

STUDIES ON PATHOGENESIS OF WAARDENBURG SYNDROME TYPE II  
AND TIETZ SYNDROME RESULTING FROM MITF GENE MUTATIONSZHANG Hua<sup>1</sup>, LI Jiada<sup>2</sup>, LUO Hunjin<sup>2</sup>, CHEN Hongsheng<sup>3</sup>, MEI Lingyun<sup>3</sup>, HE Chufeng<sup>3</sup>,  
JIANG Lu<sup>3</sup>, FENG Yong<sup>3</sup>**Abstract**

Microphthalmia-associated transcription factor (MITF) controls melanocyte survival and differentiation through directly regulating the expression of the tyrosinase (*TYR*) and tyrosinase-related proteins 1 and 2 (*TYRP1* and *TYRP2*) genes. *MITF* mutations have been reported to result in an abnormal melanocyte development and lead to Waardenburg syndrome type 2 (WS2), characterized by variable degrees of sensorineural hearing loss and patchy regional distribution of hypopigmentation. Recently, *MITF* was also indicated as a causative gene for a more severe syndrome, the Tietz Syndrome (TS), characterized by generalized hypopigmentation and complete hearing loss. However, few functional studies have been performed to compare the diseases-causing mutations. Here, we analyzed the *in vitro* activity of two recent identified WS2-associated mutation (p.R217I and p.T192fsX18) and one TS-associated mutation p.N210K. The R217I MITF retained partial activity, normal DNA-binding ability and nuclear distribution, whereas the T192fsX18 *MITF* failed to activate *TYR* promoter due to loss of DNA-binding activity, and aberrant subcellular localization. The aberrant subcellular localization of T192fsX18 MITF may be caused by deletion of a putative nuclear localization signal (NLS) at aa 213-218 (ERRRRF). Indeed, MITF with deletion of the NLS fragment failed to translocate into the nucleus and activated the *TYR* promoter. Tagging this NLS to GFP promoted the green fluorescence protein (GFP) translocated into the nucleus. The surprising finding of our study is that a TS-associated *MITF* mutation, N210K, showed comparable *in vitro* activity as WT. Thus, the possible involvement of *MITF* in TS and its underlying mechanisms still need further investigation.

**Key words:** Waardenburg syndrome; Teitz syndrome; *MITF*; mutation; haploinsufficiency

**Introduction**

Microphthalmia-associated transcription factor (*MITF*), a basic helix-loop-helix leucine zipper (bHL-HZip) transcription factor, plays a key role in survival and differentiation of melanocytes [1-4]. In melanocytes, MITF regulates the expression of major melanogenic genes such as tyrosinase (*TYR*), tyrosinase-related proteins-1 (*TYRP1*) and tyrosinase-related proteins-2

(*TYRP2*) [5-7]. MITF directly upregulates the expression of *TYR*, *TYRP1* and *TYRP2* by binding to the E-box motif (CANNTG) within the promoter [4, 7-10]. The *TYR*, *TYRP1* and *TYRP2* genes encode the three enzymes required for normal melanin synthesis or melanogenesis in melanocytes [8, 10-12]. These enzymes, specifically tyrosinase, are key players in the melanocyte differentiation, as demonstrated by the dramatic consequences of their mutations on melanin pigment production [13].

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Homozygous *Mitf* mutant mice can normally survive but are microphthalmic, deaf, and completely white due to lacking melanocytes<sup>[2, 14]</sup>, whereas heterozygous *Mitf* mutant mice show the striking phenotype of belly spotting<sup>[15]</sup>. In human, *MITF* mutations are associated with ~15% of WS2. WS is an autosomal dominantly inherited disorder of neural crest cells (NCC) characterized by sensorineural deafness and abnormal pigmentation of the hair, skin and iris<sup>[16]</sup>. Clinically, WS is divided into four types (WS1-4) based on the presence or absence of additional symptoms. Absence of additional features characterizes WS2. Moreover, mutations in *MITF* have also been shown to cause TS (albinism-deafness syndrome)<sup>[17-19]</sup>, which is inherited in a fully penetrant autosomal dominant fashion, characterized by congenital profound deafness and a uniform dilution of pigmentation instead of the patchy depigmentation seen in WS. However, no functional study has been performed to understand how different mutations in the *MITF* gene cause two diseases.

