The MITF paralog tfec is required in neural crest development for fate specification of the iridophore lineage from a multipotent pigment cell progenitor

Kleio Petratou", Samantha A. Spencer", Robert N. Kelsh", James A. Lister

1 Department of Biology and Biochemistry and Centre for Regenerative Medicine, University of Bath, Bath, United Kingdom, 2 Department of Human and Molecular Genetics and Massey Cancer Center, Virginia Commonwealth University School of Medicine, Richmond, Virginia, United States of America

Abstract

Understanding how fate specification of distinct cell-types from multipotent progenitors occurs is a fundamental question in embryology. Neural crest stem cells (NCSCs) generate extraordinarily diverse derivatives, including multiple neural, skeletogenic and pigment cell fates. Key transcription factors and extracellular signals specifying NCSC lineages remain to be identified, and we have only a little idea of how and when they function together to control fate. Zebrafish have three neural crest-derived pigment cell types, black melanocytes, light-reflecting iridophores and yellow xanthophores, which offer a powerful model for studying the molecular and cellular mechanisms of fate segregation. Mitfa has been identified as the master regulator of melanocyte fate. Here, we show that an Mitf-related transcription factor, Tfec, functions as master regulator of the iridophore fate. Surprisingly, our phenotypic analysis of tfec mutants demonstrates that Tfec also functions in the initial specification of all three pigment cell-types, although the melanocyte and xanthophore lineages recover later. We show that Mitfa represses tfec expression, revealing a likely mechanism contributing to the decision between melanocyte and iridophore fate. Our data are consistent with the long-standing proposal of a tripotent progenitor restricted to pigment cell fates. Moreover, we investigate activation, maintenance and function of tfec in multipotent NCSCs, demonstrating for the first time its role in the gene regulatory network forming and maintaining early neural crest cells. In summary, we build on our previous work to characterise the gene regulatory network governing iridophore development, establishing Tfec as the master regulator driving iridophore specification from multipotent progenitors, while shedding light on possible cellular mechanisms of progressive fate restriction.
**Introduction**

Pigmentation is a conspicuous feature of animal diversity and has broad importance for behaviour and evolution (reviewed in [1]). Much is known about the development and cell biology of melanocytes but far less is understood about the genetic mechanisms underlying the diversity of pigment cell types in non-mammalian vertebrates. Zebrafish embryos display three neural crest (NC)-derived pigment cells: melanophores, melanin-producing cells homologous to the melanocytes of mammals, and often referred to simply as melanocytes; xanthophores, yellow-orange cells bearing pteridine and carotenoid pigments; and iridophores, shiny cells containing platelets composed of guanine (reviewed in [2, 3]). Defining the fate specification mechanisms of these other cell-types is important for understanding both their genetic control and the evolutionary origins of vertebrate pigmentation, but also the much-debated process of neural crest cell (NCC) fate restriction.

The progressive fate restriction model proposes that neural crest cells (NCCs) become partially fate restricted as development progresses, giving rise to intermediate partially-restricted progenitors, each of which can generate a number, but not all of the NC derivatives [4–8]. Such a model has been strongly supported for the neural derivatives by a single cell transcriptional profiling study in mice, although, surprisingly it was unable to resolve pigment cell development [9]. Although the progressive fate restriction model has been supported by a number of studies using zebrafish, the number and identity or, indeed, even the existence of intermediate progenitors remains unclear [10–12]. Work to define the gene regulatory networks underlying fate choice provides an important context for these cellular mechanisms [11,13,14].

Characterisation of the phenotypes of mutants affecting multiple NC derivatives has been widely used to infer the identities and potencies of these progenitors. In the pigment cell field, a progressive fate restriction model has been developed based on loss- and gain-of-function studies for single genes, with both a multipotent chromatoblast and a bipotent melanio-iridoblast proposed as partially-restricted intermediates [11,14–17]. Studies of fate-specification mutants further permits elucidation of the gene regulatory networks (GRNs) governing diversification of these precursors [13,14].

To date, examination of zebrafish mutants provides evidence of complex genetic control of pigment cell development from multipotent NCCs [18]. Of the genes affected in these mutants, many have been shown to encode transcription factors that regulate fate specification of pigment cells from NCCs. Such factors may be required by either all three pigment cell lineages (e.g. *colourless*/sox10; [19, 20]) or only a single pigment cell lineage (e.g. *nacre/microphthalmia-associated transcription factor a/mitfa*, [21]). Consequently, genetic loss of several transcription factors affects one or more pigment cell types, perhaps indicating the existence of shared progenitors. For example, loss of *sox10* function results in lack of all three zebrafish lineages, but also hinders the development of peripheral nervous system components [22, 23]. Although a tripotent progenitor restricted to generating all chromatophore lineages has long been hypothesised [15], mutants with the expected phenotype (i.e. restricted to the three pigment cell lineages alone) have not been identified.

As a first step towards understanding the complex GRN governing NCC fate restriction towards pigment cell lineages, it is important to define the key components involved in fate specification of individual lineages. Of the three zebrafish pigment cells, melanocytes are the currently best studied. In this lineage, Sox10, in conjunction with Wnt signalling, is required to activate and maintain *mitfa* transcription [13, 20, 24–26]. Like its mammalian counterpart, MITF, Mitfa has been proven necessary and sufficient to upregulate numerous melanocyte differentiation genes, including those controlling melanin synthesis (e.g. *dct, silva* and *tyrosinase*).
Mitfa is thus dubbed the ‘master regulator’ of melanocyte fate choice [13, 21, 25]. However, in this role, Mitfa is supported by Tfap2, especially Tfap2a which acts as a key co-factor [27]. In contrast, Foxd3 mutants show a complex phenotype that includes delayed melanocyte and xanthophore specification and differentiation, ultimately resulting in wild-type cell numbers, and reduced iridophore numbers [16, 28–30]. These phenotypes seem to reflect roles for FoxD3 in both lineage priming [31] and, in certain contexts, repressing melanogenesis promoting the specification of other fates [16, 17].

Recent studies of key zebrafish iridophore mutants have begun to define the basic genetics of this cell type. In terms of their differentiation, heightened purine synthesis is central to the development of the guanine crystals that form the reflecting platelets. Thus, *purine nucleoside phosphorylase 4a* (pnp4a), which encodes an enzyme important in the biosynthesis of guanine, is a robust marker of mature iridophores [14, 17]. Additionally, mutations of several enzymes specific to differentiated iridophores have been shown to disrupt purine biosynthesis [32], while disruption of other proteins was found to impair iridophore survival [33–35]. Furthermore, a signalling pathway crucial to fate specification, proliferation and differentiation of iridophores has been highlighted by mutations in the gene encoding the Leukocyte Tyrosine Kinase (*Ltk*; [11, 36]) receptor tyrosine kinase, with corroboration from targeted loss of function of its ligand [37, 38]. In *shadyltk* mutants fate specification and proliferation of iridophores, but not other pigment cells, are impeded [11, 36]. Nevertheless, Ltk signalling alone is not sufficient for iridophore specification, since iridophores are eliminated in *sox10* mutants, even though *ltk* expression is strongly detectable by whole-mount in situ hybridisation [11, 14]. The function of Ltk signalling in specification of the iridophore lineage is, thus, likely analogous to that of Wnt signalling in generating melanocytes. This then leaves open the question of whether there is a ‘master’ transcriptional regulator of the iridophore lineage, analogous to the role of Mitfa in melanocytes.

The zebrafish “MiT” (Mitf/Tfe) family consists of six genes [39]; of these, *mitfa* and *tfec* are the only ones expressed in the NC. The distinctive expression pattern of *tfec* in cells along the dorsal and ventral midline in the trunk and tail and two patches over the yolk, is strongly reminiscent of differentiating iridophores [39]. Furthermore, Higdon et al. performed transcriptomic analysis to compare FACS-purified iridophores, melanocytes, and retinal pigment epithelium (RPE), and found *tfec* to be one of several transcription factor genes with enriched expression specifically in the iridophore lineage [40]. Together, these data lead to the hypothesis that Tfec might be the iridophore master regulator, equivalent to Mitfa in developing melanocytes.

In a recent detailed study, we demonstrated that, indeed, *tfec* serves as a robust marker of the iridophore lineage, allowing us to define the major stages of iridophore development [14] (Fig 1). Based on whole-mount in situ hybridisation studies of *tfec* expression patterns throughout a developmental time-course, and co-expression analysis with both *ltk* and *mitfa*, we concluded that *tfec* is first expressed extensively throughout the premigratory NC progenitors of the trunk and tail at 18 hpf, and then exclusively in the developing iridophore lineage. Focussing on cells in the posterior trunk, we distinguished a subset of *tfec*-positive cells within the premigratory domain that have downregulated the early NCC marker, *foxd3*, but do not express definitive pigment cell markers; we refer to these as early chromatoblasts (Fig 1; Cbl early). At approximately 22 hpf, *ltk* and *mitfa* are upregulated in the *tfec+*, *foxd3*+ premigratory cells of the trunk [11, 14, 21], indicating that they correspond to partially restricted progenitors, capable of generating pigment cells (Fig 1; Cbl late). By 24 hpf, *tfec* labels migrating progenitors which co-express *mitfa* and *ltk* markers, and which we consider fate-specified iridoblasts, but which likely retain at least melanocyte potential too (Fig 1; Ib(sp); [14]). From 30 hpf, *tfec*+ cells co-express *ltk*, but not *mitfa*, and we now consider these to be either
Fig 1. A progressive fate restriction model for iridophore development from multipotent NCCs. Schematic representation of the previously described model of iridophore generation from the NC, along with the expression characteristics and potential fate choices of individual arising cell types [14]. Genes actively expressed in each cell are indicated with a “+” sign. Tfec is initially co-expressed with eNCC specification factors, which gradually become downregulated (red font, vertical red arrow), while lineage-specific factors become upregulated (green font). Proteins
definitive iridoblasts (Fig 1; Ib(df), along the dorsal trunk and on the lateral migration pathway at 30 hpf), or mature iridophores (in iridophore locations from 42 hpf onwards) according to their position and state of visible differentiation. Thus, the established iridophore-associated expression pattern of tfec during zebrafish development reinforces the hypothesis that Tfec might act as the missing iridophore master regulator, but does not eliminate the possibility of additional earlier functions, either in multipotent early NCCs (eNCCs; Fig 1), or in partially restricted precursors with wider potencies.

In our previous study we used a preliminary assessment of tfec mutants to inform our derivation of a core iridophore GRN. Here, we describe in detail the generation and comprehensive characterization of the effects on NC development of mutations in tfec. Following examination of the development of a wide variety of NC derivatives in tfec mutants, using early and late molecular markers, we conclude that, although neuronal and skeletal derivatives develop normally, specification of all pigment cell fates is delayed in homozygous mutants, suggesting a common early requirement for tfec in the GRN governing specification of all three chromatophore lineages, and providing support for a common chromatoblast precursor. Finally, our previous work identified the GRN governing maintenance of tfec in the iridophore lineage [14]. In the present study, we extend this work to define tfec as a necessary regulator of iridophore specification, yet not alone sufficient to drive NCCs to adopt an iridophore fate. Importantly, we identify its upstream regulators in the multipotent premigratory NC, thus for the first time placing the transcription factor in context in the NCC specification GRN [41]. Together, these data shed light on the possible mechanism of progressive fate segregation of NCCs, and begin to elucidate the complex role for Tfec, being indispensable for iridophore development, but also playing subsidiary roles in specification of the other two chromatophores derived from the zebrafish NC.

