Acetylation of the MITF transcription factor and regulation by signaling

Acetýlering umritunarþáttarins MITF og stjórnun um boðleiðir

Katla Kristjánsdóttir

Raunvísindadeild
Háskóli Íslands
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10 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í lífefnafræði

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Útdráttur

Microphthalmia-associated transcription factor (MITF) er umritunarþáttur sem gegnir mjög mikilvægu hlutverki í öllum þroskunarstigum litfrumna (melanocytes). Vitað er að umritunarþættinum er stjórnað með fosfóryleringu í gegnum Kitl/Kit boðleiðina. Óbirtar niðurstöður rannsóknarhóps Eiríks Steingrímssonar sýna að acetylering gegnir einnig mikilvægu hlutverki í stjórnun MITF, en hvernig þetta fer fram er enn ekki ljóst. Til að varpa ljósi á þessa spurningu var MITF yfirtjáð, með og án hjálparþáttanna (co-factors) CBP/p300, í HEK293T frumum og þær síðan meðhöndlaðar með sértæku hindrunum U0126 og PD98059. Þessi efni eru sértækir hindrar gegn ákveðnum þætti Kitl/Kit boðleiðarinnar sem heitir Mek1/Mek2. Áhrif hindranna á acetýleringu MITF var síðan könnuð með ónæmisfellingu og ónæmisþrykkjum (Western blotting). Niðurstöðurnar sýna að tjáning CBP eða p300 er nauðsynleg til að acetýlering fari fram og að Kitl/Kit boðleiðin er viðriðin stjórnun á acetýleringu MITF. Þetta bendir til þess að fosfórun á annað hvort MITF sjálfu eða hjálparþáttum sé lykilskref í acetýleringu á MITF próteininu.

Abstract

The *Microphthalmia*-associated transcription factor (MITF) is a master regulator of gene expression that plays an important role in melanocyte development. MITF is known to be regulated by phosphorylation through the Kitl/Kit signaling pathway. Work in the Steingrímsson lab has led to the discovery of several acetylation sites on MITF that serve a regulatory role, however, the mechanism through which acetylation might occurr is not yet known. To address this question, MITF was overexpressed in HEK293T cells, with and without the co-factors CBP/p300, and the cells treated with U0126 or PD98059 which are inhibitors specific to a certain component of the Kitl/Kit pathway called Mek1/Mek2. The effect on acetylation was then analyzed by immunoprecipitation and Western blotting. Results show that the expression of CBP or p300 is necessary for acetylation to occur and that the Kitl/Kit pathway is involved in the regulation of MITF acetylation. This indicates that phosphorylation of either MITF or its co-factors is a key step in MITF acetylation.

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I Introduction

1.1. Structure and function of MITF

Microphthalmia-associated Transcription Factor (MITF) is a basic helix-loop-helix zipper (bHLH-Zip) transcription factor of the myc supergene family (Hodgkinson et al. 1993). Like other bHLH-Zip proteins MITF contains a basic DNA binding domain and HLH and leucine zipper domains which are both necessary for dimerization. This dimerization is a prerequisite for DNA binding. MITF forms homodimers, as well as heterodimers with TFEB, TFEC and TFE3, and together they form a subfamily of bHLH-Zip proteins (reviewed by Moore 1995). MITF is encoded by a gene which was first discovered as a mutation that causes white coat colour and microphthalmia in mice (Hertwig 1942). Since then, numerous other *Mitf* mutant alleles have been analyzed, mostly in mice. The gene has been found to be well conserved in all studied vertebrates and some invertebrates. These studies have given important insights into the development of numerous cell types, including melanocytes, the retinal pigment epithelial cells in the eye and osteoclasts (as reviewed by Steingrímsson et al. 2004). In melanocytes, MITF functions as a master regulator of gene expression and has been shown to play an important role in all stages of development, function and survival (reviewed by Widlund and Fischer 2003). It has also been suggested that it plays a role in melanoma tumorigenesis, as MITF has been shown to be amplified in over 10% of melanomas (Garraway et al. 2005).

