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# The International Journal of Biochemistry & Cell Biology

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## Phosphorylation of MITF by AKT affects its downstream targets and causes TP53-dependent cell senescence

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### ARTICLE INFO

#### Article history:

Received 19 July 2016

Received in revised form 24 July 2016

Accepted 30 September 2016

Available online 1 October 2016

#### Keywords:

MITF

AKT

Phosphorylation

TP53

Senescence

### ABSTRACT

Microphthalmia-associated transcription factor (MITF) plays a crucial role in the melanogenesis and proliferation of melanocytes that is dependent on its abundance and modification. Here, we report that epidermal growth factor (EGF) induces senescence and cyclin-dependent kinase inhibitor 1A (CDKN1A) expression that is related to MITF. We found that MITF could bind TP53 to regulate CDKN1A. Furthermore, the interaction between MITF and TP53 is dependent on AKT activity. We found that AKT phosphorylates MITF at S510. Phosphorylated MITF S510 enhances its affinity to TP53 and promotes CDKN1A expression. Meanwhile, the unphosphorylated MITF promotes TYR expression. The levels of p-MITF-S510 are low in 90% human melanoma samples. Thus the level of p-MITF-S510 could be a possible diagnostic marker for melanoma. Our findings reveal a mechanism for regulating MITF functions in response to EGF stimulation and suggest a possible implementation for preventing the over proliferation of melanoma cells.

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### 1. Introduction

Melanoma arises from melanocytes, and it is one of the most treatment-resistant and notoriously aggressive human cancers (Soengas and Lowe, 2003). MITF is crucial for the growth, differentiation and pigment production of melanoma cells (Yajima et al., 2011). In a portion of human melanomas, MITF is an amplified oncogene, and it also has an oncogenic element in human clear cell sarcoma (Levy et al., 2006). Melanogenesis is controlled by the three genes, tyrosinase-related protein 1 (TYRP-1), tyrosinase-related protein 2 (TYRP-2) and TYR, which are all downstream of MITF (Bentley et al., 1994). Among them, TYR is a rate-limiting enzyme in the process of melanin production (Korner and Pawelek, 1982). MITF was identified, via integrative genomic analyses, as a lineage survival oncogene that was amplified in malignant melanoma

(Garraway et al., 2005). Moreover, MITF activates CDKN1A expression through cooperation with retinoblastoma 1 (RB1) to regulate cell proliferation (Carreira et al., 2005a).

The PI3K/AKT signaling pathway plays a central role in regulating the proliferation and survival of both normal and melanoma cells (Palmieri et al., 2015). However, activated AKT induces proliferation arrest and senescence in several cell lines (Aistle et al., 2012). It has been reported that glycogen synthase kinase 3 beta (GSK3B), a direct target of AKT, phosphorylates MITF at S298 and enhances the expression of TYR (Takeda et al., 2000a). Many drugs that affect PI3K/AKT activity, such as baicalin and sphingosylphosphorylcholine, are involved in the process of degradation of MITF (Jeong et al., 2015; Kim et al., 2010).

TP53 is a tumor suppressor that controls the cell cycle by regulating CDKN1A, cyclin-dependent kinase inhibitor 1B (CDKN1B), and cyclin-dependent kinase inhibitor 2A (CDKN2A) transcription (Biegging et al., 2014). Too much or too little MITF causes cellular senescence in many types of melanoma cells (Carreira et al., 2005a; Strub et al., 2011). These reports indicate that regulation of cell

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proliferation may be associated with MITF and TP53 in melanoma cells.

Here, we reported that AKT phosphorylates MITF at S510, which mediates the cellular distribution and degradation of MITF. Unphosphorylated MITF enhances TYR expression. The phosphorylation of MITF promotes its association with ID4 and inhibits the expression of TYR. However, phosphorylated MITF induces CDKN1A expression and senescence in the presence of TP53 instead of ID4 binding. MITF and p-AKT are inversely correlated in human melanoma.

## 2. Material and methods

### 2.1. Antibodies and reagents

The anti-HA, anti-MITF, anti-AKT, anti-p-AKT substrate antibody, anti-AKT1, anti-p-AKT-S473, anti-p-p44/42 MAPK, anti-p44/42 MAPK, anti-PTEN, and anti-EGF Receptor were from Cell Signaling Technology. The anti- $\beta$ -tubulin was from Santa Cruz Biotech. Total S,T phosphorylation antibody was from BD Bioscience. The anti-MITF, anti-TP53, anti-TYR, anti-CDKN1A, anti-Lamin B, anti-alpha-tubulin, and anti-GSK3B antibodies were from AB Clonal Technology. PD98059, Wortmannin, PI103, and Ly294002 were from Selleck Chemicals. AKTI was from Sigma-Aldrich.

### 2.2. Cell culture and transfection

The A375, 293A, 293T, 293FT, B16, A2059, HeLa and U87 cell lines were purchased from ATCC and cultured in DMEM medium with 10% FBS and P/S in a humidified incubator at 37 °C with a 5% CO<sub>2</sub> atmosphere.

For transient expression experiments, cells were transfected with recombinant DNA using polyethylenimine. For stable cell lines were selected by puromycin or hygromycin B, respectively.

### 2.3. DNA constructs and RNA interference

The human genes with HA, mGST, Myc and Flag tags were inserted into pRK5 using the indicated restriction enzymes. The TYR promoter in pGL3 was a gift from Prof. Colin R. Goding (Oxford University, UK). The shRNA sequences for knockdown of mouse *Mitf*, *Pten* and human *AKT1* are listed as below: *shMitf*: TAACCTAT-TAATACTACACCT; *shPten*: TTATATCCCTCCTTTGTGGA; *shAKT1*: CACAATAGCCACGTCGTCAT. The sgRNA of *PTEN* (AGATCGT-TAGCAGAAACAAA) and the sgRNA of *TP53* (TGCACCAGCAGCTCCTACAC) were cloned into the modified CRISPR lentivirus vector.

## 3. Methods

The Immunoprecipitation and Western blotting and Immunohistochemical staining were processed as described in (Liu et al., 2008). The CHIP assay procedure was performed as previously reported by Dr. Saramäki (Saramäki et al., 2006). The AKT kinase assay was performed as previously reported (Hu et al., 2005). The cellular fraction and luciferase assay and GST-tag proteins purification and senescence  $\beta$ -galactosidase staining and MTT assay were performed following manufacturer's manual. The p-MITF S510 antibody was generated and purified by AB Technology.

### 3.1. Statistical analysis

Statistical significance was measured via the unpaired and two-tailed Student's test and is presented as follows: \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ . All error bars indicate the SEM.

