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Kit signaling is involved in melanocyte stem cell fate decisions in zebrafish embryos

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SUMMARY

Adult stem cells are crucial for growth, homeostasis and repair of adult animals. The melanocyte stem cell (MSC) and melanocyte regeneration is an attractive model for studying regulation of adult stem cells. The process of melanocyte regeneration can be divided into establishment of the MSC, recruitment of the MSC to produce committed daughter cells, and the proliferation, differentiation and survival of these daughter cells. Reduction of Kit signaling results in dose-dependent reduction of melanocytes during larval regeneration. Here, we use clonal analysis techniques to develop assays to distinguish roles for these processes during zebrafish larval melanocyte regeneration. We use these clonal assays to investigate which processes are affected by the reduction in Kit signaling. We show that the regeneration defect in *kita* mutants is not due to defects in MSC recruitment or in the proliferation, differentiation or survival of the daughter cells, but is instead due to a defect in stem cell establishment. Our analysis suggests that the *kit* MSC establishment defect results from inappropriate differentiation of the MSC lineage.

KEY WORDS: Clonal analysis, Fate determination, Kit, Melanocyte, Regeneration, Stem cell

INTRODUCTION

Adult stem cells play crucial roles in the growth, homeostasis and regeneration of adult tissues. Typically, adult stem cells replace themselves and produce daughters committed to differentiation. Adult stem cells can broadly be classified into two categories: stem cells that are continuously dividing, such as the intestinal stem cell (Barker et al., 2007), and those that are largely quiescent but can be recruited by injury or other physiological need to divide, such as muscle satellite cells (Schultz et al., 1978). The melanocyte stem cell (MSC) falls into the second category of quiescent stem cells (Nishimura et al., 2002). We have developed several methods to activate the MSC in zebrafish, including amputation of the fin in adult fish (Rawls and Johnson, 2000), laser ablation in larvae (Yang et al., 2004), genetic induction in the embryo (Hultman et al., 2009) and drug-induced melanocyte death followed by regeneration in the adult (O'Reilly-Pol and Johnson, 2008) and larvae (Yang and Johnson, 2006). Each of these procedures reveals one or more aspect of MSC regulation.

Regeneration output of stem cells can be conceptualized as the product of (1) the number of established stem cells, (2) the percentage of stem cells that are recruited to make differentiating daughters, and (3) the proliferation, differentiation and survival of these daughter cells. The receptor tyrosine kinase Kit can play roles in one or more of these processes in many stem cell models, including germ cells (Koshimizu et al., 1991; Manova and Bachvarova, 1991) (reviewed by Sette et al., 2000), hematopoietic stem cells (Li and Johnson, 1994; Valent et al., 1992) (reviewed by Ashman, 1999) and melanocyte stem cells (Mackenzie et al., 1997; Nishikawa et al., 1991) in mammals. Unlike in germ cells and hematopoietic stem cells, in zebrafish one of the two Kit orthologs, *Kita*, is only necessary for melanocytes (Parichy et al., 1999). Melanocytes are easily dispensable without affecting viability of

zebrafish embryos, easily quantifiable without requiring additional staining or sacrificing of the animal, and readily ablated (causing regeneration) by both chemical (Yang and Johnson, 2006) and physical means (Yang et al., 2004). During regeneration, new melanocytes incorporate legacy markers such as bromodeoxyuridine (BrdU), and larvae can be induced to regenerate multiple times (Yang and Johnson, 2006). Together, these data support the notion of an MSC in the larvae that regulates the larval pigment pattern.

The *kit* gene is also required for multiples steps in melanocyte development. We have previously isolated a temperature-sensitive mutation in *kita*, *kita*^{1le99}, hereafter referred to as *kita*^{ts} (Rawls and Johnson, 2001). We have successfully used this mutation to demonstrate the temporal requirements for *kit* during adult melanocyte regeneration following fin amputation (Rawls and Johnson, 2001) and larval ontogenetic melanocyte migration and survival (Rawls and Johnson, 2003). In contrast to the temperature sensitivity of melanocyte regeneration both following fin amputation and in larval melanocyte migration and survival, *kita*^{ts} is largely defective for regeneration even at permissive temperatures (Yang and Johnson, 2006). This suggests that larval melanocyte regeneration has a higher requirement for *kit* activity than ontogenetic development or adult regeneration after fin amputation; thus, a different technique is required to study the role of Kit in larval melanocyte regeneration.

Clonal and mosaic analyses can be used fruitfully to study the development of tissues or regeneration of larval or adult tissues. Recently, these methodologies have been used to show the contributions of specific tissue types during complex regeneration events (Gargioli and Slack, 2004; Kragl et al., 2009; Rinkevich et al., 2011; Tu and Johnson, 2011). Additionally, they have been used to elucidate contributions of individual cells within a single tissue during growth, homeostasis and regeneration (Gupta and Poss, 2012; Snippert et al., 2010; Tryon et al., 2011).