1.2. Kit mediated regulation of MITF in melanocytes

Because MITF serves so many different, important roles in melanocyte development, its regulation is quite complex. One of the major pathways that has a role in MITF regulation in melanocytes is the Kitl/Kit pathway. The activation of the tyrosine kinase Kit receptor by the Kit ligand (Kitl) causes Kit to dimerize and autophosphorylate, which activates a MAP kinase pathway involving Ras, Raf, Mek1/2 and finally the MAP kinases Erk2 and p90/Rsk, as well as other pathways (reviewed by Steingrímsson et al. 2004 and Hou et al. 2009) (Figure 1). The Map kinase pathway results in the phosphorylation of amino acid residues Ser73 and Ser409 by Erk2 and p90/Rsk, respectively. In turn, this attracts the co-activators CBP/p300 which in turn increases MITF's transactivation potential (reviewed by Steingrímsson et al. 2004). The Kitl/Kit pathway also leads to ubiquitination of MITF resulting in degradation of the protein and Ubc9 has been suggested as the Ubiquitin ligase (Xu et al. 2000) (Figure 2). Current knowledge of MITF phosphorylation therefore suggests that it both increases its activity and decreases its stability (reviewed by Steingrímsson et al. 2004). There is, however, some controversy regarding the exact effect of phosphorylation on MITF stability since contradicting data has been obtained when using different cell lines (Xu et al. 2000 and Wu et al. 2000). In Xu's experiments, using the human melanoma cell line IIB-Mel-J, the mutation of Ser73 into alanin increased

MITF stability by preventing Ubc9 mediated degradation. However, using 501 Mel melanoma cells, Wu et al. found that mutating either Ser73 or Ser409 was not sufficient to prevent degredation. They nevertheless found that a double mutant with both phophorylation sites mutated was significantly more stable.

Constitutive activation of this pathway is common in many types of cancers and mutations in B-RAF, a component of the Kitl/Kit pathway is present in 60% of malignant melanomas as well as in other cancer types. The majority of these mutant B-Raf proteins have increased kinase activity (Wan et al. 2004), presumably resulting in constitutive activation of MITF. It is therefore possible that examination of the exact mechanisms involved in post-translational regulation of MITF through this pathway might yield interesting new possibilities for melanoma treatment.

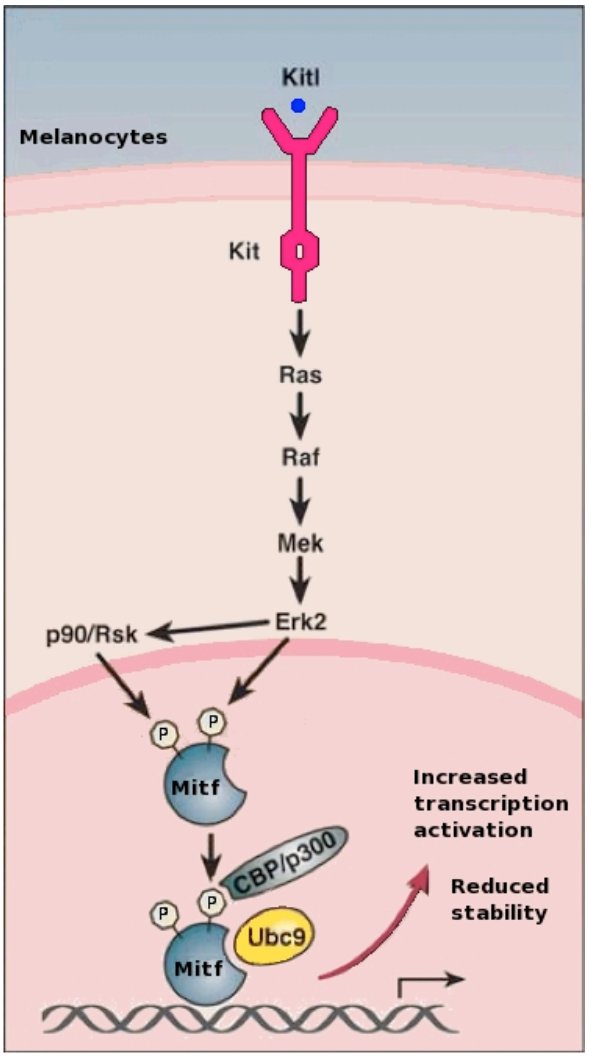


Figure 1: *A schematic figure showing the regulation of MITF through the Kitl/Kit MAP kinase pathway. (Modified from Steingrímsson et al. 2004).*

