MLANA/MART1 and SILV/PMEL17/GP100 Are Transcriptionally Regulated by MITF in Melanocytes and Melanoma

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The clinically important melanoma diagnostic antibodies HMB-45, melan-A, and MITF (D5) recognize gene products of the melanocyte-lineage genes SILV/PMEL17/GP100, MLANA/MART1, and MITF, respectively. *MITF* encodes a transcription factor that is essential for normal melanocyte development and appears to regulate expression of several pigmentation genes. In this report, the possibility was examined that MITF might additionally regulate expression of the SILV and MLANA genes. Both genes contain conserved MITF consensus DNA sequences that were bound by MITF *in vitro* and *in vivo*, based on electrophoretic mobility shift assay and chromatin-immunoprecipitation. In addition, MITF regulated their promoter/enhancer regions in reporter assays, and up- or down-regulation of MITF produced corresponding modulation of endogenous *SILV* and *MLANA* in melanoma cells. Expression patterns were compared with these factors in a series of melanoma cell lines whose mutational status of the proto-oncogene BRAF was also known. SILV and MLANA expression correlated with MITF, while no clear correlation was seen relative to BRAF mutation. Finally, mRNA expression array analysis of primary human melanomas demonstrated a tight correlation in their expression levels in clinical tumor specimens. Collectively, this study links three important melanoma antigens into a common transcriptional pathway regulated by MITF. *(Am J Pathol 2003, 163:333–343)*

MITF, a bHLHZip (*b*asic/*h*elix-*l*oop-*h*elix/*leucine-zip*per) transcription factor, is essential for the development and maintenance of the melanocyte lineage.[1] MITF binds the canonical E-box promoter sequence CACGTG as well as the non-palindromic sequence CACATG.[2,3] The three major pigmentation enzymes tyrosinase, TYRP1, and DCT all contain the MITF consensus binding site in their promoters and are thought to be transcriptional targets of MITF.[2,4,5] In humans, germline heterozygous *MITF* mutation produces, with variable penetrance, Waardenburg Syndrome IIA,[6] and Tietz syndrome,[7,8] manifesting pigmentation disturbances and deafness, the latter due to inner ear melanocyte deficiency.[9] In mice, the allele *Mitf^mi* leads to complete absence of melanocytes in the homozygous state,[10] while mice homozygous for *Mitf^vit* (*vitiligo*) are born with a nearly normal coat color, but turn white over the first few months of life as melanocytes are lost, suggesting a role for MITF in both melanocyte development and in postnatal survival.[11,12] In contrast to various melanocytic markers such as melanin or c-kit whose expression may be lost or difficult to detect in malignant melanocytic lesions,[13–15] MITF expression is usually maintained in human melanoma specimens,[16–19] and MITF is increasingly used as a histopathological marker for melanoma diagnosis.[20]

PMEL17 is the human homologue of murine *silver,* a locus whose disruption produces a significant pigmentation phenotype which resembles a silver color.[21] Although the precise function of this gene (also known as GP*100/SILV*, hereafter referred to as *SILV*) remains to be ascertained, SILV localizes to an early melanosome trafficking compartment[22] and may function in melanosome structure,[23] biosynthesis of the melanin intermediate 5,6-dihydroxyindole-2-carboxylic acid (DHICA),[24,25] or morphogenesis of premelanosomes.[26]

MLANA/MART1 (hereafter referred to as MLANA) was cloned by two separate groups using melanoma reactive cytotoxic T lymphocytes to screen a cDNA library derived from melanoma cells.[27,28] MLANA expression is restricted to melanocytes, melanomas, and retinal pigment epithelium.[27] Its function remains to be fully elucidated, but it has recently been found to localize to vesicles, including melanosomes, suggesting a role in melano-

some biogenesis.[29] Although near the loci for two murine coat color mutants, *MLANA* has been excluded as a candidate for either,[30] and its loss of function phenotype is currently unknown.

Aside from its role in pigmentation, SILV encodes antigenic epitopes which are recognized by multiple melanoma diagnostic antibodies including HMB-45, currently one of the most commonly used melanocytic markers for clinical melanoma diagnosis in humans.[31,32] The Melan-A and MART1 antibodies both recognize the MLANA gene product and are increasingly being used as markers for the diagnosis[33–36] and prognosis of melanoma.[37] Due to its early vertical (deep) growth phase, cutaneous melanomas exhibit a high propensity for local invasion and metastasis. Furthermore, melanoma may present as "metastasis of unknown primary" or relapse after considerable time intervals, often lacking differentiated features (such as pigmentation). The diagnosis of melanoma may therefore rely on combinations of immunohistochemical markers for diagnosis. Despite the common loss of many melanocyte-specific markers in melanoma, HMB-45 positivity has been seen in a high fraction of human melanoma specimens[38,39] and antibody staining for the melanocyte-melanoma lineage-specific antigen MLANA was shown to be positive in all primary malignant melanomas and nevi tested.[40,41] In subsequent studies, MLANA reactivity has been shown to largely overlap with tyrosinase[42] and HMB-45.[33,43] MITF (antibody D5) was also found to stain a high fraction of melanomas, with a pattern that included all HMB45-positive specimens.[16] A recent side-by-side examination of MITF, HMB-45, and Melan-A revealed high sensitivity and specificity for all three in melanoma diagnosis of cytologic specimens.[44]

Both SILV and MLANA have been identified as antigens recognized by tumor infiltrating lymphocytes,[28,45–47] leading to their use in vaccine protocols aimed at immunotherapy for melanoma.[48–51] Dramatic melanoma regressions were recently reported for patients undergoing intensive immunotherapy directed against SILV and MLANA.[52] In addition, it has been suggested that expression patterns of MITF and MLANA may correlate with specific clinical behaviors of melanoma, with higher expression of these markers associated with better prognosis.[17,53–55]

In this study, we demonstrate evidence of linkage between the expression patterns of the three melanoma diagnostic markers, MITF, SILV and MLANA. MITF consensus recognition sites were identified in the promoters/enhancers of both *SILV* and *MLANA*, and were found to be occupied by endogenous MITF using chromatin immunoprecipitation. Reporter assays, quantitative-PCR, Northern, and Western analysis all suggested that MITF either regulates reporters or the endogenous genes within melanoma cells and melanocytes. Protein and mRNA levels of the three antigens were found to correlate in a panel of melanoma cell culture lines as well as human primary melanocytes. Finally, we show MITF, SILV and MLANA mRNA levels are highly correlated in a primary human melanoma gene expression data set. Together, the results suggest that MITF transcriptionally regulates

SILV and *MLANA*, placing three commonly used diagnostic markers within a common transcriptional pathway.

