ABSTRACT

Spitz neoplasms are a diverse group of molecularly and histologically defined melanocytic tumors with varying biologic potentials. The precise classification of Spitz neoplasms can be challenging. Recent studies have revealed recurrent fusions involving multiple kinases in a large proportion of Spitz tumors. In this study, we generated a transgenic zebrafish model of Spitz melanoma using a previously identified ZCCHC8-ROS1 fusion gene. Animals developed grossly apparent melanocytic proliferations as early as 3 weeks of age and overt melanoma as early as 5 weeks. By 7 weeks, ZCCHC8-ROS1 induced a histologic spectrum of neoplasms ranging from hyperpigmented patches to melanoma. Given the swift onset of these tumors during development, we extended this approach into adult fish using a recently described electroporation technique. Tissue-specific expression of ZCCHC8-ROS1 in adults led to melanocyte expansion without overt progression to melanoma. Subsequent electroporation with tissue-specific CRISPR, targeting only tp53 was sufficient to induce transformation to melanoma. Our model exhibits the use of sequential mutagenesis in the adult zebrafish, and demonstrates that ZCCHC8-ROS1 induces a spectrum of melanocytic lesions that closely mimics human Spitz neoplasms.

KEY WORDS: Zebrafish, Cancer, Melanoma, Electroporation, Spitz neoplasm

INTRODUCTION

Spitz neoplasms are an uncommon group of difficult-to-classify melanocytic tumors with both benign and malignant clinical outcomes. The majority of Spitz neoplasms are benign and termed Spitz nevi. By contrast, clinically aggressive tumors with marked cytologic atypia have been variably referred to as Spitz melanoma or malignant Spitz tumors. However, the distinction between a benign Spitz nevus and a Spitz melanoma is not always possible on morphologic grounds alone, which has given rise to the terms ‘atypical Spitz tumor’ or ‘Spitz tumor of unknown malignant potential’ (Barnhill et al., 1999; Reed et al., 1975; Smith et al., 1989).

Recent advances in our molecular understanding of Spitz neoplasms have identified recurrent fusions that involve, several kinases, such as ALK, ROS1 and MAP3K8, among others (Raghavan et al., 2020; Wiesner et al., 2014). These observations have led several groups to suggest that integrating next-generation sequencing (NGS) and histologic analysis of Spitz neoplasms will provide improved risk stratification for patients (Quan et al., 2020; Zarabi et al., 2020). Despite a number of studies attempting to link clinical outcomes of Spitz neoplasms to NGS results, these tumors are uncommon, making it challenging to determine the importance of specific genetic alterations for the biologic potential of these tumors.

ROS1 fusions account for ~10-30% of kinase fusions in Spitz neoplasms and have been identified in benign Spitz nevi, atypical Spitz tumors and Spitz melanoma (Cesinaro et al., 2021; Gerami et al., 2020; Wiesner et al., 2014). ROS1 encodes a receptor tyrosine kinase, the function of which in humans is not well defined (Drilon et al., 2021). ROS1 fusion genes are best characterized in the context of lung adenocarcinoma, where they occur in 1-2% of patients (Gainor and Shaw, 2013). Numerous ROS1 fusion partners have been identified. All of these fusions result in retention of the C-terminal kinase domain of ROS1 fused to one of several N-terminal partners that are thought to provide a dimerization function, resulting in ROS1 activation and downstream signaling through the MAPK, PI3K and JAK/STAT pathways, among others (Davies and Doebele, 2013).

This study aims to characterize the role of the recurrent ZCCHC8-ROS1 fusion gene in the development of Spitz neoplasms. Given that Spitz neoplasms occur in patients of all ages, we explore the oncogenic activity of this fusion protein in development and adult zebrafish. We show that, in developing fish, expression of ZCCHC8-ROS1 alone leads to an atypical melanocytic proliferation and eventual melanoma formation that is irrespective of tumor suppressor loss. By contrast, expression of ZCCHC8-ROS1 alone results in rapid formation of pigmented patches, without overt progression to melanoma. We expand upon the later observation through sequential mutagenesis of tp53 in adult fish in order to show that subsequent tumor suppressor loss results in aggressive melanoma formation.

