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Yaqi Tang

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N°d'ordre



NNT : 2021LYSE1265

## **THESE de DOCTORAT DE L'UNIVERSITE DE LYON**

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Soutenue publiquement le 07/12/2021, par :

**Yaqi TANG**

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# **Caractérisation des mécanismes d'action du facteur de transcription ZEB1 dans le mélanome**

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Cancer cell plasticity in melanoma

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# Table of contents

<b>ACKNOWLEDGMENT .....</b>	<b>3</b>
<b>TABLE OF CONTENTS .....</b>	<b>6</b>
<b>TABLE OF ILLUSTRATIONS.....</b>	<b>8</b>
<b>INTRODUCTION.....</b>	<b>10</b>
<b>CHAPTER1. CUTANEOUS MELANOMA .....</b>	<b>11</b>
<b>A. Melanocytes .....</b>	<b>11</b>
1) Skin structure .....	11
2) Origin of melanocytes .....	12
3) Melanocyte function: pigmentation and regeneration .....	16
4) MITF expression, function and signalisation; pathology .....	19
<b>B. Melanoma .....</b>	<b>23</b>
1) Melanoma classification.....	23
2) Melanoma treatments and resistance .....	25
3) Melanoma formation .....	33
4) Melanoma models .....	36
<b>CHAPTER2. EMT-TFS FUNCTION IN CARCINOMA.....</b>	<b>40</b>
<b>A. Physiological EMT.....</b>	<b>41</b>
1) EMT definition.....	41
2) EMT in embryonic development (Type I EMT) .....	43
3) EMT in tissue repair and fibrosis (Type II EMT) .....	45
4) EMT regulation and extracellular signals .....	46
5) EMT-TFs.....	49
<b>B. EMT in carcinoma development.....</b>	<b>56</b>
1) EMT in metastasis and MET in colonization .....	56
2) Transcriptional targets of ZEB1/2 in carcinoma.....	67

---

3) EMT in treatment resistance .....	70
CHAPTER3. PHENOTYPE SWITCHING IN MELANOMA.....	73
A. <i>Phenotype switching</i> .....	73
1) Discovery and definition .....	73
2) MITF rheostat model.....	75
3) Novel phenotype switching model: intermediate phenotypes .....	77
B. <i>EMT-TFs expression switching in melanoma</i> .....	83
1) Expression and function of EMT-TFs in the melanocytic lineage .....	83
2) EMT-TFs expression switch during melanoma development .....	84
3) Control of the EMT-TFs differential expression in melanoma .....	86
4) Antagonistic functions of ZEB1 and ZEB2 in melanoma cells .....	87
5) EMT-TFs regulate phenotype switching of melanoma cells .....	88
6) Melanoma cell plasticity and treatment resistance .....	90
<b>OBJECTIVE OF THESIS .....</b>	<b>92</b>
<b>RESULTS .....</b>	<b>93</b>
<b>DISCUSSION AND PERSPECTIVES .....</b>	<b>144</b>
CHAPTER1. MODELING PHENOTYPE SWITCHING IN PATIENT DERIVED SHORT-TERM CULTURES.....	145
CHAPTER2. CELL-TYPE SPECIFIC TRANSCRIPTIONAL ACTIVITY OF ZEB1 IN MELANOMA COMPARED TO CARCINOMA MODELS.....	148
CHAPTER3. ZEB1 CO-FACTORS IN TRANSCRIPTIONAL REGULATION.....	150
CHAPTER4. ANALYSES OF ZEB2 DIRECT TRANSCRIPTIONAL TARGET GENES BY CHIP .....	152
CHAPTER5. INTRA-TUMOR HETEROGENEITY <i>IN VITRO</i> AND IN HUMAN SAMPLES.....	154
CHAPTER6. NEW CANDIDATES WITH THERAPEUTIC PERSPECTIVES .....	157
CHAPTER7. STRATEGIES TO REPRESS ZEB1 EXPRESSION AND RE-SENSITIZE CELLS TO TREATMENT.....	161
<b>APPENDIX .....</b>	<b>166</b>
A. <i>Review</i> .....	166
B. <i>Reference</i> .....	184

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## Table of illustrations

<i>Figure 1: General structure of human skin.</i> .....	12
<i>Figure 2: Neural crest cell migration in embryonic development and melanocyte development.</i> .....	14
<i>Figure 3: Signal transduction and transcription in the melanocyte lineage.</i> .....	16
<i>Figure 4: Melanosome biogenesis.</i> .....	17
<i>Figure 5: Structure of MITF isoforms.</i> .....	20
<i>Figure 6: Clinical histological melanoma subtypes.</i> .....	24
<i>Figure 7: MAPK signaling pathway and the resistance to MAPK inhibitors.</i> .....	29
<i>Figure 8: Percentage of patients responding to nivolumab +/- ipilimumab.</i> .....	32
<i>Figure 9: Different steps of melanoma formation.</i> .....	33
<i>Figure 10. Pros and cons of current melanoma models.</i> .....	36
<i>Figure 11. Examples of genetically engineered mouse models of cutaneous melanoma.</i> .....	40
<i>Figure 12. Schematic presentation of EMT.</i> .....	42
<i>Figure 13. Different types of EMT.</i> .....	42
<i>Figure 14: Different types of EMT during embryonic development.</i> .....	44
<i>Figure 15: Signaling pathways control the EMTs at gastrulation and neural crest delamination in the amniote embryo.</i> .....	47
<i>Figure 16: Common growth factor signals initiate and regulate essential EMT and wound-healing processes.</i> .....	49
<i>Figure 17: Schematic representation of the protein structures of the core EMT-TFs.</i> .....	50
<i>Figure 18: Zeb family mutually exclusive expression in embryo.</i> .....	54
<i>Figure 19. Examples of epigenetic regulators and EMT-TFs interaction and regulation.</i> .....	56
<i>Figure 20. EMT-MET model for the metastasis.</i> .....	60
<i>Figure 21. Transition between different states during the EMT process.</i> .....	61
<i>Figure 22. EMT-TFs allow the inhibition of failsafe programs and promote cell transformation.</i> .....	63
<i>Figure 23. Pleiotropic roles of ZEB1.</i> .....	67
<i>Figure 24. Recent published ChIP-seq of ZEB1 and ZEB2 in carcinoma.</i> .....	69

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Figure 25. Phenotype switching model. ....	74
Figure 26. A rheostat model for MITF function. ....	76
Figure 27. Likely relationships between the phenotypic states of melanoma cells identified in different studies. (Rambow et al 2019) .....	81
Figure 28. Potential hierarchical arrangement of the six different melanoma phenotypic states and their relative expression of MITF and SOX10. (Rambow et al 2019) .....	81
Figure 29. Potential master regulators of each melanoma cell state identified by single-cell regulatory network inference and clustering (SCENIC). ....	82
Figure 30. Expression of EMT-TFs during melanocyte lineage formation from embryonic neural crest cells. ....	84
Figure 31. EMT-TFs switching during melanoma development. ....	86
Figure 32. Proposed model of ZEB1-mediated transitional regulation of melanoma states markers. .	144
Figure 33. Western Blot analysis of C-09.10 cell line. ....	145
Figure 34. Reversible phenotype switching in GLO and Phenotypic difference between GLO and C-09.10 cell lines. ....	147
Figure 35. Comparison of ZEB1 ChIPseq peaks between melanoma and carcinoma. ....	149
Figure 36. Top 5 enriched binding motifs discovered by HOMER (Hypergeometric Optimization of Motif EnRichment) analysis in ChIPseq ZEB1 in GLO TNF $\alpha$ D14. ....	152
Figure 37. ChIP-qPCR analysis of ChIP ZEB2 in 501Mel ATCC melanoma cell line, in C-09.10 and GLO patient-derived short-term cultures. Anti-ZEB2 (ZEB2) (2 $\mu$ g, HPA003456, Sigma) or control IgG were used for the IP. Relative promoter enrichment was normalized to chromatin inputs (%). CDH4 in 501Mel, n=2. Others n=1. ....	154
Figure 38. ZEB1 and ZEB2 expression in two published scRNAseq analysis. ....	156
Figure 39. Some epigenetic regulators present ZEB1 binding sites in ChIPseq ZEB1 in GLO cell lines. ...	159
Figure 40. GLO-R is more sensitive to HDACis than GLO cell line. ....	162
Figure 41. ZEB1/2 regulate melanoma phenotype switching and resistance to treatments. ....	164

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## Introduction

The function of EMT-inducing transcription factors (EMT-TFs) of the ZEB family in melanoma has been the main focus of my PhD. Their function has been mainly studied in the context of epithelial cells and carcinoma. The introductive part will start with a general presentation of melanoma, cell of origin, development, classification and treatment. Then a second part will synthesize the extensive bibliography dealing with EMT and EMT-TFs function in the context of epithelial cells and carcinoma. The third part will finally focus on EMT-like processes in melanoma, so-called “phenotype switching”, and previous data on the expression and the role of EMT-TFs in this specific context.

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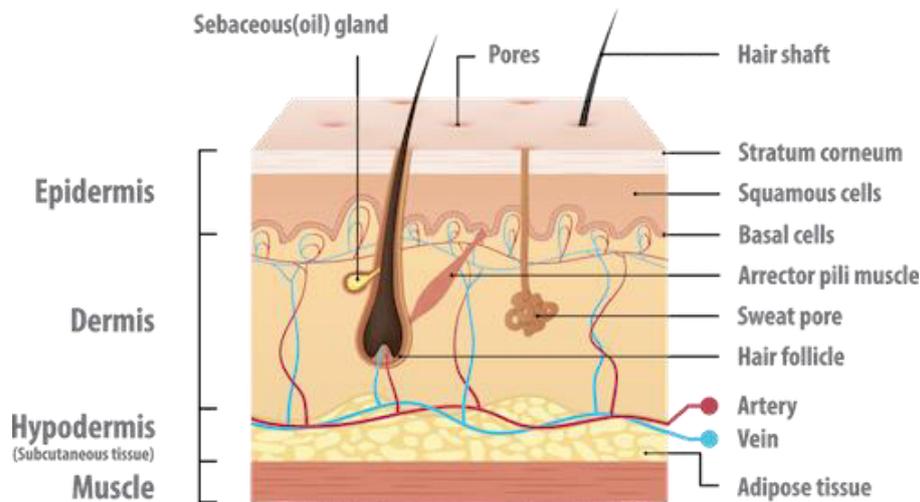
## Chapter1. Cutaneous melanoma

### A. Melanocytes

The cell of origin of melanoma is the melanocyte, which colors skin, hairs or feathers in vertebra. Melanocytes derive from neural-crest cells during embryogenesis. They have various locations including epidermis, hair follicles, as well as eyes, inner ear, nervous system, heart, digestive epithelium (Brito and Kos, 2008; Tachibana, 1999). Melanocyte produces pigmented granules called melanosomes which are rich in melanin, this pigment is capable to protect our skin from UV. Before we enter into more details in melanocyte biology, we need first to understand skin structure.

#### 1) Skin structure

The skin is the largest organ of the body; it protects us from microbes, helps regulate body temperature and permits the sensations of touch, heat and cold. The skin has three layers: epidermis, dermis and hypodermis [Figure 1]. The epidermis, the outmost layer of skin, provides a waterproof barrier, resists mechanical and chemical injury and protects against bacteria, viruses and parasites infection. There are three main groups of cells in the epidermis: keratinocytes, the main cells in epidermis, develop from the basal layer and then migrate upwards to the outer surface where they are shed; Langerhans cells are specialized immune cells that participate into body's immune response; melanocytes, melanin-producing cells that we mentioned before. Between epidermis and dermis, there is a junction called the dermo-epidermal junction, which contains the basement membrane. The dermis, beneath the epidermis, contains connective tissue, hair follicles and glands. It is composed of a dense network of collagen and elastin organized into fibers. The hypodermis, the deeper subcutaneous tissue, is made of a network of collagen fibers and adipocytes; it protects the body from external trauma and insulates from cold. There are many blood and lymphatic vessels and nerves passing through this layer.



*Figure 1: General structure of human skin.*

*The skin is composed of three main layers: the epidermis, made of closely packed epithelial cells, and the dermis, made of dense, irregular connective tissue that houses blood vessels, hair follicles, sweat glands, and other structures. Beneath the dermis lies the hypodermis, which is composed mainly of loose connective and fatty tissues. (Issued in Human Anatomy, by Matthew Hoffman)*

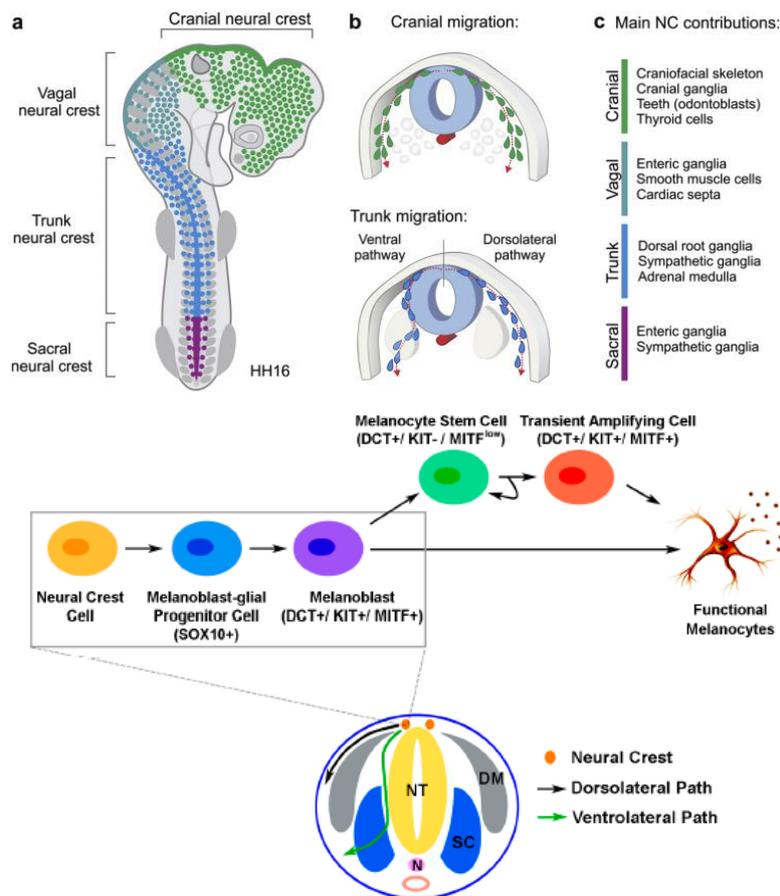
Melanocytes of the epidermis and hair follicles possess some common features, but biologically they represent different populations, which live in unique niches of the skin. The epidermal melanocytes are located among the basal layer of a stratified squamous keratinized epithelium; the hair melanocytes are between cells covering the hair papilla in the hair bulb. Stem cells of the latter are located in the region called hair bulge.

## **2) Origin of melanocytes**

Melanocyte lineage is derived from the neural crest. The neural crest is a multipotent embryonic cell population that is highly migratory; these cells are pinched off during the neural tube (precursor of the spin cord) formation, in another word, they originate in the ectoderm at the margins of the neural tube. The neural crest was initially identified by the Swiss embryologist Wilhelm His in 1868, as a group of cell localized between the neural tube and the epidermis. Neural Crest Cells (NCCs) delaminate from the dorsal-most aspect of the neural tube through a process called EMT (epithelial to mesenchymal transition), by which epithelial cells lose polarity and cell-cell

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adhesion then gain migratory and invasive properties in order to become mesenchymal cells (see part 2). Thereafter, instead of remaining as a part of the central nervous system, NCCs migrate to numerous locations in the body and contribute to the formation of diverse structures by proliferation and differentiation, mostly associated with the nervous system (Mayor and Theveneau, 2013). Before leaving the crest, according to the antero-posterior position, at which neural crest cells delaminate, their fate can be widely defined [Figure 2]. Indeed, the neural crest can be divided into five groups based on their positioning (Mull et al., 2015): the cranial neural crest gives rise to the majority of the head connective and skeletal structures, nerves and pigment cells following a dorsolateral migration; the cardiac neural crest mainly participates into heart development; vagal and sacral neural crest cells contribute to the formation of the enteric nervous system ganglia and also the parasympathetic ganglia; trunk neural crest cells migrate through two major pathways and the melanocyte, our cell of interest, is a derivative of this group. Trunk cells who leave early the crest migrating ventrally through the anterior sclerotome and become neurons and glia, while late departing cells, melanocyte precursors termed melanoblasts, migrate through a dorsolateral pathway between the ectoderm and the somites (Erickson and Goins, 1995). Melanoblasts migrate through the dermis and then into the epidermis; after that, they migrate within the epidermis and become incorporated into developing hair follicles. They continue to populate the epidermis and hair follicles, eventually becoming melanocytes that produce pigment, as well as melanocyte stem cells. As development progress, melanoblasts simultaneously differentiate, proliferate and migrate extensive distances throughout the embryo until finally populating the entire organism, together with the increase of epidermis surface; but the proliferation of epidermal melanocytes has not yet been fully terminated.



*Figure 2: Neural crest cell migration in embryonic development and melanocyte development.*

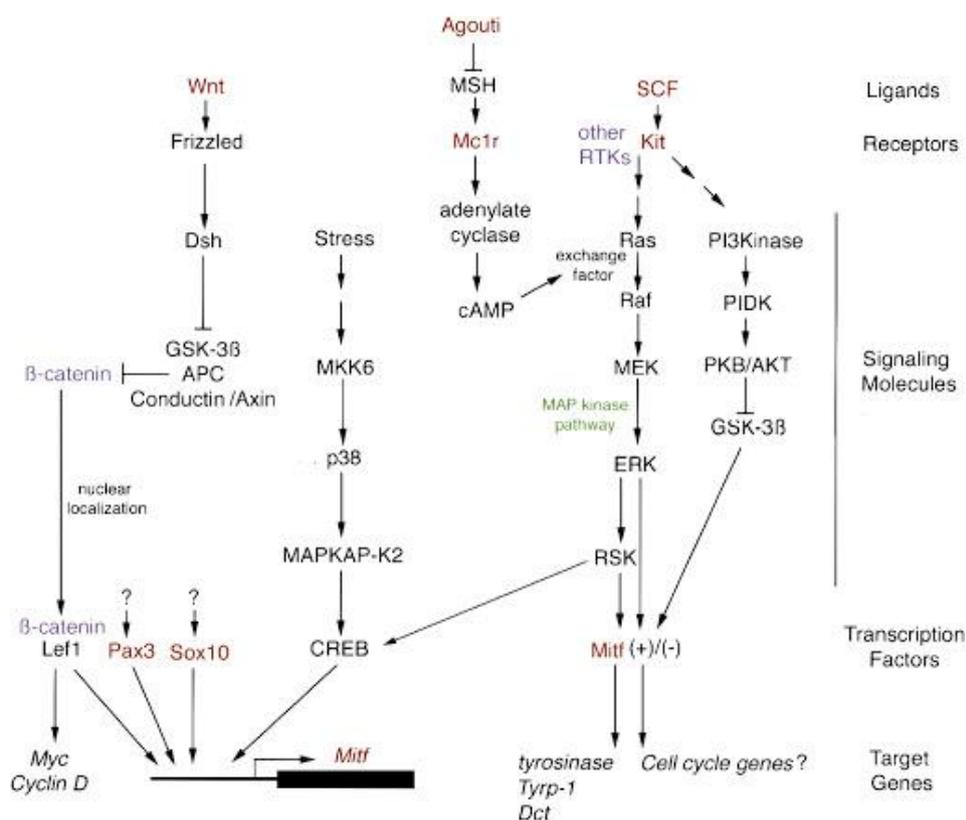
*Up: Overview of neural crest subpopulations along the anteroposterior axis. (a) Diagram of neural crest subpopulations in an HH16 chick embryo. (b) Differences in migratory patterns among neural crest subpopulations. (c) Major derivatives of neural crest subpopulations. (Rothstein et al., 2018).*

*Down: A schematic illustration of the neural crest cells migratory paths and melanocyte development. The melanoblast progenitor cells typically migrate along the dorsolateral path between the epidermis and dermomyotome.*

*NT: neural tube, N: notochord, DM: dermomyotome, SC: sclerotome (Mull et al., 2015).*

From the neural crest cells to melanoblasts, the most important regulating elements are Wnt protein, Stem Cell Factors (SCF, c-KIT ligand), endothelins, ephrins and bone morphogenetic proteins (BMP) [Figure 3] (Cooper et al., 2009; Hari et al., 2012; Hirobe, 2011; Sommer, 2011). Early melanoblasts development requires the presence of SCF and its receptor c-Kit, which activates the MAPK signaling pathway through the cytoplasmic tyrosine kinase domain. During melanoblasts migration from the region of neural tube through the dermis into epidermis, SCF is secreted by dermal and epidermal cells. Besides, SCF is also required for melanoblasts survival (Hari et al., 2012;

Hirobe, 2011). The Notch receptor is also essential for melanoblasts development; it demands contact with another cell expressing ligand molecules (Delta1, Delta3, Delta4, Jagged1 and Jagged2) on its surface. The Notch receptor then is cleaved and move to nucleus where it regulates transcription activity via for example Hes1 transcription factor (Cornell and Eisen, 2005; Moriyama et al., 2006). Wnt pathway is associated with the induction of melanocyte fate (Raible and Ragland, 2005). The ephrin and endothelin receptors allow melanoblasts to migrate along extracellular matrix containing ephrins and endothelins secreted by cells of embryo (Saldana-Caboverde and Kos, 2010). Moreover, fibrillar proteins present in the extracellular matrix, such as collagens and fibronectin, also participate in the melanocyte development, because cells adhere to one another or extracellular matrix using adhesion molecules as cadherins and integrins that act as sensors and transducers of the mechanical state of the tissue, thus regulating the migration, adhesion and proliferation of melanocytes (Lin and Fisher, 2007; Pinon and Wehrle-Haller, 2011). Finally, almost all the elements we mentioned above induce the expression of MITF (microphthalmia-associated transcription factor), a master regulator of melanocyte lineage proliferation, differentiation and survival, which activates many genes such as tyrosinase for melanin production.



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*Figure 3: Signal transduction and transcription in the melanocyte lineage.*

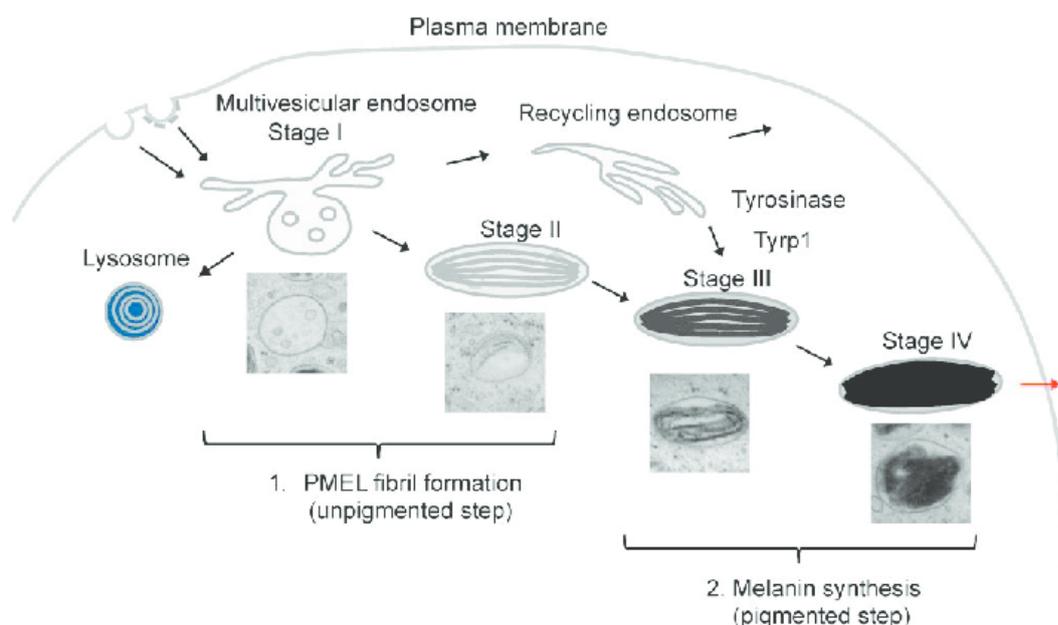
*Those signaling molecules or transcription factors that have been implicated genetically in melanocyte development are shown in red, while molecules implicated in melanoma are indicated in blue. (Goding, 2000) .*

Melanocytes in the epidermis and hair melanocytes are located in different niches. In general, melanocytes are highly differentiated cells so they lose the ability to proliferate during the maturation process. The only renewing melanocytes in adult skin are the hair melanocytes (Nishimura, 2011). Another important thing to note is the difference between mouse and human skin. Human skin contains large inter-follicle areas where exists epidermal melanocytes at the basal layer of the epidermis, while mouse cutaneous melanocytes are almost exclusively located in hair follicles and dermis, but not in epidermis, except for tail and paw skin. Mouse tail skin maintains a stable melanocyte population, including a low percentage of amelanotic melanocytes (Glover et al., 2015), this may open new insight in discovering other mechanisms sustaining inter-follicle epidermal melanocytes other than hair follicle melanocyte stem cells.

### **3) Melanocyte function: pigmentation and regeneration**

The main role of melanocytes is to synthesize melanin. Melanin is not only responsible for skin, hair and eye pigmentation, but also UV absorption, reactive oxygen species (ROS) neutralization and ions storage (Bush and Simon, 2007; Costin and Hearing, 2007; Pathak et al., 1962; Slominski et al., 2004). Melanosomes are membrane-bound organelles within which melanin is synthesized (Simon et al., 2009). This process is named melanogenesis. In response to MSH (melanocyte-stimulating hormone) secreted by pituitary gland, melanocytes produce and store melanin in melanosomes. There are four stages of melanosomes biogenesis (Costin and Hearing, 2007; Hearing, 2005; Slominski et al., 2004; Watabe et al., 2008), determined by the quality, quantity, structure and arrangement of melanin produced [Figure 4]: I, spherical form without any internal structural components; II, ellipsoidal form containing organized, structured fibrillar matrix; III, beginning of melanin production; IV, full of melanin. Specific enzymes for melanin synthesis, such as tyrosinase,

TRP1 (tyrosinase-related protein 1), TRP2 (tyrosinase-related protein 2 or DOPAchrome tautomerase DCT), as well as structural proteins for melanosomes fibrillar stroma, Pmel17 (gp100), MART1 (melanoma-associated antigen recognized by T cells), are required in melanogenesis (Hearing and Tsukamoto, 1991; Hoashi et al., 2005; Simon et al., 2009; Yamaguchi et al., 2007). As for the intracellular regulation of melanin synthesis, some transcription factors and molecules play major roles, such as MITF and cAMP (Goding, 2000; Park et al., 2009). There are several inhibitors of melanogenesis, such as sphingolipids, BMP4 and tetrahydrobiopterin (Park et al., 2009). Melanogenesis is also a process which generates many cytotoxic agents, as quinones and hydrogen peroxide for example; melanosomes are maybe a perfect place of this process in order to avoid the interaction between these agents and other cytosolic components.



*Figure 4: Melanosome biogenesis.*

*Melanogenesis takes place in special organelles named melanosomes. As first develops a vesicle (Stage I) which builds inside a fibrillar matrix formed by glycoproteins (Pmel17, MART-1) and gets tyrosinase and other enzymes of melanogenesis (Stage II). The melanosome produces melanin, which polymerizes and settles on the internal fibrils (Stage III). In the last Stage IV melanosome fulfills melanin. Each type of melanin is synthesized in separated melanosomes (Bissig et al., 2016).*

In the epidermis, melanocytes located in the basal layer cooperate with thirty to forty keratinocytes to form the epidermal melanin unit (Haass et al., 2005; Slominski et al., 2004). All the melanin produced is transferred away from melanocytes into keratinocytes; melanin granules are

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then accumulated above the nucleus of keratinocytes (Costin and Hearing, 2007; Plonka et al., 2009; Slominski et al., 2004). Melanocytes also cooperate with dermis fibroblasts. These three cell types communicate with each other via secreted factors (Haass et al., 2005; Lee et al., 2013; Tabone - Eglinger et al., 2012; Yamaguchi et al., 2007). Hormones secreted by keratinocytes regulate melanocytes proliferation and melanogenesis (Costin and Hearing, 2007; Slominski et al., 2004); this cross-talk between melanocytes and keratinocytes is also important for the maintenance of skin homeostasis (Hirobe, 2011). All the paracrine and autocrine factors secreted by these three cells can also influence the shape, dendricity, mobility and adhesion properties of melanocytes. Melanocytes are also an essential component of the skin immune system; they are capable to target keratinocytes and other epidermal cells by secreting cytokines, growth factors and hormones (Costin and Hearing, 2007; Slominski et al., 2004). Inflammatory molecules can affect directly melanocytes and may cause hypo or hyperpigmentation of the skin (Taylor et al., 2011). Melanocytes are also responsible for hair pigmentation. Hair color is a result of communication between follicular melanocytes, matrix keratinocytes and dermal papilla fibroblasts. Here, melanocytes are located in the proximal bulb of each hair follicle (Commo and Bernard, 2000; Slominski et al., 2005). Hair melanocytes are larger and more dendritic than epidermal melanocytes, but the regulation, synthesis and transfer of melanin to keratinocytes is similar.

Epidermal melanocytes proliferate extremely rarely in physiological conditions, they are very stable (Bennett, 2003; Nishimura, 2011). One molecule is essential for this longevity, BCL-2. BCL-2 is maintained at a high constitutive level so that melanocytes are strongly resistant to apoptosis (McGill et al., 2002). Moreover, the increased level of cyclin-dependent kinase inhibitors and decreased level of cyclin D1 also inhibit the cell cycle (Bennett, 2003). Different from rarely proliferative epidermal melanocytes, hair melanocytes proliferate repeatedly, migrate and undergo maturation in every hair cycle (Nishimura, 2011). Thanks to the existence of melanocyte stem cells in the hair follicle bulge, together with hair follicle stem cells (precursors for keratinocytes), hair is regenerated in each cycle. Although epidermal melanocytes number seems to be constant, from the fourth or fifth decade of life, the number of epidermal melanocytes and also hair melanocytes

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decreases progressively (Costin and Hearing, 2007). This may be due to the apoptosis of terminally differentiated melanocytes. Melanocyte stem cells also die, which results in the process of hair greying (Nishimura, 2011).

#### **4) MITF expression, function and signalisation; pathology**

MITF is the master regulator of melanocyte lineage, activated by various extracellular signals during embryonic development. This transcription factor regulates melanocytes differentiation and development and also plays essential roles in cell cycle progression, motility and survival. It is also responsible for pigment production, induced by the binding of alpha-MSH to MCR1, so that melanin synthesis is activated (Goding 2000; Goding and Arnheiter, 2019).

##### **a) MITF structure and expression**

MITF is a bHLH-Zip (basic-loop-helix-loop leucine zipper) transcription factor encoded by the *MITF* gene, which belongs to the MYC superfamily. Together with TFEB, TFEC and TFE3, MITF constitutes the MiT (microphthalmia) family (Hemesath et al., 1994). All of these transcription factors share a common bHLH-Zip dimerization motif containing a positively charged fragment involved in DNA binding, and a TAD transactivation domain. During transcription, a single *MITF* gene can produce several isoforms by using differentially alternative promoters, including MITF-A, MITF-B, MITF-C, MITF-D, MITF-E, MITF-H, MITF-J, MITF-Mc and MITF-M [Figure 5]. These isoforms demonstrate tissue-specific patterns of expression (Hartman and Czyz, 2015). The shortest isoform MITF-M is exclusively expressed in melanocytes and melanoma cells (Tassabehjil et al., 1994). MITF binds to DNA as a homodimer or heterodimer with one of the MiT proteins (Hemesath et al., 1994), but it does not form heterodimer with other bHLH-Zip proteins. The binding motif in DNA sequence of target genes involves E-box and M-box (Goding, 2000; Goding and Arnheiter, 2019; Pogenberg et al., 2012).

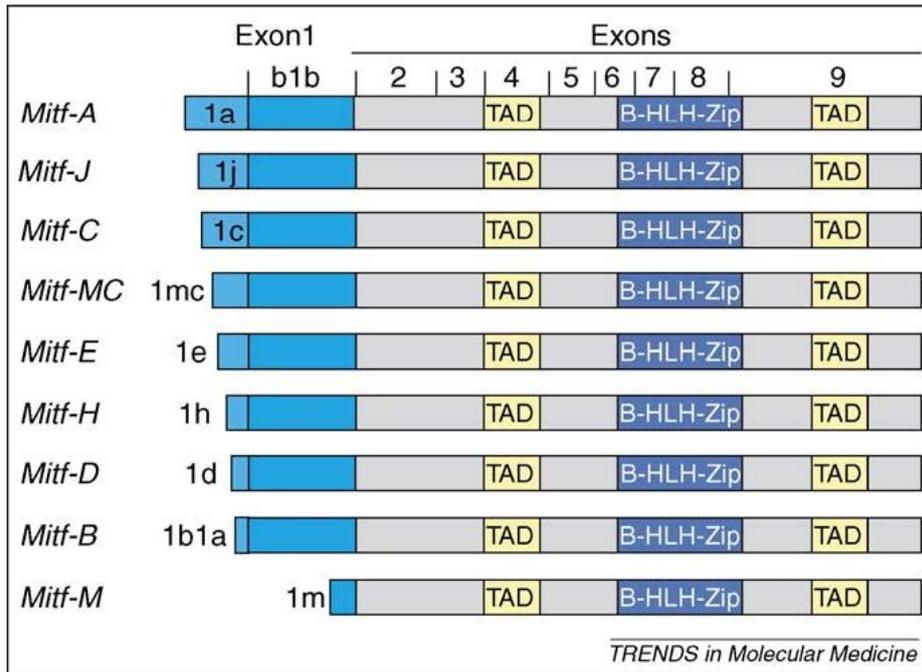


Figure 5: Structure of MITF isoforms.

Spliced mRNAs (messenger RNA) of human MITF (microphthalmia-associated transcription factor) isoforms. Each isoform has a unique promoter and a partially unique first exon. The rest parts are common to all isoforms and encode the transactivation domain (TAD) and the basic helix-loop-helix leucine zipper domain (bHLH-Zip). The M-isoform is specifically expressed in melanocyte lineages. Other isoforms expression patterns range from widely expressed to tissue specific. Expression of Mitf-Mc has not been confirmed in human cells (Levy et al., 2006).

## b) MITF regulation

MITF transcriptional control is governed by a number of regulators. SOX10 (sex-determining region Y-box 10) is the main activator of MITF (Verastegui et al., 2000). SOX proteins are one group of transcription factors characterized by a common DNA-binding domain, the Sry-box, which was first identified in Sry, a protein encoded on the mammalian Y chromosome and responsible for male sex determination (Bowles et al., 2000). SOX10 is required for survival, proliferation, and the maintenance of pluripotent NCSCs, thereby controlling the size of stem cell population and indirectly influencing the number of generated melanocyte. Melanocyte specification is also directly affected by SOX10. In MITF promoter, a SOX10 responsive element was found (Seberg et al., 2017). The nuclear localization of SOX10 is ensured by a protein tyrosine kinase TYRO3 (Zhu et al., 2009). SOX10 also cooperate with CREB (cAMP response element-binding protein) in the responsiveness of MITF to alpha-MSH-cAMP signalling (Huber et al., 2003). Moreover, MITF expression can be

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activated by two key effectors in Wnt signalling pathway: LEF1 (lymphoid enhancer-binding factor 1) and  $\beta$ -catenin (Eichhoff et al., 2011; Saito et al., 2002). MITF can also cooperate with LEF1 as a non-DNA-binding co-activator to enhance its own expression. Mediators from the alpha-MSH-cAMP signalling can also redirect  $\beta$ -catenin to CREB specific promoters including *MITF* (Bellei et al., 2011). Transcription factors involved in EMT (epithelial to mesenchymal transition), ZEB2 (zinc finger E-box binding protein 2) and SNAIL2 can also activate MITF expression and we will discuss this in the third part of the introduction. At the epigenetic level, the remodelling complex SWI/SNF also promotes MITF expression and a knock-down of members of this complex down-regulate MITF expression with a concomitant decreased expression of MITF targets and a reduced cell proliferation (Vachtenheim et al., 2010).

MITF also processes transcriptional repressors. Coming from TGF $\beta$  signalling, GLI2 is a Kruppel-like transcription factor that can bind to MITF *promoter* and inhibit MITF expression (Javelaud et al., 2011). The roles of PAX3 (paired box 3) is context-dependant: in melanocytes, PAX3 regulates positively MITF (Yang et al., 2008); Intriguingly, in melanoma, PAX3 functions independently of MITF or even repress MITF expression. MITF repression is also mediated by DEC1 (differentially expressed in chondrocytes protein 1) which is regulated by HIF1 (hypoxia-inducible factor 1) (Feige et al., 2011).

MITF transcript is also under the control of several non-coding RNAs, microRNAs (miRs), which mediate MITF mRNA degradation and protein synthesis suppression (Bell and Levy, 2011). It has been shown that miR137, miR148, miR101 and miR218 negatively regulate MITF (Luo et al., 2013a). MITF mRNA is also targeted by miR-148, miR-101 and miR-218, in melanocytes (Guo et al., 2014; Haflidadóttir et al., 2010; Luo et al., 2013b). MITF transcriptional activity is also determined by post-translational modifications and availability of cooperating partners. Generally, MITF activity is enhanced by phosphorylation; ERK1/2 phosphorylates MITF at Ser<sup>73</sup> in order to promote the interaction between MITF and its co-factor p300/CBP (Wu et al., 2000). Proteasome mediated MITF degradation is observed after double phosphorylation at Ser<sup>73</sup> and Ser<sup>409</sup> by p90<sup>RSK</sup> (Xu et al., 2000).

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MITF activity is also regulated by SUMOylation at two lysine residues (Murakami and Arnheiter, 2005). Notably, non-SUMOylable MITF mutants demonstrate increased transcriptional activity on certain target genes (Bertolotto et al., 2011).

### **c) MITF signaling and function**

In mature melanocytes, upon activation via the MSH and MC1R pathway, MITF is directly activated; it then transcribes a set of genes involving in melanin synthesis and pigmentation (Levy et al., 2006). Alternatively, MITF expression can also be induced via Wnt/ $\beta$ -catenin pathway (Anastas and Moon, 2013). MITF also directly controls the transcription of fundamental genes maintaining cell homeostasis, such as Bcl2 involved in apoptosis and CDK2 in cell cycle (Du et al., 2004). Melanogenesis is stimulated by MITF with the activation of *TYR* and other pigmentation genes including *TYRP1*, *DCT*, *PMEL* and *MLANA* (Bertolotto et al., 1998; Du et al., 2003; Yasumoto et al., 1994). However, not all MITF targets are involved in pigmentation, several MITF target genes also participate in DNA replication and repair, as well as mitosis (Strub et al., 2011). Moreover, MITF affects metabolism by activating expression of a key transcriptional regulator, PGC1 $\alpha$ , and promotes lysosomes biogenesis through activating lysosomal genes (Haq et al., 2013; Ploper et al., 2015).

Genetic defects associated with MITF in human such as Waardenburg IIA syndrome and Tietz-Albinism-Deafness syndrome are also known. Waardenburg syndrome is a group of genetic conditions that can cause hearing loss and changes in pigmentation of hair, skin and eyes. Mutations in the *EDN3*, *EDNRB*, *MITF*, *PAX3*, *SNAI2* and *SOX10* genes, which are involved in the formation and development of several cells including melanocytes, can cause this syndrome. There are four recognized types of Waardenburg syndrome, mutations in the *MITF* or *SNAI2* gene cause type II (Pardono et al., 2003; Tassabehjil et al., 1994). Missense mutation or inframe deletion of one amino acid in the basic domain of the MITF gene can cause Tietz-Albinism-Deafness syndrome (Amiel et al., 1998); these patients have a pale skin, blue eyes and light blond to white hair with white eyebrows and eyeshades. Hearing loss is always bilateral, congenital, sensorineural and

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profound. They can gradually gain some pigmentation on sun-exposed areas.

## **B. Melanoma**

Melanoma is a cancer originating from melanocytes, and the study of normal melanogenesis has shed light on some aspects of its biology. Nonetheless, this tumour is heterogeneous in terms of clinical presentation and outcome with a complex biology.

### **1) Melanoma classification**

#### **a) Morphological and histological classification**

The primary histologic subtypes of melanoma depend on six main parameters: superficial spreading, lentigo maligna, nodular, acral lentiginous, desmoplastic and amelanotic (Markovic et al., 2007) [Figure 6]. The most frequent is superficial spreading taking place in around 70% of melanoma cases, which tumor arises from the existing nevus or 30% of them without pre-existing nevus; superficial spreading is also the model that we work on in my thesis. Nodular melanoma means absence of a radical growth phase with variable presentation and robust invasion, this presents in 5% of melanoma cases. About 4% to 15% cases show lentigo maligna, which demonstrates slow progression and frequently appears in sun-exposed areas, including face, head, etc. In patients with darker skin pigmentation, acral lentiginous melanoma has high incidence and frequently occurs on the palms, soles and subungual spaces. A rare portion of melanoma shows an absence of pigmentation called amelanotic; it is also the most challenging subtype in terms of diagnosis (Kauffmann and Chen, 2014). Desmoplastic melanoma is a rare melanoma less than 4% and seen in older adults; it is characterized by scant spindle cells and minimal cellular atypia.

Subtype	Frequency	Characteristic
Superficial spreading	70%	Arises from existing nevus.
Nodular	5%	Absence of a radial growth phase, variable presentation, and robust vertical invasion.
Lentigo Maligna	4–15%	Typically demonstrates slow progression, and frequently appears in sun-exposed areas (i.e., face, head, etc.)
Acral lentiginous	5%	Has higher incidence in patients with darker skin pigmentation and frequently occur on the palms, soles, and subungual spaces.
Amelanotic	4%	Characteristic absence of pigmentation and are considered rare.
Desmoplastic	Less than 4%	Rare melanoma seen in older adults that is characterized by scant spindle cells and minimal cellular atypia.

Figure 6: Clinical histological melanoma subtypes.

Ref: *Cutaneous melanoma: Etiology and therapy. Chapter 6 Clinical presentation and staging of melanoma.*

Nowadays, the American joint committee on Cancer TNM system is used with clinical and pathological staging assignments (Amin et al., 2017). Once the lesion has been histologically considered as melanoma, other characteristics about the T (tumour) will be assessed, including tumour thickness, presence of ulceration and mitosis in lesions (Balch et al., 2009; Kauffmann and Chen, 2014). Stage 0 melanoma is *in situ* and occurs when melanoma cells are identified but with no penetration outside epidermis. N (nodal) is a parameter determined by the number of involved lymph nodes. The presence or absence of metastatic disease termed M (metastatic); if present, it will be classified by its location. In general, Stage I and II include melanoma without nodal or distant metastases; depending on the depth of vertical invasion, there are several sub-classifications in each stage. When gross or microscopic lymph node metastasis is present, this melanoma will be classified as stage III; stage IV will then be patients with distant metastasis (Balch et al., 2009; Coit et al., 2013; Kauffmann and Chen, 2014).

## b) Genetic classification

Apart from the histo-pathologic classifications, based on the occurrence of driver mutations, melanomas have been further classified into four genomic subtypes: *BRAF*-mutant, *NRAS*-mutant, *NF1*-loss and TWT (Triple wild-type) (Cancer Genome Atlas Network, 2015; de Snoo and Hayward, 2005). Driver alterations of *BRAF*, *NRAS* and *NF1* activate the MAPK

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pathway and occur generally at the earlier stages of tumour development (Shain et al., 2018). Around 45% to 50% cases are *BRAF*-mutant, principally at the V600 codon; *RAS*-mutant takes place in approximately 30% melanoma, either *NRAS*<sup>Q61</sup>, *KRAS* or *HRAS*; 10% to 15% are *NF1*-mutant and about 5% to 10% are TWT. In cutaneous melanoma, other subsequent mutations in *TERT* promoter as well as in other regulators of cell cycle, such as *CDKN2A*, which precede mutations in chromatin remodelers, the *SWI/SNF* complex for instance, and in *TP53* which are associated with later advanced stages of primary tumour progression (Shain et al., 2018). Every genomic subtype may process different clinical presentation and characteristics. *BRAF* mutations are more probably present in melanomas arising on skin with intermittent sun exposure, compared to these on chronically sun-exposed skin (Kim et al., 2016a); *BRAF* mutations are also common in younger patients, also in superficial spreading histo-pathologic subtype (Bauer et al., 2011; Long et al., 2011). In contrast, *NRAS* mutations are found in melanomas on chronic UV-damaged skin, in older patients and in nodular histo-pathologic subtype (Carlino et al., 2014; Devitt et al., 2011). Subsequent mutations also show some links with driver alterations; for instance, *BRAF*<sup>V600E</sup>-mutant melanoma tends to process fewer somatic mutations than tumours bearing other alterations, such as activating mutations in *NRAS*, *KIT* and *BRAF* non-V600E (Shain and Bastian, 2016).

## 2) Melanoma treatments and resistance

The incidence of melanoma has been increasing over the last 30 years. Treatments development has also been a great topic of research. Nowadays, different types of treatments are available for patients with melanoma.

There are five types of standard treatments: surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy. When melanoma is still in situ, surgery is the most efficient choice; it is also the primary treatment of all stages of melanoma. To remove the tumour, a wide local excision is needed as well as some of the normal tissue around. It is also important to know

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that, if cancer cells have spread into the lymph nodes, every lymph node containing melanoma cells will be removed surgically. In several cases after the surgery, chemotherapies and/or radiotherapies will be given to lower the risk of relapse. Before 2010, surgery, chemotherapy and radiotherapy were three practicable treatments for melanoma patients but were largely inefficient; from then, the therapy has been revolutionized with the advent of targeted therapy and immunotherapy which induce better responses against metastatic melanomas (Finn et al., 2012).

### **a) Targeted therapies**

In the treatment of melanoma, with the discovery of the BRAFV600 mutation in 50% of cases, targeted therapies specifically targeting this mutation have been developed. These are inhibitors blocking signals transduction in cancer cells, in order to treat some patients with advanced melanoma or tumours which cannot be removed by surgery; for instance, BRAF inhibitors dabrafenib (Medina et al., 2013), vemurafenib (Scholtens et al., 2015), encorafenib (Koelblinger et al., 2018) for mutant BRAF, and MEK inhibitors trametinib (Chopra and Nathan, 2015), cobimetinib (Eagles and Jimeno, 2016), binimetinib (Specenier, 2020) are used in combination such as dabrafenib plus trametinib (Robert et al., 2019), vemurafenib plus cobimetinib (Keating, 2016), encorafenib plus binimetinib (Holbrook et al., 2020).

The FDA approved the use of dacarbazine as chemotherapy for metastatic melanoma treatment in 1975, however, the results were unsatisfying; for example, 10% to 15% of patients do not respond positively to dacarbazine. Other therapeutic drugs such as high dose of IL-2 presented high toxicity and IFN $\alpha$ -2b did not process a significant efficiency. The launch of BRAF inhibitors in clinic use, first vemurafenib in 2011 and then dabrafenib in 2013 successfully improved the responses. Within clinical trial, the complete response to vemurafenib showed a decrease in all BRAF<sup>V600E</sup> lesions and a partial response showed a decrease of at least 30% of diameter (Bollag et al., 2012) The response rate to vemurafenib is around 50% in two weeks after treatment, which is two times higher than dacarbazine (Flaherty et al., 2010). Despite its satisfying response, vemurafenib

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also processes some side effects: around 25% of patients develop squamous cell carcinoma in around eight weeks; patients treated with vemurafenib may develop secondary melanoma in primary site from BRAF wild-type cells (Dalle et al., 2011; Zimmer et al., 2012). As a competitor to ATP fixation site in mutant BRAF, dabrafenib also blocks the BRAF signal transmission; around 65% of patients respond to dabrafenib which is better than vemurafenib. Even though this molecule showed similar side effects as vemurafenib, it does not induce the development of secondary melanoma in primary site. As mentioned before, BRAF inhibitors showed certain improve in metastatic melanoma treatment, although this is only a short-term effect; almost all patients treated with BRAF inhibitors relapsed within six months and acquired resistance (Fedorenko et al., 2011; Sullivan and Flaherty, 2013). The BRAF resistance can be classified into two types: the intrinsic resistance which means initial tumor cells are intrinsically resistant to BRAF inhibitors; the acquired resistance is developed under treatment, within which initial sensitive tumors cells become resistant through new cellular mechanisms. The intrinsic resistance is mainly due to mutations in other genes, such as *CDK2*, *CDK4*, *MITF* or *AKT3*, which can promise the cell cycle progression even under the BRAF inhibitors challenge; the loss of onco-suppressor such as PTEN in the regulation of PI3K-AKT pathway was also shown to be a reason of intrinsic resistance (Fedorenko et al., 2011; Paraiso et al., 2011). Mutation in cell cycle genes, for instance cyclin D1 overexpression, can also guaranty cell proliferation. These mutations above can also be found in some acquired resistance mechanisms.

In more detail, the mechanisms of intrinsic or acquired resistance to BRAF inhibitors can be divided into MAPK pathway-dependent and MAPK pathway-independent [Figure 7]. In about 79% of resistant melanoma, there is a reactivation of the MAPK pathway. One example is the BRAF<sup>V600E</sup> splicing variant p61 (Lito et al., 2013; Rizos et al., 2014). This variant does not possess the interaction domain with RAS, which is normally necessary in physiological context. In BRAF<sup>V600E</sup> melanoma, vemurafenib can bind to BRAF monomer so that BRAF cannot form heterodimer with RAS; instead of interacting with RAS, p61 can form homodimer to escape vemurafenib action and reactivate MAPK pathway. The amplification of *BRAF*<sup>V600E</sup> oncogene is another reason of MAPK

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activation (Shi et al., 2012). Other upstream or downstream genes in MAPK pathway can also be involved in its reactivation. Notably, NRAS mutations can activate MAPK pathway through other RAF proteins, CRAF or ARAF, instead of BRAF (Nazarian et al., 2010). Furthermore, mutations in downstream MEK1/2 also present in some BRAF inhibitors resistant patients, which can activate ERK continuously without upstream actors (Nikolaev et al., 2011). Another protein kinase COT can also activate ERK independently of RAS and RAF (Johannessen et al., 2010). Interestingly, in a heterogeneous tumor population with BRAF mutated and BRAF wild-type cells, the wild-type BRAF melanoma cells can obtain a better fitness under BRAF inhibitor treatment; these cells have no mutation so they are intrinsically resistant to vemurafenib and can proliferate better (Dalle et al., 2011; Poulikakos et al., 2010). MAPK pathway independent mechanisms use other cellular pathways to overcome the BRAF inhibitors selection. Overexpression of several tyrosine kinase receptors such as IGFR or PDGFR $\beta$  were found in melanoma cells (Nazarian et al., 2010; Villanueva et al., 2010). These receptors could then activate the PI3K-AKT pathways and cell proliferation. In other words, with an alteration of their transcriptome, melanoma cells can install other cell mechanisms to guaranty their survival under treatments. In clinic, knowing the variety of resistance, combination of BRAF inhibitors and MEK inhibitors is used in order to kill melanoma cells and to avoid or slow down the establishment of resistance.

