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Caractérisation du promoteur du gène ABCB5 et localisation subcellulaire de la protéine

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Characterization of the ABCB5 gene promoter region and Localization of the ABCB5 protein in the cell

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Résumé

ABCB5, un membre de la famille des ATP-Binding Cassette (ABC) transporteurs, est principalement exprimé dans les cellules pigmentaires, à savoir les mélanocytes, l'épithélium pigmentaire de la rétine et les neurones dopaminergiques de la substance noire. Trois isoformes principales ont été identifiées : ABCB5 Full Length (FL) encodant un transporteur complet, ABCB5β qui code un transporteur tronqué et ABCB5α codant une protéine soluble.

ABCB5β est l'isoforme la plus étudiée. Trois fonctions y sont associées. Il a été montré que ce transporteur est impliqué dans la chimiorésistance du mélanome ainsi que dans la fusion des cellules progénitrices. Enfin, il a été suggéré qu'ABCB5β est un marqueur des cellules souches du mélanome, peut-être le résultat le plus controversé à ce jour. Deux études récentes montrent que les mécanismes de chimiorésistance seraient liés à la présence d'ABCB5 FL et non pas Bêta. Malgré ces données, le transporteur ABCB5 demeure très peu caractérisé. Nous ne connaissons pas son rôle au sein de la cellule saine, ni son rôle dans la biologie de la tumeur.

L'objectif de ce mémoire est double. D'une part, il a consisté a été étudier l'activité transcriptionelle basale d'ABCB5 grâce à l'analyse de fragments tronqués de son promoteur clonés dans un système rapporteur Luciférase. Nos résultats nous ont permis d'établir une voie de régulation de la transcription d'ABCB5 impliquant une boîte TATA et une Enhancer box, ainsi que différents facteurs de transcription tels que OCT1, FOXD3 et MITF. En outre, l'analyse des sites d'initiation de la transcription nous a permis d'identifier une nouvelle isoforme qui fera l'objet d'une validation ultérieure.

D'autre part, nous nous sommes intéressés à la localisation de la protéine ABCB5 dans la cellule. Nos premiers résultats indiquent qu'ABCB5 colocalise avec la mitochondrie et le mélanosome tardif. Une colocalisation avec d'autres organelles n'est pas exclue et est en cours d'analyse. L'ensemble des résultats obtenus lors de ce travail fourni de précieuses informations quant aux mécanismes responsables de la régulation d'ABCB5 dans les cellules de mélanomes et nous a permis d'émettre plusieurs hypothèses de travail.

Mémoire de master en biochimie et biologie moléculaire et cellulaire

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Table of Contents

List of abbreviations

5’RACE: Rapid Amplification of cDNA 5’Ends
5’UTR: 5’ untranslated region
aa: amino acids
ABC : ATP-Binding Cassette
ABCB5 : ATP-Binding Cassettes, subfamily, member 5
AML: Acute Myeloid Leukemia
APD + Pi: Adenosine diphosphate + phosphate inorganique
ApE: A plasmid Editor
ATP: Adenosine triphosphate
B5: ATP-Binding Cassettes, subfamily, member 5
bp: bases pairs
BSA: bovine serum albumine
C/EBP: CCAAT/enhancer-binding protein
CGIs: CpG islands
ChIp: Chromatin Immunoprecipitation
DAPI: 4',6-diamidino-2-phenylindole
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic acid
DPE: downstream promoter element
EMSA: Electrophoretic Mobility Shift Assay
FBS: Foetal bovine serum
FL: full-length (ABCB5)
GFP: green fluorescence protein
i.e.: for example
Inr: Initiator sequence
kb: kilobases
kDA: kilodalton
LAR II: Luciferase Assay Reagent II
MAPK: mitogen-activated protein kinase
MDR: Multidrug resistance
MITF: microphtalmia-associated transcription factor
MMIC: Malignant Melanoma Initiating Cells
MOPS: 3-morpholinopropane-1-sulfonic acid
NBD: nucleotide binding domain
NHEM: Normal human epithelium melanocytes
Nt: nucleotide(s)
PBS: phosphate buffer saline
PCR: polymerase chain reaction
PEG: Polyethylene glycol
PFA: paraformaldehyde
PLS: Passive lysis solution

qRT-PCR: quantitative real-time polymerase chain reaction
RIN: RNA integrity number
RNA pol II : RNA polymerase II
RNA: Ribonucleic acid
    mRNA: messenger
    ncRNA: non coding
ROS : reactive oxygen species
RT: reverse transcription
SDS: sodium dodecyl sulfate
siRNA: silencing RNA
SSC: Saline-Sodium Citrate
TF: transcription factor(s)
TMD: transmembrane domain
TSS: transcription start site
UV: Ultra-violet
B: beta (ABCB5)

Introduction

1. ABC transporters

The ATP-Binding Cassette transporter B5 (ABCB5) belongs to the ABC transporter superfamily, which require the energy of ATP hydrolysis to move substrates across a membrane [1]–[3].

ABC transporters are present in all the kingdom of live, from prokaryotes (bacteria) to eukaryotes such as yeast, fungi, plants, and animals. Up to now, more than 1,000 ABC transporters have been discovered. Drosophila expresses 51 ABC transporters, 69 are found in E. coli, which represent 5% of its genome. In the plant, Arabidopsis thaliana has 129 ABC transporter genes, while the human genome encodes 48 of them [1].

In contrast to passive transport proteins, ATP-Binding Cassette transporters transport molecules across membranes against a chemical concentration gradient or electric potential. Those movements are possible due to the energy released by ATP hydrolysis [2]. They can transport a wide variety of substrates including hormones, lipids, ions, sugars, amino acids, xenobiotics, drugs, etc… from the inside to outside (exporters/efflux).

1.1. The Human ABC family

Juliano and Ling discovered the first human ABC transporter [4], namely ABCB1, while studying multidrug resistance *in vitro* [5]. P-glycoprotein (ABCB1), MRP1 (ABBC1) and BCRP (ABCG2) are the three most studied ABC transporters [2].

The human ABC superfamily is composed of 48 members, classified in seven families ranging from ABCA to ABCG. Even if ABC transporters are mostly studied for their role in mediating multidrug resistance in tumor tissues, each ABC transporter plays a wide variety of roles in normal tissues. They are involved in lipid homeostasis, and transport numerous molecules including ions, peptides, amino acids, sugars, etc. In the gastrointestinal tract, liver, and kidney, they protect the organism in excreting toxins, and regulate local permeability by being expressed in the blood brain barrier, blood cerebrospinal fluid, blood–testis barrier and placenta [1], [6], [7].

1.1.1. Structure and function

The structure of a typical ABC transporter consists of two nucleotide-binding domains (NBDs), and two transmembrane domains (TMDs) (**Figure 1**). The NBDs possess containhighlyconserved residues known as the ABC signature (i.e. Walker A, Walker B and C-loops). Each TMD is composed of six α-helices [1], [3], [8].

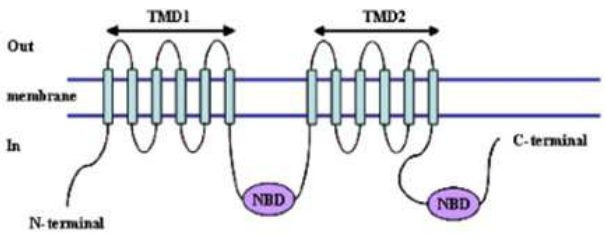

FIGURE 1 : *Structure of a typical full ATP-Binding Cassette transporter [6]*: ABC transporters are composed of two nucleotide binding domains and two transmembrane domains.

Beside typical full transporters, some members of the C family present an additional TMD composed of 5 α-helices at the amino-terminal end (i.e. ABCC1, C2, C3, and C6). Lastly,

some ABC transporters are half-transporters with one NBD either at the C-terminal side of the TMD (e.g. ABCB2, B3, B6 to B10) or at the N-terminal side of TMD (i.e. ABCG family). Those half transporters must homodimerize or heterodimerize to be functional.

The mechanism of action of an ATP-Binding Cassette transporter remains to be determined. To date, structural and biochemical data suggest an "ATP-switch model" (**Figure 2**), which consists in a switch between a high-affinity and a low-affinity state. Briefly, the cycle is composed of four steps. (1) It starts when the ligand binds to the TMD, this high affinity bound changes the conformation and opens the NDB, which increases the affinity for the ATP. (2-3) So the ATP binds to the NBD, which in turn induces a conformational changes of the TMD altering TMD's affinity to ligand, resulting in the translocation of the ligand across the plasma membrane. (4) Finally, the ATP hydrolysis and the release of ADP+Pi restore the initial conformation of the transporter [2], [3], [9].

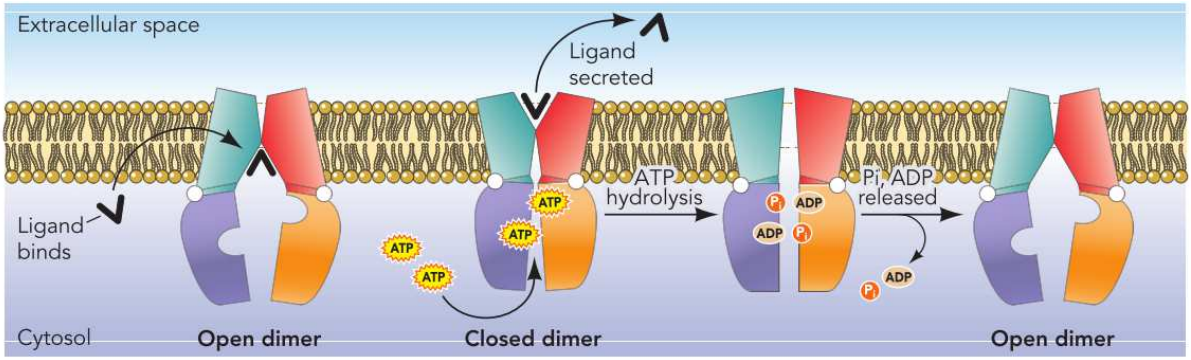

FIGURE 2: *Catalytic cycle of an ABC transporter [1]*: The proposed catalytic cycle of an ABC transporter consists of 4 steps (1) the ligand binds to the TMD, which lead to open the NBD and allow the interactions with the ATP (2-3) When the ATP binds to the NBD a new conformational change is induced, altering TMD's affinity for the ligand, resulting in the translocation of the membrane (4) ATP is hydrolyzed and its release restore the initial "open to ligand" conformation.

1.1.2. ABC transporter families

ABC transporters are classified into seven families (i.e. ABC A to ABC G). Each of those possesses its own characteristics, namely the general structure and function [1].

Due to the large variety of substrates and various localizations, mutations in an ABC transporter-encoding gene lead to severe diseases. To date, 21ABC transporters have been associated with genetic disorders including for instance cystic fibrosis (ABCC7), adrenoleukodystrophy (ABCD1), Stargardt disease (ABCA4), Tangier disease (ABCA1), immune deficiencies (ABCB2-B3), progressive familial intrahepatic cholestasis (ABCB4-B11), Dubin-Johnson syndrome (ABCC2), Pseudoxanthomaelasticum (ABCC6), persistent hyperinsulinemic hypoglycemia of infancy due to focal adenomatous hyperplasia (ABCC8), X-linked sideroblastosis (ABCB7), etc [6].

1.1.2.1. The ABCB family

The multidrug resistance family or the B family is composed of 11 members [1], [2]. The main characteristic of this family is its heterogeneity. It contains typical full transporters (i.e. ABCB1, B4, B5, and B11) and half-transporters (ABCB2, B3, B6, B7, B8, B9, and B10). They are localized in plasma membrane (ABCB1, B4, B11), in membranes of mitochondria (i.e. ABCB6, B7, B8, B10), lysosomes (ABCB9) and endoplasmic reticulum (ABCB2, B3).

They are involved in peptide transport, iron homeostasis. For example ABCB1 transports steroids, ABCB4 is implied is the transport of phosphatidylcholine, B6 and 7 carry for iron/glutathione complexes and ABCB11 transports monovalent bile salts [10].

Mutations in six of those ABC transporter-encoding genes are associated with genetic deficiencies. As expected from the name they carry, the ABC B transporters play a major role in the resistance of tumors to chemotherapy. In 2007,thirteenABC transporters (ABCA2,B1-4 and 11,C1 to 6-11 and 12 and G2) were validated as drug resistance mediators, while data were indicating that up to 30 of them may correlate with specific patterns of drug resistance [6]. Since then, additional ABC transporters have been implied in multidrug resistance (ABCA3, B5, C10 and F2) corroborating these preliminary data and assumptions.

ABCB1, a well characterized ABC transporter, has been mainly implied in multidrug resistance. Even if it plays other role in normal cell, this protein is majority known as an efflux pump able of extruding drugs out of cancer cells. In the presence of this transporter the prognostic of some cancer as acute leukemia is poor [11]. Because of its implication in multidrug resistance some clinical cancer researchers are trying to inhibit it with for example an Imatinib treatment [12]. This molecule is an antibody focus on the transporter. Up to now clinical trials weren't conclusive. ABCB5of the gene/transporter of interest of this master thesis, is an ABC transporter which possesses a lot of homology with ABCB1 (78% similarity, 54% identity). B5 is suggested to play an important role in the multidrug resistance of melanoma through a mechanism of drug sequestration into melanosomes.

1.2. The case of ABCB5

ABCB5-encoding gene is mapped on the chromosome 7 at position 7p15.3 [13]. This transporter is predominantly expressed in pigmented cells including melanocytes, retinal pigment epithelium cells and dopaminergic neurons in the *Substantia nigra pars compacta*, though it is also found in the testis [14]–[16]. Noteworthy, in situ hybridization shows that ABCB5 is expressed in brain capillaries and in Purkinje cells in the cerebellum. Interestingly, ABCB5 was not detected in the Substantia nigra, but in regions of the hippocampus and frontal cortex (data in submission for publication [17]). As one can expect from its expression in melanocytes, ABCB5 is also expressed in melanoma.

Three ABCB5 isoforms have been reported to date (**Figure 3**), the ABCB5 full-length (FL, 1257aa, 138.641 kDa), the ABCB5β (812aa, 89.9 kDa), and the ABCB5α (131aa) [13], [14]. Interestingly, our laboratory discovered two additional isoforms (manuscript in preparation).

The ABCB5FL form encodes a typical full ABC transporter and is composed of 12 transmembrane alpha helices (2 full transmembrane domains), and 2 nucleotide-binding domains [14], [18]. Sugimoto and colleagues were the first to describe this isoform that they found in the testis and in the prostate [19]. They also showed that the presence of ABCB5FL confers resistance to paclitaxel, docetaxel and doxorubicin.

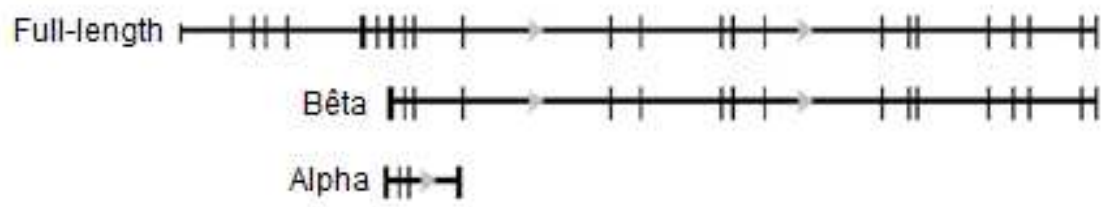

FIGURE 3: *ABCB5 isoforms*: ABCB5 can be present in three isoforms, the first one encodes a classical full-length transporter, the β form is a truncated transporter and lastly the α form is a very short transcript, and encodes a soluble protein (image from Jean-Pierre Gillet).

ABCB5β has a "one-transporter-like" structure, composed of 6 transmembrane alpha helices corresponding to one TMD and two NBDs, one of them being truncated. Moitra et al. discovered potential dimerization motifs in the N-terminal region of the beta forms [14], suggesting that ABCB5 β has to dimerize to be active. The β form is currently the most studied isoform. Frank et al. suggested that this transporter is a regulator of cell-cell fusion in normal skin progenitor cells [18] and is a mediator of resistance to doxorubicin in malignant melanoma cell lines [19]–[22]. More recently, the same group identified ABCB5β in melanoma-initiating cells [16], [22]–[25]. However, despite these reports, ABCB5, whose predicted full-length sequence is highly homologous to ABCB1, remains little characterized.

The data obtained in our laboratory indicate that ABCB5β does not mediate multidrug resistance when expressed in tumor cells and compared with mock vector transfected cells. However ABCB5FL does. Furthermore, biochemistry data reveal that ABCB5β protein show a decreased ATPase activity that may represent a futile cycle or be coupled to another function yet to be determined, while ABCB5FL protein ATPase activity falls at the expected range. In conclusion, data indicate that ABCB5β is not functional, though a native electrophoresis and western blot should contribute resolving this debate, since this transporter must form a dimer to be functional.

1.2.1. Suggested roles of ABCB5

1.2.1.1. Regulator of cell-cell fusion in normal skin progenitor cells

Studies suggest that ABCB5β is a regulator of cell-cell fusion in normal skin progenitor cells [18]. Cell fusion is observed when different cell types are co-cultured; they give rise to cell hybrids, which could generate differentiated cell. This phenomenon could have an importance in the tissue plasticity and renewal. During this fusion, plasma membrane hyperpolarization is observed. This polarization is regulated in part by ABCB5 and regulates the propensity of cells to undergo cell fusion.

1.2.1.2. Melanoma initiating cell marker

More recently, the same group identified ABCB5β in melanoma-initiating cells [16], [23], [24], [22]. The cancer stem cell model posits that tumors are hierarchically organized with a small population of tumorigenic cells that generates phenotypically diverse tumorigenic progeny in a similar manner to normal stem cell differentiation. This cell type is present in several cancers, including malignant melanoma.

First discovered as 'stem-cells like' by Fang et al. in 2005 [26], Malignant Melanoma Initiating Cells (MMICs) showed abilities to form sphere from cultured melanoma cells or

from fresh clinical specimens. Those spheres could self-renew, differentiate into various mesenchymal lineages and initiate tumors in mouse but this first experiment wasn't sufficient to prove that MMICs exist.

In 2008, Schatton et al. [27] showed that $ABCB5^+$ melanoma cells are essential for the melanoma induction and proliferation in mice [24]. This cell population represents 2 to 20% of the total tumor population. Compared to $ABCB5^-$ cells, $ABCB5^+$ have the ability to self-renew and differentiate, which are the characteristics of a stem cell.

This team wanted to see the action of a monoclonal anti-ABCB5 specific antibody. They injected it to nude mouse model and observed the apparition of neotumor and more particularly of $ABCB5^+$ cells but results were inconclusive: in some mice, $ABCB5^+$ cells were still present after using the antibody leading to regrowth of the tumor, whereas other mice remained tumor free for more than 8months following the antibody treatment [27]. They suggest that the antibody may not eradicate the totality of the $ABCB5^+$ pool of cells.

However, this paradigm was challenged by Quintana and colleagues [28] who showed that any melanoma cells, irrespective of whether they arose from melanoma-initiating cells (selected using reported markers) or not, have the capability to recapitulating a tumor in mice. They showed that around 30% of unselected melanoma cells give rise to new tumor once injected in mice. The authors also highlight the fact that both $ABCB5^+$ and $B5^-$ melanoma cells, discriminated using Frank's group antibody, were able to form tumors, in contradiction with the results obtained by Frank et al. [16].

Our laboratory is currently evaluating ABCB5 as a marker of melanoma initiating cells using transgenic mice that express ABCB5-IRESeGFP and spontaneously develop melanoma through mutation in the tumor suppressor PTEN and the BRAF oncogene.

1.2.1.3. Mediator of multidrug resistance and its implication in the treatment of melanoma

First described in 1787 by John Hunter and identified as such by René Laennec (1812) [29], melanoma is currently one of the most aggressive and frequent form of skin cancers which also include basal cell carcinoma, squamous cell carcinoma, sarcomas and rare carcinomas [30]. Melanoma arise from benign nevus, a pool of clonal melanocytes blocked in senescence, which underwent mutation by repeat DNA damages or activation of an oncogene [31].

The incidence of melanoma increases constantly since the twentieth century ranging from 1.0 per 100,000 in 1930 to 20.1 per 100,000 in 2007 in the United States. Currently, melanoma incidence is still elevate (**Figure 4**); melanoma is the seventh most common cancer in the world in women and the fifth in men [30], [32]–[34]. This worldwide increased incidence could be explain by increased UV exposure (natural and artificial) but also because of some others risk factors are as the family history, the age and the gender, the skin type and the ethnicity, the presence of dysplasic/other nevi, the body mass index, and the presence of chemicals or pollutants/ionizing radiation in the environment [32], [35], [36].

