Ras controls melanocyte expansion during zebrafish fin stripe regeneration

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SUMMARY
Regenerative medicine for complex tissues like limbs will require the provision or activation of precursors for different cell types, in the correct number, and with the appropriate instructions. These strategies can be guided by what is learned from spectacular events of natural limb or fin regeneration in urodele amphibians and teleost fish. Following zebrafish fin amputation, melanocyte stripes faithfully regenerate in tandem with complex fin structures. Distinct populations of melanocyte precursors emerge and differentiate to pigment regenerating fins, yet the regulation of their proliferation and patterning is incompletely understood. Here, we found that transgenic increases in active Ras dose-dependently hyperpigmented regenerating zebrafish fins. Lineage tracing and marker analysis indicated that increases in active Ras stimulated the in situ amplification of undifferentiated melanocyte precursors expressing mitfa and kita. Active Ras also hyperpigmented early fin regenerates of kita mutants, which are normally devoid of primary regeneration melanocytes, suppressing defects in precursor function and survival. By contrast, this protocol had no noticeable impact on pigmentation by secondary regulatory melanocyte precursors in late-stage kita regenerates. Our results provide evidence that Ras activity levels control the repopulation and expansion of adult melanocyte precursors after tissue loss, enabling the recovery of patterned melanocyte stripes during zebrafish appendage regeneration.

INTRODUCTION
Regenerative medicine is a conventional therapeutic strategy for highly renewable tissues such as blood and skin, but remains a challenging and distant goal for many human organs with poor regenerative capacity. Current attention is focused on methodology to isolate stem and progenitor cells from embryonic or adult organs, or to reprogram somatic cell types as a means of creating stem cells (Takahashi and Yamanaka, 2006). Although the promise of stem cell therapy is enormous, we know little about how stem and progenitor cell populations can be manipulated successfully to restore the correct size, pattern and function of complex tissues after organ damage. Crucial context for this degree of stem cell control can be gained by direct study of natural examples of complex tissue regeneration, during which healthy, patterned adult structures are renewed after severe injury. Among vertebrate model systems, urodele amphibians and teleost fish have tremendous capacity for organ regeneration. Together, these species are able to renew pieces of brain tissue, intestinal segments, a fully transected spinal cord, injured eye parts such as a damaged retina or dissected lens, resected heart muscle, and amputated jaws and major appendages (Brookes and Kumar, 2005; Stoick-Cooper et al., 2007). Because of the large number of community resources that are available, the zebrafish serves as an increasingly popular model for investigating regenerative events (Grunwald and Eisen, 2002). The best-studied example of regeneration in zebrafish is their rapid and virtually indelible renewal of amputated fins. Adult zebrafish fins contain several segmented fin rays of intramembranous bone. Each ray is comprised of concave, facing hemirays that are coated with bone-depositing scleroblasts and that surround mesenchymal tissue, nerves and blood vessels. The hallmark of fin regeneration is formation of the blastema, a proliferative mass of mesenchymal cells that is maintained as a zone of progenitor tissue for new structures. Regenerative growth proceeds from the blastema by progressive addition of new bone segments to the distal end of each ray, until the original length of the fin is achieved roughly two weeks after the amputation injury (Akimenko et al., 2003; Poss et al., 2003).

