Expression of Microphthalmia-associated Transcription Factor (MITF), Which Is Critical for Melanoma Progression, Is Inhibited by Both Transcription Factor GLI2 and Transforming Growth Factor-β

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The melanocyte-specific transcription factor M-MITF is involved in numerous aspects of melanoblast lineage biology including pigmentation, survival, and migration. It plays complex roles at all stages of melanoma progression and metastasis. We established previously that GLI2, a Kruppel-like transcription factor that acts downstream of Hedgehog signaling, is a direct transcriptional target of the TGF-β/SMAD pathway and contributes to melanoma progression, exerting antagonistic activities against M-MITF to control melanoma cell invasiveness. Herein, we dissected the molecular mechanisms underlying both TGF-β and GLI2-driven M-MITF gene repression. Using transient cell transfection experiments with M-MITF promoter constructs, chromatin immunoprecipitation, site-directed mutagenesis, and electrophoretic mobility shift assays, we identified a GLI2 binding site within the −334/-296 region of the M-MITF promoter, critical for GLI2-driven transcriptional repression. This region is, however, not needed for inhibition of M-MITF promoter activity by TGF-β. We determined that TGF-β rapidly repressed protein kinase A activity, thus reducing both phospho-cAMP-response element-binding protein (CREB) levels and CREB-dependent transcription of the M-MITF promoter. Increased GLI2 binding to its cognate cis-element, associated with reduced CREB-dependent transcription, allowed maximal inhibition of the M-MITF promoter via two distinct mechanisms.

TGF-β signals via serine-threonine kinase receptors. Ligand-dependent receptor activation results in phosphorylation and nuclear accumulation of proteins of the SMAD family of transcription factors, leading to target gene regulation (1, 2). Often considered a tumor suppressor pathway as it negatively controls the cell cycle in normal and premalignant cells, TGF-β/SMAD signaling also promotes cancer cell invasion and metastasis through autocrine and paracrine mechanisms. This occurs most notably via its capacity to induce an epithelial-mesenchymal transition, a phenomenon whereby epithelial tumor cells acquire a migratory and invasive, mesenchymal-like, phenotype (3). Also, TGF-β acts in a paracrine manner to favor peritumoral angiogenesis and suppress anti-tumoral immunity (4). In melanoma, constitutive TGF-β signaling through the SMAD pathway promotes invasiveness and metastatic potential of melanoma cells (5).

GLI2, a member of the Kruppel family of transcription factors, was recently identified as a direct TGF-β/SMAD target ubiquitously induced in normal and transformed cells (6, 7). Induction occurs at the transcriptional level via mechanisms that include the recruitment of both SMAD3 and β-catenin to its promoter (6). We found that GLI2 controls melanoma cell invasiveness and metastatic potential via acquisition of a mesenchymal phenotype characterized by loss of CDH1 expression and enhanced MMP2 and MMP9 secretion (8). Similar changes in gene expression were found in human melanoma tumors, suggesting that GLI2 may also represent a marker of poor prognosis for patients with melanoma (8). Recently, we determined that GLI2 expression in melanoma cells is inversely correlated with that of the melanocyte-specific isoform of the microphthalmal transcription factor (M-MITF) and its associated tran-

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5 The abbreviations used are: MITF, microphthalmia-associated transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; KLF, Kruppel-like factor.
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M-MITF, a basic helix-loop-helix leucine zipper transcription factor, is central to a variety of cellular processes in the melanocyte lineage (migration, morphology, proliferation, survival), including terminal differentiation of melanocytes leading to pigment production as well as malignant transformation and melanoma progression to metastasis (11, 12). In patients with melanoma, M-MITF may either be amplified or lost, the outcome of M-MITF function depending both on its expression levels and on the presence or absence of concomitantly occurring genetic alterations such as activating B-RAF and N-RAS mutations (11, 12). It is thought that by switching back and forth between low and high M-MITF phenotypes, melanoma is driven to progress (13, 14).

We recently identified TGF-β as a potent repressor of M-MITF expression and as an inducer of GLI2 expression in melanoma cells and found that GLI2 overexpression inhibits M-MITF promoter activity (9). In this report we have elucidated the precise molecular mechanisms underlying M-MITF transcriptional control by TGF-β and GLI2. We demonstrate that inhibition of M-MITF expression by TGF-β occurs via repression of protein kinase activity leading to reduced CREB-dependent transcription, whereas GLI2-driven transcriptional repression of M-MITF is an independent phenomenon. Distinct cis-elements within the M-MITF promoter, namely a novel Kruppel-like transcription factor (KLF)/GLI2 binding region and a proximal CREB binding site, drive M-MITF silencing by TGF-β and GLI2, respectively.

EXPERIMENTAL PROCEDURES

Cell Cultures and Reagents—Human melanoma cell lines, described previously (8, 9, 15–17), were grown in RPMI 1640 (Invitrogen) supplemented with 10% FCS and antibiotics at 37 °C and 5% CO2 in a humidified atmosphere. The expression vector encoding the constitutively active GLI2 mutant, GLI2ΔN, was a kind gift from H. Sasaki (Osaka University) (18). TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MN).

