The role of MITF in regulating human pigmentation

Christian Praetorius

Thesis for the degree of Philosophiae Doctor

August 2014

UNIVERSITY OF ICELAND
SCHOOL OF HEALTH SCIENCES

FACULTY OF MEDICINE

Hlutverk MITF við stjórnun húðlitar í mönnum

Christian Praetorius

Ritgerð til doktorsgráðu

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Document typeset with LaTeX.

ISBN: 978-9935-9200-2-7

Printing by Háskolaprent
Reykjavík, Iceland 2014

Ágrip

Þroskun litfruma og myndun sortuæxla er háð umritunarþættinum microphthalmia associated transcription factor (MITF) en hann tilheyrir basic-helix-loop-helix leucine zipper (bHLH-LZ) fjölskyldunni. MITF stjórnar tjáningu fjölda gena í litfrumum og sortuæxlum, þar með talið gena sem eru mikilvæg fyrir framleiðslu litarefnisins melanin, og gena sem stjórna frumuhringnum, frumufari og lifun. Þessi þekktu markgen skýra þó ekki að fullu hlutverk MITF í litfrumum og sortuæxlum.

Við notuðum tvískipta tjáningarrannsókn til að finna markgen MITF í sortuæxlum. Í fyrsta lagi leituðum við að genum sem fylgja tjáningarmynstri MITF gensins sortuæxlissýnum. Í öðru lagi skoðuðum við hvaða gen eru tjáð þegar MITF er yfirtjáð í sortuæxlisfrumulínunni Skmel28. Af þeim genum sem voru á báðum listunum var fjöldi þekktra markgena MITF en einnig nokkur gen sem ekki hefur verið sýnt að stjórnist af MITF svo sem umritunarþættirnir IRF4 og TFAP2alpha. Hlutverk þeirra var skoðað frekar.

Erfðamengisrannsóknir hafa sýnt að breytileiki í *IRF4* geninu í mönnum tengist freknum, ljósri húð, brúnu hári og bláum augum. IRF4 genið gegnir mikilvægu hlutverki í ónæmiskerfinu og hefur verið kallað "lineage survival oncogene" í mergfrumuæxlum. Ekki hefur áður verið sýnt hvaða hlutverki það gegnir í litfrumum. Hér sýnum við að tjáning IRF4 gensins er háð MITF. Tjáning *Irf4* gensins er næstum engin í *Mitf* stökkbreyttum músum eða þegar *MITF* genið er slegið út í sortuæxlisfrumum í rækt með notkun shRNA.

MITF stjórnar tjáningu *IRF4* gensins með því að bindast stjórnröðum í innröð 4 í *IRF4*, og er þessi örvun umritunar einnig háð umritunarþættinum TFAP2A. Breytileikinn sem tengist litareinkennum í mönnum (rs12203592-T) skarast við bindiset TFAP2A próteinsins og þegar upprunalega C-basanum er breytt í T getur TFAP2A ekki bundist lengur og tjáning *IRF4* minnkar, bæði í *in vitro* tilraunum og í litfrumum úr einstaklingum sem eru arfhreinir fyrir T samsætuna. Tilraunir sýna að MITF og IRF4 vinna saman að því að virkja tjáningu litargensins *TYR* og að þegar IRF4 bindisetum í stjórnröð MITF er stökkbreytt getur þessi samvinna ekki átt sér stað lengur. Rannsókn þessi sýnir því bein tengsl milli breytileika í innröð *IRF4* gensins og áhrifa á tjáningu ensíms sem ákvarðar litarhátt manna.

Lykilorð: MITF, IRF4, genatjáning, litarháttur, freknur

Abstract

The development of melanocytes and melanoma depends on the presence of the microphthalmia-associated transcription factor (MITF), a member of the basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor family. MITF has been shown to regulate a broad range of genes, ranging from genes important for pigment production, to genes involved in cell cycle regulation, migration and survival. Nevertheless, the known MITF target genes do not explain all the roles of MITF in melanocyte development and melanoma progression.

We have used a novel microarray approach to characterize MITF target genes in melanomas. First we identified genes which correlate with the expression of MITF in human melanoma samples. Second, we compared the genes expressed in a stably MITF-transfected line of SKmel28 cells with the genes expressed in SKmel28 cells without MITF. Third, we compared the two lists of genes to obtain a list of genes potentially regulated by MITF. A number of the genes thus identified are known MITF target genes, whereas the rest represent novel targets. Among the novel targets are two transcription factors, *IRF4* and *TFAP2α* which were analyzed further.

Genome-wide association studies have shown that a polymorphism in the human *IRF4* gene is associated with fair skin and light/blue eye color, suggesting that the gene plays an important role in melanocytes. IRF4 has been shown to play a key role in lymphoid, myeloid and dendritic cell differentiation and to act as a lineage survival oncogene in multiple myeloma. However, its role in melanocytes has not been characterized. Here we show that expression of the IRF4 gene is dramatically reduced in Mitf mutant mice and upon MITF knockdown using shRNA.

MITF activates IRF4 expression by binding regulatory sequences in intron 4 of the *IRF4* gene and depends on another transcription factor, TFAP2α for this activation. The binding site for TFAP2α is located at the same position as the rs12203592 polymorphism and when changed from C to T (as in rs12203592), TFAP2α can not bind and IRF4 expression is reduced in both reporter assays and in melanocytes from individuals homozygous for the T allele. Importantly, IRF4 and MITF cooperatively activate the expression of the pigmentation gene *TYR*; mutating the IRF4 binding sites in *TYR* eliminates this cooperative effect. Thus, a direct link has been provided where a functional polymorphism in the *IRF4* gene leads to reduced expression of a target gene encoding an enzyme essential for pigmentation.

Keywords: MITF, IRF4, gene expression, pigmentation, freckles

Acknowledgements

The research for this thesis was carried out at the Department of Biochemistry and Molecular Biology at the Faculty of Medicine of the University of Iceland. Many people have supported me directly or indirectly and I would like to thank all of them for their kindness and support.

First of all I would like to thank my supervisor Professor Eiríkur Steingrímsson for the possibility to work in his laboratory, his scientific guidance, critical and lively discussion and for the support throughout all these years, it was a great time.

I also like to thank all colleagues from the Steingrímsson Lab for their help, support and also for the critical discussions of results in the lab meetings: Kristín Bergsteindóttir, Christine Grill, Benedikta S. Hafliðadóttir, Erna Magnúsdóttir, Margrét H. Ögmundsdóttir and Alexander Schepsky. I wish to acknowledge the help and support from the other colleagues from the 5th floor at Læknagarður: Jónína Jóhannsdóttir, Sif Jónsdóttir, Guðrun Valdimarsdóttir, Pétur Henry Petersen, Stefán Þ. Sigurðsson and Hans Guttormor Þormar.

I would like to thank my PhD committee, Prof. Ólafur S. Andrésson, Prof. Zophonías O. Jónsson, Prof. Magnús Karl Magnússon and Prof. Hendrik G. Stunnenberg for their opinions on my thesis. I also thank the evaluating committee Prof. Helga Ögmundsdóttir, Prof. Zophonías O. Jónsson and Prof. Robert Kelsh for their work.

Finally I would like to thank my family, Maren, Sophie and Lisa for their patience and support, without them it would have never been possible to finish this thesis.

This work was supported by grants from the Icelandic Research Fund, the University of Iceland Research Fund and the Eimskip University Fund for doctoral studies.

Contents

List of Abbreviations

α-MSH	melanocyte stimulating hormone
aa	amino acids
ACTH	adrenocorticotropic hormone
AD	activation domain
APEX-1	APEX nuclease (multifunctional DNA repair enzyme) 1
ATP	adenosin triphosphate
BAC	bacterial artificial chromosome
bHLH-Zip	basic helix loop helix leucine zipper
cAMP	cyclic AMP
CD	cysteinyldopa
CDK2	cyclin-dependent kinase 2
CDKN2A	cyclin dependent kinase inhibitor 2A
cDNA	coding DNA
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP-sequencing
CRE	cyclic AMP response element
CREB	cAMP response element binding protein
DCT	dopachrome tautomerase
DIAPH1	diaphanous-related formin 1
DITC	dimethyl traizeno imidazole carboxamide
DNA	sesoxyribo nucleic acid
DQ	dopaquinone
E-Box	transcription factor recognition motif with the 5'-CATGTG-3' or the 5'-CAGATG-3' sequence
E-box	E-box, DNA binding motif for transcription factors
EM	electon microscopy
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
FoxD3	forkhead-box transcription factor D3
GC	germinal center

GPCR . . . . . G-protein-coupled receptor
GST . . . . . glutathion S-transferase
GWAS . . . . . genome wide association study

HAT . . . . . histone acyl transferase
HGF . . . . . hepatocyte growth factor

IFN . . . . . interferon
IL-2 . . . . . interleukin 2
ISRE . . . . . interferon stimulated response element

JAK . . . . . janus kinase
JAK/STAT . . . . . janus kinase/signal transducer and activator of transcription

kb . . . . . kilobases DNA

LEF-1 . . . . . lymphoid-enhancing factor 1

M-Box . . . . . transcription factor recognition motif with the 5'-TCATGTGCT-3' sequence
MAPK . . . . . mitogen activated protein kinase
MC1R . . . . . melanocortin 1 receptor
miRNA . . . . . micro RNA
MITF . . . . . microphthalmia-associated transcription factor
MSC . . . . . melanocyte stem cells

NCC . . . . . neural crest cells

OCA . . . . . oculocutaneous albinism
OR . . . . . odds ratio

PARP . . . . . poly ADP ribose polymerase
PAX3 . . . . . paired box 3 transcription factor
PCR . . . . . polymerase chain reaction
PDGFR . . . . . platelet-derived growth factor receptor
PFS . . . . . progression free survival
PG . . . . . pigment globules
PI3K phospho-inositol-3-kinase
PIAS3 . . . . . protein inhibitor of activated STAT3
PKA . . . . . protein kinase A

qPCR . . . . . quantitative PCR

RNA . . . . . ribonucleic acid
RPE . . . . . retinal pigment epithelial cells

RSAT . . . . . regulatory sequence analysis tool
RTK . . . . . receptor tyrosine kinase

SCF . . . . . stem cell factor
shRNA . . . . . short hairpin RNA
SL . . . . . solar lentigines
SNP . . . . . single nucleotide polymorphism
SOX10 . . . . . sry related HMG box 10 transcription factor
STAT3 . . . . . signal transducer and activator of transcription 3
SUMO . . . . . small ubiquitin-like modifier

TBX2 . . . . . T-box 2
TESS . . . . . transcription element search system
TF . . . . . transcription factor
TFAP2α . . . . . transcription factor activating protein 2α
TGN . . . . . trans-golgi network
TRAIL . . . . . TNF-related apoptosis-inducing ligand
TSS . . . . . transcription start site
TYR . . . . . tyrosinase
TYRP1 . . . . . tyrosinase related protein 1

UVR . . . . . ultraviolet radiation

WS2 . . . . . waardenburg syndrome type 2

List of Figures

List of Tables

List of Original Publications

This thesis is based on the following original publications:

Hoek, Keith S., Natalie C. Schlegel, Ossia M. Eichhoff, Daniel S. Widmer, Christian Praetorius, Steingrimur O. Einarsson, Sigridur Valgeirsdottir, Kristin Bergsteinsdottir, Alexander Schepsky, Reinhard Dummer, and Eirikur Steingrimsson (2008). **Novel MITF targets identified using a two-step DNA microarray strategy**. *Pigment Cell Melanoma Res* 21.6, pp. 665–76.

Praetorius, Christian, Christine Grill, Simon N. Stacey, Alexander M. Metcalf, David U. Gorkin, Kathleen C. Robinson, Eric Van Otterloo, Reuben S. Q. Kim, Kristin Bergsteinsdottir, Margret H Ogmundsdottir, Erna Magnusdottir, Pravin J. Mishra, Sean R. Davis, Theresa Guo, M. Raza Zaidi, Agnar S Helgason, Martin I. Sigurdsson, Paul S. Meltzer, Glenn Merlino, Valerie Petit, Lionel Larue, Stacie K Loftus, David R Adams, Ulduz Sobhiafshar, N. C. Tolga Emre, William J. Pavan, Robert Cornell, Aaron G. Smith, Andrew S. McCallion, David E. Fisher, Kari Stefansson, Richard A. Sturm, and Eirikur Steingrimsson (2013). **A Polymorphism in IRF4 Affects Human Pigmentation through a Tyrosinase-Dependent MITF/TFAP2A Pathway**. *Cell* 155.5, pp. 1022–1033.

Praetorius, Christian, Richard A. Sturm, and Eirikur Steingrimsson (2014). **Sun-induced freckling: ephelides and solar lentigines**. *Pigment Cell Melanoma Res* 27.3, pp. 339–50.

Declaration of contribution

Paper I

This article describes the analysis of gene expression in SKmel28 cells after transfection of MITF. I did the cell culture work, transfected the cells, isolated the total RNA and analyzed the RNA quality. I analyzed the data and was also involved in the preparation of the manuscript.

Paper II

The article describes the regulation of pigmentation by MITF and IRF4 and the regulation of the IRF4 expression by MITF and TFAP2α. I identified *IRF4* as an interesting target of MITF.

I analyzed the expression of *MITF* in mouse tissues and did over-expression and knock-down experiments to verify that MITF regulates *IRF4* and *TFAP2α*. To check for binding of the transcription factors to the predicted binding sites I did chromatin immunoprecipitations and also EMSA assays. I analyzed the DNA sequence to identify potential transcription factor binding sites and amplified and cloned the relevant DNA into a reporter vector. Afterwards I did the gene activation assays, to verify the potential transcription factor binding sites. For the publication I prepared all figures and participated in writing the manuscript together with my advisor.

Paper III

This article is a systematic review of the literature on ephelides and solar lentigines. For the article I did the research for references and participated in writing the article together with the others authors. I also prepared the illustrations for the article.

1. Introduction

This thesis presents studies on the Microphthalmia-associated transcription factor (MITF). MITF is an important regulator of different cellular processes including differentiation, proliferation, cell survival and apoptosis in many different cell types including melanocytes, melanoma cells and the retinal pigment epithelial cells (RPE) of the eye. This transcription factor regulates a large number of genes and cellular processes, which are both important for development, normal cell function and for the malignancy of the pigment cells. Understanding the process of expression and regulation can help to understand the processes of the melanocyte cell and also to understand how these cells develop into melanoma.

1.1. Pigmentation

Pigmentation of the human body is produced by specialized cell types, melanocytes in the hair and skin and by choroidal melanocytes and RPE cells in the eye. The synthesis of the melanin pigment itself takes place in the melanosome, a specialized organelle of the pigment cell, which is derived from the lysosome (Dell'Angelica, 2003; Schiaffino, 2010; Wasmeier et al., 2008).

1.1.1. Melanocytes

Melanocytes develop from neural crest derived pre-cursor cells originating from the embryonic ectoderm. During neurulation a group of cells undergoes an epithelial-mesenchymal transition and migrate out of the neuroepithelium. These neural crest cells (NCC), which migrate to many locations in the embryo, are multipotent in the beginning but become lineage-restricted during further differentiation (Betters et al., 2010; Ernfors, 2010).

Neural crest cells are divided by their regional distribution into four groups: Cranial, sacral, trunk and vagal, with melanoblasts mostly originating from cranial and trunk-derived NCC (Le Douarin et al., 2004). During their migration, melanoblasts multiply in number and sequentially start to express melanogenic genes. The melanocytes are located in the basal layer of the epidermis where each of them is surrounded by 30–40 keratinocytes and together form the epidermal melanin unit (Fitzpatrick and Breathnach, 1963; Jimbow et al., 1976). For details see Figure 1.1.

Melanocytes in the epidermis communicate with surrounding keratinocytes and dermal fibroblasts via secreted factors and also by cell–cell contacts. The

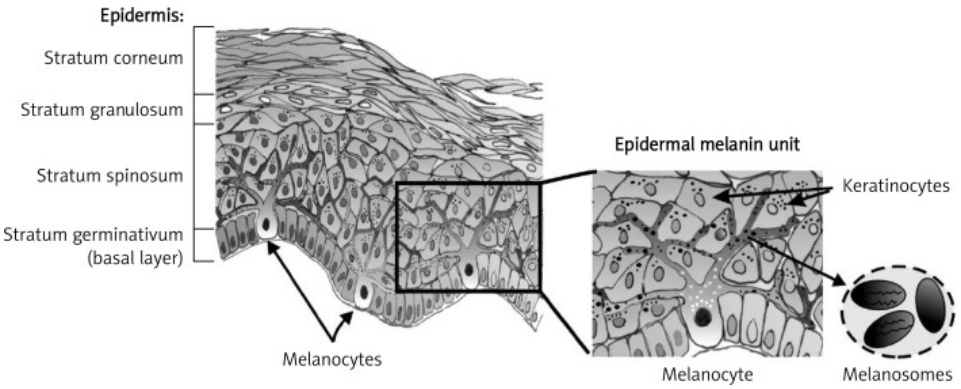


Figure 1.1.: Scheme of the epidermis structure. Melanocyte reside between the basal layer cells and through dendritic processes they communicate with about 30-40 keratinocytes in the epidermal melanin unit. Figure cited from Cichorek et al. (2013)

melanocyte itself is controlled in growth, activity and shape through paracrine hormones and cell adhesion molecules. The keratinocyte secretes α-MSH (melanocyte stimulating hormone) when the cell is exposed to UV-radiation. The hormone will then stimulate the melanocortin 1 receptor (MC1R) on the surface of the melanocyte and stimulate the synthesis of melanin (Haass and Herlyn, 2005; Yamaguchi et al., 2007).

1.1.2. Melanosome biology

Melanosomes mature in four steps from immature vesicles to fully mature and pigmented organelles. They begin as stage I pre-melanosomes which bud from the endoplasmatic reticulum (ER) (Park et al., 2009) and show a round form with no observable inner structure (Harper et al., 2008).

The trans-golgi network (TGN) trafficks structural (PMEL17/GP100) and melanosomal (TYR) proteins to the empty vesicles to form stage II melanosomes. At this stage the melanosomes get elongated and build an organized fibrillar network based on PMEL17/GP100 proteins (Theos et al., 2005). At this point the path between eumelanosomes and pheomelanosomes is parting, as pheomelanosomes do not develop further than stage II. TYR is a key enzyme for the pigmentation process, as both melanin types need it for the first step of the synthesis.

Only eumelanosomes receive TYRP1 and DCT (needed for the later stages of eumelanin synthesis) through the TGN and develop to stage III melanosomes. At this stage pigment is being produced and deposited along the matrix protein fibrils. At stage IV the pigment fills the complete melanosome (Hearing, 2005

and 2011; Schiaffino, 2010).

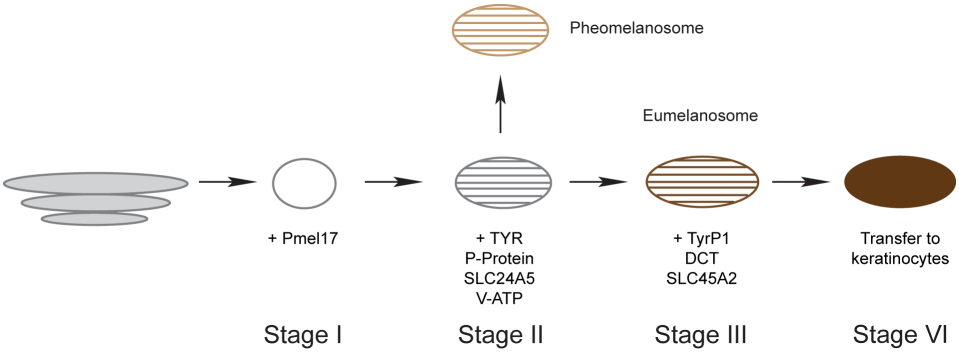


Figure 1.2.: Maturation of Melanosomes from ER to stage IV mature melanosomes.

Other important proteins are the 12-transmembrane P-protein and MATP (also known as SLC45A2) which are thought to be involved in the transport of proteins from the ER to the melanosome. Misrouting of TYR has been reported, when MATP is mutated, as well disruptions of the transport of DCT and TYRP1, thus explaining pigmentation phenotypes. Additionally, both of them are also putative proton exchangers (Smith et al., 2004).

Another factor which is important for melanin synthesis is intra-melanosomal pH. The enzymatic activity of TYR is higher when the environment is neutral than in an acidic pH. This observation led to the hypothesis that the intra-melanosomal pH is a key in the decision if a melanosome becomes eu- or pheomelanocytic (Ancans et al., 2001; Fuller et al., 2001). This idea is supported by the observation that cultured melanocytes from dark skin contain neutral melanosomes, while the cultured melanocytes from white skin are acidic (Smith et al., 2004; Watabe et al., 2004). The number of melanosomes and amount of enzyme seems to be similar in dark and white skin, but the former present a tenfold higher enzymatic activity (Iozumi et al., 1993).

The melanosomes are moved from lysosomes of the melanocytes towards the plasma membrane along microtubules using dynein proteins (Hirokawa and Noda, 2008), while they build up their melanin content. The intracellular transport is mediated by dynein and kinesin molecules on the surface of the melanosomes (Vancoillie et al., 2000; Watabe et al., 2008). Mature melanosomes loose their tyrosinase activity and are finally transported into the surrounding keratinocytes (Schallreuter et al., 1998). To explain the transport into the keratinocytes, Ando et al. (2011 and 2012) have developed a model which says that:

- Pigment globules (PG) are formed at the end of the melanocytic dendrites from multiple melanosomes and mitochondria,

- PG are then released from the melanocyte into the extracellular space,
- microvilli from the keratinocytes bind the PG,
- bound PG are incorporated into the keratinocytes in a protease-activated receptor-2 (PAR-2) dependent way,
- the membrane surrounding the PG is degraded and single melanosomes are released into the cytosol of the keratinocyte.

Besides a role in attraction (for example for colored birds) or camouflage, pigment has an important protective role (at least for humans) against UV radiation. Darker skin (more pigment) protects against the effects of the sun. Individuals with less pigmentation have a greater risk of developing melanoma. In the keratinocytes the melanosomes form a supranuclear cap around the nucleus and thus protect it from energy-rich UV radiation (Kobayashi et al., 1998).

1.1.3. Melanocytes in the hair follicle

Besides their location in the skin, melanocytes are also found in the bulb of each hair. The ratio between melanocytes and keratinocytes in the hair is 1:5 and thus more dense than in the epidermis where it is 1:30–1:40 (Jimbow et al., 1976; Slominski et al., 2005). The process of hair pigmentation is the result of the interaction of follicular melanocytes which produce the pigment, the transfer of the pigment into the keratinocytes and the formation of a pigmented hair. It is assumed that the transport of the melanin into the keratinocytes in the growing hair shaft functions in a similar way as in the skin and is mediated by the PAR-2 receptor. The melanosomes and the pigment cells in the hair bulge are bigger than the ones in the epidermis (Commo and Bernard, 2000; Slominski et al., 2005; Tobin, 2011). The differences in hair color can be explained by the quantity and the ratio of yellow-red pheomelanin and black-brown eumelanin (Ito and Wakamatsu, 2011).

The synthesis of melanin in the hair is similar to the epidermis and under the control of signalling molecules and hormones secreted by the surrounding cells, namely keratinocytes, endothelial cells and fibroblasts. While the epidermal melanocytes are long-lived cells, the melanocytes of the hair follicle die at the end of each hair cycle, which lasts 3–8 years. The process of melanogenesis is only active during the growth phase of the hair (anagen stage). Pigment production is turned off during the regression phase (catagen stage) and the differentiated melanocytes in the hair bulb go into apoptosis. Melanogenesis is completely absent in the resting phase (telogen stage). The melanocytes in the hair bulb are replenished from the melanocyte stem cell pool in the hair bulge. (Commo and Bernard, 2000; Nishimura, 2011; Tobin, 2011).

1.1.4. Synthesis of pigment

The pigmentation of hair, eye and skin in mammals is generated by the melanocytes and caused by two different bio-polymers: Eumelanin (dark, brown–black pigmentation, highly polymerized) and pheomelanin (light, red–yellow pigmentation, less polymerized). The ratio of the two melanins determines the appearance of different pigmentation phenotypes from yellow to almost black. Pigmentation is regulated by MITF, as many of the enzymes of key steps in the melanin bio-synthesis as well as later steps in the processing and transport of the pigment are under the control of MITF.

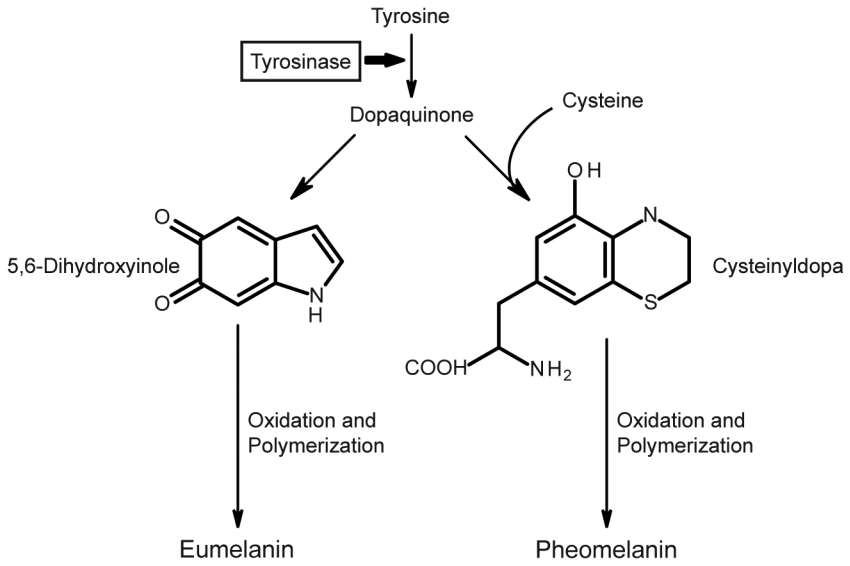


Figure 1.3.: Biosynthesis of eumelanin and pheomelanin.

In brief, the amino acid Tyrosine is converted by the enzyme Tyrosinase (regulated by MITF) to dopaquinone (DQ). This initial step of the melanin synthesis is also the rate limiting step, since both melanins need DQ for the further synthesis (Land et al., 2003). Mutations in the *TYR* gene can cause pigmentary disorders like type I oculocutaneous albinism (Oetting and King, 1994). The following reaction steps depend on whether eu- or pheomelanin is made.

For eumelanin, DQ is further processed into 5,6-indolquinone which is then oxidized and polymerized into eumelanin. The pheomelanin synthesis is different, since DQ first reacts with the amino acid cysteine to form cysteinyldopa (CD), which is then further oxidized and finally polymerized into pheomelanin (the process has been reviewed in detail by Kondo and Hearing (2011)). A schematic overview of the process can be found in Figure 1.3.

The enzymes TYR, tyrosinase related protein 1 (TYRP1) and dopachrome tautomerase (DCT) are involved in the synthesis of eumelanin whereas for pheomelanin only TYR and cysteine are necessary. The total amount of pigment depends on DQ production (which is dependent on TYR activity). The ratio between eu- and pheomelanin is dependent on three conditions: The production of the CD isomers, which is only possible when the cysteine concentration is above 0.13 µM. The next step is the oxidation of CD to pheomelanin, for which the CD concentration has to be higher than 9 µM. Eumelanin is only synthesized when the cysteine concentration is below 0,76 µM. Otherwise the synthesis is pushed by cysteinyldopa in the direction of pheomelanin (Ito and Wakamatsu, 2008; Land et al., 2003).

1.2. The microphthalmia associated transcription factor

The story of the MITF transcription factor began with the discovery of mice carrying a mutation in this gene. The first mutation in *Mitf* was discovered by Paula Hertwig in 1942 when she analyzed the offspring of mice which had been irradiated with X-rays (Hertwig, 1942). Some of these mice were completely white, with small eyes. Analysis of these mice later showed defects in a number of different cell types including melanocytes and RPE cells (the pigment cells of the eye) as well as osteoclasts and mast cells. The gene which causes the phenotype was not identified until 1993 by Hodgkinson et al. (1993).

1.3. Mutations in the *MITF* gene

Mutations in the *MITF* gene have proven very useful to study the function of the gene. These mutations cause a variety of phenotypes from altered (lighter) pigmentation to severe symptoms such as microphthalmia, complete loss of pigment, loss of hearing and bone resorption problems (in mice). In humans, mutations in *MITF* gene can lead to Waardenburg Syndrome type 2 (WS2), Tietz Syndrome (Grill et al., 2013; Takeda et al., 2000b) or melanoma (Bertolotto et al., 2011; Cronin et al., 2009).

While WS2 only affects the MITF-M isoform, the Tietz syndrome can be caused by mutations in all isoforms of MITF. The phenotype of both diseases is similar. They are characterized by sensorineural deafness and hypopigmentation of the hair, skin and iris. The Waardenburg syndrome is quite rare, it has been reported in Caucasian and black individuals, with an estimated incidence of 1:42.000 in the general population and from 1 to 2 % in the congenitally deaf (Konno and Silm, 2001).

Mutations in the *MITF* gene affect mostly conserved (functional) regions of the gene. Mutations have been reported to affect the activation domains,

the basic domain, the helix-loop-helix region and the zipper domain (for an extensive overview including references see Table 1 in Grill et al. (2013)). These mutations are connected to different diseases or phenotypes including Waardenburg Syndrome Type 2, Tietz Syndrome and melanoma. Interestingly the mutations connected to melanoma have so far only been identified in the activation domains of MITF (with one exception in the HLH-domain), while the mutations responsible for Tietz and Waardenburg Syndrome are found in the basic domain, the HLH and the zip-regions (Grill et al., 2013). The mutations in both syndromes hinder MITF either from binding DNA (for the mutations in the basic domain) or in forming a dimer (mutations in the HLH- and zip-regions). The mutations in the activation domains of MITF change the transactivation ability of the factor and thus influence the way how genes are activated or silenced (Grill et al., 2013).

Severe phenotypes arise from mutations which affect regulatory regions of the gene. Experiments in mice have revealed a number of different mutations which lead to different phenotypes. This is the case for $MITF^{mi-vga9}$, (Hodgkinson et al., 1993; Tachibana et al., 1992) or $MITF^{mi-tg}$ (Krakowsky et al., 1993). The $MITF^{mi}$ mutation, caused by the deletion of amino acid 217 (the so called ΔR217 deletion) causes mice to die at three weeks of age due to osteopetrosis and also affects melanocytes and RPE cells. Point mutations like I212N or H209R both cause severe phenotypes including white coat and microphthalmia (Hansdottir et al., 2004; Packer, 1967). Premature stop codons like in the R263stop mutation causes a white coat, pale eyes and hearing defects (Zimring et al., 1996). For an extensive review on these mutations see Steingrimsson et al. (2004).

The role of MITF in melanoma is important, since it acts as a lineage survival oncogene (Garraway et al., 2005; McGill et al., 2002). Although the factor is essential for the development of melanoma, mutations can only be found in about 10–20 % of the cases (Garraway et al., 2005), most of them are amplifications. Until very recently only acquired mutations were known, mostly inside the activation domains of the protein (Grill et al., 2013). The first known germline mutation of MITF connected to melanoma was the E318K mutation, which in-activates a SUMOylation site in the protein (Bertolotto et al., 2011; Yokoyama et al., 2011). The occurrence of this mutation leads to a genetic predisposition to melanoma in the affected families.

1.4. Mitf protein structure

MITF is a member of the basic-helix-loop-helix leucine zipper (bHLH-Zip) transcription factors and belongs to the myc superfamily of transcription factors (Ledent et al., 2002). It is a member of the *MITF-TFE* gene family which also contains *TFE3, TFEB* and *TFEC* (Hemesath et al., 1994).

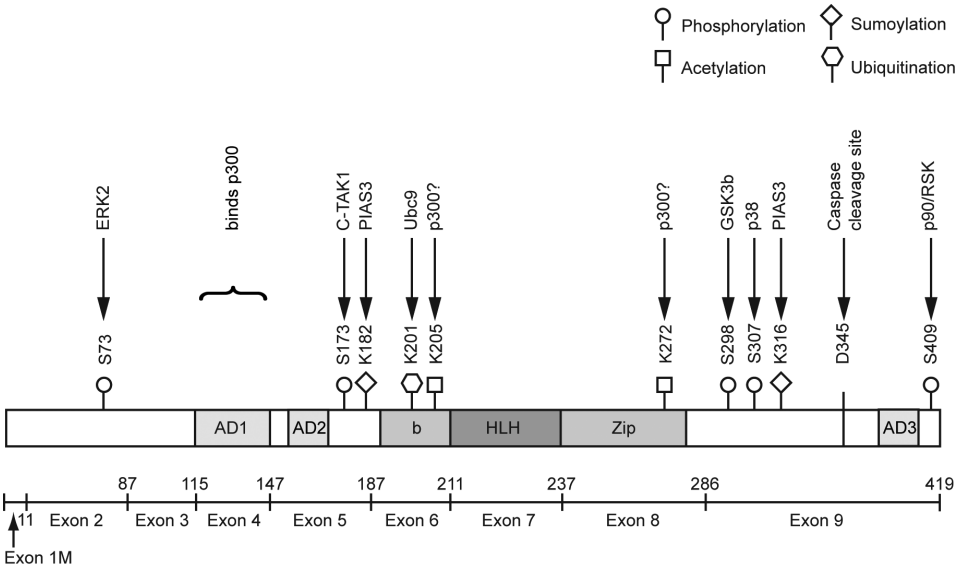


Figure 1.4.: Structure of the domains of MITF. The Figure shows the MITF-M form and the different structural domains of the protein. Amino acids which are modified post-translationally and the signal transduction pathways responsible for the modification are indicated.

This group of transcription factors shares a common domain structure which consists of the basic domain, which is responsible for DNA binding, the helix-loop-helix domain, which is important for protein-protein interaction and the leucine-zipper domain which is also involved in protein interactions (Murre et al., 1989 and 1994). All members of the *MITF-TFE* gene family share highly homologous DNA binding domains which enables the recognition of the more general E-Box (5'-CATGTG-3' or 5'-CACATG-3' sequences) (Murisier and Beermann, 2006) or the longer M-Box (5'-TCATGTGCT-3') (Yasumoto et al., 1997) motif. The bHLH-Zip domains which enable the formation of homo- and heterodimers within the family (Hemesath et al., 1994) are also highly conserved.

Beside these domains the protein contains four activation domains (AD), which have been shown to affect the transcription activation potential of MITF (see Figure 1.4) (Mansky et al., 2002b; Sato et al., 1997). The AD's are located in different parts of the protein: AD1 in exon 4, AD2 in exon 5, AD3 in exon 9 and AD4 in the alternative exon 1A. AD1 has been shown to mediate the interaction with the transcriptional co-activator p300 (Sato et al., 1997). The other ADs have not been characterized as well, but they also seem to play an important role in protein-protein interaction and MITF function.

1.5. *MITF* gene structure and splice variants

Ten different isoforms of MITF are known to exist which differ at the N-terminus of the protein, due to alternative first exons. (see Figure 1.5) (Bharti et al., 2008). These different first exons are spliced to the common exons 2–9. The resulting proteins share the DNA binding and dimerization domains as well as the activation domains. Some of the isoforms such as MITF-A, MITF-B, MITF-D and MITF-H can be found in a variety of cell types. They differ in their first exon, which is isoform specific (see Figure 1.5), which allows tissue specific function. Bharti et al. (2008) found that the expression of the MITF-A and MITF-D isoforms in the RPE cells of the eye is necessary for the correct development of the eye. Other isoforms, like MITF-M, which is specific for the melanocytic cell lineage or MITF-MC which is found in mast cells, are restricted to specific tissues (Arnheiter et al., 2006).

Each isoform has a different promoter region for regulation of transcription of the isoforms in the different tissues. Besides the alternative first exons, there are also splice variants in exons 2–9. For example, alternative splicing takes place between exon 2a/b removing a part of exon 2 and 6a/b removing a part of exon 6 (Bharti et al., 2010; Hallsson et al., 2000; Steingrimsson et al., 1994). Both alternative splicing sites lead to proteins containing or lacking internal domains. These alternatives increase the number of potential isoforms even more and to date up to 40 different *MITF* transcripts are known to exist. These different MITF isoforms may have a different specificity and may activate a different set of target genes. Shahlaee et al. (2007) have shown that the MITF-MC, MITF-E and MITF-A isoforms activate different sets of genes, but are all expressed in mast cells. It is likely that the cellular context of co-factors and other proteins necessary for the normal function of MITF influence specificity. For example, *TYR*, a gene regulated by the MITF-M isoform and one of the key enzymes in pigmentation is only expressed in melanocytes (Bentley et al., 1994).

1.6. Regulation of MITF expression

As a transcription factor which regulates downstream genes, MITF itself needs to be regulated tightly at the expression level. This is done by other transcription factors which regulate MITF expression in a positive or negative way. In some cases these transcription factors not only influence the regulation of MITF itself, they are also involved in the regulation of some of the MITF target genes.

1.6.1. Sry-related HMG box 10 (SOX10)

Sry-related HMG box 10 (SOX10) is a member of the sex-determining factor (SRY)-like, high mobility group (HMG) DNA binding proteins. It is involved in the determination of cell lineages (Wegner, 1999). Mutations of the gene

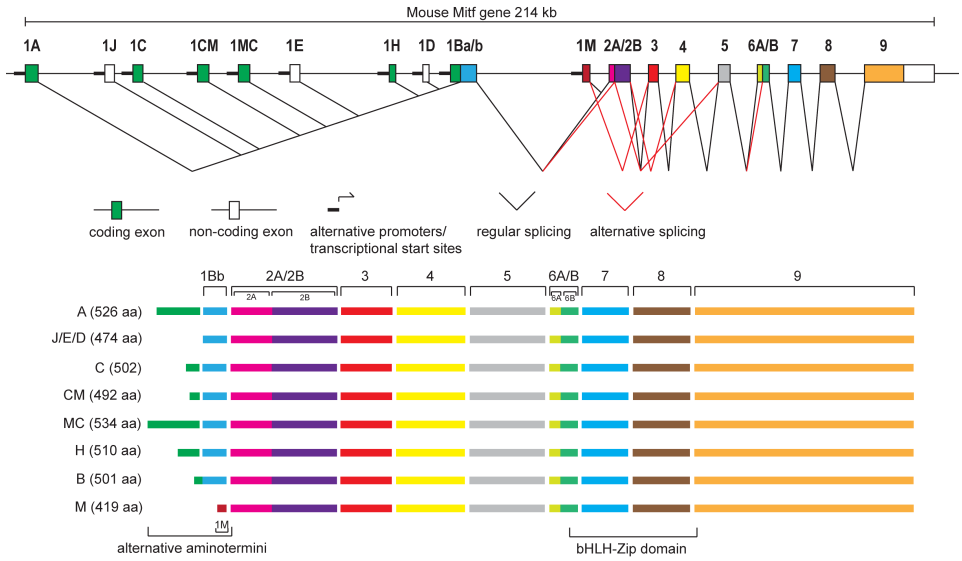


Figure 1.5.: Alternative isoforms of MITF with ten different first exons which are spliced to the common exons 2–9 and the 3' UTR and also the splice variants of the transcript. Figure modified after Bharti et al. (2008).

resulting in pigmentation disorders in mouse and human as well as different levels of hearing impairment have been identified. These phenotypes are similar to those caused by PAX3 and MITF mutations (Bondurand et al., 1999; Southard-Smith et al., 1999).

The transcription factor binds either alone to four binding sites in the *MITF* promoter and activates transcription or bind in cooperation with PAX3 to reach even higher levels of activation (Elworthy et al., 2003; Potterf et al., 2000; Verastegui et al., 2000).

