Tumour-Suppressive Roles of Transformation Growth Factor-beta in Human Cutaneous Melanoma

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List of Abbreviations

Abbreviation	Meaning
ABC	ATP-binding cassette
ALDH	Aldehyde dehydrogenase
ALK	Activin-like-kinase
ALM	Acral lentiginous melanoma
ANGPTL4	Angiopoietin-like 4
AP1	Activator protein 1
APAF1	Apoptosis activating factor-1
ARTS	Apoptosis-related protein in the TGFβ signalling pathway
ATF-3	Activating transcription factor-3
ATP	Adenosine triphosphate
AXUD1	Axin upregulated protein 1
bHLH	Basic-helix-loop-helix
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
CAF	Cancer-associated fibroblast
CBP	CREB binding protein
CD133	Cluster of differentiation 133
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
COX2	Cyclooxygenase-2
CRISPR	Clustered regularly interspaced short palindromic repeats
CSC	Cancer stem cell
CTGF	Connective tissue growth factor
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DAB2	Disabled homolog 2
DAPI	4,6-diamidino-2- phenylindole
DAPK	Death-associated protein kinase
DMEM	Dulbecco's modified Eagle medium

DMFS	Distant metastasis-free survival
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E-CADHERIN	Epithelial cadherin
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epithelial growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetra-acetic acid
EMT	Epithelial-mesenchymal transition
EMU	Epidermal melanin unit
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
G1	Gap 1 phase
G2	Gap 2 phase
GAP	GTPase-activated protein
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
GP130	Glycoprotein 130
GRK2	G protein-coupled receptor kinase 2
GSK3-β	Glycogen synthase kinase-3 β
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
hTERT	Human telomerase reverse transcriptase
ID	Inhibitor of DNA binding
IL	Interleukin
INF	Interferon
JAK	Janus kinase
JNK	C-Jun N-terminal kinase
LAP	Latency-associated peptide
LIF	Leukemia inhibitory factor

LMM	Lentigo maligna melanoma
LTBP	Latent TGFβ-binding protein
M PHASE	Mitosis phase
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin receptor 1
MEN1	Multiple endocrine neoplasia 1
MH	MAD homology
MIA	Melanoma inhibitory activity
MITF	Microphthalmia-associated transcription factor
M-MLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinases
MSC	Melanoma stem cell
mTOR	Mammalian target of rapamycin
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
N-CADHERIN	Neural-cadherin
NF-κB	Nuclear factor κb
NM	Nodular melanoma
NCSC	Neural crest stem cell
ONPG	Ortho-nitrophenyl-β-galactoside
OSM	Oncostatin M
PAI-1	Plasminogen activator inhibitor 1
PAR	Parental
PARP-1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate buffer saline
PCAF	P300/CBP-associated factor
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3- kinase
pRb	Retinoblastoma tumour suppressive protein;
PTEN	Phosphatase and tensin homolog
PTHRP	Parathyroid hormone-related protein

RANKL	Receptor activator of nuclear factor kappa-B ligand
Rb	Retinoblastoma protein
RGP	Radial growth phase
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell park memorial institute medium
R-SMAD	Receptor-regulated Smad
RT-PCR	Reverse transcription polymerase chain reaction
RUNX2	Runt-related transcription factor 2
RXR	Retinoid X receptor
S PHASE	Synthesis phase
S6K	Ribosomal protein S6 kinase
SAPK	Stress-activated protein kinase
SBE	Smad binding element
SCR	Scrambled
SDF-1	Stroma-derived factor-1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHIP	Src homology 2 domain-containing 5’ inositol phosphatase
siRNA	Small interfering RNA
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
SKI	Sloan-Kettering Institute proto-oncogene
SNON	Ski-related novel gene
SSM	Superficial spreading melanoma
STAT	Signal transducer and activator of transcription
TAK1	TGFβ-activated kinase 1
TGFβ	Transforming growth factor β
TIEG1	TGFβ-inducible early-response gene
TNF-α	Tumour necrosis factor α

TNM	Tumour-Node-Metastasis
TβRI	Type I TGFβ receptor
TβRII	Type II TGFβ receptor
uPA	Urokinase-type plasminogen activator
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase
αMSH	α-melanocyte-stimulating hormone
βME	β-mercaptoethanol

Abstract

Cutaneous melanoma is the malignant transformation of melanocytes whose incidence is the most rapidly growing in the last decades. Localized tumours could be cured through surgical excision, nonetheless, late-stage metastatic melanomas disseminating to the lymph nodes and visceral tissues show a poor prognosis. Infamous for their chemotherapeutic resistance and frequent recurrence, metastatic melanomas are the most lethal of all skin cancers. Understanding the intricate molecular mechanisms underlying the tumorigenesis, progression, and metastasis of melanoma is crucial for designing new targeted therapies. Melanomas harbour mutations in different genes that involve tumour development and progression and are comprised of heterogeneous cells including cancer stem cells that participate in the recurrence of the malignancy and its resistance to chemotherapy impacting relapse-free survival and remission of melanoma patients.

The Transforming Growth Factor beta (TGFβ) plays an important role in regulating numerous biological processes in normal or malignant tissues. The current work shows that TGFβ/Smad3 signalling mediates multifactorial tumour-suppressive effects in human cutaneous melanoma. The present work shows how TGFβ, via preferential activation of Smad3, upregulates the Axin Upregulated Protein 1 (AXUD1) as well as the multiple endocrine neoplasia type 1 protein (menin) both of which being required for the TGFβ-mediated tumour-suppressive effects in melanoma. Results show that each of the TGFβ/Smad3/AXUD1 and TGFβ/Smad3/Menin signalling cascades promoted cell cycle arrest, growth inhibition, apoptosis and autophagy, in addition to suppressing cell immortalization, cell migration *in vitro* as well as inhibiting tumour formation and tumour metastasis *in vivo*. Moreover, TGFβ/Smad3 could inhibit melanosphere formation in numerous cutaneous melanoma cell lines as well as reducing $ALDH^+$ (and possibly $CD133^+$) melanoma stem-cell-like subpopulations (MSCs) in DAUV melanoma cells. Besides, each of AXUD1 and menin could mediate this TGFβ-induced inhibitory effect on MSCs. Collectively, the work in this thesis presents a) the TGFβ/Smad3/AXUD1 and b) TGFβ/Smad3/Menin axes as new potent suppressive signalling pathways of melanoma tumorigenesis and metastasis, as well as provide original evidence for TGFβ/Smad3 signalling being a suppressor of MSCs. This work portrays TGFβ/Smad3 signalling as a significant multifaceted tumour-suppressor in human cutaneous melanoma, highlighting the importance of using TGFβ mimics as novel therapeutic approaches for treating melanoma patients as well as highlighting the value of TGFβ serum levels as a useful

clinical diagnostic and prognostic tool in melanoma patients to determine chemotherapy-responsiveness and better overall survival

Résumé

Le mélanome de la peau est la transformation maligne des mélanocytes, dont la croissance a augmenté le plus rapidement au cours des dernières décennies. L'exérèse chirurgicale permet de guérir les tumeurs localisées. Les mélanomes métastatiques à un stade avancé se propagent dans les ganglions lymphatiques et les tissus viscéraux avec un mauvais pronostic. En raison de leur résistance à la chimiothérapie et leur récurrence fréquente, les mélanomes métastatiques, sont les cancers de la peau les plus meurtriers. Notre compréhension des mécanismes moléculaires complexes causant la tumorigenèse, la progression et des métastases du mélanome est essentielle à la conception de nouvelles thérapies ciblées. Les mélanomes comprennent des mutations dans différents gènes impliquant le développement ainsi que la progression de la tumeur, et des cellules hétérogènes, notamment des cellules souches cancéreuses, qui participent à la récurrence de la tumeur maligne et à sa résistance à la chimiothérapie qui contribuent à la survie sans récidive des patients atteints de mélanome.

Le facteur de croissance transformant de type béta (TGFβ) joue un rôle important dans la régulation de nombreux processus biologiques dans les tissus normaux ou malins. Cette étude démontre que la signalisation TGFβ/Smad3 induit des effets suppresseurs multifactoriels sur le mélanome de la peau chez les humains. TGFβ, via l'activation préférentielle de Smad3, augmente Axin Upregulated Protein 1 (AXUD1) ainsi que Multiple Endocrine Neoplasia type 1 (menin) tous deux étant requis pour les effets suppresseurs de tumeur médiés par TGFβ dans le mélanome étudié. Ce travail prouve que chacune des cascades de signalisation TGFβ/Smad3/AXUD1 et TGFβ/Smad3/menin ont favorisé l'arrêt du cycle cellulaire, l'inhibition de la croissance, l'apoptose et l'autophagie, ainsi que la suppression de l'immortalisation cellulaire, la migration cellulaire *in vitro* ainsi que la formation de tumeurs et les métastases tumorales *in vivo*. En plus, TGFβ/Smad3 a inhibé la formation de mélanosphères dans de nombreuses lignées cellulaires de mélanome ainsi qu'il a réduit les sous-populations des cellules souches du mélanome (CSMs) $ALDH^+$ (et possiblement $CD133^+$). D'autant plus les résultats montrent que AXUD1 et menin sont nécessaire à l'inhibition causée par le TGFβ. Collectivement, le travail dans cette thèse présentent les axes a) TGFβ/Smad3/AXUD1 et b) TGFβ/Smad3/menin comme nouvelles voies de signalisation qui médiatisent des effets multifactoriels suppressives de la tumorigenèse et de la métastase du mélanome, ainsi que fournissent une preuve originale pour la signalisation TGFβ/Smad3 en tant que suppresseur des CSMs. Le travail ici décrit la signalisation TGFβ/Smad3

comme un important suppresseur de tumeurs dans le mélanome de la peau chez les humains, soulignant l'importance de l'utilisation de mimétiques du TGFβ comme nouvelle approche thérapeutique pour les patients atteints de mélanome, ainsi que la mise en évidence de la valeur des taux sériques de TGFβ en tant qu'outil de diagnostic et de pronostic utile chez les patients atteints de mélanome pour déterminer la réponse à la chimiothérapie et la meilleure survie.

Preface

Contribution of Author

The thesis on hand is original work conducted entirely by me and kindly reviewed by my supervisor Dr. Jean-Jacques Lebrun. This thesis is comprised of the following four chapters:

Chapter 1 is an introductory literature review of cutaneous melanoma, the role of TGFβ signalling in tumorigenesis, melanoma stem cells (MSCs), and Axin Upregulated Protein 1 (AXUD1) and Multiple Endocrine Neoplasia Protein1 (menin) and their known roles in cancer.

Chapter 2 describes the procedures and methods used in all experiments as well as all the reagents and equipment used.

Chapter 3 describes in the first part the role of TGFβ/Smad3/AXUD1 as well as TGFβ/Smad3/menin signalling in mediating various tumour-suppressive and antimetastatic effects in the DAUV human cutaneous melanoma. In this chapter, I designed, conducted, analyzed all experiments and drafted the chapter under my supervisor's guidance. Julien Boudreault generated the AXUD1 stable knockout DAUV melanoma cell line and performed the Surveyor nuclease assay and Halema Haiub performed the immunohistochemistry experiment. For the *in vivo* experiments, Dr. Ni Wang injected the mice, monitored tumour growth, sacrificed the mice, and collected the organs, and counted the secondary metastatic nodules in lungs and livers with my assistance, and I cultured the various scrambled, and knockout cell lines, prepared them for injection; and measured, photographed, fixed and stained the harvested tumours and organs. I analyzed the data, conducted the statistical analysis and prepared the figures.

Chapter 3 describes in the second part the role of TGFβ /Smad3 signalling in inhibiting melanoma stem cells, and how AXUD1 and menin mediate this inhibition process. In this part, I designed, conducted, analyzed all experiments and drafted the chapter under my supervisor's guidance.

Chapter 4 is an integrative discussion of the results and their impact on the current knowledge and therapeutic research, together with the novelties and the limitations of the presented work.

During my Ph.D. studies, I was the first co-author of the following study under Dr. Jean-Jacques Lebrun's supervision: "The leukemia factor and cyclin-dependent kinase inhibitor p21 mediate the TGFβ tumour suppressive effects in human cutaneous melanoma" by Humbert L*, Ghozlan M*, Canaff L, Tian J, Lebrun JJ. Published in BMC cancer, 2015 Mar 29; 15:200

Contribution to original knowledge

In this thesis, I demonstrated -for the first time- that AXUD1, as well as menin, are required for TGFβ-mediated multifactorial tumour-suppressive effects in DAUV human cutaneous melanoma cells, where silencing AXUD1 or menin via CRISPR/Cas9 abolished the TGFβ-induced induction of cell cycle arrest, apoptosis, and autophagy as well as the inhibition of cell immortalization and cell migration *in vitro*. Also, silencing AXUD1 or menin abrogated the TGFβ-induced inhibition of primary tumour formation and secondary tumour metastasis *in vivo*. Also, I showed that these TGFβ-induced effects are mediated through the preferential activation of Smad3 downstream of TGFβ.

Moreover, I demonstrated -for the first time- how TGFβ via preferential activation of Smad3 can suppress melanoma stem cells (MSCs) by inhibiting melanosphere formation in numerous cutaneous melanoma cell lines as well as reducing $ALDH^+$ (and possibly $CD133^+$) MSC subpopulations *in vitro*. Also, I showed that AXUD1 and menin are necessary to mediate these TGFβ-induced inhibitory effects in MSCs.

In summary, this work provides new evidence for TGFβ as a multifaceted tumour-suppressive and antimetastatic factor thus highlighting the value of TGFβ mimics to be considered in patients with metastatic cutaneous melanoma to attain better therapeutic responses and higher survival outcomes. Moreover, it highlights the clinical value of TGFβ serum levels as a useful diagnostic and prognostic marker in melanoma patients to determine chemotherapy-responsiveness and improved overall survival. Globally, the work in the thesis provides unignorable responses to questions about the role of TGFβ in human cutaneous melanoma tumorigenesis, questions that have limited and controversial answers.

Acknowledgments

The work in this dissertation would not have been possible without the guidance, the help, and the support of many individuals who by various means contributed and extended their valuable assistance in the completion of this thesis. It is to them I owe my profound gratitude.

First and foremost, to Dr. Jean-Jacques Lebrun, who undertook to be my supervisor, for his patience, his continual guidance, and enthusiasm that enabled me to develop an understanding of the subject and for providing me with an excellent environment to conduct my research.

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To our Division of Experimental Medicine, especially Dr. Elizabeth Fixman, our associate graduate program director; and Sandra Morrison, our student affairs administrator. To our Cancer Research Program team, especially Veronica Atehortua for her constant help and support and for making my life easier

To all my friends, particularly Philippe Duquette, Dr. Mina Youssef, Dr. Taghreed Ayash, and Kosta Malaperdas; to all my extended family members, especially Renée and Gilbert Nehma, Yvette and Ljubomir Teofilovic; and all those who supported and encouraged me in any respect during the completion of this research.

To my tremendously loving parents Dr. Hanaa Eid, and Dr. Mohamed Ghozlan; to my adorable brothers Dr. Hassan Ghozlan and Osama Ghozlan, for their continual unconditional love and support and their undivided devotion and commitment.

To Youssef Bacoura, for his unfaltering support in every possible means, and his encouragement every single day of my Ph.D. journey and

Above most, to God Almighty, for giving me the privilege of conducting this research and offer my humble contribution to the scientific domain.

Dedication

I heartily would like to dedicate this thesis to:

My utterly loving mom, Dr. Hanaa Eid,

and to the soul of my amazingly caring dad, Dr. Mohamed Ghozlan (June 2020),

who kept my spirits up when the muses failed me, for their continual unconditional love and support and their undivided devotion and commitment.

My beloved brothers, Dr. Hassan Ghozlan and Osama Ghozlan.

My late uncle, Moataz Eid (June 2020).

My late friend, Dr. Dina Said (May 2018).

Halema Haiub, who was my companion during five years of my Ph.D. journey, for her help that was not only confined to experimental work but also extended to encouraging and motivating me all the time, especially on those late nights in the lab.

Youssef Bacoura, to whom my words will not be enough to express my gratitude, for his unconditional encouragement and support every single minute of my entire Ph.D. journey, from beginning to end.

Epigraph

“And of knowledge, ye have been vouchsafed but little”

Quran [17:85]

Chapter One: **Introduction**

1.1 Melanogenesis

Melanogenesis is the biosynthetic process of the dark brown or black pigment melanin (particularly eumelanin) occurring in specialized neural-crest-derived cells, known as the melanocytes [1, 2] (Figure 1-1, Figure 1-2). In the basal layer of the human epidermis, melanocytes exist with basal keratinocytes in a constant ratio of 1:5 [3] under normal physiological conditions. There is a symbiotic relationship between one melanocyte and 36 associated keratinocytes [4-6], whereby together they form the epidermal melanin unit (EMU) which is preserved through a homeostatic balance maintained by the tightly-regulated division of melanocytes [7] as well as various intercellular communication such as endocrine and paracrine signalling, direct cell-to-cell interaction, or gap junctional intercellular communication [8, 9]. The dysregulation of the EMU homeostasis through disrupted intercellular communication leads to uninhibited melanocytic proliferation which, in turn, results in a type of neoplasm known as cutaneous melanoma [10, 11] (Figure 1-3).

When ultraviolet radiations (UVR) penetrate the skin, DNA dipyrimidine photoproducts result from the damaged DNA, thus initiating melanin biosynthesis [12]. This process starts by keratinocytes secreting the α-melanocyte-stimulating hormone (αMSH) which further activates its melanocortin 1 receptor (MC1R) in the melanocytes. This results in a subsequent stimulation of the expression of microphthalmia-associated transcription factor (MITF) [13-17]. The activity of MITF, being a master regulator of melanocyte function and melanogenesis, contributes to the regulation of multiple genes that control several crucial processes of melanogenesis such as melanocyte survival and motility as well as melanosome synthesis and translocation [16, 17]. Melanocytes synthesize melanin, which is packaged in melanosomes (melanin-containing vesicles) to be transferred through the melanocytic dendrites to the keratinocytes residing in the uppermost layer of the epidermis. This translocation allows the melanosomes to form a nuclear cap serving as a shield that efficiently absorbs and dissipates almost 100% of the carcinogenic UVR, thus ultimately protects the cellular DNA from damage and mutation [18-20] (Figure 1-4, Figure 1-5).

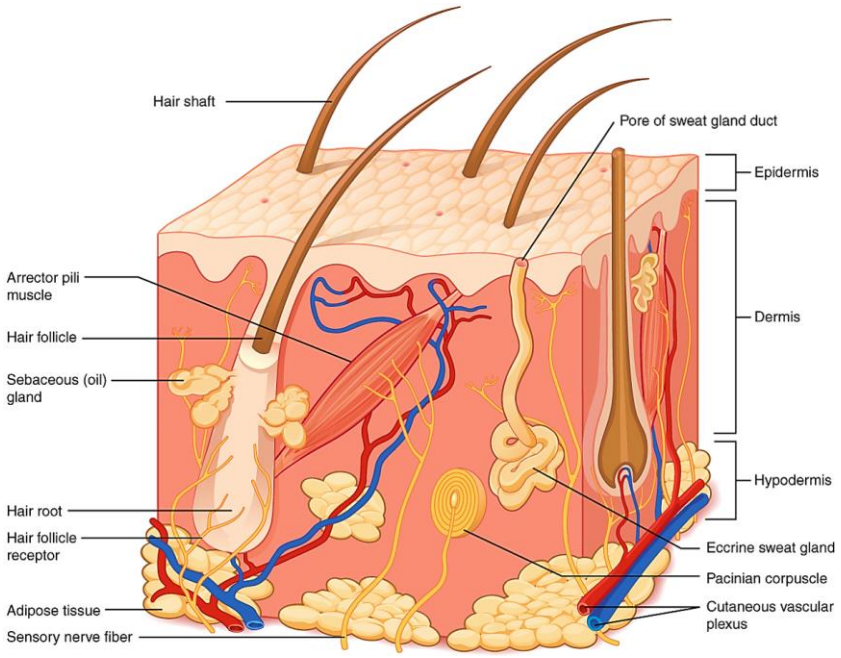

Figure 1-1: Structure of the skin.

The human skin consists of three main layers: (i) the epidermis, (ii) the dermis, and the (iii) the hypodermis (i) The epidermis is the uppermost layer, is composed of keratinized, stratified squamous epithelial cells, (ii) the dermis, which lies beneath the epidermis, is composed connective tissue containing blood and lymph vessels, nerves as well as other structures such as hair follicles and sweat glands, and (iii) the hypodermis, which lies beneath the dermis, is composed of well-vascularized, loose connective and fatty tissue. (Reproduced from Biology for Majors II, Module 24: The Integumentary System, Structure and Function of Skin, Provided by Lumen Learning, License: Creative Commons Attribution 4.0).

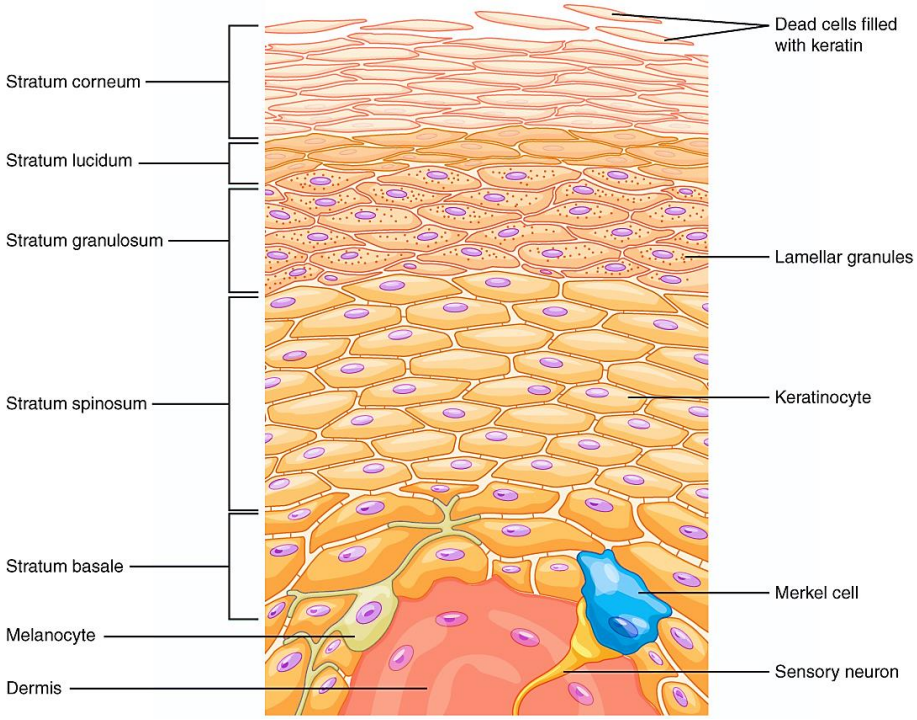

Figure 1-2: Structure of the epidermis.

The epidermis is principally comprised of keratinocytes in different stages of differentiation. It is composed of four strata, namely, i) the stratum basale, comprising the proliferating, undifferentiated basal keratinocytes; ii) the stratum spinosum and ii) the stratum granulosum, comprising the differentiating keratinocytes; and finally, iv) the stratum corneum, comprising the terminally differentiated corneocytes. (Reproduced from Biology for Majors II, Module 24: The Integumentary System, Structure and Function of Skin, Provided by Lumen Learning, License: Creative Commons Attribution 4.0).

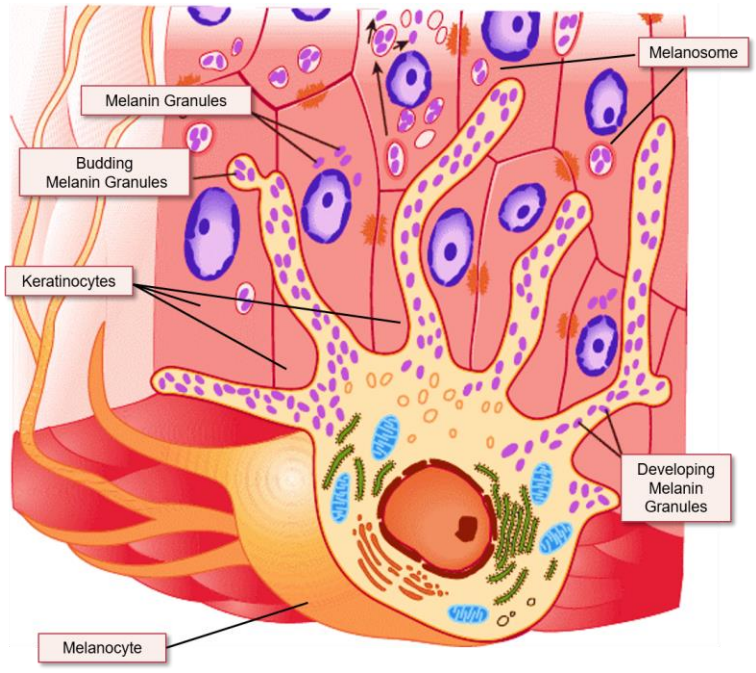

Figure 1-3: Epidermal melanin unit structure.

Melanocytes are present in the epidermal layer of the skin. During melanogenesis, the melanin pigment is packaged in the melanosomes, further translocated through the melanocytic dendrites to the uppermost layer of the skin to form a nuclear cap to protect the skin against UV radiations that could cause DNA damage. (Adapted from Diseases of the Skin, Pathophysiology of Disease: An Introduction to Clinical Medicine, 7th Edition, M. Meier, MD; Timothy H. McCalmont, MD after the model described by Fitzpatrick and Breathnach) [5].

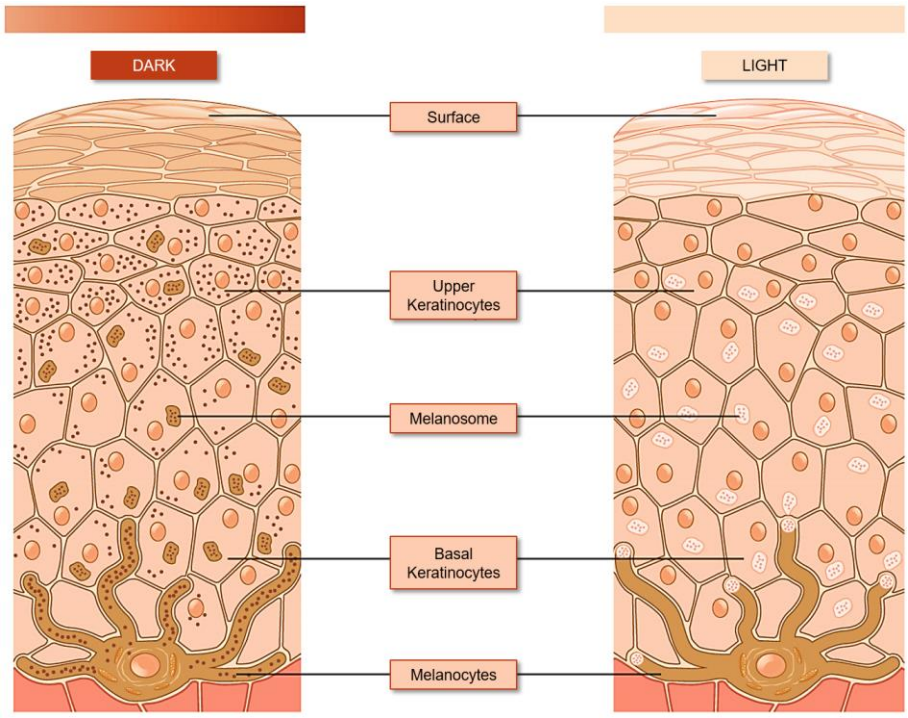

Figure 1-4: Skin pigmentation.

Skin pigmentation depends on the quantity of melanin produced by melanocytes in the stratum basale and taken up by keratinocytes (Adapted from Anatomy and Physiology-Creative Common Attribution-Rice University).

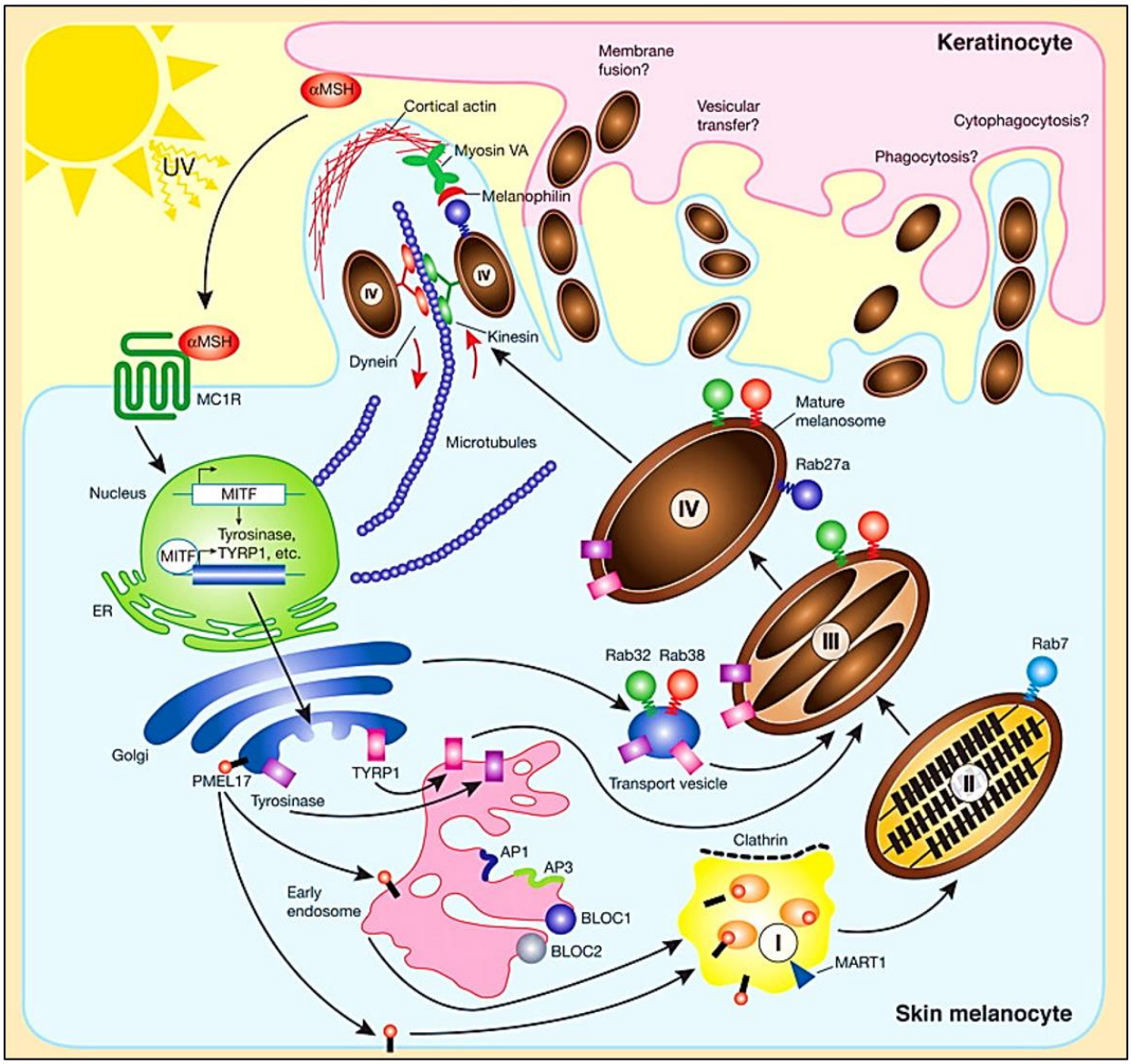

Figure 1-5: Melanogenesis.

Skin melanocytes were shown to have four morphologically-distinct stages of melanosome development as revealed by electron microscopy studies. At Stage I, pre-melanosomes are non-pigmented vacuoles that are derived from the endosomal system. At Stage II they develop characteristic internal striations. At Stage III, melanin pigment is deposited onto the striations. At Stage IV, the mature, fully melanized melanosomes arise [21] (Reproduced from Wasmeier *et al.* 2008 [22]).

1.2 Cutaneous Melanoma

Cutaneous melanoma is a type of skin cancer that originates from the malignant transformation of the melanocytes which are the pigment-producing cells located in the skin.

1.2.1 Epidemiology

The incidence of cutaneous melanoma has been rapidly increasing in most developed countries over the last few decades more than other types of cancer [23-26], displaying significantly increased mortality rates [27] despite the relative improvement in patient overall survival rates due to earlier diagnosis [28]. In 2018, cutaneous melanoma ranked globally as the twentieth most common cancer, with almost 287,723 newly-diagnosed patients and 60,712 deaths constituting 1.6% and 0.6 % of total global cancer incidence and mortality respectively [29]. In 2017, melanoma ranked as the seventh most common cancer in Canada, with about 7,200 new cases out of the 206,200 new cases of cancers and 1250 expected deaths [30]. Globally, melanoma is one of the most prevalent malignancies among young adults ageing between 20 to 35 years causing cancer mortality [31]. During their lifetime, females have a lower tendency to develop melanoma (1.5% vs. 1.2%). Noteworthy, before the age of forty melanoma incidence is higher in females, but it decreases after forty [32]. Melanoma is more common in ethnicities with lighter complexions where black people have an incidence of only 4% of that of Caucasians [33, 34]. Albeit, the black populations are more prone to develop metastatic melanoma, hence chemotherapeutic resistance and higher mortality rates [30]. Thanks to increased public awareness and early detection of benign nevi, melanoma mortality rates did not grow despite the increase of melanoma incidence [32].

1.2.2 Types

Conventionally, cutaneous melanoma is classified according to a) clinical parameters including colour, outline, nevus morphology, anatomic site, and sun exposure as well as b) histological parameters including the characterization of the intraepidermal and intradermal melanocytic proliferation [33, 35, 36] (Figure 1-6).

- Superficial spreading melanoma (SSM): accounts for about 70% of melanomas, occurring mostly on the trunk. The skin lesion is flat and asymmetric with irregular pigmentation and border [37].
- Nodular melanoma (NM): accounts for about 15% of melanomas, occurring on the trunk, head, and neck. The skin lesion grows more rapidly in thickness than in diameter. NM could arise in an existing mole or a new site. NM could be pigmented, low pigmented or unpigmented. [38].
- Lentigo malignant melanoma (LMM): accounts for 13% of melanomas, occurring in fair-skinned older individuals upon chronic sun exposure. The skin lesion is usually 3 to 6 cm or more, with a nodular area from 1mm to 2cm in width [39].
- Acral lentiginous melanoma (ALM): is a relatively uncommon subtype, occurring mainly on the nail beds, palms, and soles. The skin lesion is characterized by a flat tan, grey-brown or black macule with colour variations and irregular borders [40].

Among the other, less frequent (around 5% of melanomas) types are amelanotic, desmoplastic, verrucous, polypoid melanoma, minimal-deviation melanoma or blue nevus [41].

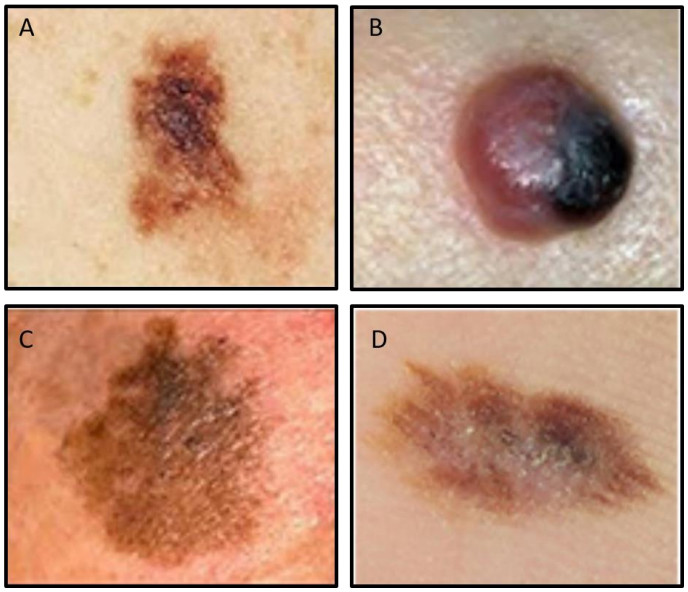

Figure 1-6: Malignant melanoma types.

(a) Superficial Spreading Melanoma. (b) Nodular Melanoma (c) Lentigo Malignant Melanoma. (d) Acral Lentiginous Melanoma. (Reproduced from Alasadi *et al*. 2017)

1.2.3 Diagnosis

Visual inspection and dermoscopy imaging are the conventional methods for melanoma diagnosis [42], using multiple criteria known as the ABCDEs of melanoma. These criteria are (A) asymmetry of lesions, (B) border irregularity, (C) colour variegation, (D) diameter of the lesion (> 6mm) and (E) evolution of lesion such as changes over time in shape, size, symptoms (itching), surface (bleeding, papular or nodular formation) and pigmentation [43]. Skin biopsy is the usual practice for the diagnosis of cutaneous melanoma [33]. Unfortunately, these criteria cannot diagnose lesions with vertical growth or amelanotic melanomas, therefore histopathological examination in addition to other methods is used (Figure 1-7).

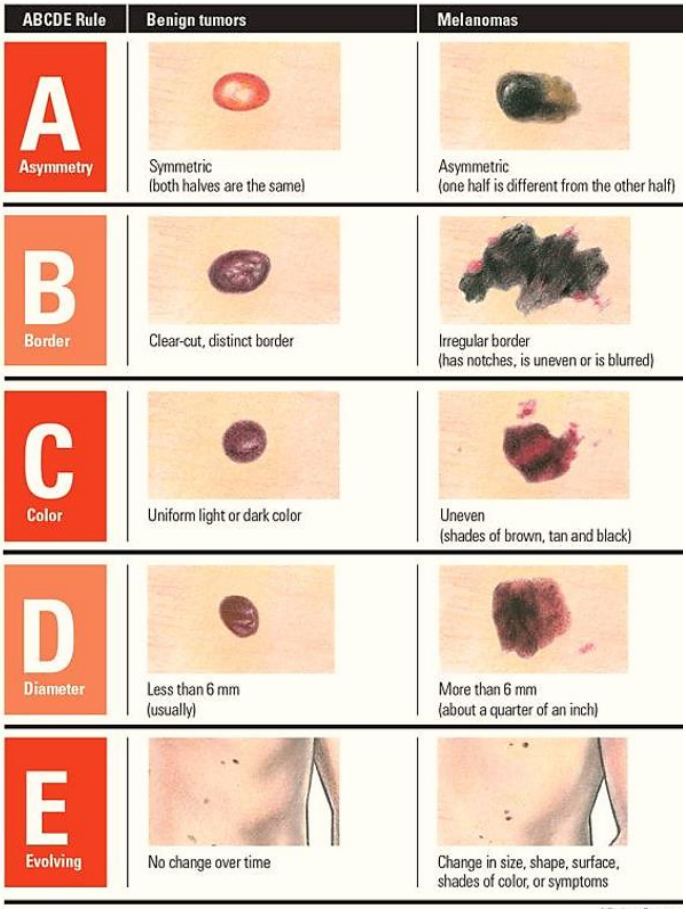

Figure 1-7: ABCDE of melanoma.

(Reproduced from **www.skincancer.org**)

1.2.4 Prognosis

At diagnosis, most patients have a confined primary tumour and are usually cured by surgical excision of the tumour [44]. However, melanomas could be highly malignant, thus metastasizing to various organs including lung, liver, brain, and bone. Patients with stage I melanoma have a 5-year and 15-year survival of ~97% and 85% respectively whereas those with stage IV melanoma drop down to 15% and 5% respectively [45-47]. Grade IV metastatic melanoma is notorious for being refractory to conventional chemotherapy [48-51] thus these patients show a very poor prognosis.

1.2.5 Clinicopathological Classification

1.2.5.1 Clark's Level and Breslow's Depth

First described in 1960, prognosis according to Clark's classification is based on measuring the depth of the melanoma invasion into the anatomic skin layer as well as the cell proliferation rate known as the mitotic index [52].

The Clark levels of invasion are divided into five levels:

- Level I: melanoma is confined to the epidermis, the outermost layer of the skin, hence the name melanoma in-situ.
- Level II: melanoma cells infiltrate past the basement membrane into the papillary dermis, the second layer of the skin, where melanoma progresses from the radial growth phase to the vertical growth phase.
- Level III: melanoma cells invade into the junction between the papillary and the reticular dermis, compressing the latter.
- Level IV: melanoma cells invade into the reticular dermis, remaining within the skin layers.
- Level V: melanoma cells infiltrate into the fat layer of the skin beneath the dermis, invading into the subcutis, the third layer of the skin.

First described in 1970, Breslow's prognostic factor [53-55] measures tumour thickness with an ocular micrometre at right angles to the surface of adjacent normal skin from the top of the granular cell layer or from the ulcer base over the deepest point of invasion to the deepest

point of invasive tumour cells [55]. Conventionally, Breslow scale is divided into four categories: i) 0 - 0.76 mm, ii) 0.76 - 1.49 mm, iii) 1.50 mm - 3.99 mm, and finally iv) > 4.00 mm (Figure 1-8).

1.2.5.2 TNM Staging System

The TNM classification of malignant tumours (TNM) staging system was developed by the American Joint Committee on Cancer (AJCC) and revised in 2018 based on the analysis of 46,000 patients in the AJCC Melanoma Staging Database [45, 56, 57].

TNM classification has an alphanumeric code system that characterizes the stage of certain cancer originating from a primary tumour where their combination characterizes four stages and nine substages (Figure 1-8):

- T: primary tumour depth as described by Breslow's thickness (expressed in millimetres).
- N: regional lymph node status including in-transit metastasis.
- M: distant metastasis including plasma levels of lactate dehydrogenase (LDH).
- Stage 0: melanomas are non-invasive and still have the integrity of the epidermal basement membrane.
- Stage I (≤ 2 mm according to Breslow’s method) and Stage II melanomas are only localized primary tumours and there is no sign tumour cell spread to lymph nodes or other parts of the body.
- Stage III is characterized by regional spread through lymphatic vessels.
- Stage IV is characterized by distant metastasis.

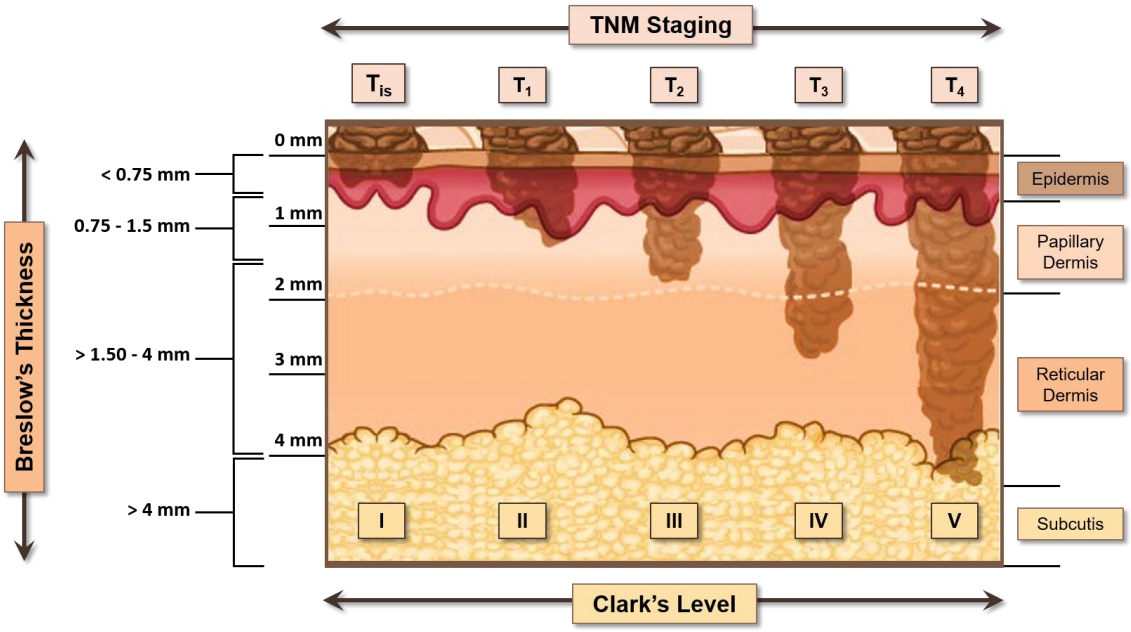

Figure 1-8: Clark's Level, Breslow's Thickness, and TNM Staging.

(Adapted from Clark Levels of Invasion of Skin Cancer https://headandneckcancerguide.org/adults/introduction-to-head-and-neck-cancer/skin-cancer/anatomy/)

1.2.6 Etiology

Numerous risk factors could lead to the development of cutaneous melanoma [58]. The leading environmental factor is Ultraviolet radiation (UVR), including both the shorter wavelength UV-B rays and the longer wavelength UV-A rays, which are both mutagenic factors contributing to the increasing incidence of melanoma [59-61]. The intensity [60], duration and frequency [62] of exposure to UVR all directly associated with the higher incidence of cutaneous melanoma. The most common types of melanoma occur in sun-exposed skin and could be classified according to their cumulative levels of exposure to UVR and their site of origins into chronically sun-damaged melanomas (CSDM) and non-chronically sun-damaged melanomas (NCSDM) [63]. Typically, CSDMs originate in the head, the neck and the dorsal surfaces of the distal extremities, showing macroscopic and microscopic signs of continuous exposure to UV radiation [64], whereas NCSDMs occur in the trunk and proximal extremities which are areas interruptedly exposed to the sun.

Moreover, multiple molecular signalling pathways are implicated in cutaneous melanoma. BRAF is the most frequently mutated gene with a frequency of ~ 66% of malignant melanomas, [65, 66]. Also, there is RAS signalling cascade (*N-RAS, K-RAS, H-RAS)* mutation [67], *NF1* loss [68], *PTEN* loss [69], as well as germline mutations in the CDKN2A locus (p16 and/or p14ARF) [70] and *MCR1* gene mutation which give rise to familial melanomas [71]. Furthermore, genetic factors that increase the risk of developing melanoma include past skin lesions, the number of existing nevi [72] or atypical nevi [73], fair complexion [74], previous family history of melanoma [75, 76] or non-melanoma skin cancer [77, 78].

1.2.7 Pathogenesis

In melanoma, the mutagenic effects due to UVR are the biggest contributor to the mutation rate. Large-scale melanoma exome data demonstrated a high base mutation rate compared to other solid tumours [79]. In CSDMs, UV-induced DNA damage results in the formation of photoproducts such as cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidone (6-4PPs) at dipyrimidine sites on the DNA via photochemical reactions [80]. UV signature mutations include mostly (≥60%) cytosine to thymine transitions (C→T) occurring at dipyrimidine sites as well as the less frequent (≥5%) tandem cytosine to thymine transitions (CC→TT) [81]. Cytosines in CPDs are unstable thus they readily undergo deamination to uracils [82]. Translesion synthesis, a DNA-damage tolerance process, could lead to the incorporation of adenosines across these uracils giving rise to C→T and CC→TT mutations [83]. These UV signature mutations are due to the orchestration between cytosine deamination, translesion synthesis, nucleotide excision repair and DNA replication. 6-4PPs show less contribution to UV signature mutations due to the efficient excision of 6-4PPs in the DNA compared to CPDs [84]. As a result, these highly mutagenic DNA could interfere with DNA replication and transcription, resulting in subsequent potential genomic mutations in coding regions of oncogenes and tumour suppressor genes. These genomic mutations could promote benign lesion formation by allowing the clonal expansion of melanocytes carrying specific mutated genes [85].

1.2.8 Development

Melanoma starts when normal melanocytes start to aberrantly grow to form a uniformly coloured, flat, benign nevus where *BRAF* or *NRAS* mutation will constitutively trigger the mitogen-activated protein kinase (MAPK) signalling cascade. This gives rise to asymmetric, multicoloured, dysplastic nevi, where *CDKN2A* or *PTEN* are lost in many familial or sporadic melanomas respectively. During the radial growth phase (RGP), a raised nevus results from increased melanocyte proliferation in the epidermis together with reduced melanocyte differentiation, due to the upregulation of the MITF, the master regulator of melanocyte development and differentiation. During the vertical growth phase (VGP), the cells invade into the dermis further migrating from the primary tumour thus metastasizing to distant locations. This is due to the increased expression of N-cadherin and αVβ3 integrin together with the concomitant loss of E-cadherin expression, eventually increasing the migration and invasion potential of melanoma cells [86-88] (Figure 1-9).

1.2.9 Progression

Melanoma progression is a complex process. Melanoma tumorigenesis is conventionally described to occur in a linear progression arising from benign nevi to dysplastic nevi, to melanomas in situ, to invasive melanomas [89]. Following the formation of a primary tumour, melanoma cells enter the lymphatic vessels, infiltrating into the proximal lymph node, then disseminating through the systemic circulation, where melanoma cells adhere to the microvasculature of a target organ, extravasate, and eventually proliferate generating a clinically relevant metastasis. In this model, the progression of individual lesions into metastatic melanomas is thought to occur via accumulating multiple genetic/epigenetic alterations in signalling pathways regulating proliferation, growth and metabolism, apoptosis, cell cycle, and replicative lifespan [79, 90-93]. Thus, melanomas can progress through various evolutionary routes, passing through or evading different stages of malignant transformation [63]. For instance, 12% of melanomas were shown to develop without identifiable cutaneous precursor lesions, suggesting non-conformity to the conventionally described linear model of progression of individual malignant melanomas [94]. The association of specific precursor lesions -harbouring specific

mutations- within certain types of melanoma could help us to understand the underlying molecular pathways involved in the melanoma tumorigenesis (Figure 1-9).

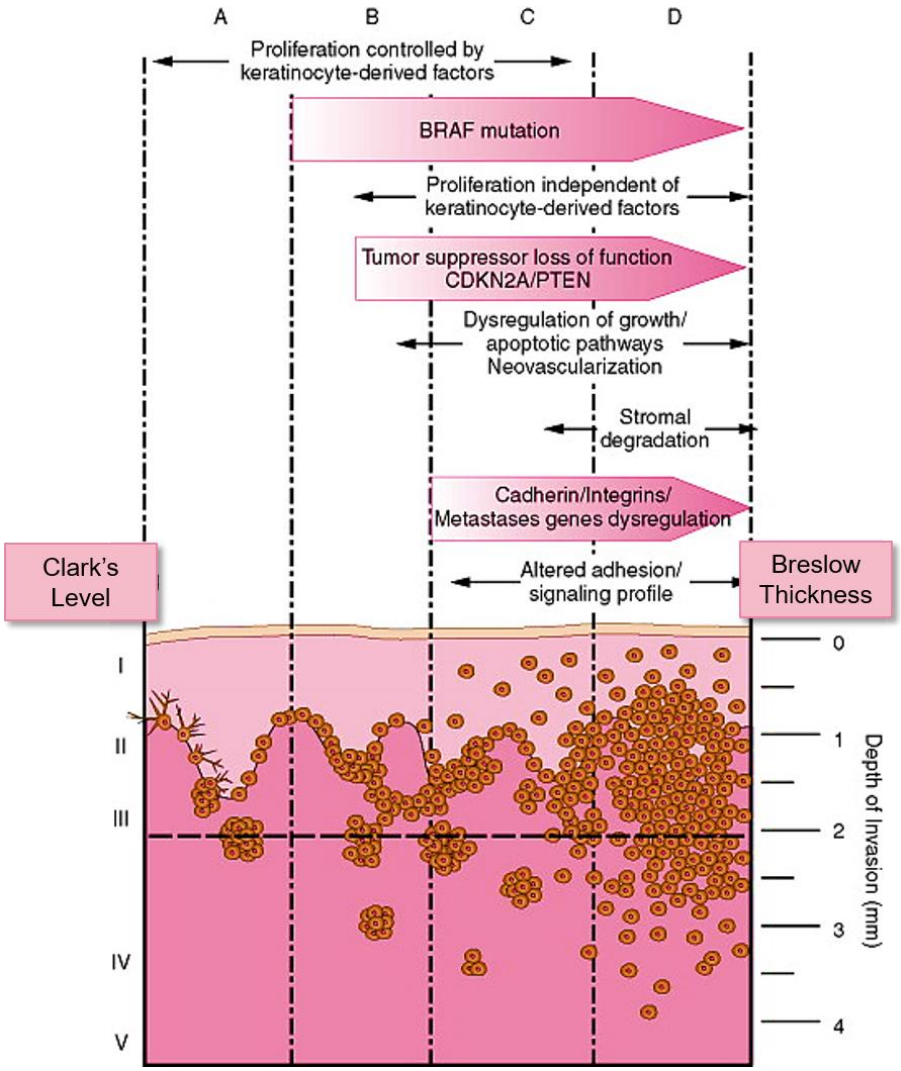

Figure 1-9: Progression of melanocytic lesions; a model correlating histologic appearance of melanocytic lesions with biologic alterations and molecular events.

An illustration of a vertical section of skin, showing the epidermis (*pink*, top, Clark's level I), the dermis (*magenta*, Clark's levels II-IV) & the subcutis (*magenta*, Clark's level V).

The Breslow thickness (right) and Clark's levels (left) are clinicopathological staging systems used by pathologists to classify the degree of invasion of a melanoma skin lesion.

The open arrows indicate the molecular events, while the arrows indicate the biological events corresponding to the histological changes in a melanoma skin lesion.

(A) Normal melanocytes, present at the dermal-epidermal junction, are arranged individually (left) or in small nests of benign nevi (right). Some melanocytes are shown with dendritic processes to demonstrate the normal physiological function of these cells which is the transfer of melanin pigment granules to surrounding keratinocytes.
(B) Dysplastic melanocytes display excessive proliferation of melanocytes along the dermal-epidermal junction (left) and bigger nests of atypical melanocytes in deeper layers of the dermis. Benign nevi have *BRAF* gene mutations that constitutively activate the MAPK pathway to drive proliferation, followed by oncogene-induced senescence. Dysplastic nevi are associated with loss of function in tumour-suppressive genes, e.g. *CDKN2A* and the *PTEN* gene, to potentially bypass senescence.
(C) Early melanoma in-situ (left) showing the proliferation of large, atypical melanocytes at the dermal-epidermal junction extending as single cells into the upper layers of the epidermis and early invasive melanoma (right) showing the transformed melanocytes extending into the upper dermis as single cells and small nests.
(D) Advanced VGP invasive melanoma is shown with large numbers of transformed cells extending deep into both the dermis and the subcutis layers.
The biological and histological alterations in cells (in C and D) are accompanied by alterations in the expression of integrins, cadherin, as well as genes and molecules mediating cell metastasis.

(Adapted from Merghoub, Polsky, and Houghton, Molecular Biology of Melanoma 2008 [95])

1.2.10 Molecular Subtypes

Melanoma had been traditionally classified into subtypes according to their respective histological origins and clinicopathological factors. Over the years, these subtypes had been investigated to identify their epidemiological, clinical and histopathological features. Recent studies have been identifying the molecular alterations of those various melanoma subtypes. For example, sequencing data from various melanomas identified UV signature mutations (about 23000 mutations across different melanoma subtypes, including both indels and SNVs) [96], whereby this signature mutation was absent in some melanomas (Berger et al. 2012; Krauthammer et al. 2012) but present in other melanomas, showing mostly C-to-T nucleotide transitions [81, 97]. Further studies used next-generation sequencing (NGS) to characterize genetic aberrations in melanoma [79, 92, 98]. Other biomarker studies focused on single high-throughput platforms of large sets of samples [79, 92, 99] as well as multi-platform analyses of a few patient samples [100, 101]. The Cancer Genome Atlas (TCGA) program conducted a systematic multi-platform identification of 333 clinicopathologically well-annotated cutaneous melanoma samples at the DNA, RNA, and protein levels, where they developed a

genomic/transcriptomic framework of classification based on the existing somatic alterations to identify their potential biological significance and clinical relevance [66]. This genomic classification could place melanomas into one of four categories according to the most prevalent significantly mutated genes: i) mutant *BRAF*, ii) mutant *RAS*, iii) mutant *NF1*, and iv) triple wild-type.

1.2.10.1 *BRAF* Subtype

The first subtype of melanoma is characterized by the presence of *BRAF* hotspot mutations. BRAF is recruited to the cell membrane upon the activation of RAS via receptor tyrosine kinases. Activated BRAF phosphorylates the protein kinase MEK, which will phosphorylate ERK, which subsequently activates - directly and indirectly- many transcription factors downstream of the MAPK pathway primordial for cell proliferation and survival [102-104].

BRAF somatic missense mutations were present in 66% of malignant melanomas, where they were found mainly in the BRAF kinase domain, with a single substitution of Valine to Glutamate at position 600 ($BRAF^{V600E}$) accounting for 80% of all such cases, being the most frequent BRAF mutation Interestingly, 82% of benign nevi show *BRAF* mutations, however, it was not enough for malignant transformation, suggesting that BRAF activation is potentially a clonal, key driving event [65, 105, 106]. The second most frequent *BRAF* mutation targeted the K601 residue Noteworthy, the patient samples harbouring $BRAF^{V600E}$ hotspot mutation showed the transversions of T-to-A due to the substitution at a specific nucleotide but were lacking common UV mutation signature resulting in UV-induced pyrimidine dimer formation [65]. Also, patients in the *BRAF* subtype were younger than patients in the other subtypes [66].

1.2.10.2 *RAS* Subtype

The second subtype of melanoma is characterized by the presence of RAS hotspot mutations predominantly including either $NRAS^{Q61K/L/R}$ or $NRAS^{G12D}$. The RAS family of GTPases comprises NRAS, KRAS, and HRAS which show similarity in structure and molecular function. In most human cancers, KRAS is the most frequently mutated member of the RAS family [107], while 15-20% of all malignant melanomas show an *NRAS* somatic mutation [108-

110]. These mutations are missense point mutations that lead to an amino acid substitution at positions 12 or 61 or less frequently 13 [107], thus resulting in the constitutive activation of NRAS and the subsequent activation of MAPK signalling. Interestingly, *RAS* mutations were found to be absent in acquired nevi yet recurrent in congenital nevi [111]. Moreover, *NRAS* mutations are more commonly present in CSDMs than other types of melanomas [93].

1.2.10.3 *NF1* Subtype

The third subtype of melanoma was characterized by the presence of frequent mutations in the neurofibromin 1 protein (NF1). NF1 is a GTPase-activating protein whose intrinsic GTPase activity reduces RAS activity, therefore *NF1* mutation resulting in its loss of function eventually results in the activation of MAPK signalling [68]. Interestingly, melanomas with *NF1* mutations show a high burden of mutation yet they possess wild-type BRAF and NRAS. Noteworthy, *NF1* mutation occurs in CSDMs or older individuals (Cancer Genome Atlas Network 2015), as well as desmoplastic melanoma [112].

1.2.10.4 Triple Wild-Type Subtype

The fourth subtype is characterized by the lack of all hotspot *BRAF*, *NRAS*, or *NF1* mutations hence the name triple wild-type (TWT) [66]. It is present in almost 11% of cutaneous melanomas and 50% of mucosal and acral melanomas. Upon analysis, TWT melanomas showed no significant difference in their UV damage signatures or overall survival compared to non-TWT melanomas [113]. Interestingly, low-frequency driver mutations other than *BRAF*, *NRAS*, or *NF1* were identified in TWT melanomas. Upon investigating SNVs in the COSMIC database (v60), TWT melanomas showed rare mutations of *GNAQ Q209P* and *GNA11 Q209L* -frequently mutated in uveal melanoma [114]- co-existing with the hot-spot mutation *SF3B1 R625H* rather than with *BAP1* mutations, which are commonly found in metastatic uveal melanomas [115]. These rare mutations were also found in *CTNNB1* and *EZH2* [66, 79]. Upon investigating somatic copy-number alterations (CNA), TWT melanomas showed more CNAs than the other three subtypes. TWT melanomas demonstrated significant focal amplifications of *KIT* accompanied with co-amplification of *PDGFRA* and *VEGFR2*, focal amplification of *CCND1*, *CDK4*, *MDM2*, *MITF*, and *TERT* as well as focal deletions of *ARID2*, *CDKN2A*, *PTEN* and *TP53* [79, 93, 113].

1.2.11 Familial Subtype

As mentioned earlier, exposure to UV radiation is the main environmental factor causing cutaneous melanoma, however other epidemiological factors such as family history, genetic and molecular factors play an important role [27, 75, 116]. Indeed, almost 3-15% of all melanoma patients have familial melanoma [117, 118], whereby almost 10% of melanoma patients reported a first- or second-degree relative to have been diagnosed with melanoma [119]. Therefore, identifying the genetic basis of the germline mutations for melanoma susceptibility is essential for early detection.

