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Mutations in *gfpt1* and *skiv2l2* Cause Distinct Stage-Specific Defects in Larval Melanocyte Regeneration in Zebrafish

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The establishment of a single cell type regeneration paradigm in the zebrafish provides an opportunity to investigate the genetic mechanisms specific to regeneration processes. We previously demonstrated that regeneration melanocytes arise from cell division of the otherwise quiescent melanocyte precursors following larval melanocyte ablation with a small molecule, MoTP. The ease of ablating melanocytes by MoTP allows us to conduct a forward genetic screen for mechanisms specific to regeneration from such precursors or stem cells. Here, we reported the identification of two mutants, *eartha* and *julie*, from a melanocyte ablation screen. Both mutants develop normal larval melanocytes, but upon melanocyte ablation, each mutation results in a distinct stage-specific defect in melanocyte regeneration. Positional cloning reveals that the *eartha* mutation is a nonsense mutation in *gfpt1* (*glutamine:fructose-6-phosphate aminotransferase 1*), the rate-limiting enzyme in glucosamine-6-phosphate biosynthesis. Our analyses reveal that a mutation in *gfpt1* specifically affects melanocyte differentiation (marked by melanin production) at a late stage during regeneration and that *gfpt1* acts cell autonomously in melanocytes to promote ontogenetic melanocyte darkening. We identified that the *julie* mutation is a splice-site mutation in *skiv2l2* (*superkiller viralicidic activity 2-like*), a predicted DEAD-box RNA helicase. Our in situ analysis reveals that the mutation in *skiv2l2* causes defects in cell proliferation, suggesting that *skiv2l2* plays a role in regulating melanoblast proliferation during early stages of melanocyte regeneration. This finding is consistent with previously described role for cell division during larval melanocyte regeneration. The analyses of these mutants reveal their stage-specific roles in melanocyte regeneration. Interestingly, these mutants identify regeneration-specific functions not only in early stages of the regeneration process, but also in late stages of differentiation of the regenerating melanocyte. We suggest that mechanisms of regeneration identified in this mutant screen may reveal fundamental differences between the mechanisms that establish differentiated cells during embryogenesis, and those involved in larval or adult growth.

**Introduction**

Regeneration and turnover of cells and tissues are essential in many vertebrates, including humans. Tissue homeostasis and regeneration have often been demonstrated to act through recruiting quiescent undifferentiated precursors or stem cells to re-enter developmental programs reminiscent of embryonic mechanisms. The mechanisms regulating these processes are critical for maintaining normal physiology. For instance, uncontrolled growth of undifferentiated precursors or stem cells has been suggested to cause many types of cancers, including leukemia and skin cancer [1–5]. However, little is known about the genes and pathways that specifically regulate these precursors or stem cells. The fact that the differentiation and physiological states of adult animals are very distinct from embryos raises the possible existence of pathways or mechanisms specific to regeneration or to those mechanisms that regulate the development of these undifferentiated cells. Such mechanisms may play roles in establishing the precursors or stem cells during embryogenesis, maintaining them throughout development, or recruiting them at the adult stages for growth, regeneration, or tissue homeostasis. Understanding the mechanisms specific to regeneration or homeostasis will provide insight into the regulation of stem cells, which may lead to the development of therapies for many human diseases.

Regeneration studies are classically performed by amputating a mass of continuous tissues, such as a limb or a portion of an organ, and investigating how organisms coordinately regenerate multiple types of cells or tissues [6,7]. Recently,
understand the cause or cure for human disease resulting
provides an opportunity to isolate the mechanisms that
complexity of the regeneration process, and second, it
advantages. First, focusing on a single cell type reduces the
and subsequent regeneration [8–11]. This approach has two
some attention has been focused on single cell type ablation
This phenotype suggests the regeneration-specific roles of the
melanocyte pattern provides an opportunity to study
aimed at isolating mutants that fail to regenerate melanocytes.
we report a melanocyte ablation screen in zebrafish
A total of 29 of these clutches and
our analyses on chondrocyte development in eartha
Importantly, after the establishment of the
larval melanocytes by MoTP allows us to conduct a forward genetic
consistent with the role for cell division during melanocyte
We identified the mutation in gfpt1 (glutamine:fructose-
However, the number of larval melanocytes remains
relatively constant throughout the following two weeks of
development with little or no turnover. This period of stable
melanocyte pattern provides an opportunity to study
ish; melanin to melanin” stage during melanocyte regeneration.
We identified the mutation as a splice-site
function in regulating various aspects of RNA metabolism in
Our in situ analysis reveals that skiv2l2 plays an
an important role in cell proliferation at postembryonic stages,
consistent with the role for cell division during melanocyte
regeneration we previously described [9].

Results

Mutations Specific to Larval Melanocyte Regeneration

To understand the genetic mechanisms underlying larval melanocyte regeneration, we conducted a parthenogenesis or
early-pressure (EP) screen for mutations that specifically
block larval melanocyte regeneration, but allow ontogenetic
melanocyte development during embryogenesis. We ablated larval melanocytes by incubating embryos in MoTP from 14
to 72 hours postfertilization (hpf) and scored their melanocyte
regeneration at 5–8 days postfertilization (dpf) when
wild-type larvae have regenerated many melanocytes (Figure
1A and 1B) [9]. Among the 648 EP clutches generated, 421
clutches had more than ten individuals survive through the
early pressure and MoTP incubation procedure for melanocyte
regeneration analysis. A total of 29 of these clutches and
their $F_1$ mothers were kept as putative mutants, because each
of these EP clutches had at least one individual lacking
melanocytes after MoTP washout. In addition to these 421
clutches, seven small clutches (fewer than ten surviving

**Author Summary**

Programs of ontogenetic development and regeneration share many components. Differences in genetic requirements between
regeneration and development may identify mechanisms specific to
the stem cells that maintain cell populations in postembryonic stages, or identify other regeneration-specific functions. Here, we
utilize a forward genetic approach that takes advantage of single
cell type ablation and regeneration to isolate mechanisms specific to
regeneration of the zebrafish melanocyte. Upon chemical ablation
of melanocytes, zebrafish larvae reconstitute their larval pigment
pattern from undifferentiated precursors or stem cells. We isolated
two zebrafish mutants that develop embryonic melanocytes
normally but fail to regenerate their melanocytes upon ablation.
This phenotype suggests the regeneration-specific roles of the
mutated genes. We further identified the mutations in gfpt1 and
skiv2l2 and show their stage-specific roles in melanocyte regener-
ation. Interestingly, these mutants identify regeneration-specific
functions not only in early stages of the regeneration process
(skin2l2), but also in late stages of differentiation of the regenerating
melanocyte (gfpt1). We suggest that mechanisms of regeneration
identified in this mutant screen may reveal fundamental differences
between the mechanisms that establish differentiated cells during
embryogenesis and those involved in larval or adult growth.

some attention has been focused on single cell type ablation
and subsequent regeneration [8–11]. This approach has two
advantages. First, focusing on a single cell type reduces the
complexity of the regeneration process, and second, it
provides an opportunity to isolate the mechanisms that
control adult stem cells from other biological mechanisms
that may be occurring during the normal development of that
cell type. Additionally, it may also provide an opportunity to
understand the cause or cure for human disease resulting
from single cell type deficits, such as loss of β cells in
autoimmune type I diabetes [12]. Several methods that ablate
single cell types have been reported in various vertebrates,
including genetic approaches, and use of lasers or chemicals
[10,13–16]. For example, Lamotte et al. [13] used transgenic
methods to introduce tissue-specific expression of a toxin to
ablate mouse pancreatic β cells. Other examples include
exposure to a dermatology laser and incubation with a small
molecule, 4-(4-morpholinobutylthio)phenol (MoTP), each of
which has been shown to specifically ablate zebrafish larval
melanocytes [9,17].

