

5-2016

Characterization of Stem Cell Turnover in a Living Epithelial Bilayer

Elizabeth Sumner

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CHARACTERIZATION OF STEM CELL TURNOVER
IN A LIVING EPITHELIAL BILAYER

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A

Thesis

Presented to the Faculty of

The University of Texas

Health Sciences Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

of the Degree of

MASTER OF SCIENCE

By

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May, 2016

Acknowledgements

First, I would like to thank my mentor Dr. George T. Eisenhoffer for his endless support and guidance during my graduate studies; I have learned an immense amount from him.

I would also like to acknowledge members of my advisory committee: Richard Behringer, Ph.D., Jichao Chen, Ph.D., George Eisenhoffer, Ph.D., Michael Galko, Ph.D., and Zahid Siddik, Ph.D. Each of them guided me to think critically about my research and aided in my overall progress and success during graduate school.

I would also like to acknowledge Adriana Paulucci, Ph.D. from the MD Anderson Genetics Department Microscopy Core for her assistance with time-lapse imaging. Thank you also to Kenneth Dunner Jr. at the UT MD Anderson High Resolution Electron Microscopy Facility for the electron microscopy data.

I also wish to thank current and past members of the Eisenhoffer lab: Sarah Cunningham, Mohammad Hamideh, Oscar Ruiz, Ph.D., Courtney Brock, and Stephen Wallin.

Abstract

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Homeostatic maintenance of epithelia requires the renewal and replacement of old or dying cells while sustaining a functional barrier. Imbalance between cell production and elimination are hypothesized to underlie many pathological conditions. However, our knowledge of cell turnover within living tissues remains largely restricted to static images due to the limited ability to study epithelia in their native context. Here we report that clearance of damaged basal stem cells promotes compensatory proliferation of neighboring stem cells to maintain overall population numbers in a bilayered epithelium. Time-lapse imaging and electron microscopy experiments reveal that dying cells are rapidly cleared as nearby basal cells engulf apoptotic debris. Remaining basal cells subsequently divide to replace lost cells, allowing for conservation of tissue integrity and function. Together, our data demonstrate the ability to study and visualize the dynamics of epithelial stem cell turnover during tissue homeostasis with *in vivo* time-lapse imaging. This approach has the ability to rapidly reveal novel mechanisms regulating epithelial tissue homeostasis and will further our understanding of how these processes become altered in disease.

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1. Introduction: The secret life of epithelial cells: Imaging birth and death in living epithelium

The ability of tissues within multicellular organisms to maintain overall homeostatic cell numbers has long eluded scientists (Cannon, 1929; Eisenhoffer and Rosenblatt, 2013; Macara et al., 2014). Epithelial tissues serve as a barrier from the outside world for all metazoans, and more specifically, bilayered epithelia provide a functional barrier for human organs despite constant exposure to external stimuli. Maintenance of epithelial tissues requires continual removal and replacement of old or dying cells throughout the life of the organism. Yet, it is not well understood how the birth and death of cells is coordinated to preserve overall cell numbers while the tissue sustains a barrier function.

Cell division, extrusion, and death can all be influenced by forces from localized areas of cellular crowding within the tissue (Frisch and Francis, 1994; Shraiman, 2005; Eisenhoffer et al., 2012). These findings highlight the inter-relatedness of cell death and division and how these processes depend on changes in cell density that are not easily detectable in standard culture assays or in current animal models. When a cell's ability to detect density changes in its neighbors is altered, epithelial pathologies can result, including decreased barrier function in the intestinal epithelium (Zeissig et al., 2007; Hering et al., 2012; Petit et al., 2012), accumulation of dangerous cells to promote carcinoma formation (de la Cova et al., 2004; Moreno and Basler, 2004), or release of cells with enhanced survival during cancer progression (Hayashi et al., 2007; Menon and Beningo, 2011; Tse et al., 2012). Carcinomas can arise in epithelial tissues that line the organs, which account for 85% of adult cancer, and altered cell turnover is correlated with carcinogenesis (Colgin et al., 2002; Rose-Hellekant et al., 2006).

The limited ability for *in vivo* perturbation and imaging of epithelia that coat internal organs has prevented a detailed characterization of cell turnover, thus hindering our understanding of how specific alterations in this process drive carcinogenesis. Emerging tools and methods for the developing zebrafish epidermis overcome these limitations and provide the ability to interrogate cell turnover in living epithelial tissue. This introduction summarizes recent tools for the zebrafish epithelium that allow non-invasive *in vivo* time-lapse visualization of cell turnover to reveal how disruptions in these fundamental processes contribute to common epithelial pathologies.

1.1 Epithelial Cell Turnover

Cell death and division have been extensively researched, yet it remains unclear how these two processes are coordinated in living tissues. Cells within epithelial layers have some of the highest rates of turnover in the body, but how overall cell numbers are maintained has not yet been fully characterized (Hooper, 1956; Pellettieri and Sanchez Alvarado, 2007). Epithelial cell turnover utilizes several processes to remove and replace old and dying cells while allowing the tissue to maintain a functional barrier. Below, we briefly discuss a few of these dynamic mechanisms driving cell turnover (Figure 1.1).

Epithelia must uphold adequate numbers of cells to maintain tissue form and function. When too many cells divide in the epithelium, crowding can occur and result in a necessity to eliminate excess cells. Excess or overcrowded cells can be removed from the tissue through a process called extrusion (Eisenhoffer et al., 2012; Kuipers et al., 2014). Extrusion requires the cell destined for removal to send signals, such as sphingosine-1-phosphate, to its neighboring cells, inducing actin- and myosin-dependent contraction around the dying cell (Rosenblatt et al., 2001). As the cell is pushed out of the tissue, those

neighbors can then efficiently close the gap left behind so a barrier function is maintained. The dynamic cytoskeletal rearrangements associated with cell extrusion and removal of defective cells must be imaged over time to truly understand the impact and sequence of such alterations during cell removal and tissue homeostasis.

Cells lost during normal homeostatic turnover must be cleared and replaced to maintain overall numbers in the tissue. Studies in *Drosophila* and *Hydra* have revealed that apoptotic cells produce WNT to promote generation of new cells, a process termed compensatory proliferation (Ryoo et al., 2004; Fan and Bergmann, 2008; Chera et al., 2009). The efficient and rapid removal of dying cells ensures that signals are only transiently produced (Ryoo et al., 2004; Fan and Bergmann, 2008; Chera et al., 2009). Inhibition of death and clearance of dying cells causes these “undead” cells to linger in the tissue, and with sustained production of WNT, results in tissue overgrowth (Bump et al., 1995; Ryoo et al., 2004; Fan and Bergmann, 2008; Chera et al., 2009). This pathway provides mechanistic insight for how balance is established between cell birth and death to maintain homeostatic cell numbers during turnover. This coordination is highly dynamic and difficult to capture in its’ entirety with static snapshots from individual time points due to the transient nature of various stages of cell removal and replacement.

Individual cells are replaced by one of two processes to promote generation and incorporation of new cells in the existing tissue. First, the plane of division orientation impacts whether the daughter cell will remain within the basal layer or move out to the apical periderm layer. Lateral division promotes symmetric division that replenishes the stem cell population in the basal layer, while division perpendicular to the basement membrane promotes stratification, differentiation, and cellular ingress into the outer layer (Lechler

and Fuchs, 2005; Macara et al., 2014). Adult homeostasis is correlated with an overall decline in mitosis but still involves perpendicular divisions to maintain a stratified epithelia, showing the importance of both symmetric and asymmetric division after development to replace cells in a bilayered epithelium (Lechler and Fuchs, 2005).

As a second mechanism of cellular incorporation, basal cells are induced to lose contact with the basement membrane and move to the outer epithelium. This intercalation involves unequal partitioning of extracellular matrix protein secretion, integrin adhesion, and growth factor signaling between the two daughter cells (Bellaiche et al., 2001; Lechler and Fuchs, 2005). In *Xenopus*, intercalation comprises rearrangement of cell borders and retraction of outer cells to accommodate an inner cell that moves into the layer (Stubbs et al., 2006). These inner cells extend and retract processes between the outer cells, mimicking the developmental stages during epiboly when cell layers are thinning and spreading; however, intercalation occurs in the opposite direction as epiboly, where cells move apically to the outer surface rather than basally (Stubbs et al., 2006). Studies of division orientation and intercalation are beginning to reveal how stem cell division replaces all cell types within stratified epithelia.

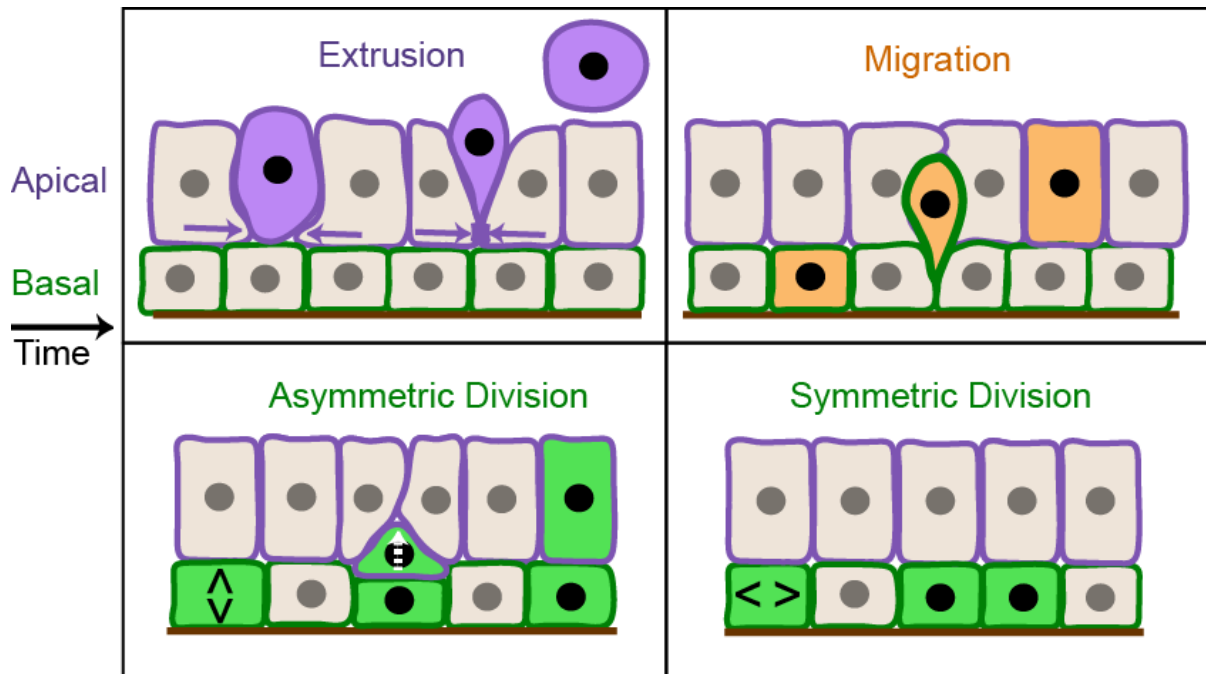


Figure 1.1 Epithelial bilayer cell turnover.