One of the most striking aspects of zebrafish fin regeneration is the accurate regeneration of pigmentation pattern after amputation. In the first several days of regeneration, new melanocytes scatter throughout the rapidly regenerating tissue. Resolution into distinct melanocyte stripes then occurs in an approximate proximal-to-distal sequence and is completed by several weeks after amputation. Although it is unclear whether the fin blastema is comprised of, or derives from, a stem cell population, the melanocytes of the regenerating fin are known to originate from unpigmented precursors (Rawls and Johnson, 2000; Rawls and Johnson, 2001; White and Zon, 2008; O’Reilly-Pol and Johnson, 2009). Recent studies revealed that two populations of melanocytes, both supplied by undifferentiated precursors, pigment adult fin regenerates. Primary regeneration melanocytes surface in the first week of regeneration, are dependent on the receptor tyrosine kinase Kita, and form the initial stripe patterns. A secondary population of regulatory melanocytes is suspected to play only a minor role during stripe regeneration. However, this population is prominent in the absence of functional Kita, where it emerges in late-stage regenerates and has the capacity to renew entire stripes (Rawls and Johnson, 2000). Adult zebrafish restore their stripe pattern after at least ten consecutive rounds of amputation and regeneration; this remarkable self-renewal capacity is indicative of an underlying stem cell population. Thus, zebrafish employ mechanisms that are largely uncharacterized at the molecular level and that control the self-renewal and patterning of adult stem cells as they restore stripes in a rapidly regenerating appendage.
Here, in attempts to identify new regulators of stem cell activity during zebrafish fin stripe regeneration, we used transgenic approaches to discover a central role for the small GTPase Ras. Most remarkably, transgenic increases in active Ras markedly amplified the number of melanocytes present during fin regeneration, hyperpigmenting new fin structures and disrupting the stripe pattern. This striking phenotype was observed even in the absence of Kita function. Our findings indicate that Ras signaling levels determine the supply of melanocyte precursors, facilitating stripe pattern recovery during zebrafish fin regeneration.

RESULTS

**Ras activity controls zebrafish fin stripe regeneration**

Under our husbandry conditions, zebrafish caudal fins recover full proximodistal extension of their stripes within ~30 days after removal of half of the appendage (Fig. 1A). Fins with more proximal amputations require additional time to complete pigmentation, indicating that, unlike the rate of bone regeneration (Lee et al., 2005), the rate of pigment regeneration is not influenced strongly by the amputation level (supplementary material Fig. S1). While testing transgenic manipulations that might influence this rate, we identified significant effects following increases in active Ras, using a transgenic zebrafish line in which a constitutively active Ras transgene is driven by a heat shock (HS)-inducible hsp70 promoter (hsp70:v-ras). v-ras mRNA was induced in a dose-dependent manner by HS, with greater mRNA levels detectable at higher HS temperatures (supplementary material Fig. S2G) (Whitman and Melton, 1992; Lee et al., 2009). Within 2 hours of the highest HS temperature, 38°C, v-ras messages were weakly detectable in many, but not all, epidermal and mesenchymal cells by in situ hybridization (supplementary material Fig. S2A-D). This protocol also increased the whole-regenerate levels of phosphorylated Erk (pErk), a primary downstream readout of Ras activation in many biological contexts (Takai et al., 2001), within 5 hours of HS (supplementary material Fig. S2E), and was shown recently to inhibit blastemal proliferation (Lee et al., 2009). By contrast, a lower-temperature (36°C) HS had no detectable effects on pErk levels, and only occasional, slight inhibitory effects on regenerative growth when applied daily during regeneration (data not shown). We suspect that we failed to detect increases in pErk after the 36°C HS because v-ras induction was low and/or mosaic, and because fins have high baseline levels of pErk (supplementary material Fig. S2F). Strikingly, animals given a daily 36°C HS during the first 5 days after amputation displayed massively hyperpigmented new structures, with most rays of each fin filled with excess melanocytes (Fig. 1B,C). Active Ras also induced hyperpigmentation in regenerating anal fins, which, unlike caudal fins, contain stripes that are parallel to the plane of amputation (Fig. 1D,E).

To determine whether amplified melanocytes in hsp70:v-ras regenerates had assumed ectopic locations, we assessed regenerates histologically. Intraray melanocytes typically align adjacent to bone within intact and regenerating fins (Fig. 1F,H). Analysis of hsp70:v-ras fin regenerates revealed a much greater number of melanocytes within the rays; however, these cells were grossly normal in morphology and were observed in the expected compartment adjacent to paired hemiray bone (Fig. 1G,I). Similarly, we did not observe unusual pigmented foci indicative of malignancy. Interestingly, Ras-induced hyperpigmentation was reversible. When hsp70:v-ras zebrafish were removed from the HS protocol for days to weeks after an initial 10-day hyperpigmentation period, the stripe pattern reverted to that typical of a wild-type clutchmate (supplementary material Fig. S3). Interstripe melanocytes in these animals contracted their dendritic processes, assuming the appearance of apoptotic cells as reported by others (Sugimoto et al., 2000; Rawls and Johnson, 2001). In summary, our results reveal that Ras activity controls melanocyte density and stripe renewal in regenerating zebrafish fins.

