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Original Article

Mechanism underlying synergic activation of Tyrosinase promoter by MITF and IRF4

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Abstract: Background: The transcription factor interferon regulatory factor 4 (IRF4) was identified to be involved in human pigmentation by genome-wide association studies (GWASs). The rs12203592-[T/C], which is located in intron 4 of IRF4, shows the strongest link to these pigmentation phenotypes including freckling, sun sensitivity, eye and hair color. Previous studies indicated a functional cooperation of IRF4 with Microphthalmia-associated transcription factor (MITF), a causing gene of Waardenburg syndrome (WS), to synergistically trans-activate Tyrosinase (TYR). However, the underlying mechanism is still unknown. Methods: To investigate the importance of DNA binding in the synergic effect of IRF4. Reporter plasmids with mutant TYR promoters was generated to locate the IRF4 DNA binding sites in the Tyrosinase minimal promoter. By building MITF and IRF4 truncated mutations plasmids, the necessary regions of the synergy functions of these two proteins were also located. Results: The cooperative effect between MITF and IRF4 was specific for TYR promoter. The DNA-binding of IRF4 was critical for the synergic function. IRF4 DNA binding sites in TYR promoter were identified. The Trans-activation domains in IRF4 (aa134-207, aa300-420) were both important for the synergic function, whereas the auto-mask domain (aa207-300) appeared to mask the synergic effect. Mutational analysis in MITF indicated that both DNA-binding and transcriptional activation domains were both required for this synergic effect. Conclusions: Here we showed that IRF4 potently synergized with MITF to activate the TYR promoter, which was dependent on DNA binding of IRF4. The synergic domains in both IRF4 and MITF were identified by mutational analysis. This identification of IRF4 as a partner for MITF in regulation of TYR may provide an important molecular function for IRF4 in the genesis of melanocytes and the pathogenic mechanism in WS.

Keywords: MITF, IRF4, TYR, synergy effects, transcriptional activation

Introduction

Pigmentation is the most visible trait in humans. Nearly 200 genes have been identified in mice that play a role in pigment system affecting various steps in the development of melanocytes, a cell population derived from the neural crest. Several common variations associated with abnormal pigmentation have been identified from these genes. In a recent genome-wide association study (GWAS), several sequence variants on interferon regulatory factor 4 (IRF4) were linked to human pigmentation [1-3].

Variants in the IRF4 have been suggested to be associated with specific pigmentation phenotypes. The rs12203592 located in intron 4 of IRF4 show the strongest link to these pigmentation phenotypes [2-5]. IRF4 belongs to the Interferon Regulatory Factors (IRFs), a family of wing-helix-turn-helix structure forms of transcription factors initially identified as downstream regulators of interferon signaling. As previously described, it is mainly express in cells of the immune system where it transduces signals from various receptors to activate or repress expression of key regulators of lym-

