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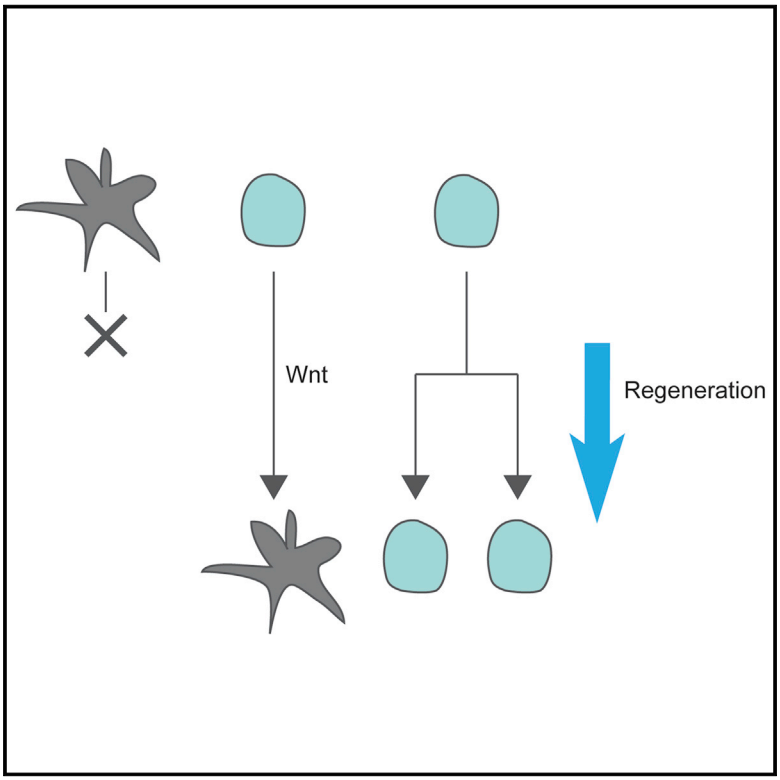
Poised Regeneration of Zebrafish Melanocytes Involves Direct Differentiation and Concurrent Replenishment of Tissue-Resident Progenitor Cells

Graphical Abstract



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In Brief

Regenerative processes must create new differentiated cells while maintaining an appropriate supply of stem or progenitor cells. Iyengar et al. show that melanocyte regeneration in zebrafish is largely accomplished by a combination of direct differentiation and symmetric divisions of progenitor cells.

Highlights

- Melanocyte regeneration is mediated by unpigmented *mitfa*-expressing progenitor cells
- Most new melanocytes arise through direct differentiation of progenitors
- Direct differentiation of progenitors requires Wnt signaling
- Symmetric divisions maintain the progenitor pool

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Poised Regeneration of Zebrafish Melanocytes Involves Direct Differentiation and Concurrent Replenishment of Tissue-Resident Progenitor Cells

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SUMMARY

Efficient regeneration following injury is critical for maintaining tissue function and enabling organismal survival. Cells reconstituting damaged tissue are often generated from resident stem or progenitor cells or from cells that have dedifferentiated and become proliferative. While lineage-tracing studies have defined cellular sources of regeneration in many tissues, the process by which these cells execute the regenerative process is largely obscure. Here, we have identified tissue-resident progenitor cells that mediate regeneration of zebrafish stripe melanocytes and defined how these cells reconstitute pigmentation. Nearly all regeneration melanocytes arise through direct differentiation of progenitor cells. Wnt signaling is activated prior to differentiation, and inhibition of Wnt signaling impairs regeneration. Additional progenitors divide symmetrically to sustain the pool of progenitor cells. Combining direct differentiation with symmetric progenitor divisions may serve as a means to rapidly repair injured tissue while preserving the capacity to regenerate.

INTRODUCTION

During regeneration, cells that are the source of new tissue must coordinate proliferation and differentiation to appropriately rebuild structures that are lost. The relationship between these processes impacts both the rate and extent to which new tissue is formed. Understanding the relative importance of proliferation and differentiation has been a long-standing goal in regenerative biology with implications not only in wound healing but also stem cell and other types of cell replacement therapies. Currently, there are efforts to manipulate regenerative proliferation and differentiation to improve clinical outcomes in hematopoietic stem cell transplantation, skin engraftment, and other tissue restorative therapies (Ballen et al., 2013; Barrandon et al., 2012).

The relationship between proliferation and differentiation defines the mode of regeneration that occurs. In tissues where sources of cells added during regeneration are known, three modes of regeneration have been described, depending on the tissue studied and the injury model used (Poss, 2010; Tanaka and Reddien, 2011). Resident stem or progenitor cells are utilized in many tissues. Typically, these are undifferentiated cells that proliferate in response to injury to generate many descendants that differentiate to generate cells needed for repair. Hematopoietic stem cells and skeletal muscle satellite cells are exemplars of this category (Sacco et al., 2008; Sherwood et al., 2004; Weissman and Shizuru, 2008). In other tissues, such as the mammalian liver, after partial hepatectomy, and zebrafish cardiac muscle, differentiated cells are the source (Jopling et al., 2010; Kikuchi et al., 2010; Michalopoulos, 2007). Here, remnant differentiated cells undergo dedifferentiation to enable their proliferation. The descendants generated differentiate into new cells of the same type that were lost. Transdifferentiation can occur in which a remnant cell type converts into a different cell type to replace lost cells. Whereas proliferation is critical in stem/progenitor cell and dedifferentiation modes of regeneration, it is thought to play little role during transdifferentiation. Although less common, important examples of transdifferentiation have been described, including the regeneration of the newt retina from pigmented retinal epithelial cells (Henry and Tsonis, 2010). Lineage tracing studies have been instrumental in defining cellular sources of regeneration, yet in many cases the steps between a source cell and its differentiated descendants remain poorly understood.

To map how cells progress through the regeneration process, we have studied melanocyte regeneration in zebrafish. Melanocytes in zebrafish have emerged as a useful cell type for studying regeneration. These cells retain melanin pigment, providing a marker to distinguish differentiated cells from their progenitors. New melanocytes are made either in the context of appendage regeneration, as when the fin is resected, or following cell-specific ablation of adult stripe or embryonic melanocytes. It is clear that new melanocytes in the fin arise from unpigmented precursors (Rawls and Johnson, 2000). Cell-specific ablations similarly implicate unpigmented precursors in regeneration of melanocytes in adult zebrafish stripes and embryos (O'Reilly-Pol and Johnson, 2008; Yang and Johnson, 2006). While some genetic regulators of melanocyte regeneration have been identified (Hultman et al., 2009; Lee et al., 2010; O'Reilly-Pol and Johnson, 2013; Rawls and Johnson, 2000, 2001; Yang et al., 2007), the source of new cells has not been defined, and the path through which source cells yield new melanocytes has not yet been described.

Here, we use a targeted cell ablation approach to define the source of regeneration melanocytes. Direct lineage determination

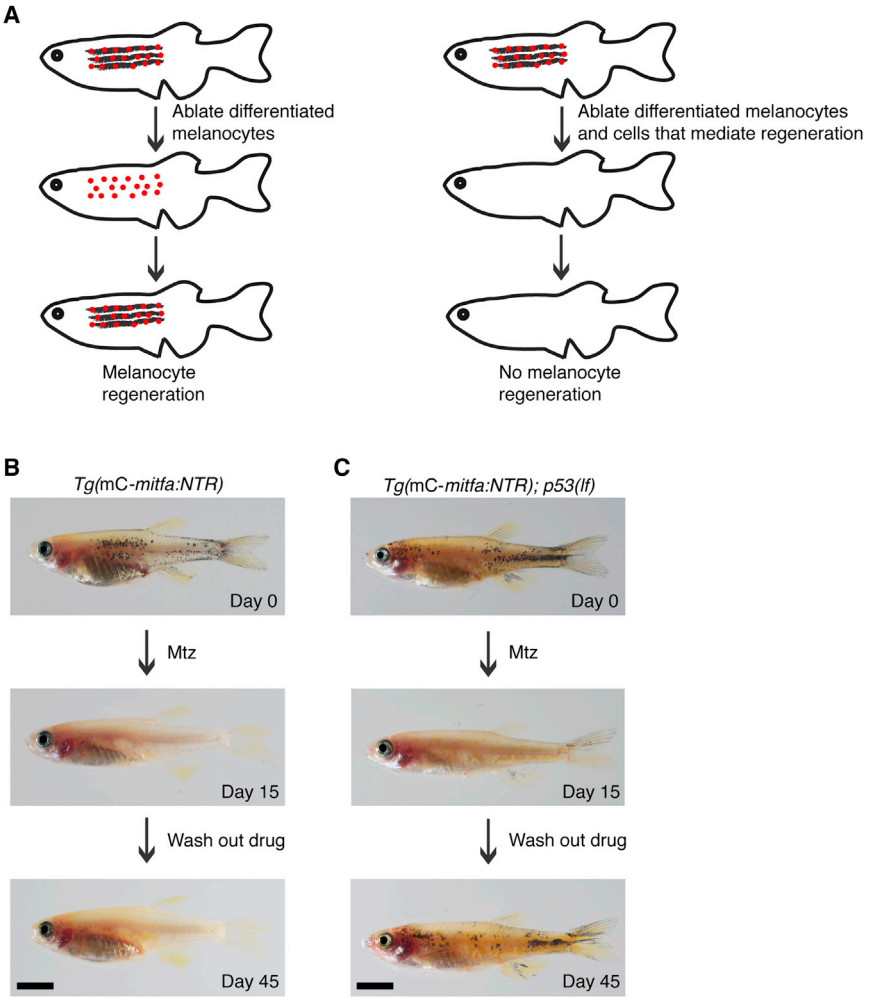

Figure 1. Melanocyte Regeneration in Adult Zebrafish Requires *mitfa*-Expressing Cells