## 4. Results

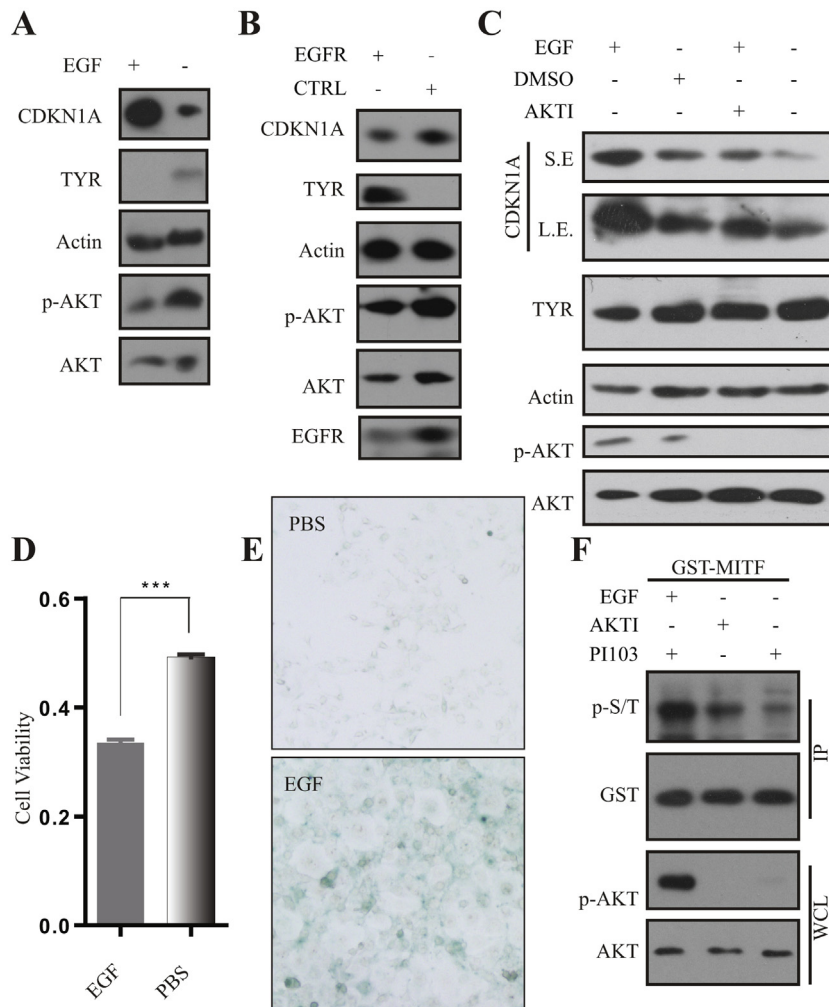
### 4.1. AKT regulates cell proliferation and melanogenesis in melanoma cells

The senescence or cell proliferation arrest of melanoma cells can be induced by EGF stimulation (Leikam et al., 2014). Here, we found that both EGF stimulation and lentiviral infection with epidermal growth factor receptor (EGFR) can induce CDKN1A expression and reduce TYR in melanoma cells (Fig. 1A, B). To confirm these results, we inhibited AKT. The abundance of CDKN1A in AKTI-treated B16 cells is less than that of cells treated with EGF. However, the abundance of TYR in AKT inhibitor-treated B16 cells is higher than that of cells treated with EGF (Fig. 1C). The MTT assay revealed that EGF stimulation prevents the proliferation of B16 cells (Fig. 1D). After treatment with EGF, the cells exhibit senescence and relatively high beta-Gal activity (Fig. 1E). AKT is associated with cell senescence (Astle et al., 2012). We hypothesized that MITF is a substrate of AKT. To confirm our hypothesis, we detected total phosphorylation of MITF after inhibition or stimulation of AKT. We found that phosphorylation of MITF depends on AKT activity (Fig. 1F). These results suggest that MITF is a target of AKT.

### 4.2. MITF is phosphorylated by AKT at serine 510

To assess whether MITF could be phosphorylated by AKT, we performed in vitro and in vivo kinase assays. GST-MITF or GST only was cotransfected with HA-AKT-CA (constitutively active AKT). A p-AKT-substrate antibody was used to detect MITF phosphorylation. A clear band was observed in the GST-MITF panel, but no signal was detected for GST only (Fig. 2A). Similar results were obtained by cotransfecting GST-MITF with HA-AKT-CA or HA vector as shown in Fig. 2B. The phosphorylation of MITF was reduced by PI3K and AKT inhibitors even with EGF stimulation. However, MITF phosphorylation was not suppressed by an ERK inhibitor (Fig. 2C). These data indicated that AKT phosphorylates MITF.

Based on the literature, AKT recognition motif is (R/K)X(R/K)XX(S/T), where X is any amino acid. Several conserved AKT substrate motifs were found in MITF. We have identified that TFEB, a MITF family member, could be phosphorylated by AKT at S467 (data not shown). We aligned the amino acid sequences of the four members of the MITF gene family. A conservative AKT substrate motif can be found in the C-terminal of this family (Fig. 2D). Alanine scanning was used to accurately identify the phosphorylation site, and S510 of MITF was found to be a precise site (Fig. 2E). The in vitro kinase assays were performed to determine whether MITF is the direct target of AKT and whether S510 is the phosphorylation site. Using the p-AKT-substrate antibody and autoradiography of  $\gamma$ -<sup>32</sup>P-ATP, the results showed that AKT could phosphorylate MITF in vitro but not the MITF S510A mutant (Fig. 2F, G). MITF WT and S510A were transfected into HEK293T cells and then treated with or without EGF. The pull-down was determined using the p-AKT substrate antibody. No signal was detected in the S510A pull-down even with EGF treatment. This suggests that S510 is a phosphorylation site of AKT in vivo (Fig. 2H). To confirm this, we generated a specific antibody that was tested by recognizing the exogenous GST-MITF WT but not GST-MITF S510A (Fig. 2I). p-MITF was detectable with this antibody after EGF stimulation in B16 cells but not in PI3K and AKT inhibitor-treated cells (Fig. 2J). Similar results were observed in A2059 cells (Fig. 2K). PTEN is upstream of AKT and inhibits the PI3K/AKT pathway. When we knockdown *PTEN*, endogenous phosphorylation of MITF is augmented (Fig. 2L). We next asked where phosphorylation of MITF by AKT occurs. To answer this question, we cotransfected GST-MITF with NLS-AKT or Myr-AKT, and the cell lysate was determined using the p-MITF antibody.



**Fig. 1.** AKT regulates cell proliferation and melanogenesis in melanoma cells.

(A) B16 cells were treated with EGF or PBS overnight. The expressions of proteins were analyzed by WB. (B) CDKN1A and TYR expression levels were determined after EGFR overexpression. (C) B16 cells were treated with or without AKTI. Then, the cell lysates were subjected to IB analysis. (D) The cell viability was determined by MITT assay after B16 cells were treated with EGF. Values are presented as the mean  $\pm$  SEM,  $n = 3$ ,  $p < 0.05$ . (E) B16 cells were treated with or without EGF and stained with beta-Gal reagent. (F) HEK293FT cells were transfected with the mGST-MITF plasmid and treated with the indicated drugs. The cell lysates were used in the IB and IP assays with the indicated antibodies.

The result showed that only Myr-AKT can phosphorylate MITF (Fig. S1A). Then AKT phosphorylates MITF in cytoplasm. All these results suggest that MITF is directly phosphorylated by AKT.

