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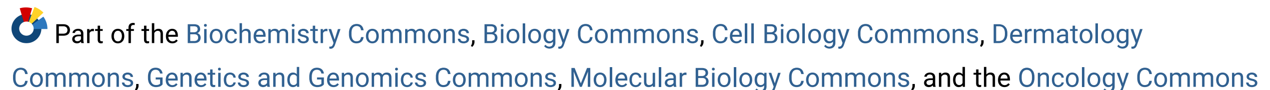
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Investigating the Redox Sensitivity of MITF Splice Variants

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Mentored by Pamela Cassidy, PhD - Leachman/Cassidy Lab

Dermatology Department, Oregon Health & Science University

May 2020

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Abstract

Within pigment-producing cells known as melanocytes, the transcription factor MITF is intimately involved in regulating genes associated with cell cycle maintenance and melanocyte differentiation. Research, however, has provided conflicting results on the relationship between the expression levels of MITF and melanocyte cell fate. To complicate matters, two splice variants of MITF exist, differing by only 18 base pairs. These variants have been observed at variable levels of expression in melanocyte and melanoma cells, raising the question as to their functional purpose. Building upon previous research by the Leachman/Cassidy lab that identified the redox sensitivity of MITF while additionally establishing a novel relationship between the antioxidant selenoprotein Thioredoxin Reductase 1 (TR1) and pigment synthesis, this work seeks to examine the redox sensitivity and function of the MITF splice variants.

Introduction

Melanocytes are specialized cells located primarily within the basal layer of the epidermis, where they synthesize photoprotective pigments known as melanin (Yamaguchi et al., 2007). Melanin is a polymer that absorbs ultraviolet (UV) radiation, protecting surrounding keratinocytes from UV-induced DNA damage by diffusing light energy (Nguyen & Fisher, 2019). Excess UV-induced DNA damage, however, can contribute to the incidence of melanoma, the cancer which results from the aberrant growth and proliferation of melanocytes. While melanoma accounts for only 1% of all skin cancer cases, it is responsible for the majority of skin cancer related deaths (Institute, 2020). Notably, melanocytes are derived from neural crest cells, a heritage that likely influences the ease of epidermal to mesenchymal transition and thus enables aggressive metastasis (Larribère & Utikal, 2019). Several genetic mutations are associated with increased melanoma risk, including the oncogenic $BRAF^{V600E}$ substitution, and mutations in the CDKN2A tumor suppressor locus (Hodis et al., 2012; Potrony et al., 2015). Although there are therapeutic treatments currently available, both intrinsic and developed drug resistance is a hallmark of melanoma (Helmbach et al., 2001), prompting ongoing research into the regulatory mechanisms of melanocyte and melanoma cell fate.

The microphthalmia associated transcription factor (MITF) is known as the "master regulator" of melanocyte differentiation and proliferation pathways (Hemesath et al., 1994; Kawakami & Fisher, 2017). This transcription factor has multiple tissue-specific isoforms that arise from different enhancer-promoters, resulting in the variable identity of exon 1 (Fuse et al., 1999). However, only the m-isoform is expressed in select cells of neural crest origin and of those, most notably in melanocytes, where MITF regulates the expression of enzymes responsible for melanin synthesis (M. Murakami et al., 2007). Additionally MITF serves as a transcriptional activator of essential cell survival genes such as MET and BCL2 (Beuret et al., 2007; McGill et al., 2002) while also regulating the expression of CDKN2A, which inhibits the cell cycle and thus maintains cell differentiation (Loercher et al., 2005). These connections to cell cycle regulation have thus made MITF a suspect of oncogenic involvement.

MITF derives its name from the phenotype resulting from mutation of the murine *Mi* locus. This phenotype consists of the complete loss of melanocytes as well as the development of small eyes (microphthalmia) (Kawakami & Fisher, 2017). Mutations in the human MITF homolog result in variable expression of Waardenburg syndrome type II, characterized by abnormal pigmentation, hearing loss, and microphthalmia (Tassabehjil et al., 1994). This

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transcription factor is a basic helix-loop-helix leucine zipper containing a transactivation domain which binds the canonical E-box motif, as well as an extended E-box termed the "m-box" (Hartman & Czyz, 2015). In its active conformation, MITF dimerizes with itself or other bHLH-Zip transcription factors, specifically TFE3, TFEB, or TFEC (Hemesath et al., 1994).

MITF activation is regulated during development by several lineage-specific factors. Notably, Sox10 and Pax3 mediate melanocyte cell survival through MITF activation, acting in synergy to promote transcription (Wellbrock & Arozarena, 2015). The Wnt pathway also alters MITF expression via β-catenin binding with LEF1, which activates the MITF promoter, an activity shown to regulate both differentiation and melanocyte growth (Chien et al., 2009; Eichhoff et al., 2011; Widlund et al., 2002). Intriguingly, MITF has also been shown to interact with LEF1 to modulate its own expression (Saito et al., 2002)**.**

Canonically, MITF is involved in melanogenesis, the skin's major defense system against ultraviolet radiation (Nguyen & Fisher, 2019). UV-induced DNA damage within keratinocytes activates transcription of POMC via p53. POMC is subsequently post-translationally cleaved into α-MSH, the signaling molecule for MC1R, a receptor located on the melanocyte plasma membrane (Hsiao & Fisher, 2014). Activation of MC1R triggers a cAMP signaling cascade that enables CREB binding to the MITF promoter, activating transcription of MITF (Yamaguchi et al., 2007). Once translated, MITF activates the expression of Tyrosinase (TYR), Tyrosinase Related Protein 1 (TYRP1), and Dopachrome Tautomerase (DCT) - enzymes essential for pigment biosynthesis (Fang, 2002; Jiao et al., 2004; Yasumoto et al., 1995). Additionally, MITF regulates the expression of PMEL, a melanosome structural protein that enables melanin deposition, and MLANA, a melanocyte-specific membrane protein (Sitaram & Marks, 2012).