We were interested in determining which of the broadly defined processes discussed above is affected by deficits in Kit signaling during larval melanocyte regeneration. We developed three assays using clonal analysis to distinguish roles for each of the broadly

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defined processes. We show that *kita*^{null/+} heterozygotes are defective for larval melanocyte regeneration, regenerating ~50% of the melanocytes compared with wild type (WT). In this genotype, we observed no defects in MSC recruitment or in the proliferation, differentiation or survival of committed daughter cells. By contrast, our clonal analysis shows that *kita*^{null/+} only establish 54% of the MSCs compared with WT embryos. Further dissection of this defect with additional clonal analysis suggests that the cells fated to become MSCs differentiate as melanocytes instead.

MATERIALS AND METHODS

Fish stocks and husbandry

Fish were housed and reared following standard protocols (Westerfield, 2007). All experiments were performed at 28.5°C except for those involving *kita*^{le99}, which were performed at 25°C and for which developmental times were adjusted according to Kimmel et al. (Kimmel et al., 1995).

Alleles of *kita*^{b5} (Parichy et al., 1999) and *kita*^{le99} (Rawls and Johnson, 2001) have been previously described, and will be referred to as *kita*^{null} and *kita*^{ts}, respectively. WT fish were either the inbred SJA line or *melanophilina* (*mlpha*) (Sheets et al., 2007) mutants. *mlpha* mutants contain contracted melanosomes, facilitating quantitative analysis, but have no other melanocyte defects (Sheets et al., 2007; Hultman et al., 2009). All clonal analysis was performed in *mlpha* or *mlpha*; *kita*^{null/+} embryos.

Melanocyte clone generation

Melanocyte lineage clones labeled with *fTyrp1*>GFP were generated as previously described (Tu and Johnson, 2010). Briefly, at the one- to two-cell stage, embryos were injected with 5–10 pg of a Tol2 plasmid bearing *fTyrp1*>GFP (Zou et al., 2006) and 5 pg of capped Tol2 transposase mRNA (Kawakami et al., 2004).

To create clones used in Fig. 4, one- to two-cell-stage embryos were co-injected with 5–10 pg *fTyrp1*>GFP transposon, 50 pg of a plasmid with a transposon containing the *Xenopus EF1a* promoter driving RFP expression (*EF1a*>RFP) (Tu and Johnson, 2010), and 5 pg of capped Tol2 transposase mRNA.

Chemical ablation of melanocytes

As previously described (Yang and Johnson, 2006), embryos were treated with 4 µg/ml 4-hydroxyanisole (4-HA; M18655, Sigma-Aldrich) to ablate melanocytes either as they developed [1–3 days post-fertilization (dpf)] or with a 24- to 48-hour treatment to ablate fully differentiated melanocytes.

Analysis of clones

Embryos were screened for GFP or RFP clones under an epifluorescence stereomicroscope (Nikon SMZ1500) after being anesthetized with tricaine (methanesulfonate). As we generally generated labeled embryos at rates of 10–40%, we used the Poisson distribution to account for the differential between the number of clones and the number of labeled animals caused by polyclonality in each injection. We used two sample *t*-tests for comparing clone sizes (Figs 2, 5), χ^2 -test to compare stem cell recruitment (Fig. 3), and Z-score to compare relative lineage numbers (Figs 4, 5).

Recruitment rate calculation

For the recruitment rate of MSCs (Fig. 3), several models were considered. The simplest model consistent with the data was a stochastic model, i.e. that MSCs are chosen randomly and independently in each round. Under this model, the recruitment rate is revealed by sequential rounds of regeneration. A general solution is that the recruitment rate is the number of clones revealed in both rounds divided by the number of clones revealed in the first (or second) round. Under this model, the probability of not seeing a particular MSC is $[1 - (\text{recruitment rate})]^2$. This model also makes predictions about the overlap between the two rounds of regeneration and the percentage of clones that appear in a given round. Specifically, when the overlap between the two rounds is high (>63.47%), the number of unrevealed MSCs is low (<5%), and the recruitment rate can be estimated by the number of clones revealed in the first (or second) round divided by all the clones revealed in the experiment (single round utilization; supplementary material Table S1). As our predicted rate of unobserved MSCs is low, we use the latter method to calculate the recruitment rate.

MSC establishment calculation

For the relative normalized GFP clones (Fig. 4), the numbers of GFP and RFP clones were calculated separately using the Poisson distribution to account for the differential of labeled animals caused by polyclonality for each clutch. The number of adjusted GFP clones was divided by the number of adjusted RFP clones to get a normalized GFP clone rate. This normalized clone rate was then used to compare clutches injected in the same experiment to calculate a relative normalized GFP clone rate.

Analysis of ontogeny and regeneration

When analyzing the ontogeny or regeneration of melanocytes in the entire animal, such as in Fig. 1, three to ten animals were counted along with WT controls and compared using Student's *t*-test. Melanocytes in the yolk stripe and over the swim bladder were ignored.

RESULTS

Dosage sensitivity of kit signaling for regeneration

Because the *kita*^{ts/ts} mutant shows an increased requirement for Kit signaling for larval melanocyte regeneration even at the permissive temperature, we explored the dosage sensitivity of other alleles of *kita*. This increased requirement of Kit signaling in the larval MSCs is similar to the increased requirement of fetal hematopoietic stem cells (HSCs) compared with the requirement of adult HSCs for Kit signaling (Bowie et al., 2007). We checked various other alleles of *kita* as heterozygotes and homozygotes and quantified their larval ontogenetic and regeneration phenotypes (Fig. 1A; supplementary material Fig. S1A). We found genotypes that had normal or nearly normal ontogeny, but had mild to severe regeneration defects (Fig. 1B; supplementary material Fig. S1B). We chose to focus on *kita*^{null/+} as it had a regeneration defect that was easily distinguishable from wild type (54% of WT; Fig. 1B), but still regenerated a significant number of melanocytes (>100 per embryo). These data confirm that larval melanocyte regeneration is dosage sensitive for Kit signaling. Interestingly, these results reveal a haploinsufficiency for *kit* in zebrafish that is consistent with the haploinsufficiency for *kit* in coat patterns of the mouse (Geissler et al., 1988).