**Results**

**tfec is a candidate for the iridophore master regulator**

As we showed previously [14], tfec is co-expressed with the established iridophore marker, ltk [11], during iridoblast fate choice and iridophore differentiation. Although Higdon et al showed that tfec expression was prominent in the RNA-seq profiles of iridophores, they also detected low levels of expression in purified melanocytes [40]. Here we used whole-mount in situ hybridisation to detect tfec in individual embryos, following photographic documentation of their individual iridophore patterns, to show definitively its presence in differentiated iridophores (Fig 2). tfec is maintained in differentiated cells (Fig 2A–2D). At these stages, consistent with our previous observations showing no overlap of tfec and the melanocyte marker mitfa in differentiated melanocytes [14], we do not detect expression in neighbouring differentiated melanocytes occupying the dorsal and ventral stripes (Fig 2A–2D). Likewise, xanthophores, which are widespread under the epidermis of the flanks of the embryos, also do not show detectable tfec expression in these expression studies (Fig 2A–2D). To confirm this, we used the xanthophore lineage marker, Pax7, detected via an immunofluorescence assay combined with simultaneous labelling of tfec transcript via whole-mount in situ hybridisation (Fig 2E–2G). In conclusion, at the detection threshold of our expression studies, tfec is a consistent marker of mature iridophores, but not of melanocytes nor xanthophores.
Loss of tfec function affects the development of all embryonic pigment cells

To assess the role of tfec in development, we induced mutations in tfec using CRISPR/Cas9 (Fig 3), selecting a target in the seventh exon of the gene, which encodes the second helix of the transcription factor’s helix-loop-helix dimerization domain. Our two laboratories independently generated identical 6 base pair deletions (the tfecba6 and tfecvc58 alleles) in two different wild-type strains, WIK and NHGRI-1, in addition to frameshifted alleles (Fig 3A). We reasoned that in this region of the gene it was likely that even indels that retained the correct reading frame (i.e., multiples of three) would likely be deleterious, because they would alter spacing of key residues and surfaces within this helix. Indeed, all of the generated alleles, when made homozygous, resulted in elimination of differentiated iridophores from the dorsal, ventral and yolk sac stripes, as well as from the lateral patches of the embryo (Fig 3B–3E). In addition, iridophores were absent from the dorsal head (Fig 3C and 3E) and the eye (Fig 3B–3E) of homozygous mutants. Moreover, both injected (G0) fish raised to adulthood, as well as a single ‘escaper’ surviving F1 adult carrying biallelic frameshift mutations, lack iridophores in patches or in whole, indicating an ongoing role in adult iridophores (S1A–S1C Fig). When concerning embryonic stages, all results presented here were produced using either the tfecba6 or the tfecvc60 alleles. The aforementioned alternative frameshift alleles were only used to describe adult phenotypes, because homozygous carriers of both ba6 and vc60 never survive to adulthood.

Quantification of iridophore numbers along the dorsal and ventral stripes of live embryos at 3 dpf illustrates the severity of the phenotype, with only very rare escaper iridophores present in homozygous tfecba6 mutant embryos (Fig 3F and S1 Table). The numbers of differentiated cells in heterozygous mutants are not significantly different from those in wild-type (WT)
Fig 3. Loss of tfec function eliminates embryonic iridophores. (A) Schematic showing the distribution of the 8 exons of tfec (orange) in relation to the functional basic helix-loop-helix-leucine zipper domains (green) of the transcription factor. The red arrow indicates the position along both the gene and protein sequences targeted for mutagenesis by the CRISPR/Cas9 system. Included are the targeted WT tfec DNA/amino acid sequence (blue, with PAM underlined), and the sequences of the examined mutant alleles, with the corresponding molecular lesions (dashes for deleted nucleotides, red font for insertions). Imaging live embryos under reflected light reveals a striking lack of iridophores in tfec mutants (D,E) compared to their WT siblings (B,C) along the dorsal (downward arrow), ventral (upward arrow), and yolk sac stripes, as well as overlying the eye (arrowhead). Iridophores are also absent on the dorsal
head (C,E, horizontal arrow) and the lateral patches. (F) Quantification of differentiated iridophores across the dorsal and ventral trunk at 3 dpf confirms a prominent lack of iridophores along the dorsal and ventral stripe of tfec mutants. (G-K) Injection of tfec cDNA can rescue the mutant phenotype. Differentiated iridophores (arrowheads) are abundant on the eye of WT embryos (G), but completely absent from the eye of a tfec\textsuperscript{-90} mutant sibling (H) at 4 dpf. Co-injection with Tol2 transposase of a construct where the tfec promoter drives transcription of the tfec cDNA sequence, leads to rescue of iridophores (arrowheads) on the eye (I) and trunk (J) of tfec\textsuperscript{-90} mutants. (K) Quantitation of rescue efficiency. Approximately 45% of mutants displayed eye iridophore rescue, 3% showed rescue in the trunk only and 6% showed rescue both in the eyes and trunk (n = 62). By contrast, iridophores were never observed in uninjected tfec\textsuperscript{-90} sibling larvae (n = 58). (L) tfec cDNA expressed from the sox10 promoter is capable of rescuing iridophores (arrowheads) in tfec\textsuperscript{-90} mutants by 4 dpf. (M) At 3 dpf, numbers of iridophores in the dorsal stripe (DS) of wild-type embryos does not significantly change between uninjected embryos and embryos injected with either of the control constructs (sox10:dsRed, ubiziGFP; orange “ns”). Embryos injected with sox10:tfec or with ubiziGFP show no significant change in DS iridophore numbers compared to both uninjected, and control injected siblings (sox10:dsRed, ubiziGFP, respectively; red “ns”). In the ventral stripe (VS), injection of the ubizi:tfec construct led to no significant iridophore number alterations, when compared to both control-injected and uninjected siblings (red “ns”). ubiziGFP-injected controls do not show differences in VS iridophores when compared to uninjected controls (orange “ns”). When injecting sox10:dsRed, a weakly significant increase in VS iridophores is observed (p = 0.04). sox10:tfec injection does not lead to significant changes compared to uninjected controls, but appears to lead to a decrease in numbers when compared to sox10:dsRed (p = 0.002). Dots indicate outliers: sgRNA, small guide RNA; DS, dorsal stripe; VS, ventral stripe; LP, lateral patches; YSS, yolk sac stripe. (B,D,G,J,L): lateral views. (C,E): dorsal views. Head towards the left. Scale bars: 200 μm. (F): spots signify outlier values*, p-value < 10\textsuperscript{-5} using t-test.

https://doi.org/10.1371/journal.pone.0244794.g003

siblings (S1 Table). The iridophore phenotype could be successfully rescued via injection of a Tol2 transposon-based plasmid containing 2.4 kb of the tfec promoter [42], guiding tissue-specific expression of full-length tfec (Fig 3G–3K). Although the WT number of iridophores was not recovered, almost half of the injected mutant embryos presented with rescue of iridophores either on the eye, the trunk, or both of those domains (Fig 3K). Moreover, successful iridophore rescue was further visible in injected fish raised to adulthood (S1D Fig). Rescue could also be achieved by tfec expression in NC progenitors using the sox10 promoter (Fig 3L). In WT embryos, overexpression using either the sox10 or the ubiquitin B (ubi) promoter did not lead to a significant increase of iridophore numbers (S3M Fig). In these results, only iridophore numbers in the ventral stripe showed a statistically significant difference when comparing injected sox10:tfec and sox10:dsRed controls, which we attribute to a likely biologically irrelevant increase in the cell numbers of sox10:dsRed control-injected compared to uninjected embryos. Supporting our conclusion that these alterations are not biologically meaningful, we detect changes neither in the number of ventral stripe iridophores between sox10:tfec-injected and uninjected embryos, nor in ubi:tfec-injected embryos.

Curiously, we found that ectopic expression of tfec in wild-type embryos using the ubi promoter led to increased numbers of melanised cells rather than iridophores as might have been expected (Fig 4A and 4B). This led us to explore whether tfec expression would rescue the melanocyte phenotype of mitfa mutants. Intriguingly, tfec expressed from either the sox10 or ubi promoters was capable of rescuing melanocytes in mitfa mutants, in a manner qualitatively comparable to sox10 promoter-driven mitfa (Fig 4C–4F).

In previous work, we showed that tfec is present in multipotent premigratory NCCs, which do not yet express definitive pigment cell markers (early NCCs, early Cbls; [14]). We further demonstrated that during early stages of specification and migration of NC derivatives, tfec expression transiently overlaps with that of mitfa in specified, but not definitive, iridoblasts (Ib (sp)). Here we report that melanogenesis is delayed in 30 hpf homozygous tfec mutant embryos when compared to WT or heterozygous siblings, supporting a functional role for Tfec during its transient expression in melanoblasts. Specifically, we observed a significant reduction in the numbers of differentiating melanocytes along the dorsal trunk, and the medial and lateral migration pathways (Fig 4G–4I and S1 Table). Melanocyte development recovers, and by 4 dpf mutant embryos present with the same number of melanised cells along their trunk as their WT or heterozygous siblings (Fig 4J–4L and S1 Table). To test whether this recovery resulted from stimulation of a regeneration response through activation of adult melanocyte stem cells, we asked whether it occurred in mutant embryos even when formation of these stem cells has been inhibited by treatment with the ErbB signaling inhibitor, AG1478.
Fig 4. Tfec can drive ectopic melanogenesis, and functions in the early stages of melanocyte development. (A,B) Tfec cDNA expressed from the ubiquitin promoter induces ectopic melanisation in wild-type embryos by 24 hpf. (C-F) Shown at 72 hpf, Tfec expressed from either the ubi or sox10 promoters (E,F) rescues large well-differentiated melanocytes (black arrows) in mitfa<sup>−/−</sup> embryos, in a manner reminiscent of sox10-driven mitfa (D), as well as apparently smaller, poorly melanised cells (blue arrows) (E,F). (G-I) Pigment cell phenotypes at 30 hpf. Compared to WT siblings (G), Tfec mutants (H) have reduced melanisation of the RPE (arrowheads), and reduced melanocytes both along the dorsal trunk (arrows) and on the migratory pathways (asterisks). (I) Quantitation of melanocytes along the dorsal trunk and migratory pathways at 30 hpf reveals a 60% reduction in both regions in Tfec mutants with respect to WT siblings. (J-L) Pigment cell phenotypes at 4 dpf. In embryos treated with melanin-concentrating hormone (MCH) to facilitate their quantitation, the
Interestingly, melanocyte recovery was not affected by AG1478 treatment, indicating that the recovered melanocytes derive from embryonic NCCs, not from adult melanocyte stem cells (S2 Fig). Furthermore, we see strikingly reduced melanisation of the RPE of homozygous mutant embryos at 30 hpf, compared to WT or heterozygous siblings (Fig 3B and 3C), suggesting an analogous role in these brain (not NC)-derived melanocytes. Surprisingly, we observed a mild, yet consistent and statistically significant increase in differentiated melanocytes on the dorsal head of mutant embryos at 4 dpf (Fig 3D, 3E and 3L and S1 Table).

