Journal of Dermatological Science 94 (2019) 321–329

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Original Article

Transcriptional repression of the tyrosinase-related protein 2 gene by transforming growth factor-β and the Kruppel-like transcription factor GLI2

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ARTICLE INFO

Article history:
Received 21 September 2018
Received in revised form 21 February 2019
Accepted 1 April 2019

Keywords:
Tyrosine-related protein 2
Microphtalmia transcription factor
Melanoma
Transcriptional control
TGF-β
Kruppel-like transcription factor GLI2
CREB

ABSTRACT

Background: Tyrosinase-Related Protein 2 (TRP2) is an enzyme involved in melanogenesis, that also exerts proliferative, anti-apoptotic and immunogenic functions in melanoma cells. *TRP2* transcription is regulated by the melanocytic master transcription factor MITF. GLI2, a transcription factor that acts downstream of Hedgehog signaling, is also a direct transcriptional target of the TGF-β/SMAD pathway that contributes to melanoma progression and exerts transcriptional antagonistic activities against MITF.
Objectives: To characterize the molecular events responsible for TGF-β and GLI2 repression of *TRP2* expression.
Methods: In silico promoter analysis, transient cell transfection experiments with 5′-end *TRP2* promoter deletion constructs, chromatin immuno-precipitation, and site-directed promoter mutagenesis were used to dissect the molecular mechanisms of *TRP2* gene regulation by TGF-β and GLI2.
Results: We demonstrate that TGF-β and GLI2-specific *TRP2* repression involves direct mechanisms that occur in addition to MITF downregulation by TGF-β and GLI2. We identify two functional GLI2 binding sites within the *TRP2* promoter that are critical for TGF-β and GLI2 responsiveness, one of them overlapping a CREB binding site. GLI2 and CREB competing for the same cis-element is associated with opposite transcriptional outcome.
Conclusion: Our results further refine the understanding of how TGF-β and GLI2 control the phenotypic plasticity of melanoma cells. In particular, we identify critical GLI2-binding *cis*-elements within the *TRP2* promoter region that allow for its transcriptional repression independently from MITF concomitant downregulation.

1. Introduction

Melanin is produced and released by melanocytes in the basal layer of the skin and protects the epidermis from damage induced by UV radiation. Tyrosinase-Related Protein 2 (TRP2) is one of the enzymes involved in the synthesis of eumelanin [1]. TRP2 plays an important role in melanocyte detoxification [2], reduces DNA damage induced by free radicals and decreases the sensitivity of melanoma cells to oxidative stress [3]. TRP2 is also involved in the proliferation and morphology of cells from the melanocyte lineage [4,5]. Moreover, TRP2 may exert anti-apoptotic functions in melanocytes and melanoma cells [6,7]. TRP2 is strongly immunogenic [8,9] and belongs to the melanoma-associated antigens recognized by cytotoxic T lymphocytes [10,11]. As such, TRP2 is a target of choice for the development of immunotherapeutic strategies against melanoma [12].

The M isoform of Microphtalmia-associated transcription factor (M-MITF, herein referred to as MITF), a b-HLH-Z transcription factor, is a central regulator of the melanocyte lineage. It controls the migration, morphology, proliferation, survival and differentiation of melanocytes, leading to the synthesis of melanin pigments [13]. While loss of MITF has been described as a marker of melanoma progression [14], the role of MITF is complex as its expression is amplified in 10%–20% of melanoma cases [15,16], 4–6% according to the TCGA database, associated with higher aggressiveness of melanoma cells.

We previously demonstrated that expression of MITF, and that of several MITF target genes implicated in pigmentation including

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https://doi.org/10.1016/j.jdermsci.2019.04.001
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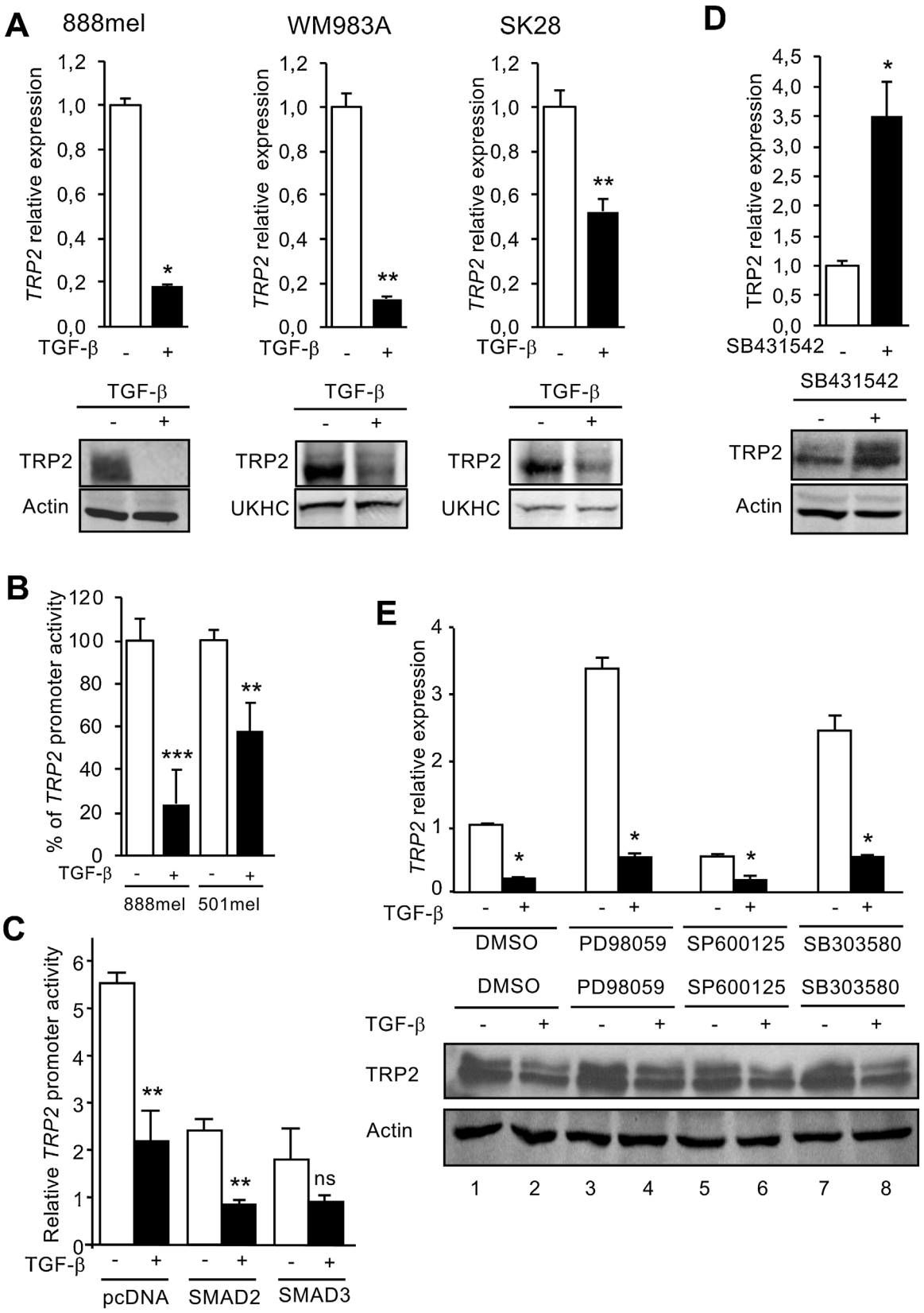