Clonal analysis

Melanocyte regeneration can be divided into three broad processes: proliferation, differentiation and survival of committed daughter cells (Yang et al., 2007); recruitment of the MSC to initially divide and produce committed daughter cells; and establishment of the MSC (Budi et al., 2008; Hultman et al., 2009) (Fig. 2A). In order to determine which of these processes contributed to the *kita*^{null/+} regeneration deficit, we used clonal analysis. To study each process we can generate clones by injecting embryos at the one- to two-cell stage with a transposon plasmid containing GFP driven by the *Tyrp1* promoter from fugu (*fTyrp1*>GFP), which is specifically activated in the melanocyte lineage. Using this protocol, we have shown that transposon integration occurs largely after the midblastula transition, centered on 6 hours post fertilization (Tryon et al., 2011). This generates melanocyte lineage clones, and we can infer the activity of the MSC and its daughters by analyzing the labeled melanocytes arising during regeneration. Prior to 3 dpf, melanocytes arise directly without going through an MSC intermediate (Hultman et al., 2009). We ablate these ontogenetic melanocytes by treatment of embryos with the melanotoxic drug 4-HA from 1 to 3 dpf. Following washout of 4-HA, melanocytes that regenerate come from MSCs, and those melanocytes that are labeled with GFP are daughters of a labeled MSC.

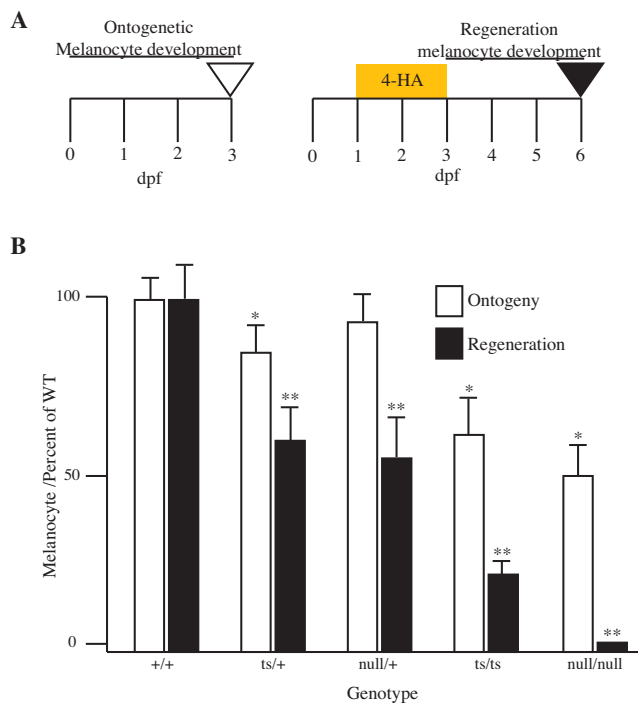


Fig. 1. Melanocyte regeneration is more sensitive to deficits in Kit signaling than ontogeny. (A) Schema of the experiments. Zebrafish were reared until 3 dpf and scored for ontogenetic melanocytes (open arrowhead) or treated with 4-HA from 1-3 dpf and scored for regeneration melanocytes at 6 dpf (filled arrowhead). (B) Comparison of *kita* mutant genotypes to WT. Three to ten embryos were compared with WT controls for ontogenetic melanocytes (white bars) and regeneration melanocytes (black bars). Experiments with *kita*^{ts} were performed at 25°C, and all others were performed at 28.5°C. Error bars indicate s.d. **P*<0.05, ***P*<0.001.

Proliferation, differentiation and survival of committed daughter cells are not affected by reduced Kit signaling

First, we used clonal analysis to analyze the proliferation, differentiation and survival of MSC daughter lineages by quantifying regeneration clone size (Fig. 2A). Following injection and melanocyte regeneration (Fig. 2B), we counted labeled melanocytes in mosaic WT and *kita*^{null/+} fish after 3 days of regeneration (Fig. 2C,D). We then adjusted for potential polyclonality (see Materials and methods). We reasoned that if Kit function affected the proliferation, differentiation and/or survival of daughter cells, we would observe smaller clone sizes in the mutant. Instead, we see that regeneration melanocyte clone size is the same for WT (2.9±0.14 melanocytes) and the heterozygous mutant (2.9±0.2 melanocytes) (mean±s.e.m.; Fig. 2E). This rules out the hypothesis that the reduced regeneration seen when Kit function is reduced is due to defective proliferation, survival or differentiation of MSC daughter cells.

