Published in final edited form as:

Pigment Cell Melanoma Res. 2009 August ; 22(4): 435–444. doi:10.1111/j.1755-148X.2009.00578.x.

Frequent Mutations in the MITF Pathway in Melanoma

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Summary

MITF (Microphthalmia-associated transcription factor) is involved in melanocyte cell development, pigmentation and neoplasia. To determine whether *MITF* is somatically mutated in melanoma, we compared the sequence of *MITF* from primary and metastatic lesions to patient matched normal DNA. In the 50 metastatic melanoma tumor lines analyzed, we discovered four samples that had genomic amplifications of *MITF* and four had *MITF* mutations in the regions encoding the transactivation, DNA binding or basic, helix-loop-helix domains. Sequence analysis for *SOX10*, a transcription factor which both acts upstream of *MITF* and synergizes with *MITF*, identified an additional three samples with frameshift or nonsense mutations. *MITF* and *SOX10* were found to be mutated in a mutually exclusive fashion, possibly suggesting disruption in a common genetic pathway. Taken together we found that over 20% of the metastatic melanoma cases had alterations in the *MITF* pathway. We show that the *MITF* pathway is also altered in primary melanomas: 2/26 demonstrated mutations in *MITF* and 6/55 demonstrated mutations in *SOX10*. Our findings suggest that altered *MITF* function during melanomagenesis can be achieved by *MITF* amplification, *MITF* single base substitutions or by mutation of its regulator *SOX10*.

Keywords

5-7 MITF; SOX10; melanoma; mutations; p21; sequencing

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Significance: Melanoma is an aggressive malignancy of the pigment-producing cells, melanocytes, and accounts for the majority of deaths resulting from skin cancer. Contributing factors to the initiation and progression of melanoma include genetic susceptibility, environmental insults such as UV exposure and somatic mutations. Identifying the cellular pathways that when altered by somatic mutations promote the progression of melanoma can provide insights into developing diagnostics and treatments. In this study we investigated whether two genes of an important melanocyte transcriptional pathway, *MITF* and *SOX10*, are somatically mutated in melanoma. We found that over 20% of metastatic melanoma tumors and over 7% of primary melanomas have somatic alterations in one of these two genes. Taken together, our data suggest a substantial fraction of melanomas acquire genetic alterations in the *MITF* pathway, occurring at both the primary and metastatic stages.

Introduction

Malignancy of pigment-producing cells known as melanocytes results in melanoma, an aggressive and often fatal cancer whose incidence is increasing worldwide. Melanocytes arise from the neural crest during embryonic development, and throughout their maturation migrate widely and proliferate extensively prior to their terminal differentiation and entry into the epidermis and hair follicle. In addition, melanocytes undergo multiple cycles of regeneration, in which melanocyte stem cells give rise to new populations of melanocytes.

Microphthalmia-associated Transcription Factor (MITF), a basic, helix-loop-helix, leucine-zipper transcription factor, plays a central role in regulation of the differentiation, growth, and survival of cells of the melanocytic lineage. MITF regulates the transcription of a large number of downstream targets that are involved in melanocyte differentiation, proliferation, and survival (Levy et al., 2006, Hou and Pavan, 2008, Steingrimsdottir et al., 2004). Therefore, changes in the expression levels of MITF and subsets of its various downstream targets can result in widely different outcomes, such as cellular growth or death.

Although melanomas are heterogeneous in nature, recent studies of gene expression indicate that melanoma progression appears to involve notable changes in the expression of genes shown to be important for melanocyte development and regeneration (Ryu et al., 2007, White and Zon, 2008). The multifaceted role of MITF in supporting differentiation, proliferation or cell death in normal melanocytes suggests that, depending on the downstream targets affected, changes in MITF signaling could result in downstream changes that would support melanoma formation. For example, *MITF* has been shown to be amplified in 10–20% of melanoma metastases and to potentially act as a dominant oncogene (Garraway et al., 2005). Conversely, MITF regulates expression of the cell cycle gene *p21*, and mutations of melanoma-associated genes that would reduce MITF stability or transcriptional activity could potentially remove MITF's tight control of the cell cycle (Carreira et al., 2005, Loercher et al., 2005). Recent melanoma gene expression profiling supported a model in which highly proliferative but weakly metastatic melanomas displayed a “neural crest-like” gene expression pattern, in which MITF expression was activated by the WNT pathway; however, melanomas that have progressed to a highly metastatic and weakly proliferative state had expression patterns that resembled TGFβ-like signaling, with a positive feedback loop and an inhibition of WNT pathway, resulting in downregulation of neural crest genes including MITF (Hoek et al., 2006).

