Elevated expression of MITF counteracts B-RAF–stimulated melanocyte and melanoma cell proliferation

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The protein kinase B-RAF is a human oncogene that is mutated in ~70% of human melanomas and transforms mouse melanocytes. Microphthalmia-associated transcription factor (MITF) is an important melanocyte differentiation and survival factor, but its role in melanoma is unclear. In this study, we show that MITF expression is suppressed by oncogenic B-RAF in immortalized mouse and primary human melanocytes. However, low levels of MITF persist in human melanoma cells harboring oncogenic B-RAF, suggesting that additional mechanisms regulate its expression. MITF reexpression in B-RAF–transformed melanocytes inhibits their proliferation. Furthermore, differentiation-inducing factors that elevate MITF expression in melanoma cells inhibit their proliferation, but when MITF up-regulation is prevented by RNA interference, proliferation is not inhibited. These data suggest that MITF is an antiproliferation factor that is down-regulated by B-RAF signaling and that this is a crucial event for the progression of melanomas that harbor oncogenic B-RAF.

Introduction

Melanocytes are pigmented skin cells that protect us from ultraviolet radiation. The processes regulating melanocyte differentiation are intensely studied because melanocytes are thought to be the precursors of melanoma, a skin cancer whose incidence is increasing in Western societies. A master regulator of melanocyte differentiation is the microphthalmia-associated transcription factor (MITF; Widlund and Fisher, 2003). Strikingly, MITF levels are reduced in spontaneously transformed melanocytes (Selzer et al., 2002), and low MITF expression correlates with poor prognosis in melanoma (Salti et al., 2000). MITF regulation is complex. For example, the differentiation factor α-melanocyte–stimulating hormone strongly increases its expression in a cAMP and cAMP response element binding protein (CREB) transcription factor–dependent manner (Bertolotto et al., 1998). Another signaling module that regulates MITF is the RAS–RAF–MEK–ERK signaling cascade, which acts downstream of the receptor tyrosine kinase cKIT to stimulate MITF phosphorylation on serine 73 (S73) and enhances its transcriptional activity (Hemesath et al., 1998). However, extracellular regulated protein kinase (ERK)–mediated S73 phosphorylation also targets MITF for ubiquitin-dependent degradation through the proteasome pathway (Wu et al., 2000; Xu et al., 2000).

There are three *RAS* (*H-RAS*, *K-RAS*, and *N-RAS*) and three *RAF* (*A-RAF*, *B-RAF*, and *C-RAF*) genes in humans. *N-RAS* is mutated in 5–20% of melanomas, and *B-RAF* is mutated in 50–70% of melanomas (Davies et al., 2002). The most common mutation in B-RAF (~90%) is a glutamic acid for valine substitution at position 600 (formally identified as V599; Wellbrock et al., 2004a), which produces a highly active kinase that stimulates constitutive ERK signaling and stimulates melanoma cell proliferation and survival (Hingorani et al., 2003; Karasarides et al., 2004).

In this study, we show that ^{V600E}B-RAF triggers MITF degradation in mouse and human melanocytes and that its reexpression inhibits proliferation. Furthermore, MITF up-regulation suppresses melanoma cell proliferation. These data suggest that high MITF levels are antiproliferative, and, therefore, its expression must be suppressed for transformation by oncogenic B-RAF.

Results and discussion

We previously described the generation of mouse melanocyte lines expressing myc-tagged versions of ^{WT}B-RAF (melan-a–B-RAF) or ^{V600E}B-RAF (melan-a–V600E [VE]; Wellbrock et al., 2004b). We demonstrated that melanocytes expressing

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Abbreviations used in this paper: 4-OHT, 4-hydroxy-tamoxifen; CREB, cAMP response element binding protein; ER, estrogen receptor; ERK, extracellular regulated protein kinase; MEK, MAPK and ERK kinase; MITF, microphthalmia-associated transcription factor; NHM, normal human melanocyte; ppERK, phosphorylated ERK; RNAi, RNA interference; VE, V600E.