1.3. CBP/p300 and the acetylation of transcription factors

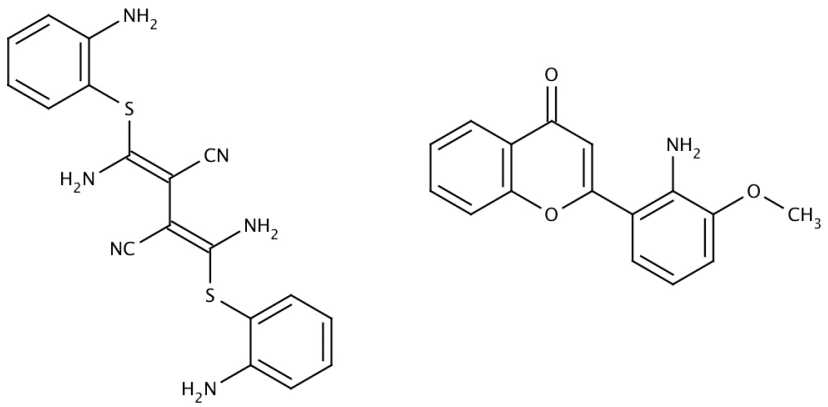
In addition to being transcription co-activatiors, CBP and p300 are histone acetyltransferases (HATs) (Ogyzko et al 1996). The two proteins are highly homologous and their function is redundant in most cases, though unique roles have been discovered (reviewed by Kalkhoven 2004). Because of the high functional overlap, CBP and p300 are often referred to as CBP/p300 where their function is interchangeable. HATs transfer acetyl groups to the amino acid lysine on the N-terminal tails of histone subunits, most commonly resulting in an increase in gene transcription due to easier access of RNA polymerase and transcription factors to genes (reviewed by Tsukiyama and Wu 2002). CBP and p300 have also been shown to acetylate non-histone transcription factors, such as TFIIEβ and TFIIF (Imhof et al. 1997), p53 (Gu and Roeder 1997) c-Myc (Vervoorts et al 2003 and Faiola et al 2005) and MyoD (Polesskaya et al 2000).

As is true for phosphorylation, the effect of acetylation on transcription factors can be quite complex. For example, Vervoorts et al found that the acetylation of c-Myc by CBP resulted in a decrease in ubiquitination and therefore an increase in c-Myc stability (Vervoorts et al 2003). However, results from Faiola et al demonstrate that CBP/p300 stabilize c-Myc independently of acetylation and that the acetylation by CBP/p300 in fact increases the c-Myc turnover (Faiola et al 2005). c-Myc is also acetylated by another HAT called mGCN5. Contrary to Faiola's results for CBP/p300, Patel et al. demonstrated that acetylation by mGCN5 increases c-Myc stability (Patel et al 2004). This demonstrates that acetylation of different sites in the same protein can have opposite effects. Another example of transcription factor regulation through acetylation is the acetylation of MyoD by CBP/p300 and PCAF (p300/CBP-associated factor). Polesskaya et al found that both PCAF and CBP/p300 acetylate MyoD *in vitro*. In this case acetylation by the HATs increases the activity of their substrate (Polesskaya et al 2000). Unpublished studies in the laboratory have shown that MITF is acetylated at certain sites and that the acetylation influences transcription activation by MITF. The transcription factors in both examples above are bHLH-ZIP proteins like MITF so it is not unlikely that CBP/p300 also may acetylate MITF.

1.4 MEK inhibitors U0126 and PD098059

Most of the cell's mechanisms are regulated through phosphorylation and dephosphorylation by a complicated network of kinases and phosphatases. It is therefore important to have ways to specifically intercept these processes at each step to study its effect on the cell. Numerous kinase inhibitors are used for this purpose to elucidate important signaling pathways. Two commonly used protein kinase inhibitors are U0126 and PD098059. Both of these inhibit the MAP kinase kinase Mek1/2, which is a downstream target of many signaling receptors, including the Kit receptor (Dudley et al. 1995 and Favata et al. 1998). Both inhibitors exert their effects by inhibiting the activation of Mek1/2 by Raf, though U0126 also inhibits the downstream activation of Erk by Mek1/2 (Davies et al. 2000). In other words, U0126 inhibits both the active and inactive form of Mek1/2 but PD98059 only inhibits the active form. U0126 also has a higher

affinity for the target, thus inhibiting more efficiently at lower concentrations (Favata et al. 1998). The chemical structures of U0126 and PD98059 are shown in Figure 2.



