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# Ovol2 factor, a novel regulator of cell migration in metastatic cutaneous melanoma

Laura Medina Cuadra

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# Ovol2 factor, a novel regulator of cell migration in metastatic cutaneous melanoma

*Le facteur Ovol2, un nouveau régulateur de la migration cellulaire dans le mélanome cutané métastatique*

**Thèse de doctorat de l'université Paris-Saclay**

École doctorale n° 577, Structure et dynamique des systèmes vivants (SDSV)  
Spécialité de doctorat : Biologie du développement  
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Thèse préparée dans l'unité de recherche **Signalisation, radiobiologie et cancer** (Université Paris-Saclay, Inserm, CNRS), sous la direction d'**Anne-Hélène MONSORO-BURQ**, Professeure et le co-encadrement de **Leonid PESHKIN**, Directeur de recherche

**Thèse soutenue à Paris-Saclay, le 22 Mars 2023, par**

**Laura MEDINA CUADRA**

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**Titre :** Le facteur *Ovol2*, un nouveau régulateur de la migration cellulaire dans le mélanome cutané métastatique

**Mots clés :** Mélanome, *OVOL2*, migration, crête neurale

**Résumé :** La transition épithélium-mésenchyme (EMT) est un processus dynamique par lequel les cellules épithéliales polarisées acquièrent un phénotype mésenchymateux, migratoire et invasif. L'EMT est cruciale au cours du développement embryonnaire, de la régénération des tissus et de la cicatrisation des plaies, mais elle participe également à la progression des tumeurs, aux métastases et à la résistance aux chimiothérapies. L'EMT est activée par plusieurs facteurs de transcription (EMT-TFs) dont *Snail1/2*, *Twist1* et *Zeb1/2*. En revanche, la régulation négative de l'EMT reste moins bien comprise. *Ovol2* encode un doigt de zinc transcriptionnel important pour le développement épithélial de la peau et un répresseur critique pour l'inhibition de l'EMT dans la peau en développement et les tissus cancéreux tels que les cancers du sein ou de la prostate. Son rôle potentiel dans le mélanome reste non décrit.

Cette thèse explore l'hypothèse qu'*OVOL2* pourrait interférer négativement avec la migration cellulaire et la formation de métastases des cellules du mélanome cutané. Les résultats montrent que *Ovol2* est exprimé dans le mélanome chez l'homme. Nous avons modulé l'expression d'*OVOL2* pendant la migration cellulaire in vitro et pendant la formation de métastases in vivo afin de comprendre les mécanismes moléculaires en jeu. La modulation des niveaux d'expression du gène *ovol2* affecte la migration des cellules de mélanome et l'expression de certains EMT-TFs, mais surtout de facteurs d'adhésion cellule-cellule, cellule-matrice et du cytosquelette. Identifier les signaux qui contrôlent l'expression du gène *Ovol2* in cellulo et in vivo pourrait permettre de proposer *Ovol2* comme une nouvelle cible clé dans l'intervention thérapeutique dans le mélanome.

**Title:** *Ovol2* factor, a novel regulator of cell migration in metastatic cutaneous melanoma

**Keywords:** Melanoma, *OVOL2*, migration, neural crest

**Abstract:** The epithelial-to-mesenchymal transition (EMT) is a dynamic process by which polarized epithelial cells acquire a mesenchymal, migratory, and invasive phenotype. EMT is crucial during embryonic development, tissue regeneration and wound healing, but also participates in tumour progression, metastasis, and chemotherapeutic resistance. EMT is activated by several transcription factors (EMT-TFs) including *Snail1/2*, *Twist1* and *Zeb1/2*. In contrast, the negative regulation of EMT remains less understood. *Ovol2* encodes a zinc finger transcriptional important for skin epithelial development and critical for EMT inhibition in cancer tissues such as breast or prostate cancers. Its potential role in melanoma remains undescribed.

This thesis explores if *OVOL2* could interfere negatively with melanoma cells metastasis. We have modulated *Ovol2* during cell migration in vitro and metastasis in vivo to understand the molecular mechanisms at work. We find that *OVOL2* is expressed in human cutaneous melanoma. Moreover, modulating *Ovol2* gene expression levels affects melanoma cells migration and expression of some EMT-TFs. However, the main transcriptional effect observed is to modulate cell-cell, cell-matrix and cytoskeletal-related factors. By identifying the signals that control *Ovol2* gene expression in cellulo and in vivo, we expect to propose *Ovol2* as a new target for future therapeutical intervention in melanoma.



## ABSTRACT

Metastasis is the cause of more than 90% of cancer-related deaths. Tumour invasion and metastasis result from complex processes involving local invasion, intravasation, transport, extravasation, and colonization of distant regions. These cellular behaviours are regulated by a delicate balance between the epithelial-to-mesenchymal transition (EMT) and the reverse program, mesenchymal-to-epithelial transition (MET). EMT occurs in physiological conditions during embryonic development, tissue regeneration and wound healing. It is a dynamic process by which polarized epithelial cells lose their cell-cell adhesions, apical-basal polarity, and epithelial phenotype to acquire a mesenchymal phenotype that enhances their migratory and invasive capability. EMT is also involved in metastasis during tumour progression and can enhance cells' resistance to chemotherapy. Multiple signalling pathways, including TGF- $\beta$ , Wnt/ $\beta$ -catenin, Notch, EGF, HGF, FGF, and HIF, are important for the maintenance of epithelia and for the EMT-MET equilibrium both in normal development and cancer. Moreover, downstream of these signals, EMT is regulated by a complex regulatory network which involves EMT transcription factors (EMT-TFs) Snail1, Snail2, Twist1, Zeb1 and Zeb2. While knowledge of the mechanisms controlling EMT grows rapidly, the pathways blocking EMT and mediating MET are far to be understood.

Among the potential negative regulators of EMT, OVOL1/2 are zinc finger-containing transcriptional repressors of the evolutionarily conserved Ovo-like protein family, identified as a critical regulators of skin keratinocyte differentiation and of wound healing. They are also described as EMT repressors in breast and prostate cancer through the direct and specific transcriptional inhibition of ZEB1 (Roca et al., 2013). OVOL2 have also shown to inhibits TGF- $\beta$  signalling-induced EMT at multiple levels in mouse and human mammary epithelial tumour cells. This includes the inhibition of SMAD4 expression and blocking the binding between SMAD4 and target DNA sequences. This produce a reduction in the expression of EMT-TFs genes such as snail1 or twist1, resulting in decreased cell migration of mouse mammary epithelial cells (R. S. Wu et al., 2017).

Moreover, in osteosarcoma, OVOL2 protein expression decrease in advances stages of the disease. However, the role of OVOL2 in neural crest-derived metastatic tumours such as melanoma or neuroblastoma have not been explored. The previous studies showing that OVOL2 inhibits EMT, and the fact that melanoma metastasis involves EMT-like mechanisms, prompted us to explore the importance of this gene in the metastatic potential and the clinical outcome of melanoma.

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## ABBREVIATIONS

**ATAC-Seq** Assay for Transposase-Accessible Chromatin using sequencing.

**AK** Actinic keratosis

**bHLH** basic helix-loop-helix

**BMP** « Bone morphogenetic protein »

**BRAF** B-Raf proto-oncogene

**CDKN2A** Cyclin Dependent Kinase Inhibitor 2A

**CDK4** Cyclin-Dependent Kinase 4

**ChIP-Seq** Chromatin immunoprecipitation sequencing

**CSC** Cancer stem cells

**cSCC** Cutaneous squamous cell carcinoma

**DNA** Deoxyribonucleic acid

**ECM** Extra cellular matrix

**EMA** European Medicines Agency

**EMT** Epithelial-mesenchymal transition

**EndMT** endothelial to mesenchymal transition

**EU** European union

**FDA** Food and drugs Administration

**FGF** fibroblast growth factor

**GDP/GTP** Guanosine triphosphate/Guanosine diphosphate

**GRB2** Growth factor receptor-bound protein 2

**GRHL**

**HNDFs** human neonatal dermal fibroblasts

**IL-2** Interleukin-2

**LC** Langerhans cells

**MAPK** « Mitogen-activated protein kinase »

**MET** Mesenchymal to epithelial transition

**MITF** « Melanocyte-microphthalmia transcription factor »

**MMPs** Matrix metalloproteinases

**NC** Neural crest

**NCC** neural crest cells

**NMuMG** mouse mammary epithelial cells

**NP** neural plate

**OAR domain**

**OMIM Online Mendelian Inheritance in Man**

**PI3K** phosphoinositide 3-kinase

**PPCD** Posterior polymorphous corneal dystrophy

**PTEN Phosphatase and tensin homolog**

**RAS** Rat sarcoma

**RNA-Seq** ribonucleic acid sequencing

**TGF- $\beta$**  *transforming growth factor beta*

**TFs** Transcription Factors



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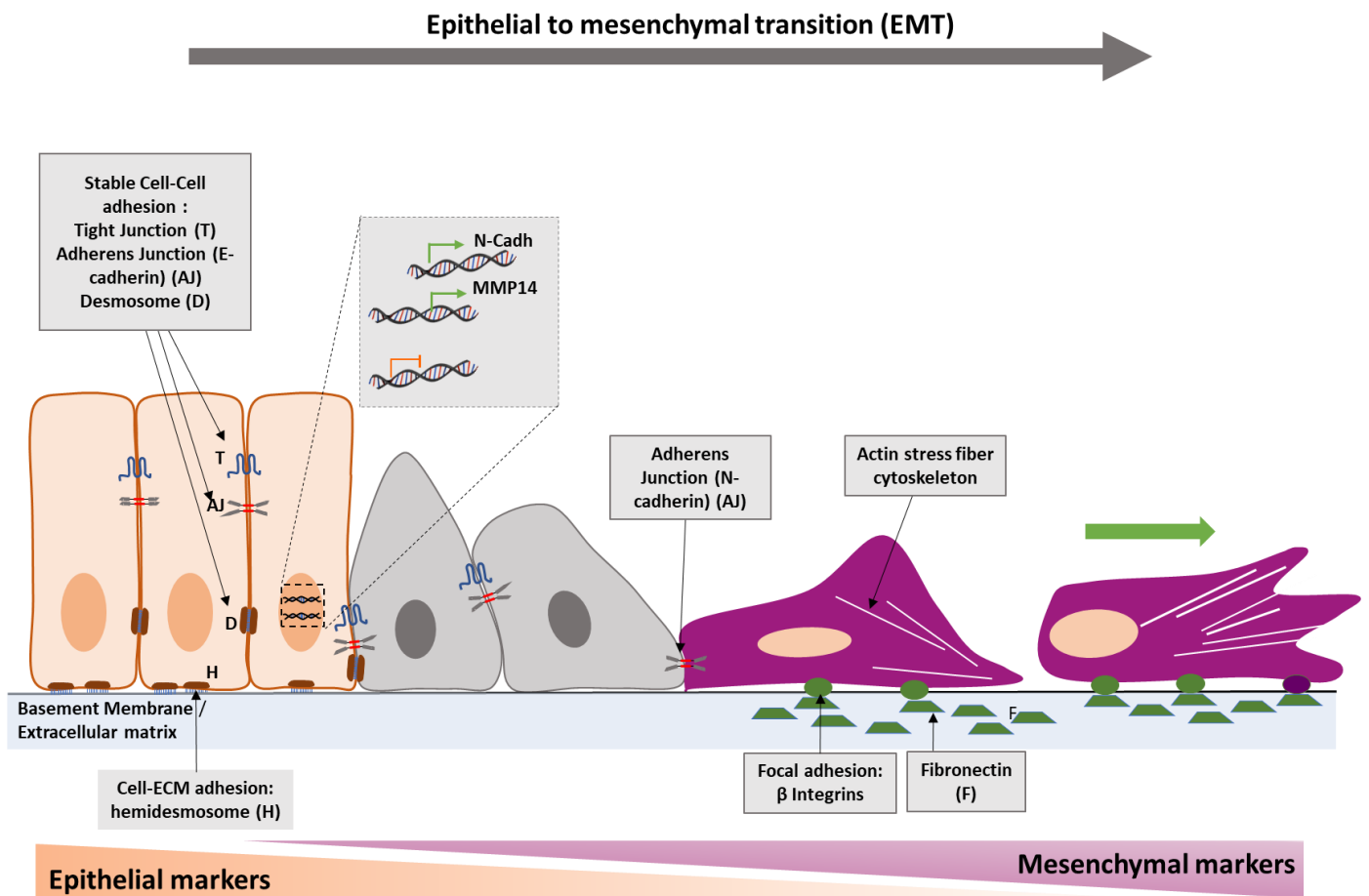
# INTRODUCTION

# 1. The epithelial-to-mesenchymal transition (EMT)

The main goal of this thesis work is to explore the regulation of EMT and cell migration by the factor OVOL2 in melanoma, a neural crest-derived cancer. For that, in this first chapter I will present the 3 types of EMT, the main actors involved in the induction of this process, and I illustrate the process of epithelial-to-mesenchymal transition (EMT) during neural crest development as a model of EMT under physiological conditions.

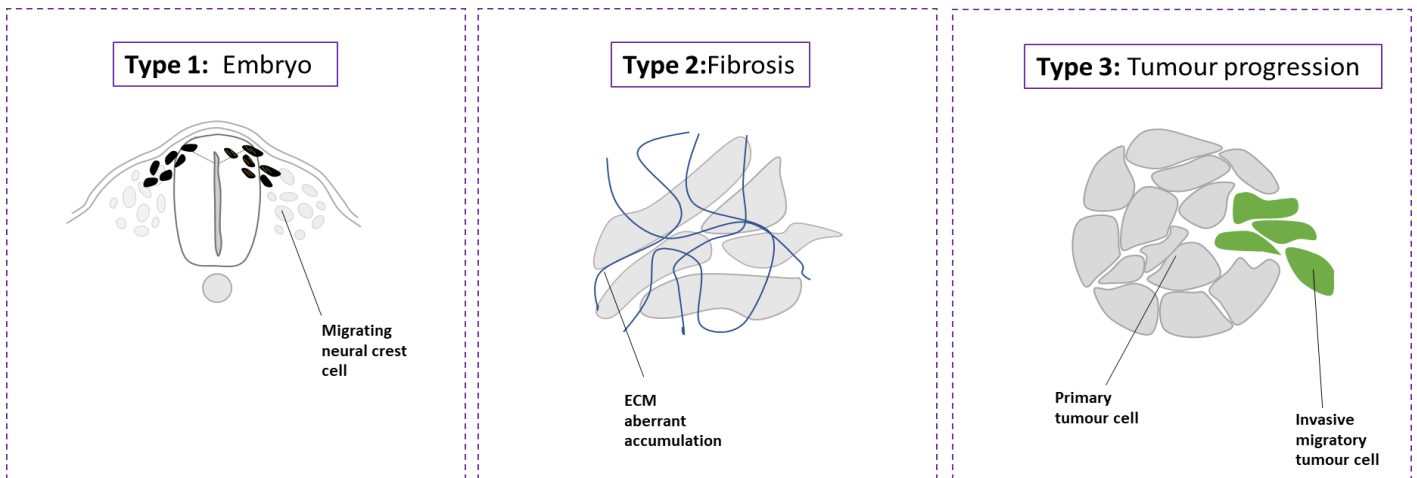
## 1.1 The different types of EMT

The epithelial-to-mesenchyme transition (EMT) is a key process by which cells with an epithelial polarized phenotype, which interact with the basal membrane by their basal surface and share multiple cell-cell adhesion contacts, undergo a series of changes that enable them to migrate by the acquisition of a mesenchyme-like phenotype (Figure 1). This process is essential for tissue patterning and organization, and it has also been implicated in a wide array of pathologies including organ fibrosis and cancer metastasis. However, the molecular pathways and signals implicated in the different EMT contexts are not clearly defined. During subsequent EMT meetings between 2007 and 2008, a proposal about the classification of EMT into three different biological types based on the biological context came out (Figure 2).



**Figure 1. Embryo development of *Xenopus laevis*.**

Before undergoing EMT, neural crest cells are situated in the neural border ectoderm forming an epithelial layer. They are in proximity between each other joined by tight and adherent junctions and present stable interactions with the extracellular matrix. During the process of EMT, the epithelial cells experience a series of transcriptional and post-transcriptional changes that progressively changes their morphology and transiently convert them into a mesenchymal-like phenotype. Cells lose epithelial markers and the stable connection like desmosomes, adherent junctions and tight junctions and start to express mesenchymal markers such as N-cadherin or fibronectin. Those changes are together with an extensive remodelling of the cytoskeleton of the cells.



**Figure 2. Types of EMT**

Type 1 EMT occurs during embryonic development. The two main examples are during gastrulation and during the delamination of the neural crest cells. Type 2 EMT occurs in organ fibrosis. This affectation occurs in epithelial tissues and is created by abnormal accumulation of inflammatory signals and molecules from the extracellular matrix such as collagens, laminins, elastin and tenascins produced or activated by fibroblast and myofibroblast differentiated from normal epithelial cells. Type 3 EMT occurs when epithelial tumour cells detach from the primary tumour mass and migrate until reaching circulation to finally metastasize in distant regions.

### 1.1.1 Type 1: EMT in NCC delamination

EMT is a crucial process during embryonic development, happening at different time-points and in different cell types. The better studied ones happen during gastrulation and neural crest cells (NCC) delamination. Ectoderm cells from the primitive streak undergo EMT during gastrulation process to form multi-layered embryo called gastrula (Acloque et al., 2009). Here I will describe the EMT process observed during NCC migration as a well-documented example of canonical embryonic EMT. During embryonic development, a subpopulation formed adjacent to the neural epithelium gives rise to migratory neural crest cells that undergo EMT at the end of neurulation (Acloque et al., 2009). Just before the closure of the neural tube, the premigratory population of NCC express the specific genes, such as *Sox8-9-10*, *snail1*, *snail2*, *cmyc* and *foxd3*. The transcription factors encoded by *snail1* and *snail2* activate EMT on those cells by inhibiting expression of cell-cell adhesion molecules (*cdh1*) and activating invasion-related genes (*cdh2*, *metalloproteases*, etc). Consequently, NCC dissociate from the neural folds, acquire motility, and navigate along stereotypic pathways throughout the embryo to reach their destination where they will differentiate among other cell types. This EMT occurring in the neural crest is triggered by the signalling pathways including Wnts, FGFs and BMPs cooperate to induce EMT (Jr et al., 2000; Karafiat et al., 2007). Finally, the formation of NCC derivatives in distinct final locations requires the opposite process also called MET. This makes neural crest cells a precious model where to study canonical EMT under physiological conditions. More details will be explained in following sections dedicated to the molecular and cellular aspect of EMT in NCC.



### 1.1.2 Type 2: EMT in fibrosis

EMT also plays an important role in different types of fibrosis, including lung and liver fibrosis. Fibrosis is caused by a dysregulated and excessive deposition and accumulation of extracellular matrix (ECM) components such as fibronectin and collagen (Distler et al., 2019). The formation of fibrotic tissue disrupts the physiological architecture of organs leading to organ malfunction and is commonly associated with high morbidity and mortality. The key cellular mediator of fibrosis is the myofibroblast, which is the main cell type responsible of the uncontrolled production of collagen and other extracellular proteins. The origin of this cell type is still controversial and has not been completely elucidated. Myofibroblasts represent a specialized fibroblast population originated from different sources, including expansion of on-site fibroblast, migration and accumulation of circulating fibrocytes derived from bone-marrow stem cells or from endothelial and epithelial cells that have undergone EndMT/EMT respectively (Piera-Velazquez et al., 2011). Moreover, some studies have proven that the contribution of EMT to fibrosis is not only due to a transition of epithelial cells into mesenchymal phenotype but also through the release of paracrine signals that contribute to the transformation of fibroblasts into myofibroblasts and to the secretion of cytokines and chemokines responsible for recruiting macrophages to the stroma (Grande et al., 2015).

### 1.1.3 Type 3: EMT in metastasis

Metastasis is principal cause of cancer morbidity and mortality. High cellular plasticity is one of the critical cell properties in cancer cells and tumour progression, enabling the cancer cells to acquire properties that provide survival advantage in adapting to different microenvironments and the acquisition of migratory properties. Tumour invasion and metastasis are the result of a complex series of processes that include local invasion, intravasation, transportation through the lymph and blood flows and colonization of distant places. Numerous studies have demonstrated that the epithelial-to-mesenchymal transition (EMT) and its reverse process (MET) play a key role in the induction and progression of cancer cell migration and metastasis (Moustakas & Heldin, 2007). As mentioned before, EMT is characterized by remarkable changes in cell-cell adhesion capacity of epithelial cells, the loss of cell polarity and the expression of epithelial markers such as E-Cadherin (CDH1) and it is believed to be a major mechanism by which cancer cells become migratory and invasive. In cancer context, when cells undergo EMT, they are able to dissociate from the primary tumour and acquire migratory properties that allow them to enter circulation. In the contrary,

mesenchymal-to-epithelial transition consists of the reverse process by which cells colonize distant metastatic places and reform a secondary tumour. A series of EMT/MET conversions is crucial, not only for the complex remodelling of embryo and organ formation during gastrulation and organogenesis as mentioned before but also are recognized as critical events for metastasis of many tumours, especially carcinomas (Saenz-Sardà et al., 2018).

Experimental support for the importance of MET in the development of cancer metastasis is increasing. This experimental information is mostly based on in vitro analysis or animals models focused on cancer metastasis studies in which MET is induced by repressing the expression of main EMT-TFs (Ocaña et al., 2012; Tsai et al., 2012). This leads to the hypothesis that blocking EMT is sufficient to induce the opposite process MET (Dongre & Weinberg, 2019). This suggests that therapeutic strategies focused on blocking of EMT may favour MET in cancer cells that have already disseminated into the bloodstream and therefore, promote secondary metastasis. Studies based on four isogenic mouse breast cancer cell lines (67NR, 168FARN, 4TO7, and 4T1) with different metastatic potential were implanted into mammary fat pads to model the steps of metastasis. However, only those that acquire epithelial properties such as high E-cadherin expression were able to form macroscopic metastasis at lung and liver, indicating the importance of the acquisition of epithelial properties at this step of metastasis formation (Dykxhoorn et al., 2009). Despite the wide knowledge about the role of EMT mediating factors in cancer, the mechanism that mediate MET are far less understood. OVOL and GRHL families, are some of the few families of genes known as MET-TFs (Figure 3). As expected, those families of genes, present a strongly positive correlation with CDH1, an epithelial marker and a negative correlation with VIM, a mesenchymal marker in 1038 CCLE (cancer cell line encyclopaedia) microarrays for cancer cell lines (Watanabe et al., 2019).

On the contrary, well-known EMT-TF genes, such as SNAI, TWIST, and ZEB families, exhibits opposite correlations. Similar studies demonstrated that among the known MET-TFs factors, OVOL2 is the only one which is strictly necessary for inducing CDH1 and the epithelial phenotype in human neonatal dermal fibroblasts (HNDFs) (Watanabe et al., 2019).

## 1.2 Key transcription factors activating EMT.

### 1.2.1 SNAIL1/2 family of transcription factors

The first member of *snail* gene family was discovered in *Drosophila melanogaster* (Grau et al., 1984). After that, many homologues have been found in different species of vertebrates. Snail genes encode zinc-finger type transcription factors that share a similar organization. The TFs are composed by a highly conserved carboxy-terminal region which contains between four and six zinc fingers and by an amino-terminal region that is more variable. The zinc-fingers acts as sequence-specific DNA-binding motifs and are structurally composed by 2  $\beta$ -strands separated by an  $\alpha$ -helix. The amino-terminal part of the  $\alpha$ -helix binds to the major groove of the DNA. Snail family members bind specifically to target genes in a consensus sequence -CANNTG- named E-Box. They can be activated by different pathways depending on the physiological context (Peinado et al., 2007). Once bound to E-boxes, they act as transcriptional repressors of epithelial genes such as E-cadherin, a transmembrane component of adherent junctions that form between epithelial cells (Batlle et al., 2000; Baulida & D, 2019). As mentioned above, SNAIL1 and SNAIL2 play critical roles in inducing EMT during embryonic development and cancer metastasis (Aybar et al., 2003; Batlle et al., 2000; del Barrio & Nieto, 2002; Jäggle et al., 2017). The lack of SNAIL1/2 in *Xenopus* embryos lead to a reduction of the neural crest markers expression and the inhibition of EMT and migration of neural crest cells (Labonne, 2000; Mayor et al., 2000).

### 1.2.2. TWIST1/2

TWIST1 and TWIST2 belong to an evolutionary conserved basic helix-loop-helix (bHLH) family. The bHLH motif is formed by a short sequence of basic amino acids followed by two amphipathic  $\alpha$ -helices that are separated by a loop of variable length. They dimerize and bind to E-boxes cis-regulatory regions (5'-CANNTG-3'), in the promotor region of their target genes. Both isoforms are key regulators in embryonic development and organogenesis. Several studies have demonstrated that Twist1 is implicated in many fibrotic diseases (Ning et al., 2018). However, the roles of TWIST2 in these diseases are not so explored. As does Snail1, Twist1 downregulates epithelial gene expression

and activates mesenchymal gene expression. In cancer cells (J. Xu et al., 2016), Twist1 can downregulate E-Cadherin expression and induce N-Cadherin expression independently of Snail1 function (J. Xu et al., 2016; Yang et al., 2010).

### 1.2.3 ZEB1/ZEB2

The Zinc finger E-box binding homeobox family is a transcription factor family that includes ZEB1 (also known as TCF8 and  $\delta$ EF1) and ZEB2 (also known as SIP1). Both proteins are composed by two flanking C2H2-type zinc finger clusters that interact with E-boxes (5'-CACCT(G)-3') in the promoter of their target genes. The zinc finger clusters are separated by a central POU-like homeodomain deprived of DNA-binding activity (Vandewalle et al., 2009). In addition to these domains, both proteins present different protein-binding domains to bind to several proteins such as Smads and CtBP (Caramel et al., 2018). Therefore, ZEB factors have the ability to act as repressor and activators. This dual activity makes this family of proteins very important in the control of many processes including EMT-induced processes in development, differentiation, and malignant mechanisms (cancer stem cell-like traits, apoptosis, cell cycle arrest and metastasis). ZEB factors are mainly associated with tumour progression and metastasis (Krebs et al., 2017; P. Zhang et al., 2015). However, ZEB2 also promotes proliferation of metastatic melanoma while reducing its invasive mesenchymal-like phenotype (Vandamme et al., 2020)

### 1.2.4 PRRX1/2

PRRX1/2 are transcription factors belonging to the paired homeobox family. PRRX1 presents two isoforms a and b. Isoform PRRX1a is usually called PRRX1 since it is the canonical isoform of PRRX1. This protein is a transcriptional activator and contains two important domains; an OAR domain of 15 amino acids and homeobox DNA-binding domain of 60 amino-acids that binds to DNA via a helix-turn-helix structure. The isoform PRRX1b shares most of the sequence with PRRX1a but lacking the OAR domain. The presence of DNA-binding sequences of those genes allows them to act as transcription factors or coactivators and regulate the expression of target genes. PRRX1 and PRRX2 are important factors for the development of mesenchymal tissues and are involved in several organogenesis processes (Higuchi et al., 2014). PRRX1 is involved in tumour metastasis and is closely correlated to the occurrence of EMT. On the other hand, the relationship of PRRX2 with cancer

metastasis is more controversial. Abnormal expression of PRRX2 has been observed in metastatic gastric cancer, acting as a potential biomarker (Feng et al., 2015). However, PRRX2 also inhibits the Wnt/ $\beta$ -catenin signalling pathway and interrupts EMT process of colon cancer cells, and consequently inhibiting their invasion and migration (W. X. Chai et al., 2019)

### 1.3. The Neural Crest: A model of canonical EMT.

#### 1.3.1 Neural crest development.

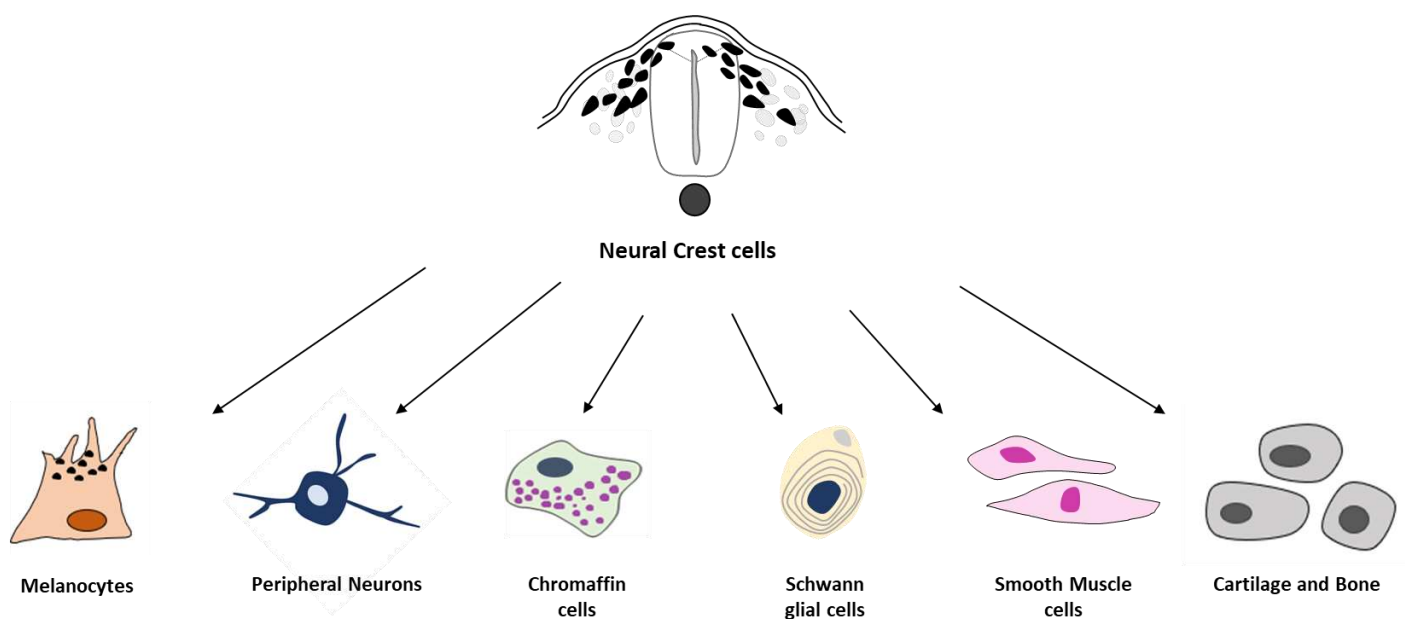
The neural crest (NC) cells form a multipotent cell population that arises from the ectoderm germ layer during early vertebrate development. This population of cells is unique for the vertebrate embryos and gives rise to a wide diversity of cell types that form part of diverse tissues and organs in body. In order to do that, they delaminate from their origin and emigrate collectively or individually throughout the body. They navigate along several different pathways where they finally differentiate into a diverse array of cell types including neurons, glia, peripheral nervous system (PNS), melanocytes, cranial cartilage and bone and others (Figure 3). In the case of humans, up to 47 cell types have been defined as NC derivatives. This physiological cellular phenomenon exemplifies the different step that are also observed during tumour invasion and metastasis and strengthen the analogies between physiological developmental EMT and EMT in cancer metastasis.

#### 1.3.2 Neural crest induction and formation

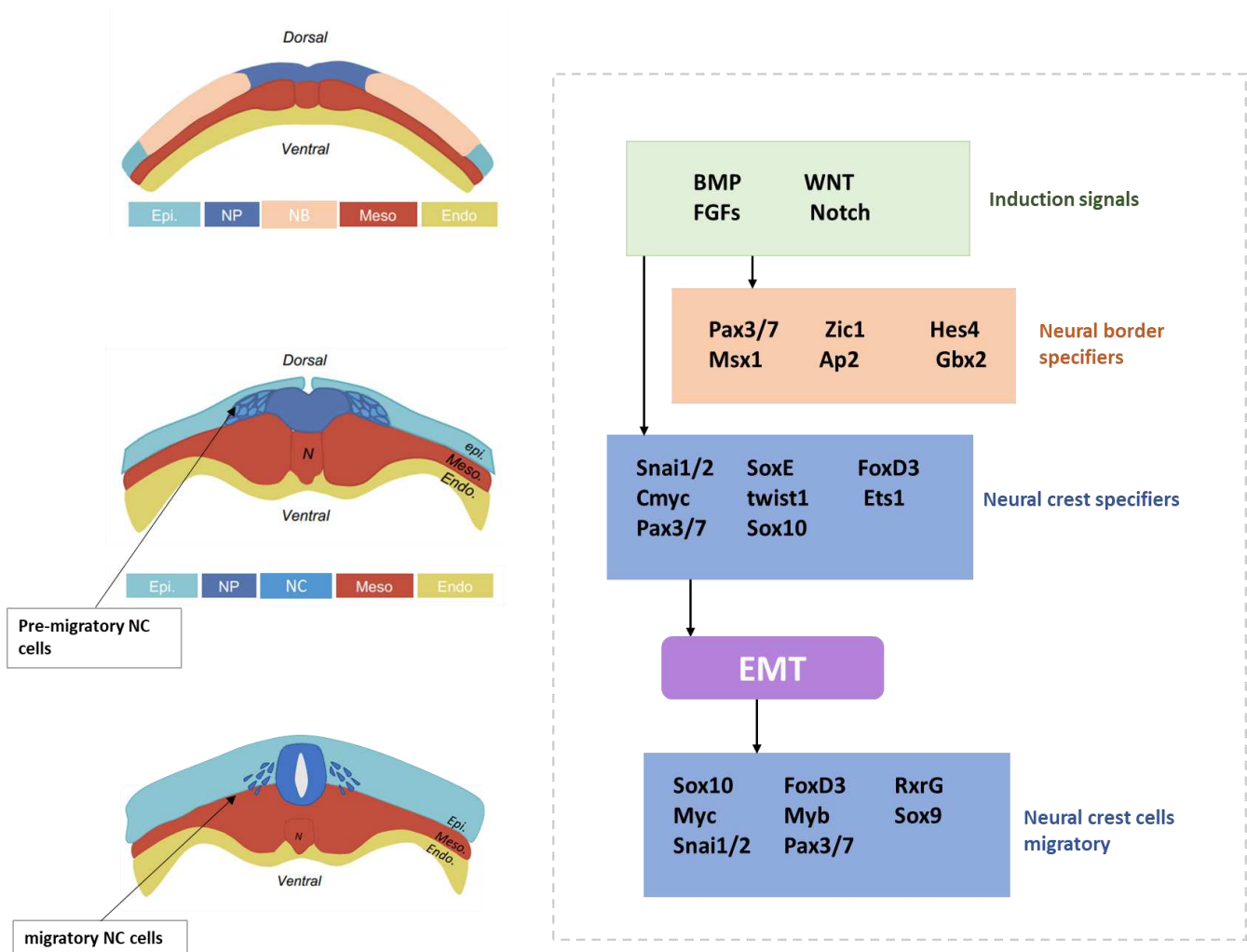
During the neurulation process, ectodermal cells form the pseudo-stratified epithelium layer of the neural plate (NP). The lateral border of the just formed neural plate that are adjacent to the ectoderm, undergo changes in cell shape and elevates giving rise to the neural folds. The neural crest cells are originally considered to originate from the lateral neural folds during the last steps of the neural tube closure. The initial experiments that explored the generation of neural crest cells were performed in amphibian and chick embryos and several types of experiments were proposed to explain the formation of this population of cells. For example embryonic experiments, in which ectoderm explants were transplanted into the developing neural plate of embryos, demonstrated that neural crest is ectopically induced by factors proceeding from the borders of adjacent ectoderm tissue (Selleck & Bronner-Fraser, 1995). Similar experiments in which neural plate tissue was co-

cultured with epidermal ectoderm, demonstrate the in vitro formation of neural crest under those conditions (Liem et al., 1995). Collectively, multiple studies have shown that NC progenitor specification takes place during gastrulation due to successive combinations of gradients of different signals. Intermediate levels of BMP and Wnt signalling activity in the ectoderm facing neural plate border are necessary for the induction and maintenance of NC cells during neurulation (Aybar & Mayor, 2002; Steventon & Mayor, 2012)(Figure 4), revealing that the tissue interactions between the ectodermal cell populations, the neural plate, the non-neural ectoderm, and the underlying mesoderm in a highly coordinated manner(Vega-Lopez et al., 2018).

Neural crest formation is controlled by multiple molecules and signalling pathways including bone morphogenic Proteins (BMPs), Wnts (Wingless) and fibroblast growth factor (FGF) (Monsoro-Burq, A. H et al., 2003).



**Figure 3. Neural crest cell derivatives.** After epithelial-to-mesenchymal transition, the motile neural crest cells delaminate from the neuroepithelium, migrate along several specific routes across the body, and reach diverse destinations where they differentiate into a wide spectrum of cell types, the main ones being illustrated here.



**Figure 4. NC development in a prototypical vertebrate (e.g. *Xenopus laevis*).**

A) Scheme of embryo development from early neurula to late neurula-tailbud in coronal section view. Same colour code is used. Nomenclature: Mesoderm (Meso.); Endoderm (Endo.); Epidermis (Epi.); Neural plate (NP); Neural Border (NB); Neural Crest (NC); Notochord (N). B) (A) Simplified diagram Neural crest gene regulatory network.

### 1.3.3 Neural crest cells EMT and migration

After NCCs have been induced, they undergo EMT (Aiello & Stanger, 2016). NCCs lose cell-cell and cell-basement membrane adhesions, lose their apicobasal polarity, and acquire mobility through cytoskeleton remodelling changes (Gougnard et al., 2018; C.-Y. Wu & Taneyhill, 2019). As NCC delamination begins, NCCs detach from the neuroepithelium and responds to environmental signals that help them to migration through the extracellular matrix (Kerosuo & Bronner-fraser, 2013). The migration of the neural crest cells differs between the different subpopulation of neural crest cells localized in different regions. While the trunk NC cells travels individually or in small groups of cells to target destinations, the cranial NC initially emigrate collectively guided by chemoattractant of the environment (Theveneau & Linker, 2017). The transition of epithelial cells into mesenchymal cells, either in development or in pathological context, follows a conserved and common programme with similar hallmarks. Nevertheless, both context presents some variations and flexibility depending on cell type, biological context, and the signals behind the activation of EMT process. Neural crest cell (NCC) migration is one of the most well-studied examples of EMT process during embryogenesis and the one in which we will enter in detail (Hutchins et al., 2020; Kim et al., 2016; Yuan et al., 2020). Migration will be better described in the following section.

### 1.3.4. EMT and cytoskeletal remodelling in Neural crest

Before they delaminate, neural crest cells are situated at the border of the neural plate as pseudo-epithelial cells. Once they are committing to EMT, cells lose their apico-basal polarity and junctions, recycle of cell-cell adhesion molecules and change their actin-myosin cytoskeleton (Lim & Thiery, 2012; Xing & Tian, 2020)One of the initial and best characterized step in the initiation of NCC EMT is the switch of expression from E-Cadherin, characteristic of epithelial cells, to N-Cadherin, which is here characteristic of mesenchymal cells (although it was initially found in neural tissues, hence its name). This switch produces a loss of cell-cell adherent junctions, that are protein complexes mediating cell-cell adhesion and interact with the cytoskeleton of the cells in order to provide mechanical links between cells within a tissue. This leads to a lower adhesiveness of the cells, the acquisition of a mesenchymal like phenotype and the cytoskeleton reorganization allowing a better



motility to migrate. Therefore, the loss of adherent junctions between cells is a critical step for cells to undergo EMT and therefore delaminate. EMT initiation also involves alteration of desmosomes (Huang & Guilford, 2012), and gap junctions. The cadherin switch is not only necessary for cell repolarization but in the development of some species as xenopus, it is also important for the acquisition of contact inhibition of locomotion (CIL) during the collective migration of Cranial NC (Scarpa et al., 2015). This process is tightly regulated so that only NCCs and not neighbouring cells become migratory. For these reasons, a full understanding of the mechanisms controlling cell motility governed by EMT process is better studied in the cell's in vivo natural environment. EMT is controlled by multiple cell signalling pathways including TGF- $\beta$ , Wnt, Notch working in context dependent ways to activate EMT-inducing factors such as Snail1, leading to adherent junction disassembling by repressing E-cadherin.

To migrate, cells develop actin-based protrusion called filopodia and lamellipodia (Lamouille et al., 2014) that facilitate the movement of the cell and act as sensory extensions of the cytoskeleton. The actin cytoskeleton dynamics during EMT is controlled by signalling pathways such as Rho family GTPases. While Rac1 and Cdc42 control actin polymerization responsible of the protrusion formation at the leading edge of the cells, Rho controls the cell retraction at the rear pole of the cell. During EMT cells also present an increase of cell contractility and the formation of actin stress fibers regulated by proteins such as moesin (Haynes et al., 2011).