Up to now the best way to treat primary melanoma remains surgery, which has high rates of recovery: 85% of patients with melanoma achieve a 20-year survival. Secondary melanomas are more difficult to treat because of the spread metastases. During the past decades cytotoxic therapies (alone or combined with immune-directed therapy) were used, but the overall

survival is dismal. Those poor results for chemotherapies can be explained by several factors such as the high heterogeneity in melanoma but also multidrug resistance.

Estimated New Cases*

Males			Females		
Prostate	238,590	28%	Breast	232,340	29%
Lung & bronchus	118,080	14%	Lung & bronchus	110,110	14%
Colorectum	73,680	9%	Colorectum	69,140	9%
Urinary bladder	54,610	6%	Uterine corpus	49,560	6%
Melanoma of the skin	45,060	5%	Thyroid	45,310	6%
Kidney & renal pelvis	40,430	5%	Non-Hodgkin lymphoma	32,140	4%
Non-Hodgkin lymphoma	37,600	4%	Melanoma of the skin	31,630	4%
Oral cavity & pharynx	29,620	3%	Kidney & renal pelvis	24,720	3%
Leukemia	27,880	3%	Pancreas	22,480	3%
Pancreas	22,740	3%	Ovary	22,240	3%
All Sites	**854,790**	**100%**	**All Sites**	**805,500**	**100%**

FIGURE 4: *Estimated new cases of cancer (USA 2013) [34]*: 31,360 new cases of melanoma in women and 45,060 cases of melanoma were expected for the year 2013 in the USA, making melanoma the 7thmore common cancer in women and the 5thin men

The underlying problem of multidrug resistance is the development of a chemoresistant cell mass. First attack with a cytotoxic drug will kill all the non-resistant cells and will leave multidrug resistant (MDR) cells alive. The cancer will regrow from MDR cells, leading to a multidrug resistant cancer [22].

The importance of multidrug resistance in melanoma has long been demonstrated [15]. Many research teams show that “side” populations of cells that are characterized by high drug efflux are present in human melanoma [37]. The most common cause of multidrug resistance in cancer is the presence of ABC transporters on those cell [2], [6], [15], [38], [39]. For example, melanoma expresses seven ATP-Binding Cassette transporters rendering it resistant to a wide range of cytotoxic drugs. ABCB5 full-length is part of the seven ABC transporters found in melanoma and it leads to resistance to taxanes, paclitaxel and doxorubicin [19], [21].

A cell growth inhibition assay was performed to examine the sensitivity of ABCB5 full-length transfectants to some drugs. Cells showed 1.5-fold higher resistance to doxorubicin, 2.3-fold higher resistance to paclitaxel and 3.0-fold higher resistance to docetaxel. To make sure that this higher resistance is associated to ABCB5, siRNAs against ABCB5 were used and as expected resistance to those chemotherapeutics agents was lower [19].

Frank et al. showed that the ABCB5β^+ cells accumulate lower drug quantities than ABCB5β^- cells under culture conditions of continuous drug exposure [21], even more ABCB5β^+ population exhibited a greater intake of doxorubicin influx.

Because of the several ABC transporters found into melanoma cells, multidrug resistance to one particular drug can be explained by more than one transporter, such as in the case of ABCB1 and B5 which have overlapping substrate specificity.

Furthermore, Chartrain et al showed that treating melanoma with chemotherapeutic drugs lead to the selection of the ABCB5-expressing cells, which are resistant to drug treatment [22].

Many clinical trials have been performed to try overcoming multidrug resistance, especially directed against ABCB1 that is found to be expressed in a subset of acute myeloid leukemia (AML) samples from patients with poor outcome [11]. The proposed strategy relied on ABCB1 inhibition, so it cannot transport drugs out of tumor cells. Although this was promising, most of the clinical trials that evaluated this strategy failed to reach a positive endpoint [12].

Those results divided the field in two groups: one group suggesting to stop those clinical trials aiming to inhibit ABC transporters [40]. The other group underlines that inhibiting ABC transporters should be considered as an individualized therapy, and so shouldn't be tested on patients who do not express the target of interest [38].

Besides inhibiting transporters, one can take advantage of their expression and test the so-called collateral sensitivity. This strategy suggests to treat drug resistant tumors with a drug that would specifically target ABC transporter-expressing cells without having any side effects on normal cells that are known to express ABC transporters.

Lastly, there is a dire need to better understand how ABC transporter expression is regulated, in our case ABCB5, to highlight potential new targets.

2. Regulation of transcription

The transcription is a part of the gene expression, more precisely the first step, in which a part of DNA containing information (gene) is copied into RNA messenger by the action of the RNA polymerase II, a specific enzyme of the transcription [41].

The starting point of the transcription is called the transcription start site (TSS) and the initiation codon is an ATG. The transcription starts when the RNA polymerase bound to the promoter, assisted by transcription factors. The RNA polymerase will produce RNA moving along the DNA strands. The RNA polymerase will produce the RNA strand by adding complementary nucleotides of the DNA strand to the RNA-in production-strand. When the RNA polymerase meet the "stop" codon it detaches from the DNA strand and the RNA is released.

2.1. Promoter and transcription factors

The promoter region is a untranscribed sequence that serves as an anchor for the RNA polymerase II, which initiates the transcription of a particular gene [42]. The minimal promoter is the minimum element assembly needed to initiate the transcription *in vitro*. It consists in at least 40 to 60 nucleotides and is composed of some elements like the TFIIB (BRE), the TATA Box, initiator elements (Inr) and downstream promoter elements (e.g. DPE, DCE and MTE). In some case, promoter located further (around 200bp) from the transcription start site have been identified [43].

In vivo, the situation is different. The core promoter region is typically situated at 30 to 60 bases from the Transcription Start Site or TSS (first transcribed nucleotide of the RNA transcript, typically an A). There is a wide variety of promoter sequences, including the TATA box, the CpG islands, the CAAT box, the DPE, etc., all these boxes are well known: their sequence is characterized as their activation pathway.

The TATA box is considered to be the core promoter element due to its major role in the initiation of the transcription [44], [45]. The DNA sequence is 5'-TATAAA-3' and is located approximately 25 bases upstream of the transcription start site. The TATA box sequence is recognized by the TATA box binding protein (TBP) and by other transcription factors such as the TFIID, A, B, F and H. This complex will initiate the transcription by helping the RNA polymerase II to bind.

CpG islands (CGIs) are regions rich in CG repeats [46]. They play a role in the transcription initiation of a lot of genes and it seems that 70% of the promoters are associated with these. CGIs are often unmethylathed, which destabilizes the nucleosome and attracts protein implied in the transcription. During her Ph.D. thesis, Chartrain showed that the ABCB5 gene isn't under the regulation of a CpG islands through methylation test.

CAAT box is another well-known promoter box [47]. C/EBP (CCAAT/enhancer-binding protein) is the transcription factor associated to this box. CAAT box is found in several gene promoters and is also present in several virus promoter regions.

Nevertheless the regulation of the transcription is not only dependent of promoter but also of distal regulatory elements which include enhancers, insulators, locus control regions and silencing elements. All those elements are often localized far from the gene that they control [48] and they allow a regulation of the transcription level of the mRNA in the cell, for example enhancers by up-regulating the production of mRNA and silencers by inhibiting it.

Enhancers are a recruit platform for transcription factors and they also act as chromatin-modifying. Those 2 actions facilitate genes transcription. Silencers act in the same way but bound inhibiting element, preventing transcription [48]. Insulators have a barrier function and prevent the interaction between an enhancer and an unrelated promoter, they are also known as "enhancer-blocking" [49].

Transcriptions factors (TFs) are proteins that assist RNA polymerase II to bind to the promoter sequence [41], [50].Once that this complex is formed, it undergoes some structural modifications, which are needed to allow the initiation of the transcription.

TFs recognize specific sequences in the upstream region of a gene, these sequences are termed transcription factor binding sites [41], [51]. Often more than one TF help the RNA polymerase to bind to the promoter, TFs interact with each other, and in this case TFs are defined as "co-activators" or "co-repressors".

Some transcription factors are essential for an efficient transcription; these are the general transcription factors (GTFs). They will assist in the loading of RNA polymerase II at the TSS. Those general transcription factors will also help to separate the DNA double strands during the initiation step, to detach the RNA polymerase II of the promoter and engaged in the elongation step.

2.2. Genetic of melanocytes and melanoma cells

There is a large variety of pigmentation phenotypes worldwide which highlights a wide regulation of the transcription network. A lot of transcription factors and proteins are implied in the regulation network of the melanocyte development and melanoma formation

(**Figure 5**). Some of them, the most characterized, are listed below, outlining the genetic of melanocytes and melanoma.

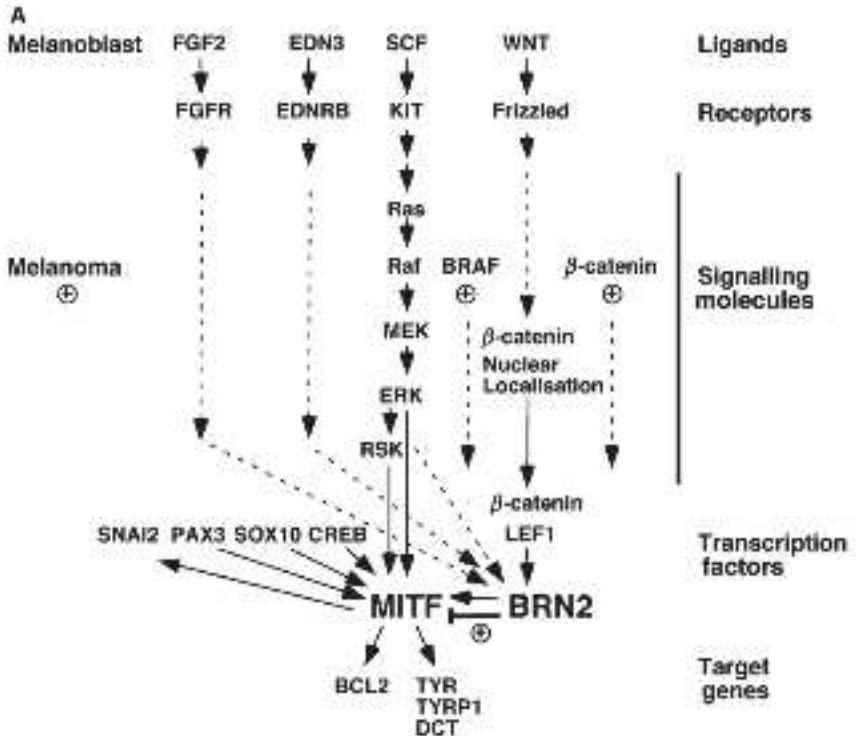

FIGURE 5: *Regulation network behind melanocytes and melanoma [64]*: Melanoma and melanocytes are regulated by a wide variety of transcription factors. The most important is MITF, the key of the regulation network behind those cells. MITF is regulated by BRN2, SOX, BRAF, PAX3 and a lot of others elements. Over expression of those TF could lead to melanoma.

2.2.1. SOX transcription factors

The SOX family is composed of approximately 20 transcription factors, which are known to be involved in the development and normal physiology of several tissues through their roles of regulation in a lot of biological process [52]–[54]. These proteins coordinate biological functions such as maintaining stem cell properties, lineage restriction and terminal differentiation through temporal and spatial expression specific to each cell type and tissue.

The role of SOX in the development of melanocytes was discovered when a mouse model for the Waardenburg-Shah syndrome was genetically characterized. In this syndrome, patients are deaf and suffer of hypopigmentation. Researchers discovered that SOX10 was truncated [55]. Moreover, SOX10 has the ability to activate the transcription of several genes involved in melanin synthesis as DCT, PMEL17 and TYRP1 [53]. SOX 10 activate the transcription of MITF which induces the transcription of those genes. Others SOX proteins are implied in the melanocyte development such as SOX5, 9 and 18 [52].

Even if SOX is mainly expressed during the embryonic life (see Harris et al. [53] for an excellent review), SOX proteins also participate to the melanocyte-life cycle after birth. For example, SOX10 is only present in melanoblasts, while SOX9 is present at high levels in melanocytes.

Both SOX 9 and 10 are present in melanoma [54], [56]–[58]. High level of SOX10 and MITF correlated with proliferative cells fit with a more invasive melanoma. Furthermore SOX10 and MITF were mutated in 7% of primary and in 20% of metastatic tumors.

Increasing SOX9 expression both *in vivo* and *in vitro* demonstrated a decrease in the proliferation of multiple melanoma cell lines [53]. A possible explanation could be that UV exposure induces expression and nuclear localization of SOX9 through the cAMP/PKC-dependent pathway which will lead to MITF up-regulation.

2.2.2. MITF

Discovered 20 years ago, microphtalmia-associated transcription factor (MITF) is the key transcription factor of melanoblasts, melanocytes and melanoma regulatory network. It regulates the transcription factors and signaling pathway behind the maturation, survival, proliferation and differentiation of both melanoblasts and melanocytes [54], [59]–[62]. Scientists proved the role of MITF in melanocytes by using MITF -/- mice which lack all pigmented cells [60], [62], [63].

Once that MITF is expressed in the cell, it may target promoter regions of different genes, which are expressed in melanocyte lineages. The principal targets of Mitf are the E-box motif characterized by the following core sequence 5'-CATGTG-3' and the M-Box, with a 5'-AGTCATGTG-3' DNA motif [62], [64]. MITF will then help to the transcription of genes present in melanocytes as TYRP1, DCT and PMEL17 [53].

Expression of MITF in melanoma is very heterogeneous, with 10 % of primary melanoma and 21 to 40% of metastatic melanoma expressing up to 10-fold higher levels of MITF [63], while in some cases MITF rates are very low. High levels of MITF exert an anti-proliferative activity in melanoma cells but low levels are associated with poor prognosis and clinical disease progression in invasive melanoma cells.

Because of its crucial role in melanocytes and melanoma, its regulatory network is more and more studied, which permit to highlight transcription factors like PAX3, SOX10, CREB, FOXD3, etc and their implication in the regulation pathway [54].

The transcription of *Mitf* is inter alia regulated by SOX10, a transcription factor already studied in melanocytes. SOX10 is known to play a role in the specification, migration and survival of a large variety of cells including melanocytes. Moreover, scientists demonstrated that four SOX10 protein binding sites are present in the Mitf gene promoter so SOX10 is a modulator of the MITF expression [54], [62].

Similarly to SOX10, PAX3 is a transcription factor able to bind the Mitf promoter region [54], [62]. Interaction between SOX10 and PAX3 was demonstrated and allow a strong expression of MITF (in co-transfected cells).

CREB is a transcription factor that binds MITF. Activated CREB binds the cAMP response element motif (present in the Mitf promoter) and increases the MITF expression.

In opposite to the 3 first TFs, FOXD3 is a TF that down-regulate the Mitf expression. This repression occurs by two ways, in the first one, the most simple, FOXD3 directly binds the Mitf promoter region. In the second point of view Foxd3 indirectly represses Mitf by binding PAX3, an up-regulator of Mitf [59], [62].

The four transcription factors listed above are examples of TFs implied in the activation network of MITF, other transcription factors are needed but to date, all of them have not been characterized yet.

The Wnt signal pathway also leads to the over-expression of MITF, as observed in melanoma. Elegant experiments in the Zebrafish showed an increase in the melanoblasts number when the Wnt signaling pathway components are over-expressed [60], [62].

2.2.3. BRAF-RAS

BRAF is an important molecule which is part of the super family of the MAPK (mitogen-activated protein kinase) signal transduction pathway. This pathway regulates cell growth, survival and invasion. BRAF belongs to the family of RAF genes. BRAF is frequently mutated in human cancers (e.g. colorectal, ovarian, thyroid, lungs…), but the highest rate of BRAF mutations have been observed in melanoma. In 90% of the cases, the mutation is a substitution of a valine with a glutamic acid at position 600 (V600E). This change activates BRAF leading to the downstream expression induction of Mitf (activator of the melanocytes growth) [54], [59], [62].

Several studies showed that the activation of BRAF-V600E is necessary but not sufficient to develop a melanoma. Transgenic zebrafish expressing BRAF-V600E present spectacular development of nevi but when BRAF-V600E was expressed in p53-deficient zebrafish, first nevi rapidly developed in invasive melanoma [59], [60].

Furthermore, the prevalence of BRAF mutations is lower in the early stage than in the late stage lesions, which seems to strongly suggest that an alteration is involved in disease progression. Data obtained about BRAF gene and melanoma suggest that this protein could be a possible target of UV-induced damage. Additional works will provides more information about interaction between UV and BRAF.

2.2.4. CDKN2A

The cyclin-dependent kinase inhibitor 2A (CDKN2A) is a gene located on chromosome 9p21 and which encodes 2 proteins p16CDKN2A and p14CDKN2A which are known to be tumor suppressors. This gene is also the major gene involved in melanoma pathogenesis and it has been studied because of its occurrence in families with multiple cases of melanoma [54], [62], [65].

Like BRAF, CDKN2A is a target of UV radiation in melanoma formation and this gene is the higher penetrance melanoma susceptibility gene. When mutated, p16CDKN2A does not check the end of the G1 step anymore, promoting cells division and mutations. If p14CDKN2A is mutated, the level of p53 decrease in the cells, allowing the cell to subdivide even in they are mutations. When one of those proteins is mutated, the senescence of the nevi is accelerated.

2.2.5. BRN2

BRN2 is a POU domain transcription factor highly over-expressed in melanoma cell lines [54], [56], [59], [66]. This TF is also expressed in specific area of the brain (hypothalamus and cortex-are required for its development region). Interactions between SOX proteins and BRN2 have been demonstrated, leading to the transcription of some genes like Krx20

(Schwann cell differentiation). SOX proteins and Brn-2 also interact with the PAX protein and more particularly PAX3. This complex could lead, for example, to the transcription of MITF protein [66].

This up-regulation in melanoma cells arise from up-regulation of 2 signaling pathway, the Wnt/β-catenin and the MAP kinase which are both deregulated in melanoma. BRN2 could act as a melanoma survival/proliferation positive regulator.

2.2.6. PTEN and AKT

PTEN is a protein that acts as a tumor suppressor by regulating cellular division, cell migration, spreading and apoptosis [54], [59], [62]. In normal conditions, PTEN has a lipid phosphatase activity which cleaves the AKT protein. AKT, at high level, conducts to stimulation of cell cycle progression, survival, metabolism and migration. PTEN regulated the cell cycle by decreasing the activity of promoter of proliferation, AKT.

When PTEN is mutated, the activation of AKT will increase by a decrease of the phosphatase activity. The activation of AKT will conduct to stimulation of cell cycle progression, survival, metabolism and migration. Moreover, AKT is a protein which activates NF-kB, a pleiotropic transcription factor involved in the control of proliferation and apoptosis in melanoma. PTEN is mutated in a large proportion of human melanomas and in a large variety of cancer, including lymphoma, thyroid, breast, prostate carcinoma.

3. Purpose of this master thesis

As a first step towards elucidating the mechanisms that regulate ABCB5 gene expression, we will characterize its promoter region and therefore highlight the regulatory elements present. This will be performed using bioinformatics through software such as Match. Experimentally, the human BAC CH17, which contains the entire ABCB5 gene region, will be used to clone the promoter region. Then, analysis of truncated fragments of the promoter cloned in a Luciferase reporter vector will be used to determine the basal transcriptional activity of ABCB5.

This will be done in a primary melanoma cell line, i.e. MelJuSo and a metastatic melanoma cell line, i.e. MelJu.

Another part of this master thesis will be to localize the ABCB5 protein in the cell. Subcellular localization will allow us to speculate on possible cell function of that transporter.

Materials and methods

1. Characterization of the ABCB5 gene's promoter region

1.1. *In silico* analysis

1.1.1. Promoter boxes

Consensus sequences in gene promoters were gathered using the literature (PubMed - http://www.ncbi.nlm.nih.gov/pubmed). Those consists in core promoter boxes including TATA box, Downstream promoter element, initiator and non-core promoter boxes such as CAAT box, CpG islands, Enhancer box (E-Box), etc. The consensus sequences of these boxes were then matched to the upstream sequence of the ABCB5 gene using the sequence analytical software ApE (A plasmid Editor, http://biologylabs.utah.edu/jorgensen/wayned/ape/).