(A) Strategy for identifying cells that mediate melanocyte regeneration: promoter-based ablation of only differentiated melanocytes results in regeneration (left) whereas promoter-based ablation of differentiated melanocytes and cells that mediate melanocyte regeneration causes failure of regeneration (right).

(B) Adult zebrafish expressing *mitfa:NTR* in miniCoopR-rescued melanocytes and lineally related cells (top). Differentiated melanocytes were ablated upon Mtz treatment (middle) but failed to regenerate after Mtz was washed out (bottom). n = 5 fish; representative images are shown. mC, miniCoopR. Scale bar, 5 mm.

(C) Adult *p53(lf)* zebrafish expressing miniCoopR-*mitfa:NTR* (top). Differentiated melanocytes were ablated on Mtz treatment (middle), and new melanocytes regenerated after Mtz was washed out (bottom). n = 5 fish; representative images are shown. mC, miniCoopR. Scale bar, 5 mm.

See also Figure S1 and Movies S1 and S2.

of source cells indicates a multifaceted regeneration process involving precursor cells that directly differentiate as well as cells that divide to yield additional lineage-restricted cells. Wnt signaling is activated during melanocyte regeneration and is important for producing new melanocytes. Coupling direct differentiation and cell division may be used in zebrafish and other metazoans to enable rapid cell replacement while preserving the capability to undergo multiple cycles of regeneration.

RESULTS

Ablation of *mitfa*-Expressing Cells Causes Failure of Melanocyte Regeneration

To study the mechanisms by which melanocyte regeneration occurs, we sought to identify the cells responsible for reconstituting adult zebrafish stripe melanocytes following injury. These cells are unpigmented, as regeneration occurs normally following ablation of all pigmented, differentiated melanocytes with the small molecule neocuproine (O'Reilly-Pol and Johnson, 2008). To identify the cells mediating regeneration, we used a promoter-based, cell-specific ablation approach. In this approach, a bacterial *nfsB* nitroreductase (*NTR*) gene was expressed in cells targeted for ablation. Expression of the *NTR* gene alone is innocuous; however, when the prodrug metronidazole (Mtz) is applied, the NTR protein processes metronidazole into toxic compounds, leading to cell death (Curado et al., 2007; Pisharath et al., 2007). Promoters were used to drive expression of the *NTR* gene in specific cells with the goal of finding a promoter that was active in the cells responsible for regeneration. In many cases, cells that mediate regeneration express lineage-specific genes that continue to be expressed once differentiated cells are generated (Muñoz et al., 2012; Nishimura et al., 2002). With this in mind, we expressed *NTR* using promoters of melanocyte lineage genes. Two outcomes from this approach were predicted (Figure 1A): (1) if the promoter were expressed only in differentiated melanocytes, then metronidazole application would lead to melanocyte ablation followed by regeneration, or (2) if the promoter were expressed in differentiated melanocytes and cells mediating regeneration, then application of metronidazole would ablate both types of cells and regeneration would not occur.

The miniCoopR system (Ceol et al., 2011) was used to express *NTR* under melanocyte lineage promoters (Figures S1A and S1B). miniCoopR animals are chimeric and express transgenes of interest in melanocytes and lineally related cells. Interestingly, when *NTR* was expressed under the control of the *mitfa* promoter and melanocyte-positive *Tg(*miniCoopR-*mitfa:NTR)* adults were treated with Mtz, melanocytes were ablated but regeneration did not occur (Figure 1B; Movie S1). To confirm that *Tg(*miniCoopR-*mitfa:NTR)* adults were capable of regeneration, we treated these melanocyte-positive adults with neocuproine and found that melanocytes regenerated from unpigmented precursors following drug washout (Figure S1C). These data suggest that the *mitfa* promoter is active

not only in differentiated melanocytes but also in the cells that mediate melanocyte regeneration.

NTR-Mediated Ablation of Unpigmented *mitfa*-Expressing Cells Is *p53* Dependent

NTR induces ablation by converting Mtz into a DNA interstrand crosslinking agent, resulting in cell death (Anlezark et al., 1992; Lindmark and Müller, 1976). To determine if NTR-induced ablation proceeded through a *p53*-dependent apoptotic program, we injected miniCoopR-*mitfa:NTR* into *mitfa(lf); p53(lf)* embryos and reared melanocyte-positive adults. Upon treatment with Mtz, differentiated melanocytes were ablated, indicating that their death could occur independently of *p53* (Figure 1C; Movie S2). However, following Mtz washout, regeneration occurred. The regenerated melanocytes were present in locations previously occupied by the ablated melanocytes, suggesting a close association between differentiated melanocytes and the unpigmented cells that give rise to new melanocytes following ablation. These data indicate that the NTR-induced ablation of cells responsible for regeneration is dependent on *p53*.

Unpigmented *mitfa*-Positive Cells Can Directly Differentiate into Melanocytes during Regeneration

The previous experiments led us to hypothesize that a pool of unpigmented, *mitfa*-expressing cells was present near differentiated melanocytes, and these unpigmented cells were activated following ablation to give rise to new melanocytes and reconstitute the zebrafish stripe pigment pattern. To directly visualize unpigmented *mitfa*-expressing cells and track their lineages, we created animals that expressed EGFP under the control of the *mitfa* promoter. An EGFP with a nuclear localization signal was used to better distinguish one cell from another as well as more accurately track mitoses. *Tg(*miniCoopR-*mitfa:nlsEGFP)* adults were generated and treated with epinephrine prior to imaging. Epinephrine promotes retrograde transport of melanosomes along microtubules within zebrafish melanocytes, leading to accumulation of these organelles in perinuclear space (Johnson et al., 1995). Melanin pigment within melanosomes absorbs UV light, and pre-treatment with epinephrine allows UV light-activated fluorophores to be readily visualized in differentiated melanocytes. Nuclei in differentiated melanocytes of *Tg(*miniCoopR-*mitfa:nlsEGFP)* adults were observed as a halo of EGFP signal surrounding the perinuclear cluster of melanosomes (Figure 2A). In addition, nlsEGFP was observed in admixed nuclei that were smaller than those of differentiated melanocytes. Coexpression of membrane-localized *mCherry-CAAX* under the *mitfa* promoter indicated that these nuclei were present in unpigmented cells distinct from differentiated melanocytes (Figure 2B). Another zebrafish pigment cell type, the xanthophore, also expresses *mitfa* (Curran et al., 2009; Lister et al., 1999). Xanthophores are mostly found in the interstripes between melanocyte stripes, but a minor population of xanthophores is also present within melanocyte stripes (Mahalwar et al., 2014). However, the unpigmented *mitfa*-expressing cells we observed were not xanthophores because they lacked the pteridine-based yellow pigment characteristic of this cell type (Figure S2).

To determine if unpigmented *mitfa*-expressing cells could be responsible for regeneration, we imaged whether these cells were present at the onset of the regeneration process. Regeneration was initiated by neocuproine-induced ablation in *Tg(*miniCoopR-*mitfa:nlsEGFP)* animals (Figure 2C) or Mtz-induced ablation in *Tg(*miniCoopR-*mitfa:NTR); Tg(mitfa:nlsEGFP); p53(lf)* animals (Figure 2D). In both backgrounds, unpigmented *mitfa*-expressing cells were spared after ablation. Taken together, these data suggest that the unpigmented *mitfa*-expressing cells present in *Tg(*miniCoopR-*mitfa:nlsEGFP)* animals are the source of regeneration melanocytes following ablation of differentiated melanocytes. Unpigmented *mitfa*-expressing cells were also admixed with xanthophores and iridophores in interstripes, but their role in these regions is presently unknown.

