Differential of Zebrafish Melanophores Depends on Transcription Factors AP2 Alpha and AP2 Epsilon

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Abstract

A model of the gene-regulatory-network (GRN), governing growth, survival, and differentiation of melanocytes, has emerged from studies of mouse coat color mutants and melanoma cell lines. In this model, Transcription Factor Activator Protein 2 alpha (TFAP2A) contributes to melanocyte development by activating expression of the gene encoding the receptor tyrosine kinase Kit. Next, ligand-bound Kit stimulates a pathway activating transcription factor Microphthalmia (Mitf), which promotes differentiation and survival of melanocytes by activating expression of Tyrosinase family members, Bcl2, and other genes. The model predicts that in both Tfaxa and Kit null mutants there will be a phenotype of reduced melanocytes and that, because Tfaxa acts upstream of Kit, this phenotype will be more severe, or at least as severe as, in Tfaxa null mutants in comparison to Kit null mutants. Unexpectedly, this is not the case in zebrafish or mouse. Because many Tfaxa family members have identical DNA-binding specificity, we reasoned that another Tfaxa family member may work redundantly with Tfaxa in promoting Kit expression. We report that Tfaxae is expressed in melanoblasts and melanophores in zebrafish embryos and that its orthologue, Tfaxe, is expressed in human melanocytes. We provide evidence that Tfaxe functions redundantly with Tfaxa to maintain kita expression in zebrafish embryonic melanophores. Further, we show that, in contrast to kita mutants where embryonic melanophores appear to differentiate normally, in tfaxae-deficient embryos partially restores melanophore differentiation. These findings reveal that Tfaxe activity, mediated redundantly by Tfaxa and Tfaxe, promotes melanophore differentiation in parallel with Mitf by an effector other than Kit. This work illustrates how analysis of single-gene mutants may fail to identify steps in a GRN that are affected by the redundant activity of related proteins.

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Introduction

An important participant in the gene-regulatory-network (GRN) that governs the differentiation of melanocytes from neural crest precursors (i.e., the melanocyte GRN) is the class III receptor tyrosine kinase Kit. In mouse embryos, binding of this growth-factor receptor by its ligand, stem cell factor (SCF), promotes the growth, survival, migration, and possibly terminal differentiation of melanoblasts [1]. Mouse embryos homozygous for hypomorphic alleles of Kit completely lack melanocytes (embryos homozygous for Kit null alleles die prior to pigmentation) [2–6]. While ligand-bound Kit stimulates many signal transduction pathways, its effects on melanocyte growth and differentiation appear to occur via the Ras/Raf/Map Kinase pathway. Activity of this pathway results in phosphorylation of Microphthalmia transcription factor (Mitf); phosphorylation of Mitf regulates its activity and stability [7,8]. Within melanoblasts, Mitf promotes a) cell-cycle exit, by activating expression of the p21(t2) gene, a cyclin-dependent kinase inhibitor [9], b) cell survival, by upregulating the expression of BCL2 [10], and c) melanin synthesis, by activating expression of Tyrosinase (Tyr), Tyrosinase-related protein 1 (Tyrp1), and Tyrosinase-related protein 2 (Tyrp2), also known as Dopachrome tautomerase, Dct [11–14]. Thus, Kit signaling is essential for normal melanocyte development, at least in part via its ability to stimulate Mitf activity. Of note, Kit levels are reported to be lower in metastatic melanoma cell lines than in benign nevi, and forced expression of Kit in these cells has been shown to induce apoptosis [15]. These findings highlight the importance of understanding the regulation of Kit expression within the melanocyte lineage.

While there is evidence that the Kit gene is dependent on direct stimulation by the Transcription Factor Activator Protein 2 alpha (TFAP2A) in melanoma, analyses of mutant model organisms indicate a more complex regulatory scenario within embryonic melanocytes. TFAP2A and other members of the TFAP2 family control cell fate specification, cell differentiation, cell survival and cell proliferation within neural crest, skin, breast epithelium, and other embryonic cell types and stem cells [16,17]. Gel shift experiments showed that TFAP2A can bind an element 1.2 kb...
neural crest-derived pigment cells, known as melanocytes, are important to an organism’s survival because they protect skin cells from ultraviolet radiation, camouflage the organism from predators, and contribute to sexual selection. Networks of regulatory proteins control the steps of melanocyte development, including lineage specification, migration, survival, and differentiation. Gaps in our understanding of these networks hamper progress in effective prevention and treatment of diseases of melanocytes, including metastatic melanoma and vitiligo. Studies conducted in tissue-culture cells and mouse embryos implicate regulatory proteins including the transcription factor TFAP2A, the growth factor receptor KIT, and the transcription factor MITF as being important for multiple steps in melanocyte development. Abnormalities in TFAP2A, KIT, and MITF expression in melanoma highlight the importance of this pathway in human disease. Here we show that a gene closely related to TFAP2A, tfap2e, is expressed in zebrafish embryonic melanocytes and human melanocytes. We provide evidence that Tafap2e cooperates with Tap2a to promote expression of zebrafish kita in embryonic melanocytes. Further we show that an effector of Tafap2a/e activity other than Kita is required for melanocyte differentiation and that this effector acts upstream or in parallel with Mitfa activity. These findings reveal unexpected complexity in the gene-regulatory network governing melanocyte differentiation.

**Author Summary**

Neural crest-derived pigment cells, known as melanocytes, are important to an organism’s survival because they protect skin cells from ultraviolet radiation, camouflage the organism from predators, and contribute to sexual selection. Networks of regulatory proteins control the steps of melanocyte development, including lineage specification, migration, survival, and differentiation. Gaps in our understanding of these networks hamper progress in effective prevention and treatment of diseases of melanocytes, including metastatic melanoma and vitiligo. Studies conducted in tissue-culture cells and mouse embryos implicate regulatory proteins including the transcription factor TFAP2A, the growth factor receptor KIT, and the transcription factor MITF as being important for multiple steps in melanocyte development. Abnormalities in TFAP2A, KIT, and MITF expression in melanoma highlight the importance of this pathway in human disease. Here we show that a gene closely related to TFAP2A, tfap2e, is expressed in zebrafish embryonic melanocytes and human melanocytes. We provide evidence that Tafap2e cooperates with Tap2a to promote expression of zebrafish kita in embryonic melanocytes. Further we show that an effector of Tafap2a/e activity other than Kita is required for melanocyte differentiation and that this effector acts upstream or in parallel with Mitfa activity. These findings reveal unexpected complexity in the gene-regulatory network governing melanocyte differentiation.

