Epithelial cell extrusion requires the sphingosine-1-phosphate receptor 2 pathway

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Introduction

Epithelia comprised of one or two cell layers cover and protect the organs that they encase. The cells making up epithelia are constantly turning over by cell division and apoptosis, yet cell death could compromise the barrier function of the epithelium. We previously found that epithelia use a process termed “apoptotic cell extrusion” to remove apoptotic cells from a layer, while preserving their barrier function (Rosenblatt et al., 2001). Specifically, an early apoptotic epithelial cell triggers formation of an actin and myosin ring in the live neighboring cells surrounding it. Contraction of this ring then squeezes the dying cell out of the epithelium. Apoptotic cell extrusion is conserved in all in vivo epithelia we have examined ranging from Drosophila to human.

We previously showed that extrusion depends on a chemical signal from the apoptotic cells, which activates the Rho pathway in the neighboring cells (Rosenblatt et al., 2001; Slattum et al., 2009). Specifically, addition of early apoptotic epithelial cells onto an epithelial monolayer induces actin assembly in the live contacted cells. Furthermore, inhibition of Rho in the cells surrounding an apoptotic cell blocks extrusion (Rosenblatt et al., 2001). We recently determined that Rho activation during extrusion requires p115 RhoGEF (Slattum et al., 2009), a protein activated downstream of the G12/13 G protein–coupled receptor (Holinstat et al., 2003). Thus, a signal on the surface of the dying cell triggers p115 RhoGEF to activate Rho-mediated actin–myosin assembly and contraction in the live surrounding cells to remove the dying cell. However, we did not know the identity of the signal produced in early apoptotic cells that activates apoptotic cell extrusion.

Here, we report that the signal produced by dying cells is the bioactive lipid sphingosine-1-phosphate (S1P), which activates extrusion by binding the S1P2 receptor in the cells neighboring a dying cell, as S1P2 knockout in these cells or its loss in a zebrafish mutant disrupted cell extrusion. Because live cells can also be extruded, we predict that this S1P pathway may also be important for driving delamination of stem cells during differentiation or invasion of cancer cells.

Results and discussion

Blocking S1P signaling inhibits extrusion of apoptotic cells

To characterize the extracellular apoptotic signal that triggers formation of the actin–myosin extruding ring, we used a modified version of our previous “cell addition assay.” In that assay,
addition of early apoptotic cells, but not late apoptotic cells or live cells, to an intact Madin Darby canine kidney (MDCK) epithelial monolayer induced actin assembly in the contacted living cells. Necrotic cells likely use the same signal that apoptotic cells use to produce an extruding ring in epithelia, as laser-ablated or mechanically wounded necrotic cells are extruded identically to apoptotic cells (Tamada et al., 2007). Therefore, we added necrotic cell fragments, prepared by scraping and needle shearing cells and found that they induced accumulation of actin in the contacted monolayer and with the same kinetics (Fig. 1). When added to a cell monolayer, ~60% of added control cell fragments (green) resulted in actin (red) accumulation in the contacted cells (Fig. 1, A and C). Pre-digestion of the dead cell fragments with trypsin did not significantly alter actin accumulation (Fig. 1 C), suggesting that the signal triggering the response is not a protein. We then postulated that the signal is a bioactive lipid.