We previously reported two mutations c.650G>T (p.R217I) and c.575delC (p.T192fsX18) in the *MITF* gene in two different families with WS2<sup>[20]</sup>. To understand the functional consequences of the two mutations, we analyzed the subcellular distribution, expression and in vitro activities of the two mutations. Furthermore, we also analyzed the in vitro activities of c.630G>C (p.N210K), a missense mutation reported in TS patients<sup>[17]</sup>.

## Materials and Methods

### Reporter and Expression Constructs

The luciferase reporter containing the *TYR* promoter (pGL3-Tyr-Luc) and the expression constructs had been described previously and were kindly provided by J. Vachtenheim<sup>[21]</sup>. The pGL3-Tyr-Luc plasmid contained the promoter region of the human *TYR* gene (-300 to +80). To generate *MITF* tagged with 3×Flag at the N-terminus (Flag-MITF), full length *MITF* cDNA (GenBank accession no. NM 000248.2) was amplified from pCMV-MITF-HA and subcloned into pCMV-3×Flag (Sigma, St Louis, WA, USA). QuikChange II Site-Directed Mutagenesis Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used to introduce R217I, T192fsX18 and N210K mutations into MITF. In addition, we generated expression plasmid encoding for a mutant MITF lacking NLS (213ERRRRF218), termed pCMV-MITFΔNLS-Flag. Furthermore, the synthetic oligonucleotide encoding the NLS (213ERRRRF218) region of MITF was subcloned into pEGFP-N1 (Sigma, St Louis, WA, USA) at EcoR I/BamH I site to add a NLS to the N-terminus of GFP. All constructs were verified by direct nucleotide sequencing.

### Cell culture, Transfections, and Luciferase Reporter Assays

The melanoma UACC903 cells or NIH3T3 cells were grown at 37°C under 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100U/ml of penicillin/streptomycin. 24 h before transfection, cells were seeded at an approximate 50% confluency in 24-well plates. Cells were transfected with 5 ng of the reporter plasmid and 20 ng of the expression plasmid, using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and 5 ng of pCMV-β-gal (BD biosciences/Clontech, Palo Alto, CA, USA) was included for normalization of transfection efficiency. The final DNA amount of each well was adjusted to 200 ng with empty vectors. At 48 h after transfection, cells were washed with phosphate buffered saline (PBS) and lysed with Reporter Lysis Buffer (Promega, Madison, WI, USA). The extracts were assayed for luciferase and β-galactosidase activity. Luciferase reporter assays were performed using Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Luciferase activities were determined using SIRIUS luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). As for competition assays, various amounts of mutant MITF plasmids (20 ng, 40 ng, 80 ng) were mixed with a fixed amount of WT MITF (20 ng) and the reporter TYR plasmid (5 ng) in the transfection. All reporter assays were conducted at least three times and performed in triplicate on different days using different batches of cells. Data were analyzed using Prism 4 software (GraphPad, Software Inc., San Diego, CA, USA).

### Western Blot Assays

The melanoma UACC903 cells were transfected with 200 ng of Flag-MITF or its mutant constructs using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in 12-well plates. At 48 h after transfection, cells were lysed in 2×SDS loading buffer, containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, St Louis, WA, USA), and 0.2 mM β-mercaptoethanol supplemented with protease inhibitor cocktail (Sigma, St Louis, WA, USA). Proteins were separated on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) in the presence of 20% methanol. The membrane was blocked in Tris-buffered saline supplemented with 5% non-fat milk for 1 h at room temperature and then incubated overnight at 4°C with mouse monoclonal anti-Flag M2 antibody (1:1000 dilution, Sigma, St Louis, WA, USA). After washing

with Tris-buffered saline supplemented with 0.1% Tween 20 (Sigma, St Louis, WA, USA), the membrane was incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary anti-mouse IgG antibody (1:10000 dilution, Sigma, St Louis, WA, USA). Detection were performed using the ECL plus Western blotting detection system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to manufacturer's instruction.