upstream of the KIT transcription start site, and expression driven by this enhancer in melanoma cells is lost when the TFAP2A binding sites are deleted [18]. Moreover, forced expression of the TFAP2A DNA binding domain, which presumably unseats endogenous TFAP2A and thus acts as a dominant negative AP2, prevents expression of KIT in these cells [18]. Mice lacking the Tafap2a gene do not live long enough to develop melanocytes, due to failure of body wall closure [19,20]. However, in embryos with Wnt1-CRE-mediated deletion of Tafap2a specifically within the neural crest, melanocytes are absent from the belly [21]. Interestingly, this phenotype resembles that of heterozygous, not homozygous, Kita loss-of-function mutants, suggesting that loss of Tafap2a leads to a reduction rather than complete loss of Kita expression. Zebrafish have two orthologues of mammalian Kita, known as kita and kith; only kita is expressed in the melanophore lineage [22]. In kita homozygous null mutants (i.e., kita mutants) relative to their wild-type counterparts, embryonic melanophores are reduced in number by about 40%, migrate less, and eventually undergo apoptosis [23]. In zebrafish ifap2a homozygous null mutants (i.e., ifap2a mutants), kita expression is reduced and embryonic melanophores exhibit reduced migration [24,25]. However, in contrast to the melanophores in kita mutants, those in ifap2a mutants do not appear to die, at least as long these animals survive [23,26]. The simplest explanation for this difference is that kita expression in melanophores is initially dependent on ifap2a but later becomes independent of it. How can the dominant negative AP2 block kita expression while loss of Tafap2a only diminishes or delays it? Because many Tafap2 family members have the same DNA binding affinity, it is possible that another such family member cooperates with Tafap2a to activate kita expression.

Here we show that Tafap2c, a homolog of Tafap2a with the equivalent DNA binding specificity, is expressed in zebrafish melanoblasts and in cultures of primary human melanocytes. With single and double knockdown studies, we show that while Tafap2c is not required for the development of embryonic melanophores, it functions redundantly with Tafap2a in maintaining kita expression in embryonic melanophores. Interestingly, in contrast to the situation in kita mutants, the melanophores in embryos doubly deficient for ifap2a/e fail to differentiate. These results imply that Tafap2 activity has targets other than kita that are important for melanophore development. We find that forced expression of mitfa partially restores melanophores in embryos lacking ifap2a and ifap2c, implying that the targets of Tafap2a/e function to stimulate Mitfa activity or act in parallel with it. These findings reveal unexpected roles for Tafap2 activity in the melanocyte GRN.

**Results**

tfap2e is expressed in zebrafish melanoblasts and cultured human melanocytes

To determine if a second Tafap2 family member is expressed in the melanoblast lineage, we identified orthologues of Tafap2h, Tafap2e, Tafap2d, and Tafap2e in a database of expressed sequence tags (www.ensembl.org), amplified partial clones of at least 1 kb from each to make a probe for in situ hybridization, and examined the expression of each in embryos that ranged in stage from 0.5 hours post-fertilization (hpf), revealing maternal expression, to 48 hpf. Expression patterns of ifap2b and ifap2c have previously been reported [27,28]. We did not detect expression of ifap2b, ifap2c, or ifap2d in melanoblasts or melanophores (Figure S1), so we did not pursue these orthologues in the context of melanophore development.

In 8-cell zebrafish embryos, maternal ifap2e transcripts were detected by both in situ hybridization and semi-quantitative RT-PCR (not shown). At 24 hpf, ifap2e expression was detected in several regions of the brain, including presumed olfactory bulb, as in mouse embryos [29,30] (Figure 1A), and also within dispersed cells in the trunk that we assumed to be a subset of migrating neural crest cells (Figure 1B and 1D). At this stage, ifap2e expression was detectable in early-differentiating melanophores close to the ear (Figure 1C), suggesting that the dispersed, non-melanized cells expressing ifap2e were melanoblasts. To test this possibility, we probed homozygous mitfa null mutant embryos (i.e., mitfa0111S), which are devoid of melanoblasts [31], and found that ifap2e expression was absent from the dispersed cells in the trunk (Figure 1E–1G). This result was consistent with expression of ifap2e in melanoblasts. However, because mitfa is co-expressed with xdh and fms, two markers of xanthophore precursors [32], it was conceivable that ifap2e was expressed in the xanthophore lineage, in an Mitfa-dependent fashion. To test whether ifap2e is expressed in xanthophores, we processed embryos to simultaneously reveal expression of ifap2e mRNA and Pax7 protein, a marker of the xanthophore lineage [33]. We did not detect overlap of the two signals, which implies that ifap2e is not expressed in xanthophores (Figure 1H). In wild-type embryos at 36 hpf, ifap2e expression was present in the forebrain and presumed optic tectum, and expanded in the hindbrain relative to earlier stages (Figure 1I and 1J). However, at this stage expression was not detected in melanophores (Figure 1K). At 48 hpf, high-level ifap2e expression was also observed in the retina (Figure 1L).

To assess if melanocyte-specific expression of TFAP2E is conserved in humans, we performed quantitative RT-PCR on cDNA generated from various human cell lines. We detected higher levels of TFAP2E message in three independent isolates of primary melanocytes, consistent with microarray data indicating expression of TFAP2E in melanocytes and melanoma cell lines [34]. Expression in melanocytes was 2–10 fold higher
than in a keratinocyte cell line, and approximately 50–100 fold higher than in a lymphocyte cell line (Figure 1M). In summary, \textit{tfap2e} is expressed in zebrafish melanoblasts and in human melanocytes.

In \textit{tfap2a} mutant embryos, \textit{kita} expression is reduced in early melanophores but normal at later stages.

As discussed in the Introduction, KIT\textsubscript{L} has been reported to be a direct target of TFAP2A, and a dominant negative AP2 variant 

Figure 1. Characterization of \textit{tfap2e} expression during embryogenesis. Wild-type zebrafish embryos, unless otherwise indicated, fixed at the stage indicated and processed to reveal \textit{tfap2e} expression by RNA \textit{in situ} hybridization. All embryos in this and subsequent figures are oriented with anterior to the left. (A) Dorsal view of the head showing \textit{tfap2e} expression in presumed olfactory placode (arrowheads), medial telencephalon (asterisk), and hindbrain (arrows). (B) Lateral view of the trunk, showing \textit{tfap2e} expression in cells migrating from the dorsal neural tube. (C) Lateral view just caudal to the ear, \textit{tfap2e} expression is seen in newly-pigmented melanophores (arrows). (D) Higher-magnification view of the \textit{tfap2e}-expressing cells of the trunk that are shown in panel B. (E-G) \textit{tfap2e} expression in \textit{mitf}\textsuperscript{−/−} homozygous mutant embryos, in (E) dorsal and (F,G) lateral views. E) Expression of \textit{tfap2e} in the head is virtually normal, (F, G) while its expression in the trunk is virtually absent. (H) Lateral view of a wild-type embryo processed to reveal \textit{tfap2e} mRNA and Pax7 protein, a marker of xanthophores. \textit{tfap2e} expression does not overlap with \textsuperscript{α}-Pax7 immunoreactivity (arrowheads). (I,J) Dorsal head views. I) At 36 hpf, \textit{tfap2e} expression is visible in the olfactory placode (arrowheads in I), in bilateral clusters in the telencephalon (asterisk); J) in the optic tectum (arrow), and in rhombomeres (arrowheads in J). (K, L) Dorsal views of the head. K) At 36 hpf expression of \textit{tfap2e} in melanophores is no longer detectable. L) At 48 hpf \textit{tfap2e} expression is detected in the retina (asterisks). (M) Quantitative RT-PCR shows that expression of TFAP2E in 3 independent primary human melanocytes (Mel 1–3) is about 2–10 fold higher than in keratinocytes (Ker), while its expression in Jurkat cells (lymphocytes, Lym) is about 10 fold lower than in keratinocytes. Scale bars: (A, B, E, F, H, I, K), 100 \textmu m; (C, D, G, J), 50 \textmu m. 

