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The Role of Melanocyte Lineage Genes in Melanoma

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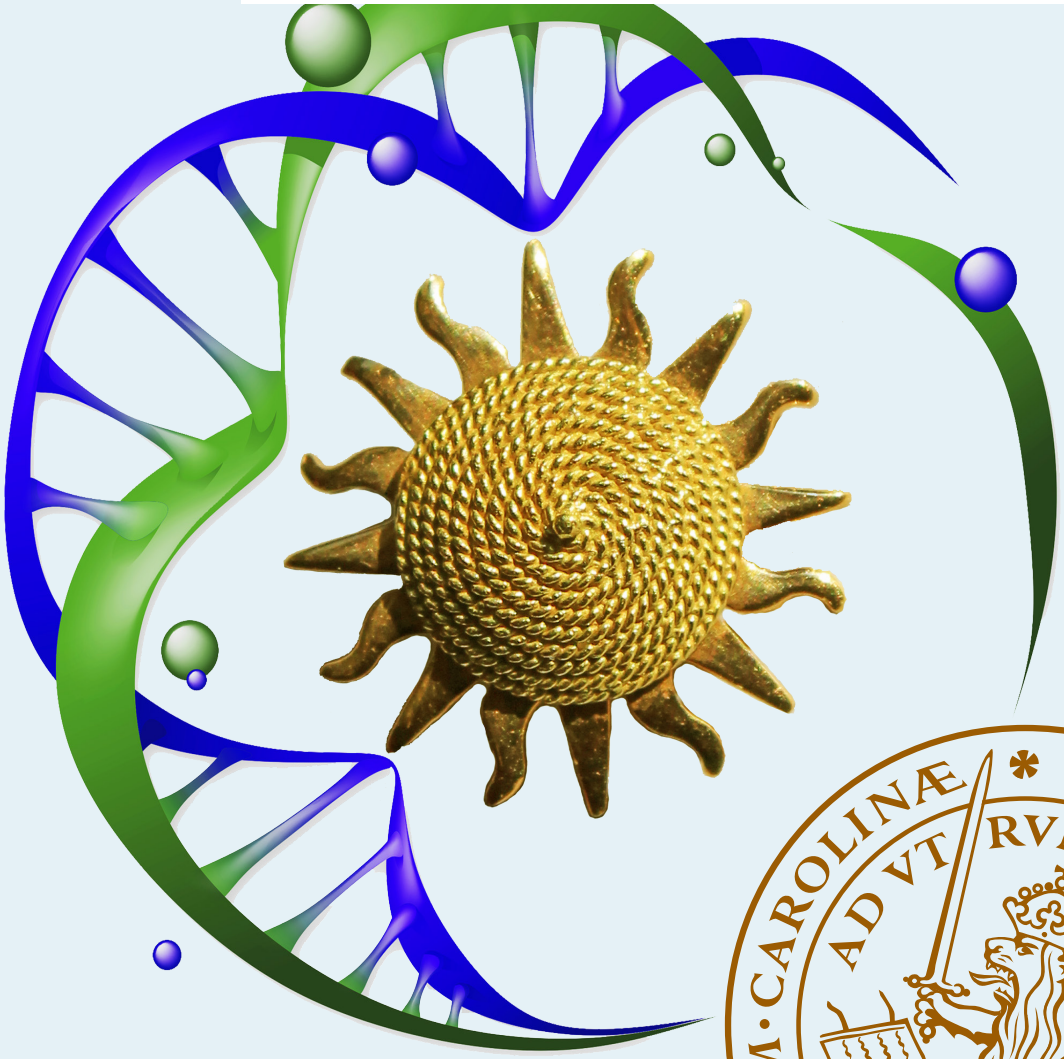

The Role of Melanocyte Lineage Genes in Melanoma

Adriana Sanna

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Abstract

Malignant melanoma accounts for the highest number of deaths among all skin cancer types, and its incidence has increased dramatically over the past decades. Despite the tremendous therapeutic advances, treatment resistant cells emerge in the vast heterogeneity of melanoma, driving tumor relapse and poor patient outcome. The aims of the studies conducted in this thesis were to contribute to the knowledge with regards to therapy-resistant melanomas, and to explore tumor heterogeneity in relation to cancer progression among the chronic sun-damaged (CSD) melanomas.

Therapy resistant cells have lost the melanocyte lineage-specific transcriptional program, which is mainly driven by the master-melanocyte regulator MITF. Paper I validated the MITF-negative ($MITF^{Neg}$) melanomas to be highly aggressive and associated with inferior patient survival compared with the MITF-high ($MITF^{High}$) lesions. We herein discovered an even more undifferentiated melanoma subtype that lacks the MITF upstream marker SOX10 ($MITF^{Neg}SOX10^{Neg}$), characterized by superior metastatic potential and resistance to targeted therapy. Importantly, we found gene methylation explaining the silencing of both *MITF* and *SOX10* in these melanomas.
To discriminate the role of *SOX10* in $MITF^{Neg}$ cells, in Paper II we engineered $SOX10^{KO}$ by CRISPR-Cas9 technology. Depletion of SOX10 in $MITF^{Neg}$ cells lead to a hyper-undifferentiated phenotype: a new distinct lineage identity state in melanoma.
Paper III uncovered a novel layer of regulation of MITF at the translational level. We showed that MITF is regulated by the RNA-helicase DDX3X. DDX3X loss in melanoma leads to decreased MITF, and results in enhanced metastasis and therapy resistance. Interestingly, *DDX3X* is located on the X-chromosome. Thus, mutations affecting *DDX3X* associate with poor prognosis in male melanoma patients, implying an exclusive window of opportunity in this gender.
Paper IV investigated the molecular features of a unique cohort of high and low CSD (CSD^{high}, CSD^{low}) melanomas. Focusing on the less investigated CSD^{high} subtype in view of cancer progression, we found no mutational difference between *in situ* or invasive phases. We further observed dissimilarity in the heterogeneity levels between CSD^{high} and CSD^{low} melanomas, which suggests distinguishable molecular entities that progress *via* different routes.

Overall, we unraveled the role of melanocyte-specific genes in defining diverse melanoma lineage states, while investigating novel biological mechanisms behind their regulation. Our findings further highlighted the variable heterogeneity in CSD melanoma subtypes, which should be taken into consideration for an improved diagnosis, and when choosing the best treatment options for melanoma patients.

Key words
Melanoma, genetic, epigenetic, heterogeneity, MITF, SOX10, CSD

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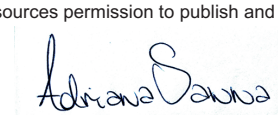
Signature 

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The Role of Melanocyte Lineage Genes in Melanoma

Adriana Sanna

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Questo è per la Gente che non posso più Scordare.
Questo è per Coloro che hanno un posto nel mio Cuore.
Questo è per la Gente che è Partita…
Questo è per i miei Fratelli,
ai miei Fratelli per la Vita.

”

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Choose the job you Love, and you will Work Hard anyway! But, surrounding yourself with Who does the same, it makes The complete difference for Your Life… and for Your Future…

Therefore, first of all, I wish to express my deepest gratitude to my Main Supervisor *Göran Jönsson*, the most brilliant and enthusiastic youngest mentor I ever had the honor and pleasure to work with. I would like to recognize your reliable leadership, invaluable advice, and especially the huge patience that you had gifted my undergraduate studies with. You should be very proud and let people celebrate your great accomplishments more often!

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This Work is Dedicated to the Loved ones We have Lost,
and in support to whom is still Fighting.
We are in This, *Together*.

Remark

I have to report that my current academic life is actually part of the plan of my adorable kitty, *MaryJane*, to conquer the world. In less than 10 years (60 felines') she has already managed a B.Sc. in Biological Sciences while enjoying aperitivos in Milan, an M.Sc. in Biomedical Sciences with a specialization in sfhjvq34d äl&5f**ja**dkva ajkdhfb&%?´l al.d*sc*213§.önjB ...Sorry, she jumped on the keyboard, specialization in Research while roaming around The Netherlands, aaand under the sun in California! Without mentioning all "business" trips she has made at least in 10 other different countries to strengthen her catwork. Worrying enough, very soon she is also aiming to award a PhD in Biomedicine, Cancer and Molecular Biology.

What will come next?! Only she knows. Meow.

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List of Abbreviations

AAD	American Academy of Dermatology
ACS	American Cancer Society
AG	Anogenital
AJCC	American Joint Committee on Cancer
ALM	Acral Lentiginous Melanoma
AM	Amelanotic Melanoma
ATCC	American Type Culture Collection
bp	Base Pair
BMIQ	Beta-Mixture Quantile Normalization
BRAFi	BRAF inhibitor
cAMP	Cyclic AMP
CAT	Computed Tomography
CCS	Clear Cell Sarcoma
chr	Chromosome
CIMP	CpG Island Methylator Phenotype
CLND	Complete Lymph Node Dissection
CMM	Cutaneous Malignant Melanoma
CNS	Central Nervous System
CNV	Copy Number Variation
COSMIC	Catalogue of Somatic Mutations in Cancer
CpG	Cytosine-Guanine Dinucleotide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	Complementary CRISPR RNA
CSD	Chronic Sun-Damaged
DCTI	Dacarbazine
ddNTP	Dideoxynucleotide
DGE	Differential Gene Expression
DM	Desmoplastic Melanoma
ECL	Enhanced Luminol-based Chemiluminescent
ECM	Extracellular Matrix
ELM	Epiluminescence Microscopy
EMP	Epithelial–Mesenchymal Plasticity
EMT	Epithelial–Mesenchymal Transition
EU	European
EWS	Ewings Sarcoma
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFPE	Formalin-Fixed Paraffin-Embedded
FPKM	Fragments *per* Kilobase of Exon Model *per* Million Reads Mapped
GEMM	Genetically Engineered Mouse Model
GEX	Gene expression
GEO	Gene Expression Omnibus
GIT	Gastrointestinal Tract
gRNA	Guide RNA
HDR	Homology-Directed Repair
HGNC	HUGO Gene Nomenclature Committee
HHC	Human Hepatocellular Carcinoma
HMG	High-Mobility-Group
HRP	Horseradish Peroxidase
IARC	Agency for Research on Cancer
IF	Immunofluorescence
IHC	Immunohistochemistry
IND	Intestinal Neuronal Dysplasia
IRES	Internal Ribosome Entry Site
IT	In-Transit Metastasis
ITH	Intratumor Heterogeneity
KD	Knockdown
KI	Knock-In
KO	Knockout
LM	Lentigo Maligna
LMM	Lentigo Maligna Melanoma
lncRNA	Long Non-Coding RNA
LOH	Loss Of Heterozygosity
MAPKi	Mitogen-Activated Protein Kinase inhibitor

MDA	Melanoma Differentiation Antigen
MEKi	MEK inhibitor
MET	Mesenchymal–Epithelial Transition
miRNA	MicroRNA
MIS	Melanoma *In Situ*
MM	Mucosal Melanoma
MRI	Magnetic Resonance Imaging
MSC	Melanocyte Stem Cell
MSLT	Multicenter Selective Lymphadenectomy Trial
NA-CMM	Non-Acral CMM
NC	Neural Crest
NCC	Neural Crest Cell
NCSC	Neural Crest Stem Cell
NES	Nuclear Exports Sequence
NGS	Next Generation Sequencing
NHEJ	Non-Homologous End Joining
NK	Natural Killer
NLS	Nuclear Localization Sequence
NM	Neuromelanin
NM	Nodular Melanoma
NMM	Naevoid Malignant Melanoma
NSG	Non-Obese Diabetic-(NOD)-Scid Gamma
NT	Neural Tube
nu/nu	Nude Mouse
ORF	Open Reading Frame
OS	Overall Survival
PCR	Polymerase Chain Reaction
PDX	Patient-Derived Xenograft
PET	Positron Emission Tomography
PNS	Peripheral Nervous System
PT	Primary Tumor
PTM	Post-Translational Modification
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Polymerase Chain Reaction
RGP	Radial Growth Phase
RNA-seq	RNA sequencing
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
S	Stage
SBS	Sequencing-By-Synthesis
sc-seq	Single-Cell RNA Sequencing
SCF	Stem Cell Factor
SCID	Severe Combined Immune Deficiency
SCP	Schwann Cell Precursor
SEER	Surveillance Epidemiology and End Results
SLN	Sentinel Lymph Node
SLNB	Sentinel Lymph Node Biopsy
SNV	Single Nucleotide Variation
sRNA	Small Non-Coding RNA
SRB	Sulforhodamine B
SSM	Superficial Spreading Melanoma
T-VEC	Talimogene Laherparepvec
TAD	Transactivation Domain
TCGA	The Cancer Genome Atlas
TF	Transcription Factor
TGX	Tris-Glycine eXtended
TIL	Tumor-Infiltrating Lymphocyte
TMA	Tissue Microarray
TME	Tumor Microenvironment
TNM	Tumor Node and Metastasis
tracrRNA	Transactivating crRNA
US	United States
UTR	Untranslated Region
UM	Uveal Melanoma
UV	Ultraviolet
UV-R	Ultraviolet-Radiation
VGP	Vertical Growth Phase
WB	Western Blot
WCRF	World Cancer Research Fund International
WES	Whole-Exome
WGA	Whole-Genome Amplification
WGS	Whole-Genome
WHO	World Health Organization
WLE	Wide Local Excision
WS	Waardenburg Syndrome
WT	Wild-Type
5-mC	5-Methylcytosine

Popular Science Abstract

Malignant melanoma is a tumor that arises from melanocytes, which are the cells that normally produce pigments in the skin in response to ultraviolet (UV) light. Melanoma cases have dramatically increased during the past three decades – particularly among young adults – and it currently ranks 19th most common cancer in men and women. The major risk factor for melanoma development is the overexposure to UV-radiation, and other risk factors include having white complexion, freckles, high number of moles, and cases of melanoma among blood relatives. Importantly, melanoma is the most dangerous skin cancer type due to high resistance to therapy, which is responsible for the death of many patients worldwide each year. Some melanoma cells are able to survive the harsh pharmacological treatments and spread to distant organs, like lungs, liver, and brain, which then cannot properly function any longer. One of the causes of resistance to therapies is represented by the high cell heterogeneity found within the tumor, as well as the heterogeneity among melanoma tumors in different patients.

The aim of the research presented in this thesis was to investigate such heterogeneity, with a focus on the relationship between the cells that do not respond to therapies and patient outcome. We established the crucial role of two melanocyte-specific genes that control different melanoma cells identities, and observed that melanoma cells that lost these genes are more aggressive and resistant to treatment than the cells which express those two genes. As a consequence, patients with tumors lacking such melanocyte-specific genes tend to have a worse outcome. In addition, we have discovered two mechanisms that can suppress these melanocyte-specific genes. Importantly, such mechanisms could be exploited in the search for new therapeutics to treat malignant melanoma.

Furthermore, we analyzed melanomas caused by chronic sun-damage (CSD), which were separated into high (CSD^{high}) or low (CSD^{low}) UV-exposure groups. We found that only the CSD^{high} melanomas do not need to accumulate gene mutations in order to progress to more aggressive stages of the disease, while the CSD^{low} do. These results highlight fundamental biological differences between low and high CSD, and reflect how important is to protect our skin and prevent overexposure from UV light.

In summary, malignant melanoma is a heterogeneous collection of tumors with different characteristics that can be used to predict the outcome for patients. Moreover, by understanding how the cells evolve in order to escape the effects of anti-cancer drugs, it will be possible to find new ways to block tumor progression and eliminate the cancer.

Sintesi Scientifica a Scopo Divulgativo

Il melanoma maligno è un tumore che deriva dai melanociti, le cellule che normalmente producono pigmenti nella pelle in risposta alla luce ultravioletta (UV). Negli ultimi trent'anni, i casi di melanoma sono aumentati notevolmente – in particolare tra i giovani adulti – e si colloca attualmente al 19° posto quale tumore più comune in uomini e donne. Il maggiore fattore di rischio per lo sviluppo del melanoma è rappresentato dall'eccessiva esposizione alle radiazioni UV; ulteriori fattori di rischio sono l'avere una carnagione chiara, lentiggini, abbondanza di nei, o una precedente diagnosi di melanoma in famiglia. È importante sottolineare che il melanoma è il tumore della pelle più pericoloso a causa di un'alta resistenza ai farmaci, ed è responsabile della morte di molti pazienti ogni anno in tutto il mondo. Alcune cellule di melanoma sono in grado di sopravvivere ai trattamenti farmacologici e hanno la capacità di diffondersi in altri organi quali ad esempio polmoni, fegato e cervello, che, se attaccati, non saranno più in grado di funzionare correttamente. Una delle cause della resistenza al trattamento nel melanoma è rappresentata da un'alta eterogeneità riscontrata sia nella composizione di ciascun tumore, sia tra i tumori di diversi pazienti.

Lo scopo della ricerca presentata in questa tesi era di indagare su tale eterogeneità, in particolare sulla relazione tra le cellule che non rispondono alle terapie e il tasso di sopravvivenza dei pazienti. Abbiamo stabilito il ruolo cruciale di due geni melanociti-specifici che controllano diverse identità delle cellule di melanoma, e abbiamo osservato che le cellule prive di questi geni sono più aggressive e resistenti al trattamento rispetto a quelle che li esprimono. Di conseguenza, i pazienti che presentano tumori sprovvisti di questi geni, tendono ad avere un esito peggiore. Abbiamo anche scoperto due dei meccanismi che possono reprimere questi geni melanociti-specifici. Tali meccanismi potrebbero essere sfruttati nella ricerca di nuove terapie per il trattamento del melanoma maligno.

Abbiamo analizzato ulteriormente i melanomi causati da danno solare-cronico (CSD) differenziati tra alto (CSD^{high}) o basso (CSD^{low}) fattore di esposizione ai raggi UV. Abbiamo scoperto che, al contrario dei CSD^{low} melanoma, solo i tumori con CSD^{high} non hanno bisogno di accumulare mutazioni genetiche per progredire agli stadi più aggressivi della malattia. Questi risultati evidenziano differenze biologiche fondamentali tra i melanomi CSD^{high} e CSD^{low}, e riflettono quanto sia importante proteggere la nostra pelle e prevenire l'eccessiva esposizione alla luce UV.

In sintesi, il melanoma maligno è una raccolta eterogenea di tumori con diverse caratteristiche che possono essere utilizzate per prevedere la sopravvivenza nei pazienti. Inoltre, comprendendo come si evolvono le cellule per sfuggire agli effetti dei farmaci antitumorali, sarà possibile trovare un nuovo modo per bloccare la progressione del tumore ed eliminare il cancro.

Abstract

Malignant melanoma accounts for the highest number of deaths among all skin cancer types, and its incidence has increased dramatically over the past decades. Despite the tremendous therapeutic advances, treatment resistant cells emerge in the vast heterogeneity of melanoma, driving tumor relapse and poor patient outcome. The aims of the studies conducted in this thesis were to contribute to the knowledge with regards to therapy-resistant melanomas, and to explore tumor heterogeneity in relation to cancer progression among the chronic sun-damaged (CSD) melanomas.

Therapy resistant cells have lost the melanocyte lineage-specific transcriptional program, which is mainly driven by the master-melanocyte regulator MITF. Paper I validated the MITF-negative ($MITF^{Neg}$) melanomas to be highly aggressive and associated with inferior patient survival compared with the MITF-high ($MITF^{High}$) cases. We herein discovered an even more undifferentiated melanoma subtype that lacks the MITF upstream marker SOX10 ($MITF^{Neg}SOX10^{Neg}$), characterized by superior metastatic potential and resistance to targeted therapy. Importantly, we found gene methylation explaining the silencing of both *MITF* and *SOX10* in these melanomas. To discriminate the role of *SOX10* in $MITF^{Neg}$ cells, in Paper II we engineered $SOX10^{KO}$ by CRISPR-Cas9 technology. Depletion of SOX10 in $MITF^{Neg}$ cells lead to a hyper-undifferentiated phenotype: a new distinct lineage identity state in melanoma. Paper III uncovered a novel layer of regulation of MITF at the translational level. We showed that MITF is regulated by the RNA-helicase DDX3X. DDX3X loss in melanoma leads to decreased MITF, and results in enhanced metastasis and therapy resistance. Interestingly, *DDX3X* is located on the X-chromosome. Thus, mutations affecting *DDX3X* associate with poor prognosis in male melanoma patients, implying an exclusive window of opportunity in this gender. Paper IV investigated the molecular features of a unique cohort of high and low CSD (CSD^{high}, CSD^{low}) melanomas. Focusing on the less investigated CSD^{high} subtype in view of cancer progression, we found no mutational difference between *in situ* or invasive phases. We further observed dissimilarity in the heterogeneity levels between CSD^{high} and CSD^{low} melanomas, which suggests distinguishable molecular entities that progress *via* different routes.

Overall, we unraveled the role of melanocyte-specific genes in defining diverse melanoma lineage states, while investigating novel biological mechanisms behind their regulation. Our findings further highlighted the variable heterogeneity in CSD melanoma subtypes, which should be taken into consideration for an improved diagnosis, and when choosing the best treatment options for melanoma patients.