Fig. 1. TGF-β represses TRP2 expression at the transcriptional level. (A) 888mel, WM983A and SKMel28 human melanoma cells were incubated for 24 h without (-) or with (+) TGF-β, after which total RNA was extracted and *TRP2* transcript levels measured by quantitative RT-PCR (upper panel). Results are mean +/- s.e. of 2 (888mel) or 3 (WM983A and SKMel28) independent experiments, TRP2 protein levels were estimated by Western blotting in parallel dishes (lower panel). Actin or UKHC in the same samples served as a loading control. A representative blot is shown for each cell line. (B), 888mel and 501mel human melanoma cells were transiently co-transfected with the -585/+344 *TRP2*-luc promoter construct and pRL-MLP. Sixteen hours later, medium was changed and cells were incubated for 24 h without (-) or with (+) TGF-β (5 ng/ml). *TRP2* promoter activity was measured as luciferase activity corrected for *Renilla*. Results are mean +/- s.e. of 3 independent experiments, each performed with triplicate dishes. (C) 888mel human melanoma cells were transiently transfected with either empty (pcDNA), SMAD2 or SMAD3 expression vectors together with -585/+344 *TRP2*-luc and pRL-MLP.

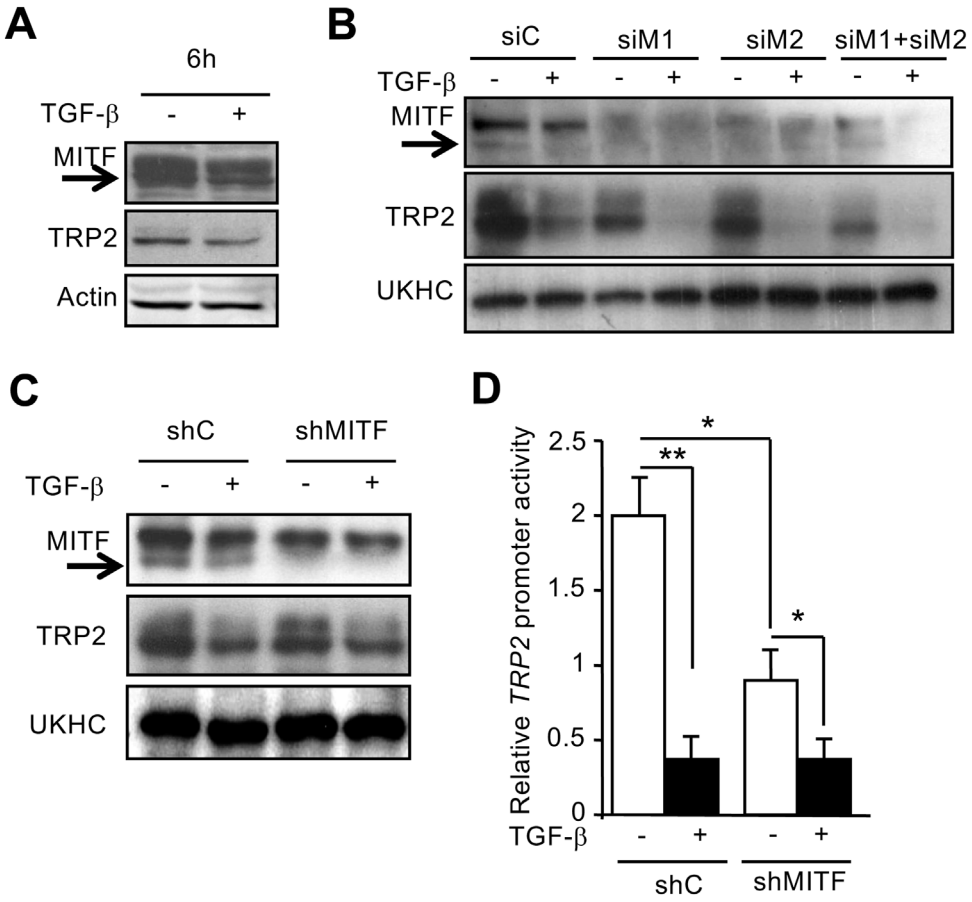

Fig. 2. TRP2 repression by TGF-β is partly independent from MITF downregulation. (A) 888mel human melanoma cells were incubated for 6 h without (-) or with (+) TGF-β (5 ng/ml), after which TRP2 and MITF protein levels were estimated by Western blotting. The arrow indicates the M-MITF isoform band. (B) 888mel human melanoma cells were transiently transfected with either control (siC) or MITF-specific siRNA oligonucleotides (siM1 and siM2), alone or in combination. 48 h later, medium was changed and cells were further incubated for 24 h without (-) or with (+) TGF-β (5 ng/ml), after which MITF and TRP2 protein levels were estimated by Western blotting. UKHC served as a control. (C) SKMel28 human melanoma cells transduced with either control (shC) or MITF (shMITF) shRNA lentiviral vectors were incubated without (-) or with (+) TGF-β (5 ng/ml) for 24 h, after which TRP2 and MITF protein levels were estimated by Western blotting. For panels A, B and C, results are from one (of three) representative experiment. (D) SKMel28 human melanoma cells transduced with either control (shC) or MITF (shMITF) shRNA lentiviral vectors were transiently transfected with the -585/+344 *TRP2*-luc construct and pRL-MLP. Sixteen hour later, medium was changed and cells were incubated for 24 h without (-) or with (+) TGF-β (5 ng/ml), after which *TRP2* promoter activity was measured. Results are mean +/- s.e. of 3 experiments, each performed with triplicate dishes. Differences between groups were estimated by unpaired Student t-test. ns: non-significant; *: $p < 0;05$; **: $p < 0.01$.

