APOPTOTIC REGULATION OF CELLULAR EXTRUSION

by

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STATEMENT OF DISSERTATION APPROVAL

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has been approved by the following supervisory committee members:

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ABSTRACT

The purpose of this dissertation is to define how the pathways of apoptosis and extrusion connect. These studies employed MDCK and HBE *in vitro* cultured epithelia as model systems to explore the ability of different components of the apoptosis pathways to participate in the activation of the extrusion response. Because of the ease of their manipulation for genetic, pharmacological and cell biology analyses but most importantly because their ability to reproduce *in vivo* apoptotic cellular extrusion, cultured epithelia provide a powerful and efficient system to study and dissect the molecular basis of apoptotic cellular extrusion.

In the first part of this dissertation, we established that both intrinsic and extrinsic apoptotic pathways activate cellular extrusion and that the contraction force that drives cellular extrusion requires caspase activity. Accordingly, we found that necrosis does not trigger the cellular extrusion response; nevertheless, necrotic cells are removed from epithelia by a passive mechanism that involves forces of the stochastic movement of epithelial cells.

The second part of this dissertation is focused on the regulation of apoptotic cellular extrusion by the anti-apoptotic protein Bcl-2. Mutations that increase Bcl-2 protein levels result in resistance to cell death, which in turn lead to several malignances in the living organism. Here, as a way to complement our studies in the first part of this dissertation,

we explore the effect of Bcl-2 on cell junctions, test the ability of HA14-1, a Bcl-2 inhibitor, to activate cell extrusion and finally we test the ability of this oncogene to regulate cellular extrusion *in vivo*.

The studies reveal the connection between apoptosis and cellular extrusion: caspase activation, a conserved point in the apoptosis pathway where all known apoptotic signals converge. The discovery of the requirement of caspases for cellular extrusion reveals a new non-apoptotic role for caspases and sets caspase activity as an important checkpoint for epithelial cellular removal. These studies also reveal a new passive mechanism by which dead cells unable to trigger cellular extrusion are removed.

To Daniel and Leonor, my parents.

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CHAPTER 1

INTRODUCTION

1.1 Cell death mechanisms

Cell death plays essential roles in living multicellular organisms during development, morphogenesis, homeostasis, tissue remodeling, and activation of inflammation during the immune response [1, 2]. Moreover, misregulation of cell death has important implications for developmental diseases, tumor initiation and progression, neurodegenerative diseases (Parkinson's or Alzheimer's diseases) and autoimmune diseases [3, 4]. Cell death can be classified in three types: Apoptosis, necrosis and autophagic cell death (Fig. 1.1). While apoptosis and necrosis are traditionally accepted as forms of cell death, the acceptance of autophagic cell death, in which cells digest themselves to death as a suicide strategy, is still on debate [5-7].

1.1.1 Characteristics of apoptosis and necrosis

During apoptosis, activated caspases, through a controlled process of proteolysis, break down and package the dying cell into apoptotic bodies. To avoid immune activation, apoptotic bodies do not undergo plasma membrane breakdown and are promptly recognized and removed by phagocytic cells. The nucleus undergoes condensation (pyknosis) and fragmentation (karyorhexis) and the chromosomal DNA is cleaved into 200 basepair inter-nucleosomal fragments [8]. The condensation and

blebbing of the dying cell requires actin and myosin dynamics, which consumes high levels of ATP [9]. In contrast to apoptosis, necrosis results from ATP depletion. Because no energy is available for the organized breakdown of the cell, necrosis is characterized by failure to regulate osmosis and cell lysis. Plasma membrane breakdown that occurs in necrotic cells allows the release of High Motility Group Box 1 protein (HMGB1), responsible for activation of the inflammatory response (Fig. 1.1) [10-12]. Necrotic cells exhibit changes in nuclear morphology due to DNA degradation, but they do not have the organized chromatin condensation and fragmentation of DNA of apoptotic cells [13]. Although necrosis has been thought to be a passive, disorganized form of cell death, it has recently emerged as an alternate form of programmed cell death, regulated by the same factors that control apoptosis (exposure to apoptotic cytokines and irradiation, Bcl-2 and heat shock proteins) [13-15]. Moreover, necrosis can be triggered during some pathological conditions such as microbial infection and cancer, or during tissue renewal and embryogenesis [14].

1.1.2 Regulation of the apoptotic machinery

Depending on the death stimulus, a cell may undergo apoptosis via two different apoptotic pathways—the extrinsic and the intrinsic pathways (Fig. 1.2) [16, 17]. Both pathways ultimately lead to the activation of executioner caspases (caspases-3, -6, and -7), which directly and indirectly physically break down the apoptotic cell [18].

To activate the extrinsic pathway, a ligand (such as TNF, Fas-L, and TRAIL) binds its respective death receptor, which recruits and oligomerizes adapter molecules that activate initiator caspases, caspase-8 and -10 (Fig. 1.2) [19]. Active initiator caspases proteolytically activate the executioner caspases, necessary to bring about apoptosis.

Type I cells can directly activate executioner caspases upon caspase-8 and -10 activation; however, type II cells must amplify the signal from the activated death receptor by targeting the mitochondrial pathway of apoptosis (Fig. 1.2) [20, 21].

On the other hand, various internal cell death cues trigger the intrinsic pathway by signaling through the mitochondrial pathway of apoptosis (Fig. 1.2) [22]. When death signals override survival signals, the mitochondrial outer membrane becomes permeable, releasing cytochrome c, the apoptosis-inducing factor (AIF), Endo G, Omi and Diablo into the cytoplasm (Fig.1.3). Once in the cytoplasm, cytochrome c forms a macromolecular complex with apaf-1 and procaspase-9 called the apoptosome, which facilitates activation of the initiator caspase, caspase-9 (Fig.1.3) [23]. While active caspase-9 activates executioner caspases, Omi and Diablo facilitates caspase activation by antagonizing the inhibitors of apoptosis proteins (IAPs) (Fig.1.3). AIF and Endo G activate DNA condensation and degradation (Fig.1.3). If caspases are genetically or pharmacologically inhibited, AIF and Endo G can still promote caspase-independent death [24, 25].

The Bcl-2 family of proteins regulates mitochondrial outer membrane permeabilization (MOMP) (Fig.1.2) [26-28]. To date, a total of 25 genes have been identified in this family [29, 30]. Based on their Bcl-2 homology (BH) domains, the gene products of this family are classified in multidomain and BH3-only proteins; and based on their function, they are classified in anti-apoptotic and pro-apoptotic (Fig. 1.4). Anti-apoptotic proteins such as Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and Bfl-1 are only multidomain, generally having four BH domains (BH1-4). Conversely, pro-apoptotic proteins contain either two or three BH domains (BH1-3) (such as Bax, Bak and Bok) or are single BH3-

only containing (such as Bim, Bid, Bad, Bik, Bmf, Noxa, Puma and Hrk) (Fig 1.4) [31, 32]. Once activated, pro-apoptotic multidomain proteins oligomerize to form pores in the outer mitochondrial membrane driving MOMP and the release of mitochondrial apoptotic factors into the cytoplasm (Fig. 1.3) [33]. To prevent MOMP, anti-apoptotic proteins bind pro-apoptotic proteins and inhibit their oligomerization (Fig. 1.2). The result of this battle between pro-apoptotic and anti-apoptotic proteins will determine whether the cell will survive or die [34]. The precise mechanism by which multidomain pro-apoptotic proteins are activated remains unclear. It is believed that BH3-only proteins activate multidomain pro-apoptotic proteins directly by binding, inducing their conformational change into their active form, or indirectly by engaging and neutralizing anti-apoptotic proteins [35]. Since inhibition of anti-apoptotic proteins is not sufficient for activation of pro-apoptotic proteins, but requires the BH3-only proteins Bid or Bim, a combination of both models may be important for apoptosis initiation [36].

1.2 Apoptotic cellular extrusion

Epithelia, formed by multiple or a single layer of cells, provide a protective barrier for the tissues and organs they encase. During development and later throughout life, the cells comprising the epithelium undergo dramatic turnover [37]. Epithelia are frequently exposed to toxins, inflammatory cytokines, or pathogens resulting in apoptosis or necrosis that could potentially damage the epithelium, and compromise its function as a protective barrier (Fig. 1.5) [38, 39]. Poor epithelial barrier function could lead to malformations in the developing embryo, edema, tissue damage, inappropriate signaling, inflammation, and severe infections or sepsis in the adult organism. However, even when a significant proportion of cells become apoptotic in the epithelia, the barrier is still

maintained in all epithelia by a process termed cellular extrusion. During extrusion, an apoptotic cell signals its epithelial neighbors to form an actin and myosin contractile ring that squeezes the dying cell out of the monolayer while simultaneously replacing the space of exiting dying cell, thereby preventing the formation of a gap in the epithelium (Fig. 1.5) [40].

Apoptotic cell extrusion occurs in all epithelia examined including the retinal pigment epithelium of chick embryos, embryonic epidermis of chick, mouse, and *Drosophila*, intestinal villi (Fig. 1.6), and can be modeled in tissue culture epithelia [40-44]. Although inducing apoptosis in epithelia activates extrusion, it is not clear how extrusion and apoptosis are connected or what factors initiate the formation and contraction of the actomyosin extrusion ring. Two models were proposed to explain how extrusion occurs during apoptosis. The cell autonomous model proposed by Mills et al. [9] suggested that extrusion results from contractile forces involved in apoptotic cell condensation and blebbing (Fig. 1.7). During apoptosis, caspases activate actomyosin regulators such as ROCK1, Gas2 and LIMK1 [45-49]. As a result, the apoptotic cell undergoes dramatic actomyosin changes forming a contraction ring inside the dying cell. The forces resulted by the contractions of this ring lead to cell blebbing and condensation. Mills et al. [9] state that these contractions also drag the neighbor cells close to fill the gap the apoptotic cell would leave. This model proposes that extrusion is absolutely dependent on the dying cell and there is no participation of the neighbor cells during this process (Fig. 1.7). On the other hand, the noncell autonomous model proposes that removal of the apoptotic cell relies on the formation and contraction of an actomyosin ring in the neighbor cells. By using a cell addition assay, where apoptotic or live cells are added to epithelial

monolayers, Rosenblatt et al. [40] found that addition of early apoptotic cells to a live monolayer can induce actin assembly in the cells they contact, suggesting that the apoptotic cell can trigger assembly of the actin extruding ring in the neighboring cells (Fig. 1.8) [40]. In addition, our lab has recently identified sphingosine-1-phosphate as the signal the apoptotic cell sends to its neighbors to execute their removal from the epithelium, further supporting this model (unpublished data).

While this model suggests that apoptotic cells can signal extrusion, it is still unclear how it does so. Additionally, is apoptosis required to initiate extrusion or can extrusion occur independently of apoptosis? If so, do both intrinsic and extrinsic apoptotic stimuli activate extrusion? Which apoptotic signals are important for activating extrusion? What component(s) of the apoptotic machinery triggers the extrusion response? Can other forms of cell death such necrosis activate cell extrusion?

In my dissertation, by testing different apoptotic stimuli, I have found that any intrinsic or extrinsic apoptotic stimulus trigger extrusion. Additionally, I found that extrusion requires caspase activation for proper formation and contraction of the actomyosin ring. Although necrotic cells, resulting from caspase inhibition, do not extrude, they are eventually cleared from epithelia by random epithelial cell movements

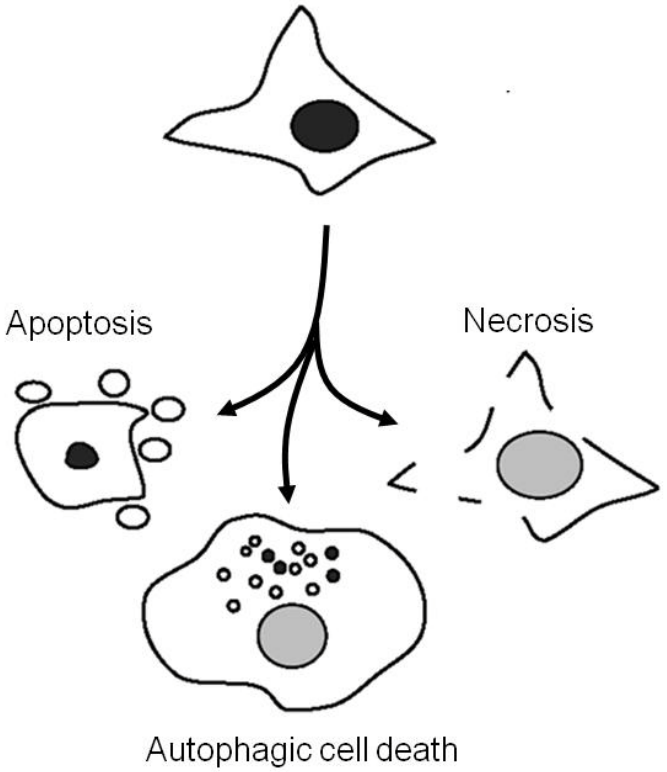

Figure 1.1 Mechanisms of cell death.
Cells can die through three types of cells death: Apoptosis, necrosis and autophagic cell death. During apoptosis, the nucleus and the cell undergo condensation and fragmentation, the plasma membrane does not become permeable and there is no release of HMGB1 (black spot). During necrosis, the plasma membrane becomes permeable and HMGB1 is released from the cell (absence of black spot in the gray nucleus). During autophagic cell death, the plasma membrane remains intact, HMGB1 is released and some autophagosomes (small circles) colocalize with HMGB1. Adapted from [50].

