The interconnected KIT receptor and Microphthalmia-associated transcription factor axis in melanoma

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Academic Dissertation

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Abstract

Melanoma is an aggressive disease that arises from deregulated signaling events in melanocytes. The KIT receptor tyrosine kinase and the Microphthalmia-associated transcription factor (MITF) are two essential components that are required for the normal physiology of melanocyte development and function. Consequently, aberrant activities of these factors are involved in the development of melanoma. To better understand the molecular basis of disease development we studied the mechanisms that gatekeep and fine-tune the activity of both KIT and MITF.

In this thesis we have identified novel KIT ligand-dependent signaling pathways that, through MITF, affect cell proliferation. Notably, we found that the specific KIT tyrosine phosphorylation sites Y721 (PI3 kinase binding site), Y568 and Y570 (SRC binding sites) affect phosphorylation status of the MITF protein. Using inhibitors against SRC, PI3K, AKT, MEK ERK and p38 during KIT activation, we identified that these signaling pathways are essential components in communicating signals between KIT and MITF.

The activity of KIT is regulated by the tetrapeptide insert in the extracellular juxtamembrane domain of the receptor. By generating receptor mutants with different insert lengths, we showed that the length of this insert is critical for the fine tuning of receptor activation.

Using mass spectrometry and phospho-site specific antibodies against MITF, we found and characterized several serine and tyrosine phosphorylation sites in the protein. More importantly, in stark contrast to previous results, we discovered that the S73 and S409 phosphorylation sites of MITF are neither dependent on KIT nor MAPK-ERK signaling. Furthermore, we show that the tyrosine phosphorylation sites of MITF are required for oncogenic KIT signaling and affect nuclear localization of MITF.

The structural properties of MITF and the KIT receptor were also clarified. By solving the crystal structure of MITF we explain the restricted heterodimerization of MITF towards TFEB, TFEC and TFE3, the binding of M-box DNA sequence and the ability to produce interallelic complementation.

In short, the detailing of KIT and MITF regulation has increased our understanding of key signaling processes that are important to understand the development of melanoma.

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To my parents

CONTENTS

LIST OF ARTICLES

I. *Differential activity of c-KIT splice forms is controlled by extracellular peptide insert length*
Bengt Phung, Eiríkur Steingrímsson and Lars Rönnstrand
Cellular Signalling (2013), doi: 10.1016/j.cellsig.2013.07.011

II. *C-KIT Signaling Depends on Microphthalmia-Associated Transcription Factor for Effects on Cell Proliferation*
Bengt Phung, Jianmin Sun, Alexander Schepsky, Eiríkur Steingrimsson, Lars Rönnstrand (2013)
PLoS ONE 6(8): e24064. doi: 10.1371/journal.pone.0024064

III. *Restricted leucine zipper dimerization and specificity of DNA recognition of the melanocyte master regulator MITF*
Vivian Pogenberg, Margrét H Ögmundsdóttir, Kristín Bergsteinsdóttir, Alexander Schepsky, **Bengt Phung**, Viktor Deineko, Morlin Milewski, Eiríkur Steingrímsson, and Matthias Wilmanns
Genes and Development 2012 26: 2647-2658

IV. *Oncogenic c-KIT D816V induces tyrosine phosphorylation of MITF which results in cytosolic retention and selective gene regulation in melanoma*
Bengt Phung, Julhash U Kazi, Alicia Lundby, Colin Goding, Jesper Olsen, Eiríkur Steingrímsson and Rönnstrand
Manuscript

V. *Biochemical characterization of MITF serine phosphorylation sites*
Bengt Phung, Alicia Lundby, Jesper Olsen, Lars Rönnstrand and Eiríkur Steingrímsson
Manuscript

LIST OF ARTICLES NOT INCLUDED IN THESIS

1. *Suppressor of Cytokine Signaling 6 (SOCS6) Negatively Regulates Flt3 Signal Transduction through Direct Binding to Phosphorylated Tyrosines 591 and 919 of Flt3*
   Julhash U. Kazi, Jianmin Sun, **Bengt Phung**, Fahad Zadjali, Amilcar Flores-Morales and Lars Rönnstrand
   Journal of Biological Chemistry 2012, 287:36509-36517

2. *Enhanced SOX10 and KIT expression in cutaneous melanoma* - Letter
   Lars Rönnstrand and **Bengt Phung**
   Medical Oncology (2013) 30:648, doi: 10.1007/s12032-013-0648-y

AUTHOR CONTRIBUTION STATEMENT

Article I

I contributed substantially to the conception and design of the study. I performed all the experiments and acquired, interpreted and carried out statistical analysis of data. I wrote the manuscript, prepared all the figures for submission. As the designated corresponding author I also had the primary responsibility for correspondence with the journal.

Article II

Same as above.

Article III

I designed and performed luciferase assay experiments which included optimization of cell transfection with various constructs to obtain optimal non-saturated signal. I acquired, interpreted and carried out statistical analysis of the data. I was involved in the drafting of the manuscript.

Article IV

Same as Article I, with the exception that the manuscript is not ready for submission. Also, I did not perform the mass spectrometry analysis.

Article V

Same as above.

LIST OF FIGURES

ABBREVIATION

Adaptor protein containing PH and SH2 domains **(APS)**
Adenosine-5'-triphosphate **(ATP)**
Alpha melanocyte-stimulating hormone **(α-MSH)**
Bacterial artificial chromosome **(BAC)**
Basal cell carcinoma **(BCC)**
B-cell lymphoma 2 **(BCL2)**
Bromodeoxyuridine **(BrdU)**
cAMP response element **(CRE)**
cAMP response element binding protein **(CREB)**
Casitas B-lineage Lymphoma **(CBL)**
CDK complex **(CDKC)**
Cellular KIT **(c-KIT)**
Colony stimulating factor 1 receptor **(CSF1R)**
Cutaneous melanoma **(CM)**
Cyclic adenosine monophosphate **(cAMP)**
Cyclin-dependent kinase 4 **(CDK4)**
Cyclin-dependent kinase inhibitor 2A **(CDKN2A)**
Dopachrome Tautomerase **(DCT)**
Endoplasmic reticulum **(ER)**
Endothelin receptor type B **(EDNRB)**
Ephrussi box **(E-box)**
Fms-like tyrosine kinase 3 **(FLT-3)**
Gastrointestinal stromal tumor (**GIST**)
Glycogen synthase kinase 3 **(GSK3)**
Heat shock protein 90 **(HSP90)**
Human embryonic kidney **(HEK)**
helix-loop-helix leucine zipper **(bHLHZip)**
Hepatocyte growth factor **(HGF)**
Histone acetyl transferase **(HAT)**
Hypoxia-inducible factor 1-alpha **(HIF1-α)**
Interstitial cells of Cajal **(ICC)**
Jun N-terminal kinase **(JNK)**
MAPK kinase **(MAPKK)**
MAPK kinase kinase **(MAPKKK)**
Matrix metalloproteinase 2 **(MMP2)**
Melanocortin 1 receptor **(MC1R)**
Microphthalmia associated transcription factor **(MITF)**
Mitf ^{transgene} **(Mitf ^{tg})**
Mitogen-activated protein kinase **(MAPK)**
Nerve-derived Schwann cell **(NSC)**
Neural crest cell **(NCC)**
P16 cell cycle inhibitor of kinase 4A **(p16ink4A)**
P38 δ **(ERK6)**
Phosphatase and tensin homolog **(PTEN)**
Phosphatidylinositide 3-kinase **(PI3K)**
Phosphatidylinositol-3,4,5-trisphosphate **(PIP_3)**
Phosphatidylinositol-4,5-bisphosphate **(PIP_2)**
Platelet derived growth factor receptor **(PDGFR)**
Pleckstrin homology **(PH)**

Pro-opiomelanocortin **(POMC)**
Protein kinase A **(PKA)**
Protein kinase B **(PKB/ AKT)**
Protein tyrosine binding **(PTB)**
Radial-growth phase **(RGP)**
Receptor tyrosine kinase **(RTK)**
Retinal pigment epithelium **(RPE)**
Son of sevenless **(SOS)**
Squamous cell carcinoma **(SCC)**
SRC **(Sarcoma)**
SRC homology-2 **(SH2)**
Stem cell factor **(SCF)**
Suppressor of cytokine signaling 6 **(SOCS6)**
Truncated KIT **(tr-KIT)**
Tyrosine-related protein 1 **(TYRP1)**
Tyrosine-related protein 2 **(see Dopachrome Tautomerase)**
Ultraviolet **(UV)**
Vertical-growth phase **(VGP)**
Viral KIT **(v-KIT)**
Western blot **(WB)**

SWEDISH SUMMARY - POPULÄRVETENSKAPLIG SAMMAFATTNING

Den farligaste formen av hudcancer, melanom, uppkommer genom okontrollerad tillväxt av melanocyter som är celler specialiserade i pigmentproduktion. Dessa celler skyddar oss från den farliga UV-strålingen med hjälp av pigment som omvandlar ljusvågor till värme. I Sverige drabbas drygt 2000 personer per år av sjukdomen. Cirka 80-85 % av dessa botas genom kirurgi men hos 15-20 % av patienterna har cancern spridit sig till andra organ vilket gör att överlevnadschansen drastiskt sjunker till 7-19 %. I denna avhandling utforskas den cellulära kommunikationen i melanocyter. Genom att förstå de molekylära mekanismerna, bland annat cellkommunikation och cellsignalering, bakom sjukdomsförloppet av melanom, kan vår utökade kunskap leda till utveckling av förbättrade läkemedel mot sjukdomen.

Cellsignalering är de processer som påverkar cellernas beteende. Till exempel kan en cell ta emot signaler från sin omgivning och startar signalfortledning (signaltransduktion) bestående av flera proteinnätverk. Genom dessa signaleringsvägar omvandlas signalerna från cellens utsida till cellulära svar med hjälp av så kallade transkriptions-faktorer som reglerar vilka gener som ska vara påslagna. Ett ökat eller minskat uttryck av vissa gener kan påverka celldelning, programmerad celldöd, tillväxt eller metabolism.

Ett annat viktigt koncept inom cellsignalering är fosforylering av proteiner. Fosforylering är en biokemisk reaktion där proteinkinaser hjälper till att fästa en eller flera fosfatgrupper på ett protein. Fosforyleringen påverkar proteinet så att dess aktivitet antingen slås på eller stängs av, hur det kan interagera med andra proteiner eller dess stabilitet.

KIT är en tillväxtfaktor-receptor som skickar signaler från utsidan till insidan av en cell. Ett av proteinerna som påverkas av signaler från KIT är transkriptions-faktorn MITF. Dessa proteiner spelar central roll i utvecklingen och funktionen hos melanocyter. Störningar i signaleringen mellan KIT och MITF och/ eller mutationer i dessa proteiner är ett vanligt förekommande fenomen i hudcancer. För att få djupare insikt i hur KIT och MITF kan påverka melanocyternas och melanoms utveckling och funktion har vi i denna

avhandling undersökt KIT och MITF med avseende på proteinstruktur, signaleringsvägar och fosforylering.

I det första arbetet undersökte vi hur proteinstrukturen hos KIT reglerar receptorsignalering. Vi tillverkade KIT proteiner med olika längder på den del av receptorn som ligger alldeles utanför cell-membranet. Vi visade att det var just längden i den regionen som påverkade KIT fosforyleringens intensitet. Graden av receptorfosforylering in sin tur påverkade melanomcellers överlevnad i närvaro av cellgifter.

I det andra arbetet kunde vi med hjälp av farmakologiska hämmare och strategiskt framkallade mutationer i KIT identifiera flera nya signaleringsvägar mellan KIT och MITF. Dessa signaleringsvägar visade sig vara viktiga för hur KIT reglerar celldelning.

I det tredje arbetet analyserade vi MITFs tredimensionella struktur genom att använda röntgenkristallografi. Strukturen vi kom fram till medförde en ökad kunskap om hur MITF selektivt binder till andra transkriptionsfaktorer och DNA och förklarade hur vissa mutationer i MITF hämmar dess funktion mer än andra beträffande utvecklingen av melanocyter.

I det fjärde och femte arbetet använde vi en molekylär metod som kallas för masspektrometri för att exakt identifierat var i MITF som fosfat binder. Tillsammans med egentillverkade antikroppar som är designade mot dessa fosforylerade platser upptäckte vi flera tidigare okända fosforyleringsmodifieringar på MITF. Hittills har dessa modifieringar visats sig vara viktiga för bland annat lokalisering av MITF till en specifik plats i cellen och reglering av genuttryck.

Sammanfattningsvis har avhandlingen bidragit med förbättrad kännedom om signaleringsvägar mellan, och struktur- och fosforyleringsförändringar av KIT och MITF som är väsentliga för den biologiska regleringen av melanocyter och melanomceller.

ICELANDIC SUMMARY - ÚTÐRÁTTUR

Sortuæxli eru krabbamein litfruma (melanocytes). Himnubundni viðtakinn KIT og umritunarþátturinn MITF (microphthalmia associated transcription factor) eru bæði nauðsynleg fyrir eðlilega þroskun og starfsemi litfruma. Prótein þessi koma líka við sögu í sortuæxlum. Því er mikilvægt að skilja betur hvernig prótein þessi starfa í litfrumum og sortuæxlum og boðleiðirnar milli þeirra.

Í ritgerð þessari eru tengslin milli KIT og MITF skoðuð á ýmsan hátt. Þær KIT-háðu boðleiðir sem senda boð til MITF og hafa áhrif á frumufjölgun voru skoðaðar. Tyrósín amínósýrurnar Y721 (sem er bindiset fyrir PI3 kínasann), Y568 og Y570 (sem er SRC bindiset) í KIT hafa áhrif á fosfórun MITF próteinsins sem bendir til að KIT sendi boð til MITF. Notkun hindra gegn SRC, PI3K, MEK, ERK og p38 kínösunum benda til að allar þessar boðleiðir sendi boð frá KIT til MITF.

Vegna valvirkar splæsingar er KIT próteinið framleitt í tveimur útgáfum, með og án fjögurra amínósýra í "extracellular juxtamembrane" svæði próteinsins. Með því að útbúa stökkbreyttar útgáfur af þessu svæði sýndum við að lengd svæðisins, frekar en röðin sjálf, er mikilvæg til að stilla af virkni KIT próteinsins.

Massagreining var notuð til að greina þau set í MITF sem eru fosfærð. Fosfó-sérvirk MITF mótefni voru síðan útbúin til að staðfesta fosfæringarnar. Fjöldi nýrra fosfæringarseta fundust. Setin Ser73 og Ser409, sem áður hafa verið talin vera undir stjórn MAPkínasa boðleiðarinnar, reyndust hvorki háð KIT né MAPkínasa boðleiðnum.

Með því að greina krystalbyggingu MITF próteinsins tókst að útskýra hvernig MITF takmarkar tvenndarmyndunargetu sína við sig sjálft, auk TFE3, TFEB og TFEC próteinanna. Krystallinn útskýrir einnig hvernig tiltekin MITF stökkbreyting veldur innangensuppbót (interalleleic complementation).

Rannsókn þessi á KIT og MITF hefur leitt í ljós að boðleiðirnar milli þessara próteina eru fleiri en áður var talið. Frekari greining þessara boðleiða er mikilvæg til að skilja hlutverk þeirra í venjulegri þroskun litfruma og í sortuæxlum.

INTRODUCTION

The receptor tyrosine kinase, KIT and the Microphthalmia-associated transcription factor (MITF) are expressed in a variety of tissues. The pigment producing cells, melanocytes, are one type of cells in which both KIT and MITF functions are critical for normal development. Dysregulation of KIT or MITF activity in melanocytes can lead to pigment-related diseases or to the development of melanoma. Thus, studying the molecular mechanisms governing the regulation of MITF and KIT activity will further our understanding in associated diseases. This thesis and the included articles aim to map out details surrounding:

- ✓ The structural properties of MITF and KIT splice forms.
- ✓ KIT ligand-dependent signaling pathways that activate MITF.
- ✓ Oncogenic KIT activity in relation to MITF tyrosine phosphorylation in melanoma.
- ✓ Serine phosphorylation-mediated MITF activities.

BACKGROUND

Melanocytes

Development

Melanocytes are the cells responsible for the production and dispersal of pigments called melanin. In vertebrates, melanocytes are distributed in, to name a few, the skin, inner ear and eyes. These pigment producing cells originate from the neural crest (NC) which consists of a transient cell population that is unique to vertebrates [5]. Recently it was shown that cranial melanocytes stem from the nerve-derived Schwann cell (NSC) precursors [6]. From the NSC and NC, melanocyte precursor cells, the melanoblasts, undergo expansion and initiate the expression of distinct molecular markers that leads to cellular differentiation. The expression of such markers occurs during the migration throughout the embryo. Notably, the expression of the melanocytic markers such as the receptor tyrosine kinase receptor KIT, the G protein-coupled Endothelin receptor type B (EDNRB), the melanogenic enzyme Tyrosinase-related protein-2 (Tyrp-2), the transcription factor SOX-10 and MITF, dictate cell fate and are therefore important steps in the development of melanocytes. Once the function of the master regulator of melanocytes, MITF, is established, the appearance of the rate-limiting enzyme Tyrosinase marks the melanoblast transition into mature melanocytes [7].