TRP2, is inversely correlated to that of GLI2, a transcription factor known as a critical mediator of the Sonic Hedgehog pathway [17], that we also characterized as a direct transcriptional target of the TGF-β/SMAD pathway [18,19]. GLI2 controls the invasive properties and the metastatic potential of melanoma cells by promoting a pseudo-epithelial mesenchymal transition characterized by loss of E-cadherin expression and increased matrix metalloproteinases (MMP-2 and MMP-9) secretion [20]. Also, GLI2 antagonizes MITF function and associated transcriptional program in melanoma cells, thus contributing to the plasticity and invasive behavior of melanoma cells whereby they switch back and forth between proliferative and invasive states to drive disease progression [17]. Also, the expression balance between MITF and GLI2 in melanoma cells is controlled in parallel by the TGF-β and PKA/cAMP pathways [17].

The *TRP2* promoter contains several critical regulatory elements, such as a preferential MITF binding site, referred to as M-box at position -138/-128, and a secondary MITF site, called E-box, at position -346/-340 [21,22]. Another critical element at position -249/-233, CREB-Binding Element (CRE)-like is involved in *TRP2* transactivation by cAMP [23]. Also, a distal enhancer enriched in HMG domains that bind LEF/TCF and SOX factors called DDE1 (Dct Distal Enhancer 1) in the -447/-416 promoter region is involved in the melanocyte lineage-specific expression of *TRP2* [24].

In this work, we have identified mechanisms of repression of TRP2 expression by TGF-β and GLI2, independent from concomitant MITF downregulation. *In silico* analysis of the TRP2 promoter identified putative GLI2 binding sites that were functionally characterized by means of 5′-end promoter deletion experiments,

Sixteen hours later, medium was changed and cells were incubated for 24 h without (-) or with (+) TGF-β (5 ng/ml), after which *TRP2* promoter activity was measured. Results are mean +/- s.e. of 2 experiments, each performed with triplicate dishes. (D), 888mel human melanoma cells were incubated for 24 h without (-) or with (+) the ALK5/TβRI inhibitor SB431542 (5μM), after which total RNA was extracted and *TRP2* transcript levels measured by quantitative RT-PCR. Results are mean +/- s.e. of 2 independent experiments, each performed with triplicate dishes. (E) 888mel human melanoma cells were incubated without (-) or with (+) DMSO, PD98059 (20μM), SP600125 (10μM) and SB303580 (20μM). One hour later, TGF-β (5 ng/ml) was added to the medium for 24 h, after which *TRP2* transcript levels were measured by quantitative RT-PCR (upper panel). TRP2 protein levels were estimated by Western blotting (lower panel). Actin served as an internal loading control. Results are mean +/- s.e. of 3 independent experiments. Where indicated, differences between groups were estimated by either two-tailed Mann-Whitney (panels A, B, D and E) or one-way Anova (panel C) tests. ns: non-significant; *: $p < 0;05$; **: $p < 0.01$; ***: $p < 0.001$.

