Generation of stable cell lines with doxycycline inducible MITF expression

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Raunvísindadeild
Háskóli Íslands
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16 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í Lífefnafræði

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Raunvísindadeild
Verkfræði- og náttúruvísindasvið
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Útdráttur

Microphthalmia associated transcription factor (MITF) er lykilprótein í þroskun litfrumna (melanocytes) og er talið vera æxlisvaldandi þáttur í sortuæxlum. Verkefni þetta fólst í því að smíða *piggyBac* genaferjur sem tjá þrjú afbrigði af MITF og útbúa stöðugar HEK293 frumurlínur sem tjá MITF undir stjórn Tet-On kerfisins. MITF afbrigðin voru $MITF^{wildtype}$ (MITF-m isoformið, 419aa), $MITF^{spotted}$ sem skortir 18 bp röð vegna splæsingar og $MITF^{spotless}$ sem auk þess að vanta 18 bp röðina ber stökkbreytinguna K316Stop. Þetta síðasta afbrigði var fundið sem stökkbreyting sem bælir svipgerð annarra stökkbreytinga í Mitf og ekki hefur enn tekist að útskýra hvernig breytingin veldur bæliáhrifum. Virkni kerfisins var sannreynd með vesturþrykksgreiningu (Western Blot) fyrir og eftir örvun með doxycycline. Með myndun þessa kerfis hefur verið útbúið öflugt tæki til samanburðarrannsókna á virkni þessara MITF afbrigða í spendýrafrumum en þannig má fá innsýn í virkni MITF próteinsins. Hér eru fyrstu skrefin í þessum samanburði tekin og samanburður gerður á stöðugleika MITF afbrigðanna eftir cycloheximide meðferð. Einnig var fosfórun serín 73 amínósýrunnar hjá MITF skoðuð. Niðurstöðurnar benda til hærri stöðugleika $MITF^{spotted}$ afbrigðisins borið saman við villigerðar og spotless afbrigðin. Einnig kemur í ljós að $MITF^{spottless}$ er fosfórýlerað á serín 73 seti, þrátt fyrir að serín 409 sé ekki til staðar.

Abstract

Microphthalmia-associated transcription factor (MITF) is a key protein in the development of melanocytes and an oncogene in a subset of human melanomas. In this project three variants of the Microphthalmia-associated transcription factor (MITF) protein were cloned into a *piggyBac* vector to set up stably transfected HEK293 cell lines with doxycycline inducible MITF expression using the Tet-On system. The variants were $MITF^{wildtype}$ containing mouse MITF-m (419aa), $MITF^{spotted}$ which is a mutant of MITF lacking the alternatively spliced exon 6A and $MITF^{spotless}$ an intragenic suppressor mutation of $MITF^{spotted}$ that in addition to $MITF^{spotted}$ contains a premature stop codon (K316Stop). An explanation for this suppressor behavior, at the molecular level has yet to be solved. The vectors were constructed and verified and stable cells generated with transfection. The function of the system was demonstrated with Western Blotting, by comparing MITF expression in stably transfected cells with and without doxycycline. By generating these cell systems a powerful tool has been produced to do comparative analysis of the different function of the MITF variants in mammalian cells. This can hopefully be used to gain insight into the function of MITF. Here, the first steps of these comparative analysis are done; comparing the stability of the MITFs variants after cycloheximide treatment of cells and comparison of the phosphorylation state of MITF. The results indicate a higher stability of $MITF^{spotted}$ compared to its counterparts. Unexpectedly serine 73 phosphorylation still takes place in $MITF^{spotless}$, despite lacking the required serine 409 phosphorylation.

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1 Introduction

1.1 MITF mutants and regulation by phosphorylation

Microphthalmia associated transcription factor (MITF) is a basic-helix-loop-helix leucine zipper (bHLH-Zip) transcription factor. Through the bHLH-leucine zipper domains MITF can bind DNA as a homodimer or as a heterodimer with other related bHLH-Zip proteins in the TFE family such as TFEB, TFE3 and TFEC (Hodgkinson et al. 1993 and reviewed by Steingrímsson et al. 2004). Mitf mutations affect the development of several cell types ranging from melanocytes, retinal pigment epithelial cells, osteoclasts and mast cells (reviewed by Steingrímsson et al. 2004). In the development of the melanocyte lineage, since the melanocytes precursors first migrate from the neural crest, Mitf is an important protein as most of the signaling molecules or transcription factors involved in melanocyte development affect either Mitf expression or its function (reviewed by Goding, C. 2000). Mitf has for this reason been termed the master regulator of melanocyte development (Steingrímsson et al. 2004). In humans MITF mutations are linked to Waardenburg syndrome type IIA (Tassabehji, M. et al. 1994), Tietz syndrome (Smith, S. et al. 2000) and MITF is an oncogene in a subset of human melanomas (Garraway, La. et al. 2005).

MITF is subjected to post-translational modifications, including phosphorylation by various kinases such as mitogen-activated protein kinase (MAPK), ribosomal s6 kinase (RSK), glycogen synthase kinase-3beta (GSK3beta) and p38 (reviewed by Levy, C. et al. 2006). Although much is known about the cause and effect of various phosphorylations on MITF, the network of MITF phosphorylations and its total effect on the activity of MITF remains to be solved. Activation of c-KIT in melanocytes results in phosphorylation of MITF at serine 73 by extracellular-signal regulated kinase 2 (ERK2) and at serine 409 by p90 ribosomal S6 kinase (p90RSK). According to unpublished lab data the serine at position 409 needs to be phosphorylated for serine 73 phosphorylation to happen (Phung, B. 2013). This phosphorylation at serine 73 causes increase in the transcriptional activity of MITF by the recruitment of the transcriptional co-activator p300. At the same time this phosphorylated MITF is targeted for ubiquitin-dependent proteolysis (reviewed by C. Levy et al. 2006). Transcriptional target genes of MITF are genes involved in various cell processes; cell-cycle progression (TBX2, CDK2), apoptosis (BCL2 and P21) and in cell differentiation by activating pigment-enzyme genes (reviewed by C. Levy et al. 2006.)

To this day, MITF mouse mutants have been used to a great extent to investigate the activity of MITF as MITF mutants have highly visible phenotypic effect on mouse. All homozygous MITF mutants affect the coat color through its effect on melanocytes, most cause microphthalmia through its effect on RPE cells and only few MITF mutants affect other cells such as osteoclasts, mastcells and natural killer cells (reviewed by Steingrimsson et al. 2004). One mutation of special interest here is the $Mitf^{spotted}$ ($Mitf^{sp}$ for short) mutation which lacks an alternative splice acceptor site in front of exon 6 which results in production of an MITF isoform lacking the following 18bp exon. This isoform is present in wild type mice and is called the $MITF^{-}$ isoform, the full length protein is called

$MITF^{+}$ (Hodgkinson et al. 1993). Homozygots for the sp allele have no visible phenotype: a visible phenotype only emerges in the heterozygous condition with another Mitf allele.