Clonal analysis during double regeneration

Our ability to estimate the recruitment rate (Fig. 3A) of the MSC is hindered by our inability to observe the MSC directly. We define MSC recruitment rate as the number of MSCs producing melanocytes divided by all available MSCs. The numerator is the number of labeled clones. The denominator requires that we estimate the number of available MSCs. We reasoned that we would

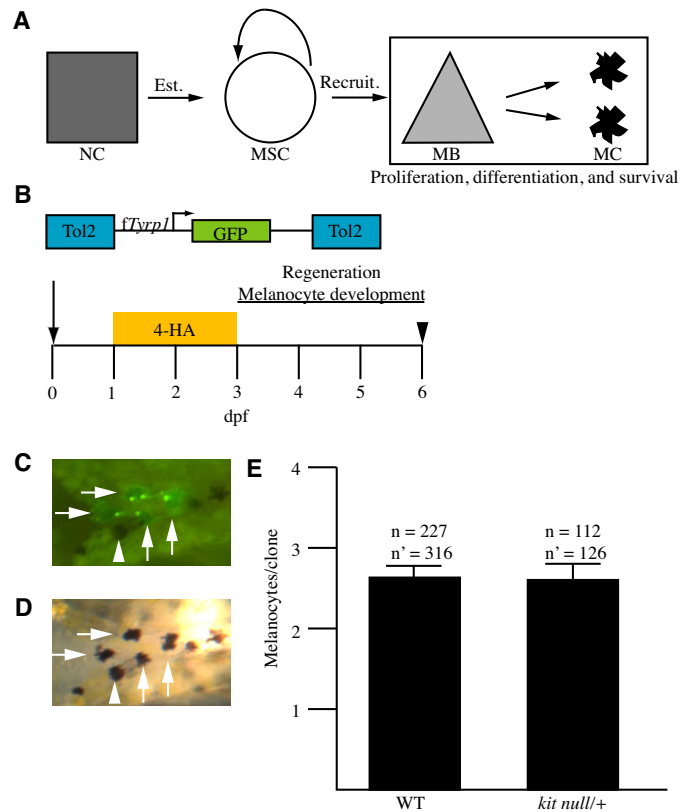


Fig. 2. MSC daughter cell proliferation, differentiation and survival are unaffected in *kita*^{null/+} embryos. (A) A general model of melanocyte regeneration. A neural crest progenitor (square, NC) establishes a melanocyte stem cell (circle, MSC), which can be recruited to produce melanoblasts (triangle, MB), which will proliferate and differentiate into melanocytes (MC). Box indicates process tested. (B) Schema of the experiment. Zebrafish embryos were injected at the one- to two-cell stage (arrow) with transposase and a transposon containing *fTyrop1*>GFP, treated with 4-HA from 1-3 dpf to ablate ontogenetic melanocytes, and scored for regeneration melanocytes at day 6 (arrowhead). (C,D) Example of a clone in WT. (C) An epifluorescent image of an embryo containing a clone. (D) A brightfield image of the embryo shown in C. Melanocytes containing GFP are marked with an arrow. Some melanocytes lack GFP, and one example is marked with an arrowhead. (E) The mean number of melanocytes per clone is the same for both genotypes in regeneration. *n*, the number of embryos containing clones; *n'*, the number of clones after adjustment for polyclonal events. Error bars are s.e.m.

need to reveal all or nearly all available labeled MSCs by regeneration. For this, we employed clonal analysis using a double (sequential) regeneration assay to assess MSC recruitment rate (Fig. 3B). We assumed a model in which MSCs were chosen to produce melanocytes stochastically, i.e. MSCs chosen in round 1 were equally likely to be chosen in round 2 (see Materials and methods). This model predicts that to observe >95% MSCs in two rounds of regeneration, the overlap between the two rounds needs to be >63.5% (supplementary material Table S1).

First, we examined recruitment in WT mosaic larvae. Of 244 WT embryos injected, 50 contained GFP-labeled regeneration melanocyte clones in the first round. We then challenged these fish to regenerate a second time and re-screened for GFP-labeled melanocytes. Three larvae failed to survive the assay. Of the surviving fish that contained GFP-labeled clones after the first round