1.2.11.1 High-Penetrance Genes

A main high-penetrance gene involved in susceptibility with familial melanoma is cyclin-dependent kinase inhibitor 2A (*CDKN2A*), where 20% of the cases presented with germline mutations in the *CDKN2A* locus. This locus encodes for the tumour suppressor proteins: inhibitor of cyclin-dependent kinase 4A (p16 INK4A) and alternate reading frame (p14ARF) [119, 120]. p16 is a tumour suppressor protein that inhibits CDK4 kinase activity, thus inhibiting the G1 phase-to-S phase transition, eventually inducing cell cycle arrest [121, 122]. p14 is a tumour suppressor protein that acts as a stabilizer of the tumour suppressor protein p53 via interacting with and sequestering the E3 ubiquitin ligase HDM2, thus inhibiting the HDM-mediated ubiquitination and inactivation of p53 [123, 124].

The second high-penetrance gene is cyclin-dependent kinase 4 (*CDK4*), although it was reported in three families only [125]. The oncogenic effect of the constitutively activated CDK4 protein is due to the phosphorylation of the Retinoblastoma protein (Rb). Rb is a nuclear phosphoprotein ubiquitously expressed in somatic cells, acting as a potent cell-growth inhibitor [126]. In early G1, the active unphosphorylated Rb is bound tightly to the nucleus, and as cells progress through G_1, Rb is phosphorylated by G_1 cyclin/CDK complexes, resulting in a phosphorylated, inactive, nuclear-unbound form of Rb [127, 128]. The CDK4-induced phosphorylated nuclear-unbound Rb (pRb) can disassociate from the transcription factor E2F1 in the cytoplasm, resulting in the subsequent translocation of cytoplasmic E2F1 inside the nucleus, which becomes capable of modulating key regulatory genes required for G1 to S phase transition in the cell cycle [129]. Upon the mutation of either p16 or CDK4, mutant p16 becomes incapable

of binding to and inhibiting CDK4-mediated phosphorylation of the Rb protein and vice versa [123, 124], thus allowing uncontrolled cell cycle progression.

1.2.11.2 Low-Penetrance Genes

Among the low-penetrance susceptibility genes that confer a lower risk for melanoma development is the melanocortin 1 receptor (*MC1R*) which regulates the ratio of the black eumelanin to the red pheomelanin in the skin [71, 74]. *MC1R* genetic mutations causing loss of function result in an increased pheomelanin production, leading to red hair, freckles, fair skin with reduced tanning ability and a subsequent lower photoprotection against UVR, thus being more susceptible to reactive oxygen species (ROS) production and DNA damage [74, 130, 131]. Other low-penetrance susceptibility genes implicated in familial melanoma were characterized including breast cancer 1 (*BRCA1*), BRCA1-associated protein 1 (*BAP1*), and telomerase reverse transcriptase (*TERT*) [132].

1.2.12 Genetic Alterations

Cutaneous melanoma development and progression are multi-step processes involving intricate signalling pathways leading to the transformation of normal melanocytes into melanoma cells that further become metastatic. Melanomagenesis implicates frequent mutations in genes that commonly regulate biological processes among the hallmarks of cancer, e.g. *BRAF*, *NRAS*, and *NF1* maintaining proliferative signalling, *PTEN* and *KIT* evading growth inhibition, *TP53* resisting programmed cell death, *TERT* enabling cell immortalization [79, 92] (Figure 1-10).

1.2.12.1 RAS-RAF-MEK-ERK Signalling Pathway

RAS signalling pathway is always subject to mutations in melanomas. *RAS* is mutated in ~ 15-20% of melanomas, *BRAF* is mutated in 82% of nevi, 66% of primary melanomas, and 40-68% of metastatic melanomas [133]. *BRAF* mutations usually exist in skin regions exposed to the sun intermittently more than those exposed regularly [109]. The most frequent mutation in melanoma is $BRAF^{V600E}$ which is a point mutation, where Valine at position 600 is converted to Glutamate resulting in the constitutive activation of *BRAF* [65]. *BRAF* mutations exist in almost all benign nevi during early melanoma development, yet -on their own- they are not enough for

malignant transformation of melanocytes [134]. As shown by constitutive *BRAF* activation in knock-in mice only nevi formation could develop, while additional *p16/ARF* deletion resulted in the development of melanoma [135]. *RAS* is mutated in 15-20% of melanomas [136]. The most frequent mutations are in *NRAS* (codon 61), but rarely in *KRAS* and *HRAS* [67]. Interestingly, *BRAF* and *NRAS* mutations are exclusive [65]. The constitutive activation of the RAS-RAF-MEK-ERK signalling cascade is essential in melanoma cell proliferation, inhibition of differentiation, tumour invasion, and metastasis [133]. *RAF* kinases inhibitors are often used in chemotherapy, yet they do not prove to be highly effective in treatment [137].

This pathway is involved in several cancers, where it is activated in 80% of cutaneous melanoma cases. This activation occurs via growth factor stimulation leading to activating mutations in either the pathway signalling components or another type of growth-factor receptors [138]. There are 3 different RAS proteins in humans including HRAS, KRAS, and NRAS. Once RAS is activated, it triggers the kinase activity of its target RAF proteins, namely ARAF, BRAF and CRAF in humans, via forming an activated complex [139, 140], which leads to the phosphorylation of mitogen-activated protein kinases (MAPK also known as ERK). In turn, activated MAPK phosphorylates nuclear transcription factors (e.g. ETS, ELK-1, MYC) or it alternatively targets intracellular signalling molecules, which will eventually regulate the expression of multiple genes involved in cell proliferation, differentiation and survival [141]. The pathway is constitutively activated by growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), glial-cell-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), stem cell factor (SCF) and vascular endothelial growth factor (VEGF) [104]. Interestingly, somatic BRAF mutations occur in 80% of melanomas in early stages in benign nevi without progression into malignant melanoma, thus suggesting that BRAF mutation could be required yet not enough to trigger melanoma malignant transformation [65]. Indeed, activated BRAF was found to induce a senescence-like state, where melanoma progression is dependent on the presence of other genetic alterations such as loss of tumour suppressor genes [105]. Different components of the MAPK signalling pathway were found to be frequently mutated in human melanoma samples and melanoma cell lines such as in MAP3K5 and MAP3K9 [142].

1.2.12.2 PI3K/Akt Signalling Pathway

The phosphatase and tensin homolog gene (*PTEN*) is a gene encoding for the phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase enzyme which negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells, therefore serving as a tumour suppressor by negatively regulating the Akt/PKB signalling pathway. AKT activation results in pro-survival signalling [143] through the inhibition of the apoptotic machinery [144, 145], the activation of NF-κB with the subsequent upregulation of pro-survival genes [146] overcoming cell cycle arrest at G1/S and G2/M checkpoints induced by DNA damage [147, 148], diminishing the tumour suppressive functions of FoxO protein (via its phosphorylation and ubiquitination-proteasome degradation [149], thus ultimately favouring proliferation, survival and cell transformation. Therefore, loss of PTEN regulation leads to loss of proliferative and apoptotic control [150]. *PTEN* mutations are found in 10-20% of primary melanoma cases [69, 96, 151]. Furthermore, epigenetic silencing, altered subcellular localization, or ubiquitination may also play a crucial role in *PTEN* inactivation and could potentially occur in almost half of the melanoma cases [133]. As a result of PTEN loss, Akt activation has been observed in a large proportion of melanomas and inversely correlates with survival [152].

PTEN dephosphorylates the focal adhesion kinase (FAK) thus inhibiting cell growth, invasion, migration, and focal adhesions in human glioma and breast cancer cells [153]. PTEN can dephosphorylate adapter proteins of the MAPK signalling pathway leading to diminished MEK activity [154]. Moreover, exogenous PTEN expression in human glioblastoma cells was shown to block the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)-mediated activation of MAPK signalling [155, 156]. Briefly, the loss of PTEN function leads to disruption of the cell growth, apoptosis, as well as cell migration, therefore promoting malignant transformation. In melanoma, loss of *PTEN* was mostly recorded as a late event, albeit dose-dependent loss of PTEN protein has been implicated in the early stages of tumorigenesis [157]. Moreover, loss of *PTEN* was shown to strongly correlate with $BRAF^{V600E}$ hotspot mutation but not with *RAS* mutations [158]. Furthermore, loss of *PTEN* on chromosome 10 was detected in 30- 60% of sporadic cases and *PTEN* mutations or deletions were detected in 30-40% of established cell lines [63] [158].

1.2.12.3 Wnt Signalling Pathway

Aberrant Wnt signalling is implicated in many human cancers due to its essential role in development since Wnt is involved in cell proliferation, differentiation, polarity, and cell fate determination [159-161]. Wnt signals through one of three different pathways: i) the canonical β-catenin pathway [162], and the non-canonical ii) $Ca2^{+}$/PKC pathway and iii) planar cell polarity pathway [163-165]. The canonical β-catenin-dependent Wnt signalling pathway was shown to promote pigment-cell formation by medial crest cells, indicating an essential role during the development of melanocytes from their neural crest precursors [166], suggesting that increased Wnt signalling could participate in the malignant transformation of melanoma cells. Indeed, nuclear β-catenin was found to be increased in primary melanomas [159-161], and Wnt/ β-catenin signalling was found to be active in 30% of melanomas [167], suggesting an important role for the canonical Wnt/β-catenin pathway in melanoma development. Furthermore, the constitutive expression of β-catenin together with an activated *NRas* oncogene was found to induce tumour progression in transgenic mouse models with high penetrance and short-latency [168].

1.2.12.4 Other Signalling Pathways

Other signalling pathways seem to be involved in melanoma progression. TGFβ has also been shown to be implicated [65, 133, 169, 170]. Besides, the TGFβ signalling pathway was shown to be implicated in melanoma tumorigenesis, in particular, TGFβ plays an important role in normal melanocytes and melanoma, eliciting tumour suppressive effects regulating growth and survival as well as migration and invasion [171, 172]. TGFβ was found to upregulate the Plasminogen Activator Inhibitor-1 (PAI-1) in melanoma cells thus decreasing plasmin generation and activity, therefore inhibiting cell migration and invasion [171]. Moreover, a study from our lab, which I first-coauthored, showed that the Leukemia Inhibitory Factor (LIF) was shown to mediate TGFβ-induced tumour suppressive effects in melanoma by inducing cell cycle arrest and cell death as well as inhibiting cell migration in a STAT3-dependent fashion [173].

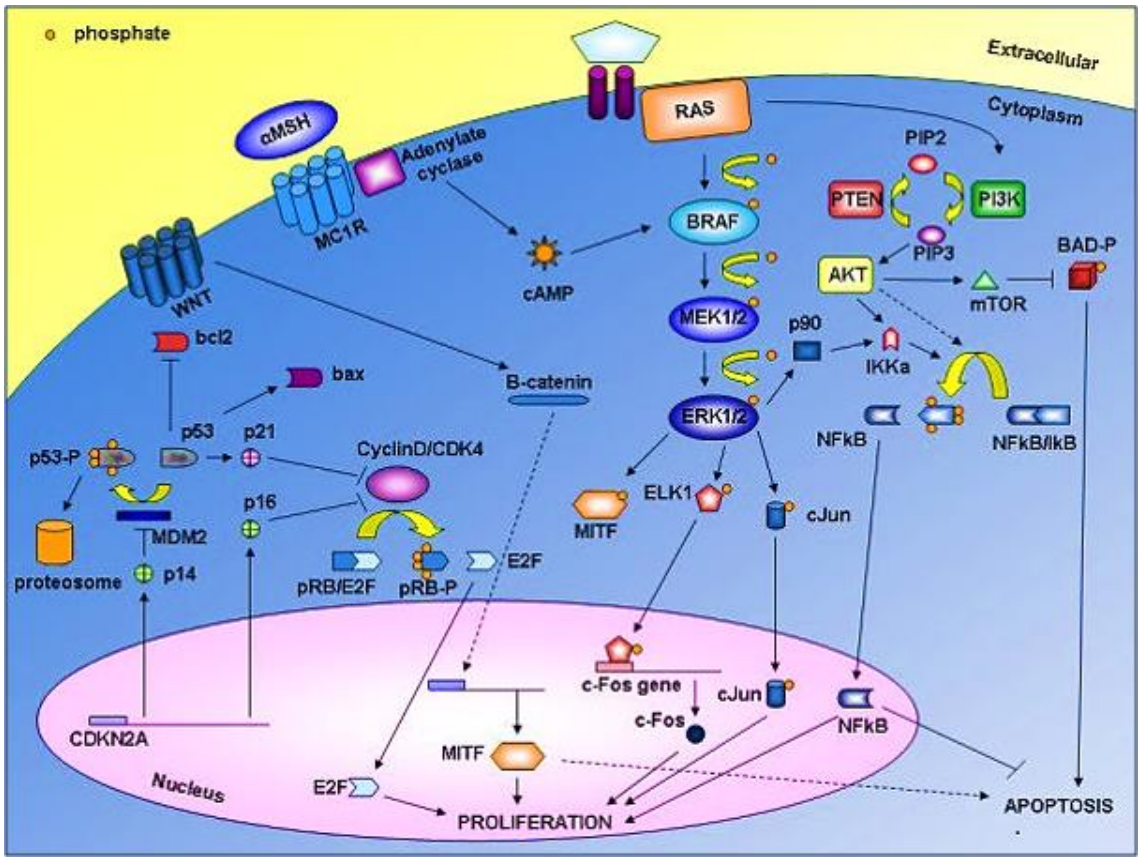

Figure 1-10: Molecular pathways involved in cutaneous melanoma.

Pathways associated with N-RAS, BRAF, and mitogen-activated protein kinase (MAPK) as well as with CDKN2A and MITF are schematically represented. Arrows, activating signals; interrupted lines, inhibiting signals. BAD, BCL-2 antagonist of cell death; cAMP, cyclic AMP; CDK4, Cyclin-dependent kinase 4; CDKN2A, Cyclin-dependent kinase inhibitor of kinase 2A; ERK1/2, Extracellular-related kinase 1 or 2; IkB, inhibitor of kB protein; IKK, inhibitor-of-kB-protein kinase; MC1R, melanocortin-1-receptor; MITF, Microphthalmia-Associated Transcription Factor; MEK1/2, Mitogen-activated protein kinase-extracellular related kinase 1/2; PI3K, Phosphatidylinositol 3 kinase; PIP2, Phosphatidylinositol bisphosphate; PIP3, Phosphatidylinositol trisphosphate; PTEN, Phosphatase and tensin homologue. (Reproduced from Palmieri *et al.* 2009 [174])

1.2.13 Melanoma Treatment

There are various therapeutic approaches for melanoma treatment, extending from surgical excision to chemotherapy, radiotherapy as well as the more recent immunotherapy, and targeted therapy. The choice of the approach depends on the stage of the tumour, its location, the genetic mutations and the degree of patient responsiveness. For patients with stage I up to stage IIIB melanoma, surgical excision is the primary treatment [44, 175-178]. Nonetheless, to enhance various survival outcomes of patients, adjuvant therapies, such as targeted therapy or immunotherapy, are usually considered [177, 179]. Similarly, for patients with solitary metastatic tumours, surgical excision of the metastatic tumour is the usual standard of care, whereby chemotherapeutic agents are commonly used to enhance survival outcomes [178, 179]. Almost 10% of melanoma patients are diagnosed at an advanced metastatic stage, presenting with a non-resectable tumour. Almost one in three patients diagnosed with stage IV melanoma is diagnosed with visceral and brain metastasis, thus demonstrating an increased likelihood to be refractory to treatment [180]. The biggest challenge facing the treatment of malignant melanoma is the low efficiency of therapies due to the refractory nature of the tumours [179] as well as the multiple severe side effects arising from the medications themselves [181-183].

Immunotherapy and kinase inhibitors are nowadays the backbones of systemic therapy, while chemotherapy is considered a second-line treatment option [184-186]. Indeed, during the past decade, several therapies were approved by the FDA for advanced metastatic melanomas such as immune checkpoint inhibitors including anti-cytotoxic T-lymphocyte-associated antigen 4 antibodies (anti-CTLA4) and anti-programmed cell death protein 1 antibody (anti-PD1), as well as selective RAF kinase inhibitors and MEK kinase inhibitors [187-195].

1.2.13.1 Dacarbazine

Dacarbazine is an alkylating agent that had been approved by the FDA in 1974 and is considered the standard chemotherapeutic agent for metastatic melanoma. Studies reported that a complete response was achieved in less than 5% and 5-year survival in 2%–6% of patients. Despite these results, dacarbazine had served as the standard of care for melanoma treatment because other single agents or combination chemotherapies did not reveal improvements in the overall survival (OS) of patients [196].

1.2.13.2 Interferon (IFN) α-2b

IFNs are cytokines secreted by leukocytes that can interfere with viral replication, thus affecting immunomodulation, angiogenesis, proliferation, apoptosis and tumorigenesis via activating various types of immune cells including T-cells, B lymphocytes, natural killer cells, and dendritic cells, while suppressing regulatory T-cells (T_{regs}) and myeloid-derived suppressor cells [197-201]. IFN α-2b had been approved by the FDA in 1995 as adjuvant therapy for the treatment of resected stage IIB/III melanoma [200, 202], whereby it could lower the high risk of relapse and improve the survival outcomes of melanoma patients [203] particularly those with ulcerated primary lesions [204].

1.2.13.3 Peg interferon α-2b (Peg-IFN)

Peg interferon is the combination of IFN α-2b with the molecule polyethylene glycol (Peg) that allows the compound to stay longer in the circulation, thus improving its therapeutic effect [205]. Peg-IFN had been approved by the FDA in 2011 as adjuvant therapy for stage III melanomas [206].

1.2.13.4 Interleukin-2 (IL-2)

IL-2 is a cytokine capable of expanding effector T-cells (T_{effs}) and T_{regs}, which had been approved by the FDA in 1998 as a treatment for metastatic melanomas [207]. Noteworthy, IL-2 treatment showed an overall response of almost 20% and a total response rate of only 4% [208]. ORR represents the sum of patients showing total and partial response to chemotherapy but does not include stable disease. Patients with total response show no detectable evidence of a tumour while those with partial response show only a reduction in tumour size over a particular period. ORR is a direct measure of the tumoristatic/tumoricidal activity of therapy rather than its efficacy. Moreover, IL-2 treatment showed multiple side effects, which include cardiac arrhythmias, hypotension, tachycardia, peripheral edema, and reversible multisystem organ failure [209].

1.2.13.5 Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) Inhibitors

CTLA-4 is an inhibitory checkpoint receptor that blocks T-cell activation and induces immune tolerance. Anti-CTLA-4 therapeutic agents antagonize the suppressive role of the CTLA-

4 checkpoint receptor, thus upregulating the immune response via increasing the production of T-cell cytokine production as well as increasing intratumoural infiltration of T-cell in regressing tumours. The anti-CTLA-4 antibody ipilimumab had been approved by the FDA in 2011 for the treatment of advanced melanomas [210-212]. Ipilimumab with IL-2 had shown no improvement in the efficacy over ipilimumab alone [213]. Ipilimumab and Peg-IFN had shown an overall response rate of 40% and median progression-free survival (PFS) of almost 6 months [214]. Ipilimumab alone had shown the highest response rates in advanced melanoma patients, followed by ipilimumab combined with gp100 peptide vaccine, followed by gp100 alone showing with median OS of 10.1, 10 and 6.4 months [187]. To be treated with ipilimumab, the patients are required to satisfy certain immune-related response criteria (irRC). The irRC are newly identified response patterns observed with immunotherapeutic agents in advanced melanoma in addition to the conventional Response Evaluation Criteria in Solid Tumours (RECIST) criteria designed to detect early effects of cytotoxic agents. The irRC include measurable new lesions into the "total tumour burden" in addition to the baseline lesions [215]. Noteworthy, ipilimumab treatment – especially at higher doses- showed immune-related side effects including -but not limited to- colitis, dermatitis, drug-related hepatitis, endocrinopathies, as well as neuritis [213].

1.2.13.6 Programmed cell death protein 1 (PD-1)/PD-1 ligand (PD-L1) Inhibitors

PD-L1 and PD-L2 bind to the PD-1 receptor to inhibit the activation of T-cells. PD-L1 and PD-L2 are expressed on the antigen-presenting cells as well as in several human tumours in addition to other cells of the tumour microenvironment in response to inflammatory stimuli [216]. Nivolumab is a high-affinity anti-PD-1 monoclonal antibody that had been approved by the FDA in 2014 for the treatment of patients with metastatic melanoma [217] whereby the disruption of the interaction between PD-1 receptors and PD-L1 and PD-L2 ligands triggers immune stimulation, thus promoting antitumour effects [218]. Nivolumab combined with ipilimumab demonstrated the highest efficacy in melanoma patients, followed by Nivolumab alone, followed by ipilimumab alone, followed by dacarbazine/paclitaxel with median PFS of 11.5, 6.9, 2.9 and 2.2 months respectively [218, 219]. Combinational therapy results in significant synergy due to the blockade of both immune checkpoints (CTLA-4 and PD-1), thus preventing any potential upregulation of compensatory pathways, hence its superiority to monotherapies. In the tumour,

the PD-1/PD-L1 pathway could cause the suppression of tumour-specific T effector cells, thus compromising the antitumour efficacy induced by CTLA-4 inhibitors (e.g. ipilimumab). On the other hand, CTLA-4 blocks the full activation of tumour-specific effector T cells, thus compromising the antitumour efficacy induced by PD-1 inhibitors (e.g. nivolumab) [220-222]. Indeed, the combination therapy of nivolumab and ipilimumab was approved in the USA for patients with a negative PD-L1 status and was approved in Europe regardless of the PD-L1 status [219]. Unfortunately, the higher efficacy is accompanied by higher toxicity, therefore, it is crucial to find the optimal combinations through proper monitoring and management to minimize the toxicity while maximizing the tumoricidal efficacy.

Pembrolizumab is an anti-PD-1 antibody that had been approved by the FDA in 2015 for the treatment of patients with advanced melanomas, showing an increase in both PFS and OS with the advantage of much lower high-grade toxicities when compared to ipilimumab [190, 223, 224]. Pembrolizumab combined with Peg-IFN demonstrated high tolerance and clinically active, especially in patients with advanced melanoma that cannot be removed by surgery [225]. The side effects related to this therapy are arthralgia, diarrhea, fatigue, headaches, infusion reactions, nausea, pruritus, and rash [178].

1.2.13.7 BRAF Inhibitors

As previously discussed, mutations in the *BRAF* gene result in the activation of the MAPK signalling pathway thus promoting tumour cell growth and proliferation [226]. Therefore, blocking MAPK signalling can result in tumour suppressive effects. Vemurafenib and dabrafenib are selective BRAF-mutant inhibitor that had been approved by the FDA, in 2011 and 2013 respectively, for the treatment of patients of non-resectable and metastatic melanomas harbouring $BRAF^{V600E}$ mutations- but not *RAS* mutations [65, 109, 227, 228] whereby 90% of those patients showed a reduction in tumour size as well as enhanced PFS, and OS in response to vemurafenib compared to dacarbazine. Unfortunately, 90% of the patients display side effects and various grades of toxicities including arthralgia, basal cell carcinoma, cutaneous squamous cell carcinoma, erythematous hyperkeratotic follicular papules, granulomatous eruption, hyperkeratosis, keratoacanthoma, photosensitivity, pruritus and pyrexia [229]. Furthermore,

patients would develop quick resistance mechanisms in response to these treatments including increased expression of various tyrosine kinases [230].

1.2.13.8 MEK Inhibitors

Similar to BRAF inhibition, inhibition of MEK1/2 decreases the growth and proliferation of tumour cells. Trametinib is a MEK1/2 inhibitor that had been approved by the FDA in 2013 for the treatment of patients with non-resectable and metastatic malignant melanomas with *BRAF* mutations, especially those not refractory to selective BRAF inhibitors as well as *NRAS* mutations whereby trametinib showed better PFS and OS responses compared to dacarbazine or paclitaxel [231-233]. Trametinib (a MEK1/2 inhibitor) combined with dabrafenib (*BRAF*-mutant inhibitor) had been approved by the FDA in 2014 for the treatment of non-resectable and metastatic melanomas harbouring *BRAF* mutations due to its improved clinical response [234, 235]. Similarly, cobimetinib (a selective MEK inhibitor) combined with vemurafenib (*BRAF*-mutant inhibitor) had been approved FDA in 2014 for the treatment of non-resectable or metastatic melanomas, harbouring *BRAF* mutations also due to its improved clinical response [235-237]. Unfortunately, whether alone or in combination with BRAF inhibitors, MEK inhibitors display side effects in patients including diarrhea, fatigue, pyrexia and vomiting [227].

1.2.13.9 Combinatorial Therapies

Studies show that it is common that cancer cells escape from immune surveillance through various mechanisms including decreased immunogenicity, immunosuppressive environment, as well as the counteraction of T-cell effector functions. Consequently, in most patients, it is unlikely to attain improved OS with a single therapeutic approach. Therefore, studying combinations of targeted therapy (e.g. BRAF inhibitors, MEK inhibitors etc.) co-administered with immunotherapy (particularly immune checkpoint inhibitors e.g. CTLA-4 inhibitors, PD-1/PD-L1 inhibitors), is necessary to reach significant progress via maximizing the chemotherapeutic effects while concomitantly minimizing toxicological adverse effects [238].

Few clinical trials testing BRAF inhibitors with CTLA-4 inhibitors in patients with either unresectable or late-stage metastatic melanoma showed promising outcomes. Among the less successful studies, a phase I trial (NCT01400451) testing vemurafenib with ipilimumab was

terminated due to grade 2/3 hepatotoxicity [239]. A phase II trial (NCT01673854) testing vemurafenib followed by ipilimumab was completed with no severe hepatotoxicity yet resulted in grade 3/4 skin adverse effects suggesting that vemurafenib possibly induces a favourable immune microenvironment before the administration of ipilimumab [240]. Similarly, a phase I trial (NCT02200562) testing dabrafenib with ipilimumab was terminated. Another phase I trial (NCT01767454) testing both dabrafenib and trametinib with ipilimumab was completed with no hepatotoxicity, however resulted in severe gastrointestinal toxicity [241, 242].

On the more positive side, a phase Ib trial (NCT01656642) testing both vemurafenib and cobimetinib with atezolizumab (PD-L1 inhibitor) was completed with a promising outcome, showing an objective response rate of 72%, thus being more favourable than monotherapies [243]. Similarly, in a phase I/II trial (NCT02130466) testing both dabrafenib and trametinib with pembrolizumab was completed with a promising outcome, showing an objective response rate (ORR) of 73% [244]. These favourable outcomes could be attributed to the lower toxicity of PD-1/PD-L1 inhibitors compared to CTLA-4 inhibitors [224]. Interestingly, another phase I/II trial (NCT02027961) testing both dabrafenib and trametinib with durvalumab (PD-L1 inhibitor) was completed with evidence of clinical activity and a manageable safety profile [245, 246]. Moreover, a recent phase III trial (NCT02967692) is currently testing both dabrafenib and trametinib with spartalizumab (PD-1 inhibitor) showing an overall favourable outcome with an ORR of 78%, although 72% of patients developed grade 3/4 adverse effects [247].

Several clinical trials focused on BRAF inhibitors with or without the co-administration of MEK inhibitors together with immune checkpoint inhibitors. To date, there are no definitive guidelines for these regimens. Therefore, multiple clinical trials are conducted to determine the optimal type, timing and sequence of administration of the inhibitors to be used in double or triple combinatorial chemotherapy required for patients with metastatic melanoma to maximize clinical effectiveness and minimize lethal toxicity. Amongst these trials, a phase III trial (*NCT02224781*) currently testing the co-administration of both dabrafenib and trametinib followed by nivolumab plus ipilimumab or the reverse both nivolumab and ipilimumab followed by dabrafenib and trametinib to determine the best regiment for patients with *BRAF*-mutated metastatic melanoma [248].

Multiple hurdles face the novel therapies for melanoma patients, despite the progress achieved. The negative outcomes in melanoma patients are due to the development of resistance to treatment that stems from the inherent intra- and intertumoural heterogeneity of the tumours, both of which need further elucidation. As mentioned above, studies showed that implicating the immune system proved beneficial to melanoma patients resulting in improved progression-free and overall survival. Ideally, an optimal synergetic effect between various therapeutic choices, e.g. chemotherapy, immunotherapy, and targeted therapy, will eliminate many of the side effects through personalized therapy. This mandates a thorough comprehension of melanoma development and progression to establish novel therapeutic tools and strategies.

1.3 Transforming Growth Factor β

1.3.1 Biological Functions

The transforming growth factor-beta (TGFβ) was isolated as a secreted factor from sarcoma virus-infected cells that conferred transient neoplastic transformation on normal fibroblasts, where they exhibited an acquired ability to grow in soft agar under anchorage-independent conditions [249-253]. TGFβ was isolated from non-neoplastic tissue indicating a significant role in normal physiological functions [253]. To date, dozens of various structurally-related cell regulatory proteins have been grouped in the TGFβ superfamily, divided into four main subgroups, the TGF-β subfamily, the bone morphogenetic proteins (BMPs) and the growth differentiation factors (GDPs), the activin/inhibin subfamily, and a subfamily comprising other members such as anti-Müllerian hormone (AMH), myostatin, and nodal among others [254, 255]. TGFβ was shown to regulate a plethora of biological processes in various cellular contexts such as the regulation of cell growth, embryogenesis, erythropoiesis, immunological response, osteogenesis, programmed cell death, tissue remodelling as well as wound healing [256-258]. Under physiological conditions, TGFβ and its receptors are almost ubiquitously expressed in all tissues and the regulatory role played by this growth factor is of central importance to human diseases. TGFβ loss of function results in hyperproliferative diseases and has been linked to cancer development, inflammatory and autoimmune diseases, while a gain of function results in immunosuppression and tumour metastasis [259-261].

1.3.2 Signalling Pathway

1.3.2.1 Smad-dependent Signalling

TGFβ signal transduction starts upon ligand binding to the type TGFβ II receptor (TβRII) which is a single transmembrane-spanning constitutively auto-phosphorylated serine/threonine kinase [257]. TβRII then recruits and transphosphorylates the juxtamembrane glycine and serine-rich region of the type TGFβ I receptor, thus activating its kinase activity which in turn phosphorylates intracellular mediators known as Smads which are the homologs of *Drosophila* gene, Mad (mothers against decapentaplegic) and *C. elegans* Sma genes [255, 262]. This phosphorylation occurs on the SxS motif (carboxy-terminal serine residue) of receptor-regulated Smads (R-Smads), Smad2 and Smad3, which subsequently results in the heterotrimerization of two phosphorylated R-Smad subunits together with one common partner Smad4 (Co-Smad) [263-265].

Smad-dependent signalling is considered the canonical TGFβ signalling pathway, whereby TGFβ and activin signal through the R-Smads, Smad2 and Smad3, whereas other TGFβ superfamily members, e.g. BMPs, signal through other R-Smads namely Smad1, 5 and 8 [254]. Afterward, the Smad heterotrimer complex translocates inside the nucleus, via importin-dependent and independent mechanisms [266, 267], where it binds -with very low affinity- to the DNA sequence CAGAC, known as the Smad binding element (SBE), in addition to some GC-rich sequences [258]. To increase their binding affinity, Smads would associate with different co-activators or co-repressors which are DNA-binding proteins functionally expressed in various types of cells, participating in the contextual cell- or tissue-dependent effect of various ligands of the TGFβ superfamily [268, 269].

1.3.2.2 Non-Smad Signalling

Multiple pathways were found to be activated downstream of the TGFβ receptors in addition to the Smads [270]. The activation of these pathways contributes to the regulation of various biological processes, for example activating the mitogen-activated protein kinase (MAPK) pathway -via triggering Src, Raf, and MEK- could activate ERK1/2 thus promoting epithelial-mesenchymal transition (EMT) [271-273]. Also, activation of the stress-activated

kinases p38 and JNK (Jun N-terminal Kinase) could trigger the ubiquitination of TRAF6 and hence MAP3K7/TAK1 activation which in turn phosphorylates and activates many MAP kinase kinases (MAP2Ks) further activating p38 MAPKs, c-jun N-terminal kinases (JNKs) thus mediating apoptosis and EMT [273-279]. Activating the Rho-GTPase pathway which is mediated through Cdc42, Rac, and RhoA triggers cell motility and EMT, hence cell migration and invasion via modifying the cytoskeleton organization [278, 280]. Activating the phosphoinositide 3-kinase (PI3K)/Akt pathway, via triggering mTOR, results in cell growth inhibition [281] and EMT induction [282, 283]. These Smad-independent pathways are implicated in the dual role of TGFβ since they mostly participate in its pro-metastatic effects (**Error! Reference source not found.**)

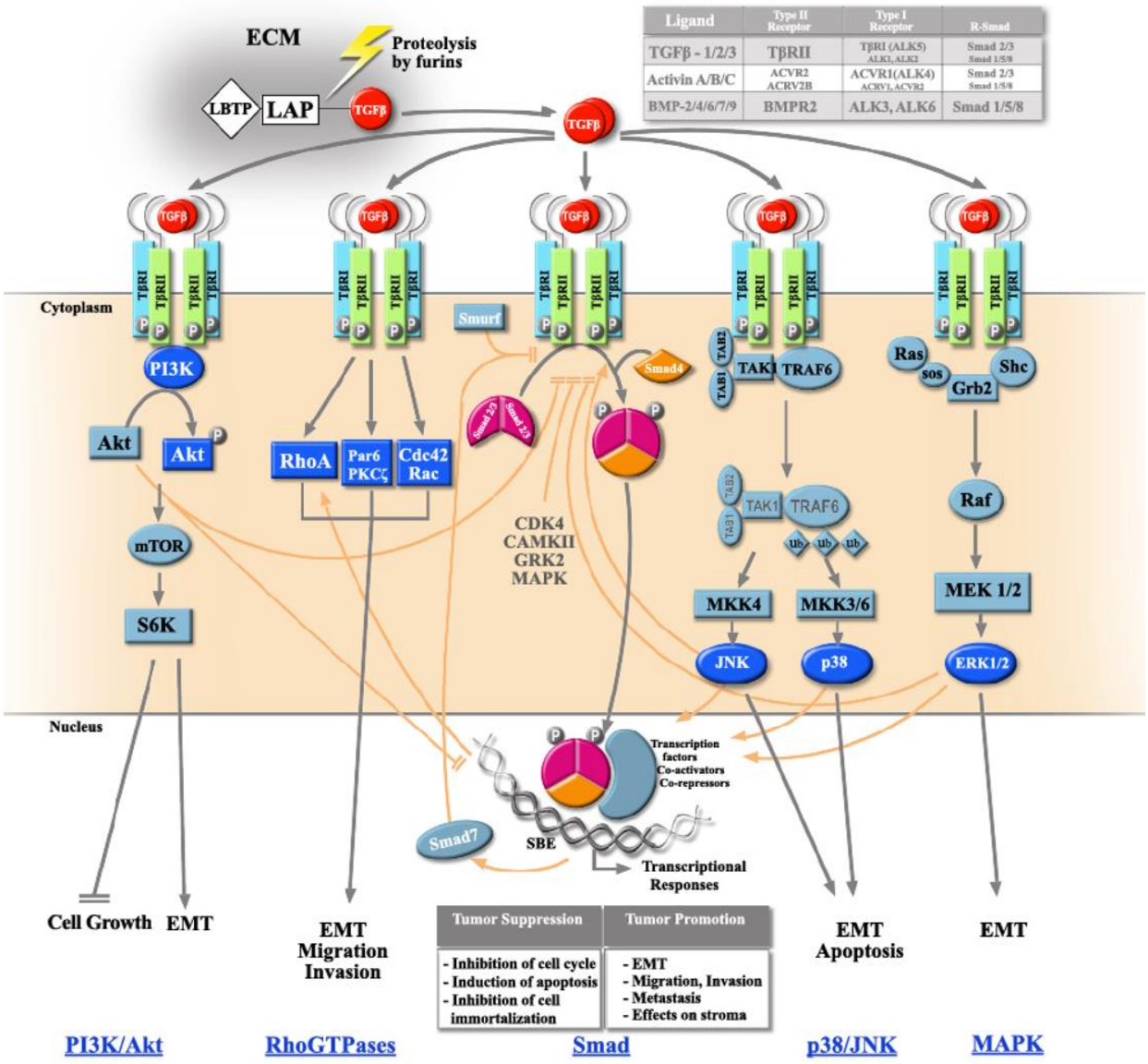

Figure 1-11: The TGFβ signalling pathways.

TGFβ relays most of its biological responses through the canonical Smad-dependent signalling pathway thus resulting in either tumour suppression (via inducing cell cycle arrest, apoptosis, and inhibiting immortalization) or tumour promotion (via increasing EMT, migration, invasion, and metastasis). Interestingly, other ligands of the TGFβ superfamily can signal through other non-Smad pathways. TGFβ can activate the PI3 K/Akt, Rho-GTPase, MAPK, and stress-activated kinase (p38/JNK) pathways. All these pathways (orange arrows) could either synergize with or antagonize the Smad-dependent signalling cascade. Smad-dependent pathways could elicit self- inhibitory roles via TGFβ-induced upregulation of Smad7or phosphorylation of the R-Smad linker (Reproduced from Lebrun 2012 [284]).

1.3.2.3 Ligands

The TGFβ subfamily comprises three ubiquitously-expressed, different isoforms namely TGFβ-1, -2, -3, each of which is encoded by a different gene [254, 257, 285, 286]. TGFβ-1 (hereunder TGFβ) is the most investigated isoform of all three isoforms, whose sequences are ~70% homologous [254, 257, 285, 286].

TGFβ exists in the extracellular matrix as an inactive dimer within a latent complex (Figure 1-12). The activity of TGFβ in the extracellular matrix is mainly controlled by the conversion of the latent TGFβ complex into its active form [287, 288]. The TGFβ precursor, also known as pre-pro-TGFβ, is a monomer containing three distinct parts: an N-terminal signal peptide required for secretion from the cell, a pro-domain region (also known as the latency-associated peptide (LAP)), and a C-terminal region containing the mature TGFβ that later becomes the active TGFβ via different cues of activation. The cleavage of the signal peptides occurs in the endoplasmic reticulum generating pro-TGFβ [289]. Next, two pro-TGFβ monomers dimerize into a dimer (usually a homodimer) via disulphide linkages. The pro-TGFβ dimer is then cleaved by the endoprotease furin in the Golgi complex [290]. This leads to the formation of an N-terminal disulphide-linked LAP dimer and a C-terminal disulphide-linked mature TGFβ dimer. Both dimers stay strongly associated via non-covalent interactions, thus forming the inactive small latent TGFβ complex (SLC) [291]. The SLC itself further associates covalently (through its LAP dimer) with the latent TGFβ binding protein (LTBP) thus forming the large latent TGFβ complex (LLC). The LTBP helps in SLC secretion and storage in the extracellular matrix. After the SLC secretion, the LAP stays linked to TGFβ to keep the latter in an inactive form. The mature TGFβ -in the SLC- needs to be released from the LAP to be activated.

The TGFβ precursor activation could be triggered by various mechanisms [292-296] including:

a) Enzymatic proteolysis either by:

i) proprotein convertases such as furin,

ii) MMPs such as MMP-2 and MMP-9,

iii) proteases such as calpain and plasmin,

iv) glycosidases, v) thrombospondin or vi) αvβ integrins

b) Physical and chemical treatments such as acids, heat and reactive oxygen species

c) Pharmacological drugs such as retinoic acid.

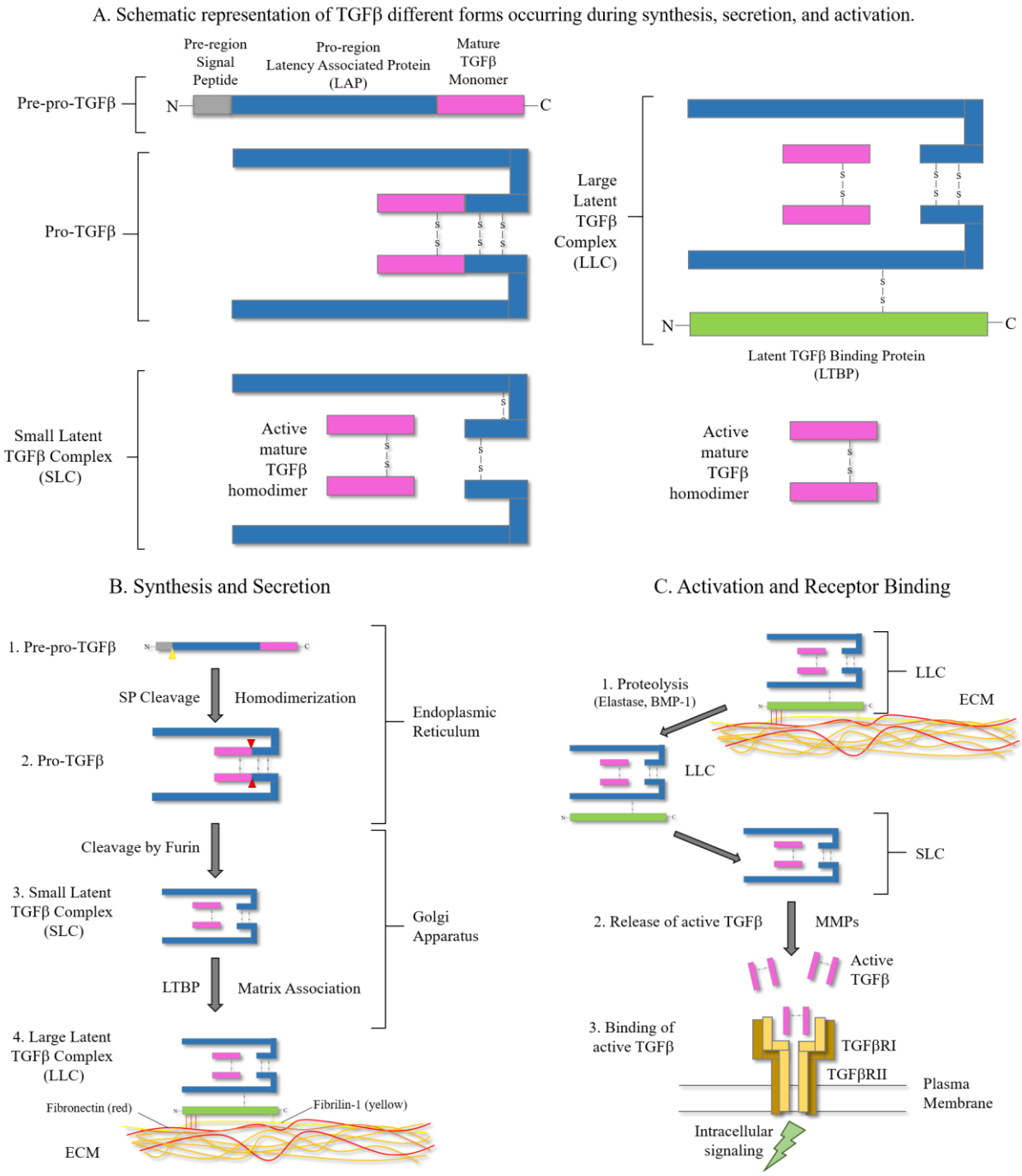

Figure 1-12: Schematic model of TGFβ synthesis, secretion, extracellular matrix association, activation and receptor binding.

(A) Schematic representation of TGFβ different forms occurring during synthesis, secretion, and activation.

(B) TGFβ synthesis, secretion, extracellular matrix association activation and receptor binding:

1. TGFβ is synthesized as a pre-pro-protein, which undergoes proteolytic processing in the endoplasmic reticulum resulting in signal peptide cleavage (yellow triangle: cleavage site).
2. Two monomers of pro-TGFβ dimerize through disulphide bridges.
3. The pro-TGFβ dimer is then cleaved by furin (red triangles: cleavage sites) to yield the small latent TGFβ complex (SLC), in which the latency-associated peptide (LAP) (blue) and the mature peptide (pink) are associated through non-covalent bonds.
4. The large latent TGFβ complex (LLC) is formed through the covalent binding of the SLC to the latent TGFβ binding protein (LTBP) (green). The N-terminal region of the LTBP interacts with extracellular matrix (ECM) components such as fibronectin (red) via covalent binding (red vertical solid lines), whereas the C-terminal region of LTBP interacts with fibrillin-1 (yellow) through non-covalent binding (yellow vertical dotted lines)

(C) TGFβ activation and receptor binding:

1. Proteolysis (mediated by elastases), as well as the activity of bone morphogenetic protein-1 (BMP1), result in the cleavage and the displacement of the LTBP and the subsequent release of LLC.
2. Matrix metalloproteases (MMP2 & MMP9) can cleave LAP (blue) to release the active mature TGFβ (pink).
3. The active mature TGFβ can then bind to its receptors, TGFBRI and TGFBRI.

(The diagram was created by me, based on ten Dijke et al. 2007, Hara et al. 2015, Poniatowski et al. 2015, Robertson 2015) [294, 297-299].

1.3.2.4 Inhibition

Through a negative feedback loop, TGFβ triggers its own inhibition via upregulating Smad7 that binds through its MH2 kinase domain to the TβRI, thus preventing R-Smad (Smad2 and Smad3) phosphorylation and activation due to steric inhibition [300, 301]. Also, Smad7 can disrupt the functional R-Smad/Smad4/DNA complex formation in the nucleus by specifically binding to the Smad-responsive element on the DNA via the Smad7 MH2 domain [302]. Moreover, Smad7 can further inhibit TβRI activity or stability through the recruitment of different enzymes such as the protein phosphatase (PP1), or the HECT-type E3 ubiquitin ligases e.g. Smurf1/2, NEDD4-2 and WWP1 [303-308]. On the other hand, Smad7 could bind to deubiquitylating enzymes [309, 310] e.g. USP15 and UCH37, thus deubiquitylating and stabilizing TβRI, therefore enhancing the TGFβ signalling in as shown in breast cancer, glioblastoma and ovarian cancer [311, 312].

Alternatively, TGFβ signalling could be negatively regulated through several non-Smad-induced mechanisms including i) receptor downregulation via endocytic internalization by clathrin-dependent or clathrin-independent mechanisms [313-315], ii) ubiquitin-proteasome-mediated degradation or dephosphorylation of the translocated nuclear Smad2 and Smad3 [316-319], or iii) phosphorylation of the linker domain of Smads by several intracellular kinases, e.g. the MAPK kinases [320, 321], calcium-calmodulin-dependent protein kinase II [322], cyclin-dependent kinase CDK2/4 [323], casein kinase [324], protein kinase C [325], and G protein-coupled receptor kinase 2 (GRK2) [326]. Furthermore, other signalling pathways, e.g. RAS-RAF-MAPK-ERK signalling, and epidermal growth factor (EGF) could disrupt the nuclear translocation of R-Smads, thus inhibiting the TGFβ-induced Smad-dependent signalling transduction [320, 327].

1.3.3 Dual Role in Cancer

In cancers, TGFβ was shown to play a dual paradoxical role, since it was reported to act both as a tumour suppressor and a tumour promoter.

1.3.3.1 Tumour-Suppressive Effects

TGFβ induces growth inhibition in cells of different lineage origins, e.g. epithelial, endothelial, lymphoid and myeloid cells [254, 328, 329]. TGFβ-mediated growth inhibitory effects highlight TGFβ as a potent tumour suppressor, inducing its effects via a) inducing cell cycle arrest, b) inducing apoptosis, c) inducing autophagy and d) inhibition of cell immortalization.

1.3.3.1.1 Induction of Cell Cycle Arrest

1.3.3.1.1.1 Cyclin-Dependent Kinase Inhibitors

Normally, cyclin-dependent kinases (CDKs) bind to their regulatory partners, the cyclins, thus becoming activated, allowing for cell cycle progression. During the G1 phase, CDK4 or CDK6 binds to cyclin D, while CDK2 binds to cyclin A or cyclin E to further induce the transcription and the expression of more cell cycle regulators such as DNA polymerases, oncogenes, etc. To negatively regulate the cell cycle, CDK inhibitors (CDKIs) inhibit the kinase

function of CDKs via competitively binding to the catalytic site of CDKs present on the cyclins, thus disrupting the CDK-cyclin complexes formation and causing cell cycle arrest [330].

Depending on the cellular context, TGFβ induces cell cycle arrest in the G1 phase, via inducing the CDK inhibitors $p21^{CIP1}$ [331, 332] or $p15^{INK4B}$ [333]. In response to TGFβ, the Smad3/Smad4 physically associates with FoxO [334] or Sp1 [335] transcription factors, where they bind to the *p21* promoter thus inducing p21 transactivation. Also, we found that TGFβ-mediated LIF upregulation could increase the transcription of p21 in a STAT3-dependent manner to induce cell cycle arrest at the G1 phase [173]. TGFβ transactivates *p15* in a Smad-dependent manner via inhibiting CDK4/Cyclin D1 [336] or via binding to the Sp1 consensus site [333, 337]. p15 inhibits CDK4 and CDK6 individually or within their complexes with cyclin D, whereas p21 inhibits CDK2 within its complexes with cyclin A or cyclin E [336, 338]. Furthermore, TGFβ-induced Smad-dependent signalling was shown to prevent the degradation of $p27^{Kip1}$ causing the accumulation of nuclear p27 thus inducing cell cycle arrest in the G1 phase [339]. Also, TGFβ wash shown to induce cell cycle arrest in the G1 phase via p53-independent upregulation of p27 which resulted in lower Cdk2 kinase activity in murine B cells [340]. Furthermore, TGFβ can downregulate the expression of the tyrosine phosphatase CDC25A, thus interfering with the dephosphorylation of CDK4 and CDK6, allowing for their sustained inhibition [341].

1.3.3.1.1.2 Growth-Promoting Transcription Factors

TGFβ represses the expression of growth-promoting factors such as cMyc [326, 342] and Inhibitor of DNA-binding Proteins namely ID1, ID2 and ID3 [343]. cMyc and ID proteins were found to be overexpressed in many types of cancers and to regulate cell growth, differentiation, and angiogenesis [343-345]. Moreover, TGFβ-induced downregulation of cMyc prevents the cMyc-mediated transactivation and transcription of ID2 [345].

TGFβ induces its antiproliferative effects via downregulating cMyc by the interaction of the Smad3/Smad4 complex with E2F4/5 and p107 [346] directly inhibiting cell growth, and indirectly by promoting the induction of p15 and p21, by reducing the cMyc and MIZ1 (zinc-finger protein)-mediated restriction of the p15 and p21 transcription [347]. TGFβ/Smad3 induces activating transcription factor-3 (ATF-3) expression, which represses ID1 [343], thus decreasing cell proliferation via inhibiting Rb and increasing the tumour suppressor $p16^{INK4a}$ [345]. Loss of the tumour suppressor menin can shut down TGFβ signalling. Indeed, in pituitary adenoma cells

menin was found to bind physically to Smad3 thus mediating TGFβ-induced effects, where silencing menin resulted in inhibiting TGFβ signalling as well as TGFβ-induced transcriptional activity through compromised Smad binding to DNA [255, 348, 349]. Similarly, menin was found necessary for activin signalling in pituitary cells [275] (Figure 1-13).

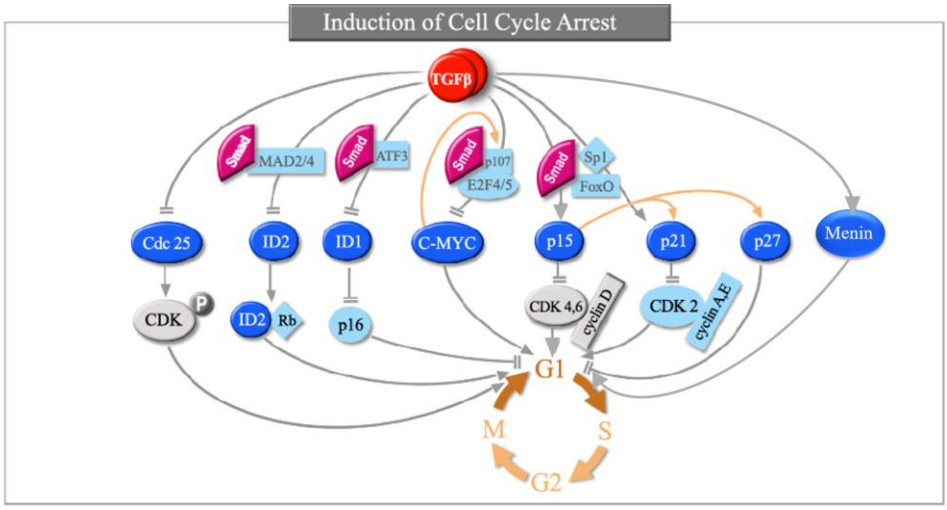

Figure 1-13: TGFβ-mediated cell cycle arrest.

TGFβ/Smad signalling induces cell cycle arrest in the G1 phase via:

- upregulating the CDK inhibitors p15, p21, and p27. p15 and p21 require the transcription factors Sp1 and FoxO. p15 can directly inhibit CDK4/6 and induce p21 and p27 to inhibit CDK2-cyclin A/E complexes (orange arrows),
- downregulating of the oncogene cMyc which requires the transcription factors E2F4/5,
- suppressing the ID1 and ID2 proteins which require ATF3 and MAD2/4 respectively,
- upregulating the tumour suppressor menin in pituitary adenomas,
- downregulating the tyrosine phosphatase CDC25A in mammary epithelial cells

(Adapted from Lebrun 2012 [284]).

1.3.3.1.2 Induction of Apoptosis

TGFβ induces apoptotic activity in different types of cells through multiple mechanisms [350-352]. Studies showed the involvement of various intermediary proteins in a cell-dependent fashion. In hepatocarcinomas, TβRII is stabilized by interacting with the adaptor death domain-associated protein 6 (Daxx), thus activating the JNK and Fas-mediated apoptotic signalling cascades [353]. Also, hepatocarcinomas showed a TGFβ-induced Smad-mediated induction of

the expression of the death-associated protein kinase (DAPK) [354], thus triggering type I programmed cell death [355]. In pancreatic epithelial cells, TGFβ increased the expression of the Krüppel-like zinc finger transcription factor TGFβ-inducible early-response gene (TIEG1), thus promoting apoptosis [356]. TGFβ induces the septin-like mitochondrial protein "apoptosis-related protein in the TGFβ signalling pathway" (ARTS), which activates caspase 3, thus inducing apoptosis [357]. TGFβ stimulates Smad3/Smad4 interaction with the JunD·FosB form of the activator protein (AP1) transcription factor [358] as well as Smad-dependent activation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signalling cascade, thus resulting in increasing the pro-apoptotic activity [359]. In B-lymphocytes and hepatocytes, TGFβ was shown to mediate Smad- and SAPK/p38-dependent transcriptional induction of the Bcl-2 Modifying Factor (Bmf) and Bcl-2 Interacting Mediator of Cell Death (Bim) from the pro-apoptotic Bcl-2 family, which further activate the pro-apoptotic factor Bcl-2 Associated X Protein (Bax) that in turn increases the mitochondrial release of cytochrome c to the cytoplasm through voltage-dependent anion channels, therefore inducing apoptosome formation, consequently triggering caspase-dependent apoptosis [360, 361]. Also, our lab found that TGFβ-mediated LIF upregulation could increase the transcription of p21 in a STAT3-dependent manner, whereby p21 could induce the expression of the pro-apoptotic factors Apaf, Bak, Bax, Bim as well as promote Caspase3/7 activity in human cutaneous melanoma cells [173].

On the other hand, TGFβ was shown to downregulate the anti-apoptotic proteins e.g. BCL2 Apoptosis Regulator (Bcl-2) and Bcl-2-Like 1 (Bcl-$X_{L)}$ in different types of cells such as B-lymphoma, hematopoietic, liver, and prostate epithelial cells [362-366]. TGFβ induces Smad3-dependent physical interaction with Akt in colon cancer [367], and recruitment of the repressive pRb/E2F4 complex to the survivin promoter in prostate epithelial cells [368], thus repressing the "PI3K/AKT/survivin" survival signalling, therefore downregulating the pro-survival protein survivin, enabling the apoptosis. In B and T lymphocytes, both TGFβ and activin can induce Smad-dependent expression of the Src homology 2 domain-containing 5′ inositol phosphatase (SHIP) [369], changing the intracellular phospholipid pools, thus inhibiting the Akt/PKB survival pathway. This leads to the activation of caspase-9, and caspase-3, as well as the upregulation of Bad, p27 together with the repression of Bcl-X_L, thus resulting in apoptosis [370]. TGFβ can induce the expression of the E2F1 transcription factor, causing the formation and binding of a transcriptionally-active pRB-E2F1-P/CAF complex that is recruited to the promoters of several

pro-apoptotic genes (Apaf1, Caspase 7, and Smac/DIABLO, p73), thus promoting apoptosis in normal and cancer cells [371]. Interestingly, these signalling proteins mainly link TGFβ-mediated signalling to the programmed cell death machinery, resulting in regulatory modifications in the expression, localization, and activation of the pro-apoptotic BCL2 and caspase families [372] (Figure 1-14).

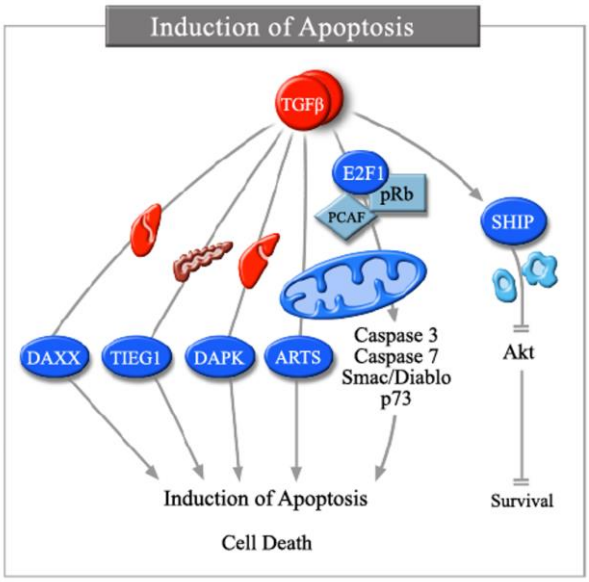

Figure 1-14: TGFβ-mediated induction of apoptosis.

TGFβ mediates pro-apoptotic effects via:

- E2F1-pRb-P/CAF-mediated signalling, leading to the upregulation of several pro-apoptotic target genes in different normal and cancer cells.
- upregulation of SHIP, leading to the blockade of the Akt-mediated survival pathways in both B and T lymphocytes.
- upregulation of the pro-apoptotic proteins DAXX and DAPK in liver cells, of the transcription factor TIEG1 in pancreatic cells, and the mitochondrial protein ARTS in prostatic epithelial cells.
- SAPK-mediated signalling, leading to the upregulation of the pro-apoptotic genes Bmf, Bim and Bax together with the downregulation of the anti-apoptotic genes Bcl-2 and Bcl-XL, resulting in triggering caspase-dependent apoptosis in hepatocytes and B-lymphocytes.
- downregulation of the pro-survival protein survivin in colon cancer (Reproduced from Lebrun 2012 [284])

1.3.3.1.3 Induction of Autophagy

Autophagy is a biological process conserved across species by which the cell self-digests its own cytoplasmic materials, including long-lived or damaged proteins and damaged organelles, through a lysosome-dependent degradation pathway as a response mechanism to different types of stress [373, 374]. On one hand, autophagy can protect cells, maintain homeostasis, prevent disease progression (via sequestering, hydrolyzing and recycling damaged protein and cellular components), thus serving as a protective pro-survival mechanism [374-377]. On the other hand, autophagy could be involved in anti-survival processes such as ageing, apoptosis, cell remodelling, and pathogenic infection [373, 374].

In cancers, autophagy could play an important role either being tumour-promoting in bladder, brain, bone, colorectal, lung, pancreatic, prostate cancer cell lines [378-381] or tumour-suppressive in brain, breast, bone, colorectal, liver cancer cell lines among others [376, 382-388]. TGFβ was found to induce autophagy, further leading to tumour suppression in various types of cells such as hepatocellular carcinoma, as well as in mammary, mesangial and renal epithelial cells [389-393]. Nonetheless, the role of TGFβ in regulating autophagy in melanoma has not been yet elucidated. In hepatocellular carcinoma cells, TGFβ was found to induce autophagy via promoting the accumulation of autophagosomes and the lipidation of LC3, increasing the degradation of long-lived proteins. LC3-II is a conventional autophagy marker in mammalian cells [394], where the cytosolic LC3-I is converted into a lipidated LC3-II localized in the autophagosome membrane. This TGFβ-mediated induction of autophagy was shown to occur through both Smad-dependent and Smad-independent signalling via activating the transcription of various autophagy-related genes, thus enhancing the tumour-suppressive effects of TGFβ [389]. Moreover, TGFβ-induced autophagosome initiation and maturation, as well as transcriptional activation of autophagy-related genes, were found to induce autophagy through the pRb/E2F1 pathway [390] (Figure 1-15).