With N-ethyl-N-nitrosurea (ENU) mutagenesis screen on homozygous $Mitf^{sp}$ mice, Steingrimsson et al. generated a novel intragenic suppressor mutation in the $Mitf^{sp}$ allele (Steingrimsson et al. unpublished article). This new mutation of MITF, called $MITF^{spotless}$ (or $MITF^{sl}$ for short) has a premature stop codon at K316, caused by a single nucleotide change, in addition to lacking the 18bp exon. Mice homozygous for the $Mitf^{sl}$ mutant appear with brownish coat color giving rise to a neomorphic phenotype compared to black coat color (wild type phenotype) seen in $Mitf^{sp}$ homozygous mice. However in mice heterozygous for $Mitf^{sl}$ with other Mitf mutations an improvement towards normal phenotype is seen compared to combinations of same mutations with $Mitf^{sp}$ (Steingrimsson et.al. unpublished article). An explanation for this behavior at the molecular level is still needed.

1.2 The *piggyBac* Transposase

The demand for mammalian cell expression systems has increased with the growing need for eukaryotic proteins in studies in fields such as cell biology, structural biology and biotechnology (Zhijie, Li. et al. 2013). The most efficient gene delivery systems to this day are based on viral vectors, though there are some drawbacks like limited cargo capacity, host immune response and the risk of insertional mutagenesis (Urschitz, J. et al. 2010). To solve these problems the use of transposon-based approaches to integrate genes has gained increased popularity over the last few years, particularly the *piggyBac* (PB) transposase for its large cargo capacity and high transposition activity (Urschitz, J. et al. 2010). The PB transposase system has been used for many different applications ranging from mutagenesis and transgenesis to gene therapy (reviewed by Kim, A. et al. 2011).

piggybac transposons belong to DNA transposons, transposable element which move between genomic sites by a cut and paste mechanism. The *piggyBac* element has a 2472bp sequence composed of an internal repeat (IR), spacer and terminal repeats (TRs) flanking a single open reading frame (ORF) that encodes transposase (reviewed by Kim, A. et al. 2011). Li X et al. 2005 examined the need for an internal domain sequence in addition to the internal repeat, spacer and TR configuration and concluded that minimal PB terminal repeats of 313 bp at the 5' terminus and 235 bp at the 3' terminus are needed for effective transformation of target genome (Li X, et al. 2005). A vector construct with these minimal PB terminal repeats flanking a cargo gene are therefore capable of integrating the cargo gene into chromosomes of a host with the enzyme PB transposase expressed in trans, for example from another plasmid.

One of the advantages of the PB transposon is the fact that it does not leave footprint in the excision event and the TTAA integration site is repaired after excision of the transposon. This enables the removal of transposons from the host genome without changing the nucleotide sequence (Fraser, MJ. et al. 1996), making the transfection event less harmful to the cells. PB transposition has been thought to be less dependent on host factors than other transposons and in 2005 Wu and colleagues showed that PB elements can effectively transpose in mouse and human cells (Wu X. et al. 2005).

The PB transposon is highly efficient as a method to insert exogenous DNA fragments into mammalian genomes, making stably transfected mammalian cells. Report from Zhijie Li et al. 2013, comparing the ability of the PB transposon system to stably transfect mammalian cells with the "Classical integration method" (based on spontaneous integration of foreign DNA into the genome) showed that the PB system has much higher

DNA integration efficiency when examining data from 14 different proteins in 3 different mammalian cells (Zhijie, Li. et al. 2013).

1.3 Tetracycline-controlled transcriptional activation

When using an effective transfection method such as the *piggyBac* system a potential problem with over-expression of the protein emerges which can inhibit cell growth, making it hard to set up a stable cell line. To address this issue the gene of interest can be kept under an inducible promoter so that the protein is only expressed when needed (Zhijie, Li. et al. 2013). The Tetracycline on (Tet-on) system is based on a chimeric protein, termed reverse-tetracycline-transactivator (rtTA), made from the DNA binding domain of the tetracycline repressor protein found in *Escherichia coli* and the transactivation domain from the herpes simplex virus VP16. This fusion protein can bind tetracycline and the formed complex can then activate transcription of a target promoter which consists of multiple tetracycline operators upstream of a core promoter (Meyer-Ficca, ML. et al. 2004). Such a system allows the regulation of expression by simply adding tetracycline or doxycycline, an analogue of tetracycline.

1.4 Introduction to the project

The goal of this project was to set up stably transfected inducible cell lines expressing each of the three MITF variants, $MITF^{wt}$, $MITF^{sp}$ and $MITF^{sl}$. The Tetracycline On system was utilized to make the expression of the MITF variants inducible and therefore controllable. Here the core promoter was the human Cytomegalovirus (hCMV) promoter and the inducer was the tetracycline derivative, doxycycline. Inducible MITF expression was achieved by using the *piggyBac* system to stably transfect HEK293 cells with the following plasmids (see Figure 1);

- pPB-hCMV*1-$MITF^{wt}$ (or $MITF^{sp}$ or $MITF^{sl}$)-pA which is the cDNA *piggyBac* plasmid containing the Mitf variants under tetracycline inducible promoter (hCMV*1).
- pPB-CAG-rtTA-Neo-pA; the rtTA *piggyBac* plasmid containing rtTA and the gene for neomycin both under CAG promoters.
- pPy-CAG-PBase-pA which is the *piggyBac* helper plasmid containing the piggyBac transposase (PBase) under CAG promoter.

The PBase will mobilize the element defined by the *piggyBac* minimal TRs on both *piggyBac* vectors into the host chromosome. As the cells proliferate, the *piggyBac* helper plasmid will decrease with each generation of cells and eventually be washed out of the culture. But the rtTA and neomycin will be stable expressed as they have been incorporated into the host genome. Selection for neomycin expressing cells with G418 will select only for cells that have rtTA + neomycin. Some of these rtTA and neomycin expressing cells will also have incorporated the Mitf construct under the tetracycline inducible promoter, so a heterozygous cell population with part of the cell culture capable of doxycycline inducible MITF expression will have emerged. The correct function of this

system for all three MITF variants was tested with Western Blotting comparing the MITF expression in HEK293 with and without doxycycline incubation.

By setting up cell systems with inducible expression of the MITF variants, an effort is made towards understanding the behavior of the three MITF proteins at the molecular level. This will in turn give more information on the molecular behavior of the MITF protein.

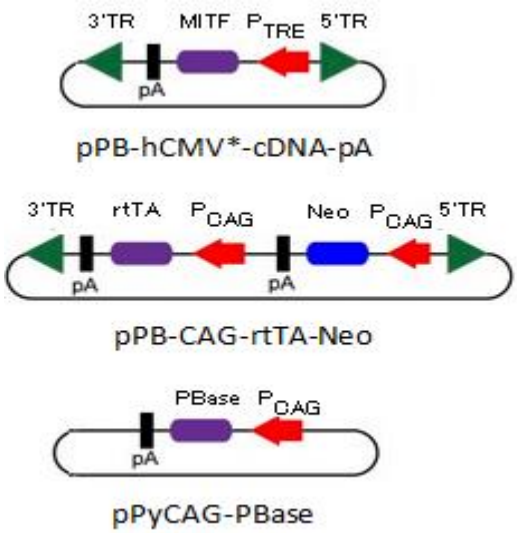

Figure 1. *Plasmids used in this experiment; pPB-hCMV* cDNA constructs, pPB-CAG-rtTA-Neo-pA; the rtTA piggyBac vector and pPy-CAG-PBase-pA, the PB transposase (PBase) vector.*

2 Materials and Methods

2.1 Construction of the MITF clones

2.1.1 Transformation

To generate sufficient vector DNA for cloning it was first necessary to make a maxiprep of the plasmid DNA. 300ng of the pPBhCMV*1-cHA-pA *piggyBac* vector (From the Laboratory of Prof. Azim Surani, Cambridge) were added to 50 µl of NEB 5-alpha competent *Escherichia coli* (NEB #C29871), kept on ice for 40 minutes, heat shocked for 30s at 42°C and again on ice for 5 minutes before adding 950 µl of SOC media (NEB #B9029S) to the competent cells. The competent cells were incubated in the SOC media at 37°C for 60 minutes. 50 and 100 µl aliquots were spread on two agar plates with ampicillin (200 µg/ml) and Luria broth (LB) media and incubated overnight at 37°C. Four colonies were picked from the plates and used to infect 5ml of LB media containing ampicillin (200 µg/ml) and incubated up to 10 hours at 37°C. Cells were grown further by adding one of the 5 ml bacteria cultures to 250 ml of LB media with ampicillin (200 µg/ml) and incubating overnight at 37°C.

