

## **THESIS / THÈSE**

MASTER IN BIOCHEMISTRY AND MOLECULAR AND CELL BIOLOGY RESEARCH FOCUS

Study of the translational regulation occurring during hepatogenic differentiation of iPSCs in vitro

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Faculté des Sciences

## STUDY OF TRANSLATIONAL REGULATION OCCURRING DURING HEPATOGENIC DIFFERENTIATION OF IPSCS IN VITRO

Mémoire présenté pour l'obtention

du grade académique de master 120 en biochimie et biologie moléculaire et cellulaire

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## Study of the translational regulation occurring during hepatogenic differentiation of iPSCs *in vitro*

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#### <u>Résumé</u>

Les cellules souches sont des cellules indifférenciées caractérisées par leur capacité d'autorenouvellement et leur potentiel de différenciation. La régulation traductionnelle est d'une importance capitale pour le remodelage du protéome au cours de leur différentiation, à la fois au niveau global et au niveau spécifique de certains transcrits. En utilisant un modèle in vitro efficace de différenciation hépatogénique, basé sur l'utilisation de cellules souches pluripotentes induites génétiquement modifiées et exposées à un protocole de différenciation métaboliquement optimisé pendant 20 jours, des données obtenues par des analyses de profilage polysomique précédemment menées dans le laboratoire ont montré qu'une régulation traductionnelle se produisait pendant la différenciation. Dans ce travail, nous avons poursuivi la caractérisation de la synthèse protéique qui a lieu au cours de la différenciation hépatogénique des iPSCs, en démontrant que la régulation traductionnelle globale est un processus en deux étapes dans lequel une stimulation précoce de la synthèse protéique est suivie par une répression globale de la traduction. Nous avons émis l'hypothèse que cette régulation traductionnelle pourrait impliquer des protéines de liaison à l'ARN (RBPs) qui modulent l'efficacité traductionnelle de transcrits spécifiques. Sur base de cette hypothèse, nous avons mené une analyse bio-informatique sur les transcrits qui sont spécifiquement régulés par la traduction au cours du processus de différenciation afin de rechercher un enrichissement de motifs d'ARN connus pour interagir avec les RBPs. Les résultats, associés à une analyse approfondie de la littérature, ont mis en évidence IGF2BP2 et IGF2BP3 comme deux candidats potentiels. Leur profil d'expression tout au long du processus de différenciation a été étudié, montrant une progressive régulation à la hausse d'IGF2BP2 et à la baisse d'IGF2BP3. Pour évaluer la fonction d'IGF2BP2 dans la différenciation hépatogénique, nous avons silencé son expression en utilisant des siRNAs. Les résultats préliminaires obtenus par immunofluorescence suggèrent que la répression d'IGF2BP2 entraîne une régulation négative de l'alpha-1-antitrypsine (AAT). Si cela est confirmé, cela suggère que la différenciation hépatogène, ou au moins l'expression de l'AAT, pourrait dépendre de l'activité d'IGF2BP2.

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## Study of the translational regulation occurring during hepatogenic differentiation of iPSCs *in vitro*

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#### Summary

Stem cells are a population of undifferentiated cells characterized by their capacity of selfrenewal and their potential to differentiate into different cell types. Translational regulation is of paramount importance for proteome remodeling during stem cell differentiation, both at the global and transcript-specific levels. Using an efficient in vitro model of hepatogenic differentiation, based on genetically modified iPSCs exposed to a metabolically optimized 20days differentiation protocol, data based on polysome profiling experiments previously conducted in the lab have shown that a translational regulation occurs during hepatogenic differentiation. In this work, we pursued the characterization of protein synthesis taking place through the differentiation of these iPSCs demonstrating that global translational regulation is a two-step process in which early stimulation of protein synthesis is followed by a global repression of translation. We hypothesize that this translational regulation may involve RNA Binding Proteins (RBPs) that modulate the translational efficiency of specific transcripts. Based on this assumption, we conducted bioinformatics analysis on transcripts that are specifically translationally regulated during the differentiation process to search for enriched RNA motifs described to interact with RBPs. The results, associated with a thorough literature analysis of the potential candidates, highlighted IGF2BP2 and IGF2BP3 as two potential RBP candidates. Their expression profile throughout the differentiation process has been investigated, showing a progressive up- and down-regulation of IGF2BP2 and IGF2BP3, respectively. To assess IGF2BP2 function in hepatogenic differentiation of iPSCs, we silenced its expression by using siRNAs. Preliminary results obtained by immunofluorescence analysis suggest that the repression of IGF2BP2 expression leads to an alpha-1 antitrypsin (AAT) downregulation. If confirmed, this suggests that hepatogenic differentiation, or at least AAT expression, could depend on IGF2BP2 activity.

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## List of abbreviations

2CLC	2-cell-like cell
AA	Amino acid
AAT	Alpha-1-antitrypsin
АСТА	Activin A
ACTB	β-actin
AFP	Alpha-fetoprotein
ALA	Alanine
ALB	Albumin
AME	Analyses of motif enrichment
ASCs	Adult stem cells
BMP4	Bone morphogenic protein 4
C/EBPβ	CCAAT enchancer binding protein beta
CHX	Cycloheximide
CYP3A4	Cytochrome P450 3A4
CYP450	Cytochrome P450
DMSO	Dimethyl sulfoxide
eEF1a	Eukaryotic elongation factor 1a
eEF2	Eukaryotic elongation factor 2
EGF	Epidermal growth factor
eIF2	Eukaryotic initiation factor 2
ELAV	Embryonic lethal abnormal vision protein
EMT	Epithelial-mesenchymal transition
ESC	Embryonic stem cells
FGF1	Fibroblast growth factor 1
FGF2	Fibroblast growth factor 2
FGF4	Fibroblast Growth Factor 4
FGFR1	FGF receptor-1
FLP	Flippase
FOXA1	Hepatocyte nuclear factor 3-alpha

FOXA2	Hepatocyte nuclear factor 3-beta
FOXA3	Hepatocyte nuclear factor 3-gamma
FRT	Flippase recombinase target
GATA3/4	Gata binding protein factor <sup>3</sup> / <sub>4</sub>
GATA6	Gata binding protein factors 6
GFI1	Growth Factor Independent 1 Transcriptional Repressor
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLY	Glycine
GrB14	Growth factor receptor-bound protein 14
GRN	Gene regulatory network
hAAT	Human alpha-1-antitrypsin
hESC	Human embryonic stem cell
HEX	Hematopoietically-expressed homeobox protein
HGF	Hepatocyte growth factor
hiPSC	Human induced pluripotent stem cell
HLC	Hepatocyte-like cell
HLX	H2.0-like homeobox protein
HMGA1	High mobility group AT-hook 1
HMGA2	High mobility group AT-hook 2
HNF1a	Hepatocyte nuclear factor 1a
HNF4a	Hepatocyte nuclear factor 4a
HnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HnRNPC	Heterogeneous nuclear ribonucleoprotein C
HnRNPL	Heterogeneous nuclear ribonucleoprotein L
HSC	Hematopoietic stem cell
HuR	Human antigen R
IGF2	Insulin growth factor 2
IGF2BP2/IMP2	Insulin-like growth factor 2 mRNA binding protein 2
IGF2BP3/IMP3	Insulin-like growth factor 2 mRNA binding protein 3

iPSC	Induced pluripotent stem cell
IPTG	Isopropyl thiogalactose
IRS	Insulin receptor substrate
KHDRBSs	KH domain containing RNA binding signal transduction proteins
KLF4	Kruppel-like factor 4
LAMB2	Laminin subunit beta 2
LIMS2	Lim zinc finger domain containing 2
LIN28A	Lin-28 homolog A
LIN28B	Lin-28 homolog B
MAPK	Mitogen activated protein kinase
MEIS1	Meis Homeobox 1
MEX-3C	MEX-3 homologous protein C
miRISC	RNA induced silencing complex
miRNA	Micro RNA
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
MSC	Mesenchymal stem cell
NANOG	Homeobox protein NANOG
NFkB	Nuclear factor kappa-B
NHEJ	Non-homologous end-joining
NPC	Neural pluripotent cell
nRNP	Nuclear ribonucleoprotein
NTCP	Na+-taurocholate cotran-sporting polypeptide
OCT3/4	Octamer-binding transcription factor <sup>3</sup> / <sub>4</sub>
PABP	Poly(A) binding protein
PEPCK	Phosphoenolpyruvate carboxykinase
PGC	Primordial germ cell
PGC-1a	Peroxysome proliferator-activated receptor gamma coactivator 1a
РНН	Primary human hepatocyte
PI3K	Phosphoinositide 3-kinase

PROX1	Prospero-related homeobox 1
PSC	Pluripotent stem cell
PTBP-2	Polypyrimidine tract binding protein 2
RBD	RNA-binding domain
RBM	RNA-binding motif protein
RBP	RNA-binding protein
RIP	RNA immunoprecipitation
RNP	Ribonucleoprotein
rRNA	Ribosomal RNA
SC	Multipotent stem cell
SERPINA1	Serine-protease inhibitor group A member 1
SG	Stress granule
sgRNA	Single guide RNA
shRNA	Short hairpin RNA
siRNA	Interfering RNA
SLUG	SNAI2
snRNA	Small nuclear RNA
SOX2	SRY (Sex determining region Y)-box2
STAT	Signal transducer and activator of transcription
TCF	T-cell factor/lymphoid enhancer factor transcription factor
TE	Translation efficiency
TF	Transcription factor
TGF-β	Transforming growth factor-β
TRBP	Tar RNA binding domain
tRNA	Transfer RNA
UBE3C	Ubiquitin-protein ligase E3C
UCP1	Uncoupling protein 1
USP10	Ubiquitin-specific peptidase 10
VEGF	Vascular Endothelial Growth Factor
Wnt3A	Wingless-type MMTC integration site 3A

# Introduction and Objectives



#### Figure 1: Classification of stem cells.

Totipotent cells found in the zygote and the 2-cell stage embryo have the capacity to selfrenew and differentiate into all cell types of an organism, including extra-embryonic tissues. Pluripotent stem cells (PSCs), including embryonic stem (ES) cells and primordial germ cells (PGCs), lose the ability to form extra-embryonic tissues and are restricted in potentiality. Multipotent stem cells (SCs) derived from PSCs can give rise to several unipotent cells, which can differentiate into multiple differentiated somatic cells (modified from Berdasco & Esteller, 2011).

### Introduction

#### 1) Stem cells

Stem cells are undifferentiated cells found in embryonic, fetal, and adult stages of life which are able to give rise to differentiated progeny that are building blocks of tissues and organs. These cells are defined by their ability to extensively proliferate (self-renewal), usually arise from a single cell (clonality), and to differentiate into multiple lineages depending on their differentiation potential (potency). The extent of these properties may differ between various stem cell types, leading to the classification of them into different categories (Fig.1) (Kolios & Moodley, 2013).

#### 1.1) Classification based on differentiation potential

The differentiation capacity is one of the main interests of stem cells, which varies from one cell to another depending on its origin and derivation. All stem cells can be classified according to the extent of their differentiation potential into 4 different groups: totipotent (or omnipotent), pluripotent, multipotent, and unipotent (Fig.1) (Smith, 2006).

#### 1.1.1) Totipotent (or omnipotent) cells

Totipotency is defined as the ability acquired by mammalian cells right after fertilization to generate a full organism. In mouse, totipotent cells are only found in the zygote and the 2-cell stage embryo where they are able to naturally develop into any cell type whether it belongs to extra-embryonic or embryonic tissues. As development proceeds, these cells progressively lose their totipotency and give way to cell differentiation (Iturbide & Torres-Padilla, 2020; Rossant, 2001).

Very little is yet known about the mechanisms underlying totipotency. Information is lacking about its establishment, its main regulatory network and how the exit from this state is regulated. Moreover, being studied mainly through investigations in the mammalian embryo, the information gathered is limited in quantity. However, cells resembling the 2-cell stage embryo, also known as 2-cell-like or 2C-like cells (2CLCs), identified in ESC cultures in 2012 has proved to be an interesting new cellular model for the study of totipotency or totipotency-like features. Since their discovery, their use proved to be useful to accelerate the advancement of the field (Iturbide & Torres-Padilla, 2020; Rossant, 2001).

#### 1.1.2) Pluripotent cells

Unlike totipotent cells, the pluripotent ones are more limited as they are unable to differentiate into extra-embryonic cell types. Pluripotent stem cells (PSCs) have the ability to differentiate into all somatic cell lineages of the three germ layers (ectoderm, mesoderm and definitive endoderm) from which all tissues and organs develop (de Miguel et al., 2010).

The first pluripotent stem cells studied were those derived from the inner cell mass of the blastocyst, so far called "Embryonic Stem Cells (ESCs)" (Evans & Kaufman, 1981). In 2006, Takahashi and Yamanaka succeeded in reprogramming somatic cells into pluripotent cells (Takahashi & Yamanaka, 2006). These cells have been called "induced Pluripotent Stem Cells (iPSCs)" and share many similarities with ESCs.

#### 1.1.3) Multipotent cells

Multipotent stem cells (SCs) are found in several tissues and differentiate into cells of a single germ layer (Ratajczak et al., 2012). Although these cells have the ability to differentiate into several cell types, those types are limited in number and are usually referred to by their tissue or germ layer origin (mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, etc.) (Spinelli et al., 2014). One of the most recognized examples of multipotent cells are Mesenchymal Stem Cells (MSCs), also called mesenchymal stromal cells. They can be derived from various tissues such as bone marrow, adipose tissue, bone, Wharton's jelly, umbilical cord blood or peripheral blood and can differentiate into various mesoderm-derived tissues such as bone, cartilage, muscle, or fat (Augello et al., 2010; Mamidi et al., 2012).

#### 1.1.4) Unipotent cells

Multipotent progenitors give rise to unipotent progenitors, which are cells with a more restricted developmental potential. Unipotent stem cells are able to self-renew and to differentiate into only one, or sometimes two specific cell types. These cells are often tissue resident stem cells allowing the maintenance of tissue homeostasis and integrity. Examples include satellite cells that give rise to mature muscle cells (Bentzinger et al., 2013).

#### **1.2)** Classification based on the origin

In addition to their classification based on their differentiation potential, stem cells can have multiple origins that bring specific advantages and disadvantages to their use for various applications. Stem cells can be classified into 3 main categories according to their origin: ESCs, adult stem cells (ASCs), and iPSCs. In general, ESCs and iPSCs are in a pluripotent state, whereas ASCs are multi- or unipotent (Fig.1) (Kolios & Moodley, 2013; Loh et al., 2015).

#### 1.2.1) Embryonic Stem Cells (ESCs)

ESCs are pluripotent stem cells originating from the inner cell mass of the mammalian blastocyst, a stage of the preimplantation embryo occurring 5-7 days after fertilization in human (Evans & Kaufman, 1981; Martin, 1981). As pluripotent stem cells, ESCs can differentiate into all somatic cell lineages of the three primary germ layers (Liu et al., 2020) but can also be maintained in an undifferentiated state *in vitro* for a prolonged period (Evans & Kaufman, 1981; Yao et al., 2006).

These cells are mainly characterized by a high capacity of self-renewal and potency (Kolios & Moodley, 2013). The key element to pluripotency is its gene regulatory network (GRN) responsible for maintaining pluripotency and for inducing specific differentiation upon



#### Figure 2: Pluripotency factors as germ layer specifiers.

Pluripotency is governed by a regime of transcription factors (TFs). These TFs are categorized as trophectodermal, ectodermal, endodermal, or mesodermal specifiers and can drive differentiation towards a specific lineage option while repressing other fate choices. Green arrows indicate lineage induction, while red arrows indicate lineage repression. In undifferentiated ESCs, each lineage option is concomitantly induced and suppressed by various pluripotency TFs (Loh et al., 2015).

stimulation. Some researchers have suggested that several key transcription factors (TFs), including the Homeobox protein NANOG, the Octamer-binding transcription factor 4 (OCT4) and the SRY (sex determining region Y)-box 2 (SOX2), are germ layer specifiers capable of stimulating differentiation to one germ layer while inhibiting commitment to the other two germ layers. A fine balance of TFs is thus required to maintain the undifferentiated state of ESCs while retaining the potential to commit to any lineage (Fig.2) (Loh et al., 2015).

However, more recently, ESCs have been stabilized from both pre- and postimplantation blastocysts and showed distinct phenotypes. They differed in their metabolic activity, epigenetic landscape and differentiation potential, leading to their classification in two distinct groups: naïve pre-implantation and primed post-implantation ESCs (Nichols & Smith, 2009).

#### 1.2.2) Adult Stem Cells (ASCs)

During the postnatal and adult periods, stem cells residing within differentiated organs are called "ASCs". They can be obtained from all tissues of the three primary germ layers and the placenta. In contrast to ESCs, they are more restricted in their capacity of self-renewal and differentiation as they lack the ability to proliferate extensively and can only differentiate into tissue-specific cells (Ilancheran et al., 2009; Passier & Mummery, 2003). The best-known examples include MSCs, which are thought to have anti-inflammatory properties and to stimulate organ damage repair (Kolios & Moodley, 2013).

The differentiation of these cells into new specialized and functional cells following transplantation has been shown in the context of bone tissue repair and ischemic heart tissue revascularization (Chimutengwende-Gordon & S. Khan, 2012; Menasché et al., 2008). Other studies have also shown that cultured ASCs secrete several molecular mediators with immunomodulatory, angiogenic, anti-apoptotic and chemoattractant properties that promote tissue damage repair (da Silva Meirelles & Nardi, 2009; Hafner et al., 2010).

The potency of these adult cells is limited at best to multipotency, but many resident stem cells are simple unipotent progenitors. This is indeed the case for tissues with a high turnover rate such as the intestinal epithelium, in which the resident stem cells will generate new epithelial cells in the intestinal crypt every  $3.48 \pm 1.55$  days (Darwich et al., 2014; He et al., 2004). These ASCs maintain a quiescent state in stem cell niches until local stimuli activate their proliferation, differentiation, migration, or apoptosis (Kiefer, 2011; Smart & Riley, 2008; Streuli, 1999). A stem cell niche is a microenvironment that influences the self-renewal and differentiation of these cells with extrinsic signals, thus playing a crucial role in stem cell homeostasis and tissue repair (Wagers, 2012; Yeung et al., 2011). The triggers that allow stem cells to move from a state of self-renewal and proliferation to differentiation have yet to be elucidated, but it appears that the niche environment consists of various signals from the extracellular matrix and soluble mediators involved in cell signaling and gene expression (Daniela et al., 2007; Tsai & McKay, 2000). This would explain why the niche is able to regulate different stem cell-specific processes.



#### Figure 3: Overview of iPSC technology.

Somatic cells can be taken from several sources, such as the skin, blood, and urine. Two categories of reprogramming strategies can be applied to these cells: integrative and non-integrative strategies. iPSCs are able to differentiate into diverse cell lines which can be used for disease modeling, drug discovery, or cell replacement therapy (Bordoni et al., 2018).

#### **1.2.3)** Induced Pluripotent Stem Cells (iPSCs)

Owing to these features, human ESCs (hESCs) could be used to treat diseases, such as Parkinson's disease or diabetes (Thomson, 1998). However, their sourcing from human embryos leads to many ethical problems as well as tissue rejection after transplantation. The major breakthrough of induced pluripotent stem cells (iPSCs), generated directly from patient's tissues, could overcome these major drawbacks.

iPSCs are stem cells produced from adult somatic cells that have been genetically reprogrammed to achieve an "ESC-like state". For the first time in 2006, Takahashi and Yamanaka reprogrammed terminally differentiated mouse fibroblasts to a pluripotent state, after transduction of 4 genes encoding key TFs, the octamer-binding transcription factor 4 (OCT4), SRY-2-related high mobility box protein (SOX2), Kruppel-like factor 4 (KLF4) and the oncoprotein c-MYC, thereby forming mouse iPSCs (Takahashi & Yamanaka, 2006). One year later, Yamanaka and his colleagues successfully generated human iPSCs (hiPSCs) from adult human dermal fibroblasts with the same 4 transcription factors. They demonstrated that hiPSCs had similar characteristics to hESCs, such as morphology, proliferation, surface antigens, gene expression profile, epigenetic status of pluripotent cell-specific genes, telomerase activity, as well as teratoma formation and differentiation potential (G. Liu et al., 2020; Takahashi et al., 2007).

Since Shinya Yamanaka and John Gurdon were awarded the 2012 Nobel Prize in Physiology or Medicine for "the discovery that mature cells can be reprogrammed to become pluripotent", the contribution of iPSCs in modern medicine became of major importance (Johnson & Cohen, 2012). In addition to their wide use in basic research, somatic cell reprogramming offers many advantages such as non-invasive and ethical cell harvesting (compared to the need to harvest a blastocyst for ESC culture) and autologous clinical use with limited rejection (Kimbrel & Lanza, 2015). Since then, iPSCs have been used as tools for drug development, disease modelling and regenerative medicine (Wernig et al., 2007). However, the use of iPSCs in clinical studies remains limited because retroviral vectors used to introduce reprogramming factors into adult cells, and oncogenes such as *c-MYC*, can cause genetic instability, leading to transformation (Daley, 2010; Ebben et al., 2011). Therefore, researchers are currently investigating new and safe reprogramming protocols to generate iPSCs without genomic manipulation. Some researchers have also tried to use non-retroviral vectors, such as chemical compounds, plasmids, adenoviruses, transposons, ... (Fig.3) (Bordoni et al., 2018; Kolios & Moodley, 2013). In addition, several new techniques described aim to avoid the use of the c-MYC oncoprotein by replacing it with the LIN28 RNA Binding Protein or NANOG, two factors sufficient to reprogram human somatic cells in addition to OCT4 and SOX2 (Fu et al., 2018; J. B. Kim et al., 2008, 2009; Yu et al., 2007).