![Fig. 1. Transgenic increases in active Ras hyperpigment regenerating zebrafish fins.](image-url)
Evidence that active Ras hyperpigments regenerating fins by amplifying melanocyte precursor cells

To determine whether the effects of active Ras on fin pigmentation required a regenerative context, we amputated one of the two caudal fin lobes and followed pigmentation in hsp70:v-ras animals that had been heat shocked for several days. This protocol hyperpigmented the regenerating lobe but had no noticeable effect on pigmentation within the intact lobe, suggesting that the effects of active Ras were regeneration specific (supplementary material Fig. S4). It remains possible that particularly long durations (>15 days) of transgenic increases in active Ras impact pigmentation in non-regenerating fins, or melanoma formation, as discussed below.

Regenerating melanocytes normally originate from undifferentiated precursors, as opposed to the division of existing differentiated pigment cells (Rawls and Johnson, 2000). Given the abundance of melanocytes caused by active Ras, it was important to distinguish whether this disturbance amplified melanocyte precursors or, instead, stimulated direct proliferation by differentiated melanocytes. To label and trace melanocytes, we blocked new melanin synthesis during regeneration with the chemical phenylthiourea (PTU), and assessed melanocytes in the regenerate for pigmentation. If the regenerates were again filled with pigmented melanocytes during v-ras expression, these could derive only from melanocytes that were existing and pigmented prior to amputation. Wild-type and hsp70:v-ras transgenic fish were incubated in PTU immediately after amputation, and given a daily 36°C HS for 5 days. We found that neither wild-type nor transgenic PTU-treated regenerates showed pigmentation through 5 days post-amputation (dpa) (Fig. 2A,B). To confirm that PTU treatment did not ablate melanocytes, we followed wild-type and transgenic

Fig. 2. Active Ras expands undifferentiated melanocyte precursors. (A,B) Low- (left) and high- (right) magnification images of PTU-treated wild-type and hsp70:v-ras animals after 5 days of regeneration and daily 36°C heat-shocks. PTU treatment blocked the appearance of pigmented melanocytes in both wild-type (A) and hsp70:v-ras (B) regenerates. (C-H) Washout experiments in wild-type and hsp70:v-ras regenerates after 5 days of regeneration, PTU treatment and heat-shocks. Pigmentation is blocked by PTU in both wild-type (C) and transgenic (F) regenerates. Removal of PTU and incubation at 26-28°C [room temperature (RT)] for 1 or 6 days enabled melanin synthesis and uncloaked melanocytes in wild-type (D,E) and hsp70:v-ras (G,H) regenerates. The same fins are shown in (C-E) and in (F-H). (I) Quantification of melanocytes in fin regenerates of wild-type and hsp70:v-ras animals after 1 day of PTU washout at 26-28°C, following 5 days of regeneration and daily 36°C heat shocks (n=8, mean ± S.E.M.; Student’s t-test, *P<0.001). (J) Dose-dependent effects of active Ras on melanocyte number. A second experiment quantifying melanocytes in fin regenerates of wild-type and hsp70:v-ras animals after 1 day of PTU washout at 26-28°C, following 5 days of regeneration and daily 35°C or 36°C heat shocks (n=10, mean ± S.E.M.; Student’s t-test, **P<0.001, significantly different from 36°C wild-type; ***P<0.001, significantly different from 36°C wild-type and 35°C hsp70:v-ras). (K-M) Representative high-magnification images at 6 dpa of animals from J.
animals for an additional day under conditions permitting melanin synthesis. Melanocytes could be visualized after a 1-day wash in aquarium water, and were punctate in appearance and more easily quantifiable (Fig. 2C-H). We observed three to four times as many melanocytes in hsp70v-ras regenerates as in wild-type regenerates after this protocol (Fig. 2J-L). The effect of v-ras was dose dependent, as a lower, 35°C HS merely doubled the density of melanocytes observed in regenerates of wild-type animals given 36°C HS treatments (Fig. 2). Our results indicate that active Ras disrupts stripe patterning by expanding a melanocyte stem or precursor cell population that exists prior to amputation, rather than amplifying existing differentiated melanocytes.