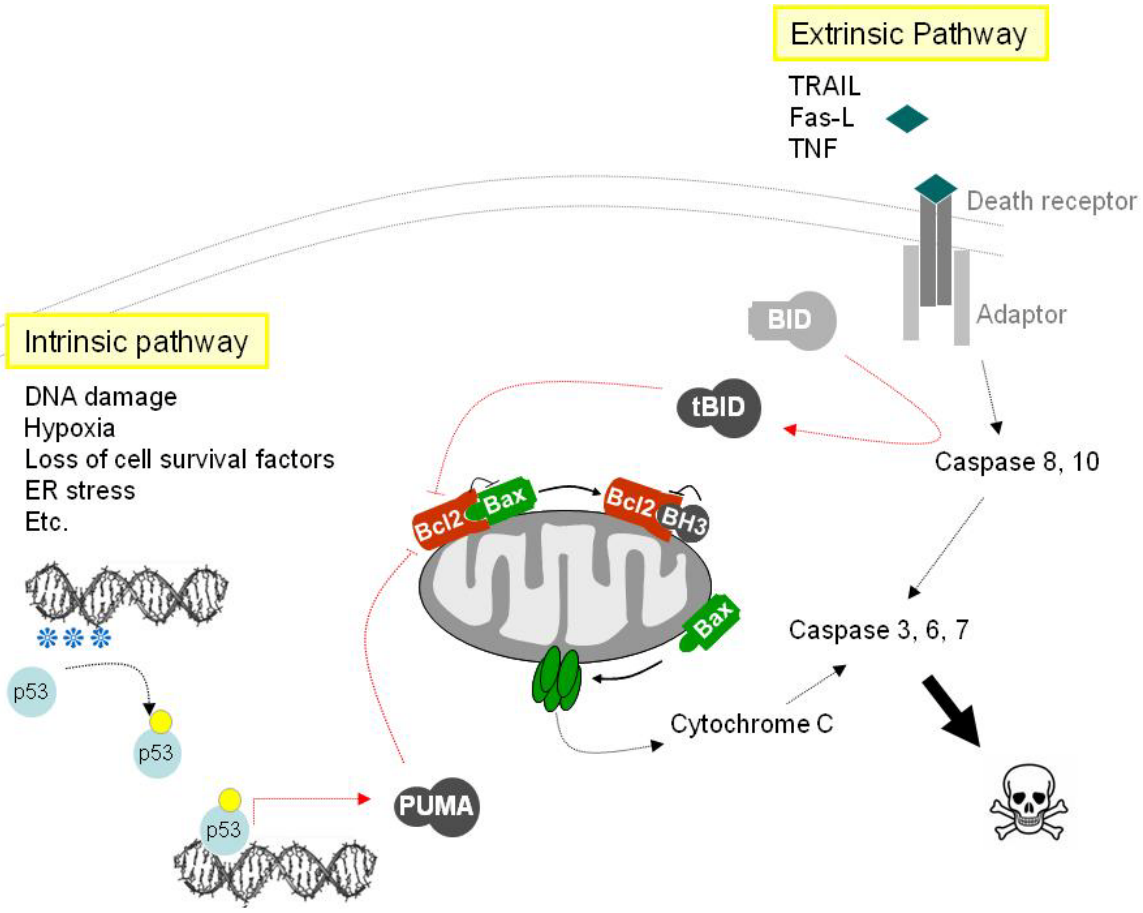

Figure 1.2 Apoptosis signaling pathways.
The intrinsic pathway senses different forms of cell stress (such as DNA damage) and targets the mitochondrial pathway for activation of apoptosis. On the mitochondria, the Bcl-2 family of proteins regulates mitochondrial permeabilization and cytochrome c release (see text for details). On the other hand, the extrinsic pathway is activated by binding of ligands on the death receptor. This signal is transduced to directly activate executioner caspases or to target the mitochondrial pathway to amplify the initial death signal.

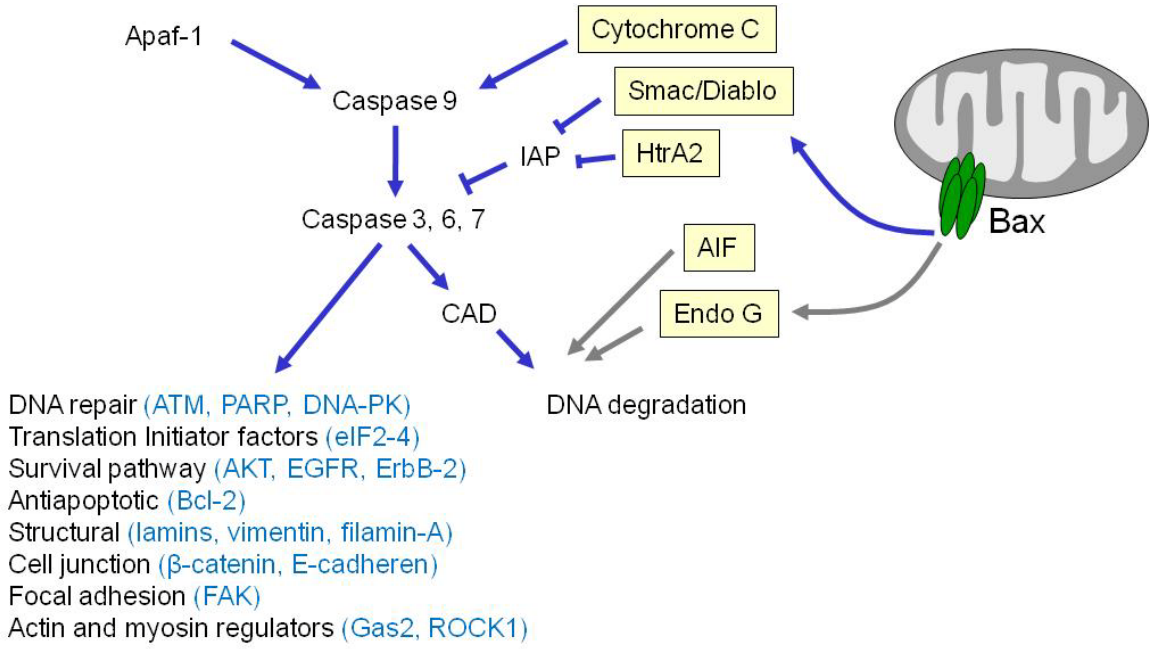

Figure 1.3 Apoptosis regulation by the mitochondrial pathway
Once the mitochondrial outer membrane is permeabilized, several factors participate in the activation of executioner caspases 3, 6 and 7. While Caspase-Activated DNase (CAD) along with AIF and Endo G participate in the degradation of DNA, executioner caspases target a set of proteins allowing apoptosis to take place.

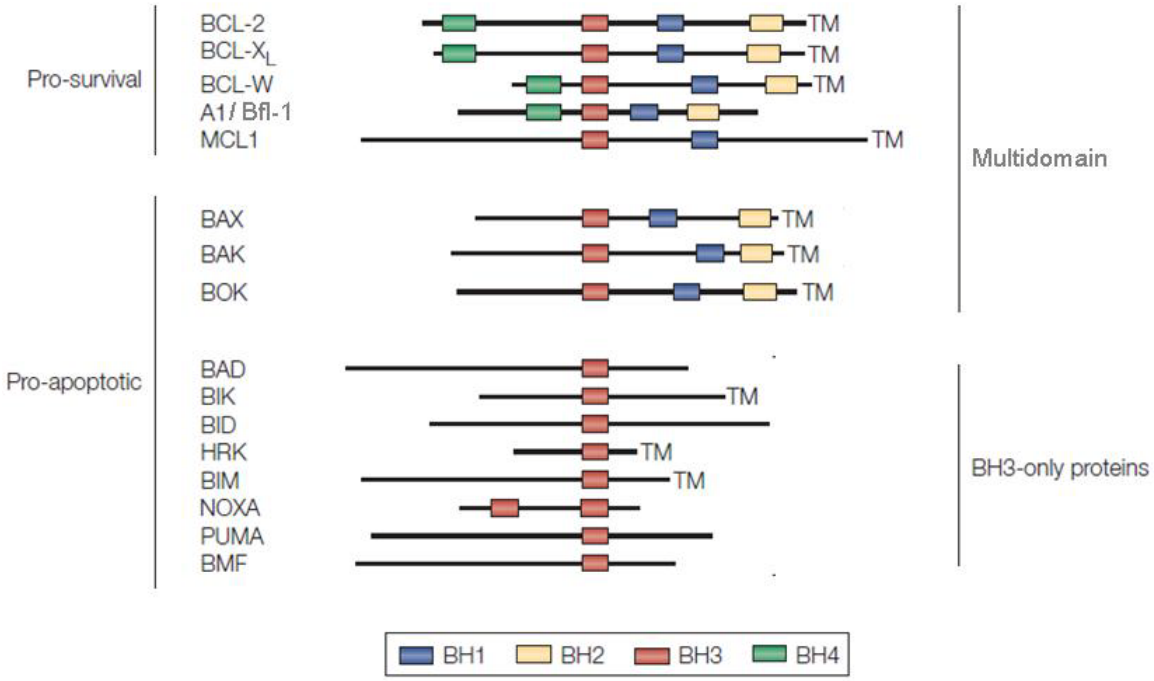

Figure 1.4 The Bcl-2 family members.
Based on their function, members of the Bcl-2 family of proteins are classified into pro-survival or anti-apoptotic and pro-apoptotic. Also based on their Bcl-2 homology domain (BH), they are classified into multidomain or BH3-only proteins. TM stands for transmembrane domain. Adapted from [51]

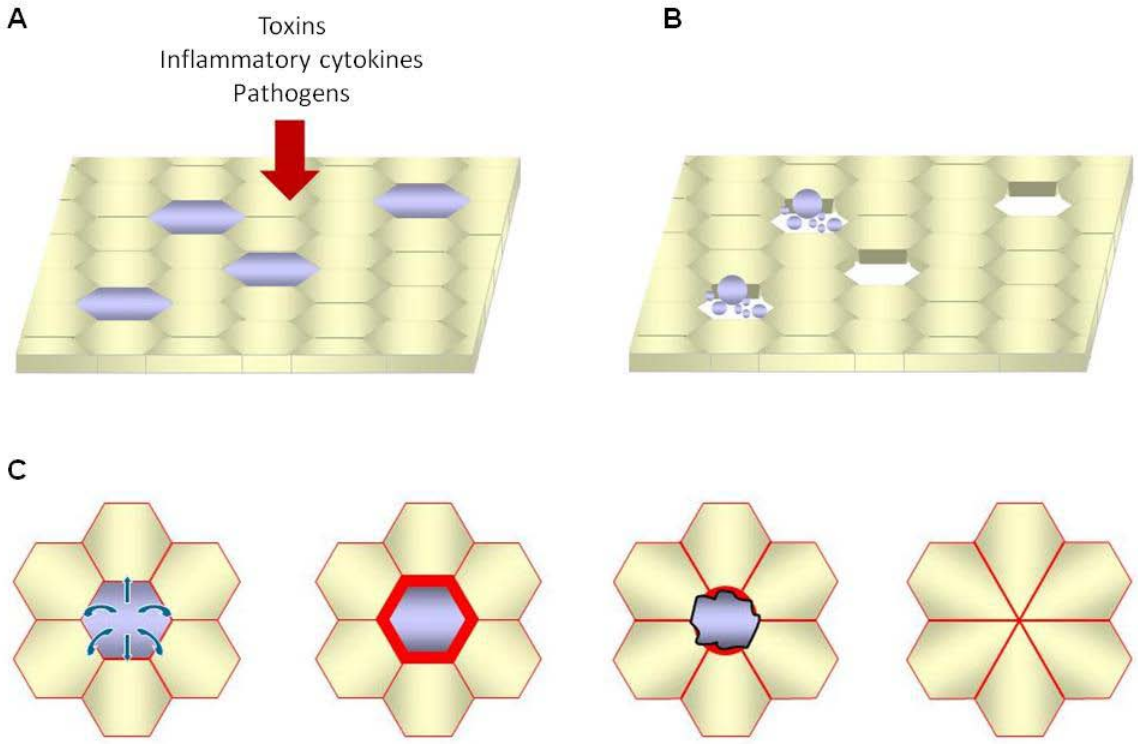

Figure 1.5 Apoptotic cellular extrusion.
Cells forming epithelia are exposed to an environment that induces cell death (a). Apoptosis could compromise epithelial protective barrier as apoptotic cells (blue) bleb and also are recognized and removed by macrophages (b). During extrusion, the dying cell signals (blue arrows) its neighbor to form actomyosin ring (red). Contraction of the ring squeezes the apoptotic cell out of the epithelium and brings the neighbor cells to seal the gap the apoptotic cell would otherwise leave (c).