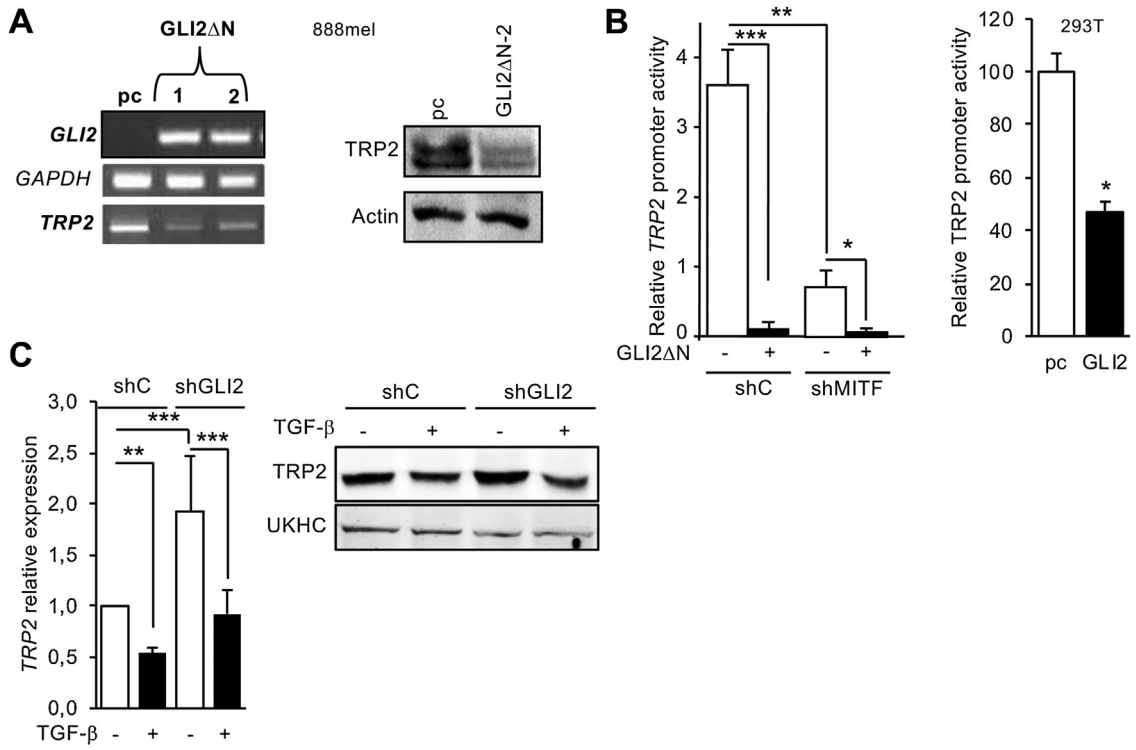

Fig. 3. TRP2 inhibition by GLI2 is independent from MITF downregulation. (A) 888mel human melanoma cells were stably transfected with either empty (pc) or constitutively active GLI2 mutant (GLI2ΔN) expression vectors. *GLI2* overexpression and *TRP2* transcript levels in the same samples were estimated by semi-quantitative RT-PCR in two distinct puromycin-resistant populations (1,2, left panel). Results were normalized with *GAPDH*. TRP2 protein levels were estimated by Western blotting in parallel dishes (right panel). Actin served as a control. Results are from one (of three) representative experiment. (B) SKMel28 human melanoma cells transduced with either control (shC) or MITF (shMITF) shRNA lentiviral vectors were transfected with either empty (-) or GLI2ΔN (+) expression vectors, the -585/+344 *TRP2*-luc construct and pRL-MLP. *TRP2* promoter activity was determined 48 h later (left panel). Differences between groups were using the unpaired Student t-test. ns: non-significant; **: $p < 0.01$; ***: $p < 0.001$. 293 T human embryonic kidney cells were co-transfected with the -585/+344 *TRP2* promoter luciferase construct without (pc) or with (GLI2ΔN) GLI2ΔN expression vector. Luciferase activity was measured 48 h later. Results are mean +/- s.e. of 3 experiments, each performed with triplicate dishes. Differences between groups were estimated using a two-tailed Mann-Whitney test. *: $p < 0;05$. (C) SKMel28 human melanoma cells transduced with either control (shC) or GLI2 (shGLI2) shRNA lentiviral vectors were incubated for 24 h without (-) or with (+) TGF-β, after which total RNA was extracted and *TRP2* transcript levels measured by quantitative RT-PCR (left panel). Results are mean +/- s.e. of 3 independent experiments, each performed with triplicate dishes. Differences between groups were estimated by one-way Anova. **: $p < 0.01$; ***: $p < 0.001$. TRP2 protein levels were estimated by Western blotting in parallel dishes (right panel). A representative blot is shown.

chromatin immune-precipitation and site-directed promoter mutagenesis.

2. Materials and methods

Human melanoma cell lines, described previously [20,25–27], were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS and antibiotics, at 37 °C, 5% CO2 in a humidified atmosphere. The MAPK inhibitors PD98059, SP600125, SB303580, and the ALK5/TβRI inhibitor SB431542 were purchased from Sigma-Aldrich (Saint-Louis, MO). TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MN).

The 5′-end deletion constructs of the human *TRP2* promoter cloned into pGL2-luc to positions -585, -475, -273, -220 have been described previously [23]. Additional 5′-end deletions to positions -412, -349 and -331 were generated by PCR and cloned as NheI/HindIII fragments into the same backbone vector (pGL2 basic, Promega, Madison, WI) with the following primers: forward (-412) 5′-CGCTGCTAGCTGGTTCTACATAAAT-3′; (-349): 5′-CTATGCTAGCA-CACATGTCAGAAAGC-3′; (-331): 5′- CTATGCTAGCCATGAGTG-CATTGTAGT-3′; reverse (+344): 5′-CGCGAAGCTTTTCTTTTCAGTAT-3′. Inactivation of the putative GLI binding site (*GACCCCCT*) at position -364/-371; of the putative C2H2 Zn Finger Transcription Factor binding site (*TCCCCA*) at -251/-246 position; and of the ubiquitous GLI putative binding site (*GAGGTCACA*) at -238/-230 position within the -412/+344 *TRP2* promoter construct were performed by point mutation (***TA*T*C*T***CCT*), (*T*T*C*T*T*A*), (***TA*TT*CACA*) respectively using the DpnI-based QuikChange site-directed mutagenesis methodology (Stratagene, La Jolla, CA). pRL-MLP vector [19] derived from pRL-TK vector (Promega), was used to monitor transfection efficiencies. The GLI-specific reporter plasmid $(GLI\text{-}BS)_8$-luc [28] was used to measure GLI2-dependent transcription.

Protein extraction and Western blotting were performed as previously described [17,29]. Mouse anti-MITF, rabbit anti-GLI2 antibodies and goat anti-TRP2 were purchased from Neomarkers (Thermo Scientific, Kalamazoo, MI), Cell Signaling (Ozyme, St-Quentin en Yvelines, France), and Santa-Cruz Biotechnology (Santa-Cruz, CA), respectively. Mouse monoclonal anti-actin and goat anti-UKHC, as well as secondary donkey anti-rabbit and anti-mouse HRP-conjugated antibodies were from Santa-Cruz Biotechnology.

For reporter assays, melanoma cells were seeded in 24-well plates and transfected at approximately 70–80% confluency in fresh medium containing 1% FCS with either Fugene™ (Roche Diagnostics, Indianapolis, IN, USA) or JetPEI (Polyplus Transfection Inc., New York, NY, USA). Luciferase activities were determined with a Dual-Glo™ luciferase assay kit (Promega), using a Fluoroskan Ascent FL (Thermo Labsystems). 888mel cells stably expressing GLI2ΔN have been described previously [17,29].

Infection of melanoma cells with lentiviral particles expressing either control, non-targeting, short hairpin RNAs (shRNAs) (shCtrl, Sigma-Aldrich SHC002 V) or shRNA targeting GLI2 (Sigma-Aldrich SHVRS clone ID TRCN0000033329 and TRCN0000033330), or