Function

The outermost layer of the human skin; the epidermis is predominated by keratinocytes. These cells act as the first level of protection against pathogens, solar ultraviolet (UV) radiation and maintain homeostasis by preventing water and heat loss to the ambient environment [8]. However, some properties such as thermoregulation and photoprotection are directly contributed by the melanin produced by the neighboring melanocytes distributed along the basal layer of the epidermis [7]. Each melanocyte is surrounded by approximately 36 keratinocytes, around 5-6 of which are in direct contact with the melanocyte. The structural arrangement known as the epidermal-melanin unit [9] is designed to facilitate distribution of melanin to protect the cells from the

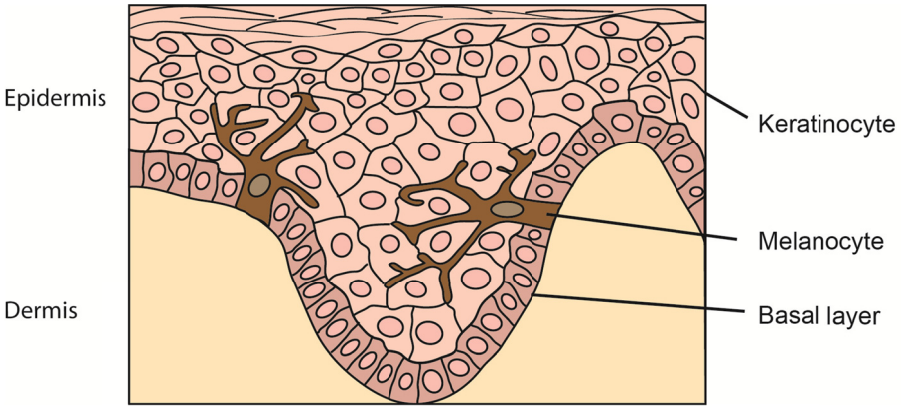

Figure 1. The epidermal-melanin unit. This unit facilitates the distribution of melanin produced by melanocytes in the basal layer of the epidermis, to the neighboring keratinocytes.

harmful UV radiation (Figure 1). When the skin is exposed to UV radiation, tanning response is initiated through factors secreted by the keratinocytes that stimulate the melanocytes to produce melanin [10]. However, UV radiation itself does not trigger the tanning response but rather DNA damage. Sensing the physical damage the keratinocytes mount a cellular response by stabilizing the transcription factor and Tumor suppressor protein TP53 [11]. Increased activity of TP53 leads to enhanced expression of the Pro-opiomelanocortin (POMC) that in turn is converted by enzymatic cleavage into the active alpha-Melanocyte-stimulating hormone (α-MSH). The mature α-MSH is then secreted by the keratinocytes to stimulate melanocytes. Upon α-MSH binding to the Melanocortin 1 receptor (MC1R), cyclic adenosine monophosphate (cAMP) is rapidly synthesized within the melanocytes, which results in the cAMP binding protein (CREB)-mediated upregulation of MITF and melanin production [11] (Figure 2). Low activity level generated by the MC1R is associated with poor tanning response and red hair and fair skin phenotype [12]. In the melanocytes, melanin is synthesized within specialized vesicular organelles called, melanosomes [5, 7]. These packages of melanin are sent back to the keratinocytes to shield the nucleus from further DNA damage induced by UV light. Although such a complex system has evolved to

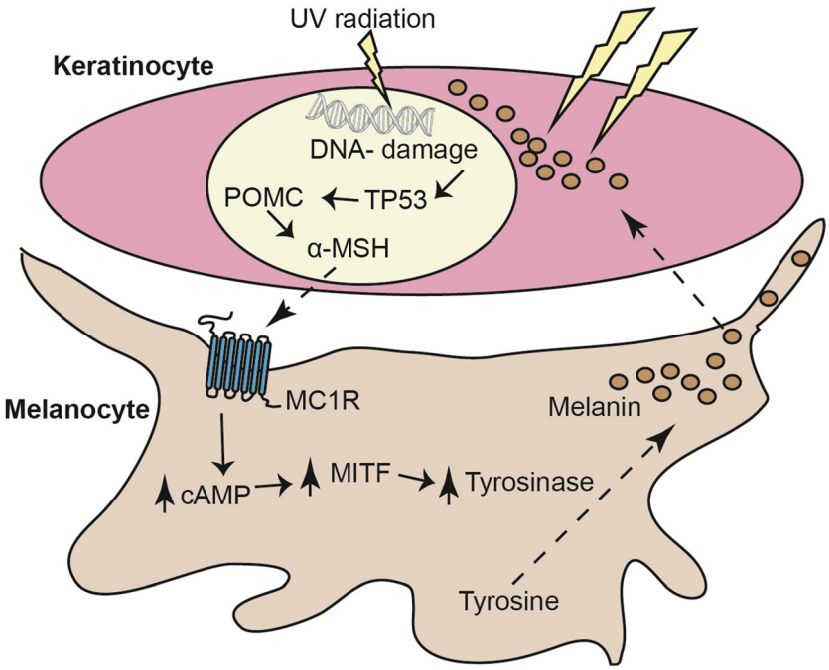

Figure 2. Tanning response. DNA damage in the keratinocyte produced by UV radiation enhances TP53-dependent production of α-MSH that initiates the pigment production pathway in the melanocytes. Illustration is adapted from [1].

specifically protect us from radiation, there are beneficial effects of UV-B radiation. Humans are critically dependent on vitamin D that is photochemically synthesized in our skin. Notably, vitamin D is essential for immune function, maintenance of bone density, blood cell formation and insulin secretion [13]. Due to the physiological importance of vitamin D, there is a mechanism built into the pigment producing system that motivates sun seeking behavior. The α-MSH produced in the keratinocytes upon UV exposure, is not the only cleavage product of the POMC polypeptide precursor [11]. The so called "endogenous morphine" or beta endorphin is another derivative of POMC, which is believed to triggers a feel-good sensation during sun exposure. On the other hand, the same chemical event could also contribute to a rare syndrome called tanning addiction of tanorexia. In

addition, excessive sun exposure is associated with increased risk of melanoma development.

Melanin

Melanin is synthesized within melanosomes. The two major types of melanin produced are the red/yellow pheomelanin and the brown/black eumelanin [14]. These are the pigment-related products that give color to feathers, hair, skin and eyes. Humans skin color is determined by the ratio of the two types of melanin, the amounts of melanin in the skin and the size and distribution of melanosomes [15]. The photoprotective capacity of eumelanin is higher than pheomelanin, which is indicated by the observation that the risk of skin cancer development is 30- to 40-fold higher in light skin compared to dark skin. Not only is pheomelanin a poor photoprotectant, it is also considered to play a role in the development of melanoma independently of UV radiation [16].

Melanoma

Epidemiology

The major forms of skin cancers are basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma. BCC is the most common type and represents about 75-80 % of all skin cancers. The second most common cancer of the skin is SCC, which represents about 16-20 % of all diagnosed skin cancer. Finally, melanoma a disease that originates from transformed melanocytes, is the rarest type and accounts for about 4 % of all diagnosed skin cancer cases [17]. Although the frequency of melanoma is very low, it is the most aggressive form of dermatological cancers and represents 80 % of all skin cancer-related deaths. To date, there is no effective cure against metastatic melanoma, which contributes to the low survival rate. Only 14 % of patients diagnosed with metastatic melanoma survive for five years [17]. Cutaneous melanoma (CM) is almost exclusively limited to the Caucasian population. In contrast, CM rarely occurs in populations with darker skin pigmentation including Africans and Asians. The incidence rate of CM is among the highest of all cancers. Epidemiology studies of the disease incidence

in the Western world indicate 3-6 cases per 100,000 individuals and year in the beginning of the 1970s and 10-18 cases per 100,000 inhabitants and year at the beginning of 2000. Moreover, the study predicts further increasing incidence rate for at least two decades ahead [18].

Causes of disease

The development of melanoma is commonly accepted to be contributed by the interplay between genetical and environmental factors. For example, exposure to UV radiation either naturally from sun light or artificially from tanning beds, is the most significant contributor [17]. The effect of UV radiation is clearly illustrated by the declining rate of CM incidence among younger people in Australia, which has been attributed to education aiming to reduce solar exposure [19]. Epidemiological studies have further identified intermittent sun exposure in contrast to chronic sun exposure, to be an important factor in CM development. In fact, some studies have proposed that chronic sun exposure is negatively associated to disease development. However, the intermittent sun exposure hypothesis is controversial, because intermittent and chronic sun exposure affect the development of CM differently depending on the anatomical site. CM found in the head and neck region is believed to be caused by chronic sun exposure whereas CM in the trunk is believed to develop as the result of intermittent sun exposure. These results demonstrate complexity of the hypothesis and indicate that melanocytes at different anatomical regions have different susceptibility to undergo malignant transformation [20].

Melanogenesis induced by UV radiation as a consequence of the release of paracrine acting factors by keratinocytes, is characterized by melanocyte differentiation or pigment production and proliferation in the epidermis. How melanocytes that are triggered into terminal differentiation by UV light are able to reenter the cell cycle and proliferate is still unclear [21]. However, the expansion of melanocytes in this event might not involve the differentiated cells, but rather the pre-melanocytic cells that are Tyrosinase-related protein 1 (TYRP1) negative and KIT receptor positive [22]. As a consequence of the proliferative response either triggered by factors secreted from keratinocytes or

stimulated by the acquisition of genetic mutation that activates the Mitogen-activated protein kinase (MAPK) signaling pathway, melanocytes form clusters that give rise to nevi or moles [23, 24]. Morphologically, melanocytes in nevi are very similar to normal melanocytes with the exception that these cells are more prone to growth due to abnormal expression of growth factors [25]. Additionally, abnormal activation of the MAPK pathway as the result of somatic mutation of the NRAS GTPase or the serine/threonine-protein kinase BRAF, also stimulates growth. However, nevus cells only grow to a certain stage before senescence, a key protection mechanism against cancer development, is triggered by the cell cycle inhibitor Cyclin-dependent kinase inhibitor 2A (CDKN2A) [26]. In other words, genetic alterations creating constitutively active BRAF or NRAS that promote growth in the combination with CDKN2A inactivation mutations can transform benign nevi into malignant cells. Indeed, mutations of the BRAF or NRAS genes or deletion of the CDKN2A gene are detected in melanomas in a frequency of around 50 %, 13-25 % and 25-40 %, respectively [27-31].

Classification

The classification of melanoma is based on histological grading and stage of progression. The melanoma progression model or the Clark's level is often used to describe the five steps of linear development of pigmented lesions [32] (Figure 3). According to this model the first event is the formation of benign nevus that consists of clustered melanocytes. Histologically, benign nevi arise from increased numbers of nested melanocytes located at the basal layer of the epidermis. The transition of benign nevi into dysplastic nevi is characterized with melanoma features including increasing size, uneven pigmentation, border irregularities and asymmetric lesions. Transition into the radial-growth phase (RGP) is marked by further melanocyte proliferation. Importantly, however, the mitogenic activity in this step is entirely confined within the epidermis. Once the lesion invades the dermis, the disease enters the vertical-growth phase (VGP). The acquired ability of cells to invade and grow into adjacent tissues at this stage is a key property to metastatic potential [32]. The

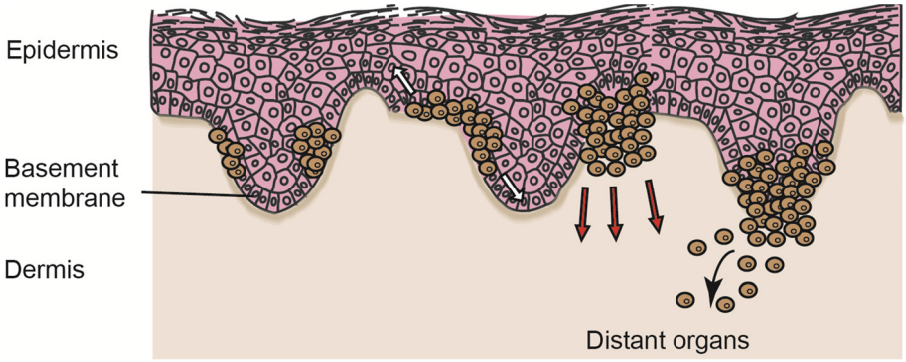

Figure 3. The Clark model for melanoma progression. The model depicts the linear development of melanoma that starts from the proliferation of cells in a benign nevus to expanded growth in the epidermis and proliferation extended to the dermis which finally transits to metastatic potential.

final step in the Clark's model is the successful spread and established growth of melanoma cells to other organs.

The model is a simplified description of development and progression of melanoma and it is important to recognize that the disease progression does not have to follow this linearized model. Both normal melanocytes and melanocytes in benign nevi can directly transit into RGP or VGP and from either of these stage cells can directly progress to the metastatic phase [33].

Molecular basis of disease progression

The histological features during melanoma progression described with Clark's level can be connected with specific gene mutations. As mentioned, mutations of the members of the MAPK cascade particularly BRAF and NRAS are often found in melanoma. The frequency of BRAF mutations and to some extent also NRAS mutations in melanoma are similar to the mutation frequency found in benign melanoma. This suggests that the RAS-RAF-MAPK pathway initiates the neoplastic growth of melanocytes, but activation of the pathway itself is not sufficient for melanoma transformation [34, 35]. Abnormal activity of these proteins generates constitutive activation of the MAPK pathway, which results in elevated melanocyte proliferation. However, melanocyte growth at this stage is prevented from progressing into melanoma by the initiation of senescence. For instance, the ectopic introduction of the

constitutively active BRAF mutant V600E into human melanocytes results in enhanced proliferation that only leads to melanocyte cluster formation resembling human nevi [36]. Senescence triggered by BRAF-mediated proliferation was in this case accompanied by the upregulation of the p16 cell cycle inhibitor of kinase 4A ($p16^{INK4A}$). Similarly, $BRAF^{V600E}$ expression did not fully transform melanocytes in zebrafish. However, in combination with a tumor suppressor TP53 deficient background, the $BRAF^{V600E}$-induced nevi were able to progress into invasive melanoma [37]. Although it was observed that $p16^{INK4A}$ is frequently upregulated in nevi and epigenetically silenced in melanoma, the removal of $p16^{INK4A}$ did not contribute to melanomagenesis in the presence of $BRAF^{V600E}$ [35, 36]. Thus, despite being a potent senescence initiator, other unknown factors are believed to cooperate with $p16^{INK4A}$ to prevent uncontrolled melanocytic growth.

After the formation of benign nevi according to Clark's model, the next step is the development of abnormal growth and the formation of dysplastic nevi. Mutations acquired at this stage affect cell growth, DNA repair and the susceptibility to cell death. Two genes frequently found to lose expression at this point are the tumor suppressor CDKN2A and the Phosphatase and tensin homolog (PTEN) [38, 39]. The CDKN2A gene encodes for two pivotal tumor suppressors $p14^{ARF}$ and $p16^{INK4A}$. Upon DNA damage, $p14^{ARF}$ inhibits the E3 ubiquitin-protein ligase Mouse double minute 2 homolog (MDM2) that targets TP53 for degradation. By inhibiting MDM2, TP53 is stabilized and consequently facilitates apoptosis and suppresses proliferation [40]. The second product of CDKN2A is the cell cycle inhibitor $p16^{INK4A}$. It is involved in the arrest of the cell cycle at the G1–S checkpoint by inhibiting the Cyclin-dependent kinase 4 (CDK4). The action of this suppressor is produced by DNA damage and aberrant activation of oncogenes [41]. However, there are mutations that can override $p16^{INK4A}$-dependent senescence. For instance, in sporadic melanoma it has been found that somatic mutations in CDK4 could block the interaction with $p16^{INK4A}$ [42, 43]. The second important tumor suppressor gene frequently mutated in dysplatic melanocytic lesions is the PTEN gene [44]. PTEN is a phosphatase that is indirectly engaged in the inhibition of the cell survival Protein kinase B (PKB/AKT)-survival pathway.

AKT facilitates cell proliferation and survival by the inactivation of cell cycle inhibitors and apoptosis initiators. Thus, lack of PTEN expression would favor hyperactivation of AKT resulting in cell survival and proliferation [45]. Although, PTEN and CDKN2a are powerful tumor suppressors in melanoma, mutation of either gene alone fails to induce melanoma in mice [46].

Cellular senescence can be divided into three different types, M0, M1 and M2 (Figure 4). The M0 type is mediated by $p16^{INK4A}$ under cellular stress such as hyperproliferation. The growth of melanocyte in a benign nevus lesion is characterized by M0 senescence [26]. Progression to dysplastic nevi occurs when $p16^{INK4A}$ function is deregulated, allowing the cells to escape from M0 [47]. In order for the lesion to further progress into melanoma in the RGP, which is characterized by unlimited hyperplasia, cellular immortalization must be achieved. Rapid cell division that is associated with melanoma in the RGP, results in shortening of the repetitive nucleotides called telomeres that protect gene degradation. As telomeres become critically short as a consequence of rapid cell division, the tumor suppressor TP53 is activated by the stress signal and induces M1 senescence. Thus, to achieve unlimited hyperplasia, cells in the RGP must overcome this barrier either through inactivation of TP53 or maintenance of telomeres. Blocking the activity of TP53 would only be adequate to avoid M1 type of senescence. As the telomeres shorten, cells eventually reach their replicative limit and senesce due to genomic instability called crises or M2 senescence. Thus, in order to avoid M1 and M2 type of senescence, the integrity of telomeres must be maintained [26]. Telomere length can be sustained by the enzyme telomerase, which is not expressed in human somatic cells but abnormally expressed in more than 90 % of human cancers [48, 49]. For instance, telomerase activity and expression in benign and dysplastic nevi is very weak whereas a significant increase of telomerase activity can be found in melanomas [50, 51].

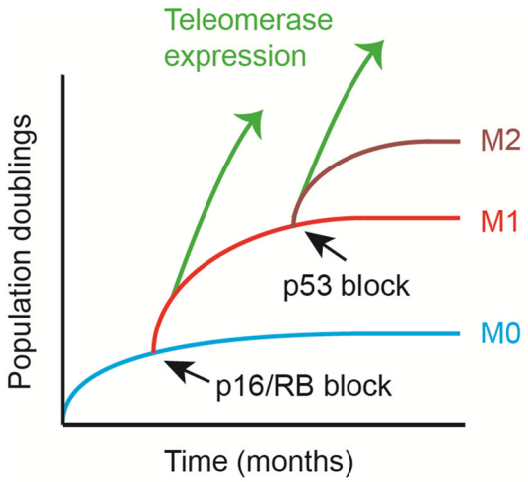

Figure 4. Melanoma growth and senescence checkpoints. Graph illustrates the different types of senescence and their dependency on molecular senescence initiators. The M0 type of senescence is started by the p16-RB pathway while TP53 is involved in the M1 phase. Finally, critical shortening of telomeres triggers M2 type of senescence. At each initiation step, senescence can be overcome by mutation of the indicated proteins, mutation of their associated pathways and acquisition of telomerase expression. Graph is adapted from [2].