### **Biotinylated DNA Affinity Precipitation**

The MITF-binding oligonucleotide, 5' -GAAAAGTCAGTCATGTGCTTTTCAG-3', probe oligo-DNA derived from the promoter of human *TYR* gene, was biotinylated at the 5' -terminus, and then annealed with its complementary strand to generate a double-stranded oligonucleotide. The melanoma UACC903 cells were transfected with Flag-MITF, Flag-R217I, Flag-T192fsX18, or Flag-N210K using Lipofectamine 2000 reagent in 6-well plates. 48 h after transfection, 293T cells were lysed by sonication in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM DTT, and 0.5% of Nonidet-P40) containing protease inhibitors. Cellular debris was removed by centrifugation. 10% cell lysates were extracted and added 2×SDS/0.2 mM β-mercaptoethanol for input immunoblotting. Remaining cell lysates were brought to a final volume of 1 ml and precleared with 30 μl streptavidin-agarose beads (Sigma, St Louis, WA, USA) with rotation at 4°C for 1 h, then incubated with or without 8 μg of biotinylated double-stranded oligonucleotide and 10 μg of poly(dI-dC).poly(dI-dC) (Sigma, St Louis, WA, USA) with rotation at 4°C for 20 h. 40 μl streptavidin-agarose beads were added and samples were incubated for another 2 h at 4°C. Beads/DNA/protein or beads/protein complexes were washed three times using cold ice HKMG buffer and resuspended in 2×SDS/0.2 mM β-mercaptoethanol. Samples were boiled at 95°C for 10 minutes and subsequently separated on 12% SDS-PAGE. Anti-Flag M2 antibody was used in the subsequent immunoblotting.

### **Immunofluorescence Assays**

The NIH3T3 cells were plated on 24-well plates and transfected with 50 ng of each MITF constructs. At 48 h after transfection, cultures were fixed in 4% paraformaldehyde for 30 min at room temperature. After permeabilized for 1 h in PBS/0.2% Triton X-100 (Bio Basic Inc, Bailey Avenue Amherst, NY, USA), they were incubated with blocking solution (PBS, 3% bovine serum albumin, 5% goat serum) for 1 h. Incubation with primary anti-Flag M2 (1:600 dilution) was performed at 4°C overnight. After washed with PBS/0.2% Triton X-100 three

times, fluorescence-labeled secondary anti-mouse antibodies (1:300 dilution; Invitrogen, Carlsbad, CA, USA) were incubated for 2 h in a dark room. After incubation of 4,6-diamino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) for 3 minutes, cells were mounted in Fluoromount medium (Sigma, St Louis, WA, USA) and fluorescence images were examined with a laser scanning confocal system installed on a Carl Zeiss microscope (Zeiss, Göttingen, Germany) with a 63 × oil immersion objective. Images were analyzed using the Metaphor software package.

## **Results**

### **Mutations of MITF in WS2 and TS Patients**

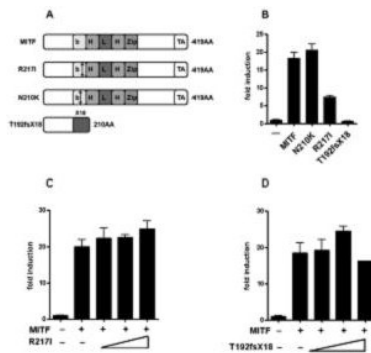
We previously identified two heterozygous mutations c.650G>T (p.R217I) and c.575delC (p.T192fsX18) in the MITF genes in three WS2 cases, presenting with deafness and heterochromia irides. The p.R217I is a missense mutation found in the basic domain of bHLHZip region of exon 7, causing an arginine to isoleucine substitution at codon 217 (Fig. 1A). The p.T192fsX18 is a frameshift mutation which generates different sequences starting at position 192 in exon 6 and introduces a premature stop codon at position 210 (Fig. 1A), resulting in truncated protein without bHLHZip domain and transactivation domain. The TS-causative missense mutation, c.630C>G (p.N210K), is located in the basic domain of bHLHZip region, resulting in substitution of lysine for asparagines in the mutant protein (Fig. 1A).

