Genetics and Genomics

Identifying *in vivo* genetic dependencies of melanocyte and melanoma development

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Abstract

The advent of large-scale sequencing in both development and disease has identified large numbers of candidate genes that may be linked to important phenotypes. Validating the function of these candidates *in vivo* is challenging, due to low efficiency and low throughput of most model systems. We have developed a rapid, scalable system for assessing the role of candidate genes using zebrafish. We generated transgenic zebrafish in which Cas9 was knocked-in to the endogenous *mitfa* locus, a master transcription factor of the melanocyte lineage. We used this system to identify both cell-autonomous and non-cell autonomous regulators of normal melanocyte development. We then applied this to the melanoma setting to demonstrate that loss of genes required for melanocyte survival can paradoxically promote more aggressive phenotypes, highlighting that *in vitro* screens can mask *in vivo* phenotypes. Our high-efficiency genetic approach offers a versatile tool for exploring developmental processes and disease mechanisms that can readily be applied to other cell lineages.

eLife Assessment

This **important** study presents a new method for generating cell-type restricted knockouts in zebrafish and it reports several interesting applications of this method to study pigmentation and melanomagenesis. The evidence supporting the conclusions is **convincing**, with rigorous characterization of several knock out mutations that provide a proof of principle. The work will be of broad interest to cell, skin, and cancer biologists.

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Introduction

Efficient cell type-specific knockout of genes is crucial for unraveling the intricate molecular mechanisms underlying cell function, development, and transformation. Zebrafish (*Danio rerio*) have emerged as a powerful genetic model organism for studying these processes. The large number of animals that can be easily generated make it advantageous (compared to other systems like mice) for querying large numbers of genetic variants that may have links to human diseases. However, most studies in this system use germline knockout, which makes it difficult to determine whether the identified gene has a specific function in a particular lineage, or whether the phenotype reflects a more general developmental defect. Furthermore, many genes which might have lineage specific functions cannot be studied using germline knockout, since embryonic lethality often accompanies global knockout of essential genes[1]. To overcome these limitations, several groups have explored tissue-specific and conditional knockout strategies in zebrafish[2–10]. For example, the MAZERATI system uses tissue-specific promoter transgenes to drive Cas9 expression, which are introduced via plasmid injection into one-cell stage embryos[3,11]. Despite these advancements, efficiency remains of paramount importance. In normal development, low-efficiency knockout of a gene can mask important phenotypes, as the mutant cells can readily be outcompeted by wild-type or inframe mutations. Efficiency is even more important in the cancer context. Mutation of a tumor suppressor (i.e., PTEN) need not be highly efficient, since clones with a Darwinian advantage will inevitably outcompete other clones. However, in situations where we want to study necessity of a tumor promoting gene *in vivo* (i.e., an oncogene), cells which have mutations of a key driver gene will easily be outcompeted by nearby clones. Such efficiency is critical as sequencing projects such as All of Us, the UK Biobank and the Cancer DepMap continue to identify hundreds of new candidate genes on a regular basis, many of which have yet to be characterized *in vivo*[12–14].

Skin color pigmentation is amongst the most heterogeneous of genetic systems, with a large number of loci linked to melanocyte development. Mutations in pleiomorphic genes such as *sox10, ednrb, jam3b, tuba8l3, meox1* and *kit* yield clear defects in melanocyte/melanophore development, but these mutants also have defects in other tissues[15–21]. Many of these genes are also expressed in melanoma, but their specific *in vivo* function in tumorigenesis remains poorly understood.

In this study, we wished to develop a highly efficient and scalable system to understand *in vivo* genetic dependencies, using melanocytes and melanoma as an exemplar. By integrating the Cas9 nuclease into the endogenous melanocyte-specific *mitfa* locus, we achieved highly efficient and specific gene disruption across the melanocyte developmental lineage. We used our *mitfa*^{Cas9} knockin animals to inactivate the pigmentation gene *albino* and the embryonic essential genes *sox10, tuba1a,* and *ptena/b* in melanocytes without additional developmental or off-target phenotypes. We also demonstrated that this system can be used to efficiently induce melanomas in wild-type fish by inactivating the tumor suppressors *tp53* and *ptena/b* within melanocytes also expressing the melanoma oncogene BRAF^{V600E}. Inactivating the neural crest transcription factor *sox10* in these tumors significantly decreased melanoma initiation but resulted in rare invasive tumors that highly expressed the *sox9* transcription factor, highlighting that unexpected *in vivo* phenotypes can emerge that aren't predicted from *in vitro* studies[22,23]. Our system can thus be used to advance our understanding of melanocyte biology and the genetic underpinnings of phenotypic switching in melanoma, an approach that can readily be used for other cell types.

Results

Targeted integration of Cas9 to the *mitfa* locus

To selectively disrupt genes in a melanocyte lineage-specific manner, we established a transgenic zebrafish line harboring Cas9 at the endogenous *mitfa* locus. *Mitfa* expression is detectable by 18 hours postfertilization (hpf) and is highly expressed in pigment cells including melanocytes, xanthophores and their progenitors. To achieve knock-in, we utilized the GeneWeld method, which enables efficient integration of DNA cassettes using homology mediated end joining (HMEJ)[24,25]. The knock-in cassette, containing Cas9 and BFP driven by the eye-specific γ-crystallin promoter, was targeted to exon 2 of the *mitfa* gene, which leads to disruption of the endogenous *mitfa* gene (**Figure 1A**). We strategically selected this genomic site for several reasons. First, this is the sole exon shared among the three *mitfa* protein-coding transcripts. Furthermore, targeting Cas9 to exon 2 offers the flexibility of replicating this knock-in strategy in *casper* zebrafish, a transparent fish widely used for melanoma studies that harbor a premature stop codon in *mitfa* exon 3[26].

The knock-in vector, along with Cas9 protein and guide RNAs targeting either the *mitfa* integration site or the sequences flanking the 5' and 3' homology arms, were injected into 1-cell stage wild-type Tropical 5D (T5D) zebrafish embryos (**Figure 1B**)[27]. An average of 19% of injected embryos had detectable ocular BFP expression at 3 days postfertilization (dpf), indicating successful integration (Figure S1A). Embryos were raised to adulthood then crossed with WT T5D fish. The resulting F1 embryos were screened for BFP+ eyes and tail clippings were sequenced to confirm precise integration of the knock-in cassette at the *mitfa* locus. We sequenced four F1 fish and detected on-target integration at the *mitfa* locus in all four fish (Figure S1B). In zebrafish, one copy of *mitfa* is sufficient for the normal development of neural crest-derived melanocytes, but loss of both copies of *mitfa* results in loss of the melanocyte lineage[26,28]. To determine the effect of a single copy of *mitfa*^{Cas9} on melanocytes, *mitfa*^{Cas9} fish were in-crossed and the resulting siblings were imaged at various time points (Figure S1C). WT and *mitfa*^{Cas9} fish exhibited indistinguishable melanocyte patterning during both embryonic and adult stages. However, as expected, fish carrying two copies of Cas9 (*mitfa*$^{Cas9/Cas9}$) resembled the *nacre* mutant (*mitfa*-/-), characterized by complete loss of melanocytes, indicating the endogenous *mitfa* gene is disrupted on knock-in alleles[28]. We used zebrafish harboring one knock-in allele (*mitfa*^{Cas9}) for all subsequent experiments to allow us to test gene function in melanocytes.

To assess whether Cas9 expression was restricted to melanocytes, we performed fluorescence in situ hybridization (FISH) on 3dpf WT and *mitfa*^{Cas9} embryos to measure *mitfa* and Cas9 mRNA expression (**Figure 1C** and S1D). In both WT and *mitfa*^{Cas9} fish, *mitfa* was detected within the embryonic melanocyte stripe regions as expected. In *mitfa*^{Cas9} fish, Cas9 mRNA was detected only within melanocyte stripe regions also expressing *mitfa*. These results provide functional validation for on-target integration and tissue-restricted expression of Cas9 to the melanocyte lineage.

Melanocyte-specific loss of the human skin color associated gene *slc45a2* results in robust loss of pigmentation

To assess the effectiveness of our stable *mitfa*^{Cas9} line, we first targeted the *albino* gene (*slc45a2*). Large scale GWAS studies have repeatedly identified SNPs in this gene as tightly linked to human skin color variation, and germline disruption leads to a complete loss of pigmentation in melanocytes[29–31]. This distinct phenotype enables the visual observation of Cas9 activity in zebrafish injected with *albino* gRNA. To test our system, we designed plasmids, denoted MG-gRNA, containing a zU6:gRNA cassette followed by *mitfa*:GFP to enable visualization of cells expressing

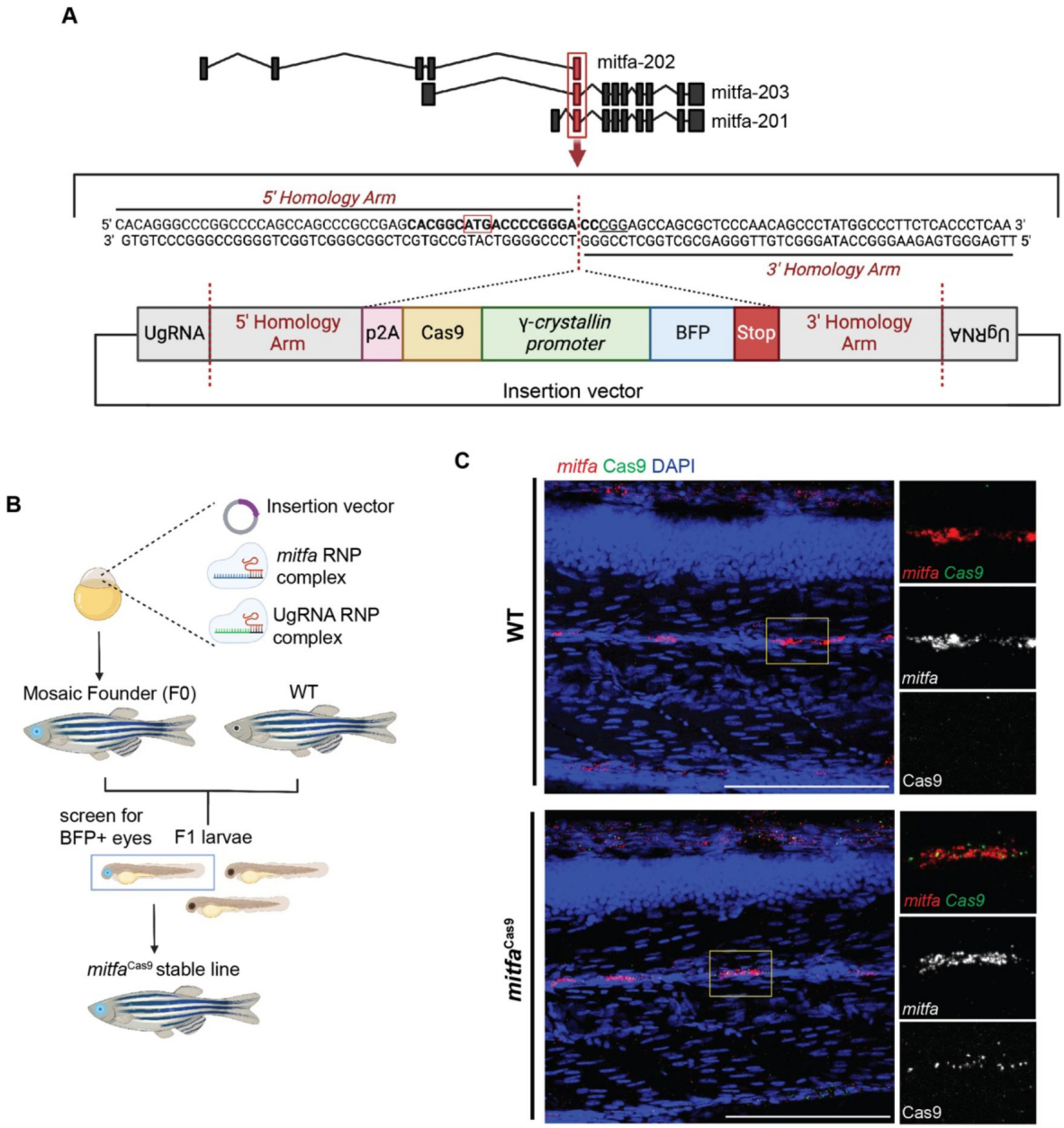

Figure 1.

