



# **Effects of the regulatory proteins MITF and TFEB on autophagy in melanoma cells**

Sólveig Hlín Brynjólfsdóttir



**Líf- og umhverfisvísindadeild  
Háskóli Íslands  
2014**



# **Effects of the regulatory proteins MITF and TFEB on autophagy in melanoma cells**

Sólveig Hlín Brynjólfssdóttir

14 eininga ritgerð sem er hluti af  
*Baccalaureus Scientiarum* gráðu í Sameindalíffræði

Leiðbeinendur  
Eiríkur Steingrímsson  
Margrét Helga Ögmundsdóttir

Líf- og umhverfisvísindadeild  
Verkfræði- og náttúruvísindasvið  
Háskóli Íslands  
Reykjavík, maí 2014

Effects of the regulatory proteins MITF and TFEB on autophagy in melanoma cells  
14 eininga ritgerð sem er hluti af *Baccalaureus Scientiarum* gráðu í Sameindalíffræði

Höfundarréttur © 2014 Sólveig Hlín Brynjólfsdóttir  
Öll réttindi áskilin

Líf- og umhverfisvísindadeild  
Verkfræði- og náttúruvísindasvið  
Háskóli Íslands  
Askja, Sturlugötu 7  
101 Reykjavík

Sími: 525 4000

Skráningarupplýsingar:

Sólveig Hlín Brynjólfsdóttir, 2014, *Effects of the regulatory proteins MITF and TFEB on autophagy in melanoma cells*, Lokaritgerð, Líf- og umhverfisvísindadeild, Háskóli Íslands, 29 bls.

Prentun: Háskólaprent ehf.  
Reykjavík, maí, 2014

# Abstract

Autophagy is a vital process in cell biology. A number of genes and proteins are necessary for regulating autophagy and the transcription factor EB (TFEB) has recently been identified as a component of the regulatory mechanism in somatic cells. TFEB belongs to the same family as MITF, which is a master regulator of melanocytes and melanoma cells. Autophagy is important, both for tumor suppression and for proliferation of cancer cells after tumor initiation. MITF is important for melanoma progression, as it regulates most aspects in the life of a melanoma cell. This project aimed to identify the role of MITF and TFEB in regulating autophagy in melanoma cells. Overexpression and knockdown experiments in human 501 melanoma cells showed interaction between TFEB and MITF. Both transcription factors had some effects on autophagy markers and proteins involved in regulating autophagy. For the first time, this study reveals a potential regulatory role for MITF in autophagy in melanoma cells.

# Útdráttur

Sjálfsát er frumunni lífsnauðsynlegt og fjöldi gena og próteina koma við sögu í stjórn sjálfsáts. Nýlega hefur verið sýnt fram á að umritunarþátturinn EB (TFEB) gegnir hlutverki við stjórn sjálfsáts í nokkrum gerðum líkamsfruma. Þessi umritunarþáttur tilheyrir sömu fjölskyldu og umritunarþátturinn MITF sem stjórnar starfsemi litfruma og sortuæxlisfruma. Sjálfsát er mikilvægur þáttur í ælisbælingu sem og lifun krabbameinsfruma þegar æxli hefur myndast. Í sortuæxlum er MITF í lykilhlutverki og hefur áhrif á flesta ferla frumanna, þar á meðal framþróun krabbameinsins. Í þessari rannsókn var miðað að því að finna hlutverk MITF og TFEB í stjórnun sjálfsáts í 501Mel sortuæxlisfrumum úr mönnum. Með því að yfirtjá og slá niður tjáningu þessara og fleiri þátta, sáust samskipti á milli TFEB og MITF. Báðir umritunarþættirnir höfðu áhrif á sjálfsáts-merkiprótein og prótein sem eiga þátt í stjórnun sjálfsáts. Rannsóknin sýnir mögulegt hlutverk MITF í stjórnun sjálfsáts í sortuæxlisfrumum.



# Efnisyfirlit

<b>Myndir</b> .....	<b>vii</b>
<b>Töflur</b> .....	<b>viii</b>
<b>Abbreviations</b> .....	<b>ix</b>
<b>Þakkir</b> .....	<b>xi</b>
<b>1 Introduction</b> .....	<b>1</b>
1.1 The importance of autophagy .....	1
1.1.1 Autophagy in cancer .....	1
1.1.2 The process of autophagy .....	2
1.2 Regulation of autophagy .....	2
1.2.1 The role of TFEB .....	3
1.3 Microphthalmia-associated transcription factor .....	4
1.3.1 Melanoma cells .....	6
<b>2 Aims of the project</b> .....	<b>7</b>
<b>3 Materials and methods</b> .....	<b>8</b>
3.1 Cell culture .....	8
3.2 Plasmids.....	8
3.2.1 Gene expression experiments .....	9
3.2.2 Transfection of human cells.....	9
3.3 Western blot .....	10
3.3.1 Protein purification .....	10
3.3.2 SDS gels.....	11
3.4 qPCR experiments.....	12
3.4.1 Purification of mRNA and reverse transcription PCR.....	12
3.5 Immunostaining experiments .....	13
<b>4 Results</b> .....	<b>15</b>
4.1 MITF downregulates itself .....	15
4.2 Expression levels of TFEB affect expression of MITF and its downstream targets .....	16
4.3 TFEB downregulates RagD.....	17
4.4 MITF and TFEB regulation of autophagy.....	18
4.4.1 MITF and TFEB influence mTORC1 .....	18
4.4.2 MITF affects autophagy markers.....	19
4.5 MITF and TFEB activity at the transcriptional level .....	22
<b>5 Discussion</b> .....	<b>22</b>
<b>References</b> .....	<b>25</b>

**Appendix .....27**



# Myndir

Figure 1 Regulation of autophagy. ....	5
Figure 2 Localization of MITF and TFEB in 501Mel.....	7
Figure 3 Treatment of 501Mel cells for immunofluorescence experiment. ....	14
Figure 4 Downregulation of MITF and TFEB. ....	15
Figure 5 The effects of TFEB overexpression.....	16
Figure 6 Alterations in protein levels of mTORC1 components.....	18
Figure 7 Overexpression of MITF affects autophagy markers.....	19
Figure 8 MITF expression affects autophagy in 501Mel. ....	20
Figure 9 MITF overexpression in qPCR.. ....	21

# Töflur

Tafla A.1 Plasmid constructs used in cell culture. ....	27
Tafla A.2 Antibodies used in western blotting.....	28
Tafla A.3 Buffers and solutions. ....	29

# Abbreviations

Amp	Ampicillin
bHLH	basic Helix-Loop-Helix
CMV	Cytomegalovirus
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
Kan	Kanamycin
LB	Lysogeny Broth
MITF	Microphthalmia associated transcription factor
mTOR	mammalian Target of Rapamycin
mTORC	mTOR Complex
Neo	Neomycin
NF $\kappa$ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
P/S	Penicillin/Streptomycin
ROS	Reactive Oxygen Species
rpm	Rounds Per Minute
RPMI	Roswell Park Memorial Institute
TFEB	Transcription Factor EB
Zeo	Zeomycin



# Acknowledgements

I would like to thank Eiríkur Steingrímsson and his team for the opportunity to do this project . Special thanks to Margrét H. Ögmundsdóttir for letting me be a part of her project and for all her help and patience.



# **1 Introduction**

## **1.1 The importance of autophagy**

Autophagy is a fundamental process in cell biology and is an essential part of normal cell function. Autophagy is a degradation pathway in which the cell digests organelles and worn-out parts of itself and is able to recycle metabolites from the digested material. This system is vital for cell clean-up, as the accumulation of old organelles and other autophagic substrates can jeopardise the life of the cell. The process of autophagy relies mostly on two organelles, the autophagosome and the lysosome. Autophagy has numerous connections to disease and defective autophagy can result in a lack of intracellular nutrient availability as well as the accumulation of protein aggregates and defective organelles.