To directly link unpigmented *mitfa*-expressing cells and regeneration melanocytes, we performed lineage analysis following treatment with neocuproine. The flanks of *Tg(*miniCoopR-*mitfa:nlsEGFP)* animals were imaged before and after neocuproine treatment, and differentiation of new melanocytes was noted by onset of melanization. Unpigmented nlsEGFP-positive cells were tracked and several gave rise to differentiated melanocytes without dividing (Figure 3). In many cases, these unpigmented cells were closely apposed to differentiated melanocytes. During regeneration, cells upregulated nlsEGFP expression as they began to melanize, and epinephrine-treated differentiated melanocytes showed robust nlsEGFP expression. Although nlsEGFP expression typically formed a halo around epinephrine-contracted melanosomes, in some cases it straddled opposite sides of the melanosomes (Figure S3A). Melanocytes with such nuclear signal arose following regeneration and through normal ontogeny, and these melanocytes, along with their resident nlsEGFP signal, were readily ablated upon neocuproine treatment (Figure S3B). During regeneration the earliest examples of direct differentiation were observed four days after neocuproine treatment. Similar results were obtained in animals expressing *Tg(mitfa:nlsEGFP)* in an otherwise wild-type background, indicating that observations using the miniCoopR system reflect the normal regeneration process (Figure S3C). Thus, a subset of *mitfa*-expressing cells is poised to differentiate into new melanocytes following ablation.

Some Unpigmented *mitfa*-Expressing Cells Undergo Mitosis during Regeneration

Since unpigmented *mitfa*-expressing cells can directly differentiate during regeneration, we wanted to determine if regeneration was mediated by a finite and exhaustible supply of progenitors. To begin to address this question, multiple cycles of melanocyte ablation were performed on wild-type adult fish. Individual fish were subjected to seven cycles of neocuproine-mediated ablation and regeneration. The stripe pigment pattern following the final round of regeneration was similar to that of untreated wild-type animals (Figure S4A), indicating little if any diminution of regenerative capacity. To determine if the number of unpigmented *mitfa*-expressing cells is decreased following regeneration, we subjected *Tg(*miniCoopR-*mitfa:nlsEGFP)* fish to neocuproine treatment and regeneration. *Tg(*miniCoopR-*mitfa:nlsEGFP)* fish were capable of regenerating their melanocytes repeatedly, and when the flanks of *Tg(*miniCoopR-*mitfa:nlsEGFP)* animals were imaged before and after melanocyte regeneration, we observed no qualitative decrease in numbers

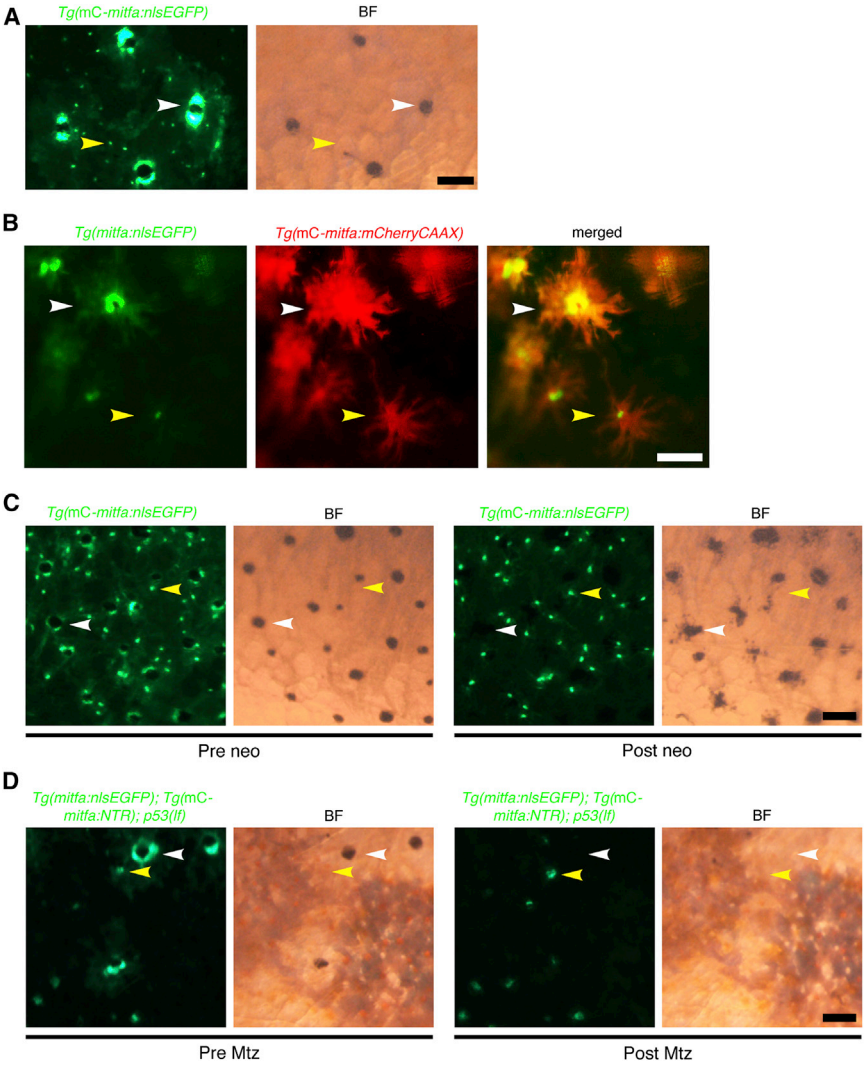

Figure 2. Unpigmented *mitfa*-Expressing Cells Are Present at the Onset of Regeneration

(A) Flank of adult zebrafish expressing miniCoopR-*mitfa:nlsEGFP*. Unpigmented nlsEGFP-positive nuclei (yellow arrowheads) were admixed with nuclei of differentiated melanocytes (white arrowheads). mC, miniCoopR; BF, brightfield. Scale bar, 100 μM.

(B) Flank of adult zebrafish coexpressing *mitfa:nlsEGFP* and miniCoopR-*mitfa:mCherryCAAX*. Unpigmented cells with nlsEGFP-positive nuclei (yellow arrowheads) were distinct from differentiated melanocytes (white arrowheads). mC, miniCoopR. Scale bar, 100 μM.

(C) Flank of adult zebrafish expressing miniCoopR-*mitfa:nlsEGFP* before (left) and after (right) neocuproine treatment. Unpigmented cells with nlsEGFP-positive nuclei (yellow arrowheads) persisted whereas differentiated melanocytes (white arrowheads) died after neocuproine treatment. n = 5 fish; representative images are shown. mC, miniCoopR; BF, brightfield; neo, neocuproine. Scale bar, 100 μM.

(D) Flank of adult *p53(lf)* zebrafish coexpressing miniCoopR-*mitfa:NTR* and *mitfa:nlsEGFP* before (left) and after (right) Mtz treatment. Unpigmented cells with nlsEGFP-positive nuclei (yellow arrowheads) were spared while differentiated melanocytes (white arrowheads) were ablated following Mtz treatment. n = 3 fish; representative images are shown. Fish were treated with epinephrine prior to imaging. mC, miniCoopR; BF, brightfield. Scale bar, 100 μM.

See also Figure S2.

of unpigmented *mitfa*-expressing cells (Figure S4B). Together, these results led us to hypothesize that, during regeneration, unpigmented *mitfa*-expressing cells must be replenished from another source to compensate for the cells that directly differentiate and become pigmented.

To determine the origin of the additional cells, *Tg(*miniCoopR-*mitfa:nlsEGFP)* animals were imaged and several unpigmented nlsEGFP cells were tracked during melanocyte regeneration. We observed that, after differentiated melanocytes were ablated, some nuclei of unpigmented *mitfa*-expressing cells divided to yield two *mitfa*-expressing daughters (Figure 4A). The earliest examples of division were observed within 2 days following melanocyte ablation. To confirm that unpigmented *mitfa*-expressing cells could actively cycle, we adapted the fluorescent ubiquitylation-based cell-cycle indicator (FUCCI) system (Sugiyama et al., 2009). This system employs two fusion proteins, mCherry-zCdt1 and mAG-zGeminin, which are expressed specifically in the G0/G1 and S/G2/M phases, respectively. We created animals coexpressing miniCoopR-*ubi:mCherry-zCdt1* and *mitfa:nlsEGFP* transgenes or miniCoopR-*ubi:AG-zGeminin* and *mitfa:nlsmCherry* transgenes. In *Tg(*miniCoopR-*ubi:mCherry-zCdt1); Tg(mitfa:nlsEGFP)* animals, nuclei of differentiated melanocytes (100%, n = 100) and unpigmented *mitfa*-expressing cells (100%, n = 142) were mCherry-positive (Figure 4B), indicating they were in the G0/G1 phase of the cell cycle. In *Tg(*miniCoopR-*ubi:AG-zGeminin); Tg(mitfa:nlsmCherry)* animals no differentiated melanocytes (0%, n = 300) or unpigmented *mitfa*-expressing cells (0%, n = 900) were AG-positive (Figure 4C), indicating they were not actively cycling. Following neocuproine-mediated ablation in *Tg(*miniCoopR-*ubi:AG-zGeminin); Tg(mitfa:nlsmCherry)* animals, some unpigmented *mitfa*-expressing AG-positive cells were observed (Figures 4C and 4D). A low, but statistically significant, percentage of *mitfa*-expressing AG-positive cells was observed at time points from 2–9 days following ablation. To estimate the scope of cell division, we treated wild-type zebrafish with EdU during neocuproine-mediated melanocyte ablation and regeneration (Figure 4E). After melanocyte regeneration, we observed EdU incorporation in 25.6% (n = 224) of unpigmented Mitfa-positive nuclei (Figures 4F and 4G). We observed little EdU incorporation in the absence of melanocyte ablation. These data indicate that a subset of unpigmented *mitfa*-expressing cells undergo mitosis following ablation of differentiated melanocytes.