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was found to block KIT expression in cultured cells [18]; however the status of kita expression in tfap2a mutants has not been fully investigated. In zebrafish tfap2a mutants or tfap2e MO-injected embryos at 28 hpf, kita expression in the melanophore lineage is reduced to undetectable levels as assessed by in situ hybridization [24,25]. However because melanophores undergo cell death in kita mutants but do not do so in tfap2a mutants, it has been proposed that kita is expressed in the melanophore lineage of tfap2a mutants at a later stage [24]. To test this prediction, we crossed heterozygote tfap2a null mutants (i.e., lockjaw, tfap2a*e2i2) and identified homozygous mutant offspring (hereafter, tfap2a mutants) at 28 hpf by virtue of their pigmentation phenotype. We fixed a fraction of these embryos at 28 hpf, and incubated the remainder in water containing phenylthiourea (PTU) to prevent melanin synthesis, until 36 hpf. We then processed all embryos to reveal kita expression. In tfap2a mutants at 28 hpf, kita expression in melanophores was undetectable by in situ hybridization (Figure 2C), as previously reported. However, at 36 hpf, kita expression was clearly visible in cells present in the dorum of these embryos (Figure 2E). Thus normal kita expression in melanoblasts at 28 hpf is dependent on tfap2a, but later becomes independent of it. To explain these observations we hypothesized that Tfap2e compensates for the loss of Tfap2a and activates kita expression by 36 hpf.

To test whether Tfap2e maintains kita expression in tfap2a mutants, we first assessed tfap2e expression in tfap2a mutants, and found that it was expressed on schedule in migrating neural crest, as in wild-type embryos (Figure S2). Next we injected embryos with a morpholinio (MO) targeting the tfap2e exon 3 splice donor site (i.e., tfap2e e3i3 MO) (Figure 2A). To confirm the efficacy of this MO towards its intended target, we harvested RNA from embryos injected with the tfap2e e3i3 MO, generated first-strand cDNA, and performed PCR using primers in exon 1 and exon 4. Sequencing of the major aberrant splice product revealed that the e3i3 MO causes deletion of exon 3 in its entirety, resulting in a frame shift and a severe truncation of the predicted protein that eliminates the DNA binding domain (Figure 2A). By semi-quantitative PCR, this MO appears to inhibit normal splicing of the majority of tfap2e transcripts at 36 hpf, but to act with greatly reduced efficiency at 3 days post fertilization (dpl) (Figure 2A). By 24 hpf, wild-type zebrafish embryos injected with tfap2e e3i3 MO showed evidence of cell death in the central nervous system (CNS), i.e., patches of opacity in the brain and spinal cord, but no other gross morphological defects; possibly this was due to non-specific toxicity of the MO to the embryo. Despite this cell death, the melanophores that developed in such embryos looked normal and were normally distributed (see below and Figure S3). tfap2e e3i3 MO-induced CNS cell death was reduced by co-injection of p53 MO, implying that Tfap2e has a role in cell survival in the CNS, or that the tfap2e e3i3 MO has non-specific toxicity towards the nervous system, which is true of many MOs (Figure S3) [35]. To preserve the morphology of embryos, in all experiments discussed hereafter we have included p53 MO with tfap2e e3i3 MO. Interestingly, in tfap2a mutants injected with the tfap2e e3i3 MO (hereafter, tfap2a/e doubly-deficient embryos), kita was absent from the dorum at 36 hpf, although kita expression was readily detected in the cloaca and pharyngeal pouches (Figure 2G and not shown). These findings imply that in absence of Tfap2a, Tfap2e promotes kita expression in the melanophore lineage.

**Simultaneous reduction of Tfap2a and Tfap2e inhibits melanophore development**

Because of the sustained loss of kita expression in tfap2a/e doubly-deficient embryos, we expected that the phenotype in these embryos would be similar to that of kita homozygous null mutants, although perhaps not as severe because MO-mediated inhibition of gene expression is transient and partial; instead, however, we detected a much more severe phenotype. At 36 hpf, compared to the embryonic melanophores in their non-mutant siblings (Figure 3A), those in kita null mutants (i.e., kita−/−) (Figure 3B) appeared normally melanized, but were reduced to about 60% of their normal numbers (because of a presumed defect in cell division) and did not migrate as extensively as their wild-type counterparts [23,36]. In control MO-injected tfap2a mutants (Figure 3C), embryonic melanophores exhibited these same phenotypes. In tfap2e MO-injected sibling embryos (Figure 3D) there was no apparent melanophore phenotype. However, in tfap2a/e doubly-deficient embryos there were far fewer melanophores than present in control MO-injected tfap2a mutant embryos. Compared with control MO-injected tfap2a mutants, tfap2a/e doubly-deficient embryos had fewer pigmented melanophores in the dorum and almost no visible melanophores on the lateral sides of the trunk or on the yolk sac (Figure 3E); this difference was still apparent at 84 hpf (not shown). In summary, whereas wild-type embryos injected with the tfap2e MO developed normally until at least 4 dpf, tfap2a/e doubly-deficient embryos displayed melanophore defects more severe than those of tfap2a or kita mutants. These findings suggest that Tfap2a and Tfap2e have partially redundant function in zebrafish melanophore development, and that this function exceeds the simple maintenance of kita expression.

To confirm the specificity of the tfap2e e3i3 MO-induced melanophore phenotypes, we co-injected mRNA encoding a glucocorticoid-fused version of Tfpaa2a (tfap2aGR), whose nuclear transport is dexamethasone-inducible, or lacZ as a control, into embryos injected with MOs— one targeting the exon 2 splice donor site (i.e., e2i2 MO) and the other the translation start site of the tfap2e gene (i.e., AUG MO) (Figure 2A). Injection of either the tfap2e e2i2 MO or the tfap2e AUG MO into wild-type embryos had no effect on melanophore development, although both induced some degree of nervous-system cell death. Upon injection of either the tfap2e e2i2 MO or tfap2e AUG MO into doubly-deficient embryos (i.e., tfap2a/e), melanophore phenotypes although it reduced nervous system cell death (not shown). These multiple tests of specificity strongly argue that the melanophore phenotypes we observe in tfap2e MO-injected embryos result from inhibition of tfap2e expression and not from off target effects.

