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UNIVERSITAT DE BARCELONA
FACULTAT DE FARMÀCIA I
CIÈNCIES DE L'ALIMENTACIÓ

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Role of the human Concentrative Nucleoside Transporter 1 (hCNT1) in oncogenesis

Memòria presentada per Hamsa Jameel Banjer

per optar al títol de doctora per la Universitat de Barcelona

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Summary

Summary

Transportome alterations have been associated with oncogenesis. The loss of human Concentrative Nucleoside Transporter 1 (hCNT1) during carcinogenesis seems to be a relatively common event in tumors and might by itself contributing to oncogenesis. In fact, hCNT1 can play a dual role in cell biology being a nucleoside and nucleoside-derived drug transporter but also an important player in the modulation of a variety of cellular functions. Accordingly, we have previously reported that hCNT1 function is able to induce physiological changes that are relevant to tumor biology in a transport independent-manner. These observations argued in favor of hCNT1 being a transceptor protein. Thus, in this thesis we focused our study on the role of hCNT1 in cell physiology beyond the mere nucleoside salvage. We found that hCNT1 is able to induce antiproliferative effects in different tumor backgrounds, and its restitution in Hepatocellular carcinoma (HCC) derived cell lines alters several different signaling cascades that are important to many physiological functions of the cell, including cell survival and cell migration. Moreover, hCNT1-induced cell death seems to be triggered by an increase in intracellular Ca^{2+} levels, which might be related to the interaction of the Ca^{2+}-binding protein S100A11 with hCNT1.

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List of abbreviations

Ad: Adenovirus

CNT: Concentrative Nucleoside Transporter

DNA: Deoxyribonucleic Acid

ENT: Equilibrative Nucleoside Transporter

FBS: Fetal Bovine Serum

GSEA: Gene Set Enrichment Analysis

HEK: Human Embryonic Kidney

KM: Michaelis-Menten constant

MOI: Multiplicity of infection

MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

PCR: Polymerase Chain Reaction

RNA: Ribonucleic Acid

RT-PCR: Real Time- PCR

SDS: Sodium dodecyl sulfate

SLC: Solute Carrier

TMD: Transmembrane

Introduction

I. Introduction

1. SLC Transporters

SoLute Carrier (SLC) transporter superfamilies are composed of more than 300 membrane-bound proteins. They facilitate various substrate transportation across biological membranes, which have important roles in several physiological processes, ranging from cellular absorption of nutrients to drug and other xenobiotic uptake. Membrane transporters are widely expressed through the body, mostly in the epithelia of major organs, for instance, the liver, kidney, intestine, and also in other organs with barrier functions such as the brain, placenta and testis. These different transporters are localized to the cell membrane, and to various subcellular organelle membranes, to ensure cellular homeostasis. Some of these transporters are only expressed in specific organs and mediate the entry and removal of endogenous and xenobiotic compounds.

Furthermore, numerous classes of approved drugs target SLC transporters. Studies on human genetics have provided a great insight into the roles of SLC transporters in both common and rare diseases, implying a wealth of new therapeutic opportunities. Additionally, knowledge on specificity and selectivity of these transporters is broad, while the biological impact of transporters on cell function beyond their mere role as substrate suppliers is less well known.

2. Nucleosides and Nucleoside transporters

Nucleosides and nucleobases are metabolites and precursors of nucleotides. Nucleotides have important roles in cell physiology both as nutrients and modulators of cellular homeostasis. Inside the cells, nucleotides are involved in many vital physiological processes, such as nucleic acid synthesis, metabolic regulation, and cell signaling. Moreover, various nucleoside and nucleobase analogs have been developed to be used as drugs in a wide variety of treatments for cancer, viral infections, and inflammatory diseases.

Nucleoside transporters are integral membrane proteins. They mediate nucleoside flux across cell membranes for exerting their physiological effects. Nucleoside transporters belong to the solute carrier families 28 and 29 (*SLC28* and *SLC29*), which encode human

Concentrative Nucleoside Transporters (hCNTs) and human Equilibrative Nucleoside Transporters (hENTs), respectively.

2.1. Importance of nucleosides

Physiological nucleosides, nucleobases, and their metabolic products have various and essential roles in numerous biological processes, including cellular division, metabolism, function, and structure. For instance, nucleotides are the molecular subunits which are essential for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis. Some nucleosides play major roles in cell regulation and metabolism, by being the key biomolecule responsible for intracellular energy transfer, i.e. Adenosine Triphosphate (ATP), or a transducer of intracellular signals as a second messenger in various cell signaling processes, e.g. cyclic Adenosine Monophosphate (cAMP) and cyclic Guanosine Monophosphate (cGMP). They are also incorporated into important cofactors of enzymatic reactions, e.g. coenzyme A and NAD^+. Furthermore, in addition to their roles in metabolism, some nucleosides play major roles in cellular regulation as signaling molecules, for example, adenosine is being a paracrine-signaling molecule by acting as a ligand for purinergic receptors.

2.1. Homeostasis of nucleotides

Since nucleotides are needed for a wide variety of metabolic function in all cells, maintenance of nucleotide levels is therefore fundamental to support cellular functions. All proliferating cells need to maintain the supply of nucleotides, whether in normal division for controlled proliferation, as well as in deregulated cell division in cancer. To guarantee correct DNA and RNA synthesis, a balanced nucleotide pool (dNTP) is essential. In fact, this balanced nucleotide pool is also essential to cellular metabolism and signaling. Additionally, dysregulation of nucleotide metabolism is commonly involved in human disease pathogenesis including cancer and diabetes.

Unsurprisingly, the nucleotide supply is tightly regulated and tied to the cell cycle through a network of interconnected biosynthetic and catabolic pathways to maintain intracellular nucleotide pools. These regulations are achieved at the gene expression level by a variety of transcription factors, and at the substrates level by a large number of enzymes involved in the synthesis of nucleotides. Under normal physiological conditions, the cellular nucleotide pool is mostly derived from the recycled nucleobases via the salvage pathway. On the other hand,

under cellular conditions requiring higher nucleotide levels, the *de novo* biosynthetic pathway is upregulated to meet the intracellular purine and pyrimidine demand (Boyle 2005; Serres (ed.) 1985).

2.1.1. *De Novo* biosynthesis

Nucleotide synthesis is regulated by various critical transcription factors, for example, MYC and E2F control the expression of nucleotide biosynthesis genes, thus, if mutated or overexpressed this would be associated with cell transformation and uncontrolled proliferation, which may lead to cancer. MYC directly controls the expression of genes that encode the enzymes of the nucleotide synthetic pathways for the production of all nucleotide precursors, it also coordinates RNA and protein biosynthesis (Mannava et al. 2008). In addition, E2F also targets the enzymes required for deoxynucleotide synthesis, such as the Guanosine Monophosphate (GMP) synthetase and the Uridine Monophosphate Synthetase. *De novo* synthesis involves a stepwise assembly of the nitrogenous purine and pyrimidine rings. It is realized through multiple enzymatic reactions using simple metabolic precursors and requires relatively high levels of metabolic energy in a form of ATP and/or GTP.

The *de novo* **purine biosynthetic** pathway is a highly conserved, energy-intensive pathway that generates the common intermediate in purine biosynthesis inosine monophosphate (IMP) from 5-phosphoribosyl 5-pyrophosphate (PRPP), which is later converted to nucleoside monophosphate (NMP) and subsequently, by the action of nucleotide kinases, to the triphosphate nucleotides. This metabolic transformation is carried out in several steps catalyzed by sequential orchestration of six enzymes along with other various amino acid substrates and cofactors as illustrated in Figure 1. The metabolic multi-enzyme complex participating in *de novo* purine biosynthesis, include three multifunctional enzymes: TGART, PAICS, and ATIC. TGART is a trifunctional protein which has glycinamide ribonucleotide synthetase (GARS), GAR transformylase (GART) and aminoimidazole ribonucleotide synthetase (AIRS) activities. PAICS is a bifunctional enzyme that has phosphoribosyl-aminoimidazole carboxylase (CAIRS) and phosphoribosyl-aminoimidazole succinocarboxamide synthase (SAICARS) activities. The other bifunctional enzyme, ATIC, has aminoimidazolecarboxamide ribonucleotide transformylase (AICART) and IMP cyclohydrolase (IMPCH) activities. Following, separate branches lead from IMP to AMP and GMP (Figure 2), with subsequent transfer of phosphoryl from ATP that converts AMP and GMP to ADP and

GDP. Later, another further transfer of a second phosphoryl from ATP converts GDP to GTP, whereas oxidative phosphorylation is primarily responsible of ADP to ATP conversion.

The *de novo* biosynthetic pathway of pyrimidine is initiated and controlled by CAD, a ~240-kDa multifunctional protein with different enzymatic domains, i.e. carbamoyl-phosphate synthetase 2 (CPS), aspartate transcarbamylase (ATC), and dihydroorotase (DHO) domains. CAD catalyzes the first three reactions of de novo pyrimidine synthesis. The pyrimidine *de novo* biosynthesis begins with carbamoyl phosphate formation in which carbon dioxide and ammonia are combined. This process is catalyzed by the cytosolic carbamoyl phosphate synthetase II enzyme. After that, carbamoyl aspartate is formed, aided by the aspartate transcarbamoylase enzyme. Further, dihydroorotate is formed by ring closure under the influence of dihydroorotase enzyme. Later, dihydroorotate is oxidized to orotic acid and attach to a ribose sugar, yielding in orotidylic acid. Following, uridine monophosphate UMP is formed by orotidylate decarboxylation. UMP then can be converted to the other pyrimidine nucleotides CMP and TMP as illustrated in Figure 3. Both pyrimidine and purine biosynthesis are energetically costly. Moreover, most of the pyrimidine biosynthetic steps reside in the cytoplasm, only one step occurs in the mitochondria, i.e. orotate dehydrogenase reaction. In opposite to pyrimidines, purine nucleotides synthesis is entirely cytoplasmic. Accordingly, intracellular mechanisms sense and regulate the pool sizes of NTPs to achieve homeostasis. Furthermore, since *de novo* nucleotide synthesis pathways are metabolically and energetically costly, the salvage pathways are often required to meet physiological needs during periods of metabolic stress, limited food supply or during growth and tissue regeneration when cells are rapidly dividing. Moreover, even in cells with *de novo* synthesis capabilities, the relative importance of salvage versus *de novo* synthesis likely depend on growth conditions and on some specific tissue.

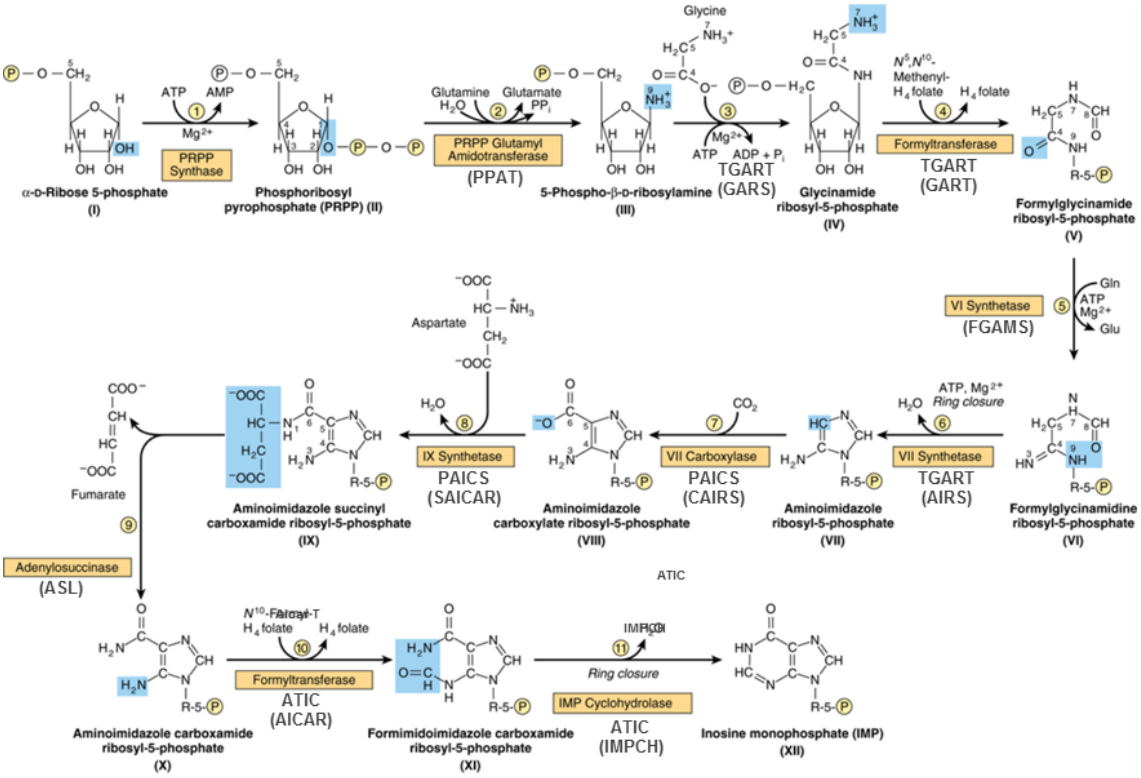

Figure 1. Purine biosynthetic pathway. Adapted from Harper's Illustrated Biochemistry, 29e: 2012.

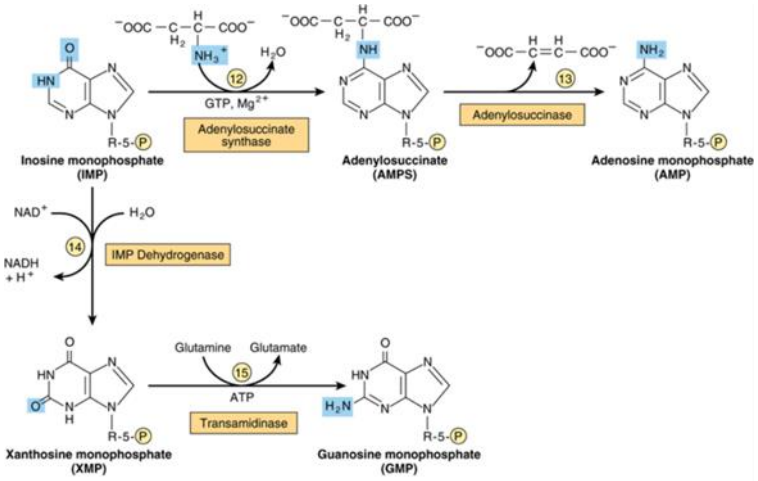

Figure 2. Conversion of IMP to AMP and GMP. Adapted from Harper's Illustrated Biochemistry, 29e: 2012.

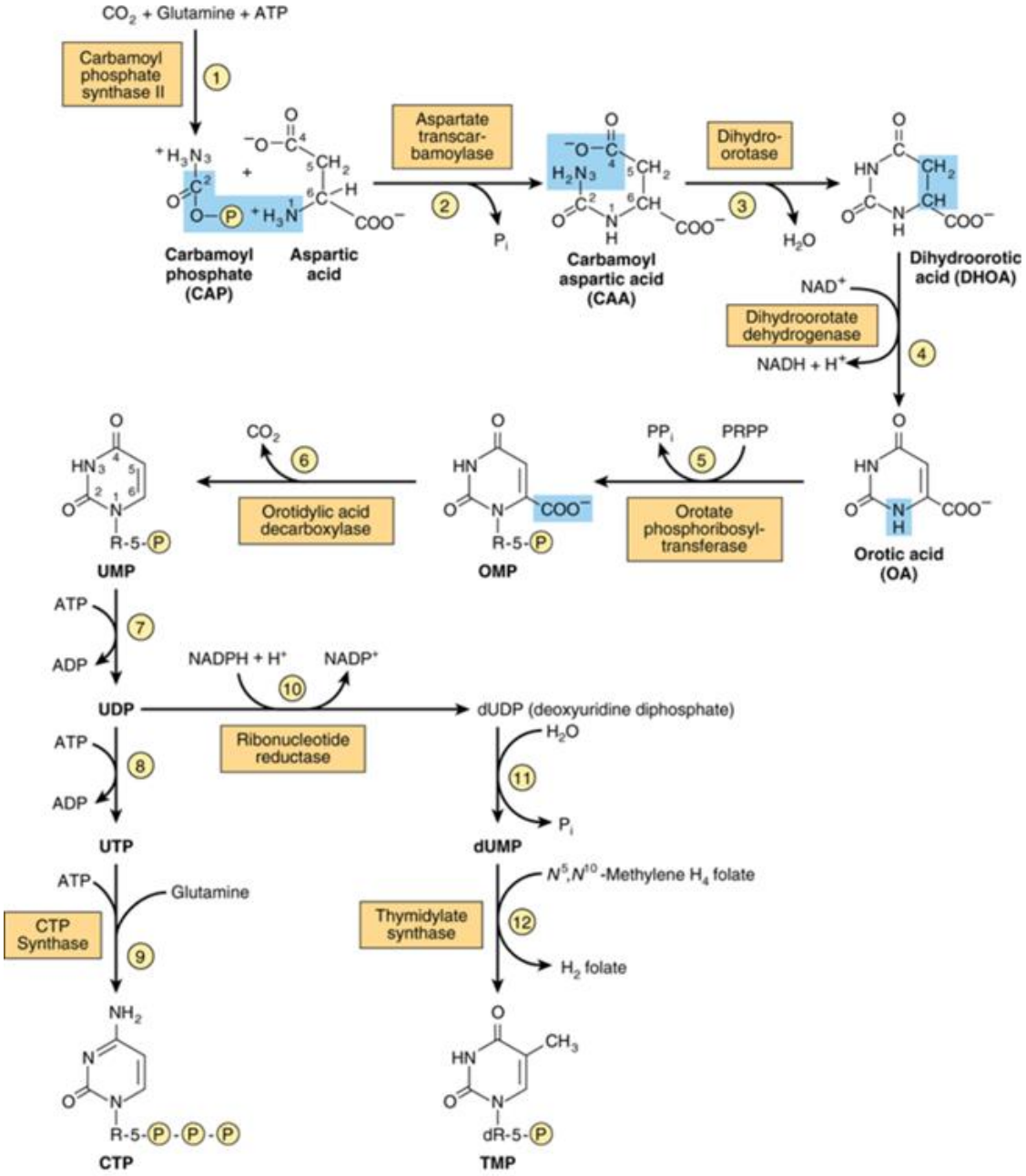

Figure 3. The biosynthetic pathway for pyrimidine nucleotides. Adapted from Harper's Illustrated Biochemistry, 29e: 2012.

2.1.2. Salvage pathway

The salvage pathway uses nucleosides generated by the breakdown of polymeric RNA and DNA into NMPs which can be recycled by the action of the nucleotide kinases. This pathway has less energetic needs than *de novo* synthesis. The salvage pathway depends on nucleosides and nitrogen bases flux from the external environment, via *SLC28* and *SLC29* transporters, into the cells and their subsequent metabolism by adding the appropriate sugar.

Cellular permeation of physiological nucleosides across plasma membranes is typically followed by a phosphorylation step by nucleoside kinases (e.g., 2'-deoxycytidine kinase, thymidine kinase, and adenosine kinase) for nucleotide production. On the other hand, nucleobases are converted to nucleotides by phosphoribosyl transferases (e.g. hypoxanthine-guanine and adenine phosphoribosyl transferases) (Figure 4). Nucleotides do not readily diffuse through plasma membranes because of the presence of negatively charged phosphate groups. As a result, phosphorylation and phosphoribosylation can serve, respectively, to trap nucleosides and nucleobases intracellularly.

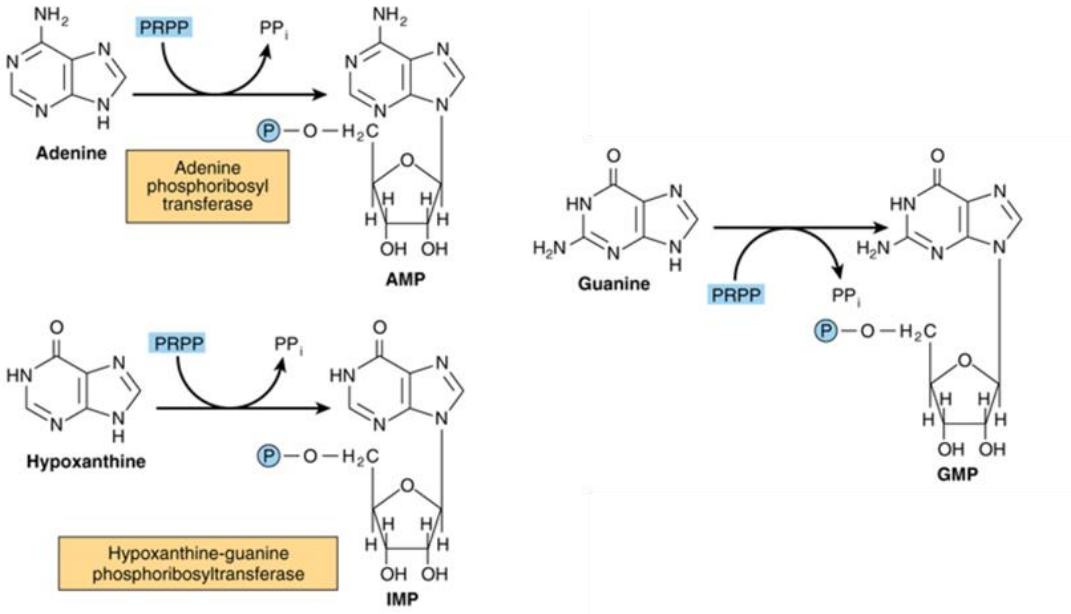

Figure 4. Phosphoribosylation of adenine, hypoxanthine, and guanine to form AMP, IMP, and GMP, respectively. Adapted from Harper's Illustrated Biochemistry, 29e: 2012.

3. *SLC28* family

The ***SLC28* gene** family in humans consists of three protein-coding genes i.e. *SLC28A1* (hCNT1), *SLC28A2* (hCNT2) and *SLC28A3* (hCNT3). These transporter proteins mediate the nucleosides unidirectional flow in an energy-costly process coupled with sodium ions influx (M. Pastor-Anglada et al. 2008). They are high-affinity transporters showing apparent Km values in the low micromolar range (10–100μM) and more restricted selectivity than equilibrative transporters (Table 1). They translocate natural nucleosides and nucleoside analogs in a sodium-dependent manner and differ in their substrate selectivity and Na^+:nucleoside stoichiometry.

Nucleosides are categorized into two major groups according to the structure of the nucleobase. Pyrimidine nucleosides are comprised of a heterocyclic aromatic benzene-like ring with nitrogen substitutions on the first and third positions. The second group, purine nucleosides, contains a heterocyclic ring comprised of fused pyrimidine and imidazole rings (Figure 5). **hCNT1** has increased preference for pyrimidine nucleosides, although it can bind adenosine with high affinity but without translocating it (Larráyoz et al. 2004). **hCNT2** has preference for purine transport. It can also translocate uridine despite being purine-preferring. Both hCNT1 and hCNT2 are able to translocate nucleosides with a 1:1 sodium: nucleoside stoichiometry. **hCNT3** transports both purine and pyrimidine nucleosides, with a sodium:nucleoside stoichiometry of 2:1.

Table 1. Concentrative nucleoside transporter kinetics and properties.

Transporter	Gene	Substrate (*Km*)	Stoichiometry (Na^+:nucleoside)
hCNT1	*SLC28A1*	Uridine (22-37 μM) Thymidine (26 μM) Cytidine (29 μM)	1:1
hCNT2	*SLC28A2*	Inosine (4 μM) Adenosine (8 μM) Uridine (40 μM) Guanosine (21 μM)	1:1
hCNT3	*SLC28A3*	Uridine (22 μM) Thymidine (21 μM) Cytidine (15 μM) Inosine (52 μM) Adenosine (15 μM) Guanosine (43 μM)	2:1

Adapted from Pastor-Anglada et al. 2008.

4. *SLC29* family

The ***SLC29* gene** family encodes for human equilibrative nucleoside transporters, a group of structurally-related proteins that mediate bidirectional nucleoside facilitated diffusion across the plasma membrane, down their concentration gradients. This family consist of four members, hENT1-4.

hENT1 and **hENT2** are expressed at the plasma membrane and have been broadly characterized and studied for the transport of natural nucleosides along with nucleoside-derived drugs. hENT1 and hENT2 can translocate both purine and pyrimidine nucleosides with a lower affinity than concentrative nucleoside transporters. **hENT3** is a pH-dependent intracellular transporter, it has a wide range of substrates and operates in intracellular lysosomes and mitochondria. Regarding **hENT4**, it is a pH-dependent transporter which functions as poly-specific organic cation transporter, hENT4 can transport adenosine efficiently at physiological pH 7.4 (Barnes et al. 2006).

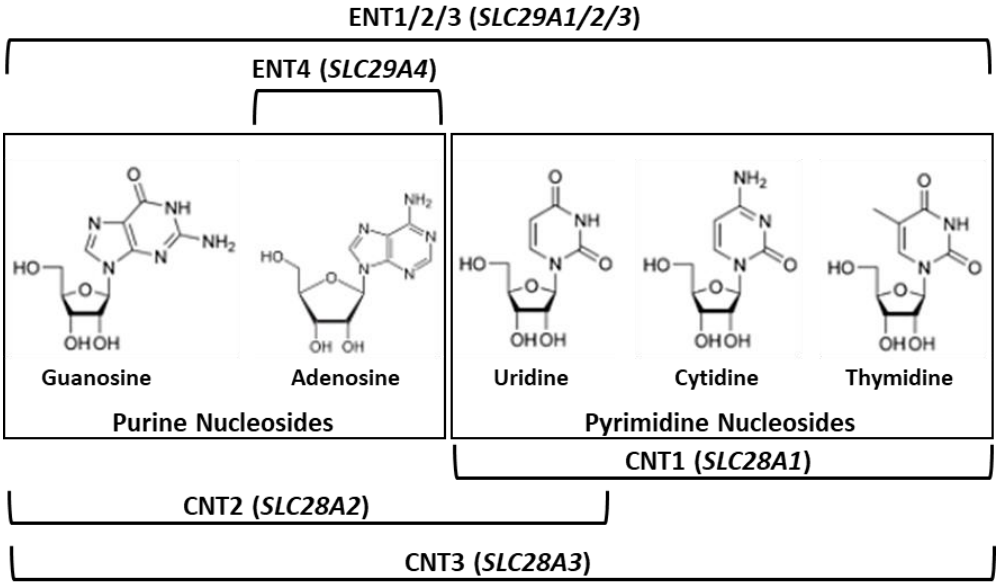

Figure 5. Structures of naturally occurring nucleosides and their affinity to *SLC28* and *SLC29* transporters. Adapted from Young et al. 2013.

5. Structure of nucleoside transporters

Due to the difficulty in the expression, isolation and crystallization of membrane proteins, there is a lack of structural information, and there is no any crystalized structure of the human

nucleoside transporters available so far. However, the present structures and topologies are established on bioinformatics prediction and methods together with biochemical validation. Determining the structure of nucleoside transporters would provide numerous information about the mechanism of substrate internalization and help to understand various physiological functions associated with them. In addition, it can also help in designing drugs to be transported in a specific manner.

5.1. Structure of hCNTs

The three members of *SLC28* family present around 50% of homology in their primary structure. Until recently, the predicted structure of hCNTs through topological models, directed mutagenesis studies and chimeric studies consisted of 13 transmembrane domain with intracellular N-terminus tail, and an extracellular C-terminus domain (Hamilton et al. 2001; M. Pastor-Anglada et al. 2008). However, the crystallization of the sodium-linked concentrative nucleoside transporter of *Vibrio cholera*, which shows high sequence homology to hCNTs, changed the predicted topology from 13 to 11 transmembrane domains. In light of the bacterial transporter being homo-trimeric with 8 transmembrane domain (Johnson, Cheong, and Lee 2012), joining the extra three transmembrane domains of the predicted hCNT structure to the N-terminus domain results in a similar structure of 11 transmembrane domains with intracellular amino terminus and an extracellular carboxy terminal end. Additionally, the sodium binding site has been identified between the tip of HP1 and the unwound portion of TM4 (Young et al. 2013) (Figure 6). Recently, we have reported the residues implicated in the additional sodium-binding site present in hCNT3, at the threonine T605 and serine S396 residues with a polarity supporting hCNT3 to host sodium inside. In opposition, S396 is substituted by alanine in both hCNT1 (A369) and hCNT2 (A374), two residues that are disrupting the possibility to bind sodium. T605 is substituted by an asparagine in hCNT1 (N578) and by serine in hCNT2 (S583), two residues that are usually found within those that participate in the coordination of sodium (Arimany-Nardi et al. 2017).

Furthermore, the homologous human CNT proteins have extended extracellular C-terminal regions containing multiple glycosylation sites that might influence their activity and location. The N-terminal end is relatively large for the whole protein (representing between 80 and 100 residues in a protein of over 600 amino acids). It consists of three transmembrane helices and a large intracellular domain which are absent in prokaryote CNTs. In fact, they are

not essential for transport activity, since truncated proteins lacking this intracellular domain, derived either from hCNT1 or from its rat homolog rCNT1 retained significant transport activity when expressed in oocytes (Hamilton et al. 2001). Hence, this intracellular domain of hCNTs is assumed to be important in intracellular protein-binding, signaling, and trafficking (Errasti-Murugarren et al. 2010; Huber-Ruano et al. 2010; Pinilla-Macua, Casado, and Pastor-Anglada 2012).

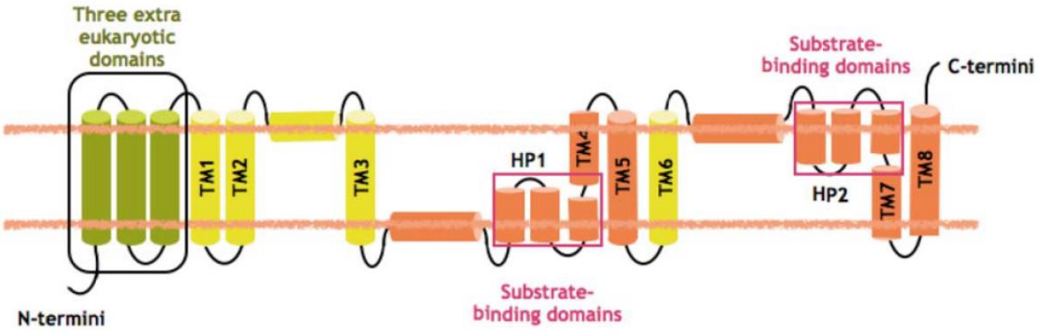

Figure 6. Predicted CNT structure model. This model is based on the *Vibrio cholerae* nucleoside transporter crystal (Johnson et al., 2012). Picture adapted from Arimany-Nardi´s PhD thesis (2013).

5.2. Structure of hENTs

The topology predicted for hENT transporters consists of 11 transmembrane domains with intracellular N-terminal and extracellular C-terminal ends. Additionally, it possesses an extracellular loop, which falls between the 1 and 2 transmembrane domains, holding glycosylation sites essential for hENT1 localization and function. Also, it consists of an intracellular loop connecting the 6 and 7 transmembrane domains (Figure 7) (Bicket et al. 2016; Sundaram et al. 2001). Chimeric studies helped identifying the function of various domains. The region between transmembrane domains 1 and 6 in hENT2 appears to be involved in nucleosides recognition while the region between transmembrane domains 5 and 6 is involved in the recognition of nucleobases (Baldwin et al. 2004). Besides, the region between transmembrane domains 3 and 6 hold residues responsible for sensitivity to NBTI and other coronary vasodilator inhibitors. Moreover, a recent study using a three-dimensional structure model detailed the molecular mechanisms whereby ENT mediate substrate transport across membranes (Valdés et al. 2014).

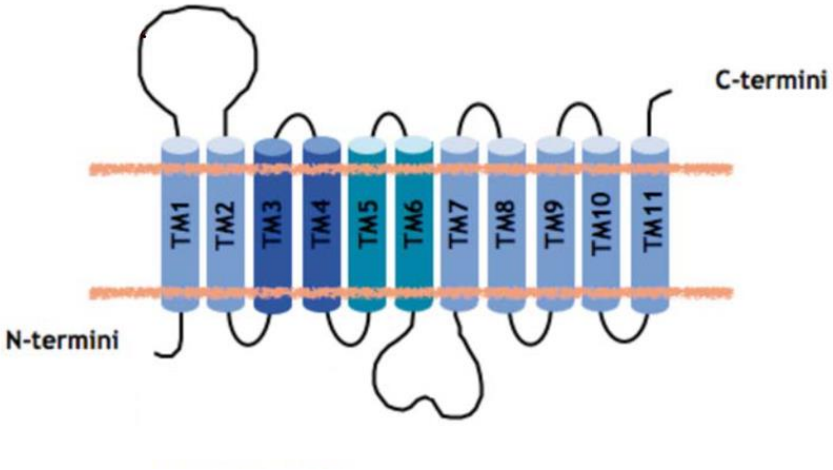

Figure 7. Equilibrative nucleoside transporters structure model. Based on the results published by Baldwin et al 2004. Picture adapted from Arimany-Nardi´s PhD thesis (2013).

6. Expression and distribution of nucleoside transporters

hCNT proteins are known to be broadly expressed but not ubiquitous, although, they were initially thought to be only expressed in polarized epithelia. hCNT1 is localized in the liver, kidney, intestine, and brain (Pennycooke et al. 2001). hCNT2 is more widely expressed in humans compared to hCNT1 and it is expressed in the kidney, heart, liver, skeletal muscle, pancreas, placenta, brain, cervix, prostate, small intestine, rectum, colon, and lung (Pennycooke et al. 2001, Gray et al. 2004). hCNT3 is broadly expressed in mammary glands, pancreas, bone marrow, intestine, liver, lung, placenta, prostate, brain, and heart tissues (Che et al. 1995, Gray et al. 2004). hCNTs are found in most of polarized epithelia and they are located at the apical membrane, thus facilitating nucleosides flux across the barriers (Figure 8) (Mangravite et al. 2001; Lai et al. 2002; Errasti-Murugarren et al. 2007).