### **1.1.1 Autophagy in cancer**

The process of autophagy has opposing effects in cancer as it can both suppress tumour formation but also promote cancer cell survival, once the tumour has formed. Under normal conditions autophagy prevents cancer initiation by degrading worn out organelles and protein aggregates and thereby maintaining homeostasis. Autophagy is also fundamental for mitochondria function and for dispensing of damaged mitochondria as old mitochondria may increase oxidative stress and ROS production. This can contribute to genome instability that can cause cancer initiation. Autophagy is important for limiting inflammation and tissue damage, through interaction with the NF- $\kappa$ B pathway and this may limit tumour growth as inflammation has been shown to promote cancer initiation (White 2012).

In normal cells, autophagy is usually at a low level and is induced under stress. In cancer cells however, autophagy is often at a high basal level and is vital for cell survival. Cancer cells depend on autophagy to compensate for other pathways that have been impaired. Furthermore, the microenvironment of cancer cells can be stressful as levels of oxygen, nutrients and growth factors are insufficient.

### **1.1.2 The process of autophagy**

In the beginning of autophagy, a double membrane encloses a part of the cytosol creating an autophagosome. How this formation is achieved and whether it is somehow selective of what is included in the autophagosome is not fully understood. The autophagosome does not start the degradation. It only isolates autophagic substrates and brings them to the lysosome (Mizushima 2007). Proper lysosomal function is a vital part of autophagy as it takes care of the degradation and is the meeting site for various proteins that control autophagy. The lysosome contains various acid hydrolases and a low pH which makes it fit for efficient degradation of a variety of substrates. The autophagosome fuses with the lysosome, creating an autolysosome or autophagolysosome. The outer membrane of the autophagosome fuses with the lysosomal membrane while the inner autophagosomal membrane gets digested along with the autophagosomal content (Mizushima 2007).

## **1.2 Regulation of autophagy**

Autophagy is a process that the cell uses under stress and when nutrients are scarce. Autophagy allows the cell to preserve energy, reuse nutrients and generate energy from disposable organelles. When resources are sufficient, autophagy is downregulated. This is dependent on many factors, such as growth factors, glucose and amino acids. The sensing mechanism is complex and involves many different genes and proteins but the mammalian target of rapamycin (mTOR) protein kinase complex is often thought of as the main regulator of autophagy (Settembre et al. 2012). This complex comes in two forms, mTORC1 and mTORC2 and both are important in promoting cell growth and proliferation and sensing nutrition availability in the cell. The serine/threonine protein kinase TOR is an essential part of the complexes and has a variety of functions in the eukaryotic cell. These two complexes are both involved in autophagy regulation although mTORC1 has been shown to be a master regulator of autophagy (Settembre et al. 2012).

The mTORC1 protein complex reacts mainly to intracellular amino acids levels, so when amino acids are abundant mTORC1 remains active and represses autophagy. The activation of mTORC1 is based on the translocation of mTOR from the cytosol to the lysosomal membrane where it resides while in an active mTORC1 complex. On the lysosomal surface the complex is activated by the small Rag GTPases. These are four different proteins that work in heterodimers. Rag A or B binds to Rag C or D, where in the



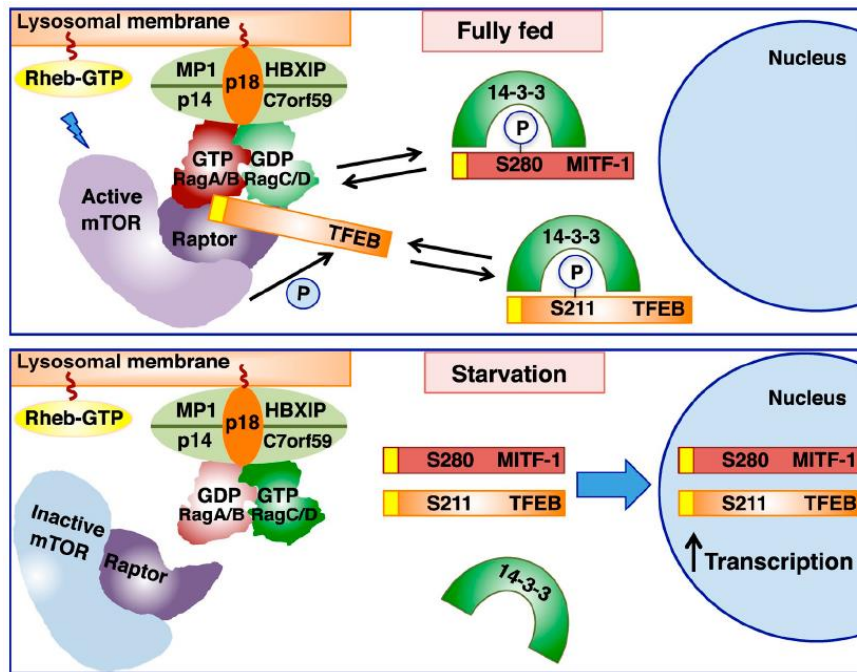
active state, A and B are GTP bound while C and D are GDP bound. When active, the heterodimers bind to Raptor, a protein specific to mTORC1. Amino acids activate these heterodimers by promoting GTP binding to RagA and RagB and therefore encourage the binding of mTORC1 to the lysosomal surface and the assembly of an active mTORC1 complex (Martina et al. 2012).

### **1.2.1 The role of TFEB**

In recent years, a new protein has been found to play a role in the regulation of autophagy. Transcription factor EB (TFEB) is a part of the basic Helix-Loop-Helix (bHLH) leucine zipper transcription factor family and has an important role in regulating lysosomal genes and lysosomal biogenesis (Settembre & Ballabio 2011). When TFEB is present in the nucleus it promotes transcription of many autophagy related genes, positively regulating autophagosome formation, lysosome biogenesis and autophagosome-lysosome fusion (Settembre et al. 2012). To suppress autophagy, TFEB has to be kept out of the nucleus, in the cytosol. While mTORC1 is active, it is responsible for keeping TFEB inactive and in the cytosol. This is achieved via the amino acid/Rag GTPase pathway (Settembre et al. 2012). Similar to Raptor, TFEB has an affinity for active Rag GTPase heterodimers. The Rags summon TFEB to the lysosomal surface where it is phosphorylated by mTORC1. An activated mTORC1 phosphorylates TFEB on various amino acid residues, including Serine 211. This phosphorylation enables TFEB to associate with the cytosolic chaperone protein 14-3-3 which causes the transcription factor to stay in the cytosol (see Figure 1), (Martina & Puertollano 2013). In fully fed cells, most TFEB protein is found bound to 14-3-3 in the cytosol but a fraction is always present on the lysosomal surface where mTORC1 keeps it phosphorylated. As soon as the amino acid signalling stops, the Rag heterodimers become inactive and mTORC1 moves back to the cytosol and becomes inactive. This causes the TFEB phosphorylation to stop and the TFEB-14-3-3 complex to dissociate so TFEB can move unhindered to the nucleus (see Figure 1). Accumulation of TFEB in the nucleus occurs rapidly under starvation, when amino acid signalling declines (Martina et al. 2012). Once in the nucleus, TFEB promotes the transcription of genes necessary for autophagosome and lysosome formation, the fusion of autophagosomes and lysosomes and the degradation in the lysosome (Settembre & Ballabio 2011).

### **1.3 Microphthalmia-associated transcription factor**

Like TFEB, microphthalmia-associated transcription factor (MITF) is a member of the bHLH leucine zipper family. MITF is found in various tissues of the human body but is most abundant in melanocytes, where it serves as a master regulator of proteins necessary for pigmentation, melanosome formation and function and melanin production. This transcription factor is especially important in melanocytes as it regulates their differentiation and development (Shibahara et al. 2001). Many different isoforms of MITF exist and some of them are homologous to the N-terminus of TFEB, where the cytoplasmic localization signal is located. Both MITF-1 and MITF-7 have this domain and have been shown to associate with Rag GTPases on the lysosome, in a similar fashion as TFEB (Ho et al., 2011; Martina & Puertollano, 2013). In response to level of nutrients, TFEB and MITF-1 show the same pattern of regulation. In fully fed cells MITF-1 is found diffused in the cytosol while under starvation it accumulates in the nucleus. This translocation has been shown to depend on mTOR inactivation (Martina & Puertollano, 2013).