Plasmid Constructs—Some of the 5’-end deletion constructs of the human M-MITF promoter cloned into pGL2-luc and the proximal cAMP-response element (CRE) mutant in −2135/+136 MMITFluc have been described previously (19, 20). Additional 5’-end deletions to positions −334 and −296 were generated by PCR and cloned as NheI/HindIII fragments into the same backbone vector (pGL2 basic; Promega, Madison, WI). Inactivation of the potential Kruppel-like binding site (CTCCTCCAAA) at position −318/−308 within the −334/+136 M-MITF promoter construct was performed by either point mutation (CTAATCTAAA) or nucleotide excision (CTCCTAAA) using the DpnI-based QuikChange site-directed mutagenesis methodology (Stratagene, La Jolla, CA) with the following primers: forward (5’-GTAGCTTTTAGATGATGTCTAATCTAAAGGGGCATTCTGC-3’) and reverse (5’-GCAGAATGGCCCTTTAGATTGACATCATCTAAAAGCTAC-3’) and forward (5’-TAGCTTTTAGATGATGTCTAATCTAAAGGGGCATTCTGC-3’) and reverse (5’-GCAGAATGGCCCTTTAGATTGACATCATCTAAAAGCTAC-3’), respectively. Site-directed mutagenesis to inactivate the CRE binding site between −140/−147 within the −334/+136 and −296/+136 M-MITF promoter constructs was carried out as described previously for the −2135/+136 construct (20). Inactivation of the KLF binding site was subsequently performed in the CRE-mutated −334/+136 M-MITF promoter construct using the same strategy as above. pRL-MLP vector (6), derived from pRL-TK vector (Promega), was used to monitor transfection efficiencies. pCRE-luc (Mercury™ pathway luciferase system, Clontech, Palo Alto, CA) and the GLI-specific reporter plasmid (GLI-BS)8-luc (21) were used to measure CREB- and GLI2-dependent transcription, respectively.

Biochemical Methods—Protein extraction and Western blotting were performed as previously described (8, 9). Mouse anti-M-MITF and rabbit anti-GLI2 antibodies were purchased from Neomarkers (Thermo Scientific, Kalamazoo, MI) and Cell Signaling (Ozyme, St-Quentin en Yvelines, France), respectively. Mouse monoclonal anti-actin and goat anti-ubiquitous kinesin heavy chain as well as secondary donkey anti-rabbit and antio-mouse HRP-conjugated antibodies were from Santa-Cruz Biotechnology (Santa-Cruz, CA). Protein kinase A (PKA) activity was measured using a commercial kit from Promega (nonradioactive PKA Assay) utilizing a fluorescent PKA-specific peptide substrate. In brief, 5 × 104 cells were incubated in medium supplemented with 1% FCS in the absence or presence of TGF-β (5 ng/ml). Cells were harvested, and samples were processed according to the manufacturer’s protocol. Phosphorylated and unphosphorylated peptides were separated by agarose gel electrophoresis and visualized under UV light. Quantitation of fluorescence was measured at 540 nm after gel dissolution into 16 m acetic acid (Sigma).

Cell Transfections—For reporter assays, melanoma cells were seeded in 24-well plates and transfected at ∼70–80% confluency in fresh medium containing 1% FCS with either the polycationic compound FuGENE™ (Roche Diagnostics) or JetPEI (Polyplus Transfection Inc., New York, NY). After incubation, luciferase activities were determined with a Dual-Glo™ luciferase assay kit (Promega) using a Fluoroskan Ascent FL (Thermo Labsystems). For stable expression of GLI2ΔN, melanoma cells were transfected with JetPEI at ∼70–80% confluency with 10 μg of either empty pcDNA3 expression vector or the same vector carrying constitutively active GLI2ΔN (18) per 100-mm diameter culture dish. Three days later, G418 (Sigma, 0.7 mg/ml) was added to the culture medium. Selection of stably transfected cell populations occurred within a 3-week period. RT-PCR and measurement of GLI-dependent transcription were used to verify expression and activity of transfected GLI2ΔN, respectively. All experiments were performed at least three times independently using triplicate dishes.

Gene Silencing in Human Melanoma Cells—Infection of melanoma cells with lentiviral particles expressing either control, non-targeting shRNA (shCtrl, Sigma SHC002V), or shRNA targeting GLI2 (Sigma SHVRS clone ID...
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TRCN0000033329 and TRCN0000033330) has been described previously (8, 9). Transduced cell populations were selected with puromycin (2 μg/ml). Efficacy of GLI2 knockdown over time was verified by real time RT-PCR and Western blotting after each cell passage.

RNA Extraction and Gene Expression Analysis—RNA extraction procedure and reverse transcription-polymerase chain reaction (RT-PCR) methodologies have been described previously together with primer sequences (8). The latter are available upon request.