On the other hand, **hENT** proteins are largely distributed, but their expression varies in abundance among cell types and organ tissues (Baldwin et al. 2004). They are expressed mainly, but not exclusively, at the basolateral side of polarized epithelial cells (Lai et al. 2002; Mangravite et al. 2003).

hCNTs and hENTs are asymmetrically distributed at the apical and basolateral poles of polarized epithelial cells. This might permit vectorial flux of nucleosides and their derivatives, thus contributing to whole body nucleoside homeostasis (Figure 8) (Errasti-Murugarren et al. 2007). Notably, the nucleoside transporter polarised distribution may differ among epithelial barriers. In human hepatocytes, hCNT1, hCNT2, and hENT1 appear to be located both in the basal (sinusoidal) and apical (canalicular) membrane domains (Govindarajan et al. 2008). However, in case of the blood brain barrier, hCNT2 seems to be the major CNT protein (J. Y. Li et al. 2001; Cansev 2006) whereas hCNT1 is the only hCNT protein expressed in syncytiotrophoblasts and it is found together with hENTs in both membranes. In general, ENT expression has been linked to proliferation (Soler et al. 2001), whereas CNT expression is commonly associated with differentiated cell types (Aymerich et al. 2005; Molina-Arcas, Casado, and Pastor-Anglada 2009; Míriam Molina-Arcas and Pastor-Anglada 2013).

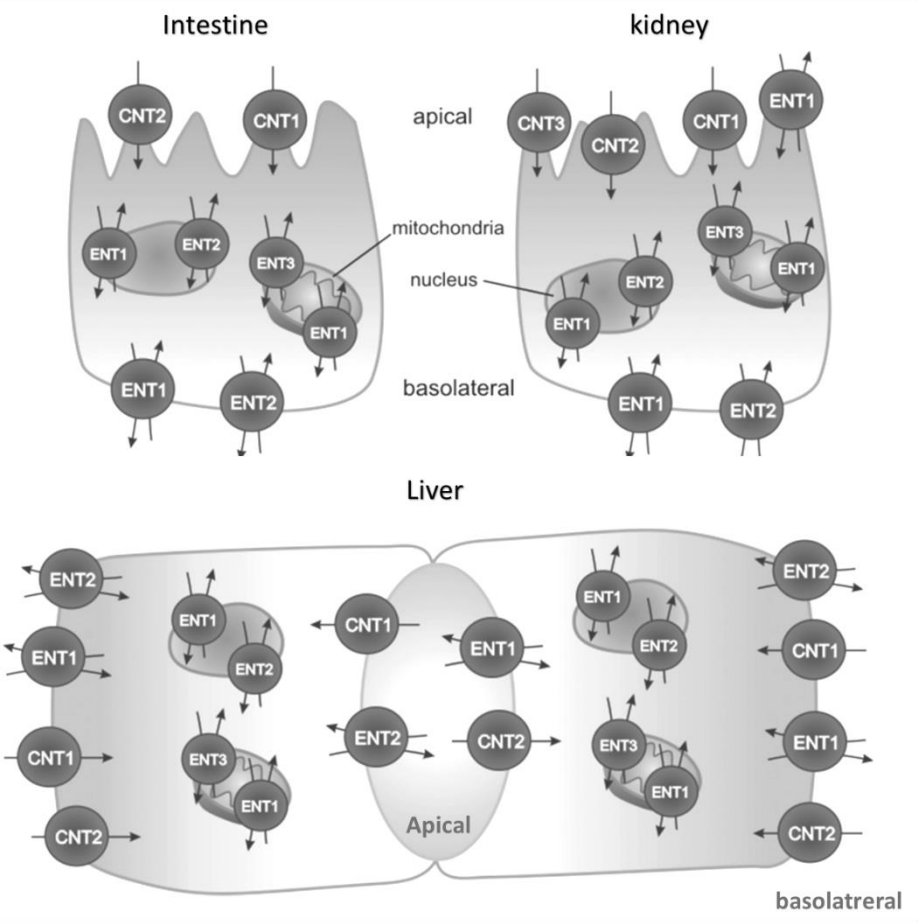

Figure 8. Nucleoside transporters distribution in polarized epithelia. Equilibrative nucleoside transporters (ENT) and concentrative nucleoside transporters (CNT) distribution in kidney, liver and intestine polarised cells. Adapted from (Molina-Arcas and Pastor-Anglada, 2013).

7. Regulation of nucleoside transporters expression

7.1. Transcriptional regulation

Several evidences of nucleoside transporters regulation have been reported. For instance, it was found that glucocorticoids, such as dexamethasone (differentiating agents), induces an increase in the mRNA and protein levels of both CNT1 and CNT2 (Aymerich et al. 2004). In bone marrow mouse macrophages, CNT1 and CNT2 are upregulated following activation by lipopolysaccharide or interferon-γ (IFN-γ) (Soler et al. 2001; Soler et al. 2003). Similarly, it was described that TGF-β transcriptionally activates the CNT2 gene (*SLC28A2*) in rat liver parenchymal cells (Valdés et al. 2006). Furthermore, the CNT1 promoter was trans-activated by the hepatocyte nuclear factor-4α (HNF-4α) in human cell lines derived from the liver, intestine, and kidneys (Klein et al. 2009).

Regarding the ENTs, studies on transcriptional regulation of the hENTs have focused largely on hENT1. Several evidences suggested that hENT1 promoter can be upregulated by the transcription factors Sp1 (zinc-finger specificity protein-1) and MAZ (myc-associated zinc protein) (Abdulla and Coe 2007). Other studies have found that the activation of JNK/c-Jun pathway negatively regulates mENT1 (Leisewitz et al. 2011) and that PPARα and γ activation or overexpression resulted in higher hENT1 transport activity (Montero et al. 2012). Moreover, in rat intestinal epithelial cell line (IEC-6), it was observed that after stimulation with TGF-α or EGF there was an increase in ENT1 mRNA (Aymerich, Pastor-Anglada, and Casado 2004).

Additionally, due to the cardio- and neuroprotective role of adenosine in ischemia or hypoxia, different research groups studied the nucleoside transporter regulation in these situations. These studies described how mENT1 and mENT2 promoters were down-regulated by hypoxia inducible factor 1 (HIF-1), as well as during cerebral ischemia (Chu et al. 2012). In both situations, ENTs downregulation promote an increasing of extracellular adenosine levels, which has a protective effect on the cell by activation of adenosine receptors (Chaudary et al. 2004; Eltzschig et al. 2005; Löffler et al. 2007).

7.2. Post-transcriptional

Aside to transcriptional regulation, nucleoside transporter regulation at protein level by post-transitional modification of some nucleoside transporters were also described. For hCNTs, in a model of chronic lymphocytic leukemia, MEC1 cell line, treatment with all-*trans*-retinoic acid (ATRA) increased hCNT3-related activity by a mechanism that involves trafficking of pre-existing hCNT3 proteins, mediated by the transforming growth factor (TGF)-β1, to the plasma membrane (Fernández-Calotti and Pastor-Anglada 2010). Additionally, a major role for RS1 in the trafficking of CNT proteins from the trans-Golgi network to the plasma membrane was supported by different data from a combination of studies on CNT proteins family (Errasti-Murugarren et al. 2012).

Moreover, regarding ENT1, there is strong evidence of its phosphorylation by the protein kinases PKC and PKA (Coe et al. 2002; Reyes et al. 2011) and in mouse by Casein kinase II (CK2) (Bone et al. 2007). It was also observed that hENT1 regulation by the presence of its substrate (cytidine) promoted its internalization (Zafar et al. 2016). In addition, a study by Nivillac and collaborators determined how the expression of hENT1 is regulated during cell cycle and the mechanisms by which it reaches the membrane and it is degraded (Nivillac et al. 2011). Similarly, a recent work by (Song et al. 2017) described the mechanism by which the erythrocyte hENT1 levels are reduced through degradation via proteasome in conditions of hypoxia and at high altitude

8. Expression of the nucleoside transporters in cancer

Protein expression is one of the processes that are commonly disturbed in cancer cells. Evidence of selective loss of nucleoside transporters in tumors was first determined in rat models of hepatocarcinogenesis (Dragan et al. 2000). Later on, in humans, some studies have evaluated nucleoside transporter expression levels in different tumors, displaying variability in nucleoside transporters expression profiles from different individuals. For instance, results of a matched normal-tumor tissue cDNA array data from different individuals showed a large variability in the profiles of the analyzed nucleoside transporters, with an overall decrease of expression in most of the tumor tissues (Pennycooke et al. 2001). Particularly, in a reduced number of pancreatic tumors and tumor cell lines, hCNT1 expression was commonly low compared to normal pancreas and pancreatic ductal epithelial cells (Bhutia et al. 2011).

Moreover, immunohistochemical analysis of hENT1, hENT2 and hCNT1 proteins in over 300 gynecological tumors (ovary, endometrium, and uterine cervix carcinomas) revealed a great variability in the expression of the nucleoside transporters. In fact, hCNT1 expression was negative in a great number of tumors. The loss of its expression was highly correlated with poor prognosis, whereas hENT1 and hENT2 expression was retained all tumors (Farré et al. 2004). Also, other studies, with different types of tumors, on nucleoside transporter expression profiles showed downregulation of hCNTs, mainly in hCNT1 as summarized in (Table 2) (Zollner et al. 2005; Lane et al. 2010; Mohelnikova-Duchonova et al. 2013; Mey et al. 2006; Marçal Pastor-Anglada and Pérez-Torras 2015).

Table 2. Nucleoside transporters expression in tumor tissues.

Transporter	Tumor	Assay	Expression	References
hCNT1	Breast	IHQ	Decreased	(Lane et al. 2010)
hCNT1	Pancreas	qRT-PCR, WB	Decreased	(Bhutia et al. 2011)
hCNT1	Ovary	IHQ	Decreased	(Farré et al. 2004)
hCNT1	Endometrium	IHQ	Decreased	(Farré et al. 2004)
hCNT1	Uterine cervix	IHQ	Decreased	(Farré et al. 2004)
hCNT1	Pancreas	qRT-PCR	Decreased	(Mohelnikova-Duchonova et al. 2013)
hCNT1	Hepatocarcinoma	qRT-PCR	Decreased	(Zollner et al. 2005)
hCNT1	Bladder	qRT-PCR	Decreased	(Mey et al. 2006)
hCNT3	Pancreas	qRT-PCR	Decreased	(Mohelnikova-Duchonova et al. 2013)
hENT1	Breast	IHQ	Decreased	(Lane et al. 2010)
hENT1	Pancreas	qRT-PCR	Decreased	(Mohelnikova-Duchonova et al. 2013)

Adapted from (Marçal Pastor-Anglada and Pérez-Torras 2015)

9. Transporters as Transceptors

Transceptors are membrane proteins that hold a solute transport as well as a receptor signaling activities. The term Transceptors comes from the contraction of **tran**sporter and rece**ptor**. New emerging roles are recently reported for a number of different solute transporters. These roles were implicated in cell signaling processes and were independent of their substrate-transporting properties. Several identified transceptors demonstrated to control multiple downstream processes. However, the separation between the transporter uptake activity and/or its substrate and other responses provoked by the substrate has led to the knowledge that the transporter might act as a sensor.

The first transceptor described was involved in the activation of the Protein kinase A (PKA) pathway. This transceptor belonged to the nutrient-sensing system of the yeast (Thevelein and Voordeckers 2009). As a matter of fact, this paradigm has facilitated further similar studies to be conducted on several candidate nutrient transceptors in other eukaryotes. Thus, many other examples of nutrient transceptors have been reported or suggested. As in yeast, most of the transceptors have been identified for their responses to the absence of their substrate or to substrate re-supplementation. Hence, these transceptors generally function in specific processes regulated by the transceptor substrate and related to some extent to the uptake and/or metabolism of the substrate (Steyfkens et al., 2018).

Moreover, transceptor function seems to be initiated by ligand-binding that leads to a conformational change of the transporter (the signal), which is transduced into a cytoplasmic effector. This signal is usually transduced through an interacting protein at the inner side of the plasma membrane, which transmits the signal and modifies gene expression accordingly, resulting in different downstream processes, including ubiquitination, endocytic turnover or signaling (Figure 9) (Kriel et al. 2011; Diallinas 2017). In fact, substrate availability is sensed by most membrane transceptors, which lead to activate cell signaling cascades when required. These transceptors include different transporters of a wide variety of substrates like amino acid, glucose, sulfur, and nitrate. Furthermore, some transporters have been linked to important pathological events such as oncogenesis, this has been reported for iodide, amino acid, monocarboxylate and nucleoside transporters (Pinilla et al. 2011; Lacoste et al. 2012; Coothankandaswamy et al. 2013; Pérez-Torras et al. 2013).

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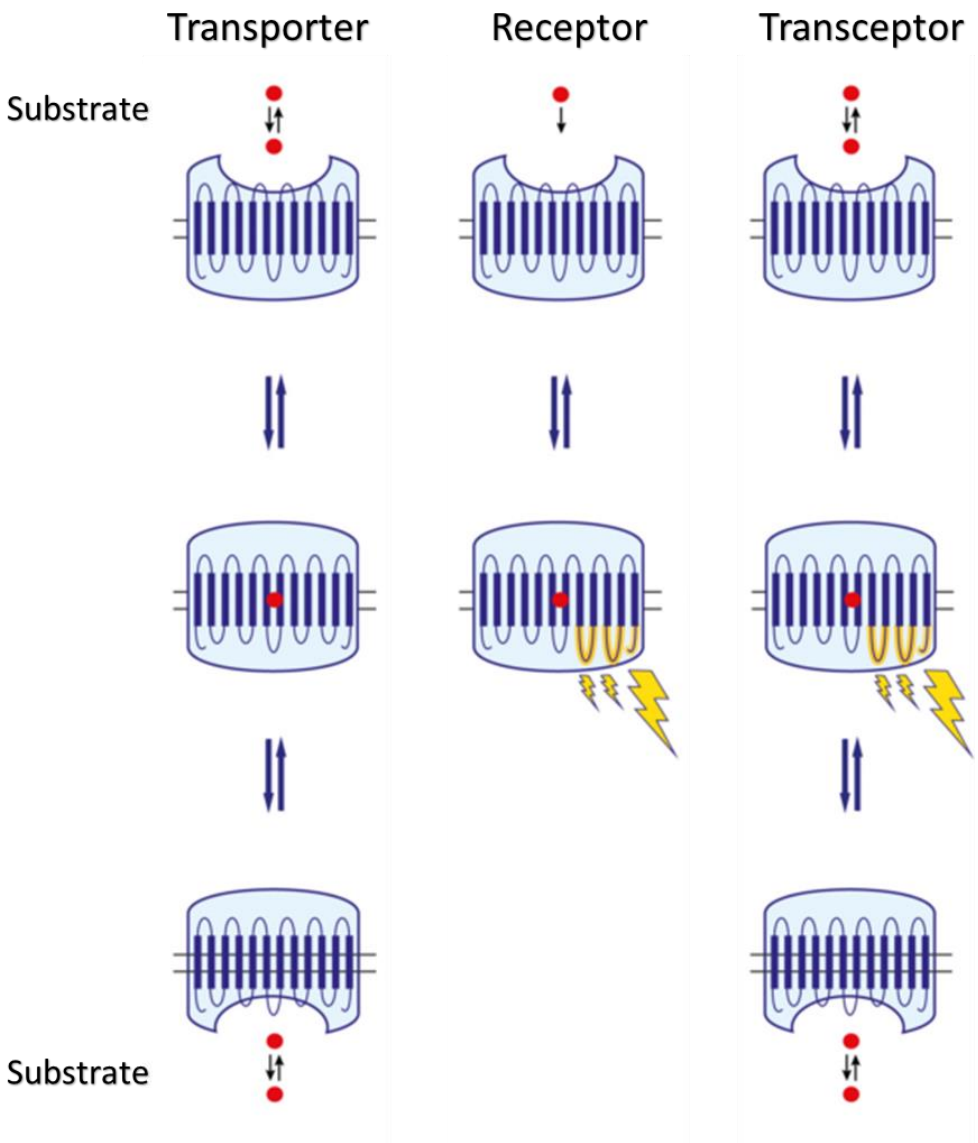


Figure 9. Illustration of transporter, receptors and transceptor proteins function. The binding to- and/or transport of- the substrates activates intracellular signaling. Adapted from Steyfkens et al., 2018.

9.1. Nucleoside transporters as transceptors

Recently, we have demonstrated that hCNT1 restoration in Pancreatic ductal adenocarcinoma (PDAC) cell lines is able to induce cell-cycle arrest, trigger changes in some intracellular signaling cascades, increase cell death by a non-apoptotic mechanism, and inhibit cell migration (Pérez-Torras et al. 2013). More importantly, all these events can also be induced when expressing a mutated hCNT1 protein that localizes to the plasma membrane but lacks the ability to translocate substrates (Figure 10). Remarkably, hCNT1 protein restoration can also inhibit tumor growth in a mouse model of pancreatic adenocarcinoma (Pérez-Torras et al. 2013). These observations argued in favor of hCNT1 being a transceptor protein. Moreover, there are some evidences suggesting the involvement of other nucleoside transporters in the regulation of some cellular processes. For example, hCNT2 function was related to the regulation of purinergic signaling and cellular energy metabolism (Duflot et al. 2004; Aymerich et al. 2006; Huber-Ruano et al. 2010). In addition, hENT1 has been reported as a modulator of epithelial-to-mesenchymal transition (EMT) in proximal tubular cells (Guillén-Gómez et al. 2012).

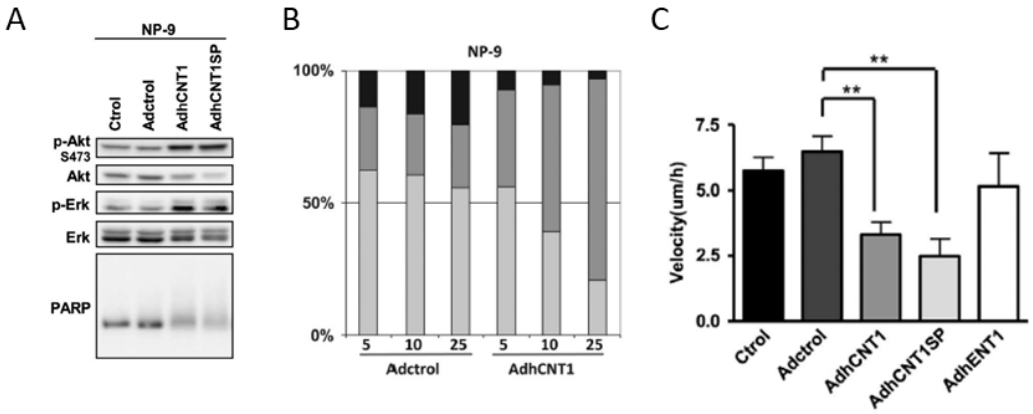


Figure 10. hCNT1 effects are translocation-independent. In PDAC cells, hCNT1 induced changes (A) in some signaling cascades and (B) cell-cycle profile (G1 (gray), S (dark gray) and G2 (black) phases). (C) It also inhibited cell migration in a translocation independent manner. Adapted from Pérez-Torras et al. 2013.

10. Other Implications of the nucleoside transporters

10.1. As drug transporters

Nucleoside transporters are involved in the cellular uptake of nucleoside analog drugs, which is a necessary step for the cytotoxic actions of most of these drugs. Nucleoside analogs are among the first chemotherapeutic agents. They are used in the treatment of malignant diseases and their activity is well established, showing a wide clinical use. Nucleoside anticancer drugs include analogs of pyrimidine and purine nucleosides.

Most nucleoside analogs share a similar mechanism of action when used for cancer treatment (Parker 2009). They internalize into cells where they are converted to nucleotide analogs by the enzymes of the metabolic pathway of pyrimidines or purines. Nucleotide drugs can then incorporate into nucleic acids and, in most cases, inhibit the DNA synthesis enzymes, causing DNA damage and induction of apoptosis. Differences in selectivity to these nucleoside analogs can also be found and they often rely upon their ability to interact with enzymes of the pyrimidine and purine salvage pathways.

Efficacy of pyrimidine analogs is shown against both solid cancer tumors and blood cancers, while purine nucleosides analogs work against hematological malignancies almost exclusively. Thus, the distribution of the transporters among different tissues has a large impact on their therapeutic effect. Gemcitabine, cytarabine, and azacytidine are pyrimidine analogs. They can be transported by hCNT1 in addition to hCNT3, hENT1 and hENT2 (Errasti-Murugarren et al. 2007; Smith et al. 2004; Arimany-Nardi et al. 2014; Mackey et al. 1999; Endo et al. 2007; Clarke et al. 2006; Errasti-Murugarren and Pastor-Anglada 2010). Moreover, purine-based nucleoside analogs such as cladribine, fludarabine, and clofarabine are substrates for hCNT2, hCNT3, hENT1, and hENT2 (Lang et al. 2001; M Molina-Arcas et al. 2005; Owen et al. 2005; King et al. 2006; Errasti-Murugarren and Pastor-Anglada 2010). In addition, some antiviral drugs are also nucleoside-derived. The management of several viral infections, including HIV, HBV, HSV, HCV, and CMV infections, can be controlled by the delivery of these nucleoside derivatives. In fact, these antiviral agents are considered potent due to their ability to inhibit viral DNA polymerases and reverse transcriptases, which play important roles in the viral life cycles. hCNT3 is one of the nucleoside transporter proteins that particularly, but not

exclusively, interact and translocate different types of antiviral drugs, such as ribavirin and zidovudine, among others (Errasti-Murugarren et al. 2007; Hu et al. 2006).

10.2. As biomarkers

Several factors may influence the transporter function, which is likely to be variable among individuals (Prasad et al. 2013; Sakamoto et al. 2013). Thus, inter-individual heterogeneity in response to therapy can be related to inherent transporter function variability among patients. It can also be related to altered transporter expression in target cells, even as a result of the disease itself. Thus, the transportome profile associated with its pharmacogenetics might prove suitable for treatment outcomes prediction and should be helpful in decision making processes, such as in treatment choices for drugs and dosages, or in anticipating drug-drug interactions when combination schedules of treatments are needed. Drug transporters can often be inhibited by other drugs or endogenous substrates, typically through the competition for recognition and binding. These interactions may even result in altered drug pharmacokinetics.

The expression of nucleoside transporters might be a biomarker of disease prognosis by itself, although all available data are still controversial. This could be explained by the fact that almost all studies done have included treated-patients. Accordingly, hENT1 itself has been related to EMT (Guillén-Gómez et al. 2012; Lee et al. 2014). Furthermore, as mentioned earlier, the loss of hCNT1 expression in numerous gynecologic tumors was observed in a high number of cases than hENT1 and hENT2. This loss was highly correlated with poor prognosis histotypes gynecological tumors (Farré et al. 2004). Moreover, hCNT1 alone has exhibited a prognostic value for disease-free survival and risk of relapse in breast cancer (Gloeckner-Hofmann et al. 2006). Therefore, it is likely that these proteins also can serve as prognostic biomarkers.

Hypothesis and objectives

II. Hypothesis and objectives

Several examples of SLC membrane transporters with additional regulatory roles have recently been reported in the literature (Tanaka et al. 2014; Lacoste et al. 2012; Pinilla et al. 2011). As for hCNT1, a side of being a nucleoside transporter, it was reported by us, to have an emerging role in cell biology. We have introduced earlier that restoration of hCNT1 in pancreatic cancer models affects the cell physiology independently of its transporting ability (Pérez-Torras et al. 2013).

Upon this background, this thesis was based on the general hypothesis that hCNT1 is a transceptor signaling to modulate a variety of cellular functions and its loss might contribute to oncogenesis.

Therefore, we aimed to:

- Explore the mechanisms implicated in hCNT1 loss in tumor cells by understanding how hCNT1 transcription is regulated.
- Ascertain the mechanisms by which hCNT1 protein modulates cell biology.
- Study the hCNT1 S100A11 putative interactor to determine how signals can be transduced inside the cell.

Results and Discussion

III. Results and Discussion

1. Expression of hCNT1 in tumors

1.1. Loss of hCNT1 expression

Human concentrative nucleoside transporter 1 is implicated in the cellular uptake of nucleosides and several nucleoside-derived anticancer drugs. Numerous studies have been done investigating the role of nucleoside transporters in cancer chemotherapy and drug resistance using anticancer nucleoside analogs. In our laboratory, several projects are now in progress to better understand how hCNT1 transcriptional and post-translational expression is regulated, how it is lost in cancer, and what roles hCNT1 might be playing in cell biology.

1.1.1. Expression of hCNT1 in digestive system tumors

As mentioned earlier, previous studies have reported reduced hCNT1 expression in solid tumors, e.g. hepatic, gynecologic and breast cancers (Farré et al. 2004; Zollner et al. 2005; Lane et al. 2010; Bhutia et al. 2011). Hence, to investigate more on hCNT1 expression in cancer, we analyzed under the doctoral research project of Clara Boces the relative mRNA expression levels of hCNT1 in paired samples taken from Colorectal cancer (CRC), PDAC, and HCC patients. By means of relative real-time polymerase chain reaction (RT-PCR), we found that hCNT1 mRNA levels were decreased in all three types of the analyzed cancer tissues, as compared with their non-tumoral counterparts (Figure 11). These results demonstrated a significant decrease of hCNT1 mRNA levels in HCC samples, while this reduction seemed less dramatic in PDAC and CRC samples.

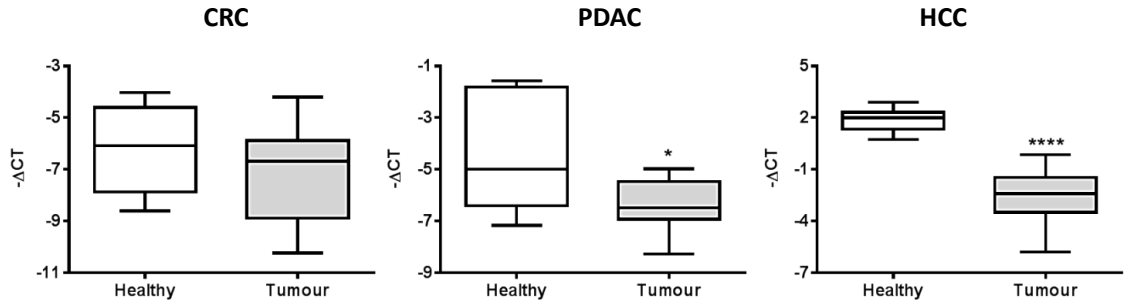


Figure 11. Endogenous hCNT1 mRNA is reduced in tumoral versus non-tumoral adjacent tissues. Relative amounts of hCNT1 mRNA determined by RT-PCR in CRC, PDAC and HCC tumors and matching adjoining non-tumoral tissue. Results are means ± S.E.M. (n=12 for CRC, n=9 for PDAC and n=22 for HCC). Statistical significance was determined with Student's t-test; *P<0.05, ****P<0.0001.

1.1.2. Expression of hCNT1 in HCC derived cell lines

Due to the dramatic decrease of hCNT1 expression in HCC samples, we decided to assess hCNT1 expression at mRNA and protein levels in two new tumor cell lines derived from hepatocellular carcinoma, BCLC3 and BCLC5, generated at the laboratory of Dr. Jordi Bruix (Hospital Clinic, IDIBAPS & CIBERehd). The analysis were performed by means of RT-PCR and immunoblotting. As expected, the results demonstrated decreased mRNA levels as compared to normal hepatocytes (Figure 12A). In addition, no protein expression was detected, in both cell lines, when immunoblotted against two different hCNT1 antibodies (Figure 12B) (hCNT1 mAb, a monoclonal antibody raised in our laboratory, and hCNT1 n-17, a commercial polyclonal antibody). HEK-hCNT1 cells (HEK293 cells stably transfected with hCNT1, generated in our laboratory) were used as a positive control for hCNT1 expression.

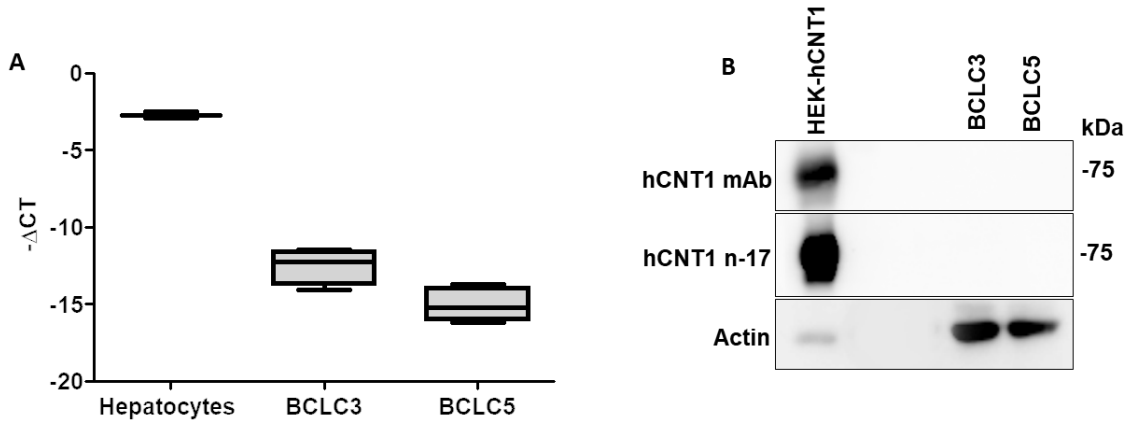


Figure 12. hCNT1 expression in HCC derived cell lines, BCLC3 and BCLC5. (A) mRNA expression of hCNT1 was determined by RT-PCR primary human hepatocytes and in BCLC cells. (B) Representative western blot of hCNT1 protein expression in BCLC3 and BCLC5 immunoblotted using two different hCNT1 antibodies. HEK-hCNT1 cell extract were used as a positive control for hCNT1 (20µg of HEK-hCNT1 and 50µg of BCLC3 and BCLC5 were loaded). Results are means ± S.E.M. (n=2 for hepatocytes), (n=3 for BCLC3 and BCLC5).

Altogether, hCNT1 expression among the analyzed CRC, PDAC, and HCC tissues including HCC cell lines, showed evident loss in both mRNA and protein levels. These results are in line with previous data showing frequent loss of hCNT1 in the human tumors (e.g. HCC and PDAC), and with the fact that CNTs expression is associated with fully differentiated cell types (Soler et al. 1998; Pastor-Anglada et al. 1998; Dragan et al. 2000). Moreover, reduced or complete loss of hCNT1 expression is likely to be, aside to multiple factors, a possible contributor to chemo-resistance in tumors treated with nucleoside analogs, causing poor drug responses.

On the other hand, reduced hCNT1 expression in tumor tissues could be a sign of the tumorigenicity linked to hCNT1 loss. This would further support the possibility of the putative tumor suppressive role of hCNT1 on cell cycle progression and proliferation (Pérez-Torras et al. 2013), which would represent an additional unique set to study the molecular events leading to oncogenesis.

1.2. Finding a cell model retaining hCNT1 expression

The frequent loss of hCNT1 in various tumors and tumor derived cell lines encouraged us to study its probable role in oncogenesis, and further investigate the relevance of hCNT1 expression changes. Hence, we planned to undertake both hCNT1 silencing and restoration of expression studies in normal and cancerous cells respectively. However, a major bottleneck here is the availability of cell lines retaining hCNT1 expression. Most immortalized and tumoral cell lines we previously investigated, including the new BCLC cells, lack hCNT1-related activity and show very low or even negligible expression of the hCNT1 protein. This fact further supports the view that hCNT1 protein is highly associated with differentiated cell types.

1.2.1. Analysis of hCNT1 mRNA expression

We analyzed a panel of cell lines to find a cell model retaining hCNT1 expression. Firstly, we performed mRNA expression analysis of hCNT1 in different human non-transformed cell lines and primary culture, in which we anticipated hCNT1 expression. Cells used were obtained from renal (i.e. ciPTEC) or hepatic (i.e. HepaRG, THLE-2 and human primary hepatocytes) origin, which are known to express hCNTs normally.

Thus, we analyzed *SLC28A1* gene expression in the hepatic cell models HepaRG and THLE-2 as well as in the ciPTEC renal cells (at proliferating ciPTEC 14 or mature ciPTEC 14.7 states). The results indicated extremely low transcript levels of hCNT1 mRNA, with cycle threshold (Ct) values higher than 35, showing levels of hCNT1 mRNA similar to those found in the HCC derived cell lines mentioned above. On the other hand, we analyzed hCNT1 endogenous expression in freshly isolated primary human hepatocytes (under the Ph.D. project of Sandra Moro). mRNA hCNT1 expression over different time points of proliferation (from Day 0 to Day 6 of isolation) showed significant high levels of hCNT1 mRNA copies (Figure 13A), with nearly 10^8 mRNA copies per one microgram of total RNA, reaching the same level as before cell

culture (Day 0). In addition, we did a comparison of the -ΔCt of the evaluated cells in Figure 13B. All cell lines showed a major decrease of hCNT1 transcriptional expression when compared to the primary hepatocyte culture cells.