We set out to investigate the early specification events that lead to the observed pigmenta-
tion phenotypes by studying expression of iridophore markers. In homozygous tfec mutants, expression of the differentiated iridophore marker, pnp4a [14, 17], was largely undetectable at 48 hpf (Figs 5E and 5F and S3), consistent with complete lack of differentiated iridophores in tfec mutants. At both 24 hpf and 30 hpf, pnp4a was expressed in a notably reduced number of cells along the dorsal trunk, the migratory pathways and overlying the eye (Fig 5A–5D). Maintenance of pnp4a in a small subset of cells at these earlier stages of chromatoblast specification was attributed to Mitfa-dependent activation ([14]; Fig 5A–5D). Notably, the early reduction of pnp4a expressing cells in the eye and trunk of homozygous tfec mutants is consistent with a defect in generating the pnp4a+ Ib(sp) in these embryos. We assessed expression of tfec itself in tfec mutant embryos, using our tfecb6 allele. At 24 hpf, presumptive homozygous mutants maintained tfec expression along the premigratory NC domain, even in anterior regions, indicating failure of a subset of NC derivatives to become fate specified, since in WT embryos fate specification to non-iridoblast fates is accompanied by loss of tfec expression in the majority of derivatives (Fig 5G and 5H). Furthermore, tfec expression was undetectable in the medial migration pathway, consistent with the absence of tfec-positive Ib(sp). At 36 hpf, the number of tfec-positive cells identified as Ib(df) [14] was strongly reduced in tfec mutant embryos compared to their siblings (Fig 5I and 5J), consistent with tfec function being fundamental for iridophore specification. Intriguingly, complete lack of differentiated iridophores (Fig 3B–3E) was not accompanied by corresponding total elimination of tfec expression at 48 hpf (Figs 5K and 5L and S3; [14]). As the remaining tfec-positive cells do not express other iridophore markers, such as pnp4a (Fig 5F) or ltk [14], we hypothesise that these correspond to early partially-restricted NC derivatives, perhaps early Cbls. Finally, we examine ltk expression in tfec mutants. Ltk expression was completely lacking on the medial migration pathway in homozygous mutants at 24 hpf (Fig 5S and 5T; [14]), consistent with an early defect in iridophore specification. However, we note that ltk expression is also missing in the premigratory Cbl domain, indicating a much earlier role for Tfec, in specification of the Cbl(late) from Cbl(early).

The delayed melanogenesis phenotype in these mutants might result from delayed specification of melanoblasts, or from slowed differentiation of normally specified melanoblasts. To distinguish between these two possibilities, we performed chromogenic in situ hybridisation at 24, 30 and 48 hpf to detect expression of the melanocyte master regulator, mitfa. Strikingly, mitfa expression was restricted to premigratory late Cbls in tfec mutants, whereas mitfa-positive melanoblasts occupied the medial and lateral migratory pathways in WT and heterozygous siblings (Fig 5M and 5N). At 30 hpf, the delay was still detectable. The trunk was occupied by a relatively small number of mitfa-positive NC derivatives, whereas in the tail of mutants cells had still not entered the migratory pathways (Fig 5O and 5P). Consistent with the live phenotype, mitfa expression in mature melanocytes at 48 hpf was unaffected in the trunk and tail of
tfec mutants, compared to WT siblings (Fig 5Q and 5R). This early retardation of mitfa expression, coupled to absence of ltk expression (Fig 5S and 5T; [14]), suggested that specification of the mitfa+; tfec+ Ib(sp) [14] from late Cbl, was hindered in the absence of functional Tfec. In addition, these data support our previous suggestion [14] that these Ib(sp) retain melanocyte potential (i.e. they can be considered both specified melanoblasts as well as specified iridoblasts), and show that melanocyte fate specification is delayed in the tfec mutant. The subsequent recovery of normal melanocyte numbers makes clear that compensatory factors allow melanoblasts to be specified, albeit with a short delay.

The tfec mutant embryos did not show obvious changes in the number and distribution of mature xanthophores (Fig 3G and 3H). However, examination of early xanthophore
specification markers by whole-mount in situ hybridisation showed that the developmental delay in producing melanoblasts is also true for generation of xanthoblasts (Fig 5U–5X). Specifically, both ao5- and gch2-positive cells appeared less abundant along the lateral migration pathway at 24 hpf. Delay in the expression of these two genes was also noted in the head of tfec mutant embryos, compared to WT siblings (Fig 5U–5X, insets). In conclusion, generation of melanoblasts, iridoblasts and xanthoblasts from multipotent NCCs is delayed in the absence of functional Tfec, pointing to an unexpectedly wide role in the specification of all chromatophore fates.

**Loss of tfec function does not affect the development of non-pigment NC derivatives**

Given the unexpected role in non-iridophore pigment cells, we then asked whether loss of tfec function affected non-pigment NC derivatives. To examine neural fates, we used a series of standard markers. The number and distribution of dorsal root ganglion (DRG) sensory neurons, as labelled by anti-Hu immunofluorescence, was unaffected by loss of tfec function (Fig 6A and 6B). Both our anti-Hu assays and traditional in situ hybridisation staining for phox2b expression (Fig 6C and 6D) indicated that development of the NC-derived enteric neurons and enteric and sympathetic progenitor cells remained unaffected in the absence of functional Tfec. Moreover, the number and patterning both of mature Schwann cells, normally residing on the posterior lateral line nerve along the horizontal myoseptum, and of satellite glial cells associated with the DRGs, remain unaffected in homozygous tfec mutants, as shown by staining for sox10 at 48 hpf (Fig 6E and 6F). Likewise, developing oligodendrocytes in the CNS appear unaffected in their numbers and distribution.

To examine whether specification of neural derivatives from multipotent progenitors is delayed in tfec mutants, similar to non-iridophore pigment cell derivatives, we conducted additional whole-mount in situ hybridisation experiments between 30 hpf and 48 hpf, when relevant early specification markers are detectable. We found that expression of pou3f1 (previously oct6) in migrating precursors of the posterior lateral line nerve (pLLn) appeared normal in tfec mutants, compared to known WT siblings (Fig 6G and 6H). Furthermore, detection of sox10 expression in batches containing WT, heterozygous and tfec mutant embryos failed to detect alterations in either the distribution or the abundance of glial progenitors migrating along the medial pathway (Fig 6I and 6J). These in situ provide evidence that premigratory NCCs appear to be present in normal numbers and show unaltered timing of downregulation of sox10 expression. In assays aiming to detect sox10 transcript at 30 hpf, we were able to confirm the presence of homozygous mutant embryos based on reduced melanisation of the RPE (Fig 6I and 6J, insets).

We then asked whether ectomesenchymal derivatives were affected in tfec mutants. We performed in situ hybridization at 30 hpf to detect dix2a transcript in migrating cranial NCCs (Fig 6K and 6L), and found no visible differences in numbers of distributions of cells between WT and tfec homozygous mutant embryos. To assess cartilage deposition, we stained tfec mutant embryos and WT siblings with Alcian Blue at 4 dpf, but did not note any phenotypic distinction in homozygous mutants (Fig 6M and 6N). For the aforementioned experiments, from 48 hpf onwards homozygous mutants, identified by their iridophore phenotypes, were processed separately from their siblings (Fig 6A–6D, 6M and 6N).

In summary, although tfec expression is prominent in the majority of, if not all, early NCCs [14, 39], we could not detect any changes at any stage of the development of NC-derived neurons, glial cells and skeletal components. This suggests that although tfec transcript is present at these early stages, Tfec is uniquely required for pigment cell fate specification, and essential for iridophore fate specification.
Fig 6. Development of skeletal and neural NC derivatives is unaffected in tfec mutants. (A-J) Peripheral nervous system derivatives develop normally in tfec mutants. (A,B) At 4 dpf, the DRG (asterisks) and enteric neurons (arrowheads) number and positioning, as revealed by immunofluorescent detection of Elav1/Hu, is indistinguishable between tfec mutant embryos (B) and WT siblings (A). (C,D) phox2b expression, detected by whole-mount in situ hybridisation, at 72 hpf. The formation and the extent of migration of enteric nervous system progenitors (region between arrowheads) are indistinguishable between tfec...
mutants and WT siblings. Likewise, expression in the earliest differentiating region of the sympathetic ganglia chain, the superior cervical ganglion (SCG), is unaffected. *phox2b* expression is also indistinguishable in the hindbrain (white asterisks) and in placode-derived neuronal progenitors in the cranial ganglia associated with the branchial arches (arrows). (E,F) At 48 hpf, *sox10* expression analysis showed indistinguishable numbers and distribution of Schwann cells (Sc) occupying the pLLn and spinal nerves. Likewise, oligodendrocyte progenitors throughout the CNS appear normal in their specification, numbers and migration (asterisks). *sox10* expression is detectable in iridophore positions (red arrowheads) and in eye iridophores (insets, white arrows), that are strongly affected in homozygous *tfec* mutants. (G,H) *pou3f1* expression analyses at 48 hpf show that glial progenitors on the posterior lateral line nerve (pLLn; area between arrowheads) develop normally in *tfec* mutants. (I,J) *sox10* staining at 30 hpf indicates no observable alterations in the migration of specified neural progenitors through the medial pathway (arrows) in *tfec* mutants, compared to WT siblings. Likewise, the number and distribution of *sox10*-positive premigratory NC progenitors (arrowheads) is unaffected. *tfec* mutants were identified by lack of RPE melanisation (I,J, insets). (K,L) At 30 hpf, *dlx2a* expression shows that formation of the three streams (s1-s3) of migrating cranial NCCs is unaffected in *tfec* mutants. Staining is also indistinguishable in the forebrain (f). (M,N) The cranial cartilage at 4 dpf is unaffected in mutants, compared to WT siblings. Likewise, expression in the earliest differentiating region of the sympathetic ganglia chain, the superior cervical ganglion (SCG), is unaffected.

### *tfec* is downstream of NC specifier genes in the NC progenitor GRN

As indicated in Fig 1, *tfec* expression appears early in NCC development, within multipotent progenitors and coinciding with *sox10* and *foxd3*, likely just after *snailb* and *sox9b* have been downregulated. To determine the upstream regulators of *tfec* in premigratory early NCCs and early Cbls of the dorsal trunk, we conducted expression studies on single and double mutants for the important vertebrate NC specifier genes *foxd3*, *sox9b*, *sox10* and *tfap2a* [41]. In these experiments, where we were using large sample sizes to ensure sufficient single and double mutant embryos, phenotypes associated with genotypes were identified using statistical testing of phenotypic ratios in comparison with expected Mendelian ratios; in the following discussion we refer to ‘presumed mutants’ in reference to their inferred genotype. The sample sizes and *p* values are detailed in S2 Table. Interestingly, at 18 hpf, all embryos from crosses of *foxd3*, *sox10* and *tfap2a* mutant carriers showed identical expression of *tfec*, strongly suggesting that early expression of *tfec* is not strictly dependent upon any one of these genes (S2 Table). In contrast, in 18 hpf presumed homozygous *sox9b* mutants, *tfec* expression does not extend towards the tail as far as in WT or heterozygous siblings (Fig 7K and 7L and S2 Table), which is likely attributable to delayed specification of early NCCs upon loss of *sox9b* function. At 24 hpf *tfec* expression in WT embryos is gradually downregulated from the majority of premigratory NCCs of the trunk in an anterior to posterior manner, strongly persisting only in Ib (sp) [14]. However, we observed a persistence of *tfec* expression in the anterior premigratory NC domain in *sox10*, *sox9b*, *tfap2a* and *foxd3* mutant embryos at this stage, consistent with retained premigratory late Cbls (Fig 7A–7D and 7M–7N). This persistence differed in severity and duration between different mutants, but homozygous mutants of each genotype show highly consistent phenotypes across experimental replicates. Specifically, as was previously reported for a single time point [14], in *sox10* mutants, where NC derivatives fail to become specified and to enter the migration pathways [11, 20], *tfec* expression is maintained in trapped late Cbls, extending to the hindbrain/trunk boundary (Fig 7A and 7B). Our results show that, at all time-points, *tfec*-positive premigratory progenitors persist in homozygous mutants (identified by their lack of *tfec* expression in Ib(sp) positions), until 36 hpf (Fig 7A, 7B, 7E–7F and 7L–7J). In each of *sox9b*, *tfap2a* and *foxd3* homozygous mutants at 24 hpf, *tfec*+ premigratory NCCs are detectable along the dorsal trunk, but not reaching the hindbrain/trunk boundary as in *sox10* homozygous mutants (Figs 7C, 7D, 7N and S4).

