

Transcriptional Regulation of the Melanoma Prognostic Marker Melastatin (TRPM1) by MITF in Melanocytes and Melanoma

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ABSTRACT

Determining the metastatic potential of intermediate thickness lesions remains a major challenge in the management of melanoma. Clinical studies have demonstrated that expression of melastatin/TRPM1 strongly predicts nonmetastatic propensity and correlates with improved outcome, leading to a national cooperative prospective study, which is ongoing currently. Similarly, the melanocytic markers MLANA/MART1 and MITF also have been shown to lose relative expression during melanoma progression. Recent studies have revealed that MITF, an essential transcription factor for melanocyte development, directly regulates expression of MLANA. This prompted examination of whether MITF also might transcriptionally regulate TRPM1 expression. The TRPM1 promoter contains multiple MITF consensus binding elements that were seen by chromatin immunoprecipitation to be occupied by endogenous MITF within melanoma cells. Endogenous TRPM1 expression responded strongly to MITF up- or down-regulation, as did TRPM1 promoter-driven reporters. In addition, MITF and TRPM1 mRNA levels were correlated tightly across a series of human melanoma cell lines. Mice homozygously mutated in *MITF* showed a dramatic decrease in TRPM1 expression. Finally, the slope of TRPM1 induction by MITF was particularly steep compared with other MITF target genes, suggesting it is a sensitive indicator of MITF expression and correspondingly of melanocytic differentiation. These studies identify MITF as a major transcriptional regulator of TRPM1 and suggest that its prognostic value may be linked to MITF-mediated regulation of cellular differentiation.

INTRODUCTION

Malignant melanoma continues to increase in incidence at a rate of ~3% per year. In 2002, there were 53,600 new cases and 7,400 deaths (1). The lifetime risk of developing melanoma in the United States is estimated currently at 1 in 87 (2). Although representing only ~4% of skin cancers, melanoma accounts for ~79% of skin cancer deaths with an annual mortality rate of 2.3 per 100,000 people (1). Thickness of the initial tumor remains one of the most important factors in predicting outcome (3). Although patients with early stage disease (Breslow thickness < 1 mm) are treated effectively with local excision, a major challenge is determining the potential for development of metastatic disease from intermediate lesions (2–4 mm). This difficulty has led to the search for prognostic markers that may guide therapeutic decisions for this group of patients.

Melastatin (TRPM1) was discovered using differential cDNA display to identify genes whose expression might be related to metastatic potential in B16 mouse melanoma. When comparing gene expression in B16-derivative cell lines with high *versus* low metastatic potential, TRPM1 was found to be down-regulated in a highly metastatic line.

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In a panel of tissues, TRPM1 expression only was documented in the eye and melanocytes (4). In the melanocyte lineage, TRPM1 transcripts are expressed at high levels in benign nevi, dysplastic nevi, and melanomas *in situ*, variably in invasive melanoma, and are absent in melanoma metastases. In addition, among primary melanoma lesions, loss of TRPM1 expression was related inversely to tumor thickness (5). Together, these data suggest that loss of TRPM1 expression may be an indicator of melanoma aggressiveness. The ability of TRPM1 staining of primary lesions to predict metastatic potential was first studied in 150 American Joint Committee on Cancer stage I and II primary melanomas. In this study, it was demonstrated that TRPM1 staining in the primary lesion predicted independently 8-year survival rates, with presence of TRPM1 staining associated with improved prognosis (6). This relationship now is being explored further in a multicenter prospective National Cancer Institute-supported protocol to study the use of TRPM1 staining as a predictor of the risk of metastatic disease.³

Several other melanoma markers also have been seen to display some correlation with melanoma prognosis, including tyrosinase (TYR), TYR-related protein-2/dopachrome-tautomerase (DCT), MLANA, and the essential melanocyte transcription factor MITF. For all of these markers, it has been shown that higher expression of mRNA and protein amounts are associated with improved prognosis (7–11). Among the growing list of clinically useful melanoma histopathologic markers, MITF remains detectable in virtually all tumors (10, 12–15). Despite variability in the level of MITF staining, it is unlike many other melanoma markers because it is present in nearly all of the samples that have been studied, perhaps because its loss is incompatible with survival of the melanocyte lineage (10, 12–15). Interestingly, the other melanoma markers, TYR, DCT, and MLANA, are thought to be transcriptional targets of MITF (16–19).

MITF appears to play dual roles in the melanocyte lineage: regulation of survival and differentiation. Hence, mutations in the *MITF* gene do not produce unpigmented melanocytes but rather complete absence of the melanocyte lineage (13). Melanocytes require the *microphthalmia* gene product MITF for their development and maintenance in the adult based on the existence of hypomorphic alleles, which produce postnatal melanocyte death and premature graying (20, 21). In humans, mutations of *MITF* produce Waardenburg syndrome, a condition associated with melanocyte deficiencies in the skin and inner ear (22). MITF is a basic helix-loop-helix leucine zipper (b-HLH-Zip) transcription factor (13) and is part of the MiT family of highly homologous transcription factors, including TFEB, TFEC, and TFE3, which are able to homodimerize and heterodimerize with each other. MITF regulates target gene expression through the binding of the canonical E-box promoter sequence CACGTG and the nonpalindromic sequence CACATG (17, 23).

The presence of potential MITF binding sites in the *TRPM1* promoter (24), the restriction of TRPM1 to the melanocyte lineage, and the loss of staining in metastatic tumors are consistent with the

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possibility that *TRPM1* may be an MITF target gene. In this study, MITF binding sites from the *TRPM1* promoter are shown to be bound by MITF using chromatin immunoprecipitation and electrophoretic mobility shift analysis (EMSA). Reporter assays and quantitative PCR analyses suggest that MITF regulates both luciferase reporters and the endogenous gene within melanoma cells and melanocytes, and mutational analyses have identified the responsible consensus binding sites. Furthermore, the dependence of TRPM1 activity on MITF is suggested by studies showing highly concordant expression levels across a panel of human melanoma cell lines, and in animals containing mutant MITF that express markedly decreased TRPM1 in the retinal pigment epithelium of the eye. Finally, in cotransfection assays, *TRPM1* promoter activity is induced at least 10-fold more strongly than two other MITF-regulated melanoma markers, suggesting that MITF regulates the transcription of TRPM1 in melanocytes and melanoma and that TRPM1 RNA levels thus are a sensitive indicator of MITF transcriptional activity.