To be able to migrate throughout the different environment of the body axis, NC cells must penetrate basement membranes and invade extracellular matrices (ECM) composed of collagens, fibronectin, laminins, and proteoglycans. To do that, NC cell may interact with the ECM before and during migration. Cells develop also other actin-based structures called invadopodia that contains an actin assembly machinery (WASP, cortactin and Arp2/3) and multimeric protein complex formed by integrins and integrin-associated proteins, around the actin-core. Invadopodia participate in the proteolytic degradation of extracellular matrix in order to facilitate the invasion of the cells through the basement membrane (Yilmaz & Christofori, 2009). ECM is proteolytically remodelled before and during the delamination of NC cells with the help of matrix metalloproteinases (MMPs). (Lamouille et al., 2014) Changes in the expression of some MMPs (ADAM13, MMP14, MMP16, and MMP9) have been shown to be important for cranial NCC migration in either Xenopus or Chick embryos (Alfandari et al., n.d.; Garmon et al., 2018).

MMP14 is one of the matrix metalloproteases that have demonstrated to be key for NCCs migration. This enzyme was described for the first time in 1994 by Sato et al (Sato et al., 1994) as a protein transmembrane is involved in the induction of tumour cells invasion and is expressed in both, pre-migratory NCCs and migratory NCCs. MMP14 is not only important in NC migration but also has been related with EMT in organ fibrosis and cancer, producing, for example, the cleavage of CD44 promoting cell migration in cancer cells (Gonzalez-Molina et al., 2019; Kajita et al., 2001; Szabova et al., 2008). MMP14 also activates other MMPs implicated in NC migration such as MMP2, however, MMP14 activity on the regulation of neural crest migration is independent of MMP2 activation (Garmon et al., 2018). Additional functions of MMP14 controlling EMT progression have been suggested since it has been observed to be present in the nucleus together with MMP28 (Andrieu et al., 2020; Gouignard et al., 2021) . Another important protein for the degradation of ECM is ADAM13, that has proven to be expressed in pre-migratory and migratory NC in *Xenopus*, and participate in the detachment of cells and cleavage of ECM barriers (Alfandari et al., 2001). Once NC cells have degraded the ECM of surrounding tissues, they produce a lax ECM in which they can easily migrate through integrin-mediated adhesions. Therefore, the ECM plays an important role in the migration of NC, acting as substrate and producing signals that facilitates neural crest cell migration (Leonard & Taneyhill, 2021).

Similar to development, EMT plays a crucial role in cancer progression and invasiveness. Both neural crest delamination and initiation of metastatic cancer cells are characterized by a high expression of EMT induction molecules (Craene & Berx, 2013). In addition, the acquisition of metastatic capacity cancer cells is highly correlated with cells undergoing EMT (Bigagli et al., 2019). In both contexts, NCC delamination and cancer cell invasion, the loss of cell-cell adhesion molecules and cell-extracellular matrix junctions is key for the ability of invade surrounding structures and migrate. In overall, molecular changes facilitating cancer cell invasion shares many similarities with the EMT process happening during neural crest delamination in development.

# 1. MELANOMA: a NC derived tumour

Melanoma results from tumour transformation of adult melanocytes and is therefore a neural crest-derived tumour. In this section we examine the composition of the skin, the different types of melanoma, the biology of melanocytes, the main pathways altered during melanoma, the various therapies currently available for the treatment of this cancer and the role of EMT in the metastatic and invasive progression of this tumour.

## 2.1 Cellular components of the skin

Skin is the largest organ in the body covering all the surface of the body. It is composed by three different layers (the epidermis, dermis, and the hypodermis) each of them anatomically and functionally different. Within the skin we can find four different cell types ordered by preponderance in the skin: keratinocytes, melanocytes, Merkel cells and Langerhans cells. Each cell type is located in specific layers and serves distinct functions.

### 2.1.1 Keratinocytes

Keratinocytes stem cells reside in the *stratum basale*, one of the sublayers that compose the most superficial layer of the skin called epidermis. *Stratum Basale* is the deepest layer of the epidermis, in close contact with the dermis. This layer is formed by a single epithelium of cells which is formed mainly by keratinocytes but also by melanocytes and Merkel cells (Figure 5). The continuous proliferation and differentiation of the keratinocytes residing in this layer of the epidermis, give rise to the rest of the sublayers in the epidermis, that receive the name of, stratum spinosum, lucidum and granulosum due to its morphological characteristics (Yousef H et al., 2022). The newest cells just generated on the *stratum basale*, generate a mechanical force on the old cells situated above, pushing them towards the upper layers until the surface of the skin. Along the different layers, keratinocytes adopt different morphologies. The stratum spinosum adopts its name due to the presence of a spiny-like phenotype of the keratinocytes. Through their migration in layers, the cells start to enter in apoptosis

and begin to flatten out (Abdo et al., 2020). Once they reach the stratum granulosum, the second layer of the epidermis, the keratinocytes that compose this layer present a flattened and densely packed morphology. On top of that, most of the cells in this layer already have gone through apoptosis and they no longer present any organelles neither nucleus. Those dead cells are mainly composed by high concentration of keratin and cell membranes, serving as a protective barrier of the epidermis.

### 2.1.2 Melanocytes

Melanocytes are a neural crest-derived type of cells that are responsible of the production of melanin. Those cells form a heterogeneous group of cells whose function can vary depend on the target place. In the epidermis, melanocytes are situated in the stratum Basale. There, they produce a pigment called melanin via melanogenesis. Melanocytes present a high number of dendrites that allow them to transfer the melanosomes containing melanin to the neighbour keratinocytes. The different phenotypes associated to colour of the skin are based on difference of the number and size of the melanosomes among others. Melanosomes are distributed in keratinocytes and then exocytosed to the cytoplasm. The melanin contained in melanosomes, has a protective function. This pigment can absorb the UV light and prevent the potential damage of the DNA of the cells, since this caused DNA damage can induce the malignant transformation of melanocytes. Melanocytes produce melanin in basal conditions. However, this production can be exogenously activated by Ultraviolet light exposure in order to protect the cells in the hypodermis (Cichorek et al., 2013). In the human body, melanocytes are well known to give colour to the epidermis, iris and hair, but they also can be found in the inner ear and nervous system (Cichorek et al., 2013).

### 2.1.3 Langerhans Cells

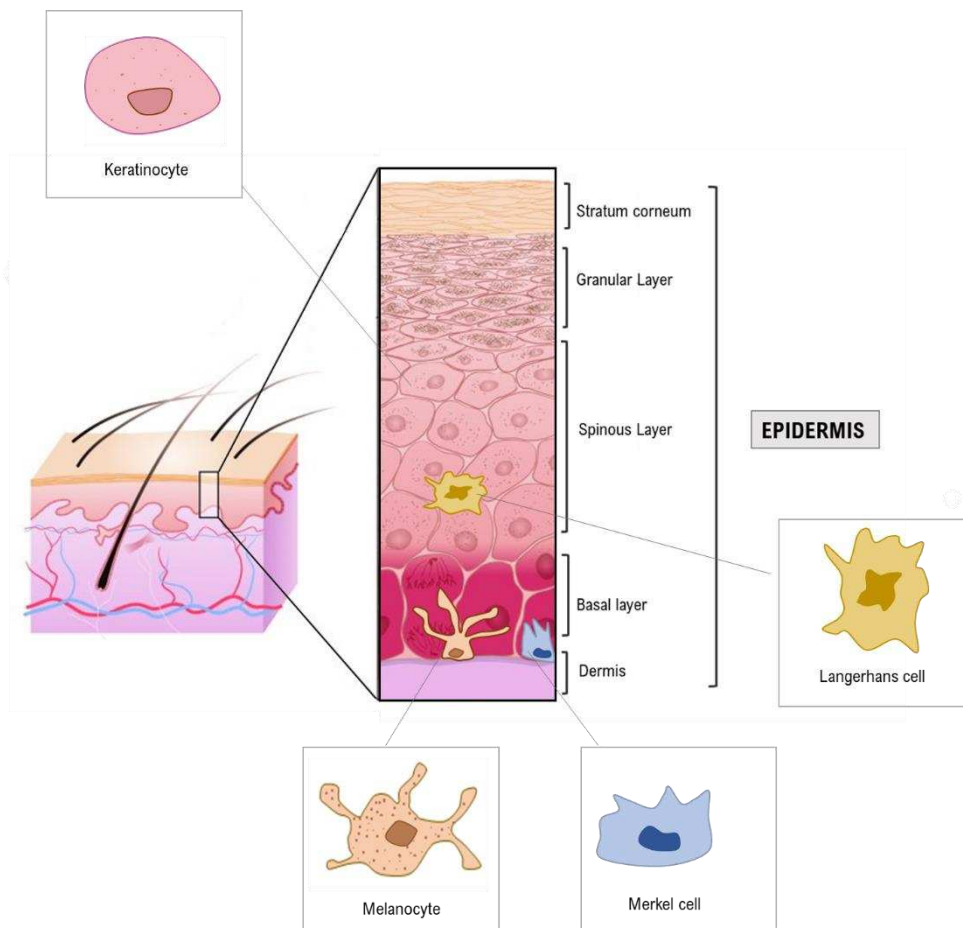
Langerhans cells (LC) is a unique population of antigen-presenting cells residing exclusively in the epidermis of the skin and other stratified epithelia such as mucous membranes (Ten Kate, A. R. et al., 1996). Those cells were described for the first time in 1868 by Paul Langerhans who proposed them as part of the peripheral nervous system (Langerhans P et al., 1868). Langerhans cells are found to be in close contact with keratinocytes. For many years, LC have been associated as Dendritic cells (DC) due to its morphology and migratory capacities through the epidermis to the skin-draining lymph nodes (Johnston et al., 2000). Later on, many studies demonstrated that LC belong to macrophage lineage (Hoeffel et al., 2012). Therefore, LC are considered as local macrophages of the skin. Due to its migratory capacities and its barely understood function in inflammation and infection,

this type of cells has awakened special interest: together with tissue resident T-cells, LC form the immunological barrier of human epidermis. On top of that, in combination with keratinocyte barrier, those immunological cells are indispensable as first line defence against external pathogens or threats (Deckers et al., 2018).

#### 2.1.4. Merkel cells

They were first named by Robert Bonnet that give them the name of the German histopathologist who described the cells for first time (Erovic & Erovic, 2013). Merkel cells are postmitotic cells that represent less than the 5% of the population of cells in the epidermis and appear as oval clear cells localized in the basal layer of epidermis. The origin of Merkel cells is still on debate. It is not clear if they differentiate from keratinocyte like epidermal cells or from migratory stem cells from neural crest origin (Szeder et al., 2003). They have a role in detecting mechanical stimuli and then communicate with sensory afferents via neurotransmission. They are distributed everywhere, skin and mucosa but mainly located in highly sensitive areas such as palms, finger pads, feet, male prepuce, clitoris and oesophagus (Hermnse et al., 1999; Moll et al., 1996). Merkel cells are difficult to observed using simple light microscopy. Hence, they may require the use of additional techniques such as ultrastructural methods. Some studies have suggested that different populations of Merkel cells can be found depending of the skin area of the body (Eispert et al., 2009).

Alterations in the homeostasis of each of those cell types will produce different cancer types including basal cell carcinoma, originated from basal cells at the bottom of the epidermis responsible for generating new skin cells, Squamous cell carcinoma, originated from squamous keratinocytes, Merkel cell cancer originated by Merkel cells and the most prevalent and aggressive one, melanoma, originated from melanocytes.



**Figure 5. Scheme of human skin**

**Adapted de L. Belote, 2016.**

A) Human skin is composed of three main layers from external to internal: epidermis, dermis and hypodermis. In the epidermis, the outer layer, we can find 4 types of cells: keratinocytes, melanocytes, Langerhans cells and Merkel cells. The dermis is the biggest layer we can find in the skin and where we can find fibroblast, blood vessels, nerves, and glands. Hypodermis is the most internal layer and is mainly composed by adipocytes and blood vessels. B) Zoom of epidermis structure. Epidermis is composed mainly by keratinocytes and melanocytes. It is structured in 4 layers: basal layer, spinous layers, granular layer, and stratum corneum. The nucleus and size of keratinocytes is reduced when advance to the outer layers of the epidermis until losing the nucleus at the stratum corneum levels which is composed by dead cells.

## 2.2 Melanocyte development

All melanocytes are derived from the neural crest. Melanocytes have been widely studied in different models' organisms including chick, zebrafish and mouse embryos (Aybar & Mayor, 2002). Melanocyte development consist in several well-understood steps, starting by the fate specification of neural crest cells into melanoblasts. As melanoblasts migrate to their destination they multiply and sequentially start to express additional melanogenic specific genes, starting with tyrosinase (*dct*) the most important one since it is the first enzyme involved in melanin synthesis.

The master regulator of melanocyte identity is the microphthalmia-associated transcription factor (MITF). Several studies in different animal models have proved the importance of MITF for the correct differentiation of melanoblasts into melanocytes. One of the studies that demonstrated this shows as mice lacking MITF expression does not develop melanocytes (Steingrímsson et al., 2004). Other study showed that the ectopic expression of this factor in embryo-derived stem cells is sufficient to induce the differentiation of those cells into melanocytes (Béjar et al., 2003). Similarly, to studies in mice, fish that lack of the ortholog of MITF, *mitfa*, do not develop melanocytes. In addition, the ectopic expression of *mitfa* can produce ectopic melanocytes (Lister et al., 2014). In the case of human, germline mutation in MITF gene can be the cause of Waardenburg syndrome, characterized by defective pigmentation and deafness (Grill et al., 2013). Therefore, the activation of MITF is a key step in the melanocyte formation. Sox10 and Pax3 also play an important role in the differentiation of melanocytes since they activate MITF-M in a cis-acting way in melanocytes and melanoma (Manderfield et al., 2014; Verastegui et al., 2000). SOX10 is critical during the formation of the melanocyte lineage since studies where animals carry Sox10 mutations exhibit pigmentary defects (Britsch et al., 2001). In other hand, a number of human disorders characterized by combination of neurocristopathies including hypopigmentation are also attributed to mutations in *SOX10* gene. Therefore, differentiated melanocytes can be easily recognized by the expression of specific markers as MITF and Tyrosinase related proteins (Tief et al., 1996). Apart from MITF and melanin-related enzymes, melanosome matrix proteins can be also used for their identification.

### 2.3 Epidemiology and melanoma types

According to the European Academy of Dermatology and Venereology (EADV) skin cancers account approximately for 1.7% of cancers in the adult European general population which means that approximately 7,304,000 of people within the European Union are estimated to develop a cancer of the skin. Although melanoma barely represent the 5% of skin cancers, it is responsible of approximately 80% of the deaths due to skin cancer (Bertolotto, 2013). Perhaps linked to their neural crest origin, those cells express many signalling molecules that promote an aggressive migration and metastasis after its transformation (Gupta et al., 2006). Melanoma rising incidence during the past years also entails an increment in mortality and the necessity of a highly elaborate care-system for patients in advanced stages of the disease.

The appearance of melanoma is most common in light-skin population, and in most world regions, the prevalence is higher in men than in women. A European study of 2020 shows that the lifetime risk of developing melanoma is 1 out of 74 in women and 1 out of 66 in men. The average age of diagnosis depends on the subtype of melanoma. Overall, it is around 65 years old. In USA, melanoma represent the most diagnosed cancer among people of 25- to 29-year-old and it is situated within the 5th most common cancers for women either in US and EU. Although the overall 5-year survival is about 90% in EU and US for melanoma patients, it falls to 30% in patients of stage IV. For this reason, it is important to fully understand the biology of melanoma progression not only for the identification of biomarkers that help us to improve the early diagnosis of the disease but also to the development of better specified and targeted treatments.

Due to the high aggressiveness of melanoma, the correct early diagnosis of the disease is crucial for the management of the patients. Clinicians use the “ABCDE rule” to assess the lesions and distinguish melanoma from benign nevus, which is a benign pigmented cutaneous round lesion. This rule is based in the presence of A: asymmetry, B: irregular border, C: heterogenous colour, D: diameters higher than 6mm, E: elevated and pronounced surface (Duarte et al., 2021). However, the diagnosis under those criteria is not always accurate which can lead to false negatives. Due to that, the development of digital systems has improved the diagnosis, including the development of automated diagnosis systems containing multiple complexes algorithms used for the classification and analysis of the characteristic of patient's lesion, leading to an effective diagnosis (Bassel et al., 2022; T. Li et al., 2021).



In most of the cases, melanoma can be classified into four main subtypes: superficial spreading melanoma (SSM), lentigo malignant melanoma (LMM), nodular melanoma (NM), acral lentiginous melanoma (ALM: acral lentiginous melanoma) (Schadendorf et al., 2013). Nevertheless, other less common melanoma subtypes have been identified such as desmoplastic melanoma and naevoid (Shaw et al., 2022; Zembowicz et al., 2001). The most common type above all is spreading melanoma, which is found approximately in 60% percent of the cases, and is present in patients of younger age compared with the other subtypes. In spreading melanoma, melanocytes present a pleomorphic epithelioid phenotype and spread laterally through the epidermis. Anatomically this subtype is presented as a flat bicoloured irregular lesion that present a slow rhythmic of growth (Scolyer et al., 2011). Lentigo malignant melanoma (LMM) usually appears in regions that have been chronically sun-exposed such as the head or neck. This subtype invade the dermis and it is characterized by the proliferations across the dermal-epidermal junction. Anatomically can be observed as a large very pigmented macule with irregular edges in median age patients.

Nodular melanomas normally appear in slightly younger patients than LMM. This subtype is very aggressive and can appear at any localization. It is characterized by a rapid growth which make hard the detection at early stages of the disease.

Last, lentiginous melanoma is melanoma that only occurs on the hands and feet, including the palms, soles, toes, fingers and nails, being most commonly found in the lower extremities. This subtype, present a high level of morbidity and mortality due to the fact that is often found at late stages of the disease.

## 2.4 Melanoma-related susceptibility genes and pathways

Melanoma present an elevated rate of somatic mutations (Davis, 2018), being far more frequent than the germline that counts for 10% of melanoma cases. It is well-known that the most common somatic mutation present in melanoma is in the gene that encode B-RAF protein. Approximately 50% of cutaneous malignant melanomas present a mutation in this gene, being the most recurrent the one that involve valine to glutamate point mutation at residue 600 (BRAF<sup>V600G</sup>), resulting in an hyperactivation of MAPK pathway (Davies et al., 2002). Others BRAF mutations such as BRAF<sup>V600K</sup>, BRAF<sup>V600D</sup>, BRAF<sup>V600R</sup>, are also found but in lower percentage of melanoma cases (Colombino et al., 2022). BRAF mutations have been observed mainly in patients who have been exposed to sun,

indicating that the intermittent sun exposure has a strong effect in inducing mutations in BRAF (Maldonado et al., 2003). Apart from BRAF, there is multiple other mutated genes that act as driver melanoma genes, producing alterations in multiple signalling pathways that acts as master contributors of melanoma development (Table 1). Among those pathways, mitogen-activated protein kinase (MAPK) pathway, protein kinase B (AKT) pathway are the two major signalling pathways implicated in melanoma. Others like Wnts and TGF-B have been also involved. All those will be discussed in more detail below.

#### 2.4.1 CDKN2A and CDK4

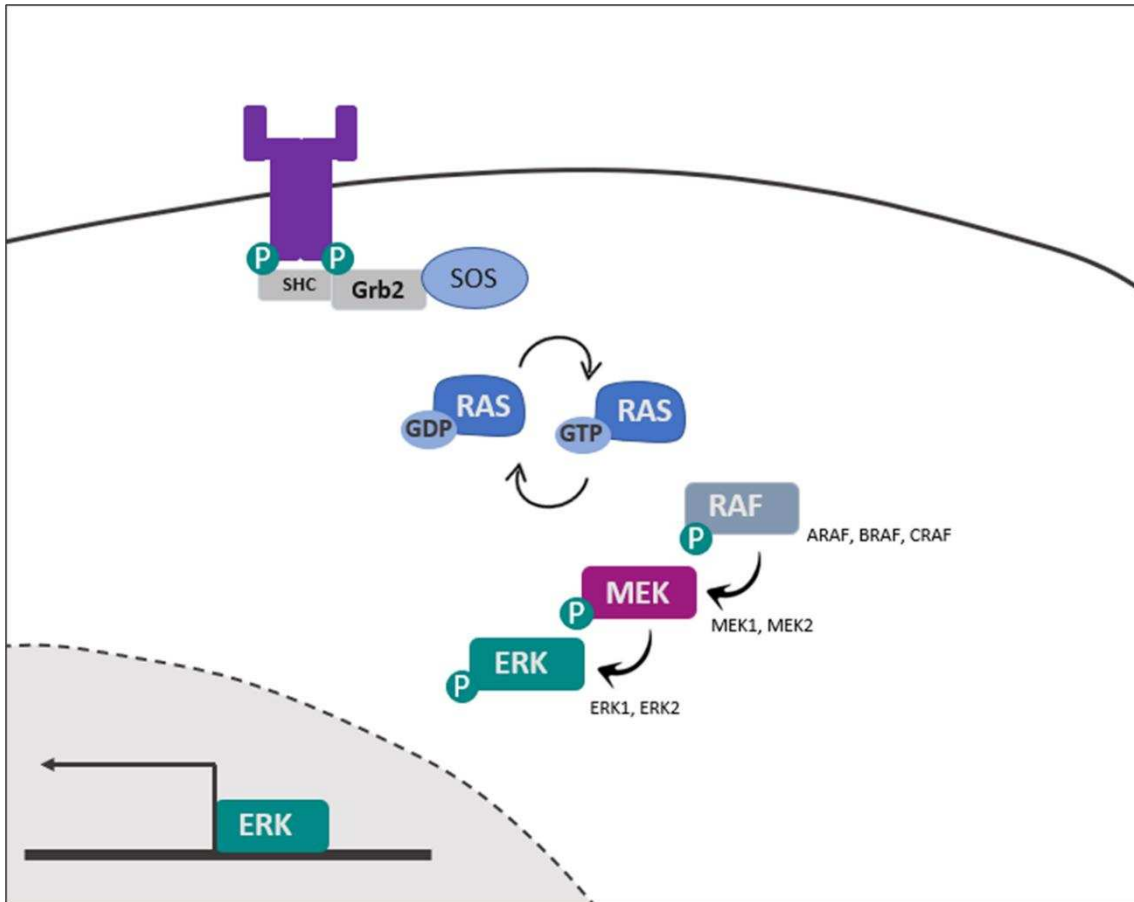
CDKN2A is a tumour suppressor gene most frequently mutated in germline of melanoma patients and the first to be associated with familial melanoma (Zocchi et al., 2021). CDKN2A gene is situated in chromosome 9 and its mutation contribute to an uncontrolled cell cycle progression of the tumour cells. CDKN2A encodes two different tumour suppressor proteins p16<sup>Ink4a</sup> and p14<sup>Arf</sup> through separate translational programs. In one hand, under physiological conditions, P16<sup>Ink4a</sup> can bind to the cyclin-dependent kinase 4 (CDK4) to control cell-cycle progression by the disassembly of the D-CDK-4 complex or CDK6-mediated phosphorylation and subsequent phosphorylation of retinoblastoma (Rb) family protein (Jyoti Motwani & Eccles, 2021). It has been proven that mice lacking this protein presented and increased tumour growth and formation, which suggested the critical role of p16INK4A in restricting aberrant cell proliferation (Krimpenfort, P et al., 2001). In the other hand, wild-type p14<sup>Arf</sup> also interferes in the control of cell-cycle by mediating the degradation of p53 by E3 ubiquitin ligase MDM2 (Vivo et al., 2015). Therefore, mutations of *CDKN2A* conduct to dysfunctional control of cell-cycle leading to an uncontrolled progression of the cells.

In other hand, CDK4 is an oncogene that belong to the cyclin-dependent kinase family and is involved in the control of cell-cycle progression and transcriptional process. In wild type condition, CDK4 form complexes with D-type cyclins. The D-CDK-4 complex is involved in the regulation of cell transition from G1 to S phase by the phosphorylation of the Retinoblastoma protein (Rb). Germline mutation in this gene, have been observed in few familial melanoma patients and have proved to cooperate with the active forms of BRAF and NRAS and to promote tumour progression.

#### 2.4.2 MAPK/ERK Pathway

Non-hereditary melanomas have been associated to the dysregulation of multiple signalling pathways associated with many different processes including proliferation, apoptosis, differentiation, and migration. One of the most associated pathways to melanoma are the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) pathway, which is composed of *TKR*, *RAS*, *B-RAF*, *MEK*, *ERK*, and *NF1* (Jyoti Motwani & Eccles, 2021) . This pathway is characterized by a cascade of phosphorylation's. In normal conditions, the signalling cascade begins with the binding of a ligand to a receptor on the cell surface. This ligand, that can be mitogens, hormones, or neurotransmitters, binds to receptor tyrosine kinases and triggers the dimerization and autophosphorylation of the receptor at the cytoplasmic side. The phosphorylated receptor produces the subsequent recruitment of proteins that allow the recruitment of GRB2, which can be also recruited by the receptor itself (Li et al., 1993). In turn, the phosphorylated SH2 domain (Src Homology 2) of the GRB2 (Growth factor receptor-bound protein 2) bring SOS (son-of-sevenless) to the protein complex at the cytoplasmic membrane side. This allows the recruitment of inactive membrane GDP-RAS and convert it to active GTP-RAS which activates kinase cascade of the MAPK pathway, with downstream effectors such as RAF, MEK1/2, ERK1/2 that can activate the expression of multiple transcription factors involved in several cellular molecular processes (Figure 5) (W. Zhang, 2002).

Genetic or epigenetics alterations that hyperactivate MAPK signalling pathway are found in more than 50% of melanoma patients (Palmieri et al., 2015). Those alterations present also mutually exclusive mutations in BRAF and NRAS. Approximately 50% of the cases present *B-RAF* mutation, 20% have NRAS mutation and less than 5% *c-KIT* mutation. The presence of *BRAF* mutations has great potential in predicting an unfavourable prognosis in melanoma patients. This importance of the BRAF pathway led to the development of specific inhibitors against the activation of the pathway, such as Vemurafenib (Cheng et al., 2017; Guo et al., 2021). However, despite the initial optimal response to the treatment, most of patients develop resistance to the treatment along the time.

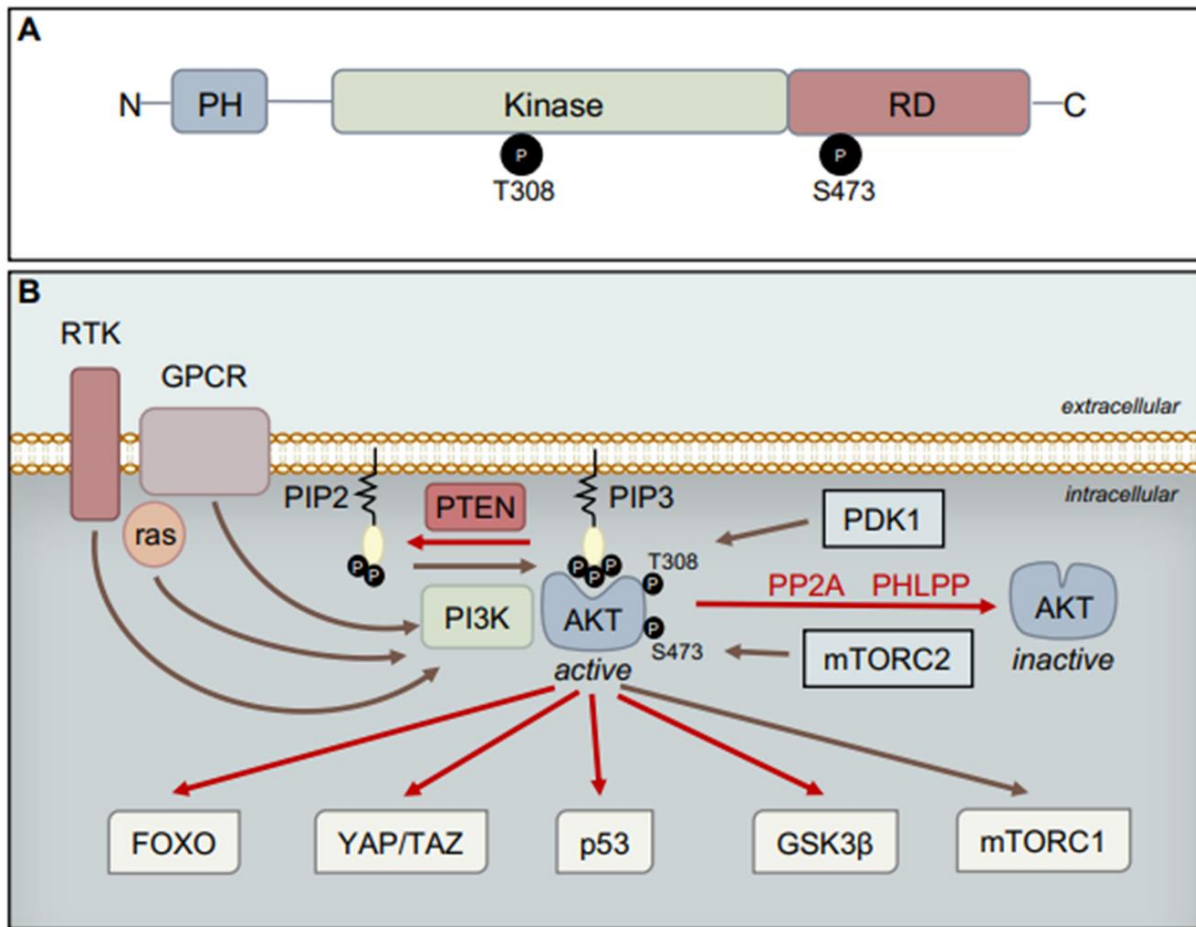


**Figure 5. Canonical MAPK pathway.**

Different stimuli cause the activation of the receptor in the membrane of the cells resulting in a phosphorylation cascade of several effectors called MAP kinases (RAS, RAF, MEK, ERK). Those MAP kinase will activate a series of genes that will contribute to proliferation, migration, senescence, or apoptosis.

### 2.4.3 PI3K Signalling Pathway

The PI3K-AKT signalling pathway is one of the most studied cascades in cancer. The pathway plays a key role in the control of many important physiological processes that are crucial for the aggressive behaviour and progression of cancer cells. PI3K is activated by multiple signals, including receptor tyrosine kinases (RTKs), RAS proteins, and cell-cell contact, among others (Palmieri et al., 2015) (Figure 6). Once active, PI3K phosphorylates the 3'-OH group of phosphatidylinositols in membrane of the cell. These 3'-phospholipids attract proteins to the cell membrane and activate them by phosphorylation. AKT is one of those target proteins attracted to the cell membrane, that is phosphorylated at two critical residues Thr308 and Ser473 by different effector complexes. Activated AKT then phosphorylates and activate several effector proteins involved in multiple key cellular processes including survival, motility and proliferation, among others (Hemmings & Restuccia, 2012). In melanoma context, PI3K-AKT pathway can be activated by multiple events. The most common reasons of PI3K-AKT activation is due to hyperactivation of the oncogene NRAS or the loss of function of the tumour suppressor PTEN (Tsao, 2007), this last being associated with quick develop of brain metastasis. However, the loss of PTEN is only present in melanoma with mutated BRAF. Therefore, NRAS mutation and PTEN loss are also mutually (Goel et al., 2006). Mutations in PTEN are normally found in the later stages of primary cutaneous melanoma. *BRAF*-mutant human melanoma cell lines lacking PTEN expression, stop proliferation when exposed to BRAF and MEK inhibitors. However, they present resistance to the apoptosis induced by same inhibitors ( Paraiso et al., 2011). Nevertheless, alterations in other important molecules such as RAC1, PIK3CA, AKT3, PREX2, and AKT1 are also responsible of the alteration of this pathway in melanoma (Cannon et al., 2020; Cherepakhin et al., 2022).



**Figure 6. Schematic representation of AKT signalling pathway (Sittewelle et al.,2022).**

A) Representative structure of the AKT protein. The protein is formed by three main domains: Pleckstrin homology (PH) domain situated in the N-terminal region, the catalytic (kinase) domain in the centre and the hydrophobic regulatory domain (RD) at the C-terminal region. AKT protein presents two main phosphorylation sites, one at the kinase domain (T308) and another one at the RD domain (S473). The phosphorylation of both domains is necessary for the full activation of AKT. B) Overview of PI3K/AKT signalling and some key downstream targets. The activation of PI3K produces the phosphorylation of phosphoinositides from the cell membrane, transforming them into 3-polyphosphoinositides (PIP3). The activity of PI3K is negatively regulated by PTEN which is able to dephosphorylate PIP3. AKT is recruited in the cell membrane by its PH domain and is activated in the membrane by PDK1 and mTORC2 which phosphorylate the two main phosphorylation sites T308 and S474, respectively. Those phosphorylation sites can be dephosphorylated by two phosphatases (PP2 and PHLPP) that return AKT to the inactive state. Once active, AKT acts over many targets, some of them represented in the scheme. Red arrows represent a negative regulation. Brown arrows represent positive regulation.

#### 2.4.4 Wnt-Signalling Pathway

Wnt pathway is an evolutionarily conserved pathway involved in a wide range of key biological processes including embryonic development and tissue regeneration (Nusse & Clevers, 2017). This pathway is composed by two main types, the canonical and the non-canonical, that carry out different functions. The non-canonical, also known as  $\beta$ -catenin-independent pathway, is have a role in the maintenance of planar cell polarity and cell motility, by the regulation and modification of actin cytoskeleton structures. The canonical pathway, also known as  $\beta$ -catenin-dependent pathway, which is involved in cell proliferation and expansion. This pathway is extremely controlled by set of protein whose function is to ensure the correct spatio-temporal expression of the target genes (Clevers & Nusse, 2012).

The causes of deregulation in the  $\beta$ -catenin-dependent pathway are due to mutations in epigenetics mechanism or elements of the pathway. Mutations are quite variable depend on the tumour origin. In most of the cases, mutations are found in the genes such as APC and AXIN, that take part of the destruction complex, and it is also frequent to find mutation in CTNNB1, encoding for B-catenin. However, in melanoma, frequencies of mutation in *APC*, *AXIN1* and *CTNNB1* are low. In general, alterations in the WNT signalling pathway are mostly due to mutations in the genes that take part of the signalling, causing either destruction of the complex or an increment of B-catenin in nucleus. In both cases, that conduce to an increment of  $\beta$ -catenin-mediated transactivation of several genes.

On top of that, WNT-signalling elements have demonstrated to interact and regulate the activity of diverse melanoma oncogenic pathways such as MAPK/ERK and PI3K as well as important transcriptional regulators as MITF (Gajos-michniewicz & Czyz, 2020).

#### 2.4.5 TGF- $\beta$ pathway

The TGF- $\beta$  superfamily of ligands is evolutionarily conserved and approximately 30 different TGF- $\beta$  members have been described in humans. TGF- $\beta$  mature ligands can form homo or heterodimers and bind to the membrane receptors that have cytoplasmatic serine/threonine kinase domain to initiate the signalling. However, other ligands also require the presence of co-receptors for the activation of the signalling. The ligand-receptor binds produce the assembly of a receptor complex in the internal cytoplasmatic side, that produce the phosphorylation of SMAD family of proteins. Some of those members can bind to the DNA and regulate the transcription of several genes controlling diverse

mechanism. TGF- $\beta$ 1 is found to be overexpressed in human melanoma cells in parallel with tumour stage and to stimulates the surrounding tumour stroma cells by the production and deposition of extracellular matrix proteins (Lopez-bergami et al., 2008). TGF-B signalling can either act as tumour suppressor at early stages through its antiproliferative potential leading the cells to cell cycle arrest or as tumour promoter at later stages of the disease by immune surveillance, epithelial to mesenchymal transition and metastasis (Javelaud et al., 2008).

In the past years, many key molecular pathways, and emerging ones, have shown to be involved in melanoma onset and progression. In addition, the metastatic outcome of the disease is typically linked with an activation of signalling pathways that are involved in embryogenesis (Kulikova et al., 2011). Nowadays it is highly appreciated that melanoma disease present complex changes in more than one signalling pathway, affecting the cell growth control, metabolism, immunity surveillance and invasive properties among others. All this also make the disease a great scenario for the development of drug targeted therapy.



Pathway	Gene	Mutation	Frequency (%)	Progression	Consequence
RAS/RAF/MEK/ERK	N-RAS	Gain-of-function RASQ61K/R	81 15–30	Congenital melanocytic nevi Melanoma	Triggers a senescence phenotype in nevi. Constitutively active MAPK pathway. Increased survival and growth.
	BRAF	Gain-of-function BRAFV600E	82 50	Benign nevi Melanoma	Triggers a senescence phenotype in nevi and transforms immortalized melanocytes. Constitutively active MAPK pathway. Increased survival and growth.
	MEK	Gain-of-function MEK1/2		Melanoma	Constitutive ERK phosphorylation and higher resistance to MEK inhibitors.
	MAPK	Gain-of-function MAP3K5 and MAP3K9	67–85 8	Metastatic melanoma Melanoma	Loss of MAPK kinase functions; decreased MAP3K pathway activity maybe can lead to chemoresistance in melanoma. Constitutive ERK phosphorylation and higher resistance to MEK inhibitors.
CDKN2A/CDK4	CDKN2A	Loss-of-function CDKN2A	40 8.2	Melanoma (familial)	Mutations affect p16INK4a, p14ARF, or both proteins by deletion, mutation or promoter silencing.
		Loss-of-function CDKN2A	56	Melanoma	Uncontrolled cell proliferation.
	p16INK4a	Loss-of-function CDKN2A (CNV)			CDKN2A loss-of-function.
		Loss-of-function p16INK4a	50	Melanoma	CDK4 and CDK6 activation and promoting of cell proliferation.
	p14ARF	Loss-of-function p14ARF	90–95	Melanoma	Loss-of-function of p53. Promoting of p53 degradation. Uncontrolled cell growth and proliferation.
	CDK4	CDK(R24C) Gain-of-function CDK4(CNV)	37	Melanoma (familial) Melanoma	Make p16INK4a unable to inhibit D1/CDK4 complex. Cell cycle progression.
PI3K/AKT	AKT3	Amplification AKT3	35	Melanoma	Negative regulation of PI3K/AKT pathway. Uncontrolled cell proliferation.
	PTEN	Loss-of-function PTEN	40–60	Melanoma	Negative regulation of PI3K/AKT pathway. Constitutive AKT activation. Uncontrolled cell proliferation.
	PI3K	PI3K	5	Melanoma	Negative regulation of PI3K/AKT pathway. Constitutive AKT activation. Uncontrolled cell proliferation.
Wnt/ $\beta$ -catenin	$\beta$ -catenin	$\beta$ -catenin stabilizing mutation in exon 3	1.5–3	Melanoma	Accumulation of $\beta$ -catenin protein in cytoplasm and nucleus, upregulation of mitogenic proteins like Myc and Cyclin D1. Uncontrolled cell growth and proliferation.
	Dickkopf-1, 2, 3	Reduced or lost expression of Dickkopf-1, 2, 3		Melanoma	Uncontrolled cell growth and proliferation.
	WIF-1	Reduced or lost expression of WIF-1		Melanoma	Uncontrolled cell growth and proliferation.

**Table 1. Some of the genetic alterations associated with melanoma progression. Adapted from Mozūraitienė et al., 2015.**

MAPK pathway is the signalling pathway more affected in melanoma. Mutated genes in this pathway are ordered by frequency.

## 2.5 Clinical strategies and therapies

Early identification of melanoma is most crucial part for the patient survival and treatment success. According to European society of medical oncology, clinicians follow a clinical practise guideline that help for the correct identification of melanoma stage of disease. As mentioned before, those guidelines are based on the size, colour, morphology, and localization of injury but in some cases, are complemented with mutation test. Different therapeutic options, including surgical resection, chemotherapy, radiotherapy, and immunotherapy among others, are used depending on the biology of the tumour and stage of the disease. In the case of primary melanomas, surgical resection is often a successful therapy option. This therapeutic strategy can be also complemented with adjuvant therapies like targeted therapy, chemotherapy or immunotherapy (Garbe et al., 2016). However, patients with metastatic melanoma the treatments options are reduced to targeted therapy, chemotherapy, and immunotherapy.

The main limitations of melanoma treatment reside on the large adverse events presented by chemo and immunotherapies and the lack of efficiency mostly due to the appearance of resistance to immune, chemo/targeted therapies, conducting to patient relapse. From 1970s to the early 2000s the standard treatment for advanced stages of melanoma was focused on the use of Dacarbazine, a chemotherapeutic alkylating agent, which acts by adding an alkyl group to the DNA, and therefore conducting to cell death. Nevertheless, this drug was not sufficient to guarantee survival prolongation along the years (Arvin S. Yang, 2014; Korn et al., 2022).

IL-2 and interferon alfa-2b were the first immunotherapy for metastatic melanoma approved first by the FDA in late 1990s and later by EMA (Rosenberg et al., 2018). The main function of immunotherapies is to stimulate the activation of the immune system. The use of the cytokine IL-2 is to expanding effector T-cells (Teffs) and Tregs meanwhile interferon alfa-2b activates multiple immunological cell types including T-cells, B lymphocytes and natural killer cells among others. Later on, an increase knowledge about the pathogenesis of the disease and the importance of immune system in tumour context allowed the increasing development of new therapeutic strategies. Since 2011, a successful new immunotherapy approach was focused on the blocking of the inhibitory effect of tumour cells against immune system (Hodi et al., 2010). Those drugs receive the name of immunological checkpoint inhibitors (ICIs). Ipilimumab was approved for the first time by the FDA in 2011 and later by the EMA, being the first treatment to demonstrate an increase of the overall survival in randomized clinical trial of metastatic melanoma (Hodi et al., 2010). This therapy is based

in the use of a human monoclonal antibody against cytotoxic-T lymphocytes antigen 4 (CTLA-4), an inhibitory molecule of cytotoxic T-lymphocytes that is able to bind the antigen presenting cells (APCs) and block the co-stimulation signalling needed to activate the T-cells (Buchbinder & Desai, 2016). With the years, similar strategies were developed including nivolumab and pembrolizumab, two antagonists of programmed cell death protein 1 (PD-1) and atezolizumab, an antagonist of programmed cell death ligand 1 (PD-L1).