Cells from the apical layer can extrude to reduce crowding (purple cell) while neighboring cells close the gap left behind to maintain barrier function (purple arrows). They can also migrate to the apical layer, away from the basement membrane (brown line) and differentiate to replace apical epithelial cells (orange cell). Basal stem cells can divide asymmetrically (green cells) to stratify and replace differentiated apical layer cells or symmetrically within the basal layer to replace basal cells.

1.2 A Brief History of Imaging and its Advances to Study Epithelial Cells

Traditional light microscopy of tissues provides static images but does not capture the transient and dynamic processes constantly occurring in living tissues. It was not until 1907 that Mikrosk Ries captured the first time-lapse film strip to study the entire process of embryonic sea urchin development (Landecker, 2009). Since Ries' work, time-lapse imaging has evolved to visualize cells and their behaviors in real time in cell culture, revealing information about individual cellular behaviors after genetic alterations. Static images, much like genome data, "alone lack spatial and temporal information and are therefore as dynamic and informative as census lists or telephone directories" (Tsien, 2003). Michael Abercrombie pioneered imaging for use as a quantitative tool to study cell motility and adhesion and also for hypothesis generation rather than only for visualization (Abercrombie and Heaysman, 1952; Dunn and Jones, 1998). While tissue culture makes cells accessible for such analysis, it lacks the ability to replicate the *in vivo* microenvironment and the tissue's native context.

In order to understand epithelial cell turnover in normal physiology, time-lapse imaging is crucial for revealing subcellular mechanisms including actin, microtubule, and biosensor dynamics in living tissue. The discovery of green fluorescent protein (GFP) in 1992 introduced the ability to fluorescently label cells and proteins *in vivo* (Chalfie et al., 1994; Tsien, 2003; Landecker, 2013). Since then, multi-photon imaging in the mouse and other techniques have been developed to visualize deep tissue; however, these strategies often require manipulation of the animal resulting in tissue damage, possibly altering natural physiology (Tsien, 2003; Brown and Greco, 2014). As technology rapidly improves the quality of fluorescent markers and microscopes, the ability to follow transient and rapid changes within a tissue becomes increasingly accessible (Landecker, 2013). Time-lapse

imaging advancement has enabled scientists to understand living tissues on a both a cellular and subcellular level; however, a model system is still needed to study epithelia in their native context in a living tissue to understand their maintenance in human health.

1.3 Zebrafish as a Model for Studying and Imaging the Epithelium

A limited ability to visualize cell division and death *in vivo* has impaired the understanding of how the two processes are linked within epithelia. The peripheral location and optical clarity of the zebrafish epidermis makes it ideal for observing division, migration, extrusion, and death of individual epithelial cells with time-lapse microscopy. The zebrafish epidermis provides an excellent model for epithelia that coat organs such as the breast, lung, and prostate due to structural and molecular similarity. The developing zebrafish epithelium is bilayered during embryonic and larval stages (Kimmel et al., 1995; Le Guellec et al., 2004) and consists of p63-positive progenitor cells in the basal layer (Lee and Kimelman, 2002) that give rise to differentiated cytokeratin 8/18-positive cells (Gong et al., 2002; Wang et al., 2006) in the outer layer (Figure 2). Additionally, gene function can be investigated using both forward and reverse genetics and several mutant lines already exist with epithelial defects during development that are caused by alterations in division and death (van Eeden, F.J.M. et al., 1996; Amsterdam et al., 2004). A wide array of cell and molecular tools in zebrafish can therefore allow investigation of mechanisms guiding cell turnover in epithelial tissues with exceptional resolution and precision.

Although these attributes made the developing zebrafish an ideal system to study a living epithelial bilayer, the tools and methods to label and visualize specific cell types within the tissue had been previously lacking. To overcome this challenge, a series of molecular tools (Eisenhoffer et al., 2012) and imaging methods (Eisenhoffer and Rosenblatt,

2011) for the epidermis of the developing zebrafish now permit researchers to monitor the dynamics of specific cell populations, track individual cellular behaviors, ablate cell types using genetically encoded toxin or enzymes (Curado et al., 2007), and overexpress genes of interest in a spatial and temporal manner. Novel GAL4 enhancer trap lines now allow for both spatial and temporal manipulation and visualization of the two most commonly studied epithelial cell types, basal stem cells and apical differentiated cells(Eisenhoffer et al., In Revision). These recent cell and molecular tools combined with time-lapse imaging are initiating a revived interest to characterize epithelial cell turnover during homeostasis, regeneration and carcinogenesis *in vivo* and in real time.

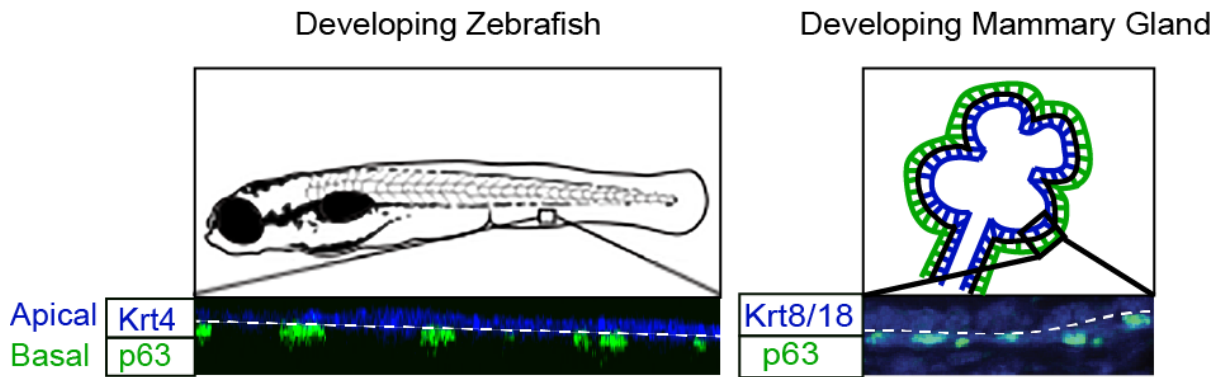


Figure 1.2 Zebrafish and mammary gland bilayer.

Zebrafish and mammary glands have a bilayered epithelium that is structurally similar, with a basal layer of p63 positive stem cells (green) and an apical layer krt4 positive differentiated epithelial cells (blue) (Modified with permission from **Macias, H., Moran, A., Samara, Y., Moreno, M., Compton, J. E., Harburg, G., Strickland, P. and Hinck, L. (2011).** SLIT/ROBO1 signaling suppresses mammary branching morphogenesis by limiting basal cell number. *Developmental cell* **20**, 827-840.)

1.4 Zebrafish as a Model for Altered Cell Turnover

Zebrafish provide a vertebrate model to study cell turnover *in vivo* and uncover novel mechanisms regulating the control of overall numbers in epithelial tissues. When these mechanisms are altered, they can drive pathogenesis in humans for diseases such as cancer. Zebrafish share approximately 70% genetic similarity with humans and the zebrafish genome contains homologues for many genes associated with human disease (Langheinrich, 2003). Both forward and reverse genetics have been used to identify genes associated with epithelial tissue homeostasis and maintenance of barrier function (van Eeden, F. J. M. et al., 1996; Amsterdam, 2006; Amsterdam et al., 2011). Using these techniques, mutants have been isolated that resemble human kidney disorders, melanoma, neurodegenerative diseases, and many other pathologies, enabling the use of drug screens to discover novel therapeutics (Santoriello and Zon, 2012).

Altered cell turnover can underlie disease progression, resulting in a loss of barrier function when epithelial tissue integrity is compromised or cancer formation due to hyperproliferation (Rose-Hellekant et al., 2006). Genes that have human homology were identified in zebrafish and when mutated, these genes impact physiological cell turnover. *Hai1* (also known as *Spint1*) leads to the death of differentiated epithelial cells and results in enhanced inflammation and epidermal hyperplasia (Carney et al., 2007). These consequences are, however, only secondary to decreased epithelial integrity within the basal layer due to E-cadherin redistribution and a loss of epithelial polarity (Carney et al., 2007). Multiple genes have also been attributed to a phenotype similar to human psoriasis when mutated, resulting in an accumulation of cells. Mutations in *lethal giant larvae (lgl2 or penner)*, *psoriasis/m14*, and *clint1* result in hyperproliferation, loss of cell polarity, and altered cell morphology

(Webb et al., 2008; Dodd et al., 2009; Reischauer et al., 2009). A difference in cell cycle regulation, cell polarity, growth regulation, or maintenance of epidermal integrity, can ultimately result in epithelial cell aggregation by altering physiological cell turnover (Webb et al., 2008; Dodd et al., 2009; Reischauer et al., 2009). Transient loss of cell-cell contacts during intercalation after division could even result in an epithelial to mesenchymal-like process with single cell motility, a feature commonly characterized in metastatic cancer (Dodd et al., 2009). These zebrafish mutants mimic human disease when cell turnover is disrupted and provide new tools to label specific cell types and observe their behavior to study disease progression *in vivo*.