Materials and Methods

Cell Culture and Media

501mel was a gift from Dr. Ruth Halaban (Yale University School of Medicine) and maintained in Ham's F10 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) or RPMI (Mediatech) with 10% FBS. SKMEL5 and A375 melanoma cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). MALME-3M, SKMEL2, SKMEL28, UACC62, UACC257 and M14 melanoma lines were all obtained from NIH (Frederick, MD). All melanoma cell lines were maintained in RPMI with 10% FBS. 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech) with 10% FBS. Human primary melanocytes were isolated as described previously[56] and maintained in TICVA medium (F10 with penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA), 7.5% FBS, 50 ng/ml TPA (Sigma), 225 μmol/L IBMX (Sigma), 1 μmol/L Na_3VO_4 (Sigma), and 1 mmol/L dbcAMP; Sigma).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays (ChIPs) were performed with cells grown in logarithmic phase. Cells were harvested by scraping and homogenized in a hypotonic buffer (10 mmol/L Tris-HCl, pH 7.4, 15 mmol/L NaCl, 60 mmol/L KCl, 1 mmol/L EDTA, 0.1% Nonidet P40, 5% sucrose, and 1X Complete proteinase inhibitor cocktail (Roche, Indianapolis, IN)) on ice using a Dounce homogenizer. The nuclei were isolated by centrifugation onto a 10% sucrose pad and then cross-linked with 1% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature with gentle shaking. Nuclei were then spun down and resuspended in ChIPs buffer (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 60 mmol/L KCl, 0.1% Nonidet P40, and 1X Complete proteinase inhibitor cocktail (Roche)) and sonicated by two 1-minute pulses on ice using a Fisher dismembranator fitted with a microtip. Rabbit antibodies against human MITF, anti-GST (Roche), and anti-Acetylated-Histone3 (Upstate, Waltham, MA) were then added to a 10-fold ChIPs buffer diluted sample and incubated on a nutator for 3 hours at room temperature. Ultralink protein-A/G-beads (Pierce, New York, NY) were added to the sample and a control sample and incubated for an additional hour. Immunoprecipitates were then washed twice with ChIPs buffer, twice with 500 mmol/L NaCl ChIPs buffer, and once with TE, pH 8.0. The immunoprecipitates were released from the beads by incubating at 65°C for 20 minutes in 1%SDS/TE and protein digested by proteinase K treatment side-by-side with an additional unprecipitated sample as input control. Cross-links were released by heating at 70°C for 10 hours and DNA recovered by extraction with phenol and chloroform at high salt (0.6 mol/L Na acetate, pH 8.0) followed by ethanol precipita-

tion. Semi-quantitative PCR was then performed on samples to amplify a fragment containing the intronic E-box (E1) or the upstream E-box (E2) in the human SILV transcription initiation region. The forward and reverse primers for the E1 region are 5′- CAT AAG ATA CCC CAT TCT TTC TCC ACT T-3′ and 5′-GAG AAT GTG GTA TTG GGT AAG AAC AC-3′, respectively. The primers for the E2 region are 5′-CAT GGA GAA CTT CCA AAA GGT GG-3′ and 5′-TAC TCT CCC CAG GGA GTA TAA GT-3′. The *MLANA* promoter and control regions were analyzed similarly. The primers for *MLANA* promoter region are: 5′-TTG GAA TAA ATT GGG CTA CGA ACT T and 5′-TGG CAG GAT CTC AGC TCA CTA CAA C. The primers for *MLANA* downstream control regions are: 5′- ATG CCT GGC CTC TAT CCA CT and 5′- GGC GAC AGA GTG AGT GAG AT.

EMSA (Electrophoretic Mobility Shift Assay)

Nuclear extracts were prepared as previously described.[57] Probe/competitor double-stranded oligonucleotides were 30 bp and spanned the individual E-boxes. A MLANA-specific double-stranded probe spanning the E2 site was prepared using the following sequences for sense oligos: wild-type probe, 5′-TTTCCATGTT*CACGTG*TGAGATATGC; mutant probe, 5′-TTTCCATGTT*GAGGTG*TGAGATATG. The SILV probe was prepared using the following sequences for sense oligos: wild-type probe, 5′- CCCAGAGCCCTTT*CAT-GTG*ATGCTCAGCT; mutant probe, 5′- CCCAGAGCCCTT-T*GAGGTG*ATGCTCAGCT. Probe labeling was performed using T4 polynucleotide kinase (New England BioLabs, Beverly, MA) and α-[^{32}P]-ATP (PerkinElmer, Boston, MA) to label the 5′ oligo before annealing. Binding reactions contained 25 *f*mol (~100,000 cpm)-labeled probe, 1 μg poly (dI-dC) (Amersham Biosciences, Piscataway, NJ), 5% glycerol, 0.1 mol/L KCl, 10 mmol/L Tris (pH 7.6), 0.2 mmol/L DTT, 1 μl (5 mg/ml) human 501 melanoma nuclear extract, and 4 to 8 μl D5 anti-human Mitf mAb (Neomarkers, Fremont, CA) in a 20 μl reaction volume. Reactions were incubated for 30 minutes at room temperature and loaded on 6% 0.5X TBE non-denaturing gels. For competition studies, indicated amounts of molar excess double-stranded mutant probes were included in the binding reactions.