As members of the same SoxE group, it is not surprising that *sox10* and *sox9b* have been shown to be functionally redundant in DRG sensory neuron development [22]. We asked whether this might also be true for *tfec* expression in early or late Cbls. Examination of embryo
batches containing sox10;sox9b double mutants at 18 hpf did not reveal elimination of tfec expression in any of the assessed embryos (S2 Table). However, tfec expression was completely eliminated from the NCC progenitor domain of genotyped tfap2a;foxd3 double mutants (Fig 7M–7O), suggesting that both these genes act together to upregulate tfec expression in premigratory NCCs of the trunk. This effect was not observed in genotyped siblings, heterozygous
for one or both alleles nor those homozygous for a single mutant allele. In conclusion, our data show key, but redundant, roles for foxd3 and tfap2a in establishing expression of tfec in premigratory multipotent NCCs.

**Mitfa represses tfec during melanocyte development**

Both tfec and the melanocyte master regulator, mitfa, are transiently expressed in the multipotent NCC domain, in late Cbls, as well as in Ib(sp). We conducted whole-mount in situ hybridisation to assess the effects of loss of mitfa function on tfec expression. Interestingly, presumed homozygous mitfa mutants show ectopic expression of tfec along the dorsal trunk and in NC derivatives along both the medial and lateral migration pathways (Fig 8A–8D). This pattern of tfec expression in mitfa mutants resembles WT mitfa expression in developing melanoblasts, therefore it is likely that Mitfa represses tfec expression in NCCs that become biased towards the melanocyte lineage. This effect persists at 30 hpf and is also observed by a complementary, and more sensitive fluorescent transcript detection technique, RNAscope; we see persistence of tfec expression in cells migrating through the lateral pathway (Fig 8E–8F'). These cells co-express the definitive lineage marker, ltk [11, 14], suggesting that they correspond to specified iridoblasts.

**Discussion**

Our previous work established tfec as a marker during NC development and iridophore fate choice [14, 39]. Interestingly, tfec was found to be co-expressed with mitfa in specified iridophore (Ib(sp)) cells, proposed to be able to at least give rise to melanocytes and iridophores, but not in mature melanocytes. Thus, Ib(sp) should also be considered as specified melanoblasts. The first aim of the present study was to determine whether iridophores are indeed the only embryonic differentiated pigment cell expressing tfec; to this end, we conducted analyses showing that tfec expression is maintained at detectable levels only in mature iridophores, but neither in melanocytes nor in xanthophores, in agreement with recently published single cell transcriptomics analyses [45].

Considering the strong sequence conservation between Tfec and the melanocyte master regulator, Mitfa, we next asked whether tfec might have a function in iridophores analogous to that of mitfa in melanocytes; i.e. as the master regulator of the iridophore lineage. We generated mutations in tfec using a CRISPR/Cas9 approach, obtaining several alleles which displayed essentially identical phenotypes. Although tfec mutants have been generated independently [46], that report did not examine NC-related defects, focusing instead on deficiencies in hematopoiesis. As with the CRISPR-generated exon 3 allele reported by Mahony and colleagues, our mutants fail to inflate the swim bladder (which, along with the caudal hematopoietic tissue, is another site of tfec expression; [39]) and die after approximately 12 days, apparently from lack of ability to feed. Potential postembryonic roles of tfec remain unclear. Nevertheless, data from mosaic adults and a single adult escaper suggest that tfec is required for iridophores throughout the lifetime of the animal (S1 Fig).

In order for tfec to be considered the iridophore master regulator, the gene must not only be necessary, but also sufficient for iridophore specification. Our loss of function data are strongly suggestive of tfec being required for iridophore development, as mitfa is for melanocytes (Fig 1). Injection of wild-type tfec CDNA under its own promoter could rescue, albeit only partially, the iridophore phenotype in tfec mutants, with rescued iridophores in the eye observed more often than in the trunk. These results show that Tfec is sufficient to rescue iridophore specification in tfec mutants. However, we show that addition of tfec failed to generate additional iridophores in wild-type fish. Our additional gain of function experiments in WT
embryos suggest that the iridophore number is not altered upon supplementation of \textit{tfec}, whether ubiquitously in the embryo using the \textit{ubi} promoter, or specifically in NCCs using the...

Fig 8. \textit{Mitfa} represses \textit{tfec} expression in melanoblasts. In WT embryos at 24 hpf (A) and at 30 hpf (B), \textit{tfec} expression is detectable in Ib(sp) and Ib(df) (arrows), respectively, and in the posteriorly regressing early NCC/Cbl domain of the dorsal posterior trunk and tail (region within arrowheads), but expression is undetectable in the lateral migration pathway. At 24 hpf (C) and at 30 hpf (D), \textit{mitfa} mutants present with an increased number of \textit{tfec}-positive cells (arrows) along the dorsal trunk, as well as in the medial and lateral (C, inset) migratory pathways. RNAscope (E,F) performed on \textit{mitfa} mutants at 30 hpf shows an increased number of \textit{tfec}-positive cells compared to the WT on the migration pathways (F, asterisks). Arrows indicate iridoblast precursors along the dorsal trunk. (F') RNAscope reveals that in \textit{mitfa} mutants ectopic \textit{tfec}-positive cells migrating on the lateral migration pathway (below the epidermis; grey and yellow dashed lines indicate the periphery and nuclear boundary, respectively, of overlying keratinocytes) co-express \textit{ltk} (arrows; nuclei indicated by purple dashed lines), and thus likely correspond to Ib(sp). ICM, intermediate cell mass; LP, lateral patches; no, notochord. Lateral views, head towards the left. Scale bars: A-D: 100 μm; E,F: 50 μm; F': 10 μm.

https://doi.org/10.1371/journal.pone.0244794.g008
sox10 promoter. This indicates that either Tfec is insufficient to alone drive NSCs to adopt the iridophore fate, or that in wild-type embryos any elevated specification of the iridophore lineage is compensated by cell regulation mechanisms. Consistently, ectopically expressing Tfec in mitfa mutant embryos failed to increase the number of iridophores. Thus, our experiments to date are indicative of an additional, uncharacterised cofactor cooperating with Tfec to generate iridophores in zebrafish embryos, and without this cofactor, Tfec is not sufficient to promote iridophore specification. The immediate MiT family candidate, Mitfa, is excluded as a potential Tfec cofactor, as it is not essential for iridophore specification [17]. Single cell transcriptomics experiments will be important to indicate candidate transcriptional regulators that might form an iridophore master regulator complex together with Tfec. Further experiments will be required to determine if the mechanism by which Tfec functions in iridophore specification is analogous to that of Mitfa in melanocytes. For example, Mitfa is able to efficiently rescue melanocyte development in the absence of sox10 through triggering both an Mitfa autoregulatory feedback loop and expression of melanocyte differentiation genes [13, 25]. We have shown a Tfec-dependent autoregulatory loop in iridophore development, which may explain why rescue of the iridophore phenotype in tfec mutants is very inefficient.

In addition to a complete absence of iridoblasts and mature iridophores our mutants also, surprisingly, presented with delayed differentiation of both NC-derived and RPE melanocytes, as well as of xanthophores. This suggests that Tfec has a subsidiary role in specification of each of the pigment cell-types. The melanocyte specification delay phenotype that we describe may also help explain the otherwise rather surprising observations of tfec-dependent melanocyte rescue upon inducing gain of function in homozygous mitfa mutants, suggesting that tfec can, when overexpressed, functionally substitute for mitfa. Work in mice has demonstrated at least partial functional redundancy between the two genes in the mammalian RPE [47] and a cooperative relationship between mitfa and tfec has also been recently indicated in the context of eye melanisation and choroid fissure closure [48]. Considered with these previous studies, our results suggest that MiT gene dosage may be an important factor in both neural crest and retinal pigment cells. The nature and origins of either the ectopic or the rescued melanocytes we describe here remain unclear. It is plausible that some, but clearly not all, of the ectopic melanised cells observed upon broad tfec overexpression (via the ubi promoter) may be of an RPE character, rather than neural crest melanocyte character. Transcriptomics analyses and detailed evaluation of the spatio-temporal requirements for melanocyte rescue, as well as Tfec-Mitfa protein-protein and DNA interactions, will be necessary to elucidate the origins of such cells, and the seemingly intricate relationship between Mitfa and Tfec during pigment cell development. Finally, work to define direct and indirect transcriptional targets of Tfec, as well as any other transcriptional co-regulators, similar to the role of Tfap2a for Mitfa [27], is needed to understand the mechanistic details of iridophore fate choice.

Nevertheless, we found that development of all non-pigmented derivatives (neurons, glia and skeletal components) is unaffected. This striking phenotype, with effects restricted to pigment cell specification, is unique amongst the characterised zebrafish pigment mutants and would be consistent with the hypothesis that the cell type defined by tfec expression is a fate-restricted tripotent precursor of the three chromatophore lineages. Decades ago, it was proposed that melanocytes share a common origin with iridophores and xanthophores from a pigment-restricted precursor [15], which we would term a chromatoblast (Cbl(late)). More recently, analysis of ltk expression in sox10 mutants indicated the presence of an ltk+ precursor of pigment cells, consistent with the chromatoblast [11]. The tfec loss of function phenotype thus provides further support for the existence of a Cbl, transiently localised within the dorsal trunk of embryos between 18 hpf and 24 hpf ([14]; Fig 9).
In a recent single cell lineage tracing study, Singh et al., identified progenitor cells proposed to generate all 3 pigment cell types throughout embryogenesis and into adulthood. However, it was shown that the vast majority of these were also able to give rise to neuronal and glial lineages. These results strongly argue for the absence of a strictly tripotent progenitor (Cbl (late)) in each of the 3 embryonic and 1 adult stages when the studies were conducted; but the hypothesis that a transient Cbl(late) cell exists during a brief time window (between 18 hpf and 24 hpf) cannot be strictly ruled out. An alternative hypothesis would be that, although tfec is expressed in a more widely multipotent progenitor (Cbl(early)), its function is only required for downstream specification of chromatophores. Such a hypothesis is further supported both by ours and others’ data on embryonic tfec expression, which is detected across the entire premigratory domain of multipotent NCCs. Based on our findings, tfec is the first zebrafish gene identified that specifically affects all chromatophore lineages without appearing to affect any non-pigment NC derivatives. The key NC transcriptional regulator, sox10, is required for each of the three pigment cell lineages, but loss of sox10 function additionally results in strong reductions or absence of all peripheral glia cells and NC-derived peripheral neurons, suggesting that neuronal and pigment lineages share a common, non-ectomesenchymal progenitor. Furthermore, this requirement of pigment cells for sox10 manifests itself apparently in different ways; sox10 is required for mitfa expression in late Cbls, and mitfa can promote melanocyte fate in the absence of sox10 if misexpressed. In contrast, sox10 mutants still strongly express tfec (as well as ltk) within trapped premigratory progenitors, yet fail to produce Ib(sp). We show here that in tfec mutants the ability to express the lineage markers of melanocytes and xanthophores is delayed but otherwise unaffected, whereas ltk expression is completely missing, even in premigratory cells, also contrasting the phenotype of sox10 mutants. Thus, Tfec is necessary for both correct and timely chromatoblast specification, either via generation of a tripotent progenitor (Cbl(late)) and/or through independent roles in each chromatophore lineage arising from a multipotent progenitor (Cbl(early)).

