

Vertebrate Epidermal Cells Are Broad-Specificity Phagocytes That Clear Sensory Axon Debris

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Cellular debris created by developmental processes or injury must be cleared by phagocytic cells to maintain and repair tissues. Cutaneous injuries damage not only epidermal cells but also the axonal endings of somatosensory (touch-sensing) neurons, which must be repaired to restore the sensory function of the skin. Phagocytosis of neuronal debris is usually performed by macrophages or other blood-derived professional phagocytes, but we have found that epidermal cells phagocytose somatosensory axon debris in zebrafish. Live imaging revealed that epidermal cells rapidly internalize debris into dynamic phosphatidylinositol 3-monophosphate-positive phagosomes that mature into phagolysosomes using a pathway similar to that of professional phagocytes. Epidermal cells phagocytosed not only somatosensory axon debris but also debris created by injury to other peripheral axons that were mislocalized to the skin, neighboring skin cells, and macrophages. Together, these results identify vertebrate epidermal cells as broad-specificity phagocytes that likely contribute to neural repair and wound healing.

Key words: axon; phagocytosis; skin; somatosensory; Wallerian degeneration; zebrafish

Introduction

The skin protects animals from environmental insults by functioning as both a physical barrier and a sensory organ. The outermost layers of skin, the epidermis, are densely innervated by axonal endings of somatosensory neurons, which intermingle with epithelial cells (for review, see Dubin and Patapoutian, 2010). Somatosensory axon endings degenerate following cutaneous injuries, leading to sensory abnormalities (Inbal et al., 1987; Healy et al., 1996; Theriault et al., 1998; Rajan et al., 2003). Repair of sensory endings in the epidermis is impaired in patients with peripheral neuropathies, such as those associated with human immunodeficiency virus or diabetes (Polydefkis et al., 2004; Hahn et al., 2007).

Clearance of cellular debris is a key step in tissue and nerve repair. In most cases, blood-derived professional phagocytes, including neutrophils, macrophages, and microglia, phagocytose neuronal debris (for review, see Vargas and Barres, 2007). Specialized nonprofessional phagocytes, such as peripheral glia (Stoll et al., 1989; Lewis and Kucenas, 2014) and the retinal pigment epithelium (RPE; Young and Bok, 1969), can phagocytose neuronal debris in specific contexts. Understanding how cutaneous sensory endings are repaired requires identifying the cell types

that clear axon debris in the skin. Although professional phagocytes play a major role in healing skin wounds, in *Caenorhabditis elegans* and *Drosophila* epidermal cells contribute to phagocytosis of apoptotic neurons and degenerating neurites (Robertson and Thomson, 1982; Hall et al., 1997; Han et al., 2014). Vertebrate epidermal cells can internalize melanosomes (for review, see Van Den Bossche et al., 2006), beads (Wolff and Konrad, 1972), bacteria (Åsbakk, 2001), and perhaps even cellular debris (Odland and Ross, 1968; Mottaz and Zelickson, 1970). However, whether they significantly contribute to phagocytosis and the degradation of debris during neural and cellular repair is unknown.

Axon degeneration and clearance in the zebrafish skin is a rapid and stereotyped process (Martin et al., 2010). If cutaneous axon degeneration is delayed, persistent axon fragments repel regenerating axons (Martin et al., 2010), implying that an understanding of the debris clearance process may ultimately suggest approaches for improving cutaneous reinnervation. Here we use the zebrafish system to provide the first description of the fate of axon debris in the vertebrate skin.

Materials and Methods

Zebrafish. Zebrafish (*Danio rerio*) were grown at 28.5°C on a 14 h/10 h light/dark cycle. Embryos were raised at 28.5°C in fish water (0.3 g/L Instant Ocean Salt, 0.1% methylene blue). The following zebrafish strains were used: AB (wild-type), *nacre* (*nac*^{w2}) (Lister et al., 1999), *leo1*^{la1186} (Nguyen et al., 2010), and *cloche* (*clo*^{m39}) (Stainier et al., 1995). The following previously described transgenes were used: *Tg(isl1[ss]:Gal4-VP16,UAS:DsRed)*^{z234} (O'Brien et al., 2009), *Tg(mpeg1:mCherry)*^{g123} (Ellett et al., 2011), *Tg(lyz:EGFP)*^{nz117} (Hall et al., 2007), *Tg(UAS:Lifact-GFP)*^{mu271} (Helker et al., 2013), *Tg(krt5:EGFP)*^{ncc100} (Hu et al., 2010), *Tg(Tru.p2rx3a:LEXA-VP16,4xLEXAop:mCherry)*^{Ja207} (Palanca et al., 2013), *TgBAC(neurod:EGFP)*^{m11} (Obholzer et al., 2008), *Tg(krt4:DsRed)*^{Ja203} (O'Brien et al., 2012), and *Tg(h2afx:EGFP-rab5c)*^{mw5}, *Tg(h2afx:EGFP-rab7)*^{mw7}, and *Tg(UAS:mCherry-rab5c S34N)*^{mw33} (Clark et

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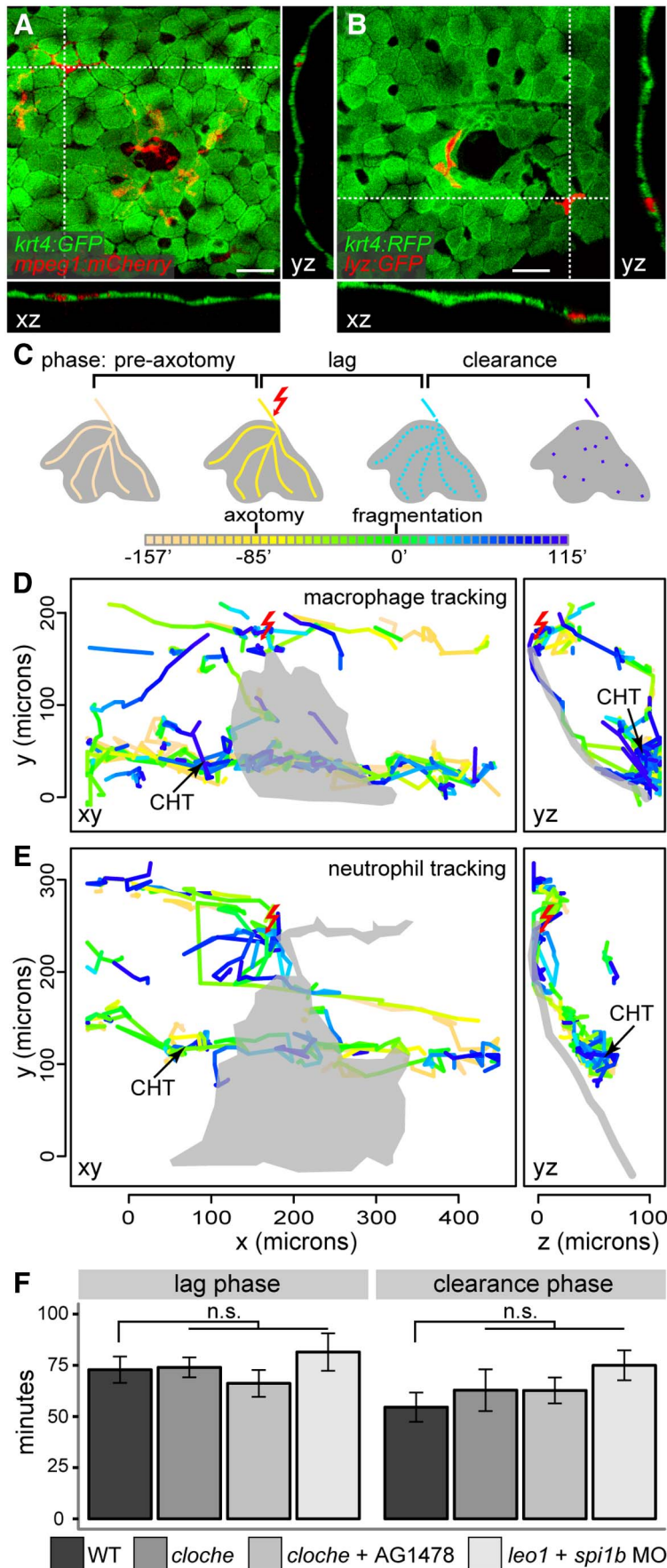
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al., 2011). Zebrafish of either sex were used for this study. All experiments using zebrafish were approved by the University of California, Los Angeles (UCLA) Chancellor's Animal Research Committee.