Based on the fact that a bioactive lipid within this cell fragment should activate Rho-dependent actomyosin assembly and contraction during extrusion, we investigated several candidate lipids and found that addition of S1P caused actin accumulation when added to monolayers, as in our cell addition assay (Fig. S1). Conversely, a total lipid extraction from Escherichia coli that did not contain S1P did not elicit the same reaction. Based on this experiment, the fact that a precursor to S1P, ceramide, is pro-apoptotic (Kolesnick and Hannun, 1999; Hannun and Obeid, 2002), and recent findings that S1P is produced in apoptotic cells (Kolesnick and Hannun, 1999; Hannun and Obeid, 2002; Gude et al., 2008; Weigert et al., 2010), we investigated whether this lipid is required for extrusion. To directly test if S1P is required for the actin reaction in the cell addition assay, we used SKI II, a specific, potent noncompetitive inhibitor of SphK that blocks conversion of sphingosine to S1P (French et al., 2003). Pre-treatment of cells with SKI II before making cell fragments significantly inhibited the formation of actin cables when added to monolayers (Fig. 1, B and C). SKI V, another SphK inhibitor, also reduced the percentage of cell fragments that induce actin accumulations with even stronger efficiency than SKI II (Fig. 1 C and Fig. S2, A and B). Therefore, we conclude that SphK is required in dying cells to trigger actin accumulation in the live cells they contact.

To test if SphK is required for extrusion of apoptotic cells from an MDCK monolayer, we pretreated the monolayer with SKI II and induced apoptosis with short-wave UV light. Extrusion was evaluated by immunostaining the resulting monolayers for active caspase-3 Ab to identify apoptotic cells, Alexa Fluor 568–phalloidin to analyze actin-based extruding rings, and Hoechst for DNA. Fig. 1 D shows a control extruding apoptotic cell, focusing only on the dying cell for the caspase-3 and DNA (which is fragmented) and in another plane on the actin ring, which is around and beneath the dying cell. Treatment with SKI II inhibited formation of the actin extrusion ring and resulted in holes in the monolayer wherever there were apoptotic cells (Fig. 1 E). Note that when a cell extrudes, DNA and caspase-3 do not exist in the same plane as its neighboring cells, whereas all nuclei are in focus when extrusion is blocked. SKI II, SKI V, and d1,3-threo-dihydrosphingosine (tDHS), a competitive inhibitor of SphKs, all dramatically increased the percentage of unextruded apoptotic cells compared with control treatment (Fig. 1 F). Inhibition of apoptotic cell extrusion by SKIs was dose dependent (Fig. S2, C–E). These results show that extrusion of apoptotic cells requires SphK activity, presumably because it catalyzes S1P synthesis.

To regulate many cellular responses, S1P is exported out of cells and binds to its receptors on the same or neighboring cells. To test whether extracellular S1P is necessary for apoptotic cell extrusion, the MDCK monolayer was treated with UV in the presence of an S1P-neutralizing mAb (Visentin et al., 2006; O’Brien et al., 2009). Formation of the extrusion ring was inhibited by the anti-S1P mAb, and consequently the apoptotic cell failed to extrude and was instead retained in the monolayer (Fig. 2, B and C). Additionally, the anti-S1P mAb also blocks actin accumulation induced by cell fragments (Fig. 2, E and F). These results suggest that extracellular S1P is critical for extrusion.

**Extrusion requires signaling through the S1P$_{2}$ receptor**

We next investigated which of the five known high-affinity cell surface S1P receptors (S1P$_{1-5}$) might mediate extrusion signaling. S1P$_{1-3}$ are ubiquitously expressed, whereas S1P$_{2}$ and S1P$_{3}$ are expressed mainly in lymphoid cells and neuronal cells, respectively. By RT-PCR we determined that mRNAs from S1P$_{1,2,3,5}$ are expressed in MDCK cells and another epithelial cell line, human bronchial epithelial (HBE) cells (unpublished data). To investigate the involvement of S1P receptors in extrusion, we treated an HBE monolayer with UV to induce apoptosis in conjunction with several antagonists that are specific for different S1P receptors, and evaluated extrusion of apoptotic cells. Neither FTY720-P (up to 10 µM), a functional antagonist of all S1P receptors except S1P$_{2}$ (Brinkmann et al., 2002; Mandal et al., 2002), nor VPC-23019 (up to 10 µM), an antagonist specific for S1P$_{2}$ and S1P$_{3}$ (Davis et al., 2005), notably affected extrusion of apoptotic cells (Fig. 3 A). In contrast, an S1P$_{2}$-specific antagonist, JTE-013 (Osada et al., 2002), increased the proportion of unextruded apoptotic cells by approximately threefold (Fig. 3 A). We obtained similar results with these S1P inhibitors in MDCK monolayers (not depicted). These results suggested that S1P signaling through S1P$_{2}$, but not the other S1P receptors, is necessary for cell extrusion.