U0126 PD98059

***Figure 2**: Chemical formulae of Mek1/2 inhibitors U0126 and PD98059.*

1.5 The project

The aim of this project was to study the regulation of MITF acetylation through the MAP kinase signaling cascade. This was done by transiently overexpressing MITF, alone and with p300/CBP, in HEK293T cells and examining the effect of the Mek inhibitors U0126 and PD98059 on MITF acetylation. Results from these experiments show that the co-activators p300/CBP are necessary for acetylation of MITF to take place. Results also indicate that acetylation is regulated through the MAP kinase signaling cascade since inhibition by U0126 eliminated acetylation of MITF in the relevant samples. PD98059 had no effect at the concentration used.

II. Materials and methods

2.1. Amplification and purification of plasmids for transfection

E. coli strains had already been transformed with several different eukaryotic expression vectors, all based on a p3xFLAG-CMV plasmid. Four different plasmids were used, one with MITF+18-FLAG, one with CBP-HA, one with p300-HA and one empty vector. Each of these strains were cultured overnight at 37°C and 250 rpm rotation in 50 mL cultures. Plasmids were then extracted using GenElute™ HP Plasmid Midiprep Kit from Sigma Life Science following the kit instructions. Plasmids were then quantified using the NanoDrop technology.

2.2. Cell culture and transfection

The human embryonic kidney cell line HEK293T was cultured at 37°C and 5% CO_2 in DMEM medium from GIBCO with the addition of fetal bovine serum (10%), 1% Pen Strep from GIBCO (+10,000 Units/mL penicillin and +10,000 µg/mL Streptomycin) and 1x GlutaMax™-1 from GIBCO. The cells were split and grown up to about 50% confluency on 10 cm^2 culture plates before transfection with the aforementioned plasmids using TurboFect™ *in vitro* transfection reagent from Fermentas and following the appropriate instructions. In each experiment four plates of cells were transfected as described in table 1.

Table 1: Experimental setup for transfection of HEK293T cells.

Plasmid	Plate 1: Empty vector	Plate 2: MITF	Plate 3: MITF / p300	Plate 4: MITF / CBP
Empty vector [µg]	17	10	-	-
MITF+18 [µg]	-	7	7	7
p300 [µg]	-	-	10	-
CBP [µg]	-	-	-	10

2.3. Kinase inhibition and harvesting

Transfected cells were allowed to grow for about 40-48 hours. Then, different MAP kinase inhibitors were added to a concentration of 10 µM and the cultures incubated for two hours. To inhibit deacetylation, sodium butyrate was added to a concentration of 10 mM and the cultures incubated for an additional hour. Cells were then harvested by removing

the medium and adding 1 mL of 75 mM KCl lysis buffer and incubating on ice for 10 minutes before scraping cells off the plate and transferring them to an Eppendorf tube. The lysates were spun at 14,000 rpm for 5 minutes and the supernatant transferred to a new eppendorf tube. A small sample (30 µL) was taken from the lysates and boiled in 30 µL SDS/β-ME loading buffer for 5 minutes and then kept at -20°C.

The 75 mM KCl lysis buffer is composed of 50 mM Tris/HCl pH 7.4, 5 mM $MgCl_2$, 0.1% Nonidet P-40 substitute, 75 mM KCl, 1 mM DTT, 0.1 mM sodium orthovanadate, 10 mM NaF, 1 µM PMSF, 2 µg/mL Aprotinin TM from Sigma, 0.3 µg/mL Pepstatin A from Sigma Aldrich, 5 mM EDTA, 10 µM sodium butyrate.

2.4. Immunoprecipitation

A FLAG tag was used to immunoprecipitate MITF from the lysate. In order to immunoprecipitate MITF, 3 µg ANTI-FLAG® M2 Monoclonal Antibody from Sigma Aldrich were added to the lysate, followed by 30 µL protein A/G beads from Thermo Fisher Scientific. The mixture was rotated at 4°C over night. Beads were then washed three times with lysis buffer and eventually boiled in 40 µL SDS/β-ME loading buffer for 5 minutes and stored at -20°C.