Construction of SILV and MLANA Reporters

A 2.8-kb fragment containing the first exon of *SILV* and flanking sequences was amplified from human genomic DNA (Roche) using primers 5′-GCA ATC CTA TGA CCA CGG CCT CCC AAA G-3′ and 5′-GTT TTC CAT GGA TTC CAC ACC ACC CTA TAC-3′. A smaller fragment was subsequently amplified from the 2.8-kb fragment with primers 5′-CCG TA CCC ACA TAA CTC CAC TCC ATG GAG-3′ and 5′-CCC CAT GGC CAG CTA ATT TTT GTA CTT TTA-3′, digested with *Kpn*I and *Nco*I, and inserted into pGL3-basic vector (Promega, Madison, WI). Subsequently, the splice acceptor of *SILV* exon 4 was amplified with primers 5′-ttC CAT GGc ctc aac ccc caa cta ml-3′ and 5′-ccC CAT GGg gct ccc tga aag ata a-3′ and

inserted into the *Nco*I site. The initiation ATG of luciferase was cloned into the comparable location of the MLANA gene to generate a luciferase reporter including 1.9 kb of the MLANA promoter upstream of the transcriptional start site. MLANA promoter was amplified with primers: 5′-ACT GTT TGG TGG TCT CTG CTG GTC TGA T and 5′-GCT GGC TGG CCG CGT GTA TGA, and inserted into the pCR4-TOPO vector (Invitrogen). The vector was then digested with *Eco*RI, the promoter fragment was filled in with Klenow, and inserted into pGL3-basic digested with *Sma*I.

SILV and MLANA Reporter Construct Mutagenesis

Site-directed mutagenesis was performed using the Quick-Change-method (Stratagene, La Jolla, CA) according to the supplier's recommendations. Mutagenesis primers were designed as follows (using the following sense strands with their corresponding complementary oligonucleotides):

SILV-E1: 5′-CCCAGAGCCCTTT*GAGGTG*ATGCTCAGCT-3′

SILV-E2: 5′-AAATCCGCCTGGA*GAGGTG*AGTGGCCTCT-3′

MLANA-E1: 5′-CAGCACCTAACCACCTCTCACACAACC-AATG-3′

MLANA-E2: 5′-GTTTCCATGTTCACCTCTGAGATATGCC-TCC-3′

Transfection and Dual-Luciferase Reporter Assay

For all reporter assays triplicate transfections of 293 cells were carried out in 24-well plates using Fugene-6 (Roche) according to manufacturer's recommendations. SILV reporter constructs were transfected together with either pRC-CMV/MITF (wt) (encoding full-length M-form human MITF)[57–59] or pcDNA3.1 (Invitrogen) and pRL-null (Promega). A total of 1 μg DNA was used for each transfection. Cell lysates were prepared 48 hours after transfection using 100 μl of passive lysis buffer as per manufacturer's recommendations (Promega). The MLANA promoter-luciferase fragment was cut from pGL3-Basic backbone with *Kpn*I and *Bam*HI, column-purified, and transfected with either pcDNA3/MITF (wt) (encoding full-length M-form human MITF) or pcDNA3.1 (Invitrogen) and pRL-TK (Promega). A total of 0.5 μg of DNA was used for each transfection. Cell lysates were prepared 20 hours after transfection using 100 μl of passive lysis buffer as per manufacturer's recommendations (Promega). All lysates were incubated for 15 minutes at room temperature. 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.

Adenovirus Infection and RNA Preparation

Adenoviruses encoding wild-type MITF, dominant-negative mutant MITF, or vector control encoding a fusion of GFP-wee1 for nuclear localization, were generated as previously described.[57] Briefly, 10^6 human primary melanocytes were plated per 100-mm plate. On the second day, cells were overlaid with 2 ml of serum-free F10 media containing 10 mmol/L $MgCl_2$ and concentrated adenovirus was added at MOI 300. The cells were incubated at 37°C for one-half hour after which virus was removed and fresh full media was added. Total RNA was isolated with RNAqueous-4PCR kit (Ambion, Austin, TX) at 68 and 80 hours after infection according to manufacturer's instructions. SKMEL5 melanoma cells were infected at MOI 100 and the RNA was harvested at 52, 68, and 80 hours post-infection. UACC62 cells were infected at MOI 200 and RNA was harvested at 72 hours post-infection.

Real-Time/Quantitative PCR

The real-time PCR primers for human SILV were 5′-TCT GGG CTG AGC ATT GGG-3′ and 5′AGA CAG TCA CTT CCA TGG TGT GTG-3′. The probe for human SILV was 5′- 6-FAM-CAGGGCAGGGCAATGCTGGGC-TAMRA-3′ (Applied Biosystems, Foster City, CA). The total volume of each reaction was 25 μl including 12.5 μl 2X Master Mix without UNG (uracil-N-glycosylase), 0.625 μl MultiScribe Reverse Transcriptase and RNase inhibitor (Applied Biosystems), 0.5 μl of each primer (10 μmol/L stock), 0.25 μl of the probe (5 μmol/L stock) and 1 μl of the template at 100 ng/μl. Reverse transcription proceeded at 48°C for 30 minutes. Then, 40 cycles of PCR reaction were carried out at 95°C for 15 seconds and at 60°C for 1 minute. Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with analysis using the integrated Sequence Detection System Software Version 1.7. Standard curves were generated for all primer sets to confirm linearity of signals over the experimentally measured ranges.

Northern Analysis

Total RNA was isolated from human UACC-62 melanoma cells using the Trizol reagent (Invitrogen). Ten μg of total RNA was resolved on a 1% agarose gel and blotted onto Hybond-N membrane (Amersham Biosciences) and cross-linked in a GS Gene Linker (Bio-Rad, Hercules, CA). The MLANA probe was prepared by random priming of the full-length reverse transcription-PCR-derived CDS for human MLANA. A probe for human GAPDH was prepared by a restriction digestion of the full-length GAPDH cDNA, followed by random priming of the purified fragment.