Aims of the Thesis

This thesis aims to elucidate the role and function of lineage genes essential for melanocyte specification in melanoma survival and development. The objectives of each paper specifically address:

Transcriptional regulation

Illustrate DNA hypermethylation mechanisms, which drive different melanoma cell states, and the relation to distinct cell phenotypes (Paper I). Define the contribution of melanocyte-specific genes towards a transcriptionally undifferentiated melanoma phenotype, by CRISPR-Cas9 selective gene deletion (Paper II).

Translational regulation

Investigate the effect of altered RNA translation that dictates melanoma reprogramming, and its association with cell metastatic potential (Paper III).

Clonal relatedness

Resolve cell clonality by ultra-deep sequencing in chronic sun-damaged melanomas, and understand tumor heterogeneity over the course of malignant transformation (Paper IV).

Introduction

The History of Cancer

New life starts with the process of embryogenesis, a finely arranged cellular program of crucial importance in the normal development of the embryo. One evolutionarily conserved program that is essential for embryogenesis is the epithelial–mesenchymal transition (EMT), and its reverse process, the mesenchymal–epithelial transition (MET). During organogenesis, the polarized and immobile epithelial cells can evolve into the migratory and invasive mesenchymal cell type. This gives them the ability to move through the extracellular matrix (ECM), and eventually to differentiate and specialize. Underlying the high plasticity of this reversible process, future organs and tissues of the newborn will be formed over sequentially orchestrated rounds of EMT–MET [1].

While this delineates the vital process that generates human beings, it can also prove to be fatal. Today the burden of cancer is increasing on a global scale and represents the second leading cause of mortality worldwide, as reported from the World Health Organization (WHO) [2]. Note that this devastating effect is not due to primary lesions, but it is a consequence of tumor growth and release of metastases in which epithelial–mesenchymal plasticity (EMP) has a pivotal role [3]. One of the greatest examples of a fatal metastatic cancer is malignant melanoma. Tumor cells can hijack the fundamental mechanism of EMP, in this case more appropriately defined as phenotype plasticity, which then enhances tumor progression and dissemination throughout the body [4, 5].

In spite of the tremendous achievements in biomedical research in the past decades, more than 80% of the advanced cancer patients will not survive at this stage, and so it has been since 1970 [6]. Therefore, it is of utmost importance for the medical community to collaborate and fight this battle against cancer.

The Melanocyte Lineage

Color traits are among the most obvious phenotypic features that characterize animals, including humans. Nevertheless, the true origin of pigmentation patterns remained unknown until the 1819, when the Italian professor of comparative anatomy and zoologist Giosué Sangiovanni discovered cells which produce melanin in squid skin. These cells had been originally named 'chromatophores' [7]. Approximately 20 years later, the presence of melanocytes in human epidermis and eyes was independently confirmed in Germany [8]. Today it is known that even if melanocytes represent a minority of the cells (from 5 to 10 %) that colonize the skin, they are easy to distinguish from non-pigmented keratinocytes, fibroblasts and immune cells. Melanocytes are mostly located in the deepest layer of the epidermis, the *stratum basale*, the uvea of the eye and in hair follicles, where their function dictates the color of our skin, eyes and hair [9]. In addition, melanocytes can be found in the inner ear and, to a minor extent, in other tissues throughout the body, e.g., gastrointestinal tract (GIT) [10] and anogenital (AG) areas [11].

In the human skin, melanocytes are highly specialized cells responsible for melanogenesis: the process which produces melanin. Melanin is a thermoregulator and photoprotective pigment that is needed to shield the cell DNA against the ultraviolet radiation (UV-R) [12]. Mechanistically, melanocytes produce melanin, it is packaged into melanosomes, and then transferred to the receiving keratinocytes by physical interaction *via* the melanocytes' dendrites.
Biologically, keratinocytes respond to a variety of stimuli, such as DNA damage due to UV light, by tumor protein 53 (TP53) stabilization and release of melanocortin molecules. These are the agonist adrenocorticotrophin hormone (ACTH) and the alpha-melanocyte-stimulating hormone (α-MSH), which are derived from the precursor molecule proopiomelanocortin (POMC). Post-translational cleavage of POMC also generates β-endorphin, which is released in high levels upon chronic UV-R exposure (**Fig. 1A**). Interestingly, high levels of β-endorphin results in analgesia and physical dependence upon continued exposure, which has been proposed to be mediated by the endogenous opioid system. Concordantly, such UV induced nociceptive effects have been shown to promote UV seeking and tanning addiction behaviors [13]. When α-MSH binds to melanocortin-1 receptor (MC1R) expressed on the surface of melanocytes, it stimulates an increase in cytoplasmic cyclic AMP (cAMP). In the cytoplasm of melanocytes, elevated levels of cAMP lead to phospho-activation of the enzyme protein kinase A (PKA), which in turn phosphorylates the cAMP response element-binding protein (CREB). Activation of this transcription factor (TF) induces its nuclear translocation and binding to DNA sequences known as cAMP response elements (*CRE*), which are also present in the promoter of the microphthalmia-associated transcription factor (*MITF*) [14] (**Fig. 1B**). In the nuclei,

MITF targets the expression of the rate-limiting melanogenic enzymes tyrosinase (TYR), together with the tyrosinase-related protein (TYRP) TYRP-1 and TYRP-2 (or dopachrome tautomerase, DCT). These enzymes synthetize melanin by converting the molecule precursors tyrosine, to levodopa (L-DOPA), to dopaquinone, and finally to melanin. Then, the melanin is packaged into melanosomes. The premelanosome protein (PMEL), also called silver locus protein (SILV), and Melan-A (MLANA), are critical for the structure of the melanosomes [15], while adaptor-related protein 3 (AP-3), biogenesis of lysosomal organelles complex 1 (BLOC-1) and melanosomal transmembrane protein 2 (OCA2) are responsible for the sorting and trafficking of the melanosomes [16]. In the context of pigmentation, *MITF* expression can also be triggered by binding of the stem cell factor (SCF) ligand to the receptor tyrosine kinase (c-KIT) on the melanocytes' membrane [17]. Besides melanin production, KIT-mediated transactivation of *MITF* plays an important role during differentiation of melanocytes.

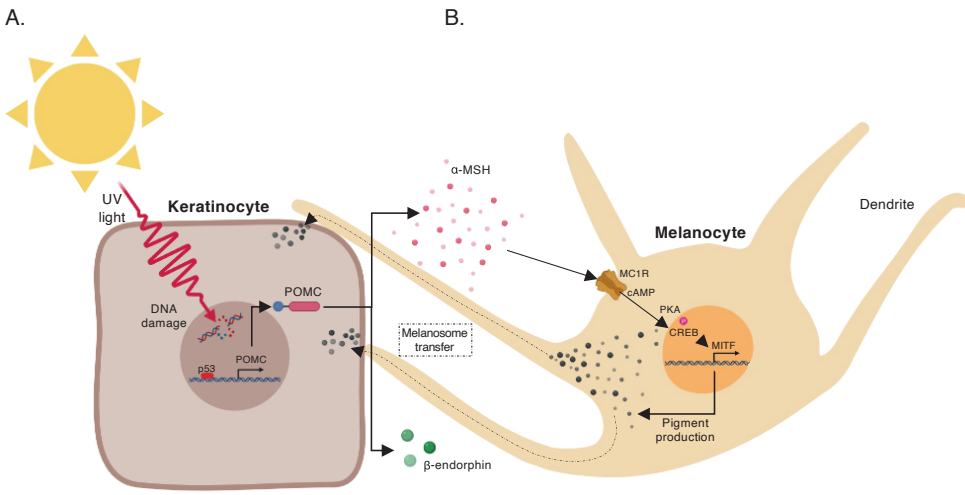

Figure 1. Cutaneous response to UV light. A. In keratinocytes, UV-R induce p53-mediated upregulation of POMC. Post-translational cleavage of POMC produces α-MSH and β-endorphin. **B.** On adjacent melanocytes, secreted α-MSH binds to MC1R leading to an increase in cAMP, activation of PKA and P-CREB. In the melanocyte nucleus, P-CREB induces *MITF* transcription. MITF downstream cascade results in synthesis of melanin, which is transferred to the keratinocytes nuclei in melanin-containing vesicles (melanosomes) *via* the melanocyte dendrites.

Melanin

Melanin are pigments that are able to absorb light and function as photoprotective agents in the human skin [18]. There are two main melanin types: the pheomelanin and the eumelanin [19]. The first can be brown or black and, in absence of other pigments, the brown appears yellow (e.g., blond hair), while the black causes grey hair, increased for example in the elderly. The yellow/reddish pheomelanin is responsible for red hair in case that brown eumelanin is also present, and it concentrates in lips or nipples.

Humans had to adapt to the different climates through their history of migration across the world. Therefore, the melanocytes in the skin have also adapted to produce the two melanin types in different proportions, depending on the availability of sunlight. Subsequently, the evolutionary process produced ethnicities' variety, which are present today [20]. For instance, people who live in sunny areas around the equator are exposed to high UV-R. Because of its superior photoprotective properties, high amount of eumelanin are produced to shield the DNA against UV damage. This results in black/brown complexion, which is typical of the ethnic groups such as African, Hispanic, Asiatic and Mediterranean [21]. By contrast, people who live in northern countries with limited sunlight exposure, have decreased need for eumelanin production, which results in lower eumelanin: pheomelanin ratio. This is one of the reasons behind fair color traits for example in Northern Europe and Scandinavia. Deficiencies in melanin production emerge as a rare congenital disorder, namely albinism. Albinism is characterized by the absence of pigmentation, and albinos can belong to any ethnic group [22, 23]. Regardless of skin pigmentation and ethnicity, a direct relationship between having darker complexion and lower risk of developing certain types of melanoma has not been ascertained [18].

Interestingly, in some brains from primates (primarily in humans), there is a third pigment that is dark and structurally similar to melanin: neuromelanin (NM) [24]. NM is synthetized from L-DOPA precursor by a distinct population of catecholaminergic neurons in specific structures of the brain (i.e., *substantia nigra*). Although the function of NM has not been fully understood, it has been shown to have a protective effect against reactive oxygen species (ROS) by accumulating in the aging brain [25]. Of clinical importance, degeneration of catecholaminergic neurons lead to lack of NM, which is thought to contribute to neurodegenerative disorders such as Parkinson disease [26].

Embryonal Origin and Development of Melanocytes

In distinction to the embryonal origin of other cells of the skin, the melanocyte lineage originates from the neural crest (NC), the early and transient ectodermal structure of the vertebrate embryo [27] (**Fig. 2**). Besides melanocytes, the multipotent NC also generates other cell types, including the neurons and the glia cells of the central nervous system (CNS) [28]. During the embryonal development, the melanocyte-precursor, melanoblasts, differentiates from the neural crest cells (NCCs). This pluripotent cell lineage specifies at the dorsal side of the neural tube (NT) *via* activation of the bone-morphogenic-protein (BMP) and the wingless-related integration site/beta-catenin (Wnt/β-catenin) signaling pathway [1]. The downstream signaling cascade results in upregulation of the RAS-homolog family member B (RhoB) and downregulation of cadherins, which render the NCCs highly motile [29]. As a consequence, NCCs are able to detach from the dorsal NT and undergo EMT, while delaminating and

dispersing throughout the embryo [30]. In this EMT transition, the NCCs express the EMT-activating TFs zinc finger proteins, SNAI1 and SNAI2, twist-related protein 1 (TWIST1), zinc-finger E-box-binding homeobox 1 (ZEB1) and ZEB2 [31]. Then, the NCCs commit to a specific cell-fate according to their migratory trajectory. For instance, the dorsolateral path implies melanoblasts specification followed by terminal melanocytes' differentiation [32]. There are specific regulatory molecules involved in this program, including paired-box 3 (PAX3), the endothelin-3/endothelin receptor type B (EDN3/EDNRB) signaling pathway and the SRY-like high mobility group box 10 (SOX10) TF. SOX10 has been shown to be essential to promote survival of the dedifferentiated NCCs prior to lineage commitment. Furthermore, it directly regulates the expression of MITF in melanoblasts [33, 34]. The finally matured melanocyte population expresses KIT, EDNRB, TYRP-2, SOX10 and MITF. Commonly, the Schwann cell precursors (SCPs) differentiate into Schwann cells and endoneural fibroblasts. However, recent data have reported that the ventral migratory NCCs, SCPs, can also represent a cellular source for differentiated melanocytes [35].

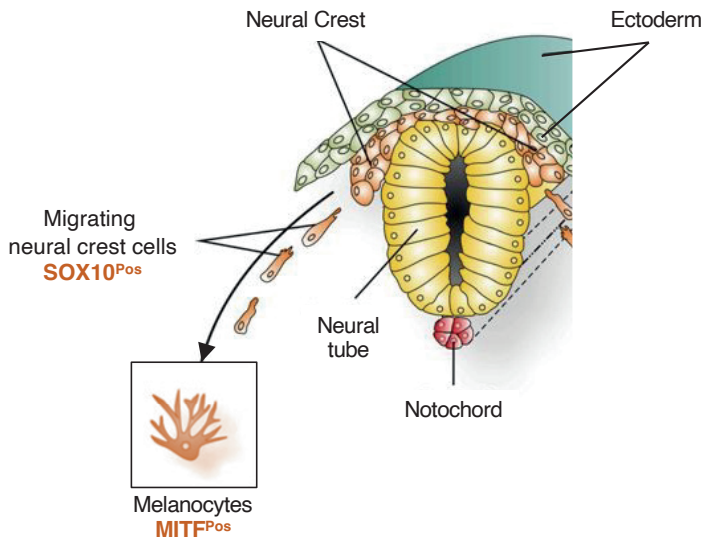

Figure 2. Embryonal origin of the melanocyte lineage. Neural crest cells arise between the newly formed ectoderm and the neural tube during the development of the vertebrate embryo. Migrating neural crest cells express SOX10 ($SOX10^{Pos}$), and the derived melanocytes express MITF ($MITF^{Pos}$). *Adapted with permission of* Kaltschmidt et al. [36].

Alterations that affect the development of the melanocyte lineage can give rise to a series of rare and uncurable disorders known as Waardenburg syndromes (WS). WS manifests with symptoms including changes in pigmentation, variable degrees of deafness and other abnormalities, depending from the gene(s) affected. To a greater extent, the accumulation of mutations in genes important to melanocytes' survival and development can contribute to malignant transformation, and eventually give raise to the cancer of melanocytes: malignant melanoma [37].

Malignant Melanoma

Epidemiology of Melanoma

Even though melanoma is less common than other malignancies on the skin, it is the deadliest form of skin cancer [38]. Melanoma incidence has risen sharply over the past few decades, almost outpacing all other tumors. Melanoma ranks now as the 19th most common cancer in men and women worldwide, and the World Cancer Research Fund International (WCRF) reports that Australia, North America, and North Europe are the most affected countries [39].

– Worldwide, a person dies of melanoma every hour, every day –

Tragically, melanoma is considered as "Australia's national cancer". There, melanoma incidence is estimated to reach up to 11% of all new cancers' cases diagnosed in 2019, which is twice as many as those registered in both America and New Zealand combined. Strikingly, by the end of 2019 melanoma was ranked the 9th most common cause of death for Australians, from being the 12th in 2016 [40].

In the US, melanoma represents the first most common diagnosed cancer in young adults between 25 and 29 years of age, and the third most common in the younger patient group, 15 to 29 years old. According to the Surveillance Epidemiology and End Results (SEER) program, only in the United States (US) almost 100,000 new melanoma cases are estimated overall in 2019. Here, this extremely high incidence is predicted to be fatal for more than 7,200 people, and in the last 10 years, the number of Americans newly diagnosed with invasive melanoma increased by 54% as compared with the previous 10-year period. Clearly, this rate results in higher health costs, which are already accounting for 3.5 billion dollars annually [41].

Although melanoma occurrence varies considerably throughout Europe, the highest incidence has been observed in the Scandinavian countries and in the UK, where it continues to increase to a worrying rate of 7% annually, in comparison to the 2.6% in the US. Overall, deaths are more than 22,000 *per* year in Europe according to WHO [42]. According to Cancerfonden, melanoma in the skin is among the most common cancer in Sweden. In the last 10 years, invasive melanoma cases increased by 5%, which correspond to more than 4,000 diagnosed in the invasive stage annually. This alarming trend has been associated with excessive sun exposure during brief period of vacations for a quick desire to tan. Such neglectful behavior often results in severe sunburns, skin genetic damage, and increased risk of developing melanoma.

Melanoma Risk Factors and Prevention

Extrinsic and intrinsic risk factors can increase melanoma incidence. The most prominent risk factor for the development of sporadic cutaneous melanoma is UV light. Two UV components (UVA and UVB) contribute differentially to skin damage and sunburn, which are both significant risk factors in sporadic melanoma [43]. Critically, the International Agency for Research on Cancer (IARC) have reported that getting sunburns during childhood or during the teen years can increase the chance of melanoma development by approximately 70% [44].

Overexposure to UV induces DNA damage in skin cells, including melanocytes, and the accumulation of mutations in the melanocytes' DNA can contribute toward their malignant transformation (melanomagenesis). It is estimated that more than 90% of the cutaneous malignant melanoma (CMM) cases arise because of an excessive UV exposure coming from the sun and/or from artificial sources including solariums and indoor sunlamps [45]. UV induced mutagenesis is one of the reasons why melanoma is characterized by a high tumor mutational burden (TMB). With an average mutation rate of 16.8 mutations *per* Mb, melanoma TMB is one of the highest among solid cancers [46]. The characteristic UV signature displays a high presence of cytosine (C) to thymine (T), CT→T and C→T mutations at di-pyrimidine sites and random CC to TT substitutions [47]. In UV induced melanomagenesis, variants of the melanogenic genes *MC1R*, *TYR* and *TYRP1*, are independent low-penetrance susceptibility genes that predispose to melanoma development [48, 49].

In the modern society, the fashionable desire of looking tan all year round leads to an unprecedented use of tanning equipment, especially in sun-deprived countries. Importantly, even in adulthood one single sunbed session can increase the risk of developing melanoma by 20%, and multiple sessions up to 75% if done before the age of 35. Concerns in regard to indoor tanning have been raised, resulting in a complete ban in both Brazil and Australia, and to a prohibitory law for teens under 18 years old in many European (EU) countries [44].

Besides UV-R, intrinsic factors are important to consider when assessing the risk of a person to develop melanoma. These include genetic-related fair complexion, freckles, high number of moles, family and personal history of cancer [50, 51]. Additionally, the American Academy of Dermatology (AAD) reports that melanoma-associated mortality is twice as much in males than in females patients [52]. This fact may be due to different reasons, including a better sun-safety behavior in women, whereas men disregard signs of cancer in the skin, or generally men are less likely to protect themselves from UV-induced skin damage. Interestingly, epidemiological studies have reported biological clues to explain the sex-related discrepancy. For example, a lower propensity for females to develop melanoma metastases in comparison to males has been observed. This may also partially explain why mortality is more marked than

incidence. Finally, similar to other cancers, older age is also associated with poorer patient prognosis in melanoma [53].

On the other hand, melanoma is one of the most preventable forms of cancer [51, 54]. In fact, protecting the skin from the sunlight with the use of sunscreen and cover up clothing, avoiding sun exposure, particularly between 10 a.m. and 4 p.m., and abstaining from any artificial UV source has been shown to greatly reduce the risk of sporadic melanoma [55]. Furthermore, regular skin self-exams followed by exams with specialists in suspicious instances are highly recommended, especially in individuals with a basal higher risk of melanoma [56].

In contrast to common beliefs, there is no such thing as a safe tan. The change in skin color, even before a visible tan is seen, is indicative of cell damage. The damage is permanent, and its cumulative effect increases the risk of developing melanoma.

– Beauty is about being comfortable in your own skin –

It is our obligation to actively raise awareness about the harm of excessive exposure to UV-R, and to challenge and extinguish the popular obsession of being tan as a sign of health and beauty.

Histopathological Subtypes in Melanoma

Melanocytes are located ubiquitously throughout the body, and thus it is not surprising that melanoma can potentially develop in several body sites. The main melanoma types are distinguished according to their dependence upon the UV-R.
There are rare forms of melanoma that arise in parts of the body, which are not usually exposed to sunlight, and thus do not depend on UV exposure [57]. These account for ~1 to 5% of all melanoma cases and bear poor prognosis for the patients because they are generally diagnosed in late stages of the disease [58]. There are three main non-UV subtypes: mucosal melanoma (MM) can develop inside the mouth, in nasal cavities, GIT tract and AG areas. MM is hard to detect, and patients have worse prognosis in comparison to other melanoma types [59, 60]; ocular melanoma, also called uveal melanoma (UM) or conjunctival melanoma, it has unknown cause, currently no effective treatment options, and high chance of recurrence [61]. Mutations in guanine nucleotide-binding protein G(q) subunit alpha (*GNAQ*) and *GNA11* are thought to drive the pathogenesis of UM [62-65], and BRCA-1 associated protein-1 (*BAP1*) mutations have been suggested to predispose to UM and other cancers [66]; acral lentiginous melanoma (ALM) arises in the palms of hand, the soles of feet or underneath the nail beds. Although ALM occurs in less than 5% of all cases in people with light skin, it is the most common melanoma subtype among people with dark complexions, reaching up to 70% of total cases. If ALM is diagnosed early, it can be easily treated [67].