Stem cells have quickly become an important tool for understanding both organogenesis and the organism's capacity for continuous regeneration (Kolios & Moodley, 2013). Once their limitations are overcome and their genetic stability is elucidated, iPSCs may be used to replace damaged tissues or even regenerate organs properly (Lodi et al., 2011), as they would bypass immune rejection and ethical issues. And while ESCs are excellent tools for understanding human development and organogenesis, iPSCs also give the possibility to create human models of disease that would improve the understanding of the pathogenic mechanisms of human

disease and improve cell therapy for degenerative disorders (Chien, 2008). Since then, these cells may become the future of cell therapy and regenerative medicine.

#### 2) Hepatogenic differentiation

The liver is a target for which the development of stem cell therapy would be of great importance. Although the liver can self-regenerate, many drugs and debilitating diseases are too damaging, finally leading to hepatocyte dysfunction and total organ failure. In most cases, liver transplantation is the only effective treatment for major liver damage. However, due to the frequent rejection of organs and the limited number of donors, alternative therapeutic approaches are needed. Stem cells, and more specifically iPSCs, could offer an unlimited and minimally invasive source of cells for hepatocyte replacement and liver regeneration (Banas et al., 2007).

Moreover, stem cells-derived hepatocytes could be perfect tools for pharmaceuticals development. Since incorrect assessment of drug hepatotoxicity is the cause of many marketing drug development halts (about 90%), there is a need for improved technologies to assess the hepatotoxicity of drugs (Dowden & Munro, 2019; Serras et al., 2021). Animal models are not well suited for the assessment of drug hepatotoxicity, as approximately 50% of the drugs responsible for hepatotoxicity in humans do not show similar toxicity in animal models, reinforcing the need for a safe *in vitro* method (van Tonder et al., 2013). For example, iPSCs-derived hepatocytes recapitulating detoxification activities like those of freshly harvested primary human hepatocytes (PHHs) would be a perfect alternative to *in vivo* models with the major advantages of indefinite culture potential and the possibility to cover a large genetic variability by using iPSCs from different donors (Boon et al., 2020).

Various research protocols, preclinical studies and clinical trials have been published on hepatogenic differentiation. Many stem cell types have been differentiated *in vivo* and/or *in vitro* to obtain iPSCs-derived hepatocytes based on different induction strategies. However, so far there is no gold-standard strategy to produce hepatocytes from stem cells and none of the protocols produce hepatocytes identical to PHHs (Snykers et al., 2009). Currently, most protocol yields immature hepatocyte resembling those of fetal origin, defined as hepatocyte-like cells (HLCs) (Boon et al., 2020). Further studies are therefore needed to achieve a maturity goal.

#### 2.1) Modelling of hepatogenic differentiation in vitro

Faithful modelling of biological processes is a key issue of any experimental biology research, as *in vivo* investigations are often impossible (especially in humans). This is typically the case for hepatogenic differentiation, which led scientists to design appropriate experimental procedures, including animal models and *in vitro* cell cultures.

Recreating the process of hepatogenic differentiation in cell culture *in vitro* requires the selection of an appropriate cell type with potential for such differentiation as well as a suitable protocol to conduct the differentiation successfully. Different cell types such as MSCs, ESCs, iPSCs or liver progenitors have already been cultured *in vitro* and exposed to different protocols

to induce their differentiation into liver lineages. However, none of these approaches allowed to drive a fully mature hepatocyte (Zabulica et al., 2019).

Early hepatogenic differentiation protocols were based on the formation of embryoid bodies of hESCs and differentiation of stem cells with the addition of specific growth factors that mimic the major steps of embryonic and fetal liver development (Baharvand et al., 2006; Basma et al., 2009). Even if differentiation appeared to be globally achieved, subtle differences in the timing and level of expression of different hepatic genes could be found. In addition, although this approach was simple and successful in inducing several hepatocyte markers, it frequently generated populations with multiple cell types (Si-Tayeb et al., 2010).

Later on, protocols performed targeted differentiation experiments using growth factors and extracellular substrates important in the natural development of hepatocytes. The protocols evolved to include a first step of specification of the definitive endoderm, since hepatocytes are of endodermal origin (Boon et al., 2020; Roelandt et al., 2010). Several compounds have been shown to be essential for committing pluripotent stem cells to the definitive endoderm, the cytokines classically used being: Activin A (ActA), Wingless-type MMTV integration site (Wnt) and Epidermal Growth Factor (EGF) (Chen et al., 2018). Classically performed during 2 or 5 days, this step is followed by a hepatic specification step aimed at driving hepatoblast differentiation. The cytokines typically used are the Bone Morphogenetic Protein 4 (BMP4), the Fibroblast Growth Factor 2 (FGF2) and the Hepatocyte Growth Factor (HGF). Finally, hepatic maturation is mainly induced by supplementation with HGF or Oncostatin M, but other culture supplements have also been shown to promote hepatocyte differentiation, such as the dimethyl sulfoxide (DMSO) and the small-molecule glucocorticoid mimetic, Dexamethasone (DEX) (Tricot et al., 2018; Vanhove et al., 2016). Currently, researchers are trying new strategies to improve hepatocyte maturation, such as replacing cytokines with small molecules (like the potent HGF mimetic N-hexanoic-Tyr, Ile-(6) aminohexanoic amide), microRNAs, or optimizing the extracellular matrix (Rashid et al., 2015; Siller et al., 2015; Zhou et al., 2017).

In addition to protocols using PSCs, MSCs from bone marrow, adipose tissue, dental pulp, umbilical cord, and cord blood have also been widely used to model hepatogenic differentiation in vitro due to their ability to trans-differentiate. Trans-differentiation is a process in which a somatic cell differentiates into another somatic cell lineage, bypassing the pluripotent or progenitor cell state (Cho & Ryoo, 2018). Under certain conditions, MSCs are able to move towards an endodermal cell fate, leading to HLCs. For example, Ishkitiev and colleagues cultured MSCs from deciduous teeth in a medium containing HGF, Insulin-Transferrin-Selenium-Ethanolamine, and Oncostatin M, and found cells differentiated into hepatocyte-like cells capable of metabolizing ammonia to urea and producing albumin. They showed a high expression of hepatocyte markers in these cells, both at the genetic and protein levels, but this expression was lower than in PHHs (Ishkitiev et al., 2010). The application of MSCs in the treatment of refractory liver diseases is currently the subject of much clinical attention due to their several advantages. Firstly, they have a low risk of tumorigenesis as they do not require the introduction of exogenes to differentiate, unlike iPSCs. In addition, they induce immune tolerance, so that immune rejection of the cells is unlikely. Finally, they can be collected in a minimally invasive manner, such as from deciduous teeth, which is a promising source of cells. MSCs are therefore considered by some to be a primary source for regenerative medicine due to the low possibility of tumorigenesis, the lack of ethical concerns and their


#### Figure 4: Overview of molecular signaling during liver development.

Embryonic liver development is based on a spatio-temporal succession of specific cytokines, including the Bone Morphogenic Proteins (BMPs), the Fibroblast Growth Factors (FGFs), the Hepatocyte Growth Factor (HGF), the Epithelial Growth Factor (EGF), Nodal, the Oncostatin M, glucocorticoids and other factors such as the Hepatocyte Nuclear Factors (FOXA1-3), the Gata binding protein factors (GATAs), the Prospero-related homeobox 1 (PROX1), the homeobox genes HEX and HLX, the Hepatocyte Nuclear Factor (HNFs), the CCAAT enhancer-binding protein-alpha (C/EBP $\alpha$ ) and the Wnt/ $\beta$ -catenin signaling (modified from Monga & Behari, 2020).

accessibility (Campard et al., 2008; Ohkoshi et al., 2018; Wanet et al., 2014). However, even if they have a high proliferation and differentiation potential, it remains lower than ESCs or iPSCs.

Several studies using different standard protocols have been conducted to generate HLCs from iPSCs (Banas et al., 2007; Boon et al., 2020). Although HLCs did express hepatic TFs and markers such as the Hepatocyte Nuclear Factors (HNF)4a, Alpha1-Antitrypsin (AAT) and Na<sup>+</sup> -taurocholate cotran-sporting polypeptide (NTCP), the level of expression and the functional properties of HLCs were far from PHHs (Boon et al., 2020; Si-Tayeb et al., 2010; Song et al., 2009). HLCs differ from PHHs at the functional, transcriptional, proteomic, and epigenetic levels, and specifically express low levels of TFs and markers (Boon et al., 2020; Toivonen et al., 2013).

In this work, we used a differentiation protocol of iPSCs-derived hepatocytes developed in the group of C. Verfaillie (KULeuven) (Boon et al., 2020) mimicking the distinct steps of mammalian hepatic development illustrated in Figure 4 (Monga & Behari, 2020).

Embryonic development of the liver is characterized by highly temporal, cell-specific and tightly regulated molecular signals that enable hepatic competence of the foregut endoderm, hepatic specification, and induction followed by hepatic morphogenesis. During gastrulation and early somite stages, the liver begins to arise from the definitive endoderm. One of the earliest known signaling factors required for definitive endoderm formation is Nodal, a member of the TGF-β signaling network. After formation of the definitive endoderm, the endoderm is patterned in an anterior to posterior manner, Wnt3a signaling promoting the proper specification and differentiation of liver progenitors from the anterior foregut endoderm (Kadzik & Morrisey, 2012). At this moment, the FOXA Hepatocyte nuclear factors (FOXA1-3) as well as the Gata binding protein factor 4 (GATA4) specify the endoderm in the process of liver competence. More particularly, FOXA2 is a transcription factor capable of opening compact chromatin to other proteins through interactions with core nucleosomal histones by replacing linker histones at enhancer and/or promoter target sites (Wang et al., 2020; Wolfrum et al., 2008). Concomitantly, the fibroblast growth factor ligands, FGF1 and FGF2 induce the mitogen-activated protein kinase (MAPK) pathway that promotes the expression of liverspecific genes in the endoderm, such as Alpha-fetoprotein (AFP) and albumin (ALB). The liver bud then migrates into the septum transversum mesenchyme under the direction of bone morphogenic protein 4 (BMP4) signaling, which is essential for hepatogenesis. BMP signaling is known to play a positive role in liver competence, since it increases histone acetylation which influences FOXA1 binding (Monga & Behari, 2020).

The hepatogenesis step is followed by the embryonic liver growth phase characterized by the expansion and proliferation of cells within the liver bud. Several transcription factors, including the Hematopoietically-expressed homeobox protein (HEX), H2.0-like homeobox protein (HLX), Gata binding protein factors 6 (GATA6) and the Prospero-related homeobox 1 (PROX1), are the first known mediators of this phase. Once the hepatic program is in full swing, liver growth continues and is now referred to as the stage of hepatic morphogenesis. The epithelial cells in this stage are now considered to be hepatoblasts and will undergo expansion while maintaining their bipotent state. Several key players at this stage include HGF and Wnt/b-catenin signaling, which are known to regulate the proliferation and survival of resident cells (Monga & Behari, 2020).

The final stage is characterized by the differentiation of hepatoblasts into mature functional cell types: hepatocytes and biliary epithelial cells. At the center of the process of hepatoblast to hepatocyte differentiation are liver-enriched transcription factors, such as



#### Figure 5: Hepatogenic differentiation protocols of iPSCs.

Schematic representation of both hepatogenic differentiation protocols. iPSCs were sequentially treated with corresponding cytokines (STD) and supplemented with doxycycline to induce expression of HNF1a, FOXA3 and PROX1 from day 4 (D4), and with amino acids from day 12 (D12) and 14 (D14) of differentiation program (HC3X). STD protocol is supplemented with DMSO from D0 to D20, whereas HC3X protocol is supplemented until D12 (see Materials and methods) (Boon et al., 2020).

hepatocyte nuclear factor transcription factors (HNF4a) and CCAAT enhancing binding protein- $\alpha$  (C/EBP $\alpha$ ). HNF4a is essential for differentiation since it binds to the promoters of nearly half of the genes associated with hepatocyte polarity, junctional integrity, and metabolic functions. Most importantly, HNF4a helps in the accumulation of hepatic glycogen stocks and in the generation of a hepatic epithelium and a normal liver architecture, including the organization of the sinusoidal endothelium (Monga & Behari, 2020; Parviz et al., 2003). Liver-enriched transcription factors enable fetal hepatocyte function by directing the expression of various genes that are classically associated with hepatocyte function at this stage, including metabolic and synthetic enzymes, such as the Cytochrome P450 (CYP450) enzymes. Again, several factors and signaling pathways have been shown to play a role in the regulation of hepatocyte maturation by regulating the expression of liver-enriched transcription factors. Some of these include Oncostatin M, glucocorticoids, HGF, the Endothelial Growth Factor (EGF), FGFs, Wnt/b-catenin signaling, and others (Monga & Behari, 2020).

Based on natural liver development, Boon and his colleagues tried to get as close as possible to PHHs by setting up a 20-days stepwise standard protocol of differentiation based on successive treatments with cytokine cocktails (Fig.5) (Roelandt et al., 2013; Tricot et al., 2018).

The first step of this protocol lasts 4 days and consists in inducing the iPSCs differentiation into anterior definitive endoderm cells thanks to the use of Activin A (ActA) and Wnt3a. In vivo, high levels of Nodal signaling drive definitive endoderm differentiation but in *vitro*, the same effect can be achieved by a high concentration of ActA (Toivonen et al., 2013). ActA is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) family of cytokines that has been recognized as a multifunctional cytokine expressed in a wide range of cells and tissues with roles in regulation of wound repair, cell differentiation, apoptosis, and inflammation (Yndestad et al., 2011). In addition to orchestrating the spatio-temporal development of multiple organs and performing various functions in the reproductive system, ActA regulates important functions in the liver, ranging from hepatocyte proliferation and apoptosis to hepatic extracellular matrix production and liver regeneration (Bloise et al., 2019). Wnt3a signaling is also able to control cell growth, differentiation, apoptosis, and self-renewal. Its canonical signaling results in the accumulation of  $\beta$ -catenin in the cytoplasm and its translocation to the nucleus, leading to its interaction with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors, and subsequent transcription activation of Wnt-target genes (Lu et al., 2017). In addition, Wnt3a has been reported to play an important role in the maturation and the differentiation of hepatocytes from stem cells (Boulter et al., 2012; So et al., 2013). Prolonged treatment with high Wnt3a concentration already commits definitive endoderm progenitors to the liver lineage during definitive endoderm induction, impairing their ability to further differentiate into pancreatic progenitors (Cho et al., 2012). Since then, the combination of ActA and Wnt3a is required for the induction of hepatogenic differentiation.

Following the anterior endoderm lineage, the next step of the protocol lasts 8 days and leads to the hepatic specification of the definitive endoderm cells. This specification can be induced by BMP4, known as a trigger for pluripotency exit and cellular differentiation in early development. During BMP4-driven differentiation of PSCs, several events take place: the cells change their morphology by elongating in size meanwhile there is extensive remodeling of gene expression programs, whereby pluripotent stem cell-associated markers like *NANOG*, *SOX2*, and *OCT4* are downregulated, and lineage-specific markers such as GATA Binding Protein 3 and 4 (*GATA3/4*) are upregulated. After this point, the probability of going back and remaining a pluripotent cell is very low (Gunne-Braden et al., 2020). FGF1 can also successfully substitute

physiological signaling for specification, which is further underlined by the observation that FGF1 directs liver cell fate commitment in evolutionarily distinct species (Gérard et al., 2017; Jung et al., 1999). However, definitive ventral foregut endodermal cells *in vivo* can also give rise to lung cells following a cell fate decision mediated by FGF1 signaling (Serls et al., 2005). The concentration of FGF1 is therefore the key determinant of lung-specific gene expression compared to liver-specific gene expression, as evidenced by the *in vitro* induction of albumin at lower concentrations of FGF1 (50-500 ng/ml) and the inhibition of albumin expression at higher concentrations (500-1000 ng/ml) (Serls et al., 2005). Similarly, microarray analyses of the effect of the FGF receptor-1 (FGFR1) inhibitor (PD161570) on hiPSC-derived differentiated endodermis showed that approximately 40 early genes are controlled by FGF1/2 signaling, several of them being involved in cell differentiation and development, like *FOXA1/2/3, HNF4a* and the CCAAT Enhancer Binding Protein Beta (*C/EBPb*) (Calmont et al., 2006; Jung et al., 1999; Twaroski et al., 2015).

The last step of the protocol lasts 8 days and consists in inducing the hepatic maturation by Hepatocyte Growth Factor (HGF) supplementation. HGF is known to play an important role in hepatocyte proliferation and liver regeneration. This factor acts by regulating cell growth, proliferation, survival, motility, and morphogenesis through the C-met receptor, which leads to stimulation of multiple downstream signaling pathways, including MAPK, phosphoinositide 3kinase (PI3K), signal transducer and activator of transcription proteins (STATs), and nuclear factor kappa-B (NF $\kappa$ B) (Garajová et al., 2015; Tangtrongchitr et al., 2020).

Even if the use of cytokines mimicking the natural liver ontology improved HLCs maturation, the cell features didn't yet match those of PHHs in terms of expression levels of functional markers, such as Albumin (ALB) and Cytochrome P450 3A4 (CYP3A4). Moreover, Boon and his colleagues observed in HLC a reduced basal oxygen consumption rate, and a glycolytic phenotype instead of the gluconeogenic phenotype of PHH, which confirmed a low basal mitochondrial activity in HLCs. This demonstrates that the standard stepwise protocol did not lead to HLCs with sufficient metabolic maturity (Boon et al., 2020). Based on numerous RNA-sequencing studies, the authors identified that three key hepatic transcription factors, the hepatic nuclear factor 1 alpha (HNF1a), the hepatocyte nuclear factor 3y (FOXA3) and the prospero-related homeobox 1 (PROX1), were expressed at a lower level in iPSC-derived hepatocyte-like cells compared to PHHs. They therefore genetically modified human iPSCs with a doxycycline-inducible cassette for the overexpression of these three key genes (Ordovás et al., 2015). HNF1a is a transcription factor highly expressed in the liver and required for the liver-specific expression of a variety of genes, including ALB and AAT (Bach et al., 1991; Cereghini et al., 1988). This transcription factor is also present in other tissues, such as kidney, intestine, and pancreatic islets, but its non-liver target genes are almost unknown (Weber et al., 1996). This molecule is activated very early in embryogenesis, plays an important role in glucose metabolism (Rajas et al., 2002) and seems necessary for the proper hepatogenic differentiation since it is involved in the transcriptional control of terminal differentiation markers such as CYP3A4 (Blumenfeld et al., 1991). The third member of the forkhead box protein superfamily, FOXA3 is an important member of the hepatocyte nuclear factor family and is abundantly expressed in the liver (Zhan & Zhao, 2021). This factor regulates the expression of liver-specific genes, such as HNF1a (Garon et al., 2017) and affects the expression of many genes involved in the glucose homeostasis, such as the Glucose transporter 2 (GLUT2) (Shen et al., 2001). Finally, PROX1 is a transcription factor involved in developmental processes such as cell fate determination, transcriptional regulation of genes,

regulation of progenitor cells in a number of organs, and cell differentiation control (Dudas et al., 2008; Elsir et al., 2012). This molecule plays a critical role in embryonic development and functions as a key regulatory protein in neurogenesis and the development of several organs such as liver, pancreas, eye, lymphatic vessel, nerve, and cardiac muscle. During liver organogenesis, PROX1 is expressed in endodermal cells, and its global inactivation impairs hepatoblast migration and results in the formation of a hypomorphic liver (Sosa-Pineda et al., 2000) (Oliver et al., 1993). There is limited information on the role of PROX1 in the adult liver, but several *in vitro* experiments have shown that it negatively regulates mitochondrial function through its association with the coregulator peroxisome proliferator-activated receptor  $\gamma$  coactivator (PGC-1 $\alpha$ ) (Charest-Marcotte et al., 2010). In addition, siRNA-mediated down-regulation of PROX1 appears to increase phosphoenolpyruvate carboxykinase (PEPCK), indicating its role in the control of hepatocyte energy metabolism (Song et al., 2006).

Despite the overexpression of these TFs and the exposure of the cells to cytokine cocktails, the expression levels of the modified iPSCs did not yet match those of PHHs, presenting only a partial metabolic maturation and gene expression signature. HC3X iPSCs were still unable to induce a gluconeogenic and oxidative phenotype, and CYP450 enzymes were only modestly increased. By studying and comparing the composition of the medium of PHHs and HLCs, they found out that the consumption of metabolites from the HC3X iPSCs culture medium was higher, especially when it comes to amino acids (AA), more precisely the glycine (GLY) and the alanine (ALA) (two strong inducers of CYP3A4). Assuming that a shortage in AAs would prevent the maturation of HC3X-iPSCs, the AA levels needed to be increased above *in vivo* physiological levels to induce full metabolic maturation. After numerous investigations, they supplemented the differentiation (Boon et al., 2020). AA supplementation would induce several metabolic signaling pathways, such as MTORC1 which leads to the induction of several transcriptional and epigenetic mechanisms that ultimately edge to a phenotypic maturation of HC3X cells (Sangüesa et al., 2019).

Finally, maturation was only achieved when DMSO was added in the medium. DMSO treatment increases the proportion of PSCs in the cell cycle G1 phase while activating a critical regulator of cell proliferation, the retinoblastoma protein (Rb). By promoting growth arrest in G1 and activating checkpoints in the PSC cell cycle, DMSO treatment prepares cells for cell fate changes. Treatment of stem cells with a low concentration of DMSO demonstrated a significant increase in the propensity of a variety of PSCs to differentiate following directed differentiation by regulating the cell cycle and priming stem cells to be more responsive to differentiation signals. This technique has been shown to be effective in different species (e.g. mouse and human) in multiple lineages, including hepatocytes (Sambo et al., 2019).

