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Functional analysis of SOX2 in melanocyte development and melanoma pathogenesis

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Declarations according to § 8 (3) b) and c) of the doctoral degree regulations:

b) I hereby declare that I have written the submitted dissertation myself and in this process have used no other sources or materials than those expressly indicated,

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Heidelberg, 10.02.2015

Kasia Weina

This PhD thesis is dedicated to my

Grandma Kutsunai

who always believed in me and

gave me the courage to reach higher

than I ever dreamed possible.

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Summary

Malignant melanoma remains the most deadly form of skin cancer due to its quick metastatic spread and the development of resistance to available treatment. The cause of melanoma is still under investigation but environmental factors, such as ultraviolet radiation, have been associated with the initiation of melanoma. Moreover, studies have revealed that the melanocytic lineage is predisposed to malignant transformation due to its developmental program. Melanocytes are derived from the embryonic neural crest, which utilizes processes such as the epithelial-to-mesenchymal transition (EMT) during their normal development to spatially migrate and complete terminal differentiation. Therefore, recent work in the field of melanoma has focused on investigating embryonic and neural crest-related genes since they may be reactivated during melanomagenesis and metastatic spread.

To date, limited studies have suggested an important role of the embryonic stem cell marker, sex determining region Y-box 2 (SOX2), in melanoma; however a lack of detailed analyses and understanding of its function remains. In this study, SOX2 was found to be highly expressed in primary melanomas compared to melanocytic nevi. Additionally, using fluorescence *in situ* hybridization analysis, genomic SOX2 was found to be significantly amplified in both primary melanomas and metastatic melanomas compared to melanocytic nevi. Gain-of-function studies revealed that melanoma cells lost differentiation marker expression upon SOX2 overexpression *in vitro*. The dedifferentiated phenotype displayed can be in part explained by SOX2 binding to the promoter region of the microphthalmia-associated transcription factor-M (MITF-M), thereby repressing its transcription. Additionally, SOX2 was shown to be required for melanoma cell migration and invasion *in vitro*. Invasion-related EMT markers were upmodulated upon SOX2 overexpression and immunohistochemical analysis revealed high SOX2 expression in deep regions of primary melanomas and in stroma-infiltrating melanoma cells. *In vitro* enhanced SOX2 expression could be induced by TGF-β, indicating TGF-β signaling as an upstream regulator of SOX2 expression in melanoma.

This study proposes that TGF-β1 induces SOX2 expression, which may lead to melanoma progression by: i) SOX2 binding and repressing the MITF-M promoter, which may influence the dedifferentiation of human melanoma cells, and ii) SOX2 inducing high expression of ZEB1 and TWIST1, which promotes a mesenchymal phenotype. In line with the described phenotypic alterations, this work revealed that SOX2 enhances melanoma cell migration and invasion and depletion of this transcription factor results in loss of cellular motility. Therefore, I identified SOX2 as a key player in the complex molecular network that governs invasion-related processes and I revealed a role for SOX2 as an invasion-related marker with potential clinical application.

Zusammenfassung

Aufgrund der starken Metastasierung und der Entwicklung von Resistenzen gegen gängige Therapien ist das maligne Melanom eine der tödlichsten Hautkrebsarten. Obwohl Umwelteinflüsse wie Exposition durch ultraviolettes Licht mit der Entstehung des malignen Melanoms in Zusammenhang gebracht worden sind, ist die Ursache bisher nicht vollständig geklärt. Studien haben jedoch gezeigt, dass die Zellen der melanozytären Linie aufgrund ihres molekularen Entwicklungsprogramms besonders anfällig für eine maligne Transformation sind. Melanozyten entstehen aus den embryonalen Neuralleisten-Zellen, die während ihrer normalen Entwicklung einen sogenannten epithelial-mesenchymalen Wechsel durchlaufen. Basierend auf dieser Entdeckung haben sich mehrere Studien der letzten Jahre auf die Untersuchung verschiedener embryonaler und neuralleisten-assoziierter Gene spezialisiert, da diese womöglich während der malignen Transformation reaktiviert werden und dadurch zur Entstehung und zur Metastasierung des Melanoms beitragen können.

Einige Studien haben in diesem Kontext dem Gen SOX2 (sex determining region Y-box 2) eine wichtige Rolle zugewiesen, es fehlen jedoch detaillierte Analysen, um dessen genaue Funktion bestimmen zu können. Die vorliegende Studie zeigt, dass SOX2 in primären Melanomen stärker exprimiert wird als in melanozytären Nävi. Zusätzlich wurde eine genomische Amplifikation von SOX2 sowohl in primären Melanomen als auch in Melanom-Metastasen im Vergleich zu melanozytären Nävi festgestellt. Anhand humaner Melanomzelllinien wurde gezeigt, dass die Expression mehrerer Differenzierungsmarker verloren geht, wenn SOX2 überexprimiert wird. Dieser Phänotyp war zum Teil durch die Bindung von SOX2 an die Promoterregion des Mikrophthalmia-assoziierten Transkriptionsfaktors M (MITF-M) und der Repression dessen Transkription zu erklären. Des Weiteren wurde gezeigt, dass die Expression von SOX2 für die Invasionsfähigkeit der Melanomzellen *in vitro* erforderlich ist. Einige Marker für den epithelial-mesenchymalen Wechsel waren hochreguliert, wenn SOX2 überexprimiert wurde und eine immunhistochemische Analyse hat ergeben, dass SOX2 in tiefen Regionen primärer Melanome und in Stroma-infiltrierenden Zellen stärker exprimiert ist als in oberflächlichen Regionen primärer Melanome. Erhöhte SOX2 Expression konnte *in vitro* durch die Stimulierung humaner Melanomzelllinien mit TGF-β1 induziert werden, was darauf hinweist, dass sich TGF-β1-aktivierte Signalwege regulatorisch auf die SOX2 Expression im malignen Melanom auswirken.

Zusammengefasst deuten die Ergebnisse der vorliegenden Studie darauf hin, dass TGF-β die Expression von SOX2 induziert und dass dies durch folgende Mechanismen zur Melanomentwicklung beitragen kann: erstens, indem SOX2 an den MITF-M Promoter bindet und dessen Transkription inhibiert, sodass es zu einer generellen De-Differenzierung der

Melanomzellen kommt, und zweitens, indem die Überexpression von SOX2 zu einer Hochregulierung der Expression von ZEB1 und TWIST1 führt, was zu einem mesenchymalen Phänotyp führt. In Einklang mit den phänotypischen Veränderungen bei starker SOX2 Expression, ist dieser Transkriptionsfaktor auch funktionell für erhöhte Invasionskapazität notwendig und seine Repression führt zu Motilitätsverlust in humanen Melanomzellen.

Ich konnte daher in dieser Studie SOX2 als einen Schlüsselfaktor identifizieren, welcher in einem hoch komplexen Netzwerk für die aggressive Entwicklung des malignen Melanoms verantwortlich ist. Außerdem weist SOX2 Potential auf, um als invasionsassoziierter Marker in der klinischen Anwendung genutzt zu werden.

List of abbreviations

°C	Degree Celsius
18S	18S ribosomal RNA
μ	Micro

A

ATP	Adenosine triphosphate

B

BiFC	Bimolecular fluorescence complementation assay
BMP4	Bone morphogenetic protein 4
bp	Base pairs
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRN2	POU domain, class 3, transcription factor 2 (POU3F2)
BSA	Bovine serum albumin

C

CCLE	Cancer cell line encyclopedia
CCND1	Cyclin D1
CD117	Tyrosine-protein kinase kit (cKIT)
CDH1	E-cadherin
CDH2	N-cadherin
cDNA	Complementary deoxyribonucleic acid
CSCs	Cancer stem cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
ctrl	Control

D

DAPI	4',6-diamidino-2-phenylindole
DCT	Dopachrome tautomerase (TRP2)
DKFZ	Deutsches Krebsforschungzentrum (German Cancer Research Center)

DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
dox	Doxycycline
dH$_2$O	Distilled water
DMSO	Dimethylsulfoxide

E

e.g.	exempli gratia (for example)
ECM	Extracellular matrix
EDN	endothelin
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
EMA	European medicines regulatory agency
EMT	Epithelial-to-mesenchymal transition
ESCs	Embryonic stem cells
ERK	Extracellular-signal regulated kinase
et al.	et alteri
EtOH	Ethanol

F

FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDA	Food and drug administration
FISH	Fluorescence *in situ* hybridization

G

GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate

H

h	Hour	
HH	Hedgehog	
hESCs	Human embryonic stem cells	
hiPSCs	Human induced pluripotent stem cells	
HMG	High-mobility box	
HPRT	Hypoxanthine phosphoribosyltransferase	
HRP	Horseradish peroxidase	
HTS	High throughput screen	

I

i.e.	*id est* (that is)
ICM	Inner cell mass
IF	Immunofluorescence
IHC	Immunohistochemistry
iPSCs	Induced pluripotent stem cells

K

KD	Knockdown
kDA	Kilo dalton

M

MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
min	Minute
MITF	Microphthalmia-associated transcription factor
mRNA	Messenger ribonucleic acid
MSA	Migration staging area
MT	Mutant
mTOR	Mammilian target of rapamycin

N

NC	Neural crest
NCCs	Neural crest cells
neo	Neomycin
NHMs	Normal human melanocytes
NRAS	Neuroblastoma RAS viral oncogene homolog
NSCLC	Non-small cell lung cancer

O

OE	Overexpression

P

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PD-L1	Programmed death 1 ligand 1
pH	potentia hydrogenii
PI	Propidium Iodide
PTEN	Phosphatase and tensin homolog

Q

qRT-PCR	Quantitative real time polymerase chain reaction (qPCR)

R

RFUs	Relative fluorescence units
RNA	Ribonucleic acid
RPM	Rotations per minute
RT	Room temperature

S

SCF	Stem cell factor
SCLC	Small cell lung cancer

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin RNA
SOX2	SRY (sex determining region Y)-box 2
SOX10	SRY (sex determining region Y)-box 10
SCC	Squamous cell carcinoma
STAT3	Signal transducer and activator of transcription 3

T

TAD	Transactivation domain
TGF-β	Transforming growth factor beta
TMA	Tissue microarray
TP53	Tumor protein 53 (p53)
TRP1	Tyrosinase-related protein 1
TRP2	Tyrosinase-related protein 2 (DCT)
TYR	Tyrosinase

U

UMM	Universitätsklinikum Mannheim (University Medical Center Mannheim)
UV	Ultraviolet

V

V	Volt

W

WT	Wildtype

Y

y2h	Yeast-2-Hybrid

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1 Introduction

Development is a complex process orchestrated by the temporal and spatial control of specific genes. Unique to vertebrates, the development of the neural crest (NC) gives rise to various lineages including melanocytes. First discovered in 1868 by Wilhelm His, the neural crest was described as a band of cells in between the neural tube and ectoderm in chick embryos [1, 2]. Nearly two centuries later, the neural crest is a well-described cell population which undergoes a complex developmental process comprised of several key steps: induction, delamination and migration. Recently, some of these processes have been implicated in the pathogenesis of the deadly skin cancer: melanoma. Since melanocytes are derived from the neural crest, one hypothesis for malignant transformation suggests the reactivation of this developmental process. Therefore, understanding the neural crest and other embryonic-related systems and molecules coordinating them may help elucidate novel mechanisms controlling the transformation of malignant melanoma.

1.1 The neural crest

In a normal developing embryo, characteristic patterning of the nervous system and exterior structures requires the motility of the pluripotent population of cells known as the NC. Neural crest cells (NCCs) are formed along the vertebrate axis in the dorsal neural tube. These pluripotent cells give rise to various lineages, including bone, neurons and melanocytes. Here, I will only discuss the development of the trunk NC, where melanocytes are derived.

1.1.1 Induction, delamination and migration of the neural crest

The cells that form the NC are induced between the neural plate, which later develops into the central nervous system (CNS) and the non-neural ectoderm that builds the epidermis. The formation of the NC begins post-gastrulation, when the neural plate folds on itself to form the neural tube, the process where the neural folds from opposite ends of the ectoderm converge and eventually fuse (**Figure 1a-c**). Following fusion, NCCs leave the neural tube during or after neural tube closure, depending on the species, and migrate throughout the body [3]. The NCCs develop as the neural tube closes on day eight of gestation in mice and day 22 in humans [4]. From the dorsal neural tube, NCCs migrate into the surrounding tissue. This process gives rise to various tissue types, including bone and cartilage, pigmented cells, neurons and glial cells of the peripheral nervous system. NCC derivatives are specified *via* spatial and temporal patterning, migration patterns, lineage-specific markers and inherent cell characteristics [4, 5]; for an overview of NC developmental stages refer to **Figure 1**.

There are several models for the induction of the neural crest, namely neural default model, variation of the neural default model and the two signal model of neural crest induction. The models differ in the potential fates of the neural plate, as well as in the nature of inductive signals and neural crest specifiers leading to the induction of neural crest effector genes. The two signal model is the most accepted model and elucidates combinations of signaling that are capable of inducing the NC. Several second signals have been identified (wingless type (WNT), fibroblast growth factor (FGF) and/or retinoic acid) and, in combination with bone morphogenetic protein (BMP) inhibitors, are able to induce snail family zinc finger 2 (SNAI2 or SLUG) expression and, in turn, NC induction [3, 6–8]. The induction of the NC potential is assayed by SNAI2 expression, a factor responsible for final emigration of NCCs. Many of the factors that are able to induce NC formation are found to be endogenously expressed in neighboring tissues at the appropriate stages during NC development. For example, the paraxial mesoderm, located adjacent to the neural tube, expresses the candidate induction signals eFGF, WNT8 and BMP4. Studies *in vivo* demonstrated, upon excision of the paraxial mesoderm from *Xenopus* embryos, SNAI2 expression was greatly reduced. The study proposed that the paraxial mesoderm and its corresponding signals are a possible requirement for NC induction [9, 10]. Additional NC-inducing signals may come from interactions between the epithelium and the neural plate [3]. Moreover, there are other potential signals that are able to induce the NC besides BMP and WNT, such as Notch/Delta signaling [11].

After the induction of the NC and formation of the neural tube, NC potential is maintained *via* BMP signaling [3, 12]. The next process to occur during NC development is delamination. Delamination is defined as the splitting of a tissue into separate populations, regardless of cellular mechanisms [13] (**Figure 1d**). Regarding the NC delamination, this process is triggered by BMP and canonical WNT signaling, accompanied by the expression of WNT1 and WNT3 and paired box transcription factor (PAX3), BMP4 and Msh homeobox 1 (MSX-1) [13–15]. The BMP and WNT signaling cascades are crucial for the G1/S cell cycle transition in NC precursor cells, since NCCs are only capable of delamination in the S-phase. However, this prerequisite is not sufficient to initiate delamination or trigger onset of migration [13].

Additionally, the activation of the BMP/WNT1 signaling cascade activates downstream mediators like a disintegrin and metalloproteinase (ADAM)-10. ADAM10 is able to cleave the extracellular domain of N-cadherin (CDH2). Therefore, the expression of ADAM10 helps to reduce cell-cell adhesion not only between NCCs but also between NCCs and neuroepithelial cells [13]. N-cadherin is then degraded or the remaining domain is further cleaved into a cytoplasmic fragment (CTF2) and activates CCND1 (Cyclin-D1). While

CCND1 promotes cell cycle progression, it does not systematically promote the G1/S transition in the neural tube [16].

The control of cadherins during delamination is conserved across species and between NCC populations at different axial levels. Typically, NCCs switch from strong cadherin-based cell-cell adhesion (N-cadherin) to a weaker type of cell-cell adhesions based on type II cadherins (Cadherin-6/6B/7/11) [13]. This process ensures the next stage in NC development: migration. Migration of individual NCCs occurs out of the neuroepithelium and into an area termed the migration staging area (MSA), located dorsally to the neural tube and underneath the ectoderm. At this stage, the premigratory NCCs are multipotent and begin to express SRY box-containing transcription factor 10 (SOX10) and SOX9 [4, 17, 18]. Studies have illustrated this by tracking the fate of individual cells and the rise of multiple lineages by injecting dye into the cells [19].

As migration occurs, the pattern of events involved in the epithelial-to-mesenchymal transition (EMT) differs among cells, but confers migration ability (**Figure 1e**). There are three major stages in the EMT process, which include the loss of cell polarity, changes in cell adhesions and increase in protease activity [20]. It is important to mention that the terms delamination and EMT are often interchangeably used in the NC field. However, it is essential to acknowledge that, while all NCCs must undergo EMT during development, the timing and completion of the EMT does not always correspond to the delamination phase. Here, I have separated the topics, but they can occur simultaneously or asynchronous from each other [13].

The first step in the EMT process is the loss of polarity or the switch from tight junctions to gap junctions between cells [20, 21]. The substitution of tight junctions with gap junctions during the first stage of the EMT process influences cell-cell interactions. The next stage, a key element in cell interactions during the EMT process, is cadherin-dependent cellular adhesion [20]. Changes in cadherins are essential, indicating the EMT process, and share an overlapping mechanism with the delamination process. As previously discussed, the shift from classical type I cadherins to type II cadherins strongly correlates to the gain of cellular motility [20, 22]. The major regulators of the cadherin switch are the members of the SNAIL gene family: SNAI1 (SNAIL) and SNAI2 (SLUG). Both were found to be expressed in the NC and are required for specification and migration of the NCCs [20, 23–25]. Gain and loss of function studies in chick embryos found that SNAI2 is an essential regulator of the EMT in the NC [26, 27].

The cadherin switch plays a key role in EMT progression in the NC but there are many migratory cells in which this step is not obligatory. However, a required and necessary step

for all cells to complete the EMT process is the ability to digest extracellular matrix (ECM). This enzymatic activity is carried out by proteins called matrix metalloproteases (MMPs). In the NC, MMPs play an important role in the last stage of development, migration. Interestingly, SNAI1 has been suggested to induce MMP expression [7]. In summary, the final step in NC development involves the initiation and completion of the EMT. The cells of the NC must lose polarity, switch from tight junctions to gap junctions, lose cell-cell adhesion and gain protease activity. The combination of these functions completes the EMT process and allows the migration of the NC cell population (**Figure 1**).

The migratory route of these NCCs form segregated streams that emerge adjacent to specific locations along the vertebrate axis [28–31]. NCCs arise along the dorsal neural tube and avoid migrating in certain areas adjacent to the neural tube [32–35]. These cells travel either ventrally, following a path between somites and the neural tube and becoming the peripheral nervous system and endocrine cells, or dorsal-laterally away from the neural tube, following a path between dermamyotome and the ectoderm and ultimately forming melanocytes [4].

There are several theories regarding melanocyte precursor migration. The traditional view is that lineage specification occurs during the early stage of development. Progenitors of neural derivatives migrate ventrally and melanocyte precursor cells enter the MSA, located close to the neural tube, before migrating on a dorsolateral pathway underneath the ectoderm [36]. However, evidence has suggested an alternative migratory pathway for melanocyte precursor cells. Studies have revealed that a small portion of melanocytes arises ventrally along the pathway containing neural precursors [37]. Studies performed in zebrafish, mouse and chick embryos found that melanocyte precursors are capable of migrating both dorsolaterally and ventrally [38, 39]. In chick embryo studies, if the dorsal neural tube and the dorsal root ganglion (DRG) were ablated after the emergence of the initial melanocytes, the number of melanocytes in the skin was significantly reduced. However, if the dorsolateral skin surface containing the first melanocytes was removed, the complete melanocyte production was hardly affected. This suggests that a large majority of melanocytes in the skin are actually derived from nerve-associated precursors instead of dorsolaterally migrating NCCs [36, 38, 39]. Studies performed in mouse embryos using the Cre/loxP system revealed that nerve cells are able to produce cells with melanocytic features *in vivo*. This *in vivo* mapping analysis showed that a large majority of dermal melanocytes found in the trunk and limbs appear to be derived from cells previously associated with nerves [36, 38]. Moreover, recent work has elucidated that there is a prenatal capacity to form pigmented cells through the generation of a glial/melanocyte progenitor cells [40, 41].

It is important to note that NC development is highly regulated by the presence of environmental cues at all stages. Several thorough studies have investigated the importance of intrinsic and local extrinsic signals from the microenvironment in the NC. These studies used tissue transplantation, cellular labeling and molecular analysis to show that the migratory streams of NCC are defined by a combination of intrinsic and extrinsic cues [42–45]. In other words, there are several intrinsic and extrinsic modulators of NC development and early melanocyte fate determination.
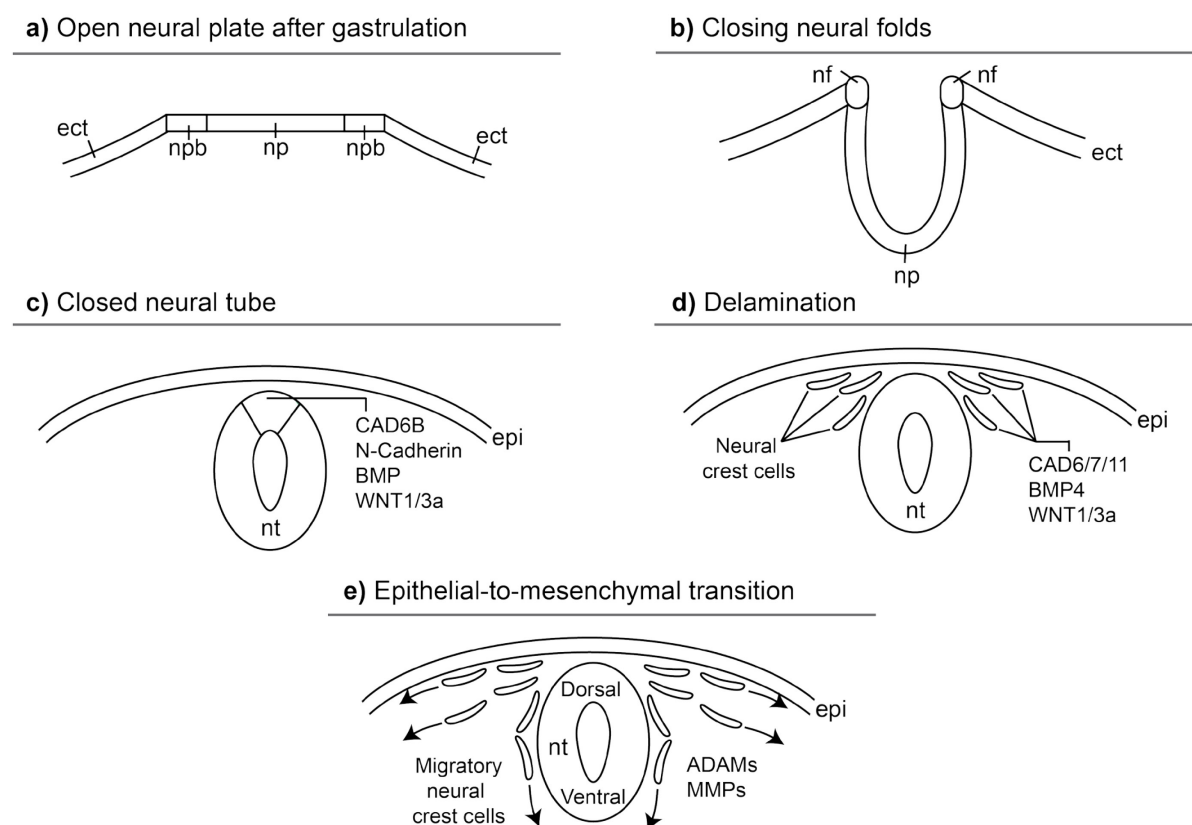
Figure 1 **Overview of neural crest development: stages, signals and regulation**

The stages and signals during NC development. **a)** The first stage of NC development occurs post-gastrulation where the neural plate remains open. **b)** During the second stage the neural folds come together to initiate the formation of the neural tube. **c)** Next, the neural plate fuses forming the closed neural tube and the epidermis. **d)** The processes of delamination occurs, where lineages begin to diverge and NCCs begin to migrate. Cells intiate the cadherin switch from type I cadherins (N-Cadherin) to type II cadherins (CAD6/7/11). This gives the cells migrative properties with the loss of cell-cell adhesions. **e)** Either during or after the completion of the delamination process, the EMT process occurs. NCCs gain protease activity and begin to express proteins such as ADAMs and MMPs. This figure was modified from Kuriyama *et al.,* 2008 [20].

CAD=cadherin; NC=neural crest; NCCs=neural crest cells; ect=ectoderm; np=neural plate; nt=neural tube; npb=neural plate border; nf=neural fold; epi=epidermis; EMT=epithelial-to-mesenchymal transition; MMPs=matrix metalloproteinases; ADAMs= a disintegrin and metalloproteinase domain-containing protein

1.1.2 Melanoblasts and terminal differentiation of melanocytes

Upon migrating away from the neural tube, some NCCs become specified into melanoblasts and continue migrating through the dermis and into the epidermis of the skin [4].

Melanoblasts are the precursors of melanocytes and are highly migratory, proliferative and terminally differentiate *en route* to their final destination in the basal epidermis and hair follicles [46]. Melanoblasts are the descendants of the multipotent NCC population and there are two theories regarding when NCCs are specified into the melanocytic lineage [4].

The first theory states that melanocyte markers, such as microphthalmia-associated transcription factor (MITF) and the proto-oncogene KIT (also known as mast/stem cell growth factor receptor (SCFR) or CD117), are expressed during NC migration. The expression of dopachrome tautomerase (DCT), also known as tyrosinase-related protein 2 (TRP2), follows soon after [4]. This theory suggests that NCCs are predetermined to become melanocytes before they migrate along the dorsal-lateral pathway and the migratory path is therefore a consequence of these pre-specifications.

The second theory acknowledges extrinsic factors in the environment which may influence the determination of the NCC population toward the melanocytic lineage. Therefore, migrating NCCs encounter factors which may have an overall impact on the terminal differentiation of the cells. To support this theory several studies found that expression of adhesion-related proteins, including extracellular matrix (ECM) proteins and cadherins, was reduced in melanoblasts upon migration initiation [8, 47–51]. The true mechanism may be a combination of both theories, where initiation of some melanocyte markers promotes initial migration and the additional expression of other genes, possibly externally induced, and further drives migration.

There are several key players involved in melanoblast specification and securing the melanocytic lineage fate. MITF is regarded as key factor involved in melanocyte specification from NCCs [36]. The expression of MITF occurs shortly after NCCs emigrate from the neural tube and in the MSA. Fate mapping studies found melanoblast survival to depend on MITF expression [52]. Moreover, MITF is responsible for initiating transcription of important downstream targets, such as TRP2. However, MITF is not required for the migratory behavior of melanoblasts [53]. Therefore, MITF plays an important role in promoting cellular survival and development of melanoblasts. However, other factors are required in combination for complete specification [36].

SOX10 and PAX3 are two important genes in melanoblast specification and activators of MITF transcription. Studies reported that SOX10 and PAX3 synergistically activate the MITF promoter *in vitro* [54, 55]. Additional work investigated the specific role of both PAX3 and SOX10 independently from each other during early stages of melanocyte development. Mouse embryos homozygous for mutated PAX3 were found to still possess TRP2-expressing migrating melanoblasts along the dorsolateral pathway [52]. In mouse embryos

negative for SOX10, the melanocytic markers TRP2 and MITF were virtually absent [54]. This analysis of both genes independent of one another suggests that PAX3 activity is not required for melanocyte specification, but is required for expansion of the melanocyte progenitor pool in early melanoblast stages. Studies have revealed that the WNT1 promoter possesses a PAX3 binding motif, allowing PAX3 to use these enhancer elements to activate WNT1. This suggests that PAX3 may be a direct regulator of WNT1 in the development of NC populations [56, 57]. Furthermore, TRP2 and MITF are direct transcriptional targets of SOX10 and are required for melanoblast specification [36, 54, 55].

Since SOX10 is crucial for melanocyte development, it is important to understand other factors that may be responsible for modulating its expression. Another member of the SOX family of transcription factors was found to be an antagonistic rather than synergistic interactor with SOX10. SOX5, a member of the SOXD subfamily, has little impact on melanocyte development alone [58]. Studies found that loss of SOX5 alone had little effect on melanocyte specification. However, when combined with SOX10 heterozygosity, the loss of SOX5 partially rescued impaired melanocyte development. This indicated that SOX10 activity is being modulated, in part, by SOX5 [36, 58].

While SOX10 is an activator of MITF expression and acts as a specifier of the melanocytic lineage, the forkhead transcription factor (FOX) D3 plays an opposing role. FOXD3 functionally represses MITF expression and melanogenesis [36]. In quail NCCs, FOXD3 expression was found in all NCCs with the exception of late-emigrating cells pre-specified for the melanocytic lineage [59]. Additional studies in quail, where FOXD3 was overexpressed, demonstrated that FOXD3 repressed MITF expression, resulted in glial marker expression instead of melanocyte markers [60]. In summary, FOXD3 is involved in controlling melanocyte fate determination and migration, but represents only one factor in a complex network orchestrating melanocyte fate in NCCs.

As described during NC induction, environmental cues are also responsible for regulating fate decision in the NC. These extrinsic signals are capable of directing NCCs towards a particular cell fate, which has been proven using clonal expansion studies. Many of these responsible factors have been identified for other lineages, including neurogenesis and gliogenesis, but inducers of the melanocytic fate remain to be discovered [36]. Nevertheless, there are several known growth factors that play a role during the melanocytic fate determination process. Among those growth factors are the members of the endothelin (EDN) family. Several studies have proven the EDN family to be important in determining the melanocytic fate. Upon exposure to EDN3 in quail NCCs, promotion and expansion of unipotent glial and melanocytic clones was observed. In addition, bipotent glial/melanocytic

clones were found [61]. Moreover, EDN3 was found to induce the generation of bipotent glial/melanocytic precursor cells from Schwann cells [40, 62]. In mouse NCCs cell cultures incubated in complex medium containing EDN, melanoblasts proliferated and successfully differentiated. However, upon the removal of EDN, melanoblast cells still emerged, suggesting that EDN signaling is not involved in melanocytic specification from NCCs [63]. Furthermore, continued migration of melanoblasts along the dorsolateral pathway requires EDN signaling. The absence of EDN signaling causes stalling of specified melanoblasts in the MSA [36, 64]. Lastly, mutations in the receptor or ligand of EDN signaling cause pigmentation defects. These defects were found to not be caused by a failure in melanocyte specification but, rather, in the later role of EDN signaling in melanocyte development [65]. Since the EDN pathway does not play a role in melanoblast survival and differentiation, this pathway is thought to be partially redundant with other pathways involved in melanocyte development.

Another pathway required for melanocyte development is tyrosine kinase receptor KIT. KIT signaling has been well-studied and found to regulate melanoblast survival, proliferation but not late melanoblast migration. Upon injection of KIT antibodies into pregnant mice at varying developmental stages, three different patterns of melanocyte pigmentation and densities were observed in embryos and adult mice [66, 67]. Mutations found in the KIT also led to hypopigmentation defects. KIT signaling is involved in melanoblast proliferation, survival and dispersal along dorsolateral pathway. It also partly mediates the melanocytic differentiation program. However, melanocyte specification in the early stages of NCC fate determination occurs independently of KIT signaling [36].

Canonical WNT/β-catenin signaling has been implicated in the early stages of melanocyte development [68]. The WNT ligand binds to its receptor, Frizzled, and β-catenin accumulates in the cytoplasm. β-catenin is translocated into the nucleus and subsequently interacts with the lymphoid enhancer-binding factor (LEF)-1/T-cell factor (TCF). This interaction modulates the transcription of several target genes, including MITF and CCND1 (Cyclin D1) [4]. In mouse studies, the directed gene transfer of WNT1 to NCCs resulted in the expansion and accelerated differentiation into pigmented cells [69]. Similarly, studies in quail NCCs demonstrated that signaling by WNT3A increased the number of melanocytes while simultaneously reducing the number of neural cells [70]. Finally, in zebrafish studies, *in vivo* overexpression of activated β-catenin promoted the formation of pigmented cells and repressed the generation of sensory neuronal and glial lineages [71]. When WNT signaling was inhibited *via* the injection of dominant negative WNT1, NCCs adopted a neural fate rather than a pigmented cell phenotype. β-catenin-deficient NCCs fail to express melanocytic markers such as MITF and TRP2. Therefore, canonical WNT/β-catenin signaling controls

expression of key melanocytic genes, MITF, and is important for melanocytic specification [36, 71, 72].

However, other studies suggest a more complex mechanism of Wnt/β-catenin signaling and melanocyte specification and development. For example, further studies in zebrafish observed that the inactivation of β-catenin inhibited the formation of both pigmented and neuronal cells [73]. Recent work tried to explain the inconsistent findings in Wnt/β-catenin signaling and melanocyte development. Using Cre cell lines, β-catenin was conditionally activated in NCCs at different developmental stages [74]. The activation of β-catenin in premigratory NCCs led to the formation of ectopic melanoblasts, while suppression of all other lineages was observed. Additionally, the activation of β-catenin in glial precursors or melanoblasts did not promote enhanced production of melanoblasts. This study demonstrated that NCC fate decisions *in vivo* are subject to strict temporal control by WNT/β-catenin, where WNT/β-catenin responsiveness is highest during NCC migration [74].

As mentioned above, the migration of melanocyte precursors is possible through two routes: dorsolaterally (traditional view) and ventrally (new theory). Different extrinsic growth factors control progenitors traveling ventrally compared to those on the dorsolateral route. However, since this route was only recently described, many mechanisms remain poorly understood. A few mechanisms have been studied. The growth factor neuregulin-1 (NRG1) is a well-established controller of axonal signaling in Schwann cells, regulating proliferation, migration and myelination. This signaling is mainly mediated by the ERBB2/3 heterodimer receptor. In mice lacking ERBB3, Schwann cells along nerves are completely abolished while melanoblast MITF expression significantly increases [38]. Other signaling mechanisms involved in regulating melanoblasts along nerves include platelet-derived growth factor (PDGF) and cytokines interleukin (IL) 3 and 5 [36]. Further investigations are required for a complete understanding of these mechanisms in regards to melanoblast formation and specification.

Visualization of melanoblast migration has been achieved in mice using a lacZ transgene with the TRP2 promoter [75]. Melanocytes differentiate from the pluripotent NCC population at embryonic day (E) 8.5 before migrating along the dorsal-lateral pathway and ventrally through the dermis. However, it is now accepted that melanocytes precursors migrate both dorsolaterally and ventrally and can be derived from nerve cells, where a dominant fraction of skin melanocytes is formed [36]. By E14.5 in mice, differentiated melanocytes populate the epidermis and developing hair follicles [46, 75].

As melanoblasts migrate, they are constantly proliferating and, as the population expands, apoptosis is also inhibited. The melanoblast population is able to migrate vast distances

through the dermis, across the basement membrane, eventually settling in the epidermis [4]. Upon reaching their final destination in the epidermis, melanoblasts become fully differentiated melanocytes.

1.1.3 Neural crest as a model system

The NC is an important and crucial part of embryonic development in vertebrates. The NC is an excellent system for studying developmental processes [3]. For these reasons, many studies have tried to recreate NC development *in vitro* for further analysis.

Earlier studies of the NC *in vivo* typically used embryos from avian, *Xenopus* or mouse species. For example, mouse embryos were used to examine melanocyte development *in vivo* and resulted in the discovery of the importance and dependence for the transcription factor MITF [76]. These studies used mouse embryos expressing wild-type (WT) MITF and MITF mutant embryos homozygous for the $MITF^{vga-9}$ or $MITF^{mi-ew}$ alleles, which encode non-functional proteins. Moreover, these studies required neural tube explants obtained from embryos at E9.5 [76]. Though these studies laid the groundwork for NC and melanocyte development and identified crucial regulators in these processes, further advancement in biology has allowed for alternative ways of studying NC development.

Recently, studies have successfully modeled the NC *in vitro* without the use of animal models. Authors developed a model using human embryonic stem cells (hESCs), where migratory cells undergo EMT to acquire properties of the NC [77]. The model used various culture conditions at different stages to promote propagation, segregation, gliogenesis and finally neurogenesis. With this model, the study identified another member of the SOX family, SOX2, involved in sensory neurogenesis [77]. This step-wise protocol was reproduced in another study as well [78]. Together, these studies gave insights into how modeling the NC will provide new perspectives without using animal models. However, the use of hESCs raises ethical concerns and is not an ideal model system.

In particular, melanoblast model systems have also been established *in vitro* for further analysis into the melanocytic lineage. One study established a cell culture system to generate melanoblast-related cells (MBrc) [79]. This method was first described by Cook *et al*. [80]. The study demonstrated that the MBrc model is a reproducible system and may provide new insights into the role of melanoblast-related genes in melanoma progression.

The most recent and groundbreaking research came with the discovery of induced pluripotent stem cells (iPSCs). Seminal research by Yamanaka and colleagues found that a terminally differentiated cell can be reprogrammed to its pluripotent state *via* the induction of four factors: SOX2, KLF4, OCT4 and NANOG [81]. This enabled further intensive research in

the field of development. The generation of iPSCs avoided ethical issues associated with hESCs and also provided research with better tools for development and disease modeling. In 2011, Ohta and colleagues successfully differentiated human iPSCs into melanocytes by supplementing culture medium with WNT3A, stem cell factor (SCF) and endothelin (ET)-3 [82].

Recently, Studer and colleagues successfully modeled NC induction and the eventual specification of melanocytes using an *in vitro* system [83]. This work provided a complete model system from iPSC to NCCs to melanoblasts and finally ending with terminally differentiated melanocytes. Upon using timed exposure to WNT, BMP, and EDN3 and under dual-SMAD inhibition culture conditions, this triggered the sequential induction of NC and melanocyte precursor fates. Moreover, the global gene expression profile was analyzed throughout the model, giving new perspectives into NC and melanocyte fate determination [83]. In summary, the future of NC development and melanocyte progenitor research lies with *in vitro* modeling systems. These models allow for efficient analysis and new insights into development that were otherwise impossible due to constraints of animal models, ethics or limited resources.

1.2   Melanocytes and their function in the skin

The skin is the largest organ of the human body and plays one of the most important roles in protecting the body and its internal organs from external harm, such as microbes and environmental stress. Moreover, the skin maintains body temperature, prevents water loss, allows for the feeling of touch and protects against harmful ultraviolet (UV) radiation from the sun. The skin is composed of several layers of ectodermal tissue. The two primary layers in the skin are the epidermis and dermis. The epidermis is the outermost layer and contains melanocytes and keratinocytes. The epidermis is divided into five sublayers: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The dermis is where connective tissue, hair follicles, sweat glands, sebaceous glands and blood vessels are mainly located. The hypodermis is not a part of the skin but is located below the dermis and responsible for attaching the skin to muscle and bone [84].

Melanocytes are pigmented cells located in the stratum basale of the skin's epidermis. These cells populate the integument, inner ear and eyes of vertebrate organisms and arise from pluripotent NCCs [4]. These cells become fully differentiated upon reaching the basal membrane of the epidermis and their development and differentiation status are regulated by a complex network of genes. Additionally, melanocytes at hair follicles are produced by melanocyte stem cells.