Given the evidence supporting a central role for *MITF* in melanoma progression reflective of its vital role in melanocyte biology, and because somatic mutations are relevant to the progression of cancer, we examined melanoma tumor DNA for the presence of somatic mutations in *MITF*. Here we demonstrate that somatic mutation of *MITF* occurs in melanoma metastases. Strikingly, genetic evaluation of the transcription factor *SOX10*, which acts upstream, activates MITF expression and also synergizes with MITF to activate downstream targets (Potterf et al., 2000, Potterf et al., 2001, Bondurand et al., 2000, Jiao et al., 2004, Ludwig et al., 2004), shows that *SOX10* is also mutated in melanoma. Our genetic analyses reveal that previously unknown mutations in *MITF* and *SOX10* occur in over 9% of primary melanoma tumors and 22% of metastatic melanoma tumors.

Results

MITF is somatically mutated in melanoma metastases

MITF has recently been shown to be amplified in 10–20% of melanoma cases (Garraway et al., 2005). However, no study has evaluated whether melanoma-specific intragenic

mutations occur in *MITF*. We therefore examined 13 *MITF* exons that encompass the full coding sequences of all six *MITF* isoforms. The exons were polymerase chain reaction (PCR) amplified and directly sequenced from genomic DNA isolated from 50 melanoma metastases. Any observed changes were evaluated against genomic DNA from patient-matched normal DNA to identify somatic, tumor-specific mutations. A total of six changes, including four non-synonymous point mutations and one splice-site alteration, were identified (Table 1 and Supplementary Figure 1). These mutations occurred at evolutionarily conserved residues within distinct functional domains.

To test whether *MITF* is altered by amplification in the same set of 50 melanoma samples, quantitative PCR was performed. Four out of the 50 samples showed amplification of *MITF* (data not shown). No *MITF* mutations were present in these 4 samples, demonstrating that *MITF* mutations and amplifications occurred in a mutually exclusive pattern (Supplementary Table 1). Taken together, these results show that *MITF* is somatically altered in 16% (8/50) of melanoma metastases.

SOX10 is somatically mutated in melanoma metastases

Since *MITF* mutations were found in the melanoma samples analyzed, we proposed that other genes in the *MITF* pathway might also be altered. *SOX10* is a transcription factor known to act upstream of *MITF* (Bondurand et al., 2000, Lee et al., 2000, Potterf et al., 2000, Verastegui et al., 2000) and to synergize with *MITF* in the transactivation of genes such as *Tyrosinase* (*TYR*) (Murisier et al., 2007), and Dopachrome tautomerase (*DCT*) (Jiao et al., 2004, Potterf et al., 2001). We therefore evaluated the presence of intragenic mutations in *SOX10* in the same 50 metastatic melanoma samples. A total of three mutations were identified in *SOX10*. Two of the mutations would be predicted to result in truncation prior to the HMG box, DNA binding domain. The third mutation, (Ser449Serfsx66), would be predicted to result in an additional 66AA of 3'UTR encoded peptide to be produced instead of the terminal 16AA of WT *SOX10* due to an 8bp nucleotide deletion (Table 1 and Supplementary Figure 2). Again, we observed that the tumor samples, which harbored these *SOX10* mutations, did not contain either *MITF* amplifications or intragenic mutations. It is also of note that the 59T cell line that contained the *SOX10* mutation (Ser449Serfsx66) was derived from a tumor in a patient exhibiting *de novo* cellular immune reactivity against *SOX10* (Khong and Rosenberg, 2002). Presumably this newly identified *SOX10* mutation was the cause for the tumor infiltrating lymphocyte (TIL) reactivity found in this patient.

BRAF and NRAS mutation status

Mutations affecting the MAPK pathway via either the serine/threonine kinase, *BRAF*, or the small guanine-nucleotide binding protein, *NRAS*, are frequently observed in metastatic melanoma (Davies et al., 2002, Demunter et al., 2001, Omholt et al., 2002, Omholt et al., 2003). Thus we next characterized the mutation status of both *BRAF* and *NRAS* in our panel of 50 metastatic tumor samples. In this panel of 50 metastatic tumors we found *BRAF* activating mutations in 38/50 (76%) of the tumors, while we observed oncogenic *NRAS* mutations in 6/50 (12%). Correlation of these results with the 11 tumors harboring mutations in the *MITF* pathway found 10 of the 11 tumors presenting with activating mutations in *BRAF* while none of the 11 tumors were found to harbor mutations in the *NRAS* (Supplementary Table 1). This correlation between mutations altering the *MITF* pathway and *BRAF* mutations is consistent with what has been observed previously, as all NCI60 cell lines harboring *MITF* amplifications were also found to harbor *BRAF* (V600E) mutations (Garraway et al., 2005).