1.1.2. Transcription factors

The upstream sequence of the ABCB5 gene was analyzed using gene-regulation.com (Match, http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) to find transcription factor-binding sites (TF). The filtering criteria that we used are as follows: the matrices were used (1) to minimize the false positives, solely, and (2) to minimize both the false positives and negatives. Only those TFs with a core match (measurement of the quality of the match between the sequence and TF. A core ratio of 1 means perfect match) greater than 0.85 were selected, and subsequently correlated with literature on PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Transcription factors were then classified into several groups.

1.2. Cell culture

Normal Human Epidermal Melanocytes - NHEM (PromoCell, Huissen, Netherlands), primary melanoma (MelJuSo) and secondary melanoma (Malme3M, MelJu and UACC257) were maintained in DMEM supplemented with 5% FBS and 100 units of penicillin/streptomycin/mL (Lonza, Verviers, Belgium) at 37°C and 5% CO_2 humidified air.

1.3. Total RNA extraction

Cells were washed with 1x PBS and total RNA extracted using TriReagent (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions. For NHEM cells, high pure total RNA isolation kit from Roche was used according to the manufacturer's instructions (Roche, Brussels, Belgium). The RNA was resuspended in DEPC water and the concentration was measured using Nanodrop (ThermoScientific, Erembodegem, Belgium). All the samples were stored at -80°C. RNA integrity was confirmed by a denaturing gel and using a bioanalyzer of Agilent (Agilent, Santa Clara, USA).

1.4. 5'RACE

Rapid amplification of the 5′ cDNA End (5'RACE) region of the ABCB5 gene expressed in UACC257 and NHEM cell lines was performed using a 5′ RACE kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. Human testis RNA (Westburg, Leusden Netherlands) was used as a positive control (**Figure 6**).
5'RACE focus on the ABCB5 full-length or on the ABCB5β were realized independently. For each focus simple and double reverse transcription step were performed.
The first-strand cDNA was synthesized from 10µg total RNA with the 5'end-phosphorylated gene-specific reverse transcription (RT) primer (Eurogenetec, Seraing, Belgium) (see supplementary data **S1**). The first strand cDNA synthesis was performed as follows: 10µg of RNA, RT Buffer, RNase inhibitor, AMV Reverse Transcriptase and RT primer were pooled and placed in a thermal cycler at 30°C for 10', 50°C for 1 hour and 80°C for 2'. For the double reverse transcription step the program in the thermal cycler was 30°C for 10', 50°C for 1 hour, adjunction of 1µL of the AMV Reverse Transcriptase, 50°C for 1hour and 80°C for 2'.
Remaining RNA was then degraded by mixing the obtained cDNA with RNase H for one hour incubation at 30°C. Ethanol precipitation and ligation reaction were performed using 5'end-phosphorylated gene-specific RT primer with the RNA ligation buffer, 40% PEG-6000 (Polyethylene glycol) and T4 RNA Ligase. Samples were then incubated overnight at 15°C.
Nested PCR were then performed to enrich the ABCB5 transcripts. The first PCR was done by using 3µL of the ligation products. The PCR program for the ABCB5 FL was as follows: a denaturation step at 94 °C for 3', followed by 35 cycles: 94 °C for 30'', 58 °C for 30" and 72°C for 1', and finally 10'at 72 °C. The PCR program focus on ABCB5β was then same expected that the elongation step was 72°C for 2'30".

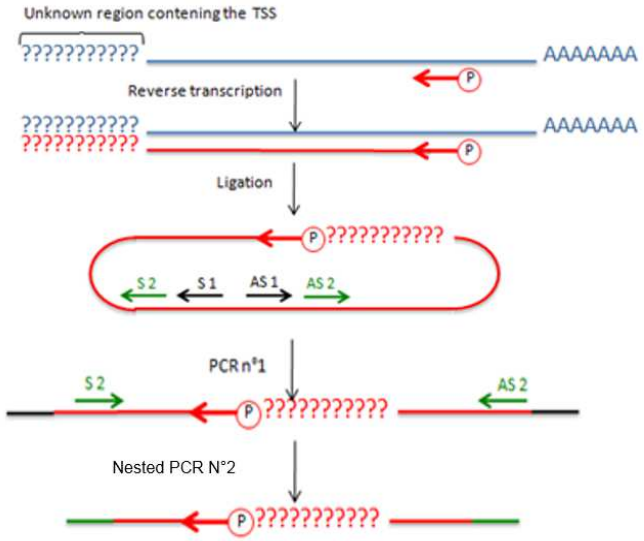

FIGURE 6: *5'RACE*: *5'RACE experiments in composed if 4 steps*: (1) reverse transcription with a phosphorylated primer (2) self-ligation of the cDNA products (3) nested PCR (4) sequencing (image from François Trouslard)

First PCR products were diluted 1:1, 1:10 and 1:100, and 3 µL of the product were used as template in the nested PCR. The latter was performed as follows: one denaturation step at 94 °C for 3', followed by 35 cycles: 94 °C for 30'', 58°C for 30'', 72 °C for 1', and a final step at 72°C for 10'. As in the first PCR, the PCR program was the same expected the elongation step (72°C for 2'30"). 5′ RACE products were separated using agarose gel (1%) electrophoresis for quality control.
PCR products were then cloned into PCR IV and PCR II vectors (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol, and the sequence of the cDNA insert was determined by sequencing (Beckman Coulter Genomics, UK). Samples of plasmids and primers were sent at a concentration of 100ng/ml and 5µg/ml, respectively. The obtained sequences were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with the ABCB5 sequence (NM_001163941.1).

1.5. Northern Blot

1.5.1. Electrophoresis gel

Northern Blotting was performed to detect the presence of ABCB5 RNAs in various cell lines and organs. After denaturing 50µg of RNA (UACC257, MelJu, MelJuSo, Malme 3M, mouse testis, human testis) with 15µL of a mix composed of 10µL of MOPS (3-morpholinopropane-1-sulfonic acid) 10X, 18µL of formaldehyde and 50µL of formamide, the solution was placed at 55°C for 15', 6X loading dye was then added.
A second run was performed with 10µg of RNA (UACC257, MelJu and Substantia nigra (Westburg, Leusden, Netherlands) mixed with 10µL of denaturing mix and put at 55°C for 15'. 2,5µL of 6X loading dye was then added.
A last run was performed in the same conditions with RNA extracted from ABCB5 non-expressing cells as negative control (COS and MCF-7, provide by Olivia Botton and Adrien Pirlot).
2, 5µL of ladder (Promega G319 A, Leiden, Netherlands) was also denatured similarly, but only 10µL of mix was sufficient and 1µL of ethidium bromide was added instead of 6X loading dye.
RNA was loaded in the following gel (900mg of agarose were dissolved in 76,2ml of DEPC water, 9ml of MOPS 10X, 4,8ml of formaldehyde and 2µL of ethidium bromide were added) for electrophoresis in MOPS 1X at 100 Volts for 1h15'. The gel was rinsed in DEPC water for 20' and soaked in SSC 20X (Saline-Sodium Citrate) for 45'. The migration could be visualized with a UV bench.

1.5.2. Transfer

Transfer of the gel was performed overnight by capillarity action. First 3-long pre-soaked Wattman papers were used to make a transfer bridge by pumping SSC 20X. Then the gel is placed on Wattman papers, followed by a Hybond membrane (Perkin Elmer NEF988001PK Boston, USA), which was soaked, beforehand, for 20' in SSC 20X. The assembly was completed using four soaked Wattman papers,eight dry Wattman papers, and 15 to 20 cm of dry paper towels. A glass plate and a weight were added on top of the assembly to optimize the transfer.
Following transfer, the Hybond membrane was recovered and dried between two Wattman papers. Once dried, RNAs were fixed on the Hybond membrane with UV during 5'.

1.5.3. Realization of the probes

Three different DNA probes were prepared by PCR (**S1**). Two probes were designed to detect ABCB5 transcripts, while the third one was prepared to detect a housekeeping gene - GAPDH.
The PCR cycles were *(i)* for the DNA probes: 94°C for 3' followed by 40 cycles: 94°C for 30", 58°C for 30" and 72°C for 1', and then a last step at 72°C for 10' *(ii)* for the GAPDH probe: the PCR program was similar, but the hybridization step was performed at 55°C. The probes were prepared in a volume of 200µL.
PCR products were gel purified. After migrating during 30' at 100 volts, DNA fragments were gathered and placed at 60°C. DNA was purified on a Wizard column (PromegaA9281, Leiden, Netherlands) by centrifugation according to manufacturer's instructions. Concentration of each sample was measured with a Nanodrop.

1.5.4. Probes hybridization

The membrane was soaked in the blocking solution for 45'minimum at 42°C before adding the labeled probes.
The labeling with ^{32}P (Perkin Elmer NEG513H, Boston, USA)was performed in several steps. The first one consisted in the denaturation of the DNA probe by mixing 25ng of DNA in 21µL of water, heated at100°C for 5'. The solution was then immediately placed on ice. Ladder was also labeled; 1µL of the ladder was denatured in 20µL of water.
Next the probe were labeled with the RadPrime DNA kit (Invitrogen, Carlsbad, USA): 1µL of dATP, 1µL of dGTP, 1µL of dTTP, 20µL of 2.5X Random primers solution and 1µL of klenow were added to the tubes and short spun. 5µL of dCTP labeled with ^{32}P were added and the mix incubated at 37°C for 20'. Then, 5µL of Stop Buffer was added into each mix and labeled probes were purified by centrifugation on a pre-equilibrated chromaspin column (Clontech, PT1300-1 Mountain View, USA) at 2700rpm for 5'.
After purification, probes were mixed with 800µL of sterile water and 150µL of DNA carrier (salmon sperm) for a total of 1ml and put at 100°C for 5' and then on ice. The probes were added to the membrane in the blocking solution and allowed hybridizing at 42°C overnight.

1.5.5. Washing

After the hybridization step, the Hybond membrane was rinsed several times to avoid unspecific background signal. A first wash was performed with SSC 2X and SDS 0, 1% at room temperature during 15'. This wash was repeated, and, followed by a step at room temperature for 15' with SDS 0,1% and SSC 0,2X and then the same washing step was repeated at 42°C. Background was checked with a Geiger counter; the membrane has to be radioactive only at the ribosomes location.
Membrane was put in a cassette with an auto-radiographic film (Super X Fujifilm 4741008389, Fujifilm, Tokyo, Japon) and placed in the -80°C freezer.

1.5.6. Revelation

Revelation of the auto-radiographic film was performed at different times. For the first time revelation was made at 7days, 1day, 8hours, 1hour and 30'. For the second gel revelation was performed at 4hours, 2 days and 5days.

1.6. qRT-PCR

cDNA synthesis was performed from 1 µg total RNA isolated from UACC257 and NHEM cell lines. A 20 µL reaction was carried out using the High Capacity cDNA RT Kit (Applied Biosystems, Gent, Belgium) according to the manufacturer's instructions. The 2X RT buffer was realized (by reaction: 2µL of RT buffer, 4, 2µL of Water, 1µL of Reverse Trasncriptase, 2µL of Random Primers and 0, 8µL of dNTP mix) then 10µL of mix were mixed with 10µL of RNA and put in a thermal cycler with the following program: 25°C for 10', 37°C for 2hours, 85°C for 5'.Once that reverse transcription was performed, cDNA was stored at -20°C.
ABCB5 expression levels were measured using TaqMan assays covering the ABCB5FL form (HS 03676539_m1, HS 03676540_m1, HS 03676541_m1, HS 03698751_m1) (Applied Biosystems, Gent, Belgium). Ct values were obtained using RQ Manager Software and normalized using 18s (HS 03003631_g1) and GAPDH (HS 02758991_g1) following the

deltadelta Ct method [67]. cDNA was mixed with 2× TaqMan Gene Expression Master Mix (Applied Biosystems), loaded on a 96-well plate, and run on an ABI Prism 7300 RT-PCR system tool (Applied Biosystems, Gent, Belgium) per the manufacturer's instructions.

1.7. Cloning of ABCB5 gene

1.7.1. Reverse transcription

RNA was extracted from different cell types (MelJu, MelJuSo, UACC257, Testis) and retro-transcribed using High Capacity cDNA reverse transcription kit (Applied Biosystems, Gent, Belgium) as explicated in the qRT PCR.

1.7.2. PCR

Different amplification reactions were performed: (a) cloning of the full-length ABCB5 cDNA with different pairs of primers (b) cloning targeting the extremities of the B5 cDNA (5' and 3'). 100 ng of each sample obtained from PCR was used to perform a nested PCR and 100 ng of a plasmid containing the ABCB5 full-length cDNA was used as positive control (see supplementary data **S1** for all the primers sequences).
The PCR program for the full-length ABCB5 cDNA was: 94°C for 3' followed by 45 cycles of 3 steps (94°C for 30", 55°C for 30", 72°C for 4'). PCR program completed with an elongation step at 72°C for 10'.
The PCR focus on the extremities was performed similarly than for the full-length cloning except that the the 3 steps were 94°C for 30'', 55°C for 30" and 72°C for 1'15.
The products of the PCR were visualized on electrophoresis gel (agarose1%).

1.8. Deletion Constructs

The region surrounding the putative transcription start site of ABCB5 was amplified by PCR from a BAC CH17 (BACPAC Resources, Oakland, USA) containing the ABCB5 promoter region. Clones were obtained using either a single restriction (5 clones: 3kb (-3003/-31); 1.5kb (-1654/-31); 1kb (-966/-31); 600bp (-609/-31) and 150bp (-190/-31)) or a double restriction (one clone: 700bp (-1500/-945)). Promoter's fragments were next added in a Luciferase vector.

1.8.1. Single restriction

Fragments were generated using primers, to which restriction enzyme sites were added to facilitate ligation. Two PCR programs were used, according to the annealing temperature of the primers.
The first PCR program (for fragments: 1kb, 900bp, 600bp and 150 bp) was as follows: 94°C for 3' followed by40 cycles composed of 3 steps : 94°C for 30'', 56°C for 30'' and 72°C for 2'15". PCR ended with an elongation step at 72°C for 10'. The second PCR program (for fragments 3kb and 1.5kb) was similar to the first one, but the 40-cycles were as follows: 94°C for 30", 54°C for 30" and 72°C for 3'30".
PCR products were gel purified using Wizard columns (Cat#A9282, Promega, Leiden, Netherlands) according to manufacturer's instructions. Fragments were then treated with the *HindIII* restriction enzyme (Promega, Leiden, Netherlands), to create the sticky ends, which are needed for the ligase step. 2µL of enzyme, 10µL of 10X appropriate buffer, 40µL of the

purified fragment and 48µL of H_2O were mixed together and placed at 37°C for 1hour. Enzyme was then inactivated at 75°C for 15'.

10µg of pGL4.10[Luc] vector (**Figure 7**) (Promega, Leiden, Netherlands) was linearized using *HindIII* at 37°C for 1hour (mix: 2µL of enzyme, 10µL of 10X buffer and H_2O up to 100µL). The enzyme was next inactivated at 75°C for 15'. Vector was then treated with phosphatase at 37°C for 1hour (SAP, Fermentas, Brussels, Belgium) to avoid self-ligation.

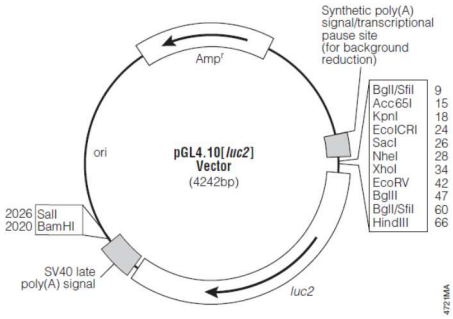

FIGURE 7: *Map of pGL4.10[Luc] vector*

Fragments were then inserted into the pGL4.10[Luc] vector using T4 DNA ligase (Cell Signaling, BIOKE, Leiden Netherlands). Materials needed for ligations were calculated using the following formula:

$$\text{X ng fragment} = \frac{(\text{fragment size in bp}) \text{ x } (\text{vector amount in ng})}{\text{vector size (4242bp)}}$$

Ligation was performed at 15°C for at least 4 hours followed by DH5α heat shock transformation as follows: 15'on ice, 42°C for 2' and 2'on ice. Bacteria were then placed at 37°C in 400 µL of culture medium (Leman Broth) for 1hour and then spread out on LB+ampicillin plates incubated at 37°C overnight.

The insertion of the PCR products in the Luciferase vector was checked with a directional PCR using a reverse primer corresponding to the fragment (19R) and a forward one matching with the Luciferase vector (RVprimer 3).

Positive clones were sequenced and then amplified in LB medium containing ampicillin to carry out a MaxiPrep (Promega, Leiden, Netherlands) according to the manufacturer's instructions. Concentration and quality ratios were checked using Nanodrop.

1.8.2. Double restriction

All the steps are similar to the single restriction procedure, but the restriction step that was performed using *KpnI* and *XhoI* restriction enzymes (Promega, Leiden, Netherlands).

The fragment (700bp) was obtained by PCR. The PCR program was as follows: 94°C for 3', 40 cycles composed of 3 steps : 94°C for 30'', 56°C for 30'' and 72°C for 1'. PCR ended by an elongation step at 72°C for 10'. Fragment was purified using Wizard columns.

Restrictions of the fragment and the vector (pGL4.10[Luc]) were performed similarly than abovementioned. Ethanol precipitation was then performed. Sodium acetate (1/10 of the DNA volume) and cold 100% ethanol (2 volumes per DNA volume) were added to the samples, mixed and placed at -80°C for 30'. Samples were then spun at maximum speed (14000rpm) during 15'. Supernatant was carefully removed and 1mL of 70% ethanol was added, mixed and spun for 10'. Supernatant was removed and pellet dried carefully.

The second restriction step was performed from the pellet obtained following precipitation supplemented with 1µL of *KpnI*, 1µL of 10X buffer and 8µL of H_2O. The steps were similar to the first restriction, but precipitation wasn't performed.

1.9. Cell Transfection

A transfection was performed using a GFP- and aABCB5-GFP-expressing constructs. This experiment was performed as positive control as well as to establish the transfection conditions using Turbofect (Thermo Scientific Fermentas, R0531, MA, USA) and the two cell lines (MelJuSo and MelJu).

1.9.1. Survival test

MelJu cells were plated at 125,000 cells/well and MelJuSo at 140,000 cells/well 24hours before de transfection in 6-well plates. Different volume of Turbofect were tested (4µL, 6µL and 8µL) with a fixed amount of DNA (4µg), and vice-versa. The fixed conditions were those provided by the manufacturer. Observations of cell survival were performed at 24, 48 and 72 hours post-transfection to determine the best conditions.

1.9.2. Transfection test

GFP imaging was performed on fixed cells. 24 hours before transfection MelJu and MelJuSo were plated in 24-well -plates at respectively 18,000 cells/well and 25,000cells/well. The transfection mix for the MelJu was composed of 2µL of Turbofect, 100µL of FBS-free DMEM and 1µg of DNA/well. The mix was incubated at room temperature for 20' before adding to the cells. The mix prepared for the MelJuSo was performed similarly, but 900ng of DNA/well only was used. Four hours post-transfection, culture medium was changed to avoid cell mortality.
Cells were fixed and observed 24, 48 and 72h post-transfection. After rinsing, cells were fixed with 4% PFA for 10', then rinsed 3X with PBS, permeabilized with 1% PBS-BSA during 5', rinsed 2X and incubated with DAPI (1µL in 10ml of DMEM) before rinsing and mounting the slides with Mowiol. Observation was performed with a confocal microscope and ratios were performed between transfected and untransfected cells based on observations.

1.10. Dual Luciferase Assay

MelJuSo and MelJu cell lines were used to compare eventually the ABCB5 activation pathway in primary and secondary melanoma.

1.10.1. MelJu cells

Cells were plated at 125,000 cells per well in 6-well plates. Each test was performed in triplicate. 24hours after platting, cells were co-transfected with our deletion Luciferase constructs (4µg/well), the Renilla vector (pGL4.74 [hRluc/TK]), 100ng/well, ratio of 1:40) and Turbofect (6µL/well). The mix between vectors and the transfection agent was carried out in serum-free DMEM and incubated at room temperature for 20'. 400µL of mix was added in each well.
An empty firefly Luciferase vector was also transfected as a control of the Luciferase activity without any promoter fragment. Two additional controls were performed: untransfected cells and Turbofect-transfected cells.