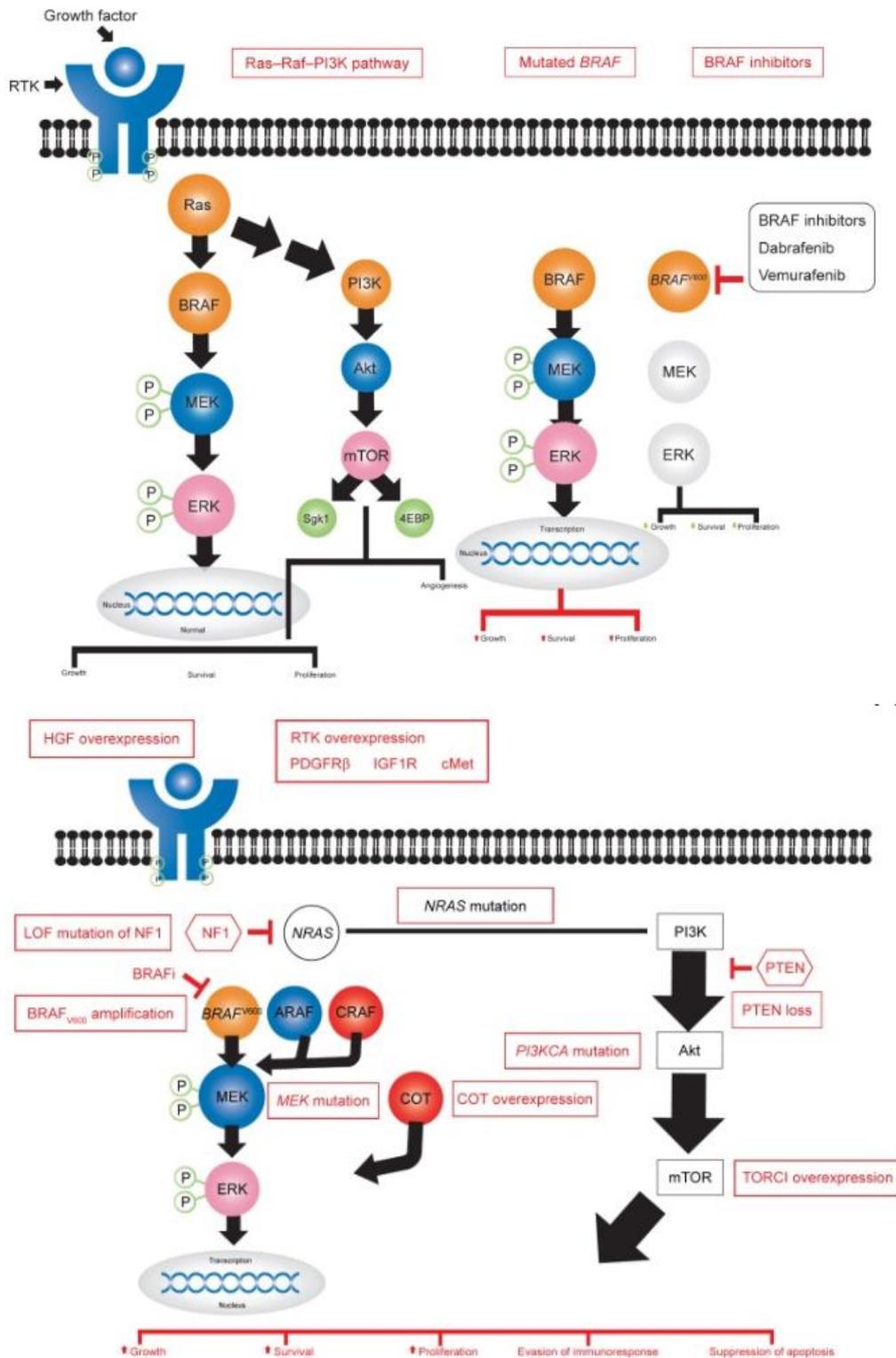


Figure 7: MAPK signaling pathway and the resistance to MAPK inhibitors.

a: MAPK-PI3K-Akt pathway and BRAFV600 mutation in melanoma. MAPK pathway in normal cells (left), where growth factors bound to RTK and further activates downstream kinases and regulates the cell growth, survival, and proliferation. BRAFV600 mutations in melanoma lead to constitutive activation of the MAPK pathway thus induce uncontrolled cell survival, growth, and proliferation in malignant melanoma (right) that might be reversed by treatment with BRAF

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*inhibitors.*

*b: Mechanisms supporting BRAF inhibitor resistance in melanoma. Genetic changes including NRAS mutation, BRAF amplification, MEK mutations, NF1 mutations, Akt amplification (genetic or epigenetic), and loss of PTEN (genetic or epigenetic), while epigenetic changes include Akt amplification, loss of PTEN, overexpression of HGF, RTK, PDGFR $\beta$ , and IGF1R. (Kakadia et al., 2018).*

## **b) Immunotherapies**

Immunotherapy is also a treatment option, which shows an upward tendency whatever the oncogenic mutation of the tumor (BRAF/NRAS/NF1/triple WT). The main idea is to boost directly the immune system against cancer. In melanoma, there are several types of immune therapies, including immune checkpoint inhibitors, interferon, IL-2 (Interleukin-2) and TNF (Tumor Necrosis Factor) therapy.

Interferon therapy has been used as adjuvant therapy in melanoma to affect cancer cell proliferation and tumour growth, additionally, IFN- $\alpha$  (Tarhini et al., 2012). IL-2 (Dillman et al., 2012) is also a molecule to treat advanced melanoma, which can boost the activity and growth of many immune cells. However it has now been widely replaced by checkpoint inhibitors because of its substantial toxicity and low efficacy. TNF- $\alpha$  has been used for locally advanced melanoma; however, the controversial role of TNF has also been demonstrated not only as a cancer cell cytotoxic molecule, but also a tumor-promoting factor (Donia et al., 2016), so several TNF- $\alpha$  inhibitors were used or tested in clinical trials, such as infliximab (Nardone et al., 2014).

Checkpoint proteins are present in immune cells such as T cell, in order to keep immune responses in check; but with a large amount of these proteins, T cells are not capable to attack cancer cells. Thus why checkpoint inhibitors can block these proteins and increase the ability of T cells to efficiently kill cancer cells. Two types of checkpoint inhibitors exist nowadays: CTLA-4 blocking antibody, which attaches to CTLA-4 in T cell surface and interrupts its binding to B7 on cancer cells. PD-1 blocking antibody attaches to PD-1 in T cell surface and blocks the interaction with PDL-1 on cancer cells.

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In 2011, anti-CTLA-4 molecule ipilimumab gave hope for the treatment of metastatic melanoma. This therapy has a long-term effect although only 20% of patients respond to it; the global survival is 10 months and may be longer in better responding patients, up to several years (Hodi et al., 2010; Jilaveanu et al., 2009; Lebbé et al., 2014). PD-1 blocking antibodies (nivolumab or pembrolizumab) are efficient in about 30% to 40% patients; similar to ipilimumab, PD-1 antibodies showed a durable effect. Anti-CTLA-4 and PD-1 antibodies have been used in the clinic and demonstrated excellent results without many side effects and increased patients overall survival (Postow et al., 2015). It is also an opportunity for BRAF or MEK inhibitors resistant melanoma. As targeted therapy, immunotherapy is also challenged by subsequent resistance of melanoma tumors. A proportion of 40% to 60% of patients do not achieve any response and some responders relapse within two years (Callahan et al., 2018; Larkin et al., 2015a, 2019) [Figure 8]. There are also two types of resistance according to the timing of occurrence: primary/intrinsic resistance and acquired resistance. Resistance can also be classified as intrinsic or extrinsic to tumor cells: when cancer cells alter intrinsically immune recognition processes, including cell signaling, gene expression and DNA damage response, or when external immune cells fail to recognize tumor cells because of non-tumor cell factors. Primary resistance to immune checkpoint blockade occurs in approximately 40% to 65% of patients treated with anti-PD1 and in more than 70% of those with anti-CTLA-4 therapy (Larkin et al., 2015b; Robert et al., 2011). Clinical pathological factors associated with primary resistance are: elevated level of baseline serum LDH (Diem et al., 2016), increased baseline tumor burden (Nishino et al., 2017), lack of PD-L1 in melanoma tissues (Taube et al., 2014), lack of T-cell infiltration (Tumeh et al., 2014), and absence of PD-1 T cells and PD-L1 macrophages in early melanoma samples (Vilain et al., 2017), low mutational burden and insufficient neoantigens (Snyder et al., 2014), absence of an interferon signature (Ayers et al., 2017). Acquired resistance develops after the immunotherapy selection of subpopulations with genetic or epigenetic traits, which allow them to escape immune system control (Restifo et al., 2016). For instance, beta-2-microglobulin, component of MHC class I molecule, can be lost in metastatic melanoma patients treated with immunotherapy (del Campo et al., 2014). PD-L1 can also be upregulated by tumor cells in order to

limit T cell function (Pardoll, 2012). To overcome this resistance, combinatorial therapies are used (anti-PD1 + anti-CTLA4) or under investigation (anti-PD1 + anti-Lag3). It is also important to note that, because of the existence of inter-patient heterogeneity, the challenge remains to determine which combination is suitable and efficient for each patient.

Variable	Nivolumab plus Ipilimumab (N=314)	Nivolumab (N=316)	Ipilimumab (N=315)
<b>Best overall response — no. (%)<sup>†</sup></b>			
Complete response	69 (22)	60 (19)	18 (6)
Partial response	114 (36)	81 (26)	42 (13)
Stable disease	38 (12)	30 (9)	69 (22)
Progressive disease	74 (24)	121 (38)	159 (50)
Unable to determine	19 (6)	24 (8)	27 (9)

Figure 8: Percentage of patients responding to nivolumab +/- ipilimumab.

Ref: Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma

### c) Other combination treatments

Besides all these treatments above, other treatments are also being studied or tested in clinical trials, such as HDACs (Histone deacetylases) inhibitors and vaccine therapy, in order to overcome the resistance to targeted therapy and immunotherapy. HDACs participate in epigenetic remodeling process; they deacetylate histone after the action of HAT (histone acetyl transferase). In uveal melanoma, HDAC inhibitor could up-regulate melanocyte differentiation markers (Landreville et al., 2012). One type of HDAC inhibitors, vorinostat, was demonstrated to be efficient to kill selectively BRAF inhibitor or MEK inhibitor-resistant melanoma cells (Wang et al., 2018). The combination of HDAC inhibitor with immunotherapy is also an idea to overcome cancer cell resistance; for example, HDAC6 inhibitor improves anti-PD1 therapy by down-regulating anti-immune protein in tumor cells and by inhibiting immune-suppressive macrophages activities (Knox et al., 2019). Another idea is about the vaccination, which means that after the analysis of tumor samples, a personalized vaccine will be generated according to melanoma cell protein fragments, which are capable to spur an immune response (Vasquez et al., 2017). Many clinical trials in melanoma treatments are ongoing or

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completed in order to determinate new treatments in different contexts.

### 3) Melanoma formation

Originated from melanocytes, different steps are required to become melanoma. This process called melanomagenesis. Here, the SSM (Superficial spreading melanoma) model will be used to present this process [Figure 9]. Approximately 70% of cutaneous malignant melanomas are SSM, they may be found on any body surface. This is also the type of melanoma that my thesis is based on.

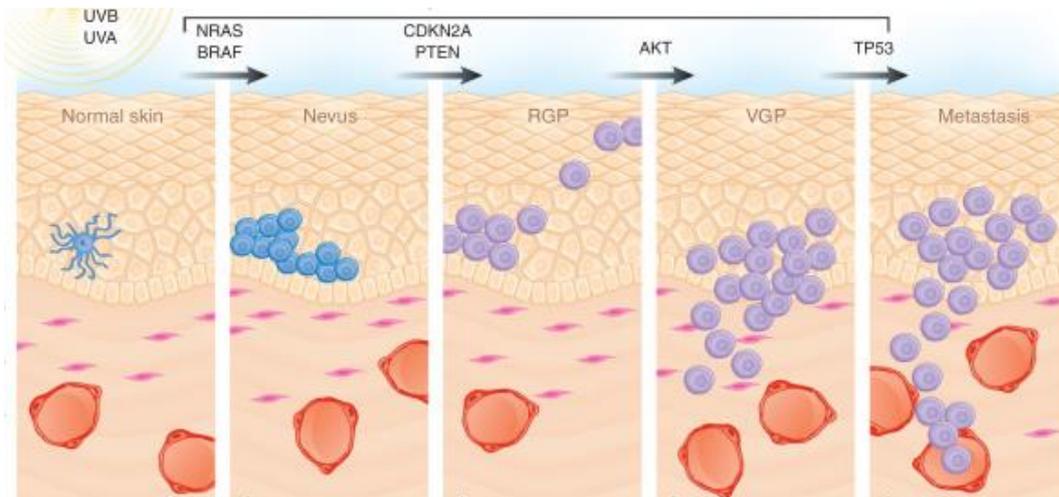


Figure 9: Different steps of melanoma formation.

From left to right side: activation of an oncogene; nevus to RGP (radical growth phase); RGP to VGP (Vertical growth phase); dissemination and metastasis (Zaidi et al., 2008).

The very first step is the aberrant activation of an oncogene. This will induce melanocyte hyper-proliferation and then cell senescence; a benign nevus will be formed. The most common mutation is  $BRAF^{V600E}$ , which presents in about 80% of nevi and 60% of SSM (Poynter et al., 2006). Mutational activation of the RAS/RAF/MEK/ERK pathway is a critical step in the development of nevi. The oncogenic role of BRAF was approved in 2004 by its capacity to induce melanocyte transformation (Garnett and Marais, 2004; Wellbrock et al., 2004). The mutation V600E makes the BRAF constantly activated independently of the extracellular stimulus, the activated MAPK pathway

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makes cells proliferate and survive, in order to initiate tumor transformation (Cantwell-Dorris et al., 2011). Without doubt, melanocytes possess a security control system, which is the senescence. High expression of p16, a cell cycle inhibitor, is a characteristic in nevi (Michaloglou et al., 2005). Other senescence markers such as lysine 9 methylation and DEC1 expression were demonstrated later on (Zhang and Herlyn, 2012).

Then, the passage from nevi to malignant tumor needs more oncogenic elements, such as the loss of a tumor suppressor which makes melanocyte stride over senescence. One important thing to note is that, a significant part of SSM does not arise from a nevus, but from direct senescence escape upon transformation of a melanocyte. Two important pathways controlling senescence need to be inhibited: the p53 pathway and the p16/RB pathway. The promoter of *CDKN2A* (p16 coding gene) may be mutated, deleted or hyper-methylated in melanoma, which results in p16 repression (Bennett, 2007; Dahl and Guldberg, 2007). Although less frequent, mutations in *CDK4* may also present; this will inhibit the CDK4-p16 interaction so that RB cannot be phosphorylated and E2F will become unrestricted to facilitate G1/S transition and S-phase (Meyle and Guldberg, 2009). As for p53 pathway, only 5% of melanomas present p53 gene mutation; in contrast, the mutation in *CDKN2A* leads to the inhibition of p14/ARF expression, so that the repression of p53 will be maintained. Another protein p21 in this pathway may also be repressed by TBX2 and TBX3 which are frequently overexpressed in melanoma (Lu et al., 2010). Besides the p16/RB and p53 pathway, several studies demonstrated also essential role of PI3K-PTEN-AKT pathway; this signaling pathway participates mainly in cell survival and cell cycle progression. One example is that AKT can inhibit GSK3 in order to release cyclin D1, which will then phosphorylate RB and guaranty cell cycle progression. AKT can also phosphorylate MDM2 in order to degrade p53 by proteasome through ubiquitinylation (Hemmings and Restuccia, 2012). Activation of PI3K-PTEN-AKT signaling has two possibilities: PTEN alteration or aberrant activation of AKT. PTEN is a negative modulator in this pathway; in 10% to 40% of melanoma, inactivation mutations in *PTEN* gene are present and it process more important role in advanced melanoma (Aguissa-Touré and Li, 2012; Conde-Perez and Larue, 2012). In 40% to 60% of melanomas, one isoform of AKT, AKT3, is over-expressed due to gene

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amplification (Stahl et al., 2004).

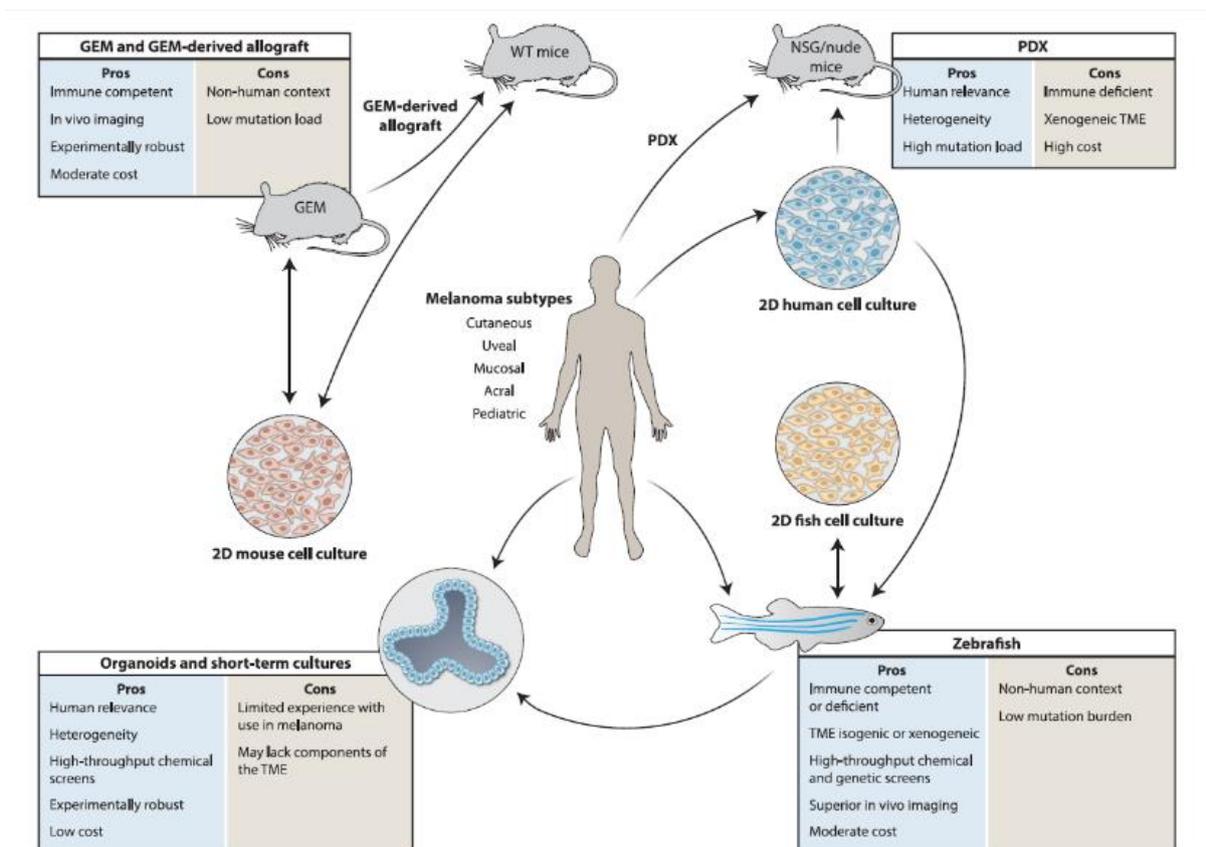
Once the oncogene-induced senescence is inhibited, tumor will become malignant and hyper-proliferative. Cells enter a radical growth phase without the physiological cell cycle control system. At this time, cells are highly proliferative but do not acquire an invasive capacity yet, so cell growth is restricted in a single plane of skin layer within the epidermis, without any penetration through the basement membrane. Surgery is sufficient to remove this *in situ* melanoma (Crowson et al., 2006). After that, some malignant melanoma cells can become invasive and grow vertically deeper in tissues. Even though melanocytes are not epithelial cells, they can pass through an EMT-like transition, and lose specific epithelial markers while acquiring mesenchymal markers, including loss of E-cadherin and gain of N-cadherin and some metalloproteases; anti-apoptotic and motility cell programs are also established. They become rarely proliferative but highly invasive to degrade basement membrane into dermis. Next, blood vessels or lymph nodes may be in contact with these cells (Mobley et al., 2012; Scatolini et al., 2010).

The last step is the dissemination. Melanoma cells enter into blood vessels or lymph circulation. Local metastasis can be generated close to the primary tumor within 2cm of the surgical scar; it can result either from the extension of the primary melanoma or from spreading via the lymphatic vessels. In-transit metastasis is melanoma deposits within the lymphatic vessels more than 2cm from the site of the primary melanoma. Nodal metastasis is metastatic melanoma involving the lymph nodes. The lymph nodes first involved are the regional lymph nodes. Usually, the involved lymph nodes become enlarged and may be able to be felt. When melanoma cells are in the bloodstream, they can travel to distant sites and seed. Melanoma cells can proliferate in any tissue but most often grows in the lungs, the liver and the brain. Many patients also develop metastases in bone, gastrointestinal tract, heart, pancreas, adrenal glands, kidneys, spleen and thyroid. At molecular level, metastatic melanoma cells express a large amount of VEGF and chemokines which are involve in cell motility (Braeuer et al., 2011; Leiter et al., 2004). In MAPK pathway, BRAF participates also in actin cytoskeleton organization to facilitate cell motility; BRAF can also influence

cellular contraction by release Ca<sup>+</sup> in cytoplasm, via the inhibition of PDE5A kinase, which fuel cell invasion (Arozarena et al., 2011).

#### 4) Melanoma models

Various melanoma models have been developed, that exhibit pros and cons depending upon the question to address [Figure 10]. *In vitro* models are suitable for mechanistic analyses, while *in vivo* models help capture the complex microenvironment and immune response that lead to the outcome of melanoma. Animal model is as similar as possible to its human counterpart, but it is also continuously improved through genetic engineering, and/or technology advances. A key developmental aspect of all animal models is that the "omics" data generated from their research can now be directly compared with comparable human data sets, providing strong correlation verification. The establishment of *in vitro* cultures generated from models and directly from patients (such as organoids) provides additional experimental possibilities. However, the most important and physiological as well as the final way is still clinical study, which is why patients cohorts are always needed to give definitive proof.



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*Figure 10. Pros and cons of current melanoma models*

*2D, two-dimensional; GEM, genetically engineered mouse; GDA, GEM-derived allograft; NSG, NOD/SCID/IL-2 $\gamma$ <sup>null</sup> immunodeficient mouse; PDX, patient-derived xenograft; TME, tumor microenvironment; WT, wild-type. Melanoma models for the next generation of therapies.(Patton et al Cancer Cell 2021).*

**a) Melanoma short-term culture**

The use of appropriate models with which to study a specific biological question is a major challenge in cancer research. Cell lines have long been used to study cellular processes and the effects of individual molecules because they are easy to use, grow rapidly, produce reproducible results and have a strong track record in research. In order to understand melanoma biology *in vitro*, melanoma cell lines are needed. Besides standard ATCC (American Type Culture Collection) melanoma cells lines such as A375, SKMEL24, which are limited in homogeneity and in genetic or phenotypic drift, other cell lines generated from patients are also important, they are closer to physiological conditions and more representatives of phenotypes *in vivo*. But these cultures are heterogeneous, require constant monitoring, are sensitive to passaging and often have slow proliferation rates. This is most likely due to the spontaneous selection for the fittest subpopulation in the *in vitro* environment, resulting in clonal expansion and eventually in an immortalized cell line (Aubert et al., 1993; Sanlorenzo et al., 2015; Thurber et al., 2011). Many melanoma short-term cultures have been generated, such as Ma-Mel-123 used in single-cell RNA-seq (Gerber et al., 2017), 11 primary melanoma cultures derived from patient biopsies were used to study melanoma progression and therapeutic target (Gembarska et al., 2012; Verfaillie et al., 2015), or GLO generated in our group to understand melanoma resistance (Richard et al., 2016a). Even though 2D cultures are now less popular than 3D cultures, they are still useful in many aspects; not all molecular analyses are possible in 3D, for example, ChIP-sequencing, which needs a large amount of 2D cultured cells and takes a large place of my thesis to investigate melanoma cells mechanisms.

**b) Melanoma 3D culture**

Nowadays, motivated by the need to work with cellular models that mimic the function of living tissues, monolayer cell culture is improved into 3D culture in order to understand disease

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biology and therapeutic efficacy. Tumor spheroids are perhaps one of the simpler and more cost-effective 3D systems talked in the literature (Fennema et al., 2013; Vinci et al., 2012). Spheroids can be generated either from a single-cell type or as multicellular spheroids representative of *in vivo* cells found in tumor of interest. Multicellular spheroids seem like avascular tumor nodules. With increasing size of spheroids, oxygen and nutrition gradients affect not only cellular RNA and protein expression but also the penetration and bioactivity of therapeutic drugs of interest (Hirschhaeuser et al., 2010). Some cells require also the addition of extracellular matrix compounds, methylcellulose or agarose. A 3D organotypic melanoma spheroid skin model was generated, melanoma cells are embedded with primary skin cell; this model portrays the *in vivo* architecture of malignant melanoma, and could facilitate drug screenings in the future (Müller and Kulms, 2018).

### **c) Human skin reconstructs**

To go further to mimic natural skin biology and to study melanoma in a physiological environment, human skin reconstructs may be a better choice. These are 3D *in vitro* models consisting of epidermal keratinocytes plated onto fibroblast-contracted collagen gels. Cells in skin reconstructs more closely recapitulate the *in situ* phenotype than cells in 2D culture. Normal melanocytes remains singly distributed at the basement membrane which separates the epidermis from the dermis, interspersed with basal layer keratinocytes. This model enables the investigation of cell-matrix and cell-cell interaction between different cell types. This model has already been used to analyze the growth pattern of melanoma cells representing different stages of tumor progression, by using normal melanocytes, radial and vertical growth phase, primary and metastatic melanoma cells, which were all incorporated into the epidermis (Meier et al., 2000). This model may allow us to investigate melanoma cell functions such as invasiveness in our study.

### **d) Melanoma mouse models**

Despite the difference between mouse and human melanocyte biology, mouse model is still a powerful strategy to understand melanoma progression and therapeutic responses, because murine model owe their ubiquity to their ease of manipulation and availability as well as existing

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knowledge base regarding their genetics. Many mouse models are used, including xenograft, syngeneic, and genetically engineered models. Xenograft models involve the culturing and engraftment of human melanoma cells into immune-deficient mice. Key pathways responsible for malignancy can be quickly identified because of their easy establishment and manipulation. Depending on different origins, there are cell line xenografts, patient-derived tumor xenografts. Syngeneic xenograft transplantation involves the induction and transplantation of melanoma cells into same species and genetic background (Bobek et al., 2010); because of the presence of immune system, this model is frequently used to evaluate immunotherapies and interactions between tumor and immune cells (Klarquist and Janssen, 2012). Genetically engineered models use transgenic mice with modified gene expression and are often used to determine the mechanisms of melanomagenesis; in such model, metastasizing melanoma either arises spontaneously or requires additional physical or chemical induction (Larue and Beermann, 2007; Pérez-Guijarro et al., 2017) [Figure 11]. Based on the identification of oncogenic mutations in melanoma, different models processing melanoma initiating mutations were generated, such as BRAF<sup>V600E</sup>, NRAS<sup>Q61R</sup>, etc., to elucidate gene function and to identify key targets for therapeutics (Combest et al., 2012; Dankort et al., 2009); RXR-mutant mice were also generated to study the UV induced melanoma progression (Coleman et al., 2014). Mouse model can allow us to investigate xenograft melanoma cells properties, such as tumor growth and metastasis in different contexts.

## Genetically engineered mouse models of cutaneous melanoma

Promoter	Genetic Modification	Carcinogen	Latency	Penetrance
Tyr	SV40 transgenic	UVB	57 wk	80%
Tyr	<i>HRas<sup>G12V</sup>, Ink4a/Arf<sup>-/-</sup></i>	None	5.5 mo	61%
Tyr	<i>HRas<sup>G12V</sup></i>	DMBA	45 wk	100%
Tyr	<i>HRas<sup>G12V</sup></i>	UVB	45 wk	20%
Tyr	<i>HRas<sup>G12V</sup>, p53<sup>+/-</sup></i>	None	65 wk	12%
Tyr	<i>HRas<sup>G12V</sup>, p53<sup>-/-</sup></i>	None	17 wk	26%
Tyr	<i>HRas<sup>G12V</sup>, p16<sup>Ink4a</sup><sup>-/-</sup></i>	None	~75 wk	40%
Tyr	<i>HRas<sup>G12V</sup>, p19<sup>Arf</sup><sup>-/-</sup></i>	None	~60 wk	~60%
Tyr	<i>NRas<sup>Q61K</sup></i>	None	>1 yr	29%
Tyr	<i>NRas<sup>Q61K</sup>, Ink4a/Arf<sup>-/-</sup></i>	None	1 yr	83%
Tyr	<i>NRas<sup>Q61K</sup>, Ink4a/Arf<sup>-/-</sup></i>	None	6 mo	94%
Tyr::CreER <sup>T2</sup>	<i>Nras<sup>61R/61R</sup>, p16<sup>Ink4a</sup><sup>lox/lox</sup></i>	None	26 wk	70%
Tyr::CreER <sup>T2</sup>	<i>Nras<sup>61R/61R</sup>, p16<sup>Ink4a</sup><sup>lox/lox</sup>, Lkb1<sup>lox/lox</sup></i>	None	22 wk	85%
Tyr-rtTA	<i>TRE-Nras<sup>Q61K</sup>; Cdkn2a<sup>-/-</sup></i>	None	15 wk	50%
Tyr::CreER <sup>T2</sup>	<i>Kras<sup>G12D</sup>, p53<sup>lox/lox</sup></i>	None	39 wk	100%
Tyr::CreER <sup>T2</sup>	<i>Kras<sup>G12D</sup>, Lkb1<sup>lox/lox</sup></i>	None	39 wk	100%
Tyr::CreER <sup>T2</sup>	<i>Kras<sup>G12D</sup>; p53<sup>lox/lox</sup>; Lkb1<sup>lox/lox</sup></i>	None	11 wk	100%
Tyr::CreER <sup>T2</sup>	<i>Kras<sup>12D/WT</sup>; p16<sup>Ink4a</sup><sup>lox/lox</sup></i>	None	36 wk	76%
Tyr	<i>Braf<sup>V600E</sup>, Ink4a/Arf<sup>-/-</sup></i>	None	~14 wk	~95%
Tyr	<i>Braf<sup>V600E</sup>, p53<sup>-/-</sup></i>	None	~21 wk	~80%
Tyr::CreER <sup>T2</sup>	<i>Braf<sup>CA</sup>, p16<sup>Ink4a</sup><sup>-/-</sup></i>	None	7 mo	80%
Tyr::CreER <sup>T2</sup>	<i>Braf<sup>CA</sup>, Cdkn2a<sup>lox/lox</sup></i>	None	14 wk	100%
Tyr::CreER <sup>T2</sup>	<i>Braf<sup>CA</sup>, Cdkn2a<sup>lox/lox</sup></i>	None	9 wk	100%
<i>Lkb1<sup>lox/lox</sup></i>	Tyr::CreER <sup>T2</sup> ; <i>Pten<sup>lox/lox</sup>Cdkn2a<sup>lox/lox</sup></i>	None	9 mo	100%
Tyr::CreER <sup>T2</sup>	<i>Braf<sup>CA</sup></i>	UVB	5.3 mo	100%
Tyr::CreER <sup>T2</sup>	<i>Braf<sup>CA</sup>, Trp53<sup>R172H</sup></i>	None	3.5 mo	100%
Tyr::CreER <sup>T2</sup>	<i>Braf<sup>CA</sup>; Pten<sup>lox/lox</sup></i>	None	5 wk	100%

Figure 11. Examples of genetically engineered mouse models of cutaneous melanoma.

Tyr, Tyrosinase; CreER<sup>T2</sup>, tamoxifen-dependent Cre recombinase 2; MT1 (Mt1), metallothionein 1; Dct, dopachrome tautomerase; rtTA, reverse tetracycline-controlled trans activator; TVA, avian leukemia virus receptor; SV40, simian vacuolating virus 40 T antigen; HRas (Hras), Harvey rat sarcoma virus oncogene; NRas (Nras), neuroblastoma ras oncogene; Ink4a/Arf (Cdkn2a), cyclin-dependent kinase inhibitor 2A; p16<sup>Ink4a</sup>, Cdkn2a isoform p16; p19<sup>Arf</sup>, Cdkn2a isoform p19; BRAF (Braf), Braf transforming gene; Braf<sup>CA</sup>, Cre-activated Braf<sup>V600E</sup> allele. Ref: Genetically Engineered Mouse Models of Melanoma.

## Chapter2. EMT-TFs function in carcinoma

Epithelial to mesenchymal transition (EMT) is a physiological cell plasticity process, tightly

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regulated in response to microenvironmental signals, which is driven by a network of EMT-inducing transcription factors (EMT-TFs), mainly represented by the SNAIL, TWIST and ZEB protein families, which interact with epigenetic regulators.

## **A. Physiological EMT**

### **1) EMT definition**

EMT, the epithelial to mesenchymal transition, is a cell plasticity process in which cells lose their epithelial features and gain mesenchymal characteristics [Figure 12]. Epithelial cells exhibit cell-cell junction as well as apical-basal polarity, while mesenchymal cells lack apical-basal polarity showing a spindle-like morphology, which exhibits a heightened motility and invasiveness (Polyak and Weinberg, 2009). This transition is tightly regulated by several micro-environmental signals, which will be detailed in the "EMT activated extracellular signals" part. The EMT paradigm was initially originated from classic experiment by Elizabeth Hay, in which mature epithelial cells were explanted into collagen I gels and they lost their polarity and migrated into the matrix as single cells with a strong morphological resemblance to embryonic fibroblasts, a mesenchymal cell type (Greenburg and Hay, 1982; Hay and Zuk, 1995). This transition is also combined with a loss of epithelium-specific gene expression such as E-cadherin, and gain of mesenchymal gene expression including vimentin and N-cadherin (Thiery, 2002). Known to have essential roles in multiple steps of embryonic morphogenesis, EMT program also plays essential role in various pathological processes, including wound healing, tissue fibrosis and cancer (Lim and Thiery, 2012; Nieto et al., 2016; Shibue and Weinberg, 2017) [Figure 13]. EMT is reversible, and the reversed process is termed MET (Mesenchymal to epithelial transition). This reversibility underscores the plasticity of certain embryonic cells and the plasticity of adult cells that are involved in disease pathogenesis (Yang and Weinberg, 2008).

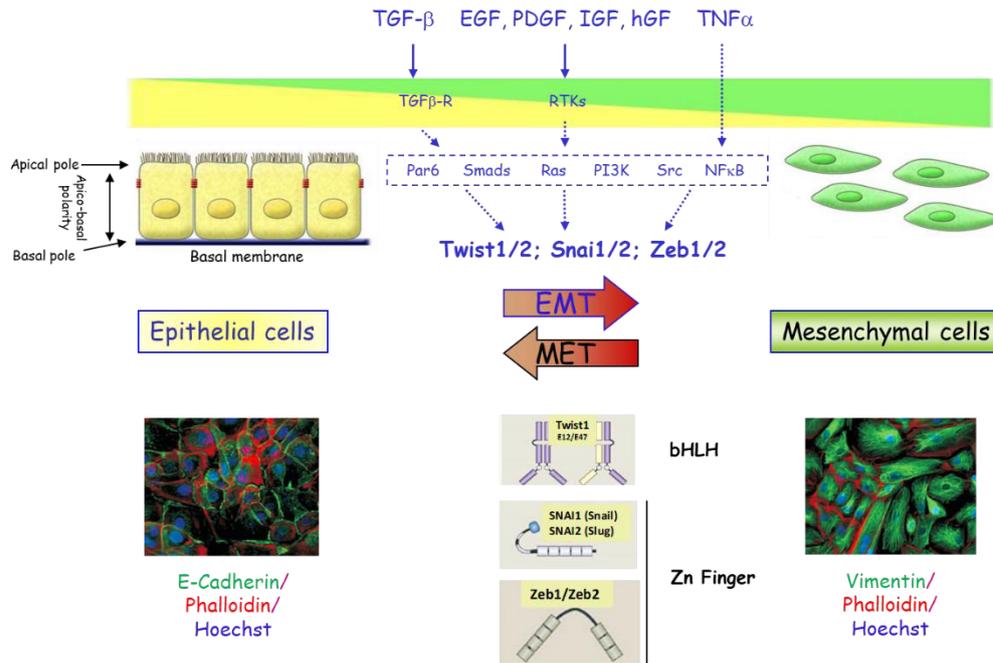


Figure 12. Schematic presentation of EMT.

Epithelial to mesenchymal transition (EMT) is a physiological cell plasticity process, tightly regulated in response to microenvironmental signals, which is driven by a network of EMT-inducing transcription factors (EMT-TFs), mainly represented by the SNAIL, TWIST and ZEB protein families.

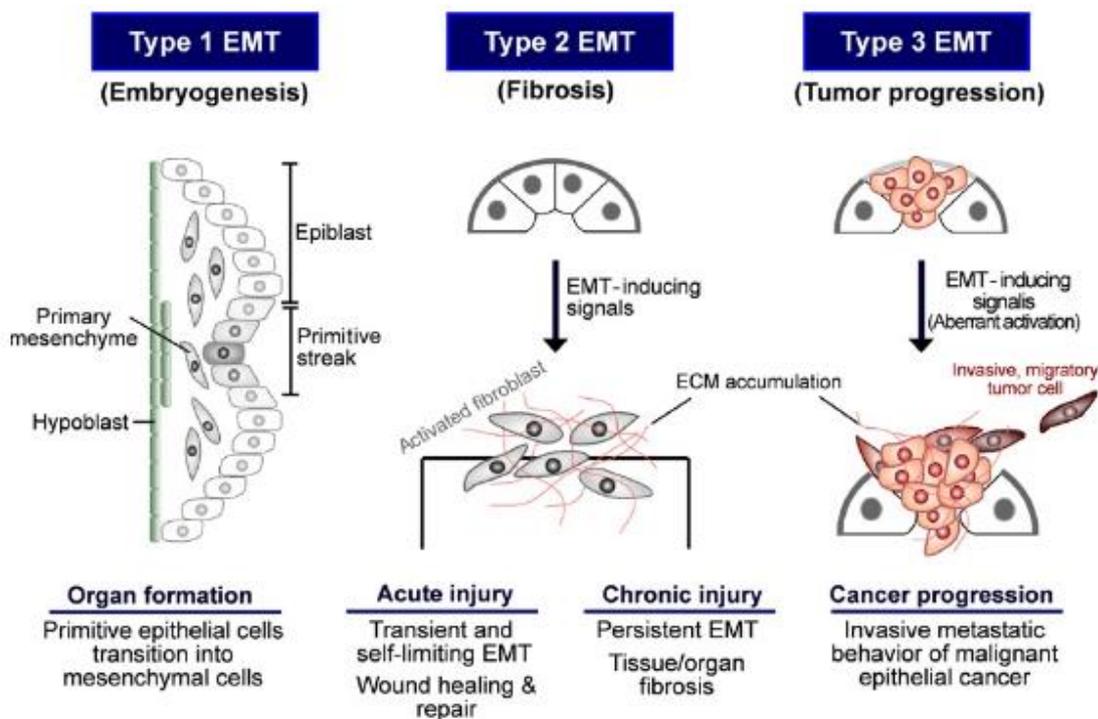


Figure 13. Different types of EMT.

Type 1 EMT is associated with gastrulation and generation of mesoderm, endoderm, and neural crest. The primitive epithelium gives rise to primary mesenchyme through an EMT. Type 2

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*EMT begins as part of tissue repair to generate fibroblasts. Type 2 EMT can contribute to organ destruction if it is persistent if inflammation insult is not attenuated. Type 3 EMT occurs in epithelial cancer cells and affects oncogenes and tumor suppressor genes which conspire with the EMT proteome to result in increased invasiveness and migration. (Lee & Nelson 2012)*

## **2) EMT in embryonic development (Type I EMT)**

The first EMT function is observed during implantation, embryogenesis and organ development, in order to generate diverse cell types which may undergo an MET to form secondary epithelial tissue.

The gastrulation, in which three definitive germ layers called ectoderm, mesoderm and endoderm are established from the initial epithelial embryonic cells, in order to form the trilaminar embryonic disk. This process situates the ectoderm in the outer layer, the mesoderm in the middle layer and the endoderm in the inner layer of the embryo. The mesoderm and the endoderm achieve their location through a process called ingression, which is an EMT process whereby outer epithelial cells detach and are internalized through the primitive streak (Shook and Keller, 2003). Neural crest cell formation is essential to form the skeletal and connective. During this process, sequential rounds of EMT and MET are needed for the cell tissue components of the vertebrate head and the ganglia of the peripheral nervous system differentiation and the 3D structure of the organs; these sequential rounds are referred as primary, secondary and tertiary EMT (Thiery et al., 2009) [Figure 14]. After implantation, the earliest primary EMT occurs in order to form mesoderm from the primitive ectoderm via gastrulation, as well as in neural crest development; this process also enables these cells to maintain multi-potentiality and to differentiate further into various cell types. A primary EMT is directed by the epithelial morphogenesis whereby the epithelial layer undergoes de-epithelialization and the epithelial cells undergo ingression, followed by the adoption of basic mesenchymal characteristics such as motility and migration. The post gastrulation is considered as the secondary EMT, which leads to the formation of neural crest within ectodermal zones, thus giving rise to various cells types. The migratory neural crest cells travel through different

routes to their final destination and differentiate into neurons, bone and connective tissues. The tertiary type of EMT can be well explained through a successive cycle of embryonic heart formation, which involves three successive cycles of an EMT and MET.

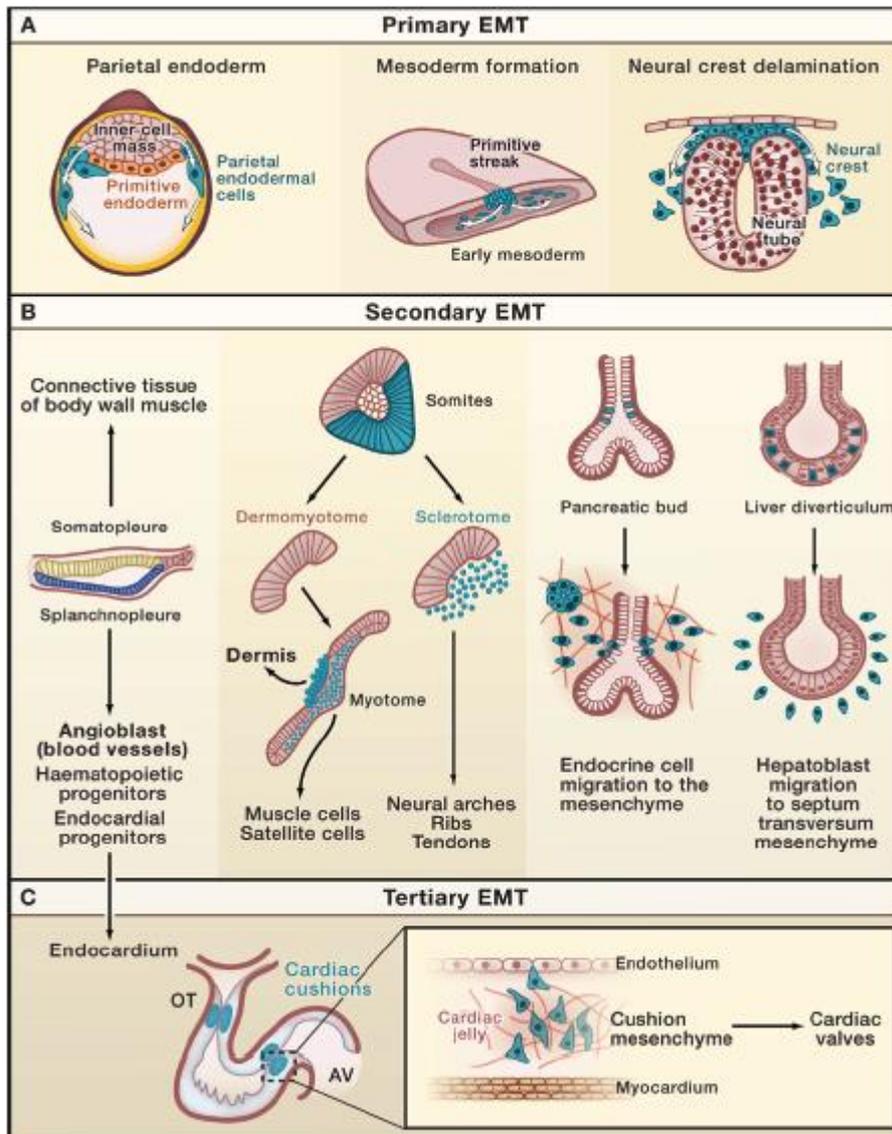


Figure 14: Different types of EMT during embryonic development.

(A) Primary EMT occurs early during embryonic development, even before implantation such as during the formation of the parietal endoderm in mice. (B) Early mesodermal cells are subdivided into axial, paraxial, intermediate, and lateral plate mesodermal cells that will condense into transient epithelial structure. These transient structures will undergo secondary EMT, leading to the generation of mesenchymal cells that differentiate into specific cell types. (C) An example of tertiary EMT arises during the formation of the cushion mesenchyme in the heart from the atrioventricular canal (AV) or the outflow tract (OT). The cushion mesenchyme is the precursor of the cardiac valves. (Thiery et al 2009)

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### 3) EMT in tissue repair and fibrosis (Type II EMT)

Type II EMT involves transition of secondary epithelial cells to resident tissue fibroblasts; it occurs during wound healing, tissue regeneration and organ fibrosis. In contrast to type I, type II EMT is induced in response to inflammation, but stops once inflammation is attenuated, especially during wound healing and tissue regeneration. Type II EMT continues to respond to inflammation during fibrosis which results in tissue destruction (López-Novoa and Nieto, 2009; Wynn, 2008).

Wound healing consists of several overlapping phases which involve an injury-induced inflammatory response which is associated with cellular proliferation, migration and ECM remodeling (Eming et al., 2014). During wound healing and tissue regeneration, the EMT process begins as a repair-associated event that normally generates fibroblasts and other related cells in order to reconstruct tissues after injury (Kalluri and Weinberg, 2009). At the wound edge, keratinocytes lose their intercellular adhesion and migrate across the wound (Coulombe, 2003). Specifically, these keratinocytes undergo changes in junction complexes including reduction in desmosomes and adherent junctions, disruption of intermediate filaments and cytoskeletal reorganization which results in the creation of intercellular gaps (Baum and Arpey, 2005; Santoro and Gaudino, 2005). These modifications enable keratinocytes to shift from cuboidal and stationary to flattened and migratory. During physiological repair, tissue integrity must be restored not only through re-epithelialization, but also through formation of a stress-resistance scar. Myofibroblasts derived from resident fibroblasts or trans-differentiated of epithelial cells through EMT are the key players in the remodeling and maturation phase of wound healing; these cells synthesize ECM components and contract the wound bed, in order to aid in the mechanical closure of the wound (Barnes and Gorin, 2011; Wynn and Ramalingam, 2012). Then, many myofibroblasts undergo apoptosis and disappear. This important process is ensured by the transcription factor Snail2, which will be better detailed in EMT transcription factors part. EMT also occurs during repair of organs other than the skin; *in vitro* healing of a mammary epithelial cell line showed that EMT-associated

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vimentin was expressed in a migration-dependent way, such as vimentin was exclusively induced in actively migrating cells at the leading wound edge, then vimentin expression subsequently disappeared when wound closure was achieved (Gilles et al., 1999).

In normal wound healing, many myofibroblasts disappear at the end; however, pathologically prolonged myofibroblasts activity can result in fibrogenesis. Indeed, persistent myofibroblast activation is a shared feature of fibrotic diseases, where the deregulation of injury-triggered EMT is believed to contribute to fibrosis of multiple organs. TGF $\beta$ 1, a critical regulator of EMT signalling as well as physiological wound healing, is also the major driver of fibrosis (details in next part). Tissue fibrosis is basically an un-abated type of wound-healing caused by persistent inflammation. A pathological EMT process resembles a physiological process in that they are both governed by similar signalling pathways. Organ fibrosis occurs in a number of glandular epithelial tissues whereby inflammatory cells and fibroblasts release various inflammatory signals and several ECM components, such as collagen, laminin, elastin and tenascin. During fibrosis, endothelial cells associated with the microvasculature contribute to the formation of mesenchymal cells via pathological EMT process called an EndMT (endothelial-mesenchymal transition) (Potenta et al., 2008).

#### **4) EMT regulation and extracellular signals**

Activated in the posterior region of the embryo, the canonical Wnt signaling pathway plays an essential role in vertebrate gastrulation, especially for mesodermal and endodermal cells to ingress at the primitive streak [Figure 15]. Besides, this signaling can also render ingressing cells to respond to extracellular signals initiating EMT (Skromne and Stern, 2001). Two downstream factors of Wnt, Nodal and Vg1, are both TGF $\beta$  superfamily members and play key roles in the initiation of the gastrulation process (Chea et al., 2005). Nodal and Vg1 cooperate to induce the primitive streak formation and cell ingression, in order to render the epiblast competent to gastrulate (Acloque et al., 2009). At the same time, FGF (Fibroblast growth factor) signaling interacts with them and maintains

their expression to ensure the gastrulation to proceed (Mathieu et al., 2004).

After the gastrulation, neural crest cells undergo an EMT process, delaminate from the neural folds and then migrate from the neural tube. They migrate into distinct regions of the embryo then undergo MET and give rise to different cell derivatives. Similar signals are shared in gastrulation initiation and during neural crest cell formation, delamination and migration. The canonical Wnt pathway participates in the induction, stabilization and delamination of neural crest cells precursors, while the non-canonical Wnt pathway is important in contact inhibition of locomotion and directional movements of neural crest cells (Carmona-Fontaine et al., 2008; De Calisto et al., 2005). The TGF $\beta$  superfamily also involve in neural crest formation, especially BMPs (Bone morphogenetic protein). For example, expression of BMP4 in neural folds can guide the epithelial cells into migratory neural crest cells via the activation of Msx1 (msh homeobox 1) and Snail2 (Karafiat et al., 2005). Neural crest cell migration timing may also be controlled by BMPs, while a BMP inhibitor, Noggin, can prevent this migration process (Burstyn-Cohen et al., 2004). Other factors involving in the neural crest formation, including SOX8, SOX9, SOX10, FOXD3, RHOB and Snail2, are also important in this process but may not be sufficient to induce a complete EMT (Acloque et al., 2009; Cheung and Briscoe, 2003; Cheung et al., 2005). Altogether, a complex and tightly regulated combination of various transcription factors is essential for the neural crest cells EMT and migration.

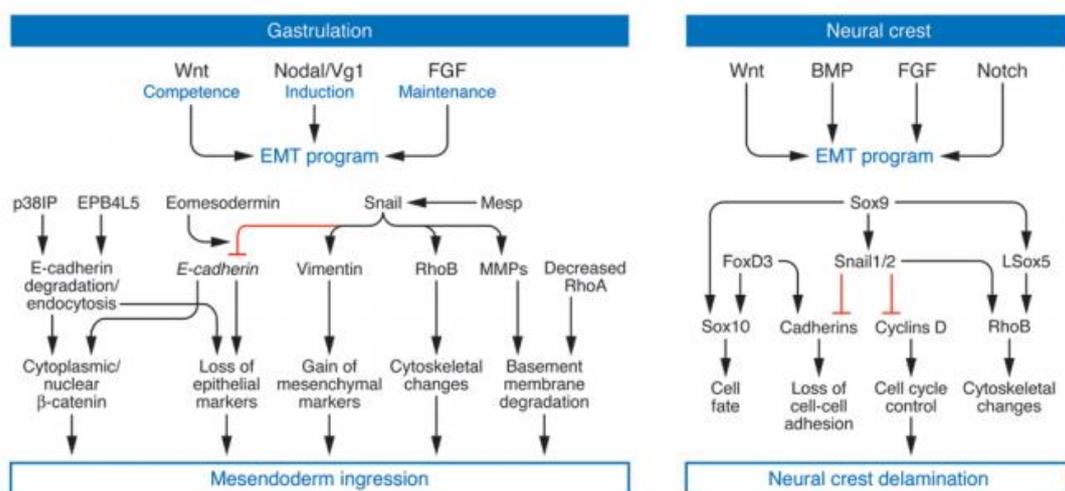


Figure 15: Signaling pathways control the EMTs at gastrulation and neural crest delamination in the amniote embryo.

TFG- $\beta$  superfamily (Nodal, Vg1, and BMPs), together with Wnt and FGF, initiate the formation

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*of the primitive streak and operate at the neural folds to drive the ingression of the mesendoderm and the delamination of the neural crest, respectively. Some downstream targets are also conserved, such as the Snail genes. While Snail factors are key regulators of the EMT program during gastrulation, the coordinated induction of several transcription factors is required to control the robust program of neural crest delamination. EPB4L5, FERM and actin-binding domain-containing band 4.1 superfamily member; p38IP, p38-interacting protein; Rho, members of the Rho family of small GTPases. (Acloque et al 2009)*

As for wound healing and tissue regeneration, the EMT process is activated by many extracellular signals. Notably, the EGF (epidermal growth factor) family represents the best characterized growth factor family in wound healing, which includes a wide variety of ligands, such as EGF, HB-EGF, TGF $\alpha$ , etc (Barrientos et al., 2008). EGF leads to the activation of several signaling pathways promoting keratinocyte migration and proliferation (Omenetti et al., 2008). EGF also aids to accomplish EMT by internalizing E-cadherin and up-regulating SNAIL1 and TWIST, and increasing cell motility through MMP-directed ECM degradation (Ahmed et al., 2006; Lo et al., 2007). EGF (epidermal growth factor) induces wound healing in ovarian surface epithelium by activating metalloproteases, ILK kinase and ERK kinase (Ahmed et al., 2006). EGF is also able to regulate the expression of transcription factors that regulate EMT. Indeed, EGF is a master regulator of EMT/Slug mediated effects; it stimulates the expression of Slug mediated by Erk5 (Kusewitt et al., 2009). Snail2 (slug) is important in wound healing, since it can regulate the metastable state of keratinocytes at the migratory front to compromise or accelerate wound healing process (Arnoux et al., 2008). Moreover, healing of excisional wounds is impaired in Slug knockout mice almost twofold in comparison to wild-type mice (Kusewitt et al., 2009). At the mechanistic level, Slug regulates keratinocyte motility during re-epithelialization by repressing E-cadherin, leading to decreased cell-cell interaction (Savagner, 2001); Slug also drives intercellular dermosomal disruption at the wound edge (Savagner et al., 2005). FGF (Fibroblast Growth Factor) family also plays a preponderant role in embryonic development and in wound healing processes. For instance, FGF2 can increase keratinocyte and fibroblast motility and stimulate fibroblasts to produce collagenase (Di Vita et al., 2006). The HGF (Hepatocyte Growth Factor) signaling is also a key regulator of wound healing processes. HGF is mainly produced by fibroblasts and binds to its tyrosine kinase receptor c-Met on the surface of keratinocytes (Toyoda et al., 2001). HGF can regulate EMT-TF SNAIL1 and Slug which

helps to break intercellular adhesion. The c-Met-PI3K/AKT pathway also influences cell cycle, proliferation and quiescence (King et al., 2015). TGF $\beta$  is well studied not only in wound healing but also in all types of EMT. In Wound healing, TGF $\beta$ 1 plays important roles in inflammation, angiogenesis, re-epithelialization and connective tissue regeneration (Ramirez et al., 2014). TGF $\beta$ /SMAD induces SNAIL1 expression in order to promote EMT process (Vincent et al., 2009). TGF $\beta$  is also a major driver of fibrosis, through its role in sustaining myofibroblast activation (Hong et al., 2007). In fibrosis, FSP1 (fibroblast specific protein 1),  $\alpha$ -SMA and collagen I are markers of an EMT induced mesenchymal cell phenotypes, especially in chronic inflammation (Zeisberg et al., 2003). TGF $\beta$  participates in renal fibrosis via Smad3 activation to induce FSP1 expression (Okada et al., 2000). In kidney, liver and intestines with inflammation, DDR2, vimentin and desmin are also markers to identify EMT (Kalluri and Weinberg, 2009)). In total, a similar set of growth factors are indispensable for wound healing and type II EMT, including FGF, EGF, HGF and TGF $\beta$  [Figure 16]. Then ERK, p38 and JNK are activated among the induced pathways that up-regulate EMT-TFs such as SNAIL, TWIST and ZEB (Tsai and Yang, 2013).