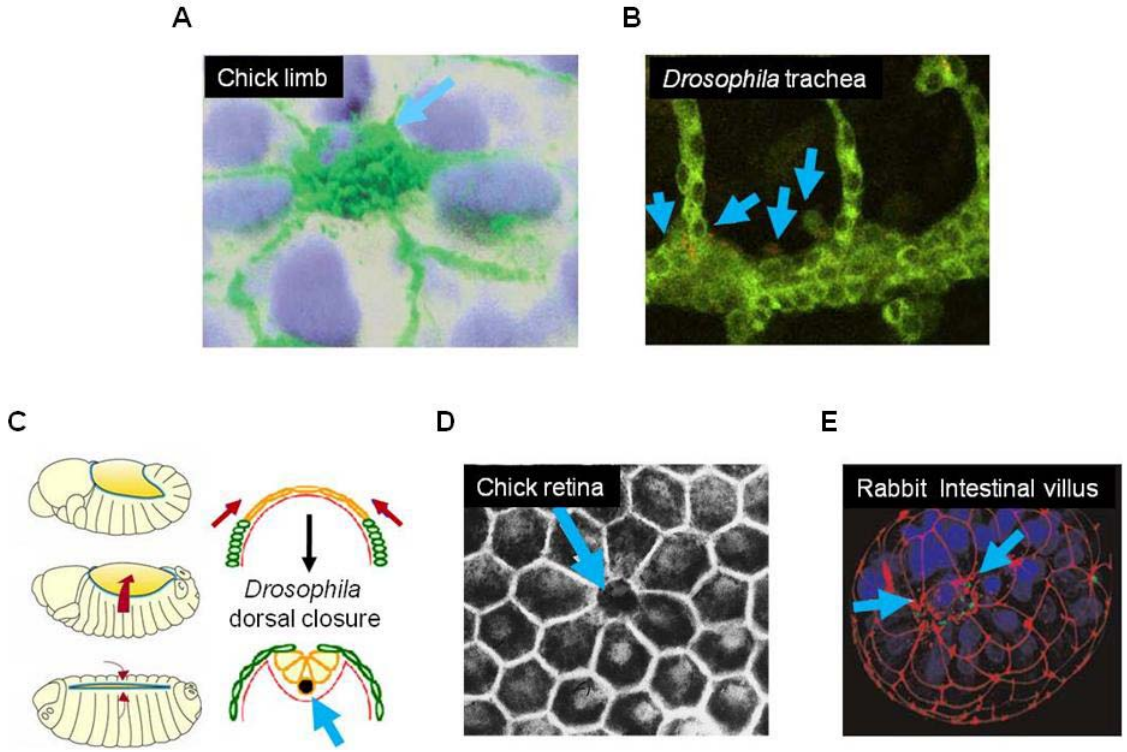

Figure 1.6 The biological roles of apoptotic cellular extrusion
Apoptotic cellular extrusion participates during developing of the limbs of chick embryos (a) and tracheal dorsal branches in *Drosophila* embryos (b). It drives dorsal closure during *Drosophila* development (c), and it maintains the proper compartment retinal pigment epithelium provides to the photoreceptors in the chick retina (d). Also during homeostasis, it maintains the protective barrier of the rabbit intestinal villus tip (e). Blue arrows point at apoptotic extruding cells. Adapted from [40, 42; 43, 52; 53].

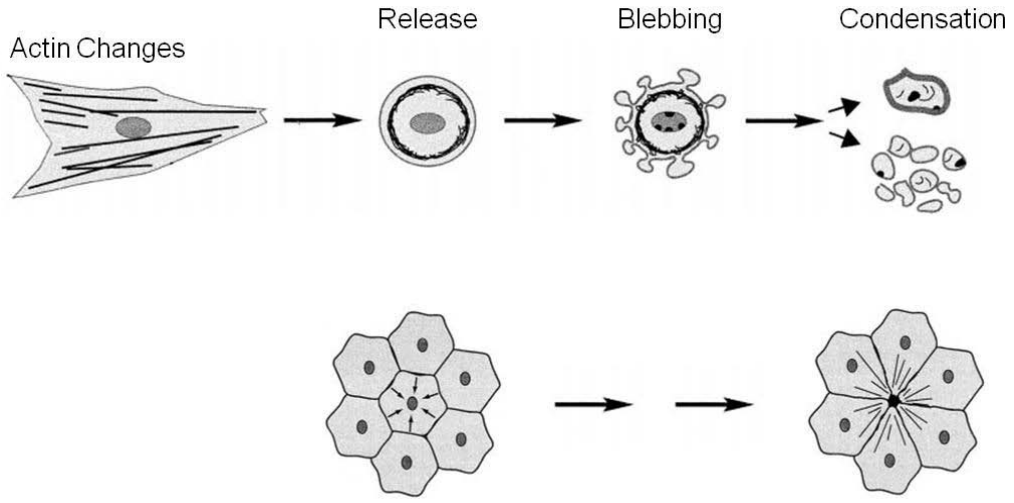

Figure 1.7 The cell autonomous model for apoptotic cellular extrusion
During apoptosis, cells undergo dramatic actomyosin changes forming a contraction ring inside the dying cell. The forces resulted by these contractions lead to cell blebbing and condensation. The cell autonomous model proposed by Mills et al. [9] states that the contractions occurring during apoptosis will drag the neighbor cells close to fill the gap the apoptotic cell would leave. This mechanism is absolutely dependent on the dying cell and there is no participation of the neighbor cells during this process. Adapted from [9]

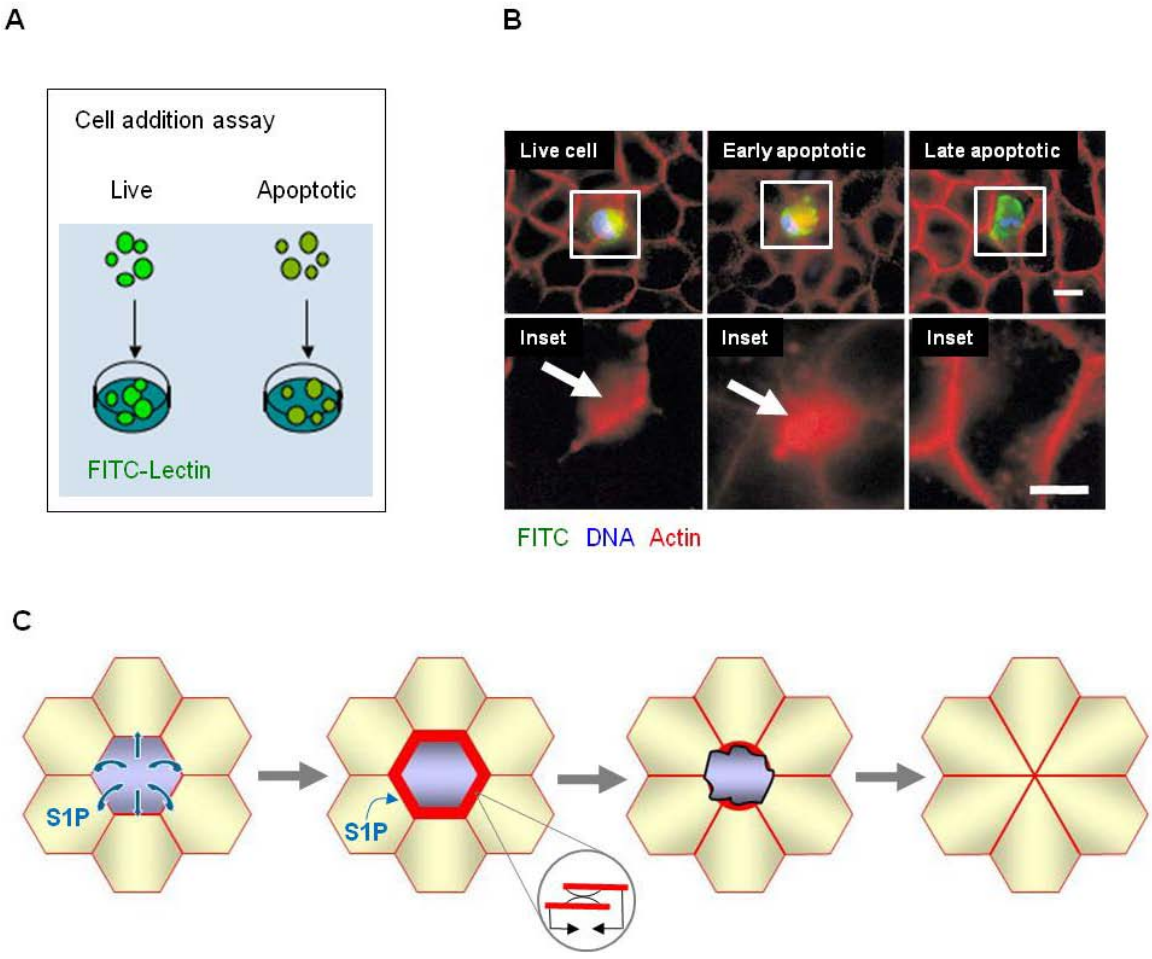

Figure 1.8 The noncell autonomous model for apoptotic cellular extrusion.
In a cell addition assay, live or apoptotic cells are added to cell epithelial monolayers (a). When live cells are added, little or none actin cables (white arrow) are formed in the monolayer where the cell added is contacting. When apoptotic cells in an early stage of apoptosis are added, a very intense spot of actin cables (white arrow) are formed in the monolayer where the apoptotic cell is contacting. No actin cables are formed when apoptotic cells in a late stage of apoptosis are added to the monolayer (b). The noncell autonomous model states that at an early stage of apoptosis, the dying cell will send a signal (sphingosine-1-phosphate [S1P]) to the neighbor cells to form a ring of actomyosin. Probably like a muscle sarcomere (inset), this ring contracts, removing the apoptotic cell out of the epithelium and bringing the neighbor cells to fill the gap the apoptotic cell would leave (c). Adapted from [40].

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CHAPTER 2

APOPTOTIC REGULATION OF CELLULAR EXTRUSION

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Apoptotic regulation of epithelial cellular extrusion

Daniel Andrade · Jody Rosenblatt

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.