Targeted integration of Cas9 to the *mitfa* locus with GeneWeld knockin system.

(**A**) Schematic depicting integration site and GeneWeld knock-in cassette. 5′ and 3′ homology arms are designed to target exon 2 of the zebrafish *mitfa* gene. The knock-in cassette includes p2A followed by Cas9 and BFP driven by the eye-specific promoter gamma-crystallin. **(B)** Pipeline to produce F1 mitfa^{Cas9} zebrafish. The GeneWeld knock-in vector and gRNAs targeting the *mitfa* genomic insertion site or specific sites on the knock-in vector (UgRNA) are co-injected into one-cell stage wild-type zebrafish embryos. Embryos are screened for BFP+ eyes marking mosaic integration. These mosaic founder fish are then raised to adulthood and crossed with wild-type fish. The resulting embryos are screened for BFP+ eyes and sequenced to confirm precise integration. **(C)** Fluorescent in situ hybridization chain reaction (FISH) on 3dpf wild-type and *mitfa*^{Cas9} embryos treated with 1-phenyl 2-thiourea (PTU). The presence of *mitfa* and Cas9 RNA was assessed by confocal microscopy at 40x magnification. Maximum intensity projections are shown. n=17 WT embryos and n=16 mitfa^{Cas9} embryos were screened. Scale bars, 100μm. Created with BioRender.com.

the *albino* gRNA. MG-*albino* and MG-NT were injected into one-cell stage embryos from crosses between *mitfa*^{Cas9} and WT fish, which express Cas9 in all melanocytes but exhibit mosaic gRNA expression (**Figure 2A**).

Embryos were screened for BFP+ eyes to identify those harboring *mitfa*^{Cas9}. Upon adulthood, 92% of F0 MG-*albino* fish had mosaic loss of pigmentation within the melanocyte stripe regions (**Figure 2B and C**). Unpigmented regions maintained GFP expression (**Figure 2B**), indicating that the breaks in melanocyte stripes arise from loss of pigmentation in melanocytes rather than loss of the melanocyte lineage (**Figure 2B**). As expected, control MG-NT clutch mates displayed normal pigmentation and GFP+ melanocytes were indistinguishable from GFP-regions on the same fish (**Figure 2B and C**).

We next aimed to determine the efficiency of *mitfa*^{Cas9} in the F1 generation, in which every melanocyte stably expresses the *albino* gRNA and *mitfa*^{Cas9}. To generate F1 lines, we outcrossed the F0 MG-gRNA fish to WT and sorted embryos for BFP+ eyes and GFP+ melanocytes. F1 MG-*albino* fish exhibited a near complete loss of pigmentation, demonstrating that our *mitfa*^{Cas9} system is highly efficient in F1 fish (**Figure 2D and E**). To study the effect of *albino* inactivation across various stages of development, F1 MG-gRNA fish were once again outcrossed to WT, generating F2 MG-gRNA fish expressing one copy of *mitfa*^{Cas9}. Imaging revealed a measurable pigmentation loss in F2 MG-*albino* melanocytes as early as 3 days post-fertilization (dpf) (**Figure 2F and G**). F2 MG-*albino* fish maintained loss of pigmentation throughout development, indicating the sustained activity of *mitfa*^{Cas9} (Figure S2A-D).

To confirm on-target cutting of the *albino* gene in the melanocyte lineage (and not other tissues), the skin from F1 MG-*albino* fish was dissected and GFP+ and GFP-cells were isolated using FACS (**Figure 2H**). GFP+ cells encompass *mitfa*-expressing melanocytes, xanthophores, and their progenitors, while GFP-cells represent other non-melanocytic, non-*mitfa* expressing skin cells, such as keratinocytes, fibroblasts, and immune cells. We performed CRISPR-seq on DNA from both populations to directly measure Cas9 efficiency and specificity, quantifying the proportion of indels at the *albino* locus. Although we have previously shown that FACS sorting melanocytic cells is possible, it is important to note that we often observe high levels of contaminating keratinocytes in FACS sorted melanocyte populations, which may lead to an underestimation of the true allelic frequency in our CRISPR-seq[32]. Despite this, robust inactivation of the *albino* gene was observed in GFP+ (*mitfa*-expressing) cells, confirming somatic inactivation (**Figure 2I**). The majority of indels were frame-shift inducing mutations. A likely PCR or sequencing artifact resulting in a “T” insertion was excluded from the frameshift indel percentage as it was observed in all conditions including WT fish with no pigmentation phenotype (Figure S2E). These results confirm that our *mitfa*^{Cas9} system allows us to inactivate genes within the melanocytic lineage *in vivo* in a highly efficient and specific manner.

The neural crest-related gene *sox10* has specific function in adult melanocyte patterning and regeneration

Having confirmed that our *mitfa*^{Cas9} system allows us to rapidly, efficiently and specifically manipulate melanocyte gene expression, we next wanted to target embryonic essential genes. Detecting “negative” gene knockout phenotypes such as cell death can be challenging *in vivo* due to the selective advantage that WT cells have over mutants. Current tissue specific knockout systems lack the sensitivity to detect negative phenotypes.

To assess the effectiveness of our *mitfa*^{Cas9} model in detecting negative phenotypes, we utilized it to target *sox10*, a transcription factor vital to the neural crest lineage and indispensable for overall survival of both zebrafish and mice[15,33]. Consequently, a global knockout approach is not feasible for studying the function of *sox10* in adult melanocytes *in vivo*. Germline knockout of *sox10* in zebrafish leads to severe defects in peripheral nerve development and the complete

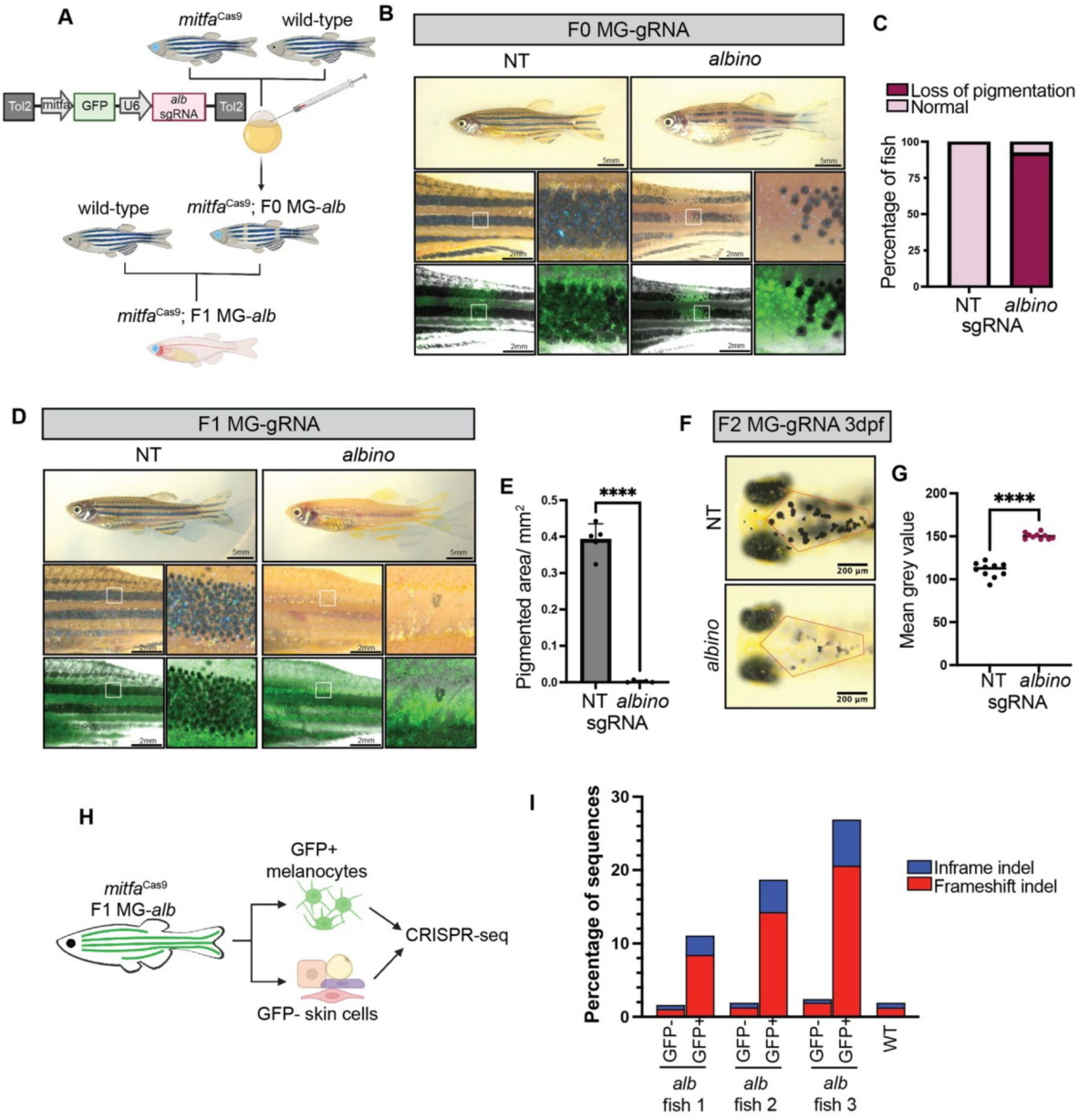

Figure 2.

Melanocyte lineage-specific knockout of *albino* using *mitfa*^{Cas9} fish.