#### 4.3. TYR and CDKN1A are regulated by phosphorylation of MITF

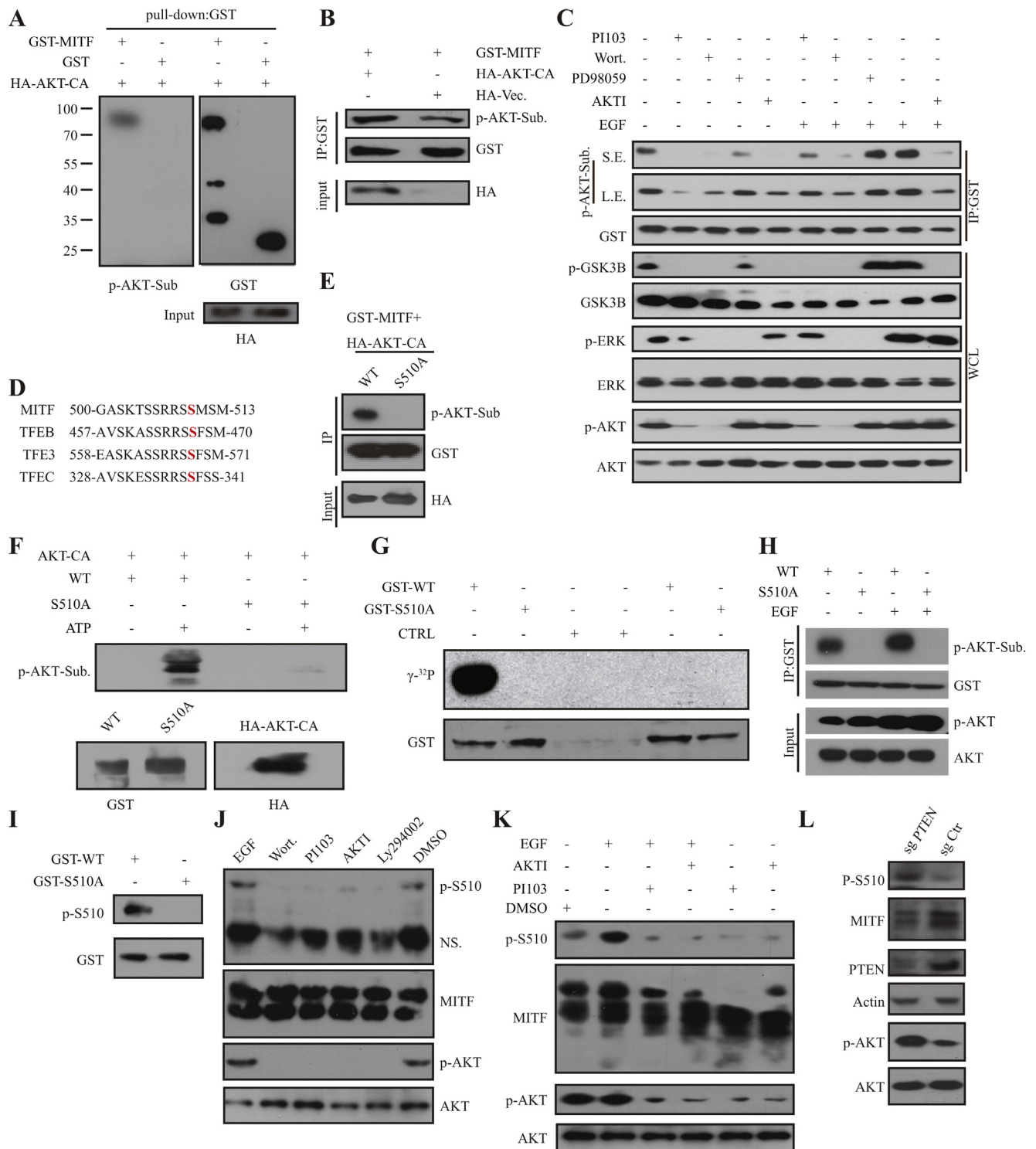
TYR and CDKN1A are the two most important genes downstream of MITF (Carreira et al., 2005a). We have already observed that the levels of TYR and CDKN1A are altered with EGF treatment. MITF is also phosphorylated by AKT; therefore, we speculated whether TYR and CDKN1A expression levels are affected by MITF phosphorylation. To determine the effect of MITF phosphorylation on TYR expression, we knocked down endogenous MITF by targeting its 3' UTR, which will not affect exogenous MITF expression with only CDS. Additionally, we rescued the expression of MITF WT, S510A, S510D mutants by transfection. Western blotting revealed that the unphosphorylatable mutant S510A enhanced TYR expression compared to MITF WT; however, the phosphorylated mimic S510D reduced TYR expression (Fig. 3A). We analyzed whether the expression of TYR corresponded to the activation of the PI3K/AKT signaling pathway. Both EGF and insulin stimulation activated the PI3K/AKT pathway and decreased TYR expression

compared to PI3K/AKT inhibition (Fig. 3B). The above results were verified by a subsequent luciferase assay, and the ability to suppress TYR expression was demonstrated by either the S510D mutant or by simulations with EGF/insulin (Fig. 3C,D). The S510A mutant or AKT inhibition increased luciferase readout compared to its counterparts (Fig. 3C,E).

To explore whether CDKN1A expression is also regulated by MITF phosphorylation, we overexpressed MITF variants in HeLa cells. Overexpression of the S510D mutant increased CDKN1A compared to its counterparts (Fig. 3F). This result was confirmed by luciferase assay (Fig. 3G). The U87 cells were infected by the indicated exogenous lentivirus expressing MITF variants. CDKN1A was increased by MITF S510D infection (Fig. 3H). These results demonstrated that phosphorylation of S510 can regulate the two most important genes downstream of MITF.

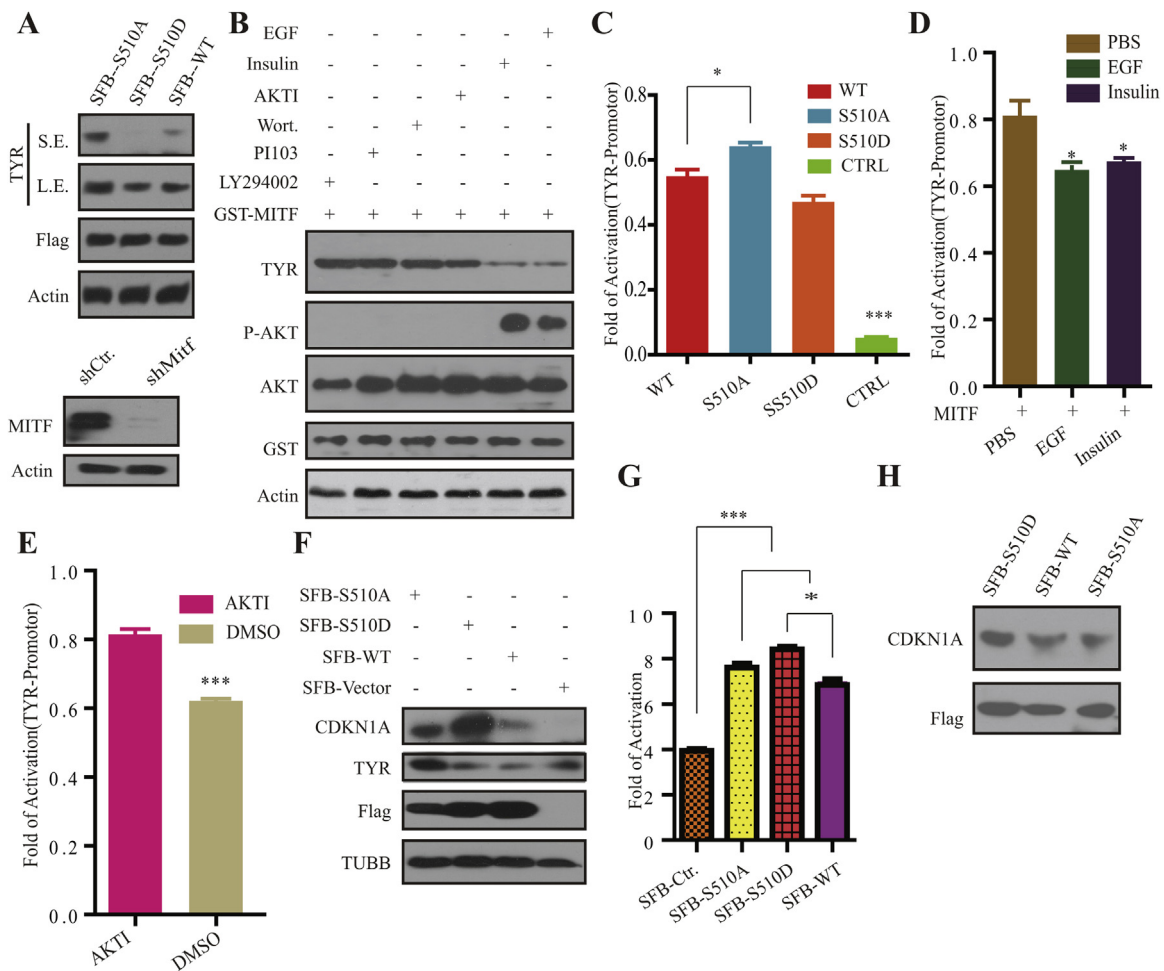
#### 4.4. Phosphorylation of MITF by AKT induces its degradation

It has been reported that phosphorylation influences the stability of proteins (Swaney et al., 2013). Therefore, we first examined the stability of MITF after its phosphorylation. The MITF WT and its variants were transfected into HEK293T cells. Then, the cells