To confirm the involvement of S1P$_{2}$ in extrusion and to determine which cells require this receptor, we used shRNA to silence S1P$_{2}$ expression in HBE cells. GFP-positive cells were FAC-sorted and knockdown of S1P$_{2}$ was confirmed by quantitative RT-PCR (Fig. 3 B). Knockdown of S1P$_{2}$ dramatically increased the proportion of nonextruded apoptotic cells, compared with control shRNA knockdown cells (Fig. 3, C and D). To control for off-target effects of hairpins, we used two additional shRNA sequences to knock down S1P$_{2}$ and obtained similar results (Fig. S3, A and B). To test which cells require S1P$_{2}$, we examined extrusion in monolayers that were mosaically knocked down for S1P$_{2}$. A dying cell with S1P$_{2}$ knockdown surrounded by cells with wild-type (WT) levels of S1P$_{2}$ extrudes successfully (Fig. 3 E, representing nine cases in three independent experiments). By contrast, complete S1P$_{2}$ knockdown of all cells...
Treating WT 3 d post-fertilization (dpf) zebrafish with apoptotic stimuli resulted in extrusion of dying cells (Fig. 3, F and H), whereas induction of apoptosis in miles apart (mil) zebrafish, which carry a loss-of-function mutation in S1P2 (Kupperman et al., 2000), resulted in apoptotic cells that do not extrude (Fig. 3, G and I, and Videos 1 and 2). Instead, brightly staining active caspase-3–positive cells remain in the plane of the epidermis with no obvious actin ring around or below them. Of 60 apoptotic cells from WT zebrafish, 59 extruded and one did not, whereas of 88 apoptotic cells within the monolayer blocked extrusion of dying cells (Fig. 3 D). These results support the conclusion that extrusion requires S1P2 in the cells surrounding a dying cell, but not in the apoptotic cell itself.

Our previous work showed that the zebrafish larval epidermis provides an excellent in vivo model system to study extrusion (Slattum et al., 2009). To test if S1P2-mediated signaling is also required for apoptotic cell extrusion in vivo, we tested if zebrafish that have a mutation in S1P2 could extrude epidermal cells. Treating WT 3 d post-fertilization (dpf) zebrafish with apoptotic stimuli resulted in extrusion of dying cells (Fig. 3, F and H), whereas induction of apoptosis in miles apart (mil) zebrafish, which carry a loss-of-function mutation in S1P2 (Kupperman et al., 2000), resulted in apoptotic cells that do not extrude (Fig. 3, G and I, and Videos 1 and 2). Instead, brightly staining active caspase-3–positive cells remain in the plane of the epidermis with no obvious actin ring around or below them. Of 60 apoptotic cells from WT zebrafish, 59 extruded and one did not, whereas of 88 apoptotic cells within the monolayer blocked extrusion of dying cells (Fig. 3 D).

Figure 1. Inhibitors of SphKs block actin assembly and apoptotic cell extrusion. (A and B) Alexa Fluor 488–labeled cell fragments (green) prepared from MDCK cells pretreated with DMSO (A) or SKI II (B) were added to an intact MDCK monolayer. Arrows point to added cell fragments. (C) The percentage of cell fragments causing actin assembly from three independent experiments; n = 100 cell fragments per experiment and error bars are standard deviations (SDs). *, P < 0.05; **, P < 0.01. (D and E) Extrusion in an MDCK monolayer in the presence of DMSO (D) or SKI II (E). Arrows point to active caspase-3–positive dying cells in each case. (F) Quantification of nonextruded active caspase-3–positive apoptotic cells with DMSO or SphK inhibitor treatment from three independent experiments; n = 100, error bars = SDs. ***, P < 0.001. Bars, 10 µm.
S1P localizes to the extruding apoptotic cell and its surrounding epithelial cells

