DOCTOR OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES

Involvement of the ABCB5 transporter in the development and progression of cutaneous melanoma

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Award date:
2019

Awarding institution:
University of Namur

UNIVERSITÉ DE NAMUR

FACULTÉ DE MÉDECINE

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Involvement of the ABCB5 transporter in the development and progression of cutaneous melanoma

Dissertation présentée par Géraldine Sana en vue de l'obtention du grade de Docteur en Sciences Biomédicales et Pharmaceutiques

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ABSTRACT

The purpose of our research was to understand the role of ABCB5 in the development and progression of melanoma, the most aggressive skin cancer, which is responsible for 65% of deaths from skin cancer. Although primary melanoma is associated with a relatively good prognosis, this latter is poor after metastases development. Metastatic melanoma is associated with a median survival time of 6 months and a 5-year survival rate of 5 to 7%. Treatment resistance remains an obstacle and mainly explains the poor clinical outcome. .

The comprehensive analysis of the *ABCB5* coding region in 640 melanoma samples identified recurrent mutations in 13.75% of the samples analysed. Mutations were located on a 3-D predicted model based on the mouse Pgp structure. Four mutations, associated with a low SIFT score and representative of the mutational pattern, were further investigated. The ATPase assays showed that these mutations resulted in a decrease in ABCB5 basal ATP hydrolysis.

The first axis of this thesis is the *in vitro* study of the involvement of the ABCB5 transporter in melanomagenesis. Overexpression of the ABCB5 mutants and the silencing of ABCB5 in melanoma cell lines led to an increase of their proliferation and migration abilities, compared with the cells overexpressing wild type ABCB5. These observations suggest that *ABCB5* is a tumor suppressor gene.

The second axis of this thesis is the investigation of the role of ABCB5 in melanoma development *in vivo*. Further analyses showed that the melanoma samples mutated in *ABCB5*, were also carrying alterations in the *NRAS* and *CDKN2a* genes (in 75 and 62,5 % of the samples analyzed, respectively). So, we performed a pilot study to assess the silencing of ABCB5 in mouse models harbouring these genetic alterations.

Lastly, we wanted to determine the molecular mechanism underlying the role of ABCB5 in melanomagenesis. To do this, we further studied its subcellular localization in melanoma cells. Using cell fractionation, preliminary data revealed an enrichment of ABCB5 in the nuclear and the microsomal fractions.

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
ADP	Adenosine diphosphate
AKT	Protein kinase B or PKB
AML	Acute Myeloid Leukemia
AP1	Adaptator Protein 1
AP3	Adaptator Protein 3
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BRAF	Member of RAF : Rapidly Accelerated Fibrosarcoma
CD	Cluster of differentiation
CDDP	Diaminedichloroplatinum II
CDK	Cyclin-dependent kinase
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
CMN	Common Acquired Melanocytic Nevi
CSD	Chronically Sun-Damaged
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CXCR6	C-X-C chemokine receptor type 6
DCT	Dopachrome Tautomerase
DHI	5,6-Dihydroxyindole
DHICA	5,6-Dihydoxyindole-2-Carboxylic
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
DTIC	Dacarbazine
E2F	E2 factor
ER receptor	Estrogen receptors
ERK	Extracellular Signal-Regulated Kinase
FDA	Food And Drug Administration
GDP	Guanosine diphosphate

GPNMB	Transmembrane glycoprotein NMB
GSH	Glutathione
GST	Glutathione-S-Transferase
GSTP1	Glutathione-S-Transferase Pi1
GTP	Guanosine triphosphate
HDM2	Human double minute 2 homolog
IFNα	Interferon-α
IL-1,6	Interleukin 1,6
IL2	Interleukin 2
LAMP	Lysosomal Membrane Proteins
LRO	Lysosome-Related Organelles
MAPK	Mitogen-Activated Protein
MART-1	Protein Melan-A
MCF7	Michigan Cancer Foundation - 7
MDR	Multidrug resistance
MEFs	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MPNST	Malignant Peripheral Nerve Tissue
mRNA	Messenger Ribonucleic acid
MRP	Multidrug Resistance-Associated Protein
MSCs	Melanoma Stem Cells
mTOR	Mechanistic target of rapamycin
MVB	Multi Vesicular Body
NBD	Nucleotide-Binding Domain
NRAS	Neuroblastoma RAS viral oncogene homolog
NSG	NOD scid gamma mouse
OCT	octamer-binding transcription factor 4
OS	Overall Survival
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein 1
PDGFR-β	Platelet-derived growth factor receptor beta

PDK1	Phosphatidylinositol 3-Dependent Kinase 1
PFS	Progression Free Survival
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
Pmel17	Premelanosome Protein 17
PTEN	Phosphatase and TENsin homolog
RILP	Rab-interacting lysosomal protein
RNA	Ribonleic acid
RPG	Radial Growth Phase
RTG	Trans-Golgi Network
SCC	Squamous Cell Carcinoma
SCID	Severe combined Immune Deficiency
SIFT	Sorting Intolerant From Tolerant
SLAC2a	Linker Protein Melanophilin
SNP	Single Nucleotid Polumorphism
SOS	Son of Sevenless
Sox 2	SRY (sex determining region Y)-box 2
TAP transporters	Transport Associated With Antigen Processing Transporters
TCGA	The Cancer Genome Atlas
TEC	Tumor Endothelial Cell
TMD	Transmembrane Domain
TNF	Tumor Necrosis Factor
TRP1	Tyrosinase-Related Protein-1
TRP2/DCT	Dopachrome Tauromerase
TYR	Tyrosinase
UV	Ultraviolet
VEGFR	vascular endothelial growth factor receptor
VPG	Vertical Growth Phase
WT	Wild type
α-MSH	Alpha-Melanocyte Stimulating Hormone

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INTRODUCTION

1 Melanoma

Melanoma is the most dangerous type of skin cancer (Lideikaitė et al. 2017). It represents 0,7% of deaths due to cancer and 1,7% of cancers newly diagnosed are cutaneous melanoma (Schadendorf et al. 2018). This malignancy is the most common in young adults between 25 and 29 years old (Gandini et al. 2011). Although melanoma represents 5% of skin cancer, it causes 65% of skin cancer deaths (Cummins 2006). It is crucial to study melanoma because its incidence and mortality are still increasing. Indeed, its incidence is projected to rise through 2030 (Gery et al. 2015). While the prognosis for patients with localized melanoma is generally good, metastatic melanoma prognosis is dismal. Melanoma is the most lethal kind of skin cancer that develops from the uncontrolled proliferation of melanocytes, which are pigment-producing cells.

1.1 The skin

The main function of the skin is the role of barrier between the “inside” and the “outside” of the organism against chemical and mechanical aggression, heat, radiation and pathogens (Baroni et al. 2012). The skin is composed of two main layers: the epidermis, an epithelial component coating on the surface, and the dermis, a dense and irregular connective tissue. These compartments are separated by the basement membrane, which provides a stabilizing and dynamic interface (Breitkreutz et al. 2009).

1.1.1 Epidermis

Epidermis is the outermost layer of the skin. It is a superficial epithelium continually renewing itself, originating from ectoderm. It is essentially composed of keratinocytes but also of melanocytes, Langerhans, Merkel and inflammatory cells (Baroni et al. 2012). Keratinocytes synthetized several structural proteins including keratin but also lipids during their maturation. During their differentiation, keratinocytes migrate toward the surface of epidermis. Their differentiation involves important changes in their structure: they become anucleated and squamous, and adopt a flattened shape (Baroni et al. 2012). They are nucleated and viable from the basal layer to the granular layer (the layers of the epidermis are explained in the next paragraph) (Proksch et al. 2008; Wickett et al. 2006). Langerhans cells are involved in the immune response of the epidermis (Cumberbatch et al. 2003) and Merkel cells play the role of touch receptor and are the main sites of mechanotransduction in the skin (Woo et al. 2015). Melanocytes are producing pigments granules in melanosomes. The pigments are transferred to keratinocytes and they protect the nucleus of epidermal cells against UV light. The role of melanocytes is fully developed on page 5.

Epidermis is composed of five layers as we can see in **Figure 1** (Gartner L. 2012; Wickett and Visscher 2006):

1. The deepest layer of epidermis, called the **basal layer** (stratum basal), which separates the epidermis (epithelium tissue) and the dermis (connective tissue). The basal layer is composed of one single layer of cylindrical cells responsible for the renewal of the epidermis. Melanocytes are located in this layer connected to numerous keratinocytes by dendrites. Merkel cells are also located in this layer, associated with afferent nerve terminals and constituting mechanoreceptors.
2. The second one is called the **spinous layer** because of the presence of desmosomes, acting as a link between keratinocytes. Langherhans cells are found in this layer and are antigen presenting cells.
3. Thirdly, the **granular layer** (stratum granulosum) is characterized by keratinocytes without nucleus and with a granular cytoplasm. These granules are called keratohyalin, which are precursors of keratin.
4. Then, the **clear layer**, only present in palms and soles, is composed of cells presenting eleidin, product of transformation of keratohyalin.

5. The outermost layer is the **cornified layer** (stratum corneum). In this layer, keratinocytes are in the final step of their differentiation: filled with keratin and without organelle and nucleus. A phenomenon of desquamation is observed in the horny layer. The cells in this layer are called “squamous cells”. This layer is a permeability barrier preventing desiccation (Madison 2003).

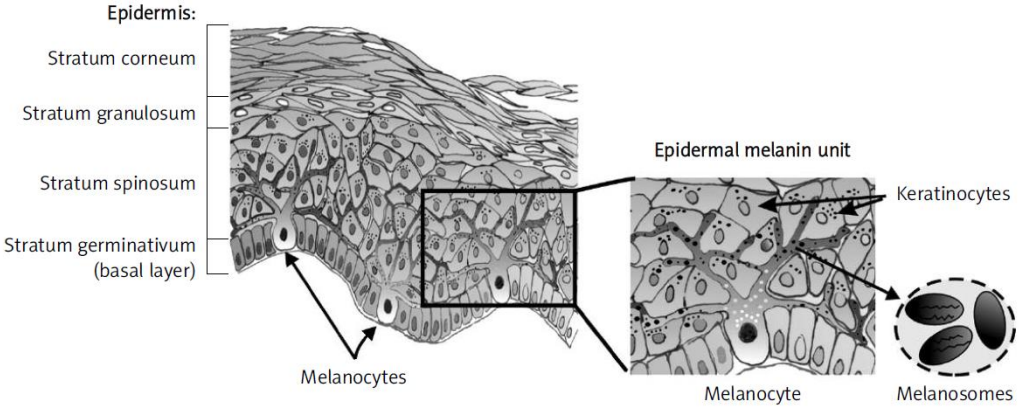

Figure 1: Layers of the epidermis. The epidermis is composed of several layers of cells: the basal, the spinous, the granular and the cornified layer. In palms and soles, there is an additional layer, called the clear layer. During their differentiation, the keratinocytes migrate toward the surface and morphological changes occur. They become anucleated and squamous and adopt a flattened shape. The melanocytes are responsible for melanin synthesis and transfer this pigment to keratinocytes by their dendrites (Mirosława Cichorek et al. 2013).

1.1.2 Dermis

The dermis is an irregular connective tissue between the epidermis and the subcutaneous tissue. The dermis is a highly vascularized connective tissue including collagen and elastic fibers. This tissue contains sweat, sebaceous glands and hair follicles (Baroni et al. 2012). It consists of two layers: the papillary dermis and the reticular layer (Gartner L. 2012). The papillary dermis is the uppermost layer containing dermal papillae. Its collagen fibers are thin and loose while the reticular layer is denser (J. Marks 2006).

1.2 Types of skin cancers and melanoma subtypes

There exists three main kinds of skin cancer: the basal carcinoma, the squamous cell carcinoma and the melanoma.

The **basal carcinoma** is the most frequent malignant tumor in the fair-skin population (Pedro et al. 2012; Peris et al. 2019). It develops from keratinocytes in the basal layer and usually appears on the sun-exposed area particularly on the head and neck. This cancer is associated with intense and intermittent UV exposure (Dessinioti et al. 2010). Generally locally invasive, its progression is slow and the occurrence of metastases is extremely rare (incidence ranging between 0,0028% and 0,55%). These metastases are lymphatic or hematogenic followed by invasion of the lungs and bones (Freitas et al. 2017).

The **squamous cell carcinoma** (SCC) is the second most frequent skin cancer (Karia et al. 2013). Developing from squamous cells and often associated with long-term UV exposure, this cancer can evolve in metastases and be lethal (Andrade 2012; Ribero et al. 2017).

Melanoma develops from melanocytes and is the most aggressive skin cancer. Most melanoma are cutaneous but there exists non-cutaneous forms as we can see in **Figure 2**. The cutaneous melanoma includes the superficial spreading melanoma, the nodular, the acral lentiginous and the lentigo malignant melanoma while the non-cutaneous melanoma can be ocular or mucosal.

The superficial spreading melanoma is the most common melanoma representing 55-65% of all melanomas (Pan et al. 2017). Its radial growth phase may last 1 to 5 years. The nodular melanoma, represents 15 to 30% of all cases, is characterized by the absence of radial growth phase, which makes its detection difficult. The acral lentiginous melanoma, representing ~5% of all cases (Ibrahim and Haluska 2009), is a rare kind of melanoma developing on the palms, soles and nails. This kind of melanoma is the most frequent in Asia and Africa. The prognosis tends to be worse than for the other melanoma subtypes (Goydos and Shoen 2016). The term lentigo melanoma was coined by Dubreuilh, describing an extensive and neoplastic skin lesion. It represents 5% of melanomas and it usually appears in sun-exposed area. This subtype of melanoma usually appears in fair-skinned population with photo damaged skin and with a greater incidence for the age of 65-80 years old (Volpini et al. 2017). This melanoma sub-type is typically located on the head and neck. The precursor lesion can be present for several years before the initiation of the vertical growth (Ibrahim and Haluska 2009).

The large majority of ocular melanoma originate from the uvea (80%), while conjunctival melanoma are less frequent. The ocular melanoma are usually primary, however cutaneous melanoma may metastasize in the ocular area (Volpini et al. 2017). The mucosal melanoma is rare and known to be associated with a poor prognosis (Tyrrell and Payne 2018).

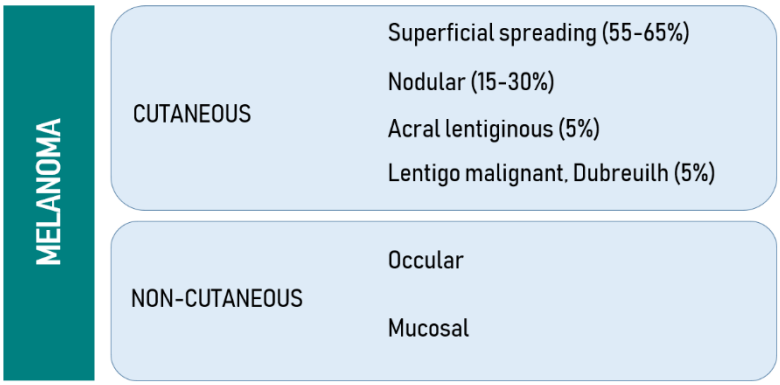

Figure 2: Subtypes of melanoma. There are different subtypes of cutaneous melanoma. The superficial spreading melanoma is the most common (55-65%) characterized by a radial growth phase. The nodular melanoma begins directly with a vertical growth phase. The acral lentiginous form, developing on palms and soles is rare and typical of Asian and African population. The lentigo malignant melanoma usually appears in sun-exposed and shows a peak incidence at the age of 65-80 years old. Melanoma can also be non-cutaneous including the ocular (from the uvea or the conjunctive) and the mucosal subtype.

1.3 Melanocytes

As previously mentioned, the melanocytes are localized in the epidermis but they are also found in hair follicle. They are responsible for melanin synthesis, a pigment responsible for the color of the skin and hair. In the skin, melanin protects keratinocytes against UV-induced DNA damage (as it is further developed page 24). They are also known to play a role in the immune system by antigen presentation to competent immune cell and by phagocytosis of invading pathogens. They also produce cytokines like IL-1β, IL6 and TNF-α as well as chemokines. These molecules alert macrophages and keratinocytes (Gasque and Jaffar-Bandjee 2015).

However, Melanocytes are also found in other organs as it is shown in the **Figure 3** : the cochlea, the inner ear, the leptomeninges, the substantia nigra and the locus coerulus of the brain, the adipose tissue, the iris, the mucosa and in the heart (Brito et al. 2008; Eichberg et al. 2019; Mahanty et al. 2017; Levin et al. 2009; Robert et al. 2015; Yajima et al. 2008; Zecca L. et al. 2003; Steel and Barkway 2008; Brenner and Hearing 2009). While Brenner and colleagues summarized the role of melanocytes in the different organs (Brenner and Hearing 2009), this manuscript focus on the cutaneous melanocytes.

All body melanocytes, except the retinal ones, derive from neural crest. The precursors of melanocytes, called melanoblast, colonize the epidermis and the hair follicle and give rise to several cellular populations including the melanocytes. The neural crest gives wide variety of other cell types like neurons, glial cells, medullary secretory cells, smooth muscle cells, and bone and cartilage cells (Kawakami and Fisher 2011; Vandamme and Berx 2019).

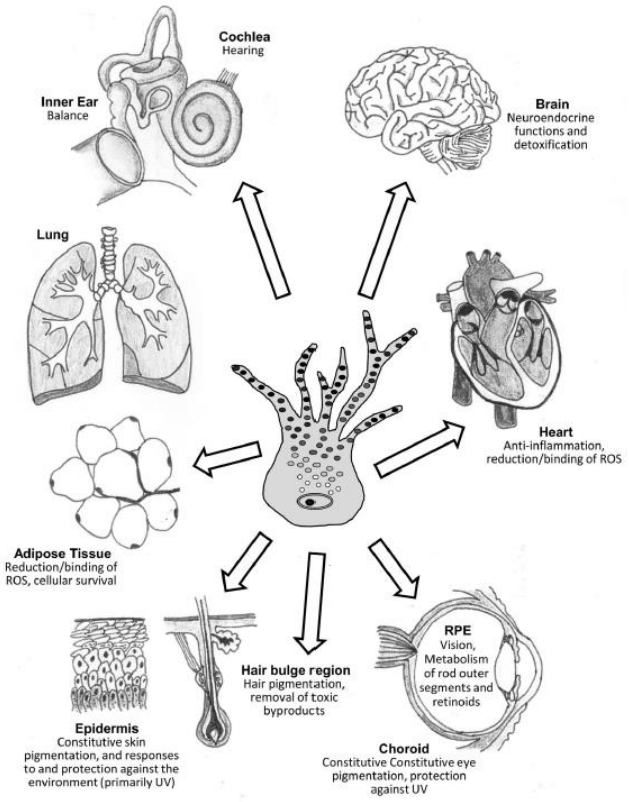

Figure 3: Distribution of melanocytes in human tissues. The location of melanocytes is not limited to the skin but melanocytes are also found in the cochlea, the inner ear, the brain, the heart, the choroid, in adipose tissue and the lung (Brenner and Hearing 2009).

1.3.1 Melanogenesis

Melanin synthesis is called melanogenesis and takes place in specialized organelles called melanosomes. This pigment protect the skin from DNA damages, which may occur from ultraviolet radiations. The skin color is not associated with the number of melanocytes but with variation in terms of the number and size of melanosomes (Gasque and Jaffar-Bandjee 2015). The gradation of the skin color is due to the amount and nature of the melanin. In human, this latter is composed of biopolymers of eumelanin (brown and black) and pheomelanin (light brown, yellow or red). Both were discovered by Prota in 1980 (Prota 1980). Following melanosomal biogenesis, which will be introduced in the next paragraph, melanosomes are transferred to keratinocytes and ensure the pigmentation and the photoprotection of epidermis (Delevoye et al. 2011).

Melanosomes are classified as lysosome-related organelles (LRO). Their size varies between 0,5 to 1µm of diameter (Tadokoro and Takahashi 2017; Wasmeier et al. 2008). They share common features with lysosomes such as the presence of identical proteins, like the lysosomal membrane proteins (LAMP-1, -2 and -3) and the hydrolases, and the internal acid environment. Lower pH is a condition for melanin synthesis (Raposo et al. 2001; Raposo and Marks 2007).

In their review, Yamaguchi and Hearing identified three kinds of proteins implicated in melanin production, explained in the next paragraph (Yuji Yamaguchi and Hearing 2009, 2014). Those are:

- The enzymes involved in the synthesis of melanin:
  The three main transmembrane enzymes implicated in melanogenesis are the tyrosinase (TYR), the tyrosinase-related protein 1 (TIRP1) and the dopachrome tauromerase (TRP2/DCT).
- Structural proteins of melanosomes:
  The protein like premelanosome protein 17 (Pmel17, also called gp100), the protein melan-A (MART-1), and GPNMB give scaffold material to enzymes allowing melanin deposition.
- Proteins implicated in the traffic of melanosome proteins or in the distribution of melanosomes microtubules such as F-actin, kinesin, Rab27a, melanophilin, myosin Va, RILP, etc.

1.3.1.1 Biogenesis of melanosomes and transfer to keratinocytes

The biogenesis of melanosomes includes four stages of maturation. During these steps, the melanosomes become denser with a structure matrix containing melanin deposit. After their maturation, melanosomes go to keratinocytes by the dendrites as explained page 11 (Delevoye et al. 2011; Wasmeier et al. 2008; Yuji Yamaguchi and Hearing 2014).

At stage I, premelanosomes are non-pigmented endosomes, common to the endocytic pathway as we can see in **Figure 5** (Delevoye et al. 2011). Pmel17 is present in the membrane of premelanosomes and in the internal vesicles as we can see in **Figure 4** (Raposo and Marks 2007; Wasmeier et al. 2008). This glycoprotein is composed of two subunits: Mα and Mβ. These ones are cleaved by a propotein convertase (of furin family), present in the lumen of melanosomes. The protein MART1 is also abundant in this stage and is required for the maturation of Pmel17 (Yuji Yamaguchi and Hearing 2014). The segregation between the endocytic pathway and the biogenesis of melanosomes occurs when premelanosomes adopt an elliptical shape, characteristic of the stage II. This particular shape is due to amyloid fibers formed by the polymerization of Mα subunits. These fibers are parallels (G. Raposo and S. Marks 2007) and will be the support for melanin fixation in further stages (Fowler et al. 2006).

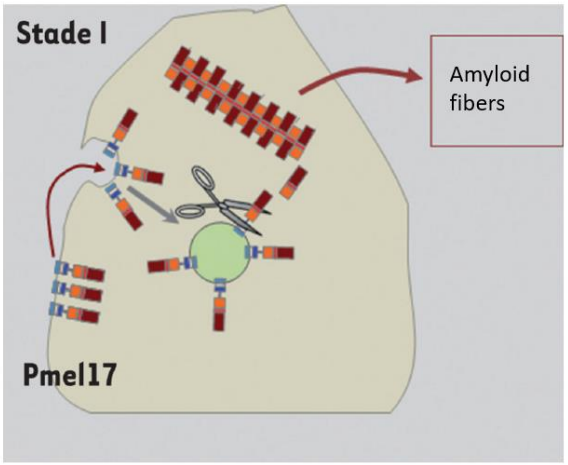

Figure 4 : Stage II of melanosome biogenesis. Pmel 17 is present in the membrane of melanosome and in intervesicle (represented in green). The protein is composed of two subunits (Mα and Mβ) which are separated by a proprotein convertase. The polymerization of Mα subunits give amyloid fibers, which will be the support for melanin fixation (Delevoye 2011).

The enzymes implicated in melanogenesis (TYR, TIRP1, TRP2) arrive in the melanosomes at stage II. The protein AP3 (adaptator protein 3) interacts with tyrosinase and is involved in its transport to melanosome while the protein AP1 (adaptator protein 1) interacts with tyrosinase and TRP1 (Delevoye et al. 2011).

The melanogenesis and melanin fixation on fibers occur at stage III. From this stage, we can actually speak about melanosomes. At stage IV, the melanosomes are completely opaque and considered as mature (Raposo and Marks 2007; Wasmeier et al. 2008). The melanogenesis pathway is described at page 11.

The protein GPNMB also plays a role in the biogenesis of melanosomes. Indeed, the silencing of this protein leads to a decrease in terms of melanosome formation (P. Zhang et al. 2012). This protein is similar to Pmel17 and is present at all melanosome stages, but is particularly enriched in late stage of melanosome biogenesis (Chi et al. 2006; Hoashi et al. 2010).

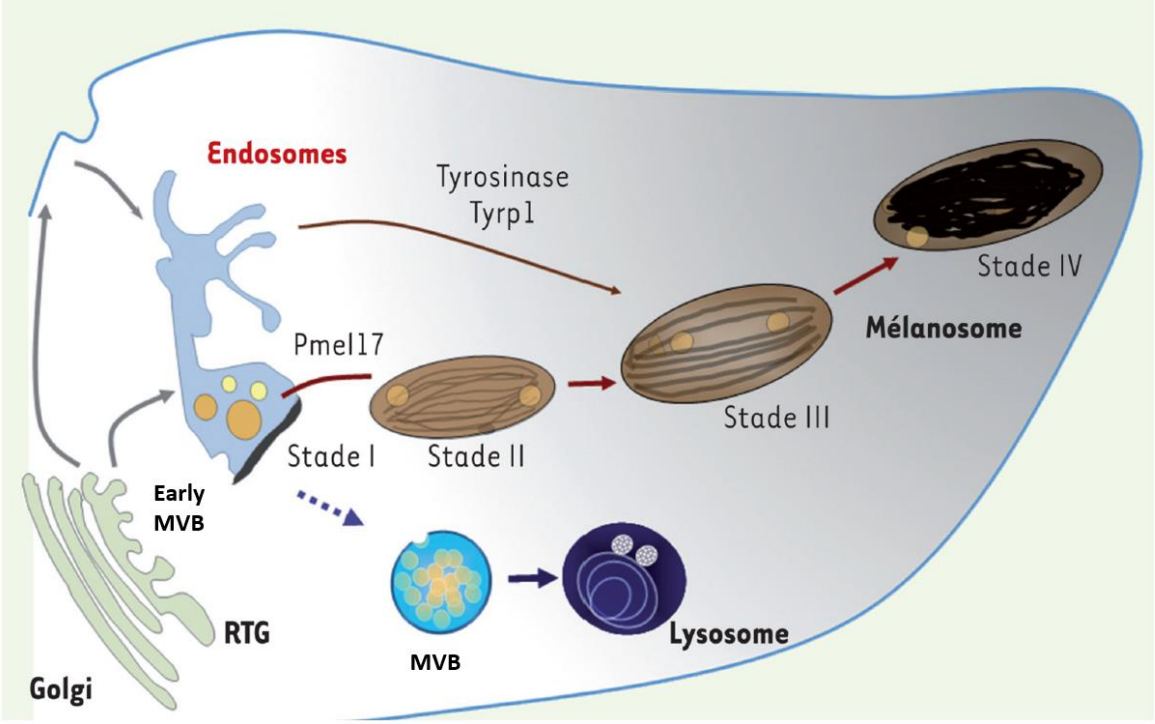

Figure 5 : Biogenesis of melanosomes. Melanosomes come from endocytic pathway. At stage I, the premelanosome is an endosome with internal vesicles containing Pmel17. The segregation between the endocytic pathway and the biogenesis of melanosomes occurs at this stage II, when premelanosome adopt an elliptical shape. Myeloid fibers are formed and are the support for melanin synthesis, which occurs at stage III and IV. The mature melanosomes are completely opaque. MVB: Multi Vesicular Body; RTG: Trans-Golgi network (Delevoye 2011).

After melanosomes biogenesis, these organelles are transferred to the end of dendrites by two phenomena:

1. The binding of the melanosome on kinesin, which will transport the melanosome in the dendrite by moving on a microtubule.
2. At the end of the dendrite, the melanosome will detach from the microtubule and fix on an actin filament under the plasma membrane. This is thought to be mediated by a complex consisting of myosin Va, the GTPase RAB27A and the linker protein melanophilin (also known as SLAC2a) (Raposo and Marks 2007; Wasmeier et al. 2008).

Then, the melanosomes can be transferred from one melanocyte to around 36 keratinocytes. The mechanism underlying this transfer is still under debate. Several models were proposed to explain the transfer of melanosomes to keratinocytes as we can see in **Figure 6**. The first one is the cytophagocytosis model suggesting that the keratinocytes phagocyte the dendrite tips filled with mature melanosomes. Secondly, the fusion of cellular membranes of melanocytes and keratinocytes was proposed. The third model implies the shedding of plasma membrane enclosing melanosomes that are phagocytosed by keratinocytes while the fourth one implies the exocytosis by melanocytes of the melanosome melanin core followed by the phagocytosis of the "melanocore" by keratinocytes (Wu and Hammer 2014). Today, the mechanisms remain to be fully understood. Tarafder and colleagues have shown the presence of "naked" melanin (melanin non-enveloped by a membrane) in the extracellular environment and inside keratinocytes (Tarafder et al. 2014).

Once in the keratinocytes, melanosomes spread around the nucleus to protect keratinocytes against DNA damages.

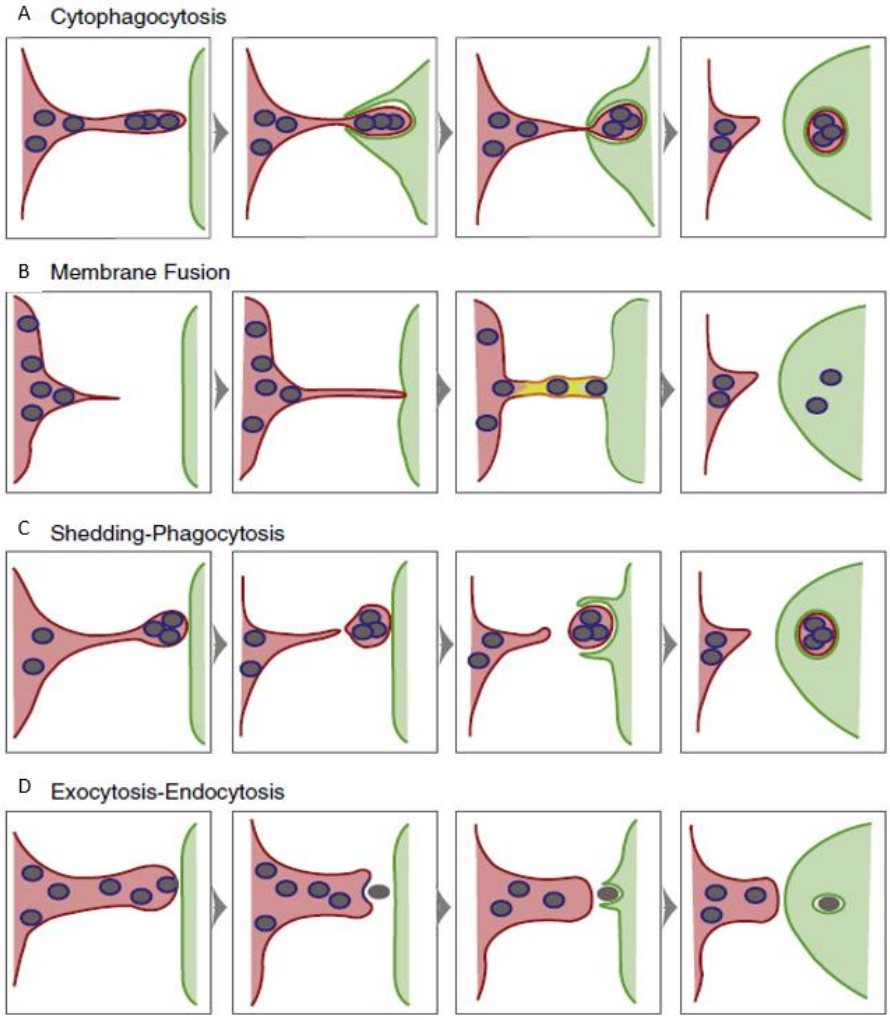

Figure 6: Transfer of the melanosomes from the melanocytes to keratinocytes. Four models were proposed. A. The first one is the cytophagocytosis model: the keratinocytes phagocyte the dendrite tips filled with mature melanosomes. B: The fusion of cellular membranes of the melanocyte and the keratinocyte, represented in yellow, would allow the cellular membranes to form a "duct" allowing the passage of melanosomes. C: The shedding of plasma membrane enclosing melanosome followed by the phagocytosis by keratinocytes. D: the exocytosis by melanocytes the melanocore and its phagocytosis by keratinocytes. The plasma membrane of the melanocyte is in red, the membrane of melanosome in blue and the plasma membrane of keratinocyte in green (X. Wu and Hammer 2014).

1.3.1.2 Melanogenesis

Both eumelanin (polymer of 5,6-dihydroxyindole (DHI) and 5,6-dihydoxyindole-2-carboxylic (DHICA)) and pheomelanin are produced from the amino acid L-Tyrosine. The pathway begins with the oxidation, catalyzed by the tyrosinase, of the L-Tyrosine in L-3,4-dihydroxyphenylalanine (DOPA), rapidly converted in DOPAquinone (**Figure 7**).

- If cysteine is available, it will react with DOPAquinone and produce either 3-S-cysteinyldopa or 5-S-cysteinyldopa. These molecules will undergo oxidation and polymerization, and give **pheomelanin** (Hennessy et al. 2005).
- On the other hand, if there is no cysteine available in the melanosome, the dopaquinone will undergo an intramolecular cyclisation, to form an orange intermediate called dopachrome. This one can be either decarboxylated or tautomerized, giving the two kinds of eumelanin. The decarboxylation is spontaneous and will form 5,6-dihydroxyindole (DHI). The Oxidation by TRP1 and TYR and polymerization of DHI will give the **DHI-melanin**, which is dark brown or black and has a high molecular weight. On the other hand, if the dopachrome tautomerase (DCT) enzyme is available, dopachrome does not lose its carboxylic acid but undergoes a tautomerization to give 5,6-dihydoxyindole-2-carboxylic, called **DHICA-melanin**. This second kind of eumelanin is lighter than DHI-melanin, is brown and has an intermediate size and solubility (Hearing et al. 2005; Pillaiyar et al. 2017).

The intermediates of melanin have toxic effects on the cells. The melanosome has a role of protection against those intermediates; it allows the melanogenesis to take place without having toxic effects for the cell (K. G. Chen et al. 2009; Hearing 2005).