The zebrafish larval melanocyte is an attractive cell type for
regeneration studies. Mature larval melanocytes can be easily
followed by the presence of black pigment; they are
dispensable for fish viability in the laboratory, and multiple
mutations have been generated that affect different aspects of
melanocyte development at different stages of the fish life
cycle [18–20]. Most importantly, after the establishment of the
larval melanocyte pattern during the first three days of fish
development, the number of larval melanocytes remains
relatively constant throughout the following two weeks of
development with little or no turnover. This period of stable
melanocyte pattern provides an opportunity to study
melanocyte regeneration once ablated. We previously re-
ported that a small molecule MoTP specifically ablates
melanocytes or melanoblasts in zebrafish larvae. The mel-
ancytotoxicity of MoTP is mediated via tyrosinase activity,
individuals) were also kept since at least one of the surviving individuals showed defects in melanocyte regeneration. In total, we found 36 clutches with possible defects in melanocyte regeneration. We then excluded 12 of these 36 clutches from further characterization because additional phenotypes in ontogenetic melanocyte development were observed in their haploid siblings. Among the remaining 24 possible melanocyte regeneration-specific mutants, we successfully rescreened 15 (62%) of them, either by a second EP screen of the F1 female or from the EP embryos of F2 outcross, indicating that we effectively screened approxi-
mately 263 (421 × 0.62) haploid genomes. Among these 15 possible mutant lines, only two repeated their melanocyte regeneration phenotypes. This high false positive noise could be due to the frequent developmental defects that were caused by the early pressure procedure. Because these mutagenesis protocols typically identify mutations in targeted loci at frequencies of approximately one per 1,000 haploid genomes (25,26), our result suggests that there may be many more genes that could be found by the further exploitation of this melanocyte ablation screen. We followed our laboratory’s cat theme for naming pigment mutants. Because the Catwoman character is regenerated by each new actress that plays the role, we chose to name our two regeneration mutations, eartha^{25c1} and julie^{24c1} after Catwoman actresses Eartha Kitt and Julie Newmar.

Both eartha^{25c1} and julie^{24c1} mutants develop ontogenetic melanocytes normally (Figure 1C, 1E, and 1G), but following melanocyte ablation by MoTP, eartha^{25c1} and julie^{24c1} mutants only regenerate 11.8% and 10.0%, respectively, of the normal complement of regeneration melanocytes at 5 dpf (Figure 1D, 1F, and 1H). Both eartha^{25c1} and julie^{24c1} mutations are recessive lethal, with most eartha^{25c1} mutants dying at approximately 12 dpf, and julie^{24c1} mutants dying at approximately 7–8 dpf. This raises the possibility that the larval melanocyte regeneration defects observed in eartha^{25c1} and julie^{24c1} mutants are the consequences of nonspecific larval-stage lethality rather than specific effects on melanocyte regeneration. To help rule out this possibility, we examined melanocyte regeneration in homozygous mutants for an early larval lethal mutation, rapunzel^{14} [27]. Although homozygous rapunzel^{14} larvae show many developmental defects including defects in jaw and fin-fold development (unpublished data), the number of melanocytes in rapunzel^{14} larvae is indistinguishable from that in wild-type larvae at 3 dpf (Figure 1G). By 5 dpf, almost all rapunzel^{14} larvae have severe edema and then die at approximately 6 dpf. Upon melanocyte ablation by MoTP (14–72 hpf), we found that rapunzel^{14} mutants regenerate approximately 70% of the number of the melanocytes that regenerate in wild-type larvae, 7-fold more melanocytes than those that regenerate in eartha^{25c1} and julie^{24c1} mutants (Figure 1H). This result rules out a strict correlation between larval-stage lethality and defects in melanocyte regeneration, and also suggests that the regeneration defects observed in eartha^{25c1} and julie^{24c1} larvae result from defects specific to regeneration mechanisms.

**eartha Mutation Specifically Affects Larval Melanocyte Regeneration after dct Stage**

The development of eartha^{25c1} embryos is indistinguishable from wild-type embryos until 3–4 dpf at which stage their melanocytes appear lighter than wild-type larvae (Figure 1C). This observation suggests that eartha plays a role in ontogenetic melanocyte darkening (Figure 2A). Additional defects appear after 5 dpf in eartha^{25c1} larvae, including a defect in jaw development, where the jaw fails to grow anterior beyond the eyes, typically described as the hammerhead phenotype (Figure 1G) [28]. eartha^{25c1} mutants develop swim bladders normally, but most die by 12 dpf, presumably a consequence of the pharyngeal skeleton defect leading to an inability to feed. Upon melanocyte ablation by MoTP treatment, only 11.8% of melanocytes regenerate at 5 dpf in

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**Figure 1. Mutations Specific to Melanocyte Regeneration**

(A–F) eartha^{25c1} (C) and julie^{24c1} mutants (E) both develop larval melanocyte patterns nearly identical to wild-type larvae (A), although melanocytes in eartha^{25c1} mutants appear to have lighter pigmentation than that in the wild-type larvae. Upon larval melanocyte ablation by MoTP (14–72 hpf), wild-type larvae (B) regenerate many melanocytes at 5 dpf (arrowhead in [B]), while eartha^{25c1} (arrowhead in [D]) and julie^{24c1} (arrowhead in [F]) mutants largely fail to regenerate melanocytes. Additional phenotypes are indicated by an arrow in (C) marking a shortened pharyngeal skeleton in eartha^{25c1} mutants and by an arrow in (E) indicating small eyes and head in julie^{24c1} mutants. (G–H) The numbers of melanocytes in the untreated (G) and MoTP-treated (H) wild-type and mutant larvae at 5 dpf.

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To further understand how the eartha mutation affects melanocyte regeneration, we examined melanocyte differentiation in eartha; dct–/– larvae during melanocyte regeneration after MoTP washout. Larval melanocytes in eartha; dct–/– larvae were ablated by MoTP treatment from 14 to 72 hpf, and regenerated melanocytes were analyzed at 7 dpf by in situ hybridization (ISH) analysis, a time when many melanocytes regenerate in wild-type larvae (Figure 2B). Although eartha; dct–/– larvae had only very few regenerated melanocytes (melanin+), we detected many dct+ melanoblasts (melanin–) in the eartha; dct–/– larvae (Figure 2C). The number of these dct+ melanoblasts and regenerated melanocytes (melanin+) in the dorsum of eartha; dct–/– mutants (30.5 ± 9.2) is virtually identical to that in the wild-type larvae (31.0 ± 7.2). This result suggests that regenerated melanocytes in eartha; dct–/– larvae develop to late-stage (dct+) but fail to produce melanin, and that eartha acts after the dct+ stage in melanocyte differentiation during melanocyte regeneration (Figure 2A).

To test the specificity of eartha’s role in melanocyte regeneration, we examined tail regeneration in eartha; dct–/– larvae. When wild-type larval tails were amputated at 3 dpf, the wound first heals by closing the injured tip of the notochord and neural tube, followed by the outgrowth of fin fold. By 9 dpf (6 d postamputation), the missing larval tails are largely reconstituted, including complete regeneration of fin-fold tissues and melanocytes. Additional lightly pigmented melanocytes appear after PTU washout, indicating that these melanocytes are newly differentiated. When similar amputations were conducted in eartha; dct–/– larvae, the larval tails regenerated identically to wild-type larvae, except that few melanocytes appear in the regenerated tail in the region immediately distal to the amputation plane (arrow in [I]). The white-dashed lines outline the fin folds. Timeline (grey) below (C) indicates the period of MoTP treatment (red) and analysis time (vertical line above the timeline). Scale bars: 250 μm.

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Figure 2. The eartha; dct–/– Mutation Specifically Affects Melanocyte Regeneration after the dct+ Stage but Has No Effect on Larval Tail Regeneration

(A) The roles of eartha in different stages of melanocyte development in ontogeny and regeneration are presented.

(B and C) Larval melanocytes in the wild-type (B) and eartha; dct–/– (C) larvae were ablated by MoTP treatment from 14 to 72 hpf, and melanocyte regeneration was analyzed at 7 dpf by ISH with dct riboprobes for late-stage melanoblasts. At this stage, many melanocytes (melanin+) have regenerated in the wild-type larvae (arrowhead in [B]). Although only few pigmented melanocytes appear in eartha; dct–/– larvae at the same stage, many dct+ melanoblasts (melanin–, stained purple) are revealed by ISH (arrow in [C]).