1.6.2. Paired box 3 transcription factor (PAX3)

Paired box 3 (PAX3) also binds to the MITF promoter (Watanabe et al., 1998) to regulate transcription. It does this together with SOX10, which leads to a strong activation of *MITF* (Bondurand et al., 2000; Potterf et al., 2000) through binding sites in the proximal promoter. In melanocyte precursor cells, PAX3 also prevents MITF from binding some of its target genes by competing for promoter binding sites (Lang et al., 2005). This might trigger a lineage-specific gene program, as it inhibits the expression of genes which induce terminal differentiation.

1.6.3. Signal transducer and activator of transcription 3 (STAT3)

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor which is located downstream of the Janus kinase (JAK) signal transduction pathway. It is activated by members of the IL-6 cytokine family. Upon activation it is phosphorylated on tyrosine residues by JAK (a receptor tyrosine kinase (RTK)) and translocated as a functional dimer into the nucleus (Darnell et al., 1994). STAT3 signalling has been reported to have anti-apoptotic effects (Bromberg et al., 1999). Constitutively active STAT3 increased the activity of MITF in a number of different melanoma lines (Dorvault et al., 2001).

1.6.4. Protein inhibitor of activated STAT3 (PIAS3)

The protein inhibitor of activated STAT3 (PIAS3) interacts specifically with MITF and STAT3 and inhibits the transcriptional activity of MITF (Levy et al., 2002 and 2003). PIAS3 is an inhibitor of STAT3 signalling and an important negative regulator of MITF. The protein binds to the zip-domain of MITF and inhibits its activation (Levy et al., 2003).

1.6.5. cAMP response element binding protein (CREB)

cAMP response element binding protein (CREB) is a basic leucine zipper transcription factor, which is activated by several signal-transduction cascades as a reaction to external stimuli like UVB-radiation or α-MSH. The signal is transferred through the protein kinase A (PKA) or the p38 signal transduction cascade which leads to activation of CREB phosphorylation. The factor then binds to its target sequence, which is located in the close proximal promoter of *MITF* (Bertolotto et al., 1998).

1.6.6. Lymphoid-enhancing factor-1 (LEF-1)

Lymphoid-enhancing factor-1 (LEF-1) is a transcription factor which is regulated by WNT signalling. LEF-1 binds to three binding sites in the *MITF* promoter and activates transcription of the factor (Saito et al., 2002; Takeda et al., 2000a). LEF-1 also interacts with the MITF-M isoform to regulate the expression of *MITF-M*. Although MITF is important in this interaction it does not bind to the *MITF-M* promoter (Saito et al., 2002; Yasumoto et al., 2002).

1.6.7. BRN2

BRN2 (or POU3F2) is a POU-Homeodomain transcription factor. The expression of the factor is inversely correlated with the expression of MITF. BRN2 is expressed in melanoblasts and malignant melanoma, but not in differentiated melanocytes (Goodall et al., 2008; Thurber et al., 2011). It regulates the

expression of MITF (and thus controls migration and proliferation of melanocytes) but binds competitively to the PAX3 binding site in the *MITF* promoter and thereby prevents the activation of expression by PAX3 (Berlin et al., 2012). BRN2 is a negative regulator of *MITF*.

1.6.8. Forkhead-box transcription factor D3 (FOXD3)

The forkhead-box transcription factor D3 (FOXD3) belongs to the fox family of winged-helix transcription factors. FOXD3 is a negative regulator of *MITF*. How this process works is still in discussion, as there is evidence for both a direct binding of FOXD3 to the *MITF* promoter as well as for indirect regulation by binding to positive regulators of *MITF* such as PAX3 and preventing them from access to the *MITF* promoter. Experiments in zebrafish mutants performed by Curran et al. (2009) showed that the inhibition works by direct interaction of FOXD3 with the *mitfa* promoter. Transcription activation assays showed specific binding to two sites in the *mitfa* promoter resulting in 13 fold reduced activation of *mitfa*. Mutating these binding sites reduced the downregulation to 2,5 fold. This can either be explained by additional, yet unknown FOXD3 binding sites or by indirect mechanisms (Curran et al., 2009). In the avian system Mitf, Thomas and Erickson (2009) showed an indirect negative regulation of MITF as FOXD3 interacted with the PAX3 protein and prevented it from activating *MITF*. Whether this regulation is also present in other species needs to be investigated further.

1.6.9. Tyro3

Tyro3 is a receptor tyrosine kinase which has been identified as an upstream regulator of *MITF*. It regulates *MITF* transcription by the nuclear translocation of the transcription factor SOX10, which activates *MITF* expression. Upon overexpression of Tyro3, more SOX10 got localized to the nucleus, whereas overexpression of a kinase-dead Tyro3 did not show this effect. Tyro3 has been shown to be elevated in human melanoma and was overexpressed in 50 % of the tested melanoma samples (Zhu et al., 2009). Co-expression of BRAFV600E with Tyro3 in primary human melanocytes led to the formation of new colonies instead of senescence. This indicates that overexpression of Tyro3 can overcome the senescence mediated by $BRAF^{V600E}$. The knockdown of Tyro3 in A2058 melanoma cells repressed tumor formation (Zhu et al., 2009).

1.6.10. micro RNA

At the transcript level, *MITF* expression is also regulated by micro RNA (miRNA), a class of small RNA molecules which bind to specific regions of the transcript and mediate degradation of the targeted mRNA. To date, the regulation is

known to be mediated through miR-148, miR-155 and miR-182 (Bemis et al., 2008; Haflidadottir et al., 2010; Philippidou et al., 2010).

Another miRNA which seems involved in the regulation of *MITF* is miR-137. When this miR is overexpressed in mice, the expression of *MITF* is decreased. Subsequently, the expression of MITF regulated genes such as TYR, TYRP1 and DCT is also reduced, leading to a lighter coat color. The relationship between miR-137 expression and the coat color of mice is inversely correlated, with mice showing low melanin production when miR-137 expression is high (Dong et al., 2012). For a review of this field see Kunz (2013).

1.7. Post-translational modifications of MITF

The activity of MITF is not only regulated at the gene expression level but also by regulating transcript stability, availability and gene accessibility. Another important method for regulating MITF is by post-translational modifications upon signalling. These modifications influence the activity and the life-time of the protein as well as target gene specificity. Post-translational modifications of MITF and the signal transduction pathways responsible for them are shown in Figure 1.4.

1.7.1. Phosphorylation of MITF

Phosphorylation of a number of conserved serine residues is mediated by different signal transduction pathways. Activation of Kit leads to phosphorylation of the residue S73 via the the RAS-ERK MAP kinase pathway, with ERK2 directly targeting S73 (Hemesath et al., 1998). The phosphorylation of S409 is also mediated via KIT, but through ERK and the serine/threonine kinase p90 RSK-1 (Wu et al., 2000). Both modifications have been shown to activate the protein, but also to promote its subsequent ubiquitination and degradation in the proteasome (Xu et al., 2000). Un-phosphorylated MITF is relatively stable but is a poor activator of transcription. *In vitro* experiments showed a complete loss of activation if either the S73 or the S409 or both serines were mutated (Hemesath et al., 1998).

MITF is also known to be phosphorylated on other sites, namely S173, S298 and S307. In osteoclasts the phosphorylation by the C-TAK1 kinase at S173 allows binding of the co-factor 14–3–3 to Mitf. In the absence of signals required for terminal osteoclast differentiation the MITF/14–3–3 complex is then localized in the cytosol (Bronisz et al., 2006). Phosphorylation of S298 by GSK3β results in enhanced binding of MITF to its target genes (Tassabehji et al., 1995). Serine 307 is phosphorylated by the p38 kinase upon activation by RANK/RANKL (Mansky et al., 2002a).

In order to determine the *in vivo* role of the phosphorylation sites, a bacterial artificial chromosome (BAC) transgene rescue strategy was used. BACs con-

taining the *Mitf* locus were modified by introducing changes at the conserved phosphorylation sites S73 and/or S409 and then injected into mouse oocytes to generate transgenic lines. The BACs where brought into the $Mitf^{mi-vga9}$ background, which expresses no MITF resulting in white coat color, microphthalmia and hearing defects. The mutated BACs were able to rescue the phenotype of the mice, except for a white belly spot (Bauer et al., 2009). This suggests that these phosphorylation sites are not essential for melanocyte development. This also raises the possibility that other, yet unknown phosphorylation sites play a role in the regulation of MITF.

1.7.2. Acetylation of MITF

The CBP/p300 complex is an important co-activator of MITF and at least in melanocytes and osteoclasts it associates with S73 phosphorylated MITF (Price et al., 1998; Sato et al., 1997), where the phosphorylation seems mandatory. How the regulation of MITF through CBP/p300 works is still unknown, but it is possible that the histone acetyl transferase (HAT) of the complex targets specific lysine residues in the MITF protein. Sequence prediction and sequence comparisons have revealed two potential acetylation sites, which are conserved in nearly all species at the lysine residues 205 and 272 (Schepsky, 2007). Whether acetylation plays a role in MITF function is not known at present.

1.7.3. Ubiquitination of MITF

Ubiquitin is a small protein, approximately 8 kDa in size, which was discovered by Goldstein et al. (1975). In the process of ubiquitination, ubiquitin is attached post-translationally to a target protein. The ubiquitination can affect a target protein in different ways. It can change its cellular location, modify the activity, stimulate or inhibit protein–protein interactions and also mark it for degradation through the proteasome (Mukhopadhyay and Riezman, 2007; Schnell and Hicke, 2003).

The ubiquitination process is carried out by a cascade of three enzymes. They perform the three major steps in the process: Activation (ubiquitin-activating enzyme (E1)), conjugation (ubiquitin-conjugating enzyme (E2)) and ligation (ubiquitin ligases (E3)). This cascade binds one or many ubiquitins (up to seven) to lysine residues of the target protein (for review see (Pickart and Eddins, 2004).

MITF is ubiquitinated on the amino acid lysine 201 following the phosphorylation by Kit on the residues S73 and S409. This leads to the proteasome mediated degradation of MITF (Wu et al., 2000; Xu et al., 2000).

1.7.4. SUMOylation of MITF

The small ubiquitin-like modifier (SUMO) proteins are a group of small proteins which are also added to proteins post-translationally to modify and regulate their function. The SUMOylation is regulated by a cascade of enzymes which is analogous to the ubiquitination. The target protein is modified on lysine residues which are part of the recognition sequence Ψ–K–X–E (Ψ stands for hydrophobic amino acids, x for any). It is involved in various cellular processes including transcriptional regulation, protein stability, progression through cell cycle or apoptosis. Unlike ubiquitination, SUMOylation does not mark the modified target proteins for degradation (Hay, 2005).

MITF contains two SUMOylation sites at the lysine residues 182 and 316. The modification of MITF at these residues does not influence the DNA binding of the transcription factor (although K182 is located directly before the DNA binding basic domain), but changes the transcriptional activity of the protein. Mutations of the SUMO acceptor sites showed that mutating either of the sites alone or both together such that they can no longer be SUMOylated leads to increased transcriptional activity. This result suggests that SUMOylation of MITF leads to decreased transcriptional activity of the protein (Miller et al., 2005; Murakami and Arnheiter, 2005). The only known heritable *MITF* mutation which has been connected to melanoma is located in one of the SUMO recognition sites, changing the amino acid 318 of MITF from glutamic acid to lysine and thus rendering the Ψ–K–X–E sequence inoperative (see section 1.10 on melanoma) (Bertolotto et al., 2011; Yokoyama et al., 2011).

1.7.5. Caspase cleavage of MITF

Binding of the TNF-related apoptosis-inducing ligand (TRAIL) to its receptor (death receptor 4 and 5) leads to activation of the caspases 3, 6 and 7 and subsequently to the cleavage of MITF at asparagine 345 into a large N-terminal fragment and a small C-terminal fragment. Although the N-terminal fragment is fully transcriptionally active, it cannot up-regulate pro-survival genes, since it is degraded rapidly. The short C-terminal fragment acts in a pro-apoptotic manner by activating caspase 3 and promoting the cleavage of poly ADP ribose polymerase (PARP) (Larribere et al., 2005). As a response to cell surface death signals, MITF is thus cleaved after amino acid aspartate 345 by the caspases 3, 6 and 7 (Larribere et al., 2005).

1.8. MITF target genes

As a transcription factor, MITF regulates a number of downstream genes. The activation of genes is dependent on the level of MITF-M in the cell and also the state of the cell (Carreira et al., 2006; Goodall et al., 2008; Javelaud et al.,

2011). As the master regulator of melanocytes MITF regulates a large number of genes involved in pigment synthesis, structural proteins of the melanosomes as well as proteins involved in the trafficking of the melanosomes. In addition it regulates genes involved in cell survival, proliferation and migration.

To identify new MITF target genes, we conducted a gene expression study comparing cells which were transfected with *MITF* to those who were not (Paper I). Another study to identify new target genes was done by Irwin Davidson's group (Strub et al., 2011). In these studies known target genes were found. Both studies also identified a large number of new potential target genes, which have not been connected to MITF regulation before. One of these genes is the Interferon Regulatory Factor 4 (IRF4), which is further explained in section 1.11.

1.8.1. Genes involved in pigmentation

Besides the enzymes which catalyze parts of the chemical reactions needed for the synthesis of melanin, other genes are important for pigmentation. Most of these genes are also regulated by MITF. Among these genes is a number of ion transporters, such as *SLC24A4* and *SLC45A2*,*V-ATPases* as well as the *P-protein*. Other target genes are important for the transport of the melanosomes to their correct destination including *MYO5A* (see also figure 1.2).

Tyrosinase, tyrosinase related protein 1 (TYRP1) and Dopachrome tautomerase (DCT)

MITF regulates the expression of the central enzymes in pigmentation. MITF has two binding sites in the proximal promoter of the *TYR* gene. MITF binds both, the more distant M-Box and the initiator E-box and induces expression of *TYR*. The binding sites have been confirmed by mutation experiments (Yasumoto et al., 1997). Tyrosinase is a copper-dependent enzyme which catalyzes the first step in pigmentation by oxidizing the amino acid tyrosine. Mutations in *TYR* cause oculocutaneous albinism type 1 (OCA1).

TYRP1 (Yasumoto et al., 1994) and DCT (Yasumoto et al., 1997) are the two other key enzymes controlled by MITF.

TYRP1 has one M-Box in the promoter region near the transcription start site which is bound by MITF. Changing the sequence of the M-Box abrogated MITF-mediated activation of this promoter (Yasumoto et al., 1997). TYRP1 has dihydroxyindole carboxylic acid oxidase (DHICA oxidase) activity and catalyzes the oxidation of 5,6-Dihydroxyindol-2-carbonic acid to melanin. Mutations in the *TYRP1* gene cause oculocutaneous albinism type 3 (OCA3) (Sarangarajan and Boissy, 2001).

The gene encoding dopachrome tautomerase has one M-Box and one LEF-1 binding site in the immediate upstream promoter. Closely upstream is also a CRE-like binding site. The M-Box and the LEF-1 binding site need to be

cooperatively bound by MITF and LEF-1, and both factors need the binding of a yet unknown factor to the CRE-like binding site to activate the expression of DCT (Yasumoto et al., 2002). DCT is a zinc-based enzyme that catalyzes the synthesis of DHICA from dopachrome.

Solute carrier family proteins

The family of solute carriers is a group of transmembrane transporter proteins which consist of 52 families with over 300 members. The carriers transport inorganic ions and charged and uncharged organic molecules through the membrane in and out of the cell (Hediger et al., 2013). Of this group, one member is known to be regulated by MITF: *SLC45A2/MATP/AIM1* (Du and Fisher, 2002). A number of other solute carriers are potential target genes of MITF, but have not been characterized further in terms of regulation: *SLC1A4, SLC7A8, SLC11A1, SLC19A2, SLC24A5* (Hoek et al., 2008). But since these proteins belong to families which are transporters for amino acids and metal ions, an integration of these in the melanosome can be hypothesized.

A mutation in the SLC24A5 gene causes the golden phenotype in zebrafish. This mutation is characterized by a lighter pigmentation of the fish caused by hypopigmentation of the melanophores in the skin and the retinal pigment epithelium (Lamason et al., 2005). A similar mutation has also been found in humans and been connected to pigmentation as well (Basu Mallick et al., 2013).

The Ala111Thr mutation (rs1426654) in the human *SLC24A5* gene has been shown to be the major reason for the lighter skin color of people of European ancestry compared with people of African-American ancestry. It is estimated that about 25–40 % of the actual skin tone difference in Europeans arises from this polymorphism (Lamason et al., 2005; Norton et al., 2007). SLC24A5 is located in the membrane of the melanosome and acts there as a potassium-dependent sodium-calcium exchanger which controls intracellular calcium level which is important for melanogenesis. Removal of the protein disrupts melanogenesis (Ginger et al., 2008).

SLC45A2 (or MATP) is also part of the melanosome. Mutations in this gene cause oculocutaneous albinism type 4 (OCA4), an autosomal recessive disorder of melanin biosynthesis that results in hypopigmentation of ocular and cutaneous tissues (Suzuki and Tomita, 2008). Melanosomes in *SLC45A2* mutant mice have reduced melanin content and are smaller compared to wild-type mice (Lehman et al., 2000). Based on similarity analysis it was proposed that SLC45A2 is a H^+/sugar symporter which is involved in the regulation of pH and osmolarity of cells (Newton et al., 2001).

Melanocortin-1-receptor (MC1R)

The Melanocortin-1-Receptor is another important gene involved in pigmentation regulated by MITF (Aoki and Moro, 2002). MC1R is a seven pass transmembrane G protein coupled receptor which is located in the cell membrane of melanocytes (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992). It binds different ligands with opposite characteristics. The ligands αMSH and adrenocorticotropic hormone (ACTH) activate signaling through the receptor, leading to increased production of cAMP which then leads to phosphorylation of the CREB, resulting in activation of expression of MITF, the melanocyte master regulator. MITF then activates the expression of several genes required for production of black eumelanin, including TYR, the enzyme that catalyzes synthesis of eumelanin (Busca and Ballotti, 2000). The agouti signaling protein (ASIP) is an antagonist of MC1R and prevents binding of αMSH and thus production of eumelanin. This leads to production of red or yellow pheomelanin, thus modulating the ratio of eumelanin and pheomelanin produced by melanocytes (Cone et al., 1996; Rees, 2003).

The majority of red-haired individuals are homozygous or compound heterozygous for variants in the *MC1R* gene (Box et al., 1997; Flanagan et al., 2000; Smith et al., 1998; Valverde et al., 1995). MC1R variants also lead to pale skin. More importantly, the *MC1R* gene has been shown to be a major contributor to the formation of freckles (Bastiaens et al., 2001; Flanagan et al., 2000; Rees, 2004). Overall this is referred to as the RHC (red hair, fair skin, lack of tanning ability and propensity to freckle) phenotype, with highly penetrant variants of the MC1R gene designated as R-alleles and lower penetrant alleles as r-alleles (Beaumont et al., 2011). More than 80 coding variants in MC1R have been described in populations of European ancestry (Gerstenblith et al., 2007; Makova and Norton, 2005). These variations lead to pigmentation traits such as fair skin, red and blond hair, UV sensitivity and freckles in both humans and animals (Kijas et al., 1998; Robbins et al., 1993; Rouzaud et al., 2006; Vage et al., 1999). The variants in MC1R affect receptor function (Beaumont et al., 2007 and 2009; Garcia-Borron et al., 2005) or the promoter region (Motokawa et al., 2008); red hair and ephelides are found in the absence of MC1R function (Beaumont et al., 2008). Activating mutations in MC1R can lead to permanent activation of the protein and dark pigmentation, which has so far only been described for animals (e.g. mice, pigs and sheep) and not for humans (Vage et al., 1999).

1.8.2. Genes involved in cell survival, cell cycle control and proliferation

MITF does not only regulate genes involved in pigmentation, it is also important for cell survival, cell cycle control as well as proliferation. This is illustrated by

the fact that the Mitf$^{mi-vga9}$ mouse line, which expresses no MITF due to an insert in the promoter region of the gene, is devoid of melanoblasts (Goding, 2000; Tachibana et al., 1992) and thus lacks hair pigmentation. It also has small eyes and the mutation leads to deafness (see Figure 1.6) (Hodgkinson et al., 1993). In Mitf^{mi-ew} (eyeless white) mice reduced numbers of melanoblasts can be detected, suggesting that these cells exist first but then either go into senescence or cell cycle arrest and do not develop further (Nakayama et al., 1998).

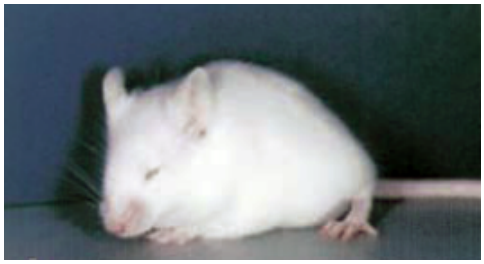


Figure 1.6.: The image shows a Mitf$^{mi-vga9}$ mouse with white coat color and microphthalmic eyes.

This observation led to the discovery that the expression of *T-box 2 (TBX2)* is regulated by MITF (Carreira et al., 2000). Factors from the T-Box family are crucial for embryonic development and have been implicated in the development of a wide range of organs such as kidneys, limbs, lung, mammary gland and heart. TBX2 is a transcription factor of the T-box transcription factor family, which blocks cellular senescence by repressing CDKN1A and CDKN2A (Jacobs et al., 2000; Prince et al., 2004; Vance et al., 2005).

Another critical pro-survival gene is the cyclin-dependent kinase 2 (CDK2), which is located close to the MITF target gene *PMEL*, but in opposite orientation. The regulatory region of *PMEL* overlaps with the promoter of *CDK2*, so binding of MITF to a specific E-box in this region is involved in the regulation of both genes (Du et al., 2004).

MITF also regulates *DIAPH1* (diaphanous-related formin 1), which in turn influences the level of CDKN1A (Carreira et al., 2006). When DIAPH1 is downregulated, SKP1 is inhibited which leads to effects on CDKN1A levels via ubiquitination and subsequent proteasomal degradation (Mammoto et al., 2004; Schulman et al., 2000).

Further prosurvival genes regulated by MITF are *HIF1α* and *APEX-1 (APEX nuclease (multifunctional DNA repair enzyme) 1)* (Busca et al., 2005; Liu et al., 2009).

1.8.3. MITF target genes involved in apoptosis

MITF regulates the expression of anti-apoptotic genes. This includes *BCL2*, which is important in both melanoma and melanocytes for cell survival. $BCL_2^{-/-}$ mice show a loss of melanocytes after birth, indicating a role of the protein in melanocyte survival. MITF binds to an E-box in the proximal promoter of *BCL2* (McGill et al., 2002).

The baculoviral IAP repeat containing 7 gene (BIRC7, also known as melanoma inhibitor of apoptosis (ML-IAP) and livin) is directly regulated by MITF by binding to the *BIRC7* promoter. The knock-down of MITF expression also disrupts the expression of *BIRC7*, showing a direct interaction at the promoter. (Dynek et al., 2008).

Further, MITF has been reported to regulate the expression of *MET*. McGill et al. (2006) used a chromatin immunoprecitation (ChIP) assay to identify a CACGTG site around position -300 in the *MET* promoter bound by MITF, but failed to confirm this finding by reporter assays. In an independent study Beuret et al. (2007) identified a CACGTG motif at a different position (around position -109) which had a transactivating effect through MITF on the *MET* promoter. Although the involvement of MITF might not be direct, the activation of MET by its ligand HGF (hepatocyte growth factor) leads to melanoma migration and the protection of melanocytes and melanoma cells from apoptosis.

1.9. Freckles and solar lentigines

Freckling is a widely seen pigmentation phenomenon which can be grouped into two different forms: Ephelides and solar lentigines (SL, which are also known as lentigo senilis, actinic lentigines, sunburn freckles, freckles in adulthood, aging spots and liver spots). They are benign pigment spots which occur mostly in persons of Caucasian and Asian descent. Despite both forms being flat macules, they are significantly different in development and morphology (see Table 1 in Paper III).

Ephelides are relatively small pigment spots (usually 1–2 mm, but they can also be larger) which are red to light brown colored. They are observed in fair-skinned and/or red-haired individuals which mostly belong to the phototypes I and II. The phototype is a classification system which describes the ability of the skin to develop tan and the risk of sunburn when the skin is exposed to the sun. The phototypes are grouped from I–VI, with I for the most sensitive skin and VI for the most robust skin against sun exposure. The phototypes I–IV occur in persons with white skin, while the phototypes V and VI are exclusive to individuals with brown and black skin (Fitzpatrick, 1988). Ephelides appear on the skin around the age of 2–3 years, their number increases during adolescence and they often disappear with increasing age (Plensdorf and Martinez, 2009).

The highest frequencies of ephelides are found on the face, arms, neck and chest (see Figure 1A in Paper III). They are more pigmented during summertime.

Lentigines are larger than ephelides, with a diameter spanning from millimeters to centimeters and are usually darker pigmented than ephelides (see Figure 1B in Paper III) (Hodgson, 1963). They get more common after the age of 50 and appear on chronic sunexposed skin such as the face, the dorsum of the hands, and anterolateral parts of the forearm) (Cario-Andre et al., 2004). Contrary to ephelides, lentigines do not change their pigmentation with the seasons.

Freckles are differently received in different cultural backgrounds, in Asia they are seen as a cosmetic problem which is often removed (Jang et al., 2000; Zhang et al., 2004). In Western culture freckles are more fashionable which is illustrated by the appearance of freckled models such as Kate Moss, in magazines and advertisements.

1.9.1. Histopathological features

Histological analysis of the skin shows that ephelides can be distinguished from the surrounding, non-freckled skin. Since only few studies have analyzed the role of melanocytes in freckled skin there are still a lot of open questions. It is so far not clear, why the melanocytes in the freckles have a higher density compared to normal skin (for an overview of the characteristics, see Figure 1 in Paper III), build up their characteristic pattern or disappear over time. Additionally the existing studies have come to some conflicting results.

Breathnach (1957 and 1958) stained epidermal sheets for melanocyte activity using DOPA, finding that ephelides contained about 40 % fewer melanocytes than the neighboring normal skin. Only few differences were found in the non-freckled skin of freckled individuals compared with non-freckled individuals. Breathnach found that the melanocytes of freckles were larger, more strongly DOPA positive, more pigmented and also showed more and longer dendrites.

Another study was performed by Rhodes et al. (1991) which used transverse skin sections of juveniles to examine the histopathology of sun-induced ephelides. They found that the rete ridges (thickenings in the epidermis that extend between the dermal papillae) of ephelides are elongated and clubbed. This leads to the accumulation of more melanocytes in the rete ridge as well as more melanin in the basal epidermal unit. The results from Rhodes et al. (1983) are contradictory to the earlier findings of Breathnach, as they found more melanocytes in ephelides than in the surrounding, non-freckled skin.

Both studies agree that there is more pigmentation present in the ephelides than in the non-freckled skin (see Figure 2 in Paper III). Electron microscopy studies confirmed this finding, which showed that melanocytes in ephelides contain multiple large melanosomes. The melanocytes of non-freckled skin contains fewer, small and less pigmented granules (Breathnach and Wyllie, 1964). This result suggests different behavior for the melanocytes in freckled individuals

depending on where they are located. They are either highly pigmented with large melanosomes in the ephelides or less pigmented with smaller melanosomes in the non-freckled skin. It is currently unclear how this different behavior can be explained, but since ephelides arise in sunexposed skin, this seems to play an important role.

Solar Lentigines show histological similarities to ephelides as they also have elongated epidermal ridges and a hyperpigmented basal layer, with keratinocytes accumulating melanin pigment (Cario-Andre et al., 2004; Noblesse et al., 2006; Unver et al., 2006). How and when the elongated ridges form is unclear since Ki67 staining did not show proliferative differences between SL and the surrounding normal skin (Unver et al., 2006; Yamada et al., 2014). SL's have been shown by Yamada et al. (2014) to contain increased numbers of melanocytes in the epidermis, melanoblasts in the hair follicular infundibulum and melanocyte stem cells (MSC) in the hair bulge. They also showed a higher expression of TYR in the melanocytes. This results in a higher number of melanocyte precursor cells in the skin and also more active melanocytes, which makes it likely that these cells respond to signals from the keratinocytes in the photodamaged skin. Results by Cario-Andre et al. (2004) show that the pigment accumulates in the keratinocytes in perilesional skin, which indicates that the accumulation of pigment happens early in the process. Electron microscopy (EM) studies showed that the melanosomes of SL are normal sized when compared to the adjacent skin. This is in contrast to ephelides where melanosomes are often bigger. The EM studies also showed that melanocytes in SL had more mitochondria and a more developed endoplasmic reticulum (Nakagawa et al., 1984; Noblesse et al., 2006).

1.9.2. Genetics

Ephelides

Ephelides have been associated with blond/red hair color and light skin and appear first around in young age, which suggests that the formation of freckles is largely genetically determined. Additive genetic effects can explain about 91 % of the variance in freckles, with no observed environmental effects (Bataille et al., 2000). In accordance with this observation, a number of different genes with an effect on the formation of freckles have been identified. They include *MC1R, IRF4, ASIP, TYR* and *BNC2* (see Table 2 in Paper III).

The gene with the biggest impact on freckling is *MC1R* (Bastiaens et al., 2001; Rees, 2004) (for details see section 1.8). The majority of red-haired individuals carries homozygous or compound heterozygous variants in MC1R (Box et al., 1997; Flanagan et al., 2000; Smith et al., 1998; Valverde et al., 1995). Carriers of one or two MC1R variants had an increased risk (3- and 11-fold) for ephelides during childhood which is independent of the individual skin or hair color. The

degree of freckling was positively correlated to the number of polymorphisms in the *MC1R* gene present in the individual (Bastiaens et al., 2001). These results were supported by Duffy et al. (2004) who found that red-haired individuals had the most freckles with a population attributable risk of 100 % when a variation was present at least on one allele of *MC1R*. MC1R not only contributes to freckling in Europeans but has also been shown to have the same effect on the Japanese population (Motokawa et al., 2007; Yamaguchi et al., 2012).

Genome wide association studies have recently associated more genes with freckling. The studies of Sulem et al. (2007 and 2008) which were done on Dutch and Icelandic populations not only found a number of SNPs in the *MC1R* gene but also in or near *ASIP, IRF4* and *TYR*. *ASIP* and *TYR* are both known to be involved in pigmentation, so their association was not a surprise. ASIP is an antagonist of the MC1R receptor, while TYR is directly involved in the pigmentation process.

The association of *IRF4* was confirmed by Han et al. (2008) which also identified the rs12203592 polymorphism in intron 4 of *IRF4* as the causative SNP. The connection between IRF4 and pigmentation is analyzed in detail in Paper II.

Solar lentigines

Solar lentigines are more influenced by environmental factors and arise from chronic photodamaged skin, where they receive paracrine, microenvironmental or cell-cell signals which determine the melanocytic growth. SL are dependent on the same melanocytic genes necessary for pigmentation as ephelides. The MC1R variants, which increase the risk for freckles by 3- to 11-fold also increase the risk for SL by 1.5- to 2-fold (Bastiaens et al., 2001). In Japanese individuals polymorphisms in the promoter as well as in the coding region of *MC1R* have been associated with SL (Motokawa et al., 2007 and 2008). Vierkotter et al. (2012) associated variations in the *SLC45A2* gene with SL. *SLC45A2* is a gene known to be involved in pigmentation. Mutations of the gene in humans cause OCA4 (see page 17).

A few other genes have been implicated in the formation of solar lentigines. Hafner et al. (2009) screened SL samples and found mutations in the *FGFR3* and *PIK3CA* genes been mutated in 17 % and 7 % of SL's respectively. They suggest that these mutations may play a role in the formation of SL. Other proteins which are probably involved include Endothelin 1 and the Endothelin B receptor, which play important roles in melanocyte development (Kadono et al., 2001); stem cell factor, the expression of which is increased in SL (Hattori et al., 2004); hepatocyte growth factor and keratinocyte growth factor (Chen et al., 2010; Kovacs et al., 2010). Goyarts et al. (2007) performed a gene expression analysis of lesional and non-lesional skin from SL individuals. They identified 23 upregulated and 17 downregulated genes in the SL samples. Among these

were no pigmentation genes, but regulatory genes of the WNT family and metalloproteases as well as genes involved in inflammation.

Solar lentigines can also form as a consequence of pleiotropic syndromes. The genetic cause of these is usually identified and are likely involved in the formation of SLs. These include the Carney complex, the LEOPARD syndrome and the Peutz-Jeghers Syndrome. These syndromes and their genetic causes have been reviewed recently (Espiard and Bertherat, 2013; Lauriol and Kontaridis, 2011; Ollila and Makela, 2011).

1.10. Melanoma

Melanoma stands for malignancy of the melanocytes. Its existence has been known for a long time. The first descriptions were passed down from the Greek physician Hippocrates in the 5th century BC. The term melanoma was first used in a publication by the French doctor René Laënnec in 1812 (for review, see Bennett and Hall, 1994) and was subsequently widely used for description of the disease.

While the disease is easily cured at the early stages by simple surgery to remove the affected skin (the 5 year survival rate here is about 95 %), the picture changes when melanoma becomes metastatic. The mean overall survival time is around 8–15 months (Jemal et al., 2010); the five year survival rate is less than 15 % (Balch et al., 2009). With metastatic melanoma accounting for less than 4 % of all skin cancers, they cause about 80 % of the deaths from skin cancers (Miller and Mihm, 2006) which shows the aggressiveness of this type of cancer.

1.10.1. Epidemiology

Melanoma incidence has been rising in the last decades in white populations of European ancestry (Chuang et al., 1999; Hall, 1999; Rhee et al., 1999) with increases in incidence rates of 3–7 % per year (Armstrong and Kricker, 1994). Especially affected are the populations of Australia and New Zealand (Dennis, 1999; Jones et al., 1999; MacLennan et al., 1992). The incidence rates vary quite dramatically between different locations, from 40 cases per 100.000 persons in Australia to below 5 cases per 100.000 persons in northern Scandinavia. The incidence rates in populations of non-European origin are less consistent, but are generally low and vary between 0.1/100.000 and 3/100.000 (Armstrong and Kricker, 1994). In addition to the rising case numbers, the death rate for melanoma has risen in the past 10 years. This is in contrast to many other tumors (Jemal et al., 2011).

1.10.2. Risk factors

Ultraviolet radiation (UVR) is the major environmental risk factor for melanoma, with an inverse proportional relation between the risk and the ability to tan. High melanin content in the skin effectively protects from sunlight (Cui et al., 2007; Evans et al., 1988; Veierod et al., 2010). Studies have confirmed that melanoma is prominent in people with pale skin, with higher rates in red-haired individuals compared to those with dark hair. The number of nevi and especially dysplastic (atypical) nevi is also associated with a significantly higher risk for melanoma, again with red-haired individuals being more likely to develop dysplastic nevi (Bataille et al., 1996; Grulich et al., 1996). The pooled analysis of the different risk phenotypes allows calculation of melanoma odds ratios (OR) for the different risk factors, as listed in Table 1.1 (Chang et al., 2009).

Table 1.1.: Odds ratios for different melanoma risk factors.

risk factor	odds ratio
fair skin	1.7
red hair	1.9
high common nevus count	7.9
$\geqslant$1 dysplastic nevi	4.0

1.10.3. Signalling in Melanoma

Signalling pathways play an important role in gene regulation in normal melanocytes. Not surprisingly, signalling is also important for melanoma development.

KIT

The receptor tyrosine kinase Kit is an important receptor in the development of melanocytes. It influences proliferation, migration, differentiation and survival. Upon binding of the ligand (Stem Cell Factor, SCF, also called steel) the receptor dimerises and autophosphorylates itself to activate downstream signalling cascades. This includes MAPK (mitogen activated protein kinase), PI3K (phospho-inositol-3-kinase) and JAK/STAT (janus kinase/signal transducer and activator of transcription)(Alexeev and Yoon, 2006). The downregulation of *Kit* seems also to be involved in an enhancement of melanoma tumorigenicity and metastasis (Huang et al., 1998; Phung et al., 2011).

Increases in copy number and/or oncogenic mutations are found frequently in *Kit*, although the rates differ largely between the studies. The rate for acral melanoma is between 13 % and 36 %, whereas for mucosal melanoma it is

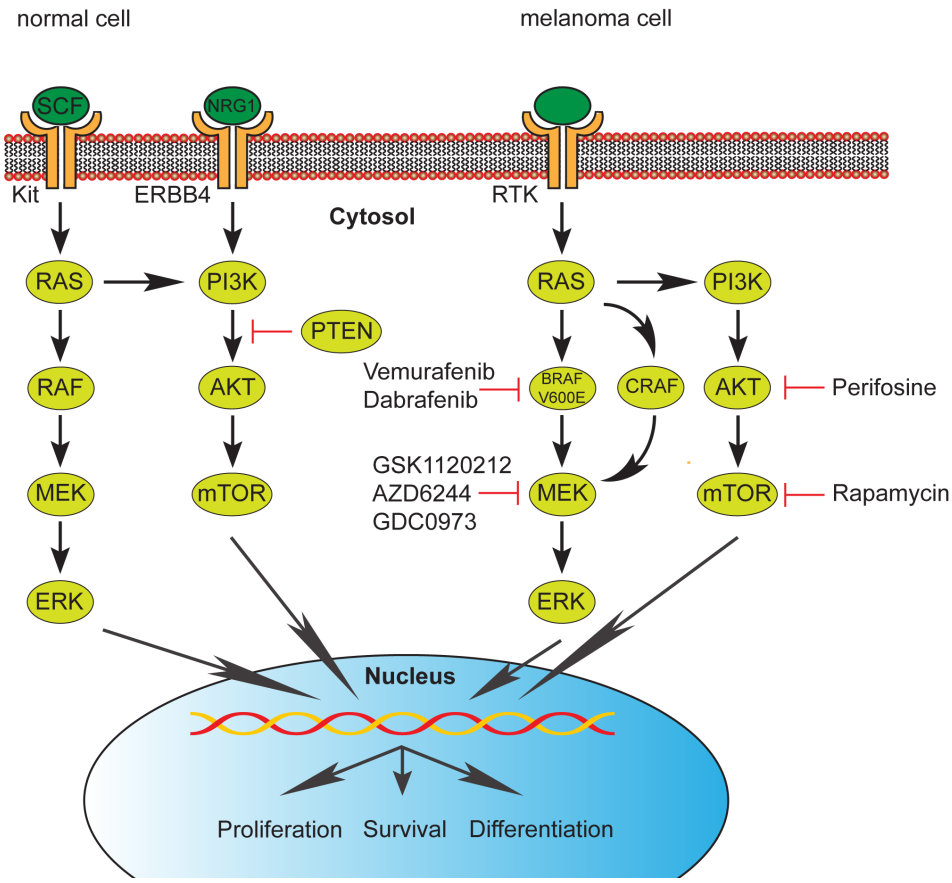


Figure 1.7.: Schematic view of the signal transduction pathways in normal cells (left side of the Figure) and melanoma cells (right side of the Figure). For the melanoma cells, mutated parts are shown together with inhibiting drugs.

between 16 % and 39 % (Beadling et al., 2008; Curtin et al., 2006; Torres-Cabala et al., 2009). Activating mutations in Kit are mostly located in the juxta-membrane region. Small molecule inhibitors (dasatinib and imatinib) are currently in clinical trials as a treatment against mutated Kit (Antonescu et al., 2007).

ERBB4

An effort to identify other protein tyrosine kinase genes involved in melanoma resulted in identification of 19 mutated genes (Prickett et al., 2009). The gene in which the most mutations where found was *ERBB4*, with 24 of 79 (19 %)

of the melanoma samples mutated. The mutations where found throughout the gene with clusters in highly conserved regions of the protein kinase domain. This region is also frequently mutated in other cancers. These mutations lead to a stronger phosphorylation of the receptor and higher kinase activity than in wild-type (Prickett et al., 2009). ERBB4 signal transduction is known to activate both MAPK and PI3K pathways (see Figure 1.7). Studies of melanoma with mutant ERBB4 have shown an increased phosphorylation of AKT which ceases when ERBB4 is knocked down with shRNA. This confirms the role of *ERBB4* as an oncogene activating AKT (Prickett et al., 2009; Rudloff and Samuels, 2010).