1.10.2. MelJuSo cells

Cells were plated at 40,000 cells per well in 24-well plates, each test was performed in triplicate. 24hours after plating, cells were co-transfected with the deletion Luciferase constructs (900ng/well), the Renilla vector (22,5ng/well, ratio of 1:40) and 2µL of Turbofect. The mix was prepared in serum-free DMEM and incubated at room temperature for 20'. 100 µL of mix was added to each well. The abovementioned controls were performed.

1.10.3. Dual Luciferase Assay

The Luciferase activity was measured according to the manufacturer's instructions (Promega, Leiden, Netherlands) as exemplified in the **Figure 8**. After washing cells with PBS, 500 µL of passive lysis solution (PLS)- MelJu - and 100µL of PLS- MelJuSo - were added per well, and plates were rocked for 15' at room temperature.
20µL of the lysate was added to 100µL of LARII (Luciferase Assay Reagent II),and the Firefly Luciferase activity was measured with a luminometre (Berthold DetectionSystem, Bielefeld, Germany). 100µL of Stop&Glow was then added to the sample to activate the Renilla Luciferase. This test was performed for each deletion construct (i.e. 6), the untransfected cells, the Turbofect-transfected cells, and the empty pGL4.10[Luc] vector. Activity was measured 24, 48 and 72 hours post-transfection.

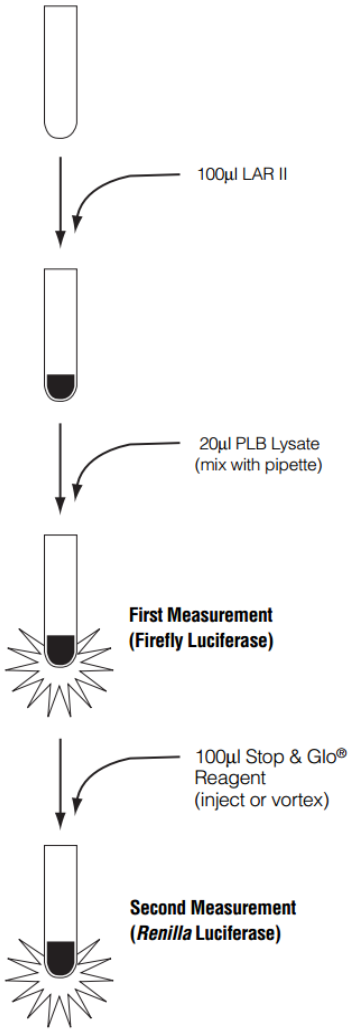

Figure 8: *Dual Luciferase Assay is based on 2 measurements (1) the Firefly Luciferase activity, corresponding to the deletion construct and (2) the Renilla Luciferase activity corresponding to the transfection control*

1.10.4. Analysis of the Luciferase values

For each condition, triplicate values were averaged and the background value (untransfected cells) was subtracted.
Relative Firefly Luciferase activity was then normalized with Renilla Luciferase activity for each construct and for the value of the empty pGL4.10[Luc]. The final ratio is obtained by dividing the Luciferase ratio by the empty pGL4.10[Luc] ratio.

$$\text{Luciferase activity} = \frac{\text{Condition Firefly Luciferase} - \text{background} \,/\, \text{Condition Renilla Luciferase} - \text{background}}{\text{pGL4.10[Luc]Firefly Luciferase} - \text{background} \,/\, \text{pGL4.10[Luc]Renilla Luciferase} - \text{background}}$$

1.10.5. *In silico* analysis of the core promoter region

The region identified with the Dual Luciferase Assay was analysed with the Match software (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) to find transcription factor-binding sites (TF) present in those particular sequences. The matrices were used to minimize both the false positives and negatives.

2. Subcellular localization of the ABCB5 protein

2.1. *In Silico* analysis of the targeting sequences

Organelle-targeting sequences were analyzed with TargetP, Geneinfinity, MultiLoc, SignalP, and SherLoc2 software to reveal potential localization(s) of ABCB5 in the cell.

2.2. Immunofluorescence

2.2.1. Cell culture

Two cell types (A375 and SK-Mel 28) stably transfected with an ABCB5-FLAG construct were used to perform immuno-fluorescence. A375 and SK-Mel 28 were maintained in DMEM supplemented with 5% FBS, 100 units of penicillin/streptomycin/mL (Lonza, Verviers, Belgium) and 3µg/ml Puromycine (Life Technologies, A1113!02, Gent, Belgium)in 37°C and 5% CO_2 humidified air.

2.2.2. Immunofluorescence

A375 and SK-Mel 28 were plated on a coverslip 48hours before fixation at respectively 45.000cells/well and 35.000cells/well in 24-wells plate. Following fixation, cells were incubated with Lysotracker Red (Life Technologies: L7528) or with MitotrackerRed (Life Technologies: M7513) at 37°C during 30'. Cells were washed with PBS and then fixed with 4% PFA10' at room temperature and permeabilized using PBS - 1% Triton X-100 for 5' at room temperature. For lysosomal colocalization (LAMP2), cells were fixed with Methanol-Acetone for 10' at room temperature. Cells were then washed with PBS-BSA 1% and incubated overnight at 4°C with the primary antibody. The antibodies were: Lysosome: LAMP2 used at a dilution of 250X (DSHB: #H4B4), Mitochondria: TOMM20 diluted 100X and 250X (Abcam: #ab115746), Early melanosomes: HMB45 diluted 250X (Abcam: #ab787), Late Melasomes: TRP1 diluted 250X (Abcam: #ab3312), Plasma membrane: β-Catenin diluted 250X and 1000X (Abcam: #ab6301) and the ABCB5 Anti-FLAG used at a dilution of 50X (Cell Signaling: #2368).
After overnight incubation, cells were washed with PBS-BSA-1% and incubated 1hour at room temperature with the secondary antibodies diluted at 1/1000. The ABCB5 FLAG-tag was stained with an antibody anti-Rabbit conjugated with Alexa 488 (A-11008, Invitrogen), while all the other staining were performed with an anti-Mouse antibody conjugated with Alexa 568 (A-11004, Invitrogen).
Coverslips were delicately washed twice with PBS-BSA-1%. Nucleus were stained with DAPI diluted 10.000X; 100µL was added in each well and incubated 5' at room temperature before washing. Coverslips were mounted on a slide with pre-heated Mowiol at 56°C and left at 4°Cin the dark overnight to harden before imaging. Imaging was performed with a Leica Microscope (Leica, Microsystems, Diegem, Belgium).

Results

1. Characterization of the promoter region of the ABCB5 gene

1.1. *In silico* characterization of the ABCB5 gene promoter region

The purpose of this study is to better understand how this gene is regulated. So, as a first step, we wanted to identify the promoter box and to compile the transcription factor binding sites in a 3 Kb-long 5' UTR region as well as a 2.4 Kb-long region including the first intron of the gene.

1.1.1. Identification of the promoter box

We have chosen to start with the identification of the promoter box of the ABCB5 gene. Gene expression is under the control of a promoter, which is defined as being located -10bp to -80 from the Transcription Start Site (TSS), and which is sufficient to activate the initiation of the transcription. The TSS is the exact site where the RNA polymerase II starts the RNA transcription. The ABCB5 gene is located on chromosome 7, and the TSS located at 20,655,245 bases. We selected a region for the analyses of the promoter flanking the transcription start site from -3002 to +2362bases.

We curated the literature to gather all the core promoter sequences (**Table 1**). The analysis showed that the ABCB5 gene is not under the control of CpG islands, nor CAAT box.

At this stage, we cannot exclude the role of a TATA box, downstream promoter element (DPE), or an Initiator (Inr) Sequence. They are present in the promoter region but are too far from the TSS or are located in multiple places. None atypical promoters such as the XCPE2 or the TRE boxes were present.

Name	*Consensus sequence*	*Present?*	*Localization (from the TSS)*
Promoters			
TATA Box	TATATA	Yes	-2873/-2069/-1705/-1088/+1332/+1401/+1600/+1941/+2069
CpG Islands	(CG)n	No	/
CAAT Box	GGCCCATCCAT	No	/
Downstream promoter element	RGWCGTG (AGTCGTG)	Yes	-2311
Inr Sequence	YYA^{+1}NWYY	Yes	-2101/-2071/-1839/-1692/-1513/-1452/-1412/-1396/-1289/-57/…
XCPE1	DSGYGGRASM	No	/
XCPE2	CCCCATTACAC	No	/
Sequence TRE (AP-1)	TGACTCA	No	/
Y Box Binding Protein (Mdr1)	CTGATTGGCCAA	No	/
X Box Binding Protein	GATGACGTG[TG]N_3[AT]T	No	/
Enhancer			
E-Box	CANNTG (palindromic)	Yes	*-2779/-2672/-2658/*-2574/-2296/-1797/-1733/*-851/-858/-795/-774/*-184

TABLE1: *Identification of promoter boxes:* Investigations was performed to identify possible promoter boxes in the ABCB5 promoter region. Tata box, DPE, INR and E-box were present but too far from the TSS.

Since there is no clear evidence for a specific promoter box, enhancer boxes were considered as they represent rational candidates. Enhancer boxes are sequences found upstream of the promoter region. It does not have to be close to the gene it regulates and so can be found up to 100,000 bases or on another chromosome. It can be bound with transcription factors that will initiate gene transcription The palindromic sequence of the E-Box was found in the sequence of ABCB5 (**Table 1**) at -2779/-2672/-2658/-2574/-2296/-1797/-1733/-851/-858/-795/-774/-184 bases from the putative TSS, but the CT repeats, which have to be 20 nucleotides (nt) before the E-Box are not present. The potential role of this enhancer in the regulation of ABCB5 gene expression has to be further investigated.

1.1.2. Analysis of the transcription factor-binding sites

In order to determine how ABCB5 expression is regulated, we have undertaken the analysis of its promoter region using the software Match (see Materials and Methods section 1.1.2.).

Analyzing one sequence of 5,364 bases generates a large amount of data. So, as a first step, we decided to analyze shorter fragments of the promoter region. Then, we analyzed the entire fragment of 5.3 Kb to avoid missing any transcription factors that could span extremities of the shorter fragments. After eliminating all the transcription factors with a core ratio (quality of the match between the sequence and the transcription factors) lower than 0.85, a literature search on each remaining TF was done.

This work allowed us to classify ABCB5 TFs into different categories: TFs linked to melanoma, TFs found in ABCB5-expressing tissues (e.g. melanocytes, retina, and testis), and unrelated TFs (**Figure 9**). Some transcriptions factors appear to be very relevant, such as SOX, PAXD3, BRN-2, AP-1, FOXJ2 and OCT-1.

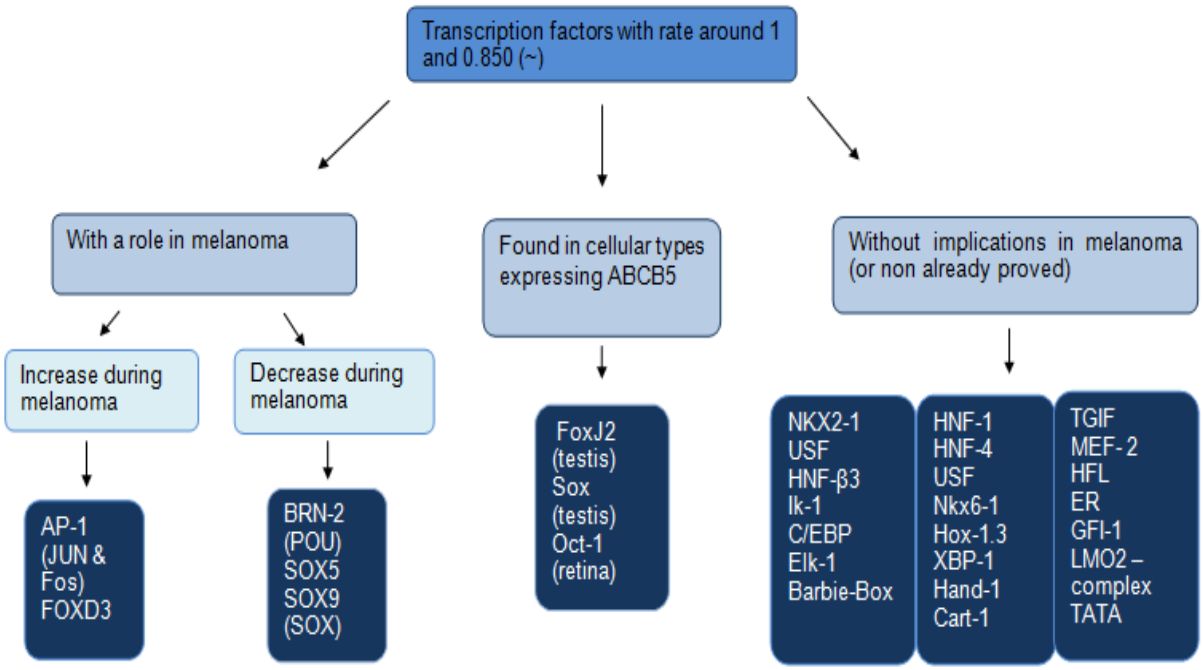

FIGURE 9: *Analysis of the transcription factor-binding sites:* The promoter region of the ABCB5 gene was analyzed with the MATCH software. Transcription factors were classified in 3 groups (1) TFs already present in melanoma, (2) TFs express in ABCB5-expressing cells and (3) unrelated TFs.

1.2. ABCB5: Identification of transcript variants and cDNA cloning

1.2.1. Determination of the transcription start site (TSS)

1.2.1.1. ABCB5 FL

As a first experimental step, we wanted to determine the transcription start site using a 5'RACE PCR experiment. This experiment allowed us to determine the TSS by a reverse transcription and ligation of the 5'end of the RNA, and a nested PCR. We isolated total RNA from two ABCB5-expressing cell lines: UACC257 and NHEM, furthermore human testis RNA was used as a positive control. Taqman-based qRT-PCR was performed to confirm ABCB5 expression. The 5'RACE nested PCR products were loaded on a 1% agarose gel (**Figure 10**), and the results revealed different bands for each cellular type.

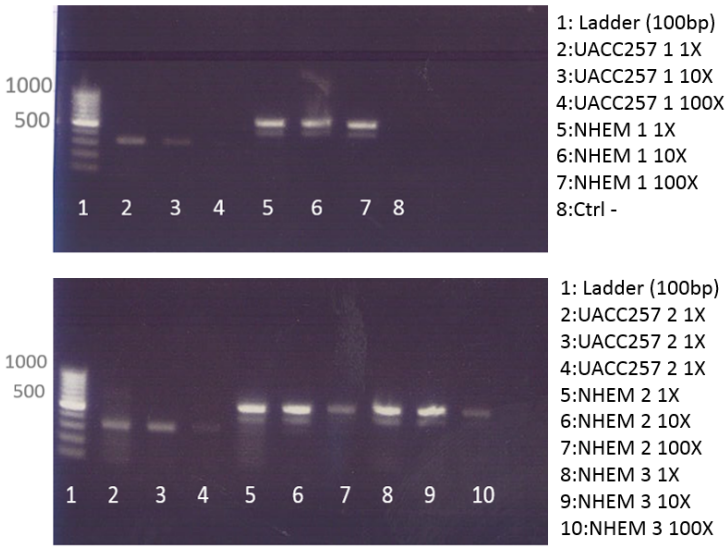

FIGURE 10*: 5'RACE focus on the ABCB5 FL (simple reverse transcription)*: 5'RACE was performed on different UACC257 (2 different and NHEM (3 different) ligation products. For the nested PCR the first PCR products were diluted 1X, 10X and 100X.

Those products were cloned into PCR IV vector to allow further analyses. All the clones were selected and classified according to the length of the insert. Samples of each group were sequenced, and results analyzed using BLAST software. Basic statistics were performed for each transcript, and each cellular type.

In the first experiment, sequencing showed that ABCB5 was present in 4 clones out of 57 obtained from the experiment performed using total RNA isolated from UACC257 cell line. This represents 7.01% of the clones.

Following localization of the phosphorylated primer as well as the primers used for the nested PCR, we were able to characterize one TSS in one out of the four positive clones. In more details, we found the RT-Primer in each sequence but the primer corresponding to the 5'end (A2) was not present in 3 clones (see **Figure 6**, in Materials and Methods section). In the positive clone, the A2 primer is very close to the Transcription Start Site. This TSS is localized in the exon 6 and could potentially indicate the existence of a new ABCB5 isoform. As for the 3 other clones, this absence of A2 primer could be explained by a problem during the self-ligation or by a problem of specificity of our primers.

ABCB5 was not found in total RNA isolated from NHEM cell line. The results of the sequencing showed that we amplified the proteasome 26S, the dodecyl sulfate transferase and the PUM2 homologue, but we did not obtain results matching with ABCB5.

Interestingly, our primers were designed on the ABCB5 full-length encoding sequence. The presence of the ABCB5β form in the cell lines that we investigated couldn't be evaluated in

the present study because of the localization of the first phosphorylated primer, which was designed before the putative TSS of the ABCB5β form.

In the second experiment, we performed the same 5'RACE experiment but the reverse transcription step was doubled to optimize the elongation of the neo-synthesized cDNA (**Figure 11**). Total RNA from human testis was used as positive control, as it was shown by Sugimoto and colleagues [19] that this tissue expressed ABCB5 full-length transporter .

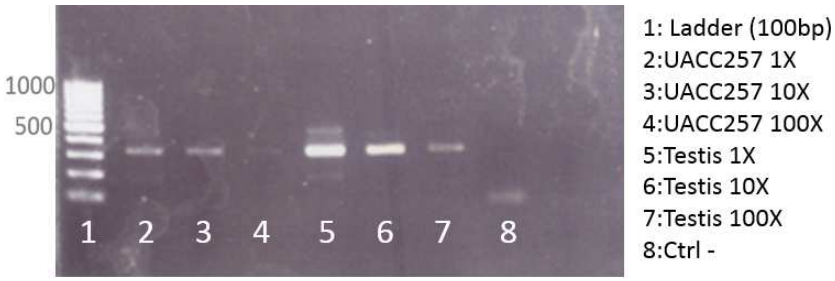

FIGURE 11: *5'RACE targeting ABCB5 FL*: 5'RACE was performed on UACC257 and Testis, but a doubled reverse transcription test was performed.

After sequencing, we could show that ABCB5 was present in 11.40% of the UACC257 clones and in 31.40% of the testis clones (**Supplementary Data S2**). Further analysis highlighted two clones (1 from UACC257 cells and 1 from testis) that corroborate the first 5'RACE results, namely a transcription start site located in the exon 6 (**Figure 12 A & B)**. However, we could not find any ATG codon that would be in frame of the coding sequence.

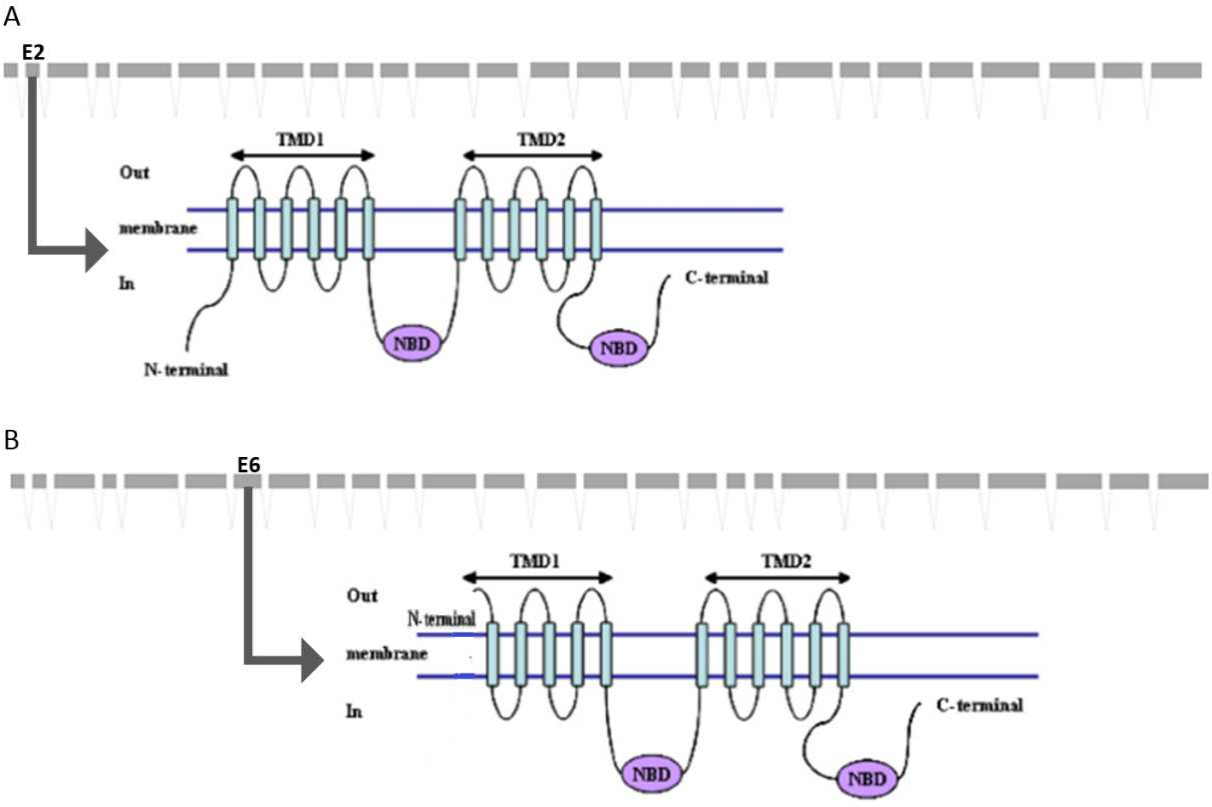

FIGURE 12: *Possible transcription start site of the ABCB5 full-length:* The putative TSS of ABCB5 FL is located in the exon 2 (A) but our 5'RACE experiments indicate a TSS in the exon 6 (B), leading to a ABCB5 full-length alike transporter, missing one α-helice in the first transmembrane domain.