(A) Pipeline to generate F0 and F1 *mitfa*:GFP;U6:gRNA (MG-gRNA) zebrafish. **(B)** Adult mitfa^{Cas9} MG-NT and MG-*albino* F0 fish. **(C)** Proportion of MG-NT (n=19) and MG-*albino* (n=25) F0 fish with loss of pigmentation phenotype. **(D)** Adult mitfa^{Cas9} MG-NT and MG-*albino* F1 fish. **(E)** Pigmented area/ mm^2 calculated for n=5 fish/ genotype within a defined rectangular region of interest (ROI) encompassing the top and middle melanocyte stripes. Two-sided student's *t*-test was used to assess statistical significance, **** $p < 0.0001$; error bars, SD. **(F)** 3dpf mitfa^{Cas9} MG-NT and MG-*albino* F2 fish. Representative embryos are shown for each genotype. **(G)** Mean grey value of head melanocytes calculated for n=10 embryos/genotype within a defined hexagonal ROI indicated as a red outline in 2F. Two-sided student's *t*-test was used to assess statistical significance, **** $p < 0.0001$. **(H)** Schematic for CRISPR sequencing protocol. **(I)** CRISPR-seq results are shown for WT and n=3 independent F1 MG-*albino* fish. Only GFP negative cells were isolated from WT fish. Results are shown as a fraction of sequences with indels calculated using CRISPRESSO. Created with BioRender.com.

absence of melanocytes, and the animals die around day 14[15,34,35]. Due to the complete absence of melanocytes and their precursor cells, melanoblasts, in *sox10*$^{-/-}$ fish, it has not been possible to study the specific role of *sox10* on these more differentiated cell types *in vivo*. One of the key unanswered questions is how *sox10* balances its role in maintaining melanoblast stemness with promoting terminal differentiation, particularly in the context of adult melanocyte regeneration.

We leveraged our *mitfa*^{Cas9} system to specifically inactivate *sox10* in the melanocyte lineage. *mitfa*^{Cas9} fish injected with a MG-*sox10* plasmid exhibited noticeable gaps in their melanocyte stripes (**Figure 3A-B**). Germline deletion of a *sox10* enhancer region similarly affects adult stripe patterning in zebrafish[36]. In contrast to the *albino* F0 fish, the gaps in the stripes of the *sox10* F0 fish were not occupied by unpigmented GFP+ melanocytes, indicating a potential defect in the survival or differentiation of melanocytes upon loss of *sox10*. This phenotype became even more pronounced in the stable F1 lines, where several gaps are visible (**Figure 3C**). Zebrafish have four dark stripes occupied by melanocytes, which alternate with light colored interstripes occupied by yellow pigmented xanthophores[37]. Typically, melanocyte stripe 1D (dorsal) is wider than the interstripe X0 directly below it, as observed in MG-NT F1 fish (**Figure 3C and D**). However, the opposite is true for MG-*sox10* F1 fish, where we observed an apparent widening of the interstripe region and narrowing of the melanocyte stripes (**Figure 3C and D**). Melanocytes were counted in fish treated with epinephrine to aggregate melanosomes, revealing a significant reduction in the number of melanocytes in the F1 *sox10* KO fish compared to F1 NT fish (**Figure 3E**). Reduction in GFP+ cells within the xanthophore interstripes (X0) suggested that *sox10* deficiency also negatively affected xanthophore populations, in line with previous work indicating a role for *sox10* in specifying both xanthophores and melanocytes in zebrafish embryos[38].

Melanocytes continually regenerate throughout life, typically from a progenitor population of melanoblasts or melanocyte stem cells. We next assessed whether *sox10* was required for this. We treated adult fish with neocuproine, a copper chelator previously shown to kill mature, pigmented melanocytes[39]. We then compared regeneration from these progenitors in MG-*sox10* and MG-NT F1 fish by counting the percentage of melanocytes that emerged after 7, 15, and 70 days (**Figure 3F**). At days 7 and 15, when the melanocytes were still in the process of regenerating from melanoblasts, there were no significant differences between the two groups. However, by the time regeneration was completed we found a significant disparity between the regenerative potential of MG-NT and MG-*sox10* fish. At 70 days post-neocuproine treatment, MG-NT fish had regenerated an average of 82% of their melanocytes, whereas only 53% of melanocytes had regenerated in MG-*sox10* fish (**Figures 3G-H**). These findings demonstrate that *sox10* is required for adult melanocyte regeneration, highlighting its requirement within melanoblast populations outside of neural crest specification.

Non-autonomous functions of *tuba1a* on melanocytes

One of the significant challenges of conventional global knockout methods is the inability to differentiate between cell-autonomous and non-autonomous phenotypes. This distinction is particularly crucial in melanocyte studies due to their direct and indirect interactions with diverse cell types. A notable example of genes with pleiotropic effects is the tubulin gene family, whose α/β tubulin heterodimers form microtubules essential for various cellular processes such as cell division, motility, and intracellular transport across various cell types[40]. Microtubules are also critical for the transport of melanosomes in melanocytic cells[41].

Mutation of tubulin genes such as *tuba8l3a* in zebrafish leads to highly pleiotropic effects including defects in melanocyte patterning, CNS abnormalities, and altered craniofacial morphology[42]. We chose to focus on the closely related α tubulin gene *tuba1a*, which is highly expressed in many cell types of the developing zebrafish including melanocytes, but whose specific function in these cells remains unexplored[43]. Non-cell type specific knockout of *tuba1a* with the Alt-R CRISPR Cas9 system results in extensive embryo abnormalities including a curved

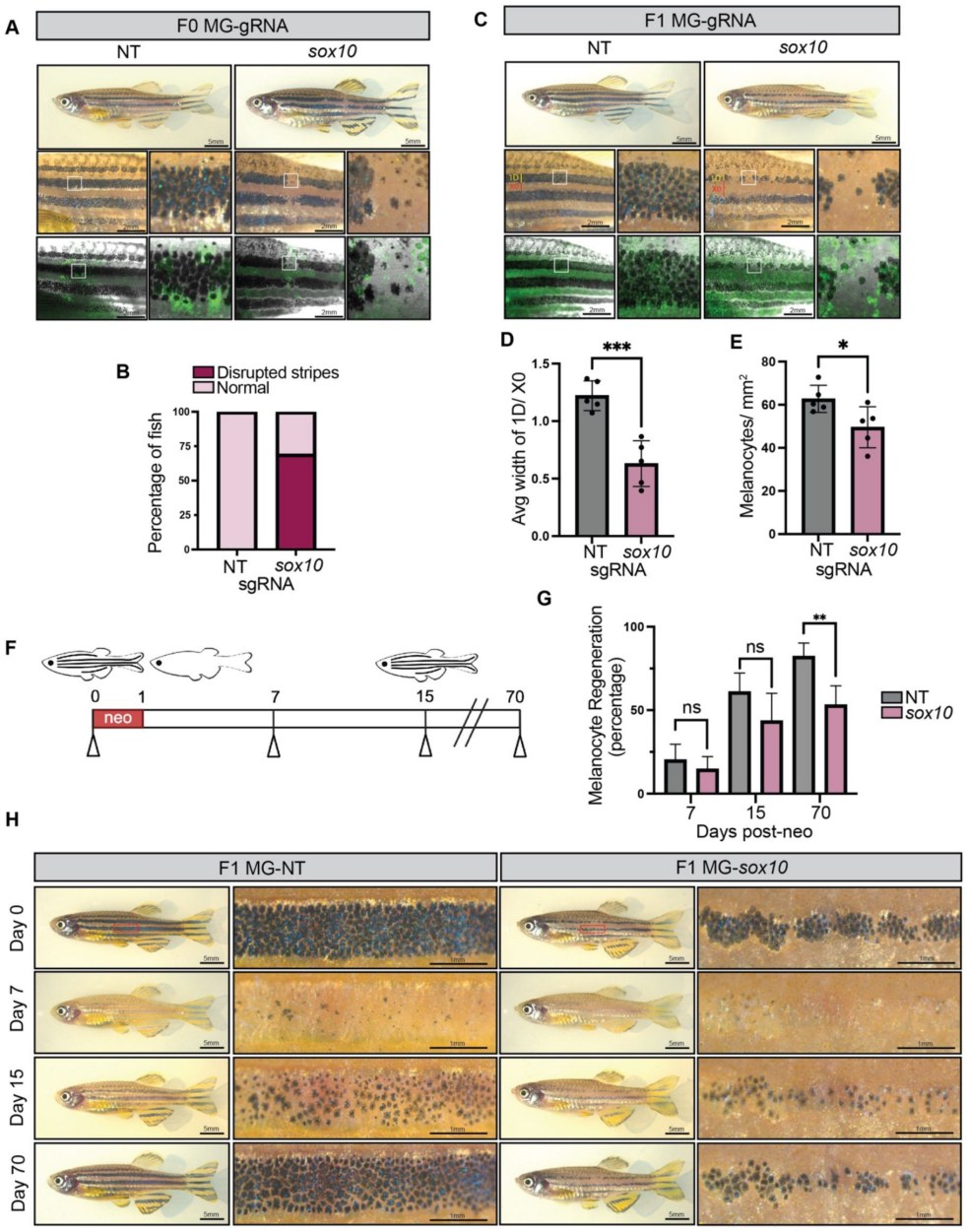

Figure 3.

Melanocyte lineage-specific knockout of *sox10* using *mitfa*^{Cas9} fish.

(A) Adult mitfa^{Cas9} MG-NT and MG-*sox10* F0 fish. **(B)** Proportion of MG-NT (n=19) and MG-*sox10* (n=16) F0 fish with disrupted stripes phenotype. **(C)** Adult mitfa^{Cas9} MG-NT and MG-*sox10* F1 fish. **(D)** The average width of melanocyte stripe 1D and xanthophore interstripe X0 was calculated for each fish by averaging 5 stripe/interstripe width measurements. N=5 fish per genotype. Two-sided student's *t*-test was used to assess statistical significance, *** $p < 0.001$; error bars, SD. **(E)** F1 MG-NT (n=5) and MG-*sox10* (n=5) adult fish were treated with epinephrine and melanocytes were counted within a defined rectangular region of interest 3.46mm x 2.54mm encompassing the top and middle melanocyte stripes. Two-sided student's *t*-test was used to assess statistical significance, * $p < 0.05$; error bars, SD. **(F)** Schematic of neocuproine (neo) experimental setup. Adult mitfa^{Cas9} MG-NT (n= 5) and MG-*sox10* (n=5) F1 fish were treated with neocuproine for 24 hours to ablate melanocytes then imaged at day 7,15, and 70 to measure regeneration of melanocytes compared to day 0. **(G)** Representative images are shown for MG-NT and MG-*sox10* fish pre- and post-neocuproine treatment. **(H)** Quantification of melanocyte regeneration. Fish were treated with epinephrine prior to imaging to enable counting of melanocytes. Two-sided student's *t*-test was used to assess statistical significance, ** $p < 0.01$; error bars, SD. Created with BioRender.com.

tail phenotype, pericardial edema, and melanocytes with more dispersed pigmentation (**Figure 4A-B**, S3A-B). We found that compared to 71% survival of NT Alt-R embryos, only 10% of embryos injected with *tuba1a* Alt-R survive to 14dpf (**Figure 4C**).

Similar to *sox10*, the embryonic lethality of global *tuba1a* knockout prevents the study of knockout phenotypes in adult fish. To overcome this limitation, we implemented our *mitfa*^{Cas9} system to specifically knock out *tuba1a* in melanocytes. Unlike global knockout of *tuba1a*, melanocyte specific loss of *tuba1a* resulted in viable adult fish with no obvious abnormalities (Figure S3C-D). Surprisingly, we did not observe the dispersed melanocyte phenotype in any *mitfa*^{Cas9} MG-*tuba1a* embryos (**Figure 4D**, E). Based on this observation, we hypothesized that *tuba1a* may be functioning in a cell non-autonomous manner on melanocytes.