MITF and SOX10 mutations occur at early stage melanoma

To evaluate whether somatic mutations in *MITF* and *SOX10* occur at earlier stages of melanoma progression, we also evaluated primary melanoma samples. This revealed a total of 2 *MITF* mutations in 26 primary melanoma samples (7.7%), both of which occurred in similar genomic locations to the mutations found in the melanoma metastases samples (Figure 1). No *MITF* amplifications were found in the primary melanomas. The *SOX10* screen in the primary melanoma samples identified six (R34Q, A361V, G413D, G413S, H414Y and A424V) *SOX10* mutations in five of 55 primary melanomas (9%) (Table 1 and Supplementary Table 2). Analysis of sequence traces across the *SOX10* amplified regions indicated that for four of the five tumors in which mutations were identified, also exhibited a loss of heterozygosity at this locus (Table 1). None of the *MITF* or *SOX10* mutations were observed in a panel of lymphocyte DNA from 150 Caucasian controls (data not shown), suggesting that these mutations arose somatically.

MITF 4TΔ2B mutation exhibits increased transcriptional activity

The positions of mutations within *MITF* implied that they were likely to alter its transcriptional activity, because no truncating mutations were observed, all the mutations were heterozygous and the mutations occurred in clearly defined domains. To test the effect these mutations have on *MITF* activity, each was cloned individually for transactivation studies. RNA derived from mutant *MITF* cell lines was amplified, and corresponding cDNA cloned into a mammalian expression plasmid. When amplifying the cDNA of mutant 4T, several transcript bands were seen (Figure 2A), and these were subsequently cloned and sequenced. Interestingly, the mutations in sample 4T (Figure 2B) had two effects on mRNA sequence. First, heterozygous guanine to adenine and adenine to guanine mutations within exon 2 resulted in a substitution of glutamine to arginine at codon 87. Second, analysis of the genomic sequence revealed that the adenine to guanine mutation was located at the splice donor site for exon 2. This mutation resulted in the abrogation of the splice donor site (AGGT), which caused deletion of exon 2B in the mutant transcript (Figure 2C). Thus full length 4T had the E87R amino acid alteration, and one of the truncated cDNA versions had a 168bp deletion corresponding to deletion of exon 2B (Figure 2D). We named this mutation 4TΔ2B.

To test the effects of these *MITF* mutations on transcriptional activity, we expressed FLAG epitope fusion constructs of wild type (WT) *MITF* or five of the identified *MITF* mutants in HEK 293T cells and assessed their transcriptional activity. We chose this non-melanocytic cell line because it does not express endogenous *MITF*, allowing us to eliminate potential confounding effects. We transfected the *MITF* constructs, together with the *MITF*-responsive promoter *HuDct-luc*, in the presence or absence of WT *SOX10*. As seen previously (Jiao et al., 2004, Ludwig et al., 2004, Potterf et al., 2001) WT *MITF* synergized with *SOX10* to activate expression of the *HuDct-luc* reporter gene, and similar levels of stimulation were observed with four out of five of the *MITF* mutants. However, expression of the 4TΔ2B *MITF* mutant conferred approximately two-fold increased activity compared to WT protein when co-expressed with WT *SOX10* (Figure 3A). Similar results were obtained when the *MITF* mutants were transfected into the retinal pigment epithelial cell line ARPE-19, where only the 4TΔ2B *MITF* mutation showed greater synergy with *SOX10* relative to WT *MITF* (Supplementary Figure 3).

To examine if the increased transcriptional activity of the 4TΔ2B *MITF* mutant was due to increased protein expression, we evaluated *MITF* levels in lysates from HEK 293T cells. The protein levels of 4 of the 5 mutant proteins were comparable to WT, while the level of *MITF* 4TΔ2B was higher than both WT *MITF* and the other *MITF* mutants (Figure 3B). The increased expression of the 4TΔ2B mutant was also observed when WT *SOX10* was co-

expressed (Figure 3C). This suggests that this variant of MITF confers increased protein stability, and may explain its increased ability to activate Hu*Dct*-luc. Of note, the lack of serine 73 (transcribed from exon 2B), which is phosphorylated by ERK2 resulting in enhanced proteasome-mediated degradation of MITF (Steingrimsen et al., 2004, Wu et al., 2000, Xu et al., 2000), may be the underlying reason for the increased protein stability.

To examine if mutations in *MITF* had any effect on other known MITF target gene promoters, we transiently transfected HEK 293T cells with WT *MITF* or mutant forms of *MITF*, and measured their transcriptional activity with either *p21*-luc or *Tyr*-luc reporter (Bentley et al., 1994) constructs. MITF has been reported to transactivate both of these reporters in cell based assays (Carreira et al., 2005). Expression of WT *MITF* resulted in a 6-fold increase in activation of the *Tyr*-luc reporter compared to empty vector control (Figure 3D). Most MITF mutants (R87G, L135V, G244R and D380N) showed activation of *Tyr*-luc similar to the WT MITF protein. However, expression of the 4TΔ2B MITF mutant conferred an increase in activation 4-fold greater than WT protein (Figure 3D). In contrast, when transactivation assays were evaluated within the context of the *p21*-luc reporter, all 5 MITF mutants showed little to no *p21*-luc activation (Figure 3E). These results suggest that while somatic mutations of MITF encode proteins that can act upon the tyrosinase promoter even in the absence of SOX10, these mutant MITF proteins appear unable to activate the *p21* promoter. These results might allow us to postulate how the lack of p21 activation by mutated MITF in melanoma cells could result in escape from p21-dependent cell cycle arrest.