1.2.1 MITF: master regulator of the melanocytic lineage

MITF is the most critical regulator of the melanocytic lineage. This gene is not only responsible for melanin biosynthesis but also regulates cellular proliferation, survival, lineage determinacy and the replenishment of follicular melanocytes in adults [85, 86]. In vertebrates, MITF is expressed in melanoblasts and melanin-containing melanocytes and retinoid pigmented epithelia (RPE) cells [85]. Moreover, loss of MITF studies performed in mice and zebrafish showed a complete loss of melanocytes, further illustrating its key function in melanocyte biology [87]. Several isoforms (assigned A, B, C, D, E, H, M and Mc) of the MITF protein have been identified and characterized. They differ in their N-terminal region due to alternative promoters and first exons. It remains unclear whether the various isoforms have specific functions [86]. However, the MITF-M gene transcript has been found to be heavily involved in melanocytic lineage and is the only isoform that will be further discussed.

The four main regulators of MITF-M transcription are CREB, SOX10, PAX3 and LEF1/TCF. Of several loci identified, the melanocortin-1 receptor (MC1R) has been found to be a major determinant of the pigmentation phenotype [46, 88]. MC1R encodes a seven-transmembrane domain G protein-coupled receptor. Once it has been bound by its agonist, typically α-melanocyte stimulating hormone (α-MSH), it activates adenylate cyclase and induces cyclic AMP (cAMP) production [89]. This production leads to the phosphorylation of cAMP responsive-element-binding protein (CREB) which further transcriptionally activates numerous other genes, including MITF.

Another crucial regulator of MITF and the melanocytic lineage is SOX10. SOX10 is also important and expressed in the NCC population [90]. Though SOX10 is not required for the development or early migration of NCCs, NCCs in mice lacking SOX10 undergo apoptosis before completing terminal differentiation [19]. Studies have shown that SOX10 is a direct regulator of MITF-M and therefore plays a crucial role in melanocyte differentiation [54, 55, 91]. Moreover, the SOX10-mediated activation of MITF can be improved by the presence of PAX3, which suggests a synergy between SOX10 and PAX3 [54, 92].

Other regulators of MITF-M transcription are the lymphoid enhancer binding factor LEF-1/TCF HMG-domain transcription factors. These factors mediate their functions in canonical WNT signaling *via* interaction with β-catenin [93, 94]. The MITF-M promoter contains LEF-1/TCF consensus binding sites and is directly regulated by WNT signaling when complexed with LEF-1 [68, 72]. Upon transcription, MITF is further regulated *via* post-translational modification by phosphorylation, which enhances the binding of MITF to the tyrosinase promoter [95–97].

Perhaps, the most crucial role of MITF in melanocyte biology is its regulation of melanogenesis or pigmentation. The pigmentation process only occurs after complete differentiation from melanoblast precursors. Studies have demonstrated that MITF is capable of transactivating the promoters of three major pigmentation-related genes: TRP1, TRP2 and TYR. Downregulation of TYR and TRP1 was observed upon MITF inhibition [98]. Despite the evidence indicating a direct effect of MITF on pigmentation-related genes, overexpression of MITF in either mouse B16 melanoma cells or human melanocytes did not result in upregulation of TYR expression [99]. Lastly, MITF is also responsible for many other target genes crucial for the melanocytic lineage, including glycoprotein (GP)-100 (PMEL17) and melanoma antigen recognized by T cells (MART)-1 [100]. Taken together, MITF can drive the activation of target pigmentation-related genes. However, this might depend on further cooperation with additional proteins.

1.2.2 SOX10: crucial regulator of melanocyte survival and specification

SOX10 has proven its importance in NC specification and terminal differentiation of melanocytes. SOX10 is capable of controlling melanocyte development on two levels. First, SOX10 is crucial for the survival, maintenance, and proliferation of NCCs. This controls the overall size of the NCC population and indirectly influences the number of melanocytes. Second, SOX10 regulates melanocyte specification by modulating melanocyte differentiation factor MITF-M *via* directly binding to the proximal promoter and activating transcription [101]. In addition to these essential roles, SOX10 is also able to influence another important factor in melanogenesis, TRP2. Studies found that SOX10 is capable of binding to the TRP2 promoter in a similar manner to that of MITF, thereby activating its transcription [102]. Moreover, SOX10-dependent activation of the TRP2 promoter could be synergistically increased by MITF [103]. The TRP2 promoter also contains CREB and β-catenin/LEF-1 binding sites, though the function of these sites and the role of SOX10 may play in this pathway remains under investigation.

1.2.3 Other important genes in melanocyte biology: PAX3, KIT and EDNs

The expression of genes involved in melanoblast and late stages of melanocyte development are often found in fully differentiated melanocytes. These include MITF and SOX10 but also PAX3, KIT and EDNs. These genes remain important regulators of the melanocytic lineage throughout the lifespan of a melanocyte.

Not only is PAX3 important for the regulation of MITF-M, it also plays a crucial role in proliferation, migration, resistance to apoptosis, lineage specificity and differentiation of melanocytes. PAX3 is important in NC and early melanocyte development and its expression continues to be essential in differentiated melanocytes for promoting and inhibiting

melanogenesis [56]. PAX3 also is capable of inhibiting TRP2 and blocking binding of MITF to the promoter of TRP2. PAX3 forms an inhibitor complex with LEF-1 and the groucho-related gene (GRG)-4. When β-catenin is present, LEF-1 switches partners, forming an activating complex composed of LEF-1, MITF, and β-catenin. This activating complex is able to displace PAX3 from the TRP2 enhancer and activate transcription [56]. PAX3 is also capable of influencing the melanocyte lineage *via* the binding to the promoter and driving expression of TRP1. Therefore, PAX3 is a crucial regulator of melanogenesis and is able to influence cellular proliferation and apoptosis.

Another key NC marker and essential melanocyte development gene is KIT, a type III receptor tyrosine kinase [104]. The function of KIT remains of importance throughout the complete differentiation and life of a melanocyte. Detailed analyses of KIT mutants found that KIT and its corresponding ligand play a complex role in final melanocyte migration from the dermis to the epidermis as well as regulating cellular survival [47]. In differentiated melanocytes, KIT signaling remains important for melanocytic survival in the skin. The ligand of KIT receptor, SCF, is a paracrine factor synthesized by various other skin cells, including epidermal keratinocytes [105].

In addition to c-KIT/SCF, there are other mechanisms likely to be involved in the late steps of melanocyte migration from the dermis into the epidermis, including the EDNs [46]. The endothelin-1 (ET1) peptide is a paracrine factor also synthesized by surrounding keratinocytes. The function of ET1 is primarily to bind and activate the endothelin-B receptor (ETBR). Upon ligand binding, a complex signaling network is activated, including the activation of protein kinase (PK) C [105]. This network, which is also important in melanoblast migration, is thought to play a role as a paracrine regulator of melanocytes, since ET1 is expressed by surrounding keratinocytes.

1.2.4 Functions of melanocytes: pigmentation and ultraviolet radiation response

Pigmentation in the skin is an essential defense mechanism against ultraviolet (UV) radiation of the sun. The pigment produced by melanocytes is termed melanin and is synthesized only by membrane surrounded organelles called melanosomes [100]. In the typical response to UV radiation, melanin-containing melanosomes are transferred to the periphery of melanocytes, where they are ultimately transported to keratinocytes [106]. The most important proteins involved in melanin catalysis include TRP1, TRP2 and TYR, whose genes are all activated by MITF-M. Another crucial protein which maintains the structural integrity of melanosomes is known as GP100, PMEL 17 or premelanosome protein (PMEL) [100].

The biochemistry of melanin production in melanosomes by the catalytic enzyme tyrosinase has been well studied. Tyrosinase converts the amino acid tyrosine into melanin *via* several straightforward reactions. The initial and critical reaction involves the hydroxylation of tyrosine into 3, 4-dihydroxyphenylalanine (DOPA). After synthesis of DOPA, this byproduct can give rise to biopolymer melanin *via* extensive reactions, termed the Raper-Mason scheme [107–109]. In brief, these reactions are a series of oxidation reactions which form indole-quinone ring structures and permit the polymerization and quick configuration of pigmented biopolymers [110–112]. These biopolymers become different forms of melanin, depending on several external factors [46, 113].

After the production of melanin in the melanosomes, these organelles are transported for protective measures against environmental responses. Melanosome transport has been well-studied in mice in which the pigmentation pathway has been altered or compromised. There are three major players in the transport of melanosomes through the cytoplasm to the periphery dendrites. These genes are Ras-related protein RAB27A, melanophilin, and myosin VA (MYO5A) [114–116]. On a molecular level, melanosomes become tethered to myosin motors which move the organelles through the cytoplasm. Upon reaching the periphery dendrites, the melanosomes are captured by actin filaments and remain there until they are delivered to keratinocytes by a process which remains poorly understood. However, recent studies have revealed that protease activated receptor 2 (PAR2) is involved in this process [117, 118]. After melanosomes reach the keratinocytes, they are distributed and, in response to UV radiation, positioned strategically over the 'sun-exposed' side of nuclei to form cap-like structures resembling umbrellas [46].

Melanocyte dendricity is not only important for the structure of the cell but also for the transfer of melanosomes to neighboring keratinocytes. Dendricity affects cell-to-cell communication, especially with keratinocytes, which stimulate melanocytes to proliferate and become more dendritic by secreting their own factors [119, 120]. Moreover, actin and RAC1/RHO have been shown to be important in the formation of dendrites. These can be externally regulated by physiological factors, such as MSH or UV radiation [121, 122].

UV radiation stimulates pigmentation in the skin by a process more commonly known as tanning. There are two main tanning processes in the skin. The first is called the immediate pigment darkening, where tanning occurs within minutes of the UV exposure. The second type of tanning process is known as delayed tanning, where the tanning response is only seen after several days [100]. The major regulator of the melanocytic lineage MITF tightly controls melanogenesis and the UV response mechanism. On a molecular level, MSH, which functions mainly through MC1R, controls the expression of MITF and its downstream targets

TYR, TRP1, TRP2, GP100 and MART1. Studies found, that regardless of racial background, similar increases in MITF expression were found within one day of exposure to UV radiation. Additionally, levels of TYR, TRP1, GP100 and MART1 were also similarly expressed between one and seven days, regardless of race [100].

Aside from the many protective responses exerted by melanocytes, UV radiation has many adverse side effects in the skin, including the induction of DNA damage. Upon characterization of melanocytes and keratinocytes after UV radiation, there is an inverse relationship between melanin content and DNA damage induced *in situ* [100]. Moreover, between individuals, there is great variability in UV-induced DNA damage. UV-induced DNA damage can also cause mutations to occur in other key regulatory genes.

1.3 Melanoma

Melanoma is a dangerous form of skin cancer derived from the malignant transformation of melanocytes. Though malignant melanoma only accounts for four percent of all dermatological cancers, it remains the most lethal by far, accounting for 80 % of all skin cancer-related deaths [106]. Additionally, the incidence of melanoma has nearly doubled in westernized countries over the past 20 years [123]. According to the Skin Cancer Foundation, melanoma kills an estimated 9,710 people in the US annually. The American Cancer Society estimates that, to date, there are more than 120,000 new cases of melanoma in the US each year. In 2014, an estimated 76,100 of these will be invasive melanomas, with about 43,890 cases in males and 32,210 cases in women [124].

If melanoma is recognized and treated early, it can be easily cured, typically by surgical resection. Approximately 80 % of melanomas are treated in this manner [123]. However, once the cancer has metastasized and spread to other regions of the body, therapy options become limited and poor prognosis is common, with a median survival rate of 6 months and 5-year survival rate of less than 5 % [123, 125]. The development of immunotherapies and targeted therapy has improved the treatment and survival of melanoma patients. However, resistance to these treatments has now become the major obstacle in melanoma therapy. Once the melanoma has spread from its initiation site to distant organs it becomes difficult to treat. The major risk factors associated with melanoma include multiple benign or atypical nevi and a family history of melanoma. Other genetic predispositions or environmental risks include sun sensitivity, immunosuppression and exposure to UV radiation [106].

The pathogenesis of melanoma was first characterized and classified in the 1970s by Clark [126]. This model describes different histological stages of melanoma, beginning from a benign melanocytic nevus to malignant melanoma *via* dysplastic nevus [127]. Although this model portrays linear progression, where each lesion is the immediate precursor to the next,

research has found that the pathways by which melanoma develops may vary [128]. Nevertheless, the model is still used and describes the first lesion type as a benign nevus or mole (small, well-defined). The next lesion is termed a dysplastic nevus, which is clinically defined as a nevus containing one or more features of melanoma, such as a larger size or irregular borders. The next phase is the radial growth-phase (RGP) of melanoma, where the lesion grows progressively but only 'radially'. This phase occurs within the epidermis only. The last phase of the Clark model is the vertical growth-phase (VGP), where the melanoma forms large, distinct nodules in the dermis, indicating the potential for metastatic spread [128].

1.3.1 Origins of melanoma

The deregulation of several pathways allows melanocytes to escape their tight regulation by keratinocytes and thereby facilitate malignant transformation. These transforming-promoting mechanisms include mutations in growth regulatory genes, production of autocrine growth factors and the loss of adhesion receptors [129]. Much research has been done to investigate the risk factors associated with melanoma initiation. One well-studied cause of malignant transformation in melanocytes is exposure to UV radiation.

Exposure to UV light is the most well-known environmental risk factor associated with melanoma initiation. UV radiation causes mutations in skin cells, increases the production of growth factors, induces the formation of DNA-damaging reactive oxygen and affects cutaneous immune function [106]. Tanning of the skin is a natural defensive and protective measure taken by the skin against UV radiation. In response to UV radiation, keratinocytes excrete factors which control several functional processes of melanocytes, including the stimulation of melanin production [123].

As seen with many other cancer types, genetic familial predisposition is also a cause of malignant melanoma in some cases. A family history of melanoma occurs in approximately 10 % of melanoma patients and increases the risk of acquiring the disease by nearly two-fold [130]. Melanoma is often considered a genetic disease due to a range of heritable risk factors such as skin complexion and eye color.

1.3.2 Familial mutations in melanoma

Due to the familial link, it has been suggested that, in melanoma, there is an inheritance of melanoma susceptibility genes. To date there have been four different genes at three different loci identified to confer susceptibility in melanoma, the genes include: $p16^{INK4a}$, $p14^{ARF}$, CDK4 and TERT.

The cyclin-dependent kinase inhibitor 2A (CDKN2A) locus, located on chromosome 9p21, is unique because it encodes two different, unrelated protein products: p16[INK4a] and p14[ARF] [131]. These two transcripts can be altered simultaneously in both familial and sporadic melanoma cases. Inactivation of the CDKN2A locus is mainly caused by deletion, mutation or promoter silencing by hypermethylation (review in [132]). Mutations in this locus are strongly related to familial history, when three or more family members are affected, 35.5 % of patients developed melanoma, while only 8.2 % of those without any family history develop melanoma [133]. Both gene products impact cellular proliferation; however *via* separate mechanisms. p16[INK4a] protein blocks cell cycle progression inhibiting the activity of cyclin D1-dependent kinase 4 (CDK4). This inhibition blocks CDK4 from phosphorylating the retinoblastoma (RB) protein and induces cell cycle arrest at G1 phase. While p14[ARF] plays an important role in stabilizing p53 by inhibiting the ubiquitin ligase murine double minute 2 (MDM2) protein responsible for degrading p53 [134]. Disrupting the degradation of p53 promotes cellular growth, since p53 normally arrests cell division at G1 to either allow DNA repair or induce apoptosis in possibly transformed cells [132].

Another melanoma susceptibility gene is CDK4, located at 12q13.6 and which encodes proteins that interact with p16[INK4a]. Mutation in CDK4 is extremely rare but has been reported in the germline of melanoma-prone families. Typically, these mutations are observed at an arginine residue (Arg 24) and eliminate regulatory interactions with p16[INK4a], leading to enhanced cell cycle progression (reviewed in [130]). It is important to note that the result of mutations in either p16[INK4a] or CDK4 seem to be functionally equivalent and both block the association between each other and hence impair the ability of p16[INK4a] to activate the RB family [135].

Recent studies identified a new melanoma susceptibility gene, the telomerase reverse transcriptase (TERT). Researchers investigated melanoma-prone families using linkage analysis and identified a germline mutation in the promoter of the gene that encodes the catalytic subunit of telomerase. This mutation is capable of creating a new binding motif which enhances its transcription two-fold [136]. Additionally, work identified that mutations in TERT can occur somatically in melanoma. The functions of TERT mutations in melanoma progression are currently being investigated [136, 137].

1.3.3   Somatic mutations in melanoma: BRAF, RAS, MITF, KIT and PTEN

Somatic mutations, like in other cancer types, are a main driving force in melanoma initiation and progression. UV-induced DNA damage can cause the formation of somatic mutations and, when these mutations are induced in key regulatory genes or oncogenes, the result is carcinogenesis. Mutations which upregulate murine sarcoma viral oncogene homolog B

(BRAF) are most common mutations in melanoma, occurring in nearly 65 % of all melanoma cases. The most common somatic mutation in the BRAF gene is a missense mutation from valine to glutamic acid at amino acid 600 (BRAF^{V600E}) in the ATP-binding region of the protein. This specific mutation comprises of nearly 90 % of all BRAF-mutated melanomas, while other variations, including BRAF^{V600K}, are rare [138]. These mutations occur somatically, since wild-type forms of both genes are also found in normal tissue of melanoma patients [139]. In fact, there is a very low incidence of BRAF mutations found in melanomas arising from non-sun exposed skin, suggesting that UV exposure plays a crucial role in inducing BRAF mutations in cutaneous melanoma [135].

Functionally, the BRAF^{V600E} mutation causes a 10-12-fold increase in its activity, triggering the hyperactivation of the mitogen-activated protein kinase (MAPK) signaling cascade leading to cellular survival and proliferation [135]. Studies in immortalized mouse melanocytes showed this connection by expressing mutant BRAF and observing the induction of extracellular signal-regulated kinase (ERK) and the initiation of malignant transformation [140]. Moreover, upon treating human melanoma cell lines with BRAF mutations with mitogen-activated protein kinase kinase (MEK)-1/2 inhibitor U0126 the blockade of MAPK signaling and cell cycle progression was observed, but had no effect on melanoma cells harboring oncogenic neuroblastoma viral oncogene homolog (NRAS). Therefore, mutated BRAF can act as a potent oncogene in early stages of melanoma progression through activated MAPK signaling. However, mutated BRAF is not required for RAS-transformed melanocytes due to the innate redundancy within the pathway [140].

The BRAF^{V600E} mutation is found in approximately 80 % of benign nevi. In zebrafish, expression of melanocyte-specific BRAF proteins induces ectopic proliferation of melanocytes, analogous to human nevi [141]. However, it is unknown why mutated BRAF leads to benign nevi formation and malignant formation. Multiple requirements are needed for transformation. A BRAF mutation alone is not sufficient to progress towards malignancy. In the zebrafish study described above, a combination of a BRAF mutation and inactivation of the tumor-suppressor gene p53 led to the malignant transformation of melanocytes [141]. Additionally, studies identified the association between mutant BRAF^{V600E} and p16^{INK4a}/p19^{ARF} loss or mutations in p53 and PTEN leading to malignant transformation [142–144]. These studies provide an explanation for how BRAF mutations exist in nevi without directly inducing malignant transformation.

The RAS family is also affected by somatic mutations in melanoma and functions as a transducer of extracellular growth factor signals in the cell. Among the RAS gene family, NRAS is the most commonly mutated and represents the second most common mutation

found in melanoma, comprising of nearly 20 % of mutations in the disease. This mutation is a point mutation of amino acid 61 from a glutamine to a lysine (NRAS[Q61K]) or arginine (NRAS[Q61R]), but there are many variations. Mutations also occur in other RAS family members but mutations in NRAS seem to be the most detrimental and oncogenic. Studies in mice investigated the different consequences of HRAS and NRAS in melanocytes. The hyperactivation of HRAS in combination with loss-of-function mutations in CDKN2A and/or p53 led to non-metastatic melanomas in mice. Activation of NRAS together with CDKN2A loss led to the production of melanomas with severe metastatic spread to both lymph nodes and distant organs in mice [135, 145, 146].

As described above, MITF plays a crucial role in melanocyte development and differentiation. The function of this gene in malignant transformation and melanoma progression has become important and remains not well understood. The role of MITF in differentiation and cell cycle arrest in normal melanocytes is known, but conversely MITF in melanoma cells does not possess the same function. A large study investigated genomic changes in melanocytes using analysis of high-density single-nucleotide polymorphisms (SNPs) and discovered an increased copy number of a region on chromosome 3 that included the MITF locus [147]. Moreover, this increase was accompanied by increased expression of the MITF protein. Upon the overexpression of both MITF and BRAF, primary human melanocyte cultures were malignantly transformed. The amplification of MITF correlates to poor prognosis and is associated with melanoma therapy resistance [147]. Taken together, these results suggest that MITF is an oncogene and a lineage-survival gene in melanoma [147–149].

There are many other somatic mutations found in melanoma [136, 137, 150–153]. For example, another gene susceptible to somatic mutations is the KIT oncogene. Approximately 1-2 % of all melanomas contain point mutations in the KIT receptor tyrosine kinase gene. Most of these mutations are found in mucosal and acral melanomas and also in constantly sun-damaged melanomas [138, 154]. An influential study observed that the MAPK signaling pathway was activated upon stimulating melanoma cells with the KIT ligand, SCF. This resulted in the phosphorylation of MITF and in the transactivation of the promoter of the pigmentation-related gene TYR [95]. This link between external signals transduced by KIT into gene regulation became an early indication for the importance of KIT in melanomagenesis.

Another gene susceptible to somatic mutations in melanoma is the phosphatase and tensin homolog (PTEN) gene. Somatic mutations in PTEN are found in 40-60 % of melanoma cell lines and in approximately 10-20 % of primary melanomas, where the majority of the

mutations occur in the phosphatase domain (reviewed in [132], [155]). However, the contrast between low mutation frequency detection and a high level of gene silencing can be explained by other mechanisms important for the inactivation of PTEN, including epigenetic silencing and altered subcellular location of this protein [135]. Several methylation sites have been found within the PTEN promoter. Hypermethylation of these sites reduce PTEN expression in melanoma [132]. Moreover, genetic studies have found that deletions in chromosome 10q, including the PTEN locus, occur at high frequencies in BRAF-mutated melanoma, while deletions in this chromosome were less common in NRAS-mutated melanomas [156, 157]. This work suggests that BRAF and PTEN may cooperate in melanoma progression. PTEN is commonly regarded as a tumor suppressor because it acts as an antagonist of the phosphatidylionositol-3 kinase (PI3K) pathway and MAPK signaling, which will be discussed further in the following sections.

1.3.4 Pathways affected by somatic mutations in melanoma: MAPK, PI3K-AKT and TGF-β signaling

The complex signaling network which initiates, maintains and drives melanoma progression is well-studied. However, the depth of complexity leaves much left to uncover. In the next section, I will only highlight the key signaling pathways involved in melanomagenesis, including MAPK, PI3K-AKT and TGF-β signaling.

The RAS/RAF/MEK/ERK signaling cascade, also known as MAPK pathway, is a serine-threonine cascade involved in the regulation of cell growth, survival and differentiation. In melanocytes, this pathway drives melanogenesis by regulating cell proliferation and survival. In addition, the MAPK pathway has been meticulously studied in melanoma, since somatic mutations in BRAF and NRAS cause an overactivation of this pathway. First, the signaling cascade starts by the binding of a growth factor (*e.g.* SCF, EGF or PDGF) to its corresponding receptor (*e.g.* KIT, EGFR or PDGFR). This binding allows for RAS (a GTPase) to exchange its bound guanosine diphosphate (GDP) for guanosine-5'-triphosphate (GTP). This exchange initiates the recruitment and phosphorylation of membrane-bound RAF proteins. Lastly, activated RAF induces downstream signaling *via* phosphorylation of MEK and, subsequently, ERK. ERK phosphorylation results in its translocation to the nucleus where it controls expression of key survival, cell-cycle progression and differentiation-associated genes [142, 158, 159].

The hyperactivation of the MAPK signaling is well-understood and strongly linked to the somatic mutation of BRAF. However, mutation of BRAF alone is not sufficient to induce malignant transformation. The MAPK signaling pathway is heavily involved in melanomagenesis by promoting cellular proliferation, inhibiting apoptosis and inducing tumor

invasion and metastasis. The overactivation of ERK controls cell proliferation by playing a role in the G1-phase to S-phase transition. The activation of ERK negatively regulates the p27/Kip1 inhibitor and upregulates c-MYC, leading to melanoma cell growth. Likewise, the inhibition of ERK leads to G1-phase cell cycle arrest mediated by the upregulation of cyclin-dependent kinase (CDK) inhibitor p27/Kip1 and hypophosphorylation of the RB protein [160]. Additionally, ERK activation targets downstream transcription of POU domain, class 3, transcription factor 2 (POU3F2 or BRN2), which then leads to an increase in cell proliferation [135].

Inhibition of apoptosis is another important effect of the hyperactivation of the MAPK signaling pathway. The overactivation of ERK mediates melanoma-specific survival signaling by regulating ribosome S6 kinase *(*RSK)-mediated phosphorylation leading to inactivation of proapoptotic Bcl-2-associated death promoter (BAD) protein. Aside from controlling apoptosis, the MAPK pathway is also important for the acquisition of the invasive phenotype. ERK activation regulates MMP production, especially the production of MMP-1. Studies found that, upon blocking ERK activity, melanoma cell proliferation and collagen degradation was inhibited [161]. Additional functions of the MAPK signaling cascade lie in its induction by α-MSH and, therefore, cAMP levels. This cAMP-induced activation of ERK induces differentiation *via* inducing MITF expression, leading to the upregulation of the differentiation-associated genes TRP1 and TRP2, though this activation is weak and transient [162].

In summary, the MAPK signaling cascade is an important pathway for maintaining melanocytic proliferation. However, the introduction of somatic mutations in RAS or RAF can cause the hyperactivation of this pathway and induce malignant transformation of melanocytes into melanoma. Although the hyperactivation of this pathway alone is not sufficient to induce transformation, the sustained activation of this pathway leads to cell proliferation, survival, inhibition of apoptosis and induction of cellular invasion. The weak and transient activation of this pathway *via* α-MSH and cAMP can also lead to cellular differentiation, further underscoring the complexity of melanomagenesis.

The phosphatidylinositide 3-kinases (PI3K) is commonly dysregulated in melanoma, typically by mutation and silencing of the tumor suppressor gene phosphatase and tensin homolog (PTEN). PTEN encodes a phosphatase that inactivates the products of PI3K. The PI3K-AKT pathway functions by first activating PI3K, which then increases phosphatidylinositol 3, 4, 5-triphosphate (PIP3) production. Conformational changes activate protein kinase B (also known as AKT), leading to cell cycle progression, survival and migration. However, PTEN antagonizes this pathway with two main biochemical functions: protein phosphatase and lipid phosphatase. The lipid phosphatase activity plays an anti-tumorigenic role by decreasing the

function of downstream AKT. PTEN dephosphorylates the cytosolic second messengers of the PI3K-AKT pathways, PIP3 and phosphatidylioninositol 3, 4-bisphosphate (PIP2) [163, 164]. The loss of function of PTEN in tumor cells leads to the accumulation of these second messenger lipids and activates AKT and decreases apoptosis, while the reintroduction of PTEN into PTEN-null cells reverts this effect [130, 132, 135].

AKT activation induces many functional changes in the cell, including cell cycle progression, survival and migration. A main activating mechanism for AKT is DNA copy gain, which is found in 40-60 % of melanomas. Mutations in the catalytic subunit of PI3K also occur in melanoma but at a low frequency (5 %). Studies have found that activated AKT promotes cellular proliferation by downregulating cyclin-dependent inhibitor p27 and upregulating CDNN1 (Cyclin D1). Additionally, the activation of AKT suppresses apoptosis *via* several mechanisms, including inactivation of pro-apoptotic proteins such as BAD [132]. Lastly, AKT activation can promote tumor migration and invasion through the induction of MMP proteins, including MMP-2 and MMP-9. Studies proposed that AKT activation leads to NF-κB binding to the promoter of MMP promoting its transcription [165].

Taken together, the PI3K-AKT pathway is extremely important in melanoma progression and survival. Mutation in the tumor suppressor PTEN enables the overactivation of AKT which further promotes cell cycle progression, inhibition of apoptosis and promotion of cellular migration and invasion. Moreover, there are activating mutations in AKT, which also lead to its overactivation in melanoma.

There are several other important signaling pathways that are crucial for melanoma progression and metastatic spread. One of those pathways is the transformation growth factor (TGF)-β signaling pathway. The TGF-β signaling is involved in regulating development and cellular growth. The TGF-β ligand, TGF-β1, binds to membrane receptors, such as transforming growth factor beta receptor (TGFBR)-2, that contain a cytoplasmic serine/threonine kinase domain. Ligand binding induces assembly of a receptor complex which phosphorylates proteins of the mothers against decapentaplegic (SMAD) family [135, 166]. Upon phosphorylation, SMAD proteins form heterodimers and they translocate into the nucleus, where they assemble complexes, bind DNA and regulate target gene transcription. Of note, there is substantial versatility in TGF-β responses. For example, there are countless combinations of interactions between the receptor and SMAD-interacting proteins, within the SMAD protein complexes and other sequence-specific transcription factors [167].

To date, studies have revealed the importance of TGF-β signaling in melanoma pathogenesis. One of the first observations was that there is increased expression and secretion of the different TGF-β isoforms in melanoma cell lines when compared with normal

melanocytes (reviewed in [168]). TGF-β was found to suppress the growth of normal human melanocytes, while melanoma cell proliferation was only slightly inhibited by TGF-β. Therefore, melanoma, cells have developed a resistance mechanism to inhibit anti-proliferative signals through TGF-β. A study found that melanoma cells express higher levels of SKI and SnoN in comparison to normal human melanocytes and that these molecules are capable of interfering with SMAD-dependent transcription of cell cycle regulator p21, therefore elucidating one possible mechanism used by melanoma cells to resist the anti-proliferative signals from TGF-β signaling [169, 170].

Additionally, TGF-β signaling is involved in melanoma metastasis. One study found the blockade of SMAD7 inhibited 1205Lu melanoma cells from forming bone metastasis [168, 171]. Moreover, tumor-stroma interactions where found to be important in melanoma, since the expression of TGF-β1 stimulated neighboring stoma cells *via* increased deposition and production of ECM proteins. This led to increased tumor cell survival and metastasis [172]. Taken together, TGF-β signaling plays an important yet complicated role in melanomagenesis and further work is required to fully understand the depth of its function in the context of this disease.

1.3.5 Current targeted melanoma therapies

The preferred treatment for melanoma is surgical resection. If detected early, melanomas can be removed surgically, with high progression-free survival. Therefore, early diagnosis remains crucial for improving clinical outcome of melanoma patients. However, once melanomas spread into the lymphatic or the circulatory system and home to distant organs, such as the liver, they become non-resectable and difficult to treat. The development of targeted therapies allows for the specific inhibition of factors responsible for the activation of mealnoma-driving pathways. To date, there are targeted therapies in melanoma inhibiting c-KIT, BRAF, CRAF, MEK1/2, mTOR, AKT and CDK4 (reviewed in [138]).

The BRAF kinase inhibitor vemurafenib was the first targeted therapy developed and shown to improve the clinical outcome of melanoma patients. Vemurafenib competes with adenosine-triphosphate (ATP) to bind the kinase domain of BRAF, leading to inhibition of BRAF-induced MEK activation [173]. Clinical trials found that, upon vemurafenib administration, patients displayed a response rate of 48 % and improved survival compared to dacarbazine, another chemotherapeutic agent [174]. This compound was approved by the Food and Drug Administration (FDA) and the European Medicines Regulatory Agency (EMA) in 2011. Soon after, a second BRAF inhibitor, dabrafenib, was developed and showed similar response rates but with different side effects [175]. The use of both of these compounds was not restricted to melanoma patients only possessing the V600E mutation. They were also

found to be effective in patients with other BRAF mutations, including V600K, V600R, p.T599del mutations and D594G [176]. Although the aforementioned compounds are generally tolerated well and considered safe, side effects associated with their use include, but are not limited to, fatigue, diarrhea and nausea [177, 178].

Another strategy for targeting the MAPK signaling pathway is the inhibition of the downstream proteins MEK1 and MEK2. The first developed MEK1/2 inhibitor, trametinib, was shown to improve the progression-free survival of stage IV melanoma patients harboring a BRAF^{V600E} or ^{V600K} mutation [179]. In 2013, this compound was approved by the FDA for the treatment of patients with BRAF^{V600E}-mutated metastatic melanoma. Phase I trials showed that trametinib may also be used in combination with paclitaxel, a mitotic inhibitor used in cancer chemotherapy [180]. Another MEK1/2 inhibitor, selumetinib, was developed and displays similar clinical activity in BRAF-mutant melanoma. Moreover, in a phase II double-blinded randomized clinical trial, this compound was tested in combination with dacarbazine in both BRAF-mutated and unknown primary melanoma, patients showed significantly improved progression-free survival compared with the control group receiving the placebo plus dacarbazine [181].

In addition to BRAF inhibition, another common mutation, observed in approximately 20-30 % of melanoma patients, is found in the NRAS gene. There are no current therapies which target NRAS directly; however, other treatments showing off-target effects on oncogenic NRAS are being used. For example, in patients harboring the NRAS mutation, treatment with MEK 1/2 inhibitors can lead to tumor regression. In a recent clinical trial, it was observed that 20 % of patients with NRAS-mutated melanoma had a partial response upon treatment with MEK inhibitor binimetinib (MEK162) [182]. Another strategy for targeting NRAS-mutated melanoma is *via* the simultaneous inhibition of MEK and CDK4/6. This is beneficial in NRAS-mutated melanoma patients because studies have revealed enhanced MAPK signaling and dysregulation of cell cycle checkpoints in NRAS-mutated melanoma [183]. Recent encouraging phase 1b/2 clinical trials showed that the combination of binimetinib (MEK162) and CDK4/6 inhibitor LEE011 resulted in partial responses in 43 % of patients [184].

Mutations in the oncogene KIT can lead to the overactivation of MAPK signaling and advance melanoma progression. Therefore, melanomas containing such regions were evaluated, leading to the development of the first targeted therapies against tyrosine kinase. One inhibitor, imatinib, showed an overall response rate of 16 %, with a response rate of 23.3 % in patients harboring a KIT mutation [185]. Other KIT inhibitors currently being

investigated in clinical trials including dasatinib, which targets the L576P mutation in KIT, and nilotinib [138].

In addition to targeted therapy, another novel approach to treating melanoma is stimulation of the immune system, known as immunotherapy. One therapy, ipilimumab, is a human IgG1 monoclonal antibody which blocks the cytotoxic T lymophocyte-associated antigen (CTLA-4). By inhibiting CTLA-4 activity, which normally is responsible for negatively regulating active T cells, results in an increase in proliferation of T cells. In clinical trials, patients with cutaneous melanoma showed an overall survival benefit when given ipilimumab [138, 154, 186]. Additionally, ipilimumab was used as adjuvant therapy in a placebo-controlled trial and showed significant improvement in recurrence-free survival in patients with stage III melanoma with a high risk of recurrence [187].

Another immunotherapy option in melanoma treatment is a monoclonal antibody which targets the programmed death receptor (PD)-1. PD-1 is responsible for downregulating the initial T cell activation by binding the immunosuppressive ligand PD-L1. By blocking this interaction, T cell activation is halted, therefore PD-1 signaling stimulates an immune response. The anti-PD-1 antibody MK3475, pembrolizumab, was first examined in patients in advanced stages of melanoma and was found to extend progression-free survival for more than seven months and sustained tumor regression was observed [188]. In September 2014, pembrolizumab (MK3475) received accelerated approval by the FDA for the treatment of patients with either advanced or non-resectable melanoma. Similarly, another PD-1 inhibitor was developed, nivolumab, and demonstrated beneficial two- and three-year progression-free survival rates [189]. In summary, the use of immunotherapy in treating melanoma has beneficial impact on overall survival and on tumor regression.

1.3.6 Therapy resistance in melanoma

The most prevalent issue with current melanoma therapy is the prompt acquisition of therapy resistance hypothesized in one theory to be acquired by selective pressure within the tumor. Typically, it is observed that the response to therapy, such as BRAF inhibitors, is transient. Therefore, mechanisms of resistance have been studied in detail.

The effectiveness of targeted therapy is limited to cancer cells which lack the ability to confer resistance. There are several general and extremely complicated resistance mechanisms that cancer cells employ in order to evade treatment, including membrane transport systems that control drug entry/export/distribution, enzymatic mechanisms that metabolize the drugs, anti-apoptotic pathways that alter cell death programs and tumor physiology associated with its microenvironment [190]. The variety of mechanisms highlight the complexity associated

with acquired resistance. Next, I will briefly provide some examples of known mechanisms associated with melanoma therapy resistance, especially with BRAF and MEK inhibitors.

The use of BRAF inhibitors showed much success in initial treatment, but the development of resistance is inevitable. Typically, resistance is acquired against targeted therapy by the development of secondary mutations. For example, resistance to KIT inhibitors, such as imatinib, may be caused by mechanisms induced by NRAS mutation and KIT copy number gain [154]. Conversely, instead of developing secondary gatekeeper mutations to reactivate BRAF, melanoma cells typically reactivate other members in the MAPK signaling cascade [191] upon BRAF inhibitor treatment. For example, patients that are resistant to BRAF inhibition have additional mutations in NRAS that reactivate the MAPK pathway (reviewed in [192, 193]). Moreover, BRAF activity is capable of being substituted by other paralogs of BRAF, such as CRAF and ARAF. Lastly, kinases that activate MAPK signaling are also involved in melanoma therapy resistance. One study identified the cobalt uptake protein (COT1) as an adaptive response mechanism to BRAF inhibition in melanoma cell lines. In the absence of activated BRAF, COT1 is able to stimulate the MAPK signaling pathway, leading to resistance [194]. Recently, work was done to better understand resistance mechanisms involved in BRAF and MEK inhibition in more detail. Studies found that suppression of SOX10 in melanoma induced the activation of TGF-β signaling, leading to the upregulation of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor-β (PDGFRβ) responsible for resistance to BRAF and MEK inhibitors [195]. Moreover, this work suggests that EGFR-positive melanoma may benefit from re-treatment of BRAF inhibitors after structured treatment suspension.

Due to the high occurrence of melanoma therapy resistance, many clinical trials have focused on combining already approved targeted therapy for synergistic and more potent effects in the clinic. Clinical studies have investigated selective BRAF inhibitors (vemurafenib, darafenib, encorafenib) in combination with selective MEK 1/2 inhibitors (trametinib, cobimetinib, binimetinib) [138]. One clinical trial investigated the effects of combining darafenib and trametinib as a first-line therapy option for patients with non-resectable or metastatic BRAF$^{V600E/K}$-mutated melanoma stage IIIC or stage IV. The trial observed an overall response rate of 67 % for the combination therapy compared to 51 % for darafenib only. Moreover, the progression-free survival rate was 93 % for the combination therapy compared to 85 % with darafenib only [196]. Correspondingly, another combinatorial phase III clinical trial found similar results when the combination of the BRAF inhibitor vemurafenib and the MEK inhibitor cobimetinib were used [197]. Lastly a recent clinical trial in metastatic melanoma patients, tested darafenib in combination with trametinib in comparison to vermurafenib monotherapy. Significantly improved overall survival was

observed in patients receiving combinatorial therapy compared to monotherapy after 12 months, with progression-free survival rates of 72 % and 65 %, respectively [198].