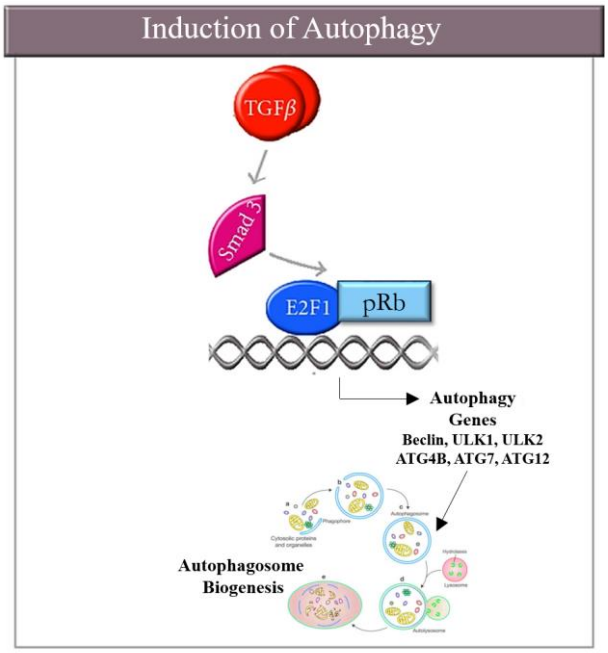

Figure 1-15: TGFβ-mediated induction of cell autophagy.

TGFβ-induced autophagosome initiation and maturation, as well as transcriptional activation of the autophagy-related gene, were found to be mediated through the pRb/E2F1 pathway (Adapted after Lebrun 2012 and Xie & Klionsky 2007 [284, 395]).

1.3.3.1.4 Inhibition of Cell Immortalization

Normal human cell populations will divide for a limited number of times during which the length of the telomeres on the ends of each chromosome slightly shorten till they reach a critical length at which cell division stops, eventually leading to cell senescence and cell death, a phenomenon known as Hayflick phenomenon [396, 397]. Human telomerase reverse transcriptase (hTERT) is the protein component and catalytic subunit of the telomerase holoenzyme and the main regulator of its activity being suppressed in somatic cells [398] but overexpressed in tumour cells, leading to cell immortalization [399] which is a hallmark of cancer [400]. Work from our laboratory and others previously reported that TGFβ could suppress hTERT gene expression in various cancers such as breast, colorectal, liver, uterine as well as normal cells as human keratinocytes, placenta and MEFs [401-403].

TGFβ/Smad3 signalling was found to involve ERK1/2, p38 kinase and histone deacetylase activities to mediate the binding of the E2F-1 transcription factor to the hTERT gene

promoter thus causing hTERT repression in normal and cancer cells [402-404]. Taken together, TGFβ acts as a significant tumour suppressor in several tissues, as a result of its total suppressive effects through inducing cell cycle arrest, apoptotic cell death, as well as inhibiting cell immortalization. TGFβ was shown to induce recruitment of E2F1 and HDAC into repressive complexes in a Smad3-dependent manner, preventing hTERT expression and inhibiting telomerase activity, thereby hindering cell immortalization in epithelial cancer cells [403] (Figure 1-16).

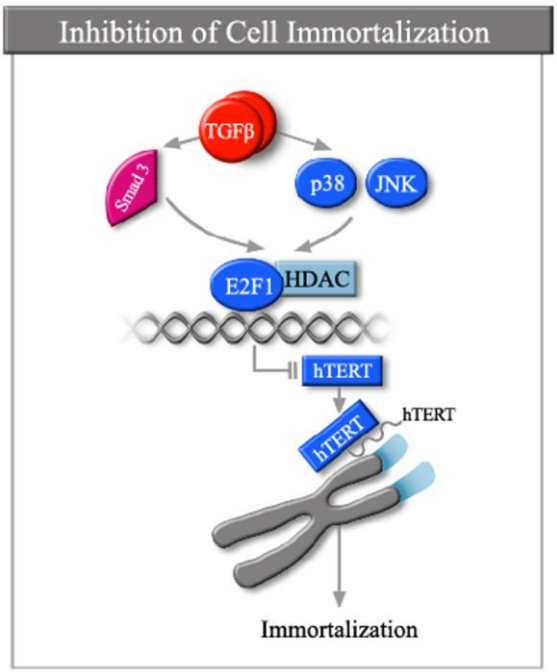

Figure 1-16: TGFβ-mediated inhibition of cell immortalization.

TGFβ mediates the inhibition of cell immortalization through the Smad, p38, and JNK pathways via recruiting histone deacetylases (HDAC) to the telomerase (hTERT) gene promoter, therefore downregulating telomerase, thus inhibiting cell immortalization. (Reproduced from Lebrun 2012 [284])

1.3.3.2 Loss of Tumour-Suppressive Effects

Tumour development and progression could result from the loss of the important TGFβ-mediated tumour suppressive effects due to both genetic and epigenetic modifications in the TGFβ signalling pathway components, as demonstrated by the inactivating mutations in TGFβ receptors or Smads causing different types of cancers [254, 284, 285, 329, 405, 406].

1.3.3.2.1 Defective TGFβ Receptors

Genetic inactivating mutations in the TβRII alleles resulting in either a truncated protein or a dysfunctional kinase domain occur in several cancers with impaired DNA mismatch repair e.g. biliary, colorectal, esophageal, gastric, head and neck, ovarian, pulmonary cancers, and to a lesser extent in breast, endometrial, hepatic and pancreatic cancers. Also, frameshift and missense mutations in the TβRI were observed in esophageal, head and neck, ovarian, pancreatic as well as metastatic breast cancers [17]. The repression of the TGFβ receptor expression could also happen due to epigenetic deregulation (through hypermethylation of their promoters) as well as aberrations in their transcription factors [407]. Experimental evidence shows that the TGFβ receptors possess a tumour-suppressive role, at least at the early stages of carcinoma, where TβRII expression inhibits growth and anchorage-independent growth in different cancers *in vivo* [408, 409] while silencing TβRII increased tumorigenicity and was associated with higher tumour grade [407].

1.3.3.2.2 Defective TGFβ Effectors

Genetic mutations in the MH2 (MAD homology 2) domain of the Smad genes (due to deletions, frameshift mutations, nonsense, and missense mutations or loss of chromosome regions) were found to occur frequently in human cancer, thus preventing Smad heterotrimer formation and loss of their transcriptional activity [269]. Noteworthy, most mutations occur in Smad2 and Smad4 genes [410, 411], whereas it is infrequent in the Smad3 gene [412]. Smad2 was found to be mutated in a small set of colorectal, hepatic and lung cancers [405, 406, 413], whereas Smad3 was deleted in gastric cancer and T-cell lymphoblastic leukemia [412]. Smad4 was found to be mutated or deleted in almost half of the human pancreatic cancers hence its name deleted in pancreatic cancer [414]. Smad7 was found to be overexpressed in pancreatic [415],

endometrial [416] and thyroid follicular [417] cancers, thus inhibiting the Smad-mediated signalling cascade. Smads could also lose their tumour suppressive role due to the high expression of their transcriptional repressors Ski Proto-Oncogene (SKI) and its closely related SKI Like Proto-Oncogene (SnoN). However, Ski and SnoN pro-oncogenic activity appear to be context-dependent, as evidence suggested a potential anti-oncogenic activity for both. [418, 419].

1.3.3.3 Tumour-Promoting Effects

TGFβ acts as a tumour suppressor in normal cells and early carcinoma, however, during tumorigenesis the TGFβ-mediated growth-inhibitory and pro-apoptotic effects are lost, enabling TGFβ-mediated tumour progression and pro-metastatic effects on both the tumour cells and their surrounding microenvironment [259, 285, 420]. Cancer cells produce and secrete TGFβ that permits the tumour cell to remodel the surrounding ECM proteins via upregulating metalloproteinases (MMP) and plasmin, further promoting ECM degradation subsequently releasing stored latent TGFβ. Enhanced TGFβ levels induce tumour angiogenesis, myofibroblast differentiation, as well as immunosuppression, thus promoting tumour progression and metastasis. TGFβ inhibits adhesion of tumour cells, induces EMT, promotes cell migration and invasion, and induces chemotaxis to distant organs, thus contributing to metastasis development [259, 285, 420] (Figure 1-17).

1.3.3.3.1.1 Angiogenesis

Angiogenesis is the synthesis of new blood vessels from pre-existing ones, therefore playing essential physiological roles (development, wound healing, and granulation) as well as being a hallmark of tumour transformation[421]. TGFβ induces angiogenesis [422] thus facilitating tumour cell intravasation to the systemic blood circulation, and the subsequent tumour cell metastasis to distant organs [423, 424].

TGFβ upregulates several angiogenic factors in epithelial cells and fibroblasts e.g. vascular endothelial growth factor (VEGF) [425], and connective tissue growth factors (CTGF) which promote cell proliferation and migration [426]. In addition, TGFβ inhibits Angiopoietin-1 protein responsible for vessel maturity and stability thus conferring the permeability of the tumour-associated blood vessels [427]. TGFβ enhances MMP expression, production and activity

thus enabling endothelial cells to be released from the basement membrane causing the dissolution of mature vessels as well as their migration and invasion [428].

1.3.3.3.1.2 Epithelial-Mesenchymal Transition

The EMT is a normal physiological process occurring during embryogenesis and development which involves - the differentiation of epithelial cells from a highly-organized, tightly-connected, immotile state into loosely-organized, motile, stem cell-like mesenchymal cells. EMT is characterized by many molecular, transcriptional, subcellular modifications that eventually result in subsequent invasive cellular behaviour [429]. TGFβ-induced Smad-dependent EMT changes include the disintegration of epithelial tight junctions and basolateral adherens junctions, the loss of epithelial cell polarity, the disruption of cell-cell adhesion and cell-substratum adhesion, the reorganization of the actin cytoskeleton into actin stress fibres, the increased secretion of extracellular proteases, increased expression of Snail and Slug, Twist and ZEB family which lead to the loss of epithelial markers (claudins, cytokeratins 8, 18 and 19, E-Cadherin, α6β4 integrins, occludins, ZO-1) and the induction of mesenchymal markers (fibronectin, N-cadherin, β1 and β3 integrins, MMPs, tenascin-C, vimentin, vitronectin) [430, 431]. TGFβ signalling pathway has cross-talk with different signalling pathways e.g. MAPK/ERK, PI3K/AKT and Rho GTPases, to regulate EMT [282, 431]

1.3.3.3.1.3 Cell Migration and Invasion

TGFβ signalling was shown to induce EMT and cell motility through ligand-dependent activation of TβRII [432] and TβRI [433]. TGFβ activates Rho-GTPases that regulate lamellipodia-mediated cell motility. TGFβ also upregulates MMPs and plasmin expression and production, thus facilitating extracellular modifications [434].

1.3.3.3.1.4 Cell Metastasis and Chemotaxis

TGFβ-mediated induction of tumour cell local invasion is considered the first event of a sequence of events leading to the formation of a secondary tumour [435]. Next, TGFβ would induce tumour cells to intravasate i.e. enter the blood circulation, disseminate throughout the body, extravasate i.e. exit the blood circulation to another organ, survive and grow in the new

microenvironment, forming a new malignant colony [258]. TGFβ directs metastasizing tumour cells to specific tissues [436-439]. Tumour cells produce TGFβ, further inducing IL-1 and IL-6 expression [440, 441] promoting the differentiation of bone-marrow-derived progenitor cells into the osteoclasts responsible for bone resorption and increased bone metastasis [442]. Additionally, TGFβ stimulates the secretion of IL-11 and parathyroid hormone-related protein (PTHrP) secretion in cancer cells, thus inducing osteoblasts differentiation into osteoclasts, leading to bone resorption and increased bone metastasis [443-445]. TGFβ could promote pulmonary metastasis via inducing the expression of angiopoietin-like 4 (ANGPTL4), cyclooxygenase-2 (COX2), epidermal growth factor receptor (EGFR) all of which contribute to both lung metastasis [438, 445] as well as brain metastasis [446].

1.3.3.3.1.5 Immunosuppression

TGFβ functions as an anti-inflammatory agent in normal tissues and early stages of cancer. Nonetheless, tumour cells have high TGFβ levels that cause them to evade immunosurveillance. Several reports showed TGFβ to suppress the differentiation and proliferation and apoptosis of T and B lymphocytes, as well as dendritic cells, lymphokine-activated killer cells, macrophages, natural killer cells, as well as neutrophils [441, 447-455].

TGFβ/Smad signalling implicates the phosphatase SHIP to suppress the Akt-mediated survival pathway, therefore promoting apoptosis in B and T lymphocytes [369, 370] and implicates the Activating Transcription Factor (ATF1) to inhibit the synthesis of five cytolytic gene products-namely, granzyme A, granzyme B, Fas ligand, interferon γ and perforin in $CD8^+$ T lymphocytes [456]. TGFβ could induce the CDKIs p21 and p27 as well as suppress IL-2 thus inhibiting the proliferation of T cells [451]. Moreover, TGFβ was shown to attenuate the major histocompatibility complex class II antigens rendering the tumour cell surface less immunogenic due to compromised tumour-specific antigen presentation ability as well as prevent the maturation of dendritic cells, which in turn fail to stimulate T lymphocytes [457-462].

1.3.3.3.1.6 Myofibroblasts

TGFβ was shown to induce the differentiation precursor fibroblasts into myofibroblasts also known as cancer-associated fibroblasts (CAFs)[463] which are mesenchymal cells with the

properties of both smooth muscle cells and fibroblasts that secrete several cytokines, ECM components, growth, pro-angiogenic, and pro-invasive factors, thus promoting tumour cells invasion [464, 465].

Through both auto-stimulation and cross-activation, both TGFβ and the stromal cell-derived factor 1 (SDF1) induce fibroblasts differentiation into myofibroblasts [466]. Also, TGFβ increases the number and invasion rate of CAFs [467] as well as increasing urokinase-type plasminogen activator (uPA) production and secretion in human breast CAFs [468].

Multiple reports indicated several potential cellular origins of CAFs including resident fibroblasts, cancer epithelial cells, endothelial cells, mesenchymal stem cells from the bone marrow, adipose tissue-derived stem cells that differentiate to CAFs through the paracrine secretion of TGFβ from tumour cells [469].

Taken together, as described above, TGFβ plays a tumour-suppressive role through inducing growth inhibition, apoptosis, and stalling cell immortalization. As the tumour progresses, these TGFβ-induced tumour-suppressive responses are gradually lost, being replaced by pro-tumorigenic effects primarily by inducing EMT thus increasing the invasiveness of tumour primary cells and distant metastasis. Moreover, excessive production of TGFβ by tumour cells equally affects the surrounding immediate and distant microenvironment, by diminishing cell adhesion, causing immunosuppression, promoting cell migration, increasing angiogenesis, hence potentiating the entire metastatic process. Thus, TGFβ plays a major role in cancer development and progression (Figure 1-18).

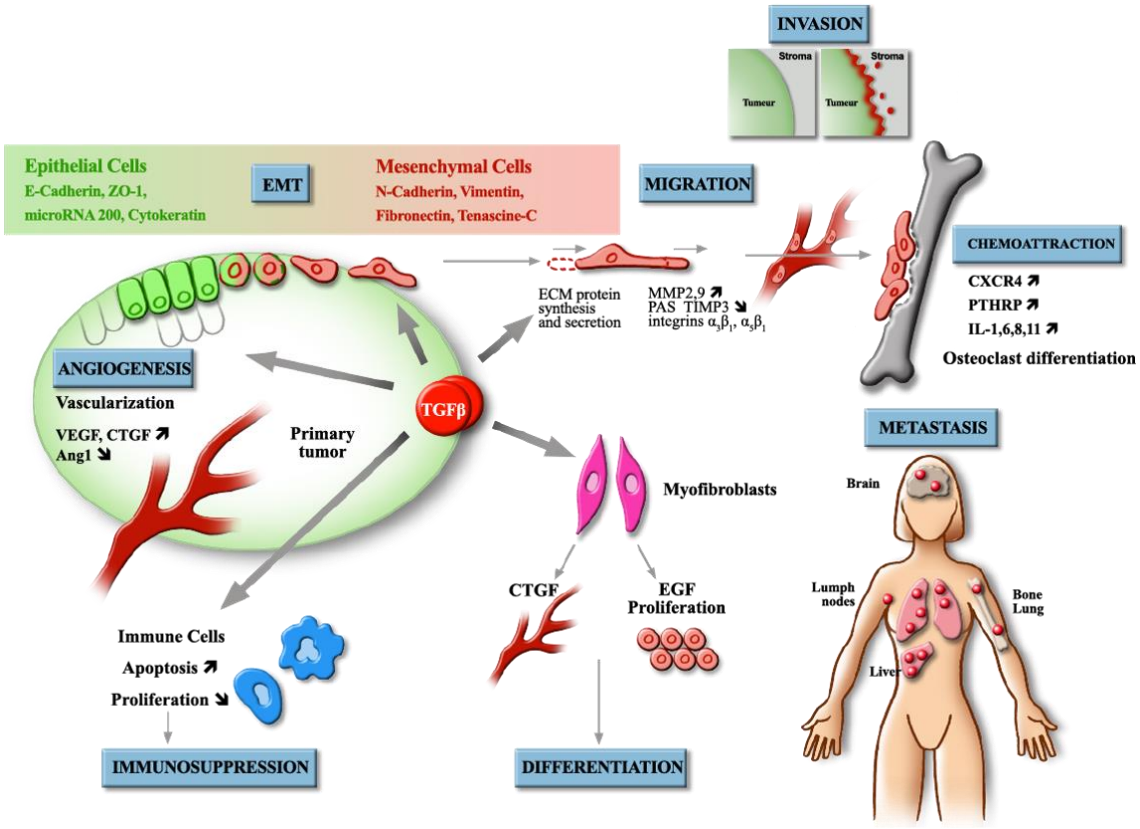

Figure 1-17: TGFβ prometastatic effects.

Cancer cells are known to synthesize and secrete TGFβ, thus eliciting both autocrine and paracrine effects that promote tumour development, growth and dissemination.

(i) Autocrine Effects: TGFβ affects cancer cells by inducing EMT, tumour cell migration and invasion as well as distant metastasis.

(ii) Paracrine Effects: TGFβ affects ECM inducing angiogenesis, vasculogenesis, blood vessel permeability, immunosuppression, as well as myofibroblast differentiation. (Reproduced from Lebrun 2012 [284]).

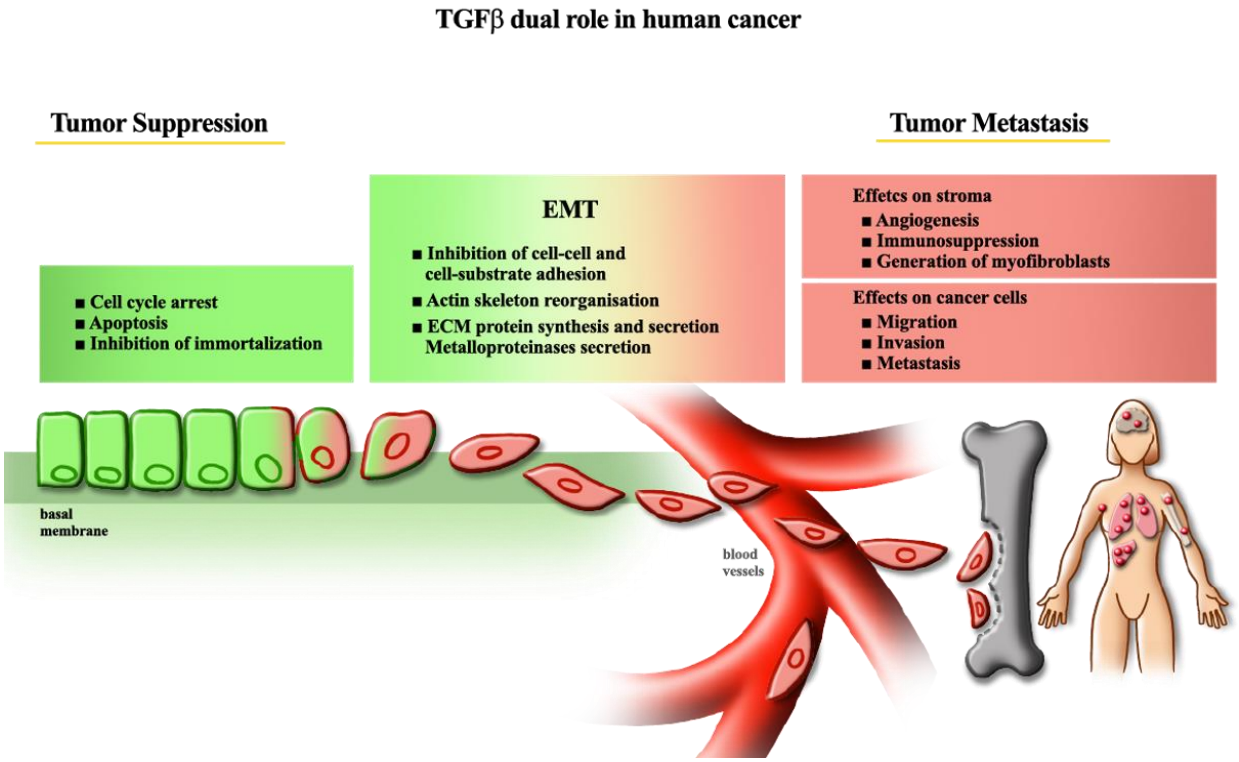

Figure 1-18: The dual role of TGFβ in human cancer.

In normal cells and early carcinomas, TGFβ plays a tumour-suppressive role, whereas in advanced cancers it plays a tumour-promoting role. TGFβ-mediated tumour suppression occurs through the induction of cell cycle arrest, apoptosis, and inhibition of cell immortalization. TGFβ-mediated tumour promotion occurs through inducing angiogenesis, immunosuppression, myofibroblast generation, chemoattraction, EMT, cytoskeletal reorganization and MMPs production all of which further promote tumour cell migration, invasion, and metastasis. Carcinomas demonstrated the decrease or loss of TGFβ-mediated tumour suppression but not tumour-promotion when the TGFβ signalling pathway is subject to genetic alterations (Reproduced from Lebrun 2012 [284]).

1.4 TGFβ in Cutaneous Melanoma

The dual role for TGFβ described above has been extensively investigated for certain cancers e.g. breast cancer, yet TGFβ showed a contextual role that is tissue or organ-specific.

TGFβ was found to inhibit the migration and invasiveness of other cancers, e.g. colon carcinoma cells [470]. TGFβ also suppressed the migration of Müller cells during retinal development, further compromising the angiogenic process [471], suppressed renal epithelial cell migration [472] and uveal melanoma cell invasion [473]. In melanoma, these TGFβ effects have not been thoroughly investigated.

1.4.1 Growth Inhibition and Melanoma Suppression

The TGFβ tumour suppressive mechanisms in melanocytes and melanoma remain controversial [474]. Many *in vitro* studies demonstrated that TGFβ elicits growth-inhibitory effects in melanocytes, whereby these effects were shown to be compromised or lost in various melanoma cell lines [475-481].

On one hand, TGFβ was shown to induce cell cycle arrest in melanoma cells, increasing the percentage of the cell population in the G0/G1 phase while decreasing those in the S or G2/M phases [482]. Similarly, in the melanoma cell lines WM35 [483] and UCD-Mel-N [484] lacking p15, TGFβ could induce cell cycle arrest via upregulating p21. In accordance, a previous study from our lab showed that the TGFβ-mediated upregulation of LIF was found to increase the transcription of p21 in a STAT3-dependent manner thus inducing cell cycle arrest [173]. On the other hand, in the melanoma cell lines 1205Lu [485] M000921, and M01081 [486], TGFβ stimulation did not upregulate p21, yet it showed significant induction of p15 [486].

Some studies reported the loss of the TGFβ-mediated growth inhibition in melanoma cells, nonetheless, TGFβ was able to induce the Smad signalling pathway in these cells, indicating that TGFβ signalling is functional and active in melanoma cells and that the reported loss of the TGFβ-mediated growth inhibition is independent of the TGFβ/Smad transcriptional regulation [479, 487]. Indeed, in melanoma, neither TβRII [488] nor TGFβ signalling molecules [170] showed any genetic mutations to justify this reported loss of the growth inhibitory effects of TGFβ.

Some mechanisms have been proposed to explain how some melanoma cells could avert the TGFβ-mediated growth-inhibitory effects [479, 488]. Reports showed that Ski and SnoN could negatively regulate the TGFβ-mediated Smad2/Smad3 activation thus disabling the TGFβ-mediated growth-inhibitory effects in melanoma cells [418, 484, 488-491]. Melanoma inhibitory activity protein (MIA) has been reported to positively regulate Ski and SnoN expression which results in downregulating Smad2/Smad3 expression in metastatic melanoma cells, thus contributing to averting the TGFβ-mediated growth inhibitory effects [492]. TGFβ-mediated growth inhibition was also attributed to aberrant TGFβ-mediated cell-cycle arrest due to the inhibition of p21 by Ski [484] or the loss of p16 and p15 as a result of the frequent genetic mutation of the *CDKN2A* and *CDKN2B* genes respectively [493, 494] or the loss of p27 [495] or

through p27 phosphorylation and mislocalization [474, 496] or the overexpression of cyclin D1 [497, 498] or the overexpression of cMyc [497, 499].

1.4.2 Migration, Invasion, Metastasis and Melanoma Progression

To date, the role of TGFβ in melanoma progression has not been entirely elucidated. Only one research group, apart from ours, produced the available studies. They reported that overexpressing the inhibitory Smad7 resulted in reducing both MMP-2 and MMP-9 secretion as well as reducing the invasion of melanoma cells in Matrigel™ without altering their migratory and adhesive capacity [500]. Besides, they reported that overexpressing Smad7 decreased bone metastasis formation of melanoma cells *in vivo*, therefore, improving patient survival. TGFβ inhibition -via Smad7 or TβRI inhibitor SB431542- were shown to cause repression of certain osteocytes genes e.g. *CXCR4*, *IL-11*, *OPN* and *PTHrP* [487]. Inhibiting the kinase activity of TβRI diminished the melanoma cell invasion through Matrigel™ and reduced the expression of the TGFβ target genes e.g. *CTGF, IL-11*, *PTHrP* and *RUNX2 in vitro* as well as delaying the development of osteolytic bone metastases and reduced their size [501].

Contrarily, TGFβ was shown to inhibit melanoma progression, where TGFβ inhibited melanoma tumour growth in murine models *in vivo* as well as potently inhibited melanoma cell invasion through Matrigel™ *in vitro*, by promoting the plasminogen activator inhibitor PAI-1 [172]. Previous studies from our research group showed that TGFβ-mediated upregulation of the Plasminogen Activator Inhibitor-1 (PAI-1) in melanoma cells could decrease plasmin generation and activity, thus reducing cell invasion and migration [171]. Moreover, we identified, in a study that I co-authored, that the TGFβ/LIF/STAT3 signalling pathway acts as a novel tumour suppressive-like pathway in melanoma via inducing cell cycle arrest, and cell death as well as inhibiting cell migration [173]. These contradictory reports indicate a significant need to investigate the role of TGFβ in melanoma progression.

1.5 Axin-Upregulated Protein1 (AXUD1)

AXUD1 encodes a 64-kDa nuclear protein containing about 589 amino acids [502], which was also termed Cysteine and Serine Rich Nuclear Protein 1 (CSRNP-1) and was characterized as a member of a new family of genes that encodes nuclear proteins comprising

cysteine- and serine-rich domains [503]. AXUD1 was cloned by Ishiguro and his research group [502] downstream of AXIN1 which encodes one of the apoptotic proteins induced by TGFβ in human hepatocellular carcinoma cells, hence its previous name TGFβ-Induced Apoptosis Protein 3 (TAIP3). AXUD1 is a downstream responsive protein for AXIN1 that negatively regulates the canonical Wnt/β-catenin pathway whose dysregulation was shown to result in tumorigenesis in several human cancers including brain [504], colorectal [505], hepatocellular [506], intestinal [507], prostate [508], skin [509], uterine [510] cancer. Indeed, while AXUD1 expression is high in normal tissues, it was found to be lower upon malignant transformation e.g. colon, kidney, liver and lung cancers [502] as well as oral squamous cell carcinoma tissues [511], thus indicating a potential tumour-suppressive role for AXUD1. In agreement, the Drosophila Axud1 orthologue (DAxud1) was found to play a tumour suppressive-like role in *Drosophila*, by inducing growth inhibition and apoptosis in imaginal cells. Knocking down *DAxud1* increased the proliferation of imaginal cells, whereas *DAxud1* overexpression impeded cell cycle progression at the mitotic phase [512]. Apart from these and other few reports, little is known about the functions and roles of AXUD1.

1.6 Multiple Endocrine Neoplasia Type 1 (MEN1)

1.6.1 MEN1 Gene

Multiple Endocrine Neoplasia Type 1 (MEN1) is a rare autosomal dominant disease due to mutations in the MEN1 gene whose most remarkable clinical features include the development of hyperplasia and/or neoplasia of parathyroid, pituitary and pancreatic islet cells [513, 514]). Hyperparathyroidism occurs in almost 90% of patients, pancreatic neuroendocrine tumours in 60% of patients, and pituitary adenomas in 40% of patients [515-517]. MEN1 is located on chromosome 11q13 in humans [518, 519] and it is highly conserved among various species such as humans, mice, rats, zebrafish and *Drosophila* [520, 521]. MEN1 mutations span over the entire genome including the intronic and promoter regions with no significant clustering or hot spots, where approximately 70% of the mutations are nonsense and frameshift mutations, leading to the truncation of the protein product menin [522].

1.6.2 Menin Protein

MEN1 gene encodes for the protein menin, which is a 67kDa nuclear protein (610 amino acids) expressed ubiquitously and abundantly throughout the body [518, 523, 524]. Menin is a putative tumour suppressor that plays a role in cell cycle progression, DNA repair, or DNA replication or transcriptional regulation [525]. Menin was shown to bind DNA in a non-specific manner, where *MEN1* mutations near the C-terminus abrogated DNA binding with a subsequent failure to inhibit cell proliferation and G2/M transition [526, 527]. Silencing menin was shown to increase proliferation, induce cell cycle progression, and inhibit apoptosis [528] while reconstituting its expression in *Men1*-deficient tumour cells could induce cell cycle arrest and apoptosis [529]. Menin was found to mediate TGFβ-induced cell cycle arrest in pituitary tumour cells [349]. Higher levels of expression were observed in actively proliferating cells, where it was colocalized with telomeres in meiotic cells but not in somatic cells [530], thus suggesting a cell-dependent regulatory role. Menin was shown to target several genes that are crucial for both cell proliferation and development, such as the cyclin-dependent kinase inhibitors p18 and p27 (*CDKN2C* and *CDKN1B)* [531-533], homeobox domain (*HOXA9 and HOXC8*), the human telomerase (*hTERT*) [534] as well as nuclear receptor target genes [521, 532, 535].

Menin was shown to interact with several nuclear partners [514] such as transcription factors [including JunD [536], NF-κB [537] and Smads [349, 538, 539]], DNA processing machinery, cytoskeleton-associated proteins as well as components of DNA repair mechanisms [524, 540-542]. Menin was shown to act as a transcriptional co-regulator, functioning as a co-activator or co-repressor via recruitment of histone-modifying enzymatic activity [521, 535, 536], where menin can act as a co-activator via regulating histone methylation [535, 543], thus enabling menin to bind to nuclear receptors and activate nuclear receptor-mediated gene transcription [521, 535]. On the other hand, menin can act as a co-repressor via tethering histone deacetylase activity to genes [544]. (Figure 1-19).

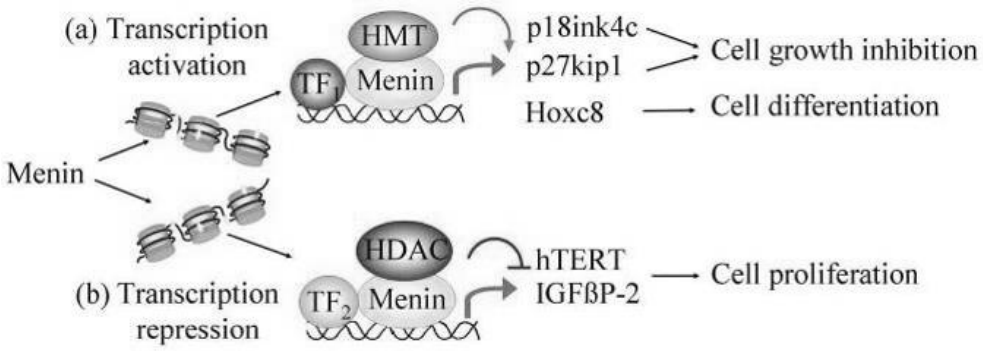

Figure 1-19: A schematic diagram for menin-mediated regulation of gene transcription.

(a) Transcription Activation: Menin binds with a specific transcription factor (TF_1), together with transcription-activating histone methyltransferases (HMTs), to target the loci of *p18*, *p27* and *Hoxc8* genes in chromatin, changing the conformational structure of the latter, thus activating the transcription of the target genes.

(b) Transcription Repression: Menin binds with a specific transcription factor (TF_2), together with a histone deacetylase (HDAC), to target the loci of the *hTERT* and *IGFBP-2* genes in chromatin, thus repressing the transcription of the target genes. (Adapted from Yang and Hua 2007 [545])

1.6.3 Menin Functions

Menin functions as a scaffolding protein in the nucleus to regulate gene transcription by coordinating chromatin remodelling [521, 525, 546]. The transcription factor JunD was shown to elicit menin-dependent cellular effects where JunD acts as a growth suppressor in the presence of menin [547], but as a growth promoter in its absence [548, 549].

1.6.3.1 Mediation of TGFβ Signalling

Reports show that menin mediates TGFβ/Smad3 signalling. Indeed, menin was found to mediate TGFβ-induced Smad3-dependent transcriptional activity through Smad3/Smad4-DNA binding at its specific transcriptional regulatory sites, whereby silencing or mutating menin would abolish those transcriptional effects [349, 550] (Figure 1-20).

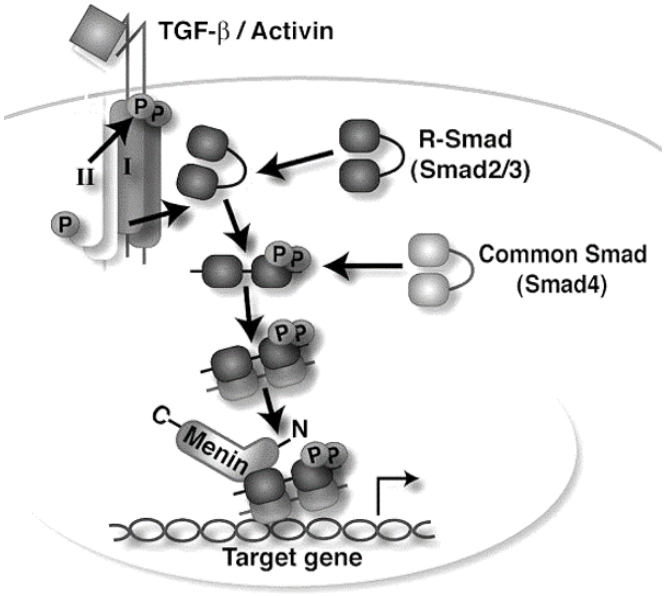

Figure 1-20: Menin and TGFβ /Activin signalling pathway.

TGFβ and activin signal through their respective Type I and Type II receptors leading to Smad2/Smad3 phosphorylation and heterotrimer complex formation with Smad4, whereby it translocates inside the nucleus to regulate the transcription of various genes. Menin binds to Smad3 inside the nucleus to facilitate Smad-mediated transcription (Reproduced from Hendy *et al* 2005 [348]).

1.6.3.2 Induction of Apoptosis

Both experimental and clinical evidence suggests the potential role of menin in the regulation of apoptosis and that menin loss of function could contribute to β-cell tumorigenesis in MEN1 patients [545, 551]. Menin overexpression in pancreatic tumour cells increased apoptosis via amplifying caspase-3 activation, increasing p53 acetylation, and enhancing p21 activation, thereby leading to promoting caspase-3 and caspase-9 activities after cell gamma-irradiation [552]. Moreover, menin was shown to induce Bax- and Bak-mediated apoptosis pathway during embryonic development and tissue homeostasis [553]. Menin overexpression induced apoptosis in murine embryonic fibroblasts, while menin deficiency abolished apoptosis in response to UV irradiation or TNF-α stimulation [554]. Menin was reported to induce apoptosis via upregulating caspase-8 expression and promoting TNF-α-mediated apoptosis [555, 556] (Figure 1-21)

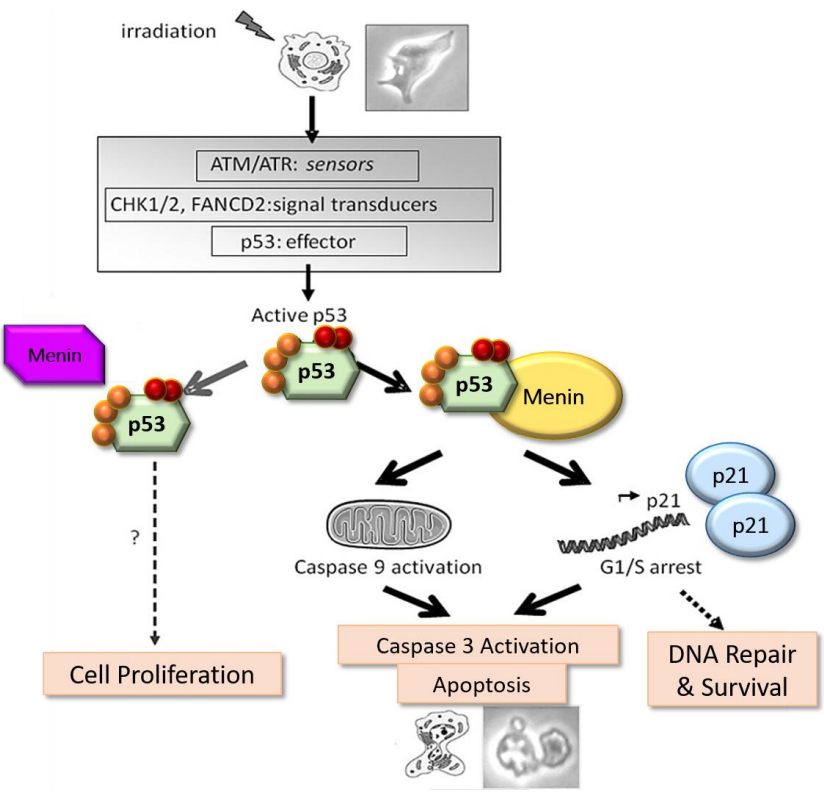

Figure 1-21: A schematic diagram of menin cross-talk with pathways activated in response to genotoxic stress.

In response to DNA damage, the sensor molecules ATM and ATR trigger p53 phosphorylation. Menin overexpression results in the acetylation of p53 causing its stabilization and activation, thus activating p21 to induce cell-cycle arrest to repair damaged DNA. Menin mediates the translocation of p53 to the mitochondria, triggering procaspase-3 cleavage, and thereby leading to apoptosis as a result of any failure of DNA repair (Adapted from Bazzi *et al.* 2008 [552]).

1.6.3.3 Inhibition of Proliferation & Cell Cycle

Menin expression was shown to inhibit cell proliferation in MEFs [557], in human endocrine tumour cells [558] and insulinoma cells [559]. Moreover, *MEN1* gene replacement therapy in *Men*$^{+/-}$ mice could re-establish menin expression in pituitary tumour cells, thus inhibiting tumour cell proliferation and growth *in vivo* [560].

Of interest, menin was found to bind directly to the double-stranded DNA in a sequence-independent fashion, where this regulates cell proliferation by blocking cycle progression at the G2/M phase [554]. Menin was shown to elicit a cytostatic effect, by repressing the expression of

cyclin D1/3 and CDK4 responsible for inducing G1/S transition in the cell cycle [561, 562], therefore inducing in G1 phase cell cycle arrest [563]. Menin is also essential for JunD-mediated inhibition of cell proliferation [548]. Menin suppresses tumorigenesis via inhibiting of G1/S transition, where silencing *Men1* in MEFs accelerated G1/S phase entry with a decrease in the expression of the CDK inhibitors p18 and p27 as well as an increase in CDK2 activity [528]. Also, menin was found to mediate TGFβ-induced growth inhibition via transcriptional regulation [523] and cell cycle control [564]. Menin was found to interact with the activator of S-phase kinase (ASK) inhibiting cell proliferation potentially via disrupting ASK-induced DNA replication [527, 565] (Figure 1-22).

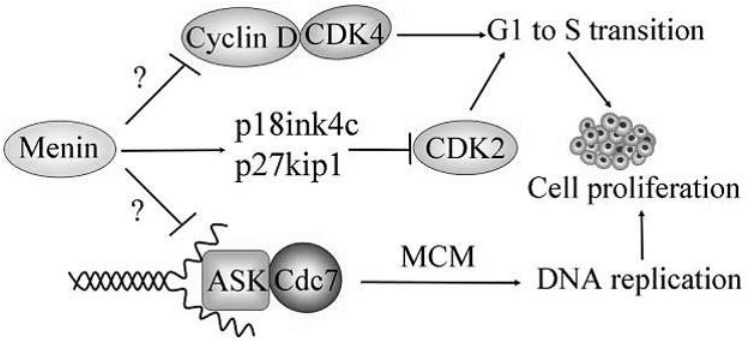

Figure 1-22: A schematic diagram for menin-mediated regulation of cell proliferation.

Menin, through either transcriptional or post-transcriptional regulation, can inhibit the kinase activity of CDK4/Cyclin D, CDK2/Cyclin A or E, and Cdc7/ASK, thus inhibiting cell cycle transition from G1 to S phase (Adapted from Yang and Hua 2007 [545]).

1.6.3.4 Parathyroid and Bones

Menin plays a role in the autocrine/paracrine TGFβ-induced inhibition of parathyroid cell proliferation and parathyroid hormone secretion, where the loss of TGFβ signalling due to menin inactivation could contribute to parathyroid tumorigenesis [539]. Menin plays a role in bone development and remodelling, and it is essential for multipotential mesenchymal stem cells commit to the more specialized osteoblasts [566] due to the role of menin in facilitating BMP signalling via Smad1/Smad5 and the transcriptional activity of the osteoblast regulator, Runx2 [539] (Figure 1-23). Moreover, menin was found to bind physically to JunD thus inhibiting the differentiation of committed osteoblasts, as well as osteoblast maturation, in part via abolishing JunD-mediated osteoblastic differentiation [567].

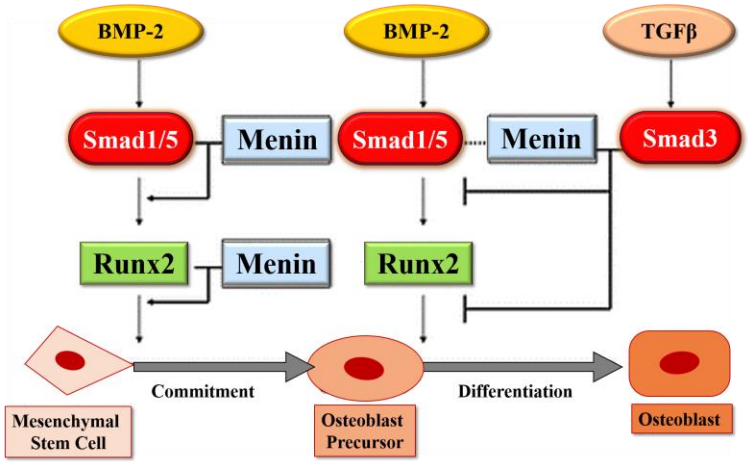

Figure 1-23: The role of menin and BMP-2 pathway/Runx2/Smad3 in osteoblastogenesis and osteoblast differentiation.

Menin interacts with Smad1/5 and the osteoblast regulator Runx2 to induce the differentiation of the multipotent mesenchymal stem cells to immature osteoblasts. Contrarily, menin interacts with Smad3 to inhibit the BMP2/Runx2 pathway to impede osteoblast differentiation at a later stage (Adapted from Kaji 2012 [568]).

1.7 Melanoma Stem Cells

1.7.1 Melanoma Stem Cells (MSCs) and Cutaneous Melanoma

In addition to exposure to UV radiation that presents the main environmental melanomagenic factor causing cutaneous melanoma, other epidemiological factors such as family history, genetic and molecular factors are also involved [27, 75, 116]. Melanoma development was once described as a process of 'de-differentiation' of mature melanocytes, thus allowing the eventual dissemination of the malignant cells [569-571]. Since most melanomas do not arise in dysplastic nevi but rather in normal skin [572, 573], theories adopting the cancer-stem-cell (CSC) concept proposed that melanomas arise due to the mutation of melanocyte stem cells or immature progenitor cells residing in the skin [574-579]. Studies even showed that neural crest stem cells (NCSCs) play a role in the initiation and propagation of melanoma cells (e.g. *Sox10* gene) due to the similarity in the gene network of NCSCs (involved in development and wound healing) and melanoma cells (involved in cancer growth and progression) [580, 581].

1.7.2 Cancer Stem Cells (CSCs)

According to the hierarchical model, CSCs are a rare biologically-distinct subpopulation of cells within a tumour, that is tumorigenic with a self-renewal potential and when tested by serial xenotransplantation they can give rise to a progeny that grows and differentiates yielding the heterogeneous lineages of the original tumour (both tumorigenic and non-tumorigenic) thus helping the tumour to sustain its propagation [582, 583] as demonstrated in the early characterized hematopoietic lineage [584, 585] and subsequently in various solid tumours such as breast [586], brain [587], and colon [588-590] cancers.

According to the stochastic model, all tumour cells are assumed to be biologically equivalent, where they could be transformed by intrinsic and extrinsic factors into a cancer cell progeny with the potential to self-renew and acquire plasticity to go from a non-stem cell to a stem cell-like precursor. Therefore, together these two models could possibly clarify tumour growth, progress, and sustained propagation [591]. Surprisingly, neither model explained the phenomenon of dormancy/quiescence, which contributes to the development of resistance to conventional chemotherapies, given that CSCs are reported to be highly refractory to drugs and cytotoxins e.g. via upregulating ABC transporters [575, 592-594], thus resulting in tumour relapse and metastasis. Therefore, it is vital to eliminate these cells to avoid potential relapse and attain a positive outcome for cancer patients [595] (Figure 1-24, Figure 1-25).

1.7.3 Melanoma Stem Cells

Similarly, different models used to characterize intra-tumoural heterogeneity of melanoma and identify melanoma stem cells are quite controversial to this date. In human malignant melanomas, considerable evidence demonstrated both inter- and intra-tumoural heterogeneity, and the presence of melanoma-initiating stem-like cell subpopulation (MSCs) [596-598]. Compared to the tumour bulk cells, MSCs were shown to have high *in vivo* tumorigenicity, high embryonic-like plasticity into multiple cell lineages, high self-renewal potential in xenografts *in vivo* and in long-term cultures *in vitro*, high metastatic potential and to develop chemoresistance [574-576, 578, 579, 582, 599, 600]. MSCs -like other CSCs- could activate different pathways to allow them to escape the patients' hostile micro-environment and evade the patient's immunological reactions [601, 602]. MSCs gave rise to non-adherent

tumourospheres (melanospheres) when cultured in a growth medium for human embryonic stem cells under low-anchorage conditions [574, 603]. MSCs could selectively grow under these culture conditions, while differentiated cells rapidly died (Figure 1-24, Figure 1-25).

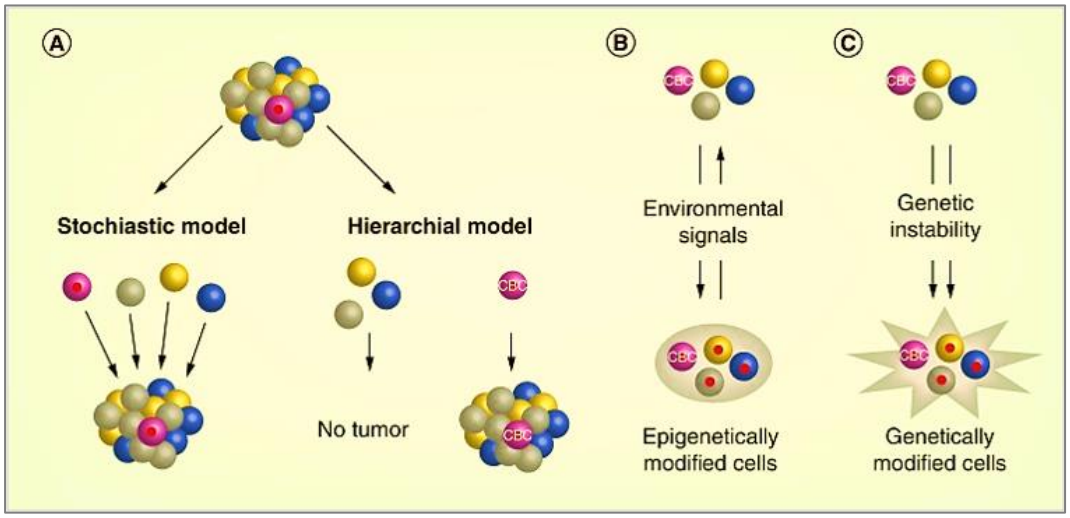

Figure 1-24: Models and dynamics of cancer stem cells.

(A) Tumour heterogeneity has been conventionally explained by two models (i) the hierarchical model that suggests the presence of a biologically-distinct population of CSCs capable of self-renewal and tumour-initiation, and (ii) the stochastic model that suggests the biological equivalence of all tumour cells, whereby any subset of cells could be transformed by intrinsic and extrinsic factors becoming capable of self-renewal and tumour-initiation. Nonetheless, neither model explains the dormancy or quiescence of tumour cells.

(B) Tumour cells are subject to reversible or transient epigenetic alteration in response to the various environmental stress factors.

(C) Tumour cells (CSCs and non-CSCs) are subject to irreversible genetic alterations, whereby these alterations are only propagated if they occur in CSCs, or if these alterations confer stem cell properties, i.e. self-renewal and differentiation, in non-CSCs.

(Adapted from Nguyen *et al.* 2015 [604]).

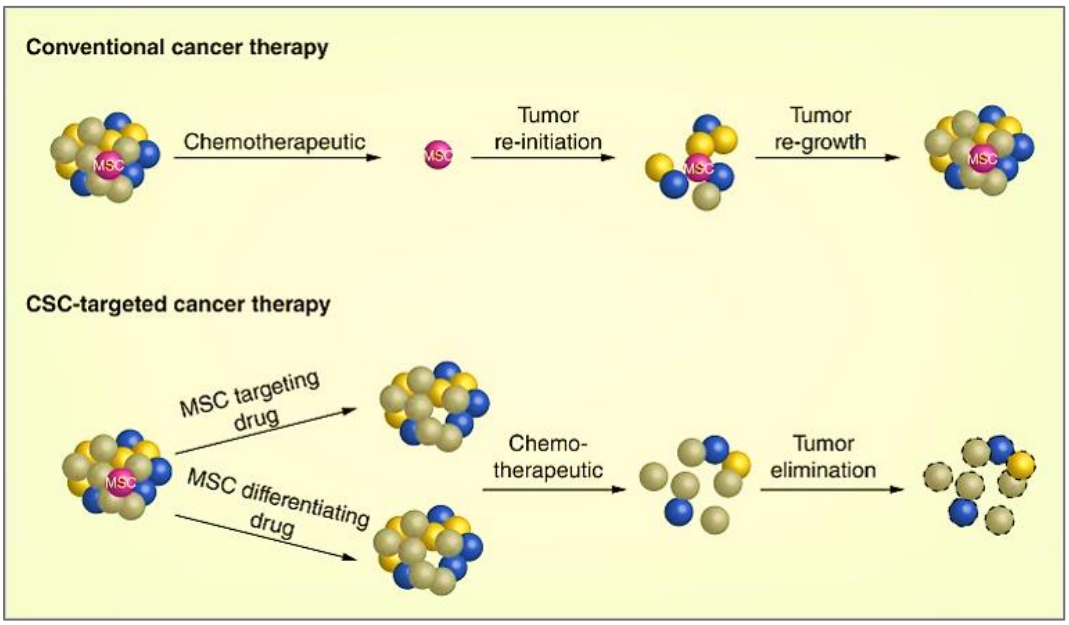

Figure 1-25: Targeting melanoma stem cells.

Conventional cancer therapy aims at targeting the rapidly-proliferating, differentiated tumour cells, but not the dormant/quiescent and slowly-proliferating MSC population responsible for tumour re-initiation and re-growth. CSC-targeted therapy aims at eliminating the MSC population, either via directly targeting the MSCs or using differentiating drugs thus preventing tumour re-initiation and re-growth (Adapted from Nguyen *et al.* 2015 [604]).

1.7.4 Melanoma Stem Cell Markers

Multiple surface markers have been identified with the MSCs including ABCB5 [575, 605], CD20 [574], CD133 [577, 606], CD166, Nestin [607], CD166 [571], CD271 [598, 608, 609] as well as ALDH [610, 611] showing self-renewal and differentiation capacities. Interestingly, ABCB5, CD271, and ALDH were shown to identify overlapping MSC populations [611, 612]. Noteworthy, these CSC markers exist in other cancers, e.g. ABCB5 in colorectal cancer patients [613] and CD271 in glioblastoma [587] and ovarian cancer [614]. Similarly, high activity of ALDH was shown in CSCs of other malignancies e.g. breast [615], colon [616], leukemia [617], ovarian [618], pancreatic [619] and prostate [620].

1.7.4.1 CD133

Neither the normal physiological function nor the role of CD133 in tumorigenesis and melanomagenesis is entirely elucidated. CD133 (Prominin-1) is a five transmembrane domain glycoprotein used conventionally to identify and purify several CSCs from their tumours. Indeed, CD133 was found to be highly expressed in neural stem cells [621], hematopoietic and endothelial progenitor cells [622] as well as CSCs of brain [587], colon [588, 590, 623] and prostate [624] cancers as well as in primary malignant melanoma cells [625]. Reports also showed a partial co-expression of CD133 with ABCB5 and CD271 in human malignant melanoma [575, 612, 626-629].

A large body of evidence described the role of CD133 in identifying MSCs and its correlation with increased potential for tumour-initiation, multipotency, self-renewal, metastasis and conferring chemotherapeutic resistance. Indeed, CD133 expression correlated with more tumour growth, progression, and metastasis [607, 630, 631]. Studies also showed that $CD133^+$ MSCs could increase melanosphere formation and induce tumorigenicity *in vivo* when injected in immunocompromised NOD/SCID mice [577]. Also, $CD133^+$ MSCs could promote tumorigenicity and perivascular niche morphogenesis via inducing vasculogenic mimicry [627], as well as being associated with lymph node and/or visceral metastasis in childhood malignant melanoma [631]. Moreover, $CD133^+$ MSCs showed not only high tumour-initiating capacity and increased lung metastasis, but also the ability to transdifferentiate into an endothelial-like phenotype *in vitro* [632]**.** Also, CD133 was shown to be upregulated in response to chemotherapy [633-636]. This presents CD133 as an important therapeutic target in melanoma patients, where indeed CD133 was shown to be an immunogenic target [625] that could help in eradicating MSCs via T-Cell-induced therapeutic antitumour immunity in melanoma [637].

Like the controversy created over CSCs in other cancer types, some reports suggest that MSCs could be a dynamic and heterogeneous population and that the expression of its "stemness" markers could be reversible and not lineage-specific where tumorigenicity could be caused by microenvironment switches from a proliferative to an invasive phenotype [485, 638-640]. For example, $CD133^+$ and $CD133^-$ melanoma cells were both shown to have the capacity to be tumorigenic in immune-deficient models [641], where similar results were found in some ovarian cancer patients [614]. Also, in metastatic melanoma, the phenotypically distinct $CD133^+$ cells

were shown to transiently exhibit a $CD133^-$ phenotype via downregulating CD133, without switching between lineages [642].

1.7.4.2 ALDH

ALDHs (aldehyde dehydrogenases) is a superfamily of enzymes involved in the biosynthesis of retinoic acid, the oxidation of intracellular aldehydes, as well as the elimination of toxic by-products from reactive oxygen species [643, 644]. ALDH was found to play a crucial role in the hematopoietic stem cell development and differentiation [645-647], due to the modulation of the retinoid signalling. This occurs via the ALDH-mediated dehydrogenation of retinol (vitamin A) into retinoic acid [645]. Notably, ALDH was proposed as a prognostic marker for many cancers including breast [648], colorectal [649], lung [650] ovarian [651] and pancreatic [619] cancers.

Compared to $ALDH1^-$ cells, $ALDH1^+$ cells were shown to contribute to higher tumour initiation, tumour metastasis, and poorer patient prognosis in many types of cancer including breast, colorectal, hematological, liver, oral squamous cell carcinoma, ovarian, pancreatic and prostate cancers [615, 616, 618, 652-660]. Moreover, $ALDH1^+$ cells were reported to confer resistance against conventional chemotherapeutic and immunological agents in different types of tumours [611, 625, 637, 661-665] possibly through increased DNA repair and decreased ROS [666, 667].

Similarly, human malignant melanoma cells displaying high ALDH activity and/or expression ($ALDH^+$) were shown to display stem-cell-like properties with enhanced *in vivo* tumorigenic capacity [610]. Compared to $ALDH^-$, human melanoma cells with $ALDH^+$ were found to be enriched 16.8-fold in tumorigenic cells in the NOD/SCID model, and 100-fold in tumorigenic cells in NSG mice, showing a melanoma-initiating cells (MIC) ratio of 1:21,000 and 1:4 respectively. NSG xenograft mouse models showed a tumour initiation capacity of 1:4 in $ALDH^+$ cells compared to 1:600 in $ALDH^-$ cells and only 1:400 in unfractionated cells. It was reported that *Aldh1a1* suppression decreased the pro-tumorigenic and pro-metastatic properties of B16F10 murine melanoma cells as well as decreasing $CD133^+/CD29^+/CD44^+$ populations, melanosphere size and the expression of the stemness/pluripotency marker Sox2 [668]. On the other hand, one study showed that in malignant aggressive melanoma, $ALDH^+$ cells were

responsible for generating tumour heterogeneity, but not the clonogenic, tumorigenic nor chemoresistance potential when compared to $ALDH^-$ [669].

1.7.4.3 ABCB5, CD271, CD20, Oct4 and Nanog

Amongst the other cell surface markers for MSCs: ABCB5, CD271, CD20 and Oct4, and Nanog. ABCB5 is a human P-glycoprotein 1 (permeability glycoprotein, multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 5), which confers chemoresistance in melanoma via its role as a drug efflux transporter [575]. In malignant melanoma cells, ABCB5 was found to be highly expressed in melanospheres and melanoma cell lines [597] and to be specifically expressed on $CD133^+$ MSCs [575, 587, 588, 590]. CD271 (the low-affinity nerve growth factor receptor) was also reported to show high tumorigenicity in $CD271^+$ melanoma cells when injected in the immunodeficient NOD/SCID mice [608, 609]. CD20 (B-lymphocyte Antigen) was shown to be highly expressed in melanospheres and melanoma cell lines [574, 670]. Oct4 and Nanog (transcription factors involved in maintaining the pluripotency and self-renewal of embryonic stem cells) were found to be highly expressed in melanospheres compared to melanoma monolayers [597].

Noteworthy, these markers cannot exclusively characterize melanoma stem cells since they might lack a biological function, or they might show unstable marker expression, or they might be also expressed in cells other than the cancer stem cells [596, 641, 671]. The lack of definitive markers for characterizing, isolating and separating tumorigenic MSCs presents a real challenge to target them therapeutically. Therefore, functional assays could serve as a better method to assess MSCs rather than mere immunophenotypic analysis [672].

1.7.5 Limitations in CSCs Characterization

Melanoma appears to be the tumour type with the highest frequency of CSCs e.g. up to 27%, while other neoplasms show a very limited presence of these cells e.g. 0.0001% [597]. Limited assays are available for identifying and defining cancer stem cells. Conventionally, xenotransplantation is considered the gold standard method to evaluate cancer stem cells, where transplanting the isolated tumour cells of interest into an immunodeficient mouse model will

demonstrate if the transplanted cells possess the ability -both- to self-renew and to generate progeny *in vivo*.

The discrepancies between the CSC behaviour are due to the removal of tumour cells from their natural environment, the varying levels of immunity available in the different murine strains as well as the difference in techniques for isolating the cell population to be tested and transplanted [670]. Studies use various strains of immunocompromised mouse models, either severe combined immunodeficient (SCID) mice with depleted T and B cells, mice with a non-obese diabetic (NOD) and SCID background having functionally immature macrophages and low level of innate immunity, or NOD/SCID/IL2Rγ^{null} mice that further lack natural killer cell activity [673]. Therefore, the current xenotransplantation models are not ideal since the immune system is usually implicated in malignant melanoma [674]. Besides, the use of Matrigel® to assist in the transplantation process was shown to enhance tumour initiation and growth [675]. Moreover, differences in isolation techniques and enzymes used (type, concentrations, incubation time) could influence the CSC behaviour [575, 596, 605, 608-610, 641]. Some argue that -in that context- the transplanted cells only demonstrate their survival through the isolation and purification process rather than the survival of the tentative cancer stem cell population. However, at present, there is little direct evidence of a separate stem cell pool within unmanipulated solid tumours. Therefore, to obtain more relevant results from the xenotransplantation model, researchers should consider the use of humanized mice or melanoma transgenic models recapitulating human disease conditions in tumour progression and metastasis [598, 676-679].