MITF undergoes an array of post translational modifications that alter its activity as a transcriptional regulator (Wellbrock & Arozarena, 2015). Principally, MITF is phosphorylated at serine 73 via MAPK, a modification shown to recruit p300, a histone acetyltransferase, which promotes melanocyte cell growth in a MITF-dependent fashion (Kim et al., 2019; Price et al., 1998). Phosphorylation of MITF was shown to increase affinity for the tyrosinase promoter, leading to speculation that phosphorylation could also modulate the transcriptional regulation of additional MITF target genes (Hemesath et al., 1998). Surprisingly, this modification was also shown to enable ubiquitin-mediated proteolysis, indicating tightly regulated post-translational control of MITF function (Wu et al., 2000)**.** MITF is also targeted by SUMOylation at two lysine residues, a modification shown to repress its transcriptional activity for some but not all target promoters (H. Murakami & Arnheiter, 2005). A germline mutation that affects the SUMO binding sequence in MITF has additionally been identified in patients with melanoma (Bertolotto et al., 2011).

In addition to the known mechanisms of transcriptional and post-translational control, two splice variants of MITF have been observed at variable expression levels in melanocyte and melanoma cell lines (Primot et al., 2009). These splice variants differ by an alternative splice acceptor in exon 6 that controls for the presence or absence (designated + or -18) of 18 nucleotides upstream of the DNA binding domain (Hodgkinson et al., 1993; Steingrimsson et al., 1994). These 18 nucleotides code for the amino acid sequence ACIFPT (Tachibana et al., 1994). Although the function of these variants is currently unknown, Primot *et al* found that the -18 isoform predominates in cultured melanoma cell lines, and variant expression is modulated by MEK inhibition (Primot et al., 2009). Additionally, MITF +18 expression was shown to elicit

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transcription of tyrosinase more efficiently, whereas other research found that this isoform has an inhibitory effect on DNA synthesis, and subsequently cell proliferation (Bismuth et al., 2005; M. Murakami et al., 2007).

Our investigation of MITF function coincides with an inquiry of melanocyte response to oxidative stress. Melanocyte cells are exposed to oxidative stress from a variety of sources, including the melanin synthesis process which generates hydrogen peroxide, and UV radiation, which induces reactive oxygen species (ROS) including superoxide anion (Meyskens et al., 2001). In general, oxidative stress incites a distinct cellular response which can be alleviated by antioxidant proteins, such as the Thioredoxin/thioredoxin reductase and glutathione/glutaredoxin redox systems, as well as additional antioxidant programs that are transcriptionally controlled by Nrf2 and the redox sensor APE-1 (Liu et al., 2009; Tonelli et al., 2018). However, melanoma cells have been reported to contain higher levels of ROS than normal melanocytes (Policastro et al., 2009). While increased oxidative stress is expected to increase the DNA mutational load and subsequent cancer potentiation of a cell, such stress may additionally dysregulate redox-mediated cell signaling pathways. Consequently, it is suggested that alterations in the redox state of melanocytes are involved in the pathogenesis of melanoma (Meyskens et al., 2001).

Previous research by Cassidy *et al* found a positive correlation between melanoma stage and Thioredoxin reductase 1 (TR1) expression, suggesting that upregulation of this antioxidant may be a factor in melanoma development (Cassidy et al., 2015). Subsequently, while further investigating the relationship between TR1 expression and melanocyte behavior, the Leachman/Cassidy lab found that melanin was significantly reduced after knocking down TR1 in immortalized melanocytes. This turned attention towards the regulator of pigment biosynthesis - MITF.

To understand the role of TR1 in pigmentation, the Leachman/Cassidy lab first examined the effects of redox stress on the network of cellular antioxidants, including TR1, its substrate Thioredoxin (TRX), and peroxiredoxin 1 (PRX1), a thiol oxidoreductase known to interact with TRX via disulfide exchange. To investigate these proteins under redox stress, cells were treated with varying concentrations of hydrogen peroxide. Following hydrogen peroxide treatment, cells were supplemented with N-ethylmaleimide (NEM) to covalently modify reduced thiols and prevent oxidized artifacts, thereby capturing the oxidation states of cellular proteins (García-Santamarina et al., 2014; Hansen & Winther, 2009). Cell lysates were then fractionated by non-reducing SDS-PAGE. By this method, proteins that engage in disulfide bonds with other proteins are anticipated to appear at higher molecular weights on a western blot, indicative of protein complexes (Stöcker et al., 2018)**.** To our surprise, probing these blots for MITF showed unexpected high molecular weight species in melanocytes treated with hydrogen peroxide (figure 1A). Additionally, when the same lysates were run under reducing conditions, MITF was restored to its expected weight of approximately 54 kD, consistent with the behavior of transient redox-sensitive complexes (Figure 1B). These experiments revealed that MITF is capable of forming disulfide linked conjugates under oxidative stress, suggesting that MITF serves a redox-mediated function within the cell. Combined with the observation that pigment synthesis is reduced in TR1 knockdown cells, these results indicate that MITF responds to TR1 regulation, potentially in a direct or indirect redox-dependent fashion. Together, these findings point towards a redox associated role for MITF in melanocyte biology and the development of melanoma.