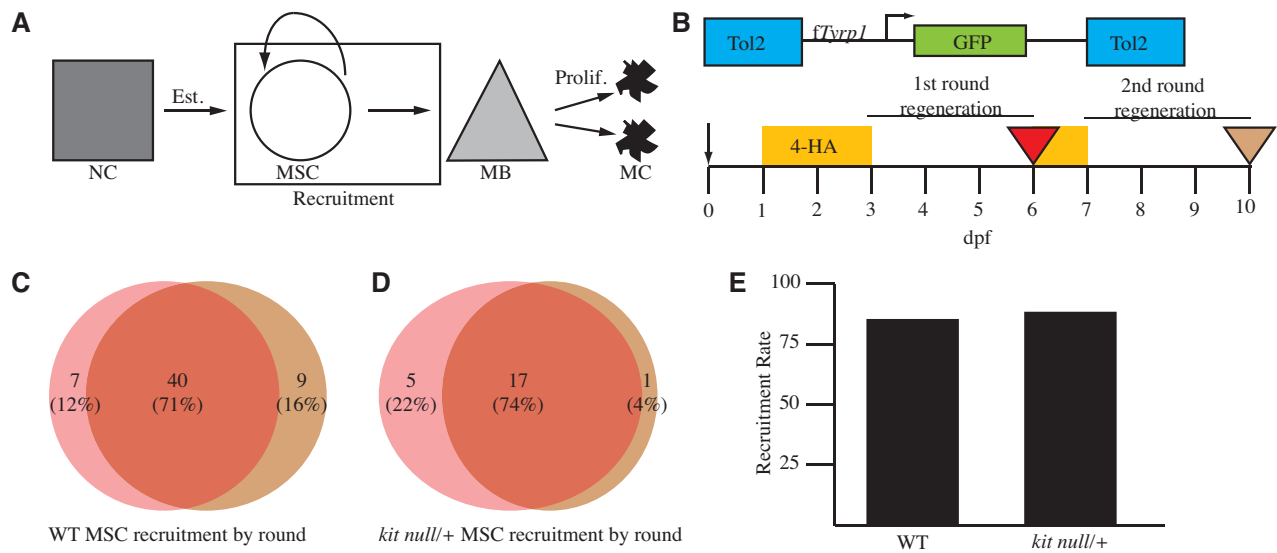


Fig. 3. MSC recruitment is unaffected in *kita*^{null/+} embryos. (A) General model for melanocyte regeneration (as described in Fig. 2 legend). Box indicates process tested. (B) Schema of the experiment. Zebrafish embryos were injected at the one- to two-cell stage (arrow) with transposase and a transposon containing *fTypr1*>GFP, treated with 4-HA from 1-3 dpf, scored for regeneration melanocytes at day 6 (red arrowhead), treated again with 4-HA from 6-7 dpf, and scored for regeneration again (brown arrowhead). (C,D) MSC recruitment rates for WT (C) and *kita*^{null/+} (D). The number of embryos (and percentage) containing a GFP clone in the first round only (light red), both rounds (dark red), and second round only (brown) are not significantly different between the genotypes ($\chi^2=3.35$, d.f.=2, $P>0.1$). (E) Recruitment rate is not different between WT and *kita*^{null/+} embryos.

of regeneration, 85% (40/47) regenerated a GFP-labeled clone after the second round of regeneration. Of the 181 surviving fish that did not contain a GFP-labeled clone after the first round of regeneration, nine regenerated GFP-labeled clones after the second round of regeneration. Thus, we observed 59 total larvae containing clones, with 50 (85%) revealed in the first round and 49 (83%) revealed in the second round (Fig. 3C). As not all larvae containing clones survived the assay, the second round recruitment rate is slightly underestimated. These stem cell recruitment rates predict that very few, if any, available MSC lineages failed to contribute to one or the other round of regeneration. These results also show that the MSC recruitment rate is 83-85%.

The high degree of overlap (>70%) between the two rounds allow us to confidently believe that nearly all available MSCs contributed to the two subsequent rounds of regeneration. If only a minority of MSCs were recruited in each round then we would not expect to see all MSCs across the two sequential rounds. Having now determined a baseline MSC recruitment rate, further studies can analyze other characteristics of MSC recruitment, including the effects of drugs or environmental factors that affect MSC recruitment, or the roles of genes in recruitment rate (below).

We then examined MSC recruitment in *kita*^{null/+} mosaic larvae. We can calculate the predicted mutant recruitment rate as follows: as described above, WT recruitment rates are ~83%, and the *kita*^{null/+} larvae regenerate 53% of the melanocytes of WT, so if the *kita*^{null/+} regeneration deficit was caused by a defect in recruitment of MSCs, we would expect to see for each round of regeneration the recruitment rate reduced to ~44% (53% of 83%). This would also be evident by a large decrease in the expected overlap between the two rounds (supplementary material Table S1). We injected 127 *kita*^{null/+} fish, of which 22 contained melanocyte clones, in the first round of regeneration. Of these fish, 77% (17/22) regenerated a GFP-labeled clone after the second round of regeneration. Of the 89 surviving fish that were not GFP labeled after the first round of regeneration, one regenerated a GFP-labeled clone after the second round of

regeneration. Thus, we observed 23 total clones, with 22 (96%) recruited in the first round and 18 (78%) recruited in the second round (Fig. 3D). Thus, the recruitment rate for *kita*^{null/+} is 78-96%. These recruitment rates are not significantly different from those observed in WT ($\chi^2=3.35$, d.f.=2, $P>0.1$), and fail to account for the 46% reduction of regeneration melanocytes seen in *kita*^{null/+} fish. Thus, we conclude that *kit* causes no deficit in recruitment of the MSC.

MSC establishment is reduced in *kita* mutants

Having excluded roles for MSC recruitment and daughter cell proliferation, differentiation and survival, we tested the hypothesis that *kita* mutants reduce the establishment of the MSC (Fig. 4A). This hypothesis predicts that fewer lineages will be used to produce the MSCs in the *kita*^{null/+} embryos. This model would be easy to test if we could visualize and quantify the MSC directly. Being unable to visualize the MSC directly, we reasoned that we could detect a deficit in MSCs by comparing the rates of labeling regeneration melanocytes in *kit* heterozygotes and WT embryos. In three independent sets of injections, we found the proportion of *kita*^{null/+}-to-WT GFP-labeled larvae was 0.40, 0.63 and 0.69 (0.57±0.15, mean±s.d.). These results support the idea that Kit signaling affects MSC establishment.