The dispersed pigmentation observed in *tuba1a* Alt-R embryos resembles that seen in blind zebrafish, which fail to contract their melanosomes in response to light[44]. This light-mediated camouflage involves retinal ganglion cells signaling hypothalamic neurons to release melanin-concentrating hormone (MCH), which then binds to MCH receptors on melanocytes, triggering melanosome aggregation via microtubules[44]. The normal melanosome dispersion observed in *mitfa*^{Cas9} MG-*tuba1a* fish suggests that rather than a cell-intrinsic defect in melanosome aggregation, *tuba1a* may indirectly influence melanocytes through potential defects in retinal and/or central nervous system cells crucial for light-mediated camouflage. Interestingly, TUBA1A mutation in humans can result in both ophthalmologic and brain abnormalities and morpholino-based knockdown of tuba1a in zebrafish inhibits CNS development[45,46].

To further test this idea, we treated *tuba1a* Alt-R embryos with epinephrine to assess their ability to contract melanosomes in response to a non-visual stimulus. *Tuba1a* Alt-R embryos exposed to epinephrine had robust aggregation of melanosomes, demonstrating that the loss of *tuba1a* did not impair melanosome transport (**Figure 4F, G**). This finding further supports a non-autonomous role for *tuba1a* in melanocytes. Utilizing the *mitfa*^{Cas9} system distinguish cell-autonomous from non-autonomous gene functions provides valuable insights into the complex dynamics of cell-cell interactions and how genes function within cellular and organismal networks.

Targeting tumor suppressors with *mitfa*^{Cas9} induces melanoma

We next turned our attention to melanoma. In humans, tumor suppressors such as *PTEN* and *TP53* are somatically inactivated in melanoma[47,48]. In contrast, the most commonly used zebrafish models of melanoma use a germline *tp53* mutation, which is not typically seen in humans with the disease and precludes our ability to discern the role of *tp53* inactivation specifically in melanocytes and melanoma[49]. While global *tp53* loss is not lethal, it does lead to a wide range of non-melanoma tumors, which can deteriorate fish health and confound melanoma studies[50]. Furthermore, current zebrafish melanoma models typically involve the utilization of MiniCoopR *mitfa* rescue cassettes into *casper* or *nacre* zebrafish, which are devoid of normal pigment cells. Although this method is highly efficient, it does not accurately represent melanoma initiation in the context of normal skin architecture and relies on artificial overexpression of *mitfa*. To mimic human melanoma initiation more accurately, we used our *mitfa*^{Cas9} model to assess tumor initiation using melanoma-specific gene knockout in animals with wild-type skin. Previous studies have shown that expression of BRAF^{V600E} and loss of *tp53* in WT (non-*casper*) fish results in relatively low tumor burden, so we decided to also target *ptena* and *ptenb*, the zebrafish orthologs of human *PTEN*[51]. *Ptena/ptenb* loss in combination with *tp53* has been shown to accelerate melanoma formation in zebrafish[52,53].

To first address the effect of *ptena/b* loss on normal melanocytes, we generated melanocyte-specific knockouts of *ptena* and *ptenb* (**Figure 5A**). Like *sox10* and *tuba1a*, germline knockout of *ptena/b* is embryonic lethal[54,55]. In our *ptena/ptenb* F0 melanocyte-specific KO fish, survival was normal. We observed an aberrant expansion of the melanocytes outside of the stripe regions

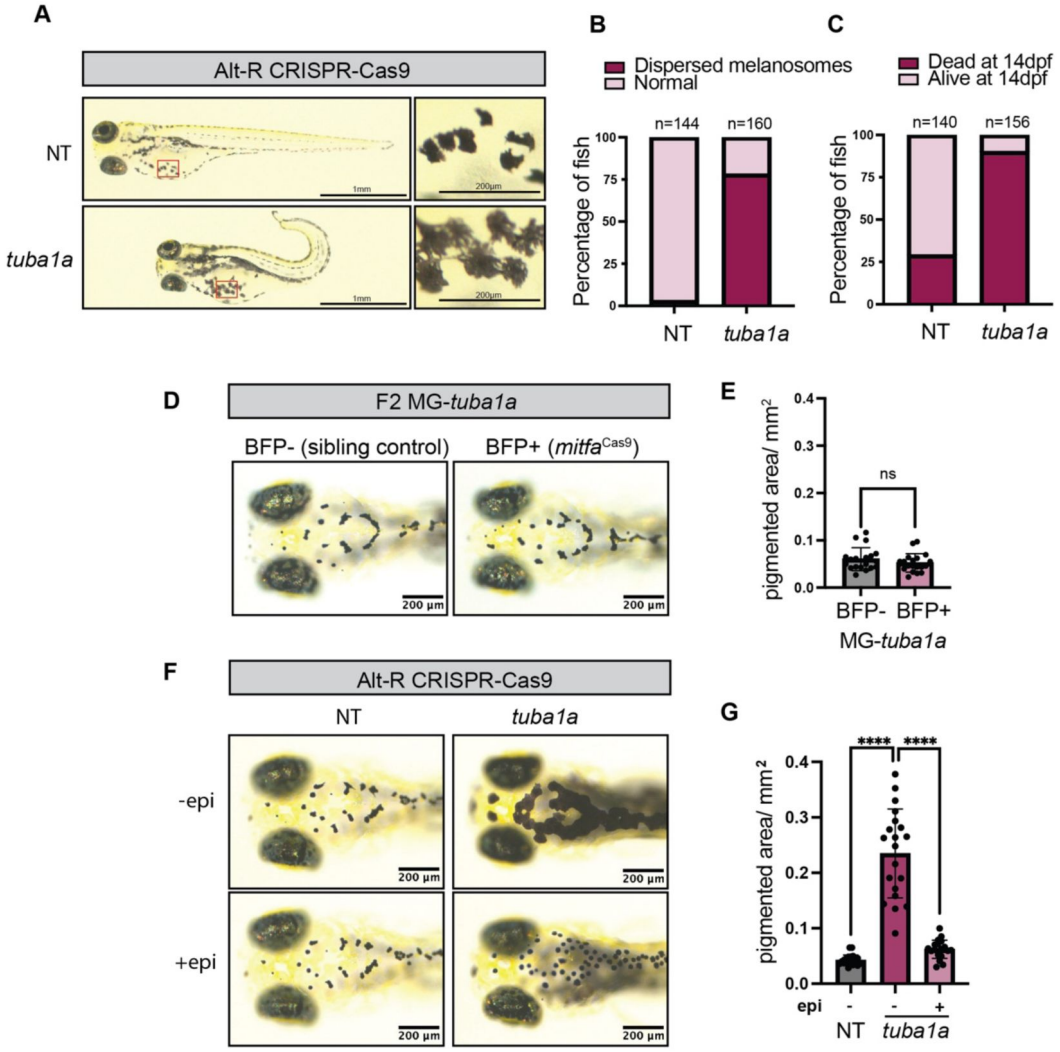

Figure 4.

Non-autonomous function of *tuba1a* on melanocytes.

(A) 4dpf zebrafish embryos injected with either NT or *tuba1a* Alt-R CRISPR Cas9 gRNAs. **(B)** Percentage of NT or *tuba1a* Alt-R zebrafish embryos with dispersed melanocyte phenotypes. N=2 independent experiments. **(C)** Survival percentage is shown for NT and *tuba1a* Alt-R embryos. Embryos were counted at 24hpf and again at 14dpf to determine survival. **(D)** 4dpf mitfa^{Cas9} MG-*tuba1a* F2 embryos (BFP+ eyes) compared to sibling controls with no mitfa^{Cas9} (BFP-eyes). Representative embryos are shown for each genotype. **(E)** Pigmented area/ mm^2 calculated for n=19 embryos/genotype from two independent MG-*tuba1a* F2 clutches. Two-sided student's *t*-test was used to assess statistical significance, ns: no significance. **(F)** 4dpf Alt-R injected zebrafish embryos imaged before and after epinephrine (epi) treatment. **(G)** Pigmented area/ mm^2 calculated for n=20 embryos/genotype from two independent clutches. Two-sided student's *t*-test was used to assess statistical significance, **** $p < 0.0001$.

they normally are restrained to, suggesting that inactivation of *ptena* and *ptenb* induces defects in melanocyte patterning (**Figure 5A and B**). However, loss of *ptena/b* alone was not sufficient to induce melanoma.

To generate tumors, we co-injected *mitfa*^{Cas9} embryos with plasmids encoding *mitfa:*BRAF^{V600E} and sgRNAs targeting *tp53* and *ptena/b* (**Figure 5C**). Larvae were sorted for BFP+ eyes, indicating mitfa-Cas9 integration, and monitored for tumors over 30 weeks. No tumors were detected in BFP- fish from any of the injection conditions (n=136). We also did not observe any tumors in BFP+ fish injected with either BRAF^{V600E} alone (n=35) or ptena/b; *tp53* guides in the absence of BRAF^{V600E} (n=17). In contrast, tumors developed in all BFP+ groups injected with both *mitfa:*BRAF^{V600E} and gRNA plasmids. Approximately 5% of fish from the BRAF;*tp53* group developed tumors (**Figure 5D**). This aligns with a previous study where 6% of *tp53*-/- fish developed tumors when injected with a BRAF^{V600E} construct [51]. Targeting of *ptena* and *ptenb* in *mitfa*^{Cas9} fish co-injected with *mitfa*:BRAF^{V600E} resulted in a higher tumor incidence of 29% (**Figure 5D**).

When all three tumor suppressors (*tp53, ptena, ptenb*) were targeted in combination with BRAF^{V600E}, 59% of fish developed tumors by 30 weeks post fertilization (wpf) (**Figure 5D**). Immunohistochemistry (IHC) revealed that all tumor samples expressed BRAF^{V600E}, while p-AKT, a hallmark of *PTEN* inactivation, was only detected in samples with *ptena/b* KO (**Figure 5E**). To confirm on-target cutting, DNA was extracted from tumors and CRISPR sequencing was performed on PCR amplicons for each tumor suppressor gene. A large proportion of reads contained indels at the target locus for each of the target genes (*ptena*, *ptenb*, and *tp53*) (**Figure 5F** and S4). Some tumors had an array of mutations, while others appeared to be composed largely of a one dominant clone (Figure S4A to C). Our system thus allows us to rapidly dissect the melanocyte-specific function of both oncogenes (BRAF^{V600E}), as well as putative tumor suppressors (*tp53*, *ptena/b*), while avoiding pleiotropic effects of global tumor suppressor loss.

