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(Leiter: Prof. Dr. med Jochen Utikal)

Role of Id3 in melanoma dedifferentiation and resistance to targeted therapy

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Sachindra

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Dekan: Prof. Dr. med. Sergij Goerdt
Referent: Prof. Dr. Jochen Utikal

Dedicated to my parents and my family
for their constant love and support

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Sachindra, Lionel Larribere, Elias Orouji, Huizi Wu, Daniel Novak, Christoffer Gebhardt and Jochen Utikal

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Sachindra, Lionel Larribere, Elias Orouji, Huizi Wu, Daniel Novak, Christoffer Gebhardt and Jochen Utikal

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Abbreviations

^{o}C	Degree Celcius
18s	18s ribosomal RNA
ACT	Adoptive cell transfer therapy
ABCB5	ATP-binding cassette sub-family B member 5
BCA	Bichinonic Acid Protein Assay
BCL2	B-cell lymphoma 2
bHLH	Basic Helix Loop Helix
BME	Basement Membrane Extracts
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
BRAF	B-Raf Proto-Oncogene, Serine/Threonine kinase
POU3F2	Pou class 3 homebox 2/BRN2
bp	Base pair
Ctrl	Control
CDKI	Cyclin dependent inhibitor
CDK4	Cyclin-dependent kinase4
CDKN2A	Cyclin Dependent Kinase Inhibitor 2
cMYC	Proto-Oncogene C-Myc
CSCs	Cancer stem cells
CTC	Circulating tumor cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
Dab	Dabrafenib
DCT	Dopachrome Tautomerase
DNA	Deoxyribonuleic acid
DMSO	Dimethylsulfoxide
DMEM	Dulbecc’s modified eagle’s medium

DKFZ	Deutsches Krebsforschungszentrum
ECM	Extracellular matrix
EMT	Epithelial-to- Mesenchymal Transition
ERK	Extracellular Signal Regulated Kinase
EDN	Endothelin
ESCs	Embryonic stem cells
EGF	Epidermal Growth Factor
EV	Empty vector
FDA	Food and Drug Administration
FOXD3	Forkhead Box D1
GAPDH	Glyceraldehyde 3-phosphate dehydrogense
GPCRs	G-protein coupled receptors
hESCs	human Embryonic Stem Cells
hiPSCs	human induced Pluripotent stem cells
Id	Inhibitor of differentiation
IHC	Immunohistochemistry
IGF	Insulin-like growth factor receptor-1
IL	Interleukin
IF	Immunoflurescence
IFN-α	Interferon alpha
iPSCs	induced Pluripotent Stem Cells
KO	Knockout
LB	Lysogeny broth
LEF1	Lymphoid Enhancer Binding Factor 1
LIF	Leukaemia Inhibitory Factor
MDM2	mouse double minute 2
mRNA	Messenger Ribonuleic Acid

MITF	Microphthalmia-Associated Transcription Factor
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse embryo fibroblast
MEK	Mitogen Activated Protein Kinase Kinase
MSX	Msh Homeobox 1
NCCs	Neural Crest Cells
NC	Neural crest
NHM	Normal Human Melanocytes
NF1	Neurofibromin 1
NGFR	Nerve Growth Factor Receptor
NOD/SCID	Nonobese Diabetic/Severe Combined Immunodeficient
NRAS	Neuroblastoma RAS viral Oncogene Homolog
PAX3	Paired box gene 3
PCR	Polymerase Chain Reaction
PDGFRβ	platelet-derived growth factor receptor β
PBS	Phosphate buffered saline
PVDF	Polyvinylidenfluorid
PD-1	Programmed death 1
PFA	Paraformaldehyde
PMEL 17	Premelanosome protein/Melanosomal matrix protein 17
PTEN	Phosphatase and tensin homolog
PI	Propidium Iodide
PIP3	Phosphatidylinositol phosphate
PI3K	Phosphatidylinositol-4,5-bisphospahte 3-kinase
PDGF	Platelet-derived growth factor
PKA	Protein kinase A
qPCR	Quantitative real-time polymerase chain reaction

Rb	Retinoblastoma
RTKs	Receptor tyrosine Kinases
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNAIL	Sanil family transcription factor
SOB	Super optimal broth
SOX	SRY (sex determining region Y)-box
TCF4	Transcription Factor 4
TCGA	The Cancer Genome Atlas
Tra	Trametinib
TRP 1	Tyrosinase-related protein 1
TYR	Tyrosinase
TWIST1	Twist family bHLH transcription factor 1
TERT	Telomerase Reverse Transcriptase
TGF-β	Transforming growth Factor β
TSP-1	Thrombospondin-1
TMA	Tissue Microarray
TNF	Tumor Necrosis Factor
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
Vem	Vemurafenib
vs	Versus
WFDC1	WAP four-disulfide core domain 1
wnt5a	Wingless-type MMTV Integration site family, member 5A
WT	Wild Type

1. Introduction

1.1 Melanoma

Melanoma, also known as cutaneous melanoma or malignant melanoma is the malignancy of melanocytes. It is one of the most aggressive forms of skin cancer. It accounts for only 4% of all skin cancers but is responsible for more than 75% of deaths due to skin cancers **(Arrangoiz et al., 2016)**. A world-wide increase in melanoma incidence has been observed among the caucasian population. Australia has the maximum rate of incidence with 50-60 new cases per 100,000 populations, which is followed by United States of America (USA) and Europe with 20-30 and 10-25 new cases per 100,000 populations respectively **(Garbe et al., 2016)**.

1.1.1 Melanoma subtypes

Classically or histologically melanoma has been grouped into four subtypes**:**

Superficial spreading melanoma is the most common subtype and accounts for approximately 70% of all melanoma cases in the white population. It is irregular in shape and spread alongside epidermis. **Nodular melanoma** is another common subtype where the melanocytes grow vertically rather that spreading horizontally. The borders of nodular melanoma are more distinct compared to superficial melanoma. The third subtype of melanoma is **lentigo malignant melanoma,** which is frequently developed on sun-exposed part of skin like forearms, face and neck of elderly people. It can also invade through the dermis. **Acral lentiginous melanoma** is the fourth subtype of melanoma which is least common in Caucasian but usually found in people with other race for e.g. African-American or Asian. Mostly, it occurs in palm, sole or under nails **(Smoller, 2006).**

1.1.2 Melanoma and Risk factors

Transformation of melanocytes to melanoma could arise due to several factors like sun exposure, family history or immunosuppression. However, exposure to the sun or ultra violet (UV) radiation has been accounted as the major cause of melanoma **(Gandini et al., 2005)**.

UV exposure can cause increased production of growth factors and reactive oxygen species which further leads to DNA damage and genetic alterations **(Huang et al., 1996)**. However, melanin, pigments present in human skin, absorbs and dissipates UV light and protects the skin from any DNA damage. Melanin is produced by melanocytes and transported to the keratinocytes so that they are similarly distributed in the skin **(Brenner and Hearing, 2008)**.

People with a large number of congenital melanocytic nevi are at higher risk of developing melanoma. The reports also suggest that chances of developing melanoma from congenital melanocytic nevi are between 20-30% (Ribeiro et al., 2016). Although melanoma can arise from the pre-existing nevus, approximately 75% of the melanoma occurs de novo. Nonetheless, these nevi can be surgically removed to reduce the risk.

Melanoma is an immunogenic tumour and some studies have found that the risk of developing melanoma increases by 20-60 times in immunosuppressed people than normal population **(Moloney et al., 2006)**. It is more common in immunocompromised people with solid organ transplant recipients (OTRs), patients with human immunodeficiency virus (HIV) infection/AIDS. **(Kubicaa and Brewer, 2012)**.

Another strong risk factor for melanoma is the family history. Around 10% of the melanoma patients have some close family members already diagnosed with melanoma. To date, two genes, cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase 4 (CDK4) are implicated in familial melanoma. Besides, CDKN2A is found to be mutated in 20-40% of members of familial melanoma **(Read et al., 2016)**.

1.2 Melanocyte to melanoma

1.2.1 Melanocyte development

Melanoma cells exploit the normal developmental process of melanocyte for its progression. But the exact mechanism behind this is not clearly understood. In the

process to illuminate this association, it is important to understand the developmental process of melanocytes.

Melanocytes are a group of special heterogeneous cells which originates from embryonic neural crest (NC) cells. Development of melanocytes encompasses several complex phases. It starts with lineage specification from neural crest cells and forms melanoblast precursors. Melanoblasts then mature, proliferate and migrate to the epidermis. Here they are incorporated into hair follicles and further differentiates to melanocytes. Eventually, melanocytes produce melanin in special organelles known as melanosomes **(O'Rahilly and Müller, 2007) (Ernfors, 2010)**. In human, melanosomes are transported to keratinocytes as a defensive measure to protect from DNA damage of skin due to sun or UV exposure. Melanin absorbs and dissipates UV light energy **(Gilchrest and Eller, 1999)**.

1.2.1.1 Pathways and genetics of melanocyte development

As I mentioned earlier that melanocytes are differentiated from highly migratory neural crest cells and during this differentiation several signalling pathways are activated. For example, BMP signalling is important for the early neural crest induction. Kanzler et al in 2000 demonstrated that knocking down BMP2 in mouse leads to the loss of neural crest derivatives. Later, the role of snail/slug family is crucial as it helps during the epithelial to mesenchymal transition (EMT) of neural crest progenitor cells. Snail/slug directly represses E-cadherin and blocks cell adhesion which ultimately allows cell migration **(Cano et al., 2000)**. Multiple roles of Notch family have been recognised during neural crest cell differentiation. In mouse, loss or gain of notch signalling could affect the neural crest cell migration, proliferation, differentiation and fate decision of early progenitors **(Mead and Yutzey, 2012)**. Another study found that notch signalling plays the determining role in melanoblasts and adult melanocyte stem cells survival **(Moriyama et al., 2006)**. Melanoblast formation is one more essential stage during melanocyte development. Study using Wnt-1 and Wnt-3a knock-out mouse suggested reduction in neural crest derivative thus deciding the fate of melanocyte maturation **(Ikeya et al., 1997).** Later, a fate mapping study showed that Wnt signalling is also critical in melanocyte formation by blocking glial and neuronal fates **(Dorsky et al., 1998).** Finally, **Dunn et**

al in 2000 illustrated that Wnt signalling is necessary for melanocyte growth and differentiation.

These signalling pathways also control several transcription factors and their downstream genes during melanocyte development from neural crest cells. The key transcription factors involved in this process are FOXD3, SOX9, SOX10, PAX3 and MITF. Activation of precise transcription factor decides the fate of the melanoblast precursor cells if it will differentiate to melanocytes or glial cells.

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix transcription factor that is considered as the master regulator of melanocyte development. It regulates three major genes (TYR, TYRP1 and DCT) which are responsible for pigmentation in melanocytes **(Levy et al., 2006)**. Mice with MITF mutations cannot form melanocytes and retinal pigment epithelial (RPE) cells couldn't develop properly **(Steingrimsson et al., 2004)**. MITF helps in the survival of melanocytes by directly regulating the key antiapoptotic gene BCL2 **(McGill et al., 2002)**. However, the expression of MITF is regulated by an array of upstream genes and these genes also play a central role in neural crest development and differentiation. It is, therefore important to highlight the role of these genes during several steps of melanocyte development. The main transcription factors which regulate MITF expression in melanocyte development are SOX10 and PAX3. In both human and mouse, a mutation in SOX10 or PAX3 gene results in Waardenburg syndrome, which is similar to the effect of mutation in MITF **(Tachibana et al., 2003)**. Mutation in these genes can lead to the abnormal melanocyte development which could alter the melanin production. In addition to this SOX10 and PAX3 work synergistically to activate MITF expression since SOX10 and PAX3 binding sites are next to each other on MITF promoter **(Potterf et al., 2000)**. Taken together these results suggest that SOX10, PAX3 and MITF are the key genes required for the melanocyte differentiation and melanin synthesis. But there are other transcription factors which are active in very early stage of embryonic development and define the fate of melanocyte development.

FOXD3 is an important transcription factor which is expressed in migrating neural crest cells that differentiate into glia and neurons but not detected in later migrating neural crest cells which give rise to melanocytes. **Kos et al in 2001** using avian

neural crest cells discovered two important roles of FOXD3 in neural crest development. FOXD3 expression was found in pre-migratory neural crest cells but was downregulated in pre-migratory melanoblast lineage. This suggests that FOXD3 assists in the segregation of the neural crest lineage from the neural epithelium. Also, knocking down FOXD3 *in vivo* led to an increase in neural crest cells differentiating into melanocyte whereas the overexpression of FOXD3 suppresses melanocyte formation suggesting the role of FOXD3 in suppressing melanogenesis. FOXD3 represses melanogenesis by inhibiting binding of PAX3 to the MITF promoter **(Thomas and Erickson, 2009)**. In another study, it was shown that Snail 2, SOX9 and FOXD3 are expressed in the dorsal neural tube suppressing melanogenesis. In later stages, the expression of these genes is lost and progress towards melanocyte differentiation **(Nitzan et al., 2013)**.

Another decisive gene which is expressed during neural crest development is SOX2. Its expression was not found in pre-migratory and migratory neural crest cells and misexpression of this gene lead to the inhibition of neural crest formation **(Wakamatsu et al., 2004)**. Therefore, the expression of FOXD3 and SOX2 decides the fate of neural crest cells if they will differentiate into melanoblast lineage or glial cells.

Several studies have discovered the role of other genes in neural crest development. Some of the important genes are MSX, cMYC, Id3, TWIST and endothelins **(Baynash et al., 1994) (Steventon et al., 2005)**.

1.2.2 Pathways and genetics of melanoma development

The mechanism behind the transformation of melanocyte to melanoma is still not clearly understood. Even though, a large body of evidence suggest the role of mutations acquired by several genes in melanoma progression. **Miller and Mihm in 2006** proposed a model explaining the different events and molecular changes during melanoma progression. The model depicted that the mutations are acquired as early as in benign nevi. During the course of transformation, more mutation burden helps in the metastasis of the melanoma cells **(Figure 1)**. Mutation in these genes alters key signalling pathway which leads to melanoma progression and

metastasis. Recently melanoma has been majorly grouped into four categories based on their mutational status- BRAF mutant, NRAS mutant, NF-1 mutant and Triple wild type **(Akbani et al., 2015)**.

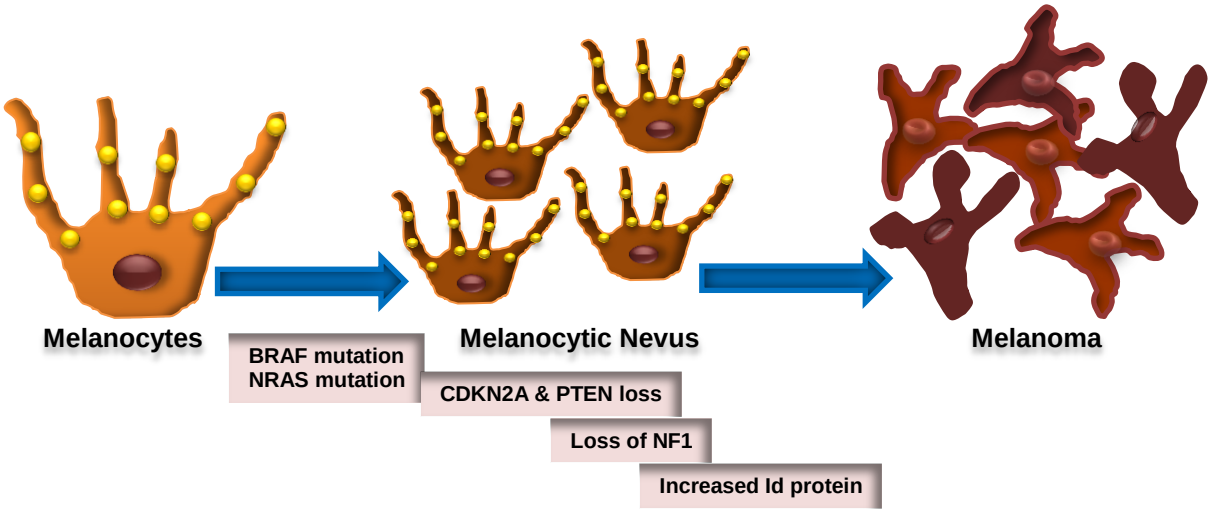


Figure 1: Melanocyte to melanoma development. BRAF is an early event during melanoma development which is followed by other mutations like loss of tumour suppressor genes like CDKN2A, PTEN and NF1. Increased in Id protein expression is also found as early as during nevi formation.

1.2.2.1 MAPK pathway

Mitogen-activated protein kinases (MAPK) pathway is an intracellular signal transduction pathway which regulates cell survival, differentiation, migration and proliferation **(Torii et al., 2006) (Dhillon et al., 2007)**. This signalling pathway can be activated via various extracellular stimuli like receptor tyrosine kinase (RTK), G-protein coupled receptors (GPCRs), mitogens and integrins **(Zhang and Liu, 2002)**. Upon activation, the downstream protein i.e. RAS, RAF, MEK1/2 and ERK1/2 are activated and regulates the above described cellular processes **(Kim and Choi, 2010)**. Mutation in any of these genes could activate the MAPK pathway and lead to uncontrolled cell growth and proliferation.

BRAF is one of the most mutated genes in this pathway. Several studies have found that approximately 40-60% of melanoma patients carry a mutation in BRAF **(Davies et al., 2002) (Akbani et al., 2015)**. It is well documented that 90% of BRAF mutation

have V600E mutation where Valine (V) is substituted by Glutamate (E) at codon 600 **(Tan et al., 2008)**. Since the mutation occurs in the kinase domain of the BRAF it constitutively activates BRAF which further lads to the activation MEK/ERK pathway. Interestingly it has been reported that BRAF mutation is more common in melanomas arising on the skin which is intermittently exposed to the sun compared to sun damaged skin **(Maldonado et al., 2003)**. Conversely, data suggests that BRAF mutation is an early event and has been found to be mutated in melanocytic nevi (82%) as well **(Pollock et al., 2003)**. Principally BRAF activates downstream MEK-ERK pathway which further leads to unchecked proliferation and growth. Through MEK-dependent activation of HIF-1α and VEGF, it also promotes metastasis and angiogenesis **(Maurer et al., 2011)**. It is also important to note that BRAF mutation alone is not able to transform the melanocyte to melanoma. For example, BRAF mutation alone in Zebra fish gave rise to nevi but not melanoma. More interestingly, Zebra fish with BRAF and additional mutation for P53 generated nevi and later formed invasive melanoma **(Patton et al., 2005)**.

Another key gene which is found to be mutated in melanoma patients is NRAS. In 1984 RAS activation was first identified in human melanoma cell lines as well in a short-term cell culture from a melanoma patient **(Padua et al., 1984) (Albino et al., 1984)**. Approximately 20% of melanoma patients carry a mutation in NRAS gene **(Jakob et al., 2012)**. The most frequent mutations found in this gene are Q61R and Q61K. Moreover, it has been found that patient with NRAS mutations are older and have chronic sun damaged skin. The tumour thickness in NRAS mutated melanoma patients has a greater Breslow index **(Devitt et al., 2011)**. The report also suggests that BRAF and NRAS mutations are mutually exclusive and less than 1% of patients carry a mutation in both genes at the same time **(Goel et al., 2006)**.

Neurofibromin 1(NF1) is a tumour suppressor gene which is significantly mutated in the MAPK pathway. Approximately 15% of melanoma patients have inactivation or loss of NF1 gene. NF1 negatively regulates RAS signalling by hydrolysing RAS-GTP (active form) to RAS- GDP (inactive form) **(Rajkumar and Watson., 2016)**. Loss or inactivation of NF1 results in unchecked RAS activation which further activates MAPK pathway. Similar to NRAS mutated melanoma patients, NF1 mutations have

been reported in older patients with chronic sun damaged skin **(Krauthammer et al., 2012) (Mar et al., 2013)**.

1.2.2.2 p53 Pathway

p53 is another tumour suppressor gene which has been reported to be mutated in several cancer types including melanoma (approximately19%) **(Hodis et al., 2012)**. In melanoma, mutations in several genes have been implicated to circumvent P53 mediated tumour suppression. CDKN2A is a well-known tumour suppressor gene which is frequently mutated in high-risk melanoma family **(Aitken et al., 1999)**. Loss of CDKN2A is significant for melanoma progression because it encodes two tumour suppressor proteins through alternative splicing i.e. p16INK4a and p14ARF. p16INK4a is a cyclin-dependent kinase inhibitor which activates retinoblastoma (RB) by blocking the CDK4. On the other hand, p14ARF regulates the level of p53 protein via mouse double minute 2 (MDM2). MDM2 is responsible for the degradation of p53 through ubiquitination but it is negatively regulated by p14ARF. Subsequently, it allows p53 to arrest the cell cycle in G2-M phase to either repair the damaged DNA or initiation of apoptosis **(Harris and Levine, 2005)**. It was also shown in vivo that loss of p16INK4a and p14ARF reduces the time to develop melanoma on exposure to UV light **(Recio et al., 2002)**. These data also clarifies that the low rate of p53 mutations in melanoma is because of loss of p14ARF.

1.2.2.3 PI3K/AKT pathway

The PI3K/AKT signalling pathway is another significant pathway for cellular proliferation, survival and migration. This pathway is more often activated by various cancer types including melanoma **(Cantley, 2002) (Davies, 2012)**. More often activation of PI3K/AKT pathway in melanoma is either due to mutation in PI3K and AKT or deletion of PTEN locus on chromosome 10 **(Davies, 2012)**. It is important to note that PI3K can also be activated by activated RAS proteins **(Mendoza et al., 2011)**. PTEN is a potent tumour suppressor which keeps in check the PI3K/AKT pathway by keeping the phosphatidylinositol phosphate (PIP_3) level low. In melanoma, loss of PTEN (20-30%) allows the accumulation of PIP_3 which further activates AKT. Phosphorylated AKT results in inactivation of Bcl-2-antagonist-of-cell-death as a result tumour cell escapes apoptosis. Two another crucial protein in this

pathway is mTOR and GSK3b which is found to be activated in metastatic melanoma and results in tumour growth **(Karbowniczek et al., 2008) (McCubrey et al., 2014)** Several in vivo and in vitro studies have shown that loss of PTEN and activation of AKT promotes growth and survival of melanoma cells **(Wu et al., 2003) (Cantley and Neel, 1999)**

1.2.2.4 Wnt signalling pathway

The Wnt signalling pathway is crucial during embryonic development and in 30% of melanoma constitutive activation of Wnt signalling has been found. However, its role in melanoma is still not clearly understood. β-catenin is the key member of Wnt signalling canonical pathway and regulates cell-cell adhesion. Interestingly 23% of human melanoma cell lines show a mutation in β-catenin. In a study researcher used a mouse model carrying PTEN loss and BRAF-V600E mutation, which can develop the tumour in 100% of the mouse within 1 month, showed that loss of β-catenin can lead to inhibition of melanoma formation **(Damsky et al., 2011).** Recently in another study, it was found that β-catenin activation promotes lung metastasis in NRAS-mouse **(Gallagher et al., 2013)**. On the contrary, expression analysis of β-catenin using immunostaining showed that only 38% of metastatic melanoma were positive compared to melanocytic nevi (95%), radial (94%) and Vertical (65%) growth phase primary melanoma **(Kageshita et al., 2001)**. These data indicates that β-catenin could be more involved during the early phase of melanoma progression.