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Heterogeneous expression of MITF in Melanoma cells has complicated the distinction between MITF and cell fate (Ennen et al., 2015; Goodall et al., 2008). A "rheostat model" has been suggested for MITF, where low levels of expression correspond to senescence or invasive properties, and higher expression to proliferation and differentiation tendencies (Goding, 2011). In line with this model, we hypothesize that a balance between MITF variant expression is involved in maintaining the associated differentiation and proliferation pathways in normal melanocytes. With the observation that MITF is sensitive to hydrogen peroxide and additional evidence that its transcriptional targets are impacted by inhibition of endogenous redox protein (Kline et al., n.d.) we suggest MITF undergoes a redox-mediated post translational modification that affects its activity within the cell, thereby attenuating transcriptional targets in accordance with cellular redox conditions. Furthermore, because the 18 base pairs retained by the +18 isoform encode an additional cysteine residue capable of oxidation, we hypothesize the splice variants may be differentially responsive to redox conditions, thus affecting their expression, protein interactions, and/or target genes. Cumulatively, a change in cellular redox state may therefore affect the expression and functional response of the MITF variants, leading to a modulated cellular response that may direct melanocytes towards a proliferative, cancerous phenotype.

To investigate this hypothesis, we designed MITF variant-specific expression vectors and transfected multiple cell lines to optimize protein expression. Transfected cells were treated with hydrogen peroxide to induce oxidative stress, and protein was extracted and analyzed under reducing and non-reducing conditions. The final expression constructs are equipped with dual tags, enabling co-immunoprecipitation of MITF with any binding partners, and their identification by mass spectrometry. This will allow us to assess whether the MITF variants interact with different proteins, potentially in a redox-dependent fashion. While this work represents only the initial stages of our intended investigation of MITF variant activity, our preliminary data corroborates earlier findings, and will enable further examination of the functional characteristics of MITF splice variants.

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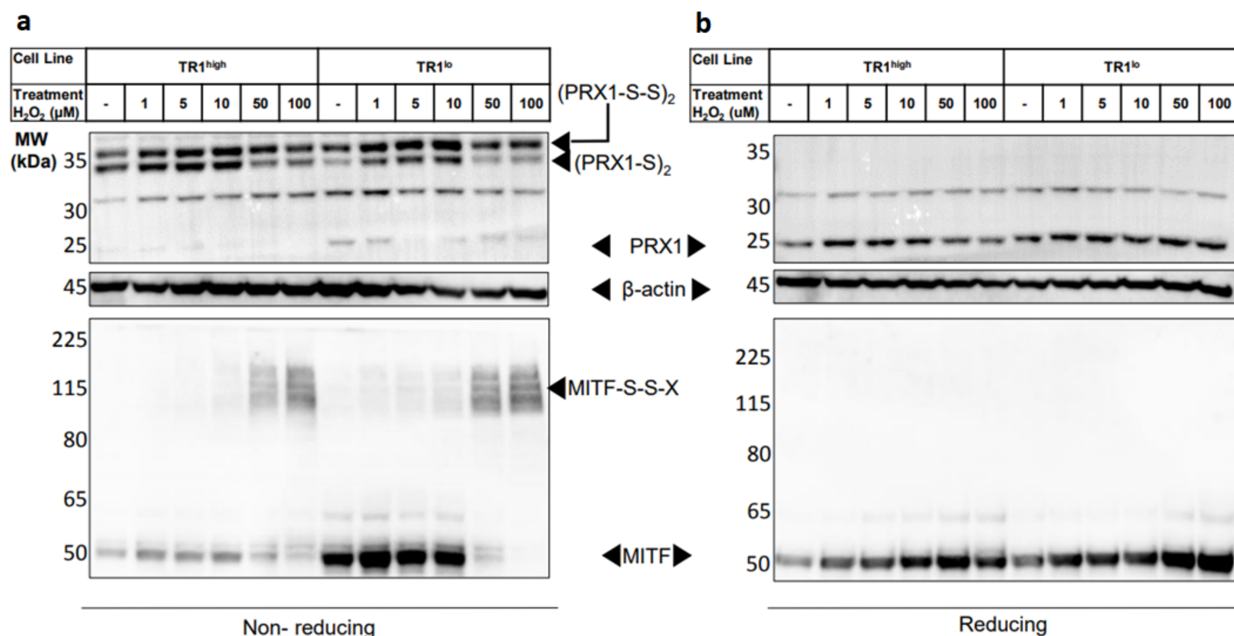

Figure 1. Western blot of PIG1 melanocytes with hydrogen peroxide treatment courtesy of Dr. Chelsey Kline, from her paper currently under review. $TR1^{lo}$ cells stably express a siRNA targeting Thioredoxin reductase 1, while $TR1^{high}$ cells express a control siRNA that does not target a eukaryotic transcript. Cells were subsequently treated with a 1 min burst of hydrogen peroxide at different concentrations, followed by treatment with 100 mM NEM to covalently modify reduced thiols. **a**) Protein lysate run on a non-reducing gel showcases MITF at high molecular weights (MITF-S-S-X) as well as the anticipated monomeric weight of 54 kD. **b**) Running the same lysates under reducing conditions shows that MITF is restored to its anticipated molecular weight of approximately 54 kD upon reduction by BME. A similar response to hydrogen peroxide is observed by peroxiredoxin 1 (PRX1), which dimerizes under non-reducing conditions (PRX1-S_2, PRX1-S-S_2).