1.7.6 TGFβ and Stem Cells

Various members of the TGFβ family play crucial roles in the maintenance of self-renewal and pluripotency in embryonic stem cells [680]. In somatic stem cells, TGFβ was shown to regulate the biological functions of the multipotent bone-marrow-derived mesenchymal stem cells, hematopoietic cells, neuroepithelial stem cells as well as melanocyte stem cells. [365, 681-684]. In cancers, TGFβ plays a complex contextual role [284]. TGFβ plays complex roles in CSCs regulation varying between suppression of those stem-like subpopulations [685-687] or their promotion [424, 688-693]. Nonetheless, the role of the TGFβ signalling pathway in regulating MSCs was not investigated and thus requires further elucidation.

Work from our lab identified TGFβ-mediated upregulation of the Plasminogen Activator Inhibitor-1 (PAI-1) in melanoma cells which resulted in decreasing plasmin generation and activity, and therefore reducing cell invasion and migration [171]. In a more recent study, that I co-authored, we characterized the TGFβ/LIF/STAT3 signalling pathway as a novel tumour suppressive-like pathway in melanoma via inducing cell cycle arrest, cell death as well as inhibiting cell migration [173]. As will be explained in chapter three, I identified the Axin Upregulated Protein 1 (AXUD1) and Multiple Endocrine Neoplasia Protein1 (menin) respectively as novel targets downstream of TGFβ in melanoma, mediating TGFβ-induced promotion of cell cycle arrest, growth inhibition, apoptosis and autophagy, as well as hindering cell immortalization, migration *in vitro*. Considering these findings which strongly support the TGFβ role as a potent suppressor of tumour formation and tumour metastasis in melanoma, and the previously stated evidence that CSCs play a significant role in mediating tumour metastasis, we hypothesized that TGFβ could similarly play a role in the regulation of CSC populations in cutaneous melanoma that is worth investigating.

1.8 Rationale

As mentioned above, TGFβ clearly plays complex and crucial roles in different types of cancer. TGFβ has been extensively studied in certain cancers e.g. breast cancer, showing a dual role, being a tumour suppressor in early stages of cancer, inducing cell cycle arrest and apoptosis, and tumour promoter and a prometastatic factor when TGFβ-mediated tumour suppressive effects are lost during cancer progression [254, 257]. Nonetheless, the regulatory role of TGFβ on tumour metastasis was shown to be tissue-specific. For instance, unlike its well-known prometastatic effect in advanced breast cancers [694-697], TGFβ was found to inhibit cell migration, invasion, and EMT in uveal melanoma [473] and retinal Müller glia [471].

In cutaneous melanoma, the role of TGFβ in tumour development and progression remains controversial. Reports showed that inhibiting TβRI with overexpressing the inhibitory Smad7 or a chemical inhibitor could promote tumour cell aggressiveness via autocrine activation of Smad signalling [487, 500]. On the other hand, TGFβ inhibited melanoma progression, where TGFβ was found to inhibit melanoma cell invasion *in vitro* and melanoma tumour growth *in vivo*, by promoting the plasminogen activator inhibitor PAI-1 [172]. In addition, reports from our research group showed that TGFβ-mediated upregulation of the Plasminogen Activator Inhibitor-1 (PAI-

1) in melanoma cells reduced plasmin generation and activity, further inhibiting cell invasion and migration [171]. Furthermore, we identified a novel tumour suppressive-like pathway mediated by TGFβ/LIF/STAT3 signalling inducing cell cycle arrest, cell death and inhibiting cell migration in cutaneous melanoma [173]. These conflicting results require further elucidation of the role of TGFβ in cutaneous melanoma progression.

1.9 Objectives

Transcriptome analysis of multiple TGFβ-responsive cutaneous melanoma cells conducted in our lab revealed several intriguing candidate genes potentially playing a role in the TGFβ signalling. I contributed to investigating the role of an interesting candidate gene LIF, where we found TGFβ-mediated upregulation of LIF expression is required for TGFβ-induced cell cycle arrest and caspase-mediated apoptosis, as well as for TGFβ-mediated inhibition of cell migration. Also, we showed how this TGFβ-mediated LIF upregulation is necessary for activating the transcription of the cell cycle inhibitor p21 in a STAT3-dependent fashion that mediates the TGFβ/LIF-induced cell cycle arrest and induction of apoptosis [173].

My objective in my thesis projects was to further elucidate the role of TGFβ in cutaneous melanoma and investigate the role of other candidate genes, namely AXUD1 and MEN1 downstream of TGFβ. For that purpose, I have used a panel of different human cutaneous melanoma cell lines to examine the role of TGFβ, AXUD1, and menin in regulating cell cycle, apoptosis, autophagy, cell immortalization and migration *in vitro* as described in chapter three. Besides, I examined the role of TGFβ in regulating melanoma stem cells via examining tumour initiation capacity, self-renewal and melanoma stem cell expansion as described in chapter four.

Chapter Two: **Experimental Methods and Materials**

2.1 Reagents and Chemicals

Recombinant human TGFβ was purchased from Peprotech (Dollard des Ormeaux, Quebec, Canada), Tissue culture medium RPMI1640 and DMEM were from Hyclone (Logan, UT, USA), Fetal Bovine Serum, Penicillin/Streptomycin were from GIBCO, MMLV reverse transcriptase and random primers were from Life Science (Grand Island, NY, USA), Flag-tagged CSRNP1 cDNA constructs were a generous gift from Dr. Sebastien Gingras (Pennsylvania University, USA), SQSTM1 (D-3): sc-28359 was a gift from Michael Dahabieh from Dr. Wilson Miller (McGill University, Montreal, Canada). Smad2 (86F7) rabbit polyclonal #3122, Smad3 (C67H9) rabbit polyclonal #9523, cleaved PARP (Asp124) rabbit polyclonal #9541, Caspase 3 rabbit polyclonal #9662 antibodies were purchased from Cell Signalling Technologies (Danvers, MA, USA), Smad2/3 (FL-425) rabbit polyclonal: sc-8332, Smad4 (B-8): sc-7966 mouse monoclonal, β-tubulin (3F3-G2): sc-53140 mouse monoclonal antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), Menin rabbit polyclonal (ab232818) antibody was purchased from Abcam Inc. (Toronto, ON, Canada), Anti-Flag M2 # F1804 mouse monoclonal antibody, branched polyethyleneimine (#408727) from Sigma Aldrich (Saint Louis, MI, USA). D-luciferin was from Roche Diagnostics (Laval, QC, Canada).

2.2 Cell Lines and Cell Culture

All cutaneous melanoma cell lines harbour a V600E mutation in the *BRAF* gene (except DAUV) and are responsive to the Smad-dependent TGFβ stimulation [171, 173]. WM278 (RRID: CVCL_6473) was derived from the primary tumour of a 62-year-old female patient and harbours a hemizygous deletion of *PTEN*. WM793B (RRID: CVCL_8787) was derived from the primary tumour of a 37-year-old male patient, harbours W274X mutation and hemizygous deletion of *PTEN* as well as a mutation K22Q of *CDK4*. 1205Lu (RRID: CVCL_5239) was derived from lung metastases of WM793B after subcutaneous injection into immunocompromised mice [698]. A375m (RRID: CVCL_B222) is the metastatic variant of A375 derived from malignant melanoma in a 54-year-old female patient [699]. SK-Mel-28 (RRID: CVCL_0526) was derived from malignant melanoma in a 51-year-old male patient, harbours a hemizygous deletion of

CDKN1A (p21) as well as a mutation R24C of *CDK4 [700-702]*. DAUV or LB33-MEL.A (RRID: CVCL_E933) was derived from a subcutaneous metastatic lesion (stage IV) in a 42-year-old female patient (WT for BRAF and NRAS) [703-705]. Cells were cultured at 37°C in RPMI1640 or DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin as antimicrobial /antimycotic under a humidified atmosphere of 5% CO_2.

2.3 TGFβ Response

In previous studies, we determined the Smad activation in various human melanoma cell lines in response to TGFβ treatment. Briefly, cells were stimulated or not with TGFβ (200pM) for 20 min, total lysates were separated by SDS-PAGE, where phospho-Smad3 by Western blot. In addition, we assessed the Smad transcriptional activity in response to TGFβ (200pM) treatment for 24 hours using a CAGA luciferase reporter construct [173].

2.4 TGFβ Treatment

Cell monolayers were grown in complete medium to 60% confluence, starved overnight in serum-free medium (0% FBS), and treated with a final concentration of 200pM of human recombinant TGFβ1 for the indicated periods.

2.5 Cell Viability Assay

Mitochondrial viability was assessed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay at 72h after TGFβ stimulation. DAUV cells were grown into 96-well plates at a density of 5000 cells per well in complete medium, then starved overnight in serum-free medium (0% FBS), replenished on the following morning with serum-low medium supplemented with 2% FBS in the presence or absence of TGFβ (200pM) (Peprotech, Dollard des Ormeaux, QC, Canada). After 72h of TGFβ treatment, 100ul of 5 mg/mL MTT in PBS was added to each well and incubated at 37°C for 2 h with, (Sigma-Aldrich, Oakville, ON, Canada). Formazan crystals were solubilized for 2h in the stop solution (50% dimethylformamide, 20% SDS, pH 4.7) and the optical density of each well was measured at 570 nm using an Epoch™ Microplate Spectrophotometer (Bio-Tek, Winooski, VT, USA).

2.6 Cell Proliferation Assay

DAUV cells grown in a 24-well plate at a density of $2.5x10^5$ cells per well in complete medium to reach 50% confluence on the following day, cells are starved in serum-free medium (0% FBS) overnight. On the following morning, the medium is replenished with serum-low medium (2% FBS) in the presence or absence of TGFβ (200pM). Triplicate wells were trypsinized after 24h, 48h, and 72h after TGFβ treatment using 100ul of Trypsin EDTA per well, then 400ul of complete medium was added, and cells were counted.

2.7 Cell Cycle Analysis

Melanoma cells were plated in 24-well plates, grown overnight in serum-free medium (0% FBS), replenished in the morning with serum-low medium and grown for 24 hours in the absence or presence of TGFβ (200 pM) in a medium containing 2% FBS. Cells were harvested, fixed in 70% ethanol for 2 hours at -20C, washed twice. Each sample (1×10^6 cells) was centrifuged; pellets of cells were resuspended in 0.5 mL of FxCycle™ PI/RNase Staining Solution (Molecular Probes, ThermoFisher Scientific, ON, Canada) stain for every flow cytometry sample and mixed well. Cells were incubated in the dark at room temperature for 30 minutes then analyzed without washing, using excitation 488-nm, 532-nm, and emission using a 585/42 band-pass filter. Cell cycle analysis was measured using the BD FACSCanto flow cytometer and analyzed by FACS Diva (BD Biosciences, Mississauga, ON, Canada) and FlowJo V10 Software (FlowJo LLC, Ashland, Oregon, USA).

2.8 Quantitative real-time PCR (qPCR)

Total RNAs were extracted using Trizol ™ (Invitrogen, ThermoFisher Scientific, ON, Canada) as per the manufacturer's instructions. One μg of RNA was reverse transcribed using M-MLV reverse transcriptase and random primers (Invitrogen) as per the manufacturer's protocol. Amplification of cDNA was performed by qPCR using SsoFast™ EvaGreen® Supermix (Bio-Rad, ON, Canada) using Rotor-Gene™ 6000 Real-time Analyzer (Corbett Life Sciences, CA, USA) and the data were analyzed with its corresponding software. The qPCR conditions were: 30 seconds at 95°C, then 40 cycles of 5 seconds at 95°C, 5 seconds at 60°C and finally 5 seconds

at 72°C. Human GAPDH was used as a housekeeping gene. Primer sequences are listed in the table below.

Gene		Sequence
Apaf1	Forward	5’-CTCTCATTTGCTGATGTCGC-3’
Apaf1	Reverse	5’-TCGAAATACCATGTTTGGTCA-3’
ATG12	Forward	5’- AGTAGAGCGAACACGAACCATCC -3’
ATG12	Reverse	5’- AAGGAGCAAAGGACTGATTCACATA -3’
ATG4B	Forward	5’- GCCGAGATTGGAGGTG -3’
ATG4B	Reverse	5’- GCCTATGGACTTGCCTTC -3’
ATG7	Forward	5’-TTTTGCTATCCTGCCCTCTG-3’
ATG7	Reverse	5’-GCTGTGACTCCTTCTGTTTGAC-3’
BCL2	Forward	5’- GAGTTCGGTGGGGTCATGT -3’
BCL2	Reverse	5’- GCCGGTTCAGGTACTCAGTC-3’
Beclin1	Forward	5’- TGTCACCATCCAGGAACTCA -3’
Beclin1	Reverse	5’- CTGTTGGCACTTTCTGTGGA -3’
Caspase3	Forward	5’-AGCGAATCAATGGACTCTGG-3’
Caspase3	Reverse	5’-CGGCCTCCACTGGTATTTTA-3’
cMyc	Forward	5’- TTCGGGTAGTGGAAAACCAG -3
cMyc	Reverse	5’- CAGCAGCTCGAATTTCTTCC-3’
GAPDH	Forward	5’-GCCTCAAGATCATCAGCAATGCCT-3
GAPDH	Reverse	5’-TGTGGTCATGAGTCCTTCCACGAT-3’
hTERT	Forward	5’-GCACGGCTTTTGTTCAGATG-3’
hTERT	Reverse	5’-CGGTTGAAGGTGAGACTGG-3’
Smac/Diablo	Forward	5’-AATGTGATTCCTGGCGGTTA-3’
Smac/Diablo	Reverse	5’-AGCTGGAAACCACTTGGATG-3’
ULK1	Forward	5’- TCGAGTTCTCCCGCAAGG -3’
ULK1	Reverse	5’- CGTCTGAGACTTGGCGAGGT -3’
ULK2	Forward	5’- GGCTCTCCTACTAAGACCACAG -3
ULK2	Reverse	5'- GACGAGTAACCAAGGCTAACAG -3'

2.9 Immunoblotting

Cells were lysed at 4°C for 15 minutes in RIPA buffer (1 mM DTT, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 50 mM Tris-HCl pH 7.41% Triton X-100) supplemented with protease inhibitors (10 μg/ml aprotinin and leupeptin, 2 μg/ml of pepstatin A, 1 mM PMSF). Cell lysates were then centrifuged at 14,000 rpm for 15 minutes at 4°C. Protein content was measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equally-loaded cell lysates (50ug) were immunoblotted via SDS-PAGE using specific commercially-available primary and secondary antibodies. Immunoreactivity was revealed by chemiluminescence using Clarity™ Western ECL Substrate and detected using ChemiDoc™ Imaging System. Densitometric analysis of protein levels was performed using Image Lab™ Software (Bio-Rad, ON, Canada).

2.10 CRISPR/Cas9 Knockout Generation

LentiCRISPRv2 (Addgene, cat. no. 52961) was digested using Esp3I restriction enzyme (ThermoFisher, cat. no. ER0451), dephosphorylated using FastAP (ThermoFisher, cat. No. EF0654), agarose gel purified and extracted using QIAquick Gel Extraction Kit (QIAGEN, cat. no. 28704). Each single-guide primer sequences below (5'-3') were phosphorylated using T4 PNK (NEB, cat. no. M0201S), annealed by slow cooling from 65°C to room temperature in T4 ligation buffer (NEB, cat. no. B0202S) and ligated in Esp3I digested lentiCRISPRv2 purified plasmid using Quick Ligase (NEB, cat. no. M2200S). Each sgRNA ligated plasmid was transformed in STBL3 chemically competent *E. coli* (ThermoFisher, cat. no. A10469) and collected from an amplified single bacterial colony using QIAprep Spin Miniprep Kit (QIAGEN, cat. no. 27104) [706, 707].

Primer sequences are listed in the table below.

Primer		***Sequence***
AXUD1	Forward	5'-CACCGTCAGCAGAGCTCGACGTCGC-3'
AXUD1	Reverse	5'-AAACGCGACGTCGAGCTCTGCTGAC-3'
MEN1sg1	Forward	5'- CACCGCATGCGCTGTGACCGCAAGA -3'
MEN1sg1	Reverse	5'- AAACTCTTGCGGTCACAGCGCATGC -3'
MEN1sg2	Forward	5'- CACCGTGACCTGCACACCGACTCGC -3'
MEN1sg2	Reverse	5'- AAACGCGAGTCGGTGTGCAGGTCAC -3'

MEN1sg3	Forward	5'- CACCGTGTAGATCTCCTCGTCTTCC -3'
MEN1sg3	Reverse	5'- AAACGGAAGACGAGGAGATCTACAC -3'
SMAD2sg1	Forward	5'- CACCGTCCCACTGATCTATCGTATT -3'
SMAD2sg1	Reverse	5'- AAACAATACGATAGATCAGTGGGAC -3'
SMAD2sg2	Forward	5'- CACCGATGTTATATATTGCCGATTA -3'
SMAD2sg2	Reverse	5'- AAACTAATCGGCAATATATAACATC -3'
SMAD2sg3	Forward	5'- CACCGCTCCAGGTATCCCATCGAAA -3'
SMAD2sg3	Reverse	5'- AAACTTTCGATGGGATACCTGGAGC -3'
SMAD3sg1	Forward	5'- CACCGCCCGATCGTGAAGCGCCTGC -3'
SMAD3sg1	Reverse	5'- AAACGCAGGCGCTTCACGATCGGGC -3'
SMAD3sg2	Forward	5'- CACCGTTCACGATCGGGGGAGTGAA -3
SMAD3sg2	Reverse	5'- AAACTTCACTCCCCCGATCGTGAAC -3'
SMAD3sg3	Forward	5'- CACCGAACGTGGAAAGGCGCAGCTC -3
SMAD3sg3	Reverse	5'- AAACGAGCTGCGCCTTTCCACGTTC -3'
SMAD4sg1	Forward	5'- CACCGAACTCTGTACAAAGACCGCG -3'
SMAD4sg1	Reverse	5'- AAACCGCGGTCTTTGTACAGAGTTC -3'
SMAD4sg2	Forward	5'- CACCGTTCTTCCTAAGGTTGCACAT -3'
SMAD4sg2	Reverse	5'- AAACATGTGCAACCTTAGGAAGAAC -3'
SMAD4sg3	Forward	5'- CACCGAATACACTTACCAGGATGAT -3'
SMAD4sg3	Reverse	5'- AAACATCATCCTGGTAAGTGTATTC -3'

2.11 Surveyor Nuclease Assay

GeneArt® Genomic Cleavage Detection Kit (Life Technologies) was used according to the manufacturer's instructions to perform the Surveyor nuclease assay on AXUD knockout DAUV cells to test for the proper indel *AXUD1* mutations. Primer sequences are listed in the table below.

AXUD1	Forward	5'-AGTGCAAAGAAGTCCCCACGC-3'
AXUD1	Reverse	5'-ACACAGTGCCCATCGCAGATTAAG-3'

2.12 Lentiviral Generation and Infection

HEK293T cells were cultured in T75 flasks to 90% confluence using a complete medium, transfected with scrambled, MEN1 shRNA, lentiCRISPRv2 AXUD1, SMAD2, SMAD3, SMAD4 and the packaging plasmids pMD2.G and psPAX2 using Opti-MEM® (Invitrogen) and branched polyethyleneimine (Sigma Aldrich). Cell culture medium with lentiviral particles was collected. DAUV cells were grown in 6-well plates to 70% confluence in antibiotic-free medium, infected with the 100µl of lentivirus in presence of hexadimethrine bromide; polybrene (8 µg/ml), incubated overnight, replenished with fresh complete medium for 48 hours. Cells were selected by 1µg/ml puromycin for 14 days followed by a maintenance dose of 0.5µg/ml puromycin. The pool of resistant cells forming the stable CRISPR knockout cells was expanded in complete RPMI medium (supplemented with 10% FBS and 1% antibiotics) and 0.5µg/ml puromycin.

2.13 In vitro Luciferase Assay

DAUV cells were plated in 6-well dishes in RPMI1640, 10% FBS (3×10^5 cells/ well) and incubated overnight. The following day, cells were replenished with 2.7 ml complete medium and transfected with 1.5 µg luciferase reporter construct, 1.5 µg of β-galactosidase (pCMV-lacZ) expression vector and 9 µg of Polyethyleneimine (PEI) 25000 each diluted in 150 µl of OptiMEM (Invitrogen). PEI mixture was added to the DNA mixture and incubated at room temperature for 30 min, PEI-DNA mixture was added dropwise and incubated. After 24 hours, cells were serum-starved in RPMI overnight (0% FBS) and cultured with or without TGFβ (200pM) for 24h. Cells were washed in PBS and lysed in 100 µl of passive lysis buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT and 1% Triton X-100) on ice. Supernatants were collected by centrifugation (14,000 rpm, 10 minutes, 4°C). 45µL of the clear cell lysates were mixed with 5µL of the cocktail buffer (0.03 M ATP, 0.1 M KH_2PO_4 pH 7.8,0.1 M $MgCl_2$) and the luciferase of each sample activity was measured after injection of 50 µl of 0.25mM D-luciferin using The Spark® Multimode Microplate Reader (Männedorf, Switzerland) where the luminescence levels were expressed as relative light units (RLU). In parallel, 5µL of lysates were mixed with 45µLof ONPG (6 mg/mL) in a β-Gal buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 50 mM βME, 10 mM KCl, 1 mM $MgCl_2$) and incubated at 37°C for 1 hour. The OD was measured at 420 nm and the

normalized luciferase activity of each lysate was calculated by dividing the RLU value of the luciferase activity by the corresponding β-galactosidase activity of the co-transfected β-gal vector.

2.14 Caspase 3/7 Luminescence Assay

Cells were plated in 96-well plates, starved overnight (0% FBS), in the absence or presence of TGFβ (200 pM) for 72 hours in a medium supplemented with 2% FBS. Caspase 3/7 activity was assessed as a measure of apoptosis by luminescence using Caspase-Glo® 3/7 (Promega, WI, USA) according to the manufacturer's instructions. The assay provides a luminogenic caspase-3/7 substrate, containing a tetrapeptide sequence DEVD, when cleaved by caspase 3/7 generates a "glow-type" luminescent signal in the presence of the Caspase-Glo® 3/7 Reagent. Luminescence was measured by The Spark® Multimode Microplate Reader (Tecan US Inc., NC, USA).

2.15 Scratch Wound Healing Migration Assay

Cells were grown to confluence in 12-well cell culture plates, a thin wound was made by scratching with sterile 200 µl pipette tips (Axygen Inc., T-200-Y, ThermoFisher), afterward, cells were washed with PBS to remove any debris, cells were replenished with serum-free medium (0%FBS) in the absence or presence of TGFβ (200 pM) for 72 hours. Images were taken using phase-contrast light microscopy with a Nikon Eclipse E600 microscope and a ToupTek Camera 5.0MP CMOS. Cell migration for every condition was calculated by subtracting the open wound area at 24 hours from that at 0 hours, subtracting the open wound area at 48 hours from that at 24 hours, and subtracting the open wound area at 72 hours from that at 48 hours. The extent of migration was calculated as the percentage of wound closure by dividing the migration (wound closure) of each condition by that of the non-treated condition of the parental cell line.

2.16 Tumorigenicity and Experimental Metastasis Assay

All mice were housed and handled following the guidelines of the Canadian Council on Animal Care (CCAC) and under the conditions and procedures approved by the Animal Care Committee of McGill University (AUP # 7497). The immunodeficient NOD-SCID IL2Rγ^{null} laboratory mice (NSG) breeders were purchased from Jackson Laboratory (California, US). NSG mice were bred and maintained in our institutional pathogen-free animal facility. Seven-week old

male mice received scrambled, Smad2, Smad3, Smad4, or AXUD1 or menin KO DAUV cells via dorsal subcutaneous injection ($2x10^6$ /mouse) to assess primary tumour formation or via intravenous injection through the tail vein ($1x10^6$ /mouse) to assess secondary metastatic tumour formation.

In the subcutaneously-injected groups, a set of 25 mice were randomized equally into 5 groups (5 mice/group) and each group received a dorsal subcutaneous injection of scrambled, Smad2, Smad3, Smad4, or AXUD KO DAUV cells to assess the role of AXUD in primary tumour formation, while another set of 15 mice were randomized into 3 groups (5 mice/group) and each group received a dorsal subcutaneous injection of scrambled, Smad3, or menin KO DAUV cells to assess the role of menin in primary tumour formation. The mice were monitored and weighed every two to three days during the first three weeks post-injection, then every day onwards, the tumour sizes were measured manually with a digital electronic calliper and recorded. Tumour volumes were calculated according to the following formula: $[(4/3) \times \pi \times (\text{Length}/2) \times (\text{Width}/2)^2]$ to generate tumour growth curves. All mice were euthanized once any mouse bore a tumour reaching 1500 mm^3. All tumours were harvested on day 37 post-inoculation of DAUV cells and photographed. Each tumour mass was divided into two parts, one of which was frozen at -80°C, while the other was fixed in 10% neutral-buffered formalin.

In the intravenously-injected group, a set of 25 mice were randomized equally into 5 groups (5 mice/group) and each group received a tail-vein intravenous injection of scrambled, Smad2, Smad3, Smad4, or AXUD KO DAUV cells to assess the role of AXUD in secondary metastatic tumour formation, and similar set 25 mice were randomized equally into 5 groups (5 mice/group) and each group received a tail-vein intravenous injection of scrambled, Smad2, Smad3, Smad4, or menin KO DAUV cells to assess the role of menin in secondary metastatic tumour formation. The mice were monitored and weighed every two or three days during the first three weeks post-injection, then every day onwards. All mice were euthanized once any mouse was extremely debilitated or rapidly lost weight. All mice were euthanized on day 35 post-injection. The livers, lungs and pancreases of the mice were harvested and photographed. The metastatic nodules in the liver were counted immediately without prior fixation of the livers, whereas the metastatic nodules in the lung were counted after being fixed in Bouin solution.

2.17 Melanosphere Formation Assay

Melanoma cell monolayers were grown in complete medium to 80-90% confluence, trypsinized using Trypsin-EDTA (0.05%), centrifuged for 5 minutes at 1200 rpm at room temperature, and resuspended in freshly prepared stem cell medium (SCM) composed of serum-free RPMI1640 or DMEM medium supplemented with 10 ng/ml EGF, 10 ng/ml bFGF and 1X B-27™ Plus Supplement. Melanoma cells were seeded at a density of 1,000 cells per well in ultra-low-attachment 24-well plates (Corning) in 1ml of freshly prepared SCM. In case of treatments with TGFβ (200 pM), Rapamycin (mTOR inhibitor, 0.1 μM), SP600125 (JNK inhibitor, 25 μM), PD169316 (p38 inhibitor, 10 μM), PD98059 (ERK1/2 inhibitor, 25 μM), SB431542 (TGFβRI/SMAD inhibitor, 10 μM), or LY294002 (PI3K inhibitor, 25 μM), all treatments were done immediately once the cells were seeded. The low-attachment plates were incubated continually -with no or minimal disruption- for 7 days at 37 °C with 5% CO_2. Secondary melanosphere formation was assessed by the enzymatic dissociation of the first passage melanospheres using Trypsin-EDTA (0.05%), where the cells were centrifuged, resuspended then seeded at a density of 1,000 cells per well into new ultra-low-attachment 24-well plates. Similarly, tertiary melanosphere formation was assessed by trypsinizing the second passage melanospheres, centrifuging, resuspending the cells, then seeding them at a density of 1,000 cells per well into new ultra-low-attachment 24-well plates. Spheroids of a diameter ≥ 25μm were counted as melanospheres [708]. The melanosphere-forming efficiency (MFE) was calculated as the percentage value of the number of formed melanospheres divided by the number of initially seeded single cells [(number of melanospheres)/ (number of seeded cells)] X 100.

2.18 Flow Cytometric Analysis

Monolayer melanoma cells were cultured in 100mm plates in complete medium, grown overnight in serum-free (0% FBS) medium replenished in the morning with serum-low (2% FBS) medium and grown for 24 hours in the absence or presence of TGFβ (200 pM). Afterward, cells were enzymatically dissociated using Trypsin-EDTA (0.05%), pellets were resuspended in complete medium and cells were counted using TC20™ Automated Cell Counter (Bio-Rad). Cells were aliquoted at a density of $0.5x10^6$ – $1x10^6$ cells per Eppendorf tube, then washed

with ice-cold 1X PBS and then resuspended in FACS Buffer which is 1X PBS supplemented with 2% bovine serum albumin (BSA). R-phycoerythrin (PE) Mouse Anti-Human CD133 antibody (BD Biosciences) were added to the cell suspension in a ratio of 1:20 (v/v), gently mixed with cells by inversion, then incubated on ice in the dark for 30 minutes. PE Mouse IgG1, κ Isotype Control were used for negative controls instead. Cells were centrifuged for 5 minutes at 1200 rpm at 4°C, washed twice with ice-cold FACS buffer, pellets were then resuspended in 300μL of FACS buffer and analyzed using the red channel (excitation 488-nm and emission using a 575/26 bandpass filter). Flow cytometric analysis was measured using the BD FACSCanto flow cytometer and analyzed by FACS Diva (BD Biosciences, Mississauga, ON, Canada) and FlowJo V10 Software (FlowJo LLC, Tree Star Inc., Ashland, Oregon, USA).

Aldehyde dehydrogenase (ALDH) enzymatic activity was determined using ALDEFLUOR™ Kit (STEMCELL™ Technologies) as per the manufacturer's protocol, briefly, as mentioned earlier, cells were dissociated, counted, aliquoted, washed and then resuspended in ALDH Assay Buffer. For samples, 5 µl activated ALDH substrate (BODIPY-amino acetaldehyde) was added to 1ml of cell suspension, 500 µl of each sample were transferred to a tube containing 5 µl diethylamino benzaldehyde (DEAB) to serve as negative controls. All tubes were incubated for 30 minutes at 37 °C with occasional gentle inversion of tubes every 10 minutes. $ALDH^+$ cells using the red channel (excitation 488-nm and emission using a 530/30 bandpass filter). Flow cytometric analysis was measured using the BD FACSCanto flow cytometer and analyzed by FACS Diva (BD Biosciences, Mississauga, ON, Canada) and FlowJo V10 Software (FlowJo LLC, Tree Star Inc., Ashland, Oregon, USA).

2.19 Data Mining

Using the online Genomic Data Commons (GDC) Data Portal of the National Cancer Institute Database of the National Institutes of Health, 469 samples were queried in the human skin cutaneous melanoma using a dataset obtained from The Cancer Genome Atlas project (TCGA_SKCM). Furthermore, using the online resource of Human Protein Atlas, I queried CD133 (PROM1) and ALDH1A1 expression levels using RNA-seq data in 17 cancer types in The Cancer Genome Atlas (TCGA) database (mRNA expression levels calculated across 17 cancer tissues including cancer tissue enriched, cancer group enriched, cancer tissue enhanced, expressed in all, mixed and not detected). In 102 sequenced melanoma cases, CD133 mean

expression was 0.6 (Max FPKM: 12.3 -Min FPKM: 0.0, Median FPKM: 0.2), while ALDH1 mean expression was 18.6 FPKM (Max FPKM: 151.3 -Min FPKM: 0.6, Median FPKM: 6.79). FPKM: fragments per kilobase per million mapped reads. Kaplan-Meier plotter graphs were generated based on datasets from the TCGA database to evaluate the correlation between CD133 (PROM1) and ALDH1A1 mRNA and patient survival.

2.20 Statistics

Data were collected from three or more independent experiments (as detailed in the table below). Values of outcomes were expressed as the arithmetic means. All the error bars represent standard errors of means (SEM). Statistical analysis was done using Student *t*-test comparing TGFβ-treated to non-treated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Experiment	Number of biological replicates	Number of technical replicates
Cell Viability Assay	For KO experiments: 3	For KO experiments: 6
	For KD experiments: 4	For KD experiments: 6
Cell Proliferation Assay	For KO experiments: 3	For KO experiments: 3
	For KD experiments: 3	For KD experiments: 3
Cell Cycle Distribution	3	2
Quantitative real-time PCR (qPCR)	3	2
Caspase3/7 Assay	3	4
Immunoblotting	3	2-8
In vitro Luciferase Assay	3TP-Lux Promotor Activity: 3	3TP-Lux Promotor Activity: 2
	CAGA-Lux Promotor Activity: 3	CAGA-Lux Promotor Activity: 2
	hTERT-Lux Promotor Activity: 3	hTERT-Lux Promotor Activity: 2
Scratch Wound Healing Migration Assay	4-6	2-3
In vivo Tumorigenicity Assay (SC Injection)	For SCR group:5 mice For Smad2 KO group:5 mice For Smad3 KO group:5 mice For Smad4 KO group:5 mice For AXUD1 KO group:5 mice For menin KO group:5 mice	
In vivo Experimental Metastasis Assay (IV Tail-Vein Injection)	For SCR group:5 mice For Smad2 KO group:5 mice For Smad3 KO group:5 mice For Smad4 KO group:5 mice For AXUD1 KO group:5 mice For menin KO group:5 mice	

Melanosphere Formation Assay	All cell lines: 4-6	All cell lines: 2
	DAUV PAR/SCR/KOs: 3	DAUV PAR/SCR/KOs: 2
	DAUV Kinase Inhibitors: 3	DAUV Kinase Inhibitors: 2
Flow Cytometric Analysis	3-4	2

2.21 Ethics

All experimental protocols and procedures were performed following McGill University regulations.

2.22 Acknowledgments

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Part I: AXUD1 and Menin

3.1.1 AXUD1 missense mutations could be deleterious in melanoma patients

To first address, whether AXUD1 could play a role in melanoma tumour formation, I initially examined the correlation between AXUD1 expression levels and melanoma patients' overall survival. Using the Skin Cutaneous Melanoma dataset of The Cancer Genome Atlas (TCGA) project of Genomic Data Commons (GDC) with a cohort of 102 patients, I found that higher AXUD1 mRNA expression correlates with better survival outcomes in melanoma patients (Figure 3-1A left panel). Kaplan-Meier Survivals Plots on Human Protein Atlas showed a significant positive correlation between AXUD1 mRNA expression levels and patient 3-year survival where patients (n=37) with higher expression levels (more than 6.4 FPKM) showed a 3-year survival of 54% compared to only 30% in patients (n=65) with low AXUD1 expression levels. Furthermore, Figure 3-1A (right panel) illustrates scatter survival plot based on these datasets of the Kaplan-Meier plots showing that 82% (24 out of 29) of the dead patients (red) had low AXUD1 expression levels, highlighting the protective role of AXUD1 and supporting its role in mediating the TGFβ-induced tumour-suppressive effects. These survival data suggest a potential role for AXUD1 in melanoma tumour suppression.

In addition, I searched large-scale cancer genomics datasets available on cBioPortal for Cancer [709, 710] to query 590 samples in 5 studies namely: Melanoma (Broad/Dana Farber, Nature 2012), Next-generation sequencing (NGS) of pre-treatment metastatic melanoma samples (MSK, JCO Precision Oncology 2017), Skin Cutaneous Melanoma (Broad, Cell 2012), Skin Cutaneous Melanoma (TCGA, Provisional), Skin Cutaneous Melanoma (Yale, Nat Genet 2012). I found that AXUD1 was altered in 10 (1.9%) of 524 sequenced cases of 590 total patient samples, revealing eight mutations found in 7 samples out of 590 samples across 5 different datasets. Most of these missense mutations showed very low SIFT scores indicating deleterious effects (either with high (0.0, 0.03) or low (0.9) confidence. SIFT (Sorting Intolerant from Tolerant) scores are the normalized probability that an amino acid substitution will be tolerated without affecting the protein function, where the score ranges from 0.0 being deleterious to 1.0 being tolerated. Mutations did not show any significant correlations with gender, age, race, lesion location, metastatic sites, or Clark's level at diagnosis. The alterations of AXUD did not score as either

Tier 1 or Tier 2 of the Cancer Gene Census, a census for the genes comprising mutations that are causally involved in cancer [711].

Moreover, using Genomic Data Commons (GDC) Data Portal of the National Cancer Institute Database of the National Institutes of Health, I queried 469 samples in the human skin cutaneous melanoma using a dataset obtained from The Cancer Genome Atlas project (TCGA_SKCM). I found that AXUD1 was altered in 12 (2.56%) of 469 sequenced cases of 10,202 total patient samples, revealing eleven mutations found in 12 samples out of 469 samples in the dataset. Almost half of these somatic mutations were missense mutations showing very low SIFT scores indicating deleterious effects (either with high (0.0, 0.02, 0.03) or low (0.9) confidence. SIFT (Sorting Intolerant from Tolerant) scores are the normalized probability that an amino acid substitution will be tolerated without affecting the protein function, where the score ranges from 0.0 being deleterious to 1.0 being tolerated. Interestingly, I could not find any correlation between menin expression levels and melanoma patients' overall survival.

3.1.2 TGFβ induces AXUD1 gene expression in melanoma cells in a Smad3-specific manner.

Given the tumour suppressor role played by TGFβ in melanoma, I then investigated whether AXUD1 could be acting downstream of TGFβ and assessed whether TGFβ could regulate AXUD1 expression in melanoma. For this, I examined the TGFβ effects on AXUD1 mRNA levels in 6 different human melanoma cell lines, which originated from different patients. As shown in Figure 3-1B, TGFβ could significantly upregulate AXUD1 mRNA levels in all melanoma cell lines, defining AXUD1 as a novel TGFβ target and further suggesting a potential role for AXUD1 in mediating the TGFβ effects in melanoma cells. Moreover, I found that TGFβ could increase AXUD1 expression levels as shown by the immunohistochemical analysis of DAUV cell lines as shown in Figure 3-1C.

To then address whether AXUD1 could modulate the TGFβ transcriptional responses, AXUD1 expression was silenced in DAUV melanoma cells using two different approaches. First, I generated an AXUD1 knockdown (KD) using a specific AXUD1 short-hairpin RNA (shRNA) and secondly, Julien Boudreault generated a stable AXUD1 knockout (KO) cell line using a CRISPR/Cas9 lentiviral approach. For the latter, 3 single-guide RNAs (sgRNAs) were generated and tested for proper indel mutation using the Surveyor nuclease assay [712] (Figure 3-1D). The most efficient sgRNA was selected for further DAUV cell line infection. Efficiencies of the

AXUD1 knockdown and knockouts were quantified using qPCR (Figure 3-1E). The effects of the AXUD1 KD and KO were then assessed using a TGFβ-specific luciferase reporter assay (3TP-lux) [713]. As shown in Figure 3-1F, silencing AXUD1 expression with both approaches resulted in nearly complete inhibition of the TGFβ-induced luciferase activity, as compared to parental and scrambled transfected cells. Together, these results strongly suggest that AXUD1 is required for TGFβ-mediated gene transcription.

Next, I further investigated the TGFβ upregulation of AXUD1 and examined whether the canonical Smad pathway was involved. While the TGFβ effects are usually triggered through its direct effectors Smad2, Smad3 and Smad4, studies have shown that the preferential activation of Smad2 versus Smad3 is context- and tissue-dependent, resulting in different roles for these 2 Smads downstream of TGFβ [346, 714-725]. For this, I generated stable Smad2, Smad3 and Smad4 knockout cell lines using a CRISPR/Cas9 lentiviral approach. The efficiency of the Smad knockouts was verified by immunoblot analysis (Figure 3-1G). Upon producing the KO cell lines, I tested three gRNAs provided by Julien and then used the gRNA with the highest knockout efficiency. Noteworthy, during the selection of KO cells, the cells were not clonal. The main reason we did not opt for a clonal selection is the fear of the subsequent propagation of one clone with atypical genetic properties that might be unrepresentative of the entire population. Therefore, instead of obtaining a stable homozygous 100% KO of one clone, we decided to obtain stable heterozygous 90%-99% KO of the entire population. Possibly, the KO cell lines comprised a heterogeneous population of cells comprising predominantly knocked out cells with a minority of cells that likely escaped the KO. Additionally, on rare occasions, an inactive protein might be detected, although the CRISPR/Cas9 system generates premature stop codon results in unstable proteins targeted for degradation.

The effect of silencing Smad2, Smad3, and Smad4 was then tested on the AXUD1 gene expression. As shown in Figure 3-1H, Smad3 and Smad4, but not Smad2 knockouts inhibited TGFβ-induced AXUD1 mRNA levels. These results indicate that the Smad pathway is required for TGFβ to induce AXUD1 gene expression and that this effect is Smad3 specific. Also, to ensure that knocking out Smads or AXUD1 did not affect the viability and growth of melanoma cells, I evaluated the growth rates of the knockout DAUV cell lines both in the presence and the absence of TGFβ (200pM) at 24, 48 and 72 hours post-treatment. As shown in Figure 3-1I-O, I found that in the absence of TGFβ stimulation all cell lines showed similar growth patterns, indicating that

knocking out these genes did not affect their growth of the melanoma knockout cell lines. Upon TGFβ treatment, only parental, scrambled and Smad2 knockout cells showed growth inhibition, whereas this TGFβ-mediated inhibitory effect was compromised in Smad3, Smad4, and AXUD1 knockout cells.

Next, I tried to rescue the effect of AXUD1 KO by AXUD1 overexpression (OE). The effect of the OE of Flag-tagged AXUD1 was demonstrated in both parental and AXUD1 KO DAUV melanoma cells (Figure 3-1P-S), where I performed functional assays using promotor luciferase reporter constructs (3TP-Lux, CAGA-lux) to assess Smad3 gene promotor activity. The results show that AXUD1 OE resulted in an increase in the TGFβ-mediated Smad activation in parental DAUV cells (Figure 3-1P, Figure 3-1Q) as well as the restoration of that TGFβ-mediated effect in AXUD1 KO DAUV cells (Figure 3-1R, Figure 3-1S), in a dose-dependent manner.

Taken together, these results indicate that AXUD1 is required for TGFβ-induced Smad3-mediated transcriptional activity.

3.1.3 TGFβ induces MEN1 gene expression in melanoma cells in a Smad3-specific manner.

To determine whether menin could also be mediating effects downstream of TGFβ in melanoma, I started by measuring the effect of TGFβ on menin mRNA levels in a panel of human melanoma cell lines with various pathological backgrounds. Figure 3-1T shows that TGFβ indeed could significantly upregulate menin mRNA levels in the tested melanoma cell lines, highlighting a potential role for menin in mediating the TGFβ effects in melanoma cells. Next, I wanted to assess whether menin could regulate the TGFβ transcriptional responses. For this, I silenced MEN1 expression in DAUV melanoma cells generating a stable menin knockout (KO) cell line via the CRISPR/Cas9 technique. I infected the DAUV melanoma cells using the lentiviral CRISPR/Cas9 construct generated from the most efficient gRNA. Efficiencies of the menin KOs were verified using qPCR and immunoblotting (Figure 3-1U). I then determined the effect of the menin KO using a TGFβ-specific luciferase reporter assay (3TP-lux) [713], where silencing menin expression resulted in almost a total suppression of the TGFβ-induced luciferase activity in comparison with the parental and scrambled melanoma cells (Figure 3-1V). These results confirm that menin is essential for TGFβ-mediated gene transcription in melanoma.

Furthermore, I evaluated the TGFβ-induced upregulation of menin and determined whether it implicated the canonical Smad pathway. TGFβ mediates its downstream effects

through its direct effectors the receptor-regulated Smad2 or Smad3 in a trimeric complex with the Co-Smad Smad4 [263-265]. Nonetheless, reports described varying roles for Smad2 or Smad3 downstream of TGFβ due to the preferential activation of Smad2 or Smad3 which happens to be context- and tissue-dependent [346, 714-725]. Therefore, using the CRISPR/Cas9 lentiviral approach, I generated stable Smad2, Smad3 and Smad4 KO DAUV melanoma cell lines to test for the Smad-dependency of menin expression in melanoma cells, where I validated the Smad knockouts efficiency using immunoblot analysis (as described and shown before). Next, I tested the effect of silencing Smad2, Smad3, and Smad4 on MEN1 gene expression, where I found that Smad3 and Smad4, but not Smad2 knockouts failed to upregulate MEN1 mRNA levels in response to TGFβ stimulation (Figure 3-1W), indicating that the Smad3-specific pathway is necessary for the TGFβ-induced upregulation of MEN1gene expression.

3.1.4 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce cell cycle arrest in human cutaneous melanoma cells.

Having shown that AXUD1 and menin are required for TGFβ-mediated gene transcription responses, I next investigated the role of AXUD1 and menin in relaying the various TGFβ tumour suppressive responses (induction of cell cycle arrest, apoptosis, autophagy, and inhibition of cell immortalization) in DAUV melanoma cells. In almost all its target tissues, TGFβ elicits Smad-dependent cytostatic effects by inducing cell cycle arrest at the G1/S phase [329], through upregulation of the cyclin-dependent kinase inhibitors p15, p21, and p27 [326, 342] as well as the downregulation of cMyc and ID1 [336, 338]. We previously reported that LIF plays an important role in the TGFβ-induced cell cycle arrest at the G1 phase in a p21-dependent manner, showing that TGFβ could inhibit cell cycle progression and inhibit cell proliferation in melanoma [173].

First, to determine whether AXUD1 or menin were required for TGFβ-mediated growth inhibitory effect, I assessed the effect of TGFβ stimulation on cell viability using an MTT assay in DAUV parental, scrambled and AXUD1 KD and KO cells, as well menin KO cells. As shown in Figure 3-2A, silencing AXUD1 (left panel) or menin (right panel) completely blocked TGFβ-mediated inhibition of cell growth. To determine the Smad dependency of these AXUD1-mediated and menin-mediated effects, I also evaluated TGFβ-mediated growth inhibition in the Smad2, 3 and 4 KOs melanoma cells and found that TGFβ-mediated growth inhibition in

melanoma cells is Smad3-specific and Smad2-independent. Interestingly, knocking out AXUD1 or menin showed similar outcomes as knocking out Smad3 and Smad4, indicating that AXUD1 and menin play a central role downstream of TGFβ. As shown in Figure 3-2B, upon examining cell proliferation by direct cell counting, I observed similar results for both AXUD1 (left panel) and menin (right panel).

To get more insight into the role of AXUD1 and menin in mediating a TGFβ-induced growth inhibitory effects, I assessed cell cycle progression by flow cytometry using propidium iodide staining in DAUV parental, scrambled and AXUD1 knockdown or menin knockout cell lines stimulated or not with TGFβ. TGFβ-induced cell cycle arrest at the G1 phase was lost upon silencing either AXUD1 (Figure 3-2C-left panel) or menin (Figure 3-2C-right panel). Finally, it was informative to further determine whether AXUD1 and menin affect p15 and cMyc (which are cell cycle regulators known to be regulated by TGFβ) thus regulating the observed TGFβ-induced growth inhibition in melanoma. As shown in Figure 3-2D, both TGFβ-induced upregulation of p15 and downregulation of cMyc were abolished in the AXUD1 KO (left panel) and the menin KO (right panel) cells compared to parental and scrambled DAUV melanoma cells. Those effects were similar to those observed upon knocking out Smad3 and Smad4 but not Smad2, where the latter was not different from the parental melanoma cells, which comes following results from cell growth and viability experiments. Taken together, these results suggest an essential role for AXUD1 and menin in the TGFβ-mediated antiproliferative effects in melanoma cells, highlighting the preferential role of Smad3 in this inhibition.

3.1.5 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce apoptosis in human cutaneous melanoma cells.

The TGFβ-mediated tumour-suppressive effects, particularly cell cycle arrest and apoptosis have been studied mostly in epithelial cancers but were not thoroughly studied in melanocytic systems [254, 284, 726, 727]. Recent work from our lab showed that TGFβ displays tumour-suppressive effects in human melanoma through TGFβ-mediated LIF upregulation, leading to both cell cycle arrest and caspase-mediated apoptosis [173].

To determine the role of AXUD1 and menin in TGFβ-mediated pro-apoptotic effects, I first analyzed the expression of several TGFβ-regulated pro-apoptotic and anti-apoptotic genes [726] using parental, scrambled, AXUD1 KO or menin KO stable cell lines. As shown, TGFβ

treatment upregulated pro-apoptotic genes such as Apaf, Bak, Bax, Bim, Caspase3 and Smac/Diablo (Figure 3-3A, B) and downregulated anti-apoptotic genes such as BCL2, BCL-XL (Figure 3-3C, D). These effects were reversed upon silencing AXUD1 (Figure 3-3A, C) and silencing menin (Figure 3-3B, D) when compared to parental DAUV cell lines.

Next, I wanted to explore which Smad mediates the AXUD1-induced and the menin-induced pro-apoptotic effects downstream of TGFβ in DAUV melanoma cells. For this I used parental, scrambled, Smad2, Smad3, Smad4 KOs, as well as AXUD1 KO and menin KO DAUV melanoma cells to evaluate the caspase3/7 enzymatic activity, as well as the levels of procaspase3 and cleaved poly (ADP-ribose) polymerase-1 (PARP-1) both of which were reported to be a hallmark of caspase-mediated apoptosis [728-733], in addition to the expression of some of the previously tested TGFβ-regulated pro-apoptotic and anti-apoptotic genes. Results showed that the TGFβ-mediated increase of caspase3/7 activity (Figure 3-3E), and decrease of procaspase3 levels (Figure 3-3F) were abolished in Smad3, Smad4, AXUD1 or menin knockout cell lines but not in the scrambled or the Smad2 KO cells when compared to parental DAUV cell lines. Moreover, TGFβ-mediated increased levels of the 89-kD catalytic fragment PARP-1 (Figure 3-3F) were compromised in Smad3, Smad4, menin knockout cell lines. Besides, the upregulation of the pro-apoptotic genes (BIM, BAX, Caspase3) and the downregulation of the anti-apoptotic gene (BCL-XL) (Figure 3-3G) were reversed in Smad3, Smad4, AXUD1 knockout cell lines. Together, these results indicate the role of AXUD1 and menin in leveraging the TGFβ-induced pro-apoptotic effects in melanoma cells and the preferential role of Smad3 –not Smad2- in this pro-apoptotic role.

3.1.6 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce autophagy in human cutaneous melanoma cells.

In neoplasms, autophagy can have dual and opposing roles, eliciting either tumour-promoting or tumour-suppressive effects in a context-dependent manner. TGFβ was shown to induce autophagy [389, 390] in Smad-dependent and Smad-independent manners in hepatocellular carcinoma as well as in mammary and renal epithelial cells, and mesangial cells [391-393], strongly suggesting a TGFβ-mediated tumour suppressive role. In melanoma, the role of TGFβ in regulating autophagy is not entirely elucidated.

I first assessed whether TGFβ could induce autophagy in melanoma and whether AXUD1 and menin could play a role in these effects by measuring the expression levels of autophagy-regulating genes involved in different stages of the autophagy process (initiation of autophagosome formation and expansion of the autophagic isolation membrane) in parental, scrambled, AXUD1 and menin KO melanoma cells. To evaluate the role of Smad2/3 in mediating this potential effect, I tested those autophagy genes in Smad2, Smad3 and Smad4 KO cells in parallel. As shown in Figure 3-4A & B, results indicate that TGFβ could increase expression of autophagy-regulating genes involved in the initiation of autophagosome formation (Beclin1, ULK1 and ULK2) and the expansion of the autophagic isolation membrane (ATG4B, ATG7 and ATG12). Interestingly, all these effects were blocked upon knocking out the AXUD1 gene (Figure 3-4A) or MEN1 gene (Figure 3-4B) in DAUV melanoma cells.

Next, I investigated the role of TGFβ/Smad in regulating the autophagy marker p62 in DAUV melanoma cells. LC3-II is a conventional autophagy marker in mammalian cells [394], where cytosolic LC3-I form is converted into a lipidated LC3-II form localized in the autophagosome membrane. Sequestosome-1 (SQSTM1), also known as the ubiquitin-binding protein p62, is necessary for both the formation and the degradation of polyubiquitin-containing bodies by autophagy. After the initiation of autophagosome formation, p62 interacts directly with LC3 facilitating the degradation of polyubiquitinated protein aggregates in the lysosomes by the autophagy machinery [734], hence the preferential degradation of p62 levels by autophagy. I found that TGFβ stimulation could decrease p62 protein levels (Figure 3-4C) in parental and scrambled melanoma cells. Moreover, I found that TGFβ-mediated downregulation of p62 was lost upon silencing AXUD1 (left panel) or menin (right panel) in DAUV melanoma cells showing similar results to Smad3 and Smad4 KO but not Smad2 KO cells, indicating the preferential role of Smad3 in this TGFβ-mediated effect.

Moreover, to determine the preferential role of Smad2 or Smad3 in the AXUD1-mediated and the menin-mediated induction of autophagy effects downstream of TGFβ in DAUV melanoma cells. Using parental, scrambled, Smad2, Smad3, Smad4 KOs, in addition to either AXUD1 KO or menin KO DAUV melanoma cells, I evaluated the mRNA expression of autophagy-regulating genes namely Beclin1 and ATG7 as genes involved in the initiation and expansion phases respectively (ATG4B, and ATG12). In agreement with previous observations,

I found that TGFβ-induced increase of Beclin1 and ATG7 expression (Figure 3-4D) was abolished the AXUD1 or menin knockout cell lines as well as in Smad3 and Smad4 KOs, yet showed no difference in the Smad2 KO or scrambled cells when compared to the parental DAUV cell lines. Taken together, these results provide evidence that TGFβ/Smad3-mediated upregulation of AXUD1 or menin is required for inducing autophagy in melanoma cells.

3.1.7 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axis are required to inhibit cell immortalization in human cutaneous melanoma cells.

A hallmark of all cancer cells is represented by cell immortalization, resulting from the reactivation of the telomerase program and the human telomerase reverse transcriptase (hTERT) [399, 400]. Previous work from our lab and others described how TGFβ downregulates hTERT gene expression in different types of cancer [401-403]. We previously described that TGFβ/Smad3 signalling could downregulate hTERT expression and suppress telomerase activity via recruiting both E2F1 and HDAC into repressive complexes, thus impeding cell immortalization of epithelial cancer cells [403]. However, the role of TGFβ in regulating hTERT in melanoma has not been explored. To address this, I examined the TGFβ effects on hTERT mRNA levels in parental, scrambled, AUXD1, menin, Smad2, 3, and 4 KO melanoma cells. As shown in Figure 3-5A, I found that TGFβ could efficiently downregulate hTERT mRNA levels in parental, scrambled, and Smad2 KO cells. This TGFβ-induced inhibitory effect was lost upon silencing Smad3 and Smad4 in melanoma cells comparable to the loss observed upon silencing AXUD1 (Figure 3-5A left panel) and menin (Figure 3-5A right panel). Moreover, I found these inhibitory effects to be mediated at the transcriptional level, where the AXUD1, menin, Smad3 and Smad4 KO cells lost the TGFβ-mediated inhibitory effect over the hTERT gene promoter fused to a luciferase gene (hTERT-Lux) (Figure 3-5B). These observations indicate that AXUD1, as well as menin, play a role downstream of TGFβ in preventing cell immortalization in melanoma cells in a Smad3-specific fashion.

Taken together, results from these previous sets of experiments all point towards an important role for AXUD1 and menin in mediating the TGFβ-induced Smad3-mediated tumour-suppressive effects in melanoma cells via inhibiting cell growth, promoting apoptosis and autophagy as well as inhibiting cell immortalization.

3.1.8 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit melanoma tumour formation in vivo.

Having shown that AXUD1 and menin are required to relay the TGFβ/Smad3 tumour suppressive effects in melanoma *in vitro*, assessment of whether silencing *AXUD1* or *MEN1* gene expression could affect melanoma primary tumour growth *in vivo* was necessary. For this, a preclinical mouse model of melanoma was used, in which subcutaneous human tumour xenografts were performed in immunodeficient NOD-SCID IL2Rγ^{null} (NSG) mice. To assess the role of AXUD1, twenty-five mice (7 weeks old) were randomized equally into five groups (5 mice/group) and each group received a dorsal subcutaneous injection of scrambled, Smad2 KO, Smad3 KO, Smad4 KO, or AXUD1 KO DAUV cells. To assess the role of *MEN1*, fifteen mice were randomized into three groups (5 mice/group) and each group received a dorsal subcutaneous injection of scrambled, Smad3 KO, or menin KO DAUV cells. Mice were monitored every two to three days during the first three weeks post-injection, then every day onwards. Tumour sizes were recorded and tumour growth curves were generated. All mice were euthanized once any mouse bore a tumour reaching 1500 mm^3, where tumours were harvested and photographed. As shown in Figure 3-6(A, B), injecting mice with AXUD1 KO cells resulted in significantly larger tumours, similar in size to those observed in animals injected with Smad3 and Smad4 knockout cells. In contrast, injection of Smad2 KO cells did not result in any change in primary tumour size, as compared to control animals (injected with the scrambled cells). Similarly, as shown in Figure 3-6(C, D), mice injected with the menin KO melanoma cells harboured significantly larger tumours than control animals. Tumours from the menin KO injected animals were as large as the one observed in the Smad3 KO group. This is consistent with our *in vitro* data, indicating that the TGFβ tumour suppressive effects in melanoma are Smad3/4-specific and Smad2-independent. The observed increase in primary melanoma tumour formation upon depletion of AXUD1, menin, Smad3 and Smad4 demonstrate their crucial role in suppressing tumorigenicity *in vivo*, further highlighting the tumour suppressive role played by the TGFβ/Smad3/AXUD1 signalling pathway in melanoma.

3.1.9 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit cell migration in human cutaneous melanoma cells.

Our research group had shown that TGFβ plays an anti-migratory role in melanoma cells [171, 173]. As a result, I wanted to determine whether AXUD1 and menin could also regulate the TGFβ-mediated suppression of cell migration in melanoma. I used a scratch wound healing assay to examine cell migration in a time-dependent manner in DAUV melanoma cells. As illustrated in Figure 3-7, results show that TGFβ could significantly inhibit melanoma cell migration at 24 and 48hrs, consistent with our previous findings [171, 173]. Noteworthy, silencing AXUD1 (Figure 3-7A, B) or menin (Figure 3-7C, D) resulted in reduction and almost complete abrogation of the TGFβ-induced effects, whereby AXUD1 or menin deficiency promoted wound closure (Figure 3-7A, C). Moreover, knocking out Smad3 and Smad4 also abolished the TGFβ-induced anti-migratory effects, further promoting cell migration. However, knocking out Smad2 showed similar effects as the parental and scrambled transfected cells, whereby the TGFβ-induced response did not change. All experiments were quantified (n=6), as shown in the lower panels.

Together, these findings suggest that AXUD1 or menin, together with Smad3 and 4, are essential for TGFβ-induced inhibition of melanoma cell migration. In agreement with the *in vitro* results on tumour suppression, these results indicate that the TGFβ-induced effects on the suppression of both tumour formation and cell migration are mediated through AXUD1 or menin, showing Smad3-specificity and Smad2-independence. Moreover, the results also suggest that AXUD1 and menin are necessary for the TGFβ-induced anti-migratory effects in melanoma cells and indicate that both could mediate an anti-metastatic role.

3.1.10 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit lung and liver metastasis of human cutaneous melanoma cells in vivo.