Similarly, in other study using mouse melanoma cell line, it was shown that Wnt3a expression is responsible for differentiation in melanoma cells and it displays slow proliferation and reduced migration. However, expression of Wnt5a in the same cell line antagonises the gene expression activated by Wnt/β-catenin signalling **(Chien et al., 2009)**. Wnt5a inhibits the canonical pathway by binding to the Fzd receptor. Wnt5a binds to Frizzled2 (Fzd2) and prevents the phosphorylation of lipoprotein receptor-related protein 6 (LRP6) by Wnt3a, therefore reducing the accumulation of β-catenin **(Sato et al., 2010).** Additionally, inhibition of β-catenin by Wnt5a results in suppression of LEF1 and upregulation of TCF4 which generates more invasive phenotype **(Eichhoff et al., 2011)**. These data suggests the effect of Wnt signalling pathway is more dependent on cellular context and more investigation is needed in this area.

1.2.2.5 Tgf-β pathway

Transforming growth factor-beta (Tgf-β) pathway is activated during various cellular processes like proliferation, differentiation, cell survival, extracellular microenvironment modification, migration. It has been well established that Tgf-β initially works as a tumour suppressor and later helps in tumour progression. In normal melanocytes only Tgf-β1 is expressed whereas in melanoma cells all 3 isoforms of Tgf-β (Tgf-β1, Tgf-β2 and Tgf-β3) are secreted **(Javelaud et al., 2008)**. As a tumour suppressor, Tgf-β checks proliferation in cells by activating cyclin-dependent inhibitor (CDKI) (p21, p15, $p27^{Kip1}$ and $p57^{Kip2}$), c-MYC and inhibitor of differentiation (Id). Experimental data shows that Tgf-β reduces melanocyte proliferation by arresting the cell cycle in G1 phase and stops differentiation by downregulating PAX3 which along with SOX10 controls melanocyte differentiation **(Yang et al., 2008)**. However, during the later stage of tumour progression, Tgf-β promotes metastasis by inducing epithelial to mesenchymal transition (EMT) and also helps tumour cell escaping immune surveillance **(Perrot et al., 2013)**. A recent finding from our laboratory shows that Tgf-β1 induces SOX2 expression to promote melanoma invasion in vitro **(Weina et al., 2016).**

1.2.2.6 TERT promoter mutation

Two independent studies in 2013 discovered that telomerase reverse transcriptase (TERT) is mutated in familial and sporadic melanoma patients. Whole genome sequencing data found two mutations in the promoter region of the TERT gene. This mutation in the promoter sequence lead to the creation of new binding motifs for the Ets- transcription factor family and increases its transcriptional activity by 2-4 folds. In human metastatic melanoma cell lines, 125 out of 168 (74%) were found with somatic mutation in TERT promoter **(Horn et al., 2013) (Huang et al., 2013)**. Horn et al did not report any TERT promoter mutation but a recent report shows low frequency mutation in benign nevi **(Horn et al., 2013) (Shain et al., 2015)**. More studies with larger data set are required to investigate the role of TERT mutation in melanoma progression.

1.3 Melanoma Treatment

Due to the heterogeneity of melanoma tumour, treatment of melanoma patient depends on several factors like stage and mutations. If detected in primary stage excision is still the first line of treatment for melanoma. Depending upon the tumour thickness and lymph node biopsy the further direction of treatment is decided. Before the arrival of 2nd generation drugs for melanoma treatment, the most commonly used cytotoxic chemotherapy was dacarbazine and immunotherapies like interleukin-2 (IL-2). These treatments can be used either as monotherapy or in combination **(Bhatia et al., 2009)**. Currently, for the treatment of metastatic melanoma, several lines of therapy can be followed and this can be grouped into chemotherapy, immunotherapy and targeted therapy.

1.3.1 Targeted therapy

A number of melanoma patients have deregulated MAPK pathway which is due to mutations in either BRAF or NRAS gene. In 2011 the first BRAF inhibitor, Vemurafenib (PLX4032), was approved by FDA for the treatment of late-stage melanoma with BRAF (V600E) mutations **(Bollag et al., 2012)**. The clinical trial data showed improved rates of overall and progression-free survival in patients treated with vemurafenib compared to patients treated with dacarbazine. Also, it was observed that patient-group treated with vemurafenib compared to dacarbazine displayed a relative reduction of 63% in the risk of death and of 74% in the risk of either death or melanoma progression (**Chapman et al., 2011)**. In 2012, another BRAF inhibitor, dabrafenib (GSK2118436), was reported to show significantly better progression-free survival compared to dacarbazine in melanoma patients with BRAF (V600E) mutations **(Hauschild et al., 2012).** Later, in the year 2013 FDA approved the use of dabrafenib for the treatment of melanoma carrying BRAF (V600E) mutation. Additionally, dabrafenib displayed similar treatment benefits on melanoma patients with the mutation on BRAF (V600K/D/R) **(Gentilcore et al., 2013) (Ascierto et al., 2013)**. However, after 6-7 months most of the patients receiving vemurafenib or dabrafenib show relapse of the tumour and develop resistance to the treatment. In another strategy to stop the activation of MAPK pathway in melanoma patients, new small inhibitors were examined to block MEK1/2 proteins. MEK inhibitors were supposed to block the MAPK pathway in melanoma carrying either NRAS or BRAF

mutation. Trametinib (GSK1120212) and Cobimetinib (GDC-0973, XL-518) are the MEK-inhibitors approved by FDA in 2013 and 2014 respectively for the treatment of melanoma patient with BRAF mutation. Trametinib functions by binding to MEK1 and MEK2 whereas cobimetinib blocks MEK1 resulting in inhibition of tumour cell growth. Later on, combination therapy of trametinib and dabrafenib was approved by FDA for the treatment of the patient with BRAF V600E/K mutation. Whereas, cobimetinib was approved to be used in combination with vemurafenib. Even though the effect of the targeted therapy was evident but adaptive resistance to these therapies limits the efficacy of the treatment.

In some cases like patients carrying a C-KIT mutation in melanoma (acral melanoma, mucosal or in chronic sunburn areas), imatinib and nilotinib can be used for the treatment **(Murer C et al., 2017)**.

1.3.2 Immunotherapy

Melanoma is one of the most immunogenic tumours and therefore immunotherapy can be used to boost the immune system of melanoma patients to attack the tumour cells. Interferon alpha (IFN-α) and IL-2 were the first immunotherapy approved for the treatment of patients with metastatic melanoma **(Bhatia et al., 2009)**. Clinical trial study with 270 metastatic melanoma patients showed that high dose of bolus IL-2 (HD IL-2) results in 16% overall objective response rate (6% complete response and 10% partial response) **(Atkins et al., 1999)**. IFN-α showed an overall response rate of 22% in phase II trial with metastatic melanoma patients **(Creagan et al., 1986)**. However, the major drawback of this approach was the heavy side effects and many patients were not able to take the high dose.

In last decade, a better understanding of tumour immunology led to the discovery of new insights for targeting the tumour cell using the immune system. An example of two such approaches is targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1). CTLA-4 is a protein receptor expressed by T-regulatory cells on their surface that inhibits T-cells and immune response. Dendritic cells present antigen produced by cancer cells to cytotoxic T-lymphocytes (CTLs) and then CTLs attack the cancer cells.

Ipilimumab is the human monoclonal antibody which binds to CTLA-4 and blocks its inhibitory action. In a randomised, double-blind, phase III trial, 676 stage III-IV melanoma patients were registered based on HLA-A*0201 positive status. These patients were assigned into 3 groups and treated with ipilimumab plus glycoprotein 100 (gp100), ipilimumab only and gp100 only. In this study, it was found that patient treated with or without gp100 showed improved overall survival compared to the patient treated with only gp100 **(Hodi et al., 2010)**. In another phase III trial with 502 patients, similar results were obtained. Patients were equally divided into two groups and either treated with ipilimumab plus dacarbazine or dacarbazine plus placebo. It was shown that the overall survival after one year in the patient group treated with ipilimumab plus dacarbazine were significantly higher (11.2 months) compared to (9.2 months) in the dacarbazine plus placebo. This study brought ipilimumab as the first line of treatment for metastatic melanoma **(Robert et al., 2011)**

PD-1 is another receptor which is expressed in activated T cells and when its ligand PDL-1 and PDL-2 binds to this receptor it promotes self-tolerance by reducing auto-immunity. In 2014 FDA approved two drugs, Nivolumab and Pembrolizumab, for metastatic melanoma that targets PD-1 receptor and augments the immune system to kill tumour cells. In a study involving 296 patients with advanced melanoma (104), non-small-cell lung carcinoma (122), castration-resistant prostate cancer (17), or renal-cell (34) or colorectal cancer (19) were given nivolumab (BMS-936558). In a cohort of melanoma patients, 26 objective responses were detected at doses ranging from 0.1 to 10.0 mg per kilogram, with response rates stretching from 19% to 41% per dose level **(Topalian et al., 2012)**. In another phase III clinical trial with 834 patients with metastatic melanoma, it was observed that patients treated with pembrolizumab showed higher progression-free survival and overall survival compared to ipilimumab. High-grade toxicity in pembrolizumab treated patient was not as much of ipilimumab treated patients (**Robert et al., 2015)**.

1.4 Drug resistance in melanoma

In recent years due to the approval of new generation drugs against metastatic melanoma, the overall survival has improved but resistance against these drugs limit

the therapeutic advantage. Data from several studies have revealed that majority of the resistance cases are associated with targeted therapy especially BRAF inhibitors but recent data also highlights resistance to the current immunotherapies. Furthermore, it was found that this resistance can be either intrinsic or adaptive. Patients with intrinsic resistance do not show any response to the therapy whereas adaptive resistant patients show early response to the treatment but later they show no effect of the therapy. This was elucidated by whole genome sequencing of BRAF positive melanoma patients before treatment and after developing resistant. Interestingly, it was found that one-fifth of melanoma patients with BRAF mutation does not respond to the therapy. Varied resistance mechanisms cultivate during target therapy but mostly involve the reactivation of MAPK pathway **(Van Allen et al., 2014)**. Earlier it was shown that the resistance to BRAF inhibitor (SB590885) was gained through overexpression of cyclin D1 and enhanced by high levels of CDK4 expression **(Smalley et al., 2008)**. In addition, loss of NF1 and increased expression of protein kinase D3 (PRKD3) has been connected to the regulation of MAPK pathway and melanoma resistance **(Whittaker et al., 2013) (Chen et al., 2011)**.

A large number of melanoma patients display significant response to the BRAF or MEK inhibitor initially but develops resistance later; this mechanism is described as adaptive resistance. Recent investigations suggest that the reactivation of ERK signalling is one of the main reasons behind the resistance in these melanoma patients. It is interesting to note that there was no secondary gatekeeper mutation found in BRAF inhibitor resistant patients but additional mutation on NRAS was identified **(Nazarian et al., 2010) (Tap et al., 2010)**. However, several studies have reported the amplification of mutant BRAF as one of the mechanisms for BRAF and MEK inhibitor resistance **(Corcoran et al., 2010) (Villanueva et al., 2013)**. Besides, it was found by random mutagenesis and biochemical studies that mutation in CRAF activates MAPK pathway which further leads to resistance **(Antony et al., 2013)**. In another study, COT kinase was identified activating ERK through MAPK and linked to resistance in BRAF and MEK inhibitor resistance **(Johannessen et al., 2010)**. COT kinase is encoded by MAP3K8 and it is overexpressed after treatment with BRAF or MEK inhibitor thus conferring resistance.

Numerous studies have indicated that additional pathways other than ERK1/2 pathway are activated in BRAF inhibitor resistant melanoma cell lines. In BRAF resistant patients platelet-derived growth factor receptor β (PDGFRβ) receptor tyrosine kinase is upregulated. Additionally, the BRAF inhibitor resistant cell lines showed inactive ERK pathway but they were dependent on PDGFRβ for growth and survival **(Nazarian et al., 2010)**. Recently another group showed the role of PDGFRβ and EGFR in adaptive resistance to BRAF inhibitor. EGFR expression was upregulated in 6 out of 16 melanoma tumours after the development of resistance to BRAF or MEK inhibitor. Also, they found that upon silencing SOX10 expression leads to activation of Tgf-β signalling which further leads to increased expression of EGFR and PDGFRβ **(Sun et al., 2014)**. One study gives experimental evidence that AXL receptor tyrosine kinase is also expressed in resistant melanoma tumours besides EGFR and PDGFRβ. The ratio between MITF and AXL ($MITF^{low}/AXL^{high)}$ expression is crucial in certain BRAF and NRAS mutated melanoma which drives the resistance mechanisms **(Müller et al., 2014)**. Another RTK, for example, insulin-like growth factor receptor-1 (IGF-1R), was identified which is constitutively active in BRAF resistant cells. Elevated IGF-1R expression in BRAF resistant melanoma cells was correlated with PI3K/AKT pathway (**Villanueva et al., 2010)**. While immunotherapy like anti-PD-1 exhibited remarkable success in terms of overall survival and efficacy, it has been also indicated that approximately 60% patients show primary resistance whereas about 25% patients tend to develop resistance subsequently **(Topalian et al., 2012) (Zaretsky et al., 2016)**. Lately, it has been found that clonal selection of tumours for JAK1 and JAK2 mutations and loss of IFN-γ signalling pathways or loss of B2M gene (decreased antigen presentation) is a possible mechanism for the development of resistance in patients with anti-PD-1 therapy **(Zaretsky et al., 2016)**. In order to circumvent these resistances in melanoma patients, several combinational therapies have been tested and approved recently.

1.4.1 Melanoma dedifferentiation

Melanoma is the malignant transformation of melanocytes and melanocytes are differentiated from nReural crest cells. The neural crest cells are transient

multipotent migratory cells. Based on several studies, it is evident that melanoma is comprised of a highly heterogeneous population of cells. Within this heterogeneous population of cells, there is a sub-population which dedifferentiates and recapitulates some of the neural crest cells phenotype. Those cells demonstrate phenotype like self-renewal, low proliferation rate, and highly resistance to therapies **(Boiko et al., 2010) (Larribere and Utikal, 2014)**. The dedifferentiation theory was also supported by **Landsberg et al in 2012**, where they have demonstrated that the pro-inflammatory cytokine, TNF-α, can lead to dedifferentiation in mouse and human melanoma cells. Here they also showed that after adoptive cell transfer therapy (ACT) the relapse of tumour in metastatic melanoma patients after significant regression was due to the gain of gene expression related to neural crest cells like P75 (also known as CD271 or NGFR) (**Landsberg et al., 2012**). A large body of work has deciphered the expression and role of numerous other genes associated with dedifferentiation during melanoma progression.

1.4.1.1 CD271 (P75/NGFR)

CD271 is a low-affinity transmembrane protein and a member of Tumor Necrosis Factor Receptor (TNFR) superfamily **(Rogers et al., 2008)**. During development, CD271 is expressed in neural crest stem cells also expressed in various adult tissues and tumours originating from neural crest or ectoderm. As reported in a study where they used CD271 as melanoma stem cell marker for identification and isolation. CD271+ and CD271− cells when injected in mouse, only CD271+ cells metastasized to liver and lung. Furthermore, the experimental data indicated that CD271+ cells either completely or partially lacked melanocytic marker like Tyr, MART-1 and MAGE **(Boiko et al., 2010)**. In accordance with previous data, **Civenni et al in 2011** confirmed that CD271+ cells are more tumorigenic compared to CD271− as well they show tumour heterogeneity similar to the parental tumour. Together, in vitro and in vivo results suggested that CD271+ cells have higher self-renewal capacity with longer propagation compared to CD271− cells. Additionally, CD271+ melanoma cell population expresses low MITF and migrate faster than CD271− cells **(Cheli et al., 2014)**. Recently it has been shown that the expression of

CD271 in melanoma cells promotes metastasis and drug resistance in a p53-dependent manner especially in BRAF wild-type cell lines **(Redmer et al., 2017)**.

1.4.1.2 ABCB5 (ATP-binding cassette subfamily B member 5)

ABCB5 is a member of ATP-binding cassette (ABC) transporter superfamily and involved in the transportation of a variety of compounds like sugar, peptides and small ions in the cell **(Chen et al., 2005)**. ABCB5 was shown to be expressed in a subset of melanoma cells which were chemoresistant and demonstrated stem cell like phenotype **(Frank et al., 2005)**. Through immunostaining, it was determined that the expression of ABCB5 was significantly higher in primary and metastatic melanoma compared to the nevus. Furthermore, ABCB5+ population showed sustained tumour growth than ABCB5− cell population and also reinstate the tumour heterogeneity. In the same study, in vivo genetic lineage tracking confirmed the role of ABCB5 in self-renewal and differentiation. (**Schatton et al 2008)**. Interestingly, the circulating tumour cells (CTC) exhibited a significantly higher frequency of ABCB5+ cells (33.4%) compared to the primary and metastatic tumours. Similar to previous findings, ABCB5+ CTC were more tumorigenic and promote metastasis **(Ma et al., 2010)**. Recently, the same group has reported the role of ABCB5 in melanoma growth and multi-drug resistance. In addition, ABCB5 promotes tumour growth by induction of IL8 through Wnt signalling pathway and also represses tumour suppressor WFDC1. At last ABCB5 was implicated in tumour growth, resistance, quiescence and melanoma-initiating cells renewal **(Wilson et al., 2014)**.

1.4.1.3 SOX2

SOX2 (sex-determining region Y (SRY) Box2) is a transcription factor which is expressed in neural crest stem cells. Several studies have established the role of SOX2 in melanoma progression. The SOX2 expression has been correlated with tumour thickness and bad prognosis **(Laga et al., 2010)**. In vitro and in vivo data suggests the role of SOX2 in cell proliferation and apoptosis. Knocking down SOX2 using shRNA resulted in smaller melanoma initiating cell spheres, whereas the

SOX2 overexpression leads to higher and bigger melanoma- initiating cells spheres **(Santini et al., 2014)**. Next, they showed that HH-GLI (HEDGEHOG-GLI) signalling pathway directly regulates SOX2 expression by binding of GLI1 and GLI2 on SOX2 promoter and induces stemness in melanoma cells. Moreover, the SOX2 expression has also been correlated with drug resistance in several cancer types **(Weina and Utikal, 2014) (Weina et al., 2016)**.

1.4.1.4 CD133

CD133 is found to be expressed in neural stem cells, cancer stem cells in brain tumour and hematopoietic stem cells **(Sanai et al., 2005) (Singh et al., 2004) (Horn et al., 1999)**. Immunostaining has revealed that CD133 expression is significantly higher in primary (39.4%) and metastatic melanoma (46.4%) compared to nevi (16.9%) **(Klein et al., 2007)**. Another group reported that less than 1% of melanoma specimens comprise CD133+ cells. More importantly, only CD133+ cells form visible tumour compared to CD133− when injected into an NOD-SCID mouse. WM115 is highly tumorigenic human melanoma cell line and almost 95% of WM115 cells express CD133 along with ABCG2. Although the expression level of these markers reduces in xenograft tumours which again increases when cultured in vitro. But interestingly WM115 cell line expresses lymphoangiogenic and angiogenic factors like VEGFR-3, LYVE-1, prox1 and podoplanin which promotes metastasis **(Monzani et al., 2007)**. Altogether these findings recommend that CD133 is essential for the maintenance and promotion of melanoma-initiating cells.

1.5 Id protein

Inhibitor of differentiation (Id) protein belongs to the helix-loop-helix (HLH) superfamily. It was first discovered in 1990 by Benezra and his colleague **(Benezra et al., 1990)**. In vertebrates four members of this family i.e. Id1, Id2, Id3 and Id4 have been identified. The members of Id family are found on different chromosomes- Id1-20q11, Id2-2p25, Id3-1p36.1 and Id4-6p21-p22; therefore, they are not isoforms rather different proteins **(Benezra et al., 1990) (Roschger and Cabrele, 2017)**. All

four members are well conserved and found in organisms ranging from drosophila to human. Although, Id protein shares the HLH domain but outside this domain, they display broad differences in their sequences **(Langlands et al., 1997)**. More importantly, Id protein cannot bind to the DNA due to lack of basic domain but they can form heterodimers with other bHLH (basic helix-loop-helix) proteins. The binding of Id protein to bHLH transcription factor leads to inhibition of its binding to the DNA. For example, E protein family (E12, E47) are the class I bHLH transcription factor which when sequesters with Id proteins cannot bind to the DNA and negatively regulates the gene expression. Therefore, the crucial biological significance, common to all four Id proteins is blocking the activity of bHLH in a tissue-specific manner **(Deed et al., 1996) (Massari and Murre, 2000)**.

1.5.1 Id protein structure and localisation

As mentioned earlier and showed in the Figure 2 , the HLH domain is highly conserved in all Id protein but the N-terminal and C-terminal of Id protein vary from each other and also comprises of crucial regulatory signals like ubiquitination, phosphorylation site etc. In a study, it was well demonstrated through mutagenesis that protein kinase A (PKA) phosphorylates Id1 at ser-5 and also stops its nuclear export **(Nishiyama K et al., 2007)**. Whereas in another study it has been shown that Cdk2 phosphorylates Id2 and Id3 at ser-5 **(Deed et al., 1997) (Hara et al., 1997)**. **Bounpheng et al in 1999** showed that Id proteins have short half-life nearly 20 min. By inhibiting the 26S proteasome it was found that Id1, Id2 and Id3 proteins are degraded via the ubiquitin-proteasome pathway. In addition, it was reported that the co-expression of E47 and Id3 protects Id3 from degradation and increases its half-life **(Deed et al., 1996)**. It was observed that Id4 degradation was not established via 26S proteasome but through E1 enzyme. **(Bounpheng et al., 1999)**. Interestingly, the Id1, Id2 and Id3 are protected from degradation by the deubiquitinase USP1 to preserve their stemness in mesenchymal stem cells USP1 **(Williams et al., 2011)**.