To visualize S1P formation during apoptotic cell extrusion, we stained UV-irradiated HBE monolayers with anti-S1P mAb and imaged them by confocal microscopy. Fig. 4 shows representative 3D projections of images of early, middle, and late stages of cells from mil zebrafish, none extruded. Addition of JTE-013 to WT zebrafish also blocked extrusion of apoptotic epidermal cells (Fig. S3 C). Of 20 apoptotic cells from WT zebrafish treated with JTE-013, one extruded and the rest did not. These data indicate that S1P signaling through the S1P₂ is required for extrusion in a number of vertebrate epithelia both in vivo and in culture.

Figure 2. An inhibitory anti-S1P mAb blocks apoptotic cell extrusion. (A and B) Extrusion in an MDCK monolayer treated with short-wave UV to induce apoptosis in the presence of a mouse IgG isotype control (A) or 10 µg/ml anti-S1P mAb (B). (C) Quantification of nonextruded apoptotic cells from three independent experiments; n = 100 active caspase-3-positive cells where error bars are SDs; **, P < 0.01. (D and E) Alexa Fluor 488-labeled cell fragments (green) prepared from MDCK cells were added to an intact MDCK monolayer in the presence of a mouse IgG isotype control (D) or 10 µg/ml anti-S1P mAb (E). (F) The percentage of cell fragments causing actin assembly from three independent experiments; n = 100 cell fragments per experiment and error bars = SDs. ***, P < 0.001. Bars, 10 µm.
Figure 3.  **Apoptotic cell extrusion requires the S1P$_2$ receptor.** (A) HBE cells induced to undergo apoptosis with UV in the presence of DMSO or the indicated S1P receptor antagonists. (B) qRT-PCR confirms shRNA-mediated knockdown of S1P$_2$ in HBE cells. (C) Quantification of nonextruded apoptotic cells in HBE monolayers expressing control or S1P$_2$-specific shRNA after UV treatment. (D and E) A dying HBE cell is not extruded by S1P$_2$-silenced cells (D, green), but is extruded successfully by normal surrounding cells (E). When S1P$_2$ shRNA is only in the dying cell (E), it extrudes and is in a higher plane than the actin ring below it, but is in the same plane when the surrounding cells are knocked down for S1P$_2$ (D). Projections of extruding and nonextruding apoptotic cells from WT (F) or *mil* (G) zebrafish larvae, respectively. (H and I) Cross sections (XZs) of an apoptotic extruding cell (H) and a nonextruding cell (I) from WT (H) and *mil* zebrafish larvae (I), respectively. For all bar graphs, each bar represents the average percentage of nonextruded apoptotic cells to total apoptotic cells with each treatment from three independent experiments; n = 100 dying cells per experiment, error bars = SDs. **, P < 0.01; ***, P < 0.001. Bars, 10 µm.
Figure 4. Apoptotic cells produce and transmit S1P during extrusion. (A–C) Confocal fluorescence images during early (A), middle (B), and late (C) stages of extrusion of apoptotic cells from an HBE monolayer. (D and E) Confocal fluorescence images of blocked apoptotic cell extrusion by SKI II (D) or the S1P2 antagonist JTE-013 (E). Each experimental sample was visualized with five (B and C) or three (A, D, and E) consecutive 3D projections (comprising 2-µm thickness each), as necessary to span the full distance from the most basal to the most apical section (second-to-bottom and top images, respectively). Note that total cell height under the different conditions varies: during early extrusion (A) and when extrusion is blocked with SKI II and JTE-013 (D and E), the dying cell is not squeezed out of the epithelium and therefore does not inhabit as great an apical-to-basal distance as when the dying cell is extruding (B and C). A, B, C, and E were obtained using a confocal microscope (TCS SP5; Leica), whereas D was taken using an inverted microscope (Eclipse TE300; Nikon) converted for spinning disc confocal microscopy. A’–E’ represent zoomed-in region (square) from each montage. (D’’) Inset denoting that the unextruded cell in D is apoptotic. Bars, 10 µm.
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migration, and invasion of cancer cells (Maceyka et al., 2002; Spiegel and Milstien, 2003; Radef-Huang et al., 2004), increased S1P signaling could enable tumors to invade by misregulating extrusion. Therefore, our work defining the signaling that drives extrusion of an epithelial cell from a layer may also be important for driving other delamination events crucial to developmental differentiation or invasion of tumor cells.