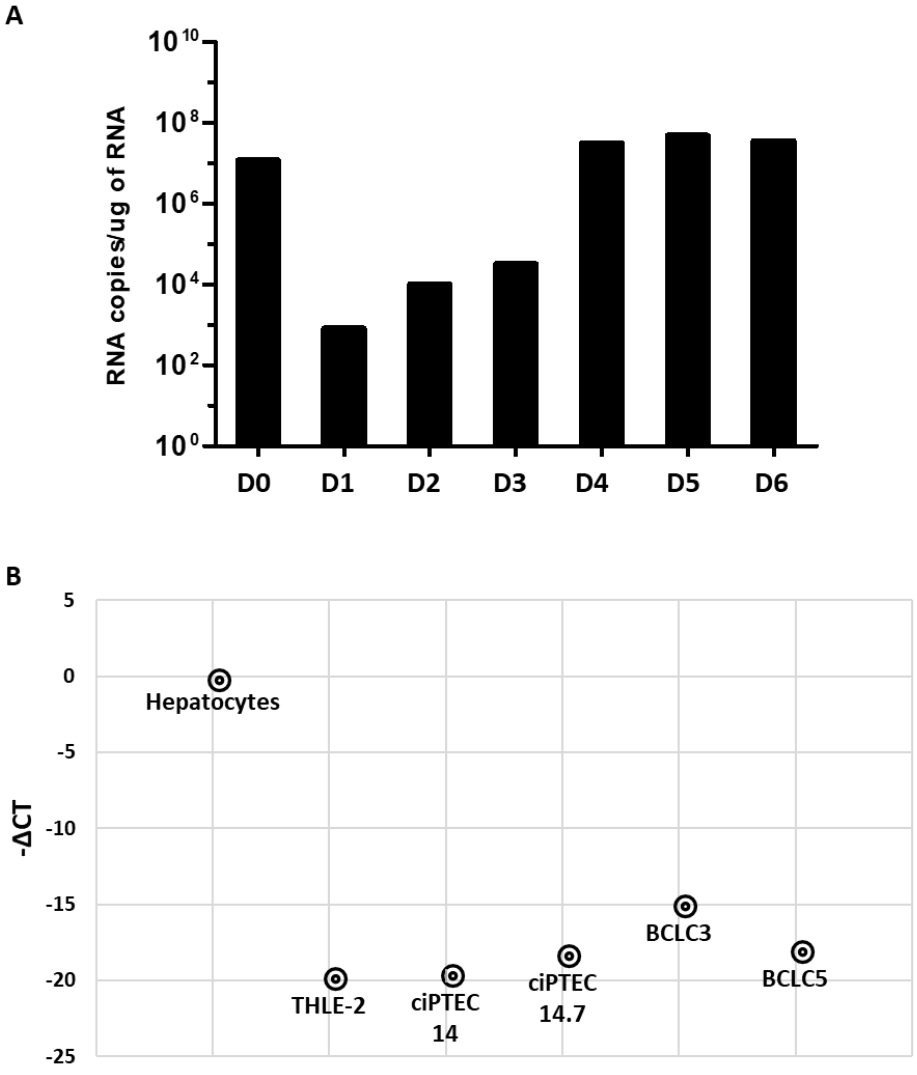


Figure 13. hCNT1 mRNA expression in a panel of cells. (A) hCNT1 mRNA copies in primary human hepatocytes (from Day 0 (D0) to Day 6 (D6) of isolation) determined by absolute quantitative RT-PCR. (B) A comparative graph of hCNT1 mRNA expression levels using the (-ΔCt) values of cells from hepatic origin (i.e. primary human hepatocytes, THLE-2, BCLC3 and BCLC5) and cells from renal origin at proliferating (ciPTEC 14) or mature (ciPTEC 14.7).

1.2.2. Analysis of hCNT1 protein expression

Regarding protein expression, ciPTEC and HepaRG cells were chosen for their ability for ''maturation'' and ''differentiation'', respectively, as we know that basal level of nucleoside transporter expression depends on the differentiation status of the cell. ciPTEC cells are conditionally immortalized human proximal tubule cells mimicking renal reabsorption and excretion. This cell line proliferates at 33°C and becomes mature at 37°C (Wilmer et al. 2010). Thus, using hCNT1 mAb antibody, we immunoblotted ciPTEC cells for hCNT1 protein in both proliferating and mature states. Unfortunately, no protein bands were observed for hCNT1 expression in both cases (Figure 14A).

Furthermore, HepaRG are human hepatic progenitor cells. These cells were one of our candidates because of their capability to be differentiated into fully functional hepatocyte-like cells (Hart et al. 2010). We have obtained HepaRG total cell protein extracts to evaluate hCNT1 protein expression at full confluence and at differentiated cell states. By immunoblotting against hCNT1, the hCNT1 endogenous protein was not detected at its reported corresponding molecular weight 72kDa, using three different hCNT1 antibodies (hCNT1 mAb and two commercial hCNT1 antibodies i.e. hCNT1 c-14 and hCNT1 n-17). Instead, in confluent cells, a protein band was detected with hCNT1 mAb at a higher molecular weight around ~ 100kDa, but then this expression appeared to be lost when cells progressed into a more differentiated state. This higher molecular weight band was not detected using the other hCNT1 antibodies (c-14, n-17). Moreover, in all the HepaRG samples, we noticed another constantly appearing protein band at a molecular weight less than hCNT1 known molecular weight (i.e. 72kDa) when using all three hCNT1 antibodies (Figure 14B). We believed these bands at 60kDa and at 100kDa might be non-specific, although, post-translational modification cannot be discarded. Thus, very recent results achieved in our laboratory revealed different molecular weights of hCNT1 protein expression due to its post-translational modification. However, even if these bands were hCNT1, functionality must be demonstrated, because up to what we know not all the post-transitional modifications of hCNTs seem to result in a functional protein. Lastly, hCNT1 protein expression in primary human hepatocyte samples was firmly expressed over different time points of proliferation (from Day 0 to Day 6 after isolation) at around the same higher molecular weight observed with HepaRG i.e. 100kDa (Figure 14C).

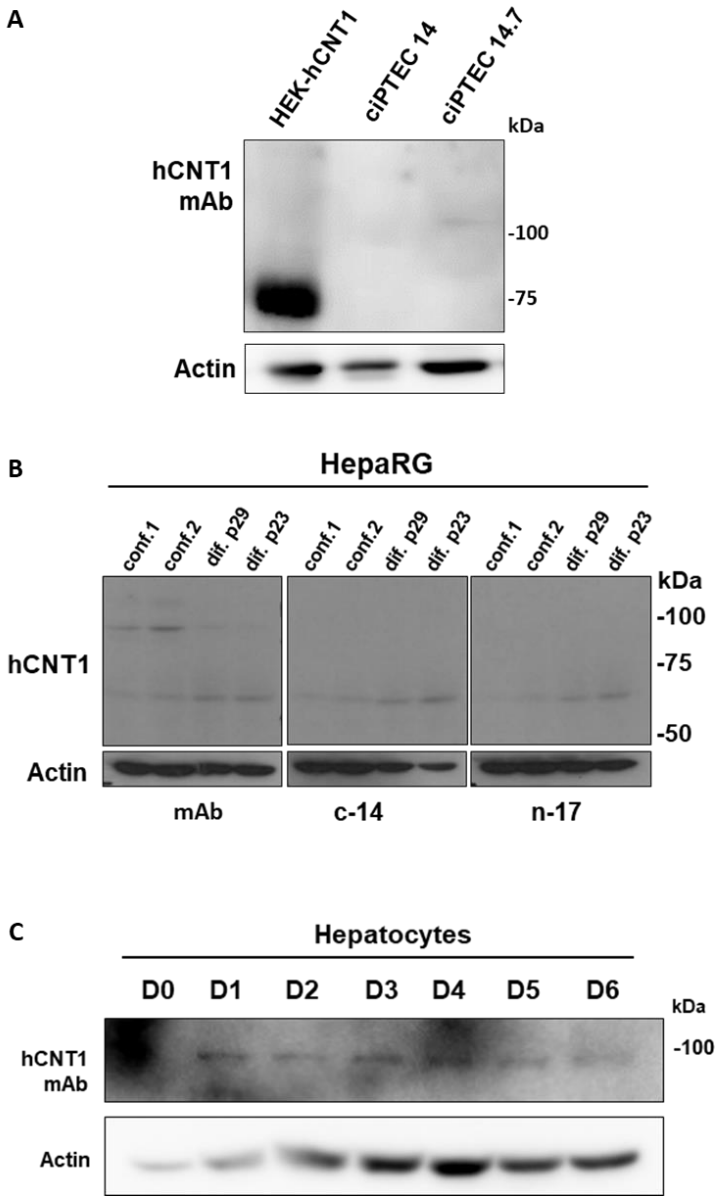


Figure 14. Endogenous hCNT1 protein expression. By immunoblotting against hCNT1, protein expression was detected in: (A) cells from renal origin at proliferating (ciPTEC 14) or matured (ciPTEC 14.7) state. HEK-hCNT1 cell extract was used as a positive control for hCNT1). And in (B) HepaRG cells at confluent (conf.1 & conf.2) and differentiated (diff. p29 & diff. p23) state. While for (C) human primary hepatocytes, samples were analyzed from Day 0 to Day 6 after isolation. hCNT1 antibody used is indicated.

Unfortunately, the results of hCNT1 expression analysis indicated very low levels of mRNA and protein, which made it difficult to carry on with our hCNT1 experiments in these candidate cell lines. This was not the case for primary hepatocytes. However, a major bottleneck was the difficulty to obtain these cells, and the inability to expand primary hepatocytes in vitro while maintaining proliferative capacity and cellular functions. Thus, we can get them occasionally to perform specific experiments, to clarify and confirm several concepts along this thesis.

Altogether, hCNT1 expression in primary human hepatocytes versus BCLC tumor cell lines demonstrated a huge decrease of expression and more likely hCNT1 function. This, again, is in line with the fact that hCNT1 expression is a characteristic of differentiated hepatocytes. On the other hand, the decrease of hCNT1 expression in ciPTEC, HepaRG and THLE-2 cell lines could be related to some kind of loss of differentiated features of cell culture. In fact, several immortalized cell lines use to lose their original differentiated functions. For instance, a traditionally encountered problem with primary hepatocyte cultures is their rapid dedifferentiation. This is reflected not only in a decrease of some liver-specific functions, but also in a dedifferentiated morphology: the cells flatten, depolarize, and lose many of the surface characteristics of normal hepatocytes *in vivo* (Arterburn et al. 1995; Runge et al. 2000). Nonetheless, we will be keeping on searching for suitable cell models with differentiated cell functions mimicking the original tissues, in which hCNT proteins are still expressed, and cells are more accurately recapitulating human disease. This would provide a superior model for pre-clinical evaluation of novel targeted therapies.

1.2.3. Transcriptional regulation of hCNT1 expression

To further understand why hCNT1 is being lost in oncogenesis, we focused a part of our study on its transcriptional regulation, which might be governed by different mechanisms, such as epigenetic regulation. The Poly [ADP-ribose] polymerase 1 (PARP-1) enzyme has been intensively described as a gene transcriptional regulator. For instance, PARP-1 can regulate chromatin structure and positively modulate RNA polymerase II activity. In addition, PARP-1 can regulate gene expression by direct interactions with DNA elements or binding factors (Weaver and Yang 2013; Schiewer and Knudsen 2014).

We have shown that hCNT1 restitution in cancer cell lines induces PARP-1 hyperactivation, concomitantly with an accumulation of poly ADP-ribose (PAR) polymers (Pérez-Torras et al. 2013). PARP-1 role in transcriptional regulation is complex, it involves the protein *per se* as well as its related PARP-1 activity (PARylation) as positive co-factors, being also a negative controller in some conditions. In this sense, specific transcription factors such as YY1, p53, CREB, Sp1, and NFkB are prevented from binding to their respective recognition sequence if PARylated (Beneke 2012). The analysis of hCNT1 promoter sequence using MatInspector software has revealed putative transcription binding sites for p53, CREB, Sp1, and NFkB. Moreover, PARP-1 activity is needed to prevent spreading of heterochromatic regions by inhibition of DNA-methyltransferase 1 (DNMT1) playing a direct role on epigenetic mechanisms (Beneke 2012). Interestingly, it has recently been reported that the expression of the sodium-iodide transporter (NIS; SLC5A5), a member of SLC family that also acts as a transceptor regulating cancer cell motility and invasiveness (Lacoste et al. 2012) is partially governed by PARP-1 (W. Li and Ain 2010; Lavarone et al. 2013). Thus, we hypothesized that PARP-1 hyperactivation, observed after hCNT1 restoration, might be a response contributing to diminish hCNT1 expression and suggesting a possible role in hCNT1 loss in tumor cells.

A. Regulation of hCNT1 expression by PARP-1

In order to study whether PARP-1 is involved in the regulation of *SLC28A1* gene expression, we have checked whether PARP-1 is hyperactivated when hCNT1 is overexpressed in the HCC cell models, BCLC3 and BCLC5. This was performed by transducing these cells with AdhCNT1 or Adctrol (Adenoviral vectors carrying the hCNT1 gene or the empty cassette, respectively) at 1, 5 and 10 MOI (multiplicity of infection). Then, cell lysates were immunoblotted against PARP-1 (Figure 15). We observed that hCNT1 overexpression actually promoted PARP-1 hyperactivation in both BCLC cell lines.

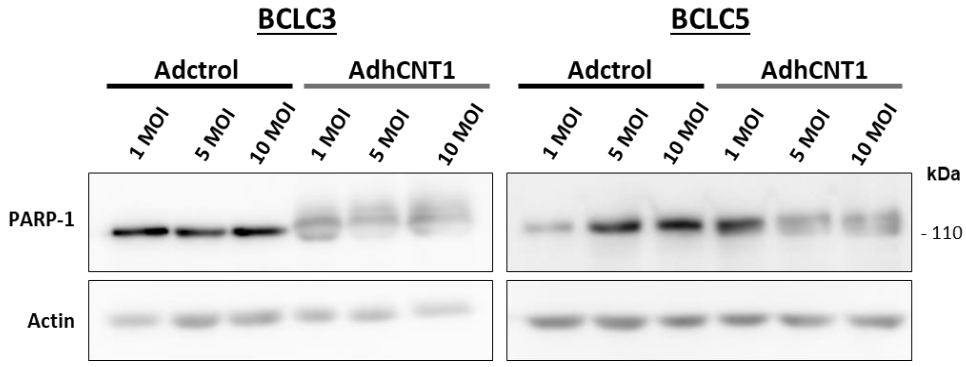


Figure 15. hCNT1 overexpression hyperactivates PARP-1. BCLC cells were infected with AdhCNT1 or Adctrol (as a negative control) at different MOIs. Total cell extracts were obtained 48h after infection and immunoblotted for PARP-1 (n = 4).

Hyperactivation of PARP-1 allowed us furtherly to investigate PARP-1 role on hCNT1 transcriptional regulation by inhibiting PARP-1 in BCLC cells, and afterwards analyzing possible changes in hCNT1 mRNA transcription levels. Thus, we started by assessing the cytotoxicity of two different PARP inhibitors on BCLC3 and BCLC5 by MTT cell viability assay. We used: (1) PJ34, a commonly used PARP-1 inhibitor, and (2) Olaparib, which is one of the recent generation PARP-1 inhibitors that has greater specificity to PARP-1 isoform (Nile et al. 2016) (Figure 16). We observed that both inhibitors did not display cytotoxic effects on any of the cell lines at concentrations up to 25µM in which minimal toxic effect on cell viability started to be observed. Thus, we used the 25µM dose along with another lower dose at 1µM of PJ34 and Olaparib to proceed with PARP-1 inhibition experiments.

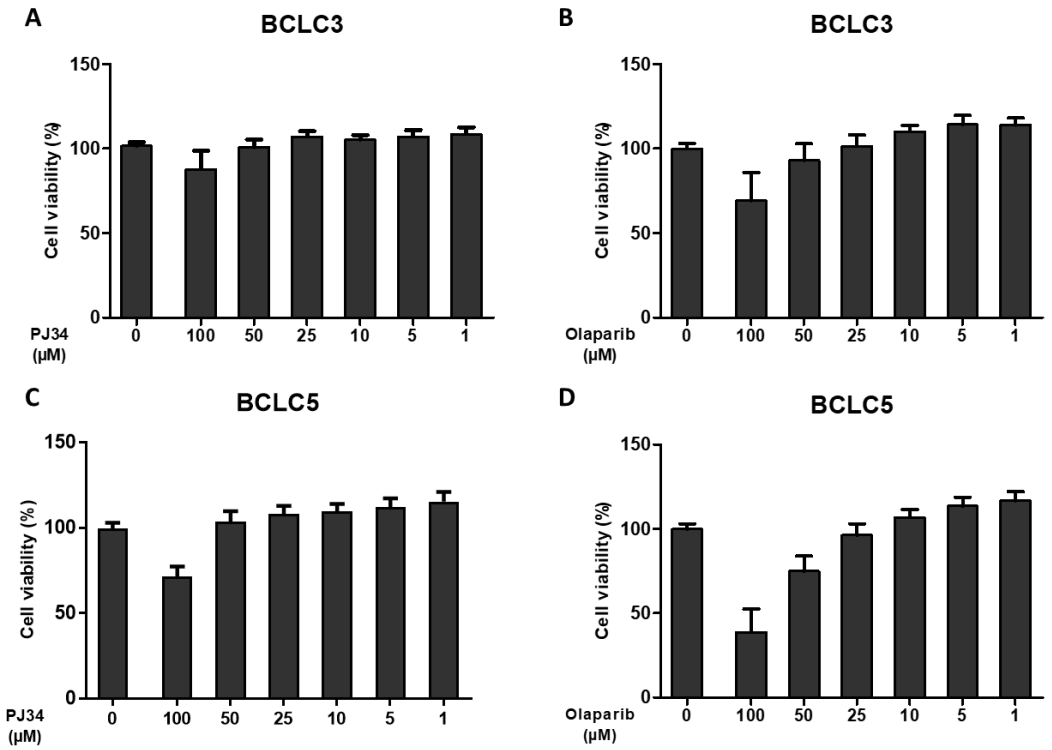


Figure 16. Cytotoxic analysis of PARP-1 inhibitors in BCLC cells. Cell viability of BCLC3 and BCLC5 cells after treatment with different concentrations of PJ34 (A & C) Olaparib (B & D) for 72h. Cell viability was assessed by MTT assay. The results are expressed as the percentage of viable cells versus non-treated control cells. Results are means ± S.E.M (n=3).

Following the approach used with PARP-1 for analyzing its transcriptional regulation of the hNIS transporter (Lavarone et al. 2013; W. Li and Ain 2010), we measured by RT-PCR the endogenous hCNT1 mRNA expression in BCLC3 and BCLC5 cell lines under basal culture conditions and following PJ34 or Olaparib treatment for three days. After normalization to the GAPDH mRNA expression, we observed different changes in the endogenous hCNT1 levels among the different conditions after PJ34 or Olaparib treatment (Figure 17). Generally, in both treated cell lines we observed a tendency to increase hCNT1 mRNA levels compared to their control cells. BCLC5 showed more sensitivity than BCLC3 to both PARP-1 inhibitors, with up to 2- to 8-fold change of hCNT1 mRNA levels in BCLC5 versus 0.5- to 1.5-fold change in BCLC3. However, we noticed that these changes in hCNT1 mRNA levels showed large variation among several independent repeated experiments, and thus the results were not statistically significant.

In addition to hCNT1, we have also examined whether PARP-1 is capable of activating *SLC28A2* (hCNT2) and *SLC28A3* (hCNT3) gene expression (Figure 18). In BCLC5 we saw up to

5-fold increase in hCNT2 mRNA expression levels under Olaparib treatment and up to 2-fold under PJ34 treatment in BCLC3. Regarding hCNT3, only BCLC5 showed an increase of expression following PJ34 treatment. For the rest of the treated conditions, hCNT2 and hCNT3 did not show much changes in their mRNA expression respect to their control cells.

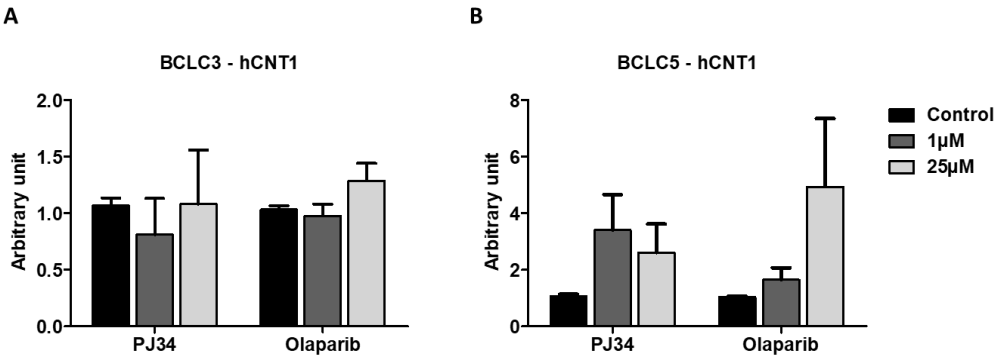


Figure 17. hCNT1 mRNA expression under PARP-1 inhibitors. (A) BCLC3 and (B) BCLC5 cells were treated with 1µM and 25µM dose of either PJ34 or Olaparib for 72h and hCNT1 expression was detected by RT-PCR. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test.

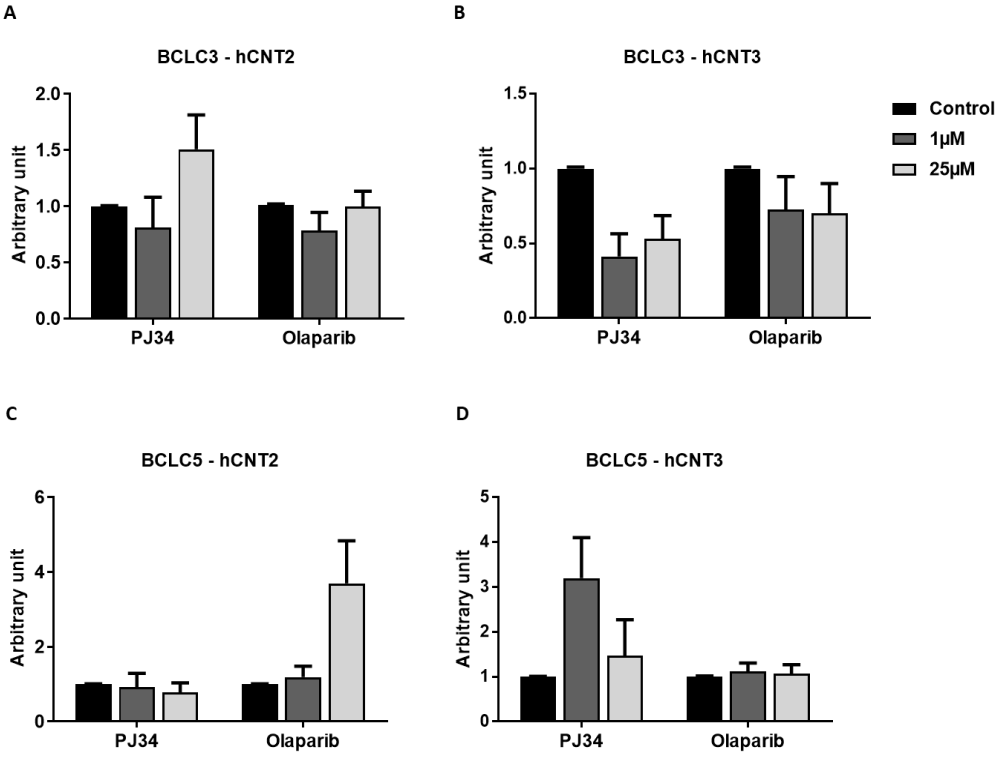


Figure 18. hCNT2 and hCNT3 mRNA expression under PARP1 inhibitors. (A and B) BCLC3 and (C and D) BCLC5 cells were treated with 1µM and 25µM dose of either PJ34 or Olaparib for 72h. hCNT2 and hCNT3 expression was detected by RT-PCR. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test.

B. PARP-1 transcriptional regulation on hCNT1 promoter

To further study whether PARP-1 is regulating hCNT1 expression through promoter association, we investigated the transcriptional activity of hCNT1 putative promoter under PJ34 or Olaparib treatment using the dual-luciferase reporter assay. We measured luciferase activities from four hCNT1 promoter reporter constructs (generated under the thesis project of Aida Mata), each with a different length covering the putative hCNT1 promoter cloned in pGL3-basic luciferase plasmid (Figure 19A).

Thus, we transiently co-transfected promoter constructs into HT29 cells (a CRC cell line in which hCNT1 promoter constructs were optimized for transfection and activity) along with Renilla luciferase plasmid (pRL-TK), and then treated them with either PJ34 or Olaparib. The results among the different conditions in this assay showed an increase in luciferase activity, after normalization to Renilla luciferase, when compared to their corresponding promoter control (pGL3-basic) of each condition (Figure 19B). On the other hand, the values obtained for the internal control activity (Renilla luciferase) to normalize the transfection efficiency among replicates, were affected by both PARP-1 inhibitors. Renilla luciferase activity under PJ34 treatment was upregulated by 2- to 5-fold over three independent experiments, whereas under Olaparib treatment a 1- to 4-fold increase was observed when comparing to non-treated condition as a control (Figure 19C).

Altogether, we noticed that the effect of PARP-1 inhibition on hCNT1 expression was highly variable among repeated experiments, whereas the results of their effect on hCNT1 putative promoters activity were interfered by the effect of both inhibitors on the activity of the internal control gene used for the dual-luciferase reporter assay.

Nonetheless, the use of Renilla luciferase plasmid (pRL-TK), as an internal control was affected by PARP-1 inhibitors, which means that it is possible that PARP-1 might interact with its regulatory elements or directly on its promoter. Choosing another Renilla luciferase plasmid compatible with our experimental conditions or using the promoterless Renilla (pRL-Null) plasmid as an internal control might overcome this problem. Additionally, other approaches of normalization might also be considered, such as the flow cytometric quantitation of GFP expression or the measurement of total protein (Gerdes and Kaether 1996; Dandekar et al. 2005).

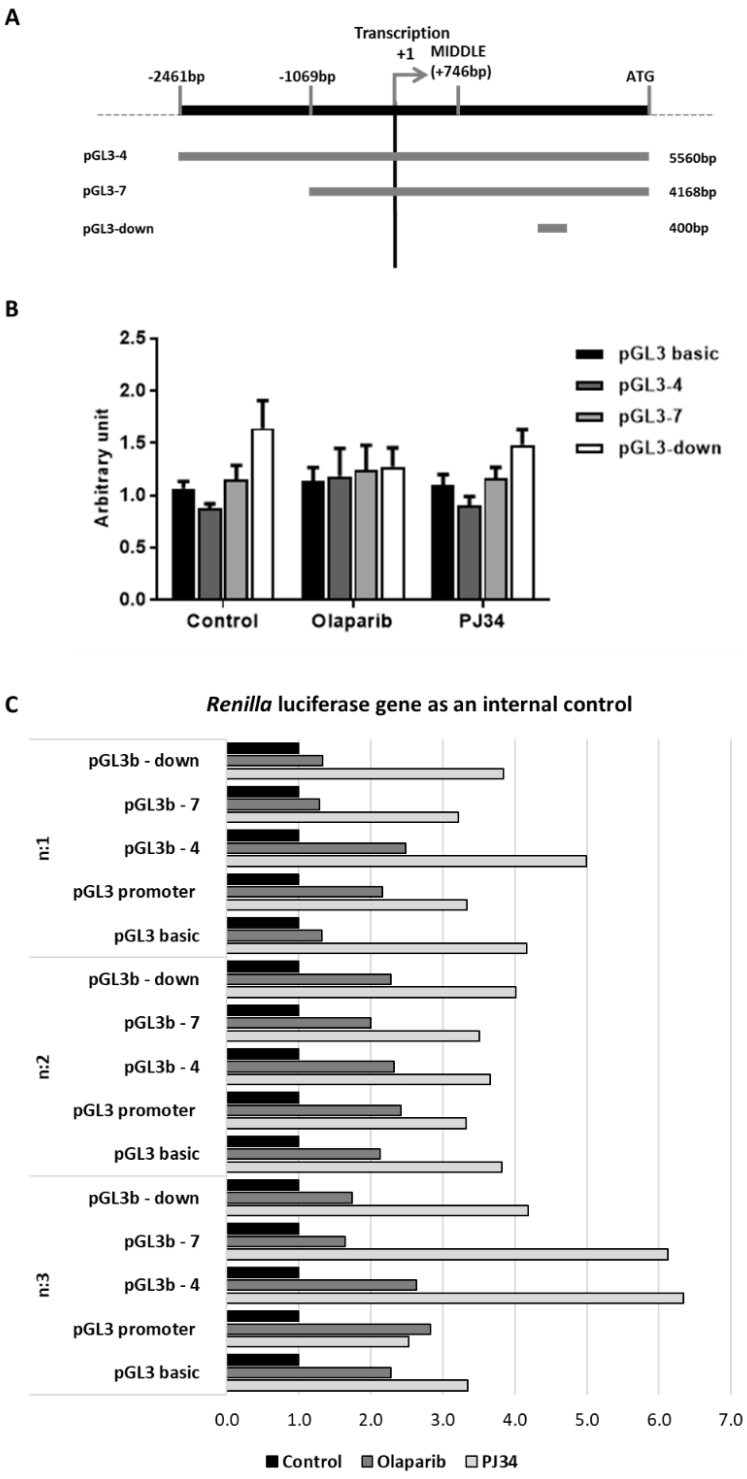


Figure 19. hCNT1 putative promoter activity under PARP-1 inhibitors. (A) A scheme of hCNT1 putative promoter constructs used for the luciferase assay. (B) Luciferase activities from three luciferase reporter constructs containing hCNT1 promoters with different lengths. These constructs were transiently transfected into HT29 cells, and then were treated with 25μM of either PJ34 or Olaparib for 48h. Luciferase activity was normalized to respective Renilla luciferase activities. (C) Effects of PARP-1 inhibitors on the activity of the internal control (Renilla luciferase gene) of the dual-luciferase reporter assay (three individual experiments).

Moreover, optimization of the test reporter plasmid by using the new series of reporter vectors the pGL4 Luciferase reporter plasmids instead of pGL3 would also be convenient. These pGL4 plasmids are expressed uniformly in host cells with minimum off-target responses and would provide a higher accurate expression yield (Dougherty and Sanders 2005).

In addition, regarding hCNT1 endogenous expression, PARP-1 effect on hCNT1 mRNA mostly presented negative regulation. However, due to the high inter-experiment variation, changes were not statistically significant. We are still not sure whether this effect is upregulating or downregulating hCNT1 expression, since the transcriptional regulation of PARP-1 can work through different repressing or activating molecular mechanisms as mentioned earlier. At the same time, despite high variation, we cannot rule out PARP-1 involvement in regulating hCNT1 expression whether directly or indirectly, as the changes in endogenous hCNT1 expression after inhibiting PARP-1 were continuously observed. Regarding hCNT1 promoter activity assay, for now, we cannot indicate whether the increase in the activity of the different hCNT1 promoter constructs after PARP-1 inhibition is truly representing transcriptional regulation by PARP-1 or not. Indeed, the alteration of the internal control gene activity induced by PJ34 or Olaparib has decreased the reliability of these results.

Moreover, many of PARP-1 functions in gene expression regulation are effected by PARP-1 binding rather than its enzymatic activity (PARylation) (Weaver and Yang 2013). Thus, a note of caution is due here since that currently available PARP inhibitors act at the catalytic site of PARP-1, which does result in some degree of altered binding capacity via changes in autoPARylation status (D'Amours et al. 1999). Accordingly, in BCLC3 or BCLC5 cells, it is possible that treatment with PARP inhibitors may not effectively inhibit specific PARP-1 interactions or may require different dosing.

Therefore, once the PARP-1 assays would be optimized, a better analysis on the mechanisms by which PARP-1 is contributing to the transcriptional regulation of hCNT1 expression could be performed. For instance, a ChIP assay using an anti-PARP1 antibody under basal culture conditions or under PARP-1 inhibitors would additionally demonstrate the association of PARP-1 to the hCNT1 promoter, and further selective deletions and site-directed mutagenesis of the hCNT1 promoter constructs would accurately map promoter regions implicated in PARP-1 regulation. A similar approach could be done as well in order to ascertain the implication of the putative transcription factors p53, CREB, Sp1 and NFkB on

hCNT1 expression, by co-transfection of plasmids encoding these transcription factors along with the hCNT1 promoter constructs.

In summary, there is a major impact of PARP-1-mediated transcriptional control on human tumor biology. In our case, PARP-1 by some means seems to be regulating hCNT1 transcriptional expression, and possibly contributing to its loss in tumors. Up to date the mechanisms responsible for hCNT1 loss during carcinogenesis are unknown, although it seems likely that epigenetic modifications either directly affecting hCNT1 transcriptional events or modulating other proteins (e.g. transcription factors) might contribute to regulate hCNT1 expression and function. Understanding how PARP-1 regulates the transcription of *SLC28A1* gene in human cancer models could help to design new therapeutic approaches.

2. hCNT1 role in the modulation of cell biology

2.1. hCNT1-mediated prevention of oncogenesis

To better understand the role of hCNT1 as a putative tumor suppressor, we attempted to explore the nature of the hCNT1-induced cell death to ascertain its possible inducing pathway.

2.1.1. hCNT1 restoration induces cell death in different solid tumor cell lines

In order to undertake hCNT1 restoration studies and assess the features of hCNT1-induced cell death in tumors, we first determined its effect on various tumor derived cell lines, in which hCNT1 expression is most likely to be reduced or lost either at mRNA or protein levels (Zollner et al. 2005; Bhutia et al. 2011). This was done by performing MTT assay at 48 hours after transducing the hCNT1 gene using AdhCNT1 virus (at 10 MOI) in tumor cell lines derived from hepatocellular carcinoma (BCLC3, BCLC5, Huh7 and Huh7.5), hepatoblastoma (HepG2), colorectal cancer (HT29) and cholangiocarcinoma (TFK-1). Pancreatic adenocarcinoma derived cell lines were also included as a positive control for hCNT1 induced cell death, as we previously reported (Pérez-Torras et al. 2013).

The results, as shown in Figure 20, demonstrated that hCNT1 restoration induced cell death among all the tested cell lines derived from different types of tumors. In hepatic cell lines, hCNT1 restoration induced a major decrease in cell viability. As well, CRC and cholangiocarcinoma cell line also demonstrated significant hCNT1-induced cell death. Cells from PDAC showed to be less affected by AdhCNT1 than the other cell lines.