The UV driven melanomas are the most common melanoma types. They can be further classified in low or high degrees of cumulative sun exposure on the chronic sun-damaged (CSD) skin [68]. The CSD^{low} melanoma typically arises in intermittently sun-exposed skin (i.e., limbs and trunk), and its incidence is higher in young people. The CSD^{high} melanoma occurs because of continuous UV exposure in the skin of mostly elderly in the neck, head and shoulders [69]. The genetic landscape and events important for CSD^{high} development and progression are still largely unknown [70].

In spite of the degree of cumulative sun exposure, CSD melanoma generally develops in the epidermis following a radial growth phase (RGP) and, if left untreated, it then progresses in a vertical growth phase (VGP) deep into the dermis. Among CSD melanomas, the non-acral CMM lesions (NA-CMM or CMM) are subdivided into three clinically distinguished histopathological subtypes: superficial spreading melanoma (SSM), nodular melanoma (NM), and lentigo maligna (LM).

SSM is the most common subtype and represents 70% of primary CSD^{low} melanoma cases. It usually arises in areas of intermitted sun exposure (CSD^{low}) from a pre-existing mole, and it is characterized by a prominent RGP [71].
NM accounts for up to 20% of melanoma cases. NM is a highly aggressive form of melanoma that appears as a bump with a rapid VGP [72]. Although NM is mostly found in CSD^{high} areas, it can actually arise in any part of the body, even in CSD^{low} and in non-UV exposed sites.
LM is a typical example of CSD^{high} melanoma. As such, it occurs in 5% to 10% of patients who are mostly elderly, and it occurs in body areas with chronic sun exposure, i.e., head, neck and shoulders. It has a slow RGP, and only 5% of LM cases are estimated to progress to VGP. Invasive LM lesions are referred as lentigo maligna melanoma (LMM) [73].
Interestingly, 1 to 8% of any of these major melanoma histopathologic subtypes can present with partial or complete lack of pigments, thus appearing as a skin-colored mark. These cases are referred to as amelanotic melanomas (AM) [74, 75].

Other well-defined UV-related melanoma types include: desmoplastic melanoma (DM) that develops in the dermis or the submucosa. DM cells have an easily recognizable histopathological feature known as 'spindle-shaped'. DM tends to have sharp VGP in CSD^{high} body sites [76]. Naevoid malignant melanoma (NMM), also known as 'small-cell melanoma', usually arises on the trunk and limbs. Macroscopically NMM can be commonly mistaken for a benign nevus, but histologically, NMM cells display dermal mitosis and nucleolar prominence [77]. Spitzoid melanomas have been observed in patients younger than 20 years old. It arises from AM nodular lesions and it is hard to detect because it closely resembles a noncancerous Spitz nevus [78].

Cutaneous Malignant Melanoma

Distinguishing a suspicious mole from any melanocyte neoplasm or other cancerous skin growth has always been a challenge in early melanoma diagnosis. While in advanced settings, the identification of melanoma metastases can be further complicated by primary tumor (PT) regression [79, 80]. Typically, upon first melanoma diagnosis, a skin biopsy is surgically removed and examined by a pathologist according to the following criteria: thickness and margins, presence of ulceration (loss of the surface of the skin), proliferative cell rate, and presence of tumor-infiltrating lymphocytes (TILs) [81]. The pathology report includes the melanoma clinic-histopathologic type/subtype, also defined by taking into account the patient individual traits (e.g., ethnicity, age, genetics and body site location involved) [82]. After the pathological exam, the primary melanoma is evaluated in view of the risk of progression and recurrence. PTs are categorized as low risk if the lesion is less than 1 mm thick, or high-risk if the PT thickness is more than 1 mm [83]. In this case, the tumor may be ulcerated and considered at higher risk of metastatic spread of melanoma cells to a sentinel lymph node (SLN). Subsequently, an SLN biopsy (SLNB) is performed for diagnosis of microscopic presence of malignant cells in the nearest draining regional lymph node [84]. In the case that the melanoma cells are found in the sentinel node(s), other testing may be needed to exclude cancer progression to distant organs. For this purpose, body radiology scans can be used, including ultrasound, computed tomography (CAT), magnetic resonance imaging (MRI), and positron emission tomography (PET) [85, 86].

Clinical Classification of Melanoma

The melanoma staging system is used to describe the location and progression of cancer cells in order to define the best treatment and to predict the patient's prognosis. The American Joint Committee on Cancer (AJCC) published the latest Cancer Staging Manual in its 8th edition in 2017 [87]. The manual uses a score based on the characteristics of the tumor, node and metastasis (TNM), as well as additional evidence-based prognostic factors. The assessment of melanoma stages is constantly refined to avoid potential pitfalls in the histological examination of the staging parameters and to ensure the best treatment option given to the patients [88-90].

Melanoma is classified in four main clinical stages (S). Melanoma *in situ* (**MIS**), is used when cancer cells are localized in the epidermis. Stage I (**SI**) is a thin melanoma still confined to the epidermal layer of the skin. It is subdivided in IA and IB, based on PT thickness and presence of ulceration. Stage II (**SII**) is used when the melanoma cells have spread into the dermis. SII subgroups (A, B, and C) follow the criteria applicable to the SI subgroups. Stage III (**SIII**) is used when the melanoma cells have metastasized locally or into regional lymph nodes. While circulating though the lymph vessels, melanoma cells can potentially grow isolated metastases in the surrounding skin. These

are known as "in-transit metastases" (IT) or "satellite metastases". There are 4 SIII subgroups (A to D) that are classified by number and size of the lymph nodes involved, the presence of IT metastases, and ulceration. Finally, Stage IV (**SIV**) is the more advanced stage where melanoma cells have disseminated to distant locations throughout the body *via* the bloodstream and into other organs. Melanoma cells most often metastasize to the lungs, the liver, the brain, the bones, and the GI tract. Further clinical evaluation divides SIV disease according to the organ(s) involved in M1a (to skin and/or soft tissue), M1b (to lungs), M1c (any other organ excluding the CNS), M1d (to the CNS) (**Fig. 3**).

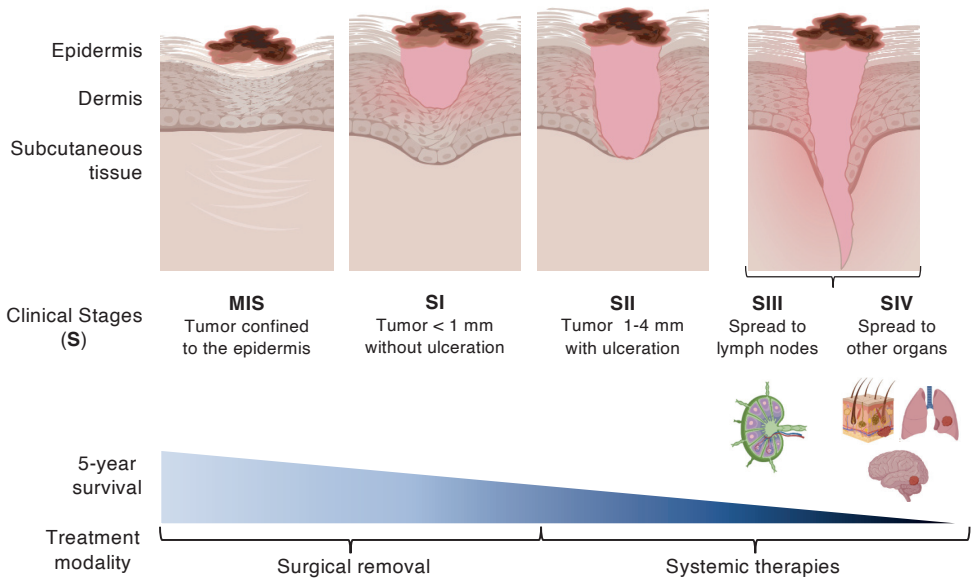

Figure 3. Clinical stages of cutaneous malignant melanoma. The figure depicts a section of the skin and melanoma progression through stages, in relation to cells invasion from the epidermis to the dermis. Decrease in 5-year survival and the treatment modality available is shown in the lower panel according to the clinical stage of the tumor.

Besides referring to melanoma stage, there are other measures that have been used to assess tumors, such as Breslow Depth (or tumor thickness) and the Clark Level [91]. The first indicates to which depth the melanoma cells have invaded through the body, and it is measured in mm. The second describes the invasion depth of melanoma as it grows through the skin, and is divided into 5 levels. While the new AJCC system takes into account the Breslow thickness, the assessment of the Clark Level has been discontinued because it does not add any prognostic value for the widely accepted TNM score for the assessment of melanoma stages [87, 92]. Although the AJCC melanoma staging system is a useful tool to stratify melanoma patients accordingly to clinic and histopathological tumor features, this classification sometimes does not correlate to prognosis and clinical outcome for the patients. Moreover, the stage itself

is not informative for therapeutic agents that may be beneficial for one melanoma type or another [93]. In this instance, gene expression (GEX) data and molecular features are critical to a proper selection of the treatment most likely to be effective in specific melanoma groups, and to better predict the patient's prognosis [94].

From a prognostic point of view, if a melanoma PT is recognized and surgically resected during the early stages (i.e., localized melanoma, MIS, SI and SII), roughly 98% of the patients will survive. However, when melanoma cells have metastasized to draining lymph nodes (i.e., SIII, regional melanoma), the patient survival rate drops to less than 65%. Dramatically, the 5-year survival of melanoma patients with distant metastases is only about 22.5%. In this advanced stage, melanoma is difficult to treat, likely to develop resistance to therapy and, subsequently, it is fatal [95].
Note that these estimations do not truly reflect the current trend. In fact, therapies available to treat advanced melanoma stages have greatly improved over the past decades, and have led to an increase in patients survival [96]. In the US, upon the food and drug administration (FDA) approval for the newest treatments, the mortality declined by 2.2% between 2016 and 2017. Following this trend, earlier this year the American Cancer Society (ACS) has reported the largest single-year decline in melanoma patients' mortality ever registered in the US [97-99].

Treatments for Melanoma Patients

The standard of care for patients with melanoma localized in the skin is the surgical removal of the PT beyond cancer margins. For melanomas with a thicker Breslow class (at least > 1 mm) usually a SLNB is performed in conjunction with wide local excision (WLE) of the scar along with the surrounding skin [100]. Until 2017, in the case of neoplastic cells spread to the SLN a complete lymph node dissection (CLND) used to be performed to eliminate nearby potentially metastatic nodes. Then, the multicenter selective lymphadenectomy trial II (MSLT-2) showed no benefit of CLND over just the "wait and see", as CLND did not increase melanoma-specific survival in patients with melanoma and sentinel-node metastases [101]. For patients with high risk of recurrence, adjuvant radiation therapy can be recommended. However, this does not appear to improve overall survival (OS), and side effects can be serious [102]. Still, palliative radiation therapy can be given to relieve symptoms in patients with melanoma localized in unresectable parts of the body [103, 104]. There are clinical trials combining radiation therapy regimes and other therapeutic options [105, 106].

Immunotherapy was recently introduced as first-line treatment for Stage III-IV melanoma patients. It mainly consists of monoclonal antibodies that target programmed cell death protein 1 (PD-1), e.g., Nivolumab and Pembrolizumab [107] and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), e.g., Ipilimumab [108]. These inhibitors have been continuously optimized and included in several clinical trials, also in combinations [109, 110]. However, in spite of the high success in view of

melanoma shrinkage, delay of recurrences, and lengthening of lifespan in responding patients, up to 50% of patients do not respond or do not tolerate the severe side effects that can be life-threatening, or even fatal [111]. Less commonly used in unresectable melanomas is the talimogene laherparepvec (T-VEC) [112].

A strategy demonstrated to be highly effective in a conspicuous fraction of melanoma patients is **targeted therapy**. Targeted therapy refers to treatment directed against the specific molecules that are mutated in that individual patient's melanoma cells, which are implicated in causing the malignancy [113]. For example, the proto-oncogene v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), is mutated in more than half of melanoma patients and gives rise to an altered serine/threonine kinase specifically in the cancer cells. Melanoma patients are screened for known cancer-related mutations and an appropriate inhibitor molecule is chosen to personalize the treatment schedule. There are two well-known examples of melanoma targeted therapies, which are remarkably powerful in combination [114, 115]. The BRAF inhibitor (BRAFi, e.g., Dabrafenib, Vemurafenib) in patients harboring a BRAF V600E or V600K mutation has been proven to shrink tumor size in the majority of cases, and increase patient survival by almost one year [116, 117]. The MEK inhibitor (MEKi, e.g., Trametinib) blocks the serine/tyrosine/threonine kinase MEK in melanoma cells and thereby impairs their growth and survival. In clinical trials, MEKi prolonged the survival of SIII and SIV melanoma patients [118].
Additional targeted inhibitor molecules that were originally developed for other cancer types could be used in less common melanoma subtypes that harbor specific driver mutations [119]. For instance, KIT inhibitors have been frequently employed to treat *KIT*-mutated melanomas, such as LMM, MM and ALM [119].

Traditionally, **chemotherapy** has been used, and it is still used in certain melanoma cases as a last-line combination therapy due to the severe side effects [120]. The only FDA-approved chemotherapy in melanoma is Dacarbazine (DCTI). Although DCTI showed tumor shrinkage, it has not been proven to prolong patient survival [121]. This has been suggested to be due to low expression levels of *BRAF* and neuroblastoma RAS viral oncogene homolog (*NRAS*) in patients, which limits the effect of the drug [122]. Other generic chemotherapeutics in melanoma treatment include cisplatin and carboplatin [123].

The most suitable therapies for different melanoma patients are often combined to create the best personalized treatment plan, and these therapies are continuously evaluated in view of cancer regression and patient survival in several clinical trials [124]. However, the inability to predict treatment efficacy and patient response remains an obstacle that is complicated further by tumor heterogeneity [125, 126]. Melanoma is in fact a notoriously heterogenous cancer, and this feature contributes to both acquired and intrinsic resistance mechanisms [127]. Therefore, novel approaches to tackle such heterogeneity are still needed.

Lastly, too often people tend to overlook the value of supportive care for cancer patients from the medical staff, the family and the society [128]. Such difficult situations need to be handled on emotional, social and financial levels, which also have a relevant role in coping with the treatments and the related side effects.

Melanoma Heterogeneity

In spite of the encouraging progress achieved in therapies available to melanoma patients, tumor heterogeneity remains the major obstacle for long-term effective treatment of malignant melanoma [127]. Generally, heterogeneity can be categorized in three hierarchical levels: *I.* Interpatient heterogeneity, which means that the same type of cancer differs among patients; *II.* Intertumor heterogeneity that occurs when a patient has multiple different tumors of the same type or metastases that bear different molecular features; and *III.* Intratumor heterogeneity (ITH), which is due to the occurrence of diverse genotypic and phenotypic characteristics among cancer cells within the tumor, referred as subclones (**Fig. 4A**).

In the melanoma field, a significant effort has been put to deeply investigate ITH, and to further understand the differential evolution of subclones during cancer progression, resistance and recurrence. Both endogenous and exogenous stimuli that act on cancer cells generate ITH *via* a process of selection that resembles Darwinian evolution [129, 130]. This dogma is applicable in the context of tumorigenesis: here, malignant transformation is driven by alterations that can successfully permit the survival of cancer cells bearing them. Subclones with the best fitness conclusively promote tumor growth and progression [131]. Melanoma cells have been shown to retain high degrees of plasticity from the embryonic precursor of melanocytes, the NCCs [5]. As a consequence, melanoma cells have the property to dynamically pass through different cell states by switching from proliferative to invasive phenotype or *vice versa* [132]. To measure the true extent of such ITH is particularly difficult, especially in bulk tumor analyses. Nevertheless, during the past decades, melanoma ITH has been widely addressed at molecular level (**Fig. 4B**). Melanoma features one of the highest mutational rates among cancers [46], and thus genomic studies aim to detect changes within the cancer genome [133]. Much effort has been put in the discovery of genes responsible for cancer development and progression [134]. Investigation of ITH at the genomic level involves, e.g., detection of point mutations (e.g., single nucleotide variations, SNVs), gene insertion/deletion, amplification, allelic losses (e.g., loss of heterozygosity, LOH) and karyotype aberrations characterizing the landscape of malignant melanoma. To complement genomic studies, ITH that define specific melanoma subclones can be investigated by integrated analyses of transcriptomic and proteomic data. While transcriptomics provide information on the mRNAs and transcripts [135], proteomics make it possible to detect and visualize proteins in the tumors that represent the true effectors of cell behavior [136]. A further layer of ITH

is due to epigenetic modifications. The expression of genes is regulated by epigenetic events that act without affecting the DNA sequences, and thus cannot be captured by genomic techniques. Epigenomic studies in melanoma investigate alterations mainly in view of DNA methylation occurring in specific coding and non-coding gene sites, and chromatin remodeling and histone organization processes. These epigenetic modifications are dynamic, reversible and adaptable depending on the circumstances to which the cancer cells are subjected. Therefore, they play a pivotal role in melanoma ITH by conferring high degrees of plasticity to cancer cells [137].

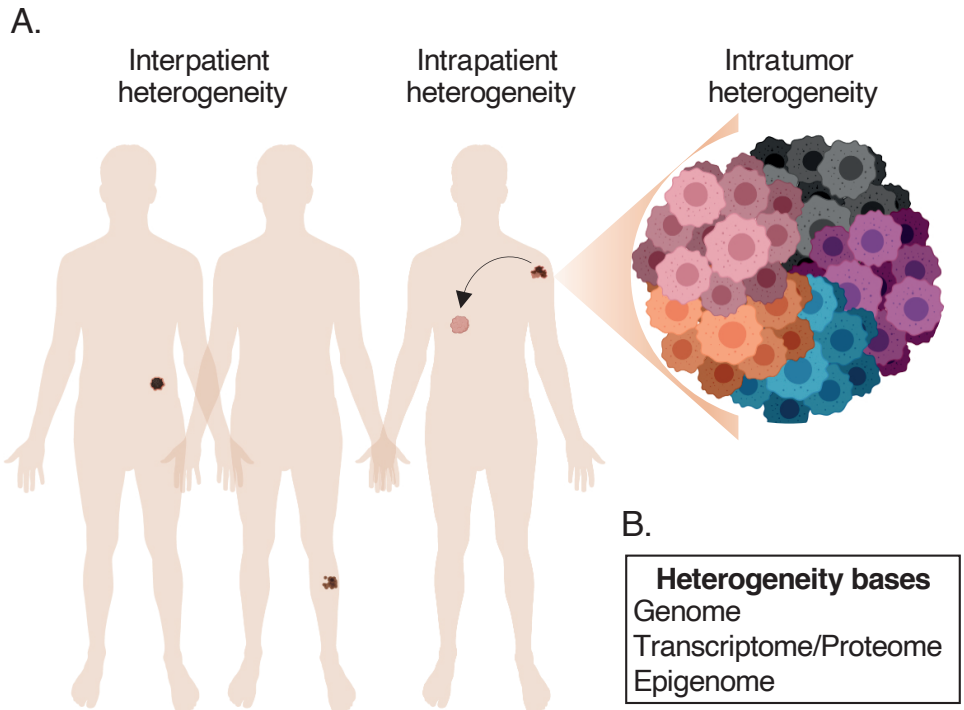

Figure 4. Heterogeneity in cancer. A. Tumor heterogeneity can be categorized in three levels: interpatient (between patients), intrapatient (between primary tumor and metastases in the same patient), and intratumor (between single cells in one tumor). **B**. Genome, transcriptome/proteome and epigenome differences generate intratumor heterogeneity.

Overall, these levels of ITH generate a complexity that is a major obstacle when treating melanoma patients. In fact, in the tumor only the sensitive cells will be efficiently targeted by specific therapeutic agents [138], while the resistant cells constitute a pool of subclones with the potential of reestablish the tumor, thus leading to recurrence [139]. In order to face this challenge, novel modern techniques, which integrate cell sorting based on biomarkers and single-cell RNA sequencing (sc-seq), are dissecting ITH at the single cell level to study cancer subclones and resistance mechanisms in melanoma in an unprecedented manner [140-143].

The Genetic Landscape of Melanoma

Cancer initiation and progression depend on genetic aberrations, known as 'drivers', which are capable of sustaining tumor growth. Finding such specific perturbations in the vast mutational landscape of melanoma is a great challenge. In fact, only a small fraction of gene mutations in the tumor represents actual drivers, while the majority of mutations are 'passengers'. Passenger mutations may contribute to oncogenesis, but they are not essential [144]. However, a recent study that characterized whole-genome data in more than 2,500 tumors from the ICGC/TCGA Pan-Cancer analysis of whole genomes (PCAWG) dataset, demonstrated that the cumulative effect of passenger mutations contributes to tumorigenesis beyond standard drivers. This suggests that aggregate putative passengers play a major role in progression of a subset of tumors. For instance, melanoma in the skin has been found to contain a large number of high impact single nucleotide variations (SNVs) [145].