Melanoma in RGP, in contrast to cells in VGP, is largely confined within the epidermis. VGP melanoma cells have the ability to penetrate the epidermis and extend its growth in the upper layer of the epidermis and the underlying dermis. Growth and growth confinement of melanoma cells in the dermis is partly regulated by keratinocytes via secreted growth factors and E-cadherins. Cadherins are glycoproteins that regulate cell-cell adhesion and cell signaling. In addition, E-cadherins are also involved in the connection between keratinocytes and melanocytes in the epidermis [52]. The transition from RGP to VGP melanoma cells is characterized by "cadherin switch" whereby E-cadherins are downregulated and N-cadherins are upregulated. Such a shift of cadherin expression has been demonstrated to be transcriptionally facilitated by BRAF activating mutations or by hyperactive PI3K-AKT signaling pathway as the result of loss of PTEN activity [53, 54]. Adding to this, the bias of N-cadherin expression further enhances the activation of AKT that favors anti-apoptotic properties [55]. More importantly, modulation of cadherin

expression allows melanoma cells to escape regulatory growth control imposed by keratinocytes and gain interaction with fibroblast in the dermis that favors melanoma growth [56]. It has been shown that N-cadherin expression in melanoma is associated with enhanced migration over fibroblasts and a more invasive phenotype during progression [55]. However, melanoma cell detachment from the epidermis is not sufficient to invade the dermis without first degrading the extracellular matrix surrounding the tumor tissue. The Matrix metalloproteinase-2 (MMP-2) and MMP-9 are highly involved in this process and the expression of these enzymes is associated with melanoma progression [57, 58]. Lastly, melanoma cell growth breaching the epidermal barrier of the skin marks the final histological phenotype of Clarks' level, the vertical growth phase.

Protein phosphorylation

Biochemically, the addition of the negatively charged phosphate group (PO_4^{2-}) to a protein is called phosphorylation. Protein phosphorylation is one type of reversible post-translational modifications utilized in prokaryotic and eukaryotic organisms to achieve activity control of proteins. More specifically, phosphorylation can temporarily enhance or reduce enzyme activity, cause conformational changes, target proteins for degradation, alter subcellular localization and facilitate or disrupt protein-protein interactions. Although, protein phosphorylation is known to occur on nine amino acids including aspartic acid, cysteine, glutamic acid, histidine, lysine, serine, threonine and tyrosine, the latter three, due to their stable nature and the availability of specific antibodies, are the most well studied [59]. The global *in vivo* site-specific phosphorylation distribution of serine, threonine and tyrosine is 79.3 %, 16.9 % and 3.8 %, respectively [60]. Phosphorylation is carried out by a group of enzymes called kinases that transfer the phosphate groups from for example adenosine-5'-triphosphate (ATP). Protein phosphatases are another group of enzymes that reverse the process by removing the phosphate group from the target protein. For the reason that phosphorylation is reversible and rapid the reaction is very well suited and heavily utilized in most cellular signal transduction pathways. Not surprisingly, owing to its central role in many

biological processes, the dysregulation of kinases and phosphatases are often found in many diseases including cancer.

The term kinome was coined by Manning and associates to describe all protein kinases in the human genome [61]. Significantly, protein kinases are one of the largest gene families in eukaryotes occupying 1.7 % of the human genome, which translates to 518 different kinases. Of these, 428 are predicted to be serine and threonine kinases and 90 are members of the tyrosine kinase family. In contrast, there are only around 200 predicted or identified phosphatases in the human genome [62]. The considerable magnitude of the kinome requires on average a single kinase to distinguish between one and a few hundred curated phosphorylation sites in a background of around 700,000 potential phosphorylation sites [63]. Such a daunting task is mitigated by the many mechanisms evolved to ensure specific interactions. The structural design of the active site of a kinase acts as the first line of specific interaction. Although the structural characteristics of the interaction sites only partially differentiates binding specificity, observations suggest that the deeper catalytic site of the tyrosine kinases and phosphatases separates these proteins from their serine-specific counterparts [64]. Another mechanism of specificity is accommodated by the modulation of overall binding energy between the phosphorylation site of a substrate and a kinase. The mere presence of a phosphorylatable residue greatly enhances the binding energy and the combination with the consensus recognition sequence further improves binding specificity [65, 66]. Co-interaction with subunits is another important mechanism that regulates target to substrate contact. In addition, a regulatory subunit can provide property for a single kinase to interact with multiple targets. For example, the CDKs as implied in their name, is dependent on cyclins to become fully active. Besides allosterically activating CDKs, cyclin also sequesters CDK substrates via a docking domain and thereby controlling the accessibility to substrates [67, 68]. Moreover, cyclins can further aid substrate specificity of CKDs by transporting them into different subcellular compartments. This mode of specificity enhancement is also utilized by the active and inactive forms of ERK2 in the rat neuroendocrine tumor PC12 cells. Depending on the type of growth factor stimulation, ERK2 either translocates

to the nucleus to drive differentiation or stays in the cytoplasm to elicit mitogenesis. Thus, by altering subcellular location, the function of ERK2 is also changed to take advantage of the different substrates [69, 70]. Finally, the use of scaffolds also provides substrate specificity. In contrast to specificity achieved by subunit interactions with kinases, the association to scaffolds is more dynamic and allows for frequent protein complex dissociation. Also unlike subunit co-binding, scaffolding proteins typically bring together kinase interactions with other kinases or phosphatases [63].

There are numerous available methods for identification of protein phosphorylation sites. One way is to combine bioinformatics with biochemistry. For instance, phosphorylation sites predicted *in silico* can be further characterized by mutating the site to a non-phosphorylatable residue. The protein is then subjected to phosphorous isotopic labeling with ^{32}P followed by enzymatic cleavage into peptide fragments that are separated in two-dimensional thin layer chromatography or SDS polyacrylamide gel electrophoresis and analyzed for loss of a phosphorylated ^{32}P-labeled peptide [71] or by Edman degradation of phosphorylated peptides. Historically, this biochemical based approach has been the gold standard for phosphorylation site identification. However, advancement in mass spectrometry has allowed for more refined detection methods. Mass spectrometry offers major advantages over the conventional method. For example, the procedure is less time consuming and isotopic labeling is not required, which eliminates the handling with radioactive material [72]. For these reasons, mass spectrometry was the chosen method for phosphorylation site identification in this thesis.

Receptor tyrosine kinase

Proteins belonging to the receptor tyrosine kinase (RTK) family are classified as single-pass, type I transmembrane proteins located in the plasma membrane. Cell surface receptors in this family are engaged in signal transduction that mediates pivotal biological processes such as growth, differentiation, migration and metabolism. Although all receptors in the family of RTK are structurally related, the family is further divided into 20

subfamilies in which each subfamily comprises of members that have even higher structural homology. Each receptor in the RTK family consists of an extracellular ligand-binding domain, a single alpha-helical transmembrane domain and a cytoplasmic portion composed of a juxtamembrane domain, protein tyrosine kinase (PTK) domain and a C-terminal tail. The kinase domain of RTKs in the Platelet derived growth factor receptor (PDGFR) subfamily is also interrupted by a kinase insert. Upon receptor activation, phosphorylation sites located in the intracellular domain of RTKs are either subjected to autophosphorylation or phosphorylation by other protein kinases [73, 74]. Without ligand binding, the receptors are in their inactive state on the cell surface either as monomers or as pre-existing dimers like the insulin receptors [73, 75]. Among the RTKs, ligand binding is diverse. For instance, KIT ligand (also known as Stem cell factor (SCF), Mast cell growth factor (MGF) and Steel factor (SF)) binds to KIT receptor in a bivalent manner. Each of the KIT ligand monomer binds to one molecule of KIT, thus a complex of KIT ligands binds to two KIT receptors and facilitates receptor dimerization [76]. Another strategy is exemplified by the receptors belonging to the subfamily of insulin receptors that exist on the cell surface in a homo- or hetero-pre-dimeric form, maintained by a single disulfide link between the monomers. Although receptor dimerization is the default state, ligand binding is still a requisite for activation [77].

After ligand binding to the receptor, a dimeric active structure is produced by conformational changes that facilitate autophosphorylation. Phosphorylated tyrosine residues in the intracellular domain allow signaling cascade activation through the interaction with downstream signaling partners. Most of the phosphorylated residues of RTKs are located in the non-catalytic part of the intracellular domain. These sites, when phosphorylated, are recognized by proteins containing the SRC (sarcoma) homology-2 (SH2) or protein tyrosine binding (PTB) domain [78].

RTKs play major roles in many cancers. In fact, more than half of the known RTKs have been found mutated or overexpressed in different malignancies [74, 79]. Malignant transformation accelerated by RTKs could occur through four major routes. First, viral transduction of the KIT receptor

was demonstrated when it was first isolated from the Hardy-Zuckerman 4 feline sarcoma virus. The high concentration of viral KIT (v-KIT) found in cat fibrosarcoma tissue indicates the transforming ability of v-KIT [80]. Second, genomic re-arrangements can produce active RTK fusion protein that generates constitutive signaling and consequently malignant transformation [81]. Third, RTK gain-of-function mutations are often detected in different tumors [82, 83]. Finally, gene amplification and overexpression of RTK have also been found in malignancies [84].

Stem cell factor receptor KIT

The first discovery of KIT was made in 1986 at which v-KIT was identified in the Hardy-Zuckerman 4 feline sarcoma virus [80]. Cellular KIT (c-KIT) was already then predicted to be a transmembrane receptor and it was believed to be expressed in human, cat and mouse. A year later, cloning of c-KIT confirmed its existence in human tissue as well as its possession of a transmembrane domain [85]. Today, the KIT mRNA is known to be expressed in many different types of tissues. For example, KIT is a surface marker for hematopoietic stem cells and progenitor cells. At the early stages of development of these cells KIT is engaged in proliferation and survival [86, 87]. However, KIT expression is lost during the differentiation of hematopoietic cells with the exception of mast cells and dendritic cells [88-90]. As mentioned, KIT is also involved in melanocyte development including cell migration, pigmentation, survival and proliferation. More specifically, the administration of the KIT blocking antibody, ACK2, during the different stages of melanocyte development identifies the requirement of KIT for melanocyte survival; during migration in the dermis, in the epidermal sheet prior to localization in the hair follicles and during the hair production growth phase, anagen [91]. Moreover, the role of KIT has also been implicated in fertility. The activation of KIT is important for the maintenance of primordial germ cells in both sexes of humans [92]. However, there are pathway specific differences between the genders. The Y719F point mutation of KIT that blocks receptor signaling to PI3K, resulted in obstruction of spermatogenesis and consequently completely sterile male mice, while impaired female follicle

development only led to reduced fertility [93]. The interstitial cells of Cajal (ICC) that regulates the movement of the gut is another tissue in which KIT serves as a marker. Inhibition of KIT activity in the ICC causes severe anomaly of gut movement in both humans and mice, indicating its importance in gut motility [94, 95]. Lastly, it is worth mentioning that KIT is also critical for function in the nervous system [96], cardiovascular system [97] and beta cells in the endocrine system [98].

Structural regulation of activation

KIT belongs to the class III receptor tyrosine kinase family and together with five other members including the PDGFR alpha and beta, Colony stimulating factor 1 receptor (CSF1R) and Fms-like tyrosine kinase 3 (FLT-3), forms the PDGFR family [99]. The class III tyrosine kinase receptors are structurally related in that they possess an extracellular domain with 5 immunoglobulin (Ig)-like domains called D1-D5 and an intracellular kinase domain that is interrupted by a kinase insert. Although, the ligand binding part of the receptors is structurally similar, their cognate ligands are not. Both helical and cysteine knot types of cytokines are involved in the activation of the receptors. More precisely, KIT, FLT-3 and CSF1R are activated by helical dimeric cytokines whereas PDGFRα and PDGFRβ bind to dimeric cysteine-knot cytokines [79].

KIT ligand interacts with the KIT receptor through the first three extracellular domains, D1, D2 and D3. The KIT ligand-KIT complex brings about homotypic receptor-receptor interactions and conformational changes that allow interactions between D4-D4 and D5-D5 of the two KIT monomers. These are critical domains for the stabilization of the two ectodomains in a KIT dimer complex. Although, a mutation of the D4 domain strongly compromises receptor activation, D4 is dispensable for ligand binding (Figure 5) [100]. Autophosphorylation of KIT initiated by KIT ligand binding occurs in trans (i.e. cross-phosphorylation between receptor monomers) and targets tyrosine residues in the intracellular domain including the juxtamembrane domain, the kinase insert, the C-terminal domain and the activation loop [101, 102]. As the juxtamembrane domain locks KIT in an

autoinhibitory state by direct binding to the N-terminal ATP-binding lobe of the kinase domain, the residues Tyr-547, Tyr-553 Tyr-568 and Tyr-570 in this region are the first residues to be phosphorylated. Subsequently, the suppressive action of the juxtamembrane domain is released by phosphorylation-dependent structural change. Once unblocked, the catalytic activity is strongly favored, which leads to rapid receptor autophosphorylation [100, 103, 104]. The second region that undergoes autophosphorylation is the kinase insert in which Tyr-703 and Tyr-721 are subjected to phosphorylation. However, as KIT receptor mutants lacking the kinase insert still retain full kinase activity this region seems to be more important for the triggering of early signal cascade initiation than for the overall activity of the receptor. Lastly, the activation loop site Tyr-823 is phosphorylated at the near completion of overall KIT phosphorylation. In protein kinases, active loop phosphorylation is a conserved mechanism of activation control [105, 106]. Significantly, the activation loop of KIT prevents the active state of the receptor to revert back to an inactive conformation. Thus, the late event of Tyr-823 phosphorylation is believed to prolong KIT signaling [101]. Once fully activated KIT is phosphorylated at the tyrosine residues Y547, Y553, Y568, Y570, Y703, Y721, Y730, Y823 and Y900 and two serine sites including S937 and S943 [101, 107, 108]. Functionally, the phosphorylated tyrosine residues of KIT are recognized by proteins containing the SH2 or PTB domain. It is believed that the sequestering and assembly of downstream signaling molecules to phosphorylated residues of KIT may act as effector platform from which multiple singling cascades and subsequently biological processes are initiated [73].

It is important to recognize that the progression of KIT phosphorylation outlined here is based on observations performed in a cell-free system without the interference from other protein kinases [101]. In a cell, the sequence of KIT phosphorylation might be different as other tyrosine kinases could influence receptor phosphorylation.

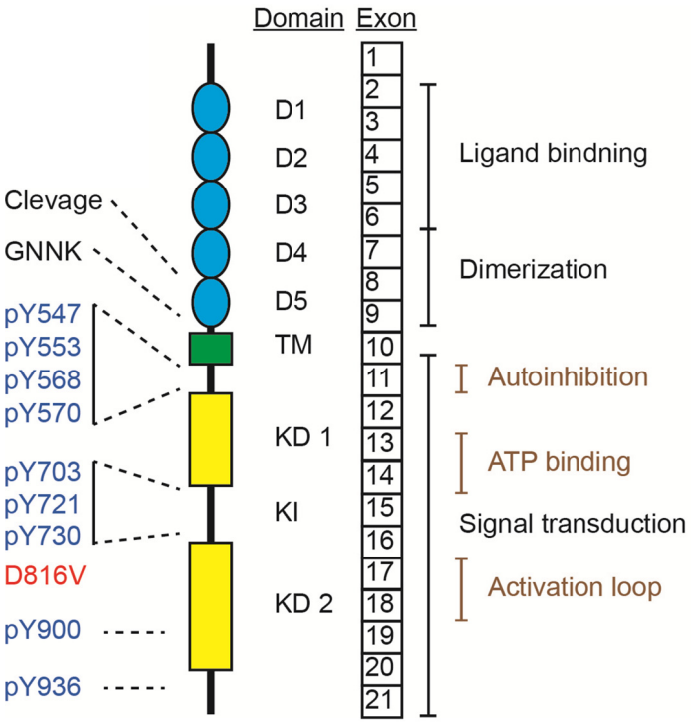

Figure 5. Schematic illustration of the structure of KIT. The extracellular ligand-binding domain of KIT (blue) consist of 5 immunoglobulin-like domains, a transmembrane domain (green) and a kinase domain (KD, yellow) that is parted by a kinase insert (KI). Tyrosine phosphorylation sites (pY) of the receptor spans from the intracellular juxtamembrane domain to KD 2. The oncogenic D816V mutation is located in KD 2. Figure not drawn to scale.