Materials and methods

Cell culture
MDCK II cells were cultured in Dulbecco’s minimum essential medium (DMEM) high glucose with 5% FBS and 100 µg/ml penicillin/streptomycin (all from Invitrogen) at 5% CO₂, 37°C. HBE cells were cultured in MEM supplemented with 10% FBS and l-glutamine in a flask coated with human fibronectin type I (BD), bovine collagen I (Pierce; Inamed biomaterials), and BSA (Invitrogen).

Drug and UV treatment
Cells were treated with 30 μM SKI II (EMD), 4 μM SKI V (Sigma-Aldrich), 40 μM dHDS (Avanti Polar Lipids, Inc.), 10 μM JTE-013 (Tocris Bioscience), 10 μM VPC23019 (Avanti Polar Lipids, Inc.), 10 μM FTY720-P (Echelon Biosciences), or 10 μg/ml murine anti-S1P mAb (ILpath) for 10 min before UV treatment. To induce apoptosis, cultured monolayers were exposed to 1,200 µJ/cm² UV254 irradiation in a UV series II (Spectroline) and incubated for 2 h before fixation.

Cell staining
Cells were fixed with 4% formaldehyde in PBS at 37°C for 20 min, permeabilized for 10 min with 0.5% Triton in PBS, blocked with AbDil (PBS with 0.1% Triton X-100 and 2% BSA) for 10 min, and incubated with primary antibody for 1 h. Antibody concentrations used for immunostaining were: 1:200 rabbit anti-actin-caspase-3 (BD) and 50 µg/ml anti-S1P mAb (ILpath Inc.). Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG were used as secondary antibodies to detect active caspase-3 and S1P, respectively. Actin was detected with Alexa Fluor 568-phalloidin (Invitrogen). DNA was detected with 1 µg/ml Hoechst 33342 (Sigma-Aldrich) or 5 µM DRAQ5 (Axxora).

Zebrafish staining
To induce apoptosis, we treated 3-d-old zebrafish larvae with 1% DMSO and lipids, Inc.) resuspended in DMEM was added to confluent MDCK monolayers cultured on glass microfuge (Eppendorf). Cells were fluorescently labeled by resuspending them in 50 µl DMEM containing 40 µg/ml FITC snail Lectin (Invitrogen) for 5 min and washed three times with 1 ml of DMEM. The labeled cell fragments were then added to confluent MDCK monolayers cultured on glass coverslips, and incubated for 90 min. The coverslips were fixed in 4% formaldehyde, 0.15% glutaraldehyde, 1 mM MgCl₂, 0.2% triton X-100, and 25 mM Pipes, pH 6.9, for 2 h, blocked in 0.25% casein overnight. Cells undergoing apoptosis were identified using an activated-caspase-3 rabbit polyclonal Ab (BD) followed by incubation with Alexa Fluor 488 anti-rabbit IgG Ab (Invitrogen). Actin was visualized using 0.1 µg/ml Alexa Fluor 568-phalloidin (Invitrogen). DNA was visualized using 1 µg/ml Hoechst 33342 (Sigma-Aldrich) or 5 µM DRAQ5 (Enzo Life Sciences).

