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A Conditional Zebrafish MITF Mutation Reveals MITF Levels Are Critical for Melanoma Promotion vs. Regression *In Vivo*

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The microphthalmia-associated transcription factor (MITF) is the "master melanocyte transcription factor" with a complex role in melanoma. MITF protein levels vary between and within clinical specimens, and amplifications and gain- and loss-of-function mutations have been identified in melanoma. How MITF functions in melanoma development and the effects of targeting MITF *in vivo* are unknown because MITF levels have not been directly tested in a genetic animal model. Here, we use a temperature-sensitive *mitf* zebrafish mutant to conditionally control endogenous MITF activity. We show that low levels of endogenous MITF activity are oncogenic with $BRAF^{V600E}$ to promote melanoma that reflects the pathology of the human disease. Remarkably, abrogating MITF activity in $BRAF^{V600E}$*mitf* melanoma leads to dramatic tumor regression marked by melanophage infiltration and increased apoptosis. These studies are significant because they show that targeting MITF activity is a potent antitumor mechanism, but also show that caution is required because low levels of wild-type MITF activity are oncogenic.

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INTRODUCTION

Driver genes that stimulate proliferation and survival are important drug targets in cancer. The discovery of $BRAF^{V600E}$ mutations in nevi and melanoma has directly led to the development of small-molecule inhibitors with clear clinical benefit (Flaherty *et al.*, 2012). Despite these dramatic improvements, drug resistance remains a critical problem, and most patients with metastatic melanoma eventually succumb to the disease within a year. It is therefore necessary to identify additional therapeutic targets in melanoma that can be used in combination with available treatments (Tsao *et al.*, 2012).

One of the important genes in melanocyte development and melanoma is the highly conserved ''master melanocyte transcription factor'' microphthalmia-associated transcription factor (MITF) (Levy *et al.*, 2006). MITF responds to multiple signaling cascades to orchestrate genes involved in melanocyte growth, differentiation, and survival (Cheli *et al.*, 2010). Although MITF mutations in development lead to similar phenotypes across species, the function of MITF in melanoma is complex and not fully understood. MITF is expressed in most melanomas, although MITF protein levels vary between melanoma specimens, with some subsets of melanoma showing high levels and others showing low levels of MITF (Flaherty *et al.*, 2012). MITF activity can also vary within an individual melanoma, such that low levels of MITF promote invasion and stem-cell like phenotypes and moderate levels of MITF activity promote cell cycle progression (Goodall *et al.*, 2008; Hoek and Goding, 2010; Cheli *et al.*, 2011; Strub *et al.*, 2011; Cheli *et al.*, 2012). The ability of MITF to activate cancer hallmark genes makes it an important mediator of oncogenic signaling in cancer. This is underscored by evidence that MITF is at least partially responsible for the oncogenic potential of BRAF in cells (Wellbrock and Marais, 2005; Wellbrock *et al.*, 2008). Studies from melanoma cells indicate that a key function of $BRAF^{V600E}$ seems to be to maintain MITF activity at a critical threshold where it promotes proliferation, invasion, and survival, without promoting differentiation (at higher levels) or apoptosis or senescence (at lower levels) (Giuliano *et al.*, 2010; Hoek and Goding, 2010; Cheli *et al.*, 2011; Strub *et al.*, 2011; Cheli *et al.*, 2012)

However, MITF may have additional cooperating functions in melanoma. MITF is amplified in some melanomas, and expression of ectopic MITF can cooperate with $BRAF^{V600E}$ to transform primary human melanocytes and neural crest cells (Garraway *et al.*, 2005; Kumar *et al.*, 2013). In addition, MITF

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mutations have been identified in $BRAF^{V600E}$ melanomas including somatic mutations with either hypomorphic or increased activity (Cronin *et al.*, 2009), and a germline SUMOylation E318K mutation that is a melanoma risk factor and confers differential gene expression of MITF target genes (Bertolotto *et al.*, 2011; Yokoyama *et al.*, 2011). Thus, MITF is important in melanoma because it is an effector of oncogenic signaling, and also because it may have additional activity that contributes to melanomagenesis.

How MITF activity contributes to melanoma development and survival in an animal is unknown. Animal models are fundamental in establishing how genetic mutations contribute to cancer *in vivo*. Although many *Mitf* mutant mouse lines exist (Hou and Pavan, 2008), they do not permit conditional control of MITF activity in melanoma development or survival. Here, we address the importance of MITF activity in melanoma *in vivo* using a conditional *mitfa* temperature-sensitive zebrafish mutant ($mitfa^{vc7}$) in which endogenous MITF activity can be altered by changing the temperature of the water (Johnson *et al.*, 2011; Taylor *et al.*, 2011). In zebrafish, there are two *mitf* genes (*mitfa* and *mitfb*), and *mitfa* is essential for the development of neural crest–derived melanocytes (Lister *et al.*, 1999). Thus, by using a *mitfa* mutant we specifically control endogenous MITF activity in skin melanocytes, and avoid the potential complication of MITF activity in other tissues, such as those described in mouse mutants (Hou and Pavan, 2008). We show that low levels of wild-type MITF activity are oncogenic with $BRAF^{V600E}$ to promote melanoma *in vivo*, and that abrogating MITF activity in melanoma leads to rapid tumor regression. These results reveal that critical thresholds of MITF lead to dramatically different melanoma outcomes, and indicate that although targeting MITF activity is a potent antitumor approach, simply reducing MITF activity is sufficient to drive melanoma in $BRAF^{V600E}$ melanocytes.