MATERIALS AND METHODS

Cell Culture and Media. MeWo and 501mel were gifts from Dr. Ruth Halaban (Yale University School of Medicine, New Haven, CT). SKMEL5 and A375 melanoma cells were purchased from American Type Culture Collection (Manassas, VA). MDA-MB435 was a gift from Dr. Kornelia Polyak (Dana-Farber Cancer Institute, Boston, MA). MALME-3M, SKMEL2, SKMEL28, UACC62, UACC257, and M14 melanoma lines were obtained from NIH (Bethesda, MD). All of the melanoma cell lines were maintained in RPMI (1640; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA), except 501mel and MeWo, which were grown in Ham's F10 (Mediatech), 10% fetal bovine serum, and penicillin/streptomycin/glutamine. HEK293 cells were cultured in DMEM (Mediatech), 10% fetal bovine serum, and penicillin/streptomycin/glutamine. Human primary melanocytes were isolated as described previously (25) and maintained in TICVA medium [Ham's F10 with penicillin/streptomycin/glutamine, 7.5% fetal bovine serum, 50 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (Sigma Chemical Co.), 225 μ M 1-methyl-3-isobutylxanthine (Sigma Chemical Co.), 1 μ M Na₃VO₄ (Sigma Chemical Co.), and 1 mM dibutyryl cyclic AMP (Sigma)].

EMSA. Nuclear extracts were prepared from 501mel as described previously (26). Probe/competitor double-stranded oligonucleotides were annealed 30-mers and spanned the individual E-boxes. Human TRPM1-specific double-stranded probes spanning the E1, E2, and E3 sites were prepared using the following sequences for sense-oligos.

E1 wild-type probe, 5'-ccc aag tgc t CACATG etc etc atg g

E1 mutant probe, 5'-ccc aag tgc t CACCTC etc etc atg g

E2 wild-type probe, 5'-taa tta gtg c CATGTG ceg cct tet c

E2 mutant probe, 5'-taa tta gtg c GAGGTG ceg cct tet c

E3 wild-type probe, 5'-ggc tga gag t CATGTG ggg etc aca c

E3 mutant probe, 5'-ggc tga gag t GAGGTG ggg etc aca c

The sense-oligo of each probe was labeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and γ -[³²P]-ATP (New England Nuclear, Boston, MA). Binding reactions were prepared with 25 fmol (~100,000 cpm) labeled probe, 1 μ g poly (dI-dC) (Amersham Biosciences, Piscataway, NJ), 5% glycerol, 0.1 M KCl, 10 mM Tris (pH 7.6), 0.2 mM DTT (Sigma Chemical Co.), 1 μ l (5 mg/ml) human 501mel nuclear extract, and 4–8 μ l D5 monoclonal antihuman MITF monoclonal antibody (Neomarkers, Fremont, CA) in a 20- μ l reaction volume. Reactions were incubated for 30 min at room temperature and analyzed by 6% (30:1) native PAGE run in 0.5 \times Tris-borate EDTA. For competition studies, indicated amounts of molar excess unlabeled double-stranded mutant probes were included in the binding reactions.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation assays were performed as described elsewhere in 501mel human melanoma cells (19). The forward and reverse primers for the human TRPM1 promoter were 5'-TGACCTCAGGCCAACTTAGAT-3' and 5'-GAGGACCTCCCTTCACCTGTGCTTACCC-3', respectively. The primers for human TRPM1 downstream control regions are 5'-AGTTCCTCCCCATTCCTCTCCCTGTG-3' and 5'-TTGCTGGTGAAGTGTAATGGTA-3', respectively.

Construction of TRPM1 Reporters. A 1.6-kb fragment upstream of the first exon of *TRPM1* was amplified from human genomic DNA (Roche, Indianapolis, IN) using primers 5'-GAAGGGTACCACTGCTCGGAGAGC-CACCTG-3' and 5'-ACCTGAGTTTGTCCACGCTTGAGTTTACC-3' and inserted into the pCR4-TOPO vector (Invitrogen). A 769-bp fragment containing 735 bp upstream of the first exon of human *TRPM1* was amplified from the cloned promoter using primers 5'-GAGAGGTACCGTTCAGGGTTT-GGGTTTT-3' and 5'-CCCTCGAGGGAATGGAGAGGGGCACAGCT-3', which contain *KpnI* and *XhoI* sites for insertion into pGL3-basic vector (Promega, Madison, WI) to generate pTRPM1 wild type (wt). All of the sequences were verified by automated sequencing.

Melastatin Reporter Construct Mutagenesis. Site-directed mutagenesis was performed using the QuickChange method (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Oligos for the mutant EMSA probes were used as sense and antisense primers to generate pTRPM-E1, pTRPM-E2, pTRPM-E3, and pTRPM-E123. All of the sequences were verified by automated sequencing.

Transfection and Dual-Luciferase Reporter Assay. All of the transient reporter assays were performed in triplicate using HEK293, B16, or 501mel cells and carried out in 24-well plates using Fugene-6 (Roche) according to manufacturer's recommendations. For all of the experiments in 293 cells, 500 ng of total vector DNA were transfected. The indicated amounts of pcDNA4-HisMAX-MITF(wt) (encoding full-length human MITF, which was PCR amplified using plaque-forming unit turbo and cloned into pcDNA4-HisMAX-B; Invitrogen) were transfected with the necessary amount of pcDNA4-HisMAX-B empty vector to keep total vector amounts constant; 100 ng of TRPM1, MLANA (19), SILV (19), or empty pGL3-basic (Promega) reporter constructs; and 100 ng of pRL-null (Promega). For experiments in B16 or 501mel, 1 μ g of plasmids were transfected. The indicated amounts of pcDNA3.1-HA-MITF(wt) [encoding full-length human MITF cloned into pcDNA3.1 (Invitrogen) carrying two consecutive HA tags at the 5' end] or pcDNA3.1-HA-MITF(DN) (27) and the necessary amount of pcDNA3.1 to keep total plasmids amounts constant, 100 ng of TRPM1 reporter construct, and 100 ng of pRL-Null (Promega) were used. Cell lysates were prepared 36 and 48 h after transfection using 100 μ l of passive lysis buffer per manufacturer's recommendations (Promega). An aliquot was used to perform luciferase assays using the dual-luciferase kit (Promega) according to the manufacturer's recommendations, and signals were normalized for transfection efficiency by the internal Renilla controls.

pTRPM1-puro Constructs and Colony Growth Assay. The pTRPM1-puro vectors were constructed by subcloning the TRPM1 promoters into the pGL3-vector, which has luciferase replaced with the puromycin-resistance gene. Colony growth assays were performed by transfecting 2 μ g of each plasmid in triplicate into B16 cells grown in six-well dishes. Cells were transfected at 20% confluence according to manufacturer's directions with Fugene-6 (Roche). Twenty-four h later, medium containing 2 mg/ml of puromycin was added. After 12 days of growth in the puromycin-selective medium, colonies were fixed with 10% methanol/10% acetic acid for 20 min and stained with 0.4% crystal violet in 20% ethanol for 20 min. Plates were washed with water and photographed. Crystal violet from triplicate transfections then was resolubilized with 10% methanol/10% acetic acid, and absorbance at 595 nm was measured.