To test the effects of the SOX10 mutations, a Hu*Dct*-luc transactivation assay was performed using NIH3T3 cells that over-express WT MITF together with either WT SOX10 or each of five SOX10 mutants identified in primary melanomas (R34Q, G413S, G413D, H414Y and A424V) and the three SOX10 mutants identified in metastatic melanoma (Pro14fsX10, Q125X, or Ser449SerfsX66). A 7- to 10-fold activation of Hu*Dct*-luc was observed when WT MITF was co-transfected with either WT SOX10 or the five primary melanoma SOX10 mutants (Figure 4) in comparison to WT SOX10 alone. With respect to the three SOX10 mutations observed in the primary melanoma samples, two mutations Pro14fsX10 and Q125X would be predicted to result in early termination of SOX10 protein prior to regions associated with nuclear localization properties (Sudbeck and Scherer, 1997). Co-expression of WT MITF with these two SOX10 mutations revealed these mutations were unable to transactivate the Hu*Dct*-luc reporter (Figure 4). Furthermore, immunofluorescence analysis of these SOX10 mutants demonstrated their aberrant localization to the cytoplasm (data not shown), consistent with previous data and underlying their reduced transactivation abilities. Ser449SerfsX66 mutation, although capable of transactivating the Hu*Dct*-luc promoter in combination with MITF does so at reduced efficiency relative to wild type (p-value = 0.0012).

Discussion

In this study we have used DNA sequence analysis to determine whether *MITF* and *SOX10* are targets of somatic mutation during melanomagenesis. Sequencing of all *MITF* and *SOX10* coding exons in a panel of primary and metastatic melanoma samples identified sixteen *de novo* mutations. In total, the primary and metastatic melanoma samples analyzed harbored non-synonymous (NS) mutations and amplifications with frequencies of 13.2% (10/76) in *MITF* and 8.6% (9/105) in *SOX10* (Supplementary Table 2). Although the sample size was insufficient to document statistical significance, the mutually exclusive nature of the *MITF* and *SOX10* mutations could indicate that their effects are functionally redundant. As many of the patients from which the metastatic tissues were derived have been treated with various chemotherapeutic regimens, the cytotoxic drugs might have caused some of the

observed mutations. However, the finding of similar mutations in some of the primary melanomas mitigates this concern. Taken together, this data provides the first evidence for the presence of NS mutations in both MITF and SOX10 in both primary and metastatic melanoma samples underscoring the involvement of the MITF transcriptional network in melanoma tumorigenesis (Carreira et al., 2005; Carreira et al., 2006; Levy et al., 2006).

It is interesting to note that unlike what has been observed for metastatic melanoma, no amplifications of MITF were observed in the primary tumor samples suggesting that amplification may be a late stage alteration taking place in a context dependent genome that can tolerate increased activity of MITF. This is consistent with prior analysis using arrays CGH primary melanomas (Curtin et al., 2005). The non-synonymous MITF mutations identified in both primary and metastatic samples were always found as heterozygous for a wild type copy of MITF. The mutations were throughout the protein, causing amino acid substitutions in conserved residues in defined functional domains (Figure 1). Two of the MITF mutations (L135V and L142F) occurred in the conserved acidic activation domain (AD1) of MITF which has been shown to be necessary for MITF transcriptional activity (Supplementary Figure 2). Two of the five MITF mutations G244R and 4TΔ2B have corresponding alleles in mouse, *Mit^b* and *Mit^{bws}* (Hallsson et al., 2000, Steingrimsson et al., 1996), respectively. In the *Mit^b* “brownish” mouse model the same amino acid, G244, is found mutated from a small, non-polar AA to a large, charged AA side chain (G244E). The amino acid location of G244 is at the junction of the loop and helix 2 where the occurrence of a small, non-charged or hydrophobic amino acid and is conserved in all basic helix-loop-helix, leucine-zipper proteins (Steingrimsson et al., 1996). Functional analysis of the *Mit^b* allele demonstrated an MITF protein with reduced DNA binding activity, either as a homodimer or as a heterodimer in combination with TFE3. The second mouse model, *Mit^{bws}*, is similar to the 4TΔ2B mutation in that it ultimately results in exon skipping and deletion of exon 2B. This animal exhibits a reduction in melanocyte cell numbers, as reflected by the black and white spotted (*bws*) coat phenotype (Hallsson et al., 2000). Within this exon is Ser73, whose phosphorylation status can mediate MITF protein turnover (Wu et al., 2000, Xu et al., 2000). Consistent with this finding, we observed increased protein expression for the 4TΔ2B allele mutated in melanoma. Subsequent analysis will be required to determine whether the MITF mutations present in these animal models, *Mit^b* and *Mit^{bws}*, confer increased frequency of melanoma metastasis as measured by intercrossing with murine melanoma-prone models.