1.2.1.2. ABCB5 beta

5'RACE experiments were also conducted to determine the transcription start site of the ABCB5β. As abovementioned, TSS was studied through reverse transcription and self-ligation of the 5'end of the RNA, and a nested PCR. The 5'RACE nested PCR products obtained from UACC257, NHEM cell lines and human testis, were loaded on a 1% agarose gel.

Those products were cloned into PCR IV vector. All the clones were selected and classified according to the length of the insert that was characterized by PCR. Samples (for each length found) were sequenced and results were analyzed using BLAST software.

As for the full-length, a first experiment was performed with a single reverse transcription step (**Figure 13**). In this experiment, total RNA isolated from UACC257 cell line was used, but two different reverse primers were assessed (RTa and RTb). ABCB5 was present in 33.40% of the clones with the reverse primer a, and in 57.30% of clones with the reverse primer b (**Supplementary Data S3**).

FIGURE 13: *5'RACE experiment targeting ABCB5β*: A first experiment was performed using UACC257 total RNA and two different reverse transcription primers (RTa and b).

After analysis of the clone sequences, only one of the A clones (obtained using RTa primer) matched with the ABCB5 sequence, and 4 of the B clones (obtained using RTb primer) do. Noteworthy, we obtained a larger range of amplicon length using reverse primer b. Therefore, the number of clones B was greater than clones A, explaining the greater rate found for B clones. The results revealed that potential TSS are located in a region of 225 bases, 88 bases the putative TSS of the ABCB5β.

The same experiment was performed on UACC257, NHEM and Testis but with a doubled reverse transcription step (**Figure 14**), to optimize the elongation of the cDNA. ABCB5 was present in 43% of the UACC257 clones, 71.40% of the NHEM and 45.80% of the Testis. Within these 71.40% of positive clones found from the NHEM RNA analysis, some of these clones were unparsed because of the absence of the RT or the nested PCR primers, in other we can found the RT primer but the sequence corresponds to the end of the RNA sequence and lastly a lot of clones had integrated the same sequence.

Three clones obtained from UACC257 and six from NHEM were present in this 225 bases region, corroborating the results obtained with the 5'RACE performed with a single reverse transcription step.

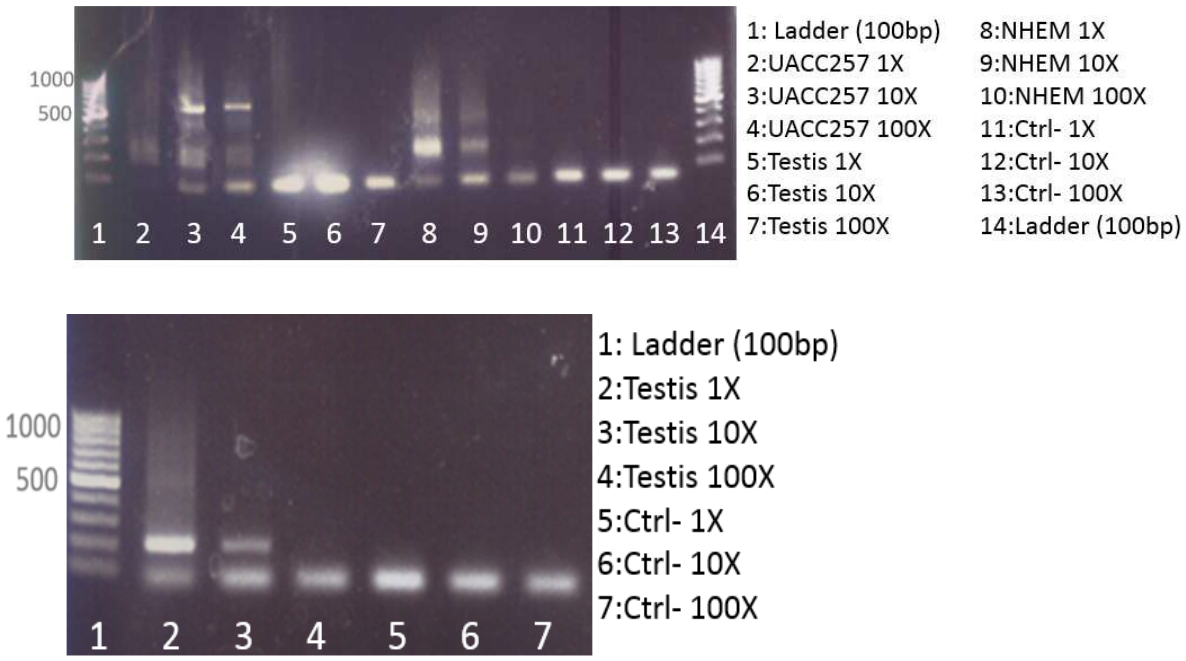

FIGURE 14: *5'RACE experiment targeting ABCB5β*: A second 5'RACE was performed on different cell lines (UACC257, NHEM and Testis) with a double reverse transcription step (A). The nested PCR on Testis cDNA product was repeated (B), allowing us to clone it as the others. To perform the nested PCR, the first PCR products were diluted 1X, 10X and 100X.

When analysis the 5'RACE performed with the total RNA extracted from human testis, we noticed that a problem during the reverse transcription occurred. After first analysis 45.80% of the clones match with the ABCB5 sequence but once that more précis analysis were performed we realized that instead of reverse transcripted the 5' extremity, it was the 3' extremity that has been reverse transcript.

In summary, the results indicate that there is a 225 base region localized 88 bases before the predicted TSS of ABCB5β that contains five potential TSS. However, further analyses did not reveal any ATG codon in that region (**Figure 15)**.

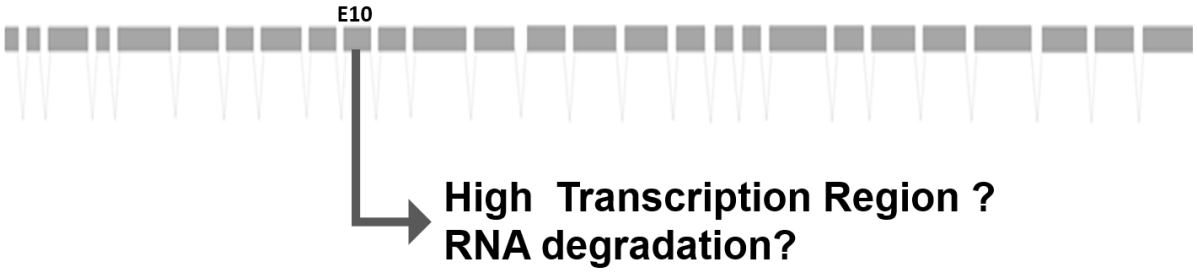

FIGURE 15: *ABCB5β5'RACE analysis:* 14 potential TSS were found to be located within a 225 nt region localized 88nt before the predicted β TSS. None ATG were found in that region.

1.2.2. PCR detection of ABCB5 isoforms

We wanted to validate by PCR the ABCB5 isoforms highlighted in 5'RACE experiment. Therefore, we targeted the ABCB5 full-length alike isoform (starting from Exon 6) as well as the predicted ABCB5 FL and Beta forms.

Total RNA was extracted from several melanoma cell lines including UACC257, MelJu, MelJuSo, Malme 3M and the primary melanocytes - NHEM as well as from human testis. Reverse transcription was performed from 1µg RNA followed by PCR using several primer sets, namely (1) 5' – amplifying a fragment 400bp long at the 5' extremity, (2) 3' - amplifying a fragment 500bp long at the 3' extremity, (3) FL – amplifying the predicted full-length-encoding sequence, (4) four combinations of primers – 5'FL, FL3', 5'-3' and 6'FL, the first three set of primers amplify the predicted FL isoform, while 6'FL amplifies the ABCB5 full-length alike isoform (**Supplementary Data S4**).

ABCB5 (**Table 2**) full-length was found to be present in all the cell lines but MelJuSo.

	5'	3'	FL	5'FL	6'FL	FL3'	5'-3'	FL/ β ?
UACC257	Yes	Yes	Yes	No	No	No	No	FL
NHEM	Yes	Yes	No	No	Yes	No	No	FL
Testis	Yes	Yes	No	Yes	No	No	No	FL
MelJu	Yes	Yes	No	No	No	No	Yes	FL
MelJuSo	No	Yes	No	No	No	No	No	Beta
Malme3M	Yes	Yes	No	No	No	No	No	FL

TABLE2: *PCR detection of ABCB5 isoforms*: PCR were performed on several ABCB5 expressing-cell lines to determine which isoforms are found to be expressed.

The results suggest that ABCB5 full-length is present in the UACC257, Malme 3M, MelJu, NHEM cell lines and testis. According to these results ABCB5 full-length is not present in the MelJuSo cell line but the ABCB5 β might be. We were not able to amplify the truncated exon 6-full-length isoform in those different cell types even in those where the ABCB5 full-length was present.

1.2.3. qRT-PCR

Taqman-based qRT-PCR was performed on the following cell lines: UACC257, Malme3M, MelJu, MelJuSo and NHEM as well as on human Substantia nigra, and testis, (**Table 3**).

ABCB5 is expressed at low level as indicated by the high ct value. qRT-PCR confirmed the presence of ABCB5 full-length isoform in all the analyzed samples.

Again, this experiment does not allow the discrimination between ABCB5 full-length and beta because the primer hybridizing on the ABCB5 beta transcript can also hybridized with the ABCB5 FL sequence.

	Exon 2-3	Exon 9-10	Exon 13-14	Exon 20-21	GAPDH
UACC257	35,7	27,1	34,6	28,5	27,7
NHEM	33,6	29,8	35,8	29,7	25,5
Subst noire	31,9	35,0	34,7	31,9	25,9
Testis	30,9	31,9	32,2	31,1	25,9
MelJu	31,1	32,4	32,4	31,1	24,6
MelJuSo	31,7	34,9	33,2	32,0	28,4
Malme3M	31,9	34,9	30,6	31,2	25,3

Table 3: *qRT-PCR*: In order to confirm the presence of ABCB5 in our B5-expressing cells qRT-PCR were performed on each cell lines. B5 is well present but expressed at low levels.

1.2.4. Northern Blot

Northern blot experiment (**Figure 16)** will allow the detection of different RNA isoforms by hybridization of specific and radioactive-labeled probes. Size of the RNA will be specific to each transcript and so this technique will indicate which isoforms of ABCB5 are expressed in the cell lines analyzed.

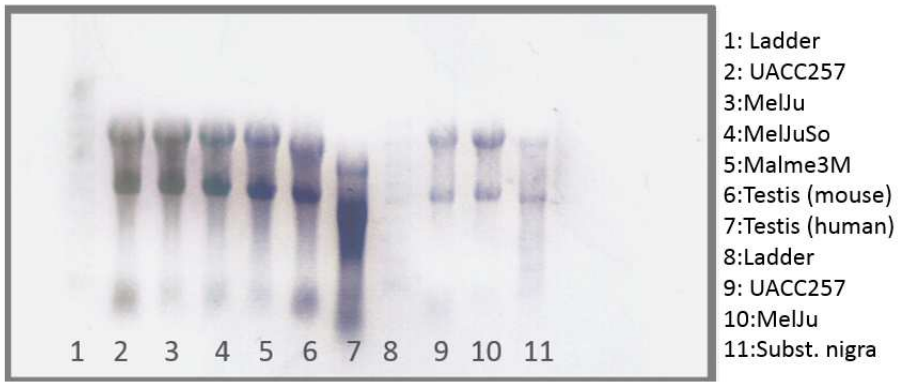

FIGURE 16: *Northern Blot*: For the 6 first samples, 50 µg of RNA were loaded and for the 3 last only 10µg were used.

We used three probes that recognize: the GAPDH, a housekeeping gene used as a control, the predicted ABCB5 isoforms: one overlapping exons 9 to 13, and the other overlapping exons 20 to 22. These two probes allow us to discriminate between the ABCB5 full-length and the beta isoforms. ABCB5 full-length is 4.5kb long, the beta form is 2.5Kb long and the GAPDH is 1.9kb long.

Hybridization of the probes was checked on cell lines that do not express ABCB5 to make sure that our probes are specific for the ABCB5 gene (**Figure 17)**. Probes were added one by one. We detected two ABCB5 isoforms in addition of the housekeeping gene, namely GAPDH. Using the probe overlapping exons 20 to 22, we could detect a shorter band appearing at approximately 150 bp.

We can say that ABCB5 full-length is present in the UACC257, MelJu, MelJuSo, Malme3M cell lines as in the human testis and the Substantia nigra. The β isoform cannot be observed on our Blot but new experiment will be performed in the upcoming months.

RNA from testis and Substantia nigra were degraded, as observed on the denaturing gel and with the RIN ratio. The presence of ABCB5 in those tissues was shown but a qRT-PCR was performed to confirm and quantify this presence.

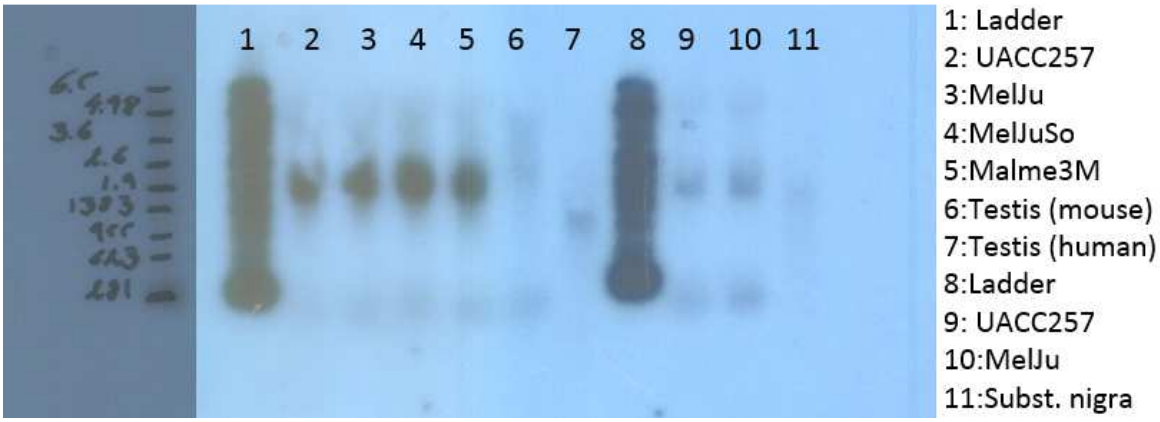

FIGURE 17: *Second Northern Blot Experiment (Revelation):* A single probe (exon 20 to 22) was added to the membrane after washing it and was revealed 48hours after. A smaller band appears on the film around 150 bp, which could fit with a small transcript discovered by J-P Gillet.

1.3. In vitro study of the promoter region of the ABCB5 gene

We cloned truncated fragments of the ABCB5 promoter region in a luciferase reporter vector to determine the basal transcriptional activity of ABCB5 (**Figure 18**). The five first clones were deleted from the upstream sequence of the promoter region. The last clone was deleted from the promoter region close to the transcription start site. All the fragments stop at 31 nucleotides ahead the Transcription Start Site because of a long TG repeats preventing a correct primer hybridization.

The constructs were checked by sequencing and amplified to obtain the purified plasmid useful for the transfection using the Dual Luciferase Assay.

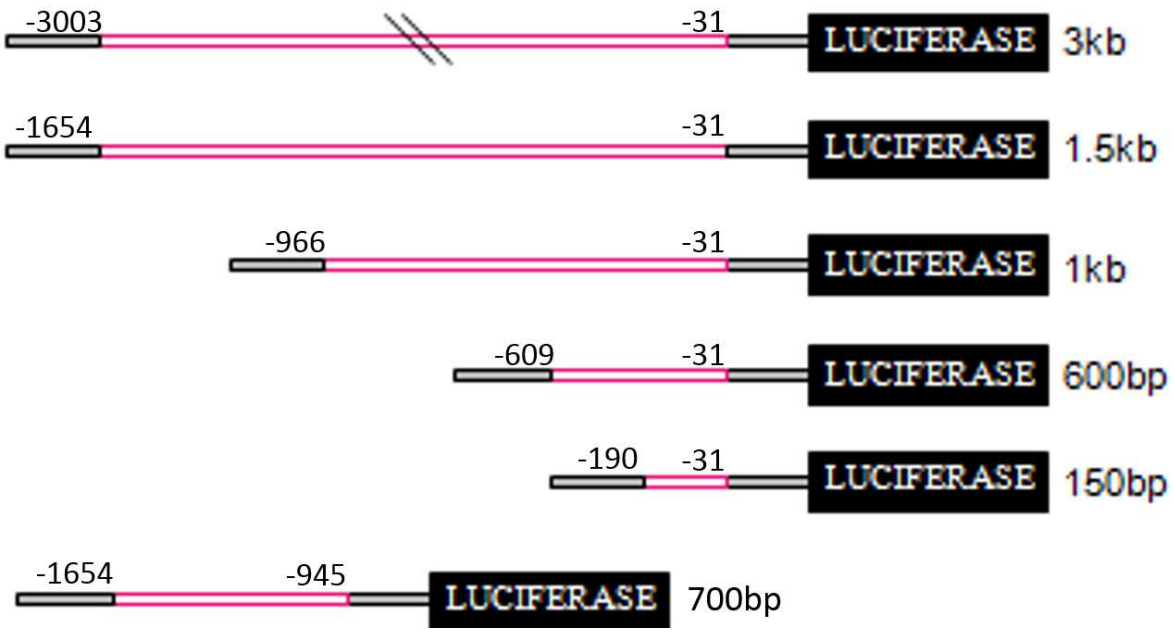

FIGURE 18: *Deletion constructs*: 6 different clones were realized: 3kb (-3003/-31), 1.5kb (-1654/-31), 1kb (-966/-31), 600bp (-609/-31), 150bp (-190/-31) and the last 700bp (-1654-/945).

As a first step, we wanted to assess the transfection efficiency of two melanoma cell lines planned to be used in the Dual Luciferase Assay, and eventually optimize the conditions using a GFP-expressing construct.

With regard to the MelJu cell line, a secondary melanoma, the conditions recommended by the manufacturer worked the best: cells were plated at 125,000 cells/well in a 6-well plate and 6µl of Turbofect were mixed with 4µg of DNA (**Figure 19**). For the MelJuSo, a primary melanoma, request more optimizations. We arrived at the conclusion that they need to be plated at 160,000 cells/well in a 6-well plate and 6µl of Turbofect were mixed with 3.6µg of DNA.