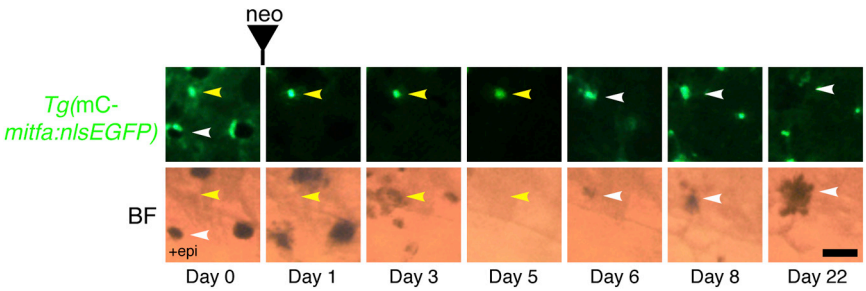

Figure 3. Unpigmented *mitfa*-Expressing Cells Give Rise to Differentiated Melanocytes by Direct Differentiation during Regeneration

Lineage tracing of unpigmented *mitfa*-expressing cells on the flank of an adult *Tg(*miniCoopR-*mitfa:nlsEGFP)* zebrafish before and after neocuproine-mediated ablation of differentiated melanocytes. Unpigmented *mitfa*-expressing cells (yellow arrowheads) directly differentiated into pigmented melanocytes (white arrowheads). Differentiation was noted by the onset of melanization. Fish were treated with epinephrine only on day 0 prior to imaging. mC, miniCoopR; BF, brightfield; neo, neocuproine. Scale bar, 50 μM. See also Figure S3.

To investigate the fates of daughter cells produced by mitoses of unpigmented *mitfa*-expressing cells we further analyzed their lineages in *Tg(*miniCoopR-*mitfa:nlsEGFP)* animals. Cells were traced for 10–12 days until melanin from regenerating melanocytes obscured EGFP signal. Following some mitoses, both daughter cells remained undifferentiated after regeneration was complete (Figure 5A). In other cases, daughter cells from the same mitosis adopted different fates with one daughter differentiating and the other daughter remaining undifferentiated (Figure 5B). The daughters that differentiated upregulated nlsEGFP expression just prior to melanization. These lineage analyses show that symmetric and asymmetric divisions of unpigmented *mitfa*-expressing cells can occur during regeneration. To quantify the relative contributions of differentiation and division, the lineages of several cells were determined (Table S1). Some cells neither differentiated nor divided. Of the cells that differentiated or divided (n = 55), 45.4% directly differentiated, 47.3% divided symmetrically, and 7.3% divided asymmetrically (Figure 5C). Divisions occurred between days 2 and 7 following ablation with the median at day 4. On average, the cells that directly differentiated became melanized at day 5.7 ± 1.4 (n = 25), whereas those that melanized following asymmetric division did so at day 7.3 ± 0.5 (n = 4). We observed no direct differentiation and little division in the absence of melanocyte ablation (Table S1), demonstrating that both processes were not constitutive but instead part of a regeneration response. These results indicate that most regeneration melanocytes are generated by direct differentiation, and the pool of unpigmented *mitfa*-expressing cells is maintained primarily through symmetric divisions.

To test whether cells generated during one round of regeneration could contribute to melanocyte regeneration in subsequent rounds, we performed an EdU incorporation experiment (Figure 5D). Fish were subjected to two cycles of melanocyte ablation and regeneration, but were treated with EdU only in the first cycle. Of melanocytes that were generated during the second cycle, 41.2% (n = 718) were EdU-positive (Figures 5E and 5F), indicating that descendants of cells that underwent S phase and division in the first round were source cells for melanocyte regeneration in the second round.

Wnt Signaling Is Activated during Melanocyte Regeneration

To identify regulators of melanocyte regeneration, we reasoned that pathways active in melanocyte ontogeny might also be important for the regeneration process. The Wnt signaling pathway is critically involved in melanocyte development, promoting the specification of melanocytes from neural crest progenitor cells (Dorsky et al., 1998; Dunn et al., 2000; Hari et al., 2002, 2012; Ikeya et al., 1997; Takeda et al., 2000). To monitor Wnt activity, we used the TOPFlash reporter, in which four consensus TCF/Lef binding sites are juxtaposed to a minimal *c-fos* promoter (Dorsky et al., 2002; van de Wetering et al., 1997). Using linearized transgenes, animals were created in which *mCherry* was expressed under *TOPFlash* control together with *nlsEGFP* under *mitfa* promoter control. The resulting *Tg(*miniCoopR-*TOPFlash:mCherry); Tg(mitfa:nlsEGFP)* adults were treated with epinephrine and imaged. mCherry signal was evident in *mitfa*-expressing differentiated melanocytes but absent from unpigmented *mitfa*-expressing cells (Figure 6A). To confirm that the mCherry signal seen was dependent upon Wnt-responsive TCF/Lef binding sites, we used the FOPFlash variant, in which these four sites are mutated to prevent binding. *Tg(*miniCoopR-*FOPFlash:mCherry); Tg(mitfa:nlsEGFP)* fish did not express mCherry in differentiated melanocytes or in unpigmented *mitfa*-expressing cells. These results indicate that Wnt signaling is active in differentiated melanocytes, potentially as a means of maintaining a melanogenic program in these cells (Rabbani et al., 2011).

To determine if Wnt signaling is activated during regeneration, *Tg(*miniCoopR-*TOPFlash:mCherry); Tg(mitfa:nlsEGFP)* adults were immersed in neocuproine solution and unpigmented *mitfa*-expressing cells were tracked longitudinally. Within one day of neocuproine exposure, differentiated melanocytes were ablated and mCherry signal was lost (Figure S5). Later, mCherry signal was observed in cells that became new melanocytes (Figure 6B). Initiation of mCherry expression shortly preceded differentiation, as *mitfa*-expressing cells melanized within 24 hr of becoming mCherry-positive. During the course of regeneration, the cells first upregulated nlsEGFP expression, began to express mCherry, and then further upregulated nlsEGFP and mCherry expression as they melanized. Thus, Wnt signaling becomes activated during melanocyte regeneration and is closely coupled to differentiation.

Inhibition of Wnt Signaling Compromises Melanocyte Regeneration

To investigate the functional significance of Wnt activation during regeneration, we treated fish with an inhibitor of Wnt signaling after ablation of differentiated melanocytes. IWR-1, a tankyrase inhibitor, has been shown to downregulate Wnt signaling in