Keywords Extrusion · Apoptosis · Contraction ring · Actomyosin dynamics · Caspases

Introduction

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

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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,

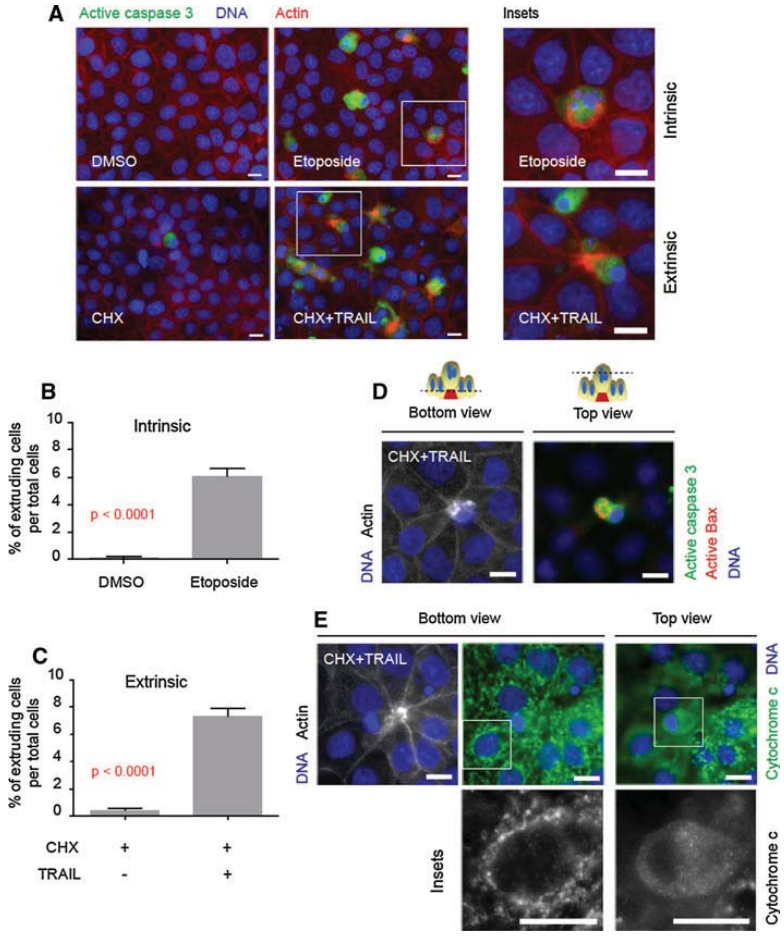

Fig. 1 Both intrinsic and extrinsic apoptotic stimuli elicit cellular extrusion in epithelial monolayers**.** MDCK monolayers treated with DMSO, etoposide, cycloheximide (CHX) or TRAIL plus CHX were fixed and stained for DNA, actin and active caspase-3 (**a**). *Insets* show a magnified view of the contraction 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

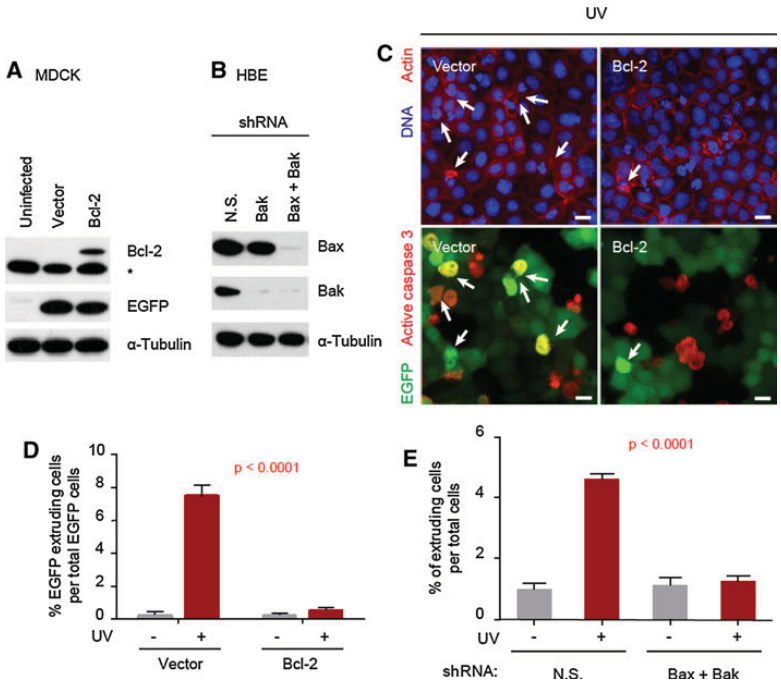

Fig. 2 Over-expression of Bcl-2 or knockdown of Bax and Bak blocks cell extrusion induced by intrinsic apoptosis stimuli. Immunoblot analysis of Bcl-2 in MDCK (**a**) or Bax and Bak in HBE (**b**) cells transduced with the indicated constructs. In (**a**), *asterisk* denotes non-specific immunogen; and 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

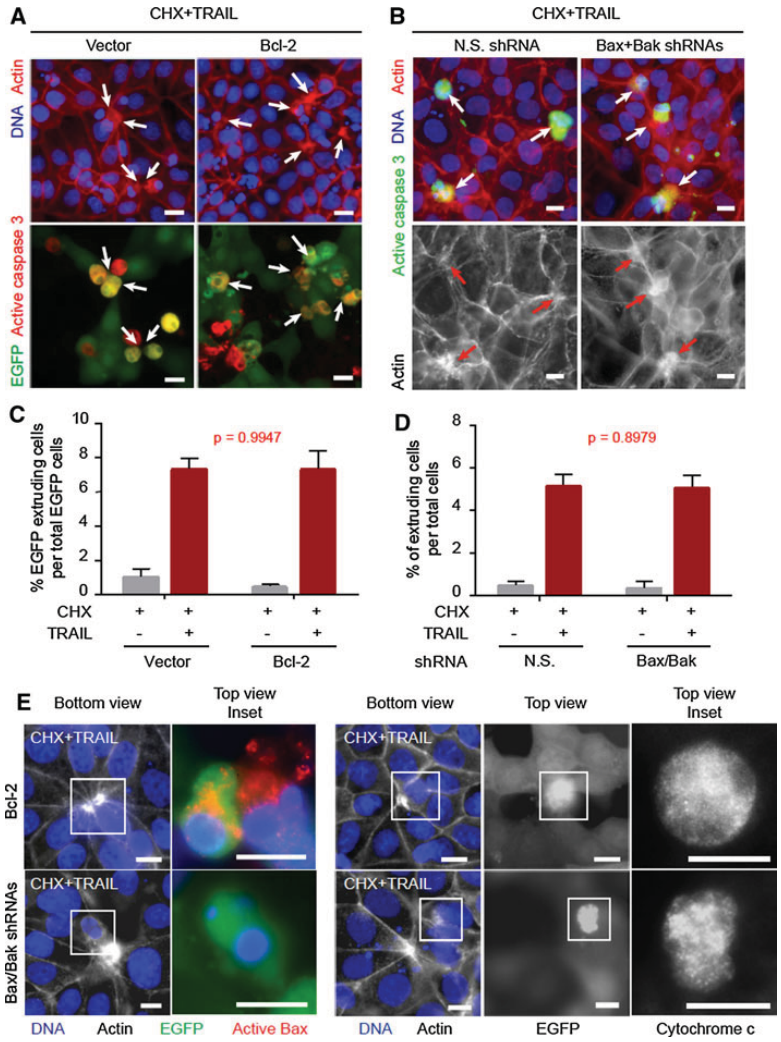

Fig. 3 Neither over-expression of Bcl-2 nor Bax/Bak knockdown blocks cell extrusion induced by extrinsic apoptosis stimuli. Extrusion in MDCK and HBE monolayers transduced with Bcl-2 or Bax/Bak shRNAs were treated with TRAIL and CHX (**a**, **b** respectively). *Arrows* in (**a**) and (**b**) show apoptotic 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 immunostained 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 immunostaining, 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],

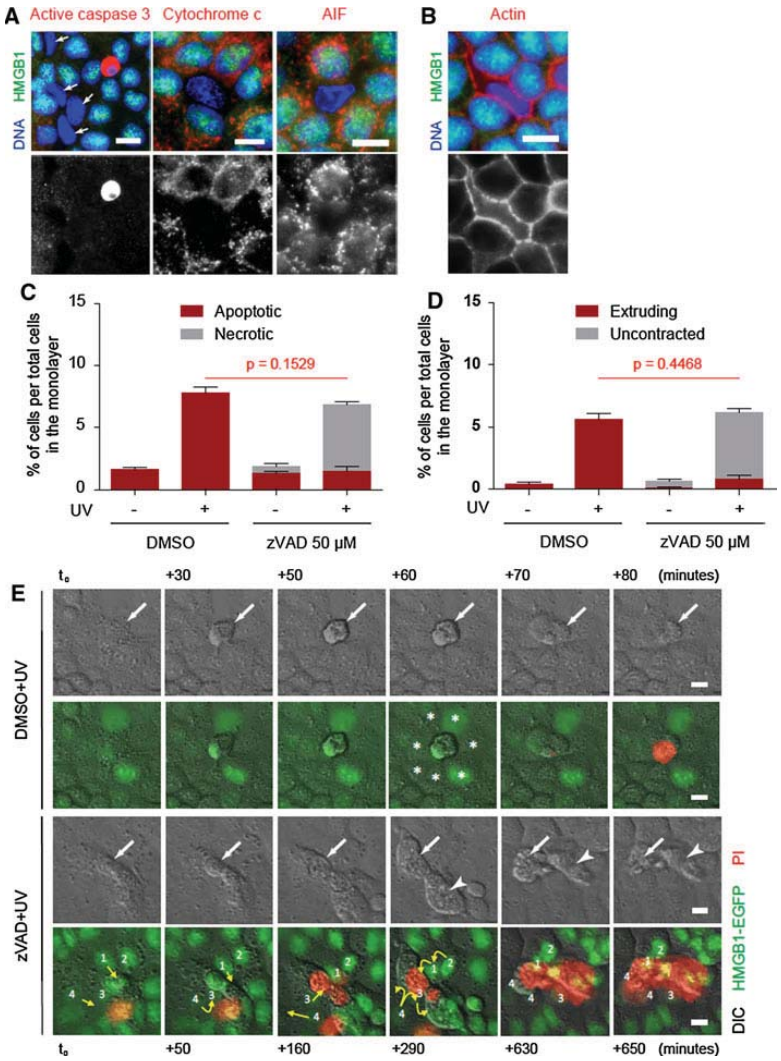

Fig. 4 Epithelial cellular extrusion requires caspase activity for proper formation and contraction of the actomyosin ring. MDCK monolayers incubated with 50 µM zVAD-fmk (caspase inhibitor) and irradiated with UV-C were immuno-stained for DNA and HMGB1 as well as active caspase-3, cytochrome c, AIF or 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

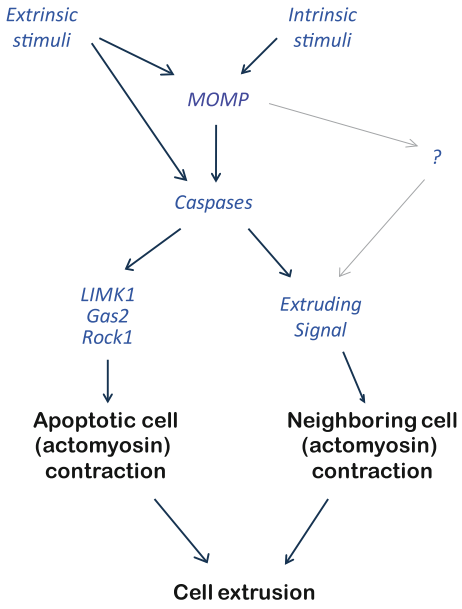

Fig. 5 A model for apoptotic regulation of cellular extrusion. This model illustrates pathways that can link apoptosis with extrusion. Here, caspases may orchestrate the removal of the apoptotic cells by activating several signals. Question mark suggests that other non-apoptotic signals may also contribute 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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CHAPTER 3

BCL-2 REGULATION OF CELLULAR EXTRUSION

3.1 Abstract

In Chapter 2, I found that Bcl-2 overexpression inhibits both apoptosis and extrusion when apoptosis is induced by intrinsic apoptotic stimuli. Accordingly, I find here that inhibition of Bcl-2 with a BH3-mimetic drug is sufficient to induce apoptotic cell extrusion. I have also examined the effects of Bcl-2 on cell junctions to rule out non-specific reasons that Bcl-2 may block cell extrusion and also find that it is required at mitochondria. Finally, I have helped develop a transgenic heat-shock inducible Bcl-2 zebrafish line to test if Bcl-2 blocks extrusion *in vivo*.

3.2 Introduction

Although pro-apoptotic and anti-apoptotic proteins have a known role in regulating apoptosis, their role is not restricted to cell death. Bcl-2 family members have also been found to play roles in other cellular processes, such as cell cycle progression, DNA repair, autophagy and glucose homeostasis [1-6]. Caspases are catalytically involved in activation and proliferation of T and B lymphocytes, cell differentiation, cell motility, cell shaping [7-9], and possibly in cell survival [10-11].

In particular, the anti-apoptotic protein Bcl-2 binds a multitude of proteins that regulate a variety of different biological processes. In the same way that Bcl-2 binds the

pro-apoptotic Bax to prevent mitochondrial outer membrane permeabilization (MOMP) and block apoptosis, Bcl-2 can also bind and inhibit Beclin 1 on the endoplasmic reticulum (ER) to prevent autophagy [5].

I found in Chapter 2 that Bcl-2 overexpression inhibits apoptotic cellular extrusion induced by an apoptotic intrinsic stimulus in cell culture. To determine if Bcl-2 inhibition is sufficient to induce cellular extrusion, I examined if the Bcl-2 inhibitor, BH3-mimetic, HA 14-1, can induce apoptotic cellular extrusion. We also investigated if the ability of Bcl-2 to inhibit cellular extrusion is only a result of loss of cell junctions by Bcl-2. By targeting Bcl-2 overexpression to the mitochondria or the ER, we find that Bcl-2 inhibition of extrusion occurs at mitochondria. In collaboration with Jean Marie Delalande in the lab, I also made a heat-shock inducible Bcl-2 transgenic zebrafish, which can be used to determine if Bcl-2 blocks extrusion *in vivo* as it does in cell culture cells.