After demonstrating that the TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes could inhibit cell migration *in vitro*, we next evaluated whether it could similarly inhibit tumour metastasis *in vivo* using a preclinical mouse model. Immunodeficient NOD-SCID IL2Rγ^{null} (NSG) mice were injected intravenously via their tail veins with different DAUV melanoma cells to evaluate the role of AXUD1 and menin downstream of TGFβ in the suppression of secondary metastatic tumour formation. For AXUD1 assessment, twenty-five mice (7 weeks old) were equally randomized into five groups (5 mice/group) and each mouse was intravenously injected

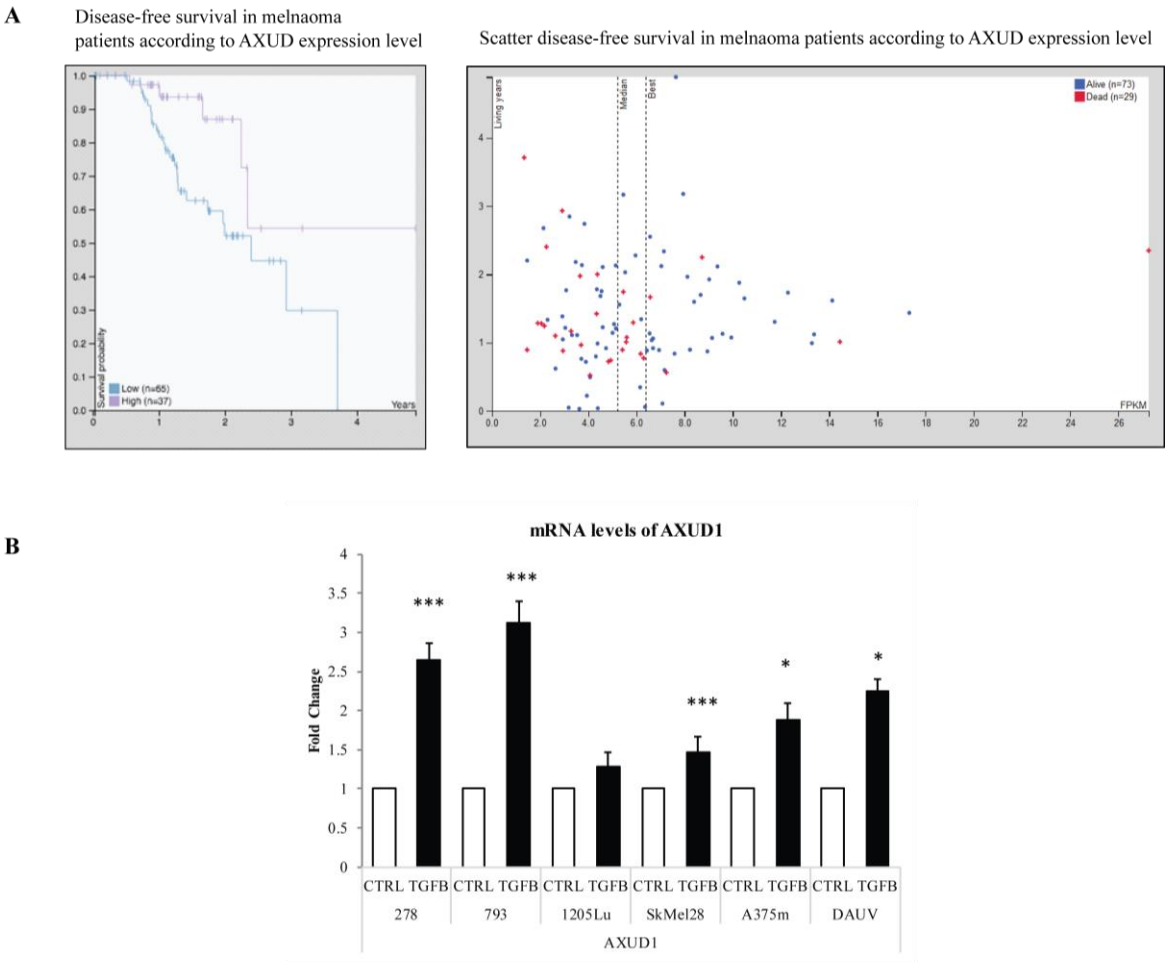
via tail vein with scrambled, Smad2 KO, Smad3 KO, Smad4 KO, or AXUD1 KO DAUV cells. Similarly, for menin assessment, twenty-five mice (7 weeks old) were equally randomized into five groups (5 mice/group) and each mouse was intravenously injected via tail vein with scrambled, Smad2 KO, Smad3 KO, Smad4 KO, or menin KO DAUV cells. All mice were monitored every two to three days, then every day starting from the third week post-injection. Once any single mouse was extremely debilitated, all mice in all groups were euthanized (on day 35 post-injection). The livers, lungs of the mice were harvested and instantly photographed. The metastatic nodules in the liver were counted immediately without prior fixation of the livers, whereas the metastatic nodules in the lung were counted after being fixed in Bouin solution.

As shown in Figure 3-8, mice injected with scrambled DAUV melanoma cells developed several metastatic nodules on both the lung and liver (indicated by the white arrows). Interestingly, the mice injected with the Smad3, Smad4 and AXUD1 KO melanoma cells, all showed a very high number (>200) of metastatic tumours in the lungs (Figure 3-8A, B) and livers (Figure 3-8C, D), while the mice injected with the Smad2 KO cells were comparable to the scrambled group, both showing no or few metastatic nodules. Similarly, the mice injected with the menin, Smad3 or Smad4 KO melanoma cells, showed a very high number of metastatic nodules in both the lungs (Figure 3-8E, F) and livers (Figure 3-8G, H) whereas those injected with the Smad2 KO cells were comparable to the scrambled group, both showing significantly lower numbers of metastatic nodules. These findings illustrate that silencing Smad3, Smad4, AXUD1 or menin results in a significant increase in lung and liver metastasis suggesting a role for AXUD1 and menin downstream of TGFβ in mediating TGFβ-induced Smad3-dependent anti-metastatic effects in DAUV melanoma cells.

Collectively, the previous results demonstrate that TGFβ acts as a strong tumour suppressor in melanoma cells, exerting its effects through inducing cell cycle arrest, apoptosis and autophagy as well as through inhibiting cell immortalization and migration *in vitro*. Moreover, TGFβ is capable of suppressing primary tumour formation as well as secondary metastatic tumours *in vivo.* Interestingly, all these arms of the TGFβ-mediated tumour suppression were found to require AXUD1 or menin and to be Smad3-specific (Smad2-independent), thus highlighting a novel function for the AXUD1 and menin as potential inhibitors of melanoma (DAUV) development and progression downstream of TGFβ.

3.1.11 Figures

3.1.11.1 TGFβ/Smad3 signalling upregulates AXUD1 and menin expression in human cutaneous melanoma cells.



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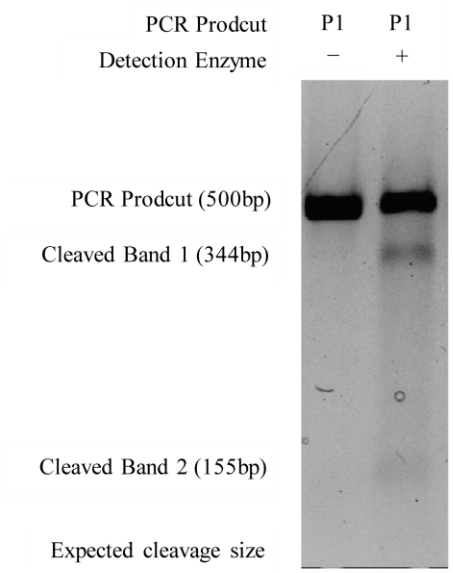
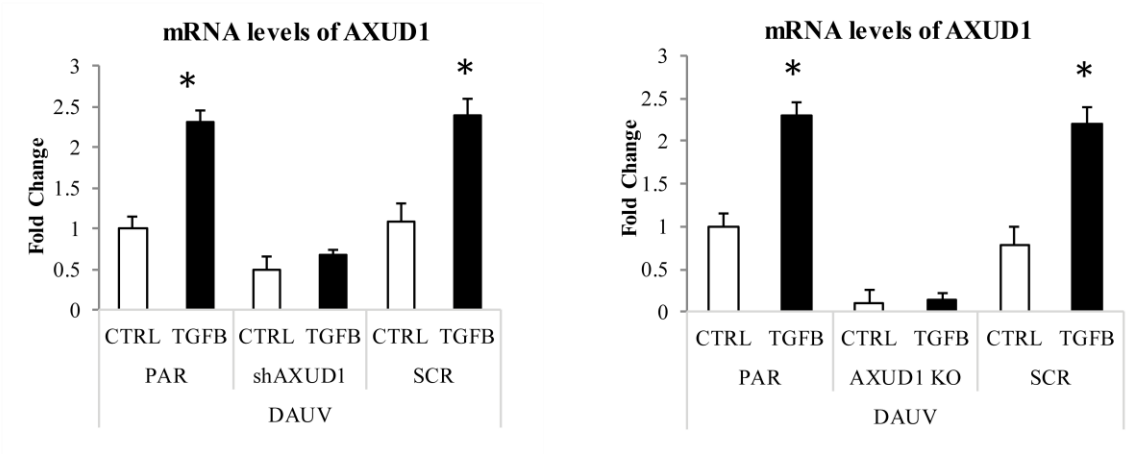
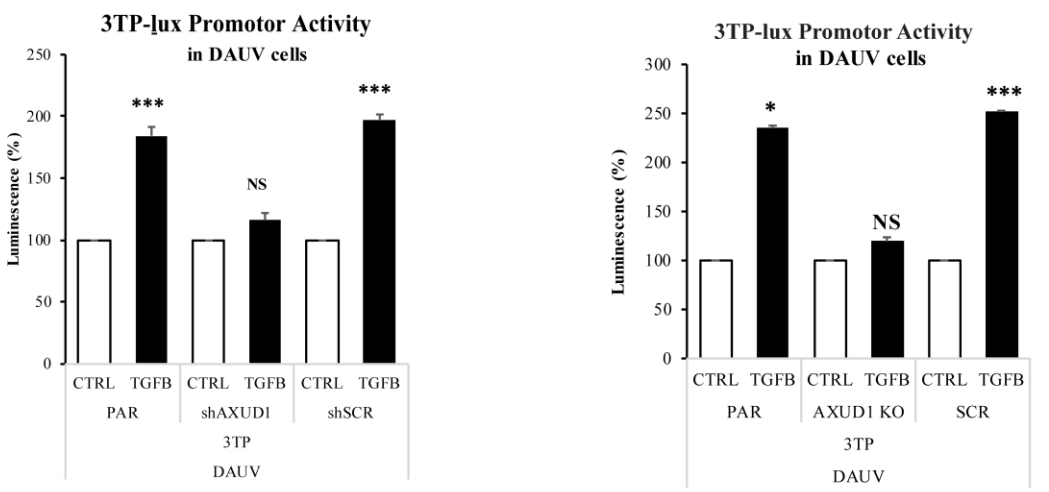

PCR Prodcut
P1
P1
Detection Enzyme
−
+
PCR Prodcut (500bp)
Cleaved Band 1 (344bp)
Cleaved Band 2 (155bp)
Expected cleavage size

E


mRNA levels of AXUD1
Fold Change
3
2.5
2
1.5
1
0.5
0
*
*
CTRL TGFB CTRL TGFB CTRL TGFB
PAR
shAXUD1
SCR
DAUV
mRNA levels of AXUD1
Fold Change
3
2.5
2
1.5
1
0.5
0
*
*
CTRL TGFB CTRL TGFB CTRL TGFB
PAR
AXUD1 KO
SCR
DAUV

F


3TP-lux Promotor Activity
in DAUV cells
Luminescence (%)
250
200
150
100
50
0
***
NS
***
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PAR
shAXUD1
shSCR
3TP
DAUV
3TP-lux Promotor Activity
in DAUV cells
Luminescence (%)
300
250
200
150
100
50
0
*
NS
***
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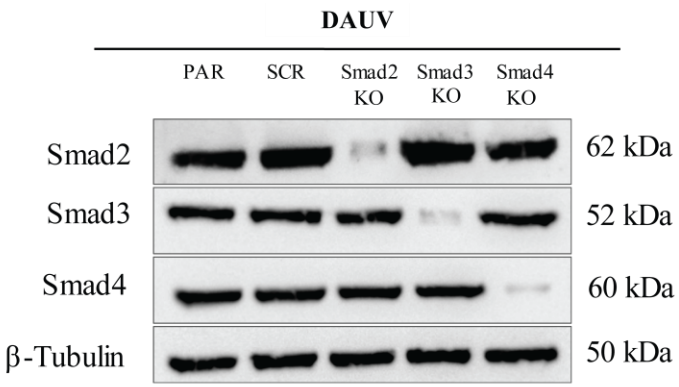
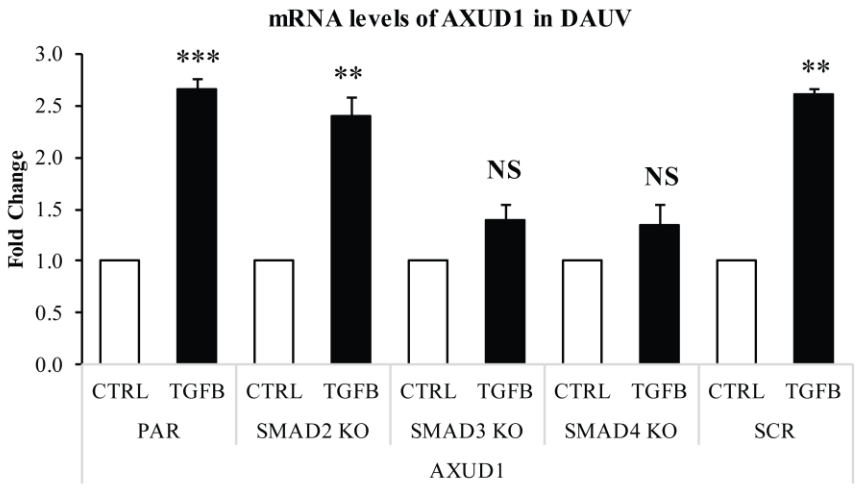
PAR
AXUD1 KO
SCR
3TP
DAUV

G


DAUV
PAR
SCR
Smad2 KO
Smad3 KO
Smad4 KO
Smad2
Smad3
Smad4
β-Tubulin
62 kDa
52 kDa
60 kDa
50 kDa

H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2.5
2.0
1.5
1.0
0.5
0.0
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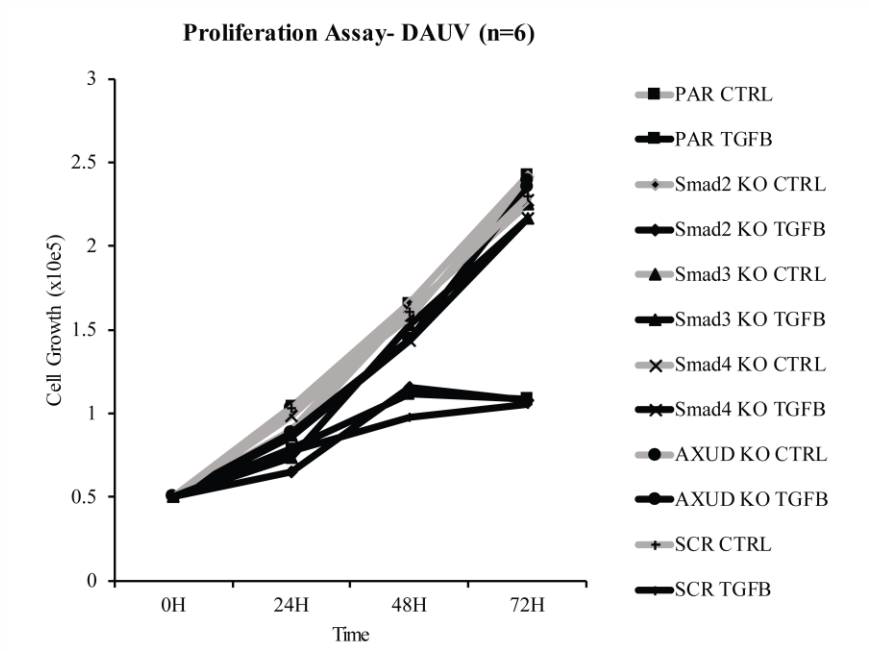
NS
NS
**
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
SCR
AXUD1

I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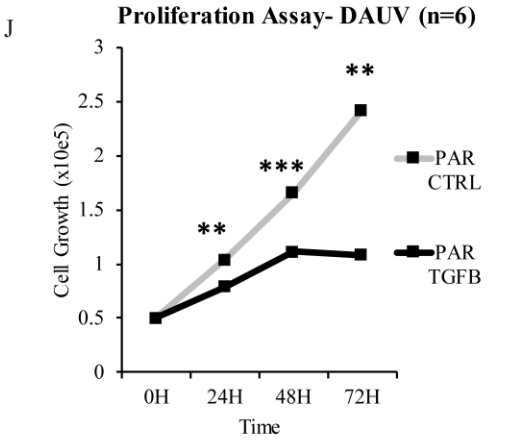
Cell Growth (x10e5)
3
2.5
2
1.5
1
0.5
0
0H
24H
48H
72H
Time
PAR CTRL
PAR TGFB
Smad2 KO CTRL
Smad2 KO TGFB
Smad3 KO CTRL
Smad3 KO TGFB
Smad4 KO CTRL
Smad4 KO TGFB
AXUD KO CTRL
AXUD KO TGFB
SCR CTRL
SCR TGFB

J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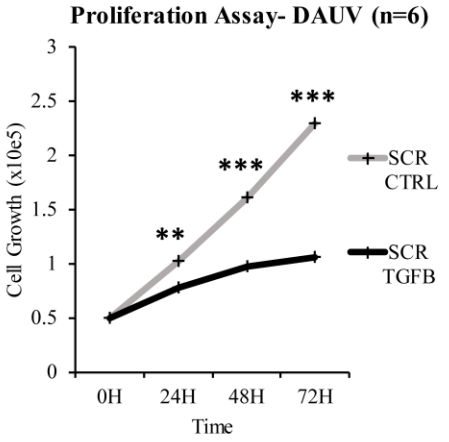
Cell Growth (x10e5)
Time
0H
24H
48H
72H
PAR CTRL
PAR TGFB
**
**
***

K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Cell Growth (x10e5)
Time
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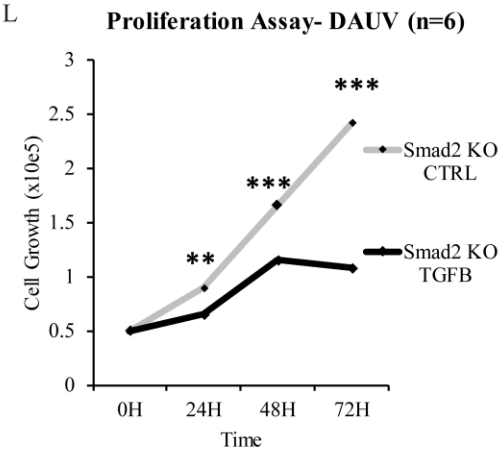
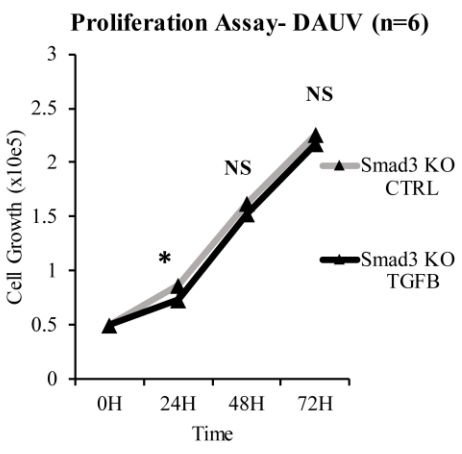
24H
48H
72H
SCR CTRL
SCR TGFB
**
***
***

L


Proliferation Assay- DAUV (n=6)
Cell Growth (x10e5)
Time
0H
24H
48H
72H
Smad2 KO CTRL
Smad2 KO TGFB
**
***
***

M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Cell Growth (x10e5)
Time
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24H
48H
72H
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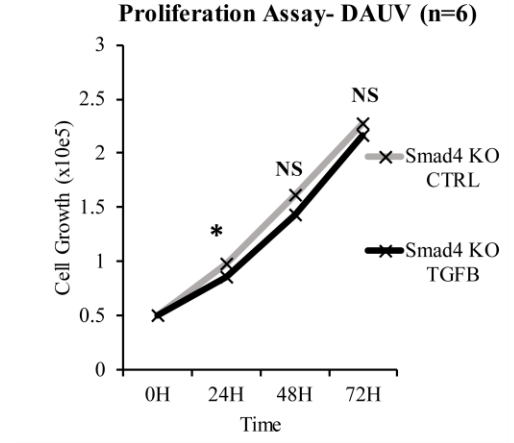
Smad3 KO TGFB
*
NS
NS

N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48H
72H
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Smad4 KO TGFB
*
NS
NS

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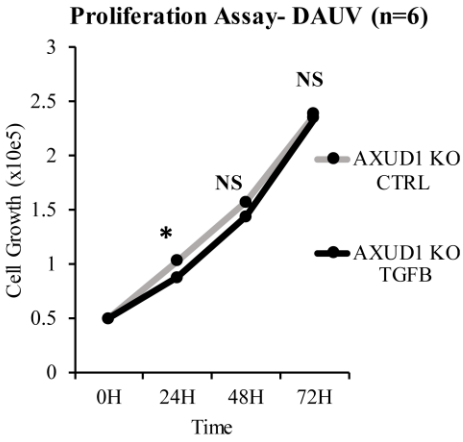
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Cell Growth (x10e5)
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48H
72H
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AXUD1 KO TGFB
*
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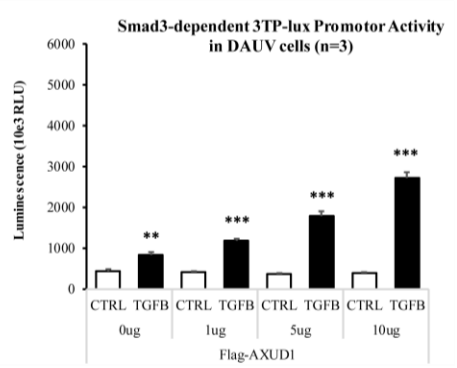
NS

P


Smad3-dependent 3TP-lux Promotor Activity
in DAUV cells (n=3)
Luminescence (10e3 RLU)
6000
5000
4000
3000
2000
1000
0
**
***
***
***
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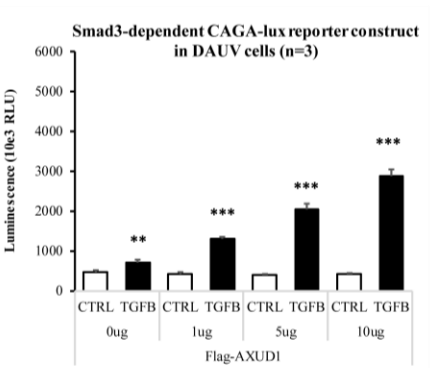
0ug 1ug 5ug 10ug
Flag-AXUD1

Q


Smad3-dependent CAGA-lux reporter construct
in DAUV cells (n=3)
Luminescence (10e3 RLU)
6000
5000
4000
3000
2000
1000
0
**
***
***
***
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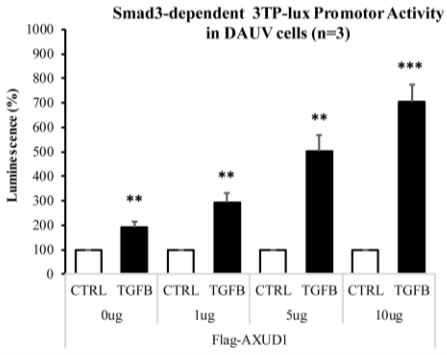
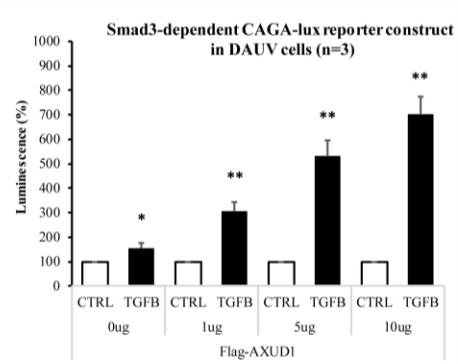
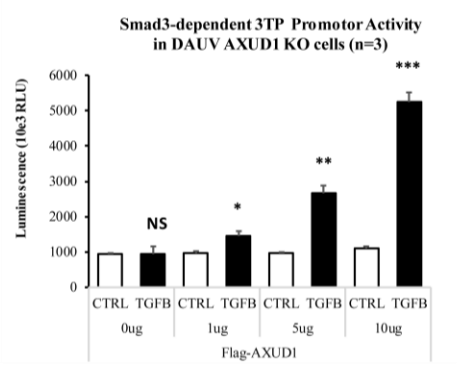
0ug 1ug 5ug 10ug
Flag-AXUD1


Smad3-dependent 3TP-lux Promotor Activity
in DAUV cells (n=3)
Luminescence (%)
1000
900
800
700
600
500
400
300
200
100
0
**
**
**
***
CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB
0ug 1ug 5ug 10ug
Flag-AXUD1


Smad3-dependent CAGA-lux reporter construct
in DAUV cells (n=3)
Luminescence (%)
1000
900
800
700
600
500
400
300
200
100
0
*
**
**
**
CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB
0ug 1ug 5ug 10ug
Flag-AXUD1

R


Smad3-dependent 3TP Promotor Activity
in DAUV AXUD1 KO cells (n=3)
Luminescence (10e3 RLU)
6000
5000
4000
3000
2000
1000
0
NS
*
**
***
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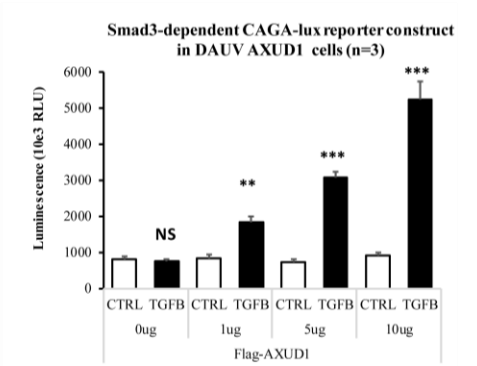
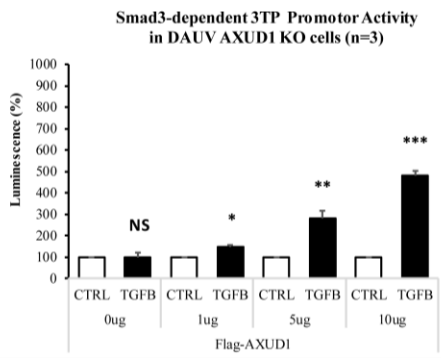
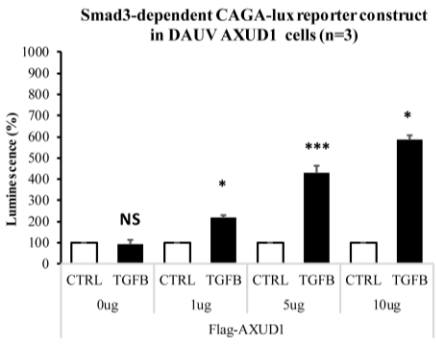
0ug 1ug 5ug 10ug
Flag-AXUD1

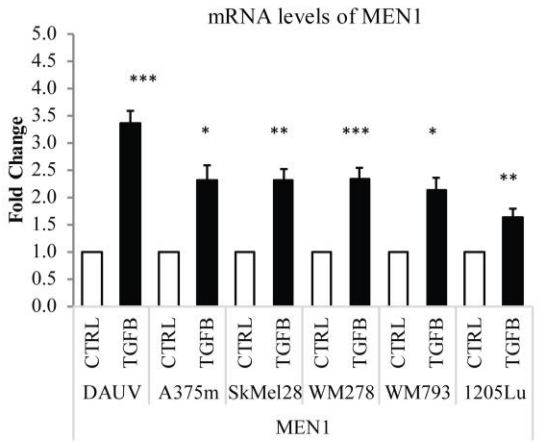
S


Smad3-dependent CAGA-lux reporter construct
in DAUV AXUD1 cells (n=3)
Luminescence (10e3 RLU)
6000
5000
4000
3000
2000
1000
0
NS
**
***
***
CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB
0ug 1ug 5ug 10ug
Flag-AXUD1

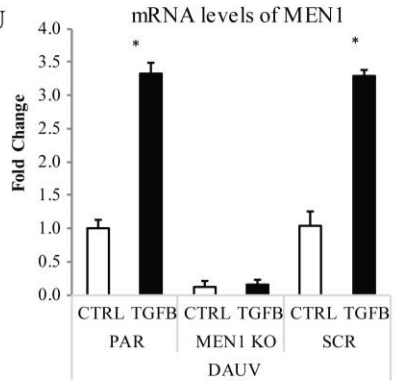

Smad3-dependent 3TP Promotor Activity
in DAUV AXUD1 KO cells (n=3)
Luminescence (%)
1000
900
800
700
600
500
400
300
200
100
0
NS
*
**
***
CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB
0ug 1ug 5ug 10ug
Flag-AXUD1

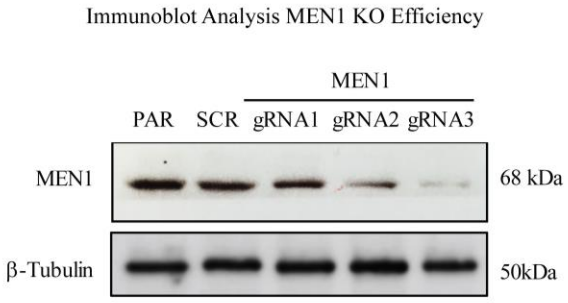

Smad3-dependent CAGA-lux reporter construct
in DAUV AXUD1 cells (n=3)
Luminescence (%)
1000
900
800
700
600
500
400
300
200
100
0
NS
*
***
*
CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB
0ug 1ug 5ug 10ug
Flag-AXUD1

T

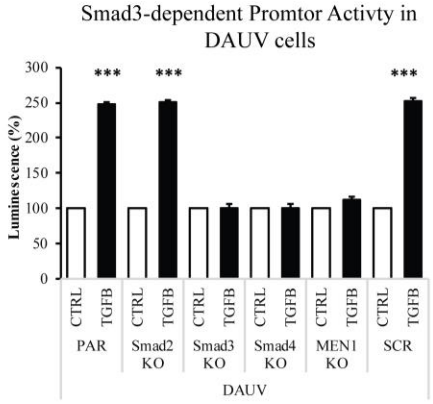

mRNA levels of MEN1
Fold Change
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
*
**
***
*
**
CTRL
TGFB
DAUV
A375m
SkMel28
WM278
WM793
1205Lu
MEN1

U


mRNA levels of MEN1
Fold Change
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
CTRL
TGFB
PAR
MEN1 KO
SCR
DAUV


Immunoblot Analysis MEN1 KO Efficiency
MEN1
PAR
SCR
gRNA1
gRNA2
gRNA3
MEN1
68 kDa
β-Tubulin
50kDa

V


Smad3-dependent Promtor Activty in DAUV cells
Luminescence (%)
300
250
200
150
100
50
0
***
***
***
CTRL
TGFB
PAR
Smad2 KO
Smad3 KO
Smad4 KO
MEN1 KO
SCR
DAUV

W

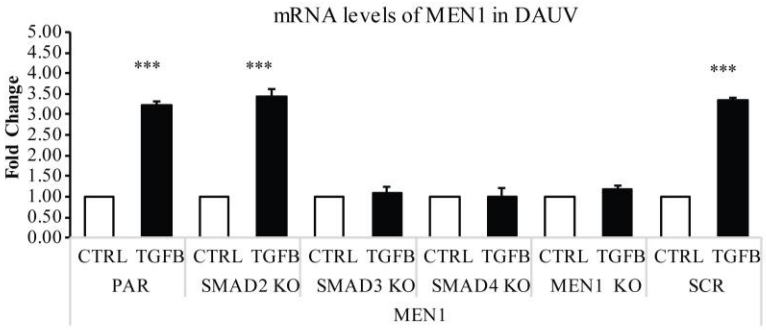

mRNA levels of MEN1 in DAUV
Fold Change
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
***
***
***
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
MEN1 KO
SCR
MEN1

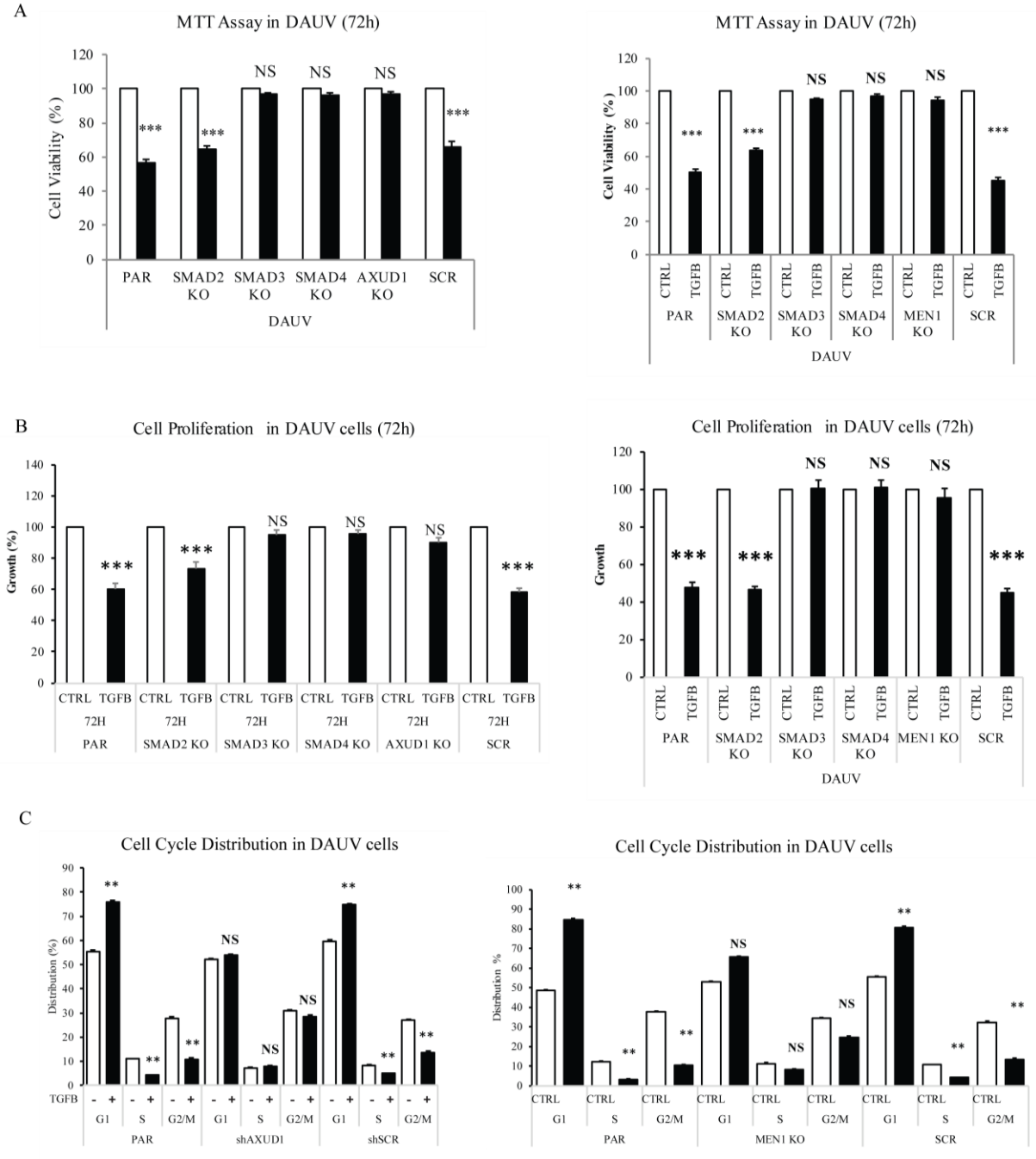
Figure 3-1: TGFβ/Smad3 signalling upregulates AXUD1 and menin expression in human cutaneous melanoma cells.

(A) Kaplan-Meier plots for disease-free survival outcome in correlation with AXUD1 expression
(B) qPCR analysis for AXUD1 mRNA levels in 6 different melanoma cell lines treated or not with TGFβ (200 pM) for 24 h. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.
(C) Immunohistochemical analysis for AXUD1 expression in parental DAUV melanoma cells treated or not with TGFβ (200 pM) for 24 h.
(D) Surveyor assay showing DAUV AXUD1 KO cleavage for Indel mutation detection. The expected cleavage band size of 344bp and 155bp is shown.
(E) qPCR analysis showing TGFβ effects on AXUD1 mRNA levels in parental, scrambled and AXUD1 knockdown (shAXUD1) and knockout (AXUD1 KO) cells. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.
(F) 3TPLux luciferase assay in DAUV parental, scrambled and AXUD1 knockdown (shAXUD1) and knockout (AXUD1 KO) cells. Data are represented as the mean of relative luciferase units/ percentage luminescence normalized to non-treated control for at least 3 independent experiments.
(G) Immunoblot analysis to assess the efficacy of the Smads-CRISPR/Cas9 knockouts in the DAUV cell line. β-Tubulin was used as a loading control.
(H) qPCR analysis showing AXUD1 mRNA levels in the Smad knockout cell lines treated or not with TGFβ. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.
(I-O) Proliferation assay showing DAUV parental (J), scrambled (K), Smad2 KO (L), Smad3 KO (M), Smad4 KO (N) and AXUD1 KO (O) melanoma cell lines that were treated or not with TGFβ (200pM) for 24, 48 and 72h in complete growth media (10% FBS) then were trypsinized and counted at each time point. Data are represented as the mean of counts or percentages normalized to non-treated control for at least 3 independent experiments.
(P-S) Luciferase assay for Smad3-dependent 3TP-Lux (P, R) and CAGA-lux (Q, S) in DAUV parental (P, Q), and AXUD1 KO (R, S) cells. Data are represented as the mean of relative

luciferase units (P-S, upper panels) or percentage luminescence (P-S, lower panels) normalized to non-treated control for at least 3 independent experiments.
(T) qPCR analysis showing mRNA levels of menin protein measured in 6 different human melanoma cell lines treated or not with TGFβ for 24 h.
(U) Knockout efficiency validation: qPCR analysis (left panel) and immunoblot analysis (right panel) showing menin levels in the generated DAUV stable knockout melanoma cell lines using CRISPR/Cas9 whereby three different guide RNAs (gRNAs) targeting specific MEN1 areas were used.
(V) 3TPLux luciferase assay in DAUV parental, scrambled, Smad2 KO, Smad3, KO, Smad4 KO, and menin KO) cells. Data are represented as the mean of relative luciferase units/ percentage luminescence normalized to non-treated control for at least 3 independent experiments.
(W) qPCR analysis showing menin mRNA levels in the Smad knockout cell lines treated or not with TGFβ in comparison to menin KO cells. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.

All error bars are standard errors of means. For statistical analysis, Student *t*-test was performed comparing TGFβ-treated to non-treated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.1.11.2 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce cell cycle arrest in DAUV human cutaneous melanoma cells.



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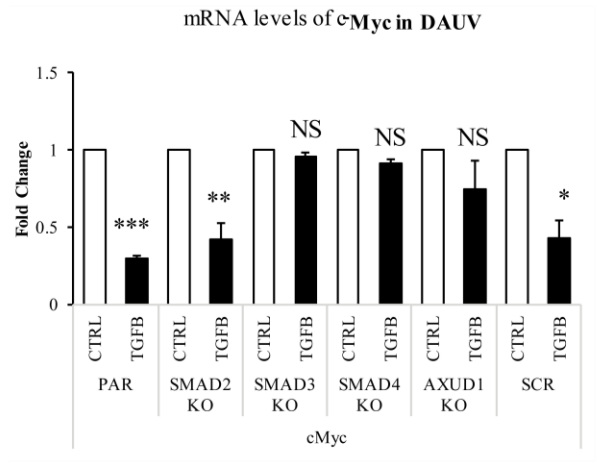
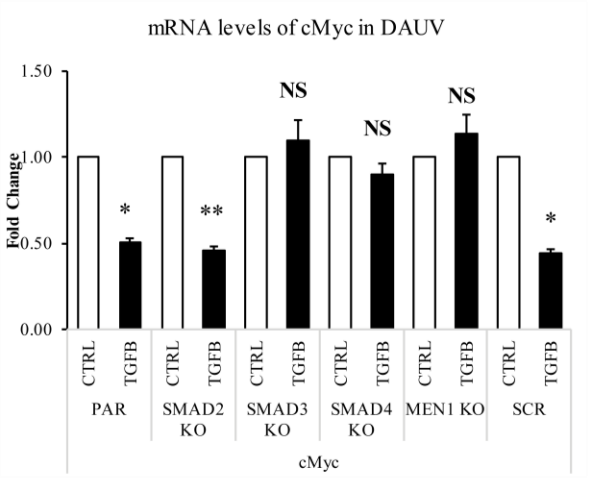
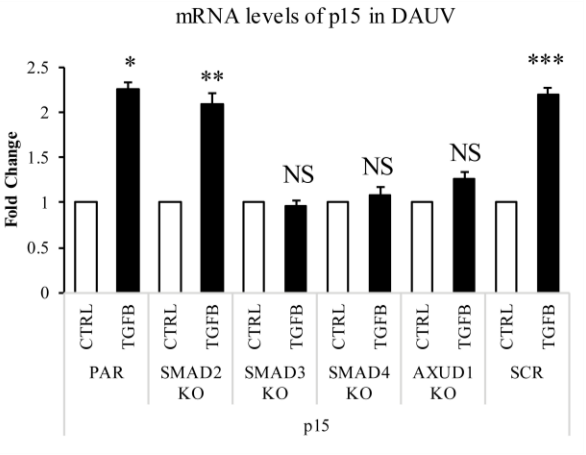

mRNA levels of c-Myc in DAUV
Fold Change
1.5
1
0.5
0
***
**
NS
NS
NS
*
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
AXUD1 KO
SCR
cMyc


mRNA levels of cMyc in DAUV
Fold Change
1.50
1.00
0.50
0.00
*
**
NS
NS
NS
*
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
MEN1 KO
SCR
cMyc


mRNA levels of p15 in DAUV
Fold Change
2.5
2
1.5
1
0.5
0
*
**
NS
NS
NS
***
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
AXUD1 KO
SCR
p15

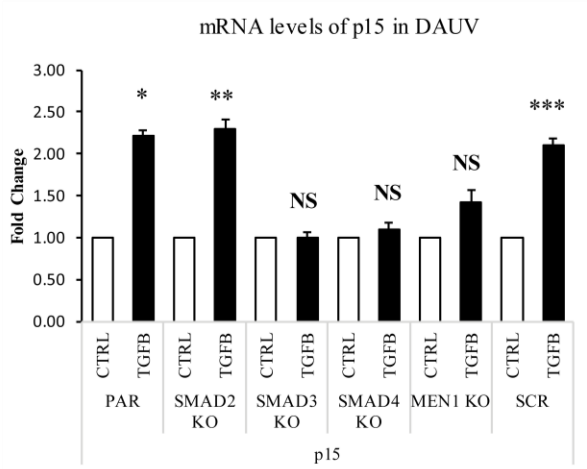
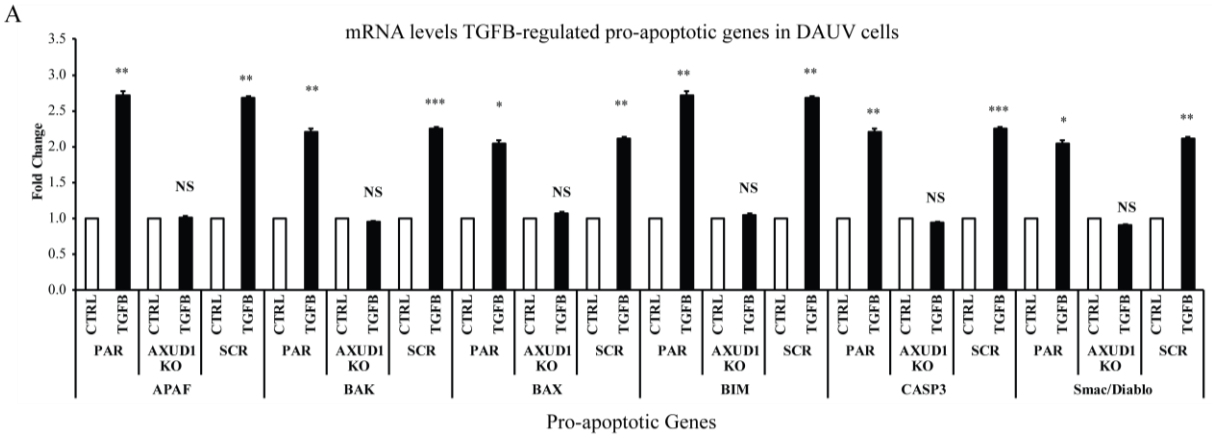

mRNA levels of p15 in DAUV
Fold Change
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
**
NS
NS
NS
***
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
MEN1 KO
SCR
p15

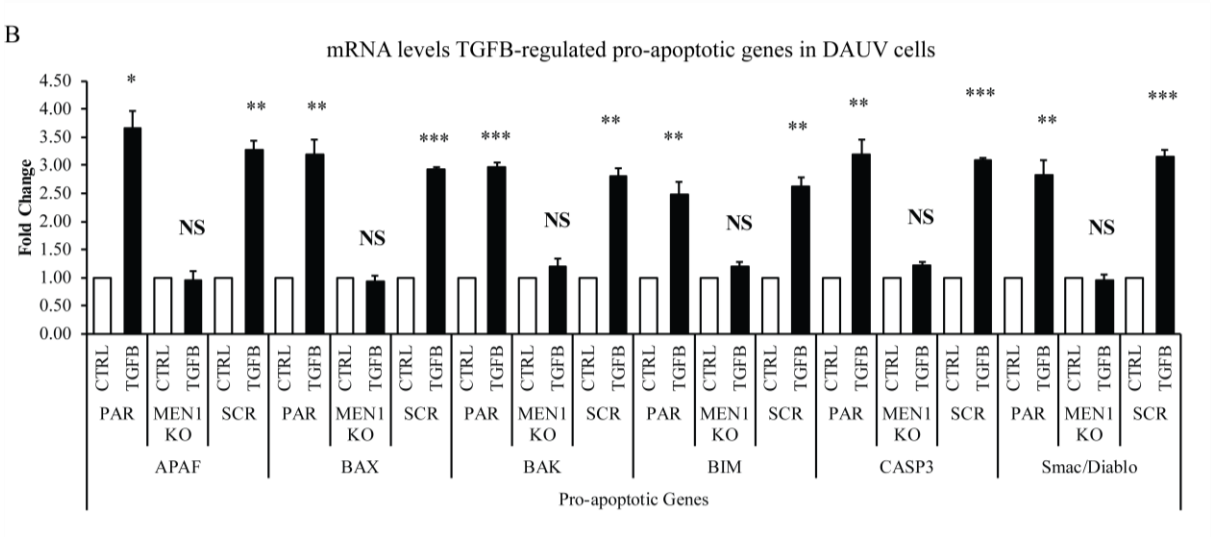
Figure 3-2: The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce cell cycle arrest in DAUV human cutaneous melanoma cells.

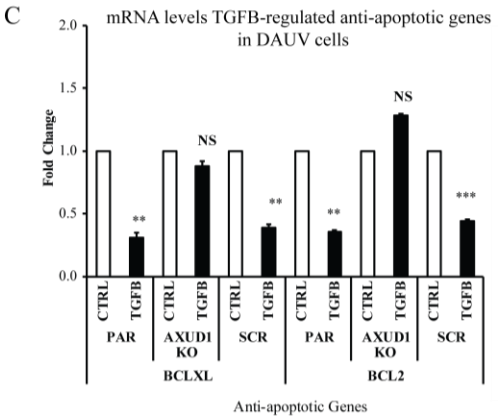
(A) MTT cell viability assay DAUV parental, scrambled, Smad2 KO, Smad3 KO, Smad4 KO and AXUD1 KO (left panel) or menin KO (right panel) melanoma cell lines were treated or not with TGFβ (200 pM) for 72h. Data are represented as the mean of percentages normalized to non-treated control for at least 3 independent experiments.
(B) Proliferation assay where DAUV parental, scrambled, Smad2 KO, Smad3 KO, Smad4 KO and AXUD1 KO (left panel) or menin KO (right panel) melanoma cell lines were treated or not with TGFβ (200 pM) for 24, 48 and 72h in serum-low media (2% FBS) before direct cell counting. Data are represented as the mean of counts or percentages normalized to non-treated control for at least 3 independent experiments.
(C) Cell distribution analysis using propidium iodide staining where DAUV parental, scrambled and AXUD1 knockdown (left panel) or menin knockout (right panel) melanoma cell lines were treated or not with TGFβ (200 pM) for 24h. Data are represented as the mean of cell population percentages for at least 3 independent experiments.
(D) qPCR Analysis: DAUV parental, scrambled, Smad2 KO, Smad3 KO, Smad4 KO and AXUD1 KO melanoma cell lines (E) were treated or not with TGFβ (200 pM) for 24 h in starvation media. mRNA levels for cMyc (left panels) and p15 (right panels) were measured and normalized to GAPDH. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.

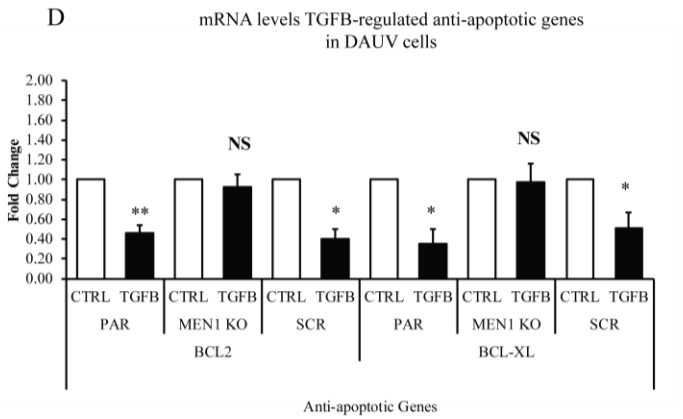
All error bars are standard errors of means. For statistical analysis, Student *t*-test was performed comparing TGFβ-treated to non-treated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.1.11.3 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce apoptosis in DAUV human cutaneous melanoma cells.

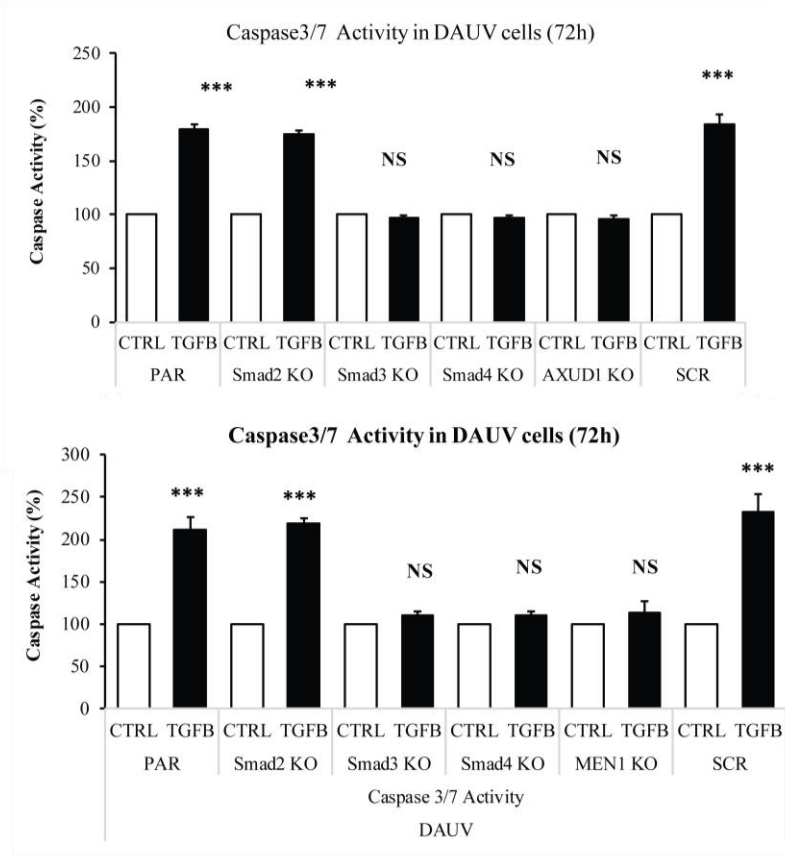




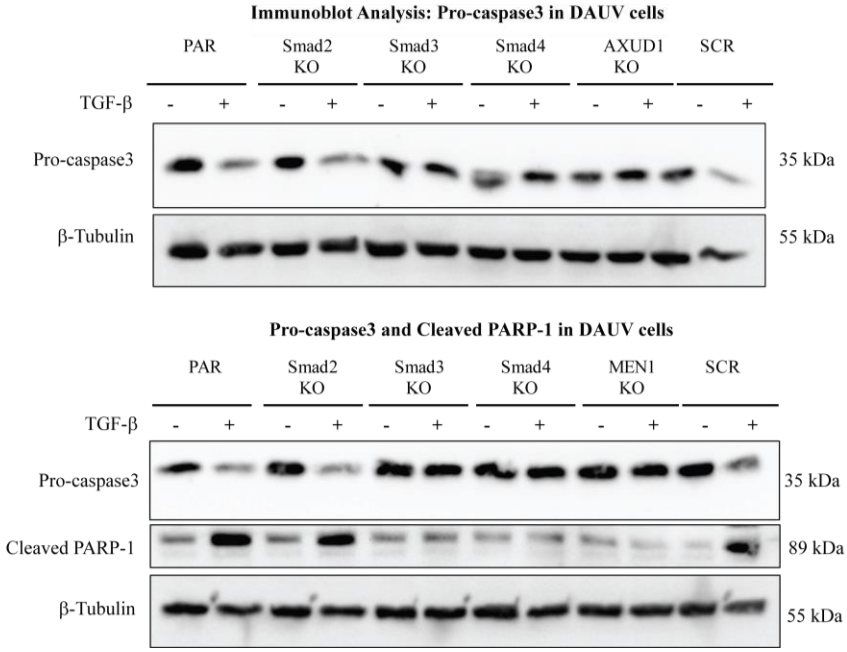




E


Caspase3/7 Activity in DAUV cells (72h)
Caspase Activity (%)
250
200
150
100
50
0
***
***
NS
NS
NS
***
CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB
PAR
Smad2 KO
Smad3 KO
Smad4 KO
AXUD1 KO
SCR
Caspase3/7 Activity in DAUV cells (72h)
Caspase Activity (%)
300
250
200
150
100
50
0
***
***
NS
NS
NS
***
CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB CTRL TGFB
PAR
Smad2 KO
Smad3 KO
Smad4 KO
MEN1 KO
SCR
Caspase 3/7 Activity
DAUV

F


Immunoblot Analysis: Pro-caspase3 in DAUV cells
PAR
Smad2 KO
Smad3 KO
Smad4 KO
AXUD1 KO
SCR
TGF-β - + - + - + - + - + - +
Pro-caspase3
35 kDa
β-Tubulin
55 kDa
Pro-caspase3 and Cleaved PARP-1 in DAUV cells
PAR
Smad2 KO
Smad3 KO
Smad4 KO
MEN1 KO
SCR
TGF-β - + - + - + - + - + - +
Pro-caspase3
35 kDa
Cleaved PARP-1
89 kDa
β-Tubulin
55 kDa

G

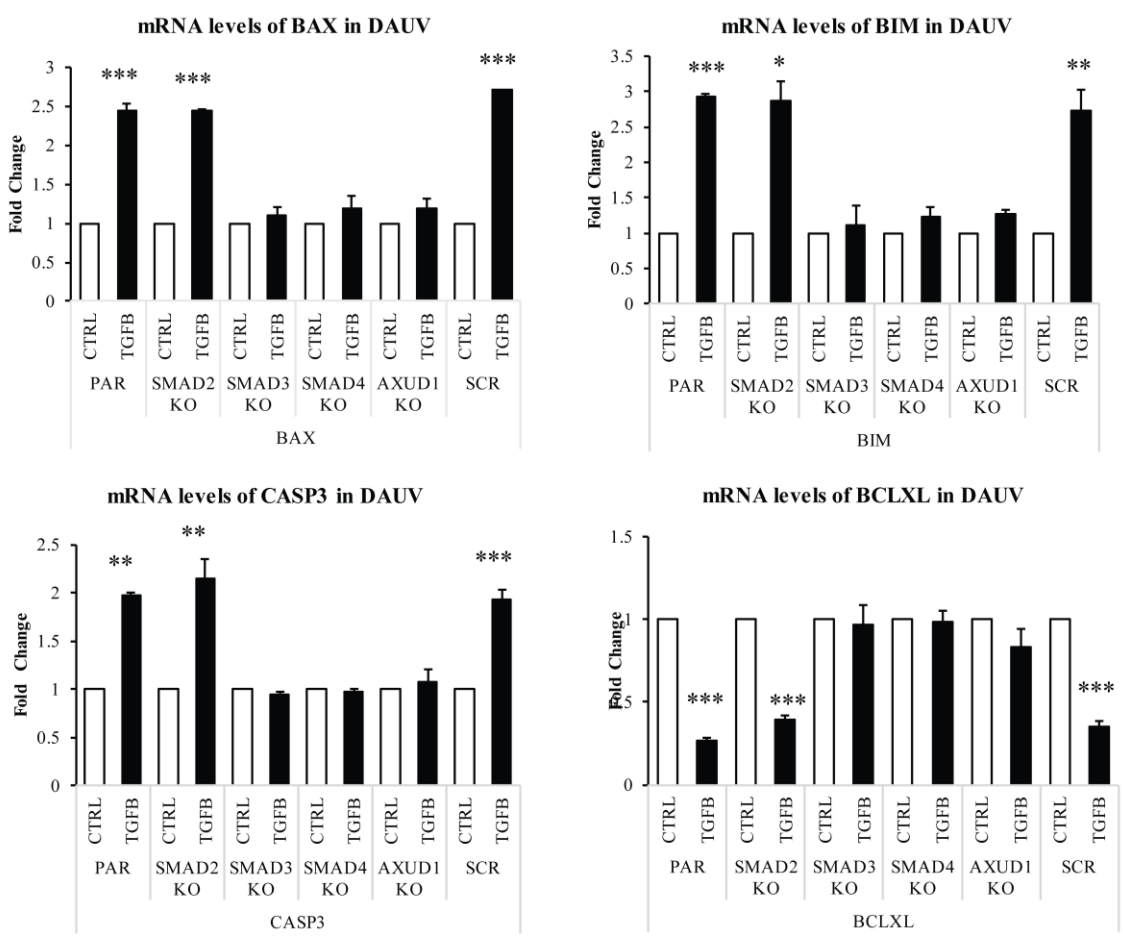

mRNA levels of BAX in DAUV
Fold Change
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
AXUD1 KO
SCR
BAX
mRNA levels of BIM in DAUV
BIM
mRNA levels of CASP3 in DAUV
CASP3
mRNA levels of BCLXL in DAUV
BCLXL

Figure 3-3: The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce apoptosis in DAUV human cutaneous melanoma cells

(A-D) qPCR analysis in DAUV parental, scrambled, Smad2, Smad3 and AXUD1 knockout (A, C) or menin knockout (B, D) were treated or not with TGFβ (200 pM) for 24 h. mRNA levels for the specified prop-apoptotic (A, B) and anti-apoptotic (C, D) genes were measured and normalized to GAPDH. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.

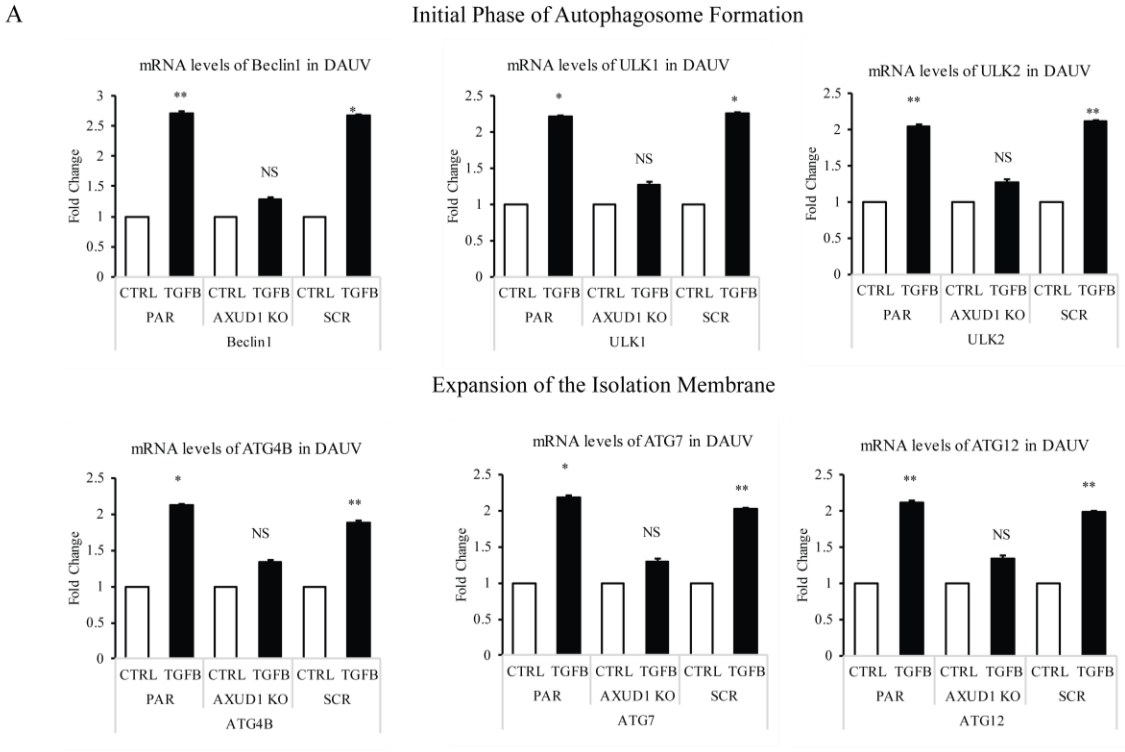
(E) Caspase 3/7 assay in where DAUV parental, scrambled, Smad2, Smad3 and AXUD1 knockout (upper panel) or menin knockout (lower panel) DAUV melanoma cell lines were treated or not with TGFβ (200 pM) for 24h. Data are represented as the percentage of the mean of relative luciferase unit normalized to non-treated control for at least 3 independent experiments.