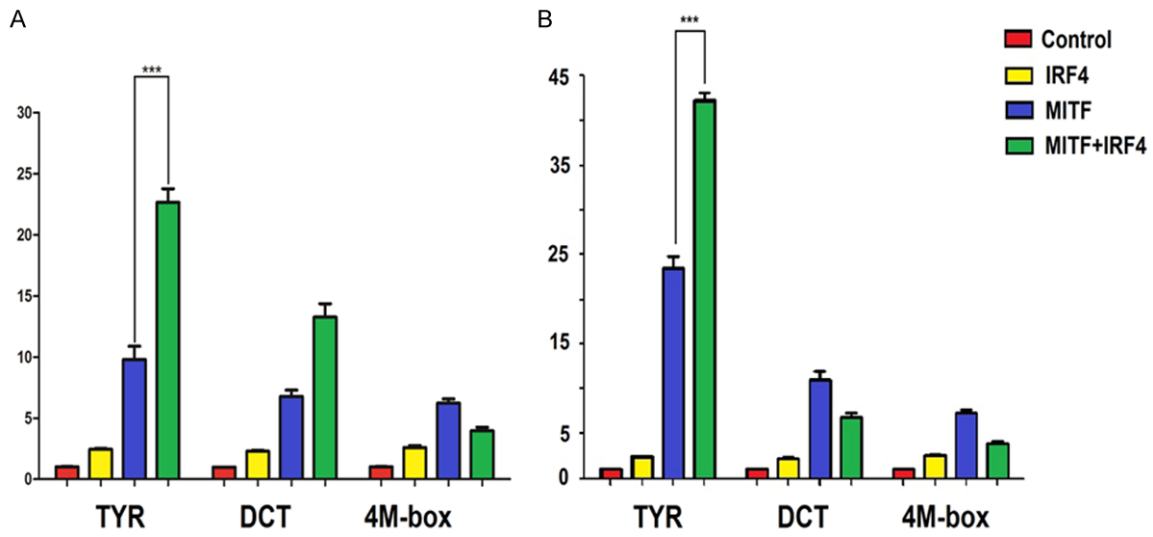

Figure 1. The effect of MITF and IRF4 on the TYR, DCT and 4M-box promoters in HEK293 cells (A) and UACC903 melanoma cells (B). HEK293T cells and UACC903 melanoma cells were transfected with MITF, IRF4 or MITF+IRF4 expression plasmids together with different report plasmids. The red bars (Control) indicate co-transfection with the empty vector as a negative control. Each value represents the mean ± SD of three replicates from a single assay. The results shown were representative of at least three independent experiments (***P<0.001 compare to the value from the MITF and MITF+IRF4, unpaired Student's t-test).

phoid, myeloid, and dendritic cell development. A few studies have mentioned the association of IRF4 with pigmentation. The expression of IRF4 was found and identified in the skin and the G361 melanoma cell lines and correlated with MITF expression in melanoma cells [6-8].

Waardenburg syndrome is a clinically rare genetic disorder, characterized by pigmentation-related syndromic deafness. Its main clinical phenotypes are deafness and pigmentation anomalies, the latter of which are majorly manifested as heterochromia iridum, white forelock, premature graying of the hair, skin hypopigmentation or hyperpigmentation [9-11]. As one of the important causing genes of WS, The transcription factor *MITF* plays an critical role in the induction of melanocytes and is also necessary for their survival and/or differentiation. MITF was shown to regulate several genes involved in pigmentation, including the tyrosinase and tyrosine-related genes, *TYRP1* and *DCT*, by binding to their promoters through an E box motif (CANNTG).

Functional analysis indicates that IRF4 and MITF cooperate to activate transcription of TYR [4], but the mechanism of this synergy still remains unclear. To date, due to the high genetic heterogeneity in WS, there are still a significant number of patients with an unidentified disease-causing gene.

Methods and materials

Plasmids constructions

MITF expression plasmid (pCMV-Flag-MITF) and TYR promoter reporter plasmid (pGL3-Tyr-Luc) were described previously [12, 13]. DCT (Another enzyme important during eumelanogenesis) and 4M-box promoter (a synthetic construct with 4 M-boxes in a row) reporter plasmid were kindly provided by Prof Hideki Murakami. The wild-type human IRF4 cDNA (NM_002460.3) was cloned into the pcDNA3.0-HA. To map the IRF4 DNA binding site in TYR promoter, a series of mutant TYR-luc constructs were synthesized by the Sangon Biotech (Shanghai) company and verified by sequencing (**Figure 2B**). To map the synergic domain of IRF4 and MITF, several mutant constructs were generated by PCR and cloned into pcDNA3 and pCMV separately. All constructs were verified by sequencing.

Transfection and luciferase assay

HEK293T (human embryonic kidney) and melanoma UACC903 cells were maintained in Dulbecco's modified Eagle medium (DMEM)