Another aim of the work presented here was to extend the GRN surrounding tfec beyond the iridophore lineage by examining its regulation in early NCCs. Initiation of tfec expression in the early NC is not affected by loss of tfap2a, foxd3 nor sox10 alone. Notably, loss of sox9b alone caused an apparent delay in induction of tfec expression in posterior NC yet, despite this alteration in the pattern, expression remained strongly present in the progenitor population. Sox10 and Sox9b have previously been reported to act redundantly during zebrafish development. Our data, however, demonstrates that tfec expression in double sox10;sox9b mutant embryos is still strongly activated in the NC. It remains to be shown whether loss of function of a single or of both sox10 alleles modifies the degree of tfec expression delay noted in homozygous sox9b mutants.

Double mutants for tgap2a;foxd3 have been shown to eliminate induction of NC. Redundant activities of T Gap2a and T Gap2c are required for NC induction and development of other non-neural ectoderm derivatives in zebrafish embryos. Consistent with this, we found that foxd3 and tgap2a are redundantly required for induction of tfec expression in early premigratory NCCs. In this context, it is interesting that tfec was recently identified as a likely direct target of T Gap2a/2c, through analysis of gene expression changes in mutants combining different numbers of tgap2a and tgap2c mutant alleles. The same study demonstrated the functional compensation of T Gap2a by T Gap2c, since in tgap2a mutants just a single copy of tgap2c was sufficient to maintain tfec expression at WT levels and rescue NC specification. Our data support the above finding, showing that transcriptional regulation of tfec via T Gap2 transcription factors is independent of them first activating sox10 or sox9b (Fig 9B). Moreover, our assays indicate that disruption of Foxd3 activity alongside T Gap2a
counteracts functional compensation by Tfap2c. Further work will be required to assess whether this is due to a role for Foxd3 in Tfap2c expression.
Our data formally establish *tfec* as a member of the GRN governing maintenance of NCC progenitors [41]. Furthermore, our data provide the first evidence for *Tfec* function in those early progenitors, since we show it is required to drive early expression of *ltk* at the Cbl stage, as well as fate specification of the iridophore lineage from these multipotent progenitors.

Although *tfap2a* and *foxd3* act redundantly to activate *tfec* expression in early NCCs, they also present with divergent ongoing effects in pigment lineages. Single mutations in *tfap2a* and *foxd3* affect the melanocyte and iridophore lineages, respectively [28, 29, 55], likely in a manner dependent upon distinct regulatory interactions with Mitfa. While *tfap2a* and *mitfa* work in parallel to promote melanocyte differentiation [27], *foxd3* has been suggested to repress *mitfa* transcription [16, 56], at least in some contexts [14]. In the absence of *foxd3*, iridophore numbers are reduced in a manner that is at least partially *mitfa*-dependent, and marker analyses and lineage-tracing experiments support the existence of a bipotent melanocyte-iridophore (MI) precursor, the fate of which is influenced by this *foxd3/mitfa* interaction [12, 17]. Our findings that *mitfa* represses *tfec* expression in melanoblasts, and that *tfec* mutants have increased melanocytes, indicate that maintenance of *tfec* activity is also key to the melanocyte versus iridophore cell fate decision. Notably, Mitfa and Tfec have the potential to physically interact as a heterodimer [57, 58], which adds an additional layer of complexity when attempting to elucidate the mechanism of cell fate choice. Interestingly, despite the similarity between *tfec* and *ltk* mutant iridophore phenotypes, *ltk* mutants do not show an analogous late increase in melanocytes [11].

Specification and differentiation of the third pigment cell type of zebrafish, the xantho-
phore, has been shown to depend upon the paired-box transcription factors Pax3 and Pax7a/b [59, 60]. Intriguingly, interactions between Pax3 and Mitf have been demonstrated in mammal melanocyte development [61]. At least some zebrafish xanthoblasts express *mitfa* [62] and we show that loss of *tfec* delays xanthoblast migration, raising the question of whether interplay between Mitfa and Tfec might be important for xanthoblast fate choice. Furthermore, it will be of interest to examine potential interactions between not only Tfec and Mitfa, but also between MiT and Pax transcription factors during chromatoblast diversification.

To conclude, our study contributes to deepening our understanding of the molecular basis of NC and pigment cell development in zebrafish, providing additional support for the progressive fate restriction of multipotent stem cells. While we show Tfec plays a key role in the GRNs underpinning NC maintenance and chromatophore specification, detailed assessment of the diversity of pigment progenitor states during embryonic stages is needed to test the hypothesis of a tripotent chromatoblast. Furthermore, focused effort on the (redundant) roles of transcription factors in xanthoblast development will be decisive in understanding pigment cell fate choice in zebrafish.

**Materials and methods**

**Ethics statement**

This study was performed with the approval of the University of Bath ethics committee and in full accordance with the Animals (Scientific Procedures) Act 1986, under Home Office Project Licenses 30/2937 and P87C67227, and in compliance with protocol AM10125 approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Zebrafish husbandry**

All embryos were obtained from natural crosses. Staging was performed according to Kimmel et al (1995) [63]. WIK or NHGRI-1 wild-type embryos served as controls as indicated in each figure. The following mutant lines were examined: *sox10* [20], *foxd3* [29], *sox9b* [64]...
mitfa<sup>vo</sup> [21], lit<sup>ka<sup>82</sup></sup> [11], tfap2a<sup>6213</sup> [66]. Adult fish were maintained in accordance to official guidelines: water temperature: 28°C, water pH: 7.4, conductivity: 800 mS, ammonia concentration: <0.1 mg/L, nitrite concentration: 0 mg/L, nitrate concentration: <5 mg/L, water hardness: 80–120 mg/L. The dark/light cycle was set at 10/14 hours. The adult stocking density was 5 fish/L, and the larval stocking density <100 fish/L.

**CRISPR mutagenesis**

Target sequences for CRISPR/Cas9 mutagenesis were identified using version 1 of the CHOP-CHOP webtool (http://chopchop.cbu.uib.no/; [67]). The template for synthesis of the guide RNA (gRNA) was generated using a previously described PCR method [68]. Primer sequences are provided in Table 1.

The tfec<sup>ba6</sup> and assorted tfec<sup>vc</sup> alleles were generated independently as follows:

- **tfec<sup>ba6</sup>**. 280 pg of CRISPR guide RNA mixed with 700 pg Cas9 mRNA per embryo were injected at the flat cell stage. RNAs were diluted in RNase-free water. Approximately 200 injected embryos were raised, from which 30–40 pairs were in-crossed to screen for germ-line transmission. Adults that transmitted mutant alleles were separated and outcrossed wild-type fish of the WIK line to generate F1 offspring. F1 siblings (which conceivably could carry different mutant alleles) were in-crossed to identify mutation carriers. 3 fish were identified, which were outcrossed again to WIK fish and the resulting F2 generation raised. For all experiments the F2 of one of the three identified F1 fish were used. Screening was based on iridophore phenotype and confirmed by a High Resolution Melt Analysis (HRMA) assay for molecular screening (see below). To characterise the mutations, F1 adult genomic DNA extracted by swabbing and genomic DNA extracted from F2 embryos were sent for sequencing.

- **tfec<sup>vc</sup> alleles**. CRISPR guide RNA was synthesized using the MEGAscriptT7 Transcription Kit (Invitrogen; Cat# AM1354) and purified using the miRvana miRNA Isolation Kit (Invitrogen; Cat# AM1560) as described by [69]. Capped Cas9 mRNA was generated from the plasmid pT3TS-nCas9n [69], a gift from Wenbiao Chen (Addgene plasmid; Cat# 46757) using the mMESSAGE mMACHINE T3 Transcription Kit (Invitrogen; Cat# AM1348). Cas9 mRNA and tfec exon 7 sgRNA were each diluted to 100 ng/μl for microinjection into one-cell embryos of the NHGRI-1 strain [70]. A fraction of the injected set was sacrificed for genomic DNA preparation [71] to evaluate the efficacy of the guide RNA using the primers cex7F and cex7R, followed by restriction digest with StuI, which cuts in the target sequence. The remaining injected embryos were raised to adulthood and intercrossed or mated to wild-type (NHGRI-1) partners. F1 carriers were identified using the PCR digest assay above, and undigested PCR products (representing mutant alleles) were purified and sequenced.

**High resolution melt analysis.** High Resolution Melting (HRM) Software V3.0.1 (Thermo Fisher Scientific) was used to detect and amplify differences between the melting temperature of 150–200 bp q-RT PCR amplicons generated from reference wild-type (WT) samples versus mutated embryos or adults. To perform q-RT PCR for HRMA, template genomic DNA was extracted using the KAPA Express Extract Kit (Sigma-Aldrich; Cat# KK7103) according to manufacturer’s instructions, and was diluted to 8 ng/μl. Amplification reactions were set up as per manufacturer’s instructions using KAPA HRM FAST reagents (Sigma-Aldrich; Cat# KK4201) and primers designed according to the relevant recommendations (Table 1). Following amplification, a continuous melt curve was generated by increasing the temperature from 60°C (1 min) to 95°C (15 sec) in 0.3°C/sec increments. To detect CRISPR/Cas9-mediated mutagenesis, at least 8 WT reference samples were included in the analysis.
Chromatophore counts

Melanocyte counts were performed at 30 hpf and at 4 dpf on anaesthetized or fixed embryos under transmitted light. Embryos at 4 dpf were treated with 2 μM melatonin directly prior to counting. To count iridophores, PTU-treated embryos were observed under incident light. Pigment cell counts were made under a Zeiss Axio Zoom.V16 fluorescence stereo zoom microscope. Statistics on cell counts were performed on Microsoft Excel using unpaired two-tailed t-tests. Means were considered statistically different if the calculated p-value was less than 0.05.