Receptor downregulation

Mechanisms to prevent prolonged KIT signaling or receptor hyperactivation are important to offset potential oncogenic activity. In addition to the structural barriers that keep the receptor in an autoinhibitory state, post-translational modification-mediated downregulation also exists to ensure appropriate signaling intensity and duration. Ubiquitination is a well-recognized post-translational process that facilitates RTK endocytosis and degradation in lysosomes or proteasomes. The process of KIT internalization is started upon KIT activation and phosphorylation of internal tyrosine residues. Ubiquitination of KIT is principally mediated by the E3 ubiquitin-protein

ligase, Casitas B-lineage Lymphoma (CBL), which interacts with KIT both directly and indirectly. The KIT phosphorylation sites Tyr568 and Tyr936 serve as docking sites and the isoleucine and leucine residues at the +3 position, respectively, direct the specificity of CBL KIT interactions [109]. The proposed direct interaction model suggests that SRC activated by KIT phosphorylates and activates CBL, which in turn catalyzes KIT receptor for ubiquitination. In addition to CBL, structural analysis show that the E3 ubiquitin ligase, the Suppressor of cytokine signaling 6 (SOCS6) is also recruited to phosphorylated KIT Tyr-568 and associates with KIT through its SH2 domain [110]. Alternatively, KIT utilizes indirect routes of interaction with ubiquitin ligases. Again the multifunctional docking sites Tyr-568 and Tyr-936 are also involved in the interaction by recruiting and binding of CBL indirectly through the adaptor protein APS. Additionally, phosphorylated Tyr-703 and Tyr-936 allow Growth factor receptor-bound protein 2 (GRB2) binding, which acts as a bridge between CBL and KIT [111]. To summarize, the process of receptor internalization is triggered by KIT ligand binding, which not only presents phosphorylated tyrosine residues for CBL interaction, but they also activate CBL in a SRC-dependent manner. Activated CBL then catalyzes the ubiquitination of KIT that is subsequently removed from the cell surface through internalization and finally targeted for proteolytic degradation in both lysosomes and proteasomes [109, 112, 113].

KIT alternative splicing

Alternative splicing of exon 9 of the KIT gene product yields two isoforms, KIT(+) and KIT(-). They differ by the presence or absence of a tetrapeptide insert consisting of the amino acids sequence GNNK (glycine-asparagine-asparagine-lysine) that is located just upstream of the transmembrane domain at amino acid position 510-513 (Figure 5). In addition, alternative splicing of the KIT transcript generates an extra insert of a serine residue in the cytoplasmic domain at position 715 [114]. All these isoforms are expressed in human normal and malignant tissues with a frequently high ratio of KIT(-) to KIT(+). There is also a truncated form of KIT (tr-KIT) lacking the extracellular and transmembrane domains as well as

the ATP-binding site of the kinase domain, which has been found in human and mouse germ cells [115, 116]. Despite lacking intrinsic kinase activity it is believed that tr-KIT could potentially act as a scaffold for protein-protein interactions. Functionally, it has been established that microinjection of tr-KIT into mouse eggs parthenogenetically activates the cells, possibly through the activation of Phospholipase C gamma by the interaction with Fyn kinase [117, 118].

The best studied isoforms are the KIT(-) and KIT(+) isoforms. The short KIT(-)isoform without the GNNK insert, display a stronger transforming potential *in vitro* than KIT(+) that possesses the tetrapeptide insert [119]. Accordingly, KIT ligand stimulation of KIT(-) elicits a higher receptor phosphorylation and downstream signaling activity than KIT(+). Although, KIT(+) activation is characterized by a weakened overall receptor phosphorylation, phosphorylation of the receptor is extended over a longer time. It has long been thought that the distinct signaling differences between the isoforms were attributed to dissimilar affinity to KIT ligand. However, saturation binding analysis indicated that they do not differ in their affinity [119]. To date it is not known whether the differences are due to insert length or sequence specificity. Studies to address the mechanisms are discussed in Article I of this thesis.

KIT-associated signaling cascades

The MAPK pathways

The mitogen-activated protein kinase pathways are composed of highly conserved signaling proteins that transduce extracellular signals into a diverse array of fundamental cellular responses such as proliferation, apoptosis, mitosis and survival. This communication highway is designed to relay signals through a three-tier kinase module: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. At present, six groups of MAPKs have been identified: Extracellular signal-regulated kinase (ERK)1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK)1/2/3 and the p38 isoforms α/β/γ (ERK6)/δ. The MAPK pathways, when deregulated, play a crucial role in the development of cancer. For instance, these signaling cascades may facilitate sustained cell

proliferation, resisting cell death, escape of growth suppressors, promote cell invasion or induce genomic instability [120]. Many if not all of the hallmarks of cancer are regulated by the MAPK pathways. Significantly, the RAS-RAF-MEK-ERK cascade of often found to be hyperactivated in the majority of melanomas in which NRAS and BRAF mutations are overrepresented with a frequency of 15-20 % and 40-50 %, respectively [33]. Consequently, this signaling cascade offers many therapeutic targets against melanoma. As we in Article II identified the p38 signaling cascade to be involved in the activation of MITF in a KIT ligand-dependent manner [121], it is worth mentioning the p38 kinase is important in the normal function of melanocytes including the initiation of pigment synthesis. For instance, it has been shown that UV radiation or signaling input by α-MSH in melanocytes activates p38 [122]. KIT ligand stimulation activates the ERK1/2, JNK, p38 and ERK5 pathways. In contrast to the ERK-MAPK pathway, p38 activation by KIT is not as well characterized. However, it is known that p38 is indirectly activated by KIT phosphorylated at Tyr-567 (corresponding to human KIT Tyr-568) through SRC family of kinases in the mouse Ba/F3 pro-B-lymphocytes. In these cells the activation of p38 induces cellular influx of calcium that promotes chemotaxis [123].

The best characterized MAPK pathway is the ERK1/2 signaling cascade which is activated by KIT through the initiation of the SRC kinase by the phosphorylated KIT residues Tyr-568 and Tyr-570. Activated SRC further phosphorylates the adaptor protein SHC whereby the guanine nucleotide exchange factors Son of sevenless (SOS) in a complex with GRB2 is recruited and indirectly interacts with KIT through SHC. This action brings the SOS/GRB2 complex close to the plasma membrane so that colocalization with RAS occurs. SOS facilities RAS to exchange the bound guanosine diphosphate (GDP) to guanosine diphosphate (GTP) and thereby allows RAS association to RAF recruited to the plasma membrane. The ensuing interaction between RAS and RAF conformational changes of RAF and initiates the phosphorylation of the dual specificity kinases MEK1/2 through which the serine/threonine kinases ERK1/2 are finally activated [107]. Interestingly, the phosphatase SHP2 that binds to Y568 of activated KIT becomes phosphorylated and

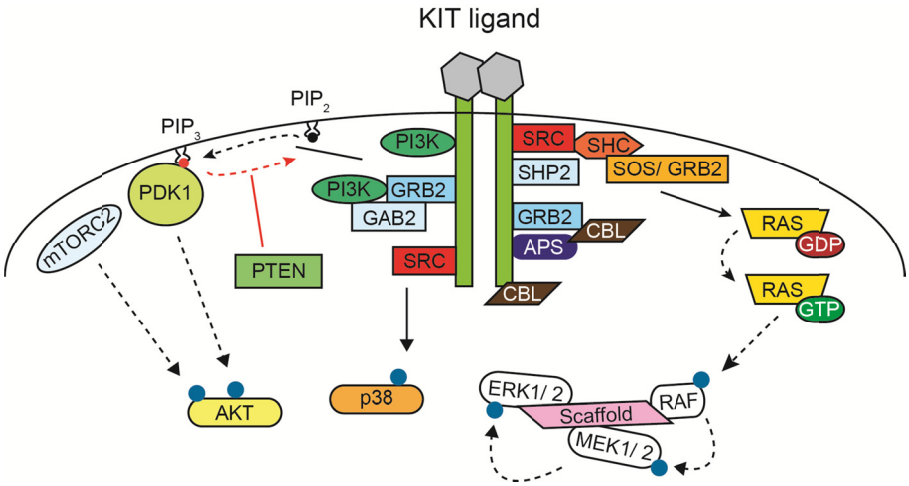

Figure 6. KIT-associated signaling cascades. Tyrosine phosphorylation of KIT initiates, among other pathways, the MAPK ERK and p38 signaling cascades and the PI3K/AKT survival pathway. Also, phosphatases and ubiquitin ligases are activated by KIT receptor phosphorylation.

enhances ERK1/2 activation, presumably by negatively regulating inhibitors of ERK [107, 124] (Figure 6).

In an individual cell, ligand-mediated RTK activation of ERK is followed by oscillating pulses of ERK phosphorylation. This phenomenon is a consequence of rapid phosphorylation followed by several mechanisms that hamper ERK phosphorylation. Examples are internalization of RTK, ERK-mediated stabilization of dual specificity phosphatases or ERK directed negative feedback of SOS, RAF or MEK. Not only is the frequency of pulses of ERK phosphorylation dose-dependently regulated by RTK ligand concentration and the amplitude of the pulses by MEK activity, but ERK signaling is also further complicated by its nonlinear nature of output to biological response (Figure 7). Accordingly, it has been shown that cells with low levels of ERK activity are very sensitive to small changes of signal intensity and minor increase of ERK signaling output elicits rapid increase of proliferation. On the other hand, cells at the high end of the dynamic range requires much higher ERK output to generate small increase in proliferation [125].

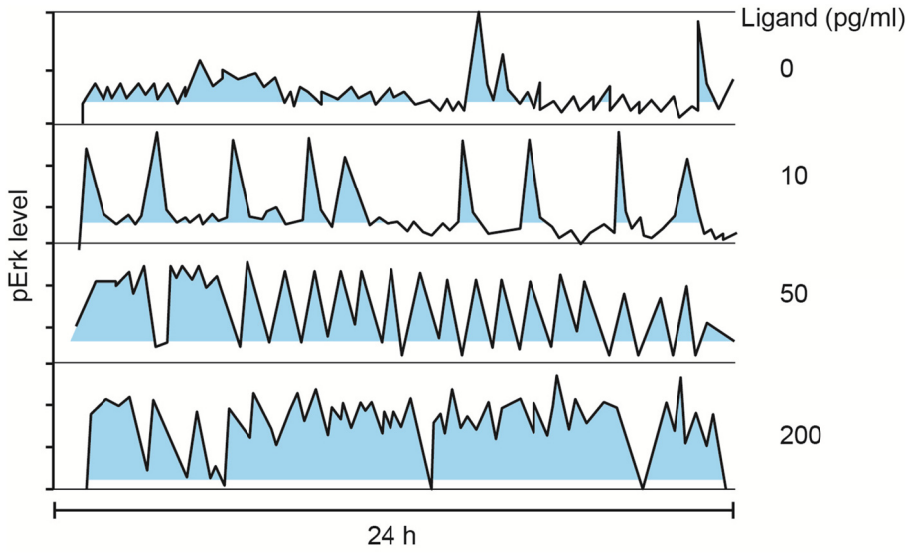

Figure 7. Ligand concentration-dependent modulation of the frequency of ERK phosphorylation. The frequency of ERK phosphorylation in an individual cell is influenced by ligand concentration. Stimulation with high concentration of Epidermal growth factor is associated with high frequency of ERK phosphorylation, whereas low Epidermal growth factor concentration elicits a lower ERK phosphorylation frequency. Figure is adapted from [125].

The PI3K pathway

The evolutionarily conserved phosphatidylinositol 3–kinase pathway is similar to the MAPK pathway in its involvement in important biological processes such as metabolism, survival, proliferation, apoptosis, growth, and cell migration. Abnormal activation of the PI3K pathway including its downstream effector Protein kinase B (PKB or AKT) is one of the most frequent events in cancer [126]. As mentioned, hyperactivation of the pathway in melanoma is often caused by loss of activity by the tumor suppressor phosphatase PTEN. In accordance, abnormal PTEN function with disturbed phosphatase activity or reduced expression caused by missense frameshift mutation that terminates translation, has been found in 10-30 % of cutaneous melanoma [44].

Kinases belonging to the PI3K family are specialized in phosphorylation of the 3'-hydroxyl group of the membrane lipids, phosphatidylinositols. The

PI3Ks are divided into three classes (I-III) based on primary structure, regulation and lipid substrate specificity [45]. Although all the classes of PI3K are essential regulators of biological function, only class I will be elaborated because of its regulation by RTKs and its frequent involvement in cancer. More specifically, the subgroup class I_A is regulated by RTKs whereas class I_B is mainly controlled by G-proteins. PI3Ks in this subclass are constitutive heterodimers that consist of a regulatory subunit and a catalytic subunit. There are three regulatory subunit isoforms (p85α, p85β and p55γ) and three catalytic subunit isoforms (p110α, p110β and p110δ). All the p85 subunits have SH2 domains that enable binding to tyrosine phosphorylated sites [45]. The direct binding of p85 to KIT occurs at phosphorylated Tyr-721 in the kinase insert of the receptor and indirectly through the GAB2 and GRB2 complex that is associated to KIT phosphorylated at Tyr-703 and Tyr-936 [127, 128]. Preceding RTK-mediated assembly of the p85 and p110 heterodimer, p85 structurally inhibits p110 through an intermolecular interaction that is relieved by conformational changes induced by SH2 domain binding to phosphorylated RTK. Recruitment and activation of p110 in the proximity to the plasma membrane also allows access to the PI3K substrate phosphatidylinositol-4,5-bisphosphate (PIP_2). PIP_2 is converted to a second messenger by phosphorylation to phosphatidylinositol-3,4,5-trisphosphate (PIP_3). However, the process can also be reversed by dephosphorylation. Namely, PTEN converts PIP_3 back to PIP_2 in order to keep a tight negative regulation of the PI3K signaling pathway. Generation and enhanced concentration of PIP_3 near the plasma membrane sequesters proteins that recognize PIP_3 via their Pleckstrin homology (PH) domain. The Phosphoinositide-dependent protein kinase 1 (PDK1) directly binds to PIP_3 and facilitates co-localization that assist the activation of AKT by PDK1-dependent phosphorylation of Thr-308 located in the activation loop of AKT (Figure 6). However, additional phosphorylation on Ser-473 by the Mammalian target of rapamycin complex 2 (mTORC2) is required to achieve full AKT kinase activation. Upon a sequence of multiple phosphorylation events, AKT drives cell proliferation, survival and metabolism by phosphorylating numerous cellular proteins. Notably, the AKT substrates

BCL2, BAD, BIM, GSK3α and ß, MDM2 and FOXO are among others to ultimately carry out cell survival and cell cycle entry.

The oncogenic KIT^{D816V}

Several mutations affecting the activation loop of KIT are known to produce constitutive receptor activation. However, mutation of the D816 residue in exon 17 is most frequently found in malignancies (Figure 5). Indeed, KIT^{D816X} (X: any amino acid) is detected in the vast majority of systemic mastocytosis cases (81-90 %) [129, 130] and in myeloid leukemia, germ cell tumors of the seminoma, sinonasal natural killer/T-cell lymphomas, intracranial teratomas and more recently also in melanoma [131-138]. By substituting the aspartic acid at 816 with a tyrosine, histidine or more commonly a valine, the structure of KIT is altered so that the inhibitory state of the activation loop maintained by the juxtamembrane domain is weakened [130]. Thus, the activation of KIT is rendered ligand- and surface localization-independent.

As a transmembrane glycoprotein KIT is dependent on N-linked glycosylation with the addition of complex carbohydrate residues on its extracellular domain for trafficking to the plasma membrane. In contrast, non-glycosylated or generically glycosylated with N-linked high-mannose oligosaccharide KIT is not expressed in the cell surface and is considered an immature protein. Unlike wild-type KIT, oncogenic KIT^{D816V} displays an extensive high-mannose–type N-glycosylation pattern which correlates with its abnormal intracellular localization around the Golgi apparatus coupled with its low cell surface expression [139]. Although not entirely understood, it has been proposed that the sub-cellular behavior of KIT^{D816V} is one of the contributing factors for its potent oncogenic property. For example, the aberrant activation of FES [140] and the splice form independent phosphorylation of SRC [141] are potentially consequences of its intra-cellular localization [142]. Also, the interference of intracellular trafficking of KIT^{D816V} from the endoplasmic reticulum (ER) to the Golgi apparatus, effectively blocked downstream signaling including AKT, ERK and the survival factor Signal transducer and activator of transcription 3 (STAT-3) [139]. However, these events were not

observed when transportation of KIT^{D816V} to the plasma membrane was blocked, further supporting the notion that localization of KIT^{D816V} in the Golgi network is essential for its oncogenic nature. Similar to the differential function of endomembrane compartmentalized RAS that activates downstream signaling pathways distinct from RAS in the proximity of plasma membrane, activation of downstream signaling proteins by KIT^{D816V} is also believed to be achieved through non-canonical routes that yield different functional outcome [143]. Surely, tagging the receptor with localization domains demonstrated that Golgi compartment anchored KIT^{D816V}, but not cell surface destined KIT^{D816V}, can transform lymphocytes. Equally important to Golgi localization is its ability to stay active independently of receptor dimerization. It was illustrated that the deletion of the entire extracellular domain including the important regions for dimerization and ligand binding terminated the ability of wild-type receptor to phosphorylate in response to KIT ligand stimulation. However, despite lacking the capacity to dimerize with the aid of the extracellular domain, mutant KIT^{D816V} was still found to be sufficiently active to transform Ba/F3 pro-B-lymphocytes in the absence KIT ligand [144].

As mentioned, the D816X mutation in particularly the substitution with a valine is frequently detected in systemic mastocytosis. In melanoma the frequency of D816H mutation among KIT mutant melanomas is only around 5 % and to date there is only one documented case of D816V found in mucosal melanoma [138]. Interestingly, the introduction of endogenous D814Y (corresponding to human D816Y) mutant KIT in mouse melanocytes did not suffice for malignant transformation. These melanocytes illustrated decreased pigmentation but contributed to larger pigmented patches in mice due to increased melanocyte migration in the epidermis [145]. In order for melanocyte transformation to occur in the presence of oncogenic KIT, secondary oncogene activation was required. It was found that hypoxia or the stabilization of the transcription factor Hypoxia-inducible factor 1-alpha (HIF1-α) in addition to an activating KIT mutation resulted in signs of cellular transformation including increased melanocyte proliferation and growth on soft agar [146].

