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# Transcriptional regulation of the human *ABCC6* gene.

**PhD Thesis**

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# 1 Introduction

## 1.1 The ABC transporter superfamily.

The ATP binding cassette (ABC) transporter family is one of the major membrane transporter families, which is present in all living organisms from prokaryotes to eukaryotes including animals, plants, and fungi (Holland and Blight 1999). It has been shown that the different members of this protein family can export or import molecules in an adenosine triphosphate (ATP) dependent fashion, and they thereby participate in various physiological processes.

In prokaryotes, the transporters are generally promoting inward movement of nutrients and outward transport of toxic compounds (Higgins and Linton 2001) showing the functional diversity of this transporter superfamily. Interestingly the outward transport of small molecular weight compounds is a common function to the different organisms, while the inward activity seems to have been eliminated during evolution, or at least to be specific to the kingdom of prokaryotes. As an example, the ABC transporters play various physiological roles by transporting molecules within the cells from cytoplasm to organelles. Besides their physiological relevance they are also involved in the export of antibiotics, antifungal agents, herbicides as well as anticancer drugs. This last transport activity highlights the economical and clinical relevance of ABC transporters. Moreover a certain number of Mendelien diseases are linked to the mutation of ABC transporter in Human (Dean and Annilo 2005) (Table 1.1).

| Gene | Mendelien disease / cancer resistance |
|---|---|
| ABCA1 | Tangier disease |
| ABCA4 (ABCR) | Stargardt syndrome, age rel. macular dystrophy |
| ABCA12 | Lamellar ichthyosis type 2 |
| ABCB1 (MDR1) | Multidrug resistance in cancer |
| ABCB3 (TAP2) | HLA class I deficiency |
| ABCB4 (MDR3) | Progressive intrahepatic cholestasis type 3 |
| ABCB4 (MDR3) | Progressive intrahepatic cholestasis type 3 |
| ABCB4 (MDR3) | Progressive intrahepatic cholestasis type 3 |
| ABCB7 | X-linked sideroblastic anemia and ataxia |

| Gene | Mendelien disease / cancer resistance |
|---|---|
| ABCB11 (sPgP) | Progressive intrahepatic cholestasis type 2 |
| ABCC1 (MRP1) | Multidrug resistance in cancer |
| ABCC2 (MRP2) | Dubin-Johnson syndrome |
| *ABCC6* (MRP6) | Pseudoxanthoma elasticum |
| ABCC7 (CFTR) | Cystic fibrosis |
| ABCC8 (SUR1) | Persistent hyperinsulinemic hypoglycemia |
| ABCD1 (ALD) | Adrenoleukodystrophy |
| ABCG2 (MXR/BCRP) | ABCG2 (MXR/BCRP) |
| ABCG5 and G8 | Sitosterolemia |

*Table 1.1: examples of ABC transporters and their clinical phenotypes*

The minimal structure of ABC transporters is composed of two transmembrane domains (TMD) that form the pathway through which the molecules are transported and give substrate specificity, and two ATP or Nucleotide Binding Domains (NBD) associated with the cytoplasmic face of the membrane and which provide energy to the transport by binding and hydrolyzing ATP molecule. These four elements can be coded in operon systems in the case of prokaryotes, while a single gene in eukaryotes can encode them. However some half transporters exist at least in human, where only one TMD and one NBD are present, and therefore they function then as homo or heterodimers (Figure 1.1.).

The NBD domain of the protein contains 2 walker A and B motifs that have been associated with ATP dependent proteins and the C motif specific to the ABC transporter superfamily (Walker, Saraste et al. 1982). Therefore the NBD domains are highly conserved among evolution and are responsible for the classification of transporter as ABC transporter (Higgins, Hiles et al. 1986). It is accepted, but not really understood, that the close cooperation between NBD and TMD allows that an ATP binding to the NBD leads to a conformational change of the TMD ultimately leading to the molecule transport (Hollenstein, Dawson et al. 2007) (Hollenstein, Frei et al. 2007).

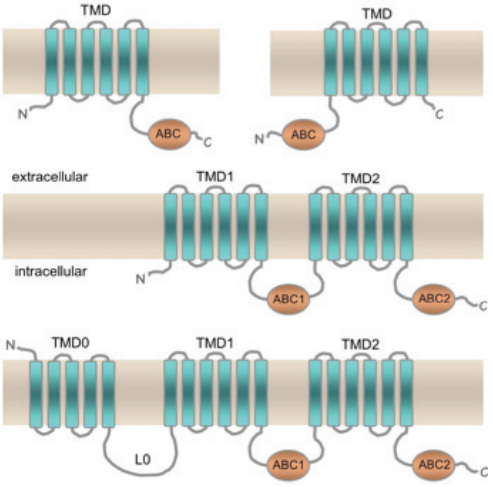
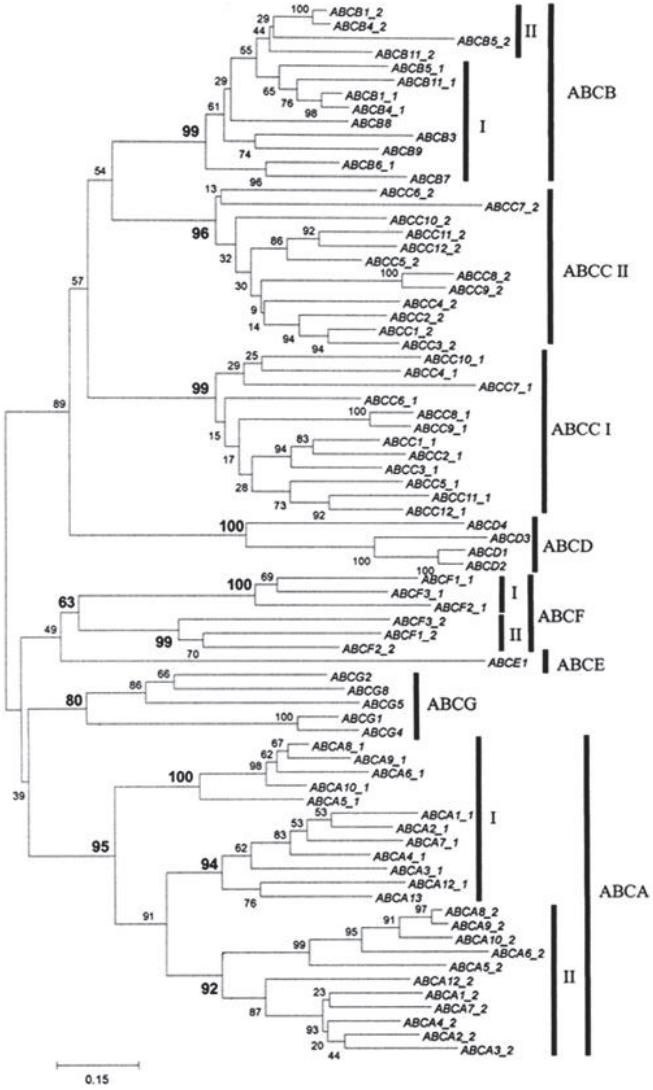



*Figure 1.1. Structural features of the ABC transporters. A. Half transporter structure as the ABCG2. These transporters are functional in dimers. B. Minimal structure of ABC transporter with two NBD and two TMD. C. MRP like structure with a supplementary L0 intracytoplasmic loop and an extra transmembrane domain. Figure adapted from Flora Szeri,*

Considering the fact that this report will focus on one single Human ABC transporter (ABCC6), I will only give a brief description of Human members of the ABC superfamily. Based on phylogenic and amino acid sequence alignment of the NBD domains, the human ABC transporters have been grouped into seven subfamilies. In each of the subfamily a high identity in the TMD organization as well as in the gene organization and the intron localization can be observed. However it is important to note that besides similarities, the functions of ABC proteins correspond poorly to the subfamilies and it is not rare to find two ABC proteins from two subfamilies sharing more similarities in substrate recognition than proteins from the same subfamily. The different ABC transporter subfamilies have been numbered from A to G. Into these 7 groups, 48 human ABC transporter members have been identified (Dean and Allikmets 2001) (Dean, Rzhetsky et al. 2001).




*The human ABC transporter subfamilies. Phylogenetic tree of the human ABC genes based on their ATP domains. Note that some proteins analyzed contain two ATP-binding domains (I and II), whereas others contain only one ATP-binding domain. (adapted from Dean 2002)*

If it is usual to see investigation of the protein function, it is important to note that transcriptional regulation studies have also been performed on some of the human ABC transporter members, usually by screening the promoter region for nuclear factor binding sites, and testing transcriptional activity of the gene after induction of specific factor by various drugs. In very few cases the promoter has been cloned and luciferase reporter gene assay has been performed to measure the promoter activity. Investigations of the methylation pattern of the promoter that is often linked with the activity of the gene, as well as epigenetic study of the gene expression were rarely performed.

After briefly introducing the different subfamilies, I will focus on the ABCC subfamily that contains the *ABCC6* gene, and I will finally describe a relevant example of ABC transporter transcriptional analysis by giving an overview of the transcriptional regulation of the *ABCC7*/CFTR gene.

## 1.2 The ABC transporter subfamilies: a quick overview.

The ABCA subfamily contains twelve full transporters that are involved for most of them in lipid metabolism. The most studied member of this subfamily is the ABCA1 transporter that is also the first described ABC transporter. ABCA1 has been associated with the Mendelian Tangier disease (Brooks-Wilson, Marcil et al. 1999), characterized by dramatic level decrease in HDL-C and Apolipoprotein A-I (Apo A-I) and accumulation of cholesteryl esters (CE) in tissues rich in macrophage (Oram 2000). It is however important to note that the significance of this disease worldwide is very limited since only 50 cases have been identified.

The ABCB subfamily consists of eleven transporters and interestingly it includes full and half transporters as well. Members of this complex subfamily can be found in many different compartimentalization / localization and its members exhibit various transport activities: bile acid secretion for ABCB4 and ABCB11, peptide transport into the endoplasmlic reticulum and a role in antigen presentation for ABCB2 and ABCB3, also called TAP1 and TAP2 respectively (Herget and Tampe 2007), iron metabolism and mitochondria localization for the ABCB6, ABCB7 ABCB8 and ABCB10 half transporters

(Zutz, Gompf et al. 2009). The most studied member of this subfamily is the ABCB1/Pgp transporter. This transporter has an important clinical impact since it has been characterized through its role in cancer resistance (Sarkadi, Homolya et al. 2006) (Szakacs, Varadi et al. 2008).

For the ABCC subfamily, see the section 1.3. below.

The ABCD subfamily consists of four half transporters that are located in the peroxisome, and are involved in the transport of very long chain fatty acids. The most studied member of this subfamily is ABCD1 since mutations in the protein lead to the X-linked adrenoleukodystrophy genetic disorder (Mosser, Douar et al. 1993). By accumulation of very long chain fatty acids this disorder affects both the adrenal glands and the white matter of the nervous system (Berger and Gartner 2006).

The ABCE and ABCF subfamilies do not have transmembrane domains, making their role in transmembrane transport unlikely. ABCE1 is the only member of the ABCE subfamily, and it has been found to block the activity of the ribonuclease L, and then to maybe be part of the innate immunity system (Vasiliou, Vasiliou et al. 2009). The ABCF subfamily contains three members that play a role in protein synthesis and it is believed that these proteins play a role in inflammatory processes (Vasiliou, Vasiliou et al. 2009).

The ABCG subfamily consists in five half transporters. Mutations in the *ABCG* genes have been implicated in sterol accumulation disorders and atherosclerosis. However among members of this subfamily the ABCG2/BCRP/MXR is the most studied. Indeed this transporter has a clinical significance since it can give resistance to a wide variety of anticancer agents (Sarkadi, Homolya et al. 2006).

## 1.3 The ABCC transporter subfamily.

The ABCC subfamily consists of twelve full transporters with various functions and transport spectrums. The *ABCC13* gene is often considered as a member of this subfamily, even if it is a pseudogene incapable of encoding a functional protein. In the ABCC subfamily, two different groups can be

distinguished within the ABCC subfamily, depending on the presence of an additional Nterminal TMD. Indeed the short ABCC members have a structure with TMD1-NBD1-TMD2-NBD2, and the long ABCC members have an extra N terminal transmembrane domain called TMD0 connected to the core by a long cytoplasmic loop called L0 (Tusnady, Bakos et al. 1997) (Klein, Sarkadi et al. 1999). Seven proteins have the long ABCC structure, ABCC1/MRP1, ABCC2/MRP2, ABCC3/MRP3, ABCC6/MRP6, ABCC8/SUR1, ABCC9/SUR2 and ABCC10/MRP7 and are considered as long (except for the SUR1 and 2), and the five remaining proteins ABCC4/MRP4, ABCC5/MRP5, ABCC7/CFTR, ABCC11/MRP8 and ABCC12/MRP9 belong to the short ABCC subfamily (except for the CFTR). Interestingly the ABCC7/CFTR, the ABCC8/SUR1 and the ABCC9/SUR2 are not transporters. Indeed, ABCC7 is an ion channel while SUR1 and SUR2 are potassium channel sensors. The other ABCC members are involved in multidrug resistance and are designated as multidrug resistance-associated proteins (MRP) (Deeley, Westlake et al. 2006).

To date, only few promoters of ABCC transporters have been consistently characterized. Thus the ABCC1, ABCC2 ABCC3 ABCC6 and ABCC7 are the only members with deep transcriptional regulation studies. Therefore there is a lack of information about the regulatory elements controlling their expression.

## 1.4 Example of transcriptional regulation studies on an ABCC transporter: ABCC7.

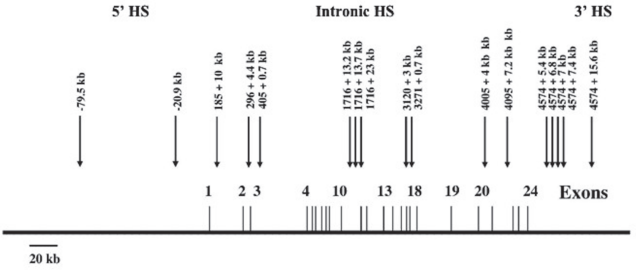
Here I will give an overview of the transcriptional regulation of the *ABCC7*/CFTR gene. This gene is a relevant example of transcriptional regulation study in our case. Indeed, *ABCC7* as *ABCC6* is a member of the ABCC subfamily, and they share sequences similarities. Moreover both genes exhibit a tissue specific pattern of expression. Finally the transcriptional regulation of *ABCC7* has been widely studied due to its important medical impact. All these reasons make the CFTR gene a good example for transcriptional studies. The cystic fibrosis (CF), a Mendelian disease, has been linked in 1989 to the chloride channel localized in epithelial cells coded by the

*ABCC7* gene (Kerem, Rommens et al. 1989) (Riordan, Rommens et al. 1989) (Rommens, Iannuzzi et al. 1989). Since this protein mediates the passive transport of small inorganic anions in both directions, the disease is characterized by a defect in chloride ion absorption and secretion due to mutations in the transporter. To date more than 1000 mutations have been described, but it is important to note that up to 90% of cystic fibrosis patients have a single mutation (ΔF508) (Lewis, Zhao et al. 2005). The regulation of the channel is mediated by phosphorylation/dephosphorylation of serine residues by the protein kinase A. The phosphorylation of the regulatory domain allows the passage of $Cl^-$ (Cheng, Rich et al. 1991) (Csanady, Chan et al. 2000).

Early transcriptional regulation studies have been performed on the *ABCC7* gene after the discovery of its role in CF. First, *in silico* analysis of a 3.8kb region upstream of the transcriptional initiation site revealed that the methylation of CpG regions correlates with a low expression level of the gene (Koh, Sferra et al. 1993). In parallel, multiple Sp1 and AP-1 binding sites, a cAMP response element (CRE), a CCAAT-enhancer binding protein (C/EBP) binding site, and several glucocorticoid response elements were identified in the promoter sequence by *in silico* analysis (McCarthy and Harris 2005). The promoter of the gene has been analyzed by using promoter deletion experiments in order to see specific regulatory region. Thus a minimal promoter has been identified between -226 and +98, as well as a negative regulatory sequence directly upstream of -227 (Chou, Rozmahel et al. 1991).

The importance of the cAMP response element, and therefore of cAMP, in the basal activity of the promoter was demonstrated by using a specific inhibitor of the cAMP-dependent protein kinase A. Using electrophoretic mobility shift assay (EMSA), a technique that allows the investigation of protein-DNA interaction, McCarthy et al. demonstrated the high binding affinity of CREB (cAMP response element binding protein) to the CRE element of the *ABCC7* promoter (McCarthy and Harris 2005). It is interesting to note that at this point no regulatory element could explain the tissue specific expression of the gene. To identify the potential regulatory elements, different groups used the

alternative DNase I Hypersensitive Assay technique. This technique allows the identification of open chromatin region generally associated with active regulatory elements such as enhancers and active promoters in the chromatin context by partial digestion with the DNase I enzyme. These sites are called hypersensitive sites (Cai, Struk et al.). Thus, using this method, more than 15 HS related to the *ABCC7* gene expression were identified (McCarthy and Harris 2005).




*Location of the identified HS across the CFTR locus. Map to scale the CFTR gene. Black arrows represent HS. Figure has been adapted from Mc Carthy and 2005*

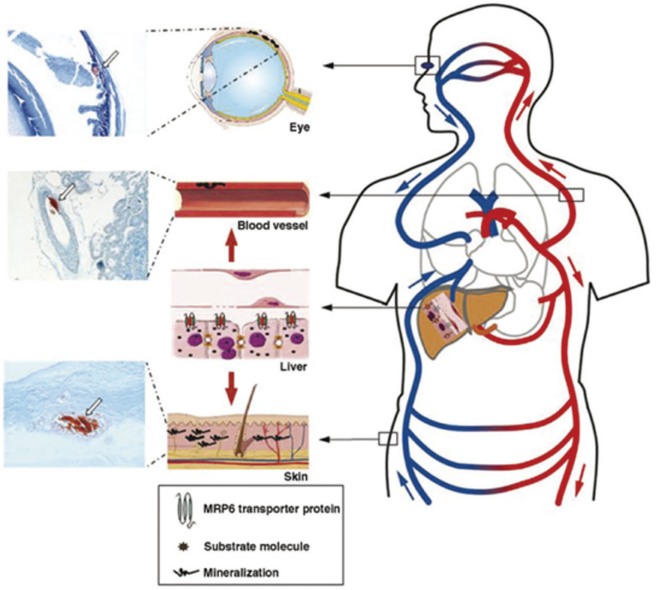
Interestingly only one hypersensitive site located in the first intron of the gene exhibits tissue specific properties. Indeed cloning of this sequence in CFTR expressing cell line led to an augmentation of the expression of the gene but it had no effect in non-expressing *ABCC7* cells (Smith, 1996 #64). Further analysis of this HS revealed by EMSA that several HNF1alpha transcription factors bind to this region and are responsible for the tissue specific expression of *ABCC7* (Mouchel, Henstra et al. 2004).

## 1.5 Pseudoxanthoma elasticum and ABCC6

Our laboratory has been involved very early in studying the *ABCC6* gene and the encoded protein, and recently we submitted a review (*ABCC6* as a target in Pseudoxanthoma Elasticum; András Váradi, Zalán Szabó, Viola Pomozi, **Hugues de Boussac**, Krisztina Fülöp and Tamás Arányi, Current Drug Targets, in press) that is a perfect introduction for the MRP6 protein and the *ABCC6* gene. I then used most of it for my introduction on *ABCC6*.

### 1.5.1.1 Pseudoxanthoma elasticum (PXE)

Pseudoxanthoma elasticum (PXE, OMIM 264800) is a recessive genetic disorder with a prevalence of 1 : 25.000 – 100.000, affecting the elastic tissues of the body, including the skin, the arteries and the elastic Bruch's membrane in the eye. Patients most commonly present with characteristic papules in the skin during late childhood or adolescence and subsequently develop angioid streaks of the retina. Angioid streaks are associated with subretinal neovascularisation, which can lead to hemorrhage and partial or complete loss of central vision. The diagnosis of pseudoxanthoma elasticum is suspected in individuals with characteristic skin and ocular findings and is confirmed by histological findings on biopsy of lesional skin in which fragmented calcified elastic fibers are visualized by use of special histologic stains (e.g. von Kossa staining).




*<u>Representation of PXE as a metabolic disease.</u> ABCC6/MRP6 is primarily expressed in the liver, and export compounds from the cell to the blood. If ABCC6 is absent of the liver, ectopic mineralization of connective tissues in the skin, the eyes and the blood vessels can be observed, due to the lack of the metabolite transported by MRP6 (adapted from Uitto J. 2007).*

The disease was first described in 1881 by D. Rigal (Rigal 1881) and Félix Balzer in 1884, but the term *Pseudoxanthoma elasticum* (PXE) was first used in 1896 by Jean-Ferdinand Darier (Darier 1896). In 1889 Robert W. Doyne was the first to describe angioid streaks, than Ester Grönblad and James Strandberg revealed the connection between PXE and angioid streaks in 1929 (Grönblad 1929).

#### 1.5.1.2 The symptoms.

**Cutaneous and mucosal manifestations**

The first sign of PXE is usually yellowish papules on the neck and other flexor surfaces. These skin lesions vary in size from 1-5 mm and may be grouped or coalesce to form larger plaques. In most of the cases the skin loses its elasticity and becomes wrinkled and redundant. In addition to the neck, plaques may also appear on other areas, such as axillae, inguinal region, antecubital and polpliteal fossae, and periumbilical area during the progression of the disease. In some cases mucosal lesions, identical to the skin lesions, can be detected on the inside of the lower lip, vagina, and all along the digestive tract mucosal membrane (Hu, Plomp et al. 2003).

The classic histological findings in PXE are ultrastructural elastic tissue abnormalities in the middle and lower dermis. In PXE patients elastin becomes fragmented and degenerated. Deposition of calcium in the abnormal elastin matrix can be visualized by von Kossa or other histological stains for calcium (Walker, Frederickson et al. 1989). Similar clinical findings may also be present is some other diseases for example in beta-thalassemia (Baccarani-Contri, Vincenzi et al. 1994), focal dermal elastosis (Limas 1999), cutis laxa (Choi, Kang et al. 2000), calciphylaxis (Nikko, Dunningan et al. 1996) or Paget's disease (Gross 1959).

However the ultra-structural histopathology is a hallmark of PXE. Even the skin findings in a highly related disease (PXE-like disorder with multiple coagulation factor deficiency) are in detail slightly different from the skin lesions found in PXE patients (Vanakker, Martin et al. 2007).

**Eyes**

Symptoms eventually appear in the eyes in all cases of PXE. The affected areas are the Bruch's membrane and the retinal-pigmented epithelium (Denson, Auld et al.).

In PXE, calcification of dystrophic elastic fibers can be observed in the elastic layer of Bruch's membrane, similar to what is seen in the skin. Pigment irregularities, called peau d'orange, may appear in the RPE. Calcification and thickening of the Bruch's membrane, as well as loss of RPE pigment granules lead to the development of angioid streaks (AS) (Finger, Charbel Issa et al. 2009).

AS are dehiscences in Bruch's membrane, forming grayish to reddish irregular lines resembling vessels, emanating from the optic disk. Due to the calcium deposition, Bruch's membrane becomes fragile, which is thought to be the major factor resulting in the dehiscences within the membrane. Later in the course of the disease fibrovascular tissue may grow through the damaged membrane, leading to choroidal neovascularization (CNV), subretinal fibrosis, atrophy of the overlying RPE and retinal hemorrhages (Dreyer and Green 1978).