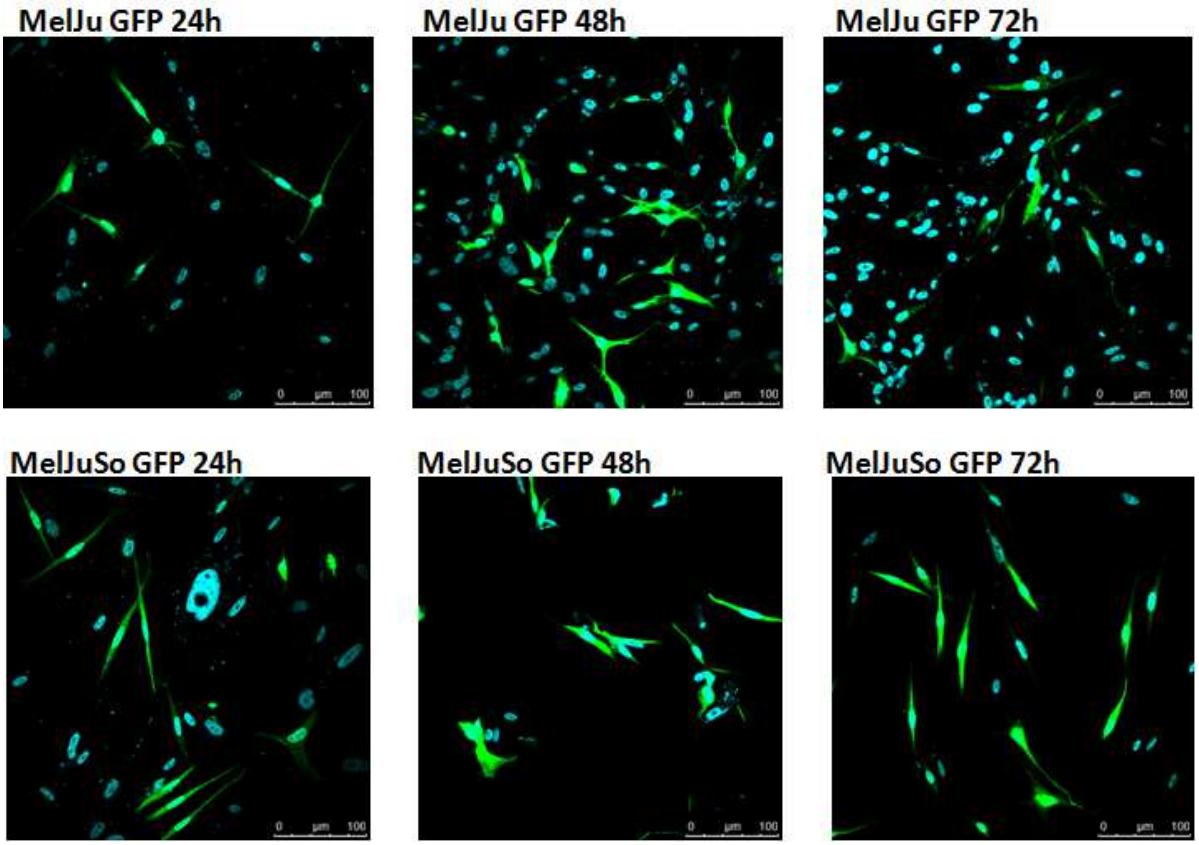

FIGURE 19: *Transfection efficiency in MelJu and MelJuSo cell lines*: a GFP plasmid was used to assess the efficiency of transfection in the two cell lines further used in the Dual Luciferase Assay. Average transfection efficiency was calculated on each picture.

The transfection efficiency in MelJu decreased over time; 31.6% of the cells expressed GFP at 24 hours, 25.08% at 48 hours and 18.87% at 72 hours. In MelJuSo, the transfection efficiency increased over time, ranging from 23.08% at 24 hours to 49.82% at 72 hours. Overall, the transfection efficiency of the two tested cell lines is sufficient to pursue with the Dual Luciferase Assay.

Dual Luciferase Assay was performed to determine which part of the promoter region is important in the transcription regulation of ABCB5.

This experiment was performed in two cell lines, MelJuSo and MelJu to compare the activation pathway of ABCB5 in primary and secondary melanoma. Deletion constructs were transfected in these two cell lines and analysis of the Firefly Luciferase activities were

performed at 24, 48 and 72 hours post-transfection. In this test, co-transfection has to be performed with a Renilla Luciferase plasmid that serves as a control of transfection and to normalize the Luciferase activity of the so called deletion constructs. Measurements of the Luciferase activity of the Firefly Luciferase vector without promoter fragment were also performed.

Luciferase activity was measured and relative Firefly Luciferase activity was then normalized with Renilla Luciferase activity for each construct and for the value of the empty Luciferase vector. The final ratio is obtained by dividing the Luciferase ratio by the empty Luciferase vector ratio.

1.3.1. Luciferase activity in both MelJu and MelJuSo cell lines, and determination of the core promoter region

Firstly the test was performed with MelJu cell line (**Figure 20**). After calculating the ratio for each fragment and time, we compared the Luciferase activities between them for each period. The trend was the same in the 3 assays: the Luciferase production increases when the first kb was deleted. The region between 3kb and 1kb before the transcription start site comprises maybe repressors.

The Luciferase activity decreases dramatically when the region between -1kb to -31 bases is deleted. Similar observations were made when the region between -1.5kb and -700 bases is deleted, leading to highlight a hot spot between -1kb and -600 bases. This region will be analyzed with Match software to identify transcription factor binding sites present in this region. The region between -175 bases and -31 doesn't seem to be relevant as only a small decrease in the Luciferase activity is detectable.

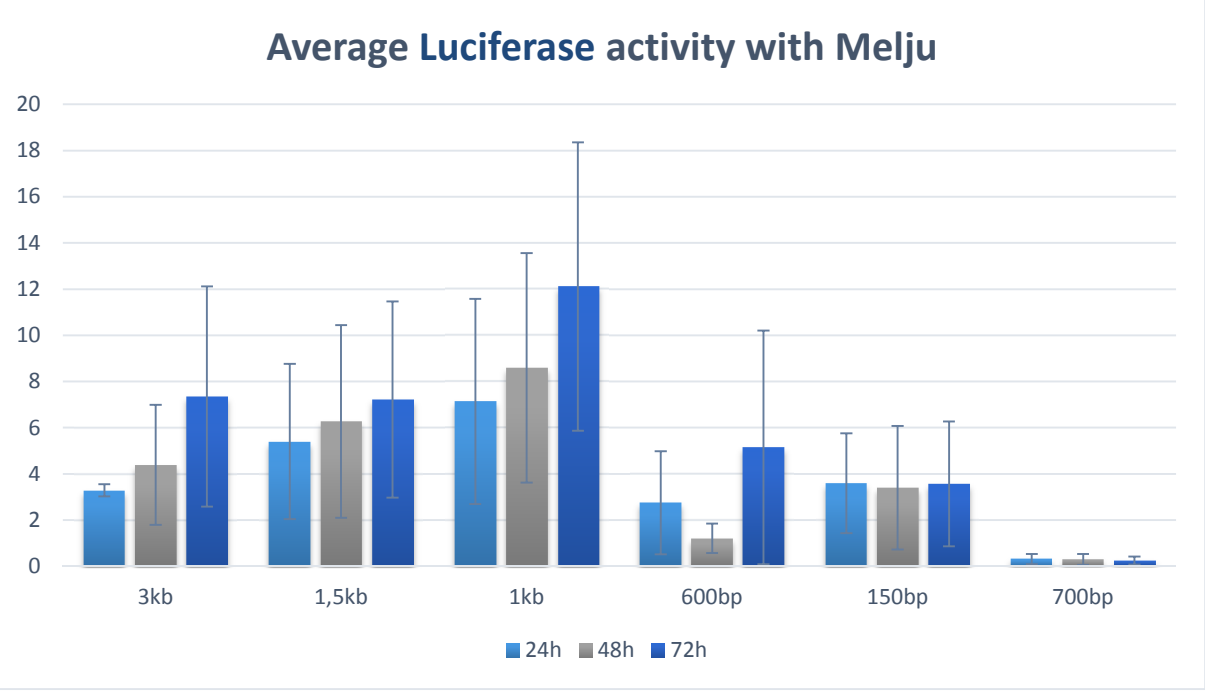

FIGURE 20: *Luciferase Activity in MelJuSo*: Luciferase activity was performed in triplicate over 72 hours (24, 48 and 72hours) allowing us to determine which part of the promoter is important.

An Anova test was done on the average value of each fragment to known if the difference observed is statistically different (**Table 4)**. We arrived at the conclusion that the fragment -1kb is statistically different from the others at the 3 period of time, while the 700bp is statistically different from the other at 48h only.

MelJu	24h	1KB vs. 700bp	0,00125	**
	48h	1KB vs. 700bp	0,000688	***
		1KB vs. 600bp	0,00166	*
	72h	1KB vs. 700bp	0,000604	***

TABLE3: *Statistical analysis of the Luciferase activity:* an Anova was performed to determine whether the difference between the Luciferase activities from the various constructs are statistically significant. The fragment 700 bp is statistical different from the 1kb at the 3 period of times, while the 600bp is only different at 48h

Constructs with shorter deletions around this hot spot will be prepared to better identify the transcription factor binding site(s) of interest.

We performed the same experiment with MelJuSo cell line, which gave us similar results than those obtained with MelJu cell line (**Figure 21** and **Table 5**). Noteworthy, the variability observed through the replicates was greater than what we observed previously with MelJu cell line.

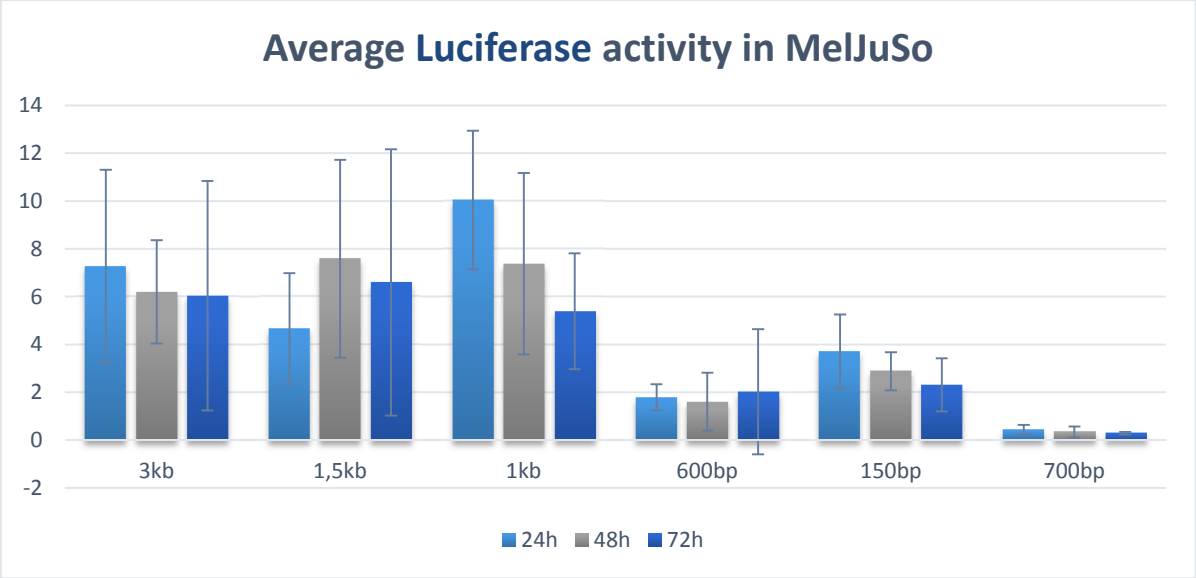

FIGURE 21: *Luciferase Activity in MelJu*: Luciferase activity was performed 3 times at three different time periods (24, 48 and 72hours) allowing us to determine which part of the promoter is important

MelJuSo	24h	1KB vs. 700bp	0,000295	***
		1KB vs. 600bp	0,00100	**
		3KB vs. 700bp	0,00382	*
	48h	Nothing		
	72h	Nothing		

TABLE4: *Statistical analysis of the Luciferase activity:* an Anova was performed to determine if the difference between the different Luciferase activity are statistical different or not. The fragment 700 bp is statistical different from the 1kb and the 3kb at 24h and the 600 bp is statistically different from 1kb at 24h. No difference was observed at 48 or 72hours, most likely due to the high variability between the replicates.

1.4. *In silico* characterization of the ABCB5 promoter hot spot

The hot spot identified with the Dual Luciferase Assay was reanalyzed with the Match software to identified transcription factor binding sites (TFBS) present in this region. TFBS were correlated with the literature.

MelJu and MelJuSo Luciferase activities highlight a hot spot comprises between -957 and -694 bases from the transcription start site. Interestingly, a TATA box is present in this region, together with OCT1, a transcription factor which help to bind the RNA polymerase II to the TATA box sequence. Furthermore FOXD3, a transcription factor implied in the down-regulation of MITF is also present in this hot spot. E-Boxes, which are not analyzed with the Match software, are also present in the Hot Spot (-851/-858/-795/-774) but the CT repeats important to its activation are not.

Based upon these results, we propose the following ABCB5 activation pathway: MITF binds to the E-box element, which triggers the transcription initiation through the RNA polymerase II and associated transcription factors associated binding to the TATA box. One of these TFs could be OCT1 (**Figure 22)**. In the case of a down-regulation, FOXD3 is expressed and binds with MITF, preventing the rest of the machinery to bind to the TATA box (**Figure 23**).

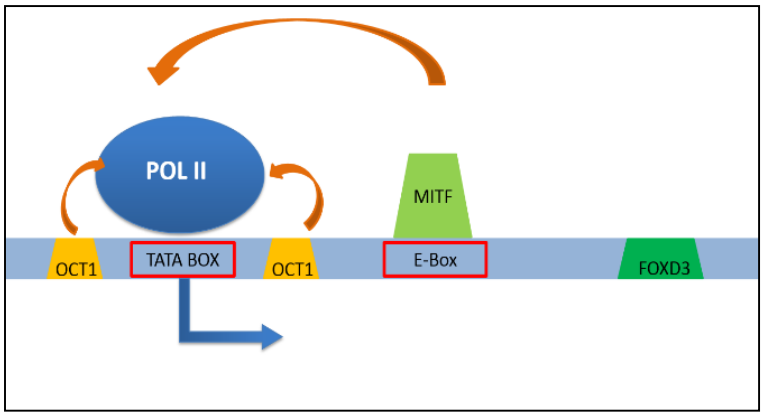

FIGURE 22*: Hypothetic activation pathway of the ABCB5 gene*: MITF binds to the E-Box, which triggers ABCB5 transcription initiation through RNA pol II and OCT1 binding to the TATA Box.

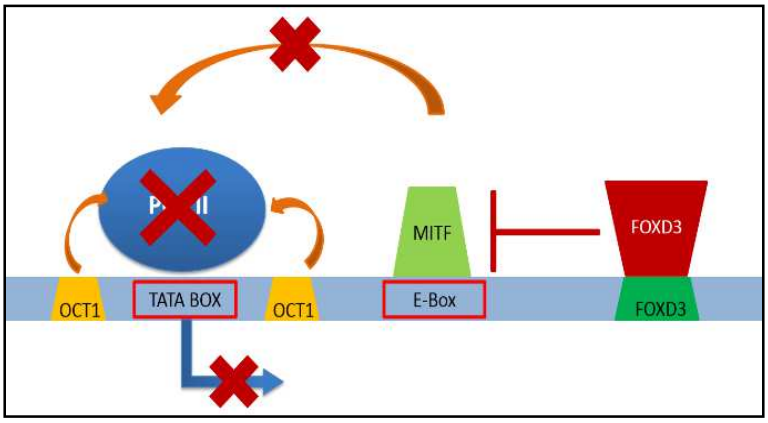

FIGURE 23: *Hypothetic inhibition pathway of the ABCB5 gene*: FOXD3 binds to MITF and as a scavenger does not allow it to bind to the E-box. It cannot bind anymore to the TATA box, the transcription is stopped.

2. Subcellular Localization of ABCB5

Localizing ABCB5 in the cell is critical to generate work hypotheses on its potential role in the cell. Furthermore knowing the exact localization of ABCB5 will help us to determine whether ABCB5, which is suggested to be involved in multidrug resistance, pumps drugs out

of cells by being expressing in plasma membrane, or pumps them in organelles (e.g. lysosomes, melanosomes).

Preliminary data (**Supplementary Data S5 A and B**) obtained by our team show the presence of ABCB5 in the mitochondria and the lysosomes (unpublished data). Those results were obtained by colocalization experiments using melanoma cells (UACC257 cell line) infected with BacMam virus. BacMam virus is a modified Baculovirus able to transiently infect mammalian cells [68]. The BacMam used in this experiment expresses a GFP-tagged ABCB5, allowing colocalization experiments.

No data can be found on a possible colocalization of ABCB5 in melanosomes i.e. organelles that are specific to melanocytes, which serve to the production, storage and transport of melanin [69]. However, as ABCB5 is expressed in primary melanocytes, one can speculate that it contributes to multidrug resistance of melanoma through melanosome-drug sequestration.

2.1. *In silico* study of the targeting sequence of ABCB5

Finding a targeting sequence in ABCB5 sequence could give us some indications on its localization. Different software's including TargetP, GeneInfinity, MultiLoc, PeroxiP, SignalP, SherLoc2 were used to analyze the RNA and the protein sequence but no targeting sequence was found.

2.2. *In vitro* localization of ABCB5

We used two melanoma cell lines (SKMEL-28 and A375), which stably express FLAG-tagged ABCB5. A FLAG tag is a short sequence of 8 amino acids (DYKDDDDK) that can be recognized by an antibody. This FLAG was introduced in the first extracellular loop of the transporter.

Colocalization was tested with the early and late melanosomes, the mitochondria, the lysosomes and the plasma membrane in the purpose of validating the preliminary results obtained by Jean-Pierre Gillet who showed that ABCB5 co-localized with the mitochondria and the lysosomes.

No colocalization was observed between ABCB5 and the plasma membrane (**Figure 24**), which corroborates previous results obtained by our team. At this stage, it does not clear whether ABCB5 colocalize or not with the early melanosomes (**Figure 25**), the lysosomes (**Figure 27 and 28**). Tiny and not in all the cells colocalization were observed between ABCB5 and early melanosomes (**Figure 26**), or the mitochondria (**Figure 29 and 30**). All the images will be run in a software to determine the colocalization ratio, to avoid any subjective bias.

Furthermore, ABCB5 expression in the nucleus and in the cytoplasm was observed in all the pictures. This raises the question of the specificity of the anti-FLAG antibody used in these experiments. Western blot and immuno-precipitation are being performed to validate this antibody. Meanwhile, we received a new validated monoclonal anti-ABCB5 antibody. Further experiments using this monoclonal antibody should contribute resolving those questions.