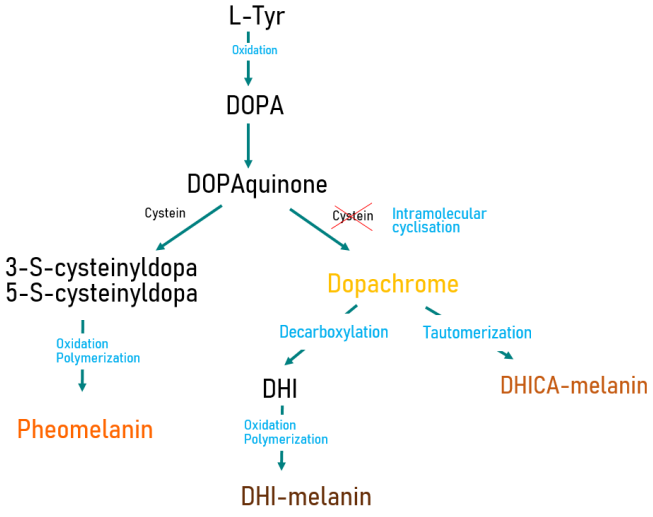

Figure 7: Melanogenesis pathway. The synthesis of both types of melanin begins with the oxidation of the amino acid L-Tyr in L-3,4-dihydroxyphenylalanine (DOPA), rapidly converted in DOPAquinone. This latter can react with cysteine, which gives 3-S-cysteinyldopa or 5-S-cysteinyldopa. These molecules will give pheomelanin after oxidation and polymerization. However, if cysteine is not available, the dopaquinone will give dopachrome, which can undergo either decarboxylation or tautomerization, respectively giving DHI-melanin and DHICA-melanin.

At least 25 genes regulate the pigmentation pathways and the function of melanosomes (Pillaiyar et al. 2017). External factors influence melanogenesis like UVB radiation, the pH, drugs but also several endogenous factors like the presence of molecules in the environment of melanocytes and keratinocytes (Hachiya et al. 2001; Videira et al. 2013). Keratinocytes are involved in the regulation of melanogenesis. After UV exposure, they express Alpha-Melanocyte Stimulating Hormone (α-MSH) and Adrenocorticotropic hormone (ACTH). These hormones bind on receptors MC1R at the cellular surface of melanocytes. This leads in the expression of MITF, which is phosphorylated by ERK1/2 and stimulates the transcription of Tyr, DCT and TRP1 (Miller et al. 2006).

1.4 Nevi

The nevi are the result of melanocytes proliferation. The majority of them do not lead to melanoma.

1.4.1 Benign nevi

The benign nevus (common acquired melanocytic nevi CMN) is a very common neoplasm in Caucasian population. They can have different histological features depending on the distribution in the epidermis and the dermis. The melanocytes of the CMN present particular features by comparison of normal melanocytes. At first, their shape is different: they are non-dendritic, rounds and compact. Secondly, they are organized in cluster and nest. Then, they have the particularity of retaining pigment and can sometimes migrate to the superficial dermis. This last feature is unusual for a benign tumor because these cells present invasion ability without architectural (circumscription and symmetry) or cytological (mitoses, maturation, uniform pigmentation) malignant features (Colebatch and Scolyer 2018). They arise very early in the life and tend to disappear after six decades (Shain and Bastian 2016).

1.4.2 Dysplastic nevi

While cancer is caused by an accumulation of genomic alteration, the dysplastic nevus represents an intermediate step between the benign nevi and melanoma (Melamed et al. 2017). Indeed, this lesion present a mixture of benign and malignant features. It can appear from a pre-existing nevus or on a new site. The architectural disorder as well as the cytological atypia and the dermal inflammatory response is often observed. The appearance of the dysplastic nevi share features with melanoma like irregular borders and the asymmetric distribution of the pigmentation (Duffy and Grossman 2013).

The definition of a dysplastic nevus is still controversial (Colebatch and Scolyer 2018; Marks 2006) The dysplastic nevi have a wider spectrum of mutations than the benign nevi (Melamed et al. 2017; Shain et al. 2015).

The diagnosis of melanocytic lesion is difficult. One raison is the sharing of histological features between the benign melanocytic nevi and melanoma.

1.5 Melanomagenesis

1.5.1 Two models explaining melanomagenesis

Two models have been proposed to explain the melanomagenesis: a more classical, which is the linear model and a melanoma stem cells model. According to the linear model, mutations occur in mature melanocytes leading to melanomagenesis, while the second model describes a tumoral sub-population with higher proliferative capacities.

1.5.1.1 The linear model

Melanoma has been classically perceived like a stepwise progression. In this linear process, the mature melanocytes subsequently acquire somatic mutations in oncogenes and tumor suppressor genes. These mutations, associated with survival and growth advantages for the cells, lead to the progression from the benign nevi to melanoma following a gradual process of several steps (Zabierowski and Herlyn 2008). Norris was the first scientist to link the nevus and melanoma in 1857. One century later, the stepwise model describing the evolution of the melanocyte to the metastatic melanoma was proposed by Clark (Clark et al. 1984). This model includes five steps presented in **Figure 7** (Colebatch and Scolyer 2018; Takata et al. 2009; Zabierowski and Herlyn 2008) :

1) The benign nevus is usually associated with *BRAF*^{V600E} mutations.
2) This benign nevus can evolve to a dysplastic nevus, characterized by cytological atypia and the acquisition of additional mutations. Mutations in *CDKN2a* and *PTEN* genes are frequent.
3) A small proportion of dysplastic nevi leads to the radial growth phase (RPG).
4) The vertical growth phase is associated with a poor prognosis and includes the invasion of the dermis. The transition to VPG is associated with the invasive phenotype, a key factor in melanoma progression.
5) The melanoma cells are then able to invade distant organs and metastases appear.

It is important to emphasize that most nevi will not lead to melanoma progression. Moreover, all the intermediate steps are not necessary to lead to melanoma (Colebatch and Scolyer 2018). The precise sequence of genetic alterations leading to melanomagenesis is not complete.

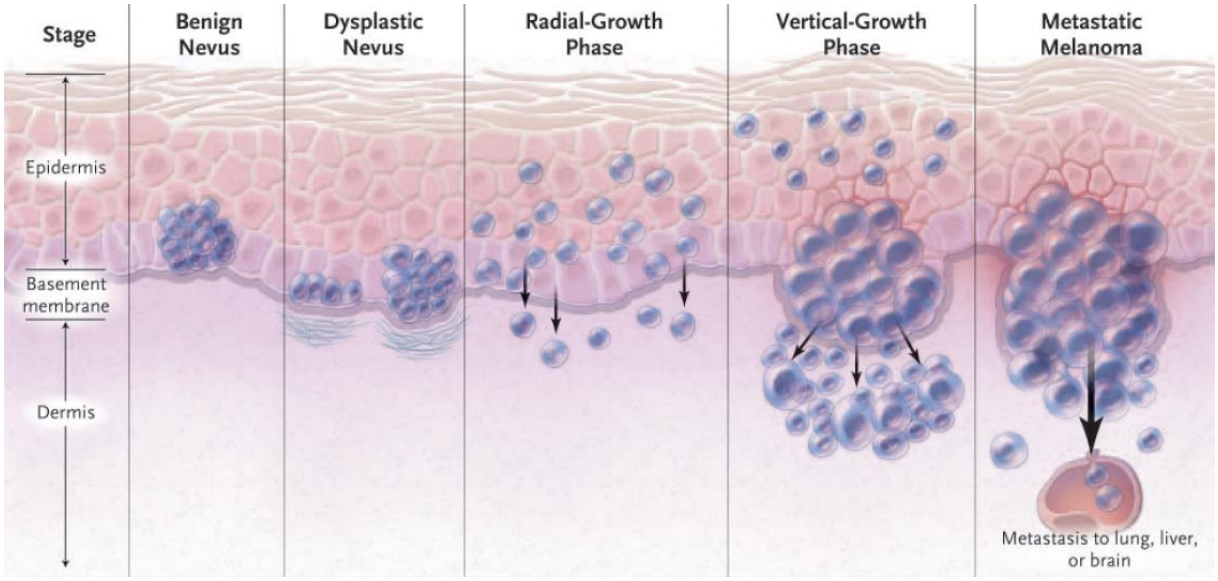

Figure 8: Linear progression of melanoma. The linear model of melanomagenesis describes several subsequent phases associated with the acquisition of mutations. The benign nevi is usually associated with $BRAF^{V600E}$ mutation and the following phase may occur with the acquisition of additional mutations. The nevus can become dysplastic. A small proportion of these nevi will follow the phase leading to melanoma, which is a radial growth phase (RPG) followed by a vertical growth phase (VPG). This VPG is associated with the acquisition of the invasive ability allowing the melanoma cells to invade distant organs (Miller et al. 2006).

1.5.1.2 Model based on the "melanoma initiating cells"

The melanoma stem cell model was proposed following the discovery of the tumoral heterogeneity. Within the tumor, there exists different gene expression profiles, which are correlated with the invasive capacity of the cell (Bittner and Meltzer 2000). Most melanoma (70%) evolve from non-appearing nevi and thus, would not follow the linear model of melanoma development (Bevona et al. 2003; Lin et al. 2015; Zabierowski and Herlyn 2008).

Studies describe a group of cells with stem cells properties even if the term "stem cells" is controversial. The term "tumor initiating cells" is sometimes preferred. The cancer stem cells is a tumoral subpopulation showing stem cell properties: the ability of self-renewal and of generating differentiated progeny. Cancer initiating cells have the ability to form tumospheres, a high invasive behavior, the ability to generate a heterogenous tumor when injected in nude mice and carry a specific set of biomarkers (Nagare et al. 2016). Firstly identified in acute myeloid leukemia (AML), they were also identified in different solid tumors such as in breast, brain, lung and colon cancers (Al-Hajj et al. 2003; Kim et al. 2005; Bonnet and Dick 1997; O'Brien et al. 2007; Piccirillo et al. 2006).

The cancer stem cells were also identified in melanoma. Fang and colleagues identified a tumoral subpopulation with stem cells properties (Fang et al. 2005). The existence of melanoma stem cells (MSCs) was confirmed by many studies (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Civenni et al. 2011; Kupas et al. 2011; Linley et al. 2012; Schmidt et al. 2011; Schatton et al. 2008). MSCs have the characteristic to form melanospheres when they are cultured in suspension and to give rise to a heterogeneous tumor after inoculation in nude mice (Nagare et al. 2016). Several MSC markers have been identified, among which we may cite CD133, CD34, CD20, CD271, Sox2, Oct3/4, CXCR6, etc. (Civenni et al. 2011; Fang et al. 2005; Klein et al. 2007; Held et al. 2010; Monzani 2007; Perego et al. 2010; Taghizadeh et al. 2010). According to this model, only melanoma stem cells are tumorigenic and are at the origin of the whole tumor.

1.5.2 Pathway involved in melanomagenesis

The transformation of the healthy melanocytes to melanoma cells is accomplished by the activation of oncogenes and the inactivation of tumor suppressor genes. The activation of oncogenes leads to a gain of function of the protein (Vicente-Dueñas et al. 2013). Such mutations are dominant. On the other hand, a genetic aberration in tumor suppressor genes may lead to a loss of function. Tumor suppressor genes can decrease cellular proliferation, promote apoptosis, be involved in checkpoint responses, in the detection and repair of DNA damages or in cell differentiation and migration (see review see C. J. Sherr 2004). The loss of function of a tumor suppressor gene can lead to cancer. However, these kinds of mutations are recessive and so must be present in both alleles to further observe the inactivation of the tumor suppressor gene (Nelson and Tsao 2009).

In melanoma, the main mutated oncogenes and tumor suppressor genes are *BRAF, NRAS* and *CDKN2a*, *PTEN*, respectively. The $BRAF^{V600E}$ mutation is found in 50-70% of melanomas, while *NRas* is mutated in 15 to 20% of melanomas (Garnett and Marais 2004; Jakob et al. 2013; Muñoz-Couselo et al. 2017). Since 10% of melanoma are familial, they show mutations in the *CDKN2a* gene in 5-20% of cases (Helgadottir et al. 2015). More explanation about familial melanomas are provided on page 26. *PTEN* disruption provokes the activation of PI3K kinas/AKT (Chin et al. 2006; Flaherty et al. 2012) and is mutated in 10-30% of melanomas. (Chin L. et al. 2006; Goel et al. 2006).

1.5.2.1 The tumor suppressor gene CDKN2A impacts the retinoblastoma and p53 pathways

CDKN2A (cyclin dependent kinase inhibitor 2A) is a tumor suppressor gene impacting the Rb and p53 pathways. Heritable deletion and mutations in the gene *Ink4a/ARF* represent the strongest genetic risk of melanoma (Nelson and Tsao 2009).

The gene *CDKN2A* produce two independent transcripts through the alternate splicing of a separate exon 1. The $p16^{INK4}$ is transcribed from the exon 1α, exons 2 and 3, while $p14^{ARF}$ is transcribed from alternate splicing of the exon 1β with exons 2 and 3. These transcripts are translated from different reading frame and give for two proteins : $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ in the case of mice). This explained that their amino acid sequence is completely different (Gray-Schopfer et al. 2007)(Nelson and Tsao 2009).

Both $p16^{INK4a}$ and $p14^{ARF}$ act as tumor suppressor genes by regulating the cell cycle. They provoke the arrest of the cell cycle or the apoptosis in various contexts including for instance DNA damages, mutations in oncogenes or old cells. These two proteins are implicated in the mechanism of senescence protecting against the apparition of cancer (Gray-Schopfer et al. 2006; V. Gray-Schopfer et al. 2007; Sharpless and Chin 2003). However, their way of action are completely different and explained in the next paragraph. The protein $p16^{INK4a}$ is implicated in the pathway of the retinoblastoma protein, while $p14^{ARF}$ is involved in the pathway of p53 as we can see in **Figure 9**.

The protein $p16^{INK4a}$ affects the cell cycle between the G1 and S phase. It binds to and blocks the catalytic activity the complex CDK4-CDK6 complex already formed. Thus, this latter is unable to phosphorylate the Rb protein, which remains unphosphorylated and active. It binds on the transcription factor E2F leading to its inactivation provoking the arrest of the cell cycle through the blockage of the S phase (Lilischkis et al. 1996; Sherr 2001). If p16 is mutated, it is not able to inhibit CDK4-CDK6, which phosphorylates the Rb protein. This inactive Rb does not bind on E2F. This one will induce S-phase genes. This can lead to melanomagenesis if it is combined with other factors (Nelson and Tsao 2009).

The protein $p14^{ARF}$ has an impact on the cell cycle through its interaction with p53. To do so, $p14^{ARF}$ binds and inhibits to the HDM2, which is an ubiquitin protein ligase promoting the degradation of p53 in proteasome (Bothner et al. 2001). So, indirectly, $p14^{ARF}$ prevents the degradation of p53 (Nelson and Tsao 2009). If $p14^{ARF}$ is inactive, HDM2 remains active leading to p53 degradation.

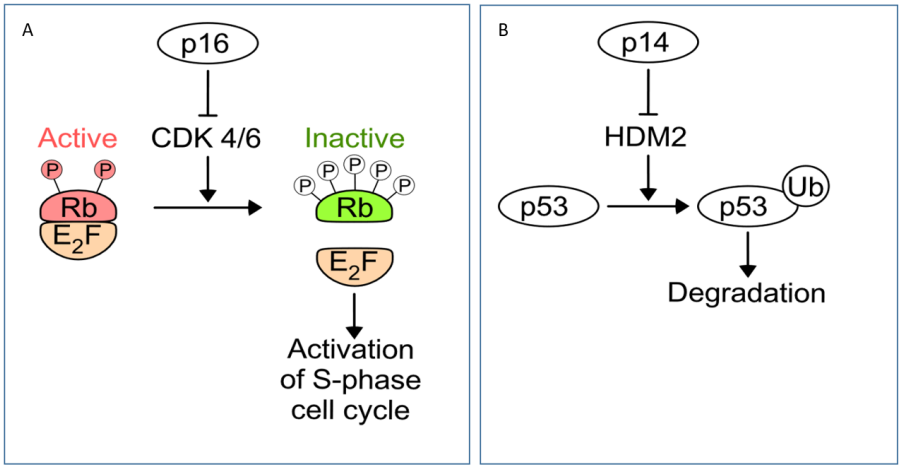

Figure 9: The gene *CDKN2a* encodes two independent protein p14ARF and p16Ink4a affecting different pathways. A. The protein p16Ink4a inhibits the complex CDK4/6, so this latter is not able to phosphorylate Rb, remaining active. It binds E2F and prevent it to activate the cell cycle. B. The protein p14ARF inhibits HDM2, which is involved in the degradation of p53.

1.5.2.2 PTEN and NRAS impact the PI3K/Akt/mTOR pathway

The PI3K pathway is also called AKT or mTOR pathway and is involved in the regulation of the cellular proliferation. It is often upregulated in melanoma resulting from activating mutations in *NRAS* gene (Giehl 2005) or inactivating mutation in *PTEN* gene (Chin L. et al. 2006; Goel et al. 2006). This pathway is summarized in **Figure 10**. Briefly, this pathway consists of the conversion of Phosphatidylinositol (4,5)-bisphosphate (PIP2) in phosphatidylinositol (3,4,5)-trisphosphate (PIP3) by PI3K. This later activates AKT, subsequently promoting cell growth and proliferation. NRAS stimulates PI3K while PTEN converts PIP3 in PIP2 by dephosphorylation. Its action, indirectly, inhibits cellular proliferation and promotes apoptosis (Chin et al. 2006; Dreyer et al. 2009).

With more details, the signaling pathway starts with binding a ligand on a tyrosine kinase receptor. This ligand can be a growth factor (such as IGF, PDGF, EGF) or a cytokine. The tyrosine kinase receptor is composed of three domains: an extracellular, a transmembrane and an intracellular domain. The binding of a ligand induces the dimerization of two receptors and the activation of their intracellular domains. This corresponds to the activation of the tyrosine kinase domain by autophosphorylation. PI3K binds on the intracellular part of the receptor (by its regulatory subunit p85) leading to its activation. PI3K is a heterodimer composed of a regulatory subunit (p85) and a catalytic subunit (p110). It can also be activated by NRas or inhibited by PTEN (Dreyer et al. 2009). Once activated, PI3K converts Phosphatidylinositol (4,5)-bisphosphate (PIP2) in phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This latter PIP3 recruits AKT near plasma membrane. This allows PDK1 (phosphatidylinositol 3-dependent kinase 1) to access and phosphorylate AKT. AKT, once activated, has many targets, it can phosphorylate other proteins such as BAD or GSK3. One of its downstream target is mTOR. By this way, it stimulates cellular proliferation or inhibits cell apoptosis (Hemmings and Restuccia 2012).

1.5.2.3 The oncogenes RAS and RAF family proteins are implicated in MAPK-signaling pathway

The upregulation of the Mitogen-activated protein MAPK pathway (**Figure 10**) is often observed in melanoma, mainly caused by *BRAF*^{V600E} or *NRAS* mutations. This pathway is also called ERK pathway (extracellular signal-regulated kinase).

The signal starts with the binding of a ligand such as an extracellular growth factor (FGF, SCF, HGF, etc.) to a tyrosine kinase receptor. This leads to the dimerization of the receptor, which triggers the intrinsic tyrosine-kinase activity. This is followed by the autophosphorylation of the intracellular protein kinase domain. These domains bind to a protein containing a SH2 domain (for example GRB2). This complex recruits the cytosolic SOS protein (son of sevenless). This protein binds a small GTPase, member of the RAS family (NRAS, HRAS or KRAS) and induce a conformational change in RAS leading to its activation (dissociation of the GDP and binding of GTP). The GTP-bound RAS is active and initiates signaling cascade of phosphorylation. RAS phosphorylates one member of the RAF family protein (c-RAF1, BRAF and ARAF), this one phosphorylating MEK, which subsequently phosphorylates and activates the MAPKs ERK1 and ERK2. This is called the MAPK cascade and provokes the translocation of ERK into the nucleus and the phosphorylation of various substrates implicated in the regulation of the proliferation, the differentiation and the cell survival (Chin 2003; Gray-Schopfer et al. 2007; Regad 2013).

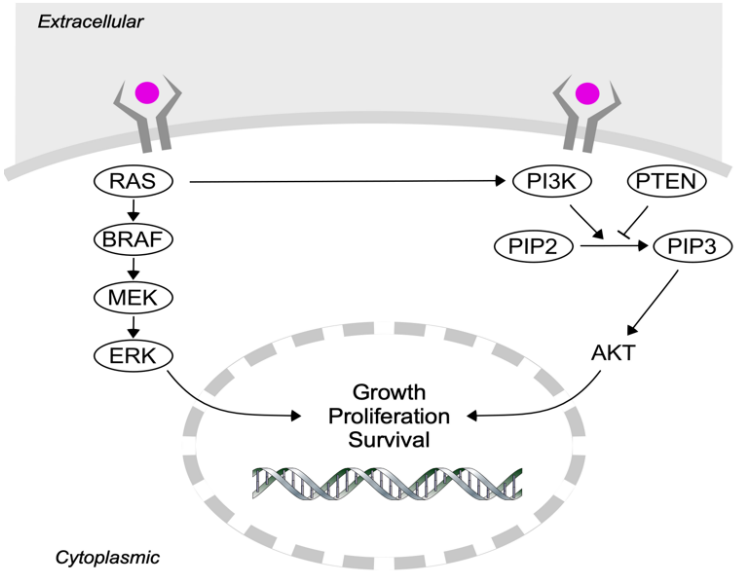

Figure 10: The MAPK and PI3K/Akt pathways. The MAPK pathway (left side) is often upregulated in melanoma mainly caused by mutation in *BRAF* and *NRAS* oncognes. This pathway begins with the binding of a ligand on a tyrosine kinase receptor leading to the activation of RAS, which initiates a cascade of phosphorylation BRAF-MEK-ERK. This one is translocated in the nucleus, where it phosphorylates various substrates stimulating growth, proliferation and cellular survival. The PI3K pathway (right side) is also uregulated in melanoma. This pathway also begins with the binding of a ligand on the receptor of Tyrosine kinase leading to the activation of PI3K. This one is responsible of the conversion of PIP2 in PIP3. This later activates AKT, subsequently promoting cell growth and proliferation. Activating mutations of NRAS stimulates PI3K while the loss of function of the tumor suppressor PTEN converts PIP3 in PIP2.

1.6. Epidemiology

1.6.1. Incidence

In fair-skinned population, melanoma incidence is increasing for decades both in men and women, as shown in **Figure 11** (F. Erdmann et al. 2013). Incidence has increased of 3% between 1982 and 2011 in countries such as Sweden, Norway, Australia, New Zealand, United Kingdom and in the caucasian population of the United States. Thanks to primary prevention, melanoma incidence is already decreasing in Australia since 2005 (Whiteman et al. 2016). This is due to the success of prevention campaigns that began in the 1980's.

Schadendorf and colleagues summarized the incidence in the different continents based on the data of Globocan 2012. The incidence is the highest in New Zealand with a number of 38,5 per 100.000 persons per year and reaches 34,9 in Australia, 13,8 in North America, 10,2 in Europe and 0,2 in South-East Asia (Schadendorf et al. 2018).

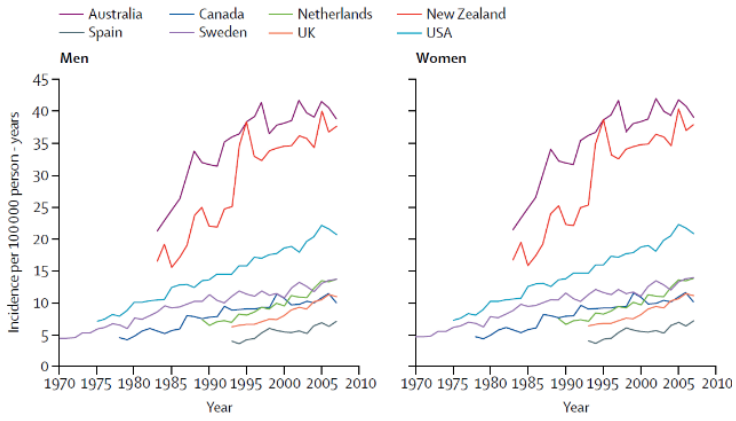

Figure 11: Incidence of melanoma in fair-skinned population between 1970 and 2007. Y-axis is the number of cases per 100.000 persons and per year age standardized. The incidence of melanoma is increasing in many countries, and is higher in New Zealand and Australia (Schadendorf et al. 2018).

1.6.2. Mortality

Schadendorf summarized Globocan data for mortality and indicates that the highest mortality is observed in New-Zealand with 4,7 deaths per 100 000 persons and per year (data age standardized), while this number reaches 1,5 in Europe and 4 in Australia (Schadendorf et al. 2018).

The survival is lower in black population developing melanoma on non-sun exposed sites. This is explained by the late diagnosis, the deeper tumor and more advanced stage of melanoma (Mahendraraj et al. 2017; Wu et al. 2011; Myles et al. 2012).

1.6.3. Risk factors

The main risk factor of melanoma is the UV exposure, the phenotypic characteristic, the presence of melanocytic and dysplastic nevi as well as their body site. The genetic predisposition also plays a role in the probability of melanoma development. A difference according to sex and age is also observed.

1.6.3.1. UV exposure

The UV exposure is the main risk factor of melanoma because the UV radiation can induce mutation in DNA (Pons et al. 2008). There are three kinds of UV radiations, knowing UV-A, UV-B and UV-C with different radiation emission spectrum. Their absorption by the ozone layer and their penetration in the skin are different. Indeed, as we can see on **Figure 12**, the UV-C are absorbed by atmospheric ozone. Thus, the UV sunlight is mainly composed of UVA (90-95%) and UVB (5-10%). Furthermore, UVA penetrate deeply in the skin and reach the dermis, while UVB are mainly absorbed by the epidermis (D'Orazio et al. 2013; Watson et al. 2016) .

The main mutation induced by UV is the replacing of C by T (C→T) at dipyrimidine site. The direct effect of UV is the formation of cyclobutane pyrimidine dimers and pyrimidine-pyrimidone adducts (Alexandrov et al. 2013; Berger et al. 2012; Shain and Bastian 2016; Sullivan and D. E. 2014). These mutations are called UV signatures. These mutations are caused by the direct mutagenic effect of UVA and UVB (Berger et al. 2012; Hodis,et al. 2012). The interaction of UVA and melanin is responsible for the production of free radicals. This indirectly provokes mutations and genetic aberration (Noonan et al. 2012; Hennessy et al. 2005).

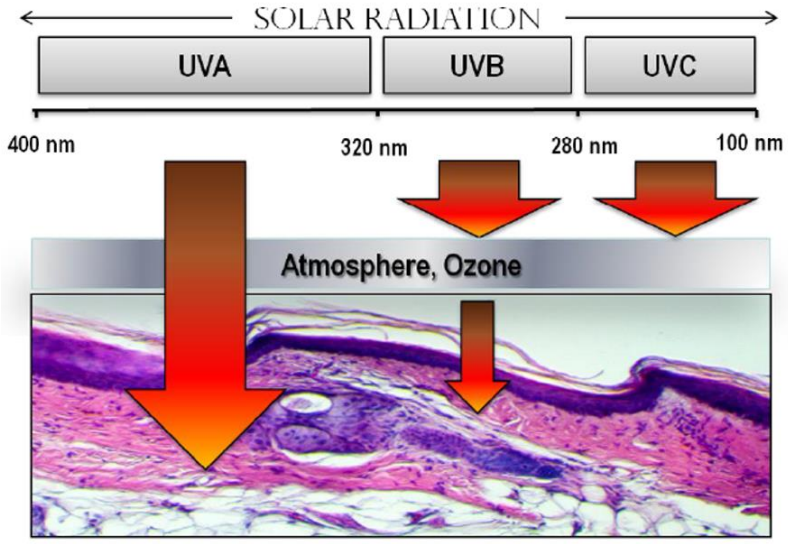

Figure 12: Penetration of the skin of the different types of UV radiations. The UVC are mostly absorbed by the ozone layer and atmosphere and do not represent a danger. By contrast, both UVA and UVB are dangerous for humans. The UVA penetrate deeply in the skin reaching the dermis, whereas UVB are mainly absorbed by the epidermis (D'Orazio et al. 2013).

The apparition of a sunburn in child increases the risk of melanoma development (Cummins 2006; Pons et al. 2008). There is a link between UV exposure in early life and melanoma development, particularly for melanoma mutated $BRAF^{V600E}$ (Thomas et al. 2007).

The impact of the sun exposure is different according the number of nevi. Persons with few nevi require repeated sun exposure to develop melanoma, preferentially on highly exposed area like the face or the neck. The persons presenting many nevi will preferentially develop melanoma on the trunk with a minimal UV radiation (Whiteman et al. 2003). The criteria of the number of nevi is developed on page 26.

We can distinguish two kinds of melanoma based on the sun exposure duration (long-term vs intermittent): the chronically sun-damaged (CSD) melanoma and the non-CSD melanoma. The CSD melanoma appears on a skin presenting microscopic and macroscopic signs of long-term UV exposure called *solar elastosis* (accumulation of abnormal elastin in the dermis). This melanoma usually appeared on the head and neck in patients older than 55 years old. By contrast the non-CSD melanoma is caused by an intermittent sun exposure, appears on a skin without solar elastosis and usually in patients younger than 55 years old (Shain and Bastian 2016).

The use of indoor tanning is clearly associated with the increased of the melanoma risk as well as carcinoma. The risk grows with the number of sunbed session and the age of the person who begins to use it (Boniol et al. 2012). Risk increases with the number of nevi, the presence of atypical nevi and lentigines. Restriction for the use of sunbeds related to age and the type of skin have been put in place in some countries of Europe (Suppa et al. 2019).

1.6.3.2. Pigmentation characteristics

The higher incidence rate of melanoma in Caucasian population is partly due to the lack of UV protection caused by reduced amount of melanin or by the kind of melanin synthetized. Indeed, **Figure 13** shows higher incidence in North America, Europe and Australia. Populations with pigmented skins have a higher protection against UVA and UVB radiations. In a darker skin, UVB radiation passing through the epidermis decrease of 50% by comparison with fairer-skin persons (Brenner M. 2009).

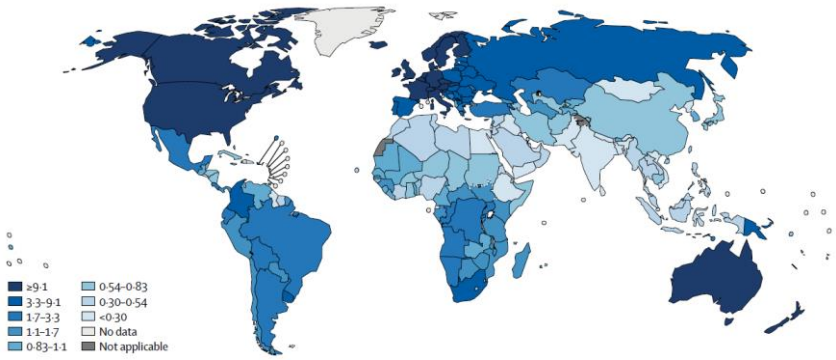

Figure 13: Worldwide melanoma age-standardized annual incidence rate by geography in 2012 (in number of cases per 100 000 persons and per year). The distribution of melanoma around the world shows the importance of of phototype. Indeed, the incidence is higher among the faire-skinned population.

In 1975, Fitzpatrick and colleagues created a numerical classification of human skin color and gave information on the UV sensitivity. It classes six different phototypes based on the color of the skin, the eyes and the hair, the presence of freckles and the reaction of the skin to sun exposure (Fitzpatrick 1975). Sitek and colleagues showed that models based on spectrophotometric variables, describing pigmentation levels skin color parameters, are a more precise predictor than phototype (Sitek et al., 2016).

1.6.3.3. Number of nevi and their body site

The number of nevi and the presence of dysplastic nevi are established risk factors for long time (Chang et al. 2009; Gandini et al. 2005; Olsen et al. 2009).

A person with more than 100 nevi has 7,6-fold risk of melanoma than a person with around ten nevi (Cummins 2006). A high number of nevi is associated with a higher melanoma risk. These persons will preferentially develop melanoma on an intermittently sun exposed body sites (Caini et al. 2009). This goes in the same direction that the hypothesis suggesting that melanomagenesis takes place following two different events : one due to intermittent sun exposure and one due to a long-term one (Whiteman et al. 2003). Classifying persons according to their number of nevi requires to take into account other risk factors like the country of residence, the body site, the gender, etc. (Cust et al. 2019). A high nevus count is an indicator of risk melanoma death, particularly in men (Li et al. 2019).

1.6.3.4.Genetic predisposition

Around 5 to 10% of melanomas are familial (Gandini et al. 2005). A positive family history means that minimum three persons in the first degree have been diagnosed with primary melanoma. However, this definition varies according to the studies. A positive family history doubles the probability to develop melanoma (Chen et al. 2014; Hawkes et al. 2016). In area with low-medium incidence of melanoma, the families with two cases of melanoma or with an individual, which has developed two primary melanomas, may benefit of genetic testing. In high incidence area, it is the case if three melanoma were diagnosed in the first or second degree, or if three primary melanoma develop among one individual (Badenas et al. 2012). *CDKN2a* and *CDK4* are identified as the first familial susceptibility gene identified. Mutation in the *CDKN2a* gene is associated with a high penetrance. The impact of alterations in this gene was already developed on the page 19. Patients harboring mutations in *CDKN2a* have a high risk of developing melanoma and other internal cancer, particularly in pancreas (Soura et al. 2016). Considering all melanoma, the incidence of germlines mutation in *CDKN2a* ranges from 0,2% to 5%. This percentage is higher in familial melanoma. *CDKN2a* is mutated in 10% of patients while it is mutated in 30-40% in families with 3 more melanoma cases (Goldstein et al. 2007). Mutations in p16 are generally missense mutations distributed on throughout the length of the protein. Mutations affecting p14ARF are insertion, splice mutations or the deletion of the entire gene (Aoude et al. 2015).

So far, mutations in *CDK4* were reported in few familial melanoma, all mutations occurring in codon 24 are supposed to be a mutational hotspot. This shows the importance of arginine, normally localized at this position, in the binding of p16 (Aoude et al. 2015).

1.6.3.5.Gender

The incidence of melanoma in adolescent and young adults is higher for women than men. The use of the indoor tanning by females is more frequent and was suggested to be the main cause of the difference observed between genders. This trend reverses after the age of 40 years old (Watson et al. 2016; Weir et al. 2011).

1.6.3.6.Age

Melanoma in children under ten years old are very rare but it increases dramatically at the time of adolescence and adulthood (Indini et al. 2018). In the high-risk populations (Australia, New-Zealand and Northern Europe), the trend is an increase with the age, with a peak at 70 and 80 years old (Coory et al. 2006)(Sneyd and Cox 2013).