2.1.2 Plasmid maxiprep

To make a large stock of piggyBac DNA, 250 ml of LB media containing transformed *E.coli* were cultured and DNA isolated using the protocol from Purelink^{Tm} HiPure Plasmid DNA Purification Kit from Invitrogen (#K2100-07). DNA concentration was measured with the spectrophotometer ND-100 Nanodrop® from Thermo Scientific.

2.1.3 Restriction enzyme digestion

In order to clone MITF in to the piggyBac vector, 1 µg of *piggyBac* vector and each of three pcDNA3 carrier plasmids containing the three MITF variants (plasmids and lab-numbers: pcDNA3-Mitf^{wt}-pA A5.5, pcDNA3-Mitf^{sp}-pA A5.6 and pcDNA3-Mitf^{sl}-pA A5.7) were digested with 0.5 µl of each restriction enzyme EcoRI (NEB #R0101S) and NotI (NEB #R0189S), 2 µl (5 µl for vector plasmid) in Orange buffer (Fermentas #BO5) and sterile Milli-Q H_2O added to a total volume of 20 µl (50 µl for vector plasmid) for 2 hours at 37°C in four separate digestion reactions.

2.1.4 Gel electrophoresis

All four restriction digestions were run on a 1% agarose gel in 1x TAE buffer with 0.5 µg/ml final concentration of ethidium bromide at 80V for 60 minutes. Fluorescence of DNA bound to ethidium bromide in UV light was visualized with G:Box from Syngene and picture taken.

2.1.5 Gel extraction

The appropriate DNA bands were cut out of the agarose gel with scalpel under UV light and placed in an Eppendorf tube, weighted and the DNA samples extracted following the protocol form the DNA gel purification kit, Nuclespin® Gel and PCR Clean Up following protocol 5.2; DNA Extraction form Agarose Gels (Macherey Nagel).

2.1.6 Ligation

The Mitf inserts were ligated to the digested *piggyBac* vector in 3:1 insert:vector molar ratios and final insert concentration not exceeding 5 μg/ml as recommended by Revie, D. et al. 1988 for asymmetrical ligation. The ligation was done with 1 μl of 10x ligase buffer (Fermentas), 0.5 μl of T4 DNA ligase (Fermentas #EL0011) with Milli-Q H_2O to a total volume of 10μl. For control (to scan background re-circularization of vector backbone) the ligation reaction was done without insert DNA. All four ligation reactions were kept at room temperature overnight. The ligation solutions were transformed into NEB5-alpha competent *E.coli* as described in 2.1.1 and spread on LB agar plates containing ampicillin (160 μg/ml) and incubated overnight at 37°C. From each plate containing MITF ligated to *piggyBac* vector, several colonies were picked to infect 5ml of LB media with ampicillin (200 μg/ml) and incubated overnight at 37°C.

2.1.7 Plasmid purification (miniprep)

To extract the plasmids from the 5 ml LB cultures the plasmid DNA purification kit Nuclespin plasmid (Macherey Nagel) was used following protocol 5.1: Isolation of high copy plasmid DNA from *E.coli.* DNA concentration was measured with spectrophotometer ND-100 (Nanodrop® from Thermo scientific).

2.2 Verifying plasmid constructs

2.2.1 Verifying plasmid constructs with gel electrophoresis

The first step to confirm that the cloning of these *piggyBac* plasmids was a success was done using restriction enzymes. This was done to confirm that these piggyBac constructs with the three Mitf variants were correctly made; pPBhCMV*1-MITF^{wt}-cHA-pA, pPBhCMV*1-MITF^{sp}-cHA-pA and pPBhCMV*1-MITF^{sl}-cHA-pA. 500 ng of all three plasmids and of one of the carrier plasmids (pcDNA3-Mitf^{wt}-pA) were cut with 0.7 μl of EcoRI and PstI (Fermentas, #ER0611) in 2 μl of Orange buffer in a total volume of 20 μl with Milli-Q H_2O. The cut plasmids and uncut pPBhCMV*1-MITF^{wt}-cHA-pA were then run on 1% agarose gel and visualized according to section 2.1.4.

The second step to confirm the plasmid construct was to sequence the inserts and part of the vector backbone.

2.2.2 Preparation for DNA sequencing; one primer PCR

PCR, using the BigDye® Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems was set up as follows. Reaction mixture: 4 μl of 2.5x Ready Reaction Premix, 1μl of 5x

BigDye Sequencing Buffer v1.1, 1µl 10pmol/µl solution of the appropriate primer, 1µl of plasmid as template and Milli-Q H_2O to 10µl final volume. The PCR reaction was as follows;

- Rapid thermal ramp to 96°C for 30s
- Repeat for 25 cycles:
    - 96°C for 10s
    - 50°C for 5s
    - 72°C for 4 min
- Hold at 4°C

Done with five different primers (see appendix) for all three plasmid constructs, a total of 15 sequencing reactions.

2.2.3 Purifying extension products

After the PCR, the extension products were purified by ethanol/EDTA precipitation: 2.5 µl of 2.5 mM EDTA and 30 µl of 100% ethanol were added to each PCR product. The solution was transferred to 1.5 ml Eppendorf tube, incubated at room temperature for 15 min, centrifuged at 3000xg for 30 min and supernatant discarded. Next, 30 µl of 70% ethanol added and centrifuged at 1800xg at 4°C for 15 min. Supernatant discarded and sample incubated at 37°C to get rid of the ethanol. Samples stored at 4°C.

2.2.4 DNA Sequencing

Purified PCR products were dissolved in 17µl of injection buffer and loaded onto a 96 well plate. The plate was placed into the ABI 3130 Genetic analyzer and the sequencing reactions started. Data was collected by Run 3130 Data collection program and read by the Sequencing analysis 5.2 program.

2.3 Generation of stable inducible cell lines

2.3.1 Thawing of frozen HEK293 cell line

A cryovial containing the frozen cells was removed from liquid nitrogen storage and immediately transferred to a laminar flow hood. The vial was heated up in hand, the cells gently poured into a 100 mm tissue culture plate and 9 ml of warm media added to the partially frozen cells. The plate was placed in an incubator at 37°C and 5% CO_2 concentration. As soon as the cells had attached, the media was changed to get rid of excess DMSO.