Methods and Results

Our initial goal was to isolate expression of the MITF + and – 18 bp variants. We constructed affinity tagged MITF expression vectors and transfected various cell lines that do not endogenously express the melanocyte-specific MITF isoform (MITF-m). The MITF - 18 expression plasmid was purchased from Addgene (pCMV Tag4a-MITF-M (wt), #33151). This plasmid encodes a carboxyl terminal flag tag, as well as a neomycin resistance gene. The MITF + 18 variant was constructed by overlap extension PCR to integrate the missing 18 bp sequence into the pCMV-Tag4a plasmid. We primarily transfected Hela cells, as the endogenous MITF expression has previously been characterized in this cell line. HeLa cells express the MITF-A, C and H isoforms, but do not express the m isoform (Fuse et al., 1999; Primot et al., 2009). We conveniently found that the MITF D9 antibody (Santacruz, sc-515925) although advertised as a MITF-m specific antibody, additionally detects the MITF-A isoform, confirmed by both western blot (Supplemental Figure 1, Appendix) and examination of the D9 epitope sequence. In contrast the MITF C5 antibody (Millipore Sigma, MAB3747-I) appears specific to the MITF-m isoform.

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The application of these two antibodies subsequently allowed us to compare the protein levels from our expression vectors against endogenous MITF levels in HeLa cells.

HeLa cells (ATCC, CCL-2) were transfected using both lipofectamine and Neon electroporation (ThermoFisher) to determine the best method of transfection. Although HeLa cells are considered an easily-transfectable cell line, we experienced inconsistent protein expression using either transfection method. Geneticin aminoglycoside antibiotic (Life Technologies, #11811023) was used to select for stable expression of MITF following transfection, however the selection process appeared to affect cell viability, further contributing to low protein yields and preventing establishment of stable cell lines. While MITF expressing non-melanocyte cell lines have been generated by other labs, reviewed literature indicates MITF was only transiently expressed in such experiments. Therefore, we hypothesize that non-melanocyte cells do not have a favorable response to stable expression of the melanocyte specific MITF isoform. Additionally, we hypothesized the carboxyl tag itself could be inhibiting protein detection, given the differences observed when probing the same samples with anti-MITF (C5) and anti-flag antibodies (Figure 2). Consequently, we later found literature that indicates MITF is cleaved by caspases to produce a 45 kD amino terminal fragment (Larribere et al., 2005). Such cleavage by caspases would explain the differences in detection between anti-MITF (C5) and anti-flag antibodies on the same membranes, as cleavage of the carboxyl terminus would result in loss of the flag tag. Typically, the melanocyte specific MITF isoform is observed around 54kD and 60kD on western blots, the higher molecular weight species being attributed to the phosphorylated form of MITF (Hemesath et al., 1998).

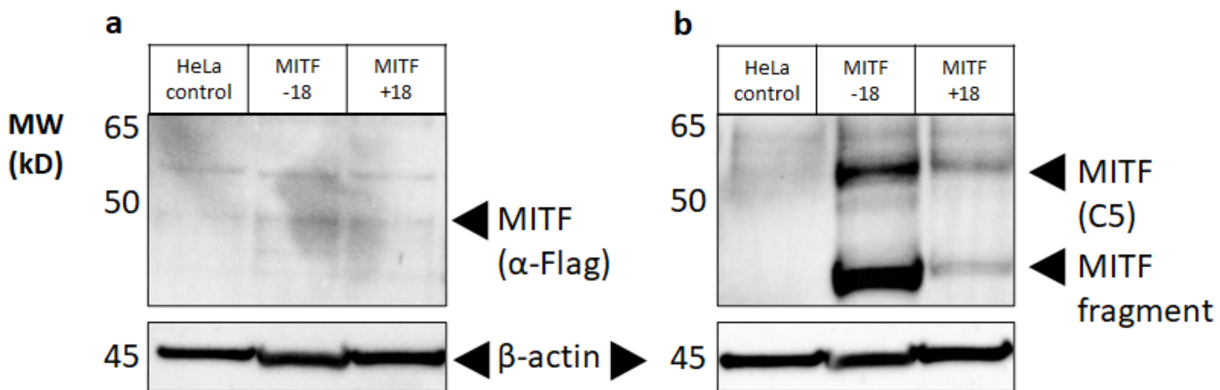

Figure 2. Western blot of HeLa cells transfected with pCMV-Tag4a MITF-m variant plasmids. Replicate membranes were blotted with anti-flag and anti-MITF C5 antibodies to compare detection between the two antibodies. **a**) The anti-flag antibody shows negligible protein expression. **b**) Results from the C5 antibody indicate MITF expression, however the -18 variant shows greater expression in comparison to the +18 variant. Furthermore, an approximately 45 kD band is recognized by the C5, but not the anti-flag antibody, supporting its identification as an amino terminal fragment of MITF, produced by caspase cleavage.