PD-1 is localized at the cell surface of activated T and B cells and natural killer lymphocytes and influence downregulation the activation of those immune molecules by binding to its ligand PD-L1. Recently in 2022, a new drug called relatlimab-rmbw which is an antagonist of lymphocyte activation gene-3 (LAG-3) (Chocarro et al., 2022).

Advances in genetics techniques revealed that an elevated percentage of cutaneous melanoma present mutations in genes of important signalling pathways associated with melanoma cell proliferation and a malignant phenotype such as BRAF gene. As mentioned before, It is estimated that approximately 50% of melanoma patients present a mutation in BRAF gene (Ascierto et al., 2012). Mutated BRAF contribute to an hyperactivation of MAPK signalling pathway, well known as regulator of cell proliferation, differentiation, and survival. This allowed the development of therapy targeting the inhibition BRAF and MEK such as *binimetinib*, *encorafenib* and *cobimetinib*. These therapies can be also play in combination to target more than one dysregulated point in a single pathway in order to have a higher efficacy specially for late-stage metastatic patients (Proietti et al., 2020).

## 2.6 Phenotype plasticity and the phenotype-switch model in melanoma

Cutaneous melanoma is initiated by the malignant transformation of normal melanocytes due to a dysregulation in homeostasis. This transformation leads the formation of primary melanoma, and often to metastatic melanoma. Melanoma cells undergo a reversible phenotype switching controlled by differences in the levels of MITF factor. This plasticity goes from a proliferative/differentiated phenotype (high levels of MITF) to an invasive/ phenotype with stem-cell-like features (Low levels of MITF). Single cell level analysis of tumour heterogeneity not only confirm this phenotype switching but also describe intermediate phenotypes that resemble neural crest cell stem cells populations (Rambow et al., 2018). This phenotypical shift observed in melanoma progression share many

molecular and phenotypic parallels with the epithelial to mesenchymal process observed during different physiological processes and in metastatic progression of epithelial derived tumours. On top of that, those states can be associated to different phenotypical properties of cell stemness, or tumour invasion. Before, aberrant activation of EMT process was related exclusively to epithelial cancer or fibrosis. However, the advances of the last years have demonstrated that EMT is also observed in non-epithelial cancers, such as glioblastoma, neuroblastoma, lymphoma, liver cancer, sarcoma, and melanoma (M. Li et al., 2021; Majc et al., 2020; Pedri et al., 2022; Tao et al., 2020). For examples, undifferentiated melanoma resembles the mesenchymal state and phenotype of epithelial cancers once they have gone through EMT.

There is a well-considered similarity between de-differentiation programs and EMT process that not only include cell morphological alterations but also extrinsically changes to the cell. Apart from cell morphological changes, EMT produces several changes in the extracellular matrix, and changes in Cell-matrix and cell-cell interactions (Leight et al., n.d.; Yilmaz & Christofori, 2009). All those changes are also observed in de-differentiated melanoma cells with an invasive and metastatic phenotype (Kaur et al., 2020) as well as in multiple epithelial cancers (Lüönd et al., 2021).

EMT-TFs also have a role in the regulation of melanoma phenotype plasticity. A reversible switch from SNAIL2/ZEB2 to TWIST1/ZEB1 expression has been observed in the progressive transition from differentiated melanocyte to malignant melanoma (Caramel et al., 2013). In addition, this reversible switch is also associated with a poor prognosis of the disease and therefore reduced patient survival (F.Z. Li et al., 2015). The expression of SNAIL2/ZEB2 have been associated with proliferative/differentiated phenotype of melanocytes with high MITF expression. On the contrary, the decrease of SNAIL2/ZEB2 expression and increase of TWIST1/ZEB1 expression promoted the invasive and dedifferentiated phenotype of melanocytes that present a low MITF expression (Caramel et al., 2013). In addition, the augmentation of ZEB1 expression and malignant phenotype switching is accompanied by the expression of specified NCSC markers such as NGFR in melanoma cells (Richard et al., 2016).

This phenotype switching displays similarities with the classic EMT program and relies on changes in the cytoskeleton, cell-cell adhesion molecules and cell-matrix interactions. A comparison study of expression pattern of epithelial and mesenchymal markers between primary cutaneous melanomas samples from patients that develop metastasis and patients who do not, shows a positive correlation between the development of metastasis and the switch E-cadherin to N-cadherin expression

characteristic of EMT (Alonso et al., 2007). However, due to closely related mesenchymal nature of many melanoma cells, there is an observed inconsistency on the expression of EMT inducers and phenotype switching which showed to be context dependent and differs along the multiple types of melanomas. The expression of Snail2 was found to be drive by SPARC and promote melanoma cell invasion (Fenouille et al., 2012). Snail2 was also found to repress E-cadherin expression in melanoma by direct activation of the factor Zeb1 (Wels et al., 2011). Contrary to those observations, in aggressive uveal melanoma the switching to a proliferative state was observed to present high levels of E-cadherin due to the loss of the E-cadherin suppressor Id2 (Onken et al., 2012). These demonstrate the transition between epithelial-like and mesenchymal-like phenotype is context dependent but the ability of switching phenotype in melanoma is associated to higher risk and aggressiveness. the EMT phenotype with the observed phenotype switching in melanoma shows a clear overlap in the external signals that are activated or play a role in both contexts. The different microenvironments to which melanoma cells are exposed include signals such as TGF $\beta$  or WNT, both able to induce the downregulation of MITF and therefore the mesenchymal/invasive phenotype in melanoma. One example of shared mechanism between EMT and melanoma invasiveness is the activation of EIF2 $\alpha$  which have a direct influence in the suppression of MITF (Goding & Arnheiter, 2019). Moreover, transcriptome analysis has shown that invasive/undifferentiated melanoma cells lose most of their melanocytic characteristic, and are closely related to mesenchymal-like cells of epithelial tumours (Hoek et al., 2008) . All these data support the idea that the molecular mechanisms that induce and control EMT are similar to the ones underlying the rise and maintenance of the melanoma phenotype switching controlling the invasive capacity of this tumour.

### 3. OVOL2: an EMT inhibitor.

The previous chapters highlight some of the cellular and molecular features shared by the neural crest and the neural crest-derived tumour melanoma. While EMT is an important physiological process for neural crest during embryonic development, it can be also observed in the promotion of metastatic state in many cancers cell types, especially for solid tumours. This process has been also described in non-epithelial tumours specially in neural crest-derived tumours such as neuroblastoma and melanoma. Thus, understanding the molecules underlying the mechanism and regulation of EMT will provide a better understanding of melanoma progression. This is why we have been interested by OVOL2 a key transcription factor in the regulation of EMT/MET. Moreover, *Ovol* genes play a role in the regulation of stem-like state of cells including cancer cells, therefore having an important role during metastatic outcome and cancer progression.

#### 3.1 Phylogenetic Conservation of OVOL2.

OVOL2 encodes a member of the evolutionarily conserved ovo-like protein family. Those proteins are members of the zinc finger protein family and acts as transcription factors in the regulation of multiple genes expression. These factors act either as transcriptional activators or repressors depending on the process and on the model organism of interest (Andrews et al., 2000; B. Li, 2002; Mével-ninio et al., 1996). *Drosophila melanogaster* OVO gene was the first to be studied and therefore, the remains best characterized. In *Drosophila* the OVO locus encodes 3 different isoforms Ovo-A, Ovo-B and Shavenbaby, which share Zn-finger DNA-binding domains in the C-terminal region and present distinct functions (Hayashi et al., 2017). It is described as a complex gene (OVO/Svb) with two different active regions that controls distinct functions and processes such as female germline development and epidermal differentiation (Kumar et al., 2012). In vertebrates, there are three different OVOL genes OVOL1, OVOL2 and OVOL3 that present similar homology to the *Drosophila* ovo/shavenbaby. In mammals, OVOL proteins also share 4 C2H2-type zinc finger motifs, and the length of the protein varies from 215 to 286 amino acids. Phylogenetic analysis based on the zinc finger motifs have confirm the orthologous relationship of *drosophila* OVO gene with mouse and human OVOL2 protein. OVOL2 and OVOL1 are closely related to each other. Little information is found about OVOL3 functions (B. Li, 2002).

The OVOL1 gene is localized on chromosome 11 (q13) in the human genome flanked by a conserved set of genes. This region is conserved across several mammals including mouse, rats, and opossum. An identical locus is found in *Xenopus tropicalis* on the scaffold\_474. No similar locus was found in avian genomes. OVOL2 gene is localized on chromosome 20 (p11.2–p12) in the human genome flanked by a conserved set of genes. This region was found to be also conserved in mice, rats, and opossum. In this case, a similar region was found in avian genome (chicken, zebra finch and turkey). In contrast, no orthologous gene was found on fish genome (Kumar et al., 2012)

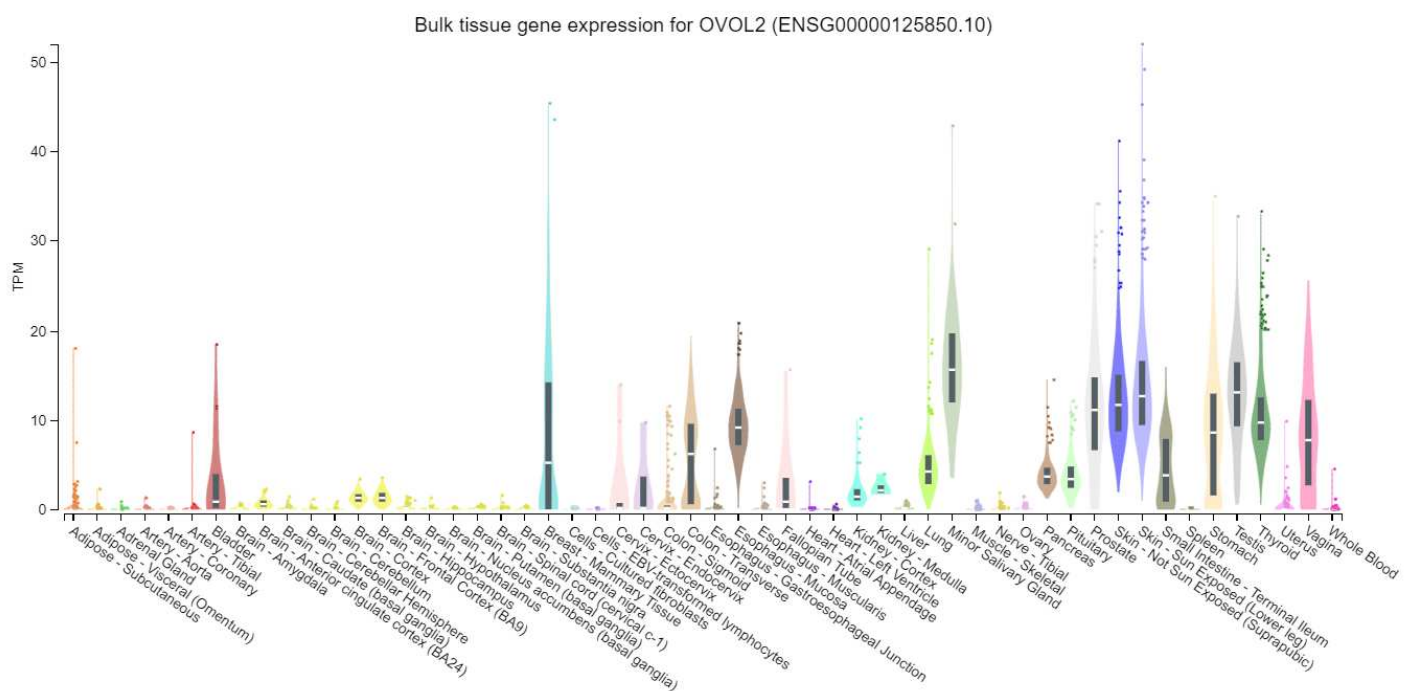
Mouse OVOL2 gene, presents different transcripts, known as OVOL2A and OVOL2B. OVOL2A is a 274-AA protein, while OVOL2B has a length of 241 AA. As mouse OVOL2, Human OVOL2 can be divided into A and B classes, and they differ on their 5' end. Human OVOL2A protein and mouse OVOL2A protein have an 89% of identity on their amino acids sequence and 98% of identity on the amino acid sequence of their zinc finger domains. Human OVOL2B also produces a shortened protein lacking one of the Zinc finger motifs situated on the N-terminal region (B. Li, 2002). The tetrad of C2H2 zinc finger motifs at the C-terminal region is highly conserved between ovo *Drosophila* and mammals. (B. Li, 2002)

Many studies demonstrated the involvement of OVOL family members in a wide range of processes, including development, differentiation, migration and recently thermogenesis (Fan et al., 2022; Mackay et al., 2006; Nair et al., 2006; Wells et al., 2009). OVOL1 and OVOL2 are crucial transcription factor for the regulation of epithelial lineage differentiation during development (Johnson et al., 2001; Mackay et al., 2006; Nair et al., 2007). Both factors play a crucial role in maintenance of epithelial state and are direct targets of GRHL2. OVOL1 represses *Ovol2* expression by binding to its promoter (Teng, 2007). OVOL2 inhibits EMT and promotes MET in different cell contexts (Hong et al., 2015; Z.-C. Liu et al., 2017; Roca et al., 2013; Watanabe et al., 2019; R. S. Wu et al., 2017).

### 3.2 Tissue specific expression

Northern blot analyses performed in human tissues showed that OVOL2 have a variable expression along different samples, presenting the highest levels of expression in skeletal muscle, thymus, and heart tissues. In the other side, OVOL2 present a different pattern of expression in mouse, with high levels of expression in testis, intestine, and stomach of adult mice. During mouse development, *Ovol2* expression was detected in the placenta, syncytiotrophoblast cells, yolk sac, chorionic plate at day

E8.5 and in the labyrinthine layer of the placenta at day E11.5 (Unezaki et al., 2007). Expression was also detected in the inner cell mass of blastocysts and in the epiblast but not in the extraembryonic region of E6.5 mice embryos. During development progression, OVOL2 is expressed in epiblast derived tissues and endoderm and axial mesoderm. Later on, OVOL2 expression is almost restricted to cephalic neural folds, ectoderm layer bordering neural plate and gut tube (Mackay et al., 2006). Unlike in humans, no expression was found in mouse skeletal muscle. In others species such as chicken embryos, OVOL2 shows a similar expression pattern than pre-neural markers such as Sox3 and neural plate markers such as Sox2 (T. Zhang et al., 2013). A complementary analysis of OVOL2 expression levels using normal RNA-human tissues was performed by GTEx Browser (<http://www.gtexportal.org/home/>). OVOL2 shows high expression in breast mammary tissue, skin, minor salivary gland, and testis. Low or no expression was observed in brain tissues, adipose tissues, or arteries, among others (figure 7).





## Figure 7. Bulk tissue gene expression for OVOL2.

Expression values are shown in TPM (transcript per million). Box plots are shown as median and 25 and 75 percentiles. Points are displayed as outliers if they are above or below 1.5 times the interquartile range.

### 3.3 Ovol2 functions

#### 3.3.1 Ovol2 in development

Despite the full conservation of their zinc finger regions, the regulation, and the role of OVOL proteins in development have not been fully studied. OVOL proteins display different functions in different species. In *Drosophila*, Ovo protein was shown to act genetically downstream of canonical Wg/Wnt signalling pathway and to be a key gene for epidermal denticle formation and female oogenesis (Garfinkel et al., 1992; Mével-ninio et al., 1996; Oliver, 1987). In mouse, OVOL1 protein was reported to also act downstream of the Wnt-B-Catenin-LEF/T-cell factor signalling (B. Li, 2002; Payre et al., 1999) and be important to kidney and male germ cells development.

In the other hand, OVOL2 is essential for correct embryonic development in mouse. Embryos lacking OVOL2 shows smaller heart that contract irregularly and present problems in angiogenesis. Those embryos died in the utero during early stages of gestation (Unezaki et al., 2007). Mutant embryos for Ovol2 display larger and rounder neural folds, lack of optic invaginations and present underdeveloped branchial arches. They also suffer failure of cranial neural tube closure and turning process (Mackay et al., 2006) . Loss of OVOL2 also produces slight alterations on the expression of signalling molecules involved in Dorsal–Ventral patterning of the neural tube. Additional experiments also shown that OVOL2 mutants embryos have defective neural crest maintenance and migration (Mackay et al., 2006). OVOL2 is also essential for placental formation in mouse. Mouse lacking OVOL2 are not able to develop placenta and therefore, the embryo die around E10.5. OVOL2-null placentas present Trophoblast with upregulated expression of Trophoblast stem (TS) cell markers such as *Id2*, *Esrrb* and *Eomes*. On the contrary, TPBA, a differentiated trophoblast marker, was barely detectable on ovol2-null placentas, suggesting a poor trophoblast differentiation in ovol2-null placenta resulted embryos. During normal Trophoblast stem (TS) cell differentiation OVOL2 expression is progressive. Due to this, experiments were performed to examine whether an ectopic increment of OVOL2 expression can drive differentiation of mouse TS cells. Results showed that increased OVOL2

expression produce an increment of some TS differentiation markers such as GCM1, SYNB, AND PRL3B1, compared to control cells, after one day of differentiation conditions (Jeyarajah et al., 2020). Supporting those evidence, OVOL2 overexpressing trophoblasts cultured in stem cell conditions present decreased levels of Eomes and Id2, two important markers of trophoblast stem state. OVOL2 act a transcriptional repressor and bind specifically upstream of the transcription start site of Id2.

Subsequently, the expression of Eomes and other stem markers decrease (Jeyarajah et al., 2020).

Studies in mouse stem cells (SCs) demonstrate the involvement of OVOL2 in cell differentiation. In mouse SCs, OVOL2 overexpression inhibits neural conversion by reducing the levels of Sox1 and Tju. In addition, this overexpression induces the mesendodermal differentiation of embryonic stem cells (ESC). On the contrary, OVOL2 knockdown promotes neural conversion and negatively affect mesendodermal differentiation of ESCs. OVOL2 is directly upregulated by BMP4 through the binding of p-SMAD1/5/8 molecules to the enhancer region of *ovol2*. BMP4 is also a direct regulator of *Ovol2* in chicken embryos. Moreover, *Ovol2* ectopic expression also inhibits the neural specification in chicken embryos (T. Zhang et al., 2013)

However, barely is known about the upstream mechanisms controlling OVOL genes expression during development. GRHL2 is one of the few known upstream regulators of OVOL1 and OVOL2 genes. During development, GRHL2 acts as trans-activator of OVOL1/2 by specifically binding to the promotor region. This is necessary to maintain the epithelial phenotype of cells of the palatal epithelium. Previous studies demonstrated that *Grhl2*<sup>-/-</sup> embryos, present altered expression of numerous EMT regulators including OVOL1 and OVOL2. Like mouse embryos lacking *Grhl2*, *Ovol2*<sup>-/-</sup> embryos die during mid-gestation, probably due to failure of cranial neural tube closure (Mackay, 2006; Rifat et al., 2010). The phenotypic similarity between these knockout embryos suggests that OVOL2 may be a key GRHL2 target during embryogenesis (Carpinelli et al., 2020). LSD1 is another upstream regulator of OVOL2 during development. In wild-type conditions, *Ovol2* expression shows a robust increase during TSCs differentiation. This progressive increment is no longer observed in *Lsd1*<sup>-/-</sup> mice. LSD1 specifically binds to a distal promoter region of OVOL2 gene, controlling its expression by demethylation of H3K4me2/1 marks. However, this interaction is exclusive for undifferentiated TSC conditions. During differentiation, LSD1 does no longer binds and control OVOL2 promoter (Zhu et al., 2014).

### 3.3.2 OVOL2 and Stem cell properties

Epithelial cells present a high plasticity and have properties that allow them to self-renew for tissue maintenance. Epidermis is one of the most well-characterized epithelial tissues. OVOL1 and OVOL2, two of the members of the OVOL family of transcription factors, play a crucial role for the maintenance of epithelial lineage specificity. There are many studies that have demonstrate the importance of OVOL1/OVOL2 in epithelial maintenance during development and for epidermal differentiation. In the mouse embryonic epidermis, depletion of OVOL1 and OVOL2 expands the epidermal basal compartment where progenitor cells reside (Lee et al., 2014). Lack of GRHL2, an upstream gene controlling *Ovol2* expression, results in defects in the nephric duct epithelium formation that present a reduced and altered lumen. The re-expression of OVOL2 in GRHL-KD cells, can restore the phenotype suggesting that the maintenance of nephric duct epithelium reside in OVOL2 function (Aue et al., 2015). In *Drosophila*, the activator form of *Ovo/Svb*, acts as a downstream effector of *wnt/EGFR* pathways, which is sufficient and necessary for inducing self-renewal and maintenance of intestinal stem cells and enteroblasts. *Ovo/svb* knockdown in adult progenitor cell results in a loss of progenitor cells caused by an increased apoptosis (Hayek et al., 2021)). In addition, the repressor form of *Ovo/svb* causes a decrease of stem cells properties through a precocious differentiation, being responsible of enterocyte differentiation (Hayek et al., 2021).

All this data identifies the role of OVOL2 as a guardian of epithelial lineage. Another remarkable function of OVOL genes is to inhibit EMT and induce the reverse process, MET, which have proved to be requisite for the reprogramming of fibroblast to induced pluripotent stem cells (R. Li et al., 2010; Samavarchi-tehrani et al., 2010).

Evidence have shown that OVOL2 expression eases fibroblast reprogramming into different epithelial cell lineages such as keratinocytes. The combination of ectopic expression of TP63/KLF4/OVOL2 in human neonatal dermal fibroblasts (HNDs) results in a dramatic increase of protein level of the keratinocyte specific marker KRT14 along the cell population. This augmentation is higher than in the condition of TP63/KLF4 expression alone, suggesting an increment of the reprogramming efficiency as the result of the combination of both genes with OVOL2 (Watanabe et al., 2019).

### 3.4 Ovol2-related pathologies.

#### 3.4.1 Actinic keratosis (AK) and cutaneous squamous cell carcinoma (cSCC)

Actinic keratosis (AK) and cutaneous squamous cell carcinoma (cSCC) are common types of precancerous and cancerous skin lesions. Those types of lesions are normally arising in elderly population due to long term exposures to sun. AK manifests by intraepidermal lesions created by atypical keratinocytes that remains in the epidermis. AK is a precursor of cSCC. However, the rate of transformation of AK to cSCC is low (around 0.1%). OVOL2 expression is present in both pathologies, AK and cSCC. Nevertheless, the levels of OVOL2 are found to be significantly decreased in patients' samples of cSCC compared to AK, suggesting that may have a role in the progression of the disease (Murata et al., 2020).

Studies carried out in human keratinocytes has demonstrated that the depletion of OVOL2 in this cell type is translated into a short-term gain of keratinocyte transient proliferation due to a direct inhibition of c-myc promoter. Additionally, the lack of OVOL2 in keratinocytes produce a loss of long-term proliferation potential and accelerates extracellular signal-induced terminal differentiation, suggesting the importance of OVOL2 to maintain the proliferation potential of keratinocytes and to suppress the differentiation of keratinocytes. The absence of OVOL2 produce an increase of K1, a terminal differentiation marker of keratinocytes by an increase of Notch1 signalling. Chromatin immunoprecipitation (ChIP) analysis has showed that OVOL2 binds Notch1 promoter in a specific manner. This repression causes a decreased activity of Hes1 promoter, a known target of Notch signalling pathway. All this together suggests that OVOL2 plays a key role in the inhibition of keratinocyte t differentiation in response to Notch signalling (Wells et al., 2009).

#### 3.4.2 Posterior polymorphous corneal dystrophy (PPCD)

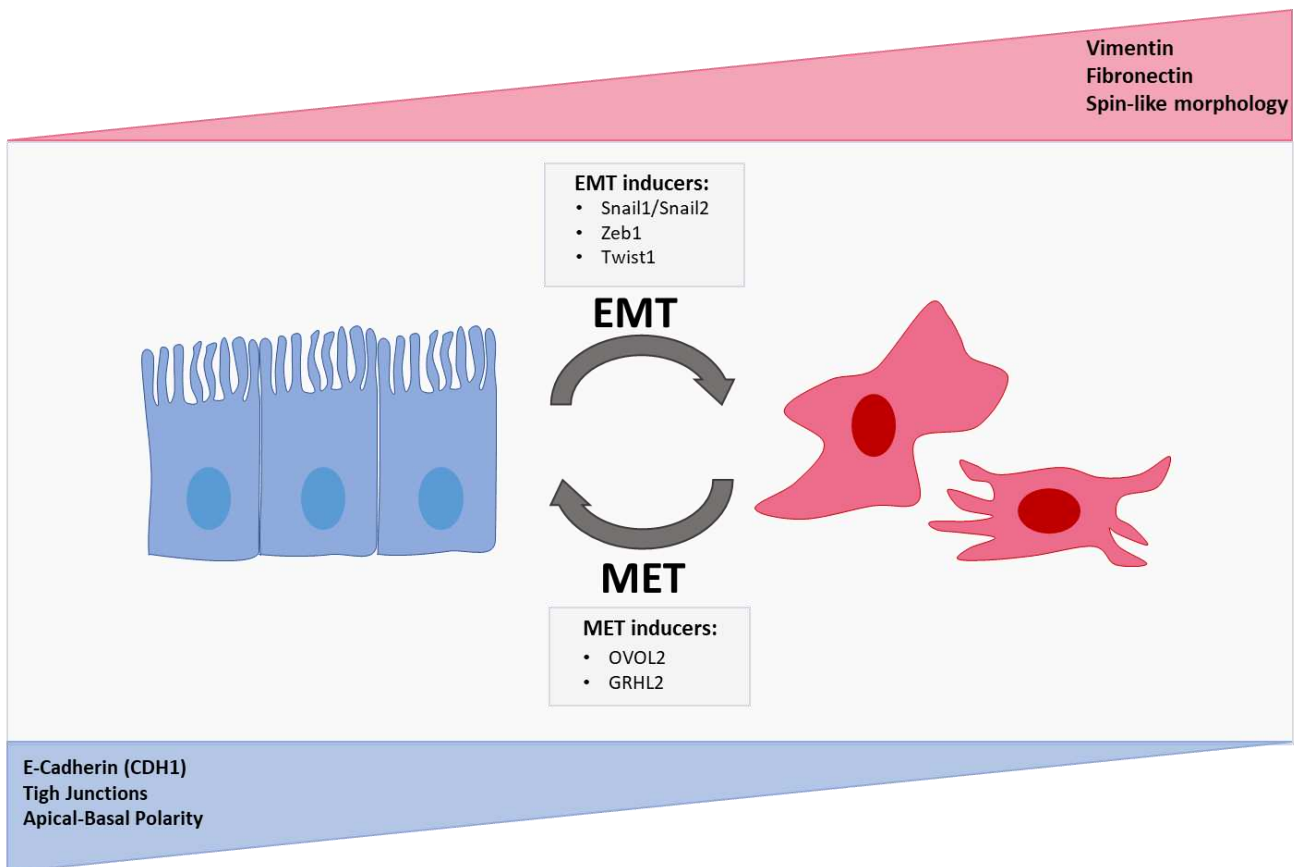
Posterior polymorphous corneal dystrophy (PPCD) is an autosomal-dominant congenital disease that mainly affects the corneal endothelium. One of the main characteristics of PPCD is abnormal corneal endothelial cell morphology and associated Descemet membrane changes. Patients present congenital altered morphology of the corneal endothelium that can show slow progression of the

abnormalities along their lives. Those patients can also present iris abnormalities, such as ectropion uveae and corectopia.

PPCD is a heterogeneous disease with a considerable percentage of the cases attributed to haploinsufficiency due to mutations or deletions of ZEB1, COL8A2 or OVOL2. PPCD1 is one of the less understood and has been associated to promoter mutations in *OVOL2* (OMIM 122000). Cell based assays have indicated that those mutations in the promoter region of *Ovol2* leads to increased *Ovol2* promoter activity. PPCD1 patient-derived findings, present a significant decrease of ZEB1, supporting the hypothesis that increased *OVOL2* expression produce PPCD1 disease by suppressing ZEB1 (Chung et al., 2019)

### 3.5 *Ovol2* in cancer: an inhibitor of the metastatic progression.

Numerous studies have demonstrated that epithelial to mesenchymal transition (EMT) and its converse process (MET) play a key role in the induction and progression of cancer cell migration and metastasis (Moustakas & Heldin, 2007). (Saenz-Sardà, X., Carrato, C., Pérez-Roca, L., Puig, L., Ferrándiz, C., Ariza, A., & Fernández-Figueras, 2016). Despite the wide knowledge about the role of EMT mediating factors in cancer, the mechanism that mediate MET are poorly understood. *OVOL* and *GRHL* families, are some of the few families of genes known as MET-TFs (Figure 8). As expected, those families of genes, present a strongly positive correlation with *CDH1*, an epithelial marker and a negative correlation with *VIM*, a mesenchymal marker. On the contrary, well-known EMT-TF genes, such as *SNAI*, *TWIST*, and *ZEB* families, exhibits opposite correlations (Watanabe et al., 2019). Similar studies demonstrated that along the known MET-TFs factors, *OVOL2* is the only one which is strictly necessary for inducing *CDH1* and the epithelial phenotype in human neonatal dermal fibroblasts (HNDFs). However, in fibroblast, functional MET induction is facilitated with the collaboration of combination of other factors such as *TP63/KLF4* and *HNF1A/HNF4A/FOXA3* (Watanabe et al., 2019).



**Figure 8. EMT/MET plasticity.**

Proposed scheme of EMT/MET plasticity. The epithelial to mesenchymal transition (EMT) is known to be induced by several TFs as Snail1/2, Twist1 and Zeb1 in most of the context. Those produce the loss of epithelial markers and phenotype including E-cadherin expression, tight junctions, and the apico-basal polarity of the cells. Both programs are controlled by the equilibrium of expression of EMT inducers and EMT inhibitors/MET inducers.

OVOL2 is one of the genes that have clearly been linked to impair cell migration through EMT inhibition in numerous cancer cell types and present a protective function of epithelial identity in epithelial cells (Fu et al., 2016; Roca et al., 2013). In osteosarcoma, OVOL2 protein expression levels negatively correlate with clinical stages being more expressed at early stages of the disease and shows a significant increase of expression in samples from patients without metastasis or recurrence (Liu J et al., 2018). In breast cancer higher Ovov2 expression is related to overall survival (OSu) and metastasis free survival of postoperative patients. (Watanabe et al., 2019). In Hepatocellular carcinoma (HCC), one of the deadliest cancers, OVOL2 expression positively correlated to E-cadherin and negatively correlated to N-Cadherin. Same set of patient's samples demonstrated that high OVOL2 levels correlated to a better prognosis than those with low OVOL2 levels (Fu et al., 2016).

In a set of tumours, including Breast cancer, Prostate cancer, and Osteosarcoma, EMT inhibition induced by OVOL2 seems to be due to a direct repression of Zeb1 expression, one of the most important EMT inducing factors. OVOL2 overexpression decreases Zeb1 expression in those tumours by binding specifically to the promoter region of Zeb1 (J. Liu et al., 2018). In addition, in the context of prostate cancer cells, ZEB1 has also demonstrated the specific inhibition of Ovov2. (Haensel et al., 2019; Hong et al., 2015; J. Liu et al., 2018; Murata et al., 2020; Roca et al., 2013). However, no influence has been observed on the expression of Snail1, Slug or Twist1. This EMT inhibition results in a significant decrease of tumour migration and invasion of metastatic cancer cells. Moreover, OVOL2 expression is concomitant with ZEB1 decrease in both cellular lines and human samples.

Other studies focused on the role of OVOL2 in EMT demonstrated that OVOL2 overexpression inhibits TGF- $\beta$  induced EMT in NMuMG mouse mammary epithelial cells. In accordance, the expression of the epithelial marker E-cadherin was retained in TGF- $\beta$  treated cells overexpressing OVOL2 and the activation of mesenchymal markers such as Vimentin and N-cadherin was suppressed. In addition, the enhanced migration and invasion induced by TGF- $\beta$  was almost completely inhibited in OVOL2 overexpressing cells (R. Wu et al., 2017). Those findings were extended in EpH4 murine epithelial cells previously transformed with H-RAS oncogene. Further studies about the effect of OVOL2 on TGF- $\beta$  signalling in tumour metastasis in vivo, demonstrate that the forced expression of OVOL2 inhibits the generation of lung metastasis of the tumours in of virgin 6-week-old BALB/c mice (R. Wu et al., 2017). More experiments carried out in NMuMG cells demonstrated that the inhibition of the TGF- $\beta$  signalling pathway was due to the inhibition of Smad4 expression, regulated by direct binding of OVOL2 to Smad4 promoter. Additionally, OVOL2 interferes with the complex formation between

SMAD4/2/3, blocking the binding of SMAD4 to Smad binding sequences of the DNA. In addition, OVOL2 activates SMAD7 expression by indirect mechanisms, which results in a decrease of the phosphorylation of SMAD2 and SMAD3 proteins (R. Wu et al., 2017).

All these observations have focused our attention on OVOL2 as a potential negative regulator of EMT and cell migration in the context of melanoma.



## OBJECTIVES

In summary, the development of the neural crest population thus shares some of the cellular and molecular mechanisms with those involved in tumour progression. The epithelial-to-mesenchymal transition (EMT) is an important dynamic process crucial during embryonic development in special for the migration of neural crest cell migration and therefore melanocyte lineage formation. EMT is not only observed under physiological conditions but also plays a role in conferring metastatic properties to many tumours including melanoma.

Previous studies have demonstrated the key role of OVOL2 in the inhibition of EMT in epithelial derived tumours such as breast cancer and prostate cancer (REF) through the transcriptional inhibition of the EMT-TF Zeb1. OVOL2 is also a critical EMT-inhibitory factor in developing skin and mammary epithelia. However, the role of OVOL2 in the regulation of EMT-like process observed in non- epithelial tumours such as melanoma have not been explored yet.

These studies prompted us to ask if OVOL2 may also have a role in the regulation of melanoma cell migration, and if yes how this role may be mechanistically linked to the metastatic potential of the tumour cells.

All these aspects taken together raise several questions that will be addressed in this thesis:

- 1) Does OVOL2 interfere with the migratory and invasive capacity of melanoma cells in vitro and in vivo?
- 2) Is OVOL2 affecting melanoma cell migration by modifications of the EMT-TFs ?
- 3) What are the molecular mechanisms underlying the regulation of melanoma cell migration by OVOL2?

Answering these questions would establish the molecular details by which OVOL2 would be able to regulate the capacity of melanoma cells to migrate and therefore the metastatic process. Furthermore, this study would be the first to explore the role of OVOL2 in the context of melanoma.



# RESULTS

# 1. Ovolutin as an inhibitor of melanoma progression

## 1.1 Introduction and summary of main results

As mentioned before, OVOL2 has been previously identified as a critical inhibitory EMT factor in different contexts including skin development and some epithelial derived cancers including breast and prostate cancer. In those studies, OVOL2 was shown to be closely related to EMT inhibition during tumour invasion by directly inhibiting one of the main EMT-TFs, Zeb1.

EMT is involved in tumour progression of epithelial derived tumour, and it is also known that specific pattern of expression of some EMT-TFs are directly involved in the phenotype switching between differentiated/proliferative and neural crest stem cell-like/invasive state observed in melanoma. Moreover, EMT factors are also considered as major drivers of intra-tumour heterogeneity and drug-resistance. This background prompted us to ask whether OVOL2 would have also an influence in the progression of melanoma. For that, we used different melanoma cell lines with different characteristic to help us better understand the influence of OVOL2 on cell migratory capacity in melanoma. Such in vitro model also allowed us to explore which mechanisms and pathways are influenced by OVOL2 during melanoma migration.

Using a plasmid encoding OVOL2 protein and siRNA (small-interfering RNA) designed against *ovol2* sequence, in this article we have shown how different levels of OVOL2 expression in different metastatic melanoma cell lines have an impact on their migratory capacity in vitro. Moreover, we explored the sequence conservation and functional conservation of the protein by introducing the expression of *xenopus laevis* protein. We observed that the function of this TFs is evolutionary conserved as we obtained similar results to the overexpression of human OVOL2 protein.

Thanks to the use of zebrafish as in vivo model, we also could observe and evaluate that increased levels of OVOL2 also impair the capacity of melanoma cell to detach from primary mass and to migrate along the body. However, the use of molecular techniques such as qPCR and western blot proved that the modulation of OVOL2 expression did not affect the RNA levels or protein levels of the main EMT-TFs. Therefore, this suggested that the phenotype observed *in cellulo* was not related to a modulation of EMT-TFs. With the use of RNA-seq methods, we could observe that OVOL2 was affecting the migratory capacity of melanoma cells by affecting genes implicated in the cytoskeleton formation, cell-adhesion, and cell polarity of the cells. The parallel use of confocal imaging techniques

of cells overexpressing OVOL2 protein also suggested that OVOL2 affects cytoskeleton and cell-matrix adhesion properties in melanoma cell lines.

## 1.2 Main results – On course to be published

### **OVOL2 transcription factor interferes with melanoma cell migration and metastatic potential.**

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**Keywords:** OVOL2, melanoma, migration, EMT, Keratins, cytoskeleton,

**Abstract:**

Great progress has been done in the study of the role of OVOL2 TFs as inhibitor of epithelial-mesenchymal transition (EMT) and migration in a variety of cancers. Nevertheless, the involvement of OVOL2 in the metastatic outcome of non-epithelial derived cancer such as melanoma has not been explored.

In the present study, we bring a potential role of OVOL2 in controlling melanoma progression. The use of patient data from public databases indicated a strong negative correlation between OVOL2 expression and metastatic progression of melanoma. Moreover, our experiments revealed demonstrated that OVOL2 expression inhibits migratory capacity of metastatic melanoma cells by an independent mechanism to EMT, by producing alteration in the cytoskeleton of the cells.

## **Introduction**

Melanoma is one of the most aggressive and invasive types of skin cancer with an increasing worldwide incidence in fair-skinned population. Initial stages of the disease can be successfully treated by surgical removal while advanced metastatic stages present lower prognosis (Garbe et al., 2022). Melanoma result from the malignant transformation of melanocytes, the neural crest-derived cells generating the melanin pigment of the skin. Despite the great advances in treatments, such as BRAF-targeted and immune modulation-based therapies, there is still a high percent of tumours showing low response to treatment or relapse after an initially successful treatment (Luther et al., 2019). Melanoma present high intra- and inter-tumour heterogeneity: various subpopulations of tumour cells coexist within the tumour lesion and express different genes associated with multiple cellular phenotypes including stem-cell like genes (Grzywa & Paskal, 2017). This property contributes to the tumour's enhanced ability to adapt to selective pressures and to survive in different microenvironments within the primary tumour and during metastatic invasion (Rambow et al., 2019). Melanoma cells' phenotype plasticity or “phenotype switching” allows them to transition in both directions from a proliferative state to an invasive one under the control of molecular cascades which focus intense research (Najem et al., 2022). Metastasis is a multistep complex process characterized by the cancer cell's ability to infiltrate the proximal tissues, to intravasate, circulate through the blood and lymph flows, extravasate and colonize distant tissues and organs. In carcinomas, the metastatic transformation includes a complex series molecular and cellular events starting with an epithelial-to-mesenchymal transition (EMT) which is a progressive switch from an epithelial state to a mesenchymal state (Yang et al., 2020). After extravasation, the formation of secondary tumours involves the reverse process of mesenchymal-to-epithelial transition (MET) (Li et al., 2015; Pedri et al., 2022; Yang et al., 2020). Although melanoma are not epithelial tumours, their invasive properties rely on molecular mechanisms related to EMT and MET. For example, previous studies demonstrated a reversible and progressive change in the expression pattern of the main EMT-TFs in melanoma. A progressive loss of ZEB2/SNAI2 and gain of TWIST1/ZEB1 expression was observed during the malignant progression of the disease and was associated with a worse prognosis (Caramel et al., 2013). Although the implication of EMT as an early driver of metastasis have been widely demonstrated in epithelial derived tumours (Du et al., 2020; Lima et al., 2016; Ye et al., 2017), its role is yet to be fully elucidated in melanoma metastasis. While knowledge of the mechanisms controlling EMT grows rapidly, the pathways blocking EMT, and mediating MET are far to be understood. Among



the potential negative regulators of EMT, OVOL1/2 are zinc finger-containing transcriptional repressors of the evolutionarily conserved Ovo-like protein family, identified as a critical regulators of skin keratinocyte differentiation and of wound healing and as EMT repressors/MET inducers in breast and prostate cancer (Roca et al.,2013).