1.6 Project Summary and Hypothesis

Despite its relevance to human disease, characterization of bilayered cell turnover remains incomplete in a vertebrate system and knowledge is largely limited to static images of only one time point in this dynamic process. Technological advances now allow for time-lapse studies to capture the entire process of cell turnover and uncover novel regulatory mechanisms. The developing zebrafish provides a unique system to image the dynamics of cell removal and replacement within a living epithelial tissue. We aim to characterize how altered homeostatic cell turnover impacts the balance of the birth and death of cells, with a focus on the basal stem cell population. We hypothesize that clearance of dying basal cells influences the rate of turnover in epithelia. To test this hypothesis, we experimentally ablate a subset of basal epithelial cells, examine clearance using *in vivo* time-lapse imaging, and assess the impact on proliferation and cell replacement. Understanding the mechanisms regulating epithelial tissue homeostasis provides new opportunities to understand how specific genetic alterations can impact the balance of cell removal and replacement and contribute to disease progression.

2. Materials and Methods

2.1 Zebrafish

Zebrafish were maintained under standard laboratory conditions with a light/dark cycle of 14 hours light and 10 hours darkness. Embryos were collected and kept in E3 embryo medium at 28.5°C and staged as described in (Kimmel et al., 1995). The following zebrafish strains were used: AB (wild-type), *zc1036a* (Eisenhoffer et al., In Revision), *Tg(KRT4:GFP)* (Gong et al., 2002), and *ΔNp63:EGFP* (Eisenhoffer et al., In Revision).

2.2 Controlled ablation of p63 positive epithelial stem cells

Embryos were sorted for mCherry fluorescence using a Leica fluorescent dissecting scope. Embryos without the fluorescent enzyme nitroreductase (NTR) were kept as enzyme negative controls. Metronidazole (MTZ) was diluted fresh for each use at a 1 M concentration diluted in dimethyl sulfoxide (DMSO) and was added directly to the E3 when embryos were 4 days post fertilization (dpf). After 4 hours, MTZ was rinsed twice from the well with E3 and embryos were either fixed or left for 20 hours to recover in fresh E3.

2.3 Zebrafish drug treatment

The chemical WNT inhibitor IWR (I0161 Sigma) was diluted in DMSO as a 10 mM stock and was added to embryos at a concentration of 10 μM. Embryos were treated with the inhibitor for 2 hours prior to MTZ treatment, during treatment, and during recovery. Fresh inhibitor was added after MTZ is rinsed and embryos are left in E3 and the inhibitor during recovery.

2.4 Embryo Fixation

Zebrafish embryos were fixed for 4 hours at room temperature or overnight at 4°C with 4% paraformaldehyde (4% PFA), rinsed once with 1X Phosphate Buffered Saline (PBS), and left in 1X PBS at 4°.

2.5 Immunofluorescence

Zebrafish embryos were washed in PBS + 0.5% TritonX-100 (PBSTX 0.5%) for 15 minutes and blocked for 1-2 hours at room temperature in 1% DMOS + 2mg/mL bovine serum albumin (BSA) + 0.5% Triton X-100 + PBS + 10% heat inactivated goat serum. Embryos were incubated at room temperature for 4 hours or overnight at 4°C with primary antibodies (Table 2.1) diluted in block solution. Primary antibody was removed and samples were washed for 2 hours in PBSTX 0.5% and incubated in block solution for 1-2 hours. Embryos were incubated for 4 hours at room temperature with secondary antibodies (Table 2.2) in block solution, rinsed with PBSTX 0.5%, and incubated with DAPI diluted in PBSTX 0.5% (1:1000) for 30 minutes. Samples were washed for 30-60 minutes in PBSTX 0.5% then left in PBS before mounting only the tail of the zebrafish embryo.

2.6 Time-lapse Imaging and Microscopy

Zebrafish were anesthetized with 0.04% tricaine in E3 and mounted in 1% low melt agarose in a 10 mm MatTek culture dish. Time-lapse imaging was performed on an existing inverted Nikon TI microscope assembled by Perkin Elmer and equipped with Yokugawa spinning disc head, three-coherent, six solid-state lasers, and 488 nm and 561 nm lines were obtained, respectively, by Argon and Krypton Melles Griot lasers. Image capture was registered by a sCMOS Hamamatsu Orca Flash 4.0 CCD camera with an effective number of pixels of 2048 by 2048 at a dynamic range of 37,000:1 at normal can 1x1. Images were captured every 6 or

10 minutes for 6-12 hours at 1 μm intervals to obtain a z-stack for each time point using Volocity 6.3 software. Objectives used were a 20x Plan Apo, 0.75 NA or an Apo 40x 1.25 NA. A Zeiss Observer upright microscope with motorized fluorescence, DIC objectives, and z motor, equipped also with an Axiocam cooled CCD camera and Zen software were also used for image capture and analysis. Both Fiji (Schindelin et al., 2012) and Imaris 7.7.2 (Bitplane Inc.) were also used for image analysis and post-capture rendering of imaging data.

2.7 Scanning Electron Microscopy

Samples were fixed in 3% glutaraldehyde + 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3). Samples were washed with 0.1 M cacodylate buffer (pH 7.3), post-fixed with 1% cacodylate buffered osmium tetroxide, washed with 0.1 M cacodylate buffer, and then in distilled water. Samples were treated with Millipore-filtered 1% aqueous tannic acid, washed in distilled water, treated with Millipore filtered 1% aqueous uranyl acetate, and then rinsed with distilled water. They were dehydrated with a series of increasing concentrations of ethanol and transferred to increasing concentrations of hexamethyldisilazane (HMDS) and air dried overnight. Samples were mounted onto double-stick carbon tabs (Ted Pella, Inc., Redding, CA), and mounted onto glass microscope slides. Samples were coated under vacuum using a Balzer MED 010 evaporator (Technotrade International, Manchester, NH) with platinum alloy for a thickness of 25 nm and then flash carbon coated under vacuum. Samples were then transferred to a desiccator until examination and imaging in a JSM-5910 scanning electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 5 kV.

2.8 Transmission Electron Microscopy

Samples were fixed in 3% glutaraldehyde + 2% paraformaldehyde + 0.1 M sodium cacodylate buffer and treated with 0.1% Millipore-filtered cacodylate buffer tannic acid. They were post-fixed with 1% buffered osmium tetroxide for 30 minutes, stained in block with 1% Millipore-filtered uranyl acetate. Samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. Samples were polymerized in an oven at 60°C for about 3 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL) and stained with uranyl acetate and lead citrate in a Leica EM Stainer. They were examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging system (Advanced Microscopy Techniques Corp, Danvers, MA).

2.9 Cell Counts

Immunostain for a marker of cell division (H3P or BrdU) or cell death (activated caspase 3) were used to visualize cell proliferation or apoptosis. H3P and BrdU cells in the epithelium were counted by hand, excluding cells over the notochord or cells that in contact with pigment cells. Activated caspase 3 was quantified using an automated macro in Fiji (Schindelin et al., 2012). After setting the scale in ImageJ, the opened image was compressed to get a maximum projection of the z-stack using the Extended Depth of Field plugin. This image was then made binary and the Analyze Particles function was used to count the number of cells that are of a size 0.05-7.00 μm and circularity of 0.25-1.00. The same analysis is run again with a line drawn around the notochord to quantify only that area and subtract it from the total number to get a quantification of only cells in the epithelium. For

low amounts of death when there are only a few cells, cells were quantified manually in ImageJ.

2.10 Statistical Analysis

Statistical analysis and graphing was done using GraphPad Prism 6. A t-test was used to test for significance and data was plotted with standard error of the mean (SEM) shown.

Table 2.1 Primary Antibodies for Immunofluorescence Staining.

Name	Anti-	Concentration	Company
Activated Caspase 3	Rabbit	1:700	BD Biosciences
Bromodeoxyuridine (BrdU)	Rat	1:100	Abcam
CDH1	Rabbit	1:100	GeneTex
Phospho-Histone H3 (H3P)	Rabbit	1:500	Abcam
Tp63	Mouse	1:500	GeneTex
Tp63	Rabbit	1:100	GeneTex
WNT3a	Goat	1:50	Santa Cruz Biotech

Table 2.2 Secondary Antibodies for Immunofluorescence Staining.

Name	Concentration	Company
Goat anti-rabbit Alexa 488	1:1:200	Life Sciences
Goat anti-rabbit Alexa 647	1:200	Life Sciences
Goat anti-mouse Alexa 647	1:100	Life Sciences
Goat anti-rat Alexa 488	1:200	Life Sciences
Donkey anti-goat Alexa 488	1:100	Life Sciences
Donkey anti-goat Alexa 647	1:100	Life Sciences

3. Results

3.1 The influence of apoptotic cell elimination on basal cell turnover in epithelia

Cell turnover in epithelia is a constant process to renew and replace old or dying cells, however, it is not yet well understood how cells are cleared from bilayered tissue and replaced, particularly the basal cells (Hooper, 1956; Pellettieri and Sanchez Alvarado, 2007). In zebrafish bilayers, epithelial stem cells self-renew and replace other epithelial cell types while residing in the basal layer, a unique location in which they are surrounded on either side by the basement membrane and or the apical surface periderm (Kimmel et al., 1995; Lee and Kimelman, 2002; Le Guellec et al., 2004). Removal of these cells would require rearrangement of neighboring cells or migration before clearance via engulfment or apical extrusion. Efficient elimination of unfit cells is essential for tissue health and function as studies in other systems have shown that apoptotic cells produce mitogenic factors to stimulate their replacement and will cause tissue overgrowth if not removed in a timely manner (Ryoo et al., 2004; Fan and Bergmann, 2008; Chera et al., 2009). When cell turnover is disrupted and dying cells linger in the tissue, subsequent inflammation and increased apoptosis can occur. Due to an absence of pro-survival signals that are normally produced during cell clearance, disorders such as chronic obstructive pulmonary disorder (COPD) can occur (Golpon et al., 2004; Henson et al., 2006).