RESULTS

Hypomorphic MITF is oncogenic with $BRAF^{V600E}$ in melanomagenesis

We sought to test whether hypomorphic levels of MITF activity could contribute to melanoma development *in vivo* using a zebrafish *mitf* temperature-sensitive mutant, $mitfa^{vc7}$ (Figure 1a–d; Johnson *et al.*, 2011; originally characterized as *fh53*) and a transgenic line expressing $BRAF^{V600E}$ in melanocytes that we have previously developed and has been effective in the identification of cooperating driver genes (Figure 1e) (Patton *et al.*, 2005; Ceol *et al.*, 2011). The $mitfa^{vc7}$allele is a splice site mutation at the intron 6 splice donor site that leads to a reduction in melanocytes when zebrafish are reared at $<26\,^{\circ}C$, and an almost complete loss of melanocytes at $>28\,^{\circ}C$ (Figure 1b–d) (Johnson *et al.*, 2011). We performed two generations of genetic crosses with the $BRAF^{V600E}$ transgenic fish to the $mitfa^{vc7}$ mutant zebrafish to generate $BRAF^{V600E/V600E}mitfa^{vc7/vc7}$ ($BRAF^{V600E}mitf$) zebrafish. As expected, $BRAF^{V600E}mitf$ zebrafish did not develop melanocytes at the restrictive temperature ($28.5\,^{\circ}C$) because there is not sufficient MITF activity to generate melanocytes (Figure 1f). Importantly, at $<26\,^{\circ}C$, $BRAF^{V600E}mitf$ zebrafish developed nevi (Figure 1g), some of which progressed to melanoma ($n=18/67$; Figure 1h–j). The $mitfa^{vc7}$ allele is a splice site mutation, and we confirmed that the $BRAF^{V600E}mitf$ melanomas expressed the mis-spliced + *intron6* variant with hypomorphic levels of correctly spliced *mitfa* (Figure 1k). As controls, neither $BRAF^{V600E}$ transgenic fish carrying wild-type *mitfa* alleles nor *mitfa* mutants lacking the $BRAF^{V600E}$ transgene developed melanoma at any temperature (Patton *et al.*, 2005; Johnson *et al.*, 2011; data not shown). We compared the incidence of melanoma in $BRAF^{V600E}mitf$ compared with $BRAF^{V600E/V600E}p53^{M214K/M214K}$ ($BRAF^{V600E}p53$) zebrafish, and found that the incidence was similar between the two genotypes ($n=48/177$; Figure 1j). These results show that hypomorphic levels of MITF activity interact genetically with $BRAF^{V600E}$ to promote melanoma *in vivo*.

$BRAF^{V600E}mitf$ melanomas display characteristic histopathological features

We wanted to know whether the *mitf* and *p53* cooperating mutations contributed to melanoma pathology. We found that most $BRAF^{V600E}mitf$ melanomas displayed a superficial spreading growth pattern with some invasion into the underlying muscle (Figure 2a; $n=22/26$). This pattern was reminiscent of superficial spreading melanoma, the most common subtype of human melanoma. A striking characteristic feature of $BRAF^{V600E}mitf$ melanomas was the presence of large, heavily pigmented cells throughout the tumor ($n=26/26$), and often found in the kidney (the site of the hematopoietic compartment in zebrafish). Macrophages laden with melanin (melanophages) are often a feature of human malignant melanoma, and express CD68. We found these large cells to correspond to CD68-positive cells in the $BRAF^{V600E}mitf$ melanomas and characterized them as melanophages (Supplementary Figure S1 online). $BRAF^{V600E}mitf$ melanomas were composed of spindle- and epithelioid-shaped tumor cells, marked by few mitoses and showing only mild nuclear pleomorphism. These histological features were characteristic of $BRAF^{V600E}mitf$ melanomas, and allowed reliable identification of these tumors on blind assessment by a clinical skin pathologist (MEM; $n=26/26$; Figure 2a). By comparison, most $BRAF^{V600E}p53$ melanomas progressed rapidly, displaying a nodular and a highly invasive growth pattern into multiple organs ($n=19/21$; Figure 2b). No melanophages were observed in $BRAF^{V600E}p53$ melanomas, and the tumors were composed primarily of epithelioid cells, with features indicative of aggressive cancers including numerous mitoses and moderate-to-severe nuclear pleomorphism.