Platelet-derived growth factor receptor (PDGFR)

PDGFR is a receptor tyrosine kinase, which is usually not mutated or changed in copy number in primary melanoma (Curtin et al., 2008). Despite this fact the upregulation of PDGFR is one of the resistance mechanisms which occurs in metastatic melanoma upon treatment with the $Braf^{V600E}$ inhibitor vemurafenib. The overexpression of PDGFR-β was found in 4 of 11 resistant tumors and the inhibition of PDGFR with siRNA resulted in growth inhibition, showing the dependence of the tumor on PDGFR-β for proliferation and survival (Nazarian et al., 2010).

NRAS

The class of RAS G-regulatory proteins (which includes NRAS) are part of the MAPK signal transduction pathway. They are activated by binding of GTP or by mutations which cause the protein to be constitutively active. Upon activation, RAS interacts with downstream pathways such as MAPK and PI3K and thus regulating cell-cycle progression, differentiation and cell survival. NRAS has been found mutated in 18 % of cutaneous melanoma and in 81 % of congenital melanocytic nevi. Melanoma with mutated NRAS are more common in skin areas which have been subjected to chronic sun damage. In NRAS most activating mutations are found in codon 61 (Q61R/K/L) and are usually exclusively found in tumors which are not mutated in $Braf^{V600E}$. This finding suggests that these mutations are functionally redundant (Bauer et al., 2007; Lee et al., 2011).

Mutations in NRAS have been found to be a negative prognostic marker in metastatic melanoma, associated with increased aggression and shorter survival time compared to patients without mutations in NRAS or BRAF. Additionally NRAS can be activated upon treatment of $BRAF^{V600E}$ cells with vemurafenib (see Figure 1.7), leading to activation of other RAF proteins such as CRAF which re-activates signalling through the MAPK pathway (Nikolaou et al., 2012).

BRAF

The RAF proteins are serine/threonine-kinases which belong to the MAPK and RAS-RAF signalling pathways. They are considered of central importance, since activating mutations in BRAF cause a constitutive activation of the downstream pathways and lead to increased proliferation and survival. Mutations in BRAF are very common in melanoma and can be found in about 41 % of cutaneous melanoma patients (Lee et al., 2011).

An exchange of the amino acid valine for glutamic acid in position 600 of the protein (V600E) is the most common BRAF mutation. It enhances the kinase activity to be constitutively active and activates MEK and ERK even if no activation through RAS takes place (Wan et al., 2004). Inhibition of the mutated kinase by the small molecule inhibitor vemurafenib initially showed promising results but also showed newly emerging resistance mechanisms as the already mentioned activation of NRAS and PDGFR (see Figure 1.7 for details) (Chapman et al., 2011).

Besides activating other pathways to get around the inhibition of $BRAF^{V600E}$ a number of other mechanisms have been found. This includes a new splice variant of $BRAF^{V600E}$ which lacks the exons 4–8 which encode for the RAS-binding domain. This isoform leads to enhanced dimerization of the kinase domains which is independent of RAS signalling and constitutively activates the MAPK pathway. Amplifications of BRAF have been found in 20 % of the patients resistant to vemurafenib. To overcome this resistance it is possible to inhibit the MEK kinase which is downstream of BRAF (and NRAS) and which is still sensitive to inhibition in $BRAF^{V600E}$ melanoma (Poulikakos et al., 2011; Shi et al., 2012).

A recent publication by Johannessen et al. (2013) showed the existence of another, as yet unknown signalling network which is active in $BRAF^{V600E}$ melanoma cells. It helps to overcome the combined treatment with different MAP, ERK and MEK inhibitors. The network is based on cyclic-AMP signalling, including G-protein-coupled receptors (GPCR), adenyl cyclase, protein kinase A (PKA) and CREB.

Upon activation, GPCR activate adenyl cyclases which then convert ATP into cAMP. This signalling molecule binds to PKA, which subsequently activates CREB. Activation of CREB leads to the expression of genes which contain a cyclic AMP response element in their promoter. Among these genes are the transcription factors MITF, c-FOS and NR4A2. This additional pathway can be inhibited by using histone-deacetylase inhibitors (Johannessen et al., 2013).

MAP kinase kinase (MEK, MAP2K)

The MAP kinase kinase (also known as MEK, MAP2K) is a protein kinase in the MAPK pathway downstream of BRAF. It phosphorylates ERK and increases

cell survival. Sequencing studies revealed mutations in MEK1 or MEK2 in about 8 % of the analyzed melanoma which frequently occurred with BRAF (60 %) or NRAS (10 %) mutations. The most common MEK mutations, MEK1-P124S and MEK1-E203K lead to a constitutive phosphorylation of ERK (Nikolaev et al., 2012). The MAP kinase pathway is shown in Figure 1.7.

Phosphoinositide 3-kinase (PI3K)

The phosphoinositide 3-kinase (PI3K) is usually important for growth and cell survival and also plays an important role in melanoma tumorigenesis. The kinase itself is not mutated in melanoma (see Figure 1.7), but the selective activation of the downstream target *AKT3* is found in 53 % of primary and 67 % of metastatic melanoma, compared to only 12 % on dysplastic nevi. The elevated expression of *AKT3* happens through different mechanisms including amplifications of the gene (Bastian et al., 1998).

Decreased expression of the tumor suppressor PTEN by genetic alteration can also lead to a constitutive activation of the PI3K pathway. Under normal conditions PTEN negatively regulates *PI3K*. Deletions and mutations of *PTEN* occur in about 30 % of melanomas and often occur together with activating mutations in BRAF (Gast et al., 2010; Tsao et al., 2004).

1.10.4. Hereditary factors in Melanoma

Most melanomas occur with no familial component, but clustering of melanoma within families has been reported. The chance of developing melanoma is twice as high with an affected parent, three times as high with an affected sibling and nine times as high if at least one parent and one sibling is affected (Hemminki et al., 2003). In about 13 % of melanoma cases, at least one first–degree relative is affected by the same disease (Hayward, 2003). The familial clustering is most likely caused by a mix of genetic and environmental reasons.

CDKN2A and CDK4

The most important high-risk melanoma locus which has been found in "melanoma families" is the cycline dependent kinase inhibitor 2A (*CDKN2A*). The gene locus is located on chromosome 9p21 and encodes for two different proteins, p_{16}^{INK4a} and p_{14}^{ARF}. Both are tumor suppressor genes and are involved in the regulation of the cell cycle.

The function of p_{16}^{INK4a} is to inhibit CDK4 (cyclin-dependent kinase 4) and CDK6. Both kinases phosphorylate the retinoblastoma protein (Rb) and the inhibition of the kinases leads to cell cycle arrest. p_{14}^{ARF} blocks the degradation of the p53 protein by inhibiting HDM2, thus enhancing apoptosis. The inactivation of either p_{14}^{ARF} or p_{16}^{INK4a} leads to uncontrolled cell growth (Snoo and Hayward, 2005).

Mutations in CDKN2A have also been associated with multiple nevi (n>100), the atypical nevus syndrome, nevi on buttocks and feet, multiple cases of melanoma in one family and multiple primary melanomas (Berwick et al., 2006; Bishop et al., 2000). In the general population, mutations of the *CDKN2A* locus are relatively rare (present in only 1,2 %), however they are common germline mutations in families with three or more cases of melanoma. In these families they occur at a rate between 20 % and 57 % (Berwick et al., 2006; Goldstein et al., 2007).

Deletion of *CDKN2A* plays a role in the development of melanoma from dysplastic nevi. While no monoallelic deletions were found in common nevi and in less than 10 % of dysplastic nevi, hemizygous deletions were found in about 19 % of the primary and in 37 % of metastatic melanoma. The loss of one allele of *CDKN2A* leads to loss of p16 expression in advanced invasive melanoma due to the epigenetic silencing of the second *CDKN2A* allele (Sini et al., 2008).

Besides inactivation via CDKN2A, a small number of oncogenic autosomal-dominant germline mutations of CDK4 have be found in familial melanoma. The CDK4 mutations identified so far are located in exon 2. The affected codon 24 is directly involved in the binding of p_{16}^{INK4a}, thus preventing the interaction between p_{16}^{INK4a} and CDK4. This in turn prevents cell cycle arrest by not inhibiting the phosphorylation of Rb. Phenotypically this mutations is identical to the CDKN2A mutations (Zuo et al., 1996).

MITF

MITF is known as the "master regulator" of melanocytes and also plays a role as a lineage survival oncogene for the development of melanoma. Ectopic expression of Mitf together with mutated $BRAF^{V600E}$ has been shown to be able to transform melanocytes into melanoma and this established it as an oncogene (Dynek et al., 2008; Garraway et al., 2005).

Somatic mutations of *MITF* are relatively rare and happen in only 8 % of the melanoma samples analyzed. In these melanomas amplifications have not been found which shows that mutations and amplifications of *MITF* are happening in a mutually exclusive manner. Mutations in *MITF* are found in the domains responsible for transactivation, DNA binding as well as in the helix-loop-helix region (Cronin et al., 2009). These mutations are most likely to change the way in which MITF recognizes target genes leading to misregulation and cancer transformation (Grill et al., 2013). Amplification of *MITF* happens in only 10 % of the primary cutaneous and 21–40 % of the metastatic tumors (Bishop et al., 2000; Garraway et al., 2005; Gast et al., 2010).

In addition to the somatic mutations, one germline mutation has been identified in *MITF*: The E318K mutation (Bertolotto et al., 2011; Yokoyama et al., 2011). This exchange renders a recognition site for SUMOylation inactive, leading to differential regulation of several MITF target genes including pigmentation genes.

The mutant MITF protein enhances the expression of the known target gene HIF1α, which plays a pro-survival role in the cell and is important in cancer (Busca et al., 2005). The mutation confers a five fold increased risk for melanoma and renal cell carcinoma. The E318K mutation in MITF has been associated with multiple primary melanoma, a family history of melanoma, increased nevus count and non-blue eye color (Bertolotto et al., 2011; Yokoyama et al., 2011).

1.10.5. Conventional treatment of Melanoma

Metastatic melanoma is a notorious, treatment resistant disease. While primary melanoma can be removed by surgery (most often this is the only treatment needed), this changes as soon as the tumors start to grow vertically and spread throughout the body. Classical approaches use chemotherapy and immunotherapy, but with only rather limited success. Classical chemotherapy uses dacarbazine (DITC), but only 15 % of all melanoma patients respond to this drug with most of the responses being partial. Also combination with other therapies (like immunotherapies) has brought no improvement (Hill et al., 1984; Lui et al., 2007).

Immunotherapies using substances such as IFN-α (Interferon α) or IL-2 (Interleukin 2) show similar or even lower response rates but are connected with a higher toxicity. With IL-2 treatment, severe side effects such as lymphocytosis immediately after treatment and long-term immunologic side effects were observed, especially vitiligo (Eggermont and Schadendorf, 2009; Phan et al., 2001).

1.10.6. Targeted melanoma therapy

The problems with treating metastatic melanoma finally led to the development of targeted therapies. These are small molecule inhibitors, targeted specifically to the mutated parts of the signalling pathways (e.g. $Braf^{V600E}$) which do not interfere with non-mutated signalling pathways.

BRAF inhibitors

41 % of the melanomas contain a mutation in the BRAF kinase (Maldonado et al., 2003). The most common BRAF mutation is the $BRAF^{V600E}$ mutation. It occurs in around 80 % of cases, with V600K being the second most common mutation followed by the more rare V600R, V600D, V600M, L597 and K601E mutations (Lovly et al., 2012). Two different inhibitors which target the V600E mutation with high specificity are available.

Vemurafenib

Vemurafenib (also known as PLX4032 or Zelboraf) is a specific BRAF inhibitor (see Figure 1.7), that has a high specificity for the V600E mutated kinase. It has been shown to inhibit cellular proliferation of tumor cells carrying the $BRAF^{V600E}$ mutation (Tsai et al., 2008) by binding to the mutant BRAF monomer and thus inhibiting it. The dimerization of wild-type BRAF molecules is not affected (Poulikakos et al., 2010). The drug was tested in Phase I and II clinical trials, which showed that it has a specific anti-tumor activity. The objective response rate was around 53 %, the responses of the tumor were very fast, leading to a stabilization of the disease in most patients (Sosman et al., 2012).

The Phase III trial demonstrated an objective response rate of 59 % with an increased 6 month overall survival rate compared with DITC (dimethyl traizeno imidazole carboxamide) (84 % vs. 64 %) and also a higher median survival than for chemotherapy (13.6 month vs. 9.7 month). The median progression-free survival (PFS) was also better for vemurafenib (5.9 vs. 1.6 month) (Chapman et al., 2011). On the downside, resistance to the therapy and progression of the tumor developed within 2 years for almost all patients, although some of the treated patients remained in remission for 24 months (Sosman et al., 2012).

Although vemurafenib is usually quite well tolerated by the patients, it has shown some side effects. They include arthralgia, fatigue, edema, nausea, and prolonged QT interval (delayed repolarization of the heart). In the first 2–3 months of the treatment, about 20 % of the patients developed secondary cutaneous squamous cell carcinomas and keratoacanthomas which often have RAS mutations (Chapman et al., 2011; Su et al., 2012).

Dabrafenib

Dabrafenib (also know as GSK2118436) is also a specific $BRAF^{V600E}$ inhibitor (see Figure 1.7). Preclinical studies show activity against melanoma *in vivo*. Phase I and II trials in melanoma patients showed objective responses in 53–69 % of the patients. The development of resistance is very similar to vemurafenib. In a phase III trial, which compared dabrafenib to DITC showed higher response rates for the inhibitor (53 % vs 16 %) and a better PFS (5.3 vs. 2.7 months). The side effects of dabrafenib differ from vemurafenib, with fevers and chills more common and a slightly lower incidence of cutaneous squamous cell carcinomas or keratoacanthomas for dabrafenib (Hauschild et al., 2012).

MEK inhibitors

The inhibition of MEK blocks the signalling to ERK in the map kinase pathway. This is interesting as both signalling from BRAF and also from CRAF (when cells develop resistance to vmurafenib/dabrafenib) target MEK. Inhibition of

MEK results in cellular proliferation and thus tumor growth in BRAF mutant melanoma. For review see Sullivan and Flaherty (2013).

Trametinib

The MEK inhibitor Trametinib (also known as GSK1120212) is a small molecule inhibitor of MEK1 and MEK2. Its effectiveness against MAP kinase signalling was shown in early trials, together with acceptable side effects (Infante et al., 2012). A phase III trial that compared trametinib with DITC in patients with BRAF mutations, the overall six month survival was improved (91 % vs. 67 %). The PFS was also higher (4.8 vs. 1.5 months); the objective treatment response rate was 22 %. The treatment caused relatively little side effects with acneiform rash, peripheral edema, diarrhea and blurred vision observed in the patients. In contrast to the BRAF inhibitors the patients did not develop squamous cell skin cancers (Flaherty et al., 2012).

1.11. The interferon regulatory factor 4

IRF4 belongs to the transcription factor family of interferon regulatory factors (for review see Paun and Pitha (2007)), a wing-helix-turn-helix family of proteins (Escalante et al., 1998; Furui et al., 1998). The gene is located on chromosome 6p25-p23 in humans and consists of nine exons (see upper part of Figure 1.8 for details). Interestingly, intron 4 of *IRF4* contains three binding sites for MITF and a single nucleotide polymorphism (SNP) (rs12203592) that has been connected to pigmentation (Han et al., 2008; Sulem et al., 2007).

The IRF4 protein consists of 451 amino acids (aa) and has a mass of approximately 52 kDa (Grossman et al., 1996). The protein consists of six different domains, as can be seen in the lower part of Figure 1.8. These are the DNA binding domain, the P-rich domain, the activation masking domain, the α–helical domain, the Q–rich domain and the auto inhibitory domain at the c–terminus (Marecki and Fenton, 2002).

The IRF family is important in the regulation of interferon–inducible genes and for the response against viral infections. It was initially characterized in lymphocytes where it plays different roles depending on whether other transcription factors are present to form a complex (Eisenbeis et al., 1995).

Although IRF4 does not depend on interferon stimulation (Grossman et al., 1996), it binds to the IFN–stimulated response element (ISRE) within IFN–responsive genes (Escalante et al., 1998). IRF4 has weak DNA binding potential which is increased in the presence of other transcription factors including PU.1 (Eisenbeis et al., 1995). IRF4 shows high homology to IRF8, another factor of the IRF family; both factors are primarily expressed in lymphocytes, macrophages and dendritic cells (DC) (Eisenbeis et al., 1995; Politis et al., 1992). Analysis of

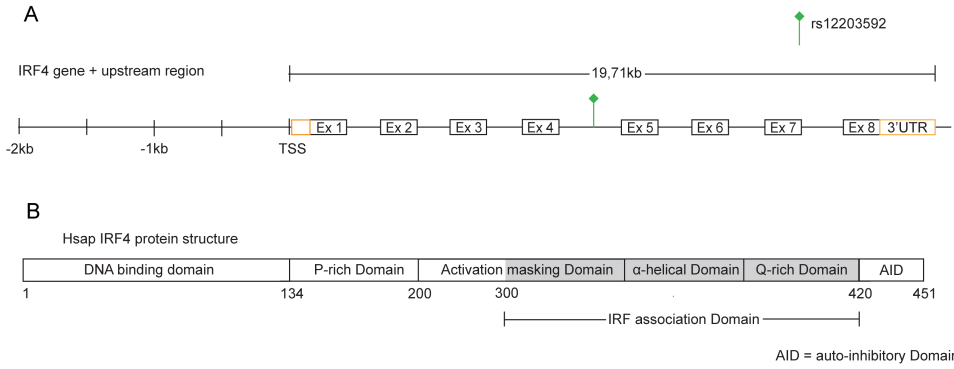


Figure 1.8.: *IRF4* gene and protein domain structure A. Structure of the *IRF4* gene locus. B. Domain structure of the human IRF4 protein. Subfigure B modified after Marecki and Fenton (2002)

plasma cells showed high expression of IRF4 while none was detectable in the rest of the germinal center (GC) B cells. The expression of IRF4 is limited to the light zone of the GC, the region where the B cells mature into plasma cells (Cattoretti et al., 2006; Falini et al., 2000).

Consequently, IRF4 plays a key role in late phases of B cell development. IRF4 mutant mice completely lack germinal centers (GC) and plasma cells and show severely reduced immunoglobulin levels in the serum (Mittrucker et al., 1997). Experiments with IRF4 knock-out mice indicated that IRF4 is not required for the generation of GC and memory B cells. Low levels of IRF4 may play a role in the maintenance of germinal centers. IRF4 plays a critical role for two processes: The class switch recombination and generation of plasma cells from the GC and also from memory B cells. In the IRF4 knock-out mice neither process is present (Klein et al., 2006). Co-expression of IRF4 with BLIMP1, which serves as the master regulator of plasma cell development, suggested that IRF4 is important for the development of plasma cells (Angelin-Duclos et al., 2000).

IRF4 also plays a critical role in multiple myeloma. In some cases the gene is translocated into the immunoglobulin heavy-chain regulatory regions and thus deregulated. These translocations have been observed also for T cell lymphoma, as well as unspecified and cutaneous anaplastic large-cell lymphoma (Shaffer et al., 2009). Besides the oncogenic potential in translocations, cancer cells can depend on IRF4 although it is neither mutated nor amplified. Here IRF4 plays a role as a lineage survival oncogene, whose presence is important for the development of cancer cells. When IRF4 is depleted from these cells with a siRNA directed against the 3'UTR of the IRF4 transcript, multiple myeloma cells stop to proliferate and die (Shaffer et al., 2008).

Polymorphisms in or near the *IRF4* gene have been connected to a number of

diseases. The intergenic rs1033180 polymorphism has been connected to celiac disease (Dubois et al., 2010). Other examples are the rs872071 polymorphism, located in the 3'UTR of *IRF4* which has been connected to chronic lymphocytic leucemia (Di Bernardo et al., 2008) and the rs12203592 polymorphism in intron 4 of *IRF4* associated with progressive supranuclear palsy (Hoglinger et al., 2011). These findings underline the importance of IRF4 outside the context of the immune system.

In addition to the association studies described above, a few reports have linked IRF4 to pigment cells. Grossman et al. (1996) found that melanocytes in the skin as well as the melanoma cell line G361 express IRF4. Furthermore, Sundram et al. (2003) have shown that IRF4 is expressed in most melanomas. However, the role of IRF4 in human pigmentation has so far not been characterized.

1.12. The transcription factor activating protein 2α (TFAP2α)

The transcription factor activating protein 2α (TFAP2α) belongs to a family of transcription factors that consists of five family members in human, mouse and rat (TFAP2α, β, γ, δ and ε) (Hilger-Eversheim et al., 2000; Wang et al., 2004; Zhao et al., 2001) while other species have less (chimpanzees have 2, chicken 4). The factor contains a highly conserved helix-span-helix dimerization motif at the c-terminus, which is preceded by central basic region and a region of less conservation which is rich in glutamine and proline (P/Q-rich domain) at the amino-terminus (see Figure 1.9). The helix-span-helix region is responsible for protein dimerization, both basic and helix-span-helix domains are responsible for DNA binding and the P/Q-rich domain is necessary for transactivation. The protein functions either as a homo- or heterodimer and does not bind to DNA using the basic domain when the helix-span-helix domain is mutated and vice versa. (Williams and Tjian, 1991a,b).

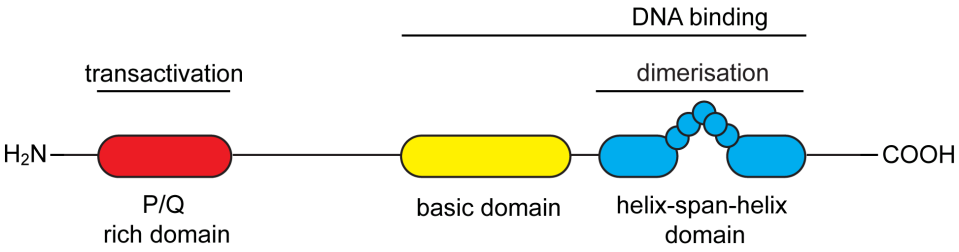


Figure 1.9.: Domain structure of the TFAP2α protein. Figure modified after Eckert et al. (2005).

The activity of the TFAP2 proteins is regulated at different levels: By regulating their subcellular localization, their transactivation and their DNA binding

(Aqeilan et al., 2004; Mazina et al., 2001; Pellikainen et al., 2004). In addition TFAP2α is regulated by post-translational modifications including PKA mediated phosphorylation (Garcia et al., 1999; Park and Kim, 1993), SUMOylation (Zhong et al., 2003) and redox regulation (Grether-Beck et al., 2003; Huang and Domann, 1998). TFAP2 proteins also interact with various other proteins (for review see Table 3 in Eckert et al. (2005)). TFAP2α and the other members of the TFAP2 family are expressed in neural crest, breast epithelium, skin and other embryonic cell types and control cell specification, differentiation, survival and proliferation (Eckert et al., 2005; Hilger-Eversheim et al., 2000). It has been shown that TFAP2α binds to a site in the *KIT* promoter and is involved in the regulation of *KIT*, which itself is involved in the regulation of *MITF*. At least in a melanocytic background the regulation of TFAP2α seems to be regulated by MITF (Hoek et al., 2008).

2. Aims

2.1. Genes regulated by MITF

1. Identify new target genes of MITF using gene expression analysis.

2.2. MITF and IRF4

1. Verify *IRF4* as a target gene of MITF.
2. Characterize the MITF binding sites in Intron 4 of *IRF4*.
3. Characterize the impact of the rs12203592 polymorphism on IRF4 expression.

2.3. Ephelides and solar lentigines

1. Analyze the available literature for information on ephelides and solar lentigines.

3. Materials and Methods

3.1. Sequences

Sequences used for the work were retrieved using the Ensembl Genome Browser (http://www.ensembl.org/index.html). The TFAP2α motif in intron 4 of *IRF4* was identified using with TESS (Transcription Element Search System, available online at http://www.cbil.upenn.edu/tess). The identification and location of the potential MITF binding sites in the *IRF4* promoter as well as in intron 4 of *IRF4* was done using the DNA-pattern tool from the RSAT (regulatory sequence analysis tool) tool box. For further details see Thomas-Chollier et al. (2011).

3.2. Cloning

For cloning intron 4 of *IRF4*, the sequence was amplified with appropriate primers (see table A.1 in the appendix) containing the recognition sites of the *Mlu*I and *Xho*I restriction enzymes (and additional bases to guarantee function of the enzyme) at the 5' and the 3' ends respectively. The product of the polymerase chain reaction (PCR) reaction was digested with the respective restriction enzyme either directly (if there was only one product band in the gel) or after clean-up (see section 3.15 for details). The pGL3basic vector (Promega) was also digested with *Mlu*I and *Xho*I. After digestion the reactions were cleaned using the agarose elution method. Afterwards the vector and the insert were mixed in a molecular ratio of 1:6 and DNA ligation buffer and T4-DNA ligase (Fermentas) added. The mix was incubated for 20 min at 20 °C and then transformed into bacteria as described in section 3.16. The bacteria were plated on LB-media and grown overnight at 37 °C. A number of clones were picked, grown on plates and checked with PCR for the presence of the intron 4 insert. From positive clones DNA was prepared and sequenced to verify the correct sequence.

Mutations were introduced by using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions. Oligo sequences used for generating the mutations are listed in table A.3. The IRF4 expression construct was provided by Dr. Singh (Brass et al., 1996) and the TFAP2A expression construct by Dr. Bar-Eli (Tellez et al., 2003).

3.3. Sequencing

For sequencing DNA the target sequence was directly sequenced from a plasmid (if there was enough material) or the sequence was amplified by PCR first (if the template DNA was not available in sufficient quantities or if the target sequence was too big, so it needed to be split in smaller, overlapping pieces). Sanger di-deoxy chain termination method was used according to the instructions provided with the BigDye 1.1 Kit from Life Technologies.

For sequencing DNA was mixed with primer, the sequencing buffer and the BigDye reaction mix as described below.

Table 3.1.: Setup of sequencing PCR reactions

For each reaction the following reagents were added to a separate PCR tube	
Reagent	Quantity
BigDye reaction mix	2 µl
BigDye sequencing buffer	2 µl
Template	Plasmid: 100–300 ng PCR-reaction: 50–100 ng
Primer [1 pmol per µl]	3.2 µl
Water	fill up to 10 µl
total	10 µl
Mix well and spin tubes briefly	

The PCR reaction was precipitated with 96 % ethanol, washed with 70 % ethanol and air-dried for 5 min. The pellet was then dissolved in 15 µl formamide and pipetted into a 96 well plate before loading on the sequencer.

3.4. Cell culture

The cell lines 501mel, SKmel28 and HEK293T were cultured in DMEM medium (Life Technologies) with the addition of 10 % fetal bovine serum (Life Technologies), 2 mM Glutamax (Life Technologies) and antibiotics (100 IU/ml penicillin and 100 µg/ml Streptomycin, Life Technologies). Cells were grown at 37 °C in an incubator in 5 % CO_2 and a humid environment. Cells were seeded into 10 cm dishes (Nunc) for cultivation at a density of 25 – 30 % confluency and grown until they reached 80–90 % density before they were split again. The 501mel cells were a kind gift from Ruth Halaban, the SKmel28 and 293T cells were obtained from ATCC.

For the overexpression of MITF in 501mel and 293T cells the cells were seeded in 6 well plates (Nunc). Then they were transfected as described above with a

construct containing the mouse *Mitf* cDNA in pCDNA3.1 and cultivated for 24 hours. After this period total RNA was extracted as described in Section 3.7, cDNA prepared and Q-RT-PCR performed with primers specific for mouse *Mitf* and for human *TYR* and *IRF4*.

3.5. Transfection of cells

Depending on the experiment cells were either seeded onto white 96 well plates (Nunc) (for luciferase experiments), six well plates (for shRNA experiments, protein isolation) or 10 cm dishes (for chromatin immunoprecipitation (ChIP) experiments). The number of seeded cells, amount of DNA and transfection reagent depended on the culture size and can be seen in the table below.

Table 3.2.: Reagents for cell transfection

experiment type	plate	cells per well	DNA	150 mM NaCl	transfection reagent (Exgen500)
luciferase experiment	white 96 well	8000	150 ng	up to 10 µl	0.5 µl
shRNA experiment	6 well	1×10^5	4 µg	up to 100 µl	9 µl
ChIP experiment	10 cm dish	5×10^5	24 µg	up to 500 µl	75 µl

The cells were split one day before the transfection and seeded on the appropriate plates. Before the transfection, medium was changed to provide optimal conditions for the cells and to remove dead cells. The appropriate amount of DNA was diluted with 150 mM NaCl, then the transfection reagent (Exgen500 from Fermentas) was added, the mixture was mixed gently and incubated for 15 min. After the incubation the mixture was added drop-wise to the cells to avoid detaching the cells from the plates. The following day, the transfection media were changed and puromycin added in a final concentration of 5 µg per ml. The selection was continued until all cells in the control well of the selection plate where dead.

3.6. shRNA mediated knockdown of genes

To downregulate specific genes in cell lines, shRNA-plasmids and controls from OpenBiosystems (Mitf V2THS_76565 and V2LHS_257541; IRF4 V3LHS_377530 and V3LHS_377531; TFAP2a V3LHS_344899, neg. control

RHS4346) were used. These plasmids express the precursor of a shRNA (short hairpin RNA), which is processed into the active form and then binds to its target sequence and downregulates the corresponding gene. The shRNA-plasmids were transfected into the cells as described in section 3.5 and the cells then allowed to grow for 24 h. After this period the medium was exchanged and puromycin added Before extraction of total RNA, the cells were grown to 80–90 % confluency and total RNA extracted as described in section 3.7 and used for cDNA preparation.

3.7. RNA extraction

For RNA extraction, heart tissues from homozygous C57BL/6J-Mitf$^{mi-vga9}$ mice as well as controls were collected and frozen in liquid nitrogen until RNA extraction. RNA was also extracted from cultures of 501mel and SKmel28 cells.

Total RNA was extracted from tissues (and cells) using Trizol (Invitrogen) according to the manufacturer's instructions. The resulting RNA was dissolved in 50 µl nuclease-free water and RNA concentration determined using Nanodrop ND-1000.

3.8. cDNA preparation

The RNA was treated with DNAseI (Qiagen) and cleaned using the RNeasy Kit (Qiagen) according to manufacturer´s instructions. The concentration of total RNA preparations (see section 3.7 for details) was measured and 2 µg RNA were used for cDNA preparation. The preparation was done with the "RevertAid First Strand cDNA synthesis kit" from Fermentas according to the protocol. The RNA was premixed with the oligo-dT primer and heated to 70 °C for 5 min for denaturation of secondary structures and cooled on ice afterwards. After adding the reaction buffer, dNTP and the reverse transcriptase the reaction tube was transferred into a PCR machine and heated for 60 min at 42 °C before heating up to 70 °C for 5 min to inactivate the enzyme. The cDNA was stored at -20 °C until it was used in qPCR.

3.9. real-time PCR

Real-time PCR reactions were prepared with cDNA. For each PCR reaction the cDNA equivalent to 50 ng of total RNA in the cDNA preparation (see section 3.8) was used.The cDNA was mixed with the Maxima SybrGreen real-time PCR master-mix from Fermentas and the appropriate primers (see table A.1 in appendix A). Reactions were set up as described in table 3.3. For all reactions master mixes were prepared. All samples were run in triplicates. Samples were run on an ABI 7500 PCR machine and analyzed afterwards with the REST2009

software package (Pfaffl et al., 2002). Expression was normalized to *GAPDH*. For gene expression analysis we assayed the following genes: *MITF, IRF4, TFAP2α*, *TYR* and *DCT*.

Table 3.3.: Setup of RT-PCR reactions

Reagent	Concentration	Volume
Master Mix	2×	5 µl
cDNA	–	0.5 µl
forward primer	10 µM	0.3 µl
reverse primer	10 µM	0.3 µl
water	–	3.9 µl
	total	10 µl

Table 3.4.: Program used for RT-PCR

Step	Temperature	Time	
Initial denaturation	94 °C	10 min	
Denaturation	94 °C	15 sec	40×
Annealing	55 °C	30 sec	
Extension	72 °C	30 sec	

3.10. Luciferase reporter assays

For co-transfection studies using the IRF4 reporter constructs, 10,000 cells were seeded onto 96 well plates. After the cells attached to the surface they were transfected with expression and reporter constructs using Exgene 500 (Fermentas). 50 ng of the reporter constructs and 15 ng of the MITF/IRF4/TFAP2A plasmids were transfected and the cells were grown for 24 h. The medium was removed completely and the cells lysed with 1× passive lysis buffer for 20 min at room-temperature. Afterwards the luciferase activity was measured using the Dual-glo luciferase kit (Promega) according to the manufacturer's instructions. Samples were measured in six replicates with three independent experiments.

3.11. Protein analysis

Cells were grown in 6 well plates in the presence of the shRNA constructs as described, grown to 80–90 % confluency and then lysed with RIPA buffer in the presence of protease inhibitors. Cell extracts where then denaturated with SDS-sample buffer and loaded on SDS-gels. For the western blots the gels where blocked with 5 % milk in PBS-T, incubated with the appropriate antibody

(Mitf: Thermo Fisher C5, TFAP2A: abcam ab52222, IRF4: Millipore 06-1047, TYR: Santa Cruz sc-7833, beta-actin: Millipore C4 MAB1501R), washed and incubated with the secondary antibody from Li-cor Biosystems (fluorescently labeled anti-mouse (IRDye 800CW Goat anti-Mouse), anti-rabbit (IRDye 800CW Donkey anti-Rabbit) or anti-goat (IRDye 800CW Donkey Anti-Goat). The blots were scanned on a Li-cor Odyssey infrared scanner. Bands were quantified using the ImageJ software package.

3.12. Chromatin immunoprecipitation

501mel cells were transfected with GST-tagged *MITF* or *TFAP2α* expression constructs. After reaching 80 % confluency, chromatin was cross-linked with formaldehyde, sonicated and the lysate pre-incubated with protein A/G-Sepharose beads (GE Healthcare). Pre-cleared chromatin samples were incubated with an antibody against the protein of interest (Mitf-GST or TFAP2α), human serum or no antibody. After overnight incubation the beads were isolated and the chromatin purified. Specific primers against the targeted regions (see table A.1) were used to analyze binding to DNA.

3.13. Annealing and radiolabeling of DNA oligonucleotides

The DNA oligos for radiolabeling have to contain a 5' G-overhang at the forward oligo so that the Klenow exo- polymerase can add a radioactive labeled cytidine on the complementary strand.

Annealing of the oligonucleotides

For the annealing 5 µl buffer (Boehringer Mannheim 10x), 250 pM of each oligo were mixed and filled up with water to 50 µl final volume. For annealing a PCR machine with the following program is used. The oligos are denaturated for 10 min at 95 °C and are then slowly cooled down at a rate of 0.1 °C/min. To check the annealing 1 µl of the annealed probe is run next to the single oligos on a 10 % native polyacrylamide gel. The gel is stained with ethidium bromide and analyzed under UV light.

Radiolabeling of the doublestranded oligonucleotides

The doublestranded oligos were labeled using P^{32}-labeled cytidine (GE Healthcare). All works with the radioactive labelled cytidine was performed applying the appropriate safety measures (shields, treatment of the waste etc.). To do so, 2.5 µl of the annealing mix were mixed with 5 µl klenow buffer, 1 µl BSA

(10 mg/ml), 1 µl DTT (0.1 M), 38 µl water. After mixing 1.5 µl of P^{32}dCTP and 1 µl Klenow exo- polymerase (Fermentas) were added. The reaction is incubated for 30 min at room temperature.

During the incubation the Sephadex column for the purification of the labeled oligo was prepared. Sephadex-G50 (equilibrated with the tenfold amount of Tris-EDTA buffer pH 8.0 (TE Buffer)) is filled into the column and the centrifugated in a 15 ml tube for 3000 rpm for 5 min. The flow-through in the tube is discarded. The probe is carefully added on the column and centrifuged for 1 min at 3000 rpm. The flow-through of the column is collected in a 1.5 ml microtube and frozen until used.

3.14. DNA binding assay

Electrophoretic Mobility Shift Assays (EMSA) were performed using proteins expressed in the TNT-T7 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's recommendations. 2 µl of *in vitro* translated proteins were analyzed by electrophoresis on 8 % SDS-polyacrylamide gels and electro-transferred to nitrocellulose (Odyssey) membranes. Membranes were incubated with antibodies against MITF (C5 , MS-771, NeoMarkers) or TFAP2A (Abcam) and then with secondary antibodies conjugated to IRDye-800 (Licor Biosciences). The blotted proteins were detected and quantified using the Odyssey infrared imaging system. Equivalent protein quantities were added to gel shift assays.

The radiolabeled oligonucleotides carry the sequence of the potential TFAP2A binding site, the same sequence including the rs12203592 polymorphism and sequence where the entire binding site was mutated were used (sequences can be found in table A.2). The probe is located at position chr6: 396309-396333 in build GRCh37.p5 of the human genome.

For determining the DNA-binding ability of MITF, the probes listed in table A.1 were used. For the MITF protein, 2 µl of TNT translated proteins were preincubated in buffer containing: 20 ng of poly(dI-dC), 10 % fetal calf serum, 2 mM $MgCl_2$, and 2 mM spermidine for 15 min on ice. The TFAP2A protein was made using the TNT system and the probes incubated in reaction buffer as described (Li et al., 2006). For supershift assays, 1 µg anti-TFAP2a antibody or 1 µg anti-MITF antibody were added and the sample was incubated on ice for 30 min. For the MITF protein we followed the procedure of Pogenberg et al. (2012). For both TFAP2A and MITF, the resulting DNA-protein complexes were resolved on 4.2 % non-denaturing polyacrylamide gels, placed on a storage phosphor screen and then scanned on a Typhoon Phosphor Imager 8610 (Molecular Dynamics).

3.15. Purification and Elution of DNA from agarose gels

The purification of DNA from agarose gels was done as described by Rogers, 1995 in the "Molecular Biology Techniques Laboratory Manual."

Briefly described the digested DNA was run on a low-melting agarose gel (Lonza), the bands of the right size were cut out and dissolved at 65 °C in a high salt elution buffer. The solution was then frozen in liquid nitrogen or a -70 °C freezer and centrifuged for 10 min at room-temperature in a bench-top centrifuge at maximum speed (14.000 rpm). The centrifugation step separates the agarose (which will form a pellet) and the solution which contains the DNA. Afterwards the ethidium bromide was removed with butanol, then the DNA was precipitated using ethanol, washed, dried and re-dissolved in TE-buffer.

3.16. Preparation and transformation of competent cells

The preparation and transformation of competent cells is described in detail in appendix B. In short the fresh grown cells where treated with a buffer containing manganese salts to reach competency. They were then shock frozen in liquid nitrogen and stored at -80 °C before use.

For transformation, cells are slowly thawed on ice. 50 {textmul of competent cells are mixed with the DNA (10–50 ng) to be transformed and incubated for 30 min on ice, followed by an incubation for 1 min at 42 °C. After this treatment 500 µl SOC media are added and the cells incubated at 37 °C for one hour before diluting and plating different dilutions on appropriate selective agar plates.

4. Results

4.1. Gene expression analysis (published in Paper I)

To find new MITF target genes, we used gene expression analysis. In order to do this, expression of genes in SKmel28 cells transfected with *MITF* was compared to untransfected SKmel28 cells. Since this cell line is a melanoma cell line, we expected to identify genes which are important in melanomas as well as in the pigment process.