Another combinatorial tactic for avoiding the development of therapy resistance is the combination of immunotherapy and targeted therapy to treat melanoma. A phase I study investigated the use of ipilimumab, an anti-CTLA-4 antibody, in combination with different doses of BRAF inhibitor vemurafenib. However, there were several adverse side effects observed, including hepatotoxicity [199]. Another phase IB study investigated the combination of ipilimumab with peginterferon alfa-2b in patients with unresectable melanoma stages IIIB/C/IV. Peginterferon alfa-2b was initially approved for the treatment of hepatitis C and, in 2011, was approved for the treatment of melanoma. Peginterferon alfa-2b modulates the JAK-STAT pathway and controls the immune response by transcribing genes, including interleukin 4 (IL4), and subsequently inducing type 2 helper T cells, stimulating B cells amplifying the immune response [200]. Combinational therapy of peginterferon alfa-2b and ipilimumab led to a clinical benefit rate of 53.8 % and response rate of 42.3 % in unresectable stage IIIB/C/IV melanoma patient population [201]. Melanoma therapy resistance remains a major roadblock for the successful treatment of melanoma patients. However, there has been some progress in understanding the mechanisms by which melanoma cells become resistant and tackling resistance using combinatorial treatment strategies.

1.3.7 Melanoma invasion, metastasis and plasticity

Activating invasion and enabling metastasis is not only a hallmark of cancer in general but also causes most cancer-related deaths, especially in malignant melanoma [202]. Invasion and metastatic spreading are processes that alter tumor cell shape and their attachment to other cells and the ECM. Alterations in cadherin expression represent a well characterized feature of invasive cells [202] and the activation of the EMT process [202].

The EMT is driven by a network of embryonic EMT-inducing transcription factors from the following gene families: SNAIL, twist family bHLH transcription factors (TWIST) and zinc finger E-box binding homeobox (ZEB). In various cancer types, aberrant expression of these genes is a common occurrence and is correlated to poor prognosis and risk of metastatic spread. The EMT leads to the in the acquisition of stem cell-like qualities such as self-renewal and reduced proliferation [203, 204]. Importantly, the EMT is flexible and transient which allows high tumor cell plasticity, driven primarily by microenvironment cues [205, 206]. In melanoma, tumor cell plasticity is mainly controlled by MITF. Unlike other epithelial tissues, melanocytes express EMT markers SNAI2 and ZEB2 and therefore one hypothesis claim this expression predisposes the lineage towards malignant transformation [207]. It is

important to note that since melanocytes do not belong to the epithelial lineage and the EMT cannot be fully attributed to malignant transformation, however melanocytes do express E-cadherin to form close contacts to neighboring keratinocytes. Recently, the EMT transcriptional network in melanoma was revealed to undergo remodeling to favor expression of TWIST1 and ZEB1 upon NRAS/BRAF activation promoting an invasive phenotype [203].

The development of the NC during normal embryogenesis requires induction of the EMT and initiation of a migratory phenotype. Many of these phenotypes exhibited by NCCs, specifically during their migration phase, have also been implicated in cancer cell invasion and metastasis. For example, some of the overlapping mechanisms include the cadherin switch from E-cadherin to N-cadherin expression, expression of members of the SNAIL superfamily and the induction of MMP expression. For these reasons, studying the NC during normal development has been of significant interest to the field of cancer research, including melanoma. The current knowledge of processes underlying melanocyte specification and lineage determination are now becoming relevant in understanding melanoma initiation, progression and invasion. Overlapping pathways between the neural crest, melanocyte development and melanoma are summarized in **Table 1**.

The melanocytic lineage has been heavily implicated in malignant transformation. Multiple behavioral and functional aspects important in NC development, including the cadherin switch and MMP expression and secretion, are also detected in melanoma (reviewed in [208]). Taken together, this indicates that the NC differentiation program can cooperate with oncogenic mutations and lead to malignant transformation. Further evidence linking the NC developmental program to malignant transformation in melanoma is provided in transplantation experiments, where melanoma cells and melanocytes were exposed to an embryonic environment. Upon transplantation into avian embryos, highly aggressive malignant melanoma cell lines were able to migrate along normal NCC routes, suggesting that melanoma cells are able to respond to environmental cues [209]. These migrating malignant melanoma cells downregulated pluripotency-associated genes while re-expressing early melanocytic and neuronal markers [210]. Interestingly, when these cells were implanted into non-NC regions, such as the embryonic optic cup, the cells gave rise to invasive malignant melanomas [211]. This data suggests that NC environmental cues are able to direct only NC-derived lineages [212]. However, these results only suggest that malignant melanoma can respond to NC-related extrinsic cues but does not implicate these cues in transformation itself.

The aberrant regulation of NC developmental genes promotes plasticity and invasiveness in malignant melanoma [213]. This ability of melanoma cells to regain NCC migrative properties

and respond to embryonic environmental cues led to further investigation of these observed phenotypes. Expression profiling of metastatic melanoma found two distinct transcriptional signatures corresponding to either proliferative or invasive phenotypes [214]. Not only did melanoma cells possess one of the two phenotypes but were also capable of switching between the two states, termed the so-called phenotype switch. Moreover, melanoma cells could generate tumors, which contained both phenotypes independent of the initial phenotype [215]. In addition, NC-related genes were expressed in the non-invasive phenotype. Further work on the effects of the environment on the phenotype switch demonstrated when proliferative melanoma cells were subjected to hypoxic microenvironments *in vivo* and that this condition was sufficient to induce de-differentiation, resulting in the phenotype switch and therefore increased invasion by the melanoma cells [216].

Table 1 | **Genes involved in neural crest development, melanocyte development and melanoma pathogenesis**

Genes/proteins involved	Neural crest process	Melanocyte development	Melanoma pathogenesis
BMPs and BMP antagonists	Induction of neural plate/neural plate border	Induces melanocyte differentiation (TYR expression)	Progression, growth, migration and invasion
WNT, FGF and retinoic acid	Induction of neural crest potential	Promotes differentiation and growth	Proliferation, tumorigenesis and differentiation
BMPs, WNT1, WNT3a, PAX3	Maintenance, proliferation, non-differentiation of NC precursors	Induction of melanocyte differentiation and specification	Initiation and progression
Notch/Delta and FOXD3	Subdivision of neural crest and neural tube lineage	Inhibitor of melanocyte specification	Growth and invasion
BMPs and SNAI2	Emigration of neural crest and delamination	Expression lost upon reaching dermis	Invasion and metastasis (EMT induction)
Cadherins,SNAI1/2 and MMPs	Migration of the neural crest	Expression lost upon reaching dermis	Cell migration, invasion and metastasis

There are many overlapping pathways and molecules between the NC, melanocytes and melanoma including: WNT, BMPs, SNAI1, MMPs. This illustrates that there are many neural crest and developmental programs that are reactivated by melanoma to not only initiate malignant transformation (WNTs) but also aid in cellular invasion and metastasis (MMPs, SNAI1/2, Cadherins).

NC=neural crest; WNT=wingless-type; BMP=bone morphogenic protein; MMP=matrix metalloproteinases; TYR=tyrosinase; FGF=fibroblast growth factor; EMT=epithelial-to-mesenchymal transition

Mechanisms regulating the differentiation status of melanoma cells have also been under investigation, since this typically indicates the aggressiveness of the tumor and potential

induction of the phenotype switch. Dedifferentiation is a common indicator and route to metastasis in cancer. One theory suggest that melanoma dedifferentiates in order to activate the NC developmental program and trigger migration and invasion [217, 218]. However, the downregulation of MITF was found to lead to the re-expression of some pluripotency markers, OCT4 and NANOG. Moreover, these melanoma cells showed an increased invasive phenotype [219]. Similarly, another study found that a melanoma cell line could switch between low and high invasive phenotypes, depending on the expression levels of MITF [214].

Taken together, melanoma cells are masters of utilizing their developmental program for many facets of malignant transformation. Their ability to use NC migration-related genes and potentially pluripotency-related genes allows melanoma cells to easily acquire migratory and invasive capabilities. Moreover, the ability of melanoma cells to switch transcriptional signatures from proliferative to invasive phenotypes *via* several mechanisms, including environmental cues and dedifferentiation, further highlights the plasticity of melanoma cells *in vivo*.

1.4 SOX proteins: Discovery, structure and function

The SOX family of transcription factors consists of crucial regulators of several known and important processes in pluripotency, development and disease. Seminal research in 1990 discovered the mammalian testis-determining factor. This gene was named SRY due to its specific location in the sex-determining region on the Y-chromosome [220, 221]. This gene received significant attention since it was found to be the sex-determining locus in mammals [222]. The SRY gene contains three major domains: N-terminal, high-mobility group (HMG) and transactivation. The HMG domain is crucial for DNA recognition and binding. In general, proteins that contain the HMG domain with amino acid similarity of 50 % or higher to the HMG domain of SRY are termed SRY-related HMG box (SOX) proteins [223–225]. These autosomal genes with similar DNA binding motifs are a part of a large family of developmentally relevant transcription factors termed the HMG superfamily [221, 226–228].

1.4.1 SOX family

The SOX family is a subfamily of the large HMG superfamily. This superfamily was said to have emerged over 1 billion years ago before the divergence of plants and animals [227, 229]. The commonality that constitutes the members of this superfamily is the essential HMG domain. The HMG domain is the fundamental domain in all members of the HMG superfamily, especially the SOX family, and is comprised of 79 amino acids, which permits DNA-SOX protein interactions [224, 230]. Nuclear magnetic resonance (NMR) analysis of the HMG domain in SOX proteins revealed that the structure and the sequence specificity of this

domain imposed restricted amino acid choices and positions for SOX proteins [224]. The HMG domain of the SOX proteins is highly homologous and recognizes only 6-7 base pairs of DNA sequence [231] in the minor groove of DNA [232, 233]. The consensus motif for SOX proteins was defined as the sequence 5 '-(A/T)(A/T)CAA(A/T)G- 3' [232]. Therefore a crucial commonality of the SOX protein family is that all family members are regulators of gene transcription.

Initially, SOX proteins were classified by the deduced amino acid sequence of their HMG domain alone [221, 224]. To date, there are 20 orthologous pairs of SOX proteins between the mouse and human genome [234]. Of these 20 SOX proteins, they are further subdivided into groups according to sequence similarities and assembled into Groups A-G, where Group A is allocated to SRY [231, 235]. Within a group, the sequence similarity of the HMG domain is ≥ 90 % for most groups, but decreases to approximately 60 % between distant groups [231]. Evidence has shown that SOX family divergence already occurred before the base of the metazoan tree and the SOX groups SOXB, SOXE and SOXF are phylogenetically old. Additionally, the diversity of the SOX family members increased, especially in the SOXB group. The SOXB group further expanded its diversity in many animal groups. In mammals, there are five SOXB genes, which are further divided into the subgroups SOXB1 and SOXB2 [223].

In 1994, the SOX2 gene, one of the SOX family members, was discovered and characterized in humans [236]. The SOX2 gene, located on chromosome 3q26.3–q27, is a member of the SOXB1 subgroup. SOX2 is comprised of three main domains: the N-terminal, the HMG and the transactivation domains (**Figure 2a**). To date, SOX2 research has heavily emphasized its crucial role in stem cell maintenance, lineage fate determination and as a necessary factor to reprogram somatic cells back towards pluripotency.

1.4.2 SOX HMG domain structure and local architecture remodeling

The structure of the SRY-type HMG domain has been described both on and off the DNA helix [237, 238]. The domain is comprised of three α-helices (I-III), like other HMG domains, which form a twisted L-shape. The overall structure of the domain is maintained by its hydrophobic core. These core amino acids remain highly conserved between SOX proteins and provide the base-specific DNA contacts. During DNA binding, the overall structure of the domain remains constant. However, a conformational change is induced on the target DNA. Therefore, the DNA bound by SRY or other SOX proteins displays a 70-85 ° bend [224, 239–241]. The HMG domain-DNA interaction is crucial for successful gene transcription. However, the DNA structural changes in the target DNA induced by this binding are also necessary for efficient transcription. A study on one SOX family member, SOX2, illustrated

that SOX2 could not only induce DNA bending in a spatially precise manner but also that its transcription depends on it [242]. The investigation was done by creating mutations directed toward the HMG domain, resulting in differential geometric alterations of DNA bending and different activation of transcription by SOX2 on the FGF4 promoter. These studies prove that precise three-dimensional architecture is necessary for successful transcriptional activity in the SOX family of transcription factors [242].

The HMG domain of SOX proteins not only restructures DNA for transcriptional purposes but can also facilitate the formation of higher-order nucleoprotein complexes [243]. There are two mechanisms by which the HMG domain-induced DNA bend assists the formation of higher-order protein-DNA structures. The first mechanism is the Compass model, where a protein regulates gene expression by bending the DNA helix to put side by side nonadjacent regulatory elements together. In this scheme, no interaction between other regulatory proteins is required for the nucleoprotein structure, since the single protein is capable of interacting with both nonadjacent sites due to the DNA bend [243]. The second mechanism is the Scaffold model (**Figure 2b**). This model occurs when DNA bending provides optimal scaffold structure for the assembly of additional proteins [243]. SOX proteins commonly act in this manner and create large nucleoprotein structures. However, these interactions do not automatically activate promoters or enhancer regions. There are two criteria which must be met before partner interaction and transcription of target genes occurs. First, both transcription factors must bind to their particular binding sequences and induce conformational changes in the DNA helix. Second, the induced conformational changes must facilitate proper spacing and angles between DNA-bound transcription factors to allow precise partner interaction to form highly potent activation complexes [244]. These interactions are highly regulated can lead to high activation of target gene transcription, For example, these conditions are impeccably met on the δ-crystallin DC5 enhancer, which contains a SOX2 binding site and PAX6 paired binding domain sites [245] and upon precise binding of both factors transcription is profoundly activated.

1.4.3 SOX protein partners and function

SOX proteins are found in all cell types but regulate distinct, variable, and cell type-dependent target genes. This specific regulation lies in the precise interactions of SOX proteins with their protein partners. Not only do SOX proteins require additional transcription factors for successful and efficient gene activation, this interaction is also highly dependent on cell type [231]. The SOX-partner combination permits specific regulation of various cell functions, including cell specification and control of embryonic development [244]. These interactions typically occur between the HMG domain of SOX proteins and the DNA binding domain of other transcription factors [246]. SOX protein interactions with specific partners are

able to determine the cellular fate of many different lineages. For example, when SOX2 partners with OCT3/4 or δEF3, the target genes FGF4 and UTF1 or CRYGD (δ-crystallin) are activated, respectively. Moreover, the partnership between SOX2 and OCT3/4 leads to the maintenance of embryonic stem cells and inner cell mass (ICM) cells, while its interaction with δEF3 is crucial for lens development [231].
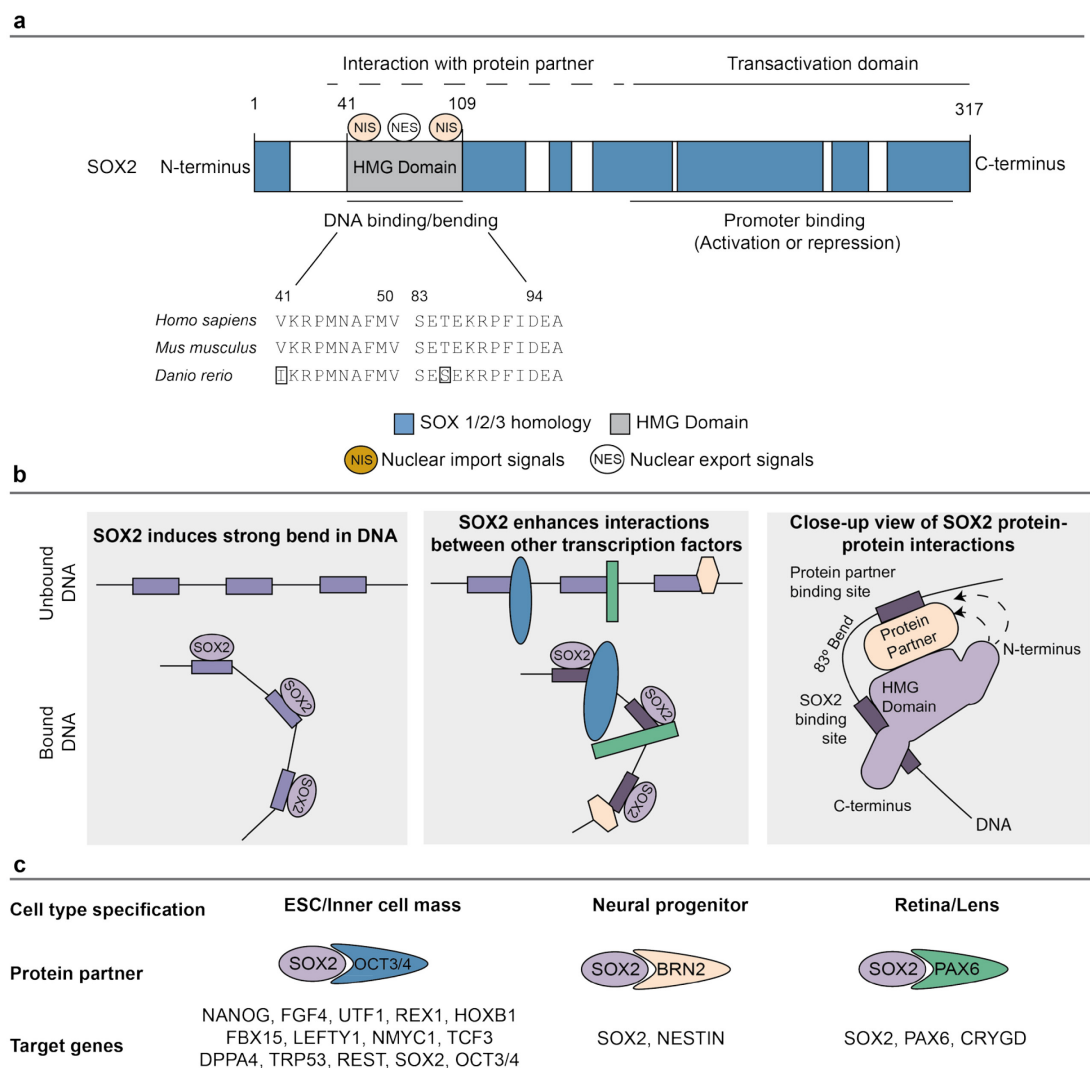
Figure 2 **SOX2 homology, structure and protein function**

a) SOX2 protein domains play several functional roles. The HMG domain of SOX2 remains fairly conserved between *Homo sapiens*, *Mus musculus* and *Danio rerio* (Swiss-Prot: P48431, P48432, Q6P0E1, respectively). The HMG domain also contains potential binding sites for protein partners. Moreover, NIS and NES sequences contained in the HMG domain regulate SOX2 expression. Lastly, the transactivation domain functions as the region responsible for promoter binding, which in turn leads to activation or repression of target genes. **b)** SOX2 proteins can bind DNA through their HMG domains and induce a strong bend in the DNA structure. This bend allows for interactions to occur between other DNA-binding proteins, which would otherwise be too distant. **c)** SOX2 has a specific partner-code depending on the cell type, which leads to differntial target gene activation.

NIS=nuclear import signal; NES=nuclear export signal; HMG=high-mobility group; ESC=embryonic stem cell; CRYGD= δ-crystallin

1.4.4 Post-translational modifications of SOX proteins

Post-translational modifications of proteins represent an additional level of regulation in the cell. These processes include altering or additional protein folding before the mature protein product is produced. Additionally, these modifications can be introduced chemically, with the addition of cofactors or in the presence of additional proteins and complexes. Some of these post-translational modifications that involve additional proteins include ubiquitination and SUMOylation. SUMOylation is the reversible covalent linkage to a small ubiquitin-related modifier (SUMO) protein (reviewed in [247]).

The mechanism of SUMOylation is similar to that of ubiquitination, where the outcome is the formation of an isopeptide bond located between the C-terminal Gly residue of the modified protein and the ε-amino group of a Lys residue of the acceptor protein [247]. Though both pathways require the activation of enzymatic cascades, there is no overlap in enzymes utilized by each pathway. Before conjugation can occur in the SUMOylation process, the immature SUMO protein must be proteolytically processed by SUMO-specific isopeptidases sentrin-specific proteases (SENPs) to reveal its C-terminal Gly-Gly motif. Once processed, the SUMO protein is mature and ready for the attachment to substrates. The first step in the attachment of mature SUMO proteins to substrates is the activation of the C-terminus *via* the SUMO-specific E1-activating enzyme heterodimer A0S1-UBA2. Next, SUMO-AOS1-UBA2 is transferred to the E2-conjugating enzyme UBC9, where a thioester linkage is formed between the Cys residue of UBC9 and the C-terminal region of SUMO. Lastly, the UBC9 transfers SUMO to its final substrate, facilitated by E3 ligases (including members of the protein inhibitor of activated STAT (PIAS) family), which catalyze the transfer of SUMO from UBC9 to the substrate [247]. This process is reversible and the substrate can be removed by SENPs.

The molecular consequence of SUMOylation on its target protein is difficult to predict. SUMOylation can influence several aspects of its target proteins function *in vivo*, such as localization, stability, activity and interaction with protein partners [247]. Since SUMOylation alters the surface of its target protein, interactions with other protein partners can either be promoted, through the addition of interfaces, or destroyed, by masking of pre-existing binding sites. Moreover, protein-protein interactions can be altered upon SUMOylation due to conformational changes in the target protein which lead to the destruction or exposure of binding sites [247].

In the SOX family, SUMOylation has been shown to be important for functional diversity in the SOXE subgroup. Studies found that in *Xenopus*, both SOX9 and SOX10 can be SUMO-modified *in vivo* [248]. In this study, two phenotypes were compared. *Xenopus* embryos

either overexpressed WT SOXE (non-SUMOylatable SOXE mutant) or a SOXE-SUMO1-fusion. The non-SUMOylatable SOXE promoted the expression of NC-related markers, while the SOXE-SUMO-1 fusion inhibited the expression of NC-related markers. Moreover, neither phenotype was able to completely reproduce the WT phenotype, which suggests that both unmodified and SUMOylated forms of SOXE proteins contribute to the WT SOXE activities *in vivo* [248, 249]. Additionally, another study observed that SUMOylation of SOX10 represses the transcriptional activity of its target genes, including MITF [250]. Recent work further investigated this topic and showed that the SUMOylation of SOXE proteins alters the recruitment of transcriptional cofactors. Specifically, SUMOylation caused the displacement of CREB-binding protein/p300 while promoting the recruitment of corepressor GRG4 [251]. In addition to the SOXE group, a study found that SOX2, when conjugated to SUMO-1, reduced its binding to the FGF4 enhancer [252].

Taken together, SOXE proteins can function as transcriptional repressors in a SUMO-dependent manner in the NC. Moreover, SOX2 conjugated to SUMO-1 also functions as a transcriptional repressor. Therefore, the role of SUMOylation in the context of SOX proteins may be crucial in further understanding their regulation in development and disease.

1.4.5 SOX2 and its role in embryonic development, disease and cancer

SOX2 has been heavily studied in many aspects of normal human development but also in areas of disease and cancer. The first function of SOX2 was identified in embryonic development and now it's well understood that SOX2 is a critical factor throughout embryogenesis.

SOX2 was first discovered in embryogenesis and development and this remains a major field of study of this protein. It plays an important role in lineage specification, proliferation, morphogenesis and differentiation of various developing tissues in a fetus (reviewed in [225]). Here, I will briefly discuss the function of SOX2 in a developing embryo.

SOX2 is initially present in both ICM and the trophectoderm (TE) but is later restricted to the ICM. Studies have shown that the zygotic deletion of SOX2 results in early embryonic lethality caused by the failure to form the pluripotent epiblast [253]. When both the maternal and zygotic SOX2 transcripts were depleted using RNAi, embryos underwent early arrest at the morula stage, leading to a complete failure of TE formation. These experiments suggest that SOX2 is required for the segregation of the TE and ICM [225, 253]. Therefore, SOX2 is critical for the maintenance of pluripotency in embryonic stem cells (ESCs). Expression levels of SOX2 heavily influence ESC self-renewal and differentiation in a dose-dependent manner. Moreover, SOX2 cooperates with other dosage-sensitive transcription factors, such as OCT4 and NANOG, to sustain the vital network responsible for self-renewal and

repression of differentiation programs in ESCs [254]. On a molecular level, SOX2 and OCT4 interact and collaborate to efficiently bind DNA and recruit other factors to initiate or repress gene transcription. Additionally, many target genes contain OCT4/SOX2 consensus binding sequences, further indicating the importance of the interaction in regulating gene transcription.

After gastrulation occurs in the embryo, SOX2 expression becomes largely restricted to the neuroectoderm, sensory placodes, branchial arches, primordial germ cells and gut endoderm [225]. In the ectoderm, SOX2 is expressed during the early stages of ESC differentiation towards the neural lineage, where SOX2 directly suppresses key regulators of the mesendodermal lineage, such as *Brachyury* [255]. Furthermore, SOX2 is important in the fate decision of bipotent axial stem cells into either paraxial mesoderm (dermis, skeletal muscle, and vertebral column) or the neural plate (central nervous system). SOX2 continues to be involved in the developing central and peripheral nervous system. Lastly, in the endoderm, SOX2 is responsible for organ specification, including the foregut [225]. In summary, SOX2 is heavily involved in key steps during embryonic development, from maintaining ESCs to fate decisions in the mesoderm and nervous system.

Many of the key regulatory features of SOX2 mentioned above are not performed by SOX2 alone. In fact, SOX2 regulates many aspects of embryogenesis in collaboration with a protein partner. The interaction of SOX2 and OCT4 is crucial for the activation of key embryonic genes responsible for ICM and ESC maintenance. Additionally, SOX2 is known to form heterodimers with transcription factor BRN2, which creates a positive feedback loop and transcribes SOX2 as well as the target gene NESTIN to maintain neural progenitor identity [244]. Another well-described interaction partner of SOX2 during lens development is PAX6. This interaction creates a positive feedback loop of both SOX2 and PAX6 transcription while also targeting the transcription of CRYGD (δ-crystallin) (**Figure 2c**). Recent work has tried to identify new interacting partners of SOX2 using proteomic analysis. However, the candidates need to be validated and investigated in a developmental context [256, 257].

Since SOX proteins play a crucial role in development and cell regulation, the mutation or deletion of SOX proteins often leads to congenital disease or developmental defects in humans [224]. For example, a heterozygous mutation of SOX2 results in an abnormal development of ectodermal and endodermal tissues, known as anophthalmia-esophageal-genital (AEG) syndrome. Individuals with anophthalmia are typically born with small eyes (microphthalmia) or even completely without eyes [258]. These patients also suffer from symptoms such as brain anomalies, hearing loss, esophageal atresia, genital abnormalities, pituitary defects and delayed motor skill development [258–260]. An interesting study in 2011

set out to further investigated SOX2 regulation in neural stem cells. Instead, they identified CHD7 as a SOX2 transcriptional co-factor [261], and revealed that SOX2 and CHD7 have overlapping genome-wide binding sites. Therefore, they are capable of regulating a common set of target genes: JAG1, GLI3 and MYCN. These genes are typically mutated in the genetic diseases Alagille, Pallister-Hall and Feingold syndromes [261]. This research further implicated SOX2 in human disease.

Current research on SOX2 has expanded focus from embryonic development to its implications in disease and cancer. SOX2 has been shown to play various roles in several cancer types and have important associations to the clinic [262, 263]. The function of SOX2 in cancer has illuminated its multifaceted capabilities in the cell. It has been shown to influence many pathways, from cellular proliferation to cancer cell invasion and metastasis (reviewed in [262]).

SOX2 amplification is induced by the multiplication of the 3q26.3 gene locus [264, 265]. Which occurs in esophageal squamous cell carcinoma (SCC), glioblastoma, oral SCC, sinonasal cancer, small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC) and lung SCC [264, 266–271]. A large study determined the copy number from 40 esophageal SCC DNA samples and 47 primary lung SCC DNA samples and found that the most significant amplification peak was located on chromosome 3q26.33 [266]. Moreover, researchers found that SOX2 was not only amplified, but that its expression was required for proliferation and anchorage-independent growth of both lung and esophageal cancer cell lines. This study identified SOX2 as a lineage-dependent oncogene in lung and esophageal SCC [266].

SOX2 has not only been shown to be amplified in some cancer types. Much work has been put into understanding how it functions in multiple cancer types. Generally, SOX2 has been shown to influence cellular migration, invasion, proliferation and self-renewal of cancer stem cells, tumorigenicity, autophagy and metastasis. This illustrates the molecular complexity of SOX2's role in cancer and further proves that SOX2 is of great importance in oncology. SOX2 typically plays differential roles, depending on the cancer type, further highlighting its intricacy in oncology. Though a few examples of SOX2's involvement in cancer are described here, the field is immense and was recently reviewed in [262].

The regulation of cellular proliferation by SOX2 was observed in various types of cancer, including breast, colorectal and pancreatic cancer. For instance, functionally inhibited SOX2 *via* cellular transfection with a tetracycline-inducible C-terminally-truncated version of SOX2, dnSOX2. Although lacking the transactivation domain, dnSOX2 could bind SOX2 recognition sites on DNA and compete with WT SOX2. This inhibition led to a decrease in cellular

proliferation in AZ-521 gastric cancer cells [272]. Numerous gain and loss of function studies in several cancer types (gastric cancer, ovarian cancer and hepatocellular carcinoma) enforced the link between SOX2 and cellular invasion and migration (reviewed in [262]). For example, the overexpression of SOX2 in the SOX2-negative glioma cell line U-87 resulted in a significant increase in the number of migratory and invasive cells [273]. Recently, SOX2's involvement in promoting invasion and migration was demonstrated in laryngeal cancer cells through the induction of MMP-2 and the PI3K/AKT/mTOR pathway [274].

SOX2 induces these functional changes in cancer by being heavily involved in various oncogenic signaling pathways. In breast and prostate cancer cells, SOX2 has shown to promote metastasis *via* the EMT through WNT/β-catenin signaling. In chromatin immunoprecipitation (ChIP) studies, SOX2 was found to bind to the promoter region of β-catenin, which further implicates a role for SOX2 in this signaling pathway [275]. Another study revealed that SOX2 was induced upon TGF-β stimulation in glioma-initiating cells. Furthermore, ChIP studies elucidated SOX4 as a direct regulator of SOX2 upon TGF-β stimulation by binding to the promoter region of SOX2 [276].

The ability to improve reliability of diagnosis and prognosis for a cancer patient can have immense impact on patient survival and contribute to overall improved responses to therapies. Since SOX2 has great influence on cancer pathogenesis, research has aimed to understand the impact of SOX2 in clinical settings. In one study, 162 esophageal squamous cancer patients were analyzed for SOX2 and OCT3/4 expression. High expression of both markers was associated with a higher histological grade or stage ($p < 0.001$ for both factors). Furthermore, a significant correlation between high SOX2 expression and decreasing patient survival was observed ($p < 0.001$) [277]. However, it is important to note that SOX2 does not correlate with poor prognosis or decreased progression free survival in all cancer types. In fact, it has been shown to correlate with a favorable prognosis in lung cancer [262, 278].

To date, little is known about SOX2 and its function in melanoma. As mentioned above, SOX proteins play a critical role during early development and the NC and only recently has been associated with melanoma progression [195, 279, 280]. Since studies have shown that the melanocytic developmental program may predispose the lineage to malignant transformation, SOX2 has raise great interest in the field of melanoma [207] Initial studies found that SOX2 was highly expressed in primary melanoma (67 %) and metastatic melanomas (80 %) compared to nevi (14 %) [281, 282]. Moreover, SOX2 expression was associated to dermal invasion, which was determined by increased tumor thickness [281]. A bioinformatic study that used the database Oncomine to investigate stem cell markers in 40 different cancer types found that SOX2 was significantly overexpressed in melanoma

metastasis patients who died after three years compared to those patients who remained alive after three years [283]. This study hinted towards SOX2 playing a role in melanoma pathogenesis.

A study in 2011 began to further investigate SOX2 in melanoma and how this protein may be functionally affecting melanoma cells [284]. Upon examining patient melanoma samples and melanoma xenografts, SOX2 was found to be highly expressed in cells which bordered and infiltrated dermal stroma. Subsequently, gain and loss of function studies were performed in melanoma cell lines and changes in invasion capacity were observed. An RT-PCR screen revealed MMP-3 to be a potential mediator of the SOX2-induced invasive phenotype of human melanoma cells [284]. In addition, one study suggested that SOX2 is able to modulate MITF in normal human melanocytes and melanoma cell lines *in vitro*, but the exact mechanism remains to be elucidated [285].

Recently, work has implicated SOX2 in the regulation of self-renewal and tumorigenicity of melanoma-initiating cells [286]. This study investigated SOX2 in melanoma-initiating cells and Hedgehog-GLI (HH-GLI) signaling and functionally revealed that the ectopic expression of SOX2 *in vitro* caused enhanced self-renewal capacity in melanoma cells. Moreover, GLI1 and GLI2, downstream transcription factors of HH-GLI signaling, were able to bind to the proximal promoter of SOX2 in primary melanoma cells in ChIP studies. These data indicate that SOX2 is regulated by HH signaling [286]. In summary, SOX2 is a critical regulator of embryonic development and is also responsible for oncogenic functionalities in various cancer types. The further understanding of SOX2 in the context of cancer will provide more insights into cancer pathogenesis and potentially be beneficial in the clinic.

Taken together, melanocytes are derived from the pluripotent stem cell population of cells known as the neural crest. The neural crest consists of several well-described stages including: induction, delamination and migration. Understanding the neural crest using *in vitro* models will aid in understanding development in depth but also becomes relevant in disease. Malignant melanoma originates from the neural crest-derived melanocytes. To date, overlapping mechanisms between the developmental neural crest and malignant melanoma, such as the role of BMP [287] and WNT signaling [288, 289] and initiation of the EMT [203], have given rise to the hypothesis that melanoma reactivates its developmental program to assist in malignant transformation (**Table 1**). For these reasons, studying neural crest and embryonic-related markers may reveal novel mechanisms that are also being reactivated in malignant melanoma.

2 Aim of study

The plasticity of melanoma cells makes them capable of switching between highly proliferative and highly invasive phenotypes depending on environmental cues, stage of disease or evasion of therapy [215]. To date, neural crest-related genes have been heavily implicated in melanoma suggesting that the melanocytic developmental program may predispose the lineage to malignant transformation [207, 283, 290]. Therefore, there is an urgency to analyze genes, which are involved in stem cell and/or neural crest processes in order to elucidate those that may be reactivated during melanoma pathogenesis. Taken together, this study aims to address the following questions:

1. Do any of the stem cell-associated genes, OCT4, SOX2 and NANOG, play a distinct role during directed melanocyte differentiation from hiPSCs?

2. Are any of the stem cell-associated genes, which are involved in directed melanocyte differentiation from hiPSCs, also linked to melanoma pathogenesis? If so, what functional roles do these genes play?

By first investigating stem cell-markers during directed melanocyte differentiation from hiPSCs, the study may identify critical genes that are reactivated during melanoma pathogenesis and melanoma plasticity. This method is a general ectodermal lineage differentiation method and therefore provides a favorable tool for identifying genes universally involved in ectodermal differentiation. Therefore, we are likely to elucidate novel genes associated with the ectodermal lineage that may be switched back on during malignant melanoma transformation. The identification of genes linked to melanoma plasticity would provide novel markers with possible clinical relevance for anti-melanoma therapy.

3 Materials

All reagents not listed below are commercially available analytical reagents or laboratory-grade materials.