2.3.2 Maintenance of cells

Cells were cultured in Dulbecco´s Modified Eagle Medium (DMEM, #10938-025) supplemented with fetal bovine serum (FBS, 10% final concentration) at 37°C and 5% CO_2

2.3.3 Splitting of cells

At about 80% cell confluency the cells where trypsinized and split; the cells were briefly washed with 1xPBS, filling the bottom of the flask with trypsin-EDTA and the flask incubated at 37°C for 3 minutes, media (with serum) added in a volume 2x that used for the trypsin-EDTA treatment. Cells were collected and centrifuged for 3 minutes at 2000 RPM, supernatant discarded and the cell pellet re-suspended in an appropriate volume of serum containing media.

2.3.4 Transfection

For generation of stable cell cultures, HEK293 cells were transfected with FuGENE HD Transfection Reagent form Promega (#E2311) following the manufacturer's instruction for HEK293 cells. HEK293 cells were counted and seeded on 6-well plates at about $5x10^5$ cells per well in 3 ml of DMEM with 10% FBS. After 1 day (70% confluency), the pPBhCMV*1-MITF^{wt}-cHA, pPBhCMV*1-MITF^{sp}-cHA or pPBhCMV*1-MITF^{sl}-cHA plasmid construct were cotransfected with the plasmid pPB-CAG-rtTA-INeo (From Laboratory of Prof. Azim Surani, Cambridge) and helper plasmid pPyCAG-PBase (From Kazuhiro Murakami) at ratio 10:10:1 or 1.57 µg:1.57 µg: 0.157 µg with 9.9 µl of FuGENE Reagent and Milli-Q H_2O to a total volume 164.9 µl. These solutions were kept at RT for 10 min and then added to the 3 ml cell cultures. As control for cell survival, another transfection was done as described above but without the pPB-CAG-rtTA-INeo plasmid.

2.3.5 Selection and maintenance of transfected cells

After 2 days and after reaching 100% confluency (80% optimal) drug selection was started with 500 µg/ml G418 in the 6 well plates. Drug selection was continued and media and G418 selection changed every 4-5 days. 10 days after transfection, control and 3 Mitf variant cell cultures were trypsinised and transferred to a new 6 well plate as an attempt to speed up the selection process by getting rid of dead and dying cells. Three days later, no live cell could be seen in the control well. On the other hand the 3 cell cultures, transfected with the plasmid constructs showed high confluency and were trypsinised and transferred to three $25cm^2$ cell culture flasks in DMEM with 10% FBS and 500 µg/ml G418.

2.3.6 Freezing and storing of stably transfected cell lines

Stably transfected HEK293 cell lines were scaled up in DMEM with 10% FBS and 500 µg/ml G418 until they reached confluency on $175cm^2$ cell culture flasks. Then the cells were frozen and stored in N_2. The cells were washed with 1xPBS, trypsinized, re-suspended in media, transferred to a sterile centrifuge tube and centrifuged at 2000 RPM at RT for 3 min. The supernatant was removed and the pellet re-suspended by adding 1ml of freezing media (10% dimethylsulfoxide, DMSO in media) per vial to be frozen. Vials were frozen for 1 month at -80°C (optimally overnight) and transferred to liquid N_2 tank for indefinite storage.

2.4 Experiments performed on the stably transfected cell lines

2.4.1 Induction of stable cell lines with doxycycline

Stably transfected HEK293 cell lines were seeded on 6-well plates at about $5x10^5$ cells per well in DMEM with 10% FBS, 500µg/ml G418 and induced for 48 hour with varying concentrations of doxycycline.

2.4.2 Stability of MITF expression

Stably transfected HEK293 cell lines were seeded on 6-well plate at about $5x10^5$ cells per well in DMEM with 10% FBS, 500µg/ml G418 and induced for 48 hours with 10µg/ml doxycycline. Cycloheximide (CHX) (#26616 from ICN Biomedicals) was dissolved in DMSO and added to each well for final concentration of 50µg/ml. Cells were harvested 0, 4 and 13 hours after CHX addition.

2.4.3 Cell harvesting

Working on ice, the media was removed from the wells on the 6-well plates and washed once with cold 1x PBS buffer. Cells were lysed with 100µl RIPA lysis buffer containing 1µl of protease inhibitor, ProteoBlock from Fermentas (#R1321). Cells were scraped off with cell scraper and the mixture transferred into an Eppendorf tube. Cell lysate was incubated on ice for 10 min, sonicated for 5 min at 4°C and centrifuged for 10 min at 14000 RPM at 4°C. The supernatant was transferred to a fresh Eppendorf tube and stored at -80°C.

2.4.4 Western blot

Total protein estimation

Bradford protein assay was used to determine protein concentration. 1ml of Bradford reagent (Sigma, #B6916-500ML) was diluted in water 1:5, mixed with 2 µl of protein solution and then absorption measurement at 595nm taken with a spectrophotometer and protein concentration estimated against a standard curve from standard solutions of 0, 0.1, 1, 5, 10 and 50 µg/ml BSA.

SDS-PAGE

The samples were prepared in the following manner. 10 µg of protein samples in a total volume of 7.5 µl of MQ H_2O mixed with 7.5 µl of sample buffer (2x) were heated for 5 min in heat block at 95°C. 12µl of sample mixture were loaded on to the SDS gel consisting of 8% polyacrylamide lower gel and 5% polyacrylamide upper (stacking) gel. Each SDS run included 3µl of pre-stained ladder (Thermo Scientific, #26616). The SDS-gel was placed in an electrode chamber with running buffer and was run at 120V for 2 hours.

Electrophoretic transfer

The proteins on the SDS-gel were transferred to an Odyssey Nitrocellose membrane in a wet transfer system using transfer buffer with 20% methanol at 100V for 1 hour at 4°C.

Antibody staining

The membrane was blocked in TBS with 5% (w/v) milk powder at RT for 40 minutes. This was followed by primary antibody staining overnight at 4°C with anti-MITF (C5) (mouse), anti-β-actin (13E5, rabbit mAB from Cell Signal, #4970S) and special antibody against serine 73 phosphorylated MITF (rabbit) (made in the Rönnstrand lab by Bengt Phung) at a concentration of 1 µg/ml in TBST with 3% milk powder. However with the antibody against serine 73 phosphorylated MITF, 4% bovine serum albumin (Sigma, #SLBF5061V) was used for blocking. Secondary antibody staining was done with Dylight-α-Rabbit and Dylight-α-Mouse in TBST with 3% (w/v) milk powder at RT for 1 hour. The membrane was washed 3 times for 10 min in TBST after primary and secondary antibody staining. Proteins were visualized using the LICOR Odyssey system.

3 Results

3.1 Verifying the plasmid constructs

3.1.1 Verifying the plasmid constructs with gel electrophoresis

All three *piggyBac* constructs and one of the carrier plasmids (pcDNA3-Mitf^{wt}-pA) were cut with EcoRI and PstI as described in 2.2.1. The results of the gel electrophoresis are shown in Figure 2.