Given the mutation spectrum and the results from corresponding mouse alleles it might be surprising that most of the mutations had minimal effects on MITF's ability to transactivate the melanogenic target genes, DCT and TYR. However, MITF provides transcriptional regulation for a wide array of genes, mediating transcriptional control over both differentiation (i.e., DCT and TYR) and cell cycle progression pathways (Bismuth et al., 2005). Our analysis suggests that these MITF mutations may lead to alterations in the capacity of MITF to regulate gene expression in a promoter specific manner, as all five of the mutations analyzed exhibited reduced transactivation activity of the p21 promoter, an MITF target gene previously shown to regulate cell cycle progression (Carreira et al., 2005). The uncoupling of MITF's ability to regulate differentiation and proliferation is consistent with studies by Bismuth et al., (2005) where they showed specific isoforms and mutations of MITF can have different effects on cell proliferation, DNA binding and transcriptional activity. It will be interesting to explore how these melanoma associated mutations affect different aspects of cell cycle and target gene regulation.

The mutation spectrum we observed for SOX10 suggests that SOX10 might be acting as a tumor suppressor gene. In metastatic melanoma samples two of the three mutations generated frameshifts predicted to result in protein truncations before the DNA binding

domain and thus a product with inactivation of SOX10 function. Consistent with this, these mutations did not transactivate the promoter of a known SOX10 target gene, DCT. The third mutation is predicted to produce a truncation and frameshift at the c-terminus of SOX10. This product demonstrated reduced activity on the DCT promoter, and in the tumor, this mutation was associated with LOH. Data from primary melanoma also support a SOX10 tumor suppressor gene model where we found that of the five samples that harbored somatic mutations, four also exhibited LOH and only retained the NS mutated allele. The fifth sample demonstrated two NS mutations, though it is not known if these are in trans or if a wild type copy remains and is expressed. All but one (R43Q) of the NS mutations in SOX10 that were identified in primary melanoma occurred within its transactivation domain, and strikingly two different melanoma samples had mutations affecting the same residue. However, these mutations did not alter SOX10's ability to transactivate DCT activity. It is possible that the melanomagenic activity for these SOX10 alleles is due to a reduced SOX10 function that is not observable on the DCT promoter under conditions used. While these data support a hypothesis of SOX10 as a tumor suppressor, further functional studies and assessment of additional mutations (for example gene amplifications) needs to be explored before a mechanistic model can be confirmed.

Alternatively, these alleles may promote differential effects on specific target genes similar to what we have shown for the MITF melanoma alleles ability to transactivate *TYR* and *p21* differently. As one of the known SOX10 target genes is *MITF* it is possible that the various genetic alterations identified in this study have a direct or indirect effect on altering MITF activity, selected for downregulation of MITF activity to adjust to a favorable tumorigenic outcome. As the mutations of MITF and SOX10 are mutually exclusive in the melanoma samples we analyzed, our results support the “biological rheostat” model (Carreira et al., 2006) where tight regulation of MITF levels is essential for balancing melanocyte growth and arrest.

In summary, this is the first study demonstrating that melanoma patients harbor somatic mutations in MITF and SOX10. Our findings indicate that a substantial fraction of melanomas acquire genetic alterations in the MITF pathway, occurring at both the primary and metastatic stages and suggest that altered MITF function during melanomagenesis can be achieved by MITF amplification, MITF single base substitutions or by mutation of its regulator SOX10.

Methods

Tumor tissues and generation of melanoma cell lines

A panel of pathology-confirmed metastatic melanoma tumor resections, paired with apheresis-collected peripheral blood mononuclear cells, was collected from 80 patients enrolled in IRB-approved clinical trials at the Surgery Branch of the National Cancer Institute, with informed consent from all human subjects. Pathology-confirmed melanoma cell lines were derived from mechanically or enzymatically dispersed tumor cells, which were then cultured in RPMI 1640 + 10% FBS at 37°C in 5% CO₂ for 5-15 passages. For all samples, matching between germline and tumor DNA was verified by direct sequencing of 26 single nucleotide polymorphisms (SNP) at 24 loci. Cytopathology was used to determine the percentage of melanoma antigen expressing cells.

PCR, sequencing and mutational analysis

Melanoma metastases samples and their matched normal samples were provided by Dr. Steven Rosenberg (NCI). Genomic DNA was isolated using DNeasy Blood & Tissue kits (Qiagen, Valencia, CA). For all samples, matching between germline and tumor DNA was

verified by direct sequencing of 26 single nucleotide polymorphisms (SNP) at 24 loci (data not shown). PCR and sequencing primers were designed using Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by Invitrogen (Carlsbad, CA). PCR amplification, sequencing and analysis was performed as previously described (Samuels et al., 2004) using the primers listed in Supplementary Table 3.