Plasmid construction. Plasmid cloning was performed using the Gateway-based Tol2Kit (Kwan et al., 2007). The following plasmids have been described previously: p5E-4xUASnr (Akitake et al., 2011), p5E-*krt4* (O'Brien et al., 2012), pME-*LEXA-VP16,4xLEXAop* (Palanca et al., 2013), pME-*EGFP*, p3E-*EGFP-polyA*, p3E-*polyA*, and pDestTol2pA2 (Kwan et al., 2007). The *neurod:mTangerine* plasmid was a gift from Alex Nechiporuk (Oregon Health & Science University, Portland, OR). The entry vectors p5E-*krt5*, p5E-*kr11c19e*, p5E-*isl1[ss]*, pME-*Gal4FF*, pME-*EGFP-2xFYVE*, pME-*lamp1*, and p3E-*tdTomato* were cloned by recombining PCR products into pDONR P4-P1R (p5E), pDONR 221 (pME), or pDONR P2R-P3 (p3E). The following oligonucleotides and templates were used in plasmid construction: p5E-*krt5* (5'-GGGGCAACTTTGTATAGAAAAGTTGGCACAACCTAACGCACTCTGC-3', 5'-GGGGACTGCTTTTTTGTACAAAAGTTGGGTGAGGATCAGAAAAAGAGCA-3'; zebrafish genomic DNA; Hu et al., 2010); p5E-*kr11c19e* (5'-GGGGACAACCTTGTATAGAAAAGTTGCAACAACAATCCACCTCAAGAGT-3', 5'-GGGGGACTGCTTTTGTACAAAAGTTGGATGGTGGTGGTGTCTTACTCT-3'; zebrafish genomic DNA; Lee et al., 2014); p5E-*isl1[ss]* (5'-GGGGACAACTTGTATAGAAAAGTTGCTCGAGCCTCGGCTCAGTT-3', 5'-GGGGACTGCTTTTGTGTACAAAAGTTGGAATCTGACACA GAATTGAATTG-3'; *isl1[ss]:Gal4-VP16, UAS:GFP* plasmid; Sagasti et al., 2005); pME-*Gal4FF* (5'-GGGGACAAGTTTGTACAAA AAAGCAGGCTGCCACCATGAAGCTAC TGCTTCTATC-3', 5'-GGGGACCACTTT

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Figure 1. Leukocytes do not associate with degenerating peripheral arbors. **A, B**, Confocal images of the trunk of transgenic zebrafish larvae at 3 dpf showing the epidermis [*Tg(krt4:RFP)* or *Tg(krt4:EGFP)*] in green and macrophages [*Tg(mpeg1:mCherry)*] or neutrophils [*Tg(lyz:EGFP)*] in red. **C**, Experimental schematic showing axotomy and degeneration of a peripheral sensory axon in the skin. The innervated territory is gray. Throughout the article, 0 min represents the time of axon fragmentation. **D, E**, Representative tracings of leukocyte behavior before and after axotomy. Leukocyte tracks are colored according to the phase of axon degeneration, as indicated in **C**. A single somatosensory peripheral axon was axotomized in 2 dpf zebrafish transgenic for either *Tg(mpeg1:mCherry)* (**D**) or *Tg(lyz:EGFP)* (**E**). The red arrow points to the axotomy site. The caudal hematopoietic tissue (CHT) is indicated. **F**, Quantification of the lag and clearance phases following axotomy of single somatosensory peripheral axons at 54 hpf in the indicated genetic backgrounds. The durations of the lag and clearance phases were unaffected by the removal of leukocytes [*cloche* or *spi1b* morpholino (MO)] or peripheral glia (AG1478 or *leo1*). Data are represented as the mean \pm SEM. n.s., Not significant ($p > 0.16$; one-way ANOVA with Dunnett's *post hoc* test). $n = 7$ –12 for each condition. Scale bars, 50 μ m.

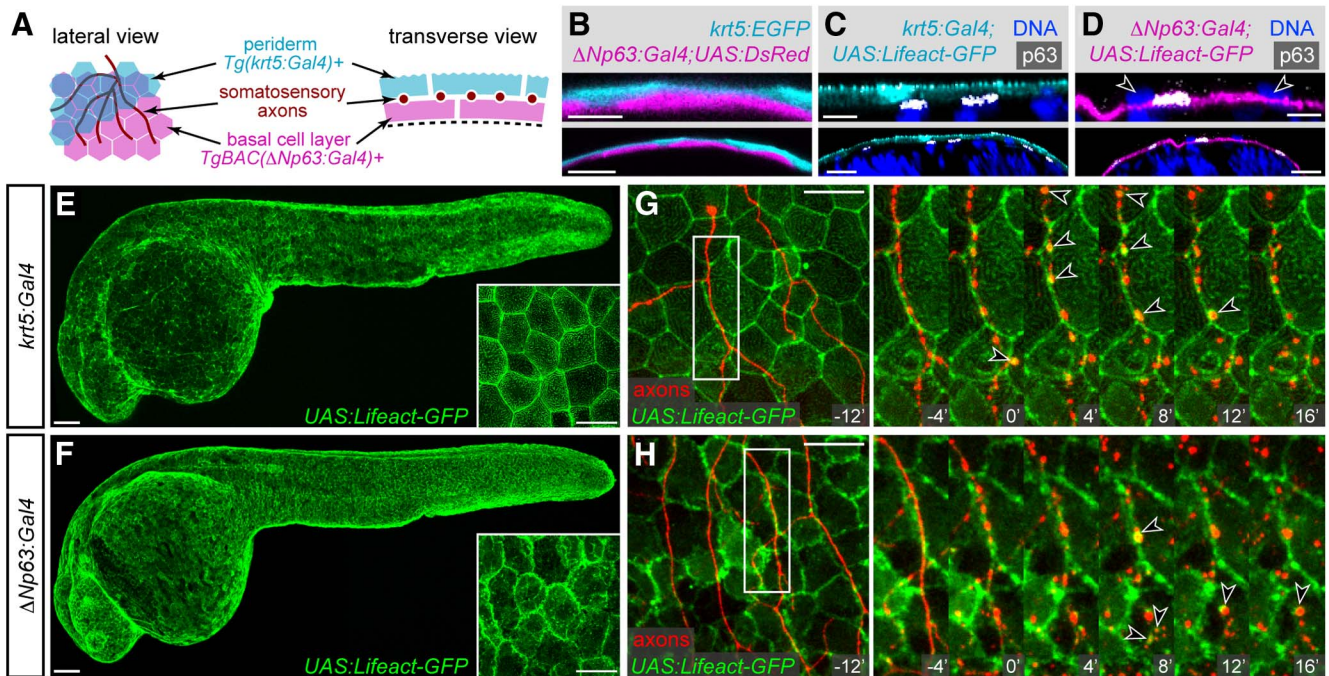


Figure 2. Epidermal actin dynamics during axon degeneration. **A**, Diagram showing larval epidermal anatomy in relation to somatosensory peripheral axons. Dashed line represents the basement membrane underlying the epidermis. **B–D**, Single optical sections showing transverse views of the periderm (cyan) and basal cells (magenta) in transgenic larvae. The top panels are higher-magnification views of the bottom panels. **C, D**, Reconstructed cross sections of animals fixed at 30 hpf and immunostained with anti-GFP and anti-p63 antibodies. p63 staining (white) shows the basal cell nuclei, and DAPI staining (blue) shows all nuclei. In **C**, note that the GFP-expressing periderm cells are superficial to the p63-positive basal cell layer. In **D**, the arrowheads indicate periderm nuclei superficial to the GFP- and p63-positive basal cell layer. **E, F**, Confocal images of transgenic larva fixed at 30 hpf and immunostained with an anti-GFP antibody. Insets show higher-magnification lateral views of the epidermis. **G, H**, Lateral confocal images from time-lapse imaging series showing F-actin dynamics in periderm (**G**) and basal cells (**H**) following transection of an axon (red) at 32 hpf. Arrowheads indicate transient enrichments of F-actin around axon debris. See also Movie 1. Scale bars: **B–D** (top panels), 10 μ m; **B–D** (bottom panels), 25 μ m; **E, F**, 100 μ m; **E, F**, Insets, 25 μ m; **G, H**, 25 μ m.