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Construction and Expression of N-terminal Flag-tagged MITF

To test the hypothesis that the C-terminal tag affects expression of MITF, we constructed N-terminal flag-tagged expression vectors. The pCMV-Tag2b vector, encoding an amino terminal flag tag, was a generous gift from Dr. Ping Xing, Oregon Health & Science University. The MITF variant inserts were isolated from the previous pCMV-Tag4a vectors by digesting with EcoRI and HindIII restriction enzymes. The pCMV-Tag2B vector was additionally digested and treated with calf intestine alkaline phosphatase, and both inserts and vector were isolated by gel electrophoresis on 1% TAE agarose gel. The DNA fragments were excised from the gel and purified using the QIAquick gel extraction kit (Qiagen, #28704). Overnight ligation at 16°C was completed using T4 DNA ligase (ThermoFisher, # 15224017) at a 3:1 insert to vector molar ratio. One Shot TOP10 E. coli (Thermofisher, # C404003) were transformed with the ligated reaction according to manufacturer's recommendations. The transformations were plated on LB plates with kanamycin (50µg/ml), and isolated colonies were selected and grown in 100 mL volumes of LB liquid culture. Plasmids were then purified from the bacterial cultures using the Qiagen Endofree plasmid maxi kit (Qiagen, #12362), quantified using a Biotek Synergy H1 microplate reader, and sequenced (Vollum DNA sequencing Core, OHSU).

Transfection with the pCMV-Tag2b MITF variant plasmids was performed using Lipofectamine 3000 lipid reagent (Fisher Scientific, L3000-08). Lipofectamine 3000 was used according to manufacturer's recommendations, with Optimem serum free media, P3000 reagent, and 2.5 µg plasmid. Solutions were added to 6-well plates with 250,000 HeLa cells/well. Twenty-four hours post transfection, cell media was removed and replaced with either 100µM H_2O_2 or PBS for 1 minute. Treatment was then removed and replaced with 100 mM NEM in PBS and incubated for 5 minutes on ice. Cells were subsequently harvested in lysis buffer (40 mM HEPES- pH7.5, 50 mM NaCl, 1 mM EDTA, 1% Triton X-100) with 100 mM NEM. Lysates were incubated for 1 hour at 37°C, sonicated, and centrifuged. Protein lysates were divided into reducing and non-reducing aliquots, whereby reducing samples were heated for 5 minutes at 95°C in NuPAGE loading buffer (Invitrogen, NP0007) with β-Mercapthoethanol (BME) at 1% final concentration. Non-reducing samples were heated in NuPAGE buffer only. Protein lysates were quantified by BCA protein assay, and 7 ug of protein per sample was run on 4-12% bis-Tris SDS-PAGE gel for 90 minutes at 100 volts. The gel was transferred to a 0.45 µm PVDF membrane on ice for 60 minutes at 30 volts. Membranes were then blocked in 5% non-fat dried milk in Tris-buffered saline overnight. Each membrane was blotted with anti-MITF C5, anti-Flag (Sigma Aldrich, # F1804), and β-actin (Cell Signaling, # F1804) with complementary secondary antibodies conjugated to horseradish peroxidase (Abcam, ab6789 and ab6721). The membranes were subsequently exposed with chemiluminescent reagent (ThermoFisher, #34076) and imaged.

Use of Lipofectamine 3000 reagent was found to increase transfection efficiency over Neon Electroporation. A time course study of protein expression indicated that 24 hours post transfection was optimum, having high expression levels without compromising cell viability. Application of NEM following hydrogen peroxide treatment preserved disulfide conjugated proteins induced by redox stress, and enabled visualization of protein conjugates on non-reducing gels (Figure 3). MITF was in fact observed at higher molecular weights in hydrogen peroxide treatment groups on non-reducing gels (Figure 3a), as previously described by Kline *et al.* In contrast, running the same protein samples under reducing conditions restores MITF to its

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monomeric molecular weight of 54 kD (Figure 3b). On additional reducing gels, MITF expression was also observed around 45kD (Figure 4). This observation is consistent with the reports of MITF cleavage by Lariberre *et al*, which produces a 45kD N-terminal MITF fragment (Larribere et al., 2005). Because we are hoping to identify binding partners of MITF through immunoprecipitation and mass spectrometry, cleavage of the protein could compromise efficient isolation of the entire polypeptide.