Adenovirus Infection and RNA Preparation. Recombinant AdEasy adenoviruses (28) expressing HA-MITF-IRES-hrGFP (AdV-MITF), control peptide-IRES-hrGFP (AdV-control), and HA-MITF(DN)-IRES-hrGFP [AdV-MITF(DN)] were used (27). Details of adenoviral construction will be presented elsewhere.⁴ For adenoviral infections, 10⁶ human primary melanocytes were plated per 100-mm plate. On the second day, cells were overlaid with 2-ml serum-free F10 medium containing 10 mM MgCl₂, and concentrated adenovirus was added at multiplicity of infection 300. The cells were incubated at 37°C for 30 min, after which virus was removed, and fresh full medium was added. Total RNA was isolated with RNeasy kit (Qiagen, Hilden, Germany) at 48 and 72 h after infection according to manufacturer's instructions. SKMEL5 melanoma cells were infected at multiplicity of infection 100, and the RNA was harvested at 24 and 36 h postinfection.

⁴ Unpublished observations.

Real-Time/Quantitative PCR. The real-time PCR primers for human TRPM1 were 5'-CACCCAGAGCTACCCAACAGA-3' and 5'-CGGATATACATGGCTTTATTGGAA-3'. The probe for human TRPM1 was 5'-6-FAM-AGTTCCTGAATTCCAGGGTGGCGG-TAMRA-3' (Applied Biosystems, Foster City, CA). The total volume of each reaction was 25 μ l, including 12.5 μ l 2 \times QuantiTect RT-PCR Master Mix, 0.25 μ l QuantiTect RT Mix (Qiagen), 1 μ l of each primer (10 μ M stock), 1 μ l of the probe (5 μ M stock), and 1 μ l of the template at 100 ng/ μ l. Reverse transcription proceeded at 48°C for 30 min, followed by 10 min at 95°C to activate DNA polymerase. Forty cycles of PCR then were carried out at 95°C for 15 s and at 60°C for 1 min. Real-time PCR was carried out using the Bio-Rad ICycler (Bio-Rad, Hercules, CA) with analysis using the integrated ICycler software. Standard curves were generated for the primer set to confirm linearity of signals over the experimentally measured ranges.

In Situ Hybridization. MITF wild-type mice (with TYR-mutated albinism to avoid the need to discriminate between melanin and hybridization signal) or *mitf*^{vga-9} homozygous mutants were generated using timed pregnancies. E14.5 embryos were collected in PBS and fixed overnight at 4°C using 4% paraformaldehyde (J. T. Baker, Paris, KY). Embryos then were cryopreserved in 30% sucrose until submerged and embedded in optimal cutting temperature compound (Sakura, Torrance, CA). Transverse sections (20 μ m) were collected on a cryostat and allowed to dry overnight. Section *in situ* hybridization was performed as described previously (29). Riboprobes labeled with digoxigenin were detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.). The MITF and DCT riboprobes were described previously (30). A 351-bp region of the murine *TRPM1* coding region was amplified from B16 melanoma genomic DNA using the primers 5'-TGGCT-GACAACGGCACC and the antisense primer 5'-GCTGATACGACTGG-GACTTGCT, cloned into TOPO-TA (Invitrogen), and sequence verified. The riboprobe for mouse TRPM1 was generated by linearizing the construct with *SpeI* (New England Biolabs) and by synthesizing an antisense riboprobe using T7 polymerase and digoxigenin-dUTP (Roche).

RESULTS

MITF Binding Elements in Mouse and Human Promoters.

Alignment of the mouse (GenBank accession no. AF084519) and the human *TRPM1* promoter regions was performed using the Basic Local Alignment Search Tool (BLAST) algorithm. As seen in Fig. 1,

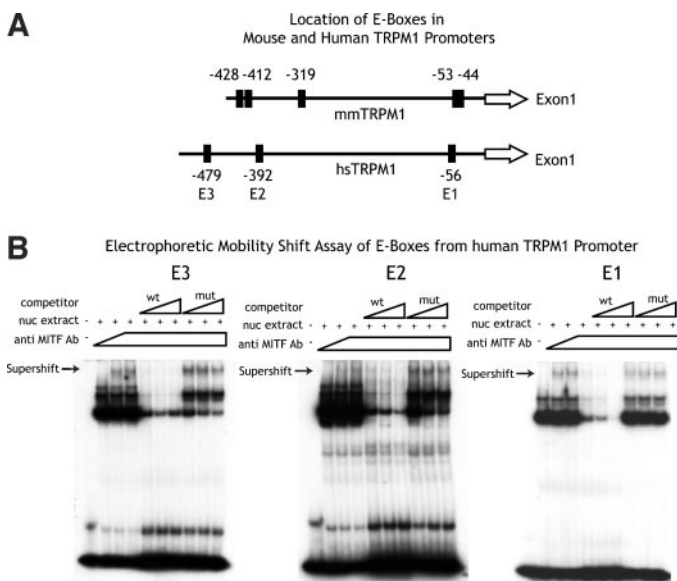


Fig. 1. *A*, The *mmTRPM1* and *hsTRPM1* promoters/enhancers. Large arrows (\Rightarrow) denote initial transcribed exons, and vertical bars represent E-boxes. *B*, binding of MITF to *TRPM1* promoter *in vitro*. Electrophoretic mobility shift analysis assays were performed on melanoma lysates using probes containing each of the three E-boxes from the human *TRPM1* promoter. Lanes 2–4 include 0, 4, and 8 μ l of anti-MITF antibody and induce a supershift that specifically is competed away by 5, 10, and 25 \times wild-type unlabeled probe in Lanes 5–7 but not by mutant probe in Lanes 8–10.