KIT-associated melanoma therapy

Initial reports described KIT as an anti-melanoma factor that caused apoptosis and diminished metastatic potential *in vitro* [147]. Further strengthening this view was the observation that loss of KIT expression correlated to melanoma development from superficial to invasive metastatic phase [147-151]. Thus, not surprisingly, initial clinical trials with the tyrosine kinase inhibitor imatinib against KIT in patients suffering from metastatic melanoma did not demonstrate any therapeutic benefits [152-154]. In the same notion, using dasatinib for KIT inhibition in a phase II trial to evaluate the benefit in unselected advanced melanoma patients illustrated minimal activity. Interestingly, both studies saw partial response of melanoma patients harboring KIT mutations, which suggests the importance of KIT in the disease [155, 156]. In all types of melanoma the frequency of KIT mutations is low (2-6 %) and the lack of selection of patients suffering from melanoma in which KIT was the driving oncogene, may have led to the misleading conclusions. However, more recently, findings have highlighted that KIT in melanoma is subtype specific and the subgrouping of melanomas into acral-, mucosal- and chronically sun-damaged subtypes facilitated the discovery of a much higher frequency (20-40 %) of KIT oncogenic mutations or copy number elevations [157-159]. Thus, previous studies suggesting the loss of KIT expression during disease development can be explained by the lack of information in regards to the specific subtype of the primary tumor. However, carefully planned studies including the selection of both patients and melanoma cells where KIT was the primary oncogenic driver have demonstrated high sensitivity to imatinib and significant clinical responses were achieved among patients with advanced melanoma harboring genetic KIT aberrations [158, 160-164].

Imatinib is a powerful therapeutic tool used to treat chronic myeloid leukemia and gastrointestinal stromal tumor, where it achieves disease control in 70-85 % [165] of patients and radically improves survival with a survival rate of 89-95 % [166, 167]. In contrast, despite positive clinical efficacy in melanoma patients, the majority of the patients will eventually progress during imatinib treatment. The mechanism of resistance includes increased MAPK signaling, PI3K/mTOR signaling, selection for N-RASQ61K mutation or the

development of a secondary KIT A829P or T670I mutations. Interestingly, the T670I mutation found in the imatinib resistance melanoma cells is also the most common secondary KIT mutation in imatinib resistant gastrointestinal stromal tumor (GIST) [168]. This indicates that natural selection for imatinib resistant cells is independent of tissue specificity.

In addition to mutations in the ATP-binding pocket of KIT (exon 13 and 14), including the gatekeeper mutation T670I that disrupts hydrogen bond formation between KIT and imatinib [169, 170], mutations located in the second part of the kinase domain (exon 17 and 18) are also resistance to imatinib. Because the efficacy of imatinib depends on targeting the inactive conformation of KIT, it is believed that the KIT activation loop mutation, D816V (exon 17) that alters the conformational equilibrium of the kinase toward the active form in a rapid rate, offers a smaller population of the molecular target to imatinib. This manifests as abrogated efficacy of the inhibitor [171]. To achieve inhibition of KIT^{D816V}, the BCR/ABL and SRC family tyrosine kinase inhibitor, dasatinib can be employed. Unlike imatinib, dasatinib is believed to bind to the active conformation of KIT and effectively inhibits KIT^{D816V} [172].

Microphthalmia-associated transcription factor

The MITF/TFE family

MITF encodes for the basic helix-loop-helix leucine zipper (bHLHZip) protein. The mouse gene located on chromosome 6 was first cloned in 1993 by Hodgkinson et al. [173] and Hughes et al. [174]. One year later the human counterpart was identified by Tachibana et al. [175] and the gene was shown to be located on the human chromosome 3p12.3-14.1 [175, 176]. It was later discovered that the expression of MITF is restricted to certain cell types and its expression plays a major role in mast cells, cardiomyocytes, osteoclasts and melanocytes [173, 177-179]. As an active form the transcription factor acts either as a homo- or heterodimer. Heterodimerization occurs with the related TFE family of bHLHZip transcription factors, TFEB, TFEC and TFE3. Together with MITF this subfamily can bind to the symmetrical DNA enhancer sequence called the Ephrussi box (E-box) as 5'-CANNTG-3. More

importantly, MITF-TFE can recognize and bind to the regulatory element in the E-Box consisting of the DNA sequence CATGTG that is highly conserved in the promoters of the pigment synthesis enzyme genes Tyrosinase, and Tyrosinase-related proteins 1 and 2 [180]. Although the MITF-TFE subfamily regulates genes in the pigment production pathway, targeted disruption of TFEB in mice also showed defects in placental vascularization. However, this effect is restricted to TFEB since homozygotes carrying mutations at the TFE3, TFEC or MITF loci do not show signs of infertility or reduced viability [181].

MITF isoforms and splice forms

To date there are more than 10 isoforms of MITF identified in various tissues. The MITF splice forms A/B/C/D/E/H/J/M/MC/CX are expressed as the result of different promoter use, while this has not been confirmed for the MITF-Mdel splice forms [3, 182, 183]. In all, the 10 characterized distinct promoters that give rise to the specific isoforms also differs in their first exons. Each MITF isoform transcript encodes an isoform-specific variable exon 1. During transcriptional processing the initial exon is spliced into that later part of exon 1B (1B1b) together with exons 2-9. The latter encodes the essential motifs of MITF including the bHLHZip, transactivation domains and important sites for post translational modifications (Figure 8). However, exon 1B1b is excluded in the MITF-M transcript and the variable exon 1M of MITF-M is directly spliced onto exon 2-9 [3]. The exon 1B1b has been proposed to be important for cytosolic retention. Thus, the lack of exon 1B1b in the MITF-M isoform is believed to be one of the causes that explain its restricted nuclear localization in melanocytic cells [184]. In addition to these promoter regulated isoforms, a number of other isoforms lacking individual exons have also been identified [185]. However, the biological significance of these is yet to be uncovered.

The majority of the MITF isoforms are broadly expressed and only the M-, CX- and MC-isoforms are tissue restricted to melanocytes [186], cervical stromal cells [182] and mast cells [187], respectively. Although, these isoform are cell type specific, cells express a variety of MITF isoforms. Moreover, the MITF isoforms A, B, E, H and J are expressed in most cell types. Although

MITF-D is mainly expressed in cells important for bone tissue removal including osteoclasts, bone marrow-derived osteoclasts and osteoclast progenitors, there is no known osteoclast-specific isoform. In human osteoclasts, the A-, E-, H-, and B-isoforms are expressed whereas the A-, C-, E-, H-, D-, and B-isoforms are expressed in mouse osteoclasts [3]. Examination of the retinal pigment epithelium (RPE) cells that express enzymes involved in the pigment production pathway, showed expression of MITF-A, MITF-C, MITF-D, MITF-H and MITF-J [3, 188, 189]. Notably, despite being the melanocyte specific isoform, MITF-M in a recent study has been found to also be expressed in human RPE cells [190].

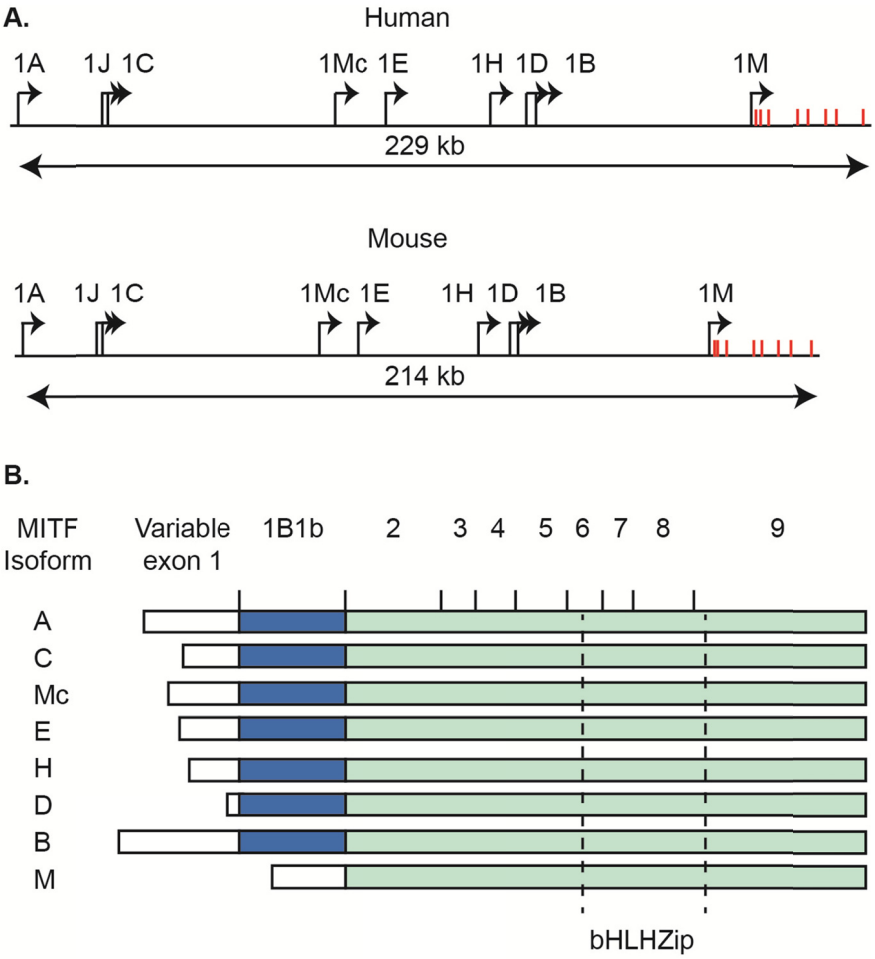

Figure 8. MITF genomic organization. A. Illustration of location of the nine 5' exons and their start and direction of transcription. Red denotes the common coding region (exon 2-9). **B.** the isoform specific exons (white) are spliced to exon 1B1b (blue) and then to the common coding region, exons 2-9. The variable region of 1M is directly spliced to exon 2-9 (green). Illustration is adapted from [3].

Diversification of the MITF protein is further supplemented by alternative splicing of exon 6a of the MITF transcript. The inclusion of 6a as the result of differential use of splice acceptor site produces the (+) isoform which includes the amino acid sequence ACIFPT (alanine-cysteine-isoleucine-phenylalanine-

proline-threonine) that is located upstream of the DNA binding basic region. Both products are expressed at similar level [173]. Importantly, these splice forms are distinct in two aspects: one, the MITF(+) has a higher binding affinity to the DNA E-box sequence [180] and two, the MITF(+) isoform exhibits a strong inhibitory effect on DNA synthesis in HEK293T cells [191]. However, the latter does not seem to be dependent on the E-box binding capacity. Evidence presented illustrates that the MITF(+)^{E213A} mutant that was unable to correctly interact with the E-box, was still capable of interfering with DNA synthesis as determined by bromodeoxyuridine (BrdU) incorporation. This suggests that direct DNA binding is not the mechanism through which DNA incorporation is facilitated [191]. In melanoma the ratio of MITF(+) to MITF(-) is regulated by the ERK-MAPK pathway. Specifically, the inhibition of the MAPK pathway excludes the 6a exon from the MITF-A/H/J/M isoforms [192]. Interestingly, although the expression ratio between the splice forms are dependent on MEK-1 and ERK-2, the mutational status of BRAF or NRAS in melanoma cells is not affecting this ratio. Consistent with the observation that MITF(-) favors proliferation in the HEK293T cells, it was found that MITF(-) is predominantly expressed in metastatic melanoma whereas MITF(+) is expressed in the surrounding epidermis [192].

MITF mutations

The *Mitf* gene has been found in many different species and determined to be highly conserved [193]. Numerous mutations have been identified in the gene and characterized in several vertebrate species. However, most of these mutations are studied in humans and mice. Mice are the best studied *in vivo* model system for *Mitf* mutations, and to date more than 25 *Mitf* mutations are known in mice whereas only 9 mutations have been found in humans [193-197]. Collectively, as MITF is the master regulator of melanocytes, all the mutations known thus far affect the function of melanocytes. Nearly all the homozygous and many of the heterozygous *Mitf* mutations produce diminished pigment in the coat and inner ear of mice. Moreover, the majority of these mutations also generate microphthalmia that is characterized by abnormal eye development, due to deficiencies in RPE cells. Some of the

mutations are also linked to hearing loss, abnormal mast cells and defective osteoclasts that could give rise to osteopetrosis associated with excess and brittle bone formation [193]. The most severe *Mitf* phenotype in mice is due to mutations in the basic domain that is important for DNA binding [198, 199]. Interestingly, the phenotypic severity of some *Mitf* mutants in heterozygotes does not necessarily produce the expected more severe phenotype in homozygous condition. For example, homozygous *Mitf* $^{Microphthalmia\text{-}white}$ (*Mitf* $^{mi\text{-}wh}$) mice are completely devoid of pigmentation and exhibit intermediate microphthalmia. Heterozygotes have grey coat color, white belly spot but normal eye development. However, *Mitf* ^{vga9}/*Mitf* ^{vga9} mice are completely white with severe microphthalmia whereas heterozygotes are perfectly normal (Figure 9). Interestingly, when these mice are crossed, creating *Mitf* $^{mi\text{-}wh}$/*Mitf* ^{vga9} mice, the mice are white but with normal eye development. This is termed interallelic complementation. [198, 199]. These inconsistencies are attributed to the dominant negative activities exerted by atypical DNA binding and the intact ability for interaction or dimerization with other proteins. The crystal structure of MITF that helped us explain the property of interallelic complementation unique to the *Mitf* $^{mi\text{-}wh}$ mutation is further elaborated in Article III. The *Mitf* $^{mi\text{-}spotted}$ (*Mitf* $^{mi\text{-}sp}$) mutation is another interesting mutation as it has no effects in either homo- or heterozygous condition except for reduced tyrosinase activity in the skin. A similarly mild allele is *Mitf* $^{mi\text{-}vitiligo}$ (*Mitf* $^{mi\text{-}vit}$) that affects melanocyte stem cells in the hair bulb niche. The loss of such cells results in gradual depigmentation of the coat over time [200].

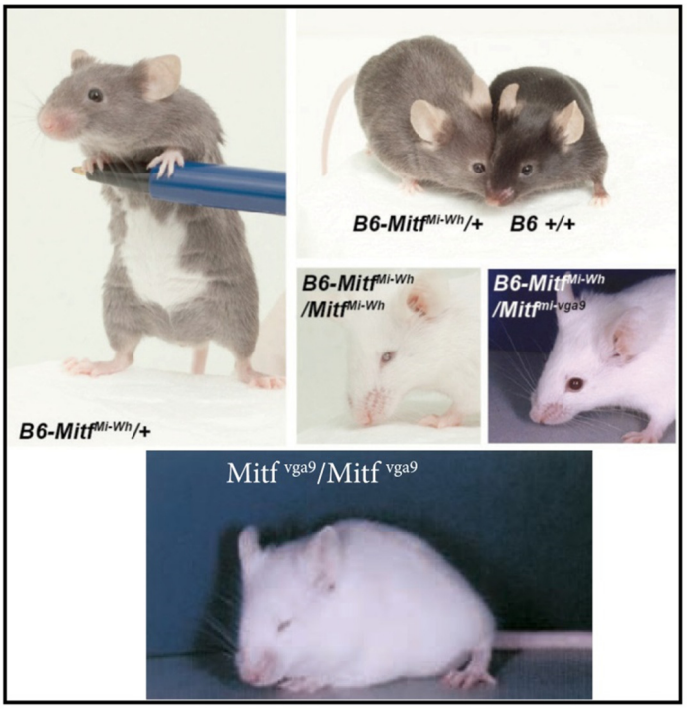

Figure 9. Interallelic complementation in Mitf $^{mi\text{-}wh}$ mice. Mice heterozygous for Mitf $^{mi\text{-}wh}$ have grey coat color, white belly spot and normal eye development and homozygous mice are completely white and have intermediate microphthalmia. In contrast, Mitf ^{vga9}/Mitf ^{vga9} mice are completely white with severe microphthalmia whereas heterozygotes are normal. Mitf $^{mi\text{-}wh}$/Mitf ^{vga9} mice are white but have normal eye development. Photographs are modified from [198, 201].

In humans, Tietz syndrome patients have been found to carry the MITF N210K-, DelR217- or R217I mutations located in the basic DNA binding region of MITF [202-205]. These MITF mutations in a heterozygotic condition are expected to homo- or heterodimerize, forming a transcription factor complex with diminished DNA binding capability that results in an overall dominant negative effect. Interestingly, the dominant negative effects of MITF produce the most severe phenotypes including deafness and albinism associated with the syndrome. Waardenburg Syndrome (WS) is another rare

genetic disorder related to Tietz syndrome and depending on the severity the clinical presentations are hypopigmentation, craniofacial deformities, deafness and microphthalmia. WS is divided into four subtypes (WS1-4) in which the core auditory and pigmentary features are shaped by haploinsufficiency of the MITF gene [194, 205]. More recently, it has been revealed that an MITF germline mutation is linked to the increased risk of the development of melanoma and renal cell carcinoma. The causative factor is the substitution mutation E318K that prevents a posttranslational modification, SUMOylation and thereby mildly hyperactivates MITF. Patients carrying the mutation had more than a fivefold increased risk of disease development [197, 206, 207]. To conclude, MITF mutations affects many different tissues with diverse biological functions that span from normal physiological development to tumorigenesis.