1.7. Treatments

When melanoma is diagnosed at an early stage, the prognosis is good and melanoma is treatable with surgical resection and with IFNα-2b (Achkar T. and Tarhini A.A. 2017). However, melanoma is an aggressive cancer, the tumor tends to metastasize and the prognosis is bad at advanced stage of the disease. When the melanoma spread to regional lymph node, the five-year survival rate is of 29% (Wu and Singh 2011) and patients presenting metastases have a median survival time of 6 months and a 5-year survival rate of 5-7% (Gray-Schopfer et al. 2007; Wu and Singh 2011). The resistance to treatment represents one of the main cause of poor outcomes associated with metastatic melanoma. This high level of resistance pushed scientists to seek newer efficient treatments. There exists a range of therapeutic options like chemotherapy, immune- and targeted therapies which are developed just below in the **Figure 14**.

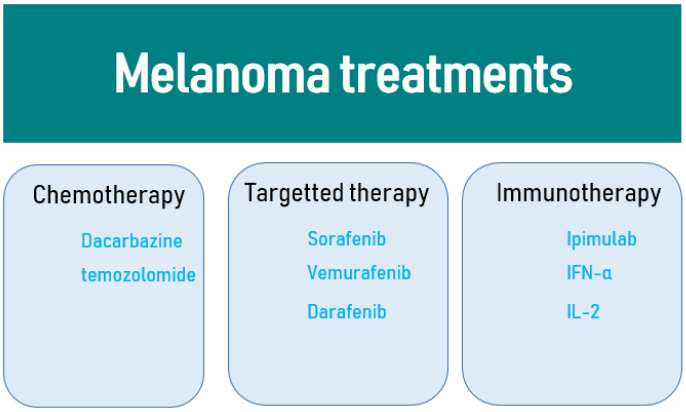

Figure 14: Melanoma treatment. Main melanoma treatment can be classified in chemotherapy, including the treatment with dacarbazine and temozolomide, immunotherapy and therapies targeting proteins or pathways. Adapted from Mishra et al. 2018.

1.7.1. Chemotherapy

Among chemotherapy, dacarbazine and temozolomide are used to treat melanoma. Dacarbazine (DTIC) was approved by the FDA in 1975 but studies show that this chemotherapeutic agent alone provides poor overall survival benefits, for review see Bhatia and colleagues (Bhatia et al. 2009). Despite its inefficacy, DTIC remains, in combination with other anti-cancer agents, the standard treatment for metastatic melanoma (Mishra et al. 2018). The temozolomide is an analogue of DTIC presenting the advantage of being able to penetrate in the CNS (Bhatia et al. 2009; Velho 2012). Studies comparing the response of these treatments conclude that there is no significant difference in terms of response to treatment (Mishra et al. 2018). Both chemotherapeutic agents are commonly used for palliative treatment. The temozolomide is well tolerated and allows to improve the quality of life of patients (Li et al. 2015).

1.7.2. Targeted therapy

The targeted therapy aim to target specifically cancer cells by blocking the action of certain protein or other molecules involved in cellular growth and proliferation. We previously developed the MAPK pathway, whose upregulation leads to melanomagenesis (page 20).

1.7.2.1.Inhibitors of BRAFV600E

Sorafenib was first developed to inhibit RAF-1, wild-type BRAF, BRAF ^{V600E} but in vitro results show that this small molecule is a multikinase inhibitor. Indeed, it targets receptor tyrosine kinases associated with tumor angiogenesis (VEGFR-2, VEGFR-3, PDGFR-β) and tumour progression (c-KIT, FLT-3) (Eisen et al. 2006). It was not associated with any benefit neither alone, nor in combined therapy (the combined therapy are developed on page 31) (Eisen et al. 2006; Mandalà and Voit 2013).

Vemurafenib is the first BRAF inhibitor which complete the phase I of clinical trial showing a clinical benefits (Mandalà and Voit 2013). However, this response is limited for patients carrying the *BRAF*^{V600E} mutation (representing 50-70% of patients with melanoma). No response was observed in patients wild type for BRAF. The phase II and III studies on vemurafenib shows its clinical superiority over dacarbazine (Mishra et al. 2018).

The dabrafenib is more potent than vemurafenib with a higher selectivity of the BRAFV600E mutant cells (Mandalà and Voit 2013) presenting the advantage to be efficient for brain metastases (G.S. Falchook et al. 2012). The phase III studies show a similar response rate than vemurafenib (Menzies and Long 2014).

While a significant increase of progression free survival (PFS) and overall survival (OS) compared with chemotherapy was observed in patients with metastatic melanoma and harboring V600E *BRAF* mutation, resistance occurs in half of the patients within 6-7 months (Hauschild et al. 2013; Sondak and Flaherty 2011). According the National Cancer institute dictionary, The PFS is defined as “the length of time during and after the treatment that a patient lives with the disease but it does not get worse”. The OS is “the length of time from either the date of diagnosis or the start of treatment for a disease that patients diagnosed with the disease are still alive”.

1.7.2.2. Inhibitors of MEK

MEK inhibitors are able to decrease the growth and to induce the death of melanoma cells mutated for *BRAF* and *NRAS* (Grimaldi et al. 2017).

Trametinib is a MEK inhibitor specifically inhibiting MEK1 and MEK2. The clinical outcome of trametinib were better than chemotherapy with an increase in terms of overall survival (OS) and progression free survival (PFS) (Gilmartin et al. 2011; Flaherty et al. 2011).

1.7.2.3. Combination of BRAF and MEK inhibitors

Several studies show the clinical benefits associated with the combination dabrafenib and trametinib (Liu et al. 2015; Queiroloa et al. 2015; Smalley and Sondak 2015). A recent meta-analysis confirmed that the combined inhibition of both BRAF and MEK increases the OS and the PFS (Chen et al. 2017). However, this is accompanied by important adverse events (Chen et al. 2017; Liu et al. 2017).

1.7.3. Immunotherapy

Immunotherapy is often used as adjuvant in addition to the surgery for melanoma of stage II and III (Aragwala S. and O'Day Steven 2011). The immunotherapy includes the use of IFNα, IL2, ipimulab, ticilimumab, nivolumab and pembrolizumab.

Ipimulab is a monoclonal antibody blocking CTLA4. It is administrated as a second line treatment and the studies in phase I/II/III show a higher median overall survival and a higher response rate (Hodi et al. 2010; Robert et al. 2011) while the ticilimumab does not confer any benefits (Franklin et al. 2017). Nivolumab and pembrolizumab are also approved by FDA and target programmed cell death protein 1(PD-1) receptors. CTLA-4 and PD1 are negative regulators of the immune system, they lead to an increased activation of the immune system (Buchbinder and Desai 2016; Erdei and Torres 2010).

The interferon-α (IFNα) are cytokines and its use was approved by FDA since 1986 as an adjuvant therapy for melanoma treatment (Erdei and Torres 2010). They have anti-proliferative, differentiation inducing, pro-apoptotic and anti-angiogenic properties (Tarhini et al. 2012). IFNα was approved by FDA in 2011 for high risk patients affected by melanoma stage II and III (Franco et al. 2017). A meta-analysis showed a reduction of 12% of the risk of death associated with the use of IFNα as an adjuvant (Rodríguez-Cerdeira et al. 2017).

The interleukin 2 (IL2), a lymphokine increasing the proliferation of T-cell, is approved for the treatment of metastatic melanoma since 1998 for metastatic melanoma. IL2 stimulates T-cell function and proliferation. The activated T-cells provokes a specific immune response (Erdei and Torres 2010; Velho 2012).

In monotherapy, the cytokines are associated with severe dose limited toxicity (Conlon et al. 2019).

1.7.4. Combined therapy

Since dacarbazine is not efficient in monotherapy, it can be combined with different compounds such as cisplatin, nitrosoureas and tubular toxins (Mishra et al. 2018; Velho 2012).

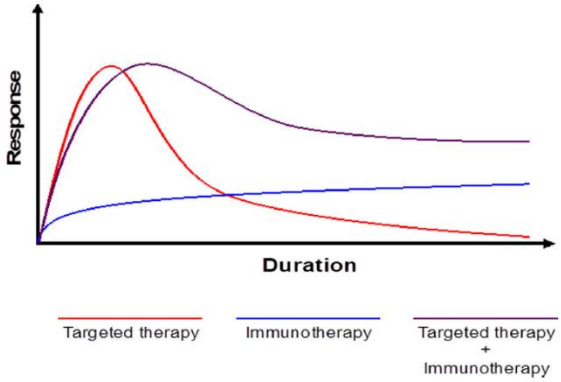

Figure 15: Combined therapy. The combination of immunotherapy and combined therapy allows to achieve a higher and more durable response (Prieto et al. 2016).

Treatments with immunotherapy are associated with a lower response rate, but their impact on the disease is more durable (Prieto et al. 2012). This suggests the interest in combining immunotherapy and targeted therapy to obtain a high and durable response as we can see in the **Figure 15** (Prieto et al. 2016). Clinical trials are underway to determine the clinical benefits of the combined therapy of BRAF-1 and IFNα by comparison with monotherapy (Rodríguez-Cerdeira et al. 2017).

Despite the advance in melanoma treatment, the prognosis of metastatic melanoma is poor and resistance to anti-cancer agent remains an obstacle. The resistance can be intrinsic (if the cancer is initially resistant to the drug) or acquired (if the resistance appeared within continuing therapy). The acquired resistance can be limited to the drug to which the patient was exposed or can concern multiple drugs with different mechanism of action (MDR) (Gottesman et al. 2016).

Several mechanisms lead to resistant to treatment like the efflux of the drug outside of the cell, the sequestration of the drug in subcellular organelles, a reduced drug uptake, altered cell cycle arrest, increased ability of DNA repair, dysregulation of apoptotic pathway,etc. (Gillet and Gottesman 2010; Gottesman et al. 2015; Kalal et al. 2017; Wu and Singh 2011). The efflux of the drug by ABC transporters is recognized as a main cause of chemotherapy failure due to multidrug resistance (Li et al. 2016).

2. ABC transporters

Expressed in all living organisms including eukaryotes and prokaryotes, the ABC transporters are a large superfamily of integral membrane proteins, transporting very diverse substrates across the membranes. They use the energy provided by ATP hydrolysis (Dean and Annilo 2005). It is the largest family of transmembrane proteins, containing 48 members in humans, classified in seven distinct families (from A to G). This classification is based on the gene structure, the organization of typical domains of ABC transporters (see page 34) as well as the sequence of these domains. The genes of ABC transporters are very dispersed in eukaryotic genome and are highly conserved between species (Dean et al. 2001).

ABCB1 was the first identified ABC transporter and is now the best-characterized one. Juliano and Ling reported the expression of a 170kDa protein expressed in hamster ovary cells resistant to colchicine (Juliano and Ling 1976). This gene was first cloned in 1985-1986 (Gros et al. 1986; Riordan et al. 1985) while Chen and colleagues provide information about the complete primary structure suggesting its role in active transport (Chen et al. 1986). Its pump activity was revealed by Horio and Gottesman in 1988 (Horio et al. 1988). Then, the second discovered was ABCC1 in 1992 (SP Cole et al.1992) and then ABCG2 was reported by three groups (Allikmets et al. 1998; Doyle et al. 1998; Miyake et al. 1999)

These latter ABC transporters were extensively studied for their role in cancer resistance. ABCB1 is involved in resistance to many anticancer agents like cisplatin, methotrexate, doxorubicin, etoposide, etc. (Chao et al. 2019; Jiang et al. 2019). It is also the case of ABCC1 transporting for example anthracycylines, vinca alkaloids, methotrexate, antifolate and etoposide (Deeley and Cole 2006). ABCG2 transport methotrexate, mitoxantrone, topotecan, irinotecan and flavopiridol (Mao and Unadkat 2015). So far, at least 15 ABC transporters were found to be involved in drug resistance (W. Li et al. 2016). In this manuscript, we will focus on the involvement of ABC transporters in melanoma resistance (see page 45) as well as their involvement in melanomagenesis (see page 52).

2.1. Structure

2.1.1. Topology

The topology of a typical full-sized ABC transporter consists of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs), which are further explained in the next section (**Figure 16A**). The transporter ABCB1 (MDR1) is an example of typical ABC transporter (Glavinas et al. 2004; Kryczka et al. 2018) Some members of ABCC family (ABC-C1 ,C2, C3, C6, C10) contain an additional TMD consisting of five alpha helices (**Figure 16B**) (Gillet et al. 2007). There also exists half transporters consisting of one NBD and one TMD. In the typical topology of half transporters, the NBD is located at the C-terminal side (for example ABC-B2,B3,B6 to B10 and ABCD1 to D4 (Gillet et al. 2007) (**Figure 16C**). However, there also exists a reverse topology of half transporter where the NBD is at the N-terminal side. It is the case of ABCG transporters (**Figure 16D**).

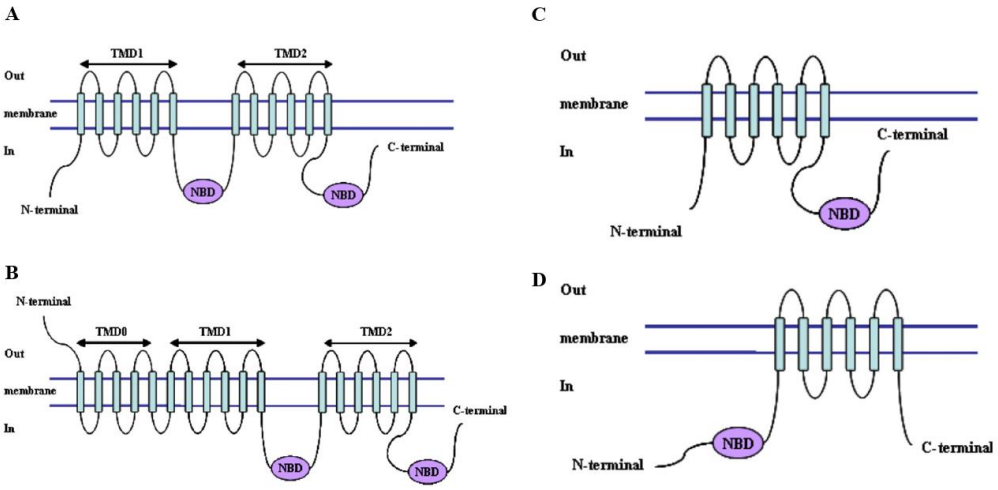

Figure 16: Topology of half transporter. A. The topology of a typical ABC transporter consists of two nucleotide-binding domains (NBD) and two transmembrane domains (TMD). B. Several ABC transporters like ABCC1, ABCC2, ABCC3 and ABCC6 contain an additional TMD. C. The half-transporters contain one NBD and one TMD The NBD can at the C-terminal side (ABCB2-B3, B6 to B10 and ABCD1) or at the N-terminal side (ABCG2) (Gillet et al. 2007)

2.1.2. The nucleotide binding domains

The NBDs are domains, located in the cytoplasm, where ATP will bind (Dean et al. 2001). These domains are highly conserved among ABC transporters and contain characteristic motifs presented in **Figure 17**. The A-loop is an aromatic residue of 25 amino acids upstream the walker A. The walker A (also called P-loop, GXXGXGKS/T where X is any amino acid) and the walker B ($\phi\ \phi\ \phi\ \phi$ D, where ϕ is a hydrophobic residue) are separated by 90 to 120 amino acids and are found in all ABC transporters as well as in ATPase. The walkers are required for ATP hydrolysis reaction. Mutations in these sequence lead to the loss of ATP driven drug efflux from cells (Rees et al. 2009; Frelet and Klein 2006). The walker A would be involved in interaction with the phosphate groups of ATP (Ambudkar et al. 2006). The C motif, also called the ABC signature or LSGGQ motif is an additional motif of the NBD located between the walker A and B. This motif is crucial for the hydrolysis of ATP and the interaction with the substrate binding site (Ambudkar et al. 2006). The NBD domains also contain a D-loop, Q-loop, a H-loop (Rees et al. 2009; Holland and Blight 1999).

We can distinguish two parts in the NBD: the catalytic core domain and the α-helical subdomain. The first one includes two β-sheets and six α-helices (including walker A and B, Q- and H-loop). The α-helical domain consists of three or four helices and includes the C motif.

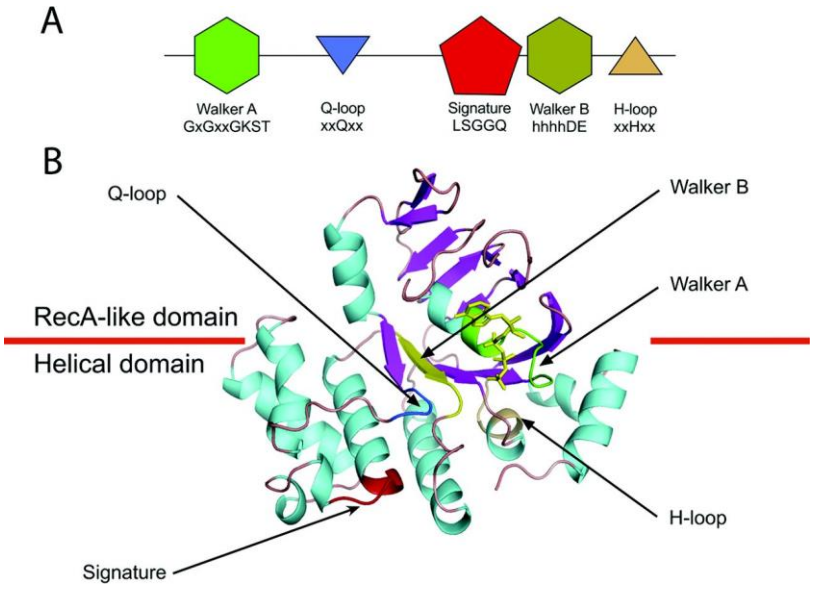

Figure 17 : The nucleotide binding domains. A. This linear representation of the nucleotide-binding domain show the main characteristic motifs. The walkers A and B are represented by hexagons. The motif C, represented as a red pentagone, is also called the ABC signature or the LSGGQ motif. B. There are two parts in the NBD: the catalytic core domain (RecA-like domain) and the α-helical subdomain. The characteristic motifs are represented with the same colors (Dassa 2011).

These walkers A, B and the motif C form a large pocket for ATP binding (Frelet and Klein 2006) and it is usually believed that both NBDs are required for ATP hydrolysis (Li et al. 2016). The NDBs are able to form a "nucleotide-sandwich dimer". ATP is then bound along the dimer interface between the walker A and B, of one subunit, and the LSGGQ motif and the D-loop of the other subunit. The adenine ring of ATP interacts with the A-loop while phosphate and magnesium interact with the waker A and B. The LSGGQ motif is involved in the formation of the NBD sandwich dimer (Ambudkar et al. 2006).

2.1.3. The transmembrane domains

The transmembrane domain (TMD) is typically composed of 6 α-helices and provides the specificity of the substrate (Dean et al. 2001; Glavinas et al. 2004). These α-helices are embedded in the membranes and traverse it several times in a zigzag fashion. They allow the translocation of the substrate across the cellular membranes. The TMDs are very heterogeneous between the different ABC transporters, reflecting the high diversity of substrates. The exporters ABC transporters are characterized by the presence of extracellular loops called ICL (25 Å) that extend the transmembrane helices (Rees et al. 2009).

2.2. Mechanisms of transport

ABC transporters allow an active transport of the substrate across the cellular membranes against a concentration gradient. This process requires energy provided by ATP hydrolysis, which is coupled with the substrate translocation. Two models describe the mechanism of transport based on ABCB1.

The first model (**Figure 18A**) starts with the binding of the substrate on the cytoplasmic side of the TMDs (which are initially in conformation inward). This leads to conformational change of the NBD increasing the affinity for ATP. The displacement of the walker A (due to signal from TMDs) was suggested to allow ATP to gain access to its binding sites. Then, two Mg-ATP molecules bind to the ATP binding pocket provoking the dimerization of the NBDs. The formation of the nucleotide sandwich dimer provokes a conformational change in the TMD from the inward to outward conformation, leading to the release of the substrate. Two sequential ATP hydrolysis, releasing 2 ADP and 2 phosphates, provide energy for the dissociation of the NBDs and then the return to the original situation. The process can repeat again (Higgins and Linton 2004).

The second model (**Figure 18B**) also requires the hydrolysis of two molecules of ATP: the first one provide the energy to efflux the drug and the second one to reset the protein to its initial state (Ambudkar et al. 2006).

This model consists of 8 steps. At first, one molecule of ATP binds on the ATP binding pocket. It is accompanied by the binding of the substrate but there is no influence between these binding events (I). The ATP hydrolysis provokes (II) a conformation change of the drug binding site decreasing the substrate affinity and leading to the release of the substrate. This conformational change also makes the NBD inaccessible to nucleotides. The steps III and IV consist of the sequential release of ADP and Pi which makes the NBDs accessible to ATP while the drug binding site remains in the low-affinity conformation.

A second molecule of ATP binds the NBD (V) and its hydrolysis (VI) provide the energy required for the transporter to return to its original conformation. The transporter can now bind to ATP and substrate.

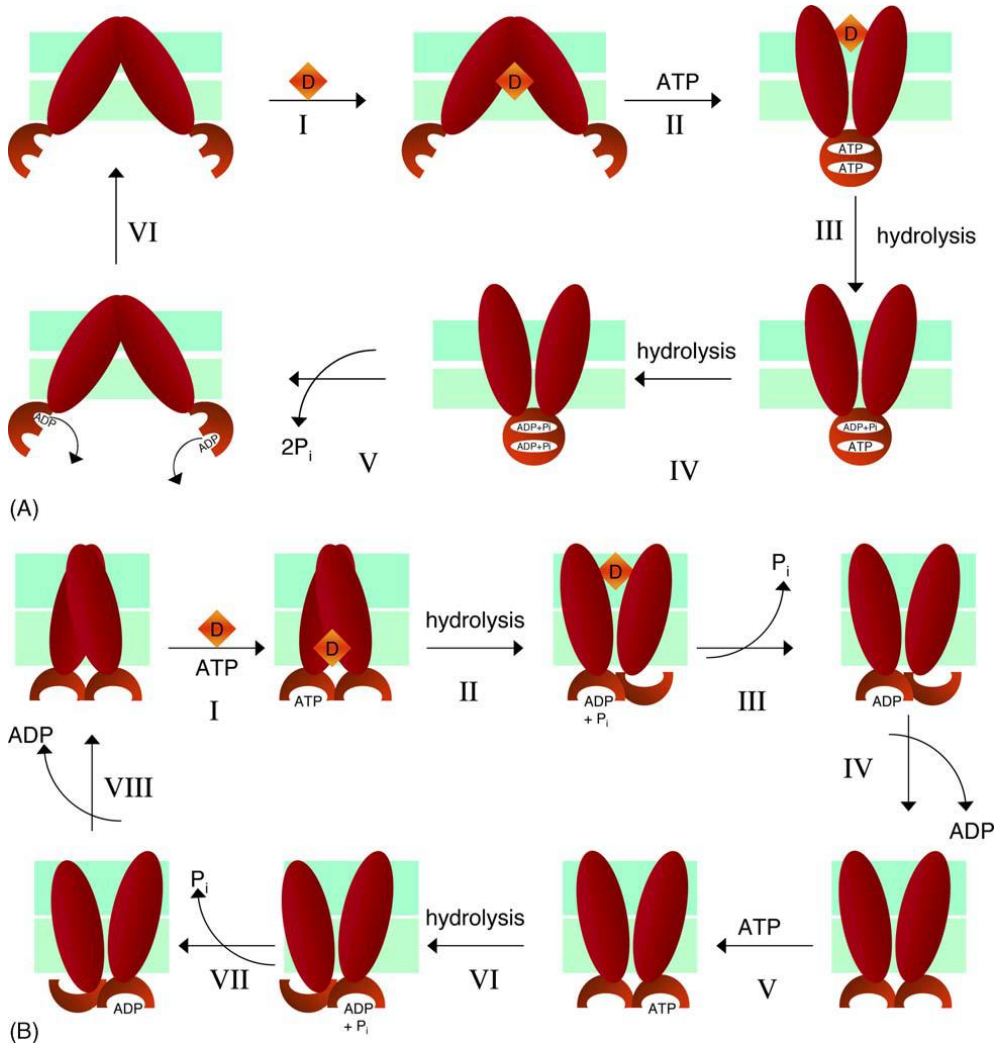

Figure 18: Two models describing the transport mechanism of ABCB1. The red ovals represent the transmembrane domains embedded in the plasma membrane (blue rectangles). The red circles and semi-circles, in the cytoplasmic side, represent the nucleotide binding domains (Ambudkar et al. 2006).

2.3. Overview of the different families of ABC transporters

The physiological role of ABC transporters are very diverse. They are responsible for the regulation of cellular level of different molecules including hormones, xenobiotic, lipids, etc. They can play a role of toxins detoxification in the liver or in the gastrointestinal tract for example. Some are implicated in the regulation of local permeability in placenta, testis in blood brain barrier and the cerebrospinal fluid, others are crucial for the immune system by peptide transport in the endoplasmic reticulum (Gillet et al. 2007; Robey et al. 2018) Below we briefly go over the role of different ABC transporters by family.

2.3.1. ABCA transporters

The twelve members of ABCA family are typical full-transporters with two NBDs and two TMDs. ABCA transporters have the particularity to have a large extracellular domain between the first transmembrane segment and the cluster of five other transmembrane segments (Illing and Molday 1997; Oram 2002) They are mainly expressed in cells of the central nervous and hematopoietic system. ABCA transporters are classified into two groups: the first group comprising ABC-A1, -A2, -A3, -A4, -A7, -A12, -A13 whose genes are located on 6 different chromosomes and the second one including ABC-A5, -A6, -A8, -A9, and -A10) whose genes are arranged in a cluster on chromosome 17q24 (Arnould et al. 2002; Broccardo et al.1999). ABCA11 is a pseudogene (Robert et al. 2009).

The substrates of main ABCA transporters are still to identified. The most studied ABC transporters in this family is ABCA1, -A2, -A3 and -A12 because they are associated with inherited disease (see **Table 1**). They are implicated in the transport of lipids across cell membrane (Molday and Zhong 2009). ABCA1 implicated in the efflux of cholesterol and phospholipid from macrophage and other cells (Oram 2002; Oram et al. 2000). The Tangier disease is an inherited disorder caused by a defective ABCA1 transporter due to a mutation in its gene (Brooks-Wilson et al. 1999). ABCA3 transport phospholipids in pulmonary surfactant biogenesis (Ban et al. 2007), ABCA4 is associated with Stargardt disease (Koenekoop 2003). ABCA12 cause harlequin and lamellar ichthyosis, link with a defective lipid transport (Akiyama et al. 2005; Lefèvre et al. 2003).

2.3.2. ABCB transporters (MDR)

The ABCB family has the particularity to contain both full-transporters (ABC-B1, -B5 Full-lenght, -B4, -B11) and half-transporters (ABCB2, -B3, -B6, -B7, -B8, -B9, -B10).

Members of this family were known to be involved in multidrug resistance. ABCB1, also called MDR1, is the best-characterized ABC transporter as previously mentioned. The transporters ABCB4 and ABCB11 were also shown to be able to transport anticancer agents. Indeed, ABCB4 was shown to transport daunorubicin, doxorubicin, vincristine, etoposide, mitoxantrone and ABCB11 to confer resistance to paclitaxel (Gillet et al. 2007).

ABCB2 and –B3 are TAP transporters (Transport Associated with Antigen Processing), respectively called TAP1 and TAP2. They heterodimerize to allow the transport of peptides in the endoplasmic reticulum and are associated with antigen processing (Herget and Tampé 2007). ABCB9 is closely related to TAP1 and TAP2 and is located in lysosomes (F. Zhang et al. 2000). So far, the physiological role of this transporter is unknown but it seemed to be implicated in phagosome maturation (Lawand et al. 2018). ABCB4 and ABCB11 are hepatocellular transporters. ABCB4 export phosphatidylcholine into bile (Zhao et al. 2015) while ABCB11 is responsible for bile salt transport (Gerloff et al. 1998). The transporter ABCB5 will be further explained in detail page 46 and page 53. ABCB6, ABCB7, ABCB8 and ABCB10 are mitochondrial ABC transporters and were associated with oxidative stress. They would homodimerize with the NBD facing the matrix (Schaedler et al. 2015).

Mutations in ABCB genes were associated with inherited disease as we can see in the **Table 1**. For example, ABCB4 was associated with cholestasis, ABCB1 with intrahepatic cholestasis of pregnancy, neonatal respiratory syndrome (Linton 2015) and ABCB7 in Sideroblastic amaemia and ataxia (Schaedler et al. 2015).

2.3.3. ABCC transporters (MRP)

The ABCC subfamily contains the higher number of drug transporters and is called the multidrug resistance protein family. The ABCC family consists of twelve full transporters. Except ABCC8-9, members of this family are called MRP (Multidrug Resistance-associated Protein).

ABCC family has the particularity that several members (ABCC1, ABCC2, ABCC3, ABCC6, and ABCC10) have an additional TMD domain called the TMD0, consisting of five alpha helices. The presence of the TMD0 is important for the function of the protein and its absence leads to the protein instability (Bera et al. 2017; Jedlitschky et al. 2006). Among this family, ABCC1, -C2, -C3, -C4, -C5, -C6 and C11 are responsible form efflux of chemotherapeutic drugs (Dean 2009). ABCC8 and ABCC9 are regulator of K+ channels without playing a role of transport but form a complex with the channel (Bryan et al. 2007). ABCC7, also called CFTR, is a very particular member because it is the only one acting as an ion channel. It also presents a regulatory domain. Mutations in this gene are responsible for Cystic fibrosis (Aleksandrov et al. 2007). Other diseases are associated with ABC transporters. Indeed, ABCC2 is associated with Dubin–Johnson syndrome, ABCC5 with Inherited hypertrichosis, ABCC6 with Pseudoxanthoma elasticum and ABCC8 and -C9 are associated with diabetes (Theodoulou and Kerr 2015).

2.3.4. ABCD family

The ABCD family contains four genes encoding for half transporters. They can homo- or heterodimerize to be functional and transport (Dean et al. 2001).

ABCD1, -D2, -D3 are localized in peroxisome and are responsible for the transport of fatty acids across the membrane. Hillebrand and colleagues show that the homodimerization predominates (Hillebrand et al. 2007). ABCD4 is localized in the lysosomal membrane and would transport vitamin B12 from lysosome to cytosol.

As we can see in the **Table 1**, mutations in ABCD1 are associated with X-linked adrenoleukodystrophy caused by an impaired peroxisomal beta-oxidation leading to accumulation of very long-chain fatty acids in plasma and tissues (Kemp and Wanders 2010). ABCD3 is associated with hepatosplenomegaly and liver disease while mutations in ABCD4 gene lead to inborn error or vitamin B12 metabolism (Theodoulou and Kerr 2015).

2.3.5. ABCE and ABCF family

ABCE and ABCF proteins do not contain transmembrane domain. This is unlikely that these proteins act as a transporter. The ABCE family only contains one member called ABCE1 which is an organic anion binding transporter (or OAPB). This protein only contains a nucleotide-binding. ABCF genes are upregulated by tumor necrosis factor-α suggesting the role of ABCF members in inflammatory process (Vasiliou et al. 2009).

2.3.6. ABCG family

ABCG family comprised five half transporters with a reverse topology, with the NBD located at the N-terminal side. ABCG1 is involved in the cholesterol homeostasis by effluxing cholesterol (Tarr et al. 2009) and is also involved in intracellular transport of cholesterol (Sturek et al. 2010).

As previously mentioned, ABCG2 (BCRP) was extensively studied for its role in drug resistance. It also plays an important role of protection in the organism. This half-transporter is expressed in the apical membrane of epithelial cells, in the liver canalicular membrane, in blood brain barrier, in placenta. It transports very diverse substrates including fate and glucuronide conjugates of sterols and xenobiotics, natural compounds and toxins, etc. (Mo and Zhang 2012). The most studied transporter of this family is ABCG2 for its implication in drug resistance.

ABCG5 and ABCG8 form heterodimers and are expressed in canalicular membranes of hepatocytes. They play a key role in biliary cholesterol transport (Velamakanni et al. 2007). ABCG4 was found in macrophage, eye, brain and spleen (Vasiliou et al. 2009).

Table 1: ABC transporters and human health and disease. ABC transporters are known to be associated with genetic disease, caused by a defect in these transporters. For example, ABCA1 is associated with Tangier disease, ABCA4 with Stargardt disease, ABCC2 with Dublin-Johnson syndrome, etc.

ABC transporter	Disease
ABCA1	Tangier disease and familial high density lipoprotein (HDL) deficiency; atherosclerosis; Alzheimer's disease
ABCA3	Neonatal surfactant deficiency and pulmonary fibrosis; congenital cataract
ABCA4	Stargardt macular degeneration
ABCA7	Alzheimer's disease
ABCA12	Harlequin and lamellar ichthyosis
ABCB1/transporter associated with antigen processing (Tap)2; ABCB2/Tap1	Immune deficiency; arthritis risk
ABCB4/MDR2	PFIC3; other types of cholestasis
ABCB7	Sideroblastic amaemia and ataxia
ABCB11/bile salt export pump (BSEP)	PFIC2; intrahepatic cholestasis of pregnancy; neonatal respiratory distress syndrome
ABCC2/MRP2	Dubin–Johnson syndrome
ABCC5/MRP5	Inherited hypertrichosis
ABCC6/MRP6	Pseudoxanthoma elasticum
ABCC7/CFTR	CF
ABCC8/SUR1	Diabetes
ABCC9/SUR2	Diabetes
ABCD1/adrenoleukodystrophy protein (ALDP)	X-linked adrenoleukodystrophy X-linked adrenomyeloneuropathy
ABCD3/peroxisome membrane protein (PMP70)	Hepatosplenomegaly; liver disease
ABCD4/PMP69	Inborn error of vitamin B12 metabolism
ABCG2/breast cancer resistance protein (BCRP)	Gout and hyperuricaemia
ABCG5; ABCG8	Sitosterolemia; coronary heart disease; gallstone disease
ABCB1/P-gp; ABCC1/MRP1, ABCG2/BCRP	Multi-drug resistance
ABCC2-6	Drug transport

2.4. ABC transporters and melanoma resistance and progression

A cluster of ABC transporters were found in melanoma cells : ABCA9, ABCB1, ABCC1, ABCC2, ABCB5, ABCB8, ABCD1, ABCG2 (Chen et al. 2009; Elliott and Al-Hajj 2009; Fukunaga-Kalabis and Herlyn 2014; Röckmann and Schadendorf 2003; Setia et al. 2012; Luo et al. 2012).