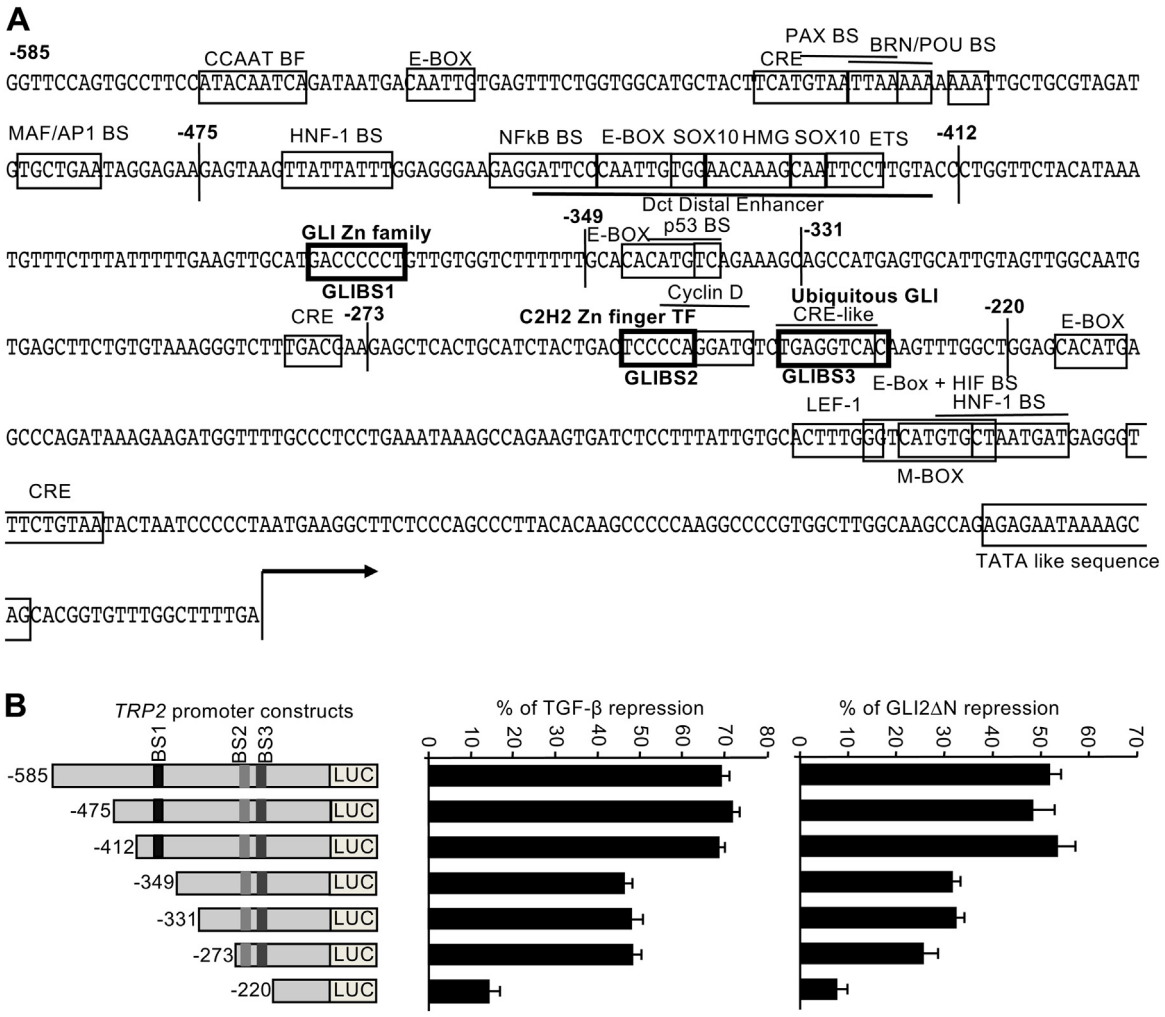

Fig. 4. TGF-β and GLI2 responsive elements within the human *TRP2* promoter. (A) Nucleotide sequence of the proximal (-585/+1) human *TRP2* promoter region and transcription factor binding sites identified with the MatInspector module of the Genomatix database (Matrix Family Library version 8.3). Note the presence of three putative GLI binding sites at positions -371/-364 (BS1); -251/-246 (BS2) and -238/-230 (BS3). (B) Effect of TGF-β (center panel) and GLI2ΔN overexpression (right panel) on the activity of a library of 5′-end deletion constructs of the human TRP2 promoter (left panel) in transient transfection experiments using 888mel melanoma cells. Luciferase activity was measured after overnight incubation. Results, expressed as % of inhibition by either TGF-β or GLI2 overexpression are mean + s.e. of 3 independent experiments, each performed with triplicate dishes.

shRNA targeting MITF (Sigma-Aldrich SHVRS clone ID TRCN0000019123) has been described previously [17,29]. Efficacy of GLI2 and MITF knockdown over time was verified by real time RT-PCR and Western blotting. Small interfering RNAs (siRNA) targeting GLI2 (Ambion/Applied Biosystems) were transfected into cells using the HiPerfect reagent (Qiagen). Control and MITF siRNAs (siM1) were previously described [30]. A second MITF siRNA (siM2) was also used to rule out the possibility of off-target effects [31].

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) methodologies have been described previously, together with primer sequences [17,20,29].

Chromatin immunoprecipitation was carried out using the ChIP-IT express kit (Active Motif, Rixensart, Belgium). Briefly, 7μg of enzymatically sheared chromatin were immunoprecipitated using 3μg of antibody against IgG or GLI2 (Santa Cruz Biotechnology), then incubated overnight at 4 °C with protein G beads. Precipitated DNA was used for PCR analysis using primers specific for -493/-253 and -272/-1 regions of the human *TRP2* promoter: forward, 5′- ATGTGCTGAATAGGAGAAGAGTAAG-3′; reverse, 5′-TCAGTAGATGCAGTGAGCTCTTC-3′ and forward 5′-ACGAAGAGCT-CACTGCATCTAC-3′; reverse 5′- ACACCGTGCTGCTTTTATTCT-3′, respectively. Amplification of GAPDH sequences was used as internal control with primers provided in the Active Motif kit. Amplimers were visualized with ethidium bromide after agarose gel electrophoresis.

For bioinformatics analyses, the Genomatix software package (Genomatix Software, München, Germany) was used. Two kb upstream and 0.2 kb downstream of the transcriptional start site of the human *TRP2* promoter [24] were extracted with help of the ElDorado module (release 4.6, Human Genome NCBI build 37/hg19). Identification of transcription factor binding sites was performed using the MatInspector module, together with Matrix Family Library version 8.3. All analyses were conducted with high threshold values (core similarity 1.0, matrix similarity >0.9/0.95).

3. Results

3.1. Canonical TGF-β signaling represses TRP2 expression

TGF-β strongly reduced the expression of TRP2 at the mRNA (Fig. 1A, upper panel) and protein (Fig. 1A, lower panel) levels in three distinct (888mel, WM983A and SKMel28) melanoma cell lines. The effect was largely transcriptional, as transient cell transfection experiments show that TGF-β represses *TRP2*

promoter activity in 501mel and 888mel melanoma cells which strongly express TRP2 [17] (Fig. 1B). Also, overexpression of SMAD2 and SMAD3 repressed *TRP2* promoter activity and enhanced TGF-β inhibitory activity (Fig. 1C). Inversely, incubation of cells with a pharmacological inhibitor of TβRI, SB431542, increased both TRP2 mRNA and protein levels, suggesting that autocrine TGF-β signaling is involved in the control of basal TRP2 levels in melanoma cells. On the other hand, pharmacological inhibition of the MAP kinase pathways ERK/MEK with PD98059, JNK with SP600125, and p38 with SB303580 had little or no effect on TGF-β-driven inhibition of *TRP2* expression, measured at both the mRNA (Fig. 1D, upper panel) and protein (Fig. 1E, lower panel) levels. Of note, basal TRP2 expression was increased upon p38 and ERK inhibition, suggesting that autocrine activity of both pathways regulates constitutive TRP2 expression.

3.2. TGF-β inhibition of TRP2 expression implicates mechanisms other than MITF downregulation

In 888mel cells treated with TGF-β for only 6 h, concomitant repression of MITF and TRP2 (84% and 64% respectively as estimated by scanning densitometry), was observed at the protein level (Fig. 2A). This is consistent with our previous work on MITF regulation by TGF-β [17]. Knockdown of MITF by means of specific siRNAs reduced TRP2 expression by up to 40%, yet allowed further inhibition of TRP2 by TGF-β, between 50–70% (Fig. 2B). This suggests that MITF repression is not the sole mechanism by which TRP2 expression is inhibited by TGF-β. Similar results were obtained in the SKMel28 cell line in which MITF levels were stably reduced by means of lentiviral expression of shRNAs directed against MITF (Fig. 2C). In the same cellular setting, reduced *TRP2* promoter activity was observed upon MITF knockdown, yet further inhibition by TGF-β was observed (Fig. 2D).

