THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 1, Issue of January 7, pp. 146–155, 2005
Printed in U.S.A.

Sumoylation of MITF and Its Related Family Members TFE3 and TFEB*

Received for publication, October 15, 2004
Published, JBC Papers in Press, October 25, 2004, DOI 10.1074/jbc.M411757200

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MITF and its related family members TFE3 and TFEB heterodimerize with each other, recognize the same DNA sequences, and are subject to many of the same post-translational modifications. We show that lysine residues within conserved small ubiquitin-like modifier (SUMO) consensus sites in these family members are subject to SUMO modification. Mutation of these sites significantly affects the transcriptional activity of MITF but does not alter dimerization, DNA binding, stability, or nuclear localization. Mutagenesis reducing the number of MITF binding sites in the promoter of TRPM1 from three to one eliminated the difference in transcriptional activity between the MITF mutants. Among other MITF target gene promoter constructs, differences in transcriptional activity between wild type and non-sumoylatable MITF were only seen in promoters with multiple MITF binding sites. These data support a synergy control model in which the functional consequences of MITF sumoylation depend on promoter context. Sumoylation, thus, provides a possible mechanism for altering the effects of MITF by affecting the target genes that it activates.

MITF is a tissue-restricted, basic helix-loop-helix leucine zipper dimeric transcription factor. It is encoded by the *mitf* locus in mice (1) and when mutated leads to defects in melanocytes, the retinal pigment epithelium, mast cells, and osteosclasts. *Mitf* mutant mice are white due to a complete lack of melanocytes, whereas heterozygotes have a white belly spot (1, 2), demonstrating a requirement for *mitf* in production of this lineage. MITF continues to be necessary in the adult based on the existence of hypomorphic alleles in mice which cause postnatal melanocyte death and premature graying (3, 4). As a transcriptional mediator of differentiation, MITF acts downstream of the melanizing hormone α-melanocyte-stimulating hormone (5) and transcriptionally regulates the expression of the enzymes necessary for melanin production in differentiated melanocytes (for review, see Ref. 6). Although these data implicate MITF in both the survival and differentiation of melanocytes, little is known about biochemical regulatory pathways that control MITF in these different roles.

MITF is part of the MiT transcription factor family whose members share significant homology and recognize the same DNA elements. Functionally, MITF binds to the canonical E-box promoter sequence CACGTG as well as to the non-palindromic sequence CACATG (7, 8). MITF functions as either a homodimer or as a heterodimer with the related MiT family transcription factors TFE3, TFEB, and TFEC (62). The related factor TFEB was recently identified as a translocated oncogene in papillary renal cell carcinoma in humans (10, 11). The structural features of these family members are so similar that MITF and TFE3 have been shown to genetically compensate for one another in regulation of osteoclast development in mice (12).

Several post-translational modifications affect members of the MiT family. In melanocytes, activation of the mitogen-activated protein kinase pathway by c-Kit leads to phosphorylation of MITF (9), triggering recruitment of the co-activator p300 (13) as well as ubiquitination and degradation of MITF (14). In osteosclasts, macrophage colony-stimulating factor signaling initiates a parallel pathway resulting in mitogen-activated protein kinase-mediated phosphorylation of both MITF and TFE3 at a conserved serine, thereby triggering recruitment of p300 (15). Other post-translational modifications that have been shown or suggested to modify MITF function include phosphorylation at serine 298 (16) and phosphorylation by p38 in osteoclasts (17). In addition MITF has been shown to be functionally repressed by the action of PIAS3 (18, 19), a component of the STAT transcriptional network that has also been implicated in the pathway of post-translational modification by SUMO[1] (20–22).

Sumoylation is the post-translational modification of proteins by the addition of the *s*mall ubiquitin-like-modifier (SUMO) (for review, see Refs. 23–25). SUMO-1 is added to substrates at lysine residues in a consensus sequence ΨKXE (26), utilizing a conjugation system analogous to ubiquitination. The E1 enzyme in this pathway is the Aos1/Uba2 heterodimer (27), the E2 enzyme is UBC9 (28), and the E3 enzymes include members of the PIAS family (29) or RanBP2 (30). There are several models describing the functional consequences of SUMO modification; it may compete with ubiquitination and affect protein stability (31), alter subcellular localization (32, 33), affect localization to pro-myelocytic leukemia

* This work was funded in part by National Institutes of Health Grants AR45662 and AR43369 (to D. E. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a National Institutes of Health Medical Scientist Training Program grant.

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[1] The abbreviations used are: SUMO, small ubiquitin-like modifier; HA, hemagglutinin; PBS, phosphate-buffered saline; Ni-NTA, nickel nitrilotriacetic acid; wt, wild type; HEK cells, human embryonic kidney cells; STAT, signal transducers and activators of transcription; C/EBP, CCAAT/enhancer-binding protein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PIAS, protein inhibitor of the activated STAT (signal transducers and activators of transcription)); E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; PML, promyelocytic leukemia protein.

protein (PML) nuclear bodies (34), or affect transcriptional activity (35–37) (for review, see Ref. 38).

In several transcription factors, including glucocorticoid receptor, androgen receptor, C/EBPα, C/EBPϵ, c-Myb, and SP3, the consensus site for sumoylation (ΨKXE) has also been identified as part of a region that negatively regulates function (39). Further analysis of this transcriptional repression by SUMO in several systems has led to the suggestion that sumoylation functions in synergy control (39–41). In this model, sumoylation of a transcription factor represses transcriptional activity at promoters with multiple binding sites (for review, see Ref. 42). In one model system using glucocorticoid receptor, mutations of several residues in a motif nearly identical to the sumoylation consensus site led to greater transcriptional activity in the mutants. Intriguingly, this effect was only apparent on promoter constructs that harbored multiple glucocorticoid response elements (41). Later work involving C/EBPα showed similar results but also suggested that this effect on synergy indeed correlates with sumoylation at the lysine residue within the synergy control motif (43).

Alignment of the MITF amino acid sequence revealed the presence of two conserved sumoylation consensus sites in the mouse and human homologues. Because MITF has also been shown to interact with UBC9 (44) and PIAS3 (18, 19), two components of the sumoylation pathway, we explored whether MITF is a substrate for sumoylation. MITF was indeed found to be subject to SUMO modification, as were the related family members TFE3 and TFEB. Mutations affecting sumoylation had significant functional consequences on transcriptional activity of MITF, which did not appear to influence dimerization, DNA binding, stability, or nuclear localization. Instead, evidence was obtained suggesting that the difference in transcriptional activity among the MITF mutants depends on the number of MITF binding sites within the promoter, fitting a synergy control model (39, 41, 43). These findings identify sumoylation as a post-translational MITF modification that affects MITF transcriptional activity in a manner dependent on the promoter elements present in MITF target genes.

EXPERIMENTAL PROCEDURES

Plasmids—The M-form MITF constructs pcDNA4HisMax-MITF (His-MITF) and pcDNA3.1HA-MITF (HA-MITF) have been described previously (45). Mutations of MITF at K182R and/or K316R were generated using the QuikChange method (Stratagene) using the following sense primers with their reverse complements: hsMITF_ K182R_S, 5′-CTT CCC AAC ATA AGA AGG GAG CTC ACA G; hsMITF_K316R_: 5′-GAA TCG GAT CAT CAG GCA AGA ACC CGT TC.