The gene expression analysis resulted in a list of 6.936 genes whose expression changed more than two fold upon transfection of MITF (see Figure 2 in Paper I). Since this list contains not only true targets of MITF but also genes which are differently expressed due to secondary effects and also false positives, it needed to be filtered further to identify the genes which correlate with MITF expression.

In order to do this, we used seven publicly available gene expression data sets from melanoma patient samples. The hypothesis was made that each gene which showed a positive Pearson correlation (with a r-value >0.5) to MITF expression in all seven datasets is likely to be regulated by MITF (see Figure 3 in Paper I). This correlation analysis led to a list of 110 genes which strongly correlated in all seven datasets with the expression of MITF.

This list of 110 genes was then compared to the list of 6.936 significantly upregulated genes obtained from the gene expression analysis, 84 genes were present on both lists and thus are likely to be target genes of MITF (see Table 2 in Paper I). The list contains 13 of 42 known MITF target genes (see table 1 in paper I). The identification of 13 target genes among the 84 genes is highly significant ($p<{}^{-18}_{10}$, hypergeometric distribution), which makes it highly likely that MITF is involved in the regulation of the 71 new potential target genes. Among the new target genes were those encoding transcription factors TFAP2α and IRF4 (Paper I).

4.2. Expression of *Mitf* and *Irf4* in mice (published in Paper II

IRF4 has been associated with pigmentation by Han et al. (2008) and Sulem et al. (2007). Thus, we hypothesized that *IRF4* is a target gene of MITF. As a first step in determining if this is true, we characterized the expression of *Mitf* and *Irf4* in mouse heart tissue, which is known to express MITF. To be able to

analyze relative gene expression, heart tissue from normal mice (B6-black) and $C_{57}BL_6/j-Mitf^{vga9/vga9}$ mice which do not express *Mitf* was harvested. Gene expression analysis after total RNA extraction, cDNA synthesis and quantitative PCR showed that *Mitf* expression is severely reduced in $Mitf^{vga9/vga9}$ mice (the small bar visible for Mitf in Figure 4.1 is a signal which is caused by self amplification of the primer in the late stages of the PCR). Furthermore expression of *Irf4* was significantly reduced in the MITF mutant mice (compared with the wild-type mice the expression of *Irf4* is reduced to 10 %).

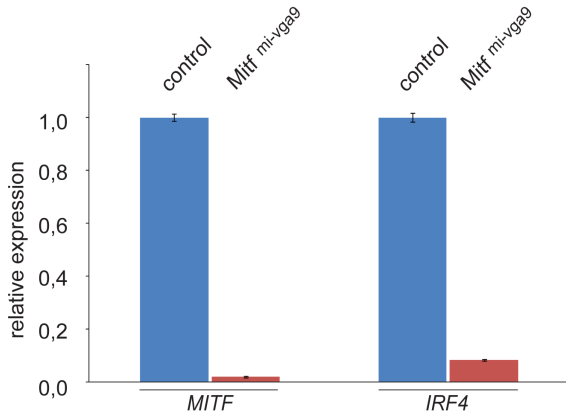


Figure 4.1.: Expression of *Mitf* and *Irf4* in the heart tissue of $Mitf^{vga9/vga9}$ and normal mice. Expression is relative to the control mice.

4.3. Overexpression of MITF in cell lines

To test the effects of MITF on *IRF4* expression, mouse *Mitf* was overexpressed in 501mel melanoma and HEK293T cell lines. Since MITF is endogenously expressed in 501mel cells, mouse Mitf was used to monitor the expression from the expression plasmid with a set of species specific PCR primers. The empty expression vector (pcDNA3.1) and no vector were used as transfection controls.

After transfection, the cells were harvested, total RNA extracted and cDNA prepared. For detection of gene expression, PCR was performed followed by agarose gel electrophoresis. PCR primers specific for mouse *Mitf*, *TYR* (as a positive control), *IRF4* (gene of interest) and *β-actin* (as a negative control) were used.

Upon transfection of *Mitf* into 501mel and HEK293T cells, expression from the construct could clearly be detected (see first lane in Figure 4.2) whereas neither transfection control showed signal. The expression of *TYR* increased in the 501mel cells compared to the transfection controls (the lanes pcDNA and

empty show basal *TYR* expression due to endogenous *MITF* expression in the cells). Expression of *TYR* is only observed in HEK293T cells when transfected with *Mitf*, but not with the controls (HEK293T cells do not express *MITF* endogenously and thus have no expression of *TYR*).

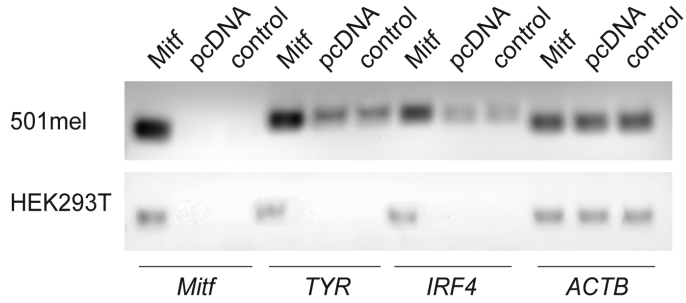


Figure 4.2.: Overexpression of *Mitf* in 501mel and HEK293T cells: Cells were transfected with either mouse Mitf, empty vector (pcDNA3.1) or with the transfection reagent only. The expression of MITF, IRF4 and β-actin (ACTB) was analyzed with PCR.

The expression of *IRF4* in 501mel cells was also increased when transfected with *Mitf*. In HEK293T cells *IRF4* was only expressed, when transfected with *Mitf*. The negative control β-actin was unaffected in all transfections, regardless of whether *Mitf* or the empty pcDNA3.1 vector was transfected in both 501mel and HEK293T cells.

4.4. shRNA mediated knockdown of genes

To verify the results from the overexpression of *Mitf* and the gene expression study, we used a shRNA based approach to specifically knock down single genes. The shRNAs were expressed from a vector which was transfected into the cells and which carries the sequence of the pre-shRNA. After transcription this pre-shRNA is processed by the cell to get a mature shRNA which then targets a specific mRNA transcript of the target gene and causes its degradation. For the experiment, the cells where transfected, total RNA extracted, cDNA prepared and then the expression of the gene of interest was measured with qPCR relative to untreated cells.

Transfection with shMITF (see the blue bars in Figure 4.3) downregulated the expression of *MITF* in the 501mel cells, to about 45 % compared to the control. It also led to the downregulation of *IRF4*, *TYR* and *DCT* transcripts (both positive controls, as they are known MITF target genes) as well as *TFAP2α* thus validating it as a new target gene for MITF.

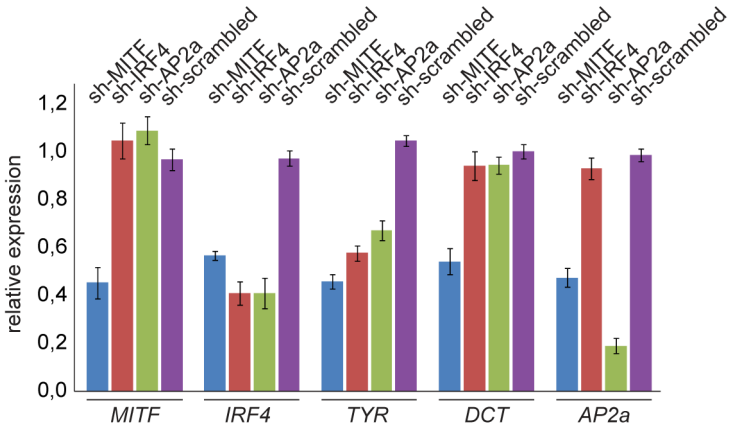


Figure 4.3.: Knock-down of *MITF, IRF4, DCT, TYR* and *TFAP2a* with shRNA transfected into cell lines. The gene expression is analyzed with qPCR and normalized to *ACTB*.

Targeting IRF4 by shRNA reduced expression of *IRF4* (red bars in Figure 4.3) to about 50 % and had no effect on expression of *MITF*, *DCT* or *TFAP2α*. Interestingly knocking down IRF4 had an effect on *TYR*, which was reduced to 50 % of its expression.

The transfection of a shRNA expression vector against *TFAP2α* reduced expression of *TFAP2α* to about 25 % compared to the control, but also reduced the expression of both *IRF4* and *TYR*. *MITF* and *DCT* where not affected by shTFAP2α. This result suggests that TFAP2α is involved in the regulation of IRF4 and TYR expression and may play a role in regulating in pigmentation.

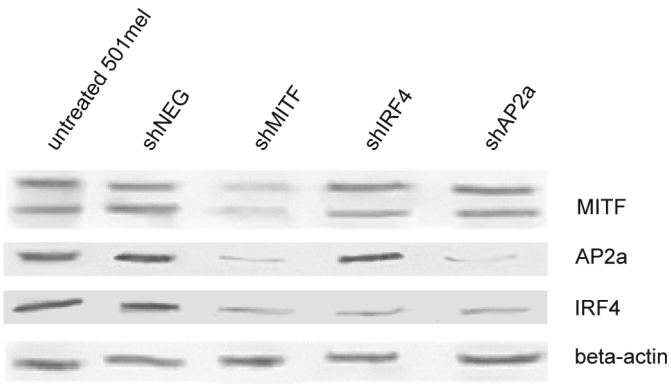
The transfection of a scrambled control shRNA expression vector did not influence the cells. This also shows that the shRNA vector itself had no effect on the transcription of the regulated genes.

4.5. Protein expression after shRNA transfection of cell lines

To verify that transfection of the cells with shRNA expression vectors also affect levels of the targeted proteins (and not only the transcript level) we did western blots with whole cell lysates from transfected cells and controls. The blots were stained with antibodies against MITF, IRF4, TFAP2α and β-actin as control.

The results confirmed the findings shown in section 4.4. Upon knockdown with sh*MITF*, the protein levels of MITF were reduced to about 41 %. The protein levels of IRF4 and TFAP2α were also reduced (to 25 % and 21 %, respectively).

Knockdown of *IRF4* only affected the IRF4 protein level and did not affect



1.00	1.03	0.41	1.00	0.92	MITF
1.00	0.95	0.21	0.94	0.21	AP2a
1.00	0.93	0.25	0.20	0.21	IRF4
1.00	1.00	1.00	1.02	1.07	beta-actin

Figure 4.4.: Protein expression of MITF, IRF4 and TFAP2α following a shRNA mediated knocked of the genes. Samples were analyzed on western blots. The table below the figure shows the band intensities measured with ImageJ and normalized to β-actin. The values are relative to the untreated control.

the protein level of MITF and TFAP2α. The transfection of shTFAP2α reduced TFAP2α to 21 % and the protein level of IRF4 to 21 %.

In untransfected cells and the cells transfected with a scrambled shRNA (negative control) the protein levels did not change (see the first two columns of figure 4.4). The protein levels of β-actin did not change throughout the experiment.

4.6. Analysis of the *IRF4* locus

To understand the results of the shRNA knockdown and to identify the potential MITF binding sites which are necessary for a direct interaction with MITF, we analyzed the *IRF4* gene locus. Using information from ChIP sequencing experiments performed in 501mel cell in Irwin Davidson's laboratory (Strub et al., 2011) we located the potential MITF binding sites in intron 4 of *IRF4*.

A number of potential binding sites for MITF are located about 2 kb upstream of the transcription start site (TSS). Three additional sites are found in intron 4 of *IRF4*. Also located in intron 4 in close proximity to the potential MITF binding sites is the rs12203592 polymorphism.

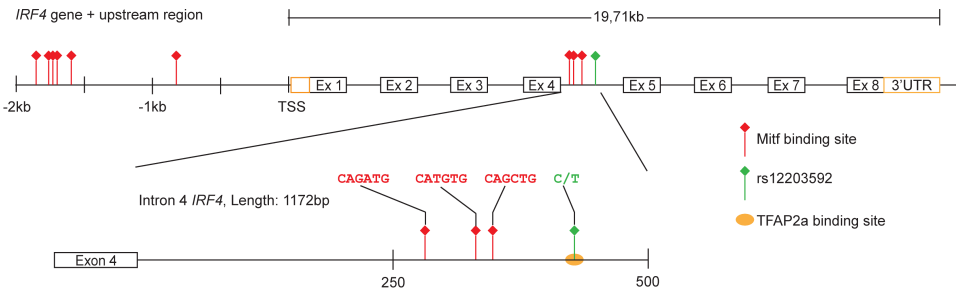


Figure 4.5.: Analysis of the *IRF4* locus with potential transcription factor binding sites and the rs12203592 polymorphism marked. Intron 4 is enlarged.

Interestingly the Transcription Element Search System (TESS) predicted a potential TFAP2α binding site at the exact location of the rs12203592 polymorphism. The *IRF4* locus with the potential binding sites is shown in Figure 4.5.

4.7. Chromatin immunoprecipitation of MITF and TFAP2α

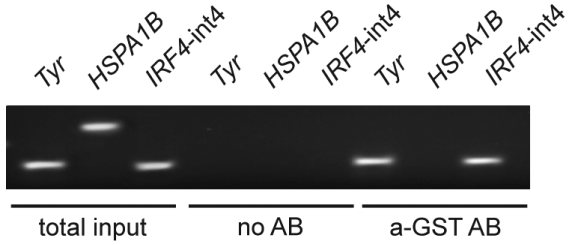


Figure 4.6.: ChIP of GST-tagged MITF transfected cells. The precipitated DNA is analyzed with PCR for the presence of intron 4 of *IRF4* (gene of interest), *TYR* (positive control) *HSPA1B* (negative control)

To test if MITF binds to intron 4 of *IRF4*, we performed a chromatin immunoprecipitation experiment with a GST-tagged MITF and a GST-specific antibody. The ChIP shows presence of all three amplified regions in the total input (see Figure 4.6): *Tyr* as a positive control, *HSPA1B* as a negative control and *IRF4-Int4* as region of interest. The negative control shows no bands indicating, that no unspecific DNA binding has taken place in the ChIP. The

GST-IP shows bands for the positive control *TYR* as well as for *IRF4*-Int4. This result confirms binding of MITF to intron 4 of *IRF4*.

To test the binding of TFAP2α to intron 4 of *IRF4* a ChIP was done with a TFAP2α specific antibody.

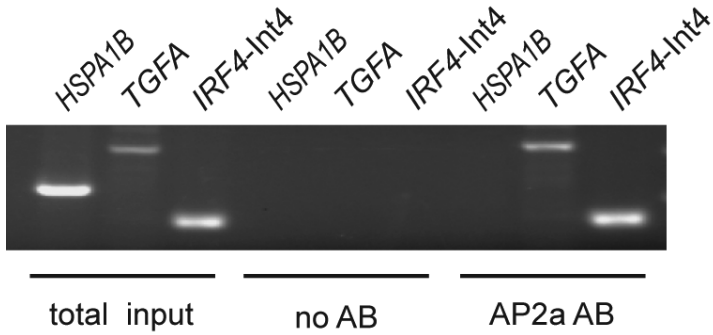


Figure 4.7.: ChIP of TFAP2α transfected into the cells. The precipitated DNA was analyzed with PCR for the presence of intron 4 of *IRF4* (gene of interest), *TGFA* (positive control) *HSPA1B* (negative control)

The ChIP of TFAP2α shows presence of all three amplified regions in the total input (see Figure 4.7). These are *TGFA* (positive control), *HSPA1B* (negative control) and *IRF4-Int4* (region of interest). For the negative (no antibody) control no signal was seen in the PCR which indicates that no DNA was enriched and precipitated in the ChIP experiment and that no unspecific amplification took place. When the TFAP2α-antibody was used, only bands for the positive control (*TGFA*) and intron 4 of *IRF4* were present. This indicates the precipitation of TFAP2α bound sequences in the ChIP and confirms the ability of TFAP2α to bind to the specific region in intron 4 of *IRF4* as shown in Figure 4.5.

4.8. EMSA with TFAP2α on intron 4 of IRF4

To characterize the TFAP2α binding site further and to test the effect of the rs12203592 polymorphism on TFAP2α binding to the *IRF4* intron sequence we used an electromobility shift assay (EMSA). The TFAP2α protein was incubated with radioactively labeled oligos containing the potential binding site, the site with the rs12203592 polymorphism and an oligo where the entire binding site was mutated.

The TFAP2α protein binds the oligo containing the wild type sequence from intron 4 of *IRF4* (see Figure 4.8). This band can be supershifted when an antibody specific to TFAP2α is added to the reaction. This confirms that the complex of protein and oligo is specific for the binding of the TFAP2α binding

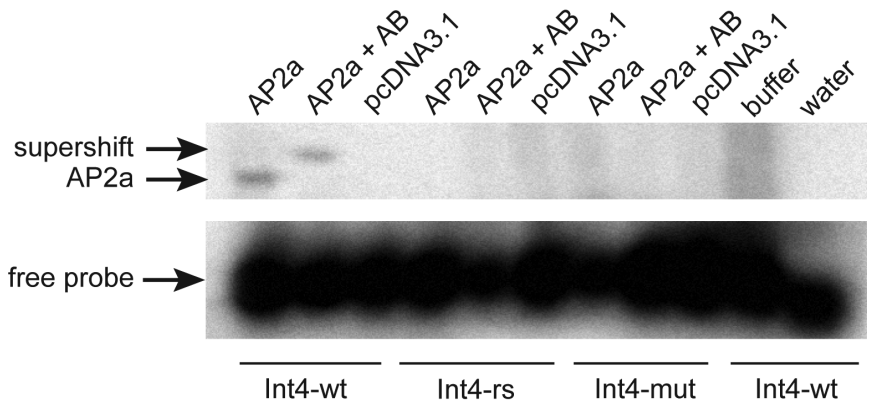


Figure 4.8.: EMSA with TFAP2α protein and radioactive labeled oligos. TFAP2α binds to the wild type oligo causing an upshift, which can be supershifted if an antibody against TFAP2α is added.

site and is not the result of unspecific binding between a protein and the marked probe.

When a probe with the rs12203592 polymorphism in the middle of the TFAP2α binding site was used, binding of the TFAP2α protein was not seen. The addition of a TFAP2α antibody did not result in a supershift when the polymorphism was present. This result shows that the polymorphism prevents TFAP2α binding.

The TFAP2α protein also did not bind to a probe with a completely mutated binding site. Also no binding occurred with EMSA reaction buffer or water only.

4.9. Gene activation assays with intron 4 of *IRF4*

To test which of the three MITF binding sites in intron 4 of *IRF4* were essential for activation, we used gene activation assays with luciferase reporter and subsequently mutated the potential MITF binding sites. For the assay a reporter construct containing the intron 4 element from *IRF4* together with an expression vector for the protein of interest were transfected into the cells. The luciferase expression from the reporter construct was assayed using the dual-Glo luciferase reporter kit with a luminometer.

Transfection of *MITF* and the reporter plasmid containing intron 4 did not activate the reporter. Similarly, neither a dominant negative MITF protein ($Mitf^{mi}$) nor TFAP2α were able to activate expression of the reporter (Figure 4.9). However, when MITF and TFAP2α expression vectors were transfected together, the reporter gene was activated about sixfold compared to the control. This activation was at background level activity when the dominant negative $Mitf^{mi}$ was used. The change in expression is significant ($p<0.0001$). Introduction

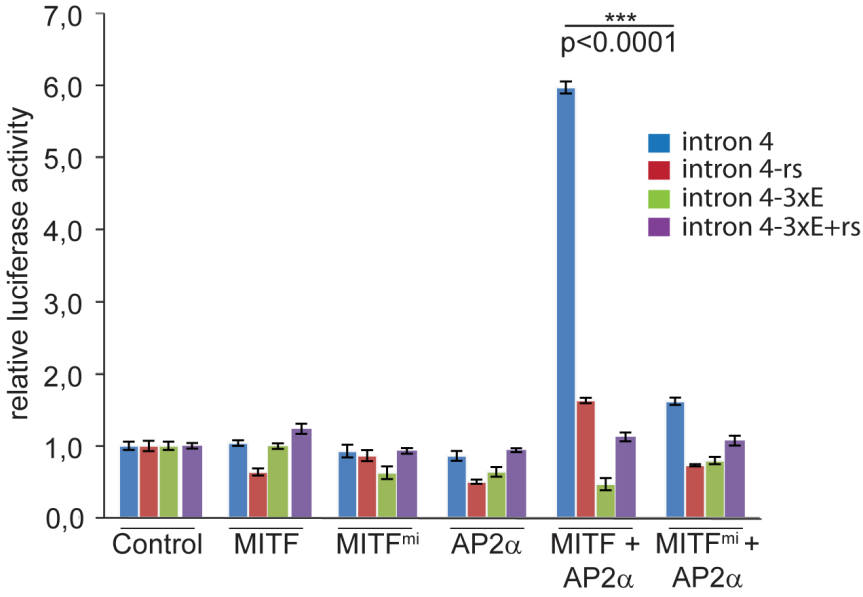


Figure 4.9.: Luciferase gene activation assays with Intron 4 of *IRF4*. The activation of intron 4 is tested using different combinations of MITF, TFAP2α and dominant negative MITF (MITF ^{mi}).

of the rs12203592 polymorphism into the reporter plasmid, mutating the E-boxes (so MITF can not recognize them anymore) or mutating all MITF and IRF4 binding sites led to background levels of reporter gene activation. These results show that for intron 4 mediated activation both transcription factors are important and the rs12203592 polymorphism has a dramatic effect on *IRF4* gene expression.

4.10. Finemapping of the E-Boxes in intron 4 of *IRF4*

For further mapping of the three MITF binding sites in *IRF4* gene activation, reporter plasmids containing single mutations (E-Box 1, E-Box 2, E-Box 3), double mutations (E-Box 12, E-Box 23, E-Box 13) and the triple mutation (E-Box 123) were used and compared to the unmutated reporter upon MITF and TFAP2α transfection.

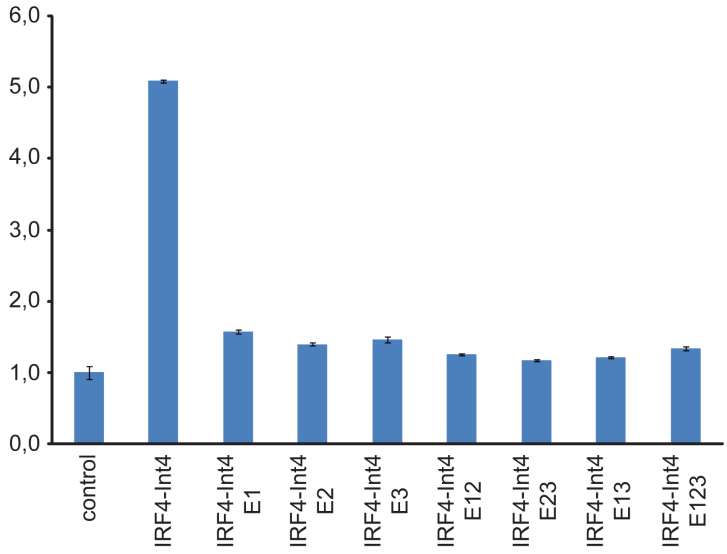


Figure 4.10.: Luciferase gene activation assays to test which E-boxes in intron 4 of *IRF4* are important.

The comparison shows that all MITF binding sites are important for the activation of intron 4 (Figure 4.10). Any single mutation or any combination of mutations disables the activation of the reporter gene. This shows that binding of MITF to all three sites is needed for activation.

5. Discussion

5.1. Discovery of new MITF target genes (Paper I)

MITF has been called the master regulator of melanocytes (Levy et al., 2006), as it is important for the development of pigment cells from their precursors as well as for the formation of the precursor cells itself. Its function has been shown in fibroblasts, which, when they were transfected with MITF expression vectors, transformed into cells which resemble melanocytes. This included changes in morphology to a more dendritic phenotype as well as the expression of melanocytic markers (Tachibana et al., 1996).

Most of the known target genes were involved in melanocytic differentiation and the pigment process, but little was known about the role of MITF in the development of the melanocytes arising from their origin in the neural crest (Ernfors, 2010).

Additionally MITF is important for the development of melanoma where it has been called a lineage survival oncogene (Garraway et al., 2005). How MITF transforms melanocytes into melanoma is also not entirely clear. It has been proposed that the processes necessary for the transformation in many ways resemble the process of melanocyte development. The transformation includes proliferation and survival of the melanoma cells and their migration to other parts of the body. The genes involved in these processes are likely to overlap with genes which are necessary for melanoblasts and melanocytes (Hoek and Goding, 2010).

Following gene expression analysis and filtering of the results (for details see section 4.1) we ended with a list of 84 genes (see Table 2 in Paper I). Ten of the identified genes have previously been reported to be expressed in melanocytes and melanoma: *BIRC7, CDK2, DCT, EDNRB, GPNMB, MLANA, RAB27A, SLC45A2, SILV* and *TYR*. The expression of these genes is correlated with MITF expression and they are upregulated when MITF is overexpressed. Furthermore three MITF target genes known to be regulated by MITF in osteoclasts were identified: *ACP5, CLD7* and *OSTM1*.

On the other hand, 29 previously known and described MITF target genes were not identified by this study. This is partly due to the fact that selection criteria were strict and thus causing false negative results. Genes were only counted as positive, if they were present in all seven datasets. If they were missing in one or more of them, they were not counted, despite the upregulation score. The most prominent example for this was *BCL2A1*, which is closely related to

BCL2, which showed a 245 fold upregulation after MITF transfection, but was only present in six of the seven datasets used for comparison. BCL2A1 has been shown by Piva et al. (2006) to be expressed in melanoma and recent results from Haq et al. (2013) showed its role as an anti-apoptotic melanoma oncogene necessary for melanoma growth. The same holds for *TRPM1* and *GPR143*, both of which showed strong upregulation (137- and 19-fold), but were counted as false negatives because they were missing in some datasets.

It is also possible that some of the known target genes require additional signalling or co-factors which were not present in the SKmel28 cells. It is known that MITF forms complexes with other transcription factors and co-factors to ensure the activation of the right genes. An example for the importance of co-factors are mouse B16 melanoma cells where MITF was not able to activate known target genes such as *TYR* (Gaggioli et al., 2003). For *Bcl2* and *TyrP1* it has been proposed that they need additional signalling via the Kit receptor (Grichnik et al., 1998; McGill et al., 2002). Some of the remaining target genes of MITF were probably missed because we used a melanocytic/melanoma background for the gene expression experiment which is most likely not favorable for MITF regulated genes which are expressed in mast cells or osteoclasts such as the genes coding for Granzyme B (Ito et al., 1998) or Cathepsin K (Motyckova et al., 2001).

The analysis also identified 71 new potential target genes of MITF (see table 2 in paper I). Three of these genes (*APOE, CA14* and *SCARB1*) have been shown to be expressed in RPE cells (Duncan et al., 2002; Ishida et al., 2004; Ochrietor et al., 2005). Another set of six genes (*CAPN3, IL6R, IRF4, LGAL3, RRAGD* and *SORT1*) have been shown to be expressed in melanoma (Chang and Schimmer, 2007; Prieto et al., 2006; Schwabe et al., 1994; Sundram et al., 2003; Truzzi et al., 2008; Wit et al., 2005). Finally four genes (*TFAP2α, HPS4, LYST* and *PSEN2*) have been associated with pigmentation, showing the overall quality of our analysis (Gutierrez-Gil et al., 2007; Hutton and Spritz, 2008; Van Otterloo et al., 2010; Wang et al., 2006).

The remaining 58 genes on the list have not previously been connected with melanocyte function or melanoma. Based on homologies or function, some assumptions can still be made. Some of the genes are involved in signal transduction (*ITPKB, PPM1H, RHOQ, SEMA6A, SGK3* and *TNFRSF14*). This is similar to some target genes (*CAPN3, EDNRB, IL6R, PSEN2* and *RAB27A*), which makes it likely that MITF is further involved in the regulation of these processes. This was confirmed by Strub et al. (2011) who also found genes also involved in signalling enriched in their study.

A number of the genes are involved in transport processes of amino acids, lipids, positively charged metal ions and protons including: *APOE, ATP1A1, ATP6V1B2, ATP6V1C1, KCNN2, LYST, SCARB1, SLC1A4, SLC7A8, SLC19A2* and *VAT*. Several known MITF target genes are involved in similar processes

such as *SLC24A5, SLC45A2* and *ACP5*. This shows an involvement of MITF in the regulation of internal pH which is important for the formation and function of melanosomes (Newton et al., 2001; Smith et al., 2004; Watabe et al., 2004). It seems also to be involved in amino acid and lipid metabolism.

Gene expression analysis on DNA microarrays is a method to analyze the expression of hundreds of different targets of transcription factors. Since the primary change in the cells (the expression of a particular transcription factor (TF)) is known, changes in the gene expression profile of the cells can be attributed directly or indirectly to that factor. However, the change in transcription does not necessarily mean a direct connection to the factor analyzed. Besides secondary effects (genes which are regulated by the TF induce further changes in expression downstream) and false positive results, changes do not have to have a biological relevance, since the overexpression of a transcription factor is a relatively artificial situation.

By comparing natural expression profiles with those generated with expression of overexpressed TFs it is possible to overcome this problem. It makes it possible to filter out secondary effects as well as biologically irrelevant target genes, and should also at least reduce the number of false positives (as these occur statistically distributed, they are unlikely to occur in all of the datasets used for comparison and thus are excluded from further analysis). What can happen though, is that known target genes are excluded due to overly strict selection criteria.

5.2. Validation and characterization of *IRF4* as a target of MITF (Paper II)

Genome wide association studies (GWAS) associate genes to a wide range of phenotypes or risk factors. Most variations identified to date are located in non-coding regions of the human genome, meaning they do not alter protein sequences. In most cases the functional consequences are not known. To understand these variations which have a phenotypic effect, they need to be characterized further. To do so, the SNP needs to be characterized on the sequence level and then the biological processes behind the phenotype needs to be identified.

IRF4 is a transcription factor which has previously been shown to regulate functions of the immune system, such as B cell maturation (Klein et al., 2006; Mittrucker et al., 1997). It has also been shown to be a lineage survival oncogene for multiple myeloma (Shaffer et al., 2008) and to be expressed in many melanomas (Sundram et al., 2003). When a polymorphism in an intron of the gene for this factor was connected to pigmentation (lighter skin and eye pigmentation, dark hair) (Han et al., 2008; Sulem et al., 2007), this was rather surprising. Together with our data from the gene expression study (Hoek et al.,

2008) identifying *IRF4* as a target for MITF, this made the factor an interesting subject for further studies. We therefore decided to further investigate *IRF4* as a target of MITF and to explore its role in pigmentation.

First we characterized *IRF4* as a target of MITF, showing that the expression in mice is dependent on *Mitf* expression. Using knockdown of *MITF* in cell lines we showed direct effects on *IRF4* expression. With this work we also established *TFAP2α* as a target of MITF. In the knockdown experiments the expression of *TYR* was not only downregulated when its known activator MITF was knocked down, but also when *IRF4* and *TFAP2α* were knocked down. This finding suggested an involvement of both IRF4 and TFAP2α in the regulation of pigmentation.

To identify a potential binding site, we used the transcription element search site to predict transcription factor binding sites in the gene and its promoter. No binding sites for MITF were found in the promoter of *IRF4*, a fact that was also confirmed by Strub et al. (2011). Further analysis revealed three potential MITF binding sites in intron 4 of *IRF4* in close vicinity to the rs12203592 polymorphism, which has been found to be cause the associated pigmentation phenotype. Located exactly at the site of the polymorphism is a potential *TFAP2α* binding site.

To test the involvement of both MITF and TFAP2α in the regulation of *IRF4*, we performed ChIP experiments. These experiments showed the binding of both factors to intron 4 of *IRF4*. Gene activation assays were used to further map the intron and to analyze which E-boxes were activated by MITF. The results of this experiment confirmed that all three binding sites are of equal importance and that the activation only happens, when all binding sites (for MITF and TFAP2α) are intact. The mutation of any site impairs activation from this enhancer element. These results explain the involvement of MITF and TFAP2α in the regulation of *IRF4*.

Further experiments showed the binding of MITF and IRF4 to the *TYR* promoter leading to a synergistic activation. MITF was able to activate the promoter alone (Yasumoto et al., 1994), but in combination with IRF4 the activation was stronger. For details of this experiment see paper II.

These results together lead to the formulation of our model describing the role of IRF4 in cooperation with MITF in the regulation of human pigmentation and how the pigmentation phenotype of the rs12203592 polymorphism arises. The model is shown in Figure 5.1.

In melanocytes, MITF and TFAP2α bind cooperatively to the enhancer element in intron 4 of *IRF4*. This activates the transcription of *IRF4*, which then binds together with MITF to the *TYR* promoter and activates transcription of *TYR*. Tyrosinase finally catalyzes the first steps of the melanin biosynthesis which leads to greater pigmentation.

The binding of TFAP2α to its recognition site is impaired by the rs12203592-T

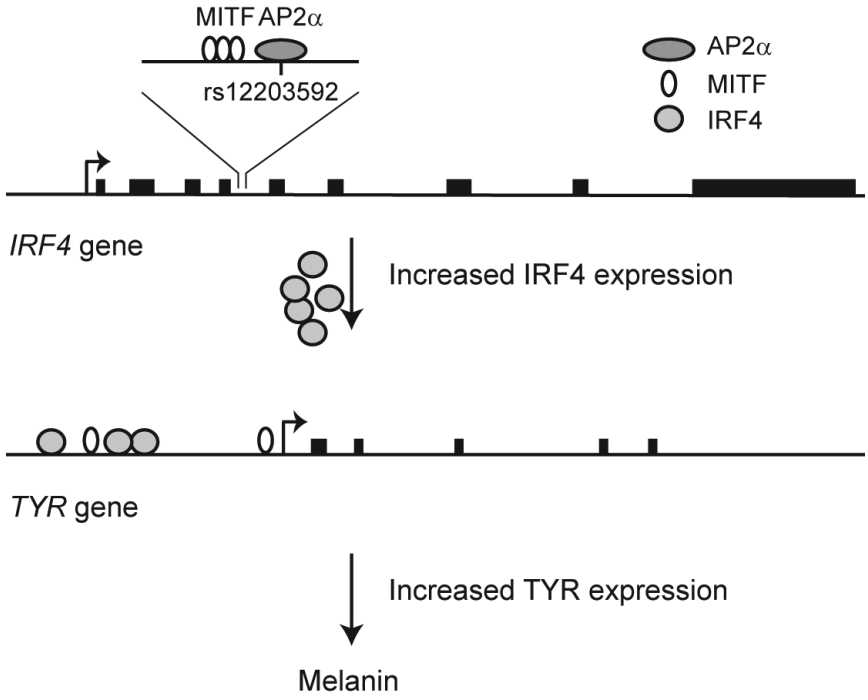


Figure 5.1.: Model of the regulation of pigmentation through the cooperative action of MITF and IRF4 on the *TYR* promoter and the regulation of *IRF4* by MITF and TFAP2α.

allele, which inhibits the activation of *IRF4* through the enhancer element and leads to reduced expression of *TYR*. This reduction in *TYR* finally leads to the pigmentation phenotypes described in the studies of Han et al. (2008) and Sulem et al. (2007). That the effects of the rs12203592-T allele are not only an *in vivo* effect seen in the laboratory. This has been shown by the experiments with primary human melanocytes and with zebrafish embryos (see Paper II). In both systems the expression is significantly lower, when the rs12203592-T allele is present. In primary human melanocytes the expression of TYR is also affected, showing the connection between IRF4 and TYR. The involvement of the polymorphism is further demonstrated by *Irf4* knockout mice which develop a significantly lighter fur pigmentation than mice with *Irf4* (see Figure 7b in Paper II). The fact that MITF also needs to be reduced to see this effect suggests some species specific differences in the regulation of the melanocytes between mouse and humans.

Interestingly, this effect has to be cell-type specific as for Burkitt Lymphoma, B cells, HEK293T and NCI-H295R cells, Do et al. (2010) reported a higher activity from the *IRF4* promoter, when the rs12203592-T allele was present. In

part, they confirmed our results by showing that TFAP2α has a higher binding affinity for the C allele than for the T allele (Do et al., 2010). It is very likely that these cell type specific effects are mediated by yet unknown transcription factors or co-factors, which are only present in melanocytes or lymphoma cells. This observation is supported by the DNAseI hypersensitivity assay, which confirms the presence of a conserved enhancer element in intron 4 of *IRF4* for cells from the melanocytic line. This peak is not present in other cell types (see Figure 2 in Paper II), indicating that the regulation differs with cell types.

In humans, pigmentation is influenced by variants in different genes involved in pigmentation. Some genes have a large effect on pigmentation e.g. *MC1R* and *ASIP*. Variants in these genes have a strong influence on freckling, sun sensitivity and red hair, whereas their effects on eye color are minor (Sulem et al., 2007 and 2008).

The T-variant of the rs12203592 polymorphism in IRF4 has strong effects on freckling, sun sensitivity and eye color (blue vs. brown). It has also been associated with brown hair color (vs. blond) (Sulem et al., 2007). The results shown here suggest that MITF regulates the major effects on pigmentation as for MC1R and ASIP. Since the regulation of the different genes, as well as of the pathways downstream of them, is different from each other, this explains differences in pigmentation between the different variants.

Our study identified only *TYR* as a target gene for the activation by MITF and IRF4. It is currently unknown, if IRF4 is involved in the regulation of other genes important for pigmentation. An analysis of other known MITF target genes involved in pigmentation, such as *DCT* and *TYRP1* did not identify any other genes with potential recognition sites for IRF4. It is possible that a broader search for possible targets can identify other genes involved in this pigmentation trait.

MITF is needed for mediating both the effects of TFAP2α on *IRF4* and also for the activation of *TYR* through IRF4. Gene-expression and ChIP-sequencing (ChIP-seq) studies suggest, that both factors are regulated by MITF (Hoek et al., 2008; Strub et al., 2011). Since TFAP2α is expressed in both mesenchymal and epithelial cells, it is possible that MITF regulates the gene throughout the development of the pigment cells from the neural crest. In zebrafish TFAP2α is important for the development of melanophores (Van Otterloo et al., 2010). How MITF and TFAP2α interact to regulate *IRF4* is currently unknown. It is possible that both proteins are part of a larger protein complex, since the binding-sites are located quite close to each other. The data shown here provide an example that transcription factors, which are expressed in a wide variety of cells can regulate very different sets of genes depending on the co-factors which are different in each cell type. This enables the expression of very different cell specific programs.

With the role of IRF4 in pigmentation known, investigating the role of IRF4

in melanoma will also be an important task for the future. IRF4 has been shown to be expressed in melanoma by Sundram et al. (2003), but Duffy et al. (2010b) and Gudbjartsson et al. (2008) found no association between the SNP in the gene and an elevated melanoma risk. There seems to be no direct involvement of IRF4 in the development of melanoma but probably an indirect. Duffy et al. (2010a) and Kvaskoff et al. (2011) found an association between IRF4 SNPs and higher nevus counts which constitute a higher melanoma risk.

Polymorphisms have been identified by several GWAS studies to be susceptibility loci through affecting the binding of transcription factors. Modifications in these loci affect the binding of these factors to DNA, leading to differences in the expression of genes located in close vicinity (Meyer et al., 2008; Pomerantz et al., 2009; Rahimov et al., 2008; Schodel et al., 2012; Tuupanen et al., 2009). Besides the association of the polymorphism with a phenotype, most of these effects have neither been analyzed for differences in gene expression nor for the biological effects of the polymorphism.