**Fig. 2.** AKT phosphorylates MITF at S510 in vivo and in vitro.

(A) MITF is phosphorylated by AKT. The indicated plasmids were transiently transfected into HEK293FT cells, followed by glutathione bead pull-down and IB. The phosphorylation of MITF was determined using p-AKT substrate antibody. (B) GST-MITF was transfected with HA-AKT-CA or HA Vector. The glutathione bead pull-down was immunoblotted with p-AKT substrate antibody. (C) HEK293FT cells were transfected with GST-MITF and pretreated with indicated inhibitors. The cells were stimulated with or without EGF, and the cell lysates were subjected to IP and IB with the indicated antibodies. (D) Schematic alignment of the protein sequences of MITF family proteins. (E) Ala scanning showed that serine 510 of MITF is the solo phosphorylation site. (F–G) HA-AKT-CA was transfected into HEK293FT cells and immunoprecipitated via HA beads. Recombinant GST-MITF and GST-MITF-S510A protein are purified from bacteria. Phosphorylation of S510 was identified by the p-AKT substrate antibody (F) or autoradiography (G). (H) GST-MITF and GST-MITF-S510A were transfected into HEK393 cells with or without EGF stimulation. Cell lysates were harvested for IP and IB analysis with the indicated antibodies. (I) WT and S510A were transfected into HEK293FT cells, and cell lysates were analyzed by IB via the p-MITF S510 specific antibody. (J–K) Identification of endogenous phosphorylation of S510 in melanoma cells. B16 or A2059 cells were pretreated with indicated inhibitors and stimulated by EGF, followed by IB analysis with the indicated antibodies (J, K). (L) A2059 cells were infected with control or sgPTEN lentivirus. Then, cell lysates were harvested for IB analysis using the indicated antibodies.



**Fig. 3.** Phosphorylation of MITF regulates TYR and CDKN1A expression levels.

(A) B16 cells were established as stable cell lines to knockdown *Mitf* by targeting the 3' UTR of *Mitf* mRNA, named shB16. shB16 cells were infected with lentivirus expressing MITF WT, S510A and S510D, and TYR expression was determined. (B) GST-MITF was transfected into HEK293FT cells and treated with the indicated drugs. The whole-cell lysates were analyzed by IB with the indicated antibodies. (C) The luciferase activities of TYR luciferase reporters were measured and normalized after overexpressions of MITF, WT, S510A and S510D, respectively. The experiments were performed in triplicate, and the data were expressed as the mean  $\pm$  SEM of 3 independent experiments. Values are presented as the mean  $\pm$  SEM,  $n = 3$ , \* $p < 0.05$ . The data was analyzed using Graph pad prism 5. (D) The cells were treated with PBS, insulin and EGF, respectively. The following measurement and data analysis are the same as in Fig. 3C. (E) The pGL3-TYR promoter was transfected into B16 cells. Then, the cells were treated with AKTI or DMSO. The analysis of the data is identical to the results that were shown in Fig. 3C. \*\*\* $p < 0.001$ . (F) HeLa cells were transfected with SFB-MITF WT, S510A, and S510D. Then, the cell lysates were analyzed by IB with the indicated antibodies. (G) A pGL3-CDKN1A promoter was co-transfected with SFB-MITF WT, S510A and S510D into HeLa cells, respectively. After 24 h, the luciferase activity was detected and calculated. Subsequent calculations and data analysis are consistent with the previous description in Fig. 3C. \* $p < 0.05$ , \*\*\* $p < 0.001$ .

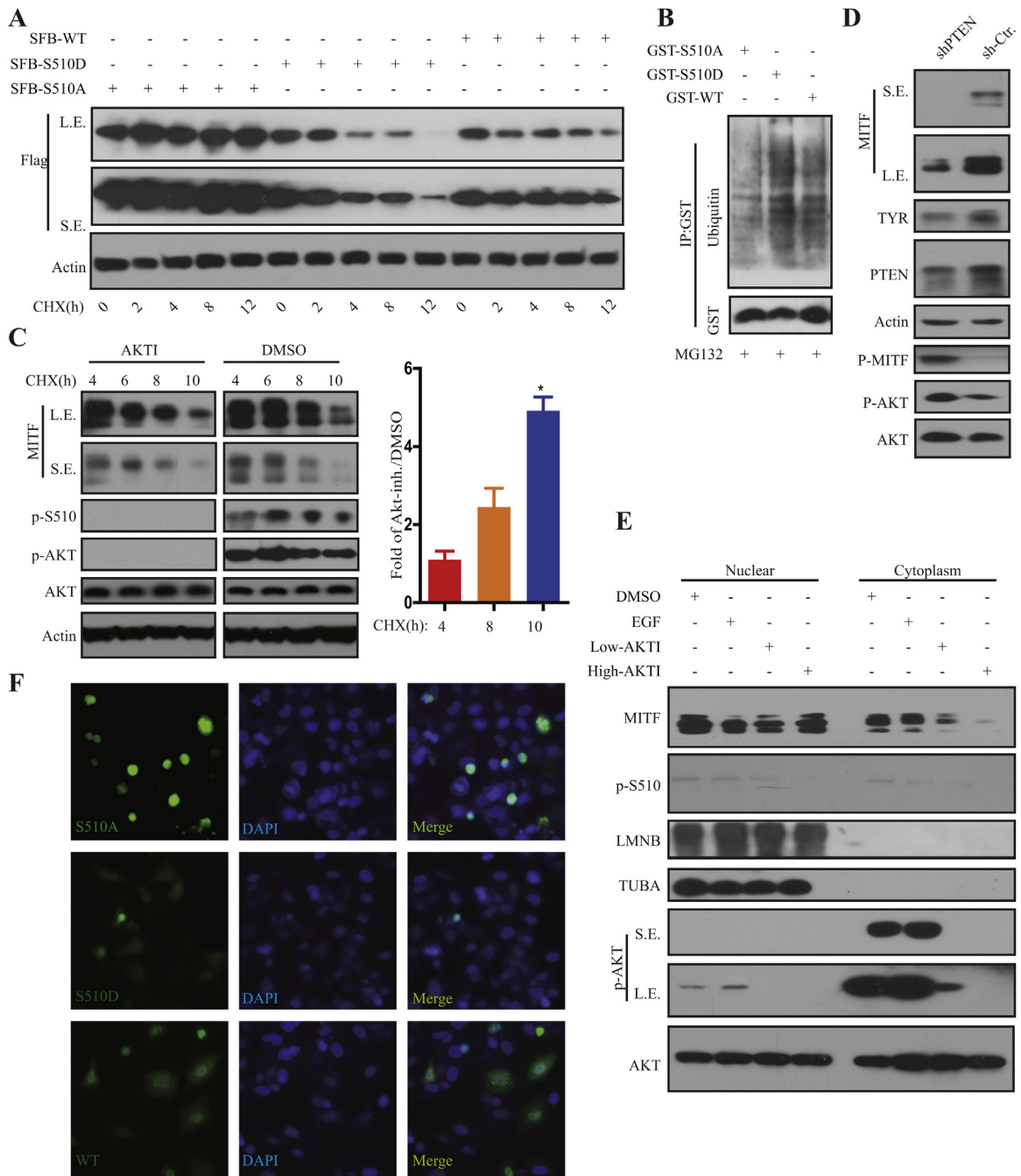
were treated with cycloheximide (CHX) for the indicated times. The cell lysate was immunoblotted with the indicated antibody. MITF S510D degraded much faster than WT and S510A (Fig. 4A, Fig. S1B). These data revealed that phosphorylation at S510 caused MITF instability. We speculated whether this instability was due to increased ubiquitination of MITF. We transfected the GST-MITF WT, S510A and S510D into HEK293T cells. The cell lysates were pulled down using glutathione beads and the pull-down complex was determined using an anti-ubiquitin antibody after MG132 treatment. The result showed that the intensity in the S510D panel was much higher than that of its counterparts (Fig. 4B). To confirm that its stability was correlated with the phosphorylation by AKT, we conducted a time-course treatment of the cells with or without AKTI combined with CHX. p-MITF and total MITF were examined. The stability of MITF was augmented when the cells were treated with AKTI (Fig. 4C). Knockdown of *PTEN* also reduced MITF and TYR (Fig. 4D). These results indicate that AKT-mediated phosphorylation of MITF led to degradation through the proteasome pathway.