The full-length human TFE3 was PCR-amplified from cDNA from day 25 primary human osteoclasts grown from normal human bone marrow using *Pfu* polymerase and GC-melt with the following primers containing engineered 5′ BamHI and 3′ EcoRV sites: 5′hTFE3 FL-ATG BamHI primer, 5′ GAG GAT CCA ATG TCT CAT GCG GCC GAA CCA GCT CGG GAT GGC; 3′hTFE3 FL-STOP EcoRV primer, 5′-CGG ATA TCT CAG GAC TCC TCT TCC ATG CTG AAG CTG CTG CGG. The human TFE3 PCR product was reamplified with *Pfu* Polymerase and GC-melt with the same primers, and the purified product was cloned into pcDNA3.1(+)-Kozak-HAHA vector (46) using the BamHI and EcoRV sites.

To create a plasmid encoding TFEB, cDNA was generated from primary human papillary renal cell carcinoma cell mRNA through reverse transcription (Superscript) after priming with oligo(dT). A fragment encoding the C-terminal portion of TFEB was isolated by PCR using a primer that incorporated an EcoRI site and sequence directing expression of the hemagglutinin epitope tag, 5′-GAC TCC TAG GTC AGG CGT AGT CGG GCA CGT CGT AGG GGT ACA GCA CAT CGC CCT CCT C, and an internal primer specific for TFEB, 5′-CAG TGA ATT CCC TGA ATG TGT ACA GCG ACC. The N-terminal fragment was generated by PCR with a primer specific for the beginning of exon 2 of TFEB, 5′ CAG TGA ATT CGG AGC CAG CGC CGG CAG CC, and an internal reverse primer, 5′-CTC CTA GGC CCT TGT TCC AGC GCA CGT CC. The PCR products were cloned and sequenced. Fragments were isolated by digestion with EcoRI and SacI or SacI and BamHI, respectively, then ligated into the EcoRI and BamHI sites of pCDNA3.1(−).

HA-SUMO-1 was a gift of Len Zon and has been described elsewhere (47). His-SUMO was generated by PCR subcloning full-length human SUMO-1 into pcDNA4.1HisMax using primers containing engineered 5′ BamHI and 3′ EcoRV sites: 5′hSUMO-1 BamHI primer, 5′-GGA TCC GAT GTC TGA CCA GGA GGC AAA ACC; 3′hSUMO-1 EcoRV primer, 3′-GAT ATC TAA ACT GTT GAA TGA CCC CCC GTT. HAHA-SUMO-1Δ GG-MITF is a fusion of SUMO-1ΔGG at the N terminus of hsMITF produced by ligating PCR fragments of HA-SUMO-1ΔGG generated using primers containing engineered BamHI and XbaI sites (5′hSUMO-1 BamHI primer 5′-GGA TCC GAT GTC TGA CCA GGA GGC AAA ACC and 3′hSUMO-1Δ GG XbaI 3′-TCT AGA CGT TTG TTC CTG ATA AAC TTC AAT) and hsMITF amplified with primers containing engineered XbaI and EcoRI sites (5′ hMITF XbaI primer 5′-TCT AGA ATG CTG GAA ATG CTA GAA TAT AAT and 3′ hMITF EcoRI primer 3′-GAA TTC CTA ACA AGT GTG CTC CGT CTC TTC). The resulting SUMO-1 and wild type MITF PCR products were ligated and cloned into the BamHI and EcoRI sites of pcDNA3.1(+)-Kozak-HAHA to produce SUMO-MITF.

PIAS1 was amplified from a human primary melanocyte cDNA library using primers engineered to contain EcoRI and XhoI sites: 5′-hPIAS1 EcoRI, 5′GCG AAT TCT GAT GGC AGT ATA TCT TGT AAA, and 3′-hPIAS1 XhoI, 3′ CCG CTC GAG TCA GTC CAA TGA AAT AAT GTC. The PCR product was cloned into pcDNA3.1. FLAG-PIAS3 has been described elsewhere (18).

The MITF-responsive reporter plasmids pTRPM1 (45), SILV promoter (pSILV) (48), cathepsin K (pCATHK) (49), and tyrosinase promoter (pTyr) (50) have been described previously. The plasmid pBABE-puro has been described previously (51). HA-tagged or His-tagged ubiquitin expression vectors were a gift from Dr. Dirk Bohmann, European Molecular Biology Laboratory, Heidelberg, Germany. All constructs were verified by automated sequencing.

Stable Lines—B16 melanoma grown in 10-cm dishes were transfected with 1 μg of pBabe-puro and 9 μg of His-SUMO-1 (His-SUMO lines) or 9 μg of pcDNA4.1HisMaxLacZ (Invitrogen) (Control cell lines) using FuGENE 6™ (Roche Applied Science) according to the manufacturer's recommendations. 48 h after transfection, cells were split 1:10 into media containing 2.0 μg/ml puromycin. Cells grew under selection for 14 days, and 24 single colonies of each line were isolated. 12 clones were assayed for SUMO-1 overexpression by comparison to control cell lines via Western blot using anti-SUMO-1 antibodies (Santa Cruz sc-9806).

Reporter Assays—B16 melanoma, COS7, and 293 cells were grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Sigma) plus penicillin/streptomycin/glutamine (Invitrogen) and transiently transfected using FuGENE 6™ (Roche Applied Science) according to the manufacturer's recommendations. For all reporter assays, cells were plated into 24-well tissue culture plates. 24 h later triplicate transfections were performed with the indicated amounts of pTRPM1, pCATHK, pSILV, or pTYR luciferase reporter plasmid, 100 ng of pRL-Null Renilla control reporter plasmid, and the indicated amounts of wild type or mutant His-MITF, HA-SUMO, or pcDNA3 control. Total amounts of DNA were kept constant in individual experiments, as indicated. 36 h post-transfection cells were lysed in passive lysis buffer for 20 min at room temperature, and an aliquot was used for reporter assays using the dual luciferase assay kit (Promega). Luciferase activity was normalized to the *Renilla* activity, and all assays were performed on three occasions.

Co-transfections and Immunoblots—COS-7 or HEK293 cells grown in 6-well dishes were transfected with the indicated plasmids (2 μg of total DNA/well). 48 hours later, cells were harvested. Whole cell lysates were prepared by rinsing cells in room temperature PBS followed by lysis in 95 °C in 2× lysis/loading buffer (125 mM Tris, pH 6.8, 4.6% SDS, 20% glycerol, and 0.04% pyronin Y) and boiling an additional 5 min. Denaturing lysis/immunoprecipitation was carried out according to previously published methods (52). Lysates were prepared for affinity pull-down assays with Ni-NTA-agarose beads (Qiagen) in denaturing conditions by lysis in 8 M urea lysis buffer (8 M urea, 50 mM sodium phosphate, pH 8.3), passage through a 25-gauge needle 5 times to shear DNA, and clearing by 5 min of centrifugation at 14,000 rpm. His-tagged proteins were purified by 1 h of incubation with Ni-NTA resin at room temperature followed by 5 washes in 8 M urea wash buffer (8 M urea, 50 mM sodium phosphate, pH 6.3), elution with 8 M urea elution buffer (8 M urea, 50 mM sodium phosphate, pH 4.3), and boiling in 6× SDS sample buffer (125 mM Tris, pH 6.8, 30% glycerol, 10% SDS, 0.6 M dithiothreitol, and0.012% bromphenol blue). Lysates were prepared for native affinity pull down assays with Ni-NTA-agarose beads by cooling

cells on ice, washing twice in ice-cold PBS, lysing in cold native lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 1% Triton X-100, pH 8.0, with protease inhibitors (Roche Applied Science)), passage through a 25-gauge needle 5 times to shear DNA, and clearing by 5 min of centrifugation at 14,000 rpm at 4 °C. His-tagged proteins were purified by 1 h of incubation with Ni-NTA resin at 4 °C followed by 5 washes in wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, 1% Triton X-100, pH 8.0), elution with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0), and boiling in 6× SDS sample buffer.