In the past, the distinction between driver and passenger mutations has been commonly based on their frequency, with the assumption that driver mutations in cancer are found statistically more often than the passengers in studies involving large cohorts [146]. However, this definition can be misleading. For example, the presence of copy number variations (CNVs) in tumor cells can over- or under- estimate mutation frequencies. Another limitation of the frequency-based approach, *per* definition, is that it cannot detect driver mutations that occur at low rates. Therefore, to better identify drivers, more recent methods that systematically take into account a gene function and network by integration of genomic data have been developed [147-149]. A notorious example is the permutation-based analysis that Hodis et al. [150] used to define more than 260 driver mutations in 21 genes in melanoma in 2012. In addition, already other studies were already about to identify key genes driving melanoma development. These tend to converge towards pathways that are essential for malignant growth and survival of melanoma cells [151-155]. To date, there are few key main established melanoma driver genes. First, *BRAF*, the gene encoding the serine/threonine kinase B-Raf, is mutated in more than half of the total melanoma cases. The point mutation V600E ($BRAF^{V600E}$) occurs most commonly (80%) and mainly in younger patients with CSD^{low} melanomas. The second mutation most commonly found is in the $BRAF^{V600K}$ (20%), which is frequent in CSD^{high} of aged patients. The least common $BRAF^{V600R}$ mutation is found in approximately 7% of melanoma *BRAF* mutant cases [156]. Normally, BRAF is activated by RAS proteins that are downstream of receptor tyrosine kinases (RTKs). Then, BRAF targets MEK1/2, which signals to ERK1/2. Altogether, the RAS-RAF-MEK-ERK pathway is known as the mitogen-activated protein kinase (MAPK) pathway. Activation of this pathway ultimately results in the transcription of genes encoding proteins involved in the regulation of cell growth, proliferation and differentiation [157]. Therefore, mutations affecting BRAF in malignancies alter their

function leading to constitutive activation of MEK/ERK signaling, and thus affecting fundamental cell functions [158]. However, $BRAF^{V600E}$ mutation has been further detected in the majority of benign nevi that do not undergo malignant transformation, suggesting that mutated BRAF alone is not sufficient to drive tumorigenesis [159, 160].

Second, alterations in *NRAS* proto-oncogene typically affect Q61R hotspot mutation. Melanoma tumor harboring *NRAS* mutations are the second most frequent (15-30% of total cases) and they are found to be mutually exclusive of *BRAF* mutations [161]. Besides involvement in the MAPK pathway, NRAS has also been shown to activate the phosphoinositide 3-kinase (PI3K) pathway, which is involved in essential cell functions. Similarly to *BRAF*, *NRAS* mutation cannot solely initiate melanoma. The other two RAS proto-oncogenes important in cancer biology are *HRAS* and *KRAS*. Although these are rarely mutated in melanoma (1%), they are known driver genes of other cancer types, such as pancreatic cancer [162] and bladder cancer [163], respectively.

Neurofibromin 1 (*NF1*) mutations are common in CSD^{high} patients classified as DM subtype, accounting for roughly 15% of melanoma cases. NF1 acts as a negative regulator of RAS, therefore its inactivation lead to constitutive activation of the MAPK pathway. These tumors present with different biological and clinical characteristics than other melanoma types, and bear a particularly poor outcome for the patients [164].

Moreover, Ras-related C3 botulinum toxin substrate (RAC) proteins are GTPase essential for cytoskeleton organization and cell motility. Among RAC1 alterations, proline-to-serine mutation at codon 29 (P29S) leads to protein dysregulation and increased cell proliferation in melanoma [165]. This mutation is recurrent in up to 9% of all tumors, representing the third most common driver mutation in sun-exposed melanomas [166].

Phosphatase and tensin homolog (*PTEN*) is a tumor suppressor gene that encodes for a key regulator of the PI3K signaling pathway. The Cancer Genome Atlas (TCGA) found *PTEN* alterations in 15% of mostly primary melanoma cases [167]. However, the prevalence of *PTEN* mutations tend to be higher in metastatic disease [168]. Mutations in *PTEN* typically cooccur with *BRAF* mutations, and contribute to tumor progression *via* activation of the PI3K signaling pathway [169]. Furthermore, methylation in the *PTEN* promoter has been shown as an independent predictor of poor prognosis in melanoma patients [170].

Telomerase reverse transcriptase (*TERT*) encodes for the telomerase enzyme, that has a crucial role in the regulation of cell senescence. Up to 90% of all melanomas harbor aberrant expression of *TERT*, which results in tumor initiation by suppression of senescence in the cancer cells [171, 172]. *TERT* has been found to cooccur with *BRAF* or *NRAS* mutations and to act as an independent factor of poor prognosis in CMM [173].

Introduced previously, *MITF* is a lineage-specific gene in melanocytes and is an oncogene in melanoma. *MITF* amplification has been found in 20% of melanoma patients with poor prognoses [174]. Besides genetic modifications, MITF is heterogeneously expressed in melanoma cells, and it plays critical roles in melanomagenesis. A comprehensive view of MITF has been provided in the dedicated chapter 'The Microphthalmia-Associated Transcription Factor: MITF'.

Finally, melanoma harboring alterations in the *KIT* proto-oncogene do not display mutations in *BRAF* or *NRAS*. *KIT* drives 3% of all melanoma tumors of the CSD^{high} subtypes, including ALM and MM [175]. Mutations detected in *KIT* include L576P and K642E, which lead to constitutive activation of KIT kinase function and therefore result in hyperactivation of both the MAPK and PI3K pathways [176, 177].

Besides alterations in these driver genes, other gene mutations are implicated in the initiation and progression of melanoma. Among the most commonly mutated gene known in human malignancies, *TP53* mutations are found only in a minority of melanomas [178]. Nonetheless, alterations in TP53 regulators such as MDM2 [179] and MDM4 [180] have been reported to contribute to melanoma progression. In familial melanoma cases, around 40% of tumors harbor frequent homozygous deletion in the tumor suppressor gene cyclin-dependent kinase inhibitor 2A (CDKN2A or $p16^{INK4a}$) at the INK4a/ARF locus, that encodes for the cyclin dependent kinase 4/6 (CDK4/6) inhibitors [181, 182]. Therefore, genetic aberrations that decrease expression of $p16^{INK4A}$ impair its tumor suppressor function and in turn promote tumorigenesis [183].

Overall, the discoveries of driver mutations obtained through advances in genomic analyses of melanoma tumors provide the basis to categorize patients accordingly to their mutation patterns. Moreover, these data have been demonstrated to be essential to generate compounds against mutated molecules and to predict patients' outcomes in melanoma. The GEX subtypes discussed below reflect the main drivers of classification. Generally, melanoma tumors are in fact defined by mutations in the *BRAF*, *NRAS*, *NF1* genes, or having none (namely triple wild-type) [184].

Melanoma Gene Expression Subtypes

The analyses of GEX data from tumors have been integrated with patient clinic and histopathological features to further improve patient prognostication in the GEX profiles. GEX profiles are a useful tool to further categorize melanoma tumors according to their complex molecular features. Already twenty years ago, a publication in Nature reported the first GEX classification of 31 CMM by using microarray technology. However, in spite of the successful identification of GEX phenotypes in this cohort, the resulting subgroups had no predictive value in terms of clinical outcome for the patients [185]. With the advent of modern high-throughput techniques, such

as whole-genome, whole-exome and next generation sequencing (NGS), large GEX datasets have been produced and several research groups attempted the identification of prognostic CMM GEX signatures in both primary and metastatic lesions. For a review on non-CMM, see Rabbie et al. [184].

Due to the lack of stored tumor material to investigate, the first study addressing GEX profiles in melanoma was published only in 2006 [186]. That study was performed on 58 primary melanomas, and results have been further validated in 176 independent lesions [186]. Here, the author found 254 genes associated with metastatic potential and poor prognosis for the patients. Two years later, John et al. [187] generated GEX profiles that could discriminate prognostic groups in 29 SIII melanoma patients. These GEX profiles directly correlated with clinical outcome for these patients who would otherwise be considered a homogeneous group. This prediction, based on the expression of 15 genes, has been successfully validated in two independent melanoma cohorts with high performances [187]. In the search for an alternative source of melanoma material, Conway et al. [188] demonstrated the feasibility of using formalin-fixed tumors for GEX data analysis by using a tissue microarray (TMA) needle. The group evaluated primary melanomas stored during two different clinical trials, for a total of 472 tumors, and successfully obtained data for 74% of the patients. In this study, the group could identify osteopontin expression as a prognostic biomarker in both melanoma cohorts [188]. Subsequently, GEX profiling has been widely employed in several other studies, such as in the separation of high and low grade primary melanoma tumors [189], to classify only metastatic tumors [190], to delineate cancer progression from dysplastic nevi to advanced melanoma [191, 192], and to predict the metastatic potential and survival of SI and SII melanoma patients [193].

Moving forward in the field, Jönsson et al. [194] in 2010 clustered 57 SIV metastatic melanomas in four GEX melanoma phenotypes that could predict patient survival. These have been named according to the phenotype that characterized the melanoma subgroups, i.e., high-immune, proliferative, pigmentation and normal-like [194]. Interestingly, that study has found that expression of MITF was particularly high in the "pigmentation" group, and low in the "proliferative" subgroup. This latter was characterized by high Ki67 and low immune infiltration, and was associated with poor prognosis for the patients. By contrast, patients belonging to the high-immune group displayed strong infiltration of immune cells in the tumors and a good prognosis. The importance of having an immune-related signature for a better prognosis in melanoma has been independently shown by another study in patients with SIII disease [195]. Here, the authors generated a 46-gene GEX signature in which a high prevalence of immune response genes has been proven to predict better survival for the patients. Shortly after these studies, the TCGA published the largest study on CMM by including 333 tumors from 331 patients. Here, the above-mentioned permutation-based approach was combined with a novel software (MutSigCV), allowing further

insights into the GEX data [167, 196]. Three melanoma groups were herein defined: the keratin, the immune and the MITF-low, which characteristics resemble the GEX subtypes previously identified by Jönsson et al. [194].

Importantly, these studies show that the GEX melanoma subtypes convene on similar phenotypes. These can be summarized by the basic features of pigmentation, proliferation, invasiveness and immune infiltration [197]. With this foundation, in 2015 Cirenajwis et al. [198] followed up the four GEX melanoma phenotypes and extensively validated the prognostic molecular signature previously found. Among 214 CMM analyzed, both the pigmentation and the proliferative groups have been found to have higher risk of developing distant metastases in comparison to the high-immune subgroup. Interestingly, while MITF is highly expressed in the pigmentation group, it is low in the proliferative group. Moreover, this latter patient group was highly enriched in target therapy resistant cases. Importantly, the molecular features that characterized the proliferative and the pigmentation melanoma patients are recapitulated in cell lines cultured *in vitro*, and therefore can be used as a suitable model to study these groups further [132].

Overall, GEX profiling represents a powerful tool that integrates tumor molecular features with histopathological and clinical data of the patients to ultimately improve prognostic assessment in melanoma.

The Genetics in Melanoma Progression

Understanding the genetic changes that allow the progression of melanoma tumor from RGP to VGP is crucial to find suitable targets to block this process [199]. As already mentioned, alterations in driver genes such as *BRAF* predispose to melanoma development, but it is demonstrated to be not insufficient for cancer initiation [200]. In fact, such mutations are widely found, for instance, in benign nevi. However, in benign lesions, these mutations cause a transient increase in melanocyte proliferation that is typically inhibited *via* $p16^{INK4}$/retinoblastoma (Rb)-mediated senescence [200]. Therefore, the outgrowth may not progress to melanoma [37]. Interestingly, non-sun induced melanoma subtypes such as ALM and MM arise *de novo*, with no recognized precursor lesion [201].

To date, no definitive orders of genetic alterations that lead to melanoma development have been established. However, several studies investigated the genetic events required for cancer progression by detecting the key mutations that occur during malignant transformation [202]. In CMMs, dysplastic nevi or MIS are considered melanoma precursors. In contrast to benign nevi, these intermediate lesions harbor activating mutations in the MAPK pathway and acquire additional mutations during cancer progression. At the early stage of malignant transformation, accumulation of mutations in *TERT* promoter immortalize cancer cells, which results in unlimited proliferative

potential [203]. Further, inactivation in *CDKN2A*, which typically occurs *via* homozygous deletion, has been shown to drive melanoma invasiveness [204]. In more advanced stages, primary melanoma lesions display loss of tumor suppressor genes such as *PTEN* and *TP53* [204]. At this point, if melanoma is left untreated, it can switch from RGP to VGP *via* an increase in cyclin D1 (CD1) and E-cadherin loss [202].

Cancer cells can further acquire a high frequency of CNVs and additional chromosomal instability due to mutations in chromatin remodeling complexes. For instance, the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex has been proposed to contribute to driving melanoma progression towards VGP [205].

In spite of these lines of evidence, melanoma progression may not necessarily follow these sequentially defined growth phases. In fact, as discussed in the chapter "Melanoma Heterogeneity", different tumors and subclones of the same tumor can emerge from diverse evolutionary pathways [131]. Nevertheless, the UV-R is the predominant mutational process, which drives melanoma initiation and progression. Thus, CMM tumors commonly display the classic UV light-induced mutational signature [45]. UV damage has its maximal contribution in the establishment of the PT and then, while in metastatic disease, its contribution tends to diminish due to an increased tissue depth [206].

Melanocyte Lineage-Specific Genes in Melanoma

A tightly controlled network regulated by the coordinated activation of TFs ensures melanoblasts survival, migration and differentiation, all of which are of crucial importance for specification of melanocytes [207]. Genes important for the specification of melanocytes have been mainly discovered by studying inherited conditions, such as the WSs. Among the melanocyte lineage-specific genes, this thesis focuses on the two fundamental TFs, *SOX10*, and its downstream target, *MITF*, in relation to their central role in melanomagenesis [208, 209].

The Sex Determining Region Y-box 10: SOX10

SOX10 expression is highly conserved among the vertebrates' kingdom, and in fact it has been originally discovered in mice, which share 98% homology with the human counterpart [34, 210]. In humans, *SOX10* is part of the SRY-related HMG-box SOX protein family, which is composed by more than 30 members [211, 212]. Together with its closest relatives, *SOX8* and *SOX9*, *SOX10* belongs to the SoxE subgroup, and they have redundant functions during NC development [213-217]. On the contrary, in melanoma SOX10 displays an antagonistic function to SOX9, and their respective genes are inversely cross-regulated [217].

The *SOX10* gene is found on chromosome (chr) 22q13.1. It is composed of 5 exons: the non-coding exons E1 to early E3 generate the 5' untranslated region (UTR), the open reading frame (ORF) is split between E3 and E5, and the terminal portion of E5 corresponds to the 3' UTR [218]. No isoform of *SOX10* has been identified, and this is probably due to the DNA-binding element, which is only translated from three coding exons. As a consequence, alternative splicing, which could generate functional isoforms of *SOX10*, is unlikely to occur [219]. As suggested by the family name, the encoded protein shares similarity with the sex determining SRY proteins. SOX10 consists of 466 amino acids (aa), starts with a Dim domain, for dimerization, and ends with two transactivation domains K2 (context-dependent) and TA (main domain). Between Dim and K2, lies the important high-mobility-group (HMG) binding domain. SOX10 HMG domain is characterized by three alpha-helical regions, two needed for nuclear localization (NLS1 and NLS2), and one for nuclear exports (NES). These allow SOX10 shuttling between the cytoplasm and the cell nucleus, where SOX10 is usually found [220]. Like other SoxE members, SOX10 HMG recognizes the general (A/T)(A/T)CAA(A/T)G binding site (monomeric and dimeric). The affinity of SOX10 binding sites is defined from specific flanking sequences in the target genes [218].

Spontaneous mutations in mouse models have provided a suitable tool to study SOX10 functions. Besides in migrating neural crest stem cells (NCSCs) of the embryo, SOX10

is also found in adult NCSC populations [221]. It is also required for the maintenance of the cell lineages in both CNS and peripheral nervous system (PNS), i.e., oligodendrocytes [222] and peripheral glial [223], respectively. Underling the important function of SOX10 in the neural tissue, *SOX10* deficiencies are demonstrated to be fatal in mice at birth due to partial impairment of the nervous system function [224]. As previously introduced, besides the nerve cells, SOX10 expression is required from precursor melanoblasts towards specification of the melanocytes [225]. Therefore, homozygous mutations in *SOX10* result in complete loss of this lineage [226-228]. Replacement of the *SOX10*-functional equivalent *SOX8* in *SOX10*-deficient mice has been shown to only partially rescue the development of the nervous system, but not of the melanocyte lineage [229]. This further emphasizes the importance of *SOX10* in the development of melanocytes.

Transcriptional Network of SOX10

Resolving the transcriptional regulatory network surrounding SOX10 is at the base of understanding its biological role. To date, multiple targets controlled directly by SOX10 have been found in several cell types [230-233]. In 2008, Lee et al. [230] combined several accessible techniques to sensitively detect SOX10-direct targets in Schwannoma, a type of tumor originating from the Schwann cell population. Here, the group identified 4 novel SOX10 target genes, including proteolipid protein (*PLP*) and nerve growth factor receptor (*NGFR*). Other studies investigating Schwann cells-specific TF demonstrated that SOX10 is necessary and sufficient for activation of the ciliary neurotrophic factor (*CNTF*), which is required for maintenance of the spinal motoneurons [231]. This study highlights the importance of direct contribution of SOX10 to tissue specificity, which has been previously reported in other cases [232, 233]. For example, myelin production is known to be regulated from an extraordinary limited number of TFs. In one study on Schwann cell precursors, the induction of myelin protein zero (*P0*) has been shown to be dependent on SOX10 both *in vitro* and *in vivo* in mouse embryos [232]. In another study in oligodendrocytes, the specificity protein 1 (Sp1) and the cyclin-dependent kinase inhibitor 1B (CDKN1B or $p27^{Kip1}$) were shown to be indispensable for the expression of the myelin basic protein (MBP). However, co-transfection of *SOX10* plasmid in overexpressing CDKN2B cells can induce MBP activation even in non-oligodendrocyte cells, indicating that SOX10 confers cell type specificity on the expression of MBP [233].

Post-Transcriptional Regulation of SOX10

SOX10 is usually found in the nucleus, where it carries out its TF function on target genes. However, SOX10 has often been observed in the cytoplasm, where it is produced and retained prior to nuclear transport. Interestingly, SOX10 is proposed to have a shuttling-dependent transactivation that is essential for its role regulated by post-translational modifications (PTMs). In this context, balancing SOX10 nucleus-

cytoplasmic shuttling can be a source of temporal and spatial advantages for optimal regulation of its function [220]. This mechanism of regulation has already been observed for other TFs e.g., for the signal transducer and activator of transcription 1 (STAT1) [234]. Therefore, it is possible that SOX10 PTMs occurring in the cytoplasm can direct its activity on target genes once transported into the nucleus, and *vice versa*. Supporting this theory, SOX10 presents many potential PTM sites. However, to date only a few have been reported to be functional.
In the past 5 years, dysregulations affecting SOX10 at the post-translational level have been linked to neoplastic transformation in many cancer types, including melanoma. Firstly in 2015, SOX10 has been observed to undergo proteasomal degradation mediated by the F-Box WD repeat domain 7 (Fbxw7α), which is part of the SKP1-cullin-F-box (S1CF) ubiquitin protein ligase complex. Here, Fbxw7α ubiquitin ligase recognizes phosphorylation of SOX10 mediated by the glycogen synthase kinase 3 beta (GSK3β), which promotes SOX10 proteolysis *via* ubiquitination [235]. Noteworthy to a pathological perspective, *Fbxw7α* mutations that impair its activity on SOX10 regulation have been shown to enhance the migratory capacities of melanoma cells. In this case, deregulation of the delicate axis Fbxw7α-GSK3β-SOX10 can contribute to melanoma progression [236]. In 2018, Cronin et al. [237] investigated SOX10 phosphorylation sites by mass spectrometry analyses in melanoma cells. The group validated the already identified phosphorylation sites of SOX10, and discovered new functional sites, including S224, S232, T240 and T244 [237]. Phosphorylation at these sites has been demonstrated to affect SOX10 protein function in melanoma, and in other cancers, i.e., triple negative breast cancer [238], glioma [239], and hepatocellular carcinoma [240]. Also in 2018, ERK2 has been discovered to phosphorylate SOX10 in two of the newly identified sites, the T240 and the T244 [241]. Importantly, this study also revealed that there is cross-regulation between SOX10 PTM sites in melanoma. For instance, sumoylation of SOX10 has been shown to interfere with its phosphorylation in mutant *BRAF* melanoma [241]. Critically, this mechanism results in cell malignant behavior due to SOX10 transcriptional activity on the TF forkhead Box D3 (FOXD3) [242]. In fact, FOXD3 mediates adaptive resistance towards RAF inhibitors in these BRAF melanoma mutants. Therefore, phosphorylation of SOX10 mediates activation of FOXD3, which is responsible for resistance to targeted therapy [243].