MITF phosphorylation and KIT-related signaling

As the one of the most potent proteins in melanocyte regulation, the expression and activity level of MITF is strictly controlled. MITF activity is tightly regulated by several post-translational modifications of which, serine phosphorylation by the MAPK signaling cascade is the best characterized. Initially, the functional interaction between MITF and the KIT receptor was predicted with the observation that mice heterozygous for both *Mitf* $^{mi\text{-}wh}$ and *Kit* $^{W\text{-}36H}$ exhibited much more extensive white spotting than heterozygous mice with a mutation in either of the genes alone [208-210]. Indeed, first reports highlighted the requirement of the KIT-ERK-MAPK pathway for Ser-73 and Ser-409 phosphorylation of MITF after KIT ligand stimulation. The activation of KIT triggers ERK2 to directly phosphorylate MITF at Ser-73 and mediates MITF Ser-409 phosphorylation via the Ser/Thr kinase p90RSK-1 [211, 212]. The MAPK-ERK signaling pathway increases MITF activity in luciferase reporter assay and the activity were observed to be phosphorylation-dependent as the introduction of constitutive active ERK mediated the activity of wild-type MITF but not the S73A or S409A MITF mutants. Moreover, phosphorylation of the serine sites also subjects MITF to polyubiquitination on Lys-201 which finally leads to protein degradation [211-213]. One of the

genes identified to be activated as a result of KIT-MITF signaling was the cell death regulator B-cell lymphoma 2 (BCL2) that implicates the survival role of KIT and MITF in the melanocyte lineage [213]. Even though the biochemical data suggest the importance of the phosphorylation sites Ser-73 and Ser-409 in melanocyte cell line, *in vivo* data supporting this notion is lacking. Rather, mice with white coat color and severe microphthalmia carrying the *Mitf*$^{mi\text{-}vga9}$ loss-of-function mutation were rescued with the bacterial artificial chromosome (BAC) containing *Mitf* mutation S73A, S409A or S73/409A [214]. The BAC *Mitf* mutants or BAC wild-type *Mitf* mice displayed normal dark coat color and did not show any degree of microphthalmia. Thus, the absence of results that link MITF phosphorylation to *in vivo* function, indicate that KIT signaling might affect additional phosphorylation sites of MITF [214]. For this reason, we decided to identify novel MITF phosphorylation sites using mass spectrometry and phospho-specific antibodies discussed in Article IV and V.

Although the transactivational activity of MITF is elevated by phosphorylation, it does not alter nuclear localization, DNA binding or dimerization. Conversely, KIT-mediated ERK activation facilitates selective recruitment of the co-activator histone acetyl transferase (HAT) p300/CBP which interacts with MITF to cooperatively enhance transactivation activity [215]. The p300/CBP enzyme is best known for its chromatin remodeling properties; however, in this case it is not known if the target is MITF itself or the chromatin of MITF target genes. Alternatively, p300/CBP might also act as a scaffolding protein that facilitates MITF activity by stabilizing the transcriptional machinery [193].

Located in a region C-terminal to the bHLHZip domain of MITF, the Ser-298 phosphorylation site was discovered to be mutated to a proline in WS2 patients [203]. Further, *in vitro* kinase assays established that the Glycogen synthase Ser/Thr kinase 3 (GSK3) directly phosphorylated MITF on Ser-298 and thereby considerably enhancing the ability of MITF to interact with the tyrosinase promoter E-box DNA element [196]. In melanoma cells, the replacement of Ser-298 with either the non-phosphorylatable residues proline or alanine impaired the ability of transactivation by more than half. The effect

does not seem to be due to protein stability, because in contrast to S73A and S409A, the S298A MITF mutant displayed expression and stability comparable with wild-type MITF [196]. Another phosphorylation site located close to Ser-298 is Ser-307 which to date is the only site that has been verified to be phosphorylated by site-specific antibodies. Signaling by the stress kinase p38 after Receptor activator of nuclear factor kappa-B ligand (RANKL) stimulation in osteoclasts leads to the phosphorylation of Ser-307. Significantly, the elevation of Ser-307 phosphorylation of MITF was observed to occur in parallel to the increase of MITF osteoclast target gene expression. This indicates that Ser-307 is important for osteoclast function. Although the study was limited to osteoclasts, the p38 stress pathway is also involved in melanocytes where it is activated by UV radiation, thus this site might also be of relevance in melanocytic cells [216].

Transcriptional regulation of MITF

In addition to the numerous post-translational modifications that modify the activity of MITF, expression regulation is another level of control to ensure proper function. The transcription factors known to directly regulate the level of MITF through promoter binding are CREB, SOX10, PAX3, and LEF1. As mentioned above, the binding of α-MSH to the seven-transmembrane MC1R protein leads to elevation of cAMP concentrations in melanocytes which switches on the bZip transcription factors CREB and ATF1. Activated CREB and ATF1 in turn bind to the CRE in the promoter of MITF, whereby transcription is initiated [217, 218]. As a consequence of MITF transcription the MITF downstream key proteins for melanin synthesis including Tyrosinase, Tyrosinase-related protein-1 and -2 (also known as Dopachrome tautomerase (DCT)) are elevated [219-221]. For the reason that the cAMP response pathway can be activated by numerous signaling cascades and is involved in many different cell types, the mechanism that keeps MITF-M expression limited to melanocytes is regulated by the SOX10 transcription factor. Specifically, it was found that ectopic expression of SOX10 in addition to cAMP signaling was required for the transactivation of CRE in the MITF promoter [222]. Although SOX10 and MITF mutations are found in different

subtypes of WS, the observation that mutations of SOX10 caused deafness and pigmentary abnormalities similar to MITF helped to identify SOX10 as a potential regulator of MITF. Later, SOX10 was shown to be essential for the development of melanocytes owing to its ability to directly regulate MITF-M via several consensus promoter and distal enhancer binding sites [223-226]. Evidence that SOX10 is a direct modifier of MITF expression was further strengthened by the use of a SOX10 dominant negative mutant found in human WS4. This mutant prominently reduced the transactivation of MITF promoter in melanoma cells [223].

The PAX3 transcription factor is mutated in the WS subtypes 1 and 3. Interestingly, symptoms such as pigmentary disorder and hearing loss of WS1 patients overlap with the MITF-related WS2 phenotype, which suggests a genetic connection between PAX3 and MITF. The epistatic link between PAX3 and MITF was confirmed by electrophoretic mobility shift assay that showed direct binding of PAX3 to the MITF promoter and luciferase assay, establishing that the transactivation of MITF promoter by PAX3 [227]. Moreover, PAX3 and MITF also share the downstream target gene Tyrosine related-protein-1 which further implicates the role of PAX3 in melanocyte development and function [228].

The WNT signaling pathway is implicated in the development of melanocytes. Several lines of observations support this view. First, knockout mice lacking the expression of the secreted paracrine acting factors WNT1 and WNT3a completely terminate that expression of Tyrosine-related protein (Trp) -2 in melanoblasts [229]. Second, overexpression of the ß-catenin transcription factor, a protein that is stabilized by WNT binding to the G protein-coupled Frizzled receptor, by mRNA injection into the premigratory neural crest cells of zebrafish promotes pigment cell formation at the expense of neurons and glia cells [230]. Third, WNT- signaling activation promotes the accumulation of ß-catenin that mediates synergistic transactivation of the early marker of melanoblast differentiation, Trp-1 promoter. Transactivation of the Trp-1 promoter is executed by the functional cooperation between ß-catenin, MITF and the Lymphoid enhancer-binding factor-1 (LEF-1). Importantly, LEF-1 also directly upregulates the expression of MITF by complexing with ß-

catenin or alternatively by the interaction between all three factors that directly transactivates the M promoter on MITF [231]. This mode of regulation establishes a feedback loop that enhances MITF expression and drives the development of melanoblasts.

MITF target genes

As the master regulator of melanocyte development, MITF is involved in essentially all aspects of melanocyte function. To date, more than 40 MITF direct target genes have been reported and verified [232, 233] and for simplification they are classified into 4 different groups.

Differentiation

Tanning response is a phenomenon in which melanocytes are triggered to produce and distribute melanin to the surrounding cells. This coordinated action requires regulation of numerous MITF-dependent genes (Figure 10). As mentioned, melanin is synthesized within specialized vesicular melanosomes. The formation of these organelles is facilitated by MITF that was identified in an electrophoretic shift assay to be bound to the promoters of the melanosome structure- and maturation-related genes SILVER and MLANA. [234]. Once the melanosomes are formed MITF transcribes the melanin producing enzymes Tyrosinase, Trp-1 and Trp-2 which catalyze the production of melanin in the melanosomes. However, in order for the desired ratio between eumelanin and pheomelanin to be produced, the pH within the melanosomes has to be critically controlled [235]. The ionic equilibrium in melanosomes is suggested to be modified by MITF through the membrane transporter Solute carrier (SLC) genes [233]. As soon as melanin is ready for distribution, MITF ensures melanosome trafficking and distribution by transcribing the RAB27A and DIAPH1 that allow melanosome interaction with the actin network and regulate the actin dynamics for the formation of melanocyte dendrites [236, 237]. Thus, many if not all essential aspects of melanocyte function and development are regulated by the transcription network of MITF.

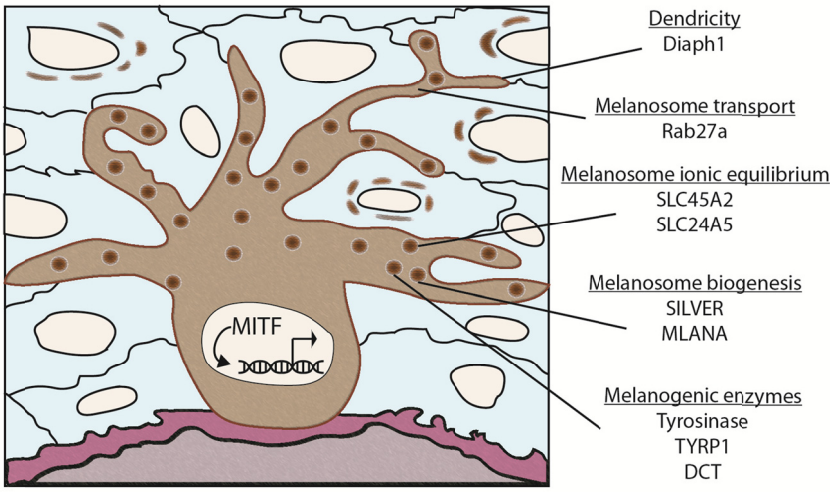

Figure 10. MITF target genes in melanocytes. MITF transcribes genes in melanocytes which are important for pigment production and transportation. Graphic is adapted from [4].

Survival

The presence of functional MITF proteins in a melanoblast during the early stages of development is critical for cell survival. The lack of *Mitf* expression in the *Mitf*$^{mi\text{-}vga9}$ / *Mitf*$^{mi\text{-}vga9}$ leads to microphthalmia, early onset of deafness and white coat color as the result of reduced melanocyte precursor proliferation and survival. As mentioned, the BCL2 gene was one of the first direct targets of MITF discovered to be important for the survival of the melanocyte lineage [213]. For example, in melanoma cells the introduction of dominant negative MITF substantially increases apoptosis. However, this process can be reversed by overexpression of BCL2, suggesting that MITF controls melanoma survival. *In vivo* studies also established the genetic interaction between MITF and BCL2. Deletion of BCL2 in mice did not affect embryonic development but visible phenotypes became evident after birth. BCL2$^{-/-}$ mice displayed massive premature loss of melanocytes shortly after birth while heterozygous mice did not demonstrate any signs of age-

dependent changes [213, 238, 239]. Moreover, genomic analysis of melanoma cell has identified MITF as an amplified lineage survival oncogene [240].

The transcription factor hypoxia inducible factor 1α (HIF1α) is stabilized during cellular oxygen deprivation and promotes genes engaged in angiogenesis. However, in melanoma it has been found to be constitutively active in normoxic conditions. The MITF target gene BCL2 prevents HIF1α degradation by involving the molecular chaperone Heat shock protein 90 (HSP90) and forming a trimeric complex consisting of BCL2, HSP90 and HIF1α [241]. Alternatively, MITF can also directly transactivate the HIF1α gene and thereby facilitate its activity [242]. In melanoma cells, HIF1α stimulates survival by interfering with the activity of caspases. In light of these observations it is tempting to speculate that the anti-apoptotic activities in melanoma cells via HIF1α are controlled by MITF via two different mechanisms. In addition to BCL2 and HIF1α, MITF also regulates the expression of melanoma oncogenes MET receptor tyrosine kinase and melanoma apoptosis inhibitor BIRC7. It has been shown that MITF directly binds to the promoters of these genes and the silencing of BIRC7 and MET reduces melanocytic cell viability and migration [243, 244]. In short, MITF controls a broad network of genes that is significantly required for melanoma cell survival.

Pro- and anti-proliferation

The acquisition of control over cell cycle proteins and overcoming senescence are hallmarks of melanoma growth. As noted, the cell cycle inhibitors and senescence mediators $p16^{INK4A}$ and $p14^{ARF}$ encoded by the CDKN2A and $p21^{Cip1}$ encoded by WAF1 are frequently mutated in melanoma cells. Also, the cell cycle mediator Cyclin D1 (CCDN1) gene is amplified in around 25 % of human melanomas [245]. All these genes are direct targets of MITF which has been found to positively mediate expression [246-248]. However, indirect regulation of the $p21^{Cip1}$ and $p19^{ARF}$ also encoded by the CDKN2A gene, is implicated by the T-Box transcription factor 2 (TBX2). This transcription factor TBX2 is overexpressed and plays an important role in maintaining proliferation and suppression of senescence in melanoma cells [249, 250].

Interestingly, TBX2 is the first characterized target gene of MITF that is not involved in melanin synthesis. Moreover, because TBX2-mediates control of E-cadherin expression it is suggested that MITF through TBX2 not only regulates melanoma growth but also plays a role during radial to vertical growth phase transition [251]. Another melanoma proliferation and invasion gene is the DIAPH1 gene which is engaged in the regulation of actin polymerization. The study performed by Carreira et al. [237] demonstrated that the depletion of MITF led to the downregulation of DIAPH1 and subsequently reorganization of the cytoskeleton via the actin network that favored melanoma invasion. In contrast, since DIAPH1 indirectly promoted the degradation of the cell cycle inhibitor $p27^{Kip1}$, depressed levels of MITF contributed to elevated $p27^{Kip1}$ which in turn put melanoma into cell cycle arrest [237].

In order to understand and describe the dual edged nature of MITF, a rheostat model was invented to reconcile the pro- and anti-proliferative roles [237]. This model describes MITF as a rheostat that dynamically fine-tunes melanoma behavior (Figure 11). Significantly, it predicts that at the lowest level of MITF activity melanoma cells are in a state of senescence and at an intermediate low level of MITF, cells possess a stem-cell like behavior but with a heightened invasive potential. Intermediate high levels of MITF generate proliferative cells. At the highest level of MITF activity, cells switch over to a differentiated pigment producing phenotype. Thus, according to this model, MITF does not simply act as an on/off switch but rather as a dynamic molecular gatekeeper that, depending on protein level and the addition of post-translational modifications, regulates the behavior of melanocyte/melanoma cells [252].

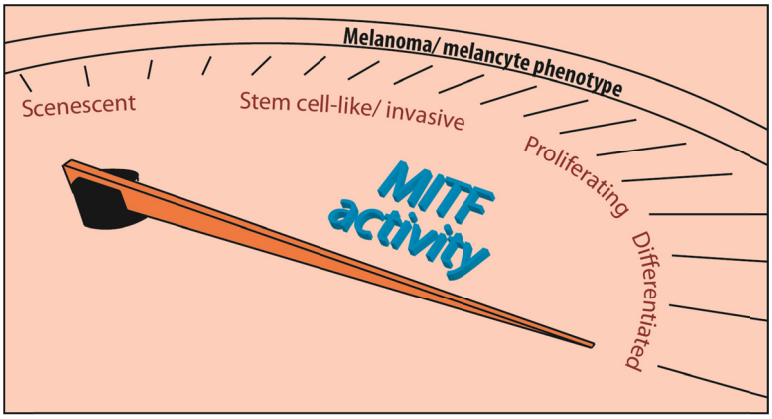

Figure 11. Molecular rheostat model of MITF activity. The activity level of MITF proteins regulated by multiple post-translational modifications, modifies melanocyte/melanoma cell behavior.

PRESENT INVESTIGATION

Summary and discussion

Article I.

Elucidation of functional differences between KIT splice forms

KIT activation is an essential component in normal physiological and oncogenic development of many different tissues [107]. Thus, delineation of mechanisms regulating receptor activation facilitates the finding of therapeutic intervention against disease in which KIT plays an important role. KIT activation by KIT ligand starts in the extracellular domain of the receptor which not only captures the ligand but also structurally stabilizes the ensuing homodimerization [100]. The extracellular domain is coupled to the transmembrane domain which in turn is joined to the kinase intracellular kinase interface [79]. Therefore, conformational changes in the extracellular region are believed to be relayed to the intracellular parts of the receptor. Illustration of this concept is demonstrated by the distinct phosphorylation pattern observed with receptor splice forms KIT(-) and KIT(+) [119]. The receptor splice forms are produced by alternative splicing of mRNA encoding KIT [114]. These protein isoforms are different in that KIT(+) possesses an amino acid tetrapeptide, GNNK, located in the juxtamembrane domain at the carboxyl-terminus of the extracellular domain. In contrast, the lack of tetrapeptide insert in KIT(-) generates increased receptor phosphorylation, ubiquitination, internalization and downstream signaling [119]. Although, the expression ratio of the splice forms are clinically relevant in diseases such as multiple myeloma [253], testicular germs cell tumors [254] and acute myeloid leukemia [255], it is unclear how the tetrapeptide insert modulated KIT. To clarify this, different KIT splice forms and mutants with different insert length were generated. In addition to the following isoforms and mutants: KIT(-), KIT(G), KIT(GN), KIT(GNN) and KIT(+), the KIT(AAAA) mutant with an alanine substituted tetrapeptide insert was also generated to identify whether the length or the specific sequence of the insert controls KIT activity. Transient

overexpression and stimulation of these receptors in mouse melanoma B16F0 cells showed that the increase of the insert length in a stepwise manner gradually reduced receptor tyrosine phosphorylation, downstream signaling, ubiquitination and receptor internalization. Likewise, the insert length also contributed to a measurable cellular output that suggested increasing insert length is associated with a corresponding sensitivity to melanoma drug treatment. Melanin extraction from B16F0 cells transfected with different KIT isoforms and insert mutants stimulated with KIT ligand, illustrated that pigment production was gradually improved by increasing KIT peptide insert length. The more rapid induction of differentiation or pigment production by longer insert length might explain the counteracting effect on survival/proliferation. Interestingly, the KIT(AAAA) mutant did not behave either like KIT(-) or KIT(+), but rather like KIT(G) or KIT(GN) in all of the mentioned experiments.