Notably, the tested cell lines have displayed different sensitivity to the same delivered amount of AdhCNT1 (10MOI). Hepatic cell lines showed the highest alteration in cell viability compared to the rest of the cell lines. Thus, hepatic cell lines seem to be more sensitive to hCNT1 restoration, especially BCLC cells, than the other cell lines derived from colorectal cancer, cholangiocarcinoma or pancreatic adenocarcinoma. Nonetheless, we must take into consideration that this higher sensitivity to hCNT1, could be related to the high level of the coxsackievirus and adenovirus receptor (CAR) expression within the liver tissue versus other organ tissues (Reeh et al. 2013). Thereby, for being the most sensitive, we have continued our studies on hCNT1 using BCLC3 and BCLC5 cells.

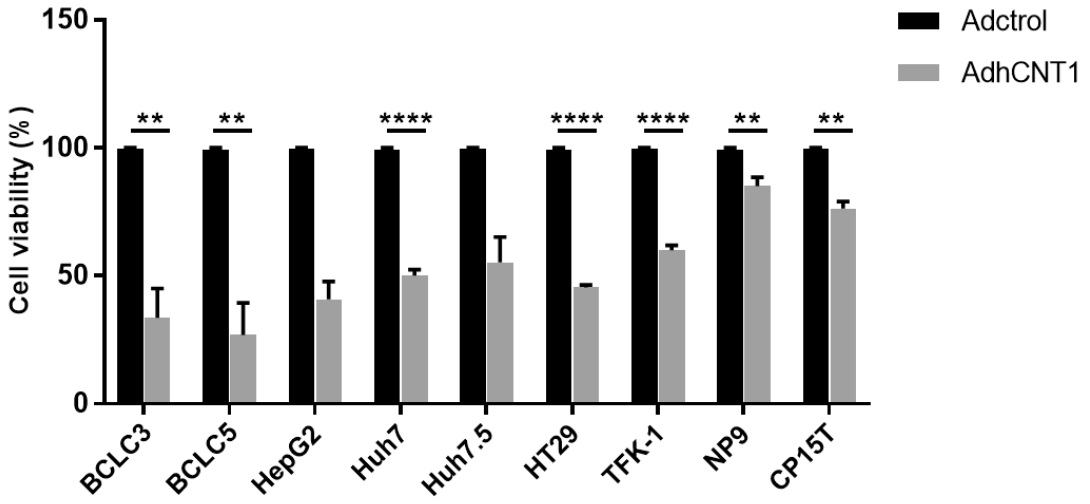


Figure 20. hCNT1 induced cell death in different solid tumor drived cell lines. Cell viability after hCNT1 restoration was assessed by MTT assay 48h following Adctrol or AdhCNT1 infection at 10MOI in tumor cell lines derived from HCC (BCLC3, BCLC5, Huh7 and Huh7.5), hepatoblastoma (HepG2), CRC (HT29), cholangiocarcinoma (TFK-1) and PDAC (NP9, CP15T). Data are expressed as percentage of viable cells relative to Adctrol infected cells of each cell line. Results are means ±S.E.M. (n=2 for Huh7.5 and HepG2 and n=3 for the rest of the cells). Statistical significance was determined with Student's t-test; **P<0.01, ****P<0.0001.

2.1.2. hCNT1-induced cell death is specific to hCNT1

We have determined whether this phenotype of altered cell cycle progression and cell death following hCNT1 restoration are induced by restoring the other two hCNT family members, hCNT2 and hCNT3, since they share functional and structural similarities as mentioned earlier.

Therefore, cell viability assays were performed on BCLC cells after infecting them with adenoviral vectors carrying either hCNT2 or hCNT3 encoding genes, at 10 MOI (Figure 21). Following 48h of incubation, cell viability in both conditions did not show any changes versus their corresponding controls. We can clearly see the difference compared to the effects of hCNT1 on cell viability. This demonstrated that hCNT1-induced cell death is transporter subtype-specific.

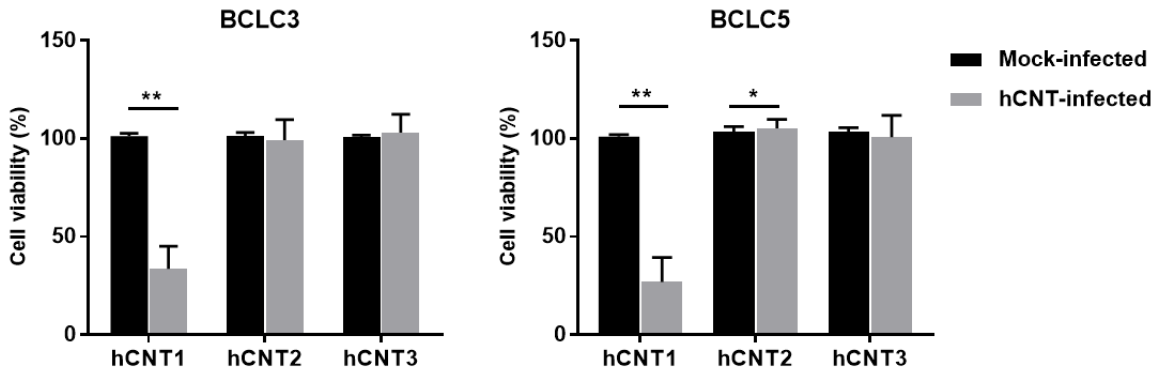


Figure 21. hCNT1 induced cell death in a hCNT subtype-specific manner. Cell viability was determined 48h after transduction of hCNT1, hCNT2 or hCNT3 at 10MOI using MTT assays. Data are expressed as percentage of viable cells relative to mock infected cells. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test; *P<0.05 and **P<0.01.

2.1.3. hCNT1-induced cell death is dose-dependent

The effect of increasing AdhCNT1 concentration on cell viability was also checked by infecting BCLC cells with different MOIs (1, 5, 10 MOI) as illustrated in Figure 22. Consequently, following 48h of infection, both cell lines displayed a significant decrease in cell viability. Gradual decrease of BCLC3 and BCLC5 cell viability was observed in correlation to the AdhCNT1 MOI used. At 10 MOI, BCLC3 and BCLC5 have shown the most significant hCNT1-induced cell death. These observations indicated a dose-dependent effect of hCNT1 restoration on cell viability.

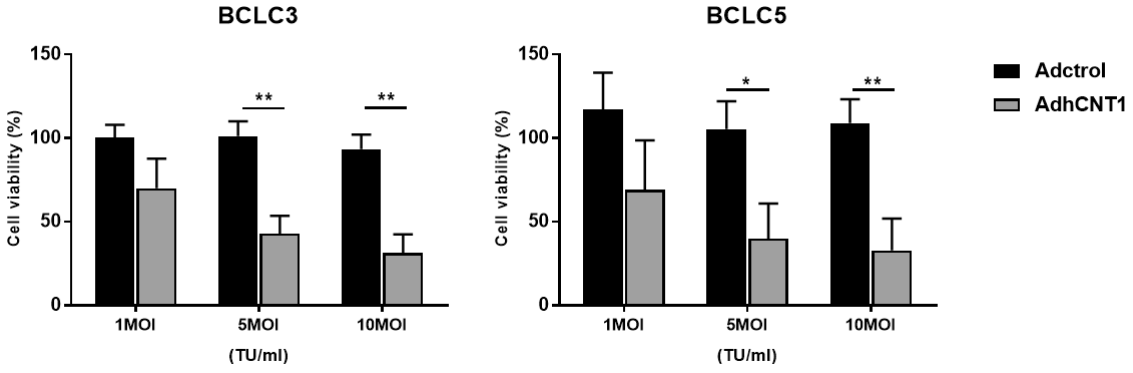


Figure 22. hCNT1 induced cell death in a dose-dependent manner. Cell viability after hCNT1 restoration was assessed by MTT assay 48h after Adctrol or AdhCNT1 infection with 1, 5, 10 MOI. Data are expressed as percentage of viable cells relative to mock cells. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's *t*-test; *P<0.05 and **P<0.01.

Moreover, imbalanced nucleotide (dNTP) pools can accumulate multiple genotoxic alterations that affect the cell-cycle progression and induce cell death (Serres (ed.) 1985; Yao et al. 2013). Thus, it might be argued about the possibility of the presence of an imbalanced dNTP pool due to the restoration of the pyrimidine-preferring transporter hCNT1. However, this can be ruled out by the fact that cell death was absent when we overexpressed hCNT2 or hCNT3 alone. This also can be supported by the fact that overexpressing the transport-null hCNT1 mutant (hCNT1SP) has also induced cell death in PDAC cells (Pérez-Torras et al. 2013). Indeed, these observations have demonstrated a specificity of this cell death to hCNT1 effects, and have further supported the fact that hCNT1 effects are independent of its role as mediator of nucleoside uptake (Pérez-Torras et al. 2013).

In summary, we have been able to go one step further in characterizing hCNT1-induced cell death. The above observations support the proposed role of hCNT1 in modulating some physiological aspects in cell biology. In addition, the significance of hCNT1 to tumor biology might help explaining its frequent downregulation in a variety of human solid tumors (Farré et al. 2004; Gloeckner-Hofmann et al. 2006; Lane et al. 2010; Bhutia et al. 2011). This in fact, further supports our hypothesis that hCNT1 loss might be contributing to oncogenesis. Thus, to better explore this hypothesis, we aimed at undertaking hCNT1 silencing experiments. Unfortunately, the lack of cells significantly expressing hCNT1 and the difficulties of obtaining primary human hepatocytes had prevented us from assessing this issue *in vitro* for the moment. On the other hand, HEK293 cells stably expressing hCNT1 have been formerly generated in our laboratory successfully. Carrying out hCNT1 silencing in such a model would not be a proper cellular context mimicking hCNT1 endogenous expression since HEK293 cells have been originated from a human embryonic kidney cell background, whereas CNT expression seems to be associated with a fully differentiated cell background (Errasti-Murugarren et al. 2007; Govindarajan et al. 2008). In fact, our group also tried to stably transfect hCNT1 in breast cancer cells (MCF-7) and in PDAC cell lines (NP9) (García-Manteiga et al. 2003), but all of the attempts did not show constant expression or activity. Additionally, repeated efforts to stably express hCNT1 in different types of ovarian cancer cell lines have induced cellular lethality (Hung et al. 2015). These observations further support the proposed role of hCNT1 in tumor suppression (Bhutia et al. 2011; Pérez-Torras et al. 2013).

2.2. Molecular events underlying hCNT1 role in cell biology

In the previous block, we have shown that hCNT1 is capable to induce cell death in different tumor cell lines. Therefore, a major focus of our investigation involved the elucidation of the molecular mechanisms that principally underlay hCNT1-induced events and cell death.

2.2.1. Ascertaining the hCNT1-induced cell death signaling pathway

A. ERK, AKT and AP-1 activation.

Our previous studies have demonstrated that hCNT1-induced cell death was accompanied by intracellular signaling responses. hCNT1 restoration affected the basal phosphorylation status of some selected key signal-transducing kinases, i.e. **PKB/AKT** and **MAPK/ERK** kinases in PDAC and breast cancer cell lines, along with **PARP-1** hyperactivation (Pérez-Torras et al. 2013). ERK and AKT are principle kinases signaling through MAPK/ERK and PI3K/AKT pathways which considered as the master mechanisms of controlling cell survival. In fact, both pathways are widely known to be mutationally activated as a common event in cancers (Liang and Slingerland 2003; McCubrey et al. 2007).

Thus, we checked AKT and ERK phosphorylation following hCNT1 restoration in the HCC cell line models. This was carried out as explained above by restoring hCNT1 expression in BCLC3 and BCLC5 cells. The expression of phosphorylated AKT (p-AKT) and ERK (p-ERK) was determined along with their total proteins, in time-course experiments using protein immunoblotting technique (Figure 23). hCNT1 restoration induced an early phosphorylation of ERK at Thr202/Tyr204 sites and AKT at both S473 and T308 sites in both cell lines, at the first time point assayed i.e. 12h, while no changes in the total ERK and AKT were observed. Notably, the effect on p-AKT at T308 seems to be secondary, as in Adctrol-infected cells it is also increased, although it is delayed in time.

Moreover, as introduced earlier, hCNT1 restoration has induced c-Jun phosphorylation in PDAC cell lines. c-Jun is a DNA binding protein belonging to the AP-1 transcription factor family. AP-1 is an early response transcription factor that binds to TRE/AP-1 elements and activates transcription. The stimulation of AP-1 is mediated, in part, by phosphorylation of c-Jun at Ser63 and Ser73 through JNK. Once phosphorylated, c-Jun can form homo- or

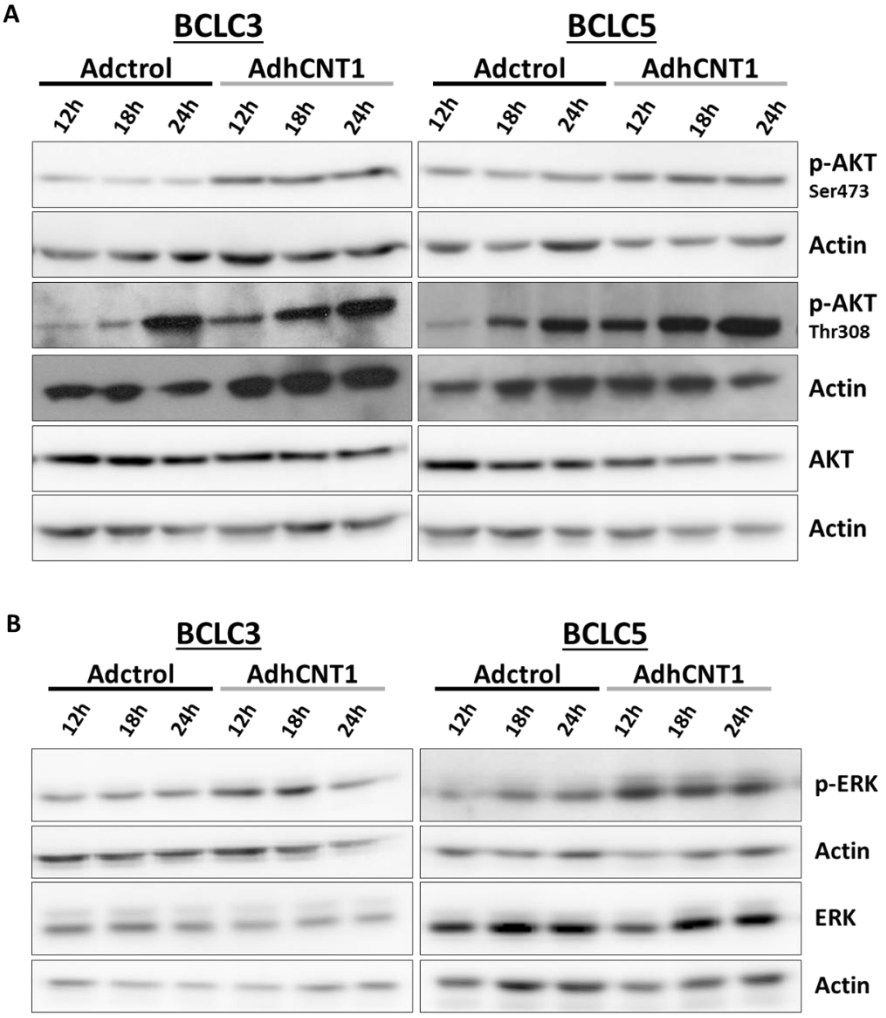


Figure 23. hCNT1 restoration activates AKT and ERK. BCLC3 and BCLC5 were infected with AdhCNT1 or Adctrol at 10 MOI. Representative Western blots of the time-course of (A) AKT and (B) ERK phosphorylation were analyzed in total cell extracts by immunoblotting against the indicated antibodies.

heterodimers with other AP-1 members, such as members of the Fos family proteins, and bind to the DNA sequence to modulate transcription.

To determine the expression of c-Jun in BCLC cells following hCNT1 restoration, we analyzed its protein expression by immunoblotting along with the phosphorylated c-Jun in time course experiments, as illustrated in Figure 24A. Western blot results showed that hCNT1 restoration affected the phosphorylation status of c-Jun (p-c-Jun) positively, starting from the first time point analyzed i.e. 12h. The expression of total c-Jun seemed to be increased in both cell lines as well.

Furthermore, the functional activity of AP-1 in a given cell relays on the relative amount of specific Jun/Fos proteins which are expressed, as well as other potential interacting proteins. Thus, as hCNT1 restoration increased c-Jun phosphorylation and expression, we questioned what happens with the expression of the Fos family proteins following hCNT1 restoration. Therefore, we analyzed the protein expression of two Fos family members, i.e. c-Fos and Fos-like antigen 1 (FRA-1) along with their phosphorylated form in BCLC3 and BCLC5, following hCNT1 restoration.

Thus, as shown in Figure 24B, phosphorylated c-Fos at Ser32 is downregulated in both cell lines. Conversely, the total c-Fos protein was increased in BCLC3 at 18h and 24h, and in BCLC5 it was increased at 18h and then downregulated at 24h. This pattern of total c-Fos expression seems to indicate a different phosphorylation site of c-Fos, other than Ser32.

Regarding FRA-1, in both cell lines, phosphorylated FRA-1 at Ser265 seems to be downregulated, while the total protein form presented different pattern of alteration. Post-translational modification could be affecting FRA-1 expression following hCNT1 restoration. FRA-1 appears to be phosphorylated at another site than Ser265. In BCLC5, increased FRA-1 expression was observed at 18h, this expression is lost at 24h, showing a similar expression pattern as c-Fos. In BCLC3, altered expression pattern of FRA-1 was observed, whereas the amount of the protein expression did not seem to be affected. Together, it seems that the c-Fos and FRA-1, members of the Fos family, are affected by the restoration of hCNT1. Both c-Fos and FRA-1 were shown to be upregulated at 18h, although, the pattern of alteration induced by hCNT1 appears to be different between BCLC3 and BCLC5 at 24h. Additionally, we checked whether the expression of c-Fos and FRA-1 proteins are affected by the expression of c-Jun. This was assessed by silencing c-Jun expression using small interfering RNA (siRNA) targeting the c-Jun gene in BCLC cells after hCNT1 restoration (Figure 24C). Immunoblotting results demonstrated that c-Fos expression seem to be correlated with the expression of c-Jun in BCLC cells, whereas FRA-1 is not.

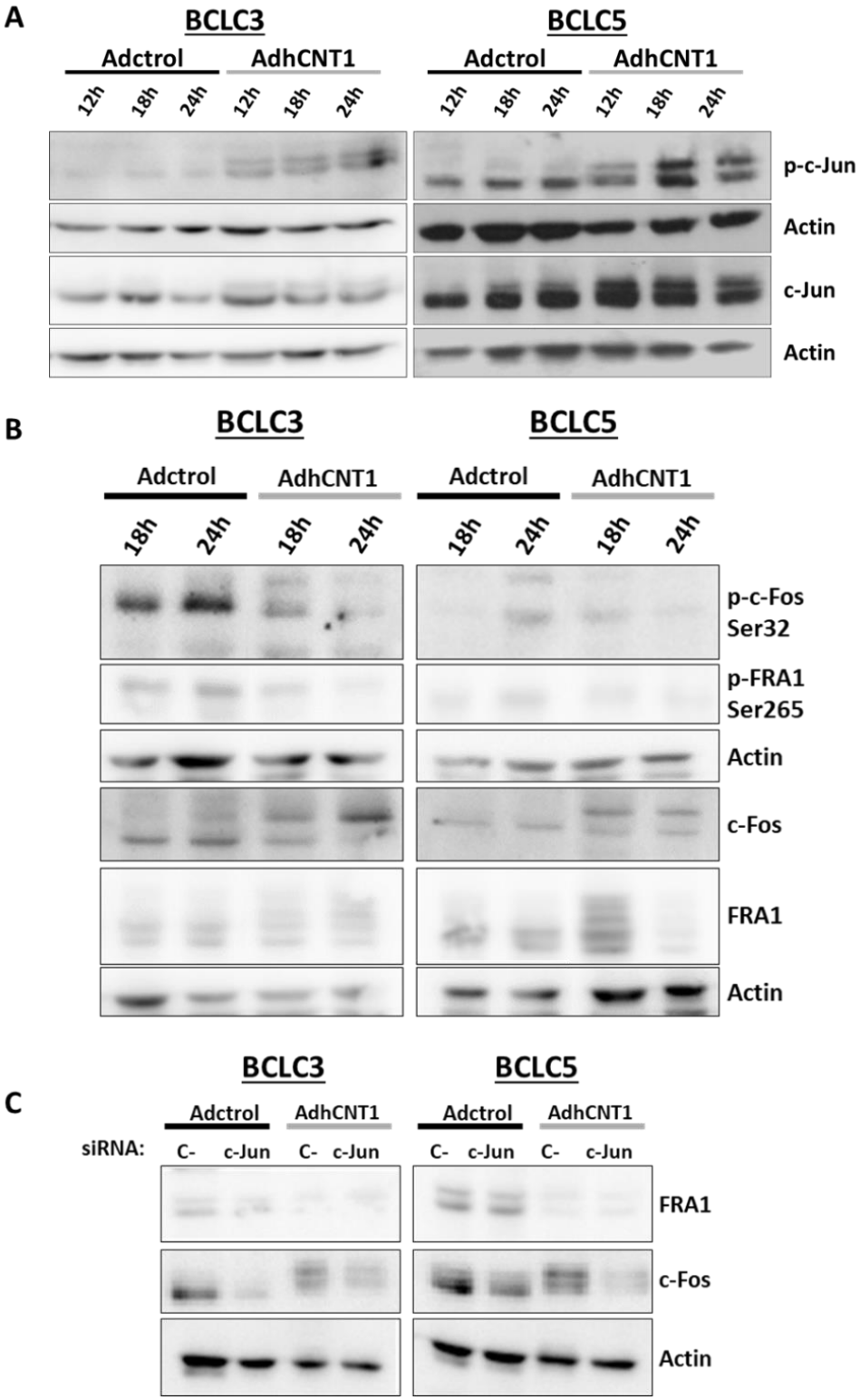


Figure 24. Expression of c-Jun and Fos proteins following hCNT1 restoration. BCLC3 and BCLC5 were infected with AdhCNT1 or Adctrol at 10 MOI. Representative western blots of the time-course of (A) c-Jun, (B) c-Fos and FRA-1 protein expression. (C) Expression of Fos proteins following c-Jun silencing, BCLC3 and BCLC5 cells were treated with 200nM of c-Jun or nontarget (C-) siRNA for 48h following AdhCNT1 or Adctrol infection. Protein expression was analyzed in total cell extracts by immunoblotting against the indicated antibodies.

Thus, these observations suggested that hCNT1 restoration is provoking changes in the expression of c-Jun, c-Fos, and FRA-1. These changes might be activated by hCNT1 signals, as a transceptor, through signaling cascades that are involved in regulating the expression of Jun and Fos family members. However, these data might suggest that hCNT1 restoration could be interfering with the activity of the early response AP-1 transcription factors. Since AP-1 activity is deeply involved in the modulation of gene expression, functional consequences of hCNT1-induced AP-1 alterations might be modifying several cellular events in which AP-1 transcription factors are involved.

Regarding cell death, AP-1 transcription factors are widely described to be contributing in different transcriptional and molecular mechanisms mediating cell proliferation, transformation, and death. Thus, it is possible that AP-1 might be mediating some cellular responses following hCNT1 restoration that might eventually induce cell death. Accordingly, two models have explained the effects of AP-1 on cell death and survival. It was suggested that the induction of AP-1 results in the activation of various genes, such as FasL, Bim, and Bcl3, whose products are either positive or negative regulators of apoptosis. The balance between the pro-apoptotic and anti-apoptotic target genes determines whether the final outcome will be survival or death. However, this balance may vary from one cell type to another, and may be dependent on the type and duration of stimulus used to activate AP-1, as well as on the activation of other transcription factors (Faris M. 1998; Na et al. 1999; Whitfield et al. 2001; Eferl and Wagner 2003). According to the second model, AP-1 functions as a homeostatic regulator that keeps cells in a certain proliferative steady state. Changes in environmental factors might affect AP-1 activity. Robust or persistent activation of AP-1 in cells containing damaged DNA causes defective replication and may trigger apoptosis through the same mechanisms that induce cell death after constitutive expression of oncogenes (Sampath Ramachandiran et al. 2002; Eferl and Wagner 2003). However, the activation of AP-1 in cells that are able to proliferate promotes cell proliferation and survival.

On the other hand, it is curious that activation of the cell survival kinases AKT and ERK following hCNT1 restoration did not prevent cells from death. Thus, we have previously determined the role of AKT on hCNT1-induced cell death, by inhibiting the phosphorylation of AKT using a PI3K inhibitor, wortmannin, in NP9 and NP29 cells (Pérez-Torras et al. 2013). However, no effect on the hCNT1-induced cell death was observed in either cell lines.

Therefore, we supposed that its phosphorylation might not be affecting cell survival and could be regulating other hCNT1-related events. Conversely, inhibition of ERK activation with PD98059 induced a slight but significant reduction in hCNT1-induced cell death in NP9, but not in NP29 (Pérez-Torras et al. 2013). As a matter of fact, there are some evidence suggesting a role for ERK in mediating cell death, depending on the cell type and/or the stimulus. For instance, ERK activity can contribute to different antiproliferative events due to prolonged ERK activation mediated by reactive oxygen species (ROS) (Sampath Ramachandiran et al. 2002; Cagnol et al. 2006; Martin and Pognonec 2010), or after treatment with some chemotherapeutic agents (e.g. Doxorubicin or Cisplatin) via the activity of p53 (DeHaan et al. 2001; Woessmann et al. 2002; Alexia et al. 2004).

Thus, we asked whether ERK and/or c-Jun activation might be mediating hCNT1-induced cell death in BCLC3 and BCLC5. Therefore, to determine the role of these different pathways in mediating hCNT1-induced cell death, we performed cell viability assays on BCLC cells following the inhibition of ERK and c-Jun phosphorylation. This was done by using PD98059, a MAP kinase/ERK kinase (MEK) inhibitor, and SP600125, c-Jun N-terminal kinase (JNK) inhibitor, either separately or in combination. In parallel, the effect of MEK and JNK inhibitors was validated via protein expression analysis by immunoblotting. Protein samples from parallel experiments to the MTT assays were analyzed against their corresponding antibody (Figure 25). Western blot analysis showed effective inhibition of p-ERK and p-c-Jun, whereas this inhibition of ERK or Jun phosphorylation did not prevent the hCNT1-induced cell death.

Moreover, in some studies, ERK was reported to activate the phosphorylation of c-Jun (Kayahara et al. 2005). Thus, we checked whether the combination of both inhibitors (PD98059 and SP600125) might show different effects on hCNT1-induced cell death than using each inhibitor alone. hCNT1-transduced BCLC3 and BCLC5 cells after the combined treatment did not show any evidence of avoiding hCNT1-induced cell death (Figure 26). In fact, in some of the treated conditions (Figure 25A&C), inhibiting ERK or c-Jun phosphorylation seemed to enhance hCNT1-induced cell death. This was observed in BCLC5 with 100µM of PD98059 and with 10µM of SP600125. In fact, this could be a rational consequence of treating cancer cells with MAPKs inhibitors, since they are widely considered

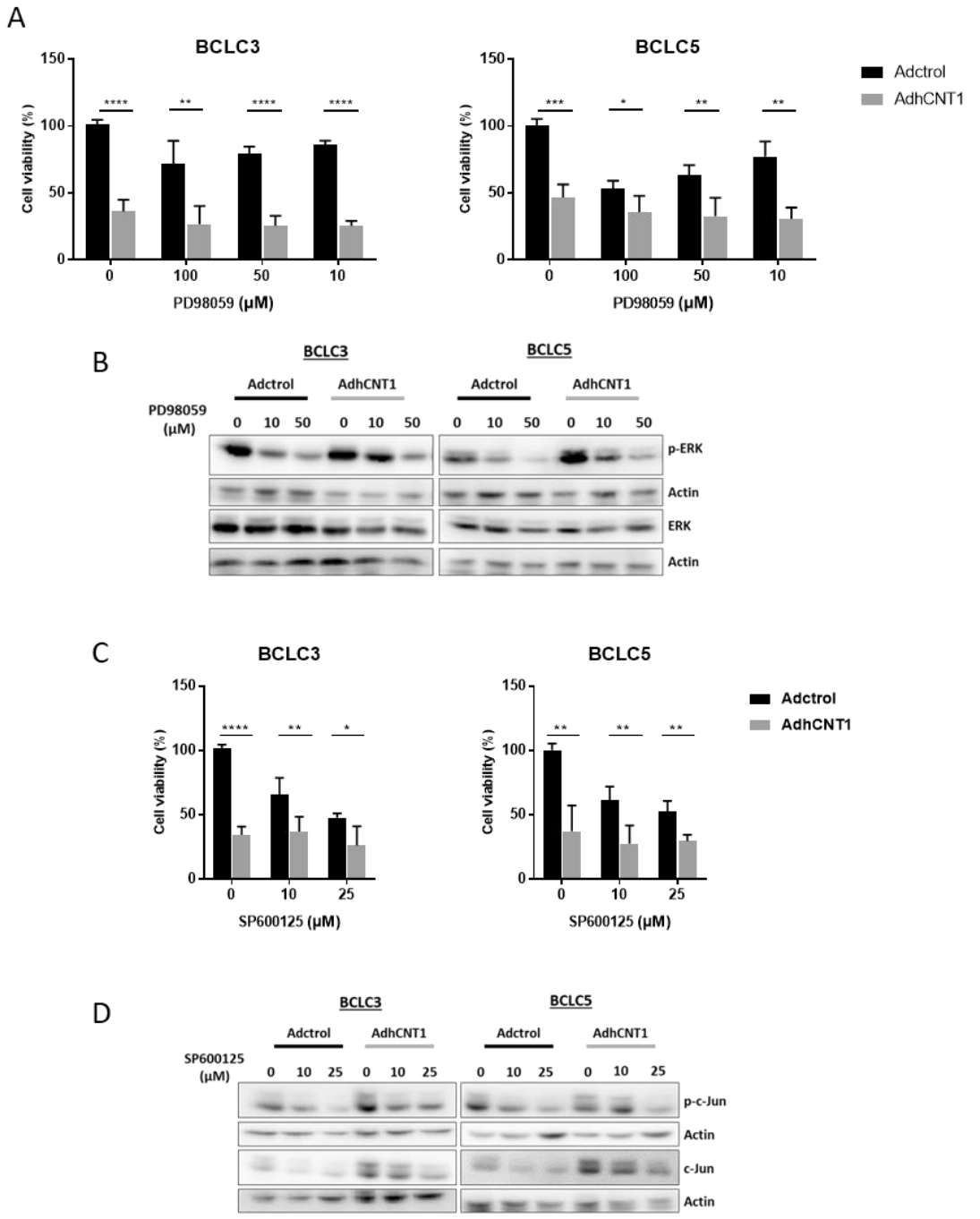


Figure 25. No effect on hCNT1-induced cell death after inhibiting ERK or c-Jun phosphorylation. BCLC cells were infected with AdhCNT1 or Adctrol at 10 MOI and incubated with the inhibitors for 48h. (A) Viability of infected with AdhCNT1 and treated with PD98059 (MEK inhibitor) at the indicated dose. (B) Total-cell lysates were analyzed by immunoblotting with the indicated antibodies for functional validation of PD98059 inhibitor. (C) Viability of infected with AdhCNT1 and treated with SP600125 (JNK inhibitor) at the indicated dose. (D) Total-cell lysates were analyzed by immunoblotting with the indicated antibodies for functional validation of SP600125 inhibitor. Cell viability was assessed by MTT assay. Data are expressed as percentage of viable cells relative to Adctrol- or AdhCNT1-infected cells. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

as anti-cancer drugs (English and Cobb 2002). These data demonstrated that the inhibition of ERK and c-Jun phosphorylation, whether separately or in combination, were unable to prevent the induced cell death following hCNT1 restoration. Thus, phosphorylation of ERK and c-Jun did not seem to mediate hCNT1-induced cell death in BCLC cells.

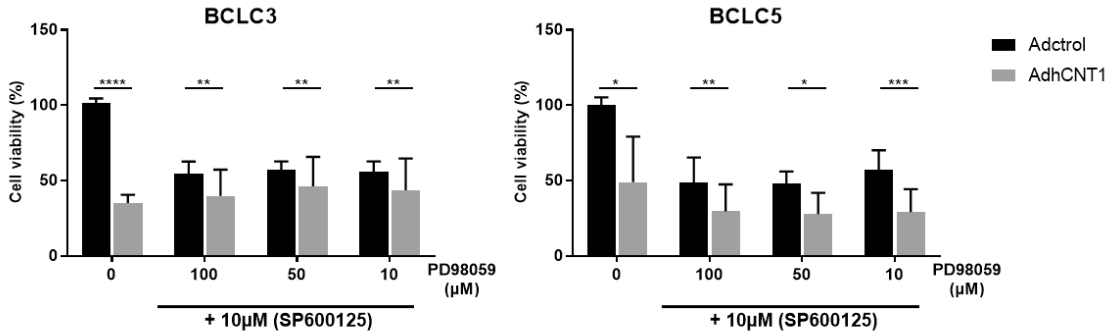


Figure 26. No effect on hCNT1-induced cell death after inhibiting ERK and c-Jun phosphorylation in combination. BCLC cells were infected with AdhCNT1 or Adctrol at 10 MOI and incubated with both PD98059 and SP600125 inhibitors for 48h. Cell viability was assessed by MTT assay. Data are expressed as percentage of viable cells relative to Adctrol- or AdhCNT1-infected cells. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test, *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

In fact, we observed that total c-Jun and Fos protein expression increased following hCNT1 restoration (Figure 24). Thus, we thought that the total protein but not its phosphorylation might be enhancing hCNT1-induced cell death. Therefore, we evaluated the effect of knocking-down the expression of c-Jun on hCNT1-induced cell death. This was performed by using siRNA c-Jun. BCLC3 and BCLC5 were infected with AdhCNT1 or Adctrol and then incubated with siRNA c-Jun or siRNA control (C-) for 48h. After that, cell viability was assessed by MTT assay. Protein samples from parallel experiments to the MTT assays were immunoblotted to validate the knockdown of c-Jun (Figure 27).