#### 4.5. The cellular distribution of MITF is affected by phosphorylation

To determine whether phosphorylation of MITF influences its localization, we took images and separated the cellular fractions with different treatments and different variants transfections. The B16 or A2059 cells were treated with EGF or AKTI, then the cells were fractionized and MITF was examined. MITF was translocated to the nucleus from the cytoplasm when AKT was inhibited (Fig. 4E; Fig. S1C). Images of exogenous GFP-MITF WT, S510A and S510D revealed that S510A was much more highly concentrated and brighter in the nucleus than WT and S510D. However, WT and S510D were distributed in both the cytoplasm and the nucleus, but S510D was much weaker (Fig. 4F). We have demonstrated that AKT inhibition or the S510A mutant induces nuclear translocation.

#### 4.6. p-MITF binds ID4 to inhibit TYR expression

We have demonstrated that the phosphorylation of MITF decreased TYR expression. We next wanted to dissect the molecular



**Fig. 4.** Phosphorylation of MITF induced its degradation and change in cellular distribution.

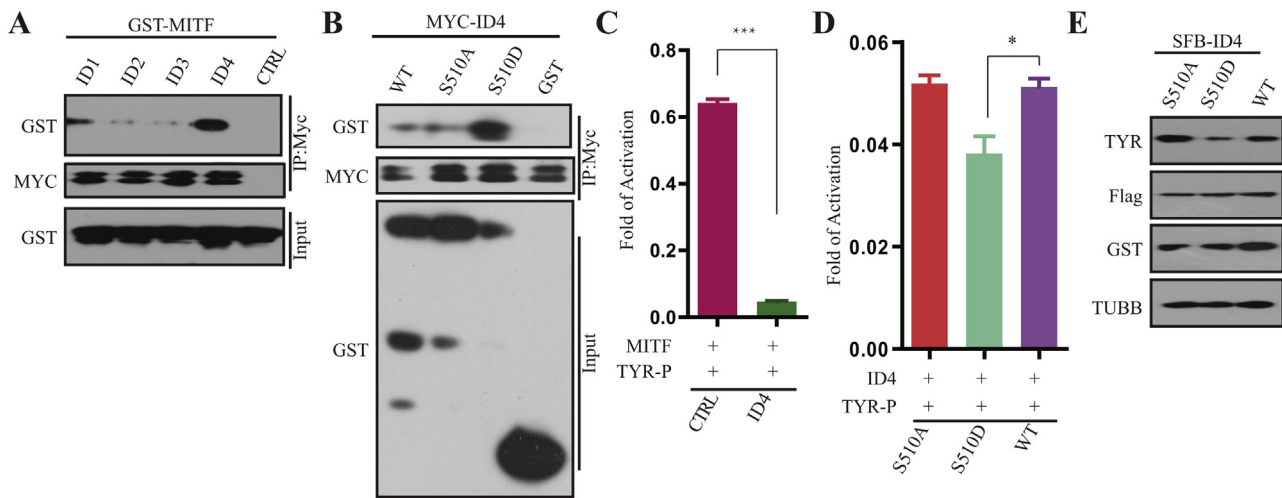
(A) SFB-MITF WT, S510D and S510A were transfected into HEK293FT cells and treated with cycloheximide (CHX) for the indicated time. Then, whole-cell lysates were harvested for the following IB experiment. (B) MITF-S510D was degraded rapidly by an ubiquitin-mediated pathway. (C) B16 cells were pretreated with or without AKTI following CHX treatment for a time course. The lysates were analyzed with the indicated antibodies. The gray scale of MITF was calculated using Image J. The numerical value of the vertical coordinates is the ratio. The data were analyzed using Graph pad prism 5. Values are shown as the mean  $\pm$  SD,  $n = 2$ , \* $p < 0.05$ . (D) MITF is reduced in the sh*Pten* B16 stable line compared with the control. (E) MITF moved into the cytoplasm when treated with an AKT inhibitor, which was the opposite effect of EGF stimulation. (F) Images of EGFP-MITF WT, S510D and S510A in HeLa cells by fluorescence microscopy.

mechanisms by which MITF phosphorylation regulates TYR expression. Inhibitor of DNA-binding/differentiation proteins are a class of proteins that bind to proteins that contain bHLH domains and inhibit the binding of bHLH to DNA and disrupt its transcriptional capabilities (Zebedee and Hara, 2001). The gene family consists of four members: ID1, ID2, ID3 and ID4. Here, we showed that the interaction between MITF and ID4 is the strongest in the ID gene family (Fig. 5A). Even S510D interacted more strongly with ID4 than S510A and WT (Fig. 5B). MITF lost its ability to transcriptionally activate

TYR when it was bound to ID4, which was shown via luciferase assay (Fig. 5C). ID4 has the strongest inhibitory effect on S510D in terms of TYR (Fig. 5D, E). These data demonstrated that p-MITF inhibits TYR expression through binding to ID4.

#### 4.7. p-MITF interacts with TP53 to regulate CDKN1A expression

A high level of MITF in melanoma cells can induce the expression of CDKN1A, and MITF is associated with cell senescence in



**Fig. 5.** Phosphorylation of MITF enhances the interaction with ID4 and reduces TYR expression.

(A) MITF binds to ID4. GST-MITF was co-transfected with ID family members into HEK293FT cells. Then, whole-cell lysates were used for IP and IB. (B) S510D binds more ID4. (C) SFB-MITF-WT and PGL3-TYR promoter were transfected into HEK293FT cells with/out Myc-ID4. The luciferase activities were assayed and analyzed. Values are shown as the mean  $\pm$  SD. \* $p < 0.05$ . The data is analyzed by Graph pad prism 5. (D) Myc-ID4 and PGL3-TYR promoters were co-transfected into HEK293FT cells with SFB-MITF WT, S510D and S510A, respectively. The following experiments and data analysis are identical to those in Fig. 5C. (E) SFB-ID4 was co-transfected into HEK293FT cells with GST-MITF WT, S510D and S510A. Then, the cell lysates were analyzed by IB.

various melanoma cell lines (Carreira et al., 2005a; Strub et al., 2011; Giuliano et al., 2010). We speculated which proteins would interact with MITF to regulate CDKN1A. We used the IP method to determine which proteins may be involved in MITF's increase CDKN1A at the transcriptional level as reported in literature, such as Retinoblastoma 1 (RB1), catenin beta 1 (CTNNB1), and TP53. We found that TP53 and RB1 can bind to MITF (Fig. 6A). But the affinity of TP53 to MITF S510D is increased (Fig. 6F). So we believed that p-MITF regulating CDKN1A is mediated by TP53. When TP53 was knocked out, CDKN1A was undetectable (Fig. 6B). We found that, of the MITF family members, only MITF can interact with TP53 (Fig. S1D). We truncated TP53 and MITF and found that the HLH domain of MITF and the DNA-binding domain of TP53 contribute to their interaction (Fig. 6C,D). Inhibition of the phosphorylation of MITF by an AKT inhibitor attenuated the interaction between MITF and TP53 (Fig. 6E). As we demonstrated above, ID4 and TP53 could interact with p-MITF, respectively. We next wanted to know whether ID4 and TP53 compete or cooperate. To answer this question we transfected GST-MITF S510D and myc-ID4 in the presence or absence of HA-TP53. The result showed that ID4 binds less MITF S510D in the presence of TP53 (Fig. 6G). This revealed that TP53 competed with ID4 to bind MITF S510D. It has been reported that TP53 binds to several regions of the CDKN1A promoter (Saramäki et al., 2006). CHIP assays were carried out using the MITF antibody and corresponding primers, which recognized different regions of the CDKN1A promoter as reported by Dr. Saramäki. The result indicated that MITF and TP53 bind to the same regions of the CDKN1A promoter (Fig. 6H). Compared to MITF WT and S510A, MITF S510D enhances the binding ability of TP53 to the CDKN1A promoter (Fig. 6I). Therefore, we concluded that, after phosphorylation by AKT, MITF is likely to interact with TP53 and leads to the expression of CDKN1A and prevents cell proliferation.