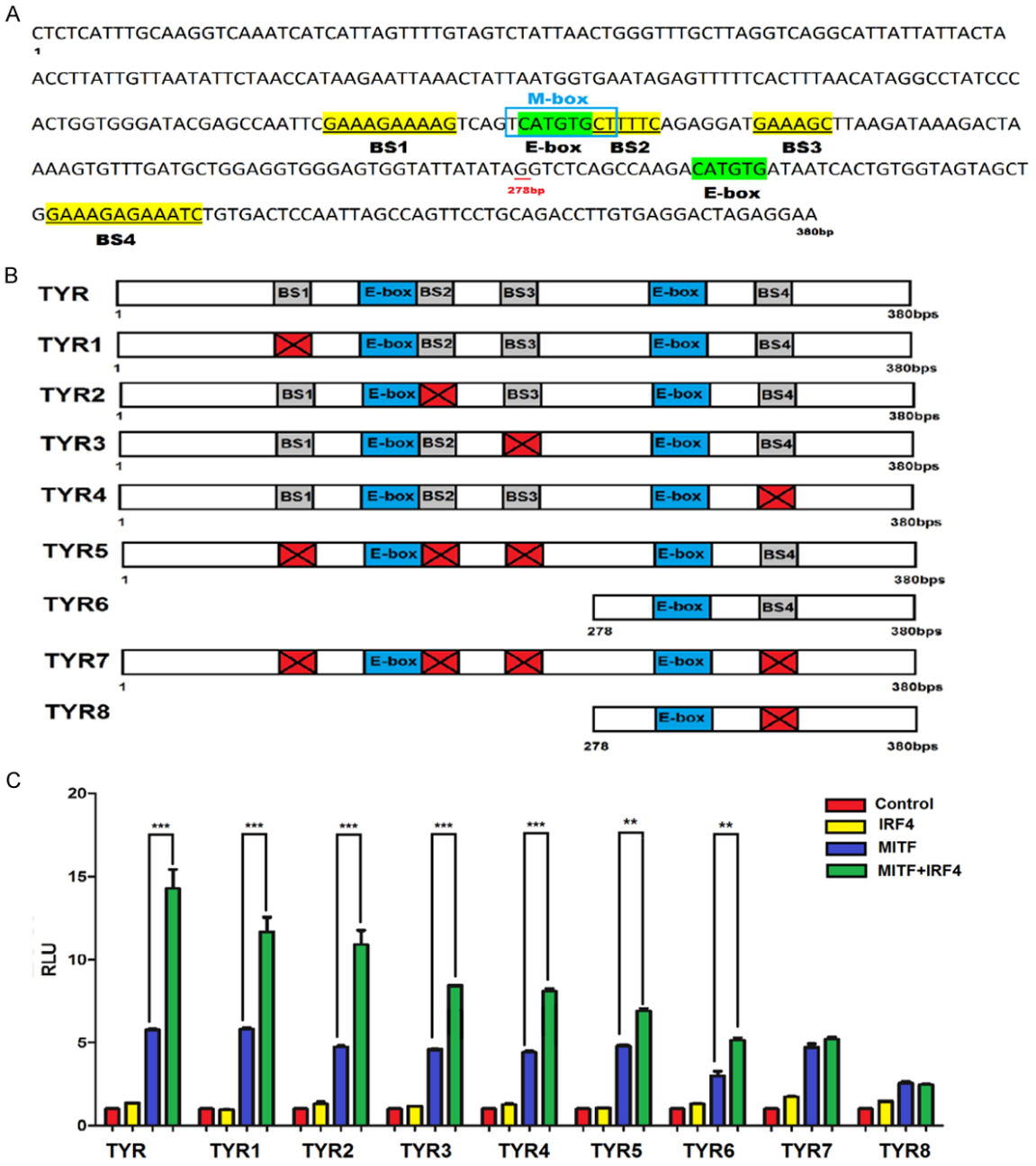

Figure 2. A. The human TYR promoter (-300 to 80) shows the sequence of the MITF binding sites (E-box and M-box) and potential IRF4-binding sites (BS1-BS4). B. Schematic view of mutant TYR promoters (TYR1-TYR8). C. Effect of MITF, IRF4 on mutant TYR promoters(TYR1-TYR8) determined by luciferase activity assays in HEK293 cells. The red bars (Control) indicate co-transfection with the empty vector as a negative control. The WT and 8 mutant luciferase reporter plasmids were respectively transfected into 293T cells in combination with MITF, IRF4 and MITF+IRF4 expression plasmids. Each value represents the mean ± SD of three replicates from a single assay. The results shown were representative of at least three independent experiments (**P<0.01, ***P<0.001 compare to the value from the MITF and MITF+IRF4, unpaired Student's t-test).

supplemented with 10% fetal bovine serum (FBS), 100 U/ml of Penicillin/Kanamycin, NEAA, Fungzone and cultured in an incubator at 37°C with 5% CO_2. HEK293T and UACC903 cells were seeded in 24-well plates and transfected with expression and report plasmids using Fugene transfection reagent (Promega) according to the manufacturer's protocol. For measur-