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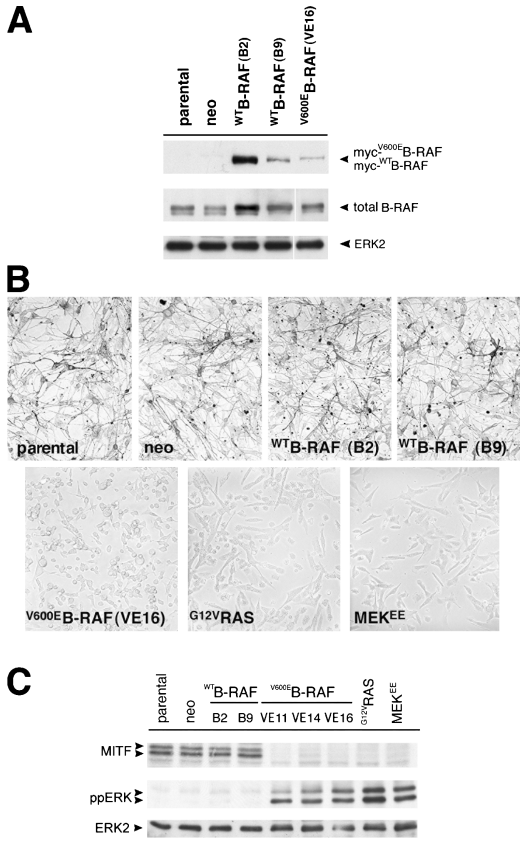


Figure 1. **MITF expression is lost in B-RAF–transformed melanocytes.** (A) Western blot analysis of melan-a cells, a neo^{R} control line, ^{WT}B-RAF–expressing clones B2 and B9, and ^{V600E}B-RAF–expressing clone VE16 probed for myc-tagged B-RAF, total B-RAF, and ERK2. (B) Bright field image of melan-a cells, neo^{R} controls, clones B2, B9, and VE16, and ^{G12V}RAS- or MEK^{EE}-transformed melan-a cells under growing conditions. (C) Western blot analysis of MITF, phosphorylated ERK (ppERK), and ERK2 in melan-a cells, neo^{R} controls, ^{WT}B-RAF–expressing clones (B2 and B9), ^{V600E}B-RAF–expressing clones (VE11, VE14, and VE16) and ^{G12V}RAS- or MEK^{EE}-expressing cells.