(D–I) eartha; dct–/– mutation specifically affects melanocyte regeneration during larval tail regeneration. Arrowheads show amputation planes at 3 dpf prior to amputation (D) and (H) and after regeneration (E–G) and (I). Wild-type larval tails (D) were amputated at 3 dpf. The wound healed in the ensuing two days by closing the injured tip of the notochord and neural tube, followed by the outgrowth of fin fold. By 9 dpf (6 d postamputation), the missing larval tails were reconstituted, including fin-fold tissues (outlined with white-dashed lines) and melanocytes (arrow in [E]). The origins of the re-established tail melanocytes were investigated by incubating larvae in PTU immediately after larval tail amputations (F). PTU prevents melanin synthesis in newly differentiated melanocytes, and thus the pigmented melanocytes that appear in regenerated tails of the PTU-treated larvae pre-existed prior to the tail amputation. At 9 dpf, few melanocytes (arrow in [F]) appear in the newly regenerated tail immediately distal to the amputation plane in the PTU-treated larvae, suggesting that these melanocytes arise from the migration of proximately differentiated melanocytes from the stump (F). Additional lightly pigmented melanocytes appear after PTU washout (arrow in [G]), indicating that these melanocytes are newly differentiated. When similar tail amputations were conducted in eartha; dct–/– larvae (H and I), the larval tails regenerated identically to wild-type larvae, except that few melanocytes appear in the regenerated tail in the region immediately distal to the amputation plane (arrow in [I]). The white-dashed lines outline the fin folds. Timeline (grey) below (C) indicates the period of MoTP treatment (red) and analysis time (vertical line above the timeline). Scale bars: 250 μm.
revealed by Alcian Blue staining, where the staining in eartha is disarranged in the hyosymplectic cartilage are arranged into a single cell wide stack (B) is significantly weaker than that in wild-type larvae (A). At 10 dpf, the chondrocytes in the symplectic (SY) region (arrowhead in (C) and (E)) of the hyosymplectic cartilage are arranged into a single cell wide stack (arrowhead in [E]) in wild-type larvae. This organization of chondrocytes is disarranged in the eartha larvae (arrowheads in (D) and (F)). Arrows in (A) and (B) indicate the hyosymplectic cartilages that are shown in (C) and (D). Arrowheads in (C) and (D) mark the SY region of hyosymplectic cartilage. (E) and (F) are enlarged images of the SY region of hyosymplectic cartilage.

Scale bars: 100 µm.

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Figure 3. The eartha Mutation Affects Cartilage Maturation
(A-F) The pharyngeal skeleton of wild-type and eartha larvae are revealed by Alcian Blue staining, where the staining in eartha larvae (B) is significantly weaker than that in wild-type larvae (A). At 10 dpf, the chondrocytes in the symplyctic (SY) region (arrowhead in (C) and (E)) of the hyosymplectic cartilage are arranged into a single cell wide stack (arrowhead in [E]) in wild-type larvae. This organization of chondrocytes is disarranged in the eartha larvae (arrowheads in (D) and (F)). Arrows in (A) and (B) indicate the hyosymplectic cartilages that are shown in (C) and (D). Arrowheads in (C) and (D) mark the SY region of hyosymplectic cartilage. (E) and (F) are enlarged images of the SY region of hyosymplectic cartilage.

Scale bars: 100 µm.

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j23e1 Mutation Affects Late-Stage Cartilage Differentiation

To further understand how eartha regulates developmental processes, we sought to examine the development of pharyngeal skeleton in eartha mutants. The pharyngeal skeleton develops from cranial neural crest cells. These arise from the dorsal part of the embryonic midbrain and hindbrain area, and migrate ventrally into the pharyngeal arches to form various cells and tissues, including cartilage. Mature cartilage includes chondrocytes and the surrounding ECM secreted by chondrocytes. At 4 or 5 dpf, the zebrafish pharyngeal skeleton and the cellular organization of chondrocytes can be robustly revealed by Alcian Blue staining, a dye that recognizes the cartilage matrix (Figure 3A). For instance, at this stage, chondrocytes in the symplectic (SY) region of the hyosymplectic cartilage are arranged into a single cell-wide stack, like a stack of coins (Figure 3C and 3E) [30,31]. eartha larvae develop shortened pharyngeal skeleton (Figures 1C and 3B). To assess the differentiation of the pharyngeal arches in eartha larvae, we use ISH with a variety of riboprobes, including dl2 and for migrating craniofacial neural crest and sox9a and type II collagen (col2a1) for differentiated chondrocytes. We found no differences in expression levels and patterns between the eartha and wild-type larvae for any of these markers above (unpublished data), indicating that chondrocytes for pharyngeal skeleton in eartha mutants are specified and then differentiate normally. In contrast, Alcian Blue staining, which marks cartilage, is significantly weaker in eartha larvae than in wild-type larvae (Figure 3A and 3B), suggesting a defect in cartilage maturation. In addition, we found eartha larvae develop all their pharyngeal cartilages, however the organization of chondrocytes is disarranged. For example, the chondrocytes in the SY cartilage fail to become arranged into the ordered cell stacks that are observed in wild-type larvae (Figure 3D and 3F). This disarrangement of chondrocytes causes the failure of pharyngeal skeleton extension into positions anterior to the eyes, accounting for the shortened head length (or hammerhead) phenotype. This observation that eartha mutants are defective in late-stage cartilage differentiation together with our analyses that indicate eartha mutation affects late-stage melanocyte differentiation during regeneration, may suggest that eartha plays a role in regulating late-stage cell differentiation.

The eartha Phenotype Is Caused by a Nonsense Mutation in gft1

To further understand how eartha regulates melanocyte regeneration, we took a positional cloning approach to identify the eartha mutation. We first mapped the eartha mutation to zebrafish Chromosome 8 by half-tetrad centromere linkage [35,36]. The eartha mutation was next mapped with a panel of 114 meioses to a 15 cM region between simple sequence repeat (SSR) markers z15786 and z51584 (Figure 4A). To further identify candidate genes, we built a physical map based on the sequences from the zebrafish genome assembly version 5 (Zv5, The Sanger Institute) by comparing sequence of the flanking markers.
z15786 and z51584 and several ESTs that were mapped within the region by radiation hybrid mapping (http://134.174.23.167/zonrhmapper/Maps.htm) [37]. Various assembly contigs were obtained and then bridged by additional fingerprinted BAC information (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish/small.shtml). Finally, the accuracy of our physical map was tested and confirmed by mapping new SSR or single nucleotide polymorphism markers that were developed from each of the assembly sequences to a selected recombinant panel derived from 1,514 meioses (Figure 4B).

By this fine mapping approach, a region containing the eartha<sup>23e1</sup> mutation, flanked by seven recombinants at the proximal end and three recombinants at the distal end, was determined across approximately 189 kb comprising four genes: ntrk2, agtpbp1, mak10, and gfpt1 (Figure 4B). A minimal critical region for the eartha<sup>23e1</sup> mutation was further determined by a single proximal recombinant with a marker in mak10 and two distal recombinants with a marker in the fourth exon of gfpt1. Sequencing of the cDNAs from the eartha<sup>23e1</sup> mutants for candidate genes agtpbp1 and mak10, which lie immediately outside the critical region, revealed no nucleotide difference from those cDNA derived from the originating nonmutagenized background (sjD strain).

In contrast, we found a nonsense mutation in the third exon of gfpt1 cDNA from eartha<sup>23e1</sup> mutants (Figure 4C and 4D). This mutation is predicted to result in an unstable RNA transcript and a truncated protein. Consistent with this prediction, we seldom detected gfpt1 RNA transcripts in the eartha<sup>23e1</sup> mutants by ISH (Figure 5B).