Targeting of lineage-specific oncogenes decreases tumor initiation but promotes progression

Our next objective was to leverage the mitfa^{Cas9} fish as a tool for identifying or validating potential genetic dependencies within melanoma. Genetic dependencies can be difficult to study *in vivo* since inactivation of a required gene inevitably leads to selection for non-mutant alleles, as has been previously shown[56]. In melanoma, the DepMap has identified *SOX10* as the top *in vitro* genetic dependency necessary for tumor cell proliferation, suggesting it acts as a lineage-specific oncogene[14,57]. In prior work in both zebrafish and mice, loss of *sox10* is clearly associated with a loss of tumor initiating potential and cell proliferation[56,57]. Yet its specific role *in vivo* remains unclear, since it is required for neural crest and melanocyte specification, making it difficult to know whether its effects reflect loss of the entire lineage[57]. Given our data above showing that *sox10* is required specifically in melanocyte patterning and regeneration, we wished to investigate its function in melanoma.

To assess this, we generated plasmids containing multiple sgRNAs driven by three distinct zU6 promoters (zU6A, zU6B, and zU6C) to simultaneously target both tumor suppressors (*tp53, ptena/ptenb*) and tumor promoting genes (*sox10*) in the same cells, which reduces the chance of selecting for non-mutant alleles[2]. We co-injected these plasmids with *mitfa*:BRAF^{V600E}, sorted fish for BFP+ eyes, and tracked tumor free survival over the course of 50 weeks (**Figure 6A and B**). Consistent with the DepMap prediction, *sox10* inactivation markedly reduced tumor incidence, which likely reflects loss of proliferation. Only 3/96 (3.1%) fish with *sox10* knockouts initiated melanomas compared to the 24/94 fish (25.5%) from the NT condition that developed tumors (**Figure 6B**). Using IHC, we validated that *sox10* KO tumors expressed lower levels of Sox10 protein compared to NT tumors (**Figure 6C** and S5A). Quantification of Sox10 intensity with immunofluorescence confirmed this finding (Figure S5B).

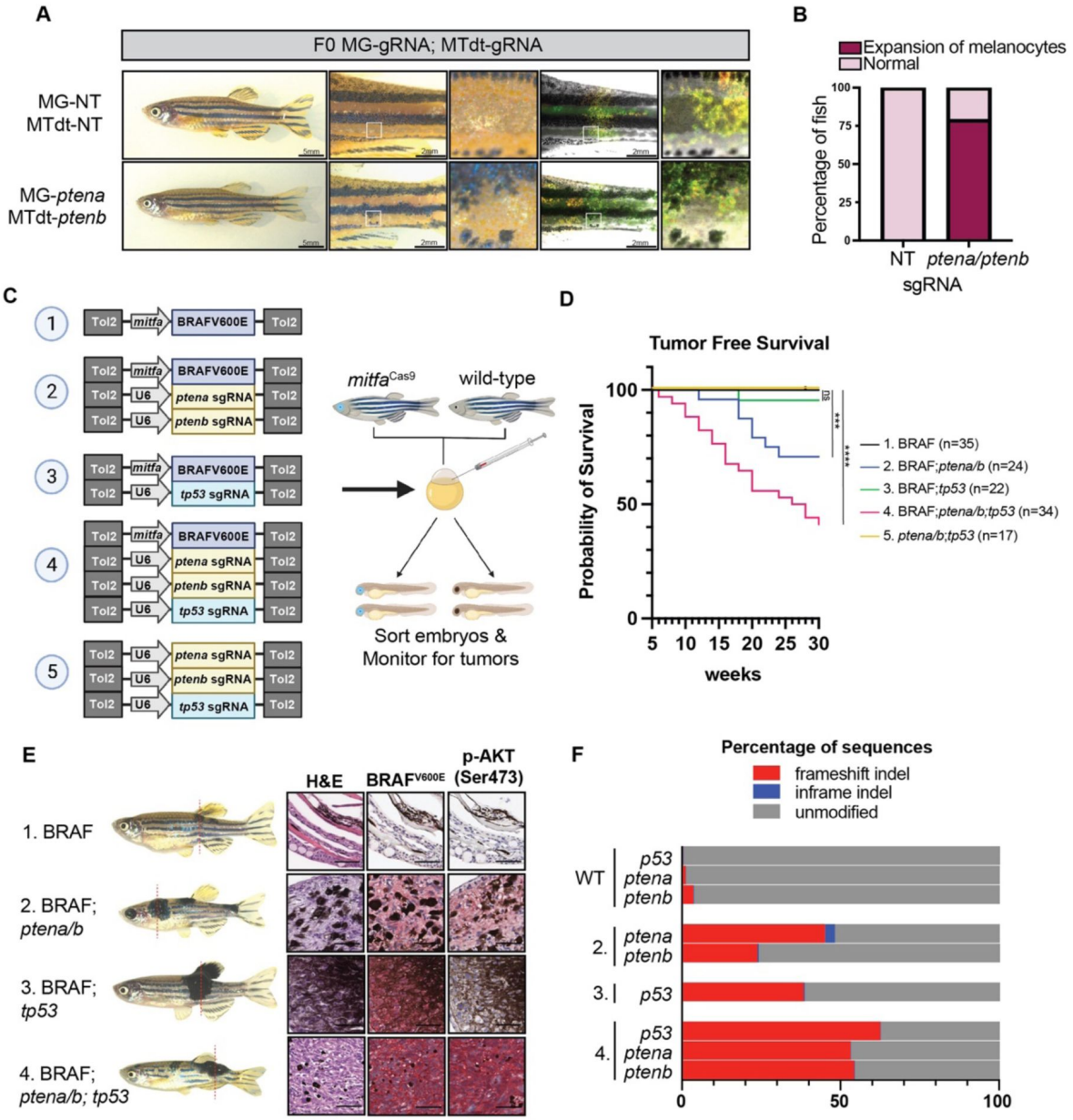

Figure 5.

Generation of a zebrafish melanoma model in *mitfa*^{Cas9} fish.

(A) Adult mitfa^{Cas9} MG-NT; *mitfa*:TdT;U6:NT (MTdt-NT) and MG-*ptena*; MTdT-*ptenb* F0 fish. **(B)** Proportion of MG-NT; MTdT-NT (n=14) and MG-*ptena*; MTdT-*ptenb* (n=14) F0 fish with disrupted stripes phenotype. **(C)** Schematic of zebrafish tumorigenesis assay. Indicated plasmids are injected into one-cell stage embryos from crosses between mitfa^{Cas9} and WT fish. The mitfa:BRAF^{V600E} plasmid includes cardiac-specific cmlc2:GFP. Embryos were sorted for GFP+ hearts and BFP+ eyes and fish were screened every 2 weeks for tumors. **(D)** Tumor free survival curve. N=2 independent experiments. Tumors were tracked over the course of 30 weeks. Log-rank (Mantel-Cox) test was used to assess statistical significance, *** $p < 0.001$; **** $p < 0.0001$; ns: no significance. **(E)** Histology was performed on one fish from each indicated injection group. Dotted lines indicate site of sectioning. Red chromogen was used for all IHC staining. Scale bars, 50μm. **(F)** CRISPR-seq results are shown for normal skin dissected from WT fish and tumors dissected from injection conditions 2, 3, and 4. Results are shown as a fraction of sequences with indels calculated using CRISPRESSO. Created with BioRender.com.

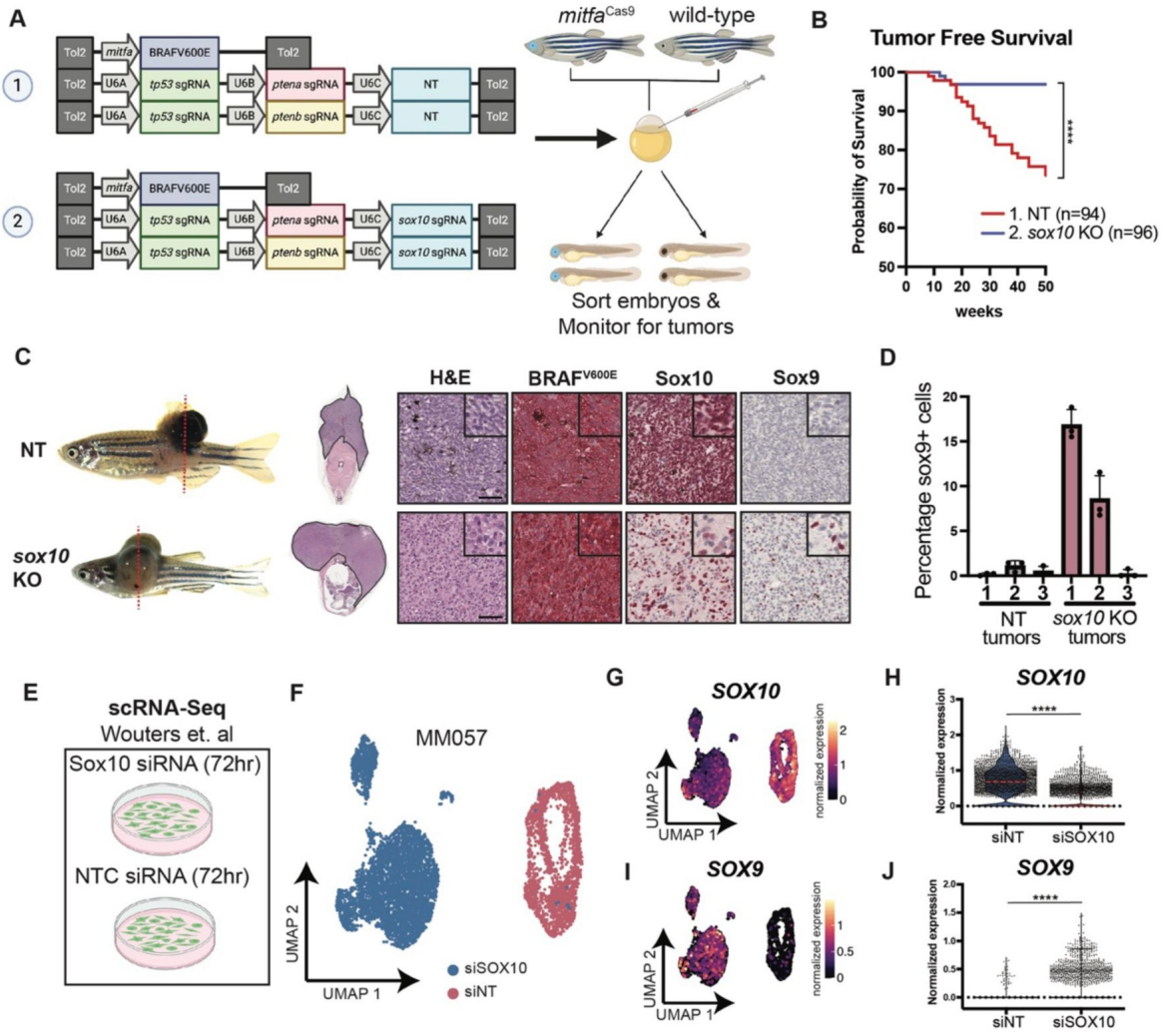

Figure 6.

Melanoma specific knockout of *sox10* reduces tumor burden and induces phenotypic switching.