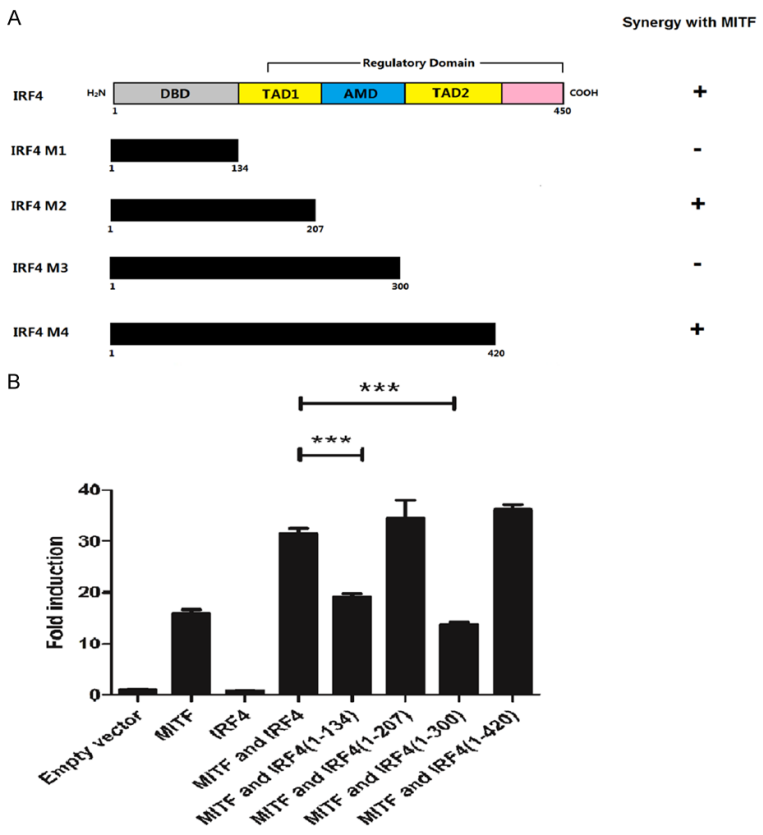

Figure 3. Identification of IRF4 sequences involved in synergic transactivation with MITF. A. A schematic representation of the full length IRF4 and various truncated IRF4 proteins. DBD, DNA binding domain. AMD, automatic mask domain. TAD, Trans-activation domain. B. HEK293T cells were transiently transfected with the TYR luciferase reporter and full length or truncated IRF4 expression plasmids including with MITF. Luciferase activity is reported as a fold increase relative to reporter alone. Each value represents the mean ± SD of three replicates from a single assay. The results shown were representative of at least three independent experiments (***P<0.001 compare to the value from the wt IRF4 and different IRF4 mutants, unpaired Student's t-test).

ing MITF and IRF4 synergy effect on different promoter study, the IRF4-DNA binding site searching study and the IRF4 and MITF synergy domain identification study, each well contains 250 ng expression plasmids with 166 ng of the reporter plasmids.

Additionally, transfection of 66 ng of pGL3 control vector (Cat.# E1741) served as a transfection control and was used to normalize luciferase activity for each well. Total amounts of transfected DNA were kept constantly at 750 ng per well by the empty vector. At 48 h post-transfection, cells were washed with PBS and lysed with 1× Reporter Lysis Buffer (Promega). The extract proteins were assayed for firefly and renilla activity. Both of the assays were performed using Duel Luciferase firefly Assay System (Promega) according to the manufacturer's protocol and determined using SIRIUS luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). All report assays were repeated at least triplicately each time. Data were analyzed using Prism 5 software (GraphPad, Software Inc., San Diego, CA, USA).

Results

IRF4 synergizes with MITF to activate the TYR promoter specifically

We first tested the ability of MITF alone or in combination with IRF4 to transactivate the TYR promoter in pigmentation cells and non-pigmentation cells. As shown in **Figure 1**, in both kinds of cells, transfection with MITF increased the TYR promoter activity as compared with that of the empty vector control. IRF4 alone did not affect TYR promoter significantly. However, IRF4 was able to augment the ability of MITF to transactivate the TYR promoter.