Based on previous observations by Bell et al. [256] on the rotation of the intracellular kinase interface of the related NEU receptor tyrosine kinase, we propose that the tetrapeptide insert of KIT modulates receptor activity in a similar fashion. The study showed that alteration of the alpha-helical structure of the receptor in the transmembrane domain by gradually increasing the length of the helix progressively shifted the orientation of the kinase interface [256]. Indeed, focus formation assays of cells transfected with the different transmembrane modified NEU mutants demonstrated that the increased length of the region was correlated to increasing foci detection to a point at which further length increments were negatively linked to foci formation. The latter was observed because rotation of the internal kinase domains until they face one another favors receptor activation. However, further lengthening of the transmembrane region of the receptor also additional rotates the kinase domains beyond optimal position of activation. In essence, the study suggests kinase regulation by structural means in the receptor transmembrane domain. In line with this, the tetrapeptide insert location in the extracellular juxtamembrane domain that is coupled to the kinase interface most likely modifies the kinase orientation of KIT. In absence of the tetrapeptide insert, the kinases of a KIT homodimer are facing one another and are consequently

in a favorable position for effective trans-phosphorylation. Thus, insertion of extra residues in this region turns the kinases away from each other and reduces trans-phosphorylation efficiency. Unlike, the NEU receptor, the tetrapeptide insert of KIT is not an alpha-helical structure. One full rotation in an alpha-helix would require 3.6 amino acids, thus the tetrapeptide GNNK would in that case be positioning the kinase interface similar to KIT receptor without the insert. However, since GNNK is predicted to be a coiled-coil structure, the tetrapeptide insert turns the kinase interfaces 180 °, hence turning them away from one another that lead to an unfavorable position for receptor activation. Unlike the GNNK tetrapeptide insert the AAAA insert consist of alanine that together are less bulky and therefore do not generate a mutant that behave like KIT(+). Instead the KIT(AAAA) mutant is similar to KIT(G) or KIT(GN), which suggests that these mutants have similar lengths. Finally, our results provide an explanation to the modulating function of the extracellular juxtamembrane domain of KIT receptor.

Article II.

Identification and characterization of MITF-activating and KIT ligand-dependent signaling pathways

The function of both MITF and KIT is essential for the development of melanocytes. It has been proposed that KIT-mediated activation of MITF regulates melanocyte differentiation and pigment production. Consequently, the loss of function mutation of *MITF* in mice results in phenotypes resembling mutations that disrupt the *KIT ligand* or the *KIT* gene. In these animals, melanocytes do not develop normally, they fail to migrate or differentiate [257-259]. To date, there are two mechanisms by which KIT is suggested to activate MITF. First, KIT ligand stimulation triggers ERK2 of the MAPK pathway to directly phosphorylate MITF at S73 that results in increased transcriptional activity and reduced protein half-life via ubiquitination and subsequent degradation of MITF [211]. Second, KIT ligand-dependent activation of ERK engages the serine/threonine kinase p90RSK-1 to phosphorylate MITF at S409 which further enhances transactivation activity. Supporting this notion was the observation that MITF

transactivational activity of the tyrosinase promoter was severely reduced by single or double mutation of S73A or S409A [212]. However, these signaling events are not evident in *in vivo* models using knock-in and BAC transgene rescue strategies to study the function of melanocyte with respect to mice coat color development. Mice with S73A, S409A or S73/409A MITF expressing melanocyte displayed a coat color phenotype similar to wild-type MITF expressing mice [214]. Thus, additional mechanisms for regulating MITF function are likely present and a mapping effort is needed to better understand the role of MITF in melanocytes.

In Western blot (WB) the MITF protein is observed as a double protein band. Upon KIT ligand stimulation the lower band undergoes a mobility shift, and the signal ratio between the two bands becomes dominated by the higher molecular weight upper band. However, the MITF mutants S73A and S409A only resolve as a single lower protein band. Thus, this distinctive shift is believed to be caused by phosphorylation of either S73 or S409. We employed the mobility shift strategy to pinpoint the specific tyrosine phosphorylation residues of KIT required to activate MITF. Using HEK293T cells and the mouse melan-a melanocytes, in Article II we showed that the KIT mutants Y568F and Y721F that are defective in SRC-RAS-RAF-MEK-ERK and p85-p110-AKT signaling, respectively, did not induce MITF mobility shift. This was further verified with inhibitors against SRC, MEK, PI3K and AKT. In addition, inhibition of the p38 stress MAPK pathway in the melan-a and HEK293T cells demonstrated that p38 is needed for MITF activation in a KIT ligand-dependent manner. The mitogenic response of the signaling events was also measured in HEK293T cells, in which co-transfection with the Y568F or Y721F KIT mutants, together with MITF resulted in lower proliferation upon KIT ligand stimulation compared to samples transfected with wild-type KIT and MITF. This indicates that the KIT ligand-dependent PI3K-AKT and SRC-MAPK signaling pathways that mediate MITF activation are important for HEK293T cell proliferation.

Overall, our article identified several signaling proteins and pathways that participate in the MITF mobility shift. The observation that the inhibition of one specific downstream protein of KIT was sufficient to completely terminate

the mobility shift, suggests the participation of all the studied KIT pathways are required for such alteration. However, as we only studied protein mobility shift which indicates change of phosphorylation, it does not provide direct measurement of MITF activity. Thus, it would be of interest to determine how these signaling pathways individually affect MITF activity, in for instance, a luciferase assay. However, the identification of the additional signaling partners downstream of KIT that engage MITF signaling is important for recognizing the complex pathways that enhance MITF phosphorylation. Moreover, our study also links the proliferative effects of specific KIT signaling cascades via MITF and establishes a biological effect that further progresses our understanding of KIT and MITF interaction at the cellular level.

Article III.

MITF crystal structure determination

MITF is one of the most important regulators of melanocyte development, survival and differentiation. In addition, MITF is also needed for the normal development and function of osteoclasts, mast cells, retinal pigment epithelium and stem cells of the hair bulge [193]. To regulate genes, MITF either act as a homodimer or as a heterodimer with the related TFE family of bHLHZip transcription factors, TFEB, TFEC and TFE3. Despite common recognition of the E-box sequence by the basic residues in their DNA-binding regions, MITF does not form heterodimers with the other bHLHZip transcription factors, MYC, MAX or USF [180]. Unlike transcription factors in the bHLHZip family MITF also binds to the distal enhancer element M-box [260]. To elucidate the restricted heterodimerization ability as well as the unique recognition of the M-box, we determined the crystal structure of MITF.

Article III shows that the dimeric apo structure of MITF consists of two interacting protomers forming a four-helical bundle. This leucine zipper assembly facilitates protein dimerization. Distinct from the other bHLHZip transcription factors, dimerized MITF is not symmetric and contains a kink that spans a length of 3 amino acids in one of the protomers. The discovery of such out-of-register structure helped to explain the selective dimerization

towards TFEB, TFEC and TFE3 but not to MYC, MAX or USF. Strategically deleting the three residues generated the MITF mutant, Δ260-262 that possessed a leucine zipper in the same register as MAX. As predicted, this mutant was observed in electrophoretic mobility assays to assemble with MAX. Moreover, the structure of MITF in complex with E-box and M-box DNA elements revealed critical residues in DNA binding recognition. His-209 was determined to aid in the overall binding of both M- and E-Box, whereas Ile-212 regulates the binding specificity towards M-box. Indeed, the H209R MITF mutant showed reduced M- and E-box binding affinity as well as increased non-specific DNA binding, whereas the I212N MITF mutant displayed decreased M-box binding, enhanced E-box binding and a slight increase in non-specific DNA binding.

Article III not only explains the structural basis of MITF that enables the selective dimerization with TFEB, TFEC and TFE3 and the M-box binding specificity mediated by Ile-212, but also the *Mitf*$^{Mi\text{-}Wh}$ (I212N) mouse phenotype. Mice homozygotes for this mutation are white with mild or intermediate microphthalmia that is a condition characterized by abnormally small eyes. Heterozygous *Mitf*$^{Mi\text{-}Wh}$ mice have large white belly spot and diluted coat color which are the most severe heterozygous phenotype of all known *Mitf* mutations. Strikingly, *Mitf*$^{Mi\text{-}Wh}$ mice crossed with the loss-of-expression *Mitf*^{vga9} mutations exhibit interallelic complementation. Homozygous Mitf^{vga9} mice displays severe microphthalmia, but *Mitf*$^{Mi\text{-}Wh/vga9}$ mice have normal eye development. As the result of reducing the *Mitf*$^{Mi\text{-}Wh}$ (I212N) concentration by half the effect of the mutation on microphthalmia is rescued. This phenotype can be explained by our structural data suggesting that interallelic complementation by these two mutations is simply the result of decreased non-specific DNA binding caused by *Mitf*$^{Mi\text{-}Wh}$ alone. Thus, reducing the dose of *Mitf*$^{Mi\text{-}Wh}$ also mitigates the destructive effects of non-specific DNA binding. In conclusion, the crystal structure of MITF helped to explain the dominant negative effects of MITF mutants and its restricted dimerization capacity towards related transcription factors.

Article IV.

Identification of KIT^{D816V}-mediated MITF tyrosine phosphorylation and gene regulation

Signaling between KIT and MITF is well established to be important for melanocyte function [209]. However, how MITF is regulated by constitutively active KIT that harbors oncogenic mutations is not well understood. Recently the D816V mutation of KIT, located in the second part of the kinase domain, has been found to control the expression of MITF through miRNAs. More specifically, the forced expression of KIT^{D816V} in mast cells suppressed the expression of miR-381 and miR-539 and thereby robustly increased the levels of MITF proteins [261]. These observations together with the fact that wild-type KIT mediates phosphorylation of MITF [211, 212], suggest a causal link between oncogenic KIT and MITF. In Article IV, we sought to identify and characterize phosphorylation sites of MITF induced by KIT^{D816V}.

Using mass spectrometry we found three tyrosine phosphorylation sites located in the N-terminus of MITF to be phosphorylated in the presence of KIT^{D816V}, namely Y22, Y35 and Y90. We made phosphorylation site-specific antibodies and verified that these tyrosine sites are phosphorylated *in vitro*. Furthermore, using general tyrosine phospho-specific antibodies we also determined that MITF tyrosine phosphorylation was completely absent when co-transfected with wild-type KIT in the presence of KIT ligand. Because phosphorylation of MITF could alter subcellular localization of the protein [262] we also studied the effects of tyrosine phosphorylation on nuclear localization. Interestingly, KIT^{D816V} but not wild-type KIT mediated cytosolic retention of MITF. This was tyrosine phosphorylation-dependent as the Y22/35/90Y MITF mutant was localized to the nucleus despite being co-expressed with oncogenic KIT. Analysis of our confocal microscopy images of subcellular localization also showed elevated co-localization between KIT^{D816V} and MITF which prompted us to investigate the possibility of direct protein interactions. Immunoprecipitation of KIT in B16F0 cells co-expressed with MITF and KIT pulled down MITF. Accordingly, MITF was only co-immunoprecipitated with oncogenic KIT but not wild-type KIT irrespective of KIT ligand stimulation. However, the specific sites of MITF that directly

interact with KIT receptor are yet to be determined. In addition to the effects on subcellular localization and direct interaction, we also observed differential regulation of MITF-dependent target genes by wild-type KIT and KIT^{D816V}. For the qPCR gene array experiments we chose to analyze the expression of genes verified to be direct targets of MITF and are involved in melanocyte/melanoma activities such as proliferation, survival, angiogenesis, cell cycle initiation, cell cycle inhibition differentiation and tumor suppression. The treatment of siRNA against MITF (siMITF) in the mouse melanoma B16F0 cells resulted in decreased expression all genes (*p35*, *TBX2*, *BCL2*, *Sox10*, *Cdk2*, *Hif1a*, *Diap1*, *Cdkn1a*, *Dct*, *Cdkn2a*, *Tyr*, *Trp1*, *Mlana*, *Runx3* and *S100a*) assayed in the study, confirming that these genes are in fact targets of MITF. Stimulation of endogenous mouse KIT by the addition of mouse KIT ligand selectively increased the expression of genes engaged in differentiation, tumor suppression and cell cycle inhibition. In contrast, cells stably transfected with KIT^{D816V} displayed a gene expression pattern in which genes important for melanocyte/melanoma proliferation, survival, angiogenesis and cell cycle initiation were upregulated and genes involved in differentiation, tumor suppression and cell cycle inhibition were suppressed. To determine the role of the tyrosine phosphorylation sites of MITF in gene regulation imparted by KIT^{D816V}, we transiently transfected B16F0 cells with either wild-type MITF or Y22/35/90F MITF triple mutant together with KIT^{D816V}. As expected, samples co-transfected with KIT^{D816V} and wild-type MITF displayed elevated expression of genes involved in proliferation, survival, angiogenesis and cell cycle initiation whereas genes involved in differentiation, tumor suppression and cell cycle inhibition did not significantly increase. Notably, this pattern of expression was severely diminished in the sample in which MITF triple mutant and KIT^{D816V} were co-expressed, indicating that intact tyrosine phosphorylation sites of MITF are required for the specific gene regulation induced by KIT^{D816V}.

In this study we are the first to report cytosolic localization of the melanocyte specific MITF-M isoform. The expression of MITF-M, as mentioned in the background of this thesis, is restricted to melanocytes (recently, also in RPE) and its subcellular localization has so far been found to

be limited to the nucleus [184, 190]. However, mutated MITF-M has been demonstrated to localize in the nucleus. A WS2-associated mutation that terminates part of the nuclear localization signal of MITF protein is so far the only reported study of MITF-M cytosolic localization in melanocytic cell lines. The specific mutation terminates part of the nuclear localization signal of MITF and was identified as the causative factor [263]. Another region of MITF thought to be important for cytosolic localization is the 1B1b exon (Figure 8) which, in contrast to other MITF isoforms, is missing from the MITF-M isoform [3]. For example, MITF expressed in monocytes, cells closely related to osteoclasts, is primarily located in the cytosol and only upon the addition of osteoblast factors CSF-1 and RANKL, is MITF translocated to the nucleus. In this case, the 14-3-3 interacting protein directly binds to MITF in the absence of CSF-1 and RANKL and keeps MITF in the cytosol [264]. However, upon differentiation CSF-1 and RANKL inactivate the interaction of MITF with C-TAK1 that is important for the MITF/14-3-3 complex formation and consequently facilitates nuclear entry of MITF [262]. Similar to our study, the cytosolic localization of MITF is phosphorylation-dependent. Ser-173 is predicted to be phosphorylated by C-TAK1 that enables MITF interaction with 14-3-3. Thus, unlike tyrosine phosphorylation, the mutation of this site keeps MITF in the nucleus. The 14-3-3 protein may potentially be interacting with tyrosine phosphorylated MITF to keep the transcription factor in the cytosol. However, to date, consensus 14-3-3 binding motifs only consist of sequences that are either threonine or serine phosphorylated [265].

As mentioned above, the oncogenic KIT^{D816V} mutation is unlike the wild-type receptor, extensively modified with the addition of high-mannose-type N-glycosylation which contributes to its intracellular expression pattern [139]. This type of post-translational modification allows KIT^{D816V} to be aberrantly localized to the Golgi-network where oncogenic activities are taking place. For instance, the unique ability of KIT^{D816V} to activate the transcription factors STAT-1 and STAT-5 is believed to be critical for mast cell accumulation typical of indolent systemic mastocytosis [266]. In accordance with this model, we believe that the intracellular expression pattern of KIT^{D816V} is essential for tyrosine phosphorylation of MITF. Supporting this notion, we observed that

KIT^{D816V} was indeed more diffusely expressed in the cells which indicates intracellular localization. More importantly, our finding that KIT^{D816V} co-immunoprecipitated with MITF further supports this hypothesis.

Not only is the localization of MITF controlled by KIT^{D816V} signaling but also the regulation of downstream target genes. In the presence of KIT^{D816V}, MITF was observed to stay both in the nucleus and in the cytoplasm. At a first glance, the fraction of MITF in the nucleus might not seem to be affected by KIT^{D816V} signaling. However, co-transfecting cells with both KIT^{D816V} and Y22/35/90F triple MITF mutant displayed a gene expression pattern that was severely reduced compared to samples transfected with wild-type MITF and KIT^{D816V}. Based on the notion that gene regulation by MITF occurs in the nucleus and that our experiments show differential regulation by MITF triple phospho-mutant, we propose that nuclear MITF in the presence of KIT^{D816V} is affected by receptor signaling. One possible explanation for the observation could be that tyrosine phosphorylation not only retains MITF in the cytosol but also changes the activity of MITF so that only a certain set of genes are transcribed.

In summary, our study reveals oncogenic gene regulation by KIT^{D816V} through MITF tyrosine phosphorylation and potentially also via changed subcellular localization. However, more studies are required to establish the melanoma cellular phenotype generated by the interaction between KIT^{D816V} and MITF.

Article V.

Biochemical characterization of MITF serine phosphorylation sites- with focus on Ser-73 and Ser-409

Serine phosphorylation is one of the best characterized post-translational modifications of MITF. It was early recognized that the activation of KIT contributed to the phosphorylation of Ser-73 and Ser-409 of MITF by ERK2 and p90RSK of the MAPK pathway [211, 212]. Interestingly, as observed in Article II of this thesis, the phosphorylation of Ser-73 and Ser-409 is believed to initiate electrophoretic mobility shift of the MITF protein. Significantly, the addition of KIT ligand causes the double protein bands of MITF as visualized

on a Western blot, to shift towards the upper band. However, the lack of convincing phosphatase treatment of MITF together with the lack of phospho-specific antibodies to support this conclusion, calls for further investigations [211].