We analyzed the activation state of the MAPK cascade in the $BRAF^{V600E}mitf$ and $BRAF^{V600E}p53$ mutant melanoma by performing immunohistochemical analysis with anti-phospho-extracellular signal–regulated kinase (ERK; Figure 2c). As expected, phospho-ERK signal was detected in the majority of melanoma cells in both $BRAF^{V600E}mitf$ and $BRAF^{V600E}p53$ melanoma, and $BRAF^{V600E}p53$ had increased levels of p53 mutant protein (Figure 2d). Both melanomas stained positively for Melan-A, a MITF target gene and marker for melanoma and melanocytes in human specimens (Du *et al.*, 2003) (Figure 2e). Increased mitotic activity in $BRAF^{V600E}p53$

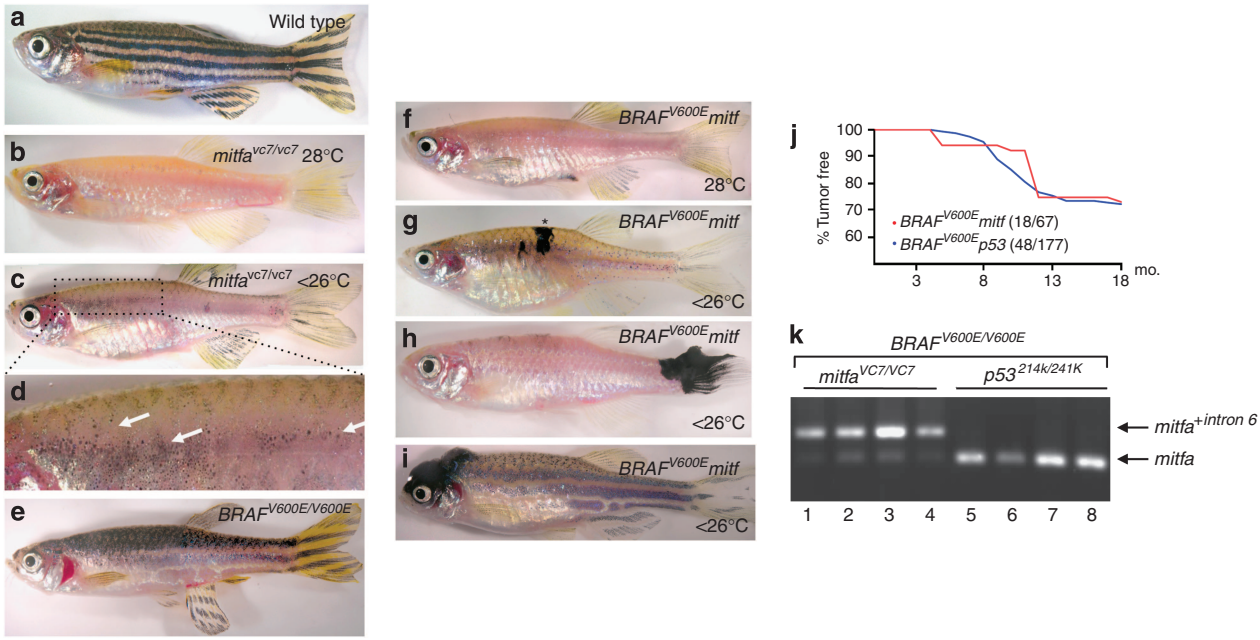

Figure 1. The microphthalmia-associated transcription factor (MITF) is oncogenic with BRAF^{V600E} in melanomagenesis. (**a**) Adult wild-type zebrafish or (**b**) adult *mitfa*^{vc7} mutant zebrafish living in water at 28 °C or (**c**, **d**) <26 °C. At <26 °C some melanocytes are visible in the body (**d**: enlarged region, white arrows). (**e**) Adult transgenic line expressing human *BRAF*^{V600E} in the melanocytes. (**f–i**) Genetic crosses of *BRAF*^{V600E}*mitf* at the semirestrictive temperatures develop nevi (*) and melanoma (on the tail of the middle fish, and on the head of the bottom fish). (**j**) Melanoma incidence curves of *BRAF*^{V600E}*p53* and *BRAF*^{V600E}*mitf* (<26 °C) genetic crosses. (**k**) Real-time PCR (RT-PCR) analysis of the *mitfa* transcript in *BRAF*^{V600E}*p53* and *BRAF*^{V600E}*mitf* melanomas.

melanomas compared with *BRAF*^{V600E}*mitf* melanoma cells was confirmed by immunostaining for phospho-histone H3, a marker of late-G2/M phase (Figure 2f and g). These results show that there is a strong genotype–phenotype correlation for cooperating mutations that can directly affect growth features and cellular histology.