Furthermore, the study of Heimler and colleagues reported the expression of ABCA5, B2,B5, B6, D3, D4, F1, F2 and F3 in melanoma were expressed using RT-qPCR. The expression level of ABCB3, B6, C2, C4 and ABCE1 was found to be high in melanoma cell lines as compared with ABCA7, A12, B2, B4, B5 and ABCD1 (S. Heimerl et al. 2007). However, Deichmann et al. did not find ABCG2 to be expressed in melanoma both at mRNA and protein level (Deichmann et al. 2005).

The **Figure 21** and the **Table 2** summarize the involvement of ABC transporters in melanoma resistance and melanomagenesis.

2.4.1. ABC transporters and melanoma stem cells (MSCs)

The melanoma stem cells were previously developed on page 16. Several ABC transporters were identified as MSCs including ABCB5β (Frank et al. 2005; Gerber et al. 2017; Suzuki et al. 2015; Schatton et al. 2008; Luo et al. 2012; Zhang et al. 2016). The ABCB5β isoform is co-expressed with other markers of melanoma initiating cells such as CD44, CD133 and CD24 (Zhang et al. 2016). ABCB1 was also found to be expressed in a subpopulation of cells representing 1 to 10% of the bulk tumor. The expression of ABCB1 increased when cells are cultured in a media for stem cells. These cells co-express ABCB5 and ABCC2 (Keshet, G. I. 2008). ABCG2 was identified to be expressed in a subpopulation of potent melanoma stem cells also expressing CXCR6 (Taghizadeh et al. 2010).

The fact that ABC transporters are considered as marker of MSC is important at two levels: for melanoma resistance and for melanomagenesis. Indeed, the cancer stem cells have the property to show a higher resistance to anti-cancer agent and to be associated with a higher tumorigenic potential.

2.4.2. ABC transporters and drug resistance in melanoma

Resistance to anticancer agents remains an obstacle for the treatment of melanoma. It represents a challenge for effective cancer therapy. One of the most common resistance mechanism observed is the efflux of the drug out of the cell by ABC transporters (Gillet et al. 2007; Szakács et al. 2006). In melanoma, drug sequestration was also suggested to be a mechanism of drug resistance (K. G. Chen et al. 2009).

2.4.2.1.ABCB1

Surprisingly, while ABCB1 was extensively studied for its role in resistance in many types of cancer, this transporter does not seem to be the key factor of resistance in the case of melanoma. However, some studies suggested its implication in melanoma resistance. The expression of its transcript was detected in the following melanoma cell lines: SK-Mel-28, SK-Mel-5 and M14 (Chen et al. 2005; Szakács et al. 2004). As previously mentioned, ABCB1 was identified as being expressed by a subpopulation of MSCs. This subpopulation was shown to efflux paclitaxel (Keshet et al. 2008; Luo et al. 2012). Joo and colleagues showed the important role of ABCB1 in brain metastatic tumor resistance. Using a murine model, they implanted the melanoma cells K1735 either in the skin or in the brain. The paclitaxel was efficient in the skin but not in the brain. Using an inhibitor of ABCB1, this brain-specific resistance disappeared. Their study confirmed the role of ABCB1 in the brain microenvironment (Joo KM. et al. 2008). This transporter was also shown to be involved in the limited distribution of trametinib in the brain (H. Vaidhyanathan et al. 2014). The tumor endothelial cells (TECs), representing an important target for the anti-angiogenic chemotherapy, are resistant to paclitaxel. This seems to be mediated by ABCB1. Indeed, the addition of verapamil (inhibitor of ABCB1) abrogated the resistance in a melanoma xenograft mouse model. This co-administration of paclitaxel and verapamil reduced lung metastasis (Akiyama et al. 2015).

2.4.2.2.ABCB5

According to AceView program, which provides a strictly cDNA-supported view of the human transcriptome and the genes, ABCB5 gene transcription gives rise to at least 11 different transcript variants (Thierry-Mieg D. and Thierry-Mieg J. 2006). Among those, three variants have been documented. In chronological order: ABCB5beta.b (812 aa, also referred to as ABCB5β) (Chen et al. 2005; Frank et al. 2003) , ABCB5.h (131 aa, also referred to as ABCB5α) and ABCB5.a (1257 aa, also referred to as ABCB5FL Full-Length). The ABCB5α mRNA encode for a soluble protein of 15kDa only containing one walker B and one C motif (Chen et al. 2005; Moitra et al. 2011). We focus here on the ABCB5β and full-length isoforms, studied for their role in melanoma.

There is a correlation between the transporter ABCB5 expression and melanoma progression and recurrence. Indeed, Gray and colleagues showed that the expression of ABCB5 is higher in melanoma circulating cells than in the solid tumor (Gray et al. 2015). The ABCB5 mRNA was also detected in sentinel metastatic lymph nodes in patients with recurrence (Suzuki et al. 2015).

ABCB5β

The ABCB5β isoform topology do not corresponds to the canonical topology of ABC transporters. Moitra and colleagues predicted ABCB5β to have a TMD composed of six α-helices flanked by two intracellular NBDs as it shown in the **Figure 19**, which is the unique feature of ABCB5β. Furthermore, the N-terminal NBD lacks Walker A motif (Moitra et al. 2011). As shown by the **Figure 19,** the conventional half-transporters possess only one NBD, either on the N- or on the C-terminal region (Gillet et al. 2007). Yet, ABCB5β might form a dimer to create a functional transporter. Potential dimerization motifs were identified in its N-terminal region (Moitra et al. 2011). This isoform is expressed in healthy cells like testis, melanocytes, retinal pigment epithelium, in the brain barrier and the uterus (Chen et al. 2005; Frank et al. 2003, 2005; Huang et al. 2004; Langmann et al. 2003). It is also expressed in many cancer cells: melanoma, breast, liver (Cheung 2011), colorectal cancer (Wilson 2011) as well as carcinoma cells and leukemia (Frank 2005, Schatton 2008, Lehne 2009).

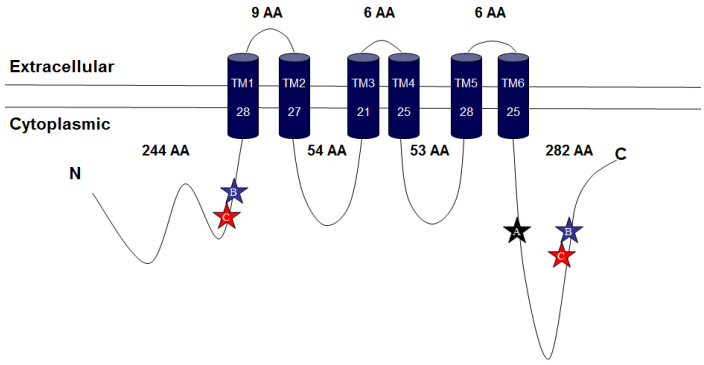

Figure 19: The topology of ABCB5β .The proposed model by Moitra and colleagues shows a topology of ABCB5β consisting of one TMD with 6 α-helices and two NBD located on the intracellular surface. The walker as is lacking in the NBD located on the N terminal side (Gillet, unpublished data)

ABCB5β was extensively studied in resistance in melanoma. It was found to be responsible for melanoma resistance to chemotherapeutic agent like doxorubicin, temozolomide, dacarbazine, vincristine, teniposide, paclitaxel, etoposide, and docetaxel in melanoma (Chartrain et al. 2012; Frank et al. 2005; Wilson et al. 2014; Zhang et al. 2016). Using shRNA against ABCB5 or an anti-ABCB5 monoclonal antibody in A375 and G3361, melanoma cells have been resensitized to multiple anticancer agents (Wilson et al. 2014). As previously mentioned, ABCB5β was identified as a marker of a subset of chemoresistant cells with stem cells properties (N. Y. Frank et al. 2005; X. Zhang et al. 2016). Observing the role of melanoma stem cells in resistance, Zhang and colleagues went further and silenced ABCB5 using VPN20009-shABCB5, previously reported to specifically target and reduce the growth of cancer stem cells. No significant difference was observed comparing VPN20009-shABCB5 and VPN-scrambled in terms of tumoral growth and survival times. The combination of VPN20009-shABCB5 and chemotherapy cyclophosphamide led to a delay of tumor growth and to an increase of survival time in the B16F10 mouse model (X. Zhang et al. 2016).

ABCB5β was also studied for its role in resistance to kinase inhibitors. Vemurafenib (PLX4032) is an inhibitor of the mutant BRAF V600E. It increases the response of melanoma to treatment and survival rate (Bollag et al. 2012; Tsai et al. 2008). However, resistance to this treatment also appears and represents a major obstacle for patients with the mutation (Chapman et al. 2011; Bollag et al. 2010). Menon and colleagues showed an increase of the expression of ABCB5 in melanoma cells, which are resistant to vemurafenib (Menon et al. 2015). Furthermore, the study of Chartrain showed that the treatment with this anti-cancer agent leads to a selection of the cells expressing ABCB5 (Chartrain et al. 2012). This suggests the role of ABCB5 in the development of resistance to vemurafenib. However, the results of Xiao et al. are less clear. They developed three resistant cell lines to vemurafenib and assessed the expression of ABCB5. ABCB5 was overexpressed in cells resistant to BRAF inhibitors SK-MEL-28PLXr and A2058PLXr but not in A375PLXr cells. However, the silencing of ABCB5 does not re-sensitive to treatment. ABCB5 may not be the mediator of resistance to vemurafenib. The overexpression of ABCB5 is associated with the activation of the p-ERK status, which may play an important role in melanoma resistance (Xiao et al. 2018).

While many studies show the transport of anti-cancer agents by ABCB5β, Keniya and colleagues showed that this isoform would not be able to confer drug resistance using yeast model. The cells expressing ABCB5β were not able to confer drug resistance alone (Keniya et al. 2014). This suggests that ABCB5β could dimerize to be functional.

ABCB5 full-length

While ABCB5β has been in the spotlight, there exists another isoform corresponding to the typical topology of a full-sized transporter. The sequence of the full-length ABCB5 cDNA was identified, in 2004, by Chen and Gottesman. Then, based on this sequence, they constructed the full-length ABCB5 cDNA which was confirmed by the clone produced by the team of Sugimoto (Kawanobe et al. 2012). This isoform is very little characterized, known to be expressed in testicular tissue (Y. Frank 2009).

From a phylogenetic point of view, Moitra and colleagues demonstrate that ABCB5 evolves as a full-sized transporter. This suggests that the function of the protein has been maintained through mammalian evolution (Moitra et al. 2011).

The full sized ABCB5 has a conventional topology of ABC transporters with two NBDs and two TMDs, each containing 6 α-helices as shown in **Figure 20** (Moitra et al. 2011). It contains 1257 amino acids and has molecular weight of 138kDa. This isoform of ABCB5 is highly homologous to ABCB1 with a homology of 73%.

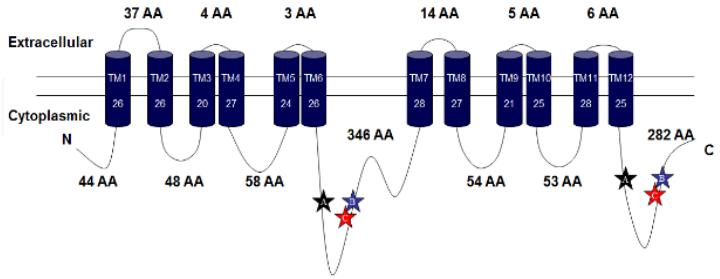

Figure 20: The topology of the isoform ABCB5 full length. The topology of ABCB5 full-length corresponds to the typical topology of full ABC transporters with two nucleoid-binding domains and two transmembrane domains (Gillet, unpublished data).

Several studies show that ABCB5 full length plays a role in resistance to anticancer agents in melanoma and demonstrate its transport functionality. Keniya and colleagues showed in Sacharomyces cerevisae that the ABCB5 full-length isoform was able to confer drug resistance to rhodamine 123, daunorubicin, tetramethylrhodamine, FK506, or clorgyline. They were not able to show an efflux activity of the transporter and thus suggested the drug sequestration in a subcellular organelle (Keniya et al. 2014). This goes in the same direction as the study of Chen suggesting the sequestration of the drug in melanosomes (Chen et al. 2006). The transport function of ABCB5 was also shown by Kawanobe using the human cancer cell line HEK293. The full length transporter led to a higher resistance of 1,5 fold to doxorubicin and to 2 to 3 folds to paclitaxel and docetaxel (T. Kawanobe et al. 2012).

2.4.2.3.Are other ABC transporters involved in melanoma resistance?

The half-transporter ABCB8 leads to resistance to doxorubicin (3 to 4 folds) by comparison with the parental ABCB8- melanoma cell lines. ABCB8 is localized in the inner membrane of mitochondria and is involved in chemoresistance by protecting the mitochondrial DNA of cells from doxorubicin induced damages (Elliott and Al-Hajj 2009).

The transporter ABCC1 alone does not seem to be involved in melanoma resistance. Indeed, inhibitors of ABCCC1 had no effect on the viability of A375 melanoma cells after treatment with antineoplastic agents. However, ABCC1 and Glutathione-S-transferase Pi1 (GSTP1) have a combined effect in resistance to etoposide. GSTP1 is the most studied form of Glutathione-S-transferase (GST) for its role in cancer resistance. The GSTs is a family of detoxification enzyme catalyzing the conjugation by the glutathione (GSH). While, the involvement of GST in resistance is still controversial, it seems that their expression must be combined with the expression of ABC transporters (Depeille et al. 2005). A link is well established between ABCC1 and the GSH. Indeed, The GSH-dependence of ABCC1 for transporting drug like vincristine was already shown (Cole 2014; Loe et al. 1998; Zaman et al. 1995). In the same family, ABCC2 is involved in resistance to cisplatin. Indeed this transporter is responsible for the decreased formation of platinum-DNA adducts and the decreased of the cell cycle at the G2 phase in melanoma cells resistant to cisplatin (Liedert et al. 2003). The subcellular localization of ABCC2 in melanoma cell is still unknown but this transporter was localized the nuclear envelope in the case of breast cancer (MacIejczyk et al. 2012) and the fallopian tube cancer (Halon et al. 2013) as well as in ovarian carcinoma also associated with cisplatin resistance (Surowiak et al. 2006). That is why we suggest its subcellular localization in the **Figure 21**. The transporters ABCB2 and ABCB3 are called "Antigen peptide Transporter" (respectively celled TAP 1 and TAP2). They were found to be down-regulated in the B16 melanoma cells. This is associated with a lack of tumor associated antigen processing, a low expression of MHC class1 and a decreased immunogenicity. Zhang and colleagues have shown that TAP1 expression restores the antigen presentation. This shows that ABC transporters can also play a role in resistance to immunotherapy (Q. J. Zhang et al. 2007).

2.4.2.4.Specialized mechanism of resistance in melanoma

In their review, Chen and colleagues proposed the ABC-M model. According to this model, the network of ABC transporters and melanogenic pathway (including melanosome biogenesis) is involved in the regulation of drug sensitivity of melanoma cells. The melanin intermediates are toxic for the melanocytes. These compounds are trapped in subcellular organelles like lysosomes, endosomes and melanosomes. In melanoma cells, the same mechanisms are used by the cell to trap the anticancer agent in subcellular organelles (K. G. Chen et al. 2009).

Melanoma cells may have developed a specific mechanism of drug resistance. Chen and colleagues demonstrated that resistance is due to the sequestration of the drug diaminedichloroplatinum II (CDDP) in melanosomes. This sequestration prevents the drug from going to the nucleus by comparison to non-melanoma cells. The sequestration of CDDP also has an impact on melanogenic pathway, on the increase of the tyrosinase activity and the intracellular pigmentation. They showed that the resistance level is more important in melanoma cells with a higher number of melanosomes (Chen et al. 2006).

Several studies have suggested the involvement of ABCB5 in melanogenesis. Lin and colleagues genotyped melanoma samples on the 7p21.1 locus and identified three SNPs. One of them was associated with the red/non red hair color. This locus was genotyped in melanoma cell lines and they noticed a non-synonymous amino acid change K115E. Further functional studies showed that the E form is associated with a lower risk of melanoma. This correlates with lower ABCB5 transport capacity and higher melanogenesis (J. Y. Lin et al. 2013). Wilson and colleagues, injected ABCB5-expressing pigmented melanoma cells G3361 in NSG mice, and they observed a difference in pigmentation between the ABCB5 expressing and non-expressing cells. The ABCB5 non-expressing cells are associated with hyperpigmentation (Wilson et al. 2014).

ABCB6 was also shown to be localized in melanosomes and lysosomes in MNT1 melanoma cells. The silencing of ABCB6 led to the accumulation of multilamellar aggregates in pigmented melanosomes (Bergam et al. 2018). It was discovered by studying Dyschromatoses, which is a rare skin disorder characterized by small irregular hyper- and hypopigmented macules (Yadalla et al. 2013).

2.4.3. Involvement of ABC transporters in melanomagenesis

While ABC transporters are mainly studied for their role in cancer resistance, they also play a role in cancer development. More and more studies showed the link between ABC transporters expression and the malignant properties of cancer cells. Indeed, the expression of these transporters has an impact on the proliferation, migration and invasion ability of the cancer cell. However, the underlying mechanisms remain to be fully understood. According to Fletcher and colleagues, they would exert this function by transporting endogenous metabolites or signaling molecules (Fletcher et al. 2016). In this section, we will focus on the role of ABC transporters in melanomagenesis.

2.4.3.1.ABCB1

The expression of ABCB1 was associated with malignant properties. Keshet et al. showed that the melanoma stem cells expressing ABCB1 are more tumorigenic than the ABCB1 knockout cells (Keshet et al. 2008). Using transplantable hamster model, the expression of the transporter ABCB1 was found to be associated with less differentiated and more aggressive tumor cells (Witkowski JM et al. 2000). *In vitro*, the ABCB1-expressing M14 melanoma cells were shown to be associated with an increase of the migration and the invasion ability of the cells by comparison with their ABCB1-knockout counterparts. Using transwell chambers, the wild-type cells showed a low capacity to go through filters, both in the presence and in the absence of the matrigel layer. The way they pass through the pores are different. The ABCB1- cells used the lamellar cytoplasmic extrusion, whereas the ABCB1+ cells elongated along the hole though globular process (this was observed by scanning electron microscopy). Molinari suggested that the interaction between ABCB1 and CD44 was associated with this more aggressive phenotype (Molinari et al. 2005). The study of Colonne aimed to clarify the involvement of ABCB1 and CD44. They showed their colocalization in certain regions of the plasma membrane. ABCB1 and CD44 were observed outside of the cells and would use the same transport vesicles. While the adhesion capacity was identical between the ABCB1+ and their ABCB1- counterparts, they confirm the more invasive phenotype conferred by ABCB1 expression.

They showed a link between ABCB1 and the MAPK pathway. The incubation with an antibody detecting ABCB1 induced the activation of ERK1/2 and p38. This activation led to a higher expression of metalloproteinase (MMP-2, MMP-3, and MMP-9) mRNAs (Colone et al. 2008).

2.4.3.2.ABCB5

ABCB5β, located in the plasma membrane, is highly expressed in several kind of cancers including melanoma (Cheung et al. 2011; Civenni et al. 2011; Kumar et al. 2013; Kupas et al. 2011; Linley et al. 2012; Schatton, et al. 2008). ABCB5β was shown to be associated with tumoral progression, resistance and recurrence in malignant melanoma (Chartrain et al. 2012; Gazzaniga et al. 2010; Ma et al. 2010; Kupas et al. 2011; Setia et al. 2012; Schatton et al. 2008).

ABCB5β is gradually expressed from the benign nevi to the invasive melanoma (Setia et al. 2012). The study of Gambichler confirmed it showing that the expression of ABCB5 is higher in primary melanoma, metastases and invaded lymph nodes than in nevi (Gambichler et al. 2016). Lin identified that a non-synonymous polymorphism (K115E) leads to a decrease of the transport function the transporter and a lower risk of melanoma (Lin et al. 2013).

As previously mentioned, ABCB5β is considered as a marker of melanoma stem cell. This transporter is co-expressed with CD133 (N. Y. Frank et al. 2005). Isolating MSC expressing ABCB5β from melanoma tissues, Schatton and colleagues showed that these cells are more tumorigenic when they are xenotransplanted to mice. They have the capacity of self-renewal and differentiation. The systemic administration of an antibody detecting ABCB5 exerted tumor-inhibitory effects (Schatton et al. 2008). This shows that ABCB5β is involved in tumor growth. Indeed, Wilson and colleagues silenced the expression of ABCB5 in melanoma cells A375 and G3361 and injected them in NSG mice. The rapidity of the tumor development was reduced in the cells non-expressing ABCB5. Furthermore, they give information about how ABCB5β plays a role in the aggressiveness of melanoma. ABCB5 represses WFDC1, acting as a tumor suppressor, and induces the production of IL8, which promotes melanoma development. They showed that ABCB5 regulates WNT pathway and controls the secretion of IL1β, an activator of IL8 (Wilson et al. 2014). Zhang and colleagues confirmed that ABCB5β plays a functional role in tumor growth. The injection of non-expressing ABCB5 cells leads to a reduction of the tumor initiating frequency and the tumor volume (X. Zhang et al. 2016).

The ABCB5β isoform was also considered as a prometastatic factor. Indeed, the ABCB5+ melanoma cells have a higher metastatic potential in comparison with the ABCB5- cells, with a higher migration and invasion ability *in vitro*. *In vivo*, the silencing of ABCB5β is associated with a decrease of the melanoma pulmonary metastases using xenograft mice. It was shown that the transporter ABCB5β activates NF-kB pathway through enhancing p65 protein stability by ubiquitination (Wang et al. 2017).

2.4.3.2.Are other ABC transporters involved in melanomagenesis?

Other ABC transporters were suspected to be involved in melanomagenesis. According to the study of Monzani, the melanoma cells expressing ABCG2 have a higher tumorigenic potential (Monzani 2007). When injected in immunodeficient mice, the ABCG2+ subpopulation leads to a tumoral mass with a size twice as large as the tumor developed in the ABCG2- cells (Taghizadeh et al. 2010). Furthermore, the expression of ABCG2 was found to be correlated with melanoma progression. Indeed, the expression level of ABCG2 was higher in stage IV patients than in stage III patients. Performing a multivariate analysis considering the age, the gender, the stage of the disease and M category (categories based on the dissemination of metastases). Speigl and colleagues showed that ABCG2 can be considered as an independent prognostic factor (Speigl et al. 2017). Lastly, we previously explained that TAP1 plays a role in the antigen processing and presentation. This transporter is often down regulated in B16F10 melanoma cell lines. This is associated with low surface expression of MHC class I. The down regulation of TAP may be responsible for the escape of the cancer cells to immunosurveillance (Zhang et al. 2007).

Table 2: Summary table of the involvement of ABC transporters in melanomagenesis

	Marker of melanoma stem cells	Drug resistance	Melanomagenesis
ABCB1	Marker of MSC vitro (Keshet 2008).	In the case of melanoma in the brain of mice, ABCB1 associated with the efficacy of paclitaxel (Joo KM. et al. 2008) Responsible for the limited distribution of trametinib in the brain (Vaidhyanathan et al. 2014) Resistance of tumor endothelial cells to paclitaxel (Akiyama et al. 2015).	Expression associated with the increase of migration and invasive abilities (Colone et al. 2008; Molinari et al. 2005).
ABCC1	/	Etoposide when combined to GSTP1 (Depeille et al. 2005).	/
ABCC2	Co-expression by a tumoral sub-population expressing ABCB1 and B5 (Keshet, G. I. 2008).	Cisplatin (Ichihashi and Kitajima 2001; Liedert et al. 2003)	/
ABCB2		Downregulated in B16 melanoma cell lines suggesting an impact on immunotherapy (Q. J. Zhang et al. 2007)	Suspected to be involved in the escape of immunosurveillance (Q. J. Zhang et al. 2007)
ABCB3			
ABCB5β	Marker of MSC(Frank et al. 2005; Gerber et al. 2017; Schatton et al. 2008).	doxorubicin, temozolomide, dacarbazine, vincristine, teniposide, paclitaxel, etoposide, and docetaxel (Chartrain et al. 2012; N. Y. Frank et al. 2005; Wilson et al. 2014; X. Zhang et al. 2016) Vemurafenib (Chartrain et al. 2012; Menon et al. 2015; Miletti-gonzalez et al. 2005; Xiao et al. 2018).	Involved in the acquisition of malignant properties (Schatton et al. 2008; S. Wang et al. 2017; Wilson et al. 2014; X. Zhang et al. 2016)
ABCB5 full length	/	Transport of chemotherapeutic agents rhodamine 123, daunorubicin, tetramethylrhodamine, FK506, or clorgyline, doxorubicin, paclitaxel and docetaxel (Keniya et al. 2014; Kawanobe et al. 2012).	/
ABCB8	/	Doxorubicin (Elliott and Al-Hajj 2009)	
ABCG2	Marker of MSC(Taghizadeh et al. 2010).	/	Expression associated with tumorigenic potential (Monzani 2007; Taghizadeh et al. 2010)

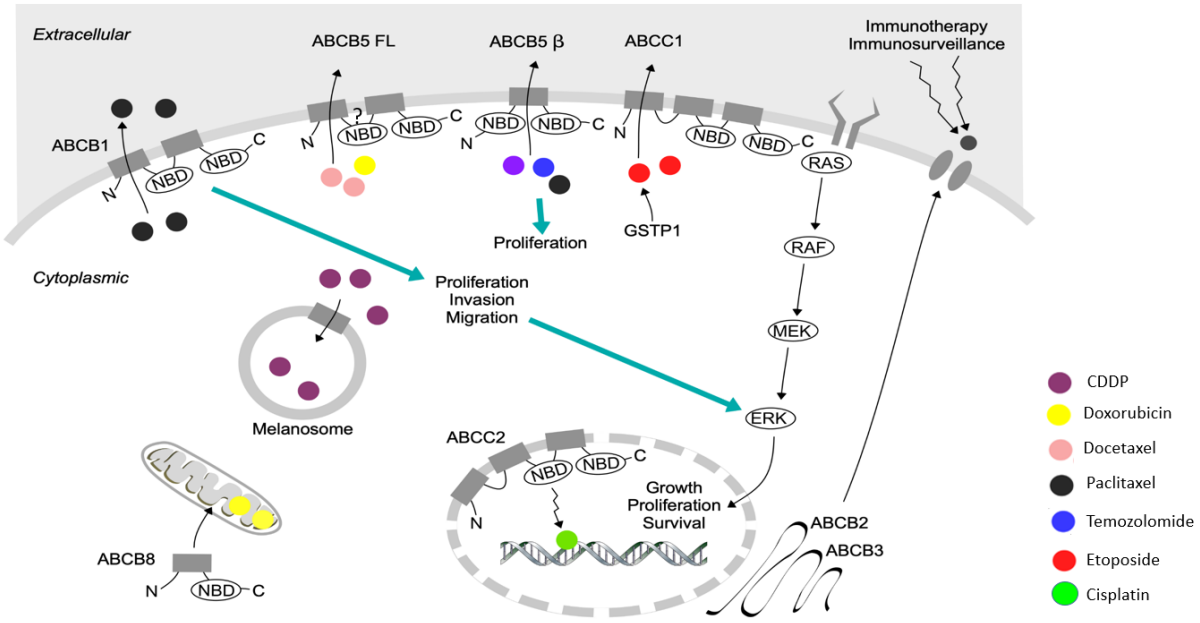

Figure 21: Involvement of ABC transporters in melanoma resistance to treatment. Both ABCB5 isoforms were shown to be involved in resistance in the case of melanoma. ABCB5β has been in the spotlight for its role in the transport of many anticancer agents including paclitaxel, temozolomide, etoposide, etc. The ABCB5 full-length isoform was less characterized. It was shown to transport paclitaxel and docetaxel. ABCC2 was shown to reduce the formation of Platinum-DNA adducts by cisplatin and ABCB8 protects the mitochondrial DNA from damage caused by doxorubicin. ABCC1 is associated with resistance to etoposide when it is combined with glutathione S transferase (GSTP1). Melanoma cells have a specialized resistance mechanism the sequestration of diaminedichloroplatinum II (CDDP) in melanosomes. ABC transporters may also be involved in this mechanism.

PRELIMINARY RESULTS

1. Explanation about the studied cohorts

To identify somatic mutations in *ABCB5*, Gillet and colleagues analyzed the coding regions in human clinical melanoma samples. All of these were non-treated to avoid mutagenic effect of chemotherapeutic agents. For each patient, the exome of the cancer cell was sequenced regarding the corresponding normal DNA (blood cell).

The first cohort analyzed of 54 clinical melanoma samples, from a previously published study by Gartner and colleagues, revealed that *ABCB5* was mutated in 14,5% ($p<0,001$) of the melanoma samples (Gartner et al. 2013). This cohort revealed 10 mutations in the ABCB5 gene, including 8 non-synonymous mutations (**Table 3**). The *in silico* SIFT analysis was performed for these ten mutations. SIFT is a software which predicts if the amino acid substitution affects the protein function (Ng and Henikoff 2003). For these samples, mutations in *ABCB5* were heterozygous. A validation was performed on six of these 10 mutations using digital PCR, which confirmed their presence at the RNA level.

Afterwards, Gillet and colleagues extended the study to an additional cohort containing 99 melanoma samples. They also reviewed the mutational data from published studies (Berger et al. 2012; Nikolaev et al. 2012; Stark et al. 2012), which were combined with exome data from The Cancer Genome Atlas (TCGA), resulting in 487 published melanoma samples.

In summary, Gillet and colleagues showed that the gene ABCB5 was mutated in 13.75% of the 640 melanoma samples analyzed.

Table 3: ABCB5 mutations in untreated clinical melanoma samples (results of Whole Exome, Whole Genome, and Sanger sequencing in a total of 54 samples).

Samples	Mutation	Mutation type	SIFT SCORE
17T	**Q187***	**Non-synonymous**	/
55T	E520D	Non-synonymous	0,21
44T	R587*	Non-synonymous	/
83T	V827I	Non-synonymous	1
12T	I828I	Synonymous	1
83T	**S830F**	**Non-synonymous**	**0**
105T	L840L	Synonymous	0,7
32T	**S1184P**	**Non-synonymous**	**0**
24T	**S1091F**	**Non-synonymous**	**0,01**
55T	Q1098*	Non-synonymous	/

2. Choice of the mutations

Among the mutations presented in the **Table 3**, Gillet and colleagues decided to focus on 4 mutations, 3 associated with low SIFT scores, S830F, S1091F and S1184P, and one stop mutation Q187*. SIFT takes into account the location where the change occurs and the type of amino acid change. The more deleterious effect will be observed for a SIFT value close to zero.

These mutation were located on a 3D-predicted model by Dr.Xia (NIH, NCI, Bethesda, MD, USA) based on the sequence alignment of the full-length ABCB5 to the mouse ABCB1, for which experimental structures are known (Esser et al. 2017; Li et al. 2014). The mutation S830F is located in the transmembrane domain (TMD2) and the mutations S1091F, S1184P are located in the nucleotide-binding domain (NBD2) as we can see in the **Figure 22**. The mutations are located in two identified hot spots.

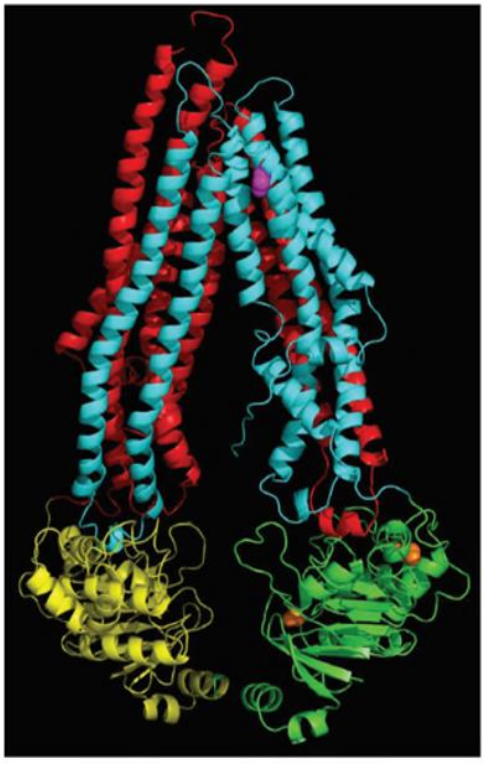

Figure 22: Molecular model of ABCB5. An atomic model of ABCB5 was constructed based on the sequence alignment of the full-length ABCB5 to the mouse ABCB1 or P-glycoprotein, for which an experimental structure is known in the absence of bound nucleotide. Ribbon diagram of the full-length ABCB5 model is given with the N-terminal transmembrane domain or TMD1 colored in red and the C-terminal TMD2 in cyan. The N-terminal NBD1 and the C-terminal NBD2 are shown in yellow and green, respectively. The location of mutations studied are shown in this 3D-model: the residue S830 in the TMD2 is represented by a magenta ball model and the two residues in NBD2 are colored in orange.

3. Effect of the mutations on the activity of the transporter

The results obtained by the SIFT prediction suggest a deleterious effect of the chosen mutations. Gillet and colleagues performed ATPase assays to determine whether these mutations alter the transporter activity. The mutants S830F, S1091F and S1184P ABCB5 and WT ABCB5 were expressed in High Five insect cells with the same expression level (**Figure 23A**). The ATPase assays showed that these mutations resulted in a decrease in basal ATP hydrolysis by ABCB5 (**Figure 23B**). The loss of ABCB5 function due to these mutations suggests that ABCB5 might indeed play a role in the development of melanoma as a tumor suppressor gene.

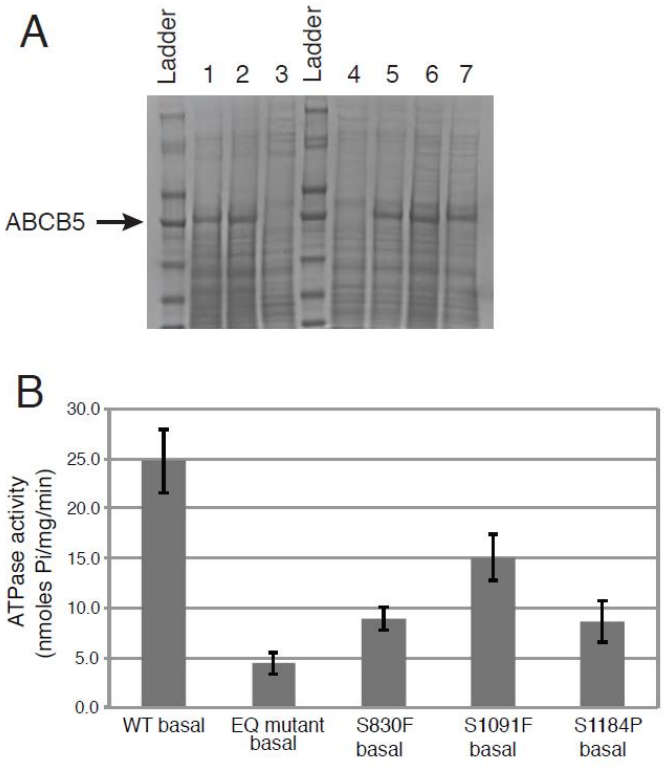

Figure 23: ATPase activity of ABCB5 WT, and mutants.