To determine whether MITF is implicated in the down-regulation of TRP2 expression by GLI2, a constitutively active truncated mutant form of GLI2 lacking the N-terminal repressor domain, GLI2ΔN, was first overexpressed in the 888mel melanoma cell line with high MITF and low GLI2 endogenous levels, as determined previously [17]. GLI2ΔN overexpression, verified by semi-quantitative Q-PCR, efficiently inhibited basal TRP2 expression (Fig. 3A), both at the mRNA (left panel) and protein (right panel) levels. Inhibition of the *TRP2* promoter by GLI2ΔN also occurred in SKMel28 cells with MITF knockdown (Fig. 3B left panel). Likewise, in human embryonic kidney 293T cells that does not belong to the melanocytic lineage and do not express M-MITF, overexpression of GLI2ΔN also inhibited *TRP2* promoter activity in transient cell transfection experiments (Fig. 3B right panel). Thus, neither M-MITF nor the cellular context of the melanocyte lineage, although biologically relevant, are necessary for transcriptional inhibition of TRP2 by GLI2 and TGF- β.

Stable knockdown of GLI2 by means of shRNA in SKMel28 melanoma cells identified a role for GLI2 to control basal TRP2 expression while it is not required for TGF-β inhibitory activity on TRP2, both at the mRNA (Fig. 3C, left panel) and protein (right panel) levels. TRP2 levels, both mRNA and protein, increased approximately 2-fold upon GLI2 knockdown, while TGF-β inhibitory effect (approximately 50% inhibition) was identical in shC and shGLI2 cells.

3.3. Identification of the TGF-β and GLI2 responsive regions of the TRP2 promoter

In silico analysis of the TRP2 promoter identified multiple putative binding sites, including, but not restricted to, an E-box, a M-box, several CRE-binding sites, a BRN/POU binding domain, as well as MAF1/AP1, HNF1 or NF-kB binding sites (Fig. 4A). Also, three putative binding sites for GLI transcription factors were identified: one at position -371/-364 (GACCCCCT), specifically identified by MatInspector as a GLI transcription factor binding site, and two others, closely positioned at -251/-246 and -238/-230, respectively labeled as a binding site for a zinc finger type transcription factor C2H2 (TCCCCA) and a binding site for ubiquitous GLI (GAGGTCACA). The latter fully overlaps a previously characterized CRE element [23].

To characterize TGF-β and GLI2 responsive regions within the *TRP2* promoter, we generated a battery of 5′end *TRP2* promoter deletion constructs (Fig. 4B, left panel). Transient cell transfection experiments identified two regions involved in the regulation of *TRP2* promoter activity by TGF-β (Fig. 4B, central panel) and GLI2ΔN overexpression (Fig. 4B, right panel). *TRP2* promoter activity was reduced by approximately 70% by TGF-β and up to 50% upon GLI2ΔN overexpression when at least 412bp upstream of the transcription start site of the promoter were present. The extent of repression by TGF-β was down to about 50%, and to 30% when overexpressing GLI2ΔN, for constructs further deleted down to position -273, and almost completely lost (less than 10%) with the shorter -220/+344 construct. This suggests the presence of at least two regulatory regions involved in the TRP2 repression modulated by GLI2 and TGF-β: one in the region -412/-349 and the other one in the region -273/-220.

3.4. GLI2 binds the TRP2 promoter

ChIP experiments were performed to determine whether GLI2 binds the two regions of interest identified above. As shown in Fig. 5A, in the 1205Lu melanoma cell line that expresses high endogenous GLI2 levels and low MITF and TRP2 levels [17], GLI2 is recruited to both the -498/-253 and -272/-1 regions of the *TRP2* promoter, not to the 1666bp *GAPDH* promoter fragment used as negative control. In SKMel28 cells, similar GLI2 recruitment was observed to both *TRP2* regions in control cells (left panel) while binding was no longer detectable in GLI2 knockdown cells (right panel). To address the possibility raised by the above *in silico* analysis that GLIBS3 overlaps with a functional CRE in the proximal region of the *TRP2* promoter and thus that GLI2 may interfere with CREB binding, we also performed ChIP assays with an anti-CREB antibody. Results shown in Fig. 5C demonstrate an absence of CREB recruitment in control SKMel28 cells while CREB is recruited to the same TRP2 promoter region in GLI2 knockdown cells (Fig. 5C). These results suggest a possible competition between GLI2 binding GLIBS3 and CREB binding the CRE-like motif, whereby GLI2 prevents CREB binding to the CRE element, resulting in reduced *TRP2* transcriptional activity.

3.5. Functional analysis of the putative GLI2-binding sites within the TRP2 promoter

Each of the three putative GLI2 binding sites identified by bioinformatics (Fig. 4A) were inactivated by point mutations and each of the mutant constructs tested in parallel transient cell transfection experiments in 888mel melanoma cells together with the wild-type construct for responsiveness to either TGF-β or GLI2 overexpression. Consistent with the results presented above, the wild-type *TRP2* promoter (-412/+344 construct) was strongly inhibited by both GLI2 overexpression (Fig. 5D) and TGF-β stimulation (Fig. 5E). Both GLIBS1 and GLIBS3 mutants were unresponsive to GLI2ΔN overexpression, while inhibition of the GLIBS2 mutant was similar to that of the wild-type promoter. This suggests therefore that transcriptional repression of the *TRP2* promoter by GLI2 occurs both through the -372/-364 (GLIBS1) and -238/-229 (GLIBS3) elements. TGF-β inhibition on the other hand was maintained even in the presence of the GLIBS1 and GLIBS2