Among PTMs, one of the crucial epigenetic mechanisms controlling gene function is DNA methylation. In normal cells, a tightly controlled methylation processes that regulates gene expression is necessary for basic biological processes such as X-chromosome inactivation in males and genetic imprinting [244]. In cancer cells, this system can be hijacked to enhance malignant behavior by upregulation of oncogenes, and downregulation of tumor suppressors, *via* hypo- or hyper- methylation, respectively [245]. Several methods have been developed to enable the detection of methylation patterns in diseases and carcinogenesis. Two popular techniques in current

use are bisulfite conversion-based DNA microarray and genome-wide DNA methylation analysis [246]. Using these methods, the methylation landscape that characterizes a variety of tumor types has been obtained, and it has been identified as CpG island methylator phenotype (CIMP) [247]. CIMP have been recently established in glioma [248], non-glioma brain tumors [249] including melanoma-brain metastasis [250] and colorectal cancer [251].

DNA methylation occurs almost exclusively in the context of cytosine-guanine dinucleotide (CpG) rich islands, at the fifth carbon of cytosines (5-methylcytosine, 5-mC) and it is catalyzed by DNA methyltransferases (DNMTs) [252-254]. The CpG island in the *SOX10* gene spans the promoter, the first two exons and the first intron. Aberrant hypermethylation at these CpG sites correlates with silencing of *SOX10* expression, and results in a variety of NCCs-related disorders and tumor types [255]. For instance, downregulation of *SOX10 via* methylation in oligodendrocytes has been reported to contribute to brain tissue dysfunction in schizophrenia [256]. Interestingly, from a clinically translational perspective, it was recently published that detection of *SOX10* promoter methylation in blood can be used as a noninvasive method for efficient diagnosis of intestinal neuronal dysplasia (IND) [257]. In many cancer types, hypermethylation of *SOX10* promoter has been shown to impact patient survival. For instance, patients with aggressive glioblastoma subtypes that harbor *SOX10* hypermethylation coupled with its downregulation, have a shorter lifespan [258]. Moreover, the CIMP characterizing gliomas with high risk of recurrence showed enrichment in *SOX10* hypermethylated cases [259]. Besides being involved in nervous system-related disorders, *SOX10* inactivation *via* methylation has been associated with multiple digestive system malignancies, including colorectal, gastric and esophageal cancers [260-262]. Furthermore, in bladder cancer patients, downregulation of SOX10 *via* hypermethylation has been linked to an increase in lymph node metastases. Strikingly, in this study the author also showed that pharmacological demethylation of *SOX10* could restore its tumor suppressor function and inhibit proliferation, invasion and migration of aggressive bladder cancer cell lines [263].

In melanoma, the importance of aberrant DNA methylation started to be appreciated as an epigenetic hallmark in recent times. Thereafter, melanoma-associated CIMP has been proposed as an indicator of tumor metastatic potential in this cancer type [264-270]. In 2009, the first unbiased effort to determine methylation patterns in melanoma was successfully performed [271]. By integrating the genome-wide promoter methylation profiles and GEX data from human melanoma cell lines and normal melanocytes in newborns and adults, Koga et al. [271] identified 76 novel methylation markers. In the same year, the methylome associated with melanoma development, and a clinically significant CIMP pattern important for cancer progression, have been published [272]. To date, more than 70 tumor suppressor genes have been found to be hypermethylated in CMM, and associated with tumorigenesis [273-275]. In view of

the debated role of SOX10 in melanomagenesis, methylation studies on *SOX10* and related genes have been limited and contradictory. For instance, several studies have reported that the melanocyte differentiation factors SOX10, PAX3 and KIT have increased expression in primary melanoma lesions, which is further followed by a gradual upregulation during tumorigenesis [276-278]. By contrast, these and other factors (i.e., *MITF* and *OCA2*) have been found to become methylated and downregulated during melanoma progression [279]. Supporting this evidence, it has been recently observed that melanoma cells harboring *SOX10* suppression are resistant to targeted therapy *via* transforming growth factor-beta (TGF-β) signal cascade [280].

In line with these findings, new biomarkers based on DNA methylation have been proposed [281-283]. However, further studies to address methylation changes supporting melanomagenesis are still warranted prior to raising the possibilities of new methylation-based therapeutic opportunities in melanoma.

Downstream Targets of SOX10

In spite of the pleiotropic role of SOX10, activation of several target genes requires synergy with other TFs. SOX10 binds to the gene promoters in a coordinated spatial and temporal way with other TFs at adjacent sites on DNA molecules. This mechanism ensures tissue-specificity of SOX10 function depending on the TF's partner in different cellular contexts [284]. The first example of SOX10 transcriptional cooperation was originally described more than 20 years ago by Kuhlbrodt et al. [285] in rats. This study showed autonomous failure of SOX10 modulatory activity in the absence of other TFs during the development of glia cells, i.e., early growth response 2 (EGR2), class III POU homeobox (POU3F) 1 and PAX3. Important for tissue-specificity, the synergy between SOX10-ERG2 observed in this study has been demonstrated to be responsible for the transactivation of the glia-specific promoter connexin32 [285]. The cooperation between SOX10 and PAX3 has been widely elucidated in other studies addressing WS. In these patients, a failed synergistic activation of the c-RET enhancer by mutations in *SOX10* or *PAX3* has been observed [286]. Besides their involvement in WS, PAX3 and SOX10 transcriptionally regulate c-RET during enteric ganglia formation, together with the NK2 Homeobox 1 (NKX2-1) and the paired like homeobox 2B (Phox2b) [287, 288]. The domain's requirements for the physical interaction between SOX10-PAX3 was discovered only in 2003 in a murine model [289]. Since mutations in SOX10 and PAX3 are associated with the emergence of WS, the demonstration that these genes can interact directly has been an important step in understanding the molecular bases of these syndromes [286]. However, alterations in *SOX10* and *PAX3* do not explain the auditory–pigmentary abnormalities seen in certain WS subtypes. Such defects are instead the result of impaired regulation of one of the most important SOX10 targets in melanocytes, *MITF* [290]. The hierarchy of these three TFs in relation to the emergence of WS has been further evaluated by systematic deletion of

gene promoter in a mouse model [291]. Already during embryogenesis, SOX10 in melanocytes can directly activate DCT. Alternatively, it can cooperate with MITF to transactivate DCT by sequential execution of a regulatory network [292, 293]. This consists of PAX3-SOX10, which synergistically activates *MITF*, which in turn cooperates with SOX10 to activate *DCT* [294]. In melanoma, the DCT antigen has been associated with tumors that are refractory to chemotherapy. Therefore, dysregulation of the PAX3-SOX10-MITF-DCT axis has important implications in therapy resistance mechanisms [295-297].

SOX10 Alterations in Diseases and Cancer

In view of the ubiquitous expression of SOX10, several germinal and somatic mutations in this gene have been discovered upon the emergence of mainly neurocristopathy disorders [298-301]. For instance, the autosomal recessive conditions, WS4, associate with *EDN3/EDNRB* mutations [302, 303] and are inherited in autosomal dominant pattern if *SOX10* is also mutated [304-306]. Other mutations in *SOX10* can cause the pigmentation anomalies seen in the Yemenite deaf-blind hypopigmentation syndrome, which can manifest with different grades of neurologic involvement [298]. Besides haploinsufficiency, alterations affecting SOX10 expression have been associated with a variety of cancers of the nervous system. For example, dedifferentiated subtypes of Schwannomas tumors show decreased SOX10 expression and harbor an aggressive tumor phenotype [307]. By contrast, SOX10 is widely expressed in gliomas (i.e., astrocytoma [308], oligodendroglioma and glioblastoma [309]). Here, SOX10 acts in synergy with the platelet derived growth factor beta (PDGFB) to induce gliomagenesis. Then, SOX10 expression is progressively lost during cancer progression [309]. Besides the nervous system, alteration of *SOX10* are involved in other tumor types. In clear cell sarcoma (CCS), the fusion Ewings sarcoma/activating transcription factor 1 (*EWS/ATF1*) genes occupy the *MITF* promoter and recruit SOX10 to enable MITF oncogenic activation [310]. Similarly, SOX10 has been recently discovered to play oncogenic functions in human hepatocellular carcinoma (HHC) by stabilizing the TCF4/β-catenin complex [240]. Finally, it is known that SOX10 promotes melanoma development, and for this reason SOX10 expression is typically used as a sensitive antigen for melanocyte neoplasms [276, 311-313]. However, recently a new role for SOX10 loss has been emerging in a fraction of aggressive melanomas. Melanoma cells that lack SOX10 are characterized by a dedifferentiated melanoma phenotype and feature high resistance to therapies [139, 314]. In fact, reduced expression of SOX10 has been observed to confer resistance towards MAPK inhibitors (MAPKi) by decreased expression of MITF and increased expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and AXL receptor tyrosine kinase signaling [315]. Low expression of SOX10 also contributes to transcriptional downregulation of ring finger protein 125 (RNF125). Low RNF125 is associated with high expression of RTKs and destabilization of the RNF125-substrate janus kinase 1 (JAK1), leading to

BRAFi-resistant melanomas [316]. In support to these findings, combinations of JAK, EGFR and BRAF inhibitors can sensitize melanoma cells *in vitro* and *in vivo* [143, 280, 316].
Overall, contradictory findings on the role of SOX10 have been reported, particularly in melanoma. This is presumably due to the analyses being done at different stages of the disease, or whether evaluating adaptive or acquired resistance mechanisms. It is important to consider that SOX10 expression and activity may be differentially regulated in melanoma cells to initiate or to sustain tumor progression, depending on the context and stimuli. Therefore, only by thoroughly understanding its regulation, functions and network, it will be possible to establish a more comprehensive view of SOX10 in melanoma.

The Microphthalmia-Associated Transcription Factor: MITF

The first observation of an *MITF* mutant was documented in 1942 by the German biologist Paula Hertwig during studies on the effects of X-rays on the offspring of irradiated mice [317]. Thereafter, many other *MITF* mutants, harboring different phenotype depending on the allele(s) affected, have been reported in several vertebrate species [318-320]. *MITF* mutants are characterized by loss of neural crest-derived melanocytes, and thus present with coat color whiteness, white spotting, deafness and microphthalmic (abnormally small) eyes [321]. The gene responsible for these traits was originally cloned less than 30 years ago in a mouse model by transgene-insertion at the *MITF* locus [319], and shortly thereafter it was shown in humans [322]. Since then, *MITF* has been one of the most extensively characterized genes within the MYC superfamily. Together with the transcription factor binding (TFE) members TFEB, TFEC and TFE3, they constitute the MiT family [323].

MITF is located on the chr 3p14.1-12.3, and it consists of 9 exons, each having its own transcriptional start site. The complex arrangement characteristic of the *MITF* gene allows the generation of multiple transcripts [324]. The exons 2 to 9 are usually found in all MITF transcripts, and they encode for the transactivation domain (TAD), the domains helix-loop-helix and leucine zipper (b-HLH-LZ) motifs, and the target sites for PTMs. The b-HLH-LZ domain shares high homologies among the MiT family, and permits functional heterodimerization of MITF with other TFE members [325].

MITF domains are common to at least ten MITF protein isoforms [326], the: MITF -A [327], -B [328], -C [329], -D [330], -E [331], -H [332], -M [319], -MC [333] -J [334] and -CX [335]. The difference among protein isoforms lies at their N-termini. These are encoded by the isoform-variable exon 1B1b, which is implicated in protein cytosolic retention. Except for the melanocyte-specific splice form MITF-M, each isoform-specific promoter is generated by alternative splicing of the first exon 1B (1B1b). Then, transcription of exon 2 to 9 encoding the above-mentioned domains follows [324]. The *MITF-M* transcript lacks the exon 1B1b. Subsequently, the exon

1M splices directly the functional exons 2 to 9. This transcript produces a shorter protein of 419aa, which weighs approximately 50 kDa and up to 65 kDa in case of PTMs. In contrast to other 1B1b-encoded isoforms, the MITF-M protein has been displayed to have higher transcriptional activity because of reduced shuttling out from the cell nuclei [324]. Although not all functions of the MITF isoforms are understood, their differential expression has been characterized in several tissue types [336-344]. Most MITF isoforms (e.g., MITF -A, -B, -E, -H, -J) are ubiquitously expressed in various cell types, while others are restricted to one cell lineage only [336]. For instance, osteoclasts express both MITF -A and -E isoforms [337, 338]; both cardiomyocytes and mast cells express the MITF-H isoform [339-341]; while the MITF-A and MITF-MC isoforms are found only in mast cells [342]; similarly, the MITF-CX isoform is expressed only in cervical stromal cells [335]. The MITF-M is an established melanocyte lineage-specific isoform, and it is therefore restricted only to melanocytes [343, 344]. However, in 2010, an MITF-M variant that harbor two deletions in exons 2 and 6 was discovered. This novel isoform is named MITF-Mdel and it has been detected in melanocytes, and proposed as candidate biomarker in melanoma cells [345]. Interestingly, and in contrast to previous beliefs that MITF-M is exclusively expressed in melanocytes [327, 329, 346], the expression of this isoform has been recently observed in the pigmented epithelial cells of the adult retina (RPE) [347].

Transcriptional Network of MITF

The role of MITF-M (hereafter MITF) in the melanocyte lineage reaches far beyond the pigmentation purpose, it is in fact an essential molecule for their entire cell biology. For this reason, MITF levels have to be stringently controlled at transcriptional, translational and post-translational levels. Additionally, MITF function is coordinated in a spatial and temporal manner, which is critical to determine appropriate cell responses [348]. Several key TFs have been reported to directly control MITF transcription in melanocytes. These can be broadly distinguished as positive and negative regulators of MITF. However, in melanoma cells the function of MITF-regulatory TFs, and in turn of MITF, is altered to promote malignant behavior [349].

Positive Regulators of MITF

A variety of TFs can bind to specific promoter regions of the *MITF* gene, i.e., the proximal promoter and the cis-regulatory elements, to induce its transcription. First, the two key factors of the Wnt signaling pathway, the lymphoid enhancer-binding factor (LEF1)/TCF4 complex and β-catenin [350], are known to induce *MITF* transcription in melanocytes [351]. The signal cascade leading to MITF activation is initiated by binding of WNT1/WNT3 to the Frizzled receptor in the melanocyte membrane, resulting in β-catenin release and transport to the cell nucleus [352]. In the melanocytes' nuclei, LEF1/TCF4/β-catenin bind *MITF* and activate its transcription [353-355]. Importantly, a study showed that differential expression of LEF1 and TCF4 in melanoma can enhance cell malignant behavior by deregulation of *MITF* translation independently from β-catenin expression [356]. Second, as described in the chapter

"The Melanocyte Lineage", *MITF* can be induced to stimulate melanin production by phospho-CREB upon α-MSH binding to MC1R [14, 357-359]. Interestingly, in this instance *MITF* transcription can be enhanced by cooperation of CREB with SOX10. Since CREB is ubiquitously expressed in several cell types, SOX10 is expressed only in certain cell types, and MITF-M is specific to melanocytes, this process represents an elegant example of cell-specific synergic transcriptional control [360]. Besides this crosstalk, SOX10 has been shown to transactivate the *MITF* promoter up to 100-fold, and it is therefore considered one of the strongest activators of *MITF* [291]. Such powerful and direct induction of *MITF* by SOX10 has been widely demonstrated in *in vivo* models, including in mouse and zebrafish [290, 291]. In humans, SOX10 has multiple putative sites for *MITF* binding. SOX10 has high affinity in the region between 268 and 262 bp of the *MITF* promoter, while it binds the 298 bp site at the *MITF* enhancer region [286, 290, 291, 361]. Additional synergic interaction of SOX10 with other key TFs to enhance *MITF* targeting has been observed, i.e., together with PAX3 [362]. The common genetic pathway linking SOX10 and PAX3 converging on *MITF*, became clear when observing the phenotypes of mutant mice and human WS counterparts [363]. Importantly, upregulation of MITF by PAX3 is required for development and survival of the melanocyte lineage during embryogenesis. Therefore, alterations affecting PAX3 reduce MITF expression, and that results in partial loss of the melanocyte functions. Mutations in the PAX3 gene only give raise to specific subtypes of human WS [364], such as WS1 and WS3. WS1 is associated with craniofacial deformities [365], and WS3 is characterized by spina bifida and exencephaly, which resembles the homologous mouse Splotch phenotype [366]. Similar outcomes in *KITL/KIT* mouse mutants underlie the contribution of these factors in the regulation of MITF expression [367]. In contrast to the role of PAX3 in melanocytes, knockdown (KD) of PAX3 in metastatic melanoma cell lines does not reduce MITF expression [368]. However, there is still an open debate on PAX3 function in melanoma cells [369]. Then, besides the previously described implication of the EMT-activating TF ZEB2 during embryogenesis in the NCCs [370], recent studies have discovered a new function for ZEB2 in differentiated melanocytes [371]. A study showed that conditional knockout of ZEB2 lead to a dramatic downregulation in expression of MITF and related downstream targets in a mouse model. These mice display congenital pigmentation loss due to impaired differentiation of the melanocyte lineage, thus demonstrating ZEB2-mediated *MITF* activation in adult melanocytes. Interestingly, the ZEB2-homologous ZEB1 has been shown to repress MITF expression in the RPE [372], indicating that a similar role for ZEB1-mediated MITF downregulation in melanocytes may be plausible. Finally, chromatin remodeling complexes have a fundamental role in the expression of genes, including *MITF*. Several studies have reported the remodeling SWI/SNF to be necessary in the induction of MITF expression in melanoma cells [373, 374]. Critically in view of melanoma progression, a study pointed out that loss of the SWI/SNF-related matrix associated actin dependent regulator of the chromatin subfamily A member 4 (SMARCA4 or BRG-1) can negatively affect the survival pathways beyond the MITF cascade in melanoma cells [375]. In view of these lines of evidence, targeting the SWI/SNF complex has been proposed as a potential strategy for melanoma treatment [374, 375].

Negative Regulators of MITF

One important TF that negatively regulates MITF expression is the TGF-β. In melanocytes, TGF-β can significantly inhibit melanin synthesis *via* downregulation of MITF and its downstream targets TYRP1/2 [376, 377]. Additionally, TGF-β plays a prominent role in the maintenance of the non-cycling melanocyte stem cell (MSC) population in the bulge niches by suppression of MITF [378, 379]. Biologically, low levels of MITF in the bulge are required to sustain the MSCs self-renewal capacities and prevent the melanocyte terminal differentiation program [380]. Besides TGF-β-mediated inhibition of MITF, the apoptotic regulator BLC2 has been proven essential to MSCs survival in the bulge niches [381, 382]. In melanoma cells, TGF-β has been identified as the direct upstream target of the MITF-antagonist, glioma associated oncogene family zinc finger 2 (GLI2). TGF-β-mediated GLI2 activation has been shown to increase the plasticity and the invasive potential of melanoma cells [383, 384].

Another molecule that plays a key role in melanocyte differentiation by modulation of MITF is POU3F2 [385]. Importantly, melanoma cells with an altered PI3K signaling pathway can direct POU3F2 to strongly repress MITF expression, which in turns enhances the invasiveness of the cancer cells [386-389]. Interestingly, it has been shown that PAX3 can control POU3F2 expression, supporting the previously reported negative regulation of MITF by PAX3 in this cancer [369, 387]. Further linking POU3F2 and MITF, inverse expression of these factors can activate or repress, respectively, the NOTCH signaling pathway in melanoma cells, which confers melanoma sphere-forming capabilities (melanospheres). In this experimental setting, melanospheres were shown to resemble more closely the heterogeneity present in tumors grown from cell lines in mouse xenograft and, most importantly, in human melanoma tumors [390].

Additional negative regulation of MITF takes place in the MSCs residing in the niches of adult tissues, in which the level of oxygen is relatively low – from 5% down to 2%. Here, cells achieve a decrease in proliferation by expressing the hypoxia inducible factor 1 alfa (HIF1α). HIF1α has been shown to inhibit MITF expression by inducing the deleted in esophageal cancer 1 (DEC1) factor. Subsequently, DEC1 binds to the *MITF* promoter and suppresses its expression [391]. Importantly, HIF1α has been observed to play a pro-survival role in melanoma cells. Here, MITF has been found to reciprocally bind HIF1α promoter and strongly stimulate its transcriptional activity to sustain melanoma progression [392].

Finally, the histidine triad nucleotide binding protein 1 (HINT1) is considered a tumor suppressor in melanoma. HINT1 has been shown to promote non-functional complexes that inhibit both MITF and β-catenin expression in melanoma cells, which reduces their malignant behavior [393].

Post-Transcriptional Regulation of MITF

Linking transcriptional and post-translational regulation of MITF, the MITF transcript can be controlled by small non-coding RNAs (sRNAs), long non-coding RNAs (lncRNAs), microRNAs (miRNAs or miR) and mRNA-binding proteins. Several molecules have been demonstrated to suppress MITF translation by binding of the MITF 3'UTR, such as miR-101 [394], miR-137 [395, 396], the TP53-dependent miR-182 [397], miR-148 [398], miR-218 [399], and the coding region determinant-binding protein (CRD-BP) [400]. Due to their important regulatory function, altered expression of these molecules can affect melanoma development and progression. For example, the two lncRNAs survival associated mitochondrial melanoma specific oncogenic non-coding RNA (SAMMSON), and the disrupted in renal carcinoma 3 (DIRC3), have been proven to regulate both MITF and SOX10 in melanoma [401, 402]. The first has a role as lineage addiction oncogene co-gained with MITF [401], and the second has been suggested to have tumor suppressor function by activation of the insulin like growth factor binding protein 5 (IGFBP5) [402]. Collectively, non-coding RNAs represent an important new class of targetable regulatory molecules proposed as candidate therapeutics in melanoma, and in other cancer types [403].