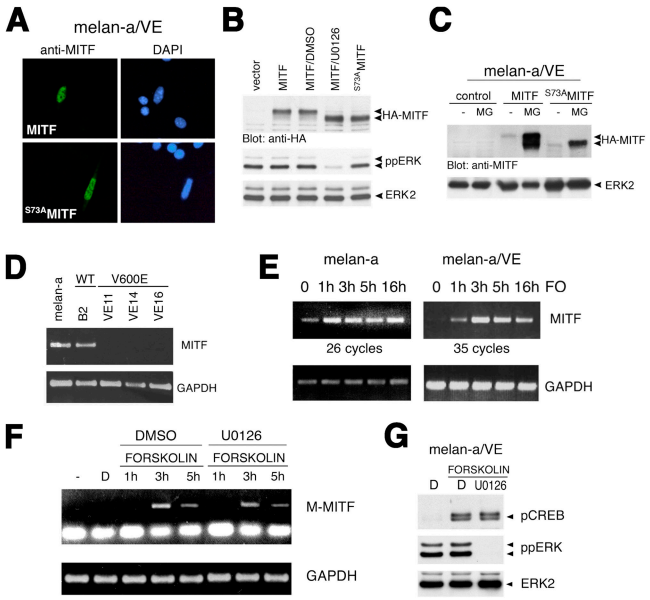


Figure 2. **Constitutive ERK activation triggers MITF down-regulation.** (A) Immunofluorescent analysis of recombinant HA-MITF and HA-^{S73A}MITF in melan-a–VE16 cells using anti-MITF antibody C5. Nuclei are counterstained with DAPI. (B) Western blot analysis of transiently expressed HA-MITF, HA-^{S73A}MITF, ppERK, and ERK2 in melan-a–VE16 cells that were either untreated or treated with 10 μM DMSO or U0126 for 2 h. Control cells were transfected with empty vector, and HA-MITF proteins were detected using anti-HA. (C) Western blot analysis of stably expressed HA-MITF or HA-^{S73A}MITF in melan-a–VE16 cells untreated or treated with 30 μM MG132 for 8 h. HA-MITF proteins were detected using anti-MITF (C5). Total ERK2 is used as a loading control. (D) RT-PCR of melanocyte-specific MITF mRNA expression in parental melan-a cells and clones B2, VE11, VE14, and VE16. GAPDH serves as a loading control. (E) RT-PCR analysis of MITF expression in melan-a and melan-a–VE cells treated with 20 μM forskolin (FO) for the indicated times. GAPDH serves as a loading control. (F) RT-PCR analysis of melanocyte-specific MITF mRNA in melan-a–VE cells treated with 20 μM forskolin for the indicated times in the presence of 10 μM U0126 or DMSO (D) for a 10-min pretreatment. GAPDH serves as a loading control. (G) Western blot analysis for phosphorylated CREB, ppERK, and ERK2 in melan-a–VE cells treated with 10 μM forskolin for 30 min, 10 μM U0126 for a 10-min pretreatment, or DMSO.

^{V600E}B-RAF show constitutive ERK signaling and proliferate in a factor-independent manner (Wellbrock et al., 2004b). Importantly, cells expressing high or low levels of ^{WT}B-RAF do not have elevated ERK activity or grow in a factor-independent manner, demonstrating that even high levels of ^{WT}B-RAF expression are not transforming. Melanocytes expressing ^{V600E}B-RAF (clone VE16; Fig. 1 A) display dramatically reduced dendricity and pigmentation, which is similar to the morphology that is observed in melanocytes expressing oncogenic RAS (^{G12V}RAS) or constitutively active MAPK and ERK kinase (MEK; MEK^{EE}; Fig. 1 B). In contrast, clones expressing low or high levels of ^{WT}B-RAF (clones B2 and B9) have a parental phenotype (Fig. 1 B). The reduction in pigmentation and dentricity that is induced by oncogenic B-RAF prompted us to examine known regulators of melanocyte differentiation. Importantly, we find that MITF is consistently down-regulated in cell lines expressing ^{V600E}B-RAF, ^{G12V}RAS, and MEK^{EE}, and this loss correlates with constitutive ERK activation (Fig. 1 C).

Previous studies have shown that ERK phosphorylates S73 of MITF, targeting it for degradation (Wu et al., 2000; Xu et al., 2000), so we analyzed whether this mechanism underlies MITF loss in our cell lines. Transiently expressed HA-tagged MITF localizes to the nucleus of melan-a–VE cells (Fig. 2 A). On SDS gels, it migrates as a single band whose mobility is increased when the cells are treated with the MEK inhibitor U0126 (Fig. 2 B); these effects were previously attributed to ERK-dependent phosphorylation on S73 (Hemesath et al., 1998). Accordingly, MITF in which S73 is mutated to alanine (^{S73A}MITF) comigrates with MITF in U0126-treated cells (Fig. 2 B). In melan-a–VE lines, ectopic MITF is expressed at low levels, but these increase when the cells are treated with the proteasome inhibitor MG132 (Fig. 2 C). This suggests that MITF is degraded by the ubiquitin pathway after S73 phosphorylation by ERK. However, when ^{S73A}MITF is expressed, it also fails to accumulate unless the cells are treated with MG132 (Fig. 2 C). This effect does not appear to be caused by mislocalization because ^{S73A}MITF also resides in the nucleus (Fig. 2 A). Although our data directly implicate the ubiquitin-mediated proteasome pathway in MITF stability in melan-a–VE cells, MG132 did not induce the accumulation of endogenous MITF in these cells (Fig. 2 C, control), suggesting that additional mecha-