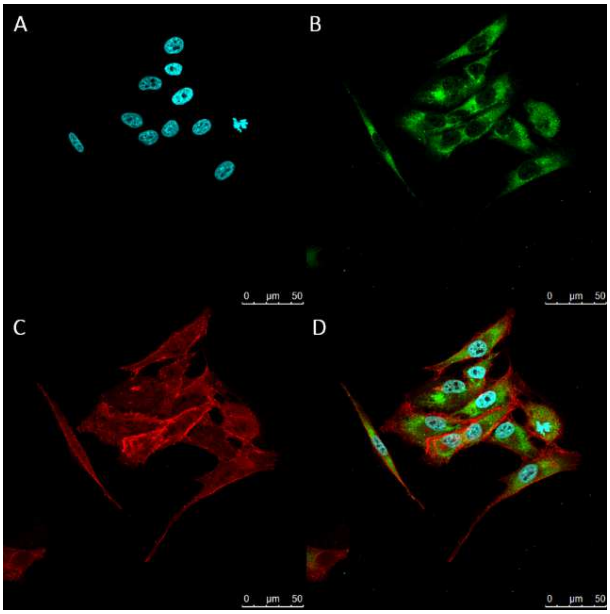

FIGURE 24: *Colocalization with the B-Catenin*:
(A) Nucleus (B) ABCB5 (C) Plasma Membrane
(D) Merged Images

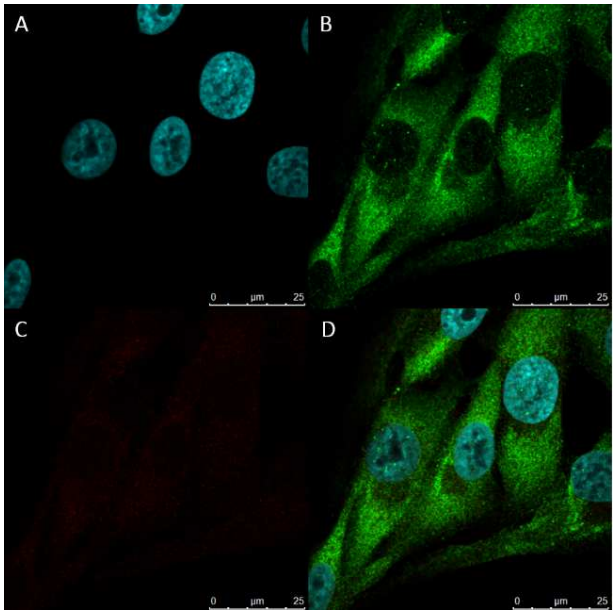

FIGURE 25*: Colocalization with the TRP-1*:
(A) Nucleus (B) ABCB5 (C) Early Melanosomes
(D) Merged Images

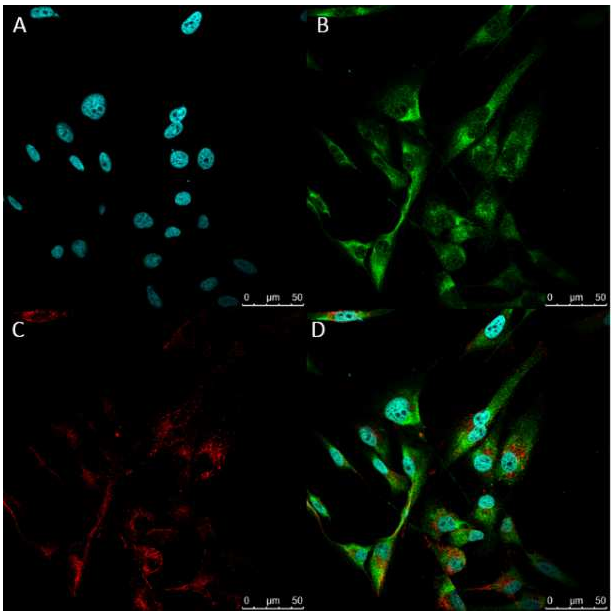

FIGURE 26: *Colocalization with HMB455*:
(A) Nucleus (B) ABCBB5 (C) Late Melanosomes
(D) Merged Images

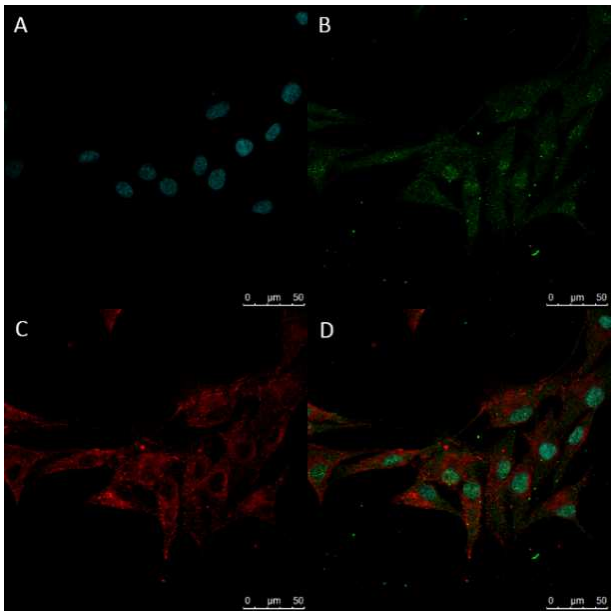

FIGURE 27: *Colocalization with LAMP2:* (A) Nucleus
(B) ABCB5 (C) Lysosomes (D) Merged Images

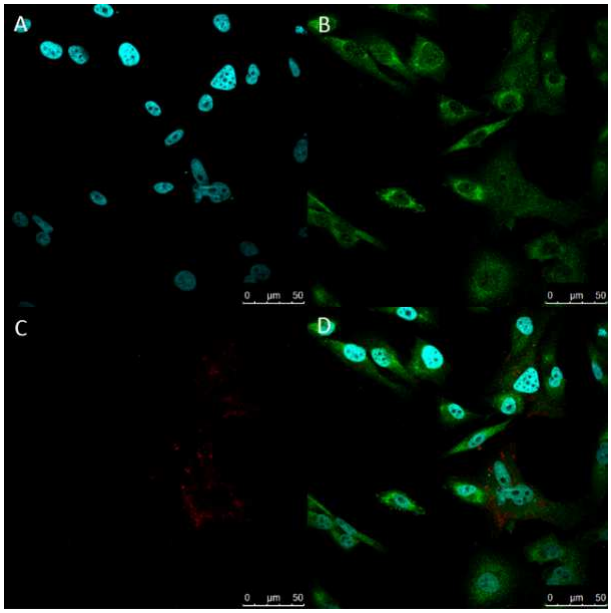

FIGURE 28: *Colocalization with the Lysotracker*: (A) Nucleus (B) ABCB5 (C) Lysosomes (D) Merged Images

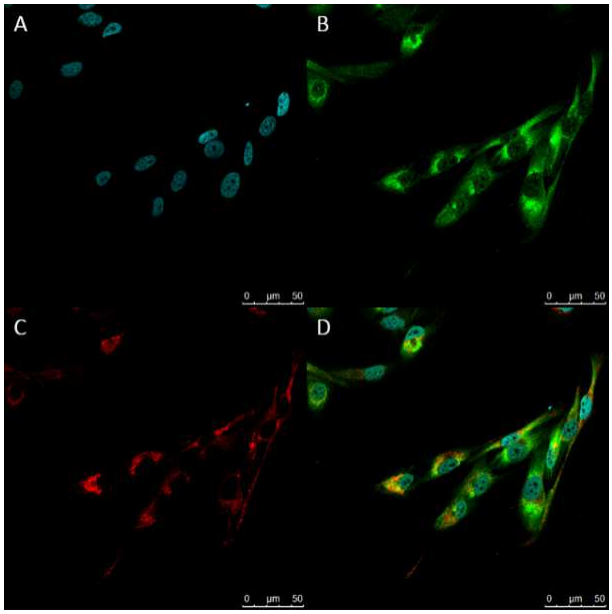

FIGURE 29: *Colocalization with the Mitotracker*: (A) Nucleus (B) ABCB5 (C) Mitochondria (D) Merged Images

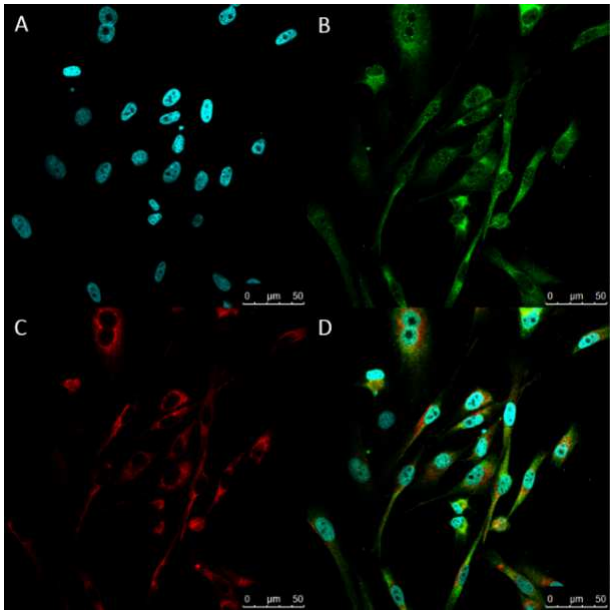

FIGURE 30: *Colocalization with TOM20*: (A) Nucleus (B) ABCB5 (C) Mitochondria (D) Merged Images

Discussion

ABCB5 is a transporter that belongs to the ATP-Binding-Cassette transporter family and more particularly the B subfamily. This latter is also known as the Multidrug Resistance Family and includes one of the most studied ABC transporter: ABCB1 (MDR1, P-gp) [5]. ABCB5 is a transporter predominantly expressed in pigmented cells (e.g. Substantia nigra, melanocytes, etc.) but also in testis [14]. Three main isoforms have been cloned [13], [19]; the so called full-length, which encodes a typical ABC transporter composed of two TMDs and two NBDs, the beta form that is truncated and composed of only one TMD, and two NBDs (one full and one truncated that cannot bind ATP). Finally, the isoform alpha that encodes a soluble protein. Its function as yet to be determined.

The literature on ABCB5 is replete with studies on the ABCB5β form, which is suggested to be the main isoform expressed in melanoma cell lines. Two findings are highly controversial: ABCB5β as a melanoma-initiator cells [22] and ABCB5β as a mediator of multidrug resistance (MDR) [19], [21]. The cancer stem cells paradigm in melanoma field was challenged by Quintana and colleagues, who demonstrated that both ABCB5β^+ and ABCB5β^- melanoma cells are tumorigenic [28]. As for the ABCB5β involvement in MDR, Sugimoto and colleagues showed that the ABCB5FL-encoding transporter, and not the atypical ABCB5β transporter, mediates resistance to taxanes and anthracyclines [19]. This is confirmed by two studies, one in revision for the Journal of Molecular Pharmacology and another in preparation arising from our laboratory [20]. Interestingly, we can detect an ATPase activity of ABCB5β, which may represent a futile cycle or be coupled to another (still unknown) function. This transporter needs to dimerize to be functional and to date, this cannot be ruled out.

Even though ABCB5 is studied because of its role in multidrug resistance, its role in normal cells remains to be determined. Analyzing the ABCB5 full-length gene promoter region and the general activation pathway will help us to better understand how ABCB5 gene is regulated. Also, it may shed some lights on the other ABCB5 transcript variants, especially, how they are regulated and eventually what role they have in the cell.

In order to characterize the promoter region, we first performed bioinformatics to identify promoter boxes and transcription factors binding-sites (TFBS). Classical and atypical promoter boxes were under investigation. The results that we obtained showed that CpG islands and CAAT box are not present in the 5'UTR region of the gene. The results were not clear for the TATA box, the downstream promoter element [71] and the Initiator sequence. They were all present in the promoter region but either too far from the transcription start site, or found repeatedly. Nevertheless, we do not rule out those possibilities, some promoter boxes being sometimes localized more than 200 nucleotides before the TSS [43].

A similar study was performed to characterize the promoter region of the ABCB1 gene in 1987 by Ueda *et al*. [72] . Because of the absence of software allowing rapid identification, they had to identify experimentally the boxes present in their promoter region. They had to construct a library containing the region of interest and to perform different experiments on this (i.e. ribonuclease protection assay and promoter activity assay). They identified a consensus CAAT sequence, two GC box-like, and no TATA box.

Due to the absence of clear evidence for a promoter box, we focused our attention on Enhancers. E-Boxes are regulatory DNA sequences capable of inducing transcription of a gene from a distant region, (**Figure 31**) which can be located upstream or downstream of the gene. They were discovered nearly 30 years ago by using a transient reporter gene assay in cultured cell lines [73]–[75]. To be efficient, they must have an affinity between the transcriptions factors that bound the enhancer(s) and the promoter(s). Furthermore this Box is recognized by transcription factors like MITF, a transcription factor mainly implicated in the regulation of melanocytes and so melanoma.

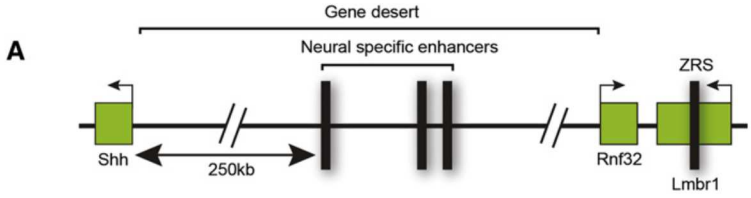

FIGURE 31: *Enhancers [74]*: Enhancers are regulatory elements localized ten to hundred bases from the gene they act on.

Enhancer sequences form clusters of transcription factor binding sites. To date, three mechanisms have been proposed in the activation of the "enhancer-gene communication" [74]. In the first one (**Figure 32**), the transcription factors bind preferentially to the enhancer region and the promoter region recruits other factors, which reorganize the chromatin between the two regions. The second mechanism is similar to the first one, but the promoter directly interacts with the enhancer regions, probably through a string of mini-chromatin loops. In the third mechanism, there is a colocalization of the promoter and the enhancer in the nucleus. They can directly interact with each other when the associated transcription factors are bound, which loops out the region. This loop allows the enhancer to interact with only one promoter region at a time. In some cases the RNA polymerase II can directly bind to the enhancer and produces a short noncoding (nc) RNA. The role of these short ncRNAs has yet to be determined.

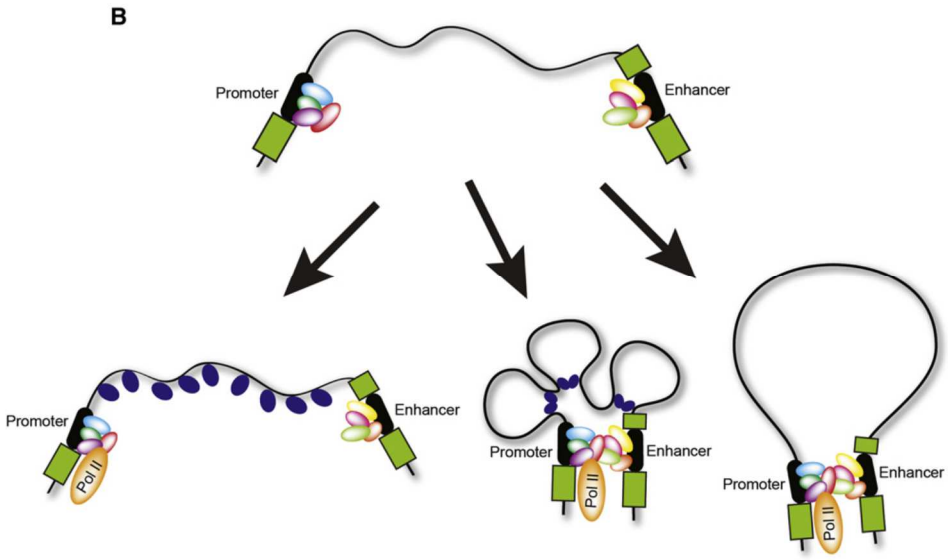

FIGURE 32*: Enhancer-gene communication [74]*: Three different mechanisms of communication between the enhancer and the gene are proposed (1) interactions via transcriptions factors, (2) interactions through mini-chromatin loops (3) normal interactions between the 2 parts in the nucleus

E-Boxes were found in the ABCB5 promoter region but it requires CT repeats, which were not found at the expected coordinates.

The presence of Enhancer in the promoter of an ATP-Binding Cassette transporter was already highlighted in 1995 by Song *et al*. [76] in the mouse ABCB1 promoter in hepatoma cells. The use of so-called deletion constructs (i.e. truncated fragments of the gene promoter region in a luciferase reporter vector) proved the importance of E-box sequences in the transcription of the gene. Indeed, they showed that the combination of two E-boxes conferred full enhancer activity, while only one alone-box was shown to be less active.

The *in silico* analysis also allowed us to study the transcription factor binding sites present in the ABCB5 promoter region. A long list of transcription factors was obtained and they were classified into different groups, which enabled us to focus on some TFs such as those already showed to be involved in melanoma, or found in ABCB5-expressing cells.

To refine experimentally this TF list, and pinpoint the important ones, truncated fragments of the ABCB5 promoter region were cloned in Luciferase vectors. We transfected the so-called deletion constructs in MelJuSo cell line, a primary melanoma, and in MelJu cell line, a secondary melanoma to highlight any potential differences in the regulation of ABCB5 in a primary and metastatic melanoma.. We haven't observed any relevant differences. However, we were able to quickly identify in both cell lines a hot spot area in the ABCB5 promoter region, localized between -1kb and -700 nucleotides before the putative transcription start site. We will further perform these experiments in normal human epidermal melanocytes (NHEM), enabling us to compare the ABCB5 regulatory pathway between tumors and a normal cell line.

Interestingly, this region contains a TATA box, OCT1 binding site, OCT1 which is a transcription factor helping the RNA polymerase II to bind the TATA box and FOXD3, a transcription factor implicated in the regulation of MITF. Furthermore, two E-Boxes are present is this hot spot. These information, even though the role of each transcription factor and boxes has not been studied, lead us to hypothesize an activation pathway. We propose that MITF binds to the E-Box and activates it [60]. This activation allows the E-Box to trigger the binding of RNA polymerase II to the TATA box, which in turn activates the transcription of the ABCB5 gene. When ABCB5 has to be downregulated, FOXD3 interacts with MITF, and prevents it to bind to the E-Box, therefore blocking the ABCB5 transcription.

These hypotheses will be tested using siRNA targeting these transcription factors. Silencing RNA will downregulate the targeted transcription factor(s), and a Luciferase activity assay could be performed with a full-promoter fragment-containing construct. As a follow-up of these experiments, Chromatin Immunoprecipitation (ChIp) Assay could be undertaken. ChIp allows to determine which protein binds to DNA and where it binds. This could be done on a few strong candidates as a validation.

Electrophoretic Mobility Shift Assay (EMSA) represent a suitable alternative to ChIp[77], [78]. In this experiment, an antibody of interest is used to shift the DNA sample that normally the transcriptions factor is bound to (hooked on the DNA). This attachment weighs the DNA and delays the DNA migration on the gel. EMSA allows focusing on one transcription factor at a time, and does not allow localizing precisely the transcription factor binding site(s).

These experiments will enable us to determine the exact role of MITF in the general activation pathway of ABCB5 in melanoma but also in melanocytes.

In addition to the identification of the promoter and transcription factors, transcription start sites of the ABCB5 gene were investigated by Rapid Amplification of cDNA 5'Ends. We highlighted two regions. The predicted TSS of the ABCB5 full-length transcript is located in the second exon but our data revealed a potential new transcription start site in the exon 6 of the ABCB5 gene. However, this potential TSS is out of frame, but another ATG, localized 20 nucleotides before this new TSS is present. This new transcript of ABCB5 would encode an ABCB5FL-alike form that is truncated at the first extra cellular loop (see **Figure 12** in the results part). We confirmed this finding in a second set of experiments in two different cell types. Beside UACC257 cell line, isolated from a secondary melanoma, we detected it in human testis.

We could not confirm the putative TSS of the ABCB5 beta isoform. Instead, we found a hot spot region of 225nucleotides long localized 100 nucleotides ahead of the predicted TSS. In this 225bp-region, we found 14 potential TSS, however, none of them could be confirmed when analyzing the sequence. Therefore, we speculate that ABCB5 Beta isoform is a degradation product rather than a transcribed isoform. This is in line with biochemistry and cell biology data strongly suggesting that ABCB5 beta protein, if expressed, is not functional. No antibody is currently available to validate this hypothesis by Western Blot.

Northern Blot is inconclusive at this stage. We detected the ABCB5 full length transcript in all the ABCB5-expressing cell types that we tested, and not in the negative controls. We were also able to detect the housekeeping gene and a short ABCB5 transcript, but not the ABCB5β isoform. This will need to be repeated before interpreting the results .A recurrent issue when working with ABCB5 is its low level of mRNA expression in positive cell lines. A strategy to improve this, could be the induction of ABCB5 expression by incubating the cells with chemotherapeutic agents that are known to be substrates of the ABCB5 transporter (e.g. paclitaxel, doxorubicin). Cytotoxic assays have to be performed to select a sub-cytotoxic dose to avoid killing too many cells, and giving rise to a drug-resistant cell line, which should express ABCB5 at a greater level.

The second objective of this study was to determine the exact localization of ABCB5 in the cell. Preliminary data obtained by our team showed a possible colocalization of ABCB5 with the mitochondria and the lysosome, but not in the plasma membrane. We wanted to confirm those results.

We first performed a bioinformatics analysis of the targeting sequences which could be present in the ABCB5 sequence but none was found. Nonetheless, all the targeting sequences are currently not known. For example, it is known that half of the mitochondrial proteins have an unknown targeting sequence [71], [72]. Also, these software only detect targeting sequences localized at the Amino- or Carboxy-terminal ends, not those localized in the center of the sequence.

Two melanoma cell lines expressing a FLAG-ABCB5 protein were used to perform the colocalization with the mitochondria, the lysosome, the plasma membrane and the early and late melanosomes. To date, there is no commercially available anti-ABCB5 monoclonal antibody that can be used. So, an anti-FLAG antibody allowed us to highlight a weak colocalization of ABCB5 with the mitochondria and the late melanosomes. However, this

remains a subjective statement as no software was utilized to measure colocalization ratio. This will be performed in the upcoming weeks through collaboration with Dr. Tanner (NIH).

However, we are not convinced of the specificity of the antibody used to detect the FLAG tag. We observed the presence of ABCB5 in the nucleus of each test and the signal was diffuse throughout the cell. Western blot and immune-precipitation are being performed to validate *in house* the anti-FLAG antibody. In the meantime, we received a monoclonal anti-ABCB5 antibody and colocalization experiments will be performed in the next weeks and should contribute resolving those questions.