A recent example of such a characterization is the connection of the rs12913832 polymorphism in intron 86 of *HERC2* which shows a strong association with human eye color and pigmentation. Since the polymorphism is located 21 kb upstream of the known pigmentation gene OCA2, it has been speculated, that the polymorphism affects binding to a distal regulatory element of OCA2, which may affect the binding of the transcription factors HLTF, LEF1 and MITF. Finally, Visser et al. (2012) showed the formation of a DNA loop between the enhancer element in *HERC2* and the *OCA2* promoter which affects the expression of *OCA2*. The binding of HLTF is prevented by the rs12913832-C allele, which in turn reduces the formation of the loop. The regulation of OCA2 is probably more complex than that, with different regulatory elements which bind different transcription factor complexes responsible for the regulation of pigmentation and eye color in the different cell types (Beleza et al., 2013). In contrast to this, we not only show the binding of the transcription factors to the regulatory elements, but also the effects on downstream targets which affect human pigmentation.

5.3. Freckles and solar lentigines (Paper III)

Freckling is an interesting human trait, showing up mostly in individuals of Caucasian and Asian descent. Although ephelides and solar lentigines look rather similar, they are different types of skin lesions. Ephelides have a strong genetic component but are dependent on UV radiation as well. Solar lentigines depend strongly on sun light and the damages induced by it accumulate over the years, but also have an underlying pigment cell genetic component. Freckles first occur in youth, while lentigines first occur in older individuals.

Freckles have so far been associated with a few genes which have a clear

connection to pigmentation and which show a dosage dependent correlation between the number of variations present and the visible phenotype. The most prominent gene involved in freckling is *MC1R*, with over 80 identified mutations in the European population (Gerstenblith et al., 2007; Makova and Norton, 2005). The presence of variations in the receptor causes fair skin, red to blonde hair and freckles in a dosage dependent manner.

Among the other identified genes which play a role in the genetic predisposition of forming freckles are *ASIP* and *TYR*. ASIP is one of the antagonists of MC1R, preventing the activation through α-MSH. The rs1015362 polymorphism is located near the ASIP gene and has been connected to freckles and sun sensitivity (or the ability to tan) (Sulem et al., 2008). Mutations in TYR influence the activity of the enzyme, explaining the effect on pigmentation (Tripathi et al., 1992). Freckling (both ephelides and solar lentigines) comprises an elevated risk for melanoma.

The association of *IRF4* as a gene which causes freckling by Sulem et al. (2007 and 2008) came as a surprise as this gene had not been known to be involved in pigmentation before. The main question was how ephelides and solar lentigines form when the genes, which are involved in pigmentation contain polymorphisms which affect the function of the proteins or the expression.

The genes associated with freckling are not only active in melanocytes but at least also in the keratinocytes surrounding them. Both cell types form the epidermal melanin unit and interact in many ways, including cell-cell contacts and paracrine signalling upon stimulation and signals from the environment. The close connection of the cells in the epidermal melanin unit makes it likely that both cell types are important for the formation of ephelides and solar lentigines.

Since the preconditions for ephelides and solar lentigines are different, there are different models for their origins. Ephelides are likely to arise from melanocytes (or MSC) which are present in the skin and are enhanced by exposure of the skin to sun light, while solar lentigines arise from melanocytes which receive signalling from a chronic UV-damaged skin. The elevated pigmentation happens in a stochastic way and thus pigmented spots can develop, while the pigmentation of the surrounding skin is normal. This hypothesis is supported by the fact that ephelides can be induced on sunburnt skin (Wilson and Kligman, 1982), that they get stronger upon exposure to the sun and weaker in the winter time. Variations in the genes relevant for the formation of ephelides influence these genes in such a way that their products are present in the pigment cells below the threshold to cause normal pigmentation. Small local changes in signalling or expression activate pigmentation locally and then cause pigment spots to form.

These small changes can be mediated through changes in local concentrations of signalling molecules secreted by the keratinocytes and which affect the melanocytes differently. Some melanocytes receive enough signalling to go over the activation threshold for inducing pigmentation, while others do not. Since

the signalling is directed eventually to MITF in most cases and since a big part of the receptors and signalling molecules are regulated by this transcription factor, this may lead to self-regulatory loop inducing more receptors and receiving more activation. At least for ephelides this loop seems to have some upper threshold which prevents the ephelides from getting very dark. For solar lentigines the signalling seems to be stronger in the first place, since these spots are bigger and darker compared to ephelides.

Among the genes which have previously been associated with the appearance of freckles is another gene, which has previously not been connected to this field: Basonuclin 2 (BNC2) (Eriksson et al., 2010). The gene has an extremely complex structure, which potentially gives rise to about 90,000 different mRNA isoforms, generating 2000 different proteins (Vanhoutteghem and Djian, 2007). The function of BNC2 is currently unknown. The protein is located in nuclear speckles in keratinocytes, which led to speculations that it might be involved in RNA-processing (Vanhoutteghem and Djian, 2006). Knockouts of BNC2 in mice have been shown to be lethal within 24 h of birth (Vanhoutteghem et al., 2009), but a truncating mutation in zebrafish showed an influence on the development of pigment cells. These develop normal in early stages but are lost in later stages of development. This results in extensive loss of pigment, the mutant fish are missing their characteristic stripes (Lang et al., 2009). BNC2 is probably regulating the pigment cells through paracrine signalling, as it has been shown to be mostly expressed in keratinocytes. The exact role of this gene in pigmentation needs to be analyzed in detail.

6. Conclusion

The fact that the MITF protein plays an important role in the development, migration, differentiation and proliferation of pigment cells has been shown in recent years. However, at the start of the project it was clear that multiple MITF target genes exist and needed to be identified. The known and characterized MITF target genes did not explain this role of the protein very well, since they were mostly related to pigmentation.

We have performed gene expression analysis to unveil new MITF target genes. This resulted in a list of previously unknown potential target genes, which needed further investigation. The list includes genes involved in gene regulation such as the transcription factor encoding genes *IRF4*, *TFAP2α* or *SOX13*, genes involved in signalling including *GPR137B* or *RRAGD*, ion carriers as *SLC1A4*, *SLC7A8* or *SLC19A2*, as well as the genes encoding for ATPases as *ATP1A1*, *ATP6V1B2* and *ATP6V1C1*. For a complete list see Table 2 in Paper II.

This gene list underlines the importance of MITF in the context of pigment cells and shows the diversity of regulated genes. We further characterized two of the new potential target genes of MITF, namely *IRF4* and *TFAP2α*.

It is currently unclear if *TYR* is the only gene in the pigmentation process which is co-regulated by IRF4. Analysis of other known MITF target genes including *TYRP1, DCT* or *PMEL*, involved in pigmentation revealed no potential IRF4 binding sites in these genes. It would be interesting to do ChIP-sequencing experiments with immunoprecipitated IRF4 to determine which genes this transcription factor binds. A correlation analysis of a ChIP-sequencing experiment with MITF in the same cell line would then allow us to analyze which sequences are bound by both factors in combination.

It is also unknown how MITF and IRF4 cooperate on the *TYR* promoter. It is possible that both proteins are part of bigger transcription regulation complexes and were at least partly obscured by other proteins which prevented co-precipitation. It is also possible that both proteins are not part of a joint transcription regulation complex, but that the proteins work in a sequential way, with one complex acting first on the sequence probably forming secondary structures which is necessary to allow the second complex to bind and activate or enhance transcription.

We then took the knowledge of molecular mechanisms one step further and analyzed the formation of pigmented lesions of the skin. Here we found out that different mechanisms underly the formation of ephelides and solar lentigines, which are both connected to the same set of genes. We presented a model for

the origin of both pigment lesions. It would be interesting to use skin sections of both ephelides and solar lentigines to analyze the expression of genes and see how they differ. It would also be interesting to stain such sections for morphological differences (ideally with samples taken from the same individual).

With all this knowledge about pigmentation, it will be interesting to analyze these genes in the context of their possible role in melanoma. It will also be interesting to find out the exact regulation of the new pigment genes and identify factors which might be either good targets for cancer therapy or of importance for the formation of malignant cells. This could then also lead to new ways of diagnosing the disease early and to analyze a possible treatment outcome.

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Paper I

Novel MITF targets identified using a two-step DNA microarray strategy

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KEYWORDS microphthalmia-associated transcription factor/microarray/melanoma/melanocyte/transcription/correlation

PUBLICATION DATA Received 20 June 2008, revised and accepted for publication 18 August 2008

doi: 10.1111/j.1755-148X.2008.00505.x

Summary

Malignant melanoma is a chemotherapy-resistant cancer with high mortality. Recent advances in our understanding of the disease at the molecular level have indicated that it shares many characteristics with developmental precursors to melanocytes, the mature pigment-producing cells of the skin and hair follicles. The development of melanocytes absolutely depends on the action of the microphthalmia-associated transcription factor (MITF). MITF has been shown to regulate a broad variety of genes, whose functions range from pigment production to cell-cycle regulation, migration and survival. However, the existing list of targets is not sufficient to explain the role of MITF in melanocyte development and melanoma progression. DNA microarray analysis of gene expression offers a straightforward approach to identify new target genes, but standard analytical procedures are susceptible to the generation of false positives and require additional experimental steps for validation. Here, we introduce a new strategy where two DNA microarray-based approaches for identifying transcription factor targets are combined in a cross-validation protocol designed to help control false-positive generation. We use this two-step approach to successfully re-identify thirteen previously recorded targets of MITF-mediated upregulation, as well as 71 novel targets. Many of these new targets have known relevance to pigmentation and melanoma biology, and further emphasize the critical role of MITF in these processes.

Introduction

Malignant melanoma is an aggressive form of cancer with a high mortality rate. Although early stage disease is easily treatable, advanced stages of the disease are highly resistant to treatment and patients rarely survive longer than 10 months. The microphthalmia-associated transcription factor (MITF) is a member of the basic helix-loop-helix leucine-zipper transcription factor family and has been shown to be a critical regulator of

Significance

While the microphthalmia-associated transcription factor (MITF) is recognized as a master regulator for both melanocyte development and melanoma progression the number of known target genes is relatively small. This high throughput study of gene expression in melanoma cell lines serves to identify with high probability novel candidates for MITF-mediated activation in a melanocytic context.

melanocyte development and survival (Steingrimsson et al., 2004). Various isoforms of MITF are also important for the development and homeostasis of other cell types including retinal pigment epithelia, osteoclasts and mast cells (Kawaguchi and Noda, 2000; Nechushtan et al., 1997; Planque et al., 2004). In melanoma, MITF is reported to be a critical factor in regulating proliferation (Carreira et al., 2006; Hoek et al., 2008), and amplification of the *MITF* gene is associated with poor patient survival (Garraway et al., 2005; Ugurel et al., 2007). Table 1 shows that MITF is reported to regulate the expression of a broad variety of genes, many of them involved in pigmentation. Despite this variety of known targets MITF must regulate multiple other genes to account for all aspects of MITF function during melanocyte development and melanoma progression. Thus, in order to further characterize the role of MITF in melanocyte and melanoma development, it is important to identify additional MITF target genes.

Endogenous expression of *MITF* is driven by signals which likely also activate other genes. Experiments where a constitutively active expression vector is used to drive *MITF* expression short-circuit this background of normally co-regulated factors. This changes the

Table 1. Verification of reported Mitf target genes

Symbol	Title	References	Cell type	Fold[a]	Co[b]
ACP5	Acid phosphatase 5, tartrate resistant	Luchin et al., (2000)	Osteoclast	209	7
BCL2	B-cell CLL/lymphoma 2	McGill et al., (2002)	Melanoma	<2	7
BEST1	Bestrophin 1	Esumi et al., (2007)	RPE	<2	7
BIRC7	Baculoviral IAP repeat-containing 7	Dynek et al., (2008)	Melanoma	214	7
CDK2	Cyclin-dependent kinase 2	Du et al., (2004)	Melanocyte	3	7
CLCN7	Chloride channel 7	Meadows et al., (2007)	Osteoclast	4	7
DCT	Dopachrome tautomerase	Yasumoto et al., (2002)	Melanocyte	218	7
EDNRB	Endothelin receptor type B	Sato-Jin et al., (2007)	Melanocyte	15	7
GPNMB	Glycoprotein (transmembrane) nmb	Loftus et al., (2008)	Melanoblast	251	7
GPR143	G protein-coupled receptor 143 (Oa1)	Vetrini et al., (2004)	Melanocyte	19	6
MC1R	Melanocortin 1 receptor	Aoki and Moro, (2002)	Melanocyte	16	4
MLANA	Melan-A	Du et al., (2003)	Melanocyte	173	7
OSTM1	Osteopetrosis-associated transmembrane protein 1	Meadows et al., (2007)	Osteoclast	5	7
RAB27A	RAB27A, member RAS oncogene family	Chiaverini et al., (2008)	Melanocyte	13	7
SILV	Silver homolog (mouse)	Du et al., 2003)	Melanocyte	217	7
SLC45A2	Solute carrier family 45, member 2	Du and Fisher, (2002)	Melanocyte	222	7
TBX2	T-box 2	Carreira et al., (2000)	Melanocyte	4	5
TRPM1	Transient receptor potential cation channel, M1	Miller et al., (2004)	Melanocyte	137	6
TYR	Tyrosinase	Hou et al., (2000)	Melanocyte	267	7
TYRP1	Tyrosinase-related protein 1	Fang et al., (2002)	Melanocyte	<2	7
CADM1	Cell adhesion molecule 1	Ito et al., (2003)	Mast cell	<2	1
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)	Carreira et al., (2005)	Melanocyte	<2	0
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	Loercher et al., (2005)	Melanocyte	<2	1
Cma1	Chymase 1, mast cell	Morii et al., (1997)	Mast cell	7	0
CTSK	Cathepsin K	Motyckova et al., (2001)	Osteoclast	<2	1
DIAPH1	Diaphanous homolog 1	Carreira et al., (2006)	Melanoma	<2	1
GZMB	Granzyme B	Ito et al., (1998)	Mast cell	10	0
HIF1A	Hypoxia-inducible factor 1, alpha subunit	Busca et al., (2005)	Melanoma	2	0
ITGA4	Integrin, alpha 4	Kim et al., (1998)	Melanocyte	3	0
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene	Tsujimura et al., 1996)	Mast cell	<2	2
Mcpt2	Mast cell protease 2	Ge et al., (2001)	Mast cell	<2	0
Mcpt4	Mast cell protease 4	Jippo et al., (1999)	Mast cell	<2	0
Mcpt9	Mast cell protease 9	Ge et al., (2001)	Mast cell	<2	0
MET	Met proto-oncogene	McGill et al., (2006)	Melanocyte	<2	3
NDST2	N-deacetylase/N-sulfotransferase 2	Morii et al., (2001)	Melanocyte	<2	0
NGFR	Nerve growth factor receptor (p75)	Jippo et al., (1997)	Mast cell	3	0
OSCAR	Osteoclast associated, immunoglobulin-like receptor	So et al., (2003)	Osteoclast	4	0
PRKCB1	Protein kinase C, beta 1	Park et al., (2006)	Melanocyte	2	1
SERPINE1	Serpin peptidase inhibitor E1 (PAI-1)	Murakami et al., (2006)	Mast cell	6	0
SLC11A1	Solute carrier family 11, member 1	Gelineau-van Waes et al., (2008)	RPE	4	1
TPH1	Tryptophan hydroxylase 1	Ito et al., (1998)	Mast cell	<2	0
Tpsb2	Tryptase beta 2	Morii et al., (1996)	Mast cell	<2	1

[a]Fold change up on transformation of SK-MEL-28 with a *Mitf*-expressing vector ($P < 0.05$).
[b]Number of data sets in which correlation exceeds 0.5 with 207233_s_at (*MITF*).
RPE, retinal pigment epithelium.

context in which MITF operates and, in the absence of co-regulated activators and suppressors, potentially activates biologically irrelevant target genes. Alternatively, identification of novel MITF targets via correlation of mRNA expression patterns is complicated by factors which are co-regulated with MITF. This potential confusion of causality and correlation also risks generating false positives. However, each of these approaches serve to complement the other. Exogenous upregulation of *MITF* is unlikely to activate endogenously co-regulated factors, and analysis of endogenous expression patterns serves to filter out biologically irrelevant transcription activated by exogenous *MITF* expression. We show here how such a two-step strategy may detect additional targets for MITF-driven transcription.

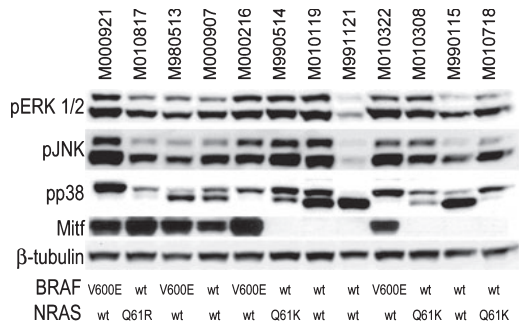

Figure 1. MAPK/JNK signalling and *MITF* expression. Cell extracts from a library of melanoma cultures were subject to western blotting against phosphorylated Erk1/Erk2, phosphorylated JNK, phosphorylated p38, and β-tubulin. MITF levels, as well as BRAF and NRAS mutation status were reported previously (Hoek et al., 2006). These data show that neither MAPK/JNK activation nor BRAF/NRAS mutation status correlate with Mitf expression in melanoma.

Results

Endogenous MAPK signalling does not affect Mitf protein levels

If a transcription factor's function is primarily regulated by post-transcriptional or post-translational mechanisms then variations in the expression of its mRNA across a range of samples will not correlate with that of its targets. For MITF there are several reports which together detail a range of post-translational modifications which in melanocytes modify its regulatory activity, including ubiquitination, sumoylation, phosphorylation, and cleavage by caspases (Hemesath et al., 1998; Larribere et al., 2005; Murakami and Arnheiter, 2005; Wu et al., 2000; Xu et al., 2000). This complex layering of multiple post-translational regulation mechanisms would seem to preclude the usefulness of correlating *MITF* mRNA with that of potential targets. However, recent gene expression data in melanoma cell lines shows significant correlation of *MITF* mRNA with the expression of many of its known targets (Hoek et al., 2006). This suggests that, for at least some genes and certain contexts, variations in post-translational modification of MITF have a lesser influence over their activation.

Because of the interest in post-translational regulation of MITF activity, particularly in the context of the $BRAF^{V600E}$ mutation, it was important that we look for a correlation between MAPK signalling events and MITF protein levels. We therefore stained melanoma cell extracts to compare endogenous levels of phospho-ERK 1/2, phospho-JNK and phospho-p38 against MITF and found no correlation between them (Fig. 1). These results concur with similar data obtained previously by Kono et al. and we agree with their assessment that if MAPK signalling in melanoma has regulatory control over MITF it is not at the level of MITF turnover (Kono et al., 2006). However, it is important to consider that we are examining homeostatic conditions and there may yet be signals to which MITF-turnover rates (and therefore MITF function) are sensitive. Nevertheless, it is clear that in unstimulated cultured melanoma cells apparent MAPK signalling status is not related to MITF protein level, and this supports the notion that in unstimulated cultures expression of *MITF* mRNA may correlate with target gene expression.

Gene expression in Mitf-transfected SK-MEL-28 cells

To identify candidate MITF target genes, we generated a stable line of the melanoma cell line SK-MEL-28 (subclone SK-MEL-28-MITF-7) which expresses a FLAG-tagged Mitf protein. Normally, the SK-MEL-28 cells express very low levels of MITF and carry the $BRAF^{V600E}$ mutation. The SK-MEL-28-MITF-7 cells proliferated slower than the parental cell line. We cultured these cells as well as the original SK-MEL-28 cells in triplicates and then isolated RNA for microarray analysis from each culture separately. By comparing the genes expressed in *Mitf*-transfected cells with the genes expressed by the untransformed cells, we obtained a list of 10348 probes (equivalent to 6936 genes) upregulated at least twofold in the presence of *Mitf* (Fig. 2, Table S1). As a positive control, four of four separate probes for *MITF* showed upregulation (18- to 24-fold) of *MITF* expression in *Mitf*-transfected cells. Among the upregulated genes were 27 of 42 genes reported to be upregulated by MITF. This represents a significant ($P < 0.005$) overlap with the known target list. However, some of the genes upregulated by *Mitf*-transfection of SK-MEL-28 are likely to be because of secondary effects and to determine which are true targets, we performed a strictly controlled correlation analysis on data obtained from multiple other studies and compared the results with those generated from *Mitf*-transfection of SK-MEL-28.

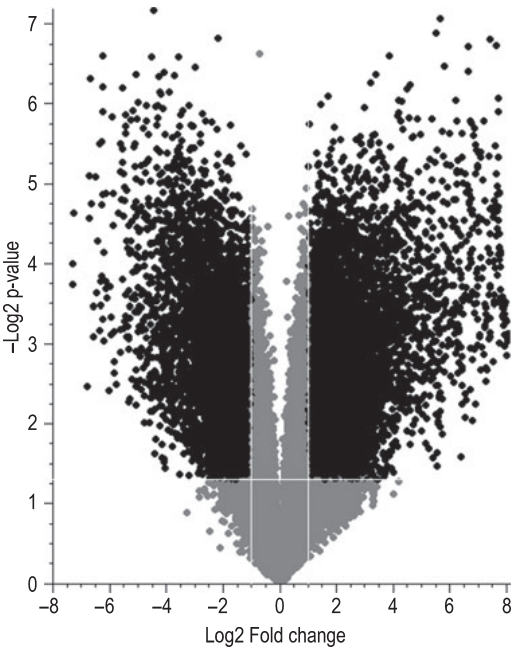

Figure 2. Exogenous Mitf drives gene expression change. A volcano plot showing the ratio of averaged untransformed and *Mitf*-transformed data reveals 9890 genes (black dots) which have significant and >2-fold change on *Mitf*-transformation of SK-MEL-28. Grey dots represent genes which did not meet these minimum criteria.

Correlation of melanocytic gene expression with MITF expression

To find genes whose expression patterns show strong and reproducible correlation with *MITF* expression it was important to consider data from independent sources. As we are primarily interested in MITF's function in a melanocytic context we restricted ourselves to selecting sources which examined gene expression in melanoma and melanocytes. Furthermore, to simplify comparison between sources, we considered only those which used the same array platform. Accordingly, we obtained from GEO data sets generated by seven different groups all using Affymetrix HG-U133 series platforms to examine in vitro gene expression in melanoma or melanocyte cultures. We assessed correlation by performing a Pearson correlation analysis on each probe set in comparison to *MITF* across all samples of each data set. If the calculated Pearson correlation coefficient (r) for a probe set exceeded 0.5 in all seven data sets then it was considered to be strongly correlated with *MITF*. We detected 154 different probe sets (110 genes; Table S2) which shared a correlation coefficient of at least 0.5 with 207233_s_at (*MITF*) in all seven data sets (Fig. 3). To determine whether or not these genes are true targets of MITF we compared this list with the list of genes identified as being upregulated by *Mitf*-transfection of SK-MEL-28.

Correlation and upregulation

To use these two different datasets to extract true MITF target genes, we compared the 110 genes which correlated with *MITF* expression in the different melanoma datasets, with the 6936 genes significantly upregulated in the transfected SK-MEL-28 cells. Of these lists, 84 genes showed both high correlation with *MITF* expression and significant upregulation of expression when SK-MEL-28 is transformed with an *Mitf*-expressing vector. Among these genes are thirteen known targets of MITF in melanocytes (Table 1). The finding that, among 84 genes, thirteen of 42 reported targets of MITF were detected has a significance (hypergeometric distribution) of $P < 10^{-18}$. Therefore it is highly likely that MITF has a direct regulatory relationship with the remaining 71 genes identified (Table 2).

Because our principal correlation criteria was very restrictive ($P < 10^{-13}$) we also performed a less strict analysis ($P < 0.004$). This determined that seven reported targets were probable false negatives in our principal study. Furthermore, we identified an additional 58 genes as being probable novel targets for MITF regulation (Table S3).

Upstream MITF recognition sequences

We looked for MITF recognition sequences in the upstream regions of strongly correlated novel candidates whose expression was increased by at least 50-fold upon Mitf transfection (*MBP, TNFRSF14, IRF4, RBM35A, PLA1A, APOLD1* and *KCNN2*). We searched both human and mouse sequences and found several instances of E- and M-boxes in the upstream regions of most of these genes (Fig. 4).

Discussion

The *MITF* gene has been termed a master regulator of melanocyte development as it can program cells towards the melanocyte lineage. For example, when fibroblasts are transfected with the *MITF* gene, they transform into dendritic cells expressing melanocyte marker genes (Tachibana, 1997). However, although a number of MITF target genes are known, most of them are involved in melanocyte differentiation and we know very little about the genes involved in the melanocyte developmental program. Similarly, as MITF is important for the development of melanomas it is of paramount interest to identify the target genes which turn normal melanocytes into malignant cells. Here we have used a dual approach to identify potential MITF target genes in melanocytes and melanoma cells, first by identifying genes upregulated by Mitf-transfection of SK-MEL-28 melanoma and then by comparing those to genes which

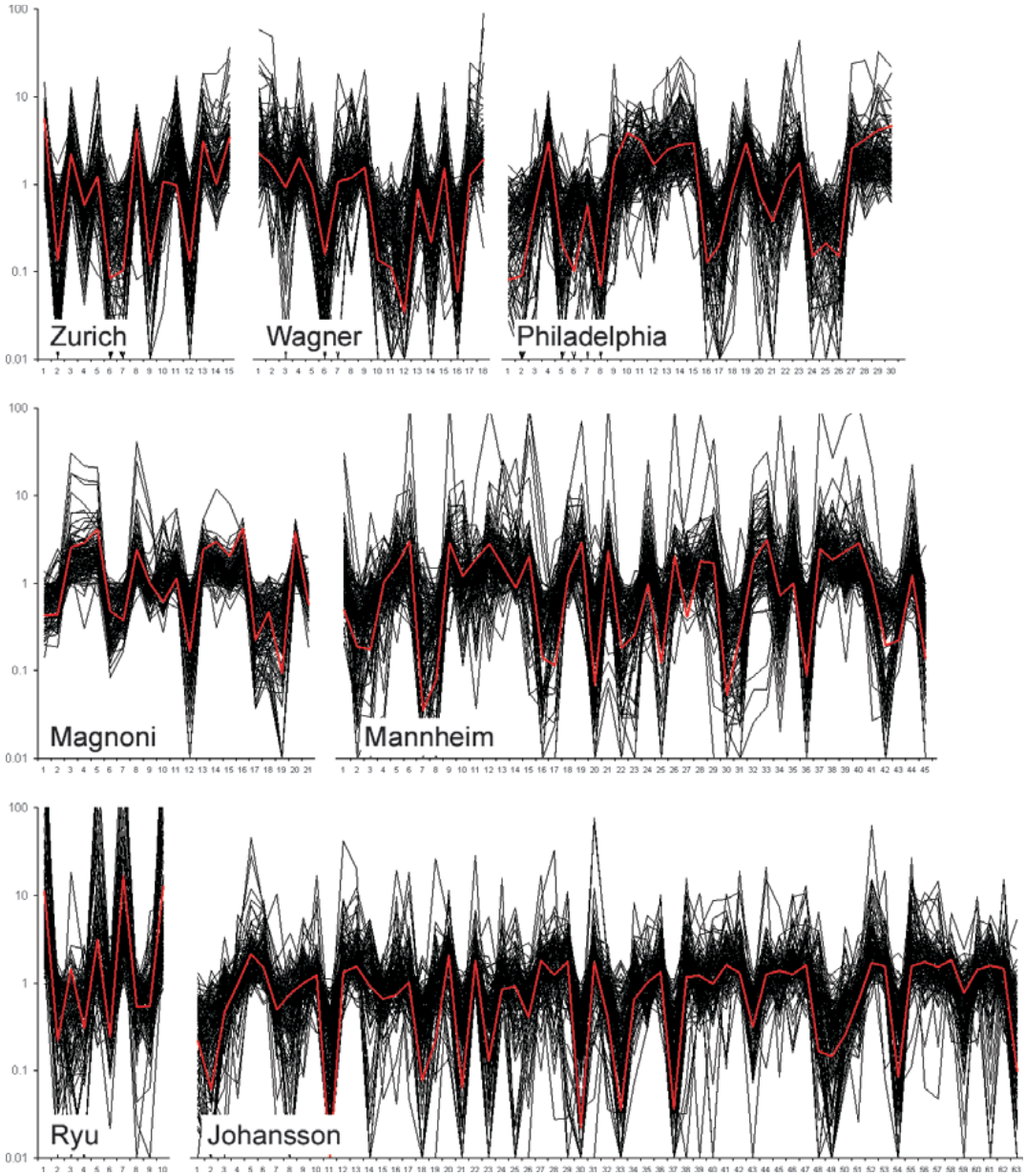

Figure 3. Correlation of gene expression with MITF. Normalized signal intensity values for 84 genes which both correlate with *MITF* expression and are upregulated by *Mitf*-transfection, are plotted against samples from the seven data sets used in the correlation analyses. *MITF* expression (207233_s_at) is plotted in red.

correlate with *MITF* expression in seven different data sets. Of the genes identified, ten (*TYR, DCT, SILV, MLANA, EDNRB, GPNMB, BIRC7, CDK2, SLC45A2* and *RAB27A*) have been previously reported as being activated by MITF in melanocytes or melanomas. All of those genes were found to be both closely correlated with endogenous MITF expression and upregulated upon transfection of SK-MEL-28 cells with an

Table 2. Novel targets for Mitf-mediated upregulation

Symbol	Title	r^a	Fold[b]
MBP	Myelin basic protein	0.82	264
TNFRSF14	Tumor necrosis factor receptor superfamily 14	0.89	259
IRF4	Interferon regulatory factor 4	0.78	170
RBM35A	RNA-binding motif protein 35A	0.88	166
PLA1A	Phospholipase A1 member A	0.71	166
APOLD1	Apolipoprotein L domain containing 1	0.71	116
KCNN2	Potassium calcium-activated channel N2	0.55	52
INPP4B	Inositol polyphosphate-4-phosphatase, type II	0.72	45
CAPN3	Calpain 3	0.91	45
LGALS3	Lectin, galactoside-binding, soluble, 3	0.77	31
GREB1	GREB1 protein	0.76	19
FRMD4B	FERM domain containing 4B	0.74	16
SLC1A4	Solute carrier family 1, member 4	0.65	15
TBC1D16	TBC1 domain family, member 16	0.84	14
GMPR	Guanosine monophosphate reductase	0.67	13
ASAH1	N-acylsphingosine amidohydrolase 1	0.83	11
MICAL1	Microtubule-associated monoxygenase calponin/LIM containing 1	0.63	11
TMC6	Transmembrane channel-like 6	0.73	10
ITPKB	Inositol 1,4,5-trisphosphate 3-kinase B	0.8	9
SLC7A8	Solute carrier family 7, member 8	0.72	9
CA14	Carbonic anhydrase XIV	0.67	8
TMCC2	Transmembrane and coiled-coil domain family 2	0.78	7
GPR137B	G protein-coupled receptor 137B	0.82	7
RIPK5	Receptor interacting protein kinase 5	0.68	7
TDRD7	Tudor domain containing 7	0.78	7
PHACTR1	Phosphatase and actin regulator 1	0.83	7
RRAGD	Ras-related GTP binding D	0.84	7
AMDHD2	Amidohydrolase domain containing 2	0.68	7
SOX13	SRY (sex determining region Y)-box 13	0.66	6
KIAA1026	Kazrin	0.78	6
SORT1	Sortilin 1	0.79	6
LYST	Lysosomal trafficking regulator	0.68	6
STXBP1	Syntaxin binding protein 1	0.7	6
USP48	Ubiquitin specific peptidase 48	0.55	5
ZFYVE16	Zinc finger, FYVE domain containing 16	0.84	5
STX7	Syntaxin 7	0.78	5
HPS4	Hermansky-Pudlak syndrome 4	0.76	5
CDK5R1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	0.7	5
PSEN2	Presenilin 2	0.69	5
RHOQ	Ras homolog gene family, member Q	0.73	5
PIR	Pirin	0.86	4
APOE	Apolipoprotein E	0.74	4
VAT1	Vesicle amine transport protein 1	0.75	4
GM2A	GM2 ganglioside activator	0.58	4
ST3GAL6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	0.81	4
IVNS1ABP	Influenza virus NS1A binding protein	0.72	4
GYG2	Glycogenin 2	0.77	4
GNPTAB	N-acetylglucosamine-1-phosphate transferase α and β	0.8	4
C14orf109	Chromosome 14 open reading frame 109	0.75	4
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	0.74	3
ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1	0.65	3
SEMA6A	Semaphorin	0.79	3
CHKA	Choline kinase alpha	0.78	3
ACSL1	Acyl-CoA synthetase long-chain family member 1	0.64	3
SGK3	Serum/glucocorticoid regulated kinase family, member 3	0.58	3
KIAA1598	KIAA1598	0.7	3
QDPR	Quinoid dihydropteridine reductase	0.75	3
IL6R	Interleukin 6 receptor	0.65	3
FAM53B	Family with sequence similarity 53, member B	0.74	3
GPM6B	Glycoprotein M6B	0.8	3

Table 2. Continued

Symbol	Title	r^a	Fold[b]
SCARB1	Scavenger receptor class B, member 1	0.67	3
MDH1	Malate dehydrogenase 1, NAD (soluble)	0.68	3
UBL3	Ubiquitin-like 3	0.73	3
ACO2	Aconitase 2, mitochondrial	0.61	2
ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	0.77	2
ATP6V1B2	ATPase, H+ transporting, lysosomal, V1 subunit B2	0.62	2
PPM1H	Protein phosphatase 1H (PP2C domain containing)	0.73	2
TFAP2A	Transcription factor AP-2 alpha	0.79	2
GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	0.63	2
SLC19A2	Solute carrier family 19 (thiamine transporter), member 2	0.77	2
DAPK1	Death-associated protein kinase 1	0.67	2

[a]*r* = median correlation with 207233_s_at (*MITF*) from seven different data sets.
[b]Fold change up on transformation of SK-MEL-28 with a *Mitf*-expressing vector.

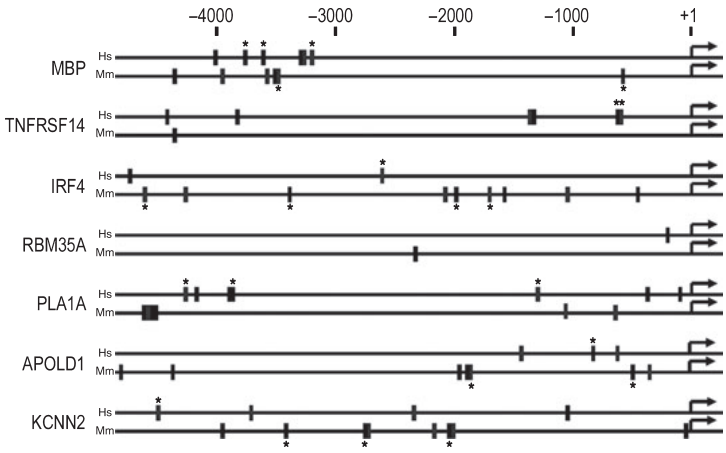

Figure 4. Upstream/promoter regions of novel MITF target genes. For both human (Hs) and mouse (Mm), upstream sequences of novel target genes with greater than 50-fold upregulation on *Mitf*-transfection of SK-MEL-28 were extracted from the UCSC Genome Browser database. These were interrogated for the presence of E-box (CAYRTG) and M-box (E-box flanked by a 5′ T or a 3′ A) sequences. M-boxes are highlighted with an asterisk.

Mitf-expressing vector (Table 1). We also identified three genes (*ACP5, OSTM1* and *CLD7*) which have previously been identified as being upregulated by Mitf in osteoclasts (Table 1).

Concomitantly, there were 29 genes previously reported to be regulated by MITF which did not meet the selection criteria (Table 2). For some of these genes it was found that the primary selection criteria was overly strict and they were omitted as false negatives. Both *TRPM1* and *GPR143* showed significant upregulation (137- and 19-fold, respectively) and failed to meet correlation criteria in only one of seven data sets. *TBX2* and *MC1R* also showed significant upregulation (3.5- and 16-fold, respectively) but missed correlation in two and three data sets, respectively. *TYRP1*, *BCL2* and *BEST1* passed the correlation criteria in all data sets but did not show significant changes in expression upon *Mitf*-transfection of SK-MEL-28 cells. Interestingly, others have shown that MITF transfection of the mouse melanoma line B16 fails to activate known targets, suggesting that MITF requires co-factors for its activity which are absent in B16 (Gaggioli et al., 2003; de la Serna et al., 2006). On the other hand, it is thought that upregulation of *TYRP1* and *BCL2* requires signalling through the KIT receptor (Grichnik et al., 1998; McGill et al., 2002), which may be inactive in SK-MEL-28 and would explain their failure to be upregulated. Intriguingly, a close *BCL2* relative, *BCL2A1* correlated with *MITF* in six of seven data sets and was upregulated 245-fold on *Mitf*-transfection (qualifying it as a probable false-negative). Like *BCL2*, this gene is involved in anti-apoptotic processes and has previously been described as expressed in melanoma (Kenny et al., 1997; Piva et al., 2006). That *BCL2A1* activation may not require KIT signalling suggests an independent mechanism for MITF to control apoptosis during melanoma progression. This is further supported by our confirmation that *BIRC7*, another anti-apoptotic factor reported to be regulated by MITF, is also upregulated 214-fold upon *Mitf*-transfection and

tightly correlates with *MITF* expression in all data sets (Table 1). For the remaining genes which were previously reported to be activated by MITF there are several possible explanations for why they were not identified here. First, we used an entirely melanocytic context for our study whereas some of the genes have only been shown to be regulated by MITF in other cell types (e.g. *CTSK*, cathepsin K, is reported only in osteoclasts (Motyckova et al., 2001)). Second, we were not able to identify genes whose activation by MITF is regulated by post-translational modifications of the transcription factor. Finally, it may also be that MITF does *not* regulate some of these genes and that the original attributions were mistaken.

We identify 71 genes as novel MITF-activated targets in melanoma and melanocytes (Table 2). Of these, three (*LYST, PSEN2* and *HPS4*) are known to be associated with pigmentation-specific processes (Gutierrez-Gil et al., 2007; Hutton and Spritz, 2008; Wang et al., 2006). Six others (*IL6R*, *IRF4, LGALS3, CAPN3, SORT1* and *RRAGD*) have previously been shown to be expressed in melanoma (Chang and Schimmer, 2007; Prieto et al., 2006; Schwabe et al., 1994; Sundram et al., 2003; Truzzi et al., 2008; Weeraratna et al., 2004; de Wit et al., 2005). Finally, three (*CA14, APOE* and *SCARB1*) have been identified in another pigment cell type, the retinal pigment epithelial cells (Duncan et al., 2002; Ishida et al., 2004; Ochrietor et al., 2005), thereby underpinning the accuracy of our approach. This left 59 genes not previously associated with melanoma or melanocytic functions. Some of these (*SLC1A4, SLC7A8, SLC19A2, ATP1A1, ATP6V1B2, ATP6V1C1, APOE, KCNN2, LYST, SCARB1* and *VAT*) are, like *SLC45A2* and *ACP5*, important for transport processes which suggests that MITF is involved in regulating cation flux as well as amino acid and lipid metabolism. Other genes (*ITPKB, SGK3, RHOQ, PPM1H, SEMA6A* and *TNFRSF14*) suggest, as do *RAB27A, IL6R, PSEN2, EDNRB* and *CAPN3*, that Mitf has important roles in regulating signal transduction.