It is therefore only by combining the addition of the cytokine cocktails, the AAs/DMSO supplementation and the induction of the 3 specific transgenes at specific time points of differentiation (see Fig.5 and Materials and Methods) that HC3X-iPSCs progeny was shown to have the ability to reach high levels of hepatogenic maturation, as illustrated by the expression of functional hepatocyte markers, such as CYP450 mRNA, drug biotransformation and hepatotoxin sensitivity (Boon et al., 2020).

### 2.2) Assessment of differentiation

Assessment of the efficiency of HC3X differentiation can be done by different methods, each one aiming at revealing key characteristics of the hepatocytes: the morphology, the expression of PHHs-specific mRNAs or proteins and the hepatocyte specific functionality.

A simple phase contrast microscopy performed on HC3X-iPSCs allows to appreciate their polygonal shape typical of hepatocytes as differentiation proceeds. Daily observation of the morphology changes is therefore an easy and quick way to monitor the differentiation process, although it's no sufficient.

The assessment of liver-specific gene expression is way more informative than morphology since classical cell biology techniques such as Western Blot (WB), RT-qPCR or immunofluorescence (IF) allow efficient characterization of both key transcription factors of hepatocytes and functional hepatocyte mRNA/proteins. Thus, the search for key specific markers to the hepatogenic differentiation of HC3X-iPSCs has been used (Boon et al., 2020). This includes stemness regulators (OCT4, SOX2, described in the section 1.2.3, whose expression is expected to decrease with differentiation), transcription factors regulating the endodermal and/or hepatic fate (GATA4, HNF4a, FOXA2, described in the section 2.1). This latter category also includes the 3 genes induced by doxycycline treatment from day 4 for direct differentiation (HNF1a, FOXA3, PROX1, described in the section 2.1). Finally, the expression monitoring of hepatic functional markers (ALB, AAT, CYP3A4) completes the picture.

ALB is the most abundant protein in differentiated liver cells and is the most abundant protein in human plasma (40 g/L of a total of 70 g/L). This molecule is exclusively synthesized in the liver and plays an important role in a number of physiological mechanisms, including the regulation of osmotic pressure. In addition, ALB is a transporter of poorly water-soluble molecules such as hormones, cholesterol, iron, calcium, bilirubin, free fatty acids and drugs, and has anti-oxidant properties (Artigas et al., 2016; Vincent, 2009).

Human AAT (hAAT), also named  $\alpha$ 1 proteinase inhibitor ( $\alpha$ 1-Pi) and serine protease inhibitor, group A, member 1 (SERPINA1) is a water-soluble and tissue-diffusible circulating glycoprotein. This molecule is mainly synthesized by the hepatocytes (over 80%), although it is also secreted by monocytes, macrophages, pancreas, lung alveolar cells, enterocytes and endothelium (de Serres & Blanco, 2014). The liver secretes large amounts of hAAT daily, making it the second most secreted serum protein after albumin, with multiple anti-inflammatory, immunomodulatory, anti-infectious and tissue repair properties (Patel & Teckman, 2018).

The last hepatic marker studied was CYP3A4. CYP3A4 is a member of the CYP450 enzymes known to metabolize over 70% of drugs in clinical use. These enzymes are primarily monooxygenases capable of mediating oxidation and hydroxylation reactions that promote hydrophilicity of CYPs' substrates, resulting in either activation of prodrugs or enhanced clearance and excretion of therapeutic agents by the kidneys. CYP3A4 is quantitatively the most important P450 enzyme in adults and is expressed to a large extent not only in the human hepatocytes (95%) but also in the small intestine, contributing to the presystemic and systemic metabolism of approximatively 30% of drugs (Lolodi et al., 2017; Werk & Cascorbi, 2014). These characteristics make it the most important drug-biotransforming enzyme of the liver.

It is therefore based on several hepatocyte characteristics, whether morphological and/or genetic, that it is possible to assess the maturity of the progeny of HC3X iPSCs.



#### Figure 6: Key steps of protein synthesis.

(1) The initiation step is the recognition of the mRNA by a ribosome. The ribosome is a ribonucleoprotein complex consisting of two distinct subunits: the small 30S subunit, consisting of 16S rRNA and 21 proteins and the large 50S subunit, consisting of two rRNAs (5S and 23S) and 33 proteins. Initially, the small subunit binds to the 5' UTR of the mRNA and then scans in a  $5' \rightarrow 3'$  way. Once the small subunit reaches the AUG start codon, the corresponding tRNA carrying the first amino acid, the methionine, binds to this subunit, followed by the large subunit recruitment.

(2) During the elongation step, tRNAs with the corresponding anticodon match the mRNA codon. A peptide bond is formed between the methionine carried by the first tRNA and the second amino acid of the second tRNA. The ribosome then shifts, again in a 3' direction, leaving a space for another tRNA to pair with its corresponding codon and allowing another peptide bond to form. This process continues with the ribosome moving all along the mRNA strand, elongating its amino acid chain.

(3) When the ribosome reaches a stop codon (UAG, UAA or UGA), a specific release factor binds to the stop codon and causes the release of the amino acid chain by catalyzing the separation of the polypeptide chain, the tRNA, and the ribosome subunits. A protein is therefore synthesized (Pearson Education, Inc, 2012).

# 3) Translational regulation

Previous studies in the laboratory have investigated the events leading to the proper differentiation of iPSCs in HLC progeny. Since the progression of cellular differentiation requires an adapted proteome, we highlighted a need for translation regulation in the process among the mechanisms studied.

#### **3.1)** Protein synthesis: Principles

The production of proteins within a cell is a complex process. Once transcription and transcript maturation are completed, mRNAs must exit the nucleus to find the translation machinery: the ribosome. Eukaryotes organize protein synthesis in a multi-step process that requires a ribosome and multiple protein factors, referred to collectively as eukaryotic initiation factors (eIFs), elongation factors (eEFs) and termination factors (eTFs). Similarly to transcription, translation can be divided into three distinct steps as shown in Figure 6: initiation, elongation and termination, with the difference that the end product is a protein and not RNA. There are three main types of RNA: messenger RNAs (mRNAs) which serve as a template for translation, ribosomal RNAs (rRNAs) which serve as a component of the ribosome, and transfer RNAs (tRNAs) which transfer amino acids during protein synthesis. However, beyond the primary role of RNA in protein synthesis, several varieties of RNA exist and are involved in post-transcriptional modification, DNA replication, and gene regulation. These include small nuclear RNAs (snRNAs), micro RNAs (miRNAs), and the long non-coding RNAs (lnRNAs) (Eddy, 2001).

### 3.2) Proteome remodelling

#### 3.2.1) Proteome's definition

Ultimately, each cell, and its identity, is characterized by a set of proteins, which is referred to as the 'proteome'. The proteome is a dynamic and complex entity since within each cell, the protein content is constantly changing, temporally and spatially. Several factors make this proteome extremely dynamic and responsive to intra- or extra-cellular signals which can lead to alternative splicing or post-transcriptional modifications such as glycosylation or phosphorylation, making different protein forms out of the same mRNA. Therefore, the proteome contains a much larger number of proteins than the genome contains genes since one gene can code for several proteins.

#### 3.2.2) Regulation of protein abundance

Protein abundance is the result of the equilibrium between protein synthesis and degradation rates (Schwanhüusser et al., 2011). Factors that can influence the rate of protein synthesis include mRNA abundance, mRNA length, translation initiation rate and translation elongation rate (Hershey et al., 2012). This suggests that mRNA abundance is not the only regulator of protein abundance, an observation that has been confirmed at the omic scale thanks to the development of high-throughput technologies able to characterize proteomes, transcriptomes and translatomes (Schwanhüusser et al., 2011).

Many studies aimed at quantifying the relative proportion of changes in protein abundance explained by transcriptional versus translational regulation have led to different conclusions. Schwanhüusser and colleagues sought to quantify cellular mRNA and protein expression levels for thousands of genes in a population of unperturbed embryonic mouse fibroblasts. Using label free mass spectrometry methods, they tracked the synthesis of the new transcripts and proteins and demonstrated that mRNA levels explained only 40% of the variability in protein levels. They proposed translation efficiency (TE), defined as the rate of mRNA translation into proteins within cells, as the single best predictor of protein level (Schwanhüusser et al., 2011). In contrast, a later study based on quantitative whole proteome analyses offered profound insights into the control of gene expression in unperturbed embryonic mouse fibroblasts used by (Schwanhüusser et al., 2011) and showed a more consistent correlation between changes in mRNA levels and protein levels. Indeed, mRNA levels would contribute between 56% and 81% to the variance in protein expression, suggesting that Schwanhüusser and co-workers had underestimated the contribution of transcription in protein abundance regulation (Li et al., 2014; Schwanhüusser et al., 2011). Those contradictory observations are explained by the concept that translational regulation is a useful mechanism for cells that respond rapidly to internal or external stimuli before a new transcription program comes into effect, whereas during the steady state, transcriptional control is considered the main regulator of protein abundance (Liu et al., 2016). Consequently, the protein abundance regulation depends on the physiological context.

Regarding stemness and differentiation, a study on molecular regulation of mESC fate change in response to the downregulation of NANOG revealed that modifications in the abundance of numerous proteins were not paralleled by a corresponding transcript profile, suggesting a role for translational regulation of stem cell fate decision (Lu et al., 2009). Indeed, stem cells generally have low rates of global protein synthesis to maintain their undifferentiated status, in contrast to differentiated cells (Saba et al., 2021; Sampath et al., 2008; Tahmasebi et al., 2019). Sampath and colleagues studied the regulation of translation during fate decisions of hESCs. They showed that, in contrast to undifferentiated cells, differentiated embryoid bodies had cellular structural features characteristic of increased translational efficiency, such as more abundant Golgi bodies and a larger cytoplasmic volume. Differentiated cells were characterized by an increase in translation that coincides with a significant increase in polysome density (i.e. the fraction of ribosomes in the active phase of translation), suggesting that mRNAs were translated more actively (Gabut et al., 2020). This low translation rate of hESCs is also observed in multipotent ASCs. Studies on various types of stem cells, including Hematopoietic stem cells (HSCs), have shown that protein synthesis is limited in the stem cell population, while differentiated hematopoietic progenitors display high levels of global protein synthesis (Buszczak et al., 2014; Signer et al., 2014). It has also been shown that the decrease in translational efficiency improves the reprogramming of somatic cells into iPSCs. This naturally brings the question of the relative contribution of translational regulation in the stemness, differentiation and reprogramming states.

#### 3.2.3) Proteome remodeling through hepatogenic differentiation

As suggested by (Gabut et al., 2020), stem cells differentiation relies on strict transcriptional and translational regulation as a mechanism to regulate the acquisition of the



Figure 7: Global translation profile during iPSCs hepatogenic differentiation.

(A and B) Western blot analysis of puromycin-labelled polypeptides in control iPSCs and HLCs at days 12 (D12), 16 (D16) and 20 (D20) (A) and days 2 (D2) and 4 (D4) (B). iPSCs left untreated by puromycin or previously treated with cycloheximide (CHX) were used as negative controls. Western blot signal intensity was quantified and normalized over  $\beta$ -Actin signal and plotted as mean +/- SD (n = 3 independent replicates).

(C) Polysome-profiling analysis during differentiation. Ribosomal subunits (40S and 60S), monosomes (80S) and polysomes from D0 iPSCs or differentiating HLCs at D12 and D20 with STD and HC3X protocols were separated on sucrose density gradient and fractionated in 24 fractions. Graphs show absorbance at 254nm along sucrose gradient (n = 4 independent replicates). Polysomal signal is colored in grey in the gradient. Bar plot represents mean +/- SD of % of polysomal signal calculated by normalizing polysomal area under the curve by total area (n = 4 independent replicates). Statistical significance is calculated by ANOVA followed by a Turkey post-test (Caruso et al, in revision).

differentiated proteome. The relative contribution of transcription versus translation in the proteomic remodeling during hepatogenic differentiation of HC3X-iPSCs has been addressed in our laboratory by transcriptomic, proteomic and polysome profiling analyses (Caruso et al., in revision). The results of these analyses allowed to conclude that hepatogenic differentiation of HC3X-iPSCs is a highly dynamic process mainly regulated by a major transcriptional rewiring while some transcripts undergo translational regulation potentially impacting the proteomic remodeling. Consequently, the ability of cells to selectively repress or induce translation of specific proteins involved in various differentiation processes contributes to the hepatogenic differentiation program of HC3X-iPSCs (Caruso et al., in revision).

From the transcriptomic analysis, the results showed a decrease in the abundance of most contributors of the protein synthesis machinery through hepatogenic differentiation (Caruso et al., in revision). Based on this information, they sought to characterize the global protein synthesis that occurs during HC3X-iPSCs differentiation using a puromycinincorporation assay. This technique consists of measuring the rate of protein synthesis, based on the incorporation of puromycin into newly synthesized proteins (see Materials and Methods). The amount of puromycin-labelled peptides is determined by Western Blot analysis using anti-puromycin antibodies and is assumed to reflect the rate of protein synthesis (Schmidt et al., 2009). The results of the Western Blot analyses performed in our lab indicated a biphasic translational rate in which early stimulation of protein synthesis during pluripotency exit of HC3X-iPSCs is followed by a global repression of translation during hepatogenic maturation (Fig.7A,B). This observation was quite unexpected as it contrasts with several reports demonstrating a clear increase in protein synthesis during PSCs differentiation (Gabut et al., 2020; Tahmasebi et al., 2019). However, it is important to note that these reports were based on the early stages of differentiation (such as embryoid body formation (Gabut et al., 2020)), rather than on the maturation stages directed towards a specific cell type. Our laboratory therefore hypothesized that the early engagement of the endodermal lineage occurring between day 0 and 4 might be associated with an up-regulation of overall protein synthesis, whereas a decrease in protein synthesis was occurring at later stages of differentiation (Caruso et al., in revision).

This global translation profile was confirmed by polysome profiling analysis in undifferentiated HC3X-iPSCs and differentiating progeny. This technique consists in analyzing polysomes by fractionating cell lysates in a sucrose density gradient followed by measuring absorbance at 254nm along the gradient. This allows to establish the distribution profiles of ribosomal subunits (40S and 60S), monosomes and polysomes (80S), which is a way of assessing global translation. Data obtained in the lab showed that at day 12 and 20 of differentiation, differentiated cells present an increased 80S peak associated with reduced polysomes (Fig.7C). This confirmed a global reduction in translation through hepatogenic differentiation, correlated with a decreased abundance of components of the translational machinery observed by proteomic analysis (Caruso et al., in revision).

In conclusion, during HC3X differentiation protocol three assays demonstrated a transient upregulation of translation upon exit from pluripotency, followed by a phase of global reduction of translation during hepatogenic maturation. However, this observation is somehow unexpected considering that hepatocytes are like 'metabolic factories' involved in carbohydrate, lipid and especially protein metabolism. Furthermore, several lines of evidence support that PSCs maintain a low basal translation rate while their differentiation induces a global



Figure 8: Characterization of specific translational reprograming occurring during hepatogenic differentiation of iPSCs.

(A) Schematic workflow of polysome profiling experiment. Cell lysates from control D0 cells and differentiated D12 and D20 cells were loaded onto a sucrose density gradient before being ultracentrifuged and fractionated. Absorbance measurement at 254nm allowed the identification of fractions containing high polysomes (polysomes with more than 3 ribosomes). For each experimental condition, samples of total RNA and high polysome fraction mRNA were extracted for analysis by RNA-sequencing. DESeq2 analysis of total RNA samples identified differentially expressed genes. Anota2seq analysis identified genes differentially translated during differentiation. Finally, pooled RNA fractions were analyzed by RT-qPCR to validate translationally regulated candidates.

(B) Scatter plot of Log2FC mRNA abundance in high polysomal fraction mRNA (X-axis) and total RNA (Y-axis) samples for control D0 iPSCs versus differentiated D12 and D20 cells following the STD or HC3X differentiation protocol. Genes are color-coded according to their mode of regulation: *Translation* (orange) for genes whose translational regulation is expected to have an impact on protein abundance, *Buffering* (blue) for genes whose translational regulation is opposite to transcriptional regulation, *Abundance* (green) for genes regulated at the transcriptional level, and *Background* (grey) for genes not regulated at either the translational or transcriptional level. For each mode of regulation, each mRNA type was separated into two categories. Dark colors indicate up-regulation (Ups) and light colors indicate down-regulation (Downs) of translation/transcription (Caruso et al., in revision).

upregulation of protein synthesis (Guzzi et al., 2018). Nevertheless, other examples of more advanced differentiation protocols have also shown that there is a decrease in overall protein synthesis. These include cardiomyocytes and neuronal differentiation, proving that this mechanism is not restricted to hepatocyte nor endodermal-derived differentiation (Baser et al., 2019; Pereira et al., 2019). Currently, the potential role of the global translational repression during the late phase of differentiation is not yet known.

Polysome profiling experiment has been used in our lab to characterize the specific translational reprogramming occurring during differentiation, and to assess its impact on the acquisition of a typical hepatocyte proteome. Polysome profiling separates highly translated mRNAs, associated with polysomes, from un/low translated ones, thanks to a sucrose gradient centrifugation. Combined with RNA sequencing, this technique allows to define the subset of transcripts that are actively translated, also called the "translatome" (Chassé et al., 2017). Using cell lysates from days 0, 12 and 20, the translatome of differentiated cells from D12 and D20 was systematically compared to the one of undifferentiated cells from D0 (Fig.8A). By comparing the level of high polysome fraction mRNAs to the level of total mRNAs, it was possible to define 4 different categories of mRNAs: "Translation", "Buffering". "Background", and "Abundance" (Oertlin et al., 2019). mRNA assigned to the "Translation" group are regulated at the translational level, either positively or negatively, compared to day 0. This is inferred by an increase/decrease in transcript abundance in the polysome fraction as compared to its abundance in the whole cell lysate. The Buffering ones concern the mRNAs which are regulated at both the transcriptional and translational levels but in opposite way, which means that a significant change in the translational efficiency of a mRNA compensates its transcriptional regulation in order to conserve similar protein abundance. Finally, Background and Abundance mRNAs correspond to mRNAs that are not regulated at the translational level. Abundance mRNAs are characterized by an increased or decreased abundance in differentiating cells in the polysome fraction that is paralleled to the cell lysate, suggesting that they are transcriptionally regulated, but not translationally. The Background transcripts are those that show no difference of abundance between D12 or D20 differentiated cells compared to D0, neither in the cell lysate nor in the high polysome fraction, which makes them neither translationally nor transcriptionally regulated (Caruso et al., in revision).

For each comparison between the D0 control iPSCs and D12 or D20 differentiated cells which had undergone either the STD or HC3X protocol, a scatter plot of Log2FC mRNA abundance in high polysome fraction mRNAs (on the Y-axis) and in total RNA (on the X-axis) showing the distribution of the colored mRNA dots according to their regulation mode was drawn (Fig.8B). In the D0 vs. D12 STD or HC3X, the Anota2seq algorithm assigned 848 and 1064 of mRNAs in the "*Translation*" regulatory mode while in the D0 vs. D20 STD/HC3X lists, it assigned 809 and 767. In addition, a very limited number of transcripts were assigned in the "*Buffering*" group (141, 364, 146 and 349 for control D0 vs. D12 STD, D12 HC3X, D20 STD and D20 HC3X differentiated cells, respectively). Comparatively, most transcripts were assigned in the "*Abundance*" group (7.239, 12.199 and 7.500, 11.959 for control D0 vs D12 STD, D12 HC3X, D20 STD, D12 HC3X, D20 STD and D20 HC3X differentiated cells, respectively) (Caruso et al., in revision).

These results demonstrated that the two differentiation protocols used were mainly accompanied by major transcriptional remodeling, while some transcripts were translationally



# Figure 9: Classical RNA-binding domains (RBDs) of different RNA binding proteins (RBPs).

(A) Several RNA-binding domains can be found within one RBP. Different RBDs include the RBD, the KH domain, the RGG box, the dsRBD, the PAZ domain, the DEAD/DEAH box, the ZnF and the PUF, all represented as colored boxes (Glisovic et al., 2008).

(B) (1) Crystal structure of RRM domain in serine/arginine-rich splicing factor 2 (SRSF2). RRMs have an average size of 90 amino acid residues and adopt a topology forming two alpha helices against an antiparallel leaflet. (2) Crystal structure of KH domain in poly(rC)-binding protein 2 (PCBP2). KH domains have an average size of 70 amino acid residues and adopt a structure in which a three-stranded  $\beta$ -sheet is packed against three  $\alpha$ -helices (Castello, 2015).

regulated, probably impacting on proteome remodeling during differentiation (Caruso et al, in revision).

### 3.3) RNA binding proteins (RBPs)

The characterization of specific translational regulation occurring during hepatogenic differentiation supports a global decrease in protein synthesis. However, as several transcripts are translationally upregulated, this naturally brings the question of the translational regulation mechanisms. Although the knowledge of the precise mechanisms regulating global translation during differentiation is currently lacking, many general mechanisms are known to control the recruitment of mRNAs for translation in order to modulate protein levels (those include regulation of translation initiation machinery, sequence-specific features of mRNAs or ribosome heterogeneity) (Hershey et al., 2019). Most of the translational regulation mechanisms described so far target the initiation step, but it is not excluded that some regulatory mechanisms target translational elongation as well as termination. Regardless the step considered, these mechanisms involve different factors, including several miRNAs and RNA binding proteins (RBPs) (de Klerk & 't Hoen, 2015). Several factors have already been discussed in the literature in the context of cell differentiation, such as the Eukaryotic Initiation Factor 2 (eIF2) (Sonenberg & Dever, 2003) or the Eukaryotic Elongation Factor  $1\alpha$  (eEF1 $\alpha$ ) (Andersen et al., 2003), but the involvement of RBPs in translational regulation remains to be fully clarified.