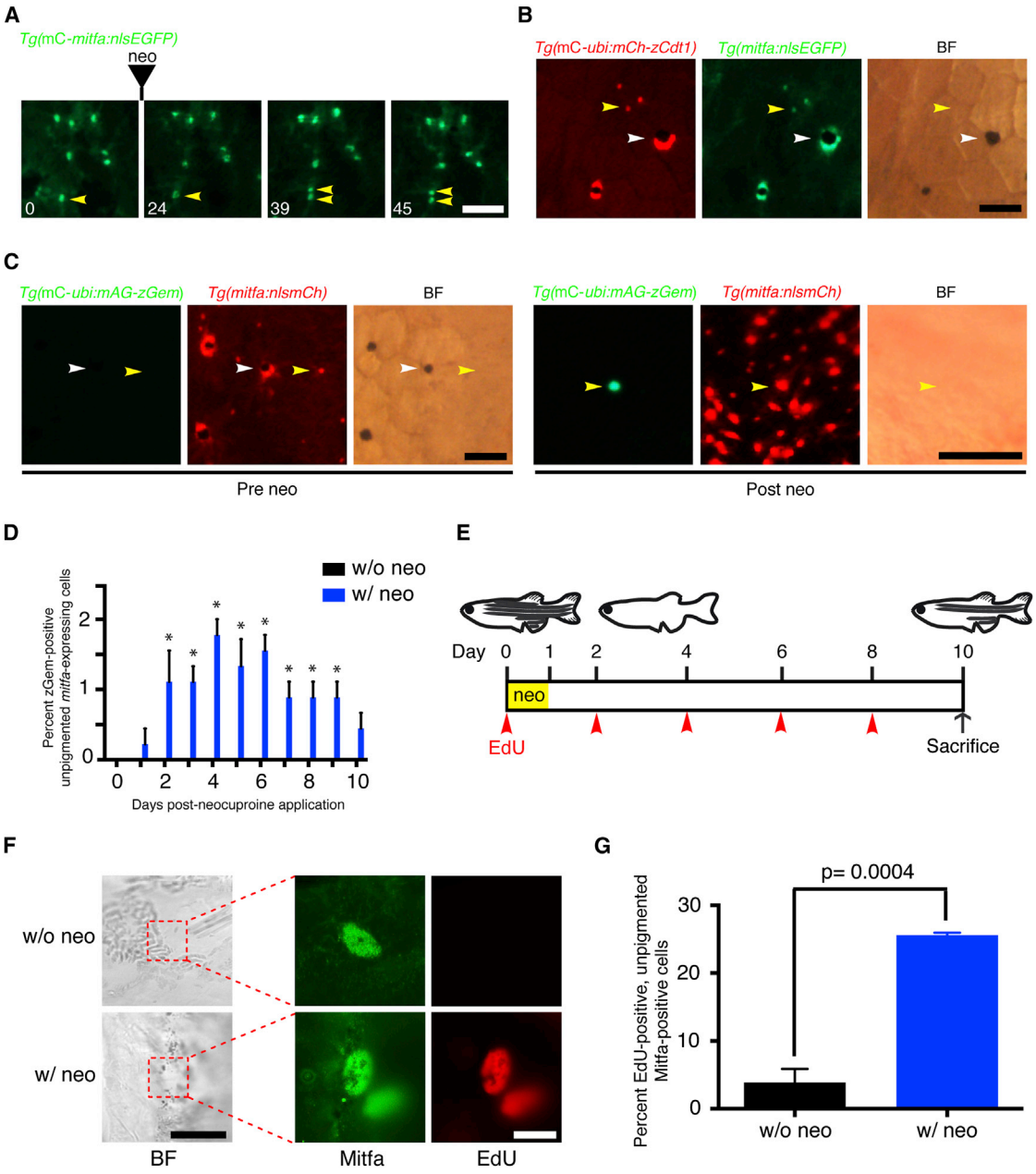

Figure 4. Some Unpigmented *mitfa*-Expressing Cells Enter the Cell Cycle upon Melanocyte Ablation

(A) Flank of an adult zebrafish expressing miniCoopR-*mitfa:nlsEGFP*. Neocuproine treatment was performed for 24 hr, and hours after onset of treatment are indicated. An unpigmented *mitfa*-expressing cell undergoing mitosis is shown (yellow arrowheads). neo, neocuproine; BF, brightfield. Scale bar, 100 μM.

(B) Flank of an adult zebrafish coexpressing miniCoopR-*ubi:mCherry-zCdt1* and *mitfa:nlsEGFP*. Differentiated melanocytes (white arrowheads) and unpigmented *mitfa*-expressing cells (yellow arrowheads) were in the G0/G1 phase of the cell cycle. n = 100 melanocytes and n = 142 unpigmented *mitfa*-expressing cells from a total of three fish; representative images are shown. Fish were treated with epinephrine prior to imaging. mC, miniCoopR; mCh, mCherry; BF, brightfield. Scale bar, 100 μM.

(C) Flank of an adult zebrafish coexpressing miniCoopR-*ubi:mAG-zGeminin* and *mitfa:nlsmCherry* before (left) and after (right) neocuproine treatment. Prior to neocuproine treatment differentiated melanocytes (white arrowheads) and unpigmented *mitfa*-expressing cells (yellow arrowheads) were not in the S/G2/M phase of the cell cycle. n = 300 melanocytes and n = 900 unpigmented *mitfa*-expressing cells from a total of three fish; representative images are shown. After neocuproine treatment unpigmented *mitfa*-expressing cells entered the S/G2/M phase of the cell cycle. The nucleus of an unpigmented AG-zGeminin-positive *mitfa*-expressing cell in the S/G2/M phase after neocuproine treatment is shown (yellow arrowheads). Fish were treated with epinephrine prior to imaging. mC, miniCoopR; mCh, mCherry; BF, brightfield; Gem, Geminin; neo, neocuproine. Scale bar, 100 μM.

(D) Quantification of AG-zGeminin-positive unpigmented *mitfa*-expressing cells. n = 150 cells from each of three fish for neocuproine-treated group, n = 300 cells from each of three fish for w/o neocuproine control group. Data are shown as mean percent positive per fish ± SEM; p values calculated by Student's t test, *$p < 0.05$. neo, neocuproine.

(legend continued on next page)

zebrafish and other systems (Chen et al., 2009; Huang et al., 2009). IWR-1 downregulated TOPFlash:mCherry signal in melanocytes, indicating it can act in this lineage to inhibit Wnt signaling (Figure S6A). To inhibit Wnt signaling during regeneration, wild-type fish were treated with neocuproine for 1 day then, after washout, were treated with IWR-1 (Figure 7A). The effect of IWR-1 during regeneration was measured by quantifying the number of differentiated melanocytes present at various time points during and upon completion of regeneration. Treatment with IWR-1 led to a reduction in the number of regeneration melanocytes: while an average of nearly 80% regeneration was observed in control animals by 15 days post-ablation, only 35% regeneration occurred in IWR-1-treated fish (Figures 7B and 7C). The effects of Wnt inhibition were specific to the regeneration process, as treatment of age-matched wild-type animals with IWR-1 in the absence of neocuproine-mediated ablation caused no change in the number of differentiated melanocytes (Figures S6B and S6C). Taken together, these data indicate that Wnt signaling plays an important role in generating differentiated melanocytes during regeneration.

DISCUSSION

Our studies have demonstrated that *mitfa*-expressing cells are required for regeneration of zebrafish melanocytes. Direct lineage determination of unpigmented *mitfa*-expressing cells following injury indicates a poised mechanism of regeneration, one that couples direct differentiation of extant progenitor cells with divisions of additional cells that replenish the progenitor population. Since Morgan's incisive discussion of the topic (Morgan, 1901), modes of regeneration have been categorized into those that utilize cell division and those in which regeneration occurs without cell division. However, a mechanism lacking any cell division, e.g., one solely dependent on direct or transdifferentiation, would likely result in impaired regeneration as the pool of source cells is expended. By coupling direct differentiation and division of lineage-restricted cells, regeneration of melanocyte stripes solves this problem, resulting in an extensive and perhaps unlimited capacity to replace this tissue.

This mechanism of regeneration is notable in two respects. First, to our knowledge, a prominent role for direct differentiation of progenitor cells has not been described in other systems. Lineage-tracing experiments have shown that regenerated cells can be derived from the progeny of tissue-resident stem or progenitor cells, the progeny of cells that have undergone dedifferentiation or, in some cases, from cells that have transdifferentiated. The extent of direct differentiation may reflect the high ratio of progenitor to differentiated cells. In systems where few progenitors must give rise to many differentiated cells, division may be more prevalent. However, in systems where a surfeit of progenitor cells is present, direct differentiation may have a predominant role. Second, the presence of a large quantity of undifferentiated precursor cells is unexpected. Enough precursor cells are present to enable most new melanocytes to arise via direct differentiation. Stripe melanocytes in the fish are important for predator avoidance and fish-to-fish recognition (Engeszer et al., 2004, 2008; Price et al., 2008), and it may be advantageous to regenerate these cells rapidly following injury. A bias toward direct differentiation from an abundant progenitor pool may enable rapid regeneration of critical tissues.