Differential MITF target gene expression between melanoma genotypes

We wanted to understand how hypomorphic MITF activity contributed to melanoma, and hypothesized that MITF target genes may be differentially expressed between the melanoma genotypes. We performed quantitative real-time PCR on MITF target genes involved in proliferation (*cdk2*), cell cycle arrest (*p16*, *p21*), differentiation (*tyr*, *dct*), and survival (*bcl-2*, *hif1α*, *c-met*) (Figure 3). Despite the differences in phospho-histone H3 staining between the genotypes, the differences in *cdk2*, *p16*, and *p21* cycle threshold (Ct) values between melanoma genotypes were not statistically significant (Figure 3a–c). Neither was there a significant difference in the cycle threshold values for expression of *p53*, *bcl-2*, or *hif1α* between melanoma genotypes (Figure 3f–h). These results indicate that despite the reduced levels of MITF activity in *BRAF*^{V600E}*mitf* melanomas, there is sufficient MITF activity to control MITF target genes involved in cell proliferation and survival. Strikingly, *BRAF*^{V600E}*mitf* melanomas expressed lower levels of differentiation genes (*tyr* and *dct*), as indicated by higher cycle threshold values (Figure 3d and e). Unexpectedly, we found that *BRAF*^{V600E}*mitf* melanomas expressed significantly higher levels of *c-met* compared with *BRAF*^{V600E}*p53* melanoma (Figure 3i). *c-met* is a MITF target gene, but is also transcriptionally regulated by Pax3 in melanoblasts and melanomas (McGill *et al.*, 2006; Beuret *et al.*, 2007; Mascarenhas *et al.*, 2010). The tumor-initiating potential of cell types can vary within a lineage and differing tumor potentials may exist within the melanocyte lineage (Kumar *et al.*, 2013). Although the tumors are heterogeneous, the low expression of differentiation genes (*tyr* and *dct*) coupled with high *c-met* gene expression pattern suggests that hypomorphic MITF activity may maintain melanocytes in a less differentiated state that is more susceptible to BRAF^{V600E} transformation.

Loss of MITF causes melanoma regression

MITF is a lineage survival oncogene in cells, but the effect of abrogating MITF activity in an animal model of melanoma *in vivo* is unknown. To develop an animal system that could directly validate MITF as a drug target, we tested the effects of dramatically reducing MITF activity on melanoma survival by increasing the water temperature to the restrictive conditions (32 °C); none of the aberrant *mitfa*^{vc7} splice products are sufficient for melanocyte development at these restrictive temperatures (Johnson *et al.*, 2011), and MITF activity is not required to maintain the activity of the *mitfa* promoter fragment driving the BRAF^{V600E} transgene (Supplementary Figure S2 online; Dooley *et al.*, 2013). *BRAF*^{V600E}*mitf*^{vc7} zebrafish were reared at <26 °C to promote melanoma development, and then the temperature of the water was raised to 32 °C to turn off MITF activity. Within 2 weeks, 8/12 melanomas had dramatically regressed (Figure 4a; fish 1 and 2). Melanoma regression was the result of the *mitfa*^{vc7}