#### 3.3.1) RBPs' characteristics

RBPs comprise a large class of over 2,000 proteins that have the ability to bind to and control RNAs at all stages of their lifetime (Grifone et al., 2020). The structures and mechanisms that RBPs use to bind and regulate RNAs are incredibly diverse, which makes them quite complicated to study and to summarize here.

Different RBPs families have been studied in the literature, but their roles and molecular mechanisms have yet to be completely discovered. The most known RBPs are the RNA Binding Motif proteins (RBMs) (Wang et al., 2019), the KH Domain Containing, RNA Binding, Signal Transduction proteins (KHDRBSs) (Sumithra et al., 2019), the Insulin like Growth Factor 2 mRNA Binding Proteins (IMPs/IGF2BPs) (Degrauwe et al., 2016), the Poly(A) Binding Proteins (PABPs) (Moore & von Lindern, 2018), the LIN28 proteins (LIN28s) (Chatterji & Rustgi, 2018), the Musashi (MSI) proteins (Kudinov et al., 2017) and the ELAV-like proteins (Hus) (Pascale & Govoni, 2012).

RBPs are defined by their capacity to bind various types of RNAs through RNA-binding domains (RBDs) that form steady secondary and tertiary structures to regulate RNA fate and function (Fig.9A,B) (Chatterji & Rustgi, 2018; Hentze et al., 2018). These RBDs consist of several types of RNA recognition and binding domains, all separated by linkers which mediate important RNA contacts as well. The flexibility of these linkers can determine whether adjacent RBDs bind independently or cooperatively (Corley et al., 2020), enabling RBPs to efficiently regulate their binding to a wide range of targets (Chatterji & Rustgi, 2018; Lunde et al., 2007). Multiple RBDs provide specificity to bind either long RNA sequences, sequences separated by many nucleotides, or two different RNAs at the same time, allowing a single RBP to bind to

hundreds, sometimes thousands different RNA targets (Smith & Valcárcel, 2000). From these multiple assemblies, almost unlimited combinations can arise, which explains the large number of functions that a single RBP can perform.

Different types of RBDs can be mentioned, the best known being the RNA Recognition Motif (RRM), the K-Homology (KH) domain, the Zinc Finger (ZnF) domain, the Pumilio homology (PUM) domain, the double stranded RNA Binding Domain (dsRBD), the RGG (ARG-GLY-GLY) box, the Piwi/Zwille (PAZ) domain, as well as the RNA helicase DEAD/DEAH box and the Puf RNA-binding repeats (PUF) (Fig.9A) (Corley et al., 2020; Mohibi et al., 2019).

The RRMs and the KHs are by far the most common domains found within RBPs and the best characterized ones (Fig.9B). Most RBPs even have several RRM and KH domains to ensure their specificity (Chatterji & Rustgi, 2018). RRMs interact with 2-8 nucleotides in single-stranded RNA (Corley et al., 2020). The combination of consecutive RRMs within a RBP greatly increases the affinity and binding specificity for a specific target. For example, in the case of the heterogeneous nuclear RNPA1 (hnRNPA1), the double bonding of RRM domains is crucial to its overall binding capacity and splicing repression function (Beusch et al., 2017). Regarding the KHs domains, they were first discovered in the heterogeneous nuclear ribonucleoprotein K (hnRNPK) (Corley et al., 2020). Compared to RRMs, KH domains typically recognize 4 nucleotides in single-stranded RNA (Valverde et al., 2008). However, as with the RRM domains, several KH domains can coordinate within the same RBP to increase the specificity of RNA binding. This is the case for the two KH domains within the MEX-3-homologous proteins C (MEX-3C) which synergistically enlarge their binding site to a 5-plus-4-nucleotide bipartite motif (Yang et al., 2017).

#### 3.3.2) Protein-RNA interactions

RNA binding sites are mainly found in the 3' UTR and 5' UTR of mRNAs, but it is not excluded that RBPs can also interact with coding sequences (Chatterjee & Pal, 2009). While our focus here is on the RBPs that are associated with pre-mRNAs and mRNAs, we note that many RBPs are associated with other classes of RNAs, including non-coding RNAs such as microRNAs (miRNAs), tRNAs, small interfering RNAs (siRNAs), telomerase RNA, small nucleolar RNAs (snoRNAs), long non-coding RNAs (lnRNAs), as well as small spliceosomal nuclear RNAs (snRNAs) (Hentze et al., 2018).

In recent years, advanced structural analyses have provided evidence of complex RBP-RNA interactions that do not require specific RBDs. Some RNA interactome capture studies have identified 'unconventional' RBPs in several organisms that have no discernible specific RBDs and no known relationship to RNA biology. Other studies have also shown that disordered protein regions may facilitate certain RBP-RNA interactions (Chatterji & Rustgi, 2018). Thus, the recognition of RNAs by RBPs would not only depend on the nucleotide sequences but also on other influencing factors, such as the di- or tri-dimensional structure of RNAs since it plays a decisive role in limiting the access of RBPs to a large subset of otherwise occupied motifs (Ganser et al., 2019; Taliaferro et al., 2016).

Regardless of the mechanisms by which RBP-RNA interactions occur, they all lead to the regulation of various RNA processes, both at the transcriptional and post-transcriptional



#### Figure 10: Schematic diagram summarizing the different roles of RBPs.

RBPs have numerous roles in transcriptional and post-transcriptional modulation of genes expression. Four such functions of RBPs are demonstrated above: RNA export, protein translation, RNA degradation, stabilization, and Alternative Splicing (Kelaini et al., 2021).

levels (Fig.10) (Chatterji & Rustgi, 2018). When bound to their target RNA, RBPs form messenger ribonucleoproteins (mRNPs) before exporting them via microtubules or other cytoskeletal structures to their intracellular destination. During transport, RBPs maintain the stability of mRNAs and silence their translation, deciding their fate once they reach their destination (Cao et al., 2018; Gerstberger et al., 2014). It is also important to note that although RBPs exhibit unique binding activity to their RNA targets, they can just as easily interact specifically with other protein partners, forming mRNP complexes (Glisovic et al., 2008). RNA-protein interactions and protein-protein interactions are therefore critical factors for the formation of these complex. Moreover, it is often the case that a specific sequence on the target RNA is recognized by more than one RBP. The complement of RBPs present at a particular location where the RNA is transcribed would affect the resulting mRNP complex, thereby modulating its downstream functional activity and dynamics (Glisovic et al., 2008).

#### 3.3.3) Functional activities of RBPs

RBPs play a critical role in the regulation of various RNA processes. They are able to modulate the transcriptional but also the post-transcriptional gene expression by taking part in RNA splicing, as well as in the export of mRNA and in the initiation, elongation and termination of translation. More precisely, these proteins can help recruiting the translation machinery to activate translation (Michlewski et al., 2008) or they are able to stabilize some mRNA and modulate subcellular localization, cleavage and polyadenylation (Fig.10) (Chatterji & Rustgi, 2018). In addition, several studies have shown that competition between RBP and miRNA for a particular binding site on an mRNA can occur, leading to antagonistic effects. For example, the ubiquitous RNA-binding protein (HuR) targets an ARE element in the 3' UTR of the cationic amino acid transporter 1 (CAT-1) mRNA, which relieves the inhibition exerted by miR-122. HuR then stabilizes the CAT-1 mRNA and enhances its translation by redirecting mRNAs to polysomes for protein synthesis (Iadevaia & Gerber, 2015).

In contrast to these antagonistic modes, RBPs and miRNAs can also cooperate to achieve repression of a common mRNA target, resulting in synergistic effects. RBPs involved in the RNA-induced silencing complex (miRISC) can lead to deadenylation, translational repression/suppression and degradation of target mRNAs (Fabian et al., 2010). For example, TAR RNA binding protein (TRBP) is an RBP capable of forming a miRISC complex in combination with Ago2. The miRISC complex binds to sequences located mostly in the 3' UTR of target mRNAs and induces changes in the stability, subcellular localization and translation efficiency (Ha & Kim, 2014). Dicer, another RBP, is one of the best-known TRBP's partner. The more TRBPs there are, the more stabilized Dicer is increasing miRNA levels and therefore lowering the protein synthesis. Being phosphorylated by the MAPK-ERK, TRBP can also modulate the processing efficiency of some pre-miRNAs and tunes the length of mature miRNAs. Its phosphorylation leads to the preferential upregulation of growth-promoting miRNAs and the downregulation of let-7 miRNAs, but the mechanism underlying the specificity remains unclear (Ha & Kim, 2014; Iadevaia & Gerber, 2015).

Finally, in addition to influencing each of these processes, RBPs also provide a link between them. The proper functioning of these complex networks is essential for the coordination of complex post-transcriptional events, and their disruption can lead to disease, including neurodegenerative disorders and various types of cancer (Harrison & Shorter, 2017;

Mohibi et al., 2019). Protein-RNA interactions are thus essential for maintaining the homeostasis of protein synthesis during early development and adult life.

#### 3.3.4) RBPs in maintenance and differentiation of stem cells

Some RBPs have been identified as occupying an important part in the global control of stem cell and differentiation specific proteomes.

For example, heterogeneous nuclear ribonucleoprotein L (hnRNP L) is essential for the survival and functional integrity of HSCs. Many genes important for HSC differentiation, such as the Growth Factor Independent 1 Transcriptional Repressor (*GF11*), the Meis Homeobox 1 (*MEIS1*), *CD34* and the Notch Homolog 1 (*Notch1*) are downregulated in hnRNPL-deficient cells. Depletion of hnRNPL is incompatible with proper hematopoietic differentiation and results in premature death as it leads to increased mitochondrial stress and initiates p53- and caspase-dependent cell death pathways in HSCs (Gaudreau et al., 2016). Furthermore, hnRNPL is considered as a critical regulator of lymphocyte homeostasis and terminal differentiation, controlling the alternative splicing or expression of genes essential for proper lymphocyte development, such as the *CD45* gene. More precisely, this RBP is a regulator of the switch from CD45RA to CD45RO, the main step of the memory T cell development (Chang, 2016).

Moreover, it seems that dozens of RBPs play a role in neurodevelopment and synaptic plasticity (Doxakis, 2014). Examples include the Polypyrimidine tract binding protein 2 (PTBP-2) and the mammalian homologs of the Drosophila embryonic lethal abnormal vision (ELAV) proteins, also called "HU proteins". An analysis performed by (Licatalosi et al., 2012) showed that the role of PTBP-2 is to maintain neural progenitor pools and prevent premature neurogenesis in the developing brain. Cross-linking immunoprecipitation followed by highthroughput sequencing (HITS-CLIP) assays showed that PTBP-2 inhibits the incorporation of alternative adult-specific exons into mRNAs that code for proteins associated with the control of cell fate, proliferation, and the actin cytoskeleton, thereby disrupting the differentiation process. A second evidence is that mice lacking PTBP-2 shows ectopic nests of neuronal progenitors, characteristic of a lack of differentiation (Doxakis, 2014). The HU proteins (HuR, HuB, HuC and HuD) are among the best characterized RBPs, with roles that span all stages of mRNA fate, including pre-mRNA splicing, transport, stability and translation. HuR is ubiquitously expressed, while HuB, HuC and HuD are neuron-specific family members. In the adult brain, all neurons express a certain set of HU mRNAs. Overall, both HuB and HuD stimulate neurite growth and neuronal differentiation in vitro. However, while HuB potentiates neural stem cell proliferation, HuD tends to negatively impact this process. As a result, mice lacking HuB contain a high number of self-renewing cells, indicating that HuD is required for neural stem cells to exit the cell cycle. The most recent evidence also suggests that HU proteins play an important role in neuronal plasticity since they are significantly upregulated in hippocampal neurons after learning tasks and after activation of glutamate receptors (Doxakis, 2014).

Those results indicate that several RBPs have already been investigated in different cell models whether to study their role in stemness maintenance or differentiation induction. However, despite an abundant literature on RBPs, their involvement in hepatogenic differentiation remains unknown.

# Objectives

Our lab has previously characterized the contribution of translational regulation in hepatogenic differentiation by designing an experimental strategy including *in vitro* hepatogenic differentiation programs of iPSCs followed by puromycin-incorporation assay and polysome profiling analysis. Results of this study indicated that hepatogenic differentiation was defined by a two-step translational rate, characterized by an increase in protein synthesis during pluripotency exit, followed by a decrease until the end of differentiation process. Considering those observations, they performed further experiments such as polysome fractionation and data mining of the translatome results to characterize the specific translational reprogramming occurring during differentiation. This analysis finally led them to conclude that hepatogenic differentiation was accompanied by major transcriptional remodeling, while some transcripts were translationally regulated, probably impacting on proteome remodeling during differentiation. Given those results, we aimed at understanding the translational regulation mechanisms occurring during hepatogenic differentiation of iPSCs by evaluating the involvement of RNA Binding Proteins (RBPs).

To address this issue, in the first part of this Master thesis project, hepatogenic differentiation programs have been conducted with the *in vitro* model of metabolically and genetically engineered iPSC differentiation, the HC3X-iPSC, with the monitoring of several stemness/differentiation markers to evaluate the differentiation efficiency. Then, as a contribution to the experiments required for the revision of the submitted manuscript (Caruso et al., in revision), a careful characterization at several time-points of the global translational occurring through differentiation was made with puromycin-incorporation assays.

In the second part of the thesis, a bioinformatics analysis of the untranslated regions of translationally regulated transcripts has been conducted in order to select RBPs potentially involved in the differentiation. Two RBPs were selected and characterized for the study: IGF2BP2 and IGF2BP3. Their abundance along the differentiation process was monitored, and a silencing strategy was designed to assess a putative role of IGF2BP2 in hepatogenic differentiation. Although performing transfection on differentiating HC3X iPSCs turned out to be technically difficult, preliminary results suggest a potential role for IGF2BP2 in the regulation of hepatogenic differentiation, or at least of AAT expression.

Materials and Methods

Reagent	Manufacturer	Reference	Final concentration
DMEM LG	Gibco	31885-023	57%
MCDB 201 Water pH 7.2	US biological	C4000-05	40%
Penicilin-streptomycin	Gibco	15140-122	1x
L-Ascorbic Acid	Sigma	A8960	0.1µM
ITS	Gibco	41400-045	0.25x
LA-BSA	Sigma	L9530	0.25x
B-mercaptoethanol	Gibco	31350-010	50μΜ
Dexamethasone	Sigma	D2915	1mM

Table 1: Liver differentiation medium (LDM)

#### **Table 2: Cytokines**

Cytokine	Manufacturer	Reference
Doxycycline	Sigma	D9891
Activin A	Peprotech	120-14E
Wnt3a	R&D	5036-WN
BMP4	Peprotech	120-05ET
aFGF	Peprotech	100-17A
HGF	Peprotech	100-39

# Table 3: HC3X differentiation medium supplements

Days of differentiation	Medium	Supplements
D0-D2	LDM	50ng/ml Activin A 50ng/ml Wnt3a 0.6% DMSO
D2-D4	LDM	50ng/ml Activin A 0.6% DMSO
D4-D8	LDM	5μg/ml Doxycycline 50ng/ml BMP4 0.6% DMSO
D8-D12	LDM	5μg/ml Doxycycline 20ng/ml aFGF 0.6% DMSO
D12-D14	LDM-AA	5µg/ml Doxycycline 20ng/ml HGF 2% DMSO
D14-D20	LDM-AAGLY	5µg/ml Doxycycline 20ng/ml HGF

# Materials and Methods

# 1) HC3X stem cells culture

#### HC3X stem cell culture surface coating

The cell culture surface used to maintain the BJ1 HC3X iPSCs inducible for HNF1a, FOXA3 and PROX1 (KUL, Belgium) was coated with hESCs qualified-matrix Matrigel (Corning, 354277) diluted in DMEM/F-12 (Gibco, 31330-038) following the lot-specific dilution factor provided by the manufacturer (1:1). Coating was performed by incubating the plates or flasks (Corning-Costar) for one hour at 37°C, immediately followed by seeding of the cells. For the HC3X differentiation experiments, the dishes were coated with Matrigel reduced in growth factor 1/62.5 (Corning, 354230) diluted in DMEM/F-12 following a similar protocol.

#### HC3X stem cell culture maintenance

BJ1 HC3X iPSCs inducible for HNF1a, FOXA3 and PROX1 (KUL, Belgium) were cultured on surfaces coated with Matrigel, a qualified matrix for hESCs, in mTESR PLUS medium (StemCells, 5825) supplemented with 1% penicillin-streptomycin (Gibco, 15140-122). The cells were passaged using accutase (StemCells, 7920) approximately every 5 days, when they reached about 80% confluency, and were plated at a 1:10 dilution in culture medium supplemented with 10 $\mu$ M ROCK inhibitor Y27632 (Axon Medchem, 1683). After the first 24 hours, the medium was refreshed, and the ROCK inhibitor was removed.

#### HC3X stem cell hepatogenic differentiation protocols

The HC3X hepatogenic protocol was performed as described in (Boon et al., 2020). Briefly,  $34.10^3$  cells/cm<sup>2</sup> were seeded on surfaces coated with reduced Growth Factor Matrigel. These cells were placed in culture medium mTESR PLUS supplemented with  $10\mu$ M ROCK inhibitor Y27632 for the first 24 hours. The cells were then cultured in regular culture medium mTESR PLUS until the colonies reached 70-80% confluency.

Hepatogenic differentiation was achieved by incubating HC3X cells with the liver differentiation medium, LDM (Table 1 for full composition) supplemented with the corresponding cytokines (Table 2). LDM-AA was also prepared by supplementing 100ml of LDM with 16ml of MEM-NEAA (Gibco, 11140-035) and 8ml of MEM-AA (Gibco, 11130-051) before adjusting the pH to 7.2-7.3. LDM-AAGLY was prepared by supplementing LDM-AA with 20g/l glycine (CarlRoth, HN07.1). Subsequently, LDM, LDM-AA, and LDM-AAGLY were supplemented with the cytokine cocktails corresponding to the day of the differentiation and a low concentration of DMSO (CarlRoth, A994.1) (Table 3).

The medium was replaced every day until a cell monolayer was obtained ( $\pm$  until day 6 of differentiation), then every two days until the end of the 20 days-differentiation process.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Source
HNF1a	ACACCTCAACAAGGGCACTC	TGGTAGCTCATCACCTGTGG	
FOXA3	ATTCTCTCTGGCATGGGTTG	AAATTCCCCACACCCTAACC	
OCT4	GATGGCGTACTGTGGGCCC	TGGGACTCCTCCGGGTTTTG	_
GATA4	TCCAAACCAGAAAACGGAAG	CTGTGCCCGTAGTGAGATGA	
HNF4a	ACTACGGTGCCTCGAGCTGT	GGCACTGGTTCCTCTTGTCT	
AAT	AGGGCCTGAAGCTAGTGGAT	TCCTCGGTGTCCTTGACTTC	_
ALB	ATGCTGAGGCAAAGGATGTC	AGCAGCAGCACGACAGAGTA	
CYP3A4	TTCCTCCCTGAAAGATTCAGC	GTTGAAGAAGTCCTCCTAAGCT	_
SOX2	GGGAAATGGGAGGGGTGCAAAAGA	TTGCGTGAGTGTGGATGGGATTGGTG	GetPrime #283157899
FOXA2	GGAGCAGCTACTATGCAGAGC	CGTGTTCATGCCGTTCATCC	GetPrime #422343050
IGF2BP2	TATCAGAGTGCCCTCTTCC	AGTTCTGCAGTTCGTTCAC	GetPrime # <u>2107097</u>
IGF2BP3	CGCCTCATTTACAGTGGGA	CAGTGTTCACTTGCTCACAG	Getprime# <u>1972373</u>
UBE3C	TTTCCCATTGCTAATGGCC	CTGATACAGCCATATCAAACGT	GetPrime #2079621

Table 4: RT-qPCR primer sequences

### 2) Cell transfection in a 24-well plate using DharmaFECT

HC3X iPSCs were plated onto 24-well plates at 34.10<sup>3</sup> cells/cm<sup>2</sup> in 500 µl of mTESR medium so that the cells reached 70-80% confluence at day 0 of differentiation. The day of transfection corresponded to the 6th day of differentiation. In a 1.5mL Eppendorf tube, 50µL volume of the siRNA in Opti-MEM serum-free medium (Gibco, 31985062) was prepared by adding 2,5 µL of 5 µM siRNA to 47,5 µL of Opti-MEM following the DharmaFECT protocol. SiRNAs used are the ON-TARGETplus® Human IGF2BP2 5µM SMARTpool siRNA (L-017705-00-0005), the siGENOME® Non-Targeting Pool #2 siRNA (D-001206-14-20) and the MISSION® Universal Negative Control siRNA (SIC003). In a second 1.5mL Eppendorf tube, 50 µL volume of diluted DharmaFECT transfection reagent (Dharmacon, T-2001-03) in Opti-MEM was prepared. Depending on the cell line and cell density, the DharmaFECT reagent amount can vary between 0.25-2.5 µL. For the tested cell line, we used the recommended volume of DharmaFECT reagent which corresponds to 1µL DharmaFECT reagent to 49µL Opti-MEM. The content of each tube was gently mixed by pipetting carefully up and down, then incubated for 5 minutes at room temperature (RT). Then, the content of tube 1 was added in tube 2, for a total volume of 100 µL. After being pipetted up and down carefully, the content of the tube was incubated for 20 minutes at RT to allow the formation of transfection complexes, then added to 400 µL of antibiotic-free complete liver differentiation medium (LDM) for a total volume of 500 µL transfection medium. The transfection medium was finally added to the cells. At 6 h post-transfection, the media containing the transfection reagent was removed and replaced with the differentiation medium provided on day 6. The cells were then analyzed at the end of the differentiation.