The transcription factor OVOL2 belongs to the OVO-like family of C2H2 zinc-finger transcription factors (OVOL1, OVOL2, OVOL3) which act as master regulators of epithelial development and differentiation during embryogenesis (Briana Lee et al., 2014; Nair et al., 2006). Moreover, OVOL2 has recently been studied as a transcriptional repressor of EMT and its low expression levels have been associated with a worse prognosis in multiple cancer types. Mechanistically, OVOL2 inhibits tumour progression by affecting EMT in several cancer types including colorectal, prostate, and breast cancer through the transcriptional inhibition of ZEB1 (Roca et al., 2013; Xia et al., 2021). Further studies in human and mouse tumour cells have shown that OVOL2 also inhibits TGF- $\beta$ -induced EMT by affecting the signalling pathway at multiple levels. This includes the inhibition of *Smad4* mRNA expression, the induction of *Smad7* mRNA expression and interference with the binding of SMAD4 binding to its target DNA sequence. All this suggests that OVOL2 may inhibits EMT by several molecular pathways (Wu RS. Et al., 2017). However, the role of OVOL2 in non-epithelial cancers such as melanoma has not been explored yet.

Here we studied the role of OVOL2 in metastatic melanoma. We first correlated the gene's expression levels with the metastatic status of the disease using public datasets. Then, using a combination of *in vitro* and *in vivo* experiments, we showed that changes in OVOL2 expression levels affects the migratory capacity of metastatic melanoma cells. This did not involve modulation of EMT-TFs expression, contrasting with its role in hepatocarcinoma, breast cancer and prostate cancer (Fu et al., 2016; Roca et al., 2013). Rather, we find that cytoskeleton organisation and expression of keratin intermediate filaments was modified. In sum, our study reveals that OVOL2 can reduce the migratory potential of melanoma cells by alternative cellular mechanisms to the ones described before.

## **Results**

### **In human cutaneous melanoma *OVOL2* expression levels anticorrelate with tumour cell migratory potential and tumour progression.**

*OVOL2* expression had not been studied in human primary and metastatic cutaneous melanoma previously. We thus explored several public human tumour transcriptome datasets to assess *OVOL2* levels and correlate them with melanoma progression. We first used R2genomic (R2: genomics Analysis and Visualization Platform (<http://r2.amc.nl>)) on two independent datasets that allowed us to compare *OVOL2* expression in 45 primary and 92 metastatic cutaneous melanoma samples (Riker et al., 2014; Xu et al., 2008). In both melanoma collections, *OVOL2* was significantly more expressed in the primary tumour compared to metastases (Figure 1A-B). Moreover, these data indicate an approximate log<sub>2</sub> intensity level for *OVOL2* in each tissue: 5.63 in primary tumours and 4.61 in the metastases, compared to an average level of 7 in normal skin (supplementary Figure 1A). A third dataset was used where we could explore *OVOL2* expression between Normal skin, Nevus and Melanoma. We observed that *OVOL2* expression is progressively decrease as disease progress from Normal skin to nevus and finally melanoma (Figure 1C). In addition, *OVOL2* expression was explored using GEPIA (Gene Expression Profiling Interactive Analysis), a valuable resource for gene expression analysis based on tumour and normal samples extracted from TCGA and GTEx databases (Tang et al., 2017). In this third dataset comprising 558 normal skin biopsies and 461 cutaneous melanoma samples (both primary and metastatic), *OVOL2* was also significantly more expressed in the normal skin compared to melanoma (Figure 1D). Together those observations indicated that *OVOL2* was clearly expressed in melanoma albeit at lower levels than in the skin. Its expression was further downregulated in metastatic cells compared to primary melanoma, suggesting that decreased *OVOL2* expression paralleled tumour progression. However, when we explored the correlation between the levels of *OVOL2* and survival prognosis for patients, using Kaplan-Meyer plots (Supplementary Figure 1B), we did not find a significant relationship between high/low *OVOL2* levels and poor/good prognosis. This suggested a relationship of *OVOL2* expression with metastatic acquisition of melanoma cells but not with the clinical outcome of the disease.

We then retrieved *OVOL2* mRNA levels using a microarray dataset studying 24 commercial cell lines of cutaneous melanoma (Rambow et a., 2015). We found lowest expression of *OVOL2* in cell lines reported to be the most invasive such as Mewo and A375M, while non-migratory cells such as MNT-1 displayed higher levels of *OVOL2* mRNA (Figure 1E). We selected 5 cell lines for further

study (Mewo, A375M, Lu1205, SK28MEL, MNT1) because they were representative of the high/low levels found in the panel of 24 cell lines. We then directly quantified OVOL2 protein levels by Western blotting and cells migration parameters *in vitro* on a fibronectin substratum by live video microscopy. Although the protein levels did not directly correlate with the published mRNA levels, we found that *OVOL2* mRNA levels correlated negatively with cell velocity *in vitro*, and positively with the duration of pausing time (Figure 1.F-G; supplemental 1 C-D). Altogether these data correlated *OVOL2* higher expression with low migratory activity and suggested that *OVOL2* transcription factor might influence of melanoma progression.

### **OVOL2 depletion enhances melanoma cell motility while increased OVOL2 levels impair metastatic melanoma cells migration *in vitro* and invasion *in vivo*.**

To test *OVOL2* function in cutaneous melanoma cell migration, we chose Mewo and A375M tumorigenic and highly metastatic melanoma cell lines because both expressed low levels of *OVOL2* RNA and protein (Figure 1E) and actively migrated on fibronectin *in vitro* (Sittewelle et al., 2022) ; Figure 1F-G). Cells were transfected with an expression plasmid encoding human *OVOL2* protein or a control plasmid encoding a Histone2b-GFP fusion construct (Supplementary 2A). Two days after transfection, 100 cells per condition were tracked by time-lapse videomicroscopy. Both Mewo and A375M cells transfected with *OVOL2* displayed strongly impaired cell migration with decreased average velocity (by 40% for A375M cells and 15% for Mewo cells, n=3) and increased pausing time (Figure 2A). Lu1205 and SKMEL28 cells showed a similar decrease of velocity and increase in cell pausing (Figure supplementary 2B-C, n=1).

Multiple sequence alignment of *OVOL2* showed that the human protein shared high amino acid sequence identity with *Ovol2* proteins from different vertebrate species including mouse (89% amino acid sequence identity), chicken, and frog (64% amino acid sequence identity with *Xenopus tropicalis* *Ovol2*; Supplemental Figure 3A). All the sequences highly differed in their C- and N-terminal domains but presented a high conservation of the zinc finger (ZF) DNA-binding motifs. Human *OVOL2* ZF motifs presented 92% and 82% of similitude with mouse and *Xenopus tropicalis* ZF motifs respectively (Supplemental Figure 3B), which suggested a high functional conservation of the transcription factor activity of *OVOL2*. To gain deeper insight into the role and functional conservation of *OVOL2*, and to track the transfected cells with GFP, Mewo and A375M cells were transfected with a plasmid encoding the *Xenopus tropicalis* protein fused to GFP (pCMV-x*Ovol2-gfp*).

GFP-positive cells were sorted using fluorescence-activated cell sorting (FACS) and seeded on fibronectin. Random *in vitro* migration of 100 cells was tracked for 16 hours. Both Mewo and A375 cells overexpressing the *Xenopus tropicalis* Ovol2 protein presented the same migratory phenotype defects as those obtained with human OVOL2: a significant decline in cell velocity and a slight increase in cell pausing (Figure 2B; n=3). Cell cycle and cell death were evaluated after OVOL2 gain-of-function to assess if the migratory phenotype was consecutive to perturbation in those parameters. Neither Mewo nor A375M cells showed notable change in proliferation or cell death levels after overexpressing either the xenopus or the human proteins (Supplementary Figure 3C).

To gain further insight into the implication of endogenous OVOL2 in metastatic melanoma cell migration, we interfered with OVOL2 expression in SK28-MEL and Mewo cell lines, both showing high and lower levels both for RNA and protein of OVOL2 respectively (Figures 1E and Supplementary 1C). We validated antisense interfering RNAs (siRNA) designed against the human sequence (Figure 3A). After OVOL2 depletion, cell velocity was significantly increased while cell pausing was reduced compared to control in both Mewo and SK28MEL cells (Figure 3B). Together, these *in vitro* gain-of-function and depletion results supported the hypothesis that enhanced OVOL2 levels reduced melanoma cell migration and indicated that this function was evolutionarily conserved.

Last we explored the effect of OVOL2 *in vivo*, using a zebrafish xenograft model of metastatic melanoma. Green, fluorescent reporter Mewo cells (Mewo-GFP) were engineered, transfected with pCMV-OVOL2 and implanted into the developing swimming bladder of 3-days old zebrafish embryos (Figure 4A). Tumour cell mass was measured after injection (t0) and 72h later using confocal microscopy. Mewo-GFP cells transfected with the control plasmid efficiently detached from the primary mass 3 days post-injection, whereas the cells overexpressing OVOL2 showed a significant reduction in the number of emigrating cells (Figure 4B-C). These results indicated that increasing OVOL2 levels in a highly metastatic cell line such as Mewo cells, impaired their ability to detach from the primary mass and to invade adjacent tissues *in vivo*.

Altogether, these observations showed that *in vivo* and *in vitro*, OVOL2 acted as a brake on cell migration, prompting us to explore the molecular mechanisms underlying this effect.

### **OVOL2 levels influence cell morphology and cytoskeleton organization in metastatic melanoma cells.**

Melanoma cells exhibit a variety of cell morphologies in culture. Mewo cells are characterized by a spindle fibroblast-like phenotype while SKMEL28 and A375M cells display a polygonal morphology with well-formed lamellipodia. To evaluate the effect of lowering OVOL2 on melanoma cell morphology and cytoskeleton organisation, we depleted OVOL2 using siRNA in SKMEL28 cells changed their morphology to a more spindled shape and exhibited an increase of filopodia formation (Figure 5A). To further study the effects of increased OVOL2 levels on melanoma cell morphology, we selected Mewo, A375M, Lu1205 and SKMEL28 cells expressing pCMV-*xOvol2-gfp*. A375M cells overexpressing OVOL2 presented reduced filopodia formation and a structure mainly based on lamellipodia. In the case of Mewo cells, characterized by fibroblast-like phenotype without lamellipodia formation, we observed a reduction on the shaped morphology (Figure 5B). Two days after transfection, positive cells were fixed and stained with phalloidin to dye filament actin (F-actin) (Figure 5C).

Using ImageJ for the analysis of cells seeded on glass, we were able to measure and compare the area and perimeter of the cells. In the case of A375M, Lu1205 and Mewo cell lines, the increased expression of OVOL2 affects the cytoskeleton structure, producing a significant decrease in the area of the cells. Only in the case of A375M and Lu1205, a significant reduction was also observed in the perimeter of the cells. Non-significant effect was found in the area or perimeter of SKMEL28 (Figure 5D). Altogether, those results suggest that changes on OVOL2 expression strongly impact melanoma cells morphology affecting the cytoskeleton.-

### **OVOL2 increases several keratin gene expressions but does not alter EMT-TFs expression in melanoma cells.**

In other cancer contexts, OVOL2 impairs cell migration by directly affecting the expression of EMT-inducing factors (Fu et al., 2016; Liu et al., 2018; Roca et al., 2013). However, in melanoma cell lines, we did not observe notable change in the expression of the EMT-inducing transcription factors (Snail1, Twist1, Snail2, Zeb1, Zeb2) and of the mesenchymal marker vimentin, after OVOL2 gain of function, either by RT-qPCR or Western blotting (Figure 6A-B). This suggested that the phenotypes observed in melanoma rely on alternative, EMT-independent mechanisms.

As OVOL2 is best known to act as a transcription factor (repressor), we performed RNA sequencing 48h after OVOL2 gain-of-function in Mewo cells to identify genes modulated soon after OVOL2 increase. Principal component analysis (PCA) analysis reveals a good quality of Mewo replicates. However, a strong batch effect was observed in A375M (Supplementary Figure 4A-B)Ovol2 levels increase of about 19-fold (Supplementary Table 1). In these conditions, OVOL2 gain for two days induced relatively limited changes in the cells' transcriptome compared to the control condition: as 37 genes were differentially expressed (Log2Fold Change > 1, p-value < 0.05; Figure 7A-B). Among those, 11 were downregulated (25.7%) and 26 (74.3 %) were upregulated suggesting direct down-regulated target genes and indirect consequences in upregulating other genes (Figure 7C). Confirming our previous results, EMT-TFs did not present any significant change after OVOL2 expression (not shown). GO-term enrichment analysis (DEVEA software, Riquelme-Perez et al., 2022) showed that the upregulated genes were involved in cell junction organization (AMIGO2, C3, CRB3, **KRT14**), cell differentiation (C3, CD74, DMBT1, IGF2, **KRT14**, **KRT80**, **KRT81**, **KRT86**, OVOL2, SEMA6B, TH), extracellular matrix composition (COL5A3, TINAGL1) and extracellular matrix binding (DMBT1, TINAGL1) among others (Supplementary table 1). In the downregulated genes we can highlight ITGB7 a member of the integrin superfamily and **SERPINE** a serine proteinase inhibitor previously associated as regulator of cell migration (Degryse et al., 2004) (Supplementary table 2). In the case of A375M, we observed a total of 33 genes differentially expressed. Among those, we highlight the upregulation of cytokeratin 13 (KRT 13, an intermediate filament that form part of the cytoskeletal and structural integrity of the cells (Supplementary table 3).

We next evaluated the expression of the upregulated targets across RNA-seq databases of melanoma patient's samples (GEPIA). We observed that WISP2, KRT14, KRT80, SH2D3A, CRB3 and TINAGL1 were significantly increased in tumour compared to normal samples (Figure 7D). The expression of differentially expressed genes were also checked between normal skin, nevus, and melanoma. Among the genes we observed that SERPINE1 expression (downregulated by OVOL2) is significantly increased in melanoma compared to nevus and normal skin. On the other hand, KRT14 expression (upregulated by OVOL2) is significantly higher in normal skin and nevus compared to melanoma. (Supplementary 4C-D).

The expression of OVOL2 upregulated genes was also explored in microarray public dataset of primary melanoma and metastatic melanoma human samples. We observed that except for SHDB2 and KRT80, all the genes are differentially increased in primary melanoma compared to metastatic

melanoma as we also found previously with OVOL2 (Supplementary 4E-H). All these proteins are involved in functions such as cell polarity, cell adhesion and cell migration. In the case of TINGL1, has also been pointed out as potential target in the inhibition of metastasis in other cancer such as non-small cell lung cancer (Umeyama et al., 2014). Several keratin genes were very highly activated after OVOL2 gain, reminiscent of OVOL2 developmental function in skin. Importantly, KRT14, a family member of intermediate filament proteins involved in cell cytoskeleton, have been previously associated in recent studies with a better prognosis of melanoma (Han et al., 2021) and with the transition from a mesenchymal to epithelial state in squamous cell carcinoma (Takaishi M et al., 2016). Altogether those results suggest that OVOL2 expression is inhibiting the detachment and migration of melanoma cells by affecting the cytoskeleton, cell-cell adhesion, and cell-matrix adhesion of melanoma cells. For validation of those findings, we are particularly interested on the protein expression of keratins since they are part of the cytoskeleton of the cells and are directly related to the migratory capacity of cells. We will use different melanoma cell lines to corroborate if the overexpression or depletion of OVOL2 in those, is traduced in changes on the protein and RNA levels of Keratins.

## Discussion

The data presented in this work reveals a novel role for *OVOL2* as important regulator of migration in melanoma. Combining *in vitro* and *in vivo* experiments, as well as the exploration of published data from cancer databases, we were able to demonstrate a clear inverse correlation of *OVOL2* expression with the metastatic and migratory potential of metastatic melanoma through mechanisms independent from EMT. Specifically, we show that *OVOL2* expression significantly decrease with the metastatic progression of melanoma. Using *in vivo* and *in vitro* models, we observed that an increment on the expression of this gene in metastatic melanoma cells is able to impair their ability to detach from primary mass and migrate.

Metastases is responsible for the greatest number of cancer-related deaths. Cancer metastasis is a complex process in which for cancer cells to disseminate to distant organs and metastasize, they detach from the primary tumour mass, enter circulation, and establish themselves into a secondary site (Gui & Bivona, 2022). In the past years, multiple evidence has demonstrated that EMT play a crucial role in the induction of invasion and metastasis of many cancers, in special epithelial, derived cancers (Cho et al., 2022; Loret et al., 2019; Ribatti et al., 2020; Veloso et al., 2022; Vijay et al., 2019). *OVOL2* is one novel protein that have been identified as regulator of EMT/MET plasticity in multiple cell contexts (Roca et al., 2013; R. Wu et al., 2017) and several studies have also revealed its capacity to inhibits EMT and migration in some cancer types by transcriptional inhibition of *Zeb1* mRNA (Haensel et al., 2019; Murata et al., 2020; Roca et al., 2013; Q.Wu et al., 2018). Although melanoma is not an epithelial-derived tumour, there is evidence that EMT-TFs are regulators of the well-defined phenotype switching observed in melanoma, that contribute to the migratory capacity/invasiveness and the emerging resistance to therapeutics.

The use of *in vitro* approaches in multiple metastatic melanoma cell lines helped us to show that *OVOL2* plays a role in the migratory capacity of melanoma cells and that its function is conserved across species. Using zebrafish as *in vivo* model of xenografts, we show that *OVOL2* gain of expression in melanoma cells reduced their capacity *in vivo* of detach from the primary mass. However, unlike what was described in other cancer types, we observed that in the case of melanoma, *OVOL2* does not control migration by controlling the expression of the main EMT-TFs (Roca et al., 2013) but rather by upregulating the expression of many genes involved in roles such as apico-basal polarity and tight junctions' formation and cytoskeleton organization and previously



associated to reduce migration in other cancer types. Among those it is worth mentioning the upregulation of KRT14, a member of the type 1 of cytokeratin family of intermediate filaments that have previously been associated with a good prognosis of the melanoma disease (Han et al., 2021; Miñoza et al., 2022). Thus, together with the observed morphological and phenotypic alterations observed after independent overexpression, suggests that OVOL2 expression interferes with the migratory capacity of melanoma cells through direct modifications of the cell cytoskeleton and the cellular structure of the melanoma cells. In comparison to previous studies in other cancer types in which OVOL2 inhibits cell migration by repressing EMT inducing factors or signalling pathways, this demonstrates that OVOL2 can also act by controlling cell migration and metastatic capacity through different mechanisms depending on the context and cell lineage. Hence, the levels of OVOL2 in melanoma cells plays a potential role in progression of the disease.

## **MATERIAL AND METHODS**

### ▪ **In silico preliminary studies**

*In silico* studies to explore OVOL2 expression in melanoma were done by using R2 Genomics Analysis and Visualization Platform (<https://r2.amc.nl>) a genomics analysis and visualization platform developed by the laboratory for experimental Oncology and Radiobiology of the Centre for experimental and molecular medicine of Amsterdam. Selected dataset was from patient melanoma samples (Matta (GSE7553) and Hynes (GSE8401)). Multiple sequence alignment was performed using Clustal Omega Multiple Alignment program provided by Bioinformatic analytical tool of EMBL (Maderia F et al., 2022)

### ▪ **Cloning, plasmids, vectors**

To ectopically express FLAG-tagged OVOL2 encoding human protein in Mewo and A375M cells, pCMV6-Entry constructs containing the complete human cDNA sequence of *Ovol2*, each linked to a 3'-sequence encoding a C-terminal FLAG tag, were obtained from OriGene (MR203678, Rockville, MD, USA). For testing protein functional conservation, a plasmid encoding the OVOL2 xenopus tropicalis sequence (clone ORFeome XB-ORF-19768813) was cloned into a destination vector containing pCMV IE93 promoter and GFP. Cloning was done following Gateway Cloning method.

OVOL2 was inserted into the pGCS-N6(eGFP) destination vector (#85723). For control plasmid plasmid encoding a Histone2b-GFP fusion construct was used.

For generating Mewo-GFP stable cell lines: Recombinant lentiviruses were produced by transient transfection in HEK293T cells (70%-80% confluence). Packaging plasmids pVSV-G (5µg), pCMVD8.91 (20µg) and the GFP Vector plasmid (20µg) were mixed with CaCl<sub>2</sub> (250µM)/ hepes solution. HEK293T cells were transfected by addition of this transfection mix dropwise. Transfected HEK293T cells were incubated 72 hours at 37°C. Filtered supernatant was collected and store at -80°C before use.

- **Cell Culture, cell transfection and cell infection.**

The well-characterized human melanoma cell lines (Mewo, A375M, MNT1, SKMEL-28) were kindly provided by Dr. L. Larue (Institut Curie). Cells were cultured in RPMI (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). All cell lines were incubated at 37°C with 5% CO<sub>2</sub>. 24H before transfection; cells lines were plates at 3 x 10<sup>5</sup> cells per well into 6-well plate. Each plasmid (pCMV6-*Ovol2*-tagged, pCMV-*xOvol-gfp*, Histone2b-GFP) were transfected into Mewo and A375M cells using Transporter™ 5 transfection reagent (Polysciences, cat n° 26008) according to the manufacturer's instructions. For siRNA experiments, cells were transfected either with a control siRNA (Stealth Negative Control Medium GC Duplex, Invitrogen) or with siOVOL2 (REF #4392420) using lipofectamine RNAimax (Invitrogen) according to the manufacturer's instructions.

- **Protein Extraction and Western blot Analysis.**

Cells were washed in PBS and lysed in RIPA buffer (10mM Tris-HCL pH 8, 150 mM NaCl, 1% NP-40, 0,1% SDS, 0,5M sodium deoxycholate) supplemented with phosphatases inhibitor (Sigma) and proteases inhibitors (Sigma) at 4°C. Protein samples resolved on 12 % SDS-PAGE gels and transferred to PVDV membranes (Biorad). After blocking in 5% skimmed milk diluted in TBS - 0,1% Tween (TBS-T), membranes were probed with primary antibodies diluted in the blocking buffer overnight at 4°C. Primary antibodies and respective dilutions are indicated in **Table SM1**. After three washes in TBS-T, membranes were probed with HRP-conjugated goat anti-rabbit or anti-mouse (1:20.000) 1 hour at room temperature. ECL signal was quantified by densitometric analyses using Image J software (<http://rsb.info.nih.gov/ij/>).

- **Migration Assay**

24h after transfection, cells were dispensed in  $3 \times 10^4$  cells per well into 12 well plates coated with Fibronectin (40ug/ml). Two-Dimensional (2D) random cell migration was monitored by time-lapse video microscopy under bright white light, with an inverted phase contrast microscope (Leica MM AF) equipped with a cell culture chamber (37°C, humidified atmosphere containing 5% CO<sub>2</sub>), an x–y–z stage controller and a charge coupled device (CCD) CoolSnap camera (Photometrics). Images were acquired each 8 min for 16 hours, with the Metamorph software (Molecular Devices). Movies were reconstructed with the ImageJ software (<http://rsbweb.nih.gov/ij/>). Cells were tracked manually, and parameters were calculated with ImageJ Manual Tracking plug-in.

- **Cell Survival in flow cytometry.**

Standard protocols were use: 48h after transfection, Cells were trypsinised and resuspended in PBS containing 0,25µg/ml of 7-aminoactinomycin (7-AAD, bdbiosciences, #559925). Cells suspensions were analysed using a BD FACSCanto<sup>th</sup> II Flow cytometer. Percent of stained (dead) cell was calculated using FlowJo software.

- **Cell cycle in flow cytometry**

48h after transfection, Cells were trypsinized, rinsed in PBS, fixed in 70% cold Ethanol and stored overnight at -20°C. After several washes, cells were resuspended in 3.5 mM Tris HCl, pH 7.6, 10 mM NaCl, 1 µg/ml 7-AAD, 0.1% NP-40, 40 U/µl RNase A, and incubated in the dark for 30 min. The cell suspension was sorted on FACSCANTO II cell sorter. The relative importance of each phase of the cell cycle was automatically calculated using standard modeling algorithms of the FlowJo software.

- **RNA extraction and Real-time PCR**

Total RNA was extracted with a RNeasy Mini kit (Qiagen) 48 h after the plasmid transfection. Reverse transcription was performed using M-MLV reverse transcriptase and SYBR Green mix (BIO-RAD) for quantitative RTPCR. mRNA expression was measured in triplicate and normalized to 18S expression levels. For primers sequences see in **TableSM2**.

- **library construction and sequencing**

Total RNA was quantified using a Nanodrop 2000 spectrophotometer. Purity and integrity of the RNA was checked in a Bioanalyzer-2100 device (Agilent Technologies, Inc., Santa Clara, CA) and submitted to the Curie NGS platform for sequencing (ngs.lab@curie.fr). Libraries were prepared using the stranded mRNA prep ligation-Illumina, according to the protocols recommended by the manufacturer. For every standard gene expression 1Unit of Novaseq –S1 –PE100 per 6 samples. Bioinformatic analyses were performed with R software and DEVEA, a Shiny application<sup>4</sup> in R<sup>3</sup> (V.4.1.1) (Riquelme-Perez M et al., 2022). Quality tests were performed by the Curie NGS platform.

- **Inmunofluorescence**

Mewo cells were plated 24h before on glass-coverslips. cells were rinsed with PBS and fixed with paraformaldehyde 4% for 15 minutes. After PBS wash, cells were permeabilized and non-specific protein binding blocked in 10% FBS and 0,1%triton in PBS for 1h at room temperature. Cells were incubated overnight at 4°C with primary antibodies respectively diluted in blocking buffer (put the composition of your buffer here), rinsed with PBS, incubated 1h at room temperature in dark with secondary antibody at a 1:1000 dilution in blocking buffer (Alexa Fluor 647/555/488-conjugated goat anti rabbit/mouse/rat). The actin cytoskeleton was stained with Alexa Fluor 647 (Invitrogen). Cell nuclei were stained by Fluoroshield with DAPI mounting medium (F6057, Sigma Aldrich). Cells were imaged with 40X or 63X oil immersion objective of a widefield microscope (DM RXA, Leica; camera CoolSNAP HQ, Photometrics), using Metamorph software.

- **Animal rearing**

Zebrafish (*Danio rerio*) strains were raised and maintained in the Fish Facility of AZELEAD under standard conditions. A partially recirculating system with a 12/12 h light/dark cycle was used for adult zebrafish, that were fed 3 times/day with fresh *Artemia salina* and dry food. Wildtype strains were crossed to obtain offspring for experimental procedures.

*In vivo* experiments on zebrafish were performed in AZELEAD's Fish Facility in accordance with the European guidelines and regulations on Animal Protection from the French Ministry of Health (F341725).

- **Embryo preparation prior to xenotransplantation**

Embryos were initially maintained at 28 °C in fish water supplemented with 0.0002% methylene blue (Sigma) as an antifungal agent. After 24h, to allow fluorescent imaging acquisition, embryos were placed in fish water containing 200 µM Phenylthiourea (PTU), preventing embryo pigmentation.

- **Xenotransplantation of human cancer cell lines**

For injections, Borosilicate glass capillaries were pulled using glass micropipette puller (Sutter Instrument). A volume of 10 µL of cells suspension was used. Injection was performed under a stereomicroscope (Leica M80 Stereo zoom microscope) using a micromanipulator (Narishige) and the manual microinjector (CellTram® 4r Oil Eppendorf) in 3 dpf larvae anesthetized in 0.16 mg/ml PBS/Tricaine (MS-222) and tidily aligned laterally. 24h before microinjection, cells were transfected with either a control plasmid or a pCMV-OVOL2 expression plasmid encoding for human OVOL2 protein. Protein levels were measured 72h after transfection in order to ensure the efficiency of transfection. Melanoma cells were transplanted in the swim bladder region. Larvae with fluorescent tumor mass in the swim bladder were selected for image acquisition using a fluorescence stereomicroscope (AXIO Zoom.V16 Zeiss) equipped with eGFP filter. Transplanted embryos were maintained at 33°C in fish water containing PTU.

- **Live-imaging acquisition of zebrafish xenograph**

Transplanted larvae were individually imaged the day of transplantation (D0) and 3 days after transplantation (D3). To study tumour migration, images were acquired using the Zeiss automated microscope system Celldiscoverer 7 (CD7) (Objetives: 5x/1). Images were acquired in z-stack mode inside a total range of 250 µm with a z-step interval of 5 µm. 470 nm LED was used to acquire green fluorescence.

- **Image analysis**

Image analysis was performed using Fiji software. To study the migratory potential of melanoma cancer cells in vivo, detached cancer cells from the primary mass were manually counted on Fiji moving through the entire Z-stack.

Antibody name/specie	Dilution	Provider	Reference
Vinculin Mouse	1/5000	Sigma	V9131
Snail1 Mouse	1:1000	Ozyme	3895S
Snail2 Rabbit	1:500	Ozyme	9585S
Twist1 (2C1a) Mouse	1:200	Santa Cruz	Sc81417
Ovol2 Rabbit	1:500	Cell Signalling	NBP2-42907
Flag-tag Mouse	1:1000	Sigma Aldrich	F3165
N-cadherin (H2) Mouse	1:1000	Santa Cruz	Sc 393933
Zeb1 Mouse	1:200	Invitrogen	14-9741-82
Actin Rabbit	1:1000	Sigma Aldrich	A2066

**Supplementary materials**

**Table SM1. qPCR primers**

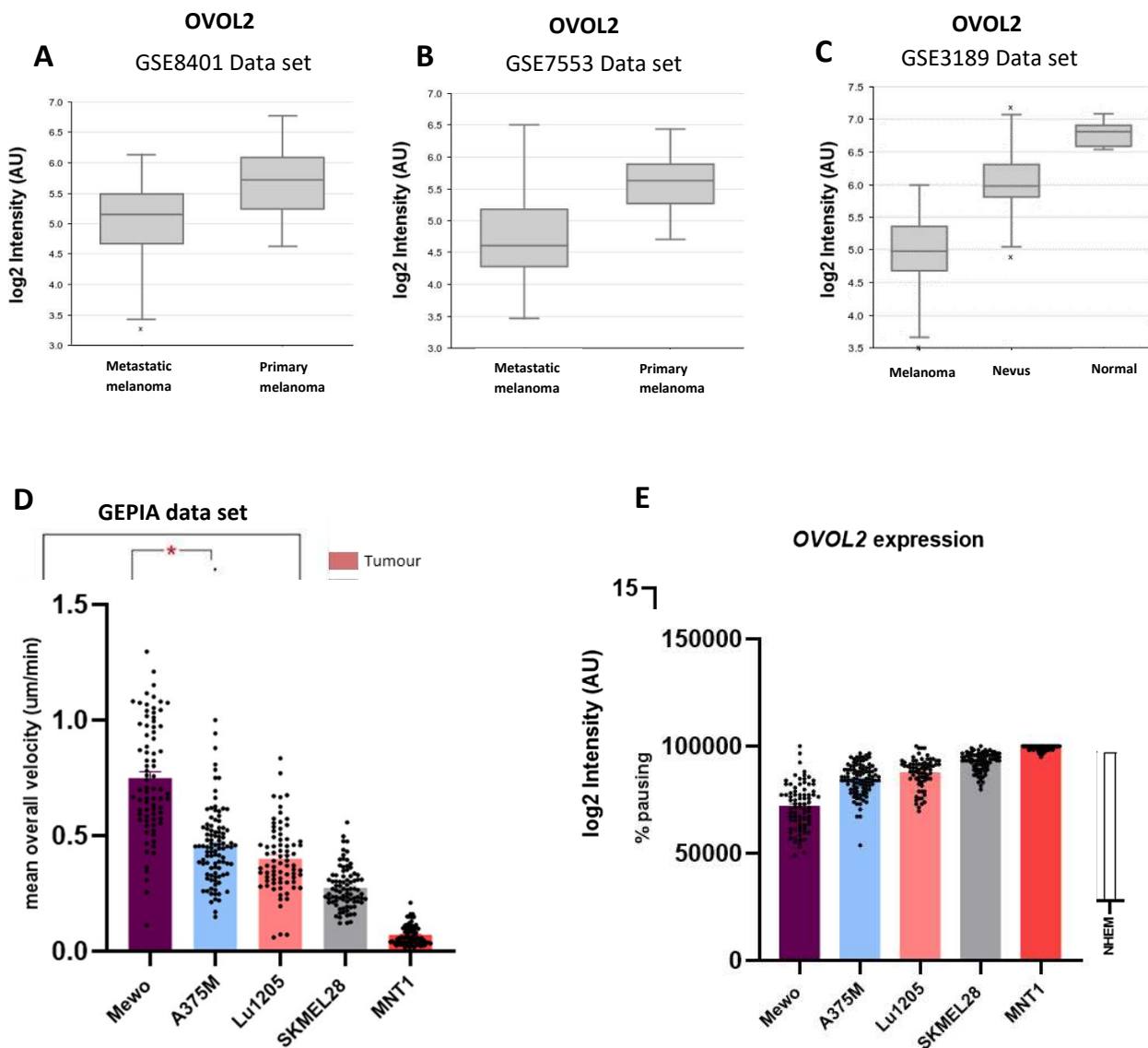
**Table SM2. qPCR primers**

Target gene	5'-3' Sequence
Snail1	Fwd: 5'- AAGATGCACATCCGAAGCC-3' Rvs: 5'- CGCAGGTTGGAGCGGTCAGC-3'
Snail2	Fwd: 5'- ATACCACAACCAGAGATCCTC-3' Rvs: 5'- GACTCACTCGCCCCAAAGATG-3'
Twist1	Fwd: 5'- GGG CCG GAG ACC TAG ATG -3' Rvs: 5' TTT CCA AGA AAA TCT TTG GCA TA -3'
Zeb1	Fwd: 5'- GATGATGAATGCGAGTCAGACA -3' Rvs: 5'- ACAGCAGTGTCTTGTGTTGT-3'
Zeb2	Fwd: 5'- AACAAACGAGATTCTACAAGCC-3'

	Rvs: 5'- TCGCGTTCCTCCAGTTTTCTT-3'
<b>Ovol2</b>	Fwd: 5'- ACA GGC ATT CGT CCC TAC -3' Rvs: 5'- CGC TGC TTA TAG GCA TAC-3'
<b>Vimentin</b>	Fwd: 5'- GAGAAGTTTGCCGTTGAAGC -3' Rvs: 5'- GCTTCCTGTAGGTGGCAATC -3'
<b>18S</b>	Fwd: 5'- GAGGATGAGGTGGAACGTGT -3' Rvs: 5'- TCTCAGTCGCTCCAGGTCT -3'

## Figures

Medina-Cuadra et al., Figure 1



**F**

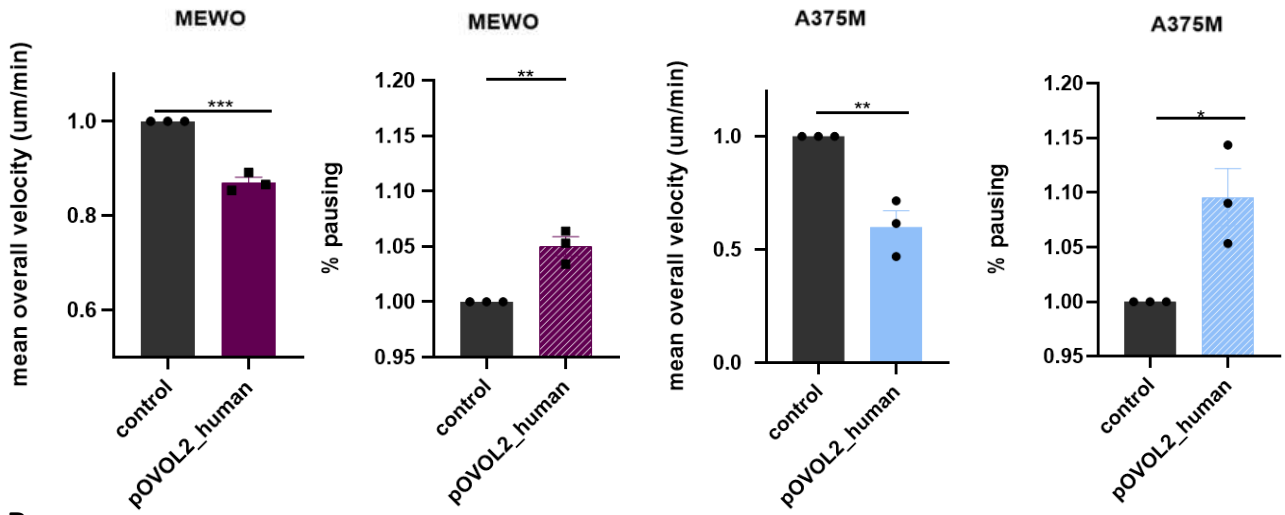
**Figure.1 OVOL2 levels in metastatic melanoma.**

A) Boxplot representing the distribution of *OVOL2* expression levels between melanoma metastatic (n =52) and primary tumour (n = 31). GSE8401 dataset. Anova test p-value = 8.22e-05. B) Boxplot representing the distribution of *OVOL2* expression levels between melanoma metastatic (n =40) and primary tumour (n = 14). GSE7553 dataset. Anova test p-value = 2.13e-05. C) Boxplot representing the distribution of *OVOL2* expression levels between melanoma (n =45), nevus (n = 18) and normal (n=7). GSE3189 dataset. Anova test p-value between melanoma and nevus = 7.74 e-08. P-value between melanoma and normal = 2.36e-15. P-value between nevus and normal = 1.48e-05. D) RNA sequencing expression data of 461 tumours and 558 normal samples from the TCGA and the GTEx projects, using a standard processing pipeline. E) *OVOL2* mRNA levels in different melanoma cell lines (Rambow et al., 2015). F-G) Mean overall velocity (um/min) and cell pausing (%) of wild type melanoma cell lines measured using time-lapse video microscopy (every 8 min during 16h). Pausing scale % \* 1000.

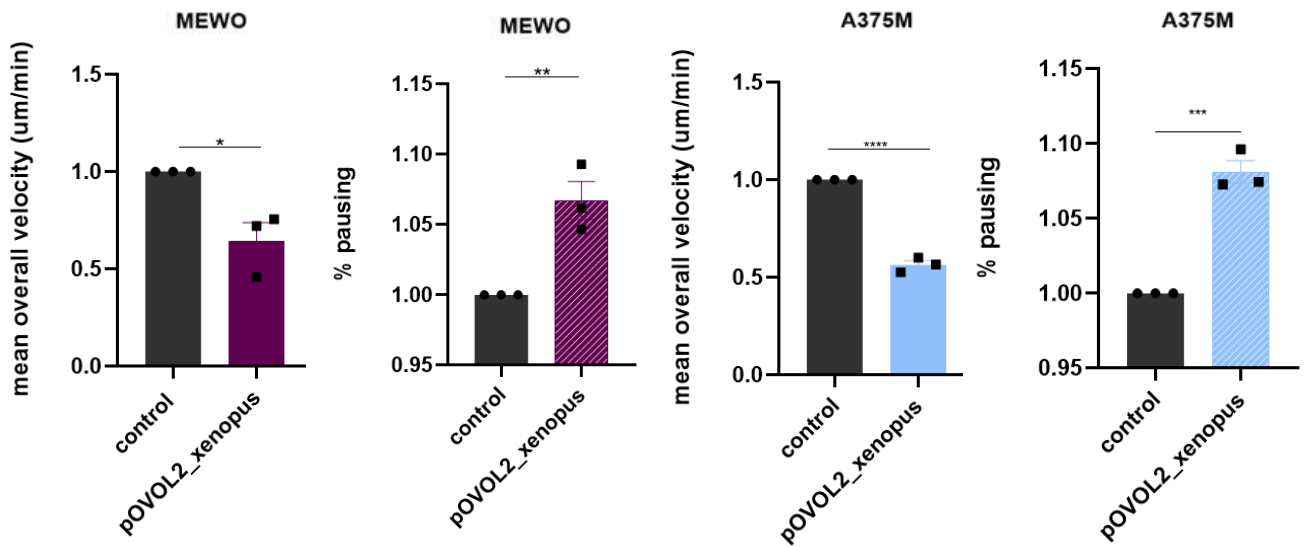


Medina-Cuadra et al., Figure 2

**A**



**B**

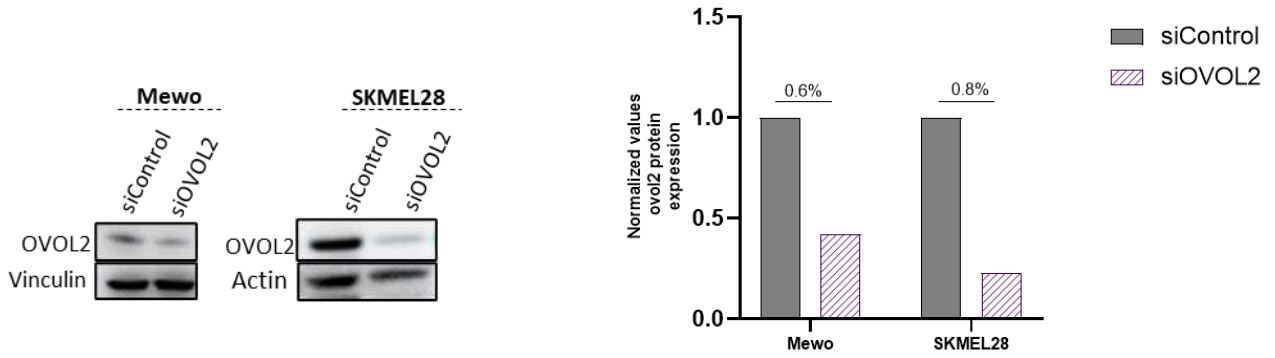


**Figure.2. Ovol2 expression changes in metastatic melanoma cell lines.**

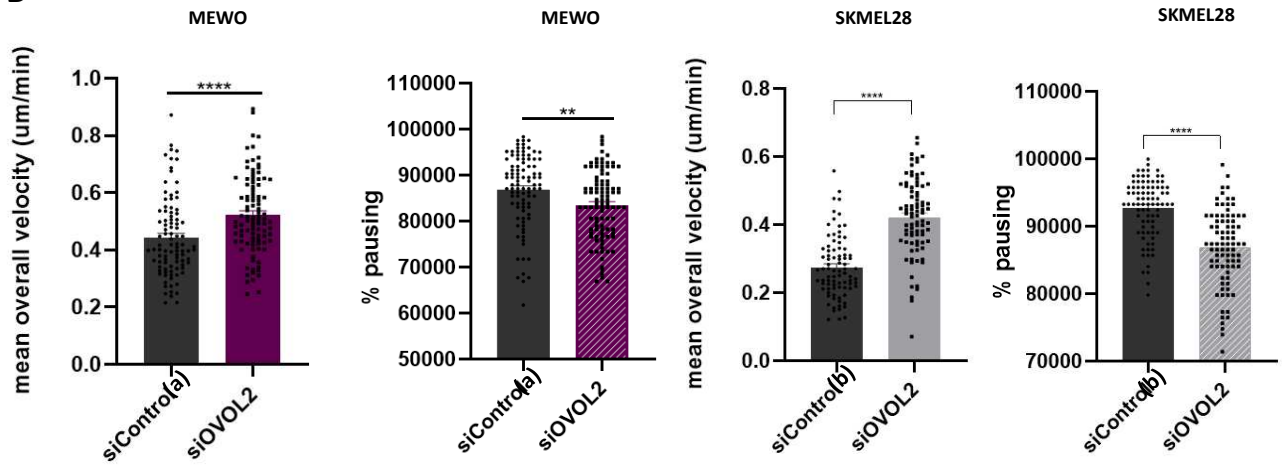
A) Cells overexpressing OVOL2 human protein present a decrease in cell speed and an increment in cell pausing when compared to control cells. Cells were tracked 48h after transfection by live microscopy in independent experimental triplicates. A total number of 100 cell were tracked in each triplicate (every 8 min during 16h). Units:  $\mu\text{m}/\text{min}$ . B) Melanoma cell lines overexpressing OVOL2 xenopus protein present a decrease in cell speed and an increase on cell pausing. Similar results were obtained in cells overexpressing OVOL2 human protein. A total number of 100 cell were tracked in each triplicate (every 8 min during 16h). Units:  $\mu\text{m}/\text{min}$ . Unpaired T-Test was used as statistical method. Significant differences are marked with \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\*for  $p < 0.001$ .