The 264-fold upregulation of *MBP*, which encodes myelin basic protein (a major structural component of myelinating tissue), and the presence of M-boxes upstream of its transcription start site prompted us to investigate whether other myelinating cell-specific genes have a measurably significant relationship with MITF. Accordingly, the major myelin component *PLP1* was upregulated 254-fold by *Mitf*-transfection of SK-MEL-28 and correlated with *MITF* in six of seven data sets. *SOX10*, a myelination critical transcription factor (Stolt et al., 2002), also correlated with *MITF* in six of seven data sets and was upregulated 128-fold by *Mitf*-transfection. Similarly, the myelin-specific connexin *GJB1* showed correlation in five of seven data sets and was upregulated 202-fold on *Mitf*-transfection. Finally, both *FABP7* and *ERBB3* correlated in four of seven data sets and were also significantly upregulated (48- and 23-fold, respectively) on *Mitf*-transfection. Upregulation of *SOX10* by *Mitf*-transfection is an interesting finding as SOX10 has long been held to be a regulator of *MITF* (Lee et al., 2000), indicating the possibility that these transcription factors regulate each other's expression. It may be that the myelinating cell genes mentioned here are detected because they are directly regulated by SOX10 (Stolt et al., 2002), while its gene is being regulated by MITF, rather than being directly regulated by MITF itself. This, nevertheless, suggests that MITF may have a role alongside SOX10 in regulating the processes of myelination.

DNA microarray analysis of gene expression is a powerful method for the parallel assessment of thousands of transcriptional variations which drive biology. The principal agent of transcriptional change is the transcription factor, the protein which binds DNA to regulate the production of RNA. The potential for using microarrays to learn which transcription factors target which genes is now coming into realization. Particularly impressive is the application of chromatin immunoprecipitation and microarray analysis to identify regions of DNA which are bound by transcription factors (Buck and Lieb, 2004). However, the finding that a transcription factor may bind a piece of DNA does not necessarily identify the relevant context of that interaction. Similarly, showing that an experimentally induced transcription factor may upregulate a potential target gene is no guarantee that the relationship exists in the biology being studied. On the other hand, by looking at natural variation in a transcription factor's expression and comparing it to expression variation in potential target genes, it may be possible to verify a regulatory relationship identified under experimental conditions. Our strategy of combining two different microarray data analysis methods, a two-step approach intended to account for both irrelevance and non-causality, works very well and is a strategy with general application to the study of other transcription factors. This may have implications in other forms of cancer where transcription factors play important roles.

Microphthalmia-associated transcription factor-regulated gene products are among the most important targets for anti-melanoma therapies employing adaptive immune responses mediated by cytotoxic T-cells or antibodies. Many trials aim to activate cytotoxic T-cell responses against MITF-regulated antigens by vaccination with peptides or dendritic cell vaccines (Dummer and Nestle, 2000). For example, *GPM6B* and *SEMA6A* are membrane proteins whose expression is typically restricted to neuronal tissues. Their identification here as MITF-regulated factors may make them suitable targets for immunotherapeutic strategies. In conclusion, our study shows that our novel approach can identify new target genes of transcription factors and may therefore have implications for further cancer treatment.

Materials and methods

Western blotting

A library of melanomas which had been previously assessed for MITF expression and BRAF/NRAS mutation status (Hoek et al., 2006) was used to determine MAPK phosphorylation status. Cells were lysed in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA), 137 mM NaCl, 10% glycerol, protease inhibitors (Complete Mini +EDTA; Roche, Basel, Switzerland) and phosphatase inhibitors (Sigma Phosphatase inhibitor cocktails 1 & 2). Proteins were separated by SDS–PAGE under reducing conditions and transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). Membranes were probed with a specific primary antibody to p-Erk1/2 (ab4819; Abcam, Cambridge, UK), p-p38 (ab4822; Abcam, Cambridge, UK) or p-JNK (9251; Cell Signaling, Danvers, MA, USA) followed by a horseradish peroxidase-conjugated goat anti-rabbit (Bio-Rad, Reinach, Switzerland) secondary antibody. Bound antibodies were detected by chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK).

Cell culturing and exogenous Mitf expression

SK-MEL-28 cell lines cells were maintained in Dulbecco minimal essential medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum, 100 U of penicillin/ml and 100 μg of streptomycin/ml and cultured in a humidified incubator at 37°C with 5% CO_2. For generating the SK-MEL-28 cell lines over-expressing *Mitf*, a neomycin-selectable vector expressing Flag epitope-tagged *Mitf* (+)(Carreira et al., 2006) was linearized by EcoRI digestion, gel purified and transfected into SK-MEL-28 cells with Exgen 500 (Fermentas, Glen Burnie, MD, USA). Individual clones were isolated following neomycin selection (Invitrogen). Positive clones were detected by Western blotting with an anti-Flag antibody (M2; Sigma-Aldrich) (data not shown) and used for further analysis. For the gene expression analysis the subclone SK-MEL-28-MITF-7 was grown to confluency and then incubated in serum-free medium for 4 h. Each experiment (SK-MEL-28 cells and the SK-MEL-28 cells stably transfected with *Mitf*) was performed in biological triplicate to ensure statistically relevant results. RNA was extracted from the cells using Trizol reagent (Invitrogen) and further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. Isolated RNA was analysed for quality using a Bioanalyser (Agilent Technologies, Palo Alto, CA, USA). cDNA was prepared using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA samples were labelled and hybridized according to Roche NimbleGen (Madison, WI, USA) standard procedures at the Roche NimbleGen Service Laboratory in Reykjavik, Iceland. The microarrays used in this experiment were NimbleGen Expression 12 × 135K microarrays. This microarray design (2007-09-12_HG18_opt_expr) comprises 12 subarrays (~8.9 mm × 6.5 mm), each containing 1 35 000 features (13 × 13 μm) on a standard 25 × 76 mm glass slide. The NimbleGen array design is based on the HG18 build from UCSC and interrogates 45034 unique transcripts. Unique sample tracking controls were added to each sample prior to loading onto 12-plex arrays to ensure the integrity of the hybridization experiment and to confirm sample identity on each array. After hybridisation the microarrays were scanned using an Axon GenePix 4000B scanner (Molecular Devices, Union City, CA, USA) at 5 μm resolution.

Fold-change analysis

All data analysis was performed using GeneSpring GX 7.3 (Agilent Technologies). Probe set data values below 0.01 were set to 0.01 and each measurement was divided by the 50th percentile of all measurements in that sample, then each probe measurement was divided by the median of its measurements across all samples. To determine gene expression patterns differentiating between sample classes, a statistical analysis (ANOVA) was used to identify probes with class-specific expression patterns. The statistical analysis used the Welch two-sample *t*-test, a P-value cut-off of 0.05 was used and the Benjamini and Hochberg false discovery rate (Benjamini et al., 2001) was employed for multiple testing correction. A two-fold change filter was then applied to identify genes undergoing changed expression on transformation with a *Mitf*-expressing vector.

Correlation analysis

We were interested in identifying the genes with expression patterns that significantly correlated with that of *MITF*. The correlation study we performed was based on the Pearson product-moment correlation coefficient and assumed both a normal distribution of data and a strictly linear relationship between *MITF* gene transcription and MITF function (i.e. target gene transcription). The risk in using this approach is that non-normal data distributions tend to degrade its efficiency and we cannot account for non-linear (e.g. multi-factorial) associations.

Ignoring multiple-testing issues, a probe set with a Pearson co-efficient (r) of 0.5 with *MITF* is considered significant ($P < 0.05$) only if it is derived using a minimum of 16 samples. However, because we analyzed correlation among 22 272 probe sets we recognized that this was a multiple-testing problem, and subsequently the minimum number of samples for an r of 0.5 to be significant becomes 81. Even with a data set of sufficient size it is desirable to consider data from multiple sources (when available) to avoid single study bias, which is an inherent risk in high-throughput analysis (Hoek, 2007). Nevertheless, even with smaller sample sizes a sufficient number of data sets will ensure that $r = 0.5$ is significant.

Therefore, our correlation analysis employed seven different sets of DNA microarray data extracted from various databases. The criteria for data set selection was that each had to comprise at least ten different samples and use a common platform. These included melanoma cell line data from GSE8332 (Wagner data set, 18 samples), GSE7127 (Johansson data set, 63 samples), GSE4843 (Mannheim data set, 45 samples), GSE4841 (Philadelphia data set, 30 samples), and GSE4840 (Zurich data set, 15 samples) (Hoek et al., 2006; Johansson et al., 2007; Wagner et al., 2007) extracted from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/), melanoma cell line data published by Ryu and co-workers (Ryu data set, 10 samples) (Ryu et al., 2007) extracted from the Public Library of Science (http://www.plosone.org), and untreated melanocyte culture data published by Magnoni and co-workers (Magnoni data set, 21 samples) (Magnoni et al., 2007). All data sets derive from experiments using HG-U133 series microarrays (Affymetrix, Santa Clara, CA, USA). Each data set was normalized as previously described and analyzed separately using GeneSpring GS 7.3 (Agilent Technologies) and identical protocols. To identify gene expression patterns which correlated with that of *MITF*, the expression patterns of 22272 probe sets were individually compared with the *MITF* probe set 207233_s_at by performing a Pearson correlation and selecting probe sets with correlation coefficients >0.5. Probe sets which failed to pass this filter in all seven data sets were discarded and for each remaining probe set the median correlation coefficient was calculated. Furthermore, for each probe set a critical t value was calculated from the correlation coefficient based on sample size and used for a t-distribution analysis to determine its P-value in each data set, which were then multiplied across the data sets and adjusted by multiple testing correction to generate a final P-value.

Our principal approach, requiring that a gene meet or exceed $r = 0.5$ in all seven data sets, ensures high significance ($P < 3 \times 10^{-13}$), provides increased confidence in interpreting identified genes as novel targets of MITF-regulation and (by identifying known targets) helps demonstrate the power of our two-step approach. However, we acknowledge that this will generate a large number of false negatives, necessitating a loosening of the correlation criteria. Statistically, if we consider only the four smallest data sets alone (Ryu, Zurich, Wagner, Magnoni) finding a gene with $r = 0.5$ in each is not significant ($P = 0.132$). However, as we are considering seven different data sets and a subgrouping of the four smallest represents only one permutation of 35 possible four-set combinations then the finding is significant ($P < 0.004$). Therefore, we considered genes which met or exceeded $r = 0.5$ in four to six (of seven) data sets as potential false negatives.

Target identification

The genes identified by assessing gene expression change resulting from transformation of SK-MEL-28 cells with an *Mitf*-expressing vector were combined with results of the correlation study. Genes which correlated with *MITF* expression but showed no significant change in expression on *Mitf*-induction were considered to be co-regulated genes (i.e. responding to the same transcriptional signals as *MITF*, but not governed by MITF). Genes which strongly correlated with *MITF* expression and showed significant change in expression on *Mitf*-induction were considered to be candidate targets for regulation by MITF.

Acknowledgements

This work was supported by grants from Krebsliga-Oncosuisse 01927-08-2006 (K.H.), Swiss National Foundation 320000-119989 (R.D. and K.H.), Julia Bangerter Rhyner Stiftung (R.D.) and research grants from the Science and Technology Council of Iceland, the University of Iceland Research Fund and from the Eimskip Fund of the University of Iceland (E.S., A.S. and C.P.). We thank Leila Virkki for proof-reading the manuscript and the Functional Genomics Center Zürich (Zürich, Switzerland) for additional computing resources.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Genes undergoing significant upregulation on transfection of SK-MEL-28 with an Mitf-expressing vector.

Table S2 One hundred and ten genes with significant and strong correlation with *MITF* expression.

Table S3 Probable false negatives (correlation in four to six of seven data sets plus significant upregulation on Mitf-transfection of SK-MEL-28).

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Paper II

Cell

A Polymorphism in IRF4 Affects Human Pigmentation through a Tyrosinase-Dependent MITF/TFAP2A Pathway

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http://dx.doi.org/10.1016/j.cell.2013.10.022

SUMMARY

Sequence polymorphisms linked to human diseases and phenotypes in genome-wide association studies often affect noncoding regions. A SNP within an intron of the gene encoding Interferon Regulatory Factor 4 (IRF4), a transcription factor with no known role in melanocyte biology, is strongly associated with sensitivity of skin to sun exposure, freckles, blue eyes, and brown hair color. Here, we demonstrate that this SNP lies within an enhancer of IRF4 transcription in melanocytes. The allele associated with this pigmentation phenotype impairs binding of the TFAP2A transcription factor that, together with the melanocyte master regulator MITF, regulates activity of the enhancer. Assays in zebrafish and mice reveal that IRF4 cooperates with MITF to activate expression of Tyrosinase (TYR), an essential enzyme in melanin synthesis. Our findings provide a clear example of a noncoding polymorphism that affects a phenotype by modulating a developmental gene regulatory network.

INTRODUCTION

Human pigmentation is a complex process involving melanocytes that synthesize the pigment melanin in melanosomes, cell organelles that are transferred to neighboring keratinocytes, where they form a cap over nuclei, thus protecting them from negative effects of UV radiation (UVR). Pigmentation is not only one of the most distinguishing features of humans but also serves an important protective role. In humans, pigmentation decreases with increasing distance from the equator, presumably due to a balance between the pressure to optimize the amount of available UVR for the generation of vitamin D3 and the protection from UVR-mediated damage, which may result in increased risk of cutaneous malignancies. This has led to positive selection for less-pigmented skin, hair, and eyes in areas distant from the equator. The major difference between dark- and light-skinned individuals is due to differences in the number, size, and density of the melanin-containing melanosomes; the number of melanocytes is roughly the same (reviewed in Sturm, 2009).

The genetics of pigmentation is complex and involves several genes and pathways. It has been best characterized in the mouse where 171 of nearly 400 loci implicated in pigmentation have been functionally characterized (http://www.espcr.org/micemut/). These pigmentation genes affect various steps in the formation of melanocytes from the neural crest (e.g., *Mgf*, *Kit*, and *Mitf*), the generation of components of melanosomes and pigment (e.g., *pMel17*/*Silver* and *Tyr*), or the transport of melanosomes along microtubules in the dendrites before delivery to adjacent keratinocytes (e.g., *MyoVa*, *Rab27*, and *Mlph*). Most genes involved in pigmentation are transcriptionally regulated by the bHLHZip transcription factor MITF, the master regulator of melanocytes (reviewed in Steingrímsson et al., 2004).

Genome-wide association studies (GWASs) have identified several SNPs involved in human pigmentation, including SNPs on chromosome 6 near the *DUSP22*, *Interferon Regulatory Factor 4* (*IRF4*), and *EXOC2* genes, none of which was previously implicated in pigmentation (Sulem et al., 2007, 2008; Han et al., 2008). The SNP showing the strongest association is located in intron 4 of *IRF4* (Han et al., 2008). IRF4 belongs to the Interferon Regulatory Factors (IRFs), a wing-helix-turn-helix family of transcription factors that regulate interferon (IFN)-inducible genes (Paun and Pitha, 2007). Although IRF4 does not depend on IFN stimulation, it binds to the IFN-stimulated response element (ISRE) within IFN-responsive genes (Grossman et al., 1996; Escalante et al., 1998). *Irf4* mutant mice completely lack germinal centers and plasma cells and show severely reduced immunoglobulin levels in the serum; no defects in pigmentation were reported (Mittrücker et al., 1997). In a subset of multiple patients with myeloma, *IRF4* is translocated downstream of the immunoglobulin heavy-chain regulatory regions (Iida et al., 1997; Tsuboi et al., 2000). Interestingly, in this disease, *IRF4* acts as a lineage survival oncogene regardless of translocation status (Shaffer et al., 2008). Polymorphisms in the *IRF4* gene are associated with chronic lymphocytic leukemia (CLL) (Di Bernardo et al., 2008) and with nonhematopoietic diseases, including celiac disease (Dubois et al., 2010), and progressive supranuclear palsy (Höglinger et al., 2011). Finally, IRF4 was recently shown to be critical for transcriptional response to nutrient availability in adipocytes (Eguchi et al., 2011).

A few reports have linked *IRF4* to pigment cells. *IRF4* is expressed in melanocytes in the skin and in the G361 melanoma cell line (Grossman et al., 1996) as well as in most melanomas (Sundram et al., 2003). Importantly, IRF4 is associated with human pigmentation (Sulem et al., 2007; Han et al., 2008), and IRF4 expression correlates with MITF expression in melanoma cells (Hoek et al., 2008). Here, we show that *IRF4* is involved in pigmentation, that the melanocyte master regulator MITF activates expression of the *IRF4* gene, and that this activation depends on the presence of the transcription factor activator protein 2α (TFAP2A). A naturally occurring sequence variant associated with human pigmentation overlaps the TFAP2A-binding site, impairs binding by this transcription factor, and consequently, lowers induction of IRF4 expression. Together, the MITF and IRF4 proteins cooperatively activate expression of the gene encoding the pigmentation enzyme Tyrosinase (TYR). This activation depends on MITF- and IRF4-binding sites in the TYR promoter. Thus, we have established a direct link between a polymorphism in an intron in the *IRF4* gene and reduced expression of an enzyme essential for pigmentation.

RESULTS

Fine Mapping of the IRF4 Locus Implicates rs12203592 as a Functional Variant Affecting Pigmentation

Several sequence variants in the *IRF4* locus have been associated with freckling, sun sensitivity, eye and hair color, and with nevus counts, with rs12203592-[T/C] showing the strongest association (Sulem et al., 2007; Han et al., 2008; Duffy et al., 2010a). The rs12203592-T minor allele is most common in individuals of European descent; it is not seen in sub-Saharan Africans or in East Asians (see Figure S1 available online). Analysis of sequenced vertebrate species shows that this position is occupied by C, suggesting that it is the ancestral allele.

In order to fine map the pigmentation trait associations at the *IRF4* locus, we used whole-genome sequencing data derived from 2,230 Icelanders sequenced to an average coverage of at least 10×. This yielded approximately 38.5 million SNP and small indel (insertion or deletion) variants genome wide, 16,280 of which were located in the region around *IRF4* (0–1 Mb on chromosome 6). Using imputation assisted by long-range haplotype phasing (Kong et al., 2008, 2009; Holm et al., 2011), we determined the genotypes of these 16,280 variants in 95,085 individuals who had been typed using Illumina SNP chips. We then tested each variant for association with eye color, hair color, freckling, and sun sensitivity, observing numerous significant signals (Figure 1). The strongest signals in the *IRF4* region came from the association of rs12203592-T with presence of freckles, brown hair, and high sensitivity of skin to sun exposure (Figure 1; Table S1). The second-most significant variant association was for rs62389424-A with freckles ($p = 1.1 \times 10^{-81}$), nearly 40 orders of magnitude less significant than the corresponding signal from rs12203592-T ($p = 2.0 \times 10^{-120}$). The rs62389424 variant is correlated with rs12203592 with an r^2 of 0.65. Two other SNPs in the region have r^2 values in excess of 0.2 with rs12203592, and they also gave association signals with similar phenotypes (Table S2). When, in a multivariate analysis, the associations were conditioned on the effect of rs12203592, the signals from the three correlated SNPs became nonsignificant (Table S2). Indeed, once rs12203592 was taken into account in the multivariate analysis, no variant in the *IRF4* region retained a significant signal after Bonferroni correction for the number of tests (Figure 1). We note in this context that the signal from the originally reported pigmentation variant at this locus, rs1540771-T (Sulem et al., 2007), was also captured by rs12203592 (Table S2). Thus, the associations at the *IRF4* locus with freckles, hair color, sun sensitivity, and eye color could all be accounted for by rs12203592, and no other variant detected by sequencing explains the effect with a similar level of significance.

Intron 4 of IRF4 Contains a Melanocyte-Specific Enhancer Element

rs12203592 is located in intron 4 of the IRF4 gene, suggesting that this SNP might alter the function of a *cis*-regulatory element. Data from the ENCODE consortium (Thurman et al., 2012) show that rs12203592 overlaps a peak of DNase I hypersensitivity

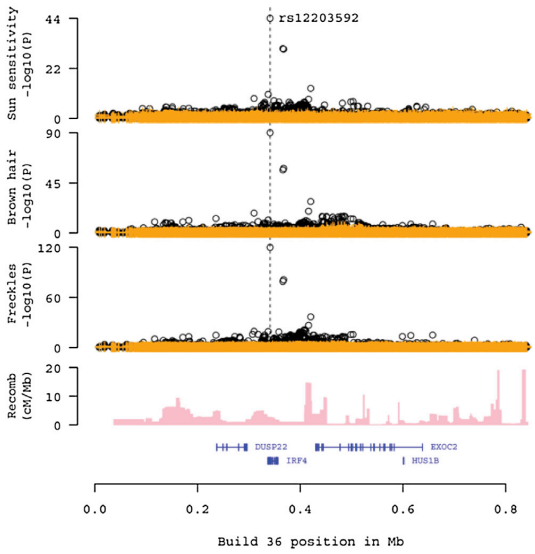

Figure 1. Association of SNPs in the IRF4 Region with Pigmentation Phenotypes

Association with pigmentation traits of variants in the IRF4 region (0–1 Mb on chromosome 6) was determined by whole-genome sequencing and imputation into SNP chip-typed individuals. Black points show the unadjusted association values; yellow points show association values adjusted for the effect of rs12203592. The x axis is the genomic coordinate (hg18 Build 36). The y axes are the association-$\log_{10}$ p values for sun sensitivity (yes/no, upper panel), brown versus blond hair color (second panel), and freckling (yes/no, third panel). The position of rs12203592 is indicated. The fourth panel shows the local recombination (Recomb) rates in cM/Mb. The lowest panel indicates the locations of known genes in the region, from UCSC. See also Table S1 and Figure S1.

(HS), a property of active regulatory elements, in human primary melanocytes and three human melanoma lines (Figure 2A). Moreover, rs12203592 does not overlap a DNase I-HS peak in 158 of 165 (96%) non-melanocyte-derived cell types examined by ENCODE, suggesting that the regulatory activity in this region is specific to the melanocyte lineage. In a subset of 49 ENCODE cell types assayed by the Crawford group (Duke University) for both DNase I-HS and global gene expression, *IRF4* is expressed at high levels only in cell types of melanocyte (n = 3) and lymphocyte origin (n = 8). The corresponding patterns of DNase I HS in these cell types suggest that *IRF4* expression in lymphocytes and melanocytes is directed by distinct sets of regulatory elements (Figure 2B). In addition, the position orthologous to rs12203592 in the mouse genome directly overlaps a melanocyte enhancer (Gorkin et al., 2012) because it is occupied by p300 and marked by H3K4me1 in the melanocyte line melan-Ink4a-Arf (Sviderskaya et al., 2002) (Figure 2C).

To confirm that the intronic sequence containing rs12203592 acts as a melanocyte-specific enhancer, we subcloned a 450 bp fragment containing the rs12203592-C allele upstream of a minimal promoter that drives luciferase expression and assayed its activity in both the mouse melan-Ink4a-Arf and human SK-MEL-28 cell lines. The fragment showed strong enhancer activity, directing >35-fold-higher luciferase expression than the minimal promoter alone in melan-Ink4a-Arf, and >200-fold in SK-MEL-28 (Figures 3A and 3B). We next asked whether the genotype at rs12203592 affects enhancer activity. In both melan-Ink4a-Arf and SK-MEL-28, the presence of the rs12203592-T allele significantly reduced the enhancer activity of the fragment ($p < 0.008$) (Figures 3A and 3B).

We further assayed the sequence containing rs12203592 in vivo using transgenic zebrafish. We engineered a vector containing the entire human *IRF4* intron 4 sequence upstream of a minimal promoter and the gene encoding GFP. We created two versions of the vector, with either the T or C allele at rs12203592; as above, the remainder of the sequence was identical in both constructs. We injected these constructs, or a negative control construct lacking any human genomic sequence, into zebrafish embryos at the two-cell stage, incubated them 48 hr postfertilization, and scored them for the presence of GFP-positive cells. In embryos injected with the negative control construct, we did not detect GFP-positive melanocytes (0 out of 40 embryos) (Figure 3C). In embryos injected with the *IRF4* intron 4 reporter construct containing the ancestral rs12203592-C allele, we detected GFP-positive melanocytes in about 20% of embryos (8 out of 40 embryos; 17 melanocytes total from the 8 embryos), consistent with the mosaicism expected in transient transgenic embryos (Figures 3C and 3D). By contrast, in embryos injected with the reporter containing rs12203592-T, only 9% of the embryos had detectable GFP-positive melanocytes (4 out of 44 embryos; only six melanocytes total). The difference in the total number of melanocytes is statistically significant ($p = 0.0023$, unpaired t test). We did not detect expression in other tissues, beyond transient, scattered expression, which was seen in all three constructs and has been reported by others (Bessa et al., 2009). We conclude that *IRF4* intron 4 contains a melanocyte enhancer, likely directing *IRF4* expression in these cells, and that the rs12203592-T allele reduces the activity of this enhancer.

MITF Activates IRF4 Gene Expression

Chromatin immunoprecipitation sequencing (ChIP-seq) and gene expression studies have suggested that MITF may be involved in regulating *IRF4* gene expression (Hoek et al., 2008, Strub et al., 2011). We analyzed *Irf4* expression in mice lacking *Mitf* and focused on the heart because cells that normally express *Mitf* are still present in the heart of $Mitf^{mi\text{-}vga9}$ homozygous mutants, whereas melanocytes are absent resulting in white coat color (Hodgkinson et al., 1993). *Irf4* gene expression was dramatically reduced in *Mitf* mutants compared to wild-type controls (Figure 4A), consistent with the possibility that *Irf4* is regulated by MITF. To determine whether *IRF4* is a target of MITF in the melanocyte lineage, we used shRNA to knock down *MITF* and *IRF4* mRNAs in human 501mel melanoma cells, which normally express these genes. Transfecting these cells with shRNA directed against *MITF* reduced *MITF* mRNA expression to 45% (Figure 4B) and MITF protein levels to 41% (Figures 4C and S2) of those seen in untreated cells or cells treated with a scrambled control shRNA. In cells treated with shMITF, the expression of *IRF4* mRNA and protein was dramatically reduced (to 50% and 25%, respectively). shRNA against *IRF4*

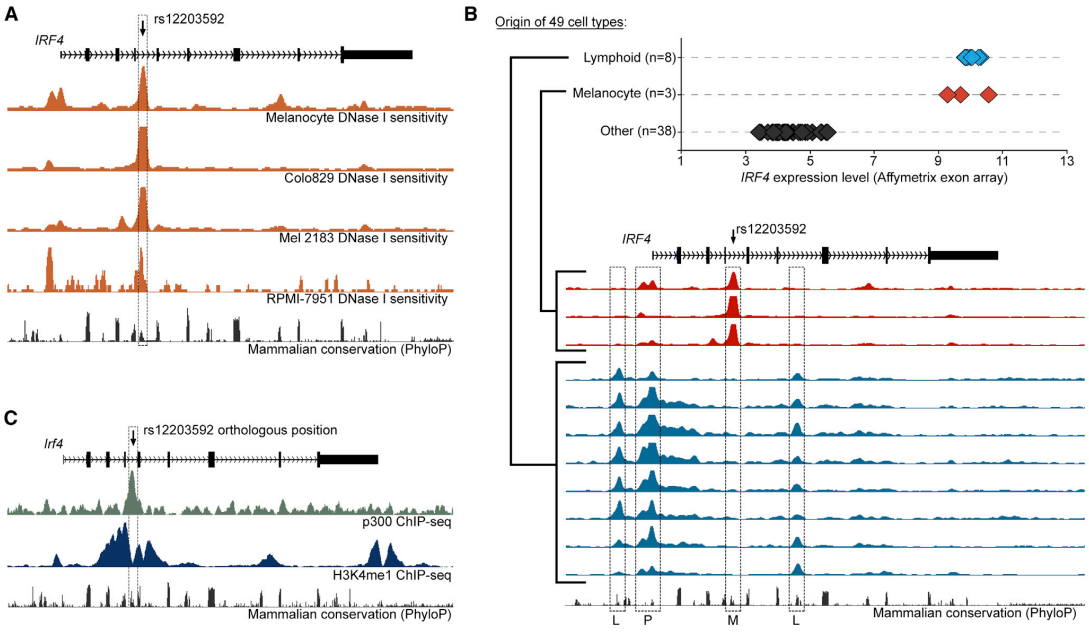

Figure 2. rs12203592 Disrupts a Conserved Melanocyte Enhancer at *IRF4*

(A) UCSC genome browser view shows 25 kb region around *IRF4* (hg19 coordinates chr6:388,750–413,750). DNase-seq signal (orange) in human epidermal melanocytes, Colo829, Mel 2183, and RPMI-7951 was generated by ENCODE.

(B) Top view is a graph showing *IRF4* expression level as measured by ENCODE in 49 cell types (Duke Affymetrix exon arrays). Bottom is the UCSC genome browser view of 25 kb region around *IRF4* (hg19 coordinates chr6:388,750–413,750) in corresponding cell lines. Melanocyte-specific DNase HS peak overlapping rs12203592 is labeled "M," lymphoid-specific DNase HS peaks are labeled "L," and DNase HS overlapping the IRF4 promoter is labeled "P." Cell types shown are (top to bottom) human epidermal melanocytes, Colo829, Mel 2183, GM12878, GM12891, GM12892, GM19238, GM19238, GM19240, GM18507, and CLL.

(C) UCSC genome browser view shows 25 kb region around *Irf4* (mm9 coordinates chr13:30,838,000–30,863,000). The ChIP-seq signals for EP300 (green) and H3K4me1 (blue) in the mouse melanocyte line melan-Ink4a-Arf are from Gorkin et al. (2012).

resulted in reduction in *IRF4* mRNA and protein expression to 40% and 20% of control levels, respectively, whereas *MITF* expression was unaffected (Figures 4C and S2). The expression of *DCT* (encoding Dopachrome tautomerase) and TYR, two known MITF target genes (Yasumoto et al., 1994, 2002), was also reduced upon shMITF treatment, whereas shIRF4 treatment only affected the expression of *TYR* (Figure 4B). TYR protein expression was reduced upon treatment with shMITF, shIRF4, and shAP2A, as well as with all shRNAs together (Figure S2). Knocking down TFAP2A did not affect MITF expression. We also tested this relationship in an overexpression assay. Untransfected 501mel melanoma cells express *MITF* but low levels of both *TYR* and *IRF4* mRNAs, whereas HEK293T cells do not express *MITF* endogenously and also lack expression of *TYR* and *IRF4*. In both cell lines, overexpression of mouse *Mitf* (*mMitf*) strongly induced the levels of *TYR* and *IRF4* (Figure 4D). Analysis of the expression of MITF and IRF4 in 22 melanoma cell lines revealed a positive correlation between MITF and IRF4 ($p = 0.0003$, Pearson correlation coefficient, Figure S3A). These results indicate that MITF directly or indirectly regulates *IRF4* expression. They also suggest that *TYR* expression depends on IRF4.

rs12203592 Alters the Function of a Melanocyte Enhancer in IRF4 through Disruption of a TFAP2A-Binding Site

The rs12203592 polymorphism is located 66 bp from three sites recently shown to be occupied by MITF in ChIP-seq studies (Figure 5A) (Strub et al., 2011). ChIP performed in 501mel and MITF-transfected 293T cells showed that MITF binds to intron 4 of *IRF4* (Figure 5B). Interestingly, the rs12203592-T polymorphism occurs in a predicted binding site (GGCAAA) for TFAP2A (Do et al., 2010), which was recently shown to be involved in melanocyte differentiation in zebrafish (Van Otterloo et al., 2010). To determine if TFAP2A can bind this sequence, we performed anti-TFAP2A ChIP in 501mel cells (homozygous for rs12203592-C) and showed that TFAP2A binds to the intron 4 element in the *IRF4* gene (Figure 5C). Furthermore, gel shift assays showed that TFAP2A binds oligos carrying the TFAP2A motif from *IRF4* intron 4 only when the oligos harbor the rs12203592-C allele. It did not bind the rs12203592-T variant, nor when the binding site was altered completely (Figure 5D). Addition of antibody resulted in a supershift only in the presence of the wild-type oligo. Binding of MITF to the neighboring MITF sites was not affected by the SNP (Figure S3B). These results show that TFAP2A binds

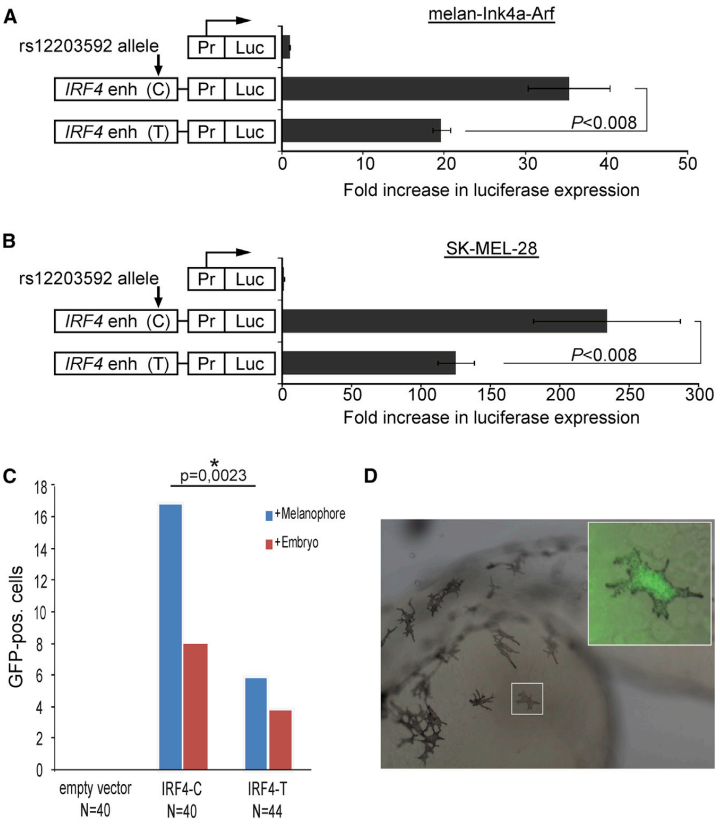

Figure 3. Intron 4 of IRF4 Drives Expression in Melanocytes

(A and B) Results of the luciferase assays in melan-Ink4a-Arf and SK-MEL-28 cells are shown. Pr, promoter; Luc, luciferase reporter gene; *IRF4* enh, 450 bp fragment from the fourth intron of IRF4 containing rs12203592. "*IRF4* enh" fragments are identical except for the base at position rs12203592 as indicated ("C" is the ancestral allele; "T" is the derived allele). The x axis shows fold luciferase activity relative to the minimal promoter alone, which is normalized to one (1). p values are calculated by Kolmogorov-Smirnov test. The result is also highly significant by other nonparametric tests (p = 0.007937 by Wilcoxon rank sum test for both melan-Ink4a-Arf and SK-MEL-28) and by the standard parametric t test (p = 0.001686 for melan-Ink4a-Arf; p = 0.01032 for SK-MEL-28). Each bar represents the average of five biological replicates per reporter construct. Error bars represent SD.

(C) Reporter constructs contain either the rs12203592-C version of the IRF4 intron 4 element (IRF4) or the rs12203592-T version (IRF4snp) upstream of the GFP reporter after injection into zebrafish. The numbers represent the number of melanophores (blue) and embryos (red) seen to be positive (pos.) for GFP. The difference between the melanophores containing the wild-type allele of intron 4 of IRF4 compared to the mutant allele (rs12203592) is statistically significant (*p = 0.0023, unpaired t test).

(D) Image shows a GFP-positive zebrafish embryo with a GFP-positive melanophore in the inset.

the ancestral sequence (rs12203592-C) in intron 4 of *IRF4* but fails to bind to the rs12203592-T variant.

To determine if TFAP2A plays a role in the activity of the *IRF4* enhancer, we performed reporter assays using intron 4 of *IRF4* as regulatory element. 501mel melanoma cells were cotransfected with the reporter constructs and with plasmids encoding MITF, TFAP2A, or both. Neither MITF nor TFAP2A alone elevated expression from this element above basal levels (Figure 5E). However, when both MITF and TFAP2A were expressed simultaneously, transcription was increased 6-fold (Figure 5E), suggesting that the proteins have cooperative effects on the activity of this enhancer. This is further supported by the shRNA experiments that showed that knocking down TFAP2A reduces IRF4 expression (Figures 4B and 4C). Removing all three MITF-binding sites together (intron 4-3xE) led to background-level expression (Figure 5E). Importantly, when the rs12203592-T variant was introduced into the reporter construct (intron 4-rs), the cooperation seen between MITF and TFAP2A was reduced to levels close to background (Figure 5E). This suggests that MITF requires TFAP2A in order to induce expression from the intron 4 element and that the rs12203592-T polymorphism abolishes the MITF-mediated activation.

To determine whether the rs12203592-T variant affects *IRF4* expression in melanocytes, RNA and protein were isolated from melanocytes differentiated from human neonatal foreskin melanoblasts from Australian subjects (Cook et al., 2009). RT-qPCR showed a significantly lower ($p < 0.01$, unpaired t test) expression of *IRF4* mRNA in cells homozygous for the rs12203592-T polymorphism than in cells homozygous for the ancestral C (Figures 5F and S3C). IRF4 protein levels in cells from T/T individuals were considerably reduced compared to that of cells from C/C individuals (Figure 5G).

To determine whether the rs12203592 polymorphism affects expression of the transcript on the chromosome on which the variation resides, we used an allele discrimination assay. We used RT-qPCR on human primary melanocytes heterozygous for the *IRF4* 3′ UTR SNP rs872071-A/G. The rs872071-G allele resides 15 kb distal to rs12203592 and is present on the haplotype on which the rs12203592-T allele arose, thus allowing for comparison of IRF4 allele-specific expression levels, controlling for potential transfactor variations that may contribute to expression level differences between individuals. We observed no difference in IRF4 allelic expression in rs12203592-C/C homozygous melanocytes, as represented by the rs872071-A/G allele ratio (Figure S4). In contrast, cells heterozygous for the rs12203592 C/T alleles exhibit a significantly higher

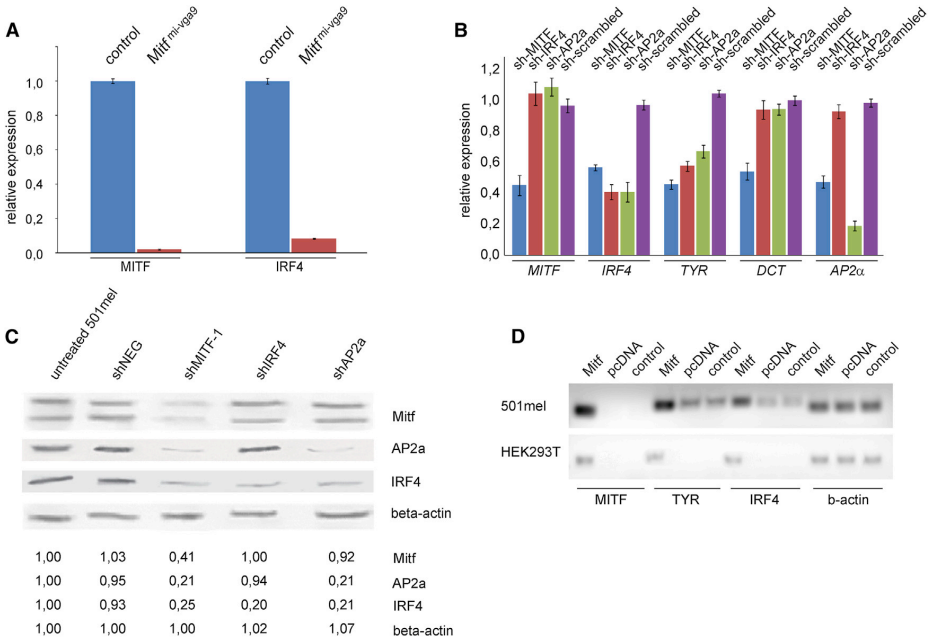

Figure 4. MITF Regulates Expression of IRF4

(A) The expression of the *Mitf* and *Irf4* mRNAs is reduced in hearts from *Mitf*$^{mi\text{-}vga9}$ mutant mice, as determined by qPCR analysis. Data are represented as mean ± SEM.

(B) Expression of the *MITF*, *IRF4*, *TFAP2a*, *DCT*, and *TYR* genes upon treatment with shRNA against *MITF*, *IRF4*, *TFAP2A*, and negative control in 501mel melanoma cells as determined by qPCR analysis is shown. Data are represented as mean ± SEM.