Several studies have examined how new melanocytes are generated in zebrafish following injury or through normal development. Regeneration of melanocytes following amputation of the zebrafish fin has been extensively studied, but the cells that give rise to new fin melanocytes have not yet been identified. It is clear that the source cells are unpigmented (Rawls and Johnson, 2000), so divisions of differentiated cells do not appear to be involved. Furthermore, clonal analyses indicate they are lineage-restricted (Tu and Johnson, 2011), arguing against transdifferentiation as a mechanism of regeneration. Two classes of progenitor cells have been found in the zebrafish fin, one class that can divide between one to three times before differentiating and another class whose cell division is less limited (Tu and Johnson, 2010). It is possible that the *mitfa*-expressing cells that give rise to new melanocytes in the flank are similarly used in fin melanocyte regeneration. However, the context of regeneration in the two injury models is different: neocuproine-mediated injury specifically ablates one cell type whereas fin amputation involves replacement of several cell types following formation of a blastema. The blastema provides an environment in which proliferative signals abound and may result in greater proliferation of melanocyte precursors during fin regeneration. That robust proliferation can occur during fin regeneration is suggested by the excess BrdU-positive melanocytes that are generated when constitutively active Ras is expressed during fin regrowth (Lee et al., 2010). Our results suggest that at least a subset of *mitfa*-expressing cells have the capacity to proliferate during regeneration. If the same cells are the source of regeneration melanocytes in both the flank and fin, the extent of proliferation may be dependent on the regeneration context. In the flank where several cells contribute to replace melanocytes the need for proliferation is low. In the fin, fewer cells are likely to contribute to regeneration and the amount of proliferation may be correspondingly increased. It is also notable that melanocyte regeneration can occur in zebrafish embryos, and nearly all new melanocytes arise through proliferation (Yang and Johnson, 2006), suggesting that fewer sources of new melanocytes are present at the time of injury. In ontogenetic development, a small number of *mitfa*-expressing cells associated with the dorsal root ganglia (DRG) of zebrafish larvae are proposed to generate adult

(E) Timeline of the experiment. Adult wild-type zebrafish were treated with neocuproine for 24 hr to ablate differentiated melanocytes and were injected with EdU every other day (arrowheads). Control animals were injected with EdU but were not treated with neocuproine. Fish were sacrificed at day 10 and were scored for EdU incorporation. neo, neocuproine.

(F) Representative images of control (top row) or neocuproine-treated (bottom row) unpigmented *mitfa*-expressing cells after melanocyte regeneration. neo, neocuproine; BF, brightfield. BF scale bar, 25 μM; Mitfa, EdU scale bar, 10 μM.

(G) Quantification of percent EdU-positive, unpigmented Mitfa-positive cells. n = 103, 83 and 38 cells from each of three fish, respectively, for neo; n = 20, 30, and 15 cells from each of three fish, respectively, for w/o neo. Data are shown as mean percent positive per fish ± SEM; p value calculated by Student's t test. neo, neocuproine. See also Figure S4.

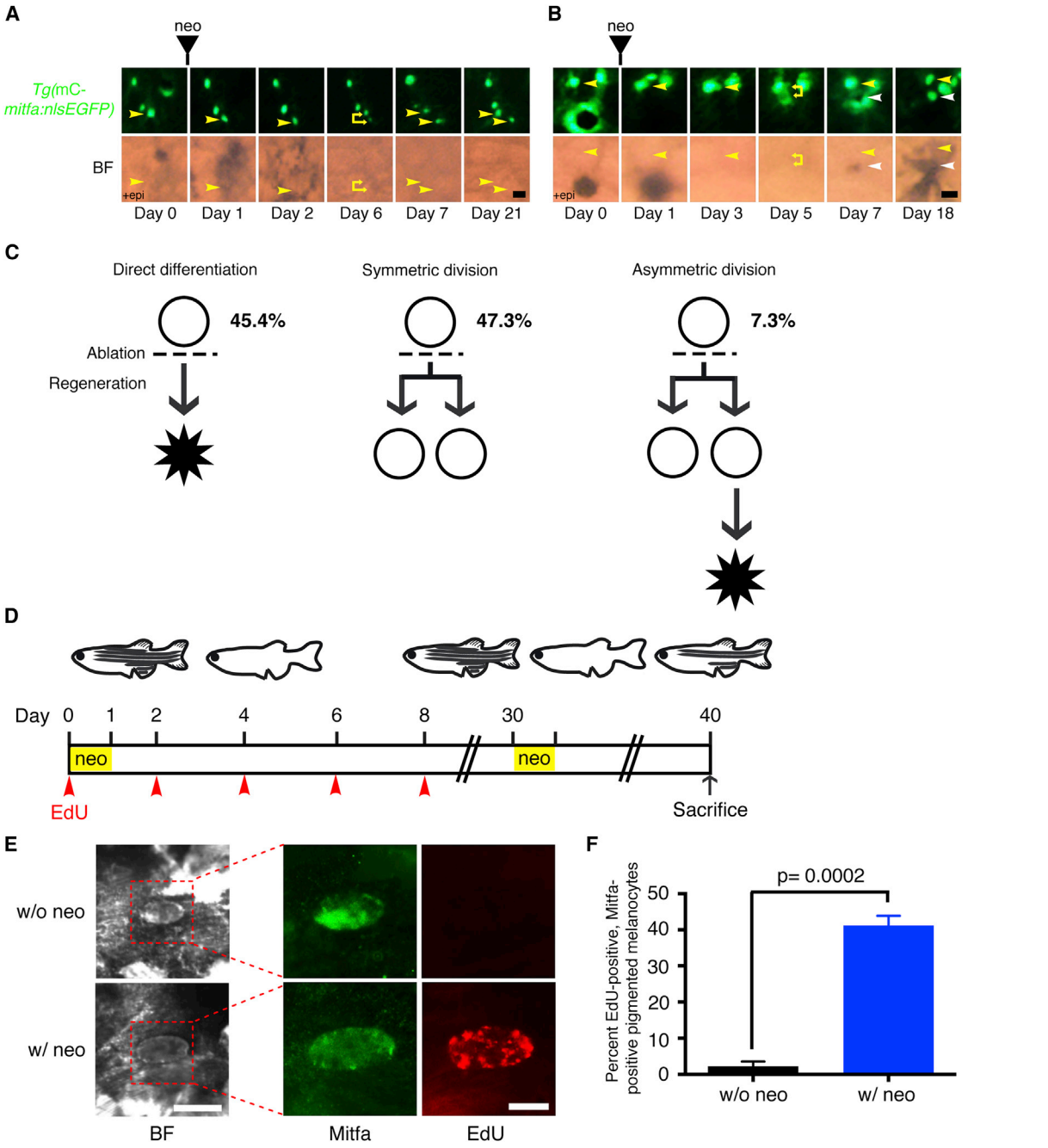

Figure 5. Divisions of Unpigmented *mitfa*-Expressing Cells during Melanocyte Regeneration

(A–C) Lineage tracing of unpigmented *mitfa*-expressing cells on the flank of adult *Tg(*miniCoopR-*mitfa:nlsEGFP)* zebrafish after neocuproine treatment.

(A) Symmetric division of an unpigmented *mitfa*-expressing cell (yellow arrowheads, days 0, 1, 2, 6). Neither daughter cell differentiated (yellow arrowheads, days 7, 21). Fish were treated with epinephrine only on day 0 prior to imaging. mC, miniCoopR; BF, brightfield, neo; neocuproine. Scale bar, 25 μM.

(B) Asymmetric division of an unpigmented *mitfa*-expressing cell (yellow arrowheads, days 0, 1, 3, 5) in which one daughter differentiated (white arrowheads, days 7, 18) and the other daughter remained undifferentiated (yellow arrowheads, days 7, 18). Fish were treated with epinephrine only on day 0 prior to imaging. mC, miniCoopR; BF, brightfield, neo; neocuproine. Scale bar, 25 μM.

(C) Schematic summarizing lineages of unpigmented *mitfa*-expressing cells that differentiated or divided following neocuproine-mediated ablation (dashed line). Percentages of each lineage are indicated.

(D) Timeline of the experiment. Adult wild-type zebrafish were treated with neocuproine for 24 hr to ablate differentiated melanocytes and were injected with EdU every other day until day 8 (arrowheads). After melanocyte regeneration fish were treated with neocuproine again at day 30 for 24 hr. Control animals were injected with EdU but were not treated with neocuproine. Fish were sacrificed at day 40 and were scored for EdU incorporation. neo, neocuproine.