Another layer of control of MITF occurs at the post-translational level. PTMs contribute to the regulation of the function of MITF, which also influences its cooperation with other TFs [348]. The activation of MITF *via* MAPK downstream signaling is among the best characterized pathways that regulate melanocyte development. Briefly, initial binding of the KIT ligand (e.g., SCF) to c-KIT receptor tyrosinase, induces the RAS-RAF-MEK-ERK1/2 cascade, which results in MITF phosphorylation at S73 and S409 sites by the ribosomal protein s6 kinase ($p90^{RSK}$) [404]. Biologically, phosphorylation at both of these sites leads to polyubiquitination and degradation of MITF, as seen in the case of UBC9-dependent ubiquitylation at K201 [405]. On the contrary, MITF deubiquitylation by USP13 has a protective effect on MITF levels [406]. Besides, phosphorylation at S73 has been identified to mediate MITF selective association to the histone acetyl transferases p300/CBP and increase MITF activity [407]. On the contrary, MITF phosphorylation at S409 has been observed to induce SUMO1 attachment in lysins K182 and K316, which results in repression of MITF [408, 409]. Other proposed sites for MITF phosphorylation include S298, which is targeted by GSK-3β in elevated cAMP levels [410, 411] and S307, which is phosphorylated in response to NF-κB ligand signaling [412].

Conclusively, a delicate balance regulates MITF protein threshold to orchestrate the multitude of functions of this master melanocyte regulator. Likewise, deregulation of MITF levels can contribute to neoplastic transformation of melanoma cells, and enhance tumor progression.

Similarly to *SOX10*, *MITF* methylation occurs at two CpG island sites in the *MITF* promoter. Hypermethylation of MITF promoter CpGs has been shown to be significantly and negatively correlated with MITF mRNA expression in melanocytes that reside in the feather bulbs in ducks [413]. Few dedicated studies have investigated the methylation patterns of genes important in cutaneous melanoma, i.e., *KIT* [414], *DIA1* [415] and *SOX9* [416]. Although methylation-mediated silencing of these genes has been linked to an enhanced MITF activity and malignant cell behavior, little is known about the direct involvement of *MITF* methylation in melanoma.

To address this lack of knowledge, in 2015, Lauss et al. [417] obtained methylation data by genome-wide DNA methylation analysis in SIV melanoma patients. In that study, the group evaluated the tumor methylation patterns in relation to matched GEX data from the tumors. The analyses identified three distinct methylation groups, namely MS1, MS2 and MS3, which to some extent resembled the melanoma phenotypes that the researchers had previously found by unsupervised hierarchical clustering of global GEX data in melanoma [194]. The MS diverged in phenotypes and in immune cell content. Interestingly, the MS1 methylation-group had a global demethylated profile, but focal promoter hypermethylation, and more importantly, patients in this group, had poor prognosis [417]. Following-up this study, the genes primarily regulated by DNA methylation in this cohort have been identified [269]. *MITF* was one of the genes found to be significantly differentially methylated in this melanoma cohort. A total of six methylated CpGs at TSS or in proximity in the *MITF* gene have been found in association with decreased MITF expression. Interestingly, the methylation patterns of MITF downstream targets, *MLANA* and *TYR* display the same trend of *MITF* methylation. Results of this study could be further validated in the TCGA dataset. Strikingly, pharmacological demethylation has been shown to partially rescue MITF activity in melanoma cell lines harboring low levels of MITF [269]. However, exogenous over-expression of MITF in MITF-hypermethylated melanoma cell lines did not induce MITF function [269], which indicates that different biological mechanisms may be controlling MITF in this cell subtype. Importantly, melanoma cells with low MITF levels have been demonstrated to be more invasive and drug resistant than the MITF-high expressing counterpart [418]. In line with this observation, acquired resistance to MAPKi therapy is associated with a decreased MITF expression in resistant cells [315, 419, 420]. Herein, *MITF* promoter methylation is seen as a plausible mechanism for cancer cells to dynamically switch towards an MITF low melanoma state, which would promote malignant behavior and allow these cells to survive inhibitor treatments. Appealing from a clinical point of view, such methylation events can be a potential target of novel melanoma therapeutics [265, 421].

Overall, these findings provide evidence that methylation has a considerable importance in the regulation of MITF and globally affects the melanoma phenotype. However, further investigation of the methylome characterizing aggressive melanoma and its implications on tumor progression and resistance to therapy are urgently needed.

Downstream Targets of MITF

The transcriptional activity of MITF is carried out by binding to the promoter region of target genes *via* the canonical DNA sequences M-box (5'-TCATGTG-3') and symmetrical E-box (5'-CACGTG-3') [422]. For efficient DNA binding, MITF can homodimerize, or form heterodimers restrictively with the family related proteins TFE3, TFEB and TFEC [325]. Similar heterodimer formations have been observed for MITF and the TFE proteins, e.g., MITF and TFE3 during osteoclast development. This indicates that heterodimeric interaction of MITF-TFE is not always essential [423].

Several potential target genes for MITF have been progressively discovered over the past 15 years [424]. MITF signals downstream towards a large variety of pathways that thoroughly control the biology of melanocytes, and this is why MITF is considered the master regulator of the melanocyte lineage [343]. Besides promoting melanocyte differentiation from unpigmented precursor melanoblasts, MITF dictates cell growth, proliferation and survival of both melanocytes [207] and melanoma cells [348, 425]. In 2008, Hoek et al. [426] used a two-DNA microarray-based approach to expand the list of the MITF target genes in melanoma cells. Analyses of GEX data identified more than 70 novel MITF targets, underling the wide range of pathways in which MITF is involved. The main downstream targets of MITF can be broadly categorized accordingly to their functional role in the cell.

The MITF melanoma differentiation antigens (MDA) target genes TYR, TYRP1/2, MLANA, aurora kinase B (AURKB), SNAI2 and PMEL play a critical role in melanocyte **differentiation** and **melanin production** [427]. The differentiation of melanocytes also depends on the cooperation of the MITF target $p21^{Cip1}$ with Rb and $p16^{INK4a}$ [428-430]. Strikingly, melanoma cells negative for MITF have concordant downregulation of MITF targets such as TYR and TYRP1/2, resulting in a further dedifferentiated phenotype biologically different from the more frequently observed MITF-high melanomas [431].

Cell cycle progression and **proliferation** of melanocytes are regulated by the MITF targets CDKs (e.g., CDK2 [432]). MITF can also target the T-Box TF 2 (TBX2) to suppress senescence *via* $p21^{Cip1}$ inhibition [433, 434]. In melanoma cells, high levels of MITF can induce DIA1 expression and increase cell proliferation. On the contrary, low MITF melanoma cells have reduced proliferation and increased invasiveness due to $p27^{Kip1}$ expression [415].

MITF controls melanocyte **survival** by targeting the antiapoptotic proteins (IAPs), B-cell lymphoma 2 (BCL2) [381], the BCL2 Related Protein A1 (BCL2A1), and the melanoma-associated IAP (ML-IAP) Livin [435]. As described previously, MITF has been shown to upregulate HIF1α in melanocytes in hypoxia [392], and to induce the apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1) in response to ROS [436].

Melanocytes **motility** can be modulated by MITF *via* MET proto-oncogene receptor tyrosine kinase (c-MET) targeting [437]. While in transformed melanoma cells, MITF upregulation of SNAI2 has been demonstrated to be critical to determine their metastatic potential [438].

Altogether, the considerable MITF downstream targeting capabilities ensure proper functional biology of the melanocyte lineage. Similar mechanisms can be controlled to enhance melanoma progression from neoplastic cells. Therefore, identification and understanding of the MITF network is at the base of finding potential targets in melanoma treatment.

MITF Alterations in Diseases and Cancer

In view of the essential role of MITF in the specification of melanocyte from precursor melanoblasts, mutations in the *MITF* gene result in abnormal melanocyte development [439].

Specifically, in humans, mutations in *MITF* can cause the allelic conditions WS2 and Tietz syndrome [440, 441]. WS2 symptoms include patchy pigmentary alteration in eyes, hair and skin and variable degrees of deafness [440]. Patients with Tietz syndrome are characterized by albinism and complete deafness disorder [441]. These syndromes are both listed in the rare disease registries in the US (Office of Rare Diseases Research [442]), and in Europe (Orphanet [443]). Strikingly, while MITF mutations typically impair MITF function in melanocytes, in melanoma cells somatic (e.g., E87R, L135V, L142F, G244R and D380N [314]) and germline (i.e., E318K [444]) mutations have been observed to enhance MITF function [445]. For instance, the only ascertained MITF germline mutation observed in melanoma is found in a putative SUMOylation site for MITF, at E318K [444]. Subsequently, mutations at this site have been demonstrated to prevent the sumo-mediated proteasomal degradation of MITF, and in turn to increase its transcriptional activity [446]. Although recurrent mutations in MITF can predispose to both familial and sporadic melanoma, these have been found in a relatively small subset of patients [444, 447]. However, MITF contribution to melanoma development has been associated with other alterations, i.e., *MITF* oncogenic amplification, which is found in 20% of melanoma cases [174].

Overall, alterations which affect MITF levels, result in deregulation of MITF function as simplified by the "rheostat model for MITF function" [448]. This model has been proposed to delineate the central role of MITF in melanoma initiation and progression. Interestingly, the MITF rheostat model implies that melanoma cells may be able to dynamically switch through proliferative-to-invasive states in response to a variety of stimuli in order to adapt to the tumor microenvironment (TME). This process, known as "phenotype switch", can promote melanoma metastatic potential and resistance to treatments [418, 449]. However, the biological mechanisms underlying this process are still widely unknown.

Melanoma Plasticity and Phenotype Switch

There is an accumulating body of evidence that melanoma complexity is driven by the ability of the single cells to undergo phenotype switch [418, 449]. This phenomenon is thought to allow cells to switch between proliferative and invasive properties [415] to escape from immune surveillance and to resist drug treatments [419]. This ability is largely dependent on the expression levels of MITF, which set the rheostat towards distinct phenotypes [450] (**Fig. 5**). According to this model, high levels of MITF are associated with a differentiated melanoma state, mid-levels promote cell proliferation, low levels generate invasive and MSCs-like phenotypes, and lastly, MITF loss induces cell senescence [451]. It has been suggested that pigmented cells have an innate ability to revert back to the state of self-renewing NC-like progenitors [452] and that is probably because of intrinsic properties retained from their pluripotent NCCs origin [5, 37, 207]. In the MITF-low side of this continuous spectrum, the melanoma subpopulation with stem cell-like properties represent a minority of cells in the tumor [139, 453]. However, these cells are found enriched in melanoma tumors that are resistant to treatment [454, 455], highly metastatic [456, 457] and in recurrent cases [458]. Importantly, this melanoma tumor subtype bears a poor prognosis for the patient [459, 460].

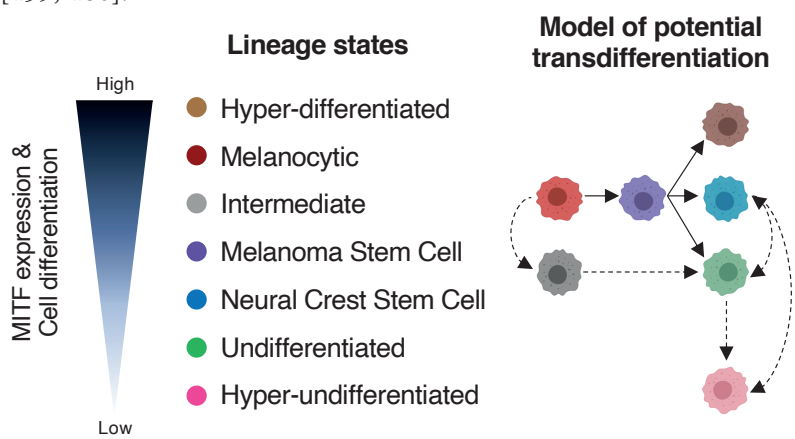

Figure 5. Melanoma differentiation model in relation to MITF expression. The activity of MITF is associated with distinct melanoma lineage states (left panel) and phenotype switch is depicted by a potential transdiffrentiation model (right panel); legend is indicated by the color of the respective cell population.

In summary, melanoma cells exhibit an exceptional phenotypic plasticity by steering the levels of the master melanocyte regulator, MITF, and related targets. Further dissection of the biological mechanisms behind the regulation of MITF and the MITF-related genes represents an important step towards a better understanding of phenotype switch in melanoma. Ultimately, this could open new therapeutic opportunities to eradicate therapeutic-resistant melanoma cells in the tumor with the aim to prevent its recurrence.

Modelling Melanoma in the Lab

Scientists can rely on a countless number of laboratory techniques that have been optimized for a variety of purposes over the course of experimental development and research. The most widely used technique to extrapolate clinical findings and address research questions is the relatively simple and direct approach of the *in vitro* assays. Although adherent cell cultures in two-dimensional (2D) systems may not be able to fully resemble the more complex *in vivo* situation, they are generally an excellent, cost-effective and an ethical alternative to gain valuable information. In melanoma, 2D culture methods are extensively used to obtain important information in regard to cell biology. This is mostly done by isolation and expansion of monolayers of melanoma cell lines collected from patients' tumors. Subsequently, the vast majority of *in vitro* assays can be performed to carefully characterize molecular and phenotypic characteristic of the cells. In particular, most studies on melanoma cells *in vitro* include assessing their aggressive potential, represented by proliferative and invasive rates, and assessing sensitivity to compounds [461]. On the downside, 2D culture systems fail to mimic the structure of solid tumors, which has been often observed to change cell responses, for example in drug screenings. Therefore, the 3D culture model evolved. A monoculture of spheroids and organoids, which have grown into matrixes, and co-culture with more than one cell type, is starting to be implemented in oncologic research [462]. Currently, there are many challenges in the use of the 3D systems due to the diverse requirements of the cells in comparison to the 2D culture, e.g., availability of nutrients and oxygen. Furthermore, downstream assays and biotechnological equipment have been manufactured for the readout of experiments in 2D settings, while they still need to be optimized in view of 3D systems.

In contrast to the *in vitro* assays, *ex vivo* assays make it easier to perform more exhaustive and biologically relevant experiments, while still taking advantage of modern and established technologies. *Ex vivo* assays, from the Latin "out of the living", are performed on intact or parts of organs freshly collected from a live organism. In this way, it is possible to investigate melanoma cells in a context that closely resembles the *in vivo* situation, with minimal alteration of the normal tissue conditions [463]. Moreover, *ex vivo* experiments are quicker to perform and undergo minor ethical restrictions compared with *in vivo* studies.
In vivo assays should be carried on with particular attention towards the importance of the welfare of the animals used in the research, by respecting the guiding principles of the 3R's – Replacement, Reduction, and Refinement – and seriously considering the use of laboratory animal models in biomedical sciences. For scientific, legal, economic, but foremost for ethic, moral and integrity reasons, all animals are always properly handled by experienced caring staff, used in minimum number, and replaced entirely if any other available technique is suitable for the scientific research scope.

Since Ancient Greece, animals have been employed as models to study human anatomy and physiology [464]. Throughout the centuries, the use of whole alive organisms in scientific experiments has almost become the gold-standard to interpret the biological significance of pre-clinical findings, prior to evaluation in humans. Such experiments are referred as *in vivo*, from the Latin "within the living", as opposed to the *in vitro*, which means "in glass". By the twentieth century, the use of animals as mammalian model organisms in the laboratory, particularly rodents, increased dramatically. Shortly thereafter, the development of inbred strains was introduced in the attempt to minimize the inherent differences between experimental settings and laboratories [465]. A milestone in this process took place in the 1980s with the advent of transgenic mouse models, and later on by genetic manipulation of other species including rats [466], cats [467], dogs [468], rabbits, pigs and sheep [469]. Nonetheless, the mouse remains the most popular animal model in current use in pre-clinical oncology research [470]. This is due to several advantages, including: a completely sequenced genome that resembles the human counterpart by 99%; animals small in size, with a relatively rapid life cycle and short generation time with numerous offspring; well-developed manipulation methods and availability of several biotechnological instruments and optimized tools for mice in the laboratories; accessibility to numerous characterized strains, with the possibility of personalized genetic manipulation and extensive supportive literature. The use of mouse models to study human disorders also has disadvantages. Even though great discoveries have been accomplished by utilizing mice in cancer research, far too often the acquired knowledge fails to be transferred into humans, simply because of basic biological limitations [471-474]. Furthermore, the most convenient or accessible laboratory mouse may not be the appropriate model for addressing certain research questions. In these cases, it is fundamental and compulsory to consider alternative models.

The Xenograft Mouse Model

Over the past decades, several genetically engineered mouse models (GEMMs) have been generated and refined to be clinically relevant for the study of human disorders [475]. These 'humanized' murine models have been extremely important by greatly facilitating translational research from the bench-to-bedside [476, 477]. One of the most used GEMM is the patient-derived xenograft (PDX). PDX benefit from having high fidelity to human diseases because patients' cancer cells are directly grafted into the PDX mouse [478]. By this principle, the tumor cells' characteristics are preserved. Melanoma PDX models have been proven to retain their phenotypic properties during PT formation and in metastatic settings [138]. A well-known example is represented by the preclinical development of BRAF inhibitors. These were first studied in the xenograft model, and those studies experienced an extraordinarily fast bench-to-bedside

success in phase II and phase III patient trials and subsequent FDA approval. Furthermore, these mice developed resistance and consequent tumor relapse, accurately mirroring patient responses and outcomes [106]. In order to avoid immunological rejection of human cells from the mouse immune system, the PDXs have to be immunocompromised, and thus this model is not suitable for studying immune response mechanisms [479]. Melanoma *in vivo* studies are typically performed by growth of melanoma cell lines and PDX tumors transplanted into immunocompromised mice [480]. Depending on the research question and on the ability of the user, different types of injections (e.g., subcutaneous, orthotopic) and sites (e.g., into organs, blood stream) can be chosen for implantation of tumor cells [481]. Although orthotopic injection may better mimic the original site of the tumor and can more accurately reproduce the TME for certain cancer types [482], it requires the animals to undergo major surgery. Another disadvantage of this method is that tumor growth can be followed up only by imaging techniques, i.e., by emission tomography [483]. On the contrary, subcutaneous injection is a simple, effective, and minimally invasive procedure at the surgical level. Another advantage of this latter site is the possibility to measure over time changes in PT volume using a caliper, without having to sacrifice the animals.

In the past decades, several immunocompromised mouse strains have been established to meet the requirements for investigating human cancers in animal models [484]. The nude mouse (nu/nu) is hairless [485, 486], and it has only 1/6 of the normal number of lymphocytes, mostly composed by B cells, and lacks T cells. This model is mostly used in cancer metabolomics studies (e.g., colorectal [487] and kidney cancers [488]). The severe combined immune deficiency (SCID) mouse lacks innate and adaptive immune responses, thus both T and B cells are lacking [489]. Therefore, this strain is ideal for human-derived hematopoietic cell transfer or isotopic labelling [490]. However, this model tends to develop spontaneous thymic lymphomas, so it typically has a shorter lifespan [491, 492]. The nonobese diabetic (NOD)-scid gamma (NOD/SCID/γ, NSG) mouse bears the severe immunodeficiency of the SCID background. Additionally, it lacks natural killer (NK) cells, and it is deficient for the gamma chain of the receptor interleukin-2 (IL-2). In addition, it has a generally nonfunctional cytokine signaling and subsequently, these mice cannot develop lymphomas [493]. Similarly, the Rag model has mutations in recombination-activating gene 1 (RAG-1) and RAG-2, which impair B and T cell development [494, 495]. Lastly, the NOD-Rag1^{null}IL-2rg^{null}NOD rag gamma (NRG) mice are NOD mice, harboring RAG-1 and IL-2 gamma mutations, and these mice are usually employed for tissue transplantation purposes because this technique requires irradiation conditioning, which these murine models can tolerate better than the NSG mice [496]. Among these strains, the NSG mice are currently widely used as a suitable model to study PT formation and metastatic potential of human melanomas *in vivo* [497]. Importantly, this murine model has been demonstrated to reproduce the clinical

progression of SIII and SIV melanoma patients. Accordingly, it has been shown that PDXs generated from melanoma patients harboring brain metastases are more likely to develop brain metastases [498].

In conclusion, animal models can be used in biomedical research to perform the pre-clinical studies needed to characterize many types of human disorders *in vivo*. This can ultimately enable the identification of biomarkers and the development and optimization of therapies required prior to evaluation in patients.