(C) Western blot shows expression of the MITF, TFAP2A, IRF4, and β-actin proteins in 501mel cells after shRNA treatment. Intensity quantification is relative to actin-loading control. See also Figure S2.

(D) Overexpression of the mouse Mitf (mMitf) cDNA (expressed from pcDNA3.1) in 501mel melanoma cells and in HEK293T embryonic kidney cells affects expression of the TYR and IRF4 mRNAs as assayed by RT-qPCR, whereas β-actin expression is unchanged. mMitf was detected with species-specific primers that only recognize the mouse *Mitf* gene. See also Figure S3.

See also Figure S2 and Table S3.

rs872071-A/G allele ratio ($p < 0.01$), consistent with reduced transcription occurring from IRF4 rs872071-G alleles in phase with rs12203592-T.

Several alternate products are made from the IRF4 gene, including transcripts that initiate in intron 4 (Figure S5A), leading to an mRNA containing internal ATGs (lacking Kozak consensus sequences) and the tentative production of a truncated protein lacking the N-terminal DNA-binding domain. To determine the major product in the melanocyte, RT-qPCR analysis with transcript-specific primers was performed on RNA isolated from foreskin melanoblasts from Australian subjects. This showed that the major *IRF4* product in melanocytes is the full-length transcript; the smaller alternative product was barely detectable (Figure S5B). Similarly, in mouse melanoblasts isolated from E15.5 and E17.5 embryos and melanocytes from P1 and P7 neonatal pups (Debbache et al., 2012), the full-length *Irf4* transcript was the only transcript detected at all developmental stages (Figure S6). We therefore conclude that the regulatory element in intron 4 affects the production of the full-length *IRF4* transcript.

IRF4 and MITF Cooperatively Regulate TYR Expression

To determine how IRF4 levels might affect pigmentation, we searched the promoter sequences of several pigmentation genes for the IRF4-binding motif. Several potential IRF4-binding motifs were found in the *TYR* promoter, on either side of known MITF-binding sites previously shown to be essential for gene activation (Figure 6A) (Yasumoto et al., 1994; Bertolotto et al., 1996). To investigate the relationship between IRF4 and the expression of several pigmentation genes, two human melanocyte strains with high expression of *IRF4* were independently transfected with siRNA targeting *IRF4* for 48 hr, followed by total RNA extraction and RT-qPCR. These results combined show that expression of *IRF4* was reduced on average to 40% of cells treated with negative control siRNA (Figure 6B). Interestingly, the expression of *TYR* was reduced to 50% compared to untreated control, whereas the expression of the pigmentation genes *SLC45A2* (*MATP*), *SLC24A5*, and *DCT* was largely unaffected (Figure 6B). Similarly, knockdown of *IRF4* affected expression of *TYR* but not of *DCT* in 501mel melanoma cells (Figures 2B and 2C). This suggests that IRF4 is involved in regulating *TYR* expression but

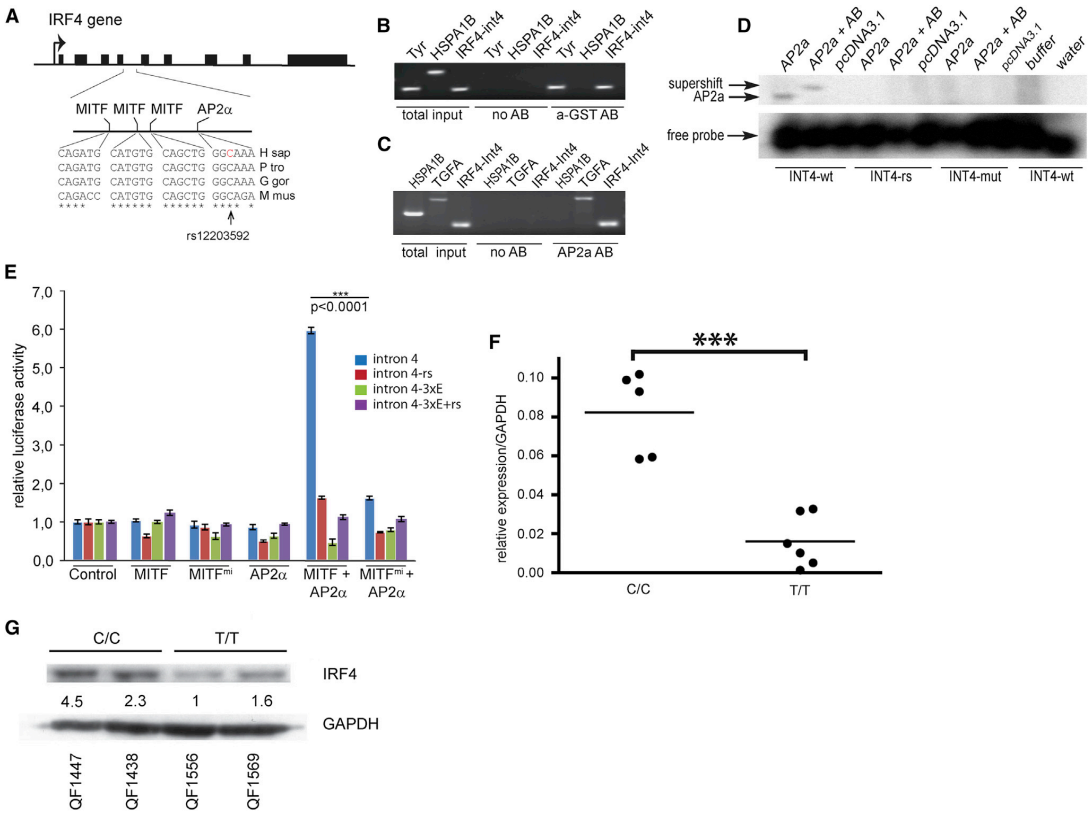

Figure 5. MITF and TFAP2A Affect *IRF4* Expression by Binding Regulatory Elements in Intron 4

(A) Schematic view presents the IRF4 gene. The sequence shows a comparison of MITF- and TFAP2A-binding sites in intron 4 of IRF4 among humans (H sap [*Homo sapiens*]), gorilla (G gor [*Gorilla gorilla*]), chimpanzee (P tro [*Pan troglodytes*]), and mouse (M mus [*Mus musculus*]). The location of the rs12203592 polymorphism is indicated.

(B) ChIP analysis of a GST-tagged MITF protein (a-GST AB) was performed in 501mel cells. Primers specific for HSPA1B (negative control), TYR (positive control), and intron 4 of IRF4 are indicated. The GST-tagged Mitf only precipitated TYR and IRF4 sequences.

(C) ChIP analysis of TFAP2A in 501mel cells is presented. PCR products specific for TGFA (positive control), HSPA1B (negative control), and intron 4 of IRF4 are shown. TFAP2A only precipitated TGFA and INT4 sequences.

(D) Gel shift analysis shows the binding of TFAP2A to the ancestral sequence (INT4-WT) in intron 4 of IRF4 (coordinates chr6: 396309–396333 in build GRCh37.p5 of the human genome), but not to the rs12203592-T sequence (INT4-RS) or to a completely mutated sequence (INT4-mut).

(E) Luciferase reporter assays were performed in 501mel melanoma cells using intron 4 of IRF4 as a reporter. The ancestral IRF4 intron 4 sequence (intron 4, blue), the rs12203592-T polymorphic sequence (intron 4-rs), a sequence where all MITF-binding sites were mutated (intron 4-3xE), and a sequence containing mutated MITF sites and the rs12203592-T polymorphism (intron 4-3xE+rs) were tested. The luciferase reporters were cotransfected with the wild-type MITF and TFAP2A proteins and with a dominant-negative version of MITF ($MITF^{mi}$). Statistical analysis was done using unpaired t test. Data are represented as mean ± SEM.

(F) Cells homozygous for the ancestral allele (CC) express significantly higher levels of IRF4 than cells homozygous for the rs12203592-T polymorphism (TT). Fold expression data are represented relative to pooled mean TT expression ± SEM. Statistical analysis was performed using ANOVA (***$p < 0.001$).

(G) A western blot shows expression of the IRF4 protein in melanocytes from CC and TT individuals. Intensity quantification is relative to GAPDH-loading control. Note the decrease of basal IRF4 protein in the TT melanocyte cell lines.

See also Figures S3, S4, and S5.

does not affect expression of SLC45A2 or SLC24A5. The expression of IRF4 correlates with the expression of TYR in 22 melanoma cell lines ($p = 0.0007$, Pearson correlation coefficient, Figure S3A).

Cotransfection assays in HEK293T cells (which do not express MITF) showed that, whereas MITF can activate expression of the *TYR* gene, the IRF4 protein is unable to do so on its own (Figure 6C). However, in the presence of both MITF and IRF4, cooperative effects were observed with increasing amounts of IRF4 (Figure 6C). A mutant MITF protein with defective DNA-binding ability resulted in less activation and reduced cooperativity

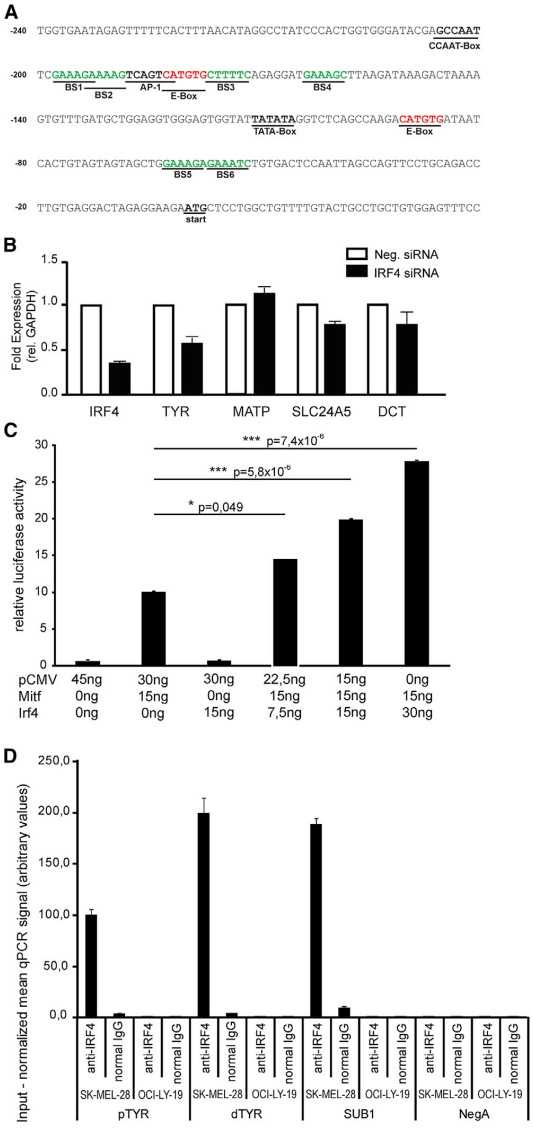

Figure 6. MITF and IRF4 Regulate Expression of TYR

(A) Schematic view of the human TYR promoter shows the sequence of the MITF and potential IRF4-binding sites.

(B) Expression of IRF4, TYR, MATP, SLC24A5, and DCT was determined by qPCR, after treatment with siIRF4 or control siRNAs. Fold expression of each gene is represented relative to cells transfected with negative siRNA (arbitrarily set to one [1]). Data represent the mean ± range of two experiments using two primary melanocytes (QF1438 and QF1424).

(C) Luciferase reporter assay used wild-type TYR promoter as reporter construct and cotransfected with constructs expressing the MITF and IRF4 proteins. Statistical analysis was performed using the t test; data are represented as average with the SD.

(D) IRF4 binds the TYR locus. ChIP real-time PCR (ChIP-qPCR) analysis shows IRF4 binding at sites proximal (around transcription start site; pTYR) and more distal (~1,800 bp upstream of transcription start site; dTYR) to TYR-coding region and other control loci in SK-MEL-28 melanoma cell line and in negative control OCI-Ly-19, a germinal center type B cell lymphoma (GCB-DLBCL) cell line previously shown to lack IRF4 expression (Yang et al., 2012). Primer pair amplifying a region upstream of the SUB1 locus was used as a positive control because this region showed strong IRF4 binding both in multiple myeloma (Shaffer et al., 2008) and activated B cell-type B cell lymphoma (Yang et al., 2012) cell lines. NegA is a region on chromosome 7, used as a negative control for IRF4 binding, due to the lack of observable IRF4 binding in previous studies with multiple myeloma and ABC-DLBCL cell lines. Error bars depict SEM. See also Figure S7.

(Figure S7A). Similar effects were observed when the E box was mutated (Figure S7B), confirming that the cooperativity is mediated through MITF. Quantitative ChIP experiments in Skmel28 (Figure 6D) and Skmel5 (Figure S7C) melanoma cells showed that IRF4 binds the proximal TYR promoter as well as a more distant region.

Irf4 Affects Pigmentation in Mice

No pigment phenotypes were reported in mice with a targeted deletion of *Irf4* (Mittrücker et al., 1997). We analyzed the expression of *Mitf*, *Tfap2a*, *Irf4*, and *Tyr* in melanoblasts isolated from E15.5, E17.5, P2, and P7 mouse embryos (Debbache et al., 2012). *Mitf* and *Tfap2a* are both expressed at the embryonic and postnatal stages, whereas *Irf4* is detected at a low level at the two postnatal stages (Figure 7A). *Tyr* is expressed at all stages, but the level of expression increases at the postnatal stages and is 10-fold higher at P7 than at the earlier stages (Figure 7A). This expression pattern is consistent with our data that MITF and TFAP2A regulate *IRF4* expression, and MITF and IRF4 regulate *TYR* expression. Because early stages of development do not express *Irf4*, this also suggests that IRF4 is mostly involved in melanocyte differentiation. To determine if *Irf4* plays a role in mouse melanocytes, mice carrying an *Irf4-flox* cassette (*Irf4 f*) (Klein et al., 2006) were crossed to mice expressing the Cre-recombinase under control of the *Tyr* promoter (*Tyr::Cre*) (Delmas et al., 2003). This led to the lack of *Irf4* expression in *Tyr*-expressing cells, but no apparent pigmentation phenotype, similar to the *Irf4* null mice (Mittrücker et al., 1997). In order to test the relationship between *Mitf* and *Irf4* in the mouse, we crossed the *Tyr*::*Cre*/^{0}; *Irf4* f/f mice to mice carrying the dominant-negative allele *Mitf*$^{Mi\text{-}White}$ (*Mitf*$^{Mi\text{-}Wh}$). Mice heterozygous for this allele (*Mitf*$^{Mi\text{-}Wh}$/+) have a partial deficiency of *Mitf* and exhibit a stable, gray coat color with white belly spot. Interestingly, the *Tyr*::*Cre*/^{0}; *Irf4* f/f; *Mitf*$^{Mi\text{-}Wh}$/+ mice showed distinctly lighter coat color than seen in *Mitf*$^{Mi\text{-}Wh}$/+ mice, which are negative for the *Tyr-Cre* transgene (*Irf4 f/f*; *Mitf*$^{Mi\text{-}Wh}$/+) (Figure 7B). This confirms that IRF4 plays a role in mouse pigmentation, like in humans, and that the effects of *Irf4* in the mouse also depend on *Mitf*. In humans, the *IRF4* variant rs12203592-T is linked to brown hair color (Sulem et al., 2007) (Table S1). Thus, reduced *IRF4* expression alone is sufficient for lighter hair color in humans, whereas in mice, MITF function also needs to be reduced to see effects on pigmentation. This suggests that there may be species-specific differences between mice and humans in terms of the requirement for *MITF* and *IRF4* in melanocytes of the hair.

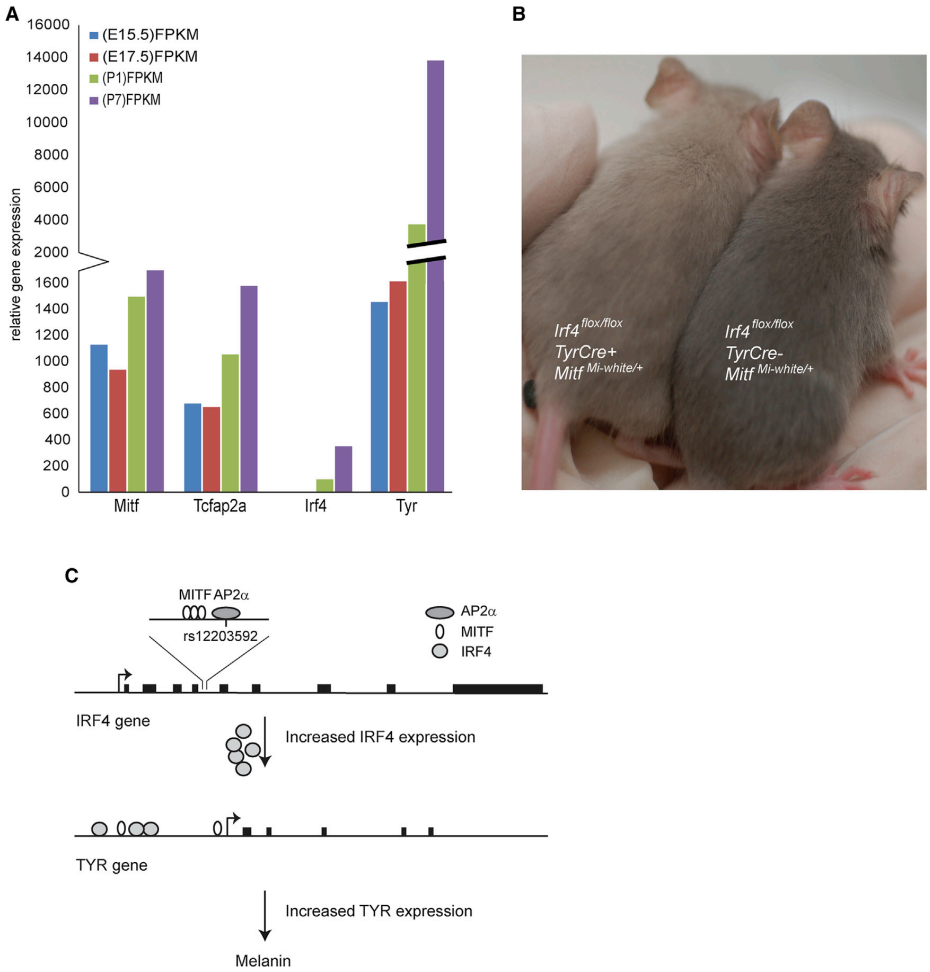

Figure 7. IRF4 Is Involved in Regulating Pigmentation in Mice

(A) The expression plot shows the presence and clear differences in the expression of *Mitf*, *Tcfap2a*, *Irf4*, and *Tyr* at different developmental time points, E15.5, E17.5, P1, and P7, measured in FPKM. Indicated expression levels of a transcript are proportional to the number of reads sequenced from that transcript after normalizing for respective transcript's length. RNA from E15.5 embryos is shown in blue bars, E17.5 embryos in red, P1 pups in green, and P7 in violet.

(B) Mice where *Irf4* has been conditionally knocked out in heterozygotes for the semidominant $Mitf^{Mi\text{-}Wh}$ mutation are presented. $Mitf^{Mi\text{-}Wh}$ heterozygotes that are simultaneously lacking *Irf4* show lighter coat color (left) than mice that are wild-type for *Irf4* (right).

(C) Model depicts the relationship among MITF, TFAP2A, and IRF4 in melanocytes and their effects on the expression of *TYR*. MITF and TFAP2A bind together to the intron 4 element in the *IRF4* gene and regulate the expression of *IRF4* from the upstream promoter. MITF and IRF4 then bind to and activate expression of TYR, leading to normal pigmentation. The rs12203592-T polymorphism leads to reduced IRF4 activation and thus reduced TYR expression, which consequently leads to sun sensitivity and blue eyes.

DISCUSSION

GWASs have yielded a long list of sequence variants correlated with a wide range of human phenotypes. Most of these variants are located in noncoding regions of the genome and do not alter the amino acid sequence of proteins or have known regulatory functions. Two steps are essential in order to characterize the biology behind such associations. First, the association signals must be fine mapped using full sequence data. Second, the biological processes involved must be unraveled experimentally. In this study, we have taken both steps to shed light on the functional consequences of the IRF4-noncoding variant rs12203592 on pigmentation.

Our work proposes a model describing how IRF4 acts in cooperation with MITF to influence pigmentation phenotypes (Figure 7C). In melanocytes, MITF and TFAP2A cooperatively activate *IRF4* expression through an enhancer element in intron 4. In turn, IRF4 and MITF cooperate to activate transcription of *TYR*. The rs12203592-T allele reduces binding of TFAP2A to its cognate site in intron 4 of *IRF4*, thereby suppressing the induction of IRF4 expression and, as a consequence, impairing the cooperative induction of *TYR*. This leads to the pigmentation phenotypes associated with the rs12203592-T allele. The effects of this variant must be cell-type specific because Do et al. (2010) have shown that it leads to increased *IRF4* promoter activity in Burkitt lymphoma B cells (Raji), HEK293T human embryonic kidney cells, and the NCI-H295R human adrenal cells. Consistent with our results, they showed that TFAP2A binds with higher affinity to the C allele than to the T allele (Do et al., 2010). Thus, the cell-type-specific effects must be mediated by transcription factors other than TFAP2A.

In humans, genetic variants in different genes have particular effects on pigmentation traits. For example, *MC1R* and *ASIP* variants have major effects on freckling, sun sensitivity, and red hair, whereas they have relatively little effect on eye color (Sulem et al., 2007, 2008). The rs12203592-T variant of *IRF4* also has major effects on freckling and sun sensitivity but is associated with brown (over blond) hair and has a notable effect on eye color (Table S1). The results presented here suggest that *IRF4*, like *MC1R* and *ASIP*, exerts its major effects on pigmentation traits through the master regulator MITF. However, some divergence in pathways must occur in order to account for the differences in effects on pigmentation traits. We have shown that *TYR* is a target of MITF-IRF4 cooperative activation. However, *TYRP1* and *DCT* are targets of MITF activation that do not appear to be responsive to IRF4. Clearly, the MITF-IRF4 cooperation only occurs in a subset of MITF-responsive genes. A broader search of MITF target genes for potential IRF4 responsiveness might shed light on the pattern of pigmentation traits affected by *IRF4*.

Freckles represent clusters of concentrated melanin in the skin, without an increase in melanocyte numbers. Freckles are either light brown or red but usually become darker and more visible upon exposure to sunlight. GWASs have shown that variations in MC1R, ASIP, TYR, and BNC2, in addition to IRF4, are associated with freckles (Sulem et al., 2007, 2008; Eriksson et al., 2010). Mutations in the *MC1R* gene have been highly associated with the formation of freckles (Bastiaens et al., 2001). In addition to regulating TYR and IRF4 gene expression, MITF is also known to regulate expression of *MC1R* (Adachi et al., 2000; Smith et al., 2001; Aoki and Moro, 2002). This suggests the possibility that, like with TYR, IRF4 cooperates with MITF in regulating expression of *MC1R* in melanocytes. Indeed, ChIP-seq studies show that MITF binds MC1R regulatory sequences (Strub et al., 2011).

The effects of both TFAP2A and IRF4 are mediated through MITF, which is needed both for the TFAP2A-mediated activation of *IRF4* and the IRF4-mediated activation of *TYR*. ChIP-seq studies suggest that MITF regulates *TFAP2A* as well as *IRF4* (Strub et al., 2011). The cooperative effects of MITF and IRF4 suggest that these proteins might physically interact on the *TYR* promoter. TFAP2A is expressed in a variety of both mesenchymal and epithelial cell types. It is not known how MITF and TFAP2A interact in the regulation of IRF4. Because the binding sites are physically close to each other, it is possible that both proteins are part of a larger complex formed at the site. Together, these data suggest that transcription factors that are expressed in multiple cell types can form cell-type-specific complexes to govern differentiation.

Several studies have shown that polymorphisms identified as susceptibility loci in GWASs affect the binding of particular transcription factors, in some cases, leading to differences in expression of a nearby target gene (Meyer et al., 2008; Rahimov et al., 2008; Pomerantz et al., 2009; Schödel et al., 2012; Tuupanen et al., 2009). However, the effects of the gene expression differences have not been characterized, and more importantly, the biological effects on the phenotype are only implied in these studies. A recent example is provided by the rs12913832 polymorphism in intron 86 of the *HERC2* gene, which shows a strong association with human eye color and pigmentation. Because the pigment gene *OCA2* is located 21 kb downstream of *HERC2*, it has been speculated that this polymorphism is located in a distal regulatory element of OCA2 and may affect binding of the transcription factors HLTF, LEF1, and MITF (Visser et al., 2012). This results in the formation of a loop between the HERC2 enhancer and the OCA2 promoter, thus affecting OCA2 expression. The rs12913832-C allele prevents HLTF from binding to the element, thus reducing loop formation. The regulation of OCA2 expression may be more complex, however, with separate regulatory elements responsible for eye and skin color (Beleza et al., 2013). Here, we have gone one step further and shown the effects of transcription factor binding on gene expression and how this gene expression difference leads to effects on human pigmentation.

EXPERIMENTAL PROCEDURES

Whole-Genome Sequence Analysis

Icelandic Population Samples and Phenotypic Information

Icelandic adults were recruited as cases, family members, or controls through a series of cardiovascular, oncology, neurology, and metabolic studies conducted by deCODE Genetics. Blood samples were taken for DNA isolation upon recruitment. The studies were approved by the National Bioethics Committee of Iceland and the Icelandic Data Protection Commission. Personal identifiers associated with phenotypic information, blood samples, and genotypes were encrypted using a third-party encryption system. Each participant completed a questionnaire that included questions about natural eye color categories (blue/gray, green, or black/brown), natural hair color categories (red/reddish, blond, dark blond/light brown, or brown/black), and the presence of freckles at any time. Sun sensitivity was assessed using the Fitzpatrick skin-type score (Fitzpatrick, 1988), where the lowest score (I) represents very fair skin that is sensitive to UVR, and the highest score represented in native Icelanders (IV) represents dark skin that tans rather than burns in reaction to UVR exposure. Individuals scoring I or II were classified as being sensitive to sun, whereas individuals scoring III and IV were classified as not being sensitive to sun.

SNP ChIP Genotyping

The Icelandic chip-typed samples were genotyped with Illumina Human Hap300, HumanHap CNV370, HumanHap 610, 1 M, Omni1-Quad, Omni 2.5, or Omni Express bead chips at deCODE Genetics. SNPs were excluded if they had (1) yield less than 95%, (2) minor allele frequency less than 1% in the population, (3) significant deviation from Hardy-Weinberg equilibrium in the controls ($p < 0.001$), (4) if they produced an excessive inheritance error

rate (over 0.001), and (5) if there was a substantial difference in allele frequency between the chip types (SNPs from a single-chip platform were excluded if that resolved all differences but otherwise from all chip platforms). All samples with a call rate below 97% were excluded from the analysis. For the HumanHap series of chips, 308,840 SNPs were used for long-range phasing, whereas for the Omni series of chips, 642,079 SNPs were included. The final set of SNPs used for long-range phasing was composed of 785,863 SNPs.

Whole-Genome Sequencing and Imputation

Sequence data were obtained from about 2,230 Icelanders using methods described previously by Holm et al. (2011). Sequencing was carried out to an average depth of at least 10×. This resulted in the identification of approximately 38.5 million SNPs and small indels that were available for imputation. Long-range phasing, imputation, and association testing (by logistic regression) were done as described previously (Kong et al., 2008, 2009; Holm et al., 2011; Stacey et al., 2011). For conditional analysis of the IRF4 region, the rs12203592-T allele count of each individual was given as a covariate in the logistic regression.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.10.022.

AUTHOR CONTRIBUTIONS

E.S. conceived and designed the study. S.N.S., A.S.H., and K.S. analyzed whole-genome sequence data and the frequency of *IRF4* variants. A.M.M., R.S.Q.K., A.G.S., and R.A.S. characterized expression of *IRF4* and other genes in cultured human melanocytes. D.U.G., A.S.M., S.K.L., D.R.A., and W.J.P. identified and characterized the enhancer element in intron 4 of *IRF4* and performed the allele-specific expression analysis. E.V.O. and R.C. performed experiments in zebrafish. C.P. analyzed the effects of MITF and TFAP2A on regulation of *IRF4* and other target genes. C.G., M.H.O., and K.B. determined the involvement of IRF4 and MITF in *TYR* regulation. E.M., U.S., and N.C.T.E. performed ChIP on the TYR promoter. P.J.M., T.G., M.R.Z., S.R.D., P.S.M., and G.M. analyzed expression of *MITF*, *TFAP2A*, *IRF4*, and *TYR* in mouse melanocytes, and V.P. and L.L. in human melanoma cells. K.C.R., L.L., and D.E.F. characterized the interaction between MITF and IRF4 in mouse pigmentation. C.P. and E.S. wrote the manuscript. All authors approved the final version of the manuscript.

ACKNOWLEDGMENTS

We thank Thorunn Rafnar, Magnus Karl Magnusson, Unnur Thorsteinsdottir, Colin R. Goding, and Thorarinn Gudjonsson for critical comments on the manuscript. This work was supported by grants from NIH (5R01 AR043369-16), Melanoma Research Alliance, Dr. Miriam and Sheldon Adelson Medical Research Foundation, US-Israel Binational Science Foundation (to D.E.F.), Ligue Nationale Contre le Cancer (Equipe labellisée) and INCa (to L.L.), the Icelandic Research Fund and the Research Fund of the University of Iceland (to E.S.), the Haskolasjodur Student Fund (to E.S., C.P., and C.G.), the Jules Verne Fund (to E.S. and L.L.), by the National Institute of General Medical Sciences (GM071648) and National Institute of Neurological Disease and Stroke (NS062972; to A.S.M.), the National Human Genome Research Institute's Intramural Research Program (to W.J.P. and S.L.), and an NSF Graduate Research Fellowship (to D.U.G.).

Received: March 11, 2013
Revised: August 19, 2013
Accepted: October 1, 2013
Published: November 21, 2013

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Paper III

Sun-induced freckling: ephelides and solar lentigines

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KEYWORDS freckles/ephelides/solar lentigines/melanocytes/stem cells

PUBLICATION DATA Received 6 December 2013, revised and accepted for publication 6 February 2014, Published online xxxxxxx

doi: 10.1111/pcmr.12232

Summary

Freckles, the lay term for ephelides and lentigines, are important pigmentation characteristics observed in humans. Both are affected by sunlight; ephelides are largely genetically determined but induced by sunlight, whereas lentigines are induced by sun exposure and photodamage of the skin. However, despite being commonly observed, we know very little about them. Here, we review the current status of knowledge about freckles and propose a model for their formation.

Introduction

Human pigmentation is determined by melanocytes, cells that arise from the neural crest during development and migrate as precursors called melanoblasts. Resident melanoblasts in the hair follicle divide into two populations whereby hair matrix melanocytes are responsible for initial hair pigmentation, and melanocyte stem cells (MSCs) enter the niche found in the bulge region of the hair follicle and are responsible for maintaining hair pigmentation in subsequent cycles (Nishimura et al., 2002). Melanoblasts remain immature and are localized in the basement membrane of the epidermis where upon signaling from neighboring cells they initiate differentiation into mature melanocytes. Notably, the behavior of melanocytes is dependent upon their location and interaction with their stromal environment, with iridial melanocytes retaining melanosomes within their cytoplasm (Sturm, 2009) as opposed to cutaneous and follicular melanocytes, which actively transfer melanosomes to keratinocytes.

The melanoblasts are unpigmented, lack functional tyrosinase activity, the critical enzyme of melanin synthesis (Hirobe, 1992; Sviderskaya et al., 1995) and contain only immature melanosomes (Kawa et al., 2000). During childhood, there is a continuous need for melanocyte proliferation to populate the expanding skin surface area that proceeds into adulthood (Herlyn et al., 2000). In the adult skin, melanocytes slowly proliferate into fully differentiated cells that reside in the basal layer of the epidermis, in so doing establishing contacts with the surrounding keratinocytes for melanosome transfer. Little is known about the genes that maintain or control progression from the melanoblast to the melanocyte in human skin (Li et al., 2010). Even their origin from the dermis is yet to be fully understood (Adameyko et al., 2009).

Within the epidermis melanocytes and keratinocytes form an 'epidermal-melanin unit' where one epidermal melanocyte makes contact with approximately 30–40 keratinocytes via the dendrites (Jimbow et al., 1976). This contact allows melanosomes to be transferred from the tips of dendrites into the surrounding keratinocytes where skin color determination and protection against UVR-induced damage occurs. This intimate association also allows extensive interaction and communications establishing a hierarchy whereby the keratinocytes can direct the cellular response of melanocytes (Hirobe, 2005; Hsu et al., 2002). Indeed, keratinocytes play a crucial role in melanocyte responses to UVR as the ligands they produce influence MC1R activation; keratinocytes are much more sensitive and reactive to UV-exposure than melanocytes (Cui et al., 2007; Gordon et al., 1989). After UV-exposure, keratinocytes express multiple ligands and produce reactive oxygen species (ROS) that are absorbed by melanocytes and the melanin pigments they produce.

Freckling is a common pigmentation characteristic that can be divided into ephelides and solar lentigines (SL, also called lentigo senilis, actinic lentigines, sunburn freckles, freckles in adulthood, aging spots and liver spots) which are benign pigment spots mainly observed in Caucasians and Asians. Although both types of pigment spots are flat

macules, they differ significantly in development and morphology (Table 1). Ephelides are generally small pigmented spots (generally 1–2 mm, but can be larger), red to light brown in color, observed in fair-skinned and/or red-haired individuals (mostly phototypes I and II) that first appear at the age of 2–3 yr, then increase during adolescence and often partially disappear with age (Plensdorf and Martinez, 2009) (Figure 1A). They are most frequently found on the face, arms, neck and chest and become more pigmented during summertime. Lentigines are larger than ephelides, ranging in size from millimeters to centimeters in diameter, and their color can be dark brown (Hodgson, 1963) (Figure 1B). They are more common after the age of 50 on chronic sun-exposed skin (mostly on the face, dorsum of the hands and anterolateral parts of the forearm) (Cario-Andre et al., 2004); their pigmentation is not affected by the seasons. In Asia, freckles are considered cosmetic disfigurations that need to be removed (Jang et al., 2000; Zhang et al., 2004), whereas in Western culture, freckles are considered fashionable and are rarely removed. Kate Moss, and many other well-known fashion models are freckled and freckled models are frequently seen in fashion magazines and advertisements. Although everyone generally recognizes freckles, surprisingly little is known about them. From reviewing the literature, it is also clear that ephelides and SLs are not always clearly distinguished, resulting in confusion in the nomenclature used. Here, we summarize the current knowledge on ephelides and lentigines with respect to histopathology, epidemiology and genetics.

Histopathological features

Histological analysis shows that ephelides have characteristics that clearly distinguish them from the surrounding non-freckled skin. However, the few studies that have been performed to date do not agree on what happens to melanocytes in ephelides. By staining epidermal sheets for melanocyte activity using DOPA, Breathnach (1957, 1958) found that ephelides contained about 40% fewer melanocytes per square mm than adjacent pale epidermis, whereas little differences were observed between pale areas of freckled subjects and of non-freckled subjects. According to Breathnach, the melanocytes of ephelides were larger, more strongly DOPA positive, more pigmented and they had longer and more numerous dendrites. Although the borders of the freckles were irregular in shape, they were sharp. Rhodes et al. (1991) also studied the histopathology of sun-induced ephelides in juveniles, this time using transverse sections. Their analysis showed that the rete ridges, (epidermal thickenings that extend between dermal papillae) of ephelides (Figure 2) are elongated and clubbed and that there is a general increase in melanocytes along the rete ridges and increased melanin in the basal epidermal unit. In contrast to Breathnach, Rhodes et al. showed that melanocytes were significantly more frequent in ephelides than in non-freckled areas. They suggested that the DOPA-incubated epidermal sheets used by Breathnach are not reliable for characterizing melanocytes in ephelides (Rhodes et al., 1983). Both the studies of Breathnach and Rhodes showed increased pigmentation in the ephelides as

Table 1. Characteristics of ephelides and lentigines

	Ephelides	Lentigines
Appearance	First visible at 2–3 yr of age after sun exposure, partially disappears with age	Accumulate with age, common after 50, stable
Areas affected	Face, neck, chest, arms	Sun-exposed skin, face, hands, forearms, chest back and shins
Effects of sun	Fade during winter	Stable
Size	1–2 mm and above	mm-cm in diameter
Borders	Irregular, well defined	Well defined
Color	Red to light brown	Light yellow to dark brown
Skin type	Caucasians, Asians, skin type I-II.	Caucasians, Asians, skin type I–III
Numbers	Few to hundreds	Few to hundreds
Etiology	Genetic	Environmental
Melanocyte number	More	1–2.2 times more
Melanocyte size	Large, more dendrites	Normal
Melanosome number	Increased	Normal
Melanosome size	Large	Normal
Epidermal rete ridges	Elongated	Elongated
Epidermal pigmentation	Increased	Increased
Other	Melanin-laden macrophages	Melanosome complexes in keratinocytes More mitochondria Better-developed ER Microinvaginations into keratinocytes 'Pendulum melanocytes'

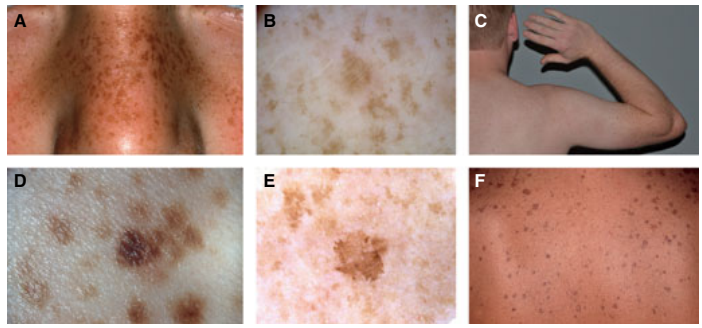

Figure 1. Examples of freckling. (A) Ephelides in a young boy. (B) A close-up of freckling in the adult shown in C. (C) Freckles on shoulder and arm showing more freckling in sun-exposed areas. (D) Lentigines in LEOPARD syndrome patient. E. Solar lentigo in a 43-yr-old female. F. Sun-induced freckles after sun-burn.

compared to non-freckled areas (Figure 2). This was confirmed using electron microscopy, which showed that ephelide melanocytes contain multiple large melanosomes characteristic of dark-skinned individuals, whereas non-freckled areas contain fewer, smaller and less-pigmented granules (Breathnach and Wyllie, 1964). This suggests that frecklers have melanocytes, which exhibit two types of behavior, namely highly pigmented melanocytes of ephelides with large melanosomes, and less-pigmented melanocytes with smaller melanosomes in non-freckled areas. Why the melanocytes behave differently in the two areas of the same individual is not clear, but as ephelides are induced by acute sunexposure in children, the origin of these melanocytes must be considered.

Similar to ephelides, solar lentigines have a hyperpigmented basal layer and elongated epidermal ridges (Cario-Andre et al., 2004; Unver et al., 2006); the epidermis is thicker and the keratinocytes accumulate melanin pigment (Noblesse et al., 2006). Montagna et al. (1980) and Cario-Andre et al. (2004) have suggested that both melanocytes and keratinocytes proliferate excessively in the lentiginous lesions, leading to extended length of the basal layer and thus longer epidermal rete ridges (Figure 2). However, Ki67 staining did not reveal proliferative differences between lentigines and adjacent normal skin (Unver et al., 2006; Yamada et al., 2014), suggesting that increased length of the rete ridges is not due to a permanent increase in proliferation in lentigines. It is not clear when in the formation of lentigines the cells in the rete ridges proliferate. Using immunohistochemistry, Yamada et al. (2014) have shown that SLs have increased numbers of melanocytes in epidermis, melanoblasts in hair follicular infundibulum and MSCs in the bulge region. In addition, they showed increased expression of TYR in the melanocytes. Thus, according to this study, the number of melanocyte precursor cells is already higher in SLs and each melanocyte is more active. It seems likely that the melanocytes and their precursors are responding to signals from photodamaged skin, in which the keratinocytes are driving the process.