Medina-Cuadra et al., Figure 3

**A**



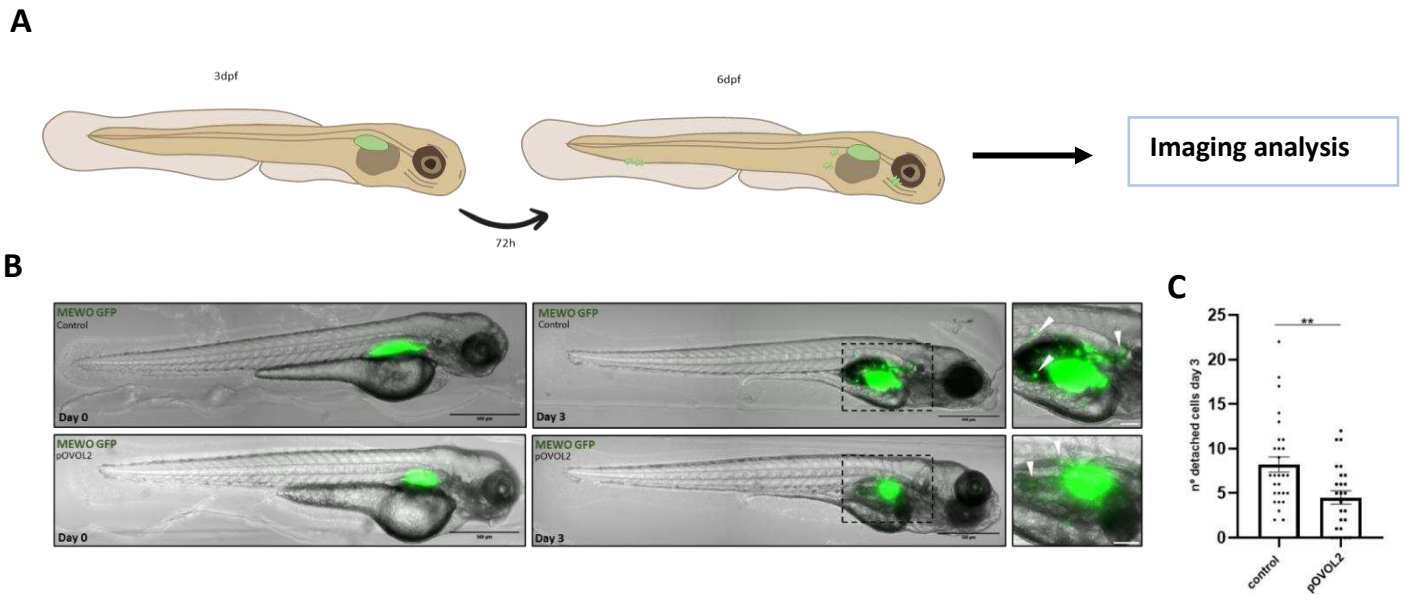
**B**



**Figure. 3 OVOL2 depletion in melanoma cell lines.**

A) OVOL2 protein levels in Mewo and SKMEL28 cells 48h after transfection with siRNA targeting OVOL2. Mewo was depleted using siOVOL(a). SKMEL28 was depleted using siOVOL2(b). B) Random cell migration was tracked for 16 hours using time-lapse video microscopy 48h after transfection. Each point corresponds to the average speed of an individual cell. Mewo and SK28MEL were transfected with siControl and siOVOL2. In each panel, a representative experiment is shown (n = 100) and displays mean  $\pm$  SEM. Experiments were performed in individual triplicates. Unpaired T-Test was used as statistical method. Significant differences are marked with \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\*for  $p < 0.001$ .

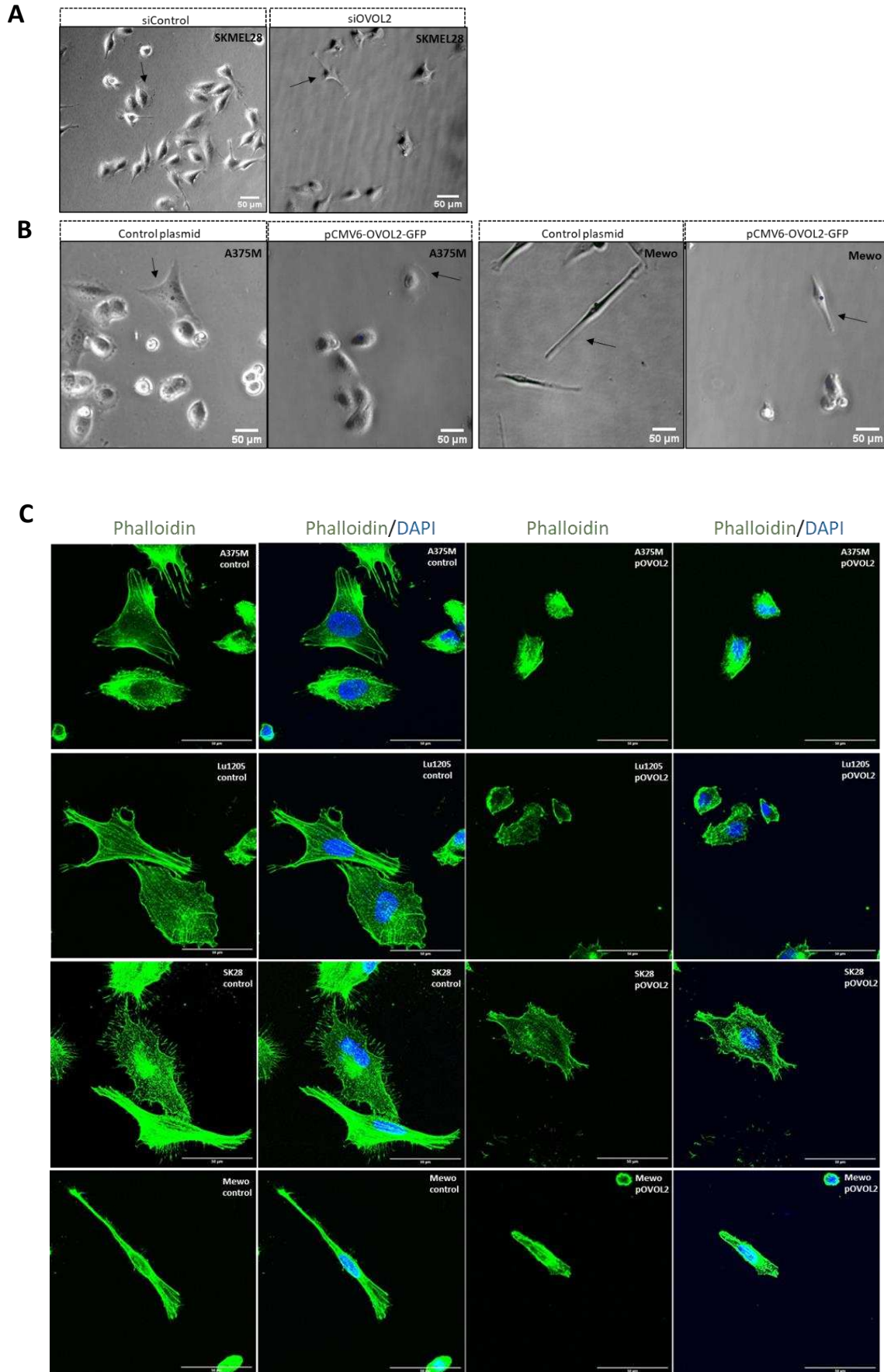
Medina-Cuadra et al., Figure 4



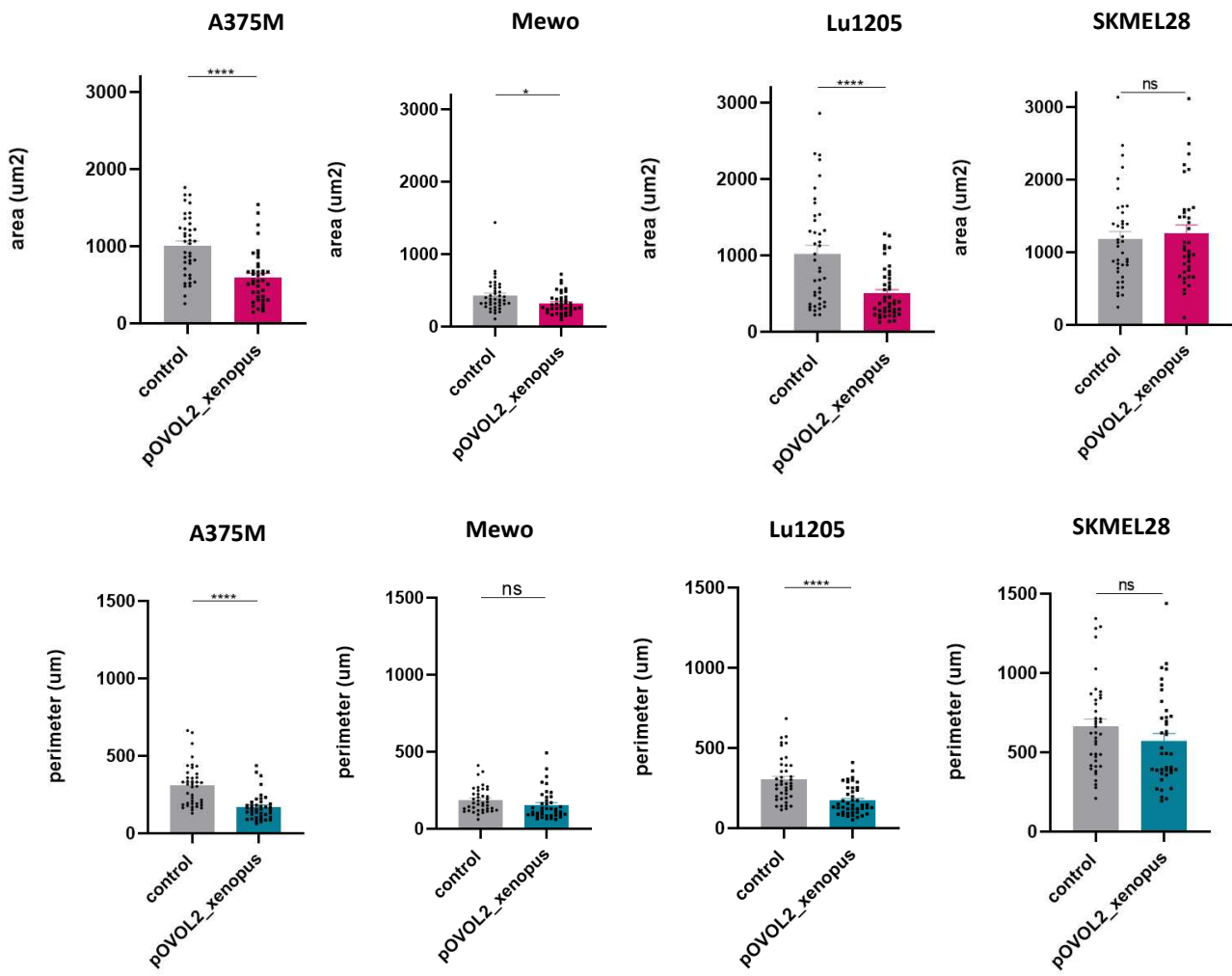
**Figure. 4 Mewo cells overexpressing OVOL2 have lower capacity of detach from primary tumour mass.**

A) Representative scheme of experimental procedure. Cells were injected in 3dfp larvae and tumour was imaged. 3 days later developed tumours were imaged to see metastatic cells. B) Arrows indicate the position of melanoma cells. Confocal Z stacks were used to visualize the cells within the body. Number of detached cells from primary tumour mass 3 days after injection (6dpf zebrafish embryos). Unpaired T-Test was used as statistical method. Significant differences are marked with \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\*for  $p < 0.001$ .

Medina-Cuadra et al., Figure 5



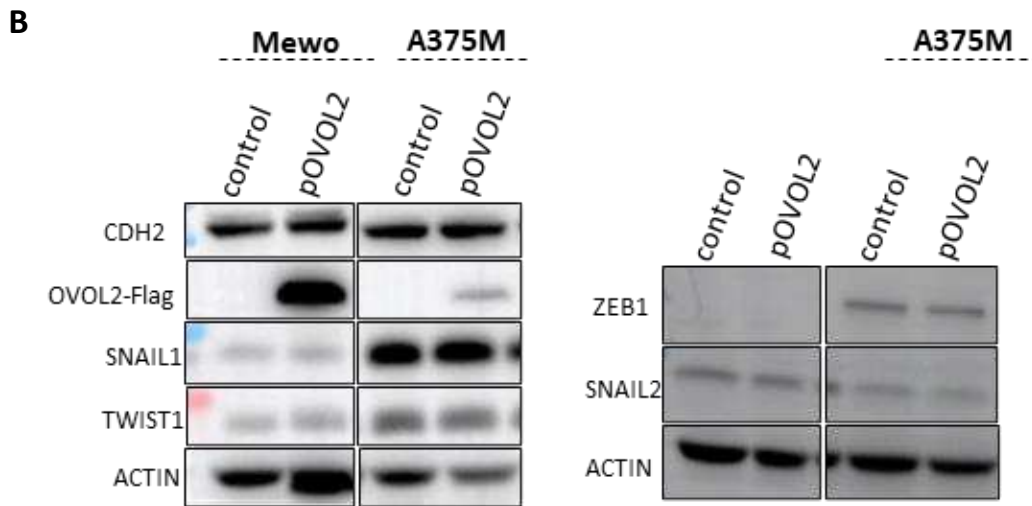
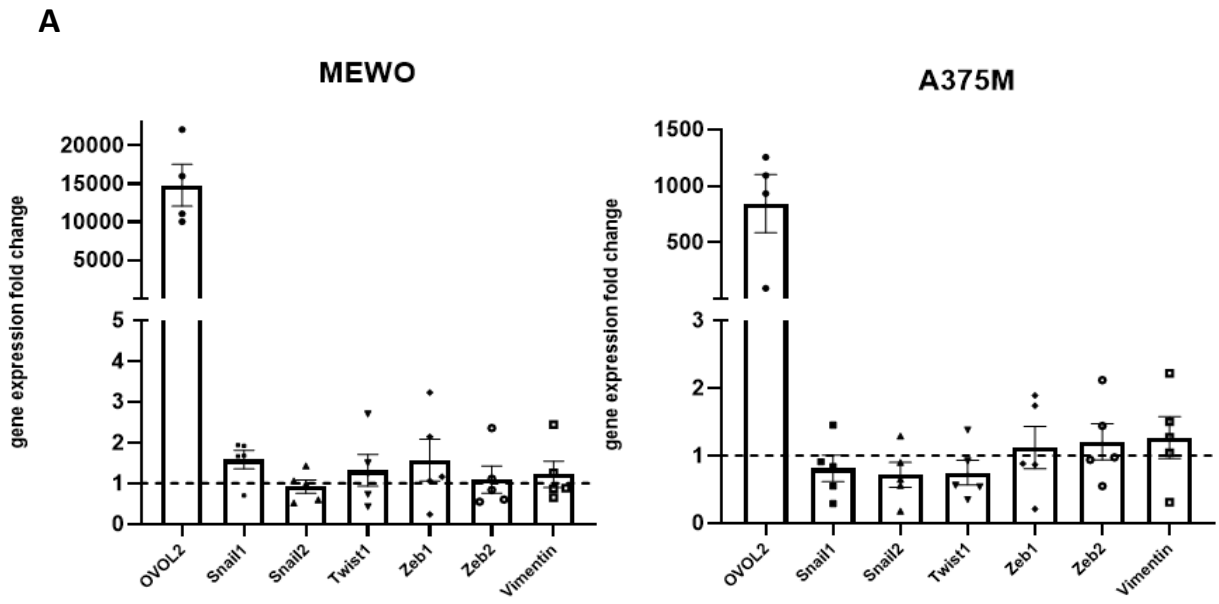
D





### Figure 5.

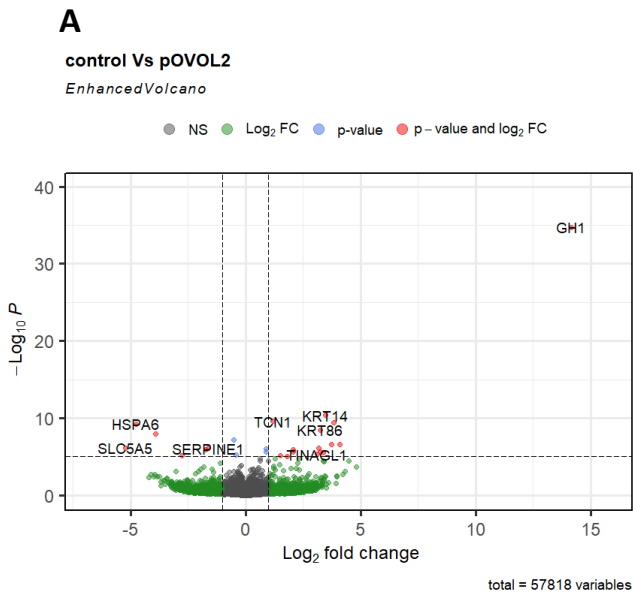
A) SKMEL28 cells 48h after transfection with siControl and siRNA targeting OVOL2. Time-lapse images observed in live microscopy in phase contrast images. B) A375M and Mewo cells 48h after transfection with control plasmid and pCMV-OVOL2 plasmid expressing for the xenopus protein. Cells were sorted 24h after transfection and re-seeded in plates coated with fibronectin. Time-lapse images observed in live microscopy in phase contrast images C) Melanoma cells (Mewo, A375M, Lu1205, SKMEL28) were transfected with control and pOVOL2\_GFP plasmid encoding for xenopus laevis protein. 24h later, positive GFP cells were sorted and seed in coverslips. Cells were cultured for another 24h and fixed with 4% PFA. Cytoskeleton was labelled with Phalloidin 647 (green). Nuclei are marked in blue by DAPI. D) Quantification of cell area and cell perimeter of melanoma cells (Mewo, A375M, Lu1205, SKMEL28) after ovol2 gain of expression. Perimeter and area of the cells were measured using ImageJ software. Cells expressing increased levels of OVOL2 showed a significantly reduce cell area and cell perimeter as compared to control cells. Nonetheless SKMEL28 does not show a decrease of cell area but present a significant decrease of cell perimeter. An average of 20 cells was measure in each individual duplicate. Unpaired T-Test was used as statistical method. Significant differences are marked with \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\*for  $p < 0.001$ .



**Figure.6 OVOL2 is affecting migration of melanoma cells by independent mechanism to EMT.**

A) Relative mRNA expression levels of the main EMT drivers measured by RT-qPCR after OVOL2 gain of expression. Normalized by the expression of 18S housekeeping gene. B) EMT drivers protein levels measured by western blotting after pCMV-OVOL2 transfection of the plasmid encoding for human protein. Actin relative quantification (optical density).

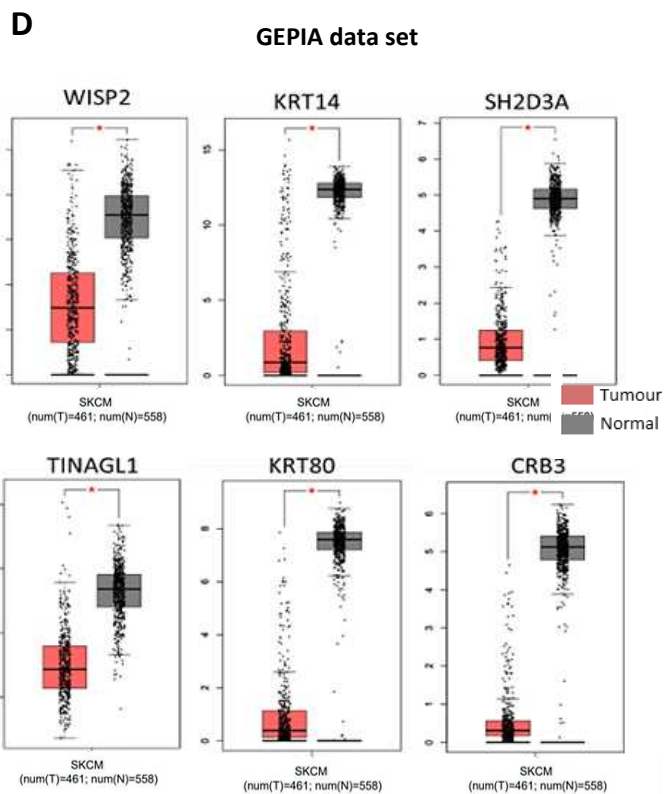
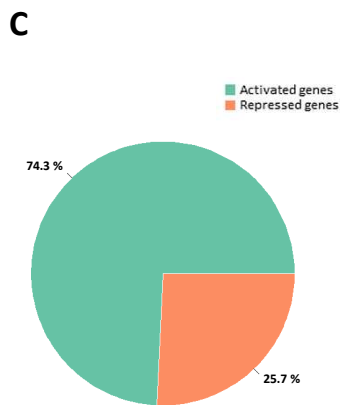
Medina-Cuadra et al., Figure 7



**B**

Gene	log2FoldChange	padj
GH1	14,17009578	4,06E-60
CRB3	4,496877283	1,63E-31
DMBT1	4,108027524	0,02602199
KRT81	3,819301675	0,00057495
FRG2B	3,745040872	1,58E-06
KRT14	3,462251324	0,00057495
PRSS33	3,415082135	2,72E-07
TH	3,38403262	0,02923615
ZNF488	3,375140253	0,02731667
CD74	3,260212729	0,00313721
KRT86	3,257276643	0,00277859
SH2D3A	3,181772194	1,05E-05
TINAGL1	3,127768141	0,0014089
IGF2	2,333372281	0,00545497
RP11-875O1	2,258948388	0,01537641
WISP2	2,07780316	0,04948939
C3	2,056276475	0,00277859
KRT80	1,819040199	0,00188928
SEMA6B	1,508544237	0,0088373

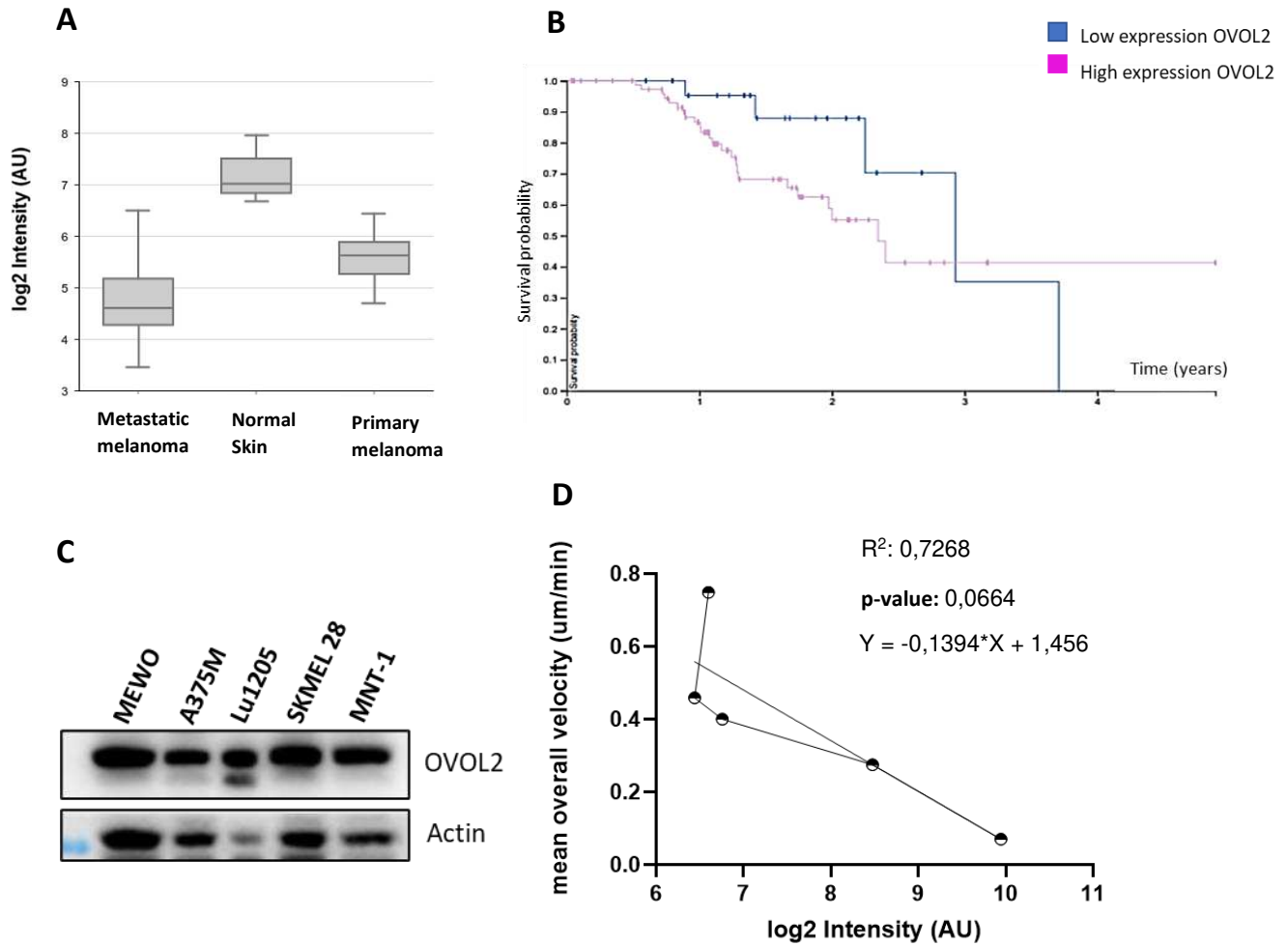
Gene	log2FoldChange	padj
SLC5A5	-5,218026024	0,0014089
HSPA6	-4,749188246	2,12E-06
XIRP1	-3,911523426	3,12E-05
ITGB7	-2,756991978	0,0070996
ARC	-1,725460528	0,00167686
SERPINE1	-1,617223488	0,0014089
CLU	-1,086842042	0,04948939
CDKN1A	-0,498170704	0,00017893
DNAJB5	-0,41827433	0,00583561



**Figure 7.**

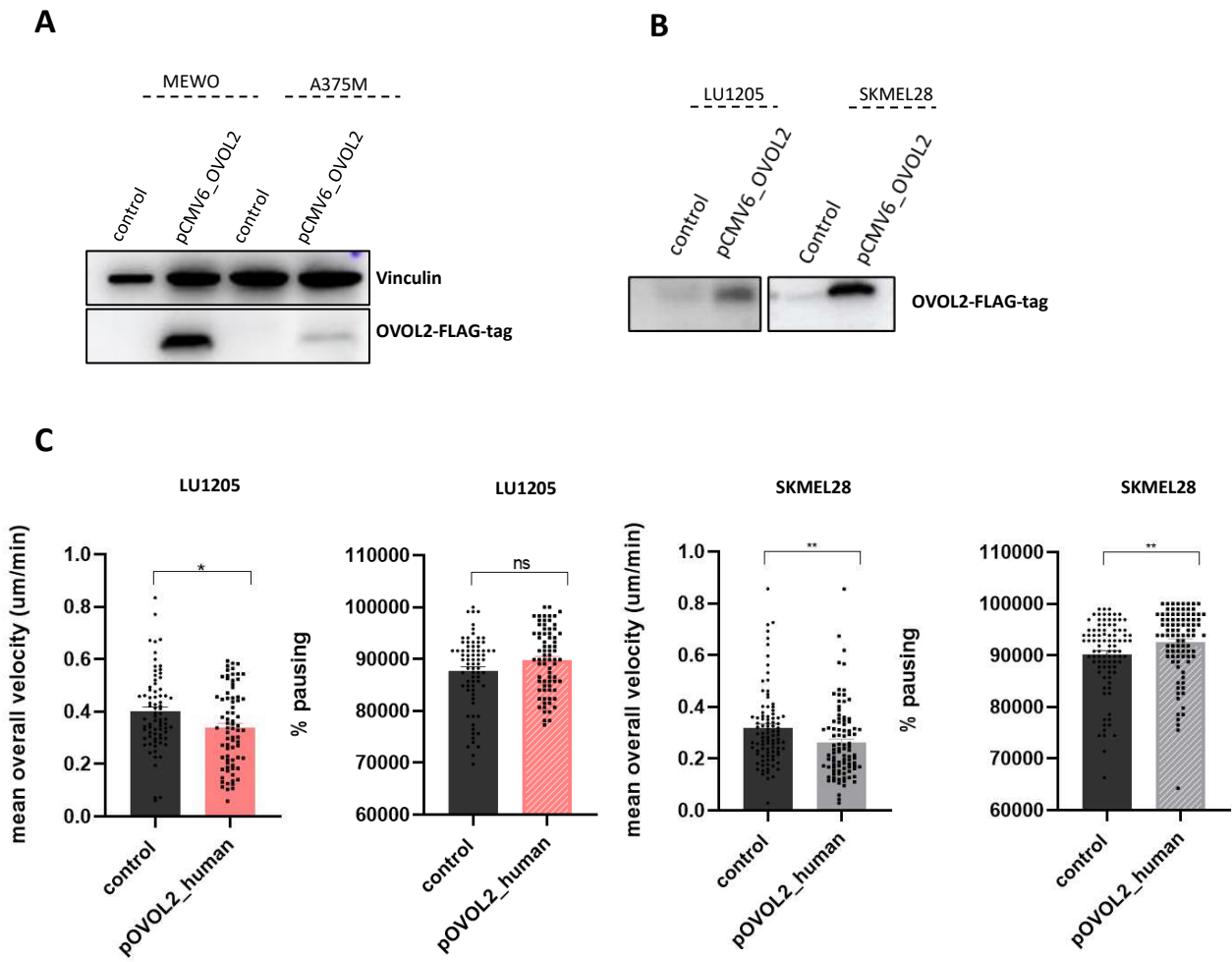
A) Volcano plot of significantly differentiated genes between cell overexpressing ovol2 and control cells. OVOL2 was removed out of the list for better representation. B) List of top upregulated genes by OVOL2 gain of function. Genes are ordered by Foldchange increase. C) Pie chart of the differentially expressed genes. D) Expression analysis of Upregulated genes by ovol2 across tumour and normal samples using GEPIA pipeline.

Medina-Cuadra et al., Supplementary Figure 1



**Supplementary Figure 1.** A) Boxplot representing the distribution of *OVOL2* expression levels between metastatic melanoma (n=40), normal skin (n=4) and primary melanoma (n = 14). GSE7553 dataset. Anova test between primary and normal skin; p-value = 1.43e-04. B) Kaplan-Meier plot summarize results from analysis of correlation between mRNA expression level and patient survival. Information extracted from protein atlas (<https://www.proteinatlas.org/>). Patients were divided based on level of expression into two groups; Low expression of *OVOL2* (under cut off = 0) or High expression of *OVOL2* (over cut off = 0). X-axis shows time in years and y-axis shows the probability of survival (1.0 corresponds to 100% of survival). P-value = 0.14 C) *OVOL2* Protein levels of melanoma cell lines. D) Regression analysis between *OVOL2* expression and mean overall velocity (um/min) of the 5 cell lines (Mewo, A375M, Lu1205, SKMEL28, MNT1).

Medina-Cuadra et al., Supplementary Figure 2





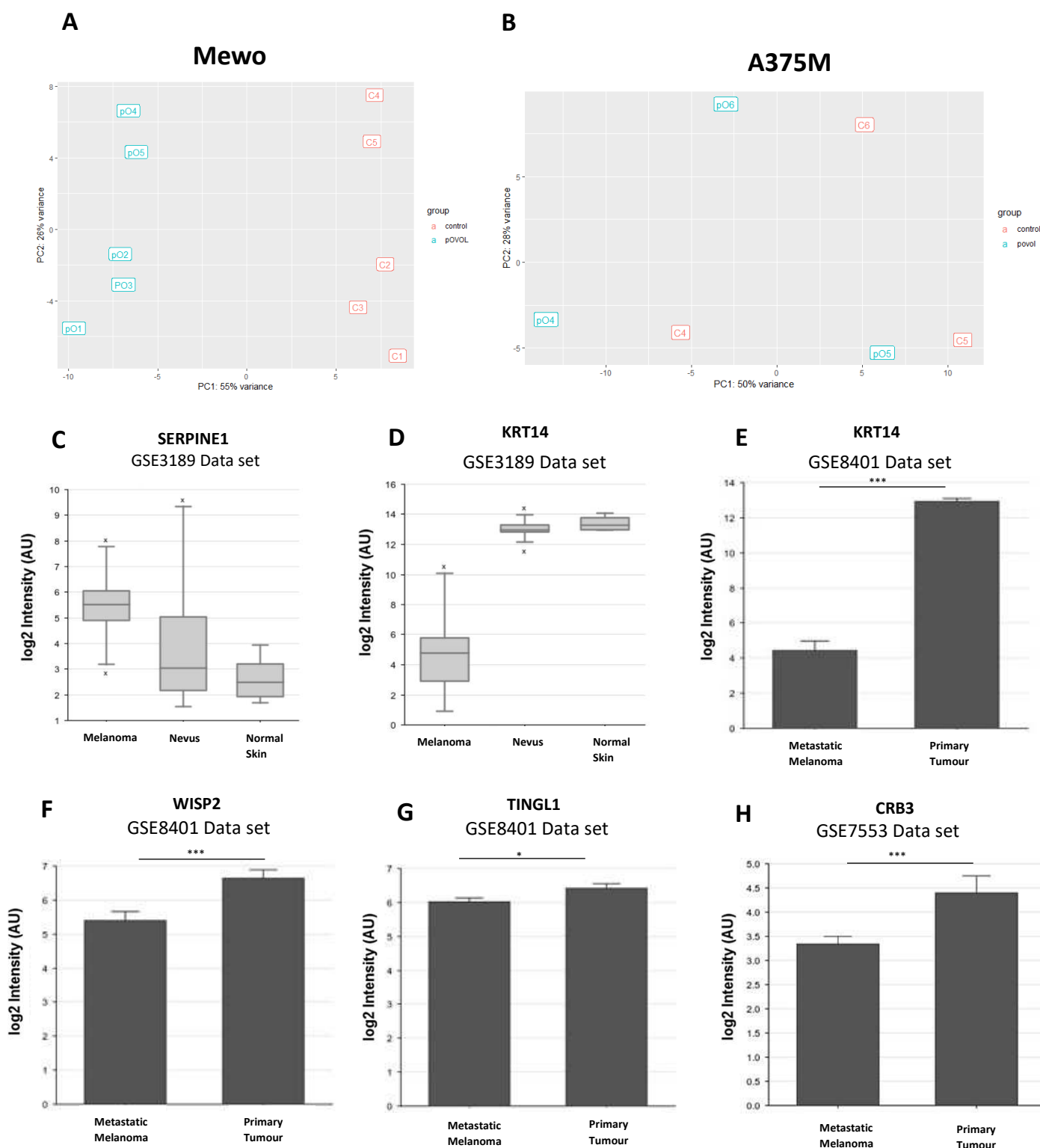
**Supplementary Figure 2.** A) OVOL2-Flag protein levels in Mewo and A375M cell lines 48h after transfection with pCMV-OVOL2-tag plasmid encoding for human OVOL2 protein. **B)** OVOL2-Flag protein levels in Lu1205 and SKMEL28 cell lines 48h after transfection with pCMV-OVOL2-tag plasmid encoding for human OVOL2 protein. **C)** Cells overexpressing OVOL2 human protein present a decrease in cell speed and an increment in cell pausing when compared to control cells. Cells were tracked 48h after transfection by live microscopy in a single experimental replicate. A total number of 100 cell were tracked every 8 min during 16h. Units:  $\mu\text{m}/\text{min}$ . Unpaired T-test was used for statistical analysis. Significant differences are marked with \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\*for  $p < 0.001$ .



### **Supplementary Figure 3. Ovol2 levels modulation does not affect cell death/cell cycle**

A) Multiple sequence alignment of the protein sequence using Clustal Omega program. References: ENSGALG00010018668, ENST00000278780.7, ENSMUST00000037423.4, CR855435.2. B ) Multiple sequence alignment of the zinc finger motifs across the different species. C) Mewo and A375M cells were stained with 7-AAD and counted after transfection with control plasmid and pOVOL2 plasmid encoding for either human or Xenopus Leavis protein. The relative number of cells in each of the phase of the cell cycle was counted. In both cell lines, curves overlapped perfectly and the relative ratio of G1 (light red), S (blue), and G2 (green) phase stay unchanged after the increment of OVOL2 expression. Likewise, in both cell lines, cell death was similar in all the three conditions. Thus, OVOL2 expression did not increase cell death rate.

Medina-Cuadra et al., Supplementary Figure 4



#### Supplementary 4.

A-B) Principal Component Analysis (PCA) plot showing the variations across the samples corresponding to control and OVOL2 overexpressing condition (pOVOL2) in Mewo and A375M. C-D) Boxplot representing the distribution of *SERPINE* and *KRT14* expression levels between melanoma (n =45), nevus (n = 18) and normal (n=7). GSE3189 dataset. E-H) Barplot showing the expression of transcriptional target f OVOL2 across primary and metastatic melanoma samples. Significant differences are marked with \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\*for  $p < 0.001$ .