GTACAAGAAAGCTGGGTTTAGTTACCCGGGAGCATATCG-3'; pCS2+_{Gal4FF}_kanR; Busmann and Schulte-Merker, 2011); pME-EGFP-2xFYVE (5'-GGGACAAGTTTGTACAAAAAAGCAGGCTA-ACCGGTGCCACCAT-3', 5'-GGGACCACCTTTGTACAAGAAA GCTGGGTTT CAGTTATCTAGATCCGGTGGATCC-3'); pEGFP-2xFYVE; Gillooly et al., 2000); pME-lamp1 (5'-GGGACAAGTTTGTACAAAAAAGCAGGCTGGACCATGGCGCGAGCTGCAGGTGTT TGC-3', 5'-GGGACCACCTTTGTACAAGAAAGCTGGGTAGATGG TCTGGTACCCGGCGTGTG-3'; zebrafish cDNA; a gift from Matt Veldman, UCLA); and p3E-tdTomato (5'-GGGACAGCTTTCTT GTACAAAGTGGGCGCCACCATGGTGAGCAAGGGCGAGGAG-3', 5'-GGGACAACCTTTGTATAATAAAGTTGTCACCTCGAGT GACCCAGATCTTCCACCGCCCTGTACAGCTCGTCCATGCC TA-3'; ChEF-tdTomato plasmid; Lin et al., 2009).

Transgene generation. The Δ Np63:Gal4FF bacterial artificial chromosome (BAC) was created by modifying BAC DKEY-263P13, which contains 117.9 kb upstream and 19.0 kb downstream of the Δ Np63 α open reading frame (ENSDART0000065137). *iTol2-amp* was recombined into the backbone of DKEY-263P13, and the predicted Δ Np63 start codon was replaced by a *Gal4FF*-*polyA*-*KanR* cassette using a previously described protocol (Suster et al., 2011). *Tg(krt4:EGFP)^{la211}*, *Tg(krt5:Gal4FF)^{la212}*, *TgBAC(ΔNp63:Gal4FF)^{la213}*, *Tg(4xUAS:EGFP-2xFYVE)^{la214}*, and *Tg(isl1[ss]:LEXA-VP16,LEXAop:tdTomato)^{la215}* were created by the injection of *tol2* mRNA and either plasmid or BAC DNA into one-cell stage embryos and screening adults for germline transmission. At least two founders were identified for each transgene. Transgenic strains have been outcrossed for at least two generations.

Immunohistochemistry and lysotracker staining. Immunohistochemistry was performed essentially as described previously (Webb et al., 2007). Briefly, embryos were dechorionated and fixed in 4% paraformaldehyde in PBS overnight at 4°C. Embryos were washed 3 \times 5 min in 0.1% Triton X-100 in PBS (PBST), blocked for 1 h in 2% heat-inactivated goat serum, 2 mg/ml BSA in PBS, then incubated for 2 h with the appropriate primary

antibody. Primary antibodies were used at the following dilutions: mouse anti-p63, 1:100 (sc-8431, Santa Cruz Biotechnology); and rabbit anti-GFP, 1:500 (TP401, Torrey Pines Biolabs). Embryos were washed 4 \times 15 min in PBST then incubated for 2 h in secondary antibody. Alexa Fluor 568-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (Life Technologies) were diluted 1:500 in blocking solution. Embryos were washed 4 \times 15 min in PBST. To visualize nuclei, embryos were incubated for 5 min in 5 ng/ μ l DAPI in PBS, followed by 4 \times 5 min washes in PBST.

For lysotracker staining, animals were immersed in 10 μ M LysoTracker Deep Red (Life Technologies) and 1% DMSO for 45 min, and then washed several times in Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.2).

Confocal microscopy. Unless stated otherwise, images are maximum-intensity projections and were acquired by time-lapse microscopy using a laser-scanning microscope. Most imaging was performed on an LSM 510 confocal microscope (Carl Zeiss) equipped with a heated stage (PeCon) set to 28°C and 20 \times air [0.5 numerical aperture (NA)], 40 \times oil (1.3 NA), and 63 \times water (0.9 NA) objectives.

Ablations. Animals were anesthetized in 0.02% tricaine and embedded in 1.2% low-melt agarose; and microscope chambers were filled with Ringer's solution and sealed with vacuum grease. Ablations were performed on an LSM 510 META microscope (Carl Zeiss) equipped with a multiphoton laser (Coherent) using either a 25 \times water (0.8 NA) or 40 \times oil (1.3 NA) objective. The ablated region was specified using a 60–100 \times digital zoom and damaged with one to three scans of 813 nm light at 12–20% power.

Plasmid and morpholino injections. The *spi1b* antisense morpholino (Rhodes et al., 2005) was purchased from GeneTools, LLC. One-cell stage embryos were injected with 1–2 nl of *spi1b* morpholino at a concentration of 0.5 mM. For plasmid injections, 3–5 nl of DNA were injected into one-cell stage embryos at a concentration of 10–20 ng/ μ l. Unless stated otherwise, somatosensory axons were visualized using *Tg(isl1[ss]:LEXA-*

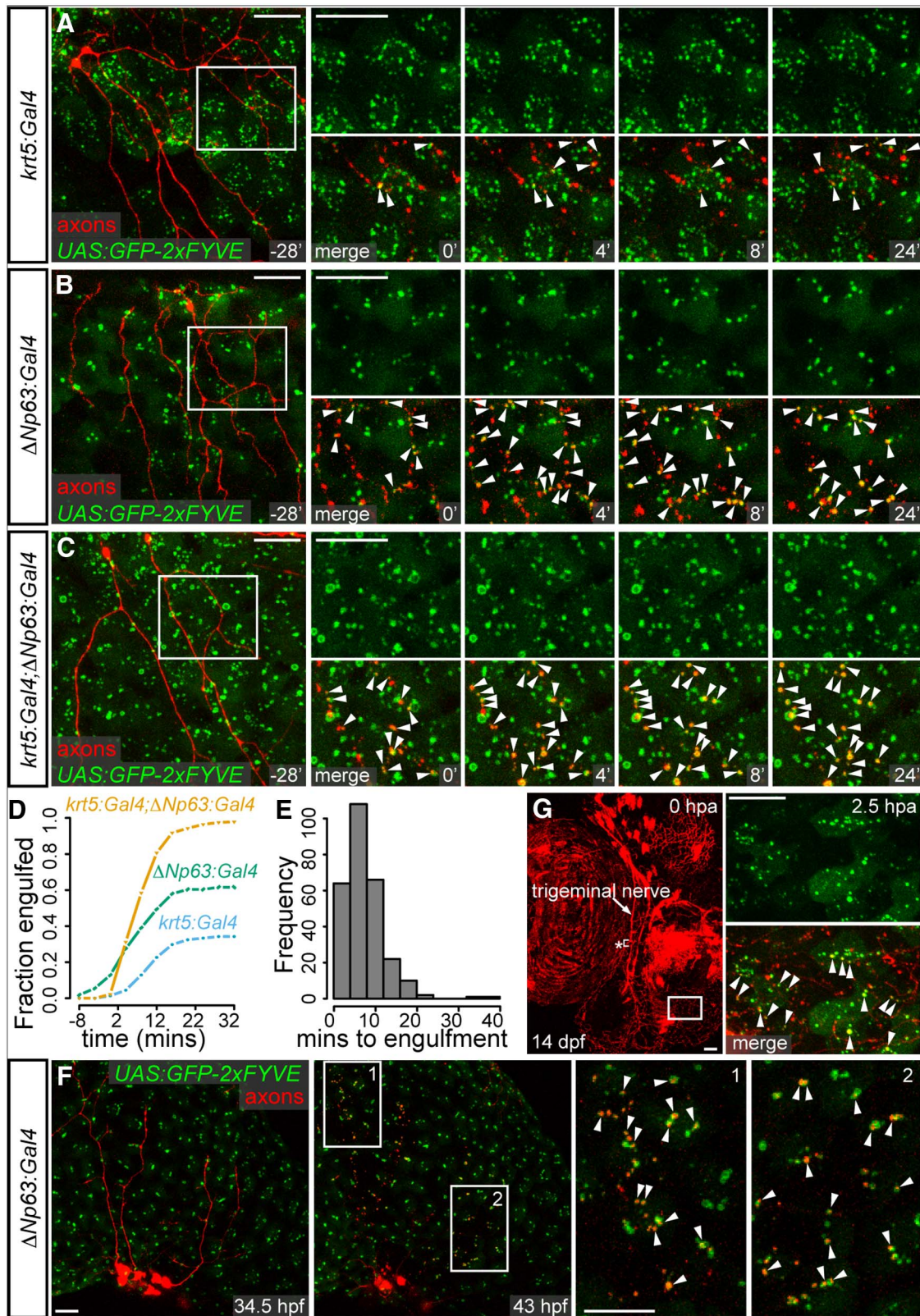


Figure 3. Both epidermal layers phagocytose axon debris. **A–C**, Confocal images of a phagosome reporter (green) expressed in periderm (**A**), basal cells (**B**), or both (**C**), following transection of an axon (red) at 32 hpf. In these and subsequent panels, arrowheads indicate phagosomes containing axon debris. See also Movie 2. **D**, The fraction of axon debris internalized, as assessed using the indicated Gal4 drivers and *Tg(UAS:GFP-2xFYVE)*. Data were pooled from at least two independent experiments and at least 164 fragments tracked per genotype. **E**, The time to internalization of axon debris by the epidermis in *Tg(krt5:Gal4)*; *TgBAC(ΔNp63:Gal4)*; *Tg(UAS:GFP-2xFYVE)* transgenic animals. **F**, Phagocytosis of axon debris following spontaneous cell death. **G**, Axotomy of trigeminal axons in a *nacre*; *Tg(Tru.p2rx3a:LEXA-VP16,4xLEXAop:mCherry)*; *TgBAC(ΔNp63:Gal4)*; *Tg(UAS:GFP-2xFYVE)* 14 dpf transgenic animal. Asterisk indicates the site of the axotomy. Right-hand panels are higher-magnification images of the boxed region. hpa, hours post axotomy. Scale bars, 25 μ m.