Electrophoretic Mobility Shift Assays (EMSAs)—Oligonucleotides spanning the regions -334/-304 and -334/-296 were radiolabeled with [32P]dCTP using Klenow polymerase and used as probes to detect DNA-protein interactions within the GLI2-responsive region of the M-MITF promoter. Nuclear extracts from 1205Lu melanoma cells were isolated using a small scale preparation (22). For competition experiments, a 100-fold excess of unlabeled consensus GLI2 (23) or Sp1 (24) oligonucleotide was used. For antibody interference experiments, nuclear extracts (15 μg) were incubated for 1 h at 4 °C with 1 μg of either anti-GLI2 (generous gift from Rüne Toftgard, Karolinska Institute, Stockholm, Sweden) or anti-TCF3 antibodies (Santa Cruz Biotechnology) before the addition of the radiolabeled probes (5 × 10⁶ cpm/lane). Binding mixtures were separated electrophoretically on native 4% acrylamide gels and visualized by autoradiography after gel drying.

Chromatin Immunoprecipitation—Melanoma cells (1205Lu, SKMel28, WM983A, and 888mel) were grown to 80% confluency on 15-cm plates. Chromatin immunoprecipitation was carried out using the ChIP-IT express kit (Active Motif, Rixensart, Belgium). Briefly, 7 μg of enzymatically sheared chromatin were immunoprecipitated using 3 μg of antibody against IgG or anti-GLI2 antibody (Santa Cruz Biotechnology), then incubated overnight at 4 °C with protein G beads. Precipitated DNA was used for PCR analysis using primers specific for region -206 of the human M-MITF promoter: forward, 5′-GTCATCCTGCAGTCGGAAGT-3′; reverse, 5′-CAGATCAAGGCCAAT-3′. Amplification of GAPDH sequences was used as an internal control with primers provided in the Active Motif kit. Amplimers were visualized with ethidium bromide after agarose gel electrophoresis.

In Silico Promoter Analysis—For bioinformatics analyses, the Genomatix software package (Genomatix Software, München, Germany) was used. Proximal promoter sequences (2 kb upstream and 0.2 kb downstream of the transcriptional start site) were extracted with help of the ElDorado module (release 4.6). Identification of transcription factor binding sites in the M-MITF promoter was performed using the MatInspector module of the Genomatix data base together with Matrix Family Library version 8.3 (October 2010) (25). All analyses were conducted with high threshold values (core similarity 1.0, matrix similarity 0.9).

RESULTS

Down-regulation of M-MITF Expression by TGF-β Implicates Multiple Mechanisms—In a first set of experiments, 888mel melanoma cells, which strongly express M-MITF and little GLI2 unless stimulated by TGF-β (9), were stably transfected with either empty (pc) or GLI2ΔN expression vector. Left panel, pc and GLI2ΔN cells were co-transfected with (GLI-BS)luc and pRL-MLP. GLI-specific transcription, measured as luciferase activity after correction for Renilla, is shown. Right panel, pc and GLI2ΔN cells were incubated for 24 h without (+) or with (+) TGF-β (5 ng/ml) after which M-MITF protein levels were determined by Western blotting. Actin content in the same protein extracts served as an internal control.

FIGURE 1. GLI2 and TGF-β cooperate to repress M-MITF expression in melanoma cells. A, shown are subconfluent 888mel melanoma cells stably transfected with either empty (pc) or GLI2ΔN expression vector. Left panel, pc and GLI2ΔN cells were co-transfected with (GLI-BS)luc and pRL-MLP. GLI-specific transcription, measured as luciferase activity after correction for Renilla, is shown. Right panel, pc and GLI2ΔN cells were incubated for 24 h without (+) or with (+) TGF-β (5 ng/ml) after which M-MITF protein levels were determined by Western blotting. Ubiquitous kinesin heavy chain (UKHC) served as an internal control. GLI2 transcript levels were measured in parallel dishes (left panel).

In a second set of experiments, SKMel28 melanoma cells, which express detectable levels of both M-MITF and GLI2 in the basal state, were transduced with either non-targeting or GLI2-specific shRNA lentiviral vectors. Quantitative RT-PCR was used to evaluate the efficacy of GLI2 knockdown; both constitutive and TGF-β-induced GLI2 expression was reduced in
shGLI2-transduced cells as compared with mock shC cells (Fig. 1B, left panel). Remarkably, GLI2 knockdown led to elevated M-MITF protein levels (Fig. 1B, right panel, lane 3 versus lane 1), indicating that endogenous GLI2 exerts an inhibitory activity against M-MITF expression. In line with the data presented in Fig. 1A, TGF-β still exerted an inhibitory effect against M-MITF expression despite GLI2 knockdown (lane 4 versus lane 3).

Next, GLI2 expression was down-regulated in 888mel cells by means of transiently transfected GLI2-specific siRNA oligonucleotides. Unlike the partial effect of lentiviral knockdown (above), this transient siRNA approach fully abolished GLI2 induction by TGF-β (supplemental Fig. S1B, left panel). Yet it did not oppose the inhibitory effect of TGF-β on M-MITF expression (supplemental Fig. S1B, right panel) as seen above after stable GLI2 knockdown. These data are consistent with the hypothesis that mechanisms other than GLI2 induction are involved to mediate M-MITF inhibition by TGF-β. Similar results were obtained in 501mel melanoma cells transfected with GLI2 siRNA (supplemental Fig. S1C).