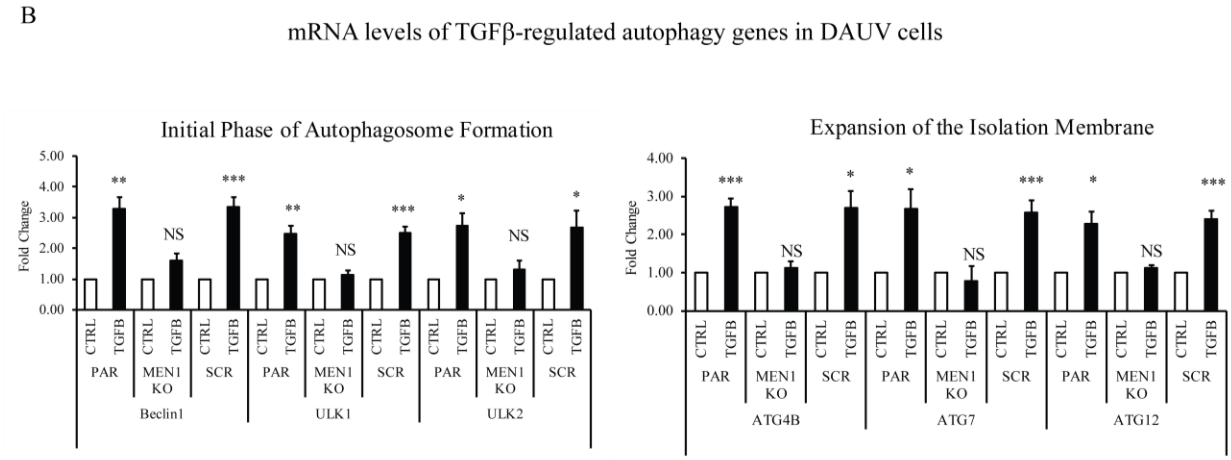
(F) Immunoblot Analysis where DAUV parental, scrambled, Smad2, Smad3 and AXUD1 knockout (upper panel) or menin knockout (lower panel) DAUV melanoma cell lines were treated or not with TGFβ (200 pM) for 24 h and the expression level of procaspase3 (upper panel) or procaspase3 and cleaved PARP-1 (lower panel) were determined. β-Tubulin was used as a loading control.

(G) qPCR Analysis where DAUV parental, scrambled, Smad2, Smad3, Smad4 and AXUD1 knockout DAUV melanoma cell lines were treated or not with TGFβ (200 pM) for 24. mRNA levels for the specified prop-apoptotic and anti-apoptotic genes were measured and normalized to GAPDH. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.

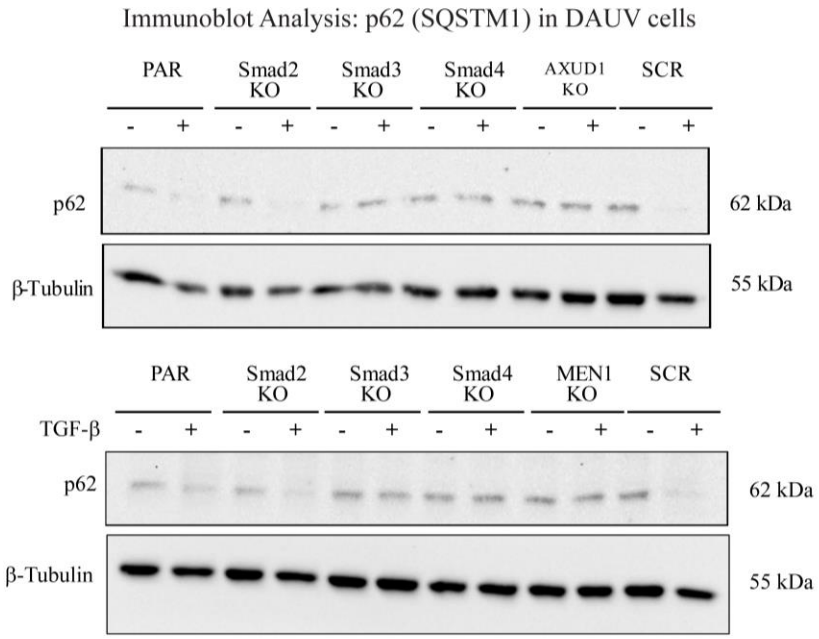
All error bars are standard errors of means. For statistical analysis, Student t-test was performed comparing treated to non-treated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.1.11.4 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce autophagy in DAUV human cutaneous melanoma cells.

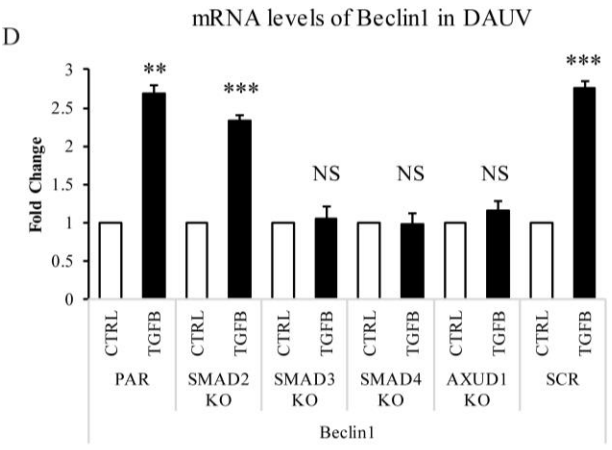


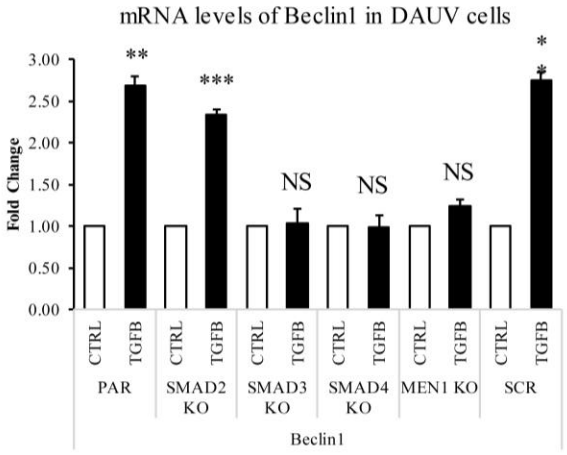


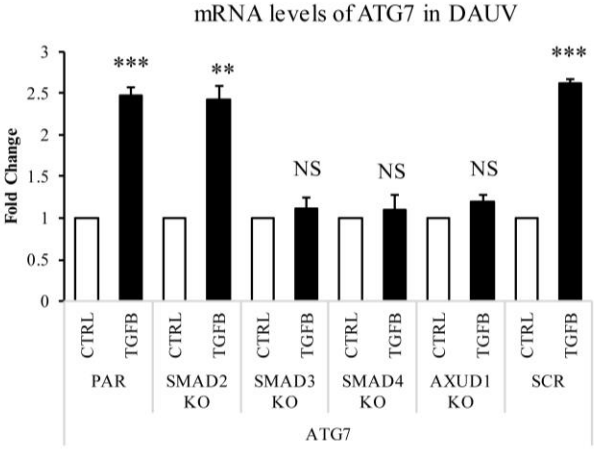
C


Immunoblot Analysis: p62 (SQSTM1) in DAUV cells
PAR
Smad2 KO
Smad3 KO
Smad4 KO
AXUD1 KO
SCR
- + - + - + - + - + - +
p62
62 kDa
β-Tubulin
55 kDa
PAR
Smad2 KO
Smad3 KO
Smad4 KO
MEN1 KO
SCR
TGF-β - + - + - + - + - + - +
p62
62 kDa
β-Tubulin
55 kDa

D


mRNA levels of Beclin1 in DAUV
Fold Change
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
AXUD1 KO
SCR
Beclin1


mRNA levels of Beclin1 in DAUV cells
Fold Change
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
MEN1 KO
SCR
Beclin1


mRNA levels of ATG7 in DAUV
Fold Change
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
AXUD1 KO
SCR
ATG7

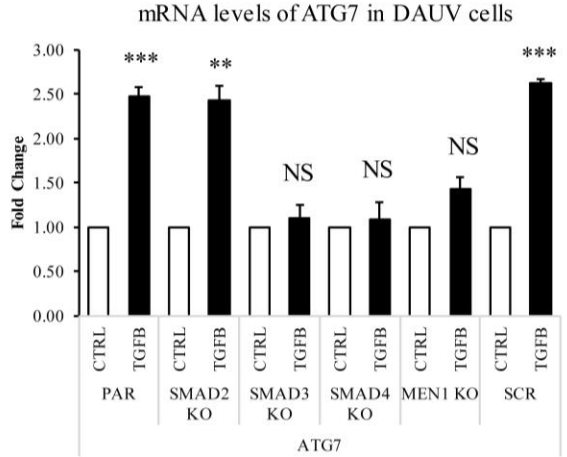

mRNA levels of ATG7 in DAUV cells
Fold Change
CTRL
TGFB
PAR
SMAD2 KO
SMAD3 KO
SMAD4 KO
MEN1 KO
SCR
ATG7

Figure 3-4: The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to induce autophagy in DAUV human cutaneous melanoma cells

(A, B) qPCR Analysis: DAUV parental, scrambled, AXUD1 knockout (A) and menin knockout (B) melanoma cell lines were treated or not with TGFβ (200 pM) for 24 h. mRNA levels for the specified autophagy genes were measured and normalized to GAPDH. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.
(C) Immunoblot Analysis: DAUV parental, scrambled, Smad2, Smad3 and AXUD1 knockout (upper panel) or menin knockout (lower panel) melanoma cell lines were treated or not with TGFβ (200 pM) for 24 h and the expression level of p62 (SQSTM1) was determined. β-Tubulin was used as a loading control.
(D) qPCR Analysis: DAUV parental, scrambled, Smad2, Smad3, Smad4 and AXUD1 (left panel) or menin (right panel) knockout melanoma cell lines were treated or not with TGFβ (200 pM) for 24 h in starvation media. mRNA levels for the specified autophagy genes were measured and normalized to GAPDH. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.

All error bars are standard errors of means. For statistical analysis, Student *t*-test was performed comparing treated to non-treated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.1.11.5 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit cell immortalization in DAUV human cutaneous melanoma cells.

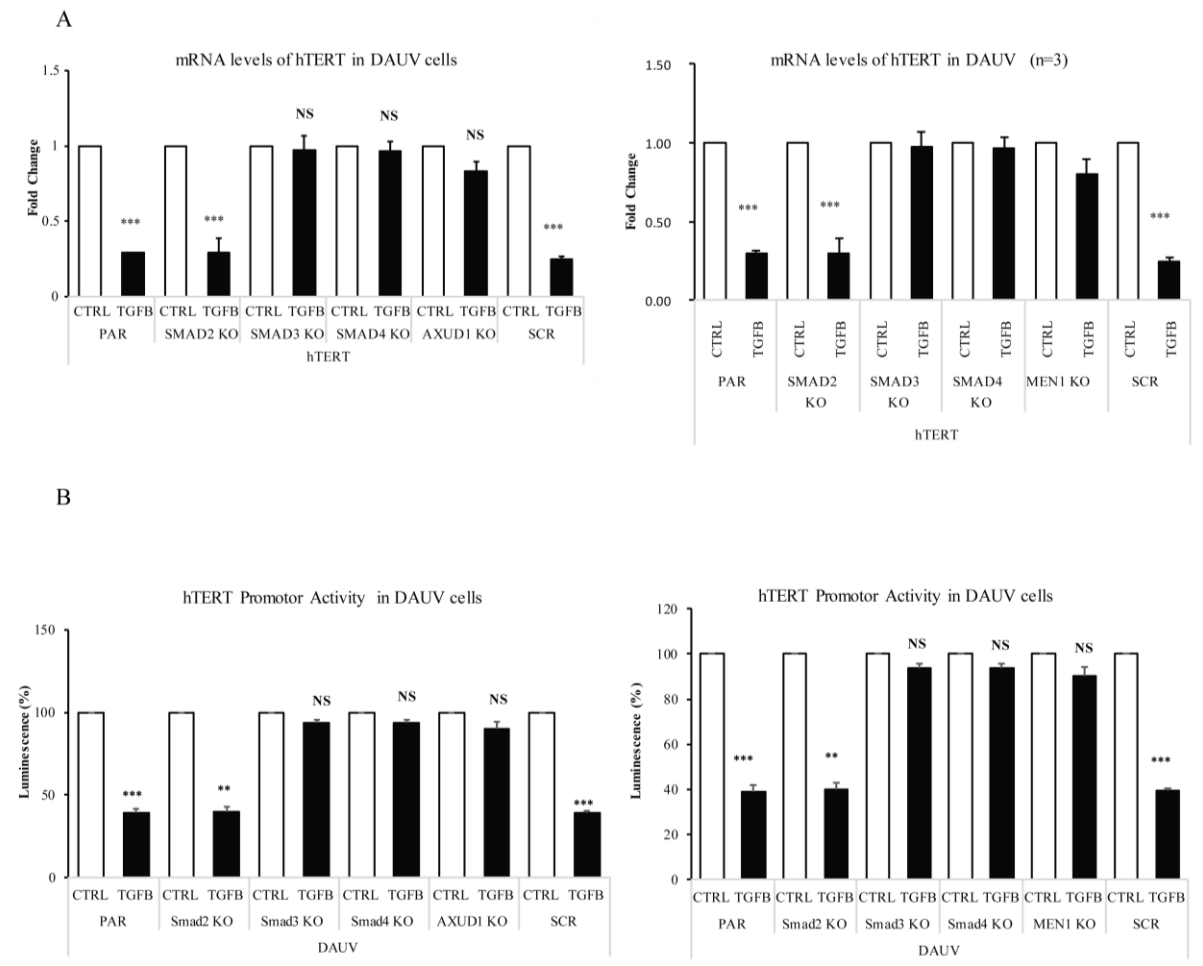
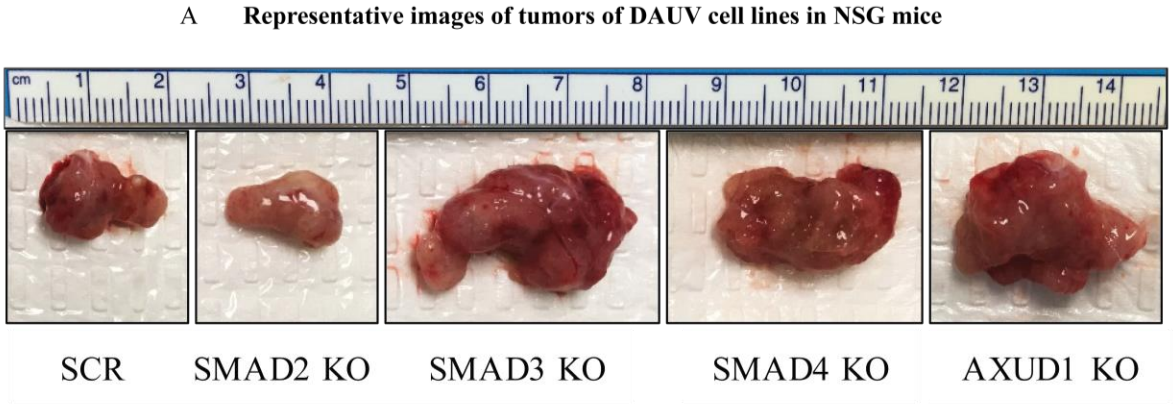


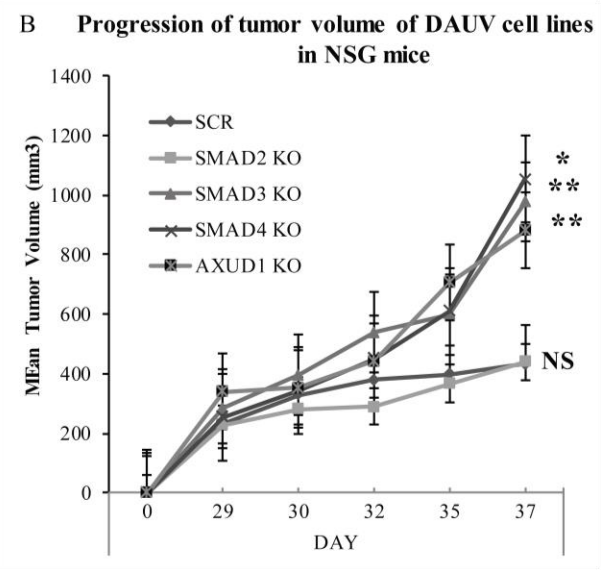
Figure 3-5: The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit cell immortalization in DAUV human cutaneous melanoma cells.

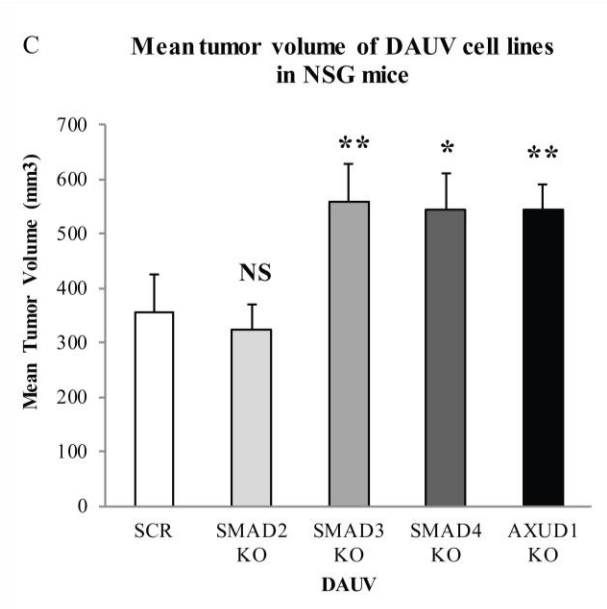
(A) qPCR Analysis: DAUV parental, scrambled, Smad2, Smad3, Smad4 and AXUD1 (left panel) or menin (right panel) knockout melanoma cell lines were treated or not with TGFβ (200 pM) for 24 h. mRNA levels for the hTERT gene were measured and normalized to GAPDH. Data are represented as the mean of fold-induction normalized to non-treated control for at least 3 independent experiments.
(B) Luciferase Assay: DAUV parental, scrambled, Smad2, Smad3, Smad4 and AXUD1 (left panel) or menin (right panel) knockout melanoma cell lines were transfected with the hTERT-lux, treated or not with TGFβ (200 pM) for 24 h. Luciferase activity was measured and represented as the mean percentage luminescence relative to the non-treated parental condition (normalized to β-galactosidase values) from 3 independent experiments.

(A, B) All error bars are standard errors of means. For statistical analysis, Student t-test was performed comparing TGFβ treated to non-treated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.1.11.6 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit primary tumour formation of DAUV human melanoma cells *in vivo*.







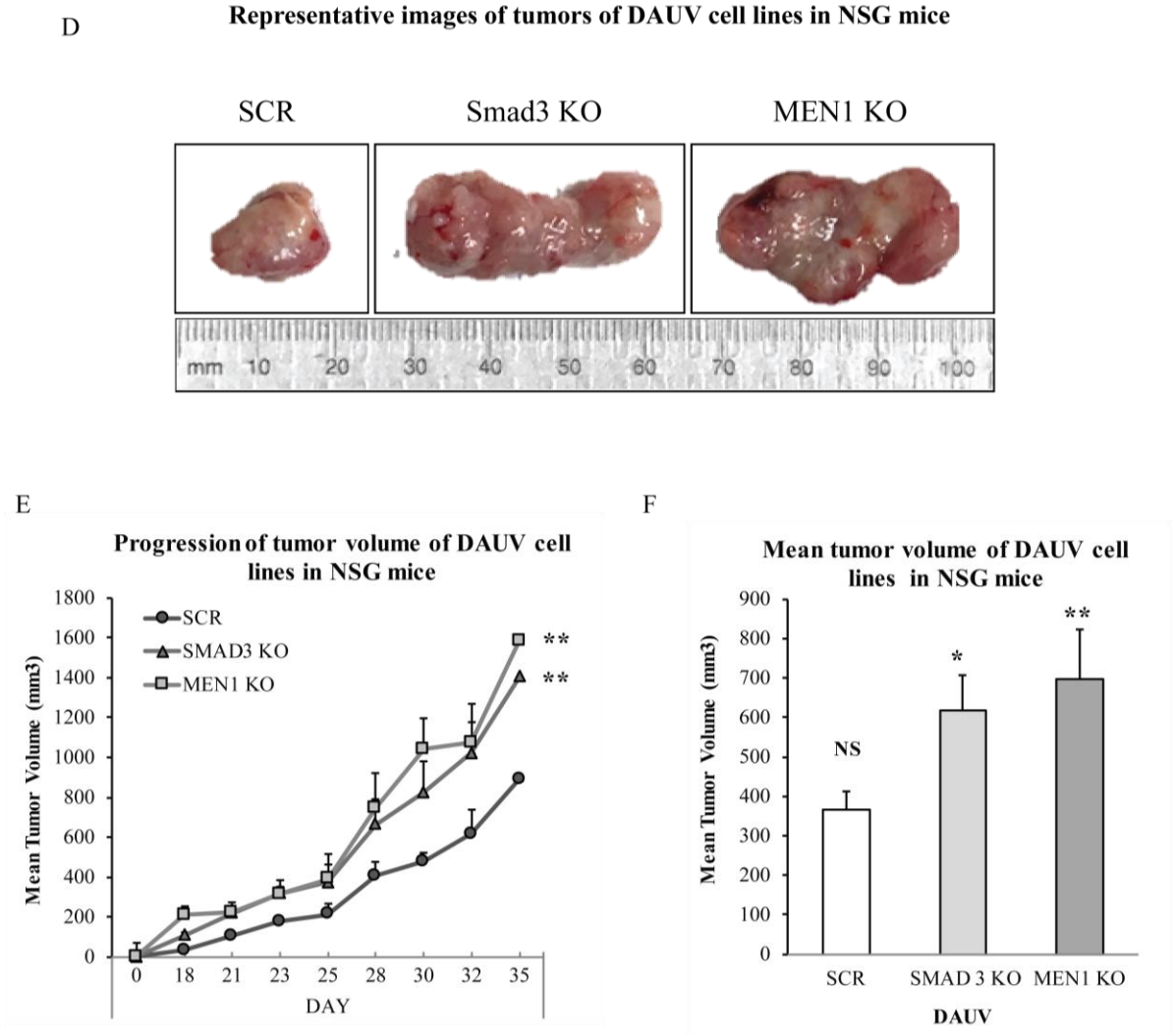


Figure 3-6: The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit primary tumour formation of DAUV human melanoma cells *in vivo*.

(A) Representative images of the tumour removed from the corresponding groups of injected immunodeficient NSG mice.

(B) Tumour Growth Curve: Five groups of NSG mice (5 mice/group) received a subcutaneous injection of 2 x10^6 of either scrambled, Smad2, Smad3, Smad4, or AXUD knockout DAUV cells.

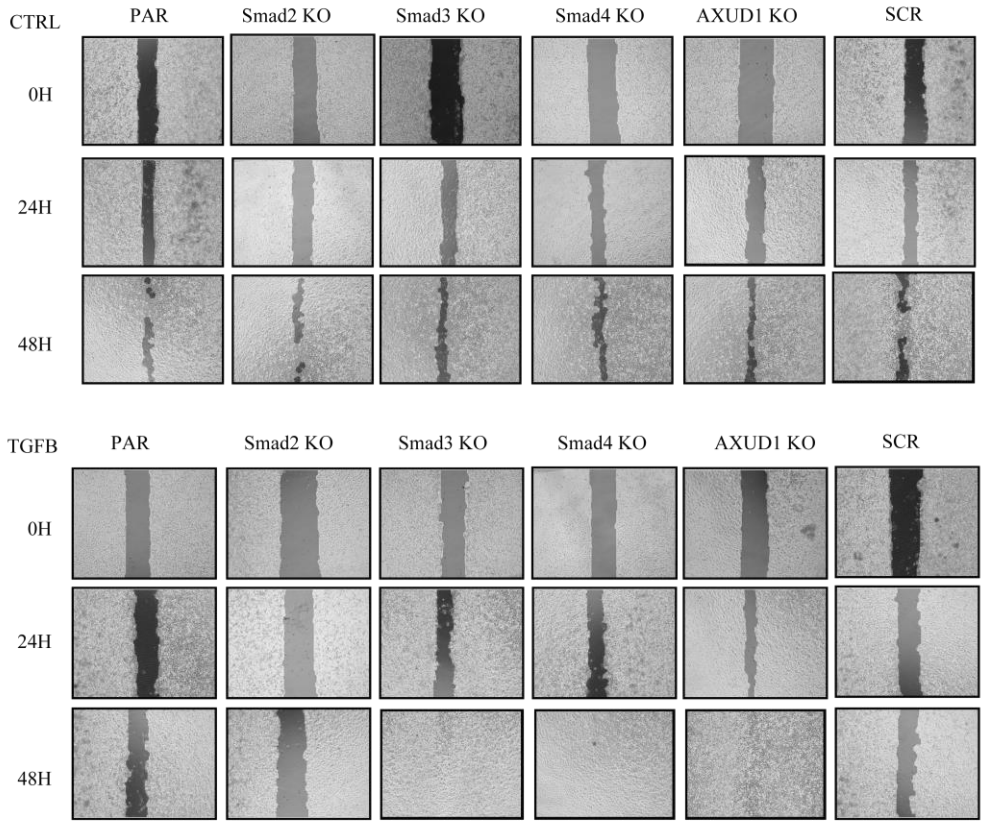
(C) Mean tumour volume in NSG mice receiving parental, Smad2, Smad3, Smad4, or AXUD KO DAUV cells (n=5 /group).

(D) Representative images of the tumour removed from the corresponding groups of injected NSG mice.

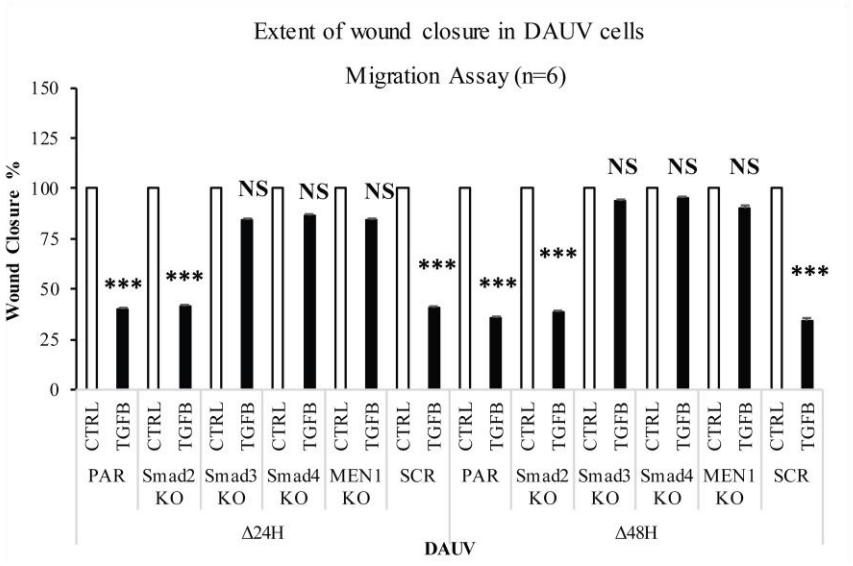
(E) Tumour Growth Curve: Three groups of NSG mice (5 mice/group) received a subcutaneous injection of 2 x10^6 of either scrambled, Smad3, or menin KO DAUV cells.
(F) Mean tumour volume in NSG mice receiving scrambled, Smad3, or menin KO DAUV cells (n=5 /group).
All error bars are the standard errors of means. Student t-test was performed comparing tumours from mice injected with KO DAUV melanoma cells to those injected with the scrambled cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.1.11.7 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit cell migration in DAUV human melanoma cells.

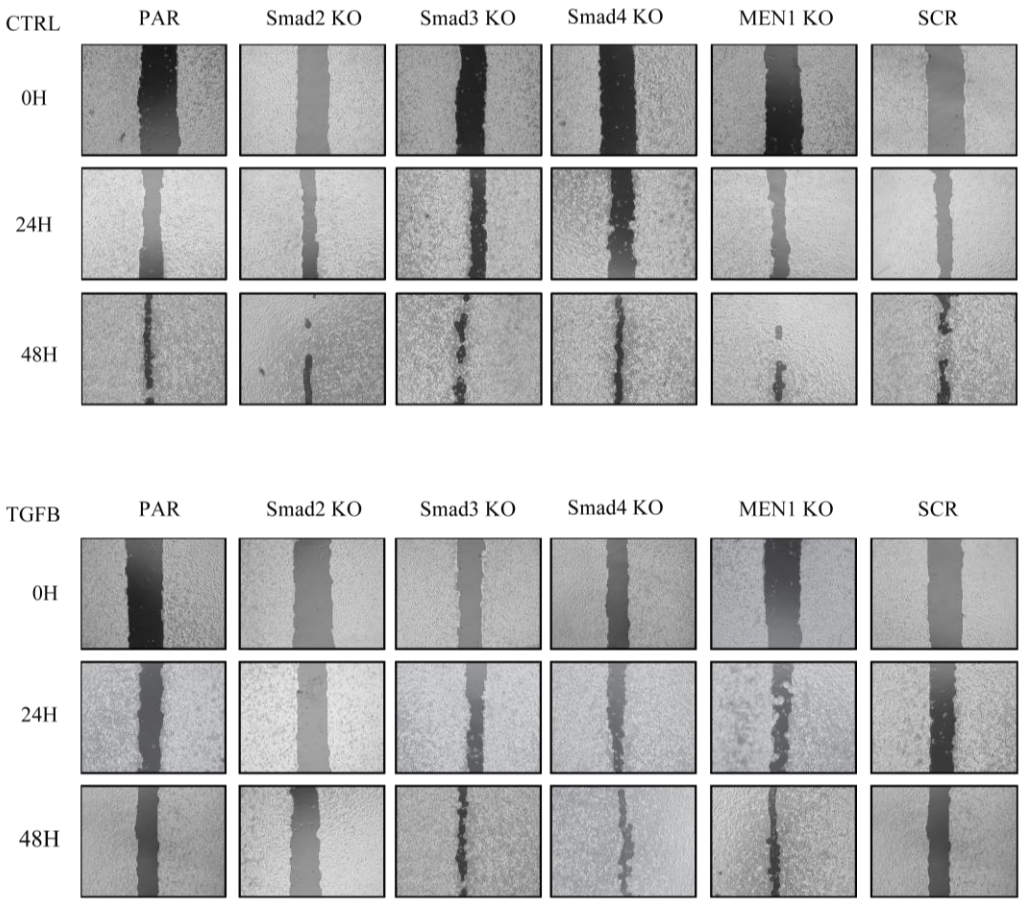
A



B



C


CTRL
PAR
Smad2 KO
Smad3 KO
Smad4 KO
MEN1 KO
SCR
0H
24H
48H
TGFB
PAR
Smad2 KO
Smad3 KO
Smad4 KO
MEN1 KO
SCR
0H
24H
48H

D

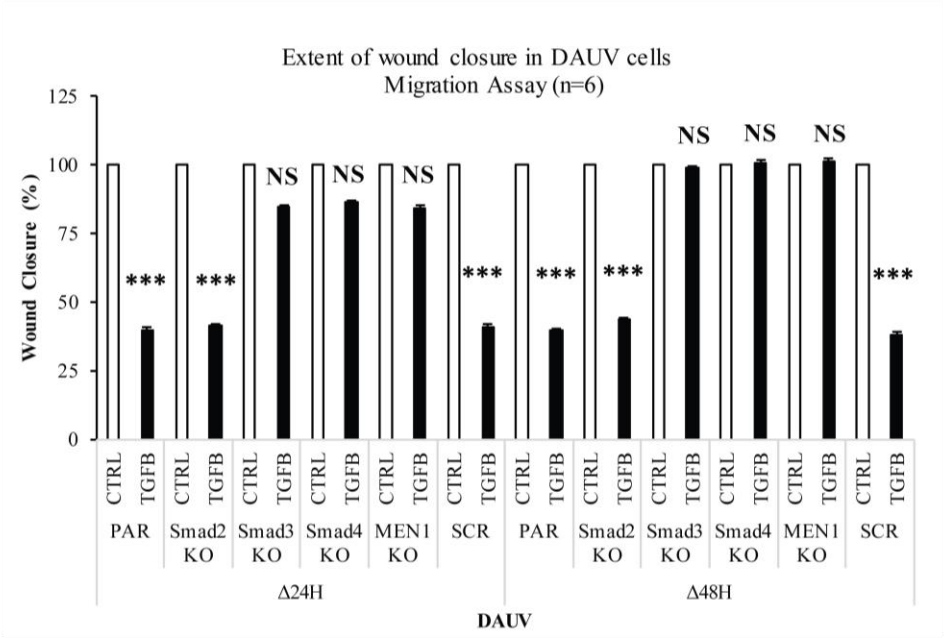
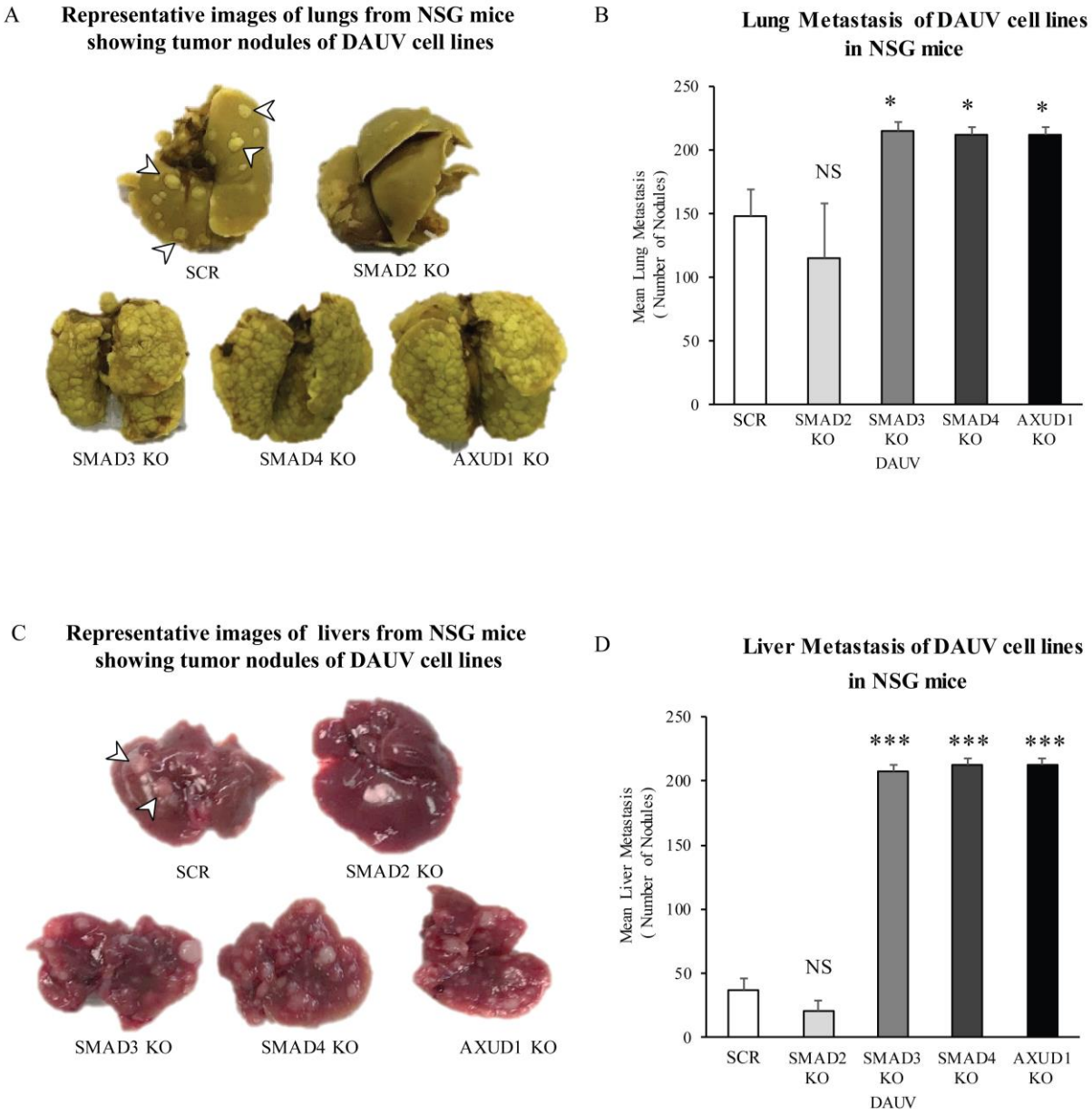

Extent of wound closure in DAUV cells
Migration Assay (n=6)
Wound Closure (%)
125
100
75
50
25
0
NS
***
CTRL
TGFB
PAR
Smad2 KO
Smad3 KO
Smad4 KO
MEN1 KO
SCR
Δ24H
Δ48H
DAUV

Figure 3-7: The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required to inhibit cell migration in DAUV human melanoma cells.

(A, C) Scratch wound healing migration assay: Representative pictures of the wound at 0, 24, and 48 hours after TGFβ treatment in DAUV parental, scrambled, Smad2, Smad3, Smad4 AXUD1 knockout (A) or menin knockout (C) DAUV melanoma cells treated or not with TGFβ (200 pM) for 72h.
(B, D) Scratch wound healing migration assay: Extent of wound-closure represented [(B) for (A), (D) for (C)] as the mean percentage closure relative to the non-treated parental condition from 4-6 independent experiments.

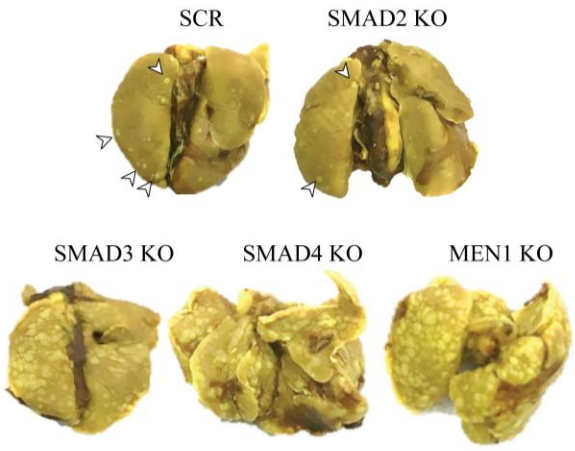
All error bars are standard errors of means. For statistical analysis, Student t-test was performed comparing TGFβ treated to non-treated control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.1.11.8 The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required for the inhibition of secondary tumour metastasis *in vivo*.

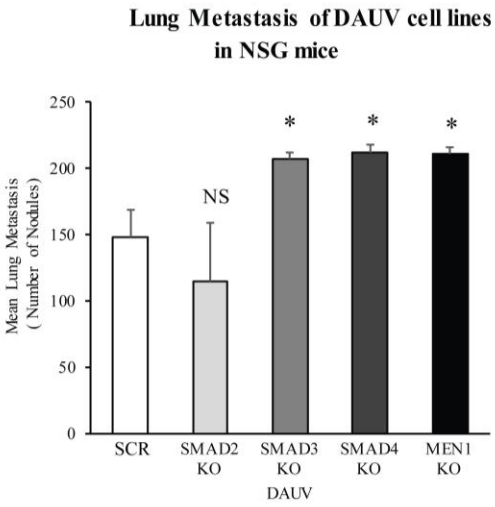


E

Representative images of lungs from NSG mice showing tumor nodules of DAUV cell lines

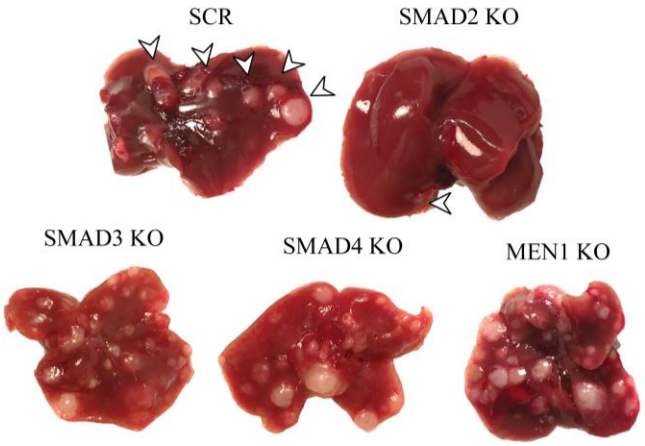


F



G

Representative images of livers from NSG mice showing tumor nodules of DAUV cell lines



H

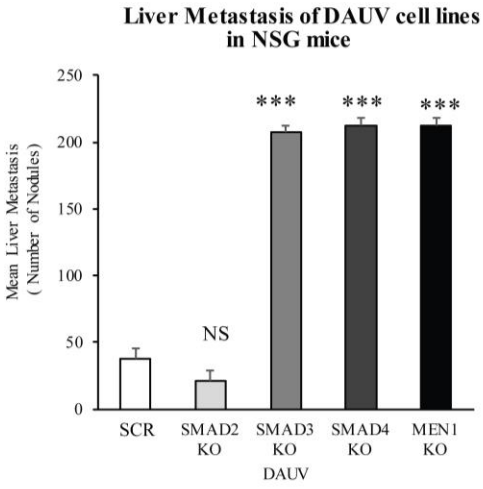


Figure 3-8: The TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin axes are required for the inhibition of secondary tumour metastasis in vivo.

(A, C) Representative images and (B, D) mean counts of tumour metastatic nodules in lungs (A, B) and livers (C, D) from five groups of NSG mice (5 mice/ group) that received an intravenous tail vein injection of $1x10^6$ cells of either scrambled, Smad2 KO, Smad3 KO, Smad4 KO, or AXUD KO DAUV cells.
(E, G) Representative images and (F, H) mean counts of tumour metastatic nodules in lungs (E, F) and livers (G, H) from five groups of NSG mice (5 mice/ group) that received an intravenous tail vein injection of $1x10^6$ cells of either scrambled, Smad2 KO, Smad3 KO, Smad4 KO, or menin KO DAUV cells.

The error bars are the standard errors of the mean. For statistical analysis, the t-test was performed compared to the parental (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

3.1.11.9 A schematic diagram for the role of AXUD1 and menin downstream of the TGFβ signalling pathway in (DAUV) cutaneous melanoma.

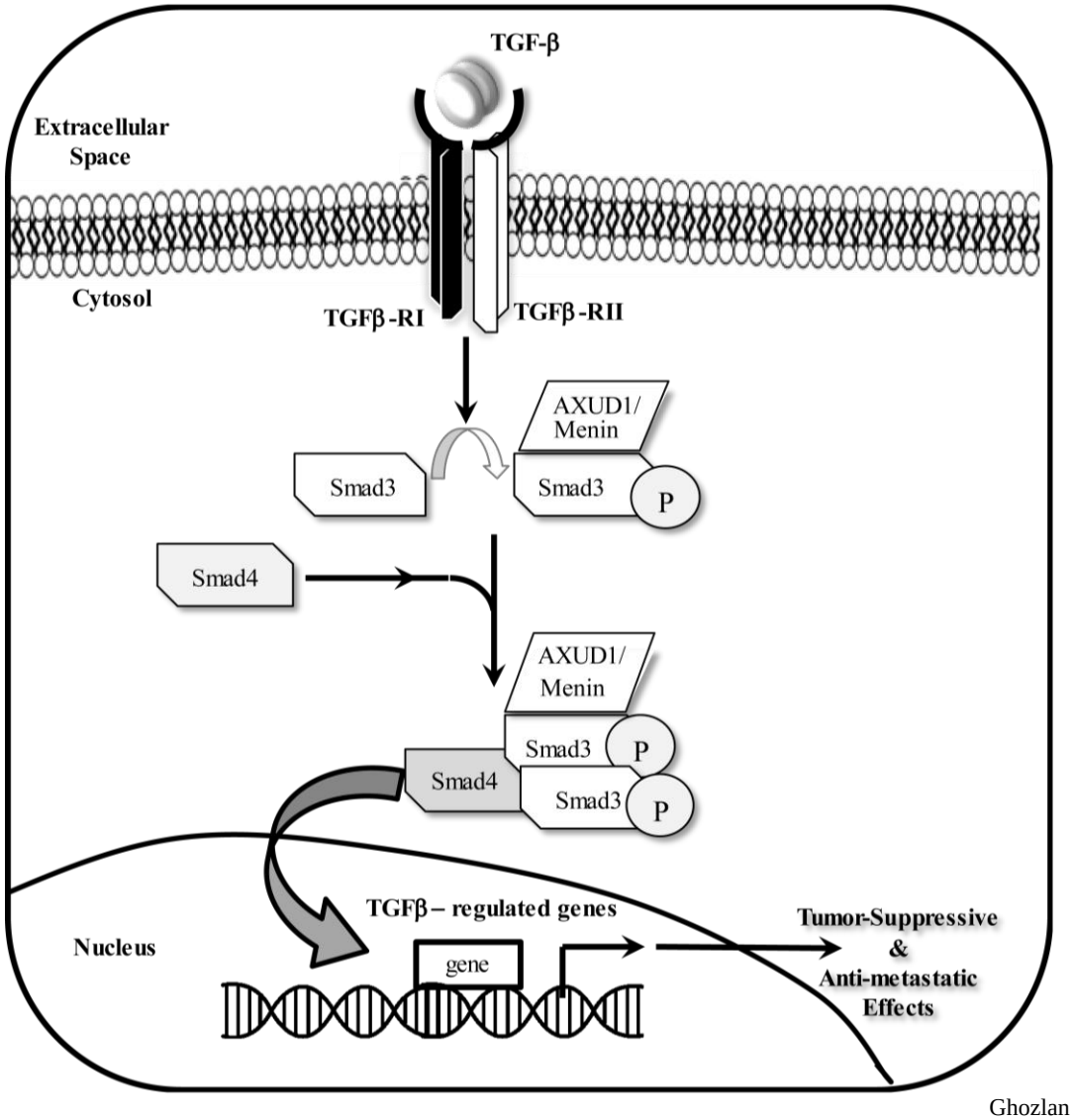


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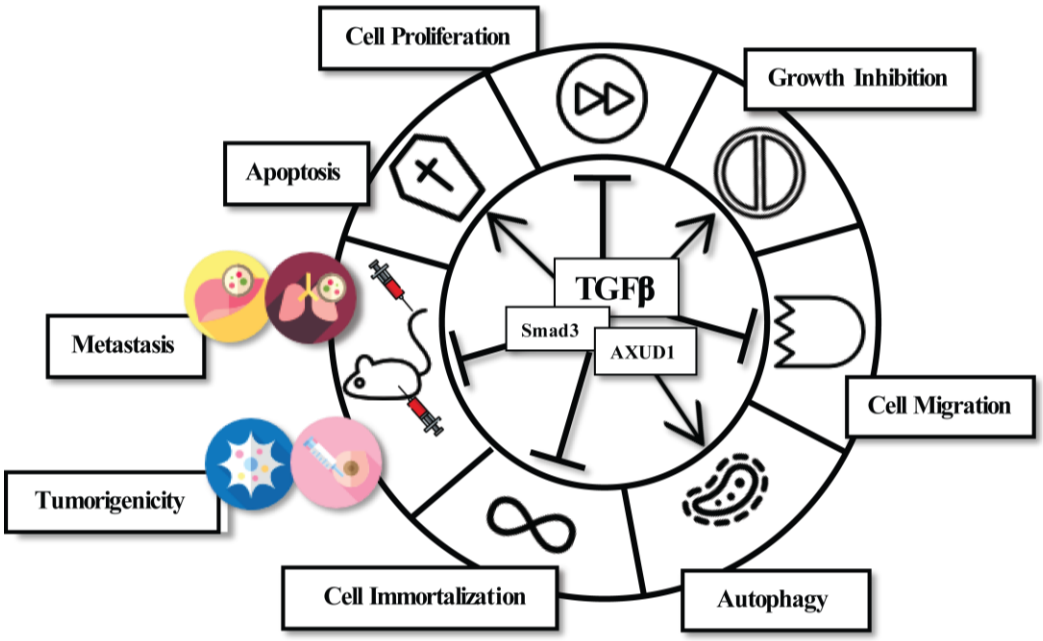
Figure 3-9: A schematic diagram for the role of AXUD1 and menin downstream of the TGFβ signalling pathway in (DAUV) cutaneous melanoma.

Upon TGFβ ligand binding, type II receptor (TβRII), a constitutively auto-phosphorylated serine/threonine kinase, recruits and transphosphorylates the type I receptor (TβRI), thus activating its kinase activity. Afterward, the activated TβRI phosphorylates the receptor-regulated Smad3, allowing subsequent heterotrimerization with its common partner, Smad4, whereby the Smad heterotrimeric complex translocates inside the nucleus where it binds to the DNA and regulate transcription, in cooperation with other transcription factors, co-activators or co-repressors. The current study suggests that TGFβ upregulates each of AXUD1 and menin to function as signalling adapters to Smad3, further promoting the Smad3-dependent multifaceted TGFβ–mediated tumour-suppressive and antimetastatic effects These effects include: the induction of cell cycle arrest, apoptosis, autophagy (as shown *in vitro*); the inhibition of cell immortalization and cell migration *in vitro*; inhibiting primary tumour formation and secondary tumour metastasis (as shown *in vivo*); and the suppression of MSC populations via reducing melanosphere formation and diminishing $ALDH^+$ (and possibly $CD133^+$) MSC subpopulations (as shown *in vitro*)

3.1.11.10 TGFβ/Smad3-mediated upregulation of AXUD1 and menin induces tumour suppressive and anti-metastatic effects in DAUV human cutaneous melanoma cells.



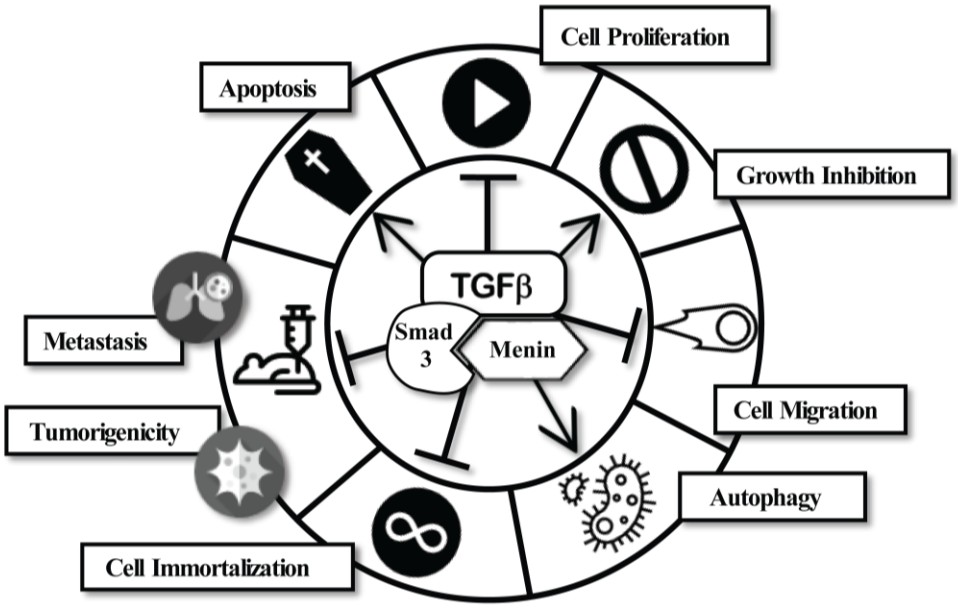


Figure 3-10: TGFβ/Smad3-mediated upregulation of AXUD1 and menin induces tumour suppressive and anti-metastatic effects in cutaneous melanoma cells.

Schematic demonstrating the TGFβ/Smad3/AXUD1 and TGFβ/Smad3/menin signalling axes as two novel tumour-suppressive axes. Each of AXUD1 and menin plays an important role in the TGFβ-induced Smad3-dependent signalling in mediating all the tumour-suppressive and anti-metastatic effects in human cutaneous melanoma *in vitro* and *in vivo*
In vitro, TGFβ could induce each of AXUD1 or menin to inhibit cell growth in melanoma, thus inducing cell cycle arrest through inducing p15 and inhibiting cMyc; could induce apoptosis through inducing pro-apoptotic genes and repressing anti-apoptotic genes as well as inducing caspase3/7 activity.; could induce autophagy by upregulating both autophagosome initiation and expansion genes; could inhibit telomerase gene expression thus inhibiting cell immortalization; could inhibit cell migration in melanoma cells, adding a new anti-migratory player downstream of TGFβ in addition to our previously identified players; PAI1 and LIF. Moreover, *in vivo*, knocking out AXUD1, menin or Smad3 in melanoma cells resulted in larger tumours and more aggressive metastasis in the lungs and livers of immunocompromised mice when compared to parental cells.

Altogether, these results highlight for the first time the potential essential role of AXUD1 and menin in the TGFβ-mediated inhibition of tumorigenesis and metastasis in melanoma in a Smad3-dependent manner.

3.2 Part II: MSCs

3.2.1 TGFβ inhibits melanosphere formation in various cutaneous melanoma cell lines.

Studies show that TGFβ exhibits contextual -sometimes contradicting- roles in CSCs regulation in different types of cancers. Nevertheless, the role of the TGFβ signalling pathway in regulating CSCs in melanoma was never studied despite its importance. For that purpose, I examined whether TGFβ could inhibit MSCs stemness, using an *in vitro* melanosphere-forming assay, a standard assay used for MSC tumour-initiating capacity and self-renewal assessment [597]. Briefly, human cutaneous melanoma cells from various clinical backgrounds (WM278, WM793, WM164, A375m, BLM, WM1232, DAUV, SkMel28 and 1205Lu, described before) were cultured for 7 days in a serum-free stem cell medium supplemented with freshly added growth factors under low-attachment conditions in the absence or the presence of TGFβ.

To assess the long-term self-renewal potential of MSC in cutaneous melanoma cells, I measured the MFE of primary (M1), secondary (M2) and tertiary (M3) melanospheres. The melanosphere forming efficiency (MFE) was calculated as the percentage value of the number of formed melanospheres divided by the number of cells per well initially seeded in each well as detailed in the methods section. As shown in Figure 3-11(A, B), the WM278, WM793, WM164, A375m, BLM, WM1232 and DAUV cutaneous melanoma cell lines were capable of forming primary (M1) and secondary(M2), but not tertiary (M3) melanospheres. However, the cell lines SkMel28 and 1205Lu did not form any melanospheres, rather they formed aggregates of cells that do not qualify to be melanospheres [708]). Interestingly, TGFβ treatment resulted in the reduction of MFE in both the primary and secondary melanospheres in all tested melanoma cell lines capable of generating melanospheres (WM278, WM793, WM164, A375m, BLM, WM1232 and DAUV), when compared to untreated control cells. This consistent suppression of melanosphere formation across various cell lines induced by TGFβ treatment strongly suggests an important TGFβ-mediated inhibitory role in regulating melanosphere formation and possibly MSC population.

3.2.2 TGFβ-induced reduction of melanosphere formation is Smad3-specific.

To determine whether the TGFβ-mediated inhibition of melanosphere formation was mediated through Smad2 or Smad3, I tested parental, scrambled, Smad2, Smad3 and Smad4 KO DAUV melanoma cells, where cells were cultivated in the presence or absence of TGFβ for 24 hours. As Figure 3-11 (C, D) shows, I found that silencing only Smad3 or Smad4, but not Smad2, reversed the TGFβ- mediated suppressive effects on melanosphere formation, where TGFβ showed a significant reduction of MFE in parental, scrambled and Smad2 KO DAUV cells, yet almost induced no inhibition in Smad3 KO and Smad4 KO DAUV cells, thus indicating the preferential specific role of Smad3 over Smad2 in mediating the TGFβ-induced inhibition of the MSC population

3.2.3 TGFβ/Smad3-induced inhibition of melanosphere formation requires menin but not AXUD1.

After confirming the inhibitory role of TGFβ on stemness in various melanoma cell lines, I were interested in investigating any potential role of menin and AXUD1 protein, mediators of TGFβ-mediated tumour suppressive effects that I previously characterized. Therefore, I tested parental, scrambled, MEN1 KO and AXUD1 KO DAUV melanoma cells cultured for 7 days in serum-free stem cell medium as previously described in the absence or the presence of TGFβ and determined MFE. Interestingly, TGFβ reduced melanosphere formation (M1 and M2) in parental, scrambled, and AXUD1 KO but not in menin KO, where only menin depletion reversed change the TGFβ-mediated inhibitory effect. Remarkably, silencing AXUD1 significantly inhibited basal melanosphere formation in DAUV KO cells compared to parental and scrambled cells as shown in Figure 3-11 (C, D). This suggests that menin could mediate TGFβ inhibition of melanosphere formation without the involvement of AXUD1 protein in the DAUV melanoma cell line. This also proposes a potential role of AXUD1 in melanosphere formation and MSC self-renewal. This latter role is supported by similar results showing that Axud1 knockdown -using antisense morpholinos- in zebrafish resulted in decreased proliferation and increased cell death in the Axud1-depleted neural progenitor cells, hence decreased neural progenitor expansion, thus resulting in smaller brain size, notably without affecting rostrocaudal patterning or differentiation within the diencephalic and mesencephalic regions [735].

3.2.4 TGFβ-induced inhibition of melanosphere formation is mediated via its Smad-dependent pathway.

Smads are considered crucial to TGFβ signalling (canonical signalling pathway), yet TGFβ could transmit its signal through other intracellular non-Smad signalling cascades. Therefore, I were interested in investigating whether any of these pathways show any cross-talk with the TGFβ signalling pathway in the regulation of melanosphere formation and thus MSC self-renewal. For this purpose, parental DAUV melanoma cells were cultured for 7 days in serum-free stem cell medium in the absence or presence of TGFβ and treated with various kinase inhibitors of ERK, JNK, mTOR, p38, PI3K, and TGFBRI, afterward, MFE was determined. As shown in Figure 3-11 (E, F), I found that only the TGFBRI kinase inhibitor could significantly reverse the TGFβ-mediated inhibition of melanosphere formation, but none of the other kinase inhibitors did. This highlights the vital preferential role of the TGFβ/Smad3 signalling cascade in negatively regulating melanosphere formation and MSC self-renewal.

3.2.5 TGFβ tends to reduce CD133+ and ALDH+ MSC populations in various melanoma cell lines in vitro

After showing that TGFβ/Smad3 reduced MSC self-renewal via reducing MFE of various melanoma cell lines *in vitro*, it was informative to determine whether TGFβ was required for the suppression of $CD133^+$ and $ALDH^+$ stem-like cell populations in melanoma cell lines. For that purpose, all melanoma cell lines (WM278, WM793, WM164, A375m, BLM, WM1232, SkMel28, 1205Lu and DAUV) were grown in monolayers in the presence or absence of TGFβ for 24 hours, then harvested to determine – using flow cytometric analysis- the proportion of $CD133^+$ using a PE-conjugated anti-CD133 antibody, and to assess the enzymatic activity of $ALDH^+$ populations using the non-immunophenotypic ALDEFLUOR™ assay. $CD133^+$ populations were gated based on the absence of the population in the absence of CD133-Phycoerythrin (PE) stain, while the $ALDH^+$ populations were gated based on the absence of the population in the presence of N,N-diethyl-amino-benzaldehyde (DEAB), a substrate and mechanism-based inhibitor for human ALDH isoenzymes.

As presented in Figure 3-14 (A, B, E, F), $CD133^+$ populations were detected in WM164, A375m, WM1232, SkMel28, and DAUV (Figure 3-14C, PAR), but not in the remaining cell

lines, whereas $ALDH^+$ populations were detected in all tested cell lines namely WM278, WM793, WM164, A375m, BLM, WM1232, SkMel28, 1205Lu and DAUV (Figure 3-14C, PAR). Interestingly, TGFβ showed a tendency to decrease $CD133^+$ population in WM164, A375m, WM1232, SkMel28, and DAUV (as shown in Figure 3-14 E) in each of the biological replicates individually although statistical analysis of all the biological replicates turned to be insignificant.

Similarly, TGFβ showed a tendency to decrease the $ALDH^+$ population in all the tested melanoma cell lines (as shown in Figure 3-14 F) in each of the biological replicates individually however upon analyzing all the biological replicates none of the cell lines was significant except for parental DAUV cells. Based on these results, there might be a potential role of TGFβ in reducing MSC in the tested human cutaneous melanoma cells, nonetheless, further experiments must be conducted, using $CD133^+$ and $ALDH^+$ as well as other surface markers for MSCs, alone or in combination.

3.2.6 TGFβ/Smad3 reduces $ALDH^+$ and possibly $CD133^+$ MSC populations in DAUV melanoma cells in vitro

Given the interesting results shown in parental DAUV cells, I investigated whether the potential TGFβ-induced effects –if any- were mediated through Smad2 or Smad3. Thus, parental, Smad2 KO, Smad3 KO and Smad4 KO DAUV melanoma cells were cultured in monolayers in the presence or absence of TGFβ for 24 hours, then harvested for measurement of $CD133^+$ and $ALDH^+$ MSC populations**.**

As shown in Figure 3-14 (C-G), while TGFβ showed barely any effect on the $CD133^+$ populations in Smad3 KO and Smad4 DAUV cells, TGFβ showed a tendency to reduce the $CD133^+$ population in the parental and the Smad2 KO DAUV cells, though this tendency was statistically insignificant. Interestingly, TGFβ showed a significant reduction in $ALDH^+$ population in parental, and Smad2 KO DAUV cells, whereas this TGFβ-induced reduction was abolished in the Smad3 KO, and Smad4 DAUV cells. Although these results are concordant with the findings found throughout the thesis that Smad3 plays a preferential role in mediating TGFβ-induced tumour-suppressive effects, they necessitate further confirmation especially TGFβ effects on $CD133^+$ populations. Other MSC surface markers will be required to determine if TGFβ regulates MSCs.