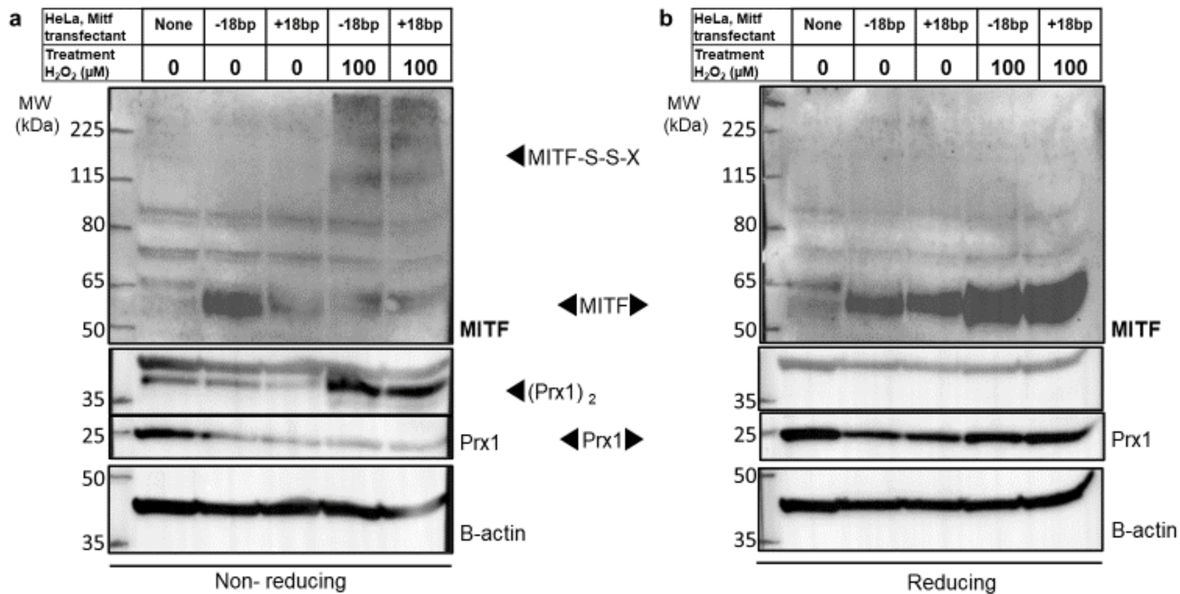

Figure 3. Western blot of HeLa cells transfected with N-terminal flag-tagged MITF variants and treated with hydrogen peroxide. Under non-reducing conditions, MITF appears at high molecular weights in hydrogen peroxide treatment groups only (MITF-S-S-X), whereas MITF in untreated cells is observed at its anticipated molecular weight between 50-65 kD (figure 4A). Under reducing conditions, MITF is restored to its anticipated molecular weight (figure 4B) as expected of transient, disulfide linked proteins. Probing these blots for Peroxiredoxin 1 (Prx1), a protein known to dimerize via disulfide bonds ($Prx1_2$), shows a similar pattern of high molecular weight species on non-reducing gel.

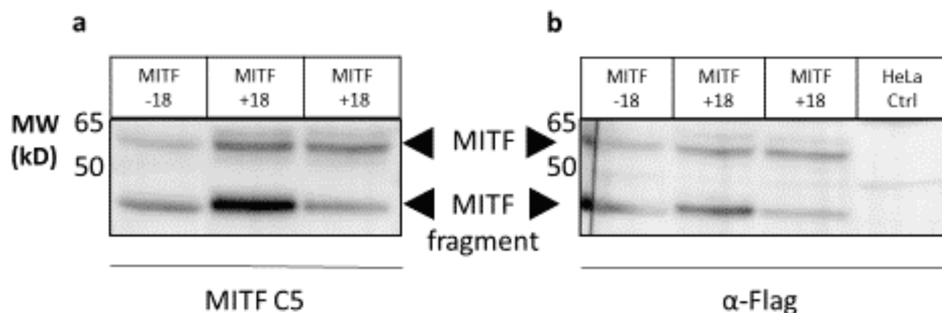

Figure 4. Hela cells transfected with N-terminal flag-tagged MITF variants, run under reducing conditions. Twenty-four hours after transfection with lipofectamine 3000 reagent, MITF is observed at two molecular weights. Probing with either C5 antibody (**a**) or anti-Flag antibody (**b**) indicates the presence of an MITF fragment of approximately 45 kD.

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Construction and expression of dual-tagged MITF transfection vector

To ensure isolation of the intact MITF polypeptide during immunoprecipitation, we designed a dual-tagged expression vector that will enable affinity purification with 2 different antibodies. A pCMV6-AC-3HA plasmid (Origene, PS100067) encoding a triple hemagglutinin (HA) carboxyl tag was utilized as the transfection vector. A BsiWI restriction site was substituted into the pCMV-Tag2B MITF variant plasmids using overlap extension PCR. By this method, the MITF variant sequences and amino-terminal flag tag could be isolated from the Tag2B plasmids by restriction digest. The pCMV-Tag2B MITF variant plasmids were amplified with CMV-F/BsiWI-R and BsiWI-F/T7-F primer pairs (Table 1, appendix) using Phusion Flash polymerase (Thermofisher, F548S). The reactions were purified using the Purelink PCR purification kit (Invitrogen, K310001), then the reaction products were amplified together using the CMV-F/T7-F primer pair. The PCR product was again purified, then digested alongside the pCMV6-AC-3HA vector using BmtI and BsiWi high fidelity restriction enzymes (NEB, R3658S and R3553S). The vector was treated with calf intestine alkaline phosphatase, then both inserts and vector were isolated by gel electrophoresis on 1% TAE agarose gel. After gel extraction and purification, insert and vector were mixed at a 3:1 molar ratio, treated with T4 DNA ligase, then incubated for 1 hour at room temperature. Transformation, plasmid purification, and sequencing were performed as previously described.

$M14^{-/-}$ and WT melanoma cells were transfected with the dual-tagged MITF expression vectors using lipofectamine 3000 reagent as previously described. The $M14^{-/-}$ cell line is a TR1 knockout generated by Dr. Chelsey Kline using CRISPR/Cas9 technology. RNAseq analysis of these cells indicates that the melanocyte specific MITF isoform is no longer expressed in these cells following TR1 knockout (Kline et al., n.d.). Given that WT M14 cells endogenously express the melanocyte specific MITF isoform, we anticipated these cells would more readily express MITF variant constructs than HeLa cells. A hydrogen peroxide assay and SDS-PAGE were run as previously described, with protein lysates fractionated under reducing and non-reducing conditions. Confirming earlier findings, both MITF variants treated with hydrogen peroxide were observed at high molecular weights on non-reducing gels (Figure 5b). In comparison, MITF was only observed at its monomeric molecular weight of 54 kD in control groups without hydrogen peroxide treatment (Figure 5a).