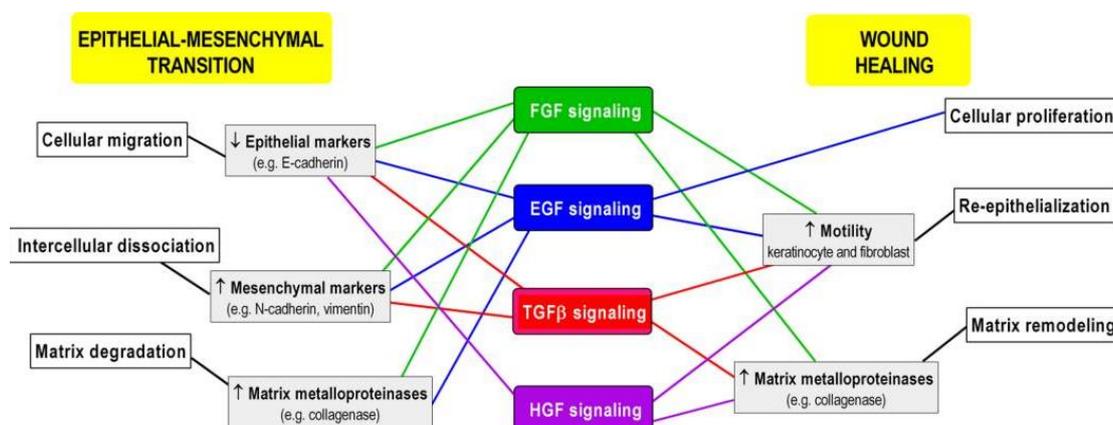


Figure 16: Common growth factor signals initiate and regulate essential EMT and wound-healing processes.

FGF, fibroblast growth factor; EGF, epidermal growth factor; TGF $\beta$ , transforming growth factor beta; HGF, hepatocyte growth factor. (Stone et al 2017)

## 5) EMT-TFs

As final effectors activated by a variety of extracellular signals, the EMT-TFs, are responsible for

EMT accomplishment. A core set of EMT-TFs, including Snail (SNAI1), Slug (SNAI2), TWIST1, ZEB1 and ZEB2 [Figure 17], mediate the EMT process as well as additional traits, such as stemness (Puisieux et al., 2014), survival and changes in cell metabolism (Huang and Zong, 2017; Sciacovelli and Frezza, 2017). Each of them can also activate specifically several traits, so they are not redundant (Stemmler et al., 2019).

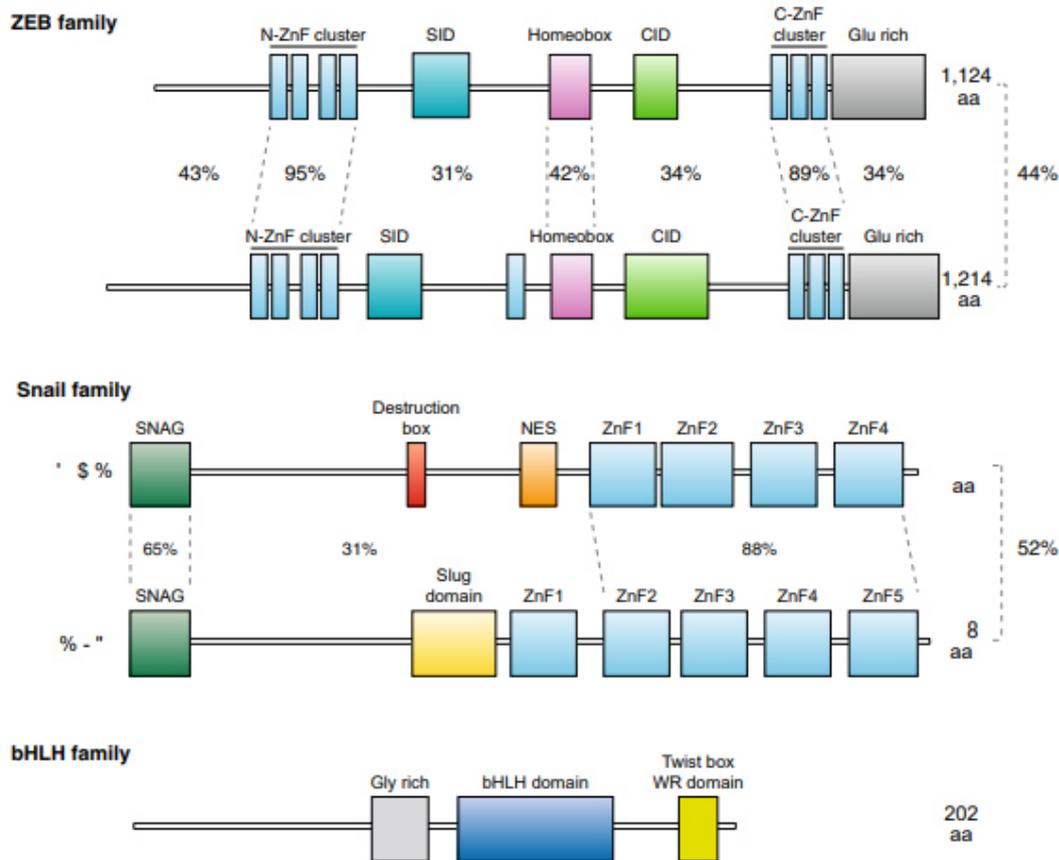


Figure 17: Schematic representation of the protein structures of the core EMT-TFs.

DNA-binding domains are represented in blue. ZEB proteins have two separate zinc-finger (ZnF) clusters, whereas the Snail proteins and TWIST1 only have one DNA-binding unit. TWIST proteins dimerize through the Twist WR domain to recognize a double E-box motif and to affect transcription. The homology is also shown (percentages). aa, amino acids; bHLH, basic helix–loop–helix; CID, CtBP interaction domain; NES, nuclear export sequence; ; CID, CtBP interaction domain; NES, nuclear export sequence; SID, Smad interaction domain; SNAG, Snail corepressor binding domain. (Stemmler et al 2019)

### a) SNAIL family

The SNAIL is the best-studied EMT-TF family. SNAI1 was first discovered as snail in Drosophila

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in 1984, SNAI1 mutant embryos display drastic alteration of segmentation, thus demonstrating its essential roles in embryogenesis (Grau et al., 1984). SNAI2 was first described ten years later than SNAI1 by Nieto et al.; SNAI2 was named as slug and was shown to be important in chick embryo mesoderm formation and neural crest migration during gastrulation (Nieto et al., 1994). SNAI1 and SNAI2 belong to the zinc finger transcription factor family; they can both recruit several co-regulators. Sharing a conserved organization, both of them are composed of four to five C2H2 type zinc fingers at C-terminal and a SNAG domain at N-terminal (Manzanares et al., 2001). A central Slug domain is present only in SNAI2. The N-terminal SNAG domain of SNAI1 has been shown to bind co-repressor, such as HDAC1/2 (Peinado et al., 2004); in SNAI2, co-repressor NCoR and CtBP interact with SNAG and SLUG domain (Molina-Ortiz et al., 2012). The EMT-TF SNAIL can bind to three E-boxes present in the human E-cadherin promoter and repress transcription of E-cadherin (Batlle et al., 2000). SNAIL family is involved in processes that imply pronounced cell movements, during both embryonic developments as well as upon acquisition of invasive and migratory properties in tumor migration. Even though similar at structural level, SNAI1 and SNAI2 have been demonstrated to display context-dependent functional roles. They are differentially expressed in normal mammary glands in which distinctly induce EMT process. SNAI1 occupies more promoters than SNAI2 (Ye et al., 2015). SNAI1 induced EMT contributes to the formation of many tissues during embryonic development. *Snai1* deficient mice die at embryonic day E8.5 with defects in left-right asymmetry. As detailed in the melanoma part, SNAI2 also displays roles in early development, *Snai2* is notably expressed in migratory neural crest cells and is necessary for melanoblast migration and survival, but not for neural crest formation (Jiang et al., 1998). *Snai2* deficient mice are viable and have a white forehead blaze, with depigmentation of the central body; it displays important roles in melanocyte stem cells, hematopoietic stem cells and germ cells (Pérez-Losada et al., 2002). Snail genes also have additional functions independently of the induction of EMT (Barrallo-Gimeno and Nieto, 2005). For instance, they protect cells from death induced by the loss of survival factors or by direct apoptotic stimuli.

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## **b) TWIST family**

After its discovery in *Drosophila*, TWIST1 has been intensively studied in field of embryonic development and subsequently in tumor development. TWIST1 belongs to the conserved bHLH family. Twist family bHLH proteins bind as dimers and recognize cis-regulatory elements called E-box. An E-box-independent mechanism of TWIST1 has also been demonstrated in the interaction between TWIST1 and p53 (Piccinin et al., 2012). Twist family form functional homodimers or heterodimers with other protein in bHLH family (Castanon et al., 2001). Twist proteins play essential roles in multiple stages of embryonic development. Mice lacking TWIST1 exhibit failure of neural tube closure and die at E11.5. TWIST1 mutation may also generate some syndrome such as Saethre-Chotzen syndrome. TWIST1 can promote EMT not only by affecting the downstream signals, such as E-cadherin repression, but also by inducing several cell changes, such as invadopodia formation and vasculogenic mimicry formation (Eckert et al., 2011; Sun et al., 2010). At the transcriptional level, different from SNAI1 and ZEB1, TWIST1 acts without direct binding to the *CDH1* (E-cadherin gene) promoter.

## **c) ZEB family**

ZEB family transcription factors are composed by two members, ZEB1 and ZEB2. ZEB1/2 are homeobox zinc finger transcription factors encoded by two distinct genes, called *ZEB1* (also *TCF8* or *DeltaEF1*) and *ZEB2* (also *SIP1*). These two proteins are similar at the structural level. The high degree of sequence identity between the two zinc finger clusters shows that the capacities of DNA binding are similar between these two proteins. Their Smad binding domain (SBD) can interact with activated R-Smad from TGF $\beta$  pathway, although the binding efficiency is different between these two proteins. The homeodomain does not bind to DNA and may participate in protein-protein interactions. They can repress the transcription of target genes through the interaction with the CtBP co-repressor. For example, ZEB1 and ZEB2 down-regulate one essential adhesion molecule in EMT, E-cadherin, via binding to E2-boxes in its promoter, so that epithelial cells lose their strong intercellular interactions and distinct polarity, which facilitate the capacity to migrate (Vandewalle et

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al., 2009). Although initially described as transcriptional repressors, ZEB1 and ZEB2 can also activate the transcription of genes, for example ZEB1 turns out to be a transcriptional activator via interaction with YAP (Lehmann et al., 2016). Knock-out of *Zeb1/2* in mouse demonstrated that they are essential in embryogenesis. The knock-out of *Zeb2* gene in mouse is lethal at E8.5, in which the murine *Zeb1* knockout did not induce embryonic lethality but caused major defects in skeletal factors and thymocytes, resulting in perinatal death. Interestingly, the expression of ZEB1 and ZEB2 is primarily complementary in mouse embryos, with overlap only in a limited number of tissues (Miyoshi et al., 2006) [Figure 18]. ZEB2 is also required for the development of neural crest cells with failure of neural tube closure involved in arrest of early migration of cranial nerve crest cells (Van de Putte et al., 2003). *Zeb2*-specific tissue inactivation early in neural crest cell development also results in embryonic death, associated with developmental abnormalities of the cranial, cardiac, and peripheral nervous systems (Van de Putte et al., 2007). Indeed, the expression of ZEB2 around E8.5 is found in the neural plate/crest and the paraxial mesoderm, while ZEB1 is absent in the neural crest and is expressed in the paraxial and limb skeletal elements up to at E12, this observation highlights the specific spatial conditioning of two ZEB family members [Figure 30].

Mutations of *ZEB1* and *ZEB2* may cause some syndromic malformations (Vandewalle et al., 2009). Some patients with Mowat-Wilson syndrome were found to have a mutated *ZEB2* allele. Frame-shift and nonsense mutations in the *ZEB1* gene were also examined in many PPCD (Posterior polymorphous corneal dystrophy) patients. Expression of ZEB1/2 is absent in epithelial adult tissues but is found in fibroblasts, in some stem cells, and differentiated cell types (some types of neurons and immune cells...). Indeed, besides displaying essential roles in development, ZEB1 and ZEB2 are also expressed in mammary stem cells (Morel et al 2018), fibroblasts, some neurons as well as immune cells including T CD8 and NK cells (Guan et al., 2018; van Helden et al., 2015).



Figure 18: Zeb family mutually exclusive expression in embryo.

a, Schematic representation of the expression of Zeb family members at embryonic day 8.5 (E8.5). The expression domains of Zeb1 and Zeb2 are largely mutually exclusive and include the ventral forebrain, rhombomeres 3 and 5 (r3 and r5, respectively), node, notochord and endoderm (Zeb1) and dorsal forebrain, neural tube (excluding rhombomeres 3 and 5) and the paraxial mesoderm (Zeb2). b, During brain development at E14.5, Zeb1 is restricted to the ventricular and subventricular zones of the forebrain (ven), Zeb2 is expressed in the superficial (sup) and intermediate (int) stratum. (Stemmler et al., 2019)

#### d) Interaction with epigenetic regulators and regulation

EMT-TFs may interact with epigenetic regulators during cell reprogramming and differentiation (Skrypek et al., 2017). One of the well-known EMT inducers, TGF $\beta$ 1, can lead to the phosphorylation of histones or epigenetic enzymes, through the activation of SMADs, PI3K/Akt and MAPK pathway. For instance, via interaction with SNAIs through their SNAG domain, or via ZEBs through their SMAD-binding domain, DNMT1 (DNA methyltransferase 1) is recruited to *CDH1* gene and hyper-methylate the CpG islands in the *CDH1* promoter which induce the repression of E-cadherin.. EMT-TFs not only regulate gene methylation via their interaction with epigenetic regulators, their expression itself is also controlled by DNA methylation. For instance, in colorectal cancer, *TWIST* promoter methylation level is inversely correlated with TWIST expression; same thing for ZEB2 in pancreatic cancers and hepatocellular carcinomas. Besides, histone modification also influences the gene transcription during EMT (Acun et al., 2011; Galván et al., 2015). SNAI and ZEB interact with LSD1 (Lysine-Specific Demethylase 1) and recruit it to their target genes. Other epigenetic regulators, such as HDAC1/2 containing complex, can also interact with SNAI2 and ZEB1, or control negatively SNAI1 and ZEB expression [Figure 19]. Pre-treat cancer cells with TSA (Trichostatin A), one of the HDAC1/2 inhibitors, could reverse almost the SNAI1 induced EMT program (Xiao et al., 2014).

Another HDAC1 inhibitor, mocetinostat, was capable to re-sensitize tumor cells to chemotherapy, by impacting ZEB1 expression and functions (Meidhof et al., 2015). More evidence was proved clinically in 2018 that HDAC inhibitor vorinostat could selectively ablate BRAF+MEK inhibitor resistant melanoma cells, which highlighted again the importance of HDAC in cancer therapy resistance (Wang et al., 2018).

**Table 1**

Enzyme	Activity	Marks	Regulated EMT-TFs	Regulatory effect	Cell type
<b>Histone methyl transferases (HTMs) and histone demethylases (HDMs)</b>					
<b>KDM6B/JMJD3</b>	HDM	H3K27m2/3	SNAI1, SNAI2, TWIST1	positive	Breast, MDCK
			SNAI1, ZEB1	negative	Colorectal cancer
<b>UTX</b>	HDM	H3K27m2/3	ZEB1, ZEB2, SNAI1	negative	Breast cancer
<b>EZH2</b>	HMT	H3K27	ZEB1, ZEB2, SNAI1	negative	Ovarian cancer
<b>PHF8</b>	HDM	H3K9	ZEB1, SNAI1	positive	Breast cancer
<b>KDM48</b>	HDM	H3K9me3	ZEB1	positive	Pancreatic cancer
<b>MLL4</b>	HMT	H3K4me2	ZEB1, ZEB2, SNAI1	positive	Breast cancer
<b>PRMT1</b>	HMT	H4R3me2as	ZEB1	positive	
<b>Histone acetyl transferases (HATs) and histone deacetylases (HDACs)</b>					
<b>SIRT1</b>	HDAC	H3K9Ac	ZEB1, ZEB2, SNAI1, SNAI2	positive	Prostate cancer
<b>P300</b>	HAT	H3K	ZEB1, ZEB2, SNAI1	negative	Breast cancer
<b>HDAC1</b>	HDAC				
<b>HDAC type I</b>	HDAC				

**Table 2**

Enzyme	Interactor	Domain of interaction	Marks	Gene targeted	Cell type
<b>Histone methyl transferases (HTMs) and histone demethylases (HDMs)</b>					
<b>LSD1</b>	ZEB1	SNAG domain	H3K4me2	Gh-repression	Pituitary development
	ZEB2			CD11b-repression	T-ALL
	SNAI1			CDH1-repression	HEK293, Breast and Colon cancer
	SNAI2			CDH1, desmoplakin, occludin -repression	HEK293, K562, HCT116
<b>SET8</b>	ZEB1		H4K20me1	Vimentin-activation CDH1-repression	Breast cancer
<b>Histone acetyl transferases (HATs) and histone deacetylases (HDACs)</b>					

<b>SIRT1</b>	ZEB1	Stabilized by MPP8	H4K16Ac/H3K9Ac	CDH1-repression	Prostate cancer
<b>P300</b>		N terminal	H3/H4	TGFβ dependent genes- activation	<i>Xenopus</i> embryos
<b>HDAC1/2</b>		Through CtBP?	H3K	CDH1-repression	Pancreatic cancer
<b>Other</b>					
<b>BRG1</b>	ZEB1	N terminal		CDH1-repression	Colon cancer

*Figure 19. Examples of epigenetic regulators and EMT-TFs interaction and regulation.*

*Table 1: Epigenetic regulation of EMT-TFs and the impact on EMT. Table 2: Interaction between epigenetic remodeling enzymes and EMT-TFs. (Modified based on Skrypek et al., 2017)*

## B. EMT in carcinoma development

We have seen the physiological roles of EMT in growth and tissue repair in previous chapters, but EMT is also involved in oncogenesis and participate to tumour progression and metastasis (Peinado et al., 2007), as well as in cell transformation and tumour initiation (Peinado et al., 2007). Similar cell signalling pathways are activated by diversiform signals in physiological and pathological EMT, but lead to different results. The abnormal expression of EMT-TFs is frequently found in many cancer types, especially in carcinoma. At invasive state, cells loss their polarity and cell-cell contacts so that EMT is activated. However, it was recently shown that MET, the reverse process (mesenchymal to epithelial transition), is required for metastasis colonization.

### 1) EMT in metastasis and MET in colonization

The very first article who described the role of EMT in cancer was based on the EMT-TF SNAIL1 (Cano et al., 2000). They demonstrated that the over-expression of SNAIL1 repressed the E-cadherin expression, induced the EMT and cells gained an invasive property. One year after, Thomas Brabletz found cells with a dedifferentiated phenotype at the invasive front of colorectal carcinomas (Brabletz et al., 2001). The cells isolated themselves in clusters and even ended up individualizing themselves by adopting a fibroblastic morphology. Interestingly they observed that the cells in the

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center of the primary tumour as well as cells present in the metastasis center had common characteristics, such as the expression of E-cadherin and an epithelial phenotype. Likewise, a similarity was observed between the invasive fronts of these same tumours and the metastases they generated; these cells lose the expression of E-cadherin, accumulated nuclear beta-catenin and this was correlated with the loss of their epithelial phenotype, and the acquisition of a more fibroblastic phenotype. Thus we found at the level of the invasive fronts, primary tumours and metastases with features of EMT.

Before 2001, according to the clonal evolution of tumour cell populations, the mesenchymal capacities were necessary for metastasis, such as cell dissociation, migration, dissemination and invasion, which were the results of a succession of secondary genetic alterations during the late stages of carcinogenesis (Nowell, 1976). The observations in colorectal carcinomas proved that the transition to a mesenchymal phenotype is reversible and transient. Thus, it would be the consequence of a major modification of the regulation of expressions of different genes and not of random and definitive genetic events. Since then, these various observations have been highlighted in many tumour models, especially in breast cancer.

Another EMT-TF, TWIST1, was then proved to participate in tumour metastasis formation by Robert Weinberg's lab (Yang et al., 2004). In a mouse model of highly metastatic mammary carcinoma, the depletion of Twist1 by interference RNA prevented the formation of metastasis to the lung. Conversely, the ectopic expression of Twist1 induces loss of E-cadherin, activation of mesenchymal markers and metastatic dissemination.

However, the involvement of EMT *in vivo* as an oncogenic process has been debated for a long time because it is a transient process and therefore difficult to observe. In addition, the tumour area is also rich in stromal cells, so it is difficult to distinguish tumour cells with mesenchymal morphology. In 2008, the use of transgenic mouse models made it possible to confirm *in vivo* the relevance of the EMT in breast cancer (Trimboli et al., 2008). In this study, transgenic mice

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expressed specifically and inducibly in epithelial cells the c-MYC oncogene as well as the lacZ operon (serving as a cell tracer). This tool allowed highlighting, after activation of the oncogene, the existence of lacZ positive mesenchymal cells, thus validating their epithelial origin and the process of *in vivo* EMT.

Besides the invasive carcinoma stage, a dissemination of isolated cells at earlier stages was detected by analysing patient carcinomas with the presence of cancer cells disseminated in the spinal cord. These cells which expressed the TWIST factor contained very few genetic alterations indicating that this dissemination had occurred at an early stage of carcinogenesis (Hüsemann and Klein, 2009). From then, the role of EMT at early stage was also observed in other type of tumours, such as in pancreatic cancer (Rhim et al., 2012). The use of a mouse model showed that an inflammatory response was established directly after activation of the oncogene KRAS in epithelial cells of the pancreas. This inflammatory response promoted EMT and the intravasation of tumour cells and therefore their dissemination from the early stages.

Since these EMT-TFs have important roles, cells also developed inhibiting systems, such as microRNAs, to repress their mRNA. The role of the ZEB / miR-200 feedback loop on the control of metastatic spread was investigated in a mouse model of *Kras*<sup>G12D</sup>*p53*<sup>R172HΔG</sup> lung adenocarcinoma. Differential expression analysis between metastases and non-metastases tumours identified members of the miR-200 family. Ectopic expression of miR-200b in tumours, which were initially capable to metastases, could negate their ability to enter into EMT, invasion and metastasis (Gibbons et al., 2009). miR-205 family also restrain the translation of ZEB1 and ZEB2 mRNAs (Vandewalle et al., 2009). In a murine model of *Kras* / *p53* mutated pancreatic ductal adenocarcinoma (KPC), deletion of the *Zeb1* transcription factor suppressed invasion and metastasis capabilities (Krebs et al., 2017). In the same model, the depletion of the EMT-TFs Snai1 and Twist1 was found to be ineffective on the alteration of the invasive and metastatic capacities of primary tumours, suggesting a specific role of Zeb1 in this model (Zheng et al., 2015). Other regulations at the protein level, such as the degradation leaded by the phosphorylation of TWIST1, also govern the

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EMT states (Hong et al., 2011).

Finally, it appears that the last step in the generation of metastases is a reversion of EMT, a mesenchymal-epithelial transition or MET, which is essential for the colonization. Analysis of TWIST expression on metastatic carcinomas showed decreased expression of TWIST1 in lymph node metastases compared to the primary tumour. The impact of EMT-TF has been precisely studied in a skin carcinoma model, in which expression of *Twist1* could be induced by the *TetOP-Twist1* transgene under the control of the keratin 5 promoter *K5*. Thus, under doxycycline treatment, the cells of the basal epidermal layer expressed *Twist1*. As the expression of *Twist1* was not sufficient for tumour formation, the induction of carcinomas was induced by DMBA / TPA treatments. Once the tumours have formed, *Twist1* was either induced in the *K5* cells of the primary tumour by topical application of doxycycline, or in all tumour cells including cells which have disseminated by treatment with doxycycline in drinking water. Maintaining the expression of *Twist1* in the second group drastically reduced the formation of metastases, demonstrating the importance of the reversible nature of EMT in the formation of metastases (Tsai et al., 2012). In another mouse model, loss of the EMT inducer *Prrx1* was shown to be necessary for metastasis after injection into the tail of immunosuppressed mice with cancer cells (Ocaña et al., 2012). Ultimately, it is the reversible succession of EMT and MET cycles but not the mere induction of a mesenchymal phenotype that allows invasion and metastasis formation, emphasizing again the importance of cell plasticity [Figure 20].

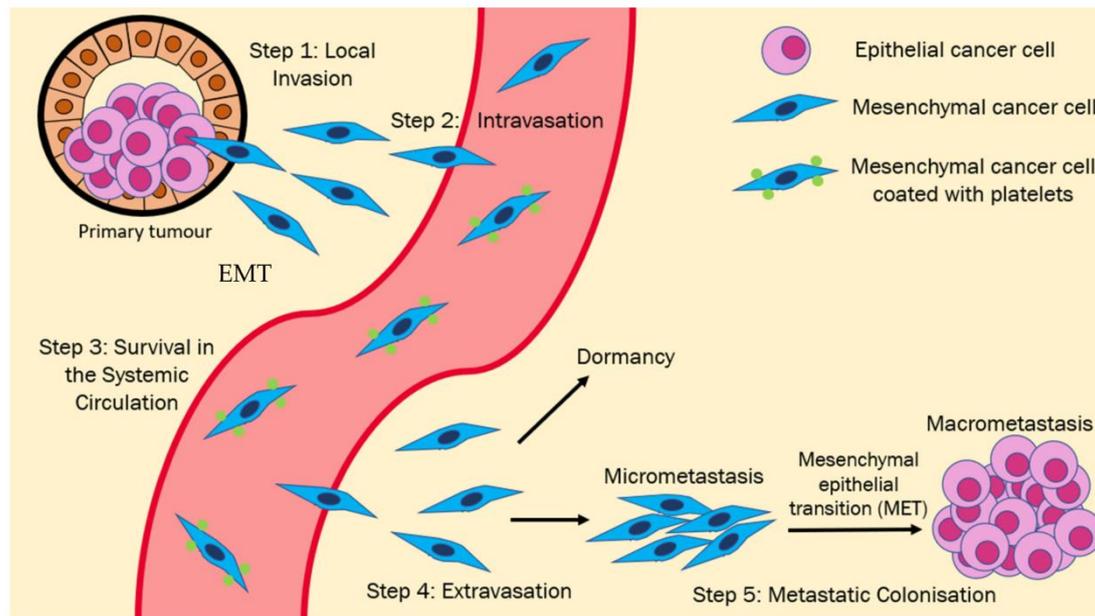


Figure 20. EMT-MET model for the metastasis.

Epithelial cancer cells undergo EMT, lose their cell-cell junctions and gain the ability to invade the surrounding tissue parenchyma (Step 1). These EMT-induced cells may intravasate into the systemic circulation (Step 2) and must survive in the circulation (Step 3) before reaching the target organ site. The cells must then extravasate into the tissue parenchyma (Step 4), following which they may either enter a state of dormancy or form micro metastases. Subsequent development into clinically detectable macro metastases requires MET activation (Step 5). (Datta et al., 2021)

All of these works illustrate the role of the reversible transition between epithelial and mesenchymal states orchestrated by EMT-TFs in invasion and metastasis formation. However, the expression of EMT-TFs has also been reported in non-invasive human and murine tumors, suggesting their involvement in other processes. In humans, for example, high levels of ZEB1 expression were observed in *in situ* pancreatic adenocarcinomas (Liu et al., 2014). At the same time, in mice, Zeb1 was found in non-invasive pancreatic ductal adenocarcinoma lesions (Zheng et al., 2015). Further experiments, including the work of Alain Puisieux's lab, demonstrated the early roles of EMT-TFs and particularly of ZEB1 in cell transformation and tumor initiation.

Finally, EMT is not a simple shift from a fully epithelial to a fully mesenchymal state, but encompasses a spectrum of intermediate states, driven by reversible transition underlying cell plasticity (Nieto et al., 2016). In order to emphasize the dynamic nature of the EMT process and the flexibility of cells in partial EMT state, the intermediate states have been described as metastable

(Tam and Weinberg, 2013). The notion of metastability comes from the field of thermodynamics, for which a metastable state corresponds to a delicate state of equilibrium outside a minimum energy. While a minimum of energy guarantees stability, requiring a large input of energy in order to change state, any other level of energy suggests a phase of transition to a state of lower energy. On this thermodynamic model, the EMT process was modelled by three stable states, the epithelial state, the intermediate state of maturation and the mesenchymal state and two metastable states [Figure 21]. Thus, the EMT process is schematized as a continuum between more or less stable states, with the progressive loss of epithelial properties and the progressive gain of mesenchymal properties. The state of stable partial EMT has been observed during embryonic development, tissue repair, or pathologies such as fibrosis or tumour progression. Depending on the tissue context or extracellular signals, epithelial cells may go through a partial EMT and display a combination of epithelial and mesenchymal traits; these cells also display a high degree of plasticity which plays a critical role in cancer metastasis (Chaffer and Weinberg, 2011; Valastyan and Weinberg, 2011). A study of a panel of surface EMT markers on primary mammary and skin tumours made it possible to identify multiple tumour populations more or less advanced in the EMT process, from pure epithelial cells to mesenchymal cells, passing through various so-called hybrid intermediate states (Pastushenko et al., 2018). The establishment of transcriptional and epigenetic mappings has revealed regulatory networks of genes, transcription factors and signalling pathways leading to different hybrid states.

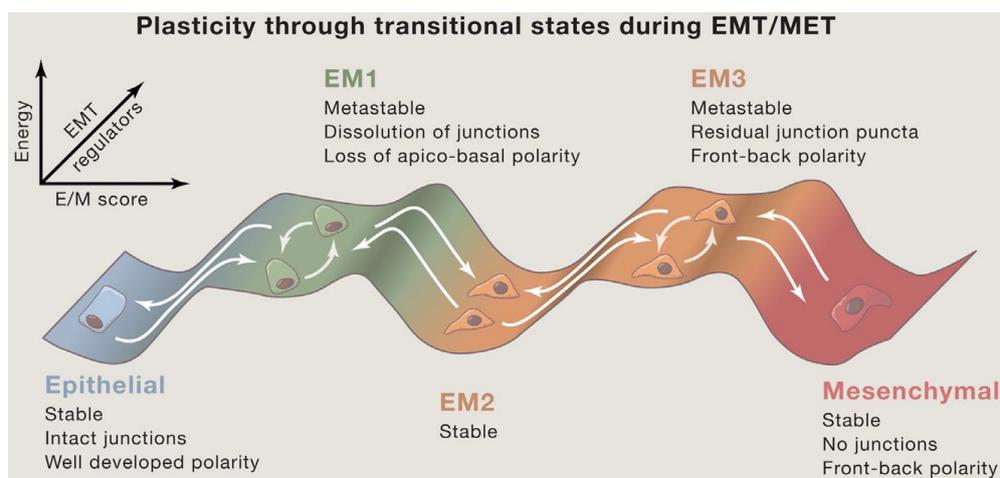


Figure 21. Transition between different states during the EMT process.

EMT is viewed as a continuum in which cells can exhibit epithelial (E), intermediate (EM), and mesenchymal (M) phenotypes. On the x-axis, the transition pattern of EMT (left to right) or MET

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(right to left). The thermodynamic energy associated with each state is plotted on the y axis with stable low energy states and metastable higher energy states. The intervention of EMT regulators during the transition is represented on the Z axis. Among these regulators, we find the transcription factors linked to EMT (SNAI1 / 2, ZEB, TWIST1, GRHL2 and PRRX1). (Nieto et al 2016)

#### a) Escape from oncogene-induced senescence

In 1997, Serrano et al demonstrated that the expression of the H-RAS oncogene in human fibroblasts lead to an engagement of cells in the process of senescence, due to an accumulation of p53 and p16, which are two major inhibitors of the cell cycles (Serrano et al., 1997). The two main onco-suppressive routes following to oncogenic activation are the p16-RB pathway (retinoblastoma) and the p14ARF-p53 pathway, which will lead to OIS (oncogene induced senescence). The work of Alain Puisieux's team, in particular, has shown that the involvement of EMT-TFs in carcinogenesis is in fact very early by promoting escape from failsafe programs, including the Rb and p53 pathways (Puisieux et al., 2014) [Figure 22]. In an HMEC immortalized mammary epithelial cell model, overexpression of TWIST1 inhibited senescence induced by the oncogene ERBB2 by inhibiting p16 and p21 (Ansieau et al., 2008). The ability of ZEB1 to inhibit EGFR-induced senescence has also been demonstrated *in vitro* in a human model of immortalized EPC2-hTERT esophageal epithelial cells. In this model, ZEB1 inhibited p16<sup>INK4A</sup> and p15<sup>INK4B</sup> (Ohashi et al., 2010). In addition, this ability is conserved in mice and has been observed in a different context, an embryonic fibroblast model. Zeb1 knockout deletion induces early cell cycle arrest in G1 / S phase following expression of p21 and p15 (Liu et al., 2008).

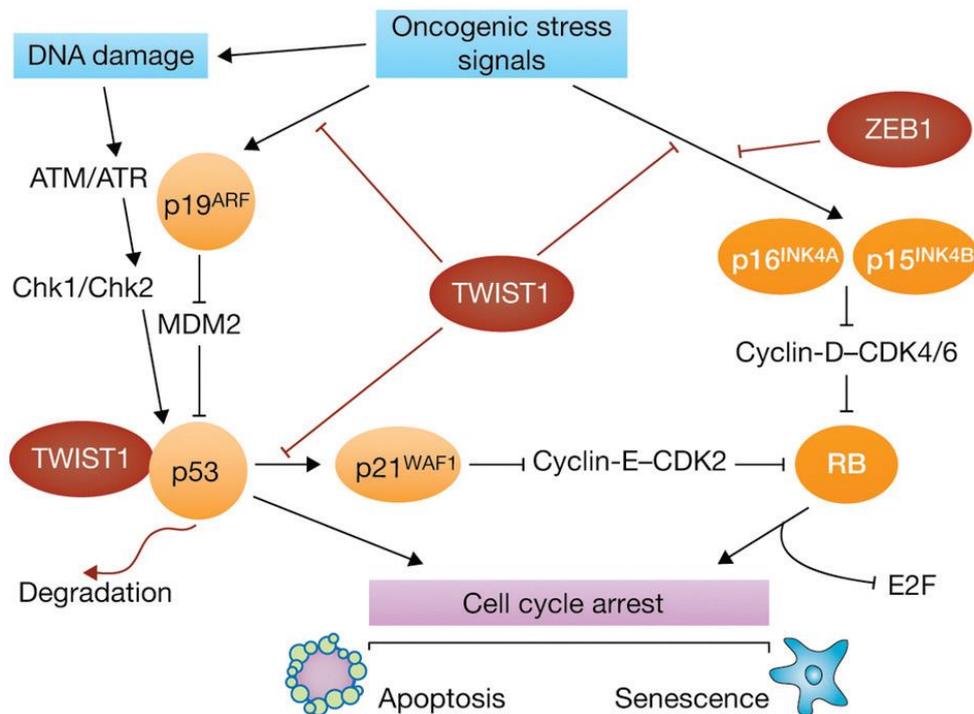


Figure 22. EMT-TFs allow the inhibition of failsafe programs and promote cell transformation.

Senescence and apoptosis represent the first anti-tumour barriers responding to oncogenic stress or DNA damage. Activation of p53 and RB pathways allows cell cycle arrest. The inducers of EMT, particularly TWIST1 and ZEB1, will prevent the implementation of p53 or RB dependent onco-suppressive programs, by inhibiting the activation of p19 and p16 proteins or by promoting the degradation of p53. (Puisieux et al., 2014)

By inhibiting the OIS, EMT-TFs promote cell malignant transformation. In the previously described HMEC immortalized breast epithelial line model, ectopic expression of ZEB1 or TWIST1 accelerated the transformation induced by the oncogene RAS through reducing the number of genetic events required. The transformation potential of these cells was proven by *in vitro* soft agar colony formation test. As for *in vivo* part, the cooperation of TWIST1 with RAS has been shown in a transgenic murine model. The Twist1 and / or K-rasG12D transgenes were mainly expressed in the mammary gland but also in the skin epithelium thanks to the MMTV promoter. While no Twist1 mice develop a malignant lesion, KrasG12D mice developed oral and anal papillomas, and mice expressing both transgenes developed papillomas which progress to carcinomas (Morel et al., 2012). Other EMT-TFs (SNAIL or ZEB2) have also been shown to be able to cooperate with oncogenic alterations to promote tumour initiation (De Craene et al., 2014; Goossens et al., 2015).

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After these works showing a synergy between oncogene induction and EMT-TFs reactivation, Cédric Blanpain's team studied the role of Twist1 in the initiation stages of skin carcinomas emerging after activation of Ras (Beck et al., 2015). In this article, experiments using a conditional knockout of TWIST1, made it possible to study the involvement of this factor in the different stages of skin carcinogenesis. Application of DMBA / TPA (potent carcinogens) induced expression of TWIST1 in epithelial cells, suggesting its early involvement in tumour development. Indeed, the activation of TWIST1 in these epidermal cells (K14<sup>Cre</sup>: Twist1<sup>fl/fl</sup>) drastically reduced the formation of papillomas following treatment with DMBA / TPA. Moreover, in a context with p53 mutation, the invalidation of two alleles of TWIST1 was able to inhibit the formation of carcinomas, while the elimination of a single allele allowed the formation of papillomas but not the transition to a malignant tumour. These results therefore indicated that a low level of TWIST1 allows the formation of benign tumours but a higher level is needed for a complete malignant progression.

In another similar study, it demonstrated the involvement of ZEB1 in the tumour initiation, and a similar dose-effect in tumour progression in a model of KRAS-induced lung cancer (Liu et al., 2014). The heterozygous deletion of ZEB1 did not compromise animal survival and no major developmental defects were observed. Even *Zeb1* +/- mice still developed adenomas following activation of KRAS, these would not progress to the malignant stage of adenocarcinoma thus showing that a haplo-insufficiency in ZEB1 expression was sufficient to inhibit tumour initiation. In addition, ZEB1 was necessary for the survival of these carcinoma cells, and a stronger level of ZEB1 was needed to stimulate metastasis. At the mechanistic level, activation of KRAS led to the activation of the transcription factor ZEB1, which in this context could directly suppress the expression of PTEN, the tumour suppresser the most expressed in these lung adenocarcinomas. Finally, in the KPC model of pancreatic ductal Kras / p53 adenocarcinoma, the major and specific role of EMT-TF Zeb1 on tumour initiation was demonstrated. Homozygous Zeb1 depletion drastically reduces the number of metaplasia and precancerous lesions while Snail1 or Twist1 depletion has no effect (Krebs et al., 2017).

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## b) CSCs generation and cell plasticity

Cancer stem cells (CSCs) are characterized by their dual capacity of self-renewal by generating phenotypic heterogeneity compared to the original tumour (Badve and Nakshatri, 2012). Initially, the hierarchical model placed CSC at the top of the tumour differentiation pyramid. CSC alone was then able to initiate a heterogeneous tumour. CSCs therefore represent a small percentage of the tumour; they are at the origin of the progression and maintenance of the tumor, supporting the tumour growth by maintaining the pool of CSCs on the one hand and generating a differentiated non-tumorigenic population on the other hand. The way of isolation and characterization of human CSCs is the limit dilution serial transplant test in immuno-deficient mice. The low number of cells injected makes it possible to assess the frequency of CSCs based on the number of tumors formed relative to the number of cells implanted. The repetition of serial transplants makes it possible to demonstrate the maintenance of tumorigenic capacities over time, excluding the presence of highly proliferating cancer cells but not CSCs. Mammosphere assays were developed to isolate and cultivate CSCs *in vitro*. As in mice, the ability to form multiple generations of mammospheres is associated with CSCs. Analysis of organoid formation efficiency, as well as expression of CD44 cluster of differentiation and the enzyme ALDH are also commonly used biomarkers for the isolation of CSCs in solid tumors (Al-Hajj and Clarke, 2004). By applying mammosphere assay, Anne-Pierre et al. demonstrated that EMT could confer properties of CSCs, more precisely through the cooperation of EMT-TFs TWIST1, ZEB1 and ZEB2 with the RAS oncogene in the acquisition of stem properties by the mammary epithelial line HMEC. The acquisition of CD44 and the loss of the CD24 differentiation cluster under combined expression of HRAS<sup>G12V</sup> with EMT-TFs validated the reprogramming of HMEC cells into cancer stem cells. Interestingly, while the expression of RAS, TWIST1 or ZEB2 was not sufficient on its own for the induction of a stem phenotype, the ectopic expression of ZEB1 alone allowed the conversion of the majority of the epithelial HMEC cells into CSC CD44 + / CD24- cells (Morel et al., 2012).

The transcriptional activity of EMT-TFs is involved in this acquisition of stem properties at different levels. For instance, the factor TWIST1 is capable to activate the expression of BMI1, a

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protein belonging to the Polycomb group, which allows repression of the INK4A locus, thus maintaining the self-renewal capacity of the cell. ZEB1, for its part, represses members of the miR-200 family which control the expression of genes associated with stem cells such as BMI1, KLF4 or SOX2 (Puisieux et al., 2014; Wellner et al., 2009). SNAIL2 can also cooperate with SOX9, an important factor for the conservation of stem cell status in adults, to induce cell dedifferentiation of breast epithelial cells and endow them with stem cell capabilities such as the ability to generate mammospheres (Guo et al., 2012).

In total, all of these data obtained mainly in cancers from epithelial origin, indicate that the aberrant re-activation of the EMT-TFs, even before allowing the cells to engage in an EMT process, can ensure tumor initiation by preventing the implementation of backup systems of cells and inducing the generation of cancer stem cells.

Overall, EMT-TFs are involved at each stage of carcinogenesis including resistance to treatment and escape from the immune system of carcinomas (Caramel et al., 2018) [Figure 23].

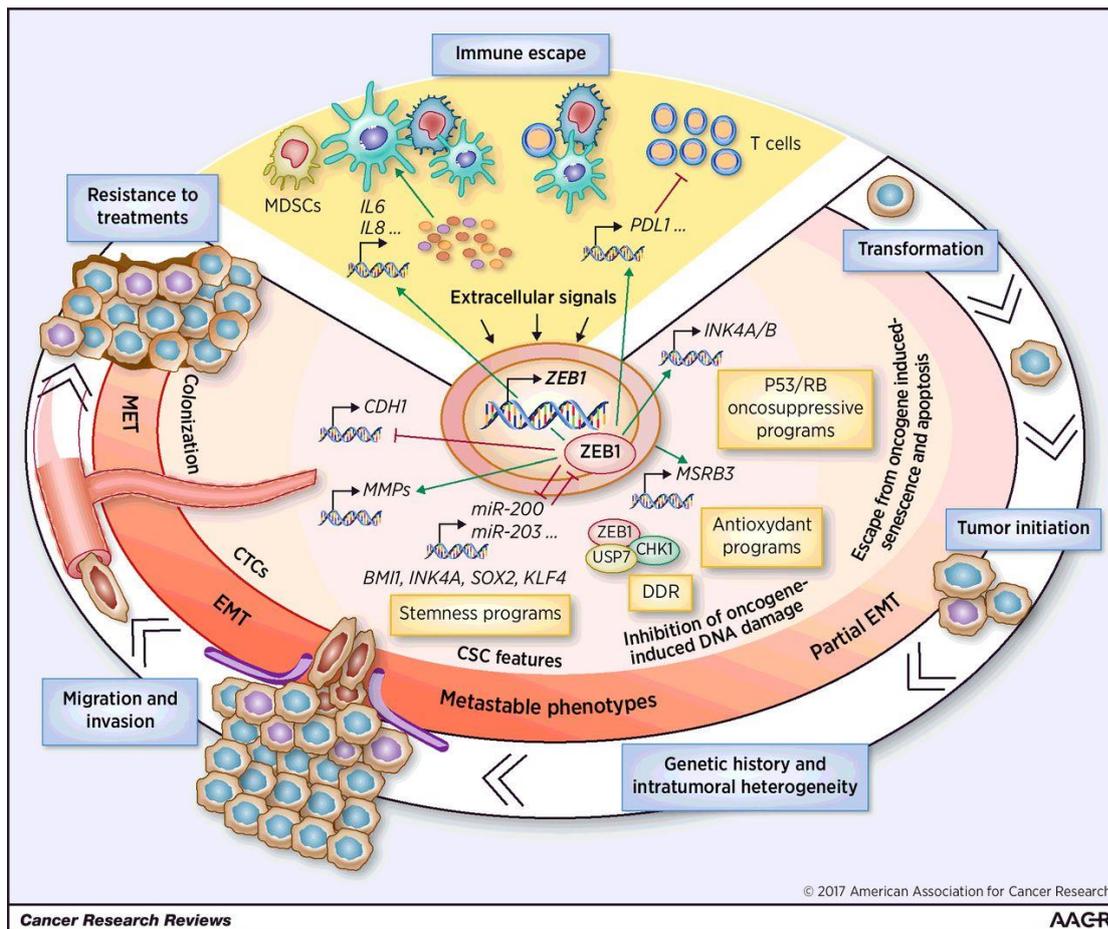


Figure 23. Pleiotropic roles of ZEB1.

Like the EMT-TFs of the SNAIL and TWIST families, ZEB1 plays a central role determining cellular fate by regulating the transcription of factors involved in the control of differentiation, proliferation, survival and mobility. Its expression supports tumor transformation and progression and contributes to treatment resistance and immune system evasion. In addition, while a large number of functions are kept from one EMT-TF to another, some functions are specific and not redundant. Finally, certain direct targets have been demonstrated by numerous ChIP-seqs produced from breast cancer lines. (Caramel et al, 2018)

## 2) Transcriptional targets of ZEB1/2 in carcinoma

EMT-TFs plays pleiotropic roles along carcinoma formation, from the tumour initiation to treatment resistance and immune escape [Figure 23], so in addition to expected epithelial and mesenchymal genes, they can regulate a large spectrum of genes.

As previously mentioned, the transcription of E-cadherin, is down-regulated by both ZEB1 and

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ZEB2 in mesenchymal cells via binding to the E2-boxes in the E-cadherin promoter, so that epithelial cells lose their strong intercellular interactions and polarity. At the same time, ZEB1 and ZEB2 also modulate the expression of other cell junction genes, mesenchymal genes and matrix metalloprotease. Furthermore, ZEB1 also induces local and temporary loss of basement membrane (BM) by modulating the gene expression of BM components. The ZEB1 transcription factor directly regulates the sulfoxide / methionine reductase MSR3, which plays a major role in the prevention of DNA damage induced by an oncogene. Thus, ZEB1 prevents the activation of p53 and allows the maintenance of genomic stability during transformation (Morel et al., 2017).

ChIP-sequencing analyses in carcinoma cells allowed to get a genome-wide, comprehensive view of ZEB1/ZEB2 target genes [Figure 24]. ChIP-seq of ZEB1 was performed in basal (MDA-231-D and Hs578T) and luminal (MCF-7) breast cancer lines. Known binding sites on the promoters of E-cadherin and ESRP2 validated the correct progress of the experiment. The signature enrichment analysis highlighted the regulation of inflammation response genes as a major target of the transcription factor ZEB1. Among the direct targets of ZEB1, there were attractants of MDSC suppressor-derived myeloid cells such as interleukins -6, -8 and -1 $\beta$  but also chemokines CXCL1 and CXCL5. The *in vivo* implementation of the gain-of-function approach of Zeb1 in a 4T1 murine mammary model made it possible to confirm the role of Zeb1 in the attraction of MDSCs to the center of the tumor, via an increased production of IL6 and IL8 by the tumor cells overexpressing Zeb1 (Katsura et al., 2017).

One year after, still in breast cancer cell line Hs578T, a ChIP-seq of ZEB1 showed that ZEB1 was associated with organ development, signal transduction, and cell communication, besides the EMT-related genes, via Gene Ontology analysis (Maturi et al., 2018). In triple-negative breast cancer cells, another genome wide study via ZEB1 ChIP-seq was also performed (Feldker et al., 2020). They first found that ZEB1 binding sites overlapped with the AP-1 factor JUN. *De novo* motif analysis highlighted the higher frequency of low affinity ZEB1 motif than canonical ZEB1 motif. They identified ZEB1 as a novel interactor of the AP-1 factors FOSL1 and JUN and showed that, together

with YAP, they form a transactivation complex, predominantly activating tumour-promoting genes. By comparing the ZEB1 only peaks and ZEB1/YAP/JUN peaks, the canonical ZEB1 motif was not indispensable to form this ZEB1 activator complex. This article proposed a model that, in addition to the well determined ZEB1 repressive function via direct binding to its E-box motif, ZEB1 is recruited by AP-1 and YAP factors and activate the tumor promoting genes transcription.

As for ZEB2, due to their structural similarity, its direct target genes are supposed to be largely the same as ZEB1 target genes, at least in mesenchymal cells. One ChIP-seq was performed in SNU398 hepatocellular carcinoma cells exhibiting high endogenous ZEB2 expression (Balcik-Ercin et al., 2018). In both SNU398 and colorectal carcinoma DLD1 cells, ZEB2 negatively regulated a GalNAc-transferase (GALNT3) which is involved in O-glycosylation. This study added another layer of complexity to the role of ZEB2 in cancer progression and metastasis. ZEB2 could negatively regulate the expression of hTERT, thus controlling partly the replicative senescence (Ozturk et al., 2006).

	Article and Data ID	Cell line	Ref antibody	Pathway
<b>ChIP-seq ZEB1</b>	Katsura et al., 2017 GSE89206	basal-type breast cancer cell lines MDA-231-D and Hs578T	Novus Biologicals, Minneapolis,NBP1-059 87	Inflammatory response
		luminal-type breast cancer cells MCF7		
	Maturi et al., 2018 E-MTAB-5241	triple-negative breast cancer cell line Hs578T	Novus Biological, Littleton, CO	Enzyme regulation activity, Cell polarity regulation, ...
<b>ChIP-seq ZEB2</b>	Feldker et al.,2020 E-MTAB-8258	triple-negative breast cancer cell line MDA-MB-231 cells	Santa Cruz, sc-25388x	(cobinding with YAP/JUN)
				Regulation of fibroblast migration
				Regulation of local adhesion assembly
<b>ChIP-seq ZEB2</b>	Balcik-Ercin et al., 2018 GSE103048	hepatocellular carcinoma cells SNU398	home-made monoclonal antibody, clone 6E5	Catalytic activity (e.g.protein O-glycosylation)

Figure 24. Recent published ChIP-seq of ZEB1 and ZEB2 in carcinoma.

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### 3) EMT in treatment resistance

Induction of the EMT process in cancer cells is associated with the acquisition of stem cell properties, and has been as well associated to treatment resistance (Shibue and Weinberg, 2017). There are two types of resistance, a so-called "de novo" resistance that can be described as innate, which means the patient does not respond to therapy. Conversely, patients who respond to treatments and then relapse represent the second type of resistance, acquired resistance. Unlike the innate resistance which normally is very specific to one drug used, acquired resistance is a more generalized resistance; in fact the cells do not respond to a whole set of drug classes, we then speak of multi-drug resistance (MDR). This process is also at the origin of the recurrence of cancers, generating new tumors that are more aggressive than the original ones. Many mechanisms can be at the origin of drug resistance, for example the activation of the signaling leading to DNA repair or alteration of drug targets; among these mechanisms there is a major one, it is the increased activation of efflux pumps which eliminate the drug ingested by cell. These pumps called ABC transporters are expressed ubiquitously and can eliminate physiologically different toxins ingested. Unfortunately, their activity is also useful in a pathological context, in which they are known to excrete a large number of chemotherapies. This increased expression of ABC transporters is associated with drug resistance in cancer (Robey et al., 2018).

In order to study the implication of EMT and its transcription factors in drug resistance, an interesting approach by analyzing the gene expression profile of different tumor lineages and comparing their sensitivity to normally used anti-tumour drugs was performed. Interestingly, it turned out that the most resistant lineages were often correlated with a gene expression profile associated with EMT (Haslehurst et al., 2012; Işeri et al., 2011). Breast cancer cell line (MCF-7), initially sensitive, was made to be resistant to drugs used to treat them (doxorubicin, paclitaxel, docetaxel); then a transcriptome analysis was performed on both sensitive lines and on resistant lines. This made it possible to show that the resistant cells presented markers of EMT, such as loss of E-cadherin and gain in expression of vimentin. In addition, these results showed that cells having

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acquired this resistance expressed EMT inducers, such as the SNAI2.