3.3 Material and methods

3.3.1 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, 2mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin (all from Invitrogen) at 5% CO_2, 37°C.

3.3.2 Induction of apoptosis

To induce apoptosis, MDCK monolayers were treated with 120 mJ/cm^2 short-wave (UV-C) light, using a Spectrolinker (Spectroline) and incubated for 2 hours after

irradiation or by treating with 15 μM HA 14-1 for 30 min. To induce apoptosis in zebrafish *Danio rerio*, we incubated four-day old zebrafish larvae with 450 μg/ml G418 (Invitrogen) in fish water (60 mg Instant Ocean mix per liter of distilled H_2O) for 2 hours.

3.3.3 Cell staining

Cells were fixed with 4% formaldehyde in PBS for 20 min, permeabilized for 5 min with 0.5% Triton in PBS, rinsed thrice 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 hour: 1:200 rabbit monoclonal anti active caspase-3 (BD pharmigen), 1:100 rabbit monoclonal anti E-cadheren (Cell siganling), 1:100 mouse monoclonal anti β-catenin (BD pharmigen), 1:400 rabbit polyclonal anti ZO-1 (Zymed), 1:100 rabbit polyclonal anti cytochrome c (Santa Cruz), 1:100 mouse monoclonal anti-Bcl-2 (Abcam) and 1:100 rabbit monoclonal anti-calnexin (Abcam). After washing coverslips 3 times in 0.1% Triton X-100 in PBS, coverslips were incubated in secondary antibodies (all diluted 1:100 in AbDil): Alexa Fluor® 488 goat anti-rabbit, Alexa Fluor® 568 goat anti-rabbit, Alexa Fluor® 568 goat anti-mouse 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).

3.3.4 Microscopy

Fluorescence micrographs of MDCK monolayers 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. Confocal micrographs were obtained using a TCS SP5 microscope (Leica). Zebrafish images were obtained using an Olympus SZX12 microscope and an Olympus S97809 camera as well as a Nikon 90i microscope with a Retiga 2000R charge-coupled device camera (Q Imaging). All images were processed using Metamorph (GE Healthcare), Photoshop (Adobe) and Illustrator (Adobe) software.

3.3.5 Immunoblot analysis

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

3.3.6 DNA constructs

Human Bcl-2 alpha isoform ORF (Open Biosystems) was PCR cloned and either inserted into the vector pEGFP-C1 (Clontech) to obtained the pEGFP-Bcl2 or recombined into the donor vector pDONR221 (Invitrogen) following company's instructions. Similarly, the Bcl-2 mutants: Bcl2-ActA and Bcl2-cb5 (Gift from Dr. David Andrews [12]) were PCR cloned and recombined with into pDONR221. The obtained entry vectors, pDONR-Bcl2-221, pDONR-Bcl2-ActA-221 and pDONR-Bcl2-cb5-221 were then recombined with the retroviral vector pMIG [13] modified to function as a

destination vector (Gift from Dr. Alana Welm [14]). The expression vectors pMIG-Bcl2, pMIG-Bcl2-ActA, pMIG-Bcl2-cb5 and the empty vector pMIG were used to produce retroviral particles.

3.3.7 Fish transgenesis

The p5E-hsp70 vector containing a 1.5-kb *hsp70* promoter for heat-shock induction (5' entry clone), the p3E-2A-EGFPCAAXpA vector containing the PTV1-2A peptide and EGFPCAAX (prenylated EGFP) plus SV40 late poly A (3' entry clone) and the destination vector pDestTol2CG2 containing the *cmlc2*:EGFP transgenesis marker were kindly provided by Dr. Chi-Bin Chien [15]. Zebrafish *Danio rerio* Bcl-2 (zBcl-2, Open Biosystems) was PCR cloned and recombined into pDONR221 (Invitrogen) to create the middle entry clone pzBcl2-221. Recombination of entry clone vectors and the destination vectors were recombined, as previously indicated [15], to produce the transgenic Tol2 vector pHsp70-zBcl2-2A-EGFPCAAX-CG2. The transgenic Tol2 vector along with Tol2 transposase mRNA was microinjected into one-cell embryos as indicated [15]. Zebrafish larvae at 2 day post-fertilization were screened for transgenic green hearts using an Olympus SZX12 microscope.

3.3.8 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).

3.4 Results

3.4.1 Bcl-2 inhibition by HA 14-1 induces cellular extrusion

I previously found that overexpression of Bcl-2 blocked cell extrusion and death when cells were induced to die with intrinsic apoptotic stimuli. To test if inhibition of Bcl-2 is sufficient to induce extrusion, we treated MDCK monolayers with the BH3 mimetic, HA 14-1, which binds and inhibits Bcl-2 to induce apoptosis [16]. After treatment with HA 14-1, I scored the number of apoptotic extruding cells in MDCK monolayers by staining for DNA, actin and active caspase 3 (Fig. 3.1). HA 14-1 increased the percentage of cells undergoing apoptotic cellular extrusion compared to a DMSO-treated control (Fig. 3.1). These results suggest that blocking Bcl-2 is sufficient to induce extrusion and that extrusion is most likely activated downstream of Bcl-2 inhibition.

3.4.2 The effect of Bcl-2 overexpression on cell junctions

Our data shows that overexpression of Bcl-2 has the ability to block both apoptosis and cellular extrusion, whereas inhibition of Bcl-2 with HA 14-1 induces apoptotic cell extrusion, suggesting that apoptosis and extrusion are activated downstream Bcl-2 inhibition. However, a report that Bcl-2 can disrupt β-catenin, E-cadherin and ZO-1 localization [17] suggests that the ability of Bcl-2 to block extrusion is only due to indirect effects of disrupting epithelial junctions that are required for extrusion. We immunostained MDCK monolayers over-expressing Bcl-2 for β-catenin, E-cadherin, and ZO-1 to examine this possibility (Fig. 3.2a). I found no difference in the localization of these junctional proteins in cells over-expressing Bcl-2 in MDCK monolayers compared to their internal control cells (untransfected cells in the monolayers) (Fig. 3.2a).

Moreover, Bcl-2 expressing cells actively participate in the formation of the actomyosin ring during extrusion of apoptotic cells (Fig. 3.2b), suggesting that their junctions are functionally intact. These results suggest that Bcl-2 does not block extrusion by soley disrupting cell-cell junctions, but rather directly regulates extrusion through other signals.

3.4.3 The effect of targeted expression of Bcl-2 on cellular extrusion

Bcl-2 localizes to the mitochondria, ER and the outer nuclear membrane [18-20]. Localization of Bcl-2 at these different organelles is due to its transmembrane domain. Removal of this domain disrupts Bcl-2 localization at these organelles and becomes soluble in the cytoplasm [12]. Exchange of this transmembrane domain for that of bacterial ActA or cytochrome b5 (cb5) targets Bcl-2 to the mitochondria or ER, respectively [12]. These Bcl-2 mutants, named Bcl2-ActA and Bcl2-cb5, were used to determine that Bcl-2 regulates autophagy only at the ER [5].

To test where Bcl-2 acts to regulate extrusion, we targeted Bcl-2 over-expression to the mitochondria or the ER using the above Bcl-2 mutants (Fig. 3.3 and 3.4). Bcl2-ActA, Bcl2-cb5, and WT Bcl-2 were cloned into a bicistronic system with EGFP where the internal ribosome entry site (IRES) allows separate expression of our gene product and the reporter EGFP. Immunoblot analysis shows that MDCK cells transduced with retroviral vectors containing these constructs express similar levels of over-expressed Bcl-2 (Fig. 3.3a). To confirm that Bcl-2 had targeted to correct subcellular sites, I tested if Bcl-2 colocalized with markers for mitochondria (cytochrome c) or ER, (calnexin) (Fig 3.3b). While WT Bcl-2 localizes to both mitochondria and ER, the Bcl-2 mutants localized to their respective targeted compartments (Fig. 3.3b). As previously established, WT Bcl-2 blocks apoptotic cellular extrusion (Fig. 3.4). While ER-targeted Bcl-2 fails to

block extrusion, mitochondria-targeted Bcl-2 inhibits cellular extrusion at the same rate as the WT Bcl-2 (Fig. 3.4b). This result suggests that Bcl-2 inhibits cellular extrusion at the mitochondria, supporting the hypothesis that the connection between apoptosis and extrusion occurs at or downstream of MOMP.

3.4.4 Bcl-2 regulation of cellular extrusion in zebra fish *Danio rerio*

In MDCK monolayers, Bcl-2 overexpression blocks apoptotic cellular extrusion, but it is not clear if this same regulation occurs *in vivo*. To test the role of Bcl-2 in zebrafish, we developed a heat-shock-inducible Bcl-2 transgenic zebrafish using the Tol2 transposon system, Tol2kit [15]. We used the inducible heat shock promoter, *Hsp70* promoter to drive Bcl-2 overexpression, as constitutive over-expression could interfere with the development of the fish (Fig. 3.5a). To monitor Bcl-2 expression after heat shock, we made a bicistronic reporter construct containing the coding sequence for zebrafish Bcl-2 (zBcl-2) and a membrane localized EGFP (EGFP-CAAX), separated by a 2A sequence (Fig. 3.5a). The 2A peptide mediates co-translational cleavage of proteins in 2A multicistronic systems, allowing equimolar protein expression (Fig. 3.5a). Since our construct has an inducible and not a constitutive fluorescent reporter, screening of transgenic fish through fluorescent microscopy would be impossible. Therefore we recombined the inducible construct into a Tol2 destination vector that carries an EGFP reporter driven by the *cmlc2* heart-specific promoter (Fig. 3.5a). The final recombination product along with Tol2 transposase mRNA was microinjected into one-cell stage embryos of *Danio rerio* zebrafish. Transgenic fish with green hearts (Fig. 3.5c, left top panel) were screened using a fluorescent microscope. To reduce abnormalities caused by the transgenesis, screened fish were outcrossed to WT fish. The F1 generation zebrafish

carrying green fluorescent hearts were then back-crossed and screen for offspring with a maximal heat shock response. A fraction of each green heart F2 population was subjected to heat shock and its zBcl-2 protein levels were analyzed by immunoblot using an anti-2A peptide antibody or an anti EGFP antibody (Fig. 3.5b). Fluorescent microscopy verified localization of the membrane EGFP-CAAX after heat shock (Fig. 3.5, right panel). Only heat shocked zBcl-2 expressing F2 fish treated with G418 to induce apoptosis did not induce apoptosis, whereas the untreated siblings had numerous cells undergoing apoptosis, as judged by active caspase-3 immunostaining (Fig. 3.6a). We are now poised to test if the Bcl-2 over-expressing fish where cell death is blocked undergo extrusion or not.

3.5 Discussion

By binding a variety of proteins, Bcl-2 regulates several cellular mechanisms such as apoptosis and autophagy. We have found that Bcl-2 also regulates cellular extrusion induced by intrinsic stimuli. Here, we show that inhibiting Bcl-2 with HA 14-1 is sufficient to induce apoptotic cellular extrusion. By evaluating the localization of junctional proteins in Bcl-2 over-expressing cells, we show that inhibition of cellular extrusion by Bcl-2 is not due to an indirect effect of merely disrupting cell junctions. We also found that Bcl-2 regulates cellular extrusion at the mitochondria, not the ER, supporting our hypothesis that the connection between apoptosis and extrusion occurs downstream mitochondria permeabilization.

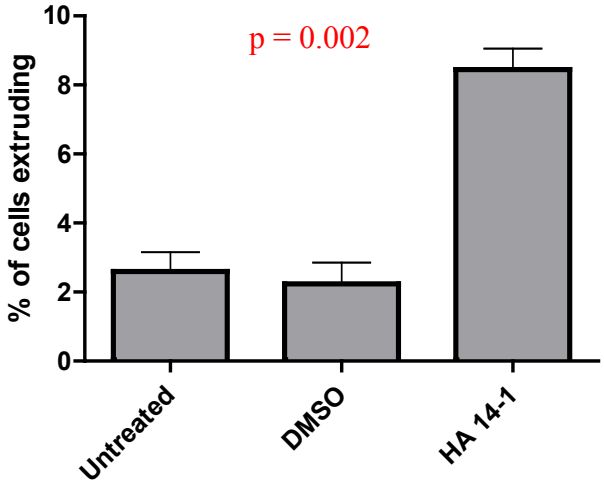

Figure 3.1 Inhibition of Bcl-2 by HA 14-1 induces apoptotic cell extrusion.
MDCK monolayers untreated, mock-treated or treated with HA 14-1 were stained for DNA, actin and active caspase 3. Then, the number of apoptotic cells were counted and analyzed from three independent experiments where the p value < 0.002.