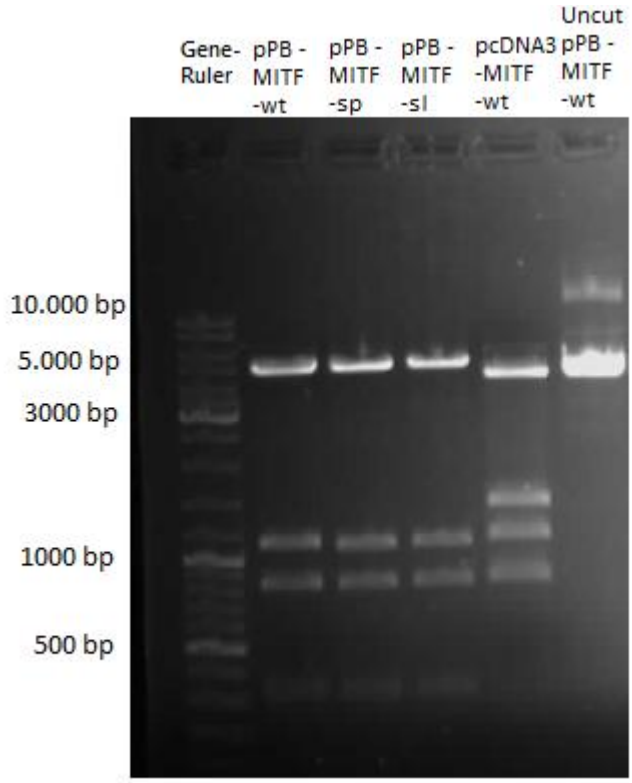

Figure 2*: Gel electrophoresis of EcoRI and PstI digested pPBhCMV*1-MITF^{wt}-cHA (lane 2), pPBhCMV*1-MITF^{sp}-cHA (lane 3), pPBhCMV*1-MITF^{sl}-cHA (lane 4) plasmid constructs, carrier plasmid pcDNA3-Mitf^{wt}-pA (lane 5) and uncut pPBhCMV*1-MITF^{wt}-cHA (lane 6). The Ladder (lane 1) was GeneRuler #SMO331 from Thermo Scientific. A clear distinction was observed in the restriction pattern between the carrier plasmid and the PB constructs, indicating successful cloning.*

For the *piggyBac* construct of all three Mitf variant (lanes 2, 3 and 4) bands were seen at approximately under 5000 bp, 1100 bp, between 800 and 900 bp and around 200 bp. For the Mitf carrier plasmid (lane 5) bands were seen at between 5000 and 4000 bp, 1200 bp and 1100bp. These results did not fit the expected results of an EcoRI/PstI digestion based on the predicted restriction pattern; at 4700 bp, 1200 bp and 1100 bp for the *piggyBac*

constructs and for the carrier plasmid at 4050 bp, 2250 bp and 1100 bp based on the knowledge of two restriction sites in *piggyBac*/pcDNA3 backbone and one in the Mitf insert. These deviations could only be explained by an extra PstI restriction site in the linker sequence of the *piggyBac* vector downstream of the insert that connect the insert and the backbone DNA. DNA sequencing actually showed two PstI restrictions sites in the downstream linker sequence (see 3.1.2.). The final map of known restriction sites for PB constructs and the carrier plasmid as shown in Figure 3 agrees with the restriction pattern seen on the gel in Figure 2. Since there was a clear distinction in the restriction pattern between the carrier plasmid and the PB constructs it was logical to move on to DNA sequencing.

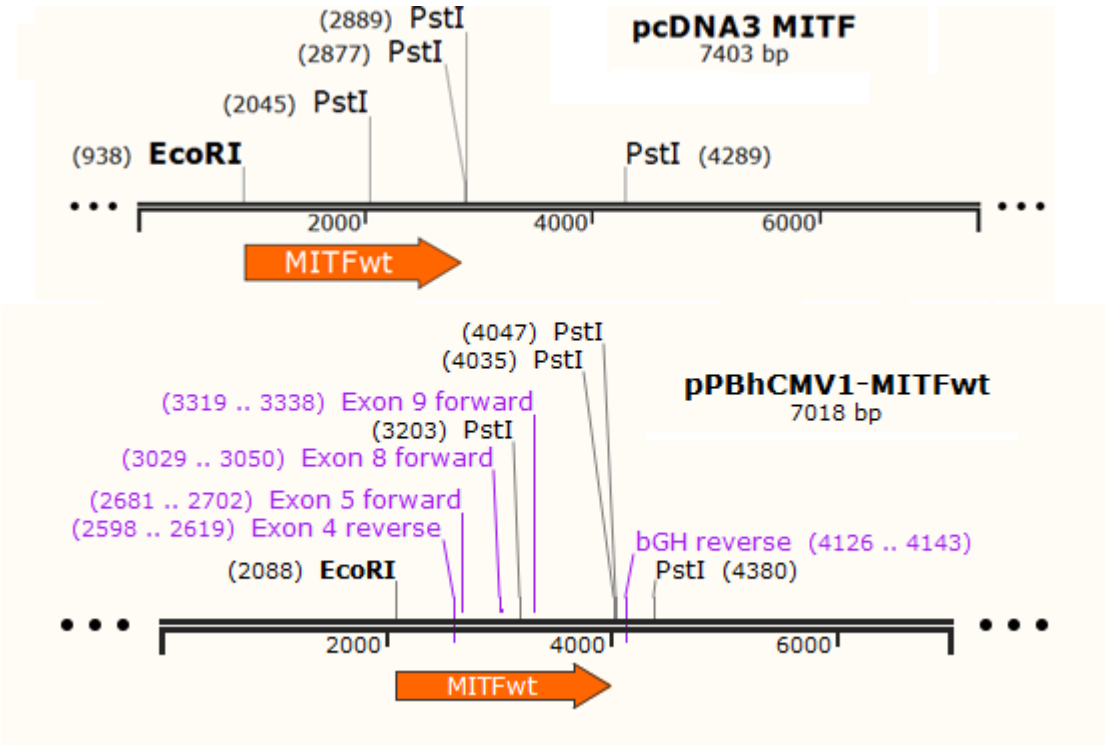

Figure 3. *Comparison of EcoRI and PstI restriction sites in PB construct and pcDNA3 carrier plasmid. Also for the PB construct, location of primers used for DNA sequencing are shown.*

3.1.2 Verifying the plasmid constructs with DNA sequencing

All three PB constructs were confirmed by DNA sequencing using the primers exon 4 reverse, exon 5 forward, exon 8 forward, bGH reverse and exon 9 forward (see Appendix for primer sequences and Figure 3 for location in vector). The alignment of the sequence resulting from exon 4 reverse primer revealed an upstream linker sequence of 9 bp between the PB vector and the Mitf insert. All three piggyBac constructs have this same 9 bp linker sequence. The sequence resulting form bGH reverse primer failed to give full sequence of the downstream linker. However, the sequence given by exon 9 forward primer managed to reveal a 58bp downstream linker sequence. This linker sequence has two PstI restriction sites within a short interval of 12 bp. This information explains the fragments of EcoRI/PstI digestion. The sequence given by exon 5 forward primer confirmed that the 18 bp sequence (5’ CGTGTATTTTCCCCACAG-3’) was missing from exon 6 in the spotted and spotless Mitf clones but was present in the wild type clone. The sequences resulting

from exon 8 and exon 5 forward primers confirmed the K316Stop mutation resulting from a A to T nucleotide change, only in the MITF^{spotless} clone.

3.2 Experiments performed on the stable inducible cell lines

3.2.1 Evaluation of the stable inducible cell lines

To evaluate the inducible expression of the system, the inducing agent, doxycycline, was added to the cell cultures of all three MITF expressing variants, at a final concentration of 10 μg/ml. After two days, induced and non-induced cells of all three systems were harvested and their total protein mass collected. Western blot of these protein collections (10 μg) were stained with antibodies against MITF and β-actin as described in section 2.4.4. The results can be seen in Figure 4.