Directly using the rate of GFP-labeled regeneration melanocyte clones ignores alternative explanations. One is the experiment-to-experiment variability in labeling efficiency, which our experience suggests is as much as twofold (not shown). Additionally, there remains the possibility that the *kita*^{null/+} stocks have an independent deficit in transposon integration. To control for these possibilities, we co-injected a transposon in which RFP is driven by the constitutive promoter *ef1a* (Fig. 4B). This promoter is expressed in all cell types, thus every integration event will be observed. We were then able to normalize the number of GFP clones to the number of RFP labeled embryos. We performed this experiment three times, and found the normalized proportion of *kita*^{null/+} to WT GFP clones was 0.58, 0.37 and 0.68 (0.54±0.16, $P<0.01$, mean±s.d.; Fig. 4C).

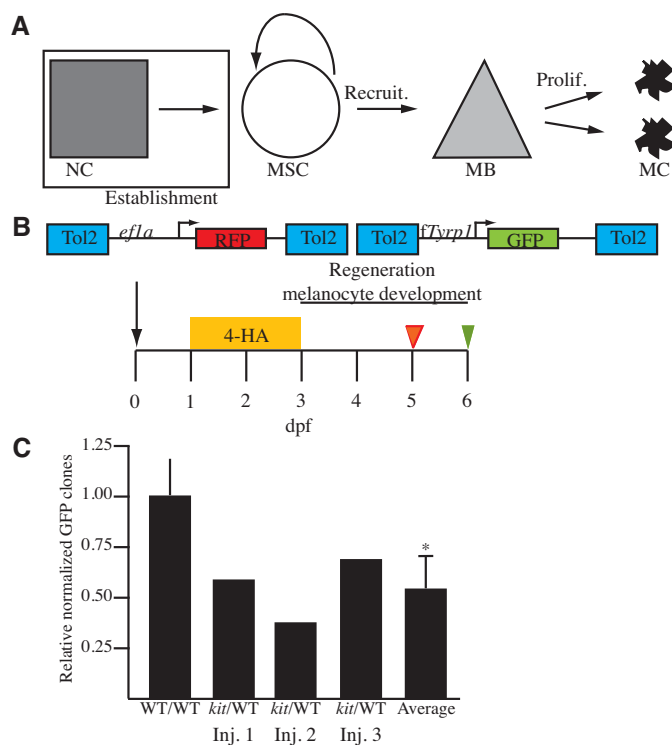


Fig. 4. MSC establishment is reduced in *kita*^{null/+} embryos. (A) A general model for melanocyte regeneration (as described in Fig. 2 legend). Box indicates process tested. (B) Schema of the experiment. Zebrafish embryos were injected at the one- to two-cell stage (arrow) with transposase, a transposon containing *efla*>RFP, and a transposon containing *fTyrr1*>GFP, treated with 4-HA from 1–3 dpf, scored for RFP at 5 dpf (red arrowhead) and scored for GFP at 6 dpf (green arrowhead). (C) Bar graph showing the relative normalized clone rate (see Materials and methods). The first bar shows a comparison of two sets of injections in WT embryos, with the line showing the deviation from expected (1.0). The middle three bars show independent experiments comparing the normalized integration rates of *kita*^{null/+} and WT. The last bar shows the mean and s.d. (0.54±0.16) of the middle three bars. This relative normalized rate of 0.54 is identical to the residual melanocyte deficit in *kita*^{null/+} embryos. **P*<0.01.

The MSC establishment deficit is the same as that seen for the *kit*^{null/+} melanocyte regeneration defect, suggesting that it explains the entire regeneration defect. Therefore, we conclude that *kit* functions in larval melanocyte regeneration to establish the MSC, but that *kita*^{null/+} does not affect recruitment or proliferation, differentiation, or survival of the daughters of the MSC.

The role of Kit signaling in MSC fate determination

Having shown that *kit* is required for MSC establishment, we next investigated what mechanism was perturbed. We have previously shown that all lineages that contain an MSC probably come from a neural crest precursor that will also produce an ontogenetic melanocyte lineage (Fig. 5A) (Tryon et al., 2011). Based on this model, we could imagine three possible ways to disrupt MSC establishment: (1) death of the neural crest precursor (Fig. 5B), (2) death of the MSC (Fig. 5C), or (3) differentiation of the cell normally fated to become the MSC (Fig. 5D). Each of these models makes a different prediction of the number of ontogenetic melanocyte lineages and ontogenetic melanocyte clone size (Fig. 5B–D) (explained in more detail below).

The MSC lineage and the directly developing ontogenetic lineage segregate at approximately the same time as the occurrence of transposon integration. This means that integration events can happen before fate segregation, generating both lineages (bipotential labelings), or after fate segregation, labeling only a single lineage. We had shown previously that ~60% of clones were bipotential, and the remainder generated only ontogenetic melanocytes or regeneration melanocytes (Tryon et al., 2011). Under Model 1 (Fig. 5B), in which some neural crest progenitors are killed in the mutants, we would expect to find similar ratios of clone classes in WT and mutant embryos. By contrast, Models 2 (Fig. 5C) and 3 (Fig. 5D) predict fewer bipotential and regeneration-only clones. As the bipotential clones appeared in far greater numbers than regeneration-only clones, we limited our analysis to the bipotential clones. We followed the established methods of Tryon et al. (Tryon et al., 2011) to test whether there were differences in the ratios of clone classes produced by *kita*^{null/+}. We found a significantly reduced rate of larvae containing bipotential clones in the mutant (WT, 19/44; *kita*^{null/+}, 12/71; *P*<0.005; Fig. 5E). This result rules out Model 1, but is consistent with Models 2 and 3.