To further test that eartha phenotypes result from loss of gfpt1 function, we attempted to rescue eartha phenotypes by injecting gfpt1 mRNA into larvae from eartha<sup>23e1</sup>/þ intercrosses. We observed that seven out of 134 larvae injected with gfpt1 mRNA (J) have some distinctly darker melanocytes (blue arrows in [G]) and more extended pharyngeal skeletons (black arrow in [H] and [I]) than those in the noninjected eartha<sup>23e1</sup> larvae (black and blue arrows in [F]).

**Figure 4.** The eartha<sup>23e1</sup> Mutation Is a Nonsense Mutation in gfpt1

(A–E) The eartha<sup>23e1</sup> mutation was mapped to Chromosome 8 between z15786 and z51584 (A). The physical map of the eartha locus was built from the zebrafish genome assembly version 5 (Zv5) with the support of recombination observed in 1,514 meioses. Shown here is a portion of this physical map delimited by seven recombinants at the proximal end and three recombinants at the distal end, containing approximately 189 kb comprising four genes: ntrk2, agtpbp1, mak10, and gfpt1 (B). Sequencing of the eartha<sup>23e1</sup> cDNA for gfpt1 revealed a nonsense mutation in the third exon, where a CAA to TAA substitution resulting in Gln to stop codon change (C) and (D). The phylogenetic tree of gfpt genes was constructed by using the neighbor-joining method with *E. coli* glmS as the out-group (E).

(F–J) gfpt1 mRNAs partially rescue the eartha phenotypes. gfpt1 mRNAs were in vitro synthesized and injected into one to four cell stage embryos. At 5–7 dpf, 22% of the eartha<sup>23e1</sup> larvae injected with gfpt1 mRNA (J) have some distinctly darker melanocytes (blue arrows in [G]) and more extended pharyngeal skeletons (black arrow in [H] and [I]) than those in the noninjected eartha<sup>23e1</sup> larvae (black and blue arrows in [F]). SIS: sugar isomerase domain.

Scale bars: 100 μm.

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eartha mutants. Taken together, with 24 unrescued eartha larvae, this result suggests that we partially rescued 22% (7/31) of the eartha mutants by injection with gfpt1 mRNA. We note that, however, the mRNA injection experiments failed to rescue the much later melanocyte regeneration phenotype, presumably because the mRNA injected at cell stage 1–4 was no longer available at the late larval stages when melanocytes were challenged to regenerate. Taken together, the high-resolution mapping, cDNA sequencing, and mRNA rescue indicate that the eartha phenotype is caused by a null mutation in gfpt1.

gfpt1 catalyzes the formation of glucosamine 6-phosphate and is the first and rate-limiting enzyme in this hexosamine biosynthesis pathway. In human, two related GFPT genes, GFPT1 and GFPT2, have been described [38,39]. In the zebrafish Zv5 genome assembly, besides gfpt1/eartha, we identified a single additional gfpt-related gene. Phylogenetic analysis (Figure 4E) revealed that this additional gene is more closely related to the mammalian glutamine-fructose-6-phosphate aminotransferase 2 (gfpt2) gene than to zebrafish and mammalian gfpt1; therefore, we defined it as the zebrafish orthologue of gfpt2. The zebrafish gfpt2 locus is located on Chromosome 21 (LG 21) near marker z28262 by our analysis of the zebrafish Zv5 genome assembly.

Expression of gfpt1 during Embryogenesis and Early Larval Development

To further explore how gfpt1 expression and function may promote larval melanocyte regeneration following melanocyte ablation by MoTP, we examined the expression of gfpt1 during early development. gfpt1 transcripts were detected as early as the four-cell stage by reverse transcription-PCR and ISH and are distributed throughout the early embryo (unpublished data). ISH analysis demonstrated the restriction of gfpt1 expression by 13 hpf, including intense expression in the notochord and the pillow (polster, arrow in [A]) at 13 hpf. At 96 hpf, gfpt1 is expressed in the lateral line neuromasts (arrowhead in [G]) and the pharyngeal arches (arrow in [G] and arrowheads in [F]). A cross section ([H]) shows the gfpt1 expression in the neuromasts.

Figure 5. gfpt1 Is Expressed in Notochord, Ear, and Pharyngeal Arches, and Acts Cell Autonomously in Melanocytes to Promote Melanocyte Darkening

(A–H) ISH reveals high expression of gfpt1 in the notochord (arrowhead in [A]) and the pillow (polster, arrow in [A]) at 13 hpf. No or very few gfpt1 transcripts were detected in the eartha23a1 mutants at 13 hpf (B). A cross section (C) shows the gfpt1 expression in notochord at 13 hpf. gfpt1 is expressed in the ear (arrowhead in [E]) and pharyngeal arches (arrow in [E]) at 36 hpf. gfpt1 expression in the sensory epithelium of the ear is shown in a cross section (D). At 96 hpf, gfpt1 is expressed in the lateral line neuromasts (arrowhead in [G]) and the pharyngeal arches (arrow in [G] and arrowheads in [F]). A cross section (H) shows the gfpt1 expression in the neuromasts.

(I–J) Chimeric animals were generated by transplanting wild-type tg(bactin:GPF) donor cells into eartha23a1 embryos at midblastula stage. A donor (GFP) melanocyte in the eartha larva is revealed following incubation in 0.1% epinephrine to induce melanosome contraction (white arrow in [I]). In the uncontracted state, the GFP (donor-derived) melanocyte appears darkly pigmented, in contrast to the lightly pigmented neighboring melanocytes (GFPr) that are derived from host (blue arrow in [J]). Yellow arrow in (I) indicates donor-derived skin cells (GFP) that are not associated with darkened melanocytes.

Scale bars: in (A), 50 μm for (A and B); in (C and D) and (H), 20 μm; in (E–G), 50 μm; in (I), 50 μm for (I and J).

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gfpt1 Acts Cell Autonomously in Melanocytes to Promote Melanocyte Darkening

To test the cell autonomy of gfpt1 for late-stage melanocyte differentiation, we took advantage of the ontogenetic defect of eartha mutants in melanocyte darkening, a late process in melanocyte differentiation. We generated chimeric larvae mosaic for gfpt1 mutation by transplanting wild-type tg(β-actin:GFP) donor cells into eartha\textsuperscript{23e1} embryos at blastula stage (Figure 5I and 5J) [41]. We identified four eartha host larvae with a total of six GFP\textsuperscript{+} (donor-derived) melanocytes. In each case, the GFP\textsuperscript{+} melanocytes were also darkly pigmented, in contrast to the neighboring lightly pigmented melanocytes (GFP\textsuperscript{−}) that are derived from host. Because the donor melanocytes show the wild-type phenotype (dark pigmentation) corresponding to their genotypes in the otherwise eartha\textsuperscript{23e1} mutation hosts, we conclude that gfpt1 acts cell autonomously to promote melanocyte darkening.

We conclude that loss of gfpt1 function results in a defect in melanocyte regeneration and that gfpt1 acts at a late stage (after dct\textsuperscript{+} stage) during melanocyte regeneration. Furthermore, chimera analysis shows that gfpt1 acts cell autonomously to promote ontogenetic melanocyte darkening. These results lead to our inference that gfpt1 acts cell autonomously in melanocytes to promote melanocyte differentiation from dct\textsuperscript{+}; melanin\textsuperscript{−} to melanin\textsuperscript{−} stage during melanocyte regeneration as well.

julie Acts prior to dct Stage during Larval Melanocyte Regeneration

julie\textsuperscript{24e1} is the second mutation identified from our melanocyte ablation screen for defects specific to melanocyte regeneration. The development of julie\textsuperscript{24e1} embryos is indistinguishable from wild-type embryos until 3.5 dpf, at which time the size of their eyes and brain appears smaller than that of the wild-type larvae, although they have normal body length. We did not observed opaque or grainy cells that are typically reliable indicators of significant levels of cell death in zebrafish embryos, in the brains of julie\textsuperscript{24e1} larvae, suggesting that this small brain and eyes phenotype may result from growth retardation (Figures 1E and S1A and S1B). The notion that julie\textsuperscript{24e1} mutants have defects in growth rather than differentiation is further supported by our histological examination of eye structure in julie\textsuperscript{24e1} mutants that reveals normal retinal cell differentiation and lamination at 4 dpf despite the smaller size of their eyes (see Figure S1C and S1D). Most julie\textsuperscript{24e1} mutants die at approximately 7–8 dpf. Upon melanocyte ablation by MoTP, julie\textsuperscript{24e1} larvae regenerate 10.0% of the normal number of melanocytes compared to wild-type larvae at 5 dpf (Figure 1F and 1H). We also note that the few regenerated melanocytes in the dorsum of the julie\textsuperscript{24e1} mutants are distributed haphazardly throughout the length of the dorsum.