Cloning and rescue by plasmid microinjection

The full-length coding sequence of tfec was subcloned in-frame into the Gateway 3’ Entry vector p3E-2A-FLAG-pA to make p3E-2A-FLAG-tfec-pA. A multisite Gateway LR+ cloning reaction was then carried out with this plasmid along with Tol2 Kit destination vector pDestTol2pA2 and entry vector pME-mCherry-no stop [72] and entry vector p5E-tfec2.4 [42]. Following bacterial transformation, correct clones were identified by restriction digest of mini-prep cultures.

The resulting plasmid (designated pDestTol2pA2-tfec2.4/mCherry-nostop/2A-FLAG-tfec-pA) at 25 ng/μl was co-injected with Tol2 transposase mRNA at 25 ng/μl into embryos from an intercross of heterozygous tfecvc60 carriers. Homozygous mutant embryos were identified between 48 and 72 hpf and scored at 96 hpf on a stereo dissection microscope under incident light for the presence of iridophores.

For overexpression experiments, the following additional plasmids were generated using multisite Gateway cloning and the entry vectors pENTR5’_ubi [73], p5E-sox10 [17], pME-EGFP [72] and pENTRDsRedEx2 [74]: pDestTol2pA2-ubi/mCherry-nostop/2A-FLAG-tfec-pA, pDestTol2pA2-ubi/EGFP/pA, pDestTol2pA2-sox10/mCherry-nostop/2A-FLAG-mitfa-pA, and pDestTol2pA2-sox10/DsRed/pA. These plasmids were injected as above at 25 ng/μl with 25 ng/μl Tol2 transposase mRNA. Iridophores were counted in the dorsal and ventral stripes of between 26 and 56 larvae.

Transcript detection in whole-mount embryos

Detailed information on the preparation of generic materials and the protocols for performing chromogenic whole-mount in situ hybridisation as well as multiplex fluorescent RNAscope can be found in Petratou et al. (2017) [75]. For whole-mount in situ hybridisation, the probes used were sox10 [20], foxd3 [76], ltk [11], pnp4a [17], aox5 [62], gch2 [62], pou3f1/oct6 [77], dlx2a [78], mitfa [21] and tfec (NM_001030105.2; [14]). For multiplex RNAscope, the following probes were used: ltk (ACD; Cat# 444641), tfec (ACD; Cat# 444701).

Embryos were observed and imaged, and the Pearson’s chi-squared test was used, as previously described [14], to process and statistically analyse results, to test the hypothesis that altered phenotypes correlated with homozygosed mutations. tfap2a and foxd3 mutant embryos
were identified using previously described genotyping protocols [29, 79] following imaging and preparation of genomic DNA [71].

**Alcian blue staining and immunohistochemistry**

Larvae from an intercross of tfec<sup>vc60</sup> heterozygous adults were sorted at 4 days post-fertilization based on the iridophore phenotype, and then were fixed in separate tubes overnight in 4% PFA. Alcian blue staining was then carried out essentially as described [80]. Samples were imaged using an Olympus SZX12 stereomicroscope with DP70 camera.

Immunohistochemistry was carried out as previously described [77]. Primary monoclonal antibodies against HuC/D (Molecular Probes) and Pax7 (Developmental Studies Hybridoma Bank) were used at 1:500 and 1:20 respectively, and goat anti-mouse secondary antibodies conjugated to Alexa 568 or Alexa 488 (Molecular Probes) were each used at 1.750 dilution. For combined tfec whole-mount in situ hybridisation/Pax7 IHC, the Pax7 antibodies were added simultaneously with the anti-Fab fragments [81]. Samples were imaged on a Zeiss Axio Imager.M2 compound microscope with Axiocam 503 colour camera, processed using ZEN software and Adobe Photoshop CC 2018 and 2019.

**Drug treatments**

Larvae from an intercross of tfec<sup>vc60</sup> heterozygous adults were treated with tyrphostin/AG1478 (LC Laboratories) at 2 μM or DMSO from 8 hpf to 48 hpf, with or without the addition of MoTP (Enzo Life Sciences) at 50 μM from 24 to 48 hpf, and examined at 4.5 dpf for recovery of melanocytes.

**Supporting information**

**S1 Fig. Loss of tfec affects adult iridophore pigmentation.** Compared to WT adult (A), G0 tfec crispant (mosaic) adult shows patches of iridophore loss on eye and flank (B). When two tfec CRISPR G0 founder fish were mated, almost all of the offspring lacking iridophores died as larvae, but one escapee survived to adulthood and the absence of iridophores persisted (C). Of the homozygous tfec<sup>vc60</sup> embryos injected with a Tol2 transposon containing the tfec promoter and cDNA and Tol2 transposase mRNA, one survived to adulthood and displayed partial rescue of adult iridophore pigmentation (D). Scale bar: A-D: 0.5 cm; D: 0.35 cm.

**(TIF)**

**S2 Fig. Recovery of melanocyte development in tfec mutants is not dependent upon regeneration from adult melanocyte stem cells.** (A,B) tfec homozygous mutant larvae treated with AG1478 (B) recover melanocytes to levels of DMSO-treated larvae (A) by 4.5 dpf. (C,D) Wild-type larvae, treated with MoTP reagent at an early stage to ablate melanocytes, and with AG1478 (D) show strongly reduced melanocyte numbers by 4.5 dpf, compared to MoTP and DMSO-treated siblings (C). Scale bar: 250 μm.

**(TIF)**

**S3 Fig. The number of tfec+ and pnp4a+ cells is significantly reduced in tfec mutants.** Positive cells were scored along the dorsal and the ventral stripe of known tfec<sup>bact/bact</sup> embryos (red bars) and of wild-type or heterozygous siblings (green bars). *: p-value < 10<sup>-3</sup>.

**(TIF)**

**S4 Fig.**

**(TIF)**
S1 Table. Additional information on the assessment of live embryonic phenotypes. For melanocyte counts at 4 dpf, to derive the average along the lateral stripe, the cells along both stripes of each embryo were independently scored. Presented p-values derived from unpaired two-tailed t-test between WT (or heterozygous for the melanocyte counts) and the genotype corresponding to each row. DS, dorsal stripe; DT, dorsal trunk; H, head; LS, lateral stripe; MP, migration paths; VS, ventral stripe; VT, ventral trunk.

(SDOCX)

S2 Table. Statistics of loss of function experiments. The Pearson’s chi-squared test for goodness of fit is used to calculate the likelihood of a non-WT phenotype, which is consistently present in a number of embryos (1st sub-column of each of the 4 developmental stages) within a batch of WT, heterozygous and homozygous mutant siblings, correlating with homozygosity of the mutant allele in those individuals. Due to the recessive nature of investigated alleles, 25% of embryos in each batch are expected to be homozygous mutants. Therefore, the p-value derived from the chi-squared test indicates whether the number of individuals with an alternative phenotype conform to the expected 25% (null hypothesis), with any deviation being attributable to random chance (p > 0.1), or whether the numbers deviate significantly from the expected (p ≤ 0.1; null hypothesis is rejected). Where observed phenotypes did not significantly correlate with expected Mendelian ratios, i.e. where there is unlikely to be a mutant phenotype, the corresponding counts and p-values are red and bold. For the data on $sox10^{t3/t3}; sox9^{b/p313/fh313}$ double mutants, the orange cells indicate how many embryos in the sample showed the known $sox9^{b/p313/fh313}$ phenotype, the green cells indicate number of embryos with the $sox10^{t3/t3}$ phenotype and blue cells indicate unexpected alternative phenotypes, likely owed to double loss of function.

(SDOCX)

Author Contributions

Conceptualization: Robert N. Kelsh, James A. Lister.

Data curation: Kleio Petratou.

Formal analysis: James A. Lister.

Funding acquisition: Robert N. Kelsh, James A. Lister.

Investigation: Kleio Petratou, Samantha A. Spencer, James A. Lister.

Methodology: Kleio Petratou, Samantha A. Spencer, James A. Lister.

Supervision: Robert N. Kelsh, James A. Lister.

Writing – original draft: Kleio Petratou, Samantha A. Spencer, James A. Lister.

Writing – review & editing: Kleio Petratou, Robert N. Kelsh, James A. Lister.

References

1. Hoekstra H. E., “Genetics, development and evolution of adaptive pigmentation in vertebrates,” Heredity, vol. 97, no. 3, pp. 222–234, 2006. https://doi.org/10.1038/sj.hdy.6800861 PMID: 16823403

2. Parichy D. M. and Spiewak J. E., “Origins of adult pigmentation: diversity in pigment stem cell lineages and implications for pattern evolution.,” Pigment cell & melanoma research, vol. 28, no. 1, pp. 31–50, 2015. https://doi.org/10.1111/pcmr.12332 PMID: 25421288

3. Schartl M., Larue L., Goda M., Bosenberg M. W., Hashimoto H., and Kelsh R. N., “What is a vertebrate pigment cell?,” Pigment Cell & Melanoma Research, vol. 29, no. 1, pp. 8–14, 2016. https://doi.org/10.1111/pcmr.12409 PMID: 26247887
4. Sieber-Blum M. and Cohen A. M., “Clonal analysis of quail neural crest cells: They are pluripotent and differentiate in vitro in the absence of noncrest cells,” Developmental Biology, vol. 80, no. 1, pp. 96–106, 1980. [doi:10.1016/0012-1606(80)90501-1] PMID: 7439536

5. Weston J. A., “Sequential segregation and fate of developmentally restricted intermediate cell populations in the neural crest lineage,” Current topics in developmental biology, vol. 25, pp. 133–53, 1991. [doi:10.1016/s0070-2153(08)60414-7] PMID: 1660392

6. Le Douarin N., Dulac C., Dupin E., and Cameron-Curry P., “Gliai cell lineages in the neural crest,” Glia, vol. 4, no. 2, pp. 175–184, 1991. [doi:10.1002/glia.440040209] PMID: 1827777

7. Serbedzija G. N., Bronner-Fraser M., and Fraser S. E., “Developmental potential of trunk neural crest cells in the mouse,” Development (Cambridge, England), vol. 120, no. 7, pp. 1709–18, 1994. PMID: 7523054

8. Calloni G. W., Le Douarin N. M., and Dupin E., “High frequency of cephalic neural crest cells shows coexistence of neurogenic, melanogenic, and osteogenic differentiation capacities,” Proceedings of the National Academy of Sciences of the United States of America, vol. 106, no. 22, pp. 8947–8952, 2009. [doi:10.1073/pnas.0903780106] PMID: 19447928

9. Soldatov R., Kaucma M., Kastriti M. E., Petersen J., Chontorotzea T., Engimaier L., et al., “Spatiotemporal structure of cell fate decisions in murine neural crest,” Science, vol. 364, no. 6444, 2019. [doi:10.1126/science.aas9536] PMID: 31171666

10. Raible D. W. and Eisen J. S., “Restriction of neural crest cell fate in the trunk of the embryonic zebrafish,” Development, vol. 120, pp. 495–503, 1994. PMID: 8162850

11. Lopes S. S., Yang X., Müller J., Carney T. J., McAdow A. R., Rauch G.-J., et al., “Leukocyte Tyrosine Kinase Functions in Pigment Cell Development,” PLoS Genetics, vol. 4, no. 3, p. 13, 2008. [doi:10.1371/journal.pgen.1000026] PMID: 18369445

12. Singh A. P., Dinwiddie A., Mahalwar P., Schach U., Linker C., Iion U., et al., “Pigment Cell Progenitors in Zebrafish Remain Multipotent through Metamorphosis,” Developmental Cell, vol. 38, no. 3, pp. 316–330, 2016. [doi:https://devcel.2016.06.020] PMID: 27453500