RESULTS

ZCCHC8-ROS1 expression leads to aberrant melanocyte development and patterning

The ZCCHC8-ROS1 fusion gene (ZROS1) has previously been identified in patient samples (Cesinaro et al., 2021; Wiesner et al., 2014). It consists of the first coiled-coil domain within the N-terminal fragment of ZCCHC8, a component of the nuclear exosome targeting complex, joined to the kinase domain of ROS1 (Fig. 1A). To test the functional role of ZROS1 in melanocyte development and melanoma progression in vivo, we introduced it into the transposon-based vector MiniCoopR (hereafter referred to as...
MCR) (Ceol et al., 2011), yielding ZROS1-expressing vector MCR:ZROS1. We used casper zebrafish, which lack melanocytes and iridophores (White et al., 2008). In this system, melanocytes do not develop, owing to a germline mutation in the mitfa gene. Introduction of the MCR:ZROS1 vector reintroduces mitfa as well as the ZROS1 fusion gene, which leads to melanocyte rescue and expression of ZROS1 protein in a tissue-specific manner via the mitfa promoter (Fig. 1B). Fish injected with either MCR:ZROS1, MCR:BRAFV600E, which overexpresses human BRAFV600E via the mitfa promoter, or MCR:Cas9:gRNA tp53, which inactivates tp53 in a tissue-specific manner, were screened for melanocyte rescue at day 4-5 and demonstrated a greatly reduced rescue efficiency in embryos injected with MCR:ZROS1 – irrespective of co-injection with MCR:Cas9;gRNA tp53, which inactivates tp53 in a tissue-specific manner, were screened for melanocyte rescue at day 4-5 and demonstrated a greatly reduced rescue efficiency in embryos injected with MCR:ZROS1 – irrespective of co-injection with MCR:Cas9;gRNA tp53 – compared to those that received MCR:Cas9:gRNA tp53 alone (P<0.001, Fig. 1C). This was similarly reflected in the reduced number of melanocytes identified when animals were screened for melanocyte rescue (day 4-5, Fig. 1D), suggesting that melanocyte development is greatly hindered by expression of ZROS1 during embryogenesis.

Despite the relative initial paucity of embryonic melanocytes, ZROS1-injected fish developed large pigmented patches by three weeks (Fig. 2A). This was not seen in controls injected with MCR vectors containing either only the 5′ fragment of ZCCHC8 or the 3′ fragment of ROS1. Upon examination of melanocytes 3 weeks post injection, we observed a highly dendritic morphology that was absent in melanocytes lacking only tp53 (Fig. 2B). These pigmented patches tended to initially form along the dorsal body surface and head. Histologic examination of these fish at 5-7 weeks demonstrated a microscopically apparent atypical melanocytic proliferation underlying the scales, which was confirmed by detection of human ROS1 and downstream activation of the MAPK pathway, as shown by immunostaining for phosphorylated ERK (phospho-ERK) (Fig. 2C). These results suggest that ZROS1 is responsible for the rapid expansion of melanocytes, despite the relative initial paucity early in development.

**ZCCHC8-ROS1 induces rapid tumor formation**

The aberrant pigmentation phenotype of the ZROS1 fish continued throughout development and gross tumors were observed as early as 5 weeks of age – in contrast to 9 weeks for the earliest tumors in BRAFV600E;tp53−/− animals (Fig. 3A). Both ZROS1;tp53−/− and ZROS1 fish had rapid tumor onset relative to BRAFV600E;tp53−/− zebrafish. In particular, ZROS1;tp53−/− showed complete penetrance of tumor formation by 20 weeks of age, whereas ZROS1 alone led to highly penetrant tumors with delayed kinetics (Fig. 3B, P<0.0001). No tumors were apparent in the first 30 weeks of observation for controls containing either the 3′-ROS1 fusion fragment or the 5′-ZCCHC8 fusion fragment. Expression of ZROS1 alone led to grossly apparent atypical melanocyte proliferations akin to atypical Spitz tumors by 5-7 weeks of age (Fig. 3C, upper panel). By contrast, at 7 weeks, ≥50% ZROS1;tp53−/− fish developed grossly apparent melanomas...
(Fig. 3B), which were highly aggressive and infiltrative (Fig. 3C, lower panel). In either case, despite likely having a small contribution from contaminating genomic DNA, the ZROS1 fusion transcript was readily detectable by qRT-PCR (Fig. 3D). Histologic examination at 7 weeks, found at least superficially invasive melanomas in almost all ZROS1;tp53−/− fish but only a small portion in ZROS1 fish. Based on morphologic analysis, the latter had a mixture of hyperpigmented areas without definite melanocytic expansion, and atypical proliferations leading to grossly apparent lesions and invasive melanomas (Fig. 3E). The melanocytes in the ZROS1;tp53−/− model tended to show a distinctly epithelioid morphology with very distinct nucleoli (Fig. 3F) compared to those in the BRAFV600E;tp53−/− model (Ceol et al., 2011), which tended to show a very non-distinct epithelioid to spindled morphology without well-defined cell borders (Fig. 3G). These findings demonstrate that ZROS1 expression leads to rapid onset of a morphologically distinct melanoma from the BRAFV600E-driven model.