**Inhibition of tfap2e does not further reduce melanophore specification in tfap2a mutants**

The reduced number of melanophores in tfap2a/e doubly-deficient embryos relative to tfap2e mutants could reflect a role for Tfap2a/e activity in the specification of melanoblasts or, alternatively, in either survival or differentiation of melanophores. To distinguish among these possibilities, we examined the expression of mitfa, an early marker of the melanoblast and xanthoblast lineages
At 29 hpf, mitfa-expressing cells are visible in the head and trunk of wild-type embryos injected with a control MO (Figure 4A). The number of mitfa-expressing cells is reduced by about half in tfap2a mutant embryos injected with a control MO (Figure 4B); this reduction results at least in part from the absence of kita in such mutants at this stage, because melanophores are reduced by this amount in kita mutants [23], as are mitfa-expressing cells (our unpublished observations). In tfap2e MO-injected, wild-type embryos, the number of mitfa-expressing cells is not grossly different from that in control MO-injected, wild-type embryos (Figure 4C). Interestingly, in tfap2a/e doubly-deficient embryos, the number of mitfa-expressing cells did not appear to be further decreased relative to that in control MO-injected tfap2a mutants (Figure 4D). To confirm these impressions, we counted mitfa-expressing cells over the hind yolk (see Materials and Methods) at 24 hpf, and compared the results for tfap2a mutants injected with control MO versus those injected with...
Tfap2 Promotes Melanophore Differentiation

Tfap2a/e activity is required for melanophore differentiation

To determine which step in melanophore development depends on Tfap2 activity, we analyzed the expression of genes involved in melanophore differentiation: tyr, tyrp1b and dct [12]. In tfap2a mutant embryos at 29 hpf, the number of cells expressing each of these melanophore markers was reduced by about half relative to that in siblings, consistent with the previously described decrease in melanophores in tfap2a mutants (Figure 5A, 5E, 5I and 5C, 5G, 5K) [24,25]. In tfap2e MO-injected embryos, the number of cells expressing each of these genes appeared to be normal (Figure 5B, 5F, and 5J), while in tfap2a/e doubly-deficient embryos their numbers were further reduced relative to that in tfap2a mutant embryos (Figure 5D, 5H, and 5L). To quantify this effect, we counted cells in embryos processed for in situ hybridization. We

discovered that the reduction in gene expression was not equal in all cases. The number of cells expressing dct was most clearly and most consistently reduced in tfap2a/e doubly-deficient embryos, i.e., by approximately 47% relative to the number in tfap2a mutant embryos (Figure 5A-5D, and 5M). The reduction in tyrp1b and tyr expressing cells was more variable, with an average reduction of approximately 30% and 23%, respectively (Figure 5E-5L, and 5M).

The results described above indicate that when the expression of tfap2a and tfap2e is reduced, melanoblasts express mitfa but fail to progress to a stage at which they express normal levels of melanophore differentiation genes, such as dct, tyrp1b, and tyr. To test this model more quantitatively, we injected mitfa:egfp transgenic embryos [37] with either tfap2a MO or both tfap2a MO and tfap2e MO, dissociated them at 29 hpf, sorted and collected GFP-expressing cells, and measured the levels of various transcripts by quantitative RT-PCR (Figure 5N). Using this method, we saw a trend similar to that observed in the histology analysis: in GFP-positive cells sorted from tfap2a MO-injected embryos relative to those sorted from tfap2a MO-injected embryos, dct expression was reduced by approximately 45%, tyrp1b expression was reduced by 17%, and unexpectedly, tyr expression was not reduced. Taken together with the cell counts, these results reveal that Tfap2 activity, redundantly provided by Tfap2a and Tfap2e, promotes the differentiation of embryonic melanophores.

Loss of Tfap2a/e activity does not result in a cell-fate switch or early cell death

We tested the possibility that the loss of differentiated melanophores in tfap2a/e doubly-deficient embryos results from
a fate switch of melanophores to xanthophores, because mitf is co-expressed with c-fms, a marker of xanthophore precursors [32]. We injected embryos with a control MO, tfap2a MO, tfap2e MO, or tfap2a/e MOs, and at 36 hpf processed them to reveal expression of anti Pax7 IR, a marker of xanthophores [33] (Figure 6A-6C and not shown). While the numbers of xanthophores in these groups did not differ significantly (Figure 6D), melanophore differentiation was clearly affected in tfap2a/e doubly-deficient embryos. These findings suggest that loss of Tfap2 activity in the melanophore lineage does not result in a cell fate switch.

We also assessed whether melanophores in tfap2a/e doubly-deficient embryos undergo cell death, i.e., despite the presence of p33 MO. First, we co-injected embryos with MOs targeting tfap2a and tfap2e and with an mRNA encoding Bcl2, an inhibitor of apoptosis [30]. Injection of bcl2 mRNA reduced the number of cells expressing a marker of programmed cell death in control embryos at 23 hpf (Figure 6E and 6F), but had no effect on the melanophore phenotype in tfap2a/e doubly-deficient embryos (Figure 6G and 6H). Secondly, embryos were incubated in acridine orange (AO), which is taken up by dying cells, from 16 hpf to 30 hpf and assessed for the presence of AO-containing cells in the dorsal neural tube and migratory neural crest. Relative to control MO-injected wild-type embryos, control MO-injected tfap2a mutants had an elevated number of such cells, but these numbers were not detectably increased in tfap2e MO-injected tfap2a mutants (data not shown). These findings suggest that loss of Tfap2 activity in melanophores does not result in either a switch in cell fate specification or promotion of cell death, but more likely in inhibition of normal melanophore differentiation.