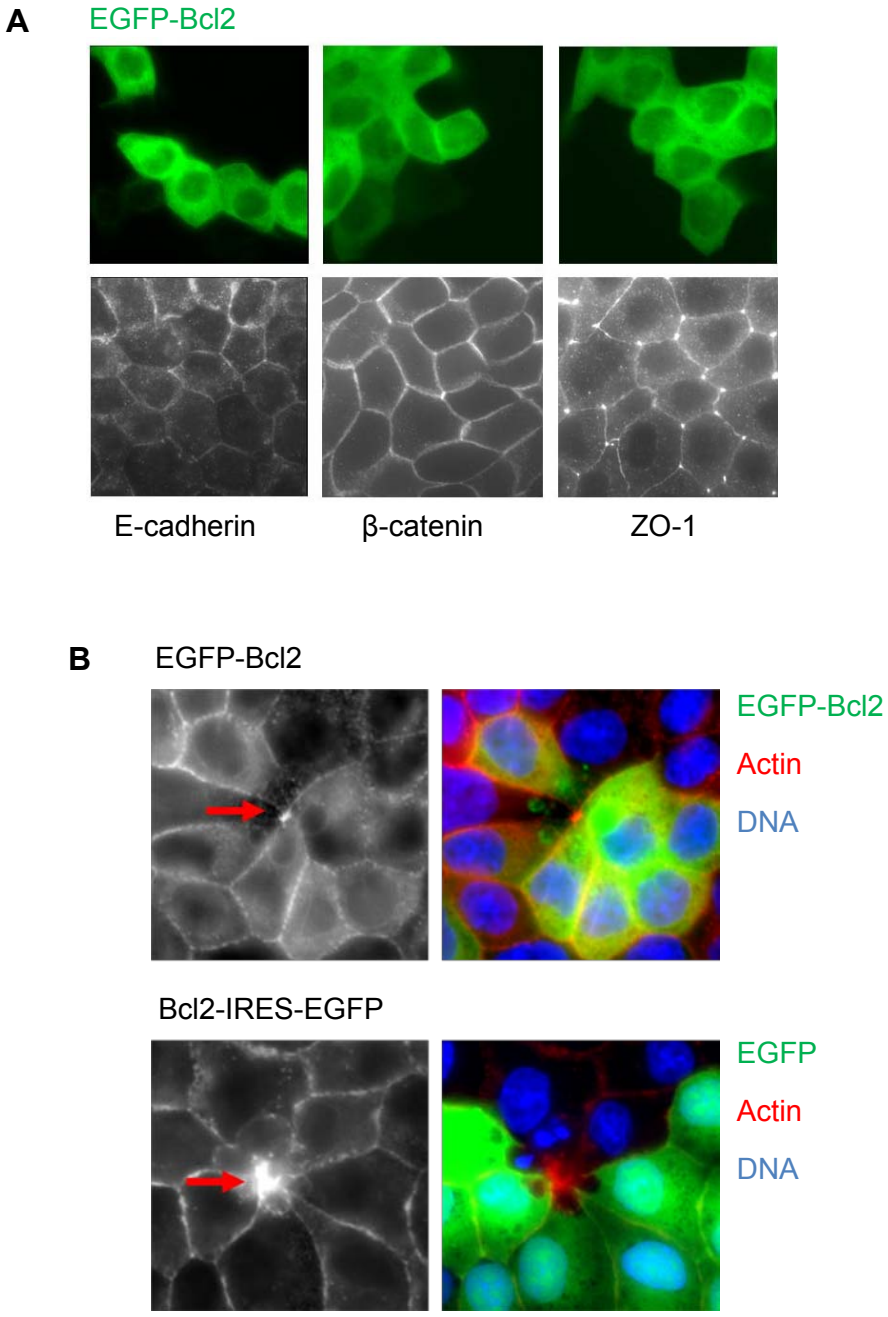

Figure 3.2 Bcl-2 over-expression does not disrupt cell junctions.
To evaluate localization of junctional proteins in Bcl-2 overexpressing cells, MDCK monolayers with cells over-expressing EGFP-Bcl2 were immunostained for E-cadherin, β-catenin and ZO-1 (a). When the neighboring cells overexpress Bcl-2, they can still participate in extrusion of dying cells, suggesting that the junctions are also functionally intact. Red arrows show closed actin extruding rings.

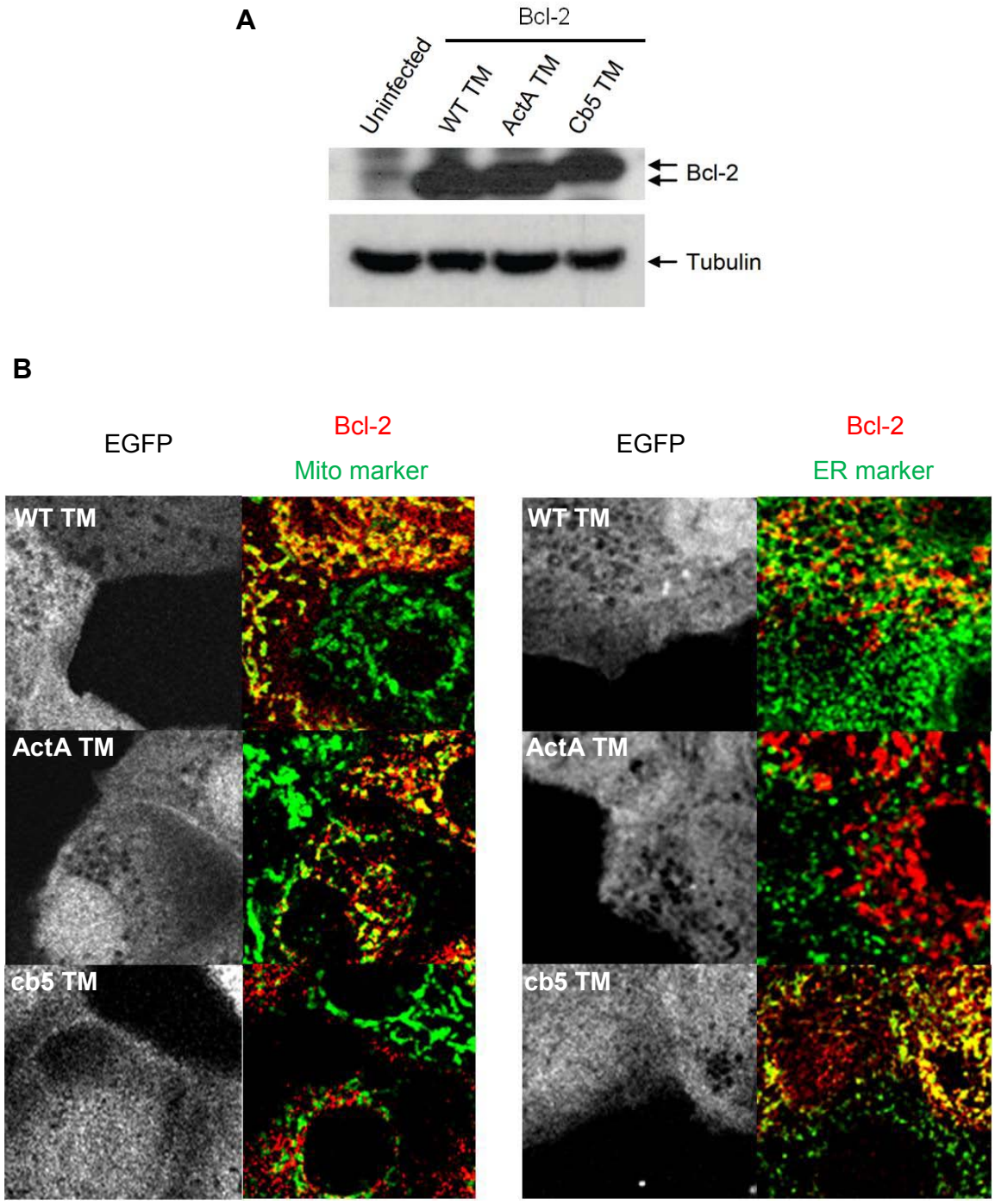

Figure 3.3 Expression levels and localization of targeted Bcl-2 mutants.
Immunoblots from MDCK cells transduced with the respective forms of Bcl-2 show that the expression levels of WT and Bcl-2 mutants are similar (a). In (b), confocal microscopy of cells in (a) shows localization of the respective forms of Bcl-2. Note that in (a) and (b) Bcl-2 antibody specifically recognizes only the transduced forms of Bcl-2 (human) and not the endogenous one (canine). ActA TM targets Bcl-2 to mitochondria and cb5 TM to ER. TM stands for transmembrane domain.

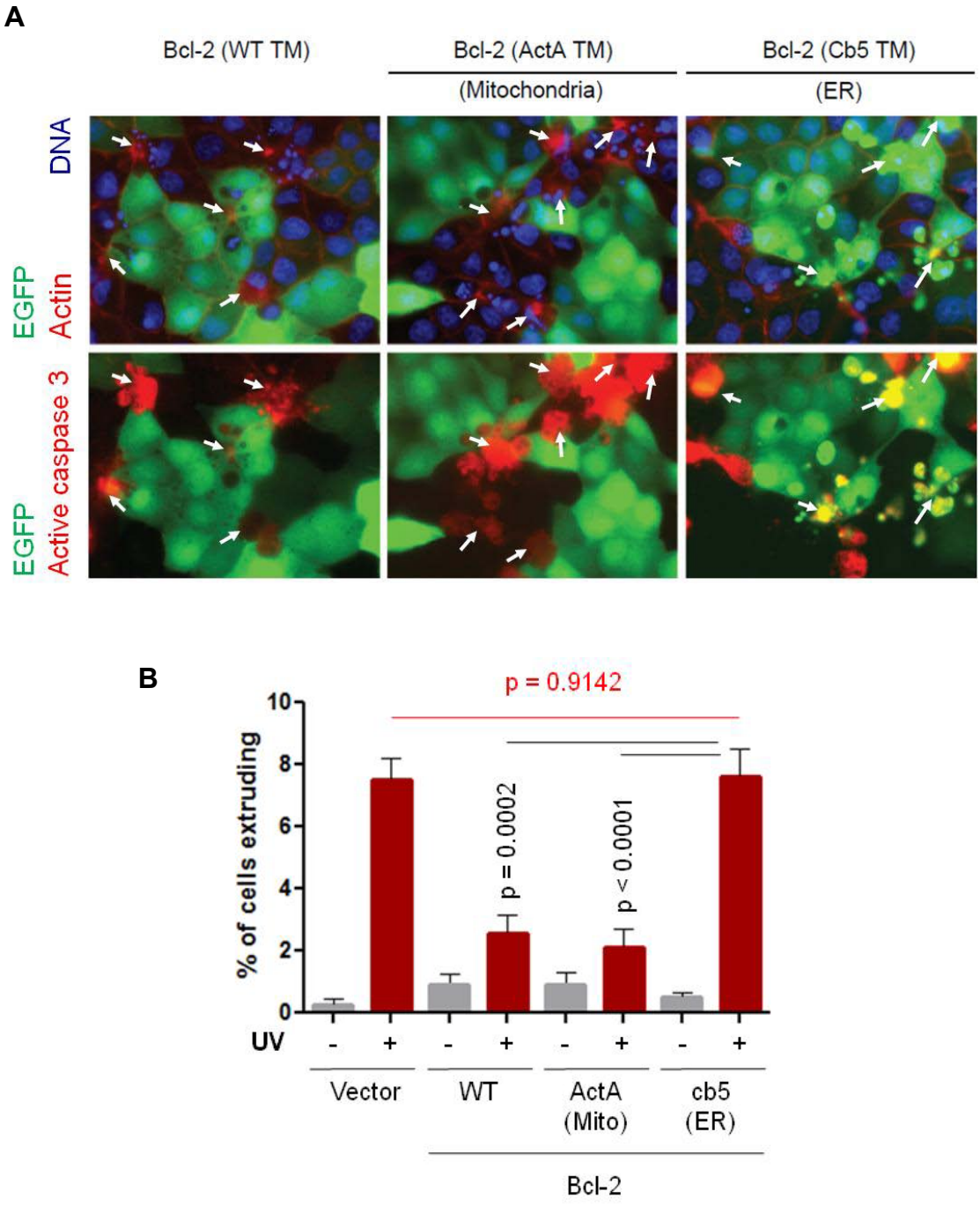

Figure 3.4 Bcl-2 blocks apoptotic cellular extrusion at the mitochondria but not at the ER. MDCK cell monolayers expressing different forms of Bcl-2 were UV-irradiated and stained for DNA, actin and active caspase 3. Arrows show actin rings and active caspase 3 staining of apoptotic extruding cells in upper and lower panels respectively (a). Note that while EGFP positive cells (Bcl-2 expressing cells) in WT and ActA are resistant to apoptotic cell extrusion, those in cb5 are not (a and b). Also, note that EGFP negative cells (non-expressing Bcl-2 cells) in all of the monolayers are not resistant to UV-induced apoptotic cell extrusion (a).