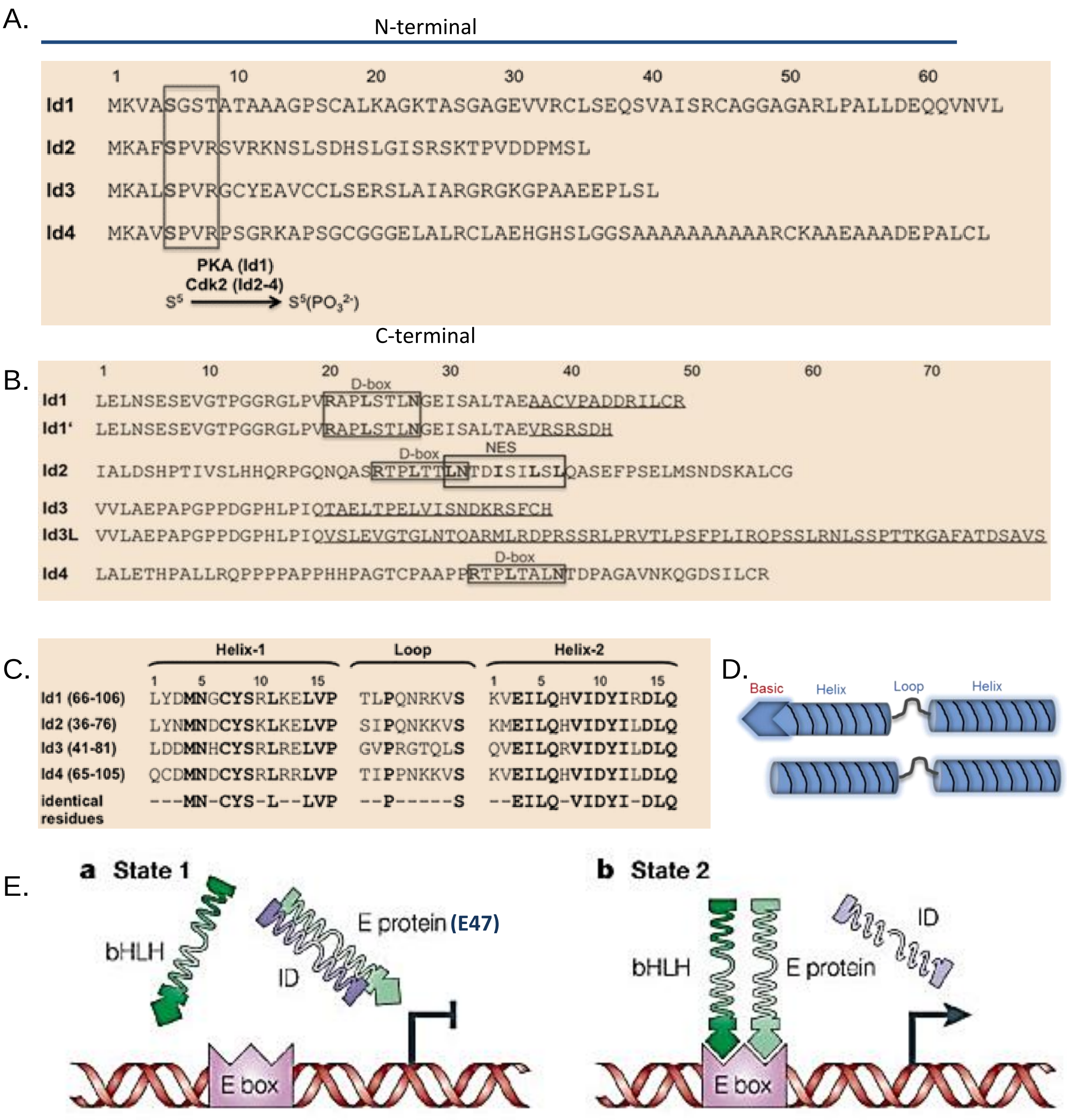


Figure 2: Human Id proteins structure and their mode of action. A, B. N-terminal and C-terminal amino acid sequence alignment of the Id proteins. Id1' and Id3L are the C terminus of human Id1 and Id3 spliced form. C. Alignment of amino acid sequence of the HLH domain of four Id proteins as well (D) the structural difference of Id proteins (HLH) with bHLH proteins. E. Schematic representation of Id protein forming a heterodimer with E-protein in state1(left panel) and inhibiting its binding to the E-box of the promoter. State 2 (Right panel) shows the activation of transcription when two bHLH forms homo or heterodimer. This figure was modified from Roschger C and Cabrele C, 2017 and Perk J et al., 2005.

Id proteins are small proteins which may be found in the cytoplasm and nucleus. Although Id proteins do not have the canonical nuclear localisation signal (NLS) motif, Id1 and Id2 have nuclear export signal (NES) motif in the HLH domain and in the C-terminus respectively **(Makita et al., 2006) (Kurooka et al., 2005)**. However, Id3 proteins have no such nuclear localisation signals like Id1 and Id2 but HLH domain is enough for its nuclear localisation. It was found that Id3 is localised more in the nucleus in the presence of E47 while found more in the cytoplasm or perinuclear region in the absence of E47 **(Deed et al., 1997)**. This finding suggests that that the NLS motif of bHLH could act as a transporter of Id protein in the nucleus.

1.5.2 Id protein regulation

As discussed earlier, Id proteins negatively regulates bHLH transcription factor functions and stops the cell from differentiation. There is overwhelming evidence which shows that the expression of Id protein is higher in undifferentiated, very proliferative and embryonic cells **(Ruzinova et al., 2003) (Engel and Murre, 2001) (Lasorella et al., 2001) (Ling et al., 2014) (Tzeng and De Vellis, 1998)**. Several studies have shown the activation of Id gene in stem cells and progenitor cells to enhance the proliferation and suppress the differentiation, but the gene expression is deactivated during lineage specification and differentiation **(Norton, 2000)**. In vitro and in vivo data suggests that BMP2/4 is instrumental in the induction of Id1, Id2 and Id3 expression in a variety of cells including embryonic stem cells **(Hollnagel et al., 1999)**. It was very recently shown using the human embryonic carcinoma cell line NTera2 that the nuclear factor Y (NFY) transcription factor directly regulates the Id1, Id2 and Id3 gene expression. The loss of NFYc was observed on differentiation which also resulted in the reduction of Id1-3 gene expression **(Moeinvaziri and Shahhoseini, 2015)**. Id1 gene has enhancer element at the 3'-end which can be activated by transcription factors C/EBPβ and STAT5. Therefore, Id1 is highly expressed in myeloid cells due to the critical role of C/EBP family in these cells. IL-3 and GM-CSF can activate STAT5 which could further result in the induction of Id1gene expression **(Saisanit and Sun XH 1997) (Xu M et al., 2003) (Cochrane, 2009)**. Another report confirmed the presence of C/EBPβ binding sites in the promoter region of Id2 and showed that it is directly regulated by C/ EBPβ **(Karaya**

et al., 2005). However, it was illustrated that in immature thymocytes, Id3 transcription was activated by Egr1 via Ras-MAPK pathway. This is a critical phenomenon for the T cell development **(Bain et al., 2001)**. In contrast, it was found that in breast cancer E2F1 recruits mutant p53 and directly binds to the Id4 promoter and activates it **(Fontemaggi et al., 2009)**.

1.5.3 Id protein and cell cycle regulation

A large body of evidence implicates the role of Id proteins in cell cycle regulation. Predominantly, Id proteins antagonise the E-proteins by sequestering them and several reports suggest the negative regulation of E2A (E12/47) on cell growth. Mainly the Id proteins inhibit the activation of genes associated with differentiation for example CDK inhibitors (p15, p16 and p21) **(Peverali et al., 1994) (Pagliuca et al., 2000)**. Id proteins are associated with the regulation of G1-S cell cycle phase. During the late G1 phase, Id2 and Id3 are phosphorylated by CDK2 restricting the dimerization of bHLH in vitro **(Deed et al., 1997) (Hara et al., 1997)**. In addition to CDK inhibitors, other cell cycle regulators like pRB (retinoblastoma) and p53 are also affected by Id protein. Only Id2 interacts with pRB directly. By crossing Id2 and Rb mutant mouse it was demonstrated that Id2 can rescue the embryonic lethality of $Rb^{-/-}$ mutant embryos **(Lasorella et al., 2000)**. Additionally, it has been shown both in vitro and in vivo that Id2 also interacts with p107 and p130 **(Lasorella et al., 1996)**. Although, Id1 and Id3 indirectly regulate the activity of pRb by binding to Ets domain proteins or bHLH transcription factors. It leads to the downregulation of p16 or p21 expression which results in the cyclin/CDK complex formation and finally hyperphosphorylation of pRb **(Ohtani et al., 2001) (Alani et al., 2001) (Ouyang et al., 2002)**. All these data highlights the importance of Id protein in cell cycle regulation.

1.5.4 Id protein and stem cell maintenance

The fact that Id protein inhibits differentiation and promotes proliferation supports their role in the maintenance of stem cell and progenitor cells. Several research groups have proven the expression of Id proteins in embryonic and somatic stem cells. For example, in mouse embryonic stem (ES) cells, it was shown that Id proteins are the target of BMP/Smad signalling which keeps the ES cells

undifferentiated and supports self-renewal **(Ying et al., 2003)**. Earlier also with in vivo data, it was shown that Id protein overexpression is correlated with inhibition of differentiation in C2C12 muscle cell line **(Jen et al., 1992)**. In hematopoietic stem cells (HSCs), Id protein roles have been similarly identified in self-renewal and stemness. The comparison between wild-type and $Id1^{-/-}$ marrow revealed the decrease in the number of long-term (LT) HSCs **(Perry et al., 2007) (Jankovic et al., 2007)**. In the same way, expression of Id1 and Id3 was found in undifferentiated human embryonic stem cells (hESCs) and human induced pluripotent stem cell (hiPSCs). Double knockdown of Id1 and Id3 in hemogenic precursors enhances differentiation into hematopoietic progenitors **(Hong et al., 2011)**. More recently, when Id3 was stably overexpressed in endothelial cells (ECs), gave rise to molecular stemness signatures like CD133+, VEGFR3+, and CD34+. Also, the Id3 overexpression significantly increased the G0-G1 phase cells compared to the wild-type cells and had phenotypes similar to glomeruloid microvascular lesions **(Das et al., 2015)**.

Furthermore, it was shown in GFAP+ astrocytes with stem cell characteristics, a high expression of Id1 which could divide asymmetrically into Id1-high stem cell and Id-low differentiated cells. The neurosphere-formation experiment substantiated that high Id1 and Id3 expression are critical for this process and double knockdown of Id1 and Id3 could reduce the secondary neurosphere formation by 50% **(Nam and Benezra, 2009)**. Consequently, it appears that higher Id expression is required to preserve the self-renewal ability and preserve the neural stem cells.

1.5.5 Id protein and development

Id proteins are expressed at several stages during the development of an organism. Over a period of time, a number of animal models with Id gene knockout (KO) have been generated to study their role in development. For example, it was observed that no embryo survived with $Id1^{-/-}$ $Id3^{-/-}$ beyond E13.5. The reason behind that was the cranial haemorrhage due to an aberration in vasculogenesis in the brain. In addition, they observed the small brain size in Id1-Id3 double KO embryos which were due to premature neural differentiation **(Lyden et al., 1999)**. It is interesting to note that embryos lacking 1-3 Id alleles survived and indistinguishable from the wild types. But the $Id1^{+/-}$ $Id3^{-/-}$ didn't support angiogenesis in tumour xenografts due to lack ok αvβ3-

integrin or MMP2. **(Lyden et al., 1999)**. Similarly, single knockout of one of the Id gene in mouse presented their role during the development. When Id1 was knocked out in mouse embryo fibroblast (MEFs), it was found that TSP-1 is the transcriptional repressor target of Id1. Due to loss of Id1, increased activity of TSP-1 was observed which was responsible for the poor angiogenesis **(Volpert et al., 2002)**. In chick embryo, through in situ hybridization, Id2 expression was shown on the dorsal cranial neural fold and neural crest. Overexpressing Id2 in chick embryo resulted in the conversion of the ectodermal cell to neural crest fate **(Martinsen and Bronner-Fraser, 1998)**. Whereas Id2 KO mouse revealed that Tgf-β induces Id2 expression and lack of Id2 can lead to a defect in the development of dendritic cells (DCs). More interestingly, $Id2^{-/-}$ mice also displayed a lack of lymph nodes and Peyer's patches **(Hacker et al., 2003)**. Another report has shown that when Id3 null mice were generated they exhibited defects in humoral immunity and B cell proliferation. However, ectopic expression of Id1 can substitute the role of Id3 in B cell proliferation. This can be explained due to the fact that Id1 and Id3 have about 69% homology and a minimal level of Id protein is necessary to stop the bHLH dimer formation which is the negative regulator of cell cycle progression **(Pan et al., 1999)**. The generation of Id4 KO mouse facilitated to study its role in neural stem cell development. The result obtained from this study shows that the $Id4^{-/-}$ mouse had smaller brain size compared to the mouse carrying Id4 wild type gene. The explanation behind this phenomenon was premature differentiation of neural stem cells and prolonged G1 to S transition in the early neural stem cells (**Yun et al., 2004).** Additionally, the $Id4^{-/-}$ mouse embryos displayed 20-30% reduction in the mitotic neural precursor cells (NPCs) and the tunnel assay further revealed an increase of 3.5 times in apoptosis of the ventricular zone (VZ). Also, the in vitro data from the neurospheres of $Id4^{-/-}$ NPCs showed reduced proliferation and increased differentiation of astrocytes in presence of BMP2 **(Bedford et al., 2005)**.

In skin, expression of Id1, Id2 and Id3 has been found in proliferating human keratinocytes which is further decreased with differentiation **(Langlands et al., 2000)**. An increasing number of studies have also found that Id proteins regulate the osteoblast proliferation and bone matrix formation **(Peng et al., 2004) (Lee et al., 2006) (Oh et al., 2012)**. Similarly, Id proteins play a crucial role during myogenesis

by regulating the differentiation of myoblasts as result of blocking E proteins activity **(Langlands et al., 1997)**.

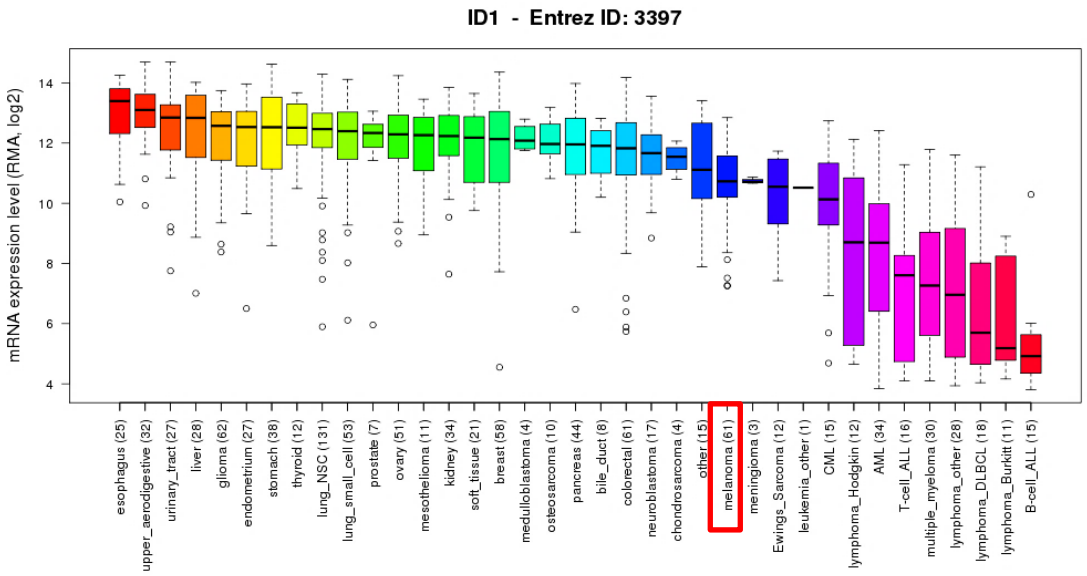
1.5.6 Id protein and cancer

A growing body of evidence shows that Id proteins play a major role in several cancers progression. Their expression has been found to be deregulated in diverse tumour types **(Figure 3)**. It becomes even important to know that Id proteins satisfy two essential criteria from the hallmarks of cancer i.e. it promotes cell growth and attenuates differentiation of the cell **(Lasorella et al., 2014)**. Even though Id proteins are involved in tumorigenesis, it doesn't fit to be classified as a tumour promoter or oncogene as there are not enough evidence which claims that Id genes are mutated or altered to transform a normal cell into the cancer cell. When Id1 and Id3 were sequenced from malignant melanoma-prone families and ovarian tumour respectively, no mutation was detected **(Casula et al., 2003) (Arnold et al., 2001)**. However a recent report put forward Id4 as an oncogene which is amplified in 32% of high-grade ovarian patients **(Ren et al., 2012)**. On contrary, the sequencing data of Burkitt's lymphoma shows that Id3 acts as a tumour suppressor, which is inactivated in 68% of the Burkitt's lymphoma samples **(Richter et al., 2012)**. But what makes Id proteins an important player during tumour development is that Id proteins are targets of numerous oncogenes like N-Myc, Ras, Notch and receptor tyrosine kinase **(Lasorella et al., 2000) (Chadwick et al., 2009) (Reynaud-Deonauth et al., 2002) (Tam et al., 2008) (Tournay and Benezara 1996) (Bain et al., 2001)**. Along with these oncogenes, there are some growth factors like TGF-β, BMP4, VEGF and FGF, which are activated in tumour cells and are positively correlated with Id protein expression and regulate cell proliferation, metastasis, angiogenesis and invasion **(Perk et al., 2005)**.

To investigate the role of Id protein in human keratinocytes, Id1 was ectopically expressed in these cells. The ectopic expression of Id1 alone resulted in the immortalization of human keratinocytes. Remarkably the immortalized cells showed reduced differentiated phenotype and furthermore activate the phosphorylation of Rb protein. They also demonstrated that ectopic expression of Id1 in human keratinocytes results in the impairment of p53 pathway. Additionally, the in vitro data showed that Id1, Id2 and Id3 all prolong the lifespan of human keratinocytes by

activating the telomerase activity and hTERT expression **(Alani et al., 1999)**. Now it is also well-established fact that Id1, which is up-regulated in many tumours including melanoma, is a transcriptional repressor of CDKN2A which encodes two important tumour suppressor genes- p16INK4a and p14ARF **(Ryu et al., 2007)**. Thrombospondin-1 (TSP-1) is another potent tumour suppressor and inhibitor of angiogenesis, which is downregulated by Id1 expression in melanoma **(Healey et al., 2010) (Straume and Akslen, 2005)**. Metastatic and invasive breast cancer cell lines show elevated levels of Id1 and upon downregulation of Id1 results in the reduction of breast cancer cell migration and invasion **(Fong et al., 2003)**.

A.



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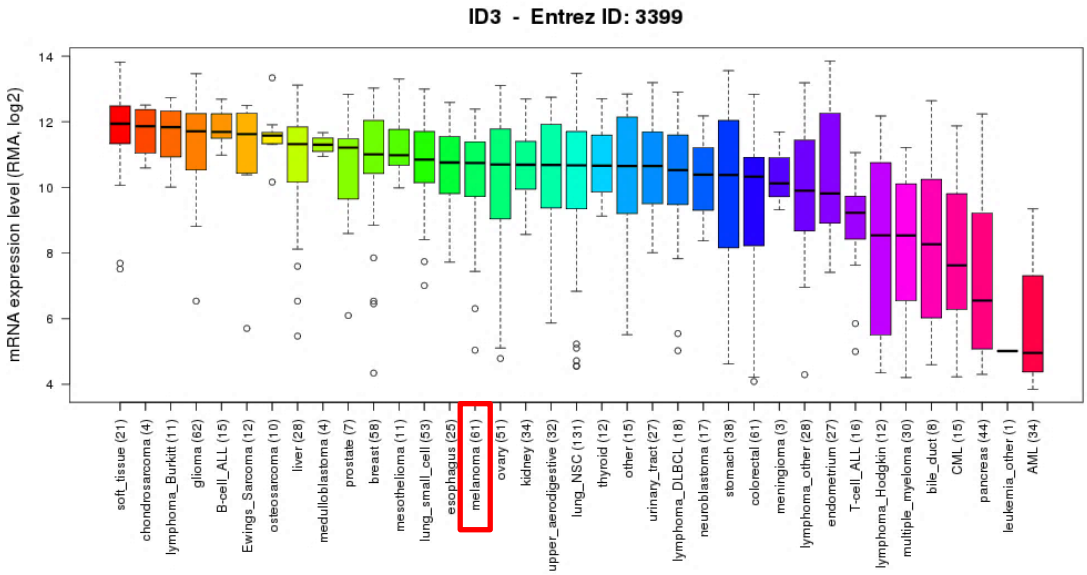

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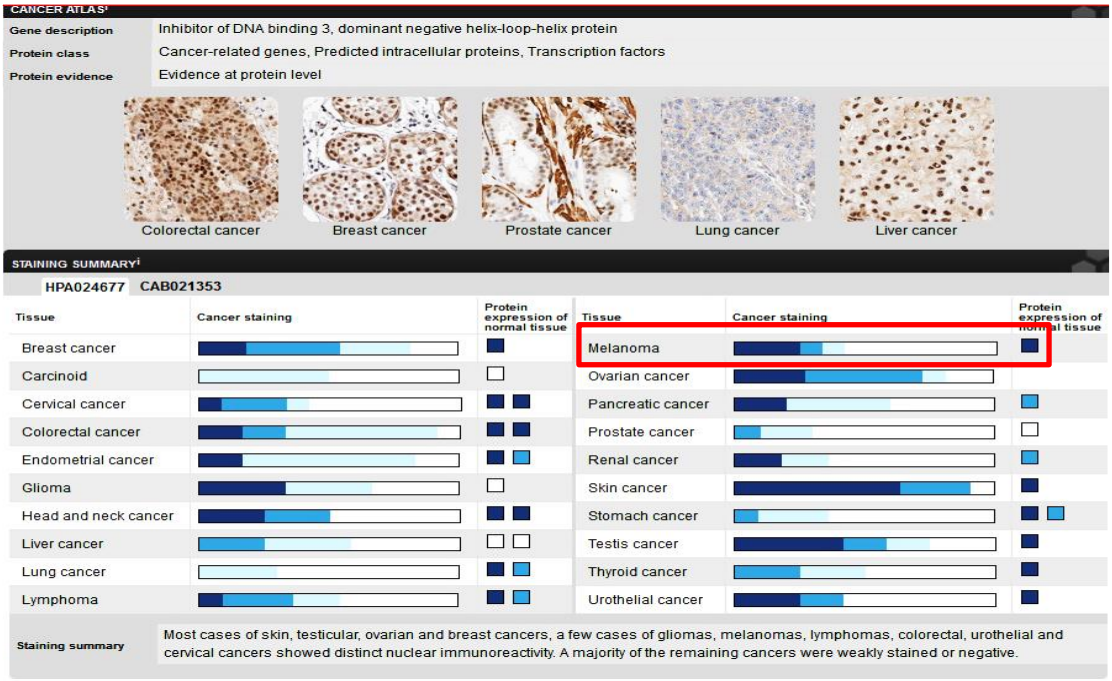


Figure 3: Id1 and Id3 expression in cancer cell lines and tissue samples: A, B. Id1 and Id3 expression in cancer cell lines including melanoma cell lines (n=61). Data used from cancer cell encyclopedia (CCLE). (C) Id3 expression in various cancer tissue samples from patients (The human protein atlas).

The role of Id1 in breast cancer was clearer when it was found that Id1 expression inhibits the binding of p53 to PTEN which further results in the activation of canonical Wnt signalling pathway via AKT phosphorylation **(Lee et al., 2009)**. The expression level of Id1 and Id3 are higher in prostate cancer tissue compared to the normal prostate tissue as well in prostate cancer cell lines. Silencing of Id1 and Id3 or Id3 alone using siRNA significantly reduces the proliferation of prostate cancer cell lines. The data further indicates that Id1 and Id3 regulate p21 and p27 cell cycle inhibitor respectively in prostate cancer cell lines **(Sharma et al., 2012)**. Knocking down Id1 and Id3 in prostate cancer cell line shows reduced Tgf-β induced migration **(Strong et al., 2013)**. Similarly, overexpression of Id3 in colorectal cancer cells affects the cell cycle and promotes cancer progression inhibiting p21. Here, Id3 was shown to be activated via AKT1/Smad5 which is the downstream target of S100A8/9 **(Zhang et al., 2015)**. In non-small cell lung cancer (NSCLC), Id1 and Id3 co-expression have been associated with the poor overall survival of the patients and low response rate

(50%) to the treatment compared to (87%) patients with no Id1-Id3 expression **(Castanon et al., 2013)**. Besides, prominent research in the field of cancer mechanism has exposed the role of Id proteins as instrumental in the progression of several cancer types including gastric cancer, hepatocellular cancer and pancreatic cancer **(Wang et al., 2004) (Sharma et al., 2016) (Kleeff et al., 1998)**.