Hemorrhages from the fragile new vessels can lead to partial or complete vision loss in PXE patients, starting usually around the third or fourth decade of life. Central vision loss has the greatest impact on the quality of life in PXE patients, but thus far no preventive measures are available. Laser therapy can be used to stop the proliferation or the bleeding of submacular neovessels, but it may cause visual loss or central scotomas due to scarring, and a high rate of recurrence has been observed (Lim, Bressler et al. 1993) (Pece, Avanza et al. 1997). A promising therapy to treat ocular symptoms of PXE may be the application of antiangiogenic drugs. There have been several publications documenting efficacy of intravitreal treatment with vascular endothelial growth factor (VEGF) monoclonal antibodies in case of age-related macular degeneration resulting in CNVs (Gragoudas, Adamis et al. 2004) (Rosenfeld, Brown et al. 2006). Similar treatment was already applied for a small group of PXE patients, showing very promising results (Finger, Charbel Issa et al. 2008).

**Cardiovascular system**

Calcification may affect the cardiovascular system, mostly the small and middle-sized arteries.

Cardiovascular symptoms include diminished peripheral pulses, angina pectoris, hypertension, mitral-valve prolapse and restrictive cardiomyopathy (Navarro-Lopez, Llorian et al. 1980) (Przybojewski, Maritz et al. 1981) (Challenor, Conway et al. 1988) (Fukuda, Uno et al. 1992) (Lebwohl, Halperin et al. 1993). One of the most common cardiovascular symptoms is intermittent claudication. Bleeding, especially gastrointestinal hemorrhages may also occur probably due to calcification of the elastic fibers in the small arteries located under the mucosa (Fah 1991). Myocardial infarction or other symptoms leading to sudden death are rare, but probably the most serious complication associated with PXE is the early onset of atherosclerosis (Hu, Plomp et al. 2003).

***ABCC6* as a potential genetic risk factor in Coronary Artery Disease (CAD)**

A strong correlation between a sequence variant of the *ABCC6* gene (c.3421C>T leading to the p.R1141X non-sense mutation) and CAD has been demonstrated in a Dutch cohort (Trip, Smulders et al. 2002). However, a surprisingly high frequency of the mutant allele was observed in the control population raising doubts about the validity of this unique study on the association of *ABCC6* mutation carrier status and CAD. An independent population genetic study on a large Hungarian cohort confirmed the findings of the previous report (Koblos, Andrikovics et al.). A significant association of carrier status and CAD was observed (5/361 carriers p=0.016 OR=10.5 95%CI 1.22-90.30). These findings mean that one non-functioning allele of *ABCC6* increases the risk of CAD significantly.

### 1.5.1.3 PXE like phenotypes.

The PXE phenotype is highly variable even within a single family where patients have the same disease-causing mutations (Hu, Plomp et al. 2003). No clear genotype/phenotype correlation has been observed to date (Li, Jiang et al. 2009). Finally, other diseases can mimic the PXE phenotype. The PXE-like

syndrome is due to mutations in the gamma-glutamyl carboxylase (*GGCX*) gene (Vanakker, Martin et al. 2007), while certain genetic hemoglobinopathies (e.g. thalassemia) lead to slowly developing phenotypes similar to PXE (Hamlin, Beck et al. 2003). Although the molecular mechanisms of the developing phenotype are not yet understood, in beta thalassemia mice a liver-specific down-regulation of *Abcc6* gene expression was observed (Edinger 2009). Christian Gotting and his co-workers identified a number of genes modifying the disease course in a German cohort. They demonstrated that the *ABCC6* c.-219A>C promoter polymorphism is significantly less frequent in patients than in the control population (Schulz, Hendig et al. 2006). They also showed that certain promoter polymorphism of the *SPP1* (secreted phosphoprotein 1, previously called: osteopontin) gene were more frequent in PXE patients than in controls (Hendig, Arndt et al. 2007). Furthermore, earlier disease onset is associated with polymorphisms of catalase, superoxide dismutase and glutathione peroxidase genes (Zarbock, Hendig et al. 2007). They also found that polymorphisms of the *VEGF* (vascular endothelial growth factor gene) are prognostic markers for ocular symptoms (Zarbock, Hendig et al. 2009). Similarly, it was observed that the c.2402C>G p.T801R polymorphism of the xylol-transferase II genes is associated with increased PXE severity (Schon, Schulz et al. 2006).

### 1.5.2 The ABCC6/MRP6 protein.

The domain architecture of the ABCC6/MRP6 shares the general features of the ABC-kingdom and is formed of the following domains: TMD0-L0-TMD1-ABC1-L1-TMD2-ABC2 (L0 and L1 are intracellular loop). ABCC6 consists of 1503 amino acids and it is known that the protein functions as an organic anion transporter (Ilias, Urban et al. 2002) (Belinsky, Chen et al. 2002). Indeed, in vitro studies demonstrated the transport of glutathione-conjugates like glutathione S-conjugated leukotriene C4 (LTC4), N-ethylmaleimide S-glutathione (NEM-GS) and S-(2,4-dinitrophenyl) glutathione, while the rat orthologue transports an anionic cyclopentapeptide (Madon, Hagenbuch et al. 2000). It has also been shown by *in vitro* assays that some missense mutations described as causative mutations in pseudoxanthoma elasticum result in the loss

of ATP-dependent transport of test substrates (Ilias, Urban et al. 2002). Compared with its sub-family members (ABCC1-5) ABCC6 is a poorly characterized transporter. The protein shows significantly lower transport rate (turnover number) than the other human ABCC-type transporters, which makes its detailed biochemical/functional characterization difficult.

It has been suggested that overexpression of ABCC6 is able to confer low levels of resistance to several commonly used natural product anticancer agents like etoposide, doxorubicin, daunorubicin and actinomycin D (Belinsky, Chen et al. 2002). However, clinically relevant ABCC6-mediated drug resistance has never been found.

#### 1.5.2.1 Homology models

No high-resolution three-dimensional structure of ABCC6 is available. However, a three dimensional homology model of ABCC6 is already published (Fulop, Barna et al. 2009), made possible by the recent publication of high resolution crystalline structures of ABC proteins (Dawson and Locher 2007) (Aller, Yu et al. 2009) (Dawson and Locher 2006). The structures representing the nucleotide-saturated, outward facing conformation show that the two nucleotide-binding (ABC) domains are in close proximity to each other in the characteristic head-to-tail orientation reflecting to the previously described "nucleotide sandwich dimer" (Smith, Karpowich et al. 2002). Newly recognized structural elements are the long "rigid" extensions of the transmembrane helices, called intracellular loops (ICL). Each half of the ABC proteins has two ICLs interacting with the ABC-domains. The coupling helices contact with their “own” as well as with the “opposite” ABC-domains, hence a special type of domain swapping can be recognized in the structure.

We have constructed two homology models of human ABCC6 protein: one of the models is based on the Sav1866 bacterial ABC transporter structure (Fulop, Barna et al. 2009) representing a nucleotide-saturated conformational state, while the other one uses the recently published mouse Abcb1 structure as template and represents the nucleotide-free (apo) conformation (Váradi et al, unpublished). By performing a statistical analysis we have found a significant

clustering of the missense PXE-mutations at the domain-domain interfaces: at the transmission interface that involves four intracellular loops (ICLs) and the two ABC domains as well as at the ABC - ABC interacting surfaces. In the nucleotide-saturated model the mutations affecting these regions are 2.75 and 3.53 fold more frequent than the average mutational rate along the protein sequence, respectively (Fulop, Barna et al. 2009). At the predicted ICL-ABC interfaces in the nucleotide-free model the mutational rate is 4.25-fold more frequent than the average mutational rate along the protein sequence (the ABC domains are distant in this conformation) (Váradi et al, unpublished). The observed significant clustering means that the domain contacts are much less permissive to amino acid replacements than the rest of the protein. These results provide a "bridge" between genetic data and protein structure and can be viewed as novel proof of the importance of the studied domain-domain interactions in the ABCC6 transporter.

### 1.5.3 Animal models

*Abcc6* knock out mouse models were generated and the critical role of *Abcc6* in ectopic mineralization/calcification has been confirmed in the *Abcc6*$^{-/-}$ mice, which recapitulates the genetic, histopathologic and ultrastructural features of PXE (Klement, Matsuzaki et al. 2005) (Gorgels, Hu et al. 2005). These findings suggest that the function of this transporter is conserved in the mouse. Calcification in the vibrissae capsules is the first symptom of the calcification phenotype detected at the 8 to 10 weeks of age and serves as an early biological marker of the disease (Klement, Matsuzaki et al. 2005). A slight alteration of plasma lipid composition of the *Abcc6*$^{-/-}$ mice has also been reported (Gorgels, Hu et al. 2005). The KO mouse models have been utilized for physiological and for pharmacological studies that are discussed elsewhere in this paper.

*Dystrophic Cardiac Calcification (DCC)* in the mouse is an autosomal recessive trait in certain laboratory strains and the *Abcc6* gene locus has been recently found as a main mediator of DCC at the Dyscalc1 locus (Meng, Vera et al. 2007) (Aherrahrou, Doehring et al. 2008). A splicing error in processing of *Abcc6* mRNA has been identified as the causative genetic event ("splice-mutation") of DCC. This mouse shows a more pronounced arterial calcification

phenotype than the one observed in the laboratory-generated *Abcc6*$^{-/-}$ mice strains (presumably due to the different genetic background) and seems to be as good model of PXE as the latter.

The zebrafish (Danio rerio) has nearly the same *ABC* gene repertoire as the human and has an accessible and well-characterized embryo. Two morpholinos were designed targeting two different regions of the *Abcc6a* gene (the *ABCC6b* gene was found to be inactive), and it was observed that they decrease *Abcc6a* expression by 54 and 81%. Both morpholinos induced a similar phenotype, cardiac edema and curled tail. Microinjecting zebrafish larvae with full-length mouse *Abcc6* mRNA completely rescued the knockdown phenotype (Qiaoli Li 2010). These recent results serve as basis of a novel knockdown animal model system. However, the results provided by this model may not be translated directly to human physiology, as the zebrafish gene appears to be essential for the development of the animal.

### 1.5.4 The ABCC6 gene

In 2000 it was discovered by positional cloning that mutations in *ABCC6* gene are responsible for the development of PXE (Le Saux, Urban et al. 2000) (Bergen, Plomp et al. 2000) (Ringpfeil, Lebwohl et al. 2000). Since the discovery of the connection between *ABCC6* mutations and PXE a large number of disease-causing mutations has been identified; the most frequent ones are p.R1141X (20 – 30%) and c.EX23_29del (5 – 15%). The high heterogeneity of PXE alleles in the population is comparable to that of other autosomal diseases. A locus-specific database has been established recently with the collection of the disease-causing mutations and other genetic variants and with link to other genetic databases [www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6].

*ABCC6* is located at 16p13.11 and codes for the ATP-binding cassette transporter protein, ABCC6/MRP6. The functional gene of 75 kb size consists of 31 exons. Two pseudogenes that are expressed at low levels and are positioned centromeric (*ABCC6-ψ1*) and telomeric (*ABCC6- ψ2*) of *ABCC6* have also been mapped (Pulkkinen, Nakano et al. 2001). Both *ABCC6-ψ1* and *ABCC6-ψ2* share a high degree of sequence similarity (~99%) with the functional gene, but are

truncated in the fourth and ninth intron, respectively. The *ABCC6* locus is located in a genomic region that was subject to segmental duplications, a series of events thought to play a crucial role in the recent evolution of *ABCC6* gene cluster (Symmons, Varadi et al. 2008). Due to this evolutionary scenario chromosomal rearrangements, gene conversion and emergence of new genes have been observed.

There are several reports indicating that the *ABCC6* locus is genetically unstable. A rare fragile site (FRA16A) has been found in close centromeric proximity to *ABCC6* (Cai, Struk et al. 2000), while the breakpoint of rearranged Chromosome 16 in the acute non-lymphocytic leukemia cell line M4Eo was localized ~0.5 Mb telomeric of *ABCC6* (Dauwerse, Wessels et al. 1993) (van Dongen, Macintyre et al. 1999). *ABCC1*, the gene located closest to *ABCC6* (8 kb apart) is frequently deleted in the drug-selected M4Eo cell line, and the concomitant amplification of *ABCC1* and *ABCC6* is observed in the SKOV3 ovarian carcinoma cell lines after multidrug selection (Buys, Chari et al. 2007).

### 1.5.5 Transcriptional regulation

The initial characterization of the transcriptional regulation of the human *ABCC6* gene identified two evolutionarily conserved regions in the 5' sequence 10kb upstream from the translation start site (Aranyi, Ratajewski et al. 2005). Both regions harbor a CpG island (CGI), potential target of DNA methylation. Analysis of DNA methylation may give clues to the location of important regulatory regions of gene expression, as methylation is stable like an imprint. Methylated regions indicate silenced and unmethylated regions design transcriptionally active sequences (Aranyi, Faucheux et al. 2005). Bisulfite genomic sequencing was carried out to analyze both the distal and the proximal CGI in different cell lines expressing and non-expressing *ABCC6* (Aranyi, Ratajewski et al. 2005). Cell-type specific DNA methylation in the proximal CpG island was detected, which inversely correlated with the expression of the gene and suggested that this region plays an important role in the tissue-specific regulation of *ABCC6*.

Based on these data luciferase reporter gene assays were performed with sequential deletion promoter constructs. One silencer (between -713 and -332 bp) and one DNA methylation sensitive activator sequence (between -332 and -145 bp) were identified. These data indicated that this region confers tissue-specificity to the *ABCC6* expression pattern. Further promoter mapping experiments confirmed these findings by identifying one tissue-specific regulator element (between -209 and -145 bp) and one further stronger activator sequence located between -234 and -209 bp (Ratajewski, Van de Ven et al. 2008).

The potential regulatory role of some transcription factors and cytokines has been suggested. The binding of the PLAG family of transcription factors and RXR has been convincingly demonstrated: they are able to transactivate the endogenous *ABCC6* gene, the binding site was determined by luciferase assay and the binding to the *ABCC6* promoter in the natural chromatin environment was demonstrated by chromatin immune precipitation. However, their functional role is still unclear (Ratajewski, Bartosz et al. 2006) (Ratajewski, Van de Ven et al. 2008). The binding of NF-κB, SP1 and TGF-β has been also suggested but their functional role and their binding to the endogenous *ABCC6* promoter have not been tested (Jiang, Matsuzaki et al. 2006).

Signal transduction pathways leading to the modulation of *ABCC6* expression have also been deciphered. Initially the activation by TGF-β and inhibition by TNF-α and IFN-γ were reported in luciferase reporter gene assays (Jiang, Matsuzaki et al. 2006). However, these effects have not yet been confirmed on the endogenous gene and the implicated signal transduction pathway was not identified.

### 1.5.6 The PXE-like syndrome

ABCC6 is predominantly expressed in the liver in the basolateral compartment of the plasma membrane of the hepatocyte (and to a lesser extent in the kidney), while the symptoms are systemic affecting various organs. This apparent discrepancy led to the hypothesis that PXE is a metabolic disease suggesting that ABCC6 is involved in secretion of a metabolite from the liver into the

circulation (Uitto, Pulkkinen et al. 2001). Recent experiments demonstrated that grafting of wt mouse muzzle skin onto the back of KO mice triggered mineralization, whereas grafting KO mouse muzzle skin onto wt mice was accompanied with no mineralization (Jiang, Endo et al. 2009). These transplantation experiments argue that PXE is indeed a metabolic disorder. Furthermore, in a parabiotic experiment the surgical pairing of Abcc6(-/-) mice with wild-type prevented the mineralization of the connective tissue in the knockout mice (Jiang, Oldenburg et al.).

There have been a few case reports of a disease that phenotypically resembled pseudoxanthoma elasticum with respect to the mineralization of soft tissues causing cardiovascular, dermal and ocular symptoms. However, these patients suffer from a vitamin K-dependent coagulation factor deficiency which is not seen in PXE (Macmillan and Vickers 1971) (Rongioletti, Bertamino et al. 1989) (Le Corvaisier-Pieto, Joly et al. 1996). The disorder is extremely rare and for decades its molecular basis remained unknown. Also, no mutations in the *ABCC6* gene could be detected in these individuals, suggesting that mutation of another gene could also cause PXE-like soft tissue calcification. The identity of this enigmatic gene was unraveled recently (Vanakker, Martin et al. 2007), and the clinical condition was classified as a novel disorder: PXE-like disease (pseudoxanthoma elasticum-like disorder with multiple coagulation factor deficiency, OMIM 610842). Six patients were found to possess compound heterozygous mutations in the gamma-glutamyl carboxylase (GGCX) gene.

The *GGCX* gene encodes the gamma-glutamyl carboxylase enzyme (GGCX), an ER (endoplasmic reticulum)-resident protein, responsible for post-synthetic carboxylation of Gla-domain containing proteins to which they confer Ca-binding properties (Berkner 2008). During the carboxylation reaction, vitamin K (VitK) is oxidized to an epoxide form, which is then re-reduced by another enzyme, Vitamin K oxido-reductase (VKORC1), thereby completing the VitK-cycle (Oldenburg, Marinova et al. 2008).

The highly similar phenotypic features of the two diseases evoked hypotheses about their overlapping patho-physiology. In PXE-like disease – due to the

mutations of the GGCX enzyme - the Gla-gammacarboxylation is reduced in the liver, thus resulting in blood coagulation abnormality, and also in the extrahepatic soft tissues where the control of calcification is impaired. In classical PXE gamma carboxylation is normal in the liver as there is no mutation in GGCX. According to the current hypothesis, in extrahepatic tissues gamma carboxylation is lower than normal as Vitamin K available for the carboxylation cycle may be limited in those tissues.

These data raises the possibility that one form of Vitamin K is transported from the liver into the circulation, and this transport is mediated by ABCC6 (and is missing in PXE due to ABCC6 mutations) (Borst, van de Wetering et al. 2008). On the other hand, the fact that PXE is a slowly progressive disease suggests that the metabolite transported by ABCC6 may only be reduced but not completely absent in the circulation of patients.

However, the key role of Vitamin K in the disease phenotypes associated with ABCC6 has not been proven.

Finally the local expression of ABCC6 has been shown in several tissues and cell types, e.g. keratinocytes, fibroblasts, smooth muscle cells and macrophages (Hendig, Langmann et al. 2008) (Beck, Hayashi et al. 2005) (Boraldi, Quaglino et al. 2003), some of those are affected in PXE. The local effect of missing ABCC6 activity may also contribute to the progression of the disease.

### 1.5.7 ABCC6 as a drug target

Two disease conditions are associated with mutations in the *ABCC6* gene: pseudoxanthoma elasticum is a recessive trait due to mutations in both ABCC6 alleles, while the loss of one functional ABCC6 allele is a genetic risk factor in coronary artery disease, CAD. As the phenotype in both cases is due to complete or partial loss of ABCC6 activity, augmentation-type gene therapy – in principle – could be an effective treatment of the disease conditions. However, even if all the safety concerns of gene therapy were solved, there are questions to be answered: is it sufficient to restore ABCC6 activity only in the liver, or it is also needed to do in other organs with lower level of expression (e.g. kidney)?

Missense disease-causing mutations can reduce the transport activity and/or the overall stability of the transporter, or may result in a slightly altered conformation that is not compatible with the normal trafficking of the protein to the plasma membrane. Indeed, defective protein trafficking caused by mutations underlies many human diseases and examples include several membrane-embedded ABC-proteins (like ABCC7/CFTR, ABCC2, ABCC8/SUR1 or ABCB11/BSEP). Efforts to identify pharmacologic compounds to correct the misfolding and/or misprocessing of mutant membrane proteins have already resulted in a few remarkable findings, and are considered as the molecular basis of allele-specific therapy of the given disease (Hayashi and Sugiyama 2007). This observation raises the possibility that pharmacological compounds (acting either as "chemical chaperones" or interfering with the quality control of the protein sorting/processing mechanism) may correct the defect causing the disease in a group of patients. Substrates and modulators of ABCB1 have been demonstrated to act as chemical chaperones thus helping the appearance of fully mature protein at the cell surface in the case of processing ABCB1 mutants (Loo and Clarke 1997). The first effort of promising correcting of folding of the delta508CFTR mutant was achieved by curcumin, which might act as a chemical chaperone (Egan, Pearson et al. 2004). High-throughput screening systems can be designed to test large chemical libraries for other "corrector" compounds. We have constructed a Hemagglutinin (HA) epitop-tagged version of human ABCC6: the antibody-reactive tag has been inserted into the extracellular N-terminal segment of the protein (Iliás and Váradi, unpublished). The protein preserved transport activity and showed cell surface expression similar to the wild type, and the HA tag recognized on the surface of intact, ABCC6-overexpressing cells provided a signal for correct trafficking. However, such an approach has inherent limitations in the case of ABCC6. In PXE – in contrast to cystic fibrosis, where deltaF508CFTR mutation is present in the majority of the patients – there is no such a high frequency mutation. It is expected, that different traffic-defect mutants are arrested at different points of the trafficking pathway and may need different "intervention".

The most frequent mutation in PXE is the p.R1141X nonsense mutation. It has been shown that aminoglycoside antibiotics like gentamicin can suppress

premature stop codon arrest of translation by inducing the ribosome to read through (or “suppress”) the nonsense mutation via insertion of a random amino acid. It was demonstrated in a muscular dystrophy mouse model that aminoglycosides could suppress stop codons not only in vitro but also in vivo (Barton-Davis, Cordier et al. 1999). However, the efficacy of the “read through” may be quite low. Enhanced production of active protein from genes with nonsense mutations can be achieved by combining treatment by inducing the promoter of the gene and the application of “read through” agents (Xi, Guan et al. 2004). These strategies can now be tested in experiments with the aim of correcting the frequent ABCC6 p.R1141X stop codon mutation.

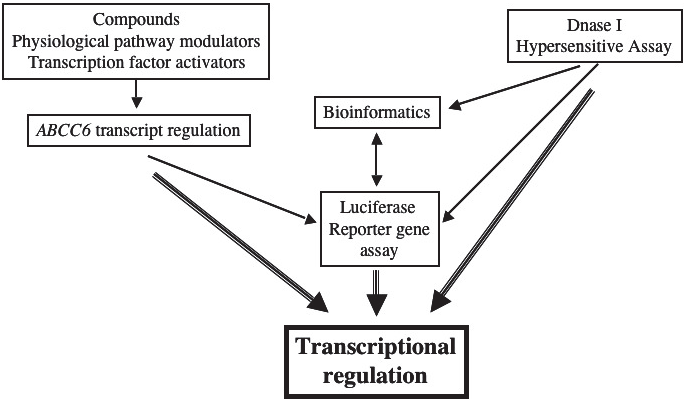
# 2 Objectives

1) We aimed to find physiological pathway regulating *ABCC6* expression. In that purpose we looked for different molecules that can increase or decrease the *ABCC6* expression. Those can be either drugs or transcription factor agonists. Our strategy was to perform a screening on the liver HepG2 cell line, and to follow *ABCC6* expression variation by quantitative RT-PCR.

2) We aimed to map the different potential regulatory regions of the *ABCC6* gene. We used the DNAse I digestion technique to identify sequences with transcriptional regulatory abilities in the endogenous chromatin environment. This technique is used in a hypothesis free context, which is a major advantage since hypothesis could lead to over or underestimation of the importance of specific sequences. This experiment was done on different cell lines to detect possible cell specific regulation profiles. Regulatory regions found by DNase I assay were then analyzed by luciferase assay and by bioinformatic tools to identify the factors potentially involved in the regulation of the gene.