Downstream Region

TRPM1 Promoter

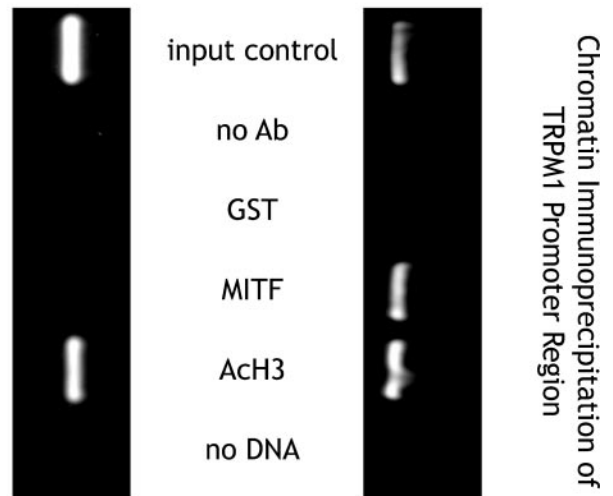


Fig. 2. Binding of MITF to *TRPM1* promoter *in vivo*. Chromatin immunoprecipitations were performed on materials isolated from melanoma cells. DNA from lysates before immunoprecipitation was used as positive input control. The *TRPM1* promoter primer set amplifies the E-box containing the promoter region, whereas the control primer set amplifies a region approximately 2 kb downstream.

upstream of exon 1 in the human and mouse genes are several E-boxes whose relative spacing is highly conserved between species. These E-boxes reside in the region of the promoter reported previously to be necessary for transcriptional activity in melanocytes (24), prompting us to explore direct regulation by MITF.

Probes containing the three E-boxes from the human promoter at –56 bp (E1), –392 bp (E2), and –479 bp (E3) from the transcriptional start were constructed and used in EMSAs to test *in vitro* binding of MITF to these sequences. Melanoma nuclear extracts were used as a source of MITF, and a monoclonal anti-MITF antibody (D5) was used for supershift analysis. Supershifted complexes were observed on addition of wild-type radiolabeled probes to the E1, E2, and E3 sites in the presence of anti-MITF antibody (Fig. 1). In each case, these supershifted bands were competed away with increasing amounts of wild-type unlabeled probe but not with identical amounts of point-mutated unlabeled probe (Fig. 1).

To determine if MITF occupies these promoter sequences *in vivo*, chromatin immunoprecipitation was carried out using 501mel human melanoma cells (Fig. 2). Primers were designed that span the *TRPM1* promoter region and a control region downstream of the gene. Immunoprecipitation with antibody against MITF revealed occupancy by MITF of the *TRPM1* promoter but not the downstream control region. Additional controls included no antibody or control species-matched polyclonal antibody (directed against GST protein). The promoter region of a previously demonstrated MITF target gene, *MLANA* (19), also was used as a positive control.⁴ Antibody directed against acetylated histone H3 represented a positive control in each reaction. Combined with EMSA evidence for MITF recognition of the conserved E-box sequences (Fig. 1), these data suggest that MITF protein occupies those elements within chromatin of living cells.

TRPM1 Promoter Activity Requires MITF and Depends on Several Conserved E-Boxes. The ability of MITF to induce the *TRPM1* promoter was tested in HEK293 cells, which do not express detectable levels of MITF. The wild-type *TRPM1* promoter only is slightly more active than control pGL3-basic. However, titration of MITF activates potently the wild-type promoter >500-fold (Fig. 3A). Individual mutants (E1, E2, and E3) and triple mutant (E123) promoter constructs were created to determine if abrogation of MITF