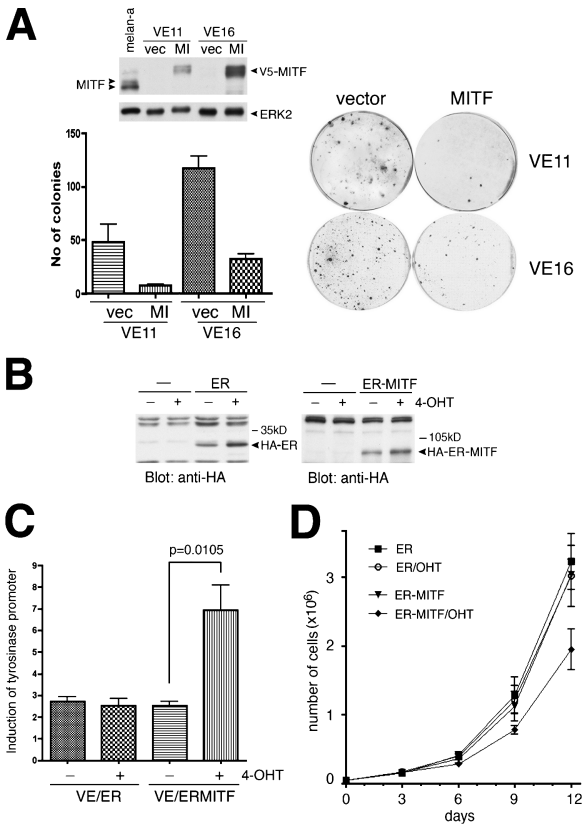


Figure 3. **MITF inhibits proliferation of B-RAF–transformed melanocytes.** (A) Melan-a–VE clones VE11 and VE16 were transfected with a Hygromycin resistance plasmid plus empty vector or the MITF expression vector. Western blot shows MITF expression 24 h after transfection in comparison with endogenous MITF in melan-a cells. The cells were then selected for Hygromycin resistance and stained for colonies 15 d after transfection. Results are means from triplicate determinations with error bars to represent the SD. A representative stained cell image is shown to the right of the graph. (B) Western blot analysis of stably expressed HA.ER and HA.ER-MITF in melan-a–VE cells untreated or treated with 200 nM 4-hydroxy-tamoxifen (4-OHT) for 24 h. HA.ER and HA.ER-MITF were revealed with the anti-HA antibody. (C) Luciferase assay of melan-a–VE HA.ER or HA.ER-MITF cells transfected with a tyrosinase promoter luciferase reporter. 24 h after transfection, cells were untreated or treated with 200 nM 4-OHT for a further 24 h. Error bars represent SD from the mean. (D) Growth curve of melan-a–VE HA.ER- or HA.ER-MITF–expressing cells in the absence or presence of 200 nM 4-OHT. Error bars represent SD from the mean.

nisms regulate MITF expression. RT-PCR analysis revealed that MITF mRNA levels are significantly lower in melan-a–VE cells than in parental or melan-a–B-RAF cells (Fig. 2 D), but the cAMP-elevating agent forskolin still induces MITF expression (Fig. 2 E). Importantly, forskolin-induced MITF expression (Fig. 2 F) and CREB phosphorylation (Fig. 2 G) are not inhibited by U0126, demonstrating that MEK–ERK signaling is not required for MITF regulation by cAMP in these cells.

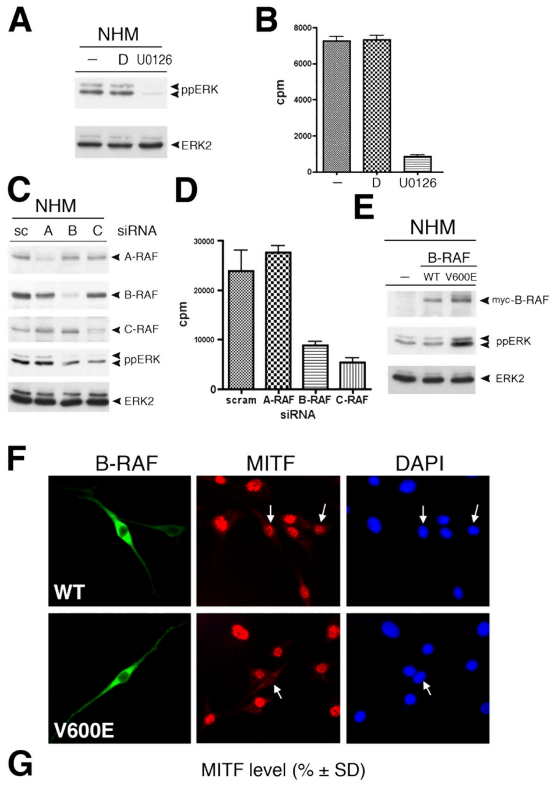
The aforementioned studies show that constitutive ERK activity that is stimulated by ^{V600E}B-RAF in melan-a cells is associated with MITF down-regulation and phenotypic transformation, suggesting that MITF loss is closely linked to melanocyte transformation. Our data are consistent with previous studies demonstrating that MITF degradation is stimulated by ERK-dependent proteasomal degradation, although the mechanism is unclear (Wu et al., 2000; Xu et al., 2000). However, S73 phosphorylation is clearly not the only mechanism regulating MITF stability, and we show that a significant component occurs through transcriptional control. A similar suppression on the transcriptional level has been described in mouse melanocytes that were transformed by oncogenic RAS or by basic fibroblast growth factor overexpression (Halaban et al., 1996). Importantly, in our cells, MITF down-regulation did not occur by promoter silencing because it was still induced by cAMP.