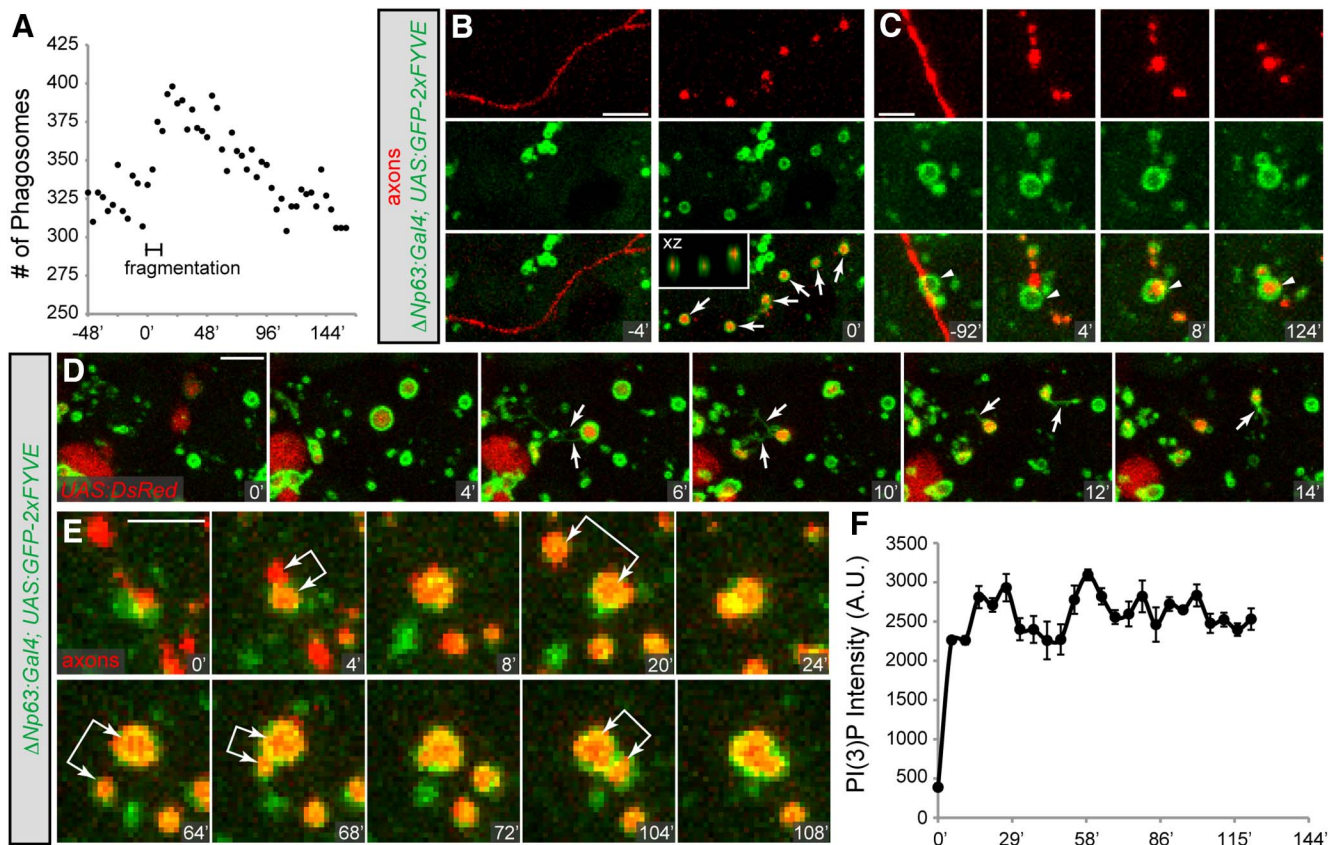


Figure 4. PI(3)P dynamics in the epidermis. Analysis of phagosomes in *TgBAC(ΔNp63:Gal4); Tg(UAS:GFP-2xFYVE)* transgenic larvae. **A**, Quantification of phagosome number in a 0.028 mm² region of the epidermis before and after axon fragmentation. **B**, Biogenesis of phagosomes (green) following axon (red) fragmentation. Arrows indicate newly formed phagosomes. Inset shows that phagosome membranes completely surround axon debris. **C**, An axon fragment (red) enters a pre-existing phagosome (green; arrowhead). **D**, Phagosomes (green) following phagocytosis of basal cell debris (red). Arrows indicate tubules extending from phagosomes following debris internalization. **E**, Fusion of phagosomes (green) containing somatosensory axon debris (red). Double-headed arrows indicate four sequential fusion events within a single cell. **F**, Quantification of PI(3)P intensity around phagocytosed axon debris ($n = 3$ fragments). Note the two peaks of PI(3)P intensity at 26.5 and 58 min are separated by a gap period (~20 min) of decreased fluorescence. Data are represented as the mean \pm SEM. A.U., arbitrary units. Ablations were performed at 32 (**A**, **C**, **E**), 50 (**F**), 56 (**B**), or 74 (**D**) hpf. Scale bars, 5 μ m.

VP16, *LEXAop:tdTomato*) or by injection of an *isl1[ss]:LEXA-VP16, LEXAop:tdTomato* plasmid.

Drug treatment. For ErbB inhibitor experiments, embryos were bathed in fish water supplemented with 4 μ M AG1478 (Calbiochem) and 1% DMSO beginning at 8 h postfertilization (hpf). Animals treated with 1% DMSO were used as controls.

Image analysis and statistics. Images were analyzed using ImageJ (Schneider et al., 2012). Leukocyte tracking in Figure 1 was performed using the TrackMate plugin and plotted using R (<http://www.R-project.org>). The whole-embryo images in Figure 2, *E* and *F*, were made using the Pairwise Stitching plugin. To assess the amount and timing of axon debris engulfment in Figure 3, *D* and *E*, individual debris fragments were followed using the Manual Tracking plugin. Only debris that could be tracked for at least 12 min was analyzed. The Squash plugin was used to count phagosomes in Figure 4*A*. Quantifications in Figures 4*F*, 5*B*, and 6, *C* and *D*, were performed by tracing a region of interest (ROI) around individual axon fragments using the Freehand tool and measuring fluorescence intensity and area within the ROI. Unless otherwise stated, at least 10 debris fragments were analyzed per time point. Statistical tests were performed using the R software package.

Results

Leukocytes are not required for clearance of cutaneous debris

Laser axotomy of cutaneous axon endings in larval zebrafish causes the severed axon arbor to fragment through a process known as Wallerian degeneration (WD), creating axon debris in the epidermis (Martin et al., 2010; O'Brien et al., 2012). Since

most studies of axon debris clearance following WD have implicated blood-derived professional phagocytes in this process, we hypothesized that these cell types also clear axon debris in the skin. To determine whether blood-derived cells can penetrate into the epidermis, we imaged larvae double transgenic for an epidermal marker and a marker of either neutrophils or macrophages. Consistent with a previous study (Herbomel et al., 2001), both types of leukocytes were found in the epidermis of unmanipulated animals (Fig. 1*A, B*).

To characterize the behavior of neutrophils and macrophages following axon damage, we axotomized cutaneous endings, and imaged leukocyte behavior before and after WD (Fig. 1*C*). As expected, both cell types responded to injury by migrating to the site of laser damage (Fig. 1*D, E*). However, neither cell type interacted significantly with degenerating peripheral arbor (Fig. 1*D, E*). To test whether blood-derived cells were required for the clearance of cutaneous debris, we examined the rate of axon fragmentation and clearance in *cloche* mutants, which lack all blood cells (Stainier et al., 1995). Consistent with our imaging results, removal of blood cells did not alter the rate of fragmentation or debris clearance (Fig. 1*F*). Because peripheral glia can also phagocytose axon debris, we repeated these experiments impairing the development of both blood cells and peripheral glia, but again detected no significant difference in the rate of debris clearance compared with controls (Fig. 1*F*). These results indicate that

neither leukocytes nor peripheral glia are primary phagocytes for cutaneous axon debris.