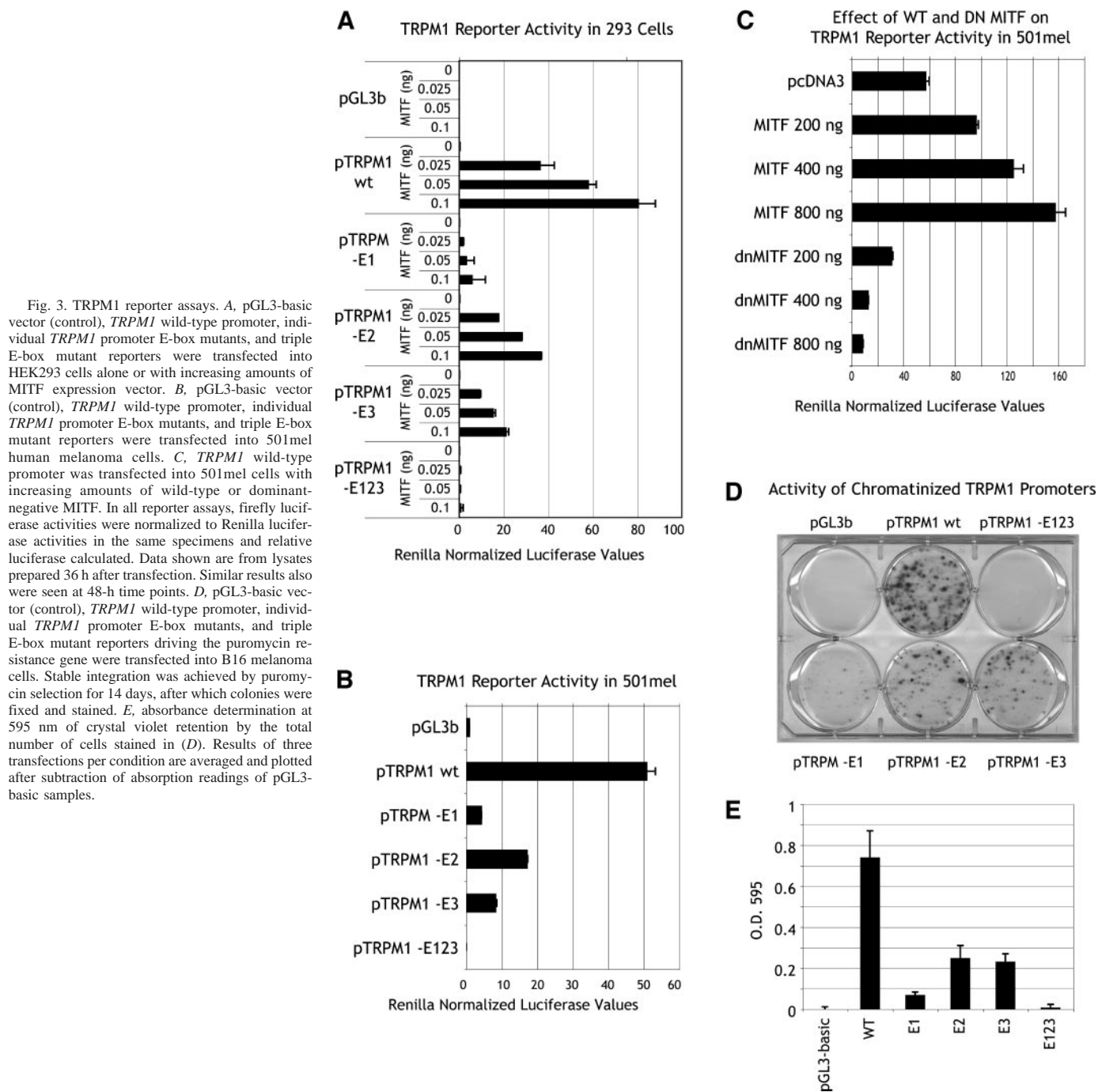


Fig. 3. TRPM1 reporter assays. *A*, pGL3-basic vector (control), *TRPM1* wild-type promoter, individual *TRPM1* promoter E-box mutants, and triple E-box mutant reporters were transfected into HEK293 cells alone or with increasing amounts of MITF expression vector. *B*, pGL3-basic vector (control), *TRPM1* wild-type promoter, individual *TRPM1* promoter E-box mutants, and triple E-box mutant reporters were transfected into 501mel human melanoma cells. *C*, *TRPM1* wild-type promoter was transfected into 501mel cells with increasing amounts of wild-type or dominant-negative MITF. In all reporter assays, firefly luciferase activities were normalized to Renilla luciferase activities in the same specimens and relative luciferase calculated. Data shown are from lysates prepared 36 h after transfection. Similar results also were seen at 48-h time points. *D*, pGL3-basic vector (control), *TRPM1* wild-type promoter, individual *TRPM1* promoter E-box mutants, and triple E-box mutant reporters driving the puromycin resistance gene were transfected into B16 melanoma cells. Stable integration was achieved by puromycin selection for 14 days, after which colonies were fixed and stained. *E*, absorbance determination at 595 nm of crystal violet retention by the total number of cells stained in (*D*). Results of three transfections per condition are averaged and plotted after subtraction of absorption readings of pGL3-basic samples.

binding to these conserved promoter elements would decrease the MITF responsiveness of the reporter. Although mutation of the most proximal E-box, the E1 site, has the greatest detriment on MITF-dependent reporter activity, no single point mutant completely abolishes activity. However, in the case of the triple-point mutant, MITF is unable to induce significantly the *TRPM1* promoter construct.

These promoters then were tested for basal activity in 501mel human melanoma, which express high levels of MITF. In 501mel, a similar pattern of E-box dependence was seen, with the E123 mutant losing nearly all activity and the E1 mutant having the greatest detriment on activity of the single E-box mutants (Fig. 3*B*). Compared with the wild-type promoter, the E1 mutant maintains 8.4% of activity; the E2 mutant maintains 33.7% of activity; the E3 maintains 16.3% of activity; and the E123 mutant maintains 0.04% of activity.

Similar results also were seen in B16 mouse melanoma.⁴ These data suggest that each E-box contributes toward the activity of this promoter and that MITF depends on these E-boxes for induction of the *TRPM1* promoter.

The ability to affect *TRPM1* promoter activity in 501mel melanoma cells through modulation of MITF activity also was tested by expressing exogenous wild-type or dominant-negative MITF. The dominant-negative allele used lacks the 5' activation domain and is mutant in the DNA binding domain. It retains the ability to dimerize with wild-type MITF and is believed to function as a dominant negative by forming complexes incapable of binding DNA or activating transcription. The wild-type *TRPM1* reporter responds to the addition of exogenous MITF (68%, 118%, and 174% increase with 200 ng, 400 ng, and 800 ng of MITF, respectively) and is repressed by dominant-negative

MITF [55%, 79%, and 85% inhibition with 200 ng, 400 ng, and 800 ng of MITF(DN), respectively; Fig. 3C]. Similar effects were seen at multiple time points.⁴

The *TRPM1* promoter region also was used to drive expression of puromycin resistance and was transfected into melanoma cells (Fig. 3D). Using colony formation to select for chromosomal integration, this assay reflects activity of a chromatinized reporter. Mutation of each individual E-box reduced sharply the number of colonies relative to wild type, whereas mutation of all three E-boxes prevented all colony growth. These effects were quantitated by measuring the crystal violet staining of surviving colonies with absorbance at 595 nm (Fig. 3E). Relative to the wild-type promoter, colony growth of the promoter mutants was 8.8%, 33.2%, 30.9%, and 0.4% for the E1, E2, E3, and E123 mutants, respectively.

MITF Regulation of Endogenous TRPM1. The ability of MITF to regulate transcriptionally the endogenous *TRPM1* gene in the melanocyte lineage was assessed using early passage human primary melanocytes and the human melanoma cell line SKMEL5. These cells were infected with the indicated adenoviruses, AdV-control, AdV-MITF, and AdV-MITF(DN); total RNA was isolated at various time points after adenoviral infections; and real-time (quantitative) PCR was performed for TRPM1 on the RNA samples. At all of the time points tested, wild-type MITF stimulated endogenous TRPM1 mRNA