To directly analyze melanocyte precursors, we performed in situ hybridization on cryosections from wild-type and hsp70v-ras 5-dpa regenerates treated with PTU, using probes that mark these cells. The transcription factor mitfa is the earliest known marker of undifferentiated melanoblasts (at 1 dpa), and is necessary for the survival and differentiation of melanocytes (Lister et al., 1999). kita is detectable by 1.5 dpa and is thought to mark a committed but undifferentiated melanoblast population. Multiple functions for kita have been proposed, including roles in melanoblast migration and survival (Rawls et al., 2001; White and Zon, 2008). We were able to identify a small number of cells that faintly expressed mitfa or kita in wild-type regenerates (Fig. 3A-C). By contrast, hsp70v-ras regenerates displayed a marked expansion of cells expressing either of these melanoblast markers (Fig. 3B-D). As was the case with differentiated melanocytes, active Ras did not alter the spatial distribution of mitfa+ and kita+ cells within regenerating mesenchyme. Although amplified, the cells remained adjacent to the hemiray bone layer (Fig. 3B,D). We also found clear increases in the presence of cells expressing xdh3.1 and gch, markers for the xanthoblast lineage (Fig. 3I-N) (Parichy et al., 2000; Pelletier et al., 2001). However, as we observed no obvious expansion of mature xanthophores, which are yellow pigment cells that normally form alternating stripes with melanocytes, the apparent impact of active Ras on xanthoblast numbers was not pursued. To test whether an excess of mitfa+ cells arose at least in part by proliferation, we labeled wild-type and hsp70v-ras animals with bromodeoxyuridine (BrdU) for the final 30 minutes of a regimen of PTU treatment, HS and regeneration. We found that many mitfa+ cells were labeled with BrdU, particularly so in hsp70v-ras regenerates (Fig. 3E-H), although this finding does not exclude increased migration as a contributor to hyperpigmentation. In total, our results indicate that levels of active Ras control the expansion of melanocyte precursor cells during fin regeneration, facilitating stripe recovery.

**Active Ras recovers primary regeneration melanocytes in the absence of Kita function**

To position Ras genetically with respect to known mediators of fin stripe regeneration, we crossed the hsp70v-ras transgene onto backgrounds that were deficient for mitfa, csf1r or kita. The null mutant of mitfa (referred to as nacre<sup>−/−</sup>) is viable throughout adulthood, but deficient of all melanocytes, including fin pigment stripes (Lister et al., 1999). Xanthophores are distributed evenly throughout the fins of these mutants. The null mutant of csf1r (referred to as fms<sup>−/−</sup>) is deficient of xanthophores, resulting in fins with low numbers of evenly distributed melanocytes (Parichy et al., 2000). Active Ras caused no recovery of melanocyte pigmentation in the absence of functional Mitfa during fin regeneration (Fig. 4A,B). Furthermore, although increases in active Ras had no effect on the xanthophore deficiency in regenerating fins that were devoid of functional Csfr1, it greatly increased the number of melanocytes (Fig. 4C,D). Together, these results indicate that excess melanocytes generated by increases in active Ras remain dependent on mitfa for differentiation and survival, and do...
not arise indirectly as a result of primary effects on the xanthophore population.