3.1 Reagents

Product Description	Company	Branch
Agarose NEEO Ultra Qualität	Carl Roth	Karlsruhe, Germany
alamarBlue®	Invitrogen	Darmstadt, Germany
APS	Carl Roth	Karlsruhe, Germany
Dimethylsulfoxide (DMSO)	Carl Roth	Karlsruhe, Germany
PhosSTOP Phosphatase Inhibitor Cocktail Tablets	Roche	Mannheim, Germany
Rotiphorese® gel 30	Carl Roth	Karlsruhe, Germany
Skim milk powder	Fluka Analytical	Steinheim, Germany
TMED($C_6H_{16}N_2$)	Carl Roth	Karlsruhe, Germany
Tween® 20	Applichem	Darmstadt, Germany
X-treme GENE®	Roche	Mannheim, Germany

3.2 Materials

Product Description	Company	Branch
DAPI	Roche	Mannheim, Germany
High performance chemiluminescence film	GE Healthcare	Buckinghamshire, UK
ibidi® 30 µ-Dish Culture Insert (35mm high)	ibidi	Munich, Germany
Immobilon-P Transfer Membrane Pore size: 0.45uM	Millipore	Schwalbach, Germany
Mini Trans-Blot® Cell	Bio-Rad	Munich, Germany
Parafilm	Sigma-Aldrich	Steinheim, Germany
Whatman chromatography paper	GE Healthcare	Buckinghamshire, UK

3.3 Antibodies

Product Description	Company	Branch
Rabbit anti-c-Jun (N)	Santa Cruz Biotechnology	Heidelberg, Germany
Mouse anti-MITF (C5)	Abcam	Cambridge, UK
Rabbit anti-SMAD2	Cell Signaling Technology	Cell Signaling Technology Europe, B.V.
Rabbit anti-SMAD3	Cell Signaling Technology	Cell Signaling Technology Europe, B.V.
Rabbit anti-SOX10	Abcam	Cambridge, UK
Goat anti-SOX2	R & D Systems	Wiesbaden, Germany
Rabbit anti-SOX2	Abcam	Cambridge, UK
Rabbit anti-TGFβ RII (C16)	Santa Cruz Biotechnology	Heidelberg, Germany
Mouse anti-TRP1	Abcam	Cambridge, UK

Mouse anti-TRP2	Santa Cruz Biotechnology	Heidelberg, Germany
Mouse anti-α-actinin	Santa Cruz Biotechnology	Heidelberg, Germany
Mouse anti-GP100	Abcam	Cambridge, UK
Anti-rabbit HRP-linked 2°Ab	New England BioLabs	Frankfurt, Germany
Anti-mouse HRP-linked 2°Ab	New England BioLabs	Frankfurt, Germany
Anti-goat HRP-linked 2°Ab	New England BioLabs	Frankfurt, Germany
Cy2-donkey anti-mouse	Dianova	Hamburg, Germany
Cy2-donkey anti-rabbit	Dianova	Hamburg, Germany
Cy3-goat anti-mouse	Dianova	Hamburg, Germany
Cy3-goat anti-rabbit	Dianova	Hamburg, Germany

3.4 Small molecule inhibitors

Product Description	Company	Branch
SB 431542	Selleckchem	Munich, Germany

3.5 Human cell lines

Cell Line	Source	Authentication	Disease	Mutation [BRAF or NRAS]
A375	ATCC	Multiplexion Heidelberg Germany	Malignant Melanoma	BRAF V600E
C32	ATCC	Multiplexion Heidelberg Germany	Melanoma, amelanotic	BRAF V600E
HT144	ATCC	DSMZ Heidelberg Germany	Malignant Melanoma	BRAF V600E
Mel 501	Ballotti lab, Nice	N/A	Melanoma	BRAF V600E
Mel STV	CNIO Madrid	IDEXX Ludwigsburg, Germany	Melanocytic cell line	N/A
MeWo	Ballotti lab, Nice	Multiplexion Heidelberg Germany	Malignant Melanoma	WT/WT
MZ-7	Thomas Tüting	Multiplexion Heidelberg Germany	Melanoma	BRAF V600E
RPMI7951	ATCC	Multiplexion Heidelberg Germany	Melanoma	BRAF V600E
SK Mel 103	CNIO Madrid	Multiplexion Heidelberg Germany	Melanoma	NRAS Q61R
SK Mel 147	CNIO Madrid	Multiplexion Heidelberg Germany	Melanoma	NRAS Q61R
SK Mel 173	CNIO Madrid	IDEXX Ludwigsburg, Germany	Melanoma	WT/WT
SK Mel 23	Cornelia Mauch	Multiplexion, Heidelberg Germany	Melanoma	WT/WT
SK Mel 28	ATCC	Multiplexion Heidelberg Germany	Malignant Melanoma	BRAF V600E
SK Mel 30	DKMZ Leibniz Institute	Multiplexion Heidelberg Germany	Melanoma	NRAS Q61R
WM2664	ATCC	Multiplexion Heidelberg Germany	Melanoma	BRAF V600D

3.6   Cell culture

Product Description	Company	Branch
2-Mercaptoethanol	Gibco® Life Technologies	Darmstadt, Germany
Adenine	Sigma-Aldrich	Steinheim, Germany
Ascorbic acid	Sigma-Aldrich	Steinheim, Germany
Bone morphogenic protein 4 (BMP4)	Promokine	Heidelberg, Germany
Cholera Toxin	Sigma-Aldrich	Steinheim, Germany
DMEM AQmedia	Sigma-Aldrich	Steinheim, Germany
Doxycyline	Sigma-Aldrich	Steinheim, Germany
Endothelin 3 (EDN3)	Millipore	Schwalbach, Germany
Epidermal Growth Factor (EGF)	Promokine	Heidelberg, Germany
Fetal Calf Serum (FCS)	Biochrom	Berlin, Germany
Gelatine	Sigma-Aldrich	Steinheim, Germany
Glutamax DMEM	Sigma-Aldrich	Steinheim, Germany
Glutamax DMEM -glucose	Sigma-Aldrich	Steinheim, Germany
HAM's F12+ DMEM	Gibco® Life Technologies	Darmstadt, Germany
Human melanocyte growth supplement (HMGS) 100x	Gibco® Life Technologies	Darmstadt, Germany
Human stem cell factor (hSCF)	PeproTech	Rocky Hill, NJ USA
Hydrocortisone	Sigma-Aldrich	Steinheim, Germany
Hydroxyurea	Sigma-Aldrich	Steinheim, Germany
Insulin	Sigma-Aldrich	Steinheim, Germany
Matrigel®	Corning	Amsterdam, Netherlands
Medium 254	Gibco® Life Technologies	Darmstadt, Germany
Mitomycin C	Carl Roth	Karlsruhe, Germany
mTeSR™ stem cell medium	Stemcell Technologies	Colonge, Germany
mTeSR™ 1 stem cell medium supplement	Stemcell Technologies	Colonge, Germany
Neomycin (G418)	Sigma-Aldrich	Steinheim, Germany
Normocin	Invivogen	Toulouse, France
Non-essential amino acids	Sigma-Aldrich	Steinheim, Germany
Penicillin streptomycin	Sigma-Aldrich	Steinheim, Germany
Puromycin	Sigma-Aldrich	Steinheim, Germany
Recombinant human TGFβ-1	PeproTech	Rocky Hill, NJ USA
Rock inhibitor	Stemgent	Bergisch Gladbach, Germany
Sodium pyruvate	Sigma-Aldrich	Steinheim, Germany
Triiodothyronine	Sigma-Aldrich	Steinheim, Germany
Trypan blue solution	Sigma-Aldrich	Steinheim, Germany
Trypsin	Sigma-Aldrich	Steinheim, Germany

3.7 Buffers and gels

Transfer buffer (pH 8.3)
25 mM Glycine
190 mM Tris
20 % SDS
20 % methanol
dH2O

Running buffer (pH 8.3)
25 mM Glycine
190 mM Tris
0.1 % SDS
dH2O

10 X TBS (pH 7.6)
150 mM NaCl
50 mM Tris
dH2O

Washing buffer (TBST)
0.02 % Tween® 20
1 x TBS

Washing buffer (PBST)
0.02 % Tween® 20
PBS

Blocking buffer (milk)
5 % Skim milk powder
Washing buffer (PBS or TBS)

Cell lysis buffer for protein isolation
1 x PhosphoStop
1 x cOmplete mini protease inhibitor cocktail
1 % Triton-X in TBS

Primary antibody (BSA)
1 x TBS
0.1 % Tween® 20
5 % BSA

10 % SDS separating gel
dH2O
30 % Acryl (Rotiphorese®)
1.5 M Tris (pH 8.8)
10 % SDS
10 % APS
TEMED

10 % SDS stacking gel
dH2O
30 % Acryl (Rotiphorese®)
1 M Tris (pH 6.8)
10 % SDS
10 % APS
TEMED

3.8 Kits

Product Description	Company	Branch
ARCTURUS PicoPure RNA Isolation Kit	Life Technologies	Darmstadt, Germany
Cultrex® 96 Well BME Cell Invasion Assay	R & D Systems	Wiesbaden, Germany
Cytoselect™ invasion assay (Basement membrane, Fluorometric Format)	Cell Biolabs	Heidelberg, Germany
ECL Prime western blotting detection reagent	GE Healthcare	Buckinghamshire, UK
Pierce BCA protein assay kit	ThermoScientific	Karlsruhe, Germany
RevertAid RT Kit	ThermoScientific	Karlsruhe, Germany
RNase-free DNase set	Qiagen	Hilden, Germany
RNeasy Mini Kit	Qiagen	Hilden, Germany
Sybr®Green PCR mastermix	Applied Biosciences	Warrington, UK

3.9 Devices

Product Description	Company	Branch
AB 7500 Real-Time PCR machine	Applied Biosciences	Darmstadt, Germany
Classic E.O.S. Developer	AGFA	Mortsel, Belgium
Nanodrop Spectophotometer ND-1000	Peqlab Biotechnologie GmbH	Erlangen, Germany
Nikon Eclipse Ti Fluorescence microscope	Nikon	Düsseldorf, Germany
Nikon Eclipse TS100	Nikon	Düsseldorf, Germany
Tecan infinite F200 PRO	Tecan	Crailsheim, Germany

3.10 Software

Product Description	Source
7500 Software	Applied Biosciences
ApE	Department of Biology, University of Utah
Chipster	Chipster Open source
DOG 2.0	University of Science & Technology of China
GENE-E	Broad Institute
Graphpad Prism	Graphpad Prism
ImageJ	NIH
Ingenuity (IPA)	Qiagen
Mendeley	Mendeley
NIS-Elements	Nikon
R	www.r-project.org
T-scratch	ETH Zürich

3.11 Online databases

Name	Website
Cancer Cell Line Encyclopedia	http://www.broadinstitute.org/ccle
GPS-SUMO	http://sumosp.biocuckoo.org/online.php
JASPAR	http://jaspar.genereg.net/
Protein Atlas	http://www.proteinatlas.org/
Uniprot	http://www.uniprot.org/

4 Methods

4.1 Cell culture

All human melanoma cell lines and NIH 3T3 feeder cells were cultured in DMEM supplemented with 10 % FCS, 1 % penicillin (100 units/ mL) and streptomycin (100 µg/ mL), 1 % mL non-essential amino acids solution (10 mM) and 0.75 % ß-mercaptoethanol, hereafter referred to as MEF medium.

Normal human melanocyte cell lines and melanocytes differentiated from hiPSCs were cultured in Medium 254 (Gibco® Life Technologies) and supplemented with human melanocyte growth supplement (HMGS), 100 x (Gibco® Life Technologies).

All cell lines were cultured in a humidified incubator with 5 % CO_2 and 95 % air.

4.2 Human induced pluripotent stem cell culture

Stables clones of human induced pluripotent stem cells (hiPSCs) were generated from primary human fibroblasts in the Utikal lab following the protocol from Sommer *et al*. [291] using a lentiviral vector system. The hiPSCs were cultivated under xeno-free cell culture on a synthetic surface matrix. Matrigel plates were prepared one day prior to use (**Section 4.2.1**). Human iPSCs were washed in PBS and undifferentiated parts were manually dissociated into small cell clusters of 50-100 cells. These aggregates were transferred to Matrigel-coated plates and cultured in mTeSR1 medium containing 20 % (v/v) mTeSR1 supplements (Stemcell Technologies) and 50 µg/mL normocin. Human iPSCs were maintained by manually removing differentiated areas using a pipette tip to ensure clean colonies.

4.2.1 Matrigel® preparation

Matrigel® plates were prepared before seeding hiPSCs. Matrigel® was thawed on ice for 1-2 h. Next, 25 mL of DMEM+F12 medium were transferred to a 50 mL falcon. Matrigel® was added to medium and 1 mL of the mixture was transferred to each well of a 6-well plate or 6 cm dish. Plates incubated for 1 h at RT and were then ready for use. For storage up to two weeks, plates were not aspirated and wrapped with parafilm and placed at 4 °C.

4.3 Melanocyte differentiation protocol

Melanocytes were differentiated from hiPSCs by following the protocol described in Nissan *et al.* [292]. In preparation for the differentiation experiment, 6 cm cell culture dishes were pre-coated with 0.2 % gelatin and left to set for 15 min at RT. 3T3 NIH feeder cells (**Section 4.3.1**) were thawed and counted. Cells were seeded at a density of 660,000 cells/6 cm dish. Feeder cells were cultured overnight in normal MEF medium. The next day, hiPSCs were manually picked (about 3 colonies per 6 cm dish) using a pipette. Cells were

centrifuged for 5 min at 900 rpm to remove excess hiPSC medium. The medium was aspirated and cells were gently resuspended in differentiation medium and placed on top of NIH 3T3 feeder cells in 10 cm cell culture dishes and supplemented with ascorbic acid (0.3 mM) and BMP4 (20 pM) (**Table 2**). By day 60, pigmented areas of the dish were manually removed using a pipette and placed into an uncoated 10 cm cell culture dish. Cells were cultured in melanocyte-specific medium 254 supplemented with human melanocyte growth supplement. Selected cells were allowed to grow and were differentially trypsinized to isolate a pure population of melanocytes derived from hiPSCs, referred to as Mel D1.

Table 2	Melanocyte differentiation medium components

Components	Concentration
HAM's F12+ DMEM	225 mL
Glutamax DMEM	225 mL
FCS	10 %
Insulin	5 µg/mL
Hydrocortisone	0.5 µg/mL
Cholera Toxin	10^{-10} M
Triiodothyronine	1.37 ng/mL
Adenin	24 µg/mL
EGF	10 ng/mL
Pen/Strep	100 x
Sodium Pyruvate	1 mM

The components of the differentiation medium used to direct and differentiate hiPSCs towards the melanocytic lineage. This protocol was first established by Nissan *et al.,* 2011 [292]. The medium is further supplemented with ascorbic acid and BMP4.

BMP=bone morphogenic protein; EGF=epidermal growth factor

4.3.1 NIH 3T3 feeder cell preparation

NIH 3T3 murine fibroblasts were trysinized and seeded in cell culture dishes in MEF medium. The following day the fibroblasts were treated with 8 µg/mL Mitomycin C in MEF medium for 4-5 h in humidified incubator. This treatment places the cells in a postmitotic state. The cells were washed in PBS (Ca/Mg) and seeded for use as postmitotic feeder cells.

4.4 Lentiviral particle production and transduction

To produce infectious lentiviral particles, HEK293T cells were transfected with the target vector in combination with the packaging plasmids VSVG and Δ8.9 using X-tremeGENE®

Transfection Reagent (Roche) according to the manufacturer's protocol. Supernatant was discarded 12 h after transduction. Viral supernatant was harvested 24, 36 and 48 h after transfection and directly used for transductions of target cells or centrifuged at 13500 rpm for 5 h at 4 °C and stored as concentrated virus, stocks.

4.5   Overexpression of SOX2 in human melanoma cell lines

Gain-of-function experiments were performed using both an inducible FU-Tet-O human SOX2 construct selectable with G418, together with a lentiviral vector encoding the M2 reverse tetracycline transactivator (kindly provided by K. Hochedlinger) (M2-rtTA, for construct design of FU-Tet-O human SOX2 and M2-rtTA see vector maps provided in **Figure S1**). As a negative control cells were transduced both constructs without doxycycline administration in order to exclude that observed effects were due to the expression of the constructs or the presence of doxycycline.

The cloning of FU-TetO-hSOX2 neomycin was performed as follows. A resistance cassette comprising the neomycin-kanamycin phosphotransferase type II under the control of the murine PGK promoter was cut with EcoRI from the MIR Neomycin vector (provided by Dr. Daniel Novak) and blunted with the Phusion proofreading polymerase. In parallel, the FU-Tet-O-hSox2 vector (kindly provided by K. Hochedlinger) was linearized with XhoI and blunted with the Phusion proofreading polymerase. Subsequently, the resistance cassette was inserted *via* blunt-end ligation into the linearized vector. The selection of the FU-TetO-hSOX2 construct was performed using geneticin, G418, since resistance to G418 is conferred by the neomycin resistance gene.

Lentiviral particles were produced following the protocol above (**Section 4.4**) and cells were transduced with supernatant containing lentiviral particles of SOX2 and M2 under S2 conditions. Cells were seeded in 6-well plates and transferred to the S2 laboratory. Cells were infected with 500 µL of each virus for each corresponding condition. Cells were incubated overnight. The next day cells were superinfected with both constructs with the same amount of virus supernatant. The following day, cells were washed three times with PBS and transferred to the S1 laboratory. Cells were then induced with 1 µg/mL doxycycline for three days before selected with G418 for one week. **Table 3** indicates the antibacterial selection concentration used for various melanoma cell lines.

4.6   Knockdown of SOX2 and SOX10 in human melanoma cell lines

shRNA constructs targeted against SOX2 were purchased from Genecopoeia (cat. #HSH017628-LvH1). Four different shRNA clones and a scrambled control (cat. #CSHCTR001-LvH1) were provided and clone 1 was used in experiments. shRNA constructs were selectable with puromycin and contained an eGFP reporter gene. Lentiviral

particles were produced as described above and transduction in human melanoma cell line SK Mel 103 was performed as described in **Section 4.4**. Transduced cells were selected using 1 μg/mL puromycin for three days (**Table 3**).

shRNA constructs targeted against SOX10 were purchased from Genecopoeia (cat. #HSH017636-LVRH1GP). Four different shRNA clones and a scrambled control (cat. #CSHCTR001-LVRH1GP) were provided and clone 2 was used in experiments. shRNA constructs were prepared and used for transduction in human melanoma cell line C32 was performed as described in **Section 4.4**. Also cells were selected using puromycin at 1 μg/mL for three days (**Table 3**).

4.7 Antibiotic selection

Cells infected with a plasmid containing a neomycin cassette, conferring G418 resistance, (SOX2 overexpression construct) were selected with defined G418 concentrations optimized for each cell line (**Table 3**). For optimization, infected cells were seeded in 12-well plates at 50 % confluence. G418 was added in six different concentrations, including a negative control without G418 (50, 5, 0.5, 0.05 and 0 μg/ml). The optimized selection concentration was defined as the minimal concentration killing all cells after one week of selection (kill curve). For long-term culture, the G418 concentration was reduced to 50 % of the optimized selection concentration. The same procedure was used to determine the antibiotic selection concentration for cells infected with a plasmid containing puromycin (SOX2 and SOX10 shRNA constructs). As described above, a kill curve was determined for the C32 and SK Mel 103 cell lines. The optimized selection concentration was defined as the concentration killing all cells after three days of selection. Optimized selection concentration was defined as the concentration killing all cells after three days of selection and the concentrations for puromycin tested were 10, 1, 0.1 and 0 μg/mL. For long-term culture, the culture medium

Table 3 | **Human melanoma cell line antibiotic selection**

Melanoma cell line	Antibiotic	Selection concentration
SK Mel 30	G418	5 μg/mL
SK Mel 173	G418	5 μg/mL
Mel STV	G418	5 μg/mL
C32	Puromycin	1 μg/mL
SK Mel 103	Puromycin	1 μg/mL

The following concentrations were used to select the corresponding human melanoma cell lines for their posession of particular construct. Optimized selection concentration was defined as the concentration killing all cells after one week of selection. For long term culture, G418 concentration was reduced to 50 % of determined selection.

was supplemented with puromycin every 10 days at 50 % of the optimal selection concentration.

4.8 Quantitative polymerase chain reaction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) or the ARCTURUS PicoPure RNA Isolation Kit (Life Technology) according to the manufacturer's protocol. Briefly, cells were lysed and RNA was purified using a column-based system. After washing and on-column DNAse treatment, RNA was eluted from the column using nuclease-free water. Concentration and integrity were measured using a Nanodrop Spectophotometer ND-1000.

Complementary DNA (cDNA) was generated from 500 ng RNA using the First Strand cDNA Synthesis Kit according to the manufacturer's protocol. For all experiments, oligo-dT primers were used for cDNA synthesis, including the optional incubation step at 65 °C. cDNA was diluted 1:10 in nuclease-free water use in quantitative PCR (qPCR).

qPCR was performed using SYBR Green in combination with the Applied Biosystems® 7500 Real-Time PCR System (Life Technologies) according to manufacturer's protocols. Primer sequences used are listed in **Table 4**. Expression of target genes was normalized to the housekeeping gene HPRT, 18S or both after testing all cells for low inter-sample variance of housekeeping gene expression (<3 cycles). All primers were validated; primers with amplification efficiencies between 80 – 120 % were defined as functional. All samples were analyzed in triplicate and data was processed using the 7500 Software and the delta (delta (Ct)) method, including the efficiency of each primer pair. Graphs were generated using GraphPad Prism software. Error bars show variance as 95 % confidence intervals calculated by the 7500 software.

4.9 Immunoblotting

Proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (Invitrogen) containing complete protease inhibitor cocktail (Roche). Protein yield was measured using the bicinchoninic acid (BCA) method (ThermoScientific) prior to protein separation on self-made 10 % Tris/glycine SDS-polyacrylamide gels (**Section 3.7**) at 70 V for 30 min through stacking gel and then at 100 V for one h or until desired separation was obtained. Proteins were transferred onto methanol-activated polyvinylidene fluoride membranes for 70 min at 100 V. Unspecific binding was blocked for 1 h using 5 % BSA in PBS or milk depending on antibody used, according to manufactures protocol. After overnight incubation with primary antibodies at 4 °C and washing three times with TBST or PBST washing buffer (at least for 10 min each), depending on antibody used, the membrane was incubated with secondary antibodies against either mouse or rabbit IgG labeled with horseradish peroxidase for 1-2 h at RT. After washing three times with TBST or PBST, signals were visualized using ECL™

Western Blotting Detection Reagents (GE Healthcare) according to the manufacturer's protocol and imaging was performed using Classic E.O.S. Developer. Protein quantification was performed using ImageJ.

Table 4 | **qPCR primer sequences**

Amplification Target	Forward Sequence	Reverse Sequence
18S	GAGGATGAGGTGGAACGTGT	TCTTCAGTCGCTCCAGGTCT
AP2	GGAGACGTAAAGCTGCCAAC	GGTCGGTGAACTCTTTGCAT
CDH1 (E-cadherin)	ATTCTGATTCTGCTGCTCTTG	AGTAGTCATAGTCCTGGTCTT
CDH2 (N-cadherin)	CTCCTATGAGTGGGAACAGGAACG	TTGGATCAATGTCATAATCAAGTGCTGTA
HPRT	CCTGCTGGATTACATCAAAGCACTG	CCTGAAGTATTCATTATAGTCAAGG
JUN	GTCCTTCTTCTCTTGCGTGG	GGAGACAAGTGGCAGAGTCC
MITF-M	GCTCACAGCGTGTATTTTCC	TCTCTTTGGCCAGTGCTCTT
p75 (CD271)	CGACAACCTCATCCCTGTCT	GCTGTTCCACCTCTTGAAGG
PAX3	CTGGAACATTTGCCCAGACT	GCTGTCGGTTCCTAGTCCAG
SNAI1 (SNAIL)	GAGGCGGTGGCAGACTAG	GACACATCGGTCAGACCAG
SNAI2 (SLUG)	GCATTTCTTCACTCCGAAGC	TGAATTCCATGCTCTTGCAG
SOX2	GTGAGGGCCGGACAGCGAAC	GCGTACCGGGTTTTCTCCATGCT
SOX2 total (C)	TACAGCATGTCCTACTCGCAG	GAGGAAGAGGTAACCACAGGG
SOX10	AGCCCAGGTGAAGACAGAGA	ATAGGGTCCTGAGGGCTG
TGFBR2	TCTGGTTGTCACAGGTGGAA	GCACGTTCAGAAGTCGGTTA
TGFBR3	CAGTGAAGCTCTCCATCAAGG	AGCTCCTGTTTAGCCACTGC
TRP1	AGCAGTAGTTGGCGCTTTGT	TCAGTGAGGAGAGGCTGGTT
TRP2 (DCT)	GGTTCCTTTCTTCCCTCCAG	CCAACAGCACAAAAAGACCA
TWIST1	TCTCAAGAGGTCGTGCCAAT	ATGGTTTTGCAGGCCAGTTT
TYR	TTGTACTGCCTGCTGTGGAG	CAGGAACCTCTGCCTGAAAG
ZEB1	AGCAGTGAAAGAGAAGGGAATGC	GGTCCTCTTCAGGTGCCTCAG
ZEB2	TCCAGAAAAGCAGTTCCCTTC	CACACTGATAGGGCTTCTCG

The following primers were used in this thesis for qPCR analysis. All primers were validated and primers with amplification efficiencies between 80 - 120 % were defined as functional.

4.10 Immunocytochemistry

Seeded cells were washed with PBS and fixed for 30 min using 4 % PFA. After several washing steps with PBS, unspecific binding sites were blocked with 2.5 % bovine serum

albumin (BSA) in PBS before the cells were incubated with primary antibody. For primary antibodies that were not directly labelled, additional washing with PBS and incubation with a fluorescence-labeled secondary antibody was performed. Cells were washed with PBS and nuclei were stained with PBS containing 1 µg/mL of DAPI for 3 min. After mounting samples with DAKO mounting medium, cells were analyzed using a fluorescence microscope (Nikon).

4.11 Immunohistochemistry

Tumor tissues provided by UMM and prepared with support from our technician, Sayran Arif-Said. The tissues were embedded in paraffin. Paraffin-embedded tissues were stained with hematoxylin and eosin (H&E) and additional indirect staining against SOX2 (1:100, ab97959 Abcam) was performed as indicated in figure legends. Using biotinylated secondary antibody, expression could be analyzed by light microscopy.

4.12 Tissue microarray analysis

All analyses involving human melanoma tissue were carried out in accordance with the ethical committee II of the University of Heidelberg. 37 nevi and 26 primary melanomas were included in the tissue microarray (TMA) used in this study. TMA was generated at the core facility of the National Center for Tumor Diseases (NCT), Department of Pathology, University of Heidelberg. TMA-slides were prepared with support from our technician, Sayran Arif-Said. TMA-slides were stained for SOX2 with anti-SOX2 (1:100, ab97959 Abcam) overnight and after washing with TBST, slides were incubated with secondary, anti-rabbit antibody (Dako EnVisionTM + System-HRP; AEC K4009) for 60 min. After 15 min incubation with AEC according to the manufacturer's protocol and additional washing steps, samples were counterstained with H&E and stabilized with mounting medium (Dako S3025) for storage and analysis. Each sample was included in duplicates to the TMA and control staining against S100B was performed to visualized tumor cells. Two blinded individuals applying a quantity/intensity- based IHC scoring system, which is displayed in **Figure S2**, performed scoring of tissue microarrays.

4.13 Migration (scratch) assay

Migration assay was performed in a manner similar to a scratch assay. However, cells were seeded using ibidi® chambers to achieve a precise 500 µm gap. The ibidi® chambers were removed from the slides, placed into a 6-well cell culture plate and allowed to adhere for at least 30 min. Next, cells were seeded at an optimized density of 70,000 cells per ibidi® side for each condition. Cells were allowed to attach for 4-5 h. The medium was then aspirated and replaced with MEF medium supplemented with 5 µM hydroxyurea (proliferation inhibitor) into the chambers. Cells were incubated overnight. The next day, the ibidi® chambers were removed very carefully. Cells were washed twice in pre-warmed medium and 2 mL of MEF

medium supplemented with 5 µM hydroxyurea were added to each well. Cells and scratch were imaged using a fluorescence microscope at the following time points: 0, 6, 12, 24, and 48 h. T-scratch software (ETH Zürich) was used to analyze images and quantify migration-covered area.

4.14 Invasion assays

The Cytoselect™ invasion assay (basement membrane, Cell Biolabs) was used according to the manufacturer's protocol to measure the invasive potential of cells. In brief, the basement membrane was rehydrated and 200,000 cells were seeded per well in serum-free medium. Next, 150 µL of media containing 10 % fetal bovine serum were added to corresponding wells of the feeder tray (bottom). The plate was covered and transferred to an incubator for 24 h. After 24 h, medium was removed and replaced with cell dissociation solution. Cells were incubated for 30 min. Next, 4 x Lysis Buffer/CyQuant® GR dye solution was added to each well and allowed to incubate for 20 min at RT. Lastly, the mixture was transferred to a 96-well plate suitable for fluorescence measurement. Fluorescence was measured at 480 nm/520 nm. Relative invasion potential was determined by comparing relative fluorescent units (RFUs).

A second assay used to determine the migration and invasion potential was the Cultrex® 96 Well BME Cell Invasion Assay (©2008, Trevigen Inc.), applied according to the manufacturer's protocol. Briefly, cells were starved in serum-free MEF-medium and transwell chambers were coated with 0.1 x basal membrane equivalent (BME) coating solution. After 24 h, cells were seeded at a density of 50,000 cells/well in coated (invasion) and non-coated (migration) wells. Cells that migrated into the bottom chamber within a 24 h-period were lysed and stained with a fluorescent dye. Fluorescence was measured at 485 nm (excitation) and 520 nm (emission). Relative migratory and invasive potential was determined by comparing RFUs.

4.15 alamarBlue® assay

Cells were seeded in triplicate into black 96-well plates and growth capacity was analyzed after 72 h. Medium was carefully removed, 10 % alamarBlue® REDOX indicator dye (Invitrogen) in medium was applied and fluorescence was measured after indicated time with an excitation wavelength of 530-560 nm and emission wavelength of 590 nm using the Tecan Infinite® 200 PRO plate reader. Fluorescence intensity was normalized to wells containing 10 % alamarBlue® REDOX indicator dye in medium only. Percentage of celluar metabolism was determined by normalizing fluorescence emission to non-treated control cells.

4.16 TGF-β stimulation experiments

Cells were seeded at 30 % confluence and allowed to attach overnight. The next day, cells were stimulated for up to one week with either TGF-β1, 10 pM, 100 pM, or 1 nM (PeproTech), or SB431542, 100 nM, 1 μM, or 10 μM of SB431542 (Selleckchem) cultured in MEF medium. SB431542 was dissolved in DMSO to a stock concentration of 10 mM, according to manufacturer's protocol. Aliquots of the inhibitors solutions were stored at -20°C and applied at the indicated concentrations. Cells were also seeded as control cells and incubated in MEF medium with 0.1 % DMSO.

4.17 Gene expressing profiling

Total RNA extracted using the RNeasy Kit (Qiagen) was submitted to the microarray unit of the DKFZ Genomics and Proteomics Core Facility. Illumina expression profiling was performed using whole genome Illumina® arrays (Human-HT12V4).

4.18 Statistical analysis

Tests for all data except microarray data were performed using GraphPad Prism version 5.00 (2007) with the appropriate tests. Significance in two-tailed t-tests was assumed for p-values < 0.05 (*), < 0.01 (**) or < 0.001 (***).

For all expression array data, significantly deregulated gene expression was determined using Chipster v2.12.0. After quantile normalization of raw data using Illumina normalization in Chipster, genes which were differentially regulated in two selected groups were extracted using the empirical bayes two-group test with Benjamini and Hochberg (BH) as the chosen p-value adjustment method [293]. A p-value cutoff of 0.05 was chosen, if not otherwise indicated.

5 Results

5.1 Establishment and validation of a protocol for the differentiation of melanocytes derived from human induced pluripotent stem cells

To date, there are several well-established models of the neural crest (NC) that allow for the critical investigation of different developmental stages such as: induced pluripotent stem cells (iPSCs), NC, melanoblast and terminally differentiated melanocytes. The human iPSCs were generated in the lab following the protocol from Sommer *et al*. [291]. The generated human iPSCs were used in the melanocyte differentiation protocol, which was first established by Nissan and colleagues [292]. In brief, hiPSCs were seeded on mitomycin C-treated, postmitotic NIH 3T3 fibroblasts in stem cell medium. After three days the medium was switched to differentiation medium supplemented with ascorbic acid and BMP4. For a complete list of medium components see **Table 2**. Pigmentation was observed by day 30 (**Figure 3**), however only after day 60 were there large enough pigmented colonies available to be manually picked and cultured in melanocyte specific medium (**Figure 3**).
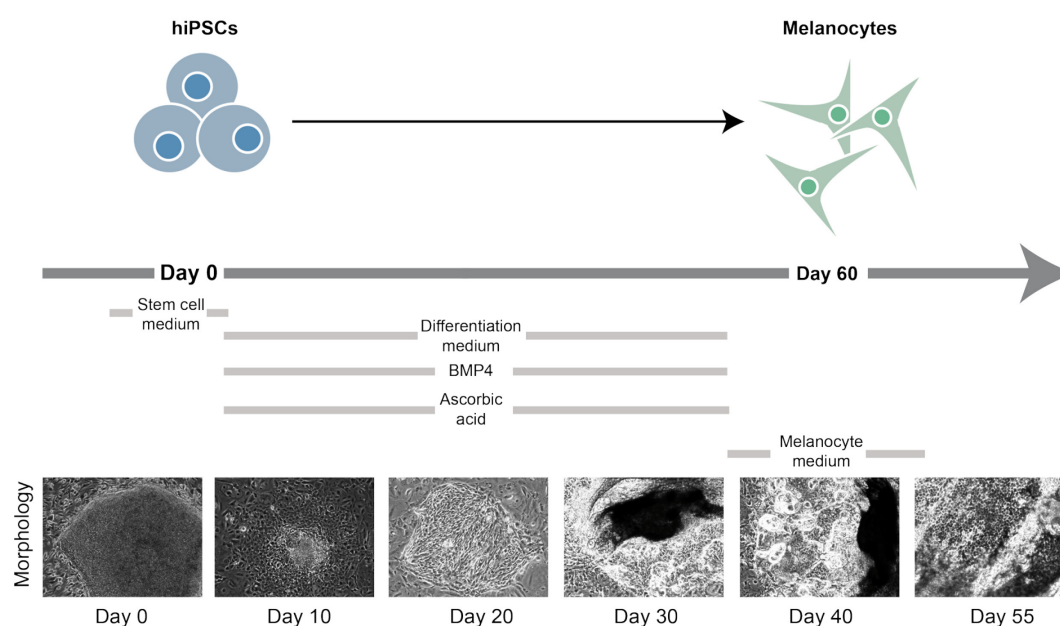
Figure 3   **Melanocyte differentiation from human induced pluripotent stem cells**

hiPSCs derived from patient fibroblasts were seeded in tissue culture dishes on top of NIH 3T3 (mitomycin C-treated) fibroblasts and allowed to recover for 2-3 days in stem cell medium. Upon recovery, the medium was replaced with differentiation medium and supplemented with ascorbic acid (0.3 mM) and BMP4 (20 pM). Medium was changed every other day. After 30 days in culture, cells began to show pigmentation and, by day 60, enough pigmented cells were present that they were manually picked and co-cultured in melanocyte-specific medium. The morphology of the cells throughout differentiation is depicted in microscopic images below. This protocol was first described by Nissan *et al.,* 2011 [292].

hiPSCs=human induced pluripotent stem cells; BMP=bone morphogenic protein

Upon successfully establishing the melanocyte differentiation protocol the melanocytes derived from hiPSCs, termed Mel D1, were characterized.

Common pluripotency markers were investigated to evaluate the differentiation protocol. For example, the expression of pluripotency markers OCT4 and LIN28 were downregulated with increasing time of differentiation (**Figure 4a**), while differentiation markers, MITF and TRP2, were upregulated with increasing time of differentiation (**Figure 4b**). The pigmentation of the Mel D1 cells was comparable to that of normal human melanocytes (NHMs) (**Figure 4c**). Additionally, the morphology of the Mel D1 was similar to that of NHMs as both melanocyte populations showed at least two dendritic processes per cells, which is a hallmark in melanocyte-specific morphology (**Figure 4d**). Next, expression of additional differentiation markers was investigated on protein-level using immunofluorescence. MITF, GP100 (PMEL17), TRP2 and TRP1 were detected in Mel D1 cells at levels comparable to NHMs (**Figure 4e**).

Mel D1 cells were further characterized using electron microscopy (EM) with the help of the DKFZ electron microscopy core facility. Mel D1 cells were seeded on special disc punches and allowed to attach and grow before they were subjected to electron microscopy (method describe in **Supplemental section 8.1.2.1**). Melanosome development was examined and the four stages were found in both the Mel D1 cells and NHMs (**Figure 5**). Melanosomes in premelanosome stage I are characterized by their spherical shape, as clearly seen in the upper panel of both the Mel D1 and NHMs (**Figure 5,** upper left panels). Those in premelanosome stage II are ellipsoidal in shape and contain perpendicular filaments; but have no notable pigmentation present. During stage III, the organelle starts to become partially pigmented or melanized. This partially melanized melanosome organelle was successfully identified in both Mel D1 and NHMs (**Figure 5**, upper middle panels). Lastly, at stage IV, the melanization process is completed and the organelle appears completely pigmented and therefore black in color [294] (**Figure 5**, upper right panels). These experiments provided substantial evidence that Mel D1 cells represent a population of successfully differentiated melanocytes with functionally essential features, *i.e.* melanosome development (**Figure 4-5**).

Mel D1 cells were further validated and characterized using whole genome expression analysis. Three independently generated Mel D1 populations were subjected to a whole genome gene expression microarray and global gene expression patterns were compared between hiPSCs, neural crest cells (characterization not shown, method described in **Supplemental section 8.1.1.2**), Mel D1 and NHMs (**Figure 6a**). Heatmap presentation illustrates the differentially up- and down- regulated genes determined by several groups testing with each row representing one gene and each column a sample (yellow indicates upregulation and blue downregulation).
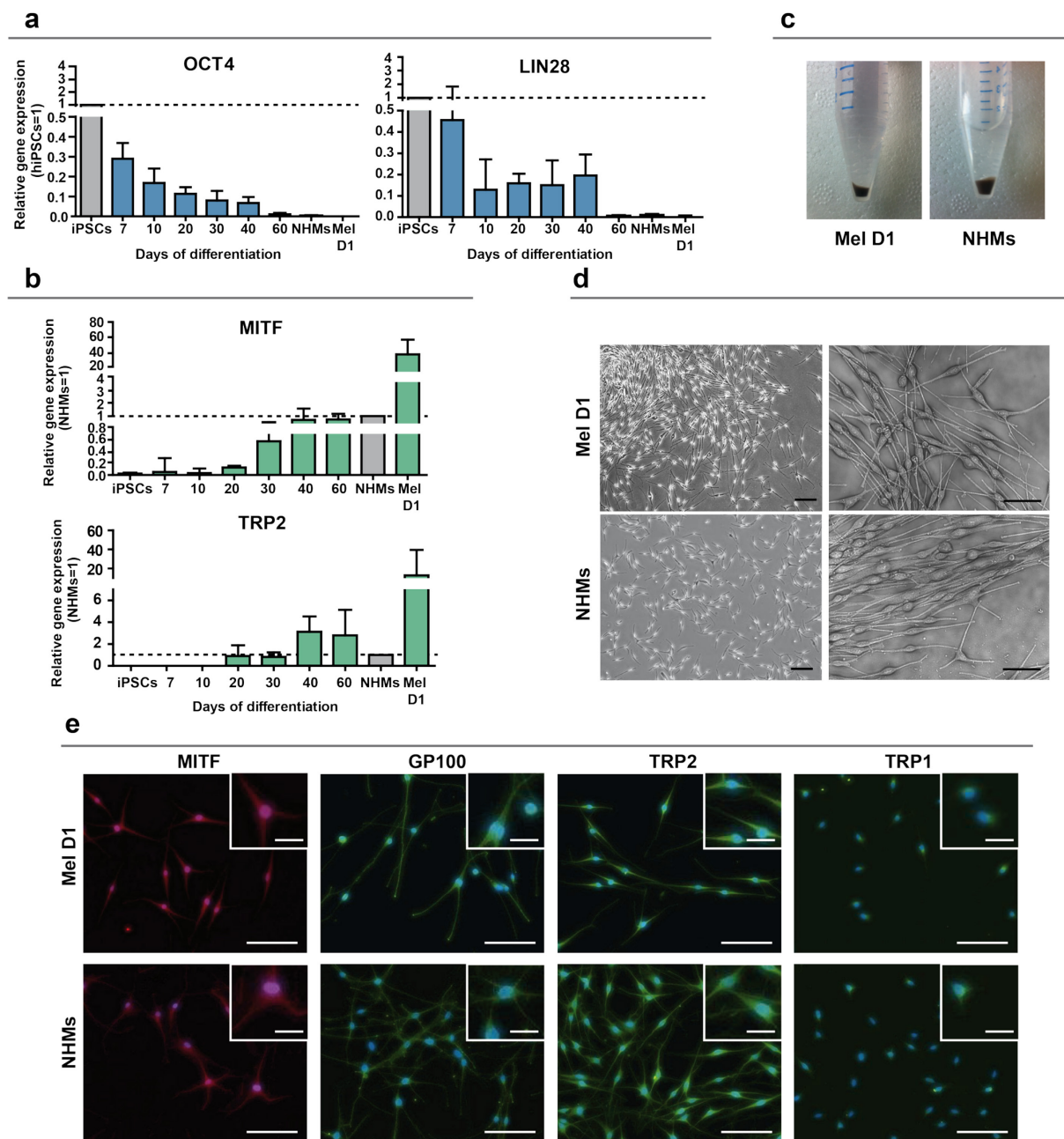
Figure 4 **Characterization of hiPSC-derived melanocytes**

a) qPCR analysis of pluripotency markers OCT4 and LIN28, HPRT and 18S were used as endogenous controls and hiPSCs as reference sample, indicated is the mean ± SD of technical triplicates. **b)** qPCR analysis of differentiation markers MITF and TRP2, where HPRT and 18S were used as endogenous controls and NHMs as reference sample, indicated is the mean ± SD of technical triplicates. **c)** Mel D1 and NHMs were pelleted and pigmented pellets are shown. **d)** Morphology of the cells was analyzed and microscopic images were taken, bar=20 μm. **e)** Immunofluorescence images of MITF (Cy3, red), GP100 (PMEL17), TRP2 and TRP1 (Cy2, green) in Mel D1 vs. NHMs, nuclei were counterstained with DAPI, upper panel bar=10 μm; lower panel bar=20 μm.