Epithelial cell turnover utilizes several processes to remove and replace old and dying cells while allowing the tissue to maintain a functional barrier. Basal cells can be cleared from the tissue either by extrusion through the apical surface or engulfment (Figure 3.1). Extrusion through the apical surface would require migration out of the basal layer and the generation of signals, such as sphingosine-1-phosphate, to neighboring cells that induce an

actin- and myosin-dependent contractile ring around the dying cell (Rosenblatt et al., 2001; Eisenhoffer et al., 2012; Kuipers et al., 2014). The cell is then pushed out of the tissue as neighboring cells efficiently close the gap left behind. Engulfment could act as another form of elimination through broad-specificity phagocytosis or entosis by neighboring epithelial cells (Overholtzer et al., 2007; Rasmussen et al., 2015). Elimination of basal cells was recently visualized in the mouse hair follicle *in vivo* and found that epithelial phagocytosis acts as a regulator of the stem cell pool (Mesa et al., 2015). Entosis is a form of engulfment led by genetic differences between cells, ultimately leading to a contractile ring of actin and myosin filaments that accumulate at the outer edge of a cell as it is engulfed (Overholtzer et al., 2007).

The peripheral location and optical clarity of zebrafish epidermis make it ideal for monitoring division, migration, extrusion and death during *in vivo* studies of cell turnover within a living epithelial tissue. Zebrafish have a bilayered epithelium during development that is structurally and molecularly similar to epithelia that coat organs such as the breast, lung, and prostate (Kimmel et al., 1995; Le Guellec et al., 2004). A vast array of cell and molecular tools in zebrafish allow for investigation of mechanisms guiding cell turnover in epithelial tissues, especially in order to visually access and image basal cells nestled within the bilayer (Eisenhoffer and Rosenblatt, 2011; Eisenhoffer et al., 2012).

By inducing damage in a subset of basal cells in the zebrafish epidermis, the dynamics of stem cell clearance and replacement were observed over time using time-lapse imaging. Our studies reveal that neighboring basal cells act as non-professional local phagocytes to clear apoptotic cell debris after damage to quickly remove dying cells from the tissue. Remaining stem cells are subsequently stimulated to divide to replace lost cells in

order to maintain homeostatic cell numbers. Basal cell removal and replacement are closely coordinated such that apoptotic cells are rapidly cleared and replaced in the epithelium by neighboring healthy basal stem cells.

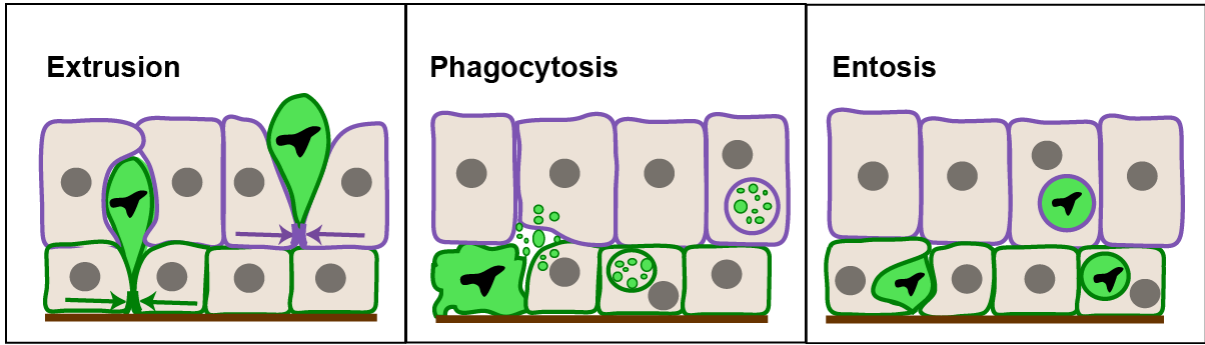


Figure 3.1 Mechanisms of basal cell clearance from a bilayered epithelium.

Basal stem cells targeted for death (green) can leave the tissue by extrusion or engulfment via phagocytosis or entosis. These processes can occur individually as shown or concurrently.

3.2 A system to spatially and temporally control ablation of a subset of p63 positive epithelial cells

To address how cell loss and proliferation are balanced while epithelia maintain a functional barrier, we developed a system to kill a subset of the basal cells while leaving many cells intact. The *zc1036a* GAL4 enhancer trap line (Figure 3.2a) drives a fluorescently tagged bacterial enzyme, nfsB-mCherry (Curado et al., 2007), in a subset of basal stem cells. The nfsB enzyme converts exogenously added Metronidazole (MTZ) into a cytotoxic DNA cross linker that induces apoptosis only in the fluorescently labeled cells. This assay therefore allows for both spatial and temporal control of cell ablation. *Zc1036* is expressed in a subset of basal epithelial cells, particularly in the median fin epithelium. Importantly, it is expressed specifically in a subset of p63 positive basal cells, as illustrated by both co-labeling with transgenic zebrafish that expresses GFP under the control of a conserved p63 enhancer element and by antibody staining to determine the localization and distribution of p63 protein (Eisenhoffer et al., In Revision). This co-localization suggests that MTZ treatment will spare many basal stem cells within the layer and allow for observation of self-renewal and regeneration (Figure 3.2b). The cells are eliminated from the epidermis in a mosaic fashion, and therefore, we termed this novel assay “controlled ablation of p63 positive epithelial cells”, or CAPEC.

We treated larvae at 4 days post-fertilization (4 dpf) with MTZ, a time in development when cell death and proliferation in the epidermis is minimal without a damaging external stimulus. Cell death after MTZ treatment was analyzed by an activated caspase 3 immunostain and revealed that apoptosis was restricted to NTR/mCherry positive basal cells (Figure 3.2c). Larvae lacking the NTR enzyme or transgenic animals that are not

exposed to the prodrug do not experience basal cell death. This result demonstrates the specificity of the GAL4/UAS system for ablation of basal cells in the epidermis. Furthermore, the induced cell death is MTZ dose dependent and increasing concentrations of the prodrug induce high amounts of cell death (Figure 3.2d).

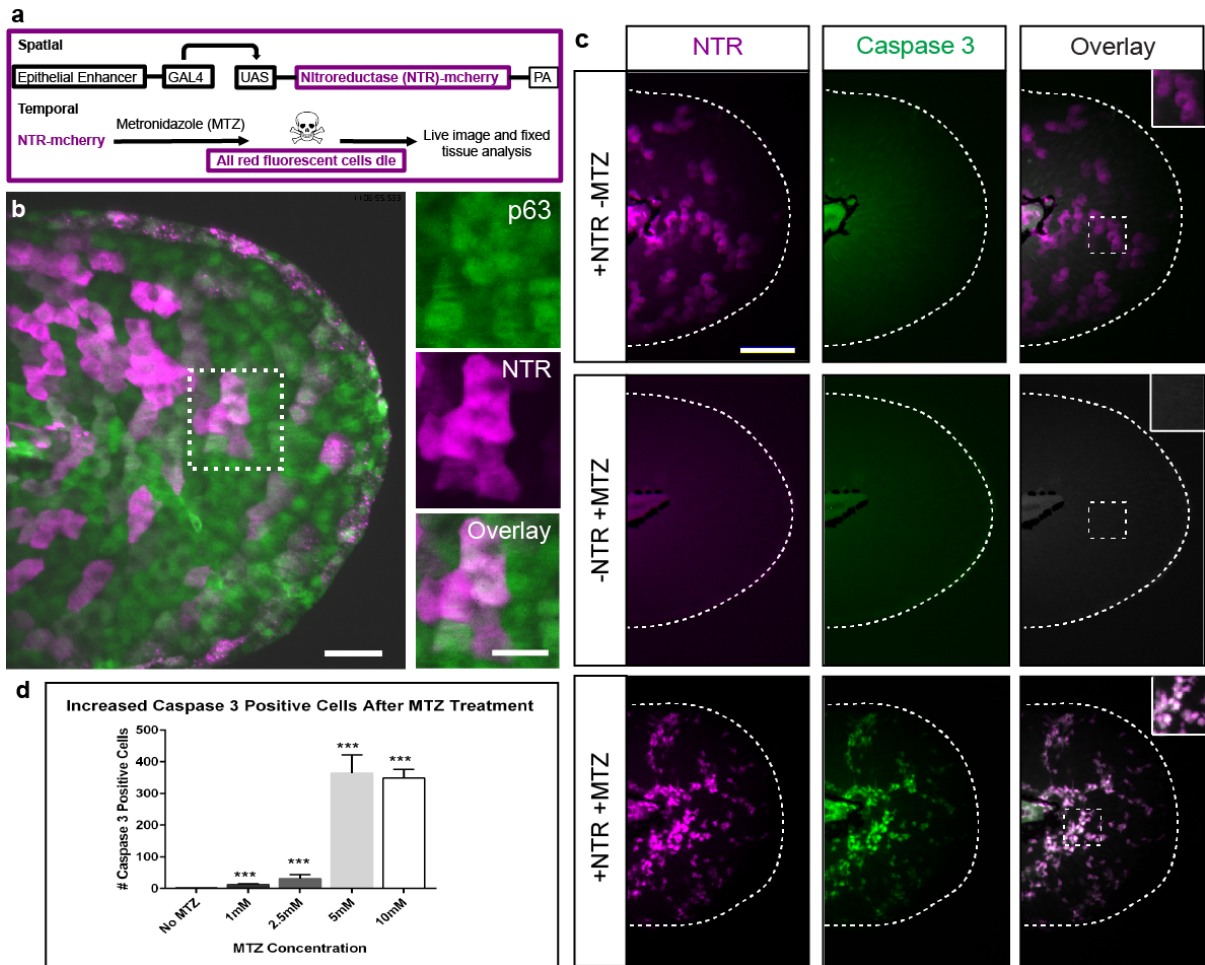


Figure 3.2. Controlled ablation of p63 positive epithelial cells.