That study contributes to a better understanding of the *ABCC6* transcriptional regulation, and we believe that it will give a better understanding of the disease linked to *ABCC6* mutations.

Before starting I need to emphasize that I carried out this project in tight collaboration with Lukasz Pulaski's group in Poland (Laboratory of Transcriptional Regulation, Institute of Medical Biology). The luciferase experiments that are part of my results were carried out in Lodz (Poland) together with or by Marcin Ratajewski. In the frame of the collaboration I went there three times to perform experiments. Moreover, the design of the constructs and of the luciferase experiments were completed following intense discussions between the Polish group and us. Of the sixteen new designed constructs used in that work, seven were cloned in Lodz, and nine were cloned in Budapest.




*Objectives representation, and the different strategies used. Thin arrows indicate the influx of information used to study the transcriptional regulation of* ABCC6 *gene. Thick arrows indicate the results leading to the understanding of transcriptional regulation of* ABCC6 *gene.*

# 3 Material and methods

## 3.1 Cell culture and treatments

HepG2 human hepatoma cell line was obtained from ATCC, and cultured according to the manufacturer's instructions in Minimum Essential Media (MEM (Advanced)) (invitrogen) in a humidified atmosphere of 5%$CO_2$/95% air. The medium was supplemented with 10% Fetal Bovine Serum (Gibco), 2mM glutamine, and 100 U/ml penicillin and 100ug/ml streptomycin. Caco2 cells were obtained from ATCC and cultured in the same condition than the HepG2, except that the media was supplemented with 20%FBS. HeLa and HEK293 cells were cultured in RPMI supplemented with 10% FBS, 2mM glutamine, and 100 U/ml penicillin and 100ug/ml streptomycin.

For HepG2 treatments, cells were maintained in 24 wells plate, and approximately $1*10^5$ cells were seeded per well. Treatments were performed 24hours after seeding the cells, and the RNA was extracted 24hours after treatment. Before treatment, cells were cultured in medium without serum, and in the case of co-treatment the different inhibitors were added 1hour before addition of the activators.

The different molecules have been used at the following concentration: (Sigma-Aldrich): hepatocyte growth factor (HGF, human, recombinant) 40ng/ml, epidermal growth factor (EGF) 100ng/ml, insulin 5ug/ml, TGFbeta 5ng/ml, phorbol myristate acetate (PMA) 100nM, menadione 5uM, tert-butyl hydroquinone (tBHQ) 75nM, (Calbiochem, San Diego, CA): U0126 2uM, bisindolylmaleimide I BIMI 500nM, LY294002 20uM.

## 3.2 DNase I hypersensitive assay

$6.10^6$ Cells were collected and resuspended in 100 µl of Ψ buffer (11 mM KPO4 pH 7.4; 108 mM KCl; 22 mM NaCl; 1 mM MgCl2; 1 mM DTT; 1 mM ATP) at (4°C), and were then treated by Dnase I during 3minutes on ice with a concentration range of DNase I. The DNase I digestion was stopped by the

addition of 2.5ml of lysis buffer (50 mM Tris-HCl pH 8; 20 mM EDTA; 1% SDS).

## 3.3 Genomic DNA extraction

The Genomic DNA was extracted by phenol and ethanol precipitation, gDNA was resuspended in TE and quantified by optical density at 260nm.

## 3.4 Southern blot

Twenty micrograms of DNase I-treated gDNA was digested overnight by the selected restriction enzyme then gel electrophoresis was performed overnight at 24V on a 1% TAE gel. The DNA was transferred to a nylon membrane and fixed by UV during 10minutes. Selected probe (P61 or P62) was labeled with $^{32}$P-dCTP using the DecaLabel™ DNA Labeling Kit (Fermentas). Membrane was incubated in hybridization solution (QuickHyb, Stratagene) 30minutes at 65°C. Then the labeled probe was added at a concentration of $1.25*10^6$cpm/ml, with 100ul herring sperm DNA previously sonicated to obtain fragment form 500bp to 1kb, and incubated 3h at 65°C. Membrane was then washed with 1X SSC washing buffer 5ml SSC 20X + 90 ml dW + 5ml SDS 10% for 10min at 37°C. Following washings were performed with more stringent condition by decreasing the concentration of SSC in the washing buffer. Thus next washing buffer contained 0.2X SSC etc… Membrane was washed until radioactivity measured on it decreases to 5 cpm/s. Membrane was exposed to hyperfilm (Hammersham) for 24-36h at -80°C. The DNase I hypersensitive sites were visualized by submitting the film to Developer and manual fixing bath solution (AGFA)

## 3.5 RNA extraction

Cells were resuspended in TRI REAGENT (Molecular Research Center, Inc.). Total RNA was extracted and purified by phenol chloroform and isopropanol precipitation. The concentration of extracted RNA was measured at 260 nm.

## 3.6 cDNA synthesis, reverse transcription.

A 20µl reaction mixture containing 500ng of total RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

## 3.7 Real time PCR

One microliter of synthesized cDNA served as template in a 10ul reaction, qPCR was performed with LightCycler FastStart DNA Master SYBR Green I (Roche) in an LC480 quantitative PCR machine (Roche). qPCR was performed at 95°C for 10min, followed by 40 cycles with denaturation at 95°C for 10s, annealing at 60°C for 5s, and extension at 72°C for 20s, fluorescence detection was performed at 87°C for 5s for the *ABCC6* gene, and at 80°C for 5s for the other genes. Denaturation curves of PCR products were determined by increasing temperature at the rate of 0.1/min from 55 to 95°C. Fluorescence of samples was continuously traced during this period. All the melting curves of PCR products gave a single peak. Agarose gel electrophoresis of representative reactions was used to confirm amplification of unique fragments of predicted lengths. All the results are expressed as the means ± S.E. of four independent experiments. The expression of target gene was normalized to the ABL gene used as an internal control according to the $2^{-\Delta Ct}$ formula.

Primer sequences are the following: *ABCC6* forward GGCCCGGGCATCCAGGTT, and reverse TTTCATCTACGCGAGCATTGTTCT, *URG7* forward TACCTCCTCTTCATCCACCACCAT, and reverse CCCTGCCTCCCCCGAACATTG, *ABL* forward GGGCTCATCACCACGCTCCA, and reverse CTGCCGGTTGCACTCCCTCA.

## 3.8 Cloning

Reporter plasmid vectors containing the *ABCC6-PXE* promoter sequences were constructed by PCR cloning. Human genomic DNA was used as template for amplification of the longest construct -332/+960, then this

construct was used as template for the amplification of the other one. The -718/+72, -332/+72, -145/+72 reporter constructs were described previously (Aranyi, Ratajewski et al. 2005).

The following sequence specific primers were designed using the Bisearch software. Forward primer: -332 TCGGTACCGATTCTTGTTGCAGGGGG; -234 CTCGGTACCCGCCTCTCTTCCCCCAT; -209 CCGGTACCTCGCCTGTTTTCACCTCC ; -145 CGGGTACCGAGCTCGAATCCCAGCCG. Reverse primers: +72 ACCAAGCTTGGAAAAGGAGAGTGGGGC; +513 CAAAAGCTTTAGCAGCATGGCACAAGG; +629 TCAAGCTTCACCGGCTGTGCAAACTT; +652 TGGAAGCTTACTCCTCCTGGTTTCACA; +688 AGAAGCTTGGACGTGGCCTCTTCAAT; +716 GTGGTACCAGCGACCACAGCAAGAGG; +817 TCAAGCTTAATCCTCCTGCCTCAGCC; +960 CAAAAGCTTTCAAGCAATCCTCCCACC. The restriction sites (KpnI site for forward primers and HindIII site for reverse primers) were added to the 5′-end of primers, and promoter sequences were amplified using high fidelity thermostable DNA polymerase (*TaqPfu*). PCR products were cloned into the pGL3-Basic vector (Promega) to construct the following plasmids phABCC6(–332/+513)Luc, phABCC6(–332/+629)Luc, phABCC6(–332/+652)Luc, phABCC6(–332/+688)Luc, phABCC6(–332/+716)Luc, phABCC6(–332/+817)Luc, phABCC6(–332/+960)Luc, phABCC6(–145/+629)Luc, phABCC6(–145/+652)Luc, phABCC6(–145/+688)Luc, phABCC6(+72/+629)Luc, phABCC6(+72/+652)Luc, phABCC6(+72/+688)Luc, phABCC6(–234/+688)Luc, phABCC6(–209/+688)Luc, phABCC6(+479/+960)Luc. The sequence of the cloned inserts was tested for the amplification of the *ABCC6-PXE* gene and the lack of errors by automated sequencing.

## 3.9 Luciferase assay

Ten thousands cells were seeded per well in a 96 well plate. After 24h, the plasmids were transfected by polyethyleneimine transfection reagent (Exgen500, Fermentas). Cells were harvested and lysed 48h after transfection. The activity of luciferase in cell lysate was determined in an EnVision luminometric plate reader (Perkin Elmer) using a commercial luciferase substrate (BD Biosciences). All cells were also transfected with a pCMV-SEAP reporter vector encoding secreted alkaline phosphatase under the control of a constitutive cytomegalovirus-derived promoter (kind gift from Dr. S. Schlatter, Zurich) as an internal control for transfection efficiency. The level of alkaline phosphatase activity was determined spectrophotometrically.

The luciferase assay treatments were carried out at the indicated concentration 24hours after transfection and for 24hours. Co-transfections of reporter constructs and plasmids carrying cDNA for selected transcriptional factors were performed analogously to normal transfection. Expression plasmids were bought from Origene Technologies Inc. (Rockville, USA).

## 3.10 Western blot

Whole cells were cultured and treated with selected compounds on a 6 well plate and were lysated using 0.5ml of the following buffer 30mM HEPES, 10mM NaCl, 5mM NaF, 1mM EGTA, 1% triton X-100, 10mM benzamindine, 1mM $Na_3VO_4$, 20ug/ml leupeptin, 1mM Phenylmethylsulphonyl fluoride. Lysates were harvested and clarified by centrifugation at 15 000g for 10 minutes at 4°C. Protein content was determined with the Lowry assay, and equal amount of protein was subjected to SDS-PAGE using 7.5%running gels. Proteins were transferred to nitrocellulose filters and membranes were blocked with 5% dry milk in TBS-Tween (TBS containing 0.05% Tween 20) overnight at room temperature. Then membranes were incubated for 120minutes with the selected primary antibody (antibodies against ERK1/2, or phospho-ERK1/2 (Thr202/Tyr204) purchased from Santa Cruz, CA. respectively K-23 and E-4) at room temperature. Membranes were then washed and incubated 60minutes with a species-specific horseradish peroxidase-labeled secondary

antibody at room temperature (anti-mouse for the anti-ERK-P (E-4), anti-rabbit for the anti-ERK (K-23)). Protein-antibody interaction was detected by using the enhanced chemiluminescence technique (ECL, Amersham Biosciences). The quantitative measure of expression level was determined by densitometry of the immunoblots.

## 3.11 Bioinformatic

For the in silico sequence analysis the following web servers were used: P-Match; Matinspector; TF-search.

## 3.12 Statistical analysis

Statistical analysis was performed using Student's t-test to compare the two groups and ANOVA was used for multiple comparisons when the three groups or more were compared. $P < 0.05$ was considered statistically significant. The results were expressed as mean ± SE. Values were analyzed using the statistical software Instat.

# 4 Results

## 4.1 *ABCC6* is regulated by a physiological pathway

### 4.1.1 Identification of drug compounds modulating *ABCC6* expression

The transcriptional regulation of the *ABCC6* gene has already been investigated. The promoter was characterized and regulatory elements of the promoter were highlighted, with an activator located between -332 and -145bp from the translational initiation site of the gene, and an inhibitor located 5'upstream -332 (Aranyi, Ratajewski et al. 2005). Additionally the Sp1, RXR and PLAG transcription factors were shown to play a role in its regulation (Jiang, Matsuzaki et al. 2006) (Ratajewski, Bartosz et al. 2006) (Ratajewski, Van de Ven et al. 2008). The chromatin context of the gene was also investigated by DNA methylation analysis of the promoter and tissue specific pattern correlation with the expression of the gene was detected in human and mice as well (Aranyi, Ratajewski et al. 2005) (Douet, Heller et al. 2007). But to date no physiological relevant physiological pathways regulating the expression of *ABCC6* were determined, and we therefore decided to investigate this issue.

*ABCC6* is mainly expressed in the liver, for that reason we chose the most relevant hepatocellular carcinoma cell line. The HepG2 cell line is highly used in the literature as a liver model and was selected for its ability to express *ABCC6* at high level.

I will use in that thesis the word "drug", as transcriptional regulation studies do, for the different compounds used to modulate the expression of the *ABCC6* gene. We performed the drug screening on the HepG2 cell line, choosing the drugs for their ability to regulate numbers of various pathways. I am going to detail only the drugs that turned out to be the most relevant ones (Table 1).

| **Nuclear factor / pathway** | **Drug used** | **Function** | **Observed *ABCC6* expression in % to the control** |
|---|---|---|---|
| Aryl Hydrocarbon Receptor (Aherrahrou, Doehring et al.) | 2,3,7,8-tetrachlorodibenzo-p-dioxin (**TCDD**) | Detoxification | 60% |
| NF-E2-related factor 2 (**Nrf2**) | Oltipraz (Kast, Goodwin et al.) | Oxidative stress response | 50% |
| **Ahr/Nrf2** | Beta naphtoflavone (**BNF**) | | 30% |
| Oxidative stress inducer | tert-Butylhydroquinone (**tBHQ**); Vitamin **K3**; Cobalt (**Co**), Desferoxamin (Schurgers, Spronk et al.), Buthionine sulphoximine (**BSO**) | | 30%<br>20%<br>50%<br>60%<br>75% |
| Peroxisome proliferator-activated receptors (**PPARg**) | Troglitazone (**TGZ**) | Lipid metabolism | 100% |
| **PPARa** | Clofibrate (**CLOF**) | Lipid metabolism | 100% |
| Retinoid X receptor **(RXR)** | 9cis Retinoic Acid (**9cis-RA**) | | 110% |
| Protein kinase C **(PKC)** | phorbol 12-myristate 13-acetate (**PMA**) | Metabolism | 30% |
| Kinase cascades | Growth factor: Hepatocyte growth factor (**HGF), Epidermal growth factor (EGF), Transforming growth factor (TGFb)** | Metabolism | 40%<br>60%<br>80% |

*Table 1.* ABCC6 *expression is inhibited by various drugs. HepG2 cells were treated with the indicated compounds (HGF, Butyrate, Oltipraz, TCDD, BNF, tBHQ) or vehicle for controls (DMSO) in serum free conditions for 24 hours. Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the ABL housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, *p<0.05.*

After the drug screening we filed the different agonist or inducers tested in two different groups. Most of the drugs showed inhibitory effect on the *ABCC6* gene expression, and others do not exhibit any effect (table 1).

### 4.1.2 **Growth factors down-regulate the expression of *ABCC6*.**

The transmission of signals from the cell membrane to their intracellular targets is dependent of a complex network of proteins present in the cytoplasm. That network transmits the signal via a number of interactions, and inter-activation processes. The kinase cascades are among these signal transmission processes (Seger and Krebs 1995). Thus, following the binding of HGF on the hepatic cell membrane, three major kinases are activated: the PI-3k, involved in glucose regulation, the PKC and the MAPK. The complex regulation processes following the activation of these cascades lead to the activation and inhibition of a set of different genes in order to complete specific actions. In the case of the MAPK activation the cell growth and differentiation are privileged while for the PI-3k the response to insulin is advantaged.

Interestingly in the case of treatment with the HGF and EGF we observed similar inhibition of the *ABCC6* expression, indicating a possible important role of the growth factors in *ABCC6* regulation, while treatment with TGFbeta, another important growth factor, did not result in a significant inhibition of *ABCC6* expression (Figure 1).

It is well known that the activation of the c-Met membrane receptor by its ligand HGF leads to the activation of three major cellular cascades in the cell which are the PI-3k, the PKC and the MEK1/2 cascades (Figure 2). We decided to investigate in more details this interesting finding.

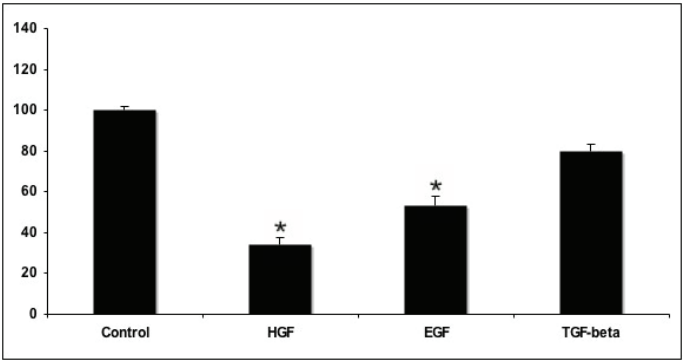



*Figure 1. Effect of growth factors on* ABCC6 *gene expression. HepG2 cells were treated with HGF, EGF and TGFbeta or vehicle for controls (DMSO) in serum free conditions for 24hours. Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the* ABL *housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, mean±SE *p<0.05.*

To understand the molecular mechanism of the regulation of *ABCC6* by HGF we decided to screen the pathways one after another. In that purpose, we used different specific inhibitors of the kinases: LY294002; BIMI and U0126 respectively for the PI-3k, the PKC and the MEK1/2 cascades.

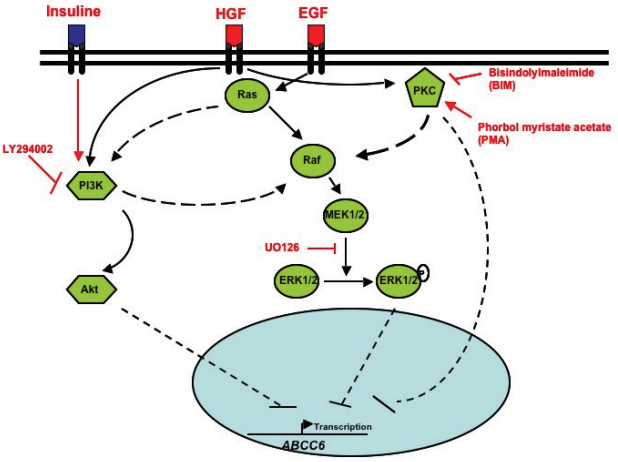



*Figure 2. HGF leads to the activation of various pathways in cells.*

The concentration of the molecules used for the treatments were chosen according to the literature as the HepG2 hepatic model cell line is a widely used cell line. It is known that the Fetal Bovine Serum usually used in cell culture contains various growth factors. Those factors can activate the kinase cascades we aimed to investigate. Therefore to avoid any uncontrolled activation by the factors contained in the serum, we maintained the cells in serum free medium. Before treatment and one hour before adding the different kinase agonists, cells were treated with the specific inhibitors (Figure 2).

First of all we considered the possible involvement of the PI-3k on the *ABCC6* expression regulation. Insulin is known to be an activator of the PI-3k cascade, but treatment with insulin did not show any inhibitory effect on the expression of *ABCC6*. However, we also performed co-treatment of HepG2 cells with the PI-3k specific inhibitor LY294002 and HGF to confirm the results observed with insulin (Figure 3). LY294002 treatment did not prevent the inhibition of *ABCC6* expression by HGF showing that the PI-3k pathway is not involved in the expression regulation of *ABCC6*.

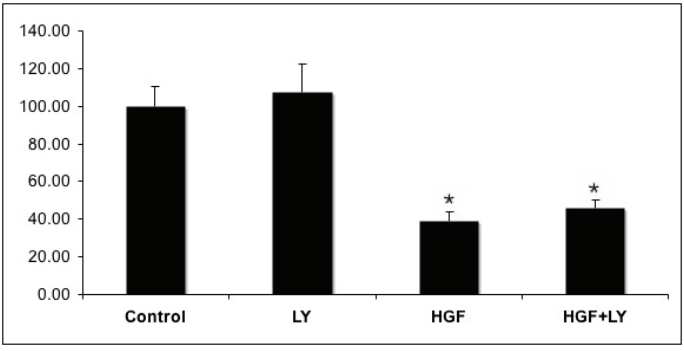



*Figure 3. PI-3kinase pathway does not modulate* ABCC6 *expression. HepG2 cells were treated with HGF or LY294002 (indicated as LY on the figure), a specific inhibitor of PI-3K or in combination or vehicle for controls (DMSO) in serum free conditions for 24 hours. When co-treatment was applied LY294002 was added to the cells 1 hour before HGF Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the ABL housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, mean±SE *p<0.05*

Next, we analyzed the PKC pathway, and in that purpose, we performed PMA treatment, a specific PKC activator, on HepG2 cells. As already described we first observed a phenotypic change of the HepG2 cells after 24hours treatment (Sipeki, Bander et al. 1999) (Gujdar, Sipeki et al. 2003), showing that the treatment was efficient. The following analysis of the *ABCC6* gene expression showed an inhibition of almost 70% of the expression of the gene, highlighting a possible role of PKC on the regulation of *ABCC6* gene expression. To further investigate the role of PKC, we performed co-treatment of PMA and BIMI a specific inhibitor of the PKC activation. Surprisingly BIMI did not prevent the inhibition of *ABCC6* expression by PMA (Figure 4).

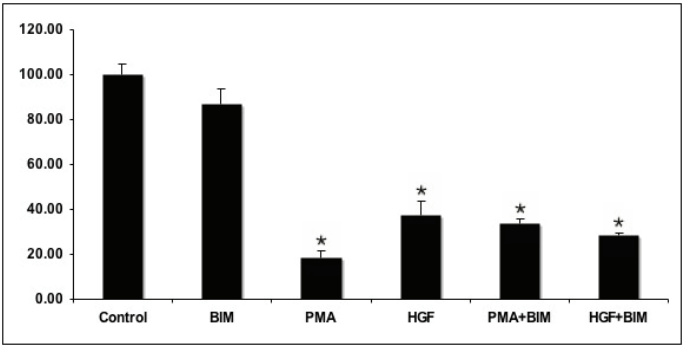



*Figure 4. Atypical PKC subfamily member(s) inhibit the expression of* ABCC6. *HepG2 cells were treated with PMA or HGF or BIM, a selective inhibitor of the PKC kinase or in combination or vehicle for controls (DMSO) in serum free conditions for 24 hours. When co-treatment was applied BIM was added to the cells 1 hour before PMA or HGF Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the* ABL *housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, mean±SE *p<0.05*

This result suggested that the PKC family was able to inhibit the expression of *ABCC6* but not all members were involved in this inhibition. Indeed the PKC family contains three subfamilies and it is known that the atypical subfamily is not inhibited by BIMI. Therefore we assumed that the atypical PKC subfamily was responsible for the inhibition observed after PMA treatment. Consequently, we asked whether that this inhibitory effect was MEK1/2 dependent since it is also known that the atypical subfamily can activate the MEK1/2 cascade (Figure 2).

### 4.1.3 **ERK1/2 dependent down-regulation of *ABCC6***

MEK1/2 is part of the ERK1/2 cascade and it is responsible for the phosphorylation and thereby the activation of the ERK1/2 kinase. In order to investigate the involvement of the ERK1/2 cascade in the regulation of *ABCC6* expression, we used U0126, a specific inhibitor of the ERK1/2 phosphorylation. We then performed a co-treatment of HGF and this inhibitor. We observed that the ERK1/2 inhibitor totally prevented the *ABCC6* expression inhibition due to HGF, pointing out the possible involvement of the ERK1/2 cascade in that inhibition.