### **Transcription Activity of Mutant MITFs**

MITF can induce the expression of target gene *TYR* and subsequently the expression of the melanocyte-specific enzyme tyrosinase<sup>[8, 22]</sup>. A luciferase reporter construct containing approximately 300 bp of the *TYR* promoter sequences was used to study if diseases-associated mutations affected MITF activity. As shown in Fig. 1B, WT MITF was able to increase the *TYR* promoter activity by about 18-fold, consistent with previous reports<sup>[21-24]</sup>. However, the transactivity of R217I MITF was dramatically reduced as compared with WT, whereas the T192fsX18 mutant failed to transactivate the *TYR* promoter, indicating that R217I mutant was partially functional while T192fsX18 mutant was loss-of-function. Surprisingly, the transcriptional activity of TS-associated N210K MITF was comparable to WT (Fig. 1B).

Some loss-of-function mutant proteins may compete with their WT protein binding to the promoter of target genes and inhibit the activity of WT proteins, ie. dominant negative effect. To determine whether the R217I and T192fsX18 MITF were able to interfere with WT

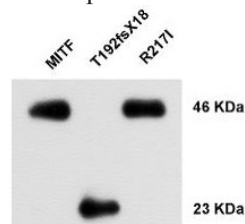
MITF function, we carried out competition assays by co-transfecting WT MITF and R217I or T192fsX18 expression plasmids together with the reporter plasmid. Neither R217I nor T192fsX18 showed inhibitory effect on the transcriptional activity of WT MITF (Fig. 1C, D).



**Figure 1.** Functional analysis of WS/TS-associated MITF. A: Schematic representation of the WT, R217I, N210K, and T192fsX18 MITF. The bHLH-Zip domain and C-terminal transcriptional activation (TA) domain are shown. R217I and N210K are missense mutants and the arrow indicates mutation site in basic domain. T192fsX18 is a frameshift mutant and the filled box represents resulting frameshift from mutation site to premature stop codon. B: Transcriptional activities of MITF and its mutants determined by luciferase activity assays. The luciferase reporter plasmid Tyr-Luc were transiently transfected into 293T cells in combination with WT or mutant MITF expression plasmids. C, D: Effect of R217I or T192fsX18 mutants on WT MITF transactivity. Increasing amounts of mutant R217I or T192fsX18 expression plasmids were cotransfected with a fixed amount of WT MITF expression plasmid and the luciferase reporter plasmid Tyr-Luc. The basal level of luciferase was set as 1. Data from all other transfections are presented as fold induction above this level. Luciferase activity was normalized by measuring  $\beta$ -galactosidase activity. Each value shown was the mean  $\pm$  s.e.m. of three replicates from a single assay. The results shown were representative of at least three independent experiments.

### Exogenous Expression of MITF Proteins

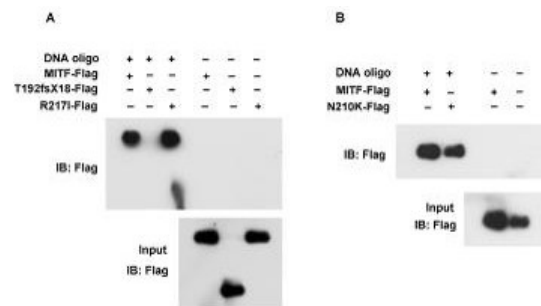
To investigate whether the disrupted function is caused by the expression level of mutant MITF, we performed Western blot analysis on protein extracts from melanoma UACC903 cells transfected with WT, R217I, or T192fsX18 MITF plasmid. As shown in Fig. 2, both WT and mutant MITF proteins were detected at the expected size. There was no apparent difference between WT and mutant MITF in the expression level.



**Figure 2.** Western blot analysis of WT and mutant MITF proteins. Melanoma UACC903 cells were transiently transfected with plasmids expressing either the WT or mutant variants of MITF. Proteins from whole cell lysates were analyzed by SDS-PAGE (12%) and the expression of MITF was visualized using monoclonal antibody against Flag epitope.

### DNA Binding Capacity of MITF Mutants

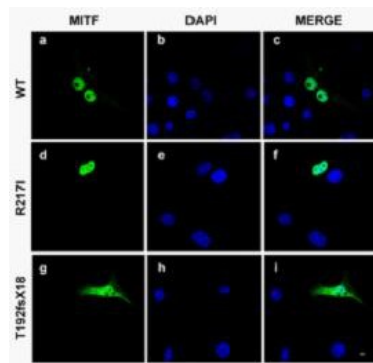
To determine the DNA-binding capacity of WT and mutant MITFs, we used a biotinylated double-stranded oligonucleotide containing MITF binding sequence to precipitate proteins from melanoma UACC903 cells transfected with WT, T192fsX18, R217I, or N210K MITF plasmid. As shown in Fig.3A and B, WT, R217I, and N210K MITFs were able to bind specifically to the double-stranded DNA, whereas T192fsX18 MITF lost the DNA-binding ability, consistent with its lack of the bHLHZip DNA-binding domain.