Tfap2a/e activity is cell-autonomously required for melanophore differentiation

In tfap2a mutants and MO-injected embryos, embryonic melanophores initially appear somewhat under-melanized [24,25]. The tfap2a gene is expressed both in skin and neural crest, and we have reported evidence based on transplant studies that Tfap2a has both cell-autonomous and cell non-autonomous effects on melanophore differentiation [25]. Because tfap2a is expressed in melanoblasts but not skin, we assumed that the even poorer differentiation of melanophores in tfap2a/e doubly-deficient embryos is primarily a consequence of a cell autonomous role for Tfap2 activity. To confirm this prediction, we created genetic chimeras by carrying out transplantations at the blastula stage. Specifically, we transplanted cells from 4 hpf wild-type donors,
which had been injected with a biotin-dextran as a lineage tracer, into 4 hpf hosts injected with tfap2a/e MO. We then reared the transplanted hosts to 48 hpf, and processed them for biotin staining to reveal the donor-derived cells. Melanophores lacking lineage tracer were indistinguishable from those seen in the untransplanted tfap2a/e MO-injected controls (Figure 7C-7F, arrows), whereas those positive for the lineage tracer were clearly darker, similar to wild-type controls (Figure 7A and 7B), indicating an increase in the level of melanin. In addition, they displayed a more normal morphology (Figure 7E and 7F, arrowheads). These findings indicate that normal melanophores can develop from wild-type cells that are flanked by tfap2a/e-deficient epidermis. This supports a cell-autonomous requirement for Tfap2a/e activity in melanophore differentiation.

Forced mitfa expression partially restores melanophores in tfap2a/e doubly-deficient embryos

Several signals are known to modulate Mitf transactivation activity [39,40]. If Tfap2a/e is required for the expression of a component of such a signaling pathway, Mitf activity might be reduced in tfap2a/e doubly-deficient embryos despite levels of mitfa mRNA being similar to those in tfap2a mutants. Alternatively, the Tfap2a/e effector required for melanocyte differentiation might be...
Figure 6. Contribution of cell fate specification and cell death to melanophore defects in tfap2a/e doubly-deficient embryos. (A–C) Lateral views of 36 hpf embryos stained with α-Pax7 to mark xanthophores. A similar number of α-Pax-7 IR positive cells is apparent in wild-type embryos injected with (A) a control MO, (B) the tfap2a MO, and (C) the tfap2a MO/tfap2e MO. (D) Average values for the number of α-Pax-7 IR positive cells counted above the hind yolk, n = 10 embryos per group. (E, F) Lateral views of 25 hpf embryos processed with the TUNEL reaction. (E) In an embryo injected with tfap2a/e MO alone there are many more TUNEL-positive cells than in (F) an embryo co-injected with an mRNA encoding a bcl2-gfp mRNA. (This effect was quantified in a parallel experiment, in which bcl2GFP mRNA was co-injected with control MO, embryos fixed at 24 hpf, and the number of TUNEL-positive cells counted: control MO, 97.7 ± 15.5; control MO + bcl2-gfp 54.4 ± 12.3, Avg ± SE, p = 0.03). (G–H) Lateral views of live 32 hpf embryos. (G) In an embryo injected with the tfap2a/e MO alone, or (H) in an embryo co-injected with bcl2-gfp mRNA, melanophores appeared similarly poorly differentiated. Insets in G and H, higher magnification views of melanophores in the respective embryos. Scale bars: (A–C, E–H) 100 μM; (Insets in G–H) 50 μM. doi:10.1371/journal.pgen.1001122.g006
co-activated by Mitf. In either of these scenarios, forced mitfa expression might rescue melanophore differentiation in tfap2a/e doubly-deficient embryos. We injected tfap2a/e doubly-deficient embryos with a plasmid in which the sox10 promoter drives mitfa expression (sox10:mitfa) [41], and found sox10:mitfa-injection increased the number of tfap2a/e doubly-deficient embryos with differentiated melanophores (compare Figure 8B to Figure 8C, 8D). We observed an increase in the number of darkly-pigmented melanophores in tfap2a/e doubly-deficient embryos injected with sox10:mitfa compared to in tfap2a/e doubly-deficient embryos alone (Figure 8E). We also quantified the mean gray value of single melanophores in these embryos (as a measure of pigment density), within a defined region, using ImageJ software. We found that there was a significant reduction in the pigment density of tfap2a/e doubly-deficient embryo melanophores, compared to control MO-injected embryo melanophores, and that this density was restored in doubly-deficient embryos co-injected with sox10:mitfa (Figure 8F).

Since sox10 is expressed throughout the neural crest, we considered the possibility that sox10:mitfa might induce a conversion of neural crest to the melanoblast lineage, and that if this were to occur in neural crest that expressed another Tfap2 family member, normally differentiated melanophores might emerge in tfap2a/e doubly-deficient embryos. However, arguing against this alternative model, we did not detect an increase in the number of melanophores in control-MO injected embryos co-injected with the sox10:mitfa plasmid (Figure 8E). Moreover, in this alternative model, tfap2b is the best candidate Tfap2 family member, as it is expressed in Rohon Beard sensory neurons [27], which are closely related to trunk neural crest [12,43]. However, we found that even in embryos triply depleted of tfap2a/b/e using MOs, co-injection of sox10:mitfa plasmid elevated the number of normal-looking melanophores (our unpublished observation). Together these observations support the model that over-expression of mitfa can compensate for the role in melanophore differentiation.
differentiation normally played by Tfap2a/e, implying that the effector of Tfap2a/e-type activity necessary for melanophore differentiation acts upstream or in parallel with Mitfa.

Discussion

The phenotype of tfap2a/e double-knockdown embryos reflects multiple roles of Tfap2 activity in the melanophore lineage

Here we have presented two new findings relevant to the gene-regulatory-network (GRN) that governs the differentiation of zebrafish embryonic melanophores. First, kita expression in embryonic melanophores is positively regulated by Tfap2e, at least when Tfap2a levels have been reduced. Expression of tfap2a is present throughout the neural crest starting at the neurula stage, while the expression of tfap2e starts at approximately the time of neural crest delamination and appears to be restricted to melanoblasts [24,25]. The relative timing of tfap2a and tfap2e expression explains why kita expression (in melanophores) in tfap2a mutants is reduced at 28 hpf, but present at later stages; Tfap2e compensates for the absence of Tfap2a but only after 28 hpf. The presence of TFAP2E expression in human melanocytes suggests
that TFAP2A and TFAP2E have redundant or partially redundant function in mammalian melanocytes, as in fish melanophores. If so it would explain the observation, mentioned in the Introduction, that the coat color phenotype in mice with neural crest-specific deletion of Tfap2a is less severe than that of Kit homozygous null mutants [21].