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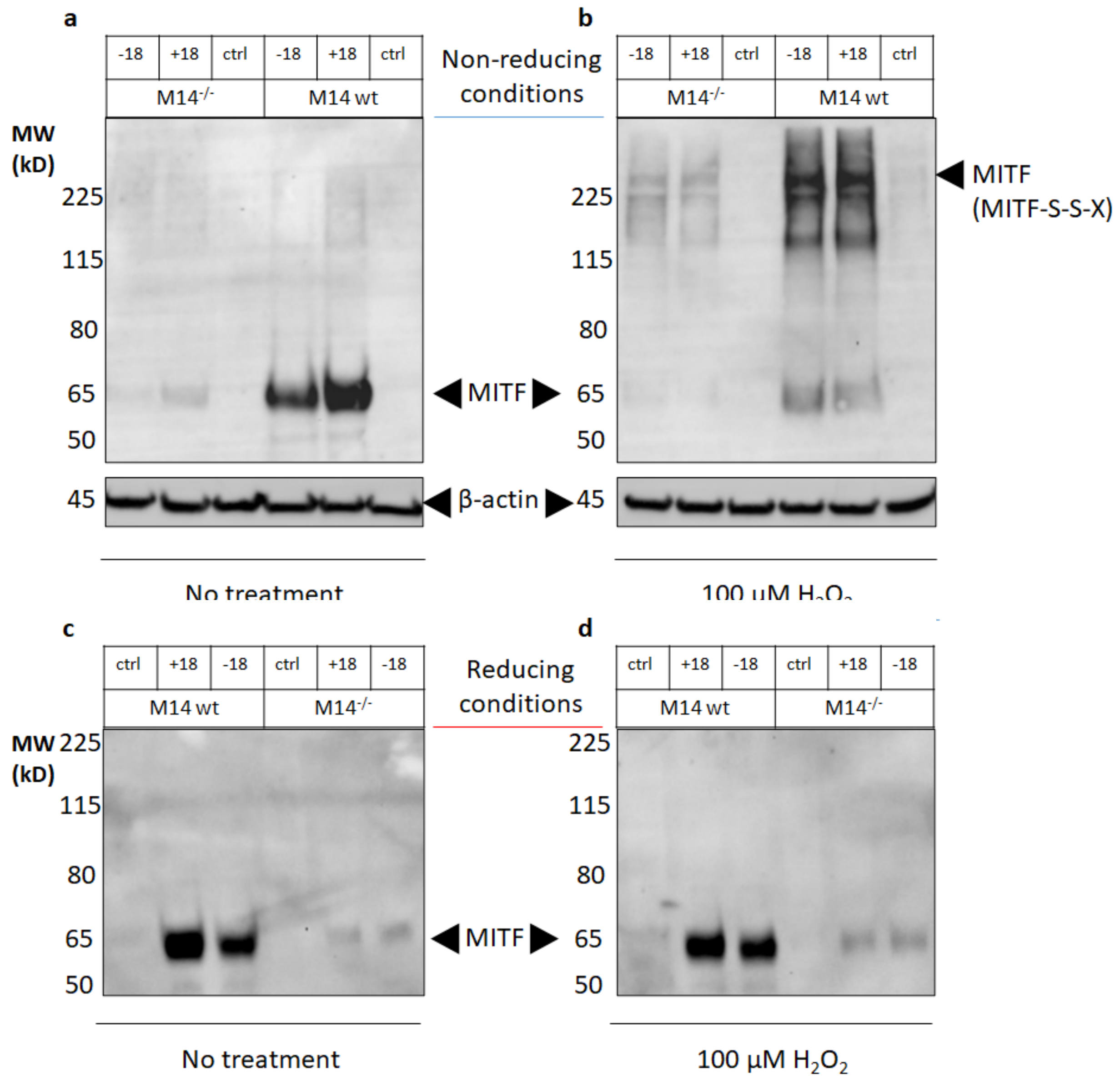

Figure 5. M14 cells transfected with dual-tagged MITF variant plasmids.
a) In control cell lysates fractionated under non-reducing conditions, MITF is primarily observed at its monomeric molecular weight, between 50-65kD. **b**) Following treatment with hydrogen peroxide, MITF is observed at high molecular weights (MITF-S-S-X) under non-reducing conditions. **c, d**) When the same cell lysates are run under reducing conditions, MITF is observed only at its monomeric molecular weight, regardless of treatment group. The restoration of MITF to its monomeric molecular weight following reduction by BME is indicative of transient, disulfide-linked protein conjugates. Surprisingly, MITF was not detected in M14 WT cells at this exposure, indicating that expression of the MITF constructs is much higher than endogenous levels of MITF in M14 cells.

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Discussion

Redox modifications have been implicated in the regulation of multiple transcription factors and signaling pathways, primarily those responsible for regulating antioxidant programs at the transcriptional level (García-Santamarina et al., 2014). For example, the transcription factor NF-κB has been identified as redox responsive, interacting with TRX to enable DNA binding activity (Hayashi et al., 1993; Toledano et al., 1993). Similarly, the activity of the Pax8 transcription factor is dependent on redox state and inhibited by glutathionylation (Cao et al., 2005; Codutti et al., 2008). The redox effector protein APE-1 (Ref-1) also co-activates transcription factors via disulfide exchange, activating both NF-κB and Hif1-α, a subunit of the oxygen homeostasis regulator Hif1 (Huang et al., 1996; Tell et al., 2009). Intriguingly, MITF interacts with some of these redox-sensitive proteins. MITF has been shown to upregulate APE-1 expression in response to cellular redox stress, while MITF additionally binds the Hif1-α promoter and strongly stimulates its transcriptional activity (Buscà et al., 2005; Liu et al., 2009). These MITF targets implicate MITF as a component of the cellular redox response.