Immunoblots

Melanoma cells and adenovirus-infected primary melanocytes were collected and lysed in 2X lysis/loading buffer (125 mmol/L Tris, pH 6.8, 4.6% SDS, 20% glycerol, and 0.04% pyronin Y), resolved by electrophoresis in 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Protran from Schleicher & Schuell, Keene, NH). All proteins were detected using chemiluminescence and antibodies to SILV (anti-PEP13h, a gift from Dr. Vincent Hearing), MLANA (NeoMarkers AB-2, Fremont, CA), MITF (C5), SOX10 (Active Motif, Carlsbad, CA), BRN2 (Santa Cruz Biotechnology, Santa Cruz, CA), CREB/ATF1 (25C10G; Santa Cruz Biotechnology), phospho-CREB/ATF1 (Cell Signaling Technologies, Beverly, MA), or α-tubulin (Sigma).

Preparation of Genomic DNA and Analysis of BRAF Status (V599E Mutation) in Melanoma Cell Lines

Genomic DNA from various melanoma cell lines was isolated with the DNeasy Tissue kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Fragments spanning the T1796A mutation were amplified with primers 5′- AAG CAT CTC ACC TCA TCC TAA CAC ATT T and 5′- CTT TCT AGT AAC TCA GCA GCA TCT CAG G from the genomic DNA. These fragments were subsequently sequenced with primer 5′- ATA GCC TCA TT CTT ACC ATC CAC AAA to determine the mutation status.

Microarray and Pearson Correlation Coefficient Analyses

Expression data on NCI 60 cell lines were collected as described elsewhere.[60] Expression profiling data on 190 human primary tumors were collected using Affymetrix Hu6800 high-density oligonucleotide microarrays as described elsewhere.[61] Expression values were given a lower threshold of 20 units and a ceiling of 16,000 units. The data set was filtered to eliminate genes whose expression levels did not vary more than fivefold or at least 100 absolute units across the data set. Of the 7129 genes on the microarray, 6466 genes passed this filter and were subjected to further analysis. The raw expression value for each gene was normalized to a mean of zero and SD of one across all samples in the data set. Degree of similarity analysis was performed using the Pearson correlation coefficient to identify genes with similar patterns of expression to MITF across the melanoma and non-melanoma collections, resulting in a sorting of the 6466 genes by their degree of correlation.

Results

It has been previously shown that MITF modulates the transcription of several key enzymes in the pigment pathway and might regulate other melanocyte-specific differentiation-associated genes in melanocytes.[62,63] Of the melanocyte-specific melanoma-associated antigens, tyrosinase, TYRP1, and DCT are widely considered to be transcriptional targets of MITF.[2,4,5] Two remaining genes
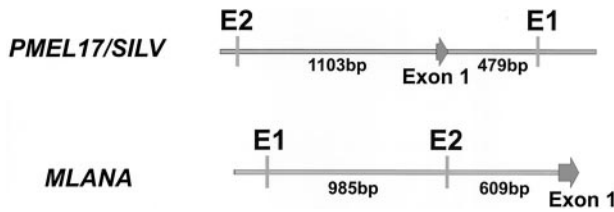
Figure 1. The *SILV* and *MLANA* promoters/enhancers. Initial transcribed exons are denoted by large **arrows** and E-boxes are represented by vertical **bars**.

are *SILV* and *MLANA*, which encode proteins recognized by the widely used diagnostic antibodies, HMB45 and Melan-A, respectively. Based on the role of MITF in the regulation of melanocyte-specific genes, we asked whether MITF might also modulate the expression of these genes.

Promoter/Enhancer Structures of SILV and MLANA

Since MITF modulates its targets through association with E-box elements in their promoters/enhancers (Figure 1), we examined human genome sequences surrounding *SILV* and *MLANA* transcriptional initiation sites. For *SILV*, one E-box (CACGTG) was found 1103 bp upstream its start and a second E-box (CATGTG) was identified in a region within the first intron. This intronic E-box contains a 5′-flanking T which was previously reported to contribute to the recognition of CATGTG by MITF.[64] For *MLANA*, two E-boxes were identified upstream of its transcriptional start site at −609 and −1594 bp, respectively. The distal site matches the consensus CACATG while the proximate site is a canonical (CACGTG) E-box that has been previously reported to be responsible for melanocyte specificity of the promoter, though the responsible transcriptional regulator was not known.[65] Murine homologues of *SILV* and *MLANA* were also examined, and were found to contain E-boxes in similar positions as the human genes (data not shown).

In Vitro *and* in Vivo *Binding of MITF to the* SILV E1 Region and MLANA *Promoter Region*

To examine *in vivo* chromatin occupancy by MITF and help establish the directness of potential transcriptional regulation, chromatin immunoprecipitation (ChIP) was carried out from human melanoma cells (Figure 2). Primers were designed which span the upstream and intronic E-boxes located within the *SILV* transcription initiation region. Immunoprecipitation with antibody against MITF did reveal occupancy by MITF of the E1 enhancer region (SILV enhancer), but not a control region located near the E2 site (Figure 1) located further upstream. Additional specificity controls included no antibody or control polyclonal antibody (directed against GST protein). An antibody directed against acetylated histone H3 represented a positive control in each reaction. For MLANA, ChIP revealed occupancy by MITF of the E-box containing
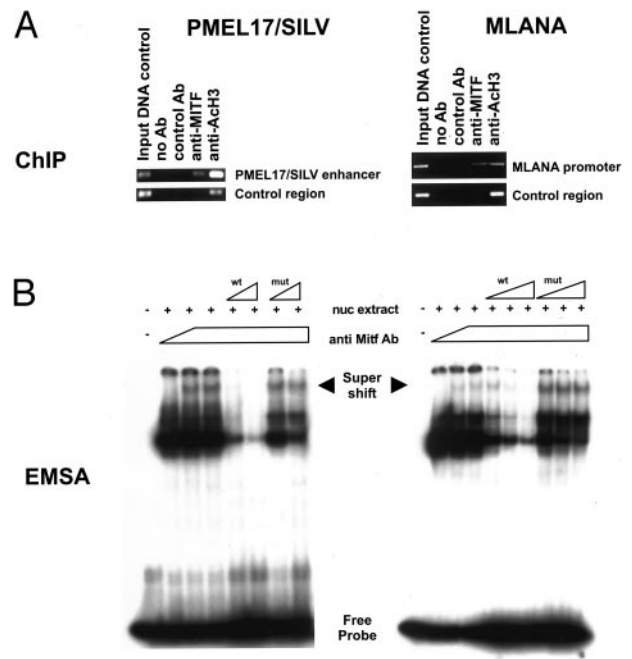