1.5.7 Id protein in cancer cell dedifferentiation

Many cancer types represent small sub-populations of cells which have the capability to initiate tumour and they show the phenotype similar to cells in embryonic development. Researchers have coined many terminologies like cancer stem cells or cancer-initiating cells to define these small sub-populations. For better comprehension I would call here these cells, dedifferentiated cells. The presence of cancer stem cells or dedifferentiated cells in cancer attributes aggressiveness and drug resistance. In order to study the role of genes involved in dedifferentiation, several models have been suggested. One of the prominent in vitro model is 3d cell culture. Very recently it was shown that Id4 is highly expressed in the melanoma spheres along with other genes related to development. Knocking down Id4 resulted in more differentiated phenotype and preventing the formation of spheroids **(Peretz et al., 2015)**. Glioblastoma and colon cancer are other two cancer types where the role of Id protein in dedifferentiation have been well studied. Id1 expression in glioblastoma has been associated with metastasis through epithelial to mesenchymal (EMT) as well proliferation and invasion **(Guo et al., 2013)**. On the other hand, a study using glioma mouse model reveals that high Id1 expression is required for the self-renewal but it doesn't affect the tumorigenic potential **(Barrett et al., 2012)**. However, in case of high-grade gliomas (HGG), a malignant glioma mouse model was created and when Id gene (Id1, Id2 and Id3) was deleted in only tumours and not the endothelial cells or tumour microenvironment resulted in tumour regression and extended survival due to rapid loss of glioma-initiating cells (GICs) from the perivascular tumour niche due to inhibition of RAP1GAP **(Niola et al., 2013)**. In colon cancer, double knock down of Id1-Id3 in colon cancer-initiating cells (CC-ICs) resulted in complete loss of tumour formation in xenografts. An immunohistochemical analysis determined that Id1-Id3 knockdown leads to induction of differentiation and reduction in micro vessel density (MVD). More importantly,

double knockdown of Id1-Id3 significantly reduced the sphere formation capacity and increased sensitivity to chemotherapy. Id1-Id3 double knockdown affect the tumour initiating potential by downregulating the cell cycle inhibitor p21 **(O'Brien et al., 2012)**.

1.5.8 Id protein and drug resistance

Resistance to therapy has been the predominant concern in most of the cancer patients getting the treatment. The efficacy of the drug is subdued due to resistance in many cases. Several studies have focused in order to determine the mechanisms behind the gain of resistance to certain drugs in cancer. The data accumulated from these studies has revealed that Id proteins play directly or indirectly a pivotal role during drug resistance. In prostate cancer, it was shown that interaction of Id1 and caveolin-1 enhances the process of EMT and reduces apoptosis induced by Taxol through activation of AKT pathway. Knocking down Id1 using siRNA makes the prostate cancer cells more sensitive to the Taxol treatment **(Zhang et al., 2007) (Wong et al., 2008)**. Besides, B-cell leukemia-3 (Bcl-3), a proto-oncogene, has been also shown to regulate Id1 and Id2 expression in prostate cancer upon exposure to IL-6 which is responsible for inhibiting apoptosis and inducing resistance to chemotherapy **(Ahlqvist et al., 2013).** Id1 expression has also been associated with glioma-initiating cells and conferring resistance to therapies. Similarly, silencing of Id2 expression in glioma cells resulted in higher sensitivity to the antineoplastic drugs used for the treatment of brain tumours **(Anido et al., 2010) (Zhao et al., 2015)**. Moreover, a comparison between tamoxifen-sensitive (TS) and tamoxifen-refractory (TR) breast cancer patients revealed that Id4 was hypomethylated in TR breast cancer **(Zhang et al., 2015)**. In colorectal cancer, overexpression of leukaemia inhibitory factor (LIF) is responsible for the chemoresistance via negatively regulating p53. To achieve this, LIF induces STAT3 expression which transcriptionally activates Id1 which further upregulates MDM2 expression and MDM2 promotes the degradation of p53 protein **(Yu et al., 2014)**. Collectively, these data highlights the involvement of Id proteins in cell survival and drug resistance in various cancer types.

2. Aims of the project

Melanoma cells show a high degree of heterogeneity and a sub-population of the tumour cells represent a dedifferentiated phenotype. Dedifferentiation in melanoma is attained by the reactivation of genes from neural crest origin. Reports from several studies suggest that dedifferentiation attributes aggressiveness to several cancers including melanoma. It is responsible for the tumour initiation, metastasis and more importantly for conferring resistance to the therapies. This study aims to find new marker for the dedifferentiation in melanoma and their role in melanoma pathogenesis. It might also help in the development of new strategies to eliminate melanoma.

The specific aim of this study is:

1. To identify genes that are expressed in neural crest cells, melanoma cell lines and are also upregulated in targeted therapy resistant cells.
2. To investigate the role of candidate gene/s in melanoma proliferation and metastasis and to find the mechanism for its functioning.
3. Finally, to examine the role of candidate gene/s in melanoma resistance to the targeted therapies.

3. Materials and Methods

3.1 Materials

3.1.1 Reagents and Kits

Product	Company	Catalog
Agarose NEEO Ultra Qualität	Carl Roth	2267.4
Alamar Blue®	Invitrogen	DAL1100
Amersham ECL prime western blotting detection reagent	GE healthcare	RPN2232
Ammonium persulfate solution (APS)	Carl Roth	9592
ARCTURUS PicoPure RNA Isolation Kit	Life Technologies	KIT0204
Complete mini protease inhibitor cocktail	Roche Diagnostics	04693159001
Endofree plasmid maxi kit	Qiagen	12362
High performance chemiluminescence film	GE healthcare	28906836
IBIDI Culture-Insert 500 µM	Ibidi	80209
Immobilion PVDF membrane pore- 0.45µM	Merck Millipore	IPVH00010
Luminata Forte Western HRP substrate	Millipore	WBLUF0500
Pierce BCA protein assay kit	Thermo scientific	23225
PhosSTOP^{TM} Phosphatase inhibitor Cocktail	Roche diagnostics	04906845001
Precision Plus	Bio-Rad	161-0394
RNase-Free Dnase set	Qiagen	79254
RNeasy plus Mini kit	Qiagen	74136
RevertAid First strand cDNA synthesis kit	Thermo Scientific	K1622
Skim milk powder	Flika Analytical	F7016605000
SYBR Green PCR Master mix	Applied Biosystems	4309155
TEMED	Carl Roth	2367.3
Tween® 20	Applichem	A13890500
TritonX-100	Carl Roth	3051.4
X-treme GENE® 9 DNA transfection Reagent	Roche Diagnostics	06365787001

3.1.2 Cell culture reagents

Product	Company	Catalog
2-Mercaptoethanol	Gibco® Life Technologies	31350010
Ascorbic acid	Sigma Aldrich	1043003
Blasticidine S	Sigma Aldrich	15205

Bone morphogenic protein 4 (BMP4)	Promokine	C-67313
DMSO	Carl Roth	A994.2
DMEM AQ media™	Gibco® Life technologies	41965-039
Fetal Calf Serum (FCS)	Biochrom	S0115
Human melanocyte growth supplement (HMGS) 100x	Gibco® Life Technologies	S002-5
Lipofectamine® RNAiMAX transfection reagent	Life Technologies	13778075
Medium 254	Gibco® Life Technologies	M254500
Non-essential amino acids	Sigma-Aldrich	M7145
Opti-MEM® I reduced serum medium	Gibco® Life Technologies	31985062
PBS	Sigma-Aldrich	D8537
Penicillin/Streptomycin	Sigma-Aldrich	P4333
Puromycin	Carl Roth	240.1
Trypan blue solution	Sigma-Aldrich	93595
Trypsin	Sigma-Aldrich	T3924

3.1.3 Human melanoma cell lines

Cell Line	Source	Disease	Mutation
A375	ATCC	Malignant Melanoma	BRAF V600E
C32	ATCC	Melanoma, amelanotic	BRAF V600E
HT144	ATCC	Malignant Melanoma	BRAF V600E
MeWo	ATCC	Malignant Melanoma	WT
Skmel 23	ATCC	Melanoma	WT
Skmel 28	ATCC	Malignant Melanoma	BRAF V600E
Skmel 30	DKMZ Leibniz Inst.	Melanoma	NRAS Q61R
Skmel 103	CNIO Madrid	Melanoma	NRAS Q61R
Skmel 147	CNIO Madrid	Melanoma	NRAS Q61R
Skmel 173	CNIO Madrid	Melanoma	WT
WM266-4	ATCC	Melanoma	BRAF V600D

3.1.4 Antibodies

Product	Source	Company	Catalog
Anti-GAPDH	Rabbit	Cell signalling	CST2118
Anti-Id1	Rabbit	Cal-bio reagent	M085
Anti-Id3	Rabbit	Cal-bio reagent	M100
Anti-MITF	Mouse	Abcam	ab80651
Anti-SOX10	Mouse	Abcam	ab181466

3.1.5 Small molecule inhibitors

Product	Company	Catalog
Vemurafenib (PLX4032)	Selleckchem	S1267
Trametinib (GSK1120212)	Selleckchem	S2673
Dabrafenib (GSK2118436)	Selleckchem	S2807

3.1.6 Buffers, Solution and Gels

Transfer buffer (pH 8.3)	Running buffer (pH8.3)	TBS 10X (pH 7.6)
25mM Glycine	25mM Glycine	150mM NaCl
190mM Tris	190mM Tris	50mM Tris
20% SDS	0.1% SDS	dH2O
20% methanol	dH2O	
dH2O		

Washing buffer (TBST)	Washing buffer (PBST)	Blocking buffer (milk)
0.02% Tween® 20	0.02% Tween® 20	5% Skim milk powder
1X TBS	PBS	Washing buffer (PBS or TBS)

Cell lysis buffer for protein isolation

1X PhosphoStop
1X Complete mini protease inhibitor cocktail
1% Triton-X in TBS

10% SDS separating Gel (10ml)	15% SDS separating Gel (10ml)
H2O (4 ml)	H2O (2.3 ml)
30% Acrylamide mix (3.3)	30% Acrylamide mix (5.0)
1,5 M Tris (pH 8.8) (2.5ml)	1,5 M Tris (pH 8.8) (2.5ml)
10% SDS (0.1 ml)	10% SDS (0.1 ml)
10% APS (0.1 ml)	10% APS (0.1 ml)
TEMED (0.004ml)	TEMED (0.004ml)

Stacking Gel (10ml)

H 2 O (6.8 ml)
30% Acrylamide mix (1.7 ml)
1,0 M Tris (pH 6.8) (1.25 ml)
10% SDS (0.1 ml)
10% APS (0.1 ml)
TEMED (0.01 ml)

3.1.7 Devices

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

3.1.8 Software

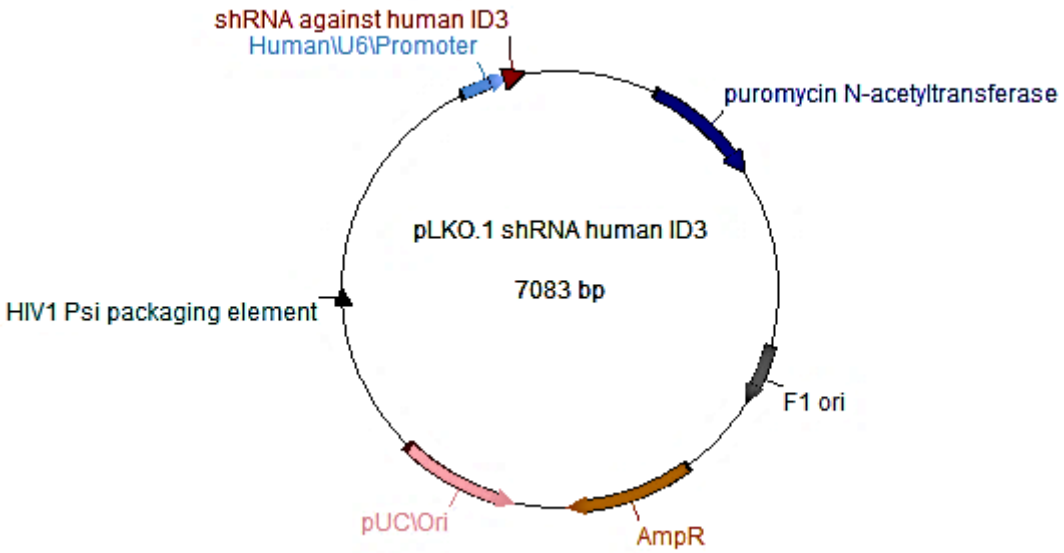
Analysis Software	Source
7500 Software v2.0.5	Applied Biosystem
ApE	M. Wayne Davis (Open Source)
BD FACSDivaTM	Biolegend
Chipster	Chipster Open source
FlowJo 7.2.2	FlowJo
Graphpad Prism	Graphpad Prism
i control 1.10	TECAN
Image J	NIH
Mendeley	Mendeley
NIS-Element	Nikon
T-scratch	CSElab

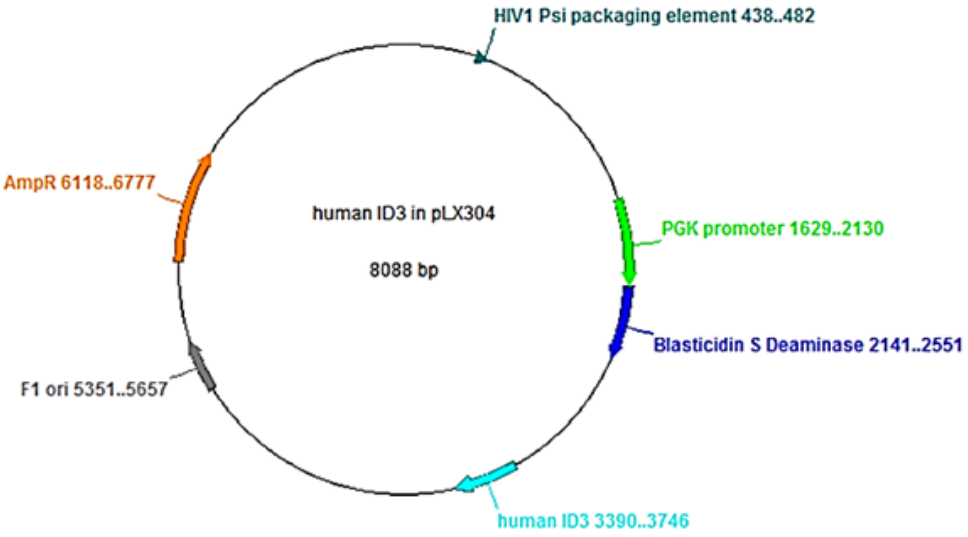
3.1.9 Online databases

Name	Website
cBioportal	http://www.cbioportal.org
Cancer Cell Line Encyclopedia	http://www.broadinstitute.org/ccle
GEO dataset	https://www.ncbi.nlm.nih.gov/gds/?term=
The Human Protein atlas	http://www.proteinatlas.org/
TRED	https://cb.utdallas.edu/cgi-bin/TRED/
Transcription factor binding sites	http://www.sabiosciences.com/

3.1.10 Plasmids

Product	Company
shRNA against human Id3 (Id3-shRNA)	Sigma-Aldrich
Non-targeting shRNA (NT-shRNA)	Sigma-Aldrich
pLX304 (Empty vector)	Addgene





3.2 Methods

3.2.1 Cell culture and cell lines

The human melanoma cell lines (A375, C32, HT144, MeWO, Skmel 28, Skmel 23, Skmel 30, Skmel 103, Skmel 147, Skmel 173, and WM266-4) were cultured in DMEM (Gibco, Life Technologies) with 10% FBS (Biochrom), 0.1mM β-mercapthoethanol (Gibco, Life Technologies), 1% non-essential amino acids (NEAA) and 1% Pen/Strep (Sigma-Aldrich).

Normal human melanocytes (NHM) isolated from donor foreskins according to the ethical regulation (Ethics committee II, University Medical Centre Mannheim, Germany) and were cultivated in medium 254 (Gibco, Life Technologies) supplemented with 100x human melanocyte growth supplement (HMGS) (Gibco, Life Technologies). Human neural crest cells were generated as described previously (Larribere L et al 2015) in the protocol for hiPSC-derived melanocyte differentiation.

All cell lines were cultured in humidified incubator at 37^0C and 5% CO2. Cell lines were sub-cultured every 3-5 days when they were about 80% confluent.

3.2.2 Lentiviral particle production and transduction

Lentivirus particles were produced in HEK293T cells. HEK 293T cells were approximately 60% confluent on the day of transfection. For one T-75 flask, 770µl of DMEM medium (without FCS) was mixed with 50µl of X-tremeGENE® (Roche) and incubated for 5 min at room temperature. Subsequently, the plasmid with the gene of interest (11µg) along with packaging plasmids VSV-G (5.5µg) and Δ8.9 (8.25µg) was mixed in the DMEM and X-treme GENE® solution. The mixture was incubated for 30 min at room temperature and then added to HEK293T producer cells. After 12h, the medium was changed. Virus supernatant was collected after 24h, 36h and 48h. The collected supernatant was filtered through a 0.45µm PVDF filter and concentrated by centrifugation for 5h 21,000g at 4°C in a high-speed centrifuge. The virus pellet was re-suspended in 100µl PBS aliquoted and stored at -80°C. The concentrated virus was used to infect the cells. The virus production was done in a Biosafety level II laboratory, according to the safety instruction.

3.2.3 Transduction with lentiviral particles

Cells were seeded in 6 well plates with 50-60% confluency and incubated for 16-18h in humidified incubator at 37^0C and 5% CO2. Fresh medium with virus (500µl-1ml supernatant or 5µl concentrated virus) was added to the cells. Cells were again transduced after 24h as described above. After 48h transduction was stopped and at least 3 times cells were washed with PBS. The Normal fresh medium was added to the cells and grown for at least 24h to let them recover before selection.

3.2.4 Antibiotic selection

Cell lines with Id1 and Id3 shRNA were selected by using puromycin (0.5-0.8µg/ml) and cell lines with Id1 and Id3 overexpression vector was selected using blasticidin (5-8µg/ml) for 4-6 days.

3.2.5 RNA isolation and cDNA synthesis

Total RNA isolation was done using RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. The RNA was treated with DNase I on the column. RNA concentration and quality were measured by NanoDrop ND1000 spectrophotometer. cDNA was synthesised using the Revert Aid First Strand cDNA synthesis kit (Thermo scientific) according to the manufacturer's protocol.

3.2.6 qPCR

Quantitative real-time PCR (qPCR) was performed using SYBR Green (Applied Biosystems, Life technologies) on a 7500 real-time PCR system (Applied Biosystems, Life technologies).In all experiments, 18s was used as the housekeeping gene and the values were normalised to it. Relative gene expressions were quantified by calculating ($\Delta\Delta$Ct). Primers used are as follow:

18s F: GAGGATGAGGTGGAACGTGT

18Sr: TCTTCAGTCGCTCCAGGTCT

Id1 F: TTCAGCCAGTCGCCAAGAAT

Id1 R: CCACGCTCTGCTCAGACA

Id3 F: GGAGCTTTTGCCACTGACTC

Id3 R: TTCAGGCCACAAGTTCACAG

SOX10 F: GGCTTTCTGTCTGGCTCACT

SOX10 R: TAGAGGGTCATTCCTGGGGG

MITF F: GCTCACAGCGTGTATTTTTCC

MITF R: TCTCTTTGGCCAGTGCTCTT

3.2.7 Western Blot

Cells were harvested and washed with PBS. Protein was extracted from the cells by using complete mini lysis buffer (Roche) with 1% Triton-X-100. The protein concentration was determined using the Pierce BCA protein assay kit (Thermo scientific). Proteins were resolved on SDS-PAGE and transferred onto PVDF membranes (Merck Millipore). Later the membrane was probed with primary antibodies and then with HRP conjugated secondary antibody. The bands were visualised using Luminata Forte western HRP substrate (Merck Millipore) according to manufacturer's protocol. The band intensities were quantified using ImageJ software (Fiji). The primary antibodies used are as follow:

Id1(cal bio), Id3(Calbio), SOX10 (Abcam), MITF (Abcam), GAPDH (Cell signalling).

3.2.8 Cell proliferation and cell cycle analysis

Cell proliferation was measured using Alamar blue (Invitrogen). Approximately 2500 or 5000 cells were seeded in triplicates in a 96 well plate. After 24 hours Alamar blue (10% of the medium) was added and the plates were incubated for 4 hours at 37^0C. Fluorescence was measured with excitation wavelength at 530-560 nm and emission

wavelength at 590 nm using the Tecan Infinite 200 Pro plate reader. Proliferation was measured for 4 to 5 days.

Cells were seeded (4-6x 10^5) in 6 well plates in triplicates and incubated at 37^0C for 18-24 hours. After that cells were washed withPBS, trypsinized and collected in tubes. The pellets were resuspended in ice-cold PBS and fixed with pre-cooled 70% ethanol. Cells were then washed with ice-cold PBS and treated with RNase for 30 min at 37^0C. Propidium iodide (50µg/ml) was used to stain the cellular DNA. Cell cycle stages were analysed in Flowcytometer Canto (Becton-Dickson).

3.2.9 Cell viability

Approximately 2500 cells were seeded in a 96 well plates. After 16-18 hours desired concentrations of Vemurafenib or Trametinib was added to the cells. After every 24 hours up to 5-6 days, cell viability was measured using Alamar blue as described above. Approximately 1X10^5 cells were seeded in 6 well plates. After 16-18 hours desired concentration of Vemurafenib and Trametinib was added and incubated at 37^0C for 72 hours. After 72 hours cells were trypsinized and washed with PBS. Total RNA was isolated and cDNA was synthesised as described previously. This experiment was performed in triplicates with DMSO as a control.

3.2.10 Migration assay

Cell migration was studied using Ibidi culture inserts. Approximately 70000 Cells were seeded inside the inserts and were starved overnight. The inserts were removed and cell migration was observed every 4 hours over 24 to 28 hours. TScratch software was used for the quantitative analysis of the data.

3.2.11 Immunohistochemistry

To perform immunostaing, 4µm sections of formalin-fixed paraffin-embedded tissue sections were stained with antibodies against Id1 and Id3 as per the manufacturer's

instruction. All analyses involving human melanoma tissues were carried out according to the principles of the Declaration of Helsinki and were approved by the medical Ethics Committee of the Medical Faculty Mannheim, University of Heidelberg.

3.2.12 Microarray expression analysis

Total RNA was isolated from primary melanocytes (NHM), human neural crest cells and melanoma cell lines A375 and C32 Vemurafenib treated and DMSO control) and purified using RNeasy kit (Qiagen). Total RNA samples were submitted for analysis to the DKFZ Genomics and Proteomics Core Facility by using HumanHT-12 v4 from ILLUMINA (Santa Clara, CA, USA). Bayes test was used as test of significance to the bead expression values of the two groups of interest. List of differentially expressed genes were selected using P-value threshold of 0.05 and log2-expression.

3.2.13 Bacterial transformation and plasmid isolation

For transformation, DH5α competent Escheria Coli (E.coli) bacteria were used. Bacteria cells were first thawed on ice and then 100µL of competent cells were mixed with 5µL of the ligation mix in a pre-chilled eppendorf tube. The samples were mixed by pipetting and incubated on ice for 30 min. The mix was subjected to heat shock for 90 sec in a 42°C water bath and incubated for 5 min on ice. Subsequently, 500µL of SOC-medium was added to each sample and again incubated for 1 hour at 37°C on a thermos-mixer with shaking at 400 rpm. After incubation, the samples were centrifuged at 6,000 rpm for 3 min, 450µL of the supernatant was removed and the pellet was resuspended. The transformed bacteria were streaked onto LB-agar plates with ampicillin (100µg/ml) and were left to incubate at 37°C overnight (approximately 18 hours) upside-down for colony formation. Next day colonies were picked and incubated in 5ml LB medium with 100µg/ml ampicillin at 37°C overnight. The plasmid was isolated as per manufacturer's guide using mini-preparation kit (Qiagen). Restriction digestion was performed to determine the correct insert and

vector. Next, I incubated the bacteria with the specific plasmid in 200ml LB medium containing 100µg/ml ampicillin at 37°C overnight. Thereafter the Qiagen Maxi kit was used to isolate the plasmid DNA. Plasmid DNA was precipitated using isopropanol and ethanol. The DNA pellet was air-dried and resuspended in TE buffer. Plasmid DNA concentration and quality was checked using Nanodrop ND-1000.