Afterwards, silencing of c-Jun was confirmed by western blot. Decrease of total c-Jun protein expression resulting in a decrease of the amount of the phosphorylated protein was observed. Cell viability assays did not show any considerable effect of this silencing on hCNT1-induced cell death. Nevertheless, a very slight prevention of cell death was observed at 5 MOI in AdhCNT1 infected BCLC3 treated with siRNA c-Jun when compared to Adctrol infected

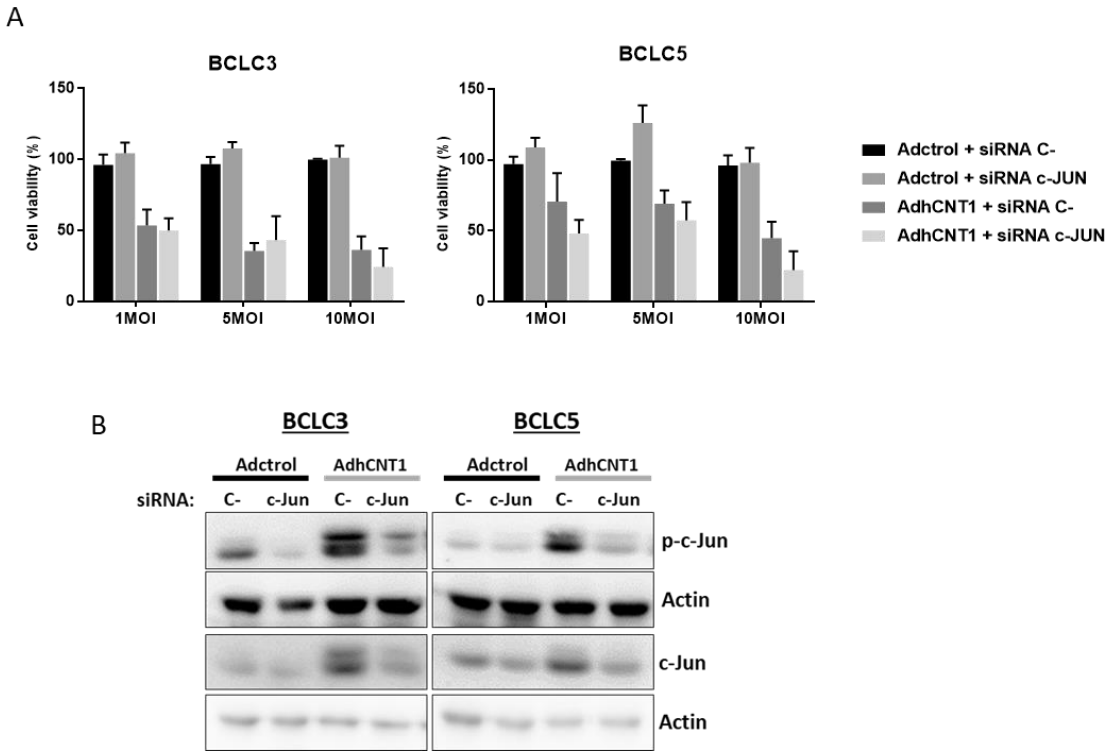


Figure 27. No significant effect of silencing c-Jun gene on hCNT1-induced cell death. BCLC3 and BCLC5 cells were treated with 200nM of c-Jun or nontarget (C-) siRNA for 48h after AdhCNT1 or Adctrol infection. (A) Cells infected with 1, 5, and 10 MOI. Cell viability was assessed by MTT assay. Data are expressed as percentage of viable cells relative to Adctrol + siRNA C- infected cells. (B) Whole cell lysates were immunoblotted against the indicated antibodies to validate of silencing efficiency. Cells were infected with 10MOIs of AdhCNT1 or Adctrol. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test.

siRNA c-Jun treated cells (Figure 27A). However, these observations suggest that the increase in the total c-Jun expression does not seem to be mediating hCNT1-induced cell death.

Altogether, hCNT1-induced cell death in HCC derived cell lines was accompanied by the early phosphorylation of the key regulatory kinases AKT and ERK along with c-Jun. These observations are in line with their activation in the PDAC cell lines following hCNT1 restoration (Pérez-Torras et al. 2013). In addition, we found that two Fos family proteins (i.e. c-Fos and FRA-1) were early upregulated.

Nonetheless, these activated pathways did not seem to be mediating hCNT1-induced cell death. Thus, it can be hypothesized that their activation might be mediating some molecular events that collectively with other hCNT1-induced effects could result in cell death. However, it can also be suggested that the activation of these pathways indeed promote cell survival.

As a matter of fact, a wide variety of events occurring downstream of JNK/c-Jun activation, ranging from cytoskeleton remodeling to regulation of cell proliferation or cell death, have been described. For example, activated JNK/c-Jun signaling cascade has been linked to cellular stress responses and DNA damage, thereby promoting cell death or DNA damage repair. These responses were observed following the exposure to a variety of genotoxic agents or following oncogene-induced replicative stress (P. Chen et al. 2010; Picco and Pagès 2013). However, the fate of cells subjected to DNA damage seem to be dependent on the genetic background of the cells and the extent of the damage.

On the other hand, regarding AKT and ERK, it could be suggested that their activation might be a kind of adaptive response to maintain the viability of the tumor cells. Likewise, several studies have reported the activation of alternative cell survival signaling pathways (e.g. ERK, AKT, EGF) following targeted treatment with different tyrosine kinase inhibitors such as mTOR and MEK inhibitors (O'Reilly et al. 2006; Chen et al. 2011; Steinway et al. 2015; Butler et al. 2017). These alternative survival pathways were considered as a compensatory escape pathway for the cancer cell to survive. Nonetheless, further functional analysis on the AKT, ERK, and AP-1 transcription factors are warranted. This would eventually elucidate the role of their activation in hCNT1-induced events.

B. p53 and the CDK inhibitors p21 and p27 downregulation.

We reported that hCNT1 restoration in PDAC cell lines induced cell-cycle alteration consistent with a S-phase arrest (Pérez-Torras et al. 2013). Thus, another significant aspect to be considered was the analysis of tumor suppressor p53 and the cyclin-dependent kinase (CDK) inhibitors p21 and p27, which act as master regulators of diverse cellular processes including cell-cycle progression. p53 is a tumor suppressor protein and a transcription factor that is widely known to mediate anti-proliferative processes and regulate diverse signaling pathways that control cell-cycle arrest, apoptosis and also cell metabolism. p21 and p27 are negative regulators of the cell cycle. They bind to cyclin-CDK complexes to inhibit their catalytic activity and induce cell-cycle arrest. p21 is known to be a downstream target of p53 that is upregulated following p53 activation. However, p21 can be induced by both p53-dependent and p53-independent mechanisms. Additionally, p27 functions were reported to be linked to the regulation of actin dynamics and cell migration. Moreover, p21 and p27 play a sequential role in gene transcriptional regulation by recruiting and regulating the activity of

specific cyclin-CDK complexes on some promoters (Orlando et al. 2015). Therefore, we determined their protein expression following hCNT1 restoration. BCLC3 and BCLC5 were infected with AdhCNT1 or Adctrol and 48h later, immunoblotting detection of p53, p21 and p27 was performed on total protein lysates (Figure 28).

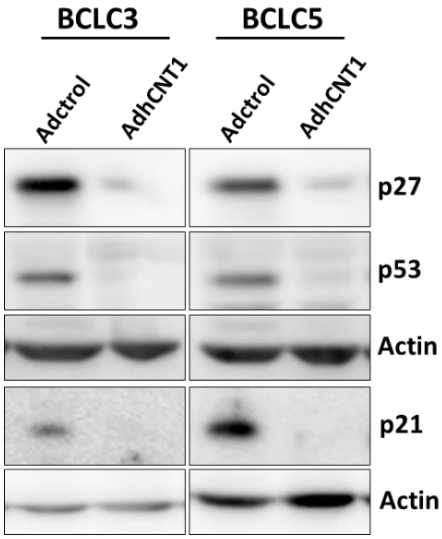


Figure 28. Downregulation of p53, p21 and p27 following hCNT1 restoration. Protein expression of p53, p21 and p27 in BCLC3 and BCL5 following 48h of AdhCNT1 or Adctrol infection with 10MOI.

As shown in Figure 28, hCNT1 restoration induced downregulation of all three proteins. Moreover, there is evidence that AP-1 proteins, mostly those that belong to the Jun group, control cell life and death through their ability to regulate the expression and function of cell cycle regulators such as Cyclin D1, p21, p19 and p16 (Shaulian and Karin 2002). As c-Jun induction was associated with hCNT1 restoration, thus, we wondered whether p21 expression is being regulated by c-Jun. Therefore, p21 expression was analyzed under the effect of silencing c-Jun, following hCNT1 restoration (Figure 29). Western blot results indicated that c-Jun seems to be affecting the expression of p21 in BCLC5 cell, either with or without hCNT1 restoration. In BCLC3, c-Jun effect on p21 was not obvious, although, there is a slight increase of p21 expression after silencing c-Jun in the Adctrol samples. These data suggest that p21 expression in BCLC cells is dependent, at least in part, on the expression of c-Jun.

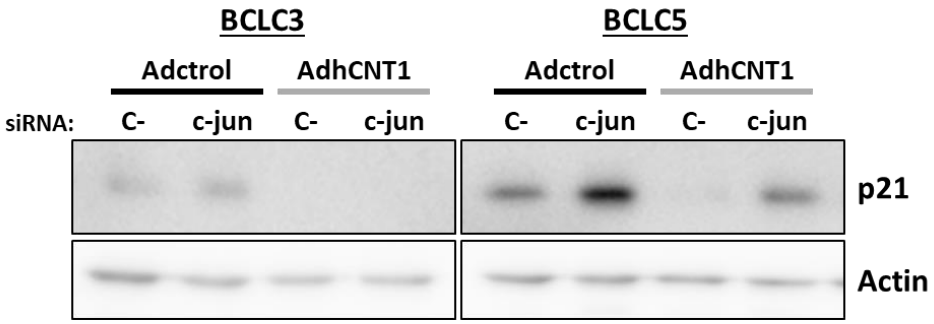


Figure 29. Effect of silencing c-Jun on p21 expression. BCLC3 and BCLC5 cells were treated with 200nM of c-Jun or nontarget (C-) siRNA for 48h following AdhCNT1 or Adctrol infection. Whole cell lysates were immunoblotted against p21 antibody.

In summary, restoration of hCNT1 induced downregulation of p53, p21 and p27, thus, hCNT1 might be mediating their transcriptional regulation or stability. In fact, p53 is subject of a complex regulation. Hence, several additional analysis are needed to elucidate the hCNT1-related pathway of its downregulation and to further understand what the consequences of this downregulation might be, especially as p53 is one of the most frequently mutated gene in human cancer. Regarding p21 and p27, mutations in these two CDKI are rare in cancers. Their activity is required for the establishment of cell-cycle checkpoints to mediate G1/S and G2/M arrest. Given that hCNT1-restoration resulted in S-phase arrest preceded by some activated cell survival signaling, it might be suggested that downregulation of both CDK inhibitors may be attenuating their inhibitory activity on CDK complexes. This could alter the integrity of the cell-cycle checkpoints in BCLC cells, thus, enhancing a further uncontrolled G1/S transition. This might result in the accumulation of DNA errors, leading to S-phase arrest. Actually, during S-phase arrest, typical S-phase markers remain present, and G1 inhibitory signals such as p21 and p27 are absent (Borel et al. 2002), to allow the proper temporal regulation of DNA replication (Cuadrado et al. 2009; Soria and Gottifredi 2010; Piccolo and Crispi 2012). These suggestions, along with the fact that p21 seems to be regulated by c-Jun expression, might be highlighting a role of the hCNT1-induced c-Jun activation in mediating cell-cycle arrest (Figure 24A).

Furthermore, we wanted to further understand hCNT1 effects by analyzing possible changes of p21 subcellular localization. In fact, there is some evidence of different or even antagonistic roles of p21 depending on whether its expression is nuclear or in the cytosol (Abbas and Dutta 2009; Galanos et al. 2016). Thus, to determine whether hCNT1 restoration,

besides decreasing p21 expression, is also changing its subcellular localization, we analyzed its expression by immunocytochemical staining in BCLC cells following 48h of hCNT1 restoration. However, images obtained by fluorescence microscopy demonstrated an overall reduction of p21 expression without any apparent change in its subcellular localization (Figure 30).

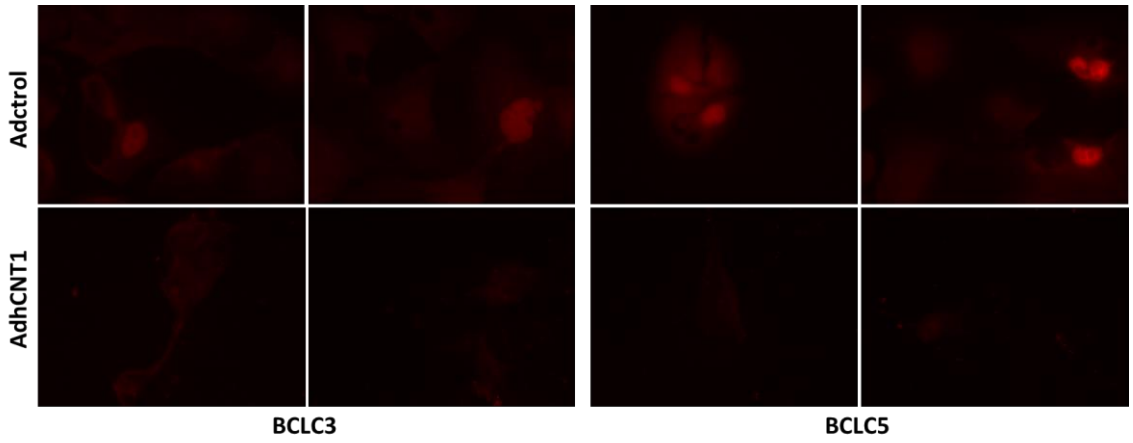


Figure 30. p21 expression in BCLC cells following hCNT1 restoration. BCLC3 and BCLC5 cells were infected with AdhCNT1 or Adctrol at 10MOI. Cells were stained by immunocytochemistry using p21 antibody. Representative images were obtained using fluorescence microscopy.

Moreover, we found that silencing hCNT1 expression in primary human hepatocytes results in the upregulation of p21 expression (Figure 31), which might be indicating a role of p21 that is mediated by the expression of hCNT1. Indeed, this might further support the role of hCNT1 in regulating some aspects of cell biology. However, this experiment was done only one time and additional validation is needed.

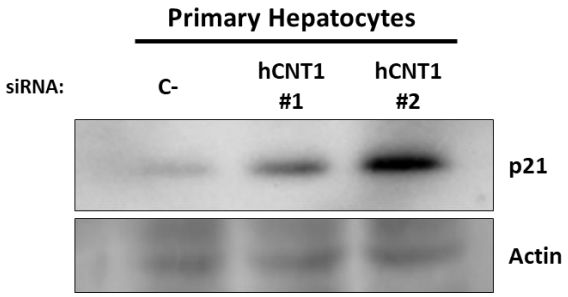


Figure 31. Effect of silencing hCNT1 expression on p21 expression. Human primary hepatocytes were treated with 100nM of two different hCNT1 siRNA (i.e. #1 and #2) or nontarget (C-) siRNA for 48h. Whole cell lysates were immunoblotted against p21 antibody.

C. PARP-1 hyperactivation.

As mentioned above, we have reported that hCNT1-induced cell death is accompanied by PARP-1 hyperactivation and subsequent accumulation of PAR polymers, with no cleavage induction or nuclear translocation of apoptosis-inducing factor (AIF), indicating a non-apoptotic and caspase-independent cell death (Pérez-Torras et al. 2013). In BCLC cells, we found that hCNT1 restoration similarly induced PARP-1 hyperactivation (Figure 32A).

PARP-1 hyperactivation was found to be cytotoxic and can initiate various cell death pathways (Andrabi et al. 2006; Robaszkiewicz et al. 2013; Zhang et al. 2014), due to the activation of poly ADP-ribosylation (PARylation) of PARP-1 itself as well as other proteins, including histones, DNA repair proteins and transcription factors (Xin Luo and Kraus 2012). All these effects can lead to cell death by various mechanisms, such as depletion of NAD+ (the substrate of PARP-1) or crosstalk between PARylation and cell death/survival kinases and phosphatases (Robaszkiewicz et al. 2013). Therefore, we assumed that this hyperactivation could be mediating hCNT1-induced cell death. Thus, we investigated whether PARP-1 inhibition is capable of sustaining survival following hCNT1 restoration. Experiments were undertaken on BCLC cells by inhibiting PARP-1 using PJ34 or Olaparib after hCNT1 restoration (Figure 32B). However, no significant effects on the decreased cell viability were observed after PARP-1 inhibition, as revealed by the MTT assays. Nevertheless, there was a slight increase in the viability of AdhCNT1-transduced BCLC5 cells treated with 10µM Olaparib when compared to the non-treated counterpart.

These results most probably suggest that PARP-1 hyperactivation does not mediate the cell death pathway and that hCNT1-induced cell death results from other effectors throughout the cell signaling cascades. Conversely, it might be that PARP-1 hyperactivation is related to other cellular processes, as suggested earlier in the first block of the results (1.3).

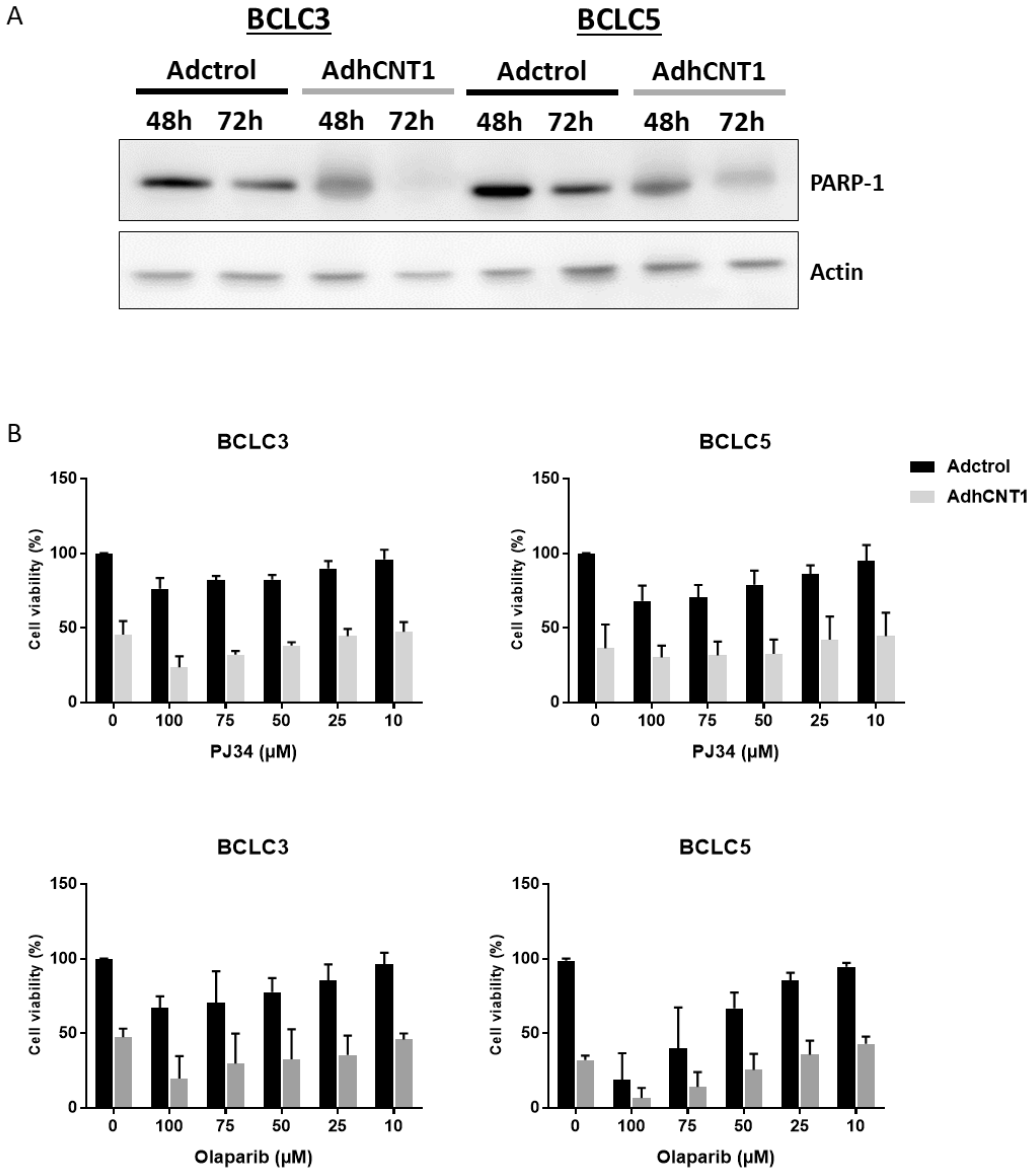


Figure 32. Effect of PARP-1 inhibition on hCNT1-induced cell death. BCLC cells were infected with AdhCNT1 or Adctrol at 10MOI. (A) PARP-1 expression following 48h and 72h of hCNT1 restoration. (B) Cell viability of hCNT1-transduced BCLC3 and BCLC5. Cells were treated with PARP-1 inhibitors PJ34 or Olaparib at the indicated dose for 48h. Cell viability were assessed by MTT assay. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test; *P<0.05, ***P<0.005.

Interestingly, it is known that PARP-1 is activated and autoPARylated by direct interaction with phosphorylated ERK2, which results in enhanced pERK2 phosphorylation of transcription factors and target genes (Cohen-Armon et al. 2007). In fact, this might be a link between PARP-1 activation and ERK signaling likely to result in increased tumor angiogenesis and metastasis, through PARP-1-mediated transcription of vimentin which enhances the activity

of critical pro-angiogenic factors including VEGF, and hypoxia-inducible factor (HIF) (Martin-Oliva et al. 2006; Pyriochou et al. 2008). This relation would further support our suggestion on ERK activation being a cytoprotective consequence to avoid or revert hCNT1 antitumoral induced effects.

2.2.2. Transcriptional Impact of hCNT1-restoration on HCC cells

To better understand the role that hCNT1 plays in cell biology and possibly in oncogenesis, and to acquire an overview of the whole altered signaling pathways, we decided to analyze the global transcriptomic alterations induced by hCNT1 restoration. BCLC cell lines were chosen due to their hepatic origin because this tissue seems to be one of the most prone to lose hCNT1 and even one of the most affected by its restoration. Thus, we infected BCLC3 and BCLC5 cells with AdhCNT1 or Adctrol for 24h, then mRNA extracts were validated for hCNT1 expression by RT-PCR (Figure 33) before proceeding with the microarray analysis using Illumina Human HT-12 V4 Bead Chip arrays.

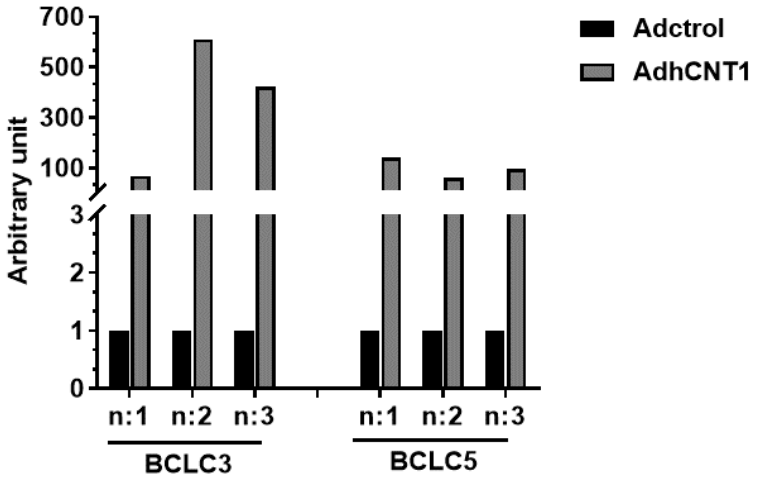


Figure 33. Verification of hCNT1 mRNA expression. BCLC3 and BCLC5 cells were infected with AdhCNT1 or Adctrol at 10 MOI, and mRNA samples from three independent experiments (n:1, n:2 and n:3) were analyzed 24h post-infection.

Afterwards, computational data analysis of the transcriptomic results was carried out comparing AdhCNT1 infected cells to their control counterpart. Interestingly, we found a huge impact of hCNT1 restoration at the transcriptional level. There were around 2000 differentially expressed genes that were significantly up- or down-regulated in both cell lines. In Figure 34, two heatmaps of the top 50 differentially expressed genes in each cell line are

illustrated. Downregulated genes are being notably more predominant than upregulated ones. Additionally, it is worth mentioning that the same experiments were carried out, in parallel, with hCNT2 transduction in BCLC3 and BCLC5. Interestingly, only few changes were observed. In fact, not more than 100 genes were shown to be altered after hCNT2 restitution. This would further indicate the specific role of hCNT1 in modulating cell physiology in a subtype-specific manner.

Interpreting such a huge transcriptomic data individually is complicated and might fail to detect by what means hCNT1 is contributing to modulate cell pathways. hCNT1-related genes might be distributed across an entire network, but changes can be subtle at the level of individual genes. Therefore, we performed a Gene Set Enrichment Analysis (GSEA). All genes were analyzed to compute a Normalized Enrichment Score (NES) for gene sets using two databases, the Reactome and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

The bioinformatic analysis (GSEA) of the differentially expressed genes following hCNT1 restoration in HCC cell lines, revealed significant enrichment of several pathways involving cell cycle, cell signaling and cell migration, which is consistent with what we know about the hCNT1 impact on cell biology. Table 3 shows the top significant downregulated and upregulated pathways, with a *p*-value <0.05 and a false discovery rate FDR <0.25. We found that several signaling pathways implicated in tumor progression were downregulated following the restoration of hCNT1. The most notable pathways were the proliferation and survival pathway, i.e. MAPK pathway, and the Focal adhesion pathway mainly responsible for cell migration and motility, along with other pathways contributing to cell survival and proliferation, i.e. transforming growth factor beta (TGFβ) and the platelet-derived growth factor (PDGF). The upregulated pathways following hCNT1 restoration were related to cell cycle, cell-cycle checkpoints and DNA replication and repair. Indeed, it seems that most of the downregulated pathways are known to be related to cell proliferation and migration and are extensively reported to be activated in cancer.

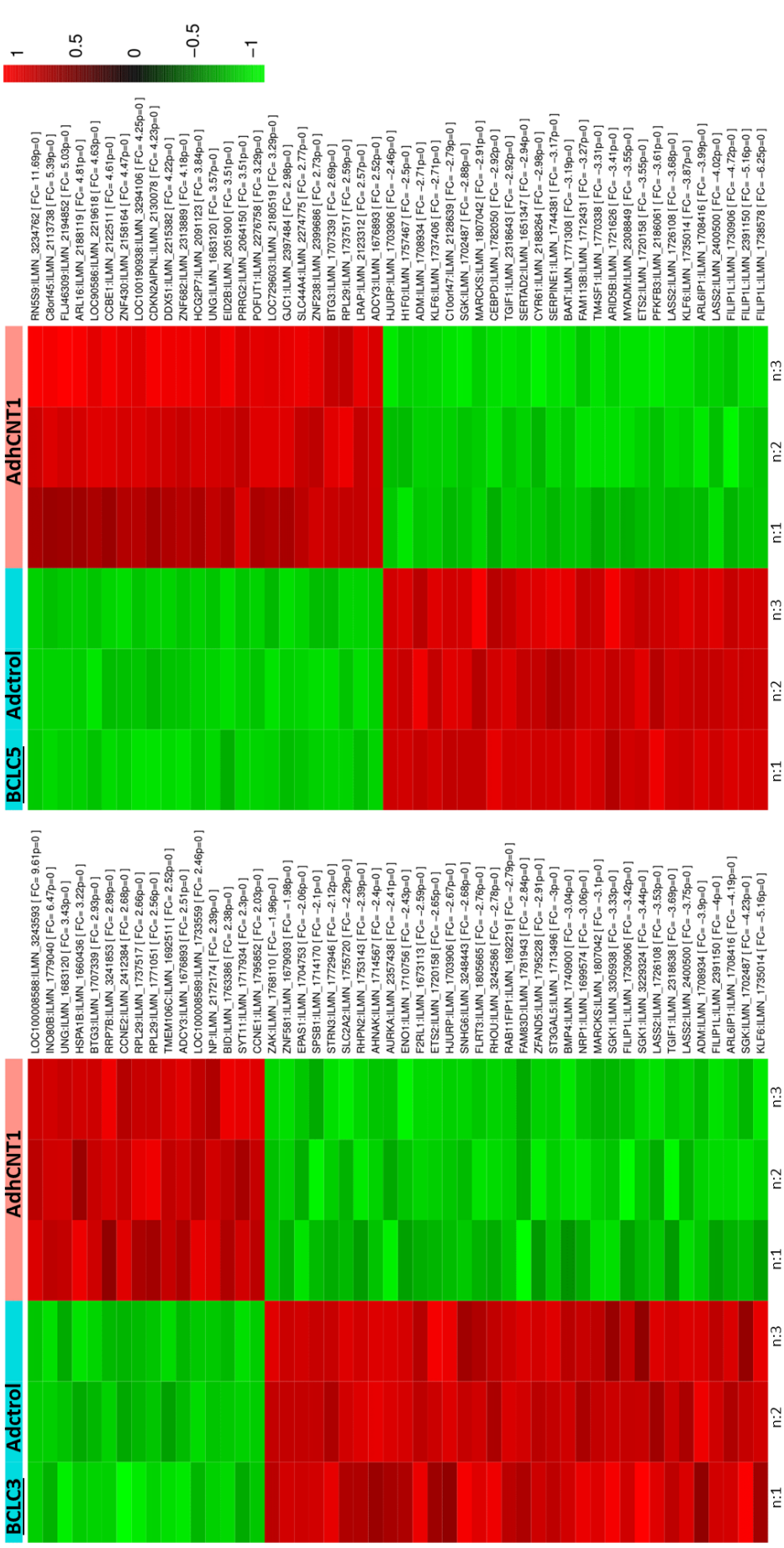


Figure 34. Heatmap of transcriptional expression changes in the 50 genes altered upon hCNT1 restoration in BCLCL3 and BCLC5. Color intensity means degree of gene expression regulation. Colors means: Green, downregulation; red, upregulation, black, no change. Heatmap columns refers to one of the three independent experiments.

Table 3. Top significant enriched pathways following hCNT1 overexpression.

DATABASE	PATHWAY NAME	NES	NOM p-val	FDR q-val	SIZE
KEGG	MAPK SIGNALING PATHWAY	-1.50	0.002	0.173	171
		-1.52	0.002	0.178	171
	FOCAL ADHESION	-1.53	0.000	0.156	137
		-1.62	0.000	0.130	137
	TGF BETA SIGNALING PATHWAY	-1.81	0.000	0.048	60
		-1.68	0.002	0.089	60
	CELL CYCLE	1.62	0.002	0.084	103
		1.51	0.004	0.160	103
REACTOME	GRB2-SOS PROVIDES LINKAGE TO MAPK SIGNALING FOR INTERGRINS	-1.58	0.024	0.130	12
		-1.79	0.002	0.047	12
	SIGNALING BY PDGF	-1.37	0.034	0.265	91
		-1.55	0.004	0.157	91
	M-G1 TRANSITION	1.96	0.000	0.003	67
		2.09	0.000	0.000	67
	G1-S TRANSITION	1.97	0.000	0.003	94
		2.05	0.000	0.000	94
	S PHASE	1.98	0.000	0.002	95
		1.97	0.000	0.001	95
	CELL CYCLE CHECKPOINTS	1.92	0.000	0.005	96
		2.02	0.000	0.001	96
	G2-M CHECKPOINTS	2.27	0.000	0.000	32
		2.17	0.000	0.000	32
	DNA REPLICATION	1.80	0.000	0.018	171
		1.60	0.000	0.089	171
	E2F MEDIATED REGULATION OF DNA REPLICATION	1.92	0.000	0.005	23
		2.17	0.000	0.000	23
	ACTIVATION OF ATR IN RESPONSE TO REPLICATION STRESS	2.45	0.000	0.000	27
		2.37	0.000	0.000	27
	SYNTHESIS OF DNA	2.12	0.000	0.000	79
		2.06	0.000	0.000	79
	DNA REPAIR	2.03	0.000	0.001	92
		2.06	0.000	0.001	92

Pathway enrichment analysis of differentially expressed genes analyzed by GSEA against gene sets of KEGG and REACTOME databases.

NES*= Normalized enrichment scores of the positively and negatively enriched genes sets.

Nom p-val= Nominal p value estimates the statistical significance of the enrichment score for a single gene set.