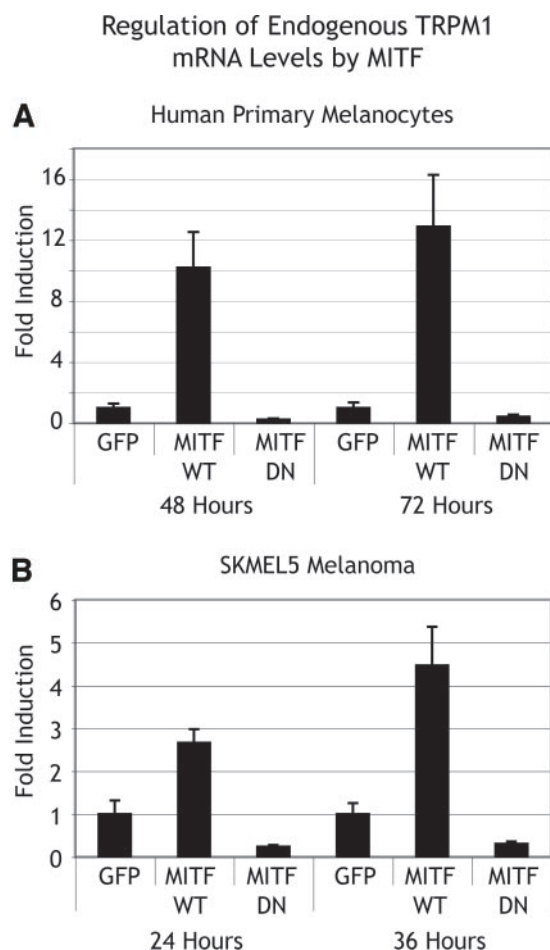


Fig. 4. Regulation of endogenous TRPM1 mRNA levels by MITF. Human primary melanocytes or SKMEL5 human melanoma cells were infected with adenovirus carrying GFP (AdV-control), MITF wild-type (AdV-MITF), or MITF dominant-negative [AdV-MITF(DN)]. TRPM1 levels were determined by quantitative real-time PCR, and data were normalized to endogenous β -actin levels. Expression levels for AdV-MITF and AdV-MITF(DN) virus-infected samples were normalized to the AdV-control samples at each time point.

Expression Patterns of MITF and TRPM1 in Human Primary Melanocytes and Melanoma Cell Lines

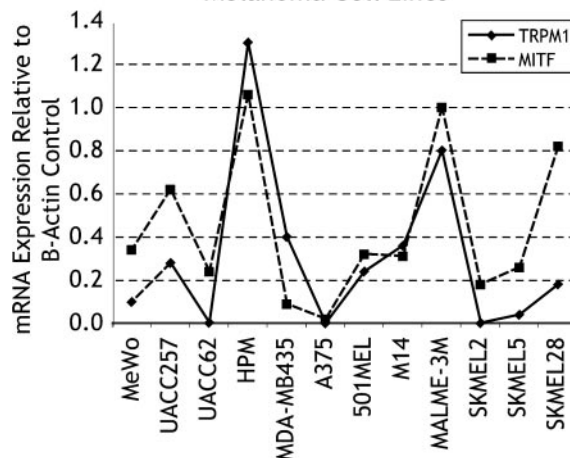


Fig. 5. Correlation of MITF and TRPM1 mRNA levels in melanoma cell lines and human primary melanocytes. Quantitative mRNA levels for MITF and TRPM1 were acquired by quantitative real-time PCR and normalized to β -actin levels.

expression (Fig. 4), whereas dominant-negative MITF suppressed significantly TRPM1 expression, findings which also were seen at multiple time points in other melanoma cell lines.⁴

Similar Expression Patterns of MITF and TRPM1 in Several Melanocyte Lineage Cell Lines. If MITF transcriptionally regulated *TRPM1*, it would be plausible that TRPM1 mRNA levels may parallel MITF mRNA levels in cell lines that express both genes. Real-time (quantitative) PCR for MITF and TRPM1 was performed on human primary melanocytes and a series of 11 human melanoma cell lines. Expression of these genes was normalized to β -actin and compared (Fig. 5). A high degree of correlation of these two markers was observed in these cell lines. Although such a correlation certainly offers no proof of a transcription factor-target relationship, the parallel expression patterns are consistent with the hypothesis that MITF modulates TRPM1 expression.

We examined TRPM1 expression in HeLa cells, which do not express detectable MITF protein, and were unable to detect TRPM1. We also tested TRPM1 expression in primary human osteoclasts, which do express MITF but were found not to express TRPM1. These observations are consistent with the previous characterization of *TRPM1* as a melanocyte-specific gene (4).

Loss of TRPM1 Expression in the RPE of MITF Mutant Mice. Using MITF wild-type mice or mice homozygous for *MITF*^{vga-9}, we investigated the genetic requirement of MITF for expression of TRPM1 in the developing retinal pigmented epithelium (RPE), a cell lineage that normally expresses MITF but remains viable in the absence of wild-type MITF. The *MITF*^{vga-9} allele is recessive and behaves like a null allele (13, 30). The pigmentation enzyme gene DCT was used as a positive staining control because its expression has been shown previously to be maintained in the RPE of MITF mutant animals (30). Analysis in melanocytes is not possible in *MITF*^{vga-9}/*MITF*^{vga-9} animals because the cells are missing completely, whereas the RPE shows nearly normal development through early stages (30).

In situ analysis at E14.5 in MITF wild-type embryos revealed similar expression patterns for TRPM1 and MITF. Both were expressed throughout the RPE with higher levels in the peripheral RPE; near the lens, where the RPE thickens at the presumptive ciliary body; and iris (Fig. 6A, C, and E). Expression of MITF and TRPM1 also was seen in the nonpigmented compartment of the presumptive ciliary

body (Fig. 6, A and C; arrowheads). Interestingly, although MITF was detectable in migrating choroidal melanocytes (arrows), no expression of TRPM1 was observed in these cells, suggesting that at this developmental stage, the presence of MITF alone is not sufficient to direct melanocyte expression of TRPM1. DCT staining served as a positive control for RPE and differed slightly because its staining intensity was greatest in the central RPE, and unlike TRPM1, it was expressed in choroidal melanocytes.

In contrast to wild-type embryos, animals homozygous for MITF^{vga-9} show essentially no MITF, whereas DCT staining was preserved (Fig. 6, B and F) as described previously (30). TRPM1 staining virtually was absent in MITF^{vga-9} mutant RPE (Fig. 6D), consistent with the model that MITF regulates expression of TRPM1 in RPE cells and melanocytes. Of note, certain MITF^{vga-9} eyes differed in displaying patchy MITF expression in the RPE, and in these cases, TRPM1 expression exhibited the same patchy distribution.⁴ These TRPM1 expression defects within MITF mutant eyes are consistent with the model that MITF regulates transcriptionally TRPM1 *in vivo*.