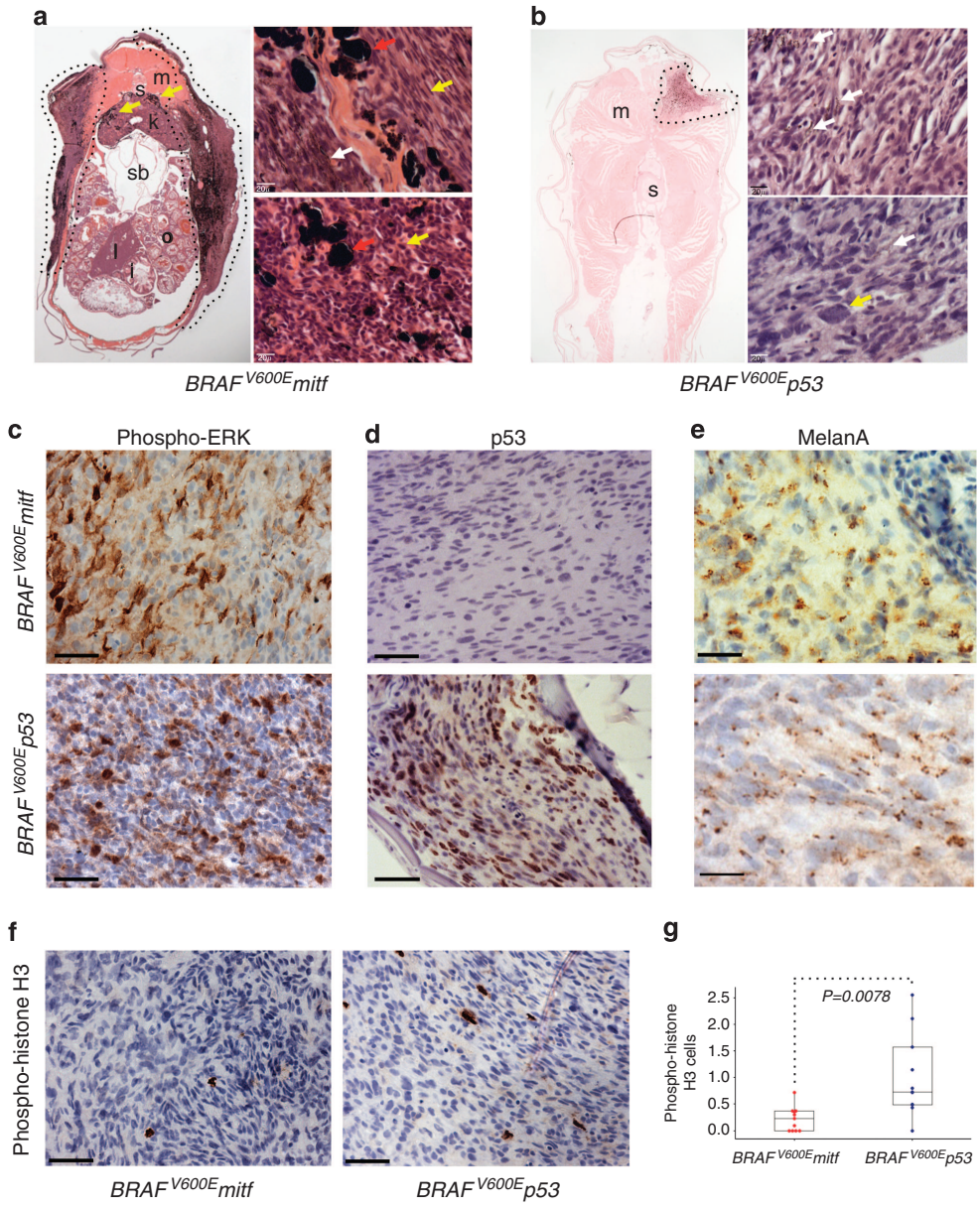

Figure 2. Comparative histopathology of BRAF^{V600E} melanomas. (**a**) Cross-section of adult *BRAF^{V600E}mitf* zebrafish with superficial spreading melanoma (dotted line). Infiltrating melanophages in the kidney are indicated (yellow arrows). i, intestine; k, kidney; l, liver; m, muscle; o, ovary; s, spinal column; sb, swimbladder. (Top and bottom panels) Hematoxylin and eosin (H&E) stain of *BRAF^{V600E}mitf* melanoma, indicating large melanophages (red arrows), spindle or epithelioid cell shapes (yellow arrows), and pigmented melanoma cells (white arrow). Scale bars = 20 μm. (**b**) Cross-section of adult *BRAF^{V600E}p53* zebrafish with invasive melanoma (dotted line). (Top and bottom panels) H&E stain of *BRAF^{V600E}p53* melanoma, indicating pigmented melanoma cells (white arrows) and nuclear pleomorphisms (yellow arrow). Scale bars = 20 μm. (**c–f**) Immunohistochemistry staining for (**c**) phospho-extracellular signal–regulated kinase (ERK), (**d**) p53, (**e**) Melan-A, and (**f**) phospho-histone H3. Scale bars = 50 μm. (**g**). Box plot of mean percentage phospho-histone H3–stained cells in *BRAF^{V600E}mitf* and *BRAF^{V600E}p53* tumors (n = 11 melanomas of each genotype). Bars represent interquartile range; Student's t-test P = 0.0078.

mutation and not just the water temperature because *BRAF^{V600E}p53* zebrafish upshifted to 32 °C for 2 weeks showed no tumor regression and even continued growth (n = 6/6; Figure 4b). By 2 months, 12/15 very large tumors showed regression, and 6 of these fish showed complete regression and even healing at the tumor site (Figure 4c; fish 3 and 4). Interestingly, despite the striking levels of melanoma regression, melanomas recurred following a temperature shift to <26 °C, indicating that a subpopulation of melanoma cells with very low MITF activity survive and are capable of repopulating the tumor site (Supplementary Figure S3 online).

To understand the process of melanoma regression, we shifted *BRAF^{V600E}mitf* zebrafish to the restrictive temperature (32 °C) for 7 days to analyze melanoma regression in progress. Histological analysis of the regressing melanomas showed evidence of tumor regression, characterized by marked loss of tumor cell density and accumulation of heavily pigmented melanophages (n = 7/7; Figure 5a and b). To address whether