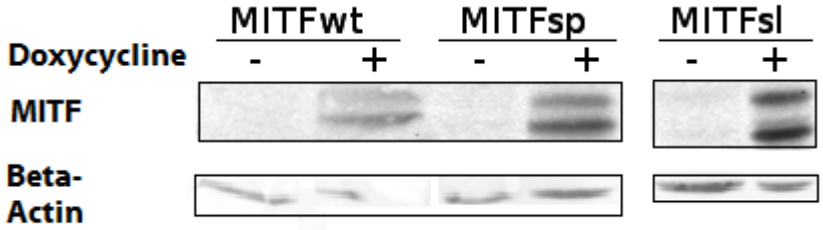

Figure 4. *The ability of doxycycline to induce MITF expression as detected by Western blotting. Comparison of stably transfected HEK293 cells with Mitf^{wt}, Mitf^{sp} or Mitf^{sl} PB constructs incubated with and without doxycyline shows that doxycycline induces MITF expression in all cell systems.*

The β-actin bands show proper sample loading on the gel. The characteristics double MITF bands are only visible when the cell cultures were incubated with doxycycline. This illustrates that doxycycline works as an inducing agent for MITF expression in all three cell systems.

3.2.2 Effects of doxycycline concentration on MITF expression

To optimize the amount of doxycycline needed to induce MITF expression, doxycycline was added into cultures of the wild type MITF expressing cells at the final concentrations of 0, 0.1, 1, 5, 10 and 50 μg/ml. After two days, the cells were harvested as described in section 2.4.3. The result of the following western blot can be seen in Figure 5.

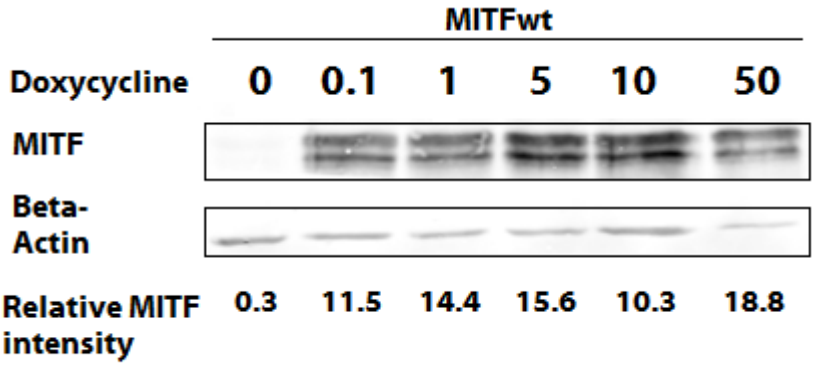

Figure 5. The effects of increasing doxycycline concentration on MITF expression, as detected by Western blotting. HEK293 cells, stably transfected with PB MITF^{wt} incubated for 48 hours in 0, 0.1, 1, 5, 10 and 50 μg/ml final concentration of doxycycline. Relative intensity of MITF to β-actin was calculated using the ImageJ program.

The intensity of the MITF bands was calculated relative to the intensity of β-actin bands using the ImageJ program. The relative intensity of the MITF signal increases with increasing concentration of doxycycline. This increase is, however, not linear and clear disruption in this pattern can be observed at the 10 μg/ml doxycycline concentration due to strong β-actin band. Although it should be noted that the system is, to some degree, leaky as there is always some background MITF expression. Full induction is observed at the lowest doxycyline concentration tested, 0.1 μg/ml which seems to be able to increase MITF expression 40-fold.

3.2.3 Estimation on stability of MITF variants

To estimate the potential difference in protein stability of the three MITF variants the cell cultures were treated with cycloheximide (CHX) as described in section 2.4.2 and induced with 10 μg/ml of Doxycycline for 48 hours. Cycloheximide is a well known inhibitor of protein synthesis and is often used as a tool to estimate protein half-life (Zhou, P. 2004). The cells were harvested after 0, 4 and 13 hours of CHX treatment and total protein mass then ran on acrylamide gel and western blotting performed as described in section 2.4.4. The results can be seen in Figure 6.

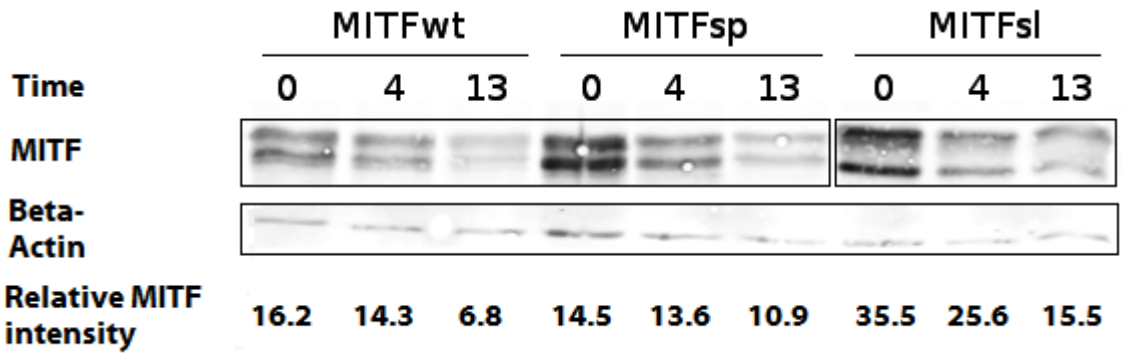

Figure 6. *The protein stability results of 0, 4 and 13 hours of cycloheximide treatment on MITF expression in stably transfected HEK293 cells expressing Mitf^{wt}, Mitf^{sp} or Mitf^{sl} as detected by Western blotting. Relative intensity of MITF to β-actin was calculated with the ImageJ program.*

The relative intensity of the MITF bands fell distinctly with increased time of CHX exposure. This indicated that the protein inhibition by CHX was working as intended. From the relative intensity of the MITF bands for the MITF variants, a rough estimate of the proteins half life can be calculated. Assuming first order reaction rate of the protein degradation, the following equation;

$$N_t = N_0 e^{-kt} \qquad \textit{Equation I}$$

describes the degradation of MITF were k is the rate constant, N_0 is the initial MITF intensity and N_t is the MITF intensity after a time, t in hours. Using the values of relative MITF intensity from Figure 6, the relationship

$$\ln\left(\frac{N_t}{N_0}\right) = -kt \qquad \textit{Equation II}$$

can be plotted to determine the rate constants for the degradation of all MITF variants (Figure 7).