To increase our confidence that the five mutations for which no matched normal DNA sample was available (L142F, A361V, G413D, G413S, A424V) did not represent germline polymorphisms, we sequenced the corresponding exons of *MITF* and *SOX10* in 149 normal DNA samples and detected no abnormalities.

DNAs from paraffin embedded primary human melanoma samples were obtained from the collaborators at the University of California, San Francisco and were part of a prior study (Curtin et al., 2005).

Construction of the *MITF* expression vectors and other expression plasmids used

Mammalian expression vectors containing either the WT or mutant *MITF* cDNAs were constructed by PCR amplification of the corresponding region from cDNA of melanoma metastases cell lines and subcloning into the Flag-Tag Vector 4 (Stratagene, La Jolla, CA) with *EcoRI* and *HindIII* restriction sites. Forward and reverse PCR primer sequences were 5' TGACCAGAATTCATGCTGGAAATGCTAGAATA 3' and 5' TGGTCAAAGCTTACAAGTGTGCTCCGTCTCTT 3' respectively. Clones were sequenced and prepped by Qiagen Maxiprep Kit (Qiagen, Valencia, CA). Tyr-luc and p21-luc constructs provided by Colin Goding.

SOX10 mutation constructs

SOX10 mutations were generated using an overlapping two-fragment PCR mediated strategy using Phusion High-Fidelity polymerase (New England Biolabs, Ipswich, MA). Minor modifications of this protocol for specific clones are indicated below. In general, forward and reverse CDS mutation-containing oligos of 21-26bp in length were designed with the corresponding mutation centrally located within the oligonucleotide. These primers were used in the two independent PCR reactions with h*SOX10*-pSport2 cDNA template as follows (1) *SOX10* 5'ATGGCGGAGGAGCAGGACCTA3' and Mutation-R primer pairs and (2) Mutation-F *SOX10*-3R 5'TTAGGGCCGGGACAGTGTCTGT3'. Primers were removed using Microcon YM-30 column (Millipore, Bedford, MA) and corresponding generated 5' and 3' *SOX10* PCR containing fragments were together used as template for PCR using ATTB1-*Sox10*–

5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGGAGGAGCAGGACCTA 3' and AttB2-*SOX10*

-5'GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGGGCCGGGACAGTGTCTGT3' PCR primers to generate full length, Gateway- BP PCR products compatible for cloning into pDonr221 entry vector (Invitrogen, Carlsbad, CA). Sequence verified entry clones were subsequently transferred into pcDNA3.1 NHA-GW using standard Gateway protocols (Invitrogen, Carlsbad, CA).

Minor modifications to the PCR cloning strategies as outlined above include: (1) For the del1346-1353 mutation the following primers modifications were used. The 3' *SOX10* primer was replaced with 1832R primer (5'TCATCAGGGCAGTGAGCCAGAC3') and the corresponding mutation primers were longer: Del 1346-1353 F-5'CAGCCCCCTCAGGGCCCCAGTCCCACACACTGGGAGCAGCC3'; Del 1346-1353 R-5'GCTGCTCCCAGTGTGTGGGACTGGGGCCCTGAGGGGCTG3'. (2) For the C373T

containing mutations a single PCR was performed using B1F-HSOX10 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGGAGGAGCAGGACCTA 3', with either B2R-HSOX10-C373T 5'GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGTCCGCGAGCTTCCTGCGCG 3', or B2R-HSOX10- C372T-;C373T 5'GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAATCCGCGAGCTTCCTGCGCG 3' as appropriate. (3) Del 44 mutation was generated using mutation primers HSOX10-del44-62 F 5'GGAGGTGGAGCTGAGCCCCGCTGCCTGTCCCCGGGGAGC3' and HSOX10-del44-62 R 5'GCTCCCCGGGGACAGGCAGCGGGGCTCAGCTCCACCTCC3'.

Destination vector pcDNA3.1 NHA-GW was generated by PCR amplification using primers RCAS YF- 5'GAGCTGACTCTGCTGGTGGC3' and RCAS-YR 5'CAGATACGCGTATATCTGGC3' to amplify a fragment containing the N-terminal HA epitope, in frame with Gateway ccdB selection cassette fragment from RCAS-Y NHA (Loftus et al., 2001) and subsequent cloning into pcDNA3.1/CTGFP-TOPO (Invitrogen, Carlsbad, CA).