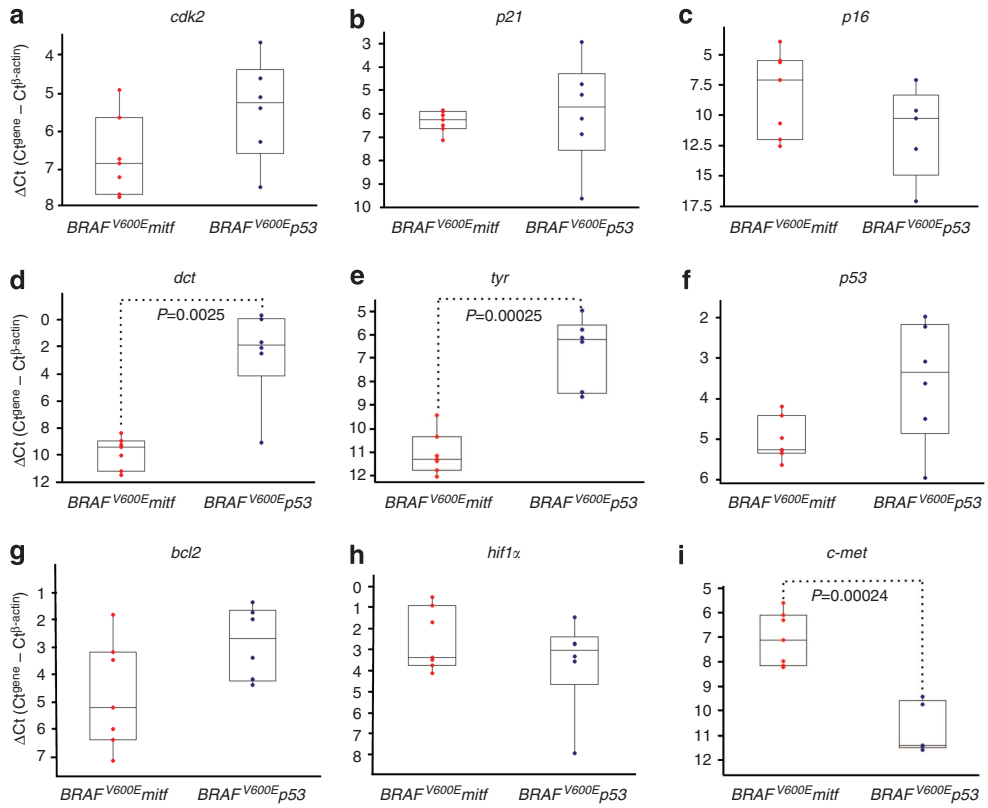

Figure 3. MITF target gene expression. (**a–i**) Box plots of quantitative real-time PCR (qRT-PCR) of MITF target genes and *p53*. The *y*-axis indicates the difference between the cycle threshold (Ct) value of the gene of interest and the Ct value of β-actin in each sample. Note that the *y*-axis is inverted for ease of interpretation. Bars represent interquartile range; *P*-values determined by Student's *t*-test. Also see Supplementary Table S1 online. MITF, microphtholmia associated transcription factor.

apoptosis contributed to regression, we stained sections of the regressing *BRAF*^{V600E}*mitf* melanomas with antibodies to detect active (cleaved) Caspase-3. We found high levels of active Caspase-3 in the regressing tumors compared with *BRAF*^{V600E}*mitf* melanomas at $<26\,^{\circ}C$ ($n=5$ in each group; Figure 5c). We conclude that there is a genetic dependency on MITF activity in *BRAF*^{V600E}*mitf* melanoma.

DISCUSSION

Identifying BRAF^{V600E} cooperating mutations that drive melanoma progression is critical for developing new therapeutic approaches and tackling drug resistance. Accumulating evidence indicates that MITF activity is a key contributing factor in melanoma (Tsao *et al.*, 2012). We now show in an animal that a low level of wild-type MITF activity is oncogenic with BRAF^{V600E} and that abrogating MITF activity in melanoma leads to tumor regression.

The BRAF^{V600E}*mitf* model is relevant to human melanoma, because for some patients, low expression of MITF is associated with disease progression and poor prognosis (Salti *et al.*, 2000; Levy *et al.*, 2006). In these contexts, exogenous expression of MITF leads to inhibition of proliferation (Selzer *et al.*, 2002; Wellbrock and Marais, 2005). This is in apparent contrast to evidence that MITF amplification is also an indicator of poor prognosis, and that MITF cooperates with BRAF^{V600E} to transform melanocytes (Garraway *et al.*, 2005). These differences in MITF activity may reflect distinct subtypes of melanoma; however, another possibility is that MITF amplification indicates the need for melanoma cells to maintain sufficient MITF activity for survival in the context of high BRAF^{V600E} signaling (Garraway *et al.*, 2005; Wellbrock *et al.*, 2008). Thus, a common feature of melanoma may involve maintaining sufficient MITF activity for survival and proliferation while at the same time restricting higher levels of MITF activity that promote cell cycle arrest and differentiation or lower levels that lead to cell cycle arrest and apoptosis (Gray-Schopfer *et al.*, 2007; Hoek and Goding, 2010). Here, the temperature-sensitive nature of the zebrafish *mitfa*^{vc7} mutant allele enables MITF activity to be varied within an individual animal by altering the water temperature, thereby revealing the role of MITF activity levels in melanomagenesis and survival *in vivo*, although we cannot exclude the possibility that the *mitfa*^{vc7} mutant has additional functions that contribute to melanoma.