(A) Schematic of zebrafish tumorigenesis assay. Indicated plasmids are injected into one-cell stage embryos from crosses between mitfa^{Cas9} and WT fish. Embryos were sorted for GFP+ hearts and BFP+ eyes and fish were screened every 2 weeks for tumors. **(B)** Tumor free survival curve. N=3 independent experiments. Tumors were tracked over the course of 50 weeks. Log-rank (Mantel-Cox) test was used to assess statistical significance, **** $p < 0.0001$. **(C)** Histology is shown for one fish from each injection group. Dotted lines indicate site of sectioning. Red chromogen was used for all IHC staining. Scale bars, 50μm. **(D)** Color thresholding was used on IHC images to calculate the percentage of nuclei that stained positive for *sox9*. NT-1 n=1222 cells, NT-2 n=1620 cells, NT-3 n=2185 cells, sox10-1 n=970 cells, sox10-2 n=594, sox10-3 n=1046. Cells from n=3 images were analyzed for each tumor. **(E)** Schematic of scRNA-seq experimental setup from Wouters et al. Patient derived cell lines were treated with siRNAs targeting SOX10 or NTC and scRNA-seq was conducted at 72hrs. **(F)** UMAP of scRNA-seq dataset for MM057 cell line from Wouters et al. Cells from siSOX10 and siNT conditions are labeled. **(G)** Normalized expression of Sox10 per cell in UMAP space. **(H)** Violin plots of normalized expression of Sox10 per cell. Median is shown as dashed red line. Wilcoxon Rank Sum Test was used to assess statistical significance, **** $p < 0.0001$. **(I)** Normalized expression of Sox9 per cell in UMAP space. **(J)** Violin plots of normalized expression of Sox9 per cell. Wilcoxon Rank Sum Test was used to assess statistical significance, **** $p < 0.0001$. Created with BioRender.com.

Although *sox10* clearly reduced tumor initiation, we noted "escapers". These tumors appeared morphologically very distinct compared to the *sox10* intact tumors. Specifically, these tumors were highly invasive with a mesenchymal morphology compared to the typical $BRAF^{V600E}$ melanoma (**Figure 6C**). This observation raised the possibility that these tumors had undergone "phenotype switching", a form of cell plasticity in which cells reversibly move between opposite extremes of proliferation versus invasive states[22,23,57,58]. In melanoma, the proliferative state is thought to be characterized by high expression of *SOX10*, whereas the mesenchymal, invasive state is characterized by high expression of *SOX9*[23]. These two highly related transcription factors have seemingly opposite and possibly antagonistic roles in melanoma. *In vitro*, knockout of *SOX10* in a variety of human cell lines is associated with acquisition of a $SOX9^{hi}$ state, which has been suggested to be linked to the invasive phenotype[23]. Re-analysis of publicly available RNA-seq data using 3 patient-derived human melanoma cell lines treated with siRNA targeting *SOX10* confirmed this observation, with the *SOX10* lines now becoming $SOX9^{hi}$ (**Figure 6E** to **J**, S5C to L)[23]. These data raised the hypothesis that the more invasive tumors we see in our escaper fish was due to gain of *sox9* expression. To test this, we stained our *sox10* CRISPR tumors for *sox9* and discovered that 2 of the 3 *sox10* KO tumors markedly upregulated *sox9* relative to control tumors expressing NT gRNA (**Figure 6C and D**). This data highlights that *in vitro* genetic studies such as the DepMap, which rely on proliferation as the readout, can mask more complex *in vivo* phenotypes.

Discussion

The ability to model genetic dependencies *in vivo* is one of the most important uses of model organisms. Forward genetic mutagenesis screens using compounds such as ENU or EMS led to the identification of many key developmental genes in organisms such as Drosophila, C. elegans, Saccharomyces, zebrafish, and many others[59,60]. These approaches have rapidly accelerated in the era of TALENs and CRISPR, readily allowing for knockout of nearly any gene in a wide variety of non-model organisms. Cre/Lox approaches have further augmented our ability to discern the cell-type specific effects of these genes, although these are still laborious and time consuming, especially in models such as mice[10].

Alongside these approaches have been remarkable advances in large scale sequencing of human tissues. GWAS-like efforts such as the UK Biobank and the US All of Us programs have identified thousands of germline variants (SNPs) that may be linked to interesting phenotype variation[12,13]. A conceptually similar approach is also happening in diseases such as cancer, in which efforts such as the TCGA, ICGC and DepMap have continued to unveil new potential somatic variants (SNVs) linked to cancer[14,61,62].

Across these efforts, in both development and disease, the large number of candidate variants makes it challenging to connect these genotypes to phenotypes. This is especially acute in melanoma, where the large number of somatic variants generated by UV radiation leads to a high number of background mutations that may or may not have pathogenic function. Thus, there is a substantial need for *in vivo* models that allow for rapid and efficient modeling of candidate genes. Our development of the *mitfa*-Cas9 knockin line provides such a method. One major advantage is that phenotypes can be readily screened either in the F0 generation (as mosaics) or in the F1 generation (3 months later). The F1 fish in particular are advantageous, since they allow for highly efficient biallelic knockout of the gene of interest, allowing for very quick identification of genes that only have recessive phenotypes (i.e., *albino*). On a per gene basis, this saves well over 3 months of work compared to germline knockouts and uses vastly fewer animals since all F1 animals (even those for recessive alleles) are informative. Additionally, this model eliminates the issue of inbreeding, promoting healthier genetic lines and facilitating the potential for outcrossing

with any available fish line. Because we demonstrate that the Cas9 is completely restricted to the melanocyte lineage, with no detectable off-target expression, this easily allows us to discern the melanocyte-specific effect of pleiotropic genes very efficiently.

One interesting observation of our melanoma studies is evidence that loss of melanoma genetic dependencies like *SOX10* (as suggested by the human DepMap data) can still lead to tumors, albeit with very different biological behaviors. In both the fish and in the human samples we analyzed, loss of *SOX10* leads to acquisition of a *SOX9*^{hi} state instead[23]. *SOX9* is increasingly recognized to be a potent mediator of tumor cell invasiveness, which is what we observe in our fish[63,64]. One implication of this finding is that clinical targeting of transcription factors like *SOX10*, long considered an ideal therapeutic approach, could lead to unexpected outcomes in which tumors might proliferate less but become more invasive or resistant to therapy. Whether this phenomenon of *SOX10/SOX9* switching is a general phenomenon, or specific to that class of TFs, remains to be determined. However, a similar phenomenon has been observed for *MITF*, another melanocytic TF, suggesting this idea of TF switching needs to be more thoroughly explored in the future[58,65,66].

Finally, while our studies clearly focused on the melanocyte lineage, highly similar approaches could be taken for virtually any cellular lineage in which master regulators or markers are known. Given the large number of already identified promoter/enhancer fragments in the zebrafish, this would be an important extension of our system to study gene variants in the context of development and other diseases[67].

Our approach has several limitations that may need to be addressed in future studies. Due to the constitutive nature of the Cas9, we have little control over the timing of its expression. Because it is knocked into the endogenous *mitfa* locus, the expression of Cas9 will vary with the endogenous regulation of that gene. In future iterations of this general approach, swapping the Cas9 for an inducible version would allow for better timed knockout of genes[68]. Because *mitfa* itself is expressed in melanoblasts, melanophores and xanthophores during different stages of development, choosing a promoter (e.g. PMEL) that is more tightly restricted to melanocytes may be advantageous. Finally, although our system was relatively efficient, we still did not see 100% knockout. This may reflect that it is relatively easy to bypass the CRISPR lesion in an exon with an in-frame mutation. The use of multiple sgRNAs, or sgRNAs that target promoter regions, rather than exons, could further augment efficiency of knockout. Alternatively, instead of targeting DNA, we could consider using RNA targeting CRISPR enzymes. Recent work has shown that RNA degradation induced by Cas13 works in zebrafish and may allow for more nuanced knockdown rather than knockout of a given candidate gene[69,70]. This may especially have advantages in the context of melanoma, where genes are commonly dysregulated rather than completely knocked out. We envision it would be relatively straightforward to knock Cas13 into the locus of interest using an analogous approach to the one we have taken here.

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Author Contributions

Conceptualization: SP, RMW

Investigation: SP, YM, MVH, JBS, ZM, JX

Visualization: SP, MVH, JBS

Supervision: TL, MM, RMW

Writing—original draft: SP, RMW

Writing—review & editing: SP, MVH, RMW

Declaration of Interests

MM has competing interests with Recombinetics Inc., LifEngine Technologies, and LifEngine Animal Health Laboratories, Inc. RMW is a paid consultant to N-of-One Therapeutics, a subsidiary of Qiagen and is on the scientific advisory board of Consano but receives no income for this. RMW receives royalty payments for the use of the *casper* zebrafish line from Carolina Biologicals. SP, YM, MVH, JBS, ZM, JX, and TL declare no competing interests.

Methods

Materials Availability

All data are available in the main text or the supplementary materials, or upon request. Plasmids, fish lines and other materials generated from this study are available upon request.

Zebrafish husbandry and ethics statement

All zebrafish experiments adhered to institutional animal protocols and were conducted in compliance with approved procedures. Fish stocks were maintained at a temperature of 28.5 °C, under 14:10 light:dark cycles, with pH set at 7.4, and salinity-controlled conditions. The zebrafish were fed a standard diet comprising brine shrimp followed by Zeigler pellets. Approval for the animal protocols outlined in this manuscript was obtained from the Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee (IACUC), under protocol number 12-05-008. Anesthesia was conducted using Tricaine (4 g/l, Syndel, Ferndale, WA, USA) from a stock of 4 g/l and diluted to 0.16 mg/ml. Adult zebrafish of both sexes were equally employed in all experiments. Embryos, collected through natural mating, were incubated in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at 28.5 °C. The wild-type strain used was Tropical 5D Zebrafish (T5D)[27].

GeneWeld plasmid construction

A combination of restriction enzyme digestion and HiFi cloning was used to construct the GeneWeld pPRISM-nCas9n, γcry1:BFP vector for targeted integration to isolate an nls-Cas9-nls knockin. A 765 bp fragment containing the Porcine teschovirus-1 polyprotein 2A peptide sequence was amplified from the pPRISM-2A-Cre, γcry1:BFP vector (Addgene #117789) with primers Ori-F and 2A-R[24,25]. The nCas9n cDNA was amplified from the expression vector pT3TS-nCas9n (Addgene #46757) with primers Cas9-F and Cas9-R[71]. A KpnI/SpeI fragment containing the pPRISM-2A-Cre, gcry1:BFP vector backbone was assembled with the 2A and nCas9n PCR amplicons using the NEBuilder HiFi DNA Assembly Cloning Kit (NEB # E5520S) following the manufacturer's instructions.

Homology arms were designed as previously described[24,25]. Briefly, 48bp homology arms complementary to the *mitfa* target site were designed using GTagHD (*http://www.genesculpt.org/gtaghd/*) for the pPRISM GeneWeld plasmid series. The two pairs of complementary oligos can be found in Table S1. 5' and 3' arms were cloned sequentially into the pPRISM-nCas9n, γcry1:BFP vector. The 3' homology arm oligos were annealed and cloned into the vector using the BspQI restriction site as previously described. Due to the multiple BfuAI sites within Cas9, instead of using BfuAI restriction cloning, we alternatively used in-fusion cloning (Takara Bio) to insert a gBlock (IDT) containing the 5' homology arm into the pPRISM-nCas9n, gcry1:BFP vector. Primers and gblock sequence used for cloning are listed in Table S1. The 5' and 3' homology arms were sequenced with primers 5'homologycheckF and R3'_pgtag_seq, respectively.