Actin dynamics in the epidermis during axon degeneration and debris clearance

The observation that neither blood-derived cells nor peripheral glia are required for cutaneous axon debris clearance led us to hypothesize that another cell type in the epidermis clears this debris. The larval zebrafish epidermis is a bilayered epithelium, consisting of an outer periderm and inner basal cell layer (Le Guellec et al., 2004), between which sensory axons arborize (Fig. 2*A,B*; O'Brien et al., 2012). We previously found that epidermal epithelial cells extend pseudopodia that contact degenerating axon debris (O'Brien et al., 2012). These pseudopodia are reminiscent of actin-rich phagocytic cups formed during phagocytosis of apoptotic corpses in *C. elegans* and opsonized beads by cultured macrophages (Swanson et al., 1999; Kinchen et al., 2005).

To visualize actin dynamics in both epithelial layers, we created Gal4 drivers specific to each and crossed them to the filamentous actin (F-actin) reporter *Tg(UAS:Lifeact-GFP)* (Fig. 2*C–F*; Helker et al., 2013). In both the periderm and basal cells, F-actin was transiently enriched around axon debris following fragmentation (Fig. 2*G,H*; Movie 1). Unlike the larval *Drosophila* epidermis, which accumulates actin around degenerating cutaneous dendrites before fragmentation (Han et al., 2014), we did not observe F-actin recruitment in either cell layer until after axon fragmentation (Fig. 2*G,H*; Movie 1), suggesting that in the zebrafish skin epidermal actin does not promote axon degeneration but may play a role in debris clearance.

Both layers of the epidermis phagocytose axon debris

The above observations revealed that the epidermis forms phagocytic cup-like structures around axon debris, prompting us to hypothesize that the epidermis phagocytoses this debris. To test this idea, we created a transgenic reporter for phagosomes, *Tg(UAS:GFP-2xFYVE)*, based on the fusion of GFP to a tandem repeat of the FYVE domain of Hrs, which has previously been shown to bind phosphatidylinositol 3-monophosphate [PI(3)P; Gillooly et al., 2000], a phospholipid enriched on the surface of endosomes and phagosomes. Crossing the periderm-specific Gal4 driver to *Tg(UAS:GFP-2xFYVE)* revealed spherical structures reminiscent of endosomes/phagosomes (Fig. 3*A*). Following injury of cutaneous axon endings, periderm cells internalized fragmented axon debris into these PI(3)P-positive phagosomes (Fig. 3*A,D*). Crossing the basal cell driver to the PI(3)P reporter demonstrated that basal cells also internalized axon debris into phagosomes (Fig. 3*B,D*). Together, the two epithelial layers ac-

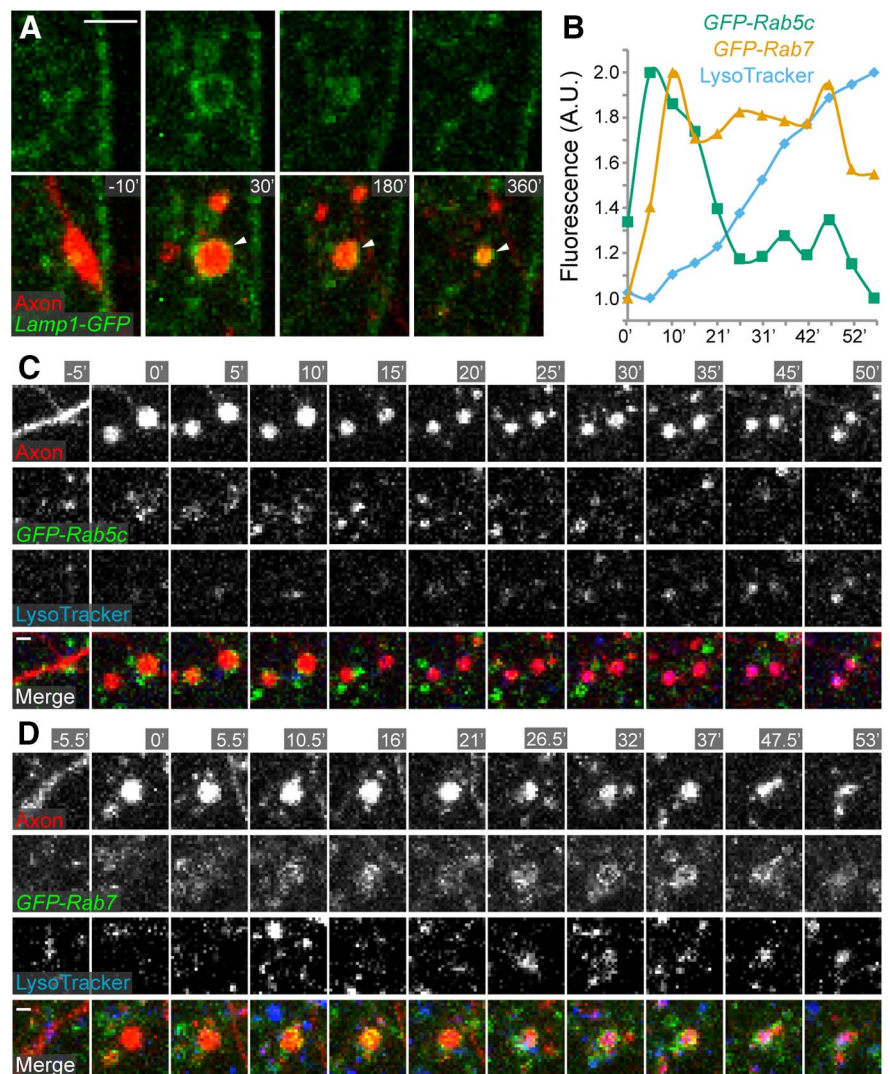


Figure 5. The endocytic pathway degrades debris in the epidermis. **A**, A periderm cell expressing *krtt1c19e:lamp1-GFP* (green) engulfs axon debris (red) in a *Tg(isl1[ss]:Gal4-VP16,UAS:DsRed)* transgenic animal. Arrowhead indicates a shrinking axon fragment that associates with Lamp1-GFP for several hours. **B**, Quantification of fluorescence intensity associated with axon debris over time. Data are represented as means. **C, D**, Confocal time-lapse images of axon debris phagocytosis in *Tg(h2afx:EGFP-rab5c)* (**C**) and *Tg(h2afx:EGFP-rab7)* (**D**) transgenic animals stained with lysotracker. Axons were transected at 32 (**A**) or 50 (**B, C, D**) hpf. Scale bars: **A**, 5 μm ; **C, D**, 1 μm .

counted for phagocytosis of at least 98% of axon debris ($n = 274/279$ fragments; Fig. 3*C,D*; Movie 2). Phagocytosis of axon debris by skin cells was rapid. The majority of debris was internalized into PI(3)P-positive phagosomes within 8 min of fragmentation (Fig. 3*E*). Axon debris created by spontaneous cell death was also internalized by the epidermis (Fig. 3*F*), indicating that this process is not specific to laser-induced injury.

A previous study found that cells with the stellate morphology characteristic of Langerhans cells, blood-derived professional phagocytes resident to vertebrate skin (Carrillo-Farga et al., 1990; Lugo-Villarino et al., 2010), populate the zebrafish epidermis by 12 d postfertilization (dpf; Wittamer et al., 2011). To determine whether epidermal cells still participate in clearance when these other phagocytic cells are present, we repeated these experiments at a later stage. Basal cells were still robustly phagocytic in 14 dpf animals (Fig. 3*G*). Together, these experiments identify epidermal cells as the primary phagocytes of cutaneous axon debris during development and following injury in zebrafish.