Proteins were resolved by electrophoresis in 7.5% SDS-polyacrylamide gels or 4–20% gradient gels and transferred to nitrocellulose membranes (Protran® from Schleicher & Schuell). All proteins were detected using chemiluminescence (PerkinElmer Life Sciences) and antibodies to MITF (C5), HA epitope (Roche Applied Science), or α -tubulin (Sigma).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as previously described (14) from COS-7 cells transfected with pcDNA3.1HAHA empty vector (control) or pcDNA3.1-MITF wt, MITF K182R/K316R, or SUMO-1-MITF. Electrophoretic mobility shift assays were otherwise performed as previously described (45).

Immunofluorescence—COS-7 or 293 cells were grown in 6-well dishes and transfected with 100 ng of MITF plasmid and 900 ng of pcDNA using FuGENE 6™ (Roche Applied Science) according to the manufacturer's recommendations. 24 h later cells were replated onto coverslips in 24-well plates. 24 h later cells were fixed for 10 min with 4% formaldehyde in PBS, washed in PBS, and permeabilized for 5 min with 0.2% Triton X-100 in PBS. Next, samples were washed 3 times in PBS for 5 min, blocked for 1 h in blocking buffer (1% bovine serum albumin, 10% fetal bovine serum, PBS), washed once with PBS, and stained overnight with monoclonal anti-MITF antibody (C5) in 1% bovine serum albumin/PBS. Samples were then washed 3 times in PBS for 5 min and stained secondarily with Alexa-488 conjugated goat-anti-mouse antibody (Molecular Probes) in blocking buffer. Cells were washed once in PBS, counterstained with 4,6-diamidino-2-phenylindole, washed 4 times in PBS for 5 min, and mounted using Fluoromount-G (Southern Biotech, Birmingham AL). Microscopy was performed at a 630× total magnification using a Zeiss Axioplan 2, and imaging was performed with a Hamamatsu ORCA-ER camera and Openlab 3.l software.

MITF Degradation and Ubiquitination—293 or COS7 cells were transfected in 10-cm dishes with 5 μg of His-MITF wild type and 5 μg of pcDNA3, 5 μg of His-MITF K182R/K316R and 5 μg of pcDNA3, or 5 μg of His-MITF and 5 μg of HA-SUMO-1 using FuGENE 6™ (Roche Applied Science) according to the manufacturer's recommendations. 24 h after transfection cells were replated into six-well dishes. After a further 24 h, cells were treated with cyclohexamide for 15 min before the addition of TPA (100 nm) at the indicated time points.

COS7 cells grown in 6-well dishes were transfected with 1 μg of HA-MITF wild type or K182R/K316R and 1 μg of His-ubiquitin. Cells were lysed under denaturing conditions, and His-tagged proteins were precipitated using Ni-NTA as described previously.

RESULTS

MITF Is Modified by SUMO-1 in Vivo—When the melanocyte (M) forms of MITF and SUMO-1 were overexpressed in COS-7 cells, additional forms were observed at molecular masses of ~20 and ~40 kDa greater than expected in addition to the regular doublet of MITF (due to phosphorylation by mitogen-activated protein kinase at serine 73 (9)) typically seen (data not shown). When cells were lysed in SDS lysis buffer at 95 °C and immediately boiled, these higher molecular mass forms were much more abundant. Because these cells do not express the A-form of MITF, which is also larger than the transfected M-form of MITF, these bands apparently represent altered mobility forms of M-MITF. To demonstrate that these forms result specifically from sumoylation, we adopted the approach of using His-tagged proteins, which can be affinity-purified under denaturing conditions using Ni-NTA resins and 8 M urea, which has previously been used to stabilize SUMO modifications (53, 54). Using His-tagged MITF and HA-SUMO-1, the same higher molecular mass species of MITF as seen in hot SDS lysates were apparent in Ni-NTA affinity purifications of His-tagged proteins (*single* and *double asterisks* in Fig. 1*A*). To further verify that this aberrant mobility occurred because of sumoylation, we reversed the tags and utilized His-SUMO-1 and HA-MITF. Similar complexes were present in the lysates from such transfections, and when His-SUMO-1-modified proteins were isolated by Ni-NTA affinity chromatography, upper bands with ~20- and ~40-kDa slower mobility than wild type MITF were detected by blotting for MITF (Fig. 1*A*). Similar results were also seen in HEK293 cells (data not shown). This result demonstrates the presence of covalent SUMO modification of MITF under conditions of co-overexpression.

We next sought to determine whether SUMO-1 modification occurs on endogenous MITF present in melanoma cells. We utilized denaturing lysis in SDS followed by dilution in radioimmune precipitation assay buffer and immunoprecipitation, as previously described (53). Using this approach, we detected forms of MITF that could be consistent with SUMO-1 modifications (Fig. 1*B*). Positions of the M-MITF and A-MITF isoforms of MITF were surmised by comparisons to recombinant expression and lysates of cells known to express these isoforms (9, 13, 15). In addition to the M-form (*M*) and A-form of MITF (*A*), several higher molecular MITF-containing species are also indicated as *X* and *XX*, which migrate at positions that would be consistent with SUMO modification. However, because these bands were identified only with antibodies to MITF, the bands could represent other modifications. Unfortunately, attempts to utilize antibodies against SUMO were unsuccessful due to nonrecognition of positive controls. Therefore, to better demonstrate SUMO-1 modification of endogenous MITF, we engineered melanoma cell lines stably expressing His-SUMO-1. Several individual clones were isolated and compared for SUMO-1 expression (data not shown). After purification of His-SUMO-1-modified proteins using Ni-NTA resin under denaturing conditions, Western blotting for MITF showed higher mobility MITF forms at similar molecular masses as those in COS-7 cells transfected with His-SUMO and HA-MITF (*single* and *double asterisks*, Fig. 1*C*), which are also of higher molecular mass than the endogenous A form present in the melanoma cell line. This result is consistent with the possibility that endogenous MITF is indeed subject to SUMO modification within melanoma cells.

Examination of the amino acid sequences of mouse and human MITF showed the presence of two conserved SUMO-1 consensus sites (ΨK*X*E) (26) at lysines 182 and 316 in the human sequence. Using site-directed mutagenesis, Lys-182 and Lys-316 were conservatively replaced by arginine residues to determine whether SUMO-1 modification indeed occurs at these residues. Whereas two SUMO-1-modified forms were readily apparent in COS-7 cells transfected with wild type MITF, only one modified form was seen in cells transfected with MITF mutated at either K182R or K316R. Mutation of both lysines (MITF K182R/K316R) prevented detectable SUMO-1 modification (Fig. 1*D*), thus localizing the site of MITF SUMO modification to these two residues. Moreover, mutation of Lys-316 more strongly abrogates SUMO modification, suggesting that Lys-316 is the more frequently SUMO-modified residue on MITF.