A. ABCB5 WT and mutants were expressed in High Five insect cells and 30 µg isolated crude membrane proteins were run in a Nupage Tris-acetate gel along with a HiMark ladder. 1. ABCB5 WT, 2. ABCB5 E1181Q mutant (non-functional transporter, - ctrl), 3. Crude membranes (- ctrl), 4. ABCB5-FLAG Q187*, 5. ABCB5-FLAG S830F, 6. ABCB5-FLAG S1091F, and 7. ABCB5-FLAG S1184P. B. ATPase activity of ABCB5 WT and ABCB5 mutants (E1181Q, S830F, S1091F, S1184P) in High Five cell crude membranes was measured by endpoint Pi assay. WT and mutant ABCB5-specific ATPase activities were recorded as beryllium fluoride (BeFx)-sensitive ATPase activity. (Error bars denote SD or SE; n= 3). For the same expression level, we observe a decrease in the ATPase activity for the cells overexpressing ABCB5 mutants by comparison with the cells overexpressing ABCB5 WT.

OBJECTIVES OF THE THESIS

In reviewing the literature, we observed that the isoform ABCB5β has been in the spotlight whereas its topology and function are still controversial. On the contrary, the isoform ABCB5 full length is little characterized. Its topology is very close to ABCB1, probably the most characterized ABC transporter, and corresponds to the typical topology of ABC transporters. The involvement of ABCB5 in melanoma resistance is developed on the page 46 and its involvement in melanomagenesis on the page 53. Several studies showed the transport functionality of the ABCB5 full length isoform. While Kawanobe and colleagues showed its activity of transport in HEK293T cells (T. Kawanobe et al. 2012), Keniya showed it in yeast model (Keniya et al. 2014) and Gillet and colleagues using insect cells (unpublished data). In this thesis, we focused on the isoform ABCB5 full length.

Preliminary results showed that *ABCB5* was mutated in nearly 15% of the clinical melanoma samples analyzed. Four mutations were selected based on the SIFT score from *in silico* analysis. These mutations have a deleterious effect on the protein function as shown by the decrease of the ATPase activity of the transporter, which in turn will affect its transport activity. This suggests that ABCB5 full length plays a role in melanomagenesis as a tumor suppressor. While ABC transporters are mainly studied for their role in cancer drug resistance, an increased body of evidence indicates that they may be involved in tumorigenesis by their transport activity.

The objective of this thesis was to investigate the involvement of ABCB5 in melanoma development and progression. To do so, the project was divided in three parts. The first one consisted of the *in vitro* study of the involvement of ABCB5 in the acquisition by the cell of malignant potential. The proliferation, migration and invasion abilities of melanoma cells overexpressing ABCB5 mutants were assessed. An alteration of these capacities would confirm the involvement of ABCB5 *in vitro*. The second axis of the project aimed to explore the role of Abcb5 *in vivo*, using transgenic mice. The involvement of ABCB5 in melanomagenesis would have an impact on melanoma incidence. Finally, the third part of this thesis had on purpose to investigate about the way of action of ABCB5 by determining its subcellular localization in melanoma cells.

RESULTS AND DISCUSSION

1. Study of the implication of ABCB5 in Melanomagenesis and melanoma progression in vitro

1.1. Objectives

Since the ATPase assays showed that the ABCB5 mutations S830F, S1091F and S1184P resulted in a decrease in basal ATP hydrolysis, we wanted to further explore their impact on the acquisition of malignant properties *in vitro*. While the inactivation of tumor suppressor genes contributes to abnormal proliferation of tumor cells, our first aim was to determine the link between the expression of ABCB5 mutants and the proliferation ability of the cells. To do so, we aimed to perform a 2D proliferation test on plastic to have a first indication. Knowing that this test presents the disadvantage to be influenced by the ability of the cells to adhere to plastic, we also wanted to perform a soft agar colony formation assay. This assay provides information relating to the cells' anchorage-independent proliferation ability, which is considered as a hallmark of carcinogenesis.

Secondly, with the aim to investigate the role of ABCB5 in melanoma progression, we decided to assess the migration and the invasion of the melanoma cells expressing the different mutants using the Boyden chambers assay.

1.2. Justification of the cellular model used

Previously to the current study, Madigan and Gillet did explore the possible functional effects of ABCB5 mutations on cell growth of A375 and SK-Mel-28 melanoma cell lines (both expressing ABCB5 WT and mutated $BRAF^{V600E}$). To do so, human melanoma cell lines were transduced to overexpress either WT ABCB5 or mutants of ABCB5 (Q187*, S830F, S1091F, S1184P). The proliferation rates on plastic of A375 WT ABCB5 and mutants were identical to parental cells. SK-Mel-28 cells overexpressing either the Q187* or S1184P mutant had significantly increased proliferation rates compared to parental cells and the cells expressing other ABCB5 mutants. Stable knockdown of ABCB5 in A375 cells had no effect on cell proliferation. In contrast, stable knockdown of ABCB5 in SK-Mel-28 cells resulted in a significantly increased proliferation rate.

Anchorage-independent growth was next assayed using a standard soft agar assay. In all cell lines examined, colony numbers were not different, but colony sizes varied. In the A375 cell line, all four ABCB5 mutant cells formed significantly larger colonies compared to parental cells. Examining SK-Mel-28 cells, only the Q187* and S1184P mutant led to significantly larger colonies compared to parental cells. In both cell lines, stable knockdown of ABCB5 resulted in significantly larger colonies in soft agar.

In both A375 and SK-Mel-28 cell lines, there was a slight trend toward increased invasion of the ABCB5 mutant cells, along with a trend toward reduced invasion in WT ABCB5 cells. However, these trends were not significant. For both A375 and SK-Mel-28 cell lines, stable knockdown of ABCB5 resulted in a significant increase in invasive capacity, suggesting that ABCB5 transporter behaves as a tumor suppressor (see article in annexes).

In this thesis project, we wanted to test the effect of the mutations on additional, perhaps more adequate cell models. To select them, further studies on the first set of 54 human melanoma samples analyzed were carried out. These studies showed mutations in the tumor suppressor *CDKN2A* gene and the *NRAS* oncogene in 62.5% and 75% of the samples, respectively, which had mutations in the ABCB5 gene. No mutation was found in the tumor suppressor *PTEN* gene, while the activating V600E mutation in the *BRAF* oncogene was found in 25% of the samples with a mutated ABCB5 gene.

Therefore, we have chosen the 17T and 63T human melanoma cell lines, both harboring the activated mutant $NRAS^{Q61K}$. The 17T cell line also harbors the heterozygous nonsense mutant $ABCB5^{Q187*}$, wild type *BRAF, PTEN* and *CDKN2A*. The 63T cell line carries WT *ABCB5*, WT *BRAF*, nonsense mutant $PTEN^{R130*}$, and knockout mutant *CDKN2*A.
Human melanoma cell lines 17T and 63T were produced that stably overexpressed either WT ABCB5 or mutants of ABCB5 (Q187*, S830F, S1091F, S1184P).

1.3. Impact of the mutations on the proliferation, migration and invasion ability of human melanoma cell lines

1.3.1. Proliferation test on plastic

To investigate the possible effects of ABCB5 on melanoma cell growth, *in vitro* proliferation on plastic was first examined. Cells were seeded in a 96-well plate and the proliferation was assayed by MTT assay at 24 hour-time points.

The 17T cells overexpressing ABCB5 mutants show a statistically highly significant increased proliferation rate compared to the cells overexpressing the wild-type ABCB5 (**Figure 24A**). The impact of the ABCB5 mutations is lower in the 63T cells except for the mutation S1184P, for which the increase of the proliferation rate is highly statistically significant when compared to the 63T cells overexpressing ABCB5 wild-type (**Figure 24B**).

This proliferation assay indicates a difference in terms of proliferation capacity of the cells. Yet, in this assay the proliferation of the cells is influenced by their ability to adhere to plastic. To address this bias, anchorage-independent growth, which is considered as a hallmark of carcinogenesis, was next studied using a standard soft agar assay.

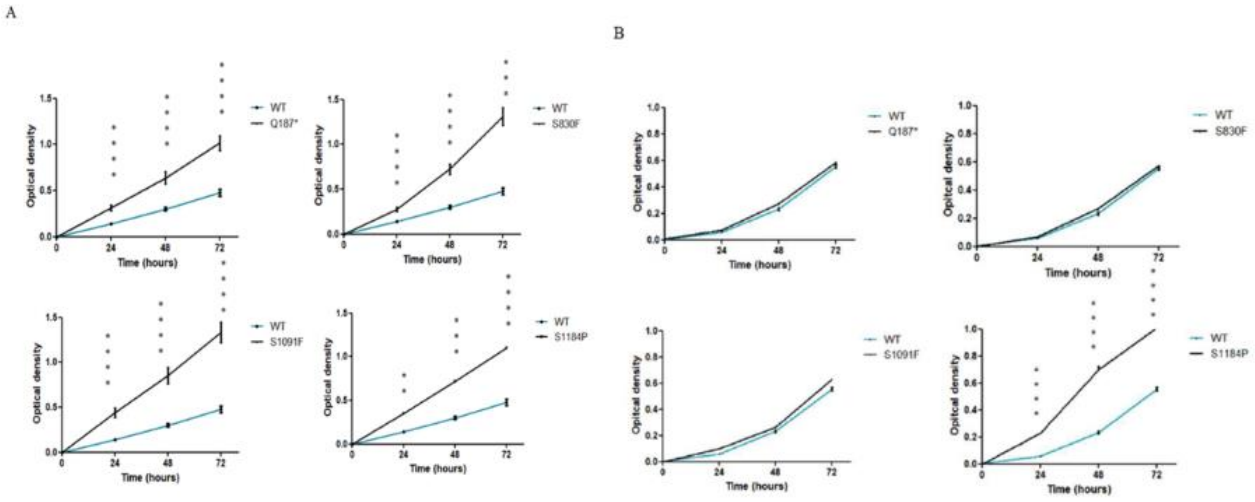

Figure 24: Effects of *ABCB5* mutations on proliferation of melanoma cells. (A) Proliferation rates of 17T cells were assayed over 3 days. All 17T cells overexpressing ABCB5 mutants had a significant increase of their proliferation rate compared to the ABCB5 WT overexpressing cells, for each time point. (B) Proliferation rates of 63T cells were measured at 0, 24, 48 and 72 hours post-seeding. The proliferation rate of the S1184P mutant only was found to be very highly statistically significant when compared to the parental WT 63T cells. *P<.05, **P<.01, ***P<.001,****P<.0001. n = 4. Values are the means ± S.E.M.

1.3.2. Soft Agar colony formation assay

The soft agar colony formation assay is an anchorage independent growth assay, considered as a hallmark of carcinogenesis. Indeed, transformed cells are able to proliferate without fixation on a substrate (Borowicz et al. 2014). To perform this assay, melanoma cells were seeded in an semi solid medium (0,33% Bacto-Agar RPMI media) on a layer of Bacto-Agar (0,5%). After three weeks at 37°C, colonies were stained and counted.

For both cell lines, colony number was significantly higher for cells expressing ABCB5 mutants compared to the WT ABCB5-expressing cells. Again for this assay, we observed a greater effect for the 17T cell line ($p<.0001$ for each mutation except for the mutation S1091F for which $p<.001$) than for the 63T cell line ($p<.0001$ for S830F and S1091F, $p<.05$ for the mutations Q187* and S1184P) (**Figure 25**).

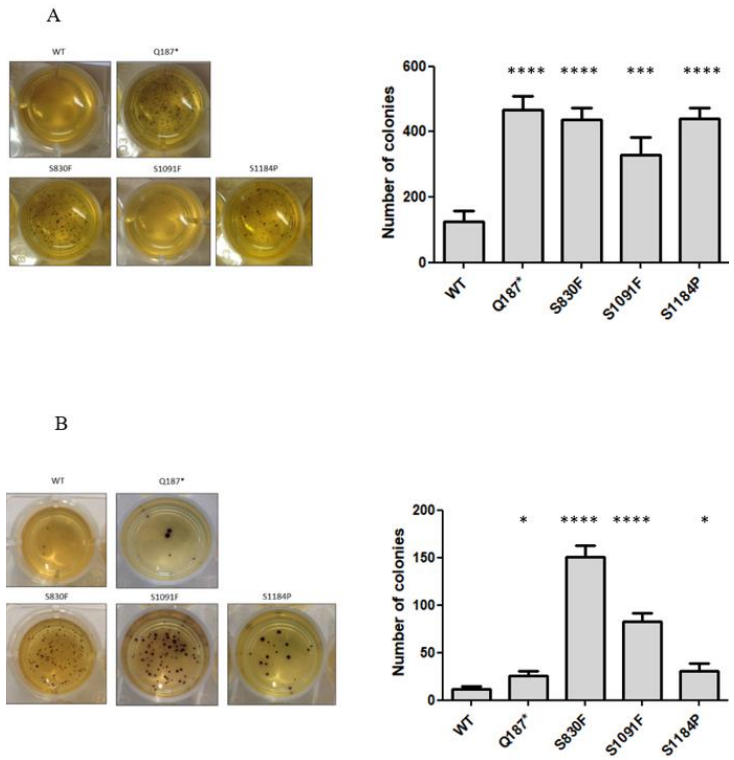

Figure 25 : Effects of ABCB5 mutations on anchorage-independent growth of melanoma cells. **A.** All 17T ABCB5 mutant cells had a highly significant increase in number of colonies by comparison with the 17T ABCB5 WT cells. **B.** The 63T mutant cells only showed a highly significant increase in number of colonies for the mutations S830F and S1091F, while the effect was lower for the Q187* and S1184P mutations. *P<.05, **P<.01, ***P<.001, ****p<.0001. n=3. Values are the means ± SEM.

1.3.3. Migration and invasion assay

The role of ABCB5 in melanoma cell migration and invasion was then examined. To do so, cells were seeded in a Boyden chamber in serum-free medium. Cells tend to migrate through the pores to reach the other side of the membrane containing serum-enriched medium. In the invasion assay, cells must additionally digest the 3D-matrigel layer located at the bottom of the Boyden chamber to reach the lower compartment. After 24 hours, the cells were stained and counted. The 17T overexpressing ABCB5 mutants showed a higher migration ability for the mutations Q187* and S1091F, while the cells overexpressing ABCB5 S830F were not able to migrate through the pores of the Boyden chamber membrane (**Figure 26A**). The migration ability of 63T cells overexpressing ABCB5 mutants was also affected and was significant for the mutations S830F, S1091F and S1184P (**Figure 26B**). Both 17T (**Figure 26C**) and 63T cells (**Figure 26D**) were not able to invade through the matrigel layer.

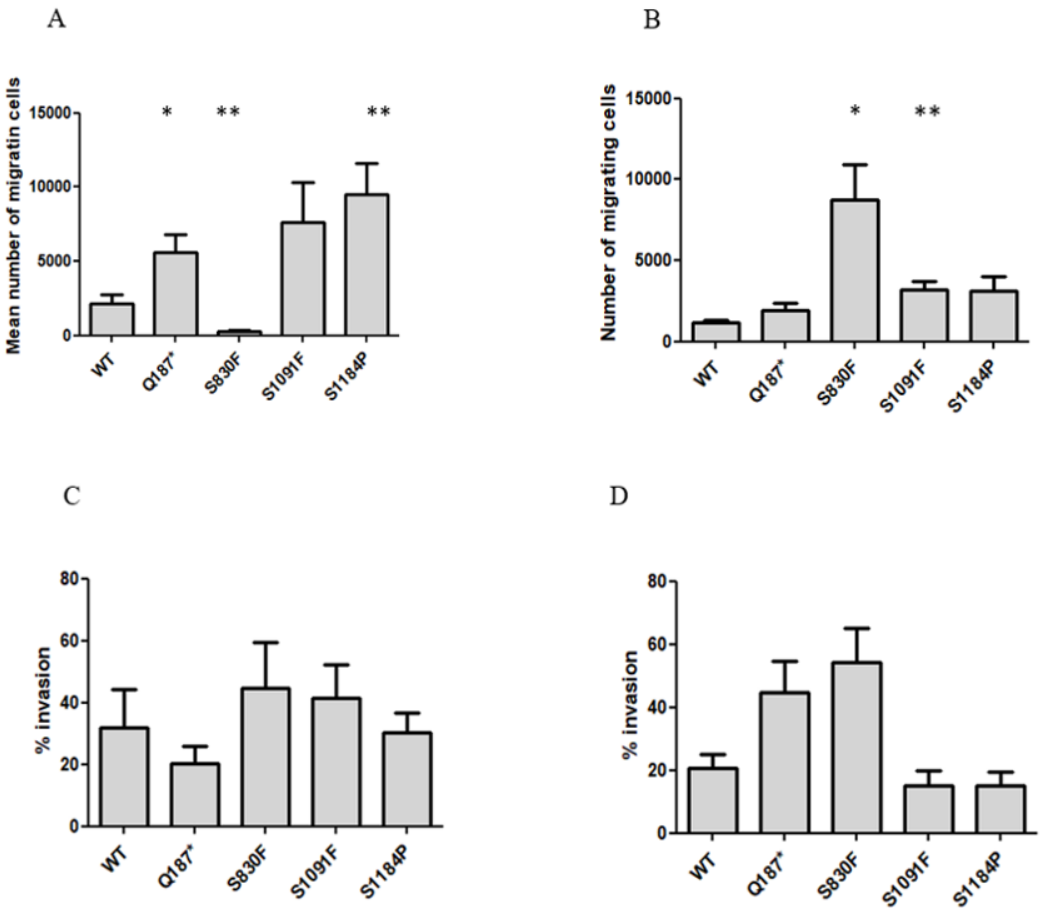

Figure 26: Effects of ABCB5 mutations on migratory and invasive abilities of melanoma cells. **A.** 17T ABCB5 Q187* and S1091F mutant cells showed a higher migration ability, while the ABCB5 S830F mutant cells was not able to migrate from the upper side of the Boyden chamber to the lower one. **B.** The migration ability of 63T ABCB5 mutant cells was also affected and was significant for the mutations S830F, S1091F and S1184P. **C.** The invasive ability of 17T ABCB5 WT and mutant cells was assessed but no significant increase in invasion was observed. The p-values (student t test) are: 0.4187 for Q187*, 0.5458 for S830F, 0.5856 for S1091F and 0.8927 for the mutation S1184P. **D.** The invasive ability of 63T ABCB5 WT and mutant cells was assessed but no significant increase in invasion was observed, but for S830F mutation. The p-value (student t test) are: 0.0885 for Q187*, 0.0458 for S830F, 0.4291 for S1091F and 0.4215 for S1184P. *P<.05, **P<.01, ***P<.001,****p<.0001. n=3. Values are the means ± SEM.

1.4. Discussion

The expression of ABCB5 mutants Q187*, S830F, S1091F, S1184P in melanoma cells is associated with an increase of their proliferation ability. This was shown by the proliferation test and the soft agar colony formation assay. All 17T cells overexpressing ABCB5 mutants have a statistically highly significant increased proliferation rate compared with the WT ABCB5 cells (**Figure 24**). The impact of the *ABCB5* mutations is lower in the 63T cells except for S1184P, for which the increase of the proliferation rate is highly statistically significant compared with the parental WT 63T cells. For both cell lines, colony number was significantly higher for cells expressing ABCB5 mutants compared with the WT ABCB5-expressing cells (**Figure 25**).

The mutations were also associated with the migration ability of melanoma cells while the invasion ability is not affected, except for the 63T S830F mutant (**Figure 26**).

1.4.1. The increase of proliferation is higher for the cell line 17T than for the cell line 63T

The proliferation test on plastic and the soft agar colony formation assay showed that the proliferation ability of the cells is higher when the cells express ABCB5 mutants. A difference is observed between the cell lines 17T and 63T. Concerning the proliferation test on plastic, the effect is highly significant for the cell line 17T for each mutation, while there is no effect for the 63T cells (except for the mutation S1184P). While the effect is significant for the 63T in the soft agar colony formation assay, the effect observed is higher for the cell line 17T. This difference could be explained by an endogenous expression of ABCB5. Indeed, the 63T cells are wild type for ABCB5 whereas the 17T cells present the heterozygous non-sense mutation Q187*. We cannot exclude that this difference of effect could be due to a difference in terms of genetic background between the cell lines 63T and 17T. While both are harboring $NRAS^{Q61K}$ mutation, the 63T cells present two additional mutations: they have the mutation $PTEN^{R130*}$ and are knock out for *CDKN2A*. We can hypothesize that the effect of the expression of ABCB5 mutants is less visible in this cellular model with other mutations of tumor suppressor genes. This could prevent us to observe the effect of ABCB5. However, the impact of these multiple mutations would be an increased proliferation of the cell line 63T. This is not the case; their proliferation rate is equivalent to that of 17T cells. Other unidentified factors can affect the proliferation ability of the cells.

1.4.2. Difference between the results of the proliferation test on plastic and soft agar colony formation assay

While the results are consistent for the cell line 17T between the proliferation test and the soft agar colony formation assay (with a highly significant effect), the results are different between these two assays for the cell line 63T. No statistical effect is observed for the proliferation on plastic test (except for the mutation S1184P) whereas there is a significant difference in the colony formation assay for each mutation. Since the difference between these assays is that the proliferation test is influenced by the ability to adhere to plastic, we may hypothesize that the absence of effect could be related to this bias. We can consider the colony formation assay as more reliable. Soft agar colony formation assay, which remains a hallmark in cancer research, is one of the most reliable assay to assess the tumor suppressive function of a protein. This assay allows a quantitative assessment of cells tumorigenecity (Borowicz et al. 2014; Du and Zhao 2017; Horibata et al. 2015). The colony formation assay mimics the proliferation conditions *in vivo* whereas it is not the case in 2D monolayer culture. It allows a quantitative assessment of the tumorigenecity (Horibata et al. 2015).

It is surprising to observe a striking difference between the results obtained for the S1184P mutation in 63T cells in the proliferation and soft agar colony formation assays. The 63T cells mutants S1184P presented the highest proliferative ability when they adhered to plastic whereas it is not the case in a semi-solid media. So far, we are still not able to explain this observation.

1.4.3. Comparison between the studied mutations

For the cell line 17T, the proliferation ability of the cells is identical for the different mutations. On the other hand, the proliferative ability of the 63T cells varies according to different mutations.

The results of the soft agar colony formation assay for the cell line 63T showed a higher effect for the mutations S830F and S1091F. This does not seem to be related to the type of domains in which the mutated amino acid is present. Indeed, the mutations S830F and S1091F have different locations, respectively in the TMD2 and in the NBD2.

After the transduction, the expression level of ABCB5 was assessed by RT-qPCR. For the 17T cell line, the ABCB5 S830F mutant was found to be 1.7 fold overexpressed when compared with its ABCB5 WT counterpart. The expression levels for the S1091F and S1184P mutants were found to be 1.6 and 1.3-fold overexpressed when compared to the ABCB5 WT expressing cells. For the 63T cell line, the ratio is higher for the mutations Q187* and S830F (1.5 and 3, respectively) and lower for the mutations S1091F and S1184P (0.80 and 0.27). This experiment was repeated but we did not manage to control this bias. This variation could explain the differences observed between the different mutants melanoma cells. This result is highly reproducible even if there is a variation in terms of expression level. Moreover, this experiment was performed on cellular population. This method reduced the heterogeneity, which could be explained using clones.

1.4.4. The expression of ABCB5 mutants (Q187* and S1184P in 17T cells and Q830F and S1091F in 63T cells) increase the migration ability of cells when compared to the ABCB5 wild type counterparts.

The results of the migration assay suggest the involvement of ABCB5 in the acquisition of melanoma cells migration capacity.

The 17T cells expressing ABCB5 mutants Q187* and S1184P have a higher migration ability than the cells expressing ABCB5 wild type. The 17T cells expressing the mutants S830F are not able to migrate through pores of the Boyden chambers. We cannot explain why these cells are not able to migrate but the same mutation is associated to the highest migration ability for the cell line 63T. Thus, one may hypothesize that this absence of migration is not associated with the mutation (**Figure 26**).

All the 63T cells expressing ABCB5 mutants have a higher migration ability than the 63T cell overexpressing ABCB5 wild type. The difference was statistically different for the mutations S830F and S1091F. The non-sense mutant Q187* is associated with the lowest migration ability. Again, this could be due to the compensation by the overexpression of other ABC transporters as it was suggested previously (**Figure 26**). Other ABC transporters were shown to be associated to the migration ability of cancer cells as explained in the next section.

At this stage, we observed that mutations in ABCB5 increased the migration ability of the cells. The migration ability is an important factor for the metastases development.

1.4.5. Other ABC transporters are involved in tumorigenesis *in vitro*

Several studies showed the involvement of ABC transporters in the acquisition of malignant potential. Recurrently, the expression of ABC transporters was shown to be associated with proliferation, migration and invasion abilities of cancer cells. However, the underlying mechanisms are often unknown. In this section, we reviewed the literature and discuss some experiments, which revealed the involvement of ABC transporters in tumorigenesis with a greater attention to ABCB1. Indeed, this transporter is very close to ABCB5 and some tracks have been explored about how this process happens. We also briefly develop ABCC4 and ABCG2.

1.4.5.1. *ABCB1*

Several studies showed that the expression of ABCB1 is associated with a more important tumorigenic potential. Already in 1999, Lehne has shown that the inhibitor of ABCB1, valspodar, provokes the arrest of the cell cycle and apoptosis in human leukemia cells (Lehne et al. 1999). This was confirmed by several studies in different cancer cell lines. In the case of colon cancer, the inhibition of the expression of ABCB1 by siRNA is associated with a decreased cell proliferation *in vitro* (Katoh et al. 2008).

In the case of breast cancer, silencing ABCB1 increased the migration of MCF7 cells when transwell migration and invasion assay were performed (Miletti-gonzalez et al. 2005). Co-immunoprecipitation of ABCB1 and CD44, a receptor on the plasma membrane responsible for adhesion, motility and metastases development, was observed in the case of breast cancer and melanoma (Colone et al. 2008; Miletti-gonzalez et al. 2005). Colone and colleagues investigated the role of ABCB1 in the invasion phenotype of melanoma cells. ABCB1 and CD44 colocalized in the plasma membrane and were associated with a more invasive phenotype. This was shown using transwell chamber invasion assay. Moreover, Colone identified a link between ABCB1 and the MAPK pathway. During the passage through matrigel, the ABCB1 expressing M14 cells adopted a different behavior than the parental M14 cells. In the ABCB1 expressing cells, the activation of ERK1/2 and p38 leaded to an increase of the matrix metalloproteinase MMP2, MMP3 and MMP9 mRNAs (Colone et al. 2008). Luciani and colleagues showed the interaction between ABCB1 and actin in human cell lines of lymphoid origin. Association of ABCB1 and actin could also explain the involvement of ABCB1 with the migration ability of these cells (Luciani et al. 2002). The interaction between plasma membrane and the cytoskeleton is involved in cell motility.

In the case of carcinoma cells, the interaction between ABCB1 and the drug transported induced the “membrane ruffling”. This phenomenon, essential for cell motility is an indicator of the metastatic potential and is due to the activation of the PI3k pathway (Yang et al. 2002). The membrane ruffling is the formation of a membrane protrusion enriched in actin (Mahankali et al. 2011).

1.4.5.2. *ABCC4*

ABCC4 has an impact on the migration ability of the cells by regulating the intracellular concentration of cyclic nucleotides, involved in cell migration. MEFS isolated from the mouse knock out for ABCC4 have a higher cAMP intracellular concentration and migrate faster than MEFS isolated from WT mice (Copsel et al. 2011; Sinha et al. 2013) These studies directly affect the probability of metastases development. Moreover, it was shown that F-actin was a downstream target of ABCC4, F-actin being involved in the formation of invadosomes (Kryczka et al. 2017; Sinha et al. 2016). The cancer associated fibroblasts play a crucial role in the invasion of many cancers including melanoma (Kryczka and Boncela 2018). Melanoma cells are able to recruit and activate fibroblasts (Flach et al. 2011). The inhibition of the expression of ABCC4 in neuroblastoma and pancreatic cancer cells is associated with a decreased cell proliferation (Henderson et al. 2011; Zhang et al. 2012). The silencing of ABCC4 also inhibits the proliferation of smooth cell muscle by cAMP independent signaling pathway (Copsel et al. 2011).

1.4.5.3. *ABCG2*

The down regulation of ABCG2 inhibits the migration and the invasion of pancreatic cancer cells (F. Wang et al. 2010). Chen identified that the silencing of ABCG2 expression leads to G0/G1 cell cycle arrest in the MCF7 and A549 cell lines. The silencing of ABCG2 is associated with the downregulation of cyclin D3 and p21. The expression of ABCG2 was also shown to be associated with the progression of the laryngeal squamous cell carcinoma. The silencing of ABCG2 inhibited tumor growth by regulating cellular proliferation and apoptosis. MAPK pathway regulates ABCG2. Indeed, inhibitors of MAPK pathway decreased the cellular proliferation and promote apoptosis by degrading endogenous ABCG2 (Xie et al. 2014).

1.4.5.4. *ABCC1, ABCA1 and ABCC7*

ABCC1 is implicated in the development of neuroblastoma. In vitro studies show that the inhibition of the ABCC1 expression is associated with an increase of apoptotic cells (Peaston et al. 2001). In cell lines overexpressing MYCN, the inhibition of ABCC1 decreased the colony formation and the migration ability (Henderson et al. 2011).

ABCA1 seems to be involved in ovarian cancer and prostate cancer biology. Indeed, its expression is correlated with a higher proliferation and migration ability of the cell (Her et al. 2013; Sekine et al. 2010).

The inhibition of the expression of ABCC7 is associated with the inhibition of the migration, invasion, proliferation and adhesion abilities in ovarian cancer (Xu et al. 2015).

1.5. Conclusion 1 about the involvement of ABCB5 in Melanomagenesis in vitro

ABCB5 seems to be involved in melanomagenesis as a tumor suppressor *in vitro* in human melanoma cells 17T and 63T both harboring the *NRas*^{Q61K} activating mutations. Indeed, the cells overexpressing ABCB5 mutants Q187*, S830F, S1091F and S1184P showed a higher proliferation ability than cells overexpressing ABCB5 wild-type. This was shown by the proliferation test on plastic and by the soft agar colony formation assay.

Furthermore, our results suggest that ABCB5 could also play a role in tumor progression. Indeed, inactivating mutations of ABCB5 leads to an increase of the migration ability of melanoma cells. However, no trend was observed for the invasive ability of the melanoma cells. The implication of ABCB5 in the migration ability suggests its involvement in the development of metastases. However, the underlying mechanism has yet to be unraveled.

ABCB5 is not an isolated case among ABC transporters. Indeed, we discussed the case of several ABC transporters involved in the acquisition by the cells of malignant properties: ABCB1, ABCC1, C4, ABCG2, etc. While the precise mechanism is still unclear, we hold that ABC transporters may impact the organization of the cytoskeleton and the signaling pathways. The involvement of ABCB1 and ABCG2 in cancer biology was associated with the MAPK pathway. This seems to be a good option to pursue, knowing that this pathway play a key role in melanoma development.

2. Study of the implication of ABCB5 in melanomagenesis in vivo

2.1. Objectives of the in vivo project

While the *in vitro* results showed that the expression of ABCB5 mutants is associated with an increased ability of proliferation and migration of melanoma cells, the second step was to investigate the involvement of ABCB5 in melanomagenesis *in vivo*.

This experiment had on purpose to assess the penetrance of *Abcb5* using a reduced number of animals. We aimed to investigate the impact of the silencing of *Abcb5* on tumor incidence. Knowing that carcinogenesis is the results of multiple genetic alterations, we aimed to combine the silencing of *Abcb5* with other genetic alterations of oncogene/tumor suppressor genes.

To do so, we decided to develop three transgenic mouse models (*Abcb5TyrNRas*^{Q61K}, *Abcb5TyrHRas*^{G12V} and *Abcb5Ink4a/Arf*$^{flox/flox}$*TyrCre*) by crossing the mouse model knock out for Abcb5 (developed by Gillet and colleagues) and pre-existing mouse models. The explanations about the genetic constructs are provided on pages 76, 79 and 82. Among the three mouse strains, we aimed to compare the incidence of melanoma between the Abcb5 knock out mice and the wild type mice.

2.2. Justification of the murine model used

As aforementioned, mutations in the *NRAS* gene are present in 20% of melanoma (Jakob et al. 2013). While *NRas* was mutated in 75% of melanoma samples mutated for Abcb5 (see in the preliminary section), we hypothesized that the activated NRas and inactivated Abcb5 could act synergistically to lead to melanomagenesis.

However, while the existing mouse model expressing NRas^{Q61K} leads to the apparition of metastases (Ackermann J. et al. 2005), it would not allow to investigate the putative involvement of Abcb5 in the apparition of metastases.

To determine the involvement of Abcb5 in melanoma progression, we aimed to determine the frequency of metastases apparition. To explore this, we used the model *TyrHRas*^{G12V} that does not lead to the apparition of metastases in distant organs.

The rationale for using the *Ink4a/Arf*$^{flox/flox}$*TyrCre* model is that, among the human melanoma samples mutated for ABCB5, 62,5% were also mutated for *CDKN2a*. This led us hypothesize that Abcb5 and Cdkn2a may act synergistically and provoke melanoma development. To answer this question, we have chosen the model *Abcb5Ink4a/Arf*$^{flox/flox}$*TyrCre*.

The mouse strain *Abcb5TyrNRas*^{Q61K} spontaneously expresses the activated NRas^{Q61K} while the mouse strains *Abcb5TyrHRas*^{G12V} and *Abcb5Ink4a/Arf*$^{flox/flox}$*TyrCre* need to be injected with 4-hydroxytamoxifen respectively to express the activated *HRas*^{G12V} or to lose the expression of the p16 and p19 transcripts.

As a negative control, four mice knock out for Abcb5 were injected with 4-hydroxytamoxifen and three with peanuts oil (used as vehicle).

2.3. Results of the different mouse models

2.3.1. The mouse strain knock out for Abcb5

Gillet and colleagues developed the Abcb5 knockout mouse strain (unpublished data). It is characterized by excision of the exon 2. No change in melanoma prevalence was observed in the case of this mouse strain.