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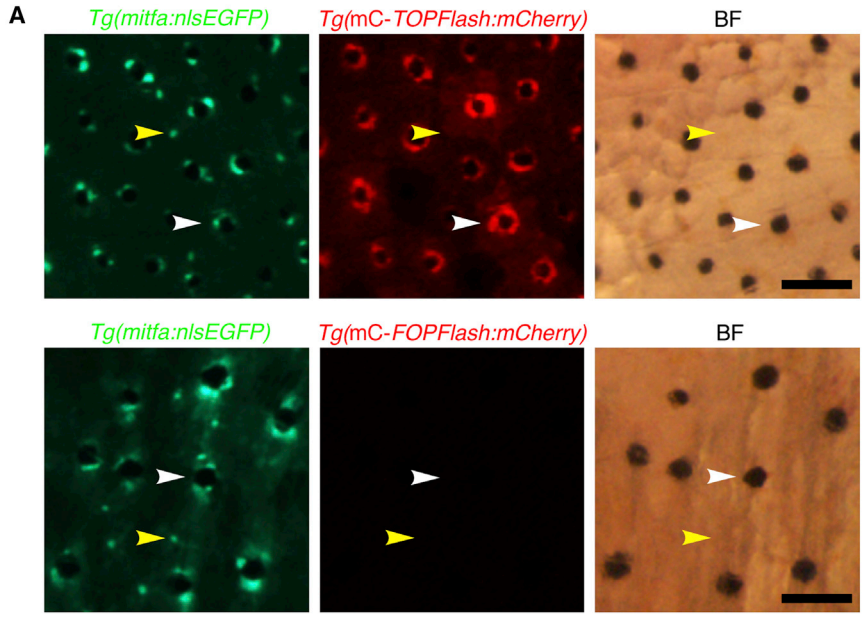

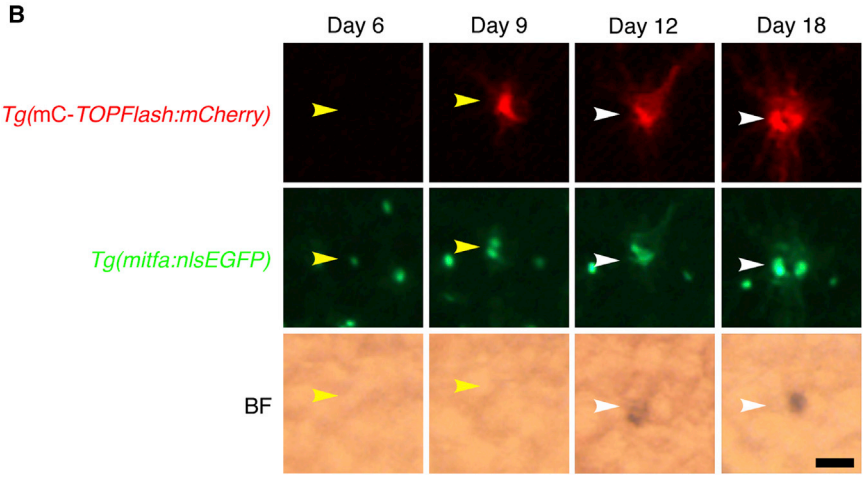

Figure 6. Wnt Signaling Is Activated during Melanocyte Regeneration

(A) Flank of an adult zebrafish coexpressing *mitfa:nlsEGFP* and miniCoopR-*TOPFlash:mCherry* (top) or *mitfa:nlsEGFP* and miniCoopR-*FOPFlash:mCherry* (bottom). In animals with *Tg(*miniCoopR-*TOPFlash:mCherry)*, mCherry signal was evident in differentiated melanocytes (white arrowheads) but not in unpigmented *mitfa*-expressing cells (yellow arrowheads). No mCherry signal was observed in Tg*(*miniCoopR-*FOPFlash:mCherry)* animals. Fish were treated with epinephrine prior to imaging. mC, miniCoopR; BF, brightfield. Scale bars, 100 μM.

(B) Lineage tracing of unpigmented *mitfa*-expressing cells on the flank of an adult zebrafish coexpressing *mitfa:nlsEGFP* and miniCoopR-*TOPFlash:mCherry* after neocuproine treatment. An unpigmented *mitfa*-expressing cell (yellow arrowheads) beginning to express mCherry shortly before differentiation (white arrowheads). mC, miniCoopR; BF, brightfield. Scale bar, 50 μM. See also Figure S5.

melanocytes that arise during metamorphosis (Dooley et al., 2013). Given their intrinsic similarities, it is possible that the DRG-associated *mitfa*-expressing cells establish the pool of unpigmented *mitfa*-expressing cells that are essential for melanocyte stripe regeneration. In future studies it will be important, through transplantation and other approaches, to determine the potential of the unpigmented *mitfa*-expressing cells and whether they are involved in melanocyte regeneration at other anatomic locations or can functionally substitute for developmental progenitors. Identifying signals that govern melanocyte regeneration is also critical. Similar to what we have shown in zebrafish stripe regeneration, Wnt signaling controls differentiation of mammalian follicular melanocyte stem cells (Rabbani et al., 2011). It will be interesting to determine additional developmental pathways that are utilized during regeneration and which, if any, initiate the regenerative process.

A poised mechanism of regeneration may enable rapid replacement of a critical cell type in other systems. For example, during follicle regrowth in mammals, epidermal hair follicle stem cells adopt different fates, depending on their position in the niche (Rompolas et al., 2013). Those closest to the growing follicle exit their niche in the bulge region of the permanent follicle region and become transit-amplifying cells, whereas those more distal retain stem cell identity. The situation with mammalian melanocyte stem cells (MSCs) is less clear. MSCs can proliferate extensively during hair follicle regrowth (Nishimura et al., 2002). However, under certain circumstances such as UV-induced interfollicular epidermal injury, MSCs can prioritize differentiation over stem cell maintenance (Chou et al., 2013). By employing cells predisposed to differentiation, poised regeneration could provide a means of repairing vital tissues and cell types with minimal delay.

EXPERIMENTAL PROCEDURES

Fish Stocks and Husbandry

Zebrafish were handled in accordance with approved institutional protocols at the University of Massachusetts Medical School. Fish stocks were maintained

(E) Representative images of control (top row) or neocuproine-treated (bottom row) Mitfa-positive melanocytes after melanocyte regeneration. neo, neocuproine; BF, brightfield. BF scale bar, 15 μM; Mitfa, EdU scale bar, 10 μM.

(F) Quantification of percent EdU-positive, Mitfa-positive melanocytes. n = 97, 256 and 365 cells from each of three fish, respectively, for neo; n = 43, 53, and 85 cells from each of three fish, respectively, for w/o neo. Data are shown as mean percent positive per fish ± SEM; p value calculated by Student's t test. neo, neocuproine. See also Table S1.