Figure 2. Binding of MITF to SILV enhancer and MLANA promoter *in vitro* and *in vivo*. **A:** Chromatin immunoprecipitations were performed on materials isolated from melanoma cells. DNA from lysates before immunoprecipitation was used as positive input control. The SILV enhancer primer set amplifies the intronic regulatory region which is important for gene expression, while the control primer set amplifies the region containing the upstream E-box which is dispensable for gene expression (negative control), based on reporter assays (see below and data not shown). The MLANA promoter primer set spanning the upstream E-boxes, while the control primer set amplifies a portion of the 3′ UTR (negative control). **B:** EMSA assays were performed on melanoma lysates using probes containing SILV intronic E-box (E1) and MLANA proximate E-box (E2), respectively. Similar binding of MITF was seen at the E1 site of MLANA as well (data not shown).

promoter region. No occupancy was seen for MITF at the 3′ UTR ("control region"), and additional controls included no antibody or comparable polyclonal anti-GST antibodies. Although ChIP does not permit accurate assessments of precise sequence elements being contacted by the transcription factor, it does suggest that MITF is bound to promoter/enhancer elements near the conserved E-boxes for both genes.

To specifically examine DNA binding by MITF of the relevant E-boxes, EMSA assays were performed using DNA probes containing these E-box elements plus melanoma nuclear extracts (as a source of MITF) and a monoclonal anti-MITF antibody (D5) for supershift analysis. Supershifted complexes were observed on addition of wild-type radiolabeled probes to the SILV E1 or MLANA E2 and E1 (data not shown) boxes in the presence of anti-MITF antibody (Figure 2). More importantly, the supershifted bands were specifically competed away with increasing titration of wild-type unlabeled probe, but not with identical titration of point mutated cold probes (Figure 2). Together, these results suggest that MITF directly binds the intronic E-box element in the *SILV* enhancer and the proximate E-box upstream of the *MLANA* transcriptional start site.

Reporter Assays:

PMEL17/SILV point mutants
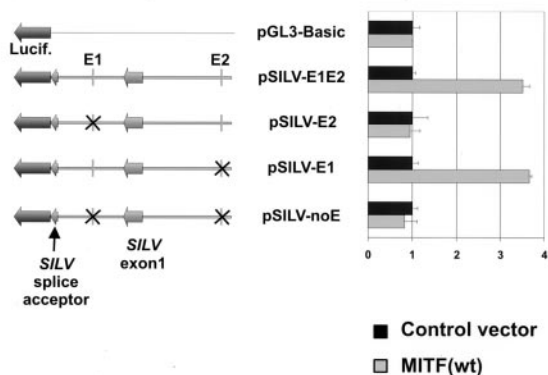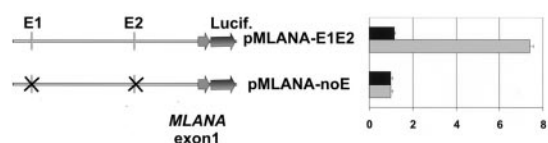

MLANA point mutants

Figure 3. Reporter assays of SILV and MLANA point mutants. SILV and MLANA reporters were transfected into 293 cells. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same specimens. Relative luciferase activities are shown. To recapitulate the putative SILV intronic enhancer, the genomic locus was incorporated into the pGL3 reporter with luciferase placed downstream of a SILV splice acceptor sequence. As described in Results, correct splicing of the reporter transcript was separately confirmed.

E-Box-Dependent Modulation of SILV and MLANA Reporters by MITF

To further test the role of specific promoter/enhancer elements in the regulation of *SILV*, luciferase reporters were constructed which recapitulate the intronic location of the E-box element for its transcription. As shown in Figure 3, the intact genomic region was used as a template for the reporter. Transcription initiation should occur at the *SILV* exon 1 site, and the intron-containing transcript should be spliced to a downstream SILV-derived splice acceptor (which is fused to luciferase). To verify that the template was correctly initiated and spliced, RT-PCR was used together with 5′ RACE of transfected cells. Sequencing of the resulting products confirmed that the luciferase reporter did correctly initiate and splice the luciferase transcript to exon 2 (data not shown). Using this reporter, wild-type MITF was found to stimulate luciferase activity (Figure 3, pSILV-E1E2), and point mutagenesis revealed an essential role for the intronic E-box element (E1) in mediating MITF-dependent transactivation. These data are consistent with the notion that the conserved E1 element may function as an intronic enhancer for MITF-regulated SILV expression.