# 3) RNA extraction and RT-qPCR analysis

Total RNA samples were extracted from HC3X iPSCs during differentiation at determined timepoints using the Reliaprep RNA Miniprep system (Promega Z6010) following the manufacturer's RNA extraction protocol. Samples RNA concentration was quantified with Nanophotometer N60 (Implen). Reverse transcription was conducted using GoScriptTM Reverse Transcriptase kit Random Primers (Promega, A2801).  $2\mu g$  cDNA was diluted 100X then analyzed by real-time qPCR using a SYBR Green GoTaq® qPCR Master Mix (Promega, A6002) on ViiA 7 Real-Time PCR system (ThermoFisher). Primer sequences of HC3X iPSCs markers are detailed in Table 4. The expression of the differentiation markers was calculated as a relative expression normalized on UBE3C (housekeeping gene), using the  $2^{-\Delta Ct}$  method.

# 4) Lysate preparation, protein assay and Western Blot analysis

HC3X cells under differentiation were rinsed twice in ice-cold PBS and then scraped in  $10\mu$ l/cm<sup>2</sup> lysis buffer made up of 20mM Tris-HCl pH7.5, 150mM NaCl, 15% Glycerol, 1% Triton X-100, 2% SDS, 4% Protease Inhibitor Cocktail (PIC) (Roche), and 4% Phosphate Inhibitor Buffer (PIB) composed of: 25 mM Na<sub>3</sub>VO<sub>4</sub> (Sigma), 250 mM PNPP (4-nitrophenylphosphate) (Sigma), 250 mM  $\beta$ -Glycerolphosphate (VWR) and 125 mM NaF (Merck) and 0.17U/µl Supernuclease.

# Table 5: Antibodies

Antibody	Dilution	Supplier	Reference
Anti-SOX2	1/1.000	Cell signaling	3579
Anti-HNF3b (-FOXA2)	1/1.000	Santa Cruz	sc374376
Anti-IGF2BP2	1/1.000	Proteintech	11601-1-AP
Anti-IGF2BP3	1/1.000	Proteintech	14642-1-AP
Anti-AAT	1/1.000	Santa Cruz	sc-166018
Anti-β-actin	1/20.000	Sigma- Aldrich	A5441
Anti-Puromycin	1/5.000	Merck Millipore	MABE343
Goat anti-Rabbit IgG	1/10.000	Li-Cor Bioscience	926-32211
Goat anti-Mouse IgG	1/10.000	Li-Cor Bioscience	926-32210

The cell lysates were incubated on vortex bloc 10 minutes at 12°C 16,000xg (Eppendorf Centrifuge 5414R) and then cleared by centrifugation 10min at 16 000xg at 12°C. Pierce 660nm Protein Assay Reagent (ThermoFischer Scientific, 22660) was used to assess the protein concentration in supernatant according to the manufacturer's instructions. A calibration curve was performed with samples containing bovin serum albumin (*BSA*) (Thermofisher Scientific) at different concentrations (from 0 to 10  $\mu$ g/ml). The absorbance was measured at 660nm using the xMARK Microplate Absorbance Spectrophotometer (BioRad).

Approximately 10-20µg of proteins were diluted in distilled water and 4x NuPAGE loading buffer (Invitrogen, NP0007) supplemented with 5% of 1M dithiothreitol (DTT, Sigma, D9163). Sample proteins were next heated for 10 min at 70°C, centrifuged for 2 min at 13 000rpm (Eppendorf) and then loaded and resolved in NuPAGE 4-12% Bis-Tris Gels (Invitrogen, NP0321) with MES running buffer (Invitrogen, NP0002) and antioxidant (Invitrogen, NP0005). The molecular weights of the proteins of interest have been determined by comparing to molecular weight maker (2 µl of Color Protein Standard Board Range (BioLabs)). The electrophoresis was performed at 200 V, 400 mA and 60 W for 45 min. The gel was then rinsed for 5 min in the transfer buffer containing 25 mM Tris, 150 mM Glycine adjusted to 1L with  $H_{20}$  MilliQ (pH = 8.3) and 400 mL of methanol (20%), all adjusted to 2L with H<sub>2</sub>0 MilliQ. Proteins were then transfered to polyvinylidene difluoride (PVDF) membrane (Merck, IPFL85R) previously activated by a one-minute bath of 100% methanol, by performing a liquid transfer at 100 V for 2 h. The membrane was then blocked with Odyssey Blocking Buffer (Licor, USA) for 1h before the incubation of primary antibodies overnight at 4°C. The next day, secondary antibodies were incubated for 1h at room temperature (RT) after 3 washes with PBS 0.1% Tween 20 (Biorad). Finally, after incubation, the membrane was rinsed 3 times for 5 min in PBS, 0.1% Tween-20 and twice for 1 min in PBS. The immunodetection was performed using a laser scanner Amersham Typhoon Scanner (Cytiva,) and analyzed with ImageQuant TL program.

Primary antibodies were diluted in Intercept blocking buffer (PBS) (Li-Cor Biosciences, 927-70001) containing 0.1% Tween-20. The infrared dye-coupled secondary antibodies were diluted in PBS containing 0,1% Tween-20 and 0,01% SDS. The antibodies used in experiments are listed in Table 5.

For the immunodetection of the  $\beta$ -actin used as a loading control, the primary and secondary antibodies have been incubated 30 min at RT. Finally, the membrane was rinsed 3 times for 5 min in PBS, 0.1% Tween-20 and twice for 1 min in PBS.

# 5) Puromycin-incorporation assay

Puromycin incorporation assay (Schmidt et al., 2009) was performed by treating the cells for 10 minutes with  $5\mu g/ml$  puromycin (invivogen) in the culture medium prior to lysis and Western Blot analysis as described below. Western Blot analysis was performed using the anti-puromycin antibody listed in Table 5. Cells that were untreated or treated with  $20\mu g/ml$  Cycloheximide (Sigma, 01810) 30 min before puromycin treatment were used as negative controls.
### Table 6: Antibodies

Antibody	Dilution	Supplier	Reference
Anti-SOX2	1/400	Cell signaling	3579
Anti-OCT4	1/100	Santa Cruz	sc-5279
Anti-HNF4a	1/100	Cell signaling	3113
Anti-AAT	1/500	Santa Cruz	sc-166018
Anti-IGF2BP2	1/200	Proteintech	11601-1-AP
Anti-IGF2BP3	1/100	Proteintech	14642-1-AP
IgG Anti-rabbit Alexa 488 nm	1/1000	Invitrogen	A11008
IgG Anti-mouse Alexa 568 nm	1/1000	Invitrogen	A11004

### 6) Immunofluorescence staining

Cells were seeded and differentiation was performed as described in section 2 but on sterilized glass cover slips coated with reduced Growth Factor Matrigel. After 2 washes with PBS, the samples were fixed for 15 minutes with 4% paraformaldehyde (Sigma, 30525-89-4), then incubated for 1 hour with blocking buffer containing 2% BSA and 0.1% Triton. The samples were then incubated in a humid chamber at 4°C overnight with a 30  $\mu$ l drop containing the primary antibodies diluted in BSA+Triton solution. The next day, after 3 washes with blocking buffer, cells were incubated in the dark for 1 hour with a 30  $\mu$ l drop of the secondary antibodies and DAPI (Sigma, 10 236 276 001) in blocking buffer. Finally, after three washes with blocking buffer, the coverslips were mounted on microscope slides with Mowiol (Aldrich, 32459-0) and analyzed on a Leica TCS SP5 confocal microscope (Leica Microsystems). The antibodies used in experiments are listed in table 6.

### 7) Statistical analyses

Quantitative results of at least 3 biological replicates were analyzed by a one-way ANOVA test followed by a Tuckey post-test for pairwise comparisons. For each comparison, P values <0.05 were considered statistically significant and symbolized as \* for p <0.05, \*\* for p <0.01 and \*\*\* for p <0.001. Statistical analyses were performed using GraphPad Prism version 6.0c software for Mac.

# Results



B

























n=4







![](_page_78_Figure_0.jpeg)

# Figure 11: Expression analysis of stemness/hepatocyte markers during hepatogenic differentiation of iPSCs.

Through hepatogenic differentiation of HC3X-iPSCs, micrographs were taken, and RNA and protein samples were harvested at days 0 (D0), 4 (D4), 12 (D12), 16 (D16), and 20 (D20) to analyze the expression of various key markers of differentiation by RT-qPCR, WB and IF.

(A) Phase contrast micrographs illustrating the morphological changes occurring during iPSCs' hepatogenic differentiation, following two different protocols (STD or HC3X), as represented in Fig.5. Scale bar =  $250 \,\mu$ m. Representative micrographs of n=11

**(B)** RT-qPCR analyses showing the relative mRNA abundance of key differentiation markers during hepatogenic differentiation of HC3X-iPSCs. Induced hepatic transcription factors (HNF1A, FOXA3), pluripotency markers (OCT4, SOX2), differentiation markers (GATA4, HNF4A, FOXA2) and hepatic functional markers (AAT, ALB, CYP3A4) are shown. Results are normalized using UBE3C, following the  $2^{-\Delta Ct}$  method and plotted as mean +/- SD of independent biological replicates (n=4).

(C) Representative immunoblots of SOX2, FOXA2 and AAT proteins using anti-SOX2, anti-FOXA2 and anti-AAT IgG antibodies. Western blot signal intensity was quantified and normalized over  $\beta$ -Actin signal and plotted as mean +/- SD of independent replicates (n= 4 for SOX2 and FOXA2, n=3 for AAT).

(D) Representative confocal micrographs of HC3X-iPSCs stained for SOX2 (green) and OCT4 (red) using DAPI (blue) as nuclear counterstain. Confocal micrographs are representative of 2 independent replicates. Scale bar =  $100 \mu m$ .

(E) Representative confocal micrographs of HC3X-iPSCs stained for HNF4a (green) and AAT (red) using DAPI (blue) as nuclear counterstain. Confocal micrographs are representative of 2 independent replicates. Scale bar =  $100 \mu m$ .

Statistical significance is calculated by ANOVA and Tukey HSD post-hoc test.

### Results

### 1) Characterization of global translational regulation of iPSCs

### 1.1) Hepatogenic differentiation models

To characterize the global translational regulation occurring through hepatogenic differentiation of iPSCs and to confirm the results previously obtained in the lab (Caruso et al., in revision), differentiation programs have been set up following the two different protocols, STD and HC3X (Fig.5). The differentiation of HC3X-iPSCs towards HLCs was systematically evaluated by phase contrast microscopy, RT-qPCR, Western Blot and immunofluorescence assays for specific markers after 0, 4, 12, 16 and 20 days of differentiation (Fig.11A-E).

For both protocols, the acquisition of the typical hepatocyte cell morphology was confirmed at day 20 of differentiation, with a more pronounced polygonal shape for the HC3X-iPSCs progeny (Fig. 11A). Cells derived from HC3X-iPSCs showed an increase in cell size and definition of cell borders compared to the cells from the STD protocol. The loss of the pluripotency markers OCT4 and SOX2 and the induction of the definitive endoderm and liver transcripts GATA4, FOXA2 (from day 4) and HNF4a (from day 12) were also confirmed. However, the expression profile of GATA4 after day 12 is unexpected since the expression level should increase until day 20 (Kimura et al., 2019). As expected, there was also a progressive increase in HNF1a and FOXA3 transcript abundance, which was enhanced by doxycycline-induction from day 4 in HC3X differentiation. In addition, there was a strong induction of hepatic functional markers AAT, ALB and CYP3A4 transcript expression (Fig.11B). Globally, the markers analyzed by RT-qPCR in differentiated cells showed a similar pattern of expression by comparison to the previous analyses obtained in the lab and those obtained in the original publication (Boon et al., 2020), even if the amplitude of relative expression might diverge depending on the gene considered.

We also studied the protein abundance of SOX2, FOXA2 and AAT by Western Blot in order to evaluate the acquisition of a hepatogenic phenotype at the protein level. Results show that protein abundance of SOX2, a stemness marker, decreases as differentiation proceeds, especially after day 4 of differentiation. The abundance of FOXA2 and AAT, a liver-specific TF and a hepatic-functional marker respectively, increase during the process, with a pronounced abundance at day 12 (Fig.11C). Immunofluorescence analyses confirmed these Western Blot results, in addition to detecting the increase in HNF4a expression from day 4 and the early-pronounced loss of OCT4 expression. The only difference resides in the protein abundance of AAT, which appears higher at day 16 and not at day 12 according to the blots (Fig.11D,E).

Together, these results confirm that HC3X-iPSCs were guided towards hepatogenic differentiation as shown by the acquisition of hepatocyte morphological phenotype, the repression of stemness markers and the induction of key hepatogenic transcription factors and functional protein expression.

![](_page_80_Figure_0.jpeg)

#### Figure 12: Global protein synthesis profile.

Western blot analysis of puromycin-labelled nascent polypeptides in iPSCs and differentiating cells at days 0, 2, 4, 8, 12, 16 and 20 from both protocols (STD and HC3X). iPSCs that were untreated or simultaneously treated with puromycin (PURO) and cycloheximide (CHX) were used as negative controls. Western blot signal intensity was quantified and normalized over  $\beta$ -Actin signal and plotted as mean +/- SD of independent biological replicates (n=4).

Statistical significance is calculated by ANOVA and Tukey HSD post-hoc test.

Total abundance of transcript	Transcript abundance in polysomes	Transcript regulatory mode	Number of transcripts in the D0 vs D12 list	Number of transcripts in the D0 vs D20 list
=	=	Background	2524	2347
קע	קע	Abundance UP	5743	5515
עע	עע	Abundance DOWN	6456	6444
עע	קע	Buffering UP	177	206
קע	תע	Buffering DOWN	187	143
=	קק	Translation UP	441	413
=	עע	Translation DOWN	623	354

Table 7: Transcript regulatory modes and abundance in both lists

#### **1.2)** Global translation profile

Our lab previously characterized the global translational rate at days 0, 4, 12 and 20 of iPSC hepatogenic differentiation following the STD or the HC3X protocol, showing that the global translation profile appeared to increase during pluripotency exit, then to globally decrease until the end of the process. Taking part in the revision of the manuscript submitted by (Caruso et al., in revision), I was led to confirm these results and finely characterize the regulation of protein synthesis taking place through the differentiation of cells undergoing both protocols. By reproducing the same puromycin-incorporation analysis as in the paper and studying several additional timepoints, the results confirmed that hepatogenic differentiation of iPSCs is clearly characterized by a two-phases process with early stimulation of protein synthesis followed by a global repression of translation. Therefore, the extra Western Blot analyses of puromycin-incorporated peptides confirmed that pluripotency exit occurring between day 0 and 4 is associated with an upregulation of global protein synthesis, followed by a decrease in protein synthesis during the hepatogenic maturation (Fig.12).

#### **1.3) RBPs' selection strategy**

The results previously obtained in the lab and confirmed in this Master thesis led us to propose that despite the overall decrease in protein synthesis during hepatogenic differentiation of iPSCs, some transcripts would be more translated and would therefore depend on a specific translational regulation. As mentioned in the introduction, translation is a complex three-stage process which requires the intervention of several regulators. Many factors have already been widely discussed in the literature concerning their contribution in protein synthesis, but the involvement of RBPs in translational regulation in the context of hepatogenic differentiation remains to be fully elucidated.

Our objective was to identify RBPs potentially able to modulate translation during hepatogenic differentiation of iPSCs following the HC3X protocol. To do so, a bioinformatics analysis was conducted by using Ensembl database and MEME suite program (Bailey et al., 2009; McLeay & Bailey, 2010) based on the previous data generated by polysome profiling followed by RNA sequencing experiments (Caruso et al., in revision). As a reminder, these analyses helped to assign mRNAs to 4 different regulatory modes by comparing the level of high polysome fraction mRNAs to the level of total mRNAs, for each comparison between D0 control iPSCs and D12 or D20 differentiating cells (Fig.8A). *Translation* and *Buffering* mRNAs are those which are translationally regulated while *Abundance* mRNAs are those which are translationally regulated, and *Background* mRNAs were assigned to the translationally regulated categories (summing up *Translation* and *Buffering* mRNAs). While comparing D0 to D20X differentiating cells, 1116 mRNAs were assigned to these groups (Table 7).

Once these lists of categorized mRNAs were defined, our goal was to find RBPs susceptible to regulate the translationally regulated transcripts (i.e., the *Translation* and the *Buffering* mRNAs). We therefore assumed that these transcripts should contain RBP recognition motifs, more than the *Background* and *Abundance* ones. To find these enriched motifs, we first focused on the mRNAs which were more translated compared to day 0, called

D0.00 D12								
DU VS D12								
				Trans	criptomic	Р	roteomic	ן
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	RBM6	MAUCCAR	2.57E-9	0,15	5,17E-79	0,68	3,43E-04	+ or -
RBP #2	IGF2BP3	AMAHWCA	1.13E-8	0,37	2,81E-13	0,78	8,51E-02	+ or -
RBP #3	SRSF1	MAGGACAV	2.48E-7	0,05	2,03E-228	0,63	3,99E-07	+ or -
RBP #4	RBFOX1	WGCAUGM	3.41E-7	0,02	2,01E-06	/	/	+
RBP #5	CNOT4	GACAGAN	4.04E-7	0,22	6,46E-03	/	/	+
RBP #6	KHDRBS1	AUAAAAR	5.14E-7	0,17	1,29E-60	0,49	1,80E-08	+ or -
RBP #7	IGF2BP2	AMAWACA	1.78E-5	1,83	1,30E-09	1,41	6,10E-04	+ or -
RBP #8	HNRNPL	ACACRAV	1.06E-4	0,19	3,61E-52	0,47	4,38E-06	+ or -
RBP #9	TIA1	UUUUUUG	1.12E-4	0,06	9,46E-139	0,67	3,16E-05	-
RBP #10	YBX1	AACAUCA	1.95E-4	0,42	2,39E-25	0,49	2,84E-06	+ or -
RBP #11	SNRNP70	RWUCAAG	2.16E-4	0,27	2,63E-24	0,39	1,25E-06	nd
RBP #12	YBX1	AACAUCA	2.73E-4	0,42	2,39E-25	0,49	2,84E-06	+ or -
RBP #13	SRSF9	AGGAGCA	1.21E-3	0,95	5,82E-01	0,46	2,33E-07	+
RBP #14	RBM41	WUACWUU	3.11E-3	2,08	1,13E-05	/	/	nd
RBP #15	PABPC3	GAAAACM	4.18E-3	/	/	/	/	+ or -
RBP #16	PABPC5	AGAAAAU	7.06E-3	0,53	5,01E-02	/	/	+ or -
RBP #17	PTBP1	ACUUUCU	8.84E-3	0,21	6,57E-120	0,78	1,26E-04	+ or -
RBP #18	SRSF2	GGRWGGA	1.03E-2	0,95	5,82E-01	0,46	2,33E-07	+
RBP #19	SRSF2	AGGAGWDR	1.08E-2	0,04	6,31E-127	0,47	1,51E-09	+
RBP #20	BRUNOL4	UGUGUGU	2.54E-2	/	/	/	/	+
RBP #21	YBX2	AACAWCD	3.51E-2	0,22	5,11E-11	/	/	+ or -
RBP #22	ENOX1	MAGACAG	8.15E-2	13,03	1,49E-04	1	1	-

D0 vs D20								
				Trans	criptomic	Prote	eomic	
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	SFPQ	GURGUKU	5.41E-13	0,05	2,38E-75	0,38	2,22E-15	+
RBP #2	BRUNOL6	UGUGDKG	2.43E-9	/	/	/	/	nd
RBP #3	MBNL1	NGCUUGC	3.33E-6	11,61	8,20E-36	/	/	+ or -
RBP #4	QKI	ACUAACM	2.16E-5	0,85	9,02E-02	/	/	+
RBP #5	MSI1	UAGUWRG	3.02E-5	1,03	8,75E-01	/	/	+
RBP #6	PABPC4	ΑΑΑΑΑΑ	1.35E-4	0,51	2,09E-02	/	/	+ or -
RBP #7	BRUNOL4	UGUGUGU	1.92E-4	/	/	/	/	+
RBP #8	CNOT4	GACAGAN	8.35E-4	1,37	6,46E-03	/	/	-
RBP #9	SART3	ARAAAAA	1.32E-3	0,16	4,51E-56	0,56	2,97E-04	+ or -
RBP #10	PABPC1	ARAAAAA	1.53E-3	0,013	4,27E-50	/	/	+ or -
RBP #11	TIA1	UUUUUUG	1.60E-3	0,065	9,46E-139	0,38	1,48E-05	-
RBP #12	FXR2	GGACRRG	2.81E-3	4,55	4,42E-32	/	/	+ or -
RBP #13	KHDRBS1	AUAAAAR	1.27E-2	0,17	1,29E-60	0,45	4,05E-07	+ or -
RBP #14	RBMS3	AUAUAUM	1.81E-2	23,22	4,38E-14	/	/	+ or -
RBP #15	RBM42	AACUAMG	1.96E-2	0,98	9,40E-01	/	/	nd
RBP #16	DAZAP1	UAGGUAR	2.51E-2	0,081	4,77E-108	/	/	+
RBP #17	PCBP1	CCUWWCC	5.18E-2	0,39	2,72E-27	0,57	3,27E-12	+ or -
RBP #18	RBMS1	UAUAUAS	7.11E-2	1,75	1,24E-05	/	/	+ or -
RBP #19	SRSF1	MAGGACAV	9.79E-2	2,59	2,21E-07	0,56	8,26E-04	+ or -

# Table 8. Lists of $\Delta$ + RBPs obtained by the MEME suite program comparing D0 to D12 and D20 of HC3X-iPSCs hepatogenic differentiation for the 5' UTR.