SD=standard deviation; DAPI=4',6-diamidino-2-phenylindole; Cy3=Cyanine3; Cy2=Cyanine2; Mel D1=melanocytes derived from hiPSCs; NHMs=normal human melanocytes; hiPSCs=human induced pluripotent stem cells

Mel D1

Stage I · Stage II · Stage III · Stage IV

NHMs

Stage I · Stage II · Stage III · Stage IV
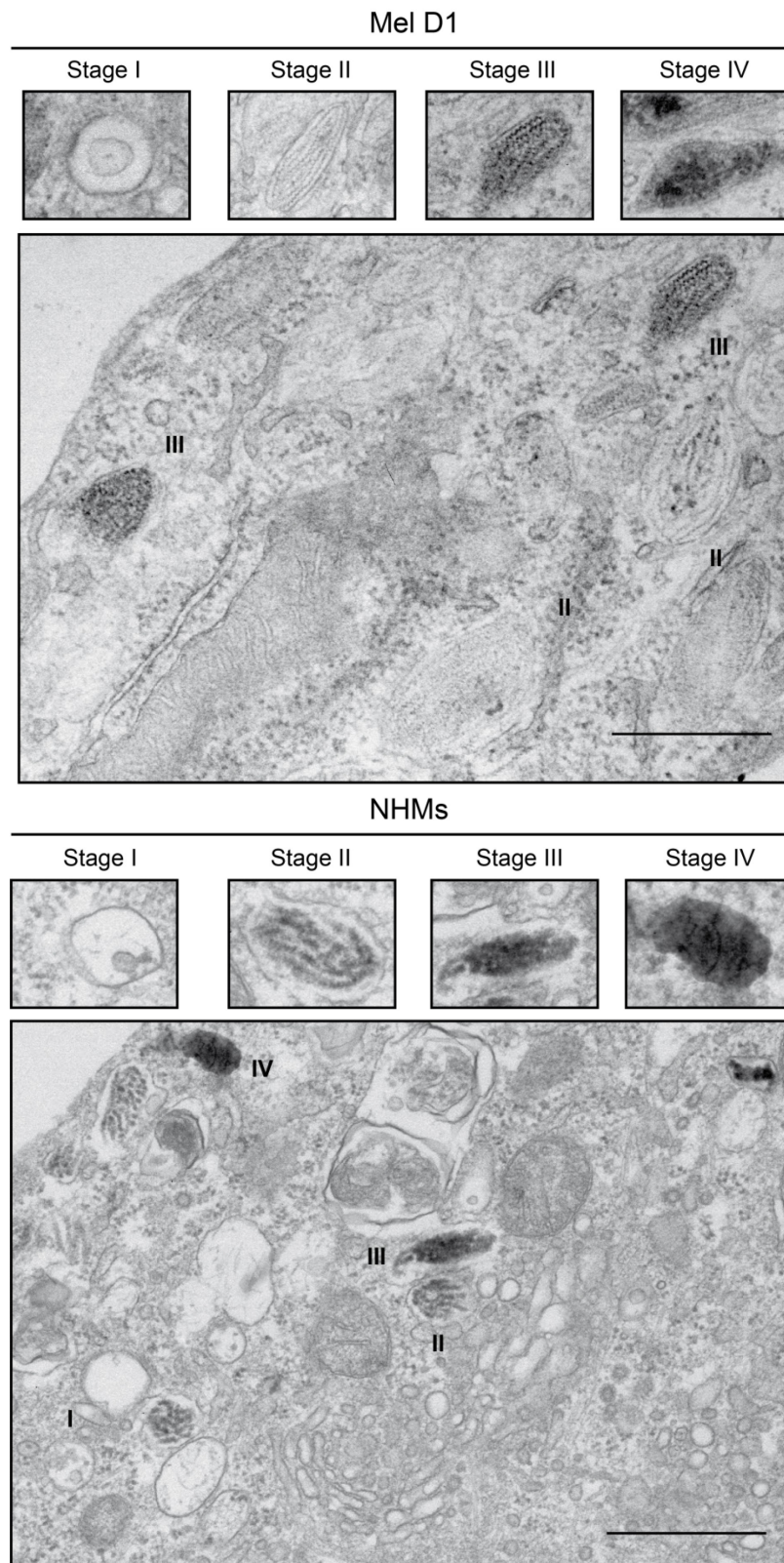
| Figure 5 | **Melanosome development in Mel D1 cells** |

Mel D1 and NHMs were seeded on ACLAR® disc punches and allowed to attach and grow. Cells were then subjected to electron microscopy. Melanosome development was examined and the four stages were observed, bar=1 µm.

Mel D1=melanocytes derived from hiPSCs; NHMs=normal human melanocytes

Pearson distance shows that Mel D1 cells have strong similarities to NHMs in contrast to their derivative hiPSCs. Upon hierarchical clustering, Mel D1 and NHMs cluster closely together and exhibit similar genome-wide expression patterns (**Figure 6a**).
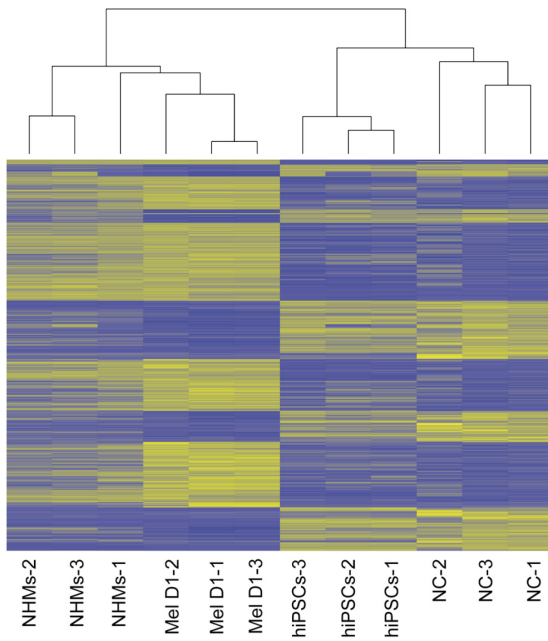
Moreover, comparable analyses were performed for Mel D1 *vs*. hiPSCs (group A) and NHMs *vs*. hiPSCs (group B). First, gene expression values for pluripotency-related markers and differentiation-related markers were extracted using Chipster software (**Figure 6b**). All pluripotency markers were similarly downregulated in both comparison groups, whereas differentiation markers were upregulated in both groups, shown as log2 [fold change]. Notably, expression of TRP2 detected by microarray analysis was higher in NHMs compared to expression in Mel D1, which is in contrast to expression analysis using qPCR (**Figure 4b**), where higher TRP2 levels were observed in Mel D1 cells. This pattern was also observed for MITF expression with higher expression levels in Mel D1 cells detected by qPCR (**Figure 4b**) but higher expression levels in NHMs using the gene expression microarray (**Figure 6b**).

Ingenuity analysis (IPA) revealed that most pathways enriched in differentially expressed genes (**Figure 6c**, +2$\leq$ fold change $\leq$-2) were similarly deregulated between Mel D1 *vs*. hiPSCs and NHMs *vs*. hiPSCs. However, a difference was observed in the pathway '*transcriptional regulatory network in embryonic stem cells*' between the two groups with a stronger enrichment in differentially expressed genes in NHMs compared to Mel D1 cells. If this has any significant meaning remains under investigation, but it is not surprising that differentiating hiPSCs towards the melanocytic lineage would utilize slightly different canonical pathways.

More detailed analysis of the canonical pathway '*melanocyte development and pigmentation signaling*' using IPA revealed that genes involved in the melanocyte pigmentation pathway were specifically differentially regulated when comparing Mel D1 *vs*. hiPSCs and NHMs *vs*. hiSPCs (**Figure 6d**). Nearly all genes that were upregulated in Mel D1 *vs*. hiPSCs were also upregulated when NHMs were compared to hiPSCs. In general, NHMs *vs*. hiPSCs pigmentation pathway was more highly upregulated compared to Mel D1 *vs*. hiPSCs. For example, TRP2 was slightly upregulated 2.35 and highly upregulated 6.6 fold in Mel D1 *vs*. hiPSCs and NHMs *vs*. hiPSCs respectively. Strikingly, genes essential for the melanin production in melanocytes, TRP1, TRP2 and TYR*,* were strongly upregulated in both NHMs and Mel D1 cells compared to hiPSCs confirming data from electron microscopy that the pigmented organelles were present in both melanocytic populations (**Figure 6d**).

a

b

Fold changes of melanocyte and pluripotency-associated genes

c

Pathways enriched in significantly deregulated genes

(+2 ≤ FC ≤ -2)
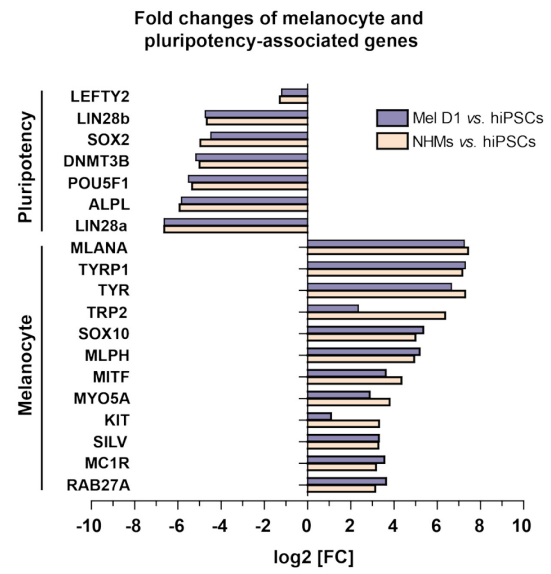
d

Figure 6	Whole genome expression analyses of hiPSC-derived melanocytes

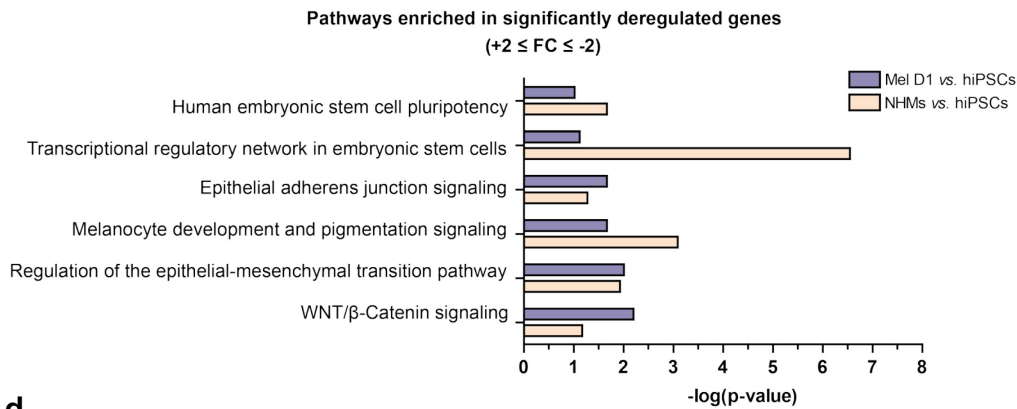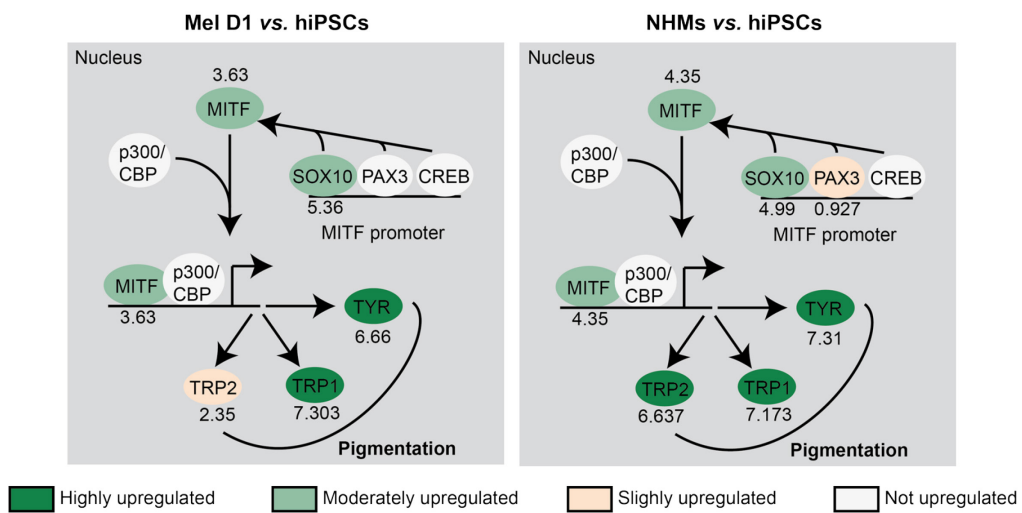**a)** Heatmap illustrating differentially up- and down- regulated genes determined by several groups testing (empirical Bayes, p ≤0.05). Each row represents one gene (3961 genes are shown) and each column a sample, n=3. Yellow indicates upregulation, blue indicates downregulation. **b)** Log2[FC] for pluripotency-related markers and differentiation-related markers were extracted using Chipster after comparing two groups: Mel D1 *vs*. hiPSCs (purple) and NHMs *vs*. hiPSCs (beige). **c)** Gene lists and log2[FC] for the two groups: Mel D1 *vs*. hiPSCs (purple) and NHMs *vs*. hiPSCs (beige) were extracted and analyzed using IPA (+2≤ FC ≤-2) to identify pathways that are significantly enriched in differentially expressed genes. **d)** Schematic of selected genes derived from pathway *Melanocyte development and pigmentation signaling* that is enriched in genes that are differentially regulated when comparing Mel D1 to hiPSCs and NHMs to hiPSCs, where highly upregulated (dark green) represents genes wtih FC≥6; moderately upregulated (light green) represents genes with FC between 3-6, slightly upregulated (beige) represents genes with FC≤3 and not upregulated (white) represents genes with FC=0.

FC=fold change; NC=neural crest; IPA=Ingenuity pathway analysis; NHMs=normal human melanocytes; Mel D1=melanocytes derived from hiPSCs; hiPSCs=human induced pluripotent stem cells

Based on these results, I conclude that I was able to successfully differentiate hiPSCs into melanocytes. For this work, the functionality of these melanocytes was not tested, for example, in an *in vitro* model of reconstituted melanized epidermis or organotypic skin reconstruction. However, this should be investigated in the future.

5.2 Role of SOX2 and SOX10 during melanocyte differentiation from human induced pluripotent stem cells

Detailed investigation of specific pluripotency genes during the established melanocyte differentiation protocol revealed a complete loss of OCT4 during the melanocyte differentiation protocol (**Figure 4a**). In addition, NANOG expression was diminished throughout the early days of differentiation (day 11-15) by day 15 (**Figure 7a**). However, SOX2 remained moderately expressed compared to hiPSCs (**Figure 7a**). This hinted that SOX2 may play a role not only in regulating pluripotency but also in further stages of ectodermal lineage development. Therefore SOX2 expression was analyzed in comparison to the neural crest marker SOX10 throughout all days of differentiation (**Figure 7b**). SOX10 expression was highest at day 20 followed by a steady decrease at the later days of differentiation (**Figure 7b**). Similarly, SOX2 expression followed the same pattern with highest expression at day 20; however relative expression was lower than SOX10 expression.

Investigating the expression of SOX2 in a NC cell population generated from Dr. Lionel Larribere from our laboratory, which was specifically enriched for neural crest cells *via* cell sorting for HNK1 and p75 (NGFR/CD271) (method described in **Supplemental section 8.1.1.2**) , revealed that SOX2 was highly expressed in the NC cell population compared to hiPSCs (**Figure 7c**). Taken together, SOX2 seems to play an important role

during melanocyte development and its regulation is important for the switch into the melanocytic lineage.
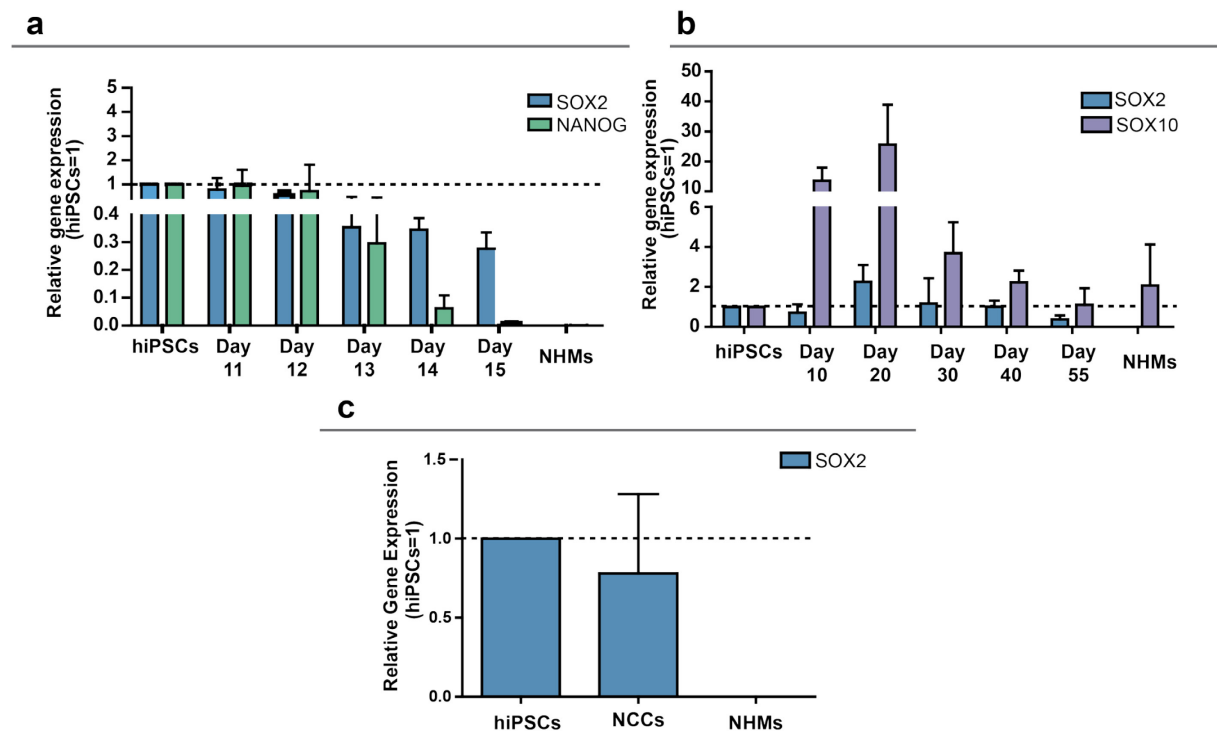
a) SOX2 and NANOG expression was evaluated using qPCR analysis during the early days of melanocyte differentiation, where 18S and HPRT were used as an endogenous controls and hiPSCs as a reference sample. **b)** SOX2 and SOX10 expression analyzed with qPCR during the full melanocyte differentiation process, where 18S and HPRT were used as endogenous controls and hiPSCs as a reference sample. **c)** SOX2 expression in NCC population using qPCR analysis. 18S and HPRT were used as endogenous controls and hiPSCs as a reference sample.

Note: for all qPCR experiments indicated are the mean ± SD of technical triplicates.

hiPSCs=human induced pluripotent stem cells; NHMs=normal human melanocytes; Mel D1=melanocyted derived from hiPSCs; NCC=neural crest cell; SD=standard deviation

5.3   Mutation-independent SOX2 function in melanoma

To date, several research groups found SOX2 to be relevant in melanoma [282, 284, 286, 295], but a detailed understanding of SOX2's role in melanoma remains unknown. First, SOX2 expression was investigated in a panel of melanoma cell lines (n=9) and 67 % of cell lines highly expressed SOX2 on mRNA-level independent of mutational status compared to NHMs (**Figure 8a**). This was also confirmed on protein-level using immunoblotting (**Figure 8b**).

To understand whether or not upregulation of SOX2 in melanoma is independent on the mutational status of BRAF, 63 melanoma cell lines were examined using the online Cancer Cell Line Encyclopedia (CCLE). BRAF-mutated melanoma cell lines (n=32) and NRAS-

mutated melanoma cell lines (n=7) were separated and relative mRNA expression of SOX2 compared to all BRAF/NRAS WT melanoma cell lines available regardless of any other mutational status (n=24). No significant difference in SOX2 expression between BRAF- or NRAS-mutated cell lines were found compared to the remaining melanoma cell lines (**Figure 8c**). This suggests that the upregulation of SOX2 in melanoma is independent of the mutational status.

Next, SOX2 expression was investigated on protein-level in melanocytic nevi, primary and metastatic melanoma samples using immunohistochemistry (IHC) (**Figure 8d**). A skin section containing a melanocytic nevus revealed that SOX2 was not expressed in nevus cells but in a sebaceous gland (**Figure 8d,** upper panel,* indicates location of dermal nevus cells). In a superficial primary melanoma, SOX2 was slightly expressed, whereas in metastatic melanoma tissue stronger staining was observed (**Figure 8d**, middle and lower panel). This indicated that SOX2 is highly expressed in both primary melanoma and melanoma metastases.

Lastly, fluorescence *in situ* hybridization (FISH) was performed with the help of Elias Orouji from our laboratory (method described in **Supplemental section 8.1.1.1**) to investigate if the SOX2 gene locus is amplified in human melanoma patient tissue (**Figure 8e**). Similar to the results observed in IHC, SOX2 was amplified in primary and metastatic tissue samples compared to melanocytic nevi, with only intact FISH signals in non-overlapping nuclei were counted (n=100). Control hybridizations were performed on metaphase spreads of euploid cells. The total number of SOX2 gene copies in relation to the reference probe copy number is indicated as SOX2 FISH ratio. Amplification of SOX2 was defined as a calculated FISH ratio > 1.3. A ratio less than 1 was considered no amplification. These values were empirically determined using Hothorn and Lausen method and could vary under different conditions [296]. Representative images of FISH analysis are shown in **Figure 8f**.

To further confirm the mutation-independent effect of SOX2 in melanoma and to understand the functional effects of SOX2 in melanoma, gain-of-function studies in the BRAF-and NRAS-WT melanoma cell line SK Mel 173 and the NRAS^{Q61R}-mutated cell line SK Mel 30 were performed. In addition, the melanocytic cell line Mel STV was transduced with the same overexpression construct. All cells were transduced using a lentiviral vector encoding for human SOX2 under the control of a tetracycline-inducible promoter in combination with a constitutively expressed neomycin selection cassette. In addition, cells were transduced with a lentiviral vector encoding for the constitutively active M2-rt-TA under the control of the human ubiquitin C promoter (**Figure 9a,** vector maps refer to **Figure S1**). Expression of SOX2 was induced by administration of doxycycline for three days before cells were selected

using G418 for one week. The cells were validated for their SOX2 overexpression on RNA-level using qPCR (**Figure 9b**). A 5-, 4- and 17-fold increase in SOX2 expression was observed in SK Mel 30, SK Mel 173 and Mel STV, respectively, when normalized to the corresponding melanoma cell line in the absence of doxycycline. Furthermore, SOX2 protein overexpression was confirmed using immunoblotting (**Figure 9b**) and stronger SOX2 bands
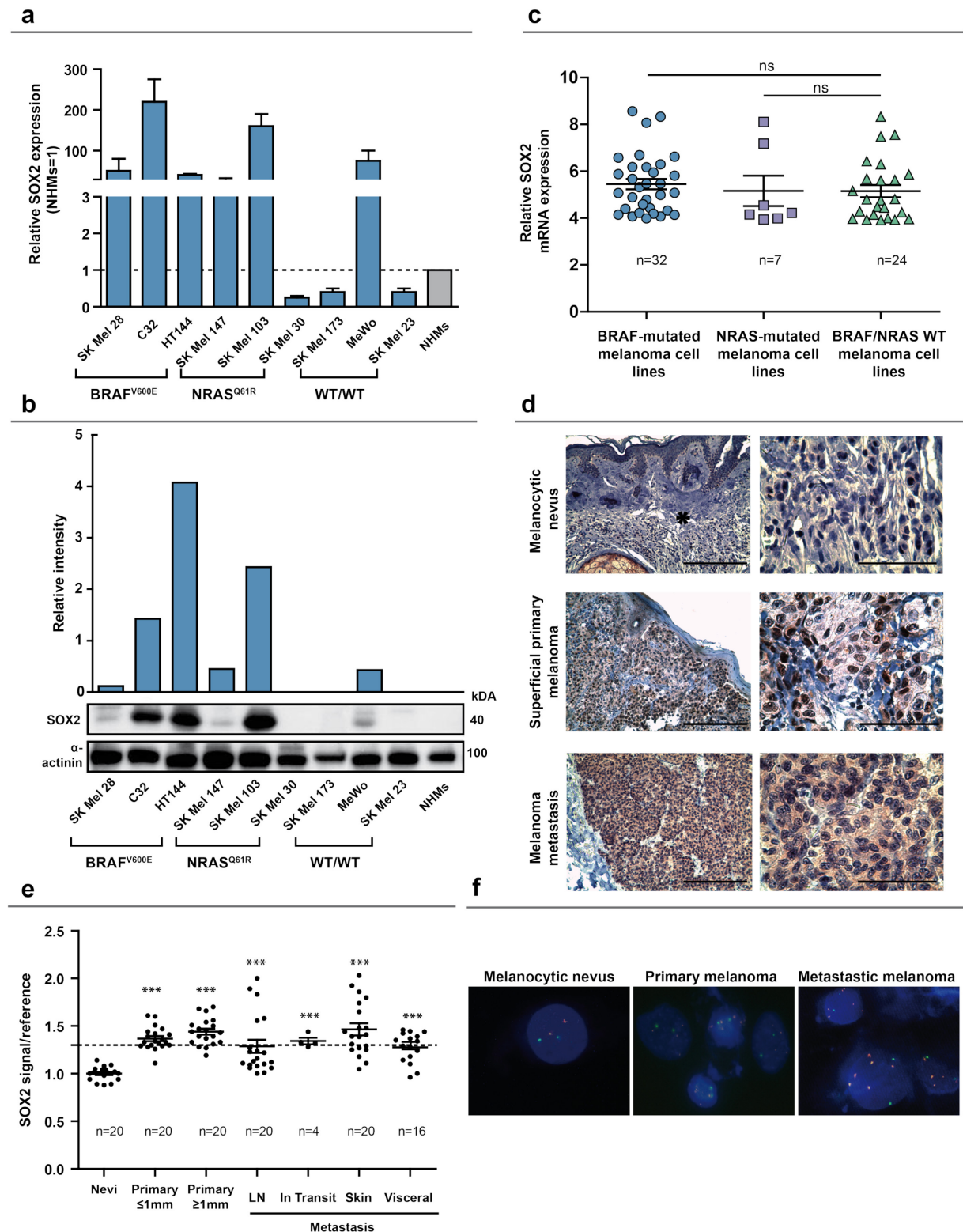
Figure 8	**SOX2 is highly expressed in primary melanomas and human melanoma cell lines**

a) qPCR analysis of SOX2 in human melanoma cell lines, 18S and HPRT were used as endogenous controls, NHMs were used as a reference sample, graph displays mean ± SD of technical triplicates. **b)** Immunoblot probed with anti-SOX2 in melanoma cell lines, α-actinin was used as loading control. Quantification of SOX2 immunoblot using ImageJ software, displaying relative intensity normalized to loading control α-actinin for 30 µg of total protein loaded. NHMs were used as reference. **c)** SOX2 expression was investigated using the CCLE database online and cell lines were sorted by mutational status and relative mRNA expression was analyzed, graph displays mean and SD of indicated n. **d)** Immunohistochemistry analysis of melanocytic nevi, primary melanoma and melanoma metastasis, stained against anti-SOX2, * indicates the location of nevus cells, bar left panel=500 µm and bar right panel=50 µm. **e)** FISH analysis of primary patient tissue for intact FISH signals, non-overlapping nuclei were counted (n=100) . The total number of SOX2 gene copies in relation to the reference probe copy number is indicated as SOX2 FISH ratio, graph displays mean and SD of indicated n. Amplification of SOX2 was defined as a ratio ≥1.3. **f)** Example images of FISH analysis in human melanoytic nevus, primary melanoma and metastatic melanoma samples where green represents SOX2 probe and red indicates reference.

IHC=immunohistochemistry score; LN=lymph node; FISH=fluorescence in situ hybridization; SD=standard deviation; NHMs=normal human melanocytes; CCLE=cancer cell line encyclopedia; WT=wildtype, significance tested using two-tailed t-test, where *** is p ≤ 0.001 and ns=not significant

were detected in doxycycline-treated cells. The morphology of the SOX2-overexpressing cells was assessed and few differences were noted (**Figure 9c**). In general, doxycycline-induced SOX2-overexpressing cells showed more dendritic-like protrusions and had a tendency to grow in smaller colonies rather than in larger clusters, (**Figure 9c**, black arrowheads).

Dedifferentiation in melanoma has been well studied in recent years and since SOX2 is a known pluripotency marker the differentiation status of the SOX2-overexpressing cells was assessed. First, qPCR analysis was performed and expression of the differentiation markers MITF and TRP2 was determined (**Figure 9d**). A reduction in expression of both differentiation markers was observed in all three SOX2-overexpressing cell lines. This loss of MITF was further verified on protein-level using immunoblotting in the SOX2-overexpressing cell line SK Mel 30 (**Figure 9f**). No difference in MITF reduction was observed upon G418 administration in the absence of doxycycline, indicating that the observed downregulation of MITF on protein-level was caused by the overexpression of SOX2 and not a side effect of selection (not shown). Band intensities revealed a 50 % loss of MITF protein (**Figure 9f**).

The strong repression of MITF on protein-level upon SOX2 overexpression led to further investigation of these two molecules. The relationship between MITF and SOX2 has been investigated by researchers in a short letter, which indicated that SOX2 may be possibly modulating MITF expression in NHMs and melanoma cells [285]. In 2012, interactions between SOX2 and MITF were investigated heavily in mouse, specifically in the cranial neural crest [297]. Authors found that not only can SOX2 bind to the proximal promoter of MITF in mouse, but was able to repress MITF-transcription in B16-F10 murine melanoma

cells. Therefore, it was of interest whether this mechanism held true in human and may partly explain the dedifferentiated phenotype observed in the gain-of-function studies in human melanoma cell lines.

In silico analysis confirmed that in human; SOX2 is capable of binding the MITF promoter. Using JASPAR, an online predictive binding site modeling database, six predicted binding sites for human SOX2 were identified on the human proximal promoter of MITF (**Figure 9e**). The database allows for manual setting of the threshold by increasing or decreasing the stringency. By setting the threshold to 85 % one particular binding site with a much higher score compared to the others was found (**Figure 9e,** red box). This site was not only the most likely predicted site by JASPAR but also matched one of the binding sites found *via* ChIP experiments in a previous publication [297, 298]. These results indicate that SOX2 is able to bind to the human MITF promoter close to the transcriptional start site. Sequence conservation of the putative SOX binding site in the MITF promoter regions between three species, *Homo sapiens*, *Mus musculus* and *Bos Tauru*s, revealed nearly 100 % conservation (**Figure 9h**) [299]. A schematic of the proximal MITF promoter is shown in **Figure 9i**. Interestingly, the confirmed binding site for SOX2 in the MITF promoter is also the identical site where SOX10 binds and activates MITF transcription. Therefore, not only does this SOX2 binding site explain a possible mechanism for SOX2-mediated repression of MITF transcription and in turn dedifferentiated melanoma cells, but also may be important in the regulation between SOX2 and SOX10.

Next, the functional effect of SOX2 on the human MITF promoter was investigated in collaboration with Dr. Corine Bertolotto's laboratory. SK Mel 30 cells overexpressing SOX2 and non-induced control cells were transfected with MITF reporter and to control transfection efficiency pCMV Gal was used and assayed for luciferase and β-galactosidase activities. Transfections were performed in technical triplicates in at least three individual experiments [300] (method described in **Supplemental section 8.1.2.4**) An approximate repression of the MITF promoter of approximately 20 % was detected upon overexpression of SOX2 in the human melanoma cell line SK Mel 30 (**Figure 9g**).

These data suggest that SOX2 is able to induce a more dedifferentiated phenotype and a mechanism, by which SOX2 may be regulating this, is *via* repressing the MITF promoter. Previous work has described that tumor dedifferentiation in melanoma, in general, is a crucial step towards tumor cell invasion [301]. It is understood that metastatic melanoma cells display a dedifferentiated phenotype with high plasticity, which allows them to invade into various microenvironments [213]. Here, the role of SOX2 was further investigated in order to

determine whether its overexpression leads to functional migrative or invasive changes in melanoma cells.

First, an improved version of the scratch assay was performed using SK Mel 30 cells overexpressing SOX2 (hereafter referred to as SK Mel 30 SOX2-OE). Cells were seeded in ibidi® chambers and cultured in medium supplemented with 5 µM hydroxyurea as a proliferation inhibitor [302]. Chambers were removed resulting in a defined 500 µm gap for measuring migration. The scratch for both, SK Mel 30 SOX2-OE cells and non-induced control cells, was imaged after 6, 12, 24 and 48 h. After 48 h, the gap was still not closed

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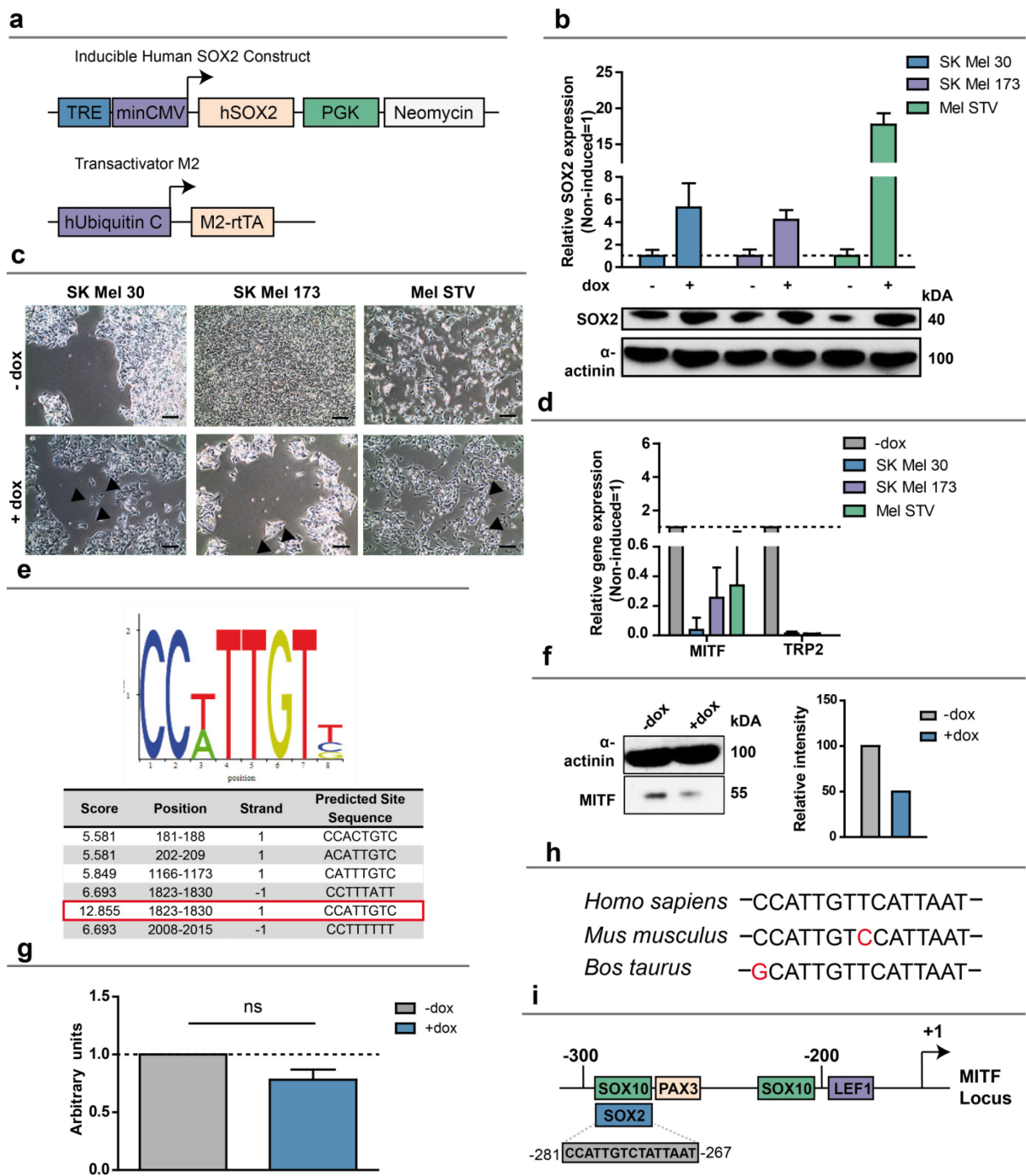
Figure 9	SOX2 overexpression in human cell lines induces a dedifferentiated phenotype *via* the repression of the MITF promoter

a) SOX2-OE experiments were performed using a lentiviral vector encoding for human SOX2 under the control of a tetracycline-inducible promoter in combination with a neomycin cassette. In addition cells were transduced with a lentiviral vector encoding for the constitutively active M2-rtTA under the control of the human ubiquitin C promoter. **b)** qPCR analysis of SOX2-OE in two different human melanoma cell lines (SK Mel 173 and SK Mel 30) and one melanocyte cell line (Mel STV), where 18S and HPRT were used as endogenous controls and each corresponding non-induced control as a reference sample. SOX2-OE was validated on protein-level using immunoblotting, where α-actinin was used as loading control for 50 µg total protein loaded. **c)** Morphology of SOX2-OE cells compared to their corresponding non-induced control cells, bar=20 µm. **d)** qPCR analysis of differentiation markers MITF and TRP2 in SOX2-OE cell lines SK Mel 173, SK Mel 30 and Mel STV. 18S and HPRT were used as endogenous controls and corresponding non-induced controls for each cell line served as a reference sample. **e)** The online database JASPAR was used to predict binding sites for SOX2 in the human MITF promoter sequence. The threshold was set to 85 % and six putative binding sites for SOX2 were identified. The binding site with the highest score, highlighted in red box, matched the binding site recognized by Adameyko *et al.,* 2012 [297] for being SOX2 binding site for the mouse MITF promoter. **f)** Immunoblot analysis of MITF in SK Mel 30 SOX2-OE cells, comparing induced and non-induced control, where α-actinin was used as loading control for 30 µg total protein loaded. The blot was quantified using Image J. **g)** SK Mel 30 SOX2-OE cells and non-induced control were seeded, transfected and assayed for luciferase and β-galactosidase activities. All transfections were repeated at least three times and performed in triplicates, graph displays mean and SD. **h)** The conservation of the predicted SOX2 binding site between species, *Homo sapiens*, *Mus musculus* and *Bos taurus*, was found to be nearly identical for this binding site sequence [299]. **i)** The predicted binding site for SOX2 in the human MITF promoter illustrated with corresponding known binding sites for other proteins, including SOX10, PAX3 and LEF1. To note, the predicted binding site for SOX2 in the human MITF promoter is the identical binding site for SOX10.

Note: for all qPCR experiments mean ± SD of technical triplicates are indicated.

OE=overexpression; dox=doxycycline; SD=standard deviation, significance tested using two-tailed t-test, where ns=not significant

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completely. For quantification, images were analyzed using the open software T-scratch and a significant increase in the migration capacity in the SK Mel 30 SOX2 OE cells compared to non-induced control cells was observed (**Figure 10a**).

After detection of enhanced migratory capacity when overexpressing SOX2 in melanoma cells, the invasive potential of these cells was assessed. A transwell chamber system was used to determine the invasive capacity of both SK Mel 30 and SK Mel 173 SOX2-OE cells through an artificial basal membrane equivalent. SK Mel 30 SOX2-OE cells demonstrated a significant increase in invasive capacity and same tendency was observed for SK Mel 173 SOX2-OE cells; however this was not significant (**Figure 10c**). This further confirmed that SOX2 is plays a role in migratory and invasive capacity of melanoma cells *in vitro*.