To examine the biological consequences of MITF regulation by B-RAF, we reexpressed MITF in melan-a–VE cells. This caused a significant (73–84%) reduction in the number of colonies that were formed by these cells (Fig. 3 A). To clarify whether this effect was caused by inhibition of proliferation or induction of apoptosis, we developed melan-a–VE cell lines expressing an estrogen receptor (ER) version of MITF (ER-MITF; Carreira et al., 2005) that can be regulated by 4-hydroxytamoxifen (4-OHT) and developed a control cell line expressing only the ER fragment. Both proteins are expressed at similar levels (Fig. 3 B). ER-MITF activates the tyrosinase promoter in a 4-OHT–dependent manner, whereas the ER fragment does not (Fig. 3 C), demonstrating that ER-MITF is functional. Critically, ER-MITF activation does not induce apoptosis in melan-a–VE but significantly impairs its proliferation (38% reduction, $P = 0.0116$; Fig. 3 D).

Because ^{V600E}B-RAF mutations occur in 50–70% of human melanomas (Davies et al., 2002), we examined whether our mouse cell studies were relevant to human melanocytes. First, we analyzed the RAF–MEK–ERK pathway in primary normal human melanocytes (NHMs). ERK inhibition by U0126 (Fig. 4 A) blocks DNA synthesis (Fig. 4 B), demonstrating that ERK signaling is essential for NHM proliferation, so we examined the contribution of individual RAF isoforms by RNA interference (RNAi). Depletion of A-RAF from these cells did not affect basal ERK activity (Fig. 4 C) or DNA synthesis (Fig. 4 D), whereas depletion of B-RAF or C-RAF suppresses ERK activity (Fig. 4 C) and significantly inhibits DNA synthesis (Fig. 4 D). Thus, A-RAF is not required for ERK-dependent melanocyte proliferation, whereas B- and C-RAF are both required. This contrasts with observations in melanoma cells harboring ^{V600E}B-RAF in which only B-RAF is required for ERK activation but all three RAF kinases are required for proliferation (Karasarides et al., 2004). The observation that B- and C-RAF both contribute to ERK signaling and proliferation in NHM can be explained by the fact that in melanocytes, these isoforms stimulate nonredundant growth signals (Wellbrock et al., 2004a).

We have demonstrated that MEK–ERK signaling is essential for NHM proliferation and that in melanoma cells, this pathway is constitutively activated by ^{V600E}B-RAF. Importantly, ^{V600E}B-RAF also induces constitutive ERK activation in NHM (Fig. 4 E), and MITF protein levels are suppressed in ~92% of cells expressing ^{V600E}B-RAF (Fig. 4. F and G). ^{WT}B-RAF does not significantly affect ERK (Fig. 4 E) or MITF protein levels in NHM (Fig. 4, F and G).

Thus, MITF protein levels are significantly reduced in melanocytes in which B-RAF–ERK signaling is elevated. However, MITF is present in most melanoma cell lines expressing oncogenic B-RAF or RAS, albeit generally at reduced



MITF	^{V600E}B-RAF	^{WT}B-RAF	control
high	8.3±3.5	96.7±2.5	100
low	5.7±3.0	0	0
absent	84.3±3.8	3.3±2.5	0