Dose-Responsiveness of TRPM1 Versus Other MITF Target Gene Promoters. Compared with two other MITF-regulated melanoma markers, MLANA and SILV, the induction of the endogenous

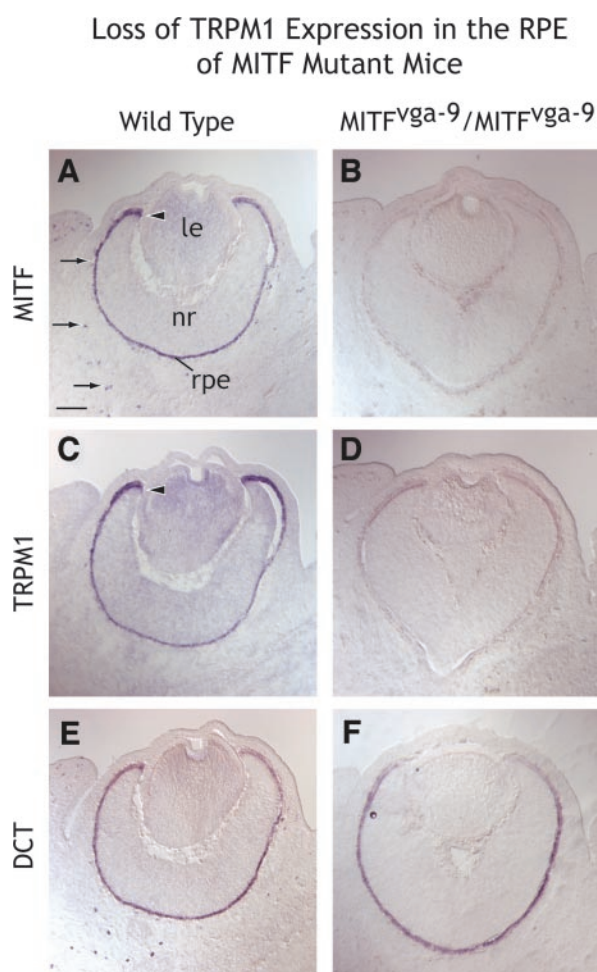


Fig. 6. *In situ* detection of MITF and TRPM1 in E14.5 embryos. Transverse sections were stained for MITF message (A and B), TRPM1 message (C and D), or dopachrometautomerase (DCT; E and F) in wild-type albinos (A, C, and E) or MITF^{vga-9}/MITF^{vga-9} homozygotes (B, D, and F). MITF in wild-type embryos (A) could be detected in the retinal pigmented epithelium (RPE) but not in the neural retina or lens. MITF also was expressed in the nonpigmented component of the presumptive ciliary body (arrowhead) and in migrating melanoblasts (arrows). TRPM1 staining was strong in the RPE (C) but absent in migrating melanoblasts. MITF levels were reduced dramatically in MITF^{vga-9}/MITF^{vga-9} homozygotes (B), and TRPM1 was undetectable except in the peripheral RPE near the lens. DCT staining served as a control (E and F). Scale bar is 100 μ M (A).

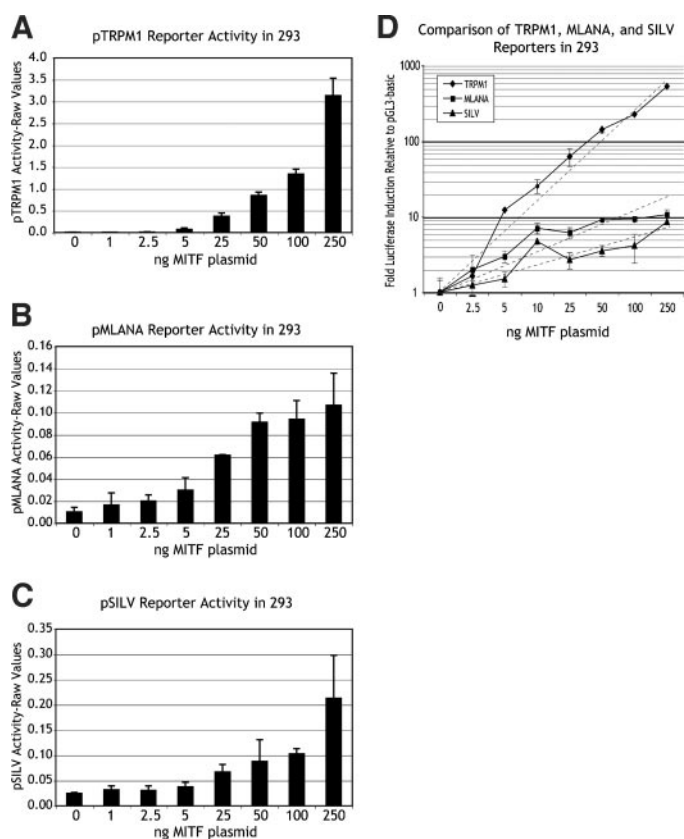


Fig. 7. MITF activity on the promoters of various melanoma markers. Increasing amounts of MITF expression vector were transfected into HEK293 cells with the indicated reporter plasmids. Cells were lysed 36 h later, and an aliquot used for luciferase assays. Another aliquot was used to confirm MITF protein expression.⁴ In all experiments, firefly luciferase activities were normalized to Renilla luciferase activities in the same specimens. A, B, and C, raw luciferase values are shown normalized to Renilla. D, values have been graphed as fold induction.

TRPM1 gene by MITF-carrying adenovirus was reproducibly considerably stronger.⁴ This observation, coupled with the strong differences in TRPM1 expression among clinically analyzed melanoma specimens, suggested the possibility that the TRPM1 promoter might exhibit a particularly steep dose-response to MITF, something which we examined and compared for two other MITF target genes, the melanoma markers HMB45/pm117/gp100/SILV and MLANA/MART1. Using luciferase-promoter constructs for TRPM1, SILV, and MLANA, we compared their inducibility across a range of MITF expression vectors ranging 1–250 ng. As shown (Fig. 7, A–C), clear induction was observed for all three genes. Moreover, the slopes of the curves for SILV and MLANA were similar, whereas the TRPM1 induction slope was significantly steeper (Fig. 7D; arbitrary units of 0.0126 for TRPM1 versus 0.0007 for SILV and 0.0002 for MLANA). Among this set of MITF target genes that also are used as melanoma markers, TRPM1 responds the most sharply to MITF levels.

DISCUSSION

The present work demonstrates regulation of TRPM1 expression by MITF in melanocytes. MITF is capable of binding multiple E-boxes from the TRPM1 promoter/enhancer region *in vitro* and the promoter/enhancer region *in vivo*. The promoter responds strongly to MITF in reporter assays and requires all three E-boxes for function in melanocytes and when introduced into nonmelanocytes. MITF is capable of activating the endogenous gene, whereas dominant-negative MITF represses expression of the endogenous gene. Levels of TRPM1 and

MITF are similar in a panel of human melanoma cell lines, and MITF mutation reduces strongly TRPM1 expression in the RPE of the eye. Several other melanoma antigens also are thought to be regulated by MITF (TYR, DCT, TRP-2, MLANA, and SILV), although none are known to display the prognostic power of TRPM1. Taken together, these data add the prognostically valuable melanoma marker TRPM1 to the list of MITF-regulated genes.