A similar study found that NSCLC lung cancer cells with mesenchymal phenotype makers were more resistant to the PI3K / AKT pathway or EGFR target treatments, than cancer cells with epithelial phenotype (Byers et al., 2013). A ZEB1-dependent phenotype promoted the acquisition of erlotinib resistance targeting the EGFR tyrosine kinase receptor in non-small cell lung cancers (NSCLC) due to activation of the AXL receptor (Zhang et al., 2012). The EMT-TFs of the TWIST and SNAIL families directly control the expression of the ABCB5 transporter, promoting resistance to chemotherapy (Saxena et al., 2011). Activation of the ZEB1-induced DNA damage response program, previously described as a feature of CSC (Morel et al., 2017), promoted resistance of breast cancer cells to radiation therapy. It is interesting to note that the mechanism identified involved ZEB1 independently of its transcriptional activity. Via interacting with the ubiquitin-specific peptidase USP7, ZEB1 increased the ability of USP7 to de-ubiquitinate and therefore stabilize the CHK1 kinase. By orchestrating faithful DNA repair by homologous recombination, CHK1 against the effects of radiotherapy (Zhang et al., 2014). In addition, the ZEB1 target microRNAs miR-203, miR-429 and miR-200c were also widely implicated in resistance to chemotherapy. For instance, epithelial ovarian cancers were found to be more resistant to cisplatin compared to those with mesenchymal characteristics. In the same cancer, resistance to cisplatin was associated with high levels of ZEB1 expression which regulated miR-429 (Miow et al., 2015; Zou et al., 2017). These two incompatible observations suggested that the resistance mechanism dependent on the ZEB1 / miR-429 feedback loop is independent from the EMT process. The expression of miR-203 was also associated with the sensitivity of mammary and pancreatic cancer cells to paclitaxel and gemcitabine (Meidhof et al., 2015). Here, Thomas Brabletz's team proposed to inhibit ZEB1 in order to restore sufficient level of miR-203 by using an HDAC inhibitor called mocetinostat. This work supports the use of epigenetic drugs to promote the epithelial phenotype, differentially sensitive to current treatments.

Although the direct link of EMT-TFs in resistance to immunotherapies has so far not been demonstrated, the EMT phenotype has been associated with resistance to immunotherapies in

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breast cancer models. In addition, the well-described role of EMT-TFs in the immune system escape from carcinomas strongly suggests that they may have an impact on the response to immunotherapies (Dongre et al., 2017). Several studies demonstrated the induction of the EMT program with resistance to lysis by cytotoxic T cells. Interestingly, various independent mechanisms promoting this resistance have been demonstrated, suggesting that a multitude of mechanisms associated with different tumor variants in EMT were able to induce resistance to the immune system attack. A study on the human mammary carcinoma cell line MCF-7 demonstrated the role of EMT in resistance to lysis by cytotoxic T cells (Akalay et al., 2013). MCF-7 cells were induced in EMT either by ectopic expression of SNAIL1 or by chronic exposure to TNF- $\alpha$ . In both cases, induction of EMT protected the mesenchymal MCF-7 from lysis by cytotoxic T cells. This protection against T lysis seemed to be linked to the activation of autophagy in mesenchymal cells. Another level of escape to the immune system is through the expression of immune system checkpoint ligands. Robust correlations between the EMT score, miR-200 and PD-L1 expression levels were observed clinically in non-small cell lung carcinomas. By repressing miR-200, ZEB1 overcome the repression of PD-L1 promoting immunosuppression of CD8 T cells (Chen et al., 2014). Finally, tumor cells are capable to recruit an immune microenvironment favorable to tumor development via the production and release of inflammatory cytokines. In a hepatic carcinoma model, under hypoxic conditions, tumour cells in EMT promoted the recruitment of monocyte-derived macrophages by producing CCL20 in a HIF- $\alpha$ -dependent manner, in order to establish an immunosuppressive microenvironment (Ye et al., 2016).

Altogether, EMT is a tightly regulated process involving various external signals, signaling pathways and EMT-TFs, its is a fundamental process necessary for normal development and tissue repair, however cancer cells display the ability to reuse this process and “hijack” EMT which can fuel oncogenesis.

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## Chapter3. Phenotype switching in melanoma

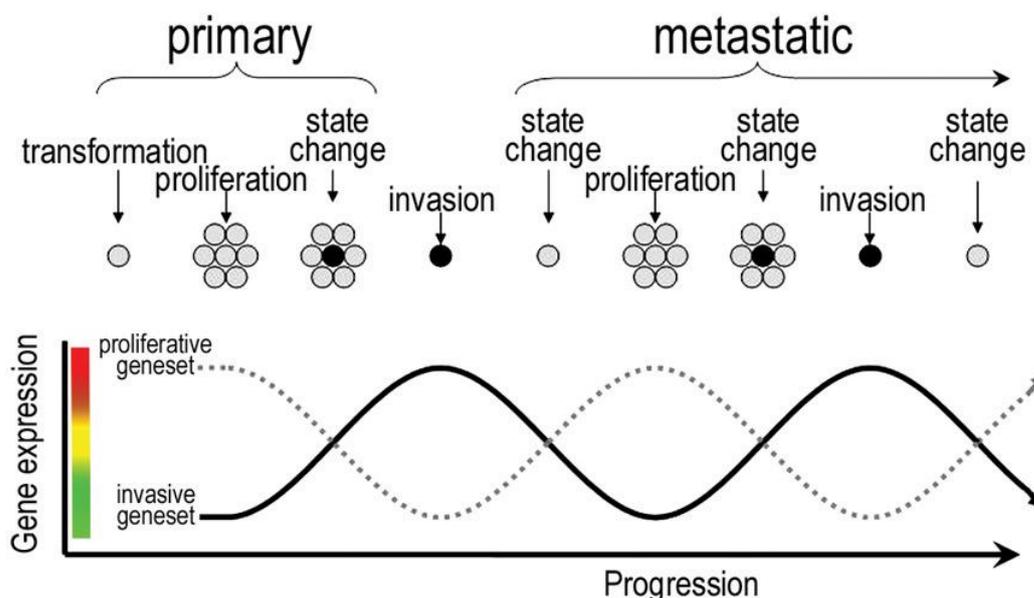
### A. Phenotype switching

#### 1) Discovery and definition

EMT is a critical step for embryonic morphogenesis and a similar process is particularly important for melanocyte lineage differentiation. It involves restructuring of the cytoskeleton, cell membrane, and cell–cell junctions. This developmental plasticity allows melanocytes to emerge from the pluripotent neural crest cells (Baker et al., 1997). Phenotype switching with similarities to the EMT program operates during development and has a recognized role in acquisition of metastatic properties in the vertical growth phase of melanoma (Bennett, 2007). A comparison of the features of primary cutaneous melanomas from the patients who develop metastasis to those who do not, revealed differences in the expression of the epithelial and mesenchymal phenotype markers. By gene expression profiling, loss of E-cadherin with increased N-cadherin and osteonectin (SPARC) was significantly associated with the development of metastases (Alonso et al., 2007).

Note that the term EMT is inappropriate for melanoma because melanocytes are not epithelial cells; instead, the term phenotype switching, which was first introduced by Hoek and colleagues in 2008, is becoming increasingly used to describe transitions between melanoma phenotypic states (Hoek et al., 2008). They proposed a model describing two phenotypic states of melanoma cell, proliferative and invasive, and the signaling pathways sustaining these transitions. They successfully tested the capacity for melanoma cells to switch between these states in an *in vivo* model, as well as the existence of both proliferative and invasive cells within heterogeneous metastatic tumors. From a previous genome-wide transcription study, they selected proliferative and invasive signature melanoma cell lines and proved their proliferation and motility *in vitro*. They then injected cell lines into immunocompromised mice to see their capacity to initiate tumors, and they found that proliferative cell lines initiated consistently tumors while invasive cell lines may be dependent on microenvironment variation. Furthermore, they found that tumors seeded with proliferative or

invasive cell lines became indistinguishable, which means the signatures of melanoma cells could change bi-directionally. This model sustains intra-tumor heterogeneity of tumors and the transition between these two phenotypes is dominated by micro-environmental conditions and is reversible. It offers a possible explanation for why mono-therapies and combinatorial therapies fail to control melanoma (for example, chemotherapy targets fast dividing cells, which represent only half of the above model) [Figure 25].



Hoek et al., 2008 - Cancer Res 68, 650-656 (Figure 6)

Figure 25. Phenotype switching model.

Early phase melanoma cells expressing the “proliferative signature” gene set proliferate to form the primary lesion. Following this an unknown signal switch gives rise to cells with a significantly different “invasive signature” gene set. Invasive signature cells escape and when reach to a suitable distal site, revert to the proliferative state in order to form a new metastasis where the cycle is repeated. (Hoek et al 2008)

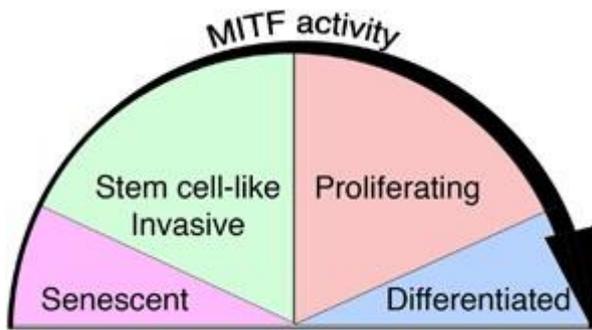
As I described before, CSCs are rare according to the cancer stem cell theory, early experiments appeared to confirm that, like normal stem cells, melanoma stem cells would represent a very minor population within the tumor. But this idea has been challenged by the observation that at least 25% of single, unselected melanoma cells derived from xenografts or directly from patient embedded in matrigel and injected into immuno-compromised mice, are able to initiate tumorigenesis (Quintana et al., 2008). In fact, different from the cancer stem cell theory, melanoma

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initiating cells present a large part of melanoma cells; they have a high plasticity and generate tumors through the phenotype switching process. Melanomas have the particularity of having an exacerbated phenotypic plasticity, allowing the simultaneous presence of multiple phenotypic subpopulations within the same tumour. Unlike irreversible genetic variation, phenotypic plasticity allows tumour cells to reversibly adapt to the pressure imposed by the ever-changing tumour microenvironment. Thus, at any given time, a melanoma cell may have a differentiated, proliferative or even invasive phenotype, allowing it to be highly adaptive to the changing microenvironment. This exacerbated plasticity implies that the majority of melanoma cells have the potential to adopt stem cell capacities (Hoek and Goding, 2010).

## **2) MITF rheostat model**

As described in previous chapter, MITF plays a critical role in melanoblast survival and melanocyte lineage differentiation. MITF induced the expression of genes which lead melanoblasts to differentiate and to synthesize melanin (Hearing, 2011). MITF is involved not only in melanocytes development and pigmentation, but also in tumorigenesis. In melanoma, MITF expression level regulates the state of melanoma cells according to the phenotype switching model: decreased expression of MITF can lead to a transition from a differentiated to a stem-like and invasive state (Hoek and Goding, 2010). In fact, the level of MITF activity is a determinant of phenotype switching in melanoma tumor cells; in order to explain the fact that MITF can both promote and inhibit the proliferation of melanoma cells, Goding et al. has proposed a model [Figure26]. This MITF rheostat model mentioned that, a high level of MITF activity promotes differentiation, mid-level activity promotes proliferation, low-level activity promotes an invasive, stem cell-like phenotype, and the absence of MITF activity causes senescence or cell death (Carreira et al., 2006; Goding, 2011; Hoek and Goding, 2010). More precisely, low MITF levels are associated with an arrested or slow G1 state, which is characterized by high levels of p27 kinase inhibitor and invasiveness. These invasive cells are predicted to have stem-like properties, and longer-term depletion of Mitf lead to DNA-damage and mitotic catastrophe-induced senescence.



*Figure 26. A rheostat model for MITF function.*

*Schematic representation of the relationship between MITF expression and melanoma cell fate. In melanoma, low levels of Mitf generate cell cycle arrested, invasive, stem-like cells. Cells expressing Mitf either proliferate or differentiate depending on the level of Mitf expression or its post-translational modification. (Goding, 2011)*

Moreover, a key study revealed that cell lines in culture reflected specific phenotypic states that could be defined by their gene expression profile (Hoek et al., 2006), in which three cohorts with low, intermediate and high level of MITF were described and could switch phenotypes in vivo (Hoek et al., 2008). As I mentioned before about the indistinguishable phenotypes of seeded proliferative and invasive melanoma cell lines after tumours removal, xenografts of either proliferative or invasive cells resulted in tumours displaying a similar level of intra-tumour heterogeneity with coexisting MITF<sup>high</sup> and MITF<sup>low</sup> cells (Hoek et al., 2008). However, the roles of MITF in melanoma seem to be a paradox: In about 10% of primary melanoma, MITF amplification is present with a higher incidence reported among metastatic melanomas (Garraway et al., 2005). Several studies showed that cutaneous melanoma with high MITF levels were well-differentiated and proliferative, as well as a favourable prognosis (Jönsson et al., 2010); those presenting a low level of MITF demonstrated an invasive phenotype, and they were intrinsically resistant to MAPK pathway inhibition (Müller et al., 2014). These observations are in line with the MITF rheostat model. After that, Harbst et al. used microarray-based gene expression profiling to classify tumours into four groups, i.e., high-immune, MITF-low proliferative, MITF-high pigmentation, and normal-like. They revealed that 50% (four out of eight) of examined tumours exhibited intra-tumour heterogeneity when considering genes determining classification into molecular groups. Moreover, the levels of mRNA of MITF and its target genes, i.e., MLANA and TYR, also displayed intra-tumour heterogeneity (Grzywa et al., 2017).

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Over the years, other specific molecular signatures and epigenetic marks discriminating the proliferative and invasive states have progressively been deciphered. Aside from MITF and AXL, other master transcriptional regulators have been identified, including SOX10 (expressed in embryonic bipotent melanoblast/glial progenitor) for the proliferative phenotype and AP1/TEAD for the invasive phenotype. The recent advent of the single-cell RNAseq technology enables the scientific community to reconcile these divergent observations and to refine the MITF rheostat phenotype switching model.

### **3) Novel phenotype switching model: intermediate phenotypes**

After the initial description of phenotype switching in melanoma, between a proliferative/differentiated and an invasive/stem-like state (Hoek et al., 2008), there is increasing evidence from single-cell gene expression analyses that additional melanoma cell states exist and the current model to account for melanoma phenotypic heterogeneity has been refined.

First, the original from Hoek et al. gene expression profiling of a large number of melanoma cell lines established that in addition to the proliferative and invasive groups A and C, a few, called group B, do not belong to the proliferation group or invasion (Hoek et al., 2006). This led to the further investigation of this unknown heterogeneous group.

The development of single-cell-scale assays is in this context a key tool for the study of intra-tumoral heterogeneity. By analysing the expression levels of 114 genes that discriminate between proliferative and invasive states, Irwin Davidson's team demonstrated the coexistence of the different states *in vitro*, in 2D culture. They characterized gene expression in two melanoma cell lines, 501Mel and 1205LU (Ennen et al., 2015). The first cell line is MITF<sup>high</sup>, which proliferates rapidly *in vitro* but has poor invasiveness, migration and tumorigenesis in nude mice. In contrast, the second was MITF-negative, which proliferated slowly *in vitro* but was invasive, motile and

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tumorigenic in nude mice. First, these cell lines were examined *in vitro*. Although the 501Mel cell line was classified as MITF<sup>high</sup> cells, low MITF expression was detected by high-throughput single-cell immune-staining and qPCR in monolayer culture. This heterogeneity was even greater in cells cultured as melanospheres. In addition, they injected 501Mel and 1205LU cells subcutaneously into nude mice. In 501Mel tumours, three groups of cells were distinguished, namely high MITF, medium MITF and low MITF, as well as its gene expression signature. In addition, a small number of cells expressed markers of invasion, for example, ZEB1, GLI2, MYOF or resistance, such as ABCB5. Immunohistochemistry revealed tumours with heterogeneous expression of MITF, CEACAM1 and POU5F1. A small population overexpressing BIRC3 was also observed. Thus, the tumours resulting from the 501Mel cell line were very heterogeneous. In 1205LU derived tumours, cells were differentiated based on high ZEB1 or low ZEB1 expression. In addition, small populations with differential expression of several genes have been observed. Unlike 501Mel tumours, POU5F1 expression was robust and uniform across all 1205LU derived tumours. In addition, they determined that there was a cluster of genes that were differentially expressed *in vitro*, in monolayers or spheres, as well as in tumours *in vivo*. Interestingly, this work has shown that heterogeneity was increased when cells were cultured in 3D or *in vivo* in tumour xenografts.

Another single-cell gene expression profiling also pointed toward an intermediate or poised state in which proliferative and invasive gene expression signatures were present, also realized by Irwin Davidson's team. RT-qPCR profiling of single cells from a series of melanoma biopsies revealed the existence of cells simultaneously expressing genes that belong to both the MITF<sup>High</sup> and MITF<sup>Low</sup> gene-expression signatures (Ennen et al., 2015, 2017).

One year after that, the work of Tirosh et al. was a major breakthrough in the study of melanoma heterogeneity and proved the coexistence of different states in patient samples. 4,645 cells isolated from fresh tumours of 19 melanoma patients were sequenced at the single-cell scale of their RNA, allowing for the analysis of intra-tumoral heterogeneity of fresh human melanoma for the first time (Tirosh et al., 2016). In this elegant study, single-cell profiling of both malignant and

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stromal cells confirmed that MITF was a key biomarker for distinct phenotypic states. MITF was correlated with a differentiation gene-expression program and anti-correlated with AXL, a tyrosine kinase receptor, associated with the invasive, dedifferentiated drug-resistance phenotype. Notably, treatment-naive tumours classified as MITF<sup>High</sup> contained some cells with the AXL<sup>High</sup>/MITF<sup>Low</sup> expression program, and treatment with BRAFi or BRAFi+MEKi led to an increased proportion of the AXL<sup>High</sup>/MITF<sup>Low</sup> population.

To continue the discovery, three recent studies at the single cell scale showed that melanoma cells were capable of transitioning between not two but a multitude of states during tumour progression, in response to signals from the microenvironment and under exposure to therapeutic agents (Rambow et al., 2018, 2019; Tsoi et al., 2018; Wouters et al., 2020).

First of all, Tsoi and colleagues refined the classification of Hoek et al. into four melanoma cell states (C1–C4) with a comprehensive study of 54 human melanoma cell lines by bulk RNA-seq. Their data obtained resulted in a split of Hoek's cohort C into two subtypes distinguished by expression of the transcription factors SOX10 (C2) and SOX9 (C1), based on a comparison of the expression profiles of the C1-C4 classes and genes expressed as the cell progresses through an *in vitro* differentiation model of embryonic stem cells to melanocytes differentiated by nerve apex and melanocyte mediators (Tsoi et al., 2018) [Figure 27]. This study also validated the existence of Hoek's cohorts A (C4) and B (C3), but it was not possible to determine from bulk RNA-sequencing analysis whether the intermediate phenotype of C3 was due to a mixed population of C4 and C1/2 cells or if it represented a distinct, stable cell state. Class C4 was the most well-differentiated, expressing MITF and a variety of MITF targets related to differentiation and was related to Hoek's group A. Class C3 was related to Hoek's group B, expresses MITF, but also has features of neural crest-like cells. Importantly, the melanoma phenotype subpopulation identified in cell lines by Tsoi et al. (2018) and Hoek et al. (2006) reflected the cellular states detected by RNAseq analysis of human tumours. The presence of cells with an intermediate phenotype between differentiation and invasion (cohort B/class C3) could arise either because each individual cell within the population

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examined expressed a gene expression profile that was truly intermediate between proliferative and invasive, or because the cell lines contained mixed but stable subpopulations each reflecting the two different phenotypic states, or because cells were unstably flipping between one state and another with a specific frequency. To conclude, this work gave a clearer molecular signature, with the presence of NGFR in the neural-crest stem cell state, and a decreased expression of SOX10 and increased expression of SOX9 to distinguish an undifferentiated state.

Next, more recent single-cell analysis was performed on human melanoma cells isolated from patient-derived xenograft (PDX) mouse models exposed to MAPK therapeutics (Rambow et al., 2018, 2019). This study revealed an even greater complexity of melanoma state heterogeneity concerning MITF expression (RNA and protein) in BRAF<sup>V600E</sup> treatment-naive tumours. Importantly, this phenotypic heterogeneity was exacerbated upon BRAF and MEK inhibition indicating that drug treatment can either impose phenotypic transitions or select for specific pre-existing phenotypic states. Significantly, different drug-tolerant cell states coexisting within the same lesion exhibited distinct transcriptional MITF-(rheostat) activities. scRNA-seq profiling of the PDX (patient-derived xenograft) tumours before, during, and after BRAFi/MEKi combination therapy identified four distinct melanoma cell states associated with MRD (minimal residual disease), and the presence of these various melanoma subpopulations in clinical specimens was confirmed by *in situ* multiplexed immunohistochemistry. One of the 4 cell states present at the MRD stage exhibited a dramatic down-regulation of an overall cancer cell metabolic signature (ccmGDB) (Kim et al., 2016b) and shared transcriptomic features of nutrient-deprived cells including an elevated expression of the fatty acid translocase CD36. Termed the starved melanoma cell (SMC), bioinformatics analysis indicated that SMCs exhibited hallmarks of an MITF intermediate state characterized by features associated with both proliferation and invasion. To conclude, they defined a SMC intermediate state, with CD36 as marker. They also proposed AQP1 and RXRG as makers of NCSC sub-state via immunostaining [Figure 28].

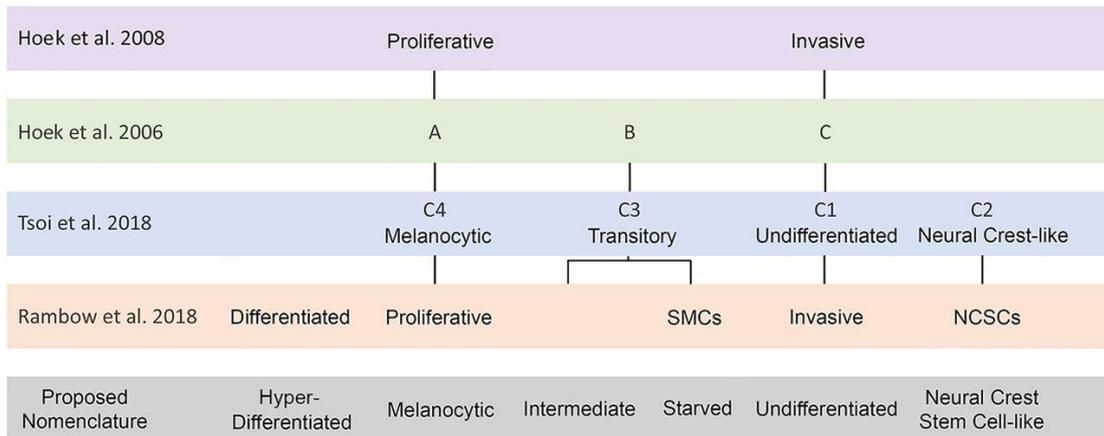


Figure 27. Likely relationships between the phenotypic states of melanoma cells identified in different studies. (Rambow et al 2019)

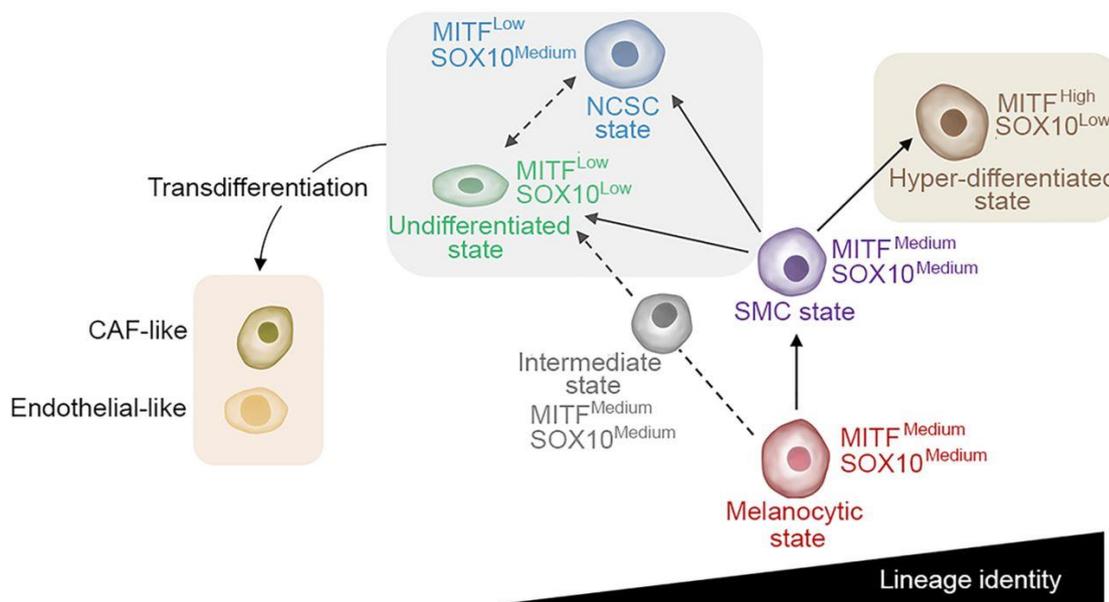


Figure 28. Potential hierarchical arrangement of the six different melanoma phenotypic states and their relative expression of MIF and SOX10. (Rambow et al 2019)

Furthermore, in this issue of Nature Cell Biology, Wouters et al. set out to distinguish between these two potential models of intermediate cellular states and to identify the gene regulatory networks that maintain phenotypic diversity in melanoma (Wouters et al., 2020). In this study, Wouters et al. used single-cell mRNA sequencing to confirm three distinct melanoma cell states in cultures obtained from patients. By combining extensive profiling of single-cell gene expression and chromatin accessibility applied to more than 39,000 cells from a cohort of patient-derived melanoma cultures, Wouters et al. identified shared gene regulatory networks that underlie the

extreme melanocytic and mesenchymal cell states as well as one stable intermediate state. The intermediate state was corroborated by a distinct open chromatin landscape and governed by the transcription factors EGR3, NFATC2, and RXRG, and exhibited intermediate MITF activity. Corresponded to the molecular signature of Tsoi et al. in 2018, melanoma cultures exhibiting high levels of SOX10 expressed additional melanocyte-specific markers such as the pigmentation genes MITF and TYR, whereas SOX9 cultures expressed genes related to EMT markers like TGFB1 and SERPINE1. The authors then applied single-cell regulatory network inference and clustering (SCENIC) to identify potential master regulators of each melanoma cell state [Figure 29]. Collectively, this study confirms that the intermediate state of melanocytes is distinct and stable, rather than a mixture of melanocytes and mesenchymal cells or an elapsed transition state. The authors discovered the gene regulatory network underlying each of the three states, suggesting that the intermediate cells express both melanoma and melanocyte gene expression tissues in addition to single regulatory mechanisms in the cell-mediated state. Overall, Wouters et al. have provided a detailed picture of the gene regulatory networks underlying melanoma cell states, which can be used in the future to design novel targeted treatments and guide phenotype switching to disarm melanomas in the clinic.

<b>Melanocytic</b>	<b>Intermediate (unique)</b>	<b>Mesenchymal</b>
<b>SOX10</b>	SOX6	SOX9
<b>TFAP2A</b>	NFATC2	JUN
<b>MITF</b>	EGR3	IRF1
<b>IRF4</b>	RXRG	FOSL2
<b>SOX4</b>	ELF1	ATF5
	ETV4	NFIB

*Figure 29. Potential master regulators of each melanoma cell state identified by single-cell regulatory network inference and clustering (SCENIC).*

*The melanocytic state was characterised by regulons of classic melanocyte TFs SOX10, TFAP2A and MITF, as well as IRF4 and SOX4. The mesenchymal state showed high activity of SOX9, JUN, IRF1, FOSL2, ATF5 and NFIB, consistent with previous reports (Fane et al., 2017; Reinhardt et al., 2017; Verfaillie et al., 2015). The intermediate state shared many regulons with both the melanocytic state and the mesenchymal state but also exhibited unique regulons, including SOX6, NFATC2, EGR3, ELF1 and ETV4. (Wouters et al., 2020).*

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## B. EMT-TFs expression switching in melanoma

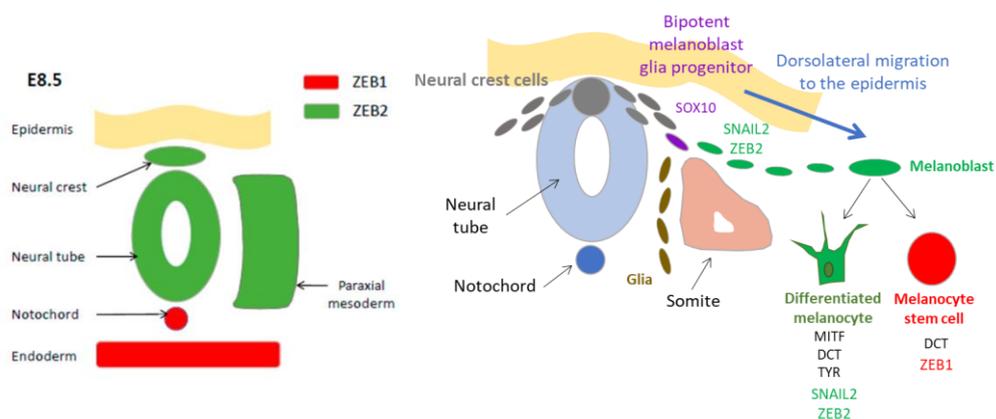
In the EMT and carcinoma part, I have already introduced physiological expression and functions of EMT-TFs, as well as their participation in carcinoma progression. In this part, I would like to talk about their roles in melanoma, with a particular attention to the two EMT-TFs I focused on during my PhD, ZEB1 and ZEB2.

### 1) Expression and function of EMT-TFs in the melanocytic lineage

EMT-TFs play a major role in the establishment of the melanocytic lineage and the function of adult melanocytes. Dorsolateral migration of melanoblasts through the embryo requires the participation of the EMT-TFs SNAIL2 and ZEB2. *Snail2*-deficient mice exhibit melanoblast migration defects that induced major pigmentation defects including a white forehead (Pérez-Losada et al., 2002). ZEB2 is also expressed in migrating melanoblasts in the mouse embryo and their precursor, neural crest cells, as described before in part "EMT-TFs". The specific deletion of *Zeb2* in the melanocytic lineage (Tyr: Cre *Zeb2* knock-out) induces a loss of pigmentation of the mouse, which is due both to poor migration of melanoblasts but also to a defect in differentiation of the melanocytes (Denecker et al., 2014). Indeed, the invalidation of *Zeb2* in primary melanocytes induces a decrease in MITF expression and differentiation defects. In addition to being expressed by melanoblasts, SNAIL2 and ZEB2 remain expressed by adult melanocytes in both humans and mice. Analysis of the expression of EMT-TFs in healthy human skin samples has demonstrated the presence of SNAIL2 and ZEB2 in differentiated melanocytes (Caramel et al., 2013). All these works highlight the key role of SNAIL2 and ZEB2 in maintaining homeostasis of the melanocytic lineage, from embryonic development through adulthood.

Conversely, ZEB1 and TWIST1 are not expressed by differentiated melanocytes in human skin (Caramel et al., 2013). However, ZEB1 expression has been observed in melanocytes stem cells (MSCs) from murine skin, suggesting its potential involvement in the generation of mature melanocytes from the stem cell pool (Denecker et al., 2014). In addition, unlike ZEB2, which

activates *MITF* transcription, ZEB1 was shown to repress *MITF* expression and alter the pigmentation of retinal pigment epithelial cells (Liu et al., 2009). A balance of ZEB2 to ZEB1 expression is thus associated with melanocyte dedifferentiation [Figure 30] even if more functional studies are required to characterize their roles.



*Figure 30. Expression of EMT-TFs during melanocyte lineage formation from embryonic neural crest cells.*

*Left : Schematic representation of ZEB1 and ZEB2 opposite expression patterns in the mouse embryo at embryonic day E8.5 .Right : The transcription factor SOX10 is expressed by the bipotent progenitor of glial and melanocytic cells. SNAIL2 and ZEB2 are required for neural crest cell delamination, melanoblast specification and dorsolateral migration. (Tang et al 2020)*

## 2) EMT-TFs expression switch during melanoma development

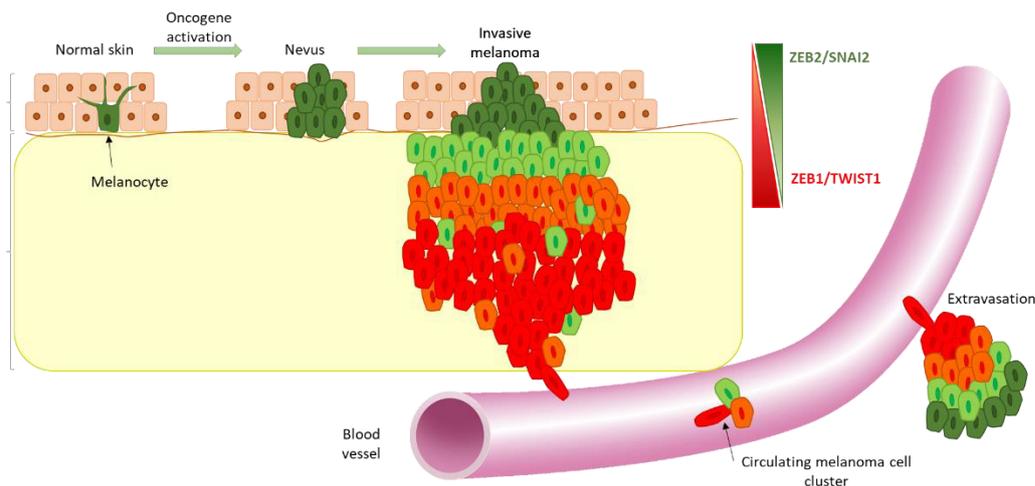
These observations of differentially expressed EMT-TFs relating to normal melanocytes raise the question of the possible and differential involvement of EMT-TFs in melanoma. The work of our team helped to better understand the role of each EMT-TFs in melanomagenesis, from the early stages of transformation (Caramel et al., 2013). Unlike carcinomas, all EMT-TFs do not exhibit the same expression profile or similar functions in melanoma. The analysis of the expressions of EMT-TFs SNAIL1, SNAIL2, TWIST1, ZEB1 and ZEB2 in a series of representative human tissues of melanoma evolutions, including normal epidermis, nevi, primary melanomas and metastases, demonstrated EMT-TFs patterns specific to the development of melanomas. As already mentioned, normal differentiated melanocytes express SNAIL2 and ZEB2 EMT-TFs and are negative for ZEB1 and TWIST1. While ZEB2 / SNAIL2 expression is progressively lost, ZEB1 / TWIST1 expression is activated

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during melanomagenesis. Within melanoma lesions, there is a gradient of EMT-TFs expression. ZEB2 and SNAIL2 are strongly expressed at the surface of the epidermis and lost in the deeper part of primary melanoma lesions while ZEB1 and TWIST1 are expressed in an inverse gradient, with stronger expressions in deeper parts, at the invasive front of melanoma [Figure 31].

The differential analysis of metastasis-free survival confirms the poor prognosis associated with the switch from ZEB2 / SNAIL2 to ZEB1 / TWIST1 expressions. Our lab demonstrated that this switch constituted a major risk factor for poor outcomes in malignant melanoma when controlling for other clinico-pathological variables. Remarkably, patients with melanoma with high ZEB2 expression and low ZEB1 / TWIST1 levels showed longer progression-free survival while almost all weak ZEB2, strong ZEB1 / TWIST1 tumors progressed rapidly (Caramel et al., 2013). Another study involving a large number of patients confirmed that high levels of ZEB2 expression improve the prognosis of patients with melanoma (Denecker et al., 2014). Furthermore, strong nuclear ZEB2 expression in human primary melanoma was associated with a lower Breslow index and increased metastasis-free survival (Vandamme et al., 2020).

Interestingly, the ZEB2 "differentiation pattern" is replicated in cortical areas of lymph node metastasis in a similar manner to that described in primary tumors, suggesting that melanoma cells can re-differentiate at the site of metastasis, reminiscent of mesenchymal epithelial processes (MET) in carcinomas (Ocaña et al., 2012; Tsai et al., 2012). Reversible switch to the proliferative phenotype may be necessary for metastasis to develop. ZEB2 may thus also contribute to the high metastatic propensity of melanoma as previously suggested for SNAIL2 (Gupta et al., 2005), in which suppression of SNAIL2 inhibited metastasis *in vivo*.



*Figure 31. EMT-TFs switching during melanoma development.*

*Schematic representation of the expression balance of EMT-TFs during the development of melanoma. ZEB2 and SNAIL2 are expressed by differentiated melanocytes within the healthy epidermis of human skin. Their expression is maintained in senescent nevi. During transformation to primary melanoma, expression of SNAIL2 and ZEB2 is gradually lost in favor of expression of TWIST1 and ZEB1. In addition, intra-tumor heterogeneity is also observed in melanoma lesions, the balance of expression of EMT-TFs being generally observed in gradient from the surface to the invasive front of the melanoma. The gain in invasive capacities through the activation of ZEB1 promotes the spread of melanoma cells. Finally, the inverse differential expression profile of EMT-TF is found at the secondary site, potentially contributing to the growth of metastasis. (Tang et al 2020)*

### **3) Control of the EMT-TFs differential expression in melanoma**

The switch in EMT-TFs expression is at least partly dependent of the MAPK pathway. Indeed ectopic expression of an activated form of BRAF in human or mouse melanocytes induces the conversion of ZEB2 / SNAIL2 to ZEB1 / TWIST1 expression profile. Indeed, the maintenance of ZEB1 / TWIST1 expression is dependent on the activation of the MAPK pathway, since the inhibition of the MAPK pathway in the A375 melanoma line induces the reverse transition, which means the loss of expression of ZEB1 and TWIST1 and the gain in expression of ZEB2. The transcription factor FRA1 (Fos-related antigen 1), member of the AP1 family, is a major actor of the gene regulatory network of the invasive phenotype in melanoma cells (Verfaillie et al., 2015). ChIP experiments showed that FRA1 controls the expression of EMT-TFs by directly binding to a region of transcriptional repression of ZEB2 and ahead of the transcription initiation sites (TSS) of ZEB1 and TWIST1.

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A double negative feedback loop involving members of the miR200 family and ZEB transcription factors is known to regulate cancer cell plasticity in carcinoma models (Brabletz and Brabletz, 2010). However, the expression level of miR200 is low in melanoma and up-regulation of ZEB1/2 has been demonstrated to be independent of miR200. In addition to FRA-1-driven oncogenic signalling, TGF $\beta$  was shown to promote the ZEB2/ZEB1 switch in melanoma cells (Vandamme et al., 2020). The transcription factor GLI2, activated by the TGF $\beta$  signalling pathway, has been shown to cooperate with ZEB1 to promote CDH1 inhibition, thereby participating in mesenchymal transition (Perrot et al., 2013). Finally, ChIP-seq targeting MITF in 501Mel melanoma line identified the ZEB1 locus as a potential target of MITF (Strub et al., 2011).

#### **4) Antagonistic functions of ZEB1 and ZEB2 in melanoma cells**

Functional assays *in vitro* and in mouse models have allowed to further characterize the oncogenic roles of EMT-TFs in melanoma. The acquisition of *Zeb1* and / or *Twist1* EMT-TFs expression is necessary for the transformation induced by the BRAF oncogene *in vitro* in melan-a murine immortalized cells, while the ectopic expression of *Zeb2* and *Snail2* drastically reduces the number of colonies formed (Caramel et al., 2013). Alteration of the switch (through inhibition of ZEB1 or ectopic expression of ZEB2) was sufficient to impair BRAF-dependent melan-a cells tumor growth *in vivo* in xenograft experiments. In addition, the ectopic expression of ZEB2 and / or SNAIL2 in established melanoma lines such as A375 also reduces the transforming potential and their tumorigenicity *in vivo*. Moreover, ectopic expression of ZEB1 increases tumorigenic characteristics of melanoma cell lines (A375, SKMEL5), while its knockdown induces significantly reduced growth of melanoma tumours *in vivo* on xenografts in nude mice (Caramel et al., 2013; Richard et al., 2016a). The deletion of *Zeb1* also reduced the transformative capacities, invasion capacities and migration capacities of B16F10 CD44 + CD133 + supposed cancer stem cells (Zhao et al., 2015). Thus, the roles of EMT-TFs appear to be completely uncoupled during melanomagenesis, where ZEB1 and TWIST1 exhibit oncogenic properties while ZEB2 and SNAIL2 rather act as tumour suppressors (Caramel et

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al., 2013).

These results support the notion that ZEB1 not only promotes invasive features but also exhibits intrinsic oncogenic functions as described in carcinoma. A tumour suppressive role for ZEB2 in melanoma was not expected, although such an activity was suggested for Zeb2 mRNA through the activation of PTEN expression (Karreth et al., 2011). Recent data in transgenic mouse models from Geert Berx lab have shown that knock-down of *Zeb2* was not sufficient to promote melanoma formation in mice and even alter NRASQ61-dependent melanoma formation (Vandamme et al., 2020). The enhanced expression of ZEB2 in this mouse model also promotes the growth of primary and metastatic tumours by promoting the proliferation of melanoma cells. Silencing ZEB2 in mouse NRASQ61 melanoma cells increases ZEB1, and induces proliferation defects (Vandamme et al., 2020).

Overall, despite a high degree of structural similarity, the zinc-finger homeodomain transcription factors ZEB1 and ZEB2 exhibit antagonistic expression patterns and functions in melanoma cells.

## **5) EMT-TFs regulate phenotype switching of melanoma cells**

Reciprocal regulations between ZEB and MITF TFs, suggested a coupling between the MITF-dependent phenotype switch and the EMT-TFs expression switch. ZEB1 is indeed one of the top-ranking genes whose expression is inversely correlated with MITF in TCGA tumors (Richard et al., 2016a; Verfaillie et al., 2015). Braf-induced transformation of melan-a cells induces decreased expression of *Mitf* and its target genes (Wellbrock and Marais, 2005). Induced expression of *Snail2* and *Zeb2* in this model restored *Mitf* expression and induced the melanocyte differentiation program described by Hoek and Goding (Hoek and Goding, 2010). Conversely, the ectopic expression of *Zeb1* and *Twist1* accentuated the *Mitf* flow invasive phenotype. In established melanoma lines, deletion of SNAIL2 and ZEB2 by interfering RNA reduced MITF expression level, in a similar way as ectopic expression of ZEB1 or TWIST1. The switch of ZEB2 / SNAIL2 to ZEB1 / TWIST1

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would therefore allow the acquisition of an invasive phenotype with low level of MITF expression (Caramel et al., 2013).

Further experiments indeed demonstrated that overexpression of ZEB1 in melanoma short-term cultures was sufficient to drive switching towards a MITF<sup>low</sup> undifferentiated phenotype bearing stem cell properties, characterized by increased expression of the neural crest stem cell (NCSC) marker NGFR, a major regulator of phenotype switching (Richard et al., 2016a). In contrast, ZEB1 knock-down induces a switch towards a MITF<sup>high</sup> differentiated phenotype. More importantly, ZEB2 knock-down in melanoma cells not only alters the expression of MITF, but also leads to induction of ZEB1. Thus, EMT-TF dependent reprogramming of melanoma cells, namely ZEB2/ZEB1 switch, is able to induce the phenotypic switch and the reversible transition between proliferative and invasive state.

ZEB2 ectopic expression activated MITF while ZEB2 knockdown resulted in a down-regulation of MITF (Caramel et al., 2013; Denecker et al., 2014). These results suggest that ZEB2 expression could mimic the rheostat pattern of MITF: ectopic expression of ZEB2 / MITF in melanoma cells would lead to differentiation and cell cycle arrest while invalidation of ZEB2 / MITF would also result in a proliferation defect. On the other hand, intermediate levels of ZEB2 / MITF would be favourable to the proliferation of melanoma cells. In this model, the aberrant expression of ZEB2 by melanoma cells would promote terminal differentiation and reduce tumorigenic capacities by xenograft in nude mice, while intermediate levels of ZEB2 in transgenic mouse models would be compatible with tumour proliferation. However, the link between the expression of EMT-TFs and proliferation remains unclear, as no effect on proliferation is observed under modulation of ZEB1 expression.

Overall, while different studies used ZEB2 and ZEB1 as additional markers to define the proliferative (MITF<sup>high</sup>/AXL<sup>low</sup>) or invasive (MITF<sup>low</sup>/AXL<sup>high</sup>) phenotypes, respectively (Müller et al., 2014), our data further demonstrated that EMT-TFs are major regulators of melanoma cell plasticity that fuel intra-tumor heterogeneity.

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## 6) Melanoma cell plasticity and treatment resistance

At the melanoma part, I already talked about the mechanisms of resistance to targeted therapies, immunotherapies and other combination treatments. Non-genetic mechanisms play a major role in the acquisition of drug resistance, since approximately 40% of BRAFi/MEKi resistant melanoma cases do not present a known genetic alteration (Hugo et al., 2015). Resistance has been attributed to drug-induced phenotypic adaptations, including the emergence of invasive traits or NCSCs, which rely on epigenetic, transcriptional, or translational reprogramming (Arozarena and Wellbrock, 2019; Shaffer et al., 2017).

Namely, the MITF<sup>low</sup>/AXL<sup>high</sup> phenotype, either innate or acquired upon treatment, has been associated with resistance to multiple targeted drugs in melanoma (Müller et al., 2014). Our lab demonstrated that ZEB1-mediated phenotype switching is associated with resistance to MAPK inhibitors (Richard et al., 2016a). More specifically, a high ZEB1 expression with low MITF expression is intrinsically resistant to MAPKi. Besides, an up-regulation of ZEB1 was observed in cell lines with acquired MAPKi resistance and in biopsies from patients suffering relapse while under treatment. Gain- or loss-of-function of ZEB1 showed that it is sufficient to promote a transition towards a NCSC/invasive state resistant to BRAFi. Single-cell RNAseq data from JC Marine lab, during BRAFi and MEKi treatment of melanoma PDXs, also highlighted the heterogeneous expression of EMT-TFs and identified an enrichment of cells in a mesenchymal-like phenotype (Rambow et al., 2018). Finally, recent data suggest that the MITF<sup>low</sup> and MITF<sup>high</sup> phenotypes may be both associated with resistance to targeted therapies (Arozarena and Wellbrock, 2019; Rambow et al., 2019). Correspondingly, we observed that high expression of ZEB1 in BRAFi-resistant tumours could be found in MITF<sup>low</sup> and MITF<sup>high</sup> context, and that knockdown of ZEB1 reduced the viability of the resistant melanoma cells in the MITF<sup>low</sup> and MITF<sup>high</sup> contexts, suggesting that ZEB1 acts partly by MITF- independent mechanisms (Richard et al., 2016b).

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To conclude, genetic and epigenetic, transcriptional, or translational reprogramming events contribute to melanoma cell plasticity as well as resistance to multi-drugs.

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## Objective of thesis

While the role of EMT (epithelial to mesenchymal transition) in carcinoma tumorigenesis has been extensively characterized, its role in melanoma, which originates from a non-epithelial cell, the melanocyte, has been poorly deciphered. However a “pseudo EMT” process occurs. Unlike cancer stem cell model in carcinoma, there is a specific switch in melanoma, termed “phenotype switching”, in which melanoma cells switch their phenotype from a proliferative/differentiated towards an invasive/stem-like phenotype, along intermediate phenotypes. The loss of MITF (major transcription factor in melanocytic lineage) induces the transition towards an invasive state (Goding and Arnheiter 2019; Rambow et al 2019). Interestingly, previous work from our team showed that EMT-TFs (EMT transcription factors) display essential functions in melanoma tumorigenesis and treatment resistance by regulating phenotype switching. Our team indeed demonstrated that ZEB2 was expressed in normal melanocytes and decreased progressively along the conversion to melanoma, while ZEB1 increased (Caramel et al 2013). ZEB2 supports the proliferation and differentiation of melanoma cells via the activation of MITF; in contrary, ZEB1 inhibits MITF expression and induces the transition towards an invasive phenotype with neural-crest stem cell (NCSC) properties, associated with NGFR activation (Richard et al 2016; Tang et al 2020). While ZEB1-dependent regulation of phenotype switching is partly mediated by the regulation of MITF expression, part of ZEB1 function was independent from this key player. Therefore, identification of additional mechanisms was required. While ZEB1 ChIP-Seq had been performed in carcinoma models, the direct target genes of ZEB1 in melanoma models had never been investigated.

The main objective of my PhD project was thus to define the direct target genes of endogenous ZEB1 during phenotype switching in a genome-wide scale. I first had to establish an *in vitro* model of phenotype switching in melanoma short-term cultures. Then, to set-up the ChIP-Seq protocol, before integrating data with RNA-seq. Finally, relevant targets were further validated upon ZEB1 gain/loss of function and in human melanoma samples through multi-immunofluorescence.

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## Results

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## **ZEB1-dependent transcriptional control of cell states transitions in melanoma cells**

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**Running title:** ZEB1 transcriptional function in melanoma

**Keywords:** melanoma; plasticity; transcription factor; ChIP-Seq

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## Abstract

Cell plasticity processes sustain tumor adaptation and treatment resistance. Intra-tumor heterogeneity in melanoma relies on reversible phenotypic transitions between a proliferative/differentiated and invasive/stem-like state. A better understanding of the transcriptomic and epigenetic mechanisms underlying these phenotypic adaptations is still required. We previously identified the ZEB1 transcription factor as a major driver of phenotype switching towards an invasive/stem-like phenotype, fostering adaptive resistance to targeted therapies. Herein, in order to precisely characterize ZEB1 function and get a comprehensive view of its direct target genes, we performed a ChIP-Sequencing combined with RNA-seq, upon phenotype switching in melanoma cells. We identified and validated ZEB1 binding peaks in the promoter of a large set of genes related to melanoma cell identity. Gain or loss of function of ZEB1 further demonstrated that ZEB1 negatively regulated proliferative-melanocytic genes and up-regulates invasive/undifferentiated genes. We further analyzed spatial intra-tumor heterogeneity of melanoma states markers according to ZEB1 expression in human samples, and validated ZEB1 expression not only in invasive cells but also in stem-like cells. Overall, our results provide a better understanding of cell state transitions and intra-tumor heterogeneity in melanoma.

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## Introduction

Cutaneous malignant melanoma is an aggressive form of skin cancer arising from melanocytes. Despite recent advances in targeted therapies and immunotherapies for the treatment of metastatic melanoma, nearly 60% of patients still develop resistance, necessitating the development of combination therapy strategies. A major mechanism of resistance to treatments relies on the ability of cancer cells to adapt. One of these plasticity mechanisms, the epithelial-mesenchymal transition (EMT), is a reversible trans-differentiation process finely regulated by a network of transcription factors (EMT-TFs) belonging to the SNAIL, TWIST and ZEB families. EMT-TFs are aberrantly reactivated in many cancers, particularly in carcinomas, where they play an oncogenic role (Caramel et al., 2018; Puisieux et al., 2014). Importantly, beyond their functions as pro-metastatic factors, the activation of ZEB1/ZEB2 plays an active role in tumor initiation by alleviating key oncosuppressive mechanisms and by endowing cancer cells with stem cell-like properties.

Although EMT cannot be formally defined in non-epithelial cancers, a related process of cellular plasticity contributes to intra-tumor heterogeneity (ITH) in melanoma and relies on reversible phenotypic transitions between a proliferative/differentiated and invasive/stem-like state (Hoek et al., 2008). Loss of Microphthalmia-associated Transcription Factor (MITF), the master regulator of melanocyte differentiation, induces a reprogramming towards an invasive and stem-like phenotype in melanoma cells (Goding and Arnheiter, 2019; Rambow et al., 2019). Gene expression analyses of tumors at the single cell level enabled to refine this phenotype-switching model, including the description of intermediate states and major molecular regulators (Jerby-arnon et al., 2018; Rambow et

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al., 2018; Tirosh et al., 2016). Reprogramming towards a Neural Crest Stem Cell-like (NCSC) phenotype was proposed as an adaptive response to targeted therapy, accounting for therapy resilience. A complete understanding of cellular and molecular mechanisms underlying phenotypic adaptations and thus, the exceptional capacity of melanoma cells to develop resistance to current therapeutic strategies, is still required.

We previously showed that ZEB2 is expressed in normal melanocytes and that its expression progressively decreases during conversion to melanoma, while ZEB1 expression increases (Caramel et al., 2013; Tang et al., 2020). ZEB2 supports melanoma cell proliferation and differentiation by activating MITF expression. ZEB1 on the contrary, inhibits MITF expression, promotes transition to an invasive phenotype with neural crest stem cell (NCSC) properties, and resistant to targeted therapies.

Herein, in order to further characterize ZEB1 function and get a comprehensive view of its direct target genes, we performed a ChIP-Sequencing approach upon phenotype switching in melanoma cells. Combined with RNA-Seq data, we define ZEB1 as a major transcriptional regulator of genes associated with phenotypic transitions in melanoma. Specific markers were validated as ZEB1 direct target genes, upon ZEB1 gain or loss of function, and their relevance *in vivo* was further addressed through spatial analyses in human samples. Intra-tumor heterogeneity of melanoma states markers according to ZEB1 expression in human samples demonstrated co-expression of ZEB1 with both stem-like and invasive markers.

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## Results:

### Modelling phenotype switching towards ZEB1<sup>high</sup>/MITF<sup>low</sup> invasive state *in vitro*

In order to study phenotypic transitions in melanoma cells, we used two patient-derived short-term cultures, established with a low number of passages after culture (GLO and C-09.10). These two short-term cultures display a ZEB1<sup>low</sup>/MITF<sup>high</sup> proliferative phenotype. C-09.10 cells are highly melanocytic, while GLO cells tend towards a transitory state, with MITF intermediate expression (Richard et al., 2016). In order to induce phenotype switching, cells were treated every 3 days, for 21 days, with the inflammatory cytokine TNF $\alpha$ , a known inducer of dedifferentiation in melanoma cells (Riesenberg et al., 2015). As expected, TNF $\alpha$  treatment (100ng/mL) decreased proliferation, but no significant cell death was observed.