3.2.14 Statistical analysis

The software GraphPad Prims version 5.00 was used for statistical analysis. Statistical significance using two-tailed paired and unpaired t-test was determined if $p < 0.05$ (*), <0.01 (**) and 0.005 (***).

3.2.15 Software Used

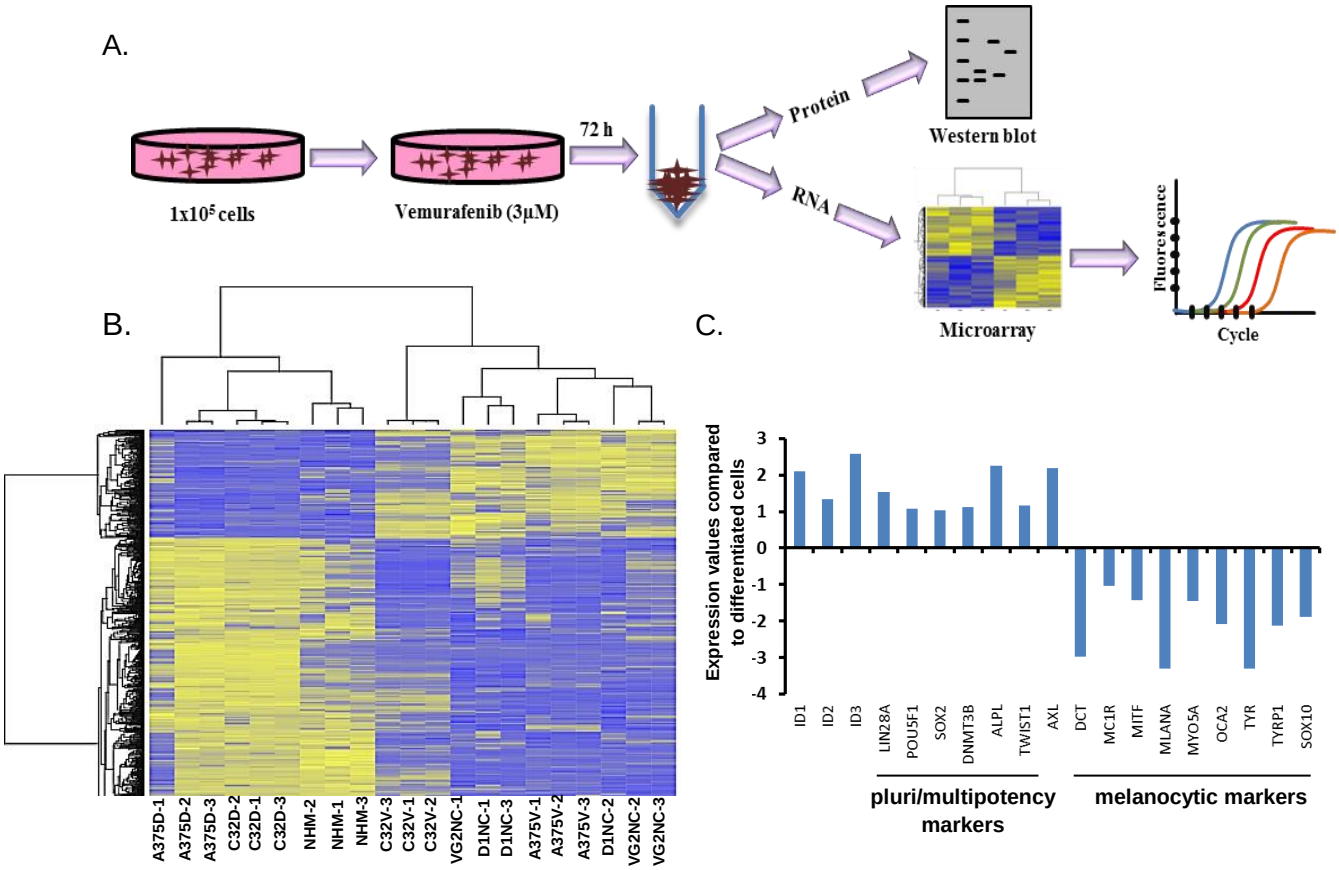
T scratch software was used to analyse the migration assay. To analyse the microarray data chipster was used.

4. Results

4.1 Identification of genes regulating dedifferentiation in Melanoma

Metastatic melanoma is a very heterogeneous cell population and during its development it reminiscences phenotype which is similar to its embryonic origin. This phenotype switch in melanoma has been considered to be responsible for resistance to several therapies **(Landsberg et al., 2012) (Roesch et al., 2016)**. To accomplish this, melanoma cells reactivates genes which were switched off during the process of differentiation. In order to trace the genes which are primarily involved in this process, induced pluripotent stem cells (iPSCs) in our lab was generated. Later, in controlled conditions, the iPSCs were differentiated into melanocytes via neural crest stage. Also, to have an insight into their role in drug resistance I treated the BRAF mutated melanoma cell lines (A375, HT144, Skmel 28 and WM266-4) with BRAF-inhibitor, vemurafenib. These cell lines were treated with a higher dose (3µM) of vemurafenib for 6h, 24h, 48h and 72h. Cells were treated with DMSO as a control for same time points. After each time points, the cells were trypsinized and harvested for RNA extraction **(Figure 4A)**. Next, the whole genome expression of melanoma cells treated with vemurafenib (72h), respective DMSO-treated control melanoma cell lines, more differentiated normal human melanocytes (NHM) and less differentiated neural crest cells derived from iPSCs was compared. Total RNA in triplicates were used and the microarray data were analysed using the chipster software. An unsupervised hierarchical clustering showed two major clusters. The more differentiated cells i.e. NHM and DMSO-treated melanoma cell lines clustered together whereas the second cluster contained vemurafenib-treated melanoma cell lines with less differentiated neural crest cells **(Figure 4B)**. Therefore, from the clustering data, it could be shown that the vemurafenib-treated cell lines are more similar to the neural crest cells or dedifferentiated. Moreover, it was also found that Id gene family- Id1, Id2 and Id3 were upregulated (log2-fold change ranging from 1 to 2.5) along with pluripotency marker genes like LIN28, POU5F1, SOX2, DNMT3B, ALPL as well as a multipotency marker gene, TWIST1, in the dedifferentiated group of cells compared to the NHM and DMSO-treated group. Similarly, the melanocyte or differentiation markers like DCT, MC1R, MITF, MELANA, SOX10, TYR and TYRP1

were down-regulated (log2-fold change ranging from -1 to -3.3) in the vemurafenib-treated melanoma cells and the neural crest group compared to the NHM and DMSO-treated group **(Figure 4C)**.



D.

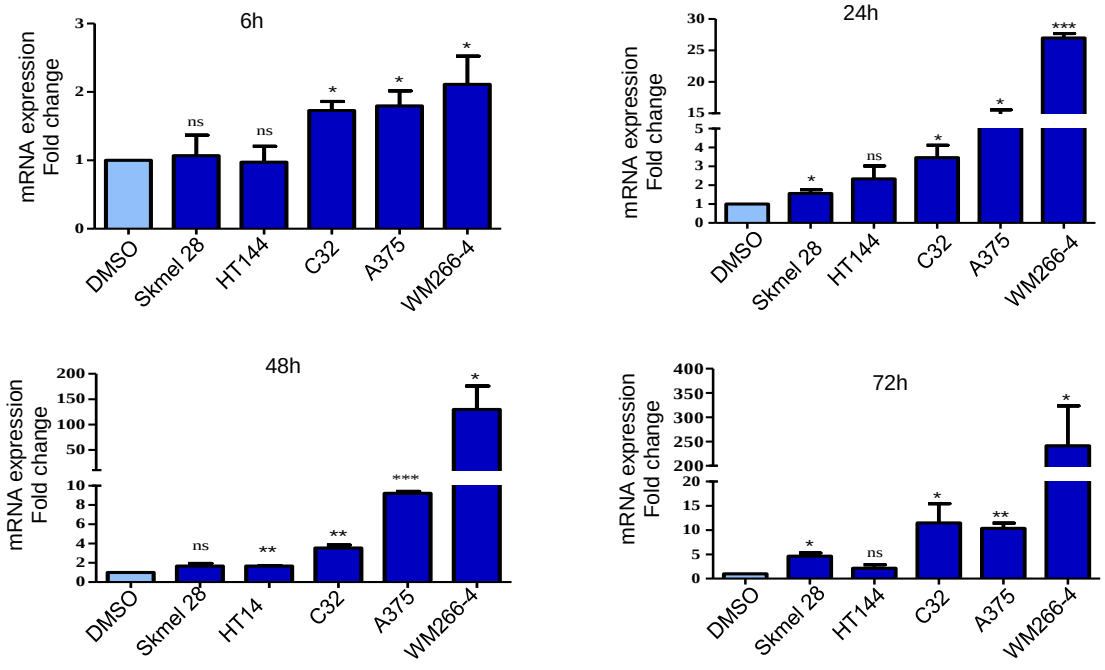


Figure 4: Id3 expression is upregulated during adaptive vemurafenib resistance in human melanoma cell lines. A. Schematic representation of human melanoma cell lines treatment with vemurafenib (3μM, 72h) followed by gene expression analysis. B. Heat map representation of unsupervised hierarchical clustering of differentially expressed genes in neural crest cells (NC), normal human melanocytes (NHM) and melanoma cell lines (A375 and C32) treated with vemurafenib (V) or with DMSO (D). C. This panel shows that the pluripotency or multipotency marker genes along with Id genes are upregulated and differentiation (melanocytic) marker genes are downregulated. D. Id3 mRNA expression in human melanoma cell lines treated with vemurafenib for 6h, 24h, 48 h and 72h. 18s was used as an endogenous expression control and DMSO treated cells were used as reference sample. The qPCR results shown here are mean of + SD of biological triplicates. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant.

Additionally, the AXL expression was found to be upregulated in the vemurafenib-treated cell lines, which is in accordance with the recent report describing low MITF/AXL expression ratio during early drug resistance in melanoma **(Muller et al., 2014)**. It was further demonstrated through qPCR that the Id3 expression was upregulated after treatment with vemurafenib. Here it was observed that a significant increase in Id3 expression starts as early as 6 hours after the treatment with vemurafenib in 3 melanoma cell lines (C32, A375 and WM266-4) and remained higher after 24 hours, 48 hours and 72 hours in all the 5 cell lines (A375, C32, WM266-4, Skmel 28 and HT144) **(Figure 4D)**. More interestingly, upregulation of Id3 in NRAS mutated cell lines (Skmel 30, Skmel 103 and Skmel 147) was detected after treatment with the MEK inhibitor (trametinib) at different time points **(Supplementary Figure 2)**. Additionally, upregulation of Id3 in three BRAF mutated cell lines (A375, C32 and Skmel28) after treatment with BRAF inhibitors (Vemurafenib and Dabrafenib), MEK inhibitor (Trametinib) or in combination (Vem+Tra, Dab+Tra) was found **(Supplementary Figure 3)**. These data suggests the potential involvement of Id3 in drug resistance is not BRAF- specific.

4.2 Expression of Id1 and Id3 in melanoma

To better understand the role of Id genes (mainly Id1 and Id3) in melanoma development, the human melanoma cell lines according to their Id1 and Id3 expression were characterised. For this, the qPCR and western blot techniques were used to show their mRNA and protein expression level in different human melanoma cell lines. This human melanoma cell panel can be divided into BRAF mutated (A375. C32, HT144, Skmel 28 and WM266-4), NRAS mutated (Skmel 30, Skmel 103 and Skmel 147) and BRAF/NRAS wild types (Skmel 23, Skmel 173 and MeWo). From the mRNA expression, it was shown that most of the cell lines express higher Id1 and Id3 compared to the NHM **(Figure 5A, B)**. 18s was used as the house-keeping gene. Similarly, the protein expression of Id1 and Id3 in most of these cell lines were higher in comparison to the NHM. Interestingly, from the results, no correlation between the Id1 and Id3 expression and their mutational status was established **(Figure 5C, D)**

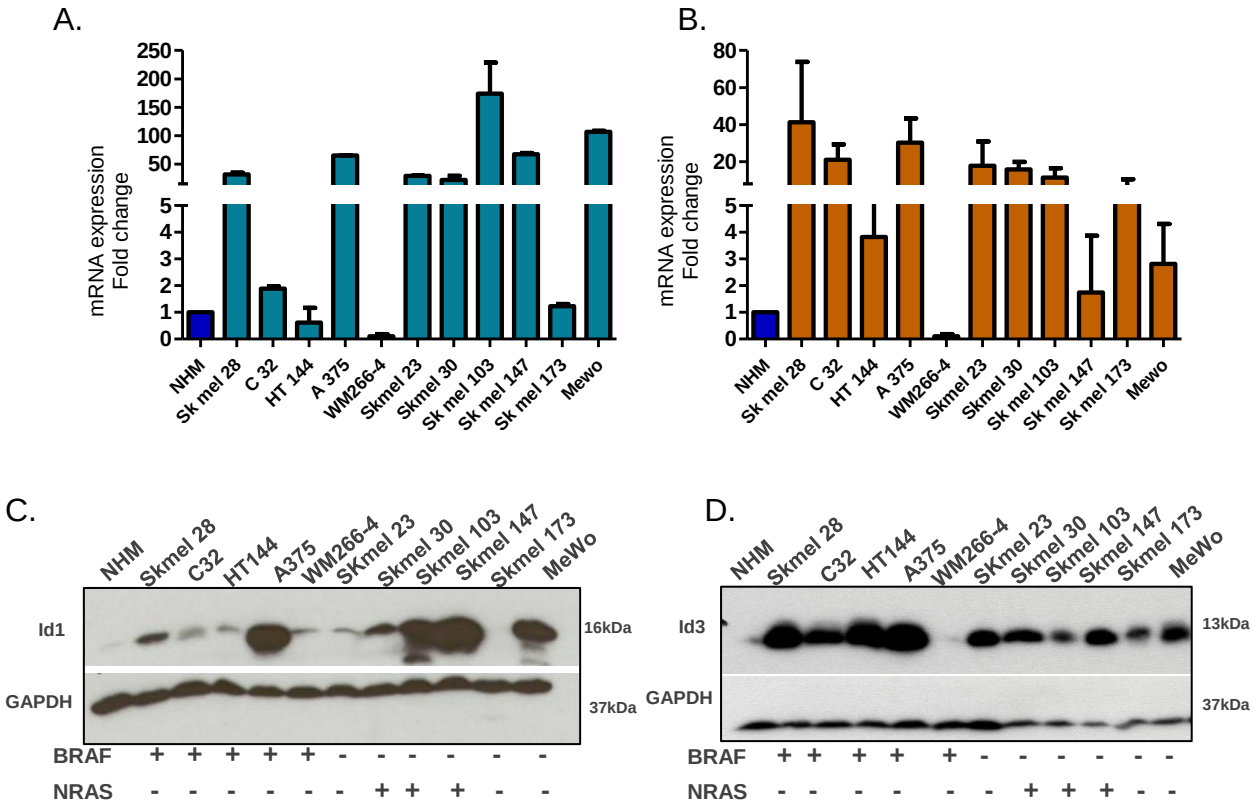
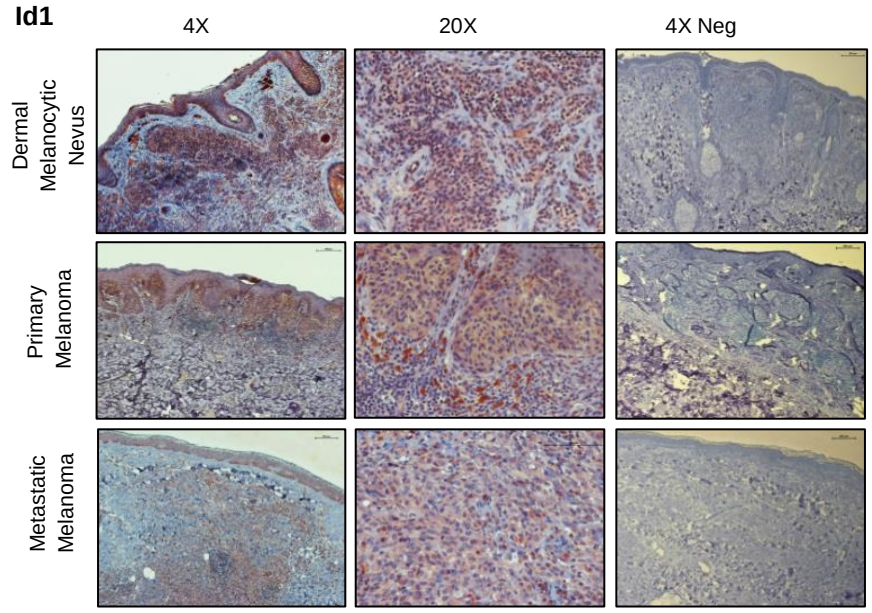


Figure 5: Id1 and Id3 expression in melanoma cell lines. A, B. mRNA expression of Id1 and Id3 in normal human melanocyte (NHM) and human melanoma cell lines. 18s was used as endogenous control and normal human melanocytes (NHM) was

used as the reference. The qPCR results showed here are mean + SD of biological triplicates. Protein expression of Id1 (C) and Id3 (D) in NHM and human melanoma cell lines.

Further, immunohistochemistry (IHC) technique was used to check the expression of Id1 and Id3 in tissue samples. For this I prepared, fixed and stained using Id1 and Id3 antibody primary melanoma, metastatic melanoma and dermal nevus tissue samples derived from patients. The staining results showed that Id1 and Id3 are highly expressed in both primary and metastatic melanoma tissues as well as in dermal nevus **(Figure 6A, B).**

A.



B.

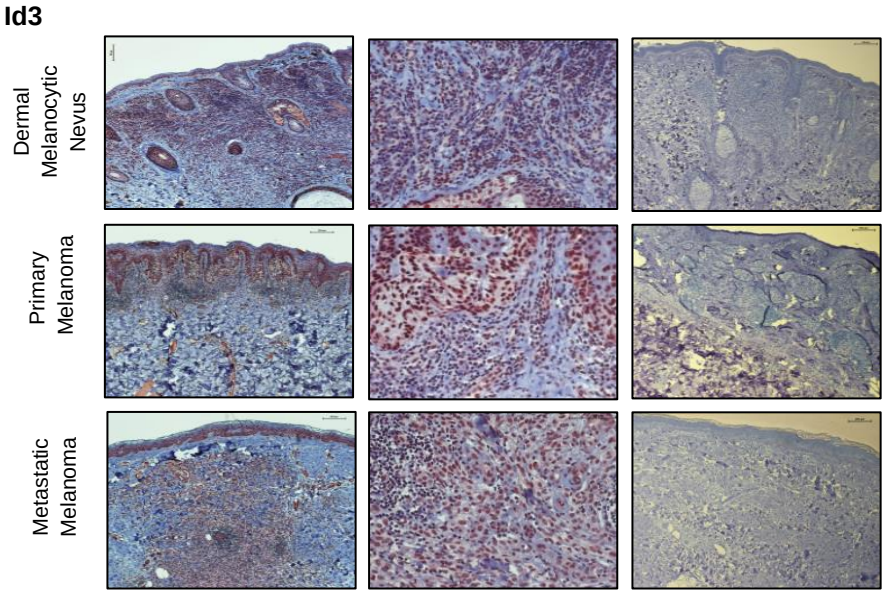


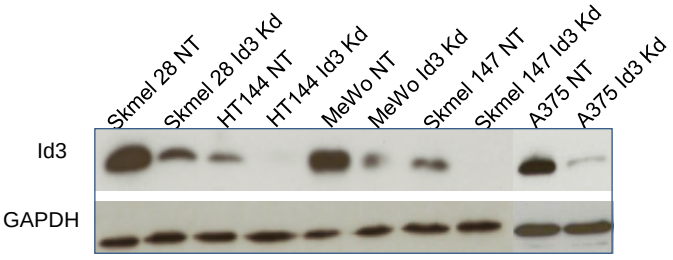
Figure 6: Id1 and Id3 expression in human melanoma patient tissue samples. A. The top panel shows the expression of Id1 in the primary, metastatic melanoma and dermal nevus. B. The bottom panel shows the Id3 expression in the primary, metastatic melanoma and dermal nevus. Images represent 4X and 20X magnification. No secondary antibody staining was used as a negative control.

4.3 Id3 Knock down in melanoma cell lines reduces cellular migration

In order to understand the role of Id3 in melanoma cell migration and proliferation, Id3 was stably knocked down using short-hairpin RNA (shRNA). The shRNA against Id3 was selected from the RNAi consortium which has 100% Specificity-Defining Region (SDR) in the coding sequence of Id3. Subsequently, I chose 5 cell lines with different mutational status and high Id3 expression i.e. A375 (V600E), HT144 (V600E), Skmel 28 (V600E), Skmel 147 ($NRAS^{Q61L}$) and MeWo (WT). Stable transfection of Id3-shRNA was performed using the lentiviral particles. Later, the cells were selected using puromycin and allowed to grow for few days.

The knocking down efficiency of Id3 was validated through western blot technique. It was found that the expression of Id3 was significantly suppressed in all the 5 cell lines compared to their respective controls with non-targeting shRNA (NT) **(Figure 7A)**. From the Image quantification data, it was determined that in 4 (A375, HT144, MeWo and Skmel 147) out of 5 cell lines the Id3 expression was more than 80% reduced, whereas, in Skmel 28 there was more than 60% reduction in the expression **(Figure 7B)**.

A.



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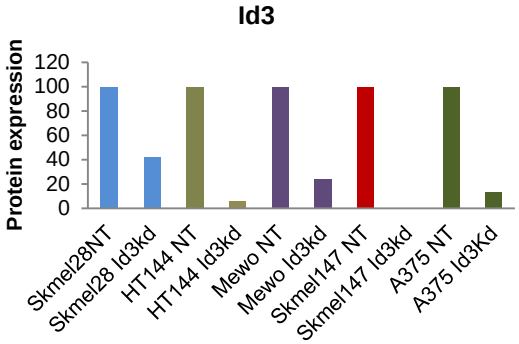
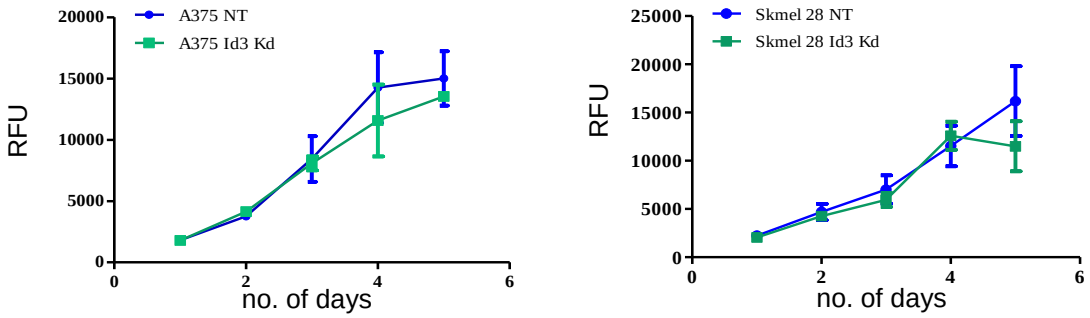


Figure 7: Id3 knockdown in melanoma cell lines. A. Id3 was knockdown in Skmel 28, HT144, MeWo Skmel 147 and A375 cell lines using shRNA against Id3. Id3 protein expression was evaluated using western blot after the knockdown. GAPDH was used as a loading control. B. Percentage of Id3 protein expression was estimated using image J.