3.2.7 TGFβ/Smad3/AXUD1 and TGFβ/Smad3/Menin reduces ALDH$^+$ and possibly CD133$^+$ MSC populations in DAUV melanoma cells in vitro

A complementary step was to determine whether AXUD1 or menin played a role downstream of TGFβ in any regulation of the CD133$^+$ and ALDH$^+$ population. For this purpose, parental, Smad2 KO, Smad3 KO and Smad4 KO DAUV melanoma cells were cultured in monolayers in the presence or absence of TGFβ for 24 hours, then harvested for measurement of CD133$^+$ and ALDH$^+$ MSC populations. As shown in Figure 3-14 (C-G), TGFβ elicited no effect on the CD133$^+$ population in AXUD1 KO and menin DAUV cells. Although TGFβ showed some tendency to reduce the CD133$^+$ population in the parental and Smad2 KO DAUV cells, this reduction was statistically insignificant. Regarding the ALDH$^+$ population, TGFβ showed a statistically significant decrease in the ALDH$^+$ population in parental, and Smad2 KO DAUV cells, however, this TGFβ-induced decrease was reversed in the AXUD1 KO, and menin DAUV cells in a manner similar to Smad3 KO and Smad4 KO DAUV cells.

These findings are consistent with the previous results demonstrated throughout the thesis indicating the preferential role of Smad3 downstream of TGFβ to relay its tumour-suppressive effects especially ALDH$^+$ population results. These results provide some preliminary evidence that the TGFβ/Smad3 signalling cascade could possibly play a role in negatively regulating the expansion of CD133$^+$ and ALDH$^+$ MSCs, and a possible implication of AXUD1 and MEN1 downstream of TGFβ. Given the insignificant results of CD133+, further confirmatory experiments -especially investigating TGFβ effects on CD133$^+$ populations- are crucial. Moreover, other MSC surface markers should be investigated in parallel to determine if TGFβ plays a role in regulating MSCs.

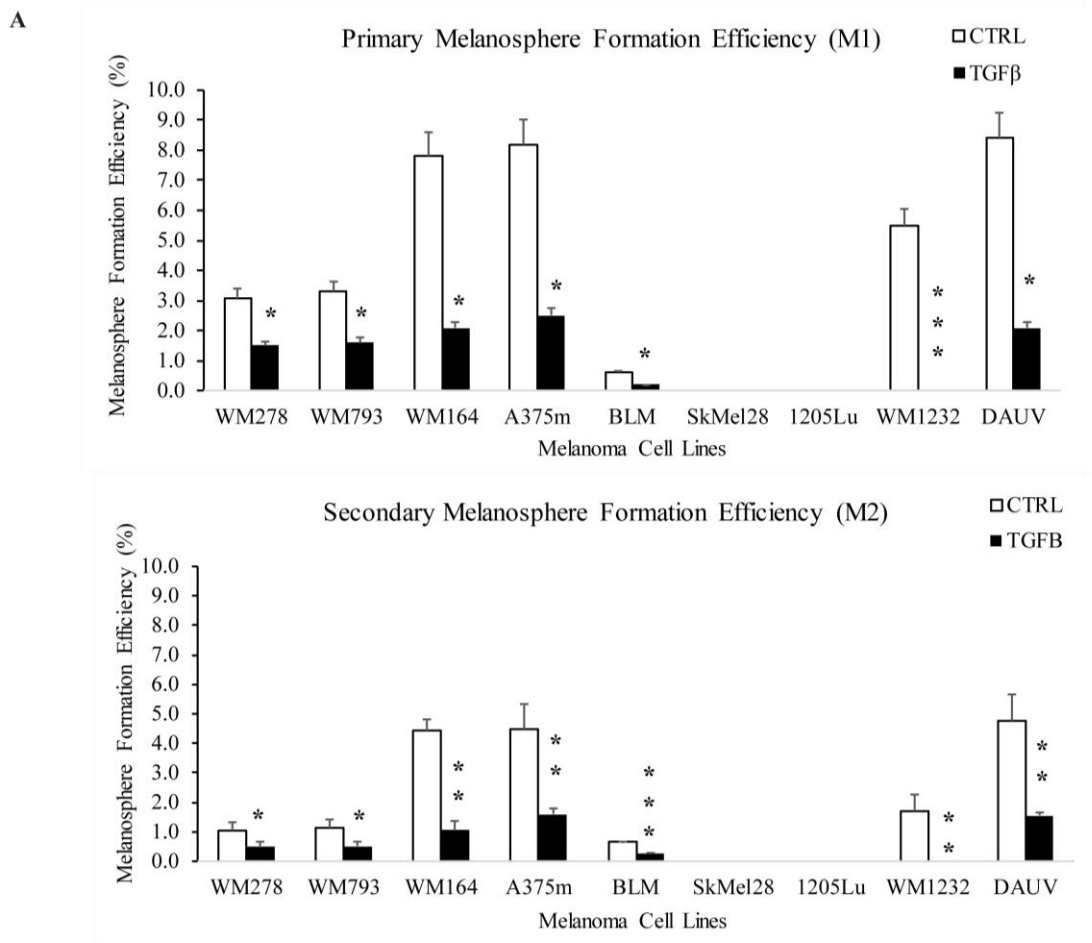
3.2.8 Low CD133 and low ALDH expression correlate with better survival outcomes in cutaneous melanoma patients.

To further evaluate whether CD133 and ALDH could play a potential role in melanoma tumorigenesis, I investigated the relation between CD133 and ALDH expression levels and the overall survival of melanoma patients. The Skin Cutaneous Melanoma dataset of The Cancer Genome Atlas (TCGA) project of Genomic Data Commons (GDC) was used, where Kaplan-Meier analyses revealed that lower CD133 and ALDH mRNA expression correlated with better

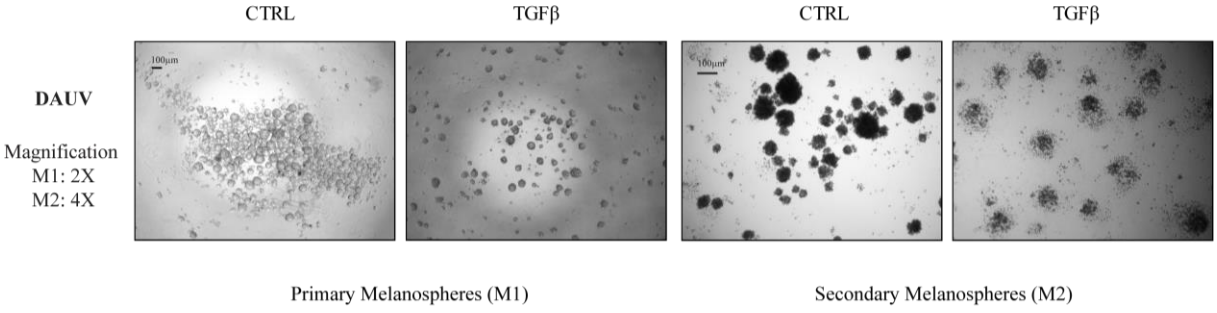
survival outcomes in melanoma patients (n=102) (Figure 3-15 A, B). High CD133 showed a 3-year survival rate of 33% versus 46% for low expression (p-value: 0.022). Similarly, high ALDH1A1 showed a 3-year survival rate of 24% versus 47% for low expression (p-value: 0.045). These survival data show the harmful role of the high expression of the markers-bearing melanoma stem cells in deteriorating survival outcomes in cutaneous melanoma patients. These correlations come in agreement with the preliminary findings I am reporting in this study (that require further confirmation). The findings show that the TGFβ/Smad3-mediated inhibition of MFE as well as the reduction of $ALDH^+$ (and possibly $CD133^+$) MSC subpopulations) could be an indication of TGFβ/Smad3-mediated of MSCs tumorigenicity and expansion, thus playing an oncoprotective role in melanoma patients.

3.2.9 Figures

3.2.9.1 TGFβ inhibits melanosphere formation in various cutaneous melanoma cell lines.



B-i Representative images of melanosphere formation in various cutaneous melanoma cell lines



B-ii Representative images of melanosphere formation in various cutaneous melanoma cell lines

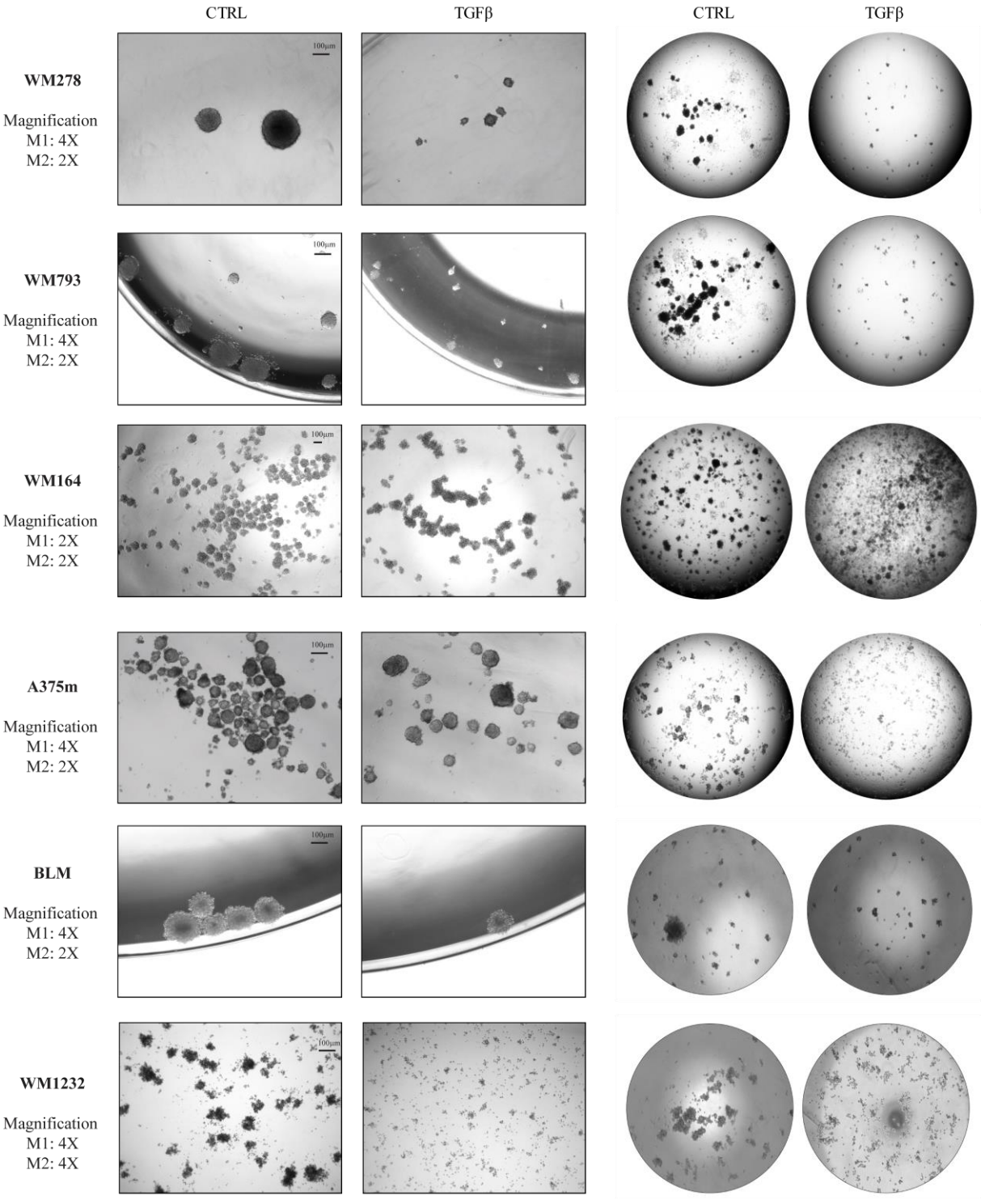
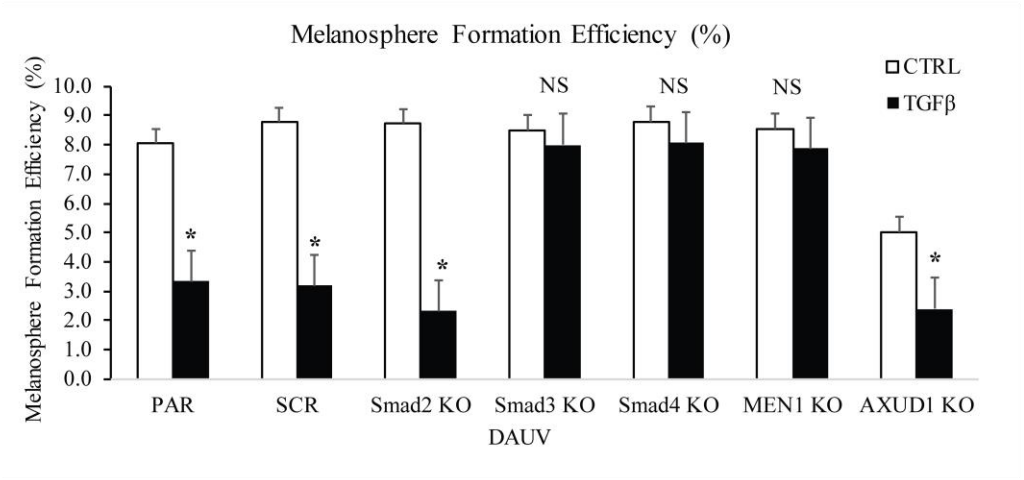


Figure 3-11(A-B): TGFβ inhibits melanosphere formation in various cutaneous melanoma cell lines.

(A) Quantification and (**B)** representative images of primary (M1) and secondary melanospheres (M2), arising from seeding 1000 cells of various cutaneous melanoma cells in the absence or presence of TGFβ (200 pM) cultured in stem cell medium (SCM) for 7 days. The magnification of the objective lens is indicated for every cell line.

3.2.9.2 TGFβ-induced reduction of melanosphere formation is Smad3-specific and the TGFβ/Smad3-induced inhibition of melanosphere formation requires menin but not AXUD1.

C



D

Representative images of melanosphere formation in DAUV cell lines

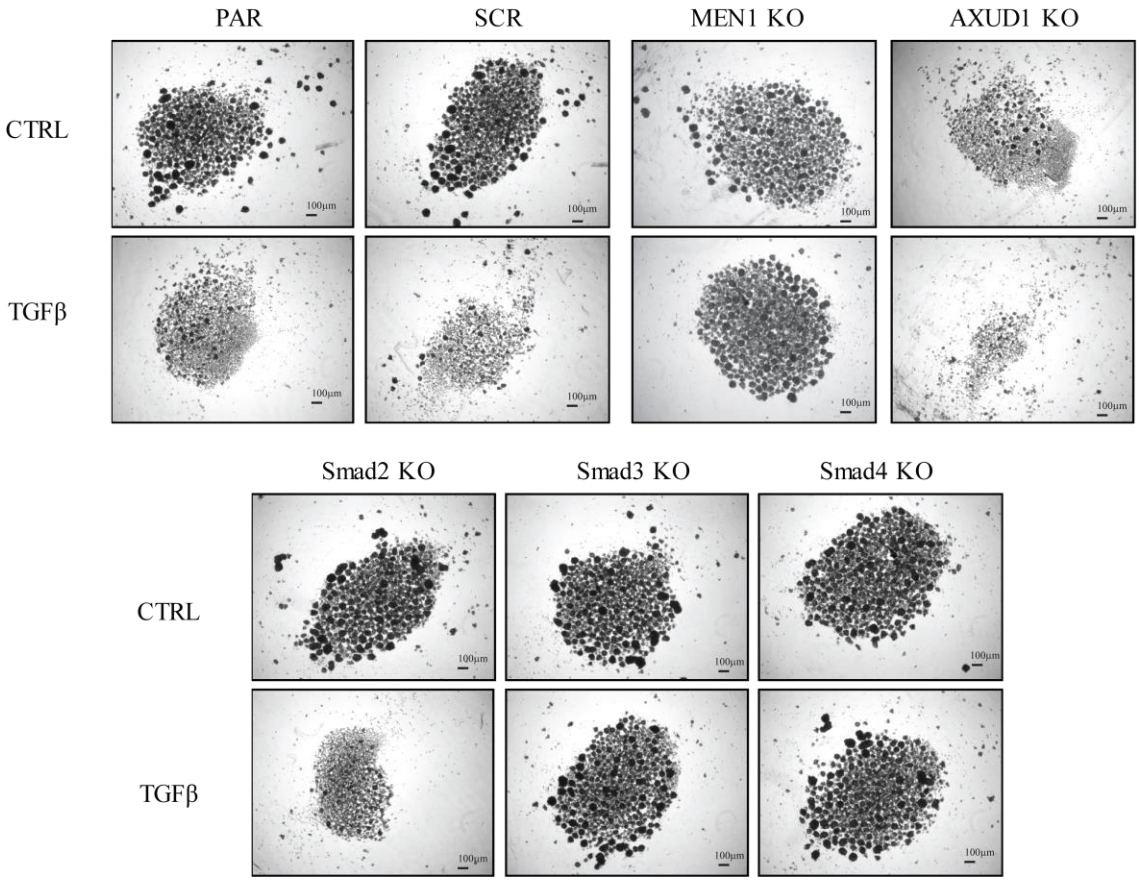
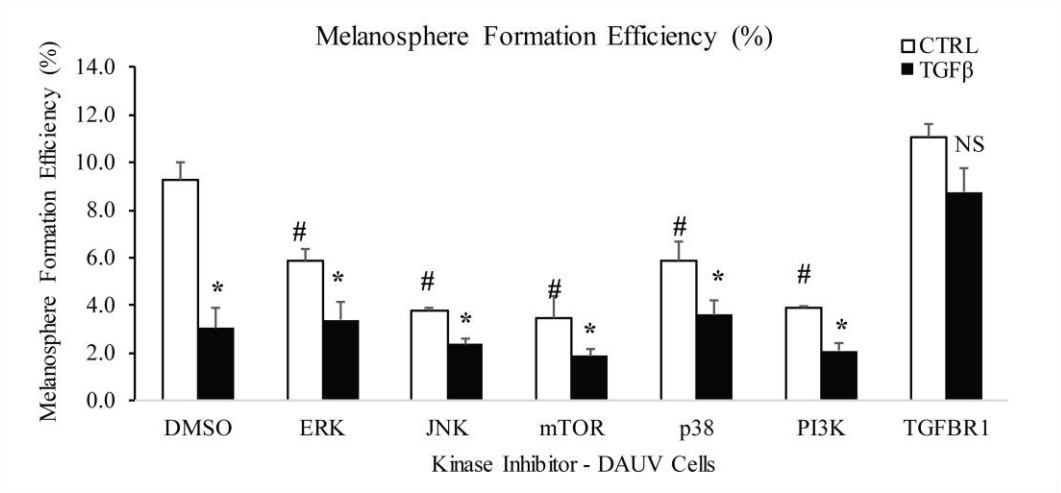


Figure 3-12(C-D): TGFβ-induced reduction of melanosphere formation is Smad3-specific and the TGFβ/Smad3-induced inhibition of melanosphere formation requires menin but not AXUD1.

(**C)** Quantification and (**D)** representative images of primary melanospheres (M1), arising from seeding 1000 cells of parental, scrambled, Smad2 KO, Smad3 KO Smad4 KO, AXUD1 KO and menin KO DAUV melanoma cells in the absence or presence of TGFβ (200 pM) cultured in SCM for 7 days. The magnification of the objective lens is 2X.

3.2.9.3 TGFβ-induced inhibition of melanosphere formation is mediated via its Smad-dependent pathway.

E



F Representative images of melanosphere formation in response to kinase inhibitors in DAUV cells

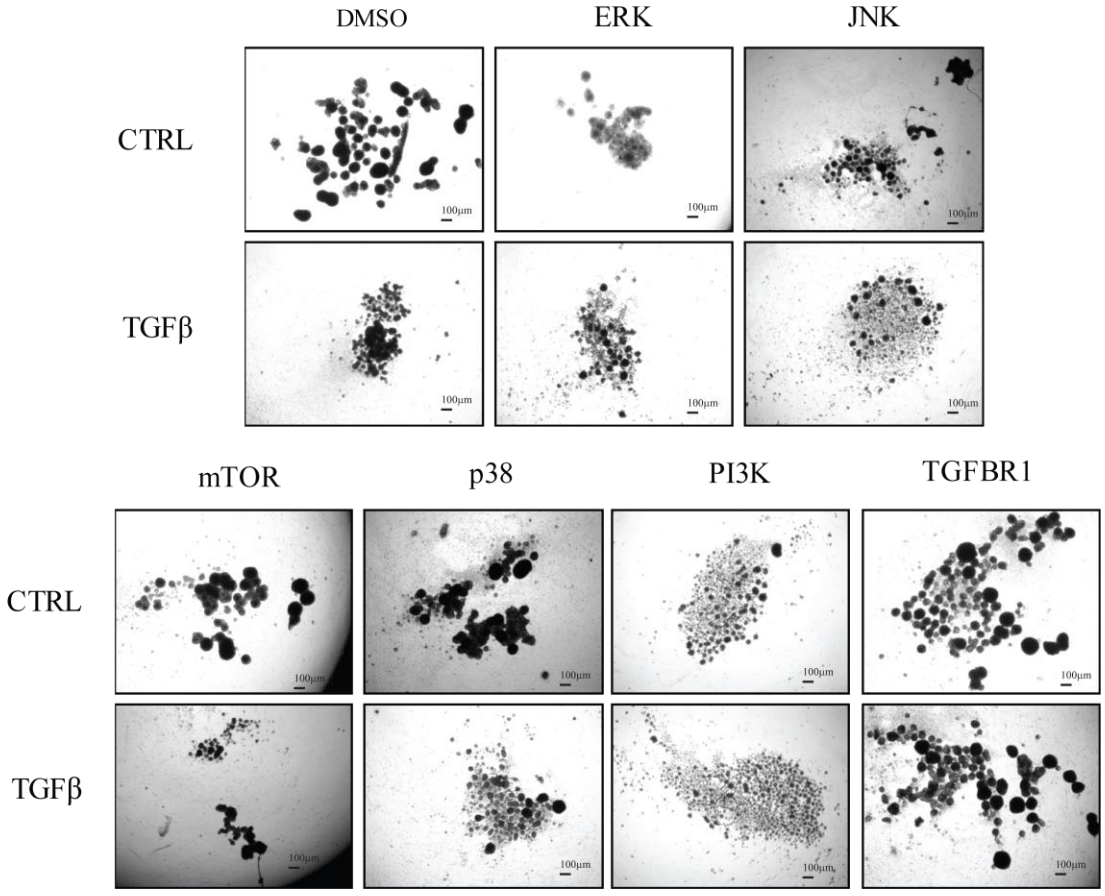


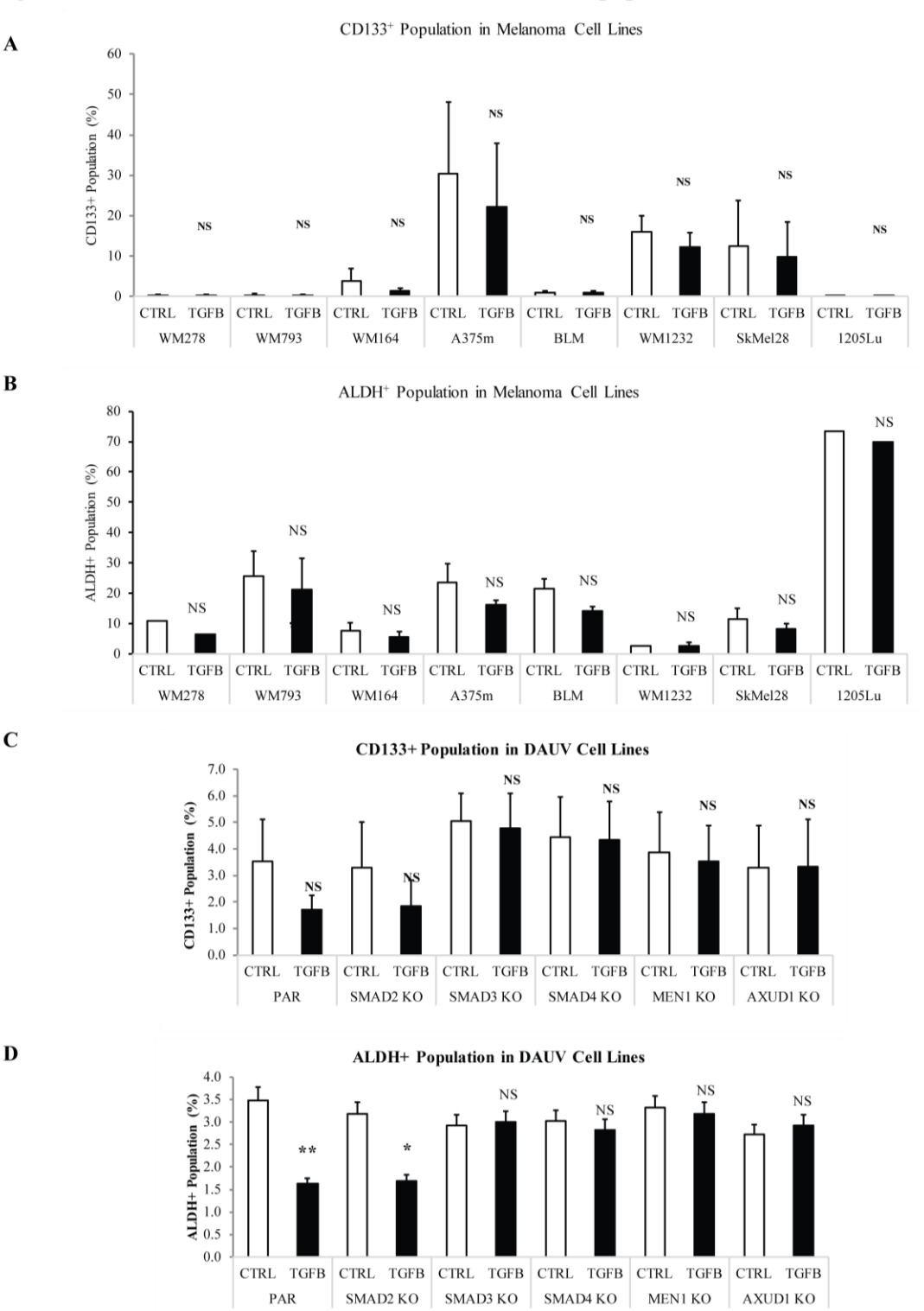
Figure 3-13(E-F): TGFβ-induced inhibition of melanosphere formation is mediated via its Smad-dependent pathway.

(**E)** Quantification and (**F)** representative images of primary melanospheres (M1), arising from seeding 1000 DAUV melanoma cells treated with various kinase inhibitors in the absence or presence of TGFβ (200 pM). The magnification of the objective lens is 2X.

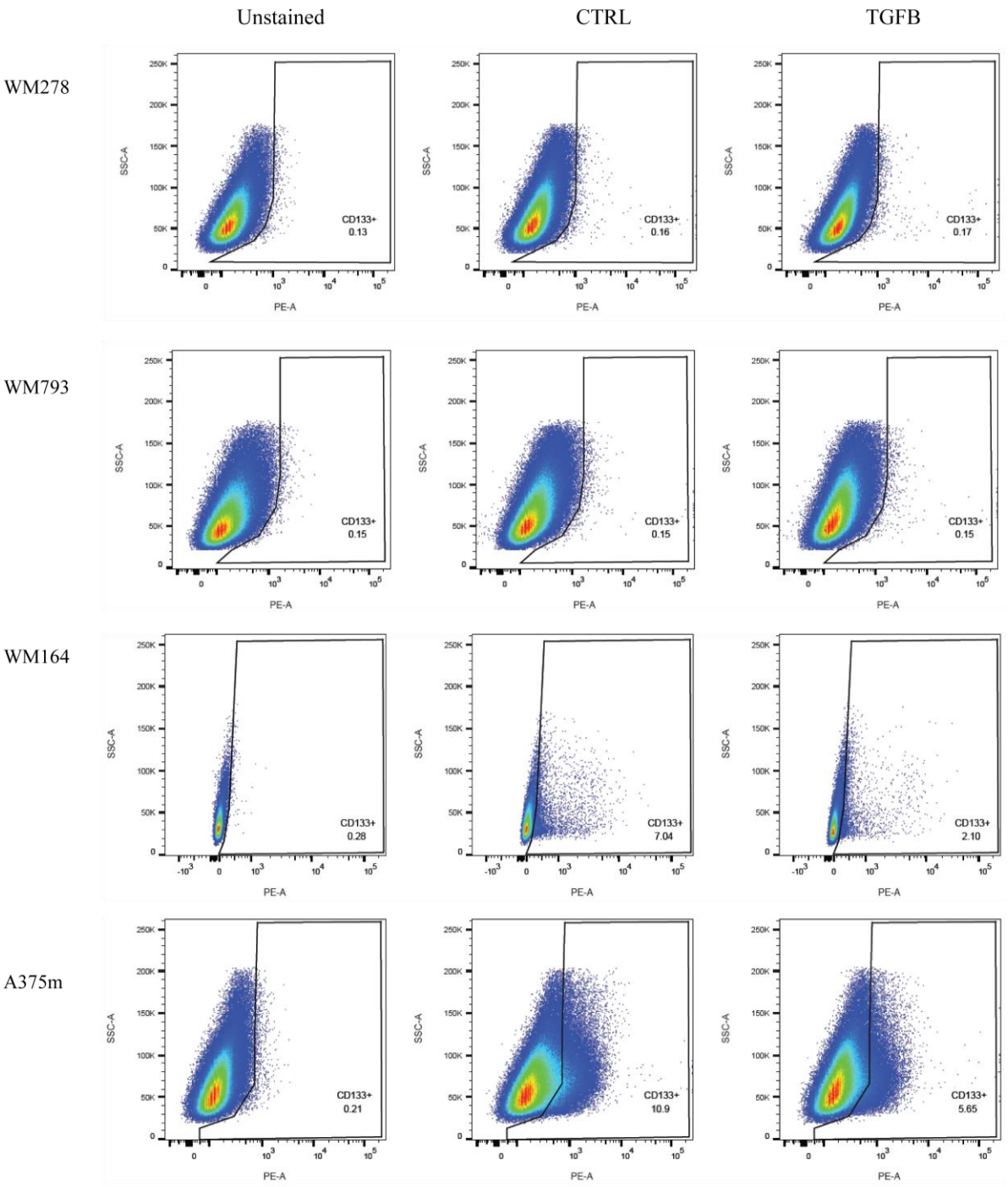
The melanosphere forming efficiency (MFE) was calculated as the percentage value of the number of formed melanospheres (>25 μm diameter) divided by the number of cells per well initially seeded in each well.

All data of Figure 3-11 are represented as the mean of MFE of every cell line for 3-5 independent experiments. All error bars are the standard errors of the mean. For statistical analysis, Student t-test was performed comparing the treated to the non-treated counterparts (***$p<0.001$, **$p<0.01$, *$p<0.05$).

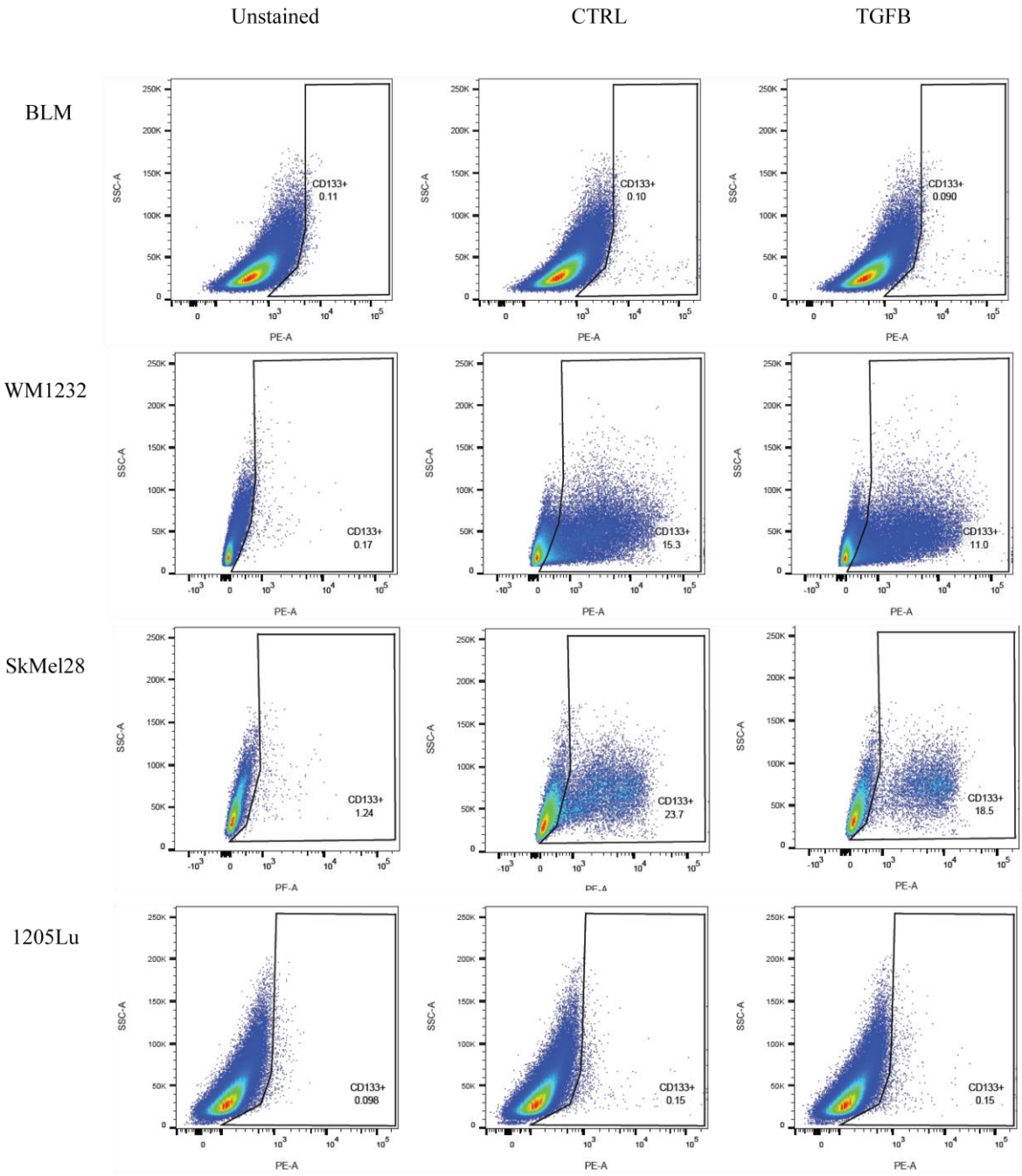
3.2.9.4 TGFβ tends to reduce $CD133^+$ and $ALDH^+$ MSC populations in various melanoma cell lines *in vitro*, and TGFβ/Smad3/AXUD1 and TGFβ/Smad3/Menin reduce $ALDH^+$ and possibly $CD133^+$ MSC populations in DAUV melanoma cells *in vitro*



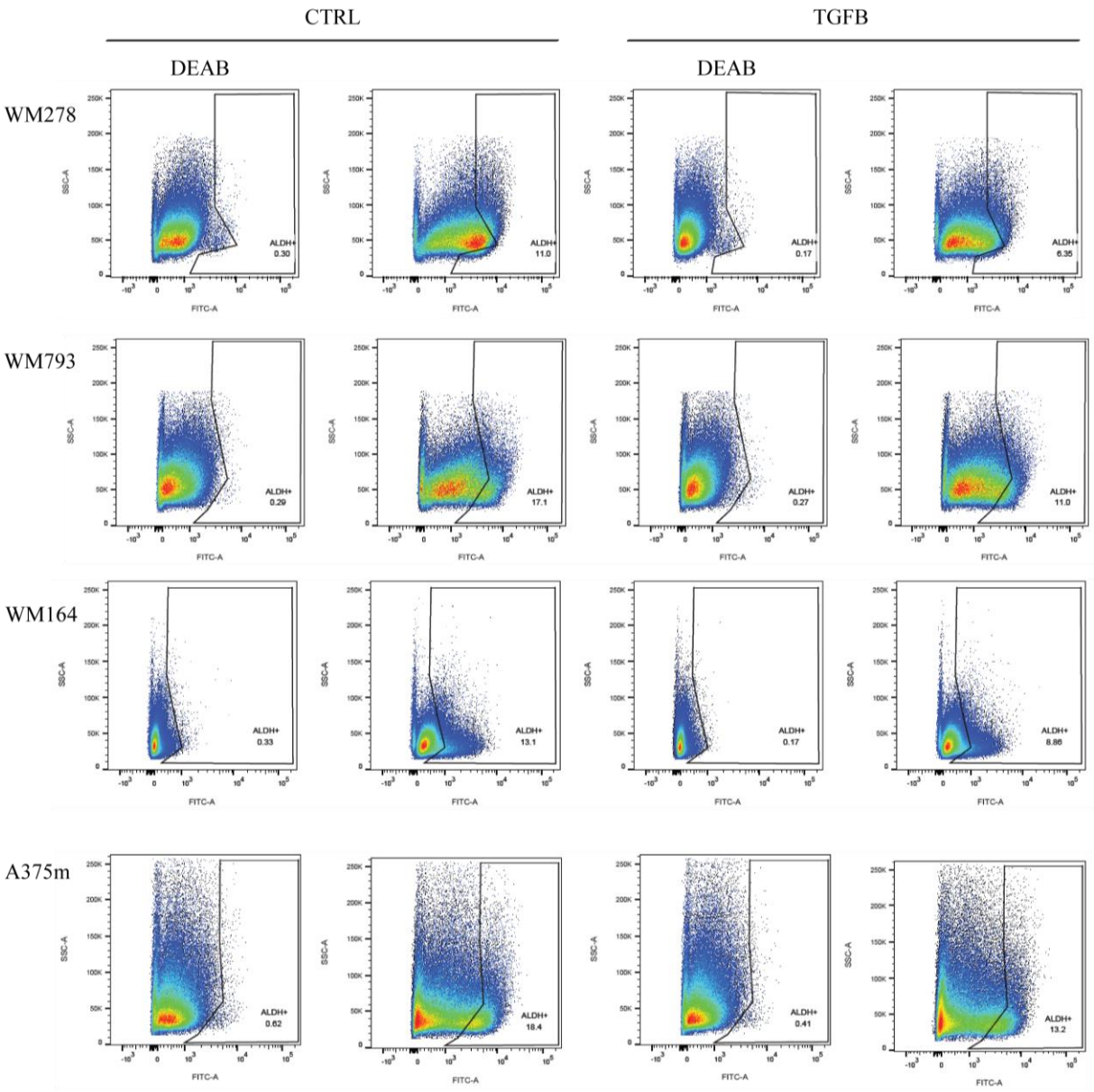
E-i Flow cytometric analysis of CD133+ populations in cutaneous melanoma cells lines.



E-ii Flow cytometric analysis of CD133+ populations in cutaneous melanoma cells lines.

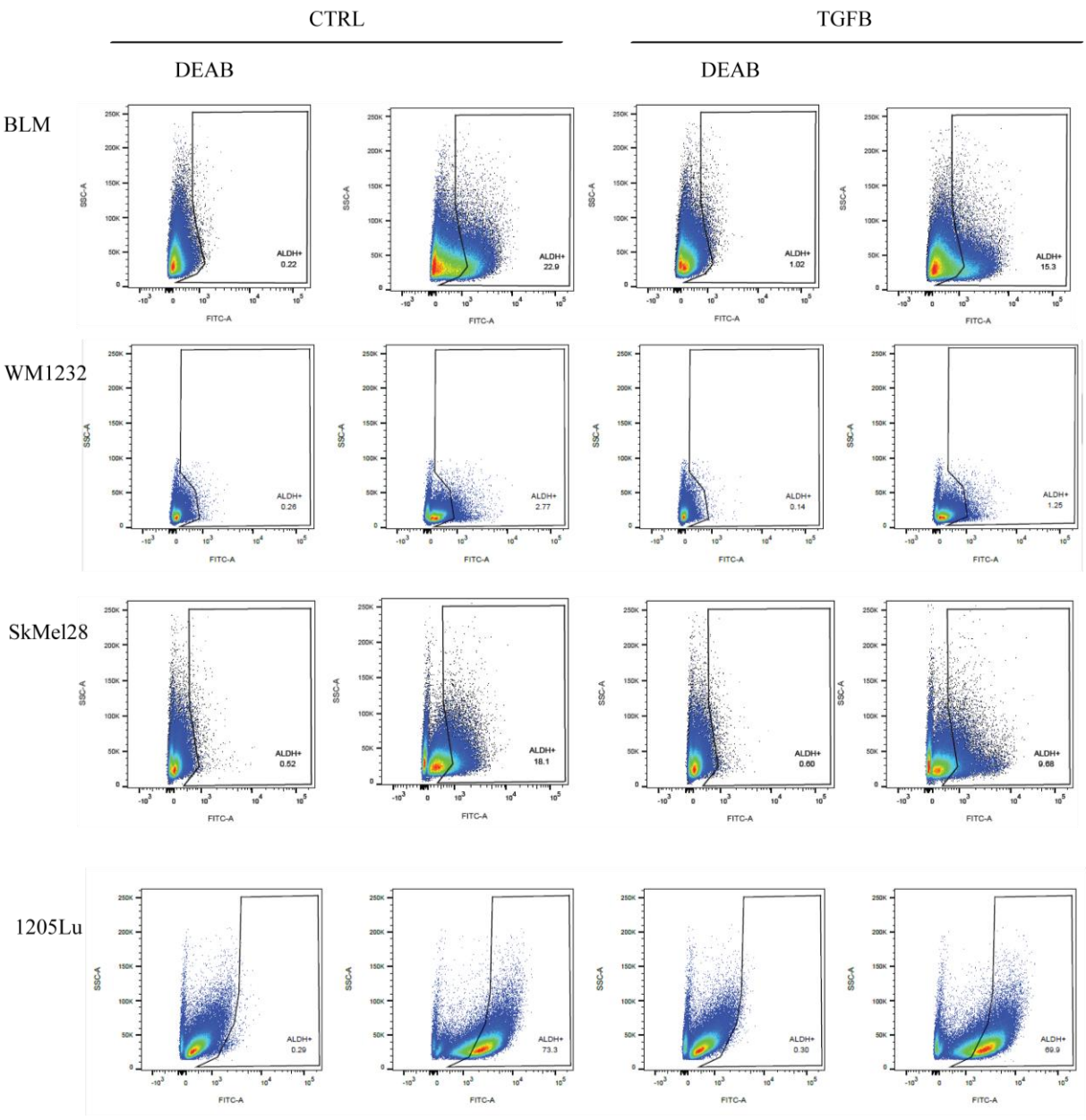


F-i Flow cytometric analysis of ALDH+ populations in cutaneous melanoma cells lines.

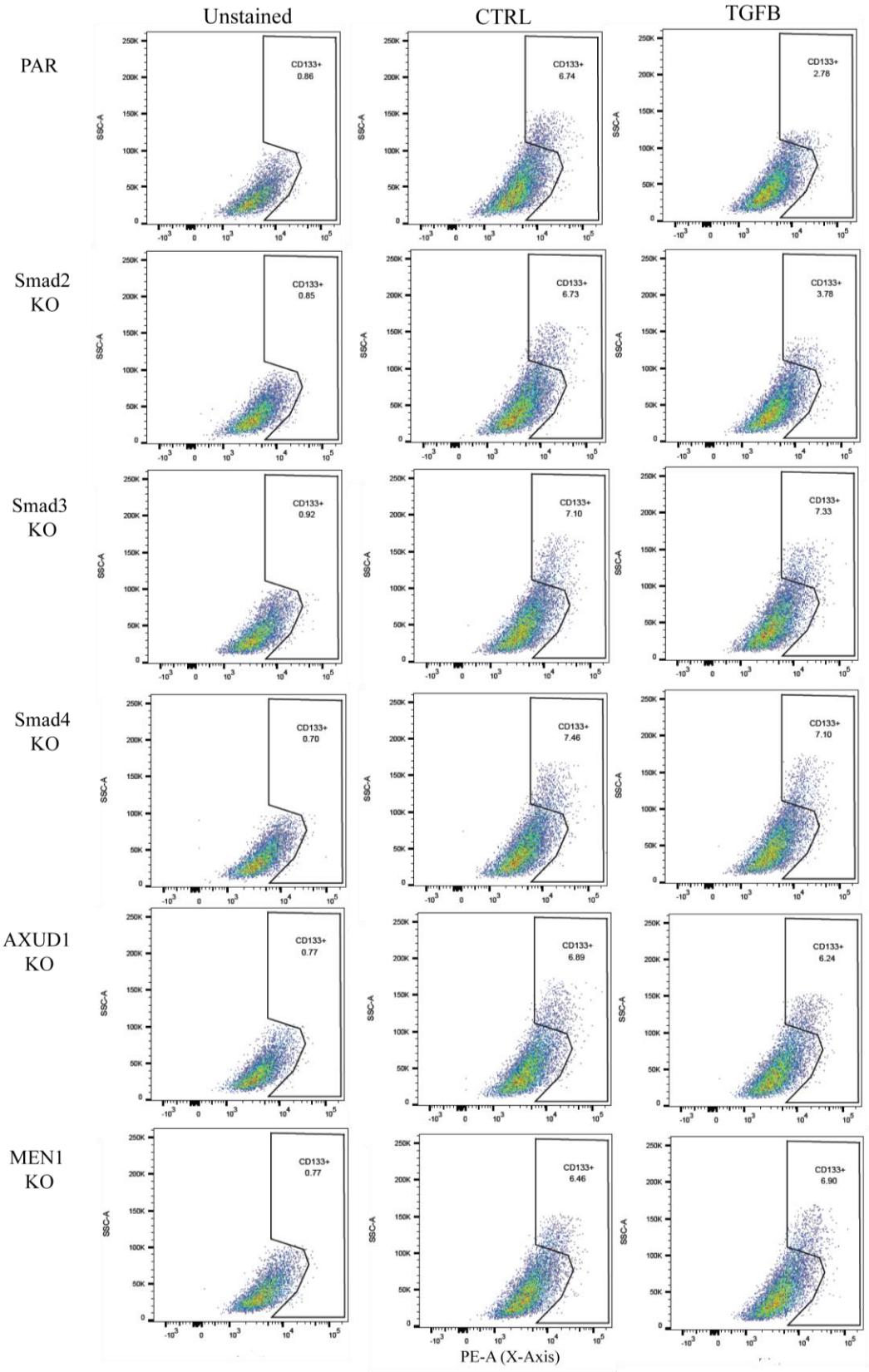


FITC-A (X-Axis)

F-ii Flow cytometric analysis of ALDH+ populations in cutaneous melanoma cells lines.



G Flow cytometric analysis of CD133+ populations in cutaneous melanoma cells lines.



H Flow cytometric analysis of ALDH+ populations in cutaneous melanoma cells lines.

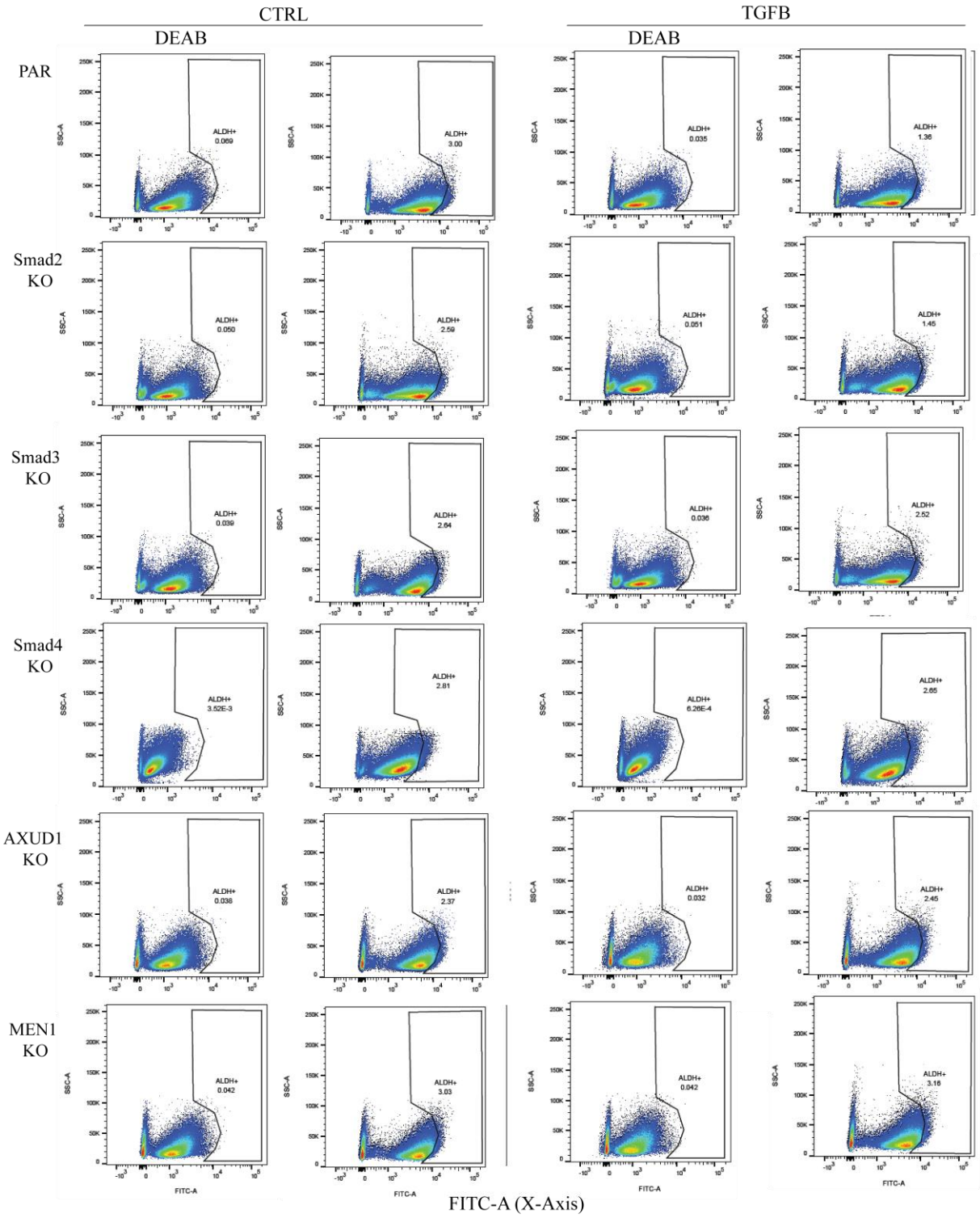


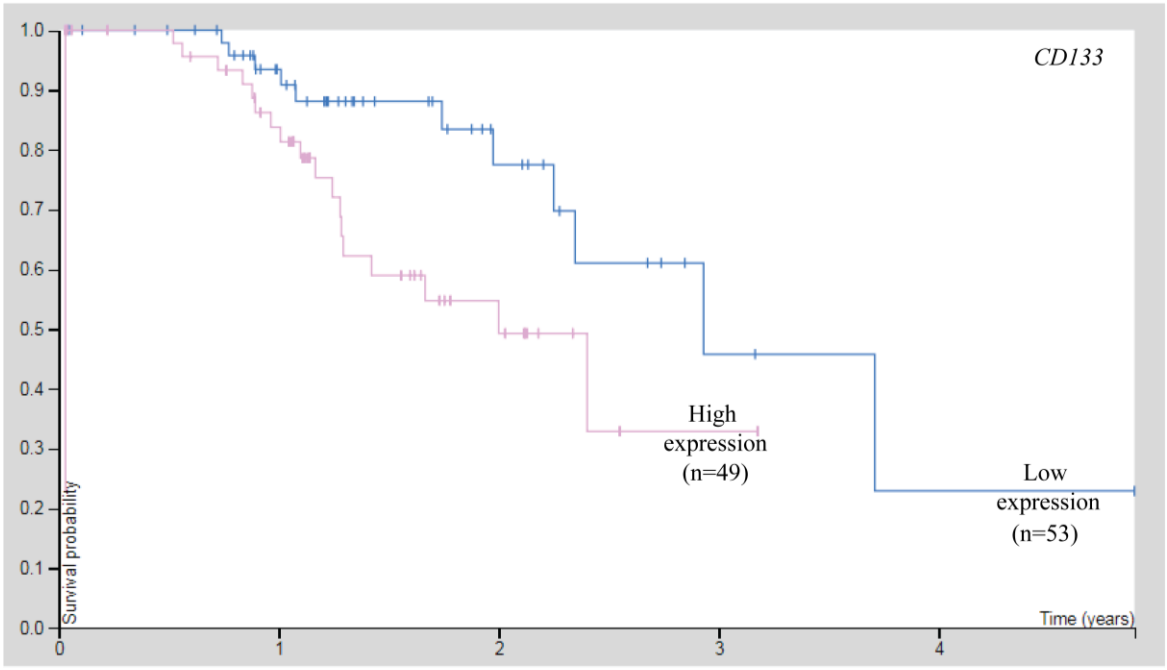
Figure 3-14:TGFβ tends to reduce CD133+ and ALDH+ MSC populations in various melanoma cell lines in vitro, and TGFβ/Smad3/AXUD1 and TGFβ/Smad3/Menin reduce ALDH+ and possibly CD133+ MSC populations in DAUV melanoma cells in vitro

(A-D) Flow cytometric quantification (percentage) of $CD133^+$ **(A, C)** and $ALDH^+$ **(B, D)** population of various melanoma cells **(A, B)** as well as in parental, Smad2 KO, Snad3 KO, Smad4 KO, and AXUD1 KO, menin KO DAUV cells **(C, D)** using a PE-conjugated anti-CD133 antibody and the non-immunophenotypic ALDEFLUOR™ assay respectively in the absence or presence of TGFβ (200pM) for 24 hours.
(E-H) Representative graphs of the flow cytometric analysis of $CD133^+$ **(E, G)** and $ALDH^+$ **(F, H)** population of various melanoma cells **(E, F)** as well as in parental, Smad2 KO, Snad3 KO, Smad4 KO, and AXUD1 KO, menin KO DAUV cells **(G, H)** in the absence and presence of TGFβ (200 pM) for 24 hours. $CD133^+$ populations were gated based on the absence of the population in the absence of Phycoerythrin (PE) stain, while the $ALDH^+$ population was gated based on the absence of the population in the presence of N,N-diethyl-amino-benzaldehyde (DEAB), a substrate and mechanism-based inhibitor for human ALDH isoenzymes.
Data are represented as the mean percentage of the $CD133^+$ or $ALDH^+$ population for at least 3 independent experiments.

All error bars are the standard errors of the mean. For statistical analysis, Student t-test was performed compared to the non-treated control (***$p<0.001$, **$p<0.01$, *$p<0.05$).

3.2.9.5 Low CD133 and ALDH expression correlate with better survival outcomes in cutaneous melanoma patients.

A Relapse-free survival in melanoma patients according to CD133 expression level



B Relapse-free survival in melanoma patients according to ALDH expression level

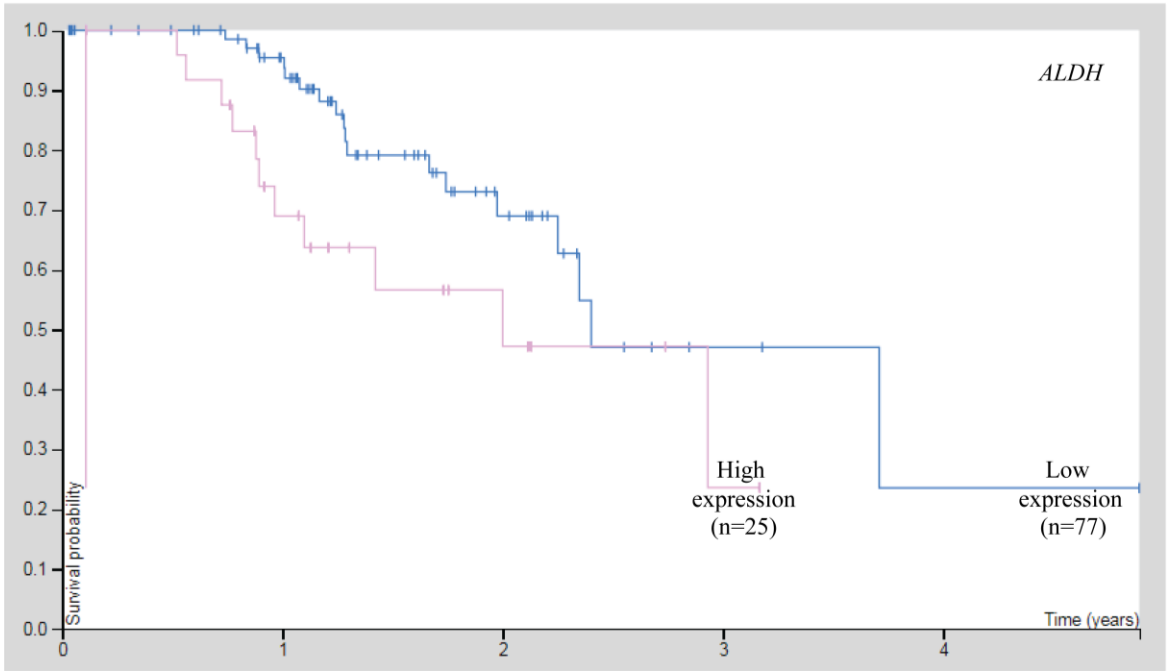


Figure 3-15: Low CD133 and ALDH expression correlate with better survival outcomes in cutaneous melanoma patients

(A): Kaplan-Meier plot, 3-year survival is 33% for cutaneous melanoma patients with high CD133 expression and 46% for patients with low CD133 expression when using best separation. The x-axis shows the number of years of survival (living years) after diagnosis while the y-axis shows the survival probability. High AXUD1 expression is represented in pink and low AXUD1 expression is represented in blue.
(B): Kaplan-Meier plot, 3-year survival is 24% for cutaneous melanoma patients with high ALDH1A1 expression and 47% for patients with low ALDH1A1 expression when using best separation. The x-axis shows the number of years of survival (living years) after diagnosis while the y-axis shows the survival probability. High AXUD1 expression is represented in violet and low AXUD1 expression is represented in blue.

Chapter Four: **Integrative Discussion**

The purpose of this thesis was to further investigate the controversial role of TGFβ in the suppression of tumour development and progression in human cutaneous melanoma. I had specific aims in my thesis which are: to investigate the role of AXUD1 and (b) menin downstream of TGFβ in regulating tumour suppression *in vitro* and *in vivo* as discussed in Part I of chapter three and (b) to evaluate the effect of TGFβ on melanoma stem cells tumour-initiation capacity in various melanoma cell lines *in vitro* as discussed in Part II of chapter three.

4.1 Discussion of the experimental work

4.1.1 Part I AXUD and MEN1

4.1.1.1 Overview

As discussed in the introduction, cutaneous melanoma is a highly aggressive and lethal malignancy, responsible for almost 80% of skin cancer-related mortality [45], where patients with stage IV melanoma have a 5-year and 15-year survival of 15% and 5% respectively [45-47]. Grade IV melanomas are the metastatic melanomas that disseminate to distant organs through the blood circulation, thus showing resistance to conventional chemotherapy [24]. Therefore, it is essential to understand the molecular and signalling mechanisms leading to melanoma development and progression, hence paving the way for more advanced, targeted therapies.

Melanomagenesis implicates frequent mutations in genes that commonly regulate biological processes among the hallmarks of cancer, e.g. *BRAF*, *NRAS* and *NF1* maintaining proliferative signalling, *PTEN* and *KIT* evading growth inhibition, *TP53* resisting programmed cell death, *TERT* enabling cell immortalization [79, 92]. While the mitogenic RAS-RAF-MEK-ERK signalling pathway is commonly mutated in cutaneous melanoma displaying *BRAF* mutations at a frequency of 50 to 80%, other signalling pathways including PI3K/AKT, Wnt, NF-κB, Jnk/c-Jun, JAK/STAT, and TGFβ have also been shown to be implicated [65, 133, 169, 170]. The TGFβ signalling pathway plays an important role in normal melanocytes and melanoma, acting as a potent tumour suppressor regulating growth and survival as well as migration and invasion [171, 172]. Previous work from our lab provided evidence that TGFβ upregulation of the Plasminogen Activator Inhibitor-1 (PAI-1) in melanoma cells reduces plasmin generation and activity, consequently inhibiting cell migration and invasion [171]. Furthermore, our lab also

identified –in a study that I first coauthored- the TGFβ/LIF signalling cascade as a tumour suppressive-like pathway in melanoma regulating cell cycle arrest, cell death and inhibition of cell migration in a STAT3-dependent manner [173].