Figure 4. **^{V600E}B-RAF activates ERK and suppresses MITF expression in human melanocytes.** (A) Western blot analysis of ppERK and ERK2 in primary human melanocytes (NHM) that were untreated or treated with 10 μM U0126 for 24 h or with DMSO control (second lane). (B) Thymidine incorporation into NHM treated with 10 μM U0126 for 24 h, DMSO, or no treatment control in the presence of melanocyte growth factor supplement. (C) Western blot analysis of A-RAF, B-RAF, C-RAF, ppERK, and ERK2 in NHM transfected with either scrambled control siRNA (sc) or siRNAs specific for A-RAF, B-RAF, or C-RAF. (D) Thymidine incorporation into NHM in the presence of melanocyte growth factor supplement 72 h after transfection with the indicated siRNAs. Error bars represent SD. (E) Western blot analysis for myc-tagged B-RAF, ppERK, and ERK2 in NHM transiently expressing either myc-^{V600E}B-RAF or myc-^{WT}B-RAF. B-RAF was revealed using the antibody 9E10. (F) Immunofluorescence analysis of transiently expressed myc-^{V600E}B-RAF and myc-^{WT}B-RAF and of endogenous MITF in NHM transfected with myc-^{V600E}B-RAF or myc-^{WT}B-RAF. B-RAF proteins are revealed with anti-myc, and MITF is revealed with C5. Nuclei are counterstained with DAPI. Arrows indicate nuclei of transfected cells expressing either wild-type B-RAF or mutant ^{V600E}B-RAF. (G) Quantification of immunofluorescence data. Means of three experiments are shown (100 cells were counted in each experiment). Vector-transfected cells served as a control.

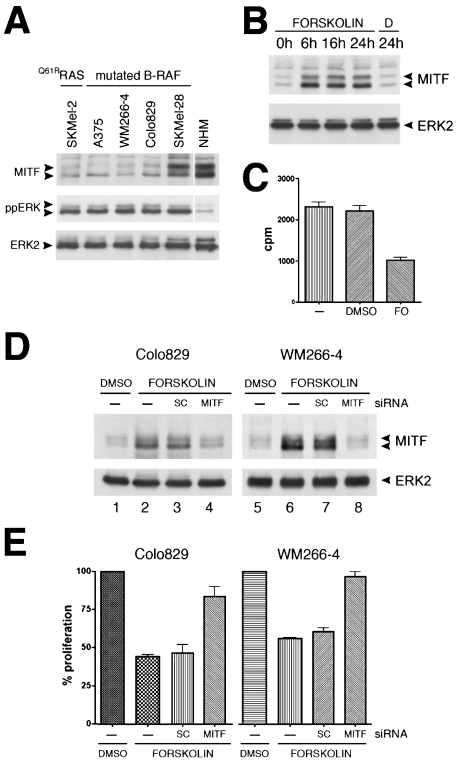


Figure 5. **Enhanced MITF expression contributes to cAMP-induced growth inhibition of melanoma cells.** (A) Western blot analysis of endogenous MITF, ppERK, and ERK2 in melanoma cell lines expressing oncogenic RAS or B-RAF compared with NHM. MITF was revealed using anti-MITF (D5). (B) Western blot of endogenous MITF (using antibody D5) and ERK2 as a loading control in NHM treated with 20 μM forskolin or DMSO for the indicated times in the absence of melanocyte growth factor supplement. (C) Thymidine incorporation into NHM in the absence of melanocyte growth factor supplement after 20 μM forskolin treatment for 24 h or DMSO treatment. (D) Western blot analysis of MITF (D5) and ERK2 in untreated or 20 μM forskolin-treated (16 h) Colo826 or WM266-4 cells in the presence or absence of either MITF-specific siRNA or scrambled control (sc). (E) Thymidine incorporation in parallel samples from D. Thymidine incorporation in DMSO-treated cells was set at 100%. Results are from triplicate assays with error bars to represent SD from the mean.

levels compared with NHM (Fig. 5 A). Our data suggests that MITF is antiproliferative and that one function of oncogenic B-RAF is to suppress its expression to overcome its growth-inhibitory activity. This model is supported by our observation that MITF expression is reduced in NHM expressing ^{V600E}B-RAF (Fig. 4, F and G) and the finding that forskolin, which up-regulates MITF (Fig. 5 B), also inhibits DNA synthesis in these cells (Fig. 5 C). Forskolin also up-regulates MITF expression in Colo829 and WM266-4 cells (Fig. 5 D, lanes 1, 2, 5, and 6) and in two melanoma cell lines that express oncogenic B-RAF (Davies et al., 2002), and this is accompanied by reduced proliferation (Fig. 5 E). Importantly, when RNAi is used to prevent MITF up-regulation (Fig. 5 D, lanes 4 and 8), forskolin does not inhibit proliferation of Colo829 and WM266-4 cells (Fig. 5 E), clearly demonstrating that elevated MITF protein levels are growth inhibitory to melanoma cells.