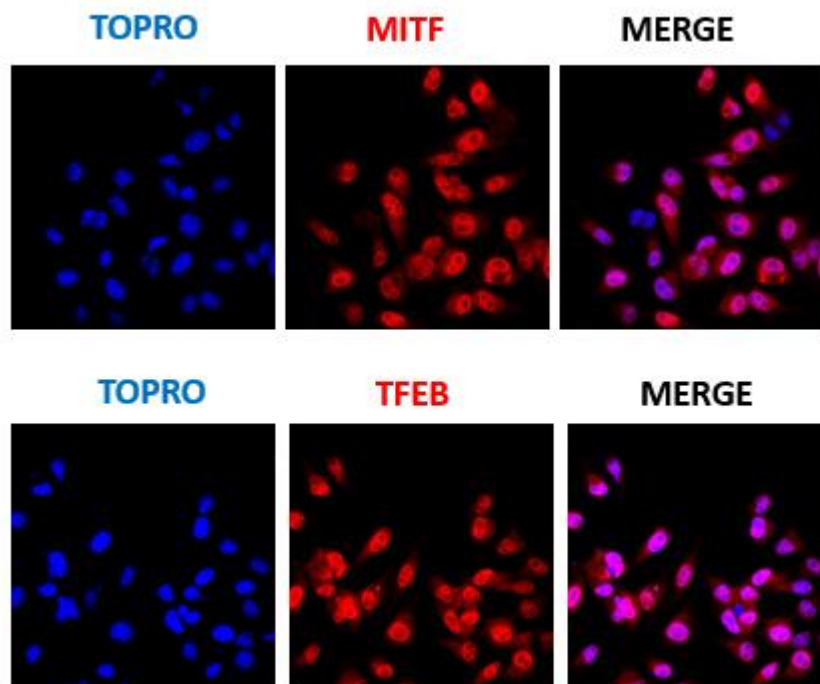
**Figure 1. Regulation of autophagy.** The mechanism by which autophagy is controlled from the lysosome. The mTORC1 complex is activated and TFEB/MITF kept in the cytosol by association with the 14-3-3 chaperone while nutrients are abundant. During starvation both mTOR and the Rag GTPases become inactive, phosphorylation stops and the transcription factors move to the nucleus (Martina & Puertollano, 2013).

The melanosome is closely related to the lysosome and certain genes and proteins have been shown to play a role in both melanosome formation and in autophagy. In melanocytes, MITF-M is in charge of regulating the transcription of proteins necessary for melanosome formation and maturation (Ho et al. 2011). This isoform of MITF does not appear to interact with the Rag GTPases in the same way as MITF-1 and MITF-7 do and it does not share the domain involved in cytoplasmic retention present in TFEB (Martina & Puertollano, 2013). However, MITF-M has been shown to react to mTOR signalling, where mTORC1 inhibition promotes MITF transcription and thereby transcription of MITF target genes involved in melanosome formation and melanogenesis (Ho et al. 2011). In MITF-M, Serine 173 is the conserved amino acid that corresponds to Ser211 in TFEB (Martina & Puertollano, 2013). Both TFEB and MITF affect transcription by binding to DNA as homodimers or working together in a heterodimer (Martina, Diab, Li, & Puertollano, 2014). Because of this overlap between the pathways for autophagy and

melanosome formation and because some isoforms of MITF seem to react to amino acid- and mTOR signalling it is possible that MITF also has a regulatory role in autophagy.

### **1.3.1 Melanoma cells**

The MITF-M isoform of MITF is melanocyte specific and is probably the most important transcription factor in melanocytes, regulating their development, differentiation and being in control of their main task, to produce melanin (Ho et al. 2011; Shibahara et al. 2001). In the deadliest form of skin cancer, melanoma, MITF plays an important role. MITF has been described as a melanoma specific oncogene and an MITF germline variant has been identified that increases the risk of melanoma (Paillerets et al. 2013). MITF has also been shown to promote proliferation of melanoma cells and levels of MITF can control invasiveness and metastasis in melanoma (Strub et al. 2011). The human melanoma cell line, 501Mel, has six copies of the MITF gene and expresses high levels of MITF and TFEB (Lionel Larue, personal communication, Strub et al., 2011). Eiríkur Steingrímsson's team has been studying autophagy in the 501Mel cell line and has shown that in these cells, TFEB and MITF are always present in the nucleus (Figure 2).



*Figure 2. Localization of MITF and TFEB in 501Mel cells. Confocal photos of untreated 501Mel cells stained for MITF and TFEB. Both transcription factors are mainly present in the nucleus. Nuclear staining is shown in blue. Experiment performed by Margrét Bessadóttir.*

When TFEB is present in the nucleus, autophagy is upregulated in somatic cells. This has not been confirmed in melanoma cells but nonetheless, 501Mel cells maintain a high basal level of autophagy. Whether the presence of MITF in the nucleus contributes to autophagy levels is not clear. On the whole, this cell line is a convenient model for looking into the role of MITF-M in regulating autophagy in melanoma cells.

## 2 Aims of the project

The main aim of this project was to study the potential role of MITF in terms of autophagy in human cancer cells. The effects of MITF expression on TFEB was studied, and vice versa, as well as the effects of both transcription factors on several genes related to autophagy. This was done by overexpressing and knocking down MITF and TFEB, as well as by knocking down mTOR in the 501Mel human melanoma cells.

The knock down of mTOR was done to see if changes in autophagy pathways would affect MITF and TFEB expression. The main research questions that we opted to answer were

Do MITF and TFEB regulate autophagy in melanoma cells?

Do MITF and TFEB affect expression of each other in melanoma cells?

Do MITF and TFEB regulate mTORC1 in melanoma cells?

## **3 Materials and methods**

### **3.1 Cell culture**

The 501Mel human melanoma cell line was taken up from storage in liquid nitrogen, thawed and cultured. These cells are anchorage dependent and grow in an adherent culture. The cells were cultured in RPMI media with 10% FBS (fetal bovine serum) but without P/S (penstrap/streptomycin) or any other antibiotic. The cells were cultured at 37°C in 5% CO<sub>2</sub>. Cells were split every 2-3 days. Subcultures were made by removing media and trypsinating cells for 1-2 min, to free them from the culture flask. The free cells were spun down in fresh RPMI for 3 min at 1400 rpm. The cell pellet was dissolved by gentle pipetting with RPMI and the cells passaged to a new flask.

### **3.2 Plasmids**

Plasmids are small DNA molecules, most commonly circular, that can replicate on their own. Plasmids are self-replicating DNA molecules that are convenient for introducing new DNA sequences into cells and thereby affect gene expression in one way or another. Changing the expression of a gene can reveal both downstream targets of the gene and other components of the pathways it plays a role in. Thereby this is a simple way to get an idea of the role of a gene in the cell. This method was used to manipulate gene expression in the 501Mel cells. A plasmid construct made to manipulate gene expression must contain the gene that is to be expressed as well as a promoter that ensures expression of the plasmid gene. Here, selection genes that provide resistance to certain antibiotics were also included in the construct so transfected cells could be easily selected for. An important

control in these experiments was to transfect with an empty vector, a plasmid that does not affect the gene expression of the cells but will show the effects of the transfection, if any.

### **3.2.1 Gene expression experiments**

Plasmids can be used to overexpress genes in cell lines when driven from appropriate promoters. We overexpressed MITF and TFEB and looked at the effect of this overexpression on expression of other genes.