The epithelial-to-mesenchymal transition (EMT) is a process strongly related to migration and invasion in both NC development and cancer. Melanoma studies found that upregulation of TWIST1 and ZEB1 indicated EMT restructuring towards a more dedifferentiated, invasive phenotype [203]. For these reasons, these EMT-related markers were investigated in SK Mel 30 SOX2-OE cells and an upmodulation of ZEB1 and TWIST1 was observed, while ZEB2

and SNAI2 expression was decreased (**Figure 10b**). Next, this SOX2-related invasive phenotype observed *in vitro* was further evaluated in primary melanoma patient samples. Moderate expression of SOX2 was observed at superficial regions of primary melanomas (**Figure 10e**, upper panel); however extremely high levels of SOX2 expression were detected in deeper regions of the same tumor (**Figure 10e**, lower panel). Next, SOX2 positive cells were observed, which invaded through the dermis and extracellular matrix supporting functional *in vitro* data (**Figure 10f**). Moreover, strong positivity was detected near an invasive border of a primary melanoma close to a nerve cell (**Figure 10g**). The nerve cell served as a positive control for SOX2 staining in IHC since SOX2 is highly expressed in this cell type (reviewed in [303]).

Melanoma research has highlighted the unique, so-called phenotype switch phenomenon, where melanoma cells oscillate between highly proliferative and non-invasive or highly invasive and low proliferative states [304]. Since a highly invasive phenotype was observed upon SOX2 overexpression, the metabolic activity of these cells was investigated to further confirm that SOX2 plays a role in the induction of the phenotype switch. The alamarBlue® assay was used to determine metabolic activity of the cells, which correlates with cellular viability and proliferation. Both SOX2-overexpressing cell lines, SK Mel 30 and SK Mel 173, were seeded into black 96-well plates. alamarBlue® was applied, three days after seeding and its conversion was measured using a fluorescence plate reader after three hours. A significant decrease in cellular metabolism in SK Mel 30 SOX2-OE cells was observed compared to the corresponding non-induced control cells (**Figure 10d**). However, only a slight decrease in cellular metabolism was detected in the SK Mel 173 SOX2-OE cells.

Next, SOX2-knockdown studies were performed in the human melanoma cell line NRAS^{Q61R}-mutated SK Mel 103. SK Mel 103 was chosen due to its high expression of SOX2 on RNA and protein-level (**Figure 8a-b**). The cells were transduced with shRNA targeting human SOX2 gene or with vectors encoding for a scrambled control that were selectable using puromycin (**Table 3**). Upon selection for three days, knockdown of SOX2 was validated for RNA expression by qPCR analysis and on protein-level by immunoblotting (**Figure 11a**). 60 % reduction of SOX2 was observed in SK Mel 103 knockdown cells and no morphological changed were evident when compared to control cells (**Figure 11a, g**). Subsequently, SOX2 knockdown (KD) cells were evaluated regarding the expression of NC markers. An increase in expression of SOX10 and a decrease in p75 expression were observed, suggesting a more differentiated state of the cells (**Figure 11b**). Moreover, an increase in differentiation-markers MITF and TRP2 was observed in the SK Mel 103 KD cells compared to the scrambled control (**Figure 11f**).
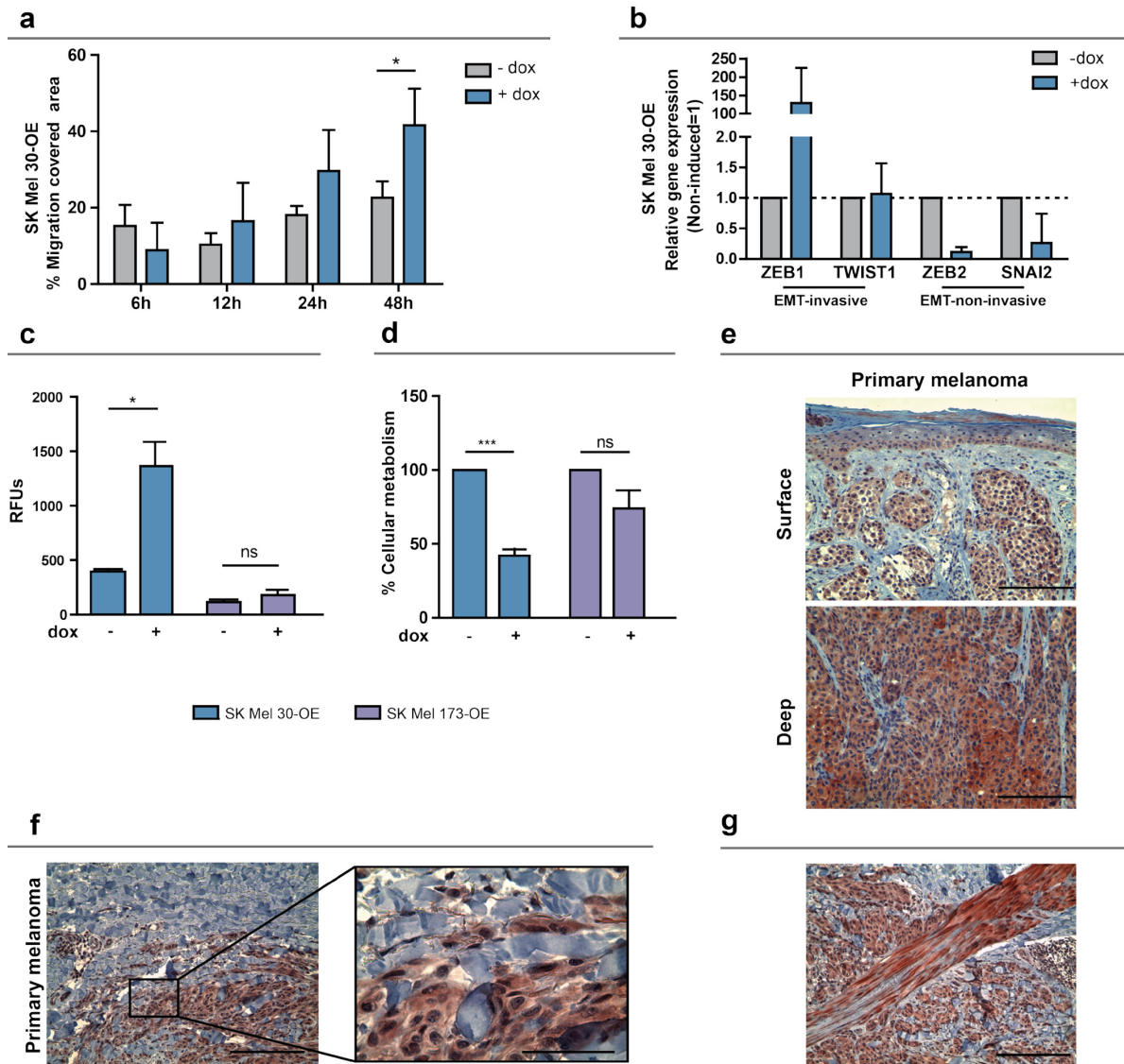
Figure 10 **SOX2 overexpression in human melanoma cell lines increases migratory and invasive capacity and induces EMT-related marker expression**

a) Improved scratch assay was performed using ibidi® chambers, which allows a precise 500 µm gap; additionally hydroxyurea was used as a proliferation inhibitor. In SK Mel 30 SOX2-OE cells and non-induced control cell migration was measured over 48 hours and microscope images were taken every hour. The images were quantified using T-scratch software, graph displays mean ± SD of technical triplicates. **b)** qPCR analysis of EMT-related markers ZEB1, ZEB2, SNAI2 and TWIST1 in SK Mel 30-OE cells. HPRT was used as endogenous control and non-induced control cells served as a reference sample, graph displays mean ± SD of technical triplicates. **c)** Invasion capacity was measured in SK Mel 30-OE and SK Mel 173-OE cells using the Cytoselect™ invasion assay and fluoresence was measured in technical triplicates, graph displays mean ± SD of biological triplicates. **d)** Cellular metabolism was measured using alamarBlue® assay, 5000 cells were seeded in black 96-well culture plates and cultured for 72 hours. 3 hours after alamarBlue® administration, fluorescence was measured. The cellular metabolism was normalized to the non-induced cells, graph displays mean ± SD of three independent experiments with technical triplicates. **e)** Displayed are microscopic images of IHC analysis using primary melanoma samples from superficial and deep tumor regions stained against SOX2, bar=500 µm. **f)** IHC analysis of primary melanoma samples shows invading cells into deep regions of melanoma stained against SOX2, bar left panel=500 µm and bar right panel=50 µm. **g)** IHC analysis of primary melanoma sample showing nerve tissue and invading cells along the nerve region stained against SOX2, bar=500 µm.

*EMT=epithelial-to-mesenchymal transition; OE=overexpression; dox=doxycycline; IHC=immunohistochemistry; SD=standard deviation, significance tested using two-tailed t-test, where *is p≤0.05, ***is p≤0.001 and ns=not significant*

Furthermore to investigate EMT-related marker expression, cadherins were investigated and upon SOX2 KD in SK Mel 103 melanoma cell line an upmodulation of E-cadherin (CDH1) was observed together with a slight downmodulation of N-cadherin (CDH2) (**Figure 11c**). Additionally, an upregulation of ZEB2 and SNAI2 was observed in the SK Mel 103 KD cells compared to the scrambled control, which suggests the cells were in a less invasive state (**Figure 11f**). Taken together, these data suggest the cells were more epithelial-like and the loss of SOX2 in the melanoma cell line SK Mel 103 leads to a more differentiated phenotype.

Following, the alamarBlue® assay was performed to assess cellular metabolic activity of the SK Mel 103 SOX2 KD cells indicating effects of SOX2 depletion on proliferation. 4000 cells were seeded per well, and three days later alamarBlue® was applied. Three hours after alamarBlue® administration, measurements were taken (**Figure 11d**). No difference was observed in cellular metabolism when the scrambled control was compared to the SOX2 KD cells.

Lastly, the invasive capacity of SOX2 KD cells was assessed using the Cultrex® 96 Well BME Cell Invasion Assay and wells were coated with 0.1 x basement membrane extract (BME). A significant decrease in invasive capacity was observed upon knocking down SOX2 in the SK Mel 103 melanoma cell line (**Figure 11e**).

5.4   Influence of SOX10 on SOX2 expression in human melanoma cells

Independently, SOX2 and SOX10 have been closely studied in melanoma. SOX10 studies have shown that its expression is required for melanoma initiation and maintenance [280]. Meanwhile SOX2, previously described in studies and the data shown, has been associated to melanoma cell invasion and regulation of melanoma-initiating cell self-renewal and tumorigenicity [284, 286, 295].

This reciprocal function of SOX2 and SOX10 was further investigated by analyzing the expression of SOX10 in SOX2-OE cell lines. When SOX2 was overexpressed in several human melanoma cell lines, SOX10 was drastically downregulated (**Figure 12a**). Moreover, when relative gene expression of SOX2 and SOX10 was analyzed in a panel of 12 melanoma cell lines an inverse correlation between the two genes was discovered (r=-0.2120) (**Figure 12b**). This data illustrated that there is inverse expression between SOX2 and SOX10 in melanoma.

To further understand the reciprocal function of SOX10 in melanoma compared to SOX2 loss-of-function studies were performed using shRNA targeting SOX10. The BRAF-mutated C32 cell line was transduced with four different shRNA clones and the scrambled control. After transduction, these cells were selected with puromycin for three days (**Table 3**). Next,

KD of SOX10 in these cells was validated on RNA-level and protein-level and a 60 % KD of SOX10 was observed in clone 2 (**Figure 12c**).
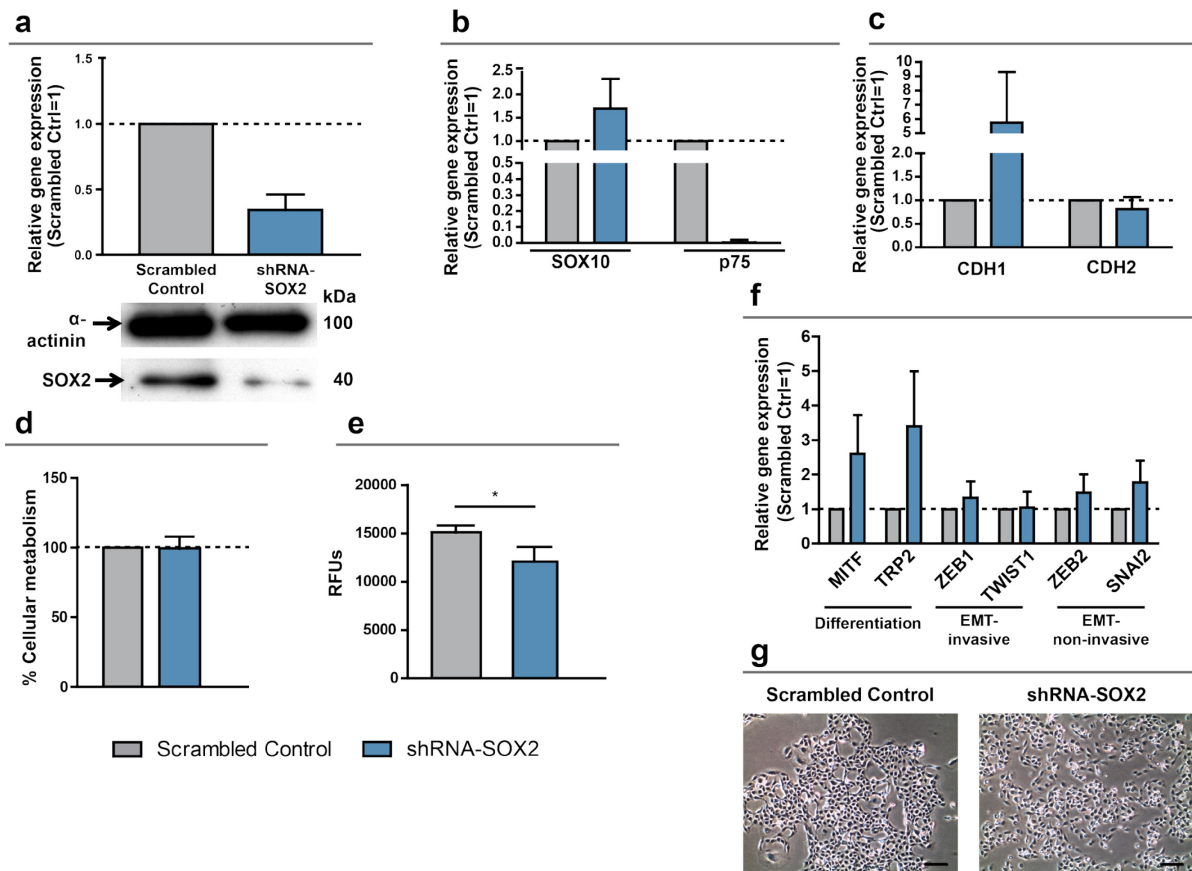
Figure 11 **SOX2 knockdown in the melanoma cell line SK Mel 103 induces a more differentiated and less invasive phenotype**

a) SOX2-KD was validated on RNA-level using qPCR, where 18S was used as endogenous control and scrambled control as a reference sample. Additionally, SOX2-KD on protein-level was analyzed using immunoblotting with anti-SOX2 antibody and α-actinin as loading control for 30 μg total protein loaded. **b)** SOX2-KD in SK Mel 103 cell line was analyzed using qPCR for marker expression of SOX10 and p75, where 18S was used as endogenous control and scrambled control as a reference sample. **c)** SOX2-KD in SK Mel 103 cell line was analyzed using qPCR for marker expression of CDH1 and CDH2, where 18S was used as endogenous control and scrambled control as a reference sample. **d)** Cellular metabolism was measured using alamarBlue® assay, 4000 cells were seeded in black 96-well culture plates and cultured for 72 hours. 4 hours after alamarBlue® administration, fluorescence was measured. The cellular metabolism was normalized to the non-induced cells, graph displays mean ± SD of three independent experiments with technical triplicates. **e)** The invasion capacity was assessed using the Cultrex® 96 well BME cell invasion assay and fluorescence was measured using a standard fluorescence reader. **f)** SOX2-KD in SK Mel 103 cell line was analyzed using qPCR for marker expression of MITF, TRP2, ZEB1, TWIST1, ZEB2 and SNAI2, where 18S was used as endogenous control and scrambled control as a reference sample. **g)** Morphological differences are shown in microscopic images, scale bar=20 μm.

Note: for all qPCR experiments mean ± SD of technical triplicated indicated.

*KD=knockdown; EMT=epithelial-to-mesenchymal transition; BME=basement membrane extract; SD=standard deviation, significance tested using two-tailed t-test, where * is p ≤ 0.05.*

In order to determine whether or not SOX2 and SOX10 play a reciprocal role in melanoma levels of SOX2 expression were checked after SOX10 KD. No increase in SOX2 expression after knocking down SOX10 was detected (**Figure 12d**, blue). In fact, a slight downregulation of SOX2 was observed on RNA-level upon SOX10 KD. Expression of the dedifferentiation marker p75 was analyzed and no increase could be detected (**Figure 12d**, purple). These results were confirmed on protein-level using immunoblotting where no difference in SOX2 protein was identified upon SOX10 KD (**Figure 12e**). These results suggest that SOX2 may operate upstream of SOX10 and that alteration of SOX10 therefore has no direct impact on SOX2 expression levels. The reciprocal effect observed between SOX2 and SOX10 is therefore a causative effect and levels of expression may determine the differential phenotype observed.

5.5 TGF-β induces SOX2 expression in melanoma *in vitro*

The mechanisms responsible for the overexpression of SOX2 and melanoma dedifferentiation, which is linked to the invasive phenotype, remain unknown. I showed that SOX2 overexpression can be induced *via* SOX2 gene amplification (**Figure 8e-f**). In order to further elucidate the mechanism behind SOX2 induction in melanoma, possible mechanisms of SOX2 induction in other cancers were investigated. Interestingly, studies found that TGF-β induces SOX2 expression in glioma-initiating cells [276]. Since TGF-β signaling is of high importance in melanoma progression and its autocrine responses includes enhanced motility and invasion, this possible mechanism of action was further investigated [168]. Therefore, melanoma cells were stimulated with TGF-β and SOX2 expression was assessed (**Figure 13**).

Upon TGF-β1 stimulation in two human melanoma cell lines, A375 and SK Mel 30, morphological differences were observed after one week compared to the ALK5 inhibitor SB431542 treated or the no treatment (0.1 % DMSO) control cells (**Figure 13a**). SB 431542 is an inhibitor of activin receptor-like kinase (ALK)5 (TGF-beta type I receptor) and functions through competitive binding to the ATP binding site, which inhibits the *in vitro* phosphorylation of SMAD3 [305, 306]. Long dendrites were noted upon TGF-β1 stimulation in both cell lines, indicated by black arrowheads (**Figure 13a**). This morphology was similar to that observed when SOX2 was overexpressed (**Figure 9c**). Next, the induction of SOX2 upon one week of TGF-β1 stimulation was investigated in SK Mel 30 and A375 melanoma cell lines. First, in SK Mel 30, a significant induction in SOX2 expression was observed compared to the no treatment (0.1 % DMSO) control or the SB431542 inhibitor treated cells (**Figure 13b**). Similarly, in the A375 cell line, a significant induction of SOX2 expression was found upon TGF-β1 treatment for one week compared to the no treatment (0.1 % DMSO) control or the SB431542 inhibitor treated cells (**Figure 13e**). These results suggest that

similar to glioma-initiating cells, SOX2 expression in melanoma cells is induced *via* TGF-β1 signaling. To further confirm that SOX2 can be induced by TGF-β1, SOX2 was checked on protein-level after stimulation with TGF-β1 for one week in SK Mel 30 melanoma cell line (**Figure 13c**). In SK Mel 30 cells stimulated with TGF-β1, increased SOX2 protein-levels were observed compared to the no treatment (0.1 % DMSO) control or the cell treated with the SB431542 inhibitor. These data further verify that *SOX2* is induced upon TGF-β treatment.
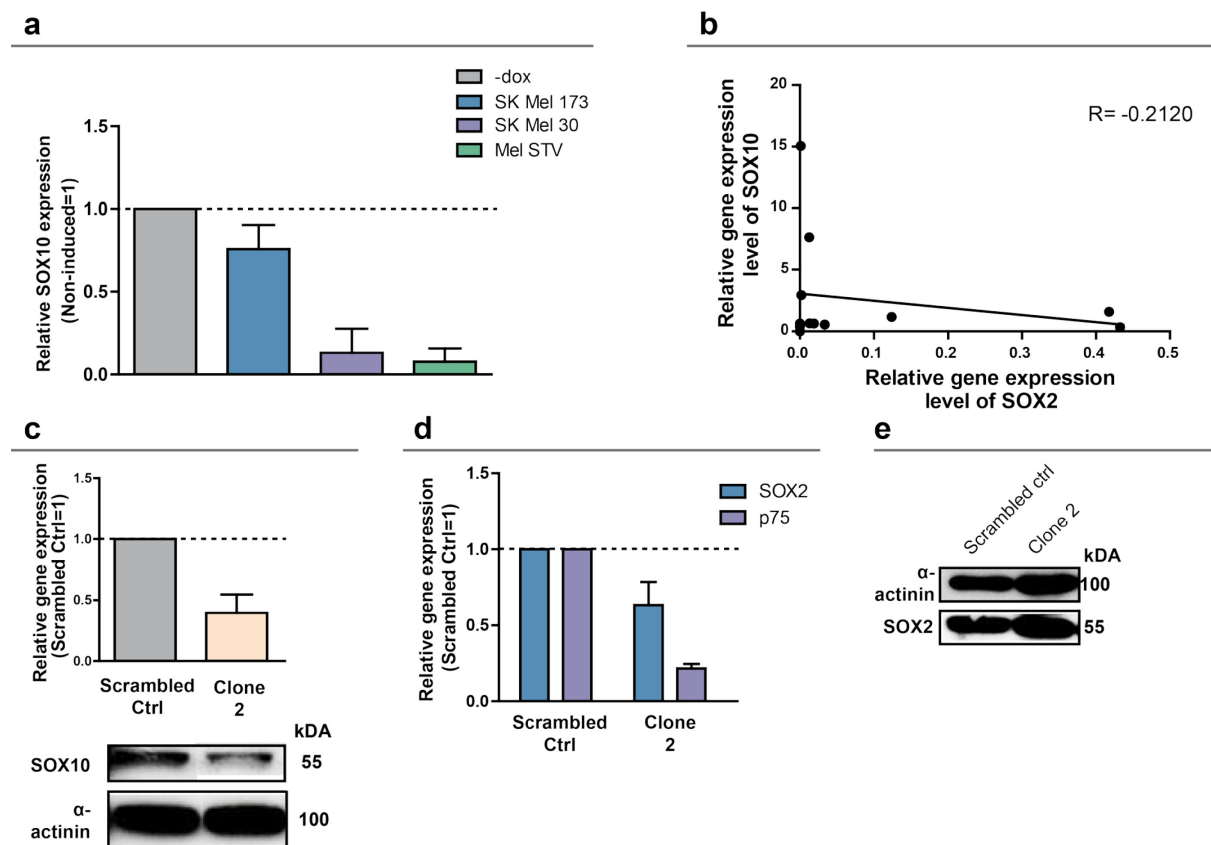
Figure 12 **SOX10 knockdown in the human melanoma cell line C32 does not induce SOX2 expression *in vitro***

a) qPCR analysis of SOX10 expression in SOX2-OE cell lines SK Mel 173, SK Mel 30 and Mel STV. 18S and HPRT were used as endogenous controls and the corresponding non-induced controls for each cell line were used as reference samples. **b)** Displayed is the correlation between SOX2 and SOX10 gene expression in 12 human melanoma cell lines (R=-0.2120). For qPCR analysis, 18S and HPRT were used as endogenous controls and data were not normalized to any reference samples. R stands for Pearson product-moment correlation coefficient. **c)** SOX10-KD was validated in the human melanoma cell line C32 on RNA-level using qPCR analysis, where 18S was used as the endogenous control and the scrambled control as a reference sample. KD was validated on protein-level using immunoblotting against SOX10, where α-actinin was loading control for 50 µg total protein loaded. **d)** qPCR analysis was performed on the SOX10-KD clone 2 examining SOX2 and p75 expression, where 18S was used as endogenous control and scrambled control as a reference sample. **e)** Immunoblotting performed in SOX10-KD clone 2 and the scrambled control against-SOX2, where α-actinin served as a loading control for 50 µg total protein loaded.

Note for all qPCR experiments indicated is the mean ± SD of technical triplicates.

*OE=overexpression; KD=knockdown; ctrl=control; SD=standard deviation*

To validate successful TGF-β1 stimulation, TGFBR2 expression and downstream c-JUN expression was investigated on protein-levels upon TGF-β1 treatment for one week in the melanoma cell line SK Mel 30. As expected, a strong increase in TGFBR2 protein expression was observed upon stimulation in combination with a slight increase in c-JUN expression (**Figure 13d**). The enhanced TGFBR2 expression upon stimulation indicates that SOX2 is induced by TGF-β signaling. Similarly, the slight upregulation of c-JUN protein expression suggests the activation of the TGF-β signaling cascade, however investigating phosphorylated c-JUN may show more drastic differences. To further confirm that the TGF-β signaling pathway was activated upon treatment and in turn induced SOX2 expression, immunofluorescence staining was performed in TGF-β1, SB431542 inhibitor and no treatment (0.1 % DMSO) control cells against SMAD2 and SMAD3 proteins (**Figure 13f, Figure S3**). These proteins form a heterodimer and become phosphorylated upon TGF-β stimulation and translocate from the cytoplasm to the nucleus. Therefore, this re-localization upon stimulation would further confirm the activation of the TGF-β signaling cascade. A cytoplasmic staining of SMAD2 in both the no treatment (0.1 % DMSO) and SB431542 inhibitor-treated cells was detected, while predominant nuclear staining was found in the TGF-β1-stimulated cells in A375 and SK Mel 30 melanoma cell lines (**Figure 13f**). Similarly, cytoplasmic distribution of SMAD3 was observed in the majority of control- and inhibitor-treated cells while translocation into the nucleus was detected upon TGF-β1 stimulation in both cell lines (**Figure S4**). These results further confirm that upon TGF-β1 stimulation, TGF-β signaling is activated and in turn induces SOX2 expression *in vitro*.

The phenotype observed upon TGF-β1 stimulation was further investigated in human melanoma cells. After one week of stimulation with TGF-β1, the RNA expression of the EMT-related markers SNAI1 (SNAIL) and CDH2 (N-cadherin) were upregulated in melanoma cell line SK Mel 30 (**Figure 13g**) indicating that the cells are undergoing EMT-like processes upon TGF-β1 stimulation. Furthermore, upon TGF-β1 stimulation CDH1 (E-cadherin) is downregulated, which further suggests that TGF-β may be involved in melanoma progression since the loss of E-cadherin expression has been implicated in cancer progression and metastasis [307] (**Figure 13g**).

In addition, the expression of several differentiation markers, including MITF, TRP2 and TYR after one week of TGF-β1 stimulation was analyzed (**Figure 13g**). All three markers were downregulated upon TGF-β1 stimulation; however TRP2 showed the most drastic difference with nearly 70 % downmodulation compared to no treatment (0.1 % DMSO) control cells. Moreover, SK Mel 30 cells overexpressing SOX2 showed an induction of TGFBR2, TGFBR3 and JUN expression using qPCR analysis (**Figure 13h**). These data suggest a distinct

mechanism, by which SOX2 regulates its own expression and TGF-β signaling in a positive feedforward loop.
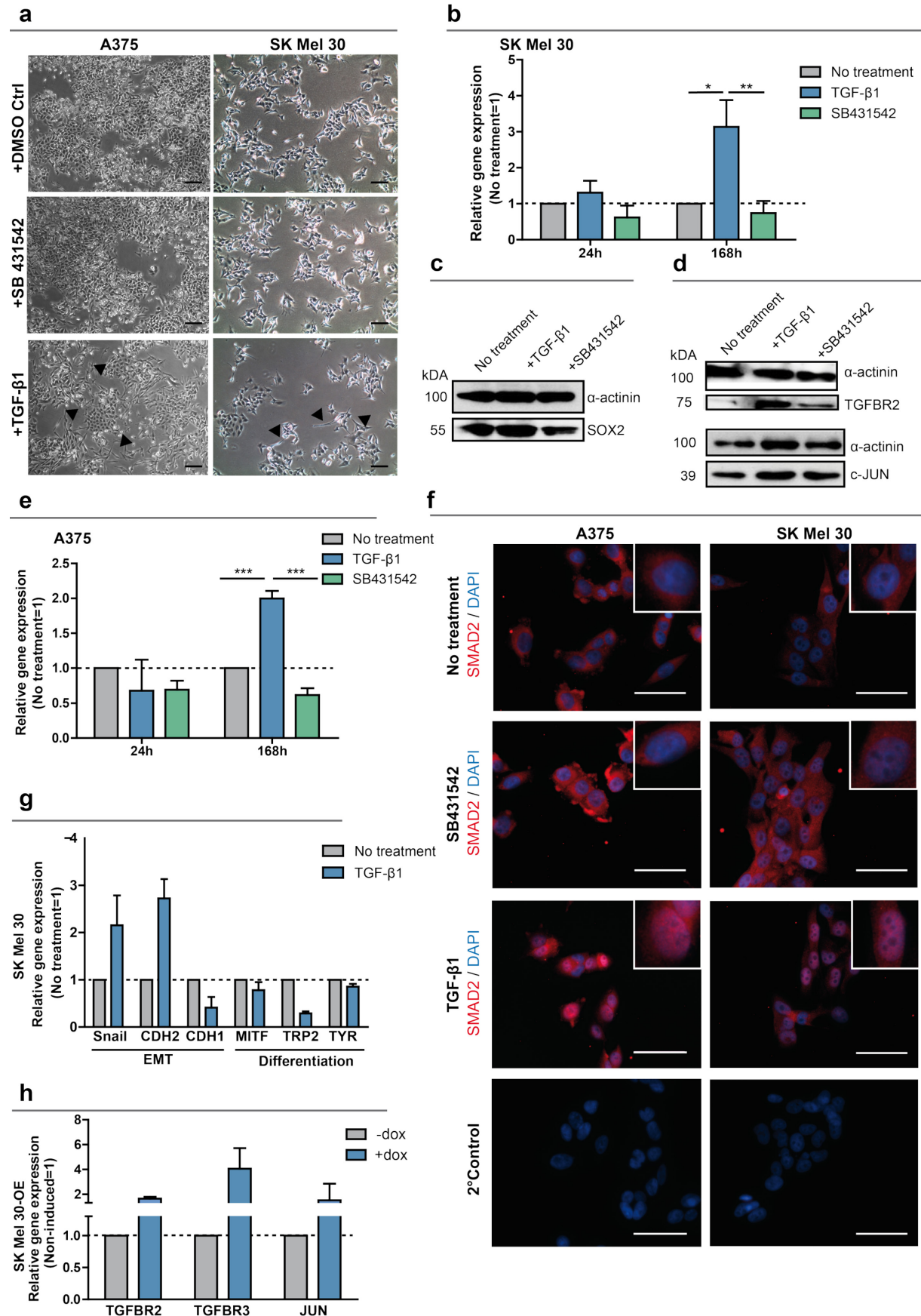
| Figure 13 | **TGF-β induces SOX2 expression in melanoma cells *in vitro*** |

a) Morphology of cells treated with TGF-β1, SB431542 or without treatment (+0.1 % DMSO) was analyzed in both A375 and SK Mel 30 and microscopic images are displayed, bar=20 μm. **b)** SK Mel 30 melanoma cell line was stimulated with TGF-β1 for 24 hours and 168 hours, and SOX2 expression was quantified using qPCR, where 18S and HPRT were used as endogenous controls and untreated cells (+0.1 % DMSO) as a reference sample. Graph displays mean ± SD of three independent stimulation experiments with technical triplicates. **c)** SOX2 protein expression was measured using immunoblotting and α-actinin was used as loading control for 20 μg total protein loaded. **d)** The expression of TGFBR2 (upper panel) and JUN (lower panel) were analyzed using immunoblotting and α-actinin was used as loading control for 20 μg total protein loaded. **e)** The A375 melanoma cell line was stimulated with TGF-β1 for 24 hours and 168 hours, and SOX2 expression was quantified using qPCR, where 18S and HPRT were used as endogenous controls and untreated cells (+0.1 % DMSO) as a reference sample. Graph displays mean ± SD of three independent stimulation experiments with technical triplicates. **f)** Displayed are IF images of SMAD2 (Cy3, red) in cells treated with TGF-β1, SB431542 and without treatment in A375 and SK Mel 30 cell lines. Nuclei were counterstained using DAPI, bar=50 μm **g)** EMT- and differentiation-marker expression was analzyed using qPCR where 18S and HPRT were used as endogenous controls and untreated cells (+0.1 % DMSO) as a reference sample. **h)** TGFBR2, TGFBR3 and JUN expression were analyzing using qPCR in SK Mel 30-OE cells. 18S and HPRT were used as endogenous controls and non-induced control cells were used as a reference sample, graphs displays mean ± SD of technical triplicates.

OE=overexpression; dox=doxycycline; IF=immunofluorescence; EMT=epithelial-to-mesenchymal transition; DAPI=4',6-diamidino-2-phenylindole; Cy3=Cyanine3; SD=standard deviation, significance tested using two-tailed t-test, where where * is p ≤ 0.05, ** is p ≤ 0.01 and *** is p ≤ 0.001

5.6 SOX2 and novel protein-protein interactions

Protein-protein interactions play crucial roles in regulating important cellular pathways. Therefore, novel potential interacting partners of SOX2 were investigated using yeast-2-hybrid (y2h) pairwise experiments and bimolecular fluorescence complementation (BiFC) experiments in collaboration with the DKFZ proteomics core facility (methods described in **Supplemental section 8.1.2.2 and 8.1.2.3**)

Using BiFC, the potential interaction between SOX2 and PAX3, SOX10, and PAX6, respectively, was investigated. PAX6 is a known interacting partner of SOX2, particularly in the retina and was used as a positive control [245]. HEK 293T cells were transfected with bait and prey vectors containing fragments of complementary fluorescent reporter proteins. Upon interaction *in vitro*, the fusion reporter protein reforms and fluoresces. A positive interaction between PAX3 and SOX2 was observed in three independent BiFC experiments, similar to that of the positive control (**Figure 14a,** left panel). The differential role of SOX2 and SOX10 was investigated in melanoma progression and therapy and it was found that SOX2 and SOX10 share a binding site on the proximal MITF promoter. Therefore, the mechanism, by which SOX2 and SOX10 may be regulated on protein-level, was of interest. Strong fluorescence was observed when SOX2-bait and SOX10-prey were transfected into HEK 293T cells, where the fluorescence was equal to positive control (**Figure 14a,** middle panel). The same fluorescence was observed when SOX10 was used as bait and SOX2 as prey (**Figure 14a**, middle panel).

a

| Bait | SOX2 | SOX2 | SOX10 | Positive Ctrl | Negative Ctrl (SOX2-LMNB1) |
| Prey | PAX3 | SOX10 | SOX2 | | |

Brightfield

RFP

Merge

b

Transformation — Transformation

TRP-Bait — Mating — LEU-Prey

Diploids in medium lacking TRP and LEU

Many other variants

c

in medium lacking HIS

Reactivated TF

B P
BD AD

HIS3 transcription (results in histidine autotrophism)

Co-expression of fusion proteins

d

3-AT: 1.0 mM

| PAX6 | TWIST1 | PAX3 | SOX10 |

SOX2-SOX2

3-AT: 2.0 mM

SOX2-SOX2

e

Prey Gene Symbol	# of times isolated Universal human cDNA library	# of times isolated Full ORF cDNA library (N-terminal)	# of times isolated Full ORF cDNA library (C-terminal)	Description
SUMO1	14	37	1	Small ubiquitin-related modifier 1
UBE2I	11	29	4	Ubiquitin-conjugating enzyme E2I
PIAS4	0	0	7	E3 SUMO-protein ligase
CBX4	0	0	15	E3 SUMO-protein ligase
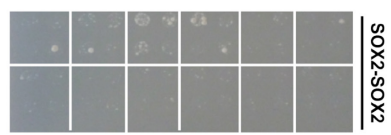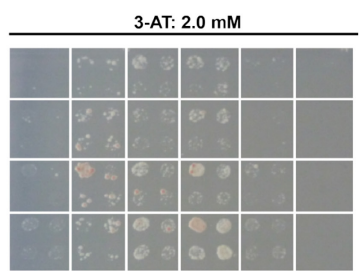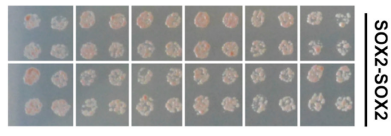
f

Position	Peptide
121	RKTKTLMKKDKYTLP
245	GSMGSVVKSEASSSP

g

Site 1
121

Site 2
245

1   41   HMG   109   180   TAD   317
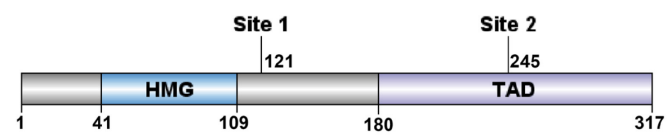
Figure 14	SOX2 protein-protein interaction studies

a) BiFC assay was performed by co-transfecting HEK 293T cells with one bait and one prey construct, both containing a gene of interest coupled to an incomplete fluorophore. Upon transfection, the cells were investigated under a fluorescence microscope. If the proteins of interest interact with each other (*i.e.* protein-protein interaction), they are in physical proximity and the fluorophore is reconstituted and fluorescence is detectable. This experiment was performed using different bait constructs (SOX2 and SOX10) and different prey constructs (SOX2, SOX10 and PAX3). Upper panel displays brightfield images, middle panel displays RFP-positive cells and lower panel displays merged images.
The y2h assay is based on the reconstitution of a functional transcription factor upon a specific protein-protein interaction. Modified yeast strains, where reporter genes lead to a specific phenotype, are used. **b)** The yeasts are first transformed with corresponding TRP-bait or LEU-prey constructs. Then the yeasts are mated and the diploids are selected *via* nutritional selection on medium lacking TRP and LEU. **c)** In the diploid yeast, the bait protein contains the protein binding domain but requires binding of the prey, which contains the activation domain, in order to initiate transcription. In this case, the HIS3 gene is transcribed and thus, the yeast can grow in medium lacking histidine. Therefore, if the proteins interact, a transcription factor will reform and inititate transcription of the HIS3 gene and the yeast cultured in histidine-deficient medium survives. **d)** y2h pairwise experiments were performed using SOX2 bait constructs with various prey constructs including PAX6, TWIST1, PAX3 and SOX10. Upon transformation of yeast with bait and prey constructs, diploids were nutritionally selected on medium lacking TRPand LEU. Positive interaction is indicated by colony growth. Bottom panels indicate SOX2 autoactivation by displaying colony growth when SOX2 is used in both, bait and prey constructs. Upper panel displays yeast growth in the presence of 1.0 mM 3-AT and lower panel displays yeast growth in the presence of 2.0 mM 3-AT. **e)** Using the y2h HTS in three independent cDNA libraries, four hits were successfully isolated: SUMO1, UBE2I, PIAS4 and CBX4. **f)** The positions where SOX2 is predicted to be SUMOylated were determined using the online database GPS-SUMO. Two sites were identified at position 121 and 245. **g)** An illustration of the SOX2 protein with corresponding HMG domain (blue) and the transactivation domain (TAD) (purple) labeled with both predicted SUMOylation sites. This graphic was constructed using the opensource software GPS-DOG 2.0 domain illustrator.

y2h=yeast-2-hybrid; B=bait; P=prey; BD=binding domain; AD=activation domain, HIS=histidine; TF=transcription factor; TRP=tryptophan; LEU=leucine; BiFC=bimolecular fluorescence complementation; RFP=red fluorescent protein; 3-AT=3-Amino-1,2,4-triazole; HTS=high throughput screen; TAD=transactivation domain; HMG=high mobility group

Next, to confirm these interactions in a second independent experiment, the y2h pairwise experiment was performed. In brief, a construct containing the bait (SOX2) coupled to an enzyme capable of synthesizing tryptophan (TRP) and the prey of our choice coupled to an enzyme capable of synthesizing leucine (LEU) were transformed into yeast. The yeasts were then mated and diploid yeast were selected *via* nutritional selection on TRP/LEU deficient medium (**Figure 14b**). Growth of the diploid yeast was only possible upon interaction of the bait, containing the protein binding domain, and the prey, containing the activation domain (**Figure 14c**). This interaction allows for the reformation of a transcription factor which initiates transcription of the integrated HIS3 gene, enabling growth on histidine-deficient media. Therefore, only diploid yeast clones in which there is an interaction between the bait and prey proteins are able to transcribe their own HIS3 and survive on histidine-deficient medium. With this selection process, one is able to elucidate potential protein-protein interactions. Another level of stringency used in y2h experiments is 3-amino-1,2,4-triazole (3-AT). This molecule is a competitive inhibitor of the product of the HIS3 gene, reducing possible autoactivation and false positives.