Addition of dead cell fragments or S1P to monolayers
To make dead cell fragments, MDCK cells were scraped from the culture dish and sheared with a 27G1/2 needle seven times. Cell fragments were transferred to a microscope tube and centrifuged for 1 min at 8,000 rpm in a microfuge (Eppendorf). Cells were fluorescently labeled by resuspending them in 50 µl DMEM containing 40 µg/ml FITC snail Lectin (Invitrogen) for 5 min and washed three times with 1 ml of DMEM. The labeled cell fragments were then added to confluent MDCK monolayers cultured on glass coverslips, and incubated for 90 min. The coverslips were fixed and stained with Alexa Fluor 568-phalloidin and Hoechst dye. For drug inhibition in the dead cell fragment addition, cells were pretreated for 10 min with SKI II at concentrations listed above before scraping and needle shearing. For S1P mAb addition, cell fragments were added to MDCK monolayers in the presence of 10 µg/ml S1P mAb. For the lipid addition experiment, 20 µg/ml S1P (Avanti Polar Lipids, Inc.) or total E. coli lipid extraction (Avanti Polar Lipids, Inc.) resuspended in DMEM was added to confluent MDCK monolayers, incubated for 1 h, and fixed and stained with phalloidin and Hoechst dye, as above.

Quantification of cell extrusion
To quantify the ratio of nonextruded apoptotic cells, we counted 100 active caspase-3-positive cells that were associated with cultured monolayers.

Apoptotic cells that possessed clearly shrunken nuclei but were not surrounded by a distinguishable actin ring were defined as nonextruded apoptotic cells. Apoptotic cells that came out of the plane of the monolayer with strong actin staining around and/or underneath the cells were defined as extruded cells. Old apoptotic cells with strong caspase-3 staining floating above the monolayer that died before we treated monolayers were excluded.

Microscopy
Fluorescence micrographs of fixed, cultured cells were obtained using a microscope (DM 600B; Leica) with an HCX PL Fluorat 63X/1.25 oil lens (Leica) and captured using a Micromax charge-coupled device camera (Roper Scientific). IP Laboratory software was used to control the camera and to process images. Fluorescence micrographs of zebrafish larvae were obtained using a microscope (model 90; Nikon) with a 40X PH.1 lens (Nikon) and captured using a charged-coupled device camera (Regeta 2000R; Q Imaging). Confocal micrographs were obtained using a microscope (TCS SP5; Leica) with a 63X oil lens or an inverted microscope (Eclipse TE300; Nikon) converted for spinning disc confocal microscopy (Andor Technologies) using a 60X Plan Fluor 0.95 oil lens with an electron-multiplying cooled CCD camera 1,000 x 1,000, 8 x 8 mm² driven by the IQ software (Andor Technologies). We used ImageJ to stack confocal sections into Z series that were then color combined and reconstructed into 3D image using MetaMorph software. For HBE cells stained with anti-S1P, we displayed five consecutive projections of 2-µm thickness each using the “montage” function on MetaMorph software. All images were processed further using ImageJ, Photoshop (Adobe), Illustrator (Adobe), and Quicktime Pro (Quicktime) software.