Other MiT Family Members Are Also Modified by SUMO-1—Sequence alignment of MITF and its closely related family members, TFEB and TFE3, shows preservation of one ΨK*X*E site at similar positions relative to the basic-helix-loop-helix of MITF. TFE3 preserves the site analogous to K182 in human MITF, whereas TFEB preserves the site analogous to K316 in human MITF (Fig. 2*A*). Coexpression of HA-TFEB or HA-TFE3 in COS-7 cells with His-SUMO-1 showed that high mobility forms of both MiT family members are obtained by Ni-NTA resin purification of His-SUMO-1-modified proteins (Fig. 2, *B*

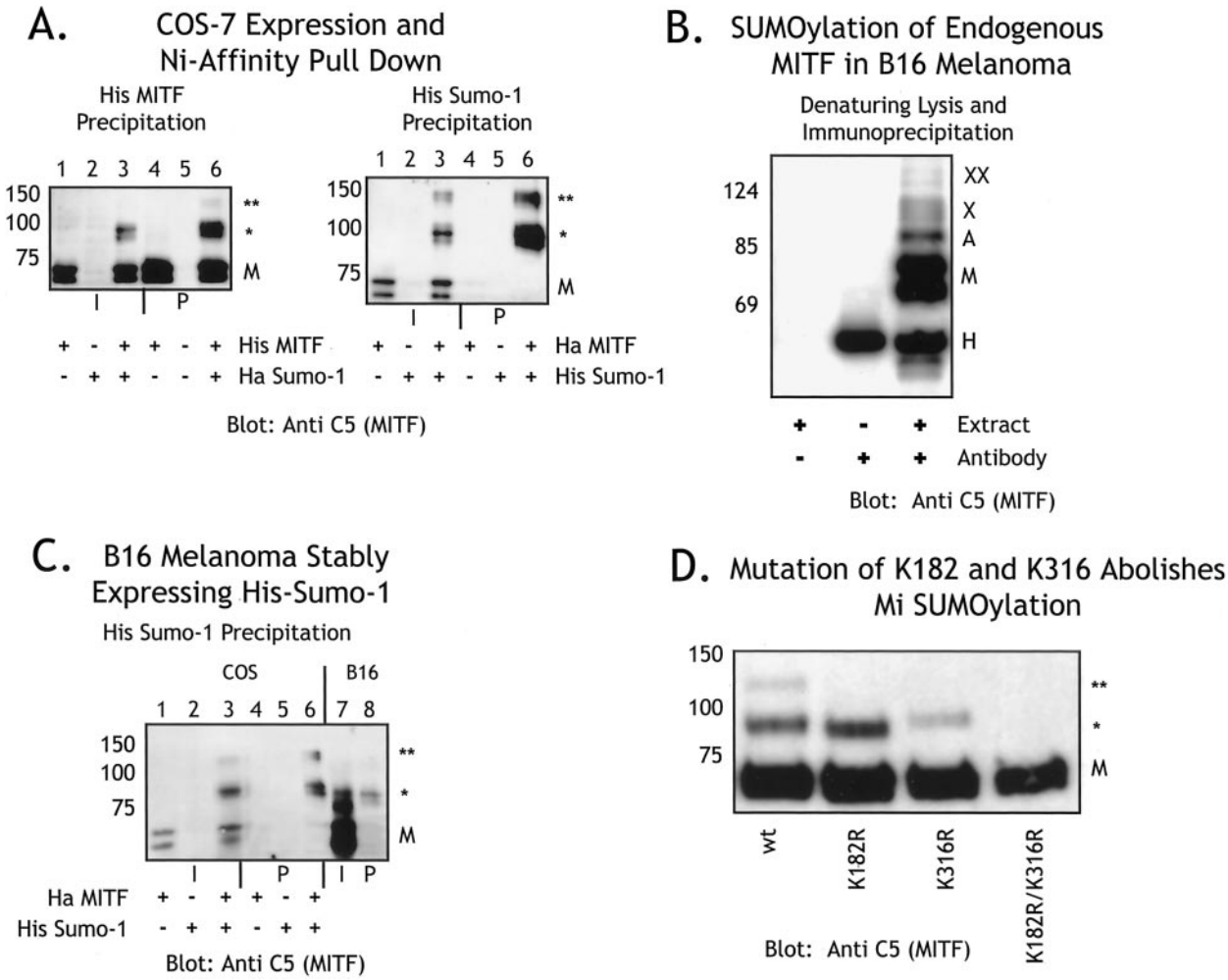

FIG. 1. **MITF is sumoylated *in vivo* at two sites.** *A*, sumoylation of MITF in COS-7 overexpression system. Cells were transfected as indicated and lysed in 8 M urea, and His-tagged proteins were precipitated under denaturing conditions. In each *panel*, the *first* through *third lanes* are lysates, representing 5% of the input (*I*), whereas the *fourth* through *sixth lanes* are Ni-NTA affinity-precipitated proteins (*P*) from 25% of the input. The MITF doublet is indicated by *M*, whereas higher molecular species are indicated by * and **. *B*, endogenous MITF is sumoylated. Denaturing lysis and immunoprecipitation were performed to preserve sumoylation in B16 melanoma. The melanocyte M-form (*M*) and more ubiquitous A-form of MITF (*A*) are indicated as well as higher molecular mass species (*X* and *XX*). *H* represents IgG heavy chain. *C*, Ni-NTA affinity purification of His-tagged SUMO-1 from a B16 melanoma cell line stably expressing His-SUMO-1. The *seventh lane* is the input lysate (*I*), and the *eighth lane* is the Ni-NTA affinity precipitation (*P*) from B16 melanoma stably expressing His-SUMO-1. The *first* through *sixth lanes* are input lysates (*lanes 1–3*) and Ni-NTA precipitations (*lanes 4–6*) from COS-7 cells transfected with His-SUMO and HA-MITF for comparison (*lanes 7* and *8*). *D*, mutation of two lysines in MITF completely abrogates sumoylation. His-tagged wild type MITF or the indicated single or double point mutants were cotransfected with HA-SUMO-1 in COS-7 cells. Mono- and doubly sumoylated forms of MITF are indicated by * and **.

and *C*). As predicted, SUMO-1 modification of TFEB and TFE3 apparently occurs at a single site, as there was not a second higher molecular mass form as there was for MITF.

Sumoylation Is Not Required for Heterodimerization—Using the COS-7 coexpression system, we expressed wild type MITF or the non-sumoylatable mutant (MITF K182R/K316R) with TFEB or TFE3 to determine whether sumoylation was required for interaction with these other family members. In both cases, wild type or mutant MITF was capable of co-precipitating either TFE3 or TFEB using Ni-NTA purification under native conditions (Fig. 3). Expression of an unrelated His-tagged protein (His-LacZ) failed to precipitate either TFE3 or TFEB (Fig. 3). Stripping and reprobing of blots with anti-C5 (MITF) antibody verified precipitation of similar amounts of wild type MITF or K182R/K316R MITF (data not shown).

PIAS3 Stimulates MITF Sumoylation—The protein inhibitor of the activated STAT (PIAS) family of proteins has been widely reported to function as E3 ligases in the SUMO-1 conjugation pathway (for review, see Refs. 22 and 55). It has been recently found that PIAS3 interacts with MITF (18, 19) and represses its transcriptional activity. We found by expression analysis[2] that PIAS1 and PIAS3 are widely expressed in melanoma cell lines (data not shown). Western blotting confirmed expression of PIAS1 and PIAS3 in a variety of melanoma cell lines (data not shown). Using the COS-7 overexpression system, we determined whether either PIAS protein was capable of enhancing SUMO-1 attachment to MITF. As seen in Fig. 4*A*, PIAS1 only very modestly induced MITF sumoylation, whereasPIAS3 strongly stimulated sumoylation (compare bands labeled as * and ** in Fig. 4*A*). Notably, PIAS3 dramatically increased the amount of MITF that had been modified at both sumoylation sites leading to a shift of ~40 kDa (indicated by ** in Fig. 4).

Because SUMO-1 modification of MITF occurs at two different lysines, we also tested whether PIAS3 acted at just one sumoylation site or both sites. Comparison of the amounts of SUMO-1-modified MITF in the presence and absence of PIAS3 shows that PIAS3 stimulates MITF sumoylation when either Lys-182 or Lys-316 was mutated. This suggests that PIAS3 is

[2] A. J. Miller, C. Levy, I. J. Davis, E. Razin, and D. E. Fisher, unpublished data.