Identification of Two Distinct Regulatory Regions Implicated in TGF-β Down-regulation of M-MITF Promoter—We used the Genomatix software MatInspector module for in silico analysis of putative transcription factor binding sites present within the −334/+1 region of the human M-MITF promoter (Fig. 2A). Results are summarized in Fig. 2A. Noteworthy, a previously undescribed putative Kruppel-like transcription factor binding site was identified at position −316.

A battery of deletion constructs of the human M-MITF promoter was assessed for transcriptional regulation by either exogenous TGF-β stimulation or GLI2N overexpression in transient cell transfection experiments with 888mel cells. As shown in Fig. 2B, efficient transcriptional repression (35–70%) of all 5′-end deletion constructs from −334 to −177 was observed in response to exogenous TGF-β (Fig. 2B, central panel). Interestingly, and in contrast to the results obtained with TGF-β, overexpression of GLI2N only inhibited the activity of constructs extending upstream of nucleotide −334, whereas further 5′-end deletions of the promoter led to complete loss of responsiveness to GLI2 overexpression (Fig. 2B, right panel).
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2B, right panel). These experiments gave matching results when repeated in 501mel cells (supplemental Fig. S2). Together, they identify the region −334/−296 of the M-MITF promoter as a GLI2-responsive region, whereas another regulatory element essential for transcriptional repression by TGF-β lies 3′ of position −177. Noteworthy, transcriptional activity of the proximal region of the M-MITF promoter downstream of −177 has been previously shown to largely depend upon the functionality of a CREB binding site at position −140 (20).

To further establish the respective implication of these two promoter regions in the context of GLI2 and/or TGF-β-mediated repression, 888mel human melanoma cells were transfected in parallel with −334/+136MMITFluc and −177/+136MMITFluc reporter constructs together with either empty pcDNA or GLI2ΔN expression vector before TGF-β stimulation. Although the activity of both constructs was dramatically repressed by TGF-β, only the longer one (−334/+136MMITFluc) was repressed by GLI2ΔN overexpression (Fig. 2C), in line with the data presented above. As expected, GLI2ΔN and TGF-β exerted an additive inhibitory activity on −334/+136MMITFluc. Also, the extent of TGF-β repression on −177/+136MMITFluc was similar in the absence or presence of GLI2ΔN overexpression. Together, these data demonstrate that GLI2 does not mediate TGF-β effect on M-MITF promoter inhibition.

Chromatin Immunoprecipitation Reveals GLI2 Binding to M-MITF Promoter—Our previous work identified melanoma cell lines with distinct levels of GLI2, directly associated with their invasive and metastatic capacity (8). Also, we determined that melanoma cell lines with high GLI2 expression have low M-MITF levels, whereas pigmented cell lines expressing M-MITF exhibit low GLI2 mRNA and protein levels (9). The latter is consistent with the above demonstration of transcriptional repression of M-MITF by GLI2. To study the possible recruitment of GLI2 to the GLI2-responsive region of the M-MITF promoter identified by 5′-end deletion studies between nucleotides −334 and −296 (see above), we performed a first set of chromatin immunoprecipitation (ChIP) experiments in three distinct melanoma cell lines previously shown to express high (1205Lu), intermediate (SKmel28), and low (888mel) levels of GLI2 protein (8). As shown in Fig. 3A, strong constitutive GLI2 recruitment to the −393/−206 region of the M-MITF promoter was observed in 1205Lu cells, although less and less detectable in SKmel28 and 888mel cells, thus following a pattern consistent with the respective steady-state expression levels of GLI2 in these cell lines.

As shown in Fig. 3B, left panel, GLI2 recruitment to the M-MITF promoter in SKmel28 cells was dramatically reduced after stable GLI2 knockdown, the latter also resulting in an up-regulation of M-MITF mRNA steady-state levels (right panel). On the other hand, in WM983A melanoma cells, in which we previously identified that TGF-β induces a solid SMAD-dependent transcriptional response (16, 17) as well as strong GLI2 induction (8), GLI2 recruitment to the M-MITF promoter was enhanced in response to TGF-β (Fig. 3C, left panel), accompanied with reduced M-MITF expression (right panel). From these experiments, we conclude that levels of M-MITF promoter occupancy by GLI2 in region −387/−296 are correlated with GLI2 expression levels.

Identification of Functional GLI2 Binding Site within Human M-MITF Promoter—Bioinformatics identified a potential binding site for KLFs; that is, CCTTCTCCAAA at position −316/−306 of the M-MITF promoter (see Fig. 2A). Given the data obtained from 5′-end promoter deletion (Fig. 2, B and C) and ChIP (Fig. 3) studies, we first hypothesized that this putative KLF binding site may bind GLI2. To address this issue, EMSA experiments were performed to analyze DNA-protein interactions within this M-MITF promoter region. As shown in Fig. 4A, incubation of nuclear extracts from 1205Lu melanoma cells, which strongly express GLI2, with a radiolabeled oligonucleotide representing region −334/−296 of the M-MITF promoter and encompassing the putative KLF binding site identified a slow-migrating band (lane 2) that was specifically eliminated by 100-fold excess unlabeled probe corresponding to a consensus GLI2 binding site (lane 3), not by a 100-fold excess consensus Sp1 oligonucleotide (lane 4). Furthermore, incubation of 1205Lu cell nuclear
extracts with an antibody against GLI2 before the addition of the radiolabeled probe in the binding mixture abrogated DNA-protein complex formation (lane 5), whereas an anti-TCF3 antibody used under the same conditions did not (lane 6). EMSA experiments performed with an additional radiolabeled oligonucleotide overlapping the KLF binding site (−334/−304) confirmed these results (supplemental Fig. S3A).