Quantitative real-time PCR for genomic DNA templates

Genome content differences were determined by quantitative real-time PCR performed on an iCycler apparatus (Bio-Rad, Hercules, CA). Each tumor DNA was quantified by comparing the *MITF* locus to the reference locus *LINE1*, a repetitive element whose copy numbers per haploid genome are similar among all human normal and neoplastic cells. PCR was done in 25 μ L with the following components: 15.2 μ L sterile tissue culture grade double-distilled water (Invitrogen, Carlsbad, CA), 2.5 μ L of 10 $\times$ PCR buffer, 2.5 μ L of 10 mmol/L deoxynucleotide triphosphates (Promega, Madison, WI), 15 μ L of DMSO (Sigma, St. Louis, MO), 0.5 μ L of SYBR Green I solution (Invitrogen, Carlsbad, CA) diluted 1:1,000 in double-distilled water, 0.5 μ L of 50 μ mol/L forward and reverse primers (Invitrogen; desalted, 25 nmol scale), 0.25 μ L of Platinum taq DNA polymerase (Invitrogen, Carlsbad, CA), and 2 μ L of purified DNA. The 10 $\times$ PCR buffer contained 670 mmol/L of Tris-HCl (pH 8.8), 67 mmol/L of $MgCl_2$, 166 mmol/L of $(NH_4)_2SO_4$, and 100 mmol/L of 2-mercaptoethanol. The reaction was monitored on an iCycler (Bio-Rad) with the following cycling conditions: (94°C, 2 min) $\times$ 1, (94°C, 10 s; 57°C, 15 s; 70°C, 15 s) $\times$ 35. The threshold cycle number was determined using Bio-Rad analysis software (version 3.0) with the PCR baseline subtracted. PCRs were done in triplicate with the following primers: 5'-AAACCCCAAGTACCACA-3' for *MITF* forward, 5'-ACATGGCAAGCTCAGGAC-3' for *MITF* reverse, 5'-AAAGCCGCTCAACTACATGG-3', for *LINE1* forward, and 5'-TGCTTTGAATGCGTCCCAGAG-3' for *LINE1* reverse. The *MITF* gene amplification rate in tumor DNA (AR_{Tu-DNA}) was determined by using the formula $AR_{Tu-DNA} = 2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T$ being [C_T (*MITF*;tumor) - C_T (average *LINE1*;tumor)]. An increase of 0.5 in the amplification rate indicates a gain of one *MITF* gene copy.

Cell Culture and transfections

Melanoma metastases lines were maintained as previously described (Chappell et al., 1999). HEK293T and ARPE-19 lines purchased from ATCC (Manassas, VA), and maintained in complete DMEM medium under standard conditions. Cells were transfected with Lipfectamine2000 reagent (Invitrogen, Carlsbad, CA). Melanoma cell lines were grown in T2 medium (RPMI-1640 supplemented with 10% fetal bovine serum, 2.5 mM HEPES, pH 7.5, 2 mM L-glutamine, 1X Pen/Strep, 10 mg/ml Ciprofloxan, 10 mg/ml Genetamicin, and 1.25 mg/ml Fungizone).

Immunoblotting

Protein extracts prepared as described previously (Samuels et al., 2005). Blots were blocked in 10% non-fat dry milk, 1×TBST (25 mM Tris, 150mM NaCl, 0.1% Tween20) for 1 hour at room temperature before incubation with primary antibody in block (1 hour to overnight). Blots were washed 2 times before the addition of HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in block. Blots were washed again before developing with Amersham ECL Blotting Kit (GE Healthcare, Piscataway, NJ). Primary antibodies used were monoclonal MITF (C5 Thermo Scientific, Fremont, CA), monoclonal α-Tubulin (Calbiochem, San Diego, CA), Anti-FLAG M2 monoclonal peroxidase-conjugate (Sigma, Saint Louis, MO).

Luciferase Assay

HEK 293T or ARPE-19 cells were cultured in 12-well plates for 24 hours prior to transfection. Cells were co-transfected with 300ng *HuDct-luc* or 100ng *Tyrosine-Luc* or *p21-Luc* reporter plasmids; 60ng-100 ng of WT or mutant HuMITF; 60ng CMV-SOX10 or pSPORT empty vector; and 10-25 ng pHRG-TK Renilla luciferase plasmid (Promega, Madison, WI). Cells were cultured for 24 hours before lysis; the resulting extracts were assayed for luciferase activity using the Dual-Luciferase reporter Assay System (Promega, Madison, WI). All experiments were done in duplicate or triplicate. NIH3T3 cells grown in a 24-well plate format, with 8 ng pCMV-RL renilla expression vector (Promega, Madison, WI), 400ng *HuDct-luc*, 400 ng SOX10 construct or empty vector, with 2ul Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) per well. Luciferase assay was performed using Dual Luciferase Reporter 1000 Assay System (Promega, Madison, WI) using a Fluoroskan Ascent FL fluorimeter (Thermo Fisher Scientific, Waltham, MA). All experiments were done in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Heinz Arnheiter, Tom Hornyak and Paul Meltzer for their helpful comments on the manuscript. Funded by the National Human Genome Research Institute and National Cancer Institute, National Institutes of Health.