(a) The *zc1036a* GAL4 enhancer trap line is expressed in a subset of basal epithelial cells. This line is used to drive expression of mCherry fluorescently tagged nitroreductase (NTR) to selectively ablate cells with the addition of metronidazole (MTZ). (b) NTR is expressed in a subset of p63 positive basal stem cells in a mosaic pattern, allowing for selective damage in only some basal cells (scale = 100 μ m, 50 μ m). (c) At 4 dpf, apoptosis (activated caspase 3 immunostain) occurs only in mCherry basal cells when the prodrug MTZ is added (scale = 100 μ m). (d) Quantification of caspase 3 positive cells reveals a steep cell death dose response for increasing concentrations of MTZ.

3.3 Rate of apoptotic basal cell clearance

Apoptosis is rapidly induced during the 4 hour MTZ treatment and results in high amounts of immediate cell death in the zebrafish (Figure 3.3a). After the CAPEC assay at 4 dpf, the prodrug is washed away and zebrafish are left to recover from the damage for two days to study how long apoptotic cells remain in the tissue. Quantification of cell death during this recovery revealed that cell clearance occurs at a fast rate, as apoptotic cells are rapidly cleared and significant declines in apoptotic cells are observed after 20 hours recovery (5 dpf) (Figure 3.3b). After 44 hours of recover (6 dpf), essentially all of the apoptotic basal cells have already been removed from the tissue. Interestingly, after apoptosis is induced, the NTR/caspase 3 positive cells no longer express p63, suggesting that this stem cell marker is turned off when the cell is damaged and undergoing death (Figure 3.3c). This system therefore allows us to control epithelial basal cell ablation and rapidly observe the dynamics of cell turnover and tissue homeostasis.

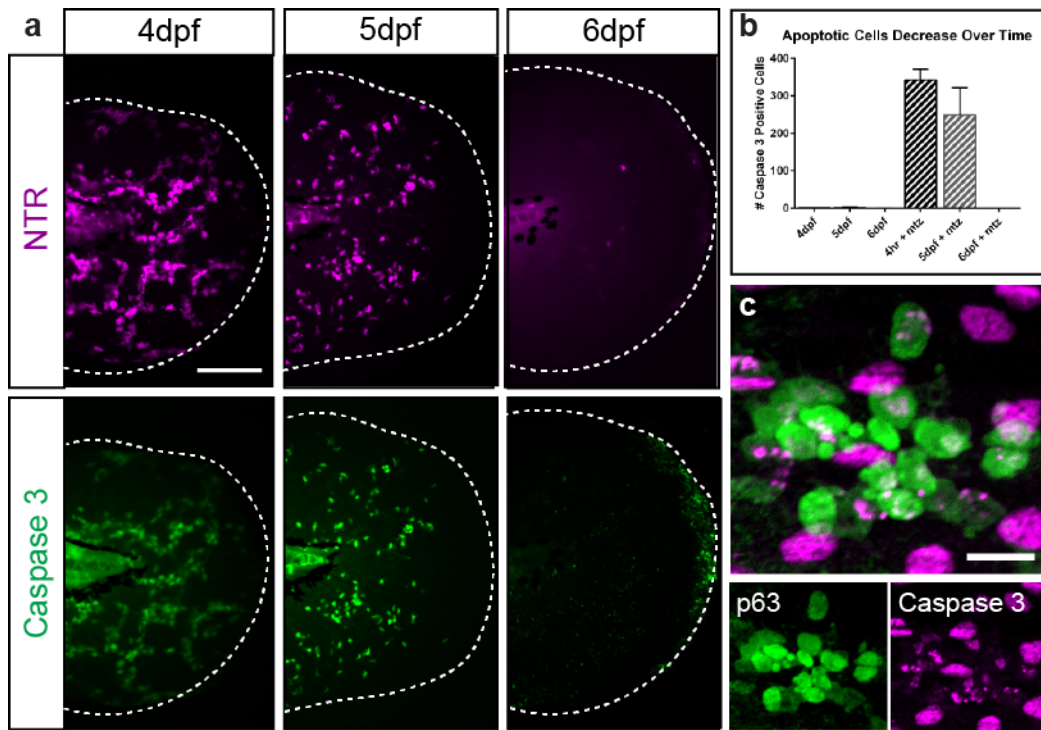


Figure 3.3 Clearance rate of apoptotic basal cells.

(a) The rate of apoptotic cell clearance can be monitored during recovery after the MTZ is washed away (scale = 100 μ m). (b) Cell death is induced immediately after prodrug treatment (4 dpf), and dying cells are cleared after 20 hours (5 dpf) and 44 hours (6 dpf) of recovery. (c) After cells are damaged and begin to undergo apoptosis, they are no longer p63 positive (scale = 15 μ m).

3.4 Dying basal cell removal from the epithelium

Apoptotic cells in the basal layer must be cleared from the tissue, possibly apical extrusion after migration through the bilayer to reach the surface epithelium or through engulfment by neighboring cells. Brightfield microscopy and scanning and transmission electron microscopy (SEM and TEM) were used to visualize the surface epithelium and the bilayer to observe the localization of apoptotic cells after the CAPEC assay. In the undamaged zebrafish epithelium, cells are organized into a smooth surface (Figure 3.4a). After damage, bulges are observed along the surface and disrupt epithelial tissue organization (Figures 3.4a, 3.4b). Yet, the surface epithelium remains intact despite the bulging and disorganization of the cells (Figure 3.4c). TEM showed that apoptotic basal cells align with this surface texture as dying cells are removed from the basal layer and push against the surface epithelium (Figure 3.4d). In order to maintain a functional barrier, the apical surface layer does not allow for apoptotic basal cell integration, as shown with E-cadherin immunostaining to visualize cell adhesion (Figure 3.5). Surface cell contacts remain intact despite the force from underlying apoptotic basal cells, and dying cells therefore reside in the interstitial space within the epithelial bilayer between the p63 positive basal cells and the krt4 positive periderm cells (Figure 3.6).

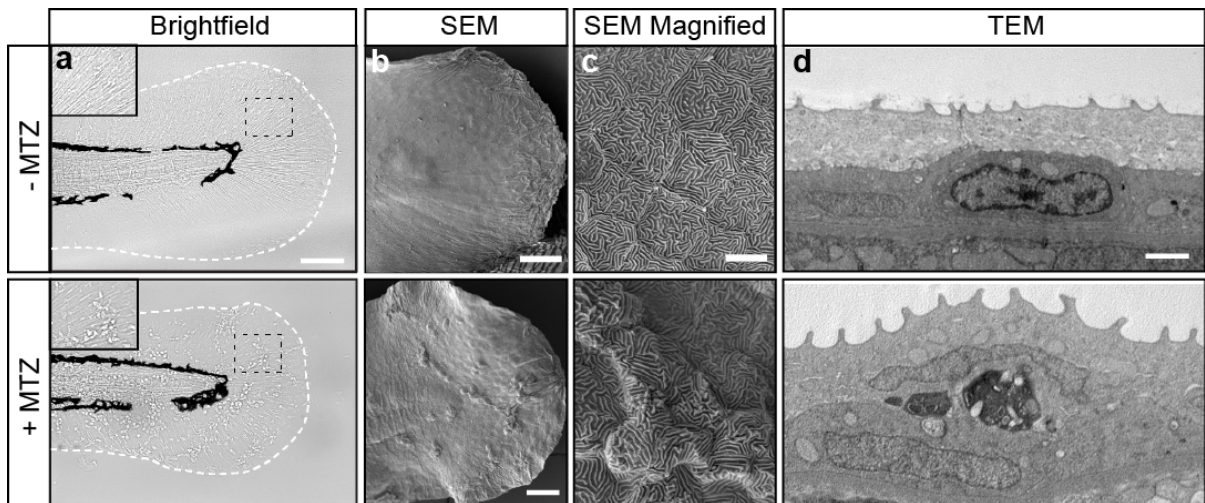


Figure 3.4 Basal cell clearance from the epithelium.

(a) Brightfield microscopy shows changes in epithelial surface texture after MTZ treatment (scale = 100 μm). (b, c) Scanning electron microscopy (SEM) reveals that the surface periderm remains intact despite swollen epithelial cells after damage (scales = 50 μm , 10 μm). (d) Transmission electron microscopy (TEM) of the epithelial bilayer shows that apoptotic basal cells push against the outer epithelium and reside between the basal layer and outer periderm (scale = 2 μm).

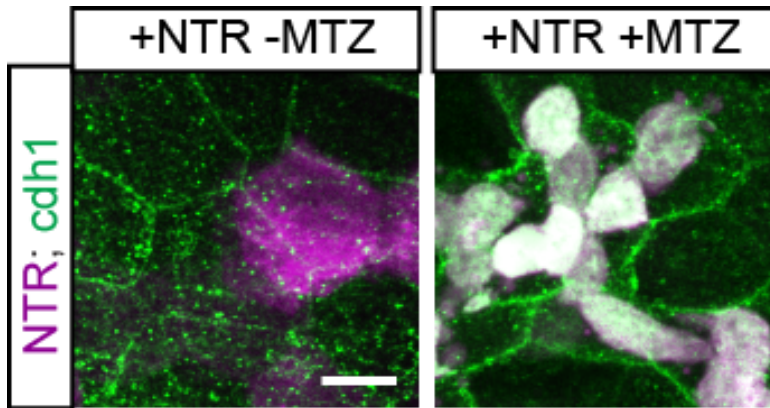


Figure 3.5 Visualization of epithelial cell adhesion.

E-cadherin immunostain (cdh1) with and without MTZ treatment to visualize cell adhesion in the surface epithelial layer (scale = 10 μ m).

