important for apoptotic extrusion—caspase activation (Fig. 5).

Caspases proteolytically inactivate or activate a variety of targets, leading to the controlled pathway of morphological changes and break down of a cell during apoptosis. Caspases also regulate several non-apoptotic processes including activation and proliferation of T and B-lymphocytes, cell differentiation during keratinization of skin and spermatogenesis, cell motility and cell shaping [39–41]. In addition, caspase-8 activity has been suggested to play a role in cell survival [42, 43]. In this work, we find that caspase activity is required to initiate the removal of dying cells from epithelia. Our findings reveal a new role for caspases in a non-apoptotic event—extrusion.

Two models were previously proposed to explain the regulation of cellular extrusion. One model proposes that the force driving cellular extrusion comes from the dying cell itself and thus it is a cell autonomous mechanism [10, 44]. The second one, a non-cell autonomous model, proposes that the contraction of an actomyosin ring formed in

the neighboring cells provides the main force for the removal of the dying cell [4]. However, because extrusion is a mechanism that requires multiple coordinated caspase-dependent events, a combination of these two models may occur (Fig. 5). During apoptosis, caspases target important actin and myosin regulators such as Gas2, LIM-kinase 1 and ROCK, which activate contractile forces required for apoptotic cell blebbing [45–49]. These forces, as initially proposed by Mills et al. [10], could be an important component of actomyosin contractions occurring during extrusion. Therefore, even though contraction of the actomyosin ring in the surrounding cells is required for extrusion, contractions in the dying cell may also be required to ensure efficient extrusion (Fig. 5).

While contractions to remove the dying cell occur, caspases could also control the reorganization of adherens and tight junctions, which are required for extrusion. This reorganization plays a crucial role during extrusion not only for the release of the dying cell from the epithelium, but also for the maintenance of the epithelial protective barrier. As shown by Kessler and Müller [50, 51], caspases trigger a progressive and orchestrated disassembly of adherens junctions. Early in apoptosis, cleavage of β-catenin promotes the removal of E-cadherin from the membrane. Later in apoptosis, all adherens junction components are removed from the membrane. At the same time, while the apoptotic cell is released, the neighboring cells form new adherens junctions, closing the gap the apoptotic cell would leave. In addition, the detachment of the apoptotic cell from the extracellular matrix is also regulated by caspases [10].

Although caspase activity is required for cellular extrusion, other upstream signals may be required to initiate early steps in extrusion activation. Indeed, we find that even when caspases are inhibited with zVAD, a non-continuous ring of actin still forms around the dying cell; however, it is unclear whether this ring forms in the live or dying cells. Therefore, other upstream signals could partially participate in the formation of the actomyosin ring, but without caspase activation, the extruding ring would not fully form or contract (Fig. 5).

In our experiments when blocking caspases inhibited apoptosis, cells instead become necrotic and did not extrude, but were eventually removed by random movements of surrounding cells. It is important to note, however, that other studies find that necrosis of single cells due to laser ablation heal by a purse-string mechanism that mimics extrusion [38, 52]. Although this mechanism might be different from extrusion, these cells may still contain extrusion-inducing signals that the necrotic cells in our study would have lost over time. Therefore, how an epithelial cell dies within the epithelium could have significant effects on the barrier function of the epithelium. If a cell

dies by apoptosis or sudden necrosis, such as a wound, the barrier will be preserved, whereas if it dies by necrosis following impaired apoptosis, the barrier of the epithelium could become compromised.

The fact that apoptosis connects with the extrusion pathway at a late point in the apoptotic pathway ensures that any apoptotic stimulus will feed into the extrusion pathway. The requirement of caspase activity also suggests that caspase activity functions as an important checkpoint in the removal of cells from the monolayer only after a cell has passed the point of no return in the apoptosis pathway. Early events in the apoptosis pathway, therefore, might not trigger extrusion as a way to give cells another chance to fix any potential threat before committing to apoptosis, such as DNA damage, protein agglomeration, ER stress, or damaged mitochondria.

Our findings establish the connection between apoptosis and cellular extrusion and bring new insights in the molecular basis of cellular extrusion. Our work also reveals another non-apoptotic role for caspases, regulation of cellular extrusion. However, further investigations will need to determine which caspases participate in the activation of extrusion and which factors targeted by caspases play decisive roles during this event.

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