2.5. SDS PAGE and Western blotting

Discontinuous SDS-PAGE with a 7% polyacrylamide resolving gel (pH 8,8) and a 5% polyacrylamide stacking gel (pH 6,8) was used to resolve the immunoprecipitated samples and lysates. The resolved proteins were transferred onto nitrocellulose membranes using wet transfer at 12 V over night and then analyzed by Western blotting. Membranes were blocked for 1 hour in blocking buffer (5% milk in PBS-T) and then incubated in primary antibody, diluted in blocking buffer, at 4°C overnight. The primary antibody for acetylated lysine was a combination of Acetylated Lysine (Ac-K-103) Mouse mAb from Cell Signaling Technology and AB387q Rb x acK from Chemicon at a 1:500 dilution each. The primary antibody for the FLAG tag was ANTI-FLAG® M2 monoclonal antibody from Sigma. Membranes were then washed three times in PBS-T and incubated in secondary antibody (Protein A/G beads from Thermo Fisher Scientific) diluted 1:10,000 in blocking buffer for 1 hour. Membranes were then washed again and proteins detected using the ECL detection system from Thermo Fisher Scientific.

III. Results

The aim of this project was to determine if phosphorylation through the Kitl/Kit pathway influences MITF acetylation. To do this MITF, alone and with p300 or CBP, was transiently overexpressed in HEK293T cells and the effects of different MAP kinase inhibitors on acetylation examined. Because of time restraints, only two inhibitors were tested; U0126 and PD98059. Both of these are specific to the same step in the Kitl/Kit pathway (Mek1/2), but have slightly different properties as discussed in chapter 1.3. U0126 has a higher affinity to the kinase and works on both the active and inactive forms of Mek 1 and Mek 2. PD98059 only works on the active form (Favata et al. 1995).

Figure 3 shows the results of these experiments. MITF was detected by Western blotting with an anti-FLAG antibody (FLAG) and an anti-Acetyl Lysine antibody (AcK) was used to detect acetylation of MITF. In the control experiment (Figure 3A), cells were treated with DMSO, the solvent in which the inhibitors were dissolved. In these blots, the amount of protein was adjusted accordingly before loading. As expected, acetylation was detected in those samples which contained MITF with p300 or CBP but not in those which contained only MITF. This indicates that the presence of at least one of these co-activators is necessary for acetylation to occur. In the U0126 experiment (Figure 3B), a 10 µM concentration of U0126 eliminated acetylation of MITF. These results strongly suggest that phosphorylation of either MITF or its co-activators through Mek1 or Mek2 is necessary for MITF acetylation. On the other hand, the same concentration of PD98059 had no measurable effect on MITF acetylation under the same conditions (Figure 3C). Protein amount was not normalized for experiments B and C.

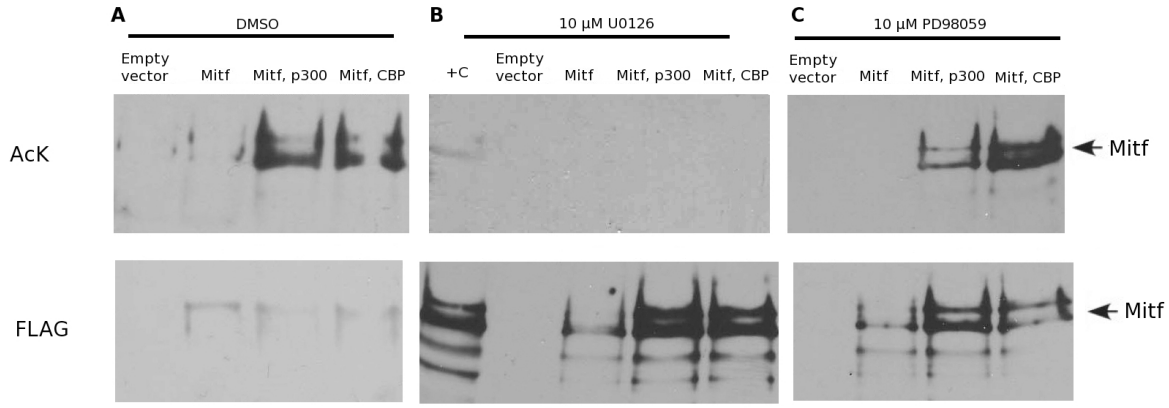


Figure 3: *The effects of selected MAP kinase inhibitors on MITF acetylation as detected by Western blotting. Western blots of FLAG immunoprecipitates from three different experiments. Cells were transfected with empty vector, MITF, MITF + p300 and MITF + CBP respectively and treated with DMSO, U0126 or PD98059. (A) Control cells, treated with DMSO. MITF was detected with anti-FLAG antibody (FLAG) and acetylation of MITF was only detected when co-transfected with CBP/p300 (AcK). (B) Cells treated with 10 µM U0126, a general Mek inhibitor. No acetylation was observed (AcK). Presence of MITF was confirmed with anti-FLAG antibody and the integrity of the anti-Acetyl Lysine antibody seems to be intact as confirmed by a positive control (+C) from experiment A. (C) Cells treated with 10 µM PD98059, a Mek2 specific inhibitor. Acetylation was observed when the cells were co-transfected with both MITF and CBP/p300 despite inhibitor treatment (AcK).*