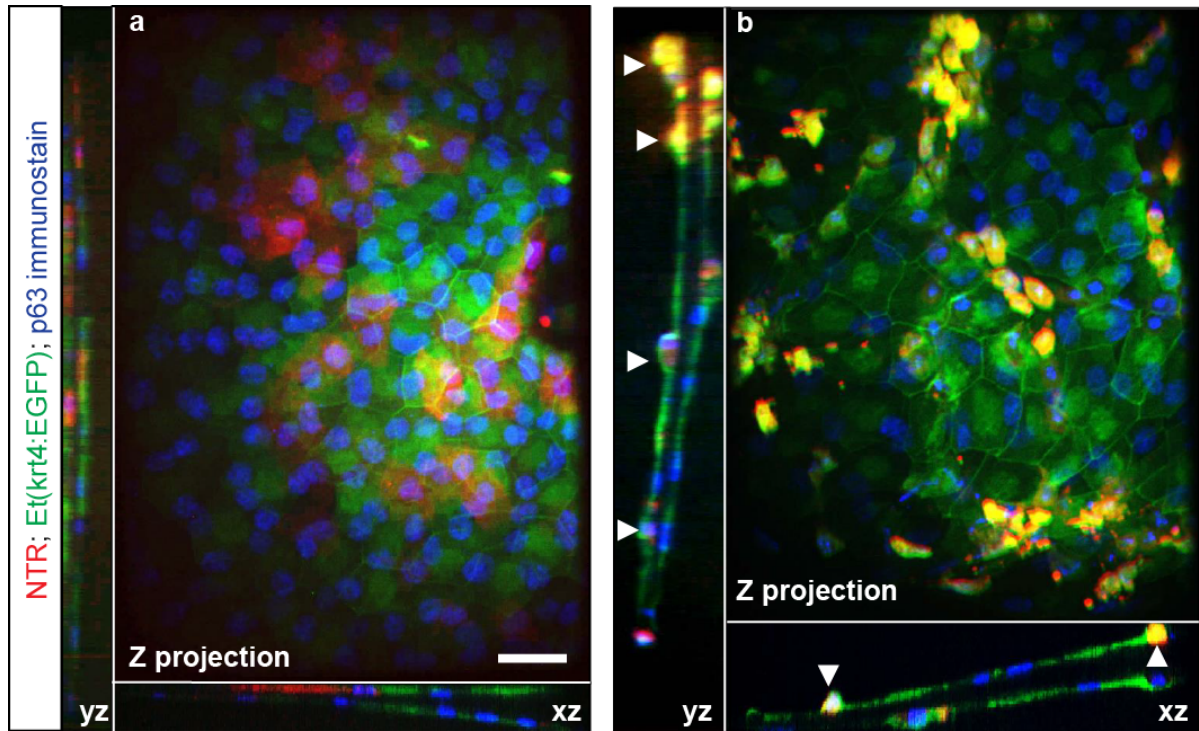


Figure 3.6 Apoptotic cell localization within the epithelial bilayer.

The *zc1036a* GAL4 line was crossed with the *Tg(KRT4:EGFP)* line which labels all apical periderm cells (Gong et al., 2002) and immunostained for p63 to label all basal stem cell nuclei. Periderm, basal, and NTR positive cells are all labeled to look for localization of NTR cells within the epithelial bilayer. (a) The untreated embryo shows the distinct bilayer and NTR cells reside in the basal layer (scale = 25 μ m). (b) The MTZ treated embryo shows NTR cells that are removed from the basal layer and push against the krt4 positive surface cells.

3.5 Visualization of clearance of apoptotic basal epithelial cells

Based on our ability to induce death within a subset of epithelia and monitor proliferation *in vivo*, we generated a transgenic line in which basal stem cells targeted and untargeted for damage were labeled simultaneously. This approach allows us to monitor the kinetics and dynamics of the entire basal layer of the epithelium. We crossed the *ΔNp63:EGFP* line expressed in all basal stem cells to the *zc1036a* GAL4 enhancer trap line expressed in a subset of basal cells (Figure 3.7a). We used spinning disc confocal microscopy to film the response after the CAPEC assay to observe how neighboring stem cells respond to damage within the basal layer. Since apoptotic basal cells remain trapped within bilayer, we used this time-lapse imaging to visualize how apoptotic cells in the tissue impact neighboring basal stem cells. Over time, several p63 positive stem cells were observed to surround neighboring mCherry apoptotic cells, indicating a role for epithelial basal cells as non-professional phagocytes (Figure 3.7b). TEM revealed the formation of autophagosomes, characterized by a double membrane, near apoptotic cells (Figure 3.7c). Apoptotic debris is engulfed by neighboring basal cells and autophagosome formation within these cells aids in clearance of debris from the tissue (Figure 3.7d). This observed engulfment supports a role for remaining basal stem cells as non-professional phagocytes to help efficiently clear the apoptotic basal cells trapped within the epithelial bilayer.

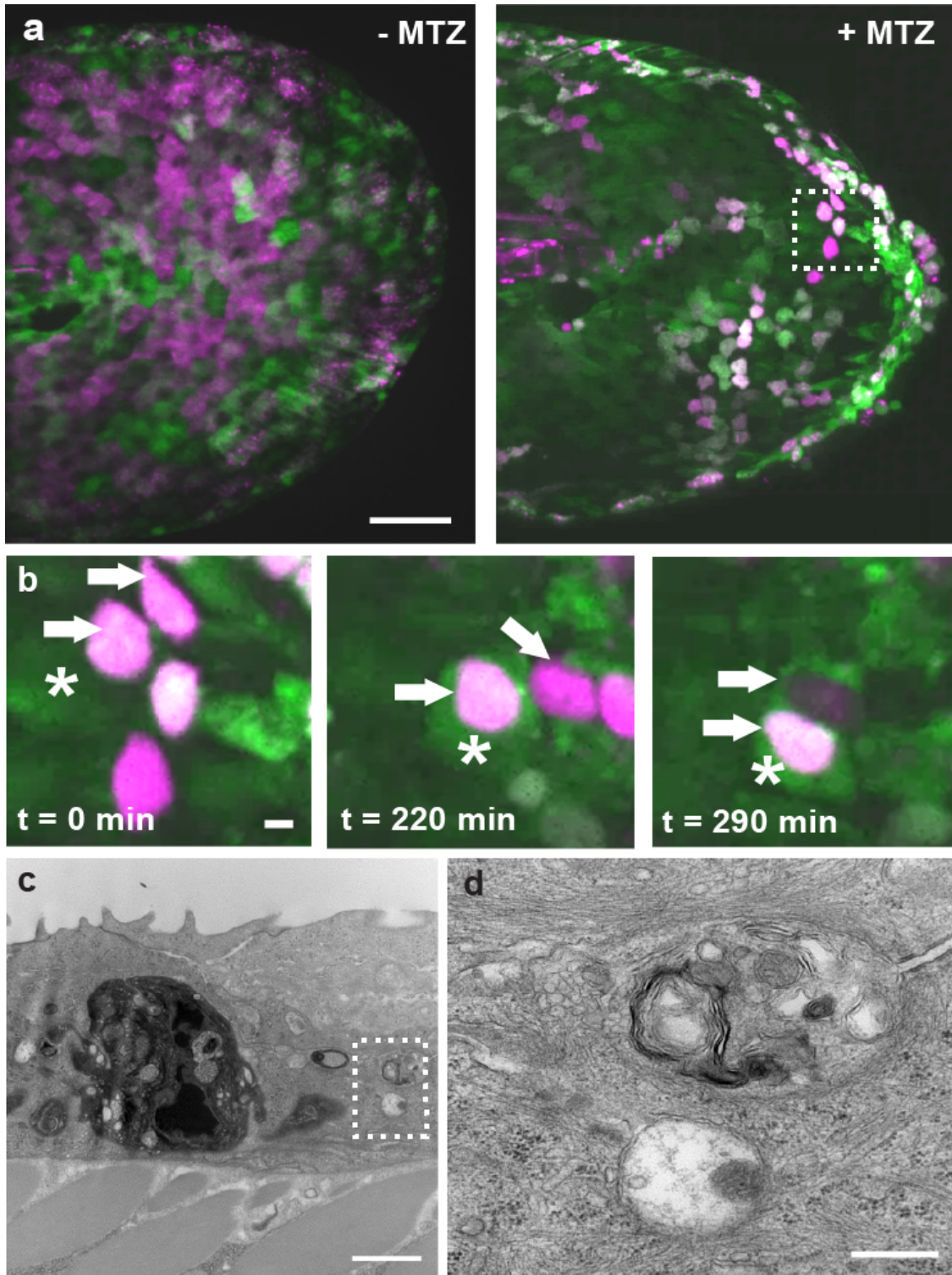


Figure 3.7 Basal cells act as broad specificity phagocytes.

(a) The *ΔNp63:EGFP* is expressed in all basal stem cells and was crossed to the *zc1036a* GAL4 enhancer trap line expressed in a subset of basal cells (scale = 100 μm). (b) *In vivo* time-lapse imaging showed a p63 positive cell (*) surrounds apoptotic mCherry basal cells (arrows) after MTZ treatment (scale = 5 μm). (c) TEM revealed formation of autophagosomes near apoptotic cells (scale = 2 μm). (d) Autophagosomes are formed by neighboring basal cells to clear engulfed apoptotic cell debris (scale = 500 nm).

3.6 Cell elimination and tissue damage promotes an increase in proliferation

Removal of the prodrug MTZ after the CAPEC assay allows for monitoring of proliferation and repair of the tissue during recovery (Figure 3.8a). A BrdU pulse-chase assay (Figure 3.8b) and H3P immunostain (Figure 3.9a) were used as cell cycle markers to quantify proliferation in the zebrafish fin epithelium during recovery from the CAPEC assay. Wild-type larvae treated with MTZ or transgenic animals lacking exposure to the prodrug have some background proliferation along the notochord area. In contrast, a significant increase in cell proliferation in the outer fin epithelium was observed 20 hours (5 dpf) after washout of the MTZ in transgenic animals. Quantification of BrdU or H3P positive cells revealed that this increased in proliferation was significant in transgenic animals after MTZ treatment (Figure 3.8c, 3.9b). Together, these results suggest the dying basal cells induce a robust proliferative response after CAPEC to replace lost cells.