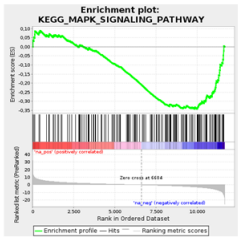
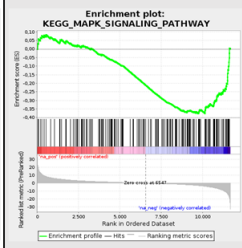
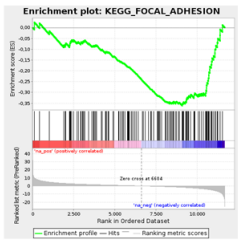
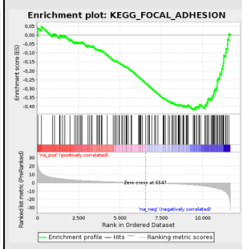
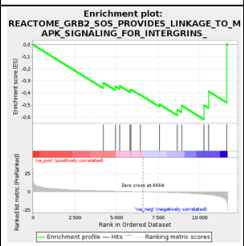
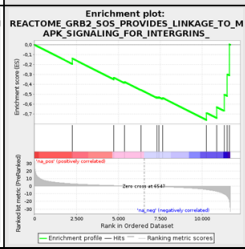
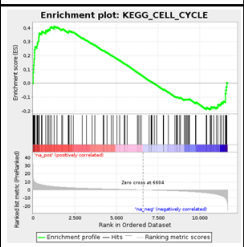
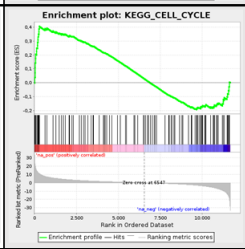
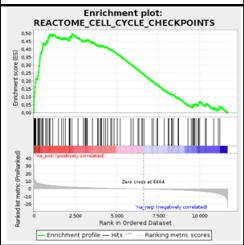
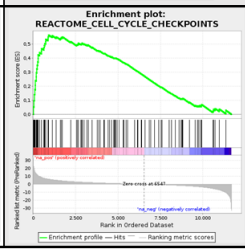
FDR q-val= False discovery rate q-value. An FDR of 25% indicates that the result is likely to be valid 3 out of 4 times.

SIZE= Number of genes involved.

*Note: A positive NES reflects enrichment of the gene set at the top of the ranked list, i.e. genes highly, positively correlated to the pathway. Gene sets with a negative NES are overrepresented at the bottom of the gene list, i.e. among negatively correlated protein-coding genes.

BCLC3 values are presented on the white rows and BCLC5 values are on the grey rows.

Table 4. GSEA-enrichment plot and core enriched genes.

	GSEA plots		Core Enrichment Genes
	BCLC3	BCLC5	
MAPK SIGNALING PATHWAY			RASA1 DUSP1 TGFBR2 CDC25B TNFRSF1A FLNB PDGFRA DDIT3 DUSP3 RPS6KA2 TGFB2 JUN NGF JUND MAP4K3 GADD45B RAPGEF2 GNG12 ZAK FOS CRKL PPM1B FLNC MAP2K4 SRF STK4 SOS1 RRAS RPS6KA5 FGF11 IKBKG HSPA1L NTF3 TAOK2 MAP3K8 PLA2G4B MAPK7 ATF2 FGF9 BDNF
			TGFB2 SRF TNFRSF1A DUSP1 RASA1 NGF JUN FLNB FOS JUND PDGFRA DUSP3 CDC25B CRKL RRAS BDNF TGFBR2 GADD45B STMN1 TAOK1 ATF2 NTF3 IKBKG DDIT3 ZAK DUSP14 MAP3K8 RAPGEF2 FLNC MAPK7 SOS1 GADD45A MAP2K1 GNG12 STK4 MYC PRKCA PPP3CB MAP2K4 TGFB3 PAK2 PDGFB SOS2 PPP3CC GADD45G
FOCAL ADHESION			CCND1 FLNB PDGFRA JUN MYL12A PIK3CB ITGAV ITGA5 TLN2 CRKL BIRC2 MYL5 ERBB2 FLNC ACTN1 ACTN4 GSK3B PIK3R1 PIK3R2 COL4A1 VEGFA SOS1 PPP1CB CAV2 SRC VASP PDGFC BCAR1 FYN COL1A1 LAMA5 VCL IGF1R ITGA2 LAMC1 PPP1CC PAK1 CAV1 LAMB3 ROCK1 THBS1 PRKCA PPP1R12A MYL7
			THBS1 JUN FLNB PDGFRA ACTN4 PPP1CB MYL12A CCND1 CRKL ITGAV ITGA5 ACTN1 PDGFC BIRC2 ERBB2 CAV1 SRC MYL7 COL1A1 GSK3B VCL PIK3R2 PPP1CC CAV2 MYL5 FLNC PIK3CB TLN2 SPP1 SOS1 MAP2K1 PRKCA PPP1R12A PIK3R1 PAK2 PDGFB SOS2 LAMB3 PIK3CD ZYX VTN BCAR1
GRB2-SOS PROVIDES LINKAGE TO MAPK SIGNALING FOR INTERGRINS			SLC38A2 SLC1A1 SNAP25 VAMP2 GLS SLC1A7 SYT1
			APBB1IP FGG FGA SRC SOS1
CELL CYCLE			CCNE1 CCNE2 E2F2 CDK2 CDC23 TFDP1 MAD2L2 PCNA CCND3 MCM2 MCM5 MCM3 PRKDC MCM6 MCM7 TFDP2 CHEK1 CDC7 MCM4 CDC25A HDAC2 PKMYT1 CHEK2 CDK4 RB1 YWHAQ CDKN2A YWHAE
			CCNE2 E2F2 CDC25A CCND3 CCNE1 TFDP1 MCM2 CDK2 MCM3 CHEK1 MCM4 PCNA MCM6 MAD2L2 MCM7 CDC23
CELL CYCLE CHECKPOINTS			CDK2 RFC5 MCM2 MCM5 MCM3 RPA1 RPA2 MCM6 MCM7 CHEK1 RFC4 RFC3 CDC7 MCM8 MCM4 CDC25A RFC2 MCM10
			CDC25A MCM8 MCM2 CDK2 MCM3 CHEK1 MCM4 MCM6 MCM7 MCM10 RFC5 RPA2 MCM5 RFC4 CDC7 RFC3 RFC2 RAD9A RPA1

Enrichment plots for the three downregulated and two upregulated pathways in BCLC3 and BCLC5 following 24h of hCNT1 restoration.The core enrichment genes are listed on the right (the core genes are the genes that mostly accounts for the gene set's enrichment signal).

Major pathways involved in hCNT1 transceptor signaling

A. MAPK pathway

This pathway consists of several sub-pathways that comprise key signaling and phosphorylation events that play a central role in cell physiology. MAPK sub-pathways regulate several cellular processes including cell growth, differentiation, proliferation, apoptosis, and migration. In tumors, the activation of these pathways is mediated by a complex network of interacting proteins, second messengers and signaling cascades that cumulatively govern all stimulated cellular processes and eventually promote tumorigenesis and cancer progression (Dhillon et al. 2007).

Restoration of hCNT1 expression in BCLC3 and BCLC5 was able to downregulate the MAPK pathway signaling. The enrichment analysis of this pathway has identified numerous differently expressed genes that are driving and contributing to the downregulation of this pathway (Table 4). The MAPK pathway was shown to be negatively altered through significant transcriptional downregulation of genes encoding some selected MAP kinases. These kinases included the ***MAP2K4*** (JNKK1), ***MAP3K8*** (MEKK8) and ***MAPK7*** (ERK5). Also, other MAPK regulatory genes such as the MAP kinase Phosphatases ***DUSP1*** and ***DUSP3***, together with several AP-1 transcription factors genes (i.e. ***JUN, FOS, JUND*** and ***ATF2***) were also leading the downregulation of the MAPK pathway.

Moreover, some important growth factor receptors genes were also contributing to the pathway downregulation i.e. ***PDGFRA, TGFBR2***. Other genes of nucleotide exchange factors, nuclear proteins and two MAPKs phosphatases were also found to contribute to the downregulation of this pathway in both BCLC cell lines. The enrichment plot and core enriched genes are illustrated in Table 4.

B. Focal adhesion pathway

This pathway regulates cell migration and adhesion by connecting the extracellular matrix (ECM) trough integrin-linked focal adhesions complexes to the intracellular signaling, whether directly or indirectly. We found that hCNT1 expression can regulate several members of the focal adhesion pathway in BCLC cells. These components include the integrin subunits ***ITGA5*** *and* ***ITGAV,*** along with their adaptor proteins such as talin (***TLN2***), α-actinin (***ACTN1/4***), filamin (***FLNB/C***) and vinculin (***VCL***). In addition, important regulatory receptors

and non-receptor tyrosine kinases genes i.e. ***GSK3B, PRKCA, PIK3CB*** and ***PAK1/2*** were shown to be transcriptionally downregulated. Moreover, the transcriptional downregulation of the AP-1 transcription factor member ***JUN*** is also contributing to the down regulation of this pathway. The detailed list of the core enriched genes of this pathway is illustrated in Table 4.

C. Integrin signaling to MAPK pathway

Notably, as shown in Table 3, the signaling cascade (GRB2-SOS) which links integrins signaling through the GRB2-SOS complex to the MAPK pathway, was downregulated too. GRB2-SOS is a complex of the nucleotide exchange factor SOS associated with the small adaptor protein GRB2. This signaling cascade links activated cell surface receptors to downstream targets and integrate cellular responses to growth factor signaling to regulate cellular proliferation, cytoskeletal reorganization and other responses necessary for cell survival in normal and cancer cells. In fact, both proteins are anticancer therapeutic target for strategies designed to prevent tumor growth, invasion and metastasis (Dharmawardana et al. 2006; Burns et al. 2014). The attenuation of this cascade, following hCNT1 restoration, was altered through the transcriptional downregulation of the nucleotide exchange factor gene ***SOS1***, and the gene of the critical non-receptor tyrosine kinase ***SRC***. *SRC* encodes proto-oncogene c-Src kinase, which phosphorylates the focal adhesion kinase (FAK) to form a complex with GRB2 and SOS, leading to the activation of Ras (Yee, Weaver, and Hammer 2008). This signaling linkage also implicates many other membrane receptor tyrosine kinases (e.g. EGFR) and not only integrins.

D. Other cell signaling pathways

hCNT1 expression was also able to induce alterations in other cell signaling pathways related to cell differentiation and proliferation, i.e. TGFB and PDGF signaling pathways. Downregulation of these two signaling cascades was observed following hCNT1 restoration in BCLC cell lines. In fact, both pathways are known to be upregulated in many types of tumors including HCC (Fischer et al. 2007).

TGFB and PDGF signaling pathways have been widely reported to signal upstream of multiple pathways, such as MAPK and Wnt/β-catenin signaling pathways. Actually, TGFB and PDGF signaling seems to contribute to the alteration of the MAPK as well as to the focal adhesion pathway. In fact, ***TGFBR2*** and ***PDGFRA*** genes, encoding for TGFB and PDGF

receptors respectively, were shown to be ones of the core enriched genes leading the downregulation of the MAPK and focal adhesion pathways (Table 4).

Overall, hCNT1 expression was able to modify the transcription of key cell signaling pathways. The MAPK and the focal adhesion signaling pathways are downstream targets of numerous cell receptors signaling, working cooperatively to regulate cell physiology. Thus, it can be suggested that the modulation of these pathways by hCNT1 expression could be a major driver to the hCNT1-induced phenotypic changes. Additionally, downregulation of all of the above mentioned pathways attenuates various cellular processes implicated in tumor progression (McLean et al. 2005; Santarpia et al. 2012). Thus, all these changes are likely to contribute to the hCNT1-induced cell death discussed in section 2.1. Moreover, the down regulation of numerous genes involved in the focal adhesion pathway could be one of the explanations for the migratory inhibition observed in the PDAC cell lines following hCNT1 restoration (Pérez-Torras et al. 2013). In fact, transcriptional downregulation of the MAPK pathway along with the focal adhesion pathway might help to clarify the underlying mechanism of hCNT1-induced phenotypic changes relevant to tumor biology.

E. Cell-cycle and checkpoint signaling, and the S-phase genes.

Restoration of hCNT1 expression also altered cell-cycle signaling, and other subsequent cell-cycle related pathways, such as cell-cycle checkpoints, DNA replication and DNA synthesis. In BCLC cell lines, different genes belonging to these pathways were upregulated following hCNT1 restoration. These alterations were accompanied by upregulation of gene sets responsible for DNA repair and ATR-kinase response to replication stress. Along with marked transcriptional increase of genes associated with S-phase of the cell cycle. In fact, the upregulation of the S-phase gene set is in line with the hCNT1-induced S-phase arrest associated with hCNT1 restoration in PDAC cell lines (Pérez-Torras et al. 2013).

The positive enrichment of the cell-cycle and the cell-cycle checkpoint pathways were mediated through the upregulation of several genes (listed in Table 4) encoding for important cell-cycle regulatory proteins. Proteins such as type E and D cyclins (***CCNE1/2, CCND1/3***), CDKs (***CDK2/4***), ***CDC25A*** and Minichromosome Maintenance protein complex (***MCM***) genes were identified in this pathway. Also, checkpoint kinases *CHEK1/2* and the proliferating cell nuclear antigen ***PCNA*** genes were also found to be hCNT1 modulated. ***CHEK1/2*** and ***PCNA*** encode for proteins (i.e. Chk1, Chk2 and PCNA respectively) that control cell-cycle checkpoints, DNA

replication and repair and cell cycle arrest to prevent damaged cells from progressing through the cell cycle. These data suggest that hCNT1 might also be involved in regulating cell cycle.

Notably, some of the upregulated pathways were related to DNA replication, DNA synthesis and ATR response to replicative stress (Table 3). "Replication stress" is a phenomenon unique to cancer cells that is described as the perturbation of error-free DNA replication and slow-down of DNA synthesis. This in turn can trigger recruitment and activation of several replication-stress response mediators that centrally involve the ATR-kinase, which is responsible for phosphorylation of the checkpoint kinase Chk1, suppression of new DNA replication origins from firing and activation of the S-phase checkpoint (Cimprich and Cortez 2008; Khurana and Oberdoerffer 2015). In fact, although replication stress induces genomic instability and fuels tumorigenesis, studies have shown this is only the case when replicative stress occurs at low to mild levels. High levels of replicative stress, however, may instead induce cancer cell death through mitotic catastrophe and therefore counteract cancer progression (Lecona and Fernández-Capetillo 2014; Berti and Vindigni 2016; J. Zhang et al. 2016). Collectively, this event can be one of the players of hCNT1-induced cell-cycle arrest.

On the other hand, it might be argued that S-phase arrest could be a consequence of DNA damage triggered by putative imbalance of dNTP pools and possible induced mutations, since we are overexpressing an active nucleoside pyrimidine-preferring transporter (hCNT1). However, as mentioned earlier, this might not be possible in this case because when we overexpressed other functional nucleoside transporters, hCNT2 or hCNT3 in BCLC cells, likely to induce dNTP pool imbalance as well, no effect on cell viability was observed (as we showed in 2.1.2). Besides, the overexpression of the transport-null hCNT1 mutant has similarly induced S-phase arrest in PDAC cell lines, as we reported previously (Pérez-Torras et al. 2013).

To summarize this section, hCNT1 restoration induced various alterations in BCLC cells physiology. We have shown hCNT1 restoration was able to induce activation of AKT, ERK and AP-1 transcription factor, along with PARP-1 hyperactivation and the downregulation of p53 and the CDK-inhibitors p21 and p27. Moreover, we have attempted to ascertain the molecular pathway underlying hCNT1-induced cell death. The data obtained indicated that the activation of AKT, ERK, AP-1 and PARP-1 hyperactivation do not seem to be crucial for hCNT1-induced cell death as individual factors. Conversely, downregulation of the CDKIs p21 and p27 were suggested to be mediating possible anti-proliferative events that lead to cell death.

On the other hand, hCNT1 expression seems to be regulating the transcription of key signaling cascades related to cell survival and cell migration i.e. MAPK and focal adhesion pathway. In addition, hCNT1 expression seems to be involved in the alterations of several cell-cycle related pathways.

An important question to be introduced here is: **How hCNT1, located at the cell membrane, is inducing all these changes?**

Considering the putative role of hCNT1 as a transceptor, it is possible that signaling occurs via protein-protein interactions. We proposed that hCNT1 is able to transduce signals inside the cell due to yet unknown stimuli. Our hypothesis is that some ligand-induced conformational changes allow hCNT1 binding to specific protein partners in order to induce these signals, which are recognized later by downstream intracellular effectors related to cell cycle, cell migration, cell survival and tumor growth regulation. In fact, our laboratory has recently identified several hCNT1 putative protein partners using two different approaches i.e. the bacterial two hybrid and the membrane yeast two hybrid (MYTH) methods to understand the mechanisms of hCNT-related modulation of cell biology. Interestingly, one of the identified putative hCNT1 protein partners is the Receptor for Activated C Kinase 1 (**RACK1**) (Pérez-Torras et al., in preparation).

RACK1 plays a key role in many signal transduction pathways. More than 100 proteins are known to interact with RACK1, either directly or as part of a complex. The MAPK and focal adhesion pathways have been continually reported to be signaling under regulatory interactions involving RACK1, such as promoting cell proliferation, cell growth, cell polarization and cell migration. The diverse functions of RACK1 are mediated by acting as a scaffold for its binding partners, that may be constitutively associated or bound in a transient stimulus-dependent manner often through interactions between RACK1 WD repeats and modular structural domains of the binding partners (Gandin et al. 2013). The disruption of several RACK1 interactions or complexes have been reported to induce contrasting effects on their associated cellular functions (Bandyopadhyay et al. 2008; Serrels et al. 2010; Ron et al. 2013).

hCNT1-RACK1 interaction has been comprehensively studied and validated in our laboratory (Pérez-Torras et al., in preparation). We proposed that hCNT1 interaction with

RACK1 might be affecting some of the diverse functions of RACK1, either by direct signaling through it or by disrupting its interactions with other regulatory proteins.

Rack1 associates with several components of cell growth and cell migration machinery including integrins, FAK, Src and the MAPK pathway core protein kinases (Adams et al. 2011; Einhorn 2013). Opposed roles of RACK1 on cell growth have been reported. On one hand, it leads to cytostasis, mainly through Src inhibition or by maintenance of an active CDK1/CyclinB complex that prevents cell cycle progression (Mamidipudi et al. 2007), while on the other hand, it induces proliferation by stimulation of the Androgen receptor activity, or through the MAPK pathway, particularly, by activating the MKK7/JNK signaling pathway via RACK1-MEKK7 interaction (Kraus et al. 2006; Guo et al. 2013).

Moreover, on cell migration, RACK1 transmit signals coming from the integrin-FAK-Src signaling to downstream effectors that regulate cell motility at several levels. It was suggested that RACK1 enhances the activation of the ERK signaling module by connecting it with the upstream activators, the FAK-Src complex and integrins, and the downstream effectors localized in focal adhesions. Thus, these effector proteins are responsible for the adhesion disassembly (Vomastek et al., 2007), regulated by a linearly organized signaling pathway consisting of integrins-FAK-Src-Raf/MEK/ERK proteins (Webb et al., 2004). Furthermore, there are at least two other mechanisms relating the integrins signaling through RACK1 and FAK in protein complexes to PDE4D5 or p190RhoGAP thereby regulating cell migration (Miller et al. 2004; Serrels et al. 2010).

3. hCNT1 as a transceptor

In previous studies from our laboratory, S100A11, a calcium-binding protein, was identified as a putative hCNT1 partner protein, using the cytosolic hCNT1 N-terminus tail as a bait in conventional bacterial two-hybrid approach that involved two cDNA libraries, from kidney and liver. This interaction was later confirmed by a targeted two-hybrid approach.

S100A11 protein is a member of the S100 family of Ca^{2+}-binding proteins. S100 proteins are regulated by Ca^{2+} binding, which allows them to act as Ca^{2+} sensors that can translate alterations in intracellular Ca^{2+} levels into cellular responses such as proliferation, differentiation, apoptosis, Ca^{2+} homeostasis, and migration through interactions with a variety of target proteins (Donato et al. 2013; Bresnick et al. 2015). Ca^{2+} binding induces a conformational rearrangement of S100 proteins that exposes a hydrophobic cleft, allowing the S100 protein to bind its cellular targets and elicit a physiological response (Haeseleer et al. 2002; Chang et al. 2007). S100A11 is located in the cytoplasm, nucleus, and in the cell periphery (He et al. 2009). There is some evidence suggesting that in normal cells, S100A11 is either fully nuclear or cytoplasmic depending on the cell status. However, localization in tumor cell is diffused (Davey et al. 2000; Cross et al. 2005; Gorsler et al. 2010). Dysregulated expression of multiple members of the S100 family is a common feature of human cancers. S100A11 has been associated with changes in cellular motility and cytoskeletal reorganization, as well as in tumorigenesis. Its overexpression is observed in a large variety of carcinomas, suggesting that S100A11 plays an important regulatory role in carcinogenesis and cell proliferation, whilst others suggest it possesses tumor-suppressing abilities (Gross et al. 2014; H. Chen et al. 2014; Xiao et al. 2017).

Notably, hCNT1-induced cell death shares similar features with glutamate-induced excitotoxicity and cadmium-induced cell death in brain cells, in terms of activated kinase pathways, i.e. ERK and AKT. Moreover, intracellular Ca^{2+} is known to play a major role in this type of cell death (Jiang et al. 2015; Gupta et al. 2013). Ca^{2+} is an ubiquitous intracellular messenger, it regulates various cellular processes by activating or inhibiting cellular signaling pathways and Ca^{2+}-regulated proteins. Upon this background, we aimed to verify whether the effect of hCNT1 could be dependent on Ca^{2+} and evaluate the role of S100A11 on hCNT1-induced cell death and also validate their putative interaction.

3.1. Intracellular calcium and hCNT1-induced cell death.

We started by analyzing the possible alteration of intracellular calcium ($[Ca^{2+}]_i$) likely induced by hCNT1 restoration. Therefore, we checked the steady-state of the $[Ca^{2+}]_i$ level in BCLC3 and BCLC5 following hCNT1 expression. This was done by imaging the $[Ca^{2+}]_i$ content using the high-affinity fluorescent Ca^{2+} indicator dye Fluo-4 AM, following 18h and 36h of AdhCNT1 infection at 10MOI. Cells were loaded with 5µM of Fluo-4 AM for 45min to allow for the dye to permeate cells. Cells were then incubated for an additional 20min with 1.5mM probenecid to avoid dye leakage. Afterwards, images were obtained by fluorescence microscopy. Interestingly, as shown in Figure 35, both cell lines exhibited an increase in Fluo-4 fluorescence in cells infected with AdhCNT1, which indicates a rise of the free $[Ca^{2+}]_i$. These results suggest a role for hCNT1 in the modulation of intracellular Ca^{2+}.

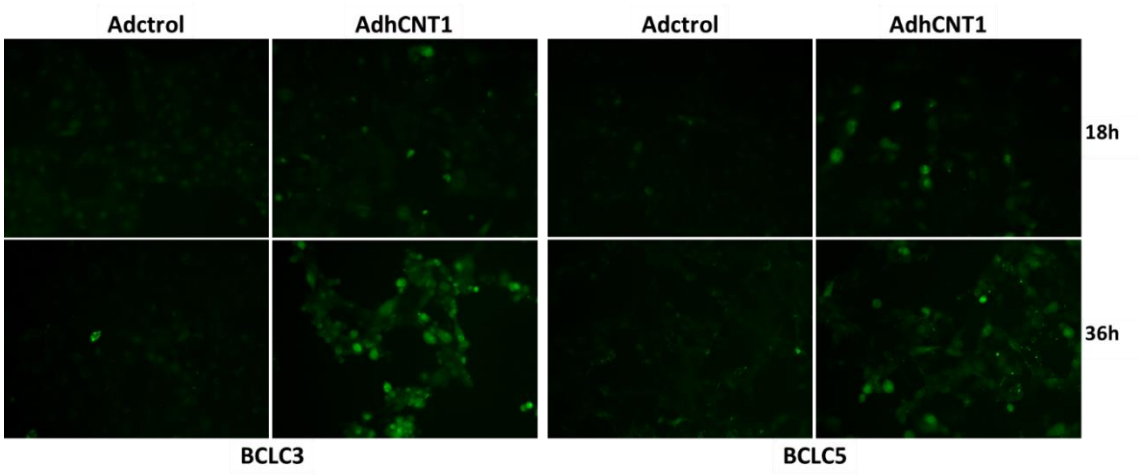


Figure 35. Increase of free intracellular Ca^{2+} content following hCNT1 restoration. BCLC cells were infected with AdhCNT1 or Adctrol at 10MOI 18h and 36h and then loaded with 5µM of Fluo-4 AM. Representative images were obtained using confocal microscopy at 10x magnification.

Based upon these observation we decided to further analyze the transcriptomic data discussed in the previous section, to identify Ca^{2+} related hCNT1-modulated biological processes using the Gene ontology (GO) platform (Ashburner et al. 2000; The Gene Ontology Consortium 2017). Table 5 presents the top significant Ca^{2+} related GO terms for biological processes. These were selected based on their calcium-containing nomenclature.

Table 5. Top significant Ca^{2+} Gene Ontology terms following hCNT1 restoration.

Gene ontology term	NES	NOM p-val	FDR q-val	SIZE
RESPONSE TO CALCIUM ION	-1.59	0.007	0.111	70
	-2.14	0.000	0.001	70
CALCIUM DEPENDENT PROTEIN BINDING	-1.72	0.006	0.060	40
	-1.69	0.002	0.068	40
DETECTION OF CALCIUM ION	-1.64	0.018	0.089	10
	-1.56	0.039	0.127	10
REGULATION OF CALCIUM ION TRANSMEMBRANE TRANSPORTER ACTIVITY	-1.47	0.039	0.171	33
	-1.53	0.011	0.141	33
POSITIVE REGULATION OF CALCIUM ION TRANSPORT INTO CYTOSOL	-1.41	0.081	0.215	23
	-1.75	0.007	0.047	23
POSITIVE REGULATION OF CALCIUM ION TRANSMEMBRANE TRANSPORTER ACTIVITY	-1.74	0.011	0.054	15
	-1.93	0.000	0.011	15

Gene Ontology enrichment analysis of differentially expressed genes analyzed by GSEA against gene sets from the GO Database.
- NES*= Normalized enrichment scores of the positively and negatively enriched genes sets.
- Nom p-val= Nominal p value estimates the statistical significance of the enrichment score for a single gene set.
- FDR = False discovery rate q-value. An FDR of 25% indicates that the result is likely to be valid 3 out of 4 times.
- SIZE= Number of genes involved.

*Note: A positive NES reflects enrichment of the gene set at the top of the ranked list, i.e. genes highly, positively correlated to the biological process. Gene sets with a negative NES are overrepresented at the bottom of the gene list, i.e. among negatively correlated protein-coding genes.

BCLC3 values are presented on the white rows and BCLC5 values are on the grey rows.

GO results showed that several calcium-related biological processes were downregulated in BCLC cells, 24h after hCNT1 restoration. Differentially expressed genes demonstrated downregulation of gene sets regulating the Ca^{2+} ion transport into the cytosol. In addition, a gene set termed as RESPONSE TO CALCIUM ION was also downregulated. This biological process term is defined as any process that results in a change in state or activity of the cell (in terms of movement, enzyme production, gene expression, etc.) as a result of a calcium ion stimulus (The Gene Ontology Consortium 2017). Moreover, cells possess specialized Ca^{2+} pumps, channels and calcium-binding proteins to maintain proper calcium levels and homeostasis (Berridge et al. 2003). The GO screening indicated downregulation of genes implicated in cellular detection and responses to calcium (Table 5). Therefore, downregulation of these biological process may result in disrupted Ca^{2+} homeostasis responses, and eventually $[Ca^{2+}]_i$ elevation and overload, as experimentally shown before.

However, the exact effector(s) underlying the increase of $[Ca^{2+}]_{i,}$ following hCNT1 restoration, have not been investigated yet. It can be suggested that the different hCNT1-induced signaling alterations (observed in the previous section 2.2 and 2.3) might contribute to the elevation of $[Ca^{2+}]_i$ levels. As a matter of fact, PARP-1 hyperactivation has been reported

to mediate $[Ca^{2+}]_i$ elevation during ROS-induced non-apoptotic cell death (F. Zhang et al. 2014). In BCLC cells, both PARP-1 hyperactivation and $[Ca^{2+}]_i$ increase were clearly detectable at later time points following hCNT1 restoration (observed in 2.2.1.C and in this section). This suggests that PARP-1 hyperactivation might be one of the factors contributing to the rise of $[Ca^{2+}]_i$ level.

Furthermore, Ca^{2+} signals are associated with different phases of cell cycle. Alterations in Ca^{2+} signaling or downstream pathways often disrupt progression of the cell cycle (Machaca 2010). Generally, disruptions in $[Ca^{2+}]_i$ homeostasis have been coupled to cell death. High and uncontrolled levels of $[Ca^{2+}]_i$ lead to cell death via different mechanisms such as necrosis, apoptosis, or auto-phagocytosis (Zhivotovsky and Orrenius 2011; Giorgi et al. 2015).

To determine whether the increases in $[Ca^{2+}]$i levels contribute to hCNT1-induced cell death, BCLC cells were transduced with hCNT1 for 18h, prior to incubation with the cell-permeable Ca^{2+} chelator, BAPTA-AM, at 5, 10 and 15µM for additional 24h. Interestingly, under these conditions, a highly significant prevention of cell death was observed in BAPTA-AM treated cells compared to non-treated cells after hCNT1 transduction (Figure 36). These results indicate that intracellular Ca^{2+} chelation attenuated hCNT1-induced cell death, suggesting that Ca^{2+} present in the cytosol plays a major role in hCNT1-induced cell death in BCLC cells.

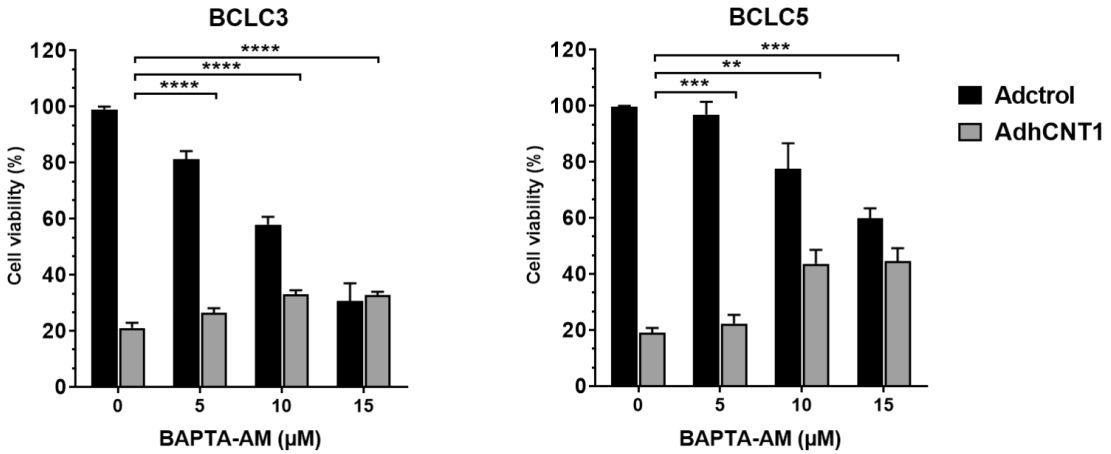


Figure 36. Intracellular Ca^{2+} chelation attenuated hCNT1-induced cell death. BCLC cells were infected with AdhCNT1 or Adctrol at 10 MOI and then treated with different concentrations of Bapta-AM at the indicated concentration, cell viability was assessed by MTT assay. Data are expressed as percentage of viable cells relative to cells infected without treatment. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test; **P<0.01, ***P<0.005 and ****P<0.0001

Increases in intracellular free Ca^{2+} play a major role in many cellular processes. Several Ca^{2+}-mediated signaling pathways are implicated in tumorigenesis and tumor progression, such as metastasis, invasion and angiogenesis (Liao et al. 2006; Cook and Lockyer 2006; Patton et al. 2003). Therefore, the apparent high increase of the free $[Ca^{2+}]_i$ following hCNT1 restoration is most likely to be resulting in $[Ca^{2+}]_i$ homeostasis disruption. This in turn would switch on multiple cytotoxic mechanisms, including mitochondrial permeability transition, disruption of cytoskeletal organization and Ca^{2+}-mediated nuclear changes (e.g. chromatin organization and gene expression) (Zhivotovsky and Orrenius 2011; Rodrigues et al. 2008). Thus, it can be suggested that the alteration of $[Ca^{2+}]_i$ following hCNT1 restoration is possibly mediating some aspects of hCNT1 antitumoral inhibitory effects in BCLC cells.

3.2. Alteration of the Ca^{2+}-binding protein S100A11.

To determine whether S100A11 expression is altered following hCNT1 restoration, we evaluated the mRNA and protein levels of S100A11, in BCLC3 and BCLC5, 24h and 48h after AdhCNT1 infection. The RT-PCR analysis showed decreased levels of S100A11 mRNA. Western blot analysis of cell lysates, of BCLC3 and BCLC5, did not show any changes on S100A11 protein expression at 24h, although, S100A11 protein levels were downregulated in both cell lines 48h after infection (Figure 37).