This mouse strain was used in our experiment as a control. Most mice knockout for *Abcb5* present relatively large and dark spots on the skin at macroscopic level. Histological sections revealed a slight melanocytic incontinence and few melanophages were identified in the dermis (**Figure 27**).

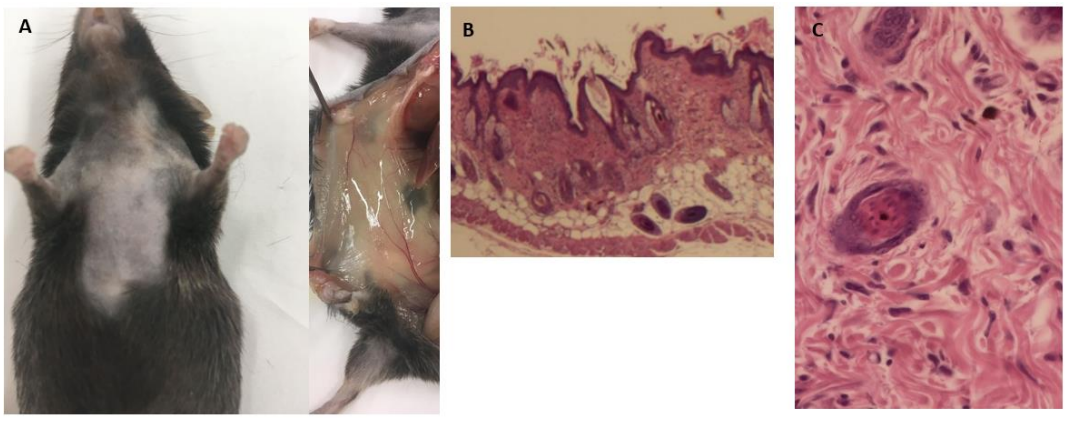

Figure 27: Slight dark spots were observed on the skin of some mice knock out for *Abcb5*. Microspically, very few melanophages were identified.

2.3.2. The mouse strain Abcb5TyrNRas^{Q61K}

2.3.2.1. Description of genetic constructs

The mouse strain *Abcb5TyrNRas^{Q61K}* was developed in our laboratory by crossing the *Abcb5* knockout mice with *TyrNRas^{Q61K}* mice developed by Ackermann and colleagues (Ackermann J. et al. 2005).

This mouse strain presents two genetic constructs. The first one will allow the excision of the exon 2 of the *Abcb5* gene and the second one, the activation of *TyrNRas^{Q61K}*. The latter is composed of the promoter of tyrosinase, followed by the *NRas* gene exhibiting the activating mutation Q61K and *SV40pA*, a polyadenylation signal. The expression of the activated NRas^{Q61K} is under the control of the tyrosinase promoter (**Figure 28**).

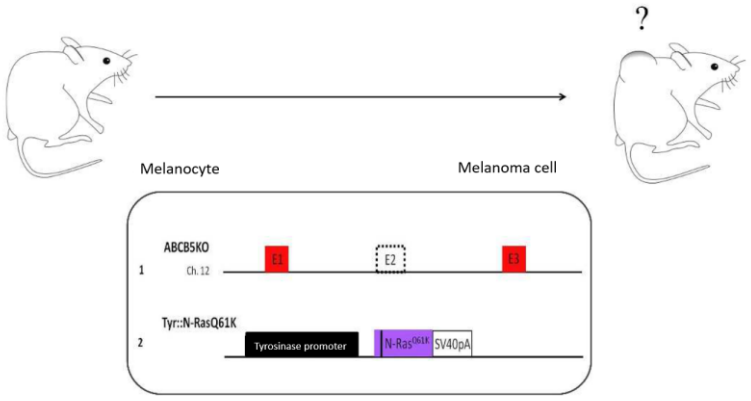

Figure 28: The mouse strain *Abcb5TyrNRas^{Q61K}* contains two genetic constructs: the excision of the exon2 of Abcb5 and the transgene *NRas^{Q61K}* under the control of the tyrosinase promoter.

2.3.2.2. Results obtained with the mouse strain Abcb5TyrNRas^{Q61K}

The mice of the mouse strain *Abcb5TyrNRas^{Q61K}* express spontaneously the activated oncogene NRas^{Q61K}. Mice were genotyped and monitored during 18 months.

The most prominent observation made on mice harboring *NRas^{Q61K}* was an hyperpigmentation of the skin as already described by Ackermann and colleagues (Ackermann J. et al. 2005). In most mice of this model, an invasion of melanocytes in the dermis was observed. At the macroscopic level, we observed little dark spots on the skin.

In tumors, melanocytes called “plump cells” and melanophages were observed. The plump cells are angular melanocytes with abundant cytoplasm densely pigmented.

In all mice, the invasion of lymph nodes was observed. At macroscopic level, no difference could be observed in other organs scrutinized.

The mice of this mouse strain are divided in two groups: the mice knockout for *Abcb5* and the mice wild type for Abcb5. We aimed to compare the tumor incidence between these groups.

Seven mice **knockout for *Abcb5*** were dissected. Among them, five mice (5/7) developed a tumor. Two tumors were sent to our collaborator specialized in anapathology Marianne Heimann. They were identified as melanoma. One mouse knockout for Abcb5 presented several tumors on the back. These tumors were melanotic. One tumor presented a melanotic part and an amelanotic part (**Figure 29**).

In the ***Abcb5* wild type** group, eleven mice were dissected. Five of them presented a cutaneous tumor. One tumor has been sent to our collaborator and the diagnosis of melanoma was confirmed. One eye was analyzed. At the level of the eyeball, there is a deposit of plump-like melanocyte in the choroid. It forms a lichenoid infiltrate throughout the posterior chamber.

Five out of seven mice knockout for Abcb5 developed tumors, while the proportion is of 5/11 for the mice wild-type for Abcb5. A fisher test, which aims to compare proportions, was performed. This statistical test did not reveal any significant difference (**Table 4**).

Table 4: Contingency table of the apparition of cutaneous tumors for the mouse model *Abcb5TyrNRas*^{Q61K}

	KO *Abcb5*	WT *Abcb5*	
Tumor	5	5	10
No tumor	2	6	8
	7	11	

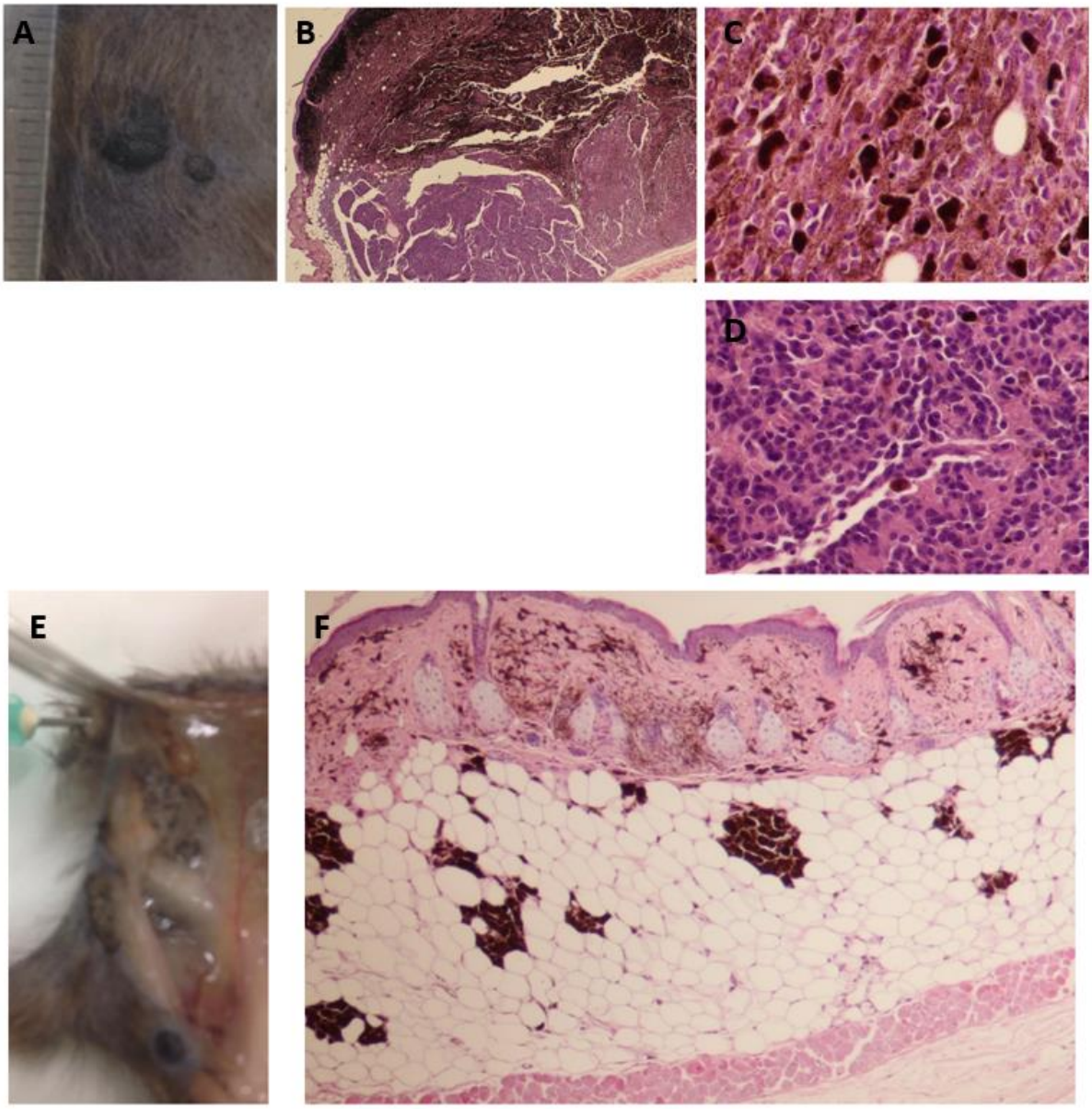

Figure 29: This mouse knock out for *Abcb5* and expressing the activated $NRas^{Q61K}$ present several melanotic tumors visible macroscopically (A). These tumors were stained with hematoxylin (B) and we distinguish a highly pigmented area where plump cells and melanophages were identified (C). Plump cells were also identified in the less pigmented area (D). All mice of this mouse strain present hyperpigmentation of the skin and most of them presented little darker spots on the skin (E). This reflect the invasion of plump cells in the dermis and hypodermis (F).

2.3.3. The mouse strain Abcb5TyrHRas^{G12V}

2.3.3.1. Description of genetic constructs

The mouse strain *Abcb5TyrHRas*^{G12V} was obtained by crossing the mice *Abcb5* knockout (Gillet and colleagues- unpublished data) and the mice carrying the *TyrHRas*^{G12V} construct (Huijbers et al. 2006).

The mouse strain *Abcb5TyrHRas*^{G12V} includes two genetic constructs (**Figure 30**). The first one to allow the deletion of the exon 2 of *Abcb5* gene and the second carrying the *Tyr-RasP1A* transgene. This one is composed of a gene *CreERΔD* flanked by two LoxP sites, followed by the mutated gene *HRas*^{G12V} and by a gene coding for the tumor-specific antigen *P1A*. The entire second construct is under the control of the tyrosinase promoter. The transgene *CreER*$^{\Delta D}$ codes for the Cre recombinase fused to a mutated estrogen receptor. Without 4-OHT (4-hydroxytamoxifen) administration, the fused Cre-ER protein is sequestrated in the cytoplasm by the HSP90 chaperone. If 4-OHT is administrated, this ER receptor binds to 4-hydroxytamoxifen with high affinity and promotes the dissociation of Cre-ER from HSP90 (Lepper and Fan 2012). Then, the recombinase is able to translocate in the nucleus and to excise the sequence flanked by LoxP sites. In the case of this mouse strain, the recombinase will excise its own coding sequence allowing the expression of the activated HRas^{G12V}.

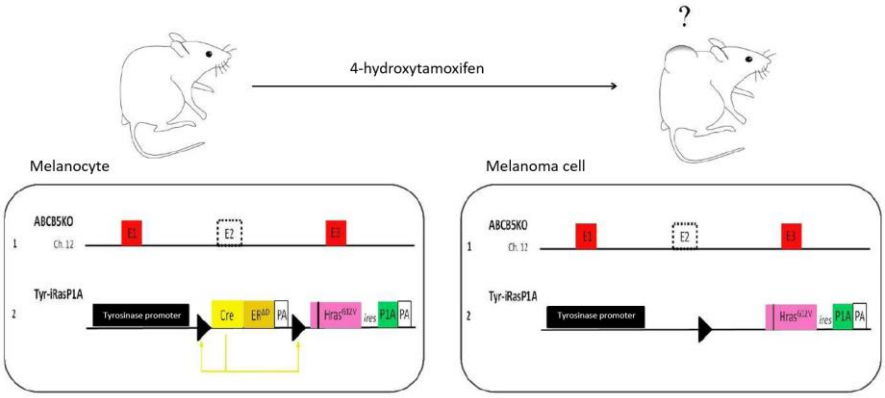

Figure 30: The mouse strain *Abcb5TyrHRas*^{G12V} contains two genetic constructs. The first one is the deletion of the exon 2 of *Abcb5* and the second is the transgene *Tyr-RasP1A* that is under the control of tyrosinase promoter. This latter transgene, floxed by two LoxP sites, contains the gene of the recombinase *CreERΔD* coding for the recombinase Cre fused with a mutated estrogen receptor. This is followed by the mutated gene *HRas*^{G12V} and by the gene of the tumoral antigen *P1A*. After administration of 4-hydorxytamoxifen, the Cre recombinase excises its own gene and allow the expression of the activated HRas G12V.

2.3.3.2. Results obtained with the mouse strain Abcb5TyrHRas^{G12V}

In the case of the mouse strain *Abcb5TyrHRas*^{G12V}, injection with 4-hydroxytamoxifen is needed to induce the expression of the activated HRas^{G12V}. Intraperitoneal injections were performed at the age of 7 weeks (four times at 48 hours interval, 2mg of 4-OHT) and mice were monitored during 18 months. Within this strain, mice are divided in two groups: the *Abcb5* knockout and the *Abcb5* wild type mice.

Nine mice **knockout for *Abcb5*** were dissected. None of them developed skin tumors (**Table 5**). Two mice presented dark spots on the skin but histological sections revealed an inflammatory process. One mouse developed hepatoma and two mice presented a cellular mass at the intestinal level. This mass was analyzed histologically and represented a lymphomatous neoplastic infiltrate.

Four ***Abcb5* wild type mice** were dissected. None of them developed a melanoma (**Table 5**). Two of them presented dark and large spots on the skin, comparable to the spots observed in the Abcb5 knockout mice.

Table 5 :Contingency table of the apparition of cutaneous tumors for the mouse model Abcb5TyrHRas^{G12V}

	Abcb5 KO	*Abcb5* WT
Tumor	0	0
No tumor	9	4

Two mice developed tumors without 4-hydroxytamoxifen injection. The first one developed metastasized melanoma without injection of 4-hydroxytamoxifen. The skin tumor was melanotic and was confirmed as melanoma by our collaborator. Plump cells were observed in the dermis. Some melanocytes similar to plump cells were found in the liver in the portal region. In the brain, pigmented cells were found in the choroid (**Figure 31**). The tumor that appeared in the second animal was identified as a schwannoma.

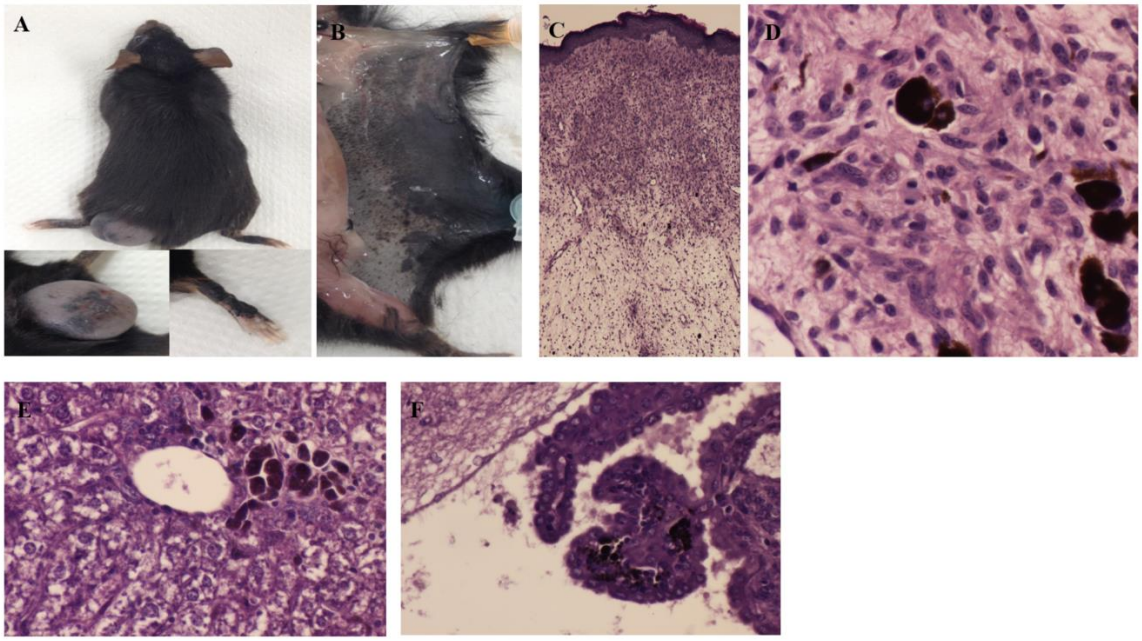

Figure 31: A mouse knock out for *Abcb5* of the mouse strain *Abcb5TyrHRas*^{G12V} develop melanoma without induction with 4-hydroxytamoxifen. The tumor was melanotic (A) and the skin of the mouse was highly pigmented (B). Inflammation was observed in the skin where plump cells and lymphocytes were identified (C, D). Melanocytes were observed in the liver in the portal region (E) and pigmented cells were found within the choroid plexus (F).

2.3.4. The mouse strain *Abcb5 Ink4a/Arf* $^{flox/flox}$*TyrCre*

2.3.4.1. Description of genetic constructs

The mouse strain *Abcb5KO Ink4a/Arf* $^{flox/}$*floxTyrCre* has three genetic constructs : the one allowing the deletion of the exon 2 of *Abcb5*, the *Tyr-Cre/ERT2-13Bos* transgene (Dankort et al. 2009) and the gene *Cdkn2a* whose exons 2 and 3 are flanked by two loxP sites (Huijbers et al. 2006). The second construct was composed by a fusion gene *CreER*$_{T2}$ under the control of the tyrosinase promoter and of its enhancer. This transgene codes for the Cre recombinase fused to a mutated estrogen receptor, selectively expressed in melanocytes (**Figure 32**).

Normally, Hsp90 sequestrates the recombinase Cre in the cytoplasm. Upon administration of 4-hydroxytamoxifen, Cre is able to excise the exons 2 and 3 of the *Cdkn2a* gene. As a reminder the gene *CDKN2A* encodes for two proteins: $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ in the case of mice). The $p16^{INK4}$ is formed by the expression of the exon 1α, exons 2 and 3, while p19 is formed from alternate splicing of the exon 1β with exons 2 and 3. Thus, the deletion of exons 2 and 3 leads to the silencing of the two transcripts.

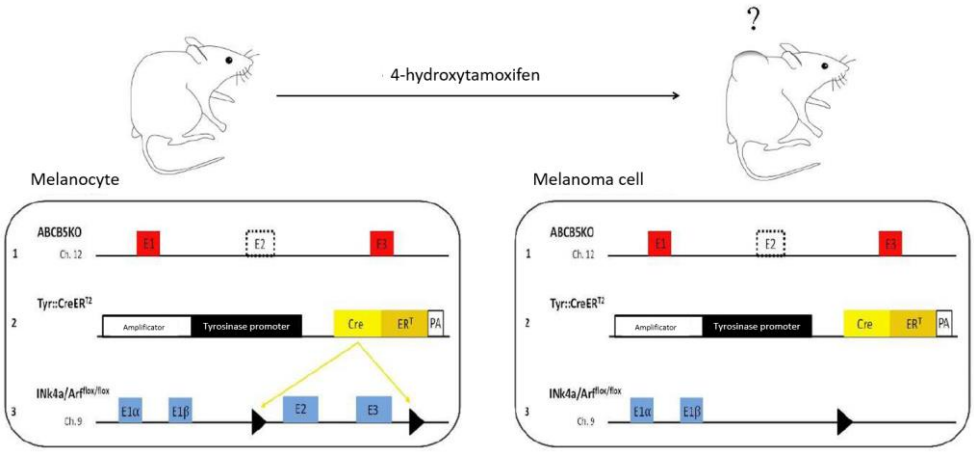

Figure 32: The mouse strain *Abcb5KO Ink4a/Arf $^{flox/flox}$TyrCre* present three genetic constructs. The first one is the deletion of the exon 2 of *Abcb5*, the second one is the gene *CreERT* under the control of the promoter of the tyrosinase. The third one is the presence of loxP sites around the exon 2 and 3 of *Cdkn2a* gene.

2.3.4.2. Results for the mouse strain Abcb5KO Ink4a/Arf $^{flox/flox}$TyrCre

In the case of the mouse strain *Abcb5KOInk4a/Arf$^{flox/flox}$TyrCre*, injection with 4-hydroxytamoxifen is needed to induce the deletion of the exon 2 and 3 of the Cdkn2a gene. Intraperitoneal injections were performed at the age of 7 weeks (four times at 48 hours interval, 2mg of 4-OHT) and mice were monitored during 18 months. Within this strain, mice are divided in two groups: the Abcb5 knockout mice and the Abcb5 wild type ones.

Mice of this model did not present any pigmentation peculiarities in the skin. The ganglia was never pigmented. Mice of this mouse strain are divided in two groups.

No melanoma development was observed in the group of eight mice knockout for *Abcb5* (**Table 6**). Three animals had dark spots on the skin but they were diagnosed as dermatitis. However, two animals developed other types of tumors. One mouse had a spleen tumor. Another mouse had a non-identified tumor, originating either from muscle tissue or from nerve sheath.

No mice in the group of four ***Abcb5* wildtype** developed a melanoma (**Table 6**). One member of this group presented a tumor in the small intestine. This tumor was analyzed by our collaborator and was identified as a lymphoma.

Table 6 : Contingency table of the apparition of cutaneous tumors for the mouse model *Abcb5TyrHRas*^{G12V}

	KO *Abcb5*	WT *Abcb5*
Tumor	0	0
No tumor	8	4

2.4. Discussion

The mice exclusively knockout for *Abcb5*, used as control, do not develop melanoma or other kinds of cancer. Among the mice *Abcb5TyrNRas*^{Q61K}, the proportion of mice developing tumors is of 5/7 in the *Abcb5* knockout group and of 5/11 in the *Abcb5* wild type group. The tumors were confirmed to be melanoma by our collaborator anapathologist (Marianne Heimann). Among the nine mice knockout for *Abcb5* of the mouse strain *Abcb5TyrHRas*^{G12V}, we did not observed any cutaneous tumors. However, we observed one case of hepatoma and two lymphomas. For the same mouse strain, no tumor was observed for the group Abcb5 wild type. We observed the development of one melanoma and one schwanoma without 4-hydroytamoxifen induction. The results for the mouse strain *Abcb5KO Ink4a/Arf* $^{flox/flox}$*TyrCre* showed one spleen tumor and one non-identified tumor over the eight mice corresponding to the knockout genotype. No melanoma was observed in the group of four *Abcb5* wild type mice, while one lymphoma was observed.

2.4.1. Tumor occurrence

The fact that mice exclusively knockout for *Abcb5* did not develop tumors does not mean that Abcb5 is not implicated in melanomagenesis. Indeed, tumorigenesis is a multistep process provoked by the “gain-of-function mutations in oncogenes and loss-of-function mutations in tumor suppressor genes”.

Many in vivo studies showing the implication of ABC transporters in tumorigenesis combined the expression of a given transporter with a mutated oncogene or tumor suppressor gene. For example to assess the involvement of *ABCB1* as an oncogene in the case of intestinal cancer, Mochida and colleagues made use of a mouse model with a truncated mutation in the tumor suppressor gene *APC* (*APC*$^{Min/+}$) (Mochida et al. 2003). The study of ABCC1 involvement in the development of neuroblastoma was performed in the mouse model expressing the oncogene *MYCN* (*Th-MYCN* mouse model of neuroblastoma) (Henderson et al. 2011).

For the ***Abcb5TyrNRas*^{Q61K} mouse strain**, an exact fisher test was performed to compare the tumor frequencies between the *Abcb5* wild type/knockout groups. This test did not allow us to draw a conclusion, this is probably due to the low number of animals that we could include in the experiment. We could also compare the experimental occurrence observed in the group *Abcb5 TyrNRas*^{Q61K} (5/7) with the occurrence of 30% reported by Ackermann for the *TyrNRas*^{Q61K} mice (Ackermann J. et al. 2005). The occurrence tends to be higher in our hands but the number of animals studied did not allow us to draw a conclusion.

In the case of the ***Abcb5TyrHRas*^{G12V} mouse strain**, no melanoma was observed; neither for the group knockout for *Abcb5*, nor for the group expressing wild type *Abcbb5*. However, other cancers developed in the group of the mice knockout for Abcb5. One hepatoma and one lymphoma were confirmed. We cannot draw any conclusion at this stage of the experiment. It is worth mentioning that it was already shown in 1995 that the only alteration of *HRas*^{V12G} is not sufficient for inducing a melanoma (Powell et al. 1995).

The ***Abcb5KO Ink4a/Arf* $^{flox/flox}$*TyrCre* mouse strain** did not show any melanoma development. Two tumors (one lymphoma and one unidentified tumor) appeared in the group of the mice knockout for *Abcb5*. One mouse wild-type for *Abcb5* developed a lymphoma.

Unfortunately, considering that tumor development was rarely observed, we cannot draw a conclusion at this stage. We started again this experiment with a number of 35 animals per group.

2.4.2. Development of tumors without induction with 4-hydroxytamoxifen

The development of tumors by two mice of the group *Abcb5TyrHRas*^{G12V} without 4-hydroxytamoxifen induction can be due to the result of the leakiness of the inducible Cre system. This means that the recombinase Cre will be translocated in the nucleus without tamoxifen induction. A phenomenon of leakiness was observed for the mouse model *BRaf*^{CA}*Pten*^{loxP}*Tyr::CreER*^{T2} developed by Jaxon Laboratory. Indeed, this strain is known to develop melanoma without induction with tamoxifen (Hooijkaas et al. 2012). The spontaneous development of melanoma in the case of this mouse strain was also observed in our laboratory (unpublished data). Leakiness was also observed in other mouse models like *RIP-CreER* (Liu et al. 2010). Steim and colleagues revealed a leakiness phenomenon using the system *Cre-ER*^{T2}*/LoxP*, performing a lineage tracking during fracture repair (Seime et al. 2015). However, in our case, the model of *TyrHRas*^{G12V} would be associated with a minor leakiness because the proportion of mice developing tumors is low. To confirm it, the recombination should be confirmed in the tumors.

2.4.3. Pigmentation of the skin

In the mouse strain **exclusively knock out for *Abcb5***, a very slight melanocytic incontinence was noticed. This could be the result of a role of the full-sized ABCB5 in melanogenesis while Chen suggests a role of ABCB5 α and β in Melanogenesis (K. G. Chen et al. 2009).

The hyperpigmentation of the skin observed in the case of the **mouse strain *B5TyrNRas*^{Q61K}** was also observed by Ackerman and colleagues. We noticed little very dark spots on the skin (**Figure 29**), a feature which was not described in the characterization of this mouse model. Microscopically, those dark spots are due to melanocytes found in the dermis. Melanophages observed in the dermis and the melanotic tumors were observed by Akermann (Ackermann J. et al. 2005).

The tumor of the mouse *Abcb5TyrHRas*^{G12V} developed without induction was melanotic.

2.4.4. Lymph nodes

The mice of the model *Abcb5TyrNRas*^{Q61K} present invaded lymph nodes by pigmented melanocytes like it was described by Ackermann for the characterization of the mouse model Abcb5TyrNRas^{Q61K} (Ackermann J. et al. 2005).

In the mouse model of Huijbers, they observed melanoma cells and melanophages in lymph nodes (Huijbers et al. 2006). In our hands, lymph node appeared to be healthy and no further analyzes were performed.

2.4.5. Metastases

Melanomas of the mouse strain *Abcb5TyrNRas*^{Q61K} metastasized in distant organs. However, while Ackerman noticed metastases in lung and liver, these organs macroscopically did not show any peculiarities in our study. Only one mouse presented dark spots on the lung. These observations indicate that we should consider an investigation on multiple organs in the rest of this study.

The absence of metastasis observed with the mouse strains *Abcb5TyrHRas*^{G12V} and *Abcb5Ink4a/Arf*$^{flox/flox}$*TyrCre* is consistent with the observation made by Huijbers and colleagues (Huijbers et al. 2006).

Among the mice of the model Abcb5TyrHRas^{G12V}, one mouse developed tumors and metastases without induction with 4-hydroxytamoxifen. Metastases were located in the liver and the brain (in the choroid plexus). While Huijbers and colleagues did not observe the development of metastasis in the model combining the activated mutation of *HRas*^{G12V} and the silencing of *Cdkn2a*, they did when they injected tumor cells with these genetic alterations in SCID mice. The metastases developed in the lung, the liver and the adrenal glands. Their hypothesis is that a step is missing in their model: the passage of the cancer cells to the blood circulation. In our study, metastases were observed in the model *Abcb5TyrHRas*^{G12V} for one *Abcb5* knockout mouse. If this is observed in other mice, we could hypothesize the implication of Abcb5 in the metastases development. Regarding the in vitro results, we could imagine the implication of Abcb5 in the acquisition by the cells of a higher migration ability.

2.4.6. Apparition of different types of cancers

The occurrence of cancers non-identified as melanoma is surprisingly high in the mouse model *Abcb5TyrHRas*^{G12V} and *Abcb5 Ink4a/Arf*$^{flox/flox}$*TyrCre*.

2.4.6.1. Lymphoma

One mouse *Abcb5Ink4a/Arf$^{flox/flox}$TyrCre* and one mouse *Abcb5TyrHRas^{G12V}* developed lymphoma. Lymphoma is a blood cancer developing from lymphocytes, which can originate from the B cells, T cells or from natural killer cells (E. N. Mugnaini 2018) . In the mouse model of Chin, the loss of the expression of Ink4a/Arf is not inducible. In this case, they observed lymphoma and fibrosarcoma (Chin et al. 1997). However, in our model, the genetic alterations are exclusively expressed in melanocytes because this is under the control of the tyrosinase promoter. To check this, we could detect the expression of the activated *HRasG12V* or the deletion of exon 2 and 3 in the *Cdkn2a* gene in the lymphoma cells.

There is an association between these malignancies. Some patients with non-Hodgkin's lymphoma develop subsequently melanoma (or non-melanoma skin cancer). This association would be due to the commonly shared genetic aberration (Brewer 2010). Lam and colleagues confirmed that patients surviving from non-Hodgkin lymphoma have a greater risk of developing melanoma. The underlying mechanisms remain unclear even if their data would indicate the role of T-cells dysfunction (Lam et al. 2015).

2.4.6.2. Schwannoma

In our study, we observed a particularly high number of schwannomas, tumors originating from Schwann cells.

One of the mouse strain *Abcb5 Ink4a/Arf$^{flox/flox}$TyrCre* developed a schwanoma as well as one mouse of the *Abcb5TyrHRas^{G12V}* strain without induction.

Studies show that there exists a link between melanocytes and Schwann cells. Both originate from neural crest (Woodhoo and Sommer 2008) and there are evidence showing that precursors of Schwann cells differentiate into melanocytes (Cichorek et al. 2013). Van Raamsdonk and colleagues point that melanocytes and Schwann cells exhibit some plasticity particularly in the process of transformation. Van Raamsdonk and colleagues claim that melanoma can show some histopathological features of glial of neural differentiation. The study of Fullen and Huttenbach showed that melanocytic nevi can follow schwannian differentiation (Fullen et al. 2001; Huttenbach et al. 2002).

However, Murali and colleagues describe melanotic schwannoma as rare, representing 1% of schwanomas (Murali et al. 2010). Melanotic schwannoma express tyrosinase and Pmel17, which are markers of melanocytes. They also contain melanosomes (Arvanitis 2010; Boyle et al. 2009). While these markers of melanocytes are also expressed in schwannoma cells, the distinction between melanoma and schwannoma appeared to be difficult. However, it is possible to determine if the tumor cell presents the recombination.

Huijbers, using the same mouse model, also observed the development of tumors described as Malignant Peripheral Nerve Tissue (MPNST). He developed arguments suggesting their melanocytic origins. The first one is that, in humans, MPNST are associated with neurofibromas or neurofibromatosis, while these kinds of lesions were not observed in its mouse model. Secondly, he also observed that tumors identified as pigmented melanomas presented areas of Schwann cell differentiation (Huijbers et al. 2006).

2.4.6.3. Studies showing the involvement of ABC transporters in melanomagenesis in vivo

Other ABC transporters were shown to be involved in tumorigenesis.

The expression of ABCB1 was shown to be associated with the aggressiveness of tumors using transplantable hamster melanoma (Witkowski JM et al. 2000). The silencing of ABCB1 in a mice xenograft tumor formation assay, inhibited tumor expansion (Katoh et al. 2008). Mochida and colleagues showed that ABCB1 was involved in tumorigenesis in the case of intestinal cancer. They silenced the expression of ABCB1 in the mouse model $APC^{MIN/+}$ (Mochida et al. 2003) .

ABCG2 was shown to be associated with a reduced median latency of breast tumor using the mouse model *BCRA1* mutated (Zander et al. 2012).

The inhibition of ABCC1 in the h*MYCN* transgenic mouse model inhibits the development of neuroblastoma. They observed a statistically significant delay in tumor progression. The oncogene *MYCN* is frequently expressed in case of neuroblastoma (Henderson et al. 2011).

2.5. Conclusion 2 and perspectives

At this stage, we cannot draw a firm conclusion on the involvement of Abcb5 in melanomagenesis *in vivo*. However, this pilot study paves the ground for further experiment on a larger set of mice

At first, as the frequency of the tumors is low, it shows us the need to increase the number of animals included in the experiment. It is now pursued with 35 mice per group.

Secondly, we should proceed to a systematic organ removal for each dissections. This is crucial to investigate the role of Abcb5 in the development of metastases. The immunohistochemistry with Antibodies recognizing Pmel17, TIRP1 or MART1 should definitively validate the diagnostic of melanoma.