To determine origin of the proliferating cells, we analyzed known markers of each cell type with the epithelial bilayer with *in vivo* timelapse imaging and immunostaining. Timelapse imaging of the *ΔNp63:EGFP* and *zc1036a* zebrafish after the CAPEC assay revealed division of p63 positive basal cells during recovery (Figure 3.8d). Immunostaining also showed that all BrdU and H3P cells were also positive for the epithelial stem cell marker p63 (20/20 cells, n = 2 fish) (Figure 3.8e). Together, these results suggest that cells within the basal layer proliferate to replace those eliminated by CAPEC.

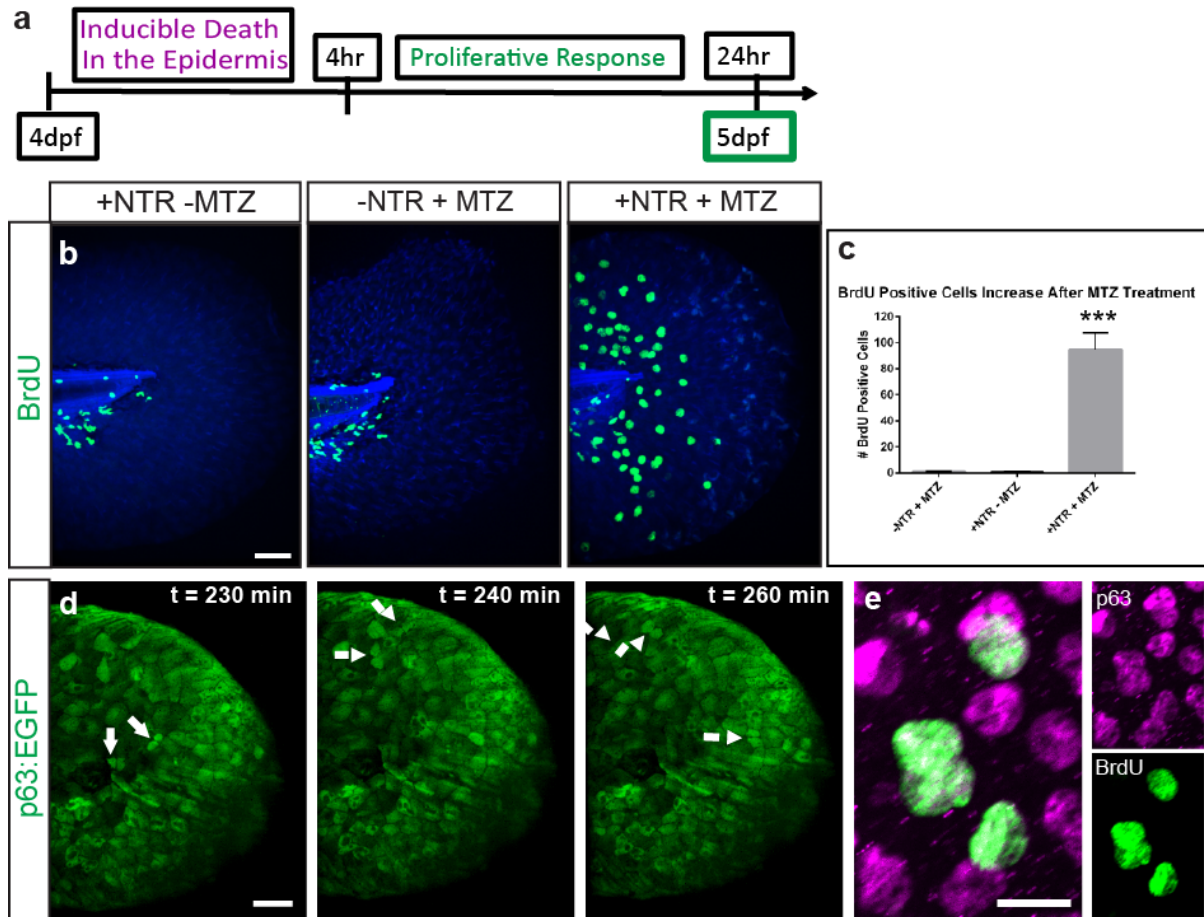


Figure 3.8 Basal stem cell proliferation replaces lost basal cells.

(a) After MTZ induced damage, the prodrug is washed away and animals are left to recover for 20 hours (5 dpf). (b) BrdU pulse-chase experiments reveal that cell division replaces lost cells during recovery (5 dpf) (scale = 200 μ m). (c) Quantification of BrdU positive cells shows a significant increase in proliferation in the outer fin epithelium. (d) *In vivo* time-lapse imaging of $\Delta Np63:EGFP$ crossed with $zc20136a$ during recovery from MTZ treatment shows p63 positive stem cell division (arrows) to replace lost basal cells (scale = 50 μ m). (e) Immunostaining showed that BrdU positive cells are also p63 positive (scale = 15 μ m).

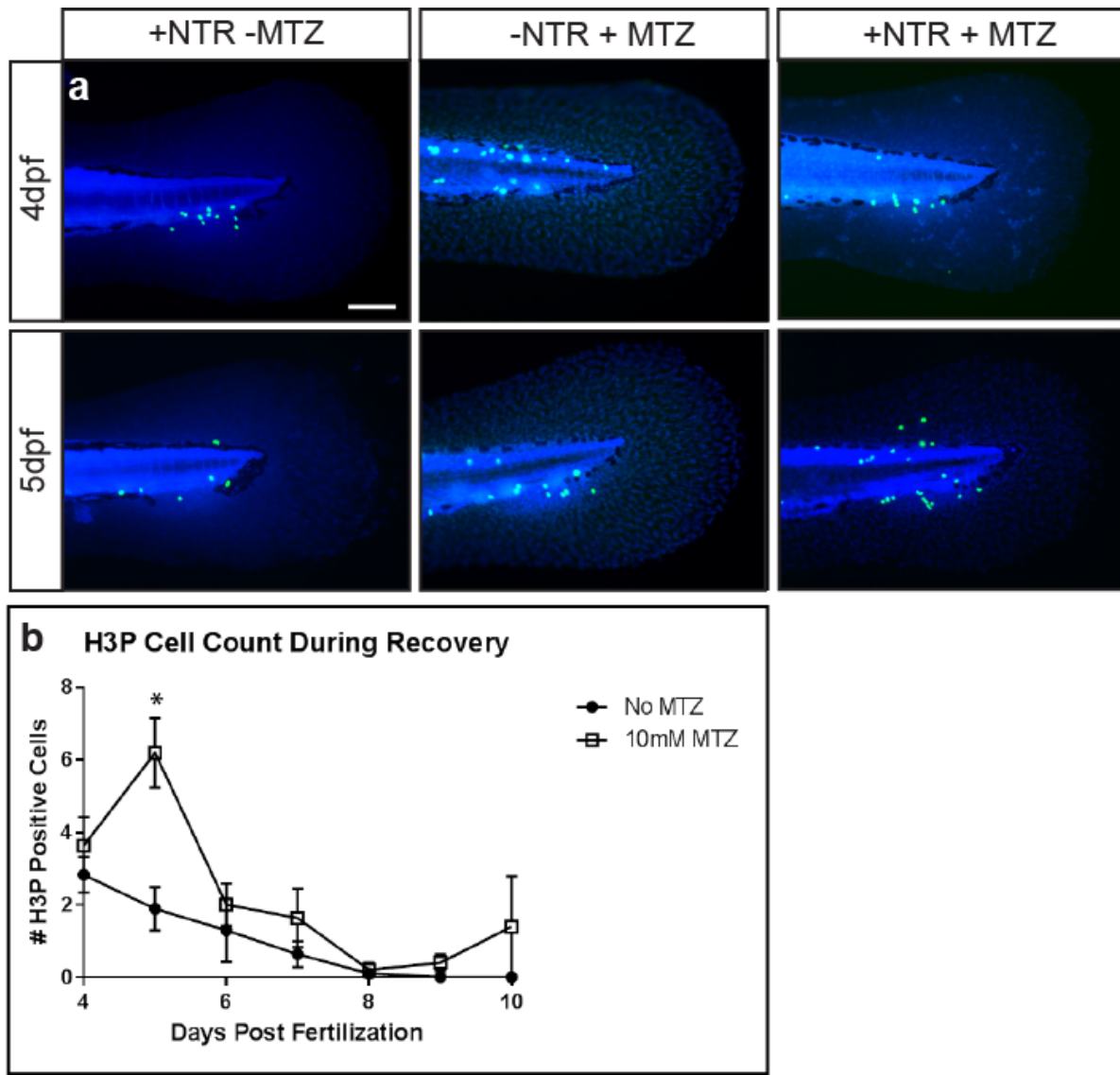


Figure 3.9 H3P as a marker of cell division.

(a) H3P was used as a marker of cell division in enzyme negative controls and in *zc1036a* embryos with or without MTZ to visualize the impact of basal cell damage on proliferation (scale = 100 μ m). (b) Quantification of H3P positive cells during recovery in *zc1036a* embryos with or without MTZ treatment.

3.7 Apoptotic cells produce mitogenic factors to promote replacement of lost cells

Studies in *Drosophila* and *Hydra* have shown that apoptotic cells can produce signals that induce proliferation in the surrounding cells of the tissue. Preliminary data reveal that after MTZ treatment, 52% of dying NTR positive cells are also positive for WNT3a, a mitogenic factor (Figure 3.10). The NTR positive cells which produce WNT have morphological characteristics of cells undergoing apoptosis. This signal may be involved in promoting the replacement of lost basal stem cells through proliferation of remaining basal cells. When the zebrafish embryos are also treated with a WNT3a chemical inhibitor during the CAPEC assay, there is 73.8% mortality, suggesting that WNT3a production is necessary for tissue maintenance after basal cell damage. WNT3a was not observed in untreated zebrafish and there was no mortality when treated with the inhibitor alone, supporting a role for the production WNT to promote tissue regeneration.