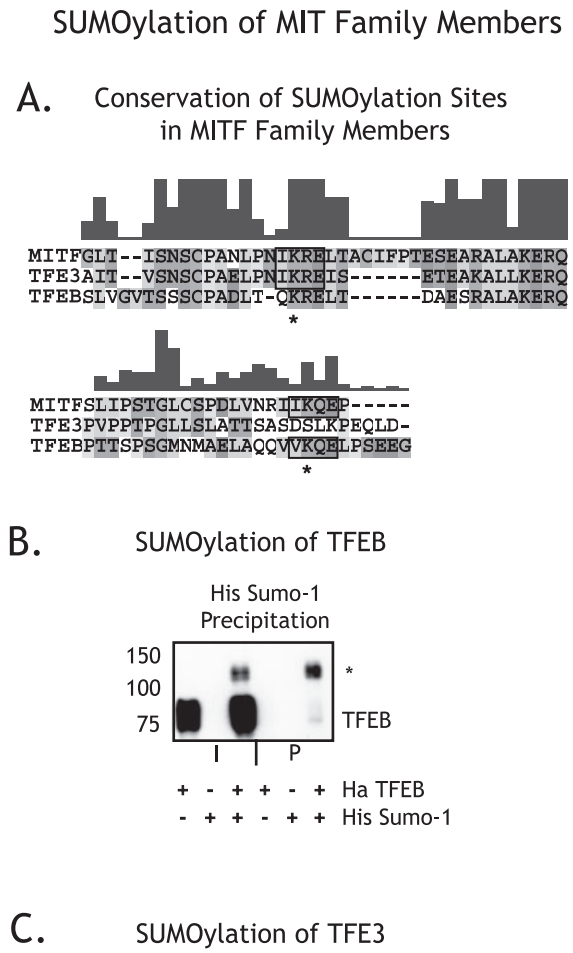

FIG. 2. **MiT family members TFE3 and TFEB are also sumoylated.** *A*, alignments of hsMITF with hsTFE3 and hsTFEB are shown for the regions surrounding the sumoylated residues in MITF. The conserved ΨK*X*E motifs are enclosed by a *box*, and the SUMO acceptor lysines are indicated by an asterisk (*). The K182 site is preserved in TFE3, whereas the Lys-316 site is preserved in TFEB. *B* and *C*, TFEB and TFE3 are sumoylated *in vivo*. COS-7 cells were transfected as indicated and lysed in 8 M urea, and His-tagged proteins were precipitated under denaturing conditions. In each *panel lanes 1–3* are lysates representing 5% of the input (*I*), whereas *lanes 4–6* are Ni-NTA affinity-precipitated proteins from 25% of the input (*P*). Higher mobility forms of TFEB and TFE3 are indicated by an asterisk (*). Western blotting was performed using anti-HA monoclonal antibody.

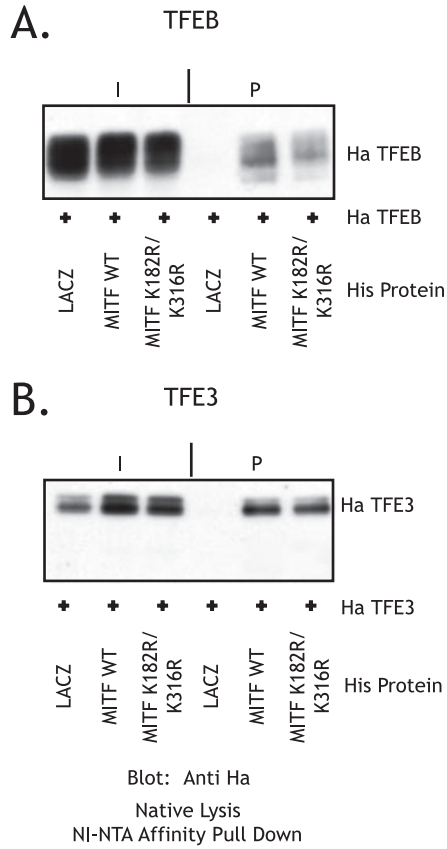

FIG. 3. **Sumoylation is not required for MITF heterodimerization.** Cos-7 cells were transfected with Ha-TFEB (*A*) or Ha-TFE3 (*B*) and pcDNA4HisMax-LacZ, pcDNA4HisMax-MITF wild type, or pcDNA4HisMax-MITF K182R/K316R mutant and lysed under native conditions, and His-tagged proteins were purified under native conditions. In each *panel*, the *first* through *third lanes* are lysates representing 5% of the input (*I*) showing similar levels of expression of indicated proteins, whereas the *fourth* through *sixth lanes* are Ni-NTA affinity-precipitated proteins from 25% of the input (*P*). Western blotting was performed using anti-HA monoclonal antibody.

capable of mediating sumoylation at either residue (Fig. 4*B*).

Effect of SUMO-1 Modification on MITF Transcriptional Activity—Coexpression of PIAS3 has been previously shown to repress MITF transcription in reporter assays (18). Because this may be due to stimulation of MITF sumoylation, we tested whether coexpression of SUMO-1 or PIAS3 repressed MITF in our system. Coexpression of SUMO-1 or PIAS3 with MITF led to a significant decrease in reporter activity. However, similar repression occurred with the MITF K182R/K316R mutant, suggesting the effect was nonspecific or independent of sumoylation (data not shown). Similar transcriptional effects have been previously reported for other PIAS family members (37, 56), and discordant findings for SUMO overexpression have also been shown (37). A large number of proteins are known to be sumoylated or to interact with PIAS family members including transcription factors, transcriptional co-activators (57), and co-repressors (58). Consequently, it has been suggested that overexpression of SUMO-1 or PIAS family members leads to a variety of nonspecific effects, necessitating a means of selectively controlling the sumoylation status of a given transcription factor (43).

Rather than using overexpression of SUMO-1 or PIAS to modulate the level of MITF sumoylation, we addressed the effects of sumoylation by comparing MITF mutants, a strategy that has been previously employed for other transcription factors (36, 59). These experiments utilized wild type MITF and mutants in the SUMO acceptor sites (K182R, K316R, and K182R/K316R) as well as a direct N-terminal fusion of SUMO-1 to MITF. Importantly, the SUMO-1-MITF fusion utilizes SUMO-1 lacking its C-terminal Gly-Gly motif, which prevents cleavage by SUMO-1 proteases. The activity of wild type MITF and mutants that lack either or both SUMO-1 acceptor sites were compared in reporter assays in HEK293 cells which lack MITF expression. The recently characterized MITF-responsive promoter of melastatin/TRPM1 (45) was utilized. At the quantities of MITF and reporter plasmid utilized, wild type

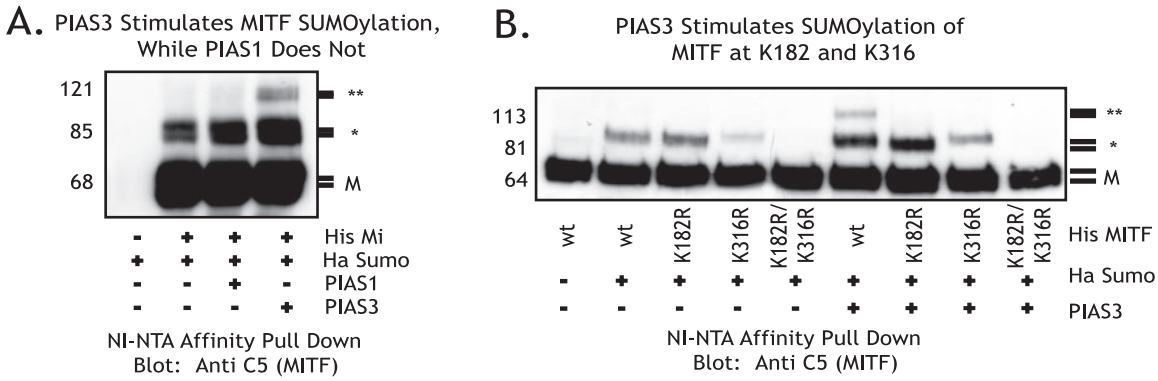

FIG. 4. **PIAS3, but not PIAS1, promotes the sumoylation of MITF *in vivo*.** *A*, co-transfection of PIAS3 stimulates MITF sumoylation, whereasPIAS1 has little effect. *B*, PIAS3 is capable of stimulating sumoylation at either lysine in MITF. The addition of PIAS3 further stimulates sumoylation at either Lys-182 or Lys-316. Sumoylated forms of MITF (*M*) are indicated by * and **.