**Rapid melanoma initiation in adult zebrafish using the TEAZ technique**

Recently, Callahan and colleagues have described Transgene Electroporation in Adult Zebrafish (TEAZ), a technique to generate melanomas within adult zebrafish in a temporally and spatially restricted manner (Callahan et al., 2018). The initial report of TEAZ used multiple transgenes to develop robust tumors and, ultimately, required protein kinase BRAFV600E, i.e. BRAF in which valine (V) at position 600 had been mutated to glutamic acid (E), in addition to loss of rb1 in a tp53-deficient background. We adapted the TEAZ technique for use in wild-type Tübingen (TU) zebrafish by taking advantage of the MCR system, the vectors of which contain Tol2 sequences (Fig. 4A). We hypothesized that we could improve the efficiency of TEAZ by leveraging these Tol2 sites by expressing Tol2 via a ubiquitous promoter introduced during the electroporation procedure. As anticipated, the addition of a vector driving expression of Tol2 (i.e. pCS2FA-Tol2) to the electroporation mixture containing MCR:ZROS1 dramatically improved the TEAZ process in wild-type TU zebrafish (Fig. 4B, \( P<0.0001 \)). The presence of pre-existing mature adult melanocytes was essential to the high efficiency of the TEAZ process because casper fish showed significantly reduced patch formation compared with that of wild-type TU fish (Table 1, \( P<0.0001 \), Chi-square test). As such, we used wild-type TU fish for all subsequent experiments. ZROS1 alone readily induced pigmented patches at the site of electroporation in adult zebrafish, which was not seen when electroporating MCR:BRAFV600E (Table 1). In contrast to ZROS1 expression during zebrafish development, these patches did not readily progress to melanoma. However, electroporation of MCR:
ZROS1 together with MCR:Cas9;gRNA tp53 to inactivate tp53, led to all fish with patches going on to develop tumors within 12 weeks (Fig. 4C,D). Histologically, these tumors recapitulate features of a progressive lesion with early patches showing a highly atypical proliferation of melanocytes that do not invade into the underlying skeletal muscle (Fig. 4E). As the lesions progress into large...
exophytic tumors (Fig. 4F), they infiltrate the underlying skeletal muscle (Fig. 4F,G). These results suggest that two genetic events are sufficient to initiate melanoma in wild-type TU zebrafish.

Given the lack of overt tumor formation in adult fish electroporated with MCR:ZROS1 alone, and the apparent progressive nature of the ZROS1;tp53−/− TEAZ fish, we hypothesized that this model represents a robust system for sequential mutagenesis in the zebrafish (Fig. 5A). To this end, we first electroporated adult TU fish with MCR:ZROS1 and waited for pigmented patches to form (at ~4 weeks post electroporation). Fish with patches were subsequently electroporated again with MCR:Cas9;gRNA tp53, resulting in tumor formation in the majority of fish that had developed pigmented patches upon electroporation of MCR:ZROS1 alone (Fig. 5B (red line), Fig. 5C). Sequencing of the tp53 CRISPR site in fish that developed tumors after sequential TEAZ demonstrated that the majority of tumors comprised single dominant CRISPR edit (12 of

Table 1. Electroporation efficiency

| Zebrafish background | Vector  | pCSFA-Tol2 | n   | Patch formation (%) | Tumor occurrence (%) |
|----------------------|---------|------------|-----|---------------------|----------------------|
| TU                   | MCR:ZROS1 | Y         | 16  | 63                  | 0                    |
| Casper               | MCR:ZROS1 | Y         | 57  | 0                   | n/a                  |
| Casper               | MCR:ZROS1 | N         | 16  | 0                   | n/a                  |
| TU                   | MCR:BRAFV600E | Y | 16  | 6                   | 0                    |

n/a, not applicable.
16) or similar variant allele fractions of two edits, suggesting a single clone (two of 16) (Fig. 5D). Two additional tumors had no detectable variant alleles at the \textit{tp53} CRISPR site. These data suggested that these tumors typically arise from a single dominant clone.