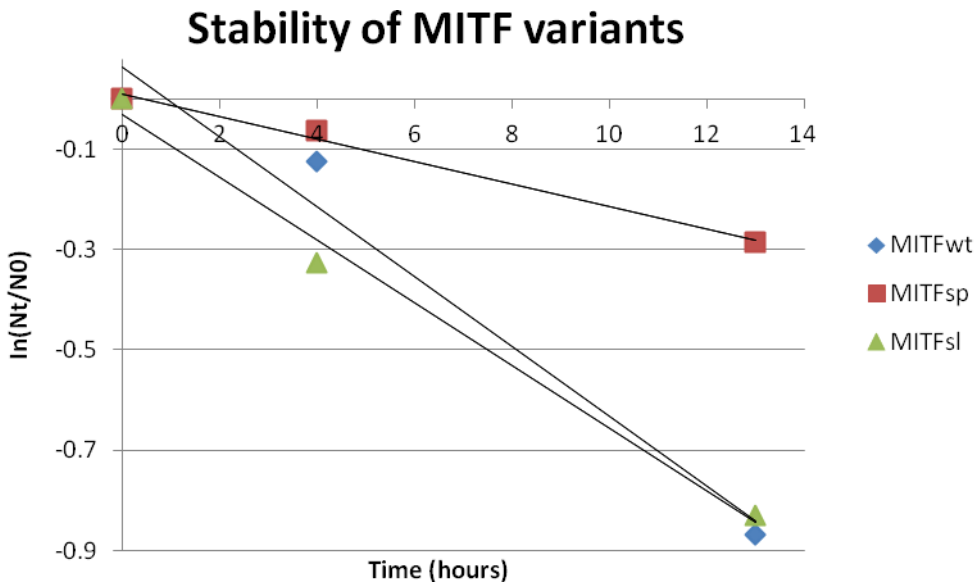

Figure 7. *Stability of Mitf variants. Natural logarithm of the ratio of MITF intensity/initial MITF intensity against time of CHX exposure, using MITF intensity values from Figure 6. The rate constants, k (hour^{-1}) are; 0.0695 MITF^{wt}, 0.244 MITF^{sp}, 0.0624 MITF^{sl}. R^2 values; 0.971 MITF^{wt}, 0.992 MITF^{sp}, 0.991 MITF^{sl}.*

By definition, half life of a protein is the time it takes the protein to be degraded by half. That is, when $N_t = N_0/2$ and is given by the equation;

$$t_{1/2} = \frac{\ln(2)}{k} \qquad \textit{Equation III}$$

From the known rate constants (Figure 7), the half life of the MITF variants can be calculated using *eq. III*. The results are presented in Table 1.

Table 1. Half life of the MITF^{wt},MITF^{sp} and MITF^{sl} proteins.

	$t_{1/2}$(hours)
MITF^{wt}	10.0
MITF^{sp}	30.9
MITF^{sl}	11.1

The half-life of all the MITFs variants are given in Table 1 and a clear difference in the stability of the MITF^{sp} protein compared to the MITF^{wt} and MITF^{sl} proteins' stability was seen. It has to be noted that this interpretation of MITF stability is limited by how weak the β-actin signals appear.

3.2.4 Phosphorylation of MITF

As seen in Figures 4-6 two protein bands are observed for MITF wild type and spotted as well as for MITF spotless. These bands appear because of the difference in their phosphorylation state as the slower migrating band is phosphorylated at serine 73 site (Hemesath, TJ. et al. 1998). According to unpublished data from the lab the serine at position 409 needs to be phosphorylated for serine 73 phosphorylation to occur, as observed when the 409 position is mutated to Ala, no phosphorylation takes place at serine 73. Thus two bands appearing for $MITF^{sl}$, which undoubtedly lacks a serine 409 site, comes as a surprise. To confirm that the upper MITF band for $MITF^{sl}$ was really phosphorylated at serine 73 the membrane with the cycloheximide treated cell samples (Figure 6.) was immunostained with special antibody against phosphorylated serine 73 of MITF. Figure 8 shows the results for this immunostaining and shows that the upper band is clearly phosphorylated.

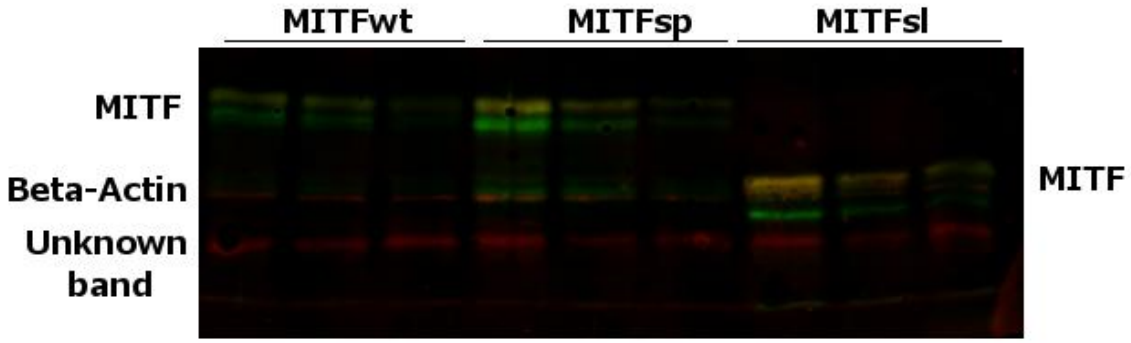

Figure 8. *Serine 73 phosphorylation of MITF. Staining with an antibody against phosphorylated serine 73 of MITF on membrane with CHX treated cell samples (Figure 6) and previously stained against MTIF and β-actin. Color code; Green: MITF, Red: β-actin,unknown band and serine 73 phosphorylated MITF. Yellow: merge of green and red.*

The membrane, containing samples from $Mitf^{wt}$, $Mitf^{sp}$ and $Mitf^{sl}$ expressing and cycloheximide treated HEK293 cells was first stained against MITF with anti-MITF (C5) antibody (mouse) and β-actin with anti-β-Actin (rabbit). Two clear MITF bands were observed in each lane (green) and only weak actin bands (red) was seen at the location marked in Figure 8 and singled out in Figure A1 (Appendix). In a second immunostaining step (with second block, TBST wash and secondary antibodies as described in 2.4.4.) the membrane was stained with the antibody against serine 73 phosphorylated MITF (rabbit). This revealed more red bands at two locations; a fast migrating unknown band and a band at the same location as the upper MITF band for all variants (Figure 2A) as indicated by the merge of red and green as yellow in Figure 8. This is seen for all three MITF variants, although the beta-actin and the upper $MITF^{sl}$ bands overlap on the gel, comparison between figures 1A and 2A shows clear band thickening at 42 kDa for the spotless samples after the second immunostaining step (Figure 2A) with the antibody against phosphorylated serine 73 on MITF, ultimately confirming the phosphorylation of serine 73 on $MITF^{sl}$.

4 Discussion

In this project the *piggyBac* vector system has been used to generate stable cells inducibly expressing Mitf^{wt}, Mitf^{sp} and Mitf^{sl}, with doxycyline as the inducible agent. All three MITF *piggyBac* constructs have been confirmed by DNA sequencing as shown in sections 3.1.1. and 3.1.2. The bands for MITF^{sl} appear lower on the gel than the wild type and spotted bands. This is consistent with the fact that the MITF^{sl} protein is about ¼ shorter than their spotted and wild type counterparts. The lack of visible MITF bands at 74 kD and 65 kD in MITF^{sl} wells can be used as a control for MITF expression of untransfected HEK293 cells (Figure 8).