RNA-Seq analyses were performed at day 7 and day 14 after TNF $\alpha$  treatment. Pathways analyses of the 4531 differentially expressed (DE) genes at day 14 compared to untreated cells ( $p < 0.001$  &  $|FC| > 1$ ) (2490 up and 2041 down), confirmed a decrease in proliferation hallmarks, as well as an enrichment in TNF $\alpha$  signaling, inflammatory response, and EMT hallmarks (Fig. 1A & Supp. Fig.1A). We next analyzed previously described proliferative and invasive melanoma signatures from (Hoek et al., 2006) and (Verfaillie et al., 2015), as well as signatures from the four states melanoma nomenclature proposed by (Tsoi et al., 2018) (melanocytic, transitory, NCL and undifferentiated). This confirmed a switch from a proliferative/melanocytic (untreated), towards a more invasive state upon TNF $\alpha$  treatment (Fig. 1A-B & Supp. Fig.1B). Both the NCSC (NCL) and the

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undifferentiated state signatures from Tsoi were activated, starting from day 7 and even more at day 14. The transitory state signature from Tsoi was also decreased in this model.

As regards to molecular markers, treatment with  $\text{TNF}\alpha$  was shown to induce a progressive switch towards a  $\text{ZEB1}^{\text{high}}/\text{MITF}^{\text{low}}$  state. ZEB1 expression progressively increased at the protein and RNA levels, while ZEB2 protein expression progressively decreased upon treatment (Fig. 1C and D). ZEB2 mRNA expression was not modified, consistent with previous reports (Vandamme et al., 2020). Drastic down-regulation of MITF expression was observed as early as 7 days after treatment. Interestingly, the expression of the Neural Crest Stem Cell like (NCSC) marker NGFR (Nerve Growth Factor Receptor) was transiently induced (day 7 and day 14), before decreasing at day 21. The receptor tyrosine kinase AXL (Müller et al., 2014) followed a similar expression pattern than NGFR. Analysis of SOX10 and SOX9 protein and RNA expression (Fig. 1C and 1D) confirmed a progressive switch, around day 14 and more strikingly at day 21, towards a putative undifferentiated state, losing NGFR and SOX10, in favor of SOX9 (Tsoi et al., 2018).

In order to more precisely monitor intra-tumor heterogeneity during phenotype transitions over time, a stable cell line was established with a *MITF* promoter-GFP reporter construct and combined with a *NGFR* membrane staining by flow cytometry. As previously mentioned, GLO cells exhibit a transitory phenotype, around half of the population present in  $\text{MITF}^{\text{high}}$  and the other in  $\text{MITF}^{\text{low}}$  state.  $\text{TNF}\alpha$  treatment decreased the proportion of the  $\text{MITF}^{\text{high}}$  population and also led to a transient emergence of a  $\text{MITF}^{\text{low}}/\text{NGFR}^{\text{high}}$  phenotype at day 7 (representing about 70 %), before transition towards a  $\text{MITF}^{\text{low}}/\text{NGFR}^{\text{low}}$  population (Fig. 2A).

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Finally, we performed functional assays in order to validate the transition towards a more invasive state. Transwell migration assays and scratch assays validated that TNF $\alpha$  treatment progressively increased the migratory capacity of GLO cells after 14 days (Fig. 2B and 2C). Consistent with their increased migratory capacities, the sensitivity of TNF $\alpha$ -treated GLO cells to BRAF inhibitor was also decreased compared to control cells, as assessed by ATP assay and Incucyte device assay (Fig. 2D). These data confirmed the transition towards a more invasive and targeted-therapy resistant state.

Importantly, a similar phenotype switching towards a ZEB1<sup>high</sup>/MITF<sup>low</sup> state could be modeled in a second melanoma short-term culture, C-09.10 cells (Supp. Fig. 2). In this model, TNF $\alpha$  (100ng/mL) was combined with TGF $\beta$  (20ng/mL) in order to ensure an efficient switching. Indeed, RNA-Seq analyses confirmed that C-09.10 cells display a more melanocytic phenotype than GLO cells (Supp. Fig. 2A). 3769 differentially expressed genes were found at day 14, upon combined TNF $\alpha$  + TGF $\beta$  treatment (2181 up and 1588 down) (that is to say still 20-25% less than in GLO cells treated with TNF $\alpha$  alone) (Supp. Fig. 2B). NCL, invasive and undifferentiated signatures were enriched at D7 and D14 (Supp. Fig. 2C-D). More specifically, ZEB1 was drastically induced, in association with MITF rapid down-regulation (Supp. Fig. 3A-B), however, the SOX10/SOX9 switch was not observed in this model. FACS analyses of NGFR in a MITF-GFP reporter line, showed as in GLO cells, a decrease in the MITF<sup>high</sup> population and a transient increase in MITF<sup>low</sup>/NGFR<sup>int/high</sup> cell population (Supp. Fig. 3C). Functional analyses validated that C-09.10 treated cells exhibited increased resistance to BRAFi (Supp. Fig. 3D-E).

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Overall, we developed two suitable *in vitro* models to study endogenous ZEB1 transcription factor direct transcriptional targets during phenotypic transitions of melanoma cells, towards NCSC-like and invasive states.

### **Determination of ZEB1 direct target genes during phenotype switching**

In order to perform Chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq) analyses, we first validated a ZEB1 ChIP-grade antibody (from Genetex, since previously described antibody from Santa Cruz was unavailable (Feldker et al., 2020)). To do so, *ZEB1* was first knocked-out in the ZEB1<sup>high</sup> A375 melanoma cell line (Supp. Fig. 4A). ZEB1 control and knocked-out clones were then used for ChIP-QPCR experiments. A significant enrichment of ZEB1 binding was found on *MITF* promoter, which was abolished in *ZEB1* knocked-out cells, thus validating the specificity of the antibody (Supp. Fig. 4B). A first ChIP-Seq was performed in A375 cells, allowing to validate the process (Supp. Fig. 4C).

We then performed ZEB1 ChIP-Seq in untreated and TNF $\alpha$ -treated GLO cells at day 14 (Fig. 3). Consistent with ZEB1 increased expression, almost 2-times more ZEB1 peaks were found in TNF $\alpha$ -treated cells than in untreated cells (Fig. 3A). 34% of ZEB1 peaks were conserved between untreated and TNF $\alpha$ -treated cells while 59.5% were specifically induced upon TNF $\alpha$  treatment (Fig. 3B). Around 70% of peaks were found in promoter regions (-1000, +500bp), the large majority overlapping with the Transcription Start Site (Fig. 3C-D).

Next, integration with RNA-seq (TNF $\alpha$  differentially expressed genes,  $p = 0,001$  and IFC $>1$  or  $<-1$ ), allowed to correlate binding of ZEB1, with up or down-regulation of the genes upon TNF $\alpha$

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treatment (Fig. 3E). 42% of TNF $\alpha$  deregulated genes exhibited a ZEB1 peak at day 14. 47% among down-regulated genes, and 38% among up-regulated genes. Pathway analyses on differentially expressed genes displaying a ZEB1 peak, demonstrated that ZEB1 directly binds to TNF $\alpha$ -down-regulated genes involved in Proliferation, Pigmentation hallmarks and to TNF $\alpha$ -up-regulated genes involved in TNF signaling, invasion/EMT hallmarks (Fig. 3F). ZEB1 was more frequently already bound in untreated cells, to genes that are down-regulated, while it was more frequently recruited *de novo* upon TNF $\alpha$  to genes that are activated.

Specific analyses of melanoma phenotype signatures from Hoek (Hoek et al., 2006) next demonstrated that ZEB1 binding peaks were present in 46,7% of proliferative signature genes, which are down-regulated upon TNF $\alpha$  at day 14 (among which MITF, PMEL, CDH1, RAB38, ASAH1, TNFRSF14) (Fig. 4A). 40,4% of invasive signature genes (which are activated upon TNF $\alpha$ ) also displayed a ZEB1 binding peak at day 14 (including ZEB1 itself, AXL, THBS1, BIRC3, ITGA2, ITGA3 and EGFR) (Fig. 4A). With respect to (Tsoi et al., 2018) signatures, ZEB1 binding peaks were found in 40% of melanocytic signature genes (including CEACAM1 and TSPAN10 which are down-regulated upon TNF $\alpha$ , and CDH3 which is up-regulated). ZEB1 binding peaks were also found in 34.8% of NCSC signature genes (among which TGFA, GLI2, NES, ANGPTL4) and in the promoter of 33% of undifferentiated signature genes (including EGFR again, SOX9, CITED2, KRT7, KRT18, KRT80, AJUBA, IL4R, TNFAIP2, ZIC2, SECTM1) (Fig. 4B). Only 17% of transitory signatures genes displayed ZEB1 binding peaks. 32% and 41% respectively of (Verfaillie et al., 2015) proliferative and invasive signatures also displayed ZEB1 peaks (Supp. Fig. 5).

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Overall, these analyses led to the identification of novel ZEB1 direct target genes, specific of the melanocytic lineage, including down-regulation of melanocytic genes and up-regulation of NCSC & undifferentiated genes.

### **ZEB1 directly regulates the expression of major melanoma state markers**

We next focused on major melanoma state markers: ZEB2, MITF, NGFR, AXL, SOX10 and SOX9 (Fig. 5A). ZEB1 was already bound, in untreated GLO cells, on the promoters of *ZEB2*, *MITF* and *SOX10*, genes the expression of which is down-regulated upon TNF $\alpha$  treatment. In contrast, a ZEB1 peak was acquired *de novo* upon TNF $\alpha$  treatment at day14 in genes, the expression of which was activated by TNF $\alpha$ , such as *ZEB1* itself, *NGFR*, *AXL* and *SOX9*. Moreover, the peaks were largely conserved in A375 cells, although not exactly at the same location (Fig. 5A). Binding of ZEB1 to the sites defined by ChIP-seq was then validated by ChIP-QPCR in both GLO and C-09.10 models, upon TNF $\alpha$  +/- TGF $\beta$  treatment (Fig. 5B-C). Consistent with ZEB1 increased expression, enrichment in the binding of this TF on the promoters of *ZEB2*, *MITF*, *NGFR*, and *SOX10* was found upon TNF $\alpha$  +/- TGF $\beta$ . Even if a peak was found in the promoter of *SOX9*, ZEB1 binding could not yet be validated by ChIP-QPCR. Binding of ZEB1 to the *AXL* promoter has to be further validated in C-09.10.

In order to reinforce the driving role of ZEB1 in the regulation of these genes, their expression was analysed upon ZEB1 over-expression in C-09.10 and upon *ZEB1* knock-out in A375 cells (Fig. 6A-B). *MITF* and *NGFR* expression upon ZEB1 deregulated expression has been previously described (Richard et al., 2016). Here, we show that *AXL* is activated by ZEB1, following the same expression profile than *NGFR*. We further demonstrate that *SOX10* expression decreased upon ZEB1

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over-expression in C-09.10, although it was not modified upon *ZEB1* knock-out in A375 cells. In mirror, *SOX9* expression increased upon *ZEB1* over-expression in C-09.10, while it decreased upon *ZEB1* knock-out in A375 cells. Overall, our data sustain a model in which *ZEB1* directly represses *MITF*, *SOX10*, and *ZEB2* and induces itself, *NGFR*, *AXL* and *SOX9*, although *SOX9* direct regulation remain to be demonstrated.

RNA-Seq analyses upon *ZEB1* over-expression in C-09.10 further demonstrated the increase in EMT/invasion pathways (Fig. 6C and Supp. Fig. 6). Only few genes were down-regulated in this model, but still associated with a decreased proliferative signature. Interestingly, the most significantly enriched melanoma signature was the NCSC one, which was almost as activated by *ZEB1* than by  $TNF\alpha$ . This further supports the conclusion that a major part of  $TNF\alpha$ -mediated transition towards a more invasive/NCSC-like state is regulated by *ZEB1*.

Interestingly, beyond well-described phenotypic markers from Fig.5, *ZEB1* binding peaks were found in other markers of melanoma identity: *RAB38*, *TGFA*, *TSPAN13*, which are down-regulated upon  $TNF\alpha$  treatment; *BIRC3*, *ITGA2*, *EGFR*, *F2RL1*, *TNFAIP2* which are rather up-regulated upon  $TNF\alpha$  (Fig. 7A-C). *ZEB1* binding on the promoters of these genes could be confirmed by ChIP-QPCR (Fig. 7B), further validating the pleiotropic roles of *ZEB1*.

### **Spatial analyses of melanoma cell intra-tumor heterogeneity in patient samples**

In order to further investigate *ZEB1* co-expression or antagonistic expression with some of its identified targets, we analyzed their expression *in situ* in patient samples. To achieve this goal we performed multi-immunofluorescence analyses (7 colors, OPAL, Perkin-Elmer) starting with *ZEB1*,

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ZEB2, MITF, NGFR, SOX10 and SOX9 to more precisely define the frequency and location of the different phenotypes, and address co-localization of ZEB1 with these melanoma states markers. A cohort of n=20 cutaneous melanoma was analyzed. Antagonistic expression of ZEB1 and ZEB2, as well as SOX10 and SOX9, validated the specificity of the stainings. Spatial reconstitution (with R) of the whole tumors for each marker revealed differential patterns of expression. Low-grade primary melanoma exhibits a ZEB2<sup>+</sup> MITF<sup>+</sup> SOX10<sup>+</sup> phenotype (and no ZEB1, SOX9 and NGFR expression). High-grade primitive melanoma or cutaneous metastases, with a high ZEB1 expression, display low ZEB2 expression, as previously described. Importantly, ZEB1 is not only expressed in the invasive front but also in the bulk. Decreased SOX10 expression could be evidenced in ZEB1<sup>high</sup> clones (Fig. 9A). NGFR expression was gained in some sub-populations of cells, and more specifically in ZEB1<sup>high</sup> cases. SOX9 expression was strongly expressed only in a few cases, and co-expression of SOX9 with ZEB1 could be evidenced. This allowed demonstrating *in situ* the presence of ZEB1<sup>+</sup>NGFR<sup>+</sup> cells and ZEB1<sup>+</sup>SOX9<sup>+</sup> cells (Fig. 9B).

## Discussion

Our study reports the first comprehensive characterization of ZEB1 transcriptional function in melanoma, providing a better understanding of the molecular mechanisms underlying phenotype plasticity and intra-tumor heterogeneity in melanoma. We identified and validated ZEB1 transcription factor direct binding to the promoter of genes specific to the melanocytic lineage or driving melanoma cell identity. Gain or loss of function of ZEB1 further demonstrated that ZEB1 negatively regulates proliferative-melanocytic genes and up-regulates invasive/undifferentiated genes. Overall, this study

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further defined ZEB1 as a major regulator of melanoma cell identity and phenotype switching, beyond its well-established role in metastasis. Although initially described as a transcriptional repressor, our data confirm previous ChIP-Seq analyses in carcinoma models (Feldker et al., 2020) showing ZEB1 capacity to mediate both transcriptional activation and repression in similar proportions. Importantly, even if some ZEB1 target genes may be shared between melanoma and breast cancer cell lines (Feldker et al., 2020), such as CDH1 and other EMT genes, our study reveals cell type specific effects of ZEB1, through the regulation of melanocytic lineage specific genes. ZEB1 notably binds to the promoter of ZEB2 and represses its expression in melanoma cells, while these two factors are co-expressed in mesenchymal cells.

Overall, we propose a model in which ZEB1 may directly repress *MITF*, *SOX10*, and *ZEB2*, and in the meantime induce itself, *NGFR* and *AXL*. At this stage, ZEB1-mediated activation of *SOX9* could be indirect, through the repression of *SOX10*, since *SOX10* and *SOX9* have been shown to repress each other (Shakhova et al., 2015). However, the *SOX10/SOX9* switch was not observed in C-09.10, maybe because of co-factors or epigenetic marks that remain to be defined.

Spatial analyses in human samples of these melanoma state markers provided further validation at the cellular level, of the co-expression of ZEB1 with *NGFR* and *SOX9*. Importantly, ZEB1 is not only expressed in the invasive front but also in the bulk, where it may sustain stem-like features and tumor-initiating properties. Although *NGFR* and *SOX9* are not sufficient to define the NCSC and undifferentiated states respectively, ZEB1 expression may be found in these two sub-populations of cells. However, our study revealed a strong inter-patient variability among the 20 samples analysed, with only few samples displaying a significant expression of *NGFR* or *SOX9*.

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Additional melanoma samples (n=10) will be analysed in order to provide a more comprehensive view of intra-tumor heterogeneity. Quantitative analyses will enable to further define the percentage of ZEB1<sup>high</sup> cells displaying lower level of SOX10, or co-expressing NGFR and/or SOX9.

While our spatial analyses in human samples clearly demonstrated the co-expression of ZEB1 with NGFR and/or SOX9, ZEB1 may not regulate these markers in the same cells, nor at the same time. Consistent with our *in vitro* analyses, an hypothesis would be that ZEB1 would activate NGFR transiently, before activating SOX9, therefore favoring the transition from a NCSC towards an undifferentiated state. Longitudinal analyses at the single cell level would be required in order to prove whether this is the same trajectory or two different routes in parallel.

As regards to MITF, ZEB1 represses MITF expression and subsequent MITF-transcriptional program. But ZEB1 also directly regulates MITF targets (PMEL, etc...). Previous MITF ChIP-Seq analyses demonstrated that MITF directly and positively regulates genes involved in DNA replication, repair and mitosis, while repressing genes involved in melanoma invasion (Laurette et al., 2015). ZEB1 and MITF may thus bind to the same genes but with different consequences.

Moreover, intermediate states, with coexistence of proliferative and invasive features, including ZEB1<sup>high</sup> / MITF<sup>high</sup> state have been described. Our previous results suggested that ZEB1 would perform some of its functions independently of MITF. Both MITF<sup>low</sup> and MITF<sup>high</sup> phenotypes may be associated to resistance to targeted therapies, suggesting a more complex model of intra-tumor heterogeneity and cell state reprogramming.

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While ZEB2 rather co-expressed with SOX10 and MITF in our *in situ* analyses, ZEB2 ChIP-sequencing analyses should reveal in the future its melanocytic lineage specific targets and cross-regulation with ZEB1.

Moreover, if we focused on markers of melanocyte cell identity, this ChIP-seq approach also revealed additional targets related with inflammatory or interferon response, consistent with the pleiotropic roles of ZEB1, that extend beyond invasion, including immune escape.

Overall, this study provides clues as to how ZEB1-driven phenotype plasticity can foster the acquisition of resistance to targeted therapies in melanoma, beyond MITF regulation. Although ZEB1 targeting is still challenging, this work should lead to evaluate the benefit of targeting cancer cell plasticity as a strategy to overcome treatments' resistance, which will, in turn, provide a strong rationale to the development of new combination therapies in the treatment of malignant melanoma.

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## Figures legends

### **Figure 1: Molecular characterization of TNF $\alpha$ -induced phenotype switching *in vitro*.**

**A.** RNA-seq analyses of GLO cells after 7 (D7) or 14 days (D14) of TNF $\alpha$  (100ng/ml) treatment. Heatmap of differentially expressed genes. The most significant Hallmarks enriched within down- and up-regulated in D14 versus untreated cells are indicated on the right. **B.** Heatmap of ssGSEA scores of the most relevant hallmarks and of melanoma states signatures from Hoek, Tsoi and Verfaillie. **C-D.** Western blot (**C**) and RT-qPCR (**D**) analyses of ZEB1, ZEB2, MITF, NGFR, AXL, SOX10 and SOX9 expression after 7, 14 and 21 days of TNF $\alpha$  (100ng/ml) treatment in GLO cell line. GAPDH was used as loading control. Histograms represent quantitative analyzes (mean  $\pm$  SEM, n=3).

### **Figure 2. Intra-tumoral heterogeneity and functional characterization of TNF $\alpha$ -induced phenotype switching in GLO cells.**

**A.** Longitudinal intra-tumor heterogeneity characterization of MITF and NGFR expression by flow cytometry in GLO pMITF-GFP cell line, upon TNF $\alpha$  (100ng/ml) treatment after 7 (D7), 14 (D14) and 21 days (D21). NGFR was marked by antibody anti-NGFR coupled with APC.

**B.** Transwell migration assays in GLO upon TNF $\alpha$  (100ng/ml) treatment after 7 (D7) or 14 (D14) days. Cells were fixed after 24h. Representative pictures of the membranes. The number of migrating cells is plotted. **C.** Scratch assay in cell culture of GLO cells, upon TNF $\alpha$  (100ng/ml) treatment after 21 days (D21). Migration distance in  $\mu$ m was measured after 24 hours. Histograms represent quantitative analyzes (mean  $\pm$  SEM, n=3).

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**D.** BRAFi Survival assays. ATP assay upon BRAF inhibitor PLX4032 treatment for 7 days. (mean  $\pm$  SD, n=3)

**E.** Incucyte assay showing the relative increase in cell death upon PLX4032 (588nM) treatment overtime.

**Figure 3. ZEB1 ChIP-sequencing analyses in GLO cells upon TNF $\alpha$  treatment.**

**A.** ZEB1 ChIP sequencing was performed in GLO cells, untreated or after 14 days (D14) of TNF $\alpha$  (100ng/ml) treatment. The number and size of peaks is indicated.

**B.** Venn diagram showing the overlapp between peaks found in untreated (green) or after 14 days of TNF $\alpha$  treatment (D14) (Red).

**C-D.** Localization of the peaks and distance to the transcription start site (**D**).

**E-F.** Integration of ChIP-Seq with RNA-seq data. **E.** Heatmap of DE genes in TNF $\alpha$ -treated cells at day14 compared to untreated cells. Presence of a ZEB1 peak in the gene is indicated by a green line (untreated) or a red line (TNF D14) on the right.

**F.** Heatmap of DE genes presenting a ZEB1 peak. The most significant Hallmarks enriched within down- and up-regulated genes in D14 versus untreated cells are indicated on the right.

**Figure 4. Specific analyses of ZEB1 binding on genes from melanoma states signatures**

Heatmap of genes from the melanoma signatures from Hoek et al (**A**), Tsoi et al. (**B**) in untreated or TNF $\alpha$ -treated GLO cells at day14. Presence of a ZEB1 peak is indicated by a green (untreated) or red (TNF D14) square. Differential expression of the gene upon TNF $\alpha$  (red:  $p < 0.001$  ; purple:  $p < 0,05$ ).

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**Figure 5. Validation of ZEB1 binding on melanoma phenotype markers by ChIP-qPCR.**

**A.** Geneviewer captures showing ZEB1 binding peaks in *ZEB2*, *MITF*, *NGFR*, *AXL*, *SOX10* and *SOX9* promoters in A375 cells and untreated or TNF $\alpha$ -treated GLO cells at day 14 (D14).

**B-C.** ZEB1 ChIP-QPCR on *ZEB2*, *MITF*, *NGFR*, *AXL*, *SOX10* and *SOX9* promoters in GLO (**B**) and C-09.10 cells (**C**) upon TNF $\alpha$  or TNF $\alpha$  + TGF $\beta$  treatment at day 14 (D14). Anti-ZEB1 (Z1) or control IgG were used for the IP. Relative promoter enrichment was normalized to chromatin inputs. N=3 except for SOX9 in GLO, and AXL in C-09.10.

**Figure 6: ZEB1-dependent regulation of melanoma markers in melanoma models.**

**A.** Western-blot and RT-qPCR analyses of phenotype markers (ZEB1, NGFR, AXL, SOX10, SOX9) in C-09.10 cell line with ZEB1 over-expression (Z1) (n=3).

**B.** Western-blot and RT-qPCR analyses of phenotype markers (ZEB1, NGFR, AXL, SOX10, SOX9) in A375 control (AS3) or ZEB1 knocked-out (AZ1) clones.

**C.** RNA-seq analyses of C-09.10 cell line upon ZEB1 over-expression (ZEB1\_OE). ssGSEA scores of Hoek and Tsoi melanoma signatures are plotted in C-09.10 cell line upon ZEB1 over-expression or upon TNF $\alpha$  + TGF $\beta$  treatment at day14.

**Figure 7. Additional ZEB1 direct targets related to melanoma phenotype transitions.**

**A.** Geneviewer captures showing ZEB1 binding peaks in *RAB38*, *TGFA*, *TSPAN13*, *BIRC3*, *ITGA2*, *EGFR*, *F2RL1*, *TNFAIP2* promoters in A375 cells and untreated or TNF $\alpha$ -treated GLO cells at day 14.

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**B.** ZEB1 ChIP-QPCR on the indicated promoters in GLO cells upon TNF $\alpha$  treatment at day 14. Anti-ZEB1 (Z1) or control IgG were used for the IP. Relative promoter enrichment was normalized to chromatin inputs.

**C.** RT-QPCR analyses showing relative expression of the genes in GLO cells upon TNF $\alpha$  treatment at 7, 14 and 21 days.

**Figure 8. Intra-tumoral analyses of melanoma states markers according to ZEB1 status in patient samples.**

7-color multi-immunofluorescence analyses of human melanoma samples with ZEB1 (red), ZEB2 (white), SOX10 (blue), SOX9 (yellow), NGFR (orange), MITF (green) and DAPI.

**A.** Representative pictures of a ZEB1<sup>low</sup> and a ZEB1<sup>high</sup> cutaneous primary melanoma.

**B.** Reconstruction of three whole tumors for each marker. Each dot represents one cell.

**Figure 9. ZEB1 antagonistic expression with SOX10 and co-expression with NGFR and SOX9 within melanoma lesions.**

**A.** Representative images showing antagonistic expression of ZEB1 and SOX10. **B.** Reconstruction of a whole tumor for ZEB1 (red) and SOX10 intensity (SOX10 high in blue, SOX10 low in yellow). The circle points a ZEB1<sup>high</sup> clone with lower SOX10 expression.

**C-D.** Representative images showing co-expression of ZEB1 with SOX9 and/or NGFR.

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**Supplementary Figures :**

**Supplementary Figure 1: RNA-seq analyses during TNF $\alpha$ -induced phenotype transition of GLO cells**

**A.** Volcano plot of differentially expressed genes in TNF $\alpha$ -treated GLO cells for 14 days, compared to untreated cells.

**B.** ssGSEA pathways analyses of collections Hallmark and C5 from Molecular Signatures Database (MSigDB) V6.2. and of melanoma signatures.

**Supplementary Figure 2: A second model of TNF $\alpha$ -induced phenotype switching in C-09.10 cells**

**A.** ssGSEA scores of melanoma states signatures in GLO and C-09.10 cells. **B.** RNA-seq analyses of C-09.10 cells upon 7 or 14 days of TNF $\alpha$  (100ng/ml) + TGF $\beta$  (20ng/mL) treatment. Volcano plot of differentially expressed genes in TNF $\alpha$ -treated cells for 14 days, compared to untreated cells. **C.** Heatmap of differentially expressed genes. The most significant Hallmarks enriched within down- and up-regulated in D14 versus untreated cells are indicated on the right. **D.** Heatmap of ssGSEA scores of the most relevant hallmarks and of melanoma states signatures from Hoek, Tsoi and Verfaillie

**Supplementary Figure 3: Intra-tumoral heterogeneity and functional assays of TNF $\alpha$ -induced phenotype switching in C-09.10 cells**

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Western blot (A) and RT-qPCR (B) analyses of ZEB1, ZEB2, MITF, NGFR, AXL, SOX10 and SOX9 after 7 and 14 days of TNF $\alpha$  + TGF $\beta$  treatment in C-09.10 cell line. GAPDH was used as loading control. Histograms represent quantitative analyzes (mean  $\pm$  SEM, n=3).

C. Longitudinal intra-tumor heterogeneity characterization of MITF and NGFR expression by Flow Cytometry in C-09.10 pMITF-GFP cell line, upon TNF $\alpha$  + TGF $\beta$  treatment after 7 and 14 days. NGFR was marked by antibody anti-NGFR coupled with APC.

D. BRAFi Survival assays. ATP assay upon BRAF inhibitor PLX4032 treatment for 7 days. (n=3).

E. Incucyte assay showing the relative increase in cell death upon PLX4032 (100nM) treatment overtime.

#### **Supplementary Figure 4: ChIP and ChIP-sequencing validation in A375 control and ZEB1 knock-out cells**

A. ZEB1 knock-out clones were established in the A375 cell line. Western-blot validation of ZEB1 loss of expression in one control (AS3) and one ZEB1 knocked-out (AZ1) clone. Actin was used as a loading control. B. ZEB1 ChIP-QPCR on the promoter of *MITF* in AS3 and AZ1 clone. Anti-ZEB1 (Z1) or control IgG were used for the IP. Relative promoter enrichment was normalized to chromatin inputs (%). N=3 C. ZEB1 ChIP-seq was performed in one control (AS3) and one ZEB1 knocked-out (AZ1) clone. Geneviewer capture of ZEB1 binding peak in the *MITF* promoter. D. ZEB1 ChIP-QPCR on ZEB2, MITF, NGFR, AXL, SOX10, SOX9 and PMEL promoters in A375 cells. Anti-ZEB1 (Z1) or control IgG were used for the IP. Relative promoter enrichment was normalized to chromatin inputs (%). N=3 except for SOX9.

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### **Supplementary Figure 5: Integration of ChIP-seq and RNA-seq data in GLO**

Heatmap of genes from the melanoma signatures from Verfaillie et al, in untreated or TNF $\alpha$ -treated GLO cells at day14. Presence of a ZEB1 peak is indicated by a green (untreated) or red (TNF D14) square. Differential expression of the gene upon TNF $\alpha$  (red:  $p < 0.001$  ; purple:  $p < 0,05$ ).

### **Supplementary Figure 6: RNA-seq analyses of C-09.10 cell line with ZEB1 overexpression (Z1).**

**A.**Volcano plot of differentially expressed genes in ZEB1 overexpressed C-09.10 cells, compared to control cells. **B.** The most significant Hallmarks enriched with ZEB1 overexpression.

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## Material and methods

### Cell culture and treatments

A375 human melanoma cell line was purchased from ATCC and cultured in DMEM complemented with 10% FBS (Cambrex) and 100 U/ml penicillin-streptomycin (Invitrogen). In order to authenticate the cell lines, the expected major genetic alterations were verified by NGS sequencing. The absence of Mycoplasma contamination was checked every 3 weeks with the MycoAlert detection kit (Lonza). Previously described patient-derived short term cultures (<10), GLO and C-09.10, established from *BRAF*<sup>V600</sup> metastatic melanomas (Richard et al., 2016), were grown in RPMI complemented with 10% FBS and 100 U/ml penicillin-streptomycin. TNF $\alpha$  (100ng/mL) and TGF $\beta$  (10ng/mL) were replaced in the culture medium every 3 days. PLX4032/vemurafenib was purchased from Selleck Chemicals (Houston, TX, USA) and reconstituted in DMSO.

### Viral infections

Generation of ZEB1-overexpressing C-09.10 cells using retroviral infection, and HA-Zeb1 in a pBabe-puro vector was previously described (Richard et al., 2016). For *Zeb1* knock-out in A375 cells, human embryonic kidney 293T cells ( $4 \times 10^6$ ) were transfected with lentiviral expression constructs (10  $\mu$ g) in combination with GAG-POL (5  $\mu$ g) and ENV expression vectors (10  $\mu$ g). The constructs allowed the insertion in a all in one manner of the Cas9 nuclease and the guide RNA scramble or targeting ZEB1 in a pLenti-Puro vector (pLenti-All-in-one-U6-sgRNA human Zeb1 (target1) or scramble -SFFV-Cas9 nuclease-2A-Puro) (Applied Biological Materials Inc., Richmond, Canada).

The sequence of the sgRNA targeting ZEB1 are the following: human 5'-CACCTGAAGAGGACCAG-3' (F = forward), 5'-TCCTCTTCAGGTGCCTC-3' (R = reverse). The MITF promoter-GFP construct was purchased from GeneCopoeia (with a Hygromycin selection). Viral supernatants were collected 48 hours post-transfection, filtered (0.45 µm membrane) and placed in contact with 2 x 10<sup>6</sup> melanoma cells for 8 h in the presence of 8 µg/mL polybrene. Forty-eight hours post-infection, cells were selected in the presence of puromycin (1 µg/mL) or Hygromycin (500µg/mL for GLO and 400µg/mL for C-09.10) (Invitrogen).

### **Immunoblot analyses**

Cells were washed twice with phosphate buffered saline (PBS) containing CaCl<sub>2</sub> and then lysed in a 100 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris pH 8.0 RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich). Loading was controlled using anti-GAPDH. Horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibodies (Glostrup) was used as secondary antibodies. Western blot detections were conducted using the Luminol reagent (Santa Cruz). Western Blot Digital Imaging was performed with ChemiDoc™ MP Imager (Bio-Rad).

	<b>Reference</b>	<b>Dilution</b>	<b>Species</b>
Anti-ZEB1	Sigma-Aldrich, HPA027524	1/500	Rabbit
Anti-ZEB2	Sigma-Aldrich, HPA003456	1/500	Rabbit
Anti-MITF	Millipore, MAB3747	1/500	Mouse
Anti-NGFR	Cell signaling, 8238S	1/1000	Rabbit
Anti-AXL	R&D Systems Bio-Techne, AF154	1/200	Goat
Anti-SOX10	Santa cruz, sc-365692	1/500	Mouse
Anti-SOX9	Genetex, GTX109661	1/500	Rabbit
Anti-GAPDH	Sigma-Aldrich, Cat# ABS16	1/20000	Rabbit

## RT-Q-PCR

Total RNA was isolated using RNeasy Kit (QIAGEN) and reverse transcribed using a high capacity reverse transcription kit following the manufacturer's instructions (Fisher Scientific). Real-time PCR intron-spanning assays were designed using the ProbeFinder software (Roche). All reactions, including no-template controls and RT controls were performed in triplicate on a CFX96 (Bio-Rad) and were analyzed with the Bio-Rad CFX manager software. Human *GAPDH* was used for normalization.

Gene	Sequence	
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
ZEB1	AACTGCTGGGAGGATGACAC	TCCTGCTTCATCTGCCTGA
ZEB2	AAGCCAGGGACAGATCAGC	GCCCACTCTGTGCATTTGA
MITF-M	CATTGTTATGCTGGAAATGCTAGA	TGCTAAAGTGGTAGAAAGGTACTGC
NGFR	ACAAGACCTCATAGCCAGCAC	TGCAGCTGTTCCACCTCTTGA
AXL	GGTGGCTGTGAAGACGATGA	CTCAGATACTCCATGCCACT
SOX10	GGC-TCC-CCC-ATG-TCA-GAT	CTG-TCT-TCG-GGG-TGG-TTG
SOX9	GTACCCGCACTTGACACAAC	TCTCGCTCTCGTTCAGAAGTC
RAB38	AGGCATCTGGAGGCTTCATC	GCACGGTTTCGTAGGATGGT
TGFA	ACATGTGTGCTGATACTGCT	CAGCAAGCGGTTCTCCCTT
TSPAN13	GTTCCAAGATGGTTTGC GGG	AGCAGCAGACTAACCAAGGTG
BIRC3	TCTGGGCAGCAGGTTTACAA	CCCGAGATTAGACTAAGTCCCTT
ITGA2	AGACGTGCTCTTGGTAGGTG	GCTGACCCAAAATGCCCTCT
EGFR	AGCAGTCACTGGGGGACTT	GGAGAGGAGAACTGCCAGAA
F2RL1	CGGCGGCGGATTCCC	AGAGGATCTACTGGTTCCTTGG
TNFAIP2	TACCCTGACTTCAGCAAAGGC	CTGACGTCCAAGATGCTCCG

## RNA-seq analyses

RNA extractions were sequenced on the CLB genomic platform, on an Illumina NovaSeq machine with a paired-end protocol (2x75bp, 32Mp reads). Libraries were prepared with the TrueSeq poly-A+ kit from Illumina. Raw sequencing reads were aligned on the human genome (GRCh38) with STAR

(v2.7.8a), with the annotation of known genes from gencode v37. Gene expression was quantified using Salmon (1.4.0) and the annotation of protein coding genes from gencode v37. RNA-seq quality controls and analyses were performed on the Gilles Thomas Bioinformatics platform.

### **Chromatin immunoprecipitation and ChIP-sequencing**

The ChIP assay was carried out according to the protocol from the iDeal ChIP-Seq Kit for Transcription Factors (Diagenode, Denville, NJ, USA). Briefly, cells from one 15-cm dish were cross-linked with 1% formaldehyde at RT for 10 min and quenched in 125 mM glycine for 5 min. The cross-linked chromatin was isolated and sonicated to generate DNA fragments averaging 200–500 bp in length by Bioruptor plus sonication device (Diagenode). Chromatin fragments were immunoprecipitated with antibodies directed against Zeb1 (1µg, Genetex, GTX105278), or IgG (1µg, Bio-Rad, PRABP01) as negative control. Immunoprecipitated DNA was purified and dissolved into 25 µl of H<sub>2</sub>O. To build the Illumina library, 5ng of input and 30pg of IP were used. Sequencing was performed on the CLB genomic platform, on an Illumina NextSeq machine with a paired-end protocol (2x75bp, 64Mp reads).

To assess the efficiency of the ChIP before sequencing, MITF (positive control) and IL1B (negative control) promoters were analyzed by qPCR. Primers were specified to amplify genomic DNA using the sequence from the peak (ChIPSeq data from MDAMB231, A375 or GLO cells). Relative promoter enrichment was normalized to chromatin inputs.

Peak sequence from	Gene	Sequence			
MDA-MB-231	ZEB2	F	GAAAACAGCCTTCTCGGTGG	R	AGACCTACAAATTCGGGGGC

	MITF	GTCAAGATCCCAGCGGGTTG	GAGGTGACTCCAAGCGAACT
	NGFR	AGCGTACCATACTTTCCCG	TGCATGGAGGGACCAGGTTA
	PMEL	TTGGGAAGAGTGTTCCAGCCG	CCCCAGGCAAGTTTGGTTCT
	SOX9	GCAAAAAGCTGTCCCTTGTGG	CAAGTCACCCCTCTGGCATT
A375	ZEB2	AATAGAGCAGGACCTCTCCCC	AGGTGTCCTTTGCTATCCAGC
	MITF	GGATTTGGAAGTCGGGGAGG	CTGCTCTTCAGCGGTTGACT
	NGFR	TACGTCTCGGGGTGCAGA	TGCGTAGCCAGAGCTGC
	AXL	CCGGGACTGTACTGTTTCTCC	CCGTTCCAGACAAGGTCAG
	SOX10	AGACTCTAGGTGGGTGCGTC	CCCACACCAAGAGACGGTTG
GLO	RAB38	CCGGTAGTGCGAAGAGAAGTT	AGTTGCTGGTGATTGGCGA
	TGFA	TCGGGGCATATCGAACGAAC	GCCAATTCTTGCCCCCTGTG
	TSPAN13	TCCGCCGGAGTCGAATTTAC	ACTGGGGATACTCACGGTGT
	BIRC3	GGGGATTTGGGTGACGCATT	ACAAGCCCAGTCTTTTCAAGC
	ITGA2	CCCTTTCTCCACCCACTTAGG	GGGCACTAGCCCTAAACCAC
	EGFR	GACAGGCCACCTCGTCG	CCGGCTCTCCCGATCAATAC
	F2RL1	GTACGCTGCTCCTTCGGTT	CTCTCACCTGCCCGAATC
	TNFAIP2	ACTATTTAGGCCTTGCGGA	TGGAAGCGGAGGTCACCTAC

### ChIP-Seq analyses

Two biological replicates from two independent experiments were performed for each ChIP-seq. Peaks calling and integration of ChIP-seq and gene expression data is detailed in supplementary information.

### Flow cytometry

To analyze the expression of the NGFR/CD271 cell surface marker, cells  $1.10^6$  cells per condition were incubated with an AlexaFluor647-conjugated anti-CD271 antibody (BD Pharmingen) for 1 h in the dark before being counted on a BD LSRFortessa™ Flow Cytometer (BD Biosciences-IN). Data were analyzed using the FlowJo\_V10 software.

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## **Migration assays**

### **Scratch Wound Assay / Collective migration test:**

70,000 GLO cells were seeded into 96-well transparent tissue culture plates and cultured to 90% confluence in RPMI supplemented with 20% FBS, with or without TNF $\alpha$  treatment. After 24h, using a 96-well wound marker (Essen Bioscience), a straight, uniform scratch was made through adherent cells. Each well was then rinsed with cell medium in order to remove all scratched cells and the plate was replaced into incubator for 24h. Photos were taken and analyzed by ZEISSZEN Microscope software. The distance was measured and shown in  $\mu\text{m}$ . Each condition was conducted at least three times.

### **Transwell migration assay / Individual migration test:**

Falcon® Cell Culture Inserts for 24-well plates were placed into a 24-well plate, and 20% FBS RPMI was added into the well and 0.5% FBS RPMI was added into inserts. The plate was then incubated for one hour for an initial equilibrium period. 200,000 GLO cells were trypsinated and rinsed with 0.5% FBS RPMI medium. 100 $\mu\text{l}$  0.5% FBS RPMI containing 200,000 GLO cells was seeded into the insert, then 600 $\mu\text{l}$  20% FBS RPMI was added into well. After 24h incubation, cells inside the insert were removed carefully and the migrated cells on the membrane were fixed and colored using 4% PFA and Brilliant Blue, then rinsed with PBS solution. When inserts were completely dry, cells were viewed using phase contrast microscopy. Photos were taken and analyzed by ZEISSZEN Microscope software. Migrated cells were counted in 3 different fields.

## **BRAF inhibitor survival assays**

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### **ATP assay:**

For short term viability assays, the CellTiter-Glo Luminescent Cell Viability Assay (ATP assay) (Promega) was used, based on quantitation of the ATP present, which signals the presence of metabolically active cells. 1000 cells in 96-well plates were treated with the indicated drugs for 72 h in a final volume of 100  $\mu$ L. Three by 3-fold PLX4032 dilutions resulted in concentrations ranging from 1 nM to 10  $\mu$ M. After 72 h, the CellTiter-Glo reaction solution (Promega) was added and luminescence was measured (Tekan). Control wells with DMSO were used for normalization.

### **Incucyte® Live Cell mortality measurement:**

30,000 cells were seeded into a 24-well plate and treated with TNF $\alpha$ +/-TGF $\beta$ . 24h after, cell medium was renewed with corresponded treatments, as well as PLX4032 and Propidium iodide (Sigma-Aldrich®, 1/3000). EssenBioScience IncuCyte Zoom Live-Cell Analysis System was used to measure and analyze cell real-time mortality every 2h. Died cells were marked with Propidium iodide. Data was then converted into Excel in order to draw graphs.

### **Human tumor samples**

Melanoma tumor samples were obtained through the Biological Resource Center of the Lyon Sud Hospital (Hospices Civils de Lyon) and were used with the patient's written informed consent. This study was approved by a regional review board (Comité de Protection des Personnes Ile de France XI, Saint-Germain-en-Laye, France, number 12027) and is registered in ClinicalTrial.gov (MelBase, NCT02828202). n = 18 cutaneous melanoma patients (from a cohort previously described in Plaschka

et al, *in revision*) were used for multi-immunofluorescence analyses. All melanoma biopsies were cutaneous, either primary melanoma (n = 7) or cutaneous metastases (n = 11).

### 7-color immunofluorescence multiplex analyses

3- $\mu$ m tissue sections were cut from formalin-fixed paraffin-embedded human melanoma specimens.

The sections underwent immunofluorescence staining using the OPAL™ technology (Akoya Biosciences) on a Leica Bond RX. A 7-color panel was designed. DAPI was used for nuclei detection.

Antibody	Reference	Dilution	Staining localization
<b>ZEB1</b>	HPA02752 Sigma	1/100	Nuclear
<b>SOX10</b>	sc-365692 Santa Cruz	1/1000	Nuclear
<b>SOX9</b>	HPA001758 Sigma	1/300	Nuclear
<b>NGFR</b>	CS 8238 Cell-signaling	1/500	Membrane
<b>ZEB2</b>	HPA003456 Sigma	1/300	Nuclear
<b>MITF</b>	284M-96 Sigma	1/200	Nuclear cytoplasmic

Sections were digitalized with a Vectra Polaris scanner (Perkin Elmer, USA). Using the Inform software (Perkin Elmer), an autofluorescence treatment of images was carried out and tissue segmentation was performed to identify epidermis, stroma and tumor. Cells segmentation was then applied to analyze expression of each marker in each cell. The matrix of phenotype containing the X- and Y- positions of each cell as well as the mean nuclear-, cytoplasmic- and membrane- intensities of

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each fluorescence staining was then further analyzed using the R software. Tumors were spatially reconstructed using the R plot() function.

For quantitative analyses, melanoma cells were classified following their expression level of each marker. The following cut-off values were defined.

Opal	Marker	Low	Intermediate	High
480	SOX10	2		5,6
520	ZEB1	3,5	7,2	11
570	MITF	0,435	0,725	1,45
620	NGFR	2		
690	SOX9	1,18	2,54	4,4
780	ZEB2	0,3	0,63	1,1

### Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, USA) or R software (version 3.6.1). All experiments were performed at least in triplicate. Data are presented as mean  $\pm$ s.d. or  $\pm$  s.e.m as specified in the figure legends. To assess significant correlation between signatures, a Pearson's correlation coefficient was performed. To determine significant differences between two groups, parametric data were analyzed using a t- or Mann Whitney- test. All statistical tests were two-tailed. The p-values obtained were considered significant  $<0.05$ .

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## **Author contributions**

YT performed and analyzed experiments with the help of SDu and MG. RP performed CHIP-Seq and RNA-Seq bioinformatics analyses. MG, VB and MP contributed to multi-IF analyses. SDa provided human samples and clinical data from the human sample experiments. JC conceived and supervised the whole project and wrote the manuscript.

**Competing interests.** The authors declare that no conflict of interest exists.

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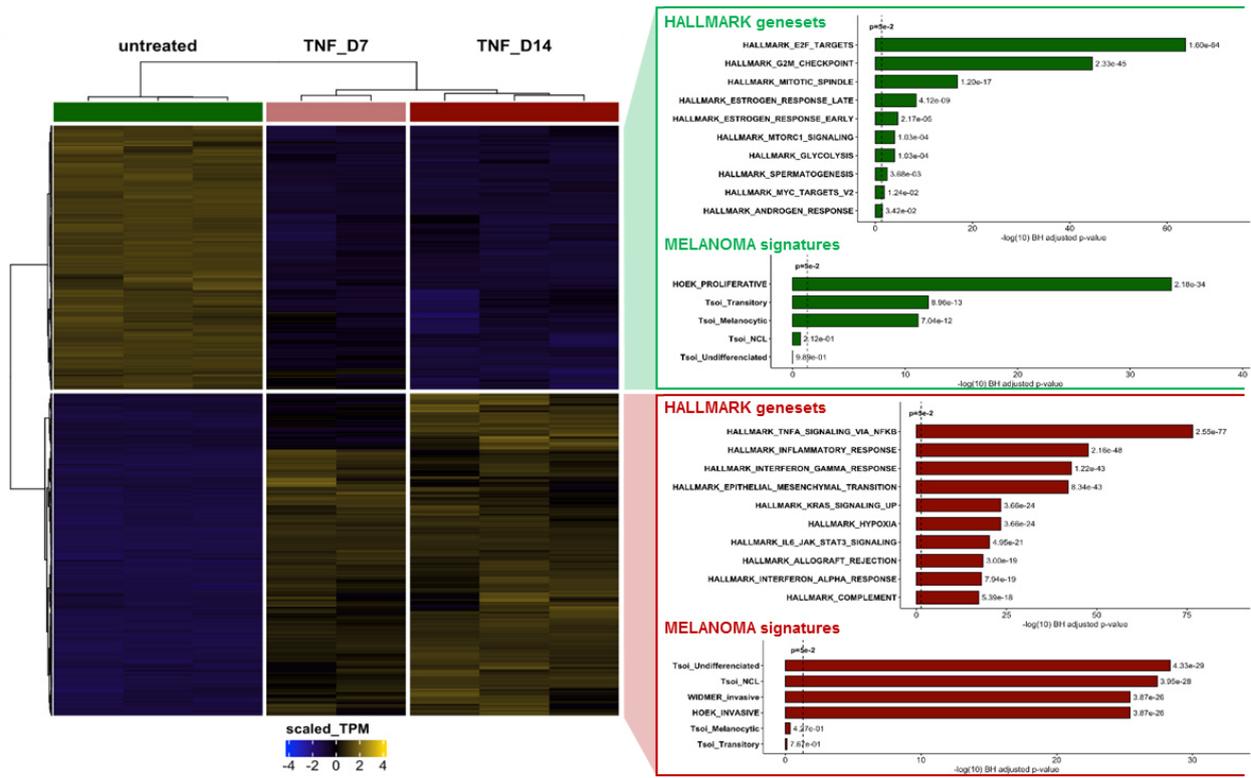
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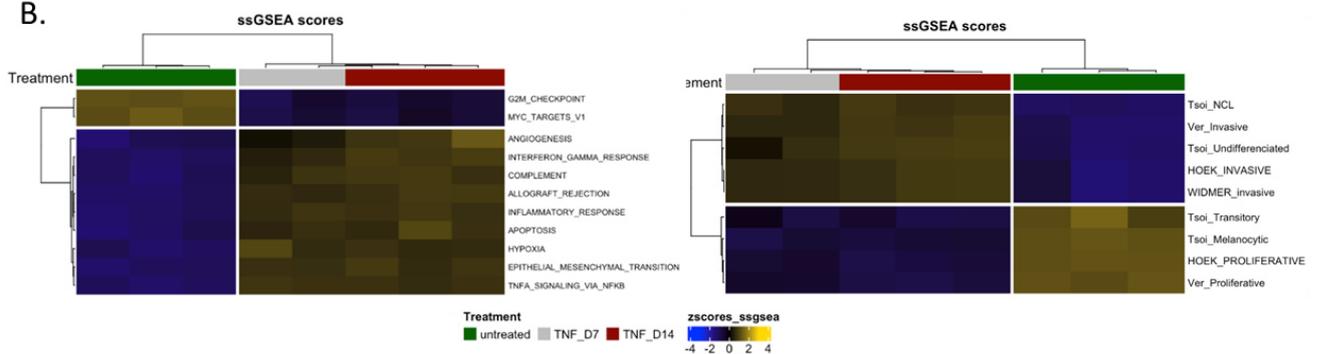
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Figure 1

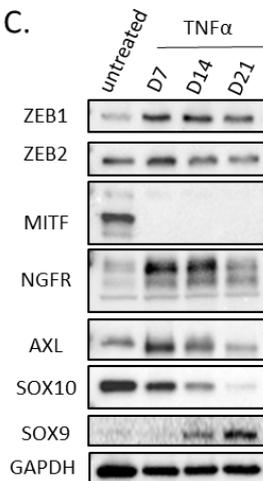
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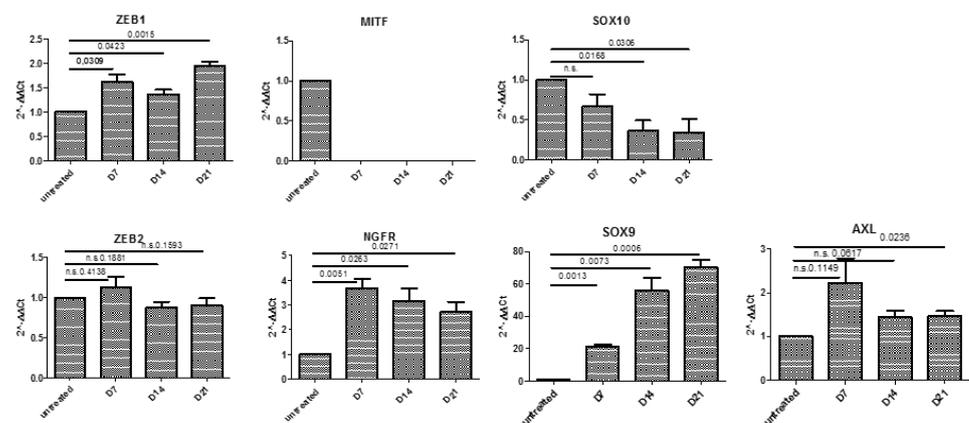


Figure 2

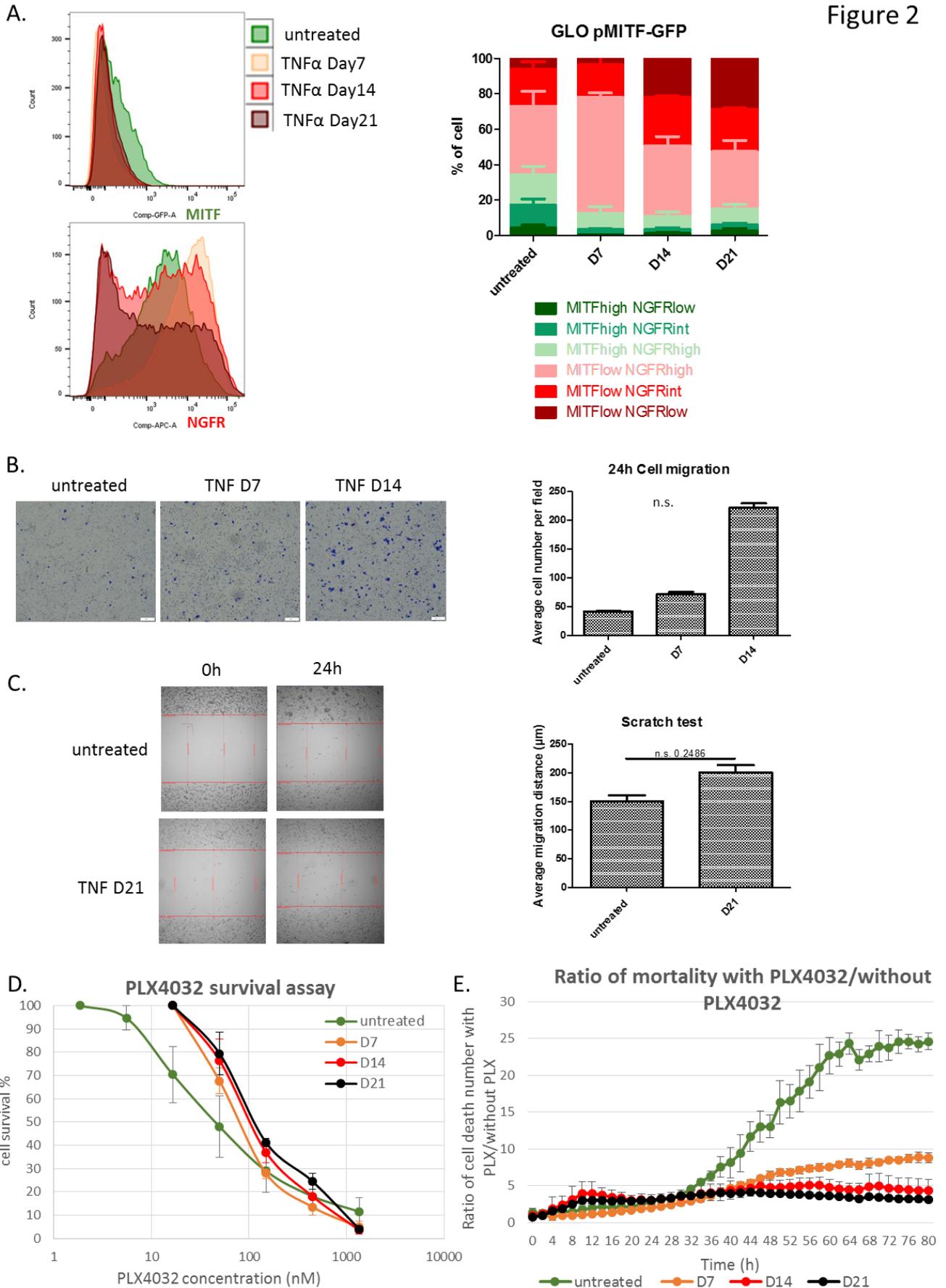


Figure 3

A.