As also shown in **Figure 1**, MITF was also able to activate 4M-box and DCT promoter alone, but did not show synergic effect on these two promoters with IRF4. Hence, the cooperative effects were specific for TYR promoter.

Analysis of the IRF4 DNA binding sites in the TYR promoter

There were four potential IRF4 binding sites (BS1-4) in the Tyrosinase minimal promoter (**Figure 2A**). To further investigate the importance of DNA binding in the synergic effect of IRF4, we generated eight reporter plasmids with mutant TYR promoters (**Figure 2B**).

Mutation one, two, three of these IRF4 DNA binding sites (TYR1/TYR2/TYR3/TYR4/TYR5)

did not disrupt the cooperative effects of MITF and IRF4. However, IRF4 failed to argument the trans-activity of MITF on the promoter with all four IRF4-binding sites mutated (TYR7). We further make a truncated reporter including E-box and BS4 only (TYR6). As shown in **Figure 2C**, MITF was still able to transactivate TYR6, indicated E-box alone was sufficient to initiate the transactivation of TYR by MITF. Importantly, IRF4 retained its synergic effect on TYR6, indicating BS4 alone was sufficient for the synergic effect. Nevertheless, The synergic effect of IRF4 was lost on TYR7 with only functional E-box, confirming IRF4-binding sites (BS1-BS4) were all required for the synergy.

Functional synergic domain in IRF4 and MITF

To map the functional domains in IRF4 that are critical for the synergic effect, we generated a series of C-terminal truncated IRF4 expression plasmids (**Figure 3A**). As shown in **Figure 3B**, full-length IRF4 (aa1-450), IRF4 (aa1-420) and IRF4 (aa1-207) showed similar synergic effect on TYR promoter. However, IRF4 (aa1-300) and IRF4 (aa1-134) lost the synergic effect with MITF.

Further, we also localized the domain of MITF that is required for its transcriptional synergy with IRF4 by a series of truncated MITF mutant constructs, which were designed based on the known functional domains of MITF (**Figure 4A**). Synergy effect with IRF4 on TYR promoter was only showed in the truncated MITF aa1-293 and aa185-419, both of which containing the bHLH/LZ domain and trans-activation domain (N-terminal or C-terminal individually). Furthermore, The mutant MITF aa1-185, aa185-293 and aa293-419 nearly completely abolished synergy (**Figure 4B**).

Discussion

MITF encodes a member of MYC superfamily transcription factor containing the bHLH-Zip domain. The basic domain used for DNA binding, whereas the HLH and Zip domains are used for homo- and/or heterodimer [14, 15]. Tyrosinase is one of the key enzymes in melanin biosynthesis. It is encoded by the *TYR* gene which is specifically expressed in differentiated melanocytes [16]. MITF alone can binds to the specific E-box motif (CANNTG) within the promoter of TYR and initiates its transcription [16, 17]. Three conserved E-box motifs have been located in the TYR promoter, named as initiator E-box (position -12 to -7), the M-box (position -104 to -99) and the TDE (tyrosinase distal element) (position -1972 to -1967) respectively. The M-box (an E-box with a flanking T at the 5' end and CT at the 3' end of the CATGTG sequence) has been shown to be essential for the activation of the tyrosinase promoter by MITF. The initiator E-box, and TDE, also act to further increase the level of tyrosinase expression [16-18]. Kluppel *et al*. found that a 270 bp upstream region is sufficient for specific expression in melanocytes and pigmented epithelium of the retina [19]. Our "minimal TYR promoter" (-300 to +80) contains M-box and initiator E-box motifs.

Quantitative ChIP experiments showed that IRF4 may bind at the sites of a proximal (pTYR; around the transcription start site) and a more distant region (dTYR; >1,800 bp upstream of transcription start site) which in both cases is consistent with the presence of E-box motifs in these positions of the TYR promoter [4].