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Figure 1. MITF and SOX10 mutations in primary and metastatic melanoma

Schematic representing the protein domains of MITF and SOX10 show mutations found in primary melanoma (blue arrows) and metastatic melanoma (red arrows). Functional domains of MITF include AD1 (activation domain 1), AD2 (activation domain 2), AD3 (activation domain 3) and bHLHZip (basic helix-loop-helix leucine-zipper domain). Functional domains of SOX10 include HMG (High Mobility Group) and TAD (transcriptional activation domain).

Figure 2. Somatic mutation in tumor sample 4T results in MITF splice variant

A. RT-PCR analysis of MITF expression in tumor samples. The product of the wild type MITF band is 1260bp, the 4T sample products are 1260bp, 1193bp and 1092bp. The 1193bp transcript represents a variant with a 67bp deletion from exon 2B that did not express protein by western (data not shown). The 1092bp transcript encodes the 4TΔ2B MITF variant, reduced in size by 168bp. **B.** Somatic mutation of the *MITF* gene in sample 4T. The top sequence chromatogram was obtained from normal tissue and the lower sequence chromatogram from the 4T melanoma sample. Arrow indicates the location of the mutation. **C.** The *MITF* mutation A to G transition in nucleotide 260 is located in the splice donor and leads to a loss of exon 2B (shaded area) and an mRNA molecule that is 168 nucleotides shorter than wild-type message. **D.** Sequence analysis of the 1260bp cDNA.

Figure 3. Mutant forms of MITF activate HuDct and Tyrosinase luciferase but not p21 luciferase
A. HEK 293T cells were transiently transfected with the indicated constructs together with the *HuDct*-luciferase reporter construct. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same samples. Ratios between firefly and Renilla luciferase activities are shown. **B.** and **C.** Immunoblots of immunoprecipitated MITF derived from transfected HEK 293T cells probed with the indicated antibodies. **D.** HEK 293T cells were transiently transfected with the WT or mutant MITF, or empty vector together with the *Tyrosinase*-luciferase reporter construct. Fold activation was calculated as in **A.** **E.** HEK 293T cells were transiently transfected with the WT or mutant MITF, or empty vector together with the *p21*-luciferase reporter construct. Fold activation was calculated as in **A.**

Figure 4. Truncated versions of SOX10 lack the ability to transactivate *HuDct*-Luciferase
 NIH3T3 cells were transiently transfected with the indicated constructs together with the *HuDct*-luciferase reporter construct. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same samples. Ratios between Firefly luciferase and Renilla luciferase activities are shown.

Mutations identified in MITF

Exon	Nucleotide	Amino acid	Functional domain	Zygosity	Sample	Primary/metastases	Histologic type
2	G259A	E87R	Activation domain 1	Heterozygous	4T	Metastases	N/A
2	A260G	E87R	Activation domain 1	Heterozygous	4T	Metastases	N/A
2	A260G	Splice site	Exon 2B donor splice site	Heterozygous	4T	Metastases	N/A
4	T403G	L135V	Activation domain 1	Heterozygous	13T	Metastases	N/A
4	G426C	L142F	Activation domain 1	Heterozygous	127	Primary	SSM
7	A/T (-3)	splice site	Basic helix loop helix (Basic domain)	Heterozygous	D196	Primary	ALM
8	G712A	G244R	Helix loop helix (helix 2)	Heterozygous	21T	Metastases	N/A
9	G1120A	D380N	Activation domain 3	Heterozygous	85T	Metastases	N/A

Mutations identified in SOX10

Exon	Nucleotide	Amino acid	Functional domain	Zygosity	Sample	Primary/metastatic	Histologic type
1	C373T	Q125X	HMG box	Heterozygous	47T	Metastases	N/A
1	44-62del	Pro14fsX10	Truncation before HMG domain	Heterozygous	68T	Metastases	N/A
1	G128A	R43Q	HMG domain	LOH	AM141	Primary	Mucosal
3	C1082T	A361V	TAD	LOH	MX41	Primary	LMM
3	G1237A	G413S	TAD	LOH	MX4	Primary	LMM
3	G1238A	G413D	TAD	Heterozygous	MX67	Primary	LMM
3	C1240T	H414Y	TAD	Heterozygous	MX67	Primary	LMM
3	C1271T	A424V	TAD	LOH	114	Primary	SSM
3	1346-1353del	Ser449SerfsX66	TAD	LOH	59T	Metastases	N/A

Exon number with nucleotide and amino acid change resulting from mutation. Nucleotide position refers to position within coding sequence, where position 1 corresponds to the first position of the start codon. Tumors 4T and MX67 contained two somatic alterations. In addition to the 16 nonsynonymous mutations recorded in the table, we detected 1 synonymous alteration. The splice site alteration in MITF was in position 3 of the donor site of exon 2B. A total number of 50 cutaneous melanoma tumors were used for the MITF and SOX10 studies. A total number of 26 primary tumors were used to screen for MITF mutations and a total number of 55 primary tumors were used to screen for SOX10 mutations. ALM, acral lentiginous melanoma, LMM, lentigo malignant melanoma, SSM, superficial spreading melanoma.