**DISCUSSION**

Investigating Spitz neoplasms has been challenging, owing to the rarity of these tumors and lack of cell lines or animal models. In this study, we describe the first animal model of Spitz tumors. \textit{ZCCHC8-ROS1} (ZROS1) fusion gene expressed under the \textit{mitfa} promoter is sufficient to drive these tumors. One of the limitations we initially encountered with this model was with embryo injections of the ZROS1 transgene limiting melanocyte rescue (~7%, Fig. 1C). This made these injections an incredibly labor-intensive enterprise that often yielded only a handful of fish with melanocyte rescue. Previous \textit{in vitro} work has suggested a role for JAK/STAT signaling, which is a known downstream target of ROS1 (Crescenzo et al., 2015), in inhibiting melanogenesis (Choi et al., 2013). This might explain the poor melanocyte rescue initially seen in ZROS1-injected embryos (Fig. 1C,D). Although initially

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**Fig. 5. Sequential mutagenesis reliably induces clonal tumors.** (A) Schematic of the sequential mutagenesis experiment in TU zebrafish. The arrowhead indicates the formation of a pigmented patch. (B) Electroporation of MCR:Cas9:gRNA tp53 into pigmented patches (black line) 4 weeks after the initial TEAZ. The red line denotes the proportion of fish with tumors generated from all fish with patches that underwent the second electroporation with MCR:Cas9:gRNA tp53. (C) Representative images of fish that had undergone sequential TEAZ (arrowhead and red dashed line denotes a pigmented patch). (D) Targeted sequencing of the \textit{tp53} CRISPR site. Most tumors show a single and dominant clonal edit (colors represent a specific mutant allele, 1D, deletion of one pair; 2D, deletion of two pairs; 7I, insertion of seven base pairs; 11I, insertion of eleven base pairs; 23I=insertion of twenty-three base pairs; n/a, not applicable (wild type sequence only).
reminiscent of zebrafish with inactivating kita mutations, which have significant reductions in early melanocytes (Hultman et al., 2007), this phenomenon was transient; large hyperpigmented patches developed on the majority of MCR:ZROS1-injected zebrafish that showed any degree of melanocyte rescue by 3 weeks of age (Fig. 2A). This suggested that these heavily pigmented patches were derived not from the initial wave of embryonic melanocytes but, rather, a subsequent wave of melanocytes probably derived from postembryonic melanophore progenitors (Dooley et al., 2013). Interestingly, we noticed a hyper-dendritic appearance to the melanocytes at the edge of these pigmented patches. One possible explanation for this morphology is the activation of downstream Rho signaling, a known downstream target of ROS1 fusion proteins and a previously described regulator of melanocyte dendricity (Kim et al., 2010; Zeng et al., 2000). These observations suggest that the dysregulation of ROS1-targeted pathways in melanocyte development can have broader implications for pigmentation, developmental patterning and melanocyte biology, which would be amenable to further study in this model system.

Over time, ZROS1-injected zebrafish almost universally develop large invasive tumors, regardless of loss of tp53 (Fig. 3B). At 7 weeks of age, ZROS1 fish had a spectrum of morphologic changes ranging from overt melanoma to hyperpigmented patches (Fig. 3E). This diversity of morphologies in the MCR:ZROS1 fish closely overlaps with the spectrum of Spitz neoplasms seen in patients (Menezes and Mooi, 2017), including a tendency towards a striking epithelioid morphology (Fig. 3F) (Cesinaro et al., 2021; Gerami et al., 2020). This is in contrast to the BRAFV600E, tp53−/− model, in which melanomas tend to have more-variable histologic appearance and, when present, a less distinct epithelioid cytomorphology (Fig. 3G) (Ceol et al., 2011). Additionally, coinjection of MCR:ZROS1 and MCR:Cas9:gRNA tp53 led to rapid tumor generation with aggressive melanomas in as few as 5 weeks (Fig. 3A,B), demonstrating that the loss of a tumor suppressor accelerates tumorigenesis. This is in keeping with TP53 mutations in humans being progressively more common – from Spitz nevi, to atypical Spitz tumors and Spitz melanomas (Dal Pozzo and Cappellosso, 2022).