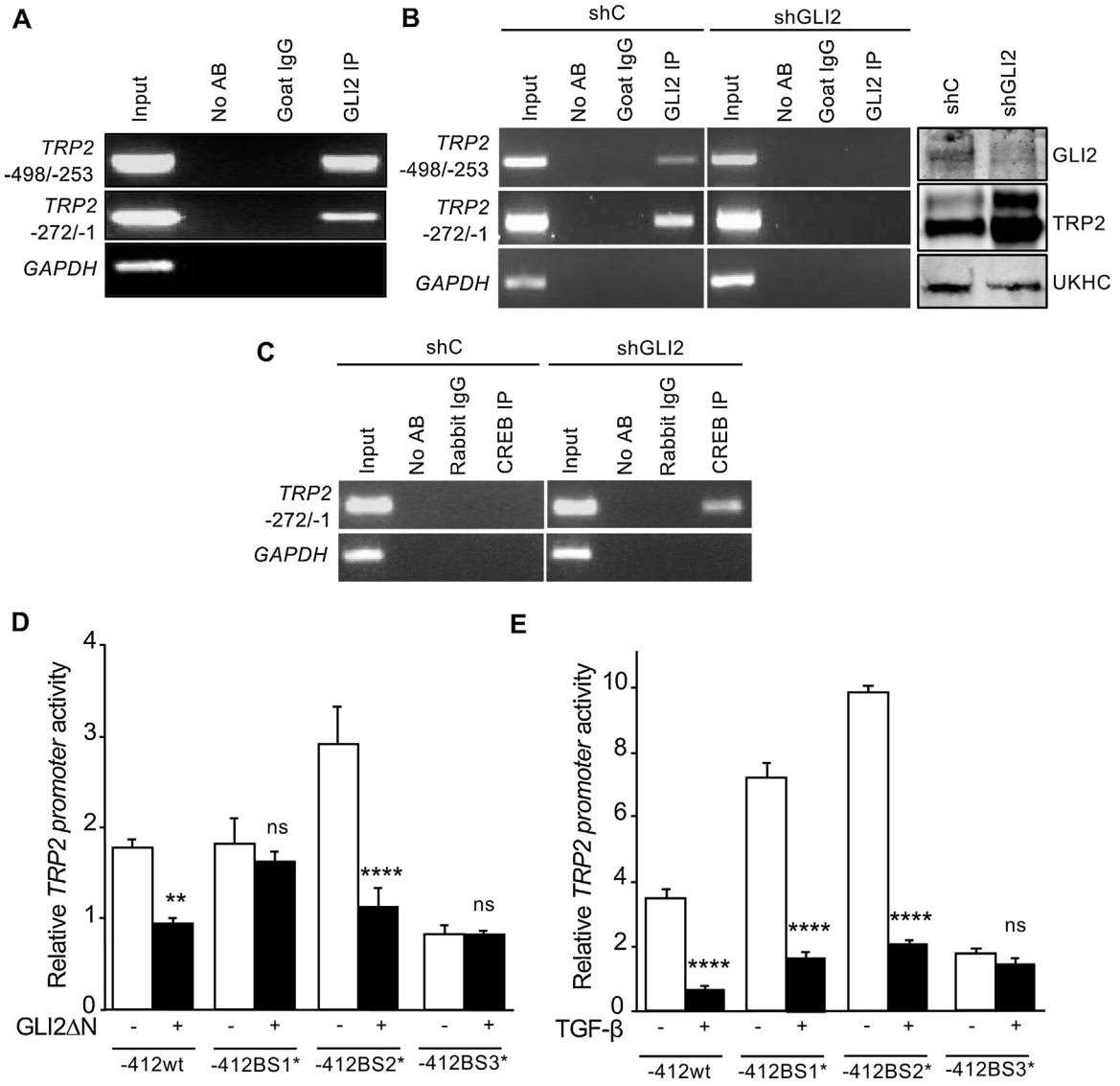

Fig. 5. Analysis of the putative GLI2 binding sites of the *TRP2* promoter. (A) Chromatin Immuno-precipitation (ChIP) analysis of GLI2 binding to the -498/-253 and to the -272/-1 regions of the *TRP2* promoter in the 1205Lu human melanoma cells (high endogenous GLI2 levels). (B) ChIP analysis of GLI2 binding to the -498/-253 and -272/-1 regions of the *TRP2* promoter in control (shC) and GLI2-knockdown (shGLI2) SKMel28 human melanoma cells (left panels). Efficacy of GLI2 knockdown was verified by Western blotting in parallel dishes (right panel). (C) ChIP analysis of CREB binding to the -272/-1 region of the *TRP2* promoter in control (shC) and GLI2-knockdown (shGLI2) SKMel28 human melanoma cells. In all experiments, amplification of a 1666-bp region of the human *GAPDH* promoter was used as a negative control, as recommended in the manufacturer's protocol. Results are from one representative (of three) experiment. (D). 888mel human melanoma cells were transiently transfected with empty pcDNA (-) or constitutively active GLI2 mutant GLI2ΔN (+) expression vector, together with either wt, BS1*, BS2* or BS3* -412/+344 *TRP2* promoter-luciferase constructs and pRL-MLP. Promoter activity was determined 48 h later. (E) 888mel human melanoma cells were transiently transfected with either wt, BS1*, BS2* or BS3* -412/+344 *TRP2* promoter-luciferase and pRL-MLP constructs. Sixteen hours later, medium was changed and cells incubated for 24 h without (-) or with (+) TGF-β (5 ng/ml). *TRP2* promoter activity was determined after overnight incubation. Results are mean +/- s.e. of 3 experiments, each performed with triplicate dishes. Differences between groups were estimated by one-way Anova. ns: non-significant; **: $p < 0.01$; ****: $p < 0.0001$.

mutations, and abolished by the GLIBS3 mutation (Fig. 5E). Also, basal activity of the GLIBS3 mutant construct, which overlaps a CRE-liked motif, has a lower basal activity than its wild-type counterpart, in accordance with the importance of the CRE for promoter basal activity [23].

4. Discussion

In melanoma, phenotypically distinct cell subpopulations have been identified, that are either highly proliferative and poorly invasive or slow-growing and highly invasive. These cells may switch from one state to the other in a phenomenon known as phenotypic switching whereby aggressive solid tumors to rapidly adapt to micro-environmental changes and escape genotoxic therapy (reviewed in [32]). In the case of melanoma, the phenotypic switching model is supported by the observation that cells expressing high levels of MITF have low invasive capacity, while cells expressing low or no MITF are highly invasive [33]. Remarkably, cells expressing high levels of GLI2 have low MITF and melanin levels, and these expression profiles appear to be independent from the BRAF or NRAS oncogenic mutation status of melanoma cells [17]. Analysis of more than 500 cell lines derived from melanoma tumors indicates that these properties are largely mutually exclusive and are defined by distinct gene expression signatures [34].

GLI2 exerts direct transcriptional repression against MITF [29] and its expression is anti-correlated with a number of markers of

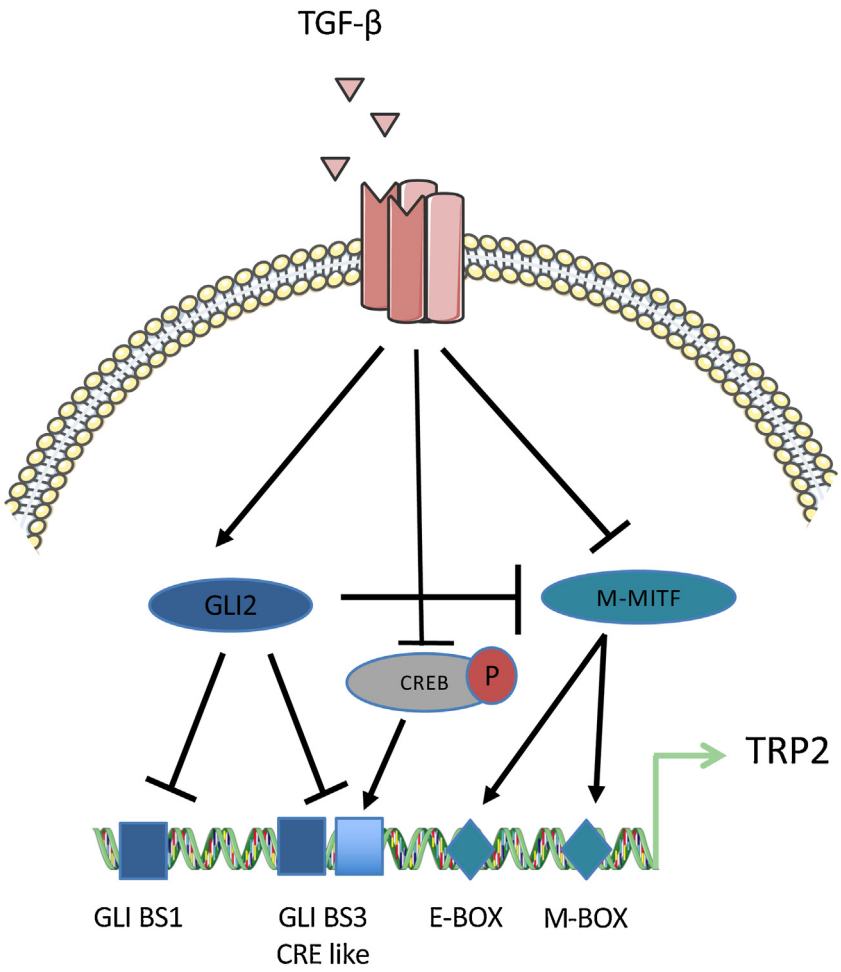

Fig. 6. Schematic representation of human *TRP2* promoter downregulation by TGF-β and GLI2. Constitutive *TRP2* promoter activity is dependent upon binding of CREB to a CRE-like cis-element and of MITF to both an E-BOX and a proximal M-BOX. TGF-β acts on CREB-driven transcription by downregulating PKA activity [29]. Also, TGF-β inhibits MITF transcription [29]. Both mechanisms may contribute to reduce TRP2 expression in melanoma cells. TGF-β induces GLI2 and GLI2 inhibits TRP2 transcription via two distinct GLI2 binding sites at positions -371/-364 and -238/-230. Endogenous GLI2 levels therefore control basal TRP2 expression. The most proximal GLI2 binding site overlaps a critical CRE-like domain [23] and GLI2 binding prevents that of CREB to the same promoter element. This Figure was created using Servier Medical Art Template (SMART, which is licensed under a Creative Commons unported license attribution 3.0 https://smart.servier.com.

melanocytic differentiation, in particular enzymes involved in the biosynthesis of melanin, including TRP2 [17]. GLI2 also upregulates the expression of the EMT-inducing transcription factor ZEB1 and represses E-cadherin expression [35]. It could therefore represent a critical regulator of the pseudo-EMT undergone by transformed melanocytes during the critical transition of melanomas from radial growth phase to vertical growth phase, leading to metastatic progression. We previously demonstrated that TGF-β represses *MITF* promoter activity via inhibition of protein kinase A, resulting in decreased CREB activity and binding to specific cis-elements within the *MITF* promoter [29]. CREB is also known to regulate TRP2 expression at the transcriptional level and it is thus highly likely that TGF-β-driven inhibition of PKA activity and MITF expression in melanoma cells contributes to its inhibitory effect on TRP2 as well. Yet, in this work, we have determined that TRP2 is not only sensitive to the cellular levels of MITF, but is also downregulated by GLI2-driven mechanisms. GLI2, induced rapidly by TGF-β, exerts a dual repressor activity on the *TRP2* promoter. GLI2 binds a consensus GLI2-specific site at position -372/-364 that allows for transcriptional repression of the promoter, and binds another element which overlaps a CRE at position -238/-230. Binding of GLI2 to the latter element prevents CREB binding, which is critical for TRP2 basal expression [23]. Thus, a complex regulatory network of transcription factors and signaling pathways is able to regulate TRP2 expression in melanoma cells, whereby TGF-β orchestrates multiple complementary mechanisms, from downregulation of MITF expression to induction of GLI2 which either directly represses *TRP2* promoter activity or interferes with CREB binding to an active positive cis-element. A schematic of the various steps leading to TRP2 gene repression is provided in Fig. 6. These data bring new insights into the transcriptional control of genes involved in melanogenesis by TGF-β. Interestingly, in the context of oncogenesis and melanoma in particular, TGF-β also acts as potent inhibitor of the anti-tumoral immune response. The immunosuppressive activity of TGF-β is driven by its ability to promote T-cell suppression, increasing T-Regs against NK cells. In turn, T-Regs interfere with T-cell priming and maintain tolerance against tumor cells (Reviewed in [36,37]). Remarkably, as TRP2 represents an important antigen for immunotherapy of melanoma [12], such transcriptional repression of TRP2 in melanoma cells by TGF-β may represent an additional mechanism that contributes to the pro-oncogenic activities of TGF-β and further potentiates tumor escape to immune clearance.

Ethical approval

Not applicable

Conflict of interest

The authors have no conflict of interest to declare

Funding

Funded by grants from Ligue Nationale Contre le Cancer (Equipe Labellisée LIGUE-2016), Institut National du Cancer (INCa MELA13-002) and Donation Henriette et Emile Goutière (to A.M), Fondation ARC (to D.J.), and institutional funding from INSERM, CNRS, Institut Curie, and Université Paris-Sud.

Acknowledgements

We thank Drs. Robert Ballotti and Corine Bertolotto (INSERM U895, Nice, France), Rune Toftgard (Karolinska Institutet, Stockholm, Sweden), and Hiroshi Sasaki (Osaka University, Japan) for sharing reagents.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.04.001.

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