Materials and Methods

Patient Material

The patient material included in Paper I originates from a population-based retrospective cohort of 177 mostly metastatic melanoma lesions. Tumor tissues were collected from treatment naïve patients at the Department of Surgery at Skåne University Hospital between 1997 and 2012. In Paper III, 124 patients with regional metastatic melanoma, all treated with vemurafenib at the University Hospital of Essen were used. For six of those 124 patients, tumor samples were available both at pre-treatment and at progression. The 72 patients included in Paper IV are part of BioMEL, a prospective cohort of early stage melanomas and other cutaneous lesions that resemble melanoma. Tumor specimens are obtained at the teaching departments of dermatology, surgery and oncology, and university hospitals in the south of Sweden. For one patient, tumor specimens from both primary melanoma and concurrent multiple satellite and in-transit metastases were collected prior to any therapy. Generally, biopsies were secured directly after surgery and appropriately stored for experimental purposes. When available, blood samples or normal skin were used as matched controls.

Clinical information and informed written consent were obtained from all participants included in this thesis. Ethical permissions for the use of the patient material in Papers I and IV were obtained by the Regional Ethical Committee (Dnr. 101/2013), and in Paper III under the ethical approval BO 11-4715.

In Papers I and III immunohistochemistry (IHC) on TMA was conducted. The TMA consisted of tissue cores (3 cores of 1 mm *per* tumor) extracted with a hollow needle from formalin-fixed paraffin-embedded (FFPE) biopsies, sectioned by a microtome, and put on a microscope slide for histological analysis by IHC staining. IHC is a detection technique to enable visualization and quantification of a target in biological tissues by selecting a primary antigen-specific antibody that recognizes it. In Paper I, the primary antibodies anti-MITF and anti-SOX10 were employed in IHC; while, in Paper III, IHC was used to assess DDX3X expression levels. Thereafter, a secondary enzyme-conjugated antibody, usually a peroxidase, binds to the primary antibody and catalyzes the reaction producing a color [499].

Early stage CMM can easily be mistaken for atypical nevi using the naked eye even by expert dermatologists because of the clinically similar appearance. The diagnosis of

melanoma has greatly improved with the advent of dermatoscopy, a non-invasive examination which evaluates the microstructures of skin lesions *via* epiluminescence microscopy (ELM). In Paper IV dermatoscopy-guided full skin tumor biopsies (diameter 1 mm) were collected by trained dermatologists, and then evaluated by specialized investigators to ensure the selection of ascertained melanoma cases only. The current standard dermatoscopic method is based on the seven-point checklist algorithm created in 1998. This method has been revised and it is currently in use to distinguish suspicious lesions in the clinic [500]. The three major criteria defining the lesions are: (1) atypical pigment network, (2) blue-whitish veil, and (3) atypical vascular pattern. While, the four minor criteria are: (4) irregular streaks, (5) irregular pigmentation, (6) irregular dots/globules, and (7) regression structure. The melanocyte lesions are scored by assigning 2 points for each major criterion and 1 point for each minor criterion, where 0-2 indicates a benign mole and 3 or more indicates a CMM. For a more detailed overview, see Argenziano et al. [501]. However, a histopathological examination is still needed to identify the truthful nature of each lesion. Upon tissue biopsy, melanoma tumors are ascertained and further classified as *in situ* or invasive. In case of invasive lesions, a description of Breslow thickness is typically included. In the process of evaluation of the skin lesions, a collaboration of cancer researchers with trained dermatologists and pathologists is essential.

This ensures the correct identification and a proper collection of melanoma tumors prior to analyses, as described in detail in Paper IV.

Nucleic Acid Extraction

In the studies included in this thesis, DNA and RNA were extracted from frozen and fixed tissues by using the AllPrep DNA/RNA Mini kit (Qiagen) after homogenization with a TissueLyser (Qiagen). DNA from blood samples was extracted using the DNeasy Blood and Tissue kit (Qiagen). Concentrations and sample purity were ascertained with NanoDrop ND-1000 (NanoDrop Products) and RNA quality was confirmed by Agilent Bioanalyzer 2100 (Agilent). Global GEX was performed only on samples with RIN > 6.

Sanger Sequencing

Prior to the NGS era, Sanger sequencing was the first commercialized and the most used method for about 40 years. Nowadays, it has been automatized and widely used for the sequencing of short regions, routine applications and NGS validations [502]. Known also as the "chain-termination method", the determination of the DNA sequence relies on: *i.* generation of various lengths of DNA fragments, which always terminates with incorporation of one of the four differently fluorescently labelled dideoxynucleotides (ddNTPs) by the DNA polymerase; *ii.* separation of DNA fragments by size, and order of the labelled-DNA fragments into capillary electrophoresis sequencers; and *iii.* excitation of the ddNTPs with a laser to produce a

chromatogram. Each ddNTP-fluorescent label corresponds to a color that is sequentially recorded. Thus, the final readout corresponds to the original DNA sequence. Although fast and cost-effective, Sanger sequencing has low sensitivity, and can only produce 300 to 1000 bp *per* run. Therefore, other variety of techniques are preferred for large-scale projects.

Sanger sequencing was used in Paper I following bisulfite conversion to detect *MITF* methylation in a melanoma cell line panel, and in Papers II and III to validate the CRISPR-Cas9-mediated gene editing of *SOX10* and *MITF*, respectively.

Next Generation Sequencing

The whole human genome comprises approximately 3 billion bases and it took over 13 years to sequence it almost completely, with a cost of 2.7 billion dollars [503]. Over the past decade, the advancements in the new sequencing techniques, referred as NGS, increased dramatically. This allowed a rapid gain in knowledge on the genome and unprecedented developments in research and discoveries [504]. By ultra-high-throughput processing, NGS revolutionized the field with the introduction of parallel sequencing of billions of fragments *per* run, simultaneously. Subsequently, the entire human genome can now be sequenced in a couple of days, for just 200$. NGS enables query at several molecular levels, such as genome (DNA), transcriptome (RNA), and epigenome (e.g., methylation) [505].

Somatic Mutation Analyses by Whole-Exome and Targeted Sequencing

Ultra-high-throughput processing techniques are used to detect mutations throughout the entire genome, i.e., whole-genome sequencing (WGS), or only part of it, e.g., whole-exome sequencing (WES), and targeted sequencing [506].
In contrast to WGS, which spans the entire DNA code, the WES allows one to focus on detection of genetic variants that impair protein-coding regions (roughly 1% of the human DNA), as only the exons are sequenced. On the other hand, the targeted sequencing approach, permits sensitive identification of somatic mutations in predefined gene panels, e.g., known cancer-associated genes. NGS methods rely on principles that are similar to the Sanger sequencing, but they utilize clonal amplification and sequencing-by-synthesis (SBS) instead. SBS is a base-by-base sequencing, where one base and a reversible fluorescently labeled terminator are added one at the time, and imaged. Subsequently, the terminator has to be cleaved prior to the incorporation of the next base. SBS is applied to sequence in parallel tens of millions of clusters, thus providing high-throughput data for genome profiling.

In Paper IV, WES was performed on multiple samples from one CSD^{high} melanoma patient. Library preparation to WES was performed as described previously by Lauss et al. [507], and libraries sequenced in Illumina HiSeq 2500. Median target coverage for the libraries ranged from 68× to 126×. Ultra-deep targeted sequencing of 40 melanoma

relevant genes were selected based on literature-documented association with average coverage of 5,758× and the sequencing was performed in Illumina TruSeq Custom Amplicon Low Input workflow and NextSeq500 on the entire cohort used in Paper IV. Processed GEX dataset is available at gene expression omnibus (GEO) under the accession number GSE139362.

Gene Expression Analyses

Shortly after NGS emergence, RNA sequencing (RNA-seq) was developed as a valuable approach to look at the GEX at the transcriptome level. This sequencing technique allows broad applications for differential gene expression (DGE) analyses on the transcriptome. The pipeline for RNA-seq starts with isolation and purification of RNA molecules; these are then used to synthesize *via* reverse transcription cDNA molecules that can be used in NGS workflow [508].

Preceding NGS, global GEX analyses could be performed on microarrays, which permit the assessment of many genes simultaneously, proving their complementary sequences or "probes". The probes are blocked onto a solid surface, to which the sample (cDNA or cRNA) is applied. Once hybridization occurs among complementary molecules, detection and quantification of the fluorescently labelled sample is possible *via* a laser scanner [509]. Another tool for assessing GEX is the NanoString technology. This method can assess GEX with high sensitivity from RNA extracted from FFPE samples, without an RNA-amplification step. It utilizes pre-designed probes and barcodes for sets of targets for hybridization, which enables microscopic imaging and automatized reads of hundreds of transcripts [510].

The Illumina Human-HT12v4.0 BeadChip arrays was used for single-channel detection of biotin-labeled cRNA antisense (12 samples/chip, 47231 probes) as previously described by Harbst et al. [190]. Mutation and clinical data of the melanoma patient cohort used in Papers I and III were downloaded from Cirenajwis et al. [198] from GEO under the accession number GSE65904. RNA-seq data from Paper III were deposited with accession number GSE131343. The custom-designed targeted-sequencing panel consisted of 1,697 cancer-associated genes, selected based on literature-documented association to cancer and the Catalogue of Somatic Mutations in Cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) and reported by Harbst et al. [190]. In Paper I, NanoString nCounter PanCancer Pathway Panel consisting of 770 cancer-associated genes was used on FFPE PT samples from the *in vivo* study at experimental termination.

Methylation Analysis

DNA methylation represents one of the most important epigenetic markers in mammals, where it plays an essential role by dynamically regulating gene expression to serve cell biology. Several tools have been developed to identify and detect DNA

methylation, which is the addition of a methyl group to the 5-carbon of the cytosine to form 5-mC. This modification occur in CpG dinucleotides and on other genetic regulatory elements of the DNA [511]. The standard technique to detect methylation is the bisulfite genomic sequencing, which allows single nucleotide resolution of 5-mC by using polymerase chain reaction (PCR) of selected DNA fragments in a quantitative way [512]. First, unmethylated cytosine in the DNA molecules are replaced with uracil, while leaving the 5-mC unmodified. Then, during the amplification step, only the cytosine-converted-uracil is amplified as thymidine, whereas the methylated, thus not converted 5-mC, is left as cytosine. Following the sequencing, the DNA products can be aligned to the genome of reference and differentially methylated sites are detected.

From this basic chemical modification, the hybridization-based Illumina Methylation Assay uses a bead-chip platform to probe methylation over the entire genome [513]. In this way, the bisulfite-converted DNA undergoes whole-genome amplification (WGA), followed by enzymatic digestion and then loading into a chip. Here, two different probes that can recognize either methylated or unmethylated sites, hybridize with the treated-DNA and label it distinctively. Finally, the chip is fluorescently stained, scanned, and read by a software that calculates the fluorescence intensity by considering the ratios between the two beads. While the Infinium HumanMethylation450 BeadChip array has a coverage over 450,000 methylation sites, the Illumina Infinium MethylationEPIC BeadChip array reaches more than 850,000 [514].

In Paper I, the melanoma patient cohort and cell lines were analyzed using Illumina Infinium Methylation EPIC BeadChip array. The patient tumor methylation dataset consisted of 788,174 probes and 196 samples. Data have been deposited into GEO with accession number GSE144487.

Statistical and Bioinformatic Analysis

All statistical analyses were performed in R and GraphPad Prism v7.0, using two-tailed tests where a *P*-value of <0.05 is considered statistically significant. The associations between categorial variables, were analyzed by Fisher's exact test, while in case of numerical and categorial variables the Mann-Whitney-Wilcoxon test, Student's t-test and ANOVA were used. In the box and whiskers graphs, the median, the 25th and 75th percentiles are indicated, while the minimum and maximum values are indicated at the extremes of the whiskers. In Papers I and III, survival analyses were performed using Kaplan-Meier plots along with log-rank test using R package 'survival'.

In Paper IV, WES data analysis including alignment, post-alignment processing, and variant calling was performed using SAREK pipeline version 2.0.0 [515, 516]. In particular, reads were mapped with bwa mem and duplicate fragments were marked using Picard MarkDuplicates. Base Quality Score Recalibration was performed using GATK. Targeted sequencing metrics were derived using Picard CollectHsMetrics. For

somatic variant calling, we used 2 algorithms: VarScan v2.4.2 (not part of SAREK) and MuTect2 (part of SAREK). VarScan call sets were further filtered using bam-readcount, VarScan processSomatic and VarScan fpfilter as recommended by VarScan developers, and annotated using Annovar [517]. Only mutations in the coding sequence of the genes were retained (i.e., exonic and splicing). The ubiquitous (trunk) mutations identified by VarScan in all tumor specimens thus constitute the core of the data set. For mutations identified by VarScan in only a proportion of samples (non-ubiquitous mutations) we further looked in MuTect2 call sets to see whether absence of such mutations was due to their variant allele frequency (VAF) <10%. Thus, we "rescued" mutations using an alternative variant caller. Non-ubiquitous mutations at sites lacking sequence coverage were excluded from the data set. An increased proportion of low variant allele frequency mutations in the private sector, as compared with the mutations found in more than one sample, was observed. Since these low VAF private mutations also displayed a different signature composition (i.e., increased proportion of C>A/G>T and decreased proportion of C>T/G>A), pointing to their potential technical artifactual nature, we decided to exclude private mutations with VAF<20% from the analysis. Mutational signatures were obtained with the R package deconstructSigs using signatures.cosmic as input matrix [518]. Copy number analysis, using Contra 2.03 [519], and phylogenetic analyses were performed as previously described [507].

In all Papers, RNA-seq data were analyzed as previously described to obtain fragments *per* kilobase of exon model *per* million reads mapped (FPKM) values [206]. Then, protein-coding genes as defined by the HUGO Gene Nomenclature Committee (HGNC) were retained.

In Paper IV, to be able to determine whether a gene has relatively low- or high expression, we used the TCGA RNA-seq data [167] as a reference set. We applied the quantile distribution of the quantile-normalized and log-transformed TCGA to each sample of the present data, and centered each gene by subtracting the median gene value of the TCGA data. In the supervised analysis of the GEX data, genes with a standard deviation > 0.3 (6699 genes) were tested for differential expression using t-test, and false discovery rate (FDR) using Benjamini-Hochberg correction was reported. Genes with $\log_2$ fold change above 1 and below -1 between the average of all specimens of the case study were submitted to GO term analysis using DAVID [520, 521].

For analyses of the Illumina methylation data in Paper I, raw idat files were processed using R (R Core Team 2016) package ChAMP [522] and background correction was performed using ssNoob [523, 524] from package minfi [525]. The type I/II probes were normalized using beta-mixture quantile normalization (BMIQ) [526] and filtered for polymorphic and off-target probes [527]. For patient samples, methylation β-values of probes that failed in 10% or less samples, were imputed using the impute.knn

function in R and default settings. While samples with a large number of failed probes (probe detection $P > 0.01$; Sample cut-off: >4% of total probes) and probes that failed in more than 10% of the remaining samples were removed. For cell lines, pre-processing of the *MITF*-promoter DNA methylation profiles was done similarly to the patient samples, with the exception that any probe above the *P*-value cut-off in one or more samples, instead of imputing their methylation β-value, was removed.

In the NanoString analysis used in Paper I, the obtained count data were normalized using the NanoString nSolver software: background thresholding was applied to set the minimum value to 20 (default), and a scaling factor, derived from the geometric mean of the positive ERCC control probes, was applied to each sample. The data were log-transformed as $\log_2(\text{data})-\log_2(20)$, and positive and negative control probes were removed.

In Papers I and III, the TCGA dataset (https://tcga-data.nci.nih.gov/tcga/) was used to validate findings. Specifically, in Paper I, methylation data obtained by Illumina HumanMethylation450 array were used. TCGA-SKCM melanoma samples consisting of 3 levels of β-values were processed as previously described by Lauss et al. [417]. In Papers I and III, TCGA matched RNA-seq data (release 3.1.14.0) were quantile-normalized and log-transformed using $\log_2(\text{data} +1)$. In Paper III, microarray data from a melanoma cohort treated with BRAFi were downloaded from the publicly available melanoma dataset GEO under accession number GSE50509 by Rizos et al. [528]. In Paper IV, an independent melanoma dataset by Cirenajwis et al. [164] was used for validation of mutation frequencies in CSD subtypes. Mutations were derived from 1,461 genes and the TERT promoter was not part of the target design.

Experimental Methods

Phenotypic Characterization of Melanoma Cells

Melanoma cell lines used in this thesis were purchased from the American Type Culture Collection (ATCC): MM383 (Papers I and II), WM852, A7 (Paper I), and HEK293T, HT144 and A2058 (Paper III); or provided by the lab of Hensin Tsao: IGR-39, LOX, WM278 (Paper I) and cultured according to ATCC guidelines.

In order to phenotypically characterize melanoma cells used in Papers I, II and III, a variety of *in vitro* assays were performed. To integrate the transcriptomic profiles, melanoma cell lines were lysed, and protein extracted for western blot (WB) analyses. The Tris-Glycine eXtended (TGX) stain-free gradient precast gels (4-20%, Bio-Rad) were used for samples run, and transferred *via* Trans-Blot Turbo (Bio-Rad) onto Polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in Blotting-Grade Blocker (5%, Bio-Rad) and stained with the primary antibodies (**Table 1**). Appropriate horseradish peroxidase (HRP)-conjugate secondary antibodies for each

primary were then applied, and the protein signal was visualized by clarity western enhanced luminol-based chemiluminescent (ECL) substrate (Bio-Rad), and images were processed in ChemiDoc MP (Bio-Rad) by ImageLab software (Rasband).

Table 1. Primary antibodies used in the thesis' Papers.

Antibodies	Source	Thesis Paper		Antibodies	Source	Thesis Paper
Anti-CDKN1B	Atlas Antibodies	II		Anti-OCT4	Atlas Antibodies	II
Anti-DDX3X	Novus Biologicals	III		Anti-SILV	Atlas Antibodies	I; II; III
Anti-DNMT1	Atlas Antibodies	I		Anti-SNAI2	Cell Signaling	II
Anti-JUN	Cell Signaling	II		Anti-SOX10	Atlas Antibodies	I; II
Anti-MITF	Atlas Antibodies	I; II; III		Anti-TCF4	Atlas Antibodies	II
Anti-MITF C5	Thermo Fisher Sci.	III		Anti-VIM	Atlas Antibodies	II
Anti-MLANA	Atlas Antibodies	I; II; III		Anti-ZEB2	Atlas Antibodies	II
Anti-NANOG	Atlas Antibodies	II		Anti-α/β-Tubulin	Cell Signaling	I; II; III
Anti-NCAD	Cell Signaling	II		Anti-β-Actin	Sigma-Aldrich	I; II; III

The cells' proliferation rates were assessed over time by three different readouts: total protein staining with sulforhodamine B (SRB); metabolic ATP levels by CellTiter-Glo (1:2 reagent to media ratio, Promega); and label-free real-time monitoring by xCELLigence real time cell analysis (RTCA) system (ACEA Biosciences). These methods were also applied to compare the viability of different cell lines upon treatment with increasing concentrations of inhibitor compounds (e.g., BRAF, MEKi).

The invasive potential of melanoma cells was measured by migration assays. These were performed by seeding fetal bovine serum (FBS)-starved cells in the upper chamber of an 8.0 µm pore polycarbonate membrane insert (Sigma-Aldrich Corning Transwell) containing low-FBS media, and cells were allowed to migrate into the lower chamber, which was filled with high-FBS media to induce cell migration.

The clonogenic ability of a single cell to grow into colonies or spheres was determined by seeding melanoma cells at low density in either treated cell culture plates, or in ultra-low adherent plates, respectively. Melanoma cells from both migration and clonogenic assays were fixed in 70% methanol, visualized by crystal violet staining (Sigma Aldrich) with 25% methanol, and quantified under a microscope.

The number of senescent cells, called senescence cell rate, in the cell lines was quantified by a cytochemical assay known as β-Galactosidase staining (Cell Signaling): when the chromogenic substrate X-Gal is cleaved by the galactosidase enzyme produced in senescent cells, a blue dye precipitates. The positive-stained cells in each well can then be counted and ratio of senescent cells estimated.

Brain-Slice Ex Vivo Assay

In Paper I, melanoma cells preference to migrate towards the brain tissue was tested by using brain-slices collected from C57BL/6 male mice originated at the Jackson Laboratory and bread at Lund University. Animals were put under isoflurane anesthesia and sacrificed by decapitation. Brains were harvested and transferred to a solution of sucrose-containing artificial cerebrospinal fluid (sucrose-aCSF) and bubbled with carbogen (95% O_2 and 5% CO_2). Whole brain-slices (300 µm) were cut horizontally by a vibratome and collected in washing solution in adjusted osmolarity (305-315 mOsm). Then, one brain-slice was placed onto a membrane insert (Corning) where melanoma cells were grown into limiting PYREX cloning cylinders (8 mm diameter, Corning). Then, the cylinders were removed, and cells were allowed to migrate freely thought the membrane.

The collection of brains from the C57BL/6 used in the *ex vivo* assays was approved by the Malmö/Lund Ethical Committee for Experimental Animals (Permit number M47-15). All concerning procedures were performed according to international guidelines for the use of research animals.

In Vivo Assay

In Papers I, II and III we used the immunodeficient NSG mouse model to follow PT growth from melanoma cell lines and to investigate their metastatic potential *in vivo*. Melanoma cells (1:1 matrigel ratio, Corning) were injected subcutaneously into the right flank of NSG mice.