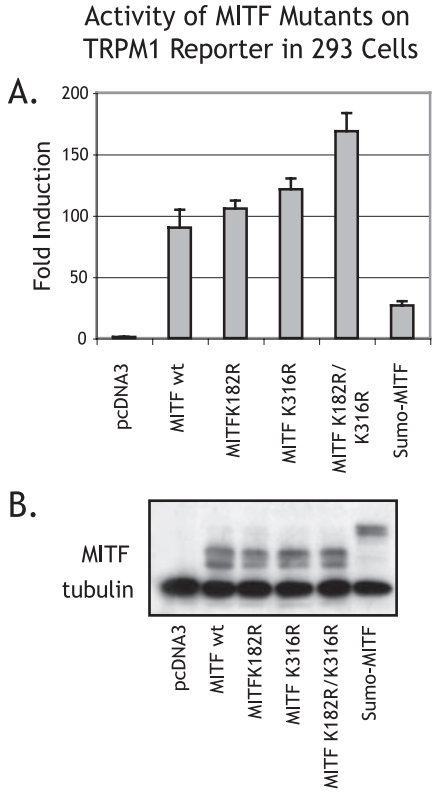

FIG. 5. **Non-sumoylatable MITF has higher transcriptional activity than wild type MITF.** *A*, HEK293 cells were transfected with promoter constructs for the MITF target gene TRPM1 with equal amounts of wild type MITF or the indicated mutants. *B*, Western blot for MITF from corresponding lysates confirms similar levels of MITF expression.

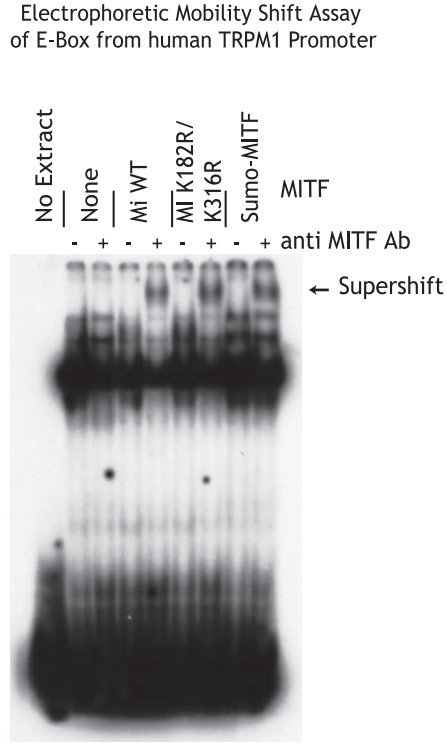

FIG. 6. **Mutants retain DNA binding activity.** COS-7 cells were transfected with pcDNA3 control plasmid, MITF (*Mi*) wild type, MITF K182R/K316R, or SUMO-MITF, and nuclear extracts were prepared. Electrophoretic mobility shift assay was performed using a probe containing a MITF binding site from the TRPM1 promoter. D5 monoclonal MITF antibody (*Ab*) was used to supershift MITF-containing protein-DNA complexes as indicated.

MITF induces this promoter nearly 100-fold (Fig. 5). Mutant MITF at either SUMO-1 acceptor site induces this promoter slightly more strongly, whereas MITF double mutant (MITF K182R/K316R) induced the TRPM promoter about 60% more strongly than wild type MITF. Compared with wild type MITF, the N-terminal SUMO-1 fusion induces the promoter much less (SUMO-MITF) but is still capable of greater than 20-fold activation. Similar effects were also seen in COS-7 cells (data not shown). In addition, this difference in activity occurred across a broad range of MITF:reporter ratios (see Fig. 8*A*).

Fusion of SUMO-1 to MITF Does Not Affect Its DNA Binding—We next sought to determine whether the inability of the SUMO-1-MITF fusion to fully induce transcription was due to a deficiency in DNA binding. Using a previously characterized probe containing a MITF consensus E-box from the TRPM1 promoter (45), we tested whether wild type MITF, MITF K182R/K316R, and SUMO-1-MITF fusion proteins could bind this DNA element (Fig. 6). MITF and mutants were expressed in COS-7 cells from which nuclear extracts prepared. Protein levels of MITF in extracts were compared by Western blotting (data not shown), and similar amounts of MITF were utilized for mobility shift assays. It is anticipated that the SUMO fusion will retain some DNA binding ability since it can activate 20-fold. The results of this assay show that each of these mutants retains the ability to bind DNA. In addition, this also shows that both the MITF K182R/K316R and SUMO-1-MITF fusion proteins are capable of dimerizing since MITF requires dimer formation for DNA binding (7).

Nuclear Localization of MITF Mutants—Another possible

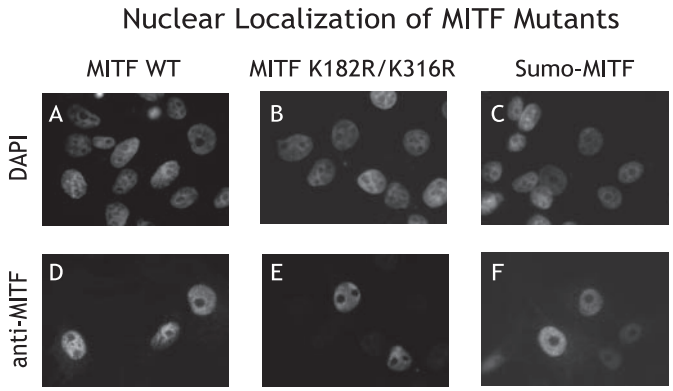

FIG. 7. **Sumoylation does not affect MITF localization.** COS cells were transfected with the indicated MITF mutants. 4,6-Diamidino-2-phenylindole (*DAPI*) staining (*upper panels*) shows nuclei, whereas MITF immunofluorescence (*lower panels*) shows MITF localization.

explanation for the different transcriptional activities of MITF wild type, MITF K182R/K316R, and the SUMO-1-MITF fusion could be altered nuclear localization or sub-localization. Sumoylation may be related to nuclear import (30) and may control the presence of proteins in PML bodies (47, 60). Immunofluorescence microscopy of COS-7 cells transfected with wild type MITF, MITF K182R/K316R, and SUMO-1-MITF fusion revealed proper localization of each to the nucleus (Fig. 7). We did not observe localization of wild type or the SUMO-1-MITF fusion into discrete nuclear dots that are suggestive of PML colocalization. Notably, the SUMO-1-MITF fusion retained the capability of localizing to the nucleus in the same manner as wild type, indicating that this fusion has no major effect on nuclear import.