For overexpression experiments, plasmid constructs for GFP-MITF, GFP-TFEB, MITF and an empty vector were used (see Table A1). These plasmids contain protein coding genes downstream of the CMV promoter and excess protein is transcribed directly from the plasmid. Constructs for GFP-tagged proteins were bought from Addgene but the empty vector and untagged MITF constructs were made by Margrét Helga Ögmundsdóttir.

GIPZ lentiviral shRNA plasmid constructs from Thermo Scientific were used for knockdown of MITF, TFEB and mTOR (see Table A1). These were bought as *E.coli* populations containing the plasmids. Cultures of *E.coli* were made to purify the plasmids. LB plates with ampicillin were prepared for selection of bacteria containing the plasmids. Bacteria were streaked out on the plates and cultured overnight at 37°C. Single colonies were picked and put in 2 ml liquid LB media with Amp selection (1:1000) and kept at 37°C for 6 h in a shaker. Of this culture, 300 µl were taken and put in 100 ml of liquid LB media with Amp selection (1:1000) and kept in 37°C in a shaker overnight. This culture was put in two 50 ml Falcon tubes and spun down at 3000 rpm, 4°C for 15 min. The media was removed, the pellets dissolved and a MidiPrep kit from Fermentas Life Sciences used to purify the plasmids. Concentration of purified plasmid solutions was determined using Nanodrop.

### **3.2.2 Transfection of human cells**

A chemical method for introducing plasmids to 501Mel cells was used, using the transfection reagent FuGene from Promega. About  $3 \times 10^5$  cells were plated in each well in 3 ml of RPMI media with 10% FBS, the day before transfection. Plating was done by the same protocol as described in section 3.1 about cell culture.

For each well, 3.3 µg DNA in 150 µl RPMI serum free media was required. To this blend, 9.9µl of Fugene were added and the blend incubated for 8 minutes at room temperature. After incubation, 150 µl of the appropriate transfection blend were added to each well of cell culture.

In knockdown experiments, medium was changed 36-48 hours after transfection and fresh RPMI + 10% FBS with puromycin (1:1000, 2 mg/ml concentration) was added to each well. The antibiotic kills off the cells that are not transfected and therefore reduces background and ensures that the cells used for sampling have taken up the plasmid and have reduced expression of the genes in question. Treated cells were lysed when all non-treated cells had died.

For overexpression experiments, transfected cells were lysed 36-48 h after transfection, without changing the media. For a few overexpression experiments, selection with neomycin (1:100, 50 mg/ml concentration) was attempted without success and neither non-treated nor transfected cells were affected by neomycin. Most overexpression experiments were done without using selection.

### **3.3 Western blot**

Western blots are a convenient way to identify and roughly quantify proteins of interest in samples extracted from cells and tissues. Protein samples from cell lysates are run on a SDS gel, which separates the proteins according to size. From the gel, the proteins are transferred to a nitrocellulose membrane where they can be labelled with specific antibodies. This detection method was used in overexpression and knockdown experiments to measure differences in protein levels of proteins of interest.

#### **3.3.1 Protein purification**

Sample preparation for western blots was carried out with the following protocol.

1. Cell plates were put on ice and all media removed.
2. Cells washed once using cold 1x PBS buffer.
3. Cells lysed with 60 µl cold RIPA lysis buffer with 1:100 protease inhibitor cocktail, ProteoBlock from Fermentas (#R1321). Cells were scraped off and dislodged using the base of a pipette tip and the mixture pipetted into an Eppendorf tube.



4. Cell lysate incubated on ice for 10 min.
5. The lysate sonicated for 5 min at 4°C.
6. Mixture centrifuged for 10 min at 14.000 RPM at 4°C.
7. Supernatant transferred to a fresh tube and mixed with 60 µl 2x Sample Buffer.
8. Samples heated to 100°C for 5 min in a heat block.
9. Protein concentration measured using Bradford reagent method and a spectrophotometer.
10. Samples stored at -20°C.

### **3.3.2 SDS gels**

All western blots were run on 8% resolving mini gels with 5% stacking gel, at room temperature in 1x Tris-Glycine running buffer. At first, 20 µl of protein sample were loaded on each well, regardless of the protein concentration. This was changed later and 20 µg of proteins were loaded on each well. This resulted in clearer bands.

Proteins were transferred from the gel to a nitrocellulose membrane using iBlot dry blotting system with the iBlot gel transfer device from life technologies.

Antibody staining was done with the following protocol

1. Blocking buffer prepared. This consisted of 5% dried milk and 1x TBS buffer. Membranes were blocked for 1h on a shaker at room temperature.
2. Membranes washed with 1 x TBST for 5 min, repeated 3 times.
3. Primary antibodies diluted in 5% BSA 1x TBST (see Table A2).
4. Membranes incubated in primary antibodies overnight at 4°C on a shaker.
5. Step 2 repeated.
6. Secondary antibodies diluted in 1xTBST, 0,01% SDS (see Table A2).
7. Membranes incubated with secondary antibodies for 35 min at room temperature, on a shaker.
8. Step 2 repeated. Membranes washed once with 1x TBS for 5 min.
9. Blots scanned on Odyssey infrared scanner.

In several experiments, membranes were stripped of their antibodies and incubated again with antibodies targeting other proteins. This was done by incubating membranes in a

stripping buffer for 30 min in a 60°C water bath. After stripping the membranes, they were blocked and incubated in antibodies, following the protocol above.

### 3.4 qPCR experiments

Real time, quantitative polymerase chain reaction or qPCR is a powerful tool for studying gene expression. Like regular PCR, genes of interest are amplified using specific primers but the amount of DNA is measured in each cycle of the reaction, in order to quantitate each gene. Since the primary product of the expression of protein coding genes is mRNA it is best to look at mRNA to evaluate expression levels of genes. For qPCR, mRNA is extracted from a sample and complementary DNA or cDNA is made from it by using reverse transcriptase. The qPCR reaction is performed on the cDNA which gives an indirect measurement of mRNA levels in the original sample.

#### 3.4.1 Purification of mRNA and reverse transcription PCR

Samples were prepared from transfected 501Mel cells (see section 3.2.2). Cells were lysed and mRNA purified using RNeasy kit from Qiagen.

Reverse transcription on mRNA to generate cDNA was done using a mastermix kit from Applied Biosystems (SKU #4368814). For each sample 2 µg mRNA were diluted in a total volume of 10 µl. Mastermix for each 10 µl sample was as follows

Reagent	Volume (µl)
10x RT buffer	2,0
25x dNTP	0,8
10x RT random primers	2,0
Reverse transcriptase	1,0
RNase inhibitor	1,0
Nuclease free H <sub>2</sub> O	3,2
Total volume	10,0

The reverse transcription PCR was done with a simple heat program in a heat block.

Heat program (°C)	Time (min)
25	10
37	120
85	5
4	∞

Both mRNA and cDNA samples were stored at -80°C.

The qPCR was done using a Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix kit (#K0221). For each reaction, 5ng RNA/cDNA were required and cDNA synthesis samples were diluted 1:100 and 10 µl used per each reaction. The qPCR was run in a 96-well plate. For each reaction a master mix was prepared as follows.

Reagent	Volume (µl)
Maxima SYBR Green/ROX qPCR Master Mix (2x)	12.5
Forward primer	0.75
Reverse primer	0.75
cDNA (1:100 dilution)	5
Nuclease free H <sub>2</sub> O	6
Total volume	25

Primers targeting MITF and GAPDH as a reference were used in the qPCR.

### 3.5 Immunofluorescence experiments

In order to see the effects of MITF on protein expression in 501Mel cells, immunofluorescence experiments were conducted. Cells are plated onto coverslips and fixed. After the fixation they can be stained with antibodies for the proteins of interest and the magnitude and localization of the respective proteins be determined using microscopy.