D0 vs D12								
				Transc	riptomic	Pro	teomic	
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	KHDRBS2	RAUAAAM	1.95E-6	0,83	5,60E-01	/	/	+ or -
RBP #2	IGF2BP3	AMAHWCA	2.08E-6	0,37	2,81E-13	0,78	8,51E-02	+ or -
RBP #3	IGF2BP2	AMAWACA	1.61E-5	1,83	1,30E-09	1,41	6,10E-04	+
RBP #4	QKI	ACUAACM	2.84E-3	0,85	9,00E-02	/	/	+
RBP #5	HNRNPL	ACACACA	3.10E-5	0,19	3,61E-52	0,47	4,38E-06	+ or -
RBP #6	SART3	ARAAAAA	3.17E-5	0,16	4,51E-56	0,61	3,05E-04	+ or -
RBP #7	PABC1	ARAAAAA	3.29E-5	/	/	/	/	+ or -
RBP #8	SRSF10	AGAGARR	4.30E-5	0,26	7,32E-22	/	/	+ or -
RBP #9	SRSF10	AGAGAAA	5.39E-5	0,26	7,32E-22	/	/	+ or -
RBP #10	KHDRBS3	GAUAAACV	9.88E-5	0,69	9,80E-03	/	/	+ or -
RBP #11	PABPC5	AGAAAAU	1.36E-2	0,53	5,00E-02	/	/	+ or -
RBP #12	PABPC3	GAAAACM	2.41E-2	/	/	/	/	+ or -
RBP #13	SRSF10	AGAGARR	2.67E-4	0,26	7,32E-22	/	/	+ or -
RBP #14	PABPN1	AGAAGAN	2.95E-2	0,21	5,52E-29	/	/	+ or -
RBP #15	RBM3	GAUACGA	3.49E-4	0,88	3,10E-01	0,59	5,60E-02	+ or -
RBP #16	KHDRBS1	AUAAAAR	3.66E-4	0,17	1,29E-60	0,49	1,80E-08	+ or -
RBP #17	HNRNPL	ACACRAV	8.12E-4	0,19	3,61E-52	0,47	4,38E-06	+ or -
RBP #18	PABPC4	ΑΑΑΑΑΑ	8.30E-4	0,84	2,40E-01	/	/	+ or -
RBP #19	SRSF9	AGGAGCA	5.28E-1	0,96	0,58	0,46	2,33E-07	+
RBP #20	HuR	υυυυυυ	1	0,32	2,64E-27	0,62	5,32E-06	+ or -

D0 vs D20								
				Trai	nscriptomic	Proteomic		1
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	IGF2BP2	AMAWACA	4.17E-30	2,7	2,45E-11	1,25	7,89E-02	+
RBP #2	RBM3	GAUACGA	7.81E-30	7,96	9,48E-69	/	/	+ or -
RBP #3	SRSF10	AGAGARR	1.26E-29	0,33	2,41E-24	/	/	+ or -
RBP #4	SRSF10	AGAGARR	1.58E-29	0,33	2,41E-24	/	/	+ or -
RBP #5	HNRNPL	ACACACA	2.24E-29	0,03	3,75E-129	0,44	2,22E-06	+ or -
RBP #6	SRSF10	AGAGAAA	2.40E-29	0,33	2,41E-24	/	/	+ or -
RBP #7	PABPC5	AGAAAAU	3.30E-29	0,77	5,50E-01	/	/	+ or -
RBP #8	KHDRBS2	RAUAAAM	5.54E-29	0,02	5,42E-16	/	/	+ or -
RBP #9	PABPC3	GAAAACM	6.11E-29	/	/	/	/	+ or -
RBP #10	KHDRBS1	AUAAAAR	8.67E-29	0,04	7,70E-134	0,45	4,05E-07	+ or -
RBP #11	IGF2BP3	AMAHWCA	9.29E-29	0,05	1,21E-55	0,59	8,02E-06	+ or -
RBP #12	PABPN1	AGAAGAN	1.07E-28	0,14	5,07E-191	0,24	4,43E-07	+ or -
RBP #13	PABPC4	AAAAAA	1.17E-28	0,14	2,43E-77	/	/	+ or -
RBP #14	PABPC1	ARAAAAA	1.26E-28	/	/	/	/	+ or -
RBP #15	SART3	ARAAAAA	1.26E-28	0,22	1,03E-61	0,56	2,97E-04	+ or -
RBP #16	KHDRBS3	GAUAAACV	5.69E-26	0,05	4,90E-154	/	/	+ or -
RBP #17	HNRNPL	ACACRAV	1.26E-30	0,04	3,75E-129	0,44	2,22E-06	+ or -
RBP #18	SRSF9	AGGAGCA	1.92E-16	1,97	5,03E-17	0,49	4,86E-04	+
RBP #19	RBM5	GAAGGAG	4.54E-3	0,22	1,42E-24	/	/	+ or -
RBP #20	RBM4	GCGCGSG	1.25E-2	0,26	3,19E-19	/	/	+ or -
RBP #21	SRSF1	GGAGGAG	3.00E-2	0,01	0	0,56	8,26E-04	+ or -
RBP #22	SRSF1	GGAGGAM	3.25E-2	0,01	0	0,56	8,26E-04	+ or -
RBP #23	FUS	UGCGCGC	4.63E-2	0,04	4,38E-107	0,3	4,12E-13	+ or -
RBP #24	SRSF1	GGAGGAN	4.76E-2	0,01	0	0,56	8,26E-04	+ or -
RBP #25	RBM4	GCGCGGG	8.19E-2	0,26	3,19E-19	1	1	+ or -
RBP #26	RBM8A	GCGCGCG	8.51E-2	0,61	4,78E-05	1	1	+ or -

# Table 9. Lists of $\Delta$ + RBPs obtained by the MEME suite program comparing D0 to D12 and D20 of HC3X-iPSCs hepatogenic differentiation for the 3' UTR.

D0 vs D12								
				_				
	[			Transcri	ptomic	Р	rotéomic	
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	PTBP1	CUUUUCU	4.73E-43	0,21	6,57E-120	0,78	1,26E-04	+ or -
RBP #2	PTBP1	ACUUUCU	2.41E-36	0,21	6,57E-120	0,78	1,26E-04	+ or -
RBP #3	U2AF2	UUUUUUC	3.69E-25	0,25	1,23E-38	0,44	1,49E-06	-
RBP #4	CPEB2	CWUUUUU	1.53E-20	349,79	1,24E-170	/	/	+ or -
RBP #5	TIA1	UUUUUUG	2.22E-20	0,06	9,46E-139	0,67	3,16E-05	-
RBP #6	ZC3H14	UUUGUUU	1.53E-16	0,73	7,43E-04	/	/	+ or -
RBP #7	HuR	UUUGUUU	2.27E-16	4,69	6,88E-24	0,62	5,32E-06	+ or -
RBP #8	RALY	UUUUUUG	8.37E-14	0,81	1,98E-02	0,6	1,69E-03	+ or -
RBP #9	TIA1	UUUUUUY	2.39E-13	0,06	9,46E-139	0,67	3,16E-05	-
RBP #10	HuR	υυυυυυ	3.18E-11	4,68	6,88E-24	0,62	5,32E-06	+ or -
RBP #11	CPEB4	CUUUUUU	3.19E-11	13,59	5,12E-99	0,65	6,11E-07	+ or -
RBP #12	YBX1	AACAUCA	1.58E-10	0,42	2,39E-25	0,49	2,84E-06	+ or -
RBP #13	HNRNPC	AUUUUUK	1.13E-9	0,2	4,20E-45	0,37	8,24E-09	+ or -
RBP #14	HNRNPCL1	AUUUUUU	2.76E-9	/	/	/	/	nd
RBP #15	ZNF638	UGUUSGU	3.89E-9	0,48	2,84E-11	/	/	+ or -
RBP #16	TUT1	CGAUACU	1.50E-8	0,37	6,88E-08	/	/	nd
RBP #17	PCBP1	CCUWWCC	1.07E-7	0,39	2,72E-27	0,64	4,08E-05	+ or -
RBP #18	HuR	UUUGUUU	3.70E-7	4,68	6,88E-24	0,62	5,32E-06	+ or -
RBP #19	PABPC5	AGAAAAU	5.06E-7	0,53	5,01E-02	/	/	+ or -
RBP #20	ENOX1	MAGACAG	1.04E-6	13,12	1,49E-04	/	/	-
RBP #21	MATR3	MAUCUUG	1.62E-6	2,00E-03	7,70E-96	0,47	3,64E-05	+ or -
RBP #22	PABPN1	AGAAGAN	4.31E-6	0,22	5,52E-29	/	/	+ or -
RBP #23	PABPC3	GAAAACM	5.47E-6	/	/	/	/	+ or -
RBP #24	CNOT4	GACAGAN	1.28E-5	1,36	6,46E-03	/	/	-
RBP #25	BRUNOL4	UGUGUGU	3.93E-5	/	/	/	/	+ or -

D0 vs D20

				Trans	criptomic	Prot	éomic	
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	PTBP1	CUUUUCU	1.93E-10	0,21	6,57E-120	/	/	+ or -
RBP #2	YBX1	AACAUCA	1.02E-6	0,42	2,39E-25	0,46	1,79E-05	+ or -
RBP #3	ENOX1	MAGACAG	3.66E-5	13,12	1,49E-04	/	/	-
RBP #4	CPEB2	CWUUUUU	1.56E-4	349,79	1,24E-170	/	/	+ or -
RBP #5	PABPN1	AGAAGAN	1.95E-4	0,22	5,52E-29	0,24	4,43E-07	+ or -
RBP #6	ZNF638	UGUUSGU	2.66E-4	0,48	2,84E-11	/	/	+ or -
RBP #7	PTBP1	ACUUUCU	2.60E-3	0,22	6,57E-120	/	/	+ or -
RBP #8	PPRC1	SSGCGCS	6.94E-3	0,01	4,57E-281	/	/	nd
RBP #9	SART3	ARAAAAA	7.97E-3	0,17	4,51E-56	0,56	2,97E-04	+ or -
RBP #10	PABPC1	ARAAAAA	9.09E-3	0,01	4,27E-50	/	/	+ or -
RBP #11	RBM42	AACUAMG	1.17E-2	0,99	9,40E-01	/	/	nd
RBP #12	YBX1	AACAUCA	1.24E-2	0,42	2,39E-25	/	/	+ or -
RBP #13	RBM5	GAAGGAA	1.46E-2	1,19	2,85E-01	0,46	1,78E-05	+ or -
RBP #14	PABPC5	AGAAAAU	1.63E-2	0,53	5,01E-02	/	/	+ or -
RBP #15	HNRNPK	CCAAMCC	1.83E-2	0,22	1,83E-32	/	/	+ or -
RBP #16	TIA1	UUUUUUG	5.71E-2	0,07	9,46E-139	/	1	-
RBP #17	CPEB4	CUUUUUU	6.99E-2	13,6	5,12E-99	/	/	+ or -
RBP #18	PABPC4	ΑΑΑΑΑΑ	7.24E-2	0,52	2,09E-02	1	1	+ or -

# Table 10. Lists of $\Delta$ - RBPs obtained by the MEME suite program comparing D0 to D12 and D20 of HC3X-iPSCs hepatogenic differentiation for the 5' UTR.

D0 vs D12								
				Tran	scriptomic	Р	rotéomic	
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	KHDRBS2	RAUAAAM	49.36E-53	0,83	5,60E-01	/	/	+ or -
RBP #2	KHDRBS3	GAUAAACV	42.07E-46	0,69	9,80E-03	0	0	+ or -
RBP #3	KHDRBS1	AUAAAAR	41.09E-35	0,17	1,29E-60	0,49	1,80302E-08	+ or -
RBP #4	IGF2BP3	AMAHWCA	4.46E-30	0,37	2,80E-13	0,78	8,51E-02	+ or -
RBP #5	IGF2BP2	AMAWACA	8.92E-26	1,83	1,29E-09	1,41	6,10E-04	+ or -
RBP #6	PABPN1	AGAAGAN	1.83E-24	0,22	5,52E-29	/	/	+ or -
RBP #7	SRSF10	AGAGARR	1.71E-23	0,27	7,32E-22	0	/	+ or -
RBP #8	PABC1	ARAAAAA	2.61E-22	/	/	/	1	+ or -
RBP #9	SART3	ARAAAAA	2.65E-22	0,16	4,51E-56	0,61	3,05E-04	+ or -
RBP #10	PABPC5	AGAAAAU	5.94E-22	0,53	5,00E-02	/	/	+ or -
RBP #11	PABPC4	ΑΑΑΑΑΑ	6.23E-22	0,84	4,00E-02	/	/	+ or -
RBP #12	RBM3	GAUACGA	1.00E-21	0,88	3,10E-01	0,59	5,60E-02	+ or -
RBP #13	HNRNPL	ACACACA	3.90E-21	0,19	3,61E-52	0,47	4,37E-06	+ or -
RBP #14	PABPC3	GAAAACM	5.82E-21	1,00	0	/	0	+ or -
RBP #15	SRSF10	AGAGARR	1.30E-20	0,26	7,32E-22	0	/	+ or -
RBP #16	SRSF10	AGAGAAA	5.17E-20	0.26	7.32E-22	0		+ or -
RBP #17	HNRNPI	ACACRAV	1.12E-15	0.19	3.61E-52	0.47	4 37E-06	+ or -
RBP #18	RBM8A	GCGCGCG	3 69F-10	0.78	7 60E-03	/	4,372-00	+ or -
DDD #10	FLIS	UGCGCGC	7 805 10	0.10	7 955 101	,	1.005.05	Lor
RBP #19	BRUNOL4	UGUGUGU	1.26F-6	6.81	1.32F-07	/	1,902-05	+ or -
RBP #21	SRSEQ	AGGAGCA	1.62E-6	0.96	0.58	0.46	,	+ or -
RBP #22	RBM24	WGUGUGA	9.08E-6	269.79	1.13E-17	/	/	+ or -
RBP #23	RBMS3	UAUAUAB	1.09E-5	23,22	4,38E-14	/	/	+ or -
RBP #24	BRUNOL5	UGUGUGU	4.12E-5	0,07	7,93E-10	/	/	+ or -
RBP #25	RBM4	GCGCGSG	1.15E-4	0.13	3.17E-35	1	/	+ or -
RBP #26	RBM5	GAAGGAG	1.83E-4	1.18	2.80F-01	,	,	+ or -
RBP #27	ZCRB1	GAMUUAAK	3.48F-8	4.79	8.96F-20	/	/	nd
RBP #28	RBM4	GCGCGGG	2.48E-3	0,13	3,17E-35	/	/	+ or -
RBP #29	RBM5	GAAGGAA	2.61E-3	1,18	2,80E-01	/	/	+ or -
RBP #30	PPRC1	SSGCGCS	3.06E-3	0,01	4,57E-281	/	/	nd
RBP #31	RBMS3	AUAUAUM	7.59E-3	23,22	4,38E-14	/	/	+ or -
RBP #32	HuR	UUGGUUU	7.99E-3	4,68	6,88E-24	/	/	+ or -
RBP #33	SNRPA	UUGCACA	2.85E-2	0,05	1,66E-125	0,67	45	-
RBP #34	RBMS1	UAUAUAS	3.09E-2	1,75	1,24E-05	/	/	+ or -
RBP #35	ZNF638	UGUUSGU	7.79E-2	0,48	2,84E-11	/	/	+ or -

D0 vs D20

								1
				Trans	scriptomic	Pro	otéomic	
	RBP's name	Consensus	adj p-value RBP	FC	adj p-value	FC	P-value	Translation
RBP #1	RBM4	GCGCGGG	5.18E-8	0,26	3,19E-19	/	/	+ or -
RBP #2	RBM4	GCGCGSG	5.98E-8	0,26	3,19E-19	/	/	+ or -
RBP #3	RBM8A	GCGCGCG	5.40E-6	0,61	4,78E-05	1	/	+ or -
RBP #4	SAMD4A	GCUGGMC	6.73E-3	2,06	3,00E-02	/	/	+ or -
RBP #5	FXR2	GGACRRG	7.57E-3	29,67	1,44E-254	1	/	+ or -
RBP #6	RBMS3	AUAUAUM	8.65E-2	1,58	4,00E-01	1	/	+ or -

# Table 11. Lists of $\Delta$ - RBPs obtained by the MEME suite program comparing D0 to D12 and D20 of HC3X-iPSCs hepatogenic differentiation of the 3' UTR.

"Ups", to study RBPs that would potentially positively regulate the translation of transcripts. The identifiers of the Ups were copied into the Ensembl database, and the 5' UTR and 3' UTR regions were retrieved since it is mostly in these regions that RBPs are recruited (Moore & von Lindern, 2018). The sequences collected from this database were loaded into the MEME suite program (https://meme-suite.org/meme/). The MEME Suite is a known motif-based sequence analysis tool used to analyze DNA, RNA, and protein sequences (Bailey et al., 2009). The main tool used in our research was AME (Analyses of Motif Enrichment). AME identifies the known motifs that are enriched in the sequences of interest (i.e., the *Translation* and the *Buffering* mRNAs), compared to the control sequences (i.e., the *Background* and *Abundance* mRNAs). Once the comparison is done, AME predicts which factors could interact with the enriched motifs (McLeay & Bailey, 2010). Lists of RBPs potentially capable of interacting with these motifs and upregulating translation have been obtained (=  $\Delta$ + RBPs). The same strategy has been applied later on to search for RBPs potentially capable of downregulating translation (=  $\Delta$ - RBPs).  $\Delta$ +/- RBPs are listed in the Tables 8-11, both for the 5' UTR and the 3' UTR comparing D12 and D20 of differentiation to D0.

From those lists, several criteria were considered to select RBPs that will be functionally investigated. The p-value of the MEME suite enrichment analysis and the expression profile of each RBPs during hepatogenic differentiation were our first selection criteria. Then, the literature was explored to find information about RBPs' role in translational regulation and possibly in stemness/differentiation. Finally, a particular attention was also given to their target mRNAs (predicted or according to MEME suite) (Bailey et al., 2009), looking for putative actors of hepatogenic differentiation. The last criterion, which was optional, was to cross-compare the lists (Tables 8-11) to find if some RBPs were recurrent.

Using this strategy, our attention was drawn on the insulin-like growth factor-2 mRNAbinding proteins (*IMPs/IGF2BPs*), more specifically on IGF2BP2 and IGF2BP3.

### 2) Features of IGF2BPs family

### 2.1) Generalities

The insulin-like growth factor-2 mRNA-binding proteins 1, 2, and 3 (IGF2BPs/IMPs) belong to a conserved family of RNA-binding proteins, which can bind mRNA and influence their fate. An important characteristic of this family is its high expression during the development and its contribution in cancer biology, which is why the proteins are often considered as "oncofetal" (Hansen et al., 2004). In adult organs, IGF2BP1 and IGF2BP3 are expressed at low levels, except for reproductive tissues. At the opposite, IGF2BP2 is expressed in various adult tissues. These proteins are known to act in various aspects of cell function, such as cell polarization, morphology, metabolism, migration, proliferation and even differentiation (Bell et al., 2013).

Mammalian IGF2BPs share a strong homology in domain organization and more than 56% identity in amino acid sequences (Bell et al., 2013). All three proteins have two RNA-Recognition Motifs (RRM) in their *N*-terminal region and four type I hnRNP-K homology

![](_page_88_Figure_0.jpeg)

#### Figure 13: Domain structure of human IGF2BPs.

Overview of the RNA-binding domains (RBDs) organization of human IGF2BPs paralogs. Family members are: IGF2BP1 (= IMP1), IGF2BP2 (= IMP2) isoform 1, isoform 2 and isoform 3 and IGF2BP3 (= IMP3). RBDs are represented by colored boxes, with RNA recognition motifs (RRMs) represented in blue and hnRNP-K homology domains (KH) represented in green and orange. Sequence identities between paralogs are indicated in % (Korn et al., 2021).

![](_page_89_Figure_0.jpeg)

#### Figure 14: Fate regulation of IGF2BPs target mRNAs.

IGF2BPs associate with other RBPs and their target mRNAs to form cytoplasmic ribonucleoprotein (RNP) complexes. From there, IGF2BPs decide the fate of the mRNAs. Either the mRNAs are destined to be degraded, translated or simply transported from one cytoplasmic location to another. Phosphorylation of IGF2BPs is crucial to the fate determination process of target mRNAs (Cao et al., 2018).

![](_page_90_Figure_0.jpeg)

#### Figure 15: Synthesized regulatory network of IGF2BP2 and IGF2BP3.

IGF2BP2 expression is controlled by HMGA2, a downstream target of the Let-7 miRNA, and promotes the stabilization of HMGA1, which in turn suppresses IGF2BP2 expression. Following mTORC-mediated phosphorylation, IGF2BP2 and IGF2BP3 promote IGF2 translation through downstream PI3K/Akt signaling. IGF2BP2 also regulates the expression of other effectors such as UCP1, LIMS2 and LAMB2, as well as the corresponding cellular functions. Regarding IGF2BP3, this RBP binds to Let-7 miRNA-responsive elements such as HMGA2 and LIN28B and inhibits their Let-7 miRNA-mediated degradation. IGF2BP3 also interacts with SLUG, a key factor in the regulation of stemness, XRN2 and USP10, thereby regulating related cellular functions (created in Biorender.com) (Mancarella & Scotlandi, 2019).

domains (KH) in the *C*-terminal region, which confer IGF2BPs the affinity towards RNA and the regulation capacity of multiple target transcripts (Fig.13) (Bell et al., 2013; Korn et al., 2021). The RNA-binding activity is primarily facilitated by the KH-domains which are important for high-affinity and specific RNA binding. In addition, the RRMs domains would play a central role in the stability of IGF2BP-RNA complexes and coordinate the interactions between the complex and other RBPs, leading to the formation of mRNPs (Farina et al., 2003; Korn et al., 2021).