It is well-documented that Id proteins play a significant role in inhibiting differentiation and promote proliferation **(Lasorella A et al., 2014)**. Therefore, the effect of Id3 knockdown on proliferation in the melanoma cell lines was first examined. Alamar blue assay (Invitrogen) was performed to study the proliferation. For this, the cells with Id3 shRNA and their controls were seeded and measured their growth after every 24 hours up to 5 days. The results from this assay displayed that there was no significant difference in proliferation between the cell lines with Id3 shRNA and their non-targeting controls **(Figure 8A)**. This observation was further verified through cell cycle analysis using flow cytometry technique. In this experiment, the cells were fixed in 70% ethanol and then stained them using propidium iodide (PI). The flow cytometry data analysis gave the percentage of cells in different cell cycle stages (G0/G1, S and G2/M). Here, no difference was observed in the percentage of cells in different cell cycle stages between Id3 knockdown cell lines and their respective controls **(Figure 9A)**. Hence, the Alamar blue assay and cell cycle analysis confirmed that there was no effect on proliferation or cell cycle after knocking down Id3 alone in melanoma cell lines.

A.



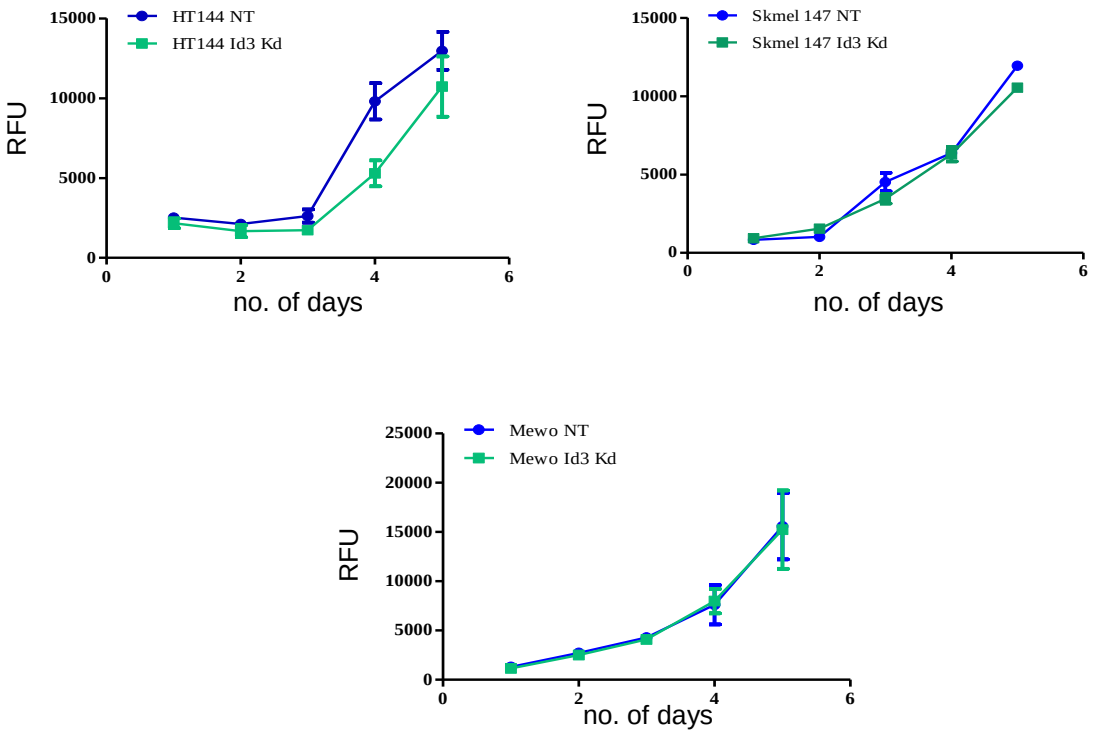
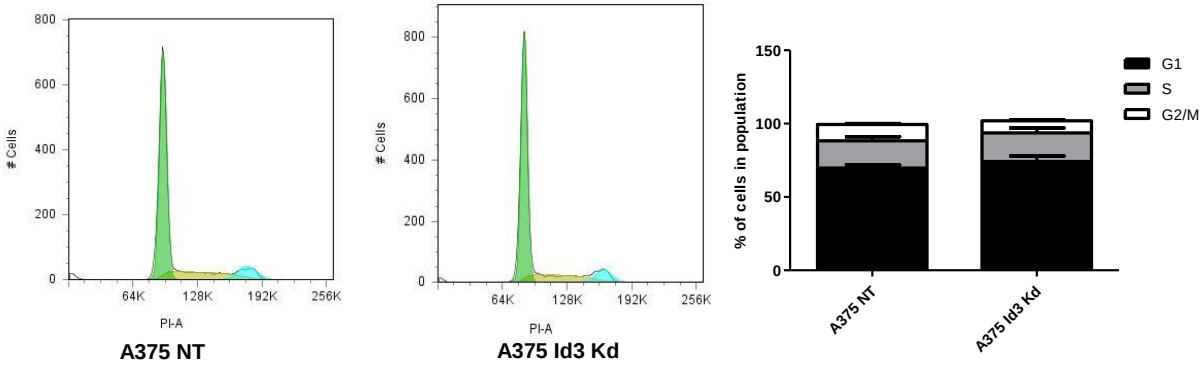


Figure 8: Id3 knockdown in melanoma cell lines does not affect proliferation: A. Alamar blue assay was used to detect the difference in cell proliferation between Id3 knockdown and control cell lines from day 1 to day 5 (A375, Skmel 28, HT144, Skmel 147 and MeWo). The cell proliferation results show the mean + SD of biological triplicates.

A.



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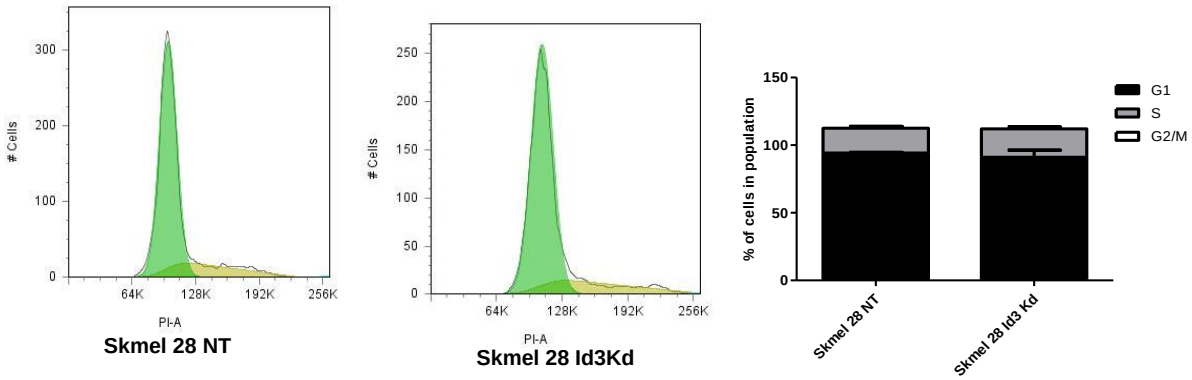


Figure 9: Id3 Knockdown does not alter the cell cycle. A, B. Analysis of cell cycle stages by flow cytometry of A375 and Skmel 28 with Id3 knockdown compared to the non-targeting control. Cells stained with PI were gated based on their DNA content (left panel) and each cell cycle stages were quantified (right panel). The cell cycle results show the mean + SD of biological triplicates.

The earlier report suggests the involvement of Id3 in various cancer metastasis but there is little known about its role in melanoma metastasis. Therefore, I thought to investigate its function during melanoma migration **(Tsuchiya T et al., 2005) (Shuno Y et al., 2010)**. For this, a scratch like assay was performed using ibidi chambers or inserts made up of biocompatible silicone. These inserts help in making a gap of approximately 500 µm in the monolayer of the cells and the closing of the gap due to cell migration was recorded over a period of time. The entire melanoma cell lines knockdown for Id3 and their corresponding controls were seeded using these inserts. After the insert was taken out, I took pictures after every four hours till the time gap was completely closed. The images were analysed using the T-scratch software. It was found that after knocking down Id3 in cell lines A375, Skmel 28 and Skmel 147 the migration was significantly impaired. A375, Skmel 28 and Skmel 147 Id3 Knockdown cells migrate approximately 20-30 % slower compared to their respective controls **(Figure 10A, B).** Whereas in HT144 and MeWo cell lines, a trend in the same direction was observed but the results were not significant (Data not shown here). These findings suggest that silencing of Id3 alone in melanoma cell lines can impair its migration in vitro.

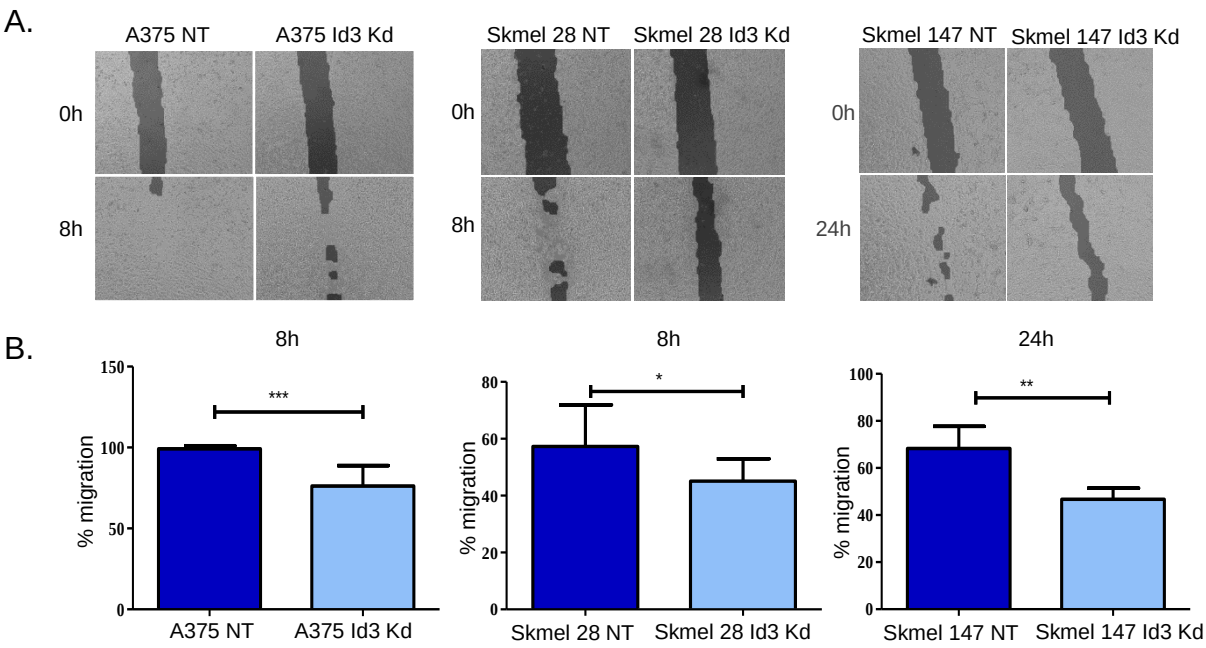


Figure 10: Id3 Knockdown in melanoma cells reduces cell migration. A. Representative images of cell migration assay. Migration of A375 and Skmel 28 cell line with Id3 shRNA (A375 Id3 Kd, Skmel 28 Id3 Kd) and non-targeting shRNA (A375 NT, Skmel 28 Id3 Kd) was measured at 0 hour and 8 hours after creation of the gap. Skmel 147 Id3 Kd and Skmel 147 NT cell migration was measured after 24 hours. B. The bottom panel represents the percentage migration of A375, Skmel 28 and Skmel 147 with Id3 shRNA and non-targeting shRNA. The graph represents the mean + SD of biological triplicates. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant.

4.4 Id3 overexpression in WM266-4 increases cellular migration

After studying Id3 loss of function, Id3 gain of function in melanoma cells were studied. For this, I first chose a human melanoma cell line (WM266-4) which has very low level of endogenous Id3 expression **(Figure 5B, D)**. Next, Id3 coding sequence was cloned in a lentiviral vector (PLX304). The cloning of Id3 was validated by restriction digestion and sequencing. Thereafter, Id3 overexpressing WM266-4 (WM266-4 Id3) cell line was generated and selected using blasticidin. The overexpression of Id3 in WM266-4 was confirmed using qPCR and western blot technique. WM266-4 with Id3 overexpressing vector showed an increase of Id3 mRNA by 1000 folds compared to the empty vector control (WM266-4 EV) **(Figure 11A)**. Equally, the western blot also showed a tremendous increase in the protein

expression of Id3 in WM266-4 cell line with Id3 overexpressing vector compared to its control **(Figure 11B)**.

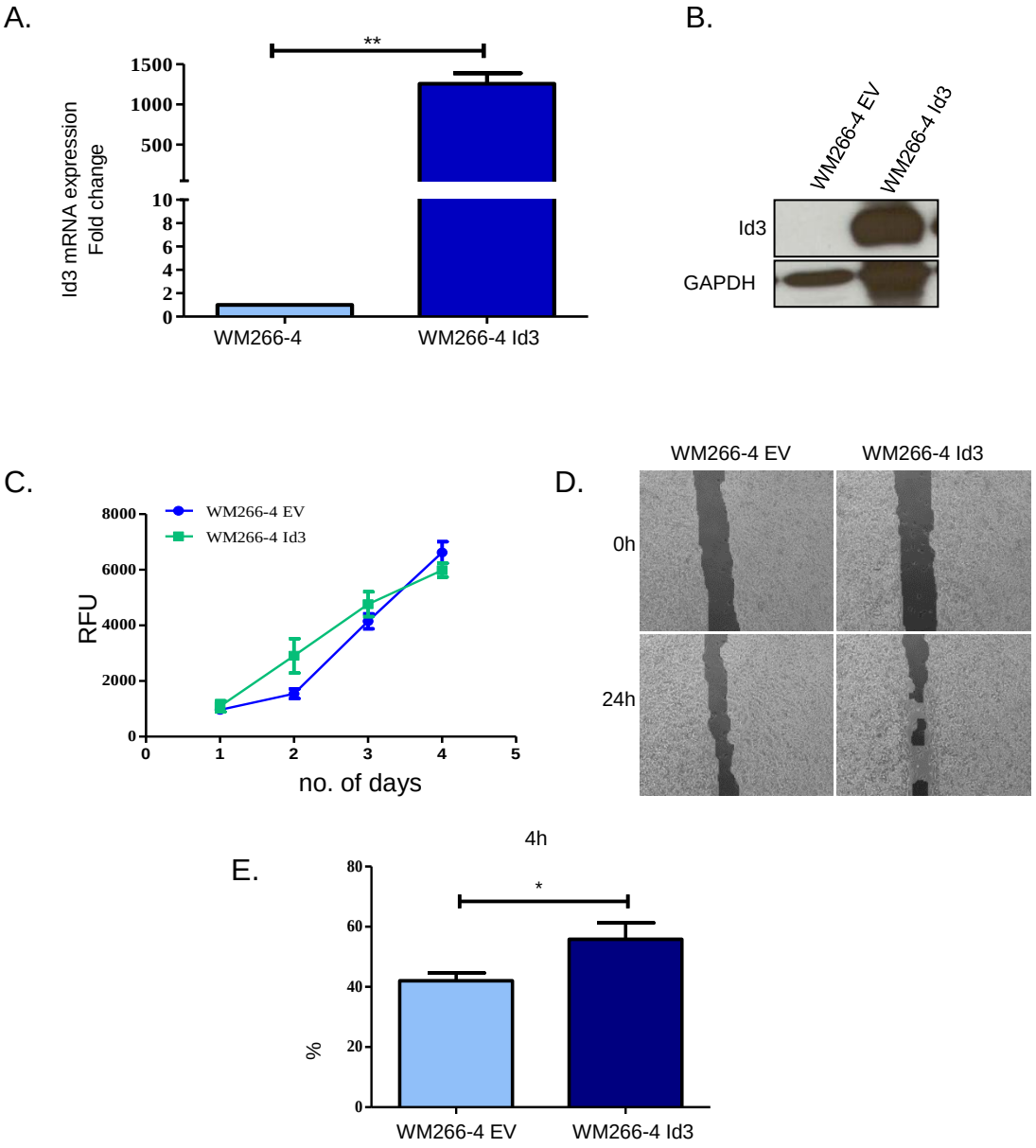


Figure 11: Id3 overexpression in WM266-4 increases cellular migration. A. mRNA expression of Id3 in WM266-4 after transfection with Id3 expressing vector. 18s was used as an endogenous control and WM266-4 with empty vector was used as the reference. The qPCR results showed here are mean + SD of biological triplicates. B. western blot data shows the Id3 protein expression in WM266-4 empty vector (WM266-4 EV) and WM266-4 Id3 overexpressing vector. C. Alamar blue assay was used to detect the difference in cell proliferation between WM266-4 Id3

overexpression and control cell lines from day 1 to day 5 (A375, Smel28, HT144, Skmel 147 and MeWo). C. Representative image of cell migration assay of WM266-4 Id3 and empty vector control (WM266-4 EV). E. Graph represents the percentage migration of WM266-4 Id3 and empty vector control (WM266-4 EV). The graph show the mean + SD of biological triplicates. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant.

To further understand if Id3 overexpression would affect the proliferation in WM266-4, Alamar blue assay was performed and analysed the cell cycle in Id3 overexpressing and control cells. Alamar blue assay results showed that there was no significant difference in the proliferation rate of Id3 overexpressing WM266-4 compared to the empty vector control **(Figure 11C)**. For cell cycle analysis, the cells were fixed with 70% ethanol and stained them with propidium iodide (PI). The flow cytometry data confirmed that there was also no difference in the percentage of cells in different cell cycle stages between WM266-4 Id3 and WM266-4 EV **(Figure 12A)**. This result was in accordance with the previous knockdown results which showed that Id3 alone cannot affect the proliferation of melanoma cells.

Now I wanted to check if the Id3 overexpression in WM266-4 cell line affects the cell migration or not. Again, the scratch like assay using ibidi chambers/inserts was performed. In **figure 11D & E**, it is evident that Id3 overexpression in WM266-4 increases the migration rate by approximately 20% compared to the empty vector control. These findings highlight that Id3 plays a critical role in melanoma migration.

A.

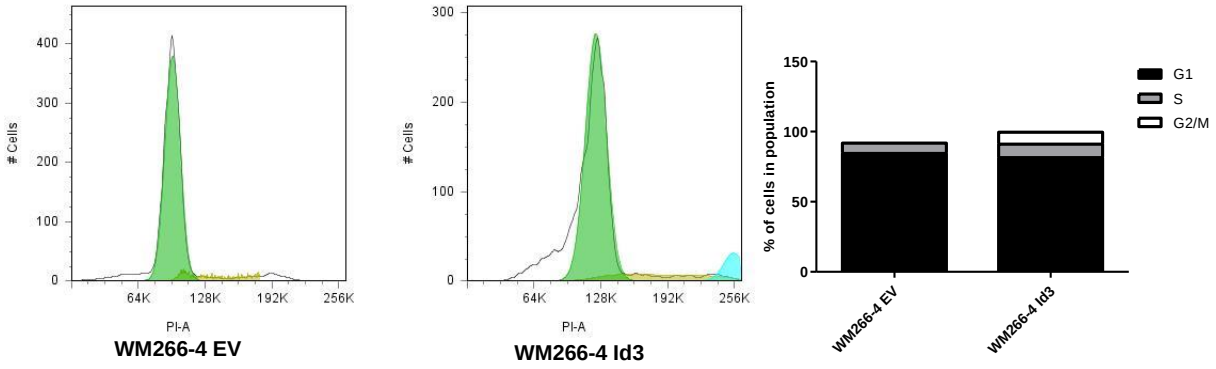


Figure 12: Id3 overexpression doesn't alter the cell cycle. A. Analysis of cell cycle stages by flow cytometry of WM266-4 with Id3 overexpressing vector compared to the empty vector control. Cells stained with propidium iodide were gated based on their DNA content (left panel) and each cell cycle stages were quantified (right panel).

4.5 Id3 expression in melanoma cells controls SOX10 expression

To determine the underlying molecular mechanism behind Id3 influencing the melanoma cell migration, a large set of genes associated with melanoma migration were screened and checked the basal protein expression of some of these genes **(Supplementary Figure 4)**. The screening result showed that the expression of Id3 in 5 melanoma cell lines is significantly inversely correlated to the SOX10 expression **(Figure 13D, left panel)**. Moreover, MITF which is the downstream target of SOX10 was also found to be inversely correlated to Id3 expression in the melanoma cell panel **(Figure 13D, right panel)**. This inverse correlation was further confirmed from the human metastatic melanoma database. A significant inverse correlation exists between Id3 and SOX10/MITF in 52 metastatic melanoma patients sample **(Figure 10 E).** Another important melanoma patient database, the cancer genome atlas (TCGA), also demonstrated an inverse correlation between Id3 and MITF by RNA sequencing results **(Akbani et al., 2015)**.