13. Greenhill E. R., Rocco A., Vibert L., Nikaido M., and Kelsh R. N., “An Iterative Genetic and Dynamical Modelling Approach Identifies Novel Features of the Gene Regulatory Network Underlying Melanocyte Development,” PLoS Genetics, vol. 7, no. 9, p. 18, 2011. [doi:10.1371/journal.pgen.1002265] PMID: 21909283

14. Petratou K., Subhankulova T., Lister J. A., Rocco A., Schwetlick H., and Kelsh R. N., “A systems biology approach uncovers the core gene regulatory network governing iridophore fate choice from the neural crest,” PLoS Genetics, vol. 14, no. 10, 2018. [doi:10.1371/journal.pgen.1007402] PMID: 30286071

15. Bagnara J. T., Matsumoto J., Ferris W., Frost S. K., Turner W. A., Tchen T. T., et al., “Common origin of pigment cells…” Science, vol. 203, no. 4379, pp. 410–415, 1979. [doi:10.1126/science.760198] PMID: 760198

16. Curran K., Raible D. W., and Lister J. A., “Foxd3 controls melanophore specification in the zebrafish neural crest by regulation of Mitf.,” Developmental Biology, vol. 332, no. 2, pp. 408–417, 2009. [doi:10.1016/j.ydbio.2009.06.010] PMID: 19527705

17. Curran K., Lister J. A., Kunkel G. R., Prendergast A., Panichy D. M., and Raible D. W., “Interplay between Foxd3 and Mitf regulates cell fate plasticity in the zebrafish neural crest,” Developmental Biology, vol. 344, no. 1, pp. 107–118, 2010. [doi:10.1016/j.ydbio.2010.04.023] PMID: 20460180

18. Kelsh R. N., Brand M., Jiang Y. J., Heisenberg C. P., Lin S., Haftter P., et al., “Zebrafish pigmentation mutations and the processes of neural crest development,” Development, vol. 123, no. 1, pp. 369–389, 1996. PMID: 9007256

19. Kelsh R. N. and Eisen J. S., “The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives,” Development, vol. 127, no. 3, pp. 2581–2590, 2000. PMID: 10631172

20. Dutton K. A., Pauliny A., Lopes S. S., Elworthy S., Carney T. J., Rauch J., et al., “Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates,” Development, vol. 128, no. 21, pp. 4113–4125, 2001. PMID: 11684650

21. Lister J. A., Robertson C. P., Lepage T., Johnson S. L., and Raible D. W., “nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate,” Development, vol. 126, no. 17, pp. 3757–3767, 1999. PMID: 10433906

22. Carney T. J., Dutton K. A., Greenhill E., Delfino-Machín M., Dufourcq P., Blader P., et al., “A direct role for Sox10 in specification of neural crest-derived sensory neurons…” Development (Cambridge, England), vol. 133, no. 23, pp. 4619–30, 2006. [doi:10.1242/dev.02668] PMID: 17065232

23. Delfino-Machín M., Madelaine R., Busolin G., Nikaido M., Colanesi S., Camargo-Sosa K., et al., “Sox10 contributes to the balance of fate choice in dorsal root ganglion progenitors,” PLOS ONE, vol. 12, no. 3, p.e0172947, 2017. [doi:10.1371/journal.pone.0172947] PMID: 28253350
24. Dorsky R. I., Raible D. W., and Moon R. T., “Direct regulation of nacre, a zebrafish MITF homolog required for pigment cell formation, by the Wnt pathway,” Genes & Development, vol. 14, no. 2, pp. 158–162, 2000. PMID: 10652270

25. Elworthy S., Lister J. A., Carney T. J., Raible D. W., and Kelsh R. N., “Transcriptional regulation of mitfa accounts for the sox10 requirement in zebrafish melanoophore development,” Development, vol. 130, no. 12, pp. 2809–2818, 2003. https://doi.org/10.1242/dev.00461 PMID: 12736222

26. Vibert L., Aquino G., Gehring I., Subkankulova T., Schilling T. F., Rocco A., et al., “An ongoing role for Wnt signaling in differentiating melanoocytes in vivo,” Pigment Cell & Melanoma Research, vol. 30, no. 2, pp. 219–232, 2017. https://doi.org/10.1111/pcmr.12568 PMID: 27977907

27. Seberg H. E., Van Otterloo E., Loftus S. K., Liu H., Bonde G., Sompallae R., et al., “TFAP2 paralogs regulate melanoocyte differentiation in parallel with MITF,” PLOS Genetics, vol. 13, no. 3, p. e1006636, 2017. https://doi.org/10.1371/journal.pgen.1006636 PMID: 28249010

28. Montero-Balaguer M., Lang M. R., Sachdev S. W., Knappmeyer C., Stewart R. A., De La Guardia A., Vibert L., Aquino G., Gehring I., Subkan kolova T., Schilling T. F., Rocco A., et al., “An ongoing role for Wnt signaling in differentiating melanoocytes in vivo,” Pigment Cell & Melanoma Research, vol. 30, no. 2, pp. 219–232, 2017. https://doi.org/10.1111/pcmr.12568 PMID: 27977907

29. Seberg H. E., Van Otterloo E., Loftus S. K., Liu H., Bonde G., Sompallae R., et al., “TFAP2 paralogs regulate melanoocyte differentiation in parallel with MITF,” PLOS Genetics, vol. 13, no. 3, p. e1006636, 2017. https://doi.org/10.1371/journal.pgen.1006636 PMID: 28249010

30. Cooper C. D., Linbo T. H., and Raible D. W., “Kit and foxd3 genetically interact to regulate melanoophore morphology and development,” Developmental Dynamics, vol. 235, no. 12, pp. 3199–3212, 2006. https://doi.org/10.1010/dvdy.2005.3212 PMID: 17013879

31. Lukosevicu M., Gavriouchkina D., Williams R. M., Hochgreb-Hagel T., Senanayake U., Chong-Morison V., et al., “From Pioneer to Repressor: Bimodal foxd3 Activity Dynamically Remodels Neural Crest Regulatory Landscape In Vivo,” Developmental Cell, vol. 47, no. 5, pp. 608–628, 2018. https://doi.org/10.1016/j.devcel.2018.11.009 PMID: 30513393

32. Ng A., Uribe R. A., Yieh L., Nuckels R., and Gross J. M., “Zebra fish mutations in gart and paics identify crucial roles for de novo purine synthesis in the retina and additional photoreceptor development,” Developmental Biology, vol. 363, no. 1, pp. 85–86, 2012. https://doi.org/10.1016/j.ydbio.2012.01.035 PMID: 223862018

33. Clancey L. F., Beirl A. J., Linbo T. H., and Cooper C. D., “Maintenance of Melanophore Morphology and Survival Is Cathepsin and vps11 Dependent in Zebrafish,” PLoS ONE, vol. 8, no. 5, 2013. https://doi.org/10.1371/journal.pone.0065096 PMID: 23862018

34. Krauss J., Astrinidis P., Astrinides P., Frohnhoer H. G., Walderich B., and Nusslein-Volhard C., “Transparent parent, a gene affecting stripe formation in Zebrafish, encodes the mitochondrial protein Mpv17 that is required for iridophore survival,” Biology open, vol. 2, no. 7, pp. 703–710, 2013. https://doi.org/10.1242/bio.20135132 PMID: 23932105

35. D’Agati G., Beltre R., Sessa A., Burger A., Zhou Y., Mosimann C., et al., “A defect in the mitochondrial protein Mpv17 underlies the transparent casper zebrafish,” Developmental Biology, vol. 430, no. 1, pp. 29–36, 2018. https://doi.org/10.1016/j.ydbio.2018.01.003 PMID: 29706315

36. Rodrigues F. S. L. M., Yang X., Nikaido M., Liu Q., and Kelsh R. N., “A Simple, Highly Visual in Vivo Screen for Anaplastic Lymphoma Kinase Inhibitors,” ACS Chemical Biology, vol. 7, no. 12, pp. 1968–1974, 2012. https://doi.org/10.1021/cb300361a PMID: 22985331

37. Fadeev A., Krauss J., Singh A. P., and Nüsslein-Volhard C., “Zebrafish Leucocyte tyrosine kinase controls iridophore establishment, proliferation and survival,” Pigment Cell & Melanoma Research, vol. 29, no. 3, pp. 284–96, 2016. https://doi.org/10.1111/pcmr.12454 PMID: 26801003

38. Mo E. S., Cheng Q., Reshetnyak A. V., Schlessinger J., and Nicoli S., “Alk and Ltk ligands are essential for iridophore development in zebrafish mediated by the receptor tyrosine kinase Ltk,” Proceedings of the National Academy of Sciences of the United States of America, vol. 114, no. 45, pp. 12027–12032, 2017. https://doi.org/10.1073/pnas.1702541114 PMID: 29078341

39. Lister J. A., Lane B. M., Nguyen A., and Lunney K., “Embryonic expression of zebrafish MIT family genes tfe3b, tfe3c, and tfec,” Developmental Dynamics, vol. 240, no. 11, pp. 2529–238, 2011. https://doi.org/10.1002/dvdy.22743 PMID: 21932325

40. Higdon C. W., Mitra R. D., and Johnson S. L., “Gene expression analysis of zebrafish melanocytes, iridophores, and retinal pigmented epithelium reveals indicators of biological function and developmental origin,” PLoS One, vol. 8, no. 7, p. e67801, 2013. https://doi.org/10.1371/journal.pone.0067801 PMID: 23874447

41. Simões-Costa M., Bronner M. E., Agarwal P., Verzi M. P., Nguyen T., Hu J., et al., “Establishing neural crest identity: a gene regulatory recipe,” Development (Cambridge, England), vol. 142, no. 2, pp. 242–57, 2015. https://doi.org/10.1242/dev.105445 PMID: 25564621
42. Miesfeld J. B., Gestri G., Clark B. S., Flinn M. A., Poole R. J., Bader J. R., et al., “Yap and Taz regulate retinal pigment epithelial cell fate,” Development (Cambridge), vol. 142, no. 17, pp. 3021–3032, 2015. https://doi.org/10.1242/dev.119008 PMID: 26209646

43. Budi E. H., Patterson L. B., and Parichy D. M., “Embryonic requirements for ErbB signaling in neural crest development and adult pigment pattern formation,” Development, vol. 135, no. 15, pp. 2603–2614, 2008. https://doi.org/10.1242/dev.019299 PMID: 18508863

44. Hultman K. A., Budi E. H., Teasley D. C., Gottlieb A. Y., Parichy D. M., and Johnson S. L., “Defects in ErbB-Dependent Establishment of Adult Melanocyte Stem Cells Reveal Independent Origins for Embryonic and Regeneration Melanocytes,” PLoS Genetics, vol. 5, no. 7, p. 13, 2009. https://doi.org/10.1371/journal.pgen.1000544 PMID: 19578401

45. Saunders L. M., Mishra A. K., Aman A. J., Lewis V. M., Toomey M. B., Packer J. S., et al., “Thyroid hormone regulates distinct paths to maturation in pigment cell lineages,” eLife, vol. 8, e45181, 2019. https://doi.org/10.7554/eLife.45181 PMID: 31140974

46. Mahony C. B., Fish R. J., Pasche C., and Bertrand J. Y., “tfec controls the hematopoietic stem cell vascular niche during zebrafish embryogenesis,” Blood, vol. 128, no. 10, pp. 1336–45, 2016. https://doi.org/10.1182/blood-2016-04-710137 PMID: 27402973

47. Bharti K., Gasper M., Ou J., Brucato M., Clore-Grenenborn K., Pickel J., et al., “A regulatory loop involving PAX6, MITF, and WNT signaling controls retinal pigment epithelium development,” PLoS Genetics, vol. 8, no. 7, 2012.