The second unexpected finding is that Tfap2 activity (provided by Tfap2a and Tfap2e) promotes the differentiation of embryonic melanophores. This was revealed by reduced expression of the dct and tyrb1 mRNAs, as well as of melanin—changes that are evident in tfap2a mutants and more pronounced in tfap2a/e doubly-deficient embryos. Does Tfap2 activity also directly neural crest cells to join the melanophore sublineage? There is precedent for such a possibility, because Tfap2 activity provided by Tfap2a and Tfap2c appears to direct ectodermal precursors to join the neural crest lineage [28,44]. In tfap2a single mutants, neural crest induction appears to occur normally, but mitfa-expressing cells, which are primarily melanoblasts, are reduced in number. This reduction may reflect a role for Tfap2 in melanophore specification or alternatively a reduction of Kita-mediated proliferation of melanoblasts. Whatever the explanation for reduced melanoblasts in tfap2a mutants, simultaneous reduction of tfap2a and tfap2e leads to a further reduction of melanoblasts to melanophores. While a reduction of melanophores without a reduction in mitfa-expressing cells might have been consistent with a cell fate change of melanophores to xanthophores (because markers of melanoblasts and xanthoblasts are briefly co-expressed [32]), xanthophore numbers are equivalent in tfap2a/e mutants, arguing against such a fate transformation. Does Tfap2 also promote survival of melanophores? We did not detect evidence of cell death of melanophores shortly after their differentiation in tfap2a/e doubly-deficient embryos. We predict that in embryos permanently deprived of both Tfap2a and Tfap2e melanophores would die as a consequence of the absence of Kita. However, because melanophores persist for several days in kita mutants, and this is longer than MOs are effective (see Figure 2A), it will be necessary to isolate a tfap2e mutant to test this prediction. Together these observations reveal that Tfap2 activity has multiple roles in melanophore development, including promoting melanophore differentiation.

Another result that will be important to revisit when a tfap2e mutant is available is the apparent heightened Tfap2-dependence of dct expression relative to tyr expression. Consistent with differential regulation of these related genes, in mice, Dct expression appears prior to Ttr expression, and this has also been suggested to be the case in zebrafish [45,46]. However, because we knock-down tfap2e expression with an MO, the stronger effect on dct expression relative to on ttr expression may simply reflect loss of MO effectiveness over time. There may be a similar explanation for the inconsistent findings regarding ttr expression between the RNA in situ hybridization and the quantitative RT-PCR analyses. The cell dissociation protocol required for quantitative RT-PCR introduces a delay in the analysis of gene expression relative to that obtained using the RNA in situ hybridization protocol, giving further time for the MO to lose efficacy. Nevertheless, these results reveal that Tfap2 activity, redundantly provided by Tfap2a and Tfap2e, promotes the differentiation of embryonic melanophores.

Tfap2 and Mita may co-activate melanophore differentiation genes

How does Tfap2 activity, mediated by Tfap2a and Tfap2e, effect melanophore differentiation? In tfap2a/e doubly-deficient embryos, melanophore differentiation fails but can be rescued by forced expression of mitfa. One model to explain these findings is that Mitfa and Tfap2 normally co-activate genes important for melanophore differentiation, but in the absence of Tfap2, elevated levels of Mitfa can suffice to do so (Figure 9A). Thus, Tfap2 family members may directly activate genes involved in melanin synthesis, such as dct, tyrb1, and possibly tyr, all of which are known to be Mitfa targets [47–49]. Consistent with this possibility, recent studies have identified conserved DNA elements adjacent to the dct and tyrb1 genes that have melanocyte enhancer activity [13], and some of these contain putative Tfap2 binding sites. Simultaneous inhibition of tyrb1a and tyrb1b blocks melanization of zebralish melanophores, suggesting that tyrb1a/b may partially mediate Tfap2a/e activity within these cells [50]. A variation of this model is that, rather than Tfap2 itself functioning as a co-activator with Mitfa, the protein product of a gene stimulated by Tfap2 does so. For instance, Tfap2 activates expression of estrogen receptor alpha (ERα) [51,52]. ERα, together with p300, interacts with Mitf to strongly activate the Dct promoter [53].

Tfap2 may indirectly promote Mitfa transactivation activity

It is also possible that the effector of Tfap2 activity is an enzyme that alters the activity, translation, or longevity of the Mitfa protein (Figure 9B). Thus, perhaps mitfa RNA levels are the same in tfap2a deficient vs. tfap2a/e deficient embryos, but Mitfa activity is reduced in the latter. For instance, the Tfap2-effector may be a receptor tyrosine kinase (RTK) whose activity results in posttranslational activation of Mitfa, i.e. similar to a proposed role of Kita [7,8]. Supporting such a possibility, Kita itself is necessary for differentiation of embryonic melanocytes in zebrafish in certain experimental conditions [54] [23]. A variety of RTKs are candidates for the Tfap2 effector in melanophore differentiation, including ErbB3 [55,56], IGF1R [57], FGFR receptor [58], c-Ret [59], and c-MET [60]. Two G-protein coupled receptors, which...
like RTKs can stimulate the MAP Kinase pathway, are also candidates. First, Endothelin receptor b (Ednrb signaling) promotes melanocyte differentiation in mammals, in part by activating MAP Kinase signaling and Mitf [61–64]. While embryonic melanophores differentiate normally in zebrafish ednrb1 mutants [65], uncharacterized ednrb homologues are present in the zebrafish genome (e.g., on chromosome 9) and may function in embryonic melanophores. Second, Melanocortin 1 receptor (Mc1r) is necessary for normal levels of pigmentation in zebrafish [66] and in mammals [67], and MC1R expression may be directly regulated by TFAP2A, because it has been shown that TFAP2A binds DNA adjacent to the Mc1r gene in HeLa cells (chromatin immunoprecipitation results) [68]. Finally, Tfap2 could normally repress expression of an Mitfa phosphate-activation of the mitfa transcript, change Mitfα translation or change Mitfα protein stability. All these scenarios would result in similar mitfa mRNA levels in situ but weaker Mitfα activity when Tfap2 levels are reduced, and would potentially be bypassed by over-expression of mitfa mRNA. The direct targets of Tfap2 in melanocytes are currently under investigation.

Materials and Methods

Fish maintenance

Zebrafish embryos and adults were reared as described previously [69], in the University of Iowa Zebrafish Facility. Embryos were staged by hours or days post fertilization at 28.5°C (hpf or dpf) [70]. Homozygous mutant embryos were generated from heterozygous adults harboring a presumed null allele of tfap2a (tfap2ats213), or tfap2e (tfap2e e2i2) [26], or kita ( kita e3i3) [23], as indicated.

Generation of cDNAs and morpholinos

First-strand cDNA was synthesized from total RNA harvested from embryos at 4 hpf and 24 hpf as described [25]. A 1.4 kb full-length zebrafish tfap2e cDNA was amplified from the wild-type cDNA using the following primers: forward, 5′-GGA TTC ATG TTA GTC GAC TTT GG-3′, reverse, 5′-TGA TTT GCG GTG CCT CTG-3′. This cDNA includes the entire open reading frame and was inserted into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). A 1.3 kb fragment of zebrafish tyrp1b cDNA was amplified from the wild-type 24 hpf cDNA using the following primers: forward, 5′-GAG GGC TGA TGA TCT CTA GAC-3′; reverse, 5′-GCC CAA TAG GCT GAG CTC-3′. This cDNA was inserted into pSC-A vector (Stratagene, La Jolla, CA).