Next, the −316/−306 KLF/GLI2 binding site present in the −334/+136 M-MITF promoter reporter construct was inactivated by either multiple simultaneous point mutations (mutKLF) or nucleotide deletion (ΔKLF). Remarkably, parallel transient transfection experiments in 888mel cells revealed that both mutant constructs had increased basal activity (2–3-fold) as compared with their wild-type counterpart (Fig. 4B, open bars). Furthermore, unlike what we observed with the wild-type −334/+136 construct, neither of these mutants was inhibited by GLI2ΔN overexpression (Fig. 4B, solid bars). In 1205Lu cells, which express high constitutive levels of GLI2, both mutant constructs exhibited an even higher activity as compared with their wild-type counterpart (Fig. 4B, right panel), suggesting that endogenous GLI2 levels in melanoma cells actively repress M-MITF transcription via the −316/−306 KLF/GLI2 binding site identified above.

Together with the ChIP and EMSA experiments presented in Figs. 3 and 4A, these data unambiguously identify binding of GLI2 to the −316/−306 KLF/GLI2 binding site of the M-MITF promoter as critical for reduced M-MITF transcription in melanoma cells. Noteworthy, neither of the KLF binding site disruptions affected transcriptional repression of the M-MITF promoter by TGF-β (Fig. 4C), consistent with our results presented in Fig. 2 that identified the critical TGF-β responsive region within the proximal −177 nucleotides of the M-MITF promoter.

TGF-β Represses M-MITF Promoter Activity via Its Proximal CREB Binding Element Through Inhibition of Protein Kinase A Activity—A CRE at position −147/−140 of the M-MITF promoter was previously found critical for cAMP-induced M-MITF expression through recruitment of the transcription factor CREB (20). PKA phosphorylation of CREB at serine 133 in response to cAMP promotes binding of CREB dimers to CREs (a conserved palindromic 8-bp sequence TGAAGTCG) and allows their association with the transcriptional coactivators CBP and p300, thereby transactivating cAMP-responsive genes (25). Interestingly, we identified antagonistic interactions between the cAMP and TGF-β/SMAD pathways (26, 27), leading to opposite regulation of M-MITF and GLI2 expression in melanoma cells (9). We thus examined whether the proximal CRE may also play a role in mediating TGF-β effect. For this purpose, M-MITF promoter constructs were generated in which the CRE was inactivated by point mutation. Parallel transfection experiments of wild-type and CRE-mutated −334/+136 constructs in 888mel melanoma cells confirmed the importance of the CRE for promoter basal activity and revealed that integrity of the CRE is critical for TGF-β responsiveness (Fig. 5A, left panel). On the other hand, inhibition of M-MITF promoter activity by GLI2ΔN overexpression was similar in the presence or absence of an intact CRE (Fig. 5A, right panel), consistent with the results presented above implicating the −316/−306 KLF binding site for GLI2 responsiveness. Similar results were obtained when the CRE mutation was generated in the context of the longer −2135/+136 promoter construct (supplemental Fig. S4A). As expected, mutation of the CRE in the −296/+136 construct (which lacks the KLF binding site) rendered the promoter not only unresponsive to GLI2 overexpression but also to exogenous TGF-β (supplemental Fig. S4B). All these experiments were repeated in the 501mel melanoma cell line and gave sub-identical results (supplemental Fig. S4C).

To gain insight into the mechanism(s) by which the CRE is involved in TGF-β down-regulation of M-MITF promoter activity, we examined the effects of TGF-β on PKA and CREB-specific transcriptional activity. As shown in Fig. 5B, left panel, incubation of 1205Lu or 888mel melanoma cells with TGF-β over a 24-h period led to a 45–55% drop in constitutive PKA activity, while no effect was observed with SP6 transcriptome (Fig. 5B, right panel). Since PKA is the only known kinase responsible for CREB phosphorylation (25), these results further support the idea that PKA activation is critical for CREB-mediated transcription of M-MITF. Together these results have identified the CRE as a novel negative regulatory element of M-MITF. This CRE is conserved in a wide variety of melanoma cell lines, and while it is only weakly conserved in non-melanocytic cell lines, it can be activated by a number of stimulatory cues (e.g. cAMP). However, in a variety of melanoma cell lines, TGF-β is the only known stimulatory cue that induces CREB phosphorylation and M-MITF repression.
activity. This phenomenon was associated with reduced CREB phosphorylation levels (Fig. 5A, center panel) and CREB-dependent transcription, as measured in transient cell transfection experiments with the CREB-specific pCRE-luc reporter plasmid in both cell lines (Fig. 5A, right panel). Similar results were obtained in the 501mel cell line (supplemental Fig. S4).