Located in the bHLHZip domain of MITF Ser-298 is phosphorylated by the Glycogen synthase kinase 3 *in vitro*. Similar to the Ser-73 and Ser-409 phosphorylation sites, Ser-298, when mutated to alanine, also negatively affects the transcriptional activity of MITF that was demonstrated to be caused by MITF binding to DNA [196]. However, recently, the WS2A and Tietz syndrome associated S298P mutation was shown to have equal DNA binding capability as wild-type MITF in addition to increased transactivation potential [267]. Thus, further studies are needed to determine the role of Ser-298. To date, the only phosphorylation site verified by phospho-specific antibodies is the Ser-307 phosphorylation site of MITF. In contrast to the other phosphorylation sites that were discovered to be biochemically important for melanocytes, Ser-307 was found to be phosphorylated in RANKL stimulated primary osteoclasts. This specific phosphorylation is critical for the regulation of genes important for osteoclast differentiation [216].

In Article V, using mass spectrometry we found Ser-100, Ser-307, Ser-384 and Ser-397 to be phosphorylated *in vitro*. In addition, we generated phospho-specific antibodies and showed that Ser-73, Ser-307, Ser-384, Ser-387 and Ser-409 were phosphorylated in melanoma cells. In line with previous predictions, our antibodies revealed that Ser-73 phosphorylation of MITF only occurs in the upper protein band. Moreover, Ser-73 phosphorylation was completely absent in the S409A MITF mutant which suggests the need of Ser-409 phosphorylation to obtain Ser-73 phosphorylation. Unlike, Ser-73 phosphorylation, Ser-409 phosphorylation of MITF was recognized in both the lower and upper protein bands. Similarly, phospho-specific antibodies against Ser-307, Ser-384 and Ser-397 also detected phosphorylation in both bands of MITF. Also unlike Ser-73 phosphorylation, these sites did not demonstrate dependency of other phosphorylation sites including, Ser-73, Ser-100, Ser-298, Ser-307, Ser-384, Ser-397 or Ser-409 of MITF.

To determine the cause of a second protein band and to characterize how an electrophoretic mobility shift occurs in the MITF protein upon KIT ligand treatment, we subjected immunoprecipitated MITF proteins to phosphatase treatment. We observed that the upper protein band of MITF disappeared after dephosphorylation. More importantly, the lower protein band that was present after the treatment migrated faster than the lower band of wild-type, S73A and S409A forms of MITF. This suggests that the migration of the lower protein band of wild-type, S73A and S409A forms of MITF in an SDS-PAGE is also influenced by phosphorylation and that the upper band of MITF is only visible in the presence of Ser-73 phosphorylation.

The transactivational activity of the different MITF serine phospho-mutants including S73A, S100A, S298A, S307A, S384, S397A, S409A, S73/409A and S73/307/409A was measured in a luciferase reporter assay. The results showed significantly higher activity of all the mutants in the HEK293T cells compared to wild-type with the exception of the S307A MITF mutant. This can be explained with the decreased expression level of this particular mutant. In the B16F0 mouse melanoma cell line that endogenously expresses MITF, the activity of all mutants was elevated except S298A and S409A. The different results between the cell lines are most likely attributed to the endogenous expression of MITF in the melanoma cells.

As our transactivation assays data conflict with previous results that showed reduced transcription activation potential of the S73A, S298A and S409A MITF mutants, we also re-analyzed the importance of ERK signaling for the phosphorylation of MITF Ser-73 and Ser-409. To do this, MITF was overexpressed in the human melanoma 501 mel cells that harbors the $BRAF^{V600E}$ mutation. A BRAF specific inhibitor, RAF-265, was used to block the ERK signaling pathway. Phosphorylation of Ser-73 and Ser-409 was still detected after 3 hours of incubation of the inhibitor. Indeed, the phosphorylation of these sites was comparable to the untreated samples which indicates that the ERK-MAPK pathway might not be involved in the phosphorylation of Ser-73 or Ser-409.

In summary, the generation of phospho-specific antibodies against MITF allowed us to determine that Ser-73, Ser-307, Ser-384, Ser-397 and Ser-409

are phosphorylated *in vitro*. More importantly, the antibodies also showed that these phosphorylation sites are constitutively phosphorylated without additional stimulation. Our results are in stark contrast to previously published data in that we showed constitutive MITF phosphorylation, MITF phosphorylation independent of the ERK pathway and phospho-mutants to have elevated transcription activity. These discrepancies can be explained by the fact that previous studies lacked phospho-specific antibodies against MITF. Although, earlier reports correctly identified the Ser-73 phosphorylation of MITF as being responsible for the upper protein band, concluding that Ser-73 phosphorylation is dependent on the ERK-MAPK pathway because inhibition of this pathway blocked the mobility shift of MITF protein, seems flawed. The differences between our transactivation data and previous results can be explained by several lines of observation we have made. For instance, neither Wu et al. [212] nor Takeda et al. [196] described the accuracy of their data. Statistical analysis, definition of error bars and the number of repetition of the luciferase assays were completely left out. More striking, is the protein migration pattern of the S73/409A MITF double mutant that showed the most significant reduction in transactivation assays performed by Wu et al. [212]. In agreement with our study, this mutant was seen as a single protein band in an SDS-PAGE. However, the protein of this double mutant migrated approximately 20 kDa below wild type MITF, which might suggest inadvertent protein truncation introduced by mutagenesis. Another line of inconsistency is also revealed by our observation that Ser-73 phosphorylation of MITF is dependent on Ser-409 phosphorylation. In other words, the S409A mutation which lacks Ser-73 phosphorylation should, in a transactivation assay, behave like the S73/409A double mutant. It is, however, also important to mention these discrepancies might be caused by different cell lines used for the assay. We used the B16F0 melanoma and HEK293T cell lines whereas Wu et al. used the NIH 3T3 fibroblasts to test S73A, S409A and S73/409A and Takeda et al. employed the NIH 3T3 cells and CV 1 monkey kidney cells to evaluate the activity of S298A and S298P mutants of MITF.

Collectively, our data strongly suggest the need of revising the current dogma of MITF phosphorylation. However, more work is needed to identify

the kinases involved in the phosphorylation of MITF and to establish the functional significance of these phosphorylation sites in melanocytes and melanoma.

Materials and methods

Cell culture conditions

The HEK293T, B16F0, 501 mel, melan-a and A375 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium and supplemented with 10 % fetal bovine serum and 100 units/ml penicillin streptomycin. TPA (200nM, Sigma-Aldrich) was also added to the culture media of the melan-a cells (Wellcome Trust Functional Genomics Cell Bank). The BaF3 pro-B (ATCC) were kept in RPMI-1640 medium with 100 units/ml penicillin-streptomycin, 10 % heat inactivated fetal bovine serum and 10 ng/ml of IL-3. All cell lines were maintained in cell culture incubator at 37 °C with an atmospheric content of 95 % ambient air and 5 % CO_2.

Transient and stable transfection of cells

TurboFect (Fermentas) transfection reagent was used in the ratio of 1 µg of DNA to 1.4 µl of reagent. Transfection reagent and DNA were mixed and incubated for 20 minutes at room temperature before adding to cells. Cells were incubated for 5 hours with the transfection complex.

JetPEI (Polyplus) transfection reagent was used in the ratio of 1 µg of DNA to 3 µl of reagent. DNA and reagent were mixed and incubated (15 minutes) at room temperature and added to suspended cells (reverse transfection). Cells were then incubated for 15-24 hours with the transfection complex. The pcDNA 3.1 vector was used for stable transfection. Transfected (JetPEI) B16F0 cells were selected with neomycin (1 mg/ml).

MITF phosphatase treatment

MITF proteins were overexpressed in the HEK293T cells and isolated with immunoprecipitation after overnight serum-deprivation. Proteins immobilized on protein G beads were washed three times with reaction buffer (10 mM, pH 7.5, Tris-HCl, 37 °C, 10 µM $MgCl_2$ and 0.1 mg/ml BSA) (Thermo Scientific). Dephosphorylation reaction was initiated using 10 units of calf intestine alkaline phosphatase (Thermo Scientific) and 4 units of shrimp alkaline phosphatase (Thermo Scientific) added immobilized MITF proteins suspended in reaction buffer. The reaction was allowed to occur at 37 °C for 40 minutes with light vortexing. In control samples phosphatase inhibitors (50 mM of β-

glycerophosphate and 50 μM of sodium orthovanadate) were added prior to phosphatase treatment.

Inhibitor treatment

The following inhibitors were used in the study: 10 μM (30 minutes) of SU6656 (CalBiochem), U0126 (Promega), LY294002 (Sigma-Aldrich) and 3 μM (30 minutes) of AKT IV (CalBiochem), 10 μM (1 hour) of dasatinib (Shanghai Yingxuan) and 5 μM (3 hours) of RAF265/CHIR-265 (Novartis). Incubation occurred in cell culture medium at 37 °C in cell culture incubator.

Conservation of MITF tyrosine phosphorylation sites

Multiple sequence alignment of MITF sequences from H. sapiens, M. musculus, B. taurus, E. caballus, C. L. familaries, D. rerio, D. melanogaster and X. laevis (obtained from NCBI) was performed using Clustal Omega.

Luciferase assay analysis

Transfection (1 ng of pRL Renilla luciferase reporter vector (Promega), 10 ng of mouse MITF-M either wild-type or mutated, 100 ng of tyrosinase reporter construct and 30 ng of KIT) was carried out in 48-well plate format. Incubation was allowed for 24 hours and cells were then serum starved prior to lysis and measurement in a luminometer (Wallac Victor 2 1420 Multilabel).

Site directed mutagenesis

Mutagenesis was performed with the QuikChange mutagenesis (Stratagene) kit. The following primer sequences were used to obtain the desired mutations:

(MITF S73A)

FWD-5'AGCGCACCCAACGCCCCTATGGCTATGCTCACTCT-3'

REV-5'AGAGTGAGCATAGCCATAGGGGCGTTGGGTGCGCT-3'

(MITF S100A)

FWD- 5'GCAGGGCAGAGGCTGAGTGCCCAGG3'

REV- 5'CCTGGGCACTCAGCCTCTGCCCTGC3'

(MITF S298A)

FWD- 5'CGCATGGACTTGCCCTTATCCCATCC3'

REV- 5'GGATGGGATAAGGGCAAGTCCATGCG3'

(MITF S307A)

FWD- 5'CCGGTCTCTGCGCGCCTGATCTGGTGAATC3'

REV- 5' GATTCACCAGATCAGGCGCGCAGAGACCGG 3'

(MITF S384A)
FWD-5'CTGATGGACGATGCCCTCGCACCTGTTGGAGTCAC3'
REV-5'GTGACTCCAACAGGTGCGAGGGCATCGTCCATCAG3'
(MITF S397A)
FWD- 5'CTGTCATCAGTGGCGCCAGGAGCTTCAAAAAC3'
REV- 5'GTTTTTGAAGCTCCTGGCGCCACTGATGACAG3'
(MITF Y22F)
FWD- 5'CCCCACCAAGTTCCACATACAGCAAGC3'
REV- 5'GCTTGCTGTATGTGGAACTTGGTGGGG3'
(MITF Y35F)
FWD- 5'GCACCAGGTAAAGCAGTTCCTTTCTACCAC3'
REV- 5' GTGGTAGAAAGGAACTGCTTTACCTGGTGC 3'
(MITF Y90F)
FWD-5'
CTGTGAAAAAGAGGCATTTTTTAAGTTTGAGGAGCAGAGC 3'
REV-5' GCTCTGCTCCTCAAACTTAAAAAATGCCTCTTTTTCACAG 3'

siRNA transfection

Predesigned siRNA primers (Mm_Mitf_7 FlexiTube siRNA) against mouse MITF and negative control siRNA (AllStars Negative Control) were obtained from Qiagen. Gene silencing was carried out using Lipofectamine 2000 (Invitrogen). B16F0 cells seeded in 60 mm well plates were incubated with reaction mixture of 30 µl of Lipofectamine 2000 dispensed in 570 µl Optimem (Invitrogen) that was mixed with 24.9 µl of siRNA (Qiagen stock concentration) in 575 µl Optimem. The gene silencing reaction was allowed to occur for 48 hours in a cell culture incubator. Cells were then serum-starved overnight prior to RNA extraction.

RNA extraction and cDNA synthesis

RNeasy kit (Qiagen) was used for RNA extraction and genomic DNA removal. The total of 1 µg of RNA was used for the cDNA synthesis reaction. The synthesis was carried out using the RevertAid First strand cDNA synthesis KIT (Thermo Scientific).

Quantitative reverse transcription PCR

SYBR green master mix (Thermo Scientific) was used for the reaction in an Applied Biosystems 7500 Real-Time PCR System. The housekeeping genes, glucuronidase beta (GUSB), hypoxanthine phosphoribosyltransferase (HPRT) and 18S ribosomal RNA (18SrRNA) were selected as normalizers for qPCR analysis and their corresponding primers were predesigned by Qiagen. The following predesigned primers were obtained from Qiagen: P35, TBX2, BCL2, SOX10, CDK2, HIF1a, DIAPH1, CDKN1a, DCT, CDKN2a, TYR, TRP1, MLANA, RUNX3 and S100A.

Heatmap construction

Heatmap of qPCR data was created using "heatmap.2" function in "gplots" package of "R". Further hierarchical clustering was obtained using the complete linkage method which sets the cluster distance between two clusters into the maximum distance between their individual values. In the clustering process, nearest two clusters were merged into a new cluster in every cycle and the process was repeated until the whole data set clustered into one single cluster.

Immunoblotting and immunoprecipitation

Cells were lysed in ice cold RIPA buffer for 15 minutes and cell debris was removed by centrifugation. Immunoprecipitation was performed using 1 µg of antibodies in 1 ml of lysate. Binding reaction was performed at 4 °C for 1.5 hours and 20 µl of protein G sepharose beads (GE Healthcare) were added and further incubated (4 °C, 30 minutes) to pull-down antigen/antibody complex. Immunoprecipitates were then washed three times in RIPA buffer before denaturing through boiling (98 °C, 5 minutes) in loading buffer. Proteins were subsequently separated in SDS-PAGE. Transfer of protein was performed in iBlot 7-Minute Blotting System (Life Technologies). Non-fat dry milk (4 %) in water was used to block (room temperature, 30 minutes) the membrane (either PVDF or nitrocellulose). After incubation with primary antibodies (either overnight at 4 °C or 1 hour at room temperature), excessive antibodies were removed by washing with PBS-T (0.05 % TWEEN 20). Secondary antibodies (either HRP coupled or IRDye (Li-Cor), 0.5 µl of stock concentrated antibodies/50 ml PBS-T) were added to the membrane

(incubation 1 hour at room temperature). Either a chemiluminescence imaging system (Bio-rad and Fujitsu) or a Li-Cor Odyssey scanner was used to develop the membrane.

Mitf mobility shift quantitation

Subsequent to protein signal quantitation by ImageJ (NIH), the value of the upper protein band was subtracted from the value of the lower protein band of MITF. The resulting positive value indicates protein mobility shift that is observed after KIT ligand (Prospec or Orf Genetics) stimulation (100 ng/ml, 15 minutes). A resulting negative value suggests the lack of mobility shift.

Statistical analysis

One way ANOVA statistical (Graphpad) test was performed on results from qPCR experiments, luciferase assays, proliferation assays and survival assays. Significant results ($P \leq 0.05$) were further evaluated by an appropriate post-hoc test (Graphpad). Output results were indicated by n.s (not significant), * ($P \leq 0.05$), ** ($P \leq 0.01$) or *** ($P \leq 0.001$).

Two tailed non-parametric sign test was performed on c-KIT phosphorylation, ubiquitination, AKT phosphorylation, ERK phosphorylation and receptor internalization experiments for Article I.

Cell proliferation assay

Four million HEK293T cells were seeded in 75 cm^2 cell culture flask, transfected with different constructs and serum-starved overnight. Cells were then trypsinized, suspended in DMEM, stained with trypan blue and proliferation analyzed with the Countess automated cell counter (Invitrogen) after 48 hours of SCF stimulation.

Cell survival assay

B16F0 cells were plated in 96 well plates in a final cell count of 3500 cells/wells. They were then treated with KIT ligand (50 ng/ml), rapamycin (Sigma-Aldrich) or dacarbazine (Santa Cruz Biotechnology) for 24 hours. To evaluate cell survival, alamarBlue (Life Technologies) was added to each well and incubated for 4 hours. Absorbance was measured (Varian Cary 50 microplate reader) at 570 nm and 600 nm.

Receptor internalization

B16F0 cells reverse transfected with KIT constructs were pre-treated (30 minutes) with Ready-Made Solution cycloheximide (100 µg/ml) (Sigma-Aldrich) prior to KIT ligand stimulation (50 ng/ml). The reaction following ligand stimulation was stopped by adding ice cold PBS to the cells. Biotinylation of cell surface proteins was performed by adding freshly prepared biotin solution containing 0.2 mg/ml EZ-Link Sulfo-NHS-Biotin (Thermo Scientific). Incubation was carried out on ice for 40 minutes. Cells were then washed with cold PBS (pH 8) and cold Tris (50 mM) was added to the cells to block (5 minutes) excessive reactive biotin. RIPA buffer was used to lyse cells which, after debris removal through centrifugation, were subjected to pull-down with immobilized Avidin Agarose (Thermo Scientific) for 30 minutes at 4 °C. Subsequent steps were carried out similar to the description outlined in the Immunoblotting and immunoprecipitation section.

Melanin production assay KIT

After stable transfection, the B16F0 cells were plated in 100 mm cell culture dishes and treated with KIT ligand in a final concentration of 50 ng/ml in complete medium for 72 hours. Cells were then collected, counted and washed with PBS. A solution of 2M NaOH with 10 % DMSO as prepared for the isolation of intracellular melanin. Cells were incubated with the solution for 2 hours in room temperature and debris was removed by centrifugation. Finally, isolated melanin was quantified by absorbance measurements at 405 nm.

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