PT were measured with a caliper over time, and mice were monitored for weight and health status every week. Once the largest PT of one of the mice reached 1 cm^3 (calculated as L x W x W x $\pi/6$), the experiment was terminated, and all mice were sacrificed. Besides PT volume, downstream analyses included micrometastases detection in mice organs. Targeting of human cells in mouse tissue (i.e., brain) was performed by quantitative PCR (qPCR) using TaqMan Assays (Thermo Fisher Scientific) with human GAPDH probe (Hs99999905_m1, Thermo Fisher Scientific), normalized by mouse GAPDH probe (Mm03302249_g1, Thermo Fisher Scientific) in ABI QuantStudio 7 Flex System.

The use of the NSG animals was approved by the regional ethics committee for animal research (approval no. M142-43). All procedures were performed according to international guidelines for the use of research animals.

CRISPR-Cas9 Gene Editing

The clustered regularly interspaced short palindromic repeats (CRISPR) system represents a powerful new tool to engineer the genome of several organisms, including humans [529]. This technology has been generated from the adaptive immune system

of prokaryotes [530], which utilize the CRISPR-guided DNA nuclease Cas9 to cleave nucleic acids of viral origin.

CRISPR-Cas9 mediated KO was used in Paper II in MM383 to engineer SOX10^{KO} melanoma cells, and in Paper III, for site directed mutagenesis of the *MITF* SL3B in A2058 melanoma cells. Mechanistically, this system uses a short non-coding guide RNA (gRNA) that consists of a target complementary CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA) to guide the nuclease Cas9 in the genome. The Cas9 enzyme works as a molecular scissors by opening the DNA strands to produce a cleavage in the target sequence. Subsequently, the DNA double-strand break is repaired by homology-directed repair (HDR) or non-homologous end joining (NHEJ). These will produce the desired gene modification, such as deletions or insertions, with higher sensitivity and reduced off-target effects, in comparison to RNA interference techniques [531] (**Fig. 6**).

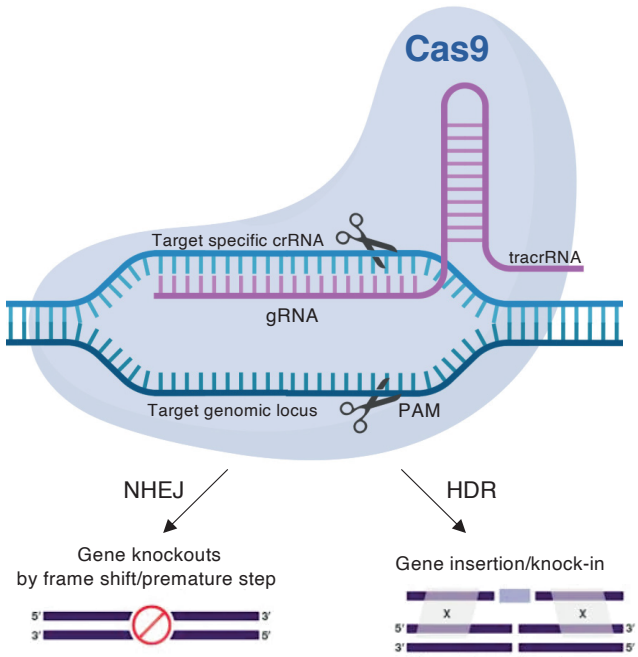

Figure 6. Schematic representation of the CRISPR-Cas9 genome editing tool. gRNA-guided Cas9 produces a cleavage in the target DNA sequence, which is followed by the DNA damage repair mechanisms, such as NHEJ or HDR, producing gene knockout or gene knock-in, respectevely. PAM, protospacer adjacent motif; gRNA, guide RNA; crRNA, CRISPR RNA; tracrRNA, transactivating crRNA; HDR, homology-directed repair; NHEJ, non-homologous end joining.

From 2012 onwards, the CRISPR-Cas9 system has been widely optimized to edit genes permanently within organisms and cell lines, and has allowed a huge variety of applications to study human disorders in an unprecedented manner [532].

Results and Discussion

Unveiling the MITF^{Low}-Methylated Melanoma Subtype

Paper I is focused on characterizing the MITF^{Low}-proliferative melanoma group that was previously identified in the classification of GEX melanoma profiles by Cirenajwis et al. [198]. The authors found that patients belonging to the MITF^{Low} melanoma subgroup had a poor prognosis. In line with this, recent studies have demonstrated that melanoma patients with tumors expressing low levels of MITF (MITF^{Low}) have worse clinical outcome than the MITF-high expressing (MITF^{High}) cases [315, 418-420].

In the current study, we scored a melanoma tumor cohort of 177 patients for the expression of MITF and other markers. By matching IHC to GEX and clinical data, our findings herein support that tumors lacking MITF (MITF^{Neg}: 17%) associate with inferior survival for the patients. Interestingly, studies employing the new sc-seq technology have recently revised the MITF rheostat model by describing four melanoma cell populations with distinctive differentiation states [533]. While melanoma cells negative for SOX10 expression (SOX10^{Neg}) have been detected, the MITF^{Neg}SOX10^{Neg} melanoma subtype has rarely been observed, and mainly captured upon enrichment of therapy-resistant subclones [139, 140, 143, 534]. Strikingly, we report that 6% of MITF^{Neg} metastatic melanoma tumors did not express SOX10 protein in our cohort, which is noteworthy treatment naïve. Although only a small fraction of the total patients had been scored double-negative for MITF and SOX10 expression, nevertheless they displayed a poor clinical outcome. Importantly, this suggests that melanoma cells in treatment-naïve metastatic tumors may also inactivate the melanocyte-specific program for a better fitness advantage.

We had further unraveled the biological mechanisms underlying MITF and SOX10 downregulation following up a recent publication by Lauss et al. [269], which had shown that MITF expression is inversely correlated with *MITF* promoter methylation in a subset of MITF^{Low} melanoma cell lines [269].

In line with this finding, by whole-genome EPIC arrays we observed that the MITF^{Neg} tumors were significantly hypermethylated at the *MITF* promoter, compared with the MITF^{High} cases. In addition, we had seen the same trend in the *SOX10* negative cases. Concordantly, when we screened a comprehensive panel of 65 melanoma cell lines, we found 23% harboring *MITF* promoter methylation (MITF^{Met}) and concurrent gene

silencing. By transcriptional profiling we have further identified two MITF^{Met} subgroups and discovered that SOX10 expression is the main discriminator between the two: MITF^{Met}SOX10^{Pos} and MITF^{Met}SOX10^{Neg}. Moreover, by analyzing the DNA methylation in these MITF^{Met} melanoma cells, we have also shown that downregulation of SOX10 in the SOX10^{Neg} subgroup is due to *SOX10* promoter hypermethylation. Strikingly, pharmacological demethylation was shown to affect only the survival of the MITF^{Met}SOX10^{Neg} melanoma cells.

Next, we characterized the MITF^{Met} subgroups further, and demonstrated that the MITF^{Met}SOX10^{Neg} melanoma cell lines exhibit an aggressive phenotype *in vitro*. This was seen as increases in proliferation, migration and colony forming capacities in the MITF^{Met}SOX10^{Neg} compared with the SOX10^{Pos} group. An additional gain in resistance towards targeted therapy in the double-negative group was also observed. Importantly, this suggests that complete lack of the melanocyte-specific gene signature can infer a further layer of dedifferentiation in melanoma cells. Such severe phenotypic dedifferentiation can be driven by methylation-mediated downregulation of SOX10 in MITF^{Met} melanoma cells.
Paper I formally establishes one of the rare melanoma phenotypic cell states described for the first time by the aforementioned sc-seq studies [139, 140, 143, 534], and recently labelled as the 'undifferentiated' phenotype [533].

In spite of the superior proliferation rate of the SOX10^{Neg} cells, SOX10^{Neg} tumors developed in NSG mice were smaller than those formed in SOX10^{Pos}-injected mice. This discordance could be due to the experimental disproportion of melanoma cells comprising the tumors' mass, which may represent a disadvantage for aggressive subclones usually found at remarkably low ratios in actual melanomas [143]. Nevertheless, tumors from both groups have shown distinctive molecular and anatomical features. More interestingly, we detected enrichment in micrometastases in the brains among the SOX10^{Neg} *in vivo* subgroup, and performed a brain-slice *ex vivo* assay that further supported our findings. This indicates that the MITF^{Met}SOX10^{Neg} melanoma cells may exploit brain tissue as a preferential metastatic site.

Finally, Paper I showed that the expression of both *MITF* and *SOX10* can be regulated by promoter methylation in treatment-naïve metastatic melanomas. Furthermore, concurrent methylation-mediated silencing of these markers associates with particularly aggressive melanoma phenotypes, recently defined as NCSC (MITF^{Low}SOX10^{Pos}) and undifferentiated (MITF^{Low}SOX10^{Low}) lineage states of melanoma [533].

SOX10 Addiction in MITF^{Met} Melanomas

In view of the ascertained importance of SOX10 in defining the lineage identity of melanoma cells [533], further corroborated in Paper I, Paper II integrates the newly updated rheostat model by investigating the role of SOX10 in the MITF^{Met}-low melanoma cell population. The MITF^{Met} melanoma cell subtype already displays a marked loss of the melanocyte lineage-specific signature due to low expression levels of MITF. Thus, to unambiguously discriminate the contribution of SOX10 to such an undifferentiated phenotype, we took advantage of the precision of CRISPR-Cas9 technology to specifically produce KO of *SOX10* in MITF^{met} melanoma cell lines.

Engineering and expanding the MITF^{Met}SOX10^{KO} cell lines proved to be extremely challenging, which indicates a high degree of dependence on SOX10 for the survival of the MITF^{Met} melanoma cells. Transcriptional analyses of the SOX10^{KO} clones revealed several differentially regulated genes compared with the parental cell line (SOX10^{WT}). Interestingly, we observed that KO of *SOX10* in MITF^{Met} melanoma cells led to a complete inactivation of the already impaired melanocyte-specific program, and subsequently pushed the cell phenotype further towards a severely undifferentiated lineage identity state. SOX10^{KO} cells were characterized by an overall decrease in EMT markers and a distinct stem-cell like signature.

Phenotypically, the MITF^{Met}SOX10^{KO} exhibited a marked decrease in proliferative and colony forming rates, presumably due to the observed increase in senescence. However, the SOX10^{KO} gained further resistance towards MAPKi than the SOX10^{WT} melanoma cells, most probably for the same reason. The SOX10^{KO} invasive capacities in the *in vitro* setting were reduced in comparison to SOX10^{WT} cells, yet, depletion of SOX10 conferred the ability of the SOX10^{KO} cells to form melanoma-spheres in an anchorage-independent manner, which associated with tumorigenic potential. Altogether, the characterization of MITF^{Met}SOX10^{KO} illustrates a melanoma phenotypic state that may be referred to as prior to both the NCSC and the undifferentiated states.

In vivo monitoring of the MITF^{Met}SOX10^{WT} and MITF^{Met}SOX10^{KO} cells in an immunocompromised mouse model recapitulated our *in vitro* findings. Here, primary tumors formed upon injection of SOX10^{WT} were large and inconsistently shaped masses, and they contained necrosis in some of the cases. In contrast, tumors developed from the SOX10^{KO} were smaller, were characterized by rounded consistent shape, and closely resembled the tumors developed from the SOX10^{Neg} melanoma cells described in Paper I. Correspondently to the *in vivo* findings of Paper I, we detected brain micrometastases in twice as many of the SOX10^{KO} mice (80%), than in the SOX10^{WT} mice. Strikingly, this suggests that depletion of SOX10 reinforces preferential invasion of brain tissue in MITF^{Met} melanoma cells.

Conclusively, the findings from Paper II validate the crucial role of SOX10 in the MITF^{Met} melanoma subtype. We have demonstrated that low expression of MITF coupled to SOX10 depletion drives an extremely undifferentiated melanoma cell phenotype, and therefore we propose the discovery and validation of a previously undefined melanoma cell state. Accordingly to the newly proposed nomenclature introduced by Rambow et al. [533], the MITF^{Low}SOX10^{Neg} phenotype may be referred as 'hyper-undifferentiated' lineage state.

Melanoma Phenotype Switch Driven by Sex-Specific Mutations

In addition to epigenetic modifications regulating *MITF* at the transcriptional level, in Paper III we unraveled an exquisite translation-based regulatory mechanism altered in a fraction of aggressive melanomas.

In this study, we have found that the RNA helicase encoding the gene *DDX3X* is frequently mutated in metastatic melanomas and associated with a poor clinical outcome for the patient. The *DDX3X* gene is found on the X-chr, and therefore male melanoma cells carrying certain *DDX3X* somatic mutations may always express an altered protein. Importantly, DDX3X contributes to the biosynthesis of several proteins *via* the IRES found on the target 5' UTR. In Paper III we have demonstrated that MITF is one key translational direct target of DDX3X helicase.

First, we have shown that loss of *DDX3X* alters MITF translation capacity, which in turn changes MITF protein levels in melanoma cells. Melanoma cell lines from males, KD for *DDX3X* (DDX3X^{KD}), exhibited a decrease in proliferative rate and an increase in invasive capacities. Such a phenotype could be rescued by ectopic expression of MITF, corroborating the DDX3X-MITF functional axis.

Second, we identified and showed that the IRES element, *SL3B*, embedded in the MITF 5'UTR, is required for efficient DDX3X-mediated translation of MITF. Subsequently, we discerned the contribution of DDX3X-mediated MITF translation in dictating the cell phenotype by CRISPR-Cas9 KO of the *MITF SL3B* site (ΔSL3B). In accordance with the results obtained in the DDX3X^{KD}, we have shown that *SL3B* deletion steers melanoma cells from a proliferative to an invasive state, and we also observed a decrease response to BRAFi-targeted therapy in the ΔSL3B cells, indicating an elevated melanoma aggressiveness.

Next, data from our *in vivo* study supported our previous findings. Here, mice transplanted with the ΔSL3B melanoma cells developed smaller primary tumors than the mice injected with the parental cells, recapitulating the ΔSL3B proliferative

disadvantage. By contrast, the lack of MITF translation in these cells significantly increased their metastatic potential. Indeed, we have detected distant metastases in the lungs of 75% of ΔSL3B transplanted mice, in comparison to the 33% found in the control group.

Finally, we had examined the levels of DDX3X mRNA in a small cohort of melanoma patients from both genders. Crucial from a clinical perspective, patients undergoing BRAFi therapy were shown to experience a reduction in DDX3X mRNA levels in case of cancer progression and in relapse biopsies, compared with matched pre-treatment samples. Concordantly with the *DDX3X* locus, these results were significant predominantly in the *DDX3X*-mutated male patients, providing a specific resistance mechanism in this gender.

In summary, Paper III illustrated how altered DDX3X-MITF translation directs melanoma phenotypic switch and dictates metastatic potential and response to targeted therapy. Importantly, these findings unveil a novel translational layer of control of MITF that may represent a unique adjuvant option for the treatment of DDX3X-mutated melanoma patients.

Tumor Heterogeneity and Clonal Evolution of CSD^{high} Melanomas

While the genetic landscape of the more common CSD^{low} melanoma subtype has been thoroughly explored [190, 198, 206], the CSD^{high} melanomas have been largely overlooked and inappropriately managed like the CSD^{low} cases [535]. It is now clear that there are important biological differences distinguishing the development and treatment response in CSD^{high} melanomas [536], which are not well understood.

To address this gap of knowledge, in Paper IV we have utilized ultra-deep targeted sequencing to depict unique genetic features that characterize the less investigated CSD^{high} melanomas. By meticulous genomic analyses, we have thereby established that the *in situ* and invasive phases of CSD^{high} are typified by different alterations in comparison to the CSD^{low} lesions. These include higher proportion of the $BRAF^{V600K}$, *NF1*, *TP53* and *KIT* mutations in the CSD^{high} cases compared with the CSD^{low} tumors, and this underlies the diverse genetic landscape between the two groups. In concordance to prolonged UV exposure as the main cause for the origin of CSD^{high} melanomas, we have observed that CSD^{high} *in situ* lesions had the most prominent TMB among all specimens. Interestingly, we have additionally shown that accumulation of mutations in *in situ* CSD^{high} lesions appear sufficient to drive the CSD^{high} invasive phase, while this was not the case for CSD^{low} tumors. These data indicate that ITH, through cancer progression in CSD^{high} melanoma, is considerably

limited as compared with CSD^{low}. Of note, a recent study reported that highly mutated melanocytes in CSD^{high} body areas may transform to melanoma in the absence of a pre-existing nevus, and therefore without passing through the canonical malignant stages, once additional mutations are acquired [537, 538].

In Paper IV we had further resolved the extent of ITH in an advanced CSD^{high} case study: a melanoma patient that presented with synchronous primary tumor (PT) and multiple secondary satellites and IT metastases. Strikingly, we had unraveled an incredibly limited ITH in the evolution of the subclones, and validated a profound molecular congruence among 5 PT and 7 IT specimens. We had indeed found over 95% of shared mutations among the biopsies, meaning that disease progression from PT to metastases was not dictated by changes in genetic mutations in this CSD^{high} patient. However, in line with the established acquisition of copy number instability during advanced evolution of melanoma tumors [539, 540], we had detected several CNVs in these tumors, in certain cases due to LOH. This could partially explain the heterogeneity observed among the PT and IT. Appealing from a treatment perspective, while therapies against cancer driver mutations often result in tumor resistance and subsequent relapse [130, 541], targeting CNVs has been shown to be a successful strategy to target aggressive cancer subclones in a translational clinical trial [542].

Overall, Paper IV uncovered the genetic landscape unique to the CSD^{high} melanoma subtype, and revealed different degrees of heterogeneity required for cancer progression in CSD^{high} compared with other CSD^{low} melanomas. This evidence provides support for the classification of CSD^{high} as a distinct molecular entity that can develop and progress *via* different evolutionary routes, and thus new therapeutic strategies are warranted.

Conclusions and Future Perspectives

Malignant melanoma accounts for the highest number of deaths among all skin cancer types, and its incidence worldwide increased dramatically during the past decades. The major obstacle in melanoma diagnosis and in the development of effective treatments is represented by a notoriously high tumor heterogeneity.

In particular, the emergence of undifferentiated melanoma subclones characterized by lack of the melanocyte lineage-specific transcriptional program, that is mainly driven by MITF and SOX10, constitutes a source of therapy resistant cells. Subsequent expansion of non-responsive subclones allows tumor progression and development of metastases, which is the primary cause of poor outcome for the patients. The contradictory findings around the role of MITF and SOX10 need to be further unraveled. More importantly, the mechanisms behind their complex regulation, including epigenetic modifications and post-translational control of these lineage-specific genes, have just started to be elucidated. Therefore, dedicated studies to tackle the newly observed undifferentiated melanoma lineages are warranted. Finally, new therapeutics should be generated in view of the opportunity to steer melanoma plasticity towards a drug sensitive state, prior to targeting the heterogenous tumor bulk. Such adjuvant therapy may represent a unique strategy for the reduction, and perhaps eradication, of tumor cells responsible for patients' relapse in melanoma.

Importantly, the distinct molecular features of CSD^{low} and CSD^{high} melanomas and the limited extent of ITH defining tumor progression in CSD^{high} cases have been just uncovered. Nevertheless, these findings outline the divergence of CSD melanomas into specific entities, each with its own characteristics, that may be exploited in view of novel treatment opportunities. Nonetheless, there is an obvious urge to expand our understanding of CSD^{high} tumors, especially in view of their malignant transformation, prior to moving forward with appropriate therapeutic interventions.

To conclude, further research in melanoma should focus on investigating the complex interplay among melanoma-specific factors responsible for cancer progression, therapy resistance, and tumor relapse. Studies on genetic mutations, molecular features and cell dynamics can contribute to a comprehensive understanding of the tumors and provide the bases for the development of novel strategies against malignant melanoma.

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About this Thesis

Malignant melanoma is the deadliest form of skin cancer, and the number of cases is escalating worldwide. In spite of the breakthrough in therapeutics made available to melanoma patients in the last decades, treatment resistance followed by tumor relapse remains the leading cause of patient death. This thesis had focused on aggressive melanoma cells that do not respond to targeted therapies due to inactivation of the melanocyte-specific transcriptional program. The current studies aimed to unravel the role of the melanocyte-lineage genes, MITF and SOX10, which loss can drive novel undifferentiated melanoma phenotypic states. Further development of drugs that can effectively target resistant melanoma cells, or steer their state towards a drug-sensitive one, represents a powerful strategy to ultimately eradicate the tumor. Additionally, heterogeneity analyses on chronic sun-damaged (CSD) melanomas revealed that high grade CSD do not undergo further genetic changes during the progression to the advanced stages of the disease, in contrast to the low CSD melanomas. This emphasizes the importance of prevention of UV overexposure. Moreover, the biological differences among CSD subtypes can be exploited for different therapeutic approaches according to the melanoma patient groups.

About the Author

Adriana Sanna is a proud Sardinian who started travelling in her teens driven by an innate passion for biological studies. After attaining her Biomedical Science degree in two world leading institutions from The Netherlands and the U.S., she was granted a prestigious European fellowship to continue her research as PhD in Cancer and Molecular Biology focused on melanoma, in Sweden. During Adriana's academic career, her beloved housekitty, MaryJane, has been loyally travelling along for the past 10 years.

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