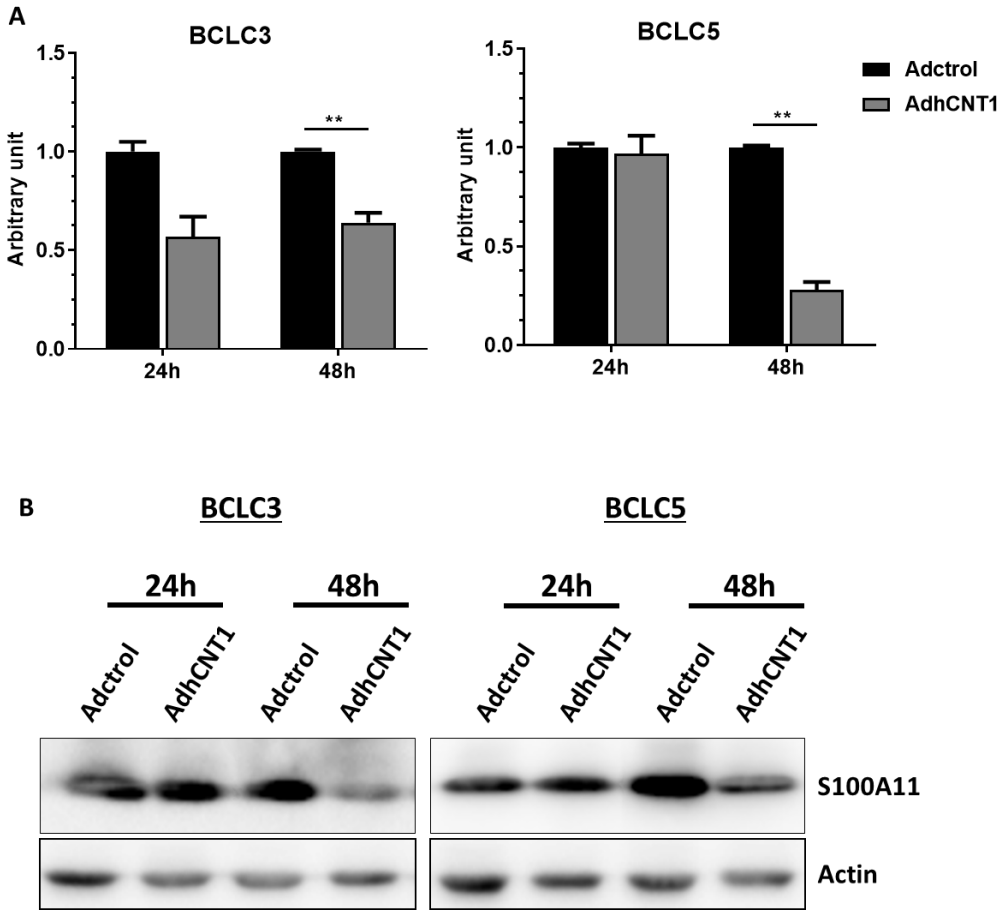


Figure 37. S100A11 expression following hCNT1 overexpression. BCLC cells were infected with AdhCNT1 or Adctrol at 10MOI. (A) S100A11 mRNA expression level following 24h and 48h of hCNT1 restoration. Results are means ± S.E.M. (n=3). Statistical significance was determined with Student's t-test; **P<0.01. (B) Western blots analyzed in total cell extracts by immunoblotting against S100A11 antibody.

S100A11 is widely expressed in multiple tissues, and its upregulated expression has been reported in several epithelial tumors. Its role in tumorigeneses has been frequently related to plasma membrane repair and tumor invasion, through its binding to Annexin A1/ A2 (Jaiswal et al. 2014; Y. Liu et al. 2015; Jin et al. 2017; L. Liu et al. 2017). Downregulated expression of S100A11 suppressed cell migration and invasion in various tumor cells, e.g. renal carcinoma cells, ovarian cancer cells and HCC cells (Luo et al. 2013; Y. Liu et al. 2015; L. Liu et al. 2017). Thus, it can be suggested that downregulation of S100A11 expression following hCNT1 restoration might contribute to and/or mediate hCNT1 antitumoral role in cell migration. This would further support S100A11 role in promoting tumorigenesis (Melle et al. 2008; Xiaoying Luo et al. 2013). Moreover, decreased S100A11 expression following hCNT1 restoration could be one of the related consequences to the downregulated cellular calcium responses in BCLC cells, as indicated by the GO annotation at 24h (Table 5). However, the exact regulation and molecular function of S100A11 under hCNT1 restoration is not well known yet and much work remains to be done to elucidate how S100A11 might be mediating hCNT1-induced effects.

3.3. Validation of S100A11-hCNT1 putative interaction

To validate S100A11-hCNT1 putative interaction, co-localization and co-immunoprecipitation (Co-IP) experiments were carried out. Using immunocytochemistry, we intended to determine the possible co-localization of hCNT1 with S100A11 protein. Co-localization assays were performed in BCLC3 and BCLC5 cells. Cells were infected with AdhCNT1 at 5MOI (to induce less cell death) and fixed after 48h of incubation. Fixed samples were then permeabilized for double immunostaining with monoclonal hCNT1 antibody along with a commercial S100A11 antibody. Then, samples were examined using laser scanning confocal microscopy. The obtained images have shown both proteins to be present at the cell membrane. Interestingly, co-localization of hCNT1 with S100A11 was observed on several points at the plasma membrane for both cell lines (Figure 38 and 39). These results suggest high chances for both proteins to be biochemically interacting.

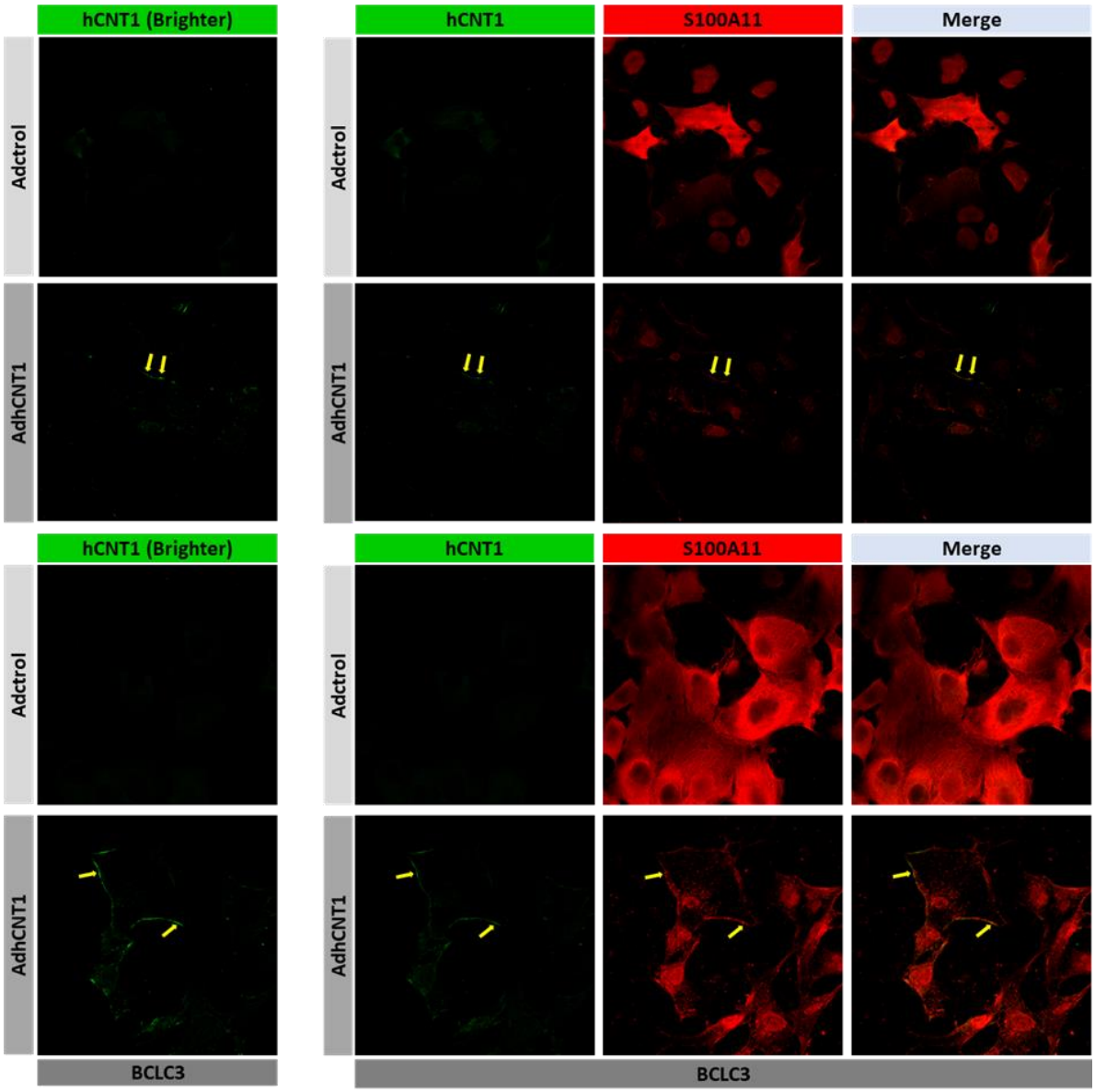


Figure 38. hCNT1 and S100A11 subcellular co-localization in BCLC3 cells. Confocal images of BCLC3 cells immunostained with hCNT1 antibody (green) and S100A11 antibody (red). All cells were infected with AdhCNT1 or Adctrol at 5MOI 48h. Images were obtained with the confocal microscope Leica SPE, objectives: 40x (upper box), and 63X (lower box). Co-localization points are indicated by the yellow arrows. Left column shows hCNT1 immunostaining at a higher brightness level for the same hCNT1 pictures on the right column.

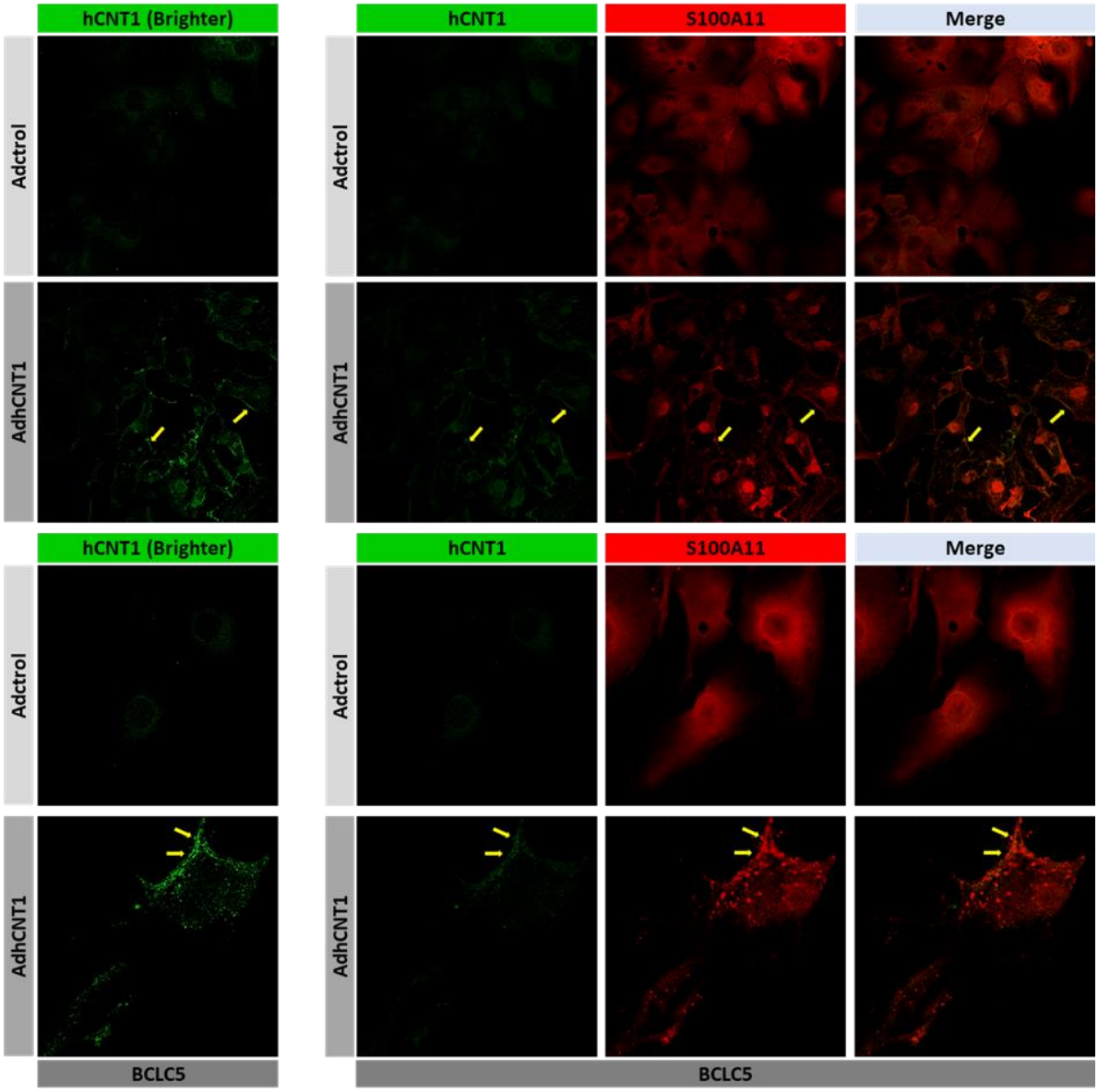


Figure 39. hCNT1 and S100A11 subcellular co-localization in BCLC5 cells. Confocal images of BCLC5 cells immunostained with hCNT1 antibody (green) and S100A11 antibody (red). All cells were infected with AdhCNT1 or Adctrol at 5MOI 48h. Images were obtained with the confocal microscope Leica SPE, objectives: 40x (upper box), and 63X (lower box). Co-localization points are indicated by the yellow arrows. Left column shows hCNT1 immunostaining at a higher brightness level for the same hCNT1 pictures on the right column.

Therefore, we performed Co-IP assays using HEK-hCNT1 cells and AdhCNT1 infected BCLC5 cells (to ensure high levels of $[Ca^{2+}]_i$ and subsequent enhanced interaction). Cell extracts were immunoprecipitated with S100A11 antibodies using the Dynabeads-magnetic system. Then, precipitated proteins were subjected to western blotting with antibody against hCNT1. As shown in Figure 40, both cell lines revealed S100A11 protein to be successfully immunoprecipitated, but hCNT1 was not detected to be co-immunoprecipitated.

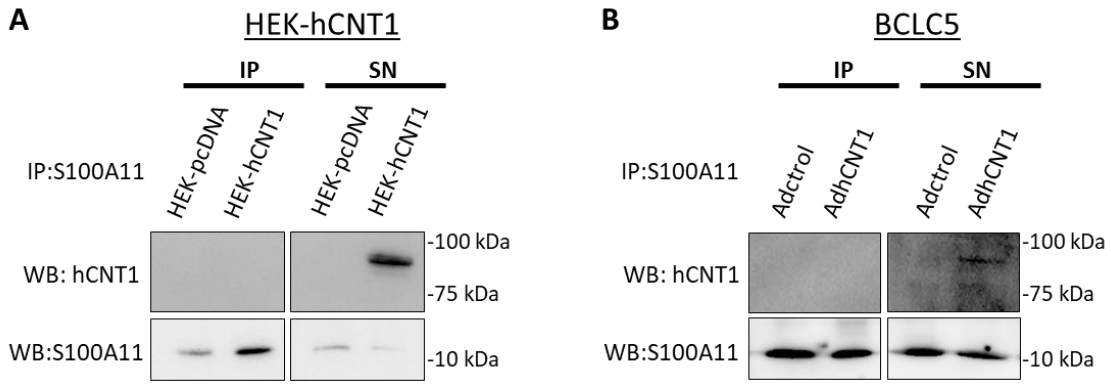


Figure 40. Co-immunoprecipitation of hCNT1 by S100A11 antibody. (A) hCNT1 Co-IP in HEK-hCNT1 stably transfected and HEK-pcDNA5 as a negative control. (B) hCNT1 Co-IP in BCLC5 infected with AdhCNT1 or Adctrol (as a negative control) at 10MOI for 48h.

Overall, we have been able to co-localize hCNT1 and S100A11 proteins, but co-immunoprecipitation assays need to be optimized. In fact, the negative Co-IP results does not rule out the possibility of protein-protein interaction. Technical errors might be interfering with the Co-IP technique. It could be that S100A11-hCNT1 interaction is affected by the calcium levels present in the Co-IP buffer. Thus, the interaction affinity is decreased due to S100 interactions are calcium-dependent (Donato et al. 2013). In addition, S100A11 is a small protein (12kDa), and binding to its antibody could induce the disruption of its interaction with hCNT1. Moreover, reversing the Co-IP by immunoprecipitating hCNT1 and detecting S100A11 was limited by the lack of availability of specific hCNT1 antibodies reliable for immunoprecipitation. On the other hand, we cannot rule out the possibility of S100A11-hCNT1 interaction to be transient. Transient protein-protein interactions readily undergo changes in the oligomeric state, whereas permanent interactions are strong and irreversible (Perkins et al. 2010).

Functional validation of S100A11-hCNT1 interaction has not been obtained yet. Further studies, which take this interaction into account, will need to be undertaken. To date, the biological and pathological functions of S100A11 have not been described in detail. However, since S100 proteins appear to lack enzymatic activity, it is supposed that they play their biological roles through binding to other proteins and changing their activity and/or cellular location (He et al. 2009). Recently, some proteins have been found to be able to associate with S100A11, and thus, the biological functions of S100A11 will be gradually revealed (Donato et al. 2013; Jaiswal et al. 2014). Therefore, in the meantime, it can be hypothesized that S100A11-hCNT1 interaction might be a Ca^{2+}-dependent regulatory mechanism controlling some aspect of hCNT1 role as a transceptor. To put it in other words, S100A11, as a Ca^{2+} sensor, could be transmitting the alterations of $[Ca^{2+}]_i$ signals to hCNT1. Subsequently, these signals are translated through hCNT1 into cellular responses. Likewise, a similar regulatory mechanism by another Ca^{2+} sensor, i.e. Calmodulin (CaM), on the nucleoside transporter hENT1 has recently been reported. Indeed changes of $[Ca^{2+}]_i$ levels modulate hENT1 function via CaM-dependent interaction with hENT1 (Bicket et al. 2016). Moreover, the regulation of membrane transport function by direct interaction of Ca^{2+}/CaM has been shown for other SLC families, such as SLC9A7, where identification and characterization of its interactome suggested Ca^{2+}/CaM regulation (Kagami et al. 2008).

S100A11 has been implicated in various cellular processes and functions by binding to an effector protein. For example, S100A11 interacts with Annexins to regulate membrane repair and cell migration (Poeter et al. 2013; Koerdt et al. 2017), with p53 to modulate its tumor suppression function (Fernandez-Fernandez et al. 2008) and with Annexin A1 to control cell growth (Sakaguchi et al. 2011). Therefore, S100A11-hCNT1 interaction could result in functional consequences on hCNT1 performance as a transceptor or even as a transporter.

As a signal transducer, hCNT1 could be signaling through S100A11. These signals could be contributing, with other hCNT1-putative protein partners, to the modulation of cell responses depending on the cell physiological context. In fact, besides RACK1 and S100A11, three novel proteins have recently been identified as putative hCNT1 partner proteins, i.e. Tyrosine-protein kinase ABL2, RhoGDI-2 and Prosaposin. All three proteins play important roles in distinct cell processes, such as cytoskeletal rearrangements, cell adhesion, cell proliferation. Regarding S100A11, not much is known about its mechanisms of signaling or its role in health

and disease. Further examination of S100A11 protein biology will be required to better define how it might contribute to proliferation, metastasis, angiogenesis and immune evasion of cancers and other diseases. This would further highlight the possible functional relevance of the S100A11-hCNT1 interaction.

Furthermore, it is known that calcium signaling and nucleoside transport are relevant to cell cycle (Warner et al. 2014; Pinto et al. 2015). A number of studies have implicated calcium as a potential regulatory component of a feedback mechanism that regulates nucleoside flux (Paes-de-Carvalho et al. 2005; Wall and Dale 2013; Bicket et al. 2016). In the rat hepatoma cell line FAO, which retain some CNT1 expression, CNT1 has been shown to be more abundant at the G1-S transition. This was interpreted as means to meet the rising demand of the cell for nucleosides during the late G1- and S-phases (Valdés et al. 2002). Nonetheless, with the knowledge provided by this thesis it is likely that changes in CNT1 expression during cell-cycle progression may not necessarily obey exclusively to nucleoside provision. Moreover, S100 proteins, including S100A11, were reported to have cell-cycle regulatory roles (Roderick and Cook 2008; Foertsch et al. 2013; H. Chen et al. 2014). Therefore, another possible suggestion is that S100A11-hCNT1 interaction might be affecting hCNT1 function as a nucleoside transporter. This could be a sort of Ca^{2+}-dependent regulatory mechanism by cell-cycle signaling pathways to regulate nucleoside uptake and meet cellular demands. To date, there is no evidence to suggest a direct link between hCNT1 and calcium, but there are several links between Ca^{2+} signaling and nucleoside transporters as a whole (Guida et al. 2002; Li et al. 2007; Bicket et al. 2016). Functional analysis of S100A11-hCNT1 interaction for nucleoside-related Ca^{2+}-dependent cell-cycle aspects would provide us with possible answers for this phenomenon.

In summary, we found that hCNT1 induces an increase of the free $[Ca^{2+}]_i$. This increase was suggested to be a result of the downregulated responses to Ca^{2+} ions following hCNT1 restoration, as shown by GSEA results (Table 5). The chelation of $[Ca^{2+}]_i$ by BAPTA-AM has significantly prevented hCNT1-induced cell death. Based upon that, we indicated that the alterations and disruption of Ca^{2+} homeostasis along with the increase of free $[Ca^{2+}]_i$ (Figure 35) are mediating hCNT1-induced cell death. Moreover, we found that the protein expression of S100A11 was downregulated following hCNT1 restoration. This seemed to be consistent with S100A11 role in promoting cell proliferation and migration. Regarding S100A11-hCNT1

interaction, we have shown that S100A11 co-localizes with hCNT1 on several points at the plasma membrane, whereas their Co-IP must be further optimized and demonstrated. Additionally, we propose that S100A11-hCNT1 interaction could be regulating or contributing to the hCNT1 transceptor function, whether as a signal transducer or even as nucleoside transporter. Finally, future research will hopefully better clarify the probable role of hCNT1 on regulating the physiological phenotype of the cell through different possible signaling of its interactome. In fact, a major breakthrough within the field might come from the elucidation of hCNT1 interactomics, aiming at linking it with other cellular events that would help in the understanding of the biological basis of their use as biomarkers of diagnosis and prognosis.

General Discussion

IV. General Discussion

Knowledge on SLC transporter selectivity and specificity is broad, but the biological impact of transporters on cell function beyond their mere role as suppliers of substrates is less well known. However, in the last years, novel roles are emerging for a growing number of these proteins. This latter evidence has led to the concept of selected transporters behaving as transceptors. Nucleoside transporters are essential for nucleoside and nucleobase uptake for salvage pathways to fuel nucleic acid synthesis. In addition, they facilitate cellular uptake of several nucleoside and nucleobase drugs used in the treatment of viral infections and cancer chemotherapy. Thus, the study of these proteins is of significant biomedical importance. For years, the study of hCNT1 has focused on the role that this protein plays in nucleoside transport and in nucleoside-derived drug efficacy and safety. In this thesis we have focused our study on the role of hCNT1 in cell physiology beyond nucleoside salvage. Thus, this work has been based upon the general hypothesis that hCNT1 is a transceptor signaling to modulate a variety of cellular functions and its loss might contribute to oncogenesis.

Our studies have shown that hCNT1 expression was downregulated in three different solid tumors from the gastrointestinal system, i.e. pancreatic ductal adenocarcinoma, colorectal carcinoma and hepatocellular carcinoma. Moreover, the restoration of hCNT1 has shown an antiproliferative effect on all the tumor derived cell lines analyzed. In general, these data support the fact that hCNT1 loss appears to be a common feature of solid tumors and are consistent with our hypothesis that hCNT1 loss is associated with oncogenesis. These data have also indicated that hCNT1-induced cell death seems to be predominantly occurring in these tumor backgrounds. In fact, this falls in line with the proposed tumor suppressive role for hCNT1 (Bhutia et al. 2011; Pérez-Torras et al. 2013), and might as well support the hypothesis of the likely tumorigenicity of hCNT1 loss in normal cells. On the other hand, this further indicates that hCNT1 expression is highly regulated. In fact, hCNT1 expression seem to be regulated and controlled at multiple levels. At a transcriptional level, we have shown that hCNT1 expression in HCC tumor cell lines could be somehow modulated by PARP-1 activity, either by directly affecting *SLC28A1* transcriptional events or modulating other proteins able to affect hCNT1 expression. This possible effect of PARP-1 on hCNT1 transcriptional expression could be explaining, in part, the diminished expression of hCNT1 in tumor cells. Nonetheless, hCNT1 transcriptional expression appears to be regulated by more

than a single molecular event. Ongoing projects in our laboratory are currently assessing various possible mechanisms of epigenetic regulation of hCNT1 expression (e.g. acetylation, methylation and miRNAs) in tumor cell lines. Moreover, hCNT1 expression seems to be also regulated by some post-translational modifications. However, further understanding of the mechanisms contributing to the loss of hCNT1 expression during oncogenesis would provide useful information and considerations significant to the potentiation of therapeutic treatments, especially in combined therapies with nucleoside-derived drugs.

To understand the role of hCNT1 in cell physiology, we attempted to explore the molecular mechanisms leading to hCNT1-induced cell death. We have demonstrated that alteration of the intracellular calcium signaling, following hCNT1 restoration in HCC derived cell lines, was a major factor mediating cell death, along with possible involvement of replication stress (observed by GSEA, although it requires further validation) that could be causing the S-phase arrest. However, the intermediate molecular events leading the calcium signaling to the cellular arrest and to cell death are still not clear. On the other hand, we initially assumed that the early activation of ERK, AKT and c-Jun was enabling a tumor suppressive role for these proteins, mediating an antiproliferative effect (Los et al. 2009; Desche et al. 2013; Tournier 2013). Conversely, none of these activations, when tested, have provided any clear evidence that these mechanisms are involved in the cell death response. Thus, one possible suggestion that was hypothetically assumed is that the activation of both kinases (ERK and AKT) and the transcription factor AP-1 might be a cytoprotective response that cells use to evade the antiproliferative effects induced by overexpressing hCNT1. However, the possibility that phosphorylation of these proteins altered additional events capable of modulating other hCNT1-related effects could not be ruled out. To address this issue, further functional validation of ERK, AKT or c-Jun -related events should be conducted. Nevertheless, a better understanding of the molecular mechanisms by which hCNT1 interactions affect the intracellular calcium signals and overcome the activated cell survival signaling, thereby resulting eventually in cell death would be of great importance. These understandings could provide further considerations for clinically useful therapeutic strategies and targets such as targeting altered Ca^{2+} signaling (Cui et al. 2017) or even DNA replication stress for cancer therapy (J. Zhang et al. 2016). Furthermore, this knowledge would shed new light on the

molecular mechanisms underlying the development and progression of HCC and contribute to developing more effective therapies in future clinical trials.

Moreover, another approach was also used to understand the role of hCNT1 in cell physiology by analyzing the global transcriptomic changes of hCNT1 restoration. The microarray data showed that hCNT1 was able to alter many signaling cascades including the MAPKs and the focal adhesion signaling pathways, together with some other cell survival signaling cascades and several cell cycle-related pathways. The involvement of hCNT1 in signaling to- and modulating such important regulatory pathways and signals could be highlighting the impact of its loss in tumorigenesis. To explain it better, alteration and downregulation of hCNT1 expression upon oncogenic transformation of differentiated cells could be contributing to deregulating several cellular processes linked to these signaling pathways (e.g. cell proliferation and migration). This would lead to a loss of regulation and enhance uncontrolled cellular functions, then possibly and ultimately contributing to oncogenesis (Ben-Ze'ev 1999; McCubrey et al. 2007; Kadio et al. 2016). However, several studies and further functional validations are needed to demonstrate these suggestions.

On the other hand, from the mechanism of cell death induction and the global transcriptomic analysis after hCNT1 restoration, we pretend to ascertain the main role hCNT1 plays in cell biology. Nonetheless, hCNT1 seems to be modulating different signaling events that are difficult to be analyzed independently in order to identify the main function of hCNT1. In fact, one of our major bottleneck to address this issue is the lack of a physiological model for hCNT1 endogenous expression *in vitro*. Thus, we had undertaken our studies using hCNT1 restoration approaches in BCLC3 and BCLC5 cells, which are originated from a cancerous background. Nonetheless, we think that the cellular responses and also the ultimate cell fate determined under hCNT1 restoration, might depend on the pathological and/or physiological context of the cell. Thus, depending on some particular cellular requirements and/or features or even the cell phase, different physiological consequences would result in response to hCNT1 signals. Actually, these suggestions might be supported, in part, by the nature of CNT1 expression in which it was found to be highly-regulated and cell cycle-dependent (Valdés et al. 2002; Duflot et al. 2002). Therefore, one possible explanation for the huge impact and signaling changes observed in the tumor cells, following hCNT1 restoration, could be due to its altered and uncontrolled signaling background. Tumor cells are continually exhibiting

altered signaling events. Restoration of hCNT1 might be inducing multiple effects that are observed at the same time, whether due to responses from a mix of several altered pathways (that might be also cross-talking) in the same cell, or a combination of responses from several cells that are individually exhibiting a different cell phase. In addition, another possible explanation could be that the amount of hCNT1 transduced into the cells might be further enforcing any possible interaction or signaling exerted by hCNT1 protein simultaneously. Likewise, some of the already identified hCNT1 putative partner proteins are usually altered and usually increased in tumors (e.g. RACK1 and S100A11). Thus, if hCNT1 signaling might depend on its interactome, its restoration in a tumor background would be furtherly inducing the signaling cascades related to each of its binding partner, all at the same time. In fact, the lack of effect on cell viability when we combined hCNT1 restoration with different inhibitors could support this hypothesis. However, in a normal background the modulation of these signaling cascades, mediated possibly by the endogenous hCNT1, should be exhibiting a normal regulated signaling, which could depend on the different physiological conditions of the cell.

Thereby, the analysis of hCNT1 behavior in cell biology in a normal background is warranted. This could better elucidate hCNT1 signaling targets and the biological consequences. As mentioned earlier, during the development of this thesis, we had undertaken our studies using hCNT1 overexpression approaches due to the bottleneck of the lack of cell lines retaining proper hCNT1 expression and the difficulty of obtaining human hepatocyte primary cultures. Nevertheless, we are currently working on silencing CNT1 expression in *in vivo* models, specifically, in mice liver. Afterwards, the impact of hCNT1 silencing on the transcriptomic profile will be monitored, and possible changes then will be validated at the protein level. In principle, silencing of hCNT1 expression would induce functional consequences, which according to our data, should be related to proto-oncogenic/proliferative phenotypes.

Furthermore, a note of caution is due here since all of the modulations and cellular responses, observed by the GSEA, was under the overexpression of a functional wt-hCNT1. Some of the indicated results could have been a consequence of an interference of a possible unbalanced nucleotide pool. Still, most of the determined data, whether by GSEA or the other approaches used along this thesis are in line with what we previously described about the

impact of hCNT1 in cell biology (Pérez-Torras et al. 2013). In fact, we are working now to further validate these data transcriptionally and functionally, using both wt-hCNT1 and the transport-null hCNT1SP mutant in order to rule out any effects that might be induced by a possible imbalanced dNTP pools interference.

Moreover, regarding hCNT1 function as a transceptor, we believe that hCNT1 signaling function and the hCNT1-related cellular events might be dependent on its interactome. Since hCNT1 is normally expressed in several differentiated cell types (Farré et al. 2004; Govindarajan et al. 2008), the identification of various important cell regulatory proteins as partners to hCNT1 have provided further explanation to the importance of its cellular redundancy. Consistently, RACK1-hCNT1 interaction was validated biochemically and functionally in our laboratory (Pérez-Torras et al., in preparation). Then, this interaction was assumed to be contributing to the modulation of the different cell signaling pathways altered following hCNT1 restoration. Likewise, S100A11 seems to interact with hCNT1, and hopefully their protein interaction will be definitely validated biochemically and functionally.

Accordingly, we have proposed that S100A11-hCNT1 interaction might be involved in the alteration of the intracellular calcium signaling, although, this still needs further characterization. Furthermore, it is indicated that what underlines the sensing function of transceptors is ligand-induced conformational alterations recognized by downstream intracellular effectors (Diallinas 2017). Thus, for hCNT1, the stimulus or ligand that is possibly inducing hCNT1-intracellular signaling is still unknown. Although, it seems logical to envisage a nucleoside binding. Interestingly, hCNT1 is known to bind adenosine with high affinity but cannot translocate it (Larráyoz et al. 2004). This has risen the question of whether this purine nucleoside may exert additional physiological roles besides its known interaction with purinergic receptors. Evidently, this fact is noteworthy, the binding of adenosine to hCNT1 might play some role in transmitting a purinergic signal that perhaps results in the hCNT1-induced effects. Notably, several studies have indicated that adenosine, via stimulation of its receptors, is involved in cell proliferation and cell death. This was mostly observed by A_3 adenosine receptor modulation of the ERK1/2 and/or the Akt signaling pathways (Merighi et al. 2006; Borea et al. 2014; Jafari et al. 2017). Thus, our data could propose a novel purinergic signaling by the pyrimidine-preferring transceptor, hCNT1. Nonetheless, this point has not been explored yet, and further studies with more focus on the rationale of adenosine/hCNT1

binding and possible subsequent signaling are warranted. As a matter of fact, further studies on hCNT1 interactome will provide important insights into the nature and the underlying mechanisms of hCNT1 signaling (from an upstream level at the cell membrane) and modulation of cellular processes. However, although the stimulating effector of hCNT1 signaling is still unknown, this change of paradigm in hCNT1 transporter biology opens new possibilities for further investigation of hCNT1 properties in cell biology.

In summary, this thesis has provided an important advance in knowledge in the Nucleoside Transporters field. Lastly, hCNT1 expression can become a suitable biomarker, as long as consistent correlations linking it with tumor progression and outcome can be proven. The elucidation of novel pathways linking hCNT1 with a variety of intracellular events would likely lead to the identification of additional novel biomarkers of cell transformation, ultimately relevant in translational medicine for prognosis, diagnosis, and even treatment.

Conclusions

V. Conclusions

- hCNT1 mRNA expression is reduced in gastrointestinal cancers. HCC seems to be the most prone to down-regulate hCNT1 expression.

- Transcriptional regulation by PARP1 seems to be one of the mechanisms governing hCNT1 mRNA expression, either directly by affecting *SLC28A1* transcriptional events or indirectly by modulating other proteins such as transcription factors.

- Restitution of hCNT1 induces an antiproliferative effect in different solid tumor backgrounds. This effect is hCNT-subtype specific.