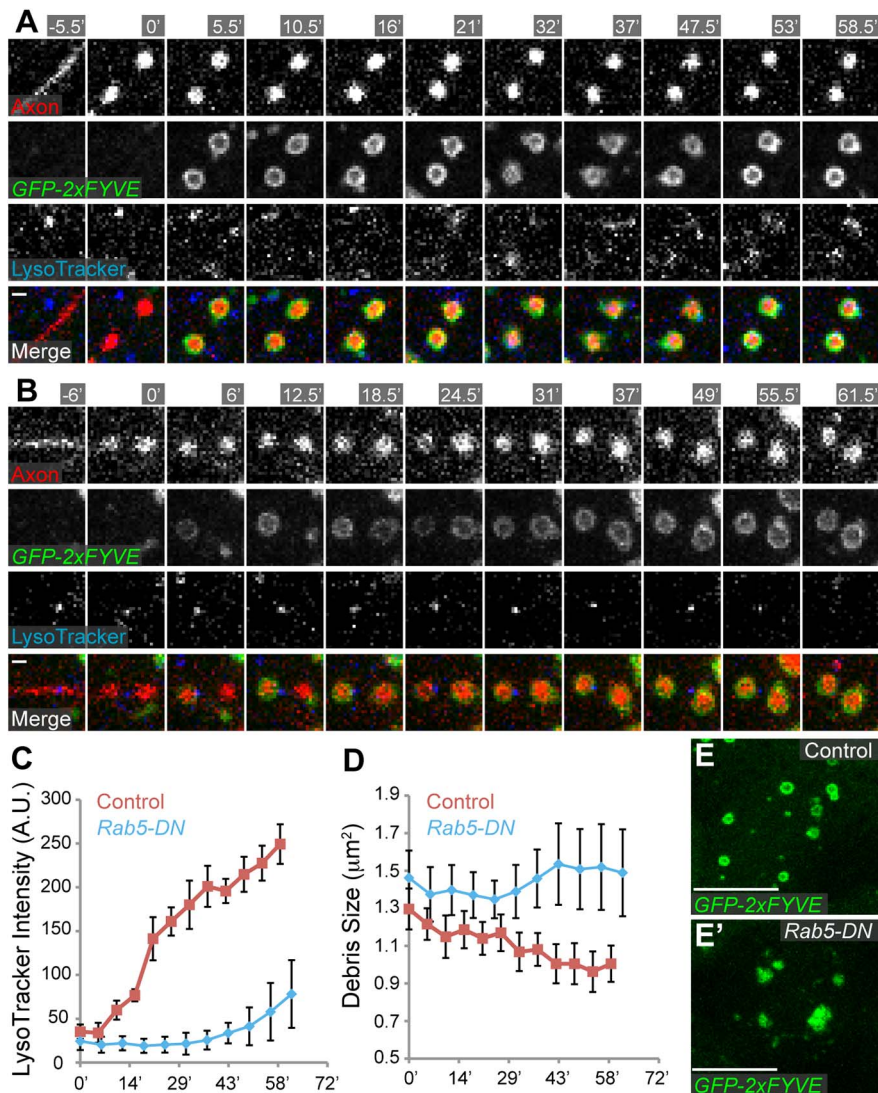


Figure 6. Rab5 activity is required for debris degradation by the epidermis. **A, B**, Confocal time-lapse images of *TgBAC(ΔNp63:Gal4); Tg(UAS:GFP-2xFYVE)* (**A**) and *TgBAC(ΔNp63:Gal4); Tg(UAS:mCherry-rab5c S34N); Tg(UAS:GFP-2xFYVE)* (**B**) transgenic animals stained with lysotracker. Axons were transected at 50 hpf. **C–E**, Analysis of phagocytosis in control and Rab5-DN animals. Transgenes are as in **A** (control) and **B** (Rab5-DN). **C, D**, Quantification of lysotracker intensity (**C**) and debris size (**D**) over time. Data are represented as the mean \pm SEM. **E**, Confocal images showing phagosome aggregation in Rab5-DN-expressing cells at 2 dpf. Scale bars, **A, B**, 1 μ m; **E**, 10 μ m.

Phagosome dynamics in the epidermis

PI(3)P-positive phagosomes are highly dynamic during phagocytosis of apoptotic corpses in *C. elegans* (Yu et al., 2008; Lu et al., 2012) but have not been well characterized in other *in vivo* systems. Our ability to perform time-lapse imaging of skin cells in live zebrafish with high temporal and spatial resolution allowed us to characterize phagosome dynamics. Similar to phagocytosis in *C. elegans*, the formation of axon debris during WD promoted the biogenesis of new PI(3)P-positive compartments, which completely surrounded axon debris (Fig. 4*A, B*). However, unlike corpse phagocytosis, debris also entered pre-existing PI(3)P-positive compartments (Fig. 4*C*). Once formed, PI(3)P-positive phagosomes often fused together (Fig. 4*E*), a process that was observed in the *Drosophila* skin in *croquemort* mutants (Han et al., 2014). Sometimes phagosomes extended dynamic tubules (Fig. 4*D*), which are similar to those observed during *C. elegans* corpse engulfment (Yu et al., 2008). PI(3)P levels on the surface of axon debris-containing phagosomes appeared to oscillate

(Figs. 4*F*, 6*A*), which is reminiscent of the two waves of PI(3)P recruitment to phagosomes observed in *C. elegans* (Lu et al., 2012). Thus, zebrafish skin cells provide a facile model for monitoring the dynamics of phagocytosis in live animals.

Trafficking of axon debris through the phagolysosomal pathway requires the small GTPase Rab5

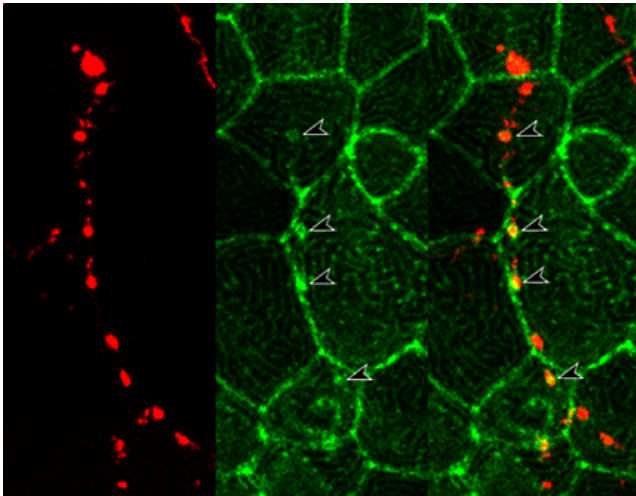
Basal skin cells in humans internalize melanosomes using a nonlytic vesicular pathway (for review, see Van Den Bossche et al., 2006). By contrast, phagosomes in *C. elegans* and mammalian macrophages mature through increasingly acidic compartments, ultimately resulting in the formation of a phagolysosome and lysis of the phagosome contents (for review, see Flannagan et al., 2012). To determine whether epidermal phagocytosis is mechanistically similar to either of these processes, we tested whether internalized axon debris associated with phagolysosomes by expressing Lamp1-GFP, a phagolysosome reporter, in epidermal cells. Lamp1-GFP surrounded axon debris as it became progressively smaller (Fig. 5*A*), indicating that skin cells degrade fragmented axons.

Phagosome maturation in invertebrate phagocytes and cultured macrophages requires sequential recruitment and activation of the small GTPases Rab5 and Rab7 (Kinchen et al., 2008; Han et al., 2014). Fluorescent fusion reporters of Rab5 and Rab7 (Clark et al., 2011) were rapidly and sequentially recruited to axon debris following fragmentation (Fig. 5*B–D*). Staining live animals with lysotracker, a marker of acidic organelles, revealed that phagosome acidification correlated with the loss of Rab5 association and a decrease in debris size (Fig. 5*B, C*). To test whether Rab5 activation is required for phagosome

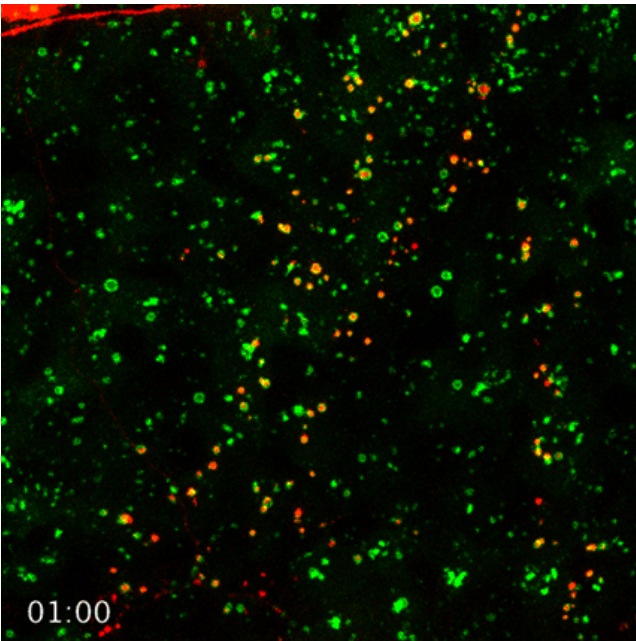
acidification, we expressed a dominant-negative version of Rab5 (Rab5-DN), engineered to have reduced GTP affinity (Clark et al., 2011), in basal cells and imaged axon debris. Rab5-DN-expressing basal cells internalized debris, but PI(3)P recruitment was inefficient (Fig. 6*A, B*). Moreover, phagosome acidification was delayed and debris size failed to decrease (Fig. 6*C, D*), although fluorescent debris eventually disappeared (data not shown). Rab5-DN expression also led to the clustering of PI(3)P-positive vesicles, consistent with a defect in phagosome fusion (Fig. 6*E*). Together, these data demonstrate that skin cells use mechanisms similar to professional phagocytes to degrade axon debris.