MLANA promoter activity was also assessed using luciferase reporters. 1.9 kb of the *MLANA* gene upstream of its transcriptional start fused to the luciferase ATG was used in reporter assays. Wild-type MITF significantly up-regulated the wild-type reporter (pMLANA-E1E2) (Figure 3). Point mu-

A. Quantitative RT-PCR for PMEL17/SILV
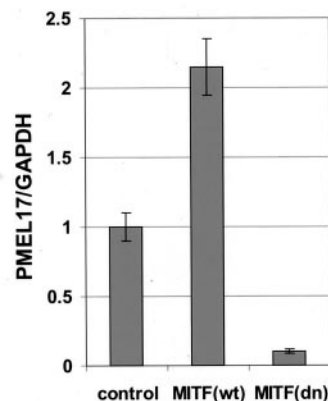
B. Northern for MLANA

Figure 4. Regulation of endogenous SILV and MLANA mRNA levels by MITF. **A:** SILV mRNA levels were assayed after adenovirus infection (**top panel**). Total RNA was isolated from melanoma cells infected with adenoviruses 72 hours after infection. Quantitative RT-PCR was performed and data were normalized to endogenous GAPDH levels. Expression levels for MITF (wt) and MITF (dn)-virus infected samples were normalized to the control samples. **B:** MLANA mRNA levels were assayed after adenovirus infection (**bottom panel**). Total RNA was harvested 72 hours after adenovirus infection and Northern analysis was performed.

tations of both E-boxes completely abolished responsiveness to MITF (Figure 3). These data suggest that the modulation of *MLANA* expression by MITF is E-box-dependent and further supports the directness of this regulation.

Transcriptional Regulation of Endogenous SILV and MLANA by MITF

To investigate whether MITF regulates endogenous SILV and MLANA mRNA levels in the melanocyte lineage, early passage human primary melanocytes and multiple human melanoma cell lines (501mel, SKMEL5, UACC-62 and A375) were infected with adenoviruses overexpressing either control, wild-type MITF or dominant-negative MITF (R218del). The R218 deletion mutant of MITF is derived from a dominantly inherited basic domain mutant mouse allele which preserves dimerization, but ablates DNA binding and thus sequesters wild-type dimerization partners in non-functional heterodimers. This allele has thus been ex-
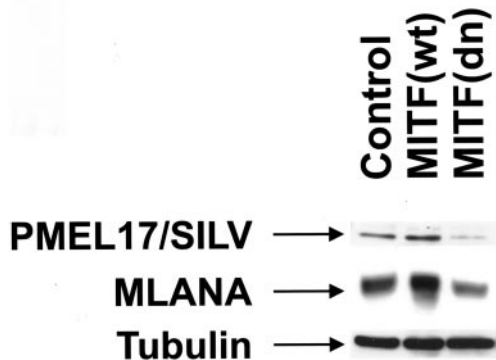
Western Analysis

Figure 5. Regulation of endogenous SILV and MLANA protein levels by MITF. Western analysis was performed on melanoma lysates harvested 72 hours after adenovirus infection.

Figure 6. Correlation between MITF, MLANA, and SILV expression and BRAF mutation status. Western analysis were performed on cell lysates harvested from various melanoma cell lines and human primary melanomas with antibodies specified in Materials and Methods. BRAF mutation status was assessed by PCR and subsequent sequencing on corresponding genomic DNA. Whereas MITF and SILV expression correlated quite closely, no simple correlation was seen between MITF and BRAF mutational state or the various transcriptional regulators shown (which have been suggested to regulate MITF expression in specific contexts).

tensively used as a specific dominant-negative mutant.[2,56,57] Adenoviral infection efficiency approached 100% in these cells, based on GFP signal (data not shown) and was shown to produce strong expression of wild-type or mutant MITF by Western blot (data not shown). Enhanced or disrupted DNA binding by MITF in nuclear extracts occurred by about 48 hours post-infection, lasting until at least 96 hours post-infection, as measured by EMSA analyses (data not shown). Total RNA was isolated after 72 hours. Real-time (quantitative) PCR was performed for SILV on the RNA samples. Wild-type MITF significantly stimulated SILV mRNA expression while dominant-negative MITF suppressed SILV mRNA levels (Figure 4A). MLANA expression was examined by Northern blot analysis (due to unknown complications with quantitative PCR). Wild-type MITF up-regulated, while dominant-negative MITF suppressed MLANA mRNA levels (Figure 4B) relative to vector control adenovirus (normalized to GAPDH as a control).

Protein levels of SILV and MLANA were also examined following up- or down-regulation of endogenous MITF using adenoviruses, as above. Western blotting for SILV and MLANA (Figure 5) revealed increases of both in the presence of wild-type MITF and decreases of both in the presence of dominant-negative MITF. These data are consistent with the hypothesis that MITF regulates expression of SILV and MLANA in the melanocyte lineage.

Comparison of MITF, SILV, and MLANA Expression in Cultured Melanomas and Melanocytes

Next, we examined protein levels of these genes in a panel of 11 human melanoma cell lines as well as primary early passage human melanocytes. As shown in Figure 6, a general pattern became apparent when comparing expression of MLANA, SILV, and MITF. A relatively clear pattern of correlated expression was seen for MITF, SILV, and MLANA in these specimens.