Although Spitz tumors are more frequent in adolescents, they occur in patients of all ages. Given the rapid development of tumors when injected into the one-cell embryo, we sought to study the ability of ZROS1 to induce tumors in adult zebrafish – in which the melanocyte pool is derived from differentiated adult melanocytes – by applying the recently described TEAZ technique (Callahan et al., 2018). Our data demonstrate that the efficiency of TEAZ can be greatly improved by co-electroporation with a vector expressing Tol2; the simple injection and electroporation process is easily learned and requires limited specialized equipment beyond the electroporator. This addition is likely to drive integration of the various MCR vectors – which contain Tol2 sites – into the zebrafish genome, leading to a greater than 10-fold enhancement of the TEAZ process efficiency in wild-type zebrafish (Fig. 4A). Interestingly, in contrast to the work by Callahan and colleagues, we did not observe an acceleration in tumor development when converting from embryo injections to TEAZ. This could be a consequence of using TU fish with pre-existing melanocytes or, perhaps, a function of the ZROS1 fusion gene itself.

Electroporation of MCR:ZROS1 alone into adult zebrafish induced variably sized hyperpigmented patches, the majority of which formed within 5 weeks of electroporation (Fig. 4C). Pigmented patches were rarely seen when electroporating MCR; BRAFV600E alone (Table 1). This raises the possibility that the pigmented patches seen with TEAZ of MCR:ZROS1 result from a phenotypic change in pre-existing adult melanocytes and progenitors, leading to an increased pigmentation and disrupted patterning without significant proliferation – as TEAZ of MCR:ZROS1 alone did not induce formation of grossly raised tumors, even when following fish out beyond 20 weeks. By contrast, co-electroporation with MCR:Cas9:gRNA tp53 led to robust and rapid formation of grossly apparent tumors in every animal that formed a pigmented patch (Fig. 4C). These data strongly suggest that cooperating loss of a tumor suppressor is needed to give rise to Spitz melanomas. Previous sequencing studies of human Spitz melanomas have made similar observations, i.e. that inactivation of tumor suppressors, such as CDKN2A, frequently occur in Spitz melanomas (Raghavan et al., 2020). The role of TP53 in the development of human Spitz melanomas is less clear. In one study of 40 RAF1-fusion melanomas, 13% (five out of 40) showed inactivating TP53 mutations. In this particular study, a 60% loss of CDKN2A (24 out of 40) and/or 62% with activating TERT promoter mutations (23 out of 37) were more common genetic events (Williams et al., 2020). Another study that sequenced nine Spitz melanomas with non-ROS1 rearranged driver mutations did not identify any TP53 mutations (Raghavan et al., 2020). Moreover, loss of tumor suppressor can also be seen in benign Spitz tumors (Quan et al., 2020), suggesting that other somatic alterations, such as the more-common mutations of the TERT promoter or the overall mutational burden, may be better markers of clinical outcomes for these patients (Lee et al., 2015).

We also believe that our work defines the minimum number of genetic elements that are needed to drive melanoma initiation in wild-type adult fish: a single oncogene and the loss of a tumor suppressor. However, we do not believe that this applies to every combination of oncogene and tumor suppressor – as previously demonstrated by Callahan and colleagues – or that it is likely to occur in zebrafish from other genetic backgrounds (Callahan et al., 2018). Additionally, we have attempted to substitute other tumor suppressors, such as cdkn2a, for tp53 in the second TEAZ step and found significantly lower penetrance. This suggests that specific oncogene and tumor suppressor combinations are able to necessitate additional ‘hits’ to initiate melanoma formation. This is evident from injections of MCR:ZROS1 into casper zebrafish embryos, which led to melanocytic lesions with a striking epithelioid morphology irrespective of targeting tp53 (Fig. 3). By contrast, TEAZ-generated tumors in TU zebrafish required additional targeting of tp53, and the resulting tumors tended to show greater pleomorphism (Fig. 4E,G). It is possible that this greater atypia is related to non-specific genomic alterations induced by the TEAZ and TOL2 insertional process. The identification of a small subset of tumors with sequential TEAZ showing no tp53 alterations suggests that tp53 loss is sufficient but not necessary for melanoma progression, raising the possibility of non-specific genetic events that result from the TEAZ/TOL2 process (Fig. 5D). By contrast, no such second hit appears to be necessary in fish injected at the one-cell stage (Fig. 3B). This may be a function of expression in ZROS1 throughout development, which includes a number of embryonic and post-embryonic progenitors that may be more susceptible to transformation than differentiated adult melanocytes and progenitors, for which loss of a tumor suppressor is likely to be necessary in order to develop melanoma.