#### 4.8. Phosphorylation of MITF causes cell proliferation arrest and senescence

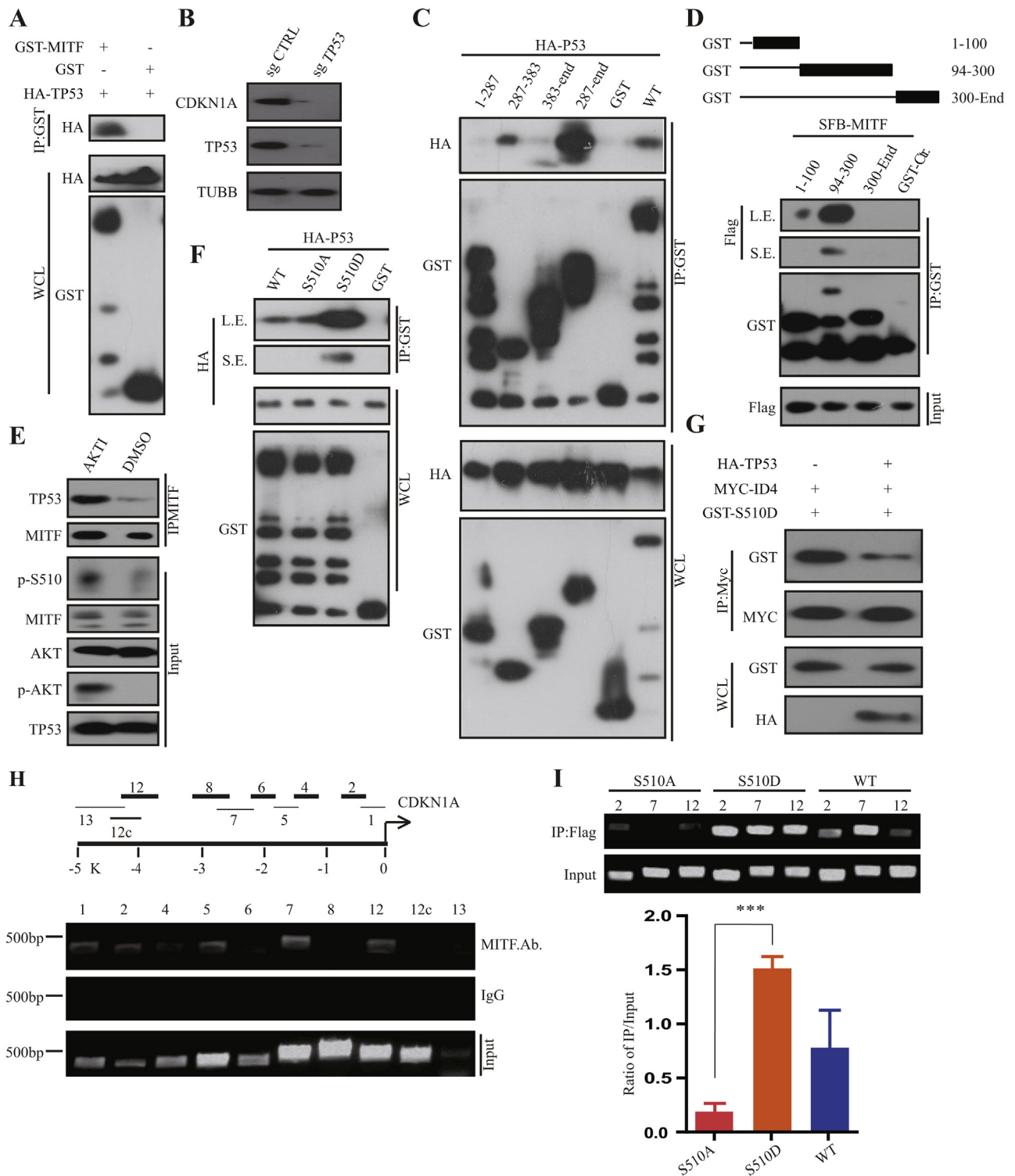
Previous data indicate that p-MITF prevents cell proliferation. We established inducible stable cell lines of the MITF variants. Cell viability was measured by the MTT assay. The results showed that all of the MITF variants could induce cell proliferation arrest; however, S510D had a stronger effect (Fig. 7A). When B16 cells

were cotransfected with lentivirus expressing MITF variants and shMitf for 72 h and beta-Gal staining was performed, much more blue staining was observed in the S510D-infected cells. This indicated that S510D induced more cell senescence (Fig. 7B). Some researchers believe that cancer is a kind of parasitic organism; to survive, they are unscrupulous. Based on this opinion, we believe that melanoma is not excluded. To survive, they chose to weaken the activity of AKT. If this is the case, p-AKT and MITF should exhibit an inverse relationship. To determine whether this is the case, we performed immunohistochemical staining of p-AKT-S473 and MITF on melanoma slices from patients. We stained samples from 16 patients who had melanoma in their skin (Supplemental Table 1). The representative images are shown in Fig. 7C. We found that 90% of the melanoma samples showed an inverse relationship between p-AKT and MITF and positive relationship between p-AKT and p-MITF (Fig. 7C). In a small number of samples, such as sample number 15-24690, some regions showed a lower MITF/p-AKT ratio relative to the negative control (Fig. 7C). This result is consistent with our observation in cell lines. This indicates that in high number of MITF melanoma cells, the AKT activity will be reduced to avoid cell senescence through phosphorylation of MITF.

## 5. Discussion

We found that EGF could cause proliferation arrest and cell senescence of melanoma cells. The genes CDKN1A and TYR were controlled by AKT. AKT directly phosphorylates MITF at S510. The phosphorylation led to proteasome-mediated MITF degradation, which was concomitant with proliferation arrest and senescence. The unphosphorylated MITF translocates to the nucleus and promotes TYR expression. The phosphorylated mimic S510D localizes to both the cytoplasm and the nucleus and enhances CDKN1A expression. We also demonstrated that MITF S510D binds to ID4 to inhibit the transcription of TYR. In the presence of TP53, TP53 would compete with ID4 to bind MITF S510D. The p-MITF/TP53 complex binds to the CDKN1A promoter and transiently enhances CDKN1A expression in response to AKT activation. Our work explains the dual role of MITF in regulating melanoma progress (Fig. 7D).