	Peak number	Mean size bp
untreated	5195	542.76
D14	11779	580.26

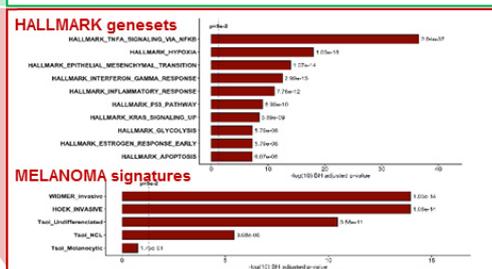
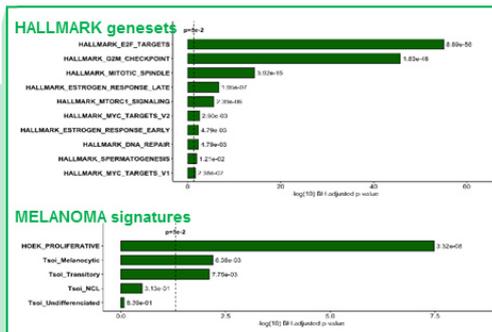
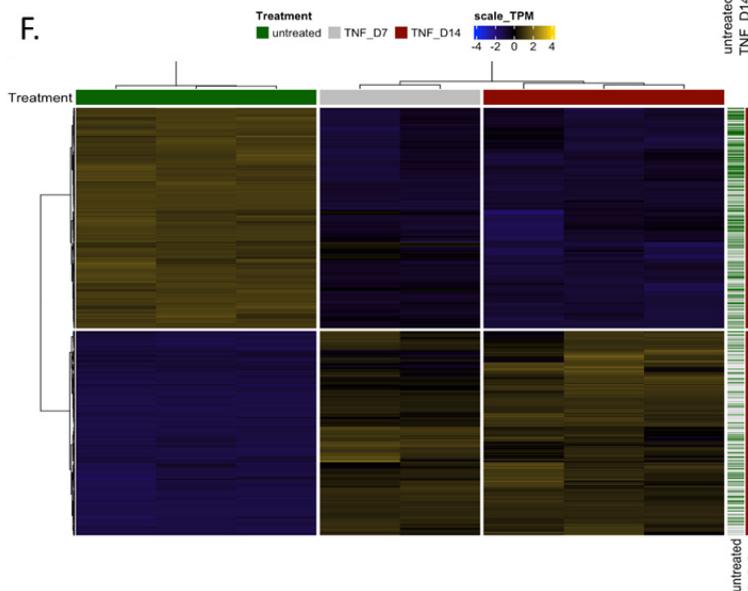
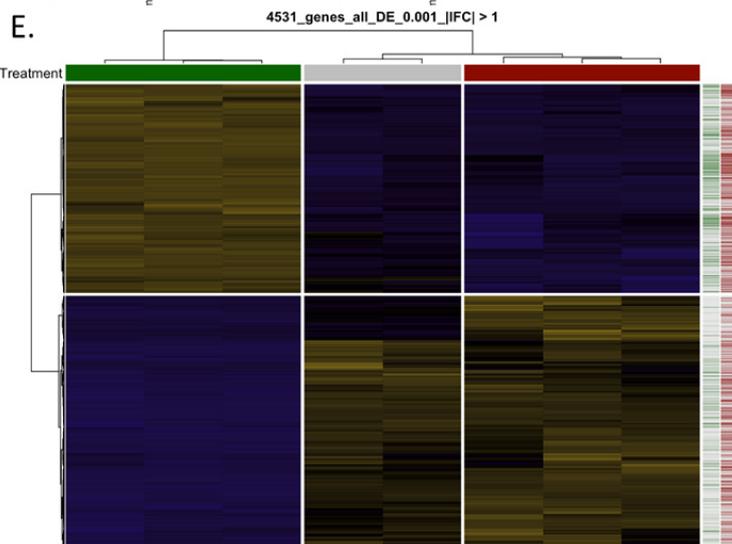
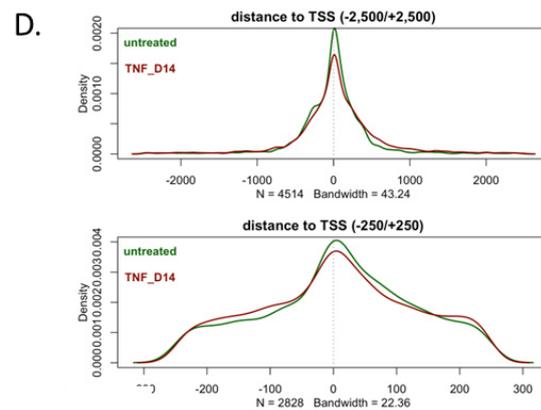
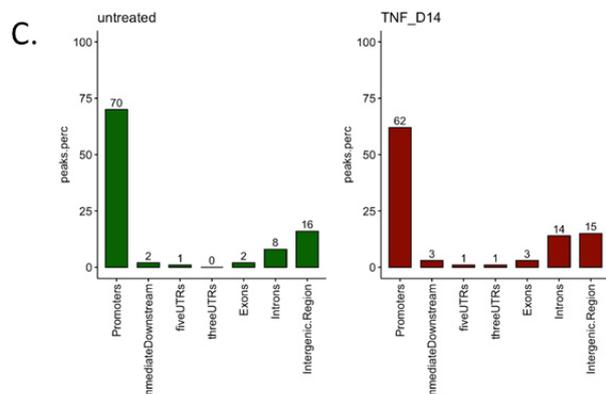
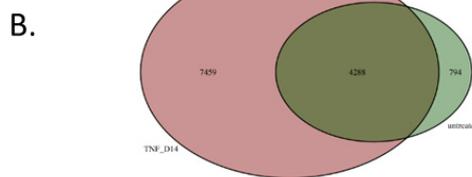


Figure 4

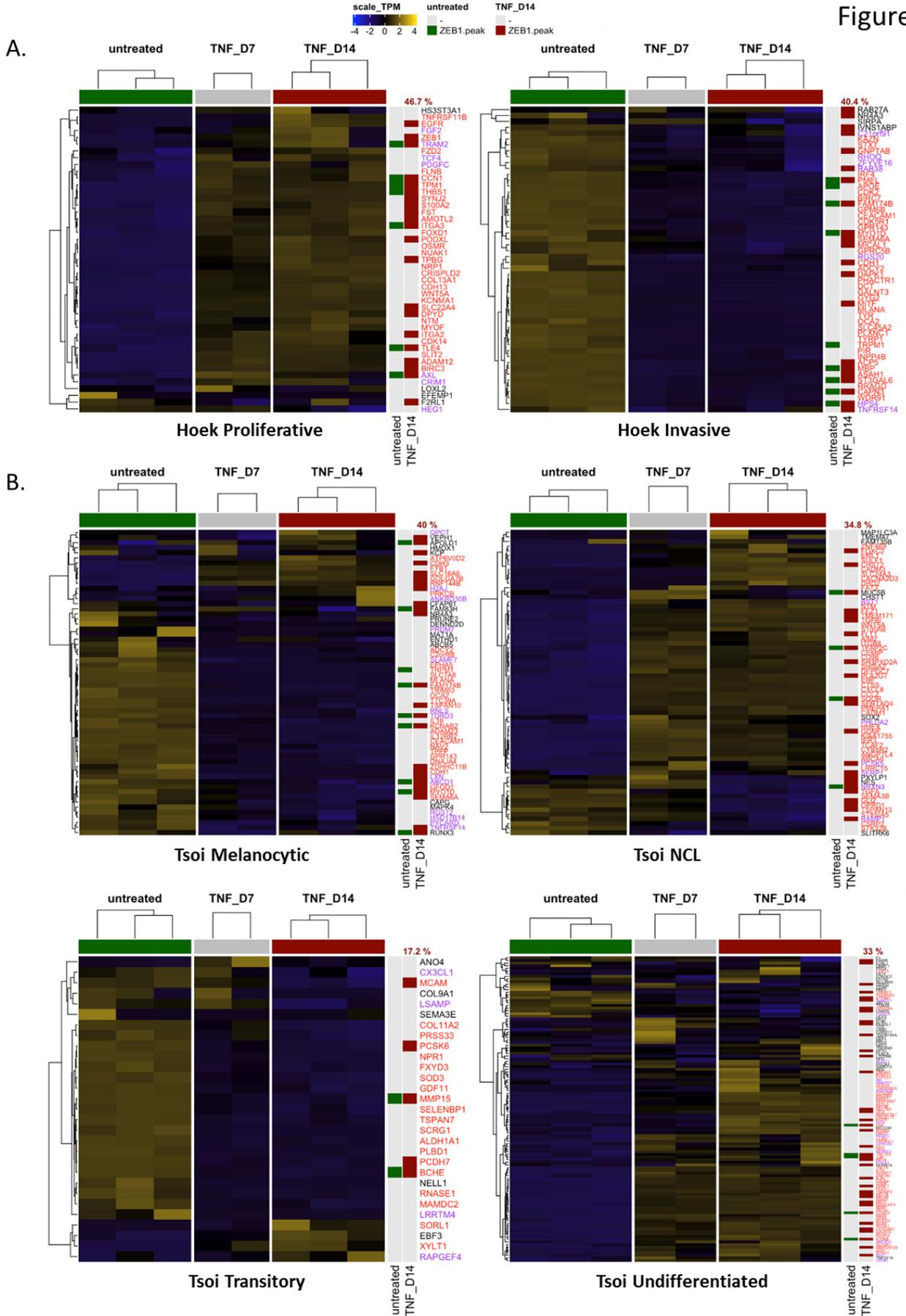
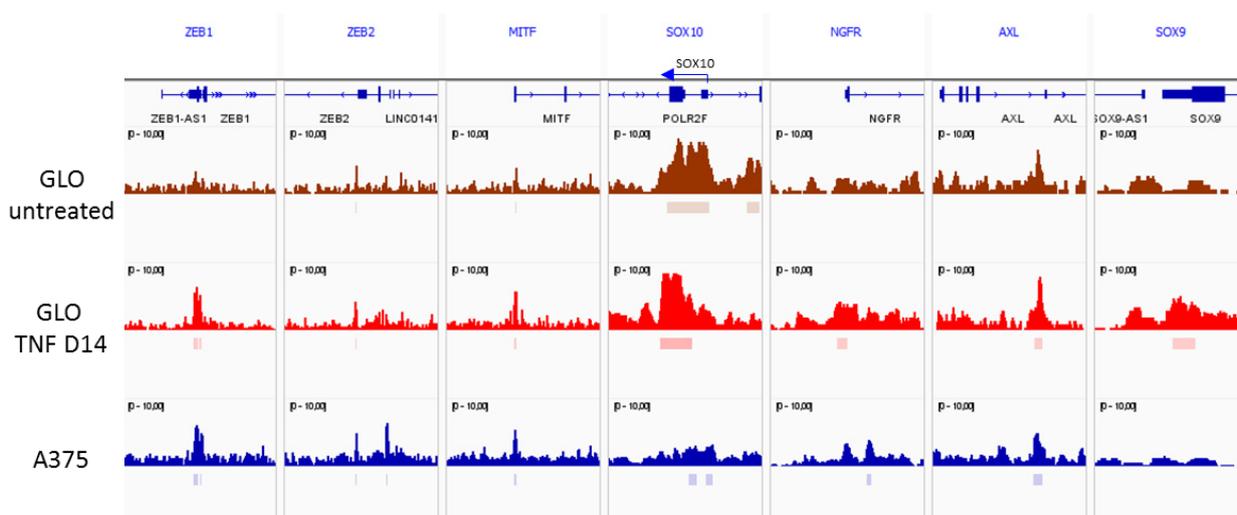
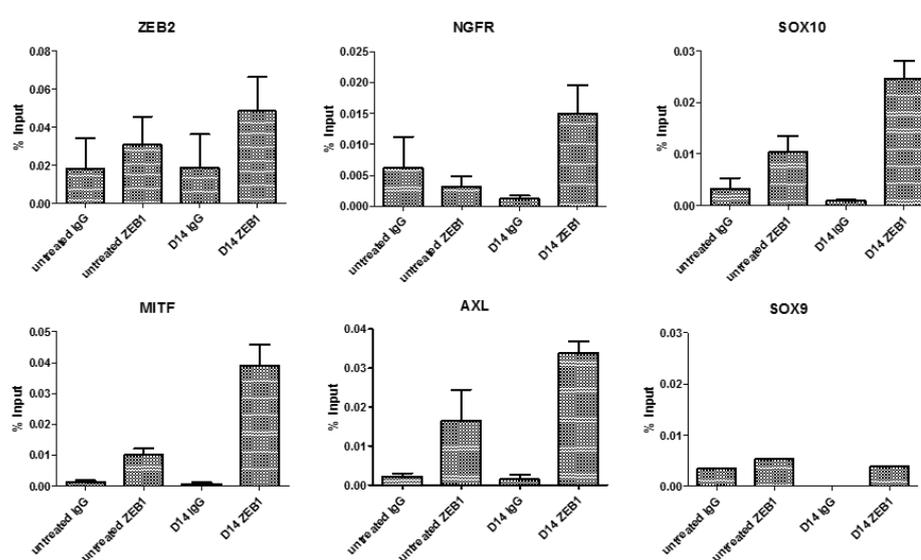


Figure 5

A.



B.



C.

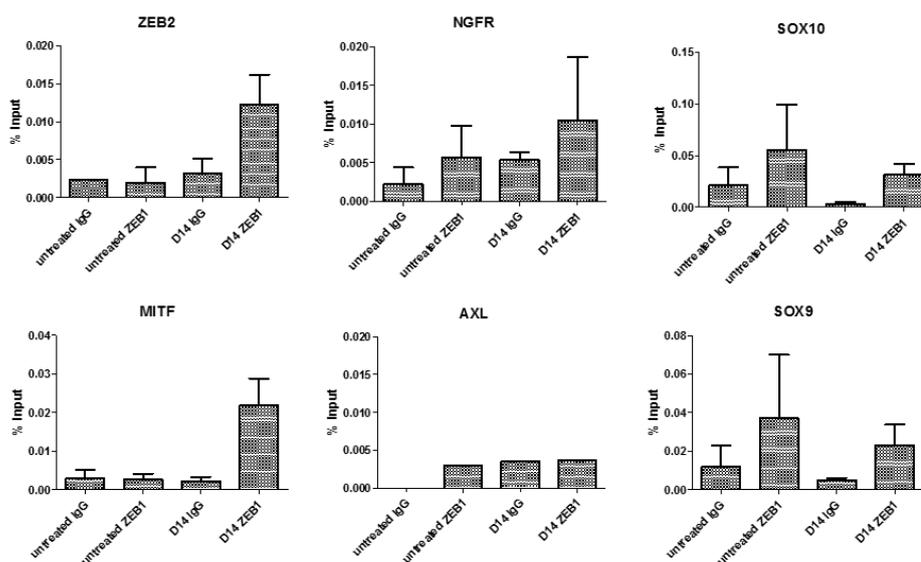


Figure 6

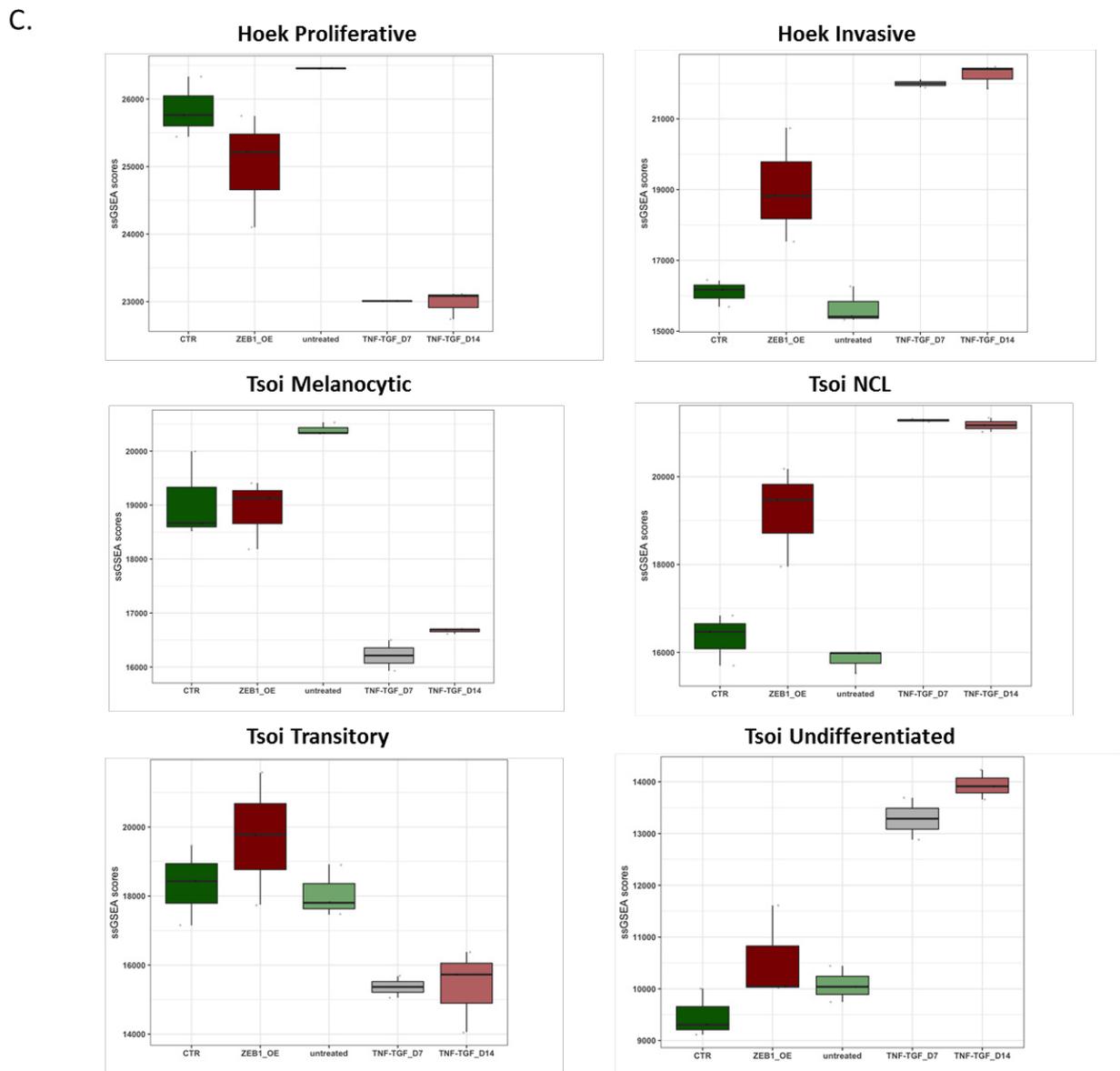
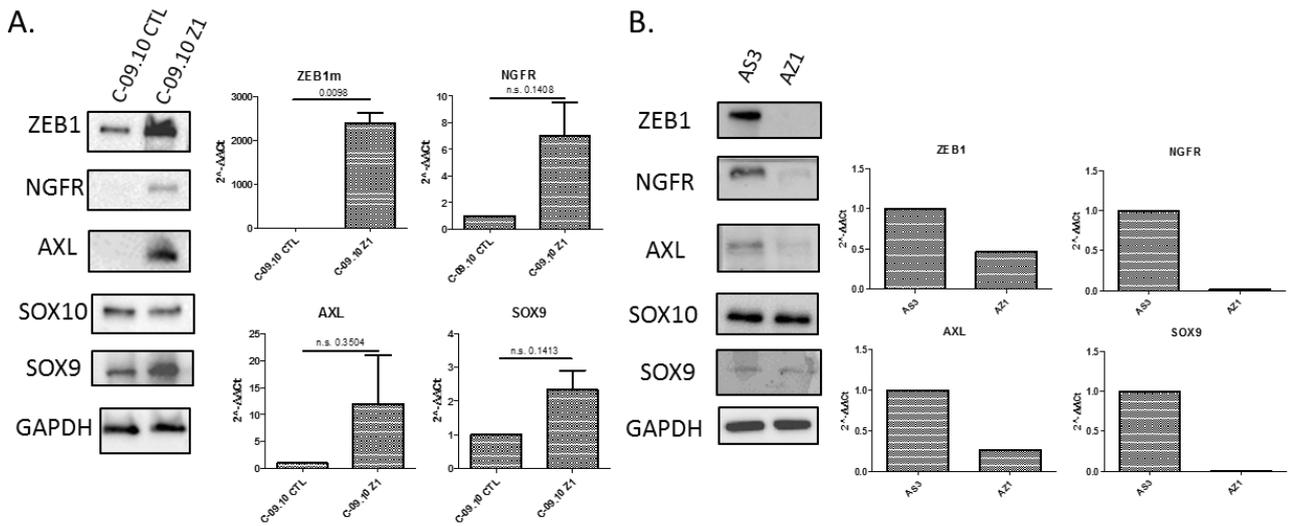
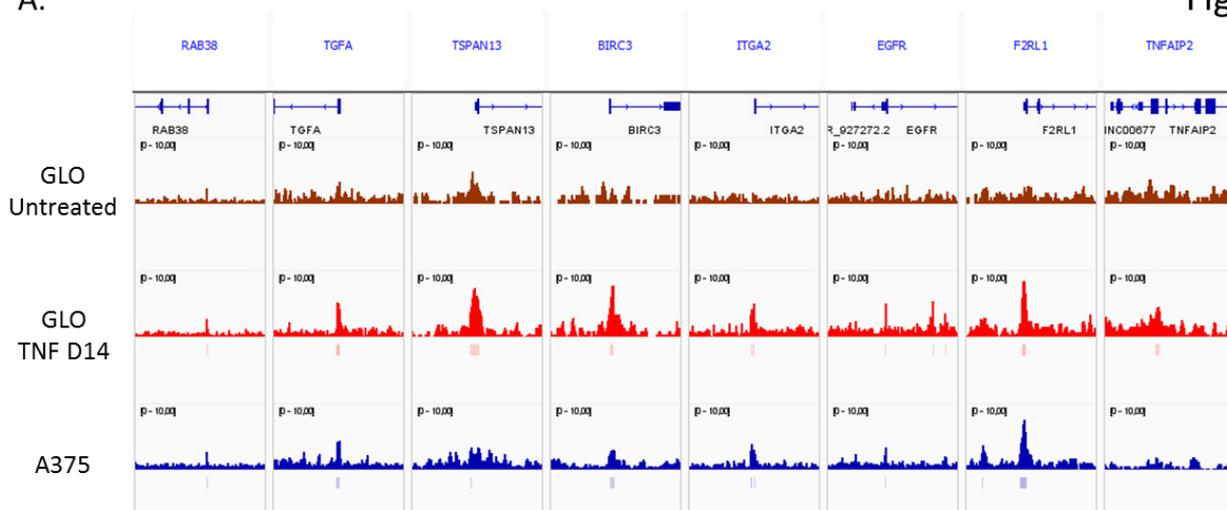
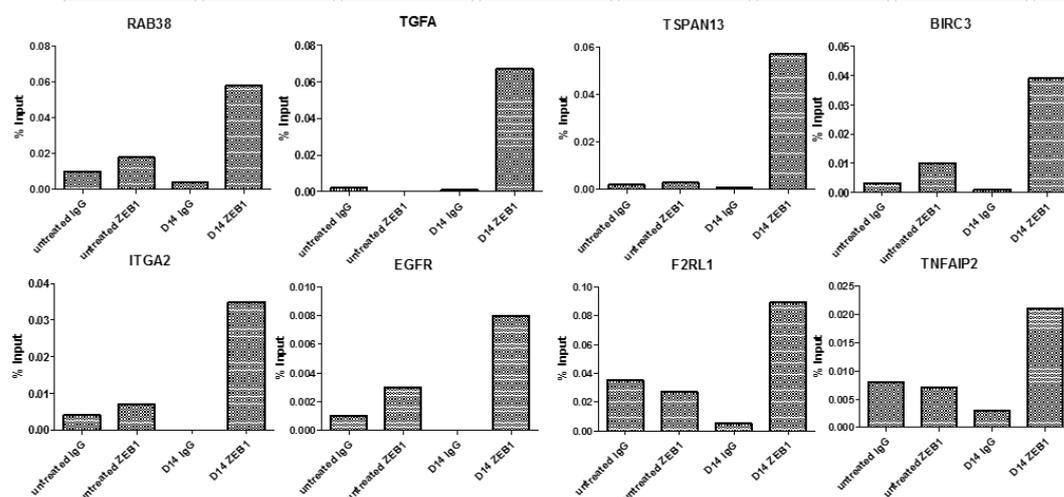


Figure 7

A.



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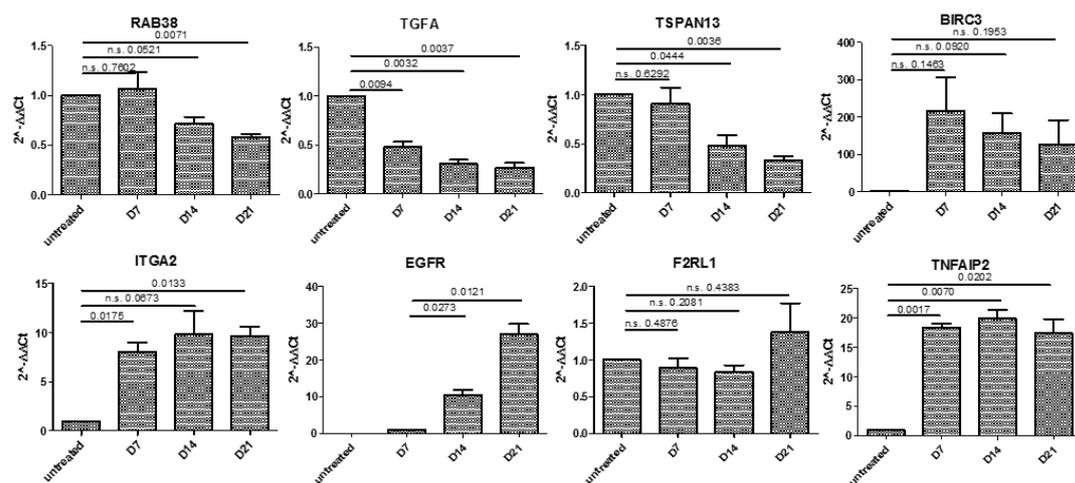
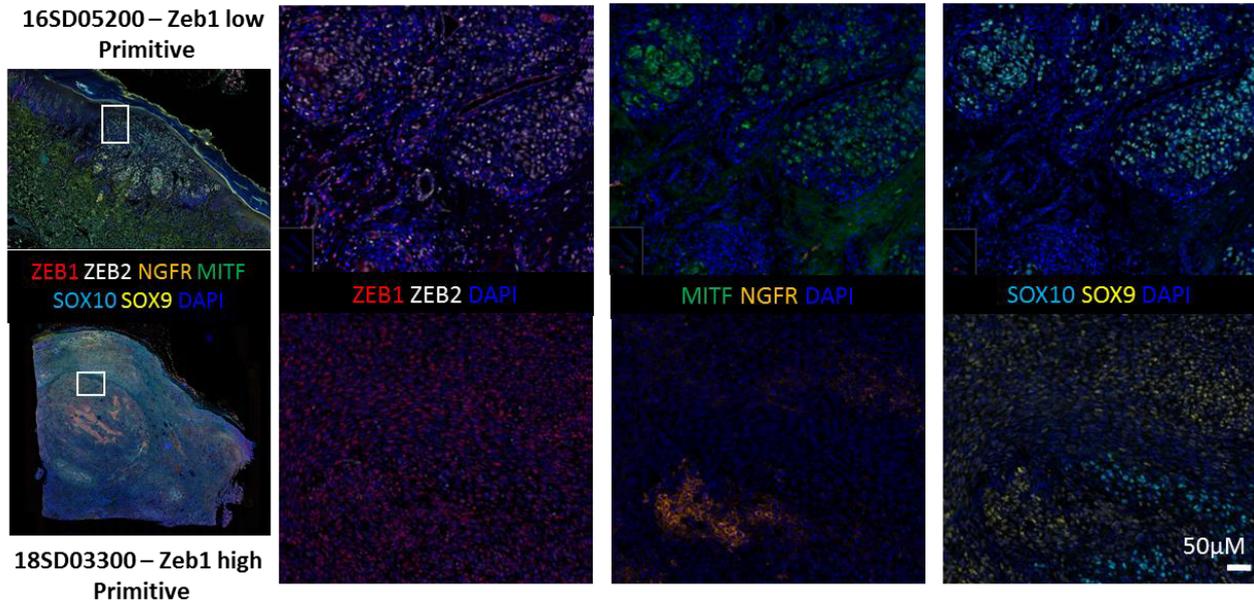
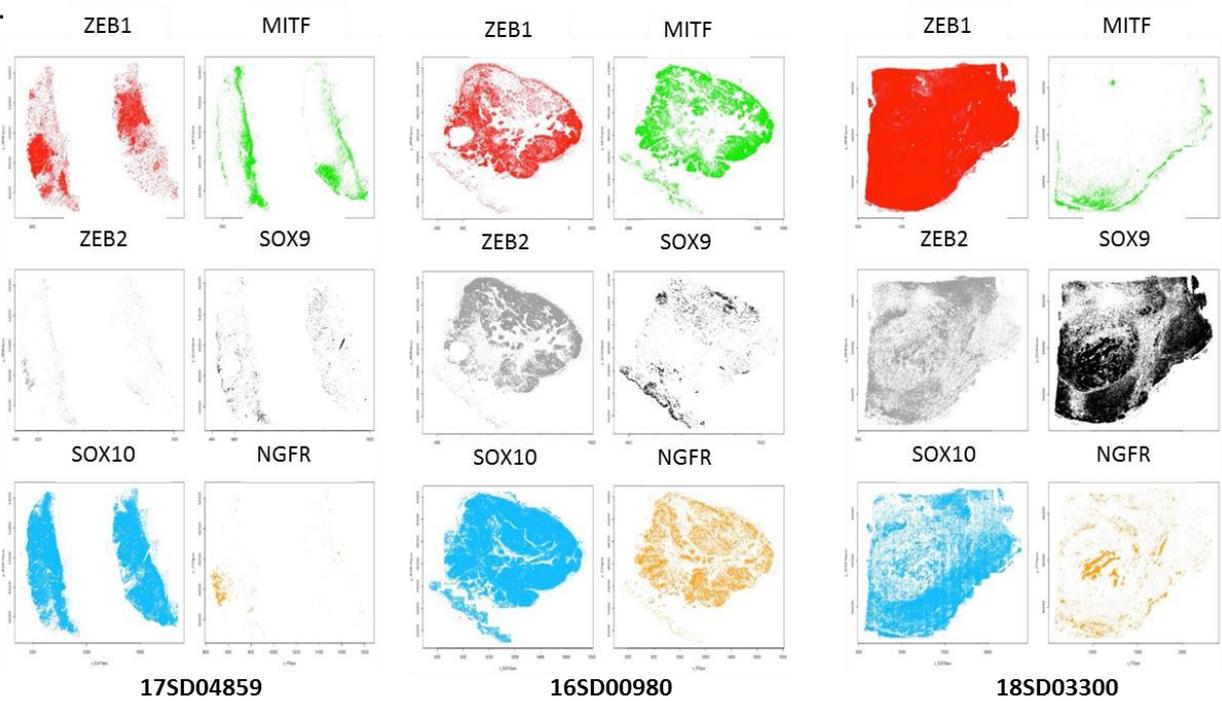


Figure 8

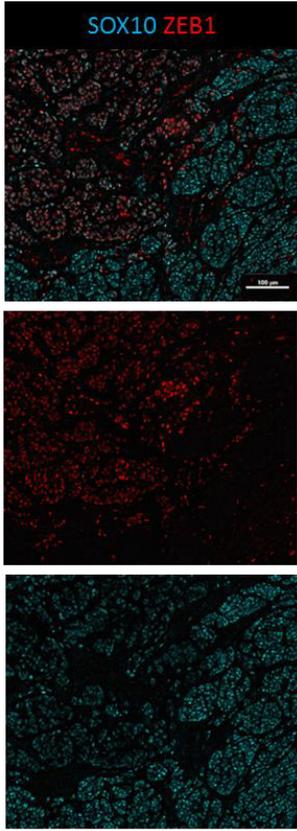
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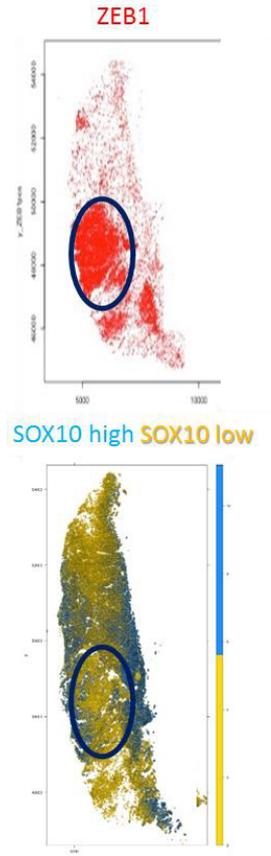
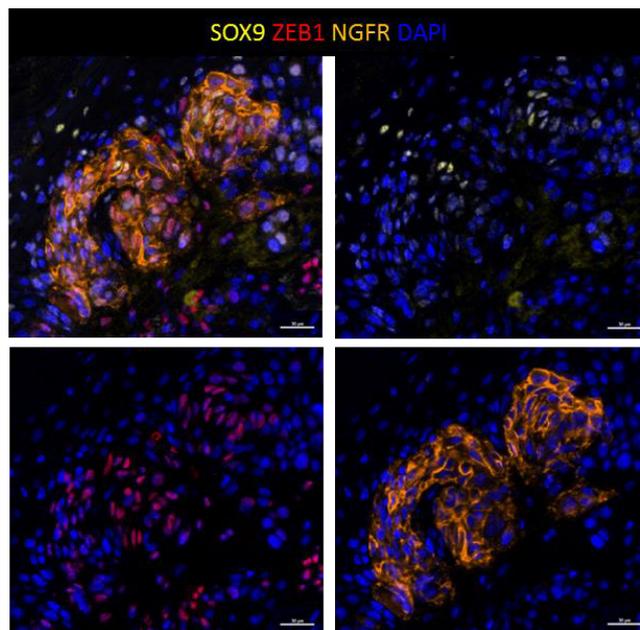
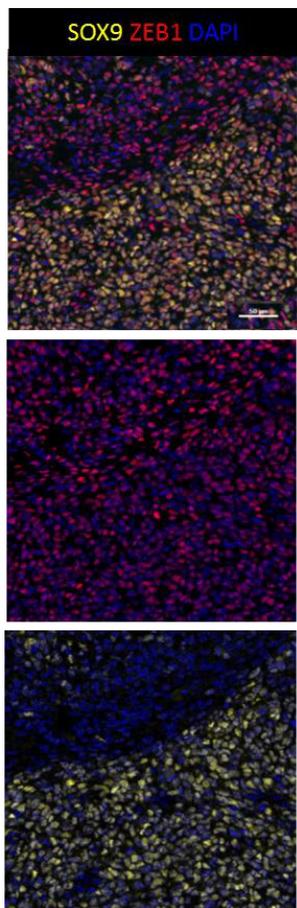


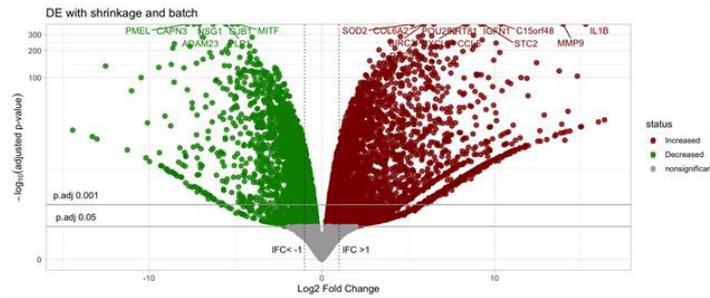
Figure 9

C.

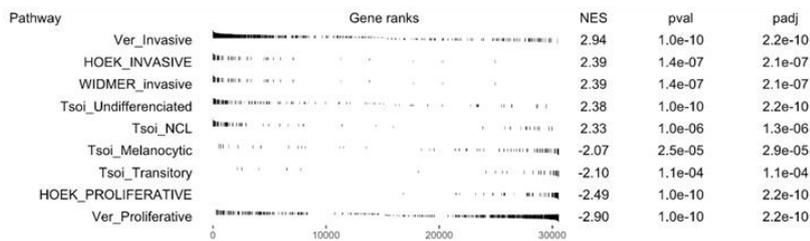
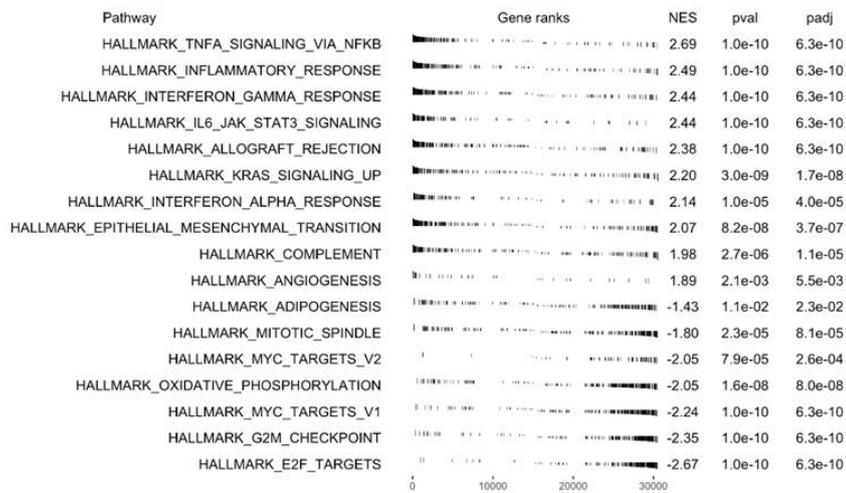


# Supplementary Figure 1

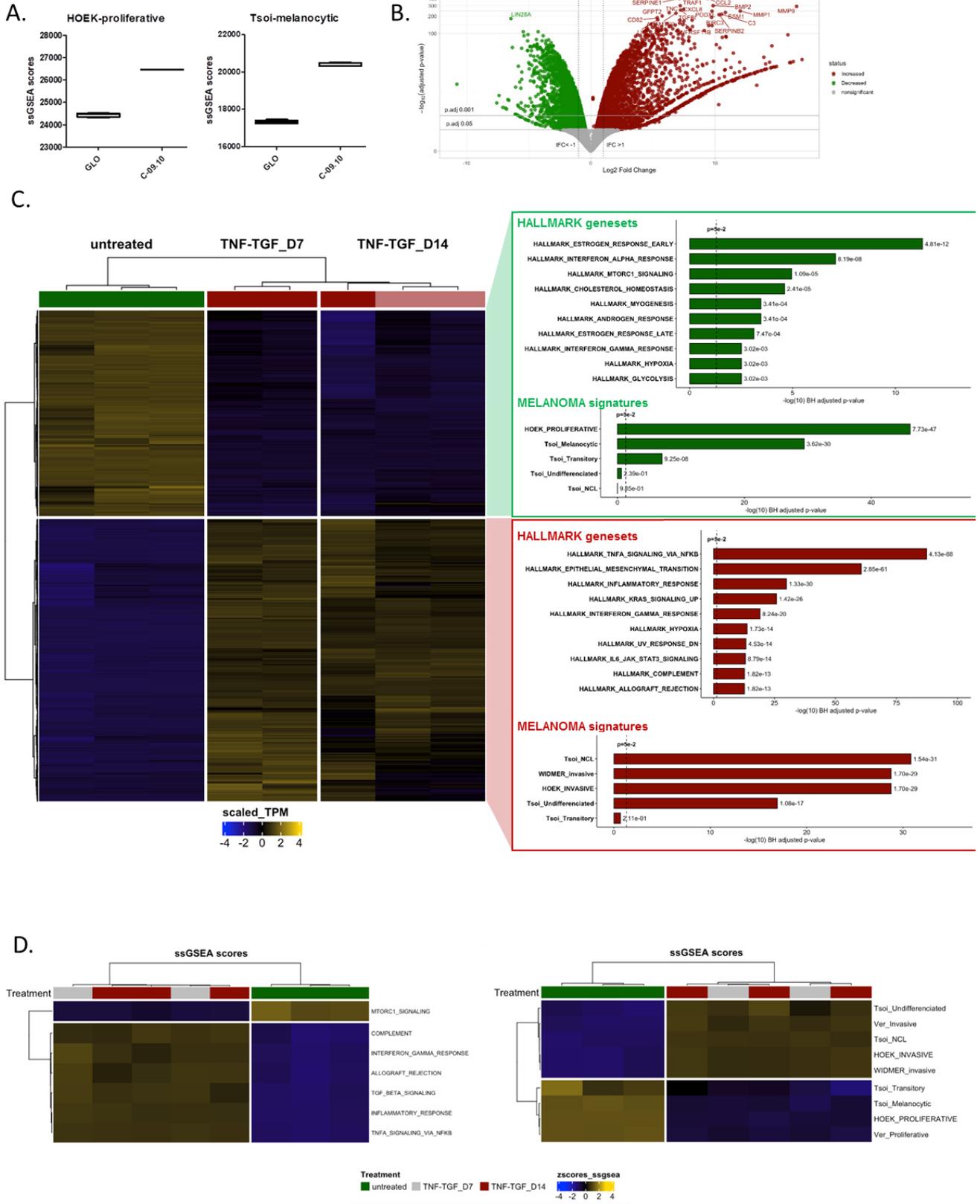
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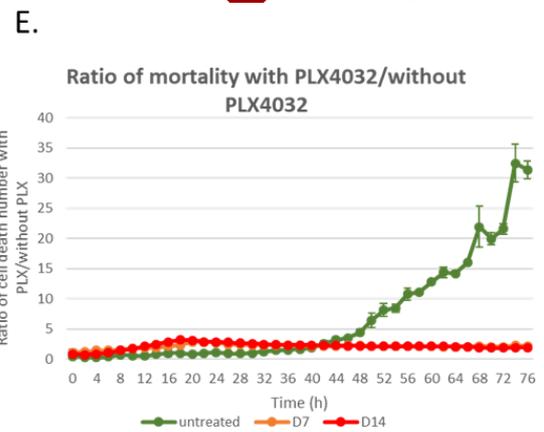
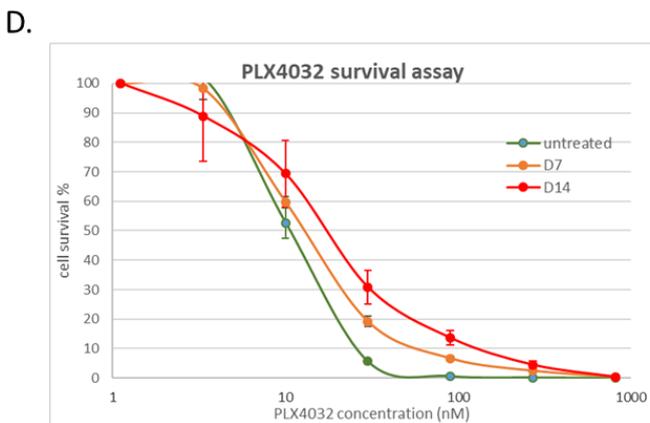
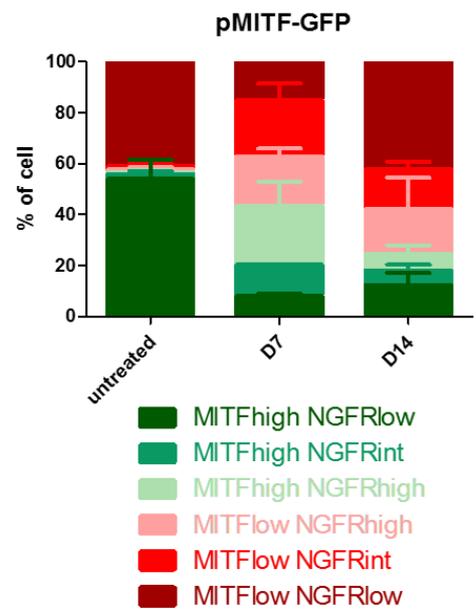
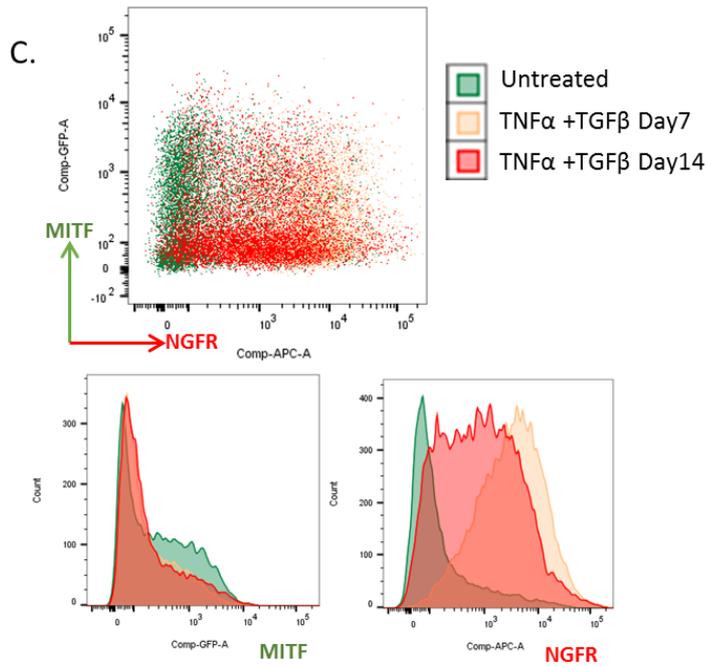
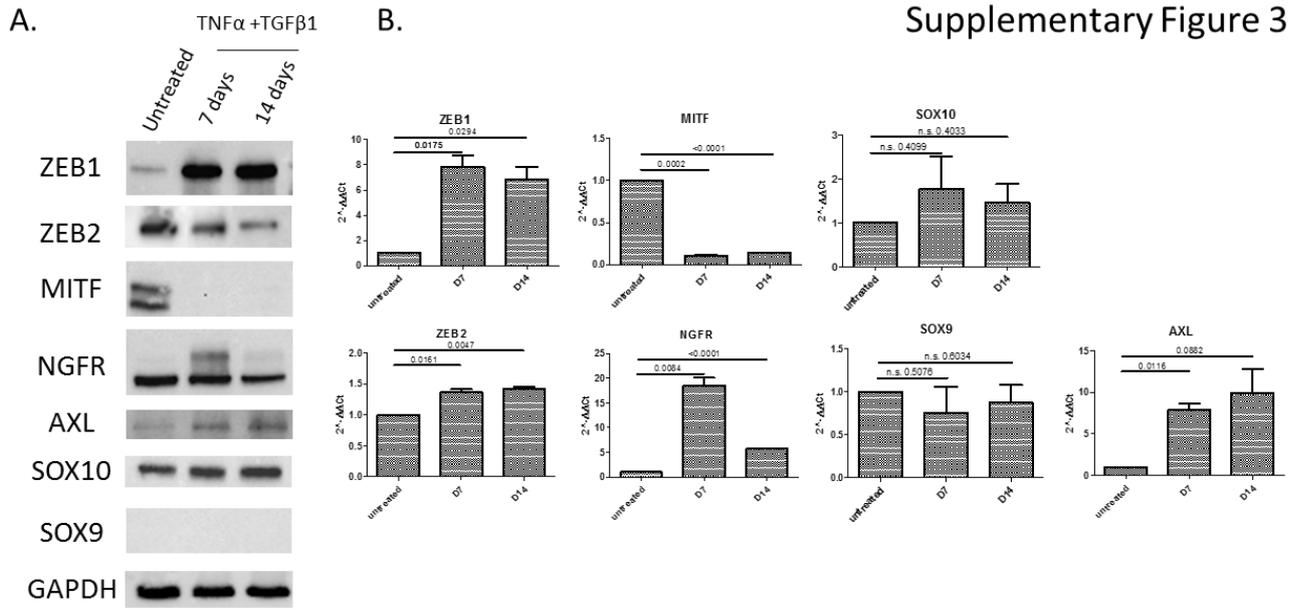
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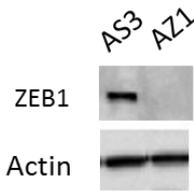
## Supplementary Figure 2



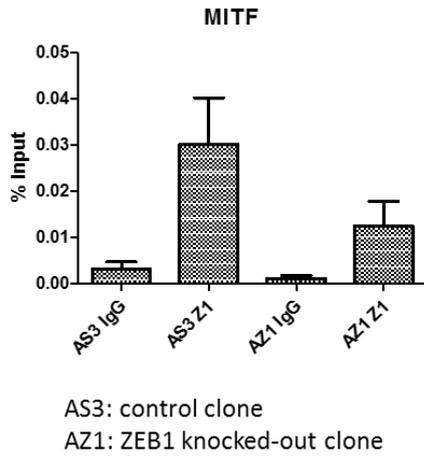
Supplementary Figure 3



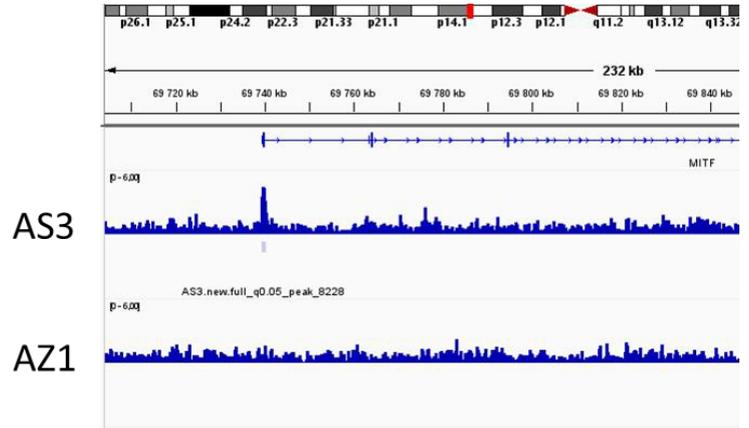
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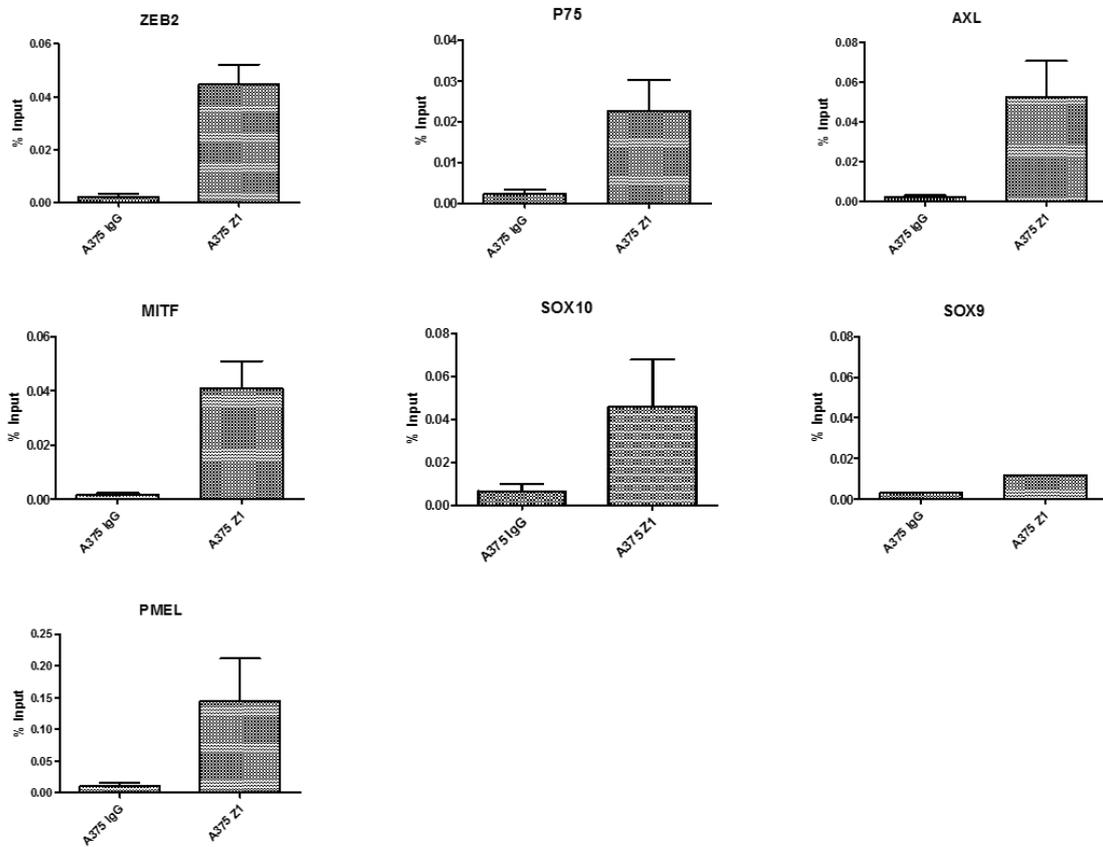
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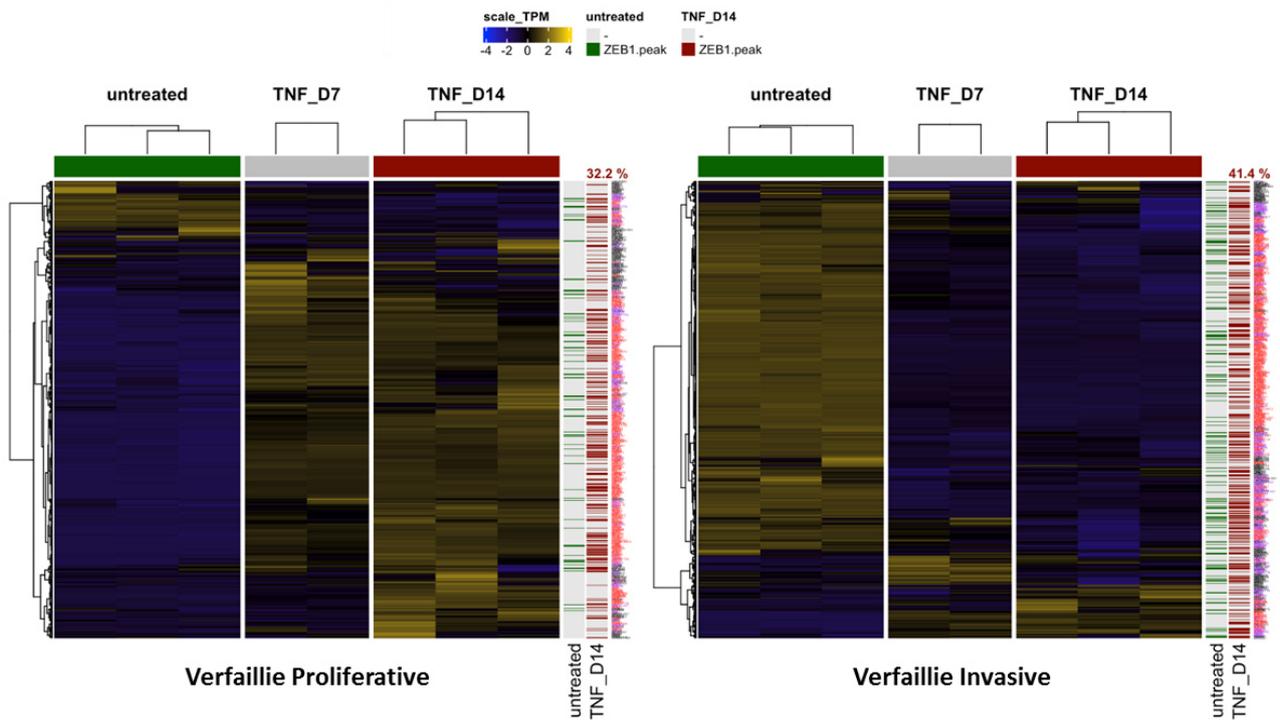
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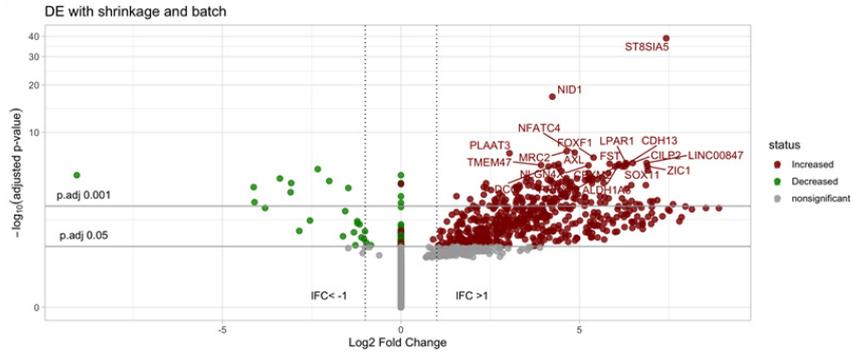


# Supplementary Figure 5

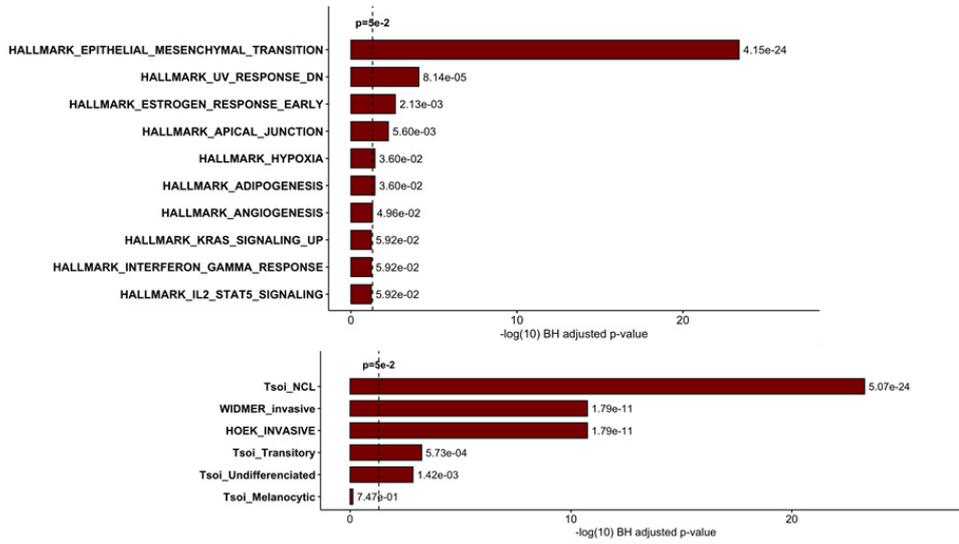


A.

Supplementary Figure 6



B.



## Discussion and perspectives

My study provides the first comprehensive characterization of ZEB1 target genes in melanoma through a ChIP-seq approach in an *in vitro* phenotype switching model [Figure 32]. Previous work from the lab demonstrated the major oncogenic role of ZEB1 aberrant reactivation in melanoma cells. ZEB1 indeed promotes phenotype switching towards a NCSC/invasive state resistant to BRAFi. We previously pointed the role of ZEB1 in repressing MITF. Herein, we further detailed the mechanisms of action of ZEB1 and demonstrated its capacity to repress ZEB2, MITF, and SOX10 as well as activate NGFR, AXL and SOX9. More ZEB1 direct target genes were identified and represent as many phenotypic markers of interest, as well as potential therapeutic targets. Meanwhile, there are still some questions, which need to be discussed and further investigated.

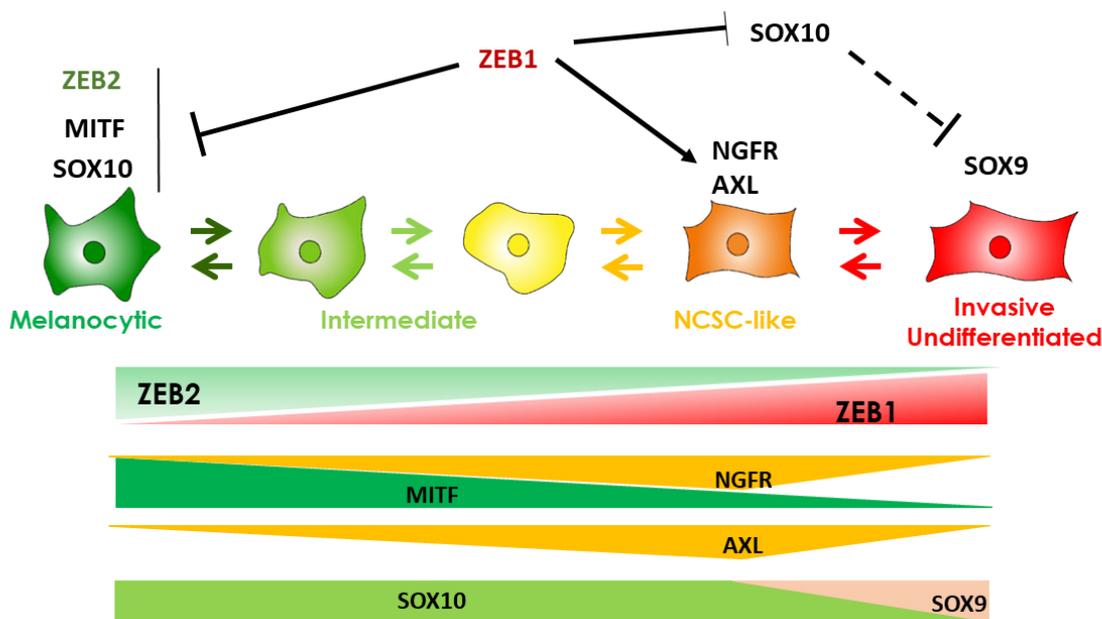


Figure 32. Proposed model of ZEB1-mediated transitional regulation of melanoma states markers.

The upper part presents the proposed regulation network of ZEB1 to melanoma state markers. ZEB1 directly represses the expression of ZEB2, MITF and SOX10, while it activated HGFR and AXL. The regulation of SOX9 may through an indirect manner via SOX10. The lower part shows the expression level of melanoma states markers at each intermediate state.

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## Chapter 1. Modeling phenotype switching in patient derived short-term cultures

In order to investigate endogenous ZEB1 function along with phenotype switching, I used two patient derived short-term cultures, C-09.10 and GLO. These models are more physiological than ATCC melanoma cell lines, which may be blocked in one phenotype, and more difficult to switch upon external signals.

In order to induce phenotype switching, I treated the cells with various cytokines, known to promote dedifferentiation or EMT (TNF $\alpha$ , TGF $\beta$ 1, but also IL6), either alone or in combination [Figure 33]. TNF $\alpha$  +/- TGF $\beta$ 1 was chosen, since it induced progressive phenotype switching of melanoma cells, towards NCSC and undifferentiated state as assessed at the molecular level, but also at the functional level, including lower proliferative rate, increased cell migration as well as reinforced resistance to BRAF inhibitor.

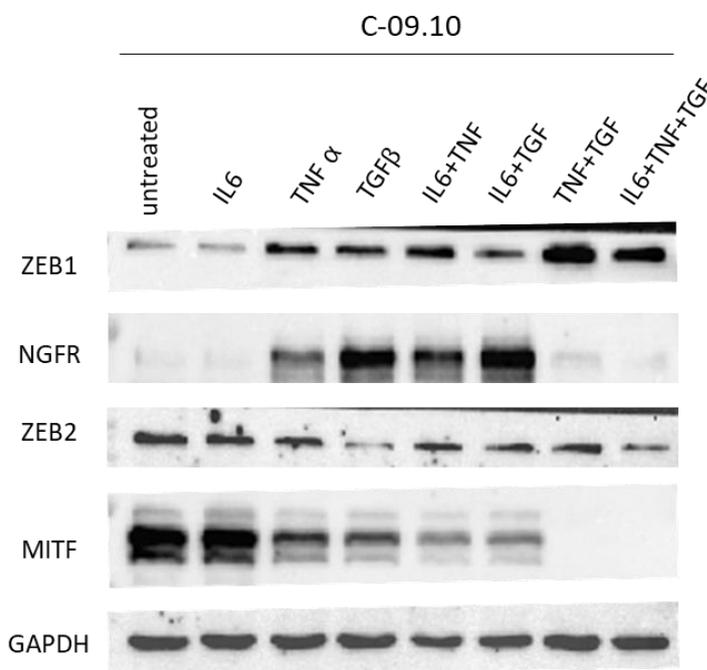


Figure 33. Western Blot analysis of C-09.10 cell line.

Cells were treated with IL6 (100ng/ml), TNF $\alpha$  (100ng/ml) and TGF $\beta$  (20 $\mu$ g/ml) for 14 days. GAPDH was used as loading control.

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I also demonstrated that this TNF $\alpha$  +/- TGF $\beta$ 1 induced phenotype switching is reversible. Indeed, after 14 days of TNF $\alpha$  treatment, TNF $\alpha$  was removed from the culture medium and GLO cells were kept in culture for another 14 days; they were then compared with untreated cells [Figure 34A]. Expression of all markers returned to basal level.

Of note, while TNF $\alpha$  was sufficient in GLO cells, a combination of TNF $\alpha$  and TGF $\beta$ 1 was the most efficient combination to promote switching in C-09.10 cells, although TNF $\alpha$ -only could also induce switching but needed a longer time. According to the differentiation model of Tsoi et al, SOX10 expression decreases progressively from melanocytic to neural-crest-like state, while SOX9 is activated in undifferentiated state. The SOX10 to SOX9 switch, which was nicely mimicked in GLO cells upon TNF $\alpha$ , was not reproduced in C-09.10.

These differences in timing and efficiency of switching upon treatments may be accounted for the baseline phenotypic differences between these two models [Figure 34B&C]. Indeed C-09.10 is more melanocytic, and displays higher ZEB2 and MITF expression than GLO. GLO is more transitory, with higher expression of ZEB1 and NGFR, thus may be more responsive to extracellular signals. Overall, since C-09.10 did not allow reproducing all the phenotypic differences, it would be interesting to test other short-term cultures, which I already started to do.

Importantly, I highlighted the role of ZEB1 in this *in vitro* induced switching model. Combined RNAseq and CHIP-Seq analyses demonstrated that 30% of TNF $\alpha$  differentially expressed genes are direct targets of ZEB1. ZEB1 knock-out in A375 cells demonstrated the role of ZEB1 in the regulation of some of these genes (including SOX9). In order to reinforce our conclusions and sustain ZEB1 requirement for at least part of TNF $\alpha$  induced phenotype switching, ZEB1 loss-of-function in GLO and C-09.10 cells would be necessary. Indeed, for now we could only provide data with ZEB1 over-expression in C-09.10 cells. This showed that part of TNF $\alpha$  function, more precisely induction of NCSC signature, is reproduced upon ZEB1 over-expression. However, ZEB1 depletion experiments with siRNA or shRNA failed to provide consistent results because of insufficient depletion and

remaining expression of ZEB1. Generation of knocked-out cells in GLO and C-09.10 cells is ongoing, as previously performed in A375 cells.

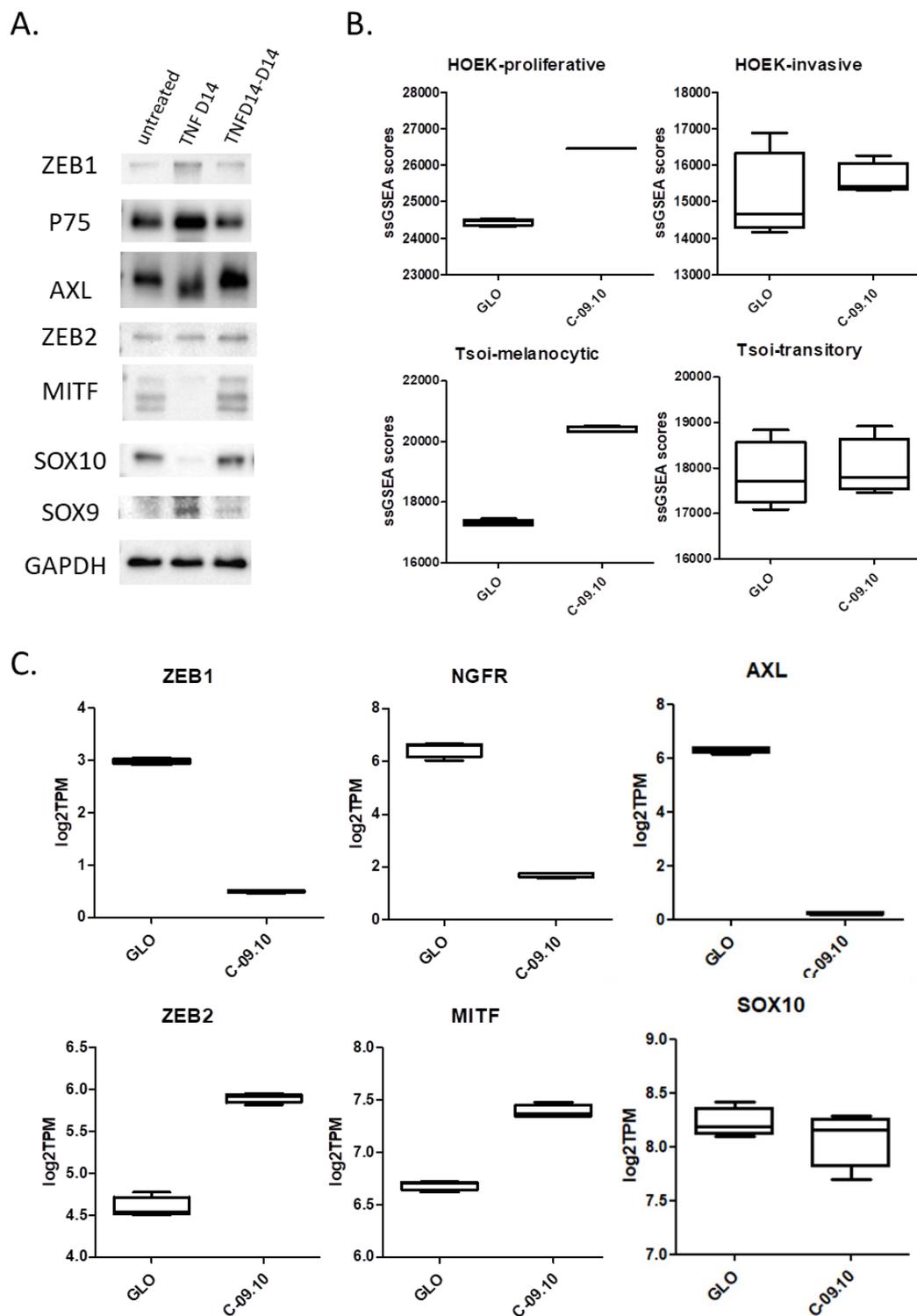


Figure 34. Reversible phenotype switching in GLO and Phenotypic difference between GLO and C-09.10 cell lines.

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A. Western Blot analysis of untreated GLO, GLO TNF $\alpha$  (100ng/ml) for 14 days, and TNF $\alpha$  was removed from cell culture for another 14 days. GAPDH was used as loading control. B. Single Sample Gene Set Enrichment Analysis (ssGSEA) from the RNAseq analysis of each gene related to different melanoma signatures of Hoek et al. and Tsoi et al. (Hoek et al., 2008; Tsoi et al., 2018). ssGSEA scores of GLO and C-09.10 cell lines are presented for each melanoma signature. n=3. C. Normalized expression of genes from RNAseq in GLO and C-09.10 cell lines. RNA expression level is presented for each gene by log<sub>2</sub>TPM (Transcripts Per Million). n=3.

## Chapter2. Cell-type specific transcriptional activity of ZEB1 in melanoma compared to carcinoma models

We herein performed the first ZEB1 ChIP-seq analysis in melanoma cells. As a well-studied EMT-TF in carcinoma, ZEB1 ChIP-seq have already been published, especially in triple negative breast cancer (TNBC) cells, MDA-MB-231 by Feldker et al., as well as by others, as mentioned in my introduction [Figure 24].

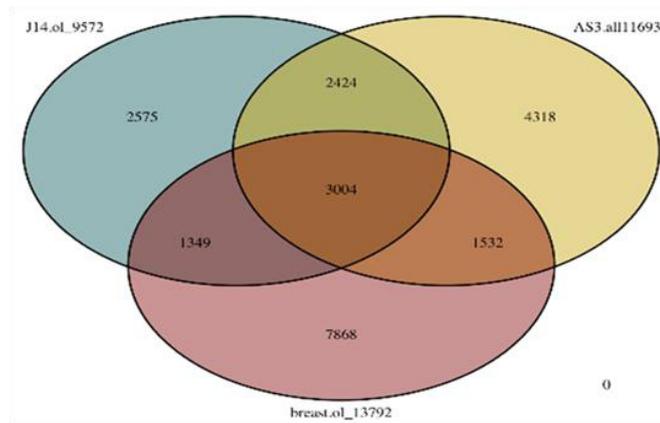
It is thus interesting to compare our results with these published datasets, in order to define cell-type specific effects. We started this comparative analysis, by analyzing the Feldker et al. dataset with the same bioinformatics pipeline as our GLO and A375 dataset, and showed that around 47% ZEB1 binding peaks were shared between GLO-TNF D14 and MDA-MB-231 [Figure 35A]. More importantly, around 53% of ZEB1 peaks were specific to melanoma compared to breast carcinoma. However, in order to be strictly comparable, a ChIP-Seq using the same ZEB1 antibody and the same ChIP-Seq protocol would need to be validated in MDAMB231 in our hands to provide a definitive comprehensive view of ZEB1 cell-type specific targets.

More precisely, ZEB1 peaks in SOX10 and NGFR were specifically found in melanoma cells, while ZEB1, MITF, SOX9 or AXL peaks were conserved in the carcinoma model, although not always exactly in the same position [Figure 35B]. SOX10, which is a lineage marker of both primary and metastatic triple-negative breast cancer (Tozbikian and Zynger, 2019), was not shown to be regulated by ZEB1 in MDA-MB-231 cell line. In contrast, SOX9 demonstrates a binding peak of ZEB1

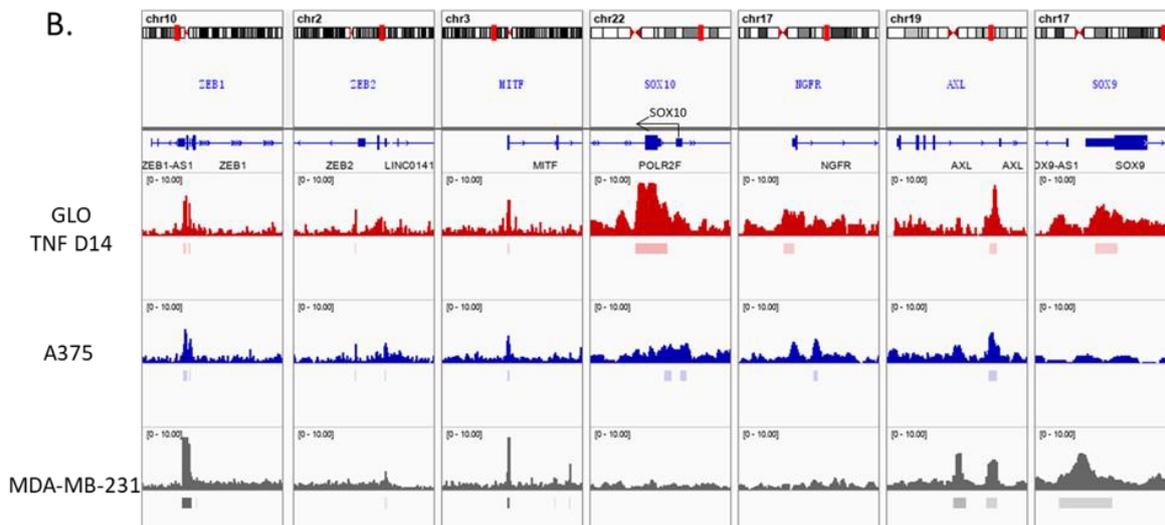
in MDA-MB-231.

Figure 35. Comparison of ZEB1 ChIPseq peaks between melanoma and carcinoma.

A.



B.



A. Number of common and specific ZEB1 peaks of ChIP-seq in GLO TNFα D14, A375 and MDA-MB-231 cell lines. B. ZEB1 peaks visualization using IGV gene viewer.

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As regards to ZEB2, the main ZEB1 binding peak in GLO cells is not present in MDA-MB-231 cells, while another one is found in a different location, the two peaks being present in A375 cells. This opens the question of whether these peaks differentially regulate transcription. Indeed ZEB2 is expressed and plays similar roles as ZEB1 in breast cancer, favoring breast cancer progression. In contrast, ZEB2 is progressively lost during melanoma progression. ZEB1 is known to activate ZEB2 expression in carcinoma models while it represses its expression in the melanocyte lineage. One hypothesis would be that this differential regulation may be accounted by the differential binding of co-factors, the recruitment of which may be lineage specific. In this respect, a similar ZEB2/ZEB1 switch has also been described in immune cell populations. Indeed, while ZEB2 leads to differentiation of CD8 T lymphocytes towards effector T cells, ZEB1 induces T memory formation (Guan et al., 2018).

### **Chapter3. ZEB1 co-factors in transcriptional regulation**

ZEB1 and ZEB2 have initially been described as transcriptional repressors, by inhibiting the expression of epithelial markers such as CDH1. According to ZEB1 structure, in addition to the co-inhibitor CtBP binding domain shared with ZEB2, there is a co-activator p300 binding domain; when associated with p300, ZEB1 enhances transcription. Different recruitment of these co-factors may determine the antagonistic roles of ZEB1 and ZEB2 in melanoma. In 2003, Postigo already showed that ZEB1 and ZEB2 acted differently to TGF $\beta$ /BMP signaling; both of them interacted with Smads coming from TGF $\beta$ /BMP signaling, when ZEB1 synergized with Smads to activate target genes transcription, ZEB2 showed an opposite effect (Postigo, 2003). They also described precisely that the antagonistic roles were due to the differential recruitment of transcriptional co-activators p300 and co-repressors CtBP (Postigo et al., 2003), in which ZEB1 bound to p300 and promoted the formation of a p300–Smad transcriptional complex, while ZEB2 acted as a repressor by recruiting CtBP.

Therefore, the validation of this co-binding may be an important step to distinct

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mechanisms of ZEB1 and ZEB2. CHIP and re-CHIP could be used to study the occupancy of two or more proteins at the same DNA binding region. CHIP-Seq for CBP could be performed, and binding sites compared genome-wide with those of ZEB1.

Next, Feldker et al., showed a co-binding of ZEB1, YAP and AP1 complex in order to induce target genes transcription in TNBC cells (Feldker et al., 2020). Using *de novo* motif analysis, they found that the second top enriched motif within the ZEB1 peaks was the consensus binding site for AP-1 transcription factors, which indicated joint binding of AP-1 factors together with ZEB1 at the same genomic regions. As there was already a published CHIP-seq data set of the AP-1 factor JUN in the same TNBC cell line, MDA-MB-231, they compared these two CHIP-seq and observed overlaps between ZEB1 and JUN binding sites; the same approach was used to investigate YAP binding sites. Together, they showed a genome-wide cooperation of these three transcription factors.

From previous works of our team, AP1 complex (and namely FRA-1) is known to regulate ZEB1 expression in melanoma (Caramel et al., 2013). It would be interesting to decipher whether AP1 factors are also major co-regulator of ZEB1 in melanoma. A CHIP-seq of YAP or AP1 could be performed in the same melanoma cell line, to allow the comparison with ZEB1 peaks location. Motifs analyses have started to be performed in our CHIP-seq data, but for now did not show a preferential enrichment in AP1 binding, rather pointing to other transcription factors such as ZBTB33 [Figure 36]. This could further point to cell-type specificities, which will need to be further analyzed in the future.

Motif	Name
	ZBTB33(Zf)/GM12878-ZBTB33-ChIP-Seq(GSE32465)/Homer
	GFX(?)/Promoter/Homer
	ZEB1(Zf)/PDAC-ZEB1-ChIP-Seq(GSE64557)/Homer
	Atf1(bZIP)/K562-ATF1-ChIP-Seq(GSE31477)/Homer
	ZEB2(Zf)/SNU398-ZEB2-ChIP-Seq(GSE103048)/Homer

Figure 36. Top 5 enriched binding motifs discovered by HOMER (Hypergeometric Optimization of Motif EnRichment) analysis in ChIPseq ZEB1 in GLO TNF $\alpha$  D14.

## Chapter4. Analyses of ZEB2 direct transcriptional target genes by ChIP

At the difference of carcinoma models, wherein ZEB1 and ZEB2 are believed to display largely similar function and target genes, antagonistic roles of ZEB1 and ZEB2 in melanoma could rely on differential regulation of specific set of genes, which remain important to be investigated. However, unlike ZEB1, there is only one published ChIP-seq of ZEB2 in carcinoma (Balcik-Ercin et al., 2018). This article used a homemade ZEB2 antibody. Given ZEB1/ZEB2 highly similar structures, many ZEB2 antibodies can also cross-react with ZEB1. For this reason, ChIP-seq grade antibodies anti-ZEB2 were not available at the beginning of my Ph.D.

The initial aim was to perform ChIP-seq of both ZEB1 and ZEB2 in the same melanoma cell line upon switching from a ZEB2<sup>high</sup> to a ZEB1<sup>high</sup> phenotype. Thanks to Irwin Davidson, we investigated an approach consisting in inserting a tag into ZEB2 sequence through a Crispr Cas9 knock-in approach, so that ZEB2 ChIP-seq could be performed easily with an anti-tag. However, this technique was for now unsuccessful because of poor recombination efficacy in these short-term cultures.

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During my PhD project, I thus tested various ZEB2 antibodies which were shown to display specificity for ZEB2, and not ZEB1, in western-blot, but which were not necessarily proven to be CHIP-grade. So I first tried to do CHIP-qPCR in one ATCC cell line, 501Mel, with high level of ZEB2, as well as in GLO and C-09.10. As controls, we used primers specific for CDH4 and CDH1 promoters, which have been described in carcinoma models [Figure 37]. Known as R-cadherin, CDH4, as well as N-cadherin are expressed in mesoderm inversely and proportionally to E-Cadherin (Inuzuka et al., 1991). Even though it has not been well studied in melanoma, in gastrointestinal cancer, CDH4 promoter is often methylated early in tumor progression, indicating a potential tumor suppressive role of CDH4 (Berx and van Roy, 2009). Encouragingly, an enrichment of ZEB2 binding on the promoter of CDH4 could be validated in these 3 melanoma models [Figure 37]. It may thus be used as a positive control for future experiments.

Interestingly, no significant binding of ZEB2 on the promoter of E-cadherin/CDH-1 was found, which is rather coherent. Indeed, contrary to the repression of E-cadherin by ZEB2 in mesenchymal cells, ZEB2 is strikingly co-expressed with E-Cadherin in melanocytes (Caramel et al., 2013), indicating that it is unable to repress E-cadherin expression.

Overall, based on these encouraging results, the specificity of this ZEB2 antibody should be validated through a knock-out of ZEB2, and the compatibility with CHIP-Seq approach will need to be evaluated. Performing ZEB2 CHIP-seq would complete our characterization of ZEB1/ZEB2 specific transcriptional regulation.

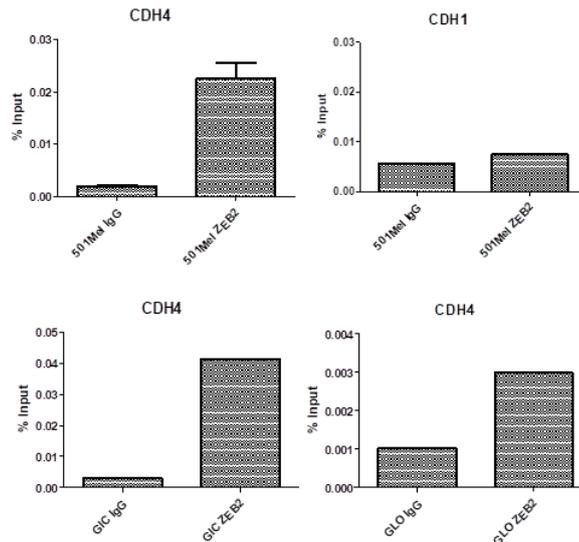


Figure 37. ChIP-qPCR analysis of ChIP ZEB2 in 501Mel ATCC melanoma cell line, in C-09.10 and GLO patient-derived short-term cultures. Anti-ZEB2 (ZEB2) (2 $\mu$ g, HPA003456, Sigma) or control IgG were used for the IP. Relative promoter enrichment was normalized to chromatin inputs (%). CDH4 in 501Mel, n=2. Others n=1.

## Chapter5. Intra-tumor heterogeneity *in vitro* and in human samples

Melanoma is a very heterogeneous tumor, so we tried to assess the heterogeneity of molecular markers expression by flow cytometry for *in vitro* experiments, and by multi-IF in patient samples.

To prevent potential staining bias of intra-cellular TF, we generated MITF promoter-GFP cell lines. Flow cytometry analyses allowed to quantify the proportion of cells in each phenotype within the population, based on MITF and NGFR expression. Although informative, these two markers are obviously not sufficient to precisely define intermediate states and scRNAseq experiments could be proposed *in vitro*.

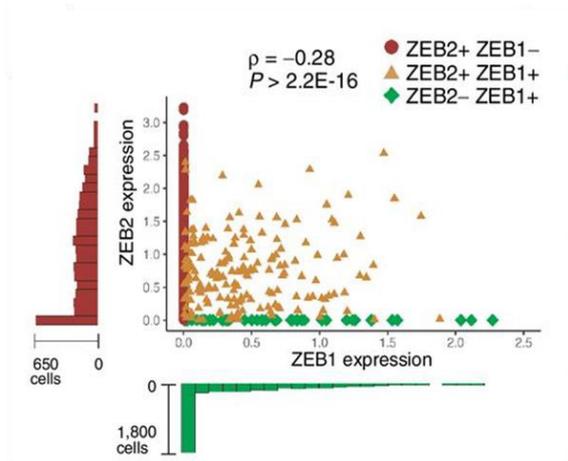
For the RNAseq / ChIP-seq analyses, we used signatures of Hoek and Tsoi, but it would be interesting to analyze other recently published signatures including Wouters et al., in which they

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identified potential master regulators of each melanoma cell state by using single-cell approach (Wouters et al., 2020) (mentioned in introduction Figure 29). Another article published by Belote et al. was also very informative (Belote et al., 2021). Different from Tsoi et al. who analyzed an *in vitro* differentiation model, this scRNA-seq was performed in human samples, in normal melanocytes across different anatomical sites, developmental age, sexes and multiple skin tones. Analysis from sorted human skin melanocytes also classified melanocytes at 4 stages: adult, neonatal, fetal and MSC (mesenchymal stem cells). By using artificial intelligence, they identified 40 to 65 genes to each cluster, in which there was also the expression of EMT-TFs, including ZEB1 in the MSCs. This study was closer to the physiological conditions, so that these signatures will be worth to be considered in the future.

scRNAseq is a powerful technique in order to precisely define molecular signatures of melanoma subpopulations in human samples. Re-analyses of previously published scRNAseq dataset already confirmed the opposite expression pattern of ZEB1 and ZEB2 (Vandamme et al., 2020), although on a limited number of cells [Figure 38A]. Wouters et al also performed scRNAseq in 9 patient-derived melanoma cultures and in A375 cell line; single-cell expression also demonstrated an opposite pattern of ZEB1/ZEB2 and SOX10/SOX9. Co-expression of ZEB1/NGFR and ZEB1/SOX9 could also be observed in several cell lines, showed that ZEB1 was also expressed in NCSC state possessing high level of NGFR, also in stem-like state marked by SOX9 [Figure 38B]. However, the information on the spatial location of the cells is lost, except going towards 10X visium technology for example.

A.



B.

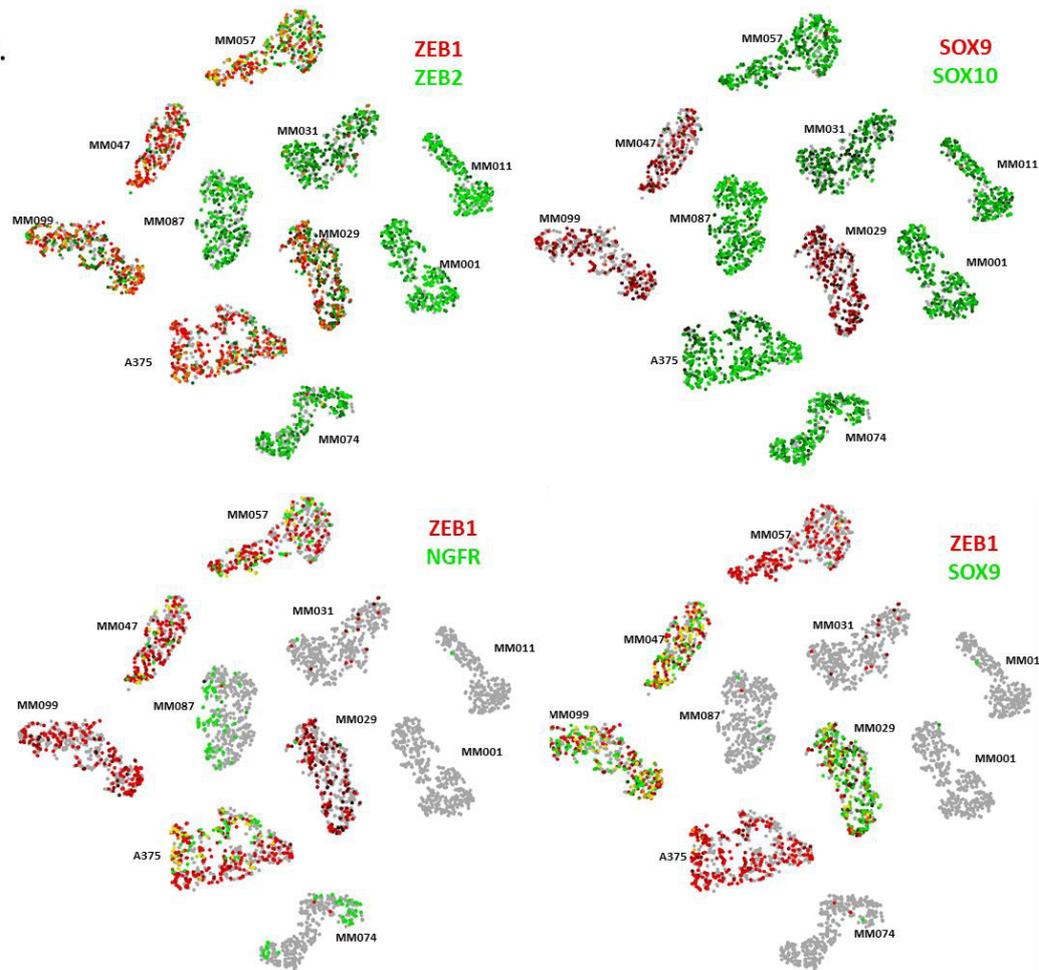


Figure 38. ZEB1 and ZEB2 expression in two published scRNAseq analysis.

A. Anti-correlated expression of ZEB1 and ZEB2 from scRNA-seq data (GSE115978) in 1,951 human malignant melanoma cells presented by Spearman correlation. Expression level was normalized in logTPM (Transcripts Per Million). The histogram next to the axis reflects the number of cells in specific ranges of expression levels for ZEB2 (left) or ZEB1 (bottom) (Vandamme et al., 2020). B. scRNA-seq analysis of 9 patient-derived MM lines and the A375 cell line (Wouters et al., 2020). The cells clustered according to their cell-line origin in a t-SNE analysis. Expression of ZEB1/ZEB2,

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*SOX10/SOX9, ZEB1/NGFR and ZEB1/SOX9 is presented here. Expression of ZEB1/NGFR and ZEB1/SOX9 shows co-expression, consistent with our multi-IF analyses.*

Multi-IF in patient samples is a highly informative technique, since it not only allows to quantify the intensity of each marker (up to 7 in our approach) in each cell, but also to visualize the spatial distribution of the sub-populations. We started with a panel containing the major phenotypic markers, which already provided some nice information regarding co-expression of ZEB1 with NCSC (NGFR) or undifferentiated (SOX9) states markers; these results were in consistent with the observations of scRNAseq in patient-derived melanoma cell lines [Figure 38B]. Additional melanoma samples are being analyzed in order to support the conclusions and quantitative and spatial (distance between cells) analyses will be further detailed. Other relevant markers could also be included in the future.

## **Chapter6. New candidates with therapeutic perspectives**

Our ZEB1 ChIP-seq data, combined with RNA-seq data, allowed highlighting novel ZEB1 direct target genes, those are up-regulated during switching towards stem-like invasive state. Their direct regulation was confirmed by ChIP-qPCR, and their expression in TNF $\alpha$  induced phenotype switching model was also assessed by RT-qPCR. BIRC3, which belongs Hoek invasive signature, has already been shown as a key actor of BRAFi resistance (Gautron et al., 2021); it was also co-expressed with SOX9 in patient derived melanoma cultures and co-expression of SOX10-SOX9/BIRC3 was also present in patient samples (Ennen et al., 2017). ITGA2 is associated with increased melanoma risk and may be a direct target of MITF (negatively) (Laurette et al., 2015; Lenci et al., 2012). EGFR appears to be involved in progression and metastasis of a subset of melanomas; EGFR activation might represent a mechanism of vemurafenib resistance in a subset of melanoma cells (Boone et al., 2011; Gross et al., 2015). Determination of these target genes can help us to optimize therapeutic, since ZEB1 was not easy to be inhibited directly. Because BIRC3 and EGFR were already shown to be associated with BRAFi resistance, their targeted therapies may re-sensitize cells to BRAFi.

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### **Enrichment of ZEB1 peaks at epigenetic regulators loci.**

In the introduction part, I already talked about epigenetic regulator and EMT-TFs in carcinoma, in which epigenetic regulators can co-operate with ZEB1/2 to repress CDH1 expression, but also can regulate ZEB1/2 expression (Skrypek et al., 2017). In our ZEB1 ChIP-seq data in GLO cell line, we interestingly found many peaks in the promoters of epigenetic drivers.

We focused on a list of 426 known epigenetic regulators, which are frequently altered across different cancer types (Halaburkova et al., 2020). More than half of them displayed a ZEB1 peak in GLO TNF $\alpha$  D14 [Figure 39A]. This suggests that, in addition to being regulated by, or cooperating with epigenetic regulators, ZEB1 can potentially control their expression, which could further participate in phenotype switching. A few examples of epigenetic regulators presenting a ZEB1 binding site are presented in [Figure 39B].

EZH2 is the H3K27me3-specific histone methyltransferase enhancer of zeste 2 polycomb repressive complex 2 subunit; it can regulate negatively ZEB1/2 expression in ovarian cancer. Several histone-modifying enzymes are also deregulated during melanoma formation, including EZH2. EZH2 is a major factor for melanoma initiation and progression. Knockout of *EZH2* in a genetically engineered *NRAS*<sup>Q61K</sup> melanoma mouse model reduced the number of melanomas and prevented metastasis formation (Zingg et al., 2015). Moreover, inhibition of EZH2 has been shown to promote dedifferentiation of melanoma cells, restore immunogenicity, and re-sensitize tumors to immunotherapy (Zingg et al., 2017). *EP300* codes for P300, a histone acetyl-transferase, which is also the co-activator of ZEB1. P300 represses ZEB1/2 expression in breast cancer, but can cooperate with ZEB1 to activate TGF $\beta$  dependent genes transcription. HDAC1 collaborates with ZEB1 to inhibit *CDH1* transcription, probably through the recruitment of co-repressor CtBP in pancreatic cancer. Overall, up or down-regulation of these epigenetic regulators by ZEB1 will next be confirmed by ChIP-qPCR as well as in our induced phenotype switching models.

The crosstalk of ZEB1 with epigenetic regulators is currently being studied by other members of the lab, in order to decipher their cooperative role in phenotype switching.

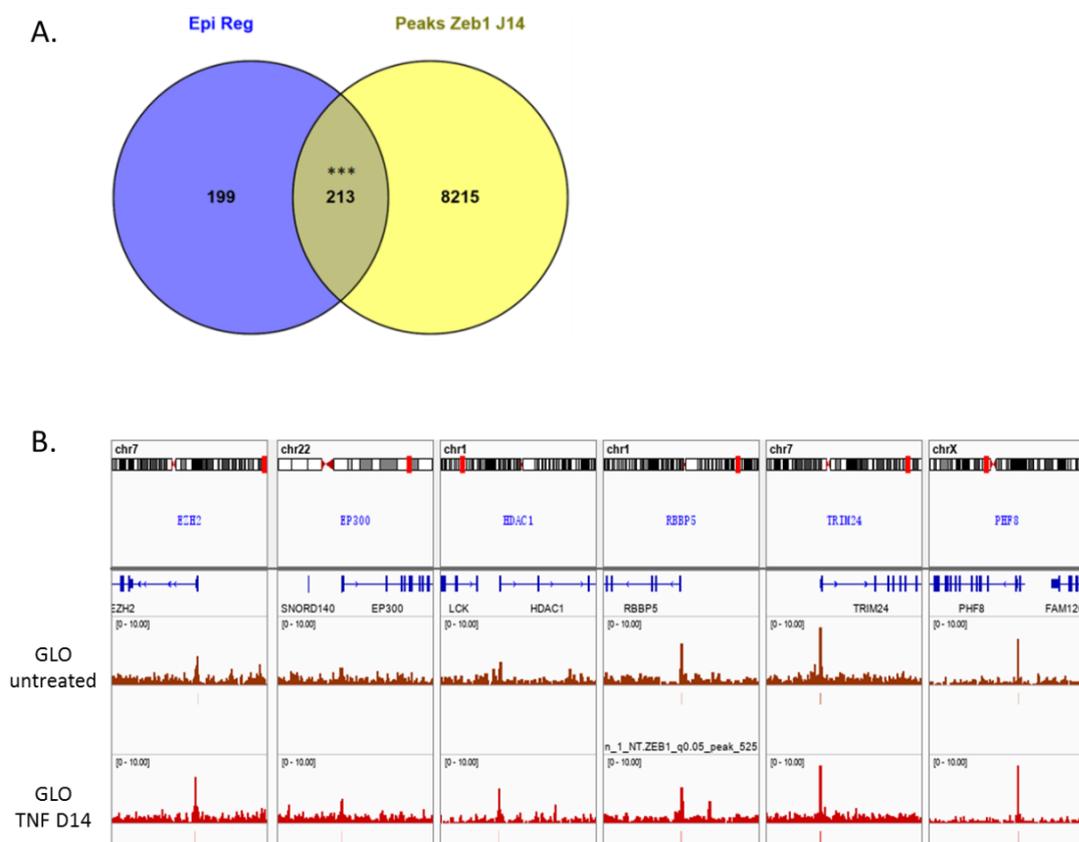


Figure 39. Some epigenetic regulators present ZEB1 binding sites in ChIPseq ZEB1 in GLO cell lines.

A. Venn diagram representing the overlap of epigenetic regulators (blue) with all the genes presenting a ZEB1 binding peak for GLO D14 (yellow). List of epigenetic regulators based on the literature (Halaburkova et al., 2020). There is a significant (Fisher's test) enrichment of ZEB1 fixation peaks on epigenetic regulators. B. Peaks visualization of some epigenetic regulators using IGV gene viewer.

Our ChIP-seq of ZEB1 also demonstrated some **immune-related direct target genes**, such as IL4R (not yet proven in ChIP-qPCR), which participate in the Th2 response. IL6 was a ZEB1 target gene in breast cancer cell lines (Katsura et al., 2017); herein I found that IL6R was a potential ZEB1 target in GLO cell line. Moreover, IL17C and IL17D showed also binding sites of ZEB1 in our context. Thus ZEB1 may participate in the regulation of immune response as in breast cancer, but need further confirmation.

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By modulating these target genes, further targeted therapies may be used to combine with immune therapies. A submitted work of our lab suggested that ZEB1 participate in the exclusion of T CD8 cells (Plashka et al., 2021 in revision). Indeed, analysis of immune infiltrate from melanoma biopsies has shown that high levels of ZEB1 expression by tumour cells are associated with low levels of infiltration by CD8 T cells. The implementation of gain and loss of function approaches in vivo in syngeneic murine melanoma models demonstrated that ZEB1 regulated tumour growth and modifies the composition of the immune infiltrate, in particular by controlling the recruitment of CD8 T to the center of the tumour. In addition, at the molecular level, a reduced production of CD8 attracting chemokines in the secretome of tumours strongly expressing ZEB1 could explain the lack of recruitment of T CD8. Finally, this remodeling of the immune microenvironment has consequences on the response to anti-PD-1. ZEB1 invalidation synergizes with anti-PD-1 treatment by inducing tumour regression of murine tumours. It will thus be important to further look at cytokines/chemokines as well as IFN responsive genes, in our CHIP-Seq data, that could provide novel mechanistic clues as regards to ZEB1-mediated immune escape.

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## Chapter7. Strategies to repress ZEB1 expression and re-sensitize cells to treatment

Identifying molecules capable to inhibit ZEB1 expression would be a way to switch phenotype back to a sensitive state. As epigenetic regulators were shown to regulate or co-operate with ZEB1/2, I tested some HDACis. Mocetinostat is a class I HDACi, it can restore cancer cell sensitivity to chemotherapy through ZEB1 expression (Meidhof et al., 2015). TSA (Trichostatin A) is a class I and II HDACi, it was shown to inhibit EMT in human epithelial cells (Nagarajan et al., 2017; Yoshikawa et al., 2007). Another HDACi SAHA (vorinostat), was shown in a clinical trial for BRAFi resistant melanoma that subsequent treatment with BRAFi then vorinostat can kill selectively BRAFi resistant melanoma cells (Wang et al., 2018).

In our lab, we have a pair of BRAFi/MEKi sensitive/resistant patient-derived cells, GLO and GLO-R. GLO-R was established from the same patient, after acquired resistance upon BRAFi/MEKi treatment. At the protein level, GLO-R exhibits higher expression levels of ZEB1 and lower expression of ZEB2, MITF and SOX10 [Figure 40A]. RNAseq analyses demonstrated that invasive, undifferentiated and NCSC signatures were enriched in GLO-R compared to parental GLO cells [Figure 40B]. SAHA survival test showed that GLO-R is a bit more sensitive than GLO [Figure40C]. Combination treatment of PLX4032 BRAFi with SAHA demonstrated higher cell mortality than with BRAFi-only. At the molecular level, three HDACi are capable to inhibit ZEB1 expression [Figure 40D]. These HDACi are thus interesting to be further investigated for reverting resistant phenotypes.

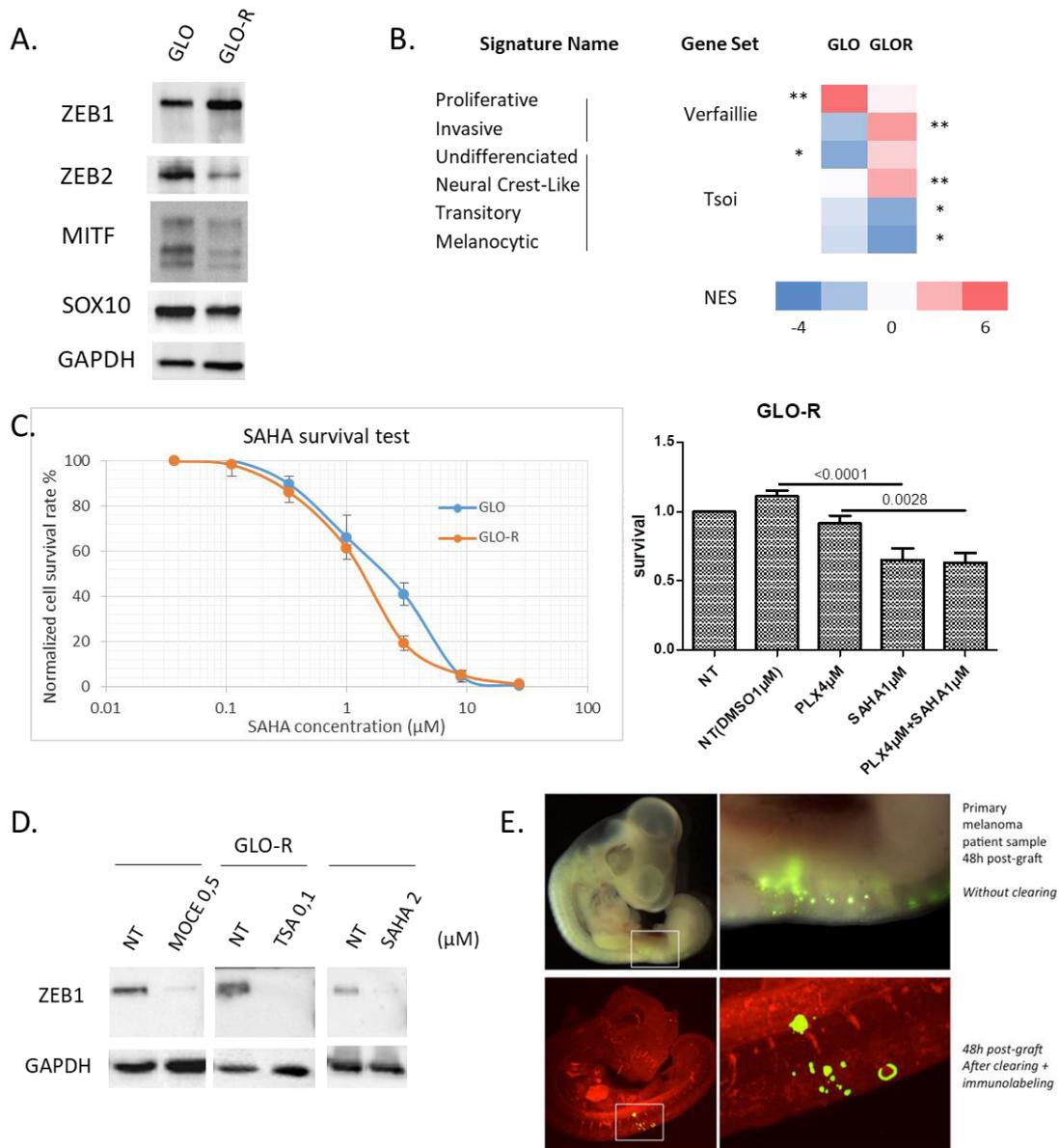


Figure 40. GLO-R is more sensitive to HDACis than GLO cell line.