IGF2BPs can bind to their target mRNAs at the 5' UTR, 3' UTR or coding regions depending on the target mRNA by recognizing specific RNA motifs such as one of the first identified, CAUH (H = A, U, C) (Hafner et al., 2010), which remains a short motif. Their first identified target is the insulin-growth factor 2 (IGF2), a member of the insulin family of polypeptide growth factors which regulate development and growth (Woźniak et al., 2015). They target many other transcripts like  $\beta$ -Actin (ACTB), CD44 and c-MYC which suggests a role of this protein family in controlling cytoskeletal organization, cell proliferation and progression of various cancers (Bell et al., 2013; Hüttelmaier et al., 2005; Korn et al., 2021).

In contrast to many other RNA-binding proteins, IGF2BPs are mostly observed in the cytoplasm, close to the peri-nuclear region, although there is evidence that IGF2BPs can enter the nucleus and associate with their target mRNAs at their site of transcription and shuttle them between the nucleus and cytoplasm (Hüttelmaier et al., 2005; Oleynikov & Singer, 2003). IGF2BPs associate with specific target mRNAs and other RNA-binding proteins to form cytoplasmic mRNP complexes. The release of mRNAs from these mRNP complexes results in mRNA degradation, mRNA transport or mRNA translation. Therefore, the role of IGF2BPs would be to ensure the execution of 'the transcript fate' by controlling the release of regulated transcripts from mRNPs. The caging of IGF2BPs transcripts is a complex process that is not totally elucidated, but usually involves the phosphorylation of IGF2BPs (see below) (Fig.14) (Bell et al., 2013; Cao et al., 2018; Dai et al., 2011).

### 2.2) Characteristics of IGF2BP2/3

Unlike the other two family members, IGF2BP2 is largely expressed in adult tissues and particularly in brain tissue, nasal cavity, lung, liver, intestine and kidney (Christiansen et al., 2009). Originally identified as an IGF2 mRNA binding protein, IGF2BP2 also targets other transcripts, such as the Laminin Subunit Beta 2 (LAMB2), LIM Zinc Finger Domain Containing 2 (LIMS2), High Mobility Group AT-Hook 1 (HMGA1) and 13 other genes encoding mitochondrial components, such as the UnCoupling Protein 1 (UCP1) (Cao et al., 2018; Dai et al., 2015). This multitargeting feature correlates with the wide range of functions of IGF2BP2 in embryonic development, neuronal differentiation, lipid metabolism, insulin resistance, and tumorigenesis (Rodriguez et al., 2006). IGF2BP2 is primarily known to facilitate trafficking of mRNAs near mitochondria, thereby enabling their localized translation. Regarding its involvement in translational regulation, phosphorylation of IGF2BP2 in the linker region between RRM2 and KH1 by mTORC1 promotes its binding to the IGF2 mRNAs, enhancing the initiation of IGF2 translation through eIF-4E- and 5' cap-independent internal ribosomal entry (Fig.15) (Cao et al., 2018; Dai et al., 2017).

![](_page_92_Figure_0.jpeg)

#### Figure 16: Schematic representation of the IGF2BP3 activity mechanisms.

In the nucleus, IGF2BP3 affects microRNA biogenesis by preventing Drosha from binding to primiRNA (1). IGF2BP3 is also able to interact with nuclear RNP (nRNP), promoting nuclear export of target transcripts (2). In the cytoplasm, IGF2BP3 acts within the ribonucleoprotein granules, leading to the control of the stability (3), the degradation (4) or the transport (5) of its target mRNAs. To ensure the stability, IGF2BP3 can either inhibit RISC activity or recruit stress granule (SG) proteins under stressful conditions. For degradation, IGF2BP3 can either recruit RISC or interact directly with enzymes, such as ribonuclease or deubiquitinase, thereby inducing microRNAdependent and microRNA-independent target degradation. For transport, IGF2BP3 directs the localization of its targets once assembled in a stress granule along the microtubules to areas of active translation. (Mancarella & Scotlandi, 2019). Currently, nothing has been described regarding a putative role of IGF2BP2 in hepatogenic differentiation, but this protein plays a role in another type of differentiation process. Indeed, silencing IGF2BP2 expression inhibits neurogenesis while promoting neural pluripotent cells (NPCs) differentiation into glial cells. During development, IGF2BP2 is expressed at a high level in NPCs in the early stages, when cells are proliferative and multipotent, but to a less extent at the later stage when the cells lose their capacity for self-renewal. The mechanism behind the progressive decrease in IGF2BP2 expression remains unclear. However, several papers argue that this downregulation results from a decreased expression of the High Mobility group AT-Hook 2 (HMGA2), an oncogenic protein known to activate IGF2BP2 expression (Fig.15). A HMGA2-IGF2BP2 axis is consequently involved in NPCs stemness control and inhibition of differentiation (Cao et al., 2018; Fujii et al., 2013).

Finally, IGF2BP2 is also recognized for its role in the mitochondrial respiratory chain. When its expression is repressed, the oxygen consumption rate and both complex I and complex IV assembly and activity are decreased, suggesting essential roles for this protein in energy metabolism (Janiszewska et al., 2012). The importance of IGF2BP2 in metabolism was confirmed by the phenotype observed in IGF2BP2 (-/-) mice. IGF2BP2 -/- mice have less birth weight and gain less weight during life compared to WT. They are more resistant to dietinduced obesity, hepatic steatosis and are also more glucose tolerant and insulin sensitive. Furthermore, brown fat cells lacking IGF2BP2 have high levels of UCP1, which maintains body temperature and reduces ATP synthesis (Fig.15). Taken together, these results highlight the important role of IGF2BP2, since its deletion increases energy consumption and provides better body protection against cold (Cao et al., 2018). Since there is an oxidative shift and mitochondrial biogenesis during hepatogenic differentiation (Boon et al., 2020; Wanet et al., 2014), this could depend, at least partially, on a contribution of IGF2BP2, whose expression would be increased as suggested by the expression profile obtained from transcriptomic and proteomic analyses performed in our group (Tables 8-11). We therefore hypothesize that an increased expression of this RBP during hepatogenic differentiation could contribute to the proper hepatogenic differentiation.

When it comes to IGF2BP3, its expression gradually recedes until the end of embryonic development, especially when adult tissues begin to be defined. This family member is known to regulate RNA stability, degradation, localization, and miRNA biogenesis (Fig.16) (Bell et al., 2013) but the exact molecular processes governing these functions are not completely elucidated.

Similarly to IGF2BP2, it has been suggested that IGF2BP3 undergoes phosphorylation by mTORC2 during translation and, importantly, that the phosphorylated status enhances IGF2BP3 binding to the 3' UTR of IGF2, leading to translation initiation of IGF2 mRNA and increased IGF2 expression, as suggested by its name (Fig.15) (Mancarella & Scotlandi, 2019).

Beyond that, IGF2BP3 may protect its target mRNAs from miRNA-dependent degradation by caging transcripts into mRNPs that do not contain RISC (Jønson et al., 2014). IGF2BP3 may also modulate the association between target transcripts and RISC and may compete with miRNAs for binding sites on the 3' UTR of target transcripts, thereby preventing their degradation (Ennajdaoui et al., 2016). Finally, IGF2BP3 may also affect miRNA biogenesis by competing with the ribonuclease Drosha to bind to pri-miRNAs in the nucleus, indirectly affecting the fate of miRNA targets. For example, IGF2BP3 has the ability to block miR-3614 maturation, leading to increased TRIM25 expression promoting breast cancer cell proliferation (Wang et al., 2019). The role of this RBP is therefore double-edged: either it

promotes translation by protecting target mRNAs from degradation, or it slows down the translational process by promoting the degradation of its target mRNAs by RISC (Fig.16). The best-known example of IGF2BP3 activity is its effect against the action of the let-7 miRNA. IGF2BP3 has been shown to confine let-7 targets, such as HMGA2 and Lin-28 Homolog B (LIN28B) transcripts, into RISC-free ribonucleoprotein granules, commonly named locasomes. This sequestration protects them from let-7-dependent degradation and provides protection from any other miRNA (Fig.15,16). Therefore, locasomes represent a cytoplasmic shelter for oncogenes that are then protected from degradation. However, the mechanism by which RISC is excluded from these granules and the full composition of locasomes remain to be discovered (Mancarella & Scotlandi, 2019).

In addition to its interference with miRNAs, IGF2BP3 also regulates the mRNAs fate by interacting with several enzymes like helicases, deubiquitinases or ribonucleases, several nuclear ribonucleoproteins and stress granule (SG)-associated proteins (Fig.16) (Mancarella & Scotlandi, 2020). In particular, IGF2BP3 interacts directly with the ribonuclease XRN2, or ubiquitin-specific peptidase 10 (USP10), leading to the degradation of EIF4EBP mRNA or p53 protein and tumorigenesis promotion. On the other hand, the interaction with the nuclear RNP (nRNP) has been shown to be crucial for the specific localization of IGF2BP3 in the nucleus, as well as the nucleus-cytoplasmic export and the stability of IGF2BP3 transcription targets (Mancarella & Scotlandi, 2019).

Finally, this family member also has other functions that made it an obvious choice in the selection process. Indeed, this RBP promotes the expression of three key regulators of stemness: HMGA2, LIN28B and SNAI2 (SLUG) (Fig.15) (Jønson et al., 2014; Samanta et al., 2016). In humans, HMGA2 and LIN28B are involved in growth and differentiation through their ability to regulate stability and translation via direct binding of various gene transcripts such as stem cell factor OCT4, growth factor IGF2, cell cycle regulators and ribosomal subunits (Shinoda et al., 2013). LIN28B blocks the production of mature let-7 miRNA, thereby avoiding HMGA2 degradation and the activation of IGF2BP2 expression. The maintenance of the pluripotent state of embryonic stem cells is therefore insured by prevention of let-7-mediated differentiation (Jønson et al., 2014). Finally, SLUG is one of the most important epithelial-mesenchymal transition (EMT)-related transcription factors. IGF2BP3 improves the transcription of SOX2 by a SLUG-dependent mechanism, contributing to the maintenance of cellular stemness (Samanta et al., 2016). We could therefore hypothesize that a decrease in the expression of this RBP during hepatogenic differentiation as suggested by the expression profile (Tables 8-11) could contribute to differentiation.

Therefore, IGF2BP2 and IGF2BP3 seem interesting to investigate in this Master thesis, especially IGF2BP2 for which we expect a decreased expression profile throughout hepatogenic differentiation according to the literature, but which seems to increase according to the transcriptomic and proteomic analyses.

![](_page_96_Figure_0.jpeg)

![](_page_97_Figure_0.jpeg)

# Figure 17: Expression analysis of IGF2BP2 and IGF2BP3 during hepatogenic differentiation of HC3X-iPSCs.

(A) RT-qPCR analyses showing the relative mRNA abundance of IGF2BP2/3 during hepatogenic differentiation of HC3X-iPSCs. Results are normalized on UBE3C using the  $2^{-\Delta Ct}$  method and plotted as mean +/- SD of independent biological replicates (n = 4).

(B) Representative immunoblots of IGF2BP2 and IGF2BP3 proteins using anti-IGF2BP2 and anti-IGF2BP3 IgG antibodies. Western blot signal intensity was quantified and normalized over  $\beta$ -Actin signal and plotted as mean +/- SD of independent replicates (n= 4).

(C) Representative confocal micrographs of HC3X-iPSCs stained for IGF2BP2 (green) and AAT (red) using DAPI (blue) as nuclear counterstain. Confocal micrographs are representative of 2 independent replicates. Scale bar =  $100 \mu m$ .

(D) Representative confocal micrographs of HC3X-iPSCs stained for IGF2BP3 (green) and AAT (red) using DAPI (blue) as nuclear counterstain. Confocal micrographs are representative of 2 independent replicates. Scale bar =  $100 \mu m$ .

Statistical significance is calculated by ANOVA and Tukey HSD post-hoc test.

![](_page_98_Figure_0.jpeg)

![](_page_98_Figure_1.jpeg)

Figure 18: Evaluation of siRNAs delivery by fluorescence microscopy using a non-target (NT) fluorescent siRNA and a non-target (NT) non-fluorescent siRNA added on day 6 on HC3X-iPSCs at day 7 (D7) of hepatogenic differentiation.

(A) Confocal micrographs taken 1-day post-transfection of HC3X-iPSCs with non-targeted (NT) control and fluorescent siRNAs (n=1). Scale bar =  $100\mu$ m.

**(B)** Confocal micrographs taken 1-day post-transfection of HC3X-iPSCs with non-targeted (NT) fluorescent siRNA (n=1). Scale bar =  $100 \mu m$ .

B

## 3) Effect of IGF2BP2/3 on hepatogenic differentiation of HC3XiPSCs

Once the RBPs of interest were selected, we evaluated their expression profile through hepatogenic differentiation. The RT-qPCR results showed a slight increase in the IGF2BP2 transcript level, especially from day 16 of differentiation, as well as a progressive decrease in the IGF2BP3 transcript abundance (Fig.17A). At the protein level, Western Blot analyses confirm a progressive increase in the IGF2BP2 abundance, as well as a slight decrease in IGF2BP3 expression (Fig.17B). To confirm these results, we next performed an immunofluorescence staining of IGF2BP2 and IGF2BP3 followed by confocal microscopy observation (Fig.17C,D). The analysis of IGF2BPs localization confirms their presence in the cytoplasm with no clear modification of abundance along the differentiation process. Therefore, it became even more interesting to know what contribution these two RBPs had in hepatogenic differentiation.

Since IGF2BP2 expression has been demonstrated as increasing throughout the differentiation process, it was interesting to investigate its putative effect on hepatogenic differentiation by using siRNAs. To our knowledge, this is the first report attempting to transfect iPSCs undergoing hepatogenic differentiation. Therefore, preliminary transfection assays were first carried out with non-target (NT) siRNAs on HC3X-iPSCs. Following the DharmaFECT protocol, HC3X-iPSCs were transfected for 6-8 hours after seeding with 5  $\mu$ M of a NT fluorescent siRNA or a NT non-fluorescent siRNA which served as a control. We had initially tried to use siRNAs from day 0, but we encountered problems as the cells did not survive to the high stress provoked by differentiation induction and transfection. Transfection is known to be an invasive and capricious process for iPSCs, but we did not expect global cell death (Chatterjee et al., 2011). We attempted to transfect the cells again, but this time at day 4, at the time of doxycycline induction. Again, the cells did not survive the transfection, probably due to cumulative stress. After these two trials, we arbitrarily defined day 6 (D6) as transfection day, when cells form a more resistant cell monolayer and do not display significant morphological changes compared to day 4.

Next, we evaluated the siRNAs delivery under confocal laser scanning microscope, one day post-transfection (D7). As shown on the Figures 18A and B, transfection with NT fluorescent siRNAs resulted in numerous granule-like fluorescent puncta in the cell culture. These live-cells micrographs were complicated to interpret since the fluorescent dots overlaying the cells did not give any information on the siRNAs' internalization. Indeed, siRNAs could be internalized in the cells or absorbed to the cell surface, while others would not even come into contact with them.

Since it was impossible to evaluate the transfection efficiency, we decided to perform transfection assays with NT fluorescent and specific IGF2BP2 siRNAs. HC3X-iPSCs were transfected on D6, for 6-8 hours with 5  $\mu$ M of a smart pool IGF2BP2-siRNA or the NT fluorescent siRNA which served as a control.

![](_page_100_Figure_0.jpeg)

n=1

A

![](_page_101_Figure_0.jpeg)

![](_page_102_Figure_0.jpeg)

# Figure 19: Effect of the silencing of IGF2BP2 with a smart pool siRNA added on day 6 on HC3X-iPSCs at days 0, 8, 12 and 20 of hepatogenic differentiation.

Through hepatogenic differentiation of untransfected ( $\emptyset$ ) or transfected on day 6 with non-targeted siRNA (NT siRNA), or with siRNA against IGF2BP2 (IGF2BP2-siRNA), HC3X-iPSCs, micrographs were taken, and RNA and protein samples were harvested at days 0 (D0), 8 (D8), 12 (D12), and 20 (D20) to analyze the expression of IGF2BP2 by WB and IF.

(A) Representative immunoblot of IGF2BP2 proteins using anti-IGF2BP2 IgG antibodies. Western blot signal intensity was quantified and normalized over  $\beta$ -Actin signal (n= 1).

(B) Representative confocal micrographs of HC3X-iPSCs transfected with IGF2BP2-siRNA and stained for IGF2BP2 (green) and AAT (red) using DAPI (blue) as nuclear counterstain (n=1). Scale bar =  $100 \mu m$ .

(C) Representative confocal micrographs of HC3X-iPSCs transfected with IGF2BP2-siRNA and stained for IGF2BP2 (green) and AAT (red) using DAPI (blue) as nuclear counterstain (n=1). Scale bar =  $50 \mu m$ .

Then, we assessed the specific mRNA markers by Western blot and Immunofluorescence analyses after days 0, 8, 12 and 20 of differentiation. Western blot analysis comparing untransfected (Ø), transfected with non-target siRNA (NT-siRNA) or with siRNA against IGF2BP2 (IGF2BP2-siRNA) HC3X-iPSCs showed that transfection of IGF2BP2-siRNA downregulated the expression of IGF2BP2 at D8 and D12 similarly to the NT-siRNA compared with the Ø group. This suggests that the transfection of IGF2BP2-siRNA was not as specific as expected, since the transfection process itself would already have an effect on IGF2BP2 abundance (Fig.19A).

Regarding the immunofluorescence results comparing only non-transfected and IGF2BP2siRNA transfected cells, and studying the hepatic functional marker AAT, it seems that transfection did influence hepatogenic differentiation (Fig.19B). At day 8, i.e. two days after transfection, there is a decrease in IGF2BP2 expression signal in some cells in the transfected condition, compared to the non-transfected one. The level of AAT expression remained similar, as evidenced by diffuse and low intensity labelling. From day 12, some transfected cells still display a low IGF2BP2 intensity, and even though the intensity of the AAT signal is still quite high, it is more diffuse than in the non-transfected cells, which have a much more defined and intense labelling. However, at higher magnification (Fig. 19C), we clearly see a difference in signal intensity between non-transfected and IGF2BP2-siRNA transfected cells, both for IGF2BP2 and AAT labelling. The areas that show a lower IGF2BP2 signal intensity also show a lower AAT signal intensity, suggesting that downregulation of IGF2BP2 would be responsible for a decrease in AAT expression. Finally, the siRNAs no longer appear to influence the cells at day 20, with IGF2BP2 expression being similar in both conditions. And the AAT labelling is comparable to the one observed in non-transfected cells (Fig.19B).

Altogether, these results show that transfection itself would down-regulate IGF2BP2 abundance, which suggest that this silencing approach is not appropriate. In addition, the silencing efficiency at the population level is not sufficient at all. However, when considering the individual cell level, thanks to immunofluorescence analysis, is seems that the abundance of IGF2BP2 is inversely correlated with the expression of the hepatogenic marker AAT. Although these data represent the result of one single experiment and are very preliminary, they are encouraging considering a potential role of IGF2BP2 in hepatogenic differentiation.

Discussion, perspectives and Conclusion

### Discussion and perspectives

The goal of this Master thesis was to characterize the translational regulation mechanisms occurring during hepatogenic differentiation of induced pluripotent stem cells in vitro. Hepatogenic differentiation of iPSCs is a highly dynamic process that requires transcriptional but also translational rewiring capable of impacting proteomic remodeling. By setting up iPSC differentiation programs followed by polysome profiling and data analyses of the translatome results, a study previously conducted in the lab showed that translation was globally repressed during hepatogenic differentiation (Caruso et al., in revision). But considering differentiation as a process mainly regulated by translational regulation is not realistic since many genes encoding functional proteins of differentiated cells are not or very modestly transcribed in stem cells. This idea is supported by the scatter plots of Log2FC mRNA abundance in high polysome samples and total mRNA in any comparison between differentiated cells and control iPSCs showing that the majority of mRNAs are distributed along the diagonal of the graph (Fig.8) indicating that the magnitude of the transcriptional regulation is stronger than translational regulation. However, although the differentiation process is mainly controlled by transcriptional regulation, the expression of some transcripts seems to depend on translational induction/repression, highlighting a potential role for translational regulation in the acquisition of the differentiated proteome. We therefore hypothesized that several factors would be able to induce or repress the expression of specific mRNAs during the differentiation process. Since there is poor information in the literature about the role of RNA Binding Proteins in different molecular processes, we decided to study the effect that some RBPs would have on the translational regulation occurring during hepatogenic differentiation (McLeav & Bailey, 2010).

Before studying the impact of RBP candidates on hepatogenic differentiation of HC3XiPSCs, a more precise timely characterization of the translational regulation profile needed to be done. To do so, a differentiation program was carried out in several replicates on STD and HC3X iPSCs and was evaluated through RT-qPCR, Western Blot and Immunofluorescence analyses. Taking our results altogether, it is reasonable to assume that the acquisition of the hepatocyte morphological phenotype, as well as the gene expression and the protein abundance of specific markers coincided with the expected results. We therefore assumed that the models were sufficiently mastered to confirm the global translational repression *in vitro*.