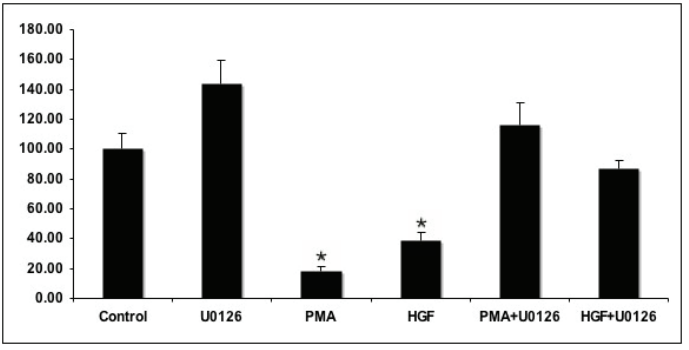



*Figure 5. Inhibition of the ERK1/2 pathway precludes the downregulation of* ABCC6 *expression. HepG2 cells were treated with PMA or HGF or U0126, a selective inhibitor of MEK1/2 kinase or in combination or vehicle for controls (DMSO) in serum free conditions for 24 hours. When co-treatment was applied U0126 was added to the cells 1 hour before PMA or HGF. Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the* ABL *housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, mean±SE *p<0.05*

Next we asked whether the previously observed inhibition by PMA was also dependent on the ERK1/2 cascade. We performed co-treatment of PMA and U0126, and observed similar results than for the HGF co-treatment suggesting that the ERK1/2 cascade is responsible for the inhibition of the *ABCC6* gene by PMA in HepG2 cells (Figure 5).

We decided to rule out the possibility that the U0126 molecule was able to act in an ERK1/2 independent manner on the expression of the *ABCC6* gene. Therefore we carried out an experiment with U0124, a compound structurally

similar to U0126 but without any activity on the ERK1/2 cascade. Simple treatment of HepG2 cells with U0124 did not show any activation of *ABCC6* expression. On the contrary, U0126 activated the gene expression confirming that the observed prevention of the inhibition by U0126 was due to the action of the molecule on the MEK1/2 kinase cascade pointing out the potential role of ERK1/2 cascade in *ABCC6* gene expression regulation (Figure 6). We considered the results obtained with U0124 sufficiently convincing not to try co-treatment of this molecule with HGF.

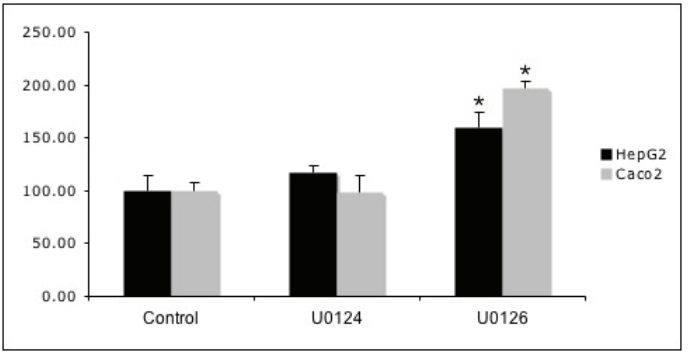



*Figure 6. U0126 molecule is not acting in an ERK1/2 independent manner on the expression of the* ABCC6 *gene. HepG2 or Caco2 cells were treated with U0126 or U0124 an inactive analog of U0126, or vehicle for controls (DMSO) in serum free conditions for 24 hours. Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the* ABL *housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated two times, mean±SE *p<0.05.*

The ERK1/2 kinase exists in 2 different forms in the cells: the phosphorylated form, which represents the active kinases (ERK1/2-P), while the non-phosphorylated form is inactive (ERK1/2). It is possible to detect by western immunoblot one or the other form with specific antibodies. Therefore, in order to further confirm the role of ERK1/2 in the observed inhibition, we tested the modification of the ratio of ERK1/2-P / ERK1/2 following treatment with the previously used molecules. In that purpose we performed a western blot and we analysed the ratio of ERK1/2-P / ERK1/2 by densitometry (Figure 7).

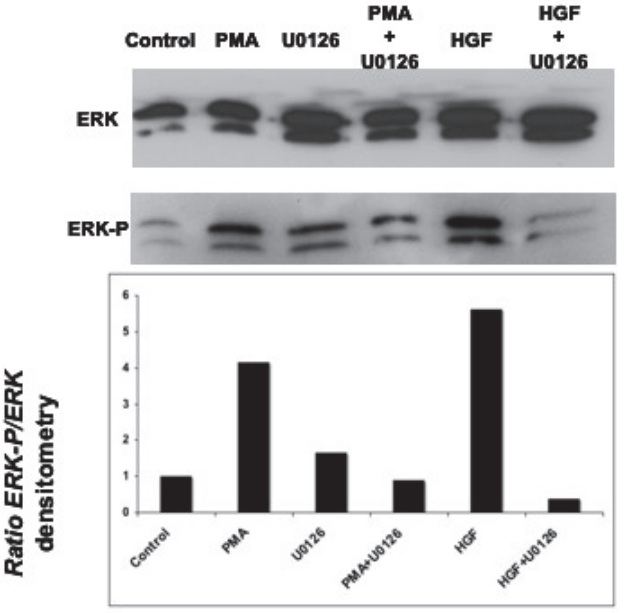



*Figure 7. Western blot analysis of ERK1/2 phosphorylation shows a correlation between the ERK1/2 activation and the* ABCC6 *transcriptional inhibition. a) HepG2 cells were treated with PMA or HGF or U0126, a selective inhibitor of MEK1/2 kinase or in combination or vehicle for controls (DMSO) in serum free conditions for 24 hours. When co-treatment was applied U0126 was added to the cells 1 hour before PMA or HGF. Proteins were extracted and 50ug protein was loaded per lane. The blotted membrane was incubated during 2 hours in the primary and subsequently 1 hour in the secondary antibody. b) Densitomentric analysis of the immunoblot shown in panel A (ERK-P/ ERK1/2 in arbitrary units).*

As expected, treatment with PMA or HGF led to more than four time increase in ERK1/2 phosphorylation in comparison to the control, while we observed an important decrease in the level of ERK1/2 phosphorylation after co-treatment with HGF or PMA and U0126, emphasizing the role of ERK1/2 in the regulation of *ABCC6* gene expression.

### 4.1.4 Oxidative stress partially inhibits *ABCC6* expression via the ERK1/2 pathway.

The ERK1/2 cascade can be physiologically activated by various conditions in the cells (Kim and Choi). Therefore we inquired whether the regulation of *ABCC6* by the ERK1/2 cascade was specific to its activation by the growth

factors or if alternative activators were also able to inhibit *ABCC6* such as the oxidative stress. We first performed treatments of HepG2 cells with different oxidative stress inducers, as tBHQ, vitamin K3, DFO, Cobalt, BNF and BSO. Interestingly, all the oxidative stress inducers led to the inhibition of *ABCC6* expression (Table 1). We then chose tBHQ and Vitamin K3 and performed co-treatments with U0126. We observed that the co-treatment with the ERK1/2 specific inhibitor at least partially prevented this inhibition (Figure 8).

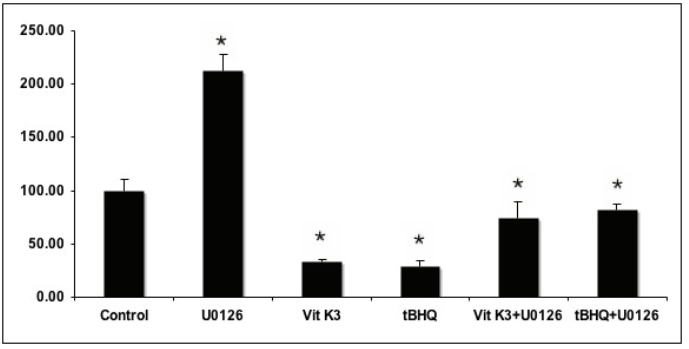



*Figure 8. Oxidative stress inhibits* ABCC6 *expression partially via the ERK1/2 cascade. HepG2 cells were treated with tBHQ or Vitamin K3 or U0126 or related co-treatment or vehicle for controls (DMSO) in serum free conditions for 24 hours. Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the* ABL *housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, mean±SE *p<0.05.*

Finally, to confirm the ERK1/2 phosphorylation/activation after treatment by the different *ABCC6* inhibitors previously used, and to further confirm the role of ERK1/2 in this inhibition, we performed a western blot experiment on cells treated with different molecules. We observed that the quantity of the ERK1/2-P form increased after treatment with tBHQ or vitamin K3, while co-treatment with U0126 partially prevented this increase (Figure 9). Due to the high background we were not able to perform quantitative densitometric analysis for any of these western blots, and therefore we were not able to determine the ERK-P/ERK ratio.

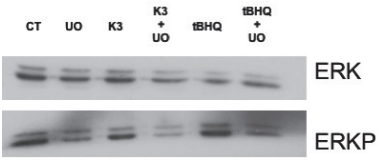



*Figure 9. Western blot analysis of ERK1/2 phosphorylation after oxidative stress induction. HepG2 cells were treated with tBHQ or Vitamin K3 or U0126 or vehicle for controls (DMSO) in serum free conditions for 24 hours. When co-treatment was applied U0126 was added to the cells 1 hour before PMA or HGF. Proteins were extracted and 50ug protein was loaded per lane. The blotted membrane was incubated during 2 hours in the primary and subsequently 1 hour in the secondary antibody.*

In conclusion our results showed that oxidative stress is an inhibitor of the expression of *ABCC6* and (at least partially) this effect is due to the activation of the ERK1/2 kinase cascade. We decided not to investigate in further details the other mechanisms leading to the inhibition of *ABCC6* expression by oxidative stress. Indeed oxidative stress can activate a multitude of different pathways in the cells that can be interconnected. We decided to focus on the interesting role of the physiological ERK1/2 pathway on the *ABCC6* gene expression.

### 4.1.5 ERK1/2 inhibits *ABCC6* expression at the transcriptional level.

Our next question was whether the observed effect was due to the down-regulation of *ABCC6* expression at the transcription initiation level, or to a post-transcriptional regulation by destabilization of the transcript. In order to rule out one of these two possibilities, we made use of a splice variant of *ABCC6* named Up Regulated Gene 7 (*URG7*). This alternative transcript of *ABCC6* was first observed in a screen for up-regulated genes following hepatitis B virus infection of HepG2 cells (Lian, Liu et al. 2001). Interestingly enough, *URG7* shares the exact same promoter as *ABCC6* but has a different 3'UTR since *URG7* ends in the second intron of the gene (Figure 10). This characteristic leads to different post-transcriptional regulatory mechanisms between *ABCC6* and *URG7* (the splicing and probably the mRNA stability too). We hypothesized that if p-ERK1/2 acts at the transcription initiation level, we should observe similar behavior of the two transcripts after treatment

with the previously tested molecules. In contrast, if p-ERK1/2 acts at the post-transcriptional level, we wouldn't observe the same inhibition of *URG7* after treatment with *ABCC6* inhibitors.

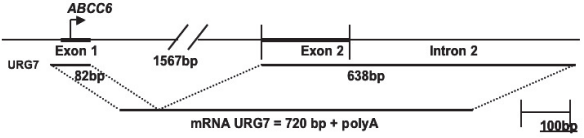



*Figure 10.* URG7 *is an alternative transcript of* ABCC6.

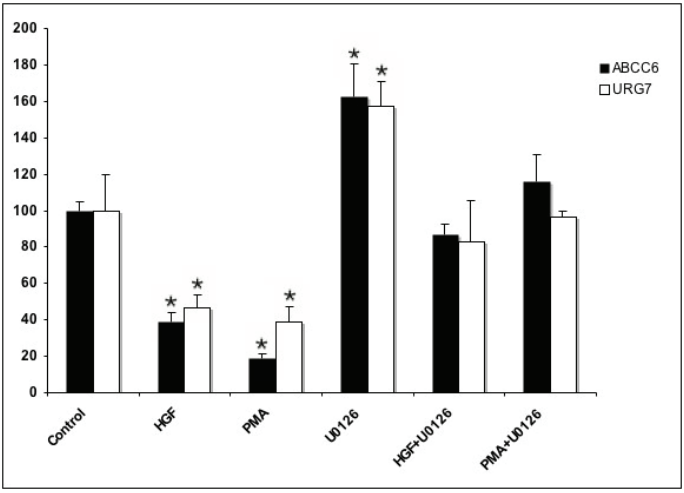



*Figure 11.* ABCC6 *expression is regulated via ERK1/2 at the transcriptional level. HepG2 cells were treated with PMA, HGF and U0126, and related co-treatment or vehicle for controls (DMSO) in serum free conditions for 24 hours. Relative* ABCC6 *and* URG7 *expression levels were determined by quantitative PCR after normalization to the* ABL *housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, mean±SE *p<0.05.*

Treatments of HepG2 cells with PMA, HGF (Figure 11), tBHQ and vitamin K3 (data not shown), and the respective co-treatments with U0126 showed the same effect on the expression level of *ABCC6* and *URG7*, strongly suggesting that the ERK1/2 cascade inhibits the expression of *ABCC6* gene at the transcription initiation level.

### 4.1.6 *De novo* protein synthesis is not needed for the down-regulation of *ABCC6* by the ERK1/2 cascade

Our results strongly suggested that the *ABCC6* expression inhibition by the ERK1/2 cascade was at the transcriptional level. In the following experiment we investigated the mechanism of this regulation, and more precisely whether the ERK1/2 action on *ABCC6* expression needs a new protein synthesis or not. To answer this question, we performed treatments of HepG2 with Cycloheximide, an inhibitor of the protein synthesis, with or without PMA selected for its ERK1/2 dependent high inhibitory effect of *ABCC6* transcription regulation. We assumed that if the ERK1/2 effect was direct then co-treatment with PMA and cycloheximide wouldn't show any differences with PMA treatment alone. In that purpose we treated HepG2 cells with cycloheximide three hours before adding PMA, and inversely. The idea was to stop the de novo protein synthesis before inducing the ERK1/2 cascade, and if de novo synthesis was needed to observe an important difference between the two conditions.

Surprisingly we observed that treatment with cycloheximide alone led to the induction of *ABCC6* expression (Figure 12). Intriguingly *URG7* expression wasn't induced but inhibited by cycloheximide. This is the first and only time that we observed regulatory differences between the two alternative transcripts. However our results also showed no difference between PMA treatment and co-treatment with PMA and cycloheximide. It is necessary to stress that the differences observed between *URG7* and *ABCC6* regulation after simple treatment with cycloheximide made impossible to conclude on that experiment, even if the co-treatment results highly suggested that the regulation of *ABCC6* expression by the ERK1/2 cascade is not dependent on *de novo* synthesis of protein in the HepG2 cell line.

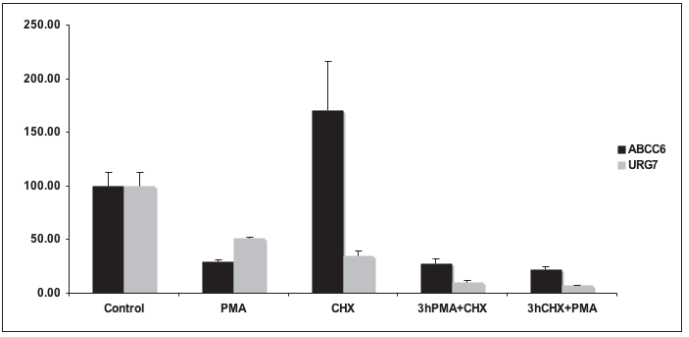



*Figure 12. ERK1/2 regulates the expression of* ABCC6 *without the needs for de novo protein synthesis. HepG2 cells were treated with PMA or cycloheximide (CHX) an inhibitor of the protein synthesis, or vehicle for controls (DMSO) in serum free conditions for 24 hours. PMA or CHX were added 2 hours before in case of co-treatment. Relative* ABCC6 *expression level was determined by quantitative PCR after normalization to the* ABL *housekeeping gene expression level as described in the methods. Expression levels are indicated as a percent of untreated control. Four parallels were used for each condition. The experiments were repeated three times, mean±SE *p<0.05.*

### 4.1.7 ERK1/2 effect is cell type specific and its response element is located in the proximal promoter of *ABCC6*.

Altogether our results showed that ERK1/2 activation in HepG2 cells seems to down-regulate *ABCC6* expression at the transcription initiation level. To identify the regulatory element(s) involved we carried out luciferase reporter gene assay. First we tested the promoter region of *ABCC6* to locate the sequence in the proximal or distal promoter. Indeed it has been shown that the distal promoter contains an inhibitory element (Aranyi, Ratajewski et al. 2005). After transfection of HepG2 cells with the promoter construct we treated the cells with PMA, or U0126. We observed that the distal and the proximal promoters showed similar variation of the luciferase activity following the treatment with the ERK1/2 inducer or inhibitor. This result clearly pointed out the role of the proximal promoter in this regulation (Figure 13).

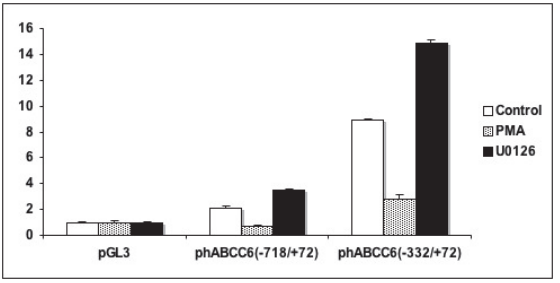



*Figure 13. ERK1/2 response element is located in the proximal promoter of the* ABCC6 *gene. Distal (-718/+72) and proximal (-332/+72) promoter constructs were transiently transfected to HepG2 cells. 24 hour after the transfection cells were treated with PMA or U0126 at the indicated concentrations or vehicle. Cells were treated with the compounds for 24 hours and promoter activity was then measured by luciferase assay. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of empty vector (pGL3-basic) in control treated cells), mean±SE (n = 5), *p<0.05.*

Next we continued by confirming the ERK1/2 dependence of that inhibition. We co-transfected our cells with the -332/+72 construct and a constitutively active MEKK1 (MEKK1ca) that phosphorylates and thus activates the ERK1/2 kinase cascade**,** or a dominant negative ERK1 (ERK1dn) that inhibits the cascade. Both MEKK1ca and ERK1dn acted on the -332/+72 construct in a dose dependent manner (Figure 14 A and B), demonstrating and confirming the role of the ERK1/2 cascade in the inhibition of *ABCC6* expression at the transcription level via the proximal promoter of the gene.

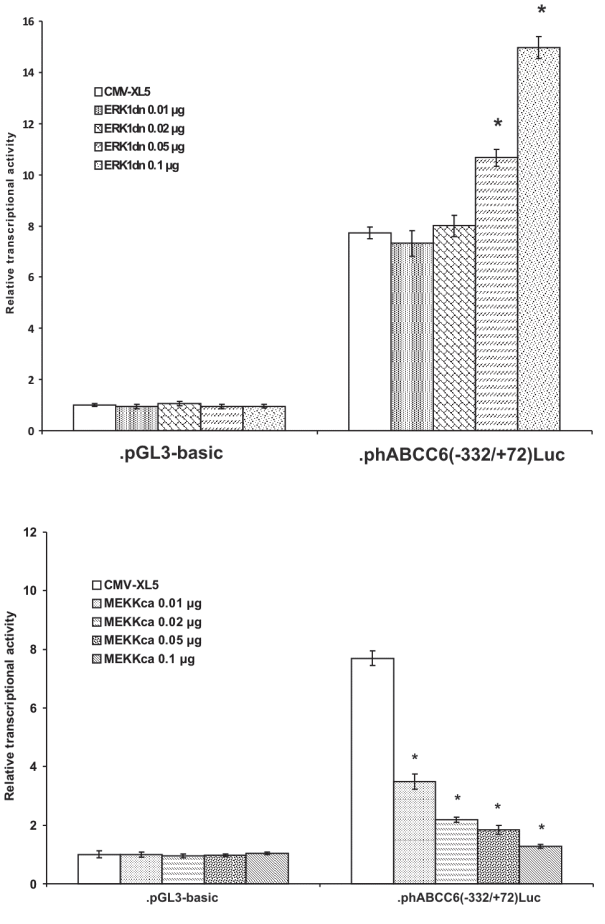



*Figure 14a. Dose-response effect of the overexpression of constitutively active MEKK1 (MEKKca) on the transcriptional activity of* ABCC6 *promoter in HepG2 cells. Results after standardization were expressed as fold induction over control value (activity of empty vector (pGL3-basic co-transfected with MEKKca or pCMV-XL5 empty expression vector), mean±SE (n=5), *p<0.05. b. Dose-response effect of the overexpression of dominant negative ERK1 (ERK1dn) on the transcriptional activity of* ABCC6 *promoter in HepG2 cells. Results after standardization were expressed as fold induction over control value (activity of empty vector (pGL3-basic) co-transfected with ERK1dn or pCMV-XL5 empty expression vector, mean±SE (n = 5), *p<0.05.*

Then we asked whether the observed effect was tissue/cell line specific or not, since it is known that *ABCC6* has tissue specific expression. In that purpose

we used HeLa cells that do not express *ABCC6* and we transfected the cells with the proximal promoter -332/+72 construct. We then performed treatments using the following protocol. Twenty-four hours after seeding HepG2 cells on a 96 well plate, we transfected them with the constructs of interest (here the -332/+72 construct). After another 24h we performed treatment with the same concentration of molecules than previously. With this protocol we performed various treatments activating the ERK1/2 cascade. We did not observe any decrease or increase in the luciferase activity after the treatments. Our results clearly demonstrated that the ERK1/2 response is tissue specific since no inhibitory effect was observed after treatment with PMA, HGF, EGF or U0126 on the HeLa model cell line (Figure 15).

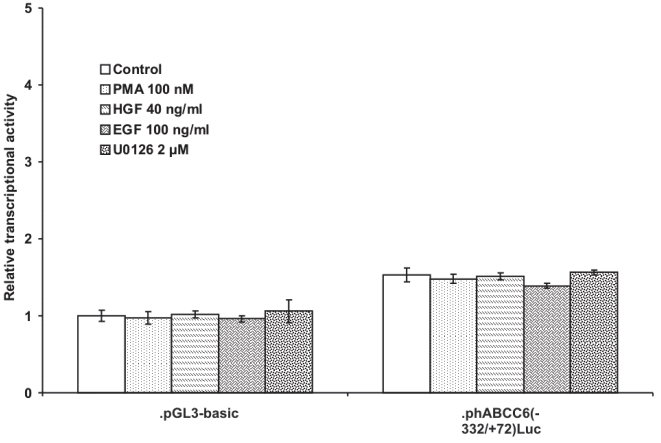



*Figure 15. the ERK1/2 response is cell type specific. Wild-type ABCC6 luciferase construct was treated with PMA, HGF, EGF, U0126 or vehicle (DMSO) at the indicated concentrations for 24hours in HeLa cells. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of empty vector (pGL3-basic) in control treated cells), mean±SE (n = 5).*

Altogether we showed in these experiments that the ERK1/2 inhibits *ABCC6* at the transcription initiation level. Also we demonstrated that the ERK1/2 dependent response element is located in the proximal promoter of the *ABCC6* gene and that this element exhibits tissue specificity.

### 4.1.8 **Localization of the ERK1/2 response element in the proximal promoter of *ABCC6*.**

In our further experiments we wished to locate the ERK1/2 response element in the *ABCC6* gene, using previously characterized constructs that map the proximal promoter of the gene (Figure 16).