Although, generally, a clinically indolent disease compared to conventional melanoma (Bartenstein et al., 2019; Quan et al., 2020),
Spitz neoplasms can be challenging to correctly diagnose. In addition, atypical Spitz tumors and melanomas occasionally give rise to metastatic tumors (Pol-Rodriguez et al., 2007; Tlougan et al., 2013). Several recent studies have suggested a dual role for both morphologic evaluation as well as next-generation sequencing to help appropriately classify these lesions (Quan et al., 2020; Zarabi et al., 2020). Beyond being the first animal model of Spitz tumors, our findings expand the utility of the TEAZ system for modeling melanoma, by providing a useful adjunct to clinical sequencing data that explores the role of specific mutations in the progression of Spitz neoplasms in either juvenile or adult zebrafish. This system is interchangeable and has the potential to model other fusion gene-driven melanomas, and to act as a test platform for targeted therapies; moreover, it is a model for performing sequential mutagenesis in a spatially and/or temporally restricted fashion.

**MATERIALS AND METHODS**

### Zebrafish modeling

All animal work was performed according to Boston Children’s Hospital Institutional Animal Care and Use Committee (protocol #20-10-4253R). Tumors were generated as outlined in Ablain et al. (2018). The MiniCoopR construct (MCR) was used to overexpress the ZCCHC8-RS01 fusion gene using HiFi assembly (New England Biolabs, Ipswich, MA, USA) with the previously identified ZCCHC8-RS01 open reading frame (Wienesser et al., 2014), which was synthesized as a gBlocks gene fragment, i.e. double-stranded 125- to 3000-bp-long DNA fragments, by Integrated DNA technologies (Corvalle, IA, USA). Among the primers were used to amplify the construct: 5′-TGGTTGACGCGCCCCCTCGAGGATGGCCCGAGGGTG-3′ and 5′-GTGGATCCTCCCGGTGCAGGAAATTITTTAATCGACGACCAC-3′, for assembly into the MCR vector that has previously been assembled using gateway multiple site cloning (Invitrogen, Waltham, MA, USA) with the mitf-promoter, a multiple cloning site and a poly-adenylation site. The vector was linearized using Sall and EcoRI prior to gateway assembly. Briefly, embryo injections were performed using: MCR:ZCCHC8-RS01 (25 pg), MCR:BRAFV600E; MCR:Cas9; gRNA tp53 or MCR:ZCCHC8-RS01:tp53 (12.5 pg each) together with Tol2 transposase mRNA (25 pg) into one-cell casper zebrafish embryos. Embryos were scored for melanocytes rescue at 4.5 days post fertilization and rescue compared by Chi-square test. Zebrafish were examined every 1-2 weeks after injection and followed for the development of melanocytic tumors. Tumor-free survival curves were scored once grossly apparent tumors, defined as a pigmented raised lesion were examined every 1-2 weeks after injection and followed for the development of Spitz neoplasms in either juvenile or adult zebrafish. The vector was linearized using the ECM 830 Square wave electroporation system (BTX, Hercules, CA, USA) using iTaq Universal SYBR green supermix (Bio-Rad) according to the manufacturer’s instructions. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using the C1000 thermocycler (Bio-Rad, Hercules, CA, USA) using iTaq Universal SYBR green supermix (Bio-Rad) with the following primers (Invitrogen): EF1a-5′-GATCAGATCAAGACGATGTCG-3′ and 5′-GACGGCTCTTGTCAGACGTTG-3′; ZCCHC8-RS01, 5′-GGCGCGGAGAAATGGAATGTTG-3′ and 5′-ACACTTCCTCAAAAGGTCTCTA-3′. Relative values were calculated using the ΔACT method (normalized to EF1a expression).

### qRT-PCR

Tumors were manually isolated from MCR:ZCCHC8-RS01, MCR:BRAFV600E;tp53−/− or MCR:ZCCHC8-RS01:tp53−/− fish, mechanically homogenized and RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized from 2000 ng of total RNA using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA) per manufacturer’s instructions. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using the C1000 thermocycler (Bio-Rad, Hercules, CA, USA) using iTaq Universal SYBR green supermix (Bio-Rad) with the following primers (Invitrogen): EF1a-5′-GATCAGATCAAGACGATGTCG-3′ and 5′-GACGGCTCTTGTCAGACGTTG-3′; ZCCHC8-RS01, 5′-GGCGCGGAGAAATGGAATGTTG-3′ and 5′-ACACTTCCTCAAAAGGTCTCTA-3′. Relative values were calculated using the ΔACT method (normalized to EF1a expression).