Overall, considerable variability was seen in MITF expression levels among the various cell sources. Yet, MITF
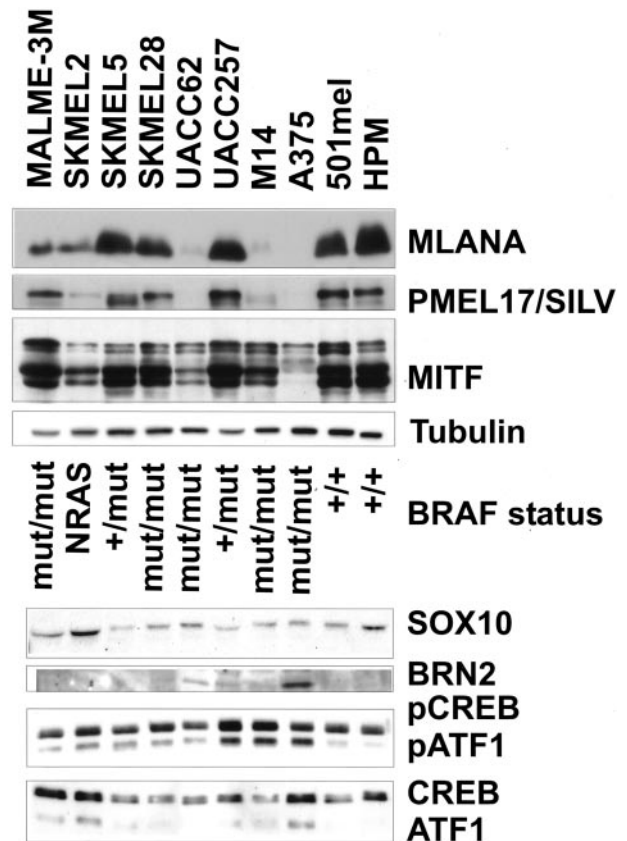
is clearly expressed in all of these samples, including A375, which contains significantly less visible protein by Western blot. To determine whether other potential upstream factors may similarly correlate with the pattern of MITF, SILV, and MLANA expression (and therefore be candidate regulators of MITF), we examined the expression of several transcription factors that have been previously demonstrated to modulate MITF expression. SOX10 binds multiple sites in the MITF promoter in melanocytes, and its mutation in humans is associated with a similar pigmentation/deafness phenotype as MITF deficiency.[9,66–68] The BRN2 transcription factor has been suggested to regulate or interact with MITF.[69,70] CREB/ATF1 have been shown to modulate MITF expression in melanocytes through recognition of a consensus CRE element, in response to cAMP signaling which is downstream of the melanocyte stimulating hormone.[71–73] As shown in the bottom panel of Figure 6, the expression levels of these factors did not correlate in a simple fashion with MITF or the other melanocytic markers.

In addition, post-translational modifications of MITF have also been observed. One of these is mediated by the MAP-kinase pathway, which leads to the phosphorylation of

Ser73 in MITF by ERK1 and ERK2.[58] This post-translational modification leads to recruitment of the p300 coactivator and concomitant degradation of MITF protein.[57] Recently, it was found that oncogenic mutant BRAF(V599E) occurs in the majority of human melanomas studied.[74] Since BRAF activates the MAP kinase pathway, it might modulate MITF expression (either post-translationally or possibly even transcriptionally by unknown intermediates). This prompted us to analyze the mutational frequency of BRAF in the panel of melanomas and see whether this could explain the varied levels of MITF protein found (Figure 6, middle). A weak trend suggested that most abundant expression of MITF, MLANA, and SILV occurs in the setting of wild-type BRAF, but expression was otherwise variable and did not correlate clearly with BRAF status. Quantitative RNA measurements similarly demonstrated no simple correlation between BRAF mutational status and MITF mRNA levels (data not shown). Thus, no simple relationship emerged from these studies of other transcription factors and regulatory pathways that might explain the variable expression of MITF, SILV, and MLANA among melanomas.

Analysis of SILV, MLANA and MITF Expression in Tumor Microarrays

Several of the cell lines used in our analysis of protein levels are part of an NCI database on which transcriptional profiling has been performed.[60] Since protein expression patterns reflect both transcriptional and post-transcriptional regulation, we sought verification at the mRNA level that MITF, SILV, and MLANA expression also correlated. Indeed, it has been demonstrated that at least one mechanism which likely confers amelanotic (unpigmented) phenotype to certain melanomas is the post-translational degradation of pigmentation factors, specifically tyrosinase.[15] As shown in Figure 7A, the levels of MITF, SILV, and MLANA fluctuate together in a similar pattern among the cell lines available for analysis.

Finally, we wished to examine whether mRNA expression levels of MITF, SILV, and MLANA correlate within primary clinical melanoma specimens (rather than cultured cell lines which have been subjected to selective pressures of unknown clinical relevance). We therefore examined microarray expression profile data from a large unbiased data set of primary human cancers, including a series of 10 melanoma clinical specimens.[61] To find genes highly correlated with endogenous MITF expression in melanomas, we identified the genes best matching the MITF expression pattern using a Pearson correlation coefficient ranking of similarity.[75] Of the 6466 genes analyzed, both *SILV* and *MLANA* were among the top 20 genes correlating with levels of MITF in the primary melanomas of this series (Figure 7). Importantly, neither gene was significantly correlated with MITF expression in non-melanoma tumors due to lack of expression of SILV and MLANA (data not shown). Although this correlation does not prove a functional relationship between these factors, it utilizes a human primary tumor data set to help validate the model that MITF transcriptionally regulates the *SILV* and *MLANA* genes.
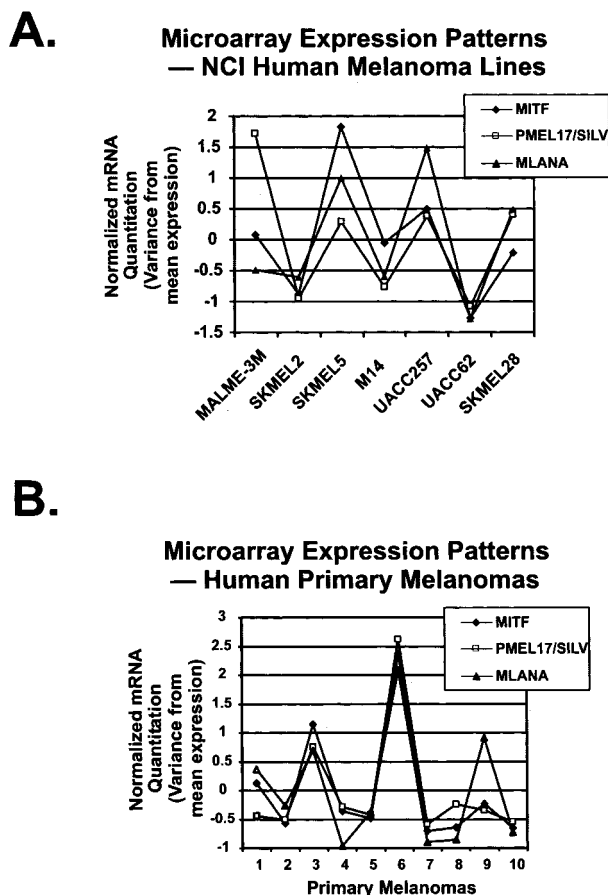

Figure 7. Correlations between MITF, SILV, and MLANA mRNA levels in cultured melanoma cell lines and human primary melanomas. NCI human melanoma cell lines (**A**) and human primary melanomas (**B**). Quantitative mRNA levels for all three genes were acquired from Affymetrix microarrays as described in Materials and Methods, and deviations from mean expression are plotted across each series.