TRPM1 Promoter/Enhancer. The fold induction of the *TRPM1* promoter by MITF is significantly stronger than any other MITF-responsive gene we have examined, whereas mutation of the MITF binding sites leads to nearly no activity in reporter assays. Each E-box was important for activity in both reporter assays and in integrated (fully chromatinized) promoters. These data have not addressed the additional possibility that MITF could alter RNA stability of the TRPM1 mRNA, although chromatin immunoprecipitation and reporter assays suggest strongly the presence of direct transcriptional regulation. Therefore, we believe that the TRPM1 promoter may be a useful model for studying the cooperativity of multiple MITF binding sites in the regulation of gene expression. In addition, the steepness of this MITF dose-response is likely to contribute to the “on” or “off” nature of TRPM1 mRNA expression, as observed within intermediate-thickness melanoma clinical specimens (4–6). For this to be true, not only must TRPM1 expression be particularly high with elevated MITF levels but also TRPM1 expression must be particularly unresponsive to low MITF levels because MITF expression may be low, but is rarely lost, even among metastatic melanomas that may entirely lack TRPM1. Because high levels of MITF are associated with melanocytic differentiation, it is plausible to conclude that melastatin is a particularly robust indicator of MITF-mediated melanocytic differentiation.

Tissue Restriction of TRPM1 Expression. Although our studies show that TRPM1 expression is strongly responsive to MITF, several lines of evidence suggest that other factors are necessary for TRPM1 expression. We were unable to detect TRPM1 expression in osteoclasts, a lineage that is known to express MITF, suggesting that other factors are necessary to permit the expression of TRPM1.⁴ This observation also was made in embryonic migrating choroidal melanocytes in which MITF was present but TRPM1 was not (Fig. 6, A and C).

Conversely, the steep dose requirement for MITF expression alternatively may suggest that TRPM1 is not expressed in those cells because MITF expression, albeit present, is not sufficient to initiate transcription at the *TRPM1* promoter. Of note, certain *MITF*^{ga9} mutant eyes were seen to exhibit patchy MITF expression, and adjacent sections exhibited parallel patchy TRPM1 expression.⁴ The presence of observable MITF in any eyes from these mutant mice suggests that they are not fully null for MITF expression, in agreement with previous observations (13). It also is apparent that melanocytes express an MITF isoform that is not present in either RPE or osteoclasts (M-MITF), and it is possible that different isoforms, which vary in their N-terminal initial exons, may modulate differentially TRPM1 expression (31).

Melanoma Markers and Prognosis. The expectation that more highly metastatic disease emerges from less-differentiated tumors is exemplified in melanoma, in which a large number of melanocytic markers have been shown to be down-regulated in metastatic disease. These include the pigmentation enzymes TYR and TYR-related protein 2 (11), the melanoma antigens MLANA (8, 9) and MDA-7 (32), the signaling molecule β -catenin (33), and the transcription factor MITF (7, 10). Notably, many of these genes are targets of MITF, which is necessary for melanocyte differentiation, suggesting that levels of these markers are reflections of the MITF-driven level of cellular differentiation. Importantly, however, MITF's role in the

melanocyte lineage is not restricted to regulation of differentiation because the consequence of MITF deficiency is complete loss of the lineage rather than an albino-like (unpigmented) phenotype. MITF's requirement for lineage survival is exemplified by the *MITF*^{vit} mutant, which turns gray postnatally because of accelerated melanocyte loss (20). Conversely, compelling evidence in support of MITF's role in differentiation comes from its position as a nuclear target of the α -melanocyte-stimulating hormone (α -MSH) pathway and at least one mouse mutant (brownish), which displays primarily a pigmentation abnormality (34).

MITF may be unique among melanoma markers because its variable expression has prognostic significance, although its expression is not often (if ever) entirely lost in metastatic lesions (10, 12, 14, 15). The prognostic value of MITF staining intensity is related probably to the level of differentiation driven by MITF. However, given the ability of post-translational modifications of MITF to affect its transcriptional activity, a measure of MITF transcriptional activity may have greater prognostic value (26). TRPM1 expression, which responds across an extremely broad dynamic range to MITF, is likely to be such a read-out. For lesions in which TRPM1 expression is maintained at higher levels, MITF may be driving a greater degree of melanocytic differentiation, whereas lesions with decreased levels of TRPM1 are at a less-differentiated state. It has been shown that treatment of cultured melanoma cell lines with an inducer of differentiation causes an up-regulation of TRPM1 transcripts (35). Multiple mechanisms likely exist for down-regulation of differentiation factors in advanced melanomas, as exemplified by the post-translational degradation of TYR (36). Such mechanisms also may contribute to modulation of TRPM1 expression, although the differences observed among clinical specimens were based on RNA (not protein) expression levels (4–6). Of note, levels of MITF are much higher relative to TRPM1 in the SKMEL28 line, potentially representing a case of an independent mechanism producing decreased TRPM1 expression.

Loss of TRPM1 Expression in Metastatic Tumors. The absence of TRPM1 expression by *in situ* hybridization in metastatic tumors is intriguing given the number of reports that MITF staining is maintained in most, if not all, metastatic tumors. The monoclonal antibodies used for this analysis of MITF expression do not discriminate between the melanocyte-specific M-form of MITF and the more ubiquitously expressed A-form of MITF. We have found that the A-form of MITF is capable of activating the TRPM1 reporter in luciferase assays, although perhaps less strongly than the M-form.⁴ The steep response of TRPM1 to MITF levels may exceed the dynamic range of the TRPM1 staining assay such that the decrease in MITF staining reported in metastatic disease manifests as a complete loss of TRPM1 staining (a clinically useful feature). Other possibilities are that some cofactor necessary for TRPM1 expression is lost selectively in metastatic lesions and that post-transcriptional events, such as RNA stability, affect TRPM1 levels.

Modulating MITF and Target Genes via α -MSH. MITF expression is regulated by the cytokine α -MSH via a well-studied signal transduction pathway (37–39). Given the relationship between prognosis and the levels of MITF and its target genes, α -MSH theoretically may have therapeutic value. In one model, α -MSH may induce differentiation of tumors into a less-aggressive state. Another possibility is that α -MSH treatment may up-regulate the expression of tumor antigens that may elicit or be recognized by an antitumor response, thereby increasing immune-mediated tumor killing, whether because of innate or vaccine-induced immunity. Additional examination of these possibilities may link the biology of MITF and melanocytes to improvements in melanoma diagnosis or therapy.

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