Effect of SUMO-1 on MITF Stability—Sumoylation has been found to block ubiquitination of IκBα (31) and to have effects on protein stability. Although expression levels of wild type MITF and K182R/K316R mutant were typically similar in all experiments, we considered whether the increased activity of the MITF K182R/K316R mutant might be due to decreased degradation after its activation by phosphorylation. Mitogen-activated protein kinase-dependent phosphorylation of MITF was previously shown to trigger MITF ubiquitination and degradation (14, 44).

We explored the effect of sumoylation on MITF stability using expression in COS-7 cells followed by stimulation with TPA, which leads to MITF phosphorylation and degradation (14). Experiments were conducted in the presence of cycloheximide to block new protein synthesis and permit measurements of decay kinetics. Whereas TPA treatment leading to MITF phosphorylation at serine 73 triggered MITF degradation, no differences were observed for either MITF wt or MITF K182R/K316R. In addition, co-expression of SUMO-1 with MITF had no effect on the half-life of wild type MITF (data not shown). These data suggest that sumoylation does not affect the process of MITF degradation that follows MITF phosphorylation. In the course of these experiments, we also noted that stimulation of MITF phosphorylation by TPA was not associated with a significant change in the level of MITF sumoylation (data not shown).

We further tested whether mutation of the SUMO-1 acceptor sites affects the ubiquitination of MITF. Wild type HA-MITF and HA-MITF K182R/K316R were co-expressed with His-ubiquitin in COS-7 cells, and ubiquitinated proteins were recovered by nickel nitrilotriacetic acid chromatography. No differences were observed in the amount of wild type or mutant MITF recovered by this method (data not shown). These data suggest that SUMO-1 does not enhance MITF stability nor are the SUMO-1 acceptor lysines required for MITF ubiquitination.

Sumoylation and MITF Synergy Control—Using the TRPM1 promoter construct (TRPM1 wt), which harbors three MITF binding sites, and a mutant in which two of the sites have been destroyed by site-directed mutagenesis (TRPM1-E1E2) (45), we explored the relationship between multiple MITF binding sites and MITF sumoylation. Using HEK293 cells, which do not express MITF, we titrated MITF plasmids from 12.5 to 800 ng with 25 ng of either reporter plasmid, representing driver: reporter ratios of 1:2 to 32:1. Across this broad range, we found that MITF K182R/K316R was consistently more active than wild type MITF on the TRPM wt promoter (3-MITF sites) but showed no difference in activity on the TRPM-E1E2 mutant promoter (single MITF site) (Fig. 8, *A* and *B*). Intriguingly, we found that the SUMO-1-MITF fusion showed greatly reduced activity on the wild type (3-MITF sites) promoter, but in the context of the single site (mutant) reporter, the SUMO-1-MITF fusion was as active as either wild type or MITF K182R/K316R (Fig. 8, *A* and *B*). As summarized in Fig. 8*C*, the differences in activity between the MITF mutants only occur in the context of the promoter with multiple MITF binding sites. These data suggest that sumoylation blocks the synergistic response of multiple MITF binding sites in a promoter construct.

We further tested this effect using a variety of other MITF-driven reporter plasmids including the pTYR (50), pSILV (48), and pCATHK (49). If the sumoylation of MITF only affects MITF transcriptional activity when multiple E-boxes are present, it would be expected that the activities of MITF wt, MITF K182R/K316R, and SUMO-MITF would be identical on promoters with one E-box such as SILV, whereas the activity on promoters with multiple E-boxes such as tyrosinase and cathepsin K would differ. Using the same HEK293 reporter assay system as before, we have found this to be the case (Fig. 8, *D*, *E*, and *F*). With both pTYR and pCATHK, MITF K182R/K316R was more active than wild type MITF, whereas the SUMO-MITF fusion was less active. In contrast, the activities of wild type MITF, MITF K182R/K316R, and SUMO-MITF fusion were nearly identical when using the promoter for SILV, which contains a single MITF binding site.

DISCUSSION

Sumoylation of MITF—We have identified MITF as a SUMO-1-modified protein and have mapped the modification sites to two consensus lysines in MITF. We have presented evidence that this modification occurs *in vivo* in a melanoma cell line as well as presented data from a melanoma cell line engineered to express His-tagged SUMO-1 that shows SUMO-1 modification of endogenous MITF. Each of the two closely related MiT family members, TFEB and TFE3, possesses a sumoylation site, and we have shown that these proteins are also sumoylated. This suggests that the effects of sumoylation on MITF may be also important for these other family members. The conservation of this modification across members of this transcription factor family suggests that it could be important for regulation of all of the MiT family members. Sumoylation of MITF affects its transcriptional activity, but this difference requires the presence of multiple MITF binding sites in the promoters of target genes. This provides a possible mechanism for fine-tuning the target gene response of MITF, which may be important in understanding the role of MITF in differentiation *versus* proliferation and survival. The magnitude of the differences in transcriptional activity between wild type MITF and the non-sumoylatable mutant are not numerically large but are nonetheless potentially capable of representing a biologically important homeostatic mechanism. For the basic helix-loop-helix leucine zipper family, which contains the MITF