Our work investigates a previously unknown aspect of MITF involvement in the cellular redox response. Previous work by the Leachman/Cassidy lab found that knockdown of the antioxidant TR1 results in reduced pigment synthesis in melanocytes, suggesting a novel association between the TR1antioxidant network and the pigment synthesis pathway. Upon examination of the cellular effects of TR1 knockdown under hydrogen peroxide-induced redox stress, MITF was observed to form disulfide linked conjugates after fractionation of cell lysates by non-reducing SDS-PAGE, illustrating its redox sensitivity. Together these findings support the hypothesis that MITF may be regulated by TR1 in a redox-dependent fashion, altering its transcriptional activity. While we suggest a discrete function for MITF redox sensitivity, additional research must be undertaken to delineate the effects of redox modification on MITF regulated genes or protein targets. In addition to the proposed redox-dependent functionality of MITF, we hypothesize the two MITF splice variants respond differently to redox regulation, according to the presence or absence of an additional cysteine residue. These variants may serve different functions in the cell, modulating cell fate according to cellular redox conditions.

To investigate the redox sensitivity of the MITF variants in vivo, we constructed affinity tagged MITF variant vectors and transfected mutant melanoma and non-melanocyte cell lines. Treatment of cells with hydrogen peroxide followed by free thiol quenching with NEM allowed us to identify high molecular weight MITF species on non-reducing SDS-PAGE that were not present under reducing conditions. This is indicative of transient, disulfide-linked protein conjugates. However, both variants engaged in these redox-mediated conjugates, leading us to conclude that analysis of MITF by western blots cannot distinguish differences in redox activity between the two variants. Given that the +18 variant retains an additional cysteine residue, it is plausible this variant responds more readily to redox regulation. In this fashion, the -18 variant could be less sensitive to cellular redox conditions, and its overexpression could serve as a mechanism of cellular escape from redox regulation, potentially driving a cancerous phenotype. To delineate these theoretical functions, we intend to perform co-immunoprecipitation of the MITF variants and assess interacting proteins by mass spectrometry. This will help elucidate whether the splice variants interact with different proteins in a redox-mediated fashion, contributing to differential activation of downstream targets.

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Given the endogenous redox stress within melanocytes and reported increase in ROS in melanomas, an explicit response to ROS is required to maintain melanocyte homeostasis. Although MITF redox sensitivity has only been preliminarily identified, confirming this novel mechanism of MITF regulation and its effect on cellular phenotype will help elucidate the multi-faceted role of MITF in melanocyte cell fate. MITF is regulated by multiple activation pathways and post translational modifications, contributing to its range of expression levels in melanocyte and melanoma cells. Although levels of MITF expression have been linked to a spectrum of cellular behavior, the implications of MITF splice variant expression have not been extensively investigated. While we are exploring the role of these splice variants in the melanocyte specific MITF isoform, these variants have been additionally reported in other MITF isoforms (M. Murakami et al., 2007). Differential response of the splice variants to redox regulation could serve as an additional mechanism of regulation in tissues that express these other MITF isoforms. Consequently, understanding the comparative function of the MITF splice variants will illuminate their role in cell fate, contributing to our understanding of cellular regulation in melanocytes and melanomas.

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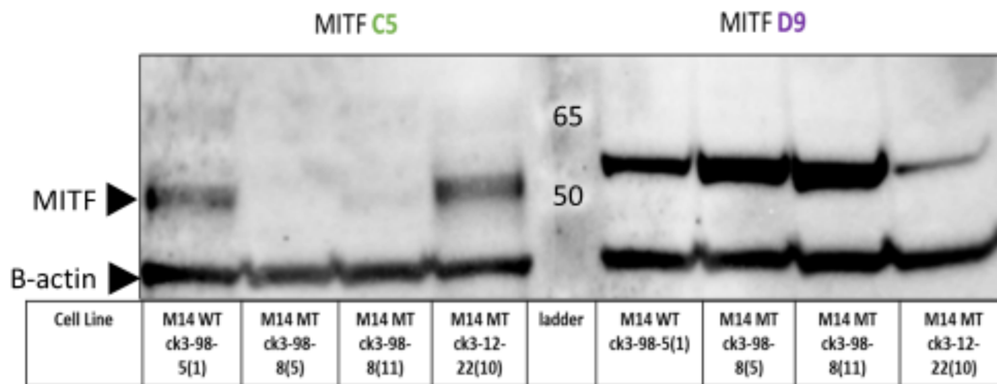
Appendix

Table 1. Primers used for overlap-extension PCR

CMV-F	5’ TAGTCATCGCTATTACCATGGTGATGC
BsiWI-F	5’ GCACACTTGTCGTACGATCGATACCG
BsiWI-R	5’ CGGTATCGATCGTACGACAAGTGTGC
T7-F	5’ CGAGTGAATTGTAATACGACTCACTATAGGG



Supplemental Figure 1. Comparison of C5 and D9 antibodies in M14$^{-/-}$ and WT cells.
The anti-MITF C5 and D9 antibodies show differences in MITF detection between M14 WT and TR1 knockout (MT) cells. C5 fails to identify MITF expression in two knockout lines (Ck3-98-8(5) and (11)). RNAseq analysis confirms these lines lack MITF-m expression but retain MITF-A expression. Additional analysis of the D9 epitope sequence confirms it is found in both the MITF-A and m isoforms, explaining why D9 detects MITF in those cells where MITF-m is not expressed.

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