In designing a tfap2e construct in which expression is disrupted, the exon 2 splice donor site and the exon 3 splice donor sites were inferred from comparison of the cDNA to the corresponding genomic sequence (http://uswest.ensembl.org/Danio_rerio/Info/Index). Morpholinos complementary to these sites were ordered: tfap2e e2i2 MO, 5′-ATA CAA GAG GAG TGA TTA ACG TCA CCT G-3′; tfap2e e3i3 MO, 5′-CAC ATG GAG GCT CTC ACC TTT CCT G-3′ (Gene Tools, Philomath, OR). In addition, a MO targeting the tfap2e translation start site (AGG MO) was designed, 5′-GCC GTA GTA GTG GAG TTA AAG GAT GTG G-3′, reverse, 5′-GCC CAA TAG GAG CGT TTT CC-3′. This cDNA was reconstituted into pSC-A vector (Stratagene, La Jolla, CA).

For immunohistochemistry, a monoclonal anti-Pax7 antibody [33] was used at a 1:25 dilution (supernatant obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, USA). The primary antibody and an anti-DIG antibody were added during routine whole mount in situ hybridization. Following development of whole mount in situ hybridization with NBT/BCIP, the embryos were blocked and then incubated with an Alexa-488 conjugated goat-anti-rabbit secondary antibody, as previously described [76]. After several washes, the embryos were mounted in 50% glycerol/PBST, and photographed. Cell counts were performed on ten embryos per group, along the entire length of the hind yolk.

Dissociation of zebrafish embryos and FACS

Live embryos were reared to an appropriate stage, homogenized with a pestle, and dissociated with PBS containing trypsin and EDTA for 30 minutes at 33°C. After dissociation, cells were resuspended in PBS plus 3% fetal bovine serum (FBS). EGFP-positive cells were counted using a Becton Dickinson FACScan. For cell sorting, cells were dissociated as previously described, and subsequently sorted, on a Becton Dickinson FACS DiVa, directly into buffer RLT and β-mercaptoethanol for subsequent RNA isolation (RNaseasy Plus Mini Kit, Qiagen, Valencia, CA). FACScan cell counting, FACS DiVa cell sorting, and data analyses were conducted at the University of Iowa Flow Cytometry Facility.

Quantitative RT-PCR

The isolation and culture of normal melanocytes and keratinocytes was performed as described previously, Mel 1 and Ker
[77,78], Mel 2.3 [79] (see Figure 1M). Total messenger RNA was isolated using an RNaseasy Plus Mini Kit (QiAGEN, Valencia, CA), along with on-column DNAse digestion according to the manufacturer’s instructions. Lymphocytes (Jurkat cells, clone E6-1) were obtained (ATCC, Manassas, VA), and total RNA was isolated using the PerfectPure RNA Kit (following manufacturer’s instructions, 5 PRIME Inc., Gaithersburg, MD). RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific) and diluted to equal concentrations. For complementary DNA (cDNA) reactions, approximately 200 ng of total RNA was added to 0.5 μg random hexamers, plus 2.5 μl of 10 mM dNTPs (Invitrogen; Carlsbad, CA), and brought to 30 μl with nuclease-free water. Reactions were heated to 65°C for 5 minutes, and cooled to 4.0°C for 5 minutes in a PTC-200 Peltier Thermo Cycler (MJ Research; Ramsey, MN). We then added 0.5 μl of a master mix containing 10 μl of 5x First-Strand buffer (Invitrogen), 5 μl of 0.1 M dithiotreitol, 20 units of RNasin (Promega, Madison, WI), and nuclease-free water to a volume of 19 μl. Reactions were incubated at 25°C for 10 minutes, and then at 37°C for 2 minutes. Then 1 μl of Moloney-murine leukemia virus Reverse Transcriptase (New England Biolabs, Ipswich, MA) or 1 μl nuclease-free water was added to each reaction. Reactions were carried out at 37°C for 2 hours, followed by incubation at 75°C for 15 minutes. PCR reactions (25 μl) were prepared with approximately 10 ng of cDNA, using the SYBR Green kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. The following primers were used at a final concentration of 200 nM in separate PCR reactions: human TFAP2E (forward: 5'-AAT GTG AGT CGT CGT ACT TC-3'; reverse: 5'-GGT CCT GAG CCA TCA AGT CT-3'); or human GAPDH (forward: 5'-AGG TCG GAG TCA ACG GAT TTG-3'; reverse: 5'-GGT ATG GCA TGG ACT GTG GT-3'). Quantitative real-time PCR was performed in Low 96-well plates (Bio-Rad, Hercules, CA) using a Bio-Rad thermal cycler (CFX96 Real-Time PCR Detection System) and following the default protocol. Primers were designed to flank large exon-intron boundaries to avoid the potential amplification of contaminating genomic DNA. Also, RNA samples not reverse-transcribed (-RT) were used as a negative control. The 2-MCT method was used to determine relative levels of gene expression between samples (normalized to GAPDH [80]). Experiments were performed in triplicate and mean and standard error were calculated. Following real-time PCR, melt-curve analysis was performed to determine reaction specificity. Similar methods were used for qRT-PCR of sorted cells, with the exception that approximately 20 ng of RNA was used for cDNA synthesis. The following primers were used at a final concentration of 200 nM in separate PCR reactions: tyr (forward: 5'-GGA TAG TTC ATG GTG CCC TT-3'; reverse: 5'-TCA GGA ACT CCT GGA CAA AG-3'); typlb (forward: 5'-TAT GAG ACA CTG GCC ACC AT-3'; reverse: 5'-CAT GCT TGC CAT GGA AAC-3'); det (forward: 5'-CCT CGA AGA ACT GGA CAA CA-3'; reverse: 5'-CAA CAC CAC CAG GAT CAA CA-3'); and β-actin (forward: 5'-CGG GCA GGA GAT GGG AAC C-3'; reverse: 5'-CAA CAG AAA CGC TGA TTT C-3'). Again, the 2-MCT method was used to determine relative levels of gene expression between samples, first normalizing both samples to β-actin, and then comparing relative gene expression levels in tfap2a/e doubly-deficient cells to those in tfap2a deficient cells.

**TUNEL staining**

Apoptotic cell death was revealed in whole embryos by terminal transferase dUTP nick-end labeling (TUNEL) as described [81]. The terminal transferase reaction was terminated by incubation at 70°C for 30 min, and embryos were processed with anti-FITC-alkaline phosphatase antibody and developed with NBT/BCIP, as for an RNA in situ hybridization.