Discussion

The results of the present work demonstrate that the essential melanocyte-specific transcription factor MITF regulates expression of the genes encoding the melanoma tumor markers MLANA and SILV. Evidence in support of this model includes the presence of conserved MITF consensus DNA binding elements within enhancer/promoter elements of both genes that are shown to be bound by endogenous MITF protein in melanoma cells. Furthermore MITF regulates luciferase reporters for these genes in a manner dependent on those DNA elements. MITF up- or down-regulation is seen to correspondingly modulate expression of MLANA and SILV in parallel directions, at both mRNA and protein levels. Across several melanoma cell lines, MITF, SILV, and MLANA correlate in expression at the protein level. Finally, expression microarray data reveal that MITF, MLANA, and SILV behave similarly in cultured cell lines as well as in primary melanoma specimens.

The *MITF* gene contains multiple alternative promoters which are capable of contributing unique initial coding exons to the common body of the gene. One of the alternative promoters is melanocyte-specific and subject

to MSH/cAMP regulation.[71–73] However, isoforms expressed from alternative promoters are present in other tissues, including osteoclasts and mast cells. As melanocyte-restricted target genes, MLANA and SILV will likely provide an opportunity to study whether their melanocyte restricted expression arises through unique activity of the melanocyte isoform of MITF or whether other transcription factors might contribute (together with MITF) to confer melanocyte-specific expression.

Levels of the Three Major Melanoma Markers Are Tightly Correlated with Each Other

Despite the generally correlated protein expression levels for MLANA, SILV, MITF, no clear transcriptional regulator upstream of MITF expression could be identified on the basis of matching expression levels. For certain transcription factors, key post-translational events (such as phosphorylation of CREB) may modulate activity more than expression levels. Another important possibility is that MITF is transcriptionally regulated in a combinatorial fashion by multiple upstream regulators, whose correlations with MITF expression would thus appear complex. This model is particularly attractive as a means of understanding how ubiquitous factors, such as CREB, can regulate tissue-restricted target genes. Through cooperative linkages with other upstream regulators at the MITF promoter, such combinatorial networks might produce highly restricted target gene expression, using relatively ubiquitous upstream factors.

We are also interested in exceptions to the simple correlations in MITF, SILV, and MLANA protein expression patterns, and have found a single melanoma cell line (not among the NCI cancer cell data set) for which MLANA expression appears under-represented relative to MITF and SILV (data not shown). Multiple models could account for this discrepancy, including the possibility that transcriptional regulators other than MITF may also be rate-limiting in specific contexts. Alternatively post-translational differences could account for such discrepancies. For example, Halaban and colleagues[15] have previously examined the mechanism underlying lack of pigment production in amelanotic melanomas, and discovered aberrant tyrosinase protein degradation. It is plausible that components of the pigmentation machinery as well as byproducts of pigmentation chemistry may be toxic to melanoma cells and therefore a selective pressure may exist to disrupt components of the pathway in selected tumors. Of note, previous studies have demonstrated that oncogenic transformation associated with dysregulation of E2F may be connected to down-regulation of MITF.[76] In fact, these studies led to the previous suggestion that MITF may transcriptionally regulate the SILV gene, as is mechanistically demonstrated here.

Melanoma Markers and Prognosis

It has been observed that low levels of MLANA and MITF correlate with a worse prognosis.[17,37,53–55] It is likely that in many of these tumors there is not a selective down-

regulation of a single antigen, but rather a coordinated decrease of many target genes, governed largely by MITF and its central regulation of pigmentation as the most observable of differentiation pathways. Thus, higher levels of antigen expression represent a more differentiated state, while lower levels represent a less differentiated state. Such regulation could be of potential therapeutic value if MITF expression were modulated.

Modulating Antigen Expression via MITF

Notably absent from the literature are cases of melanoma antigen up-regulation in metastatic disease. The trend appears to be toward an expected state of a less differentiated phenotype with loss of many markers of differentiation. While previous work has previously linked MITF to the regulation of pigment genes and work presented here shows MITF regulation of SILV and MLANA, we have shown in an earlier study that MITF transcriptionally modulates the apoptosis modulator, Bcl-2. In addition, suppression of MITF through a dominant-negative mutant triggers apoptosis in melanoma cells while overexpression of Bcl-2 at least partially rescues this effect.[56] Bcl-2 expression also correlates closely with MITF in primary melanoma expression profiles.[56] Therefore, the worsened prognosis which has been suggested for melanomas expressing lower levels of MITF, occurs despite lower expression of the anti-apoptotic Bcl-2 factor. One model to explain this observation is that lower levels of MITF expression might select for tumor cells that are less differentiated (rather than expressing higher levels of survival genes). Additional studies will be needed to examine this question. Even though MITF expression fluctuates among melanomas, some level of staining is retained in nearly all melanomas and has been demonstrated to be a specific and sensitive diagnostic marker. Together, these observations underlie MITF's apparent dual roles in regulating both survival and differentiation in the melanocyte lineage.

The cytokine α-MSH up-regulates MITF expression via a well-studied signal transduction pathway that also leads to increased pigment enzyme expression.[71–73] This pathway would therefore be expected to up-regulate MLANA and SILV expression as well. The combination of α-MSH treatment with immunotherapy directed against antigens such as MLANA and SILV could potentially lead to therapeutic benefit both by increasing tumor cell differentiation and increasing the expression of melanoma-associated antigens that have been associated with therapeutically meaningful responses.

Acknowledgments

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