This type of experiment was used to look at the effects of MITF on autophagy in 501Mel cells. Cells were plated onto two 8-well coverslips at a density of  $2 \times 10^4$  cells/well. On each coverslip, cells in four wells were transfected with a construct overexpressing GFP-tagged MITF. The transfection was done using the same protocol as described in section 3.2.2 but on a smaller scale. The day after the transfection (about 12 hours), cells were starved for 1 or 4 hours (see Figure 3). The starvation should upregulate autophagy and as a control for this treatment, cells were also starved for 4 hours with added chloroquine which blocks autophagy.

NT	No treatment	1h	4h	4h + CQ
MITF	No treatment	1h	4h	4h + CQ

**Figure 3. Treatment of 501Mel cells for immunofluorescence experiment.** Overview of treatments of the 8-well microscopy slide. NT stands for non-transfected cells in the upper four wells. Both transfected and non-transfected cells were starved for one or four hours as well as starved for 4 hours with added chloroquine (CQ).

Methanol was used to fix the cells, with the following protocol.

1. Media removed and ice cold 100% methanol put in all wells and kept at  $-20^{\circ}\text{C}$  for 15 min.
2. Methanol removed and cells washed three times with 1x PBS for 5 min.
3. Cells blocked for 1h in 1x PBS, 5% normal goat serum, 0,3% Triton x-100, at room temperature.
4. Cells incubated in primary antibodies overnight at  $4^{\circ}\text{C}$  (see Table A2).
5. Antibodies removed and cells washed three times with 1x PBS for 5 min.
6. Cells incubated with secondary antibodies for 1 hour at room temperature.
7. Washed with 1x PBS for 5 min.
8. Cells incubated with Topro3 nuclear staining in 1x PBS for 7 min.
9. Washed two times with 1x PBS for 5 min.
10. All liquid aspirated and wells removed from the coverslip. One drop of Fluroshield put on each sample and coverslips covered with microscope slides.

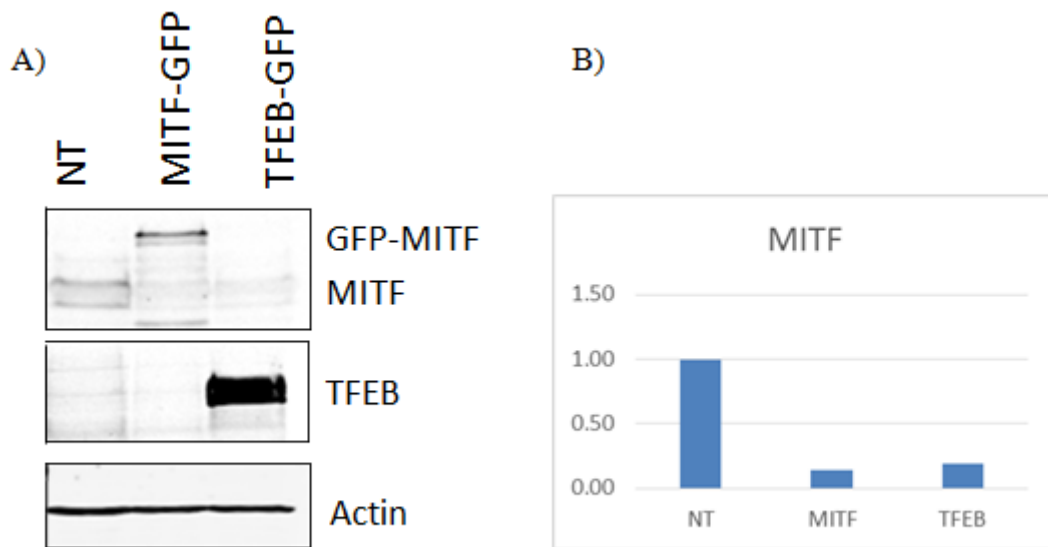
11. Samples sealed with nail polish and stored at 4°C.

The samples were examined using a confocal microscope.

## 4 Results

### 4.1 MITF downregulates itself

An MITF-GFP fusion protein was overexpressed in 501Mel cells. On a western blot this results in separate bands for GFP-tagged MITF (82 kDa) and the endogenous MITF protein (55 kDa) since the GFP-tag (27 kDa) adds weight to the protein. As previously reported, doublet MITF bands were obtained, due to phosphorylation status of the protein (Wu et al. 2000).



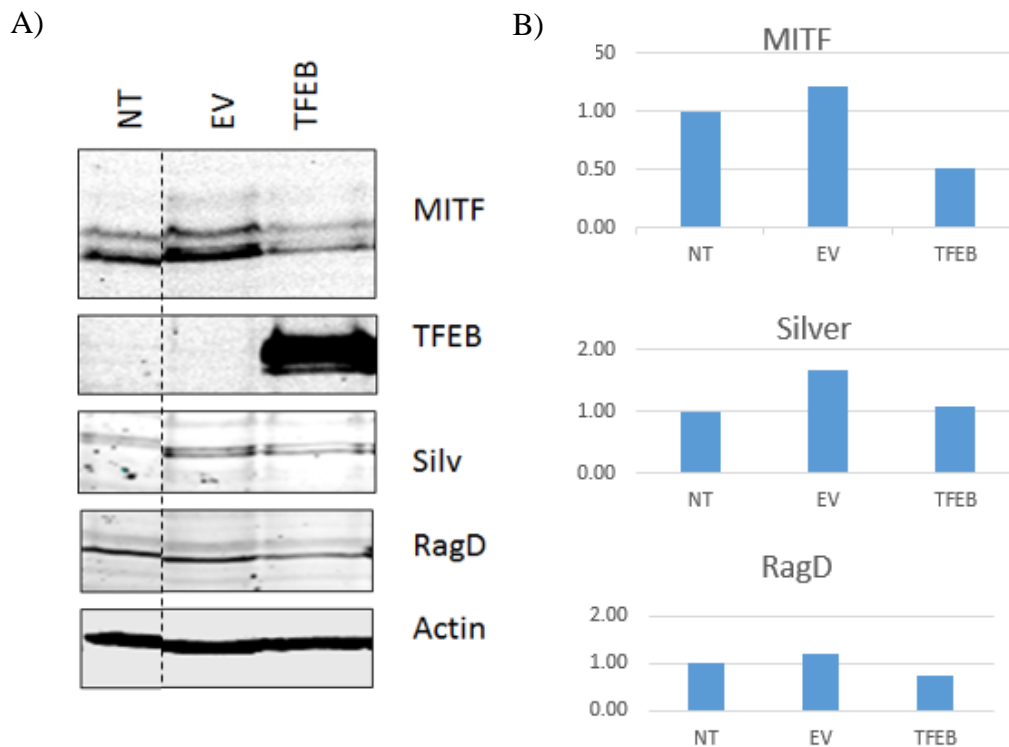
**Figure 4. Overexpression of MITF and TFEB.** A) Overexpression of GFP-tagged MITF causes downregulation of endogenous MITF. Overexpression of TFEB protein also results in lower levels of endogenous MITF. B) A quantification of the endogenous MITF bands on the blot in A) with actin as reference.

Expression of the endogenous MITF protein was reduced in cells overexpressing MITF-GFP than in non-treated cells (see Figure 4). The levels of endogenous MITF seemed to correlate with levels of GFP-MITF; more overexpression resulted in less endogenous MITF.

This translated to downstream targets of MITF. Silver is a premelanosome protein and a downstream target of MITF in melanocytes (Du et al. 2003). The protein levels of this target was reduced upon overexpression of MITF and TFEB (see Figures 5 and 7). However, this downregulation does not happen when MITF is overexpressed without the tag (data not shown). A possible explanation for this is that the protein expressed from the introduced construct has the same size as endogenous MITF and therefore a difference in endogenous levels can not be detected on a western blot. The reduced levels of endogenous MITF may be due to downregulation of gene transcription or to MITF promoting its own protein degradation. Further studies have to be carried out at the transcriptional level to see if MITF mRNA follows the same pattern as the protein (see section 4.5).