Cario-Andre et al. (2004) reported pigment accumulation in keratinocytes in perilesional skin suggesting that pigment accumulation happens early in the process. EM studies showed that, unlike in ephelides, melanosomes are of normal size in the lentigines as well as in melanocytes of adjacent regions. On the other hand, melanocytes in lentigines have more mitochondria and a better-developed endoplasmic reticulum than melanocytes from adjacent regions (Nakagawa et al., 1984; Noblesse et al., 2006). Basal keratinocytes of lentigines contain melanosome complexes (polymelanosomes) that form massive pigmented caps over the nuclei (Cario-Andre et al., 2004; Noblesse et al., 2006), suggesting efficient production and transport of melanosomes from melanocytes to neighboring keratinocytes. The EM studies also showed differences in the basement membrane of lentigines as compared to normal skin. In the lentigines, the dermal-epidermal junction was disorganized and disrupted, and the lamina densa was thinner than normal (Noblesse et al., 2006). Furthermore, microinvaginations into the keratinocytes were observed, and the melanocytes seemed to drop further into the dermis than normally, forming 'pendulum melanocytes' (Cario-Andre et al., 2004; Nakagawa et al., 1984; Noblesse et al., 2006). Andersen et al. (1997) studied 51 facial lentigines and showed that they form three classes based on morphology. The first class showed a flattened epidermis, the second exhibited hyperplasia and the third and smallest group showed characteristics of both. This variation was not observed in lentigines taken from the arm (Hodgson, 1963; Rhodes et al., 1983), suggesting that there are region-specific differences. Cario-Andre et al. (2004) have suggested, based on association with age of their subjects, that the three classes represent lesional progression.

Unfortunately, careful side-by-side comparisons of the histopathology of ephelides and lentigines, including molecular markers, are not available. It is clear that ephelides and lentigines share important characteristics, including increased melanin production and extended epidermal ridges (Table 2 and Figure 2). Differences include the increased size of the melanosomes in ephelides and the effects observed on mitochondria, endoplasmic reticulum and basement membrane in lentigines, which have not been reported in ephelides. This

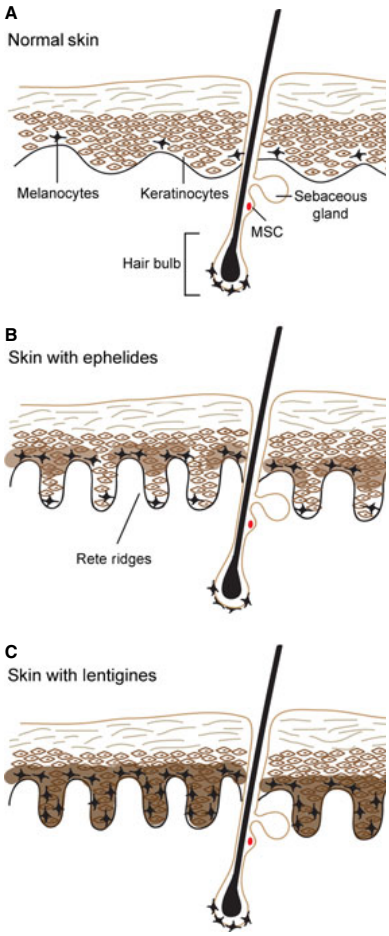

Figure 2. Schematic representations of ephelides and solar lentigines: Round red cells in the hair bulge represent melanocyte stem cells (MSC), black dendritic cells represent melanocytes and brown shading represents melanin secreted to keratinocytes. (A) Schematic view of the upper layers of normal human skin showing a hair bulb and the distribution of melanocytes. (B) Schematic view of the skin around ephelides. (C) Schematic view of the skin around lentigines.

suggests that these pigmentation traits have different origins.

Epidemiology

To characterize the epidemiology of ephelides and lentigines, Bastiaens et al. (2004) studied 962 individuals from the Leiden Skin Cancer Study. They showed that SLs on the face were associated with increased age, whereas ephelides were inversely associated with age. Pigment lesions such as skin cancer and nevi usually develop more easily in light-skinned people. Ephelides are also associated with skin type I or II and blond/red hair color (Bastiaens et al., 2004; Ezzedine et al., 2013). Lentigines, however, seem to be more associated with darker skin types. Studying 118 cases with SL on the face, and equal number of controls, all between 60 and 80 yr of age, Monestier et al. (2006) showed that having multiple senile lentigos on the face (what they called lentigo aging pattern) was associated with skin types III and IV. They speculated that this might be due to a more active melanocyte system in those skin types. A similar association with darker skin was observed by Ezzedine et al. (2013). Multiple studies suggest that the formation of SLs is linked to sun exposure in one form or another and photodamage to the skin. In the Monestier study, SLs were associated with frequent sunburns and with recreational sun exposure, but not with occupational or lifetime sun exposure. In the study of Bastiaens et al. (2004), however, SLs on the face were associated with cumulative lifetime sun exposure and not with history of sunburns, whereas SLs on the back were associated with cumulative sun exposure and with history and number of sunburns before the age of 20. Ezzedine et al. (2013) studied 523 French middle-aged women and found that SLs were associated with lifetime sun exposure but not with sunburn during either childhood or adulthood. Derancourt et al. (2007) studied 145 cases and controls and found that SLs were associated with sunburn during adolescence and this was dose-dependent. As the extent of sun exposure is self-reported in most cases, determining whether sunburns, recreational exposure and acute phototraumas or total lifetime exposure is responsible may be difficult. These studies also suggest that

Table 2. Genes involved in freckling

Gene	Phenotype	Reference
MC1R	Red hair, fair skin, UV sensitivity, freckles	Robbins et al. (1993), Kijas et al. (1998), Vage et al. (1999), Flanagan et al. (2000), Bastiaens et al. (2001), Duffy et al. (2004), Rouzaud et al. (2006), Sulem et al. (2007), Eriksson et al. (2010)
IRF4	Blue eyes, brown hair, freckles, sun sensitivity	Sulem et al. (2007), Duffy et al. (2010b), Eriksson et al. (2010)
ASIP	Red hair, freckling, sun sensitivity	Sulem et al. (2007, 2008), Duffy et al. (2010a), Eriksson et al. (2010)
TYR	Blond hair, blue eyes, freckles	Sulem et al. (2007), Eriksson et al. (2010)
BNC2	Freckles, skin color saturation	Eriksson et al. (2010), Jacobs et al. (2013)

ASIP, *agouti* signaling protein; MC1R, melanocortin-1-receptor; TYR, Tyrosinase.

there might be region-specific differences in the etiology of SL formation. No relationship was found between the formation of SLs and ephelides, suggesting that these pigment spots are etiologically unrelated (Bastiaens et al., 1999; Monestier et al., 2006).

The relationship of ephelides to sun exposure is also complicated. Freckles usually do not appear until the age of 2–3 yr of age. Ephelides respond to sunlight and can be induced, or more likely made visible in those that already carry them, by UV-irradiating non-exposed skin (Wilson and Kligman, 1982) (Figure 1A, C). The Ezzedine study showed that facial ephelides were associated with frequent sunburns. Interestingly, Garbe et al. (1994) showed that actinic lentigines were related to a tendency to develop ephelides in adolescence, suggesting that freckers are more prone to having SLs later in life. This is not, however, consistent with the studies of Bastiaens et al. (1999) and Monestier et al. (2006), nor with the observation that ephelides are more frequently observed in light-skinned individuals, whereas SLs are more observed in darker skin types. In these studies, the definition of ephelides versus SLs may not always be clear.

An increased frequency and area of pigmented birthmarks in freckled individuals have been reported in a study of Australian schoolchildren aged 7–17 yr (Nicholls, 1968). Furthermore, freckling is clearly associated with an increased risk of melanoma (Bliss et al., 1995; Dubin et al., 1990; Mackie, 1998; Titus-Ernstoff et al., 2005). Bliss et al. performed a meta-analysis of 10 case–control studies and found that all seven studies that analyzed freckles reported an association with the formation of melanoma (Bliss et al., 1995). The risk was higher the more dense the freckles were. They also found association of blond/red hair, blue eyes and light skin with melanoma. This has been confirmed in numerous studies, including a study of 423 primary melanomas and 678 controls in the US where blond/red hair, blue eyes and the presence of freckles before the age of 15 were associated with melanoma (Titus-Ernstoff et al., 2005). Clearly, light pigmentation and freckles are a risk factor for melanoma.

Pigmented spots can also be induced by drug treatment, as has been described for the so-called PUVA lentigines, which arise upon treatment of patients with psoriasis with psoralens and ultraviolet A light (PUVA). This leads to the appearance of lentigines in otherwise sun-protected skin (Abel et al., 1985; Rhodes et al., 1983). Abel et al. found atypical melanocytes containing large hyperchromatic nuclei in 57% of the PUVA lentigines and in 70% of the non-lesional PUVA-exposed skin. A comparison with 24 samples from lentigo simplex or SL patients showed only two cases of atypic melanocytic nuclei. They further discovered binucleated melanocytes and the presence of giant melanosomes in the PUVA treated patients, which can cause further complications as melanocytic dysplasia or malignancy (Abel et al., 1985). When Nakagawa et al. (1984) compared PUVA lentigines from light-protected regions (the patients had not been treated with UV radiation) to solar lentigines, they found that the melanocytes from the PUVA patients had longer and more numerous dendrites and showed more active melanogenesis. The basal keratinocytes of the PUVA lentigines showed a significantly higher frequency of large, single melanosomes.

Genetics

Ephelides

Ephelides are mostly associated with light skin and blond/-red hair color and appear during early childhood, suggesting that the formation of freckles in juveniles is largely genetically determined. Bataille et al. (2000) showed that additive genetic effects explained 91% of the variance in freckle counts, with no measurable environmental effects. Consistent with this, a number of genes have been shown to be important for the formation of freckles, including *MC1R*, *IRF4*, *ASIP*, *TYR* and *BNC2* (Table 2). The majority of red-haired individuals are homozygous or compound heterozygous for variants in the melanocortin-1-receptor (*MC1R*) gene (Box et al., 1997; Flanagan et al., 2000; Smith et al., 1998; Valverde et al., 1995). *MC1R* variants also lead to pale skin. More importantly, the *MC1R* gene has been shown to be a major contributor to the formation of freckles (Bastiaens et al., 2001; Flanagan et al., 2000; Rees, 2004). Overall, this is referred to as the RHC (red hair, fair skin, lack of tanning ability and propensity to freckle) phenotype, with highly penetrant variants of the *MC1R* gene designated as *R*-alleles and lower penetrant alleles as *r*-alleles (Beaumont et al., 2011). MC1R is a seven pass transmembrane G protein-coupled receptor, which is located in the cell membrane of melanocytes (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992). It binds different ligands with opposite characteristics. The ligands α-melanocyte stimulating hormone (αMSH) and the adrenocorticotropic hormone (ACTH) activate signaling through the receptor, leading to increased production of cyclic AMP (cAMP), which then leads to phosphorylation of the cAMP responsive element-binding protein (CREB), resulting in activation of expression of MITF, the melanocyte master regulator. MITF activates expression of several genes required for production of black eumelanin, including TYR, the enzyme that catalyzes synthesis of eumelanin (Busca and Ballotti, 2000). The *agouti* signaling protein (ASIP) is an antagonist of MC1R and prevents binding of αMSH and thus production of eumelanin. This leads to production of red or yellow pheomelanin, thus modulating the ratio of eumelanin and pheomelanin produced by melanocytes (Cone et al., 1996; Rees, 2003). More than 80 coding variants in *MC1R* have been described in populations of European ancestry (Gerstenblith et al., 2007; Makova and Norton, 2005). These variations lead to pigmentation traits such as fair skin, red and blond hair, UV sensitivity and freckles in both

humans and animals (Kijas et al., 1998; Robbins et al., 1993; Rouzaud et al., 2006; Vage et al., 1999). The variants in *MC1R* affect receptor function (Beaumont et al., 2007, 2009; Garcia-Borron et al., 2005) or the promoter region (Motokawa et al., 2008); red hair and ephelides are found in the absence of *MC1R* function (Beaumont et al., 2008). Activating mutations in *MC1R* can lead to permanent activation of the protein and dark pigmentation, which has so far only been described for animals (e.g. mice, pigs and sheep) and not for humans.

A study of 962 skin cancer patients and 385 non-skin cancer controls in the Netherlands showed that carriers of one or two *MC1R* variants had a 3- and 11-fold increased risk of ephelides during childhood, independent of skin type and hair color (Bastiaens et al., 2001). A dosage effect was observed where the degree of freckling was proportional to the number of *MC1R* variants. The population attributable risk of *MC1R* variants for freckles was 60%. Interestingly, half of the *MC1R* carriers in that study did not express freckles (Bastiaens et al., 2001), suggesting that genetic background is also important. In a comprehensive study of 2331 twins, their siblings and parents, Duffy et al. (2004) showed that red-haired individuals had the most freckles and the most extreme level of freckling. Red-haired individuals also had the lowest number of moles (Dellavalle et al., 2005; Duffy et al., 2004). This study also showed that the more freckling, the more penetrant the variant *MC1R* allele. The population attributable risk of carrying at least one *MC1R* allele was 100% in the severe cases of freckling with a reduction to 23.4% for those with less freckling limited to a single site (Duffy et al., 2004). *MC1R* is therefore the major freckling gene in individuals with extensive freckling, whereas other genes must contribute to freckling in those with less extensive freckling. The contribution of *MC1R* to freckling is not limited to Europeans as analysis of *MC1R* variants in Japanese populations has shown clear association with ephelides (Motokawa et al., 2007; Yamaguchi et al., 2012).

Recently, several additional genes have been associated with freckling. Sulem et al. (2007, 2008) performed association studies in the Icelandic and Dutch populations and showed that in addition to SNPs in the *MC1R* gene, SNPs in or near the *IRF4*, *ASIP* and Tyrosinase (*TYR*) genes are associated with the formation of freckles. Like the *MC1R* variants, the *ASIP* haplotype defined by the rs4911442*C SNP (also called ASIP AH) was significantly associated with red hair color, freckling and sensitivity to sun. Similarity in phenotype of *ASIP* and *MC1R* variants is not surprising as *ASIP* is an antagonist of the *MC1R* receptor, and this has lead to the suggestion that ASIP AH may be functionally or genetically equivalent to a *MC1R r*-allele (Duffy et al., 2010a). However, no coding variants were found in *ASIP* that explain the association (Sulem et al., 2008), suggesting that the phenotype observed is due to effects on *ASIP* gene expression, presumably leading to increased ASIP production and thus increased *MC1R* antagonism and production of phaeomelanin.

The SNP rs1540771, located between the *IRF4* and *EXOC2* genes on chromosome 6, was also shown to associate with freckles, brown hair and skin sensitivity to sun exposure (Sulem et al., 2007) and later with age specific effects on nevi number (Duffy et al., 2010b). The involvement of *IRF4* in pigmentation was confirmed by Han et al. (2008) who studied US and Australian populations and found that the SNP rs12203592, in intron 4 of *IRF4*, was strongly associated with hair, skin and eye color as well as with tanning response. They did not test for the effects on freckling but this was confirmed by the study of Duffy et al. in 2010a,b, which showed high association with freckling scores independent of nevi count (Duffy et al., 2010b). Recently, the effects of *IRF4* on pigmentation have been shown to be due to the rs12203592 polymorphism, which affects binding of the TFAP2A and MITF transcription factors to a regulatory element in intron 4 of *IRF4*, thus reducing *IRF4* expression (Praetorius et al., 2013). Importantly, the reduced expression of the IRF4 transcription factor leads to a reduction in *TYR* expression and thus effects on pigmentation (Praetorius et al., 2013). Perhaps not surprisingly, variations in *TYR* have also been shown to be associated with eye and hair color as well as freckles (Sulem et al., 2007). However, it was surprising to find that the SNP rs1042602, which results in a S192Y polymorphism in *TYR,* was only associated with freckles in the Icelandic and Dutch populations; previous analysis reported this variant to be associated with skin (Shriver et al., 2003) and eye (Frudakis et al., 2003) color. Clearly, reduced TYR expression or activity, and reduced MC1R signaling, can contribute to the appearance of freckles.

Using a web-based participant driven study to characterize multiple phenotypes, Eriksson et al. (2010) verified that *MC1R*, *IRF4*, *ASIP* and *TYR* are associated with freckling. In addition, they showed that the *BNC2* gene is associated with freckles. This gene has also been implicated as a factor contributing to skin color saturation (Jacobs et al., 2013). *BNC2* encodes the Basonuclin 2 protein, which consists of three pairs of C_2H_2 zinc fingers, a nuclear localization signal and a serine-rich region of unknown function (Romano et al., 2004). *BNC2* is related to the *BNC1* gene but is more highly conserved through evolution (Vanhoutteghem and Djian, 2004); its genomic structure is complex leading to the potential production of up to 90 000 different mRNA isoforms, generating 2000 different proteins (Vanhoutteghem and Djian, 2007). The exact function of BNC2 is presently unknown. The protein has been shown to be located in nuclear speckles in keratinocytes, leading to the suggestion that it is involved in RNA-processing (Vanhoutteghem and Djian, 2006). The *BNC1* gene, which unlike *BNC2* is uniformly present in nuclei and does not form speckles, has been implicated as a transcription factor in the regulation of expression of ribosomal RNA genes (Iuchi and Green, 1999; Wang et al.,

2006). BNC2 has also been shown to be able to bind the rRNA gene promoter (Romano et al., 2004). A mouse knockout mutation in *Bnc2* leads to lethality within 24 h of birth; the embryos exhibit cleft palate and abnormal craniofacial bones (Vanhoutteghem et al., 2009). Due to the lethality, it has not yet been possible to analyze the role of BNC2 in mouse pigmentation. However, analysis of the *bonaparte* zebrafish mutation, a truncation mutation in *Bnc2*, has led to important insights into the role of *Bnc2* in pigmentation. In *bonaparte* mutant zebrafish, early development of pigment cells is normal, whereas at later stages, they show severe disruption of the adult stripe pattern due to extensive loss of pigment cells (Lang et al., 2009). The mutant fish lack stripes in the body, whereas fins are normally striped. Genetic mosaic analysis showed that *Bnc2* acts non-autonomously to melanophores and is most likely active in cells between the myotomes and epidermis. Consistent with this, *Bnc2* is expressed in hypodermal cells adjacent to the pigment cells. This suggests that *Bnc2* affects stripe formation in the fish by providing an important environment for pigment cell survival. In fact, Patterson and Parichy (2013) have shown that *Bnc2* is involved in regulating the expression of the *Kitlg* and *Csf1* genes, both of which are required for pigment cell maintenance. Because BNC2 is primarily expressed in keratinocytes in humans, the effects of this gene on freckle formation in humans are likely to be due to similar signals from keratinocytes to melanocytes. As KITLG has been implicated in human pigmentation, it will be interesting to determine the effects of *BNC2* on *KITLG* expression in humans.

Solar lentigines

Solar lentigines are more environmentally determined than ephelides as they arise from the accumulated photodamage of the skin that occurs over time producing genetic or epigenetic changes in gene expression within keratinocytes of the lesion. Through altered microenvironmental, paracrine or cell–cell communication signals the SL keratinocytes determine melanocytic growth, dendritic and melanogenic behavior resulting in hyperpigmented spots. However, their formation is dependent on the melanocytic genes involved in pigmentation and, in fact, some of the same genes that have been shown to be involved in pigmentation and the formation of ephelides have also been implicated in SL formation. Bastiaens showed that RHC variant alleles of *MC1R* increased risk of lentigines by 1.5- to 2-fold; the risk for ephelides was increased by 3- to 11-fold (Bastiaens et al., 2001). Motokawa et al. (2007, 2008) showed that polymorphisms in the coding region as well as promoter of *MC1R* associate with SL in Japanese subjects. Like in the case of ephelides, loss-of-function *MC1R* variant alleles are associated with SLs, suggesting that melanocytes with reduced MC1R activity are likely to form this pigmented lesion. In a low-powered study of German and Japanese women, variations in *SLC45A2* were shown to associate with SLs (Vierkotter et al., 2012). *SLC45A2* is mutated in the hypopigmented *underwhite* mice and in humans with Oculocutaneous albinism type 4 (OCA4) and therefore clearly plays a role in pigmentation. The SLC45A2 protein (MATP) is a 12-transmembrane-spanning protein of unknown function and its specific role in melanocytes remains to be determined.

Other proteins have also been implicated in the formation of SLs. By screening 30 SL samples for mutations in the *FGFR3* and *PIK3CA* genes, Hafner et al. (2009) found these genes to be mutated in 17 and 7% of lentigos, respectively. They suggest that these mutant molecules may lead to the formation of SLs. Kadono et al. (2001) investigated the role of Endothelin 1 and the Endothelin B receptor, both of which play an important role in melanocyte development, in SLs. Using RT-PCR analysis and immunohistochemistry, they showed that lesional skin exhibited increased expression of both genes compared with non-lesional skin (Kadono et al., 2001). The same group showed that expression of KITLG [also known as Stem cell Factor (SCF)], but not its receptor KIT, is increased in SL samples (Hattori et al., 2004). Kovacs et al. (2010) and Chen et al. (2010) have shown that hepatocyte growth factor and keratinocyte growth factor may play a role in SLs. Increased expression of growth factors in keratinocytes or fibroblasts is likely to affect gene expression in melanocytes in the sun-damaged areas. Goyarts et al. (2007) performed a gene expression analysis on lesional and non-lesional skin from three individuals with SL. This limited dataset showed 17 downregulated and 23 upregulated genes. Although no pigmentation genes were among the genes identified, several interesting genes and gene classes were discovered in this analysis including Wnt family genes, metalloproteases and genes involved in the inflammatory response. However, this study needs confirmation as the samples were few, and no replicates were performed. The data involving the above-mentioned genes in the formation of SLs are not overwhelming and further studies are needed to validate their role in these pigmented lesions.

SLs sometimes form as a consequence of pleiotropic syndromes, and in many cases, the underlying genetic causes have been identified and may be involved in the formation of SLs. These include the Carney complex (CNC), the LEOPARD Syndrome (LS) and Peutz-Jeghers Syndrome (PJS). These syndromes, and the underlying mutant genes, have been reviewed recently (Espiard and Bertherat, 2013; Lauriol and Kontaridis, 2011; Ollila and Makela, 2011) so we will only describe them briefly here. CNC has been shown to be due to mutations in the *PRKAR1A* gene (Kirschner et al., 2000). This gene encodes the regulatory subunit 1α of the Protein Kinase A, which is known to play an important role in endocrine signaling pathways (Sassone-Corsi, 1998) and is also considered a tumor suppressor gene. The reason for the effects on pigmentation is still not understood, but it is

possible that the mechanism involves PKA activated signal transduction pathways downstream of the melanocortin receptors (Horvath and Stratakis, 2009). Approximately 85% of LS patients carry a missense mutation in the protein tyrosine phosphatase non-receptor type 11 gene (*PTPN11*) gene (Digilio et al., 2006). The *PTPN11* gene encodes a member of the protein tyrosine phosphatases and contains two tandem SRC homology-2 (SH2) domains and one protein tyrosinase phosphatase domain. The protein is a cytoplasmic signal transducer of multiple receptors for growth factors and hormones and works mainly through the RAS mitogen activated kinase (MAPK) pathway (Neel et al., 2003; Tartaglia et al., 2004). PJS is caused by mutations in the serine/threonine kinase 11 (*STK11*) gene (Hemminki et al., 1998). *STK11* is a tumor suppressor gene, which codes for a serine/threonine kinase, which regulates cellular proliferation and cell polarity (Spigelman et al., 1990). Whether any of these genes play a role in SLs is not known at present.

Model for formation of ephelides

Cario-Andre et al. (2004) have proposed a model for the formation of SLs but no model exists for the formation of ephelides. Models for ephelides need to explain why they form in individuals carrying reduced-function and loss-of-function polymorphisms in pigmentation genes. They must also explain their variegated pattern. As the genes that have been associated with ephelides can be active in either melanocytes or keratinocytes, and as many of the genes involved encode molecules involved in mediating signals between the two cell types, interactions between melanocytes and keratinocytes are likely to be important. Here, we focus our attention on these two cell types, although it is well established that other cell types also influence the melanocyte, including dermal fibroblasts, macrophages and mast cells (Wang and Herlyn, 2013). We propose that ephelides arise as a consequence of a direct growth, migration or differentiation response of melanocytes or MSCs to UV damage in juvenile or adolescent skin, whereas lentigines arise as a consequence of melanocytes responding to signals from surrounding keratinocytes upon UV damage of aged skin. Our model assumes a stochastic regional process in the activation of pigmentation, leading to pigmented spots in otherwise hypopigmented skin. According to this model, the reduced- and loss-of-function variant alleles which lead to the formation of freckles affect the activity of the respective proteins such that the cells are at, or near, a threshold of activity needed for normal pigmentation. Minor regional differences, where one region is below the threshold, whereas a neighboring region is above the threshold, might then lead to region-specific differences in melanocyte pigmentation, growth or migration. For example, BNC2 may lead to stochastic differences in KITLG or CSF1 expression in neighboring keratinocytes in the skin which then signal to their respective receptors in melanocytes, thus leading to differences in pigmentation in adjacent areas in the skin. It is possible that the expression of ASIP in keratinocytes is also regulated by BNC2, thus leading to similar stochastic differences on MC1R activation. As MC1R leads to the activation of MITF expression, which consequently activates IRF4 expression and then both MITF and IRF4 activate TYR expression, it is possible that the formation of freckles in general represents a single pathway gone awry, where signals from keratinocytes, mediated by BNC2 through MC1R, KIT and other receptors, lead to differential effects on pigment production in melanocytes, culminating in effects on TYR expression. If this were the case, we would expect MITF, KITLG and its receptor KIT to also contribute to the formation of freckling. This has not been shown to date. If freckling is truly due to a single pathway, the entire pathway is likely to have a particular threshold value. The threshold may be part of a reaction-diffusion system where activation of the pigmentary system in melanocytes also leads to production and rapid diffusion of an inhibitor, which inhibits pigmentation in neighboring melanocytes. A recently described mouse mutation, *Pied*, which affects the Adam10 zinc metalloprotease, leads to freckle-like macules in mice (Tharmarajah et al., 2012). This protein may act as an inhibitor of melanocyte expansion in the skin and may thus play an important role in the formation of ephelides. Importantly, this mutation, and the zebrafish Bonaparte mutation, may represent the first true animal models of freckling and may allow the dissection of the pathways involved. For testing, the model it will also be important to determine the expression of growth and paracrine factors in ephelides and SLs in the skin. Yamada et al. (2014) have started this work. Furthermore, it will be important to characterize somatic DNA damage and epigenetic changes in keratinocytes of the hyperpigmented spots.

Ephelides may originate from differentiated melanocytes in the skin or in MSCs derived from the bulge region of the hair follicle, or from as yet uncharacterized epidermal MSCs. Many questions still remain about the source of stem cells that lead to their replenishment in adult skin (Cramer, 2009). Notably, the migration of follicular MSCs into the epidermis after wounding and UV-treatment in a mouse model has recently been reported by Chou et al. (2013). They found MSCs leaving their hair follicle niche after wounding or UVB irradiation, migrating to the epidermis in a MC1R-dependent manner and differentiating into functional melanocytes. The ephelides-like pattern induced in vitiligo patients (Grichnik, 2008) using narrow band UV radiation in combination with a melanocortin agonist (Afamelatonide) (Grimes et al., 2013) may represent melanocytes of MSC origin. It should be recognized, however, that the repigmented spots in vitiligo patients have more regular borders than ephelides. The dependence of the MSCs on the MC1R pathway would suggest that MSCs in individuals who carry genetic variations in the MC1R pathway have

gained migratory and/or differentiation properties. MSCs in the skin, which first give rise to a local higher density of melanocytes, are then depleted with age would then explain why ephelides often fade with age. The disappearance of ephelides with age can also be explained by less p53 being active in aging keratinocytes, but p53 has been shown to be involved in regulating expression of both KITLG and α-MSH (Zeron-Medina et al., 2013).

Ephelides and solar lentigines represent different skin pigmentation states that share certain characteristics but are different in etiology and environmental response. This review highlights our limited knowledge of the freckling response to UV damage of the skin and suggests that further work is necessary to characterize these interesting features. Importantly, a consensus on terminology is needed when discussing freckles, which will be essential for further advances in the epidemiological understanding of these distinct but related phenotypes.

Acknowledgements

We thank Prof. H.P. Soyer for providing the images used in Figure 1. E.S. is supported by the Icelandic Research Fund, the Research Fund of the University of Iceland and C.P. by the Haskolasjodur Student Fund. R.A.S is a Senior Research Fellow of the Australian NHMRC.

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Appendix

A. Primers and oligonucleotides

Primers and oligos used for the experimental work. All DNA sequences are written in the 5'→3' direction.

Table A.1.: Primers used in this work

Primer (gene)	species	direction	sequence
Primer for qPCR			
Mitf	Hsap	forward	CTATGCTTACGCTTAACTCCA
		reverse	TACATCATCCATCTGCATACAG
Mitf	Mmus	forward	CAAATGATCCAGACATGCGG
		reverse	TGCTCCAGCTTCTTCTGTC
IRF-4	Hsap	forward	ACAGCAGTTCTTGTCAGAG
		reverse	GAGGTTCTACGTGAGCTG
IRF-4	Mmus	forward	CAGCTCATGTGGAACCTC
		reverse	GGAAGAATGACGGAGGGA
Tyr	Hsap	forward	CCATGACAAATCCAGAACCC
		reverse	GGACTAGCAAATCCTTCCAG
DCT	Hsap	forward	ACTGGAACTTTGCCACTG
		reverse	TCATCCAAGCTATCACAGAC
TFAP2a	Hsap	forward	GAGGTCCCGCATGTAGAA
		reverse	CCGAAGAGGTTGTCCTTGT
ActB	Hsap	forward	AAATCTGGCACCACACCT
		reverse	GTCTCAAACATGATCTGGGTC
ActB	Mmus	forward	TACCAACTGGGACGACAT
		reverse	GTCTCAAACATGATCTGGGTC
IRF4 intron 4 qPCR	Hsap	forward	ACCCACCAAAGTGGATGAAA
		reverse	CGCCTGTTGGAATATGCTTC
Primer for the amplification of Mitf/AP2a ChIP			
IRF4 intron 4 AP2a BS	Hsap	forward	CTTCAGGCTTTCTTGATGTGAA
		reverse	GGAGGATCATAAAGGACAATGG
HSPA1B promoter	Hsap	forward	CATGGAGACCAACACCTTC
		reverse	CTTTCCCCTTCTGAGCCAAT
TGFA promoter	Hsap	forward	TAGCCGCCTTCCTATTTCC
		reverse	AAAGACGCAGACTAGGCA
Tyr promoter	Hsap	forward	ACGAGCCAATTCGAAAGAAA
		reverse	CACAGATTTCTCTTTCCAGCTAC

Primer (gene)	species	direction	sequence
Primer for cloning IRF4-intron 4, with MluI/XhoI restriction sites			
IRF4-Int4 cloning	Hsap	forward	ATGGACGCGTATTTGCTATG-GCTGCTCCAA
		reverse	ATGGCTCGAGGGGTAAAGGA-GTGCAGGAGA

Table A.2.: Oligonucleotides used for EMSA, all sequences are written in 5'→3' direction.

Primer (gene)	species	direction	sequence
Oligonucleotides for EMSA			
IRF4-Int4 wildtype	Hsap	forward	GGTAAAAGAAGGCAAATTCCCCTGT
		reverse	ACAGGGGAATTTGCCTTCTTTTAC
IF4-Int4 RS12203592	Hsap	forward	GGTAAAAGAAGGTAAATTCCCCTGT
		reverse	ACAGGGGAATTTACCTTCTTTTAC
IRF4-Int4 AP2a site mutated	Hsap	forward	GGTAAAAGAATTACGATTCCCCTGT
		reverse	ACAGGGGAATCGTAATTCTTTTAC
Mbox			AAAGCTAGTCATGTGCTTTTCAGA
IRF4-Int4-wt	Hsap		TGGGAAACAGATGTTTTGTGGAAGT-GGAAGATTTTGGAAGTAGTGCCTTA-TCATGTGAAACCACAGGGCAGCTGA-TCTCTTCAGGCTTTCTTGATGTGAA-TGACAGCTTTGTTTCATCCACTTTG-GTGGGTAAAAGAAGGCAAATTCCCCTGT
IRF4-Int4-RS	Hsap		TGGGAAACAGATGTTTTGTGGAAGT-GGAAGATTTTGGAAGTAGTGCCTTA-TCATGTGAAACCACAGGGCAGCTGA-TCTCTTCAGGCTTTCTTGATGTGAA-TGACAGCTTTGTTTCATCCACTTTG-GTGGGTAAAAGAAGGTAAATTCCCCTGT

Table A.3.: Oligonucleotides used for site directed mutation of the pGL3b-IRF-Int4 plasmid, all sequences are written in 5'→3' direction.

Primer	direction	sequence
E-box 1 changed to: ACCTCA		
IRF4-Int4-E1	forward	GTTGGAATATGCTTCTCAGGTCTTCTGGGAAAACC-TCATTTTGTGGAAGTGGAAGATTTTGGAAGTAGTG
	reverse	CACTACTTCCAAAATCTTCCACTTCCACAAAATGA-GGTTTTCCCAGAAGACCTGAGAAGCATATTCCAAC
E-box 2 changed to: AGATCA		
IRF4-Int4-E2	forward	AAGTGGAAGATTTTGGAAGTAGTGCCTTATAGATC-AAAACCACAGGGCAGCTGATCTCTTCAGG
	rev	CCTGAAGAGATCAGCTGCCCTGTGGTTTTGATCTA-TAAGGCACTACTTCCAAAATCTTCCACTT
E-box 3 changed to: AGATCA		
IRF4-Int4-E3	forward	AGTAGTGCCTTATCATGTGAAACCACAGGGAGATC-AATCTCTTCAGGCTTTCTTGATGTGAATGAC
	reverse	GTCATTCACATCAAGAAAGCCTGAAGAGATTGATC-TCCCTGTGGTTTCACATGATAAGGCACTACT
E-box 2/3 changed to: AGATCA/AGATCA		
IRF4-Int4-E23	forward	GAAGTAGTGCCTTATAGATCAAAACCACAGGGAGA-TCAATCTCTTCAGGCTTTCTTGATGTGAATGACAG
	reverse	CTGTCATTCACATCAAGAAAGCCTGAAGAGATTGA-TCTCCCTGTGGTTTTGATCTATAAGGCACTACTTC
mutate AP2a binding site to rs12203592 C/T polymorphism		
Irf4-Int4-rs1220	forward	TGGTGGGTAAAAGAAGGTAAATTCCCCTGTGGTAC
	reverse	GTACCACAGGGGAATTTACCTTCTTTTACCCACCA

B. Protocols

B.1. Preparation of competent cells

Preparation

This method is based on the publication from Inoue et al. (1990). To get good results it is very important to start with fresh cells. The strain to be made competent should be plated out on LB (or minimal medium) with the appropriate antibiotics, the day before starting the liquid culture for the competent cells. Use the plate straight from 37 °C to start the cultures, storing the plates at 4 °C will lead to less competent cells. The paper warns against growing liquid pre-cultures for inoculation but we have done that and do not see a difference. After the cells have been spun down they should not leave the cold room until they are ready and frozen. Use liquid N_2 in a thermos flask or a tight styrofoam box to freeze the cells. Prepare the tubes into which the cells will be aliquoted before starting the manipulations and place them on ice.

Buffers and media for the preparation of competent cells

SOB media

- 20 g Bacto tryptone
- 5 g Bacto yeast extract
- 0.5 g NaCl
- dissolve in ~950 ml dH2O
- add 10 ml 250 mM KCl
- bring to pH 7.0 with NaOH
- adjust volume to 1 l
- autoclave and cool to 60 °C
- add 5 ml sterile 2 M $MgCl_2$ solution

SOC media

For the preparation of SOC media add 2 ml sterile 1 M Glucose solution to 100 ml of SOB media.

HTB buffer

- 0.6 g HEPEs (10 mM; PIPES, BES or MOPS buffers will work as well)
- 0.55 g $CaCl_2$ (10 mM)
- 4.66 g KCl (250 mM)
- dissolve in ~200 ml dH2O
- adjust pH to 6.7 with KOH (a few drops of 4 M solution)
- add 2.22 g (55 mM) $MnCl_2$ (Dihydrate) and dissolve
- adjust volume to 250 ml
- filtersterilize with a 0.45 µm filter
- store at 4 °C

Preparation of competent cells

This procedure describes the production of approximately 17 ml competent cells, but can be scaled up or down as necessary.

- Use about ten large colonies from a freshly grown plate to start a 200 ml culture in SOB medium. Grow the cells at 18 °C (room temperature ~22 °C works as well) with vigorous shaking. Use large flasks for good aeration. Catching the culture at the correct density is very important and growth is slow at this temperature, so start the cultures the day before the cells are to be made competent. It's advisable to make a growth curve for your strain to estimate doubling times so you don't have to harvest in the middle of the night.
- The cells should be grown to an OD_{600} of 0.45 – 0.60. 0.45 is the theoretical optimum but 0.60 gives more cells. The density must be monitored carefully. If you overshoot – start again. When the correct density has been reached the culture should be split into sterile centrifuge tubes (4 × 50 ml Falcon tubes) and placed on ice for 10 min
- Spin for 15 min at 2500 ×g and carefully discard the complete supernatant
- Resuspend in 64 ml HTB (4 × 16 ml) and place on ice for 10 min
- Centrifuge again as before and discard supernatant
- Resuspend in 16 ml HTB (each pellet in 4 ml and then pool)
- Slowly add 1.2 ml DMSO while gently swirling the cell suspension

- Aliquot immediately into convenient aliquots (88 $\times$ 200 µl in snap cap tubes) and quick freeze by throwing into liquid nitrogen. Store at - 80 °C. Avoid all temperature fluctuations.

Measurement of competence

It is not necessary to test the competence of the cells, but it is useful to know how competent the cells are when it comes to trouble-shooting problems with transformations.

- Transform 50 µl of cells with 1 µl of standard pUC19 plasmid (Invitrogen) (10 pg/µl or 10^{-5} µg/µl). This can be made by diluting 1 µl of NEB pUC19 plasmid (1 µg/µl) into 100 ml of TE buffer.
- Incubate on ice for 30 min.
- Heat shock 60 sec at 42 °C
- Add 250 µl SOC media.
- Incubate at 37 °C for 1 hour in 1.5 ml tubes with shaking (heated thermo shakers work pretty well).
- Plate 20 µl on AMP plates.
- Good cells should yield around 100 – 400 colonies per plate.
- Transformation efficiency is dilution factor (=15) $\times$ colony count $\times$ 10^5/µg DNA. The transformation efficiency should be between 5×10^8 and 5×10^9 cfu/µgDNA.

B.2. Transformation of plasmids and ligation mixtures

- thaw competent cells on ice
- add 1 µl of plasmid/ligation mixture (1 – 50 ng of DNA) to the competent cells
- Incubate for 30 minutes on ice. (do the same with the negative control)
- heat shock the cells for 1 minutes at 42 °C
- immediately place on ice and incubate there for 1 minute
- add 400 µl of LB medium into each tube and incubate at 37 °C for 30 – 45 minutes
- plate out 100 µl of each transformation reaction (dilute them 1:100 if necessary) to a LB-plate containing the appropriate antibiotics and incubate over night at 37 °C