To understand how the julie\textsuperscript{24e1} mutation affects larval melanocyte regeneration, we examined melanocyte differentiation with the late-stage melanoblast marker dct in julie\textsuperscript{24e1} larvae after MoTP washout. We detected almost no dct\textsuperscript{+} melanoblasts in julie\textsuperscript{24e1} larvae (Figure 6A and 6B), suggesting that julie\textsuperscript{24e1} mutation affects melanocyte regeneration prior to dct\textsuperscript{+} stage.

To test whether julie has a specific role in melanocyte regeneration or whether it is also required for other types of

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**Figure 6.** julie\textsuperscript{24e1} Mutation Disrupts Melanocyte Regeneration prior to the dct\textsuperscript{+} Stage and Blocks Tail Regeneration Following Amputation

(A and B) Larval melanocytes in julie\textsuperscript{24e1} and wild-type larvae were ablated by MoTP treatment from 14 to 72 hpf, and melanocyte regeneration was analyzed at 5 dpf by ISH with dct riboprobes for late-stage melanoblasts. At this stage, many dct\textsuperscript{−} melanoblasts (melanin\textsuperscript{−}, blue arrow in [A]) and melanocytes (melanin\textsuperscript{−}, black arrow in [A]) appear in wild-type larvae (A). While few melanocytes (melanin\textsuperscript{−}, black arrow in [B]) appear in the julie\textsuperscript{24e1} larvae, no dct\textsuperscript{−} melanoblast was detected by ISH (B).

(C–F) julie\textsuperscript{24e1} mutants fail to regenerate larval tails following amputation. Larval tails were amputated at 60 hpf (C and D) and tail regeneration in wild-type and julie\textsuperscript{24e1} larvae were examined at 5.5 dpf (E and F). At this stage, wild-type larval tails (E) were mostly reconstituted, including fin-fold tissues (outlined with white-dashed line) and melanocytes (arrow in [E]). In the julie\textsuperscript{24e1} larvae, the amputation wound healed but the julie\textsuperscript{24e1} mutant tail failed to regenerate (F). Arrowheads indicate the amputation planes in (C–F). The white-dashed lines outline the fin folds. Timeline (grey) above (A) indicates the period of MoTP treatment (red) and analysis time (vertical line below the timeline).

Scale bars: 250 μm.

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Figure 7. The julie\textsuperscript{j24e1} Mutation Is a Splice-Site Mutation in skiv2l2

(A) The julie\textsuperscript{j24e1} mutation was mapped close to a marker z3825 on Chromosome 10.

(B) The approximately 226.8 kb physical map of the julie locus was built from the zebrafish genome assembly version 5 (Zv5), flanked by three recombinants at the distal end and two recombinants at the proximal end from analyzing 438 meioses. A minimal critical region for the julie\textsuperscript{j24e1} mutation was determined by a single recombinant with a distal marker in dhx29 and one proximal recombinant with a marker in skiv2l2.

(C) The genomic sequencing for skiv2l2 locus in the julie\textsuperscript{j24e1} mutants revealed a splicing site mutation (GT to AT change) at the 11th exon-intron boundary (red arrowheads).

(D) Sequencing of julie\textsuperscript{j24e1} mutant cDNA for skiv2l2 revealed a 57 bp in-frame deletion at the 3' end of exon 11 resulting in a partial deletion in the highly conserved DEAD-box domain in Skiv2l2 protein.

(E) The phylogenetic tree of skiv2l genes was constructed using the neighbor-joining method with S. cerevisiae MTR4 as the out-group. doi:10.1371/journal.pgen.0030088.g007

postembryonic regeneration, such as larval tail regeneration (described above), we performed larval tail amputation on julie\textsuperscript{j24e1} larvae. We found that the amputation wound heals in the ensuing few days after tail amputation, but that julie\textsuperscript{j24e1} tails fail to regenerate (Figure 6C–6F). This defect together with the growth-deficient defect of the brain and eye suggests that julie may have a general role in postembryonic cell division or tissue growth.

The julie\textsuperscript{j24e1} Mutation Is a Splice-Site Mutation in skiv2l2

To further understand how julie regulates melanocyte regeneration, we sought to molecularly identify the julie\textsuperscript{j24e1} mutation by a positional cloning method similar to the approach described above for the cloning of eartha\textsuperscript{j23e1} mutation. We first mapped the julie\textsuperscript{j24e1} mutation close to a marker z3825 on Chromosome 10 (LG 10) by half-tetrad centromere linkage analysis (Figure 7A). Again, to build a physical map of the julie locus, we compared the sequence of the marker z3825 and several EST sequences that had previously been mapped to the region to identify various contigs from the zebrafish genome assembly version 5 (Zv5). These assembly contigs were then bridged by BAC sequences to generate the physical map shown in Figure 7B. The accuracy of this map was verified by mapping new SSR or single nucleotide polymorphism markers that were developed from the assembly sequences to a panel of selected recombinants derived from 438 meioses. The region containing the julie\textsuperscript{j24e1} mutation, flanked by three recombinants at the distal end and two recombinants at the proximal end, comprises four genes, dhx29, skiv2l2, ppap2a, and chgn, spanning over approximately 226.8 kb (Figure 7B). A minimal critical region for the julie\textsuperscript{j24e1} mutation was further determined by identifying a single distal recombinant with a marker within the dhx29 gene and one proximal recombinant with a marker within the skiv2l2 gene. Sequencing of dhx29 revealed identical nucleotide sequences between cDNAs derived from julie\textsuperscript{j24e1} mutant and that of the originating background (sjD strain). In contrast, by reverse transcription-PCR and sequencing analysis, we found a 57 bp in-frame deletion at the 3' end of exon 11 resulting in a partial deletion in the highly conserved DEAD-box domain in Skiv2l2 protein.

skiv2l2 was thought to be closely related to Saccharomyces cerevisiae SK12 [24], a DEAD-box RNA helicase in a multiprotein nuclease complex, the exosome, which mediates 3' to 5' mRNA degradation in the cytoplasm [36,42]. By our phylogenetic analysis, we found that vertebrate skiv2l2 genes are more closely related to yeast mRNA transport defective 4 (MTR4) gene (Figure 7E), an ATP-dependent DEAD-box RNA
helicase that is thought to be involved in tRNA or rRNA degradation mediated by exosome in the nucleus [43]. Besides skiv2l2, we identified another two superkiller viralicidic activity 2-like (skiv2l)-related genes in the zebrafish Zv5 genome assembly. We defined a zebrafish skiv2l orthologue and a skiv2l related gene, skiv2l3. Our phylogenetic tree analysis shows that zebrafish skiv2l3 is more closely related to vertebrate Skiv2l genes than to the Skiv2l2. This tree topology, together with our failure to find additional skiv homologues by BLAST analysis of the human or mouse genomes, suggests that the mammalian Skiv2l3 gene was lost in the human lineage, but retained in that of zebrafish (Figure 7E).