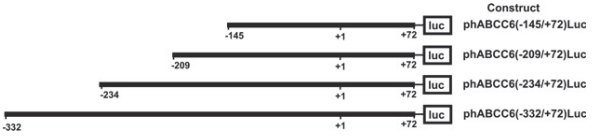



*Figure 16. Reporter plasmids containing serial deletions of the human ABCC6 gene promoter. Numbering starts at the translation start site.*

As previously observed the -145/+72 construct showed a low basal promoter activity, while the -209/+72 was slightly more active and the -234/+72 construct harbored a strong activatory element.

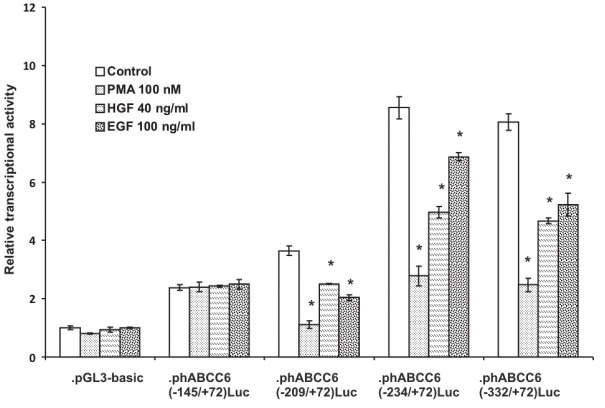



*Figure 17. The ERK1/2 cascade element is located in the proximal promoter of* ABCC6 *gene between -209 and -145 bp. Different promoter constructs were transiently transfected to HepG2 cells. 24 hour after the transfection cells were treated with PMA or EGF or HGF at the indicated concentrations or vehicle. Cells were treated with the compounds for 24 hours and promoter activity was then measured by luciferase assay. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of empty vector (pGL3-basic) in control treated cells), mean±SE (n = 5), *p<0.05.*

We then performed treatments using the same conditions than previously, and we tested the effect of HGF, PMA and EGF for their ability to inhibit *ABCC6* expression at the promoter level (Figure 17). We observed similar inhibitory effects on the constructs activity for all constructs extending in the 5' region of the promoter beyond position -145 (50%, 70% and 30% inhibition respectively). In conclusion, we identified the ERK1/2 dependent regulatory region in the *ABCC6* proximal promoter between -209 and -145bp from the translation initiation site.

Using luciferase assay we previously demonstrated the crucial role of the -209/-145bp region in the regulation of *ABCC6*. This region contains two PLAG like (one PLAG1 and one PLAGL1) transcription factor binding sites that we previously showed to play a role in the up-regulation of *ABCC6*. Moreover, we demonstrated that this region is responsible for the tissue specific regulation of *ABCC6* since mutations within the region, between the two PLAG binding sequences, led to a total loss of activity of the proximal promoter construct, in a tissue specific manner. Indeed the increased activity of the construct containing the -209/-145 region in comparison with the -145/+72 region, was observed only in the *ABCC6* expressing cell lines Hep3B and HepG2. In contrast, no difference in the luciferase activity was detectable between the different constructs in the A549 and the HEK193 cell lines that do not express *ABCC6*. Similarly, mutation between the two PLAG consensuses led to a loss of the luciferase activity in the HepG2 and Hep3B cell lines. We interpreted this effect as the PLAG factor stabilizing an unidentified transcription factor responsible for the tissue specific expression of the *ABCC6* gene. (Ratajewski, de Boussac et al. 2009).

Our experiments presented here described a tissue specific response element dependent on the ERK1/2 cascade that is located in the same 60bp region than the unidentified tissue specific factor. We hypothesized that the two factors identified in the two studies might be the same, therefore, we performed more detailed *in silico* analysis of the -209/-145 region. Several independent lines of evidence suggested that the hepatocyte nuclear factor 4α (HNF4α) might be a good candidate for the unidentified liver-specific factor. In order to examine if

the ERK1/2 cascade is exerting its activity via this site, we made use of constructs previously used of the proximal promoter region with specific mutation inside the HNF4alpha putative binding site, or in the PLAG1 binding site located next to the HNF4alpha element.

The construct m1(-332/+72) contains a mutated PLAG site, while the construct m3(-332/+72) harbors a stretch of mutation between -155 and -158 (Figure 18).

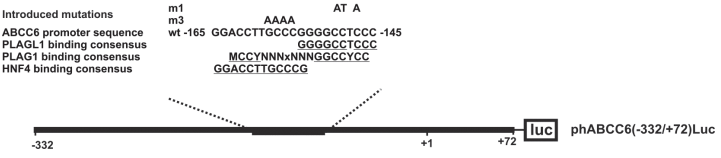



*Figure 18. Reporter plasmids containing serial deletions of the human* ABCC6 *gene promoter. Numbering starts at the translation start site.*

We performed treatment on -332/+72, m1 or m3 transfected HepG2 cells with ERK1/2 cascade modulators HGF, PMA, EGF, and U0126. Interestingly, the -332/+72 and the m1 constructs reacted in a similar way to the ERK1/2 modulators in the HepG2 cells even if the m1 construct had a decreased luciferase activity by approximately 20% in comparison to the wild type construct. In contrast, an activity similar to the -145/+72 construct, which was not responsive to the ERK1/2 cascade, could be detected with the m3 transfected HepG2 cells showing the essential role of the HNF4alpha biding site in the ERK1/2 inhibition of *ABCC6* expression (Figure 19).

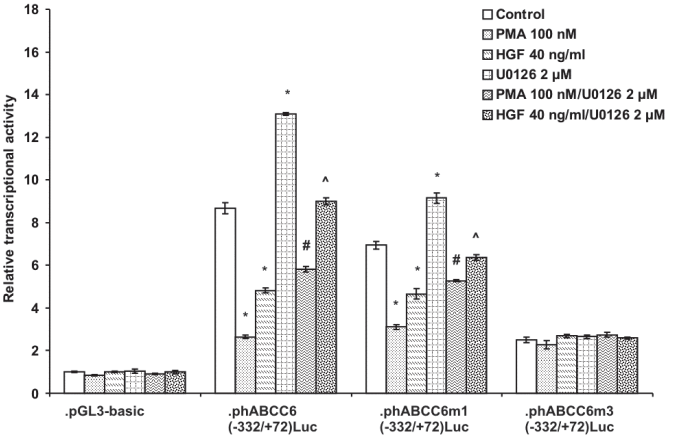



*Figure 19. A mutation in a HNF4alpha binding consensus in the proximal promoter of the* ABCC6 *gene prevent the ERK1/2 inhibitory effect. Different promoter constructs (wt, m1, m3) were transiently transfected to HepG2 cells. 24 hour after the transfection cells were treated with PMA or EGF or HGF at the indicated concentrations or vehicle. Cells were treated with the compounds for 24 hours and promoter activity was then measured by luciferase assay. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of empty vector (pGL3-basic) in control treated cells), mean±SE (n = 5), *p<0.05.*

### 4.1.9 **The ERK1/2 cascade inhibits the HNF4a induction of *ABCC6* expression.**

Finally, we tested the hypotheses that HNF4alpha is responsible for the *ABCC6* tissue specific expression, and that the ERK1/2 cascade modulates this transcription factor. It is known from the literature that HeLa cell line is expressing neither the HNF4alpha transcription factor nor the *ABCC6* gene (Sladek, Zhong et al. 1990). Therefore, we transiently transfected HeLa cells with the HNF4alpha transcription factor, and with the wild type -332/+72, the m1 or the m3 *ABCC6* proximal promoter constructs. We observed no induction of the luciferase activity after transfection of the wild type promoter construct alone (neither with m1 or m3), In contrast, and as we predicted, the HNF4alpha transcription factor increased the wild type proximal promoter luciferase activity to a similar level that the wild type proximal promoter activity in HepG2 cells (Figure 20), showing that HNF4alpha is up-regulating the expression of *ABCC6* by acting on the proximal promoter of the gene.

Moreover a similar (but reduced of about 20% compared to the wild type) induction was observed when HNF4alpha was co-expressed with the m1 proximal promoter construct (mutated at the PLAG site) showing that the PLAG site is not responsible for the HNF4alpha induction. Finally, co-expression of the HNF4alpha transcription factor, and the m3 construct (mutated at the HNF4alpha binding site) in HeLa cells did not lead to any variation of the luciferase activity compared to the control. Therefore we concluded that HNF4alpha has an important role in the *ABCC6* up-regulation.

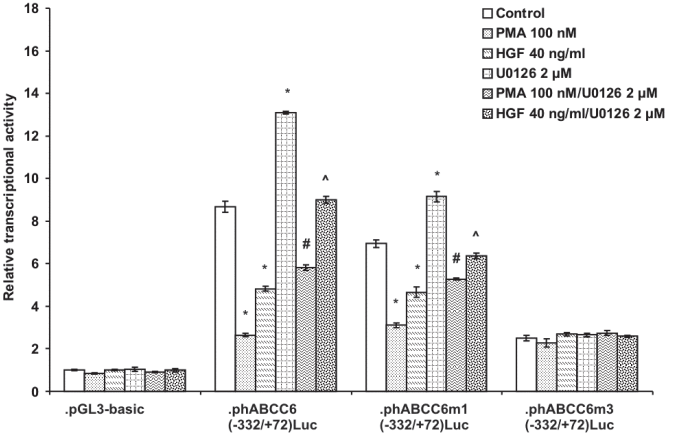



*Figure 20. HNF4a upregulates* ABCC6 *expression in an ERK1/2 dependent manner and confers tissue-specificity. Luciferase reporter gene assay in HeLa cells. Wild-type, m1 and m3 constructs were co-transfected with HNF4α, MEKKca or both or pCMV-XL5 empty expression vector. Results after standardization were expressed as fold induction over control value (activity of empty vector (pGL3-basic co-transfected with the corresponding expression vectors), mean±SE (n = 5), *p<0.05 – statistically different from cells transfected with control empty vector (CMV-XL5), #p<0.05 – statistically different from cells transfected with HNF4α expression vector.*

Next we wanted to observe in HeLa cells if ERK1/2 modulates HNF4alpha and consequently *ABCC6* gene expression. For this purpose, we co-transfected HNF4alpha, the proximal promoter construct, and the MEKKca that constitutively activates the ERK1/2 cascade. Our hypothesis was that the ERK1/2 cascade down-regulates the *ABCC6* gene expression via the HNF4alpha modulation. In such a case the co-transfection of MEKK1ca and HNF4alpha with the proximal promoter construct would lead to a decrease in

the luciferase activity compared to the HNF4alpha co-transfected alone with the proximal promoter. As expected, we observed that the constitutive activation of the ERK1/2 cascade by MEKK1ca in HeLa cells was sufficient to totally prevent HNF4alpha induction of *ABCC6* (Figure 20). This last result showed that the ERK1/2 cascade activation down-modulates the HNF4alpha transcription factor dependent regulation of *ABCC6*.

To prove the binding of HNF4alpha to the *ABCC6* proximal promoter in its chromatin environment, we performed chromatin immunoprecipitation (ChIP) (the ChIP experiment was performed in Lodz by Iwona Sachrajda). By investigating different conditions using HGF and PMA treatments, we also tested the ERK1/2 dependent binding of HNF4alpha to the promoter (Figure 21).

| Treatment | Gene promoter | Antibody | Control IgG | Anti-HNF4 |
|---|---|---|---|---|
| Control | *ABCC6* | | 2.97±0.12 | 19.97±0.49 |
| | *GAPDH* | | 1.26±0.32 | 1.38±0.31 |
| PMA 100 nM | *ABCC6* | | 3.14±0.09 | 10.72±1.02* |
| | *GAPDH* | | 1.07±0.34 | 1.39±0.26 |
| HGF 40 ng/ml | *ABCC6* | | 2.96±0.10 | 13.56±0.22* |
| | *GAPDH* | | 1.17±0.31 | 1.56±0.18 |

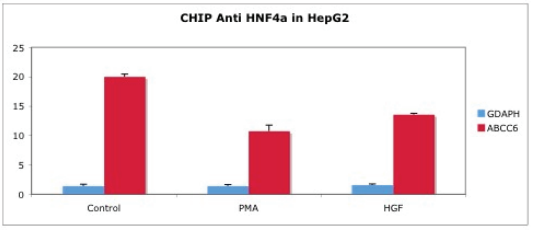



*Figure 21. Chromatin immunoprecipitation of HNF4a show the ERK1/2 dependent binding of the factor to the* ABCC6 *promoter in HepG2 cells. HepG2 cells were treated with PMA or HGF at the indicated concentrations or vehicle for 24 hours. Amount of immunoprecipitated cognate gene promoter sequences was determined by real-time PCR and expressed in arbitrary relative values, standardized against amount of input material (means±SEM., n=4). *statistically significant at p<0.05.*

As we suspected, HNF4alpha bound to the *ABCC6* promoter in control condition. In contrast, and as our previous results suggested, treatment with HGF or PMA, leading to the ERK1/2 cascade activation, reduced dramatically

(30-50%) the HNF4alpha bound protein on the *ABCC6* promoter, while no binding differences could be observed for the *GAPDH* promoter used as control. This final experiment pointed out the unique role of HNF4alpha in the regulation of *ABCC6*, and showed that HNF4alpha binding to the promoter is under the negative modulation of the ERK1/2 cascade.

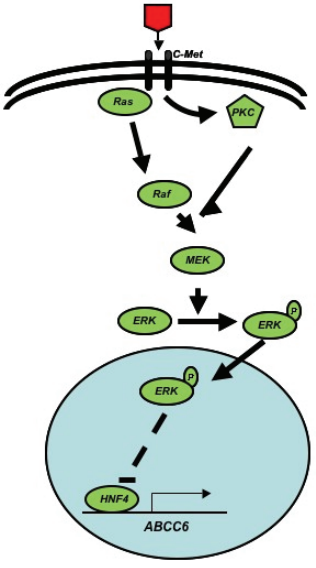



*Figure 22. Schematic presentation of* ABCC6 *expression regulation by the ERK1/2 pathway. ERK1/2 kinase pathway is represented in circles, as well as the HNF4α transcription factor. Arrows indicate positive regulations.*

In conclusion, we demonstrated that the ERK1/2 cascade down-regulates the expression of *ABCC6* at the transcription initiation level. We first identified the ERK1/2 response element in the proximal promoter of the gene and we demonstrated that this element is tissue sensitive. Next we showed that the HNF4alpha transcription factor can bind to that sequence, and activate the transcription of the human *ABCC6* gene, therefore demonstrating that HNF4alpha is a master regulator of the *ABCC6* gene. Finally we revealed that the ERK1/2 cascade negatively modulates the HNF4alpha transcription factor leading to the down regulation of the *ABCC6* gene expression (Figure 22).

## 4.2 Identification of an enhancer in the first intron of the *ABCC6* gene

### 4.2.1 DNase I technique

The essential role of HNF4alpha in the regulation of the *ABCC6* gene expression was uncovered by the study of the proximal promoter of *ABCC6*. We simultaneously completed the transcriptional regulation studies of *ABCC6* by using a different approach as well.

DNase I Hypersensitivity assay (DHA) reveals the regulatory elements as enhancers and active promoter regions by preferential digestion of open chromatin loci (Eissenberg, Cartwright et al. 1985). The major advantage of this technique is that it identifies the presence of regulatory elements in a hypothesis free context by screening a large genomic DNA (gDNA) fragment. The DNase I will first digest sensitive region of the DNA, therefore the principle is to perform a dose dependent partial digestion of the gDNA and then to reveal, using Southern blot or qPCR techniques, the appearance of different hypersensitive sites (Cai, Struk et al.).

We decided to apply this technique to the *ABCC6* promoter. *ABCC6* is in a head to head orientation with the *NOMO3* gene and they are separated by a 10 kilobase pairs (kb) region containing both proximal promoters (Figure 23). Since it has been shown that majority of the HS are located within 10 kb around the transcription initiation site (Sabo, Humbert et al. 2004) we focused on that 10kb intergenic region.

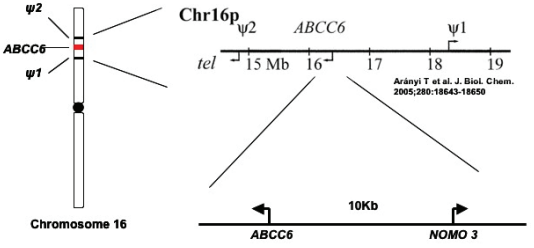



*Figure 23. Chromosomal localization of the* ABCC6 *gene.*

*ABCC6* shares more than 99% identity with its two pseudogenes in the promoter region. Our strategy was to segregate the two pseudogenes and the *ABCC6* gene by using restriction enzyme site differences between the two *ABCC6*-pseudogenes and the *ABCC6* gene promoter. More precisely, by using a probe located between the two restriction enzyme sites and in the region common to the three *ABCC6* like promoters, we would be able to segregate the pseudogenes to the *ABCC6* gene by performing double restriction enzyme digestion on the gDNA (if the pseudogenes probe is between both restriction enzymes sites), and in order to find the restriction enzymes we performed an *in silico* screen of the promoter region of *ABCC6* and the two pseudogenes (Psi1 and Psi2)

We found that the BglII restriction enzyme has a different pattern of digestion for the *ABCC6* gene and the two pseudogenes in the region of interest. Indeed the two pseudogenes contain one additional BglII restriction site in comparison with the *ABCC6* gene (Figure 24). We also located a NcoI digestion site present in all three promoters at 150bp from the BglII sites. Thus theoretically, after gDNA digestion by the two restriction enzymes and Southern blot with a specific probe recognizing the 150bp region located between the NcoI and the BglII restriction enzyme sites, we would observe a 5.6kb band representing specifically the *ABCC6* gene fragment (Figure 24).

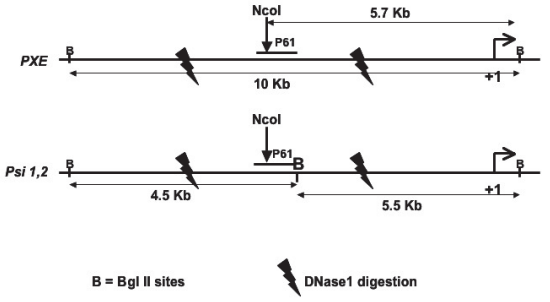



*Figure 24. Mapping of the* NOMO3 – ABCC6 *intergenic region with the BglII restriction enzyme sites and the P61 probe.*

The probe design was a challenge. Indeed the 5' upstream sequence of the *ABCC6* gene contains highly repeated sequences. Then, considering the localization restrictions due to the restriction enzyme sites and these repeated elements, we needed to limit the probe (called P61) size to a 196 bp length, and moreover, we were not able to design the probe without the NcoI restriction site, that is located at 30bp from the 5' end of P61 (figure 24).

All experiments were carried out on the *ABCC6* expressing cell line HepG2 if not specified. Please note that weak signals observed on films are very difficult to see on film photography. In our pilot experiments we first tried BglII and NcoI enzymes alone to see whether we were able to observe any hypersensitive sites. Thus we first used only the BglII digestion, with this enzyme alone we knew that a hypothetical HS site observable after Southern blot with a size of less than 4.5kb could have for origin the *ABCC6* gene or the two pseudogenes. Indeed, with this example 2 HS sites could be present on the PXE gene around the P61 probe, and then the Southern blot would reveal the fragment, while only one HS site would be necessary for the two pseudogenes. Therefore we wouldn't be able to locate and segregate the different HS sites (Figure 25). However we simply wanted to see if we were able to detect any HS sites. Southern blot performed after digestion with the BglII restriction enzymes did not reveal any clear hypersensitive site (Cai, Struk et al.) band(s).

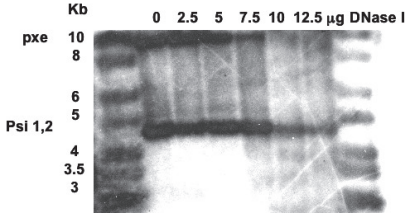



*Figure 25. DNase I digestion of HepG2 gDNA in the HepG2 cell line. Whole cells were submitted to a DNase I partial digestion during 3 minutes with 0; 2.5; 5; 7.5; 10 or 12.5 µg DNase I. gDNA was extracted and 20ug were digested with the BglII restriction enzyme. Southern blot with the P61 probe revealed a 10kb (*ABCC6*) and a 4.5kb (*ABCC6-like*) fragment.*

In the next pilot experiment we tested the NcoI digestion alone which releases a 7.2kb fragment that does not discriminate between the 3 *ABCC6*-like genes.

We assumed that if we can detect any HS with this strategy, we would next perform the double BglII/NcoI digestion to analyze the PXE gene alone. With this strategy we were able to identify a HS site at 5kb (Figure 26).

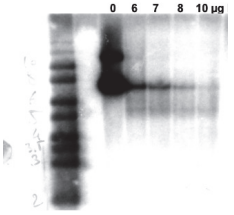



*Figure 26. Southern Blot revealed a hypersensitive site at 5.5kb in the HepG2 cell line. Whole cells were submitted to a DNase I partial digestion during 3 minutes with 0; 6; 7; 8 or 10 µg DNase I. gDNA was extracted and 20ug were digested with the NcoI restriction enzyme. Southern blot with the P61 probe revealed a 7.2kb (*ABCC6*), a 11kb (*NOMO3*) fragment and a 5.5kb hypersensitive site.*

Surprisingly, we also observed a higher band around 12kb. We thought when we designed the P61 probe that the 30bp part located on the 5' part of P61 wouldn't be sufficient to reveal a fragment by Southern blot. That 12kb extra band observed showed us that unexpectedly we were wrong. Thus, after NcoI digestion two regions were visible: the 11.7kb region on the 5' of P61 and the 7.2kb region on the 3' part of the P61. The analysis of the identified HS site at 5kb was then complicated. Indeed the HS site could be located either in the first intron of *NOMO3* if it was revealed by the 30bp in the 5'end of P61, as well as in the proximal promoter of *ABCC6* if it was revealed by the 170bp in the 3' part of P61 (Figure 27).

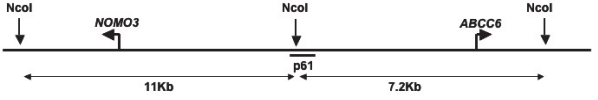



*Figure 27. Mapping of the* NOMO3 – ABCC6 *intergenic region with the NcoI restriction enzyme sites and the P61 probe.*

Considering the experimental challenges, we decided to further analyze the 5' part of the *ABCC6* gene without discriminating the three *ABCC6* like genes. After the identification of regulatory region our strategy included the following analysis of the different HS sites by Luciferase assay by cloning the

PXE promoter. Then Luciferase assay investigation would give us leads to identified transcriptional regulatory elements. The high identity between the three *ABCC6*-like genes was anyway preventing us from analyzing the *ABCC6* gene alone in a simple way.

### 4.2.2 DNase I assay revealed an active genomic region

In order to precisely map the HS sites observed in the preliminary experiment, we performed new *in silico* analysis of the region containing both NcoI HS sites previously observed, and we selected the BsmI restriction enzyme. BsmI cuts in the first intron of the *NOMO3* gene and in the second intron of the *ABCC6* gene, and liberates a 17kb fragment. We then designed a new probe P62 recognizing the 3' end of the first intron of *ABCC6*, to reveal this fragment by Southern blot. The P62 probe location in the first intron of the gene would allow us to screen *ABCC6* region with high accuracy (Figure 28).