Optimization of doxycycline showed that just as little as 0.1 μg/ml final doxycycline concentration was sufficient to cause strong increase in MITF expression. In order to estimate the stability of the different MITF mutants the cells were treated with cycloheximide. The results showed significant increase in stability of MITF^{sp} over MITF^{wt} and MITF^{sl}. This reversal of the high stability of MITF^{sp} by the MITF^{sl} mutant might be partially explained by the suppressor ability of MITF^{sl}. However, this needs to be replicated and experimentally verified. However, there are limits with this experimental setup and further optimization is needed. For example how much impact does different doxycycline incubation time (61 hours, 52 hours and 48 hours) and perhaps the following decrease in doxycycline concentration play in the declining intensity of MITF bands? Effects on cell death need to be determined and more data points are also needed to draw conclusive results.

The fact that MITF^{sl} can be phosphorylated at serine 73 despite lacking serine 409 can be viewed in the context of regulation of serine 73 phosphorylation. Possibly the carboxyl-terminus of MITF either directly or indirectly interacts with the N-terminus and in turn regulates the phosphorylation and activity of MITF. As spotless is lacking the carboxyl-terminus of MITF^{wt} and therefore the 409 site, phosphorylation of serine 73 may never be inhibited at least by the C-terminus. Although other factors independent of MITF C-terminus probably play a role in this regulation, as there is not a total shift to the upper band seen for MITF^{sl}. Further research is needed to confirm such speculations and to test if there is any interaction between the C- and N-terminus.

By generating these cell systems, a powerful tool has been produced to do comparative analysis of the different function of the MITF variants in mammalian cells. Further comparison would include qPCR of MITF target genes, subcellular location of MITF and effects of MITF over expression on cell cycle.

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Appendix

Table A1. Buffers used in this study

Buffers	Contents	Purpose
Phosphate Buffered Saline(PBS)	0.02M phosphate, 0.15M NaCl, pH 7.2	Cell splitting and harvesting
Trypsin-EDTA	0.05% Trypsin, 0.5mM EDTA, 1x PBS	Cell splitting
Resolving gel (8% polyacrylamide)	4.7 ml H_2O. 2.7 ml 30% acrylamide mix, 2.4 ml 1.5M Tris(pH 8,8), 0.1ml 10% SDS, 0.1ml 10% ammonium persulfate, 0.008 ml TEMED	SDS-PAGE
Stacking gel (5% polyacrylamide)	2 ml H_2O. 0.5 ml 30% acrylamide mix, 0.70 ml 1 M Tris(pH 6,8), 0.05 ml 10% SDS, 0.03 ml 10% ammonium persulfate, 0.003 ml TEMED	SDS-PAGE
Sample buffer(2x)	100mM TrisCl, 4%(w/v) SDS, 0.2%(w/v) bromophenol blue, 20%(v/v) glyserol, 10%(v/v) 2-mercaptoethanol	SDS-PAGE
Running Buffer(10x)	10g SDS, 144g glycine and 30.28g Tris to 1L of H_2O	SDS-PAGE
Transfer Buffer(10x)	30g Tris, 144g glycine to 1L of H_2O	Western-Blot
Tris-buffered saline(TBS)	24g Tris, 88g NaCl, pH set to pH 7.6 in 1L of H_2O	Western-Blot
TBS-tween(TBST)	10ml 10% of tween-20 added to 1L with 1xTBS	Western-Blot
Tris-acetate-EDTA(10x TAE)	48.4g Tris, 11.4 ml glacial acetic acid, 3.7g EDTA to 1L with H_2O	Agarose gel electrophoresis

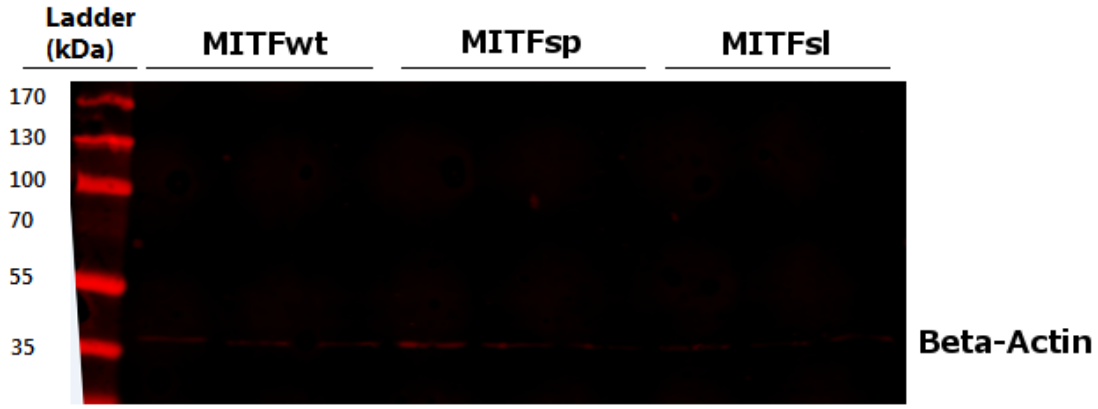

Figure 1A. *Staining with anti-β-actin on membrane with CHX treated cell samples (Figure 6), reveals band around 42 kDa for all samples.*

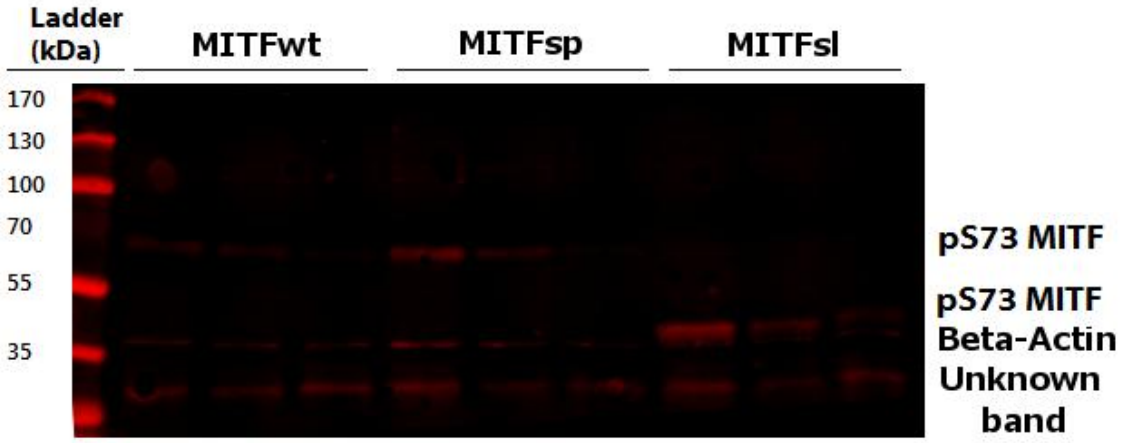

Figure 2A. *Staining with antibody against phosphorylated serine 73 on MITF on the same membrane seen in Figure 1A. Bands at two more locations were revealed, that is a fast migrated unknown band and a band at the same location as the upper MITF band for all variants (Figure 8). Clear band thickening at 42 kDa for the spotless samples is observed, confirming the phosphorylation of serine 73 on* $MITF^{sl}$.

Primers used

Exon 4 reverse primer: 5'-TAACTTGATTCCAGGCTGATGA-3'

Exon 5 forward primer: 5'-TCTGGAAACTTGATCGACCTCT-3'

Exon 8 reverse primer: 5'-CTTGAAAACCGACAGAAGAAGC-3'

Exon 9 forward primer: 5'-CTACAGCATCCCCAGGAAGA-3'

bGH reverse primer: 5'-TAGAAGGCACAGTCGAGG-3'