Finally, we examined the kita mutant, which is unable to regenerate primary melanocytes in the first week post-amputation (Rawls and Johnson, 2000). Strikingly, the kita phenotype was genetically suppressed by 5 days of active Ras expression after amputation (Fig. 4E,F). Most of the regenerated fin rays from kita; hsp70v-ras animals were densely filled with melanocytes at 5 dpa, differing notably from similarly treated, non-transgenic kita mutants. Through expression studies and lineage-tracing studies with PTU, we found that these kita mutant melanocytes regenerated by de novo differentiation from unpigmented precursor cells (supplementary material Fig. S5). Thus, Ras activity amplifies melanocyte precursors through a mechanism that does not depend on intact Kita signaling.

In the absence of Kita function, a secondary regulatory population of melanocyte precursors arrives later during regeneration and pigments regenerating fins (Rawls and Johnson, 2000). To examine the potential impact of active Ras on this cell population, we induced the expression of the v-ras transgene from 7–12 or 10–15 dpa in otherwise wild-type and kita mutants. Although transgene induction occurred in the last stages of bone regeneration, active Ras still conspicuously hyperpigmented otherwise wild-type fin regenerates (Fig. 4G–J). By contrast, by 12 or 15 dpa, kita; hsp70v-ras regenerates showed secondary melanocyte pigmentation in the proximal portions of the regenerates, but at patterns and densities that were indistinguishable from kita mutants alone (Fig. 4K–N). Thus, unexpectedly, given its suppression of the effects of the kita mutation on primary regeneration melanocytes, active Ras failed to expand the secondary regulatory population. These findings suggest that...
Ras specifically functions to regulate numbers of primary melanocyte precursors during fin stripe regeneration, although it remains possible that v-ras induction is less robust in secondary regulatory precursors. Interestingly, these results also reveal a crucial period in the first week after amputation during which regeneration melanocytes possess competence for expansion in the absence of Kita function.

**DISCUSSION**

There is a tremendous amount of excitement in the scientific community about potential therapies to combat the poor regenerative capacity of most mammalian organs. Most prominently, the discovery of induced pluripotent stem (iPS) cells has revolutionized ideas for creating patient-specific stem cells. However, these advances on their own are unlikely to achieve the full impact of regenerative medicine and must be steered by perspectives gained through the study of organogenesis in embryos, and by successful examples of adult tissue regeneration where they may exist. During the regeneration of amputated major appendages in urodeles and teleosts, cell renewal is precisely regulated such that only the appropriate structures are replaced. The experiments we describe here reveal an important mode of regulation by which pigment formation is restored to regenerating zebrafish fins, enabling stripe formation during the regeneration of complex appendage tissue. Our data indicate that the level of active Ras in regenerating zebrafish fins controls the density of melanocyte precursors and differentiated progeny within newly forming fin structures.

Although pErk levels in the hsp70v-ras transgenic fin regenerate rose rapidly after a strong v-ras induction protocol, the hyperpigmenting protocol that induced lower levels of v-ras expression resulted in no detectable changes in the amounts of pErk. That is, we could not precisely connect hyperpigmentation with the levels of Ras targets. Notwithstanding this, several findings support a model in which Ras acts as a downstream effector of Kita signaling within primary regeneration precursors, promoting their self-renewal. First, the existence of this molecular model is predicted by a number of studies that place Ras downstream of ligand binding to receptor tyrosine kinases, including c-Kit (Pazin and Williams, 1992; Schlessinger, 2000; Carlson et al., 2007). Second, in our studies, active Ras expanded a kita+ cell population during fin regeneration. Third, Kita loss of function and active Ras both affected the primary melanocyte precursor population, and showed no noticeable direct effects on secondary melanocytes. Fourth, in the absence of Kita signaling, active Ras rescued the presence of pigmented melanocytes in fin regenerates. Therefore, our data favor the idea that Ras activity is a major point of control in melanoblast amplification following Kita receptor activation. However, as Ras is involved in other aspects of regenerative growth (Lee et al., 2009), and because v-ras is induced in multiple cell types within hsp70v-ras regenerating fins, it remains possible that there are alternative or additional mechanisms by which Ras can modify the pigmentation pattern. Our analyses with PTU and with csf1r mutants indicate that such mechanisms would not include: (1) effects on melanocytes differentiated prior to injury; or (2) indirect effects caused by modulating the xanthophore population.