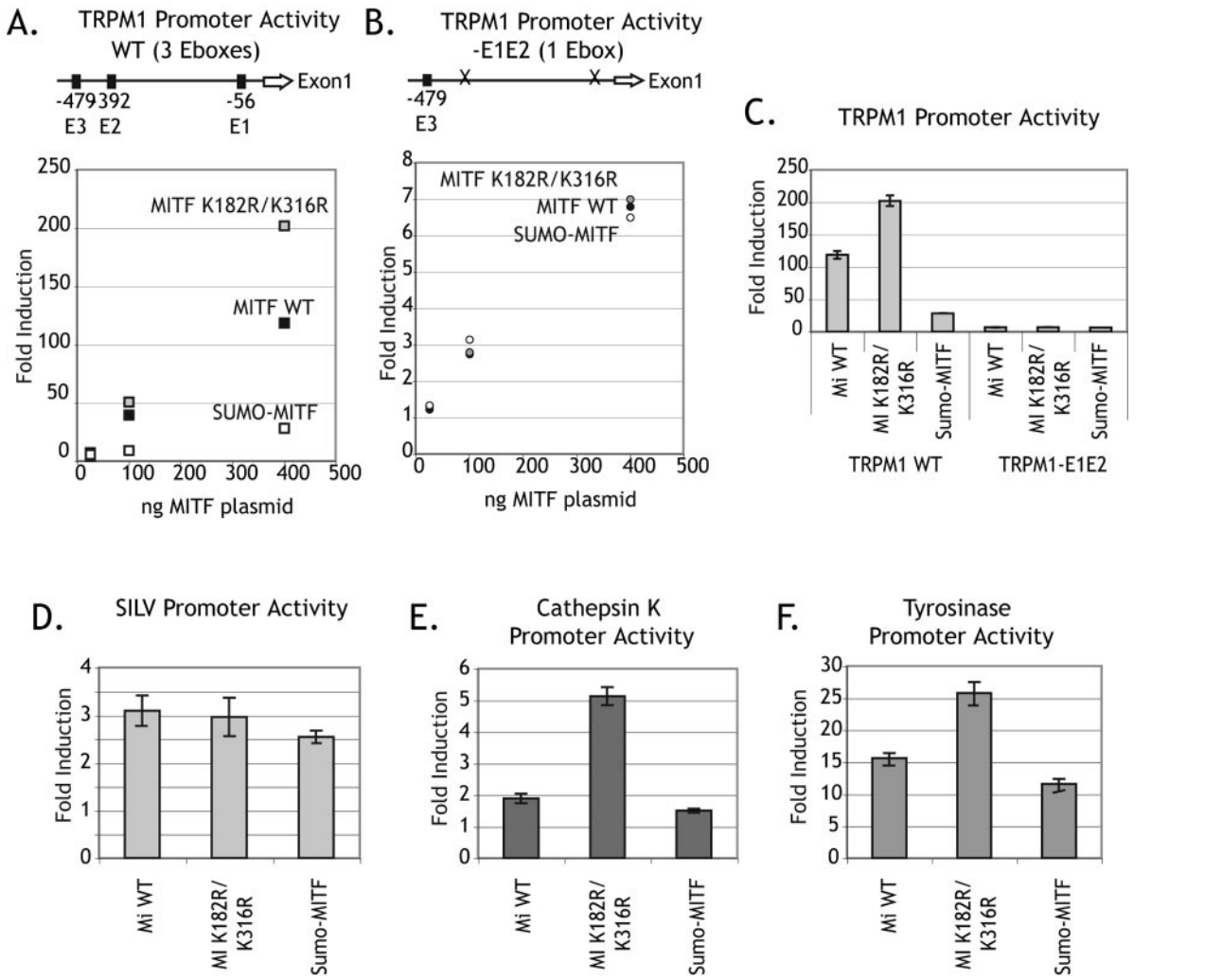

FIG. 8. **Sumoylation affects the MITF response of a promoter with multiple MITF binding sites.** *A*, HEK293 cells were transfected with 25 ng of the TRPM1 wt promoter construct, which contains three MITF binding sites, and 100 ng of pRL-Null control plasmid. 12.5–400 ng of MITF wild type, MITF K182R/K316R, or SUMO-MITF were co-transfected with the total amount of DNA kept constant by the addition of pcDNA3. *B*, the same transfection conditions were used with the TRPM-E2E2, promoter in which two MITF binding sites were mutated by site-directed mutagenesis. *C*, data from *A* and *B*, shown as -fold induction normalized to pcDNA controls. *D*, HEK293 cells were transfected with 100 ng of the SILV promoter construct, which contains one MITF binding site, 100 ng of pRL-Null control plasmid, and 400 ng of pcDNA3, MITF wild type, MITF K182R/K316R, or SUMO-MITF. Data are shown as -fold induction relative to pcDNA3 control. *E*, cells were transfected as in *D* except with the cathepsin K promoter construct, which contains four MITF binding sites. *F*, cells were transfected as in *D* except with the tyrosinase promoter, which contains two MITF binding sites.

group as well as the MYC group, it is well established that transcriptional stimulation in reporter assays such as those utilized here tend to produce modest effects that are nonetheless felt of be biologically potent. For example, MITF transcriptional up-regulation of BCL2, which was genetically validated in crosses of mutant/knockout mice, was seen to represent only a 2.5-fold difference when measured *in vitro* (63). In addition, the summed transcriptional differences of numerous transcriptional target genes which are themselves altered differentially at the level of synergy control would be anticipated to contribute significantly to the biological state of the cell, particularly for transcription factors such as MITF, which are thought to regulate the differentiation state of the melanocyte lineage.

Mechanism of PIAS3 Inhibition—We have shown that PIAS3 is capable of enhancing MITF sumoylation in COS-7 and 293 cells at two lysines within SUMO consensus sites. Mutation of these residues prevents sumoylation and leads to higher transcriptional activity. This suggests that the previously reported repression of MITF transcriptional activity by PIAS3 (18, 19) may be due to its function as an E3 SUMO ligase. However, the addition of PIAS3, SUMO-1, or both to reporter assays led to transcriptional inhibitory effects that were not specific to MITF and were, therefore, difficult to interpret. In addition, it has been suggested that PIAS3 binds directly to MITF (18), making it possible that repression occurs independently of sumoylation. Indeed, it has been recently reported that PIASγ represses androgen receptor independently of sumoylation (61). We continue to explore possible means of specifically interrogating the mechanism(s) of PIAS3 inhibition of MITF.

Synergy Control Motif in MITF; a Means of Regulating Differential Transcriptional Programs—Conservative point mutations of the SUMO-1 acceptor lysines in MITF at positions 182 and 316 to arginine led to increased transcriptional activity of the mutant proteins. MITF K182R/K316R was significantly more active than wild type. Attempting to determine the mechanism of this effect, we found that this mutation did not affect heterodimerization of MiT family members nor did it affect the nuclear localization of MITF or MITF stability after TPA-induced phosphorylation and degradation. Notably, in the reporter system the difference in transcriptional activity only occurred on a promoter with multiple MITF binding sites. Point mutations of the promoter, creating a mutant with a single

MITF binding site, abrogated the difference in transcriptional activity between wild type MITF and MITF K182R/K316R.

Stoichiometry—Based on the B16 Western blot (Fig. 1*B*), it appears that only a small fraction of MITF, perhaps 5%, is sumoylated in these cells under typical growth conditions. It will be important to further examine the stoichiometric relationship between SUMO-modified MITF and net MITF transcriptional activity in the cell, since reporter assay results suggest a more potent effect of MITF sumoylation. Possible models for superstoichiometric activity of SUMO-MITF would include its being a potent repressor through altered interactions with co-activators or co-repressors or through alterations in chromatin structure (which may not be well measured in reporter assays). Such potent repressive activity may be underscored by the strong inhibition of MITF activity by direct fusion of SUMO to MITF which, although highly artificial, does represent 100% sumoylation and does retain evidence of normal folding, as evidenced by its DNA binding.

Role of Sumoylation in MITF-regulated Transcription—Several MITF-responsive promoters harbor multiple binding sites for this transcription factor. According to a synergy control model, SUMO modification might selectively modify the expression of various MITF target genes. Sumoylation attenuates the expression of target genes such as TRPM1 but does not affect target genes controlled by a single MITF binding site such as SILV. This might allow fine-tuning of the response to extracellular stimuli such as α-melanocyte-stimulating hormone that increase the transcription of MITF itself, whereby sumoylation could lead to a relative suppression of promoters with multiple binding elements while permitting the increased levels of MITF to act upon promoters with single elements. Conversely, loss of sumoylation would allow MITF to act synergistically on promoters with multiple binding sites and lead to a dramatic increase in their transcription. Thus, sumoylation of MITF might be an important control of the relative representation of various target genes in the MITF repertoire. Such a mechanism could be particularly important for MITF, since it appears to have at least two significantly different *in vivo* functions; that is, control of melanocyte differentiation/pigmentation and control of melanocyte viability. As the identity of MITF transcriptional targets begins to emerge, it will be increasingly possible to assess the degree to which mechanisms such as synergy control by sumoylation contribute to the diversity of biological effects mediated by MITF. Similarly the expression of closely related family members, such as TFEB and TFE3, in non-melanocytes raises additional mechanistic questions regarding transcriptional target choices that could be modulated by post-translational mechanisms including sumoylation. Another major question is the means through which SUMO modification is regulated. Is sumoylation of MITF a constitutive process or a regulated one? If regulated, what signals trigger or inhibit the pathway? The fusion of SUMO to the N terminus of MITF did not affect its ability to bind DNA, localize to the nucleus, or transcriptionally activate a promoter with a single MITF binding site. Its decreased relative transcriptional activity on a promoter with multiple MITF binding sites may be due to an alteration in protein-protein interactions. It has been previously suggested that SUMO affects synergy control by recruiting repressive elements to promoters (43). Given the instability of SUMO modifications *in vivo*, the SUMO-1 fusion, which SUMO proteases cannot cleave, might provide an alternative means to elucidate the nature of such potential context-specific protein-protein interactions.

Acknowledgements—We thank the members of the Fisher laboratory for useful discussions and comments. Gabriela Motyckova provided the TFE3 construct, and Vivien Igras provided assistance with immunofluorescence. We also thank Drs. Dirk Bohmann and Len Zon for several plasmids. Dr. Gregory David provided much useful advice throughout this project.

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