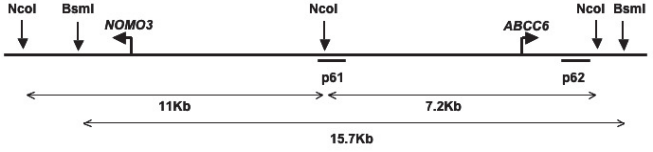



*Figure 28. Mapping of the* NOMO3 – ABCC6 *intergenic region with the BsmI and the NcoI restriction enzyme sites and the P62 probe.*

We performed Southern blot using these experimental conditions, and we observed three HS sites with bands at 11kb, 2.2kb and 1.8kb (Figure 29). We located the HS site at 11kb inside the first intron of *NOMO3* and the two other sites close to the translation initiation site of *ABCC6*.

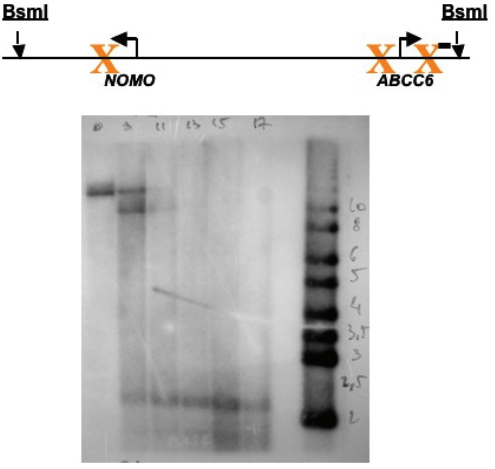
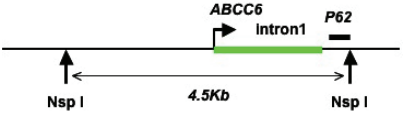



*Figure 29. Southern Blot revealed three hypersensitive sites at 11, 2.2 and 1.8Kb from the P62 probe in the HepG2 cell line. Whole cells were submitted to a DNase I partial digestion during 3 minutes with 0; 9; 11; 13; 15 or 17 µg DNase I. gDNA was extracted and 20ug were digested with the BsmI restriction enzyme. Southern blot with the P62 probe revealed a HS band at 11kb from P62 in the first intron of* NOMO3 *gene and two HS at 2.2 and 1.8kb from P62, in the proximal promoter and the first intron of the* ABCC6 *gene.*

Next we decided to investigate further these two HS sites located close to the translation initiation site (ATG) of *ABCC6*. In that purpose we found by in silico analysis another restriction enzyme (NspI) that allowed us to narrow down the screened region to a 4.7kb DNA fragment containing the first intron and the proximal promoter of *ABCC6*. DHA analyzed by this higher resolution Southern blot showed that the two HS sites previously identified around the ATG of *ABCC6* were in fact three different HS sites, represented in the Southern blot by bands at 2.2kb, 1.7kb and 1.3kb. We named the HS sites from 5' to 3', HS1 to HS3 (Figure 30).



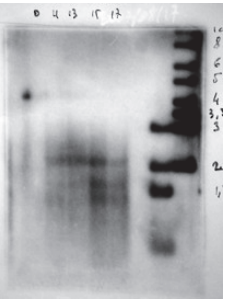


*Figure 30. Southern Blot revealed three hypersensitive sites at 2.2, 1.7 and 1.3Kb from the P62 probe in the HepG2 cell line. Whole cells were submitted to a DNase I partial digestion during 3 minutes with 0; 11; 13; 15 or 17 µg DNase I. gDNA was extracted and 20ug were digested with the NspI restriction enzyme. Southern blot with the P62 probe revealed three HS sites close to the ATG of the* ABCC6 *gene. One in the proximal promoter (HS1, band at 2.2kb) and two in the first intron of the gene (HS2 band at 1.7, and HS3 band at 1.3kb).*

Both the DNase digestion and our Southern blot conditions have a resolution of 150bp thus we located HS1 in the proximal promoter of the *ABCC6* gene between -200bp and -50bp, HS2 in the first intron of *ABCC6* between +310 and +460bp and HS3 as well in the first intron of *ABCC6* but HS3 between +600 and +750bp (Figure 31).

**GCATGT**ACACAATGGTGTACGTTCATTGTGATATTATGATATCCATAGGGTATTACAAATAATAGCAC
AGGGTGTACCCCCACTGTGATATTAGGAGTCATATCTTTCTGGGAGGTCACAGCGTGTACACGCATGGT
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TTTCACCTCCCGTGGCCTCACTCCCGCCGCGCAGCTGGACCTTGCCCGGGGCCTCCCGATCCCGGAGCTC
GAATCCCAGCCGGACCAGCCCAGCCCGACCAGCCCAGCCCCGCCTCTTCTCCGGCAGGATCGCGGCCGA
GCAGTCTGCCCAGAGACTTAGCGACAGACAGACGCTGGGACCCACGACGACAGAAGGCGC*CCGATG*GCC
GCGCCTGCTGAGCCCTGCGCGGGGCAGGGGGTGAGTGCCCCCCATCGCGCCCC*ACTCTCCTTTTCCAGGC*
*TT*GGTTTGGCTGCAGATCTCTCGGGCTGC*GATACTGGGGAGAGGAGAGA*CCCCCAAATTCCTGGACCCGG

GAAGCGAGGAGACTTACTCCGGCCCCCTCACTGCAGCGGGTTGGATTTCTTTCACCAAGCCAGCAGCCG
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*G*TTTTCCCCCTTTT*ACAGGGAAGGAAACTGAGAT*ACAGAGAGGTTAAAGTTTGCACAGCCGGTGAGTTGTG
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CGGGGCTACCTCCGGATGTCCCCACTCTTCAAAGCCAAGATGGTAGCTGCCATCCCTGGGAGCCTGGAA
CCAGGCAATGTTCGGGGGAGGCAGGGGACAGGCTGGAACCTGGTGAAGTCTTAAAGTAGACTCCTCCT
ATCGGGGTGTAGAAGGGAATCTGTTAATCAAACAGAGCAATATTAGAAAGGCTACAGAGGTCAACTCA
GTGGAACACGGTTCTCCCAAACAGATTTTGTAATTCCGAAAATCCAC**GCATGC**

*Figure 31. Genomic sequence of* ABCC6 *with the localization of HS1, HS2 and HS3.*
***GCATGT/GCATGC: NspI site; GAAC: exons;*** *TGTTTTCA: Hypersensitive sites*

### 4.2.3 Hypersensitive sites are tissue-specific

As *ABCC6* has tissue specific expression, we expected that we would be able to observe tissue specificity for those three HS sites. We used in that purpose three other human cell line models selected for their level of expression of the *ABCC6* gene. The Caco2 cell line is a colorectal adenocarcinoma cell line. This cell line is used as a model of intestinal epithelial cell. It is well known that when these cells reach confluency a differentiation process starts (Singh, Dai et al. 1996). We first demonstrated that during this process, the level of *ABCC6* expression increased by 2 to 4 times reaching the same level of *ABCC6* expression observed in the HepG2 cell line. We therefore decided to use this cell line as a model for the *ABCC6* expression regulation studies.

HEK293 cells are originating from an embryonic kidney. HeLa is an adenocarcinoma cell line. Previously we used HEK293 cells as a model (Aranyi, Ratajewski et al. 2005) but in that previous study we confirmed expression of the *ABCC6* gene by PCR, while here we analyzed its expression by quantitive PCR showing more precise results. As expected, HeLa did not express *ABCC6* at a detectable level. More surprisingly similar results were

obtained with the HEK293 cell line, while we expected this kidney cell line to express the gene (not shown). We decided to use these cell lines as negative control.

DHA performed on those cell lines showed interesting results. Indeed, while HS1, HS2 and HS3 were detected after Southern blot for the HepG2 and the Caco2 cell lines, no hypersensitive sites were observable for HeLa and HEK. We therefore performed a BsmI digestion followed by a Southern blot with the P62 in order to confirm the digestion by the DNase I for these two cell lines. Again we did not observe any HS near by the *ABCC6* ATG while the NOMO3 HS was visible, indicating that HS1, HS2 and HS3 are tissue specific (Figure 32). These data suggest a link between the observed HS sites and the *ABCC6* expression.

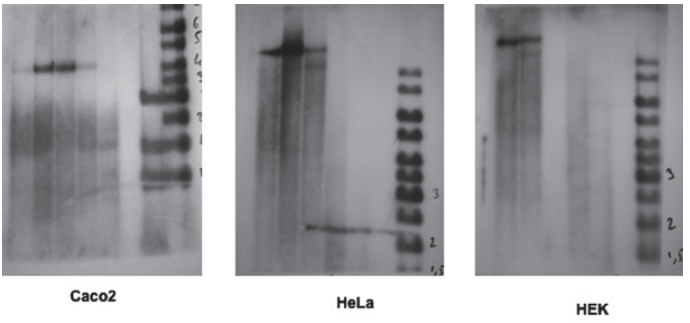


*Figure 32. The three HS sites are tissue specific. Whole cells were submitted to a DNase I partial digestion during 3 minutes with 0; 5; 10; 15 or 20 µg DNase I in the case of Caco2 cells, 0; 15; 20; 25 or 30 µg DNase I in the case of HeLa and HEK293 cells. gDNA was extracted and 20ug were digested with the NspI restriction enzyme for the Caco2 cells, and BsmI for HeLa and HEK293. Southern blot with the P62 probe revealed HS1, HS2 and HS3 for the Caco2 cell line, and do not reveal HS sites nearby the ATG of* ABCC6 *for the HeLa and HEK293 cell lines.*

### 4.2.4 Mapping of the HS sites in the proximal promoter and the first intron.

In the following experiment we investigated the functionality of the different HS sites and more specifically we focused on the two intronic HS sites by luciferase assay. Indeed we assumed from the localization of the HS1 in the proximal promoter that it marks the binding of HNF4alpha. We mapped by

luciferase assay the different HS sites, with constructs starting at -332 and progressively truncated in the 3' ends from +960 as follows: -332/+960; -332/+817; -332/+716; -332/+688; -332/+629; -332/+513 (Figure 33).

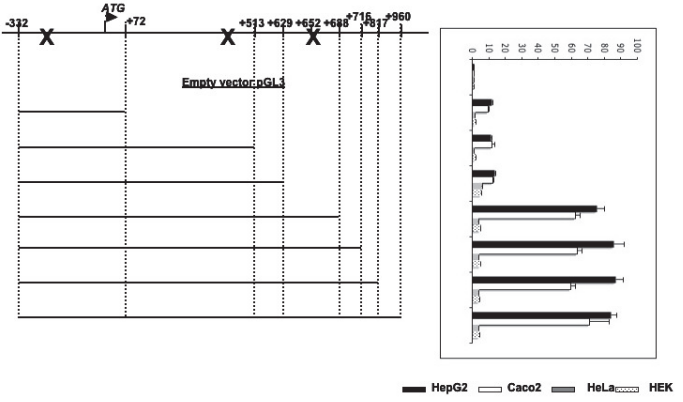


*Figure 33. HS3 is located between +629 and +688 from the ATG of* ABCC6. *Different luciferase constructs were transiently transfected to HepG2, Caco2, HEK293 or HeLa cells. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of the empty vector pGL3-basic), mean±SE (n = 5), *p<0.05*

Interestingly the activity of -332/+960 was approximately 8 times higher than the activity of the proximal promoter -332/+72. However an important loss of luciferase activity was observed with the -332/+629 construct, while the -332/+688 construct still demonstrated a similar activity to the -332/+960 construct. Finally no loss of activity was observed between the -332/+629 and the -332/+513 constructs, we then located the HS2 between +72/+513bp (Figure 33). These results suggested the localization of the different HS sites. Thus we confirmed the location of the HS2 between +310 and +460 since no loss of activity was observed 5' of the position +629. Moreover we revealed the important transcriptional induction role of HS3, overlapping with the region between +629 and +688bp.

### 4.2.5 **The intronic hypersensitive sites act as enhancers**

Next we wished to study in more details the mechanism of action of the intronic HS elements, and whether the intronic HS sites uncovered an

alternative second promoter or enhancer. We assumed that if the intronic hypersensitive sites act as strong alternative promoters, we should detect a similar activity between constructs that contained the intronic hypersensitive sites of *ABCC6* without the proximal promoter and the -332/+960 construct. Inversely if the intronic hypersensitive sites act as enhancers, the activity of these constructs should be close to the empty vector. Thus we designed the following constructs containing the HS2 site alone (+72/+629), the HS2 and HS3 sites together (+72/+688) or the HS3 alone (+479/+960) (Figure 34). We measured the luciferase activity of the constructs. No luciferase activity could be detected with any of the tested constructs in contrast to the -332/+72 construct containing the proximal promoter of *ABCC6*. We therefore concluded that the two intronic hypersensitive sites act as enhancers in the *ABCC6* gene.

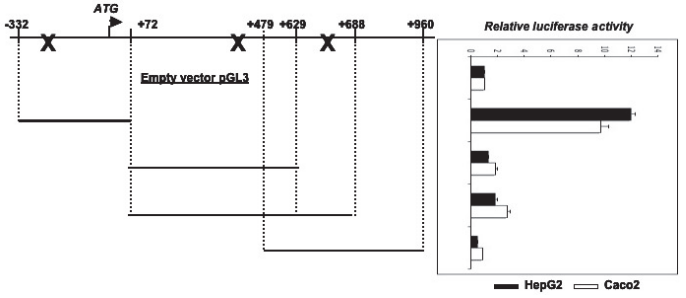


*Figure 34. HS2 and HS3 are acting as enhancers and are not marking a second promoter of the* ABCC6 *gene. Different luciferase constructs were transiently transfected to HepG2 and Caco2 cells. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of the empty vector pGL3-basic), mean±SE (n = 5), *p<0.05*

We next investigated the possible connections between the proximal promoter of *ABCC6* and the intronic hypersensitive sites. First we examined the HS2 site. As expected, the luciferase assay showed the same activity of the -145/+72 construct in HeLa and HepG2 cells and the -332/+72 constructs had higher activity in HepG2 cells (Figure 35), showing the presence of a tissue specific element in the -332/-145 region, due to the HNF4alpha binding site located around -160bp.

Interestingly, while the luciferase activity of the -332/+72 was 10 times higher in HepG2 than in HeLa, we observed comparable activity of the -332/+629 construct in both cell lines suggesting an interaction between the HS2 and the minimal -145/+72 promoter in HeLa cells (Figure 35).

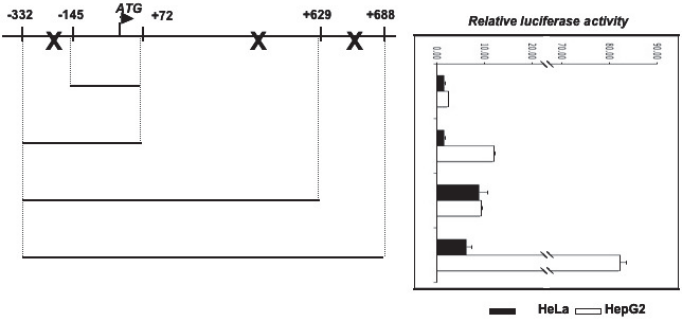


*Figure 35. HS2 is connected to the -145/+72 minimal constitutive promoter of the* ABCC6 *gene. Different luciferase constructs were transiently transfected to HepG2 and HeLa cells. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of the empty vector pGL3-basic), mean±SE (n = 5), *p<0.05*

Moreover, when we tested the activity of the following constructs, -145/+72; -145/+629 and compared them with the -332/+72; -332/+629 constructs in HepG2 and Caco2 cells (Figure 36).

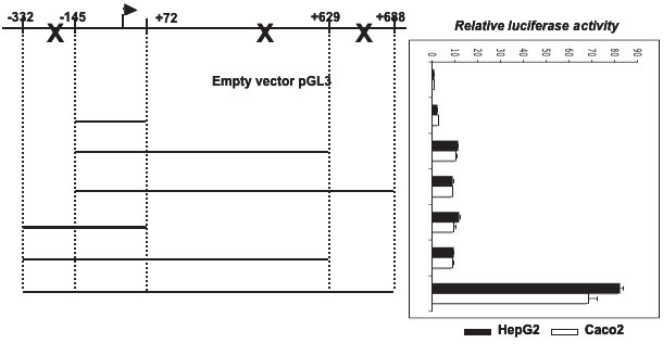


*Figure 36. HS3 is connected to the -332/-145 region of the proximal promoter of* ABCC6. *Different luciferase constructs were transiently transfected to HepG2 and Caco2 cells. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of the empty vector pGL3-basic), mean±SE (n = 5), *p<0.05*

We observed that the HS2 enhances the luciferase activity when the upstream sequence from -145 is not present since the activity of the -145/+629 construct was almost three times more important than the activity of the -145/+72 construct while no difference in the luciferase activity could be detected between the -332/+72 and the -332/+629 constructs.

Finally, these results showed that the region containing the HS2 between +72 and +629 exhibits an enhancer activity without tissue specificity. Nevertheless, the HS2 does not show any enhancer activity if the -332/-145 region is active. Finally the results suggested a connection between the minimal promoter of the gene (-145/+72) and the HS2. This last result may indicate that the HS2 takes part in the basal expression of the *ABCC6* expression.

Then we wondered about the interaction between the proximal promoter of the gene and the HS3 site. In that purpose, we compared in the HepG2 and Caco2 *ABCC6* expressing cells the constructs: -145/+72; -145/+629; -145/+688; -332/+72; -332/+629 and -332/+688 containing or not the HNF4apha binding site (Figure 36). As already shown we observed that the presence of all three hypersensitive sites together (-332/+688) greatly enhanced the luciferase activity. Indeed the activity of this construct was dramatically increased in contrast with the -145/+688 construct (Figure 36). In conclusion these results showed the important role of the HS3 on the induction of the *ABCC6* expression, and revealed the probable link between the proximal promoter of the gene and HS3.

### 4.2.6 Localization of the enhancer activity inside the 60bp of the HS3

We next wanted to clarify the organization of the HS3 site, and to identify the sites responsible for the enhancement of the promoter activity. First, we performed *in silico* analysis of the 60bp containing the HS3 site. Only few transcription factor binding sites were suggested on the sequence, and three of them were liver enriched factors (C/EBP, HNF3 and HLF) (Figure 37).


*Figure 37.* In silico *analysis of the 78bp region of the HS3. We submitted the 78bp sequence containing the 60bp region of the HS3, plus 18bp located at the 5' end of the region to different on line transcription factor screening software (P-MATCH; MatInspector; TFSEARCH). The position +652 is indicated in red.*

We first tested the different identified factors by co-expressing them with our -332/+688 construct in HeLa cells. Obviously with our current knowledge, this experiment did not work, and no transcription factor was seen as a possible candidate, but at that time we did not know about the HNF4alpha binding site on the proximal promoter of the gene and we hypothesized that in HeLa cells where those factors are not enough expressed, their co-expression with our construct will enhance the luciferase activity to a similar level than observed in HepG2.

Our next step was that we should be able to identify the factor involved by using a DNA-protein interaction screening system, and in that purpose we needed to reduce the number of factor candidates and the size of the region. Thus we designed a luciferase construct containing a truncated HS3 (-332/+652), and we compared its activity with the -332/+629 and -332/+688 constructs. To rule out one protein candidate, we designed the new construct in such a way that the HNF3 binding consensus observed by in silico analysis was cut in two parts. Thus we expected a total loss of activity for the -332/+652 construct if the enhancer was located in the +652/+688 region or was the HNF3 transcription factor. In contrast, if the enhancer protein binds to the +629/+652 region we expected a luciferase activity of the -332/+652 construct similar to the -332/+688 construct.

Surprisingly, we observed that the -332/+652 construct exhibits half of the activity observed with the -332/+688, but still 4 times more luciferase activity than the -332/+629 (Figure 38). This result can have two possible explanations: first it is possible that the +629/+688 region contains sites for multiple factors that act synergistically or additionally, or the +652 site is in

the middle of a transcription factor binding site and cuts it in two parts that can still partially bind the factor, leading to a partial decrease in the luciferase activity

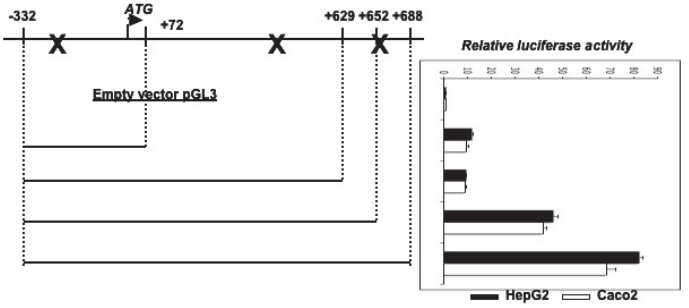


*Figure 38. HS3 mapping. Different luciferase constructs were transiently transfected to HepG2 and Caco2 cells. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of the empty vector pGL3-basic), mean±SE (n = 5), *p<0.05*

### 4.2.7 The HS3 enhancer is interacting with the -234/-209 region in the proximal promoter of the *ABCC6* gene.

In our last set of experiments we investigated the relation between the HS3 and the proximal promoter of the *ABCC6* gene. Previously we demonstrated that the HS3 was linked to the -332/-145 region in the promoter of the *ABCC6* gene, and that the proximal promoter of the gene contains a HNF4alpha binding site under the negative regulation of the ERK1/2 cascade. We hypothesized that the HS3 is linked to the HNF4alpha binding site. To confirm this hypothesis we first investigated the possible modulation of the -332/+960 luciferase activity after treatment with PMA as an inducer of the ERK1/2 kinase cascade and with U0126 as an inhibitor of this cascade. We observed a 70% inhibition after treatment with PMA, while treatment with U0126 led to an activation of approximately 160% of the -332/+960 luciferase activity (Figure 39). We previously demonstrated that the ERK1/2 cascade is inhibiting the expression of the *ABCC6* gene via a negative modulation of HNF4alpha, then this result showed that the enhancer activity of the HS3 is

modulated as well by the ERK1/2 cascade suggesting that the HS3 enhancer is linked to the HNF4alpha binding site.

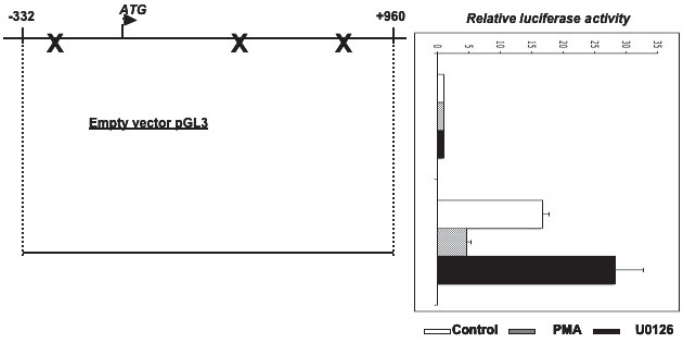


*Figure 39. The HS3 is sensitive to the ERK1/2 modulation via the proximal promoter of the ABCC6 gene. The construct containing HS1, HS2 and HS3 (-332/+960) was transiently transfected to HepG2 cells. 24 hour after the transfection cells were treated with PMA or U0126 at the indicated concentrations or vehicle. Cells were treated with the compounds for 24 hours and promoter activity was then measured by luciferase assay. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of empty vector (pGL3-basic) in control treated cells), mean±SE (n = 5), *p<0.05.*

To further confirm this link, we designed two supplementary constructs -234/+688 and -209/+688 and we compared their luciferase activity in HepG2 and Caco2 cells, with the already described -332/+68 construct. As expected the -332/+688 and the -234/+688 constructs showed a similar important activity, since all three HS sites were present. More interestingly the -209/+688 construct showed a low luciferase activity comparable to the -145/+688 activity observed previously (Figure 40), showing that the HS3 enhancer is interacting with the -234/-209 fragment and not with the HNF4alpha factor as previously suspected.