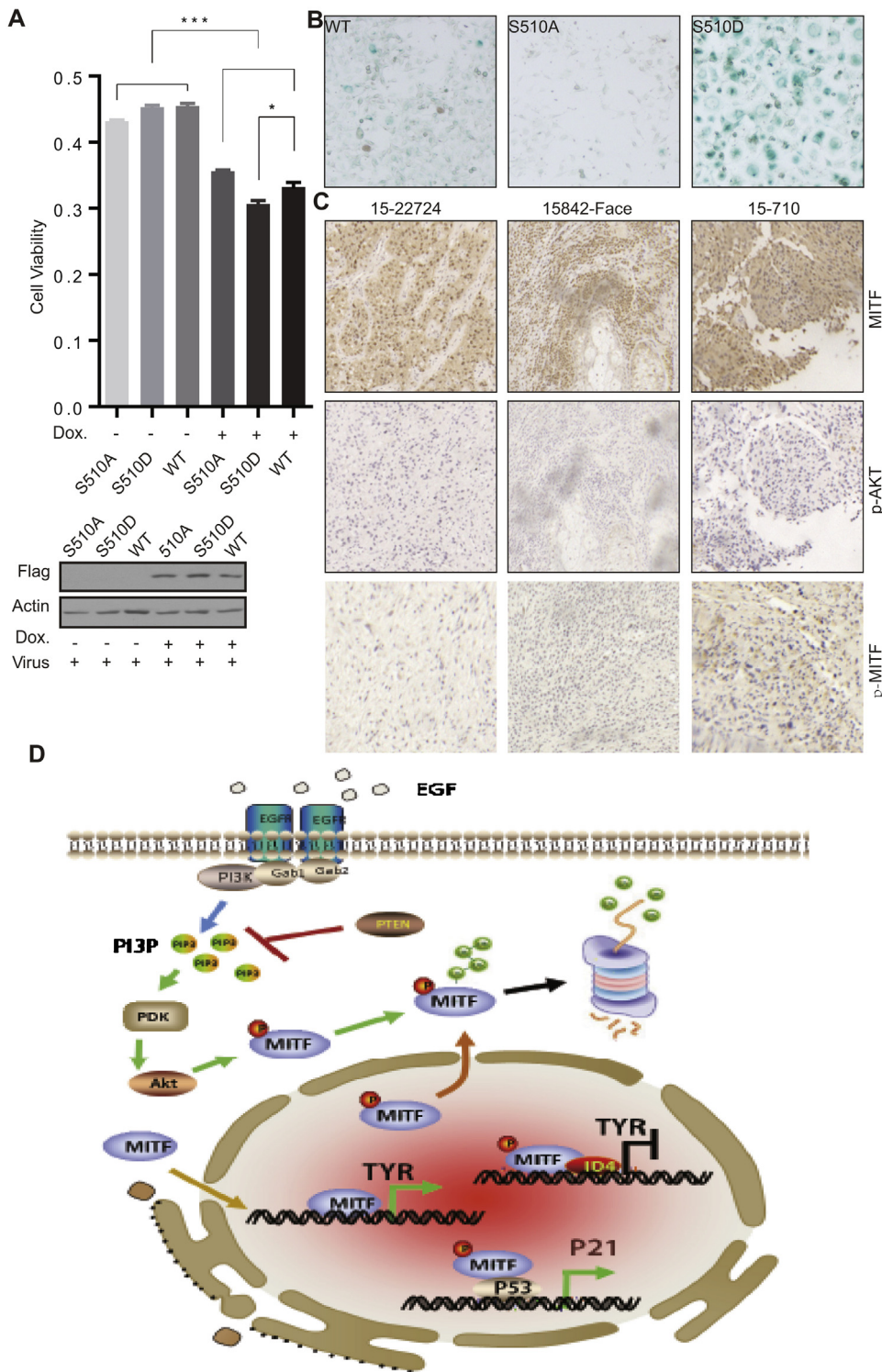
It has been reported that phosphorylation of MITF at S73 by ERK also leads to CDKN1A expression and MITF instability (Kim et al., 2010; Wu et al., 2000a; Liu et al., 2010). P90-RSK1, which



**Fig. 6.** Phosphorylated MITF interacts with TP53 and induces CDKN1A expression.

(A) MITF binds TP53 in vitro. GST-MITF and HA-TP53 were transfected into HEK293FT cells and whole-cell lysates were subjected to the indicated antibodies. (B) Deletion of *TP53* blocked the expression of CDKN1A. (C) The fragments of MITF were co-transfected with HA-TP53 into HEK293FT cells, followed by the IP and IB experiments. (D) MITF interacts with TP53 94–300. The diagram of TP53 fragments is shown in the top panel. (E) B16 cells were incubated with or without AKTI. The lysates were IPed with the MITF antibody and analyzed with the TP53 antibody. (F) MITF S510D strongly interacts with TP53. The indicated plasmids were transfected into HEK293 cells, followed by IP and IB analysis using the corresponding antibodies. (G) TP53 competes with ID4 in binding MITF. Myc-ID4 and mGST-MITF-S510D were co-transfected with or without HA-TP53 into HEK293FT cells, followed by downstream experiments and data analysis. (H) The CHIP assay was performed using the MITF antibody following the manufacturer's instructions. PCR was performed using specific primers. (I) The indicated plasmids were transfected into MGH cells, then the cells were subjected to the subsequent procedures as described in Fig. 6H. The gray scale was calculated using Image J. Then, the data were subjected to analysis via Graph pad prism 5. Mean  $\pm$  SEM. \* $p < 0.05$ .





**Fig. 7.** Phosphorylation of MITF induces senescence. (A) Stable B16 cells were infected with inducible lentivirus of MITF variants and selected by puromycin. After inducing expression of the MITF variants, the MTT assay was performed. The data analysis is identical to that presented in Fig. 1E. Values are presented as the mean ± SEM, \*p < 0.05, \*\*\*p < 0.001. (B) Images of beta-Gal staining of shB16 cells infected with MITF WT, S510D and S510A lentivirus. (C) The representative images of p-AKT and MITF in human melanomas. (D) Flowchart of the model.

is the direct target of BRAF-V600E, phosphorylates MITF at S409 and causes its degradation (Wu et al., 2000b). Our observations of phosphorylation at S510 of MITF are similar to the observations of phosphorylation of MITF at S73 and S409. Here, we found that AKT phosphorylates MITF at S510, which triggers its degradation. This

phosphorylation leads to transient expression of its downstream target CDKN1A and reduces TYR transcription.

Tyrosinase is the limiting step for melanin synthesis, which is controlled by MITF (Slominski et al., 2004). Melanin is lost during PI3K/AKT activation (Lee et al., 2012; Song et al., 2015) and TYR is downregulated by EGF treatment (Shin et al., 2014).

GSK3 $\beta$  phosphorylates MITF at S298 and blocks its transcriptional activation of TYR (Takeda et al., 2000b; Khaled et al., 2002). More melanin was produced in B16 cells when AKT was inhibited (Huang et al., 2014). All of our findings are consistent with these results, and these data are related to MITF phosphorylation. We found MITF S510A could induce melanin production by upregulating TYR expression. Thus we demonstrated the insight mechanism that AKT regulates Melanin production. Unphosphorylated MITF is much more stable than the WT and phosphorylated forms. We found that phosphorylated MITF bound more ID4, which inhibits the transcriptional activation of TYR by MITF.

Cellular senescence is an irreversible proliferation arrest that is activated by various cellular and molecular stresses (Ben-Porath and Weinberg, 2004; Campisi, 2005). Senescence or proliferation arrest could be induced by oncogenes such as KRAS mutations in normal human and mouse cells in culture (Vicente et al., 2010). PI3K/AKT is also an inducer of senescence (Astle et al., 2012; Kennedy Ras et al., 2014; Sun et al., 2014). Consistent with previous reports, we observed cell proliferation arrest as a result of PI3K/AKT activation in B16 melanoma cells. CDKN1A is a predominant inducer of cell proliferation arrest and senescence (Kuilman et al., 2010). Here, we found that CDKN1A is dramatically upregulated with EGF stimulation. The S510D mutant also increased CDKN1A expression and senescence.

Normally, cell senescence is related to RB1 and TP53 (Benhamed et al., 2012; Qian and Chen, 2013; Chen et al., 2005). Indeed, the ability of MITF to regulate CDKN1A is likely to be highly complex, with many factors contributing directly or indirectly to the regulation of CDKN1A expression by MITF (Carreira et al., 2005b, 2000). However, we did not observe any differences in the interactions between WT, S510A, S510D and RB1 (data not show). Meanwhile, MITF still upregulated CDKN1A even after knockdown of RB1. It is indicated that this phosphorylation regulating CDKN1A expression is not mediated by RB1. Actually, we discovered that p-MITF directly binds TP53 to regulate CDKN1A expression and that p-MITF does not bind ID4 in the presence of TP53.