Injection of GeneWeld reagents

Injections of GeneWeld reagents were performed on one-cell stage embryos. We used the Alt-R CRISPR-Cas9 system (IDT) for the initial knock-in of nCas9n, γcry1:BFP to the *mitfa* locus. In brief, 100 µM tracrRNA (IDT 1075928) was mixed at a 1:1 ratio with either *mitfa* crRNA or universal crRNA then incubated at 95 °C for 5 min. Recombinant Cas9 (IDT 1081059) was incubated with both sgRNAs for 10 min at 37 °C to form the RNP complex. The GeneWeld pPRISM-5'mitfaHom-nCas9n, gcry1:BFP-3'mitfaHom plasmid was then added to the injection mix along with phenol red (Sigma-Aldrich P0290). The final concentrations injected into the embryos were: 75 pg/nl Cas9, 12.5 pg/nl *mitfa* sgRNA, 12.5 pg/nl Universal sgRNA, and 5 pg/nl pPRISM plasmid.

Genotyping

Tail clips were taken from 2 month postfertilization (mpf) zebrafish and DNA was extracted using the DNeasy Blood and tissue kit (Qiagen). DNA was PCR amplified with Q5 high fidelity DNA polymerase (NEB). The forward primer MitfaPCR-F binds to the *mitfa* genomic region upstream of

the insertion site and the reverse primer 5'homology_amplify-R binds the p2A region of the insertion cassette. Expected PCR amplicon size is 269bp. Amplicons were sequenced using sanger sequencing (Azenta Life Sciences).

RNA-FISH HCR

We adapted the HCR protocol from Ibarra-Garcia-Padilla et al.[72]. Briefly, embryos were collected and treated with 0.0045% 1-phenyl 2-thiourea (PTU) (Sigma-Aldrich) after 24 hpf to block pigmentation. Embryos were harvested at 72 hpf and fixed in 4% PFA (Santa Cruz) for 24 hrs at 4 °C. Embryos were then washed with PBS and dehydrated/permeabilized with a series of MeOH (Millipore) washes. Embryos were rehydrated with a series of MeOH/PBST washes, permeabilized with acetone (Fisher Scientific) and incubated with Proteinase K (Millipore) for 30 min. This was followed by further fixation with 4% PFA. Embryos were pre-hybridized in probe hybridization buffer (PHB) before incubating them with HCR probes in PHB at concentration of 20nM. Probe sequences for *mitfa* and Cas9 can be found in Table S1. Embryos were washed with probe wash buffer before being pre-amplified with probe amplification buffer (PAB). Following pre-amplification, amplifier hairpins were thawed, snap-cooled, and mixed with PAB to a concentration of 36 nM. Embryos were incubated in the hairpin solution followed by washes with 5×SSCT and 1× PBST. Embryos were stained with 2 µg/mL DAPI in PBST and mounted on glass slides. Imaging was performed using a Zeiss LSM880 confocal microscope.

Guide RNA plasmids

The following plasmids were constructed using the Gateway Tol2kit:

zU6A:gRNA-NT;zU6B:gRNA-*ptena*;zU6C:gRNA-*tp53*/394,

zU6A:gRNA-NT;zU6B:gRNA-*ptenb*;zU6C:gRNA-*tp53*/394,

zU6A:gRNA-*sox10*;zU6B:gRNA-*ptena*;zU6C:gRNA-*tp53*/394,

zU6A:gRNA-*sox10*;zU6B:gRNA-*ptenb*;zU6C:gRNA-*tp53*/394.

In-fusion cloning (Takara Bio) was used to insert *mitfa:*GFP or *mitfa:*TdTomato into a zU6:gRNA/394 plasmid previously developed in the lab (See Table S1 for primers used). gRNAs were cloned into zU6:gRNA;*mitfa:*GFP/394 or zU6:gRNA;*mitfa:*TdTomato/394 using BsmBI sites. Other plasmids used in this study that were previously developed in the lab include zU6:gRNA-*ptena*/394, zU6:gRNA-*ptenb*/394, zU6:gRNA-*tp53*/394, *mitfa*-BRAF^{V600E};cmlc2:eGFP. All guide RNAs used in this study besides *tuba1a* have previously been validated in zebrafish[24,32,56,73].

Validation of *tuba1a* sgRNA

The Alt-R CRISPR-Cas9 system (IDT) was used to achieve non-cell type specific knockout of *tuba1a.* Guide RNAs were designed using ChopChop[74]. In brief, 100 µM tracrRNA (IDT 1075928) was mixed at a 1:1 ratio with *tuba1a* crRNA then incubated at 95 °C for 5 min. Recombinant Cas9 (IDT 1081059) was incubated with the *tuba1a* sgRNA for 10 min at 37 °C to form the RNP complex. Phenol red was added prior to injection into 1 cell-stage wild-type T5D embryos. To validate on-target cutting, 4 embryos were pooled and DNA was extracted using Quick Extract buffer (Fisher Scientific NC0302740). DNA was PCR amplified with Q5 high fidelity DNA polymerase (NEB) and primers tuba1a_seq-F and tuba1a_seq-R. Exosap IT for PCR Product Clean Up was added to PCR product prior to sanger sequencing. Primers tuba1a_seq-F and tuba1a_seq-R were also used for sequencing.

Transgenic lines

To generate MG-U6:sgRNA stable lines, one-cell-stage embryos from crossing WT and mitfa:Cas9 fish were collected and injected with the 30 pg indicated plasmid and 20 pg tol2 mRNA. Fish with GFP+ melanocytes and BFP+ eyes were selected and outcrossed with WT fish to produce the F1 generation. F1 fish were again sorted and outcrossed to generate F2 generation zebrafish. All fish were imaged on a Zeiss AxioZoom V16 with Zen 2.1 software.

Flow cytometry of melanocytes from adult zebrafish

Zebrafish were euthanized using ice-cold water and dissected using a clean scalpel and forceps. The epidermal and dermal layers of the skin as well as the fins were separated from the rest of the tissues and diced into 1-3mm pieces. Cells were dissociated with Liberase TL (Millipore Sigma 05401020001) and filtered for single cell suspensions as previously described[32]. Samples were then FACS sorted (BD FACSAria) for GFP+ and GFP-cells. WT fish were used as a GFP negative control. Genomic DNA was isolated from sorted cells using the DNeasy Blood and Tissue Kit (Qiagen).

Tumor Dissection

Fish with tumors were euthanized with ice-cold water and immediately dissected with a scalpel to isolate tumor tissue. Genomic DNA was purified from the tumor samples using the Dneasy Blood and tissue kit (Qiagen).

CRISPR sequencing

Primer pairs were designed to produce amplicons 200 to 280 bp in length with the mutation site within 100 bp from the beginning or end of amplicon (see Table S1 for primer sequences). Genomic DNA isolated from methods detailed above was PCR amplified with Q5 high fidelity DNA polymerase (NEB) and run on an agarose gel to visualize PCR products. The samples were then purified using the NucleoSpin Gel and PCR cleanup kit (Takara Bio). Deep sequencing was conducted using the CRISPR-seq platform. Sequencing data was analyzed with CRISPResso2[75]. and indel charts were generated with CRISPRVariants R package[76].

Drug treatments

Adult zebrafish were treated for 24 hr with 750nM neocuproine (Sigma-Aldrich) dissolved in fish water with a final concentration of 0.0075% DMSO as previously described [39]. Cells from the middle melanocyte stripe were counted within a rectangular region of 4mm x 1.5mm. Epinephrine hydrochloride (Sigma-Aldrich) was used at 1 mg/ml in fish water. Fish were treated for 10 minutes prior to imaging.

Zebrafish tumor-free survival curves

To generate a transgenic melanoma model, one-cell-stage embryos from WT X mitfa:Cas9 fish crosses were injected with *mitfa*-BRAF^{V600E};cmlc2:eGFP, 20 pg Tol2 mRNA, and the indicated gRNA plasmids for each condition. The total amount of plasmid per embryo did not exceed 30 pg. Embryos were screened for GFP positive hearts and sorted for BFP+ or BFP-eyes. Fish were screened for tumors using a microscope every 2 weeks starting at 4 weeks post fertilization. Tumors were only called if an elevated lesion was observed. Graphpad prism 9 was used to generate and analyze Kaplan-Meier survival curves. Statistical differences were determined using log-rank Mantel–Cox test. All screening and imaging was conducted on a Zeiss AxioZoom V16 using Zen 2.1 software.

Histology

Zebrafish were sacrificed using ice-cold water and placed in 4% paraformaldehyde (Santa Cruz 30525-89-4) in PBS for 72 h at 4°C on a rocker. Fish were then transferred to 70% EtOH for 24 h at 4°C on a rocker. Fish were sent to Histowiz, Inc (Brooklyn, NY, USA), where they were paraffin embedded, sectioned, stained and imaged. All the stainings were performed at Histowiz, Inc Brooklyn, using the Leica Bond RX automated stainer (Leica Microsystems) using a Standard Operating Procedure and fully automated workflow. Samples were processed, embedded in paraffin, and sectioned at 4μm. The slides were dewaxed using xylene and alcohol based dewaxing solutions. Epitope retrieval was performed by heat-induced epitope retrieval (HIER) of the formalin-fixed, paraffin-embedded tissue using Citrate based pH 6 solution (Leica Microsystems, AR9961) for 20 mins at 95 C. The tissues were first incubated with peroxide block buffer (Leica Microsystems), followed by incubation with the following antibodies for 30 min: BRAFV600E antibody (ab228461, 1:100), phospho-AKT antibody (CST4060, 1:50), SOX9 antibody (AB_185230, 1:1000), and SOX10 antibody (GTX128374, 1:500). This was followed by incubation with DAB secondary reagents: polymer, DAB refine and hematoxylin (Bond Polymer Refine Detection Kit, Leica Microsystems) according to the manufacturer's protocol. The slides were dried, coverslipped (TissueTek-Prisma Coverslipper) and visualized using a Leica Aperio AT2 slide scanner (Leica Microsystems) at 40X.

Quantification of Sox10 intensity

FFPE slides were deparaffinized by consecutive incubations in xylene and 100-50% ethanol. For antigen retrieval, slides were placed in 10 mM sodium citrate pH 6.2 in a pressure cooker and heated to 95°C for 20 minutes. Slides were then cooled to room temperature and blocked in 5% donkey serum, 1% BSA, and 0.4% Triton-X100 in PBS for 1 hour at room temperature. The primary antibody targeting Sox10 (GeneTex, GTX128374) was added to the blocking buffer at 1:200 concentration and incubated at 4°C overnight. Slides were then washed in PBS before incubation with the secondary antibody (donkey anti-rabbit Alexa Fluor Plus 488, Thermo Fisher Scientific #AC32790; 1:250) and Hoescht (Thermo Fisher Scientific, #62249; 1:1000) in blocking buffer for 2 hours at room temperature. Slides were mounted in Vectashield Plus (Vector Labs, H-1900). Slides were imaged on a LSM 880 confocal microscope using a 40X oil immersion objective. 3 images were captured per sample. Quantification of Sox10 intensity per cell was performed using CellProfiler.[77] and MATLAB r2023b (Mathworks). Cells were segmented in CellProfiler to obtain a nuclear mask from the Hoescht signal and using this mask the mean intensity per nucleus was quantified.