4.1.1.2 Rationale

In continuation of previous work [171, 173], we examined the TGFβ-mediated tumour suppressive effects in human cutaneous melanoma. The importance of this study stems from the limited knowledge of the role of TGFβ in cutaneous melanoma since it has not been fully investigated and the existing evidence stays controversial.

4.1.1.3 AXUD1

Transcriptome analysis of several TGFβ-responsive cutaneous melanoma cells conducted in our lab revealed several intriguing candidate genes potentially playing a role downstream of TGFβ in cutaneous melanoma. One of these genes that were upregulated by TGFβ is AXUD1. AXUD1 encodes a 64-kDa protein containing about 589 amino acids [502]. AXUD1 was also termed Cysteine and Serine Rich Nuclear Protein 1 (CSRNP-1) and was characterized as a member of a new family of genes that encodes nuclear proteins comprising cysteine- and serine-rich domains[503]. While AXUD1 expression is high in normal tissues, it was found to be lower in lung cancer, liver, kidney and colorectal carcinoma [502] as well as oral squamous cell carcinoma tissues [511], thus suggesting a potential tumour-suppressive role of AXUD1. In accordance, DAxud1 (Drosophila Axud1 homolog) was found to play a tumour suppressive-like role in Drosophila, inducing pro-apoptotic and growth-inhibitory effects in imaginal cells. Silencing DAxud1 increased the proliferation of imaginal cells, while its overexpression blocked cell cycle progression at mitosis by disrupting Cdk1, and induced apoptosis by activating JNK [512]. Concordantly, survival curves from the Human Protein Atlas and RNA-seq from TCGA dataset of cutaneous melanoma patients showed a significant positive correlation with AXUD1 mRNA expression levels, where most of the deceased patients showed low AXUD1 expression levels, while the surviving patients had higher AXUD1 mRNA expression. Taken together, these observations agree with and support the work presented in Part I demonstrating that TGFβ-mediated upregulation of AXUD1 could relay the multifactorial TGFβ-induced tumour-suppressive effects in -DAUV- human cutaneous melanoma cells

4.1.1.4 Menin

Interestingly, menin was found to leverage the TGFβ ligand family members in their signalling cascade at a transcriptional level thus facilitating their cytostatic and differentiation roles [275, 349, 736]. Menin is a protein encoded by the multiple endocrine neoplasia type 1 (*MEN1*). The *MEN1* gene is an autosomal dominant disorder affecting the endocrine system characterized by the concomitant existence of tumours in the pancreas as well as the parathyroid and anterior pituitary glands. Menin, a 610-amino-acid protein [518, 519] was found to interact with numerous protein partners including several transcription factors [525, 737] and to participate in transcriptional regulation via serving as a scaffolding protein in chromatin-remodelling complexes [521, 546]. Menin plays a significant role in regulating important genes synchronizing the cell cycle, such as cyclin-dependent kinase inhibitor (*CDKI*) genes [531, 532]. Notably, non-endocrine tumours have also been reported in *MEN1* patients. These include skin tumours of mesenchymal origin such as angiofibromas, collagenomas, lipomas, as well as malignant melanomas [738-740]. LOH in 11q13 was detected in six melanoma tumours and deletion in the *MEN1* locus found in 19 cases of sporadic metastatic melanoma. Another study implied that multiple melanoma tumour suppressors are localized in chromosome 11q, which incidentally includes the *MEN1* region, thus raising the possibility of an association between *MEN1* and melanoma [138].

Over the years, more reports show that MEN1 patients develop other malignancies different from the classical endocrine tumours including but not confined to skin tumours such as melanomas, lipomas, collagenomas, and angiofibromas [741]. Interestingly, menin was shown to play a melanoma tumour-suppressive role via stimulating the transcription of several genes involved in homologous recombination-directed DNA repair [742].

Earlier work from our laboratory showed how menin plays a tumour suppressive effect downstream of TGFβ/Smad3 signalling in pituitary adenoma cells [255, 348, 349]. Menin was found to physically bind to Smad3 in pituitary tumour cells and that silencing MEN1 expression disrupts TGFβ-induced Smad3/4 binding to the DNA at certain regulatory sites, thus preventing TGFβ/Smad3-dependent transcriptional activity [349]. Interestingly, restoring menin expression in Men1-deficient mouse embryonic fibroblasts (MEFs) resulted in G1 cell cycle arrest [528] and restored sensitivity to apoptosis, while ectopic menin overexpression induced Bak and Bax-

dependent apoptosis in MEFs [554]. Similarly, re-introducing menin in a Men1-deficient Leydig cell tumour mouse cell-line caused G1 cell cycle arrest, upregulation of the CDK inhibitors p18 and p27 as well as induction of apoptosis [529]. Moreover, menin was shown to physically bind to the hTERT promoter region, where silencing menin resulted in primary human fibroblasts immortalization [534]. These findings agree with the work presented in Part I which shows that TGFβ-mediated upregulation of menin could mediate the multifaceted TGFβ-induced tumour-suppressive effects in –DAUV- human cutaneous melanoma cells.

4.1.1.5 Cell Growth

TGFβ-mediated cytostatic effects were compromised upon silencing endogenous Smad3 thus decreasing the Smad3-to-Smad2 ratio in various TGFβ-sensitive cells such as hepatocellular carcinomas HepG2, Huh7 and the gastric adenocarcinomas SNU-16, and SNU-620 and vice versa. In agreement, TGFβ-mediated Smad3 activation was increased and TGFβ-mediated cytostatic effects were restored upon silencing endogenous Smad2 thus increasing the Smad3-to-Smad2 ratio. Moreover, Smad3 loss-of-function in mice was shown to promote adenomas and invasive carcinomas in the distal colon region [719, 722]. Moreover, another report showed that Smad3 overexpression in MCF10 breast cancer cells resulted in more and larger lung metastases while inhibiting the Smad2/3 signalling pathway inhibited their metastatic activity due to the Smad3 role in mediating cell extravasation into the lung and induction of angiogenesis [724].

These reports support the findings in Part I revealing the preferential role of Smad3 –but not Smad2- in mediating the TGFβ-mediated inhibition of cell cycle in the DAUV cutaneous melanoma cells, where silencing Smad3 abolished TGFβ-mediated induction of cell cycle arrest, in a fashion comparable to that caused by silencing AXUD1 or menin.

4.1.1.6 Immortalization

Our lab previously reported that TGFβ-mediated inhibition of cell immortalization is Smad3-specific and Smad-2-independent. These findings are concordant with previous reports showing how Smad3 plays an essential role in TGFβ-induced growth inhibition via down-regulation of c-Myc in keratinocytes [714] or breast cancer [716] mediated by a trimeric Smad3 complex involving Smad3/4, E2F4/5 as well as p107 [346, 717]. In the current study, the results provide evidence that TGFβ-mediated inhibition of cell immortalization is also Smad3-specific

and Smad2-independent, further highlighting the Smad3-dependent pathway as the preferential mediator of TGFβ-induced tumour suppression in –DAUV- human cutaneous melanoma cells

4.1.1.7 Migration

Concerning migration, invasion and epithelial-mesenchymal transition, TGFβ was shown to elicit inhibitory effects in uveal melanoma [473] as well as in retinal Müller glia [471] while showing promoting effects in advanced breast cancer [694-697]. Yet, in cutaneous melanoma, the role of TGFβ in tumour development and progression is debatable since it is still not thoroughly investigated. Initial studies showed that inhibiting TβRI with a chemical inhibitor or overexpressing the inhibitory Smad7 could promote tumour cell aggressiveness via autocrine activation of Smad signalling [487, 500], where it was suggested that malignant melanoma-associated osteolytic bone lesions were caused by TGFβ. On the other hand, other studies suggested that TGFβ might act as a suppressor of metastasis in melanoma. Indeed, previous work from our lab showed that TGFβ could significantly inhibit cell migration in human cutaneous melanoma via down-regulating plasmin generation and activity [171]. This was in agreement with an earlier study that showed that TGFβ could inhibit migration in a murine melanoma model via downregulating the plasminogen activation system [172]. The current work in this thesis suggests that AXUD1 and menin play an important role in mediating the TGFβ-induced anti-migratory effects in DAUV melanoma cells *in vitro*, as well as inhibiting secondary tumour metastasis *in vivo* in a Smad3-dependent manner.

4.1.1.8 *In vivo* work

The *in vivo* data, using preclinical models of primary tumour formation and secondary tumour metastasis, revealed that upon knocking out Smad3, Smad4, AXUD1 or menin in DAUV melanoma cells, these KO cells formed larger tumours upon subcutaneous injection and led to a high increase in metastatic nodules in the lung, liver, and spleen upon intravenous injection in immunodeficient NSG mice when compared to scrambled cells. Interestingly Smad2 KO cells were comparable to scrambled cells, indicative of a specific role of Smad3/4 and AXUD1 or menin in the TGFβ-mediated prevention of tumorigenesis and metastasis. These results come in agreement with a report showing that TGFβ-mediated cytostatic signals are Smad3-, but not Smad2-, dependent [718].

Given that silencing Smad2 in melanoma cells showed no difference than control cells, it is evident that each of AXUD1 and menin together with Smad3/4 plays a crucial role in this TGFβ-mediated suppression of tumour formation and metastasis. The data come consistent with previous studies showing how the Smad3-to-Smad2 ratio could affect the TGFβ-induced growth inhibitory function [718]. Noteworthy, the present *in vivo* work is the first step in a series of future experiments that should investigate overexpressing or reintroducing exogenous AXUD1 and menin in these mice to see if they would elicit tumour suppressive and antimetastatic effects in these mice

4.1.1.9 Initial studies from Mauviel's group

Potential concerns with the initial studies using the TβRI chemical inhibitor and overexpression of the inhibitory Smad7 are the likelihood of the indirect effects of Smad7 overexpression and/or non-specificity of the TβRI inhibitor. Another concern is that these studies were limited to one cell line 1205Lu. Interestingly, I found that the 1205Lu cell line was the least responsive to TGFβ (Figure 3-1B) amongst the panel of human melanoma cell lines tested in the present study. Thus, it may not be the best representative model to study human metastatic melanoma. Moreover, it is important to note that 1205Lu was later shown to be a problematic cell line where reports found that 1205Lu cells (WC00058) from Coriell Institute of Medical Research (Camden, NJ) showed a presence of about 80% mouse metaphases and about 20% human metaphases as well as a great percentage of mouse (H-2Kd) MHC class I positive cells, whereas the Wistar stock of 1205Lu showed more than 98% human HLA (W6/32) class I as measured by FACS [743].

4.1.1.10 TGFβ inhibitors

The results in Part I also imply that strategies based on using TGFβ inhibitors may not be as desirable, as initially suggested. This may explain, in part why a phase I study [744] testing the safety and activity of "Fresolimumab" a human monoclonal antibody neutralizing TGFβ1, 2, 3 isoforms in 28 patients with malignant melanoma, only reported 1 patient with a partial response ref. Moreover, 6 other patients showed stable disease with a median progression-free survival of 24 weeks, while other patients developed multiple drug-related skin lesions including 2 patients with eruptive keratoacanthomas (KA) and hyperkeratosis, 2 patients with cutaneous squamous

cell carcinoma (3 of whom developed a transient non-specific papular rash or hyperkeratotic lesions before KAs or SCC lesions) as well as 1 patient with basal cell carcinoma. Furthermore, 12 patients did not receive the intended 4 doses of the drug because of documented disease progression or disease-related death (1 case). Two patients died within 45 days of the last dose of the drug, where the cause of death was described to be due to disease progression or aspiration pneumonia, although they were both receiving the maximum dose (15mg/kg) of the drug. Another phase II trial [745] using FANG™ vaccine that inhibits TGFβ1 signalling in melanoma patients also ended up be non-promising. The therapy is a dual-modulatory autologous whole-cell vaccine incorporating a recombinant human granulocyte-macrophage colony-stimulating factor transgene and a bifunctional short hairpin RNAi targeting furin convertase, thus blocking TGFβ1 and β2 activation. Eight melanoma patients with stages IIIc and IV received the vaccine intradermally ($1x10^7$ cells/injection) every month for 12 months. Three-year survival showed that 7 patients were dead due to disease progression and only 1 was alive, resulting in the termination of the study. Noteworthy, adverse effects arose in patients during the treatment such as skin ulceration of tumour nodule (n=1), sacral nerve compression secondary to metastatic melanoma (n=1). In light of the present study, blocking TGFβ signalling would only produce adverse effects and would potentially aggravate the patient conditions. On the other hand, initiating new trials with strategies aiming at mimicking TGFβ/Smad3 signalling could prove very promising.

Tumorigenesis is a sophisticated multi-faceted process and TGFβ plays a delicate and complex role which is context- and tissue-dependent. The choice of signalling components to manipulate or modify for experimental studies should be determined with great care and relevance. Results from these studies raise concerns by targeting TGFβ, where test drugs could potentially aggravate the patient conditions.

4.1.1.11 Summary of findings

Briefly, results in Part I illustrate (a) how TGFβ could significantly induce cell growth inhibition, apoptosis and autophagy; as well as inhibiting cell immortalization and cell migration in the DAUV human cutaneous melanoma cells *in vitro*. Moreover, they show how TGFβ could play a tumour-suppressive role via inhibiting primary tumour formation and impeding the dissemination and development of secondary lung and liver metastatic lesions *in vivo.* Moreover, this work identified for the first time (b) AXUD1 and (c) menin as two new players through which

TGFβ mediates these effects as well as (d) the specific role of Smad3 over Smad2 as a signalling component downstream of TGFβ to mediate its tumour-suppressive and anti-metastatic effects in (DUAV) human cutaneous melanoma *in vitro* and *in vivo* (Figure 3-10).

4.1.2 Part II MSC

4.1.2.1 Overview

Melanoma development was once described as a process of 'de-differentiation' mature melanocytes, thus allowing the eventual dissemination of the malignant cells [569-571]. Since most melanomas do not arise in dysplastic nevi but rather in normal skin [572, 573], theories adopting the cancer-stem-cell (CSC) concept proposed that melanomas arise due to the mutation of melanocyte stem cells or immature progenitor cells residing in the skin [574-579]. Studies even showed that neural crest stem cells (NCSCs) play a role in the initiation and propagation of melanoma cells (e.g. Sox10 gene) due to the similarity in the gene network of NCSCs (involved in development and wound healing) and melanoma cells (involved in cancer growth and progression)[580, 581]. Evidence shows that melanoma-initiating stem-like cell subpopulation (MSCs) [596-598] – in contrast to the tumour bulk cells- have high *in vivo* tumorigenicity, high embryonic-like differentiation plasticity into multiple cell lineages, high self-renewal potential in xenografts *in vivo* and in long-term cultures *in vitro*, high metastatic potential and to develop chemoresistance [574-576, 578, 579, 582, 599, 600], high potential to evade the patient's immune system [601, 602]. Numerous surface markers were found to identify MSCs e.g. ABCB5 [575, 605], CD20 [574], CD133 [577, 606], CD166, Nestin [607], CD166 [571], CD271 [598, 608, 609] as well as ALDH [610, 611] demonstrating the MSC inherent self-renewal capacities.

4.1.2.1.1 Challenging previous reports

A huge challenge for cancer treatment is presented by CSCs, particularly quiescent CSCs, because of their inherent ability to overcome chemo- and radiotherapy party due to their drug efflux mechanisms or self-renewal capacity. Upon relapse, these CSCs principally give rise to new chemoresistant tumour cells as a result of the previous selective pressure of the chemotherapeutic drugs. [746, 747]. One challenge caused by chemotherapy is their ability to enrich CSC populations rather than depleting them in some cancers e.g. paclitaxel in lung adenocarcinoma cells [748], cisplatin or paclitaxel in ovarian cancer cells [749].

Another challenge present by current chemotherapeutic drugs is that they could expose cancer cells to cellular stress causing them to dedifferentiate to a transient CSC population which could induce tumour development and propagation. For example, breast cancer cell lines treated with valproic acid (a histone deacetylase inhibitor), in combination with ionizing radiation, induced the dedifferentiation of $ALDH^-$ cells into $ALDH^+$ cells via WNT/β-catenin pathway, increased the expansion of $ALDH^+$ population, enhanced mammosphere formation efficiency as well as promoting the tumour-initiating potential of $ALDH^-$ cells in limiting dilution assays [750, 751]. Interestingly, some pharmacological therapies proved to be useful in inducing terminal differentiation in CSCs thus eliminating the CSC pool. For example, in head and neck squamous carcinomas, all-trans-retinoic acid was reported to inhibit the Wnt/β-catenin signalling pathway thus inhibiting CSCs proliferation and stem cell markers expression *in vitro* and *in vivo* [752]. Similarly, BMP4 could induce terminal differentiation of CSCs, leading to reduced tumour initiation and tumour growth [753] in glioblastoma, in addition to inducing apoptosis and chemosensitization in the case of colorectal cancer [754]. These desirable outcomes of using differentiation-inducing drugs in CSCs of different cancer types could potentially be effective in eliminating MSCs in malignant melanomas.

4.1.2.1.2 Promising previous reports

Fortunately, in melanoma, targeting MSCs via specific suppression of MSC markers showed promising results. For example, inhibition of $ABCB5^+$ melanoma cells via the systemic use of a monoclonal ABCB5 antibody resulted in reducing tumorigenesis in xenograft nude mice [605]. Also, silencing ALDH1A was found to decrease melanoma cell viability, suppress tumorigenesis, and reduce chemoresistance [611]. Moreover, using a BCL-2 inhibitor and fenretinide (a retinoid derivative) could decrease the $ALDH^+$ melanoma cells, resulting in decreased tumorigenesis and reduced melanosphere formation efficiency [755]. Another study presented evidence for the therapeutic use of Lunasin (bioactive peptide found in soybean) in melanoma via decreasing MSCs via differentiation of $ALDH^+$ cancer stem cells into the $ALDH^-$ differentiated phenotype. Lunasin could serve as a useful chemotherapeutic tool in eliminating highly invasive chemoresistant MSCs thus blocking self-renewal and subsequent expansion of MSCs [756, 757].

Since MSCs carry specific markers (e.g. ALDH1, CD133) or antigens, therefore targeting these cells using monoclonal antibodies could help to combat melanoma growth. In human metastatic melanoma, CD133 downregulation by specific shRNAs was shown to reduce both melanosphere formation and metastasis *in vivo* and *in vitro* [630]. Further, CD133 was shown to be an immunogenic target in melanoma [625], thus serving as a means to combat MSCs via T-Cell-induced therapeutic antitumour immunity [637]. Also, $CD133^+$ MSCs treatment with Andrographolide (a diterpenoid serving as a potential cancer therapeutic agent) resulted in hindering tumour growth via disrupting CD133-dependent Notch1/MAPK pathway as well as decreasing melanoma cell migration and angiogenesis [632]. Similarly, a combination of the chemotherapeutic agents, Etoposide and Bevacizumab, showed a reduction in melanosphere formation while increasing the apoptotic activity of $CD133^+$ MSCs [758]. These reports align well with the preliminary findings in the present study that TGFβ could possibly reduce the $ALDH^+$ and $CD133^+$ MSC subpopulations, thus highlighting the protective role that could be played by TGFβ via negatively regulating the MSC subpopulation responsible for MSC expansion. Although, these results need further confirmation and validation, they still go concordantly with previous studies demonstrating the tumour-suppressive role of TGFβ in melanoma patients.

In conclusion, the present study, altogether, presents considerable evidence that can impact the choice of treatment regimens antagonizing the TGFβ/Smad signalling cascade in human melanoma. These preliminary findings -presented in Part II- suggest that using chemotherapeutic or immunotherapeutic agents acting as TGFβ mimics could prove beneficial to malignant melanoma patients. These data suggest that TGFβ can suppress MSC self-renewal and may affect the $ALDH^+$ (and possibly $CD133^+$) subpopulation responsible for MSc expansion, which -if true- could help in MSC depletion and minimization of tumour relapse. Therefore, further studies are needed to verify the role of TGFβ signalling in regulating MSC, given its potential protective role to improve relapse-free survival of melanoma patients.

Interestingly, these results concur with a [759] clinical study showing that chemotherapy-responsive melanoma patients had higher serum TGFβ compared to chemotherapy-resistant ones, Not only that but also the melanoma patients with higher TGFβ serum levels showed a more favourable overall survival rate when compared to the patients with lower levels. The findings in this thesis could explain this protective role of TGFβ in melanoma patients and suggest that the

TGFβ-induced tumour suppressive and antimetastatic effects as well as its potential role in negatively regulating MSC, are –among- the reasons why TGFβ elicits a protective role in melanoma patients. The results presented here also confirm the utility of serum levels of TGFβ as diagnostic, predictive, and likely prognostic markers in melanoma patients.

4.1.2.2 Rationale

Many TGFβ family members are involved in the maintenance of self-renewal and pluripotency in embryonic stem cells [680]. In somatic stem cells, TGFβ regulates the biological functions of multipotent bone-marrow-derived mesenchymal stem cells, hematopoietic cells, neuroepithelial stem cells, and melanocyte stem cells [365, 681-684]. However, TGFβ-mediated regulation of MSCs was not studied, thus requiring further investigation.

As mentioned earlier previous studies from our lab showed that TGFβ-mediated upregulation of the Plasminogen Activator Inhibitor-1 (PAI-1) in melanoma cells could decrease plasmin generation and activity, thus reducing cell invasion and migration [171]. Moreover, in a publication I first-coauthored, we identified the TGFβ/LIF/STAT3 signalling pathway as a novel tumour-suppressive-like pathway in melanoma, inducing cell cycle arrest and cell death as well as inhibiting cell migration [173]. As shown in Part I of the study, I identified the AXUD1 and menin proteins as new downstream targets of TGFβ in DAUV melanoma cells, where each of them could mediate the TGFβ-induced cell cycle arrest, growth inhibition, apoptosis, and autophagy, as well as hindering cell immortalization, and migration *in vitro.* Moreover, AXUD1 and menin could mediate TGFβ –induced inhibition of tumour formation and secondary tumour metastasis *in vivo*. Considering these findings which strongly support the TGFβ role as a suppressor of tumour formation and tumour metastasis in melanoma, and the previously stated evidence that CSCs play a significant role in mediating tumour metastasis, I hypothesized that TGFβ could similarly play a role in the regulation of CSC populations in cutaneous melanoma, a role that is worth investigating.

4.1.2.3 TGFβ and Stem Cells

TGFβ-family signalling plays important roles in the maintenance of self-renewal and pluripotency of both human and mouse embryonic stem (ES) cells [680], via different

mechanisms in both species involving Smad2 nuclear localization, Smad3/4 nucleocytoplasmic shuttling [760], or the Activin/Nodal cascade[761-764], promoting Oct-4 and Nanog expression [765]. During normal physiological development, TGFβ functions singularly or in concert with various environmental cues to regulate normal somatic stem cells' self-renewal and differentiation. In the TGFβ signalling, Smad complexes serve as transcriptional co-factors for the expression of several pluripotency genes such as FOXH1, NANOG, and OCT4 in response to TGFβ stimulation [764, 766-768]. TGFβ could promote the mobilization of multipotent bone-marrow-derived mesenchymal stem cells (BMMSCs) to the bone resorption sites [769] during the parathyroid hormone (PTH)-induced bone remodelling process, finely regulating the activity of chondroblasts and osteoblasts [770-772] as well as promoting the differentiation of BMMSCs into cardiomyocyte lineage [773]. Interestingly, in hematopoietic cells (HSC), TGFβ showed a dose-dependent activation of the MAPK pathway. In the absence or at low concentrations of TGFβ, HSC were highly proliferative, whereas, at higher concentrations of TGFβ, HSCs were quiescent due to the inhibition of proliferation [681-683].

TGFβ was reported to inhibit the proliferation of midbrain neuroepithelial stem cells suppressing their self-renewal [684]. On the other hand, TGFβ was shown to induce the apoptosis of melanocyte stem cell upon entry into quiescence upon silencing Bcl2 *in vivo* as well as the maintenance of melanocyte stem cell via downregulating MITF (the regulator of melanocyte differentiation) thus promoting melanocytic immaturity (undifferentiation), thus presenting the TGFβ signalling pathway as one of the central niche factors maintaining melanocyte stem cell immaturity and quiescence [365]. TGFβ plays a complex context-dependent dual role in human cancers, acting both as a tumour suppressor in normal cells and early carcinomas, and a promoter of tumour metastasis in more advanced stages of cancer [284]. Remarkably, TGFβ was also shown to play complex dual roles in CSCs regulation. In breast cancer, TGFβ was shown to inhibit tumorigenesis in a xenograft model by decreasing the putative CSC or early progenitors' subpopulations as well as inducing the differentiation of highly-proliferative committed progeny into less proliferative cells [685]. Moreover, in diffuse-type gastric carcinoma, TGFβ was shown to reduce tumour development and size *in vivo*; through inhibiting cancer-initiating cell population via downregulating ABCG2 transporters that cause active efflux of chemotherapeutic drugs [686] as well as a reducing in

the ALDH1$^+$ population that possess tumour-initiating, tumour progression and self-renewal capacity [687].

Contrarily, several reports showed that in Claudin-low human breast cancer, TGFβ was found to induce the formation of tumour-initiating cells [688]. Also, in triple-negative breast cancer, paclitaxel treatment caused an increase in autocrine TGFβ signalling leading to an IL-8-dependent expansion of CSCs leading to the development of chemotherapeutically-resistant CSCs [689]. In scirrhous gastric cancer, TGFβ was suggested to activate cancer-associated fibroblasts which increased CSC markers expression and tumourosphere formation [424]. In glioblastoma, Smad-dependent TGFβ-mediated induction of LIF promoted the self-renewal of the glioma-initiating cells as shown by increased neurospheres formation, suppressed neurospheres differentiation *in vitro* as well as increased tumour formation and size *in vivo* [690]. Moreover, TGFβ induced stemness genes via inducing the binding of its direct target Sox4 to Oct4 thus increasing Sox2 expression [691, 692]. In chronic myeloid leukemia, TGFβ was shown to regulate the phosphorylation Akt and nuclear localization of Foxo3a, required for the stemness properties of leukemia-initiating cells [693].

Interestingly, in gastric adenocarcinoma cells, TGFβ was shown to induce CD133 protein expression further triggering the phosphorylation and activation of the PI3K/ERK/p70S6K signalling pathway [774], thus increasing cancer cell motility and invasiveness resulting in a poor patient outcome [775]**.** Moreover, in hepatocellular carcinoma, Smad-dependent TGFβ signalling increased the tumorigenicity of the CD133$^+$ subpopulation *in vivo* (via inhibiting DNA methyltransferases DNMT1 and DNMT3β thus increasing the demethylation of the CD133 [776]. In squamous cell carcinoma, TGFβ signalling plays a role in generating heterogeneity in the stem cell population, inducing tumorigenicity, and increasing drug resistance [777]. Nonetheless, the role of the TGFβ signalling pathway in regulating MSCs has not been investigated and still requires further elucidation.

4.1.2.4 Melanosphere formation

Melanospheres are melanoma cells that grow into non-adherent spheroidal colonies under low-anchorage conditions and their formation reflects the self-renewal capacity and multipotency of MSC *in vitro*. Cells arising from melanospheres were shown to be highly tumorigenic when tested in immunodeficient xenotransplantation models [597, 610]. To explore the TGFβ-mediated

effect in melanoma, I used a panel of human cutaneous melanoma cell lines from different clinical backgrounds, and I measured the melanosphere formation efficiency (MFE) in these cell lines to evaluate their self-renewal capacity. The data in Part II showed that TGFβ could significantly reduce MFE in all the melanosphere-forming cell lines. Moreover, to determine the Smad2/3 specificity of this inhibitory effect, I found that upon knocking out only Smad3 and Smad4 -but not Smad2- in DAUV melanoma cells, the TGFβ-induced inhibitory effect was lost resulting in increased MFE in Smad3 and Smad4 KO cells compared to the parental and scrambled cells. This highlights the preferential role of Smad3/4 in the TGFβ-mediated suppression of melanosphere formation in DAUV melanoma cells and hence mitigating MSC tumour-initiating potential.

Noteworthy, upon testing melanosphere formation using AXUD1 and menin KO DAUV melanoma cells, only menin KO cells showed increased MFE (similar to Smad3 and Smad4 KO cells) when compared to parental or scrambled cells, whereas knocking out AXUD1 did not affect the TGFβ inhibitory effect showing decreased MFE. Furthermore, I found that silencing AXUD1 -on its own- significantly inhibited basal melanosphere formation in untreated DAUV KO cells compared to untreated parental and scrambled cells. This suggests that menin possibly plays a role in the TGFβ/Smad3-mediated inhibition of melanospheres and MSC self-renewal without the intervention of AXUD1. Moreover, this finding suggests a potential role of AXUD1 in MSC multipotency and self-renewal. Indeed, this observation agrees with a study in zebrafish showing that Axud1 depletion in neural progenitor cells (using antisense morpholinos) resulted in decreased proliferation and increased cell death, hence decreased neural progenitor expansion, thus resulting in smaller brain size, notably without affecting rostrocaudal patterning or differentiation within the diencephalic and mesencephalic regions [735].

4.1.2.5 Non-Smad Pathways

Smads are considered crucial to TGFβ signalling (canonical signalling pathway), yet TGFβ could transmit its signal through other intracellular non-Smad signalling cascades. For example, TGFβ can activate the mTOR and the phosphoinositide 3-kinase (PI3K)/Akt pathway[281-283], the mitogen-activated protein kinase (MAPK) pathway[271-273] as well as stress-activated kinases p38 and JNK (Jun N-terminal Kinase) pathways [273, 274, 276, 279, 403, 778], thus regulating different biological effects as cell cycle arrest, apoptosis, cell motility and invasion and EMT induction. Interestingly, the experiments show that TGFβ/SMAD inhibition using SB-

431542 [779] resulted in abolishing the TGFβ-mediated inhibition of melanosphere formation in DAUV melanoma cells, but not upon inhibiting ERK, JNK, mTOR, p38, PI3K. These observations indicate TGFβ-mediated inhibition of melanosphere formation in DAUV melanoma cells is Smad dependent and does not involve other Smad-independent pathways. They also come in agreement with the previous observation, this finding highlights the potential preferential role of the TGFβ/Smad3 signalling cascade in negatively regulating melanosphere formation and MSC self-renewal in DAUV melanoma cells.

4.1.2.6 $CD133^+$ and $ALDH^+$

CD133 and ALDH are two confirmed stemness markers conventionally used to isolate stem cell-like populations in human malignant melanoma cells [780], where cells with high expression of CD133 ($CD133^+$) [577] or high ALDH activity ($ALDH^+$) [610] show increased tumorigenicity and increased self-renewal capacity *in vivo* in immunodeficient mice. Interestingly, TGFβ was shown to regulate the $ALDH1^+$ population in gastric adenocarcinoma cells [687] as well as the regulating $CD133^+$ population [774] in gastric adenocarcinoma cells and in hepatocellular carcinoma [776]. As well as being involved in CSC heterogeneity, tumorigenicity, and chemoresistance in squamous cell carcinoma [777]. The two markers were used to show that $ALDH^+/CD133^+$ populations in pancreatic adenocarcinoma [781] and hepatocellular carcinoma [782] showed the highest tumorigenicity.

To determine the role of TGFβ in MSC self-renewal and expansion, I used flow cytometry to determine whether TGFβ induces the inhibition of $CD133^+$ and $ALDH^+$ stem-like cell populations in the tested panel of melanoma cell lines. Resembling its inhibitory effect on melanosphere formation results in Part II showed that TGFβ tends to reduce $ALDH^+$ and $CD133^+$ MSC populations (as shown in Figure 3-14 E and F) in different melanoma cell lines to various extents. However, the statistical analysis of all the biological replicates came as insignificant

To explore whether Smad2 or Smad3 mediates this TGFβ inhibitory effect, I found that in Smad3 and Smad4 -but not Smad2- KO DAUV melanoma cells, TGFβ lost its inhibitory effect causing an increase in $ALDH^+$ populations in Smad3 and Smad4 KO cells comparable to the parental and Smad2 KO cells. A similar tendency was observed with $CD133^+$ MSC populations, although the effects were statistically insignificant. Interestingly, upon assessment of any

potential changes in the $CD133^+$ and $ALDH^+$ populations in response to TGFβ treatment using AXUD1 and menin KO DAUV melanoma cells, results revealed that both AXUD1 and menin KO cells showed increased $ALDH^+$ (and probably $CD133^+$) populations, similarly to Smad3 and Smad4 KO cells, when compared to parental or Smad2 KO cells upon TGFβ treatment.

Not overlooking the evidence -presented in Part I- showing the preferential role of Smad3/4 in the TGFβ-induced tumour suppressive and antimetastatic effect, as well as the evidence -presented in Part II- showing the preferential role of Smad3/4 in the TGFβ-induced suppression of melanosphere formation in all tested melanoma cell lines as well as the implication of AXUD1 and menin in those TGFβ-mediated effects, these preliminary results shed the light -for the first time- on a potential role for AXUD1 and menin in the TGFβ-mediated suppression of $ALDH^+$ (and possibly $CD133^+$) melanoma stem cell subpopulations and that these effects are likely Smad3-dependent.

The role of TGFβ in regulating CSCs is majorly unknown in melanoma, however, it was studied in some other cancer. In hepatocellular carcinoma, the Smad-dependent TGFβ signalling was found to increase the tumorigenicity of the $CD133^+$ subpopulation *in vivo* [776]. In gastric adenocarcinoma cells**,** TGFβ was shown to induce CD133 protein expression [774], thus increasing cancer cell motility and invasiveness resulting in a much poorer patient outcome [775]**.** In congruence to the results presented in Part II, Kaplan-Meier survival curves obtained from the Human Protein Atlas and RNA-seq from TCGA dataset of cutaneous melanoma patients demonstrated a significant inverse correlation of ALDH1A1 and CD133 mRNA expression levels and patient survival, where surviving patients showed low ALDH1A1 and CD133 expression levels in comparison to the deceased ones. These observations align with our preliminary results which highlight the TGFβ-induced suppression of the $ALDH1A1^+$ (and possibly $CD133^+$) subpopulations in DAUV melanoma cells. Altogether these preliminary findings are consistent with findings from our previous publications and findings in the present work demonstrating the tumour-suppressive role of TGFβ in DAUV melanoma cells *in vitro* and *in vivo*, thus highlighting the protective role of TGFβ in melanoma patients,

4.1.2.7 Summary of findings

Part II of the study presents a novel role for TGFβ/Smad3 signalling cascade in melanoma tumour suppression by highlighting its role in reducing MSC self-renewal and possibly

subpopulations responsible for MSC expansion *in vitro*. These findings are of interest since the exact role of TGFβ in regulating melanoma stem cells has not been investigated. Briefly, the results present the role of TGFβ and the specific preferential role of Smad3 over Smad2 downstream of TGFβ to negatively regulate melanosphere formation of various human cutaneous melanoma *in vitro*. The current study is the first attempt -to date- to demonstrate that TGFβ could significantly inhibit melanosphere formation in seven different cutaneous melanoma cells *in vitro*. Also, it is the first to present that TGFβ plays a -Smad3-dependent- role in reducing the $ALDH^+$ (and possibly $CD133^+$) population of the MSCs in DAUV melanoma cells *in vitro*. This suggests a probable suppressive role of TGFβ over MSC tumour-initiation potential and the subpopulations essential for MSC expansion when considering previous reports from our lab as well as findings in Part I. The results in Part II present preliminary evidence for the TGFβ-mediated inhibitory role in stem-cell-like populations of cutaneous melanoma *in vitro*. These results require further confirmation and broader investigation, via measuring more MSC surface markers (e.g. ABCB5, CD20, CD166, Nestin, CD166, as well as CD271) as well as the determination of the tumour-initiation capacity and MSC expansion of isolated MSC *in vivo.*

4.2 Previous work investigating the role of TGFβ in cutaneous melanoma

TGFβ-mediated effects in tumour metastasis are tissue-specific, where on one hand TGFβ acts as a prometastatic factor in advanced breast cancer [694-697] while on the other hand acts as an antimetastatic factor in retinal Müller glia [471] and uveal melanoma [473]. Nonetheless, the role of TGFβ in tumour development and progression in cutaneous melanoma is still controversial because it is not entirely investigated.

Early reports from one research group showed that the use of a TβRI chemical inhibitor or overexpression of the inhibitory Smad7 could promote tumour cell aggressiveness by activating Smad signalling in an autocrine manner [487, 500]. The limitations of those reports include potential indirect effects of non-specificity of the TβRI inhibitor and/or Smad7 overexpression. Besides, the experiments were conducted using one melanoma cell line namely, 1205Lu, which I particularly found to be least responsive to TGFβ of all tested melanoma cell lines (Figure 3-1B). Moreover, 1205Lu may not be the best representative model to study TGFβ signalling in human metastatic melanoma, since the validity of using 1205Lu cells as a model for human melanoma

was a matter of question due to mouse contamination in 1205Lu cells (WC00058) obtained from the Coriell Institute of Medical Research (Camden, NJ) [743].

On the other hand, a pioneering study from our laboratory demonstrated that TGFβ acts as an antimetastatic agent in human cutaneous melanoma, through down-regulating the plasminogen activator [171], consistent with previous results in a murine melanoma model [172]. Moreover, in a later study from our lab, that I first co-authored, we identified LIF as an effector downstream of the TGFβ signalling cascade presenting a novel tumour suppressive-like pathway in cutaneous melanoma. We demonstrated that TGFβ -via activating its Smad-dependent signalling pathway- induces LIF secretion. LIF, in turn, phosphorylates STAT3 and promotes LIF binding to the STAT3 binding element found in the p21 promoter, with a subsequent increase in the CDKI p21 gene expression. Afterward, p21 triggers cell cycle arrest as well as Caspase3/7-dependent apoptotic activity. LIF also showed anti-metastatic effects in melanoma cell lines. Taken together, our results defined the TGFβ/LIF-mediated signalling as a novel tumour-suppressive-like pathway in cutaneous melanoma [173].

4.3 Challenges in current therapeutics

As detailed in previous sections, the results of the current study disfavour treatment strategies relying on antagonizing TGFβ in cutaneous melanoma patients. Indeed, the results in this thesis- in addition to our work- could explain why clinical trials aiming at blocking TGFβ signalling in melanoma were terminated due to their failure. In 2013, a phase II trial used the FANG™ vaccine -which blocks TGFβ1 and β2 activation- in melanoma patients was terminated, when their three-year survival analysis showed that only 1 patient survived while 7 out of 8 patients died as a result of melanoma progression [745]. A year later, a phase I study used the Fresolimumab™ antibody -which neutralizes TGFβ1, 2, 3 isoforms- in patients with malignant melanoma, was also terminated when only 1 out of 28 patients responded with a partial response [744].

4.4 The novelty in the present work

4.4.1 The role of AXUD1 downstream of TGFβ in regulating tumour suppression

In Part I of chapter three I identified TGFβ/Smad3/AXUD1 as a novel signalling axis that induces tumour suppressor and anti-migratory effects in DAUV cutaneous melanoma *in vitro* as well as an inhibitor of primary tumour formation and secondary tumour metastasis *in vivo*. Part I provides evidence presenting AXUD1 as a novel TGFβ target and the preferential role played by Smad3 but not Smad2 in mediating several TGFβ-induced tumour suppressive and anti-metastatic effects in DAUV human cutaneous melanoma *in vitro.* Further, it demonstrates how AXUD1 relays TGFβ-induced suppression of primary tumour formation and secondary tumour metastasis *in vivo* also in a Smad3-dependent manner. Since its characterization, AXUD1 was shown to play a tumour-suppressive role, where it showed high expression levels in normal tissues that dropped significantly in several cancers such as colorectal, hepatic, lung and kidney cancers [502] as well as oral squamous cell carcinoma tissues [511]. Showing an evolutionary-conserved role, the Drosophila Axud1 orthologue DAxud1 displayed tumour suppressive effects through promoting apoptosis and growth inhibition in imaginal cells. DAxud1 deficiency resulted in a high proliferation of imaginal cells, whereas DAxud1 overexpression increased JNK-dependent apoptosis and stalled cell-cycle progression at the mitosis phase via Cdk1 inhibition [512].

The present study shows how TGFβ upregulates its downstream target AXUD1 thus inducing cell cycle arrest, apoptosis, autophagy as well as preventing cell immortalization and cell migration *in vitro*, therefore serving as a significant tumour-suppressor downstream of the TGFβ signalling pathway in DAUV cutaneous melanoma cells. Furthermore, it shows how injecting AXUD1 KO DAUV melanoma cells in NSG immunodeficient mice resulted in increased primary tumour formation as well as exacerbated secondary tumour metastasis in the lungs and livers of these mice, in a fashion quite comparable to that observed upon injecting Smad3 KO DAUV cells. This indicates a crucial role played by AXUD1 downstream of TGFβ that appears to be Smad3-dependent. Indeed, several previous reports come in congruence with the present findings showing the preferential role of Smad3 in mediating TGFβ-mediated tumour suppressive effects such as the inhibition of cell immortalization [403] or the inhibition of cell growth [346, 714, 716-718]. Besides, Kaplan-Meier survival curves showed that high AXUD1 mRNA expression levels were associated with higher patient survival, further indicating the

protective tumour-suppressive effect of AXUD1 in melanoma patients suggested by the present findings. Taken together, the present results demonstrate evidence for the role of the TGFβ/Smad3/AXUD1 axis as a tumour suppressive and antimetastatic signalling axis in human cutaneous melanoma.

4.4.2 The role of menin downstream of TGFβ in regulating tumour suppression

Part I of chapter three also describes how I identified TGFβ/Smad3/menin as a novel signalling cascade that mediates tumour suppressor and anti-metastatic effects in cutaneous melanoma *in vitro* as well as an inhibitor of primary tumour formation and secondary tumour metastasis *in vivo*. Menin was highlighted as a novel TGFβ effector and its specific Smad3-dependence in mediating both TGFβ-induced tumour suppressive and anti-metastatic effects in DAUV human cutaneous melanoma cells. These results showed the tumour-suppressive role of menin downstream of the TGFβ signalling pathway in cutaneous melanoma, through inducing cell cycle arrest, apoptosis, autophagy and preventing cell immortalization and migration *in vitro*. Using menin KO DAUV melanoma cells, I showed how the TGFβ-induced inhibitory effects in melanoma cells were compromised or abolished. I also showed the preferential role of Smad3 in mediating those TGFβ effects, as observed by the loss of the TGFβ -induced inhibitory effects in Smad3 KO, but not Smad2 KO, melanoma cells. Moreover, Part I demonstrates how menin plays a role downstream of TGFβ in curbing primary tumour formation and secondary tumour metastasis *in vivo*, a role that appears to be Smad3-specific. Indeed, the results show that injecting menin KO DAUV melanoma cells in immunodeficient NSG mice resulted in higher primary tumour formation and secondary tumour metastasis in the lungs and livers of these mice. These observations were comparable to those observed when injecting Smad3 KO DAUV cells in NSG mice. Altogether, these findings indicate an essential role for menin downstream of the Smad3-dependent TGFβ signalling pathway. These data are in alignment with earlier reports -including ours- showing how Smad3 specificity in mediating the TGFβ-induced inhibition of cell growth [346, 714, 716-718] and cell immortalization [403] and that Smad3 deficiency increased adenomas and invasive carcinomas in the distal colon region in mice [719, 722].

Earlier studies from our laboratory had demonstrated the tumour-suppressive role of menin downstream of TGFβ/Smad3 signalling in pituitary adenoma cells [255, 348, 349], whereby menin physically binds to Smad3 to promote TGFβ-induced Smad3/4 binding to the DNA, thus

enhancing TGFβ/Smad3-dependent gene transcription [349]. Menin was shown to induce growth inhibitory effect, where re-establishing menin expression in Men1-deficient mouse Leydig cell tumour cell-line [529] and mouse embryonic fibroblasts (MEFs) [528] induced cell cycle arrest at the G1 phase as well as inducing apoptosis. Moreover, ectopic menin overexpression induced Bak and Bax-dependent apoptosis in MEFs [554]. Furthermore, menin was found to physically bind to and inactivate the hTERT promoter region thus preventing cell immortalization in primary human fibroblasts [534]. Collectively, these findings illustrate the essential role of the TGFβ/Smad3/menin axis as a tumour suppressive and antimetastatic effects signalling axis in cutaneous melanoma.

4.4.3 The role of TGFβ in melanoma stem cells regulation

Part II is a study where I examined -for the first time- the TGFβ-mediated role in regulating MSCs using a panel of melanoma cell lines from different clinical backgrounds. The *in vitro* data showed that TGFβ could significantly inhibit melanosphere formation efficiency (MFE) in every melanoma cell line investigated in this study. Interestingly, this TGFβ-mediated inhibition of melanosphere formation appeared to be Smad3-dependent, whereby knocking out Smad3 -but not Smad2- in DAUV melanoma cells reversed the TGFβ-mediated inhibitory effect. Similarly, knocking out menin -but not AXUD1- in DAUV melanoma cells abrogated the TGFβ-mediated inhibitory effect. Using flow cytometry, TGFβ showed a tendency –although statistically insignificant- to inhibit $CD133^+$ and $ALDH^+$ subpopulations in the tested panel of melanoma cell lines to various extents. Similar to its effect on melanosphere formation, the TGFβ-mediated inhibitory effects on $CD133^+$ and $ALDH^+$ subpopulations were found to be Smad3-dependent. Interestingly, TGFβ lost its inhibitory effect in both AXUD1 and menin KO DAUV melanoma cells (like Smad3 and Smad4 KO cells) when compared to parental or scrambled cells, suggesting a potential role for AXUD1 and menin in the TGFβ/Smad3-mediated suppression of $CD133^+$ and $ALDH^+$ melanoma cell populations and MSC expansion. Kaplan-Meier survival curves and RNA-seq of cutaneous melanoma patients obtained from the Human Protein Atlas and TCGA, whereby surviving patients showed low ALDH1A1 and CD133 expression levels were low in surviving patients and high in the deceased ones. This supports the results in Part II showing that TGFβ has an inhibitory effect over the $ALDH1^+$ and $CD133^+$ subpopulations and MSCs expansion in melanoma, therefore, suggesting a protective role of TGFβ in melanoma patients.

These findings are consistent with our earlier studies showing the tumour-suppressive effects of TGFβ in cutaneous melanoma.

4.5 The limitations and areas of improvement in the present work

4.5.1 Conducting experiments in DAUV cells

One of the limitations in the present work is the conduction of several experiments in one cell line, namely DAUV melanoma cells. Ideally, the experiments should have been conducted and verified in multiple cell lines as the experiments evaluating the AXUD1 upregulation Figure 3-1A, the melanosphere formation Figure 3-11(A, B), as well as the CD133+ and ALDH+ subpopulation Figure 3-11(E, F). Noteworthy, during the pilot experiments for this project, I conducted some of the *in vitro* experiments using WM278, WM793 and DAUV AXUD1 knockdown cells, where –similar to the knockout cells-TGFβ treatment showed tumour suppressive effects via inducing cell cycle arrest and apoptosis, as well as inhibiting cell migration. For the subsequent experiments, we determined to use KO cell lines using CRISPR/Cas9, where I later encountered difficulty (outlined below) to generate KO in cell lines other than DAUV cells. In a 2012 publication from our lab, it was shown that TGFβ treatment could induce Smad3 phosphorylation in ten different melanoma cell lines including DAUV cells. Moreover, the study showed that TGFβ could inhibit cell growth in 6 (including DAUV cells) out of 10 cell lines, as well as inhibiting cell migration in 9 (including DAUV cells) out of 10 cell lines [171]. Given these observations and those from the pilot experiments, we were comfortable to move forward using the DAUV cell line as a proof of concept.

4.5.1.1 Value of DAUV cells

While one of the limitations of the presented work is the conduction of multiple experiments in the DAUV cell line only, DAUV could represent a model for *BRAF*/*NRAS*-WT or TWT, both of which constitute an important fraction (almost 20%) of melanoma patients. As discussed in the introduction, various combinatorial therapies could prove beneficial to melanoma patients with *BRAF* or *NRAS* mutation; nonetheless, the overall survival rates of patients with wild type *BRAF* and *NRAS* (*BRAF*/*NRAS*-WT) are inauspicious. *BRAF*/*NRAS*-WT melanoma constitutes 13–26% of all cutaneous melanomas displaying other types of mutations e.g. increased

C>T mutation, *NF1* loss, *KIT* activation, or *CCND1* and *TERT* amplification [79, 783, 784]. *BRAF*/*NRAS*-WT tumours showed a higher average mutation rate (approximately five times) and were located more commonly on the head and neck ($P = 0.04$) in patients with CSDM ($P = 0.01$) when compared to tumours harbouring *NRAS* or *BRAF* mutations [784]. The broad activity of non-selective therapies targeting certain mutations could potentially be detrimental to melanoma patients with wild-type genes. Therefore, various therapeutic strategies are necessary for patients with *BRAF*-mutant, *NRAS*-mutant, or *BRAF*/*NRAS*-WT or TWT tumours based on the type of mutation [784]. The treatment of *BRAF*/*NRAS*-WT patients with dacarbazine (alone or in combination with other cytotoxic reagents) showed mediocre response rates [783, 785], similarly, treatment with dacarbazine in combination with Ipilimumab (immunotherapy) showed limited improvement in survival rates [786]. The treatment of *BRAF*/*NRAS*-WT patients harbouring KIT mutations using *KIT* inhibitors was efficacious in 10–22% of patients having *KIT* mutations [79, 784, 787]. Treating *BRAF*/*NRAS*-WT patients with trametinib in Phase 1 clinical trial showed only partial response rate requiring further validation of this targeted therapy for this subclass of melanoma patients [788]. Therefore, the results in my thesis – together with previous reports from our lab- shed the light on a different route that highlights the potential oncoprotective effects of TGFβ signalling and TGFβ mimics that deserve further investigation.

4.5.1.2 Technical challenges

At a technical level, an important reason for conducting most of the experiments in the DAUV cell line was that DAUV cells were the only cutaneous melanoma cells in the studied panel that successfully generated CRISPR/Cas9 knockout cells. As detailed in the methods section, the DAUV cells were infected with 100µl of lentivirus in presence of hexadimethrine bromide; polybrene (8 µg/ml), incubated overnight (12-16 hours), replenished with fresh complete medium for 48 hours. Afterwards, the cells were selected by 1µg/ml puromycin for 14 days followed by a maintenance dose of 0.5µg/ml puromycin. All the other tested cell lines (namely WM278, WM893, WM164, 1205Lu, and SkMel28) died either upon infection with the lentiviral particles or during the puromycin selection. I attempted to optimize the process by using various gRNAs, lowering the volume of lentivirus (50µl) reducing (4µg/ml) or eliminating polybrene, shortening the incubation period (8 hours), extending the recovery period (72 hours), and lowering (0.5µg/ml) the puromycin concentration during the selection.

4.5.1.3 Previous work from Mauviel's group

Noteworthy, the same shortcoming of conducting experiments in one cell line is present in previously published reports, from Dr. Alain Mauviel's laboratory. Their studies, which contradict our observations, were conducted in one cell line (1205Lu) whose selection was based on observations from their own work showing transcriptional response to exogenous TGFβ; invasiveness in Matrigel and tumorigenicity *in vivo* [487, 500].

4.5.2 Lack of focus on mechanisms

Another limitation is the lack of focus on "in-depth" mechanisms, where none of the biological outcomes was elucidated in depth. This is due to multiple reasons:

4.5.2.1 Controversy and limited reports

TGFβ is known to be tumour-promoting or tumour-suppressive depending on the tissue and cellular context as explained earlier. This presented work aimed to address the controversy of whether TGFβ elicits tumour suppressor or tumour-promoting effects in melanoma cells to be an addition to previous studies from our laboratory that demonstrated that TGFβ induced tumour-suppressive and antimigratory effects mediated through PAI1 or LIF [171, 173]. None of the studies conducted in Dr. Lebrun's or Dr. Mauviel's laboratory investigated all the biological processes regulated by TGFβ involved in tumorigenesis as in this thesis. Therefore, the specific aims of the present work were to demonstrate evidence that TGFβ mediates multifactorial tumour-suppressive effects in melanoma cells via modulating numerous biological processes regulating tumorigenesis (namely cell cycle arrest, apoptosis, autophagy, cell immortalization, cell migration, primary tumour formation, secondary tumour metastasis, melanosphere formation, as well as some MSC subpopulations).

Given the main aim laid out at the beginning of the thesis, the breadth of the work done, and the obstacles met, I provided evidence for the preferential role of Smad3 (over Smad2) downstream of TGFβ in mediating the tumour-suppressive and antimetastatic effects in "DAUV" melanoma cells. Nonetheless, the work presented in this thesis presents future opportunities for other colleagues to further explore and elucidate these extremely complex processes.

4.5.2.2 Detection of AXUD1

We were interested in investigating the potential role of AXUD1 downstream of TGFβ in mediating TGFβ-induced tumour-suppressive and anti-migratory effects, since a transcriptome analysis –previously conducted in our lab-revealed that *AXUD1* was upregulated in response to TGFβ stimulation. AXUD1 is an infrequently investigated protein. The available reports usually investigated its relationship with *Wnt* and its role in regulating proliferation or apoptosis during embryonic development in Drosophila or zebrafish [512, 735], or its role in cementoblasts [789] or chondrocytes [790]. In his publication [503], Dr. Sebastien Gingras characterized the AXUD1 gene as *CSRNP1*, together with two other closely-related family members *CSRNP2* and *CSRNP3*.

A major challenge that obstructed more in-depth mechanisms in the presented work was the inability to detect the AXUD1 protein. I was unable to detect AXUD1 in any of the tested melanoma cell lines (WM278, WM793, WM164, A375m, BLM, WM1232, SkMel28, 1205Lu, DAUV) or triple-negative breast cancer cells (MDA-MB-231, SUM159), prostate cancer cells (PC3) or glioma cells (U78) that were used as controls for melanoma cells. In an attempt to detect AXUD1, I used seven different anti-AXUD1 antibodies:

- Four commercial antibodies:
    - Three antibodies from Santa Cruz:
        - Anti-AXUD1 (2055E3a): a mouse monoclonal antibody (sc-81191)
        - Anti-AXUD1 (H-40): a rabbit polyclonal antibody (sc-366204)
        - Anti-AXUD1 (E-15): a goat polyclonal antibody (sc-366204)
    - One antibody from Sigma-Aldrich:
        - Anti-AXUD1 (5E8): a mouse monoclonal antibody (WH0064651M1)
- Three custom-made antibodies:
    - Anti-AXUD1 antibodies were rabbit polyclonal antibodies generously offered (Fall 2017) by Dr. Sebastien Gingras from his previous work, generated on March 19, 2005, April 28, 2008, and May 8, 2008. As per his publication [503], a peptide corresponding to the amino acids sequence 36 to 54 of CSRNP1 was synthesized, then conjugated to glutaraldehyde-activated keyhole limpet hemocyanin, and later used to immunize rabbits (Rockland Immunochemicals, Inc. Gilbertsville, USA), where the antibodies

were purified –before use- by affinity chromatography over corresponding peptide columns.

I attempted to detect AXUD1 protein by:

- Western Blotting, whether using whole-cell extract, nuclear extract, or crude extract in the presence and absence of proteasome inhibitor MG132 (using all the 7 different antibodies)
- Immunoprecipitation in the presence and absence of proteasome inhibitor MG132 (using the commercial antibodies)
- Immunofluorescent cell imaging (using commercial antibodies)

These experiments were conducted in an experimental setting comprising either:

- Parental melanoma, breast cancer, prostate cancer or glioma cells treated or not with TGFβ (200pM) with hopes that the TGFβ treatment would increase AXUD1 expression to higher levels more detectable than basal endogenous levels.
- Parental, scrambled and AXUD1 KD or KO DAUV melanoma cells treated or not with TGFβ (200pM) with hopes that the TGFβ treatment would increase AXUD1 expression to higher levels more detectable than basal endogenous levels and/or that the AXUD1 KD or KO would decrease AXUD1 expression rendering the basal endogenous levels in the parental and scrambled DAUV cells more detectable.

Noteworthy, the AXUD1 protein could not be detected (neither by immune blotting nor by immunoprecipitation) by other research assistants in our laboratory in an attempt to eliminate –my- potential technical human error. Indeed, in a discussion with Dr. Sebastian Gingras in September 2017, he confirmed to me that the AXUD1 protein is degraded extremely rapidly and they could only detect it in total thymocyte lysate, as shown in Fig. 5 G of his publication [503]. All the experiments in this study were conducted in NIH-3T3 and 293T cells ectopically expressing Flag-tagged CSRNP1. The Western blotting was eventually performed using their custom-made rabbit polyclonal antibodies.

4.6 Advancing the knowledge in the field

As discussed throughout the thesis, TGFβ is reported to play complex, essential yet contextual roles in different types of cancers. TGFβ-mediated effects had been studied extensively in some malignancies e.g. breast cancer, however, there is a gap in our knowledge regarding the

role of TGFβ in cutaneous melanoma in tumour development and progression with a few controversial reports available. The data presented in Part I of Chapter 3 could advance the knowledge in the field of melanoma and TGFβ signalling by presenting considerable evidence that TGFβ can elicit multi-faceted tumour-suppressive and antimigratory effects through modulating the biological outcomes tested in this thesis through two –unrelated- proteins namely, AXUD1 and menin. These TGFβ-mediated effects include inducing cell cycle arrest, apoptosis, autophagy and inhibiting cell immortalization and migration (as shown *in vitro*) as well as suppression of primary tumour suppression and secondary tumour metastasis (as shown *in vivo*). These results, in addition to our previous reports, indicate that TGFβ recruits various proteins (i.e. PAI1, LIF, AXUD1, menin, and probably others) to attain this tumour-suppressive and antimigratory role, probably by acting as a Smad3 signalling transduction adaptors (Co-Smads). Also, the preliminary findings –showed in Part II- suggest that TGFβ/Samd3 can negatively regulate MSC self-renewal (as indicated by the TGFβ-induced reduction in MFE in various melanoma cell lines) and can diminish the $ALDH^+$ (and possibly $CD133^+$) subpopulation responsible for MSc expansion. These findings shed the light on the potential role played by TGFβ in MSC depletion in melanoma patients, thus minimizing tumour relapse. Given the importance of this matter, the present findings need to be further investigated and confirmed.

Taken together, the findings in this thesis present considerable evidence that can impact the choice of treatment regimens antagonizing the TGFβ/Smad signalling cascade in human melanoma and suggest that using chemotherapeutic and/or immunotherapeutic agents acting as TGFβ mimics could prove beneficial to advanced melanoma patients. This is particularly true since elevated serum TGFβ levels were found to render melanoma patients more responsive to chemotherapy, with more favourable overall survival rates when compared to the melanoma patients with lower levels[759]. The findings in the thesis could further clarify this important protective role of TGFβ played in melanoma patients and present the identified TGFβ-mediated tumour suppressive and antimetastatic effects as well as the potential inhibitory role in MSC, as potential reasons why TGFβ provides a protective role in melanoma patients. The findings presented here also underline the clinical benefit of using serum TGFβ levels as diagnostic, predictive, and prognostic markers in melanoma patients.

4.7 Summary of thesis findings

In summary, the major findings of the studies comprised in this thesis are:

I. Identifying TGFβ/Smad3/AXUD1 as a novel tumour-suppressive signalling axis in "DAUV" human cutaneous melanoma cells, via inducing cell cycle arrest, apoptosis, autophagy; inhibiting cell immortalization and migration *in vitro*; as well as inhibiting primary tumour formation and secondary lung and liver tumour metastasis *in vivo*.

II. Identifying TGFβ/Smad3/menin as a novel tumour-suppressive signalling axis in "DAUV" human cutaneous melanoma cells, via inducing cell cycle arrest, apoptosis, autophagy; inhibiting cell immortalization and migration *in vitro*; as well as inhibiting primary tumour formation and secondary lung and liver tumour metastasis *in vivo*.

III. Presenting preliminary evidence for a potential role for the Smad3-dependent TGFβ signalling in inhibiting MSC populations in various human cutaneous melanoma cells *in vitro* reflected in the TGFβ-mediated inhibition of primary and secondary melanosphere formation under low-anchorage conditions, and $ALDH^+$ (and possibly $CD133^+$) MSCs.

4.8 Conclusions and Recommendations

Altogether, the studies included in this thesis provide additional evidence for the role of TGFβ in mediating tumour-suppressive and anti-metastatic effects in human cutaneous melanoma (particularly *BRAF*/*NRAS*^{WT}, and TWT), as well as negatively regulating MSC populations.

This thesis presents valuable findings that:

a) challenge the choice of current chemotherapeutic treatments that antagonize and suppress TGFβ/Smad signalling pathway in patients with metastatic melanoma advocating for the development of chemotherapeutic or immunotherapeutic agents acting as TGFβ mimics or activators. TGFβ mimics would be beneficial to those patients, due to their potential ability to hinder primary tumour formation, prevent tumour metastasis to lymph nodes and distant organs as well as depleting MSC populations, thus resulting in improved relapse-free survival of melanoma patients.

b) highlight the value of TGFβ as a diagnostic and prognostic marker for melanoma patients with better responsiveness to chemotherapy and higher survival outcome.

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