Histopathological characteristics of melanoma are determined by a number of factors, and at least some are genetically determined (Whiteman *et al.*, 2011). This is illustrated by the clinical classification of BRAF^{V600E} melanomas as a subgroup based on histomorphological features (Viros *et al.*, 2008). We show here that cooperating mutations also have an important role in determining the pathological features

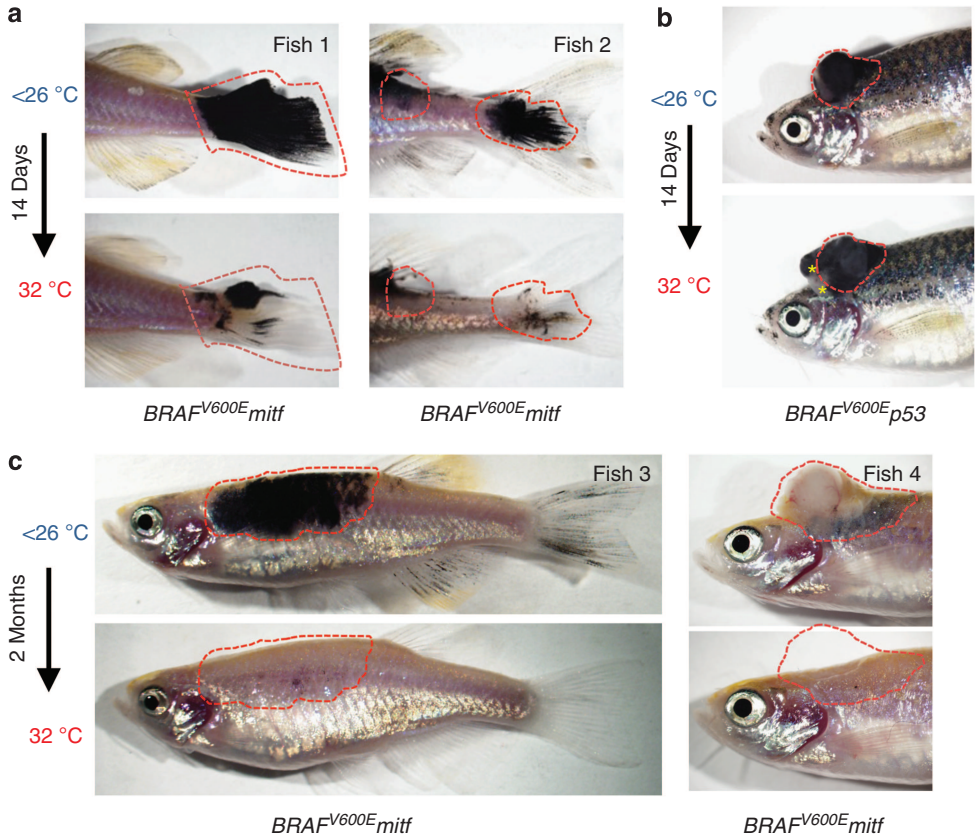

Figure 4. Abrogation of MITF activity causes melanoma regression. (**a**) Images of adult *BRAF*^{V600E}*mitf* zebrafish at <26 °C, and transferred to water at 32 °C for 14 days (bottom images). Red dotted lines outline the tumors before and after the temperature shift. (**b**) Control *BRAF*^{V600E}*p53* melanoma fish at <26 °C and at 32 °C. Areas of increased tumor growth are indicated with yellow asterisks. (**c**) Adult *BRAF*^{V600E}*mitf* zebrafish at <26 °C and after 2 months at 32 °C. MITF, microphtholmia associated transcription factor.

of melanoma. We find that low, oncogenic levels of MITF activity contribute to melanoma pathology, possibly by maintaining melanoma cells in a progenitor-like state. Notably, macrophages laden with melanin (melanophages) were a diagnostic feature of the *BRAF*^{V600E}*mitf* melanomas. Melanophages are found in human melanomas, are indicative of an immune response, and predict an improved prognosis for patients, possibly because of tumor regression through macrophage engulfment of melanoma cells (Handerson *et al.*, 2007). Thus, BRAF^{V600E} cooperating mutations can directly influence tumor morphology, as well as tumor–immune cell interactions.

The dramatic recurrence of melanomas in patients following treatment with the BRAF^{V600E} inhibitor, vemurafenib, indicates that combination therapies that target multiple pathways in melanoma may be necessary to improve patient outcome. MITF activity has been implicated as an important drug target (Flaherty *et al.*, 2012; Tsao *et al.*, 2012), and we now show that shutting off endogenous MITF activity *in vivo* leads to dramatic and rapid melanoma regression, characterized by melanophage infiltration and apoptosis. The melanomas recur at the same location following reactivation of MITF activity (Supplementary Figure S3 online), although at this stage we cannot distinguish whether this reflects incomplete tumor regression or a cancer-initiating population that can survive with low-to-no MITF activity. Notably, although melanophages are presumably participating in melanoma regression and/or clearance (Figure 5), we do not know their function in melanoma growth (Figure 2a): macrophages can lead to both melanoma regression (Nakashima *et al.*, 2012) and promotion (Zaidi *et al.*, 2011), or form melanoma–macrophage hybrids (Pawelek, 2007).

In conclusion, our zebrafish model provides *in vivo* genetic evidence that targeting MITF activity—either directly or through regulators of MITF—may be an effective approach to melanoma therapy. Critically, our studies show that although targeting MITF activity is a potent antitumor mechanism, it must be done with caution because partial or ineffective targeting of MITF is oncogenic.