Re-analysis of human melanoma scRNA-seq data

Human melanoma scRNA-seq data from Wouters et al. was accessed from GEO (GSE134432)[23]. All analysis was done in R (version 4.3.1) using the Seurat package (version 5.0.2)[78]. Counts matrices for each sample and cell line were imported into R and converted into Seurat objects before merging into one combined Seurat object. Counts were normalized using the Seurat function NormalizeData with default parameters. UMAP was calculated using the Seurat function RunUMAP with 10 dimensions.

Statistical analysis

Statistical significance was analyzed using t test, Log-rank (Mantel-Cox) test, Wilcoxon Rank Sum Test or as indicated in the figure legends. GraphPad Prism 9 software was used for data processing and statistical analysis. *P < 0.05; **P < 0.005; ***P < 0.001; ****P < 0.0001. Data are presented as means ± SD unless otherwise indicated.

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Editors

Reviewer #1 (Public review):

Summary:

Perlee et al. sought to generate a zebrafish line where CRISPR-based gene editing is exclusively limited to the melanocyte lineage, allowing assessment of cell-type restricted gene knockouts. To achieve this, they knocked in Cas9 to the endogenous mitfa locus, as mitfa is a master regulator of melanocyte development. The authors use multiple candidate genes - albino, sox10, tuba1a, ptena/ptenb, tp53 - to demonstrate their system induces lineage-restricted gene editing. This method allows researchers to bypass embryonic lethal and non-cell autonomous phenotypes emerging from whole body knockout (sox10, tuba1a), drive directed phenotypes, such as depigmentation (albino), and induce lineage-specific tumors, such as melanomas (ptena/ptenb, tp53, when accompanied with expression of BRAFV600E). While the genetic approaches are solid, the argued increase in efficiency of this model compared to current tools was untested, and therefore unable to be assessed. Furthermore, the mechanistic explanations proposed to underlie their phenotypes are mostly unfounded, as discussed further in the Weaknesses section. Despite these concerns, there is still a clear use for this genetic methodology and its implementation will be of value to many in vivo researchers.

Strengths:

The strongest component of this manuscript is the genetic control offered by the mitfa:Cas9 system and the ability to make stable, lineage-specific knockouts in zebrafish. This is exemplified by the studies of tuba1a, where the authors nicely show non-cell autonomous mechanisms have obfuscated the role of this gene in melanocyte development. In addition, the mitfa:Cas9 system is elegantly straightforward and can be easily implemented in many labs. Mostly, the figures are clean, controls are appropriate, and phenotypes are reproducible. The invented method is a welcomed addition to the arsenal of genetic tools used in zebrafish.

Weaknesses:

The major weaknesses of the manuscript include the overly bold descriptions of the value of the model and the superficial mechanistic explanations for each biological vignette.

The authors argue that a major advantage of this system is its high efficiency. However, no direct comparison is made with other tools that achieve the same genetic control, such as MAZERATI. This is a missed opportunity to provide researchers the ability to evaluate these two similar genetic approaches. In addition, Fig.1 shows that not all melanocytes express Cas9. This is a major caveat that goes unaddressed. It is of paramount importance to understand the percentage of mitfa+ cells that express Cas9. The histology shown is unclear and too zoomed out of a scale to make any insightful conclusions, especially in Fig.S1. It would also be beneficial to see data regarding Cas9 expression in adult melanocytes, which are distinct from embryonic melanocytes in zebrafish. Moreover, this system still requires the injection of a plasmid encoding gRNAs of interest, which will yield mosaicism. A prime example of this discrepancy is in Fig.6, where sox10 is clearly still present in "sox10 KO" tumors.

The authors argue that their model allows rapid manipulation of melanocyte gene expression. Enthusiasm for the speed of this model is diminished by minimal phenotypes in the F0, as exemplified in Fig.2. Although the authors say >90% of fish have loss of pigmentation, this is misleading as the phenotype is a very weak, partial loss. Only in the F1 generation do robust phenotypes emerge, which takes >6 months to generate. How this is more efficient than other tools that currently exist is unclear and should be discussed in more detail.

In Figure 3, the authors find that melanocyte-specific knockout of sox10 leads to only a 25% reduction in melanocytes in the F1 generation. This is in contradiction to prior literature cited describing sox10 as indispensable for melanocyte development. In addition, the authors argue that sox10 is required for melanocyte regeneration. This claim is not accurate, as >50% of melanocytes killed upon neocuproine treatment can regenerate. This data would indicate that sox10 is required for only a subset of melanocytes to develop (Fig.3C) and for only a subset to regenerate (Fig.3G). This is an interesting finding that is not discussed or interrogated further.

Tumor induction by this model is weak, as indicated by the tumor curves in Figs.5,6. This might be because these fish are mitfa heterozygous. Whereas the avoidance of mitfa overexpression driven by other models including MAZERATI is a benefit of this system, the effect of mitfa heterozygosity on tumor incidence was untested. This is an essential question unaddressed in the manuscript.

In Fig.6, the authors recapitulate previous findings with their model, showing sox10 KO inhibits tumor onset. The tumors that do develop are argued to be highly invasive, have mesenchymal morphology, and undergo phenotypic switching from sox10 to sox9 expression. The data presented do not sufficiently support these claims. The histology is not readily suggestive of invasive, mesenchymal melanomas. Sox10 is still present in many cells and sox9 expression is only found in a small subset (<20%). Whether sox10-null cells are the ones expressing sox9 is untested. If sox9-mediated phenotypic switching is the major driver of these tumors, the authors would need to knockout sox9 and sox10 simultaneously and test whether these "rare" types of tumors still emerge. Additional histological and genetic evaluation is required to make the conclusions presented in Fig.6. It feels like a missed opportunity that the authors did not attempt to study genes of unknown contribution to melanoma with their system.

Overall, this manuscript introduces a solid method to the arsenal of zebrafish genetic tools but falls short of justifying itself as a more efficient and robust approach than what currently exists. The mechanisms provided to explain observed phenotypes are tenuous. Nonetheless, the mitfa:Cas9 approach will certainly be of value to many in vivo biologists and lays the foundation to generate similar methods using other tissue-specific regulators and other Cas proteins.

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Reviewer #2 (Public review):

Summary:

This manuscript describes a genetic tool utilizing mutant mitfa-Cas9 expressing zebrafish to knockout genes to analyze their function in melanocytes in a range of assays from developmental biology to tumorigenesis. Overall, the data are convincing and the authors cover potential caveats from their model that might impact its utility for future work.

Strengths:

The authors do an excellent job of characterizing several gene deletions that show the specificity and applicability of the genetic mitfa-Cas9 zebrafish to studying melanocytes.

Weaknesses:

Variability across animals not fully analyzed.

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Reviewer #3 (Public review):

Summary:

Perlee et al. present a method for generating cell-type restricted knockouts in zebrafish, focusing on melanocytes. For this method, the authors knock-in a Cas9 encoding sequence into the mitfa locus. This mitfaCas9 line has restricted Cas9 expression, allowing the authors to generate melanocyte-specific knockouts rapidly by follow-up injection of sgRNA expressing transposon vectors.

The paper presents some interesting vignettes to illustrate the utility of their approach. These include 1) a derivation of albino mutant fish as a demonstration of the method's efficiency, 2) an interrogation and novel description of tuba1a as a potential non-autonomous contributor to melanocyte dispersion, and 3) the generation of sox10 deficient melanoma tumors that show "escape" of sox10 loss through upregulation of sox9. The latter two examples highlight the usefulness of cell-type targeted knockouts (Body-wide sox10 and tuba1a loss elicit developmental defects). Additionally, the tumor models involve highly multiplexed sgRNAs for tumor initiation which is nicely facilitated by the stable Cas9.

Strengths:

The approach is clever and could prove very useful for studying melanocytes and other cell types. As the authors hint at in their discussion, this approach would become even more powerful with the generation of other Cas9-restricted lineages so a single sgRNA construct can be screened across many lineages rapidly (or many sgRNA and fish lines screened combinatorially).

The biological findings used to demonstrate the power of the approach are interesting in their own right. If it proves true, tuba1a's non-autonomous effects on melanosome dispersion are striking, and this example demonstrates very nicely how one could use Perlee et al.'s approach to search for other non-autonomous mechanisms systematically. Similarly, the observation of the sox9 escape mechanism with sox10 loss is a beautiful demonstration of the relevance of SOX10/SOX9's reciprocal regulation in vivo. This system would be a very nice model for further interrogating mechanisms/interventions surrounding Sox10 in melanoma.

Finally, the figure presentation is very nice. This work involves complex genetic approaches including multiple fish generations and multiplexed construct injections. The vector diagrams and breeding schemes in the paper make everything very clear/"grok-able," and the paper was enjoyable to read.

Weaknesses:

The mitfa-driven GFP on their sgRNA-expressing cassette is elegant, but it makes one wonder why the endogenous knock-in is necessary. It would strengthen the motivation of the work if the authors could detail the potential advantages and disadvantages of their system compared to expressing Cas9 with a lineage-specific promoter from a transposon in their introduction or discussion.

Related to the above - is mitfa haplosufficient? If the mitfaCas9/+ fish have any notable phenotypes, it would be worth noting for others interested in using this approach to study melanoma and pigmentation.

A core weakness (and also potential strength) of the system is that introduced edits will always be non-clonal (Fig 2H/I). The activity of individual sgRNAs should always be validated in the absence of any noticeable phenotype to interpret a negative result. Additionally, caution should be taken when interpreting results from rare events involving positive

outgrowth (like tumorogenesis) to account for the fact many cells in the population might not have biallelic null alleles (i.e., 100% of the gene product removed).

Along those lines: in my opinion, the tuba1a results are the most provocative finding in the paper, but they lack key validation. With respect to cutting activity, the Alt-R and transgenic sgRNA expression approaches are not directly comparable. Since there is no phenotype in the melanocyte specific tuba1a knockouts, the authors must confirm high knockout efficiency with this set of reagents before making the claim there is a non-autonomous phenotype. This can be achieved with GFP+ sorting and NGS like they performed with their albino melanocytes.

The whole-body tuba1a knockout phenotype is expected to be pleiotropic, and this expectation might mask off-target effects. Controls for knockout specificity should be included. For instance, confidence in the claims would greatly increase if the dispersed melanosome phenotype could be recovered with guide-resistant tuba1a re-expression and if melanocyte-restricted tuba1a re-expression failed to rescue. As a less definitive but adequate alternative, the authors could also test if another guide or a morpholino against tuba1a phenocopies the described Alt-R edited fish.

I have similar questions about the sox10 escapers, but these suggestions are less critical for supporting the authors claims (especially given the nice staining). Are the sox10 tumors relatively clonal with respect to sox10 mutations? And are the sox10 tumor mutations mostly biallelic frameshifts or potential missense mutations/single mutations that might not completely remove activity? I am particularly curious as SOX10 doesn't seem to be completely absent (and is still very high in some nuclei) in the immunohistochemistry.

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