Expression of skiv2l2 during Embryogenesis and Larval Development

To understand how skiv2l2 expression may regulate melanocyte regeneration, we examined the expression of skiv2l2 during development by ISH. skiv2l2 transcripts were detected as early as the four-cell stage (Figure 8A) and remained ubiquitously distributed in embryos throughout early embryogenesis (Figure 8B). The expression of skiv2l2 becomes stronger in the central nervous system (arrowhead in [C]) (22 hpf) and is then restricted to pectoral fins, cranial neural crest (white arrow in [D]), the medial and posterior parts of the tectum, and cerebellum (black arrow in [D]), and ciliary marginal zone of the retina (arrowhead in [D]) at larval stages (D) (74 hpf). This larval-stage expression pattern of skiv2l2 is nearly identical to the expression of pcna (68 hpf).

(F) pcna expression is drastically reduced in julie24e1 mutants (68 hpf).

Scale bars: in (A), 100 μm for (A–C); in (D), 100 μm for (D–F).

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Figure 8. skiv2l2 Expression Is Ubiquitous during Embryogenesis and Then Restricted to Proliferation Zones at Larval Stages

(A and B) skiv2l2 transcripts were detected as early as the four-cell stage by RNA ISH (A) and remained ubiquitously distributed in embryos throughout early embryogenesis (B) (12 hpf).

(C and D) The expression of skiv2l2 becomes stronger in the central nervous system (arrowhead in [C]) (22 hpf) and is then restricted to pectoral fins, cranial neural crest (white arrow in [D]), the medial and posterior parts of the tectum, and cerebellum (black arrow in [D]), and ciliary marginal zone of the retina (arrowhead in [D]) at larval stages (D) (74 hpf).

(E) This larval-stage expression pattern of skiv2l2 is nearly identical to the expression of pcna (68 hpf).

(F) pcna expression is drastically reduced in julie24e1 mutants (68 hpf).

Scale bars: in (A), 100 μm for (A–C); in (D), 100 μm for (D–F).

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Expression of skiv2l2 during Embryogenesis and Larval Development

To understand how skiv2l2 expression may regulate melanocyte regeneration, we examined the expression of skiv2l2 during development by ISH. skiv2l2 transcripts were detected as early as the four-cell stage (Figure 8A) and remained ubiquitously distributed in embryos throughout early embryogenesis (Figure 8B). The expression of skiv2l2 becomes stronger in the central nervous system than the rest of the embryo after 16 hpf (Figure 8C) and is then restricted to pectoral fins, cranial neural crest, the medial and posterior parts of the tectum, and cerebellum, and ciliary marginal zone of the retina at larval stages (Figure 8D). The expression of skiv2l2 in these regions persists through 5 dpf (unpublished data). The medial and posterior parts of the tectum and cerebellum are proliferative zones during the brain growth at the larval stage, whereas the ciliary marginal zone is suggested to be populated with stem cells that contribute to retina growth throughout life in poikilotherms, such as amphibians and fish [44]. In addition, the expression pattern of skiv2l2 is nearly identical to the expression of proliferating cell nuclear antigen (pcna), which marks proliferating cells and tissues at this larval stage (Figure 8E) [45]. To explore the possibility that skiv2l2 promotes cell proliferation, we examined pcna expression in julie24e1 mutants and found that the expression of pcna is drastically reduced in these animals (Figure 8F), suggesting that skiv2l2 is required for cell proliferation in these regions. This result is consistent with the phenotype of small eyes and brain observed in the julie24e1 animals, as well as the failure to regenerate melanocytes following melanocyte ablation by MoTP.

We sought to examine the expression of skiv2l2 in regenerating melanocytes following MoTP washout. We were unable to detect any skiv2l2 expression in possible melanoblasts or regenerating melanocytes at two to three days postMoTP washout (5–6 dpf) despite the strong detection of skiv2l2 expression in various tissues described above. This failure could suggest the low abundance of skiv2l2 transcripts, below our ability to detect by ISH, in regenerating melanocyte lineages.

Taken together, we suggest that loss of skiv2l2 function causes the julie phenotype, and skiv2l2 regulates cell division prior to the dct+ stage during larval melanocyte regeneration.

Discussion

We report a forward genetic screen aimed at identifying regulatory mechanisms specific to regeneration. We sought to isolate mutations specific to melanocyte regeneration by conducting a F2 parthenogenesis screen in zebrafish larvae employing a small molecule, MoTP, to ablate larval melanocyte...
cytes. We specifically identified two inheritable mutations, *eartha*<sup>j23e1</sup> and *julie*<sup>j24e1</sup>, which cause defects in larval melanocyte regeneration from our screen. Because this screen was far from saturation, these results indicate that many more mutant loci might be identified by further exploitation of this melanocyte ablation screen. Notably, these two mutants are also larval lethal with other defects in jaw development (*eartha*) or general larval growth (*julie*). Thus, our findings suggest that many of the genes involved in regeneration processes are also parts of other developmental programs and that the number of genes required solely for regeneration may be small.

Because the mutations described here are late larval lethal, one possibility was that the melanocyte regeneration defects observed were a consequence of the impending death of the larvae. The finding that melanocyte regeneration occurs normally in *rapunzel<sup>14</sup>* homozygous mutants, which die between 5 and 6 dpf, indicates that larval lethality is not a sufficient cause to the defects in melanocyte regeneration and instead indicates that the genes identified here contribute specific roles in regeneration. We also note that one of the mutations, *eartha*, blocks regeneration at a late stage of differentiation, suggesting that the regeneration process may differ from ontogenetic process not only in the as yet hypothetical mechanisms that keep stem cells in check or act to recruit them back into developmental pathways, but also in those later stages in differentiation that superficially seem to be held in common between ontogenetic and regeneration processes.

**gfpt1** Promotes Melanocyte Differentiation from dct<sup>+</sup>; melanin<sup>-</sup> to melanin<sup>+</sup> Stage during Larval Melanocyte Regeneration

*eartha*<sup>j25e1</sup> mutation allows normal development of ontogenetic melanocytes (melanin<sup>-</sup>) but blocks larval melanocyte regeneration following MoTP treatment. We demonstrated that the phenotype of *eartha*<sup>j25e1</sup> mutants is caused by loss of *gfpt1* function. This conclusion is supported by several lines of evidence, including the high-resolution mapping of the *eartha*<sup>j25e1</sup> mutation, the identification of a nonsense mutation in the *gfpt1* locus, very few or no *gfpt1* transcripts detected in *eartha*<sup>j25e1</sup> mutants, and the partial rescue of the *eartha*<sup>j25e1</sup> phenotype by the injection of *gfpt1* mRNA. Our analysis of melanocyte differentiation during regeneration with the late-stage melanoblast marker *dct* reveals that a mutation in *gfpt1* blocks melanocyte differentiation after the *dct*<sup>+</sup> stage, but before they reach the melanin-positive stage. In addition, the finding that following larval tail amputation, *eartha*<sup>j25e1</sup> mutant larvae regenerate tails normally but fail to regenerate melanocytes in the tail indicates that *gfpt1* is not generally required for regeneration but instead identifies a mechanism specific to melanocyte regeneration.

*gfpt1* is the first and rate-limiting enzyme in the hexosamine biosynthesis pathway (glucosamine synthesis). The major end product of the hexosamine biosynthesis mediated by *gfpt1* is a sugar nucleotide UDP-GlcNAc, which is one of the building blocks for glycosyl side chains for glycoproteins, glycolipids, and proteoglycans [46]. Among these molecules, N-acetylgalcosamine (GlcNAc) is found most frequently in the polysaccharide side chains of glycosaminoglycans (GAGs), a type of linear polysaccharide composed of repeated disaccharides [47]. For instance, hyaluronan, the simplest GAG, is made up of a repeated disaccharide unit, GlcNAc and glucuronic acid, and is part of the ECM in cartilages [48,49]. In addition to GAGs, GlcNAc is also found enriched in the N-linked or O-linked oligosaccharides (N-glycans or O-glycans), which attach to various intracellular glycoproteins, and serves as crucial modification in various developmental processes [50,51]. We note that in addition to contributing to polysaccharides, UDP-GlcNAc serves as an obligatory substrate for monosaccharide modification on serine or threonine residues of various proteins [52,53], and this monosaccharide modification, also referred to as O-linked GlcNAcylation, modifies proteins in a manner analogous to phosphorylation, and consequently regulates the cellular and developmental functions of these proteins [54]. Although in vitro biochemical studies showed that *gfpt1* regulates UDP-GlcNAc synthesis, whether *gfpt1* is the only enzyme for UDP-GlcNAc synthesis in vivo and whether other enzymes, such as *gfpt2*, also play roles in this process are unknown. Furthermore, the developmental role of *gfpt1* in vertebrates is unexplored.