## **4.2 Expression levels of TFEB affect expression of MITF and its downstream targets**

When TFEB was overexpressed in 501Mel cells it affected the protein levels of MITF (see Figures 4 and 5). Overexpression of TFEB reduced the expression of endogenous MITF. This downregulation of endogenous MITF translated to effects on Silver, which expression was also reduced.



**Figure 5. The effects of TFEB overexpression.** A) Overexpressed TFEB causes downregulation of endogenous MITF and results in reduced levels of Silver protein, a downstream target of MITF. Overexpression of TFEB also affects the expression level of RagD. B) Quantification of protein levels of MITF, Silver and RagD bands on the blot in A, with reference to actin. All proteins show lower levels when TFEB is overexpressed.

Whether TFEB is only affecting MITF or if the interaction is going both ways and MITF is also affecting TFEB, is unclear. A band for endogenous TFEB was never observed although the bands for GFP-tagged TFEB were obtained in almost every experiment. This technical issue makes it impossible to say at present, what the effects of MITF expression are on the expression of endogenous TFEB protein.

### 4.3 TFEB downregulates RagD

The Rag GTPases are in part responsible for keeping TFEB phosphorylated and in the cytosol when there is little need for autophagy and upregulation of lysosomal activity. The Rag GTPases tether TFEB to the lysosomal membrane for phosphorylation by mTOR (see Figure 1). Since MITF-M has been shown to react to mTOR signalling, it might also have some interaction with the Rag GTPases (Ho et al. 2011). Expression of RagC and RagD

was determined using western blotting. Rag C never showed any changes upon varying MITF or TFEB expression (data not shown). However when TFEB was overexpressed, the levels of RagD lowered notably (see Figure 5) and in a few experiments this also happened when MITF was overexpressed (data not shown). This suggests that RagD may be a target of TFEB and/or MITF.

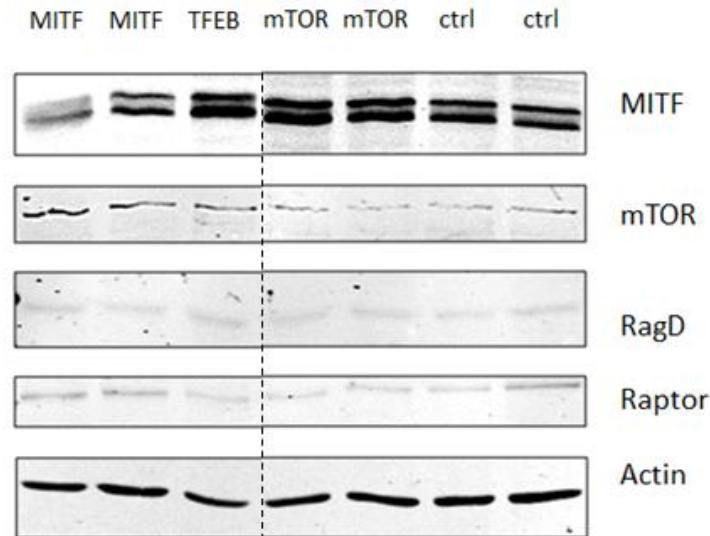
## **4.4 MITF and TFEB regulation of autophagy**

If MITF and TFEB are in fact involved in regulating autophagy in melanoma cells, their over-expression or down-regulation might be expected to alter the protein levels of mTOR as well as other autophagy related proteins including LC3, p62 and Lamp2. These are commonly used as autophagy markers. The LC3 protein binds cargo to the autophagosomal membrane and its protein levels rise with increased autophagy. The p62 protein is also bound to the autophagosome and binds cargo through interaction with LC3. The p62 protein is broken down along with the cargo in the autolysosome (White 2012).

### **4.4.1 MITF and TFEB influence mTORC1**

The expression of mTOR was knocked down in 501Mel cells using shRNA (see Table A1) to detect any difference in mTOR expression level with varying MITF and TFEB expression. No definite results were obtained from these experiments, especially since a band for the mTOR protein is difficult to obtain on western blots due to its large size of 289 kDa. Nonetheless, some changes in protein levels were detected, both mTOR responding to MITF and TFEB expression and vice versa. Expression of another component of the mTORC1 complex, Raptor, was also determined. This protein showed some response to reduced MITF, TFEB and mTOR expression (see Figure 6).



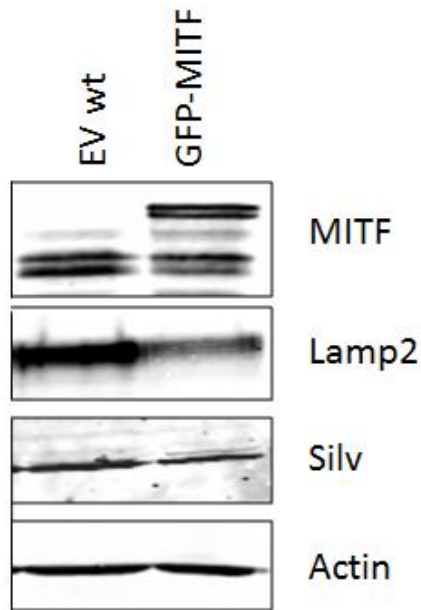


**Figure 6. Alterations in protein levels of mTORC1 components.** An example of a knock down experiment with mTOR and Raptor. Here, MITF, TFEB and mTOR expression have been knocked down and both mTOR and Raptor react to the change in expression levels.

No consistent trends were observed with Raptor and further experimenting is needed to confirm that Raptor is responding to TFEB or MITF.

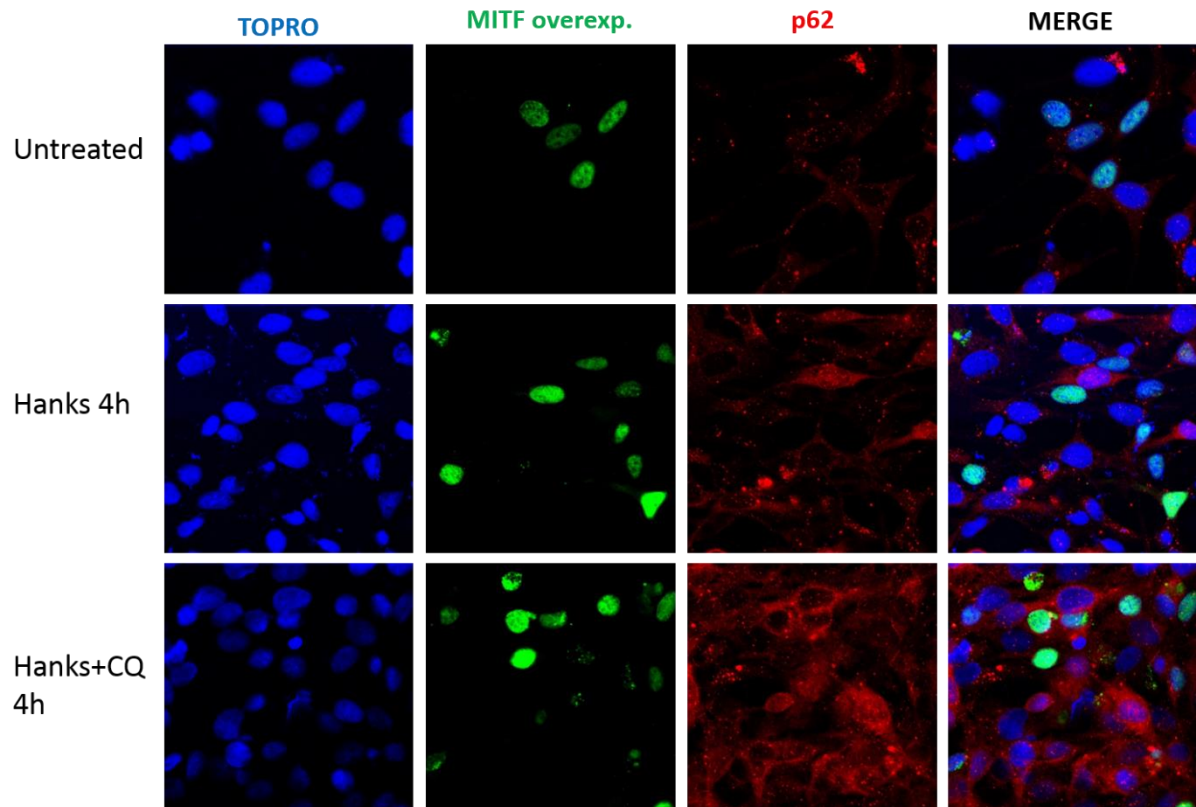
#### 4.4.2 MITF affects autophagy markers

The autophagy markers mentioned above were also detected by western blotting. Levels of Lamp2 seemed to reduce with increased expression of MITF and TFEB (see Figure 7). This protein is essential for autophagosome maturation and fusion with the lysosome.