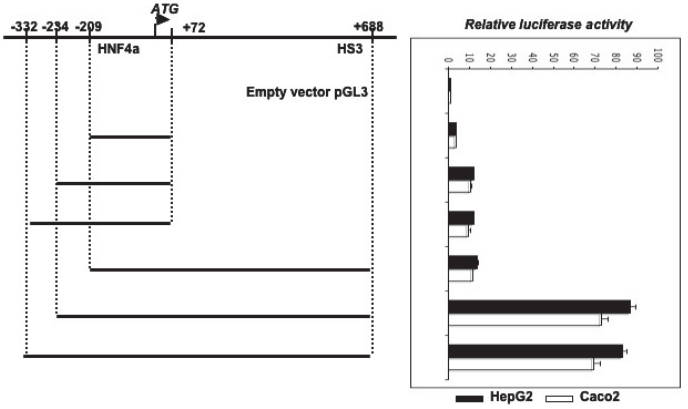


*Figure 40. HS3 is interacting with the -234/209 region of* ABCC6 *proximal promoter. Different luciferase constructs were transiently transfected to HepG2 and Caco2 cells. Results of the assay were standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of the empty vector pGL3-basic), mean±SE (n = 5), *p<0.05*

In conclusion we identified three hypersensitive sites, named HS1, HS2 and HS3, located respectively in the proximal promoter and the first intron of the *ABCC6* gene. We assumed that the HS1 marks the HNF4alpha binding site in the proximal promoter of the *ABCC6* gene. We also showed that the HS2 has a role in the basal expression of *ABCC6*, and is connected with the -145/+72 region representing the minimal proximal promoter of the gene. Last we demonstrated that these HS sites exhibit tissue specificity, and that the two intronic HS sites are acting as enhancers. Localization of the HS3 site identified a 60bp region that may contain more than one binding site for transcription factors. Finally, we demonstrated that the HS3 enhancer is connected to the -234/-209 region of the proximal promoter and has a crucial role in the enhancement of the transcription of the *ABCC6* gene in tissues expressing the gene. Moreover, we showed that the transcriptional regulation of the *ABCC6* gene is under a complex regulatory mechanism. Indeed in tissues expressing *ABCC6*, the HS3 enhances the expression of the gene and is dependent of the -234/-209 region that enhances the HNF4alpha activity (Figure 41). In contrast, in tissue non expressing *ABCC6* the HNF4alpha is

absent, and the gene expression is enhanced by the HS2, which is dependent of the -145/+72 region of the proximal promoter of the gene.

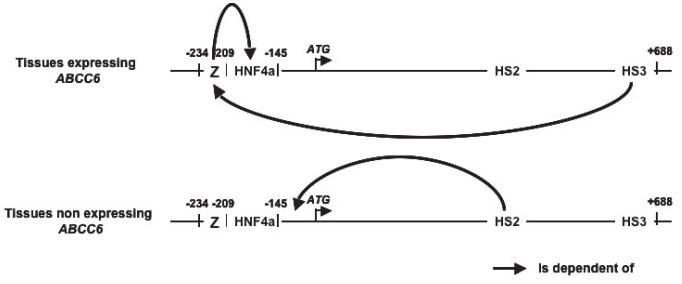


*Figure 41. Schematic presentation of the different element regulating* ABCC6 *expression and their interconnection. Arrows indicate connection/dependency between two elements.*

# 5 Discussion

## 5.1 Introduction

It has been shown that pseudoxanthoma elasticum (PXE), the Mendelian disease associated with loss-of-function mutations in the *ABCC6* gene, is a metabolic disease (Jiang, Oldenburg et al.). We think that the metabolite involved in the disease can influence the regulation of the gene. Therefore understanding the regulation of the gene, and the different intracellular processes leading to its modulation can give clues about the role of this transporter.

The goal of my PhD was to explore the regulation of the human *ABCC6* gene. First by investigating the possible physiological mechanisms leading to the regulation of *ABCC6* based on drug-screening analysis of actives molecules. With that strategy we revealed a mechanism of regulation of the *ABCC6* gene expression involving the ERK1/2 cascade and the HNF4alpha transcription factor. Second by exploring the different regulatory sites present in the natural chromatin structure by using the DNase I hypersensitive assay. This method allowed us to highlight an enhancer element in the first intron of *ABCC6*, which acts with the HNF4alpha factor and dramatically increases the expression of the gene.

## 5.2 Choice of the techniques

In the great variety of techniques options to study gene transcriptional regulation, we chose first dug screening. Indeed we consider this technique as the first step in understanding the basic regulation of a gene. In parallel we decided to investigate the possible regulatory elements present on the genomic DNA sequence of the gene by using the DNase I hypersensitive assay. The choice of this technique was made in order to screen the region in a hypothesis free context. Our idea was to obtain independent results from both techniques, in order to have a better overview of the *ABCC6* gene expression regulation.

These techniques do not give that much information by themselves and need to be completed by other studies to obtain a comprehensive picture of the gene

regulation. In that purpose we chose the luciferase assay technique. The major arguments for the choice of this technique were the multiple possibilities that allow the transfection of cells (from the simple luciferase vector transfection, to co transfection of the vector with transcription factor of interest), as well as the fact that this technique is considered as a reference technique in the study of the transcriptional regulation.

Finally, the last step of transcriptional regulation studies is usually the confirmation of the binding of the identified factor on the DNA sequence. For that two techniques are mainly used, the EMSA or the ChIP experiments, that both can reveal DNA-protein interaction. EMSA fish out the protein interaction with a selected sequence by reproducing the possible interaction in vitro, adding to the tested sequence the selected protein, and looking for a shift in the DNA migration in the case of interaction between the protein and the DNA. The other technique, the ChIP allows the confirmation of a DNA-protein interaction by immunoprecipitation of a selected protein bound to any DNA sequence where the factor is binding. Then the DNA-protein interaction is confirmed by PCR analysis of the DNA fragments co-immunoprecipitated. In our experiments we chose to perform the ChIP assay. Indeed, we believe that the results obtained with this technique are more representative of the physiological state of the cells, and ChIP has been more and more recognized during the last years as the reference technique to study protein DNA-interaction.

## 5.3 Cell lines

We first chose the in vitro system that we wanted to use for the different parts of the study. We know from the literature that the *ABCC6* gene is mainly expressed in the liver and in a lower extent in the kidney and the intestine (Kool, van der Linden et al. 1999). Therefore we started looking for relevant cellular models of these tissues, and first for a good cellular model of the hepatic tissue. We compared the two more frequently used hepatoma cell lines available in the ATCC cell catalogue, the HepG2 and the Hep3B. Both cell lines are used as liver models in different studies, but after investigating and

comparing their characteristics in the literature and on the ATCC catalogue we noticed that the Hep3B cell line is infected by the Hepatitis B virus, therefore we chose the HepG2 cells. Since *ABCC6* has also been shown to be expressed in the intestine at a low but detectable level, we looked for a cellular model of this tissue. The Caco2 cells seemed to be the perfect model since it is highly used in the literature as intestinal model for drug studies. We first demonstrated a high expression of the *ABCC6* gene in this model, and we therefore decided to use it to study *ABCC6* transcriptional regulation, showing for the first time that ABCC6 can be studied in this cell line. Finally we chose two cell lines that express *ABCC6* at very low level, the HEK and the HeLa cells. It is somehow surprising for the HEK that is originated from a kidney where *ABCC6* is normally expressed, but it is important to stress here that HEK cells are derived from an embryonic kidney where *ABCC6* is barely detectable (Beck, Dang et al. 2005), which can explain our choice.

## 5.4 HNF4alpha-ERK1/2 axis modulates *ABCC6* expression

### 5.4.1 Background

Previous reports showed that *ABCC6* transcriptional regulation was under the modulation of various factors, such as NF-kB, RXR, SP1 and PLAG (Jiang, Matsuzaki et al. 2006) (Ratajewski, Bartosz et al. 2006) (Ratajewski, Van de Ven et al. 2008) (Ratajewski, de Boussac et al. 2009). But interestingly the physiological context of this regulation has never been studied. Here we demonstrated for the first time the involvement of the ERK1/2 physiological cascade in the regulation of the *ABCC6* gene expression. Furthermore we showed that the ERK1/2 cascade is inhibiting the transcriptional activation of *ABCC6* by modulating the HNF4alpha transcription factor.

### 5.4.2 *ABCC6* expression and the ERK1/2 cascade.

Surprisingly our initial drug screening for molecules modulating *ABCC6* revealed that all the drugs tested had no, or only inhibitory effect, on the expression of the *ABCC6* gene. We interpreted that as a result of a high basal expression of *ABCC6* in the HepG2 cell line preventing us to find any inducers of the *ABCC6* expression (Table1). This hypothesis was later

supported by ChIP with the high basal binding of HNF4alpha on the *ABCC6* promoter (Figure 21).

By investigating the remarkable effect of the growth factors on ABCC6 expression and the effect of the different cascades activated by the binding of HGF to its receptor (You and McDonald 2008) (Kermorgant and Parker 2005), we clearly emphasized the major role of the ERK1/2 cascade in the regulation of the *ABCC6* gene expression. Several lines of evidence suggested that. First the ERK1/2 specific inhibitor U0126 induced *ABCC6* expression, while similar experiments performed with its biologically inactive stereoisomer U0124 remained ineffective. Moreover, inhibition of the *ABCC6* expression by HGF or EGF was totally prevented by co-treatment with U0126 (Figure 5 and 7). Second, the inhibitory effect of the vitamin K3, a strong oxidative stress inducer (Czaja, Liu et al. 2003), as well as other molecules inducing oxidative stress, were also partially prevented by co-treatment with U0126 (Figure 8). Thus, various stimuli leading to the activation of the ERK1/2 pathway had similar inhibitory effect on the *ABCC6* gene expression, strengthening the important role of this cascade on the *ABCC6* gene regulation. Third, we demonstrated a correlation between the ERK1/2 phosphorylation/activation and the inhibition of the *ABCC6* gene expression. Finally, co-transfection of a wild type promoter construct of *ABCC6* with a constitutively active MEKK1 mutant (MEKK1ca) reduced the luciferase activity, while a co-tranfection with a dominant negative ERK1 (ERK1dn) mutant increased it. Both factors acted in a dose dependent manner emphasizing the role of ERK1/2 in the regulation of *ABCC6* (Figure 14).

### 5.4.3 The PKCs and the ERK1/2 cascade

We also showed that the PKCs are involved in the regulation of *ABCC6*. Our results pointed out that PKC regulatory effect on the *ABCC6* gene is dependent on the ERK1/2 cascade. Indeed *ABCC6* inhibition by PMA treatments was insensitive to the specific PKC inhibitor BIMI while co-treatment of PMA and U0126 led to the prevention of the inhibitory effect (Figure 4 and 5). Also, BIMI is known to inhibit two subfamilies of the PKCs, while the third one, the atypical subfamily, is resistant to the BIMI treatment

(Reyland 2009). Moreover, it is also known that the atypical subfamily can activate the MEK1/2 cascade, by interacting with the Ras kinase, responsible for the MEK1/2 kinase phosphorylation/activation. Finally, it is known that PKC can down modulate the HNF4alpha factor. Our ChIP assay results showing a similar loss of binding of HNF4alpha on the proximal promoter of *ABCC6* after treatment with HGF or PMA suggest that PKC acts via the ERK1/2 kinase cascade. Altogether, these data strongly suggest the role of the four atypical PKC isoforms in the regulation of the *ABCC6* human gene.

### 5.4.4 ERK1/2 and oxidative stress.

The different oxidative stress inducers clearly inhibited the expression of the human *ABCC6* gene at the transcriptional level as we observed. Since there is a link between the oxidative stress and the induction of the ERK1/2 cascade (Ref. Czaja 2003) we tested the possibility that the observed *ABCC6* expression inhibition was due to ERK1/2 by performing co-treatment with the ERK1/2 specific inhibitor U0126. Our results only showed a partial prevention of the inhibition in presence of U0126 (Figure 8) suggesting that the observed effect was only partially due to the activation of the ERK1/2 cascade. That hypothesis was confirmed by performing a western blot to detect the ERK1/2 / ERK1/2-P ratio after treatment with selected compounds (Figure 9). Although we repeated this experiment, each time a high background prevented us to analyze the immunoblot by densitometry. However, this result emphasized the fact that we were not able to rule out another mechanism of inhibition of the gene expression by the oxidative stress and we did not investigate further the possible mechanisms involved. Nevertheless we also tested oxidative treatment performed on the -332/+72 construct in HepG2 cells. Surprisingly, these treatments did not inhibit the luciferase activity of the ERK1/2 sensitive -332/+72 construct (not shown) suggesting that oxidative stress inhibits the expression of the *ABCC6* gene via an alternative, unknown mechanism than the ERK1/2-HNF4alpha axis. To confirm that, a ChIP experiment screening for the binding of HNF4alpha on the proximal promoter of the gene after treatment with the oxidative stress inducers need to be done.

### 5.4.5 Erk1/2 regulates *ABCC6* via the -209/-145bp region.

After dissecting the role of the ERK1/2 cascade in this regulation, we investigated the inhibitory mechanism at the molecular level. We first proved that ERK1/2 regulates *ABCC6* expression at the transcriptional level using the alternative transcript of *ABCC6* called URG7 (which has the same 5'part as the *ABCC6* gene) (Lian, Liu et al. 2001) (Figure 10 and 11). Based on these data we performed luciferase reporter gene assay to identify and characterize the ERK1/2 response element in the *ABCC6* promoter. We observed a cell type specific response of the *ABCC6* wild type promoter to the ERK1/2 pathway activation (Figure 13 and 15). We located the ERK1/2 response element between -209 and -145bp from the translation initiation site (Figure 17). This finding was in accordance with our previous findings showing that a tissue specific factor is binding to the proximal promoter of the gene (Ratajewski, de Boussac et al. 2009). We previously demonstrated that this element is stabilized by the PLAG transcription factor and is binding to the -165/-150 region. As the m1 construct containing mutated PLAG binding site remained responsive to the ERK1/2 cascade activation, we ruled out PLAG as possible target of the ERK1/2 cascade.

### 5.4.6 Post transcriptional regulation of *ABCC6*

Cycloheximide (CHX), a protein synthesis inhibitor, was the only treatment where we observed a different *ABCC6* and *URG7* expression modulation. *ABCC6* was significantly up-regulated by CHX treatment alone, while the URG7 transcript was down-regulated (Figure 12).

It is known that post transcriptional regulations often occur on the 3'part of the mRNA, and we know that *ABCC6* and *URG7* transcript only share their 5' part (Figure 10). Since the two transcripts do not react equally following CHX treatment, we hypothesized that they have a different post-transcriptional regulation, consequence of a protein with a short turnover that binds to the ABCC6 transcript and destabilize it. Then our observation was due to the fact that cycloheximide treatment stopped the *de novo* synthesis of this protein leading to an increase in the *ABCC6* mRNA level.

Finally we also studied the half life of the transcripts and we observed an important differences between ABCC6 and URG7 since ABCC6 half life was found at approximately 6 hours while the URG7 half life was found at more than 22 hours (not shown), confirming the difference in mRNA stability of the two transcripts and their post-transcriptional regulation differences. Moreover, RTqPCR performed to measure *ABCC6* and *URG7* also revealed a 100fold difference between both transcripts, result of their mRNA stability difference. Nevertheless, even if the analysis is complicated by the post-transcriptional differences, our results suggest that *de novo* synthesis of a protein is not required for the ERK1/2 inhibition of *ABCC6*. Indeed even if CHX alone lead to a difference in the transcript stability, when we performed co-treatment of CHX and PMA, we observed similar inhibitory effect for the *URG7* and the *ABCC6* expression (see bellow the section 5.4.8).

### 5.4.7 HNF4alpha

*In silico* search performed on the -165/-150 identified region strongly suggested us that HNF4alpha might be a good candidate for binding to this sequence (Figure 18). A number of reasons gave credit to that idea. First, HNF4alpha is known to have a strong tissue specificity that overlaps with *ABCC6*'s expression pattern (Sladek, Zhong et al. 1990) (Kool, van der Linden et al. 1999). Second a recent study with HNF4alpha microarray identified a binding site at -166/-154bp of the *ABCC6* promoter, and siRNA against HNF4alpha significantly decreased the expression of the *ABCC6* gene in HepG2 cells (Bolotin, Liao et al.). Also, another group identified, by ChIP-on-chip analysis, the *ABCC6* promoter as a target for the HNF4alpha transcription factor (Odom, Zizlsperger et al. 2004). Finally, the predicted sequence shows a high degree of evolutionary conservation (14 in a block of 16 nucleotides are identical in four species) (Aranyi, Ratajewski et al. 2005) and HNF4alpha has already been proposed to have a role in the regulation of the murine *ABCC6* gene, where it acts on the same evolutionary highly conserved site (Douet, VanWart et al. 2006). All these data point to the potential role of HNF4alpha in the ERK1/2 dependent regulation of the *ABCC6* gene.

To confirm that hypothesis we performed experiments in HeLa cells that do not express the HNF4alpha transcription factor. In this cells co-transfection of the HNF4alpha transcription factor and the luciferase promoter construct resulted in a similar level of activity than observed in the HepG2 cells (Figure 20). On the contrary the introduction of a mutation in the HNF4alpha binding site destroyed the luciferase activity when co-transfected with HNF4alpha. suggesting that the HNF4alpha is responsible for the cell specific expression of the gene. Supported by these evidences, and the overlapping of *ABCC6* and HNF4 expression in the tissues we also believe that HNF4alpha is responsible for the tissue specific expression of the *ABCC6* gene.

### 5.4.8 ERK1/2 and HNF4alpha

After demonstrating that HNF4alpha is responsible for *ABCC6* tissue specific expression, we confirmed the link between HNF4 and the ERK1/2 cascade. By co-expressing HNF4alpha and MEKK1ca in HeLa cells (Figure 20), suggesting that ERK1/2 is modulating the expression of *ABCC6* via the regulation of HNF4alpha.

The result of the ChIP experiment showing a decrease in the binding of HNF4alpha after ERK1/2 activation in HepG2 cells (Figure 21) further supported the regulation of HNF4alpha by the ERK1/2 cascade. This could be interpreted as a decrease in the affinity of HNF4alpha for the promoter, or as a decrease in the HNF4alpha protein total amount, and finally it is also possible that the antibody used in this experiment cannot recognize a phosphorylated HNF4alpha, and that the transcription factor is present on the promoter but is inactivated.

It has been shown that HNF4alpha is under the control of PKA or PKC (Sun, Montana et al. 2007) (Viollet, Kahn et al. 1997), and few studies investigated the role of the ERK1/2 cascade in the regulation of HNF4alpha. Indeed, Reddy et al. and Hatzis et al. demonstrated that the HNF4alpha promoter is sensitive to the ERK1/2 cascade. The ERK1/2 activation leads to a decrease in the amount of HNF4alpha mRNA translated into a decrease in the protein amount (Reddy, Yang et al. 1999) (Hatzis, Kyrmizi et al. 2006).

In our system in HeLa cells, HNF4alpha is expressed under a CMV promoter, thus it is unlikely that the HNF4alpha is regulated by ERK1/2 at the transcriptional level. Therefore, in our case, the ERK1/2 cascade seems to modulate the activity of HNF4alpha protein directly and not to regulate its protein amount (this is in accordance with the CHX treatment results, see above 5.4.6). On the other hand, we cannot rule out the possibility of a double regulation of HNF4alpha (both the gene expression level and the protein activity level) in the HepG2 cell line where endogenous HNF4alpha is expressed.

All these elements point out the convincing hypothesis of the ERK1/2 cascade modulating the HNF4alpha activity, most probably via phosphorylation. Indeed, a study showed that phosphorylation of HNF4alpha can lead to a distinct subnuclear localization than normally observed (Ktistaki, Ktistakis et al. 1995).

### 5.4.9 HNF4alpha and other ABCC transporters

HNF4alpha has already been proposed to have a role in the regulation of the human *ABCC2* gene (Qadri, Iwahashi et al. 2006) (Qadri, Hu et al. 2009). We were not able to reproduce the results of that study, and our data do not suggest any involvement of the ERK1/2 cascade in *ABCC2* expression regulation since treatment of HepG2 cells with HGF or co-treatment with HGF and U0126 induced the expression of *ABCC2* in our hands (not shown). Moreover our data about oxidative stress confirmed the differences between the regulation of *ABCC6* and *ABCC2*. It is then possible that *ABCC6* regulation by the ERK1/2-HNF4alpha system is limited to *ABCC6*, and that other factor(s) is/are needed to stabilize HNF4alpha on the genomic DNA. This would explain the difference in expression observed at the mRNA level between *ABCC6* and *ABCC2*.

### 5.4.10 Perspectives

Under physiological circumstances in resting hepatocytes, the *ABCC6* gene expression reaches its highest level, and the ERK1/2 pathway has a low basal activity that contributes to the attachment of the cells to each other. However,

ERK1/2 is known to be activated by a multitude of signals (e.g. growth factors, G protein coupled receptors) during cell growth, and cell division. It is likely to think that *ABCC6* gene expression decreases in these conditions. Similarly, in oxidative stress conditions in hepatocytes, the ERK1/2 cascade is activated, and we showed that *ABCC6* gene expression is sensitive to that signal. This suggests that although in the absence of functional *ABCC6*, which leads to the development of PXE, chronic oxidative stress was observed in dermal fibroblast and other cell types (Pasquali-Ronchetti, Garcia-Fernandez et al. 2006); the physiological role of MRP6 may not be the protection of the cells from the oxidative stress. Since vitamin K3 strongly inhibited the expression of the gene it seems to be unlikely that vitamin K3 itself or one of its derivatives would be the most important physiological substrate of the MRP6 protein, in contrast to a current hypothesis (Borst, van de Wetering et al. 2008).

## 5.5 Identification of an enhancer in the first intron

### 5.5.1 Introduction

Transcriptional regulation is a highly complex mechanism involving multiple aspects of the regulation from DNA methylation to transcription factor binding to the promoter. Transcriptional regulation of the *ABCC6* gene has previously been studied using drug screening, luciferase assay and DNA/protein interaction detection techniques. We further analyzed the human *ABCC6* regulation by investigating the role of the chromatin environment using an alternative technique.

### 5.5.2 DNase I set up

The DNase I assay technique is an efficient tool used to reveal gene regulatory regions as active promoter or enhancer (Wu, Bingham et al. 1979). Enhancers have been shown to be located either very far from the gene they modulate, or also sometimes in the first introns of the genes (Hines, Mathis et al. 1988) (Smith, Barth et al. 1996). This last observation led us to investigate a 10kb upstream region from the ATG together with the first intron of the *ABCC6* gene. Accordingly we designed the probe (P61) and we chose the restriction

enzymes in order to be able to visualize and analyze these regions. After the observation of the first HS sites by Southern blot we designed a second probe P62 at the end of the first intron of the *ABCC6* gene to locate the identified HS (Figure 28). We had several arguments to do so. First, we wanted to locate more precisely the HS revealed with P61. Therefore the intronic region was the best choice to design a new probe, in order to be able to locate precisely the HS site located close to the *ABCC6* ATG. Furthermore, as I previously mentioned, the first intron of genes are regions where HS sites can be observed. Finally, the first intron of *ABCC6* does not contain repetitive elements.