A. Western blot analysis of GLO and GLO-R cell lines. B. RNA-seq analyses of GLO and GLO-R cells. ssGSEA analyses of melanoma signatures. C. SAHA (vorinostat) survival test for GLO and GLO-R. Normalized cell death was presented in histogram on the right.  $n=3$ . D. Western blot analysis of ZEB1 in GLO-R under different HDACi treatment for 5 days. E. HDACi for the treatment of BRAFi-resistant melanoma in chick embryo models. An *in vivo* paradigm to model human melanoma disease and perform fast and relevant preclinical studies. Jarrosson L, Dalle S, Costechareyre C, [Tang Y](#), Grimont M, Plaschka M, Caramel J\*, Castellani V\*, Delloye-Bourgeois C\*. *In prep, confidential*.

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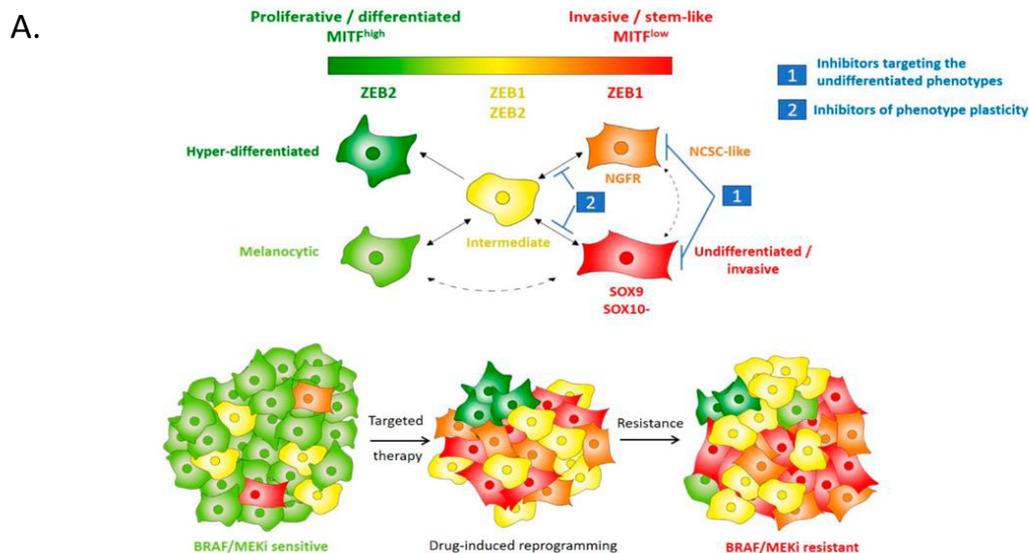
In the introduction, I mentioned some models of melanoma study, including 2D and 3D cell culture, human skin constructs as well as mouse models. Besides, in collaboration with Oncofactory, I participated during my Ph.D. in a study modelizing melanoma in chick embryos. Initially developed for neuroblastoma, this elegant miniaturized PDX model, requires only few cells, and allows testing large number of therapy combinations in only a few days (Delloye-Bourgeois et al., 2017). In this work, GLO and GLO-R cells were marked with CFSE then injected into the chick embryo. Treatments with Vemurafenib and vorinostat confirmed GLO-R increased sensitivity in an *in vivo* setting [Figure 40E].

Besides HDACis, other EMT drugs aiming to target phenotype switching [Figure 41], such as TGF $\beta$  inhibitors, were also tested clinically (Morris et al., 2014). TGF $\beta$  is a strong EMT inducer in carcinoma, as well as one of our *in vitro* phenotype switching inducers. TGF $\beta$  inhibitors turn to block the early stage of phenotype transition. However, this may become a real problem, since MET, the reverse process of EMT, participate also in tumor metastasis; TGF $\beta$  may therefore facilitate circulating or metastatic cells to colonize to a secondary site. So this approach of all EMT inhibitors needs to be well considered; it may only be used in patients at a very early disease stage.

Another approach under investigation aims at targeting the invasive/undifferentiated stage, by using antibody anti-AXL combined with BRAFi (Boshuizen et al., 2018) [Figure 41]. This combination can theoretically kill BRAFi resistant invasive cells, as well as BRAFi sensitive melanoma cells. The same idea can be realized for our new identified ZEB1 CHIP-seq targets. These new candidates may be essential to maintain one intermediate phenotype, so the combination of a specific inhibitor, plus traditional BRAFi/MEKi, as well as immune therapies, could be a good strategy to alleviate treatment resistance in melanoma cells.

Altogether, this comprehensive work allowed deciphering the role of ZEB1, the identification of its targets and a better comprehension of the phenotype plasticity in melanoma. However, it also rose new perspectives and questions and could fuel the rationale to target key actors of melanoma

plasticity in the clinic.



Strategy	References	Target	Drug/Compound	Clinical Trial	Trial ID	Cancer Type
TGFβ pathway inhibitors	[91]	TGFβ monoclonal antibody	GC1008 (fresolimumab)	Phase I	<a href="#">NCT00356460</a>	Renal Cell Carcinoma and Malignant Melanoma
AXL targeting	[92]	AXL small molecule inhibitor	BGB324	Phase I/II in combination with either dabrafenib/trametinib or pembrolizumab	<a href="#">NCT02872259</a>	Metastatic Melanoma
Epigenetic drugs	[93,94]	HDAC inhibitors	Entinostat	Phase II in combination with erlotinib	<a href="#">NCT00602030</a>	Non-Small-Cell Lung Cancer
	[95]		Vorinostat	Phase I	<a href="#">NCT02836548</a>	Resistant BRAFV600 melanoma
Metabolic inhibitors	[96,97]	Inducible nitric oxide synthase (iNOS)	L-NMMA	Phase Ib/2 in combination with Taxane	<a href="#">NCT02834403</a>	Triple Negative Breast Cancer
Ferroptosis inducers	[65,98]	GPX4 (through glutathione depletion)	PRLX 93936 (Erastin analog)	Phase I	<a href="#">NCT00528047</a>	Advanced Solid Tumors

Figure 41. ZEB1/2 regulate melanoma phenotype switching and resistance to treatments.

**A.** EMT-TFs regulate melanoma phenotype plasticity, intra-tumor heterogeneity and resistance to treatment. Up: Schematic model of putative melanoma intra-tumor heterogeneity according to a classification in five cell states, bearing various differentiation capacities, in accordance with their respective expression of MITF. Expected ZEB1 and ZEB2 expressions in the respective phenotypes are indicated. Expression of the main proposed markers of the different cell states is indicated. The invasive and stem-like phenotype actually represents two different states, both expressing high levels of ZEB1. SOX10 expression is present in all but the dedifferentiated state where it would be replaced by SOX9. The intermediate state should co-express ZEB1, ZEB2, and MITF. The intermediate state is believed to be highly metastatic and to give rise to the invasive, neural crest stem cell (NCSC) and hyper-differentiated states, especially upon targeted therapy treatment-induced adaptation. Two-way arrows indicate reversible switching between cell states. Dotted arrows indicate putative transitions. The two main therapeutic strategies under investigation are also indicated. Down: Evolution of intra-tumor heterogeneity of melanoma during treatment with BRAF/MEK

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*inhibitors-targeted therapy. The ZEB2/MITF melanocytic population is eliminated upon treatment and the tumor size decreases. Only resistant phenotypes remain with an increased proportion of NCSC-like and undifferentiated states. ZEB1-increased expression contributes to drug-induced NCSC reprogramming. Tumor adaptation ultimately leads to resistance with a regain of tumor growth and increased intra-tumor heterogeneity compared to the therapy-naive tumor. BRAF/MEK resistant tumors may display high ZEB1 expression in both MITFlow and MITFhigh clones. ZEB1 targeting can induce cell death in BRAFi-resistant melanoma cells, independently of MITF expression level. B. Shortlist of the main therapeutic strategies under evaluation to target EMT-dependent cell plasticity in cancers.*

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## **Appendix**

### **A. Review**

Review

# EMT-Inducing Transcription Factors, Drivers of Melanoma Phenotype Switching, and Resistance to Treatment

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**Abstract:** Transcription factors, extensively described for their role in epithelial–mesenchymal transition (EMT-TFs) in epithelial cells, also display essential functions in the melanocyte lineage. Recent evidence has shown specific expression patterns and functions of these EMT-TFs in neural crest-derived melanoma compared to carcinoma. Herein, we present an update of the specific roles of EMT-TFs in melanocyte differentiation and melanoma progression. As major regulators of phenotype switching between differentiated/proliferative and neural crest stem cell-like/invasive states, these factors appear as major drivers of intra-tumor heterogeneity and resistance to treatment in melanoma, which opens new avenues in terms of therapeutic targeting.

**Keywords:** EMT; transcription factors; melanocyte differentiation; melanoma development; phenotype switching; neural-crest stem cells; ZEB2/ZEB1 switch; intra-tumor heterogeneity; resistance to treatment

## 1. Introduction

The plasticity of tumors is a major source of intra-tumor heterogeneity, which underlies their capacity to adapt to the selective pressures they encounter at every stage of tumor development, from early stages of tumor initiation, through disease progression, and in response to therapy. The epithelial–mesenchymal transition (EMT) is an essential embryonic process, which provides motility properties and drives the reversible reprogramming of polarized epithelial cells into mesenchymal cells [1]. Regulated by microenvironmental signals, this cellular plasticity process is driven by a network of embryonic EMT-inducing transcription factors (EMT-TFs) mainly represented by the SNAIL, TWIST, and ZEB protein families, which interact with epigenetic regulators [2,3]. EMT is not a simple shift from a fully epithelial to a fully mesenchymal state but encompasses a spectrum of multiple states driven by reversible transitions sustaining cell plasticity [4,5]. EMT initiation is a potent source of phenotypic, metabolic, and functional cell plasticity. The expression of EMT-TFs is considered to be transient during embryogenesis, with EMT becoming latent in most adult tissues. However, in addition to fibroblasts, endogenous expression of EMT-TFs has been described in other normal adult differentiated cell types, including melanocytes, that will be the focus of this review, but also endothelial cells [6], neurons [7], and immune cells [8], such as lymphocytes [9], NK cells [10,11], or macrophages [12], suggesting major roles in cell differentiation and normal tissue homeostasis beyond embryonic development.

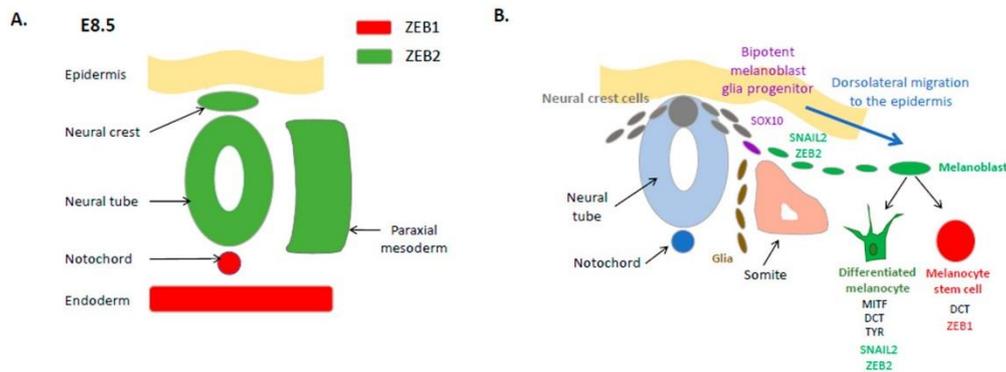
Aberrant reactivation of this embryonic process in pathological conditions, such as organ fibrosis and tumorigenesis, has been particularly studied in carcinomas [13]. While aberrant expression of EMT-TFs is known to foster dissemination and metastasis [14,15], it also plays a major role in malignant transformation and tumor initiation [16–18]. The oncogenic potential of EMT-TFs mainly relies on their ability to promote escape from oncogene-induced senescence and apoptosis by interfering with p53 and RB tumor suppressor pathways [19]. These factors also provide cells with stem-like properties [20,21]. Overall, cells committed to EMT may reside in metastable states, increasing their adaptability to the changing microenvironment that they encounter, both at the primary and distant sites [5].

While a large set of data demonstrated that aberrant reactivation of EMT-TFs in epithelial cells is oncogenic [22], the roles of these factors in non-epithelial cells have not been as thoroughly characterized. Although the term EMT *stricto sensu* cannot be formally used in the context of non-epithelial-derived cancers, a mesenchymal transition process has been observed in glioblastoma [23] or neuroblastoma [24,25], that are, similarly to melanoma, derived from neural crest cells.

Melanomas display a high degree of inter and intra-tumoral heterogeneity, supported by an exacerbated plasticity of tumor cells. Melanoma intra-tumor heterogeneity does not rely on the classical cancer stem cell (CSC) hypothesis but rather on a “phenotype switching” model, reminiscent of EMT, which is largely dependent on the expression level of the Microphthalmia-associated transcription factor MITF, the master regulator of melanocyte development [26]. According to the rheostat model proposed by Goding [27], high MITF expression maintains differentiated status of melanocytes, intermediate MITF expression sustains proliferation, while low MITF levels generate invasive and slow-proliferating cells with tumor-initiating properties. Melanoma cells were shown to shift in a reversible way between a proliferative and an invasive state [28,29]. Over the years, specific molecular signatures and epigenetic marks discriminating the proliferative and invasive states have progressively been deciphered [30,31]. Aside from MITF, other master transcriptional regulators have been identified, including SOX10 for the proliferative phenotype and AP1/TEAD for the invasive phenotype. We herein review the cell-type-specific roles of EMT-TFs in the melanocyte lineage, melanoma development, phenotype switching, and resistance to treatment.

## 2. Expression and Function of EMT-TFs in the Embryonic Neural Crest and the Melanocyte Lineage

EMT is essential for many crucial steps during embryonic development, including neural crest cell migration and melanocyte lineage formation. Neural crest cells delaminate from the dorsal neural tube through an EMT process (Figure 1A) enabling them to migrate as individual cells. While individual knock-out of EMT-TF genes can result in significant defects at the gastrulation stage, some redundancy may be observed for neural crest formation. Indeed, mice displaying *Snail1* and *Snail2* mutations still generate neural crests even though they develop multiple craniofacial defects [1]. ZEB2 is also required for neural crest cell development, since its deletion in mice causes embryonic lethality around stage E8.5, with a failure of neural tube closure associated with an early arrest of cranial neural crest cell migration [32]. Tissue-specific knock-out of Zeb2 in early neural crest cell development also results in embryonic lethality, associated with abnormalities in craniofacial, heart, and peripheral nervous system development [33]. In contrast, Zeb1 knock-out in the mouse is not embryonically lethal but induces major defects in skeletal elements and thymic T-cells, leading to perinatal death. Interestingly, expression of ZEB1 and ZEB2 is mainly complementary in mouse embryos, with an overlap in only a limited number of tissues [34]. Indeed, ZEB2 expression around E8.5 is found in the neural plate/crest and the paraxial mesoderm (Figure 1A), while ZEB1 is absent in the neural crest and expressed in the paraxial and limb skeletal elements up to E12, highlighting specific spatiotemporal regulation of the two ZEB family members.



**Figure 1.** Expression and function of epithelial–mesenchymal transition transcription factors (EMT-TFs) in the embryonic neural crest and the melanocyte lineage. **(A)** Schematic representation of ZEB1 and ZEB2 opposite expression patterns in the mouse embryo at embryonic day E8.5. **(B)** Expression of EMT-TFs during the formation of the melanocyte lineage from the embryonic neural crest. The transcription factor SOX10 is expressed in a bipotent melanoblast/glia progenitor. SNAIL2 and ZEB2 are required for neural crest cell delamination and melanoblast specification and migration through a dorsolateral pathway. SNAIL2 and ZEB2 are also required for the homeostasis of differentiated melanocytes in the epidermis, through the positive regulation of MITF expression. ZEB1 in contrast is expressed in dedifferentiated melanocyte stem cells, which have lost MITF expression. MITF: microphthalmia-associated transcription factor. DCT: dopachrome tautomerase. TYR: tyrosinase.

Neural crest cells from the trunk region generate different cells in the developing embryo, namely, melanocytes, neurons, and glia [35]. In the mouse embryo, melanoblasts are specified from a SOX10-positive bipotent melanoblast/glia progenitor (Figure 1B). Upon subsequent gain of expression of MITF, DCT (dopachrome tautomerase) and KIT in melanoblasts, these cells start their dorsolateral migration from E10.5, move into the epidermis around E11 and populate the developing hair follicle. They next separate into two populations: some melanoblasts differentiate into melanocytes and produce the melanin pigment; others dedifferentiate into non-pigmented melanocyte stem cells associated with the loss of MITF and KIT expression. These melanocyte stem cells reside in the hair follicle bulge and replenish hair follicles with new pigmented melanocytes in the subsequent hair cycle.

Melanoblast dorsolateral migration through the developing embryo requires the participation of EMT-TFs, especially SNAIL2 (SLUG) and ZEB2 (SIP1) (Figure 1B). Indeed, *Snai2*-deficient mice display defects in melanoblast specification and migration, resulting in pigmentation abnormalities, including a white forehead [36]. Similarly, in humans, SLUG mutations have been found in some patients displaying skin pigmentation abnormalities, piebaldism, or Waardenburg syndrome type 2 [37,38], a syndrome associated with melanocyte migration defects, which has notably been linked to MITF mutations. ZEB2 is also expressed in migrating melanoblasts in the mouse embryo and their precursor, neural crest cells [39]. Melanocyte-specific (Tyr: Cre) *Zeb2* knock-out induces a drastic hair pigmentation loss in mice that is due to a severe impairment of both melanoblast migration and melanocyte differentiation [39].

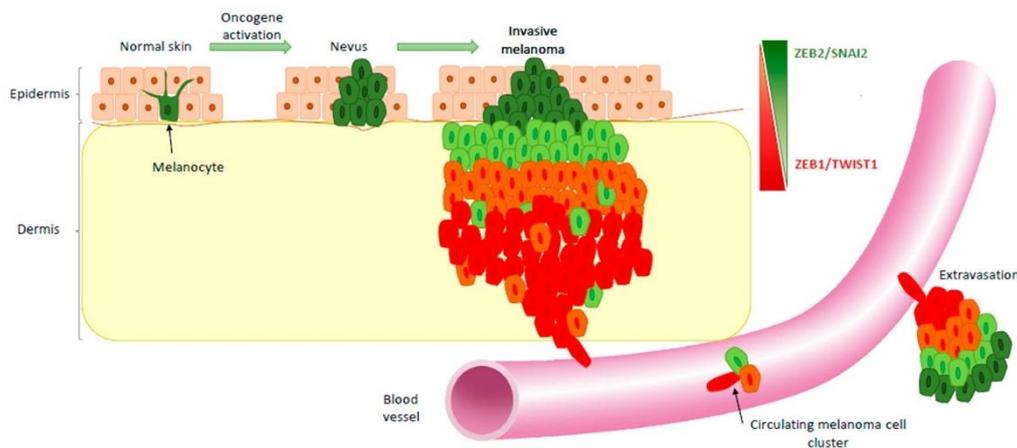
In addition to melanoblasts, SNAIL2 and ZEB2 expression is maintained in adult differentiated melanocytes in both mouse and human skin. Indeed, EMT-TF expression analyses in normal human skin samples unexpectedly showed SNAIL2 and ZEB2 expression in differentiated melanocytes, while TWIST1 and ZEB1 were not detected [40]. ZEB2 is required for melanocyte differentiation since ZEB2 knock-down in primary melanocytes induces a down-regulation of MITF and differentiation defects [39]. Overall, these data highlight a major role for SNAIL2 and ZEB2 in the regulation of the homeostasis of the melanocyte lineage, from the developing embryo to the adult life.

Interestingly, while ZEB1 is not expressed in differentiated melanocytes, it may still be involved in the homeostasis of the melanocyte lineage, since it was shown to be expressed in melanocyte stem

cells in mouse skin, and could thus be required for the renewal of mature melanocytes from the stem cell pool [39] (Figure 1B). Consistently, in contrast with ZEB2 which activates MITF transcription, ZEB1 was shown to repress MITF expression and alter pigmentation in retinal pigment epithelial cells [41]. More importantly, ZEB2 knock-down in melanocytes not only impairs MITF expression and differentiation, but also results in ZEB1 induction [39]. A switch from ZEB2 to ZEB1 is thus associated with dedifferentiation of melanocytes. Intriguingly, a similar ZEB2/ZEB1 switch has also been described in some immune cell populations. Indeed, while ZEB2 leads to a differentiation of T CD8 lymphocytes towards T effector cells, ZEB1 induces T memory formation [9]. Overall, despite their high degree of similarity at the structure level, the ZEB1 and ZEB2 zinc-finger homeodomain transcription factors display antagonistic expression patterns and functions in the melanocyte lineage.

### 3. EMT-TF Expression Switch during Melanoma Development

While EMT-TFs are essential for the development and homeostasis of the melanocyte lineage, they also play major roles during melanoma development. Cutaneous melanomagenesis is a multi-step process initiated by the transformation of a normal melanocyte following an oncogenic insult, leading to primary and metastatic melanoma, passing or not passing through a benign nevus stage (Figure 2). A few years ago, we performed a comprehensive analysis of the expression of the entire EMT-TF network by immunohistochemical staining in human samples representative of melanoma progression [40]. We unveiled a completely different scenario compared to carcinoma. Indeed, we showed that a reversible switch in the expression pattern of EMT-TFs occurs during melanomagenesis. A progressive loss of ZEB2/SNAIL2 and a gain in TWIST1/ZEB1 expression were observed along the transition from melanocytes to malignant melanoma (Figure 2). Moreover, this switch from ZEB2/SNAIL2 to TWIST1/ZEB1 was a significant factor of poor prognosis for melanoma patients. These data are consistent with other independent melanoma series showing loss of ZEB2 [39,42] or SNAIL2 [43]. This ZEB2/ZEB1 switch was also associated with reduced patient survival in an independent cohort [44].



**Figure 2.** EMT-TF switch during melanoma development. ZEB2 and SNAIL2 are expressed in differentiated melanocytes within the epidermis of normal adult skin. Their expression is maintained in senescent benign nevi. During the conversion to primary melanoma, SNAIL2 and ZEB2 expression is progressively lost in favor of TWIST1 and ZEB1. Moreover, intra-tumoral heterogeneity of EMT-TFs is observed within melanoma lesions, the EMT-TF switch being generally observed as a gradient from upper part to deeper part of invasive melanoma. Gain of invasive capacity upon ZEB1 reactivation promotes melanoma cell entry into the bloodstream, with clusters of circulating melanoma cells, which may display different differentiation expression patterns. The differentiated pattern of EMT-TFs is reproduced in the secondary site following extravasation, allowing metastatic growth.

The EMT-TF switch is partly regulated by the BRAF/MEK signaling pathway in normal melanocytes. Inhibition of the MAPK pathway with BRAF/MEK inhibitors induces the opposite reprogramming in human melanoma cells. A double-negative feedback loop involving miR-200 family members and ZEB transcription factors is known to regulate carcinoma cell plasticity [45]. However, miR-200 expression levels are low in melanoma and regulation of ZEB1/2 was shown to be largely miR-200 independent. This reversible switch in EMT-TF expression is rather controlled downstream of ERK by a member of the AP-1 complex, FRA1 (FOS related 1), a master transcription factor of the gene regulatory network of the invasive phenotype in melanoma cells [30]. More importantly, alteration of the switch (through inhibition of ZEB1/TWIST1/FRA1 or ectopic expression of ZEB2/SNAIL2) was sufficient to impair BRAF-dependent melanocyte transformation of melan-a cells both in vitro and in vivo in xenograft experiments.

These results reinforce the notion that ZEB1 and/or TWIST1 not only promote invasive features but also display intrinsic oncogenic functions. Indeed, ZEB1/TWIST1 are expressed in the bulk of primary melanoma and ZEB1 ectopic expression promotes tumorigenic features in melanoma cell lines, while its knock-down drastically decreases the tumorigenic growth of melanoma cells in vivo upon xenograft in nude mice [40,46]. In contrast, ectopic expression of ZEB2 and SNAIL2 in melanoma cells decreases tumor formation in nude mice. Therefore, while ZEB1 and TWIST1 harbor oncogenic activities in melanoma, ZEB2 and SNAIL2, which are expressed in normal melanocytes and are required for their proper differentiation, they may act as oncosuppressive proteins in this specific neural crest-derived lineage. A tumor-suppressive role for SNAIL2 and ZEB2 in melanoma was unexpected, although such an activity was suggested for *Zeb2* mRNA through the activation of PTEN expression [42]. Recent data in transgenic mouse models indicated that *Zeb2* knock-out is not sufficient to promote melanoma initiation in the mouse and even impairs NRASQ61-dependent melanoma formation [44], thus precluding a bona fide tumor-suppressor role. ZEB2-enforced expression in this mouse model also promotes growth of primary and metastatic tumors by favoring melanoma cell proliferation. ZEB2 silencing in mouse NRASQ61 melanoma cells decreases MITF, increases ZEB1, and induces proliferation defects. One can hypothesize that ZEB2 expression follows a model mirroring the MITF rheostat: ectopic expression of ZEB2/MITF in melanoma cells would result in differentiation and cell cycle arrest, while ZEB2/MITF silencing would also result in proliferation defects, an intermediate level of ZEB2/MITF would sustain proliferation of melanoma cells. Accordingly, aberrant expression of ZEB2 in melanoma cells may promote terminal differentiation and decreased tumorigenic capacity upon xenograft in nude mice, while intermediate levels of ZEB2 in transgenic mice would be compatible with proliferation. However, the link between EMT-TFs and proliferation remains unclear since we did not observe any defect in proliferation upon modulation of ZEB1 expression in melanoma cells [40,46].

A reversible switch to a proliferative phenotype may be required for metastasis to develop. Accordingly, the ZEB2/SNAIL2 “differentiation pattern” of EMT-TF expression is replicated in cortical areas of lymph node metastases (Figure 2) similarly to that described in primary tumors, suggesting that melanoma cells may re-differentiate at the metastatic site, reminiscent of the mesenchymal-to-epithelial (MET) process in carcinoma [47,48]. Indeed, reversible EMT-MET cycles are required for metastatic colonization in carcinoma. ZEB2/SNAIL2 might, in this model, equally contribute to the high metastatic propensity of melanomas as previously suggested for SNAIL2 [49] and recently demonstrated for ZEB2 in a mouse model in which ectopic ZEB2 expression facilitates the outgrowth of dormant disseminated melanoma cells and promotes the formation of successful metastases [44].

Alternatively, collective migration of clusters of proliferative and invasive cells may underlie metastatic colonization. Of importance, heterogeneous MITF expression has been detected in circulating melanoma cell clusters [50,51] (Figure 2), consistently with the phenotypic heterogeneity described in breast cancer CTCs (circulating tumor cells), where a partial EMT state sustains their migratory capacity [52]. Monitoring the number and phenotype of CTCs may thus prove highly promising for diagnosis and management of melanoma patients.

Hence, while ZEB1 and ZEB2 are largely co-expressed in mesenchymal tumor cells and display similar oncogenic roles in epithelial cell-derived carcinomas, they display opposite expression patterns and functions in melanocytes and melanoma cells, which partly rely on the antagonistic regulation of the master gene MITF.

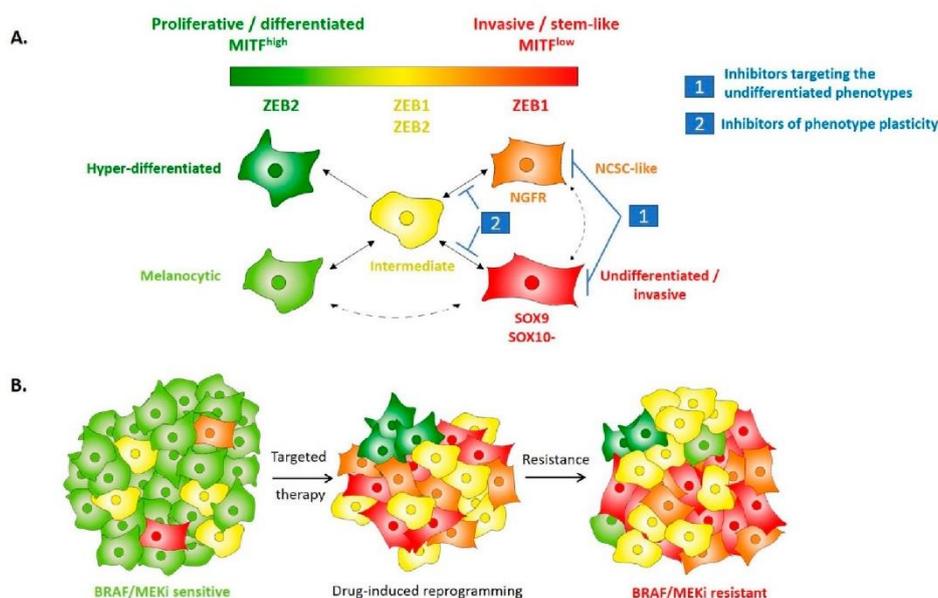
#### 4. EMT-TFs Drive Phenotype Switching of Melanoma Cells and Intra-Tumor Heterogeneity

The model based on MITF-mediated phenotype switching describes the reversible transitions of melanoma cells [27]. Gene expression analyses of melanoma short-term cultures enabled their classification into two states, proliferative or invasive [53]. Xenograft of either proliferative or invasive cells resulted in tumors displaying a similar level of intra-tumor heterogeneity with MITF<sup>high</sup> and MITF<sup>low</sup> cells [28], thus demonstrating that melanoma cells are able to shift in a reversible way between these two states [29]. With regards to EMT-TF expression, cell lines with a MITF<sup>high</sup> differentiated pattern display a higher level of ZEB2, while invasive/undifferentiated cells present an elevated ZEB1 expression. ZEB1 is one of the top-ranking genes whose expression is inversely correlated with MITF in tumors from the TCGA [30,46]. Moreover, intra-tumor heterogeneity was observed within human primary melanoma lesions by immunohistochemical analyses: opposite gradients in the expression of ZEB2/SNAIL2 and TWIST1/ZEB1 were observed from superficial to deep sites (Figure 2) [40,44]. Intra-tumor heterogeneity in the expression of ZEB2/ZEB1 was also documented in the NRASQ61 melanoma mouse model [44]. Interestingly, analyses of EMT-TF expression in the Jerby-Arnon single-cell data set [54] confirmed that ZEB2 and ZEB1 expressions are anti-correlated. More importantly, a mutually exclusive expression pattern of ZEB1 and MITF was documented in human samples with the presence of MITF<sup>high</sup>/ZEB1<sup>low</sup> and MITF<sup>low</sup>/ZEB1<sup>high</sup> subclones [46].

While different studies used ZEB2 and ZEB1 as additional markers to define the proliferative (MITF<sup>high</sup>/AXL<sup>low</sup>) or invasive (MITF<sup>low</sup>/AXL<sup>high</sup>) phenotypes, respectively [55,56], our data further demonstrated that EMT-TFs are major regulators of melanoma cell plasticity that fuel intra-tumor heterogeneity. Indeed, *in vitro* and *in vivo* experiments demonstrated that antagonistic functions of EMT-TFs in melanoma at least partly rely on their differing transcriptional regulation of MITF expression. Gene expression profiles of BRAF-activated melanocytes (melan-a cells) revealed that ZEB2/SNAIL2 activate MITF and cause a melanocyte differentiation gene signature, while ZEB1/TWIST1 repress MITF and generate an invasion-associated gene signature [40]. The converse regulation of MITF expression by ZEB1/ZEB2 may rely on differential recruitment of co-activators (p300) or co-repressors (CtBP), similarly to that described for the regulation of TGFβ/BMP target genes [57], though further mechanistic characterizations are needed to confirm this. Gain- or loss-of-function experiments of ZEB1 in human melanoma cells demonstrated that ZEB1 regulates reversible switching between the proliferative and the invasive phenotype [46]. Overexpression of ZEB1 in melanoma short-term cultures is sufficient to drive switching towards a MITF<sup>low</sup> undifferentiated phenotype bearing stem cell properties, characterized by increased expression of the neural crest stem cell (NCSC) marker NGFR, a major regulator of phenotype switching [46,58]. In contrast, ZEB1 knock-down induces a switch towards a differentiated MITF<sup>high</sup> phenotype with reduced expression of NGFR. The ectopic expression of ZEB2/SNAIL2 promotes differentiation of melanoma cells, while ZEB2 knock-down results in a reduction of MITF expression and a switch from a differentiated to an undifferentiated melanoma cell phenotype [39]. Accordingly, the switch from ZEB2 to ZEB1 in the NRAS melanoma mouse model is associated with the gain of an invasive phenotype [44]. Similarly, in the BRAF; PTEN melanoma mouse model, when tumors are induced on the tail, which better mimics the melanoma transition from a radial to a vertical growth phase [59], the loss of differentiation markers (such as MITF) in a subset of epidermal melanoma cells, before the dermal invasion, was shown to be associated with ZEB1 activation, reinforcing the role of ZEB1 in this transition. Overall, by regulating MITF-dependent phenotype switching, dysregulation of the EMT-TF network contributes to malignant progression of melanoma.

In addition to FRA-1-driven oncogenic signaling, TGF $\beta$  was shown to promote the ZEB2/ZEB1 switch in melanoma cells [44]. The transcription factor GLI2, activated by the TGF $\beta$  signaling pathway, was shown to cooperate with ZEB1 to promote *CDH1* repression, thus participating in the mesenchymal transition [60]. Overall, phenotype switching is dictated by both cell-autonomous and non-cell-autonomous mechanisms. The extensive characterization of both intrinsic and extrinsic factors interdependently contributing to EMT processes will be necessary to fully comprehend melanoma phenotype plasticity.

With the recent generation of gene expression analyses of tumors at the single-cell level, the phenotype switching model has been refined [54,61,62]. Single-cell RNAseq data of melanoma tumors from Irwin Davidson's lab [61] described, in addition to the extreme MITF<sup>high</sup>/AXL<sup>low</sup> and MITF<sup>low</sup>/AXL<sup>high</sup> states, the existence of a novel intermediate state, with the coexistence of proliferative and invasive features. This intermediate phenotype was shown to co-express both MITF and ZEB1 (Figure 3A). While cell lines preferentially adopt either a proliferative or an invasive phenotype, with a clear-mirrored expression of ZEB1 and MITF, tumors in vivo display a higher level of intra-tumor heterogeneity with a significant proportion of cells in the mixed/intermediate state. This is in accordance with our immunohistochemical staining showing co-expression of ZEB1 and MITF in some clones, especially after development of BRAFi resistance (see below and Figure 3B). Accordingly, in a *SMAD4/7* knock-out mouse model, *SMAD4* was required for tumor formation, and a low level of *SMAD7* expression promoted an invasive phenotype and metastatic spread associated with ZEB2/ZEB1 switching [63]. Interestingly, in this model, the presence of MITF/ZEB1 double-positive cells was highlighted, with both proliferative and invasive features.



**Figure 3.** EMT-TFs regulate melanoma phenotype plasticity, intra-tumor heterogeneity and resistance to treatment. (A) Schematic model of putative melanoma intra-tumor heterogeneity according to a classification in five cell states, bearing various differentiation capacities, in accordance with their respective expression of MITF. Expected ZEB1 and ZEB2 expressions in the respective phenotypes are indicated. Expression of the main proposed markers of the different cell states is indicated. The invasive and stem-like phenotype actually represents two different states, both expressing high levels of ZEB1. SOX10 expression is present in all but the dedifferentiated state where it would be replaced by SOX9. The intermediate state should co-express ZEB1, ZEB2, and MITF. The intermediate state is believed to be highly metastable and to give rise to the invasive, neural crest stem cell (NCSC) and hyper-differentiated states, especially upon targeted therapy treatment-induced adaptation. Two-way

arrows indicate reversible switching between cell states. Dotted arrows indicate putative transitions. The two main therapeutic strategies under investigation are also indicated. **(B)** Evolution of intra-tumor heterogeneity of melanoma during treatment with BRAF/MEK inhibitors-targeted therapy. The ZEB2/MITF melanocytic population is eliminated upon treatment and the tumor size decreases. Only resistant phenotypes remain with an increased proportion of NCSC-like and undifferentiated states. ZEB1-increased expression contributes to drug-induced NCSC reprogramming. Tumor adaptation ultimately leads to resistance with a regain of tumor growth and increased intra-tumor heterogeneity compared to the therapy naive tumor. BRAF/MEK resistant tumors may display high ZEB1 expression in both MITF<sup>low</sup> and MITF<sup>high</sup> clones. ZEB1 targeting can induce cell death in BRAFi-resistant melanoma cells, independently of MITF expression level.

Recent single-cell RNAseq analyses demonstrated that melanoma cells can acquire several intermediate phenotypes. A study performed on mouse patient-derived xenografts (PDXs) by the lab of JC Marine [64], identified four cell states as drug-tolerant when exposed to MAPK inhibitors. These states exhibited transcriptional signatures enriched for neural crest differentiation, response to nutrient starvation, EMT, or pigmentation. This elegant work corroborates the four-state differentiation model proposed by Graeber's lab [65]. Despite different nomenclatures, these studies pointed to a more complex model of intra-tumor heterogeneity and cell state reprogramming (Figure 3) [66]. Interestingly, ZEB2 expression progressively decreases as the cell state progresses from a differentiated to a more invasive and dedifferentiated phenotype, while a gain in ZEB1 expression is evidenced in the NCSC-like and invasive phenotype.

High levels of ZEB1/TWIST1 expression undoubtedly promote an invasive phenotype in melanoma, including decreased *E-Cadherin*, MITF, and increased expression of *Vimentin*, *SPARC*, and *MMPs* [30,40]. However, ZEB1 also activates NCSC markers (such as NGFR). We thus believe that ZEB1 is able to increase the expression of both NCSC and invasive markers, possibly not in the same cells, or at least not at the same time. This is consistent with models proposed in carcinoma, where ZEB1 may promote stemness features (partial EMT state associated with tumor initiation) but not necessarily invasive/EMT features, these two features being uncoupled [67]. Overall, ZEB2/ZEB1 may not be associated with the acquisition of a given cell state but may regulate reversible transitions of melanoma cell state in a dynamic manner.

##### 5. EMT-Like Cell Plasticity as a Driver of Resistance to Treatment in Melanoma

In recent years, melanoma has served as proof-of-concept for the development of both targeted- and immunotherapies and is the archetypal model of tumor resistance [68]. The mechanisms of resistance to BRAF and MEK inhibitors (BRAFi and MEKi) have been extensively described over the years [69]. Concomitantly to the acquisition of genetic mutations, leading to the reactivation of the MAPK pathway, increasing evidence now suggests that non-genetic mechanisms also play a major role in the acquisition of drug resistance. Indeed, approximately 40% of BRAFi/MEKi resistant melanoma cases do not present a known genetic alteration [70]. Resistance has been attributed to drug-induced phenotypic adaptations, including the emergence of invasive or NCSC features, relying on epigenetic, transcriptional, or translational reprogramming [31,71–73].

The aberrant activation of an epithelial–mesenchymal transition and the subsequent generation of a cancer stem cell (CSC) phenotype has been largely associated with resistance to therapy in carcinoma, including chemotherapy and targeted therapy such as EGFR inhibitors [74,75]. In melanoma, we provided the first demonstration that ZEB1-mediated phenotype switching is associated with resistance to MAPK inhibitors [46]. Indeed, an elevated level of ZEB1 expression, combined with a low expression of MITF, in melanoma cell lines and patient tumors is associated with an innate resistance to MAPKi. TWIST1 is frequently co-expressed with ZEB1 but is also found in some ZEB1-negative tumors, suggesting that ZEB1 is the main driver of BRAFi resistance, but that TWIST1 may complement ZEB1 when this factor is not activated. ZEB1 and/or TWIST1 expression levels may thus serve as predictive

markers of resistance, at least in a proportion of patients (about 30–50%). Furthermore, increased expression of ZEB1 was observed in patient-derived cell lines with an acquired MAPKi resistance and in biopsies from patients experiencing relapse while under treatment (Figure 3B). Gain- or loss-of-function experiments further showed that ZEB1 is sufficient to promote drug-induced reprogramming towards a NCSC state. In accordance with these results, single-cell RNAseq data highlighted the heterogeneous expression of EMT-TFs and also identified an enrichment of cells in a mesenchymal-like phenotype during the treatment of PDXs with BRAF and MEK inhibitors [64,71]. Finally, recent data suggest that both MITF<sup>low</sup> and MITF<sup>high</sup> phenotypes may be associated with resistance to targeted therapies [31,66]. Accordingly, we observed that high ZEB1 expression in BRAFi-resistant tumors could be found in both MITF<sup>low</sup> and MITF<sup>high</sup> clones [46], and that ZEB1 knock-down decreased the viability of resistant melanoma cells in both MITF<sup>low</sup> and MITF<sup>high</sup> contexts, suggesting that ZEB1 partly functions through MITF-independent mechanisms.

Overall, ZEB1, acting as a transcriptional repressor or activator, owing to its binding to specific co-factors, can down-regulate melanocyte differentiation markers and upregulate melanoma initiating cell markers that cooperate in mediating resistance to MAPKi. Altogether, these studies demonstrate that EMT-TFs are not only markers of specific cell phenotype but define EMT-TFs as part of the central actors controlling melanoma phenotype plasticity and intra-tumor heterogeneity which in turn drive tumor evolution and resistance to current therapies.

In addition to MAPK-targeted therapies, another major breakthrough in the treatment of metastatic melanoma was achieved through immunotherapies targeting negative regulatory checkpoints in immune cells, CTLA4 and PD1 [76]. Nearly 40% of sustained responses were observed with anti-PD1 (Nivolumab, Pembrolizumab), and a combination with anti-CTLA4 may further increase this efficacy [77]. However, despite impressive clinical responses, around 60% of patients still present primary or acquired resistance to these therapies. Mechanisms of resistance to immunotherapy remain unclear. They may rely on tumor microenvironmental properties, including the poor infiltration by immune cells, notably CD8+ T-cells [78]. They likely also involve tumor intrinsic mechanisms, relying on transcriptomic/phenotypic alterations of tumor cells [79]. Indeed, TNF $\alpha$ -induced dedifferentiation of melanoma cells promotes escape from T-cell lysis and resistance to adoptive cell transfer [80]. Activation of the WNT/ $\beta$ -catenin pathway [81,82] or loss of PTEN expression [83] were also shown to promote T-cell exclusion and resistance to immunotherapy in melanoma. Similarly to what was observed with resistance to targeted therapy, preliminary results in a few melanoma patients suggest that immunotherapy may also induce transcriptomic remodeling [84].

The mechanisms by which EMT-dependent cell plasticity mediates immune evasion have been well characterized in carcinoma models, including the regulation of antigen presentation, expression of MHC (major histocompatibility complex), or immune checkpoint ligands such as PD-L1 [85–87]. In melanoma, evidence also suggests that EMT-TFs may contribute to immune escape, as illustrated by the role of SNAIL in the recruitment of Treg lymphocytes [88]. Data obtained in transplanted mouse models of carcinomas also suggested that mesenchymal tumors may display increased resistance to immunotherapy compared to epithelial tumors [87]. However, the role of EMT in the resistance to immunotherapy needs to be further investigated in human tumors. Indeed, the “IPRES” (innate PD1 resistance) signature described by the team of Ribas [89] in melanoma, revealed an enrichment in a “MAPK inhibitor-induced EMT” mesenchymal signature, which includes AXL, WNT5A, or TWIST2, for example, but neither the ZEB nor the SNAIL family members. However, bulk RNAseq analyses have limitations and may be biased since EMT-TFs are expressed in many cell types in the tumor microenvironment, including cancer-associated fibroblasts (CAFs), endothelial cells, but also immune cells (T cells, macrophages, and NK cells). In this respect, inactivation of endothelial ZEB1 expression was recently shown to sensitize tumors to anti-PD1 [6]. Overall, changes in EMT-TF expression levels in tumor cells may be hidden by modulations within the tumor microenvironment, thus emphasizing the requirement for scRNAseq analyses and/or the development of in situ analyses of tumors to better tackle the issue of intra-tumor heterogeneity.

## 6. Perspectives: Targeting of Cell Plasticity to Prevent Resistance to Treatment

Despite recent progress in the treatment of metastatic melanoma, the emergence of resistance and/or toxicities to both targeted- and immunotherapies remains a major barrier to complete remission. A better understanding of cellular and molecular mechanisms underlying phenotypic adaptations, and thus the exceptional capacity of melanoma cells to develop resistance to current therapeutic strategies, may help to define biomarkers of response and new combination therapies.

We have already obtained proof-of-concept that ZEB1-knock-down re-sensitizes resistant cells to BRAF/MEK therapy [46], consistent with data in carcinoma [90]. Therefore, targeting the EMT-TF network represents an attractive treatment strategy for metastatic melanomas. However, the targeting of EMT as a highly reversible plasticity process is a challenging issue, given that EMT and MET cycles are required at different stages of tumor progression. Indeed, reverting EMT could result in the outgrowth of tumor cells already seeded in a metastatic niche. Strategies aiming at preventing the mesenchymal transition at an early stage, thus preventing dissemination, or at targeting the dedifferentiated states are still under investigation, including targeting of the TGF $\beta$  pathway [91] (Table 1 and Figure 3).

**Table 1.** Short list of the main therapeutic strategies under evaluation to target EMT-dependent cell plasticity in cancers.

Strategy	References	Target	Drug/Compound	Clinical Trial	Trial ID	Cancer Type
TGF $\beta$ pathway inhibitors	[91]	TGF $\beta$ monoclonal antibody	GC1008 (fresolimumab)	Phase I	NCT00356460	Renal Cell Carcinoma and Malignant Melanoma
AXL targeting	[92]	AXL small molecule inhibitor	BGB324	Phase I/II in combination with either dabrafenib/trametinib or pembrolizumab	NCT02872259	Metastatic Melanoma
Epigenetic drugs	[93,94]	HDAC inhibitors	Entinostat	Phase II in combination with erlotinib	NCT00602030	Non-Small-Cell Lung Cancer
	[95]		Vorinostat	Phase I	NCT02836548	Resistant BRAFV600 melanoma
Metabolic inhibitors	[96,97]	Inducible nitric oxide synthase (iNOS)	L-NMMA	Phase Ib/2 in combination with Taxane	NCT02834403	Triple Negative Breast Cancer
Ferroptosis inducers	[65,98]	GPX4 (through glutathione depletion)	PRLX 93936 (Erastin analog)	Phase I	NCT00528047	Advanced Solid Tumors

Accordingly, a promising approach was based on methotrexate-mediated upregulation of MITE, which results in the differentiation of melanoma cells, an abrogation of their migratory capacities, and sensitization to the cytotoxic agent TMECG [99]. Other therapeutic strategies aim at combining conventional therapies with drugs specifically targeting undifferentiated/invasive melanoma cells. Targeting of AXL in the invasive state, thanks to an AXL antibody linked to a microtubule-disrupting agent, cooperates with MAPK inhibitors in inhibiting tumor growth [92]. Similarly, inhibition of the retinoic receptor RXR, a key driver of the NCSC identity, synergizes with MAPK inhibitors in vitro and increases the tumor latency following xenograft [64]. Interestingly, undifferentiated melanoma cells were also shown to display an altered metabolic activity with a reduced glutathione level compared to differentiated cells [65]. The diminished glutathione quantity is correlated with an increased sensitivity to ferroptosis induced by Erastin treatment. Erastin effectively synergizes with BRAF inhibitors and decreases the number of persistent dedifferentiated melanoma cells in vitro. Accordingly, drugs affecting cell metabolism and more specifically inducing ferroptosis have also shown promising results

in other cancer models with a mesenchymal phenotype [98]. Since metabolic reprogramming is intimately linked to EMT, promising pharmacological inhibitors of metabolic pathways are currently investigated for their capacity to target EMT [96,97].

Finally, epigenetic plasticity has been proposed to account for the rapid adaptation of melanoma cells to treatment. Multiple epigenetic modifiers have been shown to regulate EMT-TF expression or to directly interact with EMT-TFs [100]. It would be of utmost interest to define the network of epigenetic regulators that cooperate with EMT-TFs in melanoma to drive epigenome remodeling and rapid phenotype switching of melanoma cells in order to identify relevant epidrugs. Notably, inhibition of the histone methyltransferase EZH2 has been shown to promote dedifferentiation of melanoma cells, restore immunogenicity, and re-sensitize tumors to immunotherapy [101]. HDAC inhibition has also been reported to increase response to immunotherapy [93,94]. Several clinical trials investigating the combination of epigenetic drugs with MAPK inhibitors or immunotherapy are ongoing and the results will reveal the feasibility of such therapeutic approaches in melanoma [95,102].

## 7. Conclusions

In conclusion, recent evidence has started to unveil the specific expression patterns and functions of EMT-TFs in the melanocyte lineage and melanoma, compared to epithelial-derived cancers. Overall, EMT-TFs appear as major regulators of phenotype switching in melanoma, between differentiated/proliferative and neural crest stem cell-like/invasive states, contributing to the emergence of resistance to current therapeutic strategies in melanoma. Further research is still needed to characterize the precise molecular mechanisms by which EMT-TFs mediate these reversible transitions, taking into account the plastic intermediate states. Single-cell approaches and in situ analyses of melanoma patient samples, upon treatment, will be of major interest in order to more precisely address EMT-TFs intra-tumor heterogeneity and their crosstalk with the immune microenvironment. Finally, future investigations will decipher the molecular crosstalk of EMT-TFs with epigenetic and metabolic regulators. This should lead to the development of novel therapeutic strategies aiming at targeting cell plasticity, which may be tested in combination with targeted- or immunotherapies.

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### **Characterization of the mechanisms of action of the transcription factor ZEB1 in melanoma**

Melanoma intra-tumor heterogeneity depends on reversible phenotypic transitions between proliferative and invasive states. This phenotype switching relies on transcriptomic and epigenetic mechanisms, which need to be better defined. Aberrant reactivation of the epithelial to mesenchymal transition inducing transcription factor (EMT-TF) ZEB1 was known to play pleiotropic roles in carcinoma development. In melanoma, a non-epithelial tumor, previous work from our team demonstrated that ZEB1 reactivation favors phenotype switching from a proliferative/differentiated towards an invasive/stem-like state, along with acquired resistance to target therapies. Herein, in order to precisely characterize ZEB1 function and get a comprehensive view of its direct target genes, we performed a CHIP-Sequencing combined with RNA-seq, upon phenotype switching in melanoma cells. Many melanoma cell identity genes were identified and validated to be directly regulated by ZEB1. Down-regulation of proliferative/differentiated genes and up-regulation of invasive/stem-like genes were confirmed through gain or loss of function of ZEB1. Intra-tumor heterogeneity was also assessed in human samples via multi-immunofluorescence approach. Spatial distribution of melanoma states markers were analyzed relying on ZEB1 expression and we found that ZEB1 was not only expressed in invasive but also in stem-like state. In summary, our results provide a better understanding of ZEB1 mechanisms of action governing melanoma cell plasticity and intra-tumor heterogeneity.

**Keywords:** melanoma, ZEB, CHIP-Seq, mechanistic, phenotype switching.

### **Caractérisation des mécanismes d'action du facteur de transcription ZEB1 dans le mélanome**

L'hétérogénéité intra-tumorale des mélanomes repose sur des transitions phénotypiques réversibles entre un état prolifératif et un état invasif. Ce changement phénotypique est contrôlé par des mécanismes transcriptionnels et épigénétiques qui demeurent à mieux caractériser. L'activation aberrante du facteur de transcription de la transition épithéliale mésenchymateuse (EMT-TF) ZEB1 était connue pour jouer des rôles pléiotropiques dans le développement des carcinomes. Dans le mélanome, d'origine non épithéliale, des travaux antérieurs de notre équipe ont démontré que la réactivation de ZEB1 favorise la transition d'un état prolifératif/différencié vers un état invasif/stem-like, ainsi que la résistance acquise aux thérapies ciblées. Afin de caractériser précisément la fonction de ZEB1, et obtenir une vision globale de ses gènes cibles directs, un CHIP-Seq combiné avec du RNA-seq a été réalisé, au cours des transitions phénotypiques dans des cellules de mélanome. De nombreux gènes contrôlant l'identité des cellules de mélanome ont été identifiés et validés comme étant directement régulés par ZEB1. La répression de gènes associés à la prolifération ou la différenciation et l'activation de gènes impliqués dans l'invasion et la « stemness » ont été confirmées par des approches de gain ou perte de fonction de ZEB1. L'hétérogénéité intra-tumorale de ces marqueurs a également été étudiée dans des échantillons humains par une approche de multi-immunofluorescence. La distribution spatiale de marqueurs du phénotype des cellules de mélanomes a été analysée en lien avec l'expression de ZEB1 et nous avons constaté que ZEB1 est non seulement exprimé dans les états invasifs mais aussi dans les états stem-like. En résumé, nos résultats ont permis de mieux décrypter les mécanismes d'action de ZEB1, gouvernant la plasticité de cellules de mélanome ainsi que l'hétérogénéité intra-tumorale.

**Mots clés :** mélanome, ZEB, CHIP-Seq, mécanistique, phenotype switching.