By studying additional time-points to those initially considered (Caruso et al., in revision), we affirmed that during hepatogenic differentiation, protein synthesis regulation follows a two-step mode where early events of pluripotency exit are accompanied by a global transient upregulation of protein synthesis, while later cell specification and maturation steps induce translational repression. These results support the observations made previously (Caruso et al., in revision) and leads to the assumption that the early endodermal lineage commitment would be accompanied by significant protein synthesis compared to hepatic specification and maturation. However, several researchers such as Gabut and Signer have shown that endoderm maintenance requires low translational regulation to control stem cell identity, whereas changes in cell fate require more protein synthesis (Gabut et al., 2020; Signer et al., 2014). Following this logic, a progressive increase in translational efficiency should be observed during hepatogenic differentiation, and not the opposite. However, although differentiation is accompanied by a global decrease in translational regulation during hepatogenic differentiation,
several transcripts have been shown to be translationally upregulated (Caruso et al., in revision). Thus, this led us to go one step further in the comprehension of the mechanisms of translational regulation during hepatogenic differentiation. Rather than studying all the factors already known to influence protein synthesis, such as the initiation, elongation et termination factors, we decided to identify RNA binding proteins (RBPs) able to regulate translation of specific transcripts.

To retrieve potential RBPs responsible for translational control, a bioinformatics analysis was conducted to find candidates based on their mRNA recognition motifs. The MEME suite program, and more particularly AME, allowed to predict which RBPs might control the translation of a subset of mRNAs by detecting enrichment of known binding motifs in the regulatory sequences of translationally regulated mRNA. This program studies motifs of variable length, but lists them as seven nucleotides consensus sequences, a convenient way to refer to the motifs, along with their corresponding RBP and the optimal enrichment p-value of the motif according to the Fisher's statistical test (https://meme-suite.org/meme/tools/ame). From there, two RBPs were selected for further study, IGF2BP2 and 3, based on the criteria already discussed. However, this type of analysis doesn't take into account the yet unknown motifs, the coding sequences nor the di-,tri-dimensional structures of mRNAs. Yet, it seems that the identification of the RNAs bound by each RBP is the key for understanding the interactions governing post-transcriptional regulation (Ganser et al., 2019; Taliaferro et al., 2016). Some RBDs are known to recognize their target sites mainly in their coding regions rather than in their 5' UTR and 3' UTR (Mancarella & Scotlandi, 2019), while some have been shown to recognize their target sites by their shape and geometry and not by their sequence content. Indeed, the secondary and tertiary structures of motifs are the major determinant of protein binding since they play a decisive role in limiting the access of RBPs to a large subset of otherwise occupied motifs (Ganser et al., 2019; Jarmoskaite et al., 2018; Taliaferro et al., 2016). Recently, many low- and high-throughput experimental methods have been developed to assess the *in vitro* sequence binding preferences of RBPs, as well as to identify the *in vivo* binding sites of RBPs in particular cellular contexts, but motif-finding methods that capture RBP-binding preferences still need to be improved (Li et al., 2014). So even if there are still many aspects to explore, we are the first to set up a project assessing the translational regulation of RBPs in the context of hepatogenic differentiation of iPSCs by focusing our study on known motifs.

Little is known about IGF2BP2 and 3, but most of the literature about these two RBPs define them as having a contribution in stemness. However, transcriptomic and proteomic analyses suggest that IGF2BP2 expression increases during the differentiation process. We therefore decided to study their expression profile through hepatogenic differentiation of HC3X-iPSCs. Our results correlated with the transcriptomic and proteomic results, showing that as differentiation proceeds, there is a slight increase in IGF2BP2 expression, as well as a progressive downregulation of IGF2BP3. Since then, it became even more interesting to study the contribution of these two RBPs in hepatogenic differentiation.

Knowing that IGF2BP2 expression increase throughout hepatogenic differentiation, we decided to assess its effect on differentiation by using specific siRNAs. According to the preliminary data, it could be proposed that IGF2BP2 influences the expression of AAT, a marker of hepatic maturation, and thus could potentially have an effect on hepatogenic

differentiation. Of note, these results are very preliminary as they are derived from a single replicate of transfection experiment. hiPSCs are among the difficult-to-transfect cell types and the delivery efficiency can be as low as 5-10% (Cerbini et al., 2015). The majority of transfection protocols used in the literature work with early passage iPSCs, when the culture is less than 80% confluent. In this work, we transfected cells at day 6 of differentiation, when cells were mostly no longer in a pluripotent state and formed a cell monolayer of 100% confluence. This high confluency would explain the poor transfection efficiency encountered.

Aiming to study the knockdown of the IGF2BP2 gene, vector-mediated expression of short hairpin RNAs (shRNAs) would also be a valuable option (Eggenschwiler et al., 2013). Like siRNAs, shRNAs may be transfected as plasmid vectors encoding shRNAs, but can also be delivered through infection of the cell with virally produced vectors (Moore et al., 2010). However, although siRNA and shRNA ultimately use a similar cellular mechanism to silence the expression of genes, plasmid vectors encoding shRNAs are capable of DNA integration. Following transcription, the shRNA sequence is exported to the cytosol, silencing its target mRNA (Taxman et al., 2010). And while siRNAs were shown to be fast-acting but transient, shRNAs have been shown to have a low degradation rate, a long-lasting knockdown effect, and are suitable for a wide range of cells, including the difficult-to-transfect cells (Eggenschwiler et al., 2013). In our silencing experiment, it would be interesting to induce shRNA expression at a key point of differentiation. To do so, shRNA expression could be controlled by the choice of an appropriate inducible promoter such as the most known inducible gene knockdown system, the isopropyl thiogalactose (IPTG)-responsive lac repressor-operator-mediated inducible RNAi expression (Wu et al., 2007). ShRNA-mediated knockdown therefore seems more appropriate than siRNAs. However, their use has been demonstrated to be limited during differentiation processes by the silencing of the shRNA expression during epigenetic remodeling (Eggenschwiler et al., 2013).

Therefore, in the future, it would be better to perform a knock-out of IGF2BP2 by using an RNA-guided CRISPR-Cas9 system. This technique is known to be much more efficient and specific than a simple silencing since iPSCs can be easily genetically modified (Sens et al., 2021; Zhen et al., 2017). Moreover, since CRISPR-Cas9 cause definitive genetic changes, its use would allow us to study the effect of IGF2BP2 through all the differentiation process (Barrangou et al., 2015). However, even though we knew that our model was likely to be difficult to transfect, we still wanted to test the siRNA transfection approach because it allowed us to test the effect of IGF2BP2 knockdown while the cells were differentiating, and not even before differentiation was initiated. It is interesting to study a long-lasting effect during differentiation, but not an effect that would already be present in the stem cell population. Indeed, IGF2BP2 has been shown to have a major role in stemness (Dai, 2020), therefore its KO would be likely to disrupt our cells even before the differentiation initiation. To overcome this putative problem, recent studies have succeeded in establishing an efficient and effective strategy to generate inducible gene knockout (iKO) hiPSC by combining CRISPR/Cas9mediated genome editing with the flippase (FLP)/FLP recombinase target (FRT) and Cre/LoxP system (Chen et al., 2015; González et al., 2014). Chen and coworkers created an hPSC line with a doxycycline inducible Cas9 expression cassette inserted into the AAVS1 site (Chen et al., 2015). AAVSI locus is known as a "safe harbor site" because its disruption does not have adverse effects on the cell, which means that it can be used for precise gene editing (Hayashi et al., 2020). After doxycycline treatment and two rounds of single guide RNA (sgRNA) transfection at specific time points, random indels are introduced into the targeted gene sites

via NHEJ, resulting in gene silencing. The advantage of their iKO system is that it is based on highly efficient FLP-recombinase-mediated excision of the DNA segment between the FRT sites, resulting in a predictable exon loss and frameshift, which leads to gene silencing in almost all cells (Chen et al., 2015). However, although this technique is extremely promising for our study, several drawbacks would be encountered. Firstly, the inducible cassette encoding Cas9 is introduced into the AAVSI locus, which is already occupied in our cell model by the three doxycycline-inducible cassettes encoding HNF1a, FOXA3 and PROX1(Boon et al., 2020). Therefore, the Cas9 cassette should be inserted into another well-known safe locus, such as the human ortholog of the mouse Rosa26 locus, one of the most preferred integration site used for transgene insertion (Irion et al., 2007). Secondly, this system uses doxycycline as the Cas9 cassette inducer. The concern is that doxycycline already induces the expression of the three cassettes encoding HNF1a, FOXA3 and PROX1 from day 4 of HC3X-iPSCs differentiation. This means that we would have a constant induction of Cas9 from day 4 onwards as doxycycline becomes a compound of the culture medium. Prolonged expression of the CRISPR/Cas9 system is known to lead to significant off-target effects, which could result in significant genetic problems during differentiation (Mout et al., 2017). Thus, rather than using doxycycline inducer, IPTG would be more useful as the Cas 9 cassette inducer. This would allow to induce the expression of the CRISPR/Cas9 system for a defined time window (usually between 24 and 48 hours) and not for a longer period. Finally, since the transfection experiment was not so easy in our cell model, transfection of sgRNAs would probably not be effective. It would therefore be preferable to also generate an IPTG-inducible single guide expression cassette and insert it into the Rosa26 locus, just like the Cas9 cassette.

Altogether, this demonstrate that we have the choice between several genome editing methods but the use of iKO by CRISPR/Cas9 system seems the most adequate to study the effect of IGF2BP2 during hepatogenic differentiation of HC3X iPSCs.

Based on the observations made from the IGF2BP2 knockdown experiment results, we can't affirm that the decrease in IGF2BP2 expression is responsible for the downregulation of AAT. In the future, it would be interesting to perform quantitative analyses, such as FACS, which would give an idea of the silencing efficiency and the number of IGF2BP2+/- and AAT+/- cells. From there, if the cells repressed in IGF2BP2 expression are also repressed in AAT expression, it would suggest that AAT expression depends on IGF2BP2 activity.

Similarly, we can't state that IGF2BP2 has a major role in hepatic differentiation by studying the effect of IGF2BP2 silencing on a single differentiation marker. To ensure that, we would need to study the expression of other markers under IGF2BP2 silencing, such as the two functional markers ALB or CYP3A4, either by WB, IF or FACS.

In this study, we did not attempt to investigate the effect of IGF2BP3 on hepatogenic differentiation of HC3X-iPSCs. Since this protein is downregulated during the differentiation process, it needs to be overexpressed to potentially counteract the differentiation. To overexpress IGF2BP3 at a specific time point of hepatogenic differentiation, we could genetically modify the initial stem cell line using a lentiviral transfer plasmid capable of introducing a fourth doxycycline-inducible cassette encoding IGF2BP3 into the *AAVS1* locus. In these cells, the IGF2BP3 would be induced from the *AAVS1* safe harbor locus from day 4 of hepatogenic differentiation until the end of the process. Then, the expression of several differentiation markers would be studied under IGF2BP3 overexpression, and the contribution of this molecule in the hepatogenic differentiation of iPSCs would be determined.

Additionally, the set-up of cell biology tools would allow to further investigate the regulating mechanisms of IGF2BP2/3 in mRNA translational control. In the literature, several articles mention the different molecular pathways in which IGF2BP2 and 3 are involved (Cao et al., 2018; Dai et al., 2015; Mancarella & Scotlandi, 2020) but it would be interesting to add as a new perspective the study of the IGF2BP2/3 interactions within HC3X-iPSCs during their differentiation. RNA immunoprecipitation (RIP) is a recent powerful technique developed to detect the association of individual proteins with specific RNA molecules. The basic workflow uses a protein specific antibody to pull down the RBP of interest (along with bound targets) from cellular lysates. Following immunoprecipitation, bound RNA is extracted, then is either coupled to RT-qPCR to quantitatively examine binding of specific targets or to high-throughput sequencing to get a global view of bound RNAs (Mukherjee et al., 2021). This assay has been successfully used to purify RNPs and knowing that RBP-RNA associations can be identified in RNP complexes, it would be interesting to investigate the interaction between IGF2BP2/3 and their target mRNAs by applying this experiment (Marmisolle et al., 2018).

Regarding the known target mRNAs of IGF2BP2 and 3, HMGA1, LAMB2 and SLUG already discussed in section 2.2 of the results are sorted in the *Translation* category of mRNAs in the hepatogenic differentiation model we use (Caruso et al., in revision). Therefore, in addition to focus our study on the RBPs of interest, it would be interesting to study their target mRNAs and their contribution in hepatogenic differentiation. By studying their expression through the differentiation process, as well as through differentiation under IGF2BP2/3 KO, we would have an idea of the IGF2BP2/3-mRNA relationship. Similarly, it would be interesting to compare the effect of IGF2BP2/3 KO vs target mRNAs KO, to deduce whether the results are similar or not. This will allow us to deduce whether the effect observed by the IGF2BP2/3 KO is due to the absence of several target mRNAs or one target mRNA.

If we want to go deeper in the study of the IGF2BP2/3 contribution in hepatogenic differentiation, another interesting perspective would be to focus on their phosphorylation status. Indeed, as mentioned in section 2.2 of the results, IGF2BP2 and 3 have been shown to be functional after being phosphorylated. The phosphorylation of IGF2BPs by mTOR is critical for post-transcriptional gene expression regulation and to coordinate cellular function and nutrient metabolism (Dai, 2020; Dai et al., 2011). Nothing has been described regarding the importance of IGF2BP2/3 phosphorylation in hepatogenic differentiation. However, the phosphorylation of some RBPs has already been shown to be essential for some differentiation processes. For example, Akt1-mediated phosphorylation of RBP-Jk tends to inhibit the Notch1 signaling pathway known to play a crucial role in determining cell fate, including cell growth, cell differentiation and cell apoptosis (Kim et al., 2019). Since then, it would be interesting to monitor the phosphorylated form of IGF2BP2/3 through the differentiation process. Western Blot and Immunofluorescence experiments using anti-pIGF2BP2/3 antibodies would be a first approach, as it would give an idea of the pRBPs/RBPs proportion. The next step would be to study the hepatogenic differentiation in absence of their phosphorylated form. Using rapamycin or shRNAs capable of reducing mTORC1 activity, studies have shown that inhibition of the mTORC1 pathway strongly reduces the dual phosphorylation of IGF2BP2 (Ser162/Ser164) as well as the individual phosphorylation of IGF2BP2 (Ser164) and leads to the inhibition of IGF2BP2 binding to its target mRNAs (Dai et al., 2011, 2017). Regarding IGF2BP3, similar inhibitors of mTORC2 pathway led to a global reduction of phosphorylated IGF2BP3 (Ser183) as well as a global inhibition of IGF2BP3 binding to its target mRNAs (Dai et al., 2013;

Mancarella & Scotlandi, 2019). Therefore, the easiest way to study the importance of pIGF2BP2/3 in hepatogenic differentiation would be to target the mTOR pathways. However, this is not as straightforward as one might hope since mTOR pathway is a crucial regulator of translational regulation (Gabut et al., 2020). Previous results obtained in the lab indicate that mTOR is not differentially activated during hepatogenic differentiation in cells undergoing the STD or HC3X protocol (Caruso et al., in revision). The fact that mTOR is not promoted during differentiation is somehow unexpected since mTOR is known to be repressed in the stemness maintenance and activated in cell fate decision (Gabut et al., 2020). Yet, although not stimulated during differentiation, Caruso and coworkers demonstrated that a basal mTOR activity was anyway required for hepatocyte differentiation since TOP mRNAs translation is largely dependent of the mTOR activity. TOP mRNAs family encompass transcripts coding for many components of the translation machinery, including all RNA proteins and several translation factors such as EEF1a and the Eukaryotic elongation factor 2 (EEF2) (Caruso et al., in revision). Their mTOR-dependent regulation is therefore needed for the translational regulation of mRNAs. Taken together, these results suggest that it would not be appropriate to target mTOR to study the effect of pIGF2BP2/3 on hepatogenic differentiation. Moreover, we can't exclude that other kinases may phosphorylate these proteins. Thus, rather than targeting kinases, it would make more sense to target directly the RBPs of interest by inducing the mutation of Serines to Alanines and thereby preventing their phosphorylation.

Regarding the model used, it is important to note that the progeny of HC3X-iPSCs will never be considered as true primary human hepatocytes (PHHs). Although it is a representative model of these primary cells, HC3X-iPSCs grow in a 2D in vitro environment, as a cell monolayer, which means that even if they adopt a maturation and a phenotype similar to PHHs, they do not have the capacity to become completely identical. Being in a single cell type culture, they do not have the capacity to interact with other cell types, such as those found in the liver, notably intrahepatic cholangiocytes, Kupffer cells, hepatic progenitor cells, hepatic sinusoidal endothelial cells and hepatic stellate cells. Yet, those interactions would improve their maturation. Indeed, sinusoidal endothelial cells represent a well-organized vascular matrix that provides the structural and biochemical environment in which liver cells live and interact (Marrone et al., 2016). It is through this particular structure that liver cells regulate each other precisely by secreting mediators such as peptides, hormones and cytokines, and help each other to reach complete maturation (Sato et al., 2019). Thus, although our model is representative of PHHs, it's an experimental model mimicking a portion of the biological process studied that sacrifice a part of reality. Therefore, a key perspective of this work would be to validate our results in improved models, such as matrix-guided three-dimensional (3D) organoids (Olgasi et al., 2020) or in in vivo models (Wei et al., 2021) where characterization of IGF2BP2/3 expression by immunohistochemistry in embryonic, fetal or adult livers would allow to determine to which extent our results are applicable.

In addition, it is important to note that the karyotype is strongly impacted in prolonged iPSCs culture. Several studies have demonstrated that over time, iPSCs acquire chromosomal abnormalities and changes in gene expression and cellular functions (Elliott et al., 2012). Since genomic alterations present potential risks in the application of iPSCs, it is important to monitor their genomic integrity. Karyotype analysis of iPSCs is the examination of chromosomal morphology. Through this analysis, changes in size, centromere position, and banding patterns are the main criteria studied and allow to deduce whether iPSCs have undergone genetic remodeling. In this Master thesis, it would have been best to karyotype our cells to ensure their

stability. However, karyotyping is an expensive, time-consuming, and complex analysis, and due to lack of time, could not be performed (Noto et al., 2014).

Finally, other RBPs potentially able to regulate hepatogenic differentiation could be studied. Although we were mainly interested in the IGF2BPs family, the Human antigen R (HuR) was also a potential candidate, which responded positively to our selection criteria. Indeed, the p-value of the MEME suite enrichment analysis was in each list < 0.05 and while transcriptomic analysis shows an increase of gene expression during differentiation, proteomic analysis shows a decrease of protein abundance comparing D0 vs D12 but there is a lack of information regarding D0 vs D20.

HuR is a ubiquitously expressed member of the ELAV (embryonic-lethal abnormal visual in Drosophila melanogaster) family of RNA-binding proteins. HuR is able to bind with high affinity and specificity to adenines and uracils-rich elements (AREs) usually found in the 3' UTR of a variety of mRNAs, such as those encoding the Vascular Endothelial Growth Factor (VEGF), p21, cyclin A, cyclin B1 and the glucose transporter type 1 (GLUT-1) and is believed to increase mRNA stability and mRNA translation (W. Wang et al., 2002). While the precise mechanisms regulating HuR function in mRNA stabilization remain largely unknown, researchers demonstrated that the mRNA-stabilizing influence of HuR requires its AMPKdependent translocation to the cytoplasm. In hepatocytes, AMPK activation after HGF stimulation has been shown to promote translocation of HuR from the nucleus to the cytosol, thereby stabilizing several cell cycle genes such as cyclins A2 and D1 and promoting hepatocyte proliferation (Gomez-Santos et al., 2012). Martinez-Chanter and coworkers have demonstrated that by inhibiting AMPK phosphorylation, HuR could not be transported to the cytoplasm and stabilization of target genes could not take place, thus blocking the HGF-induced proliferative response (Martínez-Chantar et al., 2006). The involvement of HuR is therefore necessary for HGF-induced liver growth.

Regarding cellular differentiation process, HuR is known to have a putative role in the differentiation of specific cellular lineages, such as myocytes, spermatocytes, and adipocytes, while its role in hepatogenic differentiation is currently investigated (Gomez-Santos et al., 2012). HuR critically influences the cellular S-Adenosylmethionine (SAM) content, the principal methyl group donor in the mammalian cell. Since its discovery, SAM has emerged as a key molecule that plays a central role in numerous hepatic processes. SAM and HuR were reported to execute a modulation on the proliferative response of hepatocytes as well as on the hepatic differentiation program, since the S-Adenosylmethionine content varies depending on the status of the cell, being lower in immature than in adult hepatocytes. Similarly, there is a predominantly nuclear localization of HuR in actively proliferating undifferentiated cells, but remarkably abundant in the cytoplasm during the induction of differentiation, returning to a nuclear presence at the end of the cell differentiation process. However, the mechanisms underlying this modulation remain to be discovered (Gomez-Santos et al., 2012). In order to deeply examine the functioning and regulation of HuR and to elucidate the signaling pathways involved, it would be interesting to use in vitro models, such as HC3X-iPSCs, which is capable of reproducing the physiological events related to hepatocyte differentiation.

## Conclusion

In the end of this Master thesis, we were able to identify two RBPs, IGF2BP2 and 3, potentially able to influence the translational regulation of specific transcripts during hepatogenic differentiation of HC3X-iPSCs. We had the opportunity to investigate their expression throughout the differentiation process and to test the effect of IGF2BP2 by silencing. The results were not as conclusive as expected as the transfection was not sufficiently efficient to conclude that IGF2BP2 contributes to hepatogenic differentiation marker AAT, which is an encouraging result regarding the impact of IGF2BP2 inhibition by siRNA on differentiation process. Therefore, even if this result needs to be confirmed and deepened in the future, it leads to numerous future perspectives that may one day allow us to understand the differentiation.

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