**Rescue experiments**

For tfap2aGR mRNA rescue experiments, approximately 5 nL of 0.075 mg/mL tfap2aGR or los2 encoding mRNA, transcribed in vitro (mMessage mMachine kit, Ambion, Austin, TX) was injected into one of four cells of embryos previously injected with tfap2a/e or p33 MOs (similar concentration as indicated before). Embryos were raised until they reached approximately 75% epiboly, at which point dexamethasone (dissolved in EtOH) was added to the fish water at a final concentration of 40 μM. For DNA rescue experiments, 5 nL of a 0.025 mg/ml plasmid encoding 4.9 Kb of the sox10 promoter driving full length mitfa [41] was injected at the one cell stage, followed by co-injection of various MO combinations (control MO and p33 MO or tfap2dΔ10/mitfaMO). Embryos were then raised until approximately 36 hpf and fixed in 4% paraformaldehyde overnight. Finally, embryos were rinsed in PBST, mounted in 3% methylcellulose, and photographed.

**ImageJ analysis**

To analyze the mean gray value of melanophores, embryos were first fixed at the appropriate stage in 4% paraformaldehyde overnight. Embryos were then rinsed in PBST and mounted in 3% methylcellulose, and images of single melanophores were taken near the otic vesicle at 40x. All lighting conditions remained constant throughout image capturing. 6-10 melanophores were imaged per embryo, and 10 embryos were analyzed per group (roughly 70–80 melanophores per group). Images were converted to a 32 bit gray image and then processed using the auto threshold function in ImageJ software (Version 1.40 g, National Institutes of Health, Bethesda, MD), creating an outline of the melanophore being analyzed. After application of the auto threshold function, a selection was created of the pixels highlighted, and a measurement reporting mean gray value for the given area was taken. An inverse of the selection was then created, highlighting the background (area not occupied by the melanophore), and a similar measurement was taken, reporting the mean gray value of the surrounding background. The difference was then calculated between the mean gray value of the melanophore and the surrounding background, resulting in the normalized mean gray value of the melanophore. Averages were then calculated for all melanophores measured per group, and standard deviation was calculated.

**Cell counts**

For mitfa-positive and TUNEL-positive cell counts, the entire region overlying the hind yolk was counted. For melanophore cell counts in sox10/mitfa rescue experiments, the total number of melanophores in the embryo body (excluding yolk and hind yolk) were counted. Embryos were fixed in 4% paraformaldehyde overnight, washed in PBST, and mounted in 3% methylcellulose for counts. Embryos were mounted and then counted blindly by an independent observer.

**Supporting Information**

Figure S1 Expression of tfap2d is absent from developing melanophores. Lateral views of wild-type zebrafish embryos, fixed at the stage indicated and processed to reveal tfap2d expression by RNA in situ hybridization. (A) At 24 hpf, an embryo shows tfap2d expression within specific regions of the midbrain, (B) which persists until 36 hpf. Importantly, tfap2d expression is not detected within the trunk of these embryos. Embryos were treated with low levels of PTU to decrease melanin production to allow better...
visualization of potential expression within melanophores. Scale bar: 50 μM.

Found at: doi:10.1371/journal.pgen.1001122.s001 (4.16 MB TIF)

**Figure S2** Expression of *tfap2e* in *tfap2a* mutants. Lateral views of zebrafish embryos, fixed at the stage indicated and processed to reveal *tfap2e* expression by RNA *in situ* hybridization. (A) A sibling embryo at 28 hpf with *tfap2e* expression within melanoblasts, located throughout the trunk of the embryo, as described earlier. (B) A *tfap2a* mutant, in which *tfap2e* expression is detected within melanoblasts near the dorsum of the embryo; it is evident that fewer than normal numbers of *tfap2e*-expressing cells (presumably melanoblasts) have migrated ventrally. (C) Sibling and D) *tfap2a* mutant embryos at 34 hpf; *tfap2e* expression is detected in the posterior trunk of both sibling and mutant embryos, although fewer *tfap2e*-expressing cells have migrated ventrally in the *tfap2a* mutant. Embryos were treated with low levels of PTU to better visualize expression within melanophores. Scale bar: 25 μM.

Found at: doi:10.1371/journal.pgen.1001122.s002 (6.76 MB TIF)

**Figure S3** *p53* MO blocks nervous system necrosis but does not affect melanophore development. Lateral views of live zebrafish embryos at 36 hpf. Insets show higher magnification of melanophores contained in white boxes. (A) A wild-type embryo shows embryos at 36 hpf. Insets show higher magnification of melanophores contained in white boxes. (A–E) Sibling embryos injected with A), control MO, (B,C) *tfap2a* MO; all of these *tfap2a* mutant embryos exhibit normally pigmented melanophores. (B) A *tfap2a* mutant embryo injected with a control MO, with a reduction in melanophore numbers and melanophore migration, and slightly less than normal melanization. (D,F) *tfap2a* mutant embryos injected with (D) a *tfap2e* e2i2 MO or (F) a *tfap2e* *AUG* MO. These embryos display a further reduction in darkly pigmented melanophores, throughout the embryo. (G,H) Dorsal views of embryos at 36 hpf, anterior to the left. Embryos were first injected with *tfap2a/e MO*, followed by injection of miRNA encoding either (G) 13lacZ or (H) a dexamethasone-inducible version of *tfap2a* (tfap2aGR). Following injections, embryos were incubated in dexamethasone (Dex). The embryo injected with tfap2aGR shows rescue of pigmented melanophores whereas that injected with lacZ did not. Scale bars: 25 μM.

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**Figure S4** Specificity of *tfap2a/e* doubly-deficient melanophore defects. (A–F) Lateral views of live zebrafish embryos at 36 hpf. Insets show higher magnification of melanophores contained in the white boxes. (A–E) Sibling embryos injected with A), control MO, (C) *tfap2e* e2i2 MO, or (E) *tfap2e* *AUG* MO; all of these embryos exhibit normally pigmented melanophores. (B) An *tfap2a* mutant embryo injected with a control MO, with a reduction in melanophore numbers and melanophore migration, and slightly less than normal melanization. (D,F) *tfap2a* mutant embryos injected with (D) a *tfap2e* e2i2 MO or (F) a *tfap2e* *AUG* MO. These embryos display a further reduction in darkly pigmented melanophores, throughout the embryo. (G,H) Dorsal views of embryos at 36 hpf, anterior to the left. Embryos were first injected with *tfap2a/e MO*, followed by injection of miRNA encoding either (G) 13lacZ or (H) a dexamethasone-inducible version of *tfap2a* (tfap2aGR). Following injections, embryos were incubated in dexamethasone (Dex). The embryo injected with tfap2aGR shows rescue of pigmented melanophores whereas that injected with lacZ did not. Scale bars: 25 μM.

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**Author Contributions**

Conceived and designed the experiments: EVO WL GB RAC. Performed the experiments: EVO WL GB KMD RAC. Analyzed the data: EVO WL GB KMD RAC. Contributed reagents/materials/analysis tools: MYH RAC. Wrote the paper: EVO RAC.

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