To distinguish between the remaining models, we again turned to clonal analysis. As the *kita*^{null/+} mutant has nearly the same number of ontogenetic melanocytes as WT (266±20 melanocytes for WT; 249±22 melanocytes for *kita*^{null/+}; *P*>0.1; Fig. 5F), we reasoned that a change in the number of ontogenetic lineages should be reflected by a change in the number of ontogenetic melanocytes per lineage (clone size). Under Model 2, the death of the MSC should leave the ontogenetic clone size unaffected, as the number of ontogenetic lineages is unchanged (Fig. 5C). Under Model 3, direct differentiation of the presumptive MSC precursor into melanocyte(s) should lead to a decrease in mean ontogenetic clone size as the number of ontogenetic lineages has increased (Fig. 5D), with no corresponding change in the total number of differentiated melanocytes (Fig. 5F).

We injected *fTyrr1*>GFP at the one- to two-cell stage and screened for ontogenetic clones at 3 dpf. The clone size for WT embryos, 3.7±0.3 melanocytes, is significantly larger than the clone size in *kita*^{null/+} embryos, 3.0±0.2 melanocytes (mean±s.e.m., *P*<0.05; Fig. 5G). This result is inconsistent with Model 2, but consistent with Model 3. Therefore, we suggest that cells normally fated as MSCs instead differentiate into ontogenetic melanocyte lineages when Kit signaling is reduced.

DISCUSSION

In this study, we took advantage of the dosage sensitivity of melanocyte regeneration for Kit function to explore the role of Kit signaling in MSC regulation. We developed clonal analysis methods that allowed us to show that the melanocyte regeneration deficit observed in *kita*^{null/+} is a defect in establishing the MSC, rather than recruiting the MSC to generate new melanocytes, or in the proliferation, differentiation or survival of the MSC daughters. Clonal analysis also suggests that in the absence of sufficient Kit signaling, cells destined to become MSCs instead directly differentiate as melanocytes. The clonal analysis methods developed in this study could be used to study other defects in larval melanocyte regeneration, or regulation of other adult stem cells.

The finding that Kit signaling is important for MSC establishment was unexpected. Although *kit* has been shown to be important for stem cell establishment in other systems (Manova and Bachvarova, 1991), we had previously shown by reciprocal shifts with the *kita*^{ts} mutation that Kit signaling was not involved in the establishment of a different MSC population used in adult fin regeneration, but is involved in the recruitment and/or proliferation, differentiation or