The validation of the recombination should be performed. In the case of the mouse model *Abcb5 Ink4a/Arf* $^{flox/flox}$*TyrCre*, a PCR with three primers will be performed with DNA extracted from the tail and the tumor. Two primers are flanking the loxP site downstream of exon 3 and one additional primer is located upstream the other loxP site. The size of the PCR product will be different if the recombination takes place. Without recombination, the size of the PCR product will be of 350bp and of 427bp if the recombination takes place (Ackermann J. et al. 2005).

In the case of the *Abcb5TyrHRas*^{G12V} mouse strain, the expression of the transgene *Tyr-RasP1A* will be validated by southern blot on the DNA fragment isolated from the tumor cell lines and from the tail, digested with EcoRV and hybridized with *P1A* probe. The observation of the transgene at the size of 3,5kb will confirm the recombination.

3. Study of the subcellular localization of the transporter ABCB5 in melanoma Cells

3.1. Objective of the study of the subcellular fractionation

As previously described, our in vitro results point the involvement of ABCB5 in melanomagenesis. If this study is confirmed *in vivo*, it will be of paramount importance to assess the molecular mechanism underlying the role of ABCB5 in melanomagenesis. The subcellular localization of any given protein is classically an important step towards the elucidation of its function. With this idea in mind, we decided to start the exploration of the intracellular localization of ABCB5 in A375 melanoma cells. A375 appeared to be an adequate cellular model because these cells constitutively express ABCB5 and are non-pigmented.

Two main approaches can be used to study the subcellular distribution of any given protein. One approach relies on the use of microscopy and co-localization studies performed by immunofluorescence. Alternatively, a biochemical approach can be used, based on subcellular fractionation by ultracentrifugation. Typically, the aim of the subcellular fractionation is to use a fractionation protocol giving rise to characterized subcellular fractions specifically enriched in given organelles. From there, it becomes possible to compare the distribution profile of any protein of interest to the distribution profiles of resident proteins of the classical classes of organelles. The main asset of such biochemical approach is the fact that it can be monitored through a rigorous bookkeeping and is easily prone to quantitative measurements. On the other hand, if the protein of interest cannot be detected through the measurement of its biological activity, establishing its distribution requires the availability of a quality antibody leaving no doubt about its specificity.

With this idea in mind, we decided to start the first steps of the biochemical analysis of ABCB5 subcellular localization in A375 melanoma cells, hence to establish a protocol of fractionation allowing the recovery of subcellular fractions suitable to discriminate between the distributions of the major subcellular compartments: nuclei, mitochondria, lysosomes, endoplasmic reticulum, plasma membrane and cytosol.

3.2. Results of Subcellular fractionation

The protocol used for the fractionation of the A375 cells by differential sedimentation was adapted from the method described by de Duve and colleagues (de Duve et al. 1955). Its principle is to break open the plasma membrane to obtain a so-called "homogenate" hence a suspension of organelles with minimal leakage of their soluble protein content. This homogenate is then submitted to several steps of centrifugations at increasing centrifugal forces (increasing g x min). At each step, pellets are collected, which allow the recovery of subcellular structures characterized by their decreasing sedimentation coefficients (**Figure 33**).

When applied to murine liver, this method gives rise to 5 fractions. The first fraction to sediment is the nuclear fraction (N), enriched in nuclei but containing also some intact cells and large size fragments of the plasma membrane, while the supernatant is the cytoplasmic extract (E). This latter fraction is then further fractionated, which gives rise to the "heavy mitochondrial fraction" (M) containing the bulk of mitochondria, lysosomes and peroxisomes, the "light mitochondrial fraction" (L) enriched in lysosomes and peroxisomes and the microsomal fraction (P) containing microsomes, hence small size vesicular structures formed during the homogenization of the plasma membrane, Golgi and endoplasmic reticulum. This fraction also contains small size vesicular structures belonging to the family of endo-lysosomes. The last fraction (S) is the final supernatant containing the cytosol and all the subcellular structures with a coefficient of sedimentation such that they do not sediment at the centrifugal force used to obtain the P pellet.

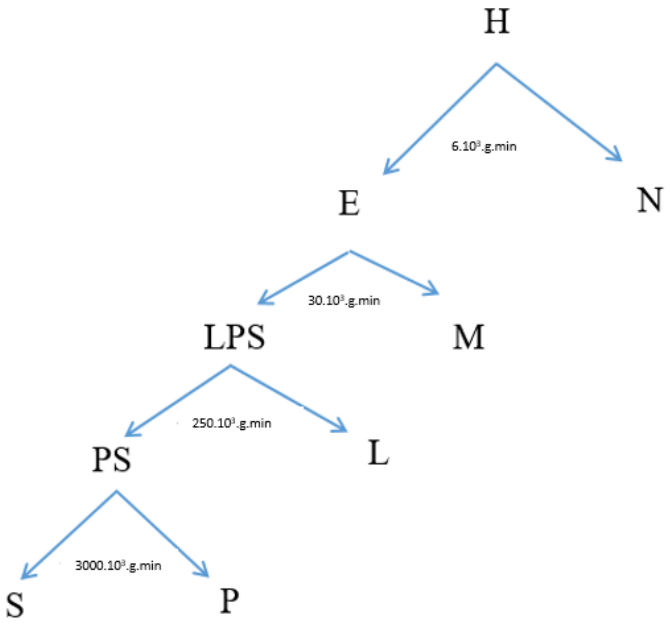

Figure 33: Simplified scheme of the subcellular fractionation protocol. The first centrifugation of the homogenate at 6.10^3.g.min will give the nuclear fraction (N) and the supernatant is the cytoplasmic extract (E). This one undergoes a series of centrifugation at higher and higher speed giving the heavy mitochondrial fraction (M) at 30.10^3.g.min the light mitochondrial fraction (L) at 250.10^3.g.min, and the microsomal fraction at 3000.10^3.g.min. The soluble fraction (S) contains all cell component non-sedimentable at the speed of 3000.10^3.g.min. LPS and PS are supernatants containing the corresponding fractions.

3.2.1. Distribution profile of marker proteins

The behavior of cells with respect to homogenization and fractionation can differ greatly from cell type to cell type. Our first goal was therefore to establish a method (homogenization and fractionation) suitable to A375 cells, that would give rise, as much as possible, to the clear-cut distributions profiles that have been extensively characterized in liver (de Duve et al. 1955). In order to establish the distribution profiles of subcellular structures, we mainly relied on measurements of enzymatic activities of well characterized "marker enzymes". We assayed acid beta-galactosidase (lysosomes), alkaline alpha-glucosidase (endoplasmic reticulum), alkaline phosphodiesterase (plasma membrane), cytochrome oxidase (mitochondria) and lactate dehydrogenase (cytosol). Moreover, we assessed the distribution of nuclei through a western blotting detection of histone H1 by loading the same amount of protein for each fraction. The western blot signal was quantified using the software Image J.

The distribution profiles of these markers were presented as histograms (**Figure 34**) in which the Y-axis represents the relative specific activity (the ratio of the relative activity measured in the fraction to its relative protein content) and the X-axis represents the cumulative percentages of proteins in the 5 fractions. Plotted this way, the histograms provide a clear view of two important parameters: the height of each rectangle represents the enrichment of the protein in the fraction, while its surface indicates the relative activity (the percentage of activity recovered in the fraction relative to the total activity present in the homogenate). These percentages are presented in the tables next to histograms.

When a protein is detected by wester blotting as in the case of histone H1, the same procedure applies except that the enrichment factor is assessed through the measurement of a "relative specific signal intensity" instead of the relative specific activity used for the marker enzymes. The quantification of the signals obtained by western blotting allowed us to establish a distribution profile of the nuclei.

As stated previously, an asset of such biochemical approach is that it can give rise to quantitative measurements and a strict bookkeeping follow-up. For each assay, a recovery was calculated, comparing the activity recovered in the whole cell (E+N) to the sum of the activities measured in the 5 subcellular fractions (N+M+L+P+S). Credits were given to any distribution provided the percentage of recovery of the marker protein was found between 80 and 120 %. The lysosomal marker enzyme β-galactosidase was mainly detected in fractions M, L and P while the relative specific activity showed the highest enrichment of lysosomes in fraction L. Alkaline α-glucosidase was most abundant in the P fraction (41,66%) which also showed the the highest enrichment of this enzyme present in the endoplasmic reticulum. The distribution profile of the plasma membrane marker alkaline phosphodiesterase was similar to that of α-glucosidase with an enrichment in the P fraction. Around 6% of alkaline phosphodiesterase was found in the P fraction. The distribution profile of plasma membrane and endoplasmic reticulum were thus very similar under these conditions of fractionation. The mitochondrial marker enzyme cytochrome oxidase was enriched in M and L fractions while these two fractions contained the bulk of the activity (51,5 % in M, 25,3 % in L). Lactate dehydrogenase, representing the cytosol, was, as expected, mainly present in fraction S (79,5 %). The western blot, allowing the detection of histone H1, confirmed that the nuclei were mainly recovered in the N fraction (relative abundance of 8%) with an enrichment of 6x over the homogenate.

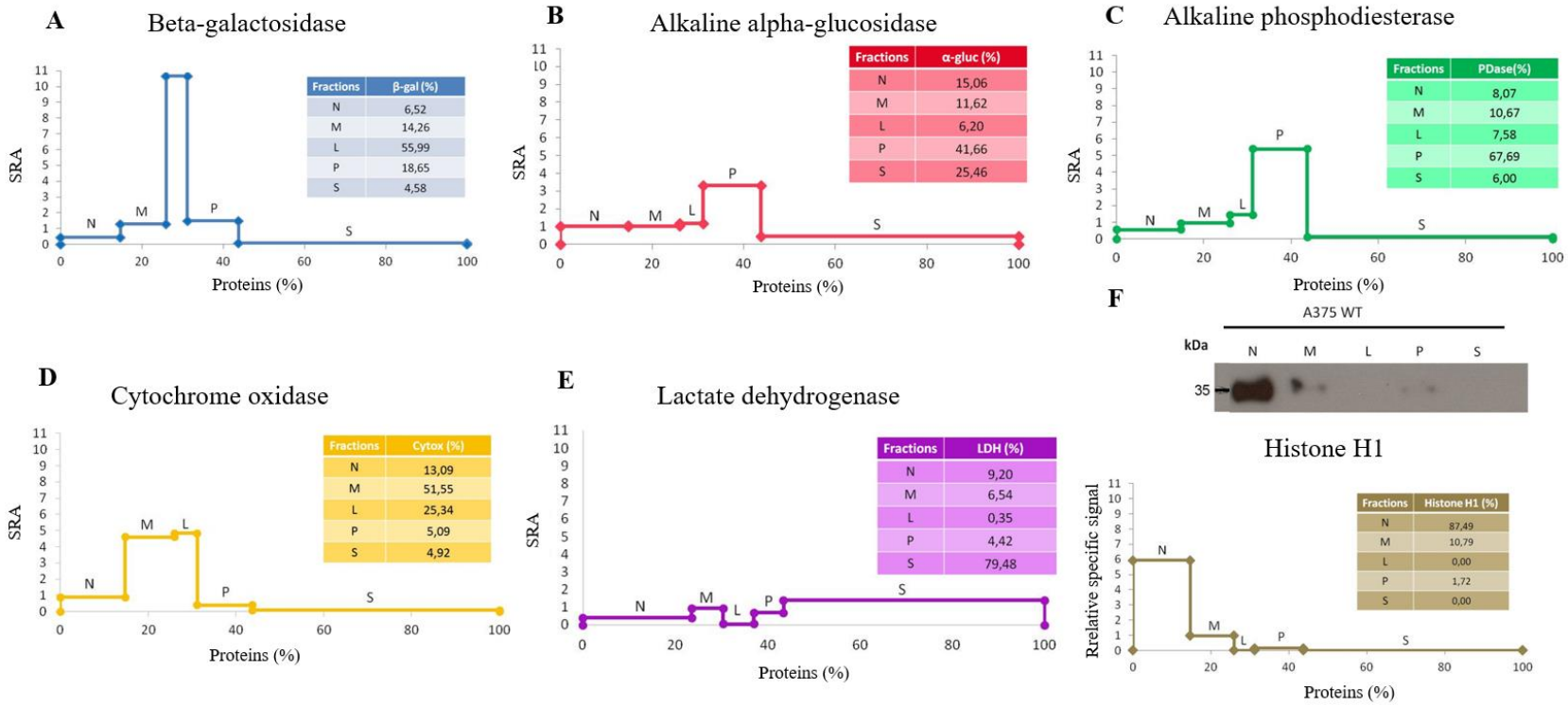

Figure 34: Distribution profiles were obtained by the assay of different marker enzymes in the fractions N,M,L,P and S. Distribution profiles are presented in histogram. The X-axis is the protein percentage and the Y-axis is the specific relative activity (SRA).The beta-galactosidase is a marker enzyme of the lysosomes (A), the alkaline alpha-glucosidase of the endoplasmic reticulum (B), the alkaline phosphodiesterase of the plasma membrane (C), the cytochrome oxidase of the mitochondria (D) and the lactate dehydrogenase of the cytosol (E). Histone H1 was detected in the fractions by western blot, the same amount of protein was loaded and the signal was quantified using the software Image J (F).

3.2.2. Distribution profile of ABCB5

To determine the distribution profile of ABCB5, the transporter was detected by western blotting using the polyclonal antibody from Rockland (Limerick, USA). The same amount of proteins was loaded for each fraction. As a control, we used crude membranes prepared from ABCB5-transfected High 5 cells (Hi5 ABCB5 CTL+) and their counterpart mock-transfected cells (Hi5 CTL-). The insect cells were transfected and the membranes prepared by Gillet JP. Crude membrane of A375cells were also loaded.

We observed a strong signal in the nuclear (N) and microsomal (P) fractions, at the size of approximately 140 kDa, corresponding to the size of ABCB5 full-length. This signal is stronger in the nuclear fraction than in the microsomal one. The western blotting was performed by loading the same amount of proteins for each fraction. Thus, the signals observed in the different fractions, and quantified using Image J software, represent the enrichment of the protein over the homogenate. As in the case of marker proteins, the results were presented as an histogram (**Figure 35**). The distribution profile showed the presence of the protein in fractions N and P, with the highest enrichment in the former. The abundance was also higher in the nuclear fraction (67,9 % vs 23,7 % in the microsomal fraction).

It should be noted that we also detected a signal at the size of around 90 kDa in the microsomal fraction and in the crude membrane of the A375 cells.

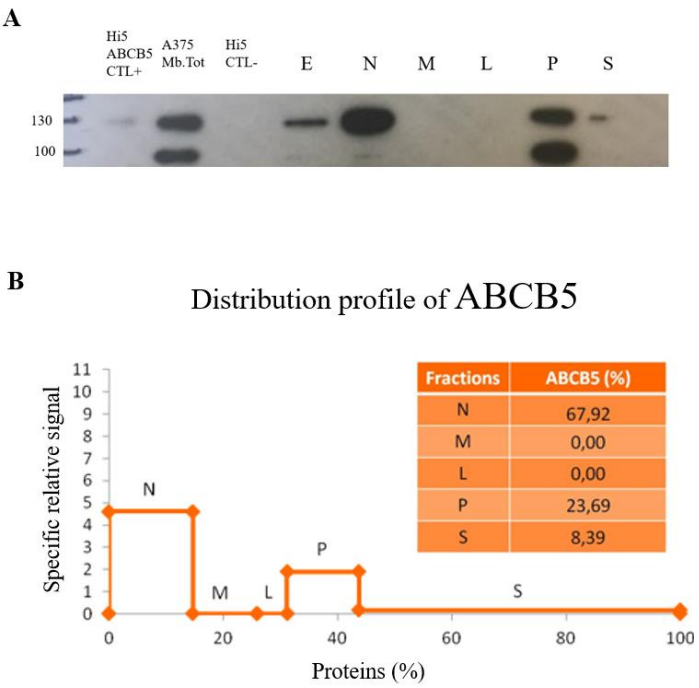

Figure 35 : ABCB5 was detected by western blot using the polyclonal antibody of Rockland. At the size of the isoform full length, we observed a signal in the nuclear and microsomal fraction (A). The intensity of the signal was quantified using the program Image J and allow to obtain the histogram of the distribution profile of ABCB5 full length. The percentage of proteins is presented in the X-axis and the Y-axis is the specific relative signal. The height of the rectangle represents the enrichment of ABCB5 in the fraction, which is higher in the nuclear fraction than the microsomal fraction.

3.3. Validation of the polyclonal antibody of Rockland

The Rockland antibody recognized ABCB5 full length in Hi5 insect cells. We observed a band in the Hi5 insect cells with ABCB5 full-length construct while this band was not present in their counterpart mock-transfected cells (**Figure 36**).

We wanted to validate the results obtained and confirm that the bands observed at the size of 138 kDa in A375 fractions correspond to ABCB5 full length. We have done it to by two ways. First, a western blot was performed with the cancer cell lines MCF7, KB31, H1299, showing a low expression level of ABCB5 mRNA in terms of RT-qPCR. By western blotting, the band was present at the size of +/- 140kDa with the same intensity (**Figure 36**). The second way was a western blot with the cell line 63T used for the first part of the project. The first band corresponds to the 63T cells transduced to overexpress ABCB5 wild type. This band disappeared in the 63Tcells expressing the ABCB5 mutants Q187*, a non-sense mutation.

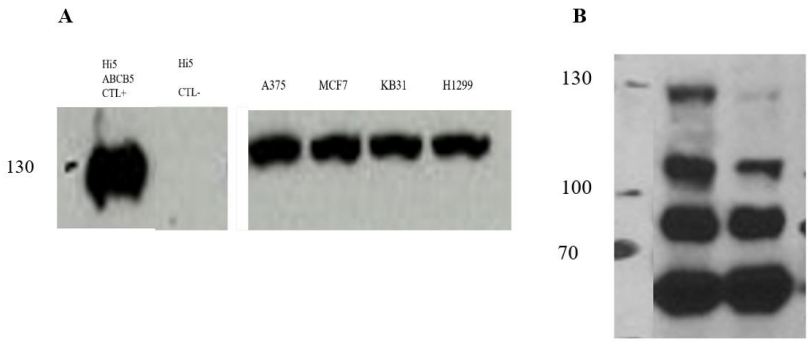

Figure 36: A. The Rockland antibody detect ABCB5 in insect cells transfected to overexpress ABCB5 (Hi5ABCB5 CTL+) showing the specificity of the antibody. In human cells, a signal is observed in the cell lines MCF7, KB31 and H1299 associated with low expression of ABCB5 mRNA. B. The first band corresponds to the 63T cells transduced to overexpress ABCB5 wild type. This band disappeared in the 63Tcells expressing the ABCB5 mutants Q187*, a non-sense mutation.

3.4. Discussion

The specific aim of this part of the project was to obtain some information about the intracellular localization of ABCB5. We first applied a subcellular fractionation procedure by differential centrifugation of the homogenate of A375 cells. The distribution profiles of the main subcellular compartments were shown to be quite specific except for the endoplasmic reticulum and plasma membrane both showing a very similar microsomal profile. The Rockland antibody was used to detect ABCB5 in the different fractions. A strong signal was observed in the N and P fractions at the size of 140kDa and in the P fraction at the size of 90kDa.

3.4.1. Conditions of fractionation

The distributions obtained informs us about the conditions of fractionation and demonstrate that the protocol used is suitable for this cell line. To evaluate this, the distribution profile of the lysosomal marker enzyme β-galactosidase is interesting because the lysosomal membrane is very sensitive to homogenization. The low percentage of β-galactosidase recovered in the nuclear fraction (6,5%) indicates that only a very low fraction of the cell population, if any, remained intact after homogenization. On the other hand, the distribution of β-galactosidase also demonstrates that the homogenization conditions were not too harsh because the percentage of this soluble lysosomal protein recovered in the S fraction is very low (4,9%).

Globally, the distribution profiles obtained are quite similar to the distribution of organelles obtained in rat liver by de Duve (described on page 92) and indicate that future works requiring subcellular fractionation of A375 cells could take advantage of this protocol.

3.4.2. Validation of the antibody

The Rockland antibody recognizes ABCB5. Indeed, a signal was observed in the insect cells transfected to overexpress ABCB5, while this signal was absent in the negative control. Results obtained with the melanoma cell line 63T go in the same direction. Indeed, while a band was observed at 140kDa in the 63T cells transduced to overexpress ABCB5 wild type, this band attenuates significantly when the 63T cells were transduced with the mutant Q187*, a non-sense mutation.

However, in our attempt to validate this antibody in human cells, we also performed a western blot with the MCF7, KB31 and H1299 cell lines with a low expression level of ABCB5. In western blot, the intensity of the signal was identical between the cell line A375 expressing ABCB5 and the cell lines with low expression level.

We cannot rule out that the antibody recognizes another protein with a similar size. In order to fully validate this antibody, mass spectrometry will be performed on digested proteins isolated from a silver stained polyacrylamide gel. The development of a reliable negative control should also be required to validate the antibody. In this purpose, the CRISPR-Cas9 is being developed in the laboratory to generate an ABCB5 KO melanoma cell line. Actually, two cell lines are being engineered. A first one, which will not express any ABCB5 transcript isoforms. The entire gene being removed. To date, we obtained a heterozygous cell line for the ABCB5 knockout. A second engineered cell line, which is homozygous for the ABCB5β knockout, allowed us to determine that the antibody can detect the ABCB5β isoform at the expected size of 90 KDa.

3.4.3. Presence of ABCB5 full length in the nuclear and microsomal fraction

3.4.3.1. Interpretation of the distribution profile of the signal at 140 kDa

Classically, when using analytical subcellular fractionation, the intracellular localization of a protein is inferred from the comparison between its distribution among the different fractions and the distributions of known marker proteins of subcellular organelles. Here, regarding the distribution of the signal observed at 140kDa, one should be very careful and keep in mind that the identification of this signal as ABCB5 full length needs further validation.

The distribution profile of the signal at 140kDa presents the highest enrichment in the nuclear fraction N and in the microsomal fraction P. This profile is completely different from the distribution profile of the β-galactosidase, marker enzyme of the lysosomes. This would exclude a lysosomal localization of ABCB5. We can also exclude the location ABCB5 in mitochondria because the distribution profile of the cytochrome oxidase is also completely different with the majority of the enzyme recovered in M and L fractions. The same reasoning can be applied to the lactate dehydrogenase distribution profile excluding the presence of ABCB5 in the cytosol.

Considering the distributions of alkaline α-glucosidase, alkaline phosphodiesterase and histones H1 is less straightforward. The distribution profile of ABCB5 exhibits two strong signals in fractions N, where the bulk of nuclei are recovered, and in the microsomal fraction P containing most of the plasma membrane and endoplasmic reticulum microsomes. Hence we cannot exclude the possibility that ABCB5 would exhibit some mixed localization, in the nucleus and in the endoplasmic reticulum and/or the plasma membrane.

Based on these results we are left with two possibilities. Either the distribution profile of ABCB5 is indicative of a mixed localization, or ABCB5 is located in some organelle whose distribution has not been assessed by the panel of marker proteins that we selected.

3.4.3.2. Other ABC transporters were found in the nucleus

It may seem surprising to detect ABCB5 in the nucleus. However, leaning into literature, we found that several ABC transporters were localized in the nuclear envelope, in particular ABCB1, which is very close to ABCB5.

ABCB1 was primarily located in the plasma membrane, which is associated with the classical “MDR phenotype”. However, it was also detected in the nuclear envelope. ABCB1 was detected in the nucleus for the first time in 1995 by Baldini (Baldini N et al.1995). It was also localized in the nuclear envelope of rat brain cells (Babakhanian et al. 2007). Szaflarski and colleagues showed the expression of ABCB1 in the resistant cell line LoVo as well as its implication in the efflux of doxorubicin from nucleus to cytoplasm (Szaflarski et al. 2013).

ABCC1 was also shown to be located in the nucleus in mucoepidermoid carcinoma (Cai et al. 2011) and ABCC2 in breast cancer (MacIejczyk et al. 2012) and non-Hodgkin's lymphomas (Szczuraszek et al. 2009).

While the transporter ABCG2 is generally localized in the plasma membrane, the studies of Bhatia and Habicht et al. showed its localization in the nucleus of glioblastoma cells (Habicht et al. 2013; Bhatia et al. 2012).

3.4.3.3. Other ABC transporters were localized in the microsomal fraction

The distribution profile of the protein signal at 140kDa suggests the presence of ABCB5 in the microsomal fraction. This fraction was shown to be enriched in fragments of plasma membrane and endoplasmic reticulum (**Figure 35**).

Considering that many ABC transporters are located in the plasma membrane, the presence of ABCB5 in the microsomal fraction is not surprising. However, the band observed in the P fraction could also correspond to other organelles. Indeed, some ABC transporters were shown to be located in the endoplasmic reticulum and in the Golgi apparatus.

The ABCB1, ABCG2, ABCA1 transporters and the transporters associated with antigen processing (ABCB2 and ABCB3 so called TAP transporters) were shown to be located in the endoplasmic reticulum (Fu et al. 2004; Ifergan et al. 2005; Karttunen et al. 2001; Krishnamachary and Center 1993; Yamauchi et al. 2015).

The microsomal fraction should also be enriched in fragments of the Golgi apparatus, whereas this assumtpion be confirmed using a protein marker of this organelle. ABCB1 was detected in breast cancer cell lines and inhibitors of this transporter decreased the sequestration in Golgi apparatus (Merlin et al. 2000). According to Molinari, ABCB1 was also detected in Golgi apparatus in melanoma cell lines, while this transporter is not expressed in the plasma membrane (Molinari A et al. 2002).

Going further into the interpretation of the microsomal distribution and in particular trying to discriminate between a residence of ABCB5 in the plasma membrane, in the endoplasmic reticulum, in the Golgi apparatus (or in endosomes, see bellow) would require to sub-fractionate the fraction P by using several orthogonal methods of separation such as isopycnic centrifugation on various density gradients.

3.4.4. Presence of ABCB5β in the microsomal fraction

As mentioned in the introduction, there exists several isoforms of ABCB5. The most studied isoform is ABCB5β. While the topology of ABCB5β is controversial and does not correspond to the typical topology of a half-transporter, it has been studied for its role in drug resistance and was identified as a marker of melanoma initiating cells (N. Y. Frank et al. 2005).

Since data obtained in the laboratory after completion of our experiments confirmed that the Rockland antibody specifically recognizes ABCB5β in human melanoma cells, the distribution profile of this isoform can be considered as reliable.

One band at the size of 90kDa, corresponding to ABCB5β, was observed in the microsomal fraction (P) (**Figure 35**). This isoform is enriched in the microsomal fraction P. Considering the distribution profiles of the marker enzyme tested, this result could point a localization of ABCB5β in the plasma membrane or in the endoplasmic reticulum.

However, one should take into account that the P fraction most likely contains the microsomes formed during the homogenization of the Golgi and some vesicular structures of the endo-lysosomal system, such as endosomes and melanosomes. Again, discriminating between such putative localizations of the ABCB5β form would require to further fractionate the microsomal fraction P and to measure the distribution profiles of marker proteins of the organelles of interest.

The distribution profile of ABCB5β obtained is compatible with a location in the plasma membrane. This conclusion would be consistent with the results obtained by Frank and colleagues. Indeed, their studies showed a surface plasma membrane localization for this protein (N. Y. Frank et al. 2003).

3.5. Conclusion 3 and perspectives

The results of fractionation obtained showed us that A375 melanoma cells behaved quite typically. Indeed, the organelles distribution observed is similar to the distribution observed by de Duve fractionation of rat liver (de Duve et al. 1955).

While it is necessary to further validate the Rockland antibody, the distribution profile of ABCB5 full length showed an enrichment in the nuclear and the microsomal fractions. This distribution appears to be comparable to that of other transporters. Other avenues can be explored to confirm the localization of ABCB5 full length in the nucleus. The efflux of doxorubicin from nucleus to cytoplasm could be observed by fluorescence microscopy. ABCB5 was shown to transport doxorubicin in insect cells (Gillet and colleagues, unpublished data) and in HEK293T transfected to overexpress ABCB5 full length. This experiment would require a ABCB5 knockout cell line. The identification of the organelles in the microsomal fraction expressing ABCB5 full length requires sub-fractionation steps.

We showed the enrichment of the beta isoform in the microsomal fraction. This result is compatible with a plasma membrane localization suggested by Frank and colleagues. A isopycnic gradient of the P fraction followed by the detection of marker proteins in the sub fractions would confirm us the localization in the plasma membrane.

While melanoma are highly resistant to chemotherapy, melanoma cells could have a typical resistance mechanism. Chen hypothesizes that a mechanism of resistance would be the sequestration of the drug in melanosomes (K. G. Chen et al. 2009). It would be interesting to determine the distribution of these organelles.

DISCUSSION

1. Mutations in the ABCB5 gene appear to be cancer driver mutations.

The *ABCB5* gene was mutated in 13,75% of the 640 melanoma samples analyzed. Such a high mutation rate suggests that cells harboring *ABCB5* mutations are selected. This means that these mutations contribute to cancer initiation and/or progression. The observed ratio between the non-synonymous to synonymous of 3,7:1 is higher than the ratio predicted for non-selected passenger mutations, which is of 2,5:1 (Sjoblom et al. 2006). Together, these data indicate that mutations in *ABCB5* gene are cancer diver mutations and should contribute to melanomagenesis.

2. We have chosen to focus on four mutations: Q817*, a non-sense mutation, and three missense mutations S830F, S1184P and S1091F

The first two cohorts of melanoma analyzed allowed us to identify 10 mutations: 3 non-sense mutations (Q187*, R587* and Q1098*), 5 missense mutations (E520D, V827I, S830F, S1184P and S1091F) and 2 synonymous mutations (I828I and L840L) (**Table 3**).

Among the three non-sense mutations, we have chosen to focus on Q187*, potentially leading to the shortest gene product. However, the study of the other non-sense mutations could also be interesting, especially the R587* mutation. Indeed, this mutation was present in 3/640 melanoma samples (information that we did not have at the early stage of the study).

To assess the impact of the missense mutations, the in silico analysis SIFT score was performed. SIFT (Sorting Intolerant From Tolerant) is a prediction tool relying solely on sequence homology. It allows to obtain a score between 0 and 1. This score predicts if a missense change would alter the protein function. A score below 0.05 suggests a deleterious effect. We have chosen the mutations associated with the lowest values. Mutation S830F and S1184P are associated with a null SIFT score and S1091F with a score of 0.01. By contrast, the non-synonymous mutations E520D and V827I, were associated with scores higher than 0.05 (respectively 0.21 and 1). The synonymous mutations I828I and L840L were associated with the values of 1 and 0.7 respectively. This means that these synonymous mutation are predicted to have no impact on ABCB5 function.

We are well aware that the choice of the mutations is based on a prediction tool. Obviously, no conclusion on the real impact of these mutations on ABCB5 function can be drawn based on the SIFT analysis. We could have enriched this analysis by using another software like Polyphen-2, taking into account the domains and the three-dimensional structure. Other software exist such as Align-GVGD, Hansa, MAPP, MutPred, PROVEAN, etc.

A study of Flanagan and colleagues showed that the main limitation of SIFT and Polyphen was their low specificity (respectively of 13 and 16%) (Flanagan et al. 2010). It is important to keep in mind that these prediction tools are only prioritizing changes with higher probability to alter the protein function. These data must be interpreted with caution. That is why we generated experimental data to support or refute the effect on function associated with the missense mutations.

In this regard, we could also have studied synonymous mutations. Kimchi-Sarfaty and colleagues demonstrated that SNP leading to the same amino acid can alter the protein structure and function of ABCB1. Their hypothesis is that a rare codon affects the cotranslationnal folding and the insertion of ABCB1 in the membrane (Kimchi-Sarfaty et al. 2007). Tsai an colleagues suggest that this phenomenon could be explained by a long ribosomal pause times-scales, which may lead to an alternate folding pathway (Tsai et al. 2008).

3. Are those mutations in ABCB5 observed in melanoma associated with a gain of function or with a loss of function?

3.1. ABCB5 mutations are distributed throughout the gene.

The mutational pattern of a gene observed in cancer cells may help to determine if the disease is associated with a gain or a loss of function of the protein. Mutations in tumor suppressor genes, usually associated with loss of function, are often dispersed along the length of the gene. By contrast, oncogene activating mutations have specific locations, leading to the activation of the protein (Vogelstein et al. 2013). In our case, the mutations are dispersed along the gene (**Figure 1 –JID** annexed) suggesting that loss of function of ABCB5 contribute to tumorigenesis. However, we observed two sites where mutations occur recurrently (at amino acids 587 and 830). The Vogelstein's 20:20 rule indicates that a gene is predicted to be an "oncogene if 20% of all missense mutations occur at a single position in the sequence" (Baeissa et al. 2016). In our case, we observed a lower percentage: 6,7% and 8,3% are corresponding to the amino acid 587 and 830, respectively.

Overall, the distribution of mutations that we observed in the *ABCB5* gene is more typical of a tumor suppressor gene. However, this is only a prediction requiring experimental validation.

3.2. The ABCB5 gene mutations are heterozygous.

The mutations in the *ABCB5* gene were found to be heterozygous. Nevertheless, we observed that overexpression of ABCB5 mutants led to an increase of the proliferation ability of melanoma cells, when compared to overexpression of ABCB5 wild type. A soft agar colony formation assay was performed in 17T and 63T melanoma cells and showed an increase of the proliferation ability in both cell lines for all the mutants. The migration ability was also shown to be increased for the Q187* and S1184P ABCB5 mutants in the 17T cells and for the S830F and S1091F ABCB5 mutants in the 63T cells. The silencing of ABCB5 in the melanoma cells A375 and SK-Mel-28 using shRNA leads to an increase in terms of the size of colonies. All the experiments were performed in biological and technical triplicates, and the results were reproducible. Overall, the *in vitro* data indicate that ABCB5 has a tumor suppressor function whereas the mutations were heterozygous in melanoma cells.

More than forty years ago, Knudson coined the two-hit model, in which homozygous loss of function mutations of tumor suppressor genes are necessary to confer a selective advantage to cancer cells (Knudson 1971). However, there are now sufficient evidence that support a role for both complete and partial tumor suppressor inactivation in the tumorigenesis process.