As shown in **Figure 1**, Unlike the TYR promoter, Wild type MITF is able to transactivate the DCT promoter and the artificial promoter containing 4 copies of the M-box (4M-box), but no cooperative effects could be observed with IRF4. IRF4 contains an N-terminal DNA-binding domain that recognizes GAAA/TTTC core motifs [20]. Four potential IRF4 binding sites (BS1-4) in the our Tyrosinase minimal promoter were identified by sequencing (**Figure 2A**). Since there is no IRF4 DNA binding site on DCT or 4M-box promoter by sequencing. Mutating analysis also showed that synergistic effects of MITF and IRF4 on the TYR promoter are mediated through all these IRF4 binding sites. All these results indicated that the DNA binding of IRF4 is required for synergy.

Map the IRF4 and MITF functional synergic domains

The IRF4 protein can be basically divided into two parts: DNA binding domain (DBD), aa1-134 and Functional regulatory domain, aa135-450 (**Figure 3A**). The DBD contains five conserved tryptophan residues [21]. The Functional regulatory domain contains two transactivation domains (TAD): TAD1, aa134-207 and TAD2, aa300-420 and an auto-masking domain (AMD), aa207-300 [20].

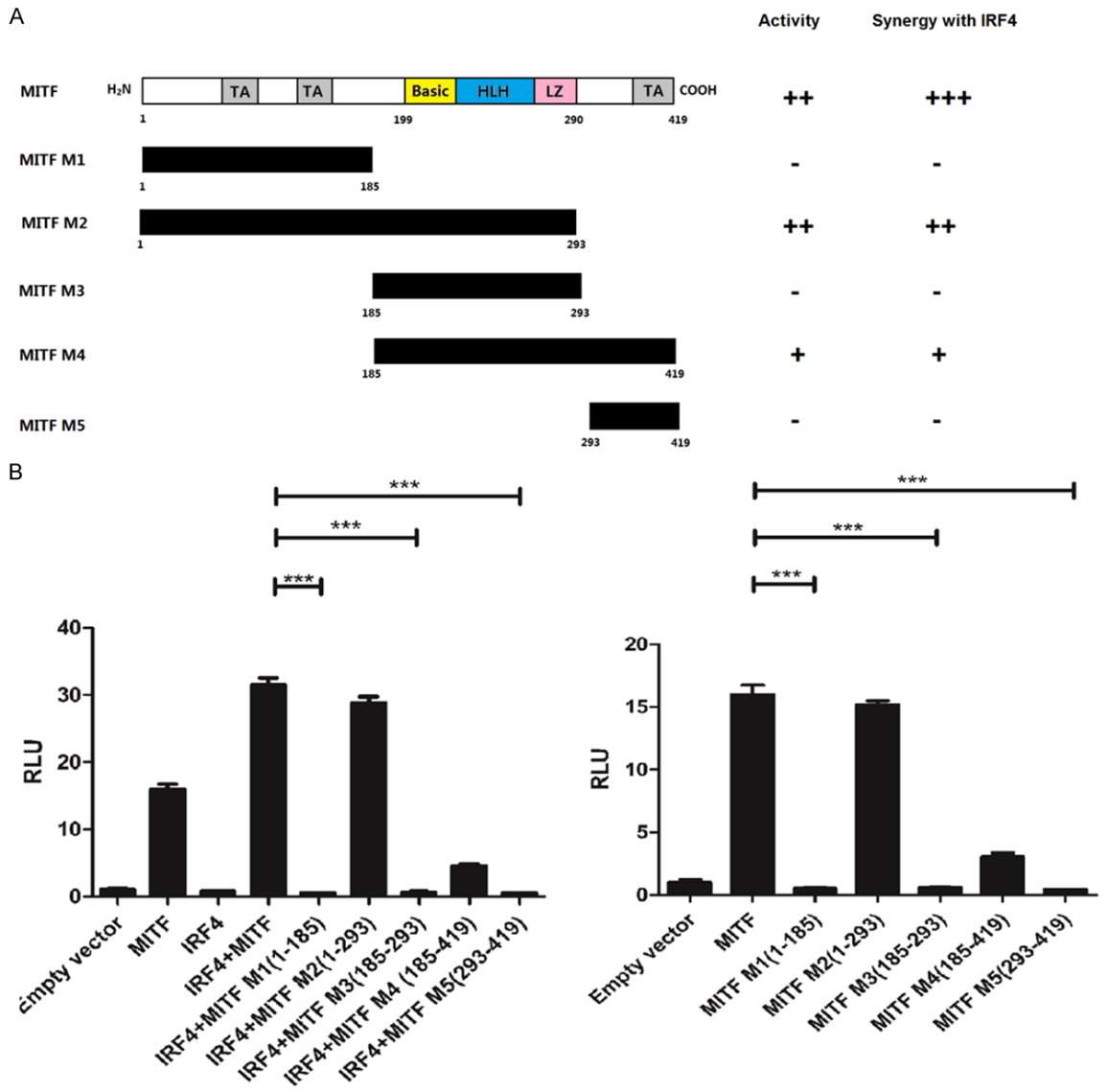

Figure 4. Identification of MITF sequences involved in transactivation with/without IRF4. A. A schematic representation of the full length MITF and various truncated MITF proteins. Basic-HLH-LZip, region of the basic HLH leucine zipper domains. TA, Transactivation domain. B. HEK293T cells were transiently transfected with the TYR luciferase reporter and full length or truncated MITF mutant expression plasmids with/without IRF4. Luciferase activity is reported as a fold increase relative to reporter alone. Each value represents the mean ± SD of three replicates from a single assay. The results shown were representative of at least three independent experiments (***P<0.001 compared to the value from the wt MITF with/without IRF4 and different MITF mutants with/without IRF4, unpaired Student's t-test).

Our results indicated that TAD1 (aa134-207) and TAD2 (aa300-420) of IRF4 participate in the synergic trans-activation with MITF, However the AMD (aa207-300) may serve as a masking region. Our results are consistent with previous reports regarding the cooperation between c-Src and IRF4 [22]. In the study of the interaction of c-Src and IRF4, the region spanning aa255-412, rich in α-helix structure, was identified to an important domain which could inhibits the cooperational activity.

The bHLH-LZ domain contains a basic domain which is used for DNA binding and HLH and Zip domains that are used for homo- and/or heterodimer formation. Moreover, As important functional domains for the transactivation, two Tyrosinase related different activation domains