Epidermal phagocyte specificity is determined by proximity

Phagocytic cells other than blood-derived professional phagocytes are sometimes termed “nonprofessional” phagocytes and are thought to be specialized to recognize specific types of debris (for review, see Rabinovitch, 1995). Do molecular signals specify



Movie 1. F-Actin dynamics in the epidermis during axon degeneration. F-actin dynamics in a *Tg(krt5:Gal4); Tg(UAS:Lifeact-GFP)* transgenic animal (green) following axotomy of a somatosensory peripheral axon (red) at 32 hpf. Arrowheads indicate enrichments of F-actin around axon debris. Frames were acquired every 4 min for 128 min.



Movie 2. Both epidermal layers phagocytose virtually all axon debris. Debris from a degenerating somatosensory peripheral axon (red) is engulfed into epidermal cell phagosomes (green) in a *Tg(krt5:Gal4); TgBAC(ΔNp63:Gal4); Tg(UAS:GFP-2xFYVE)* transgenic animal following axotomy at 32 hpf. Time is displayed as HH:MM (hours:minutes).

debris/phagocyte interactions, or is specificity simply determined by accessibility? To address this question, we devised experiments to test whether epidermal cells can phagocytose debris from axons they do not normally encounter, and, conversely, if mislocalized somatosensory axon debris can be phagocytosed by other types of cells. In control animals, the posterior lateral line nerve (PLLn) is associated with Schwann cells that position it below the epidermis (Fig. 7A; Raphael et al., 2010). Leukocytes phagocytose PLLn debris during injury-induced WD (Villegas et al., 2012). Consistent with this finding, basal skin cells did not phagocytose PLLn debris in control animals (Fig. 7B). Inhibition of the ErbB signaling pathway blocks peripheral glial development, causing the PLLn to remain in close association with the epidermis (Fig.

7C; Lyons et al., 2005; Raphael et al., 2010). Following ErbB inhibition and PLLn axotomy, basal cells phagocytosed debris from injured PLLn axons (Fig. 7D), suggesting that skin cells are not limited to phagocytosing debris only from somatosensory axons.

To determine whether somatosensory axon debris can be cleared by phagocytes other than skin cells we overexpressed a dominant-negative version of the PTPRFb receptor (PTPRFb-DN) specifically in somatosensory neurons (Wang et al., 2012). This manipulation impairs sensory axon guidance to the skin, causing some peripheral axons to innervate internal tissues (Wang et al., 2012). Degeneration of PTPRFb-DN-expressing peripheral axons, whether within the skin or below the skin, exhibited similar axon fragmentation and debris clearance kinetics (Fig. 7E,F). This result suggests that epidermal cells are not required for fragmentation of touch-sensing neurites, as has been proposed in *Drosophila* (Han et al., 2014), and that nonepidermal phagocytes, likely leukocytes, are capable of clearing somatosensory axon debris. Thus, specificity of phagocytes for axon debris, at least for skin cells and somatosensory axons, is determined by proximity rather than by dedicated molecular determinants.

Epidermal cells phagocytose a broad range of debris

The ability of skin cells to phagocytose debris from at least two axon populations prompted us to investigate whether they could also phagocytose debris from other kinds of cells. First, we tested whether they could cannibalize debris of other epidermal cells by laser-ablating basal skin cells mosaically expressing DsRed in transgenic fish that expressed the PI(3)P reporter in all basal cells. Strikingly, much of the debris from these cells was internalized into phagosomes in neighboring basal cells (Fig. 8A). Similarly, debris from macrophages that were ablated in the skin was also phagocytosed by basal cells (Fig. 8B). Epidermal cells thus have broad phagocytic capabilities, suggesting that they may play roles not only in neural repair but also in wound healing.

Discussion

Following developmental pruning or laser axotomy, cutaneous axon debris is rapidly removed from the zebrafish skin (Martin et al., 2010). The speed and reproducible timing of degeneration and clearance make zebrafish an excellent model for studying cutaneous repair. Surprisingly, we found that blood-derived phagocytes do not significantly interact with debris and are not required for debris removal, despite penetrating into the epidermis and responding to the site of injury. Instead, epithelial cells of the skin phagocytose axon debris. Combined with studies in *C. elegans* and *Drosophila* (Robertson and Thomson, 1982; Hall et al., 1997; Han et al., 2014), our results demonstrate that the ability of epidermal cells to phagocytose neuronal debris is conserved between vertebrates and invertebrates.

Roles of vertebrate epidermal cells in neurite degeneration and clearance

The zebrafish epidermis shares many similarities with the mammalian epidermis and is significantly more complex than invertebrate skin. While invertebrate skin is typically a monolayer, zebrafish skin is initially bilayered and becomes increasingly stratified with the addition of suprabasal cells as the animal ages (Le Guellec et al., 2004). By developing transgenic tools specific to each layer of the larval epidermis, we found that both epithelial layers are phagocytic, with the basal cell layer eating the majority of the debris. Do suprabasal cells also eat axon debris in older animals? Recent studies found that stratification of zebrafish skin is remarkably similar to mammalian skin (Guzman et al., 2013;

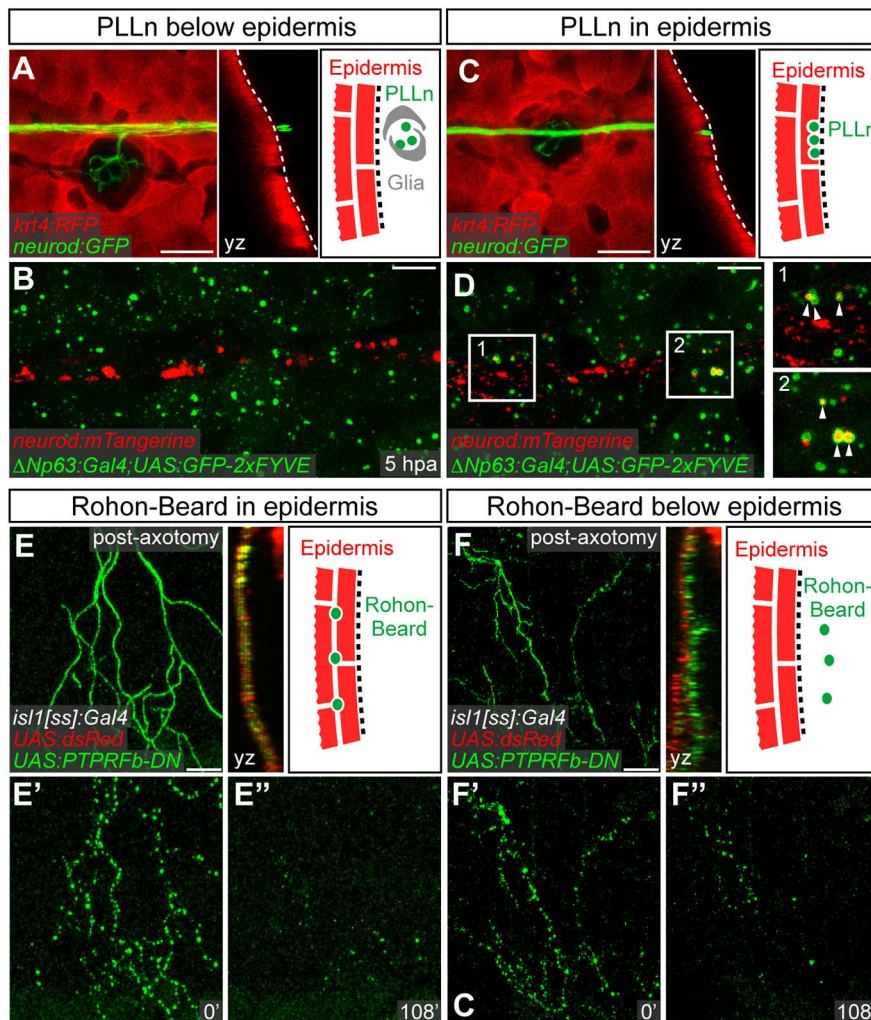


Figure 7. Epidermal cells phagocytose multiple types of neuronal debris, and neuronal debris is recognized by multiple phagocytes. **A, C**, Confocal images of the PLLn and epidermis at 3 dpf. Dashed line represents the basement membrane underlying the epidermis. **B, D**, Confocal images showing phagosomes (green) 5 hpa of the PLLn (red). Axotomy was performed at 74 hpf. **E, F**, Confocal images of single somatosensory axons expressing *PTPRFb-DN-GFP* following axotomy at 52 hpf. The duration of the lag phase (mean \pm SD) was 126.2 ± 23.4 min for axons in epidermis ($n = 9$) and 135.4 ± 16.2 min for axons below ($n = 10$; $p = 0.342$, unpaired two-tailed Student's *t* test). The duration of the clearance phase was 130.0 ± 44.9 min for axons in epidermis ($n = 9$) and 150.8 ± 61.2 min for axons below ($n = 10$; $p = 0.408$, unpaired two-tailed Student's *t* test). Scale bars: **A, C, E, F**, 25 μ m; **B, D**, 10 μ m.