**Figure 3.** DNA binding capacity of WS/TS-associated MITF. The lysates of melanoma UACC903 cells transfected with WT, R217I, T192fsX18, and N210K MITF plasmids were incubated with or without biotinylated double-stranded oligonucleotides of the MITF binding region at the TYR promoter. And the DNA/protein complex was pulled down with streptavidin-agarose beads. The precipitated proteins were separated on SDS-PAGE and analyzed by immunoblotting using anti-Flag M2 antibody. DNA precipitation demonstrates that R217I and N210K mutant retain the DNA-binding activity (A,B), whereas T192fsX18 mutant lose capability to bind to DNA (A).

### Subcellular Distribution of WT and Mutant MITF Proteins

As a transcription factor, MITF is localized in the nucleus. Then, we attempted to investigate if mutations altered the subcellular distribution of MITF protein. Immunofluorescence was used to detect the exogenously expressed proteins. As shown in Fig. 4, WT and R217I MITF were only localized in the nucleus, whereas T192fsX18 MITF showed localization in both cytoplasm and nucleus. These results indicated that WT and R217I MITF produced in NIH3T3 cells were normally transported into nuclei while T192fsX18 MITF was defective in the nuclear translocation.

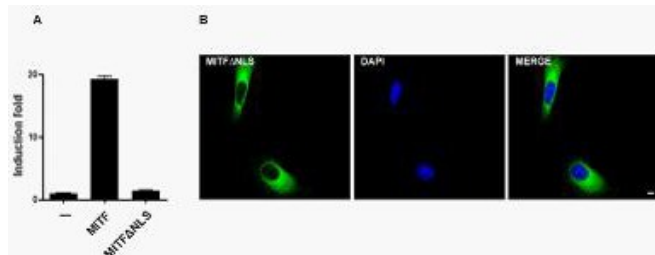


**Figure 4.** Subcellular localization of WS-associated MITF proteins. WT MITF and its mutant proteins (a, d, g) were shown in green, DAPI (b, e, h) revealing nucleus was shown in blue, and the merged images (c, f, i) were shown. Scale bar: 5 $\mu$ m.

### Functional Analysis of NLS of MITF

Protein localized in nucleus contains specific NLS and can be recognized by transport protein to cross nuclear pore complex (NPC) into nucleus to exert its function. We speculate that the abnormal cytoplasmic distribution of T192fsX18 MITF may be due to its loss of NLS. Indeed, MITF contains a putative NLS (NLIERRRRFNIN) between aa210-221 of the C-terminal basic domain<sup>[23]</sup> (<http://cubic.bioc.columbia.edu/predictNLS/>). To examine whether the NLS is necessary for the MITF function, we made a MITF construct with deletion of aa213-218 (ie. MITF  $\Delta$  NLS). As shown in Fig.5A, MITF  $\Delta$  NLS failed to transactivate the *TYR* promoter. Importantly, the MITF $\Delta$ NLS protein was localized exclusively in the cytoplasm (Fig.5B).

To examine whether the NLS sequences of MITF is sufficient to render cytoplasmic protein into nuclei, we tagged the NLS sequence of MITF (ERRRRF) at the N-terminus of green fluorescence protein (GFP). After transfected into the NIH3T3 cells, about 15% WT GFP was distributed in the nucleus, 30% was localized in the cytoplasm, whereas about 55% was seen in both cytoplasm and nucleus (Fig.5C). However, NLS-tagged GFP significantly increase the nuclei localization to about 80%, and about 20% of NLS-tagged GFP was observed in both cytoplasm and nuclei, while none of them was seen in the cytoplasm only (Fig.5C).