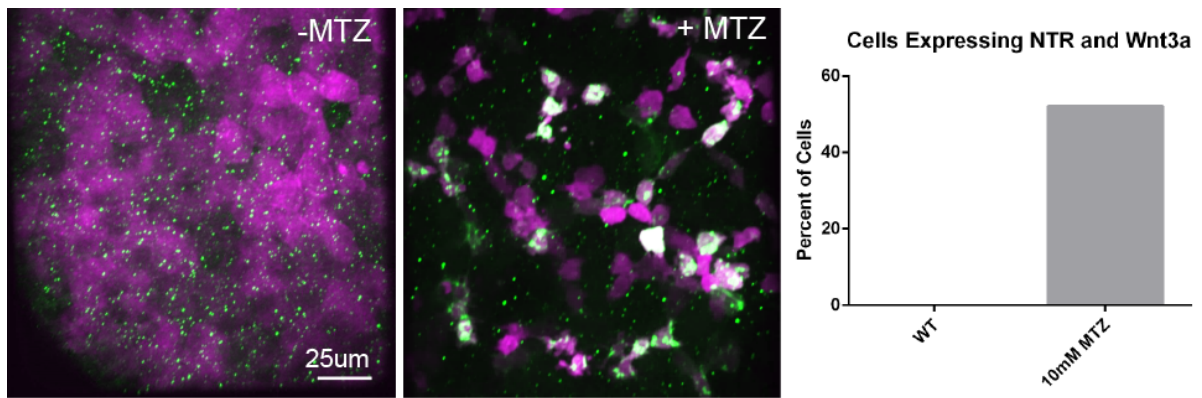


Figure 3.10 Apoptotic basal cells produce WNT3a.

After MTZ treatment, 52% of dying NTR cells (mCherry) are also positive for WNT3a (green immunostain) (n = 2-3 fish, 231 cells counted) (scale = 25 µm).

4. Discussion and Future Directions

The peripheral location and bilayered structure of the developing zebrafish epidermis makes it an ideal model for studying cell turnover *in vivo*. Little is known about how epithelial stem cells are renewed and cleared from the tissue and our results indicate that these basal cells can be cleared by engulfment. Epithelial cells can act as non-professional phagocytes to clear axon debris or basal cells within the epithelium (Mesa et al., 2015; Rasmussen et al., 2015). Tissue damage then promotes proliferation of remaining basal cells to replace those that are lost and maintain homeostatic cell numbers. In this study, we show that apoptotic basal cells remain trapped within a bilayered epithelium and autophagosomes form around the debris (Figure 4.1). Neighboring basal stem cells then engulf these autophagosomes to clear apoptotic cell debris. These same cells also divide to replace those that are lost while the tissue maintains a functional barrier. Based on our findings, WNT3a is present in apoptotic cells to provide the mitogenic signal driving compensatory proliferation. Controlled ablation of a subset of epithelial basal cells with the CAPEC assay allows us to study *in vivo* how cells are removed from the tissue and how they are replaced in a specific and targeted manner.

Since engulfment is an essential component of proper cell clearance, a mechanistic understanding of epithelial cell phagocytosis may reveal mechanisms that are conserved between species. Engulfment genes identified in *C. elegans* (Figure 4.2) present a list of possible genes to investigate in zebrafish in relation to cell turnover (Chung et al., 2000; Shen et al., 2013). In the case of our model system, apoptosis is induced after the CAPEC assay, and phagocytosis-activating signals associated with apoptotic corpses identified in other model systems may prove to identify common genes across species. Cell death corpses

are removed rapidly and in this way can prevent an inflammatory response, tissue damage, and autoimmunity (Chung et al., 2000; Reddien and Horvitz, 2004).

When death is blocked and apoptosis cannot proceed, what is the impact on neighboring basal cells in the tissue? Studies in *Drosophila* and *Hydra* show that when death is inhibited, these apoptotic “undead cells” linger in the tissue and continue to stimulate stem cell division, ultimately resulting in tissue overgrowth (Ryoo et al., 2004; Fan and Bergmann, 2008; Chera et al., 2009). Rapid clearance is therefore important to achieve an appropriate amount of compensatory proliferation to replace lost cells. These studies from mono-layered epithelium likely reflect similar effects on stem cell proliferation in response to damage in bilayered epithelium. This hypothesis could be tested by blocking apoptosis in the developing zebrafish epithelium after the CAPEC assay to establish “undead cells” that remain trapped within the tissue. Inhibition could be accomplished either genetically or with chemical inhibitors to prevent apoptosis from proceeding in the damaged basal cells that are targeted for death.

If engulfment and damaged cell clearance is impaired, what is the impact on proliferation, cell turnover, and overall tissue maintenance? When epithelial cells are exposed to and engulf nearby apoptotic cells, there is an increase in transcription factors relating to cell growth and angiogenesis, including VEGF (Golpon et al., 2004). These factors help the wound healing process by promoting survival and proliferation of remaining cells; however, if not kept in check, these signals could instead lead to apoptosis-resistance (Golpon et al., 2004). The opposite scenario is also problematic if there is a decrease in survival factors and therefore increased apoptosis, as seen in patients with chronic obstructive pulmonary disease (COPD) (Henson et al., 2006). COPD patients have greater

numbers of apoptotic cells in comparison to healthy individuals or smokers without COPD, and this increased cell death persists after smoking cessation (Henson et al., 2006). Typically, cell turnover involves scattered apoptosis and cell replacement throughout the tissue, but in COPD patients, clearance is less effective and apoptotic cells are not easily recognized for engulfment (Henson et al., 2006). Without engulfment, stimulation for cell replacement is absent and lingering apoptotic cells can proceed to lyse and induce an inflammatory response and subsequent tissue damage (Henson et al., 2006). When apoptotic cell clearance via engulfment or cell replacement is disrupted, the tissue cannot properly renew and replace cells during homeostatic tissue maintenance, leading to disease progression.

In summary, these findings indicate novel mechanisms for stem cell clearance and replacement as well as the connection between these two processes. When a subset of basal stem cells is targeted for death in bilayered epithelia, they are cleared from the tissue through engulfment by neighboring basal cells. As these cells are undergoing apoptosis, they produce a mitogenic factor, WNT3a, to stimulate remaining basal cells to proliferate. Knowledge of basal cell homeostasis and how the epithelium maintains a barrier function contributes to our understanding of cell turnover and how disruptions in this process can result in epithelial pathologies and diseases.

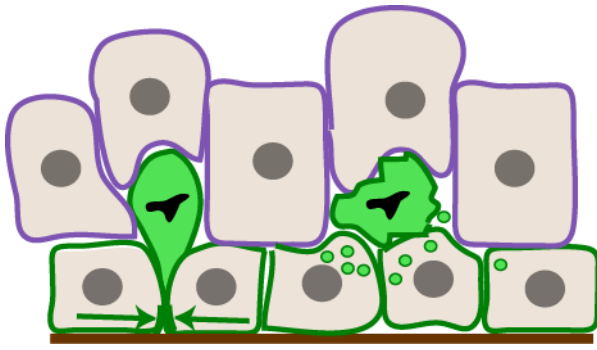


Figure 4.1 Cell clearance from the basal layer.

Apoptotic basal cells move out of the basal layer and toward the apical surface periderm.

They remain trapped in the tissue between the two layers of the epithelial bilayer and autophagosomes form around apoptotic cell debris. Autophagosomes are then engulfed by neighboring basal cells.

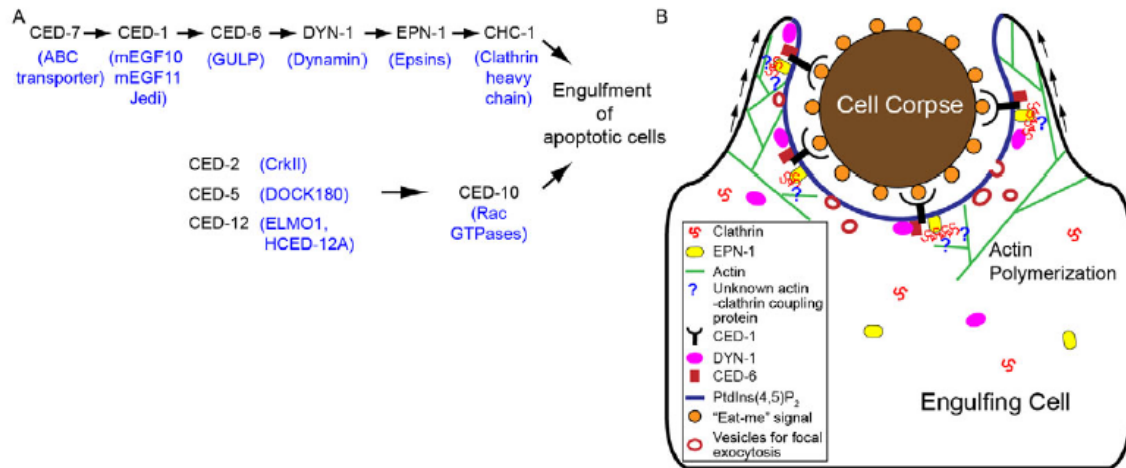


Figure 4.2 *C. elegans* cell corpse engulfment genes.

Genes involved in apoptotic cell engulfment in *C. elegans* with human homologues shown in parentheses (Modified with permission from **Shen, Q., He, B., Lu, N., Conradt, B., Grant, B. D. and Zhou, Z. (2013).** Phagocytic receptor signaling regulates clathrin and epsin-mediated cytoskeletal remodeling during apoptotic cell engulfment in *C. elegans*. *Development* **140**, 3230-3243.

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Vitae

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