48. Sinagoga K. L., Larimer-Picciani A. M., George S. M., Spencer S. A., Lister J. A., and Gross J. M., “Mitf-family transcription factor function is required within cranial neural crest cells to promote choroid fissure closure,” Development, p. dev. 187047, 2020. https://doi.org/10.1242/dev.187047 PMID: 32541011

49. Arduini B. L., Bosse K. M., and Henion P. D., “Genetic ablation of neural crest cell diversification,” Development (Cambridge, England), vol. 136, no. 12, pp. 1987–94, 2009. https://doi.org/10.1242/dev.033209 PMID: 19439494

50. Cheung M. and Briscoe J., “Neural crest development is regulated by the transcription factor Sox9,” Development (Cambridge, England), vol. 130, no. 23, pp. 5681–93, 2003. https://doi.org/10.1242/dev.00808 PMID: 14522876

51. Betancur P., Bronner-Fraser M., and Sauka-Spengler T., “Genomic code for Sox10 activation reveals a restricted leucine zipper dimerization and specificity of DNA recognition of the melanocyte master regulator MITF,” Genes and Development, vol. 26, no. 2, pp. 568–583, 2012. https://doi.org/10.1101/gad.198192.112 PMID: 23207919

52. Li W. and Cornell R. A., “Redundant activities of Ttap2a and Ttap2c are required for neural crest induction and development of other non-neural ectoderm derivatives in zebrafish embryos,” Developmental Biology, vol. 304, no. 1, pp. 338–354, 2007. https://doi.org/10.1016/j.ydbio.2006.12.042 PMID: 17258188

53. Wang W. Der, Melville D. B., Montero-Balaguer M., Hatzopoulos A. K., and Knapik E. W., “Ttap2a and Foxd3 regulate early steps in the development of the neural crest progenitor population,” Developmental Biology, vol. 360, no. 1, pp. 173–185, 2011. https://doi.org/10.1016/j.ydbio.2011.09.019 PMID: 21963426

54. Dooley C. M., Wali N., Sealy I. M., White R. J., Stemple D. L., Collins J. E., et al., “The gene regulatory network distinguishing PAX6, MITF, and WNT signaling controls retinal pigment epithelium development,” PLoS Genetics, vol. 5, no. 7, p. 13, 2009. https://doi.org/10.1371/journal.pgen.1000544 PMID: 19578401

55. Knight R. D., Javidan Y., Nelson S., Zhang T., and Schilling T., “Skeletal and pigment cell defects in the lockjaw mutant reveal multiple roles for zebrafish tta2a in neural crest development,” Developmental Dynamics, vol. 229, no. 1, pp. 87–98, 2004. https://doi.org/10.1002/dvdy.10494 PMID: 14699580

56. Ignatius M. S., Moose H. E., El-Hodri H. M., and Henion P. D., “colgate/hdac1 Repression of foxd3 expression is required to permit mitfa-dependent melanogenesis,” Developmental Biology, vol. 313, no. 2, pp. 568–583, 2008. https://doi.org/10.1016/j.ydbio.2007.07.045 PMID: 18068699

57. Hemeshah T. J., Steingrimsson E., McGill G., Hansen M. J., Vaught J., Hodgkinson C. A., et al., “microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family,” Genes & development, vol. 8, no. 22, pp. 2770–80, 1994. https://doi.org/10.1101/gad.8.22.2770 PMID: 7958932

58. Pogenberg V., Ögmundsdóttir M. H., Bergsteinsdóttir K., Schepsky A., Phung B., Deineko V., et al., “Restricted leucine zipper dimerization and specificity of DNA recognition of the melanocyte master regulator MITF,” Genes and Development, vol. 26, no. 23, pp. 2647–2658, 2012. https://doi.org/10.1101/gad.198192.112 PMID: 23207919

59. Minchin J. E. N. and Hughes S. M., “Sequential actions of Pax3 and Pax7 drive xanthophore development in zebrafish neural crest,” Developmental Biology, vol. 317, no. 2, pp. 508–522, 2008. https://doi.org/10.1016/j.ydbio.2008.02.058 PMID: 18417109
60. Nord H., Dennhag N., Muck J., and Von Hofsten J., “Pax7 is required for establishment of the xanthophore lineage in zebrafish embryos,” Molecular Biology of the Cell, vol. 27, no. 11, pp. 1853–1862, 2016. https://doi.org/10.1091/mbc.E15-12-0821 PMID: 27053658

61. Lang D., Lu M. M., Huang L., Engleka K. A., Zhang M., Chu E. Y., et al., “Pax3 functions at a nodal point in melanocyte stem cell differentiation,” Nature, vol. 433, no. 7028, pp. 884–887, 2005. https://doi.org/10.1038/nature03292 PMID: 15729346

62. Parichy D. M., Ransom D. G., Paw B., Zon L. I., and Johnson S. L., “An orthologue of the kit-related gene fms is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio,” Development, vol. 127, no. 14, pp. 3031–3044, 2000. PMID: 10862741

63. Kimmel C. B., Ballard W. W., Kimmel S. R., Ullmann B., and Schilling T. F., “Stages of embryonic development of the zebrafish,” Developmental Dynamics, vol. 203, no. 3, pp. 253–310, 1995. https://doi.org/10.1002/aaja.1002030302 PMID: 8589427

64. Manfroid I., Ghaye A., Naye F., Detry N., Palm S., Pan L., et al., “Zebrafish sox9b is crucial for hepato-pancreatic duct development and pancreatic endocrine cell regeneration,” Developmental Biology, vol. 366, no. 2, pp. 268–78, 2012. https://doi.org/10.1016/j.ydbio.2012.04.002 PMID: 22537488

65. Delous M., Yin C., Shin D., Ninov N., Debrito Carten J., Pan L., et al., “sox9b is a key regulator of pancreaticobiliary ductal system development,” PLoS Genetics, vol. 8, no. 6, 2012. https://doi.org/10.1371/journal.pgen.1002754 PMID: 22719264

66. Schilling T. F., Piotrowski T., Grandel H., Brand M., Heisenberg C. P., Jiang Y. J., et al., “Jaw and branchial arch mutants in zebrafish I: Branchial arches,” Development, vol. 123, pp. 3031–3044, 1996. PMID: 9007253

67. Montague T. G., Cruz J. M., Gagnon J. A., Church G. M., and Valen E., “CHOPCHOP: A CRISPR/Cas9 and TALEN web tool for genome editing,” Nucleic Acids Research, vol. 42, no. W1, 2014. https://doi.org/10.1093/nar/gku410 PMID: 24861617

68. Talbot J. C. and Amacher S. L., “Supplementary methods: A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles,” Zebrafish, vol. 11, no. 6, 2014.

69. Jao L. E., Wente S. R., and Chen W., “Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system,” Proceedings of the National Academy of Sciences of the United States of America, vol. 110, no. 34, pp. 13904–13909, 2013. https://doi.org/10.1073/pnas.1308335110 PMID: 23918387

70. Lafave M. C., Varshney G. K., Vemulapalli M., Mullikin J. C., and Burgess S. M., “A Defined Zebrafish Line for High-Throughput Genetics and Genomics: NHGRI-1,” Genetics, vol. 198, no. 1. Genetics Society of America, pp. 167–170, 11-Apr-2014. https://doi.org/10.1534/genetics.114.166769 PMID: 25009150

71. Meeker N. D., Hutchinson S. A., Ho L., and Trede N. S., “Method for isolation of PCR-ready genomic DNA from zebrafish tissues,” BioTechniques, vol. 43, no. 5, pp. 610–614, 2007. https://doi.org/10.2144/000112619 PMID: 18072590

72. Kwan K. M., Fujimoto E., Grabher C., Mangum B. D., Hardy M. E., Campbell D. S., et al., “The Tol2kit: A multisite gateway-based construction Kit for Tol2 transposon transgenesis constructs,” Developmental Dynamics, vol. 236, no. 11, pp. 3088–3099, 2007. https://doi.org/10.1002/dvdy.21343 PMID: 17937395

73. Mosimann C., Kaufman C. K., U.P., Pugach E. K., Tampii O. J., and Zon L. I., “Ubiquitous transgene expression and Cre-based recombination driven by the ubiquitin promoter in zebrafish,” Development, vol. 138, no. 1, pp. 169–177, 2011. https://doi.org/10.1242/dev.059345 PMID: 21138979

74. Villefranc J. A., Amigo J., and Lawson N. D., “Gateway compatible vectors for analysis of gene function in the zebrafish,” Developmental Dynamics, vol. 236, no. 11, pp. 3077–3087, 2007. https://doi.org/10.1002/dvdy.21354 PMID: 17948311

75. Petratou K., Sosa K. C., Al Jabri R., Nagao Y., and Kelsh R. N., “Neural Crest Methodologies in Zebrafish and Medaka: Transcript and protein detection methodologies for neural crest research on whole mount zebrafish and medaka.” Springer, 20-Dec-2017. https://doi.org/10.1111/pcmr.12580 PMID: 28182333

76. Odenthal J. and Nüsslein-Volhard C., “fork head domain genes in zebrafish,” Development and Evolution, vol. 208, no. 5, pp. 245–58, 1998. https://doi.org/10.1007/s004270050179 PMID: 9683740

77. Lister J. A., Cooper C., Nguyen K., Modrell M., Grant K., and Raible D. W., “Zebrafish FoxD3 is required for development of a subset of neural crest derivatives,” Developmental Biology, vol. 159, no. 1, pp. 50–59, 2006. https://doi.org/10.1016/j.ydbio.2005.11.014 PMID: 16364284

78. Akimenko M. A., Ekker M., Wegner J., Lin W., and Westerfield M., “Combinatorial expression of three zebrafish genes related to distal-less: Part of a homeobox gene code for the head,” Journal of
79. Knight R. D., Nair S., Nelson S. S., Afshar A., Javidan Y., Geisler R., et al., “lockjaw encodes a zebrafish tfap2a required for early neural crest development,” Development, vol. 130, no. 23, pp. 5755–5768, 2003. https://doi.org/10.1242/dev.00575 PMID: 14534133

80. Walker M. B. and Kimmel C. B., “A two-color acid-free cartilage and bone stain for zebrafish larvae,” Biotechnic and Histochemistry, vol. 82, no. 1, pp. 23–28, 2007. https://doi.org/10.1080/10520290701333558 PMID: 17510811

81. Van Otterloo E., Li W., Bonde G., Day K. M., Hsu M.-Y., and Cornell R. A., “Differentiation of zebrafish melanophores depends on transcription factors AP2 alpha and AP2 epsilon,” PLoS genetics, vol. 6, no. 9, p. e1001122, 2010. https://doi.org/10.1371/journal.pgen.1001122 PMID: 20862309