**Figure 5.** Functional analysis of NLS of MITF. A: Transcriptional activities of WT MITF and MITF $\Delta$ NLS determined by luciferase

activity assays. The luciferase reporter plasmid Tyr-Luc were transiently transfected into melanoma UACC903 cells in combination with WT MITF or MITF $\Delta$ NLS expression plasmid. B: Subcellular localization of MITF  $\Delta$  NLS in transfected cells was shown in green, DAPI revealing nucleus was shown in blue, and the merged images were shown. Scale bar: 5 $\mu$ m. C Frequency distribution of GFP and GFP-NLS in transfected cells.

### Discussion

*MITF* encodes a 419-amino-acid transcription factor containing bHLHZip structure which is considered to bind DNA by the basic domain, to dimerize via the HLH domain, and to be stabilized by the Zip domain<sup>[1, 2]</sup>. The carboxyl terminus of MITF contains a transcriptional activation domain and plays an important role in defining the target genes<sup>[9]</sup>. The *TYR* gene, a melanocyte-specific gene, encodes tyrosinase, is a key enzyme for melanin synthesis. *MITF* binds to the specific E-box motif (CAT-GTG) within the promoter of *TYR* and initiates its transcription<sup>[8, 9, 22]</sup>. Few functional studies have been performed on human diseases-associated *MITF* mutations. Two nonsense mutations of *MITF* causing WS2 were shown to abrogate DNA binding and transactivation of the *TYR* promoter<sup>[24]</sup>. Another WS2-associated mutation, c.892T>C (p.S298P), impaired MITF function due to loss of the phosphorylation site by GSK3 $\beta$ <sup>[25]</sup>. We have shown here that WS2-associated T192fsX18 and R217I mutations attenuated the transactivity of MITF on *TYR* promoter. R217I MITF retained the DNA-binding activity as well as nuclear localization. However, T192fsX18 mutation, lacking of NLS, resulted in abnormal cytoplasmic distribution. Moreover, T192fsX18 MITF, without bHLHZip and transactivation domains, was not able to bind to the *TYR* promoter. Nevertheless, none of WS2-associated MITF mutants showed dominant negative effect. Haploinsufficiency may be the underlying mechanism for WS2 pathogenesis. The surprising finding of our study is that a TS-associated N210K MITF showed comparable *in vitro* activity as WT MITF. TS patients show a more severe phenotype of complete penetrance including profound deafness and generalized hypopigmentation, whereas WS patients only show mild phenotype such as patchy depigmentation and incomplete penetrance [17]. It thus has been hypothesized that the N210K mutation may exert a dominant negative effect. However, N210K MITF behaved similarly as WT in both *in vitro* transcriptional activity and DNA-binding assays. Therefore, the possible involvement of MITF in the pathogenesis of TS still needs further investigation, especially in animal models and neural crest cells such as melanocytes.

Protein localized in the nucleus frequently contains specific NLS that can be recognized by NLS-binding protein<sup>[26, 27]</sup>, and be transported through the nuclear pore

into the nucleus. The basic domain of MITF may contain a putative NLS (210NLIERRRRFNIN221)<sup>[23]</sup> (<http://cubic.bioc.columbia.edu/predictNLS/>). The T192fsX18 mutant protein lacks NLS, however, it may diffuse to the nucleus due to its small molecular mass (predicted to be 23 kDa) as proteins below 40 kDa are known to cross the NPC via passive diffusion<sup>[28, 29]</sup>. The R217I mutation in the NLS did not impair the nuclear localization potential of MITF, consistent with the mice study carried out by Takebayashi *et al*<sup>[23]</sup>. They found that *Mitf<sup>or</sup>*-MITF encoded by the mouse *Mitf<sup>or</sup>* allele, with a R216K substitution in the basic domain, showed normal subcellular localization. The function of MITF NLS was further demonstrated by loss-of-function and gain-of-function studies. In the loss-of-function study, MITF with deletion of NLS localized exclusively in the cytoplasm and failed to activate the *TYR* promoter. Conversely, addition of this NLS to the N-terminus of GFP enhanced the translocation of GFP into nucleus.

In summary, we have shown that two recent identified WS2-associated mutations attenuated the transcriptional activity of MITF. As both mutations were in the heterozygous form and lack of dominant-negative effect, haploinsufficiency is the most reasonable mechanism, where the normal MITF protein resulted from one copy of the wild allele does not reach the threshold level necessary for full function of the protein. However, the TS-associated mutation, N210K, showed comparable activity as WT MITF, implying an alternative mechanism for the TS pathogenesis.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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