**Table supplementary 1**

Term	p-value	genes	url
C5a anaphylatoxin chemotactic receptor binding	0,00120937	C3	GO:0031714
CSL2 anaphylatoxin chemotactic receptor binding	0,00120937	C3	GO:0031715
MHC class II protein binding, via antigen binding groove	0,00120937	CD74	GO:0042658
zymogen binding	0,00120937	DMBT1	GO:0035375
tyrosine 3-monooxygenase activity	0,00120937	TH	GO:0004511
extracellular matrix binding	0,00200483	DMBT1, TINAGL1	GO:0050840
macrophage migration inhibitory factor binding	0,00241733	CD74	GO:0035718
prolactin receptor binding	0,00241733	GH1	GO:0005148
protein binding	0,00391565	AMIGO2, C3, CCN5, CD74, CDC42BPG, C	GO:0005515
tetrahydrobiopterin binding	0,00482904	TH	GO:0034617
MHC class II protein binding	0,00723516	CD74	GO:0042289
cytochrome-b5 reductase activity, acting on NAD(P)H	0,00723516	CYB5R2	GO:0004128
keratin filament binding	0,00723516	KRT14	GO:1990254
dopamine binding	0,00843613	TH	GO:0035240
oxidoreductase activity	0,00843613	TH	GO:0016714
hormone activity	0,00948376	GH1, IGF2	GO:0005179
CD4 receptor binding	0,01083387	CD74	GO:0042609
oxidoreductase activity, acting on NAD(P)H, heme protein as acceptor	0,01083387	CYB5R2	GO:0016653
growth hormone receptor binding	0,01083387	GH1	GO:0005131
signaling receptor binding	0,01183911	C3, CCN5, CD74, GH1, IGF2, SEMA6B	GO:0005102
ferric iron binding	0,01203065	TH	GO:0008199
cobalamin binding	0,01203065	TCN1	GO:0031419
integrin binding	0,01267494	CCN5, IGF2	GO:0005178
protein-containing complex binding	0,01487588	CCN5, CD74, COL5A3, IGF2, KRT14	GO:0044877
catecholamine binding	0,01561267	TH	GO:1901338
growth factor activity	0,01627009	GH1, IGF2	GO:0008083
heparin binding	0,01665107	CCN5, COL5A3	GO:0008201
nitric-oxide synthase binding	0,01680391	CD74	GO:0050998
extracellular matrix structural constituent	0,01781686	COL5A3, TINAGL1	GO:0005201
intermediate filament binding	0,01799375	KRT14	GO:0019215
insulin-like growth factor receptor binding	0,01918222	IGF2	GO:0005159
receptor ligand activity	0,02023231	GH1, IGF2, SEMA6B	GO:0048018
MHC class II protein complex binding	0,02036931	CD74	GO:0023026
signaling receptor activator activity	0,02089309	GH1, IGF2, SEMA6B	GO:0030546
insulin receptor binding	0,02628408	IGF2	GO:0005158
receptor regulator activity	0,02635617	GH1, IGF2, SEMA6B	GO:0030545
pattern recognition receptor activity	0,02746291	DMBT1	GO:0038187
semaphorin receptor binding	0,02746291	SEMA6B	GO:0030215
glycosaminoglycan binding	0,03075964	CCN5, COL5A3	GO:0005539
ferrous iron binding	0,03099119	TH	GO:0008198
MHC protein complex binding	0,03099119	CD74	GO:0023023
chemorepellent activity	0,03216456	SEMA6B	GO:0045499
laminin binding	0,03450719	TINAGL1	GO:0043236
insulin-like growth factor binding	0,03450719	CCN5	GO:0005520
sulfur compound binding	0,03909575	CCN5, COL5A3	GO:1901681
FAD binding	0,03917613	CYB5R2	GO:0071949
proteoglycan binding	0,04266361	COL5A3	GO:0043394
protein folding chaperone	0,04266361	CD74	GO:0044183
oxygen binding	0,04613893	TH	GO:0019825
protein serine/threonine kinase activator activity	0,04613893	IGF2	GO:0043539
MHC protein binding	0,04729468	CD74	GO:0042287
extracellular matrix structural constituent conferring tensile strength	0,04844908	COL5A3	GO:0030020

## Table supplementary 2

Term	p-value	genes	url
misfolded protein binding	5,29E-05	CLU, HSPA6	GO:0051787
sodium:iodide symporter activity	0,000387	SLC5A5	GO:0008507
unfolded protein binding	0,00091457	CLU, HSPA6	GO:0051082
iodide transmembrane transporter activity	0,0011606	SLC5A5	GO:0015111
ubiquitin protein ligase binding	0,00529863	CLU, HSPA6	GO:0031625
anion:sodium symporter activity	0,00540605	SLC5A5	GO:0015373
ubiquitin-like protein ligase binding	0,00598722	CLU, HSPA6	GO:0044389
anion:cation symporter activity	0,00848372	SLC5A5	GO:0015296
low-density lipoprotein particle receptor binding	0,00886784	CLU	GO:0050750
protein-containing complex binding	0,00955629	CLU, ITGB7, XIRP1	GO:0044877
lipoprotein particle receptor binding	0,0107865	CLU	GO:0070325
lipopolysaccharide binding	0,01231909	H2BC11	GO:0001530
protein folding chaperone	0,0138496	HSPA6	GO:0044183
tau protein binding	0,01690439	CLU	GO:0048156
signaling receptor binding	0,02067511	CLU, ITGB7, SERPINE1	GO:0005102
solute:sodium symporter activity	0,02753107	SLC5A5	GO:0015370
virus receptor activity	0,02904093	ITGB7	GO:0001618
exogenous protein binding	0,02941807	ITGB7	GO:0140272
amyloid-beta binding	0,0320545	CLU	GO:0001540
enzyme binding	0,03266813	CLU, HSPA6, SERPINE1	GO:0019899
serine-type endopeptidase inhibitor activity	0,03730856	SERPINE1	GO:0004867
solute:cation symporter activity	0,03768289	SLC5A5	GO:0015294
chaperone binding	0,03843118	CLU	GO:0051087
protein binding	0,0408798	ARC, CLU, H2BC11, HSPA6, ITGB7, SERPINE1, SLC5A5, XIRP1	GO:0005515
heat shock protein binding	0,04662876	HSPA6	GO:0031072
protease binding	0,04959451	SERPINE1	GO:0002020

**Table supplementary 3**

Gene	log2FoldChange	padj
GH1	12,4732547	1,43E-21
ALPPL2	6,90862753	0,00108656
S100A9	6,14660582	0,03058944
KRT13	5,27011479	0,04443217
TRIM43B	4,61094357	0,00639022
RP11-432M8	4,23587486	0,00117232
TRIM49B	4,22374147	0,04687646
RP11-432M8	4,12645849	0,00236085
TRIM43	3,49109033	0,00757182
ZSCAN4	3,41356718	0,0018515
ATG9B	3,16664129	0,00236085
RP11-432M8	2,97887663	0,00728552
RP11-432M8	2,73921106	7,88E-09
TCN1	1,75766419	0,04101758
UCA1	1,53427116	0,04815993
RP11-189B4, (	1,06618323	0,00728552
CDC42BPG	1,06242	0,04990843
SPINT1	1,00143935	0,04815993
LINC00641	0,95435604	0,01826909

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## DISCUSSION AND CONCLUSION

As a result of this work, we have demonstrated that OVOL2 plays a role in the migratory capacity of melanoma cells in vitro and in their ability to detach from primary mass and invade the surrounding tissue in vivo. We have also shown that the high protein sequence evolutionary conservation underlie a functional conservation at least between amphibians and human, since *xenopus tropicalis* OVOL2 protein produces the same inhibitory effect on migration of human cells as the one observed with human OVOL2.

We investigated OVOL2 expression in human melanoma samples using in silico public data. Using data from independent studies we observed that nevi and primary melanoma have significantly higher levels of OVOL2 than metastatic melanoma.

With the use of imaging techniques, we observed the effects of OVOL2 gain of function on the morphology of metastatic melanoma cells in vitro. We observed that OVOL2 expression led to a reduction in the area and perimeter of the cells, suggesting that it has a specific effect in the cytoskeleton that leads to changes in cell shape and migratory characteristic.

We also studied the gene expression differences after OVOL2 ectopic expression in melanoma by RNA-seq. The data obtained show that OVOL2 expression led to an increase in the expression of proteins involved in cell adhesion, cell-matrix adhesion, and cytoskeleton, while other genes encoding ITGB7 and SERPINE1 were decreased. All this supported the previous observations that OVOL2 is an important regulator of cell morphology in melanoma.

Last, we have also shown that in melanoma, OVOL2 is not controlling migration through inhibition of EMT as was observed in epithelial cancers (Roca et al., 2013; R. S. Wu et al., 2017; Xia et al., 2021). We observed that none of the main EMT-TFs or markers were altered after OVOL2 ectopic expression. We also observed that OVOL2 is affecting the cytoskeleton of melanoma cells.

All those findings could potentially provide insights into the role of OVOL2 in regulating the cytoskeleton and cell adherence processes in melanoma cells. Further research would be needed to fully understand the mechanism by which OVOL2 affects the cytoskeleton and therefore the metastatic potential of those cells.

The following pages of this chapter are dedicated to the discussion and perspective of the results obtained during this thesis.

## **1. Role of OVOL2 controlling the metastatic potential of melanoma.**

OVOL2 plays a role in different crucial processes including embryonic development, cell differentiation and cell migration (Haensel et al., 2019; Mackay, 2006; Roca et al., 2013; Watanabe et al., 2019). Among those functions, OVOL2 is critical to control epithelial to mesenchymal transition during the metastatic outcome of many cancer types, including breast cancer, prostate cancer, hepatocellular carcinoma and colorectal cancer among others (J. Liu et al., 2018; Roca et al., 2013; Xia et al., 2021). In most of the cases OVOL2 inhibits the expression of the EMT transcription factor ZEB1 by directly binding the promoter regions, and therefore having a direct impact on the morphology and migratory capacity of cancer cells (Roca et al., 2013). In other context, OVOL2 controls EMT in mammary tumour metastasis by antagonizing the TGF- $\beta$  signalling pathway. However, there is no information about the role of OVOL2 controlling the migratory and metastatic potential of non-epithelial cancer cells. In this study we investigate the potential biological role of OVOL2 controlling the metastatic capacity and outcome of melanoma. Using a combination of in vitro and in vivo analysis we show that OVOL2 expression is negatively correlated with the invasiveness capacity of metastatic melanoma and plays a role in the regulation of the migratory potential of melanoma cells.

### **In silico analysis**

The expression of OVOL2 was studied in human melanoma samples using publicly available data in silico (Riker et al., 2015; Talantov et al., 2005; L. Xu et al., 2008). Here we could analyse OVOL2 expression across melanoma patients' samples provided by different independent studies. We could observe that across all datasets, OVOL2 shows a negative correlation with the metastatic progression of melanoma, since OVOL2 mRNA levels significantly fall in metastases. Hereby, OVOL2 expression can act as a good prognosis factor of the disease. However, Kaplan-meier analysis does not show a significant correlation between the levels of OVOL2 and the percentage of survival in 5 years. This indicates that OVOL2 expression has a role in the retention of migrating cells from the primary mass. However, it is not involved in the mechanism of survival of the tumour such as the response to treatments or immune system evasion.

OVOL2 expression was previously correlated with disease prognosis by other studies in other types of cancers. In osteosarcoma protein expression levels of OVOL2 were examined across patients' samples of different clinical stages using Immunohistochemistry (ICC).

In this study, protein expression levels of OVOL2 have a negative correlation with the clinical progression of the disease, meaning that OVOL2 show a significant increase of expression in samples from patients without metastasis or recurrence. In hepatocellular carcinoma (HCC), low OVOL2 expression was positively correlated with HCC poorly differentiated, microvascular invasion, and cirrhosis. All those are clinical indicators of poor prognosis factors of the disease. The use of Kaplan-Meier analysis also demonstrated that patients with HCC and low OVOL2 expression had significantly worse overall survival, suggesting that OVOL2 deletion is a strong predictor of poor prognosis in HCC. (Fu et al., 2016). Additionally, in those tumours OVOL2 expression is significantly lower in tumour tissues compared to the adjacent non-cancerous tissues. Similar to the observed differences in OVOL2 expression between Normal skin, nevus and melanoma. On the side, they observed a link between the levels of OVOL2 and EMT markers expression, with a positive correlation with E-cadherin levels and a negative one with N-Cadherin expression. Here, in addition to patient data, we also used a published dataset (Rambow et al., 2015) from immortal melanoma cell lines bulk RNA sequencing in order to compare the mRNA levels of OVOL2 across different melanoma cell lines and correlate those with the cell's migratory capacity in vitro. In coherence with previous results, the expression level of OVOL2 across those cell lines was inversely correlated to the migratory capacity of those cells in vitro. Cells highly metastatic such as MEWO and A375M present lower levels of expression than cells which do not migrate such as MNT1. All of this is suggesting that OVOL2 expression have a correlation with the metastatic potential of cancerous cells, and the clinical outcome and not only in epithelial derived cancers. Nevertheless, the information and correlations done in melanoma are based on RNA levels of the transcription factor. Further analysis focused on the protein expression levels of this gene across different stages of the disease and in comparison with healthy non-tumorigenic tissue would be an asset to solidify the correlations found in RNA values in melanoma. This study is in progress. Moreover, through collaboration, we recently had access to patient's melanoma samples and are currently assessing OVOL2 expression in in situ and vertical stage melanoma (Carmit Levy's laboratory, Tel Aviv, Israel).

### **In cellulo analysis**

The study of OVOL2 in cell lines of metastatic melanoma confirmed the association observed between OVOL2 expression levels and the migratory capacity of the melanoma cells without affecting the viability or cell cycle of the cells. Among the melanoma metastatic cell lines used (A375M, Mewo,

SKMEL28) the manipulation of the levels of OVOL2 have an impact on their migratory capacity that correlate with the observations done in public melanoma patients' dataset. Thus, the metastatic cell lines have a decrease in migration after OVOL2 ectopic expression and an increase of their migratory capacity after OVOL2 depletion. The use of OVOL2 *xenopus tropicalis* protein for similar experiments, not only confirmed the phenotype observed after ectopic OVOL2 expression but also confirm the functional conservation of the gene already suggested by previous works. To expand the study of OVOL2 expression impact on the metastatic potential of melanoma we used a zebrafish xenograft model of metastatic melanoma. This model allows time-effective and dynamic quantification of tumour progression. The transparency of the embryos makes this model ideal for the study of tumour metastatic progression using non-invasive and high-resolution microscopy techniques. After implantation of melanoma metastatic cells in the 3dpf zebrafish embryos, engrafted cells were able to successfully detach from primary mass in the nearby area within the embryo at the third day post-transplantation (6dpf). The manual quantitative analysis of the confocal scanning images of 6dpf anesthetized living embryos revealed difference between control melanoma and melanoma cells previously transfected with OVOL2, with OVOL2 overexpressing cells having a significant decrease on primary mass detached cells. Altogether those results support the observed in cell migration in vitro assays and in silico studies, accentuating the importance of OVOL2 expression in the metastatic potential of melanoma malignant cells.

It should be noted that OVOL proteins have been identified as transcription inhibitors that suppress gene expression by binding to DNA sequences within the promoter's region (Dai et al., 1998). In accordance, multiple studies have revealed that OVOL2 is able to inhibit migration and EMT in different cancer contexts by the transcriptional inhibition of ZEB1 mRNA expression (Briana Lee et al., 2014; Roca et al., 2013). The process of phenotype switching, which has similarities to EMT program, plays an important role in the acquisition of metastatic properties during the vertical growth phase of melanoma disease. However, in our present study OVOL2 is controlling the migratory and potential of melanoma cells by a mechanism independent of EMT, since none of the main EMT-TFs and EMT-markers were altered neither at RNA nor protein levels. Therefore, we analysed by RNA-sequencing the gene expression changes induced by OVOL2 gain of function, relative to control metastatic melanoma cells (Mewo). From this analysis, we have identified a set of genes that changed over 2-fold after OVOL2 expression. OVOL2 induced the expression changes in approximately 37 genes, from which 26 are upregulated and 11 downregulated. RNA-sequencing data showed that in melanoma

OVOL2 expression regulates transcriptional program affecting cytoskeleton formation, cell junction organization and epithelial cell differentiation. Within those upregulated genes we found *Crumbs Cell Polarity Complex Component 3* (CRB3), *Deleted In Malignant Brain Tumors 1* (DMBT1), *Keratin 14* (KRT14) and *WNT1 inducible signalling pathway factor* (WISP2) that plays various roles such as apico-basal polarity and tight junctions formation and cytoskeleton organization and have been previously associated to reduce migration in other cancer types. For example and interestingly, although *zeb1* was not decreased in our dataset, CRB3 was found upregulated after the silencing of ZEB1 in colorectal carcinoma cells and breast tumour cells (Aigner et al., 2010; Spaderna et al., 2008). Addition, reduction in CRB3 expression can promote carcinogenesis of kidney epithelia in mice (Karp et al., 2008; P. Li et al., 2017). Upregulation of CRB3 after OVOL2 expression in melanoma suggests a first indication that CRB3 may participate in cell polarity reorganisations incompatible with active cell migration. In other examples, DMBT1 was associated to inhibition of proliferation, migration, and invasion in ovarian cancer (Ma, 2020); KRT14 expression is of good prognosis in melanoma patients (Han et al., 2021); and WISP2 exhibits antitumour activity in oesophageal cancer cells (ESCC) and colorectal cancer (CRC) (D. Chai et al., 2019; Frewer et al., 2013). Moreover, the exploration of those genes in public dataset of melanoma showed a correlation with OVOL2 expression, upregulated gene expression also being significantly more expressed in primary melanoma samples. Altogether, these data reinforce our main hypothesis of OVOL2 negative role in the metastatic progression in melanoma.

In terms of limitations of the study and futures perspectives, the development of stable or inducible cell lines expressing OVOL2 at a lower level, would allow a better understanding of the morphological and phenotypical changes observed. Stables cell lines would be an asset to better confirm that EMT-TFs are not affected by OVOL2 expression, since our model is based in short term and high levels modifications that may not show some expression changes that would require more time to be observed. Futures perspectives will be oriented to reproduce in vitro and in vivo the model using additional metastatic melanoma cell lines and the validation of RNA-seq analysis target genes. We would validate functionally OVOL2 targets, both repressed genes (may be directly repressed) and upregulated ones. A simple approach would be to complement the current results with analysis after OVOL2 depletion; and see if the genes up or down-regulated exhibit the converse regulation. Moreover, re-expressing depleted genes or depleting upregulated ones in the context of OVOL2 depletion would allow assessing the importance of each target in the phenotype observed. In



addition, the modalities of *ovol2* regulation in melanoma remain to be explored: BMP pathway or *Grhl2* are ones of the few regulators of OVOL2 already identified (Aue et al., 2015; Wang et al., 2020; T. Zhang et al., 2013). Therefore, the study of the upstream regulator controlling OVOL2 expression in melanoma would be beneficial, not only for ensuring the phenotype observed during genetic manipulation but also will put the study closer to potential therapeutic strategies.

## **Conclusion**

Our observation in the main study of this thesis also demonstrates that metastatic capacity of tumour cell is affected when we knocked down OVOL2 expression in metastatic melanoma cell lines. We expected that OVOL2 exerts this function mainly through EMT inhibition, as described in other works. However, the current study shows that in the context of melanoma, OVOL2 inhibits migration by affecting the cytoskeletal and adhesive properties of the cells. These observations suggest that OVOL2 may adopt alternative mechanisms, depending on the origin of the cell context. Although OVOL2 has been proposed as a potential EMT regulator in many cancers by several important studies, our work provided important findings that enhanced the understanding of OVOL2 functions in melanoma in three regards: (1) We explored methodically the expression levels of OVOL2 in public databases that shares information of melanoma patients, observing a negative correlation of OVOL2 expression with the metastatic outcome of the disease. (2) Using in vitro and in vivo approaches we found that OVOL2 expression correlates with migratory capacity of melanoma cells and their ability to detach from primary mass. (3) Importantly, we observed that in melanoma OVOL2 regulates migration by changes in their cytoskeleton and adhesion properties and not by EMT as we expected. Taken together, our data demonstrate that OVOL2 re-expression counteracts melanoma aggressiveness by acting on cytoskeleton dynamics.



## SUMMARY IN FRENCH

### INTRODUCTION

Les métastases sont la cause de plus de 90% des décès liés au cancer (Welch & Hurst, n.d.). L'invasion tumorale et les métastases sont le résultat de processus complexes impliquant l'invasion locale, l'extravasation, le transport, l'extravasation et la colonisation de régions éloignées. Ces comportements cellulaires sont régulés par un équilibre délicat entre la transition épithéliale-mésenchymateuse (TEM) et le programme inverse, la transition mésenchymateuse-épithéliale (MET). La TEM est un processus biologique très important qui, dans des conditions physiologiques, se déroule au cours du développement embryonnaire, de la régénération des tissus et de la cicatrisation des plaies. Ce processus est essentiel pour la migration des cellules de la crête neurale, qui vont ainsi former de multiples tissus de type cellulaire différent. La TEM est un processus hautement dynamique par lequel des cellules épithéliales correctement polarisées et liées les unes aux autres perdent leurs adhésions cellule-cellule, leur polarité apicale-basale et leur phénotype épithélial pour acquérir un phénotype mésenchymateux qui les rend migratoires et invasives. L'activation de ce processus peut également être observée dans des conditions pathologiques telles que le développement de métastases au cours de la progression tumorale, l'augmentation de la résistance des cellules à la chimiothérapie et la fibrose des organes. De multiples voies de signalisation, notamment TGF- $\beta$ , Wnt/ $\beta$ -caténine, Notch, EGF, HGF, FGF et HIF, sont importantes pour le maintien épithélial et l'équilibre TEM-MET dans le développement normal et le cancer. En outre, en aval de ces signaux, la TEM est régulée par un réseau de régulation complexe impliquant les facteurs de transcription de la TEM (TEM-TFs) Snail1, Snail2, Twist1, Zeb1 et Zeb2. Bien que la connaissance des mécanismes contrôlant la TEM progresse rapidement, les voies qui bloquent la TEM et médient la MET sont loin d'être comprises.

Parmi les régulateurs négatifs potentiels de la TEM, les OVOL1/2 sont des répresseurs transcriptionnels contenant des doigts de zinc de la famille des protéines Ovo-like conservées dans l'évolution. Les protéines de type OVO sont cruciales pour la formation de l'épithélium au cours du développement (Saxena et al., 2022) ; elles sont identifiées comme des régulateurs essentiels de la différenciation des kératinocytes de la peau, de la cicatrisation des plaies et comme des répresseurs/inducteurs de la TEM dans certains types de cancer, par exemple le cancer du sein et de la prostate (Roca H. et al., 2013).

Parmi eux, le rôle d'OVOL2 comme inhibiteur de la TEM dans plusieurs contextes cellulaires est remarquable. Des études portant sur l'expression d'OVOL2 dans de multiples lignées cellulaires cancéreuses ont montré que l'expression d'OVOL2 est corrélée positivement avec l'expression de marqueurs épithéliaux tels que l'E-cadhérine (CDH1) et négativement avec les marqueurs mésenchymateux tels que la vimentine (VIM). En outre, les facteurs induisant la TEM tels que SNAI1/2, TWIST1 et ZEB1/2 présentent un rôle inverse de OVOL2 sur CDH1 et VIM. En d'autres termes, ils présentent une corrélation négative avec CDH1 et positive avec VIM (Watanabe et al., 2019).

D'autres études axées sur les fonctions d'OVOL2 ont démontré que ce facteur de transcription inhibe le processus de la TEM dans les cellules cancéreuses, y compris le cancer de la prostate et du sein, par l'inhibition transcriptionnelle directe et spécifique de ZEB1 (Roca et al., 2013).

D'autres études sur ce facteur de transcription ont permis d'identifier que la voie de signalisation TGF- $\beta$  est également inhibée au cours du processus TEM dans le contexte des cellules tumorales mammaires humaines et murines. Ceci suggère que plus d'une voie de signalisation peut être contrôlée par OVOL2. Ces études démontrent que OVOL2 inhibe la signalisation de TGF- $\beta$  à plusieurs niveaux, y compris l'inhibition de

l'expression de l'ARNm de Smad4 et l'induction de l'expression de l'ARNm de Smad7. En outre, dans les cellules épithéliales mammaires de souris, la liaison OVOL2-SMAD4-spécifique inhibe la liaison de SMAD4 à ses gènes cibles et interfère avec la formation de complexes entre SMAD4 et SMAD2/3. Cela conduit à l'expression de gènes inductibles de la TEM tels que snail1 ou twist1, ce qui entraîne une diminution de la migration des cellules épithéliales mammaires de souris (Wu RS. Et al., 2017). D'autre part, plusieurs études ont associé l'expression d'OVOL2 à un pronostic favorable dans la maladie tumorale. Dans le cas du carcinome hépatocellulaire et de l'ostéosarcome, des niveaux plus élevés d'expression de la protéine OVOL2 sont corrélés avec des stades cliniques plus précoces et un meilleur pronostic de la maladie (Fu et al., 2016 ; Liu et al., 2018).

Cependant, il n'y a actuellement aucune étude explorant le rôle d'OVOL2 dans les tumeurs métastatiques dérivées de la crête neurale, comme le mélanome. Comme décrit ci-dessus, les études précédentes montrant que OVOL2 inhibe de la TEM nous ont conduit à nous interroger sur l'importance de ce gène dans le potentiel métastatique et le résultat clinique du mélanome.

## **OBJECTIFS**

Comme décrit ci-dessus, le développement de la population de la crête neurale partage donc certains des mécanismes cellulaires et moléculaires avec ceux impliqués dans la progression tumorale. La transition épithélio-mésenchymateuse (TEM) est un processus dynamique important, crucial au cours du développement embryonnaire, notamment pour la migration des cellules de la crête neurale et donc pour la formation de la lignée mélanocytaire. La TEM n'est pas seulement observée dans des conditions physiologiques, mais il a également été démontré qu'elle joue un rôle en conférant des propriétés métastatiques à de nombreuses tumeurs, dont le mélanome. Des études

antérieures ont démontré le rôle d'OVOL2 dans l'inhibition de de la TEM dans les tumeurs dérivées de l'épithélium telles que le cancer du sein et le cancer de la prostate (Roca et al., 2013) par l'inhibition transcriptionnelle de de la TEM-TF Zeb1. OVOL2 s'est également révélé être un facteur inhibiteur critique de de la TEM dans les épithéliums cutanés et mammaires en développement. Cependant, le rôle d'OVOL2 dans la régulation du processus de type TEM observé dans les tumeurs non épithéliales telles que le mélanome n'a pas encore été exploré.

Ces études nous ont amenés à nous demander si OVOL2 a également un rôle dans la régulation de la migration des mélanomes et, dans l'affirmative, si ce rôle est mécaniquement lié au potentiel métastatique des cellules tumorales.

Dans ce travail, nous avons également décidé d'explorer de manière préliminaire quel est le rôle d'OVOL2 en relation avec la formation de la crête neurale et le processus TEM que ce type de cellule effectue pendant le développement embryonnaire en utilisant *Xenopus laevis* comme modèle d'étude.

L'ensemble de ces aspects soulève plusieurs questions qui seront abordées dans cette thèse :

Dans le contexte de la capacité migratoire des mélanomes :

- OVOL2 interfère-t-il avec la capacité migratoire et invasive des cellules de mélanome in vitro ?
- Comment OVOL2 affecte-t-il la régulation de la migration des cellules de mélanome ?
- Quel est le mécanisme qui sous-tend la régulation par OVOL2 de la migration des cellules de mélanome ?

- OVOL2 affecte-t-il l'invasivité des cellules de mélanome in vivo ?

Dans le contexte de de la TEM pendant le développement embryonnaire :

- Où OVOL2 est-elle exprimée pendant le développement embryonnaire ?

- Le contrôle spatio-temporel d'OVOL2 est-il nécessaire pour la formation correcte des cellules de la crête neurale et le processus TEM ?

La réponse à ces questions permettrait d'établir les mécanismes moléculaires par lesquels :

1) OVOL2 est capable de réguler la capacité de migration des cellules de mélanome et donc le processus métastatique. En outre, cette étude serait la première à explorer le rôle d'OVOL2 dans le contexte du cancer dérivé de la crête neurale et également du mélanome.

2) Comprendre l'importance du modèle d'expression d'OVOL2 pendant la formation et la migration de la crête neurale pendant le développement et son implication dans l'équilibre TEM/MET pendant le développement embryonnaire.

## RÉSULTATS

### **1. Résultats principaux : OVOL2 dans le mélanome.**

L'expression d'Ovol2 dans les mélanomes humains primaires et métastatiques n'a pas été étudiée auparavant. Ainsi, nous avons évalué l'expression d'OVOL2 dans le mélanome en utilisant des bases de données publiques compilées à partir de plusieurs ensembles de données sur les tumeurs humaines (REF). Dans deux ensembles de données indépendants (Xu, L., et al., 2008 ; Riker, A. I. et al., 2008), OVOL2 était significativement plus exprimé dans le mélanome primaire que dans les métastases. Un troisième ensemble de données a été utilisé comprenant n=558 tissus normaux, n=461 tumeurs ; nous avons également constaté que OVOL2 était significativement plus exprimé dans les tissus normaux de la peau par rapport aux échantillons de mélanome tumoral.

Deuxièmement, nous avons recherché les niveaux d'ARNm de l'expression d'OVOL2 dans les lignées cellulaires de mélanome en utilisant les données de microarray fournies par Rambow et al, 2015. Parmi ces 24 lignées cellulaires de mélanome, nous avons observé que les propriétés migratoires des cellules étaient négativement corrélées avec les niveaux d'expression de l'ARNm OVOL2, telles que Mewo ou A375M, présentent de faibles niveaux d'OVOL2, tandis que les lignées cellulaires non migratoires, telles que MNT-1, présentent des niveaux plus élevés d'expression d'OVOL2. Prises ensemble, ces données soutiennent notre hypothèse de travail selon laquelle les niveaux d'expression d'OVOL2 influencent le potentiel migratoire et métastatique des cellules de mélanome.

Deux lignées cellulaires de mélanome métastatique ont été évaluées in vitro après transfection avec un plasmide d'expression pCMV-OVOL2 codant pour la protéine humaine et un plasmide pCMV-OVOL2-GFP codant pour la protéine de *xenopus tropicalis*. 48h après la transfection, nous avons observé que les cellules surexprimant



OVOL2 avaient une migration cellulaire altérée avec un taux diminué (um/min) dans les deux lignées cellulaires. Les paramètres de pause des cellules ont également été affectés. Des analyses complémentaires ont été effectuées sur 2 autres lignées cellulaires de mélanome (Lu1205 et SKMEL28) dans un seul réplicat expérimental dans lequel les mêmes résultats ont été obtenus. Pour s'assurer du phénotype observé, le cycle cellulaire et la mort cellulaire ont également été analysés après le gain de fonction d'OVOL2.

Pour explorer l'effet d'OVOL2 dans un contexte in vivo, nous avons utilisé le modèle de xénogreffe de mélanome métastatique du poisson zèbre, qui nous permet de visualiser et de suivre la migration cellulaire in vivo dans un court laps de temps. Des cellules rapporteuses de fluorescence verte Mewo (mewo-GFP) générées au laboratoire et préalablement transfectées avec le plasmide d'expression pCMV-OVOL2 ou le contrôle, ont été implantées dans la vessie natatoire en développement d'embryons de poisson zèbre âgés de 3 jours. Les tumeurs ont été mesurées juste après et 72 h après l'injection par microscopie confocale, dans le but de visualiser le nombre de cellules capables de se dissocier de la masse tumorale et de commencer à envahir les tissus environnants. Il a été observé que les tumeurs qui surexpriment OVOL2 présentaient une réduction significative du nombre de cellules détachées de la masse tumorale primaire, indiquant que l'augmentation des niveaux d'OVOL2 dans les lignées cellulaires hautement métastatiques, telles que Mewo, est capable de diminuer sa capacité à se détacher de la masse primaire et migrent in vivo.

Nous avons également observé que les changements dans l'expression d'OVOL2 ont un effet sur la morphologie des cellules de mélanome. Les cellules SMEL28 avec une expression réduite d'OVOL2 ont montré des changements significatifs dans la morphologie, montrant une altération d'un phénotype polygonal à un phénotype fusiforme et une augmentation de la formation de filopodes.

D'autre part, les cellules A375M surexprimant OVOL2 ont montré des changements de morphologie caractérisés par une réduction de la formation des filopodes et une augmentation des lamellipodes. Dans le cas de Mewo, nous avons observé une réduction de la morphologie conformationnelle. Les changements morphologiques ont été quantifiés et une réduction significative de la surface et du périmètre des cellules a été observée pour A375M, Lu1205 et Mewo suite à une augmentation de l'expression d'ovol2.

Pour comprendre le mécanisme sous-jacent au phénotype observé après la surexpression d'OVOL2, nous avons décidé d'effectuer une RNA-seq pour explorer l'expression différentielle des gènes entre les cellules témoins et les cellules surexprimant la protéine OVOL2 humaine. Dans le cas de Mewo, quelques 37 gènes ont été observés comme étant exprimés de manière différentielle. Parmi les gènes qui ont été régulés à la hausse, on trouve des gènes impliqués dans l'organisation de la jonction cellulaire (AMIGO2, C3, CRB3, KRT14), la différenciation cellulaire (C3, CD74, DMBT1, IGF2, KRT14, IGF2, KRT14, KRT14), et la différenciation cellulaire (C3, CD74, DMBT1, IGF2, KRT14, KRT80, KRT81, KRT86, OVOL2, SEMA6B, TH), les constituants structurels de la matrice extracellulaire (COL5A3, TINAGL1) et la liaison à la matrice extracellulaire (DMBT1, TINAGL1), entre autres. Ces données suggèrent donc que dans le mélanome, l'expression d'OVOL2 régule le programme transcriptionnel affectant la formation du cytosquelette, l'organisation de la jonction cellulaire et la différenciation des cellules épithéliales.

## **2. Annexe : OVOL2 en cours de développement.**

Pour aborder la question de la fonction d'OVOL2 dans l'embryon en développement, nous avons d'abord utilisé Ectomap, un ensemble de données de dépôt public construit à partir du transcriptome de régions disséquées de l'ectoderme d'embryons

en développement (Plouhinec JL et al., 2017). Cet outil nous permet d'explorer l'expression spatiale des gènes dans la neurula en développement de *Xenopus leavis*. En utilisant Ectomap, nous avons pu observer que OVOL2 est exprimé au stade gastrula et que son expression diminue de moitié au début du stade neurula. L'information spatio-temporelle montre qu'au stade de la gastrula précoce (stg12.5) OVOL2 est fortement exprimé dans la frontière neurale antérieure (ANB) et dans la zone de la future frontière neurale. Une fois dans le stade neural (stg14) l'expression est restreinte à la frontière neurale, étant plus forte dans la partie antérieure à latérale. Peu ou pas d'expression est observée dans la plaque neurale. L'analyse de l'expression moyenne a également montré que OVOL2 est le plus fortement exprimé dans l'ectoderme de l'embryon en développement. OVOL2 est également exprimé le long de la frontière neurale située dans la partie antérieure et postérieure. L'expression est faible dans la plaque neurale antérieure et aucune expression n'est détectée dans la plaque neurale postérieure.

Pour explorer l'expression d'ovol2 au niveau de la cellule unique, nous avons utilisé SPRING, un outil permettant de dévoiler la structure hautement dimensionnelle des données d'expression de la cellule unique. Nous avons analysé les stades 12 à 18, couvrant les processus de gastrula et de neurula. Au stade 12, l'expression est limitée à l'ectoderme non-neurula. Une fois que l'embryon entre dans la neurulation, au stade 14, OVOL2 est exprimé dans les cellules placodiques antérieures et postérieures, comme nous l'avons précédemment trouvé dans d'autres ensembles de données. Au stade 16, l'expression est limitée aux cellules de la placode antérieure et aux progéniteurs ciliés épidermiques et épidermiques.

## **DISCUSSION ET CONCLUSION**

Les pages suivantes de ce chapitre sont consacrées à la discussion et à la mise en perspective des résultats obtenus au cours de cette thèse.

### ***Ovol2 dans le mélanome***

Grâce à ce travail, nous avons montré que OVOL2 est impliqué dans la capacité migratoire des cellules de mélanome in vitro et dans leur capacité à se détacher de la masse primaire et à envahir les tissus environnants in vivo. Nous avons également montré que la séquence hautement conservée déjà étudiée par d'autres études se traduit également par une conservation fonctionnelle de la protéine au moins entre les amphibiens et les humains, puisque la protéine OVOL2 de *xenopus tropicalis* produit le même effet inhibiteur sur la migration des cellules humaines que celui observé avec la séquence OVOL2 humaine.

Nous avons étudié l'expression d'OVOL2 dans des échantillons de mélanome humain en utilisant des données publiques in silico. En utilisant les données d'études indépendantes, nous avons observé que le mélanome primaire présente des niveaux significativement plus élevés d'OVOL2 et que ces niveaux baissent lorsqu'ils sont examinés dans le mélanome métastatique.

En utilisant des techniques d'imagerie, nous avons observé l'effet d'OVOL2 sur la morphologie des cellules de mélanome métastatique in vitro. Nous avons observé que l'expression d'OVOL2 conduisait à une réduction de la surface et du périmètre des cellules, ce qui suggère qu'elle a un effet spécifique sur le cytosquelette conduisant à des changements de la forme des cellules et de leurs caractéristiques migratoires.

Nous avons également exploité les différences d'expression génique après l'expression ectopique d'OVOL2 dans le mélanome en utilisant RNAseq. Les données obtenues montrent que l'expression d'OVOL2 a conduit à une augmentation de l'expression des

protéines impliquées dans l'adhésion cellulaire, l'adhésion cellule-matrice et le cytosquelette. Cela confirme les observations précédentes sur la morphologie des cellules.

Par séquençage de l'ARN, nous avons observé que OVOL2 ne semble pas contrôler la migration par l'inhibition des facteurs d'induction comme cela est observé dans de nombreux cancers dérivés de l'épithélium. Nous avons observé qu'aucun des principaux TEM-TFs ou marqueurs n'est altéré suite à l'expression ectopique d'OVOL2. Nous avons également observé que OVOL2 affecte le cytosquelette des cellules de mélanome.

Tous ces résultats pourraient donner un aperçu du rôle d'OVOL2 dans la régulation du cytosquelette et des processus d'adhésion cellulaire dans les cellules de mélanome. Des recherches supplémentaires sont nécessaires pour comprendre pleinement le mécanisme par lequel OVOL2 affecte le cytosquelette et donc le potentiel métastatique de ces cellules.

### ***Ovol2 en développement***

On sait qu'au cours du développement embryonnaire, des cycles séquentiels de la TEM et de MET sont nécessaires pour la différenciation finale des types de cellules spécialisées et la formation de la structure tridimensionnelle des organes. Nos analyses ont montré qu'au cours du développement, l'expression d'OVOL2 est limitée aux zones de la frontière neurale, une région comprenant les progéniteurs de quatre populations cellulaires clés : les cellules de la plaque neurale, les cellules de la crête neurale, les placodes crâniennes et l'épiderme. Ceci a été confirmé par hybridation in situ (ISH) dans des embryons fixes de *xenopus laevis* aux stades de la gastrulation, de la neurula précoce et de la neurula tardive. L'analyse d'OVOL2 dans l'ensemble de données de

cellules uniques de *xenopus tropicalis* a révélé que l'expression de ce facteur de transcription est principalement limitée aux futures cellules progénitrices placodiques et épidermiques et qu'aucune expression n'a été observée au niveau de la crête neurale à n'importe quel stade. Ces données sont conformes à ce que nous attendions de la littérature, car les cellules de la crête neurale doivent subir une TEM pour migrer le long de l'axe du corps et donner naissance à un large éventail de dérivés dans l'embryon. Ces données suggèrent que OVOL2 peut également agir comme un inhibiteur important de la TEM pendant la neurulation dans le développement embryonnaire en empêchant la migration des cellules de la crête neurale à partir des tissus adjacents. De futures expériences pourraient viser à manipuler l'expression d'OVOL2 au niveau des tissus de la crête neurale chez *xenopus laevis*. Par la suite, l'observation des tissus de la crête neurale et des placodes par des marqueurs spécifiques au cours des différentes étapes du développement. Ces études nous aideront à comprendre le rôle d'OVOL2 dans la régulation de l'équilibre TEM/MET pendant le développement embryonnaire et ses effets possibles sur le développement de différentes structures.



## ANNEXES





# 1. Preliminary results: Exploration of OVOL2 function in neural crest cells migration

## 1. 1 Introduction

Neural crest cells are a transient population of multipotent cells that have the ability to give rise to large population of different cells during development. During development, the induction and specification of this population take place in the population of cells that reside in the border between the neural plate and the adjacent epidermis and is known to be controlled by certain combination of bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Notch and Wnt signals received from the ectoderm and mesoderm. Once the neural tube is closed, those cells go through a process named EMT by which they detach from their epithelial neighbours' cells and migrate to distant sites along the developing embryo to finally differentiate into a wide range of derivative in different sites. Therefore, the correct embryonic development highly depends on the morphological and phenotypical change from epithelial to mesenchymal of those cells and the reverse process once they reach their destination. This plasticity and cellular dynamic between epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) have been recognised along many years as the basic and main fundamental process driving embryonic development. The induction of NCC by the signals mentioned before also activate a series of EMT promoting transcription factors (EMT-TFs) known to be responsible for the changes in epithelial cells and therefore EMT. Snail1/2, Twist1, Zeb1/2 are some of the main TFs known for repressing epithelial genes and subsequently activate the expression of many mesenchymal genes. During the years, many studies have been focused on the understanding and patterning of Gene regulatory network controlling neural crest formation, migration, and differentiation. EMT inducing factors play an important role for neural crest cells and have been widely studied in several models including Chicken, xenopus, zebrafish, and mouse (Hutchins et al., 2020; Milet et al., 2013; Rocha et al., 2020). On the opposite, little is known about the factors controlling the opposite process and stop the migratory capacity of neural crest cells. As mentioned before OVOL2 have been characterized as EMT inhibitor or MET inducer in few contexts including cancer human cell lines. However, the role of this gene during the EMT-MET plasticity observed during NCC development have not been explored yet. Due to highly conservation of the protein observed across several species, we proposed *Xenopus laevis* as a good model in which be able to study the expression pattern and involvement of *ovol2* in EMT/MET plasticity during embryonic development.