In normal skin cells, p-AKT and MITF are moderately expressed (<http://www.proteinatlas.org>), and we believe that MITF is in a controlled transcriptional state to maintain normal physiological function. Here, we identified an important role of MITF in response to EGF stimulation and dissected the mechanism by which MITF regulates CDKN1A and TYR. MITF and p-AKT exhibit an inverse relationship in patient samples. p-MITF-S510 is an alternative diagnostic marker for human melanoma. Our results help us to understand the multiple roles of MITF in melanoma and open up a new avenue for the treatment of melanoma.

### Competing financial interests

The authors declare no competing financial interests.

### Author contributions

Z. L. designed the experiments and wrote the manuscript; C. W. processed the experiments and wrote the manuscript; L. Z., Y. W. and S. G. provided the slices of human melanomas; Q. S. and H. W. completed experiments; X. F. classified the slices of human melanoma after staining; H. C. provided material and suggestions.

### Acknowledgements

We thank Prof. Colin R. Goding at University of Oxford for kindly gift of constructs. The authors have no competing financial interests to declare. This work was supported by grants from the Ministry of Science and Technology of China (973 Program; 2014CB910500)

and the National Natural Science Foundation of China (81172231) to Z. Liu.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2016.09.029>.

### References

- Astle, M.V., et al., 2012. AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: implications for targeting mTOR during malignancy. *Oncogene* 31 (15), 1949–1962.
- Ben-Porath, I., Weinberg, R.A., 2004. When cells get stressed: an integrative view of cellular senescence. *J. Clin. Investig.* 113 (1), 8–13.
- Benhamed, M., et al., 2012. Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. *Nat. Cell Biol.* 14 (3), 266–275.
- Bentley, N.J., Eisen, T., Goding, C.R., 1994. Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. *Mol. Cell. Biol.* 14 (12), 7996–8006.
- Bieging, K.T., Mello, S.S., Attardi, L.D., 2014. Unravelling mechanisms of p53-mediated tumour suppression. *Nature reviews. Cancer* 14 (5), 359–370.
- Campisi, J., 2005. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 120 (4), 513–522.
- Carreira, S., Liu, B., Goding, C.R., 2000. The gene encoding the T-box factor Tbx2 is a target for the microphthalmia-associated transcription factor in melanocytes. *J. Biol. Chem.* 275 (29), 21920–21927.
- Carreira, S., et al., 2005a. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. *Nature* 433 (7027), 764–769.
- Carreira, S., et al., 2005b. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. *Nature* 433 (7027), 764–769.
- Chen, Z., et al., 2005. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 436, 725–730.
- Garraway, L.A., et al., 2005. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* 436 (7047), 117–122.
- Giuliano, S., et al., 2010. Microphthalmia-Associated transcription factor controls the DNA damage response and a lineage-specific senescence program in melanomas. *Cancer Res.* 70 (9), 3813–3822.
- Hu, Y., et al., 2005. Akt phosphorylates acinus and inhibits its proteolytic cleavage: preventing chromatin condensation. *EMBO J.* 24 (20), 3543–3554.
- Huang, H.-C.C., et al., 2014. [6]-Shogaol inhibits  $\alpha$ -MSH-induced melanogenesis through the acceleration of ERK and PI3K/Akt-mediated MITF degradation. *BioMed Res. Int.* 2014, 842569.
- Jeong, H.-S.S., et al., 2015. Baicalin-induced Akt activation decreases melanogenesis through downregulation of microphthalmia-associated transcription factor and tyrosinase. *Eur. J. Pharmacol.* 761, 19–27.
- A.L. Kennedy Ras, D. Adams, J. Morton, PI3 K/Akt and senescence Ras, PI3 K/ Akt and senescence 2014.
- Khaled, M., et al., 2002. Glycogen synthase kinase 3beta is activated by cAMP and plays an active role in the regulation of melanogenesis. *J. Biol. Chem.* 277 (37), 33690–33697.
- Kim, D.-S.S., et al., 2010. Sphingosylphosphorylcholine inhibits melanin synthesis via pertussis toxin-sensitive MITF degradation. *J. Pharm. Pharmacol.* 62 (2), 181–187.
- Korner, A., Pawelek, J., 1982. Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. *Science* 217 (4565), 1163–1165.
- Kuilman, T., Michaloglou, C., Mooi, W.J., 2010. The essence of senescence. *Genes* 24, 2463–2479.
- Lee, M.-S.S., et al., 2012. Dioxinohydroeckol inhibits melanin synthesis through PI3K/Akt signalling pathway in  $\alpha$ -melanocyte-stimulating hormone-treated B16F10 cells. *Exp. Dermatol.* 21 (6), 471–473.
- Leikam, C., et al., 2014. Cystathionase mediates senescence evasion in melanocytes and melanoma cells. *Oncogene* 33 (6), 771–782.
- Levy, C., Khaled, M., Fisher, D.E., 2006. MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol. Med.* 12 (9).
- Liu, Z., et al., 2008. Neuroprotective actions of PIKE-L by inhibition of SET proteolytic degradation by asparagine endopeptidase. *Mol. Cell* 29 (6), 665–678.
- Liu, F., et al., 2010. Mitf links Erk1/2 kinase and p21CIP1/WAF1 activation after UVC radiation in normal human melanocytes and melanoma cells. *Molecular* 9 (214).
- Palmieri, G., et al., 2015. Multiple molecular pathways in melanomagenesis: characterization of therapeutic targets. *Frontiers in oncology* 5, 183.
- Qian, Y., Chen, X., 2013. Senescence regulation by the p53 protein family. *Cell Senescence Methods Protoc.* 965, 37–61.
- Saramäki, A., et al., 2006. Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. *Nucleic Acids Res.* 34 (2), 543–554.
- Shin, J.-M.M., et al., 2014. Nrf2 negatively regulates melanogenesis by modulating PI3K/Akt signaling. *PLoS One* 9 (4).

- Slominski, A., Tobin, D.J., Shibahara, S., 2004. Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiological* 84 (4), 1155–1228.
- Soengas, M.S., Lowe, S.W., 2003. Apoptosis and melanoma chemoresistance. *Oncogene* 22 (20), 3138–3151.
- Song, Y.C., et al., 2015. Berberine regulates melanin synthesis by activating PI3K/AKT: ERK and GSK3 $\beta$  in B16F10 melanoma cells. *Int. J. Mol. Med.* 35 (4), 1011–1016.
- Strub, T., et al., 2011. Essential role of microphthalmia transcription factor for DNA replication, mitosis and genomic stability in melanoma. *Oncogene* 30, 2319–2332.
- Sun, C., et al., 2014. Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. *Nature* 508 (7494), 118–122.
- Swaney, D.L., et al., 2013. Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. *Nat. Methods* 10 (7), 676–682.
- Takeda, K., et al., 2000a. Ser298 of MITF: a mutation site in Waardenburg syndrome type 2, is a phosphorylation site with functional significance. *Hum. Mol. Genet.* 9 (1), 125–132.
- Takeda, K., Takemoto, C., Kobayashi, I., 2000b. Ser298 of MITF, a mutation site in Waardenburg syndrome type 2, is a phosphorylation site with functional significance. *Hum. Mol.* 9 (1), 125–132.
- Vicent, S., et al., 2010. Wilms tumor 1 (WT1) regulates KRAS-driven oncogenesis and senescence in mouse and human models. *J. Clin. Invest.* 120 (11), 3940–3952.
- Wu, M., Hemesath, T.J., Takemoto, C.M., 2000a. c-Kit Triggers Dual Phosphorylations, Which Couple Activation and Degradation of the Essential Melanocyte Factor Mi. *Genes Dev.* 14 (3), 301–312.
- Wu, M., et al., 2000b. c-Kit triggers dual phosphorylations: which couple activation and degradation of the essential melanocyte factor Mi. *Genes. Dev.* 14 (3), 301–312.
- Yajima, I., et al., 2011. Molecular network associated with MITF in skin melanoma development and progression. *J. Skin Cancer* 433 (7027), 764–769.
- Zebedee, Z., Hara, E., 2001. Id proteins in cell cycle control and cellular senescence. *Oncogene* 20, 8317–8325.