Our data suggest that high levels of MITF hinder cell cycle progression, and it has been suggested that MITF-induced cell cycle exit is necessary for melanocyte differentiation (Loercher et al., 2005). Consistent with this, melanogenesis-inducing factors such as α–melanocyte-stimulating hormone and forskolin, which up-regulate MITF expression (Bertolotto et al., 1998), also block melanoma cell proliferation. Moreover, MITF regulates transcription of the cell cycle inhibitors p16^{Ink4a} and p21^{Cip1} (Carreira et al., 2005; Loercher et al., 2005). However, Colo829 and WM266-4 cells are p16^{Ink4a} negative (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200505059/DC1), and we did not observe increased p21 expression in response to forskolin (not depicted), sug-

gesting that MITF inhibits growth through alternative mechanisms in these cells.

The antiproliferative effects of high MITF levels in human melanoma is supported by the fact that MITF mRNA expression is frequently low or undetectable in human melanoma cells (Vachtenheim and Novotna, 1999). Importantly, MITF reexpression in transformed MITF-negative human melanocytes and melanoma cells reduces their tumorigenecity in vivo (Selzer et al., 2002), suggesting that elevated MITF is incompatible with melanoma progression. Notably, low MITF levels are linked to reduced survival rates and increased metastases in patients with intermediate thickness melanoma (Salti et al., 2000). Furthermore, MITF target genes such as melan-a/MART-1 or melastatin/TRPM1 are generally down-regulated in more advanced melanomas (Duncan et al., 1998; Hofbauer et al., 1998), which is consistent with MITF not being expressed or being nonfunctional. Our data have clear clinical implications, suggesting that MITF has important prognostic value in melanoma, particularly if used in conjunction with B-RAF mutation status, which is an area that needs urgent investigation.

Previous studies have suggested that MITF protein levels are regulated by ERK-induced degradation (Wu et al., 2000; Xu et al., 2000) and, in agreement with this, we observe that MITF expression is significantly reduced in melanocytes in which ERK is constitutively active as a result of oncogenic B-RAF expression. However, we note that in melanoma cell lines and clinical samples of melanoma, MITF expression is not completely suppressed. We propose that MITF function cannot be completely abolished in melanoma and that low level expression must be maintained to stimulate survival and/or proliferation, possibly by regulating BCL2 (McGill et al., 2002) and CDK2 (Du et al., 2004) expression. Presumably, mechanisms exist to counteract the suppression of MITF expression by oncogenic B-RAF such as maintaining its expression at a level that is compatible with tumor progression but insufficient to suppress cell growth. We are currently developing genetic approaches to test this hypothesis. Our data demonstrate that MITF expression is carefully regulated in melanocytes and melanoma cells and that the regulation of its expression by oncogenic B-RAF warrants further study.

Materials and methods

Cell culture and transfection
Melan-a cells expressing B-RAF, ^{V600E}B-RAF, MEK^{EE}, and ^{G12V}RAS were described previously (Wellbrock et al., 2004b). Melan-a cells (gift of D. Bennett, St. George's Hospital Medical School, London, UK) and melan-a–B-RAF cells were grown in RPMI/10% FCS supplemented with 200 nM TPA and 120 pM cholera toxin. Melan-a–VE and melan-a–VE-derived cells were cultured in RPMI/10% FCS. Melan-a–VE/ER-MITF cells expressed MITF that was fused to the ligand-binding domain of the ER (ER-MITF; gift from C. Goding, Marie Curie Research Institute, Oxted, UK; Carreira et al., 2005), and melan-a–VE/ER cells expressed only the ER fragment. They were created by transfecting melan-a–VE11 cells with pRK5HA.ER or pRK5HA.ER-MITF and 1:10 of pCDNA3.1/Hygro and were selected in 0.5 mg/ml Hygromycin for 1 wk. pRK5ER and pRK5ER-MITF were generated by cloning HA.ER and HA.ER-MITF as EcoRI fragments from pBABEpuroHA.ER and pBABEpuroHA.ER-MITF (Carreira et al., 2005) into pRK5. Human melanoma cell lines were grown in DME/10% FCS. NHM were cultured in medium 154 with HMGS-2 (Cascade Biologics, Inc.) and transfected with 5 μg DNA using a Nucleofector according to the manufacturer's protocols (Amaxa).