First, we may hypothesize haploinsufficiency. This means that the inactivation of only one allele can have a selective advantage for the cell. Indeed, it that been shown that heterozygous mutations of tumor suppressor genes can contribute to tumorigenesis by conferring an intermediate phenotype (Santarosa and Ashworth 2004). Many tumor suppressor genes exhibit haploinsufficiency. For example, this was observed for p53. The study of Venkatachalam and colleagues showed that the loss of both p53 alleles is not required for tumorigenesis. Indeed, the heterozygous mice *p53*+/- also develop tumors, but at a later stage in their life by comparison with the homozygous *p53*-/- mice. The heterozygosity leads to an intermediate phenotype (Venkatachalam et al. 1998). The tumor suppressor *27kip1* was also shown to be haploinsufficient for tumor suppression. Both p27-/- and p27+/- lead to tumor development in multiple tissues (Fero et al. 1998). *TGFbeta* was also shown to be haploinsufficient for tumor suppression. However, TGFbeta is a secreted protein functioning as a non-cell autonomous fashion (Tang et al. 1998). We cannot exclude that ABCB5 may also have a non-cell autonomous function. Haploinsufficiency is also the case of other tumor suppressor genes such as *Apc*, *BRCA1/2*, *Cdkn1a/1b/2c*, *Pten*, etc. Other examples are given in the review of Payne and Kemp (Payne and Kemp 2005). Haploinsufficiency can be tissue specific and context dependent.

A second hypothesis, which could explain heterozygous mutations in tumor suppressor genes, is the existence of a negative dominant effect. This means that a mutation in one allele results in an altered gene product, which affects the function of the wild-type gene product. In this case, the heterozygous mutations can give a survival advantage by comparison with homozygous mutations. For example, it is the case for the tumor suppressor gene *GRIN2a* in melanoma. The mutant GRIN2a inhibits the tumor suppressive phenotype of wild type GRIN2a in melanoma. Somatic mutations in *GRIN2a* gene results in a loss of complex formation between wild type GRIN1 and wild type GRIN2a (Prickett et al. 2014).

The hypothesis of a dominant negative effect seems less likely because this phenomenon is usually described for multimeric proteins. This is not the case of ABCB5 full length. Indeed, its topology corresponds to the typical topology of full ABC transporters, which are monomeric proteins (see introduction page 48). However, we cannot exclude that the mutated ABCB5 will have a dominant negative effect on the wild type ABCB5. In **Figure 25**, we observed that the non-sense mutation Q187* does not correspond to the highest effect by comparison with the other mutations. This could be explained by dominant negative effect caused by the ABCB5 mutant S1184P, S1091F and S830F, which could not take place in the case of the short truncated protein Q187* ABCB5. To address this possibility, it could be interesting to investigate whether this truncated protein is expressed. The addition of a tag on the N terminal side to the truncated protein would allow its detection by western blot. If this protein is not detected, we may hypothesize that either this protein or the RNA is unstable. RNA extraction from ABCB5 Q187* mutant cells, followed by the cDNA synthesis, its sequencing and the assessment of the ABCB5 mutant expression level would allow us to have an idea of the RNA stability.

The possibility of the dominant negative could also concern the beta isoform of ABCB5. Indeed, the mutations S830F, S1091F and S1184P are common for both beta and full length isoforms. We cannot exclude that ABCB5 beta could act as follows: the mutant ABCB5beta would dimerize with the WT ABCB5beta, preventing ABCB5beta function. However, no study showed that ABCB5beta would be a tumor suppressor. By contrast, studies of the group of Frank showed that it would contribute to tumorigenesis as an oncogene (Schatton et al. 2008). Assessing the effect of ABCB5 mutants S830F, S1091F and S1184P on the proliferation, migration and invasion abilities of melanoma cells would also be an interesting. We cannot exclude that ABCB5 isoforms have completely different functions. For example, it is the case for Tapp73, acting as a tumor suppressor, while ΔNp73 displays oncogenic properties (Rufini et al. 2011).

To determine if heterozygosity could be sufficient to induce a growth advantage, we could use the CRISPRCas9 technique to obtain *ABCB5* +/- and -/- cells. After obtaining cell lines, we could compare their ability to proliferate and migrate. If the ABCB5 +/- cells display increased proliferation ability when compared to the WT cells, we could conclude that the heterozygosity would be sufficient to confer a phenotype.

The development of the cell line knock out for *ABCB5* (-/-) would also be an interesting tool to complete our *in vitro* observations and confirm that the silencing of ABCB5 leads to an increase of the proliferation ability. Indeed, we have data obtained on the melanoma cell lines A375 and SK-Mel-28 (harboring $BRAF^{V600E}$ mutation), which were transduced with ABCB5-shRNA. We observed in soft agar colony formation assays a higher number of colonies larger than 400µm and 200µm, respectively. Unfortunately, we did not manage to reproduce the transduction with shRNA for the cell lines 17T and 63T. Another strategy to study the impact of these mutations would be the introduction of the Q187*, S830F, S1091F and S1184P mutations by CRISPR Cas9. However, this method includes the bias of the variability between the clones. This experiment should be performed on several independent clones. It is also possible to insert loxP sites around the ABCB5 gene and to add tamoxifen with the advantage that we can perform it on a cell population.

Main results agree for a loss of function and suggest that ABCB5 has a tumor suppressor function.

4. How ABCB5 could be involved in melanomagenesis?

At this stage, the mechanisms underlying the increase of the proliferation and migration abilities observed for mutated ABCB5 cells compared with ABCB5 wild type expressing cells are still unknown. The contribution of ABC transporters to cancer biology was shown, so far, to be due to the transport of metabolites and signaling molecules (see page 70).

To understand how ABCB5 could be involved in tumorigenesis, it may be important to expand the definition of the tumor suppressor. There are three main types of tumor suppressor genes: the gatekeepers, the caretakers and the landscapers.

The gatekeepers are known to control the cellular proliferation by encoding protein associated with the cell cycle. The APC, CDKN1B and Rb proteins are examples of gatekeepers. Caretakers are also called stability genes, promoting tumorigenesis when they are mutated. They are responsible for repairing mistakes made during normal DNA replication or induced by exposure to mutagens. Caretakers can be also responsible for the process involving large portion of chromosomes such as those responsible for mitotic recombination and chromosomal segregation. Inactivation in caretakers lead to genomic instability and mutations in other genes that occurs at higher rate. All the genes will be affected including oncogenes and tumor suppressor genes. Only mutations in oncogenes/tumor suppressor genes lead to an increase of the net cell growth (Kinzler and Vogelstein 1997; Vogelstein and Kinzler 2004). The third kind of tumor suppressors, called the landscapers, are responsible to create an environment controlling cellular proliferation (Stratakis 2003).

It seems unlikely that ABCB5 could be a gatekeeper, having a direct impact on the cell cycle. However, we can hypothesize that ABCB5 has an indirect impact on the cell cycle. For example, ABCB5 could transport a substrate having an impact on a gatekeeper or on a molecule involved in a pathway leading to melanomagenesis (MAPK, PI3K/Ak, Rb and p53 pathways, developed in page 17 of the introduction). For example, we could hypothesize that ABCB5 could transport a substrate inhibiting CDK4/6, HDM2 or PI3K, etc. However, these actions can be very diverse and concern all the molecules involved in the cell cycle. We could investigate whether there is a link between ABCB5 and the pathway leading to melanomagenesis by correlation studies between ABCB5 expression and the detection of the phosphorylated proteins of the MAPK pathway. Using antibodies recognizing the phosphorylated proteins, Colone and colleagues established a link between ABCB1 and the MAPK pathway (Colone et al. 2008). We could also use inhibitors of the MAPK pathway, which could have an impact on the ABCB5 expression. This approach performed in the case of the transporter ABCG2 (Xie et al. 2014). We could hypothesize that ABCB5 can efflux out of the cell a negative regulator of the proliferation, which will limit the cellular growth of the other cells. If this transporter is located in a subcellular organelle, it could sequestrate a positive regulator of the cell cycle. In both cases, a defective transport due to the mutations would increase cell proliferation.

ABCB5 might also act as a caretaker by transporting a molecule having a role in the maintain of the genome stability. However, in this case, we should have observed genomic instabilities when ABCB5 is mutated.

We also may hypothesize that ABCB5 acts as a landscaper. Indeed, we could envision that ABCB5 efflux a compound out of the cell having an impact on the microenvironment, preventing uncontrolled proliferation. This hypothesis supposes a localization of ABCB5 in the plasma membrane. However, the subcellular localization of this transporter remains to be precisely resolved. The determination of the subcellular localization and the study of its substrates are crucial.

It is not always possible to classify the tumor suppressor gene in distinct categories. Some can act as a gatekeeper and as a caretaker. It is the case of p53, which regulates the cell cycle and is involved in DNA repair (Rubbi and Milner 2005).

The *in vitro* results showed that the increase of proliferation was higher using cell lines with *NRAS*^{Q61K} background (17T and 63T) than with cell lines with a *BRAF*^{V600E} background (A375 and SK-Mel-28). This could be due to a link between NRAS and ABCB5 that could be further investigated.

Kondo and colleagues showed that the cells transfected to overexpress ABCB5 are more resistant to buthionine sulfoximine (BSO) than the control cells. BSO is an inhibitor of the GCL, an enzyme implicated in the biosynthesis of the glutathione (GSH). They also showed a higher GSH content in the cells expressing ABCB5 when compared to the control cells and that GSH was not a substrate of ABCB5. In their experiment, they showed that BSO and GSH are not transported by ABCB5. GSH being a tripeptide of glutamic acid, cysteine and glycine, they measured the amino acid content of the cells and observed a higher cellular content of glutamic acid in the ABCB5 transfected cells. Their results suggest the possible effect of ABCB5 on the cellular amino acid content (Kondo et al. 2015).

GSH has several functions including detoxification and elimination of the reactive oxygen species, but is also responsible for the detoxification of xenobiotics and of some endogenous compounds. These are electrophiles, which form conjugates with GSH (Traverso et al. 2013).

These data support the role of ABCB5 as a tumor suppressor. Indeed, GSH is involved in the detoxification of the reactive oxygen species commonly known to lead to cancer. However, ROS play a dual role in cancer. Moderate ROS contribute to the increase of cellular proliferation and survival by activating signaling pathways, while excessive accumulation of ROS results to cell death (Bansal and Simon 2018). GSH also plays a dual role in cancer. On one hand, the detoxification of carcinogen leads to protection against cancer (Jakóbisiak et al. 2003). On the other hand, in many tumors, the increased GSH level is associated with the cell cycle progression and with a higher proliferation rate (Traverso et al. 2013). The link between the GSH status and cell growth was established in melanoma. The GSH content regulates the metastatic behavior (Carretero et al. 1999). So far, the mechanism of how GSH modulates cellular proliferation remains speculative (Traverso et al. 2013).

ABCB5 seems to perturb the redox homeostasis in the HEK-293 cells. However, the study of Kondo does not explore the effect of the higher GSH cellular content on the cellular growth. To address this question, we could investigate if the expression of ABCB5 in 17T and 63T melanoma cells affect the cellular content in GSH. We could also explore this for the ABCB5 mutants Q187*, S1184P, S1091F and S830F.

5. The *in vivo* study of the involvement of ABCB5 in melanomagenesis

The use of animal models is crucial to understand how cancers develop. In our project, we have chosen to use the mouse model, which is commonly used to study melanoma. So far, the genetically engineered mouse models were very useful to characterize melanomagenesis. Both in human and mice, melanocytes are originating from neural crest. Many mouse models have been developed. Mouse models are adequate to study the initiation of the tumor (Pérez-Guijarro et al. 2017). Transgenic mouse models allow studying the frequency of melanoma development as well as the apparition of metastases and there was pre-existing models to our study. Indeed, the team of Van den Eynde had developed the inducible mouse model with the expression of $HRas^{G12V}$ and the deletion of the exon 2 and 3 of the gene *Cdkn2a* (Huijbers et al. 2006). We also relied on the construction developed by Ackermann and colleagues, which is the spontaneous expression of $NRAS^{Q61K}$(Ackermann J. et al. 2005). However, the mouse model presents limitations. Indeed, there are differences in terms of the morphology of the skin between human and mice, which can affect the initiation of melanoma. The human and murine melanocytes do not share the same localization. The murine melanocytes are exclusively present in the hair follicle and at the bottom of the hair follicle (Pérez-Guijarro et al. 2017).

The zebrafish model is also used to study melanoma. Xenograft of human cells can be used as well as transgenic models. The zebrafish have melanocytes called melanophores, in which the melanin is accumulated by contrast with human in which the melanin is transferred to keratinocytes (Kauffman 2016). It is possible to observe tumoral progression because tissues are transparent (Mione and Trede 2010). A zebrafish harboring the mutation $NRAS^{Q61K}$ was developed by Dovey and colleagues. The tumors described are very similar to human tumors (Dovey et al. 2009). The zebrafish model allows studying mutagenesis. Indeed, N-ethyl-N-nitrosourea induces punctual mutations in zebrafish (Grunwald and Streisingert 1992). In the way to pursue the investigation of ABCB5 involvement in melanomagenesis, we could introduce the mutations Q187*, S830F, S1184P and S1091F in *Abcb5* gene in zebrafish. This model has allowed identifying several tumor suppressors such as *ptena, ptenb, apc, mlh1, msh2, msh6, brca2* ou *tp53* (Völkel et al. 2018).

CONCLUSION

ABCB5 is an ATP-binding cassette (ABC) transporter, known to be expressed in pigmented cells, and associated with low-level resistance in melanoma. While ABC transporters are mainly studied for their role in drug resistance, many studies show that they also play a role in tumorigenesis. *ABCB5* was identified as the sixth most frequently mutated gene in melanoma (Krauthammer et al. 2015). Meanwhile, Gillet and colleagues provide a more systematic analysis of the nature and function of these mutations. The exome sequencing of 640 clinical melanoma samples revealed that *ABCB5* was mutated in nearly 15% of cases. This percentage suggests this gene to be a cancer "driver" gene and the distribution of the mutations throughout the gene would evoke a priori a loss of function. Moreover, the 3,7:1 ratio of non-synonymous on synonymous mutations is higher than the ratio predicted by Sjobolm and colleagues for non-selected passenger mutations, which is of 2,5:1 (Sjoblom et al. 2006). We aimed to investigate the role of ABCB5 in melanomagenesis. Among the recurrent mutations identified, four were selected, representative of the mutation pattern and associated with low SIFT scores: Q187*, S830F, S1091F and S1184P. At biochemical level, these mutations were associated with a decrease in basal ATP hydrolysis.

Two melanoma cell lines (17T and 63T), harboring $NRAS^{Q61K}$ mutations, were stably transduced to overexpress ABCB5 mutants ($ABCB5^{Q187*,}$ $ABCB5^{S830F}$, $ABCB5^{S1091F}$ and $ABCB5^{S1184P}$). The overexpression of these mutants has an impact on the cellular proliferation, by comparison with the cells overexpressing the wild type ABCB5. The proliferation test on plastic showed a highly significant increase of the growth ability, for the 17T cell line and for the 63T expressing the mutant S1184P. Because this latter assay is biased by the ability of the cells to adhere to plastic, we additionally performed the anchorage-independent soft agar colony formation assay. This one showed a significant effect for both cell lines and each mutation. The soft agar colony formation assay being a hallmark, we can definitively conclude that these ABCB5 mutants lead to an increase of proliferation ability. The anchorage independent growth was also increased when the expression of ABCB5 was silenced using shRNA in melanoma cell lines A375 and SK-Mel-28.

In order to determine if ABCB5 is involved in melanoma progression, we also assess the migration and invasion ability by performing Boyden chambers assay. These tests revealed a significant increase in the proliferation ability for the 17T cells expressing the mutant Q187* and S1184P and for the 63T cells expressing the mutants S830F and S1091F. By contrast, the invasion test did not show any trend. These results suggest that mutations in the *ABCB5* gene could have an impact on melanoma progression through the migration ability.

The *in vitro* data showed that the expression of ABCB5 mutants and the silencing of its expression increase the proliferation and migration ability of melanoma cells. These observations, combined with the distribution of the mutations throughout the gene, suggest that ABCB5 act as a tumor suppressor. However, this contradicts the "two-hits" model of Knudson because the identified mutations were heterozygous. We made two assumptions: haploinsufficiency and the dominant negative effect. Further experiments like the CRISPR Cas9 would allow us to assess the impact of heterozygosity.

Mice experimental results would allow investigating whether ABCB5 acts as a tumor suppressor *in vivo*. In addition to the ongoing study, it also appears important to assess the impact of heterozygosity in mice. In this project, we performed a pilot study to assess the penetrance of the gene *Abcb5* using three mouse models: *Abcb5TyrNRas*^{Q61K}, *Abcb5TyrHRas*^{G12V} and *Abcb5Ink4a/Arf*$^{flox/flox}$*TyrCre*. For each mouse strain, we compared the tumor occurrence between wild type (WT) and knock out (KO) Abcb5 mice. Among the mice *Abcb5TyrNRas*^{Q61K}, five out of seven (5/7) KO mice and five out of eleven (5/11) WT mice developed melanoma. The proportion of tumor development is slightly higher in the KO group. This study is currently carried on with a larger number of mice to allow us to draw a conclusion. For the other two mouse strains, which are tamoxifen inducible model, no melanoma developed but other cancers appeared. In the KO mice of the model Abcb5TyrHRas^{G12V}, we observed one hepatoma and one lymphoma. Among the mouse strain Abcb5Ink4a/Arf$^{flox/flox}$TyrCre, we observed one spleen tumor and one lymphoma. Considering the low tumor occurrence, this experiment is currently underway with more animals.

The mechanisms underlying the involvement of ABCB5 in the proliferation and migration ability of the cell remains to investigate. We could hypothesize that ABCB5 would transport a signaling molecule, or a molecule having an impact on a signaling molecule, involved in pathway associated with melanomagenesis. While the clinical human melanoma samples mutated for *ABCB5* gene were very frequently mutated for *NRAS* (75%) and *CDKN2A* (62,5%), ABCB5 could have an impact on several pathways such as the MAPK, PI3K/Akt, retinoblastoma or p53 pathways. The determination of its subcellular localization is crucial for going further on the study of ABCB5 function.

This thesis allowed optimizing the conditions of de Duve fractionations of the melanoma cell line A375. Performing western blot, we observed an enrichment of this transporter in the nuclear and the microsomal fraction. However, further validations of the Rockland antibody are still needed. The western blot signal should be confirmed as corresponding to ABCB5 and further experiment could precise the subcellular localization. The presence of ABCB5 in the nucleus cannot be ruled out. Indeed, other transporters were localized in the nuclear envelope like ABCB1 and ABCC1 (Szczuraszek 2009; Szaflarski et al. 2013). The microsomal fraction contains fragments of plasma membrane, Golgi apparatus and endoplasmic reticulum. In order to identify the organelle enriched in ABCB5, we should perform the subfractionation of this fraction by using several orthogonal methods of separation, such as isopycnic centrifugation on various density gradients.

The identification of tumor suppressors have therapeutic applications. Indeed, targeting tumor suppressors may represent promising therapies. Strategies were developed for p53. For example, it is possible to reactivate the expression of the wild type p53 or to selectively kill p53 mutant tumor cells (Morris et al. 2015).

This shows the importance of studying tumor suppressor genes. It is crucial to investigate the potential link between ABCB5 and pathway associated with the proliferation and cellular survival as well as studying the function of this transporter. After establishing the potential pathway linked with ABCB5, this one could be considered as a target.

MATERIAL AND METHODS

1. Cell Culture

The human melanoma cell lines 17T and 63T were cultured in RPMI media supplemented with 10% Fetal Clone Serum, 1% penicillin/streptomycin, 1% L-Glut and HEPES (Life Technologies, Carlsbad, CA). The human melanoma cell lines A375, H1299 and KB31 were cultured in MDEM media supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

2. Lentiviral ABCB5 Wildtype and Mutated ABCB5 Production

Lentiviral ABCB5-FLAG tag plasmid DNA's (ABCB5-FLAG tag WT - M01, ABCB5-FLAG Q187* - M02, ABCB5-FLAG S830F - M03, ABCB5-FLAG S1091F - M04 and ABCB5-FLAG S1184P - M05) were supplied by the Protein Expression Laboratory Cloning and Optimization Group (Frederick National Laboratory for Cancer Research, MA). The HEK293T cells were co-transfected with the lentiviral envelope plasmid (pMD2.G, Addgene number 12259), the lentiviral packaging plasmid (psPAX2, Addgene number 12260), and one of the five lentiviral ABCB5-FLAG tag plasmid to generate lentivirus particles. The melanoma cell lines were infected with these lentivirus particles to overexpress either ABCB5 wild-type or one of the four mutated ABCB5. Cells were selected using 5 μg/mL puromycin. Sequencing was performed to assess the presence of WT or mutated ABCB5.

3. Proliferation Assay

4,500 cells per well of 17T and 63T cells were seeded into 96-well plates in complete RPMI media. At 24-hour time points (0, 24, 48 and 72 hours), growth media was removed and replaced with complete RPMI media solution containing a working concentration of 0.5 mg/mL MTT and incubated for 3 hours. Media with MTT was removed and cells were solubilized in DMSO. Absorbance was measured at 570 nm on a SpectraMax i3 plate reader (Molecular Devices, San Jose, CA).

4. Soft Agar Colony Formation Assay

Per well of a 24-well plate, 4,000 cells (17T, 63T), for all cell lines mutants, were suspended in 0.33% Bacto-Agar (Sigma-Aldrich, Saint-Louis, MO), diluted in complete RPMI media. This layer was plated on top of a layer of 0.5% Bacto-Agar, diluted in complete RPMI media. Plates were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 3 weeks. Colonies were stained using 2mg/mL MTT for 3 hours, and then counted.

5. Transwell Migration and Matrigel Invasion Assay

$12{,}5 \times 10^3$ cells for 17T and 63T cell lines were suspended in serum-free RPMI and pipetted into a Transwell insert (BD Biosciences, San Jose, CA) to assess their migration ability. The insert was placed into a well of a 24-well plate containing complete RPMI media and incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. The same manipulation was performed for the invasion assay with a Biocat Matrigel Invasion chamber. The inserts were washed with PBS (to remove the non-migrating and non-invading cells from the interior of the inserts) and stained using a Hema3 staining kit (Fisher Scientific, Waltham, MA). The migrating and invading cells were counted under a light microscope and the percent invasion was calculated by the ratio between the mean number of invading cells and the mean number of migrating cells.

6. Statistical Analysis

Data analysis was done by an unpaired Student's *t* test or Welch's *t* test when samples have unequal variances. Values are the means ± S.E.M. *P* values < 0.05 were considered statistically significant. Statistics and graphing were done using Prism software (GraphPad, La Jolla, CA).

7. Mice genotyping

7.1.DNA extraction

The DNA was extracted from mice ear-tip, lysed with 200µl of lysis buffer (100mM Tris HCl pH 8,8, 500mM KCl, 10% Tween20) with the action of proteinase K, at 95°C during 5 minutes and overnight at 55°C. The centrifugation at 15000g during 5 minutes was performed twice, at first in isopropanol and in a second time in ethanol 70%. The DNA was then suspended in water.

7.2. Amplification of the gene WT or mutant Abcb5 alleles

The amplification of the WT or mutant Abcb5 alleles was performed using the forward primer 5'-GTTAACTCCAACTCTCAGCTAAG-3' and the reverse primer 5'-CTCAACAATTTCTATAGCAATTACC-3' (final concentration of 500nM). The PCR program includes the heating at 94°C during 3 minutes, 29 cycles -30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C- and heating at 72°C during 3 minutes. An amplicon size of 512bp would be associated with the wild-type genotype, while the size if 112bp indicated the excision of the exon 2 of Abcb5.

7.3. Amplification of the Ink4a/Arfflox/flox allele

To amplify the Ink4a/Arf$^{flox/flox}$ allele, the forward primer 5'-CCTGACTATGGTAGTAAAGTGG-3' and the reverse primer 5'-ACGTGTATGCCACCCTGACC-3' (final concentration of 1µM) were used. The PCR program consists of 3 minutes at 94°C, 35 cycles of 94°C – 40 seconds, 60°C for 30 seconds and 72°C for 50 seconds, followed by 2 minutes at 72°C. The size of the amplicon was of 280bp for the wild-type allele and the size of 350bp indicates the presence of loxP sites flanking the exon 2 and 3 of Cdkn2a.

7.4. Amplification of the allele transgeneTyrCreERT2

To amplify the transgene TyrCreER^{T2}, a PCR with the primers 5'-GCGGTCTGGCAGTAAAAACTATC-3' and 5'- GTGAAACAGCATTGCTGTCACTT-3' at a final concentration of 500mM was performed. This PCR includes an internal control using two additional primers, 5'- CTAGGCCACAGAATTGAAAGATCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3'. The PCR program consists of 3 minutes at 94°C, 35 repetitions of -30 seconds at 94°C, 1 minute at 51,7°C et 1 minute à 72°C- followed by 2 minutes at 72°C. The amplicon corresponding to the transgene appeared at the size of 100bp, while the size corresponding to the internal control corresponds to 324bp.

7.5. Amplification of the transgene TyrHRas

The amplification of the transgene TyrHRas was performed using the forward primer 5'-AACTGCAGTGGGCAGGTAAGTATCAAGG-3' and 5'-CGCATAACCAGTGAAACAGC-3' at a final concentration of 1µM. The PCR program was the heating at 94°C during 3 minutes followed by 35 cycles of -40 seconds at 94°C, 30 seconds at 60°C, 50 seconds at 72°C- and 2 minutes at 72°C. The amplicon has the size of 586bp.

7.6. Amplification of the transgene TyrNRas

The amplification of the transgene TyrNRas using the forward primer 5'-AACTGCAGTGGGCAGGTAAGTATCAAGG-3' and the reverse prime 5'-CGCATAACCAGTGAAACAGC-3' at the concentration of 1µM was performed. The PCR program is 4 minutes at 94°C, 35 cycles of -94°C during 30 seconds, 30 seconds at 58°C, 30 seconds at 72°C- and 5 minutes at 72°C. The amplicon is visible at the size of 180 bp.

8. Dissolution and administration of 4-hydroxytamoxifen

The injections of 4-hyrdocytamoxifen were performed for the Abcb5Ink4a/Arf$^{flox/flox}$TyrCre and Abcb5TyrHRas^{G12V} mouse strains.

The dissolution of 4-hydroxytamoxifen to a concentration of 10mg/ml is a critical step. 100µl of ethanol was added at 10mg of 4-hydroxytamoxifen without vortexing the tube. This one was sonicated until the solution became clear. At that moment, 900µl of 45°C pre-heated peanuts oil was added to this tube, directly vortexed. The dissolved 4-hydroxytamoxifen was kept at the temperature of 37°C until its administration. The 8 weeks old mice received an intraperitoneal injection of 2mg 4-hydroxytamoxifen. This injection was performed four times with 2-days interval.

9. Mice monitoring

After injection, mice were monitored during 18 months or until the tumor reached a size of 0.5 cm^3. At this moment, the mice were sacrificed by cervical dislocation and dissected. Tumors or other remarkable organs were fixed with 4% paraformaldehyde and embedded in paraffin. They were sent to our collaborator Marianne Heimann who performed sections and hematoxylin staining.

10. Cell fractionation

Differential centrifugation was performed according de Duve and colleagues (de Duve et al. 1955) with the modifications proposed by Remacle and colleagues. The de Duve fractionation scheme allows the separation of the different organelles in six different fractions from the cell homogenate. The fraction E corresponds to the cytoplasmic extract excluding nucleus, the fraction N is the nuclear fraction, M the heavy mitochondrial fraction, L the light mitochondrial fraction, and P the microsomal fraction. The last supernatant corresponds to the cytosolic fraction S (de Duve et al. 1955). The A375 melanoma cells have undergone a differential centrifugation according to the de Duve protocol. Each step of this experiment was performed at 4°C to keep maintain the enzymatic activity. The cells were rinsed two times with sacharose 0,25M. Cells were scraped and mechanically homogenized in ice cold 0,25M sucrose by 15 passages in dounce homogenizer. The homogenate was centrifuged at 6.10^3.g.min. The pellet was rinsed with sucrose, homogenized and centrifuged again at the same speed. This second centrifugation gives the nuclear fraction (N), which is the pellet resuspended in saccharose and homogenized again by 5 dounce passages. The supernatant of these two centrifugations are pooled and correspond to the cytoplasmic extract (E). For the next steps, the ultracentrifuge Beckman L7-35 was used and a Beckmann rotor 50Ti. For each centrifugation, the duration was determined based on the volume. The cytoplasmic extract (E) is centrifuged at 30.10^3.g.min, the pellet obtained was washed with sucrose 0,25M and centrifuged again at the same speed. The pellet obtained corresponds to the M fraction. The supernatant obtained after these two centrifugations were pooled and centrifuged at 250.10^3.g.min. Again, this centrifugation was repeated to obtain the pellet corresponding to the L fraction. The centrifugation of the remaining supernatant at 3000.10^3.g.min allowed obtaining the pellet corresponding to P fraction (resuspended in sucrose and centrifuged once more) the supernatant, which is S fraction.

11. Enzymatic assay

11.1. Dosage of beta-galactosidase

The substrate of beta-galactosidase 4-MU-β-D-galactopyranoside (Carl Roth, Karlsruhe, Germany), was stored at -20°C in DMSO (50mM) was dissolved at 5mM in citrate buffer (50mM citric acid monohydrate, 50 mM tri-sodium citrate deshydrate) at pH4,5 and containing 0,05% of triton-100 (Promega, Madison, WI, USA).The different diluted fractions were added to this substrate solution. After 7 hours of incubation at 37°C, the reaction was stopped by the addition of glycine NaOH 0,1M pH 10,3. The absorbance was measured at 495 nm using fluorimeter VersaFluor™ (BioRad, Hercules, CA, USA).

A blank was used for this experiment. The fraction sample was replaced by sacharose 0,25M. For each fraction, two dilutions were used.

11.2. Dosage of alkaline alpha-glucosidase

The substrate of alkaline alpha-glucosidase, 4-MU-Alpha-D-glucopyranoside, was freshly prepared before performing the assay. It was dissolved in a solution at 37°C in 50% ethanol at 3,38mg/ml. The diluted fractions were incubated with a glycin buffer 0,1M at pH9 and 0,05% of Triton-X-100 (Promega, Madison, WI, USA). After an incubation of 4 hours, the reaction was stopped by the addition of a solution of glycine-NaOH 0,1M at pH 10,3. The fluorescence was measured at 495nm using fluorimeter VersaFluor™ (BioRad, Hercules, CA, USA). A blank was used for this experiment for which the fraction sample was replaced by saccharose 0,25M. For each fraction, two dilutions were used.

11.3. Dosage of alkaline phosphodiesterase

The activity of alkaline phosphodiesterase was measured using its substrate “Thymidine 5” monophosphate p-nitrophenyl ester sodium salt” (Sigma-Aldrich, MO, USA). The substrate was dissolved in a solution of glycine buffer 0,1M pH 9,6, 4mM of zinc acetate and 0,1M NaOH to obtain a final concentration of substrate of 1,6mM. The diluted fraction were added to this substrate solution and incubated at 37°C until this solution turned yellow. The reaction was then stopped by adding NaOH 0,1M and the absorbance was measured at 400nm using a spectrophotometer Lambda 10 (PerkinElmer, Waltham, MA, USA). A blank was used for this experiment. The fraction sample was replaced by sacharose 0,25M. For each fraction, two dilutions were used.

11.4. Dosage of cytochrome oxydase

The activity of the cytochrome oxidase was measured using its substrate « Cytochrome c from equine heart » (Sigma-Aldrich, Saint-Louis, MO, USA). The substrate solution must be freshly prepared before the assay. The substrate was dissolved (final concentration of 0,27mg/ml) in a solution 0,03M phosphate buffer pH7,4 ($NaH_2mgO_4.2H_2O$ 0,5 M ; Na_2HPO_4 0,5 M (Carl Roth, Karlsruhe, Germany)), 1mMM of EDTA pH 7,4. The fractions were diluted in a solution of phosphate buffer 1mM pH 7,4 ($NaH_2PO_4.2H_2O$ 0,5 M ; Na_2HPO_4 0,5 M) (Carl Roth, Karlsruhe, Germany), 1 mM d'EDTA pH7,4 (Carl Roth, Karlsruhe, Germany) and Triton X-100 0,04% (Promega, 29 Madison, WI, USA). The addition of ferrocyanure to substrate solution (oxidized cytochrome) and of dithionite (reduced cytochrome) was useful to calibrate the spectrophotometer. The zero corresponds to the completely oxidized cytochrome, while the absorbance value of the reduced substrate solution should be around 0,36 using spectrophotometer Lambda 10 (PerkinElmer, Waltham, MA, USA). 50µl of the fraction was added to 1ml of the substrate solution and the absorbance at 550 nm was measured during 30 seconds. The measure was performed in triplicate for each sample and underwent a logarithmic transformation.

11.5. Dosage of lactate deshydrogenase

The activity of the lactate deshydrogenase was measured by using its substrate: the pyruvic acid (Sigma-Aldrich, MO, USA). The substrate solution contain pyruvic acid 1,25mM, 63mM Tris (Carl Roth, Karlsruhe, Germany). In cuvettes containing 800µl of this solution, 100µl of fractions were added and 100µl of NADH (Sigma-Aldrich, MO, USA). The absorbance was measured during 3 minutes at 340 nm using a spectrophotometer Lambda 10 (PerkinElmer, Waltham, MA, USA).

11.6. Western blot

For ABCB5 detection in western blot, the fractions and homogenates were diluted in Laemmli's sample buffer containing 100 mM of DTT and heated at 37°C for 20 minutes. 15 µg of proteins were loaded and separated by SDS-PAGE. The separated proteins were electrophoretic ally transferred onto a polyvinylidene fluoride membrane (PVDF) membrane (Bio-Rad, CA, USA) at 110mV during 1 hour. The blotted membrane was blocked during 25 minutes, with 10% fat-free milk in in PBS containing 0,01% Tween 20.

The protein of interest was detected by incubation overnight with the Rockland antibody (Limerick, USA) 1,29x10-3 µg/µl diluted in BS/Tween 0.1% containing 0.2% fat-free milk. The membrane was then washed three times with PBS containing 0,01% Tween 20. The bound antibodies were detected using perroxidase-conjugated anti-rabbit Ig secondary antibody $8{,}3x10^{-5}$µg/µl) (Dako, Glostrup, Denmark) followed by ECL detection system. The membrane was incubated for 5 minutes in SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, MA, USA). The revelation was performed using Amersham Hyperfilm™ ECL (GE Healthcare, Fairfield, USA) and an imager Fujifilm FPM 100A Desktop Processor (Fujifilm, Minato-ku, Tokyo, Japan).

For the detection of histone H1, the same procedure was used. The primary antibody was used at the concentration of $2x10^{-4}$ µg/µl (Thermo Fisher Scientific, MA, USA) and the secondary mouse-Ig antibody at $8{,}3x10^{-5}$µg/µl (Dako, Glostrup, Denmark).

11.7. Quantification of western blot results

The intensity of the western blot signals of ABCB5 and histone H1 were quantified using the Image J software. In the distribution profile of ABCB5 and histone H1, the intensity of the signal corresponds to the Y-axis “the relative specific signal”.

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