The isolation and analyses of *eartha*<sup>j25e1</sup> mutants may provide insight into the roles of *gfpt1* in vertebrate development. Alcian Blue is a cationic dye that binds negatively charged sites on the GAGs, such as hyaluronan [55,56]. Since cartilage is largely composed of GAGs, Alcian Blue staining reveals the presence of these long chain sugar molecules. Our findings that chondrocytes in *eartha*<sup>j25e1</sup> mutants commit to their cell fates and develop to *col2a1<sup>+</sup>* stage, but display weaker Alcian Blue staining, suggest defects in formation of GAGs in the ECM of the cartilages. These results indicate possible defects in the ECM of the connective tissues and raise the possibility that the lightly pigmented phenotype of the ontogenetic melanocytes and the melanocyte regeneration defect we observed in the *eartha*<sup>j25e1</sup> mutants was also the consequence of defects in the ECM. Our finding by chimera analysis that wild-type or donor-derived melanocytes have normal pigmentation in the *eartha*<sup>j25e1</sup> mutant hosts indicates that this particular function is not mediated through ECM, but instead reflects a cell autonomous, presumably intracellular, function for *gfpt1* in melanocyte development. Although we have not yet explored the autonomy for *eartha* in melanocyte regeneration, it now seems likely that the role for *gfpt1* in melanocyte regeneration is autonomous as well.

Although the in vitro biochemical studies suggest a general role for *gfpt1* in the UDP-GlcNAc production, our analyses suggest that this pathway has only a handful of easily discerned and diverse consequences when disrupted in zebrafish embryos. Thus, the defect in jaw morphogenesis may indicate a role in regulating the proper formation of the ECM in chondrocyte development, while different roles in modulating intracellular targets may be revealed by the defects in melanocyte darkening during ontogeny or defects in progression from the *dct*<sup>+</sup>; melanin<sup>-</sup> to melanin<sup>+</sup> stage during melanocyte regeneration.

One question remaining is how a mutation in *gfpt1* specifically affects the progression from *dct*<sup>+</sup>; melanin<sup>-</sup> stage to melanin<sup>+</sup> stage in the regenerating melanocytes but has no similar effects on this stage of ontogenetic melanocyte development. One possibility is that the *gfpt1* mutation reveals a function specific to melanocyte regeneration that is not shared by embryonic melanocyte development.
However, our observation that ontogenetic melanocytes in *eartha* j25e1 mutants at 4–5 dpf have lighter pigmentation than those in wild-type larvae suggests that *gfpt1* also plays a role in embryonic melanocyte darkening. These findings may indicate that the two different phenotypes are due to the contribution of *gfpt1* from two distinct mechanisms, for instance that two distinct GlcNAc modified factors are responsible, respectively, for the two different stages of melanocyte development. The alternative possibility is that defects in a shared mechanism cause distinct phenotypes in ontogenetic and regeneration melanocytes. Differences between the roles in ontogenetic melanocyte development and larval melanocyte regeneration have been described for the kit receptor tyrosine kinase. Ontogenetic melanocytes differentiate in the absence of kit receptor tyrosine kinase function, but melanocytes in kit receptor tyrosine kinase mutants largely fail to differentiate when challenged to regenerate [9,17,57]. Therefore, the difference between ontogenetic and regeneration stages of melanocyte development in the *eartha* j25e1 mutants may either reflect the effect of *gfpt1* on modified oligonucleotides from zygotic expression (Figure 5B), tending to argue against maternal perdurance. Moreover, morpholino oligonucleotides detected some maternal *gfpt1* transcripts are derived from zygotic expression (Figure 5B), tending to argue against the effect of maternal messages in promoting melanocyte development, but melanocytes in kit receptor tyrosine kinase mutants largely fail to differentiate when challenged to regenerate [9,17,57]. Therefore, the difference between ontogenetic and regeneration stages of melanocyte development in the *eartha* j25e1 mutants may either reflect the effect of *gfpt1* on modified oligonucleotides from zygotic expression (Figure 5B), tending to argue against maternal perdurance.

A less likely explanation that we cannot yet formally exclude is that the ability of ontogenetic melanocytes to reach a relatively early stage (prior to 22–24 hpf when ontogenetic melanocytes begin to melanize) is the perdurance of *gfpt1* maternal message or protein. Such a model would suggest a maternal role in melanocyte differentiation at approximately 22–24 hpf when ontogenetic melanocytes begin to melanize. This stage of melanocyte development is far beyond the midblastula transition (4 hpf) [58,59], when zygotic transcription begins, and many maternal transcripts are degraded [60]. Furthermore, although we detected some maternal *gfpt1* transcripts in four-cell-stage embryos, by 13 hpf, most or all *gfpt1* transcripts are derived from zygotic expression (Figure 5B), tending to argue against maternal perdurance. Moreover, morpholino oligonucleotides directed against the ribosome binding site of the *gfpt1* transcript (ATG blocking morpholinos), which might be expected to block the translation of maternal transcripts, cause embryonic melanocyte darkening defects similar to those observed in the *eartha* mutants, but no defect on the initial development and pigmentation of embryonic melanocytes (unpublished data). Although such results tend to argue against a role for maternal *gfpt1* transcripts in promoting embryonic melanocyte development, these experiments do not rule out the possibility of maternally supplied protein or other post-translational products being responsible for the embryonic melanocytes that are observed in the *eartha* mutants.

**skiv2l2** Regulates Cell Division during Larval Melanocyte Regeneration

We identified a second mutant, *julie* j24e1, which develops normal ontogenetic melanocytes but fails to regenerate melanocytes upon ablation by MoTP. The high-resolution mapping of the *julie* j24e1 mutation reveals that *skiv2l2* is the best candidate in the *julie* j24e1 critical region and excludes the possibility that the *julie* j24e1 mutation is in the coding sequence of immediately flanking genes *dbx29* and *ppap2a* (Figure 6B). Furthermore, the identification of a splice-donor site mutation in the *skiv2l2* locus further supports the assertion that *skiv2l2* is the mutated gene in *julie* j24e1 mutants. This splice-site mutation causes a 57 bp in-frame deletion in the helicase domain, which presumably leads to the disruption of the *skiv2l2* function [61].

Mammalian *skiv2l2* is originally thought to be closely related to yeast RNA helicase SKI2, which is known to be part of the cytoplasmic exosome complex mediating 3’ to 5’ mRNA degradation of selective mRNAs [62]. Our phylogenetic analysis (Figure 7E), however, reveals that vertebrate *skiv2l2* are the orthologues for a different yeast RNA helicase MTR4. Yeast MTR4 was originally suggested to function as mRNA chaperones to unwind RNA duplexes and pack RNAs into stable structures for mRNA transport out of the nucleus [63,64]. More recently, MTR4 was found to be part of a nuclear complex TRAMP that polyadenylates noncoding RNAs to trigger their 3’ to 5’ degradation by the nuclear exosome complexes [65,66]. This exosome-mediated nuclear RNA degradation was proposed as a surveillance mechanism to control the quality and quantity of various nuclear RNAs, including rRNA, sn/snoRNA, and tRNA, and Mtr4p is a crucial factor for this degradation [43,65,67,68]. This mechanism is essential for 60S ribosomal unit biogenesis and also for yeast to grow [69]. However, the cellular function of *skiv2l2*, a vertebrate orthologue of MTR4, is unexplored.