have been identified in the MITF protein (TA in **Figure 4**) so far [23-25]. As shown in the **Figure 4B**, MITF (aa1-185) and MITF (aa293-419), which are lack of bHLH/LZ domain (DNA binding domain), completely lost the transcriptional activation on TYR promoter and so does the synergic effect with IRF4. MITF (aa185-293), which is lack of transcriptional activation (TA) domains, lost the transcriptional activation on TYR promoter and synergic effect with IRF4 similarly. Notable, Full length MITF, MITF (aa1-293) and MITF (aa185-419) show obvious transactivation of the TYR promoter, synergy with IRF4 also can be observed, all of which contain the bHLH/LZ domain (DNA binding domain) and trans-activation domains in N-terminal and/or C-terminal. Our results indicated that the bHLH/LZ domain and transactivation domain of MITF are both required for the transactivation and synergic effect with IRF4 on TYR promoter.

IRF4 usually as a positive regulator of gene transcription for many cofactors. Some of the Mechanism of IRF4 synergy with other protein has been characterized in the previous studies. IRF4 and PU.1 can form a stable ternary complex to regulate the target gene dependent on DNA [26]. IRF4 can also functionally cooperate with the transcription factor NFATC2 to synergistically regulate the IL4 promoter in T cells without binding DNA [27].

Since IRF4 poorly binds to DNA by itself as a weak transcriptional activator which previously research described [26] and cannot activate the TYR promoter independently. These domains could either form as interaction surface for a co-activator or as a component for the transcription apparatus.

The mechanism of the MITF and IRF4 relative domains in mediating synergy is proposed. MITF may lead a conformational change of IRF4, which lead to strengthen the ability of binding DNA and get expose TAD sequences necessary for trans-activation of TYR. Moreover, the conformational changes in both proteins are also could be involved in this synergy mechanism. Therefore, the formational changed IRF4 may act as a enhancer and induce an MITF conformation which facilitates DNA binding and lead to the increasing production of TYR promoter.

Here we showed that IRF4 potently synergizes with MITF to activate TYR promoter, which is dependent on DNA binding of IRF4. The synergic domains in both IRF4 and MITF were also identified by mutational analysis. This will help to better understand the role of IRF4 in the pigment system and the pathogenic mechanism in WS.

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Disclosure of conflict of interest

None.

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