- hCNT1 restitution in tumor cells triggers a massive transcriptomic response. Transcriptomic changes impact on several different but inter-related signaling cascades, which are relevant to many cell biological processes, among them those known to be associated with hCNT1 restitution, such as cell migration and survival.

- hCNT1-induced cell death seems to be triggered by an increase in intracellular Ca^{2+} levels.

- The interaction of S100A11 with hCNT1 might be one of the mechanisms by which the hCNT1 transceptor is modulating cell biology, whether to respond to a Ca^{2+} signal or to exert an intracellular Ca^{2+}-dependent signal.

Materials and Methods

VI. Materials and Methods

1. Cell culture

Cell culture is the process by which cells are grown ex vivo under controlled conditions in order to maintain their original physiological, biochemical and genetic properties utmost. Experimenting with cell culture provides notable advantages to the biomedical research since it allows the study and analysis of relatively homogeneous cell populations. Primary cultures are those obtained directly from tissues of a specific organism and they have a limited lifespan. Likewise, established or immortalized cell lines can be generated by acquiring the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. Numerous cell lines were well established as representative of particular cell types. Cell lines are better conserved than primary cell culture and they also allow to perform long-term experiments. Nevertheless, as they do not have proliferation control they may lose cellular characteristics which lead them to a less differentiated state than the original tissue.

Working with cell culture entails the maintaining of strict sterile conditions. Therefore, it is required to work in a laminar flow hood previously sterilized with UV light and all material used have to be sterile as well as all media and reagents.

1.1. Cell maintenance, seeding and preservation.

Cells were cultured in an incubator at 37°C with 5% CO^2 and 95% humidity. All cell lines used were split every two to three days using Trypsin/EDTA. Trypsin is a serine protease which cleaves proteins bonding the cultured cells to the dish, so that the cells can be suspended whereas EDTA chelates the calcium involved in cell-cell adhesion. Following a 1 step of PBS buffer washing, cells were incubated with trypsin/EDTA for 2-5 min at 37°C to detach cells. The proteolytic cleavage reaction by trypsin was stopped by adding new inactivated fetal bovine serum (FBS) containing medium to the cells. At this point, cells were ready for passaging, seeding or freezing.

- **For passaging,** a small number of cells were transferred to a new dish and fresh medium was added.
- **To use the cells in further experiments**, cells were counted using the Invitrogen cell counter and then seeded at the required density depending on the experiment.
- **For freezing and thawing**, cryoprotectors such as DMSO (dimethylsulfoxide) are required to ensure cell viability after freezing. Thus, trypsinized cells were centrifuged for 4 minutes at 1200rpm. The supernatant was removed, then cells were resuspended in freezing medium (FBS + 10% DMSO) and aliquoted in 2mL cryovials. Subsequently, these vials were stored in the cell freezing container which provides the 1°C/min cooling rate required for successful cryopreservation of cells. This container was placed in a -80°C freezer for 2 days to 1 month and afterward the cryovials were stored in a liquid nitrogen tank at -196ºC. Later on, thawing frozen cells was done by placing the vial in a 37ºC bath and transferring the cell suspension for centrifugation for 4 minutes at 1200rpm. Then cells were resuspended in fresh medium with 20% FBS and plated in a new dish.

Materials:

- PBS buffer: 137mM NaCl, 2.7mM KCl, 1.4mM KH2PO4 and 4.3mM Na2HPO4 at 7.3 pH.
- Fetal bovine serum. FBS was inactivated by incubation at 56°C for 45 minutes (min).
- 0.05% Trypsin-EDTA from Life Technologies.
- Cell culture Medium from Lonza

1.2. Cell lines used

1- **BCLC cell lines.** BCLC3 and BCLC5 cell lines were obtained from Dr. Jordi Bruix group (Hospital Clinic, IDIBAPS & CIBERehd). They were generated from human hepatocellular carcinoma. BCLC3 were originated from a 67-year-old male patient. BCLC5 were originated from a 48-year-old female patient. The medium used for these cell lines is DMEM F-12 supplemented with 5% of inactivated FBS, 1% of 200mM sodium pyruvate and 1% non-essential amino acids. The BCLCs were continually sub-cultured before reaching full confluence in a dilution of 1:5-1:6 for BCLC3 and 1:4 to 1:5 for BCLC5.

2- **HEK293-hCNT1/pcDNA**. These are human embryonic kidney 293 cells (HEK293) stably transfected with hCNT1 gene or pcDNA5 empty vector generated at our laboratory by Dr. Medina-Pulido with the Flp-InTM system. DMEM medium supplemented with 5% of inactivated FBS were used for the maintenance of these cell lines. These cells were continually sub-cultured before reaching full confluence in a 1:10 dilution.
3- **HepG2 cell line.** Epithelial HepG2 cells derived from a well-differentiated HCC of a 15-year-old caucasian male from Argentina. DMEM medium supplemented with 5% of inactivated FBS was used for the maintenance of this cell line.
4- **Huh7 and Huh7.5 cell lines.** A well-differentiated hepatocyte-derived cellular carcinoma cell line that was originally obtained from a liver tumor of a 57-year-old Japanese male in 1982. DMEM medium supplemented with 5% of inactivated FBS were used for the maintenance of these cell lines.
5- **NP9 and CP15T cell lines**. This cell line was derived from human pancreatic adenocarcinomas that had been perpetuated as xenografts in nude mice. DMEM F-12 medium supplemented with 5% of inactivated FBS were used for the maintenance of these cell lines.
6- **HT29 cell line**. A human colorectal adenocarcinoma cell line with epithelial morphology originated from a 44-year-old female patient. DMEM medium supplemented with 5% of inactivated FBS was used for the maintenance of this cell line.
7- **TFK-1 cell line**. This cell line was originated from a surgical specimen extracted from a 63-year-old Japanese man with extrahepatic bile duct carcinoma (cholangiocarcinoma). RPMI 1640 medium supplemented with 5% of inactivated FBS was used for the maintenance of this cell line.

- All media were supplemented with 1% antibiotics (penicillin/streptomycin).

2. Cell culture treatments

2.1. Adenovirus transduction

Viral vectors are commonly used tools to deliver genetic material into cells. This process can be performed in cell culture (*in vitro*) or inside a living organism (*in vivo*). Delivery of genes

by a virus is termed transduction and the infected cells are described as transduced. Viruses have specialized molecular mechanisms to efficiently transport their genomes inside the cells they infect. Adenoviral vectors provide the most efficient gene transfer among other viral vector systems for a wide variety of cell types. Especially, they can transfer genes into both proliferating and quiescent cells. Moreover, they do not integrate into the host cell genome so that there are low disturbances in genes or cellular processes.

For these experiments, cells were seeded 24h before adenovirus infection. Viral stocks were diluted to reach the desired MOI in medium supplemented with 5% of inactivated FBS. This was added to the cell monolayer. Infection was stopped after 4 h by replacing the medium.

Recombinant adenovirus for Ad5CMV-hCNT1 (AdhCNT1) was previously generated in our laboratory by double recombination in bacterial cells. Briefly, hCNT1 were cloned into a pShuttle-CMV plasmid, then recombinant adenoviruses were generated by cotransforming each transfer vector with the viral DNA plasmid p3602 into Escherichia coli strain BJ5183. The recombinant adenoviral construct was then transfected into HEK293 cells to produce viral particles. Individual viral plaques were isolated and amplified in HEK293 cells, and recombinant adenoviruses containing hCNT1 was identified by restriction enzyme digestion and PCR. The control adenovirus lacking an insert, Ad5CMV-1613 (Adctrol) was generated similarly. All adenoviruses were propagated in HEK293 cells, and functional transduction units were determined using the hexon protein-staining technique.

2.2. Transfection with TransIT-siQuest

Genetic material, such as supercoiled plasmid DNA or siRNA constructs, are transfected into animal cells by typically opening transient pores in the cell membrane to allow the material uptake. Transfection is the process of deliberately introducing nucleic acids into cells, specifically for non-viral methods in animals cells. TransIT-siQUEST® is a broad spectrum transfection reagent for siRNAs, it enables the delivery of siRNA with high efficiency and knockdown of target gene expression in many cell types. Transfections with TransIT-siQUEST do not require medium changes and can be carried out in serum-containing medium. Following 4h of AdhCNT1 and Adctrol infection, the medium was removed and replaced by

fresh new medium. The transfection mixtures were prepared according to Table 6. Therefore, in a sterile tube, TransIT-siQUEST® and the desired siRNA were mixed with serum-free medium and incubated at room temperature for 20 minutes. This mixture was distributed on the cell culture medium of each plate. Then cells were assayed within 48 hours after transfection.

Table 6: Recommended starting conditions for siRNA transfections with TranslT-siQUEST Transfection Reagent.

Culture vessel	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate	10-cm dish	T75 flask
Surface area	0.35 cm^2	1.0 cm^2	1.9 cm^2	3.8 cm^2	9.6 cm^2	59 cm^2	75 cm^2
Complete growth medium	92 µl	263 µl	0.5 ml	1.0 ml	2.5 ml	15.5 ml	19.7 ml
Serum-free medium	9 µl	26 µl	50 µl	100 µl	250 µl	1.5 ml	1.9 ml
*Trans*IT-siQUEST Reagent	0.3 µl	0.8 µl	1.5 µl	3 µl	7.5 µl	46 µl	59 µl
siRNA (10 µM stock) 25 nM final	0.25 µl	0.7 µl	1.4 µl	2.8 µl	6.8 µl	42.5 µl	54 µl

2.3. Transfection with lipofectamine

Lipofectamine® 2000 transfection is based on the formation of complexes from a cationic lipid and DNA producing liposomes. Those liposomes will fuse with the cell membrane internalizing the genetic material into the cytoplasm. The mortality rate of that technique is even higher than using calcium-phosphate, therefore initial culture should be at 75-90% of confluence before transfection.

Once cells were ready to be transfected, first step was removing the old culture medium and replace it by fresh new non-supplemented medium, since antibiotics and serum might interfere in the liposomes formation. In one sterile tube, lipofectamine® and non-supplemented medium are mixed and incubated at room temperature for 5 minutes. In another sterile tube, DNA is added to non-supplemented medium. Both preparations were eventually mixed adding the lipofectamine® to the DNA tube. After mixing, this transfection mix was incubated for 20 minutes at room temperature and added to the cell culture. Cells

were incubated for 4 hours at 37°C. Afterwards, transfection mix is replaced by fresh medium properly supplemented. Cells then were assayed within 48 hours after transfection.

2.4. Bapta-AM treatment

BAPTA, AM is a cell-permeant chelator, which is highly selective for Ca^{2+} over Mg^{2+}, and it can be used to control the level of intracellular Ca^{2+}. BAPTA is more selective for Ca^{2+} than EDTA and EGTA, and its metal binding is also less sensitive to pH.

For this treatment, cells were seeded 24h before adenovirus infection. Viral stocks were diluted to reach the desired MOI in medium supplemented with 5% of inactivated FBS. This was added to the cell monolayer. Infection was stopped after 4h by replacing the medium. Next day, at 18h after infection, cell medium was changed with a fresh medium containing the desired concentration of Bapta-AM (Abcam), then cells were incubated for additional 24h and after that cells were assayed.

3. Protein expression techniques

3.1. Protein extraction

Cellular lysates were obtained from cells seeded and treated on 60mm or 100mm plates. The growing medium from each plate was removed and plates were washed twice with PBS making sure no medium traces were left. Then, plates were snap frozen in liquid nitrogen and stored at -80ºC or proceeded to directly to protein extraction protocol.

To proceed with the protein extraction protocol, cell lysis buffer was added to the cells (50-60µl for 6 well plate, 70-100µl for 60mm dish, 250µl for 100mm dish). From this point all the process was done on ice or at 4ºC to avoid protein degradation. Using a plastic cell scraper, adherent cells were scrapped off the plate and suspension were collected in a 1.5mL tube which was kept for 5 to 10 minutes on ice.

Then, cell suspension was passed through a 1ml syringe with a needle to ensure homogenization. After that, all samples were centrifuged at 13,000rpms at 4ºC for 15 minutes. The resulting supernatants containing the total proteins were then transferred into

a clean 1.5ml tube, and proceeded to protein quantification using the Bradford protocol, and then stored at -20ºC.

Materials:

- **Lysis buffer**:
    - **NP-40**:
        - 50 mM Tris (pH 7.4)
        - 250 mM NaCl
        - 1% NP-40
        - 5 mM Pyrophosphate sodium
        - 50 mM NaF
        - 1 mM sodium ortovanadate
        - Proteases cocktail of inhibitors (Complete Mini, Roche)

    - **Phosphorylated proteins buffer**:
        - 10 mM Tris (pH 7.4)
        - 400 mM NaCl
        - 5 mM NaF
        - 10% glycerol
        - 1 mM EDTA
        - 1 mM sodium ortovanadate
        - 0.5% NP40
        - 4 mM dithiothreitol (DTT)
        - Cocktail of protease inhibitors (Complete Mini,Roche)
        - Cocktail of phosphatase inhibitors (PhosSTOP, Roche).
        - This buffer preserves the phosphorylation state of the proteins.

3.2. Protein quantification

Protein concentration was measured by using Bio-Rad Bradford reagent (Bio-Rad) and bovin serum albumin (BSA) as standard protein. The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). BSA stock solution at 1 mg/ml were used to prepare the dilutions of the protein

standard curve. Bradford Reagent was used at 1:5 dilution. Absorbance was measured at 595 nm.

3.3. Electrophoresis and immunoblotting

3.3.1. SDS-PAGE electrophoresis

Electrophoresis is a technique used for protein separation. When separated on a polyacrylamide gel, the procedure is called SDS-PAGE (Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis). SDS is an anionic detergent applied to protein sample to impart a negative charge to linearized proteins. The binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N-methylenebisacrylamide (Bis). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with TEMED. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The gel is composed of two parts, the stacking gel, which has a low acrylamide percentage (5%) and allows the alignment of the samples before they get in the resolving gel which will be in charge of the separation. The acrylamide concentration can be from 8% to 15% depending on the size of the protein of interest being gels of higher percentage more suitable for smaller proteins.

Materials:

- **Electrophoresis buffer**:
  - 250mM Tris-base
  - 1.91M glycine
  - 1% SDS
  - in distilled water.

- **Loading buffer (5X)**:
  - 62.5mM Tris-HCl; pH 6.8
  - 2% SDS; 20% glycerol

- 0.01% bromophenol blue.
- Before using, 100µL of β-mercaptoethanol per 1mL of loading buffer is added to reduce the di-sulfide bonds.

- To prepare the gel, the separating gel is loaded first in the electrophoresis apparatus, and then, the stacking gel was added once the separating gel is polymerized.

  - **Separating gel**:
    - For 1 gel (10mL) of a 10% acrylamide:
      - 3.3mL 30% acrylamide/bis-acrylamide
      - 2.5mL Tris 1,5M pH 8.8
      - 0.1mL 10% SDS; 0.1mL 10% ammonium persulfate; 4µL TEMED, and water were added up to 10mL
  - **Stacking gel**:
    - For 1 gel (4mL):
      - 0.67mL 30% acrylamide/bis-acrylamide
      - 0.5mL Tris 1M pH 6.8
      - 40µL 10% ammonium persulfate
      - 40µL 10% SDS
      - 4µL TEMED
      - water was added up to 2.7mL

3.3.2. Sample preparation and gel running and transfer

The amount of sample to be loaded depends on the experimental purpose. Considering the total amount loaded (20-25µL), protein sample, loading buffer (5X) and water are mixed. Before loading them into the gel, proteins were denatured by incubating the samples for 30 minutes at 37ºC for hCNT1 detection and for 5 minutes at 95ºC for the rest of the proteins. These samples were then loaded and separated on 8%, 10% or 12% SDS–PAGE gels, along with a molecular weight marker (dual colour- Biorad). The gel was run at 40mA. After that, proteins were transferred to an activated PVDF membrane. Activation is done by incubating the membrane for 15 seconds in absolute methanol, followed by a 2-minute wash with

distilled water. The transferring of the protein was prepared following the order shown in Figure 41. A sandwich of gel and membrane was made for transferring the proteins from gel to membrane It is very important to be sure that no air bubbles are formed as they would affect the correct transfer. Then the transfer is run at 100V for 90 minutes. An ice block is added to the buffer to prevent excessive heating.

Materials:

- **Transfer buffer (10x)**:
    - 250mM Tris-base
    - 1.91M glycine.
    - before using, 100mL of this buffer were dissolved in 700mL water and 200mL methanol.

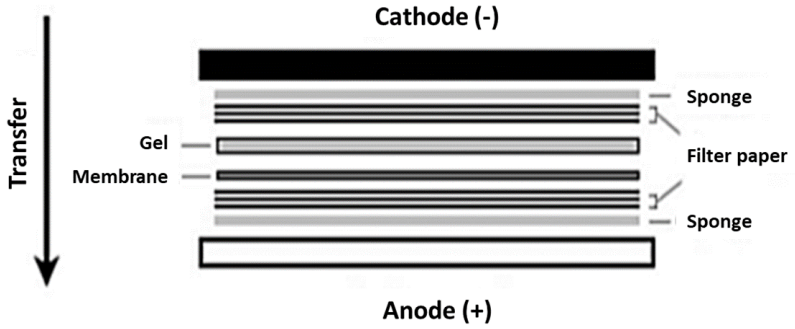


Figure 41. Gel and membrane setup for electrophoretic transfer.

3.3.3. Blocking and immunoblotting

After transfer was finished, membrane was stained with Ponceau S to check whether transfer was uniform and proteins were equally loaded. For blocking the unspecific sites on the membrane where antibodies can bind, 5% milk blocking buffer was added to the membrane and incubated for 1 hour at room temperature or overnight at 4°C. This was followed by overnight incubation with appropriate dilution of primary antibody at 4°C (for more details on dilution of primary antibodies, refer to Table 7). Next day the primary antibody was washed 3 times for 5 min with the washing buffer (TBST). After that the membrane was incubated with the secondary antibody for 2 hours, then antibody was washed 3 times for 5 min with the washing buffer before developing the membrane. For both

primary and secondary antibodies, milk or BSA blocking buffer was used for their dilutions according to the manufacturer's indication.

Materials:

- **TBST buffer**:
  - 20mM Tris-base
  - 150mM NaCl
  - 0.1% Tween at 7.6 pH
- **5% Milk blocking buffer**:
  - 50mL TBS-Tween
  - 5% of non-fat milk powder
- **5% Bovine serum albumin (BSA) blocking buffer**:
  - 50mL TBS-Tween
  - 5% w/v BSA

3.3.4. Membrane development and imaging

The horseradish peroxidase linked to the secondary antibody can react and cause chemiluminescence which can be captured. To do so, ECL (Biological Industries) was used, following manufacturer's instructions. Briefly, solution A and solution B are mixed 1:1 preparing a total volume of 500µL per membrane. The membrane is covered with the mix and incubated for 1-3 minutes at room temperature. The membrane is then developed using a LAS3000 camera or on an x-Ray film.

3.4. Co-Immunoprecipitation

Immunoprecipitation is the technique by which a target protein or antigen is precipitated from a solution using an antibody specific for that target. In addition to isolating a specific target protein from a sample, IP can also be used to isolate protein complexes from cell extracts by targeting a protein or antigen believed to be part of the complex. Co-Immunoprecipitation was done using Dynabeads Co-Immunoprecipitation Kit (Life technologies) following the manufacturer's instructions. It allows easy coupling of an antibody of your choice to the surface of uniform, 2.8 µm superparamagnetic Dynabeads M-270 Epoxy

beads. Following immobilization of your antibody, use the coupled beads and the buffers supplied in the kit for co-immunoprecipitation of proteins or intact protein complexes, and to elute those complexes from the beads.

4. Immunocytochemistry

Immunocytochemistry (ICC) is a common laboratory technique that uses antibodies that target specific peptides or protein antigens in the cell via specific epitopes. These bound antibodies can be detected using secondary antibodies conjugated to different fluorophores (different Alexa Fluor® in our case), which are detected in a fluorescence or confocal microscope. To perform ICC assays, cells were previously seed on 12 mm coverslips contained in a 24-well plate (one coverslip per well). Cells then were transduced with AdhCNT1 or Adctrol and incubated for 48h.

Then the following steps were carried out:

- **Fixation**:
  1- Cells were fixed with 4% Paraformaldehyde in PBS buffer for 15 min at room temperature (400µl/well)
  2- After that, a one-step incubation with cold absolute acetone was done on ice for 5 min exactly (400µl/well).
    - The paraformaldehyde and acetone were disposed in the non-halogenated residues tanks according to the lab´s chemical wastes rules and regulations. The materials used in this steps were also discarded in Biohazardous waste blue container.
  3- Following, the cells were washed 3 times with PBS on a slow shaker for 5 min at room temperature (500µl/ well).
    - At this step, the plate can be kept with PBS at 4ºC for 1 week.

- **Blocking and permeabilization**:
  Cells were incubated for 30 min at room temperature (500µL/ well).
  - **Blocking and permeabilization buffer**:
    - 5% BSA

- 0,3% Triton X-100 (detergent)
- In 1x PBS
- (e.g. for 10mL: 0,5g BSA + 30µL Triton X-100 +10mL PBS)
- Saponin can be also used at 0.1% for permeabilization when detecting membrane proteins

- **Immunostaining**:

1- Coverslips containing the cells were incubate inverted (face down) on paraffin film with the diluted primary antibody overnight at 4°C in a humid chamber with 30µl/coverslip. All antibody used are lined out in Table 7.

- **Antibody dilution buffer**:
  - 1% BSA
  - 0,3% Triton X-100
  - In 1x PBS
  - e.g. for 10mL: 1mL PBS 10x + 0.1g BSA +30 µL Triton X-100 + distilled H_2O up to 10m

2- Next day, coverslips were returned to the plate and washed 3 times with PBS on a shaker for 5 min at room temperature with 500µl/well.

3- Then, coverslips with cells were incubated (inverted on paraffin film) with the diluted secondary antibody for 1-2h at room temperature in a dark humid chamber 30µl/coverslip. All antibody used are lined out in Table 7.

4- After that, coverslips were returned to the plate and washed 3 times with PBS on a shaker for 5 min at room temperature with 500µl/well in dark.

5- Nucleus can be counterstained with Hoechst (a DNA Stain) diluted according to manufacturer's instruction in the dark, followed by 3 washes with PBS on shaker .

6- Then coverslips were mounted on a glass slide with 3 µl of the mounting media (Prolong®Gold antifade reagent) to minimize photo-bleaching and left to dry for 24h at room temperature in dark.

7- The slides then were conserved at 4°C in the dark.

Table 7: Antibodies and dilutions used.

	Antibody	Application technique: Dilution	Comercial Brand	Antibody species	Molecular weight (kDa)
Primary Antibodies	AKT	WB: 1/1000	Santa Cruz	Goat	62
	p-AKT Ser473	WB: 1/1000	Cell Signaling	Rabbit	62
	p-AKT Thr308	WB: 1/1000	Cell Signaling	Rabbit	62
	ERK	WB: 1/1000	Cell Signaling	Rabbit	42-44
	p-ERK	WB: 1/1000	Cell Signaling	Rabbit	42-44
	Jun	WB: 1/1000	Cell Signaling	Rabbit	43-48
	c-Jun	WB: 1/1000	Cell Signaling	Rabbit	43-48
	c-Fos Ser32	WB: 1/1000	Cell Signaling	Rabbit	42-62
	p-c-Fos	WB: 1/1000	Cell Signaling	Rabbit	62
	FRA-1	WB: 1/1000	Cell Signaling	Rabbit	40
	p-FRA1	WB: 1/1000	Cell Signaling	Rabbit	40
	hCNT1 mAb	WB: 1/500, ICC:1/50	In-house	Mouse	72
	hCNT1 n-17	WB: 1/500	Santa Cruz	Goat	72
	hCNT1 c-14	WB: 1/500	Santa Cruz	Goat	72
	Actin	WB: 1/2000	Sigma Aldrich	Rabbit	42
	S100A11	WB: 1/1000, ICC:1/300	ProteinTech	Rabbit	12
	p21	WB: 1/1000, ICC: 1/100	Cell Signaling	Rabbit	21
	p27	WB: 1/1000	BD Biosciences	Rabbit	27
	p53	WB: 1/1000	Calbiochem	Rabbit	53
	PARP-1	WB: 1/1000	Cell Signaling	Rabbit	24-89-116
Secondary Antibodies	Anti-mouse	WB: 1/2000	Bio Rad	Rabbit	
	Anti-rabbit	WB: 1/2000	Bio Rad	Goat	
	Anti-goat	WB: 1/5000	Dako	Rabbit	
	Anti-mouse Alexa-488	ICC:1/300	Invitrogen	Goat	
	Anti-rabbit Alexa-546	ICC:1/300	Invitrogen	Goat	

5. RNA expression techniques

5.1. RNA extraction

To purify RNA the "SV Total RNA Isolation System" (Promega) was used following the manufacturer's protocol. It is based on the combined effects of guanidine thiocyanate, beta-mercaptoethanol and SDS which allow the nucleic acids to be isolated essentially free of proteins such as RNases.

Cells were washed twice with PBS, the lysis buffer then was added. In brief, after the centrifugation of the cell lysate, the RNA is selectively precipitated and purified with a silica column in which it is attached. Then, after several washings, the bound RNA was eluted with nuclease free water. Once eluted the RNA can be immediately used or stored at -80°C to avoid its degradation.

Purity of extracted RNA was estimated by measuring the 260/280 nm absorbance ratio using a NanoDrop ND-1000.

5.2. cDNA synthesis from RNA

Complementary DNA (cDNA) synthesis was carried out using the M-MLV Reverse Transcriptase system. Briefly, 1 µg of RNA was incubated at 65°C for 5 minutes. After that, the RNA is left on ice and the reaction mix is added. The samples were then incubated for 2h at 37°C followed by 10-minute incubation at 65°C. The samples then were ready to be further used or stored at -20°C.

Materials:

- **Reaction mix** contains per sample:
  - 1µM DTT (Life Technologies)
  - 625µM dNTPs (Promega)
  - 250ng random primers (Life Technologies)
  - 1x Buffer first strand (Life technologies)
  - 6U M-MLVRT (Invitrogen)
  - 0,8U RNAsin (Promega).

- The samples were then incubated for 2h at 37ºC followed by 10 minute incubation at 65ºC. Then stored at -20ºC

5.3. Real-time PCR

The Real-time PCR technique (RT-PCR) is used to amplify and simultaneously quantify a targeted cDNA molecule. The quantity can be either an absolute number of copies or a relative amount when normalized to endogenous reference. There are two common methods for the detection of products in quantitative PCR: (1) nonspecific fluorescent dies that intercalate with any double-stranded DNA such as SYBR Green and (2) sequence-specific DNA probes, consisting of specific oligonucleotides labelled with a fluorescent reporter. From these two options the latter is the most specific, giving more reliable and reproducible results and its the one used in this thesis.

The oligonucleotides used present two types of molecules linked to their structure: a fluorescent marker (reporter) at 5' and a quencher at 3'. When the probe is intact the proximity between the reporter and the quencher reduces the fluorescence of the reporter due to FRET (Förster resonance energy transfer). When the probe binds downstream one of the primers at the target sequence, it is degraded due to the 5'- nuclease activity of the Taq DNA polymerase separating the reporter form the quencher and subsequently increasing the fluorescence signal of the reporter. In each PCR cycle more reporter molecules are liberated, increasing the fluorescence in a quantity-related manner. One advantage of this technique is that the probe has to bind specifically to the target to generate a fluorescent signal. Thus, not generating any signaling if the probe binds incorrectly to the wrong sequence.

The primers used (hCNT1, hCNT2, Hfor the RT-PCR were purchased at Life technologies. In each well of 96 multi-well plate samples were loaded as following :

- 2µL cDNA
- 7µL water
- 10µL TaqMan master mix 10X (Life technologies)
- 1µL probes 20X
- A well containing water instead of cDNA is used as negative control to discard any

contamination.

The RT-PCR conditions used are:

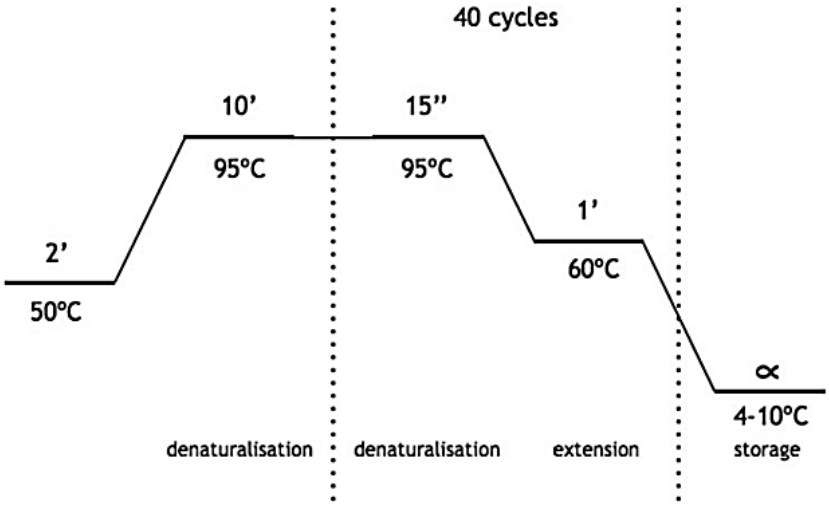


Figure 42. RT-PCR conditions.

5.3.1. Data analysis

The RT-PCR results give us the Ct value. The Ct value is the number of cycles required for the fluorescent signal to exceed the background level. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. Ct results were first normalized with the endogenous control (GAPDH) and then compared to the control sample.

The calculations carried out are:

1- Calculate average Ct of each sample and the endogenous control.

2- Calculate ΔCt as Ct_{sample}-$Ct_{endogenous\ control}$

3- The ΔCt standard error (SE) can be calculated as √(SE_{sample})-($SE_{endogenous\ control}$)

4- Calculate ΔΔCt as ΔCt_{sample}-$\Delta Ct_{contol\ sample.}$

5- To determine the expression of the sample related to the control sample in arbitrary units it is needed to calculate $2^{-\Delta\Delta Ct}$.

5.4. DNA Microarray

For this assay, cells were seeded at 500,000 cell/60mm plate. Next day, cells were infected with 10 MOI of AdhCNT1 or Adctrol and incubated for 24h. Plates were snap frozen at 24h. After that RNA were extracted using the miRNeasy Mini Kit, Qiagen.

Then, the mRNA extract was sent to Genome Analysis Platform, where the samples with proper mRNA concentration were analyzed using the Illumina Human HT-12 V4 Bead Chip arrays.

5.5. Luciferase assay

Luciferase reporter assay allows to investigate promoter activity by measuring light output from luciferase enzyme that is expressed under the control of your promoter of interest. Thus, cells were seeded in 24-well plates. Each condition consisted of 3 replicates. Cloned pGL3 luciferase reporter plasmids were co-transfected with the pRL (Renilla luciferase control reporter plasmid) into cells using Lipofectamine® 2000. The plates were incubated for 48h. Then cells were lysed according to the manual of ''Dual-Luciferase® Reporter Assay System - Promega''. Once the cells were lysed, the expression of luciferase was quantified by measuring the luminescence units with a luminometer. The data of fire fly signals from luciferase reporter plasmid were normalized to Renilla luciferase activity.

6. Cytotoxicity assay

Cell viability was measured in this thesis using the MTT method, a quantitative and colorimetric assay. The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5diphenyltetrazolium bromide), from a yellow tetrazole to its insoluble purple formazan in living cells. This assay measures cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and reflects the number of viable cells. MTT assays can be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of different compounds and drugs.

Cells were seeded (5000 cells/well) in 96-well plates. Next day, the cells were transduced and treated according to the treatment protocols (mentioned above). The plates then were incubated for 24h-48h depending on the experiments. The viability was measured by adding 100µL/well of 7.5mg/mL MTT dissolved in PBS and supplemented-culture medium (1:9). This solution is photosensitive, so it has to be kept protected from the light. Cells are incubated with the solution for 45-60 minutes depending on the cell line, at 37°C. After this time a purple precipitate can be observed. The medium is removed and 100µL/well DMSO is added in order to dissolve the formazan crystals. The absorbance was measured at 550nm.

7. Fluo-4 AM calcium imaging

Fluo-4, AM is a membrane permeant probe that can be passively loaded in cells by simple incubation. Once inside the cell, Fluo-4 becomes fluorescent in the presence of free Ca2+.

Cells were seeded into 6-well plates (100,000 cells/well). Next day, the cells were transduced with 10MOI of AdhCNT1 or Adctrol and incubated for 18h or 36h. Then, cells were washed 3 times with 1x Hank's Balanced Salt Solution (HBSS++). Following, cells were incubated in a dark place for 45 min with 1 mL of Fluo-4 AM (5 µM; Genecopoeia) in HBSS++ buffer at room temperature. After incubation, cells were washed three times with HBSS++ buffer and incubated for another additional 30 min with HBSS++ containing the organic anion-transport inhibitors probenecid at 1.5 mM to reduce leakage of the de-esterified indicator. Finally, cells were imaged under fluorescence microscopy.

Material:

- **HBSS++ buffer :**
  - 400mg KCl
  - 60mg of KH2PO4
  - 350mg NaHCO3
  - 8 g NaCl
  - 48mg Na2HPO4 anhydrous
  - 140mg CaCl2
  - 100mg MgCl2-6H2O
  - 100mg MgSO4-7H2O

- 1 g D-Glucose (Dextrose)
- in 1000 mL distilled water

- Fluo-4 AM stock solution were preperd in dimethylsulfoxide (DMSO) or 20% pluronic F-127/DMSO.

8. Statistics and data representation

Data processing has been done with the Microsoft Excel 2016. The graphic representation and the statistic tests have been performed with the GraphPad Prism 5.0 program. Results shown in this thesis are the mean ± standard error of n experiments. Routinely, in each experiment three to four replicates per conditions have been measured. Statistic differences have been measured by t-Student tests, calculating the p value.

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