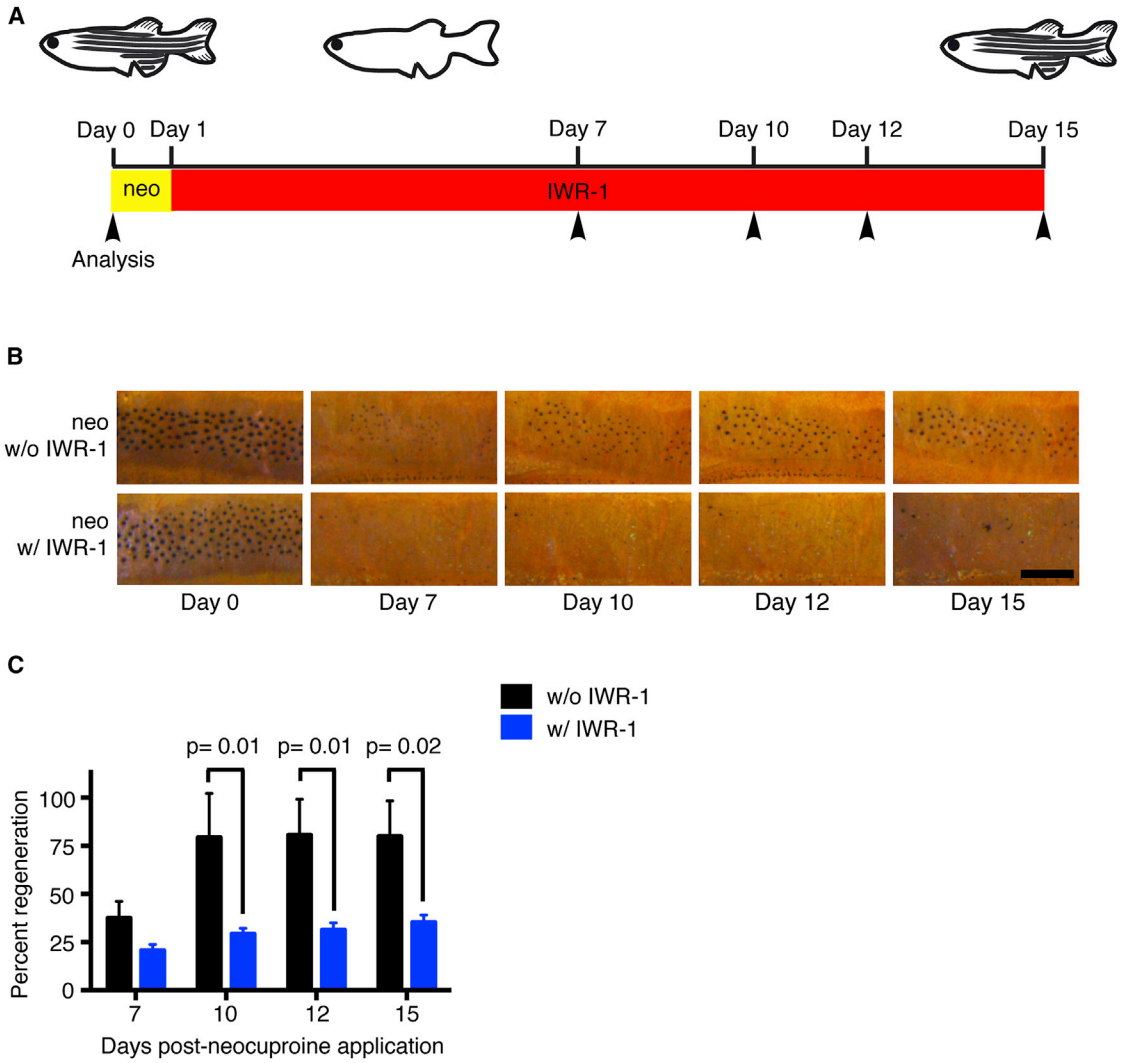

Figure 7. Wnt Signaling Regulates Melanocyte Regeneration

(A) Timeline of the experiment. Adult wild-type zebrafish were treated with neocuproine for 24 hr to ablate differentiated melanocytes and were then treated with the Wnt inhibitor IWR-1 for 14 days. Control animals were treated with neocuproine and then DMSO alone. Fish were scored for regeneration melanocytes at 7, 10, 12, and 15 days (arrowheads) after neocuproine treatment. neo, neocuproine.

(B) Representative images of control (top row) or IWR-1-treated (bottom row) animals during melanocyte regeneration. Fish were treated with epinephrine prior to imaging. neo, neocuproine. Scale bar, 500 μM.

(C) Quantification of melanocyte regeneration. Data are shown as mean ± SEM; p values calculated by Student's t test, Day 7 p = 0.36.

See also Figure S6.

at 28.5°C on a 14L:10D light cycle (Westerfield, 2007). The wild-type strain used was AB. Alleles used in this study were *mitfa*^{w2} (Lister et al., 1999) and *p53*^{zdf1} (Berghmans et al., 2005).

DNA Constructs

sox10, *dct*, *pax3a*, and *erbb3b* promoters were PCR-amplified from zebrafish genomic DNA or BAC clones, the *TOPFlash* reporter promoter containing Tcf/Lef binding sites was PCR-amplified from plasmid TOPdGFP and cloned into pDONRP4P1r (Life Technologies). The *NTR and NTRmCherry* from plasmid UNM, *mCherry-zCdt1* and *mAG-zGeminin* from plasmids *ZD:mCherry-zCdt1(1/190)/pT2KXIGΔin* and *ZB:mAG-zGeminin(1/100)/pT2KXIGΔin*, respectively, were PCR amplified and cloned into pDONR221 (Life Technologies). Oligonucleotides used are described in Supplemental Experimental Procedures. To create the *FOPFlash* reporter promoter, a gBlock (IDT) containing *FOPFlash* (*TOPFlash* with mutated Tcf/Lef binding sites) (van de Wetering et al., 1997) was used for cloning into pDONRP4P1r (Life Technologies). The construction of miniCoopR and pENTRP4P1r-*mitfa* was previously described (Ceol et al., 2011). pENTR5′_*ubi* (Mosimann et al., 2011) and the following components of the Tol2Kit (Kwan et al., 2007) were used: pME-*nlsEGFP*, pME-*mCherryCAAX*, p3E-*polyA*, pDestTol2pA2, pCS2FA-*transposase*. Using the entry clones described above, multisite Gateway cloning (Life Technologies) was used to make the following constructs for injection (a *polyA* signal was used as the 3′ element in all cases): miniCoopR-*mitfa:NTR*, miniCoopR-*mitfa:NTRmCherry*, miniCoopR-*sox10:NTRmCherry*, miniCoopR-*dct:NTRmCherry*, miniCoopR-*pax3a:NTRmCherry*, miniCoopR-*erbb3b:NTRmCherry*, miniCoopR-*mitfa:nlsEGFP*, miniCoopR-*mitfa:mCherryCAAX*, miniCoopR-*ubi:mCherry-zCdt1*, miniCoopR-*ubi:mAG-zGeminin*, miniCoopR-*TOPFlash:mCherry*, miniCoopR-*FOPFlash:mCherry*, Tol2pA2-*mitfa:nlsEGFP*, Tol2pA2-*mitfa:nlsmCherry*. For

metronidazole-mediated ablation either miniCoopR-*mitfa:NTR* or miniCoopR-*mitfa:NTRmCherry* transgene was used.

Microinjection and Transgenic Fish

For transposon-mediated integration, 25 pg of a construct was injected along with 25 pg Tol2 transposase mRNA into one-cell embryos. For injections of two constructs, constructs were linearized and 25 pg of each coinjected into embryos. When injected into zebrafish embryos, linearized transgenes cosegregate such that transgenic cells in resulting chimeric animals contain both transgenes (Langenau et al., 2008). The miniCoopR system was used to generate transgenic animals (Ceol et al., 2011). Using this system, transgenes were juxtaposed to a *mitfa* minigene in the miniCoopR vector, and the resulting constructs injected into melanocyte-deficient *mitfa(lf)* mutant animals. The *mitfa* minigene is capable of rescuing melanocytes in *mitfa(lf)* mutants cell autonomously, causing any transgene juxtaposed to the *mitfa* minigene to be present in rescued melanocytes and cells lineally related to them.

Lineage Analysis

Adult zebrafish were treated with neocuproine in a beaker for 24 hr and then kept individually in tanks after neocuproine was washed out. Prior to imaging, fish were anesthetized with 0.17 mg/ml tricaine and were placed on their sides in a plastic Petri dish. The same locations on the fish were identified using patterns of miniCoopR rescue as a landmark and were imaged three times or once daily before and after neocuproine treatment. Fish were viewed with a Leica M165FC stereomicroscope and images captured with a Leica DFC400 camera. The fish were allowed to recover in fish water.

EdU Incorporation Assay and Histological Methods

Wild-type fish injected with EdU were sacrificed, fixed for 2 hr in freshly prepared 4% PFA at room temperature, and 14-μm cryosections were cut. The sections were stained with Mitfa primary antibody (1:100) (Ceol et al., 2011) followed by Alexa Fluor 488 secondary antibody (Life Technologies). EdU development was performed (Click-iT EdU Alexa Fluor 594 Imaging Kit; Life Technologies), and sections were stained with DAPI and mounted (Fluoromount G; SouthernBiotech).

Drug Treatments

Metronidazole (Sigma-Aldrich) was dissolved to a final concentration of 5 mM in fish water containing 0.02% DMSO. Adult zebrafish were treated for three cycles of 3 days on followed by 3 days off drug. Neocuproine (Sigma-Aldrich) was used at 750 nM in fish water containing 0.0075% DMSO. Neocuproine treatment was performed as described previously (O'Reilly-Pol and Johnson, 2008). Epinephrine (Sigma-Aldrich) was used at 1 mg/ml in fish water, and treatments were performed for 10 min immediately prior to imaging. IWR-1 (Sigma-Aldrich) was used at 10 μM in fish water containing 0.1% DMSO. Ten microliters EdU (Life Technologies) at 10 mM in 0.9× PBS containing 0.1% DMSO was injected intraperitoneally at indicated time points.

Imaging and Quantitative Analysis

Whole-fish images were captured using a Nikon D90 camera equipped with SB-R200 wireless remote speed lights. Fish with fluorescent markers were viewed with a Leica M165FC stereomicroscope or a Leica DM550B compound microscope and images captured with Leica DFC400 or DFC365FX cameras, respectively. Brightfield images were adjusted for contrast and color balance for clarity. Sections were viewed with a Leica DM550B compound microscope and images were captured with DFC365FX or DMC2900 cameras. To quantify the effect of IWR-1 on melanocyte regeneration, flanks of fish were imaged and melanocytes counted on the left side center stripe of each fish in a rectangular region delimited as a 5.8 mm × 1.2 mm window with its left boundary 1 mm posterior to the edge of the operculum. Percent regeneration was calculated as a ratio of the number of melanocytes within the region at the specified time point to the number of melanocytes within the region prior to neocuproine treatment. Student's t tests were performed using Prism 6.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, one table, and two movies, and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.04.025.

AUTHOR CONTRIBUTIONS

S.I. and C.J.C. designed and interpreted the experiments. S.I. performed the experiments. M.K. assisted with genotyping and cryosectioning. S.I. and C.J.C. wrote the manuscript.

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