**Figure 7. Overexpression of MITF affects autophagy markers.** Overexpression of GFP-tagged MITF has an impact on Silver protein levels, a downstream target of MITF. High expression of MITF also downregulates Lamp-2, a marker for autophagy.

The p62 protein never showed any changes in levels and therefore seems to be unaffected by MITF and TFEB expression (data not shown). Expression of LC3 was also determined but a proper band was never detected. This protein is very small (about 15 kDa) and the transfer protocol of the Western blotting needs to be optimized to detect this protein. These targets were looked at by immunofluorescence experiments where MITF was overexpressed in cells under starvation and changes in p62 and LC3 in the cells validated (see Figure 8). The protocol for LC3 staining needs to be optimized and data is not shown for that experiment.

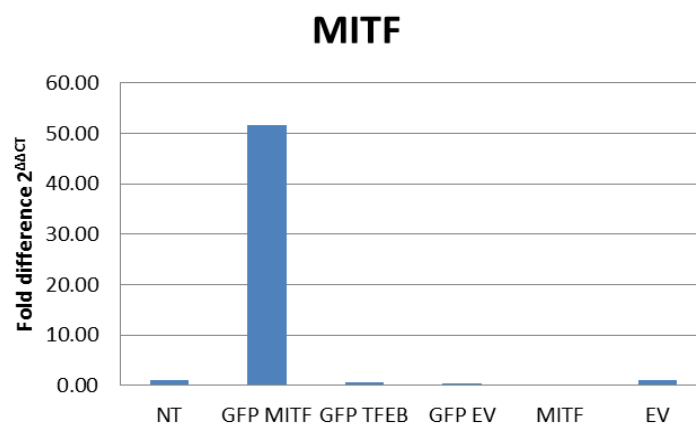


**Figure 8. MITF expression affects autophagy in 501Mel.** A comparison of untreated, starved (Hanks) or starved with added chloroquine (Hanks + CQ) 501Mel cells where MITF has been overexpressed. The autophagy marker p62 accumulates upon starvation but autophagy is blocked. Cells with overexpressed MITF show different patterns of p62 accumulation.

The autophagy marker p62 is located on the inner membrane of the autophagosome and plays a role in binding cargo. This protein is broken down along with the cargo so when autophagy is upregulated, degradation of p62 is also increased. However, if chloroquine is added into media it inhibits autophagy so degradation cannot happen and p62 accumulates. This trend is visible in Figure 8 where little change in p62 levels is visible between untreated and starved cells but a clear accumulation occurs when autophagy is blocked in starved cells. p62 expression is upregulated without any increase in break down. In cells where MITF is overexpressed this pattern is altered and p62 does not accumulate in starved cells, although it does in cells that are not overexpressing MITF. When autophagy is blocked in starved cells, p62 becomes visible in cells overexpressing MITF. These results might indicate that MITF is actively involved in autophagy pathways in melanoma cells, although further research is needed to verify this.

## 4.5 MITF and TFEB activity at the transcriptional level

Future work includes analysing the activity of MITF and TFEB at the transcriptional level by qPCR. In this project samples were prepared from 501Mel cells for qPCR experiments (see section 3.4). One overexpression experiment was performed where both MITF and TFEB were overexpressed. In this experiment only MITF was targeted and for GFP-tagged MITF a good overexpression was obtained (see Figure 9).



**Figure 9. MITF overexpression in qPCR.** Fold difference in mRNA levels of MITF from a qPCR experiment where MITF and TFEB were overexpressed. A significant overexpression was obtained from the GFP-tagged MITF construct.

Samples are ready for further transcriptional level analysis that would include the same markers as have already been targeted at the protein level.

## 5 Discussion

This project has provided some clues that MITF and TFEB may influence autophagy and affect each other in melanoma cells. Experiments with overexpressing and knocking down the expression of MITF and TFEB in 501Mel cells showed that these transcription factors are altering the protein levels of each other as well as of other proteins known to play a role in regulation of autophagy.

When MITF is overexpressed it downregulates the endogenous levels of itself. Overexpression of TFEB has the same impact on MITF but it is not clear at what level MITF is reduced, since experiments were only done at the protein level. Further experiments need to be carried out to investigate whether endogenous MITF is being degraded or if its gene expression is being altered.

The downstream target of MITF, Silver, showed changes in expression in response to increased MITF and TFEB. Bearing in mind that MITF and TFEB affect each other, it is not yet clear if the effect of TFEB on Silver is direct or via effects on MITF. We did not detect endogenous TFEB on the western blot and therefore, were not able to assess the effect of knocking down TFEB on downstream targets. This will be important to optimize in order to compare the effects of knocking down MITF and TFEB or both MITF and TFEB on downstream targets.

Since mTORC1 is a master regulator of autophagy it is likely that proteins that influence protein levels of the components of this complex are in some way involved in regulating autophagy. In cells other than melanoma cells, the role of TFEB in autophagy and its interaction with mTORC1 has been well established. Here we found that TFEB and MITF are influencing RagD, a key component of mTORC1. Indeed, Chip-seq data in 501Mel cells shows that MITF binds two sites within 600 bp of the transcription start site of the RagD gene (Strub et al. 2011). This suggests that MITF is really involved in regulating RagD expression.

Whether MITF and TFEB are interacting with components of the mTORC1 complex is not yet clear and needs to be examined. Some alterations were seen in protein levels of mTOR and Raptor when expression of the two transcription factors was increased or reduced but no clear trends were diagnosed. To verify the impacts MITF and TFEB have on mTORC1, RagD and downstream targets of MITF in melanoma cells, experiments at the transcriptional level are needed. This project has however provided indications that these transcription factors are in fact, involved in regulating autophagy in melanoma cells.

Autophagy has recently been studied as a possible target for cancer therapy. It is a tempting possibility to be able to shut down cancer cells through autophagy but unfortunately this would have numerous side effects as all somatic cells use autophagy to some extent and need to be able to tidy up their cytosol, get rid of worn out organelles and

so forth. In melanoma cells, however, it appears that autophagy is differently regulated compared to other cell types. It is possible that MITF has a major role in autophagy in melanoma cells. Should this be the case, it could offer the possibility of shutting down autophagy specifically in melanoma cells, through MITF-M. This might reduce side effects as this transcription factor is essential in melanocytes and not in every cell of the body.

To date, no efficient treatment is available for melanoma cancer. This calls for a better understanding of melanoma and a better characterization of individual melanoma tumours. This is ongoing research and some very interesting discoveries have been made. One of those is that half of all melanoma tumours harbour an activating mutation in the BRAF kinase. This has been utilized in therapy and patients with these kinds of tumours are treated with BRAF inhibitors which works well for some months, but soon the tumours develop resistance to the inhibition (Johannessen et al. 2013). How this resistance develops is not known but one hypothesis is that other mechanisms are activated and take over the pathway, bypassing the inhibition. A vast study on BRAF inhibition resistance mechanisms showed importance of MITF, as well as TFEB and p62 came up as resistance mechanisms, which could point in the direction of autophagy (Johannessen et al. 2013). In addition to this, it has been shown that levels of MITF can determine invasiveness and metastasis of melanoma tumours (Strub et al. 2011).