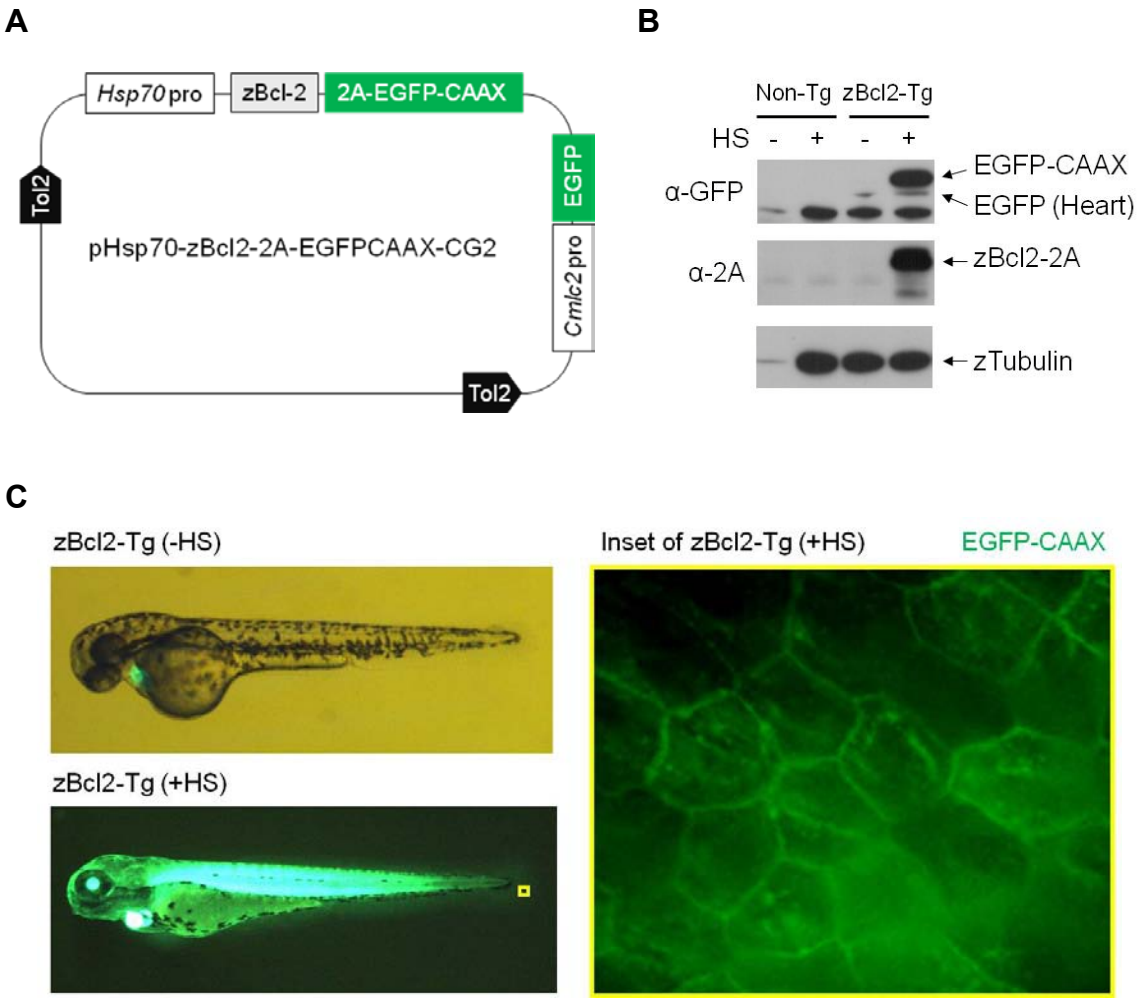

Figure 3.5 Inducible zBcl-2 transgenic zebrafish.
The pHsp70-zBcl2-2A-EGFPCAAX-CG2 vector (a) along with Tol2 transposase mRNA were microinjected into one-cell embryos. Fish larvae with green fluorescent heart were screened by fluorescent microscopy (c, left top panel). F2 transgenic and nontransgenic fish were heat-shocked and analyzed by immunoblotting to evaluate expression of zBcl2-2A and its EGFP-CAAX reporter (b). Transgenic heat-shocked fish were also analyzed by fluorescent microscopy to evaluate heat-shock response (c, left panels) and EGFP-CAAX localization (c, right panel).

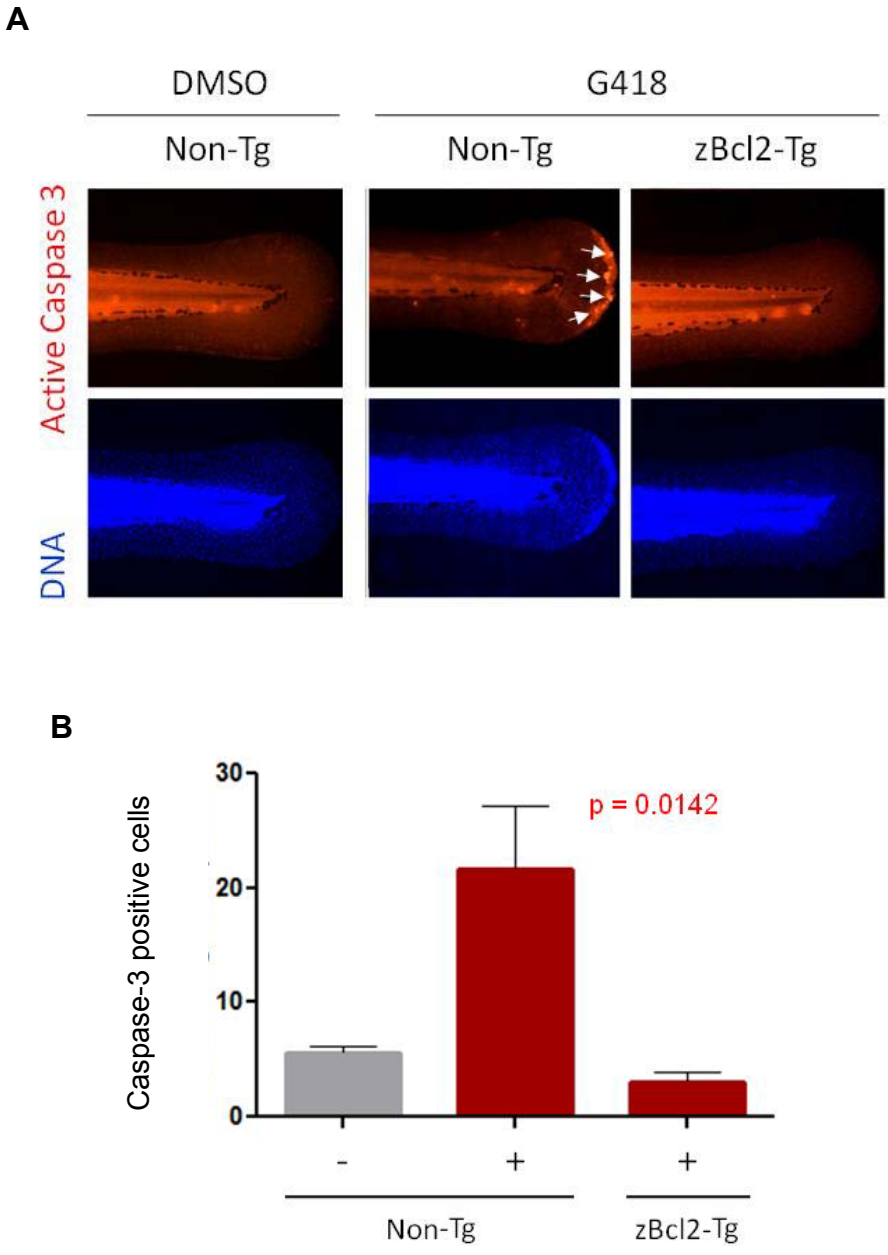

Figure 3.6 Bcl-2 inhibits apoptotic cell extrusion *in vivo*.
F2 zBcl-2 transgenic and nontransgenic fish were incubated in G418 or DMSO and stained for DNA and active caspase 3 (a). Arrows in (a) show cells undergoing apoptosis. The number of apoptotic cells in the fin was counted from six fish for each treatment (b).

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CHAPTER 4

CONCLUSIONS

During development and throughout adulthood, cellular extrusion plays crucial physiological roles: ensuring the maintenance of the epithelial protective barrier during limb and trachea development and homeostasis, driving dorsal closure during *Drosophila* embryogenesis and maintaining proper compartment for the photoreceptor in the retina of the eye [1-6]. Several studies show that cellular extrusion is linked to apoptosis *in vivo* and *in vitro*. Moreover, in all observed epithelia, induction of apoptosis triggers cellular extrusion. However, little was it done to understand this regulation.

Two models that seem to contradict each other were proposed to explain how extrusion occurs during apoptosis. While the cell autonomous model suggests that the dying contracting cell drags its neighbors to fill in the gap [7, 8], the non-cell autonomous model proposes that the dying cell signals its surrounding cells to form and contract an actomyosin ring, which drive its removal from the monolayer [3]. These models constitute an important step for understanding how apoptosis initiates the extrusion response; however, several questions still remain. Which apoptotic signals are important for activating extrusion? Is extrusion activated in response to both intrinsic and extrinsic apoptotic stimuli? What component(s) of the apoptotic machinery triggers the extrusion

response? Is apoptosis required to initiate extrusion or can extrusion occur independently of apoptosis? Can other forms of cell death such necrosis activate cell extrusion?

In this work, I found that several intrinsic (UV irradiation, etoposide, camptothecin and serum deprivation) and extrinsic (Fas-L and TRAIL cytokines) apoptotic stimuli trigger cellular extrusion in two different cell lines (MDCK and HBE), suggesting a universal connection between apoptosis and extrusion (Fig. 4.1). Also, by investigating different steps in the apoptosis pathway, I found that caspase activation is required for extrusion to take place. This finding explains the universal response of extrusion to different apoptotic stimulus as all apoptotic pathways funnel into activation of caspases (Fig. 4.1). It also shows a new role for caspases, the regulation of cellular extrusion. Caspases appear as an important checkpoint for cell removal, as only cells crossing the point of no return in the apoptosis pathway (caspase activation) will be removed from the epithelium, giving cells a second chance to fix any potential threat (DNA damage, ER stress, etc). By identifying the component of the apoptosis machinery that initiates the extrusion response, my work finally establishes the connection between apoptosis and extrusion: activation of caspase. Also, because actomyosin contractions triggered by caspases in the dying cell, as previously suggested, may be essential for extrusion [7], and because caspase activity is required for proper formation and contraction of the actomyosin ring, my work reconciles two models that for long seemed to be mutually exclusive (Fig. 4.1).

I also found that necrotic cells resulting from caspase inhibition do not extrude. They remain in the epithelium and become permeable, potentially compromising the epithelial protective barrier. Nevertheless, necrotic cells are still cleared from epithelia by a passive

mechanism that involves random movements of cells in the epithelium (Fig. 4.2). Although cells in epithelial monolayers are connected to each other with no room left around, they do not remain static. Cells die and drag neighbor cells to fill that gap, they also push their neighbors away when they grow and divide. As a result, cells in epithelial monolayers constantly move in different directions resembling people in a rock concert. These stochastic movements of epithelial cells exert forces on the necrotic cell until it burst. Simultaneously, the adjacent cells exerting pressure take over its place, sealing the gap the necrotic cell has left (Fig. 4.2). This mechanism is different from extrusion as it is not executed by contraction of an actomyosin ring. Instead, the removal of necrotic cells relies on the stochastic movement of the epithelial cells and, unlike extrusion, no rosette arrangement of the neighbor cells take place (Fig. 4.2). Because of that, the time for removal of necrotic cells from monolayers is very random and varies dramatically when compared to the time of removal of apoptotic cells by extrusion. The requirement of caspase activity for extrusion suggests that other forms of cell death different from apoptosis might lack the ability to trigger cellular extrusion. Instead, cells dying through these mechanisms might be removed similarly to necrotic cells in our studies.