## 1.2 Results

### **1. Exploration of OVOL2 expression in different tissues and developing stages of xenopus embryos.**

To address the question about the role of OVOL2 in developing embryo, we first used Ectomap, a dataset built by our team with the transcriptome of dissected regions of ectoderm of developing embryos (Jean-Louis Plouhinec, 2017). This tool allows us to explore spatial gene expression in the developing neurula of *Xenopus laevis*. Using Ectomap we could observe that OVOL2 is expressed at gastrula stage and its expression is decreased to the half at the beginning of neurula stage (Figure 1A). Spatial-temporal information shows that at early gastrula stage (stg12.5) OVOL2 is highly expressed in anterior neural border (ANB) and in the future neural border area. Once in neural stage (stg14) we can see that the expression is restricted to the neural border, being strongest at the anterior and lateral part. Slight or no expression is observed at the neural plate (FIGURE 1B). Mean expression analysis also showed that *ovol2* has the highest expression in the ectoderm of developing embryo. OVOL2 is also expressed along the neural border being found at the Anterior and posterior part. Low expression is found in the anterior neural plate and no expression is detected at the posterior neural plate (FIGURE 1C).

### **2. Exploration of OVOL2 expression across cell population**

To explore the expression of *ovol2* at later stages at a single cell level we used SPRING, a tool for unveiling high dimensional structure of single cell expression data. We analysed from 12 to 18 stage, covering gastrula and neurula process. At stage 12, the expression is restricted to the non-neural ectoderm. Once the embryo enters neurulation, at 14 stage, OVOL2 was found to be expressed in cells of the anterior and posterior placode, such as we found before in other datasets. At stage 16 the expression is limited to the anterior placode cells and the epidermal progenitors and ciliated epidermal progenitors (Figure 2).

### **3. Ovol2 expression was checked in xenopus laevis embryos.**

To observe OVOL2 expression, digoxin labelled antisense probes were made using a plasmid containing OVOL2 sequence as template. In situ hybridization was made in embryos of gastrula (12.5 stg) neurula (14stg) and pre-migratory NC stage (18-19 stg). No good signal was detected in early gastrula stage. In neurula stage we could observe slight expression all over the developing epidermis. At late neurula stage (18-19) ovol2 expression is low and restricted to the epidermis and not expressed at the neural plate (Figure 3), such as we observed previously in Ectomap data.

### **4. Manipulation of OVOL2 expression during embryonic development and neural crest cell formation.**

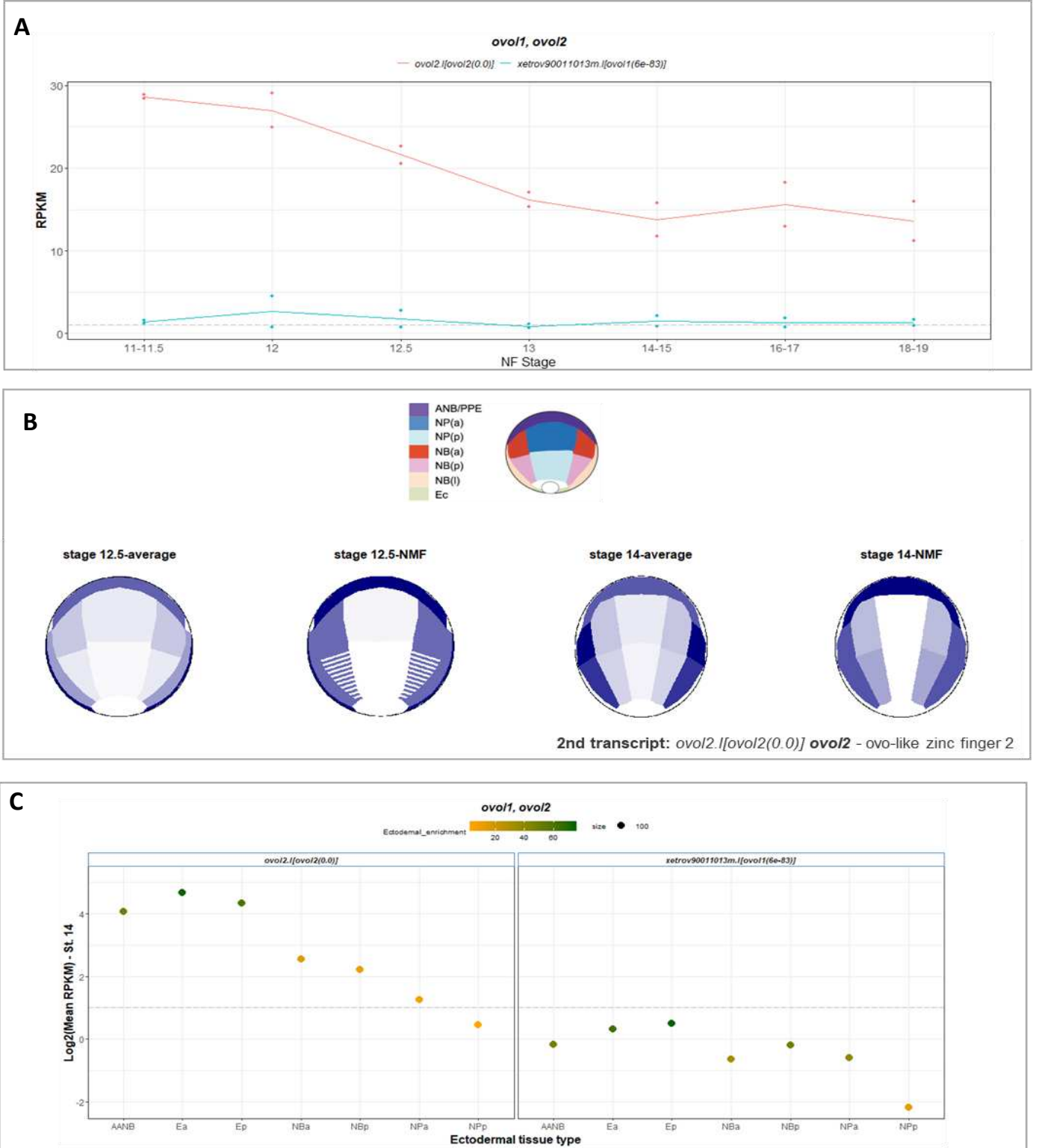
As we observed OVOL2 which have been associated as EMT inhibitor, is slightly expressed, or no expressed at neural plate and later in neural crest during embryonic formation. This suggest us that no expression of this TF at neural plate during development may be crucial for the correct formation and migration of neural crest cells. To explore that, we designed a series of experiments to follow. The first experiment in our plan is to modify Ovol2 expression, by gain of function (GOF) as loss of function (LOF) in *xenopus laevis* embryos. First, to develop the tools needed for the GOF, we created a plasmid containing OVOL2 *xenopus tropicalis* protein under CMV promoter. For that we performed Gateway cloning from a pDNOR vector that contains *Xenopus tropicalis* OVOL2 sequence to a pCS+ vector that already include CMV promoter and GFP (Figure 4A). After that we need to inject a gradient of concentration into 2 cells embryos and perform ISH of NC and placode markers at pre migratory and migratory stages (Figure 4B).

In order to also study how the absent of Ovol2 affect migration and formation of neural crest/placodes, we designed a morpholino sequence (Figure 4C) that target the start codon of Ovol2. A gradient of concentration was done in injections in 2 cells stage and measure the pattern of neural crest/placode at pre-migratory and migratory stages (Figure 4D).

Unfortunately, those experiments were halted due to lack of time. Tools are designed and prepare ready for use.

It is known that Ovol2 have a role in the inhibition of EMT at in vitro cellular level, especially in cancer related contexts. However, nowadays there is no information about the role of this gene controlling EMT in a physiological context as embryonic development. As we know, neural crest cells is a highly valuable population of cells and the disruption of those during embryonic development normally present a dramatic outcome resulting in different diseases under the group of neurocristopathies. Therefore, those experiments are key to understand the role of OVOL2 in the correct neural crest and placode formation. They Will also can be solid evidence of the conservation of EMT process between the physiological and pathological context.

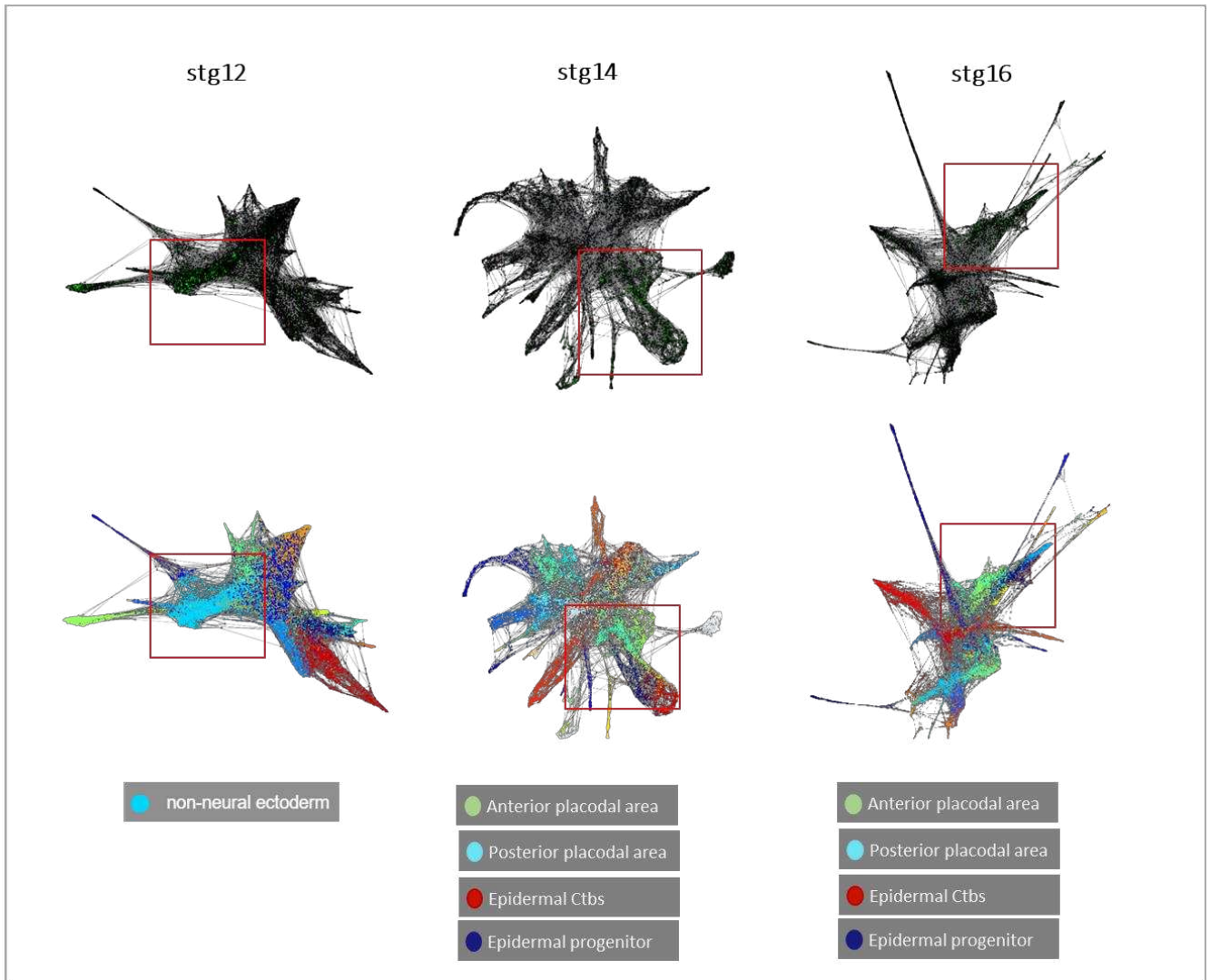
**Figure 1**



**Figure 1. Ovov2 RNA expression data in xenopus laevis developing embryos.**

A) Gene normalized RNA expression (RPKM) of OVOL2 and OVOL1 in xenopus laevis whole embryo from early neurula stage until promigratory/late-neurula stages. B) Spatio-temporal expression of OVOL2 in xenopus laevis developing embryo in gastrula (12.5 stg) and Neurula (stg14) stages. NMF: C) Average counts (log<sub>2</sub>mean RPKM) of OVOL2 and OVOL1 for each dissected region of whole embryo. An average count above the grey line indicates the enrichment of the gene in that area in comparison to the whole embryo expression. Color indicates the gene's ectoderm enrichment relative to the rest of the genes expressed in that region and goes from yellow/orange (no enrichment) to green (highly enriched). Gene enrichment value is ranked by comparing it to the one of all the other genes expressed in that region. Rank is scaled between 0 (poorly enrich/specific) to 100 (highly enrich/specific)

**Figure 2**

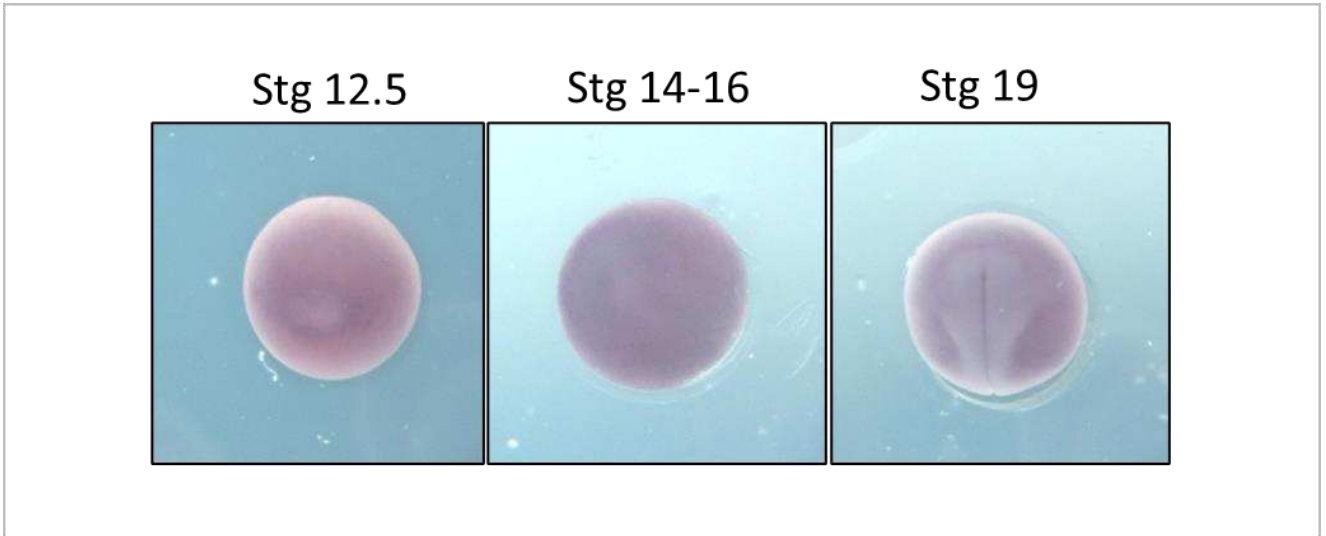




**Figure 2. Single cell expression data in xenopus tropicalis developing embryos.**

Catalog of single cell expression data of whole xenopus tropicalis embryos through early gastrulation (stg 12) to late-mid neurulation (stage16).We can see in a single cell level the distribution of OVOL2 expression across different cell types during different stages of development. For analysis we used a tool developed by Briggs, J. A. et al., 2018. Data is published and interactive analysis can be done using: [tinyurl.com/scXen2018](http://tinyurl.com/scXen2018).

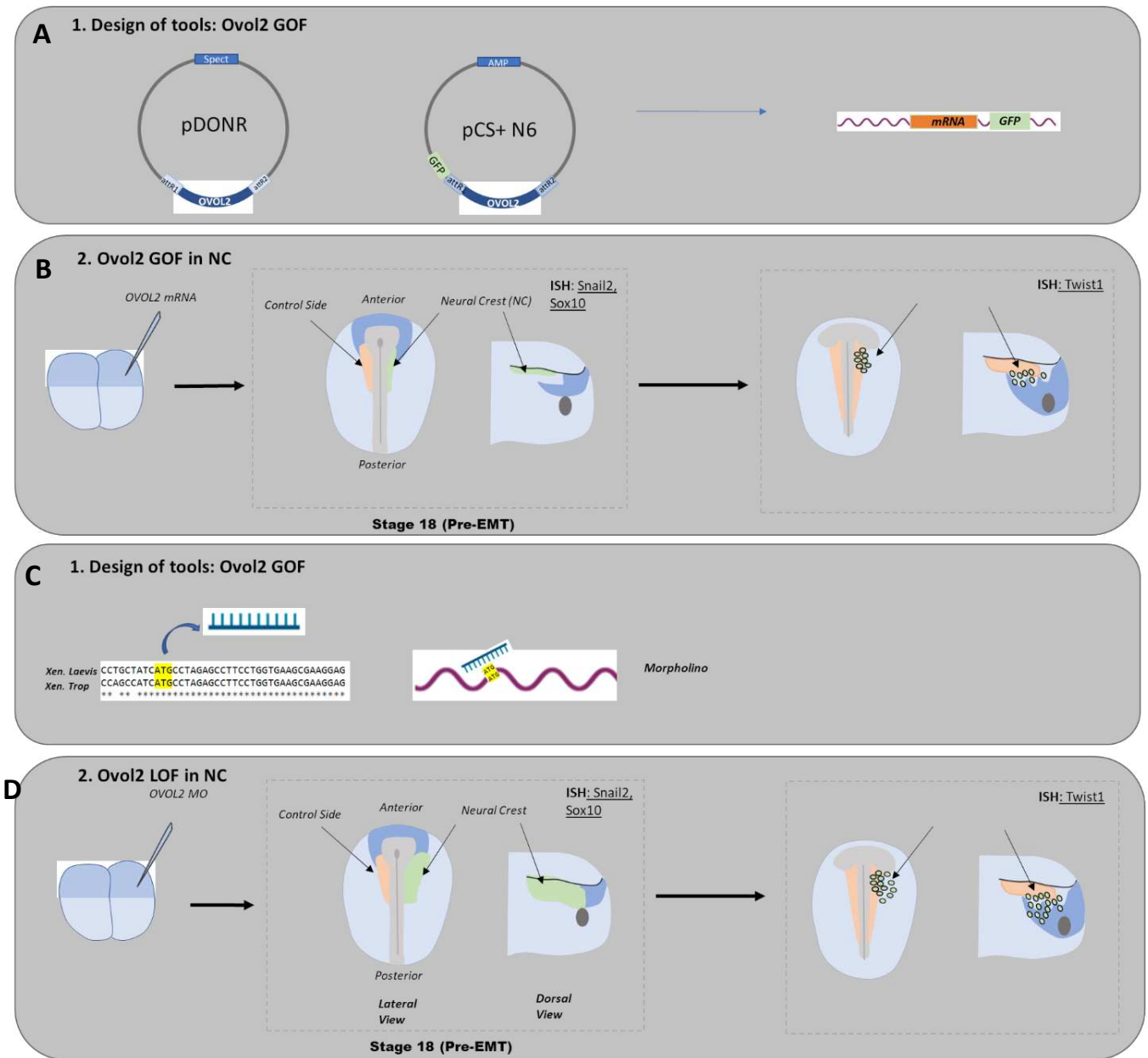
Figure 3



**Figure 3. OVOL2 expression pattern in xenopus laevis embryos.**

In situ hybridization of OVOL2 at stages 12, 14-16 and 19 respectively. OVOL2 is expressed in the developing epidermis and neural border. Absence of OVOL2 expression is found in the neural plate as we expected by the previous analysis on Ectomap.

**Figure 4**



**Figure 4. Design of strategy to follow to study the implication of OVOL2 in EMT during development and neural crest formation.**

A) Design of plasmid expressing OVOL2 *xenopus tropicalis* protein and tagged with GFP. The sequence was cloned from donor plasmid into a PCS+ plasmid containing GFP. B) Plan: injection of mRNA of OVOL2 into 2 cell stage embryos targeting the area forming the neural crest in the future. ISH of neural crest marker at pre-migratory and migratory stages. C) Morpholino design targeting OVOL2 for *xenopus laevis* embryos. D) Plan: injection of gradient concentrations of OVOL2 targeting morpholino into 2 cell stage embryos targeting the area forming the neural crest in the future. ISH of neural crest marker at pre-migratory and migratory stages.

2. Publication 1: *Xenopus*, an emerging model for studying pathologies of the neural crest.



# *Xenopus*, an emerging model for studying pathologies of the neural crest

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## Abstract

Neural crest cells are a multipotent embryonic stem cell population that emerges from the lateral border of the neural plate after an epithelium-to-mesenchyme transition. These cells then migrate extensively in the embryo and generate a large variety of differentiated cell types and tissues. Alterations in almost any of the processes involved in neural crest development can cause severe congenital defects in humans. Moreover, the malignant transformation of one of the many neural crest derivatives, during

childhood or in adults, can cause the development of aggressive tumors prone to metastasis such as melanoma and neuroblastoma. Collectively these diseases are called neurocristopathies. Here we review how a variety of approaches implemented using the amphibian *Xenopus* as an experimental model have shed light on the molecular basis of numerous neurocristopathies, and how this versatile yet underused vertebrate animal model could help accelerate discoveries in the field. Using the current framework of the neural crest gene regulatory network, we review the pathologies linked to defects at each step of neural crest formation and highlight studies that have used the *Xenopus* model to decipher the cellular and molecular aspects of neurocristopathies.

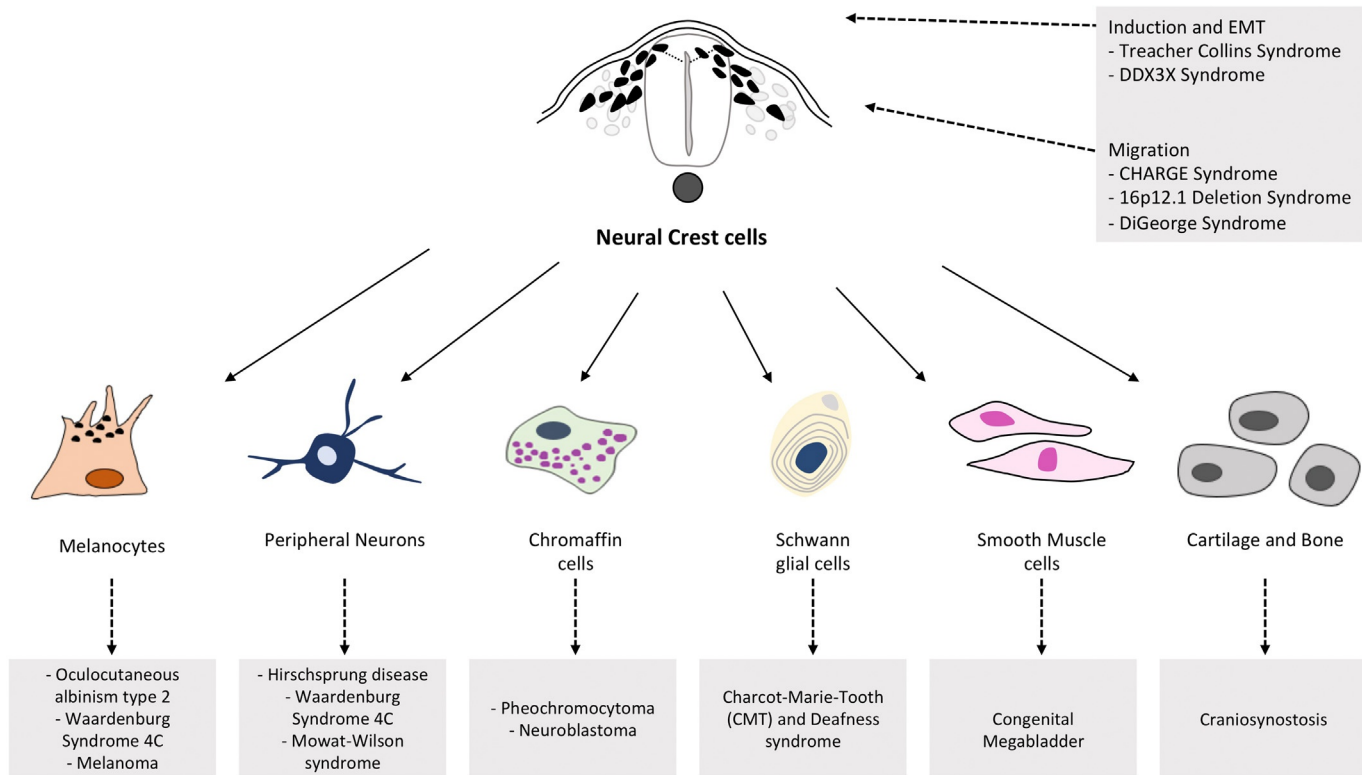


## 1. Introduction

### 1.1 The neural crest cells

The neural crest (NC) cells form a multipotent cell population that arises from the ectoderm germ layer during early vertebrate development. They give rise to an exceptionally rich variety of cell types forming diverse tissues and participating to the development of many organs. This includes typical ectodermal and neuro-ectodermal derivatives (e.g., neurons, glia), pigment cells, as well as ectomesenchymal derivatives, which are cell types formed by the cranial NC cells in the head and neck (e.g., smooth muscle, cartilage, bone) while these cell types typically arise from the mesoderm germ layer in the trunk (Alkobtawi & Monsoro-Burq, 2020; Le Douarin & Kalcheim, 1999). At the end of neurulation, when the neural tube is about to close, NC cells undergo an important cellular remodeling process known as the epithelium-to-mesenchyme transition (EMT). During gastrulation and neurulation, NC progenitors are induced in the epithelium adjacent to the neural plate ectoderm, so that in the head, as the cephalic neural folds elevate, NC precursors lie on both sides of the future brain while they are located at the tip of the dorsal neural folds in the trunk. EMT allows NC cells to exit the ectoderm epithelium, to acquire motility, to migrate toward internal or superficial tissues and to finally differentiate into more than 30 cell types throughout the body. The cranial NC cells initiate emigration collectively while the trunk NC cells tend to travel individually or in very small groups toward the target tissues, guided by anatomical or chemical cues provided by the environment (Theveneau & Linker, 2017). NC cells navigate along several stereotyped pathways according to their prospective differentiation into neurons and glia of the peripheral nervous system (sensory, autonomous, or enteric nervous system), pigment cells of the skin and internal organs, ectomesenchyme in the head, cardiac and pulmonary outflow tract, and secretory chromaffin cells in adrenal medulla (Fig. 1).





**Fig. 1** After undergoing a stereotypical epithelium-to-mesenchyme transition, the motile neural crest cells delaminate from the neuroepithelium, migrate along several specific routes across the body, and reach diverse destinations where they differentiate into a wide spectrum of cells types, the main ones being illustrated here. Severe or milder alterations during the delamination and migration processes, in the cell guidance parameters, or during the differentiation of each lineage can result into a broad range of human pathologies and complex syndromes. A subset of those diseases, classified by the cell process affected or a neural crest-derived cell type affected for each pathology, is presented here and detailed in [Table 1](#).

## 1.2 Neural crest-linked congenital defects and other pathologies of the neural crest derivatives

During embryonic development, any mild temporal or functional alteration of the molecules and signals involved in NC cells induction, EMT, migration or differentiation is likely to produce a significant developmental disruption. These defects lead either to the failure of embryonic development (embryonic lethality) or to one of the diseases and syndromes collectively named neurocristopathies (NCPs; [Bolande, 1974](#)). Therefore, understanding the details of NC cells development and function has immense medical implications. NCPs encompass a broad spectrum of congenital malformations and diseases affecting individual or multiple NC derivatives, as well as the tumors arising from the malignant transformation of one of the NC-derived cells. It is estimated that 1/4th to 1/3rd of the human congenital defects involve NC cells derivatives. With tumors formed by NC-derived cells during childhood or adult life, collectively these diseases constitute a heavy societal burden. However, individual NCPs are clinically classified by their specific phenotypes which can cover a range from relatively frequent to very rare pathologies: for instance, Hirschsprung disease is described in 1/5000 live birth in human ([Amiel & Lyonnet, 2001](#)); Charge syndrome in 0.1–1.2/10000 human live birth ([Bajpai et al., 2010](#)) and Waardenburg syndrome is found in 1/42000 infants at birth ([Song et al., 2016](#)).

NCPs were subdivided into four main categories by Bolande: (1) isolated tumors, (2) tumor syndromes, (3) congenital malformations, (4) other NC-linked pathologies ([Bolande, 1974](#)). Recently, a new classification has proposed to group NCPs according to the stage of NC development affected at the onset of the disease ([Vega-Lopez et al., 2018](#)). This proposal takes advantage of the tremendous progress made in understanding the sequential steps of the NC gene regulatory network (GRN) during the last 15 years, using a variety of vertebrate animal models *in vivo*, including the clawed frogs *Xenopus laevis* and *tropicalis*, the chick *Gallus gallus*, the zebrafish *Danio rerio*, and the mouse *Mus musculus*. To this list of models suitable to explore NC biology, research of the last decade has added human cells derived from induced pluripotent stem cells (iPSCs) reprogrammed into NC cells ([Barrell et al., 2019](#); [Leung et al., 2016](#)). In this chapter, we will use this novel classification for NCPs and will thus define four main groups of diseases: NCPs arising from defects primarily occurring during NC induction, specification and EMT (group 1), NC migration (group 2), NC differentiation (group 3) and tumorigenesis (group 4). [Table 1](#) references selected

**Table 1** Selected neurocristopathies with studies using *Xenopus* frogs as a model organism.

<b>Disease name</b>	<b>OMIM reference</b>	<b>Mutations identified in patients</b>	<b>References using <i>Xenopus</i> as a model</b>
<i>Group 1: NC induction and EMT</i>			
Branchio-Oto-Renal (BOR) syndrome	#113650	EYA1	Li, Manaligod, and Weeks (2010) and Moody, Neilson, Kenyon, Alfandari, and Pignoni (2015)
Chromosome 16p12.1 deletion syndrome	#136570	–	Lasser et al. (2020)
DDX3 syndrome	#300160	DDX3X	Perfetto et al. (2021)
Diamond Blackfan Anemia	#105650	RPS19, RPS24, RPS17, RPL35A, RPL5, RPL11, RPS7, RPS10, RPS26, RPL26, RPL15, RPS29, TSR2, RPS28, RPL27, RPS27, RPL18, RPL35, RPS15A	Griffin, Sondalle, Del Viso, Baserga, and Khokha (2015) and Robson, Owens, Baserga, Khokha, and Griffin (2016)
Mowat-Wilson syndrome	#235730	ZEB2	Verstappen et al. (2008)
Treacher Collins syndrome	#154500	TCOF1, POLR1C, POLR1D, POLR1B, DDX21	Gonzales, Yang, Henning, and Valdez (2005), Robson et al. (2016) and Calo et al. (2018)
<i>Group 2: NC migration defects</i>			
Auriculo condylar syndrome	#602483	GNAI3	Marivin et al. (2016)
Charge syndrome	#214800	SEMA3E, CHD7	Schulz et al. (2014), Ufartes et al. (2018) and Bajpai et al. (2010)
Chromosome 16p12.1 deletion syndrome	#136570	–	Lasser et al. (2020)
DiGeorge syndrome	#188400	TBX1	Tazumi, Yabe, and Uchiyama (2010) and Alharatani et al. (2020)
Hamamy syndrome	#611174	IRX5	Bonnard et al. (2012)

*Continued*

**Table 1** Selected neurocristopathies with studies using *Xenopus* frogs as a model organism.—cont'd

<b>Disease name</b>	<b>OMIM reference</b>	<b>Mutations identified in patients</b>	<b>References using <i>Xenopus</i> as a model</b>
Hypohidrotic ectodermal dysplasia	#305100	EDA	<a href="#">Essenfelder et al. (2004)</a>
Kabuki syndrome	#147920	KMT2D	<a href="#">Schwenty-Lara, Nehl, and Borchers (2020)</a>
Noonan syndrome	#163950	PTPN11, KRAS, SOS1, RAF1, NRAS, BRAF, RIT1, SOS2, LZTR1, MRAS, RRAS2, SHOC2, PPP1CB, CBL	<a href="#">Popov et al. (2019)</a>
Wolf-Hirschhorn syndrome	#194190	–	<a href="#">Mills et al. (2019)</a>
<i>Group 3: Differentiation defects</i>			
Axenfeld-Rieger syndrome	#180500; #602482	PITX2, FOXC1	<a href="#">Cha et al. (2007)</a>
Bamforth-Lazarus syndrome	#241850	FOXE1	<a href="#">El-Hodiri et al. (2005)</a>
Blepharocheilodontic syndrome 2	#617681	CTNND1	<a href="#">Alharatani et al. (2020)</a>
Charcot-Marie-Tooth (CMT) and deafness syndrome (Schwann cell differentiation)	#118300	PMP22, PRPS1, GJB1	<a href="#">Tae, Rahman, and Park (2015)</a> and <a href="#">Bae et al. (2014)</a>
Craniosynostosis (cartilage and bone differentiation)	#123100 #604757 #600775	MSX2, TWIST1, FGF receptors	<a href="#">Twigg et al. (2015)</a>
Dyschromatosis Symmetrica Hereditaria (melanocyte differentiation)	#127400	DSRAD	<a href="#">Wagner, Smith, Cooperman, and Nishikura (1989)</a>

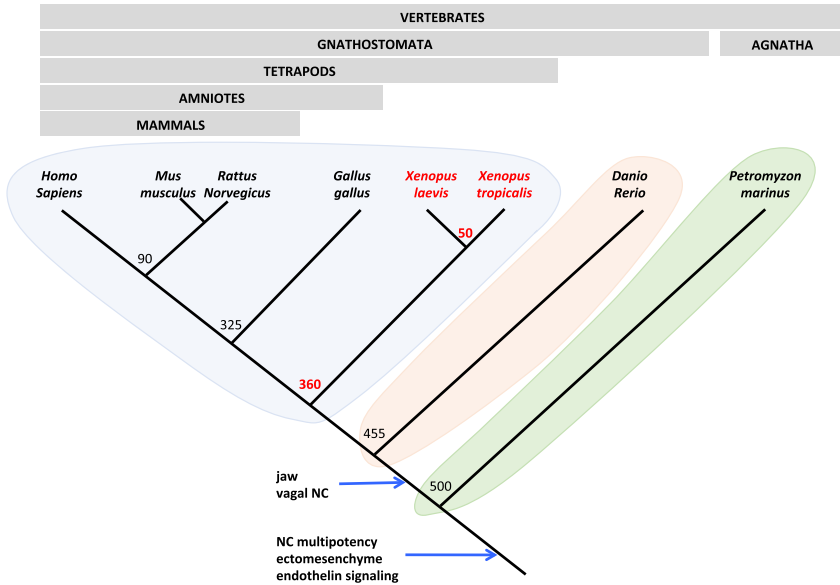
Hermansky-Pudlak syndrome 6	#614075	HPS6	Nakayama et al. (2017)
Hirschsprung disease (enteric nervous system)	#142623	RET, EDNRB	Li et al. (2018)
Klippel-Feil syndrome	#118100 #214300 #613702	GDF6, MEOX1, GDF3	Martens et al. (2020) and Tassabehji et al. (2008)
Megabladder, congenital (cardiac NC cell differentiation defects)	#618719	Myocardin	Wang et al. (2001)
Oculocutaneous albinism II (melanocyte differentiation)	#203200	OCA2, MC1R, TYR	Shi et al. (2019) and Barsh, Gunn, He, Schlossman, and Duke-Cohan (2000)
Waardenburg syndrome, type 2E, type 4C (pigment cell, enteric neurons)	#277580	SOX10, EDNRB, EDN3	McGary et al. (2010)
<i>Group 4: Tumors</i>			
Melanoma	#155600	Multiple	Kuznetsov et al. (2019), Kuznetsoff et al. (2021), Fürst et al. (2019) and Plouhinec et al. (2017)
Neuroblastoma	#256700	KIF1B, PHOX2B, ALK	Corallo et al. (2020) and Gonzalez Malagon et al. (2018)

Diseases were selected using both the OMIM database (search with “neural crest” and “*Xenopus*” as key words, 39 hits were further sub-selected if they matched with a NC-linked defect) and the neurocristopathies listed in [Vega-Lopez, Cerrizuela, Tribulo, and Aybar \(2018\)](#) further sub-selected for the availability of functional studies in *Xenopus* (using both Xenbase and PubMed websites). When such studies were not available, some gene expression studies of particular interest for neurocristopathies were mentioned. When the molecular and cellular mechanisms underlying the pathology were available, the disease was listed under one of the four following groups: (1) “induction and EMT,” (2) “migration,” (3) “differentiation” and (4) “tumors.” Within each group, alphabetical order is used to classify diseases. The genes identified in human patients are listed in column 2 and the main references using *Xenopus* biology to understand the disease are listed column 3. We apologize to the authors that could not be listed here due to space constraints and hope that the indicated references will point toward their work.

NCPs which have been explored using *Xenopus* frogs as animal models, either by a functional study or, at minimum, by the expression analysis of a gene involved in the human pathology and has shown expression in the developing NC. This table indicates the main gene(s) involved in humans when this is known. References for studies conducted in other animal models can be found elsewhere (Vega-Lopez et al., 2018; Watt & Trainor, 2014). Using the sequential steps of the current NC-GRN is very useful to establish a hierarchical sequence of molecular causes for each disease. However, this new classification does not fully describe the complexity of these pathologies, as most NCPs are not exclusive members of a single category. The frequent overlap of multiple defects occurring at distinct steps of NC formation renders NCP description and understanding very complex. This facet of neural crest biology is probably one of the main scientific challenges of the next decade.

### 1.3 Conservation of the neural crest gene regulatory network in vertebrates

In this context, the multiplicity of vertebrate animal models used in developmental biology brings many options for genetic or experimental approaches to decipher the biology of NCPs. Cellular models further increase the range of possibilities to explore the details of NC cells behavior *in vitro* and in controlled environments (Scarpa et al., 2015). Collectively, animal models and human-derived NC cells present complementary advantages to tackle almost any question in NC and NCPs biology (Leung et al., 2016; Tchieu et al., 2017). Importantly, the recent comparison of the NC-GRN across various vertebrate animal models, both amniotes and non-amniotes, highlights the high degree of conservation of the global architecture of this gene network and of the associated signaling pathways, starting from the base of the vertebrate evolutionary lineage. The main steps and gene families identified in the animal models classically used for NC studies such as bird (amniotes, tetrapod; chick, quail), mouse (amniote, tetrapod, mammal) or frogs (non-amniote, tetrapod) are found conserved in lamprey (cyclostome; non-amniote, non-tetrapod; Hockman et al., 2019; Nikitina, Sauka-Spengler, & Bronner-Fraser, 2008; Sauka-Spengler, Meulemans, Jones, & Bronner-Fraser, 2007) and in bony fishes (jawed vertebrates, non-amniote, non-tetrapod) (Fig. 2). Additionally, specific features of the NC, such as its high multipotency, jaw formation and the recruitment of specific signaling pathways, have emerged at various evolutionary time points, supported by genetic innovations unleashed by the whole genome



**Fig. 2** A simplified vertebrate evolutionary tree presents the main groups, with a highlight on the clawed frogs *Xenopus laevis* and *Xenopus tropicalis* among the Tetrapod superclass. The early evolutionary acquisition of characteristic features of the neural crest, at the base of the vertebrate tree (multipotency, endothelin signaling, ectomesenchyme potential) or just after early-derived vertebrates (jaws and vagal neural crest derivatives), is reflected in the high conservation of the gene regulatory network governing neural crest development. The main scaffold of this network is highly comparable across vertebrates, yet subtle species/class-specific variations also modulate the fine-tuning of neural crest developmental molecular regulations. Tetrapods are grouped together and highlighted in blue, to emphasize the close evolutionary position of *Xenopus* frogs compared to mammals. Jawed fishes are highlighted in beige and the jawless vertebrates in green. Median divergence times are indicated in million years.

duplications that occurred in vertebrate evolution. These genetic novelties have allowed a range of molecular modulations on top the main NC-GRN scaffold. Hence the acquisition of multipotency, of the ectomesenchymal potential, and endothelin signaling are found at the base of the vertebrate tree (Scerbo & Monsoro-Burq, 2020; Square et al., 2020), but the acquisition of jaws, odontoblasts and vagal neural crest derivatives likely appeared in gnathostomes (Martik et al., 2019; Fig. 2). This high degree of conservation of the NC-GRN, taken with the caution of controlling for species-specific modulations, allows the efficient use of several animal models to explore the mechanisms of human NCPs. For instance, a survey of the steps leading human iPSCs from pluripotency to premigratory NC specification has

highlighted the recapitulation of the key elements defined for the frog NC-GRN (Kobayashi et al., 2020; Pla & Monsoro-Burq, 2018). Moreover, during later stages of NC development, craniofacial malformations are obtained when gene expression causing NCPs in human are experimentally altered in frog embryos (e.g., Bajpai et al., 2010).