On the whole, it is evident that MITF is a dominating factor in melanoma development and is a master regulator of melanoma cells as well as melanocytes. Future research and therapeutic studies will have to look in the direction of MITF and dissect its role in melanoma tumours. The plausible function of MITF in autophagy in melanoma cells is one way to gain insight into the extensive role of MITF in melanoma cells and in the end understand and treat melanoma.

# References

- Du, J. et al., 2003. MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *The American journal of pathology*, 163(1), pp.333–43. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1868174&tool=pmcentrez&rendertype=abstract>.
- Ho, H. et al., 2011. WIPI1 coordinates melanogenic gene transcription and melanosome formation via TORC1 inhibition. *The Journal of biological chemistry*, 286(14), pp.12509–23. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3069453&tool=pmcentrez&rendertype=abstract> [Accessed February 19, 2014].
- Johannessen, C.M. et al., 2013. A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. *Nature*, 504(7478), pp.138–42. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24185007> [Accessed March 20, 2014].
- Martina, J. a et al., 2012. MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy*, 8(6), pp.903–14. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3427256&tool=pmcentrez&rendertype=abstract>.
- Martina, J. a et al., 2014. Novel roles for the MiTF/TFE family of transcription factors in organelle biogenesis, nutrient sensing, and energy homeostasis. *Cellular and molecular life sciences : CMLS*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24477476> [Accessed February 25, 2014].
- Martina, J. a & Puertollano, R., 2013. Rag GTPases mediate amino acid-dependent recruitment of TFEB and MITF to lysosomes. *The Journal of cell biology*, 200(4), pp.475–91. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3575543&tool=pmcentrez&rendertype=abstract> [Accessed February 3, 2014].
- Mizushima, N., 2007. Autophagy : process and function. *Genes & Development*, 21, pp.2861–2873.
- Paillerets, B.B., Lesueur, F. & Bertolotto, C., 2013. A germline oncogenic MITF mutation and tumor susceptibility. *European journal of cell biology*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24290354> [Accessed February 18, 2014].
- Settembre, C. et al., 2012. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *The EMBO journal*, 31(5), pp.1095–108. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3298007&tool=pmcentrez&rendertype=abstract> [Accessed January 22, 2014].

Settembre, C. & Ballabio, A., 2011. TFEB regulates autophagy: An integrated coordination of cellular degradation and recycling processes. *Landes Bioscience*, (November), pp.1379–1381.

Shibahara, S. et al., 2001. Microphthalmia-associated transcription factor (MITF): multiplicity in structure, function, and regulation. *The journal of investigative dermatology.*, 6(1), pp.99–104. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11764295>.

Strub, T. et al., 2011. Essential role of microphthalmia transcription factor for DNA replication , mitosis and genomic stability in melanoma. *Oncogene*, pp.1–14.

White, E., 2012. Deconvoluting the context-dependent role for autophagy in cancer. *Nature reviews. Cancer*, 12(6), pp.401–10. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3664381&tool=pmcentrez&rendertype=abstract> [Accessed March 4, 2014].

Wu, M. et al., 2000. c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi. *Genes & Development*, 14, pp.301–312. Available at: <http://genesdev.cshlp.org/content/14/3/301.short> [Accessed March 18, 2014].



# Appendix

Tables containing information about plasmid constructs, antibodies and solutions used in the study.

*Table A1. Overview of plasmid constructs used for cell culture experiments, see section 3.2.*

Construct	Plasmid ID	Vector backbone	Resistance	Lab database no.	Concentration (ng/ $\mu$ l)
MITF-GFP	38131	pEGFP-N1	Neo Kan	K 4.1	950
TFEB-GFP	38119	pEGFP-N1	Neo Kan	K 4.2	490
MITF	-	pcDNA 3.1	Amp Neo	C 4.8	2332
EV	-	pcDNA 3.1	Amp Neo	C 5.5	1741
MITF	257541	pGIPZ	Zeo Amp	H 2.1	305
MITF	76565	pGIPZ	Zeo Amp	H 2.2	355
TFEB	332992	pGIPZ	Zeo Amp	K 5.3	633
TFEB	332994	pGIPZ	Zeo Amp	K 5.4	644
mTOR	644694	pGIPZ	Zeo Amp	K 4.8	568
mTOR	635008	pGIPZ	Zeo Amp	K 5.2	513
Ctrl	4480	pGIPZ	Zeo Amp	H 2.4	560
Ctrl	4346	pGIPZ	Zeo Amp	H 2.3	746

**Table A2.** Antibodies used in western blotting experiments, see section 3.3.

Antigen	Host	Product ID	Producer	Concentration
MITF C5	Mouse	MS 771	Thermo	1:2000
TFEB	Mouse	120432	MyBio Source	1:100
mTOR	Rb	7C10 2983	Cell Signaling	1:1000
RagC	Rb	9480	Cell Signaling	1:1000
RagD	Rb	4470	Cell Signaling	1:1000
Raptor	Rb	2280	Cell Signaling	1:1000
$\beta$ -actin	Rb	4970	Cell Signaling	1:1000
p62	Mouse	M162-3	Mbl	1:1000
LC3	Rb	12741	Cell Signaling	1:1000
pS6K	Mouse	9206	Cell Signaling	1:1000
p4EBP1	Rb	2855	Cell Signaling	1:1000
$\beta$ -actin	Mouse	MAB 1501	Millipore	1:20000
Tyrosinase	Rb/Goat	Sc-7833	Santa Cruz	1:200
Silv	Rb	AV46802-100UG	Sigma	1:800
Lamp2	Mouse			1:2000
Mouse	-	5257	DyLight	1:15000
Rabbit	-	5366	DyLight	1:15000

**Table A3.** Recipes for buffers and solutions used in this study. All solutions were made with MilliQ water.

Solution	Ingredients	Volume
10x Running Buffer	5g SDS, 72 g Glycine, 15 g Tris	0.5 L
10x TBS	12 g Tris, 44 g NaCl, pH set on 7.4	0.5 L
TBST	1x TBS, 0.5 ml Tween-20	1 L
10x PBS	40 g NaCl, 1 g KCl, 7.2 g Na <sub>2</sub> HPO <sub>4</sub> , 1.2 g KH <sub>2</sub> PO <sub>4</sub> pH set on 7.4	0.5 L
4x LTB	181.65 g Tris, 4 g SDS pH set on 8.8	1 L
4x UTB	60.6 g Tris, 4 g SDS pH set on 6.8	1 L
2x Sample Buffer	4 g SDS, 10 ml β-mercapto ethanol, 27 ml 50% glycerol, 1.51 g Tris 20 mg bromophenolblue	0.1 L
Stripping Buffer	12.5 ml 1M Tris pH 6.8, 40 ml 10% SDS, 1ml β-mercapto ethanol	0.2 L
8% Resolving Gel	2.1 ml 40% Acrylamide, 2.6 ml LTB, 100 μl 10% APS, 10 μl TEMED	10 ml
5% Stacking Gel	630 μl 40% Acrylamide, 1.25 ml UTB, 50 μl 10% APS, 5 μl TEMED	5 ml
LB media	10 g Bacto-Tryptone, 5 g Bacto-Yeast, 10 g NaCl, pH 7.0	1 L
LB Amp plates	LB media, 15 g Bacto-Agar, 100μg/μl Ampicillin	
RIPA lysis buffer	50mM Tris pH 8.0, 150 mM NaCl , 1% Igepal CA-630, 0,5% sodium, Deoxycholate, 0,1% SDS	