Although necrotic cells resulting from caspase inhibition during apoptosis fail to extrude, other studies find that necrosis of single cells due to laser ablation heal by a purse-string mechanism that mimics extrusion [10, 11]. Although this mechanism might be different from extrusion, it is possible that necrosis induced by laser irradiation activates a caspase-independent pathway that leads to extrusion. The existence of a caspase-independent pathway might explain extrusion occurring in some live cells or where caspase activity is inhibited [3, 12-14]. In fact, in our work, when caspases are

inhibited during apoptotic cellular extrusion, a non-continuous actin ring is still formed in the interface between the necrotic cell and its neighbors, supporting this possibility. During necrosis caused by caspase inhibition, the caspase-independent pathway might be only partially activated and is not sufficient to complete extrusion. However, during extrusion of live cells or necrosis induced by laser ablation, activation of this pathway could be sufficient to drive cellular extrusion. Nevertheless, future studies need to be performed to explore this hypothesis. EGFP-actin expressing cells along with untransfected cells in a mosaic arrangement should be use to determine whether the non-continuous actin accumulation around the uncontracted cells, resembling a contraction ring, is formed in the neighbor cells. That is, an untransfected cell undergoing necrosis surrounded by EGFP-actin expressing cell would help to determine whether the necrotic cell is inducing the formation of this actin ring-like structure. Staining for myosin II, another component of the extruding ring [3] would further support this hypothesis. To develop the caspase-independent pathway activated during extrusion of live cells or laser-irradiation-induced necrosis, identification of actomyosin regulators should be performed. This will allow sequential identification of their activators and so on.

In this dissertation, I show that caspase activity drives the removal of apoptotic cells by triggering both the formation and contraction of an actomyosin ring in the neighbor cells and the actomyosin contractions occurring in the dying cell. However, the importance of caspase activity during other aspects of extrusion, the release of the apoptotic cell from the epithelium, has been explored by others. During extrusion, while the dying cell is been squeezed to be removed out of the monolayer, cell junctions between the dying cell and its neighbor cells as well as that between the dying cell and

the extracellular matrix need to be disrupted in an organized and coordinated manner in such a way that the epithelial protective barrier is maintained. Using *Drosophila* as a model, Kessler and Müller [15, 16] demonstrated that caspases trigger a progressive and orchestrated disassembly of adherens junctions. During early stages of apoptosis, caspases cleave β-catenin, promoting partial removal of E-cadherin from the membrane. Later, all adherens junction components are removed from the membrane. At the same time, while the apoptotic cell is being released, the neighboring cells form new 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 [7]. In this way, my work complements other's findings and put together a major scope of the regulation of apoptotic cellular extrusion by caspases, the release of the dying cell and the contraction forces that drive removal of the cell from the monolayer.

At the completion of this dissertation, we have established the connection between apoptosis and extrusion, caspase activity. We have also found a passive mechanism by which dead cells that fail to activate extrusion are cleared from epithelia. However, although our work brings new insight into the molecular basis of apoptotic cellular extrusion, several questions remain. For example, which caspases participate during apoptotic cell extrusion? During apoptosis, three executioner caspases (caspase 3, 6 and 7) target several proteins for proteolysis, leading to the morphological changes associated with apoptosis. Out of this group, caspase 3 is required for DNA degradation and blebbing [17, 18]. This is not unexpected as caspase 3 proteolytically activate critical actomyosin regulators such as Gas2, gelsolin, and Rock1, whose roles have been previously established in actin-myosin dynamics during apoptotic cell blebbing [19, 20].

As initially proposed by Mills et al [7], these contractions are important for extrusion because allow dragging of the neighboring cells by the contracting apoptotic cell, which is one of the component forces during extrusion (Fig 4.1). However, these actomyosin regulators, activated by caspase 3 during apoptosis, could also play important roles in the contraction of the actomyosin ring, the second component force during extrusion (Fig 4.1). As previously showed, inhibition of Rock1 and myosin light chain kinase (MLCK) by the drugs Y-27632 and ML-9 respectively blocks extrusion of the apoptotic cell from the monolayer, supporting the importance of these regulators for the contraction of the extrusion ring [3]. Nevertheless, because Rock1 and MLCK are key regulators of apoptotic cell blebbing [21], it is necessary to understand whether they are required for contractions in the apoptotic cell, the extrusion ring or both. For that, the use of mosaic epithelial monolayers, where an untrasfected cell surrounded by cells expressing myosin or MLCK shRNAs with an EGFP reporter or vice versa, will help to better understand the role of these myosin regulators during extrusion. We believe that these myosin regulators are important for both component forces. In the apoptotic cell, Rock1 and MLCK are directly activated by caspases, inducing the first extrusion component force, contractions in the apoptotic cell. Caspase activation will lead to the formation and translocation of sphingosine-1-phosphate (S1P). Once translocated into the neighbor cells, S1P will induce the activation of Rock1 and MLCK for contraction of the extrusion ring (unpublished data).

Therefore, the crucial role of caspase 3 for the activation of actomyosin regulators during apoptosis makes it an important candidate during apoptotic cellular extrusion, not only for driving the contraction forces in the dying cell, but also for the formation and

translocation of the extruding signal that will trigger the formation and contraction of the actomyosin ring in the neighbor cells. The role of the caspase 3 during extrusion can be further explored by using specific caspase 3 inhibitors such as z-DEVD-fmk, or shRNA oligos to downregulate caspase 3 levels. In addition, an active form of caspase 3, under the regulation of an inducible promoter, could be used to test the sufficiency of caspase 3 activity during cellular extrusion. Using a proteomics approach, cells expressing the active form of caspase 3 along with cells where caspase 3 is downregulated could be used to identify potential candidates targeted by caspase3 during cell extrusion.

Although caspase 3 might be required to trigger the actomyosin contractions leading to removal of the dying cell, other caspases might coordinate the release of the dying cell from its neighbors and the extracellular matrix. As previously mentioned, caspases orchestrate the release of the dying cell during extrusion [7, 15, 16]. Cells lacking caspase 3 do not contract but are still released [7], suggesting that the activity of other caspases is sufficient to coordinate the release of the dying cell during extrusion. It is possible that caspase 3 also participates in the release of the dying cell during extrusion but its role is dispensable or not required.

A better understanding of extrusion during apoptosis and non-apoptotic cell contexts will allow us to explore its role in other biological processes such as wound healing, cell fate during differentiation and its potential role as a tumor suppressor pathway by removing epithelial apoptotic resistant cells.

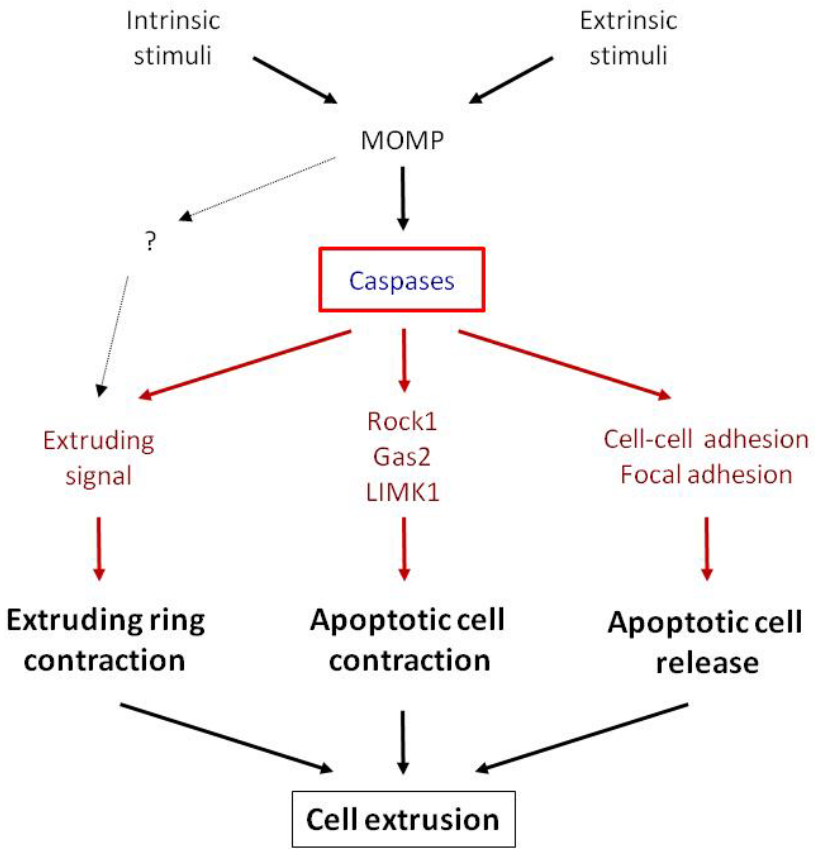

Figure 4.1 Apoptotic regulation of epithelial cellular extrusion.
The proposed model shows the connection between apoptosis and extrusion: caspases. It illustrates the different tasks caspases perform during cellular extrusion: 1) formation and contraction of the extruding ring in the neighboring cells, 2) contractions in the dying cell and 3) remodeling of the cell junctions to release the apoptotic cell while maintaining the epithelial protective barrier. Question mark (?) points a potential alternative pathway to trigger formation and contraction of the extruding ring. MOMP stands for Mitochondrial Outer Membrane Permeabilization.

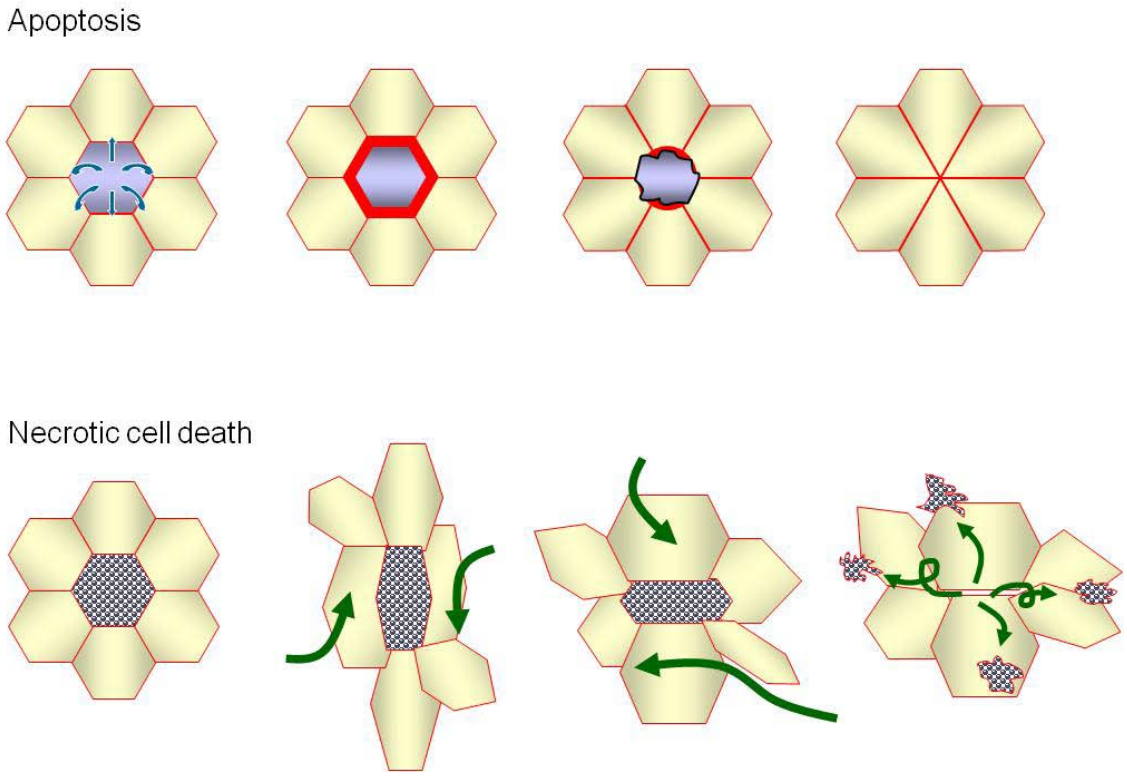

Figure 4.2 Mechanism of epithelial cell removal
Cells dying through apoptosis signal its neighbors to trigger its removal through an active mechanism of cell removal: cellular extrusion. In contrast, cells dying through necrosis do not trigger the extrusion response. Instead, they become permeable, potentially compromising the epithelial protective barrier. Eventually, the constant and random movements of cells in the epithelial exert enough pressure on the necrotic cell for them to burst.

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