We next determined the kinetics of TGF-β effect on both PKA activity and GLI2 expression in 888Mel melanoma cells. A rapid and dramatic (up to 75%) reduction in PKA activity was observed 30 min after TGF-β addition (Fig. 5C) followed by a partial and transient recovery at 2 h followed by prolonged secondary inhibition of PKA, around 50% of the initial enzyme activity, from 8 to 48 h. In parallel, maximal GLI2 mRNA induction was found at 8 h (~10-fold) and remained high (at least 6-fold above basal expression levels) over the course of the experiment, as measured by quantitative RT-PCR.

Simultaneous Inactivation of Both KLF Binding Site and CRE of M-MITF Promoter Is Required to Abolish Both TGF-β and GLI2 Inhibitory Activities—To definitely establish the functionality of both KLF and CREB binding sites within the M-MITF promoter, simultaneous inactivation of both sites was performed in the 334/136 M-MITF promoter constructs, which contains sufficient regulatory sequences to confer inhibition in response to either TGF-β or GLI2 (see Fig. 2). Responsiveness of the double mutant (mutKLF/mutCRE) promoter to TGF-β or GLI2N overexpression was compared with that of constructs in which only one of the two regulatory elements was nonfunctional (mutCRE or mutKLF). As shown in Fig. 6A, wt and mutKLF constructs were inhibited by TGF-β, whereas mutCRE and mutKLF/mutCRE were not. On the other hand, wt and mut-

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We next determined the kinetics of TGF-β effect on both PKA activity and GLI2 expression in 888Mel melanoma cells. A rapid and dramatic (up to 75%) reduction in PKA activity was observed 30 min after TGF-β addition (Fig. 5C) followed by a partial and transient recovery at 2 h followed by prolonged secondary inhibition of PKA, around 50% of the initial enzyme activity, from 8 to 48 h. In parallel, maximal GLI2 mRNA induction was found at 8 h (~10-fold) and remained high (at least 6-fold above basal expression levels) over the course of the experiment, as measured by quantitative RT-PCR.
CRE were inhibited by GLI2ΔN overexpression, not mutKLF or mutKLF/mutCRE (Fig. 6B). Thus, only the double mutant construct was unresponsive to both TGF-β and GLI2ΔN overexpression.

Together with the data presented in Fig. 5, these experiments suggest that TGF-β effect on M-MITF promoter activity occurs primarily via the proximal CRE. The repressed state of the M-MITF promoter may be maintained over time by additional mechanisms involving GLI2 acting through the KLF binding site. The integrity of the latter is not required for M-MITF repression by TGF-β.

**DISCUSSION**

Interest in studying the transcriptional control of M-MITF expression is motivated by the critical role played by this transcription factor at all stages of normal and pathological development of the melanocyte lineage (11, 12). Most critically, M-MITF is involved in various steps of melanoma progression. In particular, M-MITF is essential for melanoma cell proliferation through the control of cell cycle progression. In this study we have dissected the molecular mechanisms by which TGF-β down-regulates M-MITF gene transcription in melanoma cells. We identify two distinct mechanisms involved in TGF-β and GLI2 action. Using transient transfection assays and site-directed mutagenesis to inactivate the proximal CRE binding site of the M-MITF promoter, we determined that the latter is absolutely critical for inhibition of M-MITF transactivation by TGF-β. Inhibition of PKA activity by TGF-β results in reduced CREB phosphorylation, an essential step for full transcriptional activity of the CREB transcription factor. Another mechanism is implicated in the down-regulation of M-MITF expression by the GLI2 transcription factor. Using a combination of ChIP, EMSA, site-directed mutagenesis, and transient cell transfection assays, we found that GLI2 exerts a negative regulatory activity on M-MITF transcription via direct binding to a previously uncharacterized KLF/GLI2-specific cis-element located in region −324/−294 of the M-MITF promoter. We previously identified GLI2 as a transcriptional target of TGF-β signaling whereby gene transactivation occurs in response to TGF-β-dependent recruitment of both SMAD and β-catenin to the GLI2 promoter (6). GLI2, however, was not required for TGF-β effect on M-MITF transcription. A schematic representation of our findings is provided in Fig. 7. Yet, GLI2, which can accumulate in melanoma cells after TGF-β stimulation and possibly in response to other stimuli that remain to be identified, contributes to the long-term maintenance of low M-MITF levels in certain melanoma cell lines. Indeed, we have shown previously that melanoma cells that constitutively express high levels of GLI2 have very low M-MITF expression, whereas on the other hand, cell lines with high M-MITF levels express little GLI2 (9).

Although these findings represent a leap forward in the identification of novel mechanisms of M-MITF silencing in melanoma cells, the transcriptional repressors associated with GLI2 remain to be identified. One possibility may be that the oncoprotein c-Ski or its homolog, SnoN, abundantly expressed in melanoma cells (28, 29), may function as co-repressor for GLI2. They have previously been characterized as transcriptional corepressors of the SMAD transcription factors, as they recruit a repressor complex comprising N-CoR SMRT, Sin3A, and HDAC-1 to the target gene promoters, leading to gene silencing (30, 31). A two-hybrid screen with the N-terminal fragments of both GLI2 and GLI3 has found that both c-Ski and SnoN interact with GLIs and repress GLI-dependent transcription (32). Work is in progress to further characterize the mechanisms implicated in GLI2-driven M-MITF transcriptional repression and the possible implication of c-SKI/SnoN. The high functional redundancy of c-Ski and SnoN makes it a difficult task, as we have not been able thus far to perform efficient and concomitant knockdown of both proteins in melanoma cells.