In this chapter, we will present some regulatory mechanisms affected in human NCPs that have been explored using the clawed frog *Xenopus laevis* or *tropicalis* models. We will focus on several examples illustrating the advances made using this versatile amphibian tetrapod model, ideally suited for studying the earliest stages of NC development, NC migration and various aspects of NC differentiation, either *in vivo* or *in vitro*. We will further describe the use of this animal model to understand NCPs and NC-derived tumors, using high throughput screening strategies *in vivo*.



## 2. General considerations about *Xenopus* frog embryos as models for neural crest developmental studies

*Xenopus*, the African clawed frog, has been widely used in cell and developmental biology since early 1950s. The advantages of *Xenopus laevis* and *tropicalis* for experimental and genetic research, and the high conservation of developmental genes and molecular mechanisms from amphibians to human have promoted this important model for biomedical research. However, perhaps because it has been used for the best classical embryological studies for a long time, researchers using other models sometimes do not fully appreciate the amazing possibilities of the frog models to understand complex disease-related molecular networks. With modern genetics, epigenetics, single cell approaches, imaging and other tools, combined to the rich heritage of experimental embryology, *Xenopus* research must be highlighted for its important contributions to the better understanding of human pathologies, through the detailed molecular studies of candidate genes associated with human diseases (Fortriede et al., 2020). Similar to other aquatic laboratory models such as the zebrafish *Danio rerio*, *Xenopus* also respects the ethical needs for limiting the experimentation on mammalian models. Moreover, *Xenopus* frogs are tetrapods with lungs and a three-chambered heart, presenting a closer evolutionary relationship with humans than bony fishes for example (Fig. 2). The *Xenopus* genome shares approximately 80% of the genes known to cause human diseases, listed under an easily searchable repository linked to the OMIM database (Nenni et al., 2019; [www.xenbase.org](http://www.xenbase.org)). From an experimental point of view, *Xenopus* embryos are robust and



generated in large numbers by a simple hormone injection and these externally fertilized embryos form large cohorts of siblings allowing sound statistical analyses. Wildtype, inbred and transgenic frog lines are available at Resource Centers in Europe and the United States of America (EXRC, NRC, listed at [www.xenbase.org](http://www.xenbase.org)). NC-related reporter transgenic lines have been developed (Alkobtawi et al., 2018; Li et al., 2019). Relatively large size eggs can be injected at early stages of development and embryos remain accessible at all subsequent stages, including gastrulation and neurulation, the critical stages of neural crest induction, which are poorly accessible in mammalian models. Gene expression can be easily disrupted with morpholino-mediated knockdown or CRISPR/cas9-based approaches. The spatial and temporal control of gene expression is also possible using hormone-inducible constructs, targeted injections or tissue grafting. The studies of frog NC cell migration have extensively explored the mechanisms of collective cell migration and cell motility parameters *in vitro* and *in vivo*. At later stages, *Xenopus* tadpoles are transparent and suited for imaging similar to zebrafish larvae, allowing a follow-up of neural crest-derived differentiated cells. Given all these advantages, the *Xenopus* animal model counts among the current key models to approach neural crest-derived diseases both for basic and biomedical research.



### 3. Congenital disorders caused by defects in early formation of the neural crest

It is very unlikely that genetic alterations affecting deeply the induction and specification steps of the neural crest cells be found in live human births. As they would affect a large set of NC derived cells with critical functions during organogenesis, such defects would most often result in early embryonic lethality. For example, *pax3* and its paralog *pax7* have been identified as critical regulators of neural crest induction at the neural border of frog and chick embryos during gastrulation and neurulation (Basch, Bronner-Fraser, & García-Castro, 2006; Milet, Maczkowiak, Roche, & Monsoro-Burq, 2013; Monsoro-Burq, Wang, & Harland, 2005). The lack of *pax3* gene expression in mouse embryos severely affects cardiac and trunk neural crest cell formation, resulting in failure of heart development and death of the mutant mouse embryos (Morgan et al., 2008). Interestingly, cranial NC cells seem to form normally in those mouse mutants, whereas cephalic NC depleted for *pax3* is virtually not induced in the frog animal model (Monsoro-Burq, 2015; Monsoro-Burq et al., 2005). There are

however, multiple alleles for *Pax3* mutation in mouse: in the spontaneous *Pax3* mutant *Splotch*, homozygotes present an open neural tube, severe neural crest defects and limb muscle anomalies, resulting in embryonic lethality at various time points (Epstein, Vekemans, & Gros, 1991). Interestingly in mouse, *pax7* is also expressed in the neural crest cell lineages and the mouse *pax7* mutants exhibit craniofacial phenotypes of varying severity in different genetic backgrounds, suggesting complex and still incompletely understood specific and redundant functions for *pax3/7* in mice (Mansouri, Stoykova, Torres, & Gruss, 1996; Murdoch, DelConte, & García-Castro, 2012; Zalc, Rattenbach, Auradé, Cadot, & Relaix, 2015). In humans, *PAX3* mutation (*PAX3* (2q36.1)) causes Waardenburg syndrome type I and III, with type III being homozygous or heterozygous, and type I being heterozygous (Pingault et al., 2010). The main features of Waardenburg syndrome include congenital hearing loss, mild craniofacial anomalies and pigmentation defects in the skin, hair and eyes. Waardenburg syndrome Type III or Klein-Waardenburg syndrome is exceedingly rare, suggesting that in humans too, the complete lack of *PAX3* gene function causes embryonic lethality. Interestingly, Klein-Waardenburg syndrome also involves upper limb anomalies due to muscle defects, as observed in the *splotch* mice, consistent with the development of mesoderm-derived limb muscle cells, which depend on early *PAX3* gene functions. This exemplifies the complexity of NCPs syndromes, due to multiple functions of a given gene during development. Moreover, in the same cell lineage, here the neural crest lineage, a same gene may display successive functions at different steps of the NC-GRN. In the case of *PAX3*, heterozygous mutations in Waardenburg syndrome type I (or Shah-Waardenburg syndrome) cause defects in melanocyte biology, indicating anomalies in the later control of NC-derived melanoblast differentiation by *Pax3*.

The example of the Waardenburg syndrome illustrates three key points in NCPs analyses.

- Early regulators of the NC-GRN are essential for the global development of all NC cell populations throughout the embryo. In consequence, their null mutations will likely cause embryonic lethality. Only milder alterations of such genes would be found in human live births, with more subtle phenotypes than the ones usually studied in animal models. Those alterations can include several situations with reduced (but not absent) protein levels, such as found with heterozygous gene mutations, mutations resulting in a partially active protein or affecting protein stability, or when the epigenetic regulation of elements controlling the gene

expression levels is altered. Finally, and more complex to understand, human syndromes may result from the alteration of the protein's co-factors, not the gene itself.

- Animal models are useful to understand NCPs, but a careful analysis of paralog genes activity has to be validated in each model, as a developmental usage swapping between genes with closely related functions has been described: for example *pax3* and *pax7* display both similar and distinct functions in frogs and chick (Basch et al., 2006; Maczkowiak et al., 2010; Monsoro-Burq et al., 2005). According to the genetic background, *Pax3* mutations have various outcomes in the mouse. Moreover, genetic duplications in vertebrates may create redundant activities masking the role of individual genes, but providing with interesting indications for safeguard modalities created during evolution, as it is the case for the four paralogs *pax3a/3b*, *pax7a/7b* in zebrafish (Minchin & Hughes, 2008).
- Altered development of the NC-derived cells is often accompanied by alterations in cell types formed from other germ layers. This can be due to the use of a given gene/protein—in particular transcription factors—for the development of unrelated cell types. In this instance, the developmental trees established from whole embryos single cell transcriptomes allow tracing the expression of a given gene in each cell type and better understand phenotypic alterations observed after mutation of this gene in human. Such a comprehensive resource is available during gastrulation and neurulation in frog embryos (Briggs et al., 2018).

To further illustrate these points, another example can be taken from the TFAP2 family of transcription factors. Of the five paralogs of this family, four (*tfap2a*, *b*, *c* and *e*) are expressed during ectoderm and neural crest patterning. TFAP2a has been involved in non-neural ectoderm formation and in early NC induction in mouse and frog embryos (Luo, Lee, Saint-Jeannet, & Sargent, 2003; Luo, Matsuo-Takasaki, Thomas, Weeks, & Sargent, 2002; Schorle, Meier, Buchert, Jaenisch, & Mitchell, 1996). Moreover, in *Xenopus laevis* *tfap2a* displays several successive functions in the NC-GRN: TFAP2a first initiates neural border patterning in response to canonical WNT signaling, then TFAP2a collaborates with the key transcription factors Pax3 and Zic1 to specify neural crest, and TFAP2a is then necessary during NC cell EMT and migration (De Crozé, Maczkowiak, & Monsoro-Burq, 2011). TFAP2 factors act as dimers on the target DNA motifs. A key switch between TFAP2a/c and TFAP2a/b dimerization controls the onset of NC specification at the neural border in chick neurulas (Rothstein & Simoes-Costa, 2020). In zebrafish, gene redundancy between

*tfap2a* and *tfap2c* ensures robust NC induction, but their individual mutation also reveals which target genes are most sensitive to *tfap2* gene dosage (Dooley et al., 2019; Li & Cornell, 2007). In humans however, the recorded NCPs involving TFAP2 transcription factors comprise Branchiocardiofacial syndrome (TFAP2a) and Char syndrome (TFAP2b) which are found in live births, indicating that NC cells did develop in those patients, albeit with severe alterations of their derivatives, including various craniofacial and heart anomalies associated with malformation of tissues unrelated with the NC lineage (e.g., limbs).



#### **4. Congenital disorders caused by defects in neural crest EMT and migration**

Embryonic development requires dramatic morphogenetic changes, at tissue-level and cell-level during gastrulation, neurulation and organogenesis. Among the most notorious changes in tissue architecture, EMT is a process by which an epithelial cell acquires a mesenchymal phenotype, a state often accompanied by the acquisition of cell motility and the ability to migrate along specific paths of the body. The reverse process, mesenchyme-to-epithelium transition (MET) is involved during diverse tissues and organs formation, and allows migratory cells to settle into distant epithelial derivatives. For example, sequential rounds of EMT and MET control gastrulation (in chick embryos), somite formation, and craniofacial development. While EMT and MET are tightly orchestrated in the embryo, the uncontrolled reactivation of EMT in adults is exploited by cancer cells to disseminate and establish secondary tumors in distant regions of the body (Brabletz, Kalluri, Nieto, & Weinberg, 2018). EMT is also involved in tissue healing and fibrosis in adults.

At the end of neurulation (in the head) and during organogenesis (in the trunk), the NC cells emigrate from the neural border area by a stereotypical EMT with a strictly controlled spatiotemporal onset along the anterior-posterior body axis. Chick and *Xenopus* embryos have been instrumental for the identification of the molecular actors driving EMT and the precise dissection of NC EMT controls. Although much remains to be understood in this complex process, NC development provides one of the best settings to understand EMT *in vitro* and *in vivo*.

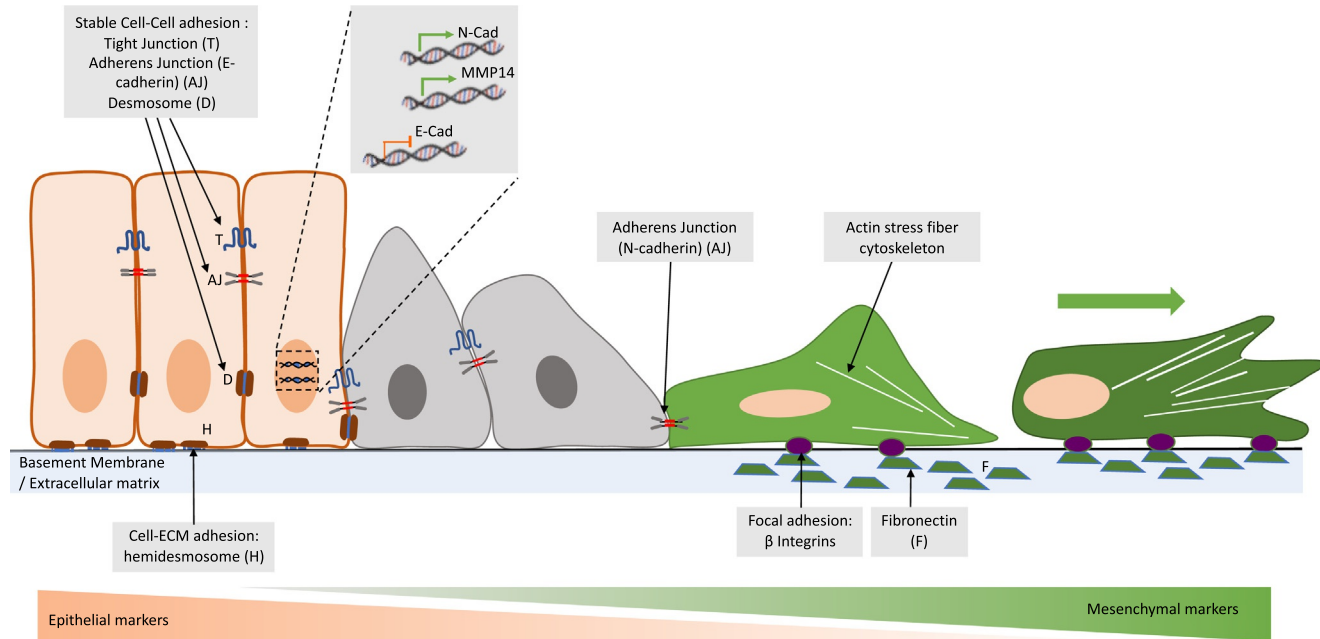
## 4.1 The epithelium-to-mesenchyme transition in *Xenopus* neural crest cells

### 4.1.1 General cellular features of EMT

As described in other vertebrates, frog premigratory NC cells initiate EMT by the loss of epithelial markers such as Cadherin 1 (*cdh1*, Ecadherin), a transmembrane adhesion molecule localized in adherens junctions of the cell basolateral membrane (Fig. 3). In parallel, NC cells gain expression of mesenchymal markers such as the cell-cell adhesion molecule Cadherin 2 (*cdh2*, N-cadherin), the intermediate filament Vimentin and the extracellular matrix adhesion molecule Fibronectin (Kalluri & Weinberg, 2009). As cells lose their E-cadherin-based complexes and their apical-basal polarity, the stable epithelial-type cytoskeleton is reorganized into the dynamic actin and microtubule system of motile cells. Further changes in the repertoire of cadherins and adhesion molecules accompany the graded steps of EMT and the onset of NC cell migration.

### 4.1.2 Direct molecular control of EMT

The cellular transdifferentiation from an epithelial to a mesenchymal phenotype is activated by a core set of transcription factors (TFs) that regulate cell-cell adhesion, cell polarity and the onset of cell migration. Collectively, EMT-inducing TFs repress the expression of the genes and the function of the proteins maintaining the epithelial shape and activate mesenchymal ones. The three main groups of EMT-TFs comprise the zinc-finger transcription factors Snail 1 and Snail 2, the basic helix-loop-helix (bHLH) factor Twist 1, the zinc-finger E-box-binding homeobox factors Zeb 1 and Zeb 2, and the Prrx 1 and Prrx 2 factors. Most of these factors have been discovered in chick and *Xenopus* embryos during the 1990s by degenerate cloning aimed at finding vertebrate orthologs of *Drosophila melanogaster* genes. Their expression in the premigratory NC immediately caught researcher's attention: *snail 1* (*snai1*, initially named *xsna*), *snail 2* (*snai2*, *xslu*), *twist 1* (*xtwi*), *prrx 1* (*prx-1*) and *zeb 1/zeb 2* (initially named *deltaef1* and *sip-1*, respectively) (Hopwood, Pluck, & Gurdon, 1989; Mayor, Morgan, & Sargent, 1995; Nieto, Bennett, Sargent, & Wilkinson, 1992; Nieto, Sargent, Wilkinson, & Cooke, 1994; Takahashi et al., 1998; Van Grunsven et al., 2006). These factors display non-redundant roles and their interactions are still not fully understood (Fazilaty et al., 2019; Stemmler, Eccles, Brabletz, & Brabletz, 2019). NC model still remains essential to identify



**Fig. 3** The epithelium-to-mesenchyme transition (EMT). When located in the neural border ectoderm prior to EMT, the neural crest cells display an epithelial phenotype. They are closely attached to one another by tight and adherens junctions, and display stable adhesion complexes with the extracellular matrix. During the process of EMT, the epithelial cells experience a series of transcriptional and post-transcriptional changes that progressively convert them into cells with a mesenchymal phenotype. Premigratory NC cells lose the expression of epithelial markers such as E-cadherin (Cdh1), encoding a transmembrane adhesion molecule localized in adherens junctions and gain the expression of mesenchymal markers, such as N-Cadherin (Cdh2) and fibronectin. An extensive remodeling of the cytoskeleton accompanies these changes in cell-cell and cell-matrix adhesion and yields to a motile cell phenotype.

novel features of EMT-TFs nowadays. For example, the cooperation between EMT-TFs *in vivo* has been studied in frog NC cells, showing that Twist 1 controls post-translational modifications of Snail1/2 proteins, thereby modulating their activity (Lander et al., 2013). Further general interactions between the EMT-TFs at different stages of premigratory and migratory NC development remain largely unknown. In addition to the transcriptional regulations directly triggered by the EMT-TFs, complex epigenetic regulations participate to the repression/activation of either epithelial or mesenchymal genes, respectively, involving polycomb repressor complexes (PRCs), histone deacetylases (HDACs), and histone lysine demethylases (KDMs) (reviewed in Tam & Weinberg, 2013). The details of these regulations, on each of the genes forming the NC-GRN remain to be understood, as are the details of the direct and indirect transcriptional regulations of the network. In sum, although tremendous progress has been done in the last 10 years to establish the EMT-GRN in development and cancer, there is still a vast horizon to be explored.

#### **4.1.3 Control of EMT-TF gene expression: Deciphering the early NC-GRN**

Snail 1 and Snail 2 are the earliest core EMT-TFs expressed during NC formation *in vivo*. Their expression is first detected in NC progenitors at the end of gastrulation (stage 11.5–12 in *Xenopus laevis*) (Mayor et al., 1995; Plouhinec et al., 2017). This means that the discovery of the upstream regulators of EMT-TFs has been conducted very efficiently in frog embryos, as they are highly suitable for experimental and genetic manipulations during gastrulation. Hence, the early NC-GRN was first drafted in 2005 using combined gain and loss of function both in *Xenopus laevis* embryos *in vivo* and in dissected animal caps grown *in vitro* (a well-established frog spheroid model of pluripotent blastula-stage stem cell ectoderm, extensively used for the last 30 years to discover new genes and new genetic regulation). This first NC-GRN displayed the epistasis relationships between the secreted BMP, FGF8 and canonical Wnt signals, the transcription factors acting at the neural border Pax3, Zic1 and Msx1, and the EMT-TF Snail 2 (Monsoro-Burq et al., 2005). In this case, the robustness of the experimental assays established in *Xenopus* has allowed the simultaneous gain or depletion of several genes *in vivo*. The short development time of this embryo has then allowed the analysis of hundreds of conditions including multiple gene combinations at different doses and different developmental time points. Combined, these unique features of the frog model were the key, used by many since, to successfully build a complex vertebrate GRN during early development

(Buitrago-Delgado, Nordin, Rao, Geary, & LaBonne, 2015; De Crozé et al., 2011; Gutkovich et al., 2010; Macrì et al., 2016; Milet et al., 2013; Scerbo & Monsoro-Burq, 2020). For a review of the current NC-GRN upstream of EMT-TFs expression with focus on the regulations identified using the frog model, please see Pla and Monsoro-Burq (2018).

#### **4.1.4 Mutation of EMT-TFs in human disease**

As noted for their upstream regulators in the neural border, Pax3 and TFAP2a, the complete loss by mutations in the core EMT-TFs would affect either gastrulation or whole NC formation. The human syndromes related to mutations in Snail 2 (Waardenburg syndrome type 2D) or Twist 1 (Saethre-Chotzen syndrome) found in live births, thus correspond to later functions of these genes for example in the differentiation NC-derived of skull bones (Saethre-Chotzen syndrome) or pigmentation phenotypes (WS2D).

#### **4.1.5 Mutations causing altered NC survival upon EMT**

An important feature of EMT is the acquisition of enhanced cell survival properties, resistance to apoptosis and to various cellular stresses. In consequence, several genes with a “housekeeping” function, in addition to be important for all cell types of the embryo, display a particularly acute phenotype when they are altered in the premigratory neural crest. This is the case in particular for factors monitoring the global cellular homeostasis, which trigger cell death when cell parameters are altered.

A first example is the AKT signaling pathway, a central hub of the cell homeostasis acting in almost all cell types. Disrupted AKT signaling was recently described to alter ectoderm patterning and NC induction (Pegoraro et al., 2015) as well as NC EMT and migration (Bahm et al., 2017; Figueiredo et al., 2017). In both cases, upstream regulators of the NC-GRN, PFKFB4 and PDGFRa, respectively, stimulated the appropriate levels of AKT signaling in premigratory NC cells. In particular, the PFKFB4/AKT signaling deficiency prevented *ncad/cdh2* activation, leading to EMT failure. Interestingly, DDX3, the ortholog of the human gene DDX3X which, when mutated, is involved in the intellectual disability and craniofacial dysmorphogenesis, was studied in *Xenopus tropicalis* (Perfetto et al., 2021). Similar to PDGFRa and PFKFB4, DDX3 is important for NC induction, by sustaining high AKT levels in NC progenitors.

Another example is the Treacher Collins syndrome (TCS), an autosomal dominant disorder causing severe craniofacial anomalies (Dixon et al., 2006). In human, TCS is caused by a mutation in TCOF1, POLR1C or

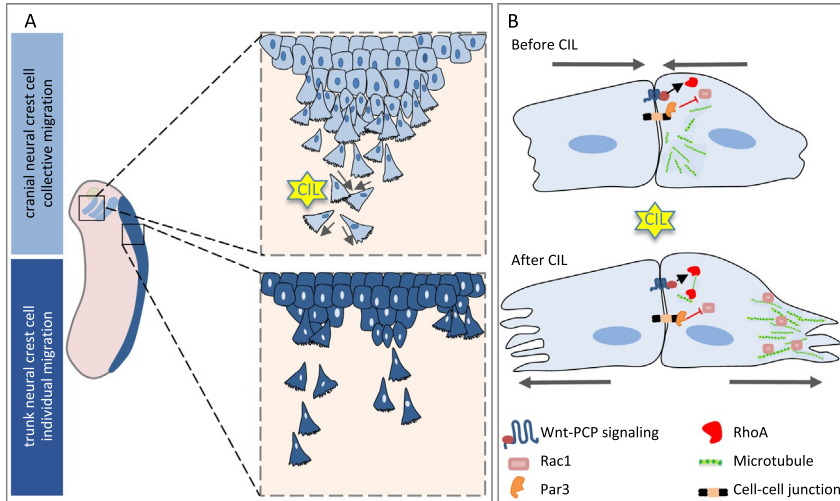


PLOR1D, encoding proteins involved in ribosome biogenesis. Altered ribosome biogenesis causes nucleolar stress and triggers apoptosis. Mouse models reproduce the human phenotype and have shown that mutant NC cells undergo extensive p53-dependent apoptosis in the neural folds upon EMT (Dixon et al., 2006). In frogs, the genes causing Treacher-Collins syndrome have not yet been studied in NC, although their function in ribosome genesis is conserved (Gonzales et al., 2005). Interestingly, the detailed analysis of 13 genes mutated in various ribosomopathies conducted in *X. tropicalis* revealed a surprising diversity of those genes expression patterns including several enriched in NC cells (Robson et al., 2016). This study suggests a fine-tuned and dynamic control of ribosome biogenesis in early NC formation that may be conserved with other vertebrate models and creates an opportunity to develop new models of ribosomopathies in frog (Griffin et al., 2015).

## 4.2 *Xenopus* neural crest cell migration

### 4.2.1 Mechanisms of cranial neural crest collective migration

In frog, cranial NC (CNC) cells are the most accessible and most studied NC subpopulation during neurulation and organogenesis, because trunk NC cell delaminate at later tailbud stages in smaller numbers (Fig. 4). During delamination, CNC cells move collectively as cohesive population and undergo a progressive dissociation into smaller cell clusters then separate as individual cells as their migration progresses further toward target tissues (Fig. 4A). The complex dynamics of CNC cell migration is controlled by several complementary mechanisms, which have been deciphered mainly using *Xenopus laevis* as a model (reviewed in Shellard & Mayor, 2019). These include individual cell-cell interactions, cell adhesion properties within a moving tissue and cell-environment interactions precisely guiding the migration. Interactions between two migrating CNC cells involve contact inhibition of locomotion (CIL) and co-attraction. Cells located in the larger sheet of migrating CNC regulate the adhesion with their neighbors in a dynamic fashion, involving LPAR2 and N-cadherin-dependent remodeling of the cell-cell junctions. Collectively, the population of migrating cells efficiently moves by creating a contractile supracellular ring of actomyosin at the rear of the cellular group creating a net forward force. Lastly, the environment presents chemical and physical cues guiding the migration, involving chemotaxis toward specific neighboring tissues (e.g., CXCR4-SDF-1 signaling with adjacent placode ectoderm) and repulsive cues such as Ephrin signaling, semaphorins, and extracellular matrix components which shape and constrain the stream of migrating CNC cells. Although



**Fig. 4** (A) Cranial (light blue) and trunk (dark blue) neural crest cell migration in *Xenopus* embryo. (A) Cranial neural crest cells migrate collectively, as a cell population with dynamic cell-cell and cell-environment relationships. On the free edge of the migrating population, forward movement is promoted by Rac1 expression in the protrusion side, and forces resulting from a supracellular contractile actomyosin ring formed at the back end of the group of cells. Active Rac1 stabilizes the microtubule cytoskeleton and therefore promotes lamellipodia formation and cell migration in the forward direction. (B) When two cells collide Rac1 activity is inhibited at the site of collision by the activation of RhoA, which is recruited by the WNT/PCP signaling pathway. After collision, Par3 is activated at the cell-cell adhesion complexes and inhibits Rac1 activity resulting into a microtubule catastrophe. This generates a new gradient of RhoA-Rac1 activity in the cell and repolarizes the cytoskeleton. The cells form new protrusions away from the contact site and migrate in opposite directions.

molecular mechanisms controlling CNC migration remain incompletely understood, tremendous advances were brought by studies using frog CNC cells migrating *in vitro* on a fibronectin substratum or using elegant microchannels approaches (Scarpa et al., 2015; Szabó et al., 2016).

As an example, CIL is the mechanism by which a cell-cell collision causes the arrest of migration, cell repolarization and a change in the direction of migration (Carmona-Fontaine et al., 2008; Scarpa et al., 2015) (Fig. 4B). Importantly, this process is also involved in metastatic dissemination and the mechanisms discovered in NC studies have highlighted several important mechanisms also activated during cancer progression. The non-canonical Wnt/PCP signaling pathway controls both CIL and the directionality of cell movements during migration (Carmona-Fontaine et al., 2008; De Calisto, Araya, Marchant, Riaz, & Mayor, 2005). Disheveled, a central

cytoplasmic element of the non-canonical Wnt/PCP signaling is recruited at the plasma membrane at the level of the cell-cell contacts. This activates the small Rho GTPase RhoA and recruits Par3, a protein associated with cell-cell adhesion complexes which promotes the collapse of the microtubule at the level of the cell-cell contacts sites through the inactivation of Rac1/TRIO interaction (Moore et al., 2013). At the opposite end of the cell, the small Rho GTPase Rac1 is active and promotes the formation of membrane protrusions and focal adhesion contacts with the extracellular matrix. Several parameters, including the E-to-N cadherin switch upon EMT and PDGFR/Akt signaling favor active Rac1 polarization in the new direction of migration (Bahm et al., 2017; Scarpa et al., 2015). In sum, by controlling the gradient of RhoA and Rac1 activity at two opposite poles of the cell, CIL leads to the dispersion of the CNC cells. In addition, Cadherin-11 initiates the formation of filopodia and lamellipodia *in vivo* (Kashef et al., 2009). Inhibiting Cadherin 11 or the processing of Cadherin 11 by the ADAM13 metalloprotease affects CIL and the directionality of migration (Abbruzzese, Becker, Kashef, & Alfandari, 2016; Becker, Mayor, & Kashef, 2013). Importantly, in chick NC, Cadherin-11 also controls p53-dependent cell death, suggesting complex roles of such adhesion molecules at the onset of NC cell emigration as well (Manohar, Camacho-Magallanes, Echeverria Jr, & Rogers, 2020). As our understanding of CNC cell migration deepens, the new molecular actors will help understanding how human mutations create NCPs caused by defective NC migration.

#### **4.2.2 Human syndromes affecting neural crest cell EMT and migration**

*In vivo* and *in vitro* analyses of NC migration are thus a strong point of studies using frog models. The consequences of altered NC migration can be detected morphologically at differentiation stage, for example at head levels by the abnormal/reduced shape of the craniofacial cartilages or at trunk levels by the altered repartition of melanoblasts/melanocyte. Two examples that benefited from studies in frog, CHARGE syndrome and 16p12.1 deletion syndrome are detailed below, but other examples can be found in Table 1.

CHARGE syndrome is a rare congenital syndrome characterized by a variable combination of multiple anomalies, mainly eye coloboma; choanal atresia; peripheral nervous system defective cranial nerves; characteristic ear anomalies (known as the major 4C's), combined with conotruncal heart defects and craniofacial anomalies. Several other syndromes share close

similarities with CHARGE, such as the KABUKI syndrome. CHARGE is caused by the heterozygous loss of function mutation in the chromodomain helicase DNA-binding CHD7, a chromatin remodeler (Bajpai et al., 2010). Chd7 frog ortholog are highly similar to the human protein (Linder, Cabot, Schwickert, & Rupp, 2004). Knockdown studies in *Xenopus* phenocopy the main features of the pathology and show that NC specification is affected (defective *sox9*, *snail2* and *twist1* expression) together with NC cell migration (Bajpai et al., 2010). Moreover, comparison with human iPS cells has helped identifying the action of CHD7 on regulatory elements controlling *sox9* and *twist1* (Bajpai et al., 2010). Putative CHD7 targets, including *ephrin* and *semaphorin* genes, were identified in a screen of mutant mouse embryos. Those targets were validated in *Xenopus* embryos *in vivo*. Semaphorin 3a (*sema3a*) expression was altered in Chd7 morphant frog embryos (Schulz et al., 2014). The functional and evolutionary conservation of the molecular mechanisms causing the CHARGE disease were validated by the phenotypic rescue of the frog morphants after co-injection with human Chd7 mRNA (Ufartes et al., 2018). Interestingly, SEMA3f is mutated in KABUKI syndrome (Schwenty-Lara et al., 2020). Collectively, these results uncover that defective CNC cell migration contributes to the pleiotropic alterations seen in CHARGE patients, and involves guidance molecules of the Semaphorin family. Moreover, other syndromes related to CHARGE have involved other causative mutations, such as in the p120 catenin gene CTNND1 (Alharatani et al., 2020). It will be interesting to explore further these candidates as targets of CDH7. Lastly, all these studies gather excellent examples of the combined use of *Xenopus* assays and mammalian models to explore the molecular mechanisms of NCPs.

Complex or multigenic syndromes can also be very effectively explored using *Xenopus* assays. For example, the hemizygous deletion of the p12.1 region of Chromosome 16 causes a series of neurodevelopmental defects, including severe craniofacial malformations. The four genes involved in this deletion encode proteins with very different biological functions, *polr3e* (encodes RNA Polymerase III; synthesis of small RNAs), *mosmo* (encodes a modulator of Smoothed; Sonic Hedgehog signaling), *uqcrc2* (encodes a component of mitochondrial respiratory chain; ATP biosynthesis), and *cdr2* (encodes an oncoprotein) were systematically studied at the different steps of the NC-GRN *in vivo* (Lasser et al., 2020). This study found different roles for each candidate genes in NC specification (*mosmo*, *polr3e*), NC motility *in vitro* (*polr3e*, *uqcrc2*, *cdr2*), NC migration (all) altogether resulting in craniofacial anomalies similar to the 16p12.1 syndrome malformations in

human. Importantly, the availability of a large number of stage-matched sibling embryos to conduct parallel analyses for the different genes has allows a direct and systematical comparison of these complex phenotypes, at several sequential steps of the NC-GRN.

### 4.3 Pathologies linked to defective differentiation of neural crest derivatives or their tumoral transformation

#### 4.3.1 Studies of neural crest differentiation in NCPs

As the development of frog NC cells in the cranial area is better understood than in the trunk, it is not surprising that few studies focus on the defects in the trunk NC derivatives related to human NCPs (Table 1). For instance, the expression of *pmp22*, the gene encoding a myelin protein causing defective Schwann myelin protein function in the Charcot-Marie-Tooth syndrome, is controlled by Pax3 and Zic1 in the frog NC-GRN during early organogenesis (Bae et al., 2014; Tae et al., 2015). The expression of other genes causing altered differentiation of glial cells, such as in the Congenital Central Hypoventilation syndrome (altered sympathetic glia) was described in frog embryos, but not with a focus on glial differentiation (*phox2a/b*, Talikka, Stefani, Brivanlou, & Zimmerman, 2004; *gdnf*, Kyuno & Jones, 2007; *ret*, Carroll, Wallingford, Seufert, & Vize, 1999; *edn3/ednr*, Square, Jandzik, Cattell, Hansen, & Medeiros, 2016). This means that some molecular tools have been developed and could be directly used in NCP studies. Moreover, studies which independently explore the developmental signaling driving the migration and the differentiation of derivatives such as melanoblasts and melanocytes or trunk sensory neurons as readouts will be valuable to further understanding NCPs (e.g., Nakano et al., 2003; Valluet et al., 2012).

However, although this remains seldom used, *Xenopus* tadpoles are fully suitable for the analysis of trunk NC derivatives formation. For example, Hirschsprung disease is caused by mutations in RET, GDNF, and several other genes, resulting in the defective colonization of the caudal parts of the digestive tract by NC progenitors of the enteric nervous system. Li and colleagues have studied the impact of retinoic acid (RA) signaling and CREB-binding protein on the differentiation of the enteric nervous system in *Xenopus laevis* tadpoles (Li et al., 2018). They show that RA signaling is important for NC induction globally and affects ENS formation in particular. Although RA signaling has been studied in-depth in many studies of early NC patterning, this analysis includes an interesting late differentiation step.

In contrast, the differentiation of the cranial skeleton has received high attention in the frog model, as size, morphology and differentiation of the cartilage elements forming the visceral skeleton are readouts easily examined in *Xenopus* tadpoles. Most alterations in the NC-GRN affecting CNC cells are tested to alter ectomesenchyme formation (e.g., Monsoro-Burq et al., 2005; Scerbo & Monsoro-Burq, 2020). Moreover, frog craniofacial morphogenesis was studied in relationship with cleft palate and other common human craniofacial malformations (reviewed in Dickinson, 2016). These studies involved for instance p120-catenin (Alharatani et al., 2020), Zic1/Zic4 transcription factors (Twigg et al., 2015) and Msx2 (Kennedy & Dickinson, 2012). While MSX2 is also involved in craniosynostosis in human, the exact molecular mechanisms involving the same gene in the two species remain to precisely be compared, since in slightly different cell contexts or different steps of development, the same transcription factor may act with different partners for a different function. Two of the genes causing DiGeorge syndrome, *tbx1* and *tbx6*, are another example where frog craniofacial cartilage biology uncovered detailed mechanistic information (Ataliotis, Ivins, Mohun, & Scambler, 2005; Tazumi et al., 2010). While the initial induction of NC appeared normal, and the NC cell migration only slightly delayed, the chondrocyte progenitors failed to maintain *sox9* gene expression and consequently did not differentiate appropriately. Thus, an important aspect of DiGeorge pathology was explored, while other NC derivatives such as cardiac NC affected in the pathology await further analysis. In sum, the analysis of craniofacial cartilage differentiation is one of the major assets of the frog model. Since recently, it includes the possibility to develop quantitative approaches in a more systematic manner with Optical Coherence Tomography (OCT), which will be invaluable for the robust statistical analyses of the frog models of human craniofacial malformations (Deniz et al., 2017).

#### **4.3.2 Studies of tumorigenic transformation of neural crest derivatives tadpoles or adult frogs**

How frog studies have advanced our understanding of NC cell migration is highlighted above. These studies display an intricate crosstalk with studies on human cancer metastasis, as the molecules involved in NC migration have later been found in cancer and *vice-versa*. The biology of matrix metalloproteases of the MMP family brings a series of such examples. MMP14 is a transmembrane protein triggering tumor cells invasion of the extracellular matrix (Sato et al., 1994). MMP14 is also important for NC migration

and EMT in cancer and organ fibrosis (Gonzalez-Molina et al., 2019; Szabova, Chrysovergis, Yamada, & Holmbeck, 2008; Xiong et al., 2017). Details of MMP14 activity, such as its control of the levels of cadherin expression, have been explored in frog (Garmon, Wittling, & Nie, 2018). Moreover, the use of transplanted NC cells into host embryos allowed to test which tissue relies on MMP14 expression or on other MMPs such as MMP2, and showed that NC cells depend on MMP14 while MMP2 is needed in the surrounding craniofacial mesenchyme (Garmon et al., 2018).

As far as tumor formation is concerned, NC derivatives form aggressive tumors such as melanoma, neuroblastoma or schwannoma. About 220 studies have used frog models as part of the analysis of mechanisms driving melanoma or neuroblastoma. For instance, BRCA-1-associated protein BAP-1 is found mutated in uveal melanoma, a pigment cell type derived from neural crest as are cutaneous melanoma cells. In several cancers, BAP-1 displays tumor suppressor functions. Partly because of its closer relationship with the human protein (*Xenopus* Bap1 protein shares 92% similarity and 71% identity with human BAP1, compared to 85% and 66%, respectively, for zebrafish), Kuznetsov and colleagues conducted a thorough study of *bap1* gene expression, regulation and function in *Xenopus* development (Kuznetsov et al., 2019). They show NC-restricted expression and identify its function as a regulator of the histone deacetylase HDAC4. They further confirm BAP1 function on HDAC4 nucleo-cytoplasmic shuttling in a human uveal melanoma cell line. Moreover, a following study from the same authors, described below, exemplifies how the frog model can both be used to decipher complex mechanisms of tumor biology, and for screening chemical therapeutic compounds (Kuznetsoff et al., 2021).

### 4.3.3 Drug screening for cancer studies

Both *Xenopus laevis* and *tropicalis* have proven excellent models for the screening of pharmacological compounds targeting various signaling pathways and developmental mechanisms. Those screens take advantage of the availability of large embryos number, of their fast external development, and of their easy manipulation in multi-well plates. Hence, pigment cell development readout was used for a screen looking for chemicals involved in melanocyte migration and differentiation (Tomlinson, Rejzek, Fidock, Field, & Wheeler, 2009), very similarly to the strategies used in zebrafish studies (White et al., 2011). Moreover, gastrulation and NC migration in *Xenopus* embryos were used to screen 100 novel chemical compounds and identify those inhibiting cell migration. This *in vivo* screening identified

nine novel molecules that inhibited cell migration without embryonic lethality. When those molecules were tested on cancer cell line invasion, two significantly suppressed tumor cell invasion in 3D-collagen/matrigel gels and reduced melanoma and glioma tumor size in nude mice. Both components disrupted microtubule assembly and caused a dose-dependent increase in apoptosis (Tanaka et al., 2016). Lastly, *Xenopus* was recently used to identify non-toxic compounds rescuing the loss of the tumor suppressor BAP-1 *in vivo*. A two-step strategy has found a HDAC inhibitor selectively rescuing BAP-1 loss in uveal melanoma (Kuznetsoff et al., 2021).



## 5. Conclusion

In this review, we have attempted to highlight several fields in which the study of *Xenopus* frogs can contribute to understand neural crest cell biology and disease. With a selected series of examples, we have illustrated how biomedical research has tremendously benefited from the in-depth analyses that can be conducted in these models, both *in vivo* and *in vitro* using a variety of assays for cell migration and differentiation. Our understanding of NCPs, such as CHARGE and Hirschsprung syndromes, but also of neurodevelopmental disorders more generally such as the Bainbridge-Ropers syndrome and of other pathologies (Blackburn et al., 2019; Lichtig et al., 2020; Singh et al., 2020) will certainly include results obtained using this animal model more and more in the near future. With increasing usage of CRISPR/Cas9-based mutagenesis in *Xenopus tropicalis* (and to a lesser extent in *Xenopus laevis*), many novel mutants and variants can be studied faster than in mammalian models and in complement to studies using human or mouse approaches (Marquez et al., 2020). Novel tools including NC-centered genomic resources (Plouhinec et al., 2017) and transgenic frog lines are developed for NC reporter genes (Alkobtawi et al., 2018; Li et al., 2019). Complex syndromes, involving several gene alterations can be explored in a systematic manner (Lasser et al., 2020; Lasser, Pratt, Monahan, Kim, & Lowery, 2019). Lastly, cancer studies are increasingly aware of the advantages of this animal model (Hardwick & Philpott, 2018; Kuznetsoff et al., 2021), opening avenues to explore the neural crest-derived tumorigenesis as well as developmental neurocristopathies.

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## Authors contribution

L. M.-C. and A. H. M.-B. wrote the manuscript and designed the original figures. Both authors contributed to the article and approved the submitted version.

## Declaration of interest

The authors declare no competing interests.

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