### 5.5.3 Identification of the HS sites and significance of the HS1

By using P62 in Southern blot, we clearly observed a hypersensitive band that we were able to locate in the proximal promoter of the *ABCC6* gene around the previously identified HNF4alpha binding site, and two other HS in the first intron of *ABCC6* (Figure 30 and 31). We first considered the HS1 as a mark of open chromatin structure linked to the presence of the HNF4alpha transcription factor. Independent clues led us to that conclusion. First, HS marks active regions of promoters and we demonstrated the important role of HNF4alpha in *ABCC6* regulation (see Figure 22), the location of the HS1 is in accordance with the binding site of HNF4alpha. Moreover, we observed a tissue specific pattern of the three identified hypersensitive sites including HS1 (Figure 33). Since HNF4alpha has the same pattern of expression as HS1 this other evidence emphasized the connection between the HS1 and the HNF4alpha binding site. However, we also identified different regions of the proximal promoter that harbored transcriptional activity. We demonstrated that the -145/+72 region has a basal activity (Figure 35), while the -209/-145 region shows tissue specific activity due to the presence of the HNF4alpha transcription factor, and finally the -234/-209 region is connected to the HNF4alpha and the HS3 site (Figure 40). These new evidences made us think that the HS1 might represent the -332/-145 region activity instead of only a part of it.

### 5.5.4 Tissue specificity and HS1

The tissue specificity observed with the construct containing the three hypersensitive sites is most probably due to the presence of the HS1 in the construct. Indeed, the -145/+72 (as the -145/+629) construct does not show any tissue specific activity since it harbors an equivalent activity in different cell types (Figure 35). On the other hand construct containing the HS1 site exhibit tissue specific activity (Figure 33). It is probable that the tissue specificity observed with the construct containing the HS3 enhancer is due to the presence of the HNF4alpha factor in the proximal promoter. Nevertheless we have no data about the tissue specificity of the factor(s) binding to HS3, it is then possible that this/these factor(s) is/are more widely expressed, and that the presence of HNF4alpha determines the tissue specificity, or that these factors are tissue specific as the HNF4alpha.

### 5.5.5 Role of HS2

Investigating the two other HS sites highlighted the interesting effect of HS2. We demonstrated that HS2 seems to have a role in the basal expression of the gene. Indeed the luciferase activity of -145/+629 demonstrated an increase in comparison with the -145/+72bp construct (Figure 36). We can't clearly conclude on the role of HS2, but we assume that the factor, or the element revealed by DHA interacts with the -145/+72 region of the *ABCC6* gene, and it leads to the enhancement of the minimal promoter activity. It has been shown that Sp1 and RXR can bind to this region of the promoter (Ratajewski, Bartosz et al. 2006) (Jiang, Matsuzaki et al. 2006), therefore it is possible that the HS2 is acting synergically with one or both of these factors.

It should also be noted that the presence of the -234/ -145 region hides the effect of HS2 on the minimal promoter. Indeed, the luciferase activity of the -332/+72 construct is equivalent to the activity of the -145/+629 and the -145/+688 constructs (Figure 36). Similarly, the -209/+688 construct exhibits equivalent activity than the -145/+688 while the -234/+688 has the maximum luciferase activity (Figure 40). This observation suggests that the presence of HNF4alpha itself is not sufficient for the hiding effect of -234/-145 on HS2.

Our data indicate that the presence of the factor binding to the -234/-209 region is required for that effect.

With our current understanding we cannot explain this mechanism completely, It is possible that the recruitment of the HNF4a transcription factor, together with the unidentified factor binding to the -234/-209 region, leads to the release of the factor(s) binding on the minimal promoter, therefore preventing the enhancement of HS2 on the construct. Moreover the interaction of the -234/-209 region with HS3 may also lead to the release of the factor binding on the -145/+72 region.

### 5.5.6 Role of HS3

The most interesting hypersensitive site that we found was undoubtedly HS3. We believe that this element enhances the expression of the *ABCC6* gene, and different indications meet up with this hypothesis. First, the addition of HS3 to the two other hypersensitive sites leads to a dramatic increase in the luciferase activity if compared to the HS1 or the HS1+HS2 constructs (Figure 33). We also ruled out the possibility of the presence of a second promoter in the first intron by designing a construct containing HS3 alone, and we couldn't observe any luciferase activity (Figure 34), showing that this element cannot act on its own, and thereby it should be considered as an enhancer.

### 5.5.7 Factors binding to HS3

It is very difficult to predict the binding of a protein on a DNA sequence if this one is too long. Furthermore, the predictions of factor binding sites are not taking into account the cellular environment and the reality of the regulation. In order to identify the location of HS3 we narrowed down the functional part of this element by cloning different sizes of the region, and we identified the +629/+688bp region as the core containing the binding site (Figure 33).

We then performed *in silico* analysis of the HS3 site (Figure 37) but to be certain to do not miss the possible candidate we increased the size of our HS3 sequence. Indeed it is possible that the position +629, which marks the 5'end of the HS3 region, is part of a binding site. In such a case the -332/+629

construct would behave as a mutated construct of this hypothetical site. Inversely the -332/+688 construct harbored a full activity showing that any potential binding sites are located 5' of the +688 position. Consequently, for the *in silico* analysis of this region we incorporated ten base pairs on the 5' part of the sequence to include any potential supplemental binding site.

*In silico* analysis gave us potential candidates for this 60bp region and we tested them by cotransfection with the -332/+688 construct in the HeLa cells (not shown). Not surprisingly with our current knowledge none of the transcription factors was able to increase the luciferase activity of our -332/+688 construct. However, at this time we have not yet identified the HNF4alpha factor as responsible for the tissue specific expression of the gene, and we thought that the tissue specific expression of the *ABCC6* gene was due to the binding of a tissue specific factor on HS3. Then we expected an enhancement of the luciferase activity in HeLa cells after that co-transfection.

Since we did not obtain any results with the co-transfection assay, we decided to narrow down the HS3 site in order to limit the number of possible binding factors to the sequence using the -332/+652bp constructs (Figure 38). We observed a 50% decrease of the luciferase activity for the -332/+652 construct compared to the -332/+688. To explain this surprising result, and since activity of the -332/+652 construct was still four times more important than the -332/+629, we hypothesized that more than one factor can bind to the -629/+688, and then a factor is binding to the +629/+652 and another one to the +652/+688 region. Alternatively, it is possible that only one factor binds to HS3. Indeed, the -332/+652 construct truncates the HNF3 binding site present in this region, which can lead to a partial binding of the factor and therefore a partial activity of the construct. Therefore, we still consider HNF3 as a potential candidate for the binding to the HS3 site. To verify that hypothesis we are currently co-expressing HNF4alpha, the selected transcription factor and our -332/+688 construct in HeLa cells.

### 5.5.8 A unified view

We investigated the regulation process of the *ABCC6* gene expression, and our

results showed the complexity of this regulation. We demonstrated that the HS2 enhancer located in the first intron of the gene is acting on a basal promoter located between -145/+72bp. The presence of this basal promoter in every cell type, is suggesting a minimal expression of *ABCC6* in tissues different than the liver. However, when we compared the amount of transcript between HepG2 and HeLa, we observed a 200-fold difference while only 7 fold difference was observed in the luciferase activity between he -145/+629 (minimal promoter + HS2) and the -332/+688 (tissue specific) constructs. Moreover tissue specific methylation of the proximal promoter of the *ABCC6* gene was previously observed. It is therefore possible that the basal promoter observed in luciferase has no significant effect in the cells but we cannot conclude on the relevance of this observation. A possible explanation of the basal expression of *ABCC*6 in other tissue could be a role of the protein in detoxification processes, acting in parallel with other ABC transporters, but in that case the presence of the protein need to be confirmed.

Finally, we described different regulatory element of the *ABCC6* gene expression and we demonstrated the connections existing between them (Figure 42).

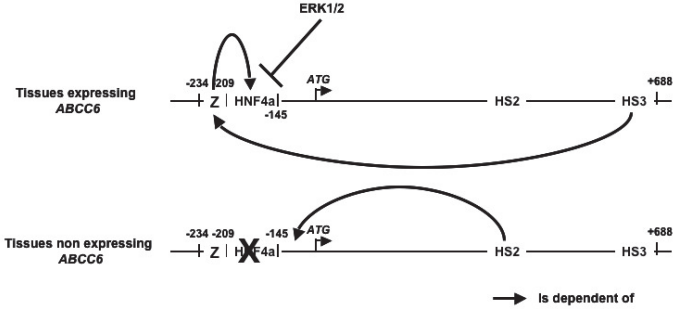


*Figure 42.Synthetic scheme representing the different element regulating* ABCC6 *expression and their inter-connection. Arrows indicate connection/dependency between two elements. ERK1/2 down-modulates the HNF4alpha activity.*

We showed that HNF4alpha is a key mediator of the tissue specific expression of *ABCC6*. We proved that the HS3 enhancer is dramatically increasing the expression of the gene by interacting with the -234/-209 region of the

proximal promoter, and we believe that this region needs the presence of the HNF4alpha transcription factor to be active.

In conclusion we described a complex regulatory mechanism of the *ABCC6* gene expression, leading to tissue specificity and involving the presence of different factors for a full *ABCC6* expression in tissue expressing the gene.

In this study we pointed out different regulatory elements of the *ABCC6* gene expression, and we think that this may have consequences with respect to PXE.

Indeed some of the PXE-causing *ABCC6* mutations may result in only partial loss of function of the protein. Induction of expression of such a low activity mutant – in theory – could potentially be therapeutic in these cases. The induction of *ABCC6* expression could be achieved at distinct levels: increased transcription, increased RNA stability or increased protein stability. However, we have almost no information to date about the regulation of *ABCC6* RNA and protein stability. Therefore, currently the only way to develop a hypothesis-driven therapy based on increased *ABCC6* expression is to intervene at the transcriptional regulation of the gene. As we showed, the role of ERK1/2-HNF4 pathway has been analyzed in a potential physiological context. As previously mentioned, HNF4 is a *bona fide* activator of the gene, while the activation of the ERK1/2 pathway inhibits HNF4 and thereby the expression of *ABCC6*. Moreover the future identification of the enhancers will also give more potential drug targets for a hypothetical treatment.

However, from this study, an option to increase the expression level of the *ABCC6* gene would be the inhibition of ERK1/2 activation in hepatocytes. Due to the myriad of ERK1/2 activating factors, the low basal activity and the fast turnover of the signals, inhibitory stimuli of ERK1/2 are not easy to find. Some of the anticancer therapies are targeting the ERK1/2 pathway and promising results have been obtained both *in vivo* and *in vitro* in inhibiting cancer progression (Roberts and Der 2007) (Friday and Adjei 2008), It is highly probable that new generations of these molecules will become available. These molecules might have beneficial effect in some patients

because only a slight inhibitory effect would be necessary in the case of PXE symptoms, the toxic effect of the molecules on other tissues would be diminished. Furthermore, in some cases patients are suffering from chronic oxidative stress and develop secondary PXE (e.g. in beta thalassemia) (Hamlin, Beck et al. 2003). These patients treated with ERK1/2 inhibitors and/or an anti-oxidative stress therapy might also receive therapeutic benefit. Similarly, the *ABCC6* mutation carriers also suffer from mild oxidative stress (Pasquali-Ronchetti, Garcia-Fernandez et al. 2006) (Zarbock, Hendig et al. 2007), a condition potentially inhibiting the expression of the gene from the remaining allele. These patients could presumably benefit from the same therapy to prevent the development of CAD.

ERK1/2 has a wide variety of targets. One of them is HNF4, which is inactivated either directly or indirectly by the kinase (Reddy, Yang et al. 1999) (Hatzis, Kyrmizi et al. 2006). We have found that HNF4 transcription factor is a major regulator of the expression of *ABCC6* and also a master regulator of metabolism in hepatocytes. By influencing the global metabolic state of the liver one can induce the expression and/or the activity of HNF4. Accordingly, it has been demonstrated that the functional HNF4 level is increased in fasting states in mouse (Martinez-Jimenez, Kyrmizi et al.). Similarly, it has been shown, that HNF4 is up-regulated by glucocorticoid hormones (Bailly, Torres-Padilla et al. 2001). All these conditions might contribute to a higher *ABCC6* expression level and diminishing or eliminating the PXE symptoms in some PXE and probably the beta thalassemic patients.

Approximately 10% of the PXE patients have no identified mutation, and we presume that some other patients do not have a complete loss of the ABCC6/MRP6 protein function. Therefore we can imagine that for these patients, increasing the expression of the gene by treatment targeting the factor involved in the gene regulation might decrease the severity of the PXE phenotype. Moreover it has been shown that heterozygous carriers have an increased risk in coronary artery disease (Koblos 2010, Trip 2002), and beta thalassemia patient can develop PXE-like symptoms (Hamlin beck 2003). These two groups may get benefits from a treatment to increase the *ABCC6*

expression that may diminish the complication. Thus, according to this study, treatments activating HNF4alpha, or the HS3 enhancer, when it will be identified, would lead to *ABCC6* expression induction, and then a reduction of the PXE associated phenotypes. Finally an other option would be to target the ERK1/2 cascade using specific inhibitors of this pathway that are available as anticancer drug, but the benefit effect may be less than the side effects.

At last, open questions still remained, and are currently under investigation in our laboratory: what are the different factors binding in the +629/+688 and the -234/-209 regions? What is the mechanism of regulation of the RNA at the post-transcriptional level? Is there polymorphism related to the PXE disease in the HS3 region?

# 6 Conclusions

We have investigated the transcriptional regulation of the *ABCC6* gene, using different techniques. By examining the gene modulation following treatment with various factors involved in major physiological pathway in the cells, we described that the ERK1/2 cascade inhibits its expression. Further analysis by luciferase reporter gene assay revealed that the proximal promoter of the gene contains the response element to the ERK1/2 cascade. Moreover we showed that ERK1/2 modulatory effect on the *ABCC6* gene was cell type specific. Indeed while expression of *ABCC6* in HepG2, which express the *ABCC6* gene, was sensitive to ERK1/2 modulation, no differences was observable in the HeLa cell line, which do not express *ABCC6*. This last observation led us to the conclusion that the ERK1/2 cascade must modulate a factor involved in the tissue specific expression of the gene.

We then identified the response element and locate it between -166 and -154bp from the transcription initiation site. This element was identified as a degenerate HNF4alpha binding site. Co-expression of HNF4alpha and the *ABCC6* promoter construct in HeLa cells, showed that HNF4alpha is responsible for the tissue specific expression of *ABCC6*. Next we demonstrated that the ERK1/2 cascade is inhibiting the HNF4alpha induction of *ABCC6*. Indeed in HepG2 cells, a mutated construct for the HNF4alpha binding site did not react anymore the ERK1/2 modulation by drugs. Moreover co-expression of HNF4alpha and the mutated construct in HeLa cells showed similar results. A last set of experiment confirmed these results since co-expression of HNF4alpha and MEKK1ca in HeLa cells lead to the inhibition of the *ABCC6* luciferase activity. Altogether these results emphasized the crucial role of HNF4alpha in the regulation of *ABCC6* expression, but also highlighted the potentially important role of the ERK1/2 cascade in this modulation.

We revealed an important mechanism of the *ABCC6* regulation, and we wanted to further examine the possible different elements involved in this regulation. In that purpose we used the DNase I hypersensitive assay

technique that reveals open chromatin region, known to be located in active promoter or enhancer region. We identified four hypersensitive sites in a 12kb region nearby the translational initiation site of *ABCC6*. One was located in the first intron of the *NOMO3* gene that is located close to the *ABCC6* gene. More interestingly we identified three hypersensitive sites in a 4.5kb region around the *ABCC6* translation initiation site and named from the 5' to 3' HS1, HS2 and HS3.

We showed that HS1 is located in the proximal promoter of the gene, and that this hypersensitive site probably marks three active regions of the proximal promoter of the gene from 5' to 3', the -234/-209 region, the HNF4alpha binding site and the -145/+72 region.

Next we described the HS2 in the first intron of the *ABCC6* gene, and we showed that it enhances the activity of the minimal basal promoter of the gene located in the -145/+72 region. We demonstrated that the HS2 enhancer plays a role in the constitutive expression of the gene since the construct containing the HS2 and the minimal promoter do not exhibit any tissue specific pattern.

Finally we demonstrated that the HS3 enhances dramatically the expression of the *ABCC6* gene, and that this enhancement is dependent of the -234/-209 region in the proximal promoter of *ABCC6*. We narrowed down the sequence containing the enhancer region to 60bp located in the first intron of *ABCC6* in the +629/+688 region, and we determined that it is possible that more than one factor is binding to this sequence, but we were not able to identify the factor(s) involved. Altogether we identified crucial regions of the *ABCC6* gene that are involved in its regulation, and we described for the first time the complex mechanism of regulation of this gene by demonstrating the interconnection between the different transcriptional regulatory elements present on the promoter and the first intron of *ABCC6*.

# 7 Summary

The *ABCC6* gene encodes an organic anion transporter ABCC6/MRP6. It has been shown that mutations in the gene lead to the development of pseudoxanthoma elasticum a rare recessive genetic disease characterized by the calcification of elastic fibers. Although *ABCC6* is mainly expressed in the liver, PXE symptoms are dermatologic, ocular and cardiovascular, which confirms the theory of a metabolic disease due to the lack of export in the blood of a liver specific metabolite. The transcription regulation of the gene has been studied but no physiological cascade has been described as a potential regulator of the gene. We believe that understanding the *ABCC6* gene regulation can lead to the understanding of the gene function and maybe the identification of the unidentified metabolite.

Here we investigated the regulation of the gene using two different strategies. First by performing a drug screening we identified growth factors as potential inhibitor of the expression of the gene. We demonstrated that the ERK1/2 cascade is responsible for the inhibitory effect observed. Using luciferase reporter gene assay we showed that HNF4alpha is activating the expression of the gene and is responsible for its tissue specific expression pattern. We finally proved that the ERK1/2 cascade is down modulating the activity of HNF4alpha leading to the down regulation of the *ABCC6* gene.

Parallely, we investigated the transcription regulation of the gene in a hypothesis free context, using the DNase I hypersensitive assay. We identified three hypersensitive sites, and located HS1 in the proximal promoter and HS2 and HS3 in the first intron of *ABCC6*. We characterized the functional role of these HS sites by luciferase assay. We demonstrated the enhancer activity of the intronics HS. HS2 acts on the minimal basal promoter of the gene mapped by luciferase between -145 and +72bp from the ATG in tissues where *ABCC6* is barely detectable. Finally we showed that HS3 enhances the expression of *ABCC6* in tissues expressing the gene. The activity of HS3 is dependent on the presence of an unidentified factor binding to the -234/-209 region. The binding of this unknown factor depends on the presence of HNF4alpha.

# 8 Összefoglalás

Az *ABCC6* gén egy organikus anion transzportert, az ABCC6/MRP6 fehérjét kódolja. A génben bekövetkező mutációk a pseudoxanthoma elasticum kialakulásához vezetnek, amely a rugalmas rostok meszesedésével járó ritka recesszív genetikai betegség. Bár az *ABCC6* elsősorban a májban expresszálódik, a PXE tünetei a bőrben, szemben és a keringési rendszerben jeletkeznek. Ez igazolja a metabolikus betegség hipotézist, azaz hogy egy máj-specifikus metabolit vérbe történő exportjának a hiánya vezet a betegség kialakulásához. A gén traszkripciós szabályozását régóta vizsgálják, de még nem írtak le olyan fiziológiás kaszkád-rendszert amely felelős lehet a gén szabályozásáért. Az *ABCC6* gén szabályozásának a megértése hozzásegíthetne bennünket a gén funkciójának a megértéséhez, és talán a jelenleg még ismeretlen metabolit meghatározásához is.

A gén szabályozásának tanulmányozása során két különböző stratégiát alkalmaztam. Először biológiailag aktív vegyületek szűrésével azonosítottam olyan növekedési faktorokat, amelyek lehetséges gátlószerei a gén expressziójának. Kimutattam, hogy az ERK1/2 kaszkád felelős a megfigyelt gátló hatásért. Azonosítottam egy konzervált HNF4alfa kötőszekvenciát és bizonyítottam, hogy az ERK1/2 hatás létrejöttéhez ez a szekvencia szükséges. További kísérleteimben igazoltam, hogy ehhez a szekvenciához a HNF4alfa kötődik, ami meghatározza a gén szövetspecifikus kifejeződését. Végezetül kísérleteim során megfigyeltem, hogy az ERK1/2 hatás a HNF4alfa transzkripciós faktor aktiváló hatásának szabályozásán keresztül jön létre.

Ezzel párhuzamosan egy hipotézis-mentes megközelítéssel is vizsgáltam a gén transzkripciós szabályozását, a DNáz I hiperszenzitivitási esszét alkalmazva. Három hiperszenzitív helyet azonosítottam, a HS1-et a proximális promóteren lokalizáltam, a HS2-t és HS3-at pedig az *ABCC6* első intronján. Ezt követően luciferáz esszével karakterizáltam ezeket a HS helyeket. Kimutattam az intronikus HS helyek enhancer aktivitását. Azokban a szövetekben, ahol az *ABCC6* csak kis mértékben detektálható, a HS2 a gén minimál promóterén hat, amelyet luciferáz kísérleteimmel az ATG-től számított -145 és +72 bp közé térképeztem. Végül kimutattam, hogy az *ABCC6*-ot expresszáló szövetekben a HS3 növeli a gén expresszióját. A HS3 aktivitása függ egy még egyenlőre nem azonosított faktor jelenlététől, amely a -234/-209 régióhoz kötődik. Ennek az ismeretlen faktornak a kötődése pedig függ a HNF4alfa jelenlététől.

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# 10 Abbreviations:

ABC : ATP-binding cassette;

ABCC6 : ATP-binding cassette protein, family C, number 6;

AS : angioid streak;

CAD : coronary artery disease;

CGI : CpG island;

ChIP : chromatin immunoprecipitation;

CNV : choroidal neovascularization;

DCC : dystrophic cardiac calcification;

DHA : DNase I hypersensitive assay

DHS : DNase I hypersnsitive site

EGF : epidermal growth factor

ERK1/2 : extracellular signal- regulated kinase 1/2;

ERK1dn : ERK1 dominant negative mutant

GGCX : γ-glutamyl carboxylase;

HGF : hepatocyte growth factor

HNF4 : hepatocyte nuclear factor 4;

HS : hypersensitive site

K3 : vitamin K3/menadione;

KO : knock out;

MAPK : mitogen-activated protein kinase;

MEKK1ca : MEKK1 constitutively active mutant

MRP : multidrug resistance-associated protein;

PMA : phorbol miristate actetate

PXE : pseudoxanthoma elasticum;

RPE : retinal pigmented epithelium;

tBHQ : tert-butyl hydroquinone

VEGF : vascular endothelial growth factor;

Wt : wild type.

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# 12 Publication list

**Hugues de Boussac**, Marcin Ratajewski, Iwona Sachrajda, Gabriella Köblös, Attila Tordai, Lukasz Pulaski, László Buday, András Váradi, Tamás Arányi. ***The ERK1/2 – hepatocyte nuclear factor 4α axis regulates the human ABCC6 gene expression in hepatocytes.*** j biol chem First Published on May 12, 2010, doi: 10.1074/jbc.M110.105593

András Váradi, Zalán Szabó, Viola Pomozi, **Hugues de Boussac**, Krisztina Fülöp and Tamás Arányi. ***ABCC6 as a target in Pseudoxanthoma Elasticum,*** Current Drug Targets, in press

Marcin Ratajewski, **Hugues de Boussac**, Lukasz Pulaski. (2009). ***Liver specific enhancer in ABCC6 promoter - Functional evidence from natural polymorphisms.*** Biochem Biophys Res Commun. 2009 May 22;383(1):73-7.