### CRISPR sequencing

Tumor tissue was isolated from MCR:ZCCHC8-RS01:tp53−/− tumors that had been generated in casper zebrafish using sequential TEAZ and genomic DNA isolated using the Qiagen DNeasy Blood and Tissue Kit. Genomic DNA was amplified with Phusion high-fidelity DNA polymerase (New England Biolabs) with the following primers (Invitrogen): tp53, 5′-CTGGTTGGTCGACGGAGACCTTT-3′ and 5′-TATGGTGGTTGATCGGCTTTTG-3′. PCR products were purified using the QIAquick PCR purification kit (Qiagen). Sequencing was performed at the MGH DNA core and analyzed using CRISPResso2 (https://crispresso.pinellolab.org/) (Clement et al., 2019).

### Histology and immunohistochemistry

Zebrafish were euthanized and fixed in 10% formalin, processed, paraffin-embedded and sectioned (5 µM) after brief surface decalcification. Using standard techniques, Hematoxylin and Eosin staining was performed by the Dana-Farber/Harvard Cancer Center Specialized Histopathology core. Photomicrographs were acquired on an Olympus BX43 microscope with a DP27 camera (Olympus Corporation, Tokyo, Japan). Histologic sections reviewed by two pathologist (J.K.M. and G.F.M., the latter a dermatopathologist). Melanocytic lesions were classified as (1) hyperpigmented, when no atypical melanocytes were identified; (2) atypical proliferations, when there was mild-to-moderate cytologic atypia without evidence of local tissue invasion or; (3) melanomas, when highly infiltrative lesions contained overt cytologic atypia.

Histology was performed by the Dana-Farber/Harvard Cancer Center Specialized Histopathology core with 5-µm-thick formalin-fixed, paraffin-embedded tissue sections using the Bond III automated staining platform (Leica Biosystems, Wetzlar, Germany). Antibody against phosphorylated ERK (p44/p42 MAPK, clone D13.14.4E, cat #4370, 1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) was utilized after citrate antigen retrieval. The antibody for ROS1 (clone D4D6, cat #3287, 1:250 dilution, Cell Signaling Technology) was utilized after EDTA antigen retrieval. For both, the Refine Detection Kit (Leica Biosystems) was used.

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### Competing interests

L.I.Z. is a founder and stockholder of Fate Therapeutics, Inc., Scholar Rock, Camp4 Therapeutics, Inc. and Amagma Therapeutics, Inc.

### Author contributions

Conceptualization: J.K.M., L.I.Z.; Methodology: J.K.M.; Formal analysis: J.K.M., M.C.W., G.F.M.; Investigation: J.K.M., M.C.W., A.C., G.F.M.; Resources: L.I.Z.; Data curation: J.K.M.; Writing - original draft: J.K.M.; Writing - review & editing: J.K.M., M.C.W., A.C., G.F.M.; Visualization: J.K.M.; Supervision: L.I.Z.; Funding acquisition: L.I.Z.
References

Abdallati, S., Tepen, S., Zhang, Y. M., Lindsay, H., Burger, A., Campbell, N. R., Bartenstein, D., From, L., Glass, L. F., Maize, J. C., Mihm, M. C., Jr, Barnhill, R. L., Argenyi, Z. B., From, L., Glass, L. F., Maize, J. C., Mihm, M. C., Jr, Dooley, C. M., Mongera, A., Walderich, B. and Nusslein-Volhard, C. (1999). Atypical Spitz nevi/tumors: lack of consensus for diagnosis, discrimination from melanoma, and prediction of outcome. Hum. Pathol. 30, 513-520. doi:10.1016/S0363-4689(99)00075-1

Crescenzo, R., Abate, F., Lasorsa, E., Tabbo, F., Gaudiano, M., Chiesa, N., Di Clement, K., Rees, H., Canver, M. C., Gehrke, J. M., Farouni, R., Hsu, J. Y., Cole, M. A., Liu, D. R., Joung, J. K., Bauer, D. E. et al. (2013). ROS1 fusion Spitz neoplasms. Mod. Pathol. 34, 348-357. doi:10.1038/s41379-020-00658-w

Hultman, K. A., Bahary, N., Zon, L. I. and Johnson, S. L. (2007). Gene Duplication of the zebrafish kit ligand and partitioning of melanocyte development functions to kit ligand a. PLoS Genet. 3, e17. doi:10.1371/journal.pgen.0030017

Kim, M. Y., Choi, T. Y., Kim, J. H., Lee, J. H., Kim, J. G., Sohn, K. C., Yoon, K. S., Kim, C. D., Lee, J. H. and Yoon, T. J. (2010). MKK6 increases the melanocyte dendritcity through the regulation of Rho family GTPases. J. Dermatol. Sci. 60, 114-119. doi:10.1016/j.jdermsci.2010.08.006