IV. Discussion

4.1. The co-activators CBP or/and p300 are necessary for MITF acetylation

The results of the experiments performed show that either CBP or p300 need to be expressed alongside MITF for acetylation to take place (Figure 3A). It is well established that these co-activators have a role in MITF activation (Sato et al. 1997) and that they are capable of mediating the acetylation of general transcription factors (Imhof et al. 1997). It is therefore likely that CBP and p300 have a role in mediating the acetylation of MITF in connection with a Map kinase pathway.

4.2. Inhibition with U0126 inhibits MITF acetylation in HEK293T cells

As shown in Figure 3B, inhibition of Mek by U0126 eliminates acetylation of MITF in HEK293T cells that overexpress MITF and either p300 or CBP. This supports the hypothesis that phosphorylation is necessary for MITF to be acetylated and that Mek is a part of the pathway through which the phosphorylation takes place. There are a few different ways in which phosphorylation might affect MITF acetylation. Since MITF is known to be phosphorylated through the Kitl/Kit pathway, one possible model is that MITF itself is phosphorylated, giving a signal for acetylation. However, data from unpublished research by Dr. Alexander Schepsky suggests that this is not the case as a double mutant with both phosphorylation sites, Ser73 and Ser409, mutated to alanin can still be acetylated. Another possible model is that the co-activators, p300 and CBP, are the target for phosphorylation. This could stimulate their acetyltransferase activity, causing them to acetylate MITF. Two experiments need to be performed to validate this hypothesis. First, p300 and CBP need to be checked for phosphorylation. Second, phosphorylation sites on both co-factors need to be mutated and the effect on MITF acetylation needs to be examined. If p300/CBP are phosphorylated and mutations of their phosphorylation sites inhibits MITF phosphorylation, it is likely that p300/CBP are the acetyltransferases responsible for MITF acetylation.

4.3. Inhibition with PD98059 does not affect MITF acetylation in HEK293T cells

Since PD98059 affects the same step in the Kitl/Kit MAP kinase pathway as U0126 it might come as a surprise that their effects on MITF acetylation are not the same. Both inhibitors affect Mek1/2 but their affinities to Mek1/2 are different and the mechanisms of inhibition are not exactly the same as was discussed in chapter 1.3. U0126 not only blocks the activation of Mek1/2, inhibiting the activation of the downstream target called Erk

indirectly, it also directly inhibits the activation of Erk by Mek1/2. Thus, there are several possible explanations for this difference in effects on MITF acetylation. One explanation is that a higher concentration may be needed due to the affinity difference. Another explanation is that the difference in the inhibition mechanism allows for an alternative pathway to still be intact despite PD98059 inhibition, through which acetylation may be induced. A third possibility is that the inhibitor was not working properly in these experiments. To test the integrity of the inhibitors, the lysates from immunoprecipitations were checked for Erk, in it's phosphorylated and unphosphorylated forms. If Mek1/2 was being inhibited, only unphosphorylated Erk would be detected. This proved unsuccessful, probably due to problems with the antibodies used. This experiment therefore needs to be reproduced with new antibodies.

4.4. Next steps

Apart from reproducing the entire experiment for verification, the immediate follow up to these experiments would be to reproduce the Western blot of the lysates using new Erk antibodies to verify that the MAP kinase cascade is indeed inhibited. If PD98059 is not inhibiting Erk phosphorylation at this concentration, different concentrations could be tested. As discussed in chapter 4.2, CBP/p300 phosphorylation should be checked and the influence of mutating their phosphorylation sites on MITF acetylation should be tested. This would give important information regarding CBP/p300's involvement in MITF acetylation.
To further define the signaling pathway controlling MITF acetylation it would be interesting to test inhibitors for other components of the cascade, such as the B-RAF inhibitor NVP-RAF265 and the p38 inhibitor SB203580. To verify that the expressed proteins are working normally, immunohistochemistry experiments could be performed to check the entry of the expressed proteins into the nucleus. Finally it would be interesting to try the same experimental setup on different cell types, for example, melanoma cells like 501 Mel, and see if it yields the same results.

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