**CONCLUSION**

We previously demonstrated the pro-invasive and pro-metastatic role played by TGF-β and GLI2 in various experimental settings to address melanoma cell behavior both in vitro and in vivo (16, 29, 33–35). This work extends our understanding of the transcriptional control of M-MITF expression by TGF-β and GLI2. In particular, we provide important new information regarding the fine-tuning of M-MITF promoter regulation by TGF-β and GLI2 and unveil the independent contribution of a known proximal CRE and that of a novel KLF/GLI regulatory element to, respectively, allow efficient transcriptional repression of M-MITF by TGF-β and GLI2.

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**REFERENCES**

1. Javelaud, D., and Mauviel, A. (2004) Mammalian transforming growth factor-β. Smad signaling and physiopathological roles. Int. J. Biochem. Cell Biol. 36, 1161–1165
2. Massagué, J., Seoane, J., and Wotton, D. (2005) Smad transcription factors.
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Genes Dev. 19, 2783–2810
3. Massagué, J. (2008) TGFβ in Cancer. Cell 134, 215–230
4. Meulmeester, E., and Ten Dijke, P. (2011) The dynamic roles of TGF-β in cancer. J. Pathol. 223, 205–218
5. Javelaud, D., Alexaki, V. I., and Mauviel, A. (2008) Transforming growth factor-β in cutaneous melanoma. Pigment Cell Melanoma Res. 21, 123–132
6. Dennler, S., André, J., Verrecchia, F., and Mauviel, A. (2009) Cloning of the human GLI2 promoter. Transcriptional activation by transforming growth factor-β via SMAD3/β-catenin cooperation. J. Biol. Chem. 284, 31523–31531
7. Dennler, S., André, J., Alexaki, V. I., Li, A., Magnaldo, T., ten Dijke, P., Wang, X. J., Verrecchia, F., and Mauviel, A. (2007) Induction of sonic hedgehog mediators by transforming growth factor-β. Smad3-dependent activation of GLI2 and Glil1 expression in vitro and in vivo. Cancer Res. 67, 6981–6986
8. Alexaki, V. I., Javelaud, D., Van Kempen, L. C., Mohammad, K. S., Dennler, S., Luciani, F., Hoek, K. S., Jüreà, P., Goydos, J. S., Fournier, P. J., Sibon, C., Bertolotto, C., Verrecchia, F., Saule, S., Delmas, V., Ballotti, R., Larue, L., Saiag, P., Guise, T. A., and Mauviel, A. (2010) GLI2-mediated melanoma invasion and metastasis. J. Natl. Cancer Inst. 102, 1148–1159
9. Javelaud, D., Alexaki, V. I., Pierrat, M. J., Hoek, K. S., Dennler, S., Van Kempen, L., Bertolotto, C., Ballotti, R., Saule, S., Delmas, V., and Mauviel, A. (2011) GLI2 and M-MITF transcription factors control exclusive gene expression programs and inversely regulate invasion in human melanoma cells. Pigment Cell Melanoma Res. 24, 932–943
10. Javelaud, D., Alexaki, V. I., Dennler, S., Mohammad, K. S., Guise, T. A., and Mauviel, A. (2011) TGF-β/SMAD/GLI2 signaling axis in cancer progression and metastasis. Cancer Res. 71, 5606–5610
11. Levy, C., Khaled, M., and Fisher, D. E. (2006) MITF. Master regulator of melanocyte development and melanoma oncogene. Trends Mol. Med. 12, 406–414
12. Steingrimsson, E., Copeland, N. G., and Jenkins, N. A. (2004) Melanocytes and the microphthalmia transcription factor network. Annu. Rev. Genet. 38, 365–411
13. Hoek, K. S., and Godin, C. R. (2010) Cancer stem cells versus phenotype-switching in melanoma. Pigment Cell Melanoma Res. 23, 746–759
14. Widmer, D. S., Cheng, P. F., Eichhoff, O. M., Belloni, B. C., Zipser, M. C., Schlegel, N. C., Javelaud, D., Mauviel, A., Dummer, R., and Hoek, K. S. (2012) Systematic classification of melanoma cells by phenotype-specific gene expression mapping. Pigment Cell Melanoma Res. 25, 343–353
15. Alexaki, V. I., Javelaud, D., and Mauviel, A. (2008) INK supports survival in melanoma cells by controlling cell cycle arrest and apoptosis. Pigment Cell Melanoma Res. 21, 429–438
16. Javelaud, D., Mohammad, K. S., McKenna, C. R., Fournier, P., Luciani, F., Niewolna, M., André, J., Delmas, V., Larue, L., Guise, T. A., and Mauviel, A. (2007) Stable overexpression of Smad7 in human melanoma cells impairs bone metastasis. Cancer Res. 67, 2317–2324
17. Rodeck, U., Nishiyama, T., and Mauviel, A. (1999) Independent regulation of growth and SMAD-mediated transcription by transforming growth factor-β in human melanoma cells. Cancer Res. 59, 547–550