Kwan, K. M., Fujimoto, E., Grabher, C., Mangum, B. D., Hardy, M. E., Campbell, D. S., Parant, J. M., Yost, H. J., Kanki, J. P. and Chien, C. B. (2007). The Tozl2kit is a multisite gateway-based construction kit for Tozl2 transposon transgenesis constructs. Dev. Dyn. 236, 3088-3099. doi:10.1002/dvdy.21343

Lee, S., Barnhill, R. L., Dummer, R., Dalton, J., Wu, J., Pappo, A. and Bahrami, A. (2015). TERT promoter mutations are predictive of aggressive clinical behavior in patients with Spitzoid melanocytic neoplasms. Sci. Rep. 5, 11200. doi:10.1038/srep11200

Menezes, F. D. and Mooi, W. J. (2017). Spitz tumors of the skin. Surg. Pathol. Clin. 10, 281-298. doi:10.1016/j.pathcl.2017.01.004

Pol-Rodríguez, M., Lee, S., Silvers, D. N. and Celebi, J. T. (2017). Influence of age on survival in childhood spitzoid melanomas. Cancer 109, 1579-1583. doi:10.1002/cncr.22584

Quan, V. L., Zhang, B., Zhang, Y., Mohan, L. S., Shi, K., Wagner, A., Kruse, L., Taxter, T., Beaubier, N., White, K. et al. (2020). Integrating next-generation sequencing with morphology improves prognostic and biologic classification ofSpitz neoplasms. J. Invest. Dermatol. 140, 1599-1608. doi:10.1016/j.jid.2019.12.031

Raghavan, S. S., Peternel, S., Mulyy, T. W., North, J. P., Pincus, L. B., LeBoit, P. E., McCalmon, T. H., Bastian, B. C. and Yeh, I. (2020). Spitz melanoma is a distinct subset of spitzoid melanoma. Mod. Pathol. 33, 1122-1134. doi:10.1038/s41379-019-0445-z

Reed, R. J., Ichinose, H., Clark, W. H., Jr. and Mihm, M. C. Jr (1975). Common and uncommon melanocytic nevi and borderline melanomas. Semin. Oncol. 2, 119-147.

Smith, K. J., Barrett, T. L., Skelton, H. G., III, Lupton, G. P. and Graham, J. H. (1989). Spindle cell and epithelioid cell nevi with atypia and metastasis (malignant Spitz nevus). Am. J. Surg. Pathol. 13, 283-291. doi:10.1001/jamadermatol.2013.1124

Taxter, T., Beaubier, N., White, K. et al. (2020). Integrating next-generation sequencing with morphology improves prognostic and biologic classification ofspitz neoplasms. J. Invest. Dermatol. 140, 1599-1608. doi:10.1016/j.jid.2019.12.031

White, R. M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., Dovey, M., Goessling, W., Burns, C. E. et al. (2008). Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2, 183-189. doi:10.1016/j.stem.2007.11.002

Wiesner, T., He, J., Yelensky, R., Esteve-Puig, R., Botton, T., Yeh, I., Lipson, D., Otto, G., Brennan, K., Murali, R. et al. (2014). Kinase fusions are frequent in Spitz tumours and spitzoid melanomas. Nat. Commun. 5, 3116. doi:10.1038/ncomms4116

Williams, E. A., Shah, N., Montesion, M., Sharaf, R., Pavlick, D. C., Sokol, E. S., Alexander, B. M., Venstrom, J. M., Elvin, A. J., Ross, J. S. et al. (2020). Melanomas with activating RAF1 fusions: clinical, histopathologic, and molecular profiles. Mod. Pathol. 33, 1466-1474. doi:10.1038/s41379-020-0510-7

Zarabi, S. K., Azzato, E. M., Tu, Z. J., Ni, Y., Billings, S. D., Arbesman, J., Funchain, P., Gastman, B., Farkas, D. H. and Ko, J. S. (2020). Targeted next generation sequencing (NGS) to classify melanocytic neoplasms. J. Cutan. Pathol. 47, 691-704. doi:10.1111/jcpp.13695

Zeng, L., Sachdev, P., Yan, L., Chan, J. L., Trenkle, T., McClelland, M., Weish, J. and Wang, L.-H. (2000). Vav3 mediates receptor protein tyrosine kinase signaling, regulates GTPase activity, modulates cell morphology, and induces cell transformation. Mol. Cell Biol. 20, 9212-9224. doi:10.1128/MCB.20.24.9212-9224.2000