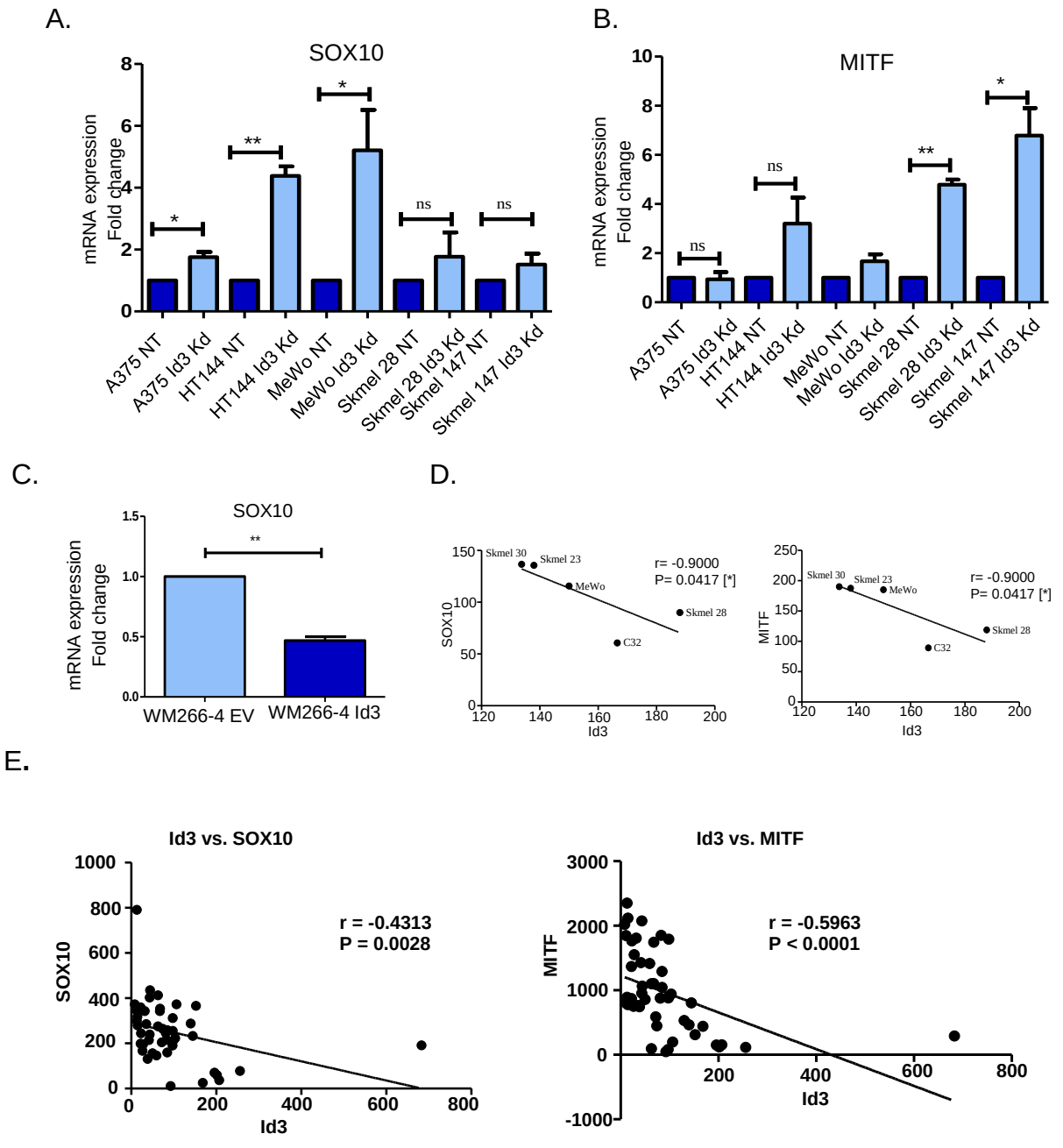


Figure 13: Id3 knockdown or overexpression regulates SOX10 and MITF expression in melanoma cell lines. A. Quantitative real-time PCR analysis of SOX10 expression in Id3 knockdown melanoma cell lines (A375, HT144, MeWo, Skmel28 NT, Skmel 147). B. Quantitative real-time PCR analysis of MITF expression in Id3 knockdown melanoma cell lines (A375, HT144, MeWo, Skmel28 NT, Skmel 147). 18s was used as endogenous control and cell lines with non-targeting shRNA (NT) were used as reference control. C. qPCR analysis of SOX10 expression in WM266-4 Id3 overexpressing cell line. 18s was used as endogenous control and WM266-4 with empty vector was used as a reference. The qPCR results shown here

are mean of + SD of biological triplicates. D. Protein expression of Id3 in melanoma cell lines is inversely correlated to SOX10 and MITF. E. mRNA expression of Id3 in metastatic melanoma patients is inversely correlated to SOX10 (left panel) and MITF (right panel) (GEO dataset- GDS3966). *P<0.05, **P<0.01, ***P<0.001 and ns = not significant. (Spearman correlation =r)

Finally, the expression of SOX10 and MITF in Id3 knockdown and overexpressing cell lines was examined. Indeed, the expression of SOX10 was higher in cell lines (A375, HT144, MeWo, Skmel28 and Skmel147) with Id3 knockdown compared to the control cell lines **(Figure 13A)**. Whereas its expression is almost reduced by 50% in WM266-4 with Id3 overexpression compared to WM266-4 with empty vector **(Figure 13 C)**. Additionally, the qPCR data validated 3-5 fold increase in the mRNA expression of MITF in 3 out of 5 Id3 knockdown cell lines **(Figure 13 B)**.

A. B.

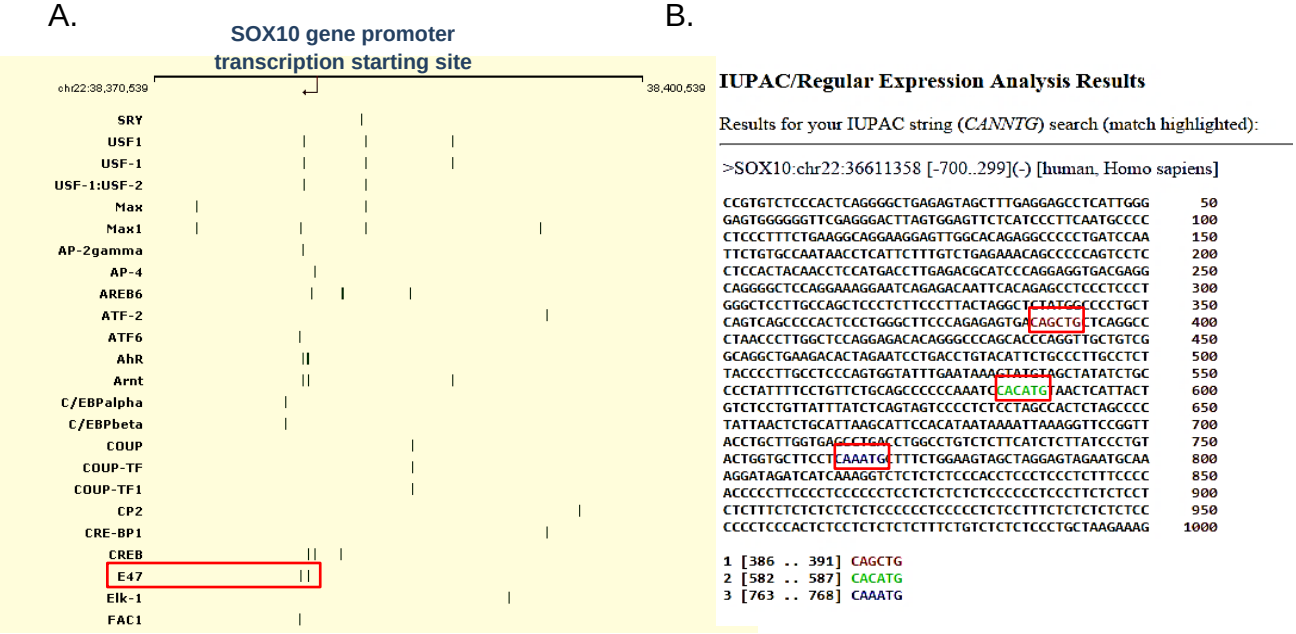


Figure 14: Probable E47 binding site on SOX10 promoter sequence. A. Two E47 binding sites (red box) on SOX10 promoter sequence predicted using Qiagen prediction tool. B. Transcriptional Regulatory Element Database (TRED) shows three E47 binding sites on SOX10 promoter sequence.

To better understand the correlation between Id3 and SOX10 I did some in silico search. For this, I looked for the transcription factors that regulate SOX10 expression. Using the prediction tool from Qiagen for the transcription factor binding site it was found that SOX10 has an E47 binding site in its promoter site **(Figure 14A)**. This data was further validated using Transcriptional Regulatory Element Database (TRED) where three CANNTG sequence in the SOX10 promoter sequence was found **(Figure 14B)**. It has been well documented that Id3 negatively regulates E47 by heterodimerizing and E47 mainly binds to the E- box motif (CANNTG) in the promoter region. Above findings suggest that Id3 could regulate the SOX10 expression by preventing E47 from binding to the SOX10 promoter.

4.6 Id3 Knockdown sensitizes melanoma cells to the targeted therapy.

In order to verify the effect of vemurafenib or trametinib on Id3 knockdown or overexpressing melanoma cells, the cell viability assay was conducted. For this, the BRAF mutated cell lines with different concentration (0.001, 0.01, 0.1, 1 and 10µM) of BRAF inhibitor (Vemurafenib) and the NRAS mutated cell line with MEK-inhibitor (Trametinib) were treated. The cell viability was tested using Alamar blue. It was found that Id3 knockdown cell line, A375, after 48 hours of treatment showed greater sensitivity to the vemurafenib compared to the non-targeting control. Similarly, Id3 knockdown cell line Skmel 28 and HT144 were more sensitive to vemurafenib after 96 hours of treatment in comparison to their non-targeting controls. Interestingly, Skmel 147 Id3 knockdown cell line were more sensitivity to the trametinib after 96 hours compared to the Skmel 147 non-targeting cell line **(Figure 15A)**. Lastly, it was also found that upon Id3 overexpression in WM266-4 the cell survival rate after treatment with vemurafenib was increased significantly compared to the WM266-4 cells with empty vector **(Figure 15B)**. These results indicate that Id3 expression in melanoma cells have profound effect during tumour progression and drug resistance.

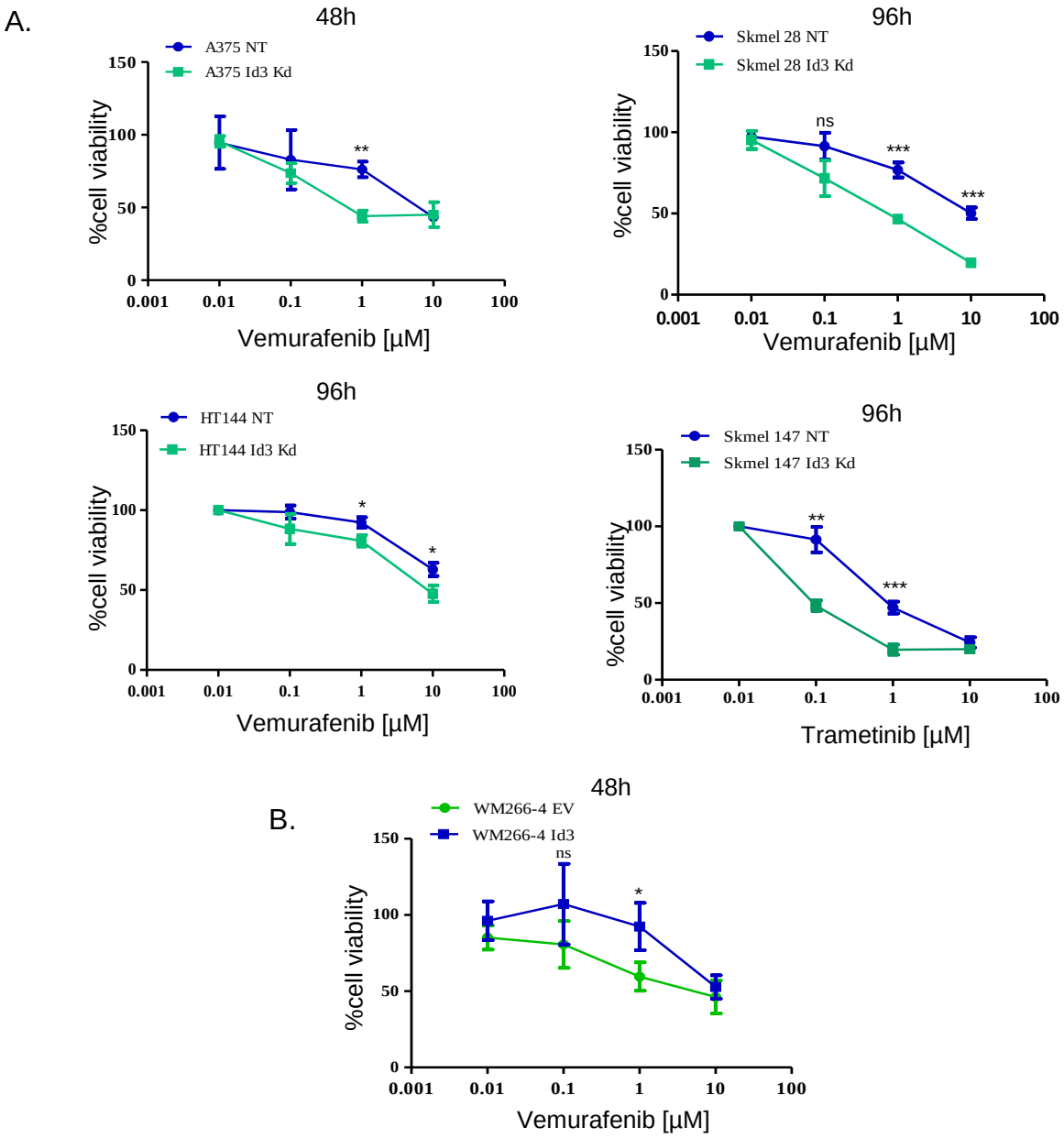


Figure 15: Id3 expression in melanoma cell lines provides resistance to the targeted therapy. A. The graph represents the effect of vemurafenib treatment (0.01-10 µM) after 48 hours (A375) or 96 hours (HT144, SKmel 28 and Skmel 147) on the viability of Id3 knockdown or control cell lines, assessed by Alamar blue staining. The results are shown as mean + SD of biological triplicates. B. The graph represents the effect of vemurafenib treatment (0.01-10 µM) after 48 hours (WM266-4) on Id3 overexpressing cell lines viability. The results are shown as mean + SD of biological triplicates. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant.

5. Discussion

Currently, different approaches have been involved in the treatment of melanoma patients as melanoma tumour displays numerous complexities at genetic as well as at immune level. The new treatments exhibit remarkable efficacy but resistance developed in patients restricts the benefits from these approaches. To identify the mechanism behind the gain of resistance, different theories and research models have been presented. A majority of publication supports that the resistance shown by melanoma patients, especially to the targeted therapies, is due to dedifferentiation. Like in other cancer types, it has been found that tumour cells in melanoma patients also retain some of the phenotypes similar to multipotent cells **(Landsberg J et al., 2012) (Lehraiki et al., 2015) (Fallahi-Sichani et al., 2017)**. Melanoma cells are transformed from melanocytes which are differentiated from the neural crest cells. The neural crest cells are multipotent, transient and migratory cell population developed during the neural tube formation. Melanoma cells regain some of these features of neural crest cells in order to metastasize and acquire resistance from the therapies. Here, I have identified new dedifferentiation marker in melanoma cells which could potentially lead to metastasis and resistance to the melanoma therapy.

5.1 Identification of novel marker for dedifferentiation and resistance in melanoma

To date, several markers like CD271, CD133 ABCB5 and ALDH have been associated with the melanoma dedifferentiation. The dedifferentiated population of melanoma cells shows the embryonic phenotype **(Boiko et al., 2010) (Monzani et al., 2007) (Frank et al., 2005)**. They are more migratory, invasive and resistant to the therapies. Due to ethical limitations in human it is not possible to isolate the multipotent neural crest cells and study the link with melanoma pathogenesis.

The reprogramming of somatic cells using Yamanaka factors provides a new approach to study the role of dedifferentiation in cancer cells. Besides, this technique opens a new insight in the study of developmental biology by subsiding the ethical issues (of using human embryonic stem cells). Previously in our lab, human induced

pluripotent stem cells (HiPSCs) were generated and differentiated into neural crest cells under controlled conditions. The neural crest cells were identified using two different cell surface markers (CD271 and HNK1) **(Larribere and Utikal, 2014) (Larribere et al., 2015)**.

On the other hand, melanoma cell lines were treated with a high dose of BRAF-inhibitor for a period of 72hours **(Figure 4A)**. This process helped in the selection of most resistant and dedifferentiated cell population as the inhibitor can easily eliminate the normal tumour cells at this drug concentration **(Supplementary Figure 1)** but not the dedifferentiated population. This was also shown in the gene profiling data where the neural crest cells clustered together with the vemurafenib-treated cells **(Figure 4B)**. This shows that the cell survived after vemurafenib treatment was more dedifferentiated than the non-treated or DMSO-treated human melanoma cells. This was strongly supported by another finding where it was shown that the pluripotency or multipotency markers (LIN28, SOX2, DNMT38, TWIST1and POU5F1) were upregulated in the dedifferentiated group of cells. In addition, the melanocytic or differentiated markers (DCT, MC1R, MITF, TYR, SOX10 and TYRP1) were downregulated in the dedifferentiated group of cells **(Figure 4C)**. It is well known that the expression of SOX2, LIN28 and POU5F1 has been implicated in dedifferentiation of pancreatic cancer cells, Müller glia and embryonic stem cells respectively **(Herreros-Villanueva et al., 2013) (Ramachandran et al., 2010) (Niwa et al., 2000)**. Indeed the melanocyte differentiation from neural crest cells requires the expression of SOX10 and its downstream gene MITF, DCT, TYR and TYRP1. Interestingly, TWIST1 is another gene associated during embryonic development. It is upregulated in many cancers and promotes metastasis through epithelial to mesenchymal transition (EMT) **(Yang et al., 2006)**. Neural crest cells are migratory in nature and express TWIST 1 during entire migratory pjase **(Vincentz et al., 2013)**. Furthermore, the findings show that AXL expression was upregulated in the vemurafenib-treated cells which are in accordance with the earlier findings where it was described that high AXL and low MITF expression is an early indication of drug resistance in BRAF and NRAS mutated cell lines **(Müller et al., 2014)**. Contrastingly, many reports suggest an elevated level of MITF in drug resistant melanoma cells **(Johannessen et al., 2013) (Van Allen et al., 2014)**. This discrepancy could arise due to the difference in the experimental procedures. As I have used BRAF inhibitor

PLX4032 (vemurafenib) and not PLX4720. Additionally, the difference of drug concentration for the treatment in this study could also affect the end results.

Inhibitor of differentiation (Id) proteins is expressed in stem cells and maintains the stemness by inhibiting the differentiation. In Xenopus embryo, Id3 was shown as an essential gene for the proliferation and survival of the neural crest cells **(Kee and Bronner-Fraser et al., 2005)**. It has been described to play a quintessential role in cancer-initiating cells and drug resistance. This was reflected in the result as well where Id1, Id2 and Id3 expression were upregulated in vemurafenib treated cells. Id1 and Id2 expression have been well studied in melanoma progression and associated with the suppression of tumour suppressor genes. Here, the expression of Id3 was found to be elevated in the dedifferentiated cells. In my results, I also demonstrated the elevated mRNA expression of Id3 in BRAF mutated cell lines (Skmel 28, HT144, C32, A375 and WM266-4) after treatment with high dose of vemurafenib. Remarkably, the upregulation of Id3 initiates as early as 6 hours after treatment and retained after 24hours, 48hours and 72 hours of vemurafenib treatment. Although HT144 cell line does not show significant upregulation of Id3 after 72 hours of vemurafenib treatment **(Figure 4D)**. This could be because of another resistance mechanism involved in this cell line and further investigation is required. This was also true for the NRAS mutated cell lines as the Id3 expression increases after treatment with the MEK-inhibitor (Trametinib). Formerly, Id3 role in drug resistance has been well documented in colon cancer and Non-small cell lung cancer **(O'Brien et al., 2012) (Castañon et al., 2013)**. These findings suggest that Id3 expression is higher in dedifferentiated melanoma cells and upregulated during adaptive resistance.

5.2 Id3 expression mediates migration in melanoma cells

Id proteins are highly expressed during development but usually found to be low or downregulated in terminally differentiated cells. Although their expression in several cancer types is dysregulated and supports the tumour cells in proliferation and metastasis. For example in hepatocellular cancer Id1 expression is upregulated compared to the normal liver cells **(Sharma et al., 2016)**. Similarly, in melanoma

cells, Id1 is expressed in the tumour cells but melanocytes do not show any expression of Id1 **(Straume and Akslen, 2005)**. Id2 and Id3 expression was variable in melanoma cell lines but very low or not expressed in human melanocytes **(DiVito et al., 2014)**. Tumour suppressor genes are more often inactivated in cancer, Id1 transcriptionally inactivates p16INK4a in melanoma whereas Id2 suppresses Rb protein in neuroblastoma **(Polsky et al., 2001) (Lasorella et al., 2000)**. In this study, it was demonstrated that both at mRNA and protein level Id1 and Id3 expression was elevated in most of the human melanoma cell lines compared to the normal human melanocytes (NHM). In addition, Id1 and Id3 expression in dermal nevus, primary and metastatic melanoma patient samples were found **(Figure 5 and 6)**. However, their expression in dermal nevus could indicate that Id1 and Id3 are essential during the early transformation of melanocyte to melanoma. To date not enough data has been published which explains the mechanism of Id3 in melanoma progression. I performed functional experiments to study the role of Id3 in melanoma pathogenesis. Melanoma cell lines (A375, Skmel 28, HT144, Skmel147 and MeWo) upon knocking down Id3 using shRNA do not show any effect on proliferation **(Figure 8)**. This result was further confirmed by cell cycle analysis where I didn't see any difference in the percentage of cells in the different phase of cell cycle **(Figure 9)**. Also, WM266-4 cell lines displayed no alteration in proliferation or cell cycle phases upon overexpression of Id3 **(Figure 11C and 12)**. Taken together these results suggest that Id3 alone is not essential for proliferation and cell cycle in melanoma cell lines. The probable explanation could be that Id1 expression in Id3 knockdown cell lines compensate for the loss of Id3 since they share 69% homology **(Supplementary Figure 5)**.

However, melanoma cell lines (A375, Skmel 28 and Skmel 147) showed significantly reduced cellular migration upon knocking down Id3 using shRNA **(Figure 10)**. In accordance, a significant increase in cellular migration was observed in WM266-4 cell line on ectopic expression of Id3 **(Figure 11 D, E)**. These results indicate that Id3 expression in melanoma cell lines is essential for the cellular migration.

5.3 Id3 confers dedifferentiation in melanoma cells by regulating SOX10 and MITF expression

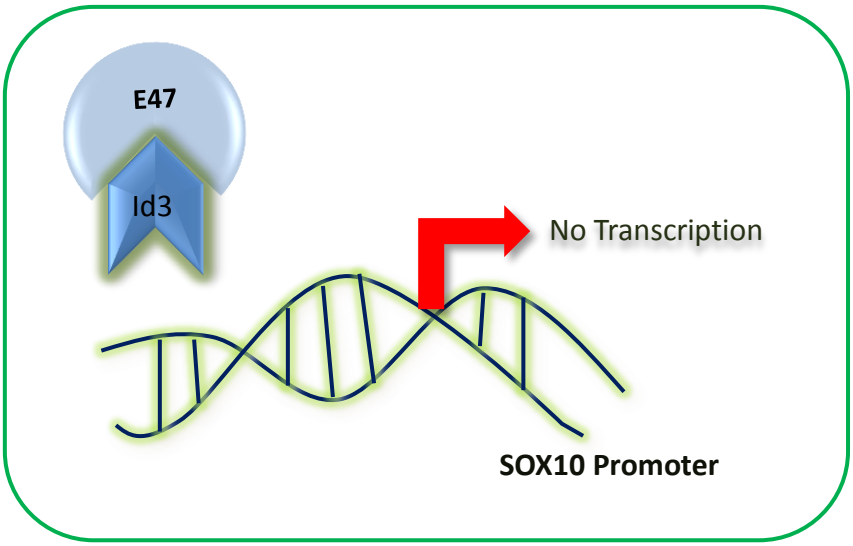
Dedifferentiating cells are more migratory in nature compared to the terminally differentiated cells. Here, it was shown that melanoma cells upon Id3 knockdown migrate slower compared to the melanoma cell line with Id3. Several factors are involved in melanoma migration and to analyse the mechanism behind melanoma migration involving Id3, a large set of melanoma associated genes were screened. Interestingly, in this screening, it was identified that SOX10 gene which was inversely correlated with Id3 expression. Furthermore, MITF, downstream target of SOX10, was also inversely correlated to Id3 in melanoma. MITF transcription factor is a master regulator of melanogenesis which directly controls the activation of important enzymes like DCT, TYR and TYRP1 **(Bondurand et al., 2000)**.