It is interesting that we observed hyperpigmentation, but not melanomas, in regenerates expressing oncogenic Ras. This contrasts with a recent report describing zebrafish that were injected with a construct directing the expression of the same v-ras cassette from the mitfa promoter (Michailidou et al., 2009). In that study, 60% of mosaic animals injected with the v-ras construct developed patches of ectopic melanocytes, with 30% showing tumor nodules by 12 weeks of age. We suspect that this difference can be explained by the lower levels of active Ras, and a shorter observation time, in our study. Nevertheless, despite the major expansion of melanoblasts and melanocytes caused by active Ras during fin regeneration, these cells remain within their normal niche adjacent to hemirays. The stimulation of precursor renewal without oncogenesis by low levels of activated Ras may be relevant to stem or progenitor cell proliferation in other regenerative contexts, or to strategies to boost mammalian regeneration.

Our data indicate that, consistent with previous reports (Rawls and Johnson, 2000; White and Zon, 2008), the transcription factor Mitfa is required for the development of adult melanoblasts to a stage where they are competent for expansion through Kita and active Ras. Then, Kita and Ras are crucial for rapidly populating the fin regenerate with a burst of primary regeneration melanocyte precursors. However, these factors do not appear to participate directly in the resolution of scattered pigment cells into stripes. Pigment patterning in adult fish is known to be dynamic and highly regulative, occurring through community interactions among the different differentiated pigment cell types (Parichy and Turner, 2003; Yamaguchi et al., 2007). The proposed mechanism for autologous generation of spacing between two populations of skin pigment cells invokes the reaction-diffusion model first proposed by Turing, in which melanocytes and xanthophores exert stimulatory or antagonistic effects depending on their distance from each other (Turing, 1952; Nakamasu et al., 2009). The signaling networks defining pigment patterning remain to be elucidated at the molecular level, and it is likely that the same mechanisms become activated within fin rays after amputation and complex tissue regeneration. We observed that active Ras induction regularly filled regenerated ray structures with melanocytes; yet, within days of withdrawal of v-ras expression, the dispersed and over-represented melanocyte pigmentation resolved into a typical striped pattern. Interestingly, expanded melanocyte populations also regressed in csf1r mutants without xanthophores or a defined melanocyte stripe pattern (supplementary material Fig. S6). Thus, the evidence to date indicates that the Kita/Ras pathway supplies a sufficient amount of precursors and differentiated melanocytes for newly regenerated tissue, after which their numbers and organization are refined by the supplementing effects of secondary regulatory precursors and the depleting effects of reciprocal patterning interactions. This tight regulation of melanocyte number during fin regeneration, by the supply of two forms of precursors and by the culling of differentiated cells, provides a glimpse into the complexity of the regenerative process that is relevant to tactics for changing limitations in the regenerative capacity of human tissues.

**METHODS**

**Zebrafish and fin amputations**

Mature adult zebrafish that were 4-6 months of age were used for all of the experiments, and were maintained at a density at which they display little or no animal growth (Wills et al., 2008). Wild-type animals were of the outbred Ekkwill (EK) strain. Mutant and transgenic stocks of nacre2, csf1r, kitb5 mutant and hsp70v-ras...
Melanocyte expansion during regeneration

Transgenic zebrafish have been described previously (Lister et al., 1999; Parichy et al., 1999; Parichy et al., 2000; Lee et al., 2009). The full name of the hsp70:v-ras line used for this study is Tg[hsp70v-ras]pa66. Fin amputations and HS treatment of transgenic animals were performed as described previously (Lee et al., 2009). hsp70v-ras animals, in various backgrounds, were typically given a single, daily 36°C HS for 5 days. Each treatment involved a gradual increase in aquarium temperature over 3 hours, at which point the tanks were maintained at the target temperature for around 30 minutes before the heat source was shut off. In some experiments, a different temperature was used for decreased or increased induction of the transgene. All experiments with zebrafish were performed in accordance with animal use protocols that were approved by Duke University.