MATERIALS AND METHODS

All zebrafish work was done in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act (1986) and approved by the University of Edinburgh Ethical Review Committee, and in the United States in compliance with protocol AM10415, approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. The temperature-sensitive *mitfa*^{vc7} mutant is described in Johnson *et al.* (2011), and the *mitfa*^{vc7} phenotypes were first inadvertantly ascribed to the *mitfa*^{fh53}mutant.

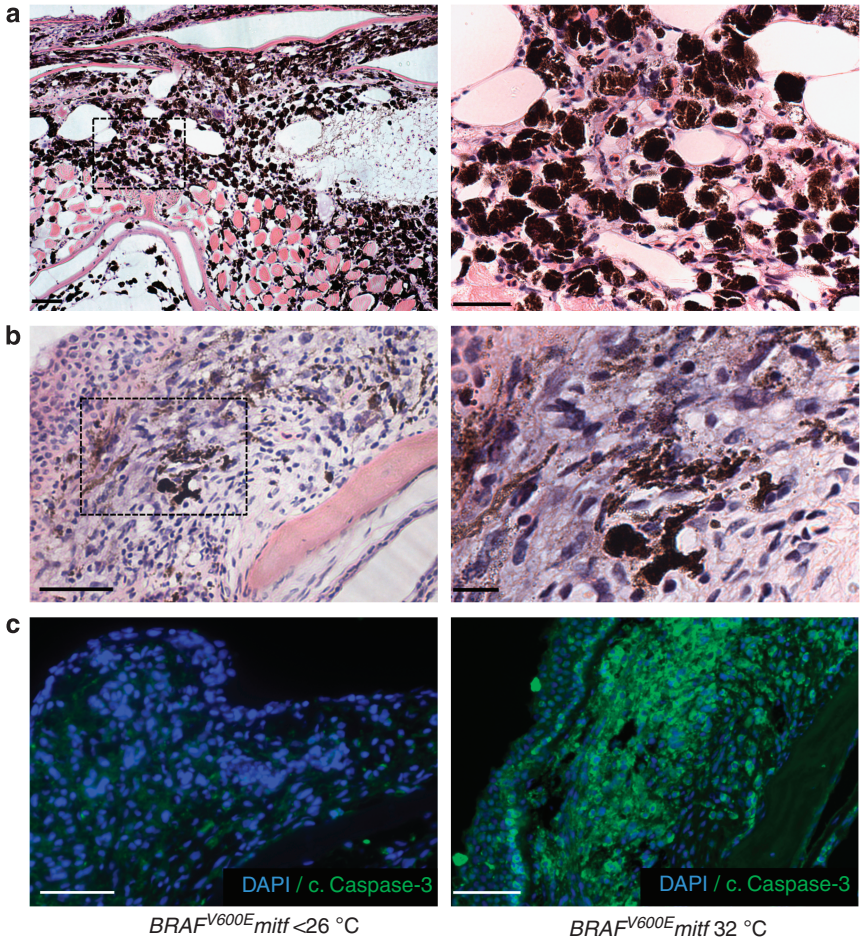

Figure 5. Melanoma regression is associated with melanophage and apoptotic activity. (**a**) Hematoxylin and eosin (H&E) staining of a regressing *BRAF*^{V600E}*mitf* melanoma showing almost total regression with prominent melanophages (scale bar = 200 μm). Boxed region is enlarged in right panel (scale bar = 100 μm). (**b**) A regressing tumor, showing subtotal regression with melanoma cells present (scale bar = 100 μm). Boxed region is enlarged in right panel (scale bar = 50 μm). (**c**) Images of nonregressing (<26 °C) and regressing (32 °C) *BRAF*^{V600E}*mitf* melanomas (as shown in **b**), stained with an antibody to detect cleaved-Caspase-3 (scale bar = 50 μm).

Histopathology

Adult zebrafish were prepared for histopathology as described previously (Patton *et al.*, 2011). Antibodies and antigen retrieval methods were as follows: anti-phospho-extracellular signal–regulated kinase 1/2, 1:1,000, EDTA buffer (Cell Signaling Technology, Danvers, MA); p53 5.1, 1:500, citrate buffer; phospho-histone H3, 1:1,000, citrate buffer (Cell Signaling Technology); Melan-A, 1:75, citrate buffer (DAKO, Cambridge, UK). For the proliferation analysis, the total melanoma cell population in each of the six images was counted (between 1,000 and 3,000 cells) and the percentage of phospho-histone H3–stained cells calculated.

PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Paisley, UK). First-strand complementary DNA was synthesized from 1 μg of total RNA in a 10 μl reaction using SuperScript III Reverse Transcriptase (Invitrogen). Complementary DNA was then amplified by PCR using primers covering the alternative splicing region in the *mitfa*^{vc7} gene. Quantitative real-time PCR was performed using SYBR Green Jumpstart Taq Readymix for high-throughput real-time PCR (Sigma, St Louis, MO). Reactions were run in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Paisley, UK) using the SYBR Green protocol. The zebrafish *β-actin* gene was used as reference. Primers sequences are presented in Supplementary Table S1 online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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