18. Sasaki, H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H. (1999) Regulation of GlI2 and GlI3 activities by an amino-terminal repression domain. Implication of GlI2 and GlI3 as primary mediators of Shh signaling. Development 126, 3915–3924
19. Bertolotto, C., Bille, K., Ortonne, J. P., and Ballotti, R. (1996) Regulation of tyrosinase gene expression by cAMP in B16 melanoma cells involves two CATGGT motifs surrounding the TATA box. Implication of the microphthalmia gene product. J. Cell Biol. 134, 747–755
20. Bertolotto, C., Abbe, P., Hemesath, T. J., Bille, K., Fisher, D. E., Ortonne, J. P., and Ballotti, R. (1998) Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. J. Cell Biol. 142, 827–835
21. Sasaki, H., Hui, C., Nakafuku, M., and Kondoh, H. (1997) A binding site for Gli proteins is essential for HNF-3β floor plate enhancer activity in transgens and can respond to Shh in vitro. Development 124, 1313–1322
22. Andrews, N. C., and Faller, D. V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. 19, 2499
23. Kemp, E., Ji, J., Wernli, M., Häusermann, P., and Erb, P. (2008) GlI2 up-regulates cFlip and renders basal cell carcinoma cells resistant to death ligand-mediated apoptosis. Oncogene 27, 3856–3864
24. Verrecchia, F., Rossert, J., and Mauviel, A. (2001) Blocking sp1 transcription factor broadly inhibits extracellular matrix gene expression in vitro and in vivo. Implications for the treatment of tissue fibrosis. J. Invest. Dermatol. 116, 755–763
25. Mayr, B., and Montminy, M. (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev. Mol. Cell Biol. 2, 599–609
26. Schiller, M., Dennler, S., Anderegg, U., Kokot, A., Simon, C. J., Lugar, T. A., Mauviel, A., and Böhm, M. (2010) Increased cAMP levels modulate transforming growth factor-β/Smad-induced expression of extracellular matrix components and other key fibroblast effector functions. J. Biol. Chem. 285, 409–421
27. Schiller, M., Verrecchia, F., and Mauviel, A. (2003) Cyclic adenosine 3′,5′-monophosphate-elevating agents inhibit transforming growth factor-β-induced SMAD3/4-dependent transcription via a protein kinase A-dependent mechanism. Oncogene 22, 8881–8890
28. Chen, D., Lin, Q., Box, N., Roop, D., Ishii, S., Matsuizaki, K., Fan, T., Hornyk, T. J., Reed, J. A., Stavnezer, E., Timchenko, N. A., and Medrano, E. E. (2009) SKI knockdown inhibits human melanoma tumor growth in vivo. Pigment Cell Melanoma Res. 22, 761–772
29. Javelaud, D., van Kempen, L., Alexaki, V. I., Le Scolan, E., Luo, K., and Mauviel, A. (2011) Efficient TGF-β/SMAD signaling in human melanoma cells associated with high c-SKI/SnoN expression. Mol. Cancer 10, 2
30. Luo, K., Stroschein, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) The Ski oncoprotein interacts with the Smad proteins to repress TGFβ signaling. Genes Dev. 13, 2196–2206
31. Sun, Y., Liu, X., Eaton, E. N., Lane, W. S., Lodish, H. F., and Weinberg, R. A. (1999) Interaction of the Ski oncoprotein with Smad3 regulates TGFβ signaling. Mol. Cell 4, 499–509
32. Dai, P., Shinagawa, T., Nomura, T., Harada, J., Kaul, S. C., Wadhwa, R., Khan, M. M., Akimaru, H., Sasaki, H., Colmenares, C., and Ishii, S. (2002) Ski is involved in transcriptional regulation by the repressor and full-length forms of GlI3. Genes Dev. 16, 2843–2848
33. Mohammad, K. S., Javelaud, D., Fournier, P. G., Niewolna, M., McKenna, C. R., Peng, X. H., Duong, V., Dunn, L. K., Mauviel, A., and Guise, T. A. (2011) TGF-βRI kinase inhibitor SD-208 reduces the development and progression of melanoma bone metastases. Cancer Res. 71, 175–184
34. DiVito, K. A., Trabosh, V. A., Chen, Y. S., Chen, Y., Albanese, C., Javelaud, D., Mauviel, A., Simbulan-Rosenthal, C. M., and Rosenthal, D. S. (2010) Smad7 restricts melanoma invasion by restoring N-cadherin expression and establishing heterotypic cell-cell interactions in vivo. Pigment Cell Melanoma Res. 23, 795–808
35. Javelaud, D., Delmas, V., Möller, M., Sextius, P., André, J., Menashi, S., Larue, L., and Mauviel, A. (2005) Stable overexpression of Smad7 in human melanoma cells inhibits their tumorigenicity in vitro and in vivo. Oncogene 24, 7624–7629