RT-PCR
Total RNA was isolated from cultured cells using the RNeasy kit (QIAGEN) and reverse-transcribed using the SuperScript III first strand synthesis kit (Invitrogen) with random hexamers according to the manufacturer’s guideline. PCR detection of S1P2 was conducted as described previously (Estrada et al., 2008). In brief, PCR amplification of the targeted fragments was performed with 30 cycles of denaturation at 95°C (30 s), annealing at 58°C (30 s), and extension at 72°C (30 s). PCR primer pairs used were: S1P; sense, 5'-GCACAAACACCATCACTCTC-3', antisense, 5'-TGGTCCCTCTCTTCTCTG-3'; S1P2; sense, 5'-CAAGTCCTACTCGGACAGT-3', antisense, 5'-TAGAGGAGCCTGAAGACAGAGG-3'; S1P3; sense, 5'-TCAGGGGCAATGTGC-3', antisense, 5'-GAGTAGAGGGGCGAGGAGTTG-3'; S1P4; sense, 5'-AGCCTCTGCCCTCTTACTCT-3', antisense, 5'-ATGACGACCCTGCTTCCAGCA-3'; and S1P5; sense, 5'-ACAACATACCCGGCAGCTC-3', antisense, 5'-GCCCGAGCTAGGATTGT-3'. Quantitative real-time PCR was performed using a LightCycler480 (Roche) and the SYBR mix (SABiosciences). Relative mRNA expression was quantified using the comparative threshold method with the content of actin mRNA as internal control.

shRNA-mediated gene silencing of S1P2
We designed sense and antisense hairpin oligonucleotides specific for S1P2 (shS1P2-1) according to a published work (Estrada et al., 2008). Two additional pairs of hairpin oligonucleotides were used to knockdown the S1P2 receptor. The oligonucleotide sequences were: shS1P2-2; sense, 5'-GGGCCTCAATGTTGGGAAA-3', antisense, 5'-TTTCCCCACAAATGGGCCG-3'; shS1P3; sense, 5'-GCAAGITCCACTCGGCAAT-3', antisense, 5'-ATGGCAGATTGGAACACTGC-3'. The sense and antisense oligonucleotides were annealed and cloned into a plasmid 5.0-based lentiviral vector. Transducing lentiviral particles carrying the shRNA oligonucleotides were produced by packaging in 293T cells. Lentiviral particles carrying nonspecific shRNA oligonucleotides were used as a control. HBE cells were transduced with lentiviral particles for 6 h in the presence of 2 µg/ml polybrene, washed, and replaced with fresh medium. Stably transduced cells were FAC-sorted based on GFF fluorescence.

Statistics
The statistical analysis was performed using an unpaired t test. Values of P < 0.05 were considered significant.

Online supplemental material
Fig. S1 shows that S1P directly induces actin assembly at the apex of an MDCK monolayer. Fig. S2 shows that inhibition of actin assembly and apoptotic cell extrusion by inhibitors of SphKs is dose dependent. Fig. S3 shows that apoptotic cell extrusion requires the signaling mediated by S1P1. Video 1 shows a filling 3D projection movie of two apoptotic cells that do not extrude from the epidermis of a mutant zebrafish embryo. Video 2 shows the Z planes of an apoptotic cell that extrudes normally from the
epidermis of a WT zebrafish embryo and an apoptotic cell that fails to extrude from the epidermis of a mil zebrafish embryo. Videos 3–5 show the localization of S1P during early, middle, and late stages of apoptotic cell extrusion. Video 6 shows that an apoptotic HBE cell that produces S1P or extrudes in the presence of SKI II. Video 7 shows that an apoptotic HBE cell that generates high levels of S1P fails to extrude in the presence of JTE-013. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201010735/DC1.

We thank Dr. Jean Marie Delalande and Amy Carr for help on zebrafish experiments and to Mark Metzstein, Katie Ullman, and Thomas Marshall for helpful comments on our manuscript. We also thank Dr. Diana Staffinelli for helpful advice regarding lipid signaling. Dr. Carl Thummler for use of his ISM confocal microscope, and Dr. James Bear for providing the lentiviral constructs used for shRNA experiments.

This work was supported by a National Institutes of Health Innovator Award no. DP2 OD002056-01 to J. Rosenblatt and P30 CA042014 awarded to The Huntsman Cancer Institute for core facilities. R. Sabbadin has stock options in iPath, Inc.

Submitted: 14 October 2010
Accepted: 15 April 2011

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