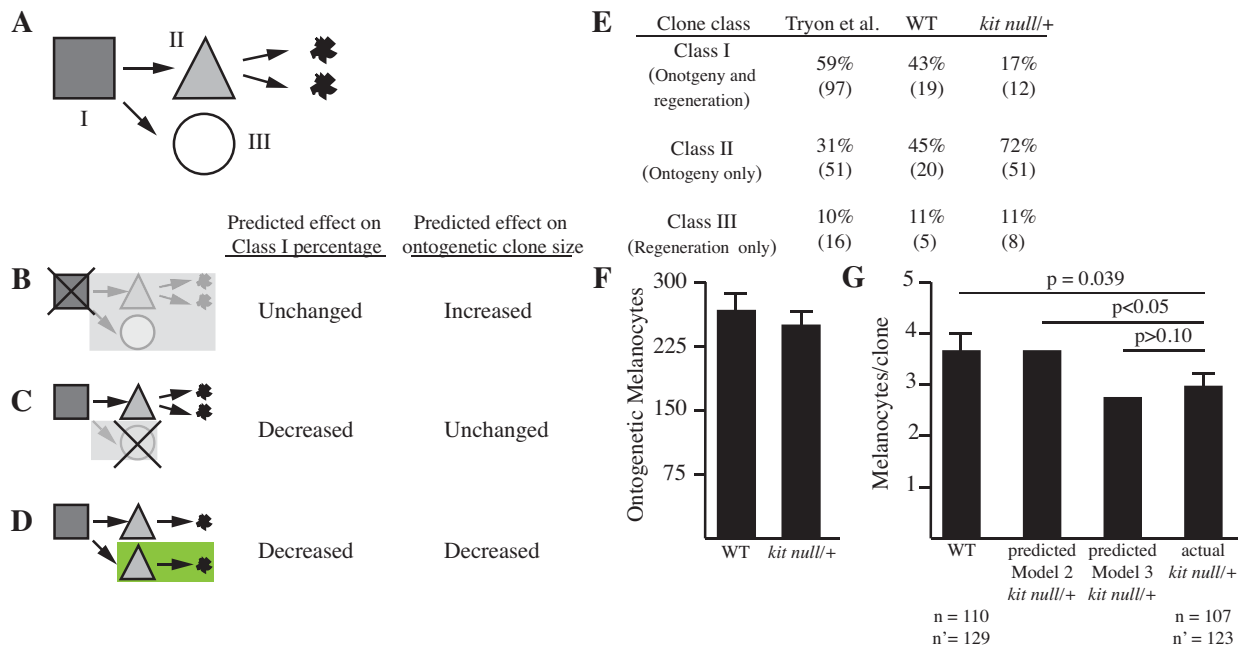


Fig. 5. Reduction of Kit signaling leads to an increase in ontogenetic melanocyte lineages. (A) A neural crest progenitor cell (square) produces an MSC (circle) and a melanoblast (triangle) followed by melanocyte differentiation. Roman numerals indicate clone classes caused by varying times of transposon integration. (B-D) Possible models explaining the effect of *kit* deficit on MSC establishment. Models include the predicted effect of *kit* deficit for each model on the probability of a Class I integration and ontogenetic melanocyte clone size. Cell death in B and C is represented by a cross and faint pathways indicate consequences of cell death. Green shaded box in D indicates inappropriate differentiation of MSC. (E) Effect of *kit*^{null/+} on generation of different clone classes. Shown is data from Tryon et al. (Tryon et al., 2011) (first column), WT data from this study (second column) and *kit*^{null/+} data from this study (third column). WT data from this study is not significantly different from that generated by Tryon et al. ($\chi^2=4.9$, d.f.=2, $P>0.05$). Clone class distribution in *kit*^{null/+} is significantly different ($\chi^2=59$, d.f.=2, $P<0.001$). The difference is due to a reduction in ontogeny and regeneration clones co-occurring (Class I) (Z-score=3.1, $P<0.005$). (F) The number of ontogenetic melanocytes is not significantly different between WT and *kit*^{null/+}. (G) The mean number of ontogenetic clones is smaller for *kit*^{null/+} than WT, indicating an increase in the number of lineages of ontogenetic melanocytes. *n*, the number of embryos containing clones; *n'*, the number of clones after adjustment for polyclonal events. Error bars are s.e.m.

survival of the daughter cells (Rawls and Johnson, 2001). Differential roles for Kit in different melanocyte lineages are summarized in Table 1. We did not find any evidence in our clonal analyses that suggested a role for *kit* in larval MSC recruitment or daughter cell proliferation, survival or differentiation. Thus, Kit performs different functions in each of the different melanocyte lineages that require *kit*. This emphasizes the folly of pigeonholing the function of a gene from only one of its requirements in the organism. However, we note that our analysis here is limited to the dosage sensitive or haploinsufficient roles for *kit* in melanocyte regeneration. Although these assays clearly show the role of *kit* in establishing the MSC, we cannot use them to exclude an effect of complete loss of Kit signaling on MSC recruitment or daughter cell proliferation, differentiation or survival.

Some ambiguity is possible in our analyses of the contributions of the different processes involved in regeneration. For example, if the daughter cells of the MSC died before they had a chance to divide, by our assay this would appear as a defect in recruitment, rather than in proliferation, differentiation or survival. In this study, as *kit*^{null/+} shows no defect in either MSC recruitment or proliferation, differentiation or survival of the daughter cells, we can confidently conclude that neither process is affected.

Although the adult stem cell in some systems, such as the intestinal stem cell (Barker et al., 2007), is constitutively active, the MSC is usually quiescent (Nishimura et al., 2002; Hultman and Johnson, 2010). Although quiescent stem cells can be put through multiple injurious events to cause multiple rounds of regeneration,

Table 1. Differential roles of Kit in different melanocyte lineages

Role	Embryonic and larval				Adult fin melanocytes	
	Direct-developing melanocytes		Regeneration melanocytes		<i>kit/kit</i>	<i>kit/+</i>
	<i>kit/kit</i>	<i>kit/+</i>	<i>kit/kit</i>	<i>kit/+</i>		
MSC establishment	N.A.	N.A.	Yes*	Yes*	No ^{§,¶}	No
Melanocyte differentiation	No*	No [‡]	N.D.	No*	Yes ^{§,¶}	No
Melanocyte migration	Yes [‡]	No [‡]	N.D.	No*	N.A.	N.A.
Melanocyte survival	Yes [‡]	No [‡]	N.D.	No*	No [§]	No

*This study.
[‡]Rawls and Johnson, 2003.
[§]Rawls and Johnson, 2001.
[¶]Tu and Johnson, 2010.
 N.A., not applicable, N.D., not determined.

observing the individual rounds often requires the sacrifice of the animal or other laborious means to score. This has left open the question of how many stem cells contribute to a single regenerative event. A thorough understanding of this question may be important to tune endogenous adult stem cells that are artificially activated. We find that most (~83%) but not all MSCs respond during any given regenerative event. As far as we are aware, this is the first quantitative analysis of quiescent stem cell activation.

It remains unclear what role Kit plays to avoid the inappropriate differentiation of cells normally fated to become MSCs take place. Signaling from Kit could be required as early as the segregation of the MSC lineage from the directly developing ontogenetic lineage (~6 hours post fertilization). However, we first observe *kit* expression in neural crest cells between 14 and 18 hours (Parichy et al., 1999), suggesting a role for Kit in migration of the presumptive MSC from the neural crest, or in taking up its position in a yet to be identified niche. Our data now sets the stage for addressing this question.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.088112/-DC1>

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