The same interactions, PAX3/SOX2 and SOX10/SOX2, identified by the BiFC assay were further examined in y2h experiments (**Figure 14d**). However, difficulties were observed when performing these experiments due to the strong autoactivation tendencies of SOX2. Therefore, the effect of autoactivation could not be completely ruled out in y2h pairwise experiments. Nonetheless, weak colony growth in the PAX3/SOX2 mating at the highest stringency of 2.0 mM 3-AT (bottom panels) was observed; however between replicates (column) there was great variability (**Figure 14d**, left panel). Next, the SOX2/SOX10 potential interaction was investigated in the y2h system; however the overexpression of both SOX2 and SOX10 proved to be lethal in yeast even in the lowest stringency tested (**Figure 14d**, upper panels). Therefore, the possible interaction between SOX2 and SOX10 was not confirmed in yeast (**Figure 14d,** right panel).

TWIST1 was further investigated as a potential interactor with SOX2. TWIST1 is known to be implicated in cell lineage determination and the EMT process. Since SOX2 plays important roles in both pathways possible protein-protein interactions between these two proteins were investigated. Unfortunately, TWIST1 analyses could not be performed using BiFC experiments due to cloning difficulties. Nonetheless, y2h pairwise experiments for TWIST1 were performed. Although autoactivation-mediated effects could not be completely ruled out in all y2h pairwise experiments, weak colony growth in the TWIST1-SOX2 mating was observed at the highest stringency of 2.0 mM 3-AT (bottom panels); however between replicates (columns) there was great variability (**Figure 14d,** middle panels). In conclusion, SOX2 may form potential protein-protein interactions with PAX3, SOX10 and TWIST1; however since the y2h pairwise experiments were unreliable due to strong autoactivation of SOX2 and therefore these experiments need to be repeated with SOX2 construct lacking the HMG domain to eliminate autoactivation tendencies. Furthermore, these interactions should be confirmed in co-immunoprecipitation experiments *in vitro*.

5.6.1 SOX2 forms protein-protein interactions with novel candidates related to SUMOylation

In addition to y2h pairwise experiments, y2h high-throughput screen (HTS) was performed. We performed the screen on multiple libraries (human universal, full ORF N-terminal, full ORF C-terminal) to eliminate as many artifacts as possible. The concentration of 3-AT used depends on the strength of the interaction between the bait and prey used and is usually determined in preliminary experiments (**Figure S5a**). During this HTS, 3-AT was used at a concentration of 4 mM to eliminate as many promiscuous and artificial preys. The experimental setup of the screen is identical to the basics of y2h experiments, as described in **Figure 14b-c**. The main difference between the standard y2h pairwise experiments and the HTS is that in the screen, the prey proteins are not pre-selected but contained in a cDNA

library. Therefore, the mating occurred between a particular bait of interest (SOX2) and the prey offered by the cDNA library. Upon the reformation of the transcription factor, reporter genes were transcribed allowing fluorescence readout. After mating, the yeast were dispensed onto ten microtiter plates and allowed to grow (**Figure S5b**). Fluorescence was measured and the positive wells collected. Next, PCR was performed and the bands were sequenced and analyzed.

Three independent HTSs were performed using three different cDNA libraries: universal human, full ORF library (N-terminal) and full ORF library (C-terminal). Several hits were isolated in at least two of the three screens. These hits included SUMO1, UBE2I, PIAS4, PCFG2, and CBX4 (**Figure 14e**). For example, small ubiquitin-related modifier 1 (SUMO1) was isolated 14 times from the universal human library and 37 times from the full ORF library. Ubiquitin-conjugating enzyme E2I (UBE2I) was isolated 11 times from the universal human library and 29 times from the full ORF library (C-terminal) (**Figure 14e**).

The first hit, SUMO1, was particularly interesting since this protein is heavily involved in protein SUMOylation or post-translational modifications [252]. Literature research revealed that the conjugation of SOX2 to SUMO1 has already been investigated [252]. Moreover, it was discovered that upon conjugation of SUMO1 to SOX2, inhibition of the DNA binding activity by SOX2 was observed. This work not only suggests that SUMOylation has a functional effect on SOX2 but also validates that the y2h HTS likely identified true protein-protein interactions. Likewise, the next hit, ubiquitin-conjugating enzyme (UBE) - 2I, is an enzyme responsible for performing SUMO conjugation is related to the SUMOylation process. Further hits, E3 SUMO-protein ligase (PIAS4) and Chromobox protein homolog 4 (CBX4), function in the SUMOylation process by stabilizing the interaction between UBE2I and the substrate [247]. The identification of potential interactions occurring between SOX2 and proteins related to the SUMOylation process suggest SOX2 can be SUMOylated.

To determine whether SOX2 can be SUMOylated, the GPS-SUMO online database was used to predict SUMOlyation sites within the SOX2 protein (**Figure 14f**). The database identified two predicted SUMOylation sites for SOX2; one located at amino acid position 121 and the other at 245. Interestingly, the publication which reported conjugation of SOX2 to SUMO1 describes a conjugation at lysine 247 [252]; but the exact positions where SOX2 is SUMOylated *in vitro* needs to be confirmed. Lastly, the SOX2 protein domains and its predicted SUMOylation sites were constructed using the open source software DOG 2.0 (**Figure 14g**).

Taken together, y2h HTS experiments identified four key players in the SUMOylation process, SUMO1, UBE2I, PIAS4 and CBX4, which may form protein-protein interactions with SOX2.

5.7 SOX2 in the clinic

The final part of the current study involved investigating the role of SOX2 in the clinic. After showing that SOX2 is highly involved in regulating melanoma cell invasion and migration *in vitro* and SOX2 expression was found to be highly overexpressed in deep melanoma tumor regions and in stroma-infiltrating melanoma cells (**Figure 10e-f**). Detailed analyses were performed regarding SOX2 expression and corresponding clinical data.

A tissue microarray (TMA) was used to further investigate SOX2 expression in primary melanoma patient tissues and melanocytic nevi samples. Representative TMA images are shown in **Figure 15a**, where primary melanoma samples are displayed in the bottom panel and melanocytic nevi in the upper panel. Upon scoring the TMA (method described in **Figure S2**), a significant increase in SOX2 expression was found in primary melanomas compared to melanocytic nevi (**Figure 15b**).
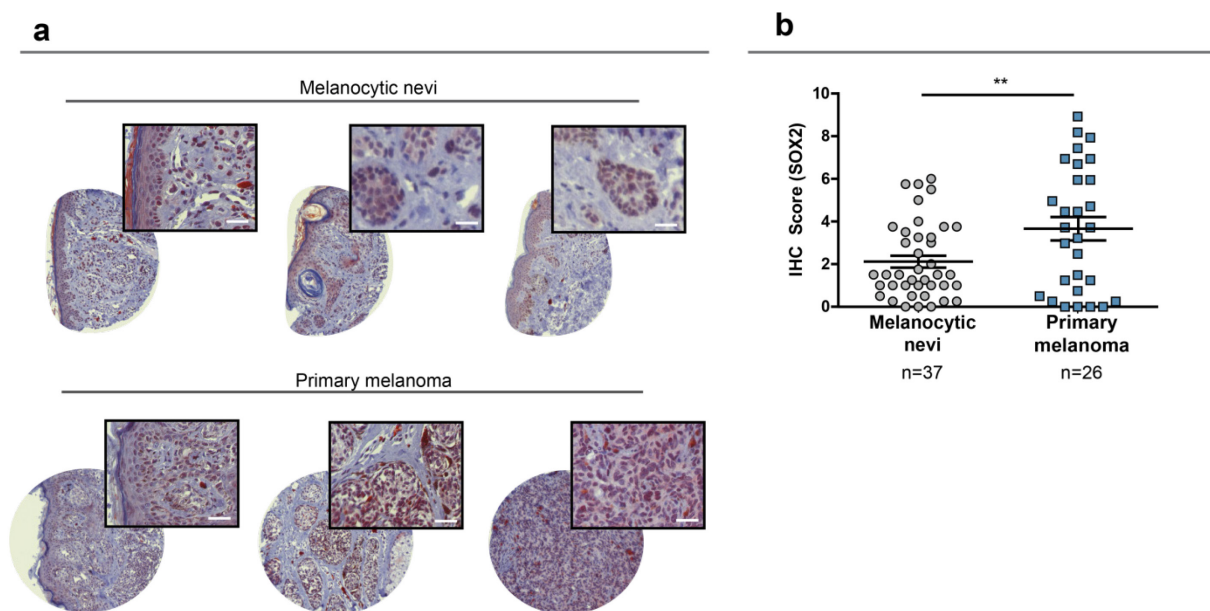
Figure 15   **SOX2 is upregulated in primary melanoma samples compared to melanocytic nevi**

a) Tissue microarray samples of melanocytic nevi (upper panel) and primary melanomas (lower panel) stained for SOX2, bar=25 µm. **b)** IHC score for SOX2 staining is plotted for melanocytic nevi and primary melanomas; graph displays mean and SD of indicated n.

*IHC=immunohistochemistry score; SD=standard deviation, significance tested using Student's t-test, where **is p≤0.01*

To summarize these results, we have identified SOX2 to play a role in a melanocyte differentiation protocol, to be important in melanoma pathogenesis and possibly forming

novel protein-protein interactions with SUMO-related proteins. SOX2 was identified to be expressed at low levels during the established melanocyte differentiation protocol from human iPSCs. Using FISH analysis, we demonstrated SOX2 amplification in primary melanoma tissue compared to melanocytic nevi. The functional analysis of SOX2 in melanoma revealed the requirement for SOX2 in melanoma cell invasion *in vitro* and was linked to EMT-marker induction. Furthermore, dedifferentiation in melanoma cell may be influenced by SOX2 binding and repression of the MITF-M promoter. Additionally, we found that SOX2 was induced by TGF-β1 *in vitro*. Finally, in protein studies using y2h HTS, we identified potential new interacting partners of SOX2 related to the SUMOylation process.

6 Discussion

The resistance of advanced melanoma to current targeted therapies, unavailable prognostic tools and lack of knowledge about the molecular mechanisms underlying the pathogenesis of this cancer together highlight the deficiencies in the melanoma field. To date, research has identified alterations in key regulators in both, tumor suppressor and oncogenic pathways, which are responsible for disturbed signaling in mitogen-activated protein kinase (MAPK) pathway [308], inhibition of the RB tumor suppressor pathway [309] and further activation of downstream tyrosine kinases [310]. In melanoma, these modifications cause cellular proliferation and uncontrolled growth which can progress quickly into metastatic cancer. Accordingly, widespread dissemination of melanoma cells to distant organs is a common occurrence in melanoma progression.

There are several proposed mechanisms for malignant transformation in melanoma. One mechanism is through the acquisition of genetic aberrations that lead to malignant transformation, such as the acquisition of mutant BRAF^{V600E} and p16^{INK4a}/p19^{ARF} loss or mutations in p53 and PTEN [142–144]. The acquisition of these aberrations can occur through various means including exposure to environmental risk factors, such as UV radiation. Aside from somatic mutations, it has been suggested that, in melanoma, there is an inheritance of melanoma susceptibility genes. To date there have been four different genes at three different loci identified to confer susceptibility in melanoma, the genes include: p16^{INK4a}, p14^{ARF}, CDK4 and TERT. Lastly, melanocytes are derived from embryonic, migratory neural crest cells (NCCs). Throughout development, the NCC population gives rise to various lineages including bone and cartilage, pigmented cells, neurons and glial cells of the peripheral nervous system. Upon the induction of the neural crest (NC) and formation of the neural tube, NCCs migrate dorsolaterally by acquiring migratory features through the epithelial-to-mesenchymal transition (EMT), before becoming fully differentiated melanocytes when reaching the epidermis [4]. Due to their developmental program, which gives the lineage invasive, metastatic qualities [207], melanocytes may be intrinsically predisposed to transformation and metastasis. Taken together, melanoma has several routes towards malignant transformation and it's likely a combination of routes that ultimately confers malignancy.

6.1 Embryonic stem cell marker expression during melanocytic differentiation from human induced pluripotent stem cells

In this study, a protocol was established to terminally differentiate melanocytes from human induced pluripotent stem cells. For this approach differentiation medium (**Table 4**) supplemented with BMP4 and ascorbic acid was used; which directed differentiation into all ectodermal lineages (**Figures 3**). Further cultivation in melanocyte medium successfully

selected a pure population of melanocytes, which were characterized in depth for their differentiation- and pluripotency-marker expression (**Figure 4a-b,e**), melanosome development (**Figure 5**), and whole genome expression array analyses (**Figure 6**) compared to normal human melanocytes (NHMs). Minor differences were noted when comparing the hiPSC-derived melanocytes (Mel D1) compared to NHMs, such as the observed difference in the Ingenuity-derived pathway '*transcriptional regulatory network in embryonic stem cells*' where there was stronger enrichment in differentially expressed genes in NHMs compared to Mel D1 cells. If this has any significant meaning remains under investigation, but it is not surprising that differentiating hiPSCs towards the melanocytic lineage would utilize slightly different pathways (**Figure 6c**).

To date, there are several well-established models of the NC that allow for the critical investigation of different developmental stages such as: iPSCs, NC, melanoblast and terminally differentiated melanocytes. Of note, Studer and colleagues recently modeled the NC using a complete and comprehensive *in vitro* system [83]. Upon using timed exposure to WNT, BMP, and EDN3 and under dual-SMAD inhibiting culture conditions, the sequential induction of the NC and melanocyte precursor fates was triggered. Moreover, the global gene expression profile was analyzed throughout the differentiation process, giving new perspectives into the NC and melanocyte fate determination [83]. In comparison, our established melanocyte differentiation protocol did not include the usage of the particular factors driving a specific route of melanocyte differentiation. It cannot be excluded that also other factor combinations may induce the development of neural crest cells or melanocyte differentiation. The Studer protocol permits for the investigation of defined stages throughout melanocyte development which makes it an ideal model for studying processes such as the NC; while the protocol used in this study is a more general approach that directs cells into the ectodermal lineage. Although the Studer protocol allows in-depth analyses of each processes throughout melanocyte development, both protocols end with terminally differentiated melanocytes which indicates that either can be used for this purpose.

The lack of characterization throughout the differentiation process allows limited interpretation of the particular differentiation stages; however, the protocol used in the current study generated terminally differentiated melanocytes at a reasonable efficiency. The protocol used was not necessarily established for neural crest modeling purposes by Nissan and colleagues [292] but rather to obtain fully functional melanocytes. The protocol used in this study was based on a study that differentiated human embryonic stem cells (hESCs) into another ectodermal derivative: keratinocytes [311]. Researchers found that high concentrations of BMP4 and ascorbic acid controlled and mediated pluripotent stem cell differentiation along the ectodermal lineage resulting in keratinocyte lineage differentiation in

approximately 50-60 % of cells, while the remaining cells formed clusters of pigmented cells. Consequently, Nissan and colleagues hypothesized that these pigmented cells may contain melanocytes or neuroectodermally-derived RPE cells [312] and adapted the protocol for the derivation of terminally differentiated melanocytes [292].

In the current study, this protocol was utilized due to its high efficiency in generating terminally differentiated melanocytes. After the completion of the differentiation protocol, a pure population of hiPSC-derived Mel D1 cells was achieved without any keratinocyte or RPE cell contamination. A pure population was generated by culturing the pigmented colonies from the differentiation protocol in separate culture dishes with a melanocytes specific medium, which only permits growth of melanocytes.

One would assume that after placing human iPSCs in differentiation medium containing high levels of BMP4 and ascorbic acid, expression of all pluripotency markers would diminish at a rapid pace. This holds true for both OCT4 (**Figure 4a**) and NANOG (**Figure 7a**) expression. Interestingly, SOX2 remained expressed after an initial decrease upon the initiation of differentiation (**Figure 7a-b**). Moreover, SOX2 was found to be highly expressed in NC-derived cells (**Figure 7c**). The expression of SOX2 in cells that were enriched for NCCs by sorting for HNK-1 and p75 expression (method described in **Supplemental section 8.1.1.2**), suggested that SOX2 may play a role in the developing NC (**Figure 7c**). To date, there have been several important studies, which have investigated the role and function of SOX2 in NC development; one of which used an *in vitro* model for NC characterization to demonstrate a key role for SOX2 is neurogenesis [77]. Moreover, an *in vivo* system for modeling NC development examined tumor cell invasion by transplanting human metastatic melanoma cells into chick embryonic NC environment [210]. These transplantation experiments revealed that the invasion and migratory pathways employed by melanoma cells were heavily influenced by NC microenvironmental signals. In addition to *in vivo* modeling, laser capture microdissection (LCM)-assisted gene profiling was utilized to identify novel interactions and signaling networks. Cells were collected from distinct locations, including migrating NCCs, from the *in vivo* model and analyzed using qPCR [210]. Pluripotency markers were analyzed and SOX2 showed the highest expression in the migratory cells, supporting the model used in the current study that also suggests a role for SOX2 in the NC.

Although the spatiotemporal expression of SOX genes in the NC has not been fully investigated, some of these genes have been analyzed in detail, *e.g.* SOX10 [313]. SOX10 is expressed in early migrating NCCs [314, 315]. In detailed investigations of NC-derived cells *in vitro*, SOX10 was required for survival and glial differentiation [316]. High expression of SOX10 was observed throughout the days of differentiation towards the ectodermal lineage

in our melanocyte differentiation protocol (**Figure 7b**), which suggests that this protocol not only follows a NC-like path but that SOX10 is also important for this lineage determination.

During the differentiation process, SOX2 expression levels remain lower than SOX10 expression levels but remain detectable even at late differentiation stages, suggesting that complete ablation of SOX2 is not required for successful ectodermal lineage differentiation. Moreover, these experiments were normalized to hiPSCs, therefore SOX2 expression levels remain equivalent or slightly to that of hiPSCs (**Figure 7b**).This is in agreement with the fact that the majority of terminally differentiated cells derived from the ectoderm requires the expression of SOX2, such as neurons (**Figure 7b**) (reviewed in [303]).Conversely, SOX2 has been reported to inhibit NC formation and subsequent EMT in avian studies [313]. Moreover, SOX2 is actually downregulated in both early premigratory and migratory NCCs [11, 313]. In neural plate explants and in the embryonic ectoderm, SOX2 expression was aberrant and mutant forms of SOX2 were used to reveal that SOX2 is an inhibitor of NC formation [313]. This may explain the lower levels of SOX2 expression seen throughout the melanocyte differentiation protocol compared to SOX10 expression (**Figure 7b**). Moreover, the observed downregulation of NC-related markers, such as SOX10, upon the overexpression of SOX2 in the human melanoma cell lines (**Figure 12a**) might be explained by the inhibitory effect SOX2 has on the neural crest during development. The patterning of SOX10 *vs*. SOX2 observed during melanocyte differentiation from hiPSCs is in line with this hypothesis (**Figure 7b**). Since SOX2 was suggested to be an inhibitor of the induction of NCCs, the expression of SOX2 found in NC-derived cells remains under investigation (**Figure 7c**). However, the expression of SOX2 in this NCC- population may provide evidence that further characterization of these cells is needed to ensure the population contains cells in the pure NC-state.

In summary, the temporal expression of SOX2 and SOX10 during the ectodermal-specific differentiation supports developmental studies showing the importance of SOX10 expression and lower relative levels of SOX2 for successful ectodermal lineage development and progression. Several studies, including the current one, reveal that NC development and the characterization of melanocyte progenitors can be successfully simulated using *in vitro* modeling systems. These model systems require full characterization to allow for comprehensive analyses of melanocyte development that was otherwise impossible to study due to constraints of animal models, ethics or limited resources.

6.2   Functional analysis of SOX2 in melanoma

The function of stem cell-associated markers in cancer has been investigated in several studies (reviewed in [317]). One study found significant overexpression of at least one out of

the four pluripotency factors OCT4, SOX2, c-MYC or KLF4 in 45 % of cancer types investigated using the Oncomine online database [283]. Another study identified three stem cell markers, CD166, CD133 and NESTIN, and observed increased expression of these markers in metastatic melanoma samples, where at least one of the markers was expressed in all metastatic samples [290]. Taken together, these studies illustrate the importance of stem cell-associated markers in cancer, including melanoma.

To date, limited research has been done on SOX2 in the context of malignant melanoma. The 3-year median survival for patients with SOX2-positive metastatic melanomas decreased by 145 days compared to patients with SOX2-negative metastatic melanomas [283, 284], implicating role for SOX2 in melanoma progression. Further functional studies have been performed linking SOX2 to melanoma cell invasion, growth and to NESTIN expression [282, 284, 295, 318]. Recently, it has been demonstrated that SOX2 is crucial for regulating self-renewal capacity and tumorigenicity in melanoma initiating cells *via* Hedgehog (HH) signaling [286]. The complete understanding of SOX2 in melanoma pathogenesis including its induction, regulatory mechanisms involved in SOX2 expression in melanoma and potential protein binding partners of SOX2 remains not well understood. The current study set out to confirm defined key functions of SOX2 in melanoma pathogenesis.

6.2.1 Genomic SOX2 amplification in melanoma

Tumor formation and development occurs when a cell accumulates genetic aberrations that alter normal cell cycle control. There are several means of genetic aberrations including gene deletions or gene amplifications. Gene amplification is defined as a copy number increase of a particular chromosomal region and SOX2 amplification is caused by multiplication of the 3q26.3 gene locus [264, 265]. SOX2 amplification has been widely studied in several cancer types including glioblastoma, SCLC and many forms of SCC [264, 266–269, 271, 273, 319]. (reviewed in [262]). To date SOX2 amplifications have not been investigated in melanoma, however the amplification of SOX2 in SCC is well established [269]. In order to understand if amplification is one possible mechanism, by which the SOX2 gene is upregulated in melanoma, fluorescence *in situ* hybridization (FISH) analyses were performed in primary melanoma tissue using previously described protocols (**Figure 8e-f**, method describe in **Supplemental section 8.1.1.1**) [320]. Notably, SOX2 was significantly amplified in both low-risk (≤1mm) and high-risk (≥1mm) primary melanoma compared to nevi samples (**Figure 8e**). Additionally, all metastatic samples from lymph-nodes, in-transit (metastasis moving towards lymph-nodes), skin and visceral organs, were significantly amplified when compared to melanocytic nevi (**Figure 8e**). A common drawback to this technique is the high false-positive rate due to the evident co-localization of two signals, which occurs when viewing a three-dimensional nucleus in two dimensions [321]. However, the location of the investigated locus

and the clear pairs of reference signals per nuclei confirm the amplification observed (**Figure 8f**).

Amplification typically correlates with expression levels and thus these data suggest accelerated SOX2 expression in both low- and high-risk primary melanoma and metastatic melanoma compared to melanocytic nevi samples (**Figure 8e-f**). Correspondingly, these results correlate with the significant increase of SOX2 expression in primary melanoma compared to melanocytic nevi in the TMA analyses (**Figure 15**) and, in IHC analyses, metastatic melanoma, displayed strong immunopositivity of SOX2 compared to slight staining of primary melanoma and no staining in the melanocytic nevi (**Figure 8d**). Furthermore, SOX2 was seen to be highly expressed in 67 % of human melanoma cell lines (n=9) (**Figure 8a-b**). Taken together, the amplification of SOX2 in melanoma likely enhances SOX2 expression in primary and metastatic melanoma compared to melanocytic nevi.

6.2.2 SOX2 expression induces dedifferentiation of melanoma cells *in vitro*

The differentiation status of tumor can have drastic impacts on the phenotype and aggression of the disease. In fact, tumor cell dedifferentiation is a crucial step towards the acquisition of invasive properties and even subtle changes in the differentiation status allows tumor progression [301]. In epithelial cancers, *i.e.* carcinomas, both primary tumors and corresponding metastasis display similar heterogeneous organization, where dedifferentiated tumor cells are observed along the invasive front [218]. These regions of dedifferentiated cells possess hallmarks of EMT and the acquisition of this process is now heavily associated with tumor cell dissemination. In melanoma, the high heterogeneity allows for subpopulations to switch into a more dedifferentiated phenotype, known in melanoma as the 'phenotype-switch' (reviewed in [217]). The phenotype-switch is a phenomenon where melanoma cells switch from a proliferative to an invasive state, which is a process that resembles the EMT and permits the acquisition of therapy resistance [322]. The dedifferentiated subpopulations within melanomas retain stem cell-like properties and dedifferentiation is a key step in switching towards an invasive phenotype that also correlates to therapy resistance. In regards to therapy resistance, differentiation-related markers were downregulated and the dedifferentiation-related marker p75 (also known as NGFR and CD271) was upregulated upon TNF-α treatment, which mimics immunotherapy-induced tumor inflammation [323]. Moreover, in B16 murine melanoma cells, the inhibition of MITF led to the upregulation of stem cell-associated markers, OCT4 and NANOG, and promoted an invasive phenotype [219]. This agrees with work revealing that MITF expression regulates the phenotype switch and controls dedifferentiation in melanoma [214, 215, 304].

Although SOX2 expression is low in terminally differentiated melanocytes the observed expression of SOX2 in melanoma may be caused by insufficient silencing of SOX2 in melanocyte precursors which facilitates tumorigenic potential; this supports the theory that cancers arise from early progenitor or cancer stem cells. Conversely, SOX2 may be expressed in melanoma but not in melanocytes due to early molecular events during the initiation of transformation process which leads to the re-expression of SOX2 in melanoma cells. However, to date, the reactivation of SOX2 in human melanoma and its influence on melanoma dedifferentiation remains unknown.

Studies in murine melanoma cells revealed that SOX2 is capable of binding directly to the promoter of MITF-M [297], suggesting a connection between SOX2, MITF expression and dedifferentiation of melanoma cells. Here, binding of human SOX2 to the human promoter of MITF-M promoter was suggested using a computer-based DNA sequence analysis with JASPAR [298]. One putative binding site that matches results from the murine study was confirmed (**Figure 9e**). Furthermore, there is high conservation between mouse and human for this particular binding site (**Figure 9h**) [299], which further supports the identification of a putative binding site for SOX2 on the MITF-M promoter *in silico*. However, future chromatin immunoprecipitation experiments are required to confirm this *in vitro*. Consistent with results from the mouse study, I observed a functional repression of the MITF promoter upon SOX2 expression using luciferase reporter assays (**Figure 9g,** method described in **Supplemental section 8.1.2.4**). However, in the murine work an approximate 80 % repression was observed [297], in contrast to 20 % repression that was observed using SOX2 overexpression in human melanoma cells (**Figure 9g**). There are several explanations for this variance regarding the repressive effect of SOX2 expression on MITF-promoter activity between the human and murine system. First, the difference in species may represent one reason for the difference in functionality of SOX2-mediated repression of the MITF-M promoter activity. However, due to the high conservation of the SOX2 binding site of interest, it is unlikely that the difference in species influences functional assays to such an extent. Secondly, another explanation might be the experimental setup used in the current work compared to the murine studies. In the B16 murine melanoma study, a MITF reporter was used that contains a fragment of the full-length MITF promoter including the regions between -515 and +90 [60], which contained the SOX2 binding site. This setup omitted further upstream activation elements including additional SOX10 binding sites [297]. In contrast, the reporter system used in the current study includes the full-length MITF-M promoter and therefore possesses all activation sites for SOX10 upstream from the SOX2 binding site [300]. In other words, the slight repression of the human MITF-M promoter observed in

SOX2-overexpressing human melanoma cells is likely weakened due to potential SOX10-activation sites located upstream, which were eliminated in the murine studies.

The implications of SOX2 binding to the MITF-M promoter in human melanoma cells would explain the downregulation of MITF upon SOX2 overexpression and upregulation upon SOX2 knockdown (**Figure 9d-f**, **Figure 11f**). To confirm the loss of MITF protein expression was due to SOX2 overexpression and not doxycycline or G418 side effects SK Mel 30-OE cells were cultured in the absence of doxycycline, treated with G418 or in the presence of both and immunoblotting of MITF revealed no difference compared to the non-selected cells (not shown). Therefore, the downregulation of MITF was caused by the upregulation of SOX2. For further experiments, only non-induced cells were used for control. Furthermore, the location of the putative binding site for SOX2 in the human MITF-M promoter revealed that this site is identical to a SOX10 binding site. This suggests that SOX2 and SOX10 compete for binding at this particular binding site to either repress or activate the MITF-M promoter, respectively. It is of interest whether or not competitive binding assays will confirm this hypothesis. Nevertheless, the current study suggests one mechanism, by which SOX2 may be contributing to melanoma dedifferentiation through binding and repressing the MITF-M promoter *in vitro*.

6.2.3 SOX2 is required for melanoma cell invasion and induces EMT-marker expression

Activating invasion and enabling metastasis is not only a hallmark of cancer in general but also causes most cancer-related deaths, especially in malignant melanoma [202]. Invasion and metastatic spreading are processes that alter tumor cell shape and their attachment to other cells and the extracellular matrix (ECM). Alterations in cadherin expression represent a well characterized feature of invasive cells [202]. Typically, invasive cells lose their expression of E-cadherin (CDH1), which is a key cell-to-cell adhesion molecule. In contrast, N-cadherin (CDH2) is upregulated in invasive tumor cells and gives the cells a more meschenchymal phenotype [202]. In 2003, a depiction of the connections between invasive properties of cancer cells and metastatic spreading was described by Fidler [324, 325]. There are different stages in tumor cell invasion and metastatic dissemination. First, local invasion is detectable, followed by intravasation of tumor cells into neighboring blood and lymphatic vessels. The tumor cells are transported through these circulation systems, then escape from lumina into distant tissues by extravasation and finally, they form small nodules of tumor cells, *i.e.* micrometastases [202, 324, 325].

The EMT has been implicated in regulating both, tumor cell invasion and metastatic spread. This developmental regulatory process allows tumor cells to gain abilities to resist apoptosis,

invade and disseminate from the primary tumor [202, 218]. A set of transcription factors, mainly SNAI1 (SNAIL), SNAI2 (SLUG), TWIST and ZEB1/2 are responsible for orchestrating the EMT process and many of these factors have been implicated in melanoma invasion and metastasis [218]. These transcription factors are responsible for several hallmark features of invasive cancer cells including: loss of adherent junctions, changes in morphology and polarity [202].

To date, many associations have been made between SOX2 expression and tumor cell invasion (reviewed in [262]). For example, SOX2 has been implicated in cellular migration and invasion *in vitro* by regulating MMP-2 in colorectal cancer cells [326]. Likewise, the overexpression of SOX2 in a glioma cell line resulted in significant increase in migratory and invasive properties of cells [273]. This association has also been demonstrated in melanoma. A SOX2 knockdown study in the human melanoma cell line A375 showed a 4.5-fold decrease in invasive capacity [284]. Results from this current study confirm this by demonstrating that both, migratory and invasive capacities were significantly increased in the human melanoma cell line SK Mel 30 upon SOX2 overexpression (**Figure 10a & c**) In agreement, shRNA-mediated knock down of SOX2 expression in the human melanoma cell line SK Mel 103 confirms a significant decrease in invasion capacity (**Figure 11e**). Taken together, the current study confirms that SOX2 is required for melanoma cell invasion, which is in line with results from previous studies.

SOX2 expression has also been investigated in melanoma patients. In preliminary analyses SOX2 immunopositivity correlated with dermal invasion and was found in close proximity to invasive-fronts of a melanoma [282, 284]. Here, using immunohistochemistry analysis, high expression of SOX2 in primary and metastatic melanoma patient samples compared to melanocytic nevi was confirmed (**Figure 8d**). Moreover, a drastically increased immunopositivity of SOX2 was found in deeper regions of primary melanoma samples (**Figure 10e**). In line with previous studies and the functional data, high SOX2-positive cells were observed invading into the extracellular matrix (**Figure 10f**), between fat cells (**Figure S4b**), into the epidermis **(Figure S4a)** and along nerve cells (**Figure 10g**). These results further indicate an important role for SOX2 in melanoma cell invasion, not only *in vitro*, but also in melanoma patients *in vivo*.

Tumor cell invasion is mainly regulated and orchestrated by a set of transcription factors (TFs) governing the EMT process. SOX2 has been demonstrated in many cancer types to be associated with these EMT-TFs. For example, in colorectal cancer cells, SOX2 induced EMT-TFs which led to the downregulation of E-cadherin [327]. Melanoma represents a unique case in EMT-TF expression because normal melanocytes express SNAIL2 and ZEB2

and these markers activate the MITF differentiation program resulting in tumor-suppressive signaling. However, upon melanoma initiation, either by BRAF or NRAS hyperactivation, the EMT-TF network undergoes dramatic restructuring and the expression of TWIST1 and ZEB1 are favored [203]. Moreover, after this reorganization, melanoma cells are more dedifferentiated and invasion is enhanced. Results from the current study are in agreement with this work since SOX2 leads to melanoma cell dedifferentiation and is required for melanoma cell invasion (**Figure 9-10**). Furthermore, upon the overexpression of SOX2 in the human melanoma cell line SK Mel 30, an upregulation of both ZEB1 and TWIST1 expression was observed (**Figure 10b**). Additionally, in the knockdown of SOX2 in SK Mel 103 melanoma cell lines, a slight increase in SNAI2 and ZEB2 expression was demonstrated (**Figure 11f**). Taken together, this suggests that SOX2 may be involved in regulating several aspects of the melanoma invasive switch, by controlling dedifferentiation which may in part be caused *via* repressing the MITF-M promoter, being required for melanoma invasive capacity and enhancing EMT-TFs, TWIST1 and ZEB1, identified to be involved in melanoma progression.

Linking SOX2 to these aspects further implicates SOX2 in the melanoma phenotype switch. This switch describes highly proliferative cells with low invasion capacity, which is converted into highly invasive and slow proliferating cells during melanoma progression. This switch provides melanoma cells with mechanisms in progression, maintenance and evasion of melanoma therapy [304]. After investigating SOX2 in melanoma cell invasion, the effect of SOX2 overexpression on melanoma cell metabolism was investigated. Thereby, a significant decrease in melanoma cell metabolism in the SOX2 overexpressing cells was revealed (**Figure 10d**) although knockdown experiments had little effect on the metabolism (**Figure 11d**). It is important to mention that the alamarBlue® assay is a metabolism-based method and therefore one cannot rule out that metabolic changes may lead to altered reduction of the dye without true impact on the cell cycle. Further cell cycle analysis using fluorescence-activated cell sorting (FACS) is needed to reveal whether SOX2 overexpression had a cytostatic effect or induced apoptosis resulting in cellular cytotoxicity. Furthermore, one explanation why there was not a significant increase in invasion and in turn significant decrease in cellular metabolism in the SK Mel 173-OE may be explained by the lower levels SOX2 overexpression observed in this cell line compared to the high levels of SOX2 in the SK Mel 30-OE (**Figure 9b**). Therefore, the effect seen is only a slight tendency but not significant. Nonetheless, these studies suggest that SOX2 overexpression induces a highly invasive, slowly proliferative phenotype and taken together with the knockdown studies, SOX2 is required for melanoma cell invasion *in vitro*, but not cellular metabolism.

In summary, SOX2 is a key regulator in melanoma cell plasticity. This stem cell-marker dedifferentiates human melanoma cells by repressing the MITF-M promoter, is required for melanoma cell invasion and upregulates EMT-TFs related to dedifferentiation and an invasive phenotype.

6.3 SOX2 expression is induced by TGF-β signaling

The mechanisms of melanoma cell invasion and metastatic spreading are governed by several signaling pathways, mainly TGF-β and WNT/β-catenin signaling. The initiation of invasive properties is predominantly induced by the EMT process. EMT-like processes are typically initiated in epithelial precursor cells in response to extrinsic TGF-β stimulation. TGF-β has been described as a potent inducer of the EMT [328, 329] *via* SMAD-related or SMAD-independent pathways [330], and the blockade of transforming growth factor receptor-I (TGFBRI) led to the induction of a more epithelial phenotype [204]. Furthermore, in human carcinomas, characteristic features of the EMT were observed at the invasive front of the tumors, an area containing high levels of stromal TGF-β [166]. In melanoma, the common routes towards tumor cell invasion heavily involve TGF-β signaling. A reduction in colony formation, invasion and expression of MMP-2 and MMP-9 was observed after the overexpression of the inhibitory SMAD protein, SMAD7 [331]. Furthermore, studies in mice demonstrated that similar to breast cancer bone metastasis [332, 333], SMAD7 overexpression blocked melanoma bone metastasis [171].

For these reasons, an understanding of the mechanisms controlling TGF-β signaling is needed to prevent invasion and metastatic spreading. In glioma-initiating cells, TGF-β was found to induce the expression of SOX2 *in vitro*. This mechanism was confirmed in melanoma in two independent human melanoma cell lines after one week of TGF-β stimulation (**Figure 13b & e**). Moreover, a more mesenchymal phenotype was observed upon stimulation, where cells stimulated with TGF-β had long protrusions and loss of the polygonal body shape (**Figure 13a**). In accordance with this, drastic upregulation of N-cadherin expression and corresponding downregulation of E-cadherin was found (**Figure 13g**), demonstrating the key characteristics of the activation of tumor cell invasion [202].