Lee et al., 2014), but markers do not yet exist to specifically label suprabasal cells in zebrafish. Akin to other vertebrates, the zebrafish epidermis contains resident Langerhans cells (Lugo-Villarino et al., 2010). We found the epidermis is still robustly phagocytic after the differentiation of Langerhans cells, suggesting that the proliferation of Langerhans cells seen in experimental models of neuropathy and humans with small-fiber neuropathy (Lauria et al., 2005; Siau et al., 2006; Casanova-Molla et al., 2012) may be primarily related to tissue inflammation rather than debris clearance.

Studies in *Drosophila* have proposed that phagocytes not only clear cutaneous neurite debris, but also participate in neurite destruction (Williams and Truman, 2005; Han et al., 2014). A potential destruction mechanism involves the corpse engulfment receptor cell death abnormal-1 (CED-1)/Draper-dependent concentration of epidermal F-actin around neurites before fragmentation (Han et al., 2014). By imaging F-actin dynamics in the zebrafish skin, we found that F-actin did not associate with so-

matosensory axons before fragmentation. Moreover, axon fragmentation proceeded with normal kinetics after repositioning somatosensory axons below the skin. Thus, cutaneous neurite destruction may involve distinct mechanisms in vertebrates and invertebrates.

Dynamics of phagocytosis in the epidermis

Phagocytosis of cellular debris requires specific particle recognition molecules, rearrangement of the phagocytic membrane, and pathways for internalization and trafficking. Phagocytes can use several types of receptors to recognize cellular debris (for review, see Flannagan et al., 2012). For example, the CED-1 family receptor Draper is used by *Drosophila* glial and epidermal cells to recognize neurite debris (Hoopfer et al., 2006; MacDonald et al., 2006; Han et al., 2014), whereas vertebrate RPE cells use the MerTK receptor to recognize photoreceptor debris (D'Cruz et al., 2000). We have yet to identify the receptor used by vertebrate epidermal cells to recognize debris, but since these cells can clear several different kinds of cellular debris, the receptor likely recognizes a universal signal of degenerating membranes.

A previous ultrastructural study (O'Brien et al., 2012) found that pseudopodia project from zebrafish epidermal cell membranes toward degenerating axon debris. Consistent with this study, live imaging revealed that epidermal cells concentrate F-actin in ring-like structures around axon debris shortly after fragmentation. These results indicate that epidermal cells rearrange their membranes into phagocytic cup-like structures around axon debris before internalization, similar to the phagocytosis of beads by cultured macrophages. Following particle recognition, the mechanisms used to internalize debris depend on particle size. For example, inhibition of PI3K (phosphatidylinositol 3-kinase) only affects phagocytosis of beads at least 3 μ m in diameter (Araki et al., 1996; Cox et al., 1999). Because WD generates cutaneous axon fragments of varying sizes (~ 0.5 –4 μ m in diameter; data not shown), it will be interesting to determine whether the mechanisms used to internalize axon debris are also size dependent.

Whereas professional phagocytes are highly motile, epidermal cells are not, making it possible to visualize the dynamics of progression through the phagolysosomal pathway *in vivo* with high resolution. The most extensively characterized *in vivo* system for phagocytosis is the clearance of apoptotic corpses during embryonic development in *C. elegans* (for review, see Li et al., 2013). We found that phagocytosis of axon debris shares many similarities with corpse clearance, including the generation of new phagosomal compartments upon debris internalization, oscillations of PI(3)P on the surface of phagosomes, and a requirement for Rab5 for the efficient recruitment of PI(3)P to phagosomes and debris

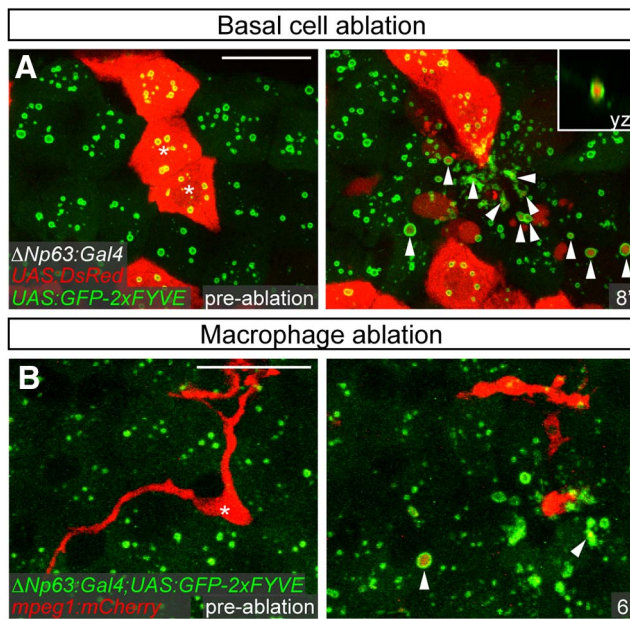


Figure 8. Epidermal cells are broad-specificity phagocytes. **A**, Confocal images before and after ablation of two basal cells mosaically expressing DsRed. Asterisks indicate ablated cells. Arrowheads indicate phagosomes containing basal cell debris. Inset shows that phagosome membranes completely surround basal cell debris. **B**, Confocal images before and after ablation of a single macrophage (red) in the epidermis. Asterisk indicates ablated cell. Arrowheads indicate phagosomes containing macrophage debris. Ablations were performed at 74 hpf. Scale bars, 25 μ m.

degradation. Phagocytes in the *C. elegans* embryo typically internalize only one apoptotic cell at a time; by contrast, epidermal cells in zebrafish can phagocytose multiple axon fragments per cell, and we observed the repeated fusion of phagosomal compartments containing axon debris. An upper limit on the amount of debris that individual skin cells can clear may exist, since a previous study found that creating large amounts of axon debris in the epidermis slowed debris degradation (Martin et al., 2010).

Many cell types contribute to phagocytosis of neuronal debris

Although the phagocytic ability of Schwann cells and the RPE has long been recognized (Young and Bok, 1969; Stoll et al., 1989), recent work has shown that several other cell types, including astrocytes (Chung et al., 2013; Tasdemir-Yilmaz and Freeman, 2014), perineurial glia (Lewis and Kucenas, 2014), and the epidermis (this study; Han et al., 2014), play major roles in nervous system homeostasis. The ability of these nonprofessional phagocytes to process neuronal debris may have evolved as a specialization in response to the widespread death and remodeling of neurons during development and/or damage to neurons by injury later in life. Alternatively, phagocytosis may be an underappreciated, but near universal ability of many cell types. Since PLLn axon debris, which is normally cleared by leukocytes, was cleared by skin cells when genetically mispositioned to the skin, and, conversely, somatosensory axon debris was likely cleared by leukocytes when mispositioned below the skin, phagocytosis of specific types of neuronal debris is not limited to a single cell type. These observations are consistent with several paradoxical findings that removal of the primary phagocyte does not necessarily delay axon debris clearance (Perry et al., 1995; Villegas et al., 2012; Niemi et al., 2013).

The free endings of somatosensory axons are extremely large and complex, often orders of magnitude larger than other axonal

endings in the skin (Wu et al., 2012). Understanding the mechanisms involved in maintaining these somatosensory endings is important for the prevention and treatment of peripheral neuropathies. The ability to visualize and manipulate somatosensory debris clearance in a living, intact vertebrate may lead to improved regenerative treatments. Moreover, since the phagocytic nature of the skin is not limited to neuronal debris, it may have important implications for other forms of tissue repair.

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