Thymidine incorporation, long-term growth, and colony formation assay
Cells were incubated with 0.4 μCi/ml ^{3}H-thymidine for 4 h before harvesting and were quantified by liquid scintillation counting. For long-term growth, melan-a–VE/ER and melan-a–VE/ER-MITF cells were seeded at 0.5×10^6 cells per 10-cm dish in 5% FCS (+/− 4-OHT), counted every 3 d, and replated at 0.5×10^6 cells per dish. For clonogenic survival, cells were transfected with pCDNA3.1/V5-HisMITF (provided by H. Arnheiter, Porter Neuroscience Research Center, Bethesda, MD) or pCDNA3.1/V5-His plus 1:10 of pCDNA3.1/Hygro and were selected in Hygromycin for 5 d. After a further 10 d without Hygromycin, colonies were stained with crystal violet.

Cell lysis, Western blotting, and antibodies
Western blot analysis was performed by standard protocols with the following antibodies: A-RAF (C-20; Santa Cruz Biotechnology, Inc.), B-RAF (F-7; Santa Cruz Biotechnology, Inc.), C-RAF (Transduction Laboratories), phosphorylated ERK (ppERK; MAPK-YT; Sigma-Aldrich), myc tag (9E10), HA tag (12CA5), ERK2 (C-14; Santa Cruz Biotechnology, Inc.), MITF (C5 and D5; Neomarkers and provided by D. Fisher, Dana-Farber Cancer Institute, Boston, MA), rabbit anti-myc (Abcam), and phosphorylated CREB (Cell Signaling).

RNAi
Cells were transfected as described previously (Wellbrock et al., 2004b) using 20–120 nM A-RAF, C-RAF, scrambled (Karasarides et al., 2004), B-RAF (5′-AACAGUCUACAAGGGAAAGUG-3′), or melanocyte-specific MITF (5′-AGCAGTACCTTTCTACCAC-3′) siRNA oligonucleotides.

Luciferase assays
2.5×10^5 cells were transfected with 0.6 μg pGL2 or pGL2htyr (Hemesath et al., 1998) with 0.4 μg pSV–β-galactosidase (Promega) using LipofectAMINE (GIBCO BRL) and were treated with 4-OHT after 24 h for a further 24 h before lysis in reporter lysis buffer (Promega) for luciferase activity analysis. Experiments were performed in triplicate, and luciferase activity was corrected for β-galactosidase expression.

RT-PCR
RNA was isolated using TRIzol (GIBCO BRL), and first-strand cDNA synthesis was performed with 1 μg of total RNA and random hexanucleotides. Specific genes were amplified under conditions in which amplification was still linear. Primers that were used are listed as follows: *gapdh* (5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′) and melanocyte-specific *mitf* (5′-ATGCTGCAAATGCTAGAATACAGTCACTA-3′ and 5′-GTTGCTGTAGAGGTCGATCAAGTTTCC-3′).

Immunofluorescence
Cells that were grown on glass coverslips were fixed in methanol/acetone, blocked with 1% BSA/PBS, and incubated with rabbit anti-myc (Abcam) and/or anti-MITF (clones C5 and D5; Neomarkers). Staining was revealed by using secondary antibodies that were conjugated to Cy2 or Cy3 (Dianova), and nuclei were counterstained with DAPI. Cells were mounted in DABCO-glycerol.

Microscopy and image analysis
Fluorescence images were acquired at RT with a 40×/0.55 long working distance objective lens on a microscope (model TS-100; Nikon) with fluorescence optics. Bright field images were acquired with a 20×/0.4 long working distance objective lens on the same microscope. A camera (model DN-100; Nikon) was used for image processing using Nikon acquisition software.

Online supplemental material
Fig. S1 shows Western blot analysis of p16^{INK-4a} in melanoma cell lines SKMel-2, A375, WM266-4, Colo829, and SKMel-28 and in NHM. ERK2 serves as a loading control. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200505059/DC1.

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