As described in **Section 6.2.3**, melanoma cells undergo dramatic reorganization of EMT-related TFs upon tumor initiation [203]. Moreover, this reorganization operates upstream of MITF and controls dedifferentiation and melanomagenesis. The EMT-TFs ZEB1 and TWIST1 were found to induce TGF-β and, in turn, melanoma cell invasion [203]. In line with these findings, data from the current study suggest that SOX2 is playing a critical role in this process. This study proposes that TGF-β1 induces SOX2 expression, which may lead to melanoma progression by: i) SOX2 binding and repressing the MITF-M promoter, which may

influence the dedifferentiation of human melanoma cells, and ii) SOX2 inducing high expression of ZEB1 and TWIST1, which promotes a mesenchymal phenotype. In line with the described phenotypic alterations, this work revealed that SOX2 enhances melanoma cell migration and invasion and depletion of this transcription factor results in loss of cellular motility. The overall phenotype observed upon the overexpression of SOX2 matches parts of EMT-TF restructuring and the dedifferentiation phenotype in melanoma. Therefore, I identified a key player in this complex network that is responsible for several mechanisms observed in aggressive melanoma. Future experiments will solidify these finding, such as chromatin immunoprecipitation (ChIP) experiments to elucidate the possible direct regulation of EMT-TFs by SOX2 promoter binding. This has been demonstrated in pancreatic cancer, where SOX2 was demonstrated to bind to the promoter of SNAI1 [327]. Furthermore, in affinity capture mass spectrometry experiments, SOX2 was found to potentially form a protein-protein interaction with ZEB2 [334], which may be another mechanism by which SOX2 controls the restructuring of the EMT-TF network in melanoma to promote the invasive phenotype switch.

Interestingly, the results from the current study also suggest a unique role for SOX2 in regulating itself and possibly parts of the TGF-β signaling pathway. The induction of several TGF-β-related markers: TGFβR2, TGFβR3 and JUN in SK Mel 30 SOX2 overexpressing cells was demonstrated (**Figure 13h**). This suggests that SOX2 may be involved in a positive feedforward loop in regulating first, TGF-β signaling-related genes and second, itself since SOX2 expression is also induced by TGF-β. Furthermore, this has been demonstrated in pluripotency and SOX2 was found to contain SOX2 binding sites for its own autoactivation [335]. It is of great interest to understand this mechanism in more detail and further studies need to be done to confirm this, such as determining if SOX2 can bind the promoters of genes involved in TGF-β signaling using ChIP analysis.

6.4 SOX2 may form potential interactions with several melanocyte- and SUMO-related proteins

Understanding expression levels of particular gene sets gives an impression of the cellular state, activated signaling pathways and possible phenotypes. However, there are many levels of regulation that are involved in controlling cellular functions, such as protein-protein interactions. SOX2 is essential in embryogenesis and development, and there are many different partners that can associate with SOX2 (**Figure 2c**). The recent association between SOX2 expression and tumorigenesis has led to investigations attempting to identify novel SOX2-interacting partners in the context of cancer. Mass spectrometry studies identified several novel heterogeneous nuclear ribonucleoprotein family proteins, including

HNRNPA2B1, HNRNPA3, as well as other ribonucleoproteins, DNA repair proteins and helicases as potential interacting partners of SOX2 if glioblastoma [257].

Next to mass spectrometry, the bimolecular fluorescence complementation (BiFC) assay can be used to determine novel interactions between proteins. [336]. The principle of the BiFC assay is the reformation and fluorescence of two fragments of a single fluorescent protein upon interaction. Using BiFC experiments, interactions between three proteins, ERNI, BERT, and Geminin, were found to modulate the choice of repressors and regulate SOX2 expression in early neuronal development [337]. The experiments performed in the current study investigated potential interactions between SOX2 and PAX3, and between SOX2 and SOX10, respectively, using BiFC experiments. PAX3 was chosen since it is known to be important for the melanocytic lineage and responsible for co-activating the MITF promoter [56, 92, 338, 339]. Moreover, studies discovered that PAX3 interacts with SOX10 using its paired domain that binds to the HMG domain of SOX10 [92]. Therefore, it is likely that SOX2 may be using its HMG domain to form an interaction with the paired domain of PAX3.

The interactions between SOX family members remain not well studied. However, the interaction between SOX2 and SOX10 would have immense impact on further understanding the regulation between the two. Results from the current study suggest that in melanoma, SOX2 operates upstream of SOX10, since knockdown of SOX10 had no effect on the expression of SOX2 (**Figure 12**). Therefore, further investigations are needed to understand the reciprocal role and possible interactions between these two proteins and what functional effect this has *in vitro*.

Upon co-transfection of SOX2-bait and either PAX3- or SOX10-prey constructs into HEK293T cells, a positive fluorescent signals was observed when using both combinations **(Figure 14a**), with fluorescence at levels similar to the positive control. This experiment was performed three times independently with a reproducible outcome indicating true interaction between SOX2 and PAX3 or SOX10, respectively. However, these results must be confirmed using parallel BiFC analysis of proteins containing mutated interaction interfaces. It is important to note that although these studies are performed *in vitro*, the prospect of false positives is still possible since the overexpression of proteins may not represent proteins concentrations reached *in vivo*.

Yeast-2-hybrid (y2h) pairwise experiments were performed to verify these observed interactions in a different system. PAX3-SOX2 interactions were analyzed in the y2h pairwise setup with PAX6 a positive control (known interactor of SOX2) [245]. However, difficulties were observed because SOX2 showed strong autoactivation tendencies. Therefore, it cannot completely be ruled out that the effect of autoactivation in the y2h pairwise experiments

influences the results. Nonetheless, weak colony growth was observed in the PAX3/SOX2 mating at the highest stringency of 2.0 mM 3-AT, though there was great variability between replicates (column) (**Figure 14d,** bottom panels). Another level of stringency used in y2h experiments is 3-amino-1, 2, 4-triazole (3-AT). This molecule is a competitive inhibitor of the product of the HIS3 gene, reducing possible autoactivation and false positives.

Furthermore, we aimed at confirming the interactions between SOX2-SOX10 in pairwise y2h experiments; however the overexpression of both SOX2 and SOX10 proved to be lethal in yeast even at the lowest 3-AT concentrations tested (**Figure 14d**, upper panels). Therefore, possible interactions between SOX2 and SOX10 could not be confirmed in yeast (**Figure 14**).

Since the yeast-2-hybrid (y2h) pairwise experiments could not independently confirm these interactions, these experiments should be repeated using SOX2-bait construct lacking the HMG domain to remove autoactivation tendencies. The autoactivation function of the SOX2 protein has been thoroughly studied, as previously mentioned, and SOX2 itself contains SOX2 binding sites to regulate its own transcription. In the field of pluripotency this has been well described and SOX2 has been shown to autoactivate itself in a complex and well-described pluripotency circuit [335, 340].

TWIST1 is known to be implicated in cell lineage determination and the EMT process. Therefore, the interaction between SOX2 and TWIST1 would further implicate SOX2 in the regulation of the EMT process. The interaction between SOX2 and TWIST1 was found in affinity capture-mass spectrometry [261], but was not the focus of the study. This work suggests that SOX2 and TWIST1 are likely to interact but further *in vitro* studies need to confirm this. Nonetheless, y2h pairwise experiments for TWIST1 were performed but due to the strong SOX2 autoactivation, false positive results cannot be completely ruled out. Nevertheless, weak colony growth in the TWIST1/SOX2 mating setup was observed at the highest stringency of 2.0 mM 3-AT; however between replicates (column) there was great variability, due to technical problems (**Figure 14d,** bottom panels).

In conclusion, SOX2 may form potential protein-protein interactions with PAX3, SOX10 and TWIST1; however this needs to be confirmed by further protein studies, such as co-immunoprecipitation.

In addition to protein-protein interactions, cellular regulation can also occur through post-translational modifications. These modifications include protein cleavage or additional protein folding, which can allow additional co-factors to bind or complexes to form. SUMOylation is a

type of post-translational modification by which a SUMO molecule is attached by reversible covalent linkage (reviewed in [247]).

In the SOX family, SUMOylation has been shown to be important for functional diversity in the SOXE subgroup. Studies found that in *Xenopus*, both SOX9 and SOX10 can be SUMO-modified *in vivo* [248]. Non-SUMOylatable SOX9 and SOX10 promoted the expression of NC markers and the SOX9/10-SUMO-1 fusion inhibited the expression of NC markers. Moreover, neither phenotype was able to completely reproduce the wildtype (WT) phenotype, which suggests that both unmodified and SUMOylated forms of SOXE proteins contribute to the activities of WT SOXE family members *in vivo* [248, 249]. Additionally, another study observed that SUMOylation of SOX10 represses the transcriptional activity of its target genes, including MITF [250]. In addition to the SOXE group, a study found that SOX2 reduced its binding to the FGF4 enhancer element when conjugated to SUMO-1 [252].

In accordance to these studies, four SUMO-related proteins were identified that may form protein-protein interactions with SOX2 in y2h high throughput screens (HTS) (**Figure 14e**). In detail, Small ubiquitin-related modifier 1 (SUMO1), Ubiquitin-conjugating E2I (UBE2I), E3 SUMO-protein ligase (PIAS4), and chromobox homolog 4 (CBX4), which is also an E3 SUMO-protein ligase were found (**Figure 14e**). Interestingly, SOX2 was shown to bind to SUMO1 in another study using y2h HTS and bioinformatics analyses [341]. Furthermore, other hits were previously identified, including SOX2 interaction with CBX4, using affinity capture mass spectrometry [334]. These studies support results derived from the y2h HTS used in the current study and suggests that true interaction partners of SOX2 and not false-positives were identified. Validation of these hits and the function of their interaction with SOX2 remain under investigation.

In summary, this work suggests that SOX2 may be SUMOylated and this could have further functional implications. There may be important functional effects of SUMOylation on SOX2 in melanoma that remain unknown, since one study found the conjugation of SOX2 with SUMO1 decreased the transcriptional activity of the protein [252]. Furthermore, I could predict two SUMOylation sites within the SOX2 protein using GPS-SUMO online database; one located close to the HMG domain and the other one located in the transactivation domain (**Figure 14f-g**). This further supports results from the y2h HTS. Taken together, the identification of four SUMO-related proteins and identification of putative SUMOylation sites in the SOX2 protein suggests that SOX2 can be SUMOylated. Therefore, the role of SUMOylation in the context of SOX2 may be crucial in further understanding its regulation in melanocyte development and melanoma.

6.5   SOX2 in the clinic

Malignant melanoma remains a challenging disease to treat due to the acquisition of resistance and fast metastatic spread. Therefore, the identification of new markers for prognostic and therapeutic intervention would greatly benefit melanoma patients. SOX2 has proven to be clinically relevant in many different cancer types. Not only is SOX2 highly expressed in many different cancer types but it has also shown potential as a useful biomarker in the clinic for some cancer types, for the identification of cancer stem cell populations and in tumor staging (reviewed in [262]). For example, SOX2 was found to be overexpressed with SALL4, another stemness regulator, in esophageal cancer samples. Moreover, the co-expression of these markers correlated with depth of tumor invasion and occurrence of metastasis [342].

To date, only a few studies have investigated the impact of SOX2 in the clinic. Studies found that SOX2 and NESTIN expression are able to distinguish between melanocytic nevi and metastatic melanoma samples [282, 318]. Moreover, SOX2 was discovered to be present in up to 67 % of primary and 80 % of metastatic melanomas compared to only 14 % in melanocytic nevi samples [281, 284]. This work is in line with the tissue microarray (TMA) data form the current study where a significant increase in expression of SOX2 was observed in primary melanoma compared to melanocytic nevi samples (**Figure 15b**). Furthermore, bioinformatic studies investigating embryonic stem cell-associated markers in cancer analyzed mRNA expression using the Oncomine database. They revealed that the 3-year median survival was decreased by 145 days in SOX2-expressing metastatic melanoma patients compared to those patients with SOX2-negative metastatic tumors [283]. Taken together, these data illustrate the potential ability of SOX2 to become a powerful diagnostic tool in melanoma.

For the most part, therapeutic strategies targeting SOX2 remain unrealistic due to the functional nature of the protein and its importance in many normal processes. Nonetheless, recent studies have attempted to investigate SOX2 as a therapeutic target. For example, one study in oligodendroglioma, examined the use of SOX2 peptides for immunotherapy treatment in mice [343]. C57BL/6N mice were injected with WT oligodendroglioma cells from an established mouse model and, upon vaccination with SOX2 peptides, a significant delay in tumor growth was observed (reviewed in [262]). In another study, an experimental DNA vaccine against SOX2 was developed and, upon injection, found to significantly induce SOX2-specific lymphocyte activation. The vaccine was able to significantly reduce tumor growth but not prevent development in mice [344].

Though a few studies have provided experimental evidence that direct inhibition of SOX2 may have a therapeutic benefit in mice, targeting SOX2 directly remains impractical in humans, since the proper function of this protein is also important in many aspects of cellular function. Furthermore, SOX2 remains highly expressed and important in the brain and nervous system throughout adulthood and targeted therapy will likely cause many unwanted complications (reviewed in [262]). Nonetheless, targeting up- or-downstream of SOX2 may prove beneficial in anticancer therapy. In NSCLC CSCs, SOX2 is highly expressed and relies heavily on EGFR signaling in order to promote self-renewal [345]. Anticancer therapies targeting EGFR currently available on the market include gefitinib and erlotinib. These molecules may prove useful in inhibiting SOX2's role in self-renewal capacity of NSCLC CSCs. However, resistance acquisition to anti-EGFR therapies is common in cancer cells and therefore it may be useful to further target SOX2-signaling in EGFR-mutant cancer cells with PI3K/AKT inhibitors to yield better results (reviewed in [262], [345]).

My work suggests that SOX2 plays a pivotal role in the melanoma phenotype switch by dedifferentiating melanoma cells, enhancing invasion and inducing expression of EMT-TFs. Since highly invasive, slowly proliferating cells are more prone to melanoma therapy resistance, work has been done to investigate if switching these cells back to a highly proliferative, poorly invasive phenotype might be a successful anti-melanoma therapy option [322]. A directed phenotype switching method demonstrated the sensitization of melanoma cells to lineage-specific therapies [346]. Upon methotrexate (MTX) treatment, cells expressed MITF, showed differentiation-associated marker expression and inhibited invasiveness. Furthermore, after treatment with MTX, cells were sensitized to treatment with the tyrosinase-processed antifolate prodrug 3-O-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG) [346]. This strategy shows the potential of inducing a phenotype switch as an effective, tissue-specific treatment for aggressive melanoma both *in vitro* and *in vivo*. However, successful pre-clinical and clinical trials need to be performed before this therapy can be applied to patients in the clinic.

In light of these new results suggesting that SOX2 can be induced by TGF-β (**Figure 13**), several new therapeutic options are now being investigated to be utilized for the treatment of melanoma. The complexity of TGF-β signaling, as discussed above, also presents intricacies in therapy targeting TGF-β. The inhibition of TGF-β signaling through antagonists is currently under development in both pre-clinical and clinical studies (reviewed in [347]). To date, there are several options to target this pathway, including ligand traps [348], antisense olignonucleotides (ASOs) [349], peptide aptamers [350] and small molecule receptor kinase inhibitors [351]. Ligand traps, including anti-ligand neutralizing antibodies and soluble decoy receptor proteins, are able to capture excess TGF-β ligand produced by tumor cells and

fibroblasts, blocking the progression of tumor development [166, 352–354]. Similarly, ASOs can reduce the bioavailability of TGF-β ligands in the local microenvironment by directly blocking TGF-β synthesis and leading to enhanced mRNA degradation [355]. Another strategy is blocking the activity of TGF-β receptor signaling by using small kinase inhibitors that competitively block ATP catalytic activity. Moreover, additional targets include the intracellular SMAD proteins, which can be inhibited by using peptide aptamers that target, bind and interfere with SMAD downstream function (reviewed in [347]).

All TGF-β therapeutic strategies face complex and problematic overlapping mechanisms that are essential for normal physiological function (reviewed in [347]). For instance, outgrowths of drug-resistant carcinomas was observed in a mouse study after long-term suppression of the TGF-β signaling pathway using the TβRI/II kinase inhibitor, LY2109761, [356]. However, with correct dose and duration of treatment with TGF-β therapies, beneficial outcomes may follow especially in malignant melanoma.

Current pre-clinical and clinical trials using the therapeutic strategies targeting TGF-β signaling mentioned above are ongoing. Of these, the ligand traps are fully developed, with humanized pan-TGF-β monoclonal neutralizing antibodies, including Lerdelimumab [357, 358], Metelimumab [359] and GC1008 or Fresolimumab [360]. In 2014, Genzyme sponsored a clinical trial for investigating GC1008 in patients with advanced renal cell carcinoma (RCC) and malignant melanoma. 25 % of malignant melanoma patients exhibited stable disease or improvement upon receiving therapy. Of those responders, 21 % had stable disease with a median progression-free survival of 24 weeks and one patient achieved partial repsonse [360]. Another pre-clinical study investigated the kinase inhibitor of TGF-β receptor I kinase (TβRI), SD-208, and revealed a reduction in both, progression and frequency of bone metastases in mice [361]. Further clinical trials will help to gain final approval of anti-melanoma treatment using TGFβ-targeted therapy.

The benefit of targeting TGF-β may improve the therapeutic prospects of success of melanoma patients in the future. Whether or not this tumor suppressive effect, which is seen when TGF-β signaling is impeded, is partially due to the downregulation of SOX2 remains unknown and future investigations are required to solidify the role of SOX2 in TGF-β signaling and anti-melanoma therapy.

7   Conclusions

In the present study, I investigated the function of SOX2 in both, melanocyte differentiation from human induced pluripotent stem cells (hiPSCs) and melanoma progression. This work highlights the importance of this transcription factor during the differentiation from hiPSCs *via* the ectodermal lineage into fully differentiated melanocytes. It also outlines that SOX2 may regulate key processes in melanoma pathogenesis.

The ectodermal germ layer specifies different lineages such as neuronal cells, retinoid pigmented epithelial cells and melanocytes. Interestingly, upon directed ectodermal lineage differentiation from hiPSCs, expression of all pluripotency markers decreased while SOX2 remained moderately expressed, which suggests its role in this specific germ layer. Since SOX2 plays a role in the development of neurons its expression in cells derived *via* ectodermal-directed lineage differentiation is expected. However, this work proposes the investigation of early ectodermal markers in ectodermal-derived tumors, since these genes may also be reactivated during carcinogenesis. To date, melanoma research has focused on the predisposition of the lineage to malignant transformation due to its neural crest developmental program; however this work suggests that it may also be the reactivation of early ectodermal markers that are responsible for malignant behavior

In-depth functional analyses of SOX2 in melanoma pathogenesis revealed multiple facets of SOX2 function emphasizing the importance of investigating ectodermal lineage markers in melanomagenesis. Upon the overexpression of SOX2 in melanoma cells, a change of the differentiation state in human melanoma cells was observed *in vitro*, which I demonstrated, may be achieved through its binding and repressing of the MITF-M promoter. Dedifferentiation in melanoma correlates to the aggressive behavior of melanoma cells and in agreement with that, SOX2 also demonstrated to enhance melanoma cell invasion and migration functionally, in combination with upregulated epithelial-to-mesenchymal transition (EMT) marker expression. In line, its depletion led to a loss of motility and EMT-marker expression. The invasive melanoma phenotype seems to rely heavily on the upregulation of SOX2 in order to orchestrate invasion-related processes including the induction of the EMT and repression of differentiation-related markers

The regulation of SOX2 in melanoma remains unknown even though it plays a crucial role in melanoma pathogenesis. This study found additional genomic SOX2 copy numbers in primary and metastatic melanoma samples compared to melanocytic nevi, which correlated to SOX2 overexpression in tissue samples. On a molecular level, TGF-β1 was found to induce SOX2 expression in human melanoma cell lines. This suggests TGF-β to be an upstream regulator of SOX2 in melanoma, and furthermore SOX2 may play a role in a

positive feedforward loop, which regulates members of the TGF-β signaling cascade and itself. This has recently been shown in TGF-β signaling in kidney cells, where a study observed the promotion of TGF-β signaling through a positive feedback loop mediated by a microRNA [362]. It is of further interest, whether or not SOX2 is required for TGF-β-mediated invasion capacity in melanoma cells.

In addition, I identified novel specific protein-protein interactions between SOX2-PAX3 and SOX2-SOX10 and analyses of mutated protein interfaces will help to determine specific interactions sites. Additionally, *in vitro* experiments, such as co-immunoprecipitation studies will help to confirm this interaction in the context of melanoma or the melanocytic lineage.

Moreover, I revealed SUMOylation-related proteins potentially interacting with SOX2, proposing a further mechanism of SOX2 protein regulation. In depth analyses such as co-immunoprecipitation experiments are needed to investigate whether these interactions are also formed in melanoma cells and what functional effect SUMOylation has on SOX2 protein in the context of melanocyte development and melanoma pathogenesis.

This study highlights the complex networks SOX2 may be involved in during melanoma pathogenesis. Here, SOX2 is suggested to play a role in melanoma cell dedifferentiation, motility and invasion. Moreover, gene amplification may cause SOX2 overexpression in melanoma and TGF-β induces its expression in melanoma cell lines. Therefore, I identified a key player in melanoma pathogenesis that is responsible for several invasion-related processes observed in aggressive melanoma. For these reasons, targeting TGF-β or inhibiting the induction of phenotypic alterations, *i.e.* dedifferentiation, may be beneficial for indirectly impeding enhanced SOX2 expression and therefore inhibiting the progression of aggressive, metastatic melanoma. However, as the TGF-β inhibitor used in this study only showed little effect on inhibiting SOX2 expression, a combination of TGF-β inhibition, which leads to melanoma differentiation, together with conventional anti-melanoma therapy may prove beneficial for melanoma patients.

8 Supplemental material

The following data and extended experimental procedures are listed below.

8.1 Extended experimental procedures

The following experimental procedures were performed in collaborations or by others from the Utikal laboratory and are mentioned accordingly. Note these methods are only mentioned in brief.

8.1.1 Experimental procedures performed by others in the Utikal laboratory

The below procedures were performed with the help of other members in the Utikal laboratory.

8.1.1.1 Fluorescence *in situ* hybridization

FISH experiments were performed by a colleague in the Utikal laboratory, Elias Orouji, with support from our technician, Sayran Arif-Said. In brief, the CTD-2348H10 BAC clone, hybridizing to the locus 3q26.33, was used to detect human SOX2. The RP11-286G5 BAC clone, hybridizing to 3p22.3-3p22.2, was used as a reference probe purchased from Invitrogen). DNA was isolated using a MAXIprep (Qiagen) from *E. coli* containing the BAC clones. Nick translation was performed using different fluorochromes for dUTPs to prepare the reference and gene probes (Nick Translation System, Life Technologies, Catalog Number 18160-010). Probe size was determined by agarose gel electrophoresis. FISH was performed on samples using previously described protocols [320]. Control hybridizations were performed on metaphase spreads of euploid cells. To analyze the slides, only FISH signals of intact, non-overlapping nuclei (n=100) were counted. The total number of SOX2 gene copies in relation to the reference probe copy number is indicated as the SOX2-FISH ratio. Amplification of SOX2 was defined as a ratio > 1.3. This cutoff value was determined using Hothorn and Lausen method and using a package developed in the R program [296].

8.1.1.2 NC differentiation protocol from human induced pluripotent stem cells

The neural crest cell population was differentiated in a different manner than melanocytes. This protocol was established and performed by Dr. Lionel Larribere from the Utikal lab. In brief, hiPSCs were trypsinized and seeded into cell culture dishes coated with Matrigel® and cultured in mTeSR™ stem cell medium with Rock inhibitor. The day after the seeding, the medium was changed to differentiation medium supplemented with cytokines (BMP4, ascorbic acid, hSCF, EDN3 and WNT3A). Cells were cultured in the above medium for 11 days and sorted by fluorescence-activated cell sorting (FACS) using HNK1 and p75 expression.

8.1.2 Extended experimental procedures performed in collaborations

The following procedures were performed in collaboration with other groups or core facilities at the DKFZ.

8.1.2.1 Electron microscopy

Electron microscopy was performed in collaboration with the DKFZ Microscopy Core Facility. Cells were seeded on ACLAR® cover slips and cultured for one week before submission to the Microscopy Core Facility, where they were fixed, sliced and imaged.

8.1.2.2 Yeast-2-Hybrid experiments

Yeast two-hybrid (y2h) experiments were performed in collaboration with the DKFZ Proteomics Core Facility. Constructs were provided by the core facility or cloned in our laboratory with the help of Dr. Daniel Novak. In brief, yeast were transformed with bait and prey vectors of our choice. Bait- and prey-transformed yeast were mated and successful diploids were nutritionally selected on medium lacking tryptophan and leucine. The y2h high-throughput screen was performed similarly to aforementioned experiments, expect that only the bait construct was needed and the prey constructs were provided in selected cDNA libraries. Positive matings were detected *via* fluorescence. These signals were further analyzed by PCR and sequencing to determine potential new interacting partners.

8.1.2.3 Bimolecular fluorescence complementation assay

The bimolecular fluorescence complementation (BiFC) assay was performed in collaboration with the DKFZ Proteomics Core Facility. In brief, HEK293T cells were seeded in 96-well plate format and transfected with both bait and prey constructs containing the gene of interest plus a partially fluorescent fluorophore. Upon transfection, cells were visualized and imaged under fluorescence microscope.

8.1.2.4 Luciferase reporter assay

Luciferase reporter assay was performed in the lab of Dr. Corine Bertolotto at the INSERM institute in Nice, France. Briefly, The luciferase reporter plasmids pMITF and the expression vector encoding the wild-type or the dominant negative form of MITF were previously described [363]. The SK Mel 30 SOX2-overexpressing cells and non-induced control were seeded in 24-well dishes and transient transfections were performed the following day using 2 µL of lipofectamine and 0.5 g of total DNA plasmid. pCMV Gal was transfected with the test plasmids to control the variability in transfection efficiency. After 48 h, cells were harvested in 50 µL of lysis buffer and assayed for luciferase and galactosidase activities. All transfections were repeated at least three times and performed in triplicate [300].

8.2 Supplemental figures

The following figures are supplemental to this doctoral work.

a

b
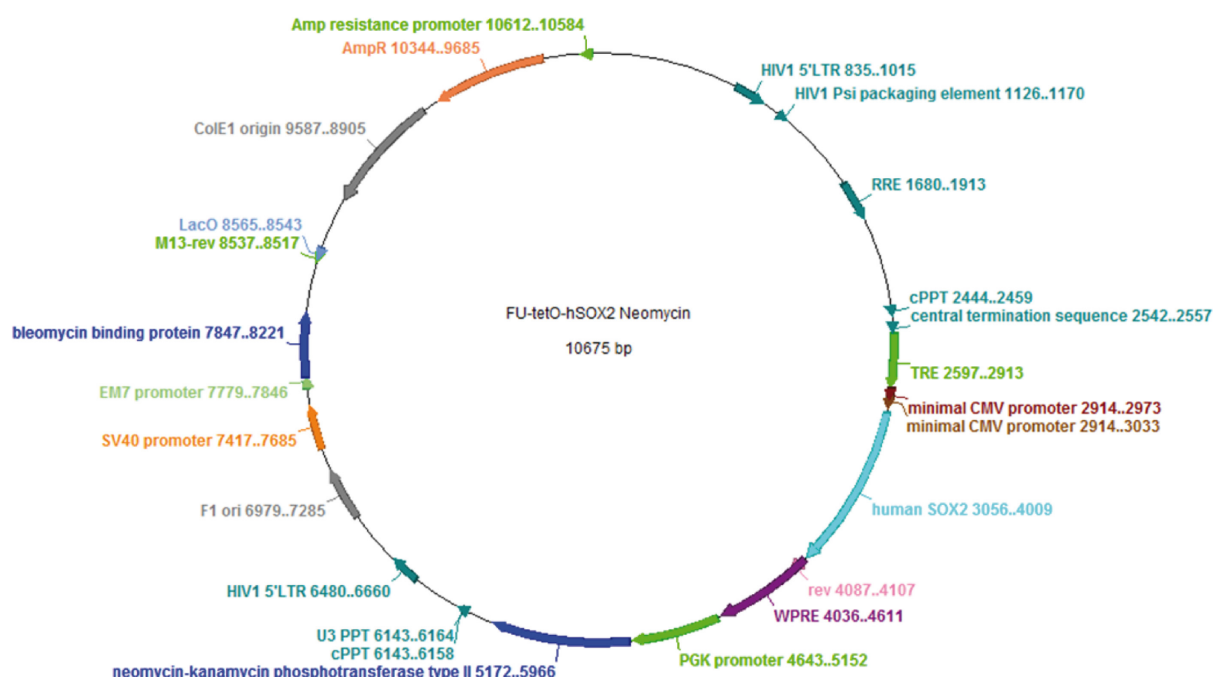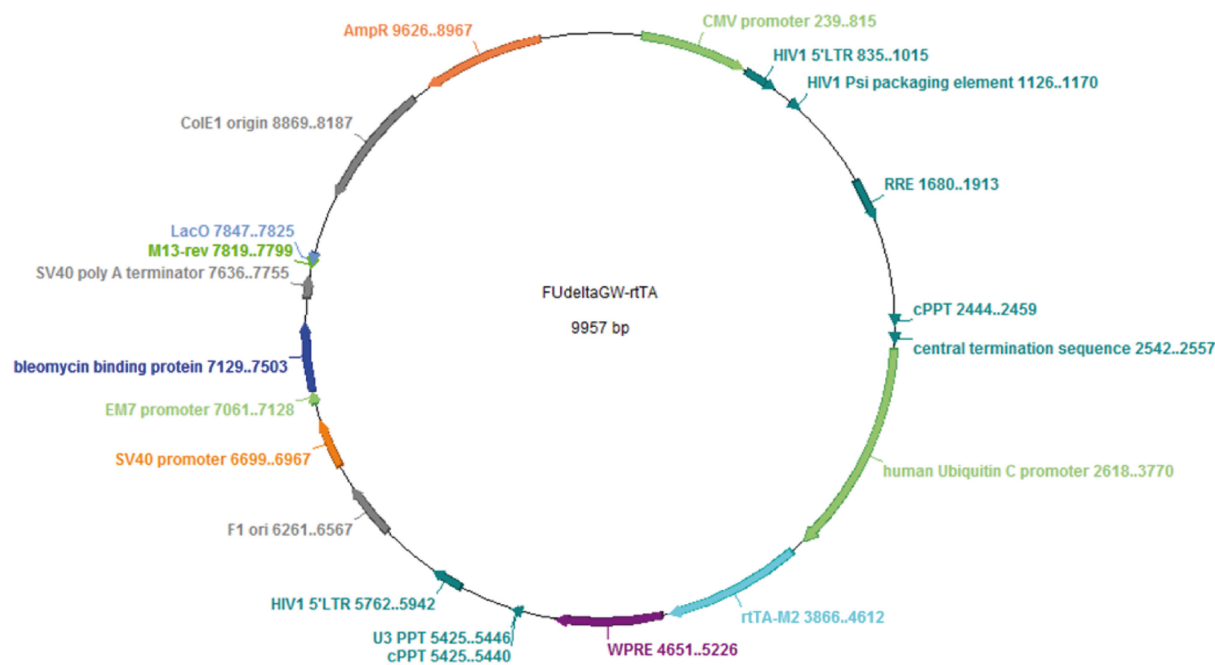
Supplemental figure S1 **Vector maps of FU-Tet-O human SOX2 and M2-rtTA**

a) Inducible FU-Tet-O human SOX2 construct selectable with G418 cloned in our laboratory by Dr. Daniel Novak. **b)** Lentiviral vector encoding the M2-rt-TA reverse tetracycline transactivator (kindly provided by K. Hochedlinger).

a

Negative control
(epidermis)

IHC Score 0   IHC Score 1

Negative control
(primary melanoma)

IHC Score 2   IHC Score 3

b

Percentage positive cells	Score
0	0
1-25 %	1
26- 50 %	2
51-75 %	3
76-100 %	4
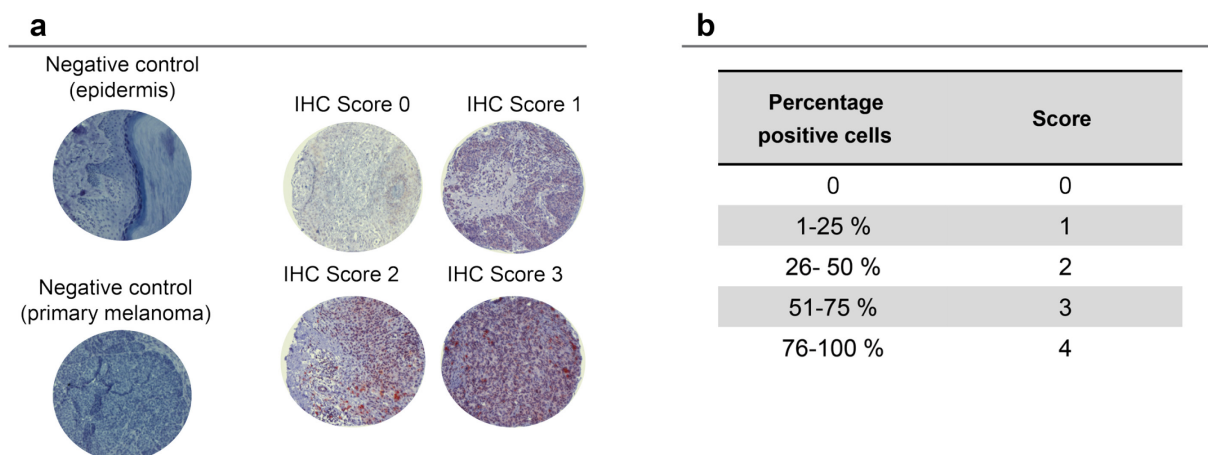
Supplemental figure S2   **Tissue microarray analysis and scoring**

Immunohistochemistry scores were calculated by multiplying intensity and quantity scores. **a)** Intensity score of SOX2-staining. Shown are samples representing different stainings used to score the intensity of SOX2 apperance in different patient samples. Negative controls displayed in left panel lack primary antibody against SOX2, different staining intensities and corresponding scores are displayed in right panel. **b)** Table showing the defined quantity scoring.

A375   2° control   SK Mel 30   2° control

No Treatment
SMAD3 / DAPI

SB431542
SMAD3 / DAPI
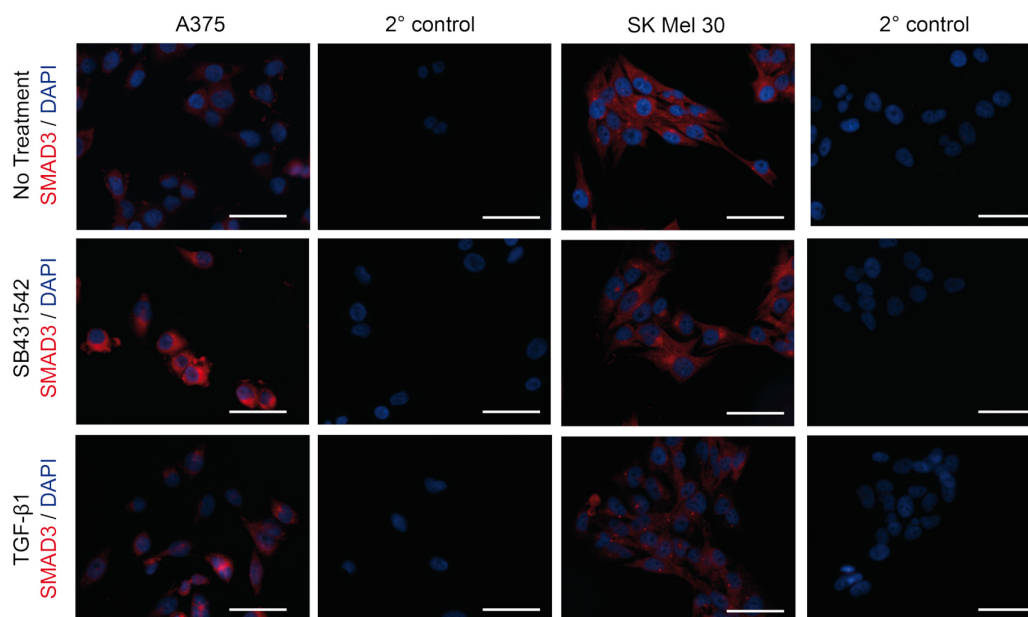
TGF-β1
SMAD3 / DAPI

Supplemental figure S3   **Immunofluorescence analysis of SMAD3 in A375 and SK Mel 30 human melanoma cell lines**

Displayed are IF images of anti-SMAD3 (Cy3, red) in cells treated with TGF-β1, SB431542 and without treatment (+0.1% DMSO) in A375 and SK Mel 30 cell lines. Nuclei were counterstained using DAPI, bar=50 µm.

IF=immunofluorescence; DAPI=4',6-diamidino-2-phenylindole

a

b
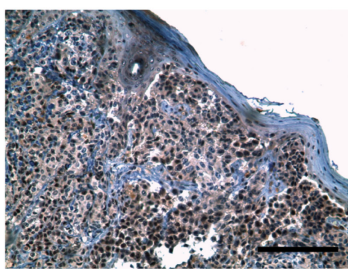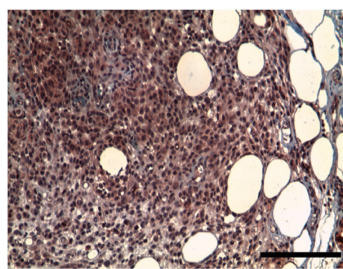
Supplemental figure S4   **Immunohistochemistry analysis of SOX2 in invasive melanoma tissue**

a) IHC analysis against SOX2 in primary melanoma samples shows epidermal invasion of melanoma cells, bar=500 μm. **b)** IHC analysis against SOX2 in primary melanoma samples shows invading melanoma cells between fat cells, bar=500 μm.

IHC=immunohistochemistry

a

b
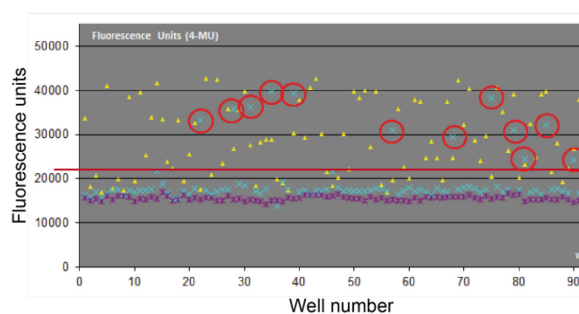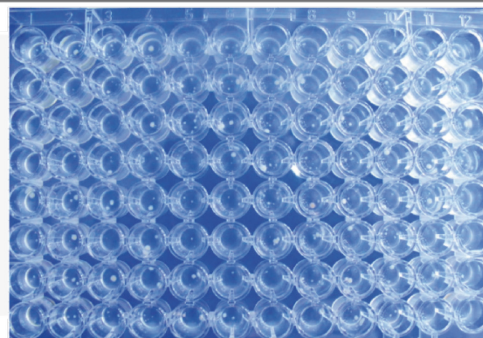
Supplemental figure S5   **Yeast-2-Hybrid high throughput screen bait validation**

A y2h HTS was performed in collaboration with the DKFZ proteomics core facility. An initial experiment was performed to test if the bait (SOX2) was suitable for the y2H HTS format. This was examined by increasing the stringency *via* the addition of higher concentrations of 3-AT. The red line indicates an example of the threshold that would be set in the HTS and the red circled hits are examples of which hits would be further analyzed for potential interaction partners of SOX2. Note the output of the HTS is fluorescence. **b)** Example image of a microtiter plate after yeast have successfully mated (white dots).

y2h=yeast-2-hybrid; HTS=high throughput screen; 3-AT=3-Amino-1,2,4-triazole

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