In the panel of human melanoma cell lines, it was shown that the endogenous level of Id3 expression is inversely correlated to SOX10 and MITF. This result was further confirmed in the Id3 knockdown and overexpressing cell lines. Three out of five cell lines (A375, HT144 and MeWo) showed a significant increase in the SOX10 expression after Id3 Knockdown **(Figure 13A)**. Conversely, WM266-4 cell line after Id3 overexpression displayed almost 50% reduction in SOX10 expression **(Figure 13C)**. Since MITF has SOX10 binding sites on its promoter sites, I also checked the expression of MITF in Id3 knockdown cell lines. Here it was found that MITF expression was upregulated in three out of 5 cell lines **(Figure 13B)**. Lastly, the inverse correlation of Id3 with SOX10 and MITF was confirmed using the in silico data analyses of RNA expression from metastatic melanoma patients **(Figure 13E)**. As discussed earlier that MITF is required for the differentiation of melanocytes whereas Id3 is the inhibitor of differentiation, my result suggests that in melanoma Id3 stops the differentiation by downregulating MITF and SOX10 expression.

Id3 lacks the basic domain in its structure and it cannot bind directly to the DNA or promoter of any gene. However, it binds to other basic helix-loop-helix transcription factor and inhibits them from binding to the promoter of the target gene. It is well documented that Id3 binds to the E2A (E47/E12) protein and stops it from binding to E-box of DNA **(Loveys et al., 1996)**. This protein-protein interaction is known to stop

differentiation in cells. Taking this in consideration I again performed some in silico search and found that SOX10 has E47 binding sites in its promoter region.

State-1



State-2

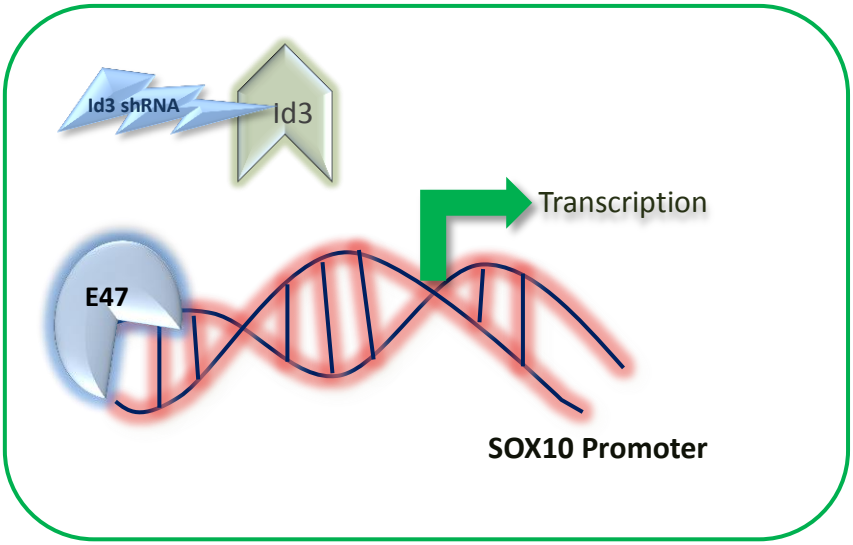


Figure 17: Id3-mediated regulation of SOX10. State-1 represents when Id3 is present in excess inside the nucleus where it binds to the E47 transcription factor and inhibits the SOX10 activation by inhibiting E47 from binding to the promoter of SOX10. State-2 represents when Id3 is knocked down using shRNA and E47 can bind to the promoter of SOX10 which starts the transcription and further activates the downstream genes like MITF.

E47 binds to the E-box motif (5-CANNTG-3) on promoter sequence and using another database search it was found that SOX10 promoter has three CANNTG motifs (CAGCTG, CACATG and CAAATG) **(Figure 14)**. Taking together these observations I propose that Id3 regulates SOX10 expression in melanoma by inhibiting E47 from binding to SOX10 promoter and stopping the differentiation. This is described by a schematic representation but further research is required to be done to formally prove it **(Figure 17)**.

5.4 Id3 in melanoma induces drug resistance to target therapies

This study presents a direct connection between Id3 expression in melanoma cells and resistance to the targeted therapy. The role of Id1 in retaining chemoresistance has been well documented before but to our understanding, Id3 role in resistance to melanoma target therapy has never been explored. In my results, it was presented that BRAF mutated cell lines (A375, Skmel 28 and HT144) are more sensitive to the vemurafenib after knocking down Id3 gene **(Figure 15A)**. Whereas, WM266-4 cell lines show more resistance to the vemurafenib treatment after the overexpression of Id3 gene **(Figure 15B)**. On the other hand, it was found that NRAS mutated cell line SKmel 147 also become more sensitive to the MEK-inhibitor (Trametinib) after silencing Id3 gene **(Figure 15A)**.

This is the first time shown that Id3 gene expression in melanoma cell lines helps in maintaining the dedifferentiated state by regulating the expression of SOX10 and MITF, the most significant genes for the melanocyte differentiation. Although SOX10 involvement in resistance to BRAF and MEK inhibitor has been described before. **Sun et al in 2014** found that after knocking down SOX10 in melanoma cells, EGFR and PDGFRB expression were upregulated via Tgf-β and conferred resistance.

As it was highlighted in the introduction, ABCB5 is the drug efflux transporter and is crucial for stem cells maintenance. It is also upregulated in several cancers and confers resistance to the therapy. Here, using the GEO data set it was found that after stably knocking down ABCB5 in A375, the Id3 expression is significantly downregulated compared to the vector control. Whereas, the expression of melanocyte differentiation markers i.e. SOX10 and MITF significantly increases after

ABCB5 suppression **(Supplementary Figure 6) (Wilson et al., 2014)**. These data are in agreement with the findings where it was shown that Id3 is upregulated upon treatment with BRAF or MEK inhibitors and inversely correlated with the expression of SOX10 and MITF.

In conclusion, this study reveals that Id3 expression in melanoma is crucial for the dedifferentiation which is responsible for the resistance to the targeted therapy like BRAF and MEK inhibitors. In addition, it was found that Id3 also regulates SOX10 and MITF expression (potentially via E47) to keep the cells in the more dedifferentiated state.

6. Summary

Melanoma is the deadliest form of skin cancer which is transformed from the melanocytes. Sunburn is one of the leading causes of melanoma. Patients acquire various mutations during melanoma pathogenesis which are responsible for the tumour progression, metastasis and invasion. Additionally, a subpopulation of melanoma cells is dedifferentiated due to the recapitulation of expression pattern also found in neural crest cells. Despite several targeted and immunotherapies have been approved for the treatment of metastatic melanoma, the gain of resistance reduces the efficiency of these drugs. A number of reports suggest the role of dedifferentiation in resistance to the therapies but the exact mechanism behind this is poorly understood. Therefore, in this study, a new protein was identified which plays role in dedifferentiation and resistance in melanoma cells.

In this project, it was shown that Id3 expression is upregulated in melanoma cells compared to the human melanocytes. Also, it was found that Id3 expression is higher in primary and metastatic melanoma patient samples as well as in dermal melanocytic nevus cells. By means of gain and loss of function studies, it was shown that Id3 alone does not alter the proliferation or cell cycle in melanoma cells. However, Id3 expression in melanoma cells promotes cellular migration. On screening a large set of melanoma associated genes it was revealed that Id3 expression is inversely proportional with the melanocyte differentiation marker gene-SOX10 and MITF.

It was also demonstrated that Id3 expression is upregulated in the BRAF and NRAS mutated cell lines upon treatment with vemurafenib and trametinib respectively. Also, from the expression analysis, it was shown that higher Id3 expressing cell lines after treatment with inhibitors display dedifferentiated phenotype. Further, I confirmed the role of Id3 expression in vemurafenib or trametinib resistance by showing that after knocking down or overexpressing Id3 in melanoma cell line sensitizes or confer resistant respectively to the treatments. These findings highlight the importance of Id3 in melanoma resistance against targeted therapy and inhibition of Id3 clinically in metastatic melanoma patients could increase the efficacy of the current treatment.

7. References

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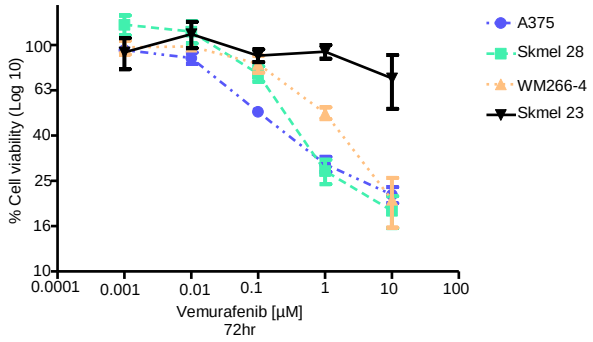
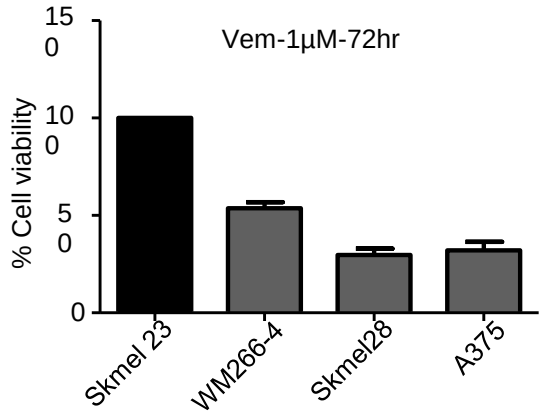
8. Appendix:

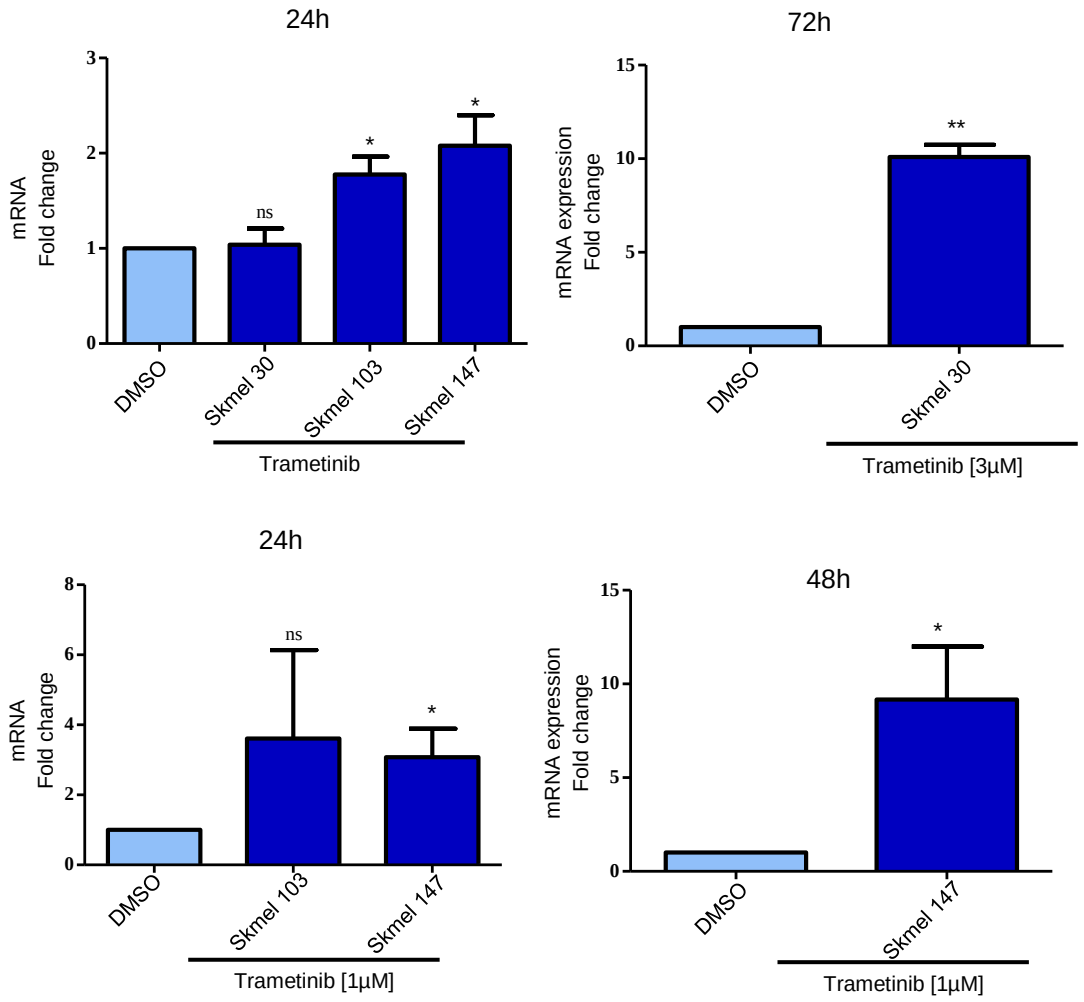
A.



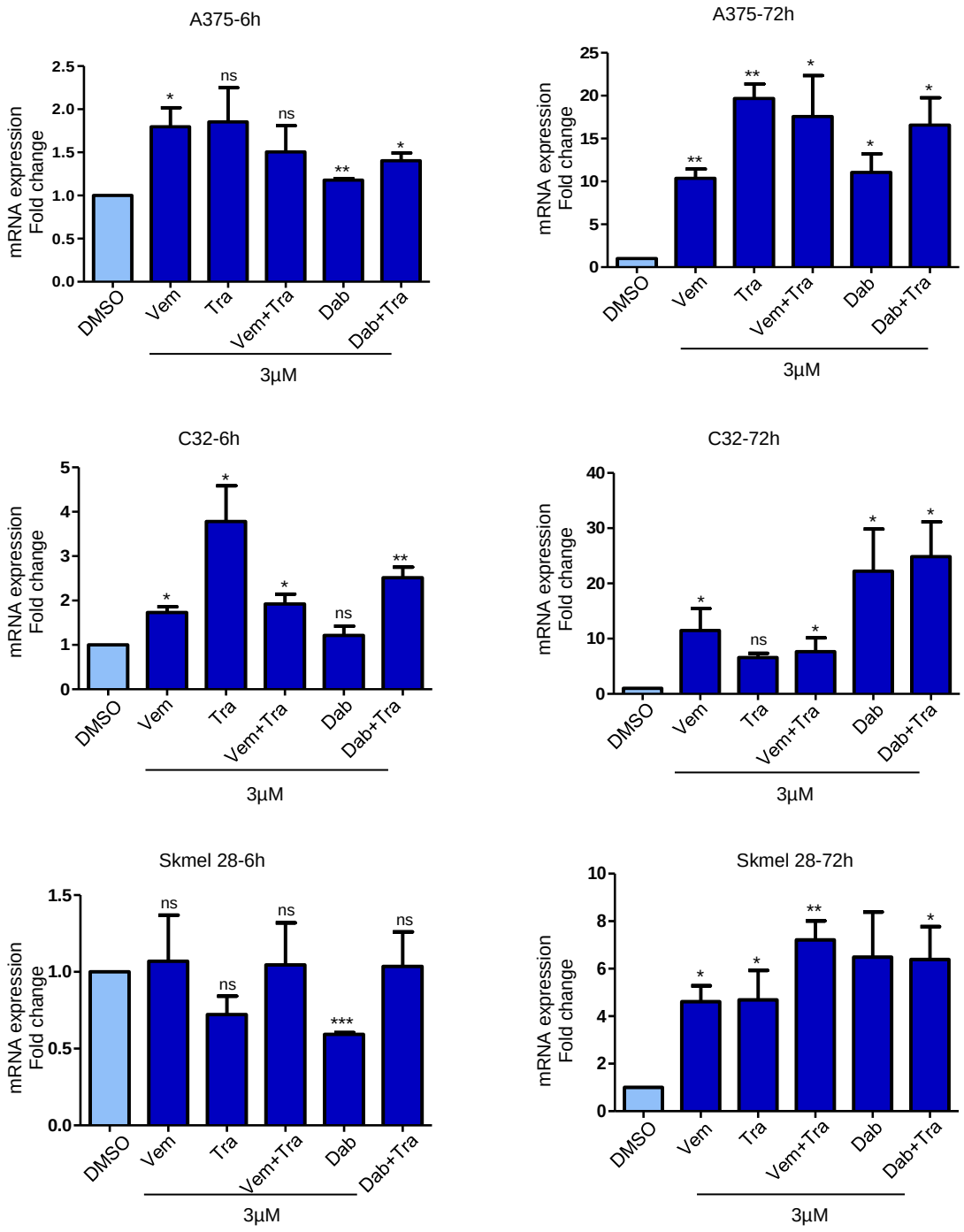
B.



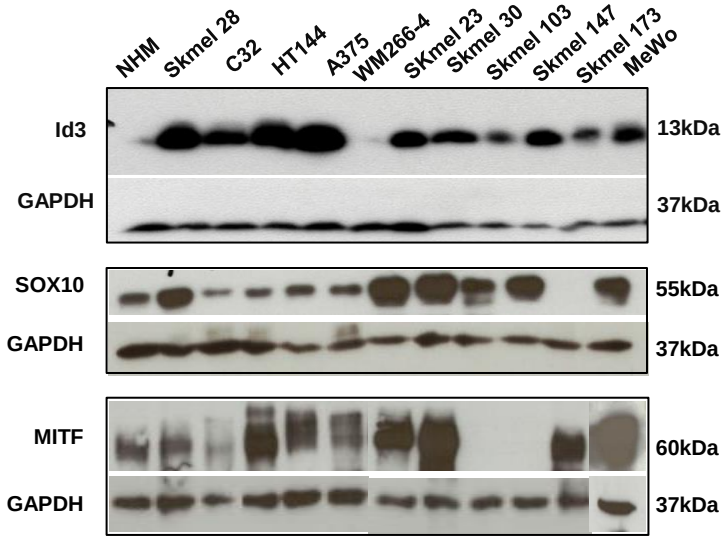
Supplementary Figure 1: Efficacy of vemurafenib treatment. A. Curve represents the viability of BRAF mutated melanoma cell lines (A375, Skmel28 and WM266-4) and BRAF wild type cell line (Skmel 23) after vemurafenib treatment (0- 10 µM) after 72h. B. Graph represents the percentage of cells survived after 72h of treatment with vemurafenib (1µM). Skmel 23 was used as a negative control. The cell viability results shown here are mean of +SD of biological replicates.



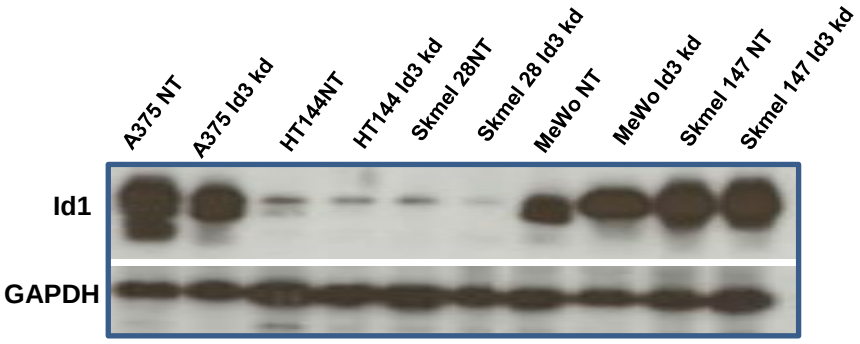
Supplementary Figure 2: Id3 expression is upregulated during adaptive resistance. Graph represents the mRNA expression of Id3 in NRAS mutated cell lines (Skmel 30, Skmel 103 and Skmel 147 after treatment with Trametinib (3µM or 1µM). 18s was used as an endogenous expression control and DMSO treated cells were used as reference sample. The qPCR results shown here are mean of + SD of biological triplicates. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant.



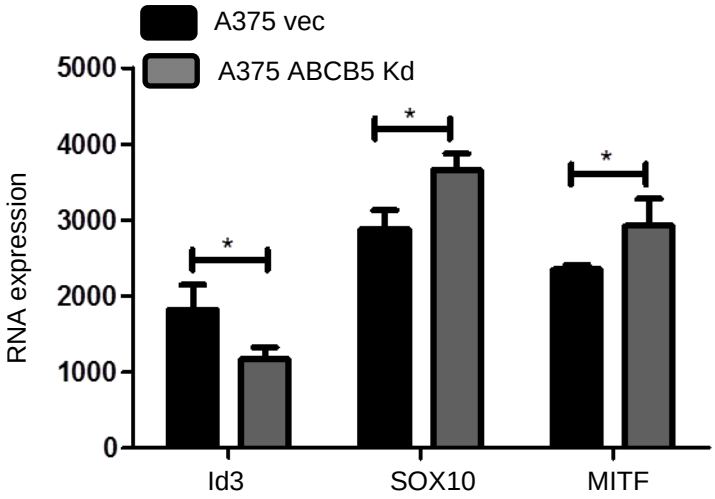
Supplementary Figure 3: Id3 expression is upregulated during adaptive resistance. Id3 mRNA expression in BRAF mutated melanoma cell lines (A375, C32 and Skmel 28) after 6 hours and 72 hours of treatment with BRAF or MEK inhibitor alone or in combination. 18s was used as an endogenous expression control and DMSO treated cells were used as reference sample. The qPCR results shown here are mean of + SD of biological triplicates. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant.



Supplementary Figure 4: Id3, SOX10 and MITF protein expression. Protein expression of Id3, SOX10 and MITF in NHM and human melanoma cell lines. GAPDH was used as a loading control.



Supplementary Figure 5: Unaffected Id1 expression in Id3 knockdown cell lines. Protein expression of Id1 in melanoma cell lines with Id3 knockdown and control cell lines. GAPDH was used as loading control.



Supplementary Figure 6: Melanocyte differentiation marker is upregulated in drug sensitive cells. Dataset used was from GSE38290. mRNA expression of Id3, SOX10 and MITF in A375 cell line after knocking down ABCB5 gene. The results are shown as mean + SD of technical triplicates. *P<0.05, **P<0.01, ***P<0.001 and ns = not significant.

9. Curriculum Vitae

Personal details:

Name:	Sachindra
Date of Birth:	25th September 1986
Gender:	Male
Nationality:	Indian
Marital Status:	Married
Father:	Arun kumar sinha
Mother:	Shanti sinha

Eduacation:

2012-2013:	Masters in Translational Medical Research, University of Heidelberg, Germany.
2004-2008:	Bachelor in Technology, Biotechnology, Allahabad Agricultural Institute-Deemed University, Allahabad, India.
2001-2003:	Intermediate in Science, B.N. College, Patna University, Patna, India.
1990-2001:	DAV Public School, Ara, India.

WORK EXPERIENCE

Mar 2011-Jul 2012	Application Specialist for Cell and Imaging at Imperial life sciences Pvt. Ltd., Gurgaon, India
Feb 2010-Feb 2011	Technical Specialist for In Vivo imaging system at National Center for Cell Sciences (NCCS), Pune, India
Feb 2009-Aug 2009	Junior scientist animal house department at Raj biotech Pvt. Ltd., Pune, India
July 2008-Jan 2009	Training manager at Biobrainz life sciences, Lucknow, India

10. Acknowledgments

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My PhD would be incomplete without thanking my group members for their great help, support and fun. I would especially express my deepest appreciation for **Dr. Lionel Larribere**, who always mentored me and made an enormous contribution to my work through his insightful comments. Also thanks for all the laughs and fun we shared in the office. I am particularly thankful to **Dr. Elias Orouji** for his generous support, encouragement and suggestions during my work. I am grateful for the assistance given by **Dr. Daniel Novak** for cloning and all the fun moments (sports, you tubing etc. etc.), we shared during my thesis.

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