Quantitative PCR
hsp70:v-ras transgenics and clutchmate controls were given a single HS at 35°C, 36°C or 38°C for 40 minutes at 4 dp, or were left at room temperature. Total RNA was extracted, using TRI reagent (Sigma), from regenerates at 2 hours after the completion of HS. First-strand cDNA was transcribed from 2 μg of total RNA using the Transcriptor First-Strand cDNA synthesis kit oligo dT protocol (Roche). Quantitative PCR was performed in triplicate for each gene using the Lightcycler 480 SYBR green DNA master mix (Roche). Primers that were specific for the transgene were designed and verified (sequence below). Amplification of β-actin1 served as a control. Normalization of the samples was performed using the formula described previously (Yin et al., 2008). The primers were:
- v-ras forward primer, 5’-AATCTCGCAGGCTCAGGACC-3’; v-ras reverse primer, 5’-GCGGGGCCACTCTCATCA-3’.

In situ hybridization and immunohistochemistry
In situ hybridization on cryosections of 4% paraformaldehyde-fixed fins was performed as described (Poss et al., 2002b) using digoxigenin-labeled probes produced with mitfa and kita DNA templates (Rawls and Johnson, 2000). To remove pigmentation, wild-type and hsp70v-ras animals were simultaneously incubated in 0.2 mM PTU and given daily 36°C heat shocks prior to fin collection. To obtain sections of fin regenerates, fins were mounted in 1.5% agarose/5% sucrose and then saturated in 30% sucrose overnight at 4°C. Frozen blocks were sectioned at a thickness of 10 μm and in situ hybridization was performed with serial sections.

The analysis of BrdU incorporation was performed as described previously (Poss et al., 2002a). The BrdU solution was injected intraperitoneally 30 minutes prior to collection, and fins were then collected and fixed. For the analysis of mitfa expression and BrdU incorporation, cryosections of fin regenerates from BrdU-injected animals that had been stained for mitfa expression by section in situ hybridization were stained for BrdU as previously described (Lepilina et al., 2006).

Hematoxylin staining was performed as described previously (Poss et al., 2000). Longitudinal and transverse fin cryosections of wild-type and transgenic animals were stained with hematoxylin for 30-45 seconds.

Western blotting
Fin regenerates were collected from 4 dp of wild-type or hsp70v-ras transgenic animals given a single HS 5 hours prior to tissue collection, or from uninjured and regenerating wild-type fins. The tissue was lysed in a solution of 10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100 containing protease inhibitor (10 μM/ml). Lysates were analyzed by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated with rabbit anti-Erk (Sigma) and mouse anti-phospho Erk antibodies (Cell Signaling), with an enhanced chemiluminescence (ECL)–horseradish peroxidase (HRP)-linked secondary antibody (Amersham). HRP activity was detected by ECL treatment (Pierce Protein Research).

PTU treatment
Animals were transferred to aquarium water containing PTU (0.2 mM), and the solution was changed daily. For heat shocks...
simultaneously with PTU treatment, a 1.5-liter aquarium with one liter of 0.2 mM PTU was placed in a 3-liter aquarium using the regular HS system described previously (Lee et al., 2005). To wash out the PTU, wild-type and hsp70cr-ras transgenic animals were incubated in aquarium water at 26-28°C. The number of newly differentiated melanocytes was quantified at 24 hours post-washout with the aid of Openlab software.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Y.L. performed the majority of experiments, analyzed data and prepared the manuscript. G.N. performed immunoblotting and quantitative PCR. P.W.K. analyzed pigmentation during regeneration in wild-type and transgenic animals. D.H. generated transgenic animals. K.D.P. directed the study, evaluated data and prepared the manuscript.

SUPPLEMENTARY MATERIAL

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