The isolation of the *julie* j24e1 mutation from our melanocyte ablation screen and our following characterization reported here may now shed light on the functional roles of *skiv2l2* in vertebrate development. Our finding of reduced *pena* expressions in the proliferation zones of the eye and brain in the *julie* j24e1 larvae suggests that *skiv2l2* plays a role in cell division. Furthermore, the finding of virtually no *det* melanoblasts in the *julie* j24e1 mutants after MoTP washout suggests that *skiv2l2* may regulate melanocyte cell division at a relatively early stage (prior to *det* stage) during melanocyte regeneration. If the zebrafish *skiv2l2* has an equivalent biochemical function as that proposed for yeast MTR4 in transporting mRNA from the nucleus, it is possible that certain as yet unidentified mRNAs regulating cell proliferation may fail to be transported out of the nucleus in the *julie* j24e1 mutants. This, consequently, would deplete their activities, thereby reducing cell proliferation in *julie* j24e1 mutants. Alternatively, *skiv2l2* could function in the surveillance mechanism postulated in yeast to control the quality and quantity of various nuclear RNA species. This surveillance mechanism could be essential for proper ribosome assembly for protein translation, which is usually active in dividing cells. The defect in this surveillance mechanism could consequently lead to deficits in cell division in *julie* j24e1 mutants. Finally, several DEAD-box RNA helicases in the exosome complex have been implicated in microRNA processing of transcripts for genes that subsequently regulate cell division, differentiation, and apoptosis during various aspects of organismal development including brain morphogenesis in zebrafish [70–73]. Whether *skiv2l2* plays a role in any of these aspects of RNA metabolism is an intriguing question for further investigation.

A remaining question is how a mutation in *skiv2l2* specifically affects melanocyte regeneration but not ontogenetic melanocyte development. One possible model is that
**Materials and Methods**

**Fish stocks and rearing.** Wild-type fish stocks used for this manuscript were the sjC (C32) or AB inbred strain. The sjD inbred strain for mutagenesis was previously described by Rawls et al. [77]. Wild-type fish stocks used for this study were the sjC (C32) or AB inbred strain. The sjD inbred strain. The requirement for first generation (F1) was cloned into the *mMESSAGE mMACHINE*, Ambion, http://www.ambion.com). We injected 40–100 pg of mRNAs with 1% phenol red into one to four cell stage embryos [84]. At 5–7 dpf, mRNA-injected larvae were assayed for rescue phenotype by measuring tail length. We injected a 5% solution of sodium tetraborate for 1 h. The pigmentation of the larvae in prewarmed 0.05% trypsin dissolved in a saturated solution of sodium tetaborate for 1 h. 8. The pigmentation of the larvae was bleached in a solution of 3% H2O2/1% KOH for one-hour incubation. The skeletal preparations were finally mounted in glycerol [28].

**Mapping.** The *eartha* and *j24e1* mutations were localized on LG 8 and LG 10, respectively, by half-tetrad centromere-linkage analysis [35,36] using SSR markers by early pressure treatment. In the *eartha* and *j24e1* larvae, we stained the larvae with Alcian Blue (Sigma, http://www.sigmaaldrich.com), a dye that binds to the ECM associated with chondrocytes. Larvae at various stages were fixed at 4% PFA at room temperature for 24–48 h and then transferred into a solution of 0.1% Alcian Blue dissolved in 7:3 ethanol/glacial acetic acid. After 24 h staining, the larvae were rinsed with 7:3 ethanol/glacial acetic acid and subjected to a series of dehydration to distilled water. Tissues were cleared by incubating the larvae in prewarmed 0.05% trypsin dissolved in a saturated solution of sodium tetraborate for 1 h. The pigmentation of the larvae was bleached in a solution of 3% H2O2/1% KOH for one-hour incubation. The skeletal preparations were finally mounted in glycerol [28].

**Parthenogenesis screen.** Adult sjD inbred strain males were mutagenized by incubating them in 3 mM N-ethyl-N-nitrosourea (ENU), a chemical mutagen, for four one-hour periods over the course of six weeks [25,26]. The mutagenized sjD males were bred with sjC females to create a pool of F1 carriers with random mutations. Clutches of eggs from individual F1 females were collected and fertilized with UV-inactivated sperm. To exclude mutations that also cause defects in ontogenetic melanocyte development from our analysis, approximately ten to 20 of these fertilized eggs were set aside and allowed to develop into haploid embryos for the examination of their ontogenetic melanocyte development at 3 dpf [77]. The remaining fertilized eggs were subjected to an early pressure protocol to generate half-tetrad F2 diploid embryos [80] for the melanocyte regeneration assay.

At 14 hpf stage, F2 diploid embryos were placed in 14 µg/ml MoTP for approximately a 58-hour incubation to ablate developing melanocytes. Washed into egg water, then scored for melanocyte regeneration at 5–8 dpf [9].

**Whole-mount ISH.** ISH with antisense DIG-labeled riboprobes was performed as described [81], using 68 °C hybridization stringency washes, alkaline phosphatase-conjugated secondary antibodies, and NBT/BCIP (nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) for color development (Roche, http://www.roche.com). Fish embryos were fixed in 4% paraformaldehyde (PFA). Probes for *det* [82], *dlx2* [32], *sox9a* [33], and *col2a1* [34] were previously described. The full-length cDNAs of *gfp1*, *skiv2l2*, and *pcna* were amplified from a cDNA pool of 3 dpf larvae and cloned into a pBluescript II vector (Strategene, http://www.stratagene.com). All the riboprobes were synthesized by 17 RNA polymerase (Promega, http://www.promega.com). To better visualize NBT/BCIP precipitates in otherwise dark mature melanocytes, some embryos and larvae were treated with 10 mM PTU to partially inhibit melanin synthesis. For ISH on larval stage 3 dpf, 1% DMSO was added to the 4% PFA to increase the tissue permeability, and riboprobes were fragmented to approximately 300 bp by alkaline hydrolysis [75]. For cryostat sections, embryos with larval staining were fixed, embedded in 1.5% agarose, frozen in liquid nitrogen, and cut into 7–10 µm sections as previously described [78].

**Larval tail regeneration.** Tail amputations were performed on 3 dpf larvae with razor blades under a dissecting microscope. The tails were cut immediately posterior to the turn of caudal vessel, and amputated tissues included neural tube, somites, and larval tail. For cryostat sections, embryos with larval staining were fixed, embedded in 1.5% agarose, frozen in liquid nitrogen, and cut into 7–10 µm sections as previously described [78].
death in zebrafish embryos, are observed in the brains of \textit{ju}l\textsuperscript{46\textalpha}\textbullet\textsuperscript{1} larvae (arrowheads in [A and B]). (C and D) Paraffin sections stained with hematoxylin (H) and eosin (E) show the normal retinal cell differentiation and lamination in the \textit{ju}l\textsuperscript{46\textalpha}\textbullet\textsuperscript{1} larvae (D) despite the smaller size of the eyes than that of wild-type (C). Scale bars: in (A), 250 μm for (A and B); in (C), 20 μm for (C and D).

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**Accession Numbers**

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the genes described in this paper are gftp1 (DQ173929), skiv21 (BC061156, pena (BC064299), dbx29 (XM_692941), ppaq2a (XM_692415), and chgn (XM_001339600); gftp-related gene (DQ173929, CR735104); skiv21 orthologue (XM_694641); and skiv21-related gene, skiv23 (XM_685014).

The GenBank accession numbers for protein sequences used in the phylogenetic analyses are as follows: E. coli glmS (NP-418185); S. cerevisiae GFA1 (NP_012818); D. rerio Gfpt1 (NP_001030153) and Gfpt2 (NP_001029903); and Homo sapiens GFTPT1 (NP_002047) and GFTPT2 (NP_005101); S. cerevisiae Sk12 (NP_015050) and MTR4 (NP_012465); D. rerio Skiv2 (BC061156), Skiv21 (XM_694641), and Skiv23 (XM_001339603); and H. sapiens Skiv21 (NP_056175) and Skiv22 (NP_008860).

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**Author contributions.** CTY and SLJ conceived and designed the experiments. CTY, AEH, and KAH performed the experiments. CTY and SLJ analyzed the data and wrote the paper.

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**References**