Once we will have confirmed the localization of ABCB5, the data will be validated by subcellular fractionation and Western blot. If the presence of ABCB5 in mitochondria is confirmed, we will have to identify in which membrane of the mitochondria, either inner or outer, ABCB5 is localized. To do that digitonin will be used to solubilize mitochondrial membranes. Briefly, digitonin allows separating the outer and the inner mitochondrial membranes by displacing the cholesterol of the outer membrane. The outer membrane will be solubilized while the inner will be precipitated. Western blot is then performed on each fraction to localize ABCB5.

A possible hypothesis about the role of ABCB5 in cells in physiological and tumoral situations is to control the level of reactive oxygen species (ROS) produced by the mitochondria. In melanocytes, melanosomes produce melamine but also protect the cell from the ROS produced by sunlight and cellular metabolism. In melanoma this process is altered, melanosomes produce the ROS combined to the intracellular processes leading to high level of ROS. This ROS production has been associated to multidrug resistance in cancer; ROS leading to the activation of proto-oncogenes pathways. Scientists are trying to divert this high production of reactive oxygen species against the cell itself by inhibiting the scavenging of those species or increasing the levels of ROS. Those two actions will lead to the death of the cells by apoptosis [79], [80].

Some members of the ABC transporter B subfamily, such as ABCB10, localized in the inner mitochondrial membrane [81], are already known to have a role in the mitochondrial export of molecules that constitute a pathway to protect cells from oxidative stress. Those molecules are still unidentified. ABCB7 is involved in the iron-sulfur transport in *C. elegans* and it has also been implicated in an oxidative stress pathway; when mutated it will lead to the accumulation of iron-sulfur cluster assembly and the increase of oxidative stress in the cell [82].

We speculate that ABCB5 plays a role in the protection of cells against reactive oxygen species. This hypothesis will be challenged by measuring ROS levels in cells using UV stress, which induces high production of ROS, in ABCB5-overexpressing melanoma cell lines plus and minus ABCB5 shRNA [79]-[81], [83].

In conclusion, the data generated in this study allowed us to hypothesize a potential regulation pathway of the ABCB5 gene expression. Furthermore, we also identified a potential new isoform encoding an ABCB5FL-alike transporter, which is truncated at the first extracellular loop. This warrants further analysis to validate this new isoform, especially by Northern Blot. We also performed colocalization experiments that are being validated further using a newly developed anti-ABCB5 monoclonal antibody.

Overall, this study contributes in better understanding the role of ABCB5 in normal and tumor cells. In long term, this work paves the ground for the development of new therapy to treat melanoma [84].

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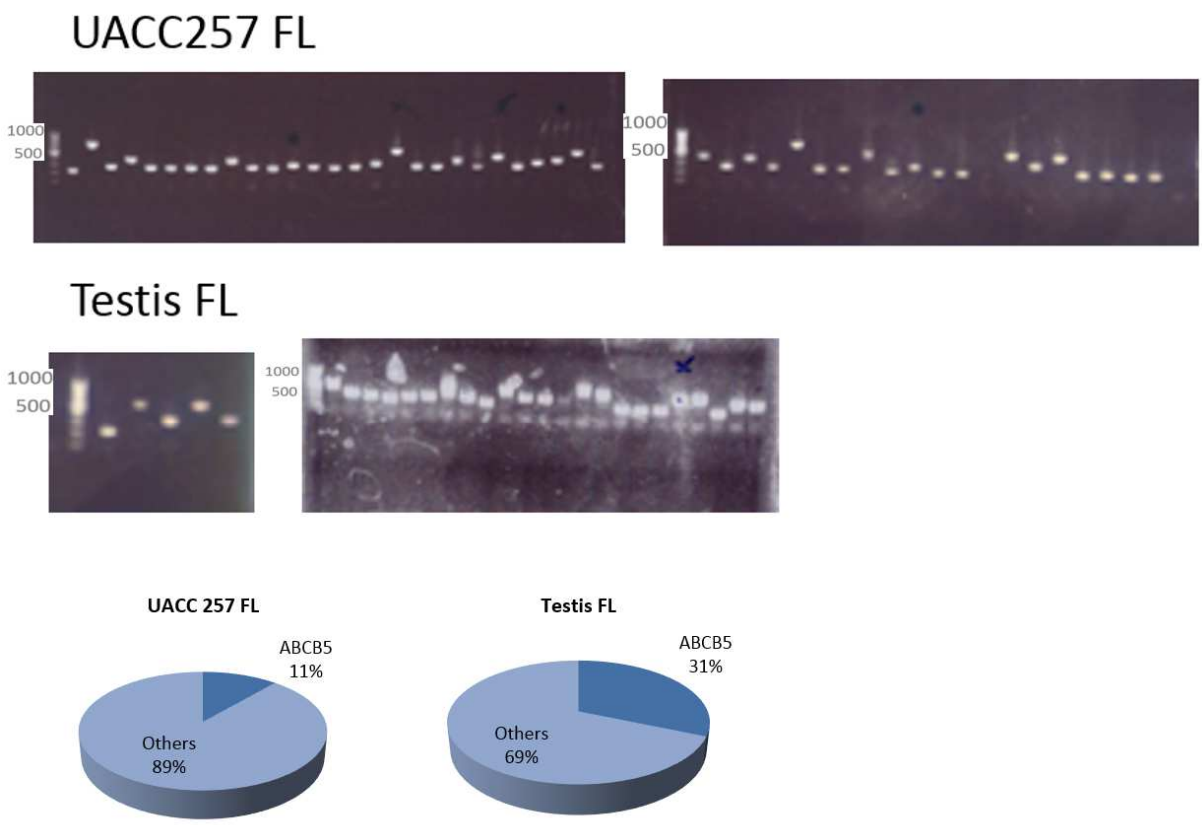
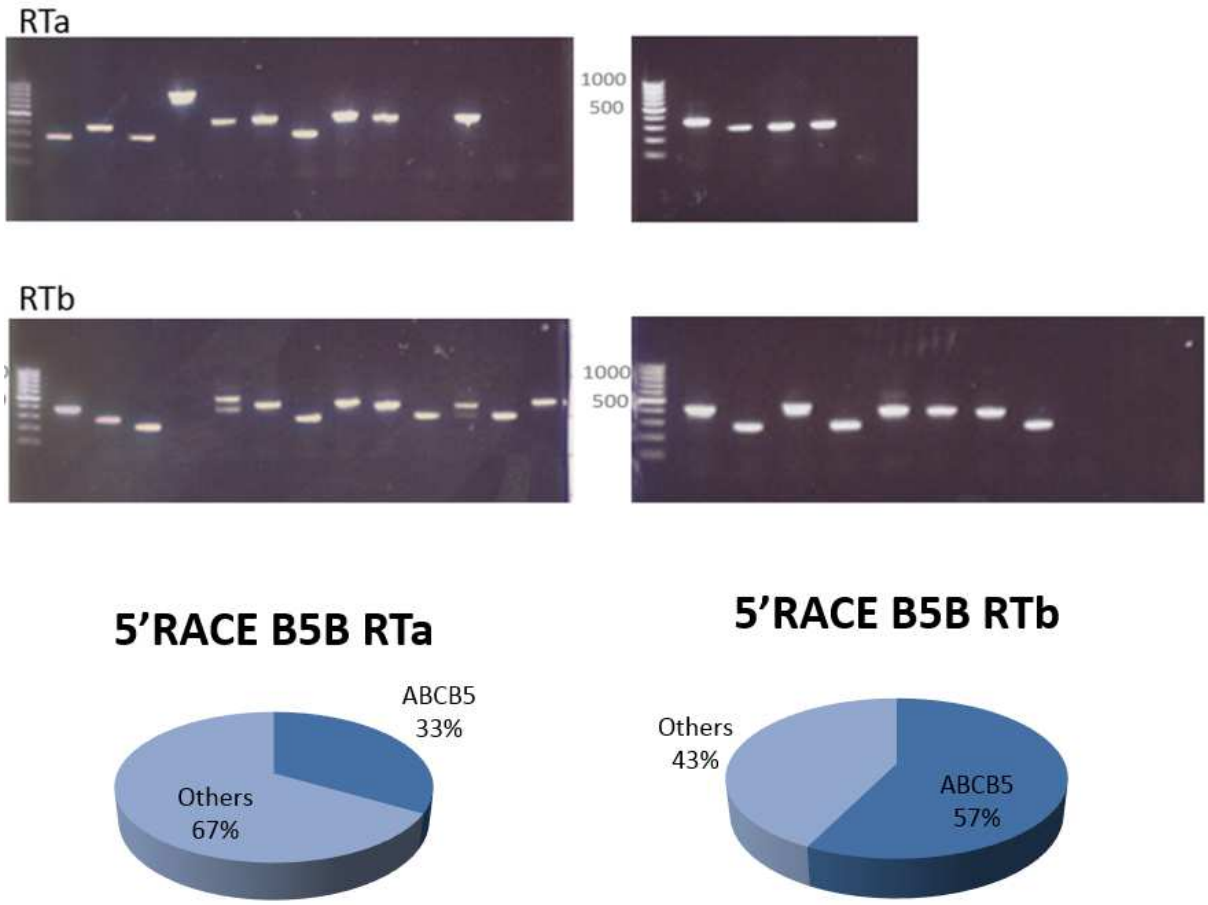
Supplementary data

PRIMERS FOR 5'RACE	
ABCB5 FL	
RTB	GATAGAGTCACTAGGGTGAG
A1B	CTTGAGACTGAGTACAGTTC
S1B	ATATGACTCTGTTGACCCTG
A2B	CTAGACATCCACTAATAAGG
S2B	TTGGAATAGGTGTTGCTGCC
P16_B5_S1	AAGATATGACTCTGTTGACC
P16_B5_A1	GAGACTGAGTACAGTTCTGA
P17_B5_A2	GGACTAGACATCCACTAATA
P17_B5_S2	TGTTGGAATAGGTGTTGCTG
ABCB5B	
RACE JPS1	GTGGATGAGAATGACATCAGAGC
RACE JPS2	GAGACCATATTGGAGTGGTTAGTC
RACE JPA1	GAAGCTGGACTACCGTACTCTTC
RACE JPA2	CAAGGCGACTGTCTCTCCAGAC
RACE JP RTA	CTCCATCTCTTCATCAGTCACATCATCTCGTC
RACE JP RTB	CTCCATCTCTTCATCAGTCACATC
PRIMERS FOR NORTHERN BLOT PROBES	
P22_NB_E9-13_F	CAATAGCCCGAGGAGCTGC
P22_NB_E9-13_R	CGAGCAATTGCGATCCTCTG
P23_NB_E20-22_F	GCACAGGAGGCTTGACAACA
P23_NB_E20-22_R	GCATGGCTGAATGCATAACAG
PRIMERS FOR CLONING	
5'F	GAGCTGAAGAAATGCAAGAAAAT
5'R	CGATGTCCTGTGCCAAAACTGAATGAAA
3'F	TTGCCGTGACAGGAATGATTG
3'R	TCTGGCTTTTTCCCTTCT
FLF	ACAGCCAAAACTGAGAAAGGAAGC
FLR	ATGCTACTGTCTTTCCTCGCTC
EXON6F	GGATTATAACTGCAGCACGAC
PRIMERS FOR CONSTRUCTION LUCIFERASE	
SINGLE RESTRICTION	
LIGLUC P1F	GCTAAGCTTTGGAGCAAGTGGACCTCTCA
LIGLUCP4F	GCTAAGCTTTGGATGGCTCTGCTCTAGAAG
LIGLUCP6F	CCTAAGCTTCCTTGTCCTCTTTACCTACC
LIGLUCP7F	GCTAAGCTTCATCCTCATCTGTGGGCGAAAC
LIGLUCP19F	GCTAAGCTTCCTGTCCATTGCGGCATTAT
LIGLUCP19R	CCTAAGCTTCACACACGGCCTATTGATTC
RVPRIMER3	CTAGCAAAATAGGCTGTCCC
DOUBLE RESTRICTION	
P19F	GGTACCCCTGTCCATTGCGGCATTAT
P3R	CGCTCGAGTTCTAGAGCAGAGCCATCCA

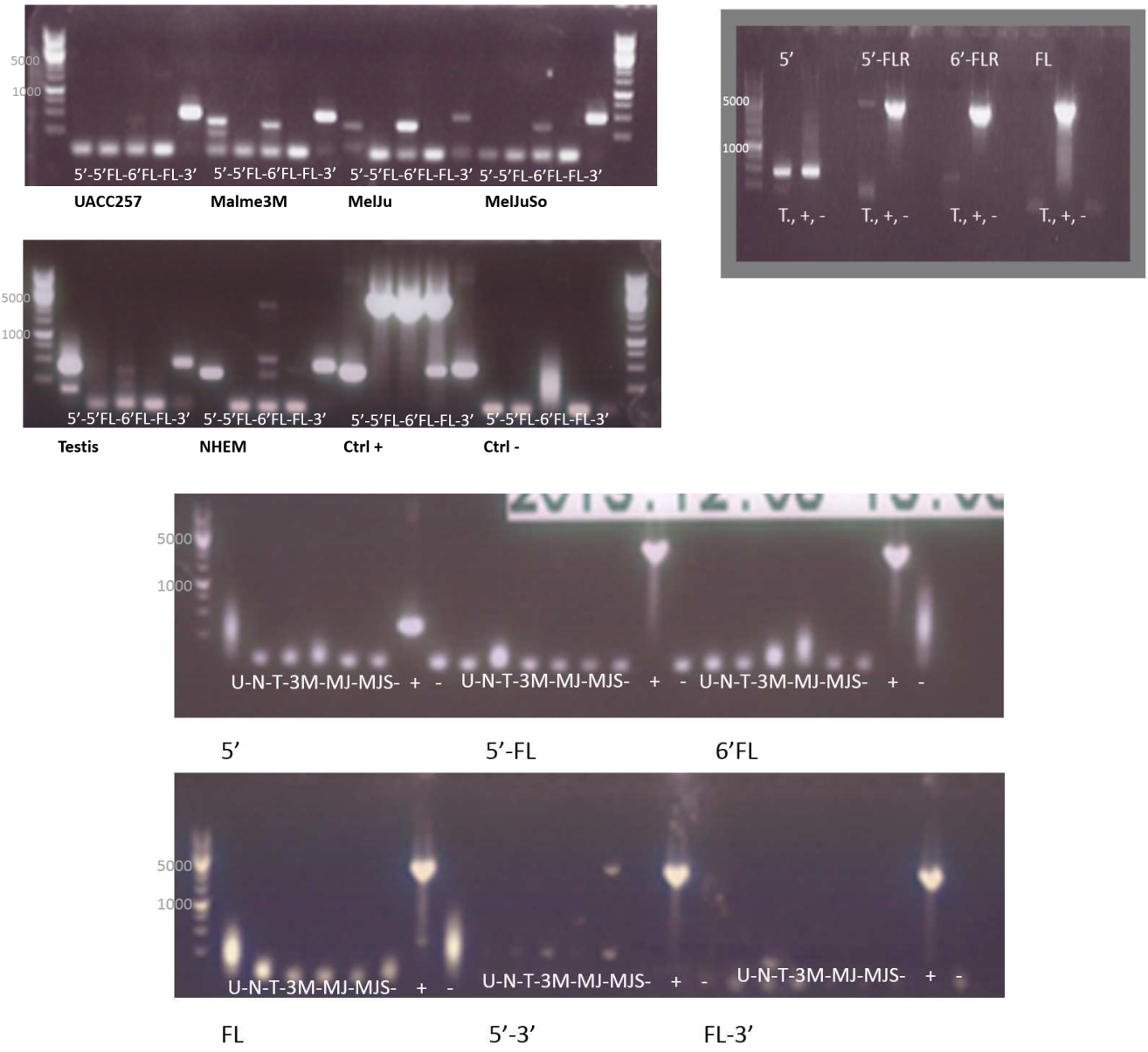
SUPPLEMENTARY DATA 1: PRIMERS USED FOR ALL THE EXPERIMENTS



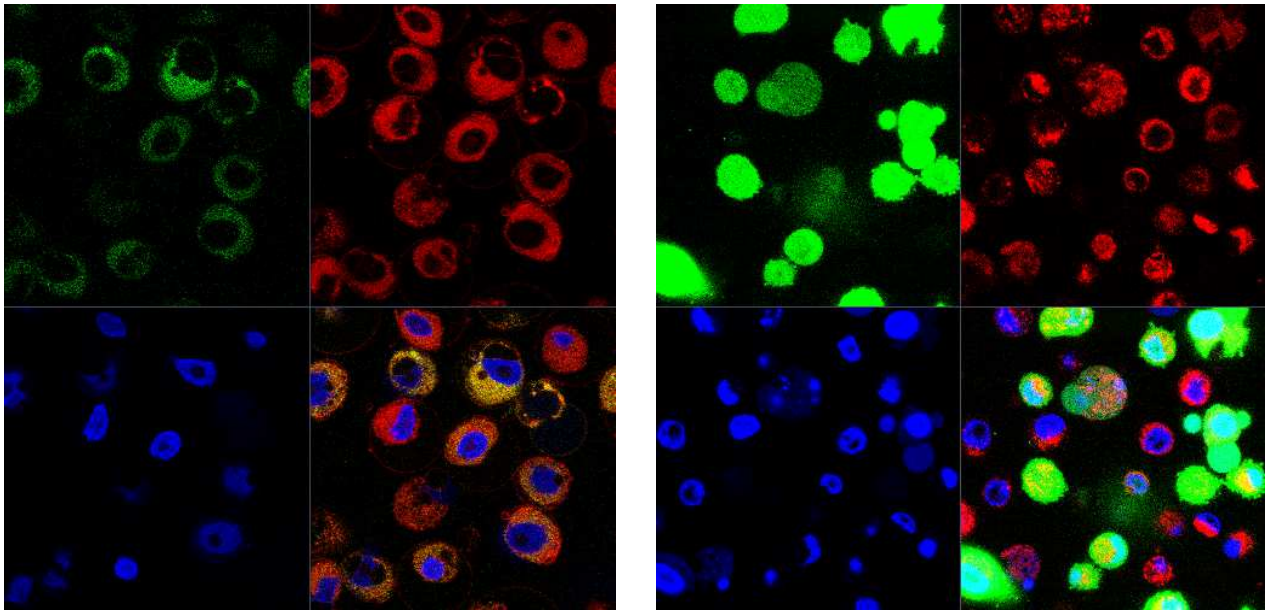
SUPPLEMENTARY DATA 2: CLONES OF THE 5'RACE EXPERIMENT TARGETING THE ABCB5 FULL LENGTH WITH A DOUBLE REVERSE TRANSCRIPTION STEP. AFTER THE SCREENING OF THE CLONES OBTAINED FROM UACC257 CELL LINES AND THE TESTIS WE ESTABLISH CHART PIES. ABCB5 WAS PRESENT IN 11% OF THE UACC257 CLONES AND IN 31% OF THE TESTIS CLONES.



SUPPLEMENTARY DATA 3: CLONES OF THE 5'RACE EXPERIMENT TARGETING THE ABCB5 BETA WITH A SIMPLE REVERSE TRANSCRIPTION STEP. AFTER SCREENING OF THE CLONES OBTAINED FROM THE UACC257 CELL LINE WITH THE RTA AND RTB WE CAN ESTABLISH CHART PIES. ABCB5 WAS PRESENT IN 33% OF THE UACC257 RTA CLONES AND IN 43% OF THE UACC257 RTB CLONES.



SUPPLEMENTARY DATA 4: DIFFERENT PCR WERE PERFORMED ON THE cDNA OF UACC257, MELJUSO, MELJU, MALME3M, NHEM CELL LINES AND TESTIS TO DETECT THE PRESENCE OF THE ISOFORMS FULL LENGTH AND BETA. PCR WERE TARGETING THE 5’ AND 3’ EXTREMITIES AND THE B5 FL.



SUPPLEMENTARY DATA 5: PRELIMINARY DATA OBTAINED FROM THE COLOCALISATION OF ABCB5 IN UACC257 CELL LINES WITH THE MITOCHONDRIA. BACMAM WERE USED TO INFECT CELLS (A) COLOCALIZATION WITH AN ABCB5-GFP AND MITOCHONDRIA (B) CONTROL WITH A GFP CONSTRUCT AND STAINING OF THE MITOCHONDRIA.