DOCTOR OF SCIENCES

Translational regulation of hepatogenic differentiation

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Translational regulation of hepatogenic differentiation

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Table of contents

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Abstract

Translational regulation is of paramount importance for proteome remodeling during stem cell differentiation both at the global and transcript-specific levels. Previous results generated by our group in a model of human bone-derived mesenchymal stem cells (hBM-MSCs) hepatogenic differentiation suggest that translational regulation could participate to hepatocyte differentiation. In this work, we took advantage of a more robust induced pluripotent stem cells (iPSCs) model to characterize translational remodeling during hepatogenic differentiation by polysome profiling. We demonstrate that protein synthesis increases during exit from pluripotency, and is then globally repressed during later steps of hepatogenic maturation. This global downregulation of translation is accompanied by a decrease in the protein abundance of components of the translation machinery, which involves a global reduction in translational efficiency of terminal oligopyrimidine tract (TOP) mRNA encoding translation-related factors. Despite global translational repression during hepatogenic differentiation, key hepatogenic genes remain efficiently translated, and the translation of several transcripts involved in hepato-specific functions and metabolic maturation are even induced. We conclude that, during hepatogenic differentiation, a global decrease in protein synthesis is accompanied by a specific translational rewiring of hepato-specific transcripts.

List of abbreviations

4EBP1	eIF4E-binding protein 1
4EBP1	eIF4E-binding protein 1
ABC	ATP-binding cassette
ACC1	acetyl-CoA carboxylase 1
ADH6	Alcohol dehydrogenase 6
AFP	Alpha-fetoprotein
AGO2	Argonaute
AHR	Aryl hydrocarbon receptor
AKT	Protein kinase B
ALD	Alcoholic liver disease
APP	Acute Phase Proteins
APR	Acute Phase Response
ARG-1	Arginase I
ASC	Adult stem cells
ASL	Arginosucinnate lyase
ASS	Arginosuccinate synthetase
AUF1	ARE/poly(U)-Binding/Degradation Factor 1
BFC	7-benzyloxy-4-trifluoromethylcoumarin
BM	Bone Marrow
BMP	Bone morphogenic protein
BSEP	Bile salt export pump
BSH	Bile salt hydrolase
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor
CCM2	Cerebral cavernous malformation 2
CDK1	Cyclin-dependent kinase 1
CK19	Cytokeratin-19
CPS-1	Carbamoyl phosphate synthetase I
CREB	cAMP response element-binding protein
DCA	Deoxycholic acid
DPF	Days post fertilization
ECM	Extracellular matrix
EEF	Eukaryotic elongation factor
EFNB1	EphrinB1
EGF	Epidermal growth factor
eIF4E	Eukaryotic initiation factor 4E
eIF4G1	Eukaryotic initiation factor 4G1
eIF4G2	Eukaryotic initiation factor 4G2
eIF4G3	Eukaryotic initiation factor 4G3
ELOV1	Elongation of very Long chain Fatty acids protein1

EMT	Epithelial to mesenchymal transition
ESCs	Embryonic Stem Cells
ESRRB	Estrogen Related Receptor Beta
FA	Fatty acid
FAS	Fatty acid synthase
FBP1	Fructose-1,6-bisphosphatase
FGF	Fibroblast Growth Factor
FGF19	Fibroblast Growth Factor 19
FGL1	Fibrinogen-like protein 1
FOXO1	Forkhead box protein O1
FXR	Farnesoid X receptor
G6Pase/G6PC	Glucose-6-phosphatase
GCK	Glucokinase
GH	Growth hormone
GLUT2	Glucose transporter 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
GOBP	Gene Ontology Biological function
GOCC	Gene Ontology Cellular Compartment
GOMF	Gene Ontology Molecular Function
GPBAR-1	G protein-coupled bile acid receptor 1
GRN	Gene regulatory network
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3
hBM-MSCs	Human Bone-marrow-derived mesenchymal stem cells
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HEG1	EGF- like domain containing gene heart of glass
HGF	Hepatocyte Growth Factor
HHEX	Hematopoietically expressed homeobox
hiPSCs	Human induced pluripotent stem cells
HLC	Hepatocyte-like cells
HNF-4α	Hepatocyte Nuclear Factor 4 Alpha
HNF1B	Hepatocyte Nuclear Factor 1B
HP	High polysome
HPC	Hepatic Progenitor Cell
HSC	Hepatic stellate cell
ICM	Inner Cell mass
IGF-1	Insulin-like growth factor 1
iPSCs	Induced Pluripotent Stem Cells
KEGG	Kyoto Encyclopedia of Gene and Genomes
KLF4	Kruppel-like factor 4

KO	Knock-Out
LaM	La module
LARP1	La-related protein 1
LCA	Lithocholic acid
LDL	Low-density lipoprotein
LDLR	LDL receptor
LXR	Liver X receptor
MDR1	Multidrug resistance protein 1
mESC	Mouse embryonic stem cell
MHC	Major histocompatibility complex
miRNAs	Micro RNAs
MMP-14	Matrix metalloproteinase 14
MRP2	Multidrug resistance-associated protein 2
MSCs	Mesenchymal Stem Cells
MSI	Musashi
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin complex 1
NAFLD	Non-alcoholic fatty liver disease
NANOG	Homeobox protein NANOG
NASH	Non-alcoholic steatohepatitis
NTCP	Na^+-taurocholate cotransporting polypeptide
OAT2	Organic anion transporter 2
OATP	Organic anion-transporting polypeptides
OCT1	Organic cation transporter 1
OCT4	Octamer-binding transcription factor 4
ORA	Over representatino analysis
ORF	Open Reading Frame
OSM	Oncostatin M
OTC	Ornithine transcarbamylase
PABP	Poly-A binding protein
PAMP	Pathogen associated molecular pattern
PAS	Periodic acid Schiff
PE	Primitive endoderm
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferative activated receptor-g co-activator-1α
PHH	Primary human hepatocytes
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKFL	6-phosphofructokinase liver isoform
PKLR	Pyruvate kinase isozymes R/L
PP2	Protein phosphatase 2

PPARα	Peroxisome proliferator-activated receptor α
PROX1	Prospero homeobox protein 1
PSC	Pluripotent Stem Cells
PUS7	Pseudouridine synthetase 7
PXR	Pregnane X receptor
PYGL	Glycogen Phosphorylase Liver form
RAPTOR	Regulatory-associated protein of mTOR
RBP	RNA-binding protein
ROS	Reactive Oxygen Species
RP	Ribosomal protein
RRM	RNA recognition motif
SCD-1	Stearoyl-CoA desaturase-1
SFRP5	Secreted frizzled-related protein 5
SOX2	SRY (sex determining region Y)-box 2
SREBP-1c	Sterol regulatory element-binding protein 1c
StarD1	Steroidogenic acute regulatory protein
SULT1A2	Sulfotransferase 1A2
T3	Triiodothyronine
TCF7L2	Transcription factor 7-like 2
TDO	Tryptophan 2,3-dioxygenase
TE	Translational efficiency
TEM	Transmission electron microscopy
TF	Transcription factor
TFR1	Transferrin Receptor 1
TGFα	Transforming growth factor-□
TIA1	T-cell intracellular antigen
TLR4	Toll-Like receptor 4
TOP	Terminal Oligopyrimidine Tract
UDCA	Ursodeoxycholic acid
uORF	Upstream Open Reading Frame
uPA	Urokinase plasminogen activator
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VLDL	Very Low-Density Lipoprotein
VMEL	Ventral midline endodermal lip
YY2	Yin-Yang 2

Introduction

This work has been focused on the interplay between two contemporary field of cellular and molecular biology corresponding to “Stem cells differentiation and development” and “Protein synthesis and translational control”. More precisely, experiments, results and conclusions described in this manuscript include the study of *in vitro* hepatogenic differentiation of cultured stem cells by investigations focused on the global and specific regulation of protein synthesis which finally led to the study of the translational regulation of a particular class of mRNAs: the TOP mRNAs. The goal of this introduction is therefore to lay the foundation for the understanding of the project by reviewing generalities about the liver structure and function and the global concept of translation as well as presenting the state of the art regarding liver development (with a particular emphasis on hepatocyte differentiation) and the mechanisms of translational control (with a focus on TOP mRNA translational regulation). The introduction finally describes the current discoveries regarding the role of protein synthesis and translational regulation in the stem cells field.

1. The liver: generalities

1.1. Structure

Accounting for a significative proportion of the adult body weight, the liver is the heaviest abdominal organ as well as the largest gland of the body. The plethora of biological roles performed by the liver relies on a very well-coordinated tissular organization.

- *Liver vascular and biliary trees*

Hepatic vasculature is unique since the organ receives a dual blood supply from both the hepatic artery, responsible for approximately 25-30% of oxygenated blood supply, and the portal vein, a venous valveless and low-pressure circuit connected in series with the gastrointestinal tract and the spleen, responsible for approximately 70-75% of nutritive blood supply.

At the histological level, arterial and portal venous blood enter the liver parenchyma through hepatic artery and portal vein and follow vascular tree divisions until reaching hepatic arterioles and portal venules, respectively. As explained later, the biliary tree structure is very similar and a bile duct thus lies close to hepatic arteriole and portal venule. Altogether, these three structures form, in the liver parenchyma, the “portal triad”, located in the portal space which is surrounded by a monolayer of hepatocytes constituting the limiting plate. Of note, portal space also contains lymphatic vessels and small nerves.

Branches of the hepatic arteriole and portal vein emerge and perforate the limiting plate to conduct blood in an anastomosing network of fenestrated capillaries called sinusoids (Fig. 1). Arterial and venous blood are thus mixed in sinusoids prior to drainage by a central venule carrying blood toward hepatic vein and, ultimately, toward inferior vena cava. Sinusoids are separated from each other by single rows of hepatocytes, the hepatocyte plates (or cords). Thereby, hepatocyte plates lie from the limiting plate toward the central venule.

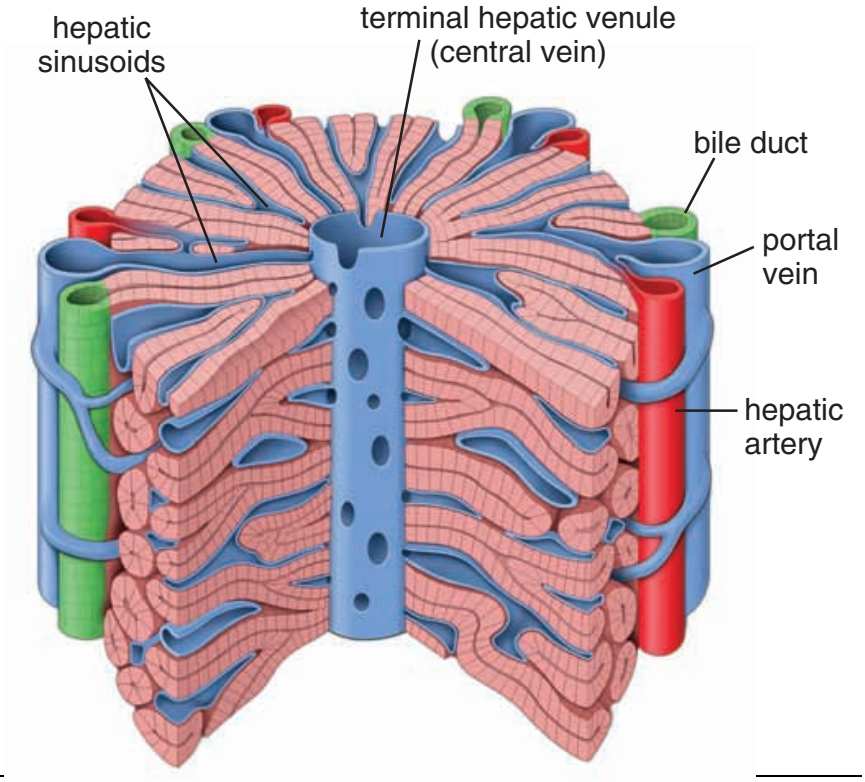

Figure 1: The liver lobule (from Histology, Wojciech Pawlina).
Schematic representation of the liver lobule showing the global organization of hepatic cords around the central vein with portal triads in periphery.

Liver sinusoidal endothelial cells constituting the sinusoids are not directly in contact with the sinusoidal surface of hepatocytes, defining a perisinusoidal space called Space of Disse. The Space of Disse corresponds to the bidirectional interface between hepatocytes and sinusoidal blood, allowing uptake of blood compounds by hepatocytes as well as secretion toward circulation. In addition, another cell type is also resident of the space of Disse: the hepatic stellate cells (also known as perisinusoidal cells or Ito cells).

Excepted for the sinusoidal surface (that accounts for 70% of the total hepatocyte surface), hepatocytes are lined by intercellular surfaces between adjacent hepatocytes of the plate. Some of these surfaces (apical membranes) form the bile canaliculus along hepatocyte cords. The bile secreted by hepatocytes will thus flow into the lumen of those canaliculi toward the canal of Hering. The canal of Hering crosses the limiting plate and allows the bile to be carried toward the bile duct in the portal space. Thus, the direction of bile flux along hepatocyte cords is opposite to the blood flow in the sinusoid capillaries. As the first histological structure lined by cholangiocytes (corresponding to a cell type specialized in bile synthesis), the canal of Hering is thus the starting point of the biliary tree.

- *Functional organization*

Different concepts defining the functional structure of the highly organized liver histology are classically used to describe the link with liver physiology (Fig. 2). First, the hepatic lobule

is a roughly hexagonal structure centered on a central vein draining surrounding sinusoids irradiating from different peripheric portal tracts. Second, the liver acinus describes the complete parenchymal structure receiving blood from a single terminal arteriole. The structure is thus centered on a sinusoid emerging from portal space toward central veinule. Interestingly, the acinus allows delimitation of three zones, named I, II, III, from portal space to the central vein. Mixed blood originating from terminal arteriole and portal veinule has the higher oxygen concentration in zone I while it progressively decreases with blood flowing toward next zones. This results in the so-called liver zonation that gives rise to hepatocytes functional specialization. Hepatocytes of the zone I synthetize glycogen and proteins while hepatocytes of the zone III are involved in detoxification processes. The specification from one end to the other is progressive, as observed in zone II hepatocytes that are characterized by intermediate phenotypes. Finally, the portal lobule, defined as a triangular structure centered on a portal triad, correspond to a biliary functional unit. Indeed, all the biliary canals included in the parenchyma of a portal lobule are converging toward the bile duct at the center of the lobule.

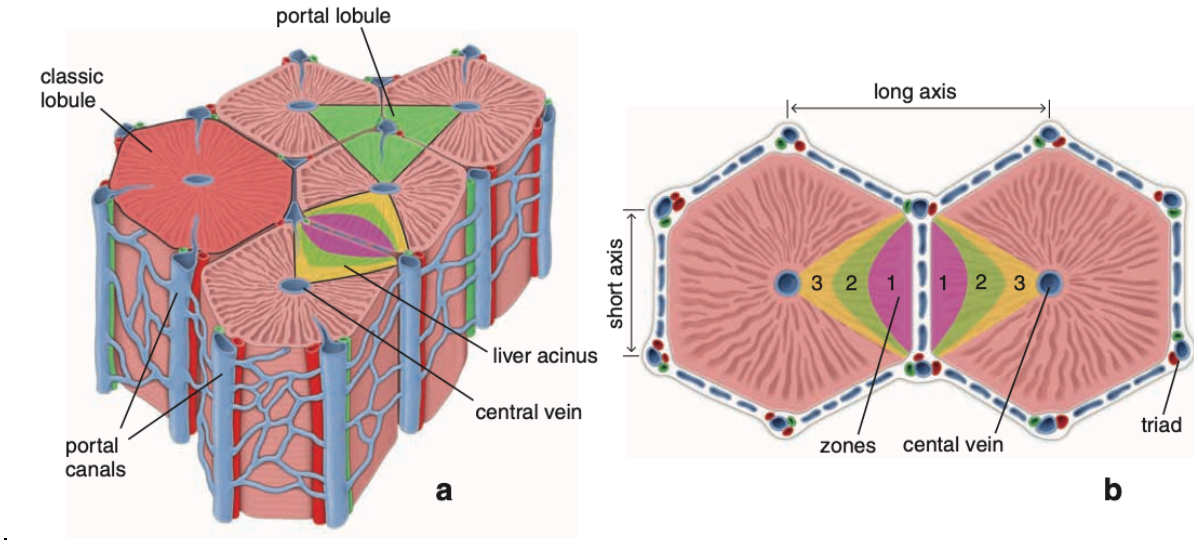

Figure 2: Functional structure of the liver lobule (from Histology, Wojciech Pawlina).
Schematic representation of the functional structure of the liver lobule showing (a) Different functional models are illustrated: the classic lobule, the portal lobule (centered on a central vein), the portal lobule (centered on a portal vein) or liver acinus (encompassing hepatic cords from a portal triad to a central vein). (b) Liver acinus colored for zone 1, 2 and 3 of liver zonation.

Of note, while those structural concepts constitute interesting supports for the comprehension of liver biology, there is no histological outlines of such structures *in vivo*.

1.2. The liver cell types

1.2.1. Parenchymal cells: hepatocytes

Hepatocyte is the main cell type of the liver with up to 80% of total liver mass of the organ. These cells present a typical cuboidal shape with a central nucleus, although some hepatocytes are binucleated or present polyploid nucleus characterized by 1 or 2 well defined nucleolus.

Hepatocyte ultrastructure is characterized by a predominant mitochondrial network, smooth and rough endoplasmic reticulum as well as glycogen and lipid inclusions.

As epithelial cells, they also present a polarized cytoplasm surrounded by specialized plasma membrane domains (Schulze *et al.*, 2019). Apical membrane presents ATP-binding cassette (ABC) and bile acid efflux transporters supporting the formation of bile canaliculus on these membranes. Basolateral membrane, the major part of hepatocyte membrane, functions as a sinusoidal interface presenting microvilli with surface protein channels and receptors such as EGF, low-density lipoprotein (LDL) receptor or transferrin receptor and bile acid uptake transporters. This structure supporting the different roles of hepatocytes requires an adapted highly efficient trafficking machinery including endocytic membranes, endosomes and a proper cytoskeletal architecture.

Characterization of hepatocytes at the "omic" level allowed identification of a group of 477 transcripts presenting increased expression in liver samples compared to other tissues of the body (Kampf *et al.*, 2014). Interestingly, numerous transcripts encoding plasmatic proteins secreted by the liver (such as Albumin) or mitochondrial proteins were part of the top 30 most abundant liver transcripts while Gene-ontology (GO) based overrepresentation analysis of this group of transcripts indicated a significative enrichment of the GO tree hierarchical level 2 terms "Response to stimulus", "Immune system process" or "metabolic process". This is in line with the fact that the liver is considered as the most metabolically active tissue of the body while being also able to globally regulate cellular functions based on external signals, and participate to immunity through plasmatic protein secretion. In addition, further analysis of enriched terms from all hierarchical levels of GO biological process (GOBP) or Kyoto Encyclopedia of Gene and Genomes (KEGG) database allowed to identify numerous hepato-specific biological processes such as "complement and cascade coagulation", "retinol metabolism" or "drug metabolism" as significantly enriched in the group of liver-specific transcripts.

1.2.2. Cholangiocytes

Cholangiocytes line the biliary tree from Canal of Hering to the end of bile duct in the duodenum. Cholangiocytes present a typical epithelial phenotype characterized by a polarized cytoplasm separating apical (luminal) from basolateral (vascular system and tight junctions) plasma membrane domains. The apical membrane surface is greatly extended by microvilli and hosts all the receptors and transporters required for bile homeostasis.

Cholangiocytes are functioning as key actors of bile homeostasis, not only by driving bile along the biliary tree, but also by regulating bile composition and actively contributing to bile production. Up to 20-30% of the bile volume is indeed produced by these cells while they only account for 5% of liver cell population. Beside this major function, cholangiocytes are also involved in liver immunobiology by secretion, in the bile, of IgA and several antimicrobial peptides (such as lactoferrin and cathelicidin) as well as by constitutively expressing Toll-like receptors responding to conserved PAMPs (pathogen associated molecular patterns) and used in case of biliary infections (Yoo, Lim and Choi, 2016; Banales *et al.*, 2019).

1.2.3. Non-parenchymal cell types of the liver

- *Liver sinusoidal endothelial cells*

Liver endothelial cells are the most abundant non-parenchymal cells of the liver and constitute a highly-fenestrated vascular endothelium with loosely organized cell junctions that drive the mix of arterial and portal blood through the liver sinusoids (Shetty, Lalor and Adams, 2018). Cells present small pores of 100 to 150 nm of diameter functioning as an interface between vascular lumen and space of Disse (Sørensen *et al.*, 2015). Importantly, these cells not only communicate with immune cells such as Kupffer cells, but they are also actively participating in blood clearance thanks to their highly active endocytic machinery. As an example, this allows to regulate lipoprotein traffic toward hepatocytes. Finally, they are also actively secreting signaling molecules impacting hepatocyte development and biology (see 2.1).

- *Hepatic stellate cells*

Hepatic stellate cells (HSCs) are resident mesenchymal cells of the space of Diss presenting Vitamin A-containing lipid droplets (Higashi, Friedman and Hoshida, 2017). These fibroblastic cells are resting in a quiescent state in physiologic conditions, while interacting via soluble mediators and cytokines/chemokines with hepatocytes, cholangiocytes, Kupffer cells and liver sinusoidal endothelial cells. Importantly, upon liver injury, signals from damaged hepatocytes or immune cells induce activation of HSCs toward myofibroblasts able to remodel the extracellular matrix as well as to secrete signaling molecules to promote liver regeneration (Yin *et al.*, 2013). However, chronic activation of HSC activation may drive to excessive ECM remodeling and collagen deposition. These first signs of fibrosis might further evolve toward cirrhosis and hepatocellular carcinoma.

- *Resident liver macrophages*

With a blood stream originating directly from intestinal tract, liver is the first target of ingested harmful materials. As a first line of hepatic defense, resident liver macrophages plays a major anti-inflammatory role by clearing immunoreactivity substances from sinusoidal blood (Dixon *et al.*, 2013). Upon different damaging conditions, resident liver macrophages can be activated and promote immune response. Indeed, like other macrophages, Kuppfer Cells can undergo M1 or M2 activation states upon stimulation by cytokines or microbial stimuli and this can lead to the secretion of pro- or anti-inflammatory cytokines. Importantly, those liver cells have initially been termed as Kuppfer Cells and considered as circulating monocytes originating from BM precursors that homed in the liver but advances in the field now provide a different view (Blériot and Ginhoux, 2019). Indeed, resident liver macrophages encompass a family of heterogeneous macrophages including Kupffer cells (corresponding to the vast majority of resident macrophages with a fetal origin) further complemented by variable amount of blood-recruited monocyte-derived macrophages as well as recruited peritoneal macrophages originating from previous inflammatory events.

1.3. Functions

"The hepatocyte is the favorite cell of the biochemist"
Prof. M. Jadot, Biochemistry class, University of Namur, 2012

Functions of the liver mostly rely on the ability of hepatocytes to function as metabolic factories. Liver will thus appear as the master regulator of energetic and homeostatic metabolisms through regulation of carbohydrates, lipids, proteins and amino acids metabolism. In addition, metabolic capacities of hepatocytes also confer the liver an important role in digestion (through bile synthesis) and detoxification of exogenous compounds. This part describes the main functions of adult human liver with a particular emphasis on carbohydrate metabolism.

1.3.1. Metabolism of Carbohydrates, lipids and amino acids

The liver can be considered as a real metabolic factory which ultimate goal is to ensure energetic and metabolic homeostasis of the body. This is highlighted by its particular vascularization (which is directly connected to digestive tract through portal circulation) together with the plethora of biosynthetic pathways supported by hepatocytes and the close endocrine communication network with other metabolic organs (such as muscle and adipose tissues, pancreas or thyroid). In line with this, a huge number of hepatocyte-specific and non-specific enzymes involved in glucose, lipid and amino-acid metabolism are expressed by hepatocytes, giving the liver numerous metabolic and energetic homeostasis functions. Those encounters the regulation of glucose and amino acid blood concentration, urea excretion or ketone bodies production.

- *Carbon metabolism in the hepatocyte (Lehninger Principles of Biochemistry, Seventh Edition)*

Although glycolysis (the 10 enzymatic step-conversion of glucose into pyruvate) is performed in all cell types, gluconeogenesis (the anabolic conversion of pyruvate and other 3- or 4-carbon compounds to glucose) is mainly performed in the liver, and to a lesser extent in the renal cortex and the intestinal epithelial cells.

Despite an apparent "bidirectional carbon backbone pathway", glycolysis and gluconeogenesis are not identical pathways running in opposite directions. Even if these two metabolic pathways share 7 enzymatic reactions, 3 glycolytic as well as 4 gluconeogenesis reactions are irreversible (underlined in Fig. 3). The regulation of the enzymes that catalyze these irreversible reactions allow the control of metabolic flux in hepatocyte.

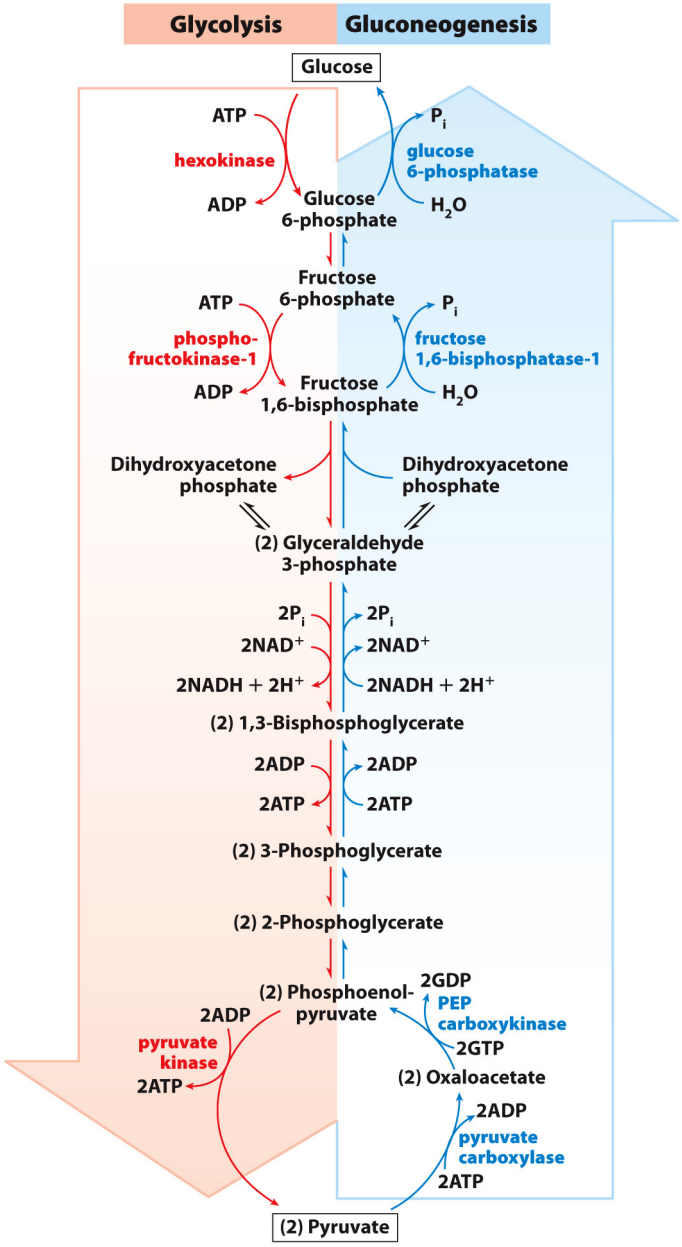

Figure 3: Glycolysis and gluconeogenesis biochemical pathways (Lehninger Principles of Biochemistry, Seventh Edition).

Schematic representation of glycolysis and gluconeogenesis biochemical pathways. Specific enzymatic reaction of the glycolysis and gluconeogenesis are underlined in red and blue, respectively.

Importantly, this "bidirectional backbone pathway" is closely related to many other metabolic pathways. Glucose-6-phosphate connects this backbone to the bidirectional glycogen pathway (whose purpose in the liver and muscular tissue is either to store glucose as glycogen, or to catabolize glycogen to produce glucose) and to the pentose-phosphate pathway (which aims is to synthetize precursors for nucleotides and aromatic amino acids). Dihydroxyacetone phosphate connects the backbone to the glycerol metabolism in liver and adipose tissue. Finally, pyruvate catabolism into Krebs cycle intermediates connects this backbone to the metabolism of amino acids and fatty acids (which, together with glycerol,

connects the backbone to the triglyceride metabolism).

Importantly, despite glycolysis is a ubiquitous biochemical pathway, several key enzyme isoforms are specifically expressed in the liver (although sometimes present in other tissues, such as glucokinase which is present in pancreatic β cells). Altogether, the regulation of rate-limiting steps of connecting biochemical pathways as well as the regulation of specific enzymes of glycolysis and gluconeogenesis allow the hepatocyte to control carbon and energetic homeostasis.

The liver is also in charge of the regulation of glycemia (i.e. blood glucose concentration) which requires uptake and storage of nutritive glucose during the post-prandial phase (when glycemia is increasing higher than the normal concentration range) as well as hepatic glucose production during fasting (when glycemia is decreasing below the normal concentration range) (Postic, Dentin and Girard, 2004; Raddatz and Ramadori, 2007). The glucose homeostasis is based on the pancreatic β cells – hepatocyte interaction in which β cells act as sensor of blood glucose concentration and trigger endocrine secretion of insulin (when high glycemia is detected) or glucagon (when low glycemia is detected). Both these hormones have opposite effects on the regulation of glucose metabolism pathways in the hepatocytes. Result of these regulations allows to regulate glucose concentration in hepatocyte that, through its bidirectional diffusion via GLUT2 (glucose transporter 2), regulates glycemia.

- *Hepatocytic lipid metabolism* (Postic, Dentin and Girard, 2004; Liu *et al.*, 2017)

Lipid metabolism is closely related to energetic metabolism since lipids can both be used as energetic substrates or as energy storage molecules. Hepatocytes, as major regulators of energy homeostasis, play an important role in lipid metabolism by regulating fatty acid, ketone bodies, and triacylglycerol synthesis and degradation.

Acetyl-CoA, which is obtained through oxidative decarboxylation of pyruvate and can be used as substrate for Krebs cycle, is also a metabolite that connects lipid and carbohydrate metabolism. Indeed, acetyl-CoA is a precursor for liver *de novo* fatty acid synthesis and allows the hepatocyte to store energy upon excess of glucose (after high-carbohydrate diet). Enzymes involved in these pathways are acetyl-CoA carboxylase 1 (ACC1) that converts acetyl-CoA to malonyl-CoA and the fatty acid synthase (FAS) that grafts malonyl-CoA on the acetyl-CoA primer (or the neosynthesized $_{-2C}$fatty acid). This allows synthesis of palmitate, the most abundant fatty acid with 16 saturated carbons. Additional enzymes such as the acyl-CoA elongase family (Elov1) or desaturases (such as stearoyl-CoA desaturase-1, SCD-1) also participate in the elongation or desaturation of fatty acids. Finally, fatty acids can be stored in the hepatocytes as triacylglycerols, corresponding to esters of 3 fatty acids and glycerol. Of note, this further connects lipid and carbohydrate metabolism since glycerol can be interconverted to dihydroxyacetone, a glycolysis-gluconeogenesis intermediate metabolite. Fatty acids and triacylglycerols can be shared between hepatocytes and adipocytes through blood circulation after being packed with proteins and secreted as Very Low-Density Lipoprotein (VLDL). Additionally, digestive lipolysis allows enterocytes to import fatty acids and to transfer them toward hepatocytes via portal circulation as chylomicrons containing triacylglycerol, cholesterol and proteins. Oppositely, during a fasting phase, fatty acids are

catabolized through β-oxidation in mitochondria (excepted for long-chain fatty acid whose oxidation occurs in peroxisomes) in order to produce energy. First, oxidation itself produces reduced coenzymes (NADH and $FADH_2$) that can feed the oxidative phosphorylation to produce ATP. Secondly, the acetyl-CoA generated by oxidation can enter the Krebs cycle as substrate and generate reduced coenzymes. Finally, in a context of low carbohydrate availability, fatty acids are catabolized toward acetyl-CoA which serves as precursor for ketone bodies acetoacetate and β-hydroxybutyrate (this also promotes the production of acetone from spontaneous breakdown of acetoacetate). Ketone bodies are secreted by hepatocytes and serves as energetic substrate by peripheric organs (such as heart, nervous tissue and renal cortex).

Regulation of hepatocytic lipid metabolism is mediated by two key transcription factors: Peroxisome proliferator-activated receptor α (PPARα) and SREBP1-c. While the first controls key enzymes of fatty acid oxidation pathways, the second induces expression of enzymes involved in fatty acid synthesis such as ACC1, FAS and SCD-1. Importantly, SREBP-1c is induced by insulin, which regulates connected carbohydrate and lipid metabolism.

- *Amino acid metabolism*

Unlike carbohydrates and lipids, amino acids are not stored in cells and the amino acid homeostasis consists in the tight regulation of the circulating pool of free amino acids in the body (Schutz, 2011). Factors influencing this pool are: amino acid intake from food, amino acid metabolism (amino acid catabolism and utilization of its end-products as precursors for other biosynthetic pathways and non-essential amino acid synthesis) and the protein turnover (i.e. release of amino acid from degraded proteins, or amino acid consumption for protein synthesis). Among these processes, the use of amino-acid catabolites as precursors for biosynthetic pathways and the amino-acid catabolism are specific to the hepatocyte, making the liver a key regulator of amino acids homeostasis.

The amino acid catabolism requires transamination followed by deamination. This is mostly performed through reversible aminotransferase activities that transfer an amino group from a donor amino acid to a recipient 2-oxoacid, giving a new 2-oxoacid (from donor) and a new amino acid (from recipient) (Liu *et al.*, 2017). Thereby, many amino acid catabolic pathways use 2-oxoglutarate as amino group acceptor, forming glutamate. Glutamate can then be deaminated in the mitochondria by glutamate dehydrogenase, releasing ammonia (see next paragraph for further explanation about ammonia metabolism through urea cycle). On the other hand, the neosynthesized 2-oxoacid is then catabolized toward end-products that connect the amino acid catabolism to other metabolic pathways. Most amino acids (excepted lysine and leucine) are glucogenic, meaning that their catabolism leads to glycolysis or Krebs cycle intermediate metabolites, which allows their use as precursors for gluconeogenesis in the liver. Similarly, amino acids whose catabolism produces acetyl-CoA are considered ketogenic since acetyl-CoA can be used as precursor for ketone bodies synthesis (or more largely, lipid biosynthesis) in the liver (for example, the 2-oxoacid of alanine is pyruvate).

The hepatic amino acid metabolism is reported to be regulated at two different levels. First, at short-term, the metabolism is regulated by substrate abundance. Second, at long-term, amino acid uptake and metabolism is controlled hormonally. Glucagon, more than simply being the counter part of insulin in glucose homeostasis, is an important activator of amino acid

catabolism (as well as urea cycle) and participates in a feedback loop regulating circulating amino acid concentrations (Thiessen, Gunst and Van Den Berghe, 2018; Janah *et al.*, 2019).

- *Urea cycle* (Wang, Ran and Jiang, 2014)

An additional function of liver is the metabolization of ammonia, the nitrogenous waste of amino-acid catabolism toward urea. Indeed, the urea cycle allows the transformation of ammonia, an alkaline and cytotoxic compound, to urea, $CO(NH_2)_2$, a polar, charge neutral, water soluble molecule that is delivered in blood stream prior elimination by the kidney in the urine.

The cycle more generally participates to the body nitrogen balance. The metabolic pathway is cyclic (Fig. 4) and starts in mitochondria with a first rate-limiting enzyme carbamoyl phosphate synthetase I (CPS-1) which synthetizes carbamoyl phosphate from ammonia, CO_2 and H_20. Carbamoyl phosphate is then integrated in the cycle through its reaction with ornithine to produce citrulline (catalyzed by ornithine transcarbamylase, OTC). Citrulline is exported in the cytosol and used by arginosuccinate synthetase (ASS, the cytosolic rate-limiting enzyme) together with aspartate (that brings the second amino group) to synthesize arginosuccinate. Then, arginosuccinate lyase (ASL) catalyzes the transformation of arginosuccinate in arginine and fumarate. Importantly, these two reactions are connecting the urea cycle to several other metabolic pathways such as the Krebs cycle (since fumarate is an intermediate metabolite of the cycle) and glucose metabolism (since aspartate is part of the malate-aspartate shuttle, which carries reducing equivalents through mitochondrial membrane through interconversion of malate and aspartate to oxaloacetate, respectively, allowing to connect glucose metabolism and Krebs cycle). Finally, urea exits the cycle through transformation of arginine to ornithine, a reaction catalyzed by arginase I (ARG-1). While urea cycle cytoplasmic enzymes are present in several other tissues in order to synthetize entities such as polyamine, hepatocytes (and to a lesser extend enterocytes) are the only cells to express all enzymes of the cycle.

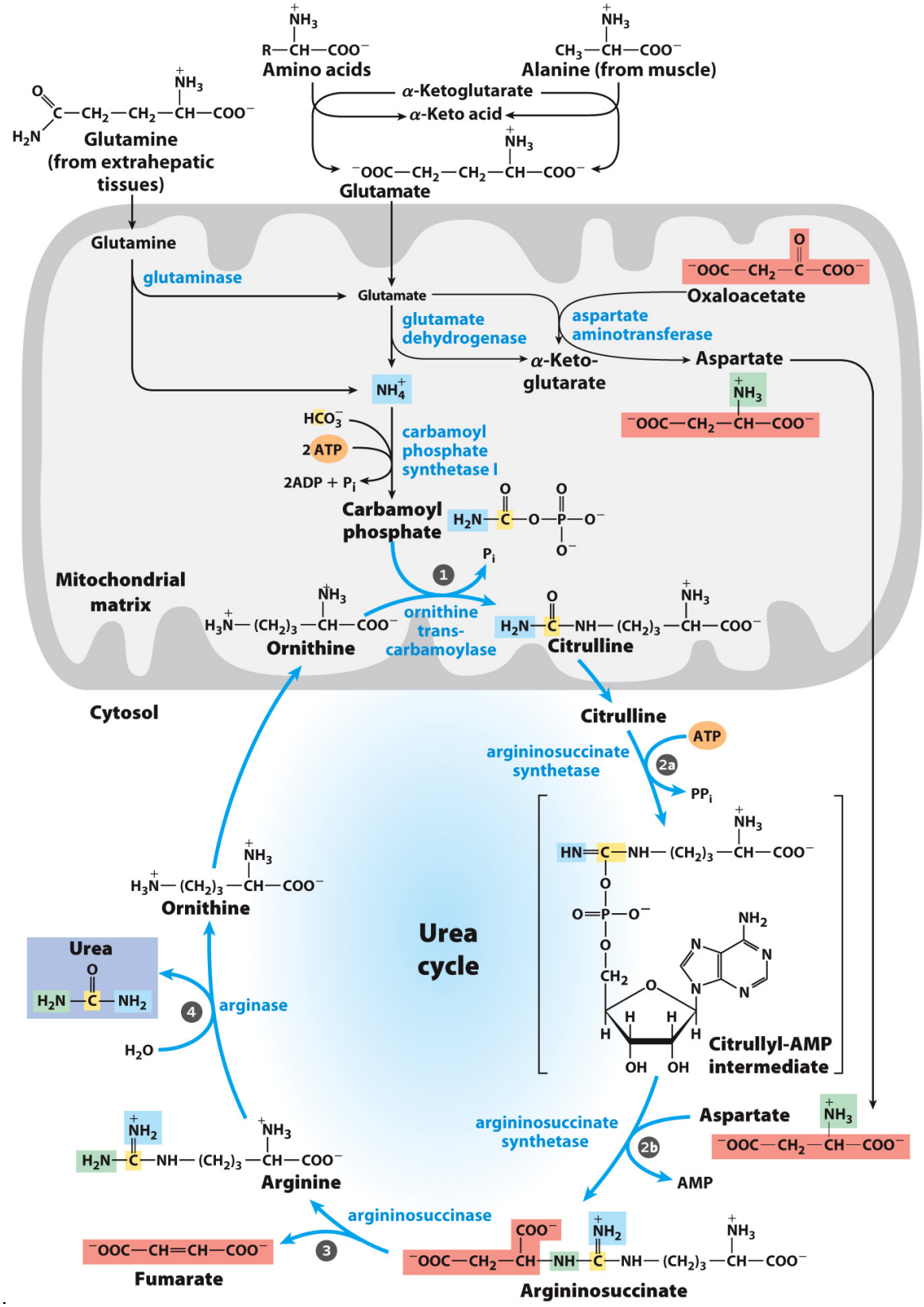

Figure 4: Urea cycle (Lehninger Principles of Biochemistry, Seventh Edition).
Biochemical pathways of the urea cycle. The complete cycle is indicated by blue arrows.

Multiple regulations of the urea cycle are exerted by hormones affecting the level of key

enzymes of urea cycle such as CPS-1, OTC, ASS and ARG-1. Thereby, insulin globally reduces urea synthesis rate while glucagon and glucocorticoids have opposite effects. Of note, several liver pathologies such as cirrhosis, hepatoma or fatty liver diseases are responsible for a decrease in urea cycle enzyme expression and a subsequent defect in circulating ammonia regulation.

1.3.2. Bile synthesis and secretion (Di Ciaula *et al.*, 2017; Liu *et al.*, 2017; Chiang and Ferrell, 2018)

Bile secretion is a hepatocyte-specific function that allows the liver to undergo a variety of endocrine, exocrine and metabolic functions. Bile is mainly composed of cholesterol-derived bile acids excreted by active transport in bile canaliculus by hepatocytes (via canalicular bile salt export pump, BSEP) as well as bilirubin and cholesterol itself. Bile is conducted into the biliary tree, the common hepatic duct and cystic duct before reaching the gallbladder where it is concentrated and stored. After meal intake, secretion of cholecystokinin by the pancreas induces contraction of the gallbladder and secretion of bile through common bile duct toward duodenum. In the upper intestinal tract, a small portion of bile acids are passively absorbed while most of bile acids are actively reabsorbed in the ileum. This allows hepatocytes to recover approximately 95% of secreted bile acids through portal circulation. The remaining 5% of bile acids are either lost in feces (0.5g/day) or spilled over into systemic circulation (0.5mg/day) and finally excreted into urine. Parallelly, an equivalent of approximately 0.5g/day of bile acids are *de novo* synthetized in hepatocytes to maintain a constant bile acids pool of 3g. This *de novo* bile acid synthesis from cholesterol corresponds to the main pathway for the cholesterol catabolism.

Two different bile acid synthesis pathways co-exist in the adult liver: the classic and alternative bile synthesis pathways, both composed of different CYP-450 and non-CYP450 enzymes. The classic pathway includes several cholesterol modifications in the mitochondria and in the peroxisome, relying on several enzymes for the formation of chenodeoxycholic acid and cholic acid. Besides, the alternative bile synthesis pathway (also called acidic pathway) converts cholesterol to different oxysterols by CYP27A1. CYP27A1 is ubiquitously expressed and localized in the inner mitochondrial membrane and the pathway is therefore not completely restricted to hepatocytes. These oxysterols are important signaling molecules that regulate cholesterol and lipid metabolism but are also cytotoxic for the cells if accumulated.

Prior to secretion, both chenodeoxycholic acid and cholic acid are conjugated to glycine or taurine on C_{24}, giving T/G-chenodeoxycholic acid and T/G-cholic acid. These modifications allow to an increase of bile acid ionization, amphipathic properties and solubility; bile acids are thus forming Na^{2+} salts. Additional conjugations to glucuronides and sulfates are also performed in hepatocytes prior to secretion. Importantly, primary conjugated bile acids that reached the digestive tract are unconjugated by gut microbial bile salt hydrolase (BSH) and further converted to secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA) or ursodeoxycholic acid (UDCA), by other gut bacterial enzymes.

Through bile synthesis, liver takes part in a large set of functions related to digestion, gut microbiota or cholesterol, lipid and glucose metabolism. Of note, bile is also the excretion pathway of bilirubin and some metabolized drugs.

1.3.3. Drug metabolization (Almazroo, Miah and Venkataramanan, 2017; Liu *et al.*, 2017)

In the human body, a large set of enzymes mainly expressed in hepatocytes, together with the portal vascular system, confers the liver a central role in xenobiotics clearance (i.e. elimination of compounds that are not synthetized by the organism itself). This is mainly done through modification of lipophilic compounds to more hydrophilic molecules, facilitating their excretion into the bile or by the kidneys. Since they need to pass through the portal system and liver before reaching the systemic circulation, orally administered drugs undergo a first row of metabolization, reducing their bioavailability. This is known as the "first pass effect". However, certain chemicals designed as prodrugs take advantage of this effect since liver metabolization converts them to their pharmacologically active metabolites prior release in bloodstream, increasing their bioavailability.

Drug metabolization can be regarded as a three-phase process. Phase I enzymes involve basic chemical modification of the drug, Phase II enzymes conjugate hydrophilic endogenous compounds of the phase I metabolites and Phase III transporters export the metabolites out of the cells. Most common Phase I enzymes are part of a large superfamily of proteins: the cytochrome P450 protein family. More than 115 genes coding for CYP proteins (from CYP1A1 to CYP51P3) mainly located in endoplasmic reticulum and mitochondria are part of the superfamily. The most abundant CYP450 proteins in human liver are CYP3A4 (approx. 22%), CYP2E1 (15%) and CYP2C9 (15%). These proteins with different substrate affinities catalyze different reactions such as oxidation or reduction while non CYP450 phase I enzymes can catalyze hydroxylations. Of note, some non CYP450 enzymes are also part of the Phase I enzymes such as alcohol dehydrogenase, responsible for oxidation of ethanol to acetaldehyde. Enzymes of the Phase II metabolism are known as "transferases" and involve, among others, glucuronidation, sulfation, acetylation and methylation. Finally, Phase III transporters include ATP-binding cassette (ABC, relying on ATP for the uptake or efflux of the drug or its metabolites through cell membrane) and solute carrier (SLC, which couples the transport of solutes to those of solutes or ions drove by their electrochemical gradients). In the liver, these transporters serve for the uptake of drugs into hepatocytes: main uptake transporters are Na^+-taurocholate cotransporting polypeptide (NTCP), organic cation transporter 1 (OCT1), organic anion transporter 2 (OAT2) and organic anion-transporting polypeptides (OATP1B1, OATP1B3, OATP2B1). In addition, they also serve for the efflux out of the hepatocytes (toward bloodstream or bile): multidrug resistance protein 1 (MDR1, also known as P-glycoprotein, P-gp or ABCB1), bile salt export pump (BSEP, or ABCB11) and multidrug resistance-associated protein 2 (MRP2 or ABCC2).

Regulation of drug-metabolizing enzymes is under control of xenosensors, i.e. transcription factors coordinating the expression of phase I, II and III enzymes after substrate recognition (Köhle and Bock, 2009). The mechanisms can involve binding of xenobiotics to the transcription factor in cytoplasm inducing its translocation to nucleus and its subsequent target-gene activation. Three sensors are generally described: Ah receptor (AHR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR).

1.3.4. Plasma protein secretion (Kuscuoglu *et al.*, 2018)

Hepatocytes are also responsible for secretion of plasma proteins and liver will thereby produce approximately 10-20g of plasma proteins daily. On one hand, this requires an efficient protein production machinery, including folding, processing and trafficking. While in another hand, it can lead to important protein accumulation upon dysfunction of the proteostatic machinery.

Plasma proteins cover a large set of functions and thus can be classified as carrier proteins, homeostatic proteins and hormones. The first group is largely the most abundant in the blood and includes proteins helping the transport of different compounds such as Albumin (the most abundant plasma protein accounting for 55% of total plasma proteins) which binds fatty acids, hormones and drugs but is also very important in osmotic regulation of blood, and Transferrin that binds and carries iron and apolipoproteins responsible for formation and transport of blood lipid particles. The group of homeostatic proteins includes coagulation cascade factors such as fibrinogen (that polymerizes after cleavage to form fibrin clots), protease inhibitors (such as α_1-antitrypsin (an inhibitor of several serine proteases) and complement factors (C1 to C9) (that are part of the innate immune system and play different immunologic roles such as induction of phagocytosis or bacteria cell wall rupture). Finally, as an endocrine gland, the liver also secretes different hormones such as hepcidin and thrombopoietin that regulate iron metabolism and platelet production, respectively.

1.3.5. Iron homeostasis

Liver is responsible for systemic iron homeostasis (Schulze *et al.*, 2019). Since the only source of iron is dietary, ingested iron is absorbed and stored by hepatocytes and macrophages through the transferrin system. Circulating iron binds to the hepatocyte secreted circulatory protein Transferrin and is internalized by hepatocyte via the ubiquitous Transferrin Receptor 1 (TFR1). After being transported out of endosomes, multiple atoms of iron are bound to Ferritin for storage. Additionally, hepatocytes are (together with erythroid cells) also expressing TFR2, a low-affinity Transferrin receptor playing a sensing role for global circulating iron. Upon high circulating levels, TFR2 promotes import and storage of iron by hepatocytes and macrophages.

1.4. Liver regeneration

In order to produce plasma proteins and play multiple roles as hematological filters, Hepatocytes require to be literally bathed in a mix of arterial and veinous blood. This particularity also put liver at risk of damaging from excessive exposure to fat, alcohol, drugs, toxins as well as host pathogens (hepatitis viruses most particularly). Hepatocytes are indeed among the first cells exposed to all the compounds absorbed by the gut, regardless of whether they are nutritious or toxic.

Different pathological conditions can lead to a common set of liver pathological manifestations which can eventually cause death (Zhou, Zhang and Qiao, 2014). Indeed, liver pathologies represents approximatively 4% of all deaths worldwide during years 2010 and 2016 (with Europe presenting the largest burden of those conditions) (Byass, 2014; Naghavi *et al.*, 2017; Pimpin *et al.*, 2018; Xiao *et al.*, 2019).

Upon various threats such as drug induced (including alcohol) hepatocyte toxicity,

hepatitis viral infection or fatty liver disease and metabolic syndrome, liver will encounter common pathological features. Chronic exposure to these factors firstly leads to chronic inflammation triggering progressive liver fibrosis mediated by the activation of HSCs toward myofibroblast-like cells that participate in ECM remodeling and excessive collagen deposition. Fibrosis is also sustained by progressive defenestration and capillarization of sinusoidal endothelium, as well as by Kupffer cell activation. Chronic liver diseases can further evolve toward cirrhosis which includes degeneration and necrosis of hepatocytes, progressive replacement of liver parenchyma by fibrotic tissues, and continuous inflammation ultimately leading to liver failure. Finally, the continuous progress of chronic liver disease also constitutes a favorable environment for development of hepatocellular carcinoma (HCC).

Homeostasis of liver mass and functions is tightly regulated during mammalian adult life in order to maintain a liver to body weight ratio and constant liver function (Michalopoulos and Bhushan, 2021). Additionally, particular physiological and pathological conditions can regulate the liver mass such as pregnancy, or partial hepatectomy. Rat livers are indeed capable of retrieving normal hepatic mass after up to two-thirds of liver removal (Higgins and Anderson, 1931), while livers transplanted from smaller to bigger animals (baboon-to-human) increase in size (Starzl *et al.*, 1993). This homeostatic process aiming at maintaining liver function has been termed "hepatostat" (Michalopoulos, 2007).

The purpose of liver regeneration is to retrieve a sufficient amount of hepatic functions by compensating the loss of hepatocytes occasioned by acute or chronic liver disease (reviewed in (Kiseleva *et al.*, 2021)). This can be achieved by two different mechanisms: cellular hypertrophy (consisting in the increase of remaining hepatocytes size toward 150% of normal hepatocyte size) and hyperplasia (which corresponds to cell proliferation). Hyperplasia itself is variable since newly populating liver cells can originate from different sources. Indeed, typical liver regeneration is characterized by phenotypic fidelity, which means that each cell type of the liver can proliferate and make similar cell types. Alternatively, in certain conditions, liver regeneration will rather be ensured by the differentiation of Hepatic Progenitor Cells (HPCs) that give rise to hepatocytes. During liver regeneration, contribution of both processes is believed to be dictated by the nature and magnitude of the liver damage (Ko *et al.*, 2020). Thus, HPCs differentiation is only involved in massive acute liver injuries or chronic liver diseases when proliferation of hepatocytes is blocked.

Partial hepatectomy has been widely used as a model for the "typical liver regeneration", allowing the description of a multistep process (reviewed in (Kiseleva *et al.*, 2021; Michalopoulos and Bhushan, 2021)). Typical liver regeneration starts with a priming phase triggered by portal pressure increase (a result of the immediate decrease in liver mass) and an increase in urokinase plasminogen activator (which converts plasminogen to plasmin). This allows the breakdown and remodeling of liver ECM through the activation of metalloproteinases. Importantly, ECM remodeling allows the release of different factors in the bloodstream, including Hepatocyte Growth Factor (HGF), which ultimately leads to a transition of the hepatocyte cycle from phase G1 to S. This phase in which mitogens and auxiliary mitogens induce parenchymal cell proliferation, is also characterized by the secretion of pro-mitotic cytokines (vascular endothelial growth factor (VEGF), angiopoietins 1, 2, transforming growth factor-α (TGFα), Fibroblast Growth Factor 1 and 2 (FGF1, FGF2) and granulocyte-macrophage colony-stimulating factor (GM-CSF)) by proliferating hepatocytes to

support the proliferation of other liver cell types When initial liver mass is recovered, hepatocytes gradually acquire the terminally differentiated quiescent phenotype and ECM is restored.

Beside the phenotypic fidelity of the typical liver regeneration, particular pathological conditions can also trigger alternative regenerative pathways. The precise mechanisms of those alternative pathways are still debated in literature; interested reader can refer to (Michalopoulos and Khan, 2015) for further details. The existence and precise characterization of bipotent "hepatic progenitor cells" is of central importance regarding alternative liver regeneration in which failure in hepatocytes or cholangiocytes regeneration by proliferation results in the need for alternative cell sources. Suppression of hepatocyte proliferation triggers the expansion of progenitor's cells from the ductular regions (a process called ductular reactions). The precise origin of HPCs remains debated, although different hypotheses have been proposed and probably co-exist *in vivo*, including trans-differentiation to HPCs from cholangiocytes, hepatocytes or resident progenitor cells of the liver.

2. Development of the liver

The development of the liver during embryogenesis and fetal life in mammals is a process aiming at (1) the organization of tissue morphogenesis with all required cell types and correct histological structures from organ bud and (2) the cell fate specification and differentiation of the hepatic cell types. This chapter will therefore review the *in vivo* liver development including early embryogenesis, liver morphogenesis and hepatocyte differentiation prior description of key concepts of stemness and *in vitro* modelling of hepatocyte differentiation.

2.1. *In vivo* liver development

This section introduces key concept of early embryogenesis taking place before the onset of liver development prior to a more in-depth description of liver development.

2.1.1. Overview of early embryogenesis

Early embryogenesis is a complex process aiming at recreating, from only two gametes, an embryonic organism with a collection of precursor tissues for latter formation of a complete adult.

- *Establishment of the primary germ layers adapted from (Solnica-Krezel and Sepich, 2012; Vijayraghavan and Davidson, 2017; Wamaitha and Niakan, 2018)*

First step of development starts when two gametes, the oocyte (the female gamete) and sperm cell (the male gamete) meets in the mother fallopian tube in a process called fertilization that yields the "zygote". At this step, the zygote relies exclusively on maternal mRNA for translation. This allows major genome reorganization that ultimately leads to totipotency, an important feature required for the complete development of a new individual (see next section

for further detail about potency). Pioneering work in the field of developmental biology paved the way for such a concept by showing the possibility to form developing embryos after somatic nuclear transfer into zygotes (Gurdon, 1962).

The onset of zygote cell division giving rise to the 2-cell and 4-cell stage, respectively, arrives at day 1 and 2 post-fertilization (1, 2 DPF), and is thus followed by mitotic divisions that increase the number of cells in the embryo, without increasing the total amount of cytoplasm (as shown by the fact that embryo size remains unchanged at this step) (Fig. 5). This led to a progressive decrease in cell size accompanied by an increase in nuclear to cytoplasm ratio. Importantly, at 2-2.5 DPF, maternal to zygotic transition is achieved and consists in the embryo genome activation. Combination of cell cleavage without growth and increase in tight-junctions of the blastomeres (the cells of the embryo) triggers compaction of the embryo around the 8/16-cell stages (3-4 DPF) that leads to the formation of the morula stage.

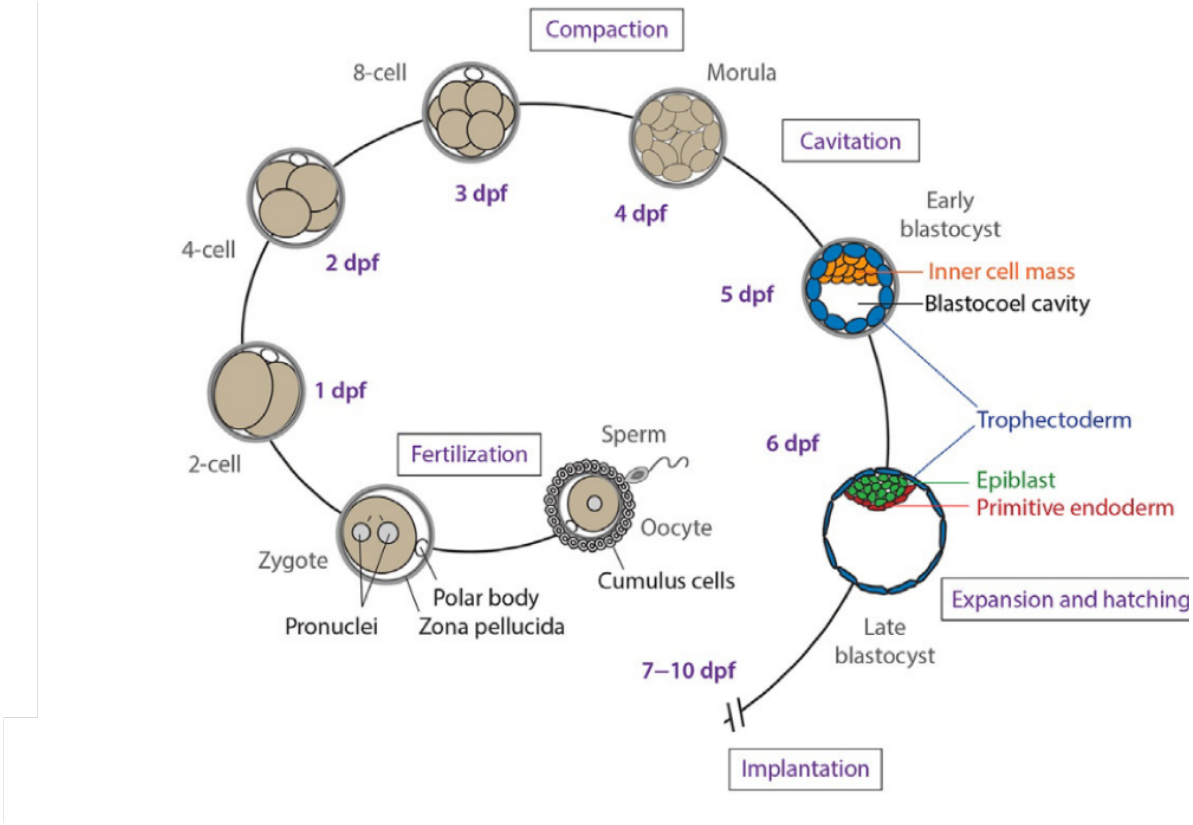

Figure 5: Human preimplantation development (adapted from Wamaitha and Niakan, 2018). Schematic representation showing the first step of human development from fertilization to implantation.

At 3 DPF (8/12-cell stage), the blastomeres starts to polarize and present apical domains with microvilli and gap junctions. The polarization is further followed, at the 8/16-cell stage by asymmetrical cell division leading to the formation of two cell populations: outer cells conserve the apical domain, which allows them to further reestablish polarity and inner cells which remain apolar. This constitutes the first event of cell fate decision in development. Importantly, both cell populations will further segregate during the next blastocyst formation: embryo will induce cavitation, at 5 DPF, forming a central fluid-filled cavity (the blastocoel)

containing the Inner Cell Mass (ICM, with embryonic progenitors) and surrounded by an outer layer of cells of the trophoectoderm. Between 5 and 6 DPF, ICM will further segregate by achieving the second cell fate decision of embryo. This generates the primitive endoderm as a single layer constituting the border between ICM and blastocoel, and the pluripotent epiblast progenitor cells (surrounded by primitive endoderm on one side, and by trophoectoderm on the other).

After blastulation and subsequent segregation of embryonic and extra-embryonic cell lineages, the blastocyst is ready for implantation, a process of nesting in uterine endometrium (7-10 DPF). Blastocyst will first proceed with "Hatching" defined as the emergence of blastocyst from the zona pelucida, the glycoprotein layer surrounding it prior to apposition and attachment to the endometrium and invasion of uterine by trophoectoderm. This is further followed by proliferation and differentiation of trophoectoderm cells leading to the formation of two functionally distinct layers: the cytotrophoblast, a single sheet of cells surrounding extraembryonic mesoderm, and the syncytiotrophoblast, formed by the fusion of external trophoectodermal cells toward a multinucleated tissue which constitutes the primary interface between the embryo and the mother. Finally, epiblast will cavitate to form the primitive amniotic cavity, while cavitation of primitive endoderm leads to formation of the yolk sac. Together, these events produce a bilaminar disk constituted by the embryonic disk (epiblast epithelium) lying on the PE endoderm (called hypoblast). The embryonic disk is thus the source of pluripotent stem cells that will become the future individual.

Gastrulation, the next step of development consists in the establishment of the three germs layers namely endoderm (embryonic precursor of thyroid, lungs, pancreas and liver), mesoderm (embryonic precursor for a large variety of cells and tissues including smooth, cardiac and muscular muscle, kidney, reproductive organs, connective tissue, bone, cartilage, dermis, blood cells, …) and ectoderm (which is the precursor for neuroectoderm and surface ectoderm will ultimately will give rise to nervous system and skin, respectively) (Fig. 6) (Ghimire *et al.*, 2021). This is also the moment for determination of antero-posterior, dorso-ventral and left-right axes. Gastrulation starts with the formation of a groove progressively invading epiblast from the caudal side and cranially to the middle of the embryo. This structure that allows determination of symmetry axis of the embryo is called primitive streak. A node is formed at the end of the primitive streak and progressively extend caudally. This constitutes a gateway for internalization of epiblasts cells undergoing epithelial to mesenchymal transition (EMT) prior to delamination and migration down/into the primitive streak. Those mesenchymal cells will therefore colonize and replace the hypoblast to form the first germ layer: the endoderm. Similarly, cells migrating from epiblast will thus fill the space between epiblast and endoderm to form the second germ layer: the mesoderm. Finally, remaining cells of the epiblast constitutes the third germ layer: the ectoderm, in which primitive streak will progressively decrease and completely disappear.

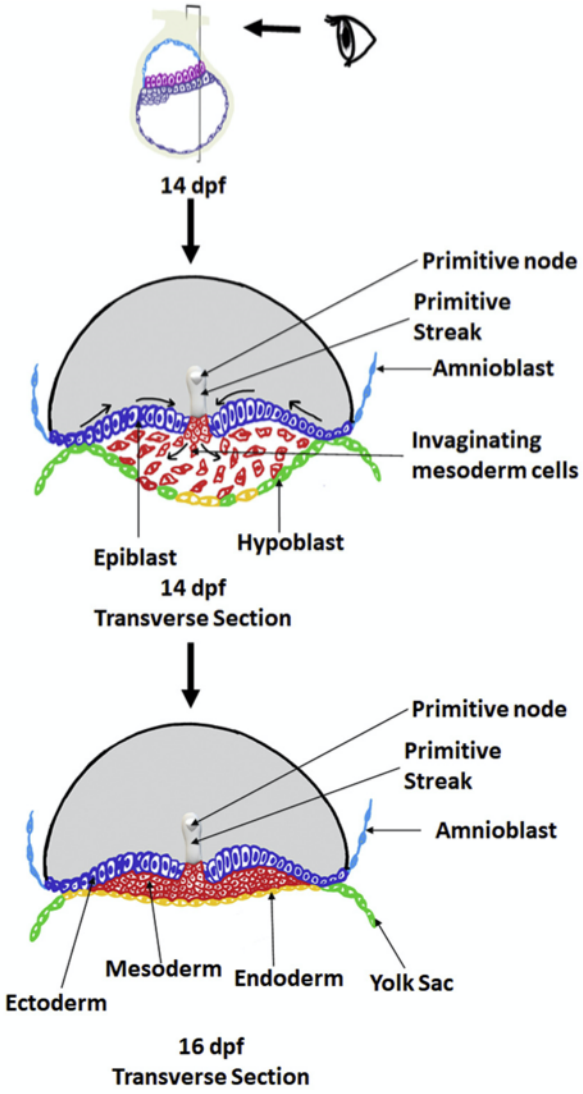

Figure 6: schematic representation of the human gastrulation (adapted from Ghimire et al., 2021)

Views represents transversal sections through the human embryo at indicated dpf during gastrulation. Migration of invaginating mesoderm cells are indicated as well as the formation of the three germ layers

The next steps of embryonic development encompass formation of the notochord (a longitudinal structure that will serve both as a mechanic support of development and as a signaling actor participating in the establishment of cranio-caudal axis), and the somites from mesoderm (those allows the segmentation of the body), as well as neurulation (formation of the neural plate from ectoderm and its subsequent folding toward a neural tube).

- *Gut tube formation and endoderm patterning* (Zorn, 2008)

Parallelly with the establishment of the germ cell layers and embryo primitive structures, the embryo will undergo different folding events resulting in the conversion of definitive endoderm from a disk to a tube. At this step, the amniotic cavity continues to grow while yolk sac does not. This induces the progressive engulfment of the complete embryo by amniotic

cavity bringing ectoderm all around the embryo. This movement brings a part of the yolk sac surrounded by definitive endoderm inside the embryo that will be the future gut tube. The epithelial gut tube is surrounded by mesoderm which allows, through different secreted factors, patterning of the gut tube on the cranial-caudal axis. This gave rise to three different gut regions: namely foregut, midgut and hindgut.

2.1.2. Liver morphogenesis

Liver morphogenesis is a complex process allowing the establishment of a fetal liver including all hepatic cell types with the appropriate histological structure. *In vivo* study of human liver morphogenesis is currently not possible regarding ethical consideration linked to the use of human embryos for research. This led scientists to develop different models allowing the study of mammal liver development. Mice will be the reference model used to run through liver morphogenesis in this work, unless stated otherwise.

- *Liver progenitor cells*

The liver is mainly composed of parenchymal hepatocytes and cholangiocytes differentiated from a common bi-potent progenitor called hepatoblast. Hepatoblast originates around E8.25 from bilateral populations of lateral endoderm cells merging at the ventral midline during the process of gut tube formation (Fig. 7) (Gualdi *et al.*, 1996; Ober and Lemaigre, 2018). A second population of progenitors arises from the ventral midline endodermal lip (VMEL) and largely contributes, in mice and chicken at least, to the liver. However, the participation of these cells in liver formation has not been confirmed in other mammals. Both cell populations give rise to different parts of the liver: lateral endoderm epithelial cells will respond to FGF signaling to form the posterior part of liver bud while VMEL epithelial cells will respond to Bone Morphogenic Protein (BMP) signaling, move caudally and contribute to the anterior part (Wandzioch and Zaret, 2009a; Wang *et al.*, 2015). Hepatoblasts of the foregut epithelium are lined by a basement membrane surrounded by mesenchymal cells.

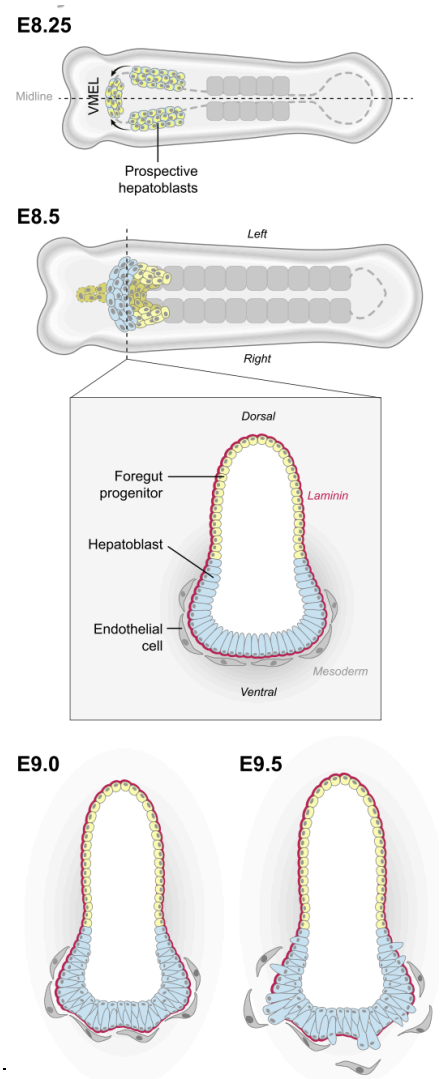

Figure 7: First steps of liver development (Ober et al., 2018).
Schematic representation of the first step of mouse liver development showing specification of hepatoblast from progenitors.

- *Organ bud morphogenesis*

The next steps of organ bud morphogenesis is triggered by a combination of morphogenetic processes including (1) cell shape changes, (2) cell proliferation and (3) migration of hepatoblasts (Ober and Lemaigre, 2018). Thanks to signaling from adjacent mesoderm, the cuboidal foregut endoderm thickens toward a columnar epithelium and then toward a pseudostratified epithelium of hepatoblasts. The epithelium thickening occurs synchronously with the triggering of hepato-specific markers expression by hepatoblasts such as AFP or HNF4α. Additionally, hepatoblasts forming the pseudostratified epithelium proliferate with nuclei entering S-phase at the cell basal side followed by mitosis after nuclear migration toward apical cell side (Bort *et al.*, 2006). The epithelium finally breaks down and hepatoblasts delaminate and invade the adjacent septum transversum mesenchyme. This migration is promoted by nascent endothelial cells originating from mesodermal cells surrounding the hepatoblast basement membrane, which will ultimately differentiate toward functional blood

vessels of the liver (Matsumoto *et al.*, 2001). Such delamination and migration require different processes such as loss of contact between hepatocytes (mediated from downregulation of E-Cadherin) or ECM-remodeling systems (expression of metalloproteinase (MMP-14) for example) allowing liver expansion and growth (Margagliotti *et al.*, 2008). Altogether, the hepatoblast morphological specification, proliferation and migration are necessary steps for liver outgrowth.

Importantly, organ bud morphogenesis is also the developing step ensuring onset of liver asymmetry. While precise mechanisms governing liver asymmetry in mammals are not well understood, two simultaneous processes participating in asymmetry have been identified in zebrafish (Ober and Lemaigre, 2018). First, communication between hepatoblasts and the adjacent right lateral plate mesoderm mediated by hepatoblast transmembrane protein EphrinB1 (EfnB1) and its receptor EphB3b expressed by mesodermal cells, is required for hepatoblast migration and establishment of asymmetry (Cayuso *et al.*, 2016). Second, asymmetric morphological changes of the right and left lateral plate mesoderm participate in liver asymmetry, potentially by bringing mechanical forces that further drive hepatoblasts migration. During this step, the left lateral plate mesoderm migrates dorsally to the hepatoblast endoderm while the right lateral plate mesoderm moves ventrolaterally (Horne-Badovinac, Rebagliati and Stainier, 2003).

- *Organ development*

Liver bud formation is followed by organ growth, which will ultimately produce the definitive lobes of the liver. Proliferation and differentiation patterns of growing liver chicken showed that localized growth zone allows formation of the lobes with increased proliferation at the periphery of the lobe, and reduced proliferation at the center. Additionally, an inverse distribution is observed concerning differentiation, since hepatoblast maturation will progressively decreases toward periphery of the lobe during organ growth (Ober and Lemaigre, 2018).

Importantly, growing bud is also an important site of fetal hematopoiesis (a liver function that is lost in adult life). Haematopoietic cells are indeed colonizing the liver bud at E10.5 and accompany hepatocytes proliferation to produce blood cells.

- *Hepatic parenchyma maturation*

At the histological level, the mouse liver parenchyma is composed of a mixture of bipotent hepatoblasts and fetal hematopoietic stem cells until E17-21, followed by a progressive decline of fetal hematopoietic stem cells (Ober and Lemaigre, 2018). This is further accompanied by formation of hepatic cords associated with an increase in cellular adhesion of the hepatic cells and the formation of short bile canaliculi. An important feature of such hepatic parenchyma maturation is the differentiation of non-polarized bipotent hepatoblasts toward fully polarized hepatocytes, which allows at E18, the formation by apical membranes of the canalicular network connected to bile ducts (Tanimizu *et al.*, 2016). Different mechanisms participate in the establishment of polarized hepatic epithelium structure.

Expansion and maintenance of hepatic cords require a controlled mechanism of cell proliferation allowing preservation of the apical polarity axis along the epithelium, thus

different mechanisms of hepatocytes proliferation have been reported (Ober and Lemaigre, 2018). First, the asymmetric hepatocyte division segregating apical membrane to produce a non-polarized daughter cell that can undergo polarization and form de novo apical domain with its mother cell (Slim *et al.*, 2013). Second, the symmetric division of hepatocytes dividing their apical membrane has also been observed *in vitro* in micro-niches, although whether this mechanism participates in liver development *in vivo* is not yet known (Li *et al.*, 2016).

More than proliferation mechanisms, ECM signaling is also an important controller of hepatic epithelium polarity and structure. During mid-gestation stages, ECM is mainly composed of fibronectin, laminin, collagen type I and IV (while fibronectin and laminin levels decrease after birth) (Baloch *et al.*, 1992). Communication with ECM through integrins is therefore required for suitable parenchyma organization, with several important signaling pathways of hepatocyte differentiation converging toward β1-integrin (Weinstein *et al.*, 2001).

Finally, an important controller of hepatic microanatomy structure is the bi-cellular interaction between differentiating hepatoblasts and sinusoidal endothelial cells. In zebrafish, regular disposition of hepatocytes is regulated by *cerebral cavernous malformation 2* (*ccm2*) and *EGF- like domain containing gene heart of glass* (*heg1*) expressed by endothelial cells (Sakaguchi *et al.*, 2008). On the other side, secretion of VEGF by hepatoblast of the early developing liver attract a cells of the endocardium of the sinus venosum to form a third of the endothelium lining the sinusoid (Ober and Lemaigre, 2018).

2.1.3. Hepatocyte differentiation

At the cellular level, hepatocyte differentiation is a key process playing roles in liver macro and micro structure formation as well as in liver functional maturation. To better understand hepatocyte differentiation, biologists have extensively studied the intercellular communications and signaling effectors responsible for hepatocyte specification or maturation as well as the gene regulatory networks (composed of a network of transcription factors regulating the progression toward mature hepatocyte and expression of hepatocyte-specific genes) controlling cell fate decisions (Gordillo, Evans and Gouon-Evans, 2015; Gérard, Tys and Lemaigre, 2017).

2.1.3.1. Regulatory gene network regulating *in vivo* hepatocyte differentiation

- *Hepatic competency*

Hepatic specification is the initiation step of hepatic gene expression. This could be described as a two-step process consisting first in the acquisition of competence for hepatic gene expression from endodermal and second, in the setting up of a signaling pathway network orchestrating hepatic gene expression (Gérard, Tys and Lemaigre, 2017).

At the step of differentiation preceding hepatic specification, ventral foregut cells of the posterior domain of the midline of embryo trigger the expression of the pioneer transcription factors GATA4/6 and Forkhead box (Fox)A1/A2 (also called Hnf3a and Hnf3b). GATA4 and 6 are zinc-finger transcription factors with largely overlapping functions (while also presenting

non-redundant functions as demonstrated by the differential lethality age of KO mouse embryos) (Arceci *et al.*, 1993; Borok, Papaioannou and Sussel, 2016). FoxA transcription factors (TFs) encompass FoxA1/2/3 that also presents redundancy. Interestingly, as pioneer factors, these TFs expressed in definitive endoderm cells have the ability to bind DNA and trigger chromatin opening. This makes these factors participating in the decompaction of chromatin at hepatic-specific loci (Cirillo *et al.*, 2002; Lee *et al.*, 2005). FoxA2 has been reported to displace the linker histone H1, keeping enhancer nucleosomes accessible on hepatic-specific genes (such as AFP) while their expression is not yet induced (Xu *et al.*, 2012; Iwafuchi-Doi *et al.*, 2016). GATA-4 is indeed the first factor to bind to the very compacted Albumin gene in endoderm cells, which further allows the enhancer to be available for binding to transcription factors (Bossard and Zaret, 1998). Altogether, these mechanisms allow endoderm cells to become competent for hepatic specification.

- *Hepatic specification*

The next step of hepatic specification, which corresponds to the definitive endoderm cell fate decision toward hepatic lineage is induced by signaling of adjacent cells of the lateral mesodermal plate. The goal of this step is to trigger intracellular signaling pathways converging toward expression of hepatic specific genes which DNA conformation has been opened during hepatic lineage competency acquisition. Fibroblast Growth Factors (FGFs) secreted by adjacent cardiac mesoderm, Bone Morphogenic Proteins (BMPs) secreted by adjacent septum transversum mesenchyme cells and Wingless-type MMTV integration site (Wnt) proteins have been reported to play such roles during hepatic specification (Jung *et al.*, 1999; Deutsch *et al.*, 2001; Rossi *et al.*, 2001).

Precise identification of the role of each individual FGF protein remains elusive, partly because of the supposed relative functional redundancy between members of the family. However, FGF1 and FGF2 can successfully replace physiologic signaling for specification, which is further emphasized by the observation that FGF drives hepatic cell fate commitment in evolutionary distinct species (Jung *et al.*, 1999; Gérard, Tys and Lemaigre, 2017). Importantly, definitive endoderm cells of the ventral foregut also give rise to lung cells upon cell fate decision mediated by similar FGF signaling (Serls *et al.*, 2005). The concentration of FGF, potentially varying with the proximity with mesoderm, is the determinant of lung-specific versus liver-specific gene expression as shown by the *in vitro* induction of Albumin at lower FGF concentration (50 to 500ng/ml) and inhibition of albumin expression together with lung marker Nkx2.1 at higher concentrations (500-1000 ng/ml). Finally, microarray analysis of the effect of FGFR inhibitor PD161570 on differentiating hiPSCs-derived endoderm allowed to identify the RAS-RAF-MAPK and PI3K-AKT pathways as acting downstream of FGFR, further confirming the previously observed role of MAPK signaling actors ERK1/2 in Albumin expression (Calmont *et al.*, 2006; Twaroski *et al.*, 2015). This study identified approximately 40 early genes whose expression was controlled by FGF and involved in different biological processes such as cell differentiation and development including several TFs and naked cuticle homolog 1 (NKD1), an inhibitor of Wnt canonical pathway that binds to Disheveled (DVL1).

BMP signaling originating from septum transversum induces activation of Smad1,5,8 that are able to bind to Smad4 to participate in hepatic gene expression such as Alb or Prox1 in

competent endodermal cells (Wandzioch and Zaret, 2009b). Interestingly, BMP signaling also induces DNA remodeling during hepatic specification through recruitment of P300 (an histone acetyltransferase) on hepatic genes by Smad4 (Xu *et al.*, 2011).

The Wnt signaling pathway is a key signaling pathway involved in different aspects of hepatic differentiation, including foregut endodermal specification. However, inhibition of the canonical Wnt/β-catenin pathway is required for hepatic specification since overactivation of the pathway suppresses the induction of hematopoietically expressed homeobox (Hex) transcription factor required for endoderm maintenance and hepatic specification (Wild *et al.*, 2020). The expression of Wnt antagonist secreted frizzled-related protein 5 (Sfrp5) has been identified in *xenopus* embryos as participating in the silencing of the pathway (Li *et al.*, 2008). This has also been confirmed in mouse and human ESCs, while co-culture with endothelial cells indicated that those could be responsible for Wnt inhibition (Han *et al.*, 2011). In line with those results, our team previously reported the participation of a transcriptional pathway characterized by a downregulation of Wnt/β-catenin effector Transcription factor 7-like 2 (TCF7L2) in modulating the expression of PGC1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) during hepatic specification of hBM-MSCs *in vitro* (Wanet *et al.*, 2017). Interestingly, this further allows PGC1α to connect the mitochondrial biogenesis associated with metabolic shift observed during differentiation with signaling pathways controlling differentiation (Wanet *et al.*, 2014).

These pathways converge toward the expression of several key TFs that are specific of hepatoblasts such as GATA4, FOXA2, Hepatocyte nuclear factor 4α (HNF4α), HNF1α, HNF1β, HNF6, HHEX, Prospero homeobox protein 1 (PROX1). Those factors are deeply interconnected with mutual induced expression creating a network that promote the expression of the specific hepatic proteins during the final maturation steps.

- *Liver bud morphogenesis*

Following hepatic specification, hepatoblast will accomplish the hepatic bud growth previously described and characterized by thickening of epithelium and cell migration toward adjacent mesenchyme. At the molecular level, key TFs Hhex, Prox1, Hnf6 (Onecut-1) and Onecut-2 (OC-2) are required for this step, as shown by the impaired delamination of hepatoblast upon conditional suppression of these genes (Sosa-Pineda, Wigle and Oliver, 2000; Bort *et al.*, 2006; Margagliotti *et al.*, 2007). The mechanisms precisely linking those transcription factors with this step of development need further investigation, but it is tempting to hypothesize that they control ECM remodeling necessary for cell migration (Zorn, 2008). Prox1 has indeed been shown as regulating many MMPs, which suppressed activity is responsible for the inhibition of *in vitro* hepatoblast migration (Papoutsi *et al.*, 2007; Margagliotti *et al.*, 2008). Finally, prior vascularization of the liver bud at E9, hepatoblasts are in close contact with endothelial precursors (themselves lying on septum transversum). Communication from these cells also participates in liver bud morphogenesis, as shown by the impaired migration of hepatoblasts in embryo lacking endothelial cells (through mutation of vascular endothelial growth factor receptor gene Vegfr-2) (Matsumoto *et al.*, 2001).

- *Liver bud growth*

The liver bud growth observed during E9.5 and E15 is triggered, at the molecular level, by several signaling pathways. First, beside their role in earlier process of liver development, FGF and (through the PI3 kinase pathway) BMP and HGF signalings promote liver bud growth (Jung *et al.*, 1999; Calmont *et al.*, 2006; Berg *et al.*, 2007). Additionally, Wnt/ß-catenin signaling pathway, while repressing hepatoblast specification, promotes liver bud growth. TGFß ligands are also expressed in liver bud and embryos bearing depletion of Smad2 and Smad3 show hypoplastic liver.

- *Hepatocyte maturation*

Finally, around E13, bi-potent hepatoblasts expressing both hepatocyte (such as HNF4α, Albumin) and cholangiocytes (cytokeratin-19 (CK19)) as well as fetal liver markers (α-fetoprotein (AFP)) start to differentiate toward mature hepatocytes and cholangiocytes, the last cell fate decision of the liver development. At the molecular levels, Oncostatin M (OSM), HGF, glucocorticoid hormones have been shown to promote hepatocyte maturation.

Oncostatin M is an interleukin-6 family cytokine secreted by CD45+ hematopoietic cells of the fetal liver. This allows, paracrine signaling pathways involved in morphological (including tight-junctions formation), genetic and functional maturation of hepatocytes (Kamiya *et al.*, 1999; Imamura *et al.*, 2007). Hepatoblast gp130 is the receptor for OSM and signaling pathways involving Stat3 or K-Ras have been identified as mediating the pro-differentiation effect of OSM (Ito *et al.*, 2000; Matsui, Kinoshita, Morikawa, *et al.*, 2002). Stat3 promote cyclins D1, D2 and D3 repression during maturation, which results in proliferation arrest of developing hepatocytes (Matsui, Kinoshita, Hirano, *et al.*, 2002). HGF signaling through C-met receptor similarly promotes expression of hepato-specific markers (Kamiya, Kinoshita and Miyajima, 2001). Finally, glucocorticoid dexamethasone has also been shown to induce the expression of several key hepatic TFs such as HNF4α or C/EBPα while suppressing hepatocyte growth (Michalopoulos *et al.*, 2003).

At the transcriptional level, key hepatic TFs such as HNFs or FoxAs are already expressed in bipotent hepatoblasts. The goal of hepatocyte maturation is therefore to reinforce and stabilize the hepatocyte-specific transcriptional network constituted by HNF4α, HNF1α, HNF6, HNF1ß, FoxA2 and C/EBPα as well as their targets' gene expression (Fig. 8) (Gérard, Tys and Lemaigre, 2017). This stabilization of the GRN is believed to be mediated by an increase in cross-interactions observed between TFs (the fact that key hepatic TFs stimulate each other's transcription). Pioneering work in this field indeed showed that promoter region of those genes are increasingly occupied by the liver GRN TFs along differentiation of hepatocytes (Kyrmizi *et al.*, 2006). HNF4α appears even more important for hepatocyte maturation, as seen by its increased recruitment in nearly all of the key hepatic TFs during differentiation. Importantly, positive feedback loop between TFs and micro-RNA miR-122 (an hepato-specific microRNA) also reinforce expression of key hepatic TFs, as shown by the miR-122-mediated induction of HNF4α and FoxA1 in differentiating ESCs (Deng *et al.*, 2014). The stabilization of the GRN in hepatocyte is thus responsible for the expression of hepatic genes

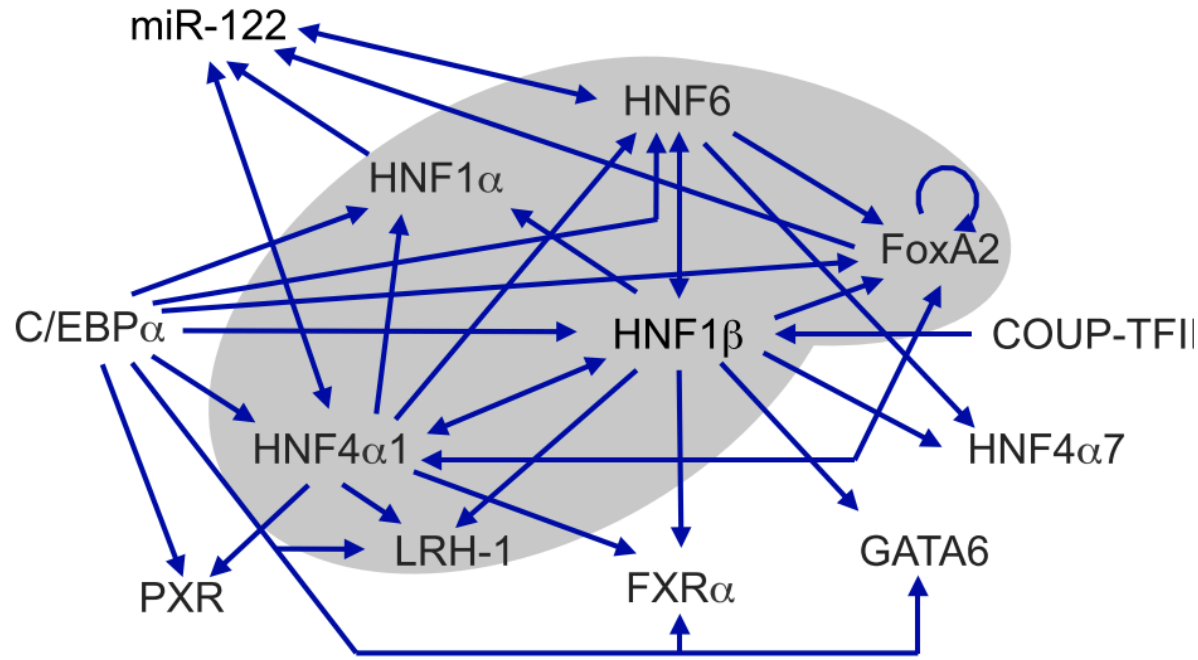

Figure 8: Gene Regulatory Network of differentiating hepatocyte (Gérard et al., 2017).
Schematic representation of the GRN in E18.5 mouse differentiating hepatocytes showing the formation of a stable network of transcription factors mutually stabilizing hepatocyte gene expression.

2.2. *In vitro* hepatocyte differentiation

2.2.1. Stem cells

As observed during early embryogenesis, development requires both cell proliferation and commitment toward cell lineages, a critical role ensured by stem cells and defined by their ability of self-renewal and cellular differentiation. Both these particular abilities of stem cells make them the master cell type of development during embryonic and fetal lives but also in tissue maintenance and regeneration during adult life. Various types of stem cells can be classified according to their potential for commitment to cell lineage (called potency) or according to their origin.

2.2.1.1. Potency

The potency is defined as the ability of stem cells to fate toward a new fully differentiated cell type (reviewed in (Weissman, 2000; Loh, Lim and Ang, 2015). During development, stem cells will progressively commit to cell lineage in a progressive differentiation process which is accompanied by gradual restriction of the potency for downstream lineage. Therefore, this progression defines different stages of potency: Totipotent stem cells are able to differentiate toward embryonic and extra-embryonic tissues (such as placenta). *In vivo*, totipotent stem cells include the zygote and the first cells of the blastocyst prior the initial cell decision segregating

ICM and TE cells. Next, ICM further giving the epiblast is composed by pluripotent cells that retain potency to differentiate toward all three germ layers. Those pluripotent cells are thus very interesting for clinical and research use since they can virtually be differentiated toward any adult cell type. Following gastrulation, pluripotent stem cells will be committed to one of the three germ layers, giving rise to endodermal, mesodermal and ectodermal progenitors considered as multipotent stem cells since they can only further differentiate in multiple cell types of a common lineage. Finally, end of development and adult life are characterized by progenitors simply able to self-renew and differentiate in a single cell type mainly for tissue homeostasis; those are called unipotent. Throughout development this concept can be seen as a hierarchical tree starting from the totipotent cell and further dividing at each cell fate decision toward fully differentiated cells.

2.2.1.2. Origin of stem cells

Stem cells have various origins and this dictates the potency status of the cell type. This feature makes stem cells of different types largely unequal regarding their self-renewal (defined by the parallel proliferation and maintenance of similar potency level) or differentiation potential (Loh, Lim and Ang, 2015). Altogether, this brings specific advantages and disadvantages for the use of certain stem cell types for various applications.

- *Embryonic Stem Cells*

The *in vitro* culture of pluripotent cells starts in 1981 upon successful isolation and maintenance of mouse embryonic stem cells (mESCs) (Evans and Kaufman, 1981; Martin, 1981). Pluripotency of these cells originating from mouse blastocyst ICM, was shown as both presenting the capacity to self-renew in culture as well as the ability of teratoma formation *in vivo*.

A key element of our comprehension of pluripotency encompasses the deciphering of the gene regulatory network (GRN) responsible for pluripotency maintenance (reviewed in (Loh, Lim and Ang, 2015). Current hypotheses suggest that several key TFs including NANOG, OCT4 and SOX2 constitute germ layers specifiers able to stimulate differentiation toward a germ layer while inhibiting commitment to the two other germ layers. Altogether, subtle equilibrium of TFs is required for maintenance of undifferentiated state still retaining the potential to engage toward any lineages.

Finally, regarding precise characterization of the potency state of ESCs, it should be noted that it is now well established that phenotypes of pre-implantation and post-implantation ESCs differ, further segregating this stem cells' family. Both types have been called "Naïve ESCs" (pre-implantation) or "Primed ESCs" (post-implantation) following the observation that during the first steps of development, a ground state (Naïve state) is firstly established followed by cell preparation toward germ layers commitment (Primed state) (Nichols and Smith, 2009).

- *Induced pluripotent stem cells*

Together with the improvement of our comprehension and culture capacity of pluripotent ESCs, many efforts have been made to set up somatic cell reprogramming toward pluripotency.

In 2006, Yamanaka and Takahashi, encouraged by prior observation of the fact that differentiated nucleus can be successfully reprogramed when injected in an egg cytoplasm, successfully identified TFs able to restore pluripotency in fibroblast upon TFs overexpression (Gurdon, 1962; Takahashi and Yamanaka, 2006). Indeed, exogenous expression of POU5F1, Kruppel-like factor 4 (KLF4), SRY (sex determining region Y)-box2 (SOX2) and proto-oncogene c-MYC (MYC), called the OKSM cocktail or Yamanaka factors allowed acquisition of the so-called "Induced Pluripotent Stem Cells" (iPSCs). iPSCs are morphologically indistinguishable from ESCs, present similar *in vitro* differentiation capabilities and participate in the development of an embryo when injected in the ICM.

Advantages offered by the reprogramming of somatic cells are numerous and include: non-invasive and ethical collection of cells (compared with the need to harvest blastocyst for ESCs culture) and autologous clinical utilization limiting rejections (Kimbrel and Lanza, 2015).

- *Adult stem cells*

As mentioned previously, the body also contains adult stem cells (ASCs) that plays important roles in tissues homeostasis. The high proliferative potential and the capacity to differentiate into a subset (or a single) cell type of ASCs allow them to regenerate new tissues in response to injuries or diseases (Bozdağ, Yüksel and Demirer, 2018). Importantly, the potency of such adult cells is restricted, at best, to multipotency, while numerous resident stem cells are simple unipotent progenitors. This is indeed the case for high-turnover tissues such as intestinal epithelium, in which resident progenitors will generate new epithelial cells in the intestinal crypt.

ASCs are found in most tissues and persist throughout life in a quiescent state. This results in a strong dependency of ASCs to their environment, described as Stem cells Niche (Ferraro, Lo Celso and Scadden, 2010). It appears that different stem cells niches (from different location, or different organism) present common features, allowing identification of conserved components. Those include (1) stromal supportive cells (composed of cell-cell adhesion and secreted molecules in close proximity of the ASCs), (2) ECM as a mechanical scaffolding unit to transmit stem cell signaling, (3) circularization?? allowing nutritional support and systemic signals as well as participating in the recruitment of circulating stem cells from and to the niche and (4) Neural inputs favoring the mobilization of ASCs out of their niches. The bone marrow constitutes the perfect example of niche offering those features.

Mesenchymal stromal/stem cells are well studied ASCs found in diverse tissue such as bone-marrow, adipose tissue, dental pulp or umbilical cord (Ding, Shyu and Lin, 2011). General definition of MSCs include, according to the 2006 ISCT criteria, expression of markers CD105, CD73, CD90 and lack of expression of HLA-DR, CD14, CD19, CD34, CD45 and CD79α (Dominici *et al.*, 2006). The fact that MSCs lacks traditional Major histocompatibility complex (MHC) allows them to be used across MHC barrier and thus opens the clinical possibilities of these cells to allogeneic cell therapies.

Importantly, original description of MSCs properties emphasized their mesodermal multipotent feature that allowed MSCs to be *in vitro* differentiated toward mesoderm-derived cell types or even trans-differentiated toward other cell lineage including endoderm-derived hepatocyte differentiation (Wanet *et al.*, 2014; Viswanathan *et al.*, 2019). The initial

nomenclature of MSCs with the S corresponding to Stem cells has been subject of debate and the reassessment of their *in vivo* function as immunomodulators rather than progenitors (reviewed in (Caplan, 2017)) prompted the community to name MSCs Mesenchymal Stromal Cells (Viswanathan *et al.*, 2019).

2.2.2. Modeling hepatocyte differentiation *in vitro*

Robust and efficient modeling of biological processes is a central issue of any experimental biology research question since *in vivo* investigations are often impossible (especially in human). This is typically the case for hepatocyte differentiation and more largely, for human development. This led scientists to design suitable experimental procedures allowing such investigations, including animal models and *in vitro* cell culture. Importantly, most of the currently used protocols only yield cells presenting an immature phenotype with fetal features (Boon *et al.*, 2020).

2.2.2.1. Objectives of *in vitro* hepatocyte differentiation

- *Understanding liver development and disease*

Technological advances in hepatocyte differentiation modelling has been widely used, together with animal models, to improve our comprehension of liver development and biology.

On another hand, different hepatotropic viruses including HCV, HBV and HEV are capable of infecting PSCs derived hepatocyte-like cells (HLCs) (Yoshida *et al.*, 2011; Shlomai *et al.*, 2014; Helsen *et al.*, 2016; Xia *et al.*, 2017). Therefore, *in vitro* hepatogenic differentiation constitute an appealing model for the study of hepatitis virus life cycle.

- *Cultured hepatocytes as tools for drug development*

The development of new drugs is a very long and costly process and presents a very low success rate (less than 10%) (Dowden and Munro, 2019). Incorrect drug hepatotoxicity assessment is believed to be responsible for many drug development arrest both prior and after reaching the market (Serras *et al.*, 2021). Therefore, improved technologies allowing drug hepatotoxicity assessment are required. Animal models are not well suitable for drug hepatotoxicity assessment since about 40-50% of drug responsible for hepatotoxicity in human does not present similar toxicity in animal models, further reinforcing the need for *in vitro* alternative methods (van Tonder, Steenkamp and Gulumi, 2013). PHH could represent perfect models of liver detoxification since they truly recapitulate human liver functions and offers the possibility to cover a large genetic variability by using PHH banks originating from multiple donors. However, as mentioned earlier, rapid dedifferentiation of PHH upon *in vitro* culture as well as low recovery post-cryopreservation, compromise this possibility. Thus, PSCs-derived hepatocyte mimicking the detoxifying activities of primary PHH freshly harvested would be the perfect alternative since they present the advantage of prolonged *in vitro* culture. This perspective has been, so far, limited by the maturity levels of PSCs-derived hepatocyte like cells (HLCs). Constant improvement of *in vitro* hepatocyte differentiation protocols offers great promise. Indeed, optimization of culture medium with supplementation of amino acids

beyond the level of nutritional need promote hepatocyte maturation to yield HLCs presenting similar detoxifying capacities as freshly isolated PHH (Boon *et al.*, 2020).

- *Hepatocyte transplantation*

As described previously, the liver presents a very high potential of regeneration, by the reactivation of hepatocyte proliferation, induction of hepatocyte hypertrophy or differentiation of liver progenitor cells. However, prolonged liver damage induces progressive fibrosis eventually leading to cirrhosis which impairs the process of hepatocyte proliferation, resulting in liver failure. Since the first attempts of liver transplantation (performed on dogs in 1952 and in humans in 1963) orthotopic liver transplantation has become the gold standard treatment to cure liver failure (Starzl *et al.*, 1963; Busuttil *et al.*, 2012; Zarrinpar and Busuttil, 2013). Unfortunately, the number of deceased or living liver donors is far from sufficient compared to the growing number of potential recipients added to the transplant waiting list. Paradoxically, the evolution of transplantation technology allows the progressive enlargement of pathological conditions suitable for liver transplantation, which further worsen the disequilibrium.

Another approach, firstly described in 1977 in rat models, consists in the direct engraftment of hepatocytes (Groth *et al.*, 1977). Hepatocytes have indeed the capacity of homing into the liver, even when injected in the spleen. The precise mechanism remains poorly understood, but it is believed that hepatocytes aggregation in sinusoids triggers portal hypertension that activates Kupffer cells and improve vascular permeability. This allows hepatocytes to migrate through the endothelium and join the parenchyma (Joseph *et al.*, 2002; Benten *et al.*, 2005). The technique has been largely studied using PHH in different animal models (reviewed in (Fisher and Strom, 2006)). However, hepatocyte transplantation suffers from the same limitations as liver transplantation as for instance the availability of donors, and this cannot be circumvented by *in vitro* proliferation since cultured PHH rapidly dedifferentiate. This prompted many teams to look for alternative sources of hepatocytes (reviewed in (Najimi, Defresne, Sokal; 2016) (Tricot, De Boeck and Verfaillie, 2020). Criteria for the perfect candidate include the possibility to expand the cells *in vitro*, an advanced matured phenotype (immature hepatocytes are very less efficient at repopulating livers), low risk of teratoma formation and autologous origin. There is currently a lack of such candidate and no alternative source of hepatocytes has been robustly shown as capable of efficient liver repopulation. However, the constant improvement of directed differentiation from hiPSCs offers promise since the matured phenotype of such cells would be one of the last barriers to cross in order to widely apply this approach.

2.2.2.2. Methods to promote hepatogenic differentiation *in vitro*

Recreating the hepatocyte differentiation process *in vitro* requires two essential components: first, the choice of a suitable cell type presenting potential for such differentiation together with an adapted protocol successfully driving differentiation. Cell types such as MSCs, ESCs, iPSCs or hepatic progenitors were indeed *in vitro* cultured and exposed to

different protocols to induce their differentiation toward the hepatic lineages.

The journey toward characterization of an *in vitro* protocol yielding fully mature hepatocytes, while not finished yet, starts with complementation of hESCs embryoid body formation by growth factor inducing hepatocyte differentiation (Baharvand *et al.*, 2006). While successfully inducing several hepatocyte markers, this approach also generated multiple cell types and protocols further evolved by including a first definitive endoderm specification step (Roelandt *et al.*, 2010; Si-Tayeb *et al.*, 2010; Boon *et al.*, 2020). Classical cytokines used to commit PSCs to definitive endoderm include Activin A (an activator of TGF-β receptor), Epidermal Growth Factor (EGF) and WNT. This step is classically achieved after 2 or 5 days and further followed by a hepatic specification step aiming at driving hepatoblast differentiation. Cytokines used include BMP-4, FGF-2 and HGF. Finally, hepatocytic maturation is induced by supplementation with HGF or Oncostatin M. Additional culture supplements have also been shown as promoting hepatocyte differentiation, such as DMSO or Dexamethasone, (Michalopoulos *et al.*, 2003; Vanhove *et al.*, 2016; Tricot *et al.*, 2018).

Besides protocols starting from PSCs, MSCs have also been largely used to model hepatocyte differentiation *in vitro* through their ability to *trans*-differentiate toward an endodermal-lineage cell fate. MSCs originating from Bone Marrow (BM), adipose tissues (AT), dental pulp (DP), umbilical cord (UC), liver or cord blood (CB) have been differentiated toward HLCs (Campard *et al.*, 2008; Wanet *et al.*, 2014; Alizadeh *et al.*, 2016; Ohkoshi *et al.*, 2018). (Najimi et al, 2007, Khuu et al, 2011)

Different strategies have been developed to further improve hepatocyte maturation such as replacement of cytokines by small molecules, microRNAs, ECM optimization, mixed cell population embryoids (Hashemi *et al.*, 2009; Siller *et al.*, 2015; Takebe *et al.*, 2015; Zhou *et al.*, 2017). None of the approaches cited before allowed to drive a fully mature hepatocyte maturation and most protocol yields immature hepatocytes resembling fetal hepatocytes (Zabulica *et al.*, 2019; Boon *et al.*, 2020).

Of note, in this work we firstly modelized hepatocyte differentiation by using a protocol that was previously published (Najimi et al, 2007) to study mitochondrial biogenesis during hepatogenic differentiation of hBM-MSCs (Wanet *et al.*, 2014). The results show that hepatogenic differentiation of hBM-MSCs is characterized by mitochondrial biogenesis and a metabolic shift toward oxidative phosphorylation-based metabolism. Further RNA-seq analysis allowed identification of the PGC1α-TCF7L2 signaling pathway as a connector of commitment to differentiation and mitochondrial biogenesis (Wanet *et al.*, 2017).

However, most of the experiments presented in this manuscript have been performed on different models corresponding to iPSCs hepatocyte differentiation protocols presented in (Boon *et al.*, 2020). This study describes the development of an improved version of a previously published PSCs hepatogenic differentiation protocol (which will be referred to as Standard (STD) and HC3X protocols in the present manuscripts, respectively) (Roelandt, Vanhove and Verfaillie, 2013; Tricot *et al.*, 2018). First, comparison of freshly isolated PHHs with HLCs differentiated with the STD protocol allowed to identify FOXA3, HNF1α and PROX1 as key hepatic TFs presenting insufficient expression at the end of the protocol. Thus, PSCs where genetically engineered to overexpress those TFs under the control of a Doxycycline-inducible promoter. Second, medium was supplemented with considerable

concentrations of amino-acids during hepatocyte specification and maturation. Those improvements allowed to further drive HLCs toward a more mature phenotype presenting significant levels of CYP3A4 mRNA expression and metabolic functions including drug biotransformation and liver-toxin sensitivity closer to PHHs.

2.2.2.3. Assessment of differentiation efficiency

Assessment of differentiation efficiency in HLCs can be undergone by several approaches converging toward key features of hepatocytes: the morphology, the expression of liver-specific protein or mRNAs and ultimately, hepatocyte specific functionality (Sancho-Bru et al, 2009).

The morphology is the most straightforward since simple phase contrast microscopy performed on freshly isolated PHH allows to appreciate their typical cuboidal shape with enhanced cell border definition when cultured in 2D (Olsavsky Goyak, Laurenzana and Omiecinski, 2010). Control of successful acquisition of this phenotype is therefore an easy and quick way to control differentiation on a daily basis.

Assessment of liver-specific gene expression is more informative than morphology since classical cellular biology techniques (such as western blot, immunofluorescence or RT-qPCR) allows efficient characterization of both key transcription factors of the hepatocyte GRN and functional hepatocyte mRNAs/proteins. Thus, probing for definitive endoderm TFs (FOXA2, SOX17, GATA4), or hepatocyte markers (HNF4α, AAT, ALB, NTCP, TDO2, …) has been widely used. Importantly, while simple qualitative induction of those markers is described in many protocols, quantitative comparison of markers expression is more and more used in order to precisely evaluate the matured phenotype of HLCs. This approach has been applied by our collaborators to determine, by using RT-qPCR, the relative expression of hepatocyte markers normalized on a house keeping gene in HLCs and by comparing this results with those obtained on freshly isolated PHH (technically, this correspond to 2^{-dct} for the gene of interest) (Boon *et al.*, 2020). This approach has been performed on a wider scale by assessing 62 commonly used hepatocyte markers in a group of 17 fetal and 25 adult liver samples, constituting reference values for the assessment of differentiation (Zabulica *et al.*, 2019).

Finally, the ultimate way to evaluate hepatocyte differentiation efficiency is the use of hepatocyte-specific functional assays (reviewed in (Sancho-Bru et al, 2009) (Olsavsky Goyak, Laurenzana and Omiecinski, 2010). Those include evaluation of glycogen storage by Periodic Acid Schiff staining, plasma protein and urea secretion assays or chemical compounds biotransformation assay.

3. Translational regulation: principles

From a biochemical point of view, protein synthesis is a simple condensation reaction to form the peptide bond linking the carboxyl group of one amino acid to the amino group of the other one, involving nucleophilic attack of the amino group. However, organisms organize protein synthesis by a complex and multistep process requiring a ribosome and multiple initiation and elongation factors. Advantages of this complex process is therefore to synthetize

a protein based on an mRNA template through the genetic code. The complexity and the multiple proteins and RNAs required make translation the most energy consuming process of the cell (Jackson, Hellen and Pestova, 2010). Interestingly, the complexity of the translation machinery comes together with the potential to specifically regulate the pool of mRNAs to be translated. This regulating process that impacts protein levels through control of protein synthesis is commonly described as "Translational regulation".

Translation can be divided in four steps namely: initiation, elongation termination and ribosome recycling. Most of the translational regulation mechanisms described so far targets the initiation step thus, this section will firstly start by a paragraph giving an overview of the major steps of translational initiation, prior to describe some mechanisms of translational regulation, its relevance on proteome remodeling and the methods commonly used to investigate it. Finally, since results of this work led us to investigate the translational regulation of TOP mRNAs, the last part of this section will be dedicated to a more in-depth description of this particular group of mRNAs and their mode of regulation.

3.1. Mechanism of translational initiation

Canonical translation initiation aim is to recruit a complete 80S ribosome on the translation start site of mRNAs (Fig. 9). The ribosome is composed of 79 ribosomal proteins (RPs) and 4 ribosomal rRNAs (rRNAs) forming together two subunits: the 40S small ribosomal subunit (formed by 33 RPs of the small subunit (SSU) RPSs and 18S rRNA) and the 60S large ribosomal subunit (LSU) (formed by RPs of the large subunit RPLs and 5S, 5.8S and 28S rRNA) (Merrick and Pavitt, 2018). Translation initiation starts with the formation of the 43S pre-initiation complex (PIC) composed of 40S SSU bond by eukaryotic initiation factors (EIFs) 1, 1A and 3 as well as EIF2-GTP-Met-tRNA^{met}. During classical Cap-dependent translation, the EIF4F complex is required to attach the PIC to mRNA. Indeed, the EIF4F complex is composed of EIF4G scaffold protein bound by a Cap-binding protein EIF4E (that will bind to capped mRNAs), dead-box helicase EIF4A and EIF3. EIF4F also binds poly-A binding protein (PABP) via EIF4G. Altogether, the EIF4F complex thus "circularizes" mRNAs that are both recruited by their 5'UTR (through their Cap) and 3'UTR (through their poly-A terminal sequence). Binding of EIF3 to the PIC thus allows the recruitment of mRNA, followed by the PIC scanning the 5'UTR in a 5' to 3' direction in order to find the translation start site. This step consists in two linked processes: the progressive movement of PIC along 5' UTR together with the EIF4A-mediated unwinding of mRNA secondary structures. Generally, the first "AUG" start codon found in an optimum context (defined as the Kozac consensus sequence GCCPuCCAUGG) allows, under control of EIF1, the codon-anticodon base pairing with Met-tRNA^{met} and a modification of the 40S SSU toward a closed conformation that locks the mRNA. Commitment of ribosomes to translation is thus controlled, upon start codon-anticodon base pairing by the hydrolysis of EIF2-GTP, impairing its binding with Met-tRNA^{met} and triggering its partial release from PIC, which is now the 48S initiation complex. Next, EIF5B-GTP binding terminates the release of partially detached in EIF2-GTP but also promotes release of other initiation factors. This allows the recruitment of the 60S LSU that is further followed by the hydrolysis of GTP bound EIF5B and its release. The 80S ribosome is now ready for translation.

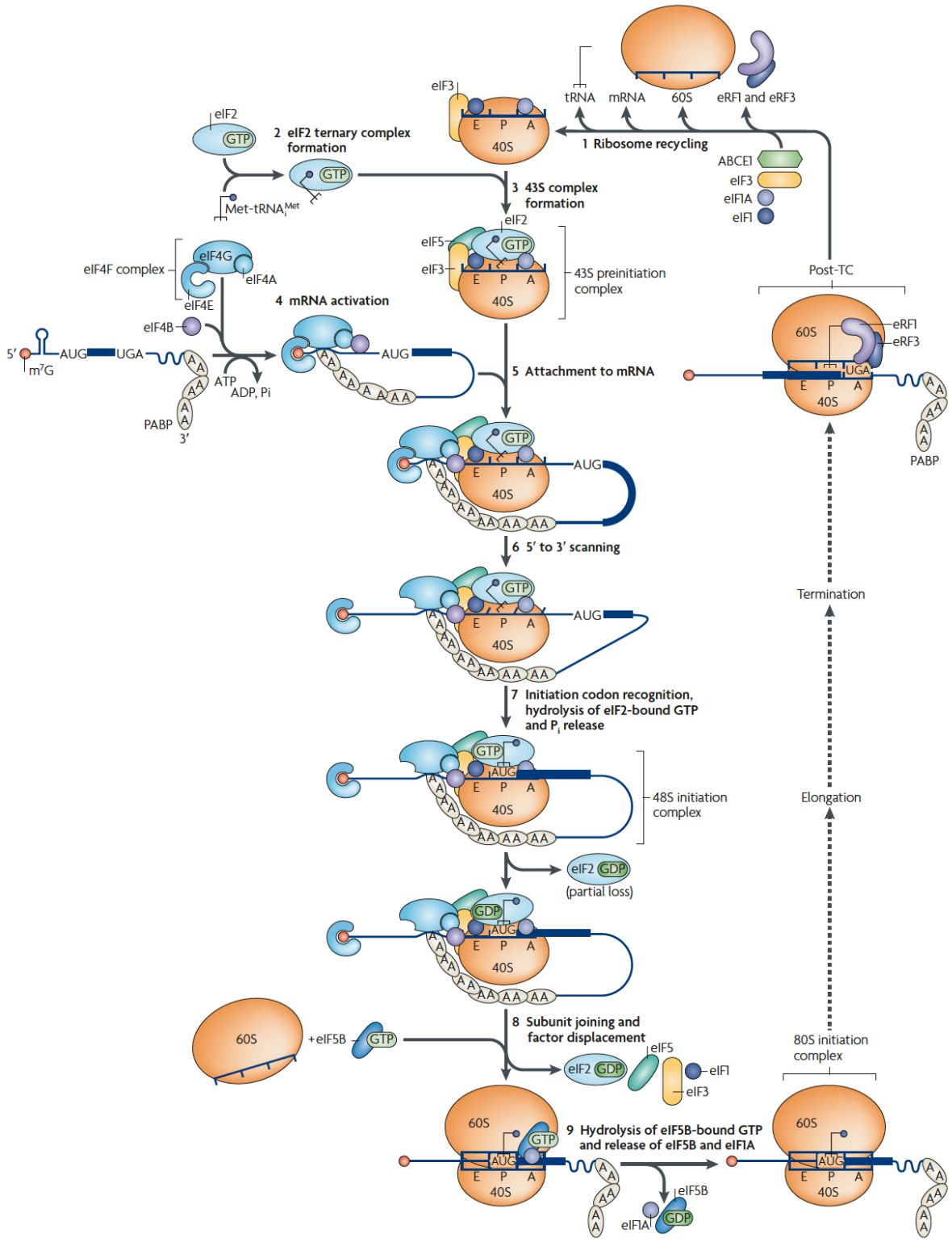

Figure 9: Translational initiation (Jackson et al., 2015).
Schematic representation of major steps of translation initiation.

3.2. Importance of translational regulation for proteome remodeling

Protein synthesis abundance is the result of the equilibrium between protein synthesis and degradation rates. Factors influencing protein synthesis rate include mRNA abundance, mRNA length, translational initiation rate and translation elongation speed (Hershey, Sonenberg and Mathews, 2019). This indicates that mRNA abundance is not the sole regulator of protein abundance regulation, an observation that has been confirmed at the omic scale thanks to the development of high-throughput technologies characterizing proteomes, transcriptomes and translatomes (defined as the pool of mRNA that are translated) (Schwanhüusser *et al.*, 2011; Kuersten *et al.*, 2013). Actually, the poor correlation between protein and mRNA abundance regulations, together with the stronger conservation of protein expression compared to mRNA expression across species suggests that post-transcriptional control explains a large percentage of protein abundance variance (Gygi *et al.*, 1999; Maier, Güell and Serrano, 2009; Schwanhüusser *et al.*, 2011; Vogel and Marcotte, 2012; Aviner, Geiger and Elroy-Stein, 2013). This highlights the capacity of the translation machinery to impact protein abundance by specific selection of transcripts to be translated. Together with the global regulation of protein synthesis rate, this constitutes "the translational regulation".

It is important to note that studies aiming at quantifying the relative proportion of protein abundance variations explained by transcriptional versus translational regulation reached different conclusions. Schwanhüusser and colleagues, by monitoring synthesis of new transcripts and proteins by a pulse labelling approach highlighted a major role for translational regulation, while other groups described more consistent correlation between mRNA level variations and protein levels (with mRNA abundance variation explaining up to 81% of protein abundance variation) (Schwanhüusser *et al.*, 2011; Li, Bickel and Biggin, 2014). Those contradictory observations are explained by the concept that cellular status dictate the relative contribution of translational and transcriptional regulation. Indeed, translational regulation is a useful tool for cells fast response to internal or external stimuli before a new transcription program comes into effect, while during steady state or after long-term processes such as differentiation, transcriptional control is considered the main regulator of protein abundance (Liu, Beyer and Aebersold, 2016).

3.3. Mechanisms of translational control

Translational regulation ensures on one hand, that global protein production, consuming most of the cell energy, does not exceed the demand and in another hand, that necessary proteins are made in the right amount. Since initiation is the rate-limiting step of translation, many mechanisms control the recruitment of mRNAs for translation in order to finally modulate protein levels (those include regulation of translation initiation machinery, sequence-specific features of mRNAs or ribosome heterogeneity, detailed hereafter). Importantly, translation of a transcript can also be inhibited upon strong slow-down of elongation caused by ribosome stalling and ribosome heterogeneity. Principal mechanisms controlling the initiation of mRNA translation are detailed here.

- *Regulation of the translation initiation machinery*

The translation initiation machinery is regulated at multiple levels but the most described

mechanisms include EIF4F complex and EIF2α regulation (Proud, 2019).

A major aim of translational control is to regulate EIF4F complex assembly. Fine-tuning EIF4F complex assembly allows to control translation both globally, such as global control of cap-dependent translation, and in a transcript-selective manner, as is the case for TISU mRNAs, for instance, detailed in the next section (Masvidal *et al.*, 2017). EIF4E-binding proteins (4EBPs) are able to sequestrate EIF4E from EIF4G, impairing complex assembly. Transcripts particularly sensitive to EIF4F inhibition encompass pro-tumoral mRNAs, mitochondrial mRNAs, TOP mRNAs, TISU mRNAs (Morita *et al.*, 2013; Musa *et al.*, 2016). This regulation is under the control of mammalian target of rapamycin (mTOR) since 4EBP1 is a target of mTORC1 (mTOR complex 1) (Gingras, Raught and Sonenberg, 2003; Qin, Jiang and Zhang, 2016). mTOR, the core protein of the mTORC1 complex, is a Ser/Thr kinase of the phosphatidyl-3-kinase (PI3K) family which can bind to different proteins for the formation of two different complex, mTORC1 and mTORC2, respectively. Importantly, both complexes are distinguished by their ability to phosphorylate different substrates. Besides mTOR, the core components of mTORC1 include mammalian lethal with SEC13 protein 8 (mLST8) and the scaffold protein regulatory-associated protein of mTOR (RAPTOR) (reviewed in (Liu and Sabatini, 2020)). RAPTOR, plays several important roles in the complex such as substrate selectivity (through the recruitment of TOR motif on substrate proteins), the correct regulation of subcellular localization of the complex, and the binding to the accessory factor proline-rich AKT substrate 40Da (PRAS40). Regulation of mTOR activity is quite complex and is ensured by a small G protein Ras homologue enriched in brain (Rheb), which is itself inhibited by the Tuberous Sclerosis Complex 1 (TSC1)-TSC2. Thus, phosphorylation of TSC2 by AKT induce the dissociation of the TSC1-TSC2 complex, finally resulting in mTORC1 activation (Proud, 2007). Upon mTORC1 activation, 4EBP1 is phosphorylated at multiple levels, impairing its binding with EIF4E that is thus free to participate in EIF4F complex assembly. Oppositely, inhibition of mTORC1 activity represses EIF4F complex assembly and global cap-dependent translation. Of note, additional mechanisms aiming at controlling EIF4F complex or other members of the initiation machinery also exist. As an example, those include EIF4E or EIF2α (interested reader can refer to (Proud, 2019)).

- *mRNA sequence- specific translational regulation*

Translational regulation can be seen as the integrated result of a regulated translation machinery and a specific mRNA. On the mRNA side, several sequences and structural determinants confer to groups of mRNAs the sensibility to different translational regulation mechanisms. mRNA 5'UTR are at particular importance considering their role in translational initiation through the scanning process (Hinnebusch, Ivanov and Sonenberg, 2016). During scanning, EIF4F complex needs to unwind the 5'UTR in order to reach the translating start site and initiate protein synthesis. This step, performed by EIF4A, is not equally required for translation of all mRNAs. Indeed, translation of mRNAs with long 5'UTR capable of forming stable secondary structures of G-quadruplex structures is preferentially repressed upon treatment with Silvestrol, an EIF4A inhibitor (Rubio *et al.*, 2014; Wolfe *et al.*, 2014). Oppositely, mitochondrial-encoding mRNAs contain a 5' TISU sequence (Translation Initiator of Short 5'UTRs) corresponding to a short and non-structured 5'UTR that confers maintained

translation even upon low energy levels (and EIF4F complex inhibition) thanks to the fact they do not require EIF4A for translation (Sinvani *et al.*, 2015). Consequently, regulation of helicase activity and/or availability of EIF4F complex will differentially impact mRNAs.

Finally, beside structural considerations of the 5' UTR, specific sequences located in 5' and 3' UTR are also of major importance to possibly modulate mRNA degradation, localization and translation by RNA-binding proteins or microRNAs (Hinnebusch, Ivanov and Sonenberg, 2016). An example of RBP-mediated regulation of mRNA biology including stability and translation through both 5' and 3'UTR sequence recognition is further detailed in the next section. Concerning microRNAs, with their ability to target specific transcripts thanks to their seed sequence, are also able to control mRNA translation and stability (Fujita and Crist, 2018).

- *Ribosome heterogeneity*

As translation, the ribosome itself is traditionally seen as a monolithic entity functioning as a housekeeper. However, observations of ribosome heterogeneity has long been achieved by the characterization of ribosomes presenting differential RPs compositions in bacteria grown under different conditions (Van Duin and Kurland, 1970; Deusser, 1972). This led Mauro and Edelman to postulate the "ribosome filter hypothesis" in 2002 (Mauro and Edelman, 2002). According to this hypothesis, the pool of ribosomes is heterogeneous and different ribosomes have differential capacity to associate with specific mRNAs for translation. Thus, controlling the ribosome heterogeneity allows the control of translational regulation.

Features underlying ribosomal heterogeneity include variation in the stoichiometry of RPs on the ribosome, alternative or paralogous RPs, RP modifications or rRNA modifications (reviewed in (Sauert, Temmel and Moll, 2015). Example of translational regulation conferred by a paralogous RP includes the RPL3-RPL3l1 (RPL3-like protein 1) couple (Chaillou, Zhang and Mccarthy, 2016). RPL3L1 is dominantly expressed in striated muscle but, upon induction of muscle hypertrophy, expression of RPL3l1 is strongly decreased and further accompanied by an increase in RPL3. Expression of RPL3l1 actually inhibits myotube growth. Additionally, other examples encompass the modifications of rRNAs. 2'-O-methylation is the most abundant modification of rRNAs and recent technological advances now allow the high-throughput quantification of those rRNA modifications (Birkedal *et al.*, 2014; Marchand *et al.*, 2016; Ayadi, Motorin and Marchand, 2018). Interfering with the modification pattern of 2'O-methylations confers translational selectivity to ribosomes, further confirming the role of rRNA modifications in translational regulation.

3.4. Methods for the investigation of translational regulation

Translatomic (i.e. the high-throughput characterization of translatomes) received limited attention by the scientific community, has demonstrated by the relatively poor number of studies investigating translation at genome-scale (when compared with similar studies of transcriptomes or proteomes) (Zhao *et al.*, 2019). This can be explained by the fact that translatomic investigations comes together with technical difficulties including the combination of specialized and expensive lab equipment together with transcriptomics technologies (King and Gerber, 2016). However, the progressive recognition of the great

involvement of translation on proteome remodeling has been accompanied by the development of sophisticated strategies for the study of translation at the global scale. Considering that translation initiation is the rate-limiting step of translation, together with the global conservation of translational elongation rate, variation in the ribosome density on a particular mRNA has been used as a reliable measure of translational regulation. Ainsi, most of the translatomic methods aims at measuring association between ribosomes and mRNAs.

This section will describe the most popular methods for translatomic, including polysome profiling (the strategy at the center of this thesis) and ribosome profiling (Fig. 10).

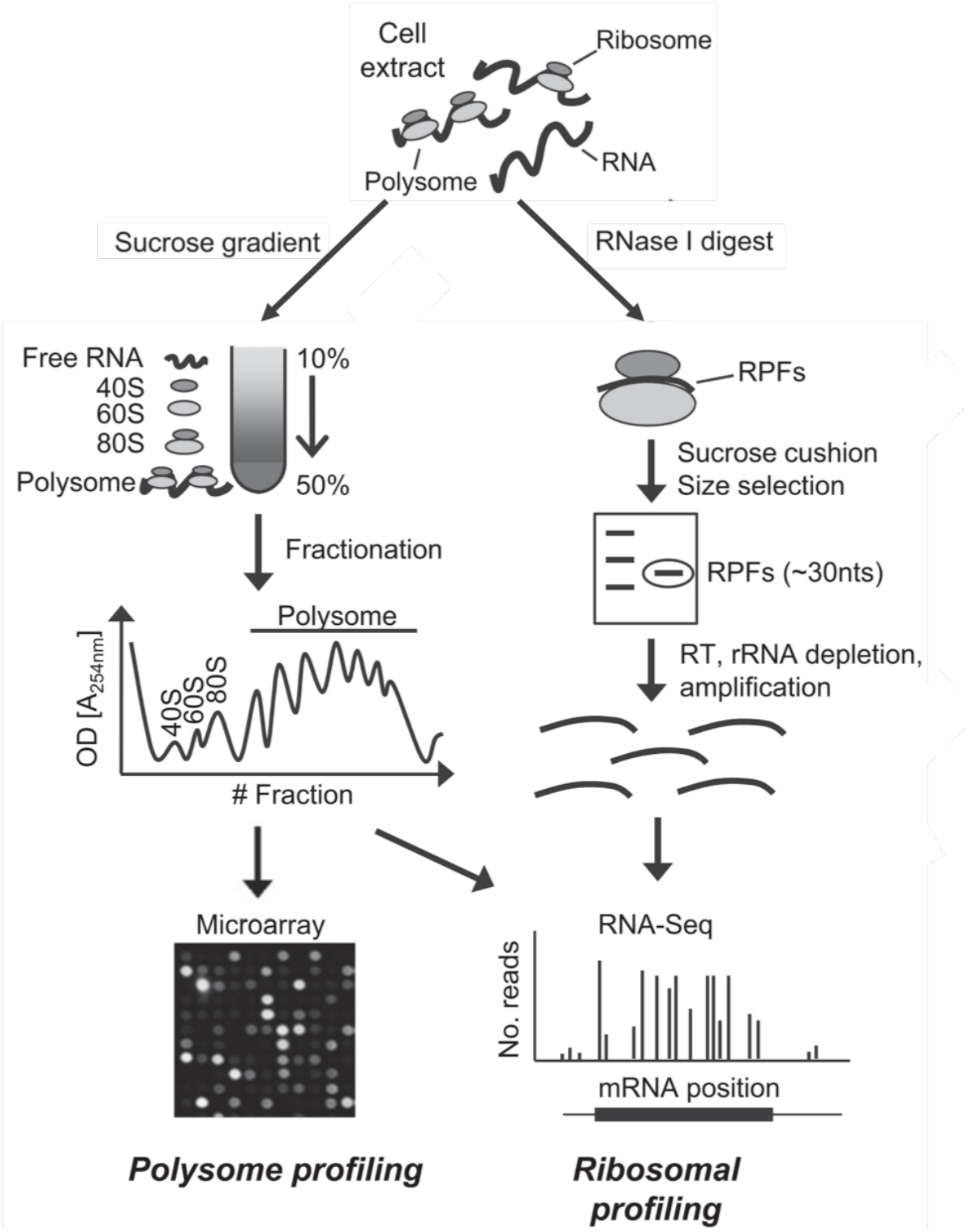

Figure 10: Polysome and ribosome profiling (adapted from King et al., 2016).
Schematic representation of the major steps of polysome profiling (left) and ribosome profiling (right).

- *Polysome profiling*

Polysome profiling (Fig. 10) starts by an initial step of polysome fractionation. This

consists on the immobilization of ribosomes on mRNA by translation elongation inhibitors (such as cycloheximide) followed by lysis and separation by ultracentrifugation on 10-50% continuous sucrose density gradient (Gandin *et al.*, 2014). Fractionation of the gradient with simultaneous measurement of absorbance at 254 nm allows the characterization of the polysome profile. Typical polysome profiles encompass (from lower to higher sucrose concentrations) subsequent signal peaks corresponding to 40S and 60S ribosomal subunits, 80S monosomes and polysomes of increasing number of ribosomes, respectively. This allows the isolation, by RNA extraction, of different types of mRNAs from "free mRNAs" (considered as non-translated) to "light-polysome mRNAs" (lowly translated) and "high-polysome mRNAs" (i.e. mRNAs covered by >3 ribosomes, considered as actively translated). Finally, quantification of High-polysome mRNA together with total mRNA originating from the initial lysate by high-throughput transcriptome methods allows the characterization of the translational efficiency of a given mRNA and thus, the calculation of a translational efficiency fold change for a given mRNA in different experimental conditions.

Advantages of this method include the possibility to simultaneously characterize the global polysome profile of a given sample. It is also possible to validate the results by precisely visualizing the distribution of a specific mRNA throughout the gradient by RT-qPCR. Finally, the fact that full length mRNA are analyzed during polysome profiling (oppositely to mRNA fragment in ribosome profiling, see next paragraph), allows the adaptation of the sequencing depth in order to reach transcript-level mapping, which can be helpful to analyse the impact of alternative splicing on translational regulation (Floor and Doudna, 2016). However, on the negative side, the experiment requires specific and expensive equipment such as ultracentrifuges and gradient fractionation system (King and Gerber, 2016) and are labor intensive (which is further complicated by the requirement to work under RNAse-free conditions). Importantly, this method also requires very large amount of starting material.

- *Ribosome profiling*

Ribosome profiling, initially developed in 2009, also aims at measuring ribosome density on mRNAs (Ingolia *et al.*, 2009). However, the principle behind ribosome profiling differs from polysome profiling since the strategy to measure ribosome density differs (Fig.10). Indeed, following ribosome stalling by translation elongation inhibitors, digestion of mRNAs by RNAse I allows the preparation of ribosome-protected fragments. Thus, deep sequencing of the fragments allows to characterize ribosome density for each given mRNAs and calculate translational efficiency fold change by comparing experimental conditions. Moreover, this approach also adds positional information of ribosomes on mRNAs, which further extend the information gathered (Zhao *et al.*, 2019). Indeed, identifying ribosomes positions allows the deduction of start codon positions, codon usage bias, upstream ORFs as well as translational pausing sites (Ingolia, 2014). Similarly, to polysome profiling, ribosome profiling presents the disadvantage of the requirement for a specialized equipment. Moreover, the methods is also very labor intensive and requires very deep sequencing (which increases the cost and the need for extensive bioinformatic analysis of the small sequenced fragments).

3.5. Regulation of TOP mRNAs

A well-known example of translational regulation downstream of mTOR includes the regulation of 5' Terminal Oligopyrimidine Tract mRNAs (TOP mRNAs). TOP mRNAs family encompasses transcripts coding for many components of the translation machinery, including all RPs and several translation factors such as EEF1A and EEF2 (eukaryotic elongation factors 1A and 2). The story of 5' TOP mRNA translational regulation starts approximately 35 years ago, when different studies identified differential recruitment of RP encoding mRNAs into polysomal fractions in diverse biological contexts such as development (in Xenopus, drosophila or dictyostelium), or growth stimulated/arrested cells (such as glucocorticoid treated lymphosarcoma cells, or serum-stimulated fibroblasts) (reviewed in (Mager, 1988)), suggesting a hypothetical role of the particular 5' end of those RPs (bearing an invariable C as first nucleotide followed by a stretch of pyrimidines). This was later confirmed in the early 90ies by assessment of translational regulation of chimeric mRNAs containing TOP sequences (Levy *et al.*, 1991). Since then, many studies were conducted to better understand TOP mRNA regulation, with particular emphasis on the definition of the TOP *cis*-regulatory element, which was concomitant with the identification of the TOP mRNA repertoire, and the regulating actor(s) involved (Meyuhas, 2000; Meyuhas and Kahan, 2015; Cockman, Anderson and Ivanov, 2020).

3.5.1. Regulatory mechanisms of TOP mRNAs

TOP mRNAs have long been described as regulated downstream signaling pathway of mTOR but the direct actors mediating this effect have been debated. Different RNA binding proteins (RBPs) have been investigated, including La, La-related protein 1 (LARP1), T-cell intracellular antigen (TIA1) or TIA-related protein (TIAR) (Thoreen *et al.*, 2012; Cockman, Anderson and Ivanov, 2020). More recently, LARP1 appeared to be a central player of TOP mRNA translational regulation, while current models of regulation fail to explain all the observations made so far (Berman *et al.*, 2020).

- *mTORC1-LARP1axis*

LARP1 is an RBP presenting different domains: a La module (LaM), a RNA recognition motif-like (RRM-l), a PAM2-like domain and a DM15 motif (Tcherkezian *et al.*, 2014). The DM15 motif resembles HEAT repeats (helical protein-binding structures) and recognizes TOP sequences, allowing binding of LARP1 to TOP mRNAs in a LaM-independent manner (Lahr *et al.*, 2015). Importantly, the binding is not limited to RNA and the m^7GTP cap is required for appropriate binding (Lahr *et al.*, 2017). Finally, structural analysis of the DM15 motif showed that it can exist in two conformations: the collapsed state and the ligand-ready state indicating that the region could be regulated as a molecular switch between binding or dissociation from TOP mRNAs (Cassidy *et al.*, 2019). Additionally, LARP1 also binds TOP and non TOP mRNA through direct 3' end binding or through its binding with Poly-A binding protein (PABP) (Tcherkezian *et al.*, 2014; Fonseca *et al.*, 2015). The PAM2-like motif encompassing 11 amino acids within the LaM mediates PABP binding.

It is difficult to establish a model for the LARP1-mediated regulation of TOP mRNAs since diverse contradictory observations have been made. On one side, LARP1 has initially

been showed as involved in the stabilization of TOP mRNAs (Aoki *et al.*, 2013). Additional experiment with LARP1-depleted cells indicated that LARP1 associates with TOP mRNAs and 40S ribosomal subunit to selectively stabilize TOP mRNAs, thereby constituting a cell reservoir of TOP mRNAs (Gentilella *et al.*, 2017). This is also supported by a preprint study showing that LARP1 binding to the 3' UTR sequence of TOP mRNAs preserves mRNA in a long polyadenylated state, participating in its stability (Ogami *et al.*, 2019).

On the other side, impact of LARP1 on TOP mRNA translation has also been reported. Early investigations in the story of LARP1-mediated TOP mRNA translational control include parallel differential recruitment of LARP1 protein and TOP mRNAs in polysome fractions upon mTOR regulation (which indicates a potential positive role of LARP1 in TOP mRNA translation) (Tcherkezian *et al.*, 2014). Contrarily, Fonseca and colleagues showed that LARP1 interacts with TOP mRNAs in an mTORC1-dependant manner by showing that mTORC1 pharmacological inhibition inhibits TOP mRNA release by LARP1, thus repressing their translation and this has been further confirmed in different studies (Fonseca *et al.*, 2015; Lahr *et al.*, 2017; Philippe *et al.*, 2018).

Since regulation of TOP mRNA and mTORC1 activity have been showed as closely linked, LARP1 was suspected to be the mTORC1 target mediating this effect. Different phospho-proteomic studies identified LARP1 as a highly phosphorylated protein with rapamycin or Torin1-sensitive phosphorylation sites (Hsu *et al.*, 2011; Yu *et al.*, 2011; Kang *et al.*, 2013). Direct interaction with mTORC1, and more precisely with RAPTOR (regulatory-associated protein of mTOR), was later confirmed (Fonseca *et al.*, 2015; Hong *et al.*, 2017). An important next step in the deciphering of mTOR-LARP1 control of TOP mRNAs is the identification of the target residue(s) phosphorylated by mTORC1. There is currently no consensus regarding this and different residues have been considered such as S766 or S770/S979 (Kang *et al.*, 2013; Hong *et al.*, 2017).

At the molecular level, the mechanism of TOP mRNA translational repression by LARP1 is not fully understood and a complete model reconciliating all the experimental observations is currently lacking. The current hypotheses of LARP1-mediated regulation of TOP mRNAs have been recently discussed (Berman *et al.*, 2020). First, LARP1 could be an mTORC1-dependent switch of TOP mRNA biology (Fig. 11): in this model, LARP1 binds TOP mRNA both through PABP and the 5'TOP sequence (with the PAM2-like motif and DM15 motif, respectively). This prevents interaction between mRNA cap and EIF4E, leaving the TOP mRNA in a translationally repressed state. Upon mTORC1 activation, phosphorylation of LARP1 induces conformational changes that inhibit the TOP sequence recognition by the DM15 domain of LARP1 and the cap is free to bind EIF4E and undergo translational initiation. Additionally, PABP binding is not impaired upon LARP1 phosphorylation and this allows LARP1 to remain on 3' UTR of TOP mRNAs and to participate to the conservation of a long poly-A tail resulting in an increased mRNA stability. This model can be further refined when considering that the LaM domain has also been reported to bind TOP sequences on its own (Al-Ashtal *et al.*, 2019). In this updated model, mRNAs are circularized by the presence of LARP1 independently of the DM15 binding. The impact of the circularization isn't clear and it could be argued that circularization by LARP1 not associated with DM15-cap binding activity would be beneficial for translation, since circularization is required for translation initiation. Alternatively, circularization with parallel DM15 binding would be responsible for

translational initiation and recruitment of TOP mRNAs in stress granules and p-bodies (Nykamp, Lee and Kimble, 2008).

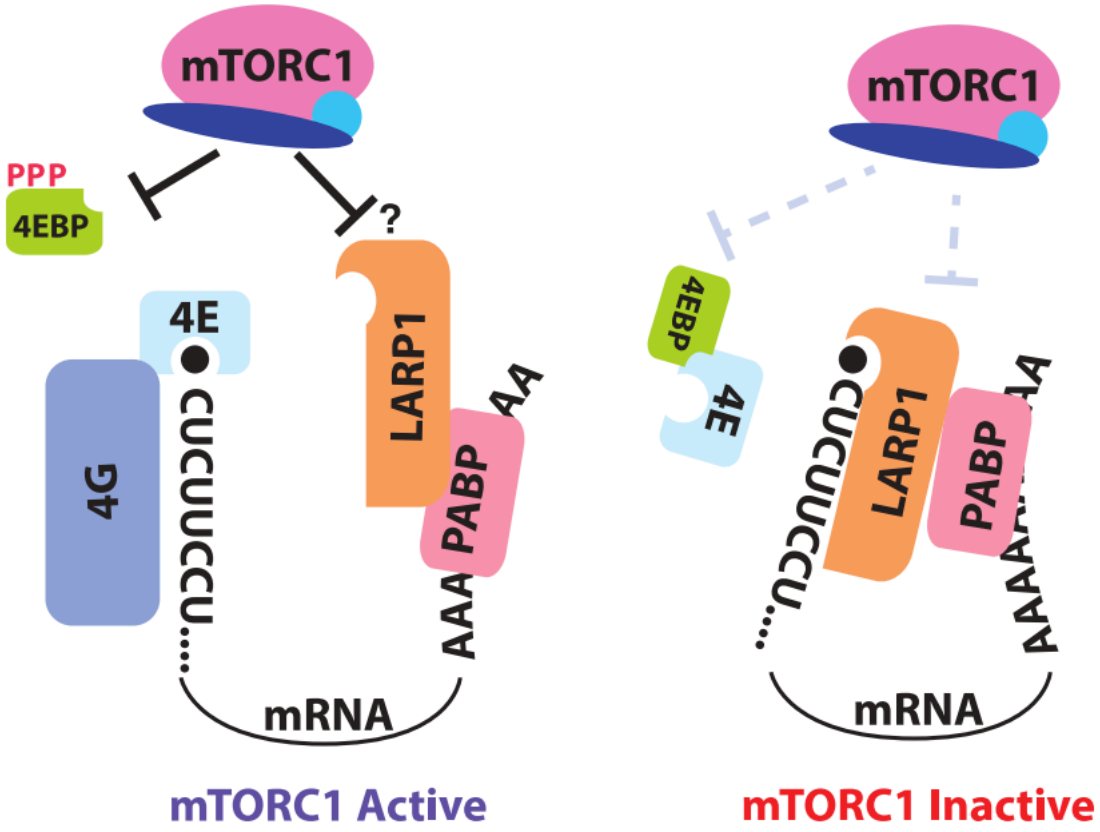

Figure 11: Model for LARP1 two-step regulation of TOP mRNA translation (Phillipe et al., 2018).

- *Other regulations*

Beside the mTOR-LARP1 axis, one can't exclude that other pathways are also responsible for translational regulation of RPs. The potentialities include (1) a TOP mRNA translational regulation mediated by other RBPs, or (2) an alternative signaling pathway regulating LARP1 function. (1) Other RBPs such as Cellular Nucleic Acid Binding Protein (CNBP), TIA1, TIAR or ARE/poly(U)-Binding/Degradation Factor 1 (AUF1) have been shown to bind to 5' TOP sequences, although robust evidences of their role in the regulation of TOP mRNA translation is lacking (reviewed in (Cockman, Anderson and Ivanov, 2020). (2) Is in line with chemical profiling of RP translational inhibitor showing mTOR independent candidates (Li *et al.*, 2018) as well as recent identification of Cyclin Dependent Kinase 1 (CDK1) as a regulator of LARP1 translational repression activity (Haneke *et al.*, 2020).

3.5.2. The 5' TOP *cis*-regulatory element

Initial description of the 5' TOP cis-regulatory element is based on the observation of mRNAs encoding RPs (Levy *et al.*, 1991; Yoshihama *et al.*, 2002). This led to the identification

of particular 5'UTR of RP-encoding mRNAs presenting a first cytidine followed by a pyrimidine tract of 5-25 nucleotides. Of note, those particularities are highly conserved between species. Following this, efforts have been made to better characterize the transcripts group, but this appeared challenging for different reasons. First, many studies of the "pre-LARP1 era" took advantages of the mTORC1-mediated control of TOP mRNA in order to identify differentially translated mRNA upon mTORC1 inhibition (Hsieh *et al.*, 2012; Thoreen *et al.*, 2012; Fonseca *et al.*, 2015). This approach presents a major limitation since mTORC1 translational regulation is not restricted to TOP mRNAs. Second, the presence of the TOP sequence is not the sole determinant of the translational status of a mRNA. This is the case of human β1 and β2-tubulin mRNAs that present all the hallmarks of TOP mRNAs, while their translation regulation is not occurring similarly than the one of other TOP mRNAs. Oppositely, the first 53 nucleotides of their mRNAs are sufficient to confers a TOP-like regulation of translation, indicating that other regulatory elements of their mRNAs could be responsible for their exclusion from the TOP mRNA group (Avni, Biberman and Meyuhas, 1996). This led to the identification of the 92 "classical" TOP mRNAs including all RPs, five translation elongation factors, three translation initiation factors, PABP1 and few other proteins (Meyuhas and Kahan, 2015).

More recently, Thoreen's team aimed at the global characterization of TOP mRNAs by ribosome profiling combining mTORC1 differential activation and LARP1 depletion (Philippe *et al.*, 2020). This allowed to confirm the need for a +1 C followed by a series of pyrimidine in mRNAs sensitive to LARP1 regulation. They also firstly introduced the "TOP score", shifting the TOP mRNA concept from a discrete feature toward a continuous one. Additionally, they also showed that alternative transcription start sites can offer tissue-specific sensitivity to TOP mRNA regulation. Finally, this allowed the identification of a "core" set of TOP mRNAs robustly regulated in tissues.

3.5.3. Biological context of regulation

The function of TOP mRNA translational regulation can be regarded to participate in different regulating processes: biogenesis of the translation machinery is highly energy-consuming (Granneman and Tollervey, 2007). This implies that upon unfavorable conditions (such as amino acid or oxygen deprivation), cells need to reduce the production of RPs to save energy. Oppositely, growth and proliferation require an increase in protein synthesis requiring a parallel increase in the translation machinery. Finally, a last context of TOP mRNA translational regulation includes ribosomal stress, in which perturbations of the ribosomal biogenesis are associated with increased TOP mRNA translational efficiency (Fumagalli *et al.*, 2009).

Links between TOP mRNA regulation and growth/proliferation have long been identified and examples of TOP mRNA translational inhibition upon cell cycle arrest include different biological contexts such as chemically arrested cells in G_0, G_1/S or G*2*/M phases (Stolovich *et al.*, 2005). Oppositely, upon increase of cell mass (cell growth), TOP mRNA translation is activated as demonstrated during induction of neurite outgrowth in quiescent PC12 cells by nerve growth factor, insulin-induced hypertrophy of adipocytes, or hypertrophic

myocardiocytes (Stolovich *et al.*, 2002; Tuxworth *et al.*, 2008; Patursky-Polischuk *et al.*, 2009). Selective inhibition of TOP mRNA translation is also described upon energy deprivation such as amino-acid deprivation or hypoxia (Tang *et al.*, 2001; Miloslavski *et al.*, 2014).

The link between TOP mRNA translation and proliferation is also highlighted by study of cancer cells. Interestingly, LARP1 expression can be used as negative prognosis marker for different cancer such as HCC, colorectal cancer, Non-Small Cell Lung Cancer or ovarian cancer (Xie *et al.*, 2013; Mura *et al.*, 2015; Ye *et al.*, 2016; Xu *et al.*, 2017). Additionally, depletion of LARP1 has been reported to reduce proliferation of different tumor cells (Burrows *et al.*, 2010; Xu *et al.*, 2017).

4. Translational regulation and stemness/differentiation

Stemness and differentiation are biological conditions that are not exception of the poor correlation observed when comparing transcriptome and proteome. Indeed, comparison of transcriptome and proteome showed poor correlation both in ESCs or iPSCs (Munoz *et al.*, 2011). Additionally, following Nanog depletion in mESCs, modifications in the abundance of numerous proteins are not paralleled by a corresponding transcript profiles, suggesting a role for translational regulation of stem cell fate decision (Lu *et al.*, 2009). Those observations have prompted the stem cell researcher community to investigate the role of translational regulation in stemness and differentiation over the past decades and this led to the identification of translation regulatory mechanisms controlling the self-renewal and differentiation of stem cells. Both global protein synthesis and specific activation of key regulating transcripts translation have been identified as regulating different aspects of stem cells' biology (reviewed in (Tahmasebi, Amiri and Sonenberg, 2019; Gabut, Bourdelais and Durand, 2020; Li and Wang, 2020)).

4.1. Translational control in stem cells maintenance

- *Regulation of global protein synthesis in stem cells*

Stem cells tightly regulate global protein synthesis in order to maintain an undifferentiated self-renewal capacity and perturbations of basal protein synthesis is detrimental for their homeostasis. Indeed, increased protein synthesis downregulates POU5f1 expression and induces a fibroblast-like phenotype in ESCs (Easley *et al.*, 2010). In addition, increased global protein synthesis by inhibiting PUS7 (a pseudouridine synthetase that drives the formation of translation inhibiting rRNA fragments), drives the induction of lineage-specific markers impairing proper differentiation (Guzzi *et al.*, 2018). Oppositely, repression of global protein synthesis by depletion of small subunit processome components (a ribonucleoprotein complex responsible for small ribosomal subunit assembly), or the use of 4EGI-1 (an EIF4F complex inhibitor) also impairs ESCs identity (You, Park and Kim, 2015). Finally, it has been shown that inhibiting translation impairs the permissive global euchromatin state associated with hypertranscription observed in mESCs (Bulut-Karslioglu *et al.*, 2018). Those observation suggest that precise regulation of global protein synthesis is required for proper maintenance

of stem cells.

More than relying on a precise regulation of global translation, stem cells are also believed to maintain a repressed translational status with low protein synthesis compared with their differentiated counterparts (reviewed in (Tahmasebi, Amiri and Sonenberg, 2019). This assumption is based on numerous observations of increased global protein synthesis upon differentiation of both pluripotent and adult stem cells. A pioneering study by Sampath and colleagues indeed showed that ESCs differentiation toward embryoid bodies is accompanied by an increase in 35S methionine incorporation and increased cytoplasm/nucleus ratio as well as higher levels of total RNA, protein and rRNA copy number (when normalized to gDNA) (Sampath *et al.*, 2008). Others have also confirmed those results in PSCs or in adult stem cells by comparing their global protein synthesis with their differentiated progeny in different models (including germline stem cells, muscle stem cells or hair follicle stem cells) and confirmed the stimulation of translation (Easley *et al.*, 2010; Blanco *et al.*, 2011; Sanchez *et al.*, 2016; Zismanov *et al.*, 2016; Guzzi *et al.*, 2018). Of note, the hypothesis is also reinforced by the observation that hematopoietic stem cells have a significantly lower protein synthesis rate than other hematopoietic cells, and perturbating this by a modest increase or decrease is sufficient to impair the maintenance of hematopoietic stem cells (Signer *et al.*, 2014, 2016).

Importantly, the concept of low translational status as a hallmark of stemness is challenged by contradictory observations. Indeed, ESCs translation has been demonstrated as required to maintain the global euchromatin state in stem cells (Bulut-Karslioglu *et al.*, 2018). More precisely, these authors suggest that some regulators of chromatin compaction are very unstable proteins that rely on high translational status and translational inhibition drives decrease of chromatin accessibility at developmental enhancer loci. Additionally, using both embryoid body differentiation and retinoic acid-treated mESCs, You and collaborators propose that ESCs require to maintain a high translational activity for expression of labile key pluripotency markers (such as Nanog and Essrb), while they describe a decrease of bulk protein synthesis upon EBs differentiation (an opposite result of what is observed in (Sampath *et al.*, 2008)) (You, Park and Kim, 2015). Similar observations have been made in other models including cardiomyocyte differentiation (Pereira *et al.*, 2019) and neural stem cells differentiation (Baser *et al.*, 2019).

Those results indicate that global protein synthesis is tightly regulated during stem cells maintenance and differentiation. How the precise dynamics of regulation occur during differentiation and how this process is applicable to any differentiating cells remain to be confirmed.

- *Specific translational control mechanisms regulating stemness*

In addition to global protein synthesis, specific translational mechanisms are also involved in the maintenance of stemness and pluripotency in PSCs. Diverse translational regulation mechanisms ensuring the expression of key pluripotency markers have been identified including uORF-, mTORC1- or EIF2α-mediated translational regulation. c-Myc and Nanog have been shown to be translationally regulated through a uORF-dependent mechanism relying on differential EIF2α phosphorylation. Indeed, c-Myc and Nanog mRNAs present multiple uORFs in their 5'UTR sequences and translation of uORFs is reduced by 25% upon embryoid

body differentiation (Ingolia, Lareau and Weissman, 2011). This is controlled by EIF2α since the decrease in P-EIF2α/EIF2α ratio during differentiation represses c-Myc and Nanog translation (Friend *et al.*, 2015). Similar regulation of EIF2α is required for muscle stem cells (satellite cells) activation and exit from quiescence (Zismanov *et al.*, 2016). Finally, Sonenberg team described an elegant synchronized network of alternative splicing and EIF4F-mediated translational regulation that controls mESCs self-renewal and pluripotency (Tahmasebi *et al.*, 2016). Yin-Yang 2 (YY2) is a transcription factor involved in the control of pluripotency gene expression such as POU5F1 and Essrb. Combination of alternative transcription start sites and alternative splicing results in the existence of 4 different transcripts, with two of them retaining introns in their 5'UTR that decrease their translational efficiency. The decreased translational efficiency is relieved by mTORC1-mediated increase in cap-dependent translation. Altogether, combining the mTORC1 activation status with the alternative splicing of YY2 allows to dictate its translational efficiency and control cell fate.

4.2. Translational control regulates stem cells fate decision and differentiation

Similarly, as for stemness maintenance and self-renewal, differentiation is also relying on translational regulation as a mechanism to regulate differentiated proteome acquisition. This naturally brings the question of the relative contribution of transcription versus translation in the proteomic remodeling during differentiation. This has been addressed by systems biology approach consisting in the comparison of transcriptional and proteomic remodeling and allowed to conclude that, upon differentiation, proteomic rewiring is primarily controlled by translational regulation rather than transcriptional regulation (Lu *et al.*, 2009; Kristensen, Gsponer and Foster, 2013).

The capacity of the cell to selectively repress or induce the translation of key proteins playing roles in various differentiation processes has therefore been identified and literature contains various examples of such mechanisms. Here are various examples of translational regulation mechanisms modulating different types of differentiation processes. MicroRNAs play an important role in translational regulation of the myogenic program and conditional KO of Dicer (which process microRNAs) in the myotome (embryonic structure at the origin of myoblasts) strongly reduces the amount of muscle in mice (O'Rourke *et al.*, 2007). Further studies allowed the identification of "MyomiRs" (including miR-206, miR-1 and miR-133) which correspond to microRNAs specifically expressed during differentiation and controlling by several ways the balance between proliferation, growth and differentiation of myoblasts (reviewed in (Fujita and Crist, 2018)). RBP-mediated translational regulation by the Musashi protein family of RBP (encompassing MSI1 and MSI2), which has been described as promoting translation, repressing translation, regulating pre-mRNA processing or alternative splicing and is required for the maintenance and self-renewal of stem cells (reviewed in (Chagas *et al.*, 2020)). MSI1 is indeed strongly expressed in neural precursor stem cells and its expression is required to confers the cells the capacity to undergo asymmetric cell divisions necessary for the generation of neurons and glia (Sakakibara *et al.*, 1996). MSI1 regulates miRNA processing in neural precursor cells and translational regulation of target genes

(Kawahara *et al.*, 2011; Shibata *et al.*, 2012).

Those results indicate that translational regulation plays a major role in the remodeling of proteome upon differentiation and different mechanisms of specific translational regulation controls the establishment of differentiation program during development.

Objectives

Our group has previously characterized the mitochondrial remodeling and metabolic shift in a model of hBM-MSCs *in vitro* hepatogenic differentiation (Wanet *et al.*, 2014), leading to conduct RNA-seq on hBM-MSCs undergoing hepatic specification (at D3 and D5 of the protocol) (Wanet *et al.*, 2017). Results of this analysis (presented in the Results section in 1.1) indicated that several transcripts encoding protein of the translation machinery were differentially expressed upon differentiation as compared to control conditions. Considering those results, together with the growing number of observations linking translational regulation (both at the global and specific levels) and differentiation in the literature, we decided to investigate the potential involvement of translational regulation in the proteome remodeling of stem cells upon hepatocyte differentiation. In this work, our objectives are (1) to characterize the regulation of global protein synthesis rate and (2) to identify differentially translated mRNAs upon hepatogenic differentiation. For this latter objective, we applied polysome profiling, a RNA-seq-based analysis of total and high-polysome mRNAs that allows the high-throughput quantification of mRNAs translational efficiency. Importantly, performing polysome profiling on differentiated hBM-MSCs turned out to be technically not possible. Therefore, we changed our experimental cell model and rather used two different protocols of iPSCs *in vitro* hepatogenic differentiation yielding hepatocyte-like cells with different maturation phenotypes.

A better understanding of hepatocyte differentiation is currently required in order to improve our ability to differentiate cells *in vitro* for further clinical applications such as cell therapy or drug toxicity assays. On another hand, the stem cell field is currently investigating how translational regulation is connected with differentiation. We hope that our work will further bring helpful piece of knowledge for the comprehension of both axes.

Results

1. Translational control of hBM-MSCs hepatogenic differentiation

1.1. hBM-MSCs hepatogenic differentiation provides indications of translational control

The transcriptional remodeling accompanying hepatogenic specification in an *in vitro* model of differentiated hBM-MSCs has been previously characterized by our team. This work highlighted the role of the TCF7L2-PGC1α axis as a link between stem cells commitment to differentiation and mitochondrial remodeling (Wanet *et al.*, 2017). RNA-seq data from this study also suggested a potential role for translational regulation in hepatogenic differentiation of hBM-MSCs (Fig. 12). Indeed, Ingenuity Pathway Analysis (IPA) of differentially expressed genes in D3 and D5 differentiated cells versus control expanding cells and undifferentiated cells highlighted "EIF2 signaling" "mTOR signaling" and "Regulation of EIF4E and P70S6K signaling" pathways as enriched (Fig. 12A). Additionally, 4EBP1 mRNA abundance, encoding a major translation regulator downstream of mTOR, showed a two-fold decrease upon hepatogenic specification step at D5 (Fig. 12B). The downregulation of 4EBP1 protein abundance during hepatogenic specification (at D5 and D12) was further confirmed by western blot. Furthermore, we also showed that 4EBP1 protein abundance is re-induced at D22 of differentiation (Fig. 12C). Finally, investigations of mitochondrial biology during differentiation also highlighted an increase in the abundance of proteins, such as mtHSP70, while related mRNA levels were decreased (Fig. 12D).

These results support a potential role for translational regulation since both EIF2 and mTOR signalings are major regulators of translation (Proud, 2019). Additionally, 4EBP1, whose protein abundance is decreased during hepatogenic specification, is well known for its inhibitory role on EIF4F complex formation resulting in a decreased translation of mRNAs encoding mitochondrial protein (Morita *et al.*, 2013). Altogether, this prompted us to further investigate the translational remodeling occurring during hBM-MSCs differentiation.

A

Ingenuity Canonical Pathways	-log(p-value)	Downregulated genes	Upregulated genes
EIF2 Signaling	1,13E01	16/172 (9%)	50/172 (29%)
mTOR Signaling	6,01E00	32/182 (18%)	41/182 (23%)
Regulation of eIF4 and p70S6K Signaling	4,42E00	21/142 (15%)	31/142 (22%)

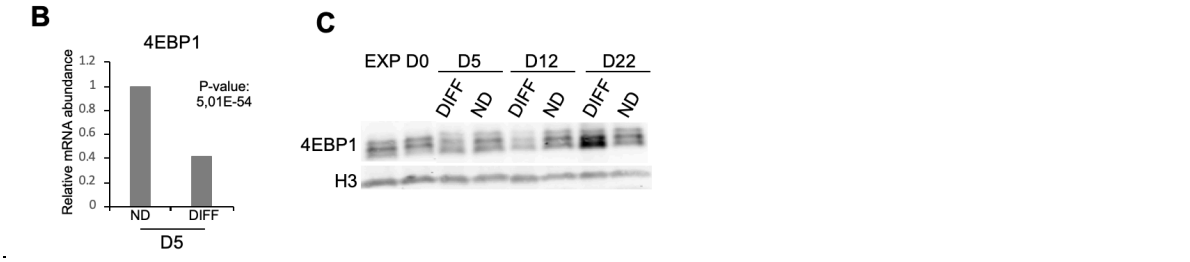

Figure 12: Data supporting a potential translational regulation during hBM-MSCs hepatogenic differentiation

(A) Translational regulation-related enriched terms of Ingenuity Pathway Analysis showing -log(P-value) of enrichment as well as proportion of downregulated and upregulated mRNAs for comparison of control and differentiating hBM-MSCs at D5 (Wanet et al. 2017)

(B) RNA-seq results of 4EBP1 mRNA abundance fold change between control (expanding cells, EXP or non-differentiated cells, ND) and differentiating (DIFF) hBM-MSCs at D5 (Wanet *et al.,* 2017).

(C) Western blot analysis of 4EBP1 protein abundance regulation during hBM-MSCs hepatogenic differentiation showing a downregulation of protein abundance during hepatic specification (D5 and D12 DIFF) followed by an upregulation of protein abundance during hepatogenic maturation (D22 DIFF).

(D) Relative abundance of mtHSP70 protein and transcript during hBM-MSCs hepatogenic differentiation showing opposite modifications of expression at the mRNA and protein levels (Wanet *et al.,* 2017).

1.2. hBM-MSCs hepatogenic differentiation model

We firstly aimed at confirming the acquisition of hepatocyte features in the model of hBM-MSCs *in vitro* hepatogenic differentiation previously used in our lab (Wanet *et al.*, 2014, 2017). hBM-MSCs were differentiated during 22 days by sequential incubation with three cytokines cocktails driving endodermal differentiation (EGF and FGF2 for 2 days), hepatic specification (FGF2, HGF, nicotinamide and ITS for 10 days) and hepatic maturation (OSM, nicotinamide and ITS for 10 days) (Fig. 13A). Differentiated cells were compared with different controls such as expanding hBM-MSCs, control cells collected at D0 and undifferentiated cells collected at similar timepoints as differentiated cells while left untreated in control medium containing only 1% of fetal bovine serum. Regarding cellular morphology, hBM-MSCs showed a fibroblastic phenotype while D12 timepoints showed cell shrinkage prior to acquisition of a polygonal-shape cell phenotype at D22, supporting the induction of hepatogenic morphological differentiation (Fig. 13B). PAS staining showed accumulation of a granular signals in D22 differentiated cells indicative of glycogen accumulation (Fig. 13C). Finally, at the transcriptional level, SOX9 mRNA abundance suppression was confirmed and hepatic markers TDO2 and AAT were induced during differentiation as previously described

on other differentiated MSC (Najimi et al, 2007; Paganelli et al, 2013; Campard et al, 2008). TBX3, a regulator of liver bud formation, was slightly induced during hepatogenic specification at D12 (Fig. 13D).

Experiments done on hBM-MSCs originating from different healthy donors and cultivated between passage 1 and 7 were systematically controlled by phase contrast microscopy, PAS staining and qPCR to monitor differentiation efficiency.

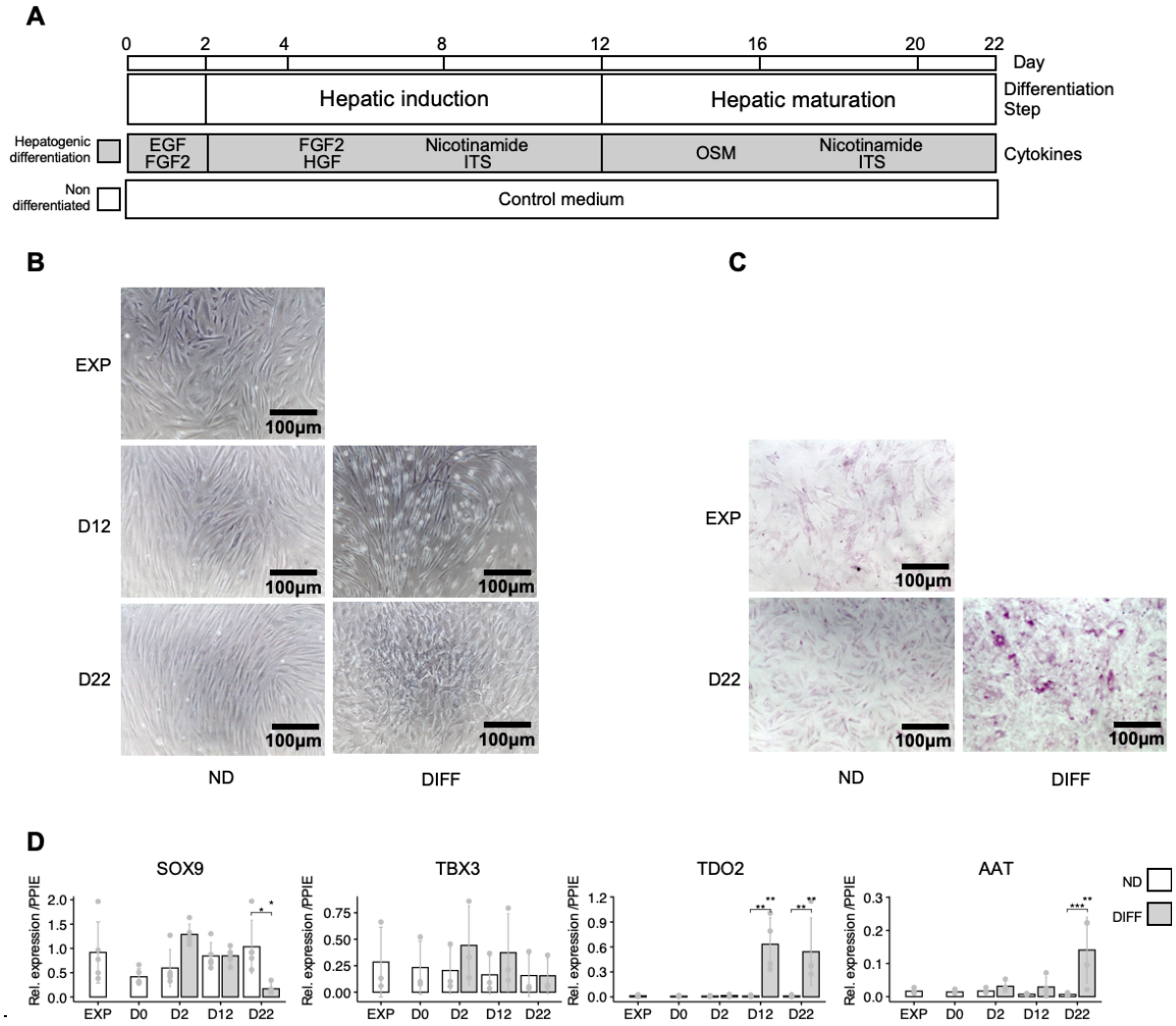

Figure 13: Characterization of hBM-MSCs *in vitro* hepatogenic differentiation model

(A) Schematic representation of culture protocols showing the 3 steps of *in vitro* hepatogenic differentiation. hBM-MSCs were sequentially treated with the corresponding cytokines for a total of 22 days. For experiments involving this model, differentiating cells (DIFF) at different differentiation steps were harvested together with control cells left untreated (ND) during similar timepoints (0, 2, 12 and 22 days, corresponding to D0, D2, D12 and D22) as well as control expanding hBM-MSCs (EXP).

(B) Phase contrast micrographs illustrating the morphological changes occurring during hepatogenic differentiation of hBM-MSCs (scale bar = 100 µm).

(C) Phase contrast micrographs of PAS-stained control and differentiating hBM-MSCs showing a granular signal in differentiated cells indicative of glycogen accumulation (scale bar = 100 µm).

(D) qPCR analysis showing the relative mRNA abundance of key differentiation markers during hepatogenic differentiation of hBM-MSCs.

Results were normalized on PPIE and plotted as means +/- SD of independent biological replicates (n=5 for SOX9, n=4 for TDO2, n= 3 for TBX3 and AAT). Statistical significance was calculated by ANOVA and Tukey's HSD post-hoc test.

1.3. Setup of polysome fractionation protocol

In order to characterize the translational regulation occurring during hBM-MSCs

hepatogenic differentiation, we chose to analyze D5 differentiating cells undergoing hepatogenic specification by polysome profiling. Polysome profiling is a technique to measure mRNA translation by analyzing the efficiently translated mRNAs (i.e. mRNAs with >3 bound ribosomes, called hereafter "High-polysome", HP) (Gandin *et al.*, 2014). Practically, polysome distribution profiles are used to identify HP-containing fractions from which RNA is extracted and analyzed by RNA-seq (in parallel with corresponding total mRNA). Protocol optimization steps showed that at least 1,800 cm^2 of cultured cells were required to generate a polysome lysate of 10-15 OD (absorbance at 260 nm) required to detect a polysome profile in our experimental conditions (Fig. 14). Signal of 254 nm absorbance across 10-50% sucrose gradient for expanding, control and differentiating cells at D5 showed a first triad of peaks in fractions 7, 9 and 11, approximately. These peaks that are usually observed in polysome profiles correspond to the small and large ribosomal subunits (40S and 60S) and the monosomes (80S), respectively. Typical polysome profiles usually present a major 80S peak preceded by two smaller peaks of 60S and 40S ribosomal subunits and followed by increasing waves of polysome signals, thus the profiles obtained here is rather unexpected. We thus sought at confirming the identity of these peaks. Western blot analysis of fractions from D5 differentiated condition showed a strong enrichment of the small RP RPS5 in fractions 7-8 in which the 40S peak was also detected while large RP RPL13A signal was also overlapping with 60S peak. Similarly, agarose gel electrophoresis of RNAs extracted from the 22 fractions showed higher signal for 18S and 28S rRNAs in fractions 7-8 and 9-12, respectively. Finally, both large and small ribosomal subunit protein and RNA members were found in fractions 10-22, confirming the presence of 80S monosomes and polysomes in these fractions.

These results indicate that we successfully fractionated the polysomes of differentiated and control hBM-MSCs while their profiles showed very limited monosomes and polysomes signals. However, despite successful fractionation of polysomes from a considerable amount of differentiated cell lysates, RNA extraction on polysomes-containing fractions yielded poor RNA amounts that did not allowed RNA-seq analysis. Moreover, this was further complicated by technical restrictions inherent of the hBM-MSCs hepatogenic differentiation model that restricts the final amount of differentiated cells that can be generated: (1) hBM-MSCs in culture are slowly proliferating, (2) they should be differentiated before passage 6 to achieve sufficient differentiation efficiency, (3) differentiation efficiency is donor-dependent and certain donors did not differentiate, (4) the culture history of each hBM-MSCs culture strongly impacts differentiation efficiency (we observed a loss in the potential for differentiation in several conditions including culture after multiple cell freezing or culture after over/under confluency). Those technical restrictions did not allow us to robustly conduct polysome profiling on this model and we thus decided to shift toward an hiPSCs-based *in vitro* protocol.

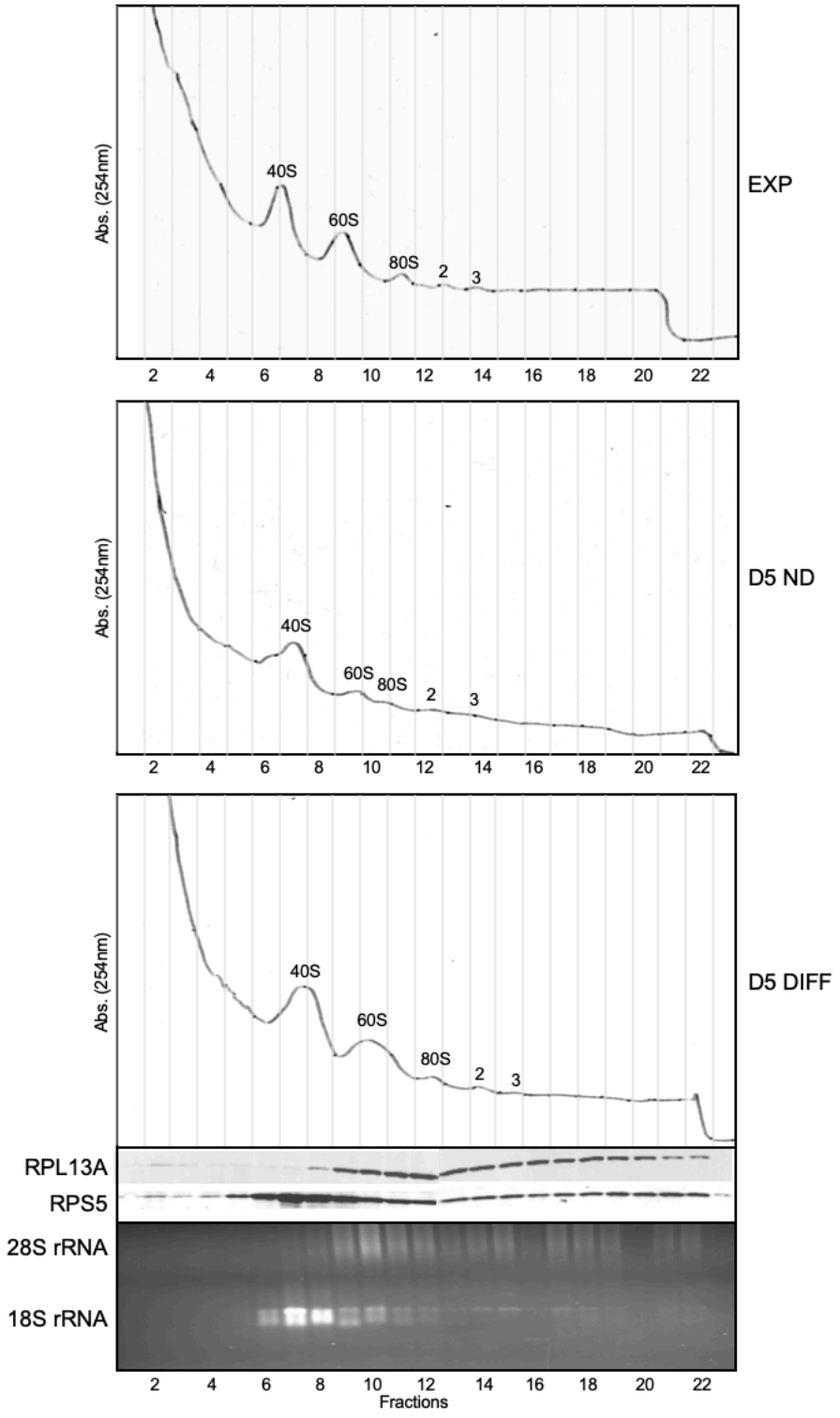

Figure 14: Polysome fractionation of hBM-MSCs

Polysome fractionation profiles of EXP, D5 ND and D5 DIFF hBM-MSCs in 10-50% sucrose density gradients. 254 nm absorbance signal is used to identify fractions containing the 40S small ribosomal subunits, the 60S large ribosomal subunits, the 80S ribosomes and successive peaks of polysomes containing 2 and 3 ribosomes. For D5 DIFF condition, proteins were extracted from each fraction and analyzed by Western Blot of ribosomal protein of the large subunit RPL13A and ribosomal protein of the small subunit RPS5. Similarly, RNA extracts from fractions were analyzed by agarose

gel electrophoresis using Ethidium Bromide to reveal 28S and 18S rRNA distribution across the gradient.

2. Characterization of translational regulation of hiPSCs pluripotent stem cells

Results presented here are part of our publication "The global downregulation of protein synthesis observed during hepatogenic differentiation is associated with a decrease in TOP mRNA translation" (Caruso et al., 2021, under reviewing by Stem Cell Reports).

2.1. iPSCs hepatogenic differentiation models

For hepatocyte differentiation, we used two different protocols. In a first protocol, we applied the methods described in (Roelandt, Vanhove and Verfaillie, 2013) (Fig. 15A) called hereafter the "standard" (STD) protocol. In this scheme, we used sequential treatments with cytokine cocktails driving definitive endoderm formation, hepatic specification and hepatic maturation after 4, 12 or 20 days of differentiation, respectively. This protocol induces differentiation of pluripotent stem cells toward Hepatocyte-Like Cells (HLCs) expressing key hepatogenic transcription factors and functional markers as well as displaying the susceptibility to hepatotropic virus infections (Tricot *et al.*, 2018). A second protocol consisted of an improved differentiation scheme in which PSCs undergo both genetic engineering (doxycycline-induction of three hepatic transcription factors (HNF1A, FOXA3 and PROX1)) from day 4 of differentiation; termed HC3X cells) and differentiation is further optimized by metabolic engineering of the culture medium (supplementation of 3.7 g/l amino acid cocktail from day 12 of differentiation and 20 g/l glycine from day 14 of differentiation) (Fig. 15A) (Boon *et al.*, 2020). The HC3X protocol yields HLCs presenting a mature phenotype closer to freshly isolated hepatocytes in terms of both metabolic activity and hepatocyte specific functions, as demonstrated by measurement of glucose uptake/secretion and 7-benzyloxy-4-trifluoromethylcoumarin metabolic assays (Boon *et al.*, 2020).

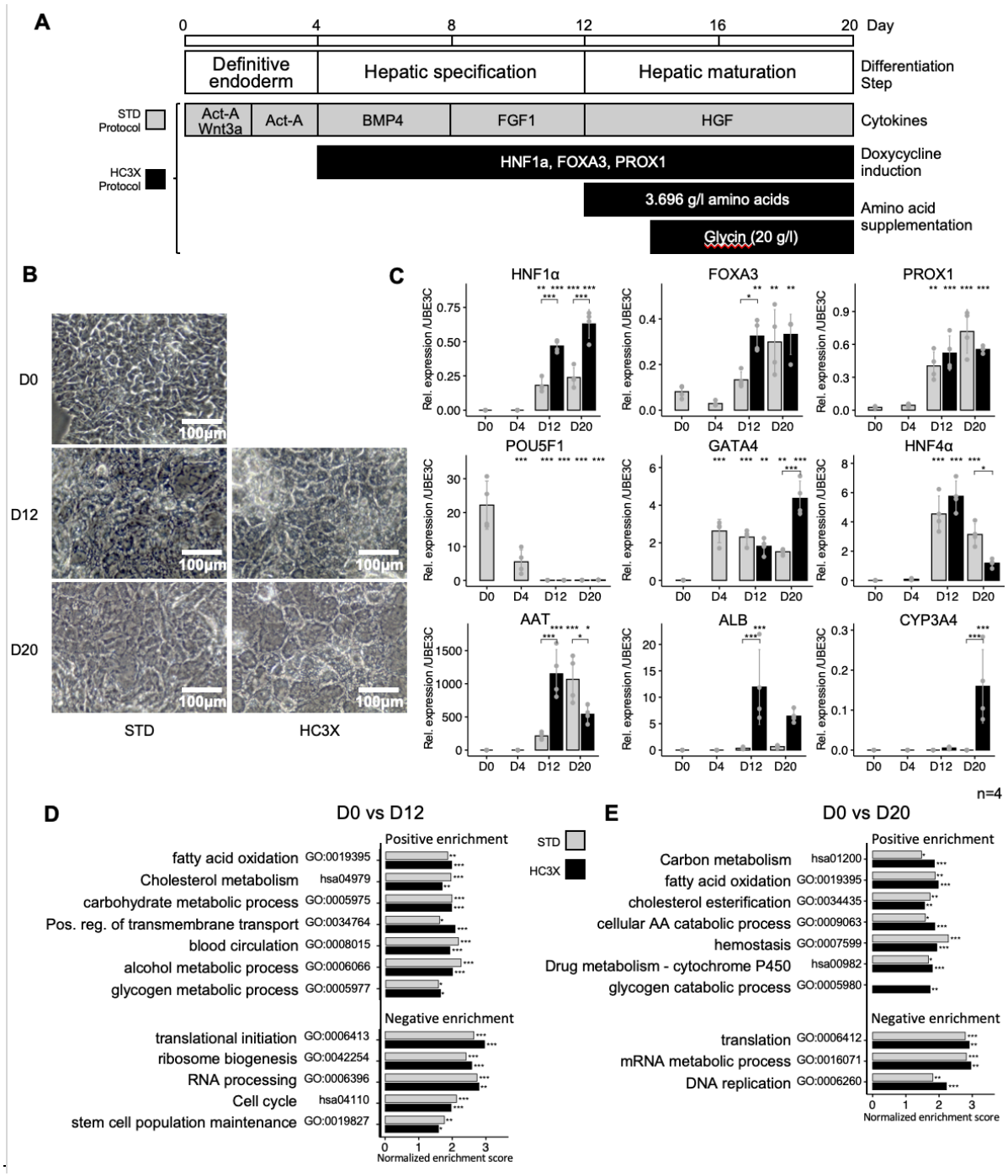

Figure 15: iPSCs hepatogenic differentiation protocols: STD and HC3X

(A) Schematic representation of culture protocols showing the 3 steps of *in vitro* hepatogenic differentiation. For the STD hepatogenic differentiation protocol, iPSCs were sequentially treated with the corresponding cytokines for a total of 20 days. For HC3X hepatogenic differentiation protocol, standard differentiation medium was supplemented with doxycycline to induce expression of HNF1A, FOXA3 and PROX1 from D4 of differentiation and with amino acids supplementation from days 12 and 14 of differentiation. For the majority of experiments, control cells were harvested at day 0 and differentiating cells (with STD or HC3X protocol) were harvested at D12 and D20.
(B) Phase contrast micrographs illustrating the morphological changes occurring during hepatogenic differentiation of iPSCs (scale bar = 100 µm).
(C) qPCR analysis showing the relative mRNA abundance of key differentiation markers during hepatogenic differentiation of iPSCs.
Induced hepatic transcription factors (HNF1A, FOXA3, PROX1), pluripotency marker (POU5F1),

differentiation markers (GATA4, HNF4A), hepatic functional markers (ALB, AAT, CYP3A4) are shown. Results are normalized on UBE3C and plotted as means +/- SD of 4 independent biological replicates. Statistical significance was calculated by ANOVA and Tukey's HSD post-hoc test.
(D and E) Ontology analysis of proteomic data. Log2 fold changes of protein abundance obtained by label-free quantitative proteomic are used to produce a ranked protein list analyzed by GSEA using GO Biological process and KEGG annotation databases. Normalized enrichment score of selected upregulated and downregulated terms for both STD and HC3X protocols at two timepoints: D12 (D) and D20 (E) compared to D0 (terms with pValue < 0.05 were considered significantly enriched).

In our study, the differentiation of iPSCs toward HLCs was systematically evaluated by phase contrast microscopy and RT-qPCR assays for specific mRNA markers (Fig. 15B-C). For the STD and HC3X protocols, the acquisition of the typical hepatocyte polygonal-shaped cell morphology was confirmed on day 20 of differentiation (Fig. 15B). HC3X protocol progeny showed an increased cell size and cell border definition compared to cells that underwent the STD protocol. The loss of pluripotency marker *POU5F1* and induction of definitive endoderm and hepatic transcripts *GATA4* and *HNF4A* (from day 4 and 12 of differentiation, respectively) were also confirmed (Fig. 15C). As expected, the progressive increase in *HNF1A, FOXA3* and *PROX1* transcript abundance observed in the STD protocol was further enhanced by doxycycline induction of these transgenes from day 4 in HC3X differentiation condition. In addition, while expression of hepatic functional marker Alpha 1 anti-trypsin (*AAT*) was significantly induced in both protocols, only HC3X progeny showed a strong induction of Albumin (*ALB*) and cytochrome P450 3A4 (*CYP3A4*) transcripts. Globally, the markers analyzed by RT-qPCR in differentiated cells showed a similar pattern of expression by comparison to freshly isolated hepatocytes (see (Boon *et al.*, 2020) for details).

Next, we analyzed differentiated cells by label-free mass spectrometry in order to establish the acquisition of a hepatogenic phenotype at the protein level. Proteomic analysis comparing samples at D0 with D12 STD, D12 HC3X, D20 STD and D20 HC3X allowed quantification of 1274, 1220, 1028 and 1154 proteins, respectively. Among those, 33%, 33%, 38% and 33% proteins showed a significant (pVal < 0.05) decrease in abundance (Fold Change, FC < 0.5) while 16%, 24%, 19% and 27% of the protein showed a significant increase in abundance (FC > 2) (when comparing D0 with D12 STD, D12 HC3X, D20 STD and D20 HC3X, respectively). Log2 fold change (Log2FC) of protein abundance in control day 0 (D0) iPSCs versus differentiated cells at day 12 (D12) (Fig. 15D) or day 20 (D20) (Fig. 15E) for cells undergoing either the STD or HC3X protocol was used for Gene Set Enrichment Analysis (GSEA). At the end of hepatic specification (D12), the data showed a significant positive enrichment of several GO Biological Process and KEGG pathways terms linked to lipid and carbohydrate metabolisms, indicative of a metabolic maturation. We also found hepatic specific groups of enriched terms linked to glycogen metabolism, plasma protein secretion and cellular detoxification. Finally, relevant terms in the context of hepatogenic differentiation such as cholesterol metabolism and transmembrane transport were also significantly enriched. For both comparisons between control iPSCs and differentiated cells at D12 or D20, we found negative enrichment of terms related to cell cycle and, interestingly, to protein synthesis including numerous RPs.

These results confirm that iPSCs were successfully guided toward hepatogenic

differentiation in STD and HC3X protocols as shown by the induction of key hepatogenic transcription factors and acquisition of a hepatocyte morphological phenotype. Additionally, the HC3X progeny acquires a greater level of hepatic maturation, as shown by the higher expression of functional hepatocyte markers, as previously described (Boon *et al.*, 2020). Perhaps more unexpectedly, the proteomic analysis of differentiated cells, indicates a decrease in the abundance of many components of the protein synthesis machinery during hepatogenic differentiation.

2.2. Hepatogenic differentiation induces a global decrease in protein synthesis rate

As the proteomic analysis revealed a negative enrichment for several proteins related to the translation machinery, we next sought to characterize global protein synthesis during iPSC differentiation using a puromycin-incorporation assay (Fig. 16A). Western blot analysis of puromycin-labelled peptides showed that global protein synthesis appeared to decrease during hepatogenic maturation (D20) in cells undergoing either the STD or HC3X protocol. This quite unexpected observation contrasts with several reports demonstrating an increase in protein synthesis upon stem cells differentiation (reviewed in (Tahmasebi, Amiri and Sonenberg, 2019; Gabut, Bourdelais and Durand, 2020)). However, these reports only focused on the early steps of differentiation (such as embryoid body formation (Sampath *et al.*, 2008)), corresponding to pluripotency exit and stem cells commitment to differentiation, rather than on maturation steps directed toward a precise cell type. This led us to hypothesize that early endodermal lineage commitment might indeed be associated with an upregulation of global protein synthesis, while a decrease in protein synthesis would follow during the subsequent stages of differentiation. Thus, puromycin-incorporation assay was performed on differentiating cells at D2 and D4 (Fig. 16B). This indicated that global protein synthesis was transiently upregulated during endodermal lineage commitment since puromycin signal at D2 showed a 1.5-fold increase when compared to D0 (although this result, performed on 4 biological replicates, was not statistically significant). These results thus suggest a two-step process of global translational regulation whereby early stimulation of protein synthesis is followed by a global repression of translation during hepatogenic maturation.

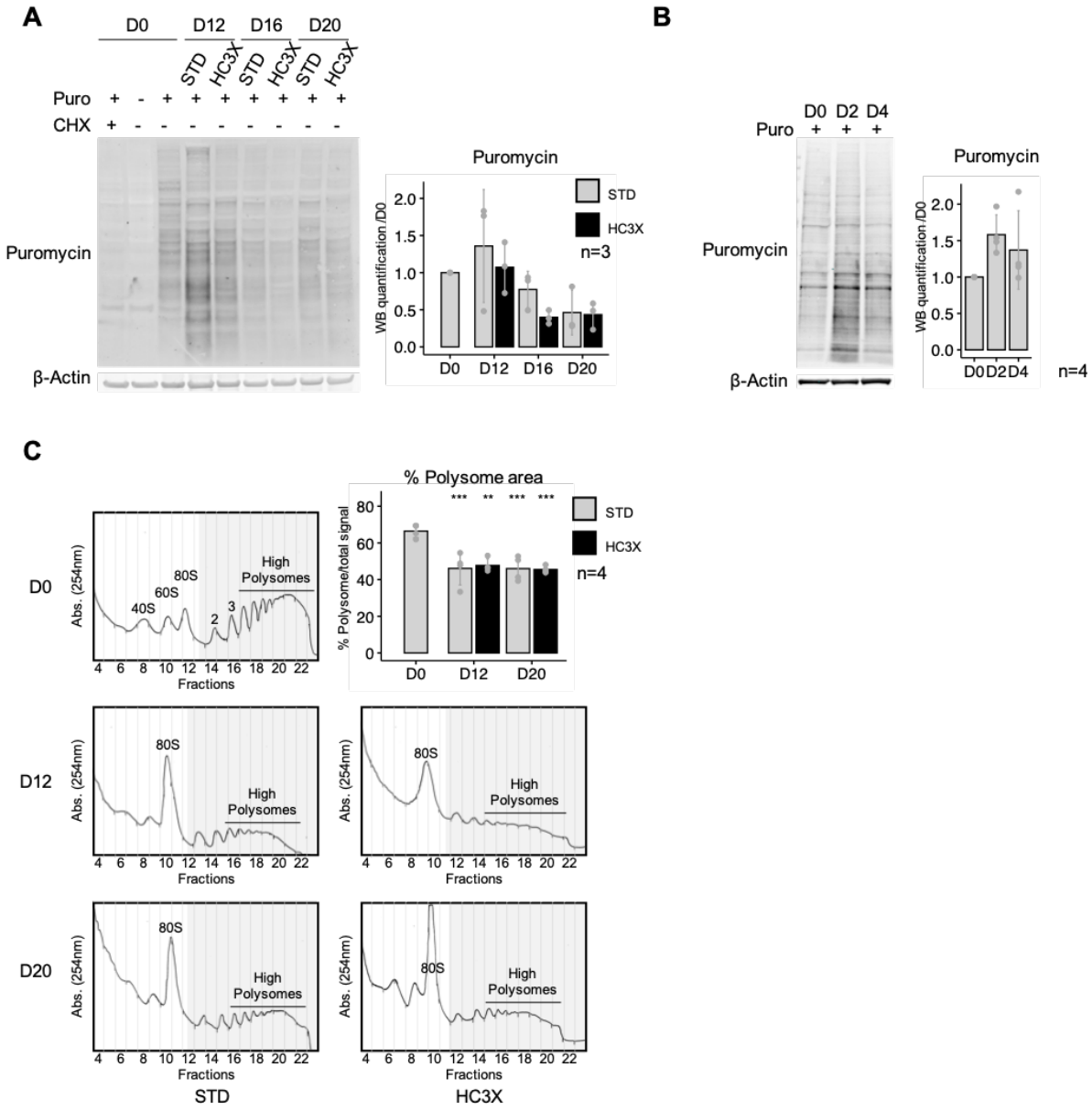

Figure 16: Differentiation induces a global decrease in protein synthesis

(A and B) Western blot analysis of puromycin-incorporated nascent polypeptides in control iPSCs and differentiating cells at day 12, 16 and 20 (A) and D2 and D4 (B). iPSCs left untreated by puromycin or simultaneously treated with puromycin and cycloheximide (CHX) were used as negative control. Western blot signal intensity was quantified and normalized over β-Actin signal and plotted as means +/- SD of 3 independent replicates.

(C) Polysome fractionation profile during differentiation. Ribosomal subunits (40S and 60S), monosomes (80S) and polysomes from control D0 iPSCs or differentiating cells at D12 or D20 with STD and HC3X protocols were separated on sucrose density gradient and fractionated in 24 fractions. Graphs show absorbance at 254 nm across sucrose gradient and are representative of 4 biological replicates. Polysomal signal in the gradient is colored in grey. Bar plot represents means +/- SD of % of polysomal signal calculated by normalizing polysomal area under the curve by total area under the curve for 4 independent biological replicates.

Statistical significance was calculated by ANOVA and Tukey's HSD post-hoc test.

The global translation profile was further characterized by polysome profile analysis in

undifferentiated iPSCs and differentiated cells (Fig. 16C). Polysomal analysis consists in the fractionation of cellular lysates in sucrose density gradients followed by measurement of absorbance at 254 nm across the gradient. This allows to establish the distribution profiles of ribosomal 40S and 60S subunits, 80S monosomes and polysomes, which is a mean to assess global translation. The data show that at D12 and D20, differentiated cells undergoing either the STD or the HC3X protocol display an increased 80S peak associated with reduced polysomes, indicating a global reduction of translation.

In conclusion, using two models of hepatogenic differentiation, our data are in accordance with those of the literature concerning the previously described upregulation of translation during pluripotency exit, but they also reveal that this effect is transient and that the increased protein translation phase is followed by a global reduction of translation during hepatogenic maturation. This conclusion was correlated with a decreased abundance of components of the translational machinery as observed by proteomic analysis (Fig. 15C and D; negative enrichment).

2.3. Polysome profiling experiment of differentiated iPSCs

The global downregulation of protein synthesis observed upon hepatogenic differentiation was quite unexpected since hepatocytes are generally considered as “biochemical factories” actively involved in multiple metabolic pathways and dedicated to plasma protein production. Polysome profiling experiment was thus used to characterize the specific translational reprogramming occurring during differentiation, and to assess its impact on the acquisition of a typical hepatocyte proteome (See Fig. 17A for a schematic representation of polysome profiling). RNA-seq raw counts of total and HP mRNAs were thus analyzed with the R package Anota2seq. Anota2seq analysis of RNA-seq data quantifies the Log2 Translational Efficiency fold change (Log2TE FC) of mRNAs, and integrates mRNA abundance regulation and translational regulation to assign each mRNA to a regulatory mode “Translation”, “Buffering”, “Abundance” or “Background” (Oertlin *et al.*, 2019). mRNAs assigned to the “translation” group display modified abundance in the HP samples that are not paralleled by corresponding total RNA levels, supporting a differential TE that impacts the protein level. mRNAs with an opposite modification for HP and total RNA abundances are assigned in the “buffering” group. This regulation mode is currently interpreted as a mechanism by which a change in the TE of a mRNA compensates its transcriptional regulation in order to conserve similar protein abundance (Lorent *et al.*, 2019). The ”abundance” group include transcripts characterized by equivalent regulation of their HP and total RNA abundances, indicating a change in mRNA abundance not associated with differential TE. Finally, the remaining genes, which are neither regulated at translational nor at the transcriptional levels were assigned in the “Background” group.

For each comparison between D0 control iPSCs and D12 or D20 differentiated cells which had undergone either the STD or HC3X protocol, scatter plot of Log2FC mRNA abundance in HP (on Y axis) and in total RNA (on X axis) shows the distribution of mRNAs colored by regulatory mode (Fig. 17B). The Anota2seq algorithm assigned 5.34 %, 6.70 %, 5.30 % and 5.00 % of mRNAs in the regulatory mode “Translation” for each comparison between control D0 and D12 STD and D12 HC3X, or D20 STD and D20 HC3X differentiated cells,

respectively. Additionally, a very limited number of transcripts were assigned in the "Buffering" group (0.89 %, 1.08 %, 0.89 %, 0.90 % for D0 versus D12 STD, D12 HC3X, D20 STD and D20 HC3X, respectively). Comparatively, numerous transcripts were assigned in the "abundance" group (45.61%, 51.03%, 49.09%, 54.69% for D0 versus D12 STD, D12 HC3X, D20 STD and D20 HC3X, respectively). These results confirm that both differentiation protocols used are accompanied by a major transcriptional rewiring while a limited number of transcripts undergo translational regulation potentially impacting proteome remodeling.

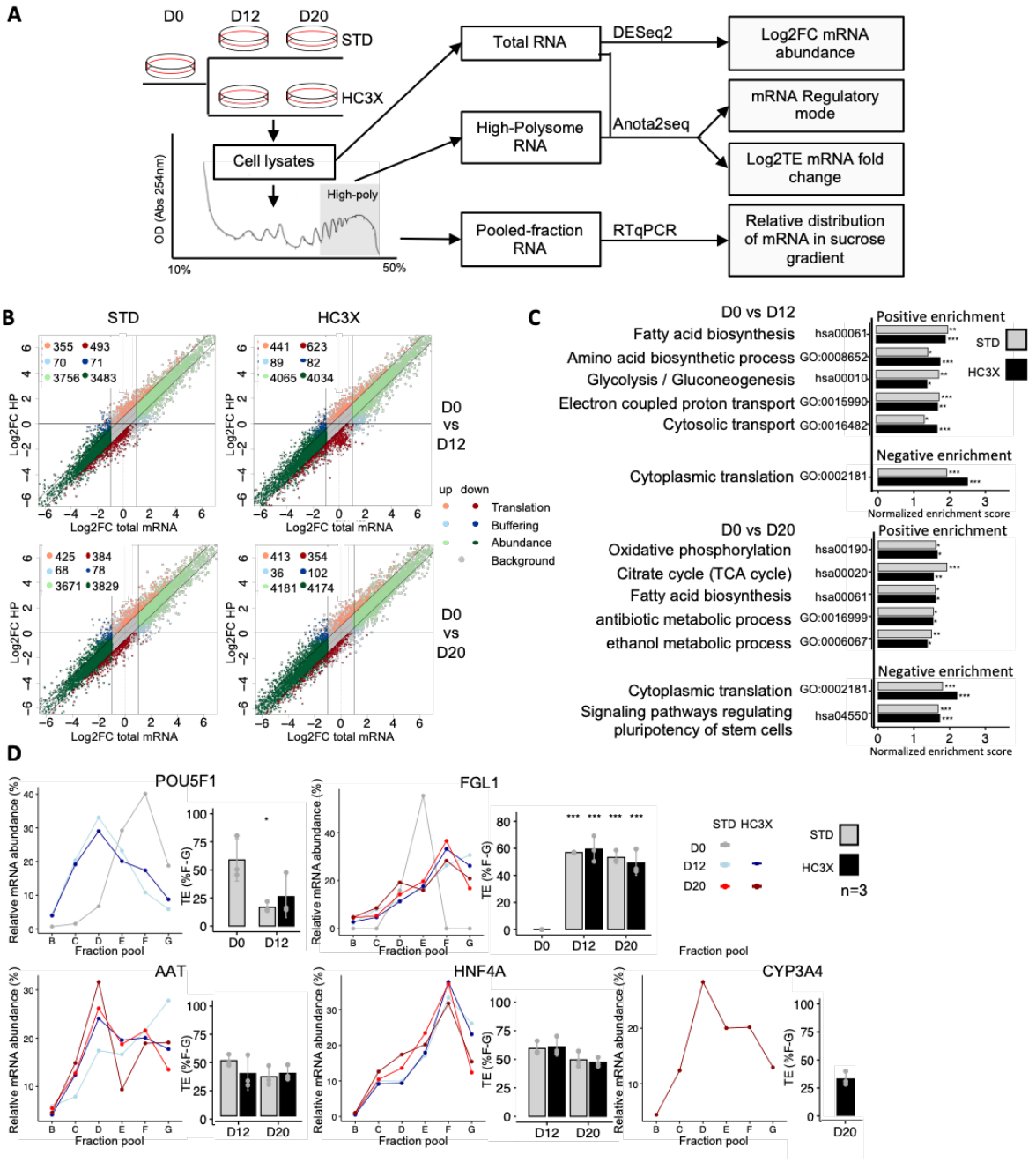

Figure 17: Characterization of specific translational reprograming during hepatogenic differentiation

(A) Schematic workflow of the polysome profiling experiment. Cell lysates from control D0 and differentiating cells were loaded on sucrose density gradients prior to ultracentrifugation and fractionation. Measurement of 254 nm absorbance allows to identify HP containing fractions (polysomes with >3 ribosomes). For each experimental condition, total RNA and HP RNA samples were extracted (from cell lysates and HP fractions, respectively) and analyzed by RNA-seq. DESeq2 analysis of total RNA samples identifies differentially expressed genes while Anota2seq analysis identifies differentially translated genes during differentiation. Equal volumes of fractions were pooled three by three prior to RNA extraction of 8 pooled-fractions (A to H) covering the sucrose gradient. Pooled RNA fractions are analyzed by RT-qPCR to validate translationally regulated candidates.

(B) Scatter plot of Log2FC mRNA abundance in HP (X axis) and total (Y axis) mRNA samples for control D0 iPSCs versus differentiating cells at D12 and D20 with STD or HC3X differentiation protocol. Genes are classified by regulatory modes: Translation (orange) for genes whose

translational regulation is expected to impact protein level, Buffering (blue) for genes whose translational regulation is opposite to transcriptional regulation, mRNA abundance (green) for genes regulated at the transcriptional level and background (grey) for genes not regulated at translational nor transcriptional level. For each regulatory mode, light colors indicate upregulation and dark colors indicate downregulation.
(C) Ontology analysis of polysome profiling data comparing control and differentiated cells by GSEA of Log2TE FC ranked gene list using GO Biological Process and KEGG annotation databases. Results are shown for several hepatocyte differentiation relevant terms and expressed as Normalized Enrichment Score (terms with pValue < 0.05 where considered significantly enriched).
(D) RT-qPCR analysis of hepatic transcripts of the "translation group" (POU5F1, HNF1B and FGL1) and key hepatic genes (HNF4A, AAT and CYP3A4) showing the relative distribution of mRNAs in pooled fractions of sucrose density gradients. Pooled fractions B-C-D and F-G correspond to subpolysomal and HP fractions, respectively. Results are normalized by a spike-in exogenous luciferase RNA and expressed as mean of % mRNA abundance in each fraction from 3 independent biological replicates. Boxplots represents means +/- SD TE corresponding to mRNA abundance in HP fractions (F-G). Statistical significance was calculated by ANOVA and Tukey's HSD post-hoc test.

2.4. Characterization of specific translational regulation during hepatogenic differentiation

We next performed a more in-depth analysis of the Anota2seq results to investigate how the translational reprogramming of differentiated cells could specifically affect the acquisition of hepatocyte features. GSEA using the GO Biological Process and KEGG annotation databases was performed on the transcript list ranked on Log2TE FC for each comparison. Comparison of D0 control cells with D12 differentiated cells highlighted several groups of terms linked to metabolism as positively enriched (Fig. 17C). Those included fatty acid, amino acid, glucose and energy metabolism. In addition, GSEA also identified several terms related to cellular transport as significantly enriched, suggesting that translational regulation could contribute to the establishment of hepatocyte intracellular trafficking machinery, an important feature that supports hepatocyte interactions with apical and basolateral lumens (Schulze *et al.*, 2019). Finally, among the negatively enriched terms, we found a significant number of terms related to protein synthesis and translation, as previously observed in the proteomic GSEA (Fig. 17D and E). These observations support a strong contribution of translational regulation in the downregulation of protein abundance involved in the translational machinery. When comparing D0 control cells with D20 differentiated cells from the STD or HC3X protocol (Fig. 17C), numerous metabolism-related terms (associated with fatty acid and OXPHOS metabolism) were, again, observed as well as some terms more specifically associated with hepatogenic differentiation such as cellular detoxification. Translation-associated terms were also strongly negatively enriched when comparing D0 and D20 differentiated cells. Detailed results of GSEA analyses comparing D0 with D12 and D20 differentiating cells including the list of mRNA candidates differentially translated are presented in the appendix section as Table 7 and 8, respectively. These results indicate that translational regulation might contribute to the metabolic maturation that occurs during hepatogenic differentiation but is also involved in the reduction of translation-related protein synthesis as revealed in the proteomic dataset.

It is important to underline that a significant number of hepato-specific transcripts were

excluded from Anota2seq analysis. Indeed, calculation of TE FC requires the comparison of enrichment of a transcript in HP fractions compared to total RNA in both experimental conditions included in the comparison. However, several hepato-specific transcripts were not detected in HP fractions of day 0 control iPSCs, preventing Anota2seq algorithm to calculate the Log2TE FC for these transcripts. Indeed, when retrieving raw count results for a group of 40 transcripts commonly used as hepatocyte differentiation markers (Zabulica *et al.*, 2019), about half of the candidates (52.5 %) presented 0 count at least in one sample, excluding them from the analysis (data not shown). We thus combined Over Representation Analysis (ORA) using the GO Biological Process and KEGG database and manual scanning of the "Translation" set of genes to find relevant candidates regulated at the translational level. This allowed identification of different mRNAs encoding proteins involved in the endodermal lineage differentiation such as Hepatocyte Nuclear Factor 1B (*HNF1B*, Log2FC TE of 0.96 and 0.88 for STD and HC3X protocol) or β-Catenin (*CTNNB1*, Log2FC TE of 0.61 and 0.74 for STD and HC3X protocol) as translationally upregulated in D0 vs. D12 comparisons. Interestingly, the key pluripotency gene *POU5F1*, while strongly down-regulated at the mRNA level during differentiation (see Fig. 15C) was also assigned in the "Translation" group for the D0 vs D12 comparison with Log2FC TE of -1.26 and -0.67 for STD and HC3X protocol. The distribution profile of *POU5F1* transcript in sucrose gradients obtained by RT-qPCR (Fig. 17D) showed enrichment in HP fractions (fractions F-G) in D0 condition while *POU5F1* mRNA distribution shifted toward non-polysomal fractions in D12 differentiated cells, confirming that this mRNA presents decreased translation efficiency during differentiation. This constitutes an additional proof for the previously documented *POU5F1* translational efficiency downregulation upon differentiation (Tahmasebi *et al.*, 2016). For D0 vs D20 comparisons, Anota2seq analysis classified several genes involved in cellular detoxification in the "Translation" group, including Alcohol dehydrogenase 6 (*ADH6*, with Log2TE FC of 1.56 and 1.25), Aldehyde dehydrogenase family 3 member B1 (*ALDH3B1*, with Log2TE FC of 2.56 and 2.49, Sulfotransferase 1A2 (*SULT1A2*, with Log2TE FC of 0.88 and 1.08). Finally, the hepatokine Hepassocin (also known as Fibrinogen-like protein 1, *FGL1*) was identified as translationally regulated in all differentiating conditions (with Log2TE FC of 1.26, 1.77, 1.22 and 1.08 for D12 STD, D12 HC3X, D20 STD and D20 HC3X, respectively). *FGL1* mRNA distribution across the sucrose gradient also confirmed the induction of mRNA translation as shown by the recruitment of FGL1 mRNA in HP-containing fractions in differentiated cells.

Next, we assessed the RT-qPCR-based TE of key hepatic markers excluded from the Anota2seq analysis in differentiated cells to evaluate how efficiently these genes were translated during differentiation. mRNA distribution profile of hepatocyte transcription factor *HNF4A* and the serum protein *AAT* showed consistent TE with approximately 50% of mRNA in HP fractions of differentiated cells in all conditions (Fig. 17D). *CYP3A4* mRNA profile at D20 after HC3X protocol (the only condition expressing sufficient amount of mRNA) showed a rather modest TE with approximatively a third of mRNA distribution in HP fractions.

Altogether, the characterization of specific translational regulation occurring during hepatogenic differentiation supports a global contribution of translational regulation to metabolic maturation of differentiating cells. Additionally, while differentiating cells undergo a global decrease in protein synthesis, several transcripts involved in hepato-specific differentiation showed translational upregulation while different hepatocyte-specific markers

were shown to be efficiently translated in differentiated cells.

2.5. Translation of TOP mRNAs is decreased upon hepatogenic differentiation

Our results showed that numerous proteins involved in translation are less abundant during differentiation while mRNA encoding those proteins are less efficiently translated. mRNAs encoding RPs and several translation factors, such as those involved in initiation and elongation, display a terminal oligopyrimidine tract (TOP) sequence in their 5' UTR allowing the control of their translation (Jefferies *et al.*, 1994; Avni, Biberman and Meyuhas, 1996). Thus, we hypothesized that translation of TOP mRNAs might be specifically repressed during hepatogenic differentiation. A core set of TOP mRNAs has recently been defined (Philippe *et al.*, 2020). For these transcripts, the heatmap of transcriptomic (Log2FC), translatomic (Log2TE FC) and proteomic (Log2FC) results associated with Anota2seq regulatory modes is presented (Fig. 18A).

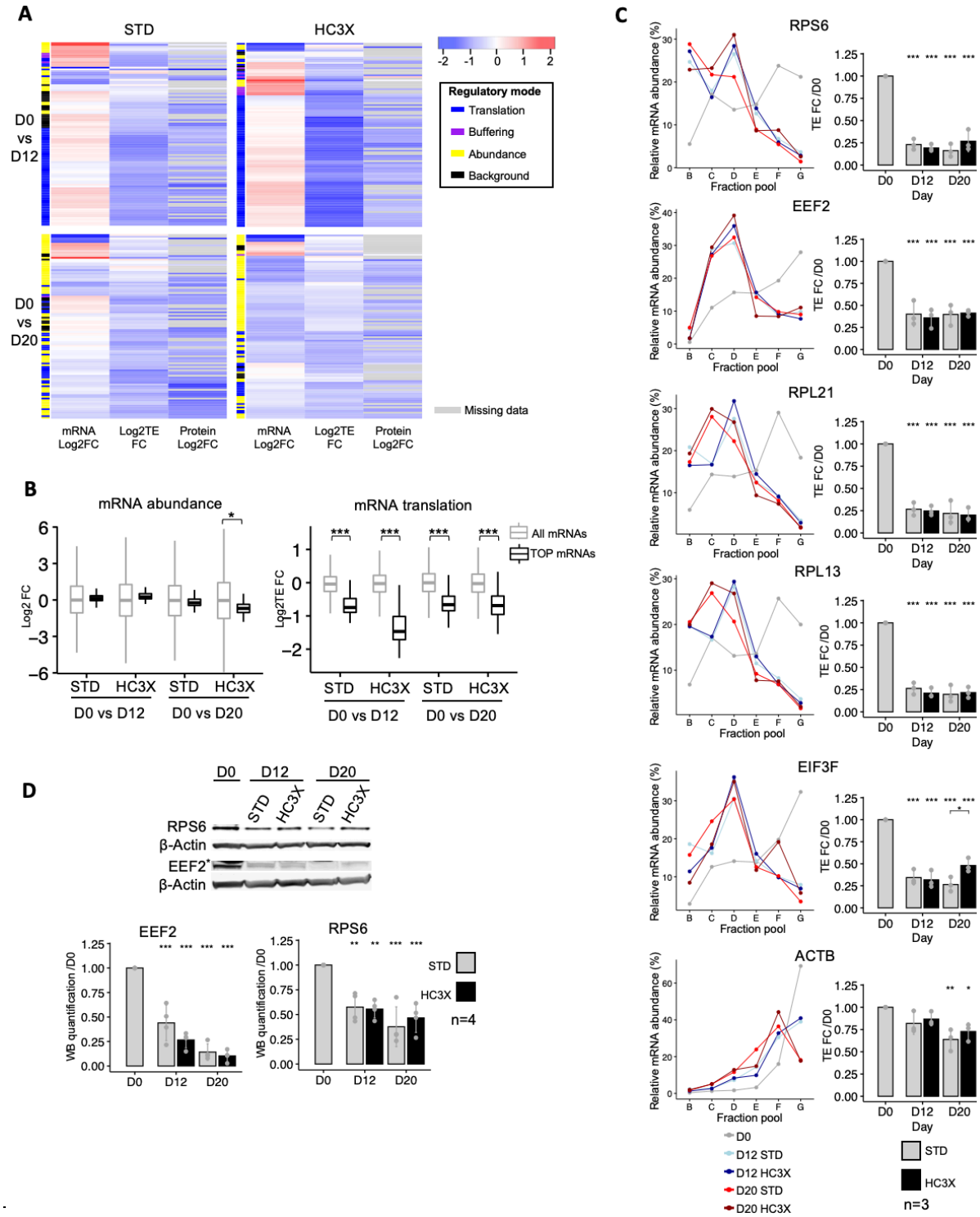

Figure 18: TOP mRNAs translation is downregulated during hepatogenic differentiation

(A) Heatmaps of omics results for core TOP mRNAs on differentiating vs control D0 iPSCs. For each comparison, results are expressed as transcriptomic Log2FC (mRNA abundance), Log2TE FC (mRNA translation) and proteomic Log2FC (protein abundance) (for first, second and third column, respectively). Labels correspond to Regulatory mode obtained by Anota2seq. (Grey corresponds to missing values from the proteomic dataset).

(B) Boxplots of means Log2FC (mRNA abundance) and Log2TE FC (mRNA translation) of core TOP mRNAs or all analyzed mRNAs for D0 versus differentiating cells. Statistical significance was calculated by Student T-test.

(C) RT-qPCR analysis of TOP mRNA candidates showing the relative distribution of mRNAs in

pooled fractions of sucrose density gradients. Pooled fractions B-C-D and F-G correspond to subpolysomal and HP fractions, respectively. Results are normalized by a spike-in exogenous luciferase RNA and expressed as means of % mRNA abundance in the fraction from 3 independent biological replicates. Boxplots represent means +/- SD TE FC corresponding to fold changes of mRNA abundance in HP (F-G) fractions relative to control iPSCs at D0. Statistical significance was calculated by ANOVA and Tukey's HSD post-hoc test.
(D) Western blot analysis of TOP mRNA-encoded proteins RPS6 and EEF2 (* indicates non-specific signal). Graphs shows quantification of western blot signals normalized to β-Actin and expressed as means +/- SD of FC over D0 at D12 and D20 with STD and HC3X protocol. Statistical significance was calculated by ANOVA and Tukey's HSD post-hoc test.

Our proteomic results confirmed the global downregulation of TOP mRNA-encoded proteins during hepatogenic differentiation. At the transcript level, TOP mRNA abundance remained unchanged at all timepoints, excepted for D22 with the HC3X protocol, where a small but significant downregulation was observed (Fig. 18B). Finally, polysome profiling results confirmed the global decrease in TOP mRNAs TE during differentiation at all timepoints, as confirmed by their mean Log2TE FC (Fig. 18B). Thereby, Anota2seq analysis massively assigned TOP mRNAs in the "Translation" group at D12 for both protocols. For hepatic maturation timepoints with the HC3X protocol, the superposition of transcriptional repression onto the reduction of TE shifted many transcripts in the "abundance" group while a significant number of TOP mRNAs remains in the "translation" group. These results suggest that translational repression of TOP mRNAs might be an important driver of the decrease in RP abundance observed during differentiation.

We next selected several TOP mRNA candidates (*RPS6, EEF2, RPL21, RPL13* and *EIF3F*) to validate the translatomic results by RT-qPCR analysis of pooled fractions from gradients (Fig. 18D). All the TOP mRNAs selected presented a distribution profile enriched in HP fractions (fractions F-G on the graphs) in D0 control iPSCs. However, TOP mRNAs clearly shifted toward non-polysomal fractions upon hepatogenic differentiation. Calculating an RT-qPCR-based FC TE by comparing percentage of mRNA abundance in fractions F-G confirmed at least a two-fold decrease in TE for all TOP mRNAs studied upon differentiation, confirming their translational repression. Finally, western blot analysis of RPS6 and EEF2 confirmed the progressive downregulation of protein abundance during hepatogenic differentiation.

In conclusion, these results show that TOP mRNAs are globally less efficiently translated during hepatogenic differentiation while their mRNA abundance is also downregulated during hepatogenic maturation. This argues that the downregulation of protein abundance of the translation machinery is firstly controlled at the translational level during hepatogenic differentiation while repressed at the transcriptional level in experimental conditions displaying the most advanced hepatogenic phenotype (D20 HC3X).

2.6. Control of TOP mRNA translation

TOP mRNA translation is largely considered as regulated by the mTORC1/LARP1 regulatory axis (Berman *et al.*, 2020). Strikingly, western blot analysis demonstrated that abundance of LARP1 is progressively decreased during hepatogenic differentiation (Fig. 19C). Additionally, detecting mTOR and P-mTOR (S2448, indicative of mTORC1 activation)

protein abundance together with phosphorylation status of its targets 4EBP1, S6K1 and S6K1 target RPS6 indicated that mTORC1 is not activated during hepatogenic differentiation (Fig. 19A).

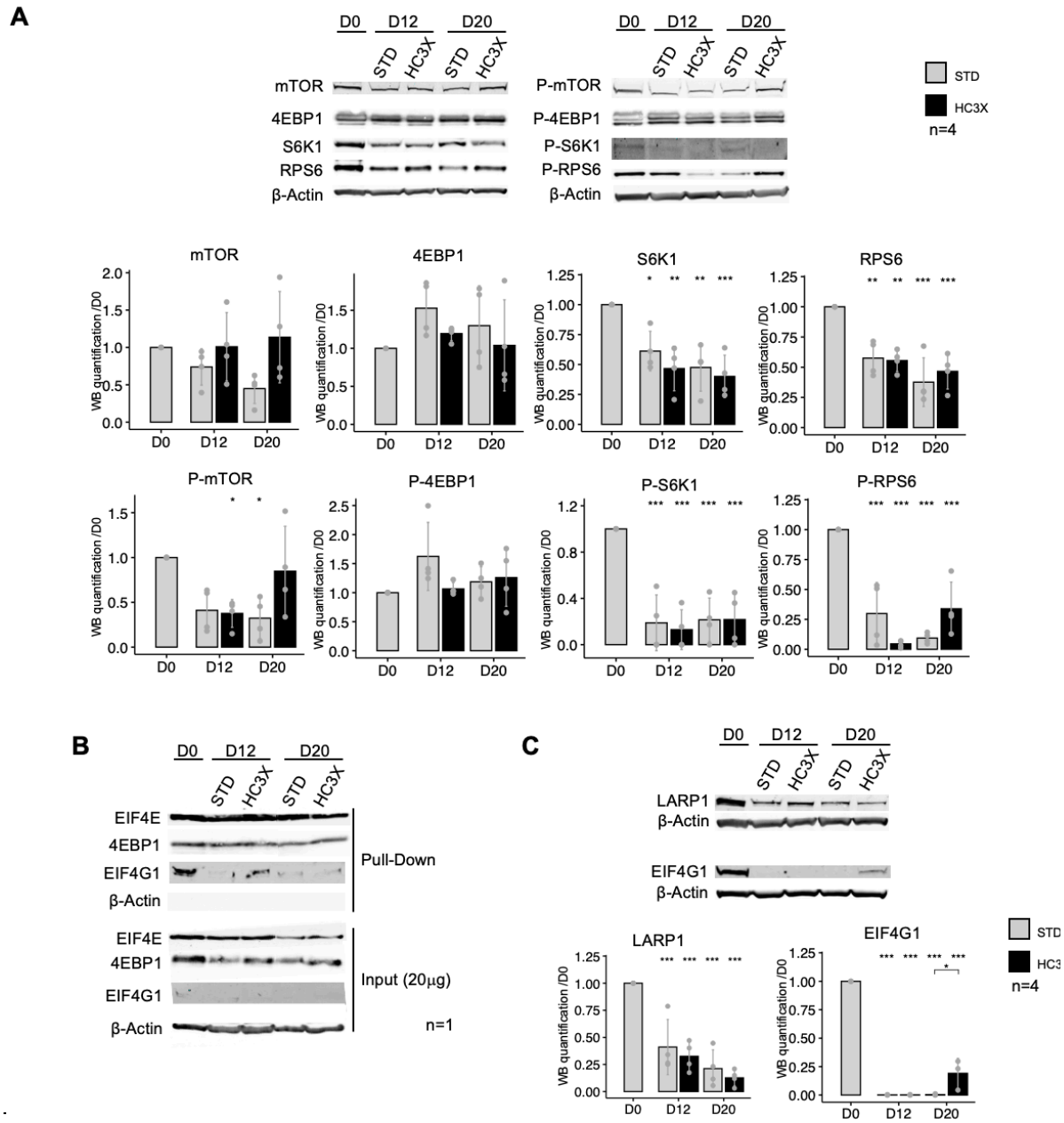

Figure 19: Characterization of TOP mRNAs regulators

(A) Western blot analysis of mTOR pathway actors showing total mTOR, 4EBP1, S6K1 and RPS6 protein levels in control D0 iPSCs and differentiating cells at D12 and D20 after STD and HC3X protocols. Corresponding phospho-proteins levels are also showed (S2448 mTOR, T37/46 4EBP1, T389 S6K1, S240/244 RPS6). Graphs show quantification of western blot signals normalized to β-Actin and expressed as means +/- SD of Fold change over D0 at D12 and D20 with STD and HC3X protocols for 4 biological replicates. Statistical significance was calculated by ANOVA and Tukey's HSD post-hoc test.

(B) Western blot analysis of cap-binding assay showing EIF4E pulled-down by cap-coated beads protein abundance as well as EIF4E binding partners 4EBP1 and EIF4G1 in 1 biological replicate. β-Actin was detected as negative control. 20µg of proteins from total lysates were simultaneously analyzed as input control.

(C) Western blot analysis of TOP mRNA translation regulator LARP1 and EIF4F complex scaffold protein EIF4G1 in control D0 iPSCs and differentiating cells at D12 and D20 after STD and HC3X protocols. Graphs show quantification of western blot signals normalized to β-Actin and expressed as means +/- SD of Fold change over D0 at D12 and D20 with STD and HC3X protocols. Statistical

significance was calculated by ANOVA and Tukey's HSD post-hoc test.

To further confirm the absence of mTORC1 regulation and assess the EIF4F complex formation, we pulled-down EIF4E by m7-GTP-coated beads and analyzed the abundance of co-precipitated 4EBP1 and EIF4G1 using western blot (Fig. 19B). This assay allows to estimate the relative binding of EIF4E with either EIF4G1, the scaffold protein responsible for the assembly of the EIF4F translation initiation complex, or 4EBP1, the inhibitory binding partner. A single replicate of this experiment shows that, for constant levels of pulled-down EIF4E, a comparable abundance of 4EBP1 was detected in differentiated conditions as compared to D0 cells, confirming the absence of regulation of 4EBP1 binding to EIF4E by phosphorylation. Interestingly, EIF4G1 was poorly detected in pulled-down samples from differentiated conditions, while showing a strong signal in D0 condition. Further western blot analysis of EIF4G1 protein abundance showed a strong decrease upon hepatogenic differentiation with both STD and HC3X protocols. This is an interesting result since a report previously showed a decrease of global protein synthesis accompanied by selective repression of TOP mRNA translation upon EIF4G1 knock-down (Thoreen *et al.*, 2012). Whether LARP1 and/or EIF4G1 play a role in the control of TOP mRNA translation during hepatogenic differentiation requires further investigations.

In conclusion, our results show that the previously observed reduction in TOP mRNA TE may not be controlled by the mTORC1 regulating pathway. However, the decrease in protein abundance of both LARP1 or eIF4G1 might potentially be responsible for TOP mRNAs translational regulation.

Discussion and perspectives

The goal of this PhD thesis project was to characterize the contribution of translational regulation in the stem cells hepatogenic differentiation. In order to address this question, we designed an experimental strategy including *in vitro* hepatogenic differentiation followed by polysome profiling and data mining of the translatome results. The establishment of such experimental approaches has required an extensive technical optimization including the setup of polysome fractionation and the use of an optimal model of *in vitro* hepatogenic differentiation. This has finally led us to characterize the translational remodeling at the "omic" scale. We have chosen to further investigate TOP mRNAs translational regulation since those mRNAs largely encode members of the translation machinery, while global protein synthesis is repressed during differentiation.

1. Suitable model for the study of translational regulation in hepatogenic differentiation

In vitro hepatogenic differentiation is a tool that has been developed both for the modelling of hepatocyte biology, including development and diseases, and for clinical applications (such as hepatocyte transplantation or drug discovery). Both utilizations are interconnected since a better understanding of the molecular mechanisms regulating hepatocyte biology based on *in vitro* models is a useful piece of knowledge that can be used to further develop clinical applications. The purpose of the present work falls in the first category since we aimed at understanding a very fundamental research question regarding the molecular mechanisms regulating hepatocyte differentiation. The project thus naturally led us to extensive discussions about hepatocyte differentiation modelling.

"All models are wrong, but some are useful"

Experimental models can be defined as "a simplified and accessible reflection of a part of reality". Indeed, a majority of biological questions does not allow direct *in vivo* investigations: this is typically the case for human development, for obvious ethical reasons. Thus, experimental modelling can be seen as an experimental protocol mimicking a portion of the biological processes studied but sacrifices a part of reality in order to offer the opportunity for investigations. Important features determining the quality of a model thus encompass reproducibility (the capacity of a model to generate replicated data with low variance), robustness (the capacity of a model to remain constant under perturbation or uncertainty), and fidelity (the amount of reality that is modelled). Besides that, important technical considerations influence the relevance of a model to a biological question including: technical possibilities and compatibility with specific assays.

Early data of this project were generated on *in vitro* hBM-MSCs hepatogenic differentiation. While this model was successfully used to characterize the mitochondrial remodeling during *in vitro* hepatogenic differentiation (Wanet *et al.*, 2014) and to identify the involved signaling pathways (Wanet *et al.*, 2017), we did encounter several technical problems

for polysome profiling analyses, preventing us to draw any conclusion about translational regulation in this model.

First, hBM-MSCs yield very few amounts of biological material upon lysis. Protocols used in the literature (mostly from cancer and embryonic cells) describe the lysis of 300 cm^2 of cultured cells as sufficient to prepare a lysate of 20 OD 254 nm (Gandin *et al.*, 2014). In our hands, control and differentiated hBM-MSCs only yielded approximately 0.01 OD/cm^2 of cultured cells, forcing us to culture 1,800cm^2 of control or differentiating hBM-MSCs for polysome profiling. Oppositely, iPSCs yielded 0.06 OD/cm^2, allowing the preparation of a sufficient amount of material from 300cm^2, as mentioned in the literature. Of note, the profile obtained from hBM-MSCs was different from those classically reported in textbooks (see Fig. 10 for a schematic example of classical polysome profile), with a very poor polysome/subpolysome signal ratio (Fig. 14), which probably reflects a biological specificity of hBM-MSCs rather than technical issues, since (1) western blot and agarose gel electrophoresis confirmed the expected distribution of ribosomal subunits throughout the gradient and (2), other reports of polysome profiles generated from MSCs also present non classical profiles (Spangenberg *et al.*, 2013).

Second, beside those technical restrictions, the model itself presents limitations. The hBM-MSCs used in this work are primary cells originating from healthy donors, which constitutes a double-edged sword since it offers the potential to cover a larger genetic variability than a cell line but comes with a huge variability in the cell response to a common protocol: cells originating from certain donors did simply not differentiate when suggested to the hepatogenic protocol. Additionally, we noticed that "the culture history" of each hBM-MSC batch strongly influenced their potential to differentiate: in our hands, passaging (>p6), cell freezing (> 1 cryopreservation) or over-confluency dramatically impacted their hepatogenic differentiation potential. Of note, those observations reflect the current fate of MSCs in clinical trials. Indeed, while MSCs offer immunomodulatory properties, most of the clinical trials failed to confirm preclinical promises and sourcing, manufacturing and storage of MSCs are believed to introduce heterogeneity responsible for their poor efficacy in clinics (Levy *et al.*, 2020).

Altogether, the poor reproducibility and robustness, associated with the need to prepare considerable amount of input material led us to move toward a more suitable model of iPSCs-derived hepatogenic differentiation offering better reproducibility and robustness and suitable for the polysome profiling analysis.

Finally, considering experimental models as only recapitulating a fraction of the reality implies a direct consequence: multiplying the number and the nature of models supporting a hypothesis is required to increase confidence in this hypothesis. In this work, two different models of iPSCs hepatogenic differentiation (STD and HC3X) have been used and the large majority of the results gathering our attention regarding global protein synthesis and the regulation of TOP mRNA translation displayed similar patterns in both protocols. However, the great improvement of hepatogenic maturation observed in the HC3X protocol when compared with the STD protocol offers an interesting experimental scheme. Indeed, HC3X progeny displays a closer mitochondrial metabolic phenotype to freshly isolated PHH as well as an improved hepatic functionalities (Boon *et al.*, 2020). Thus, analysis of our data by comparing STD and HC3X protocols at D12 and D20 would allow to decipher whether translational regulation participates in the improvement of hepatocyte maturity phenotype of

HC3X cells. Moreover, our work is restricted to *in vitro* differentiation of iPSCs and confirmation of our observations in different models constitutes a very important perspective. This could be achieved by characterizing whether the correlation between LARP1 or EIF4G1 protein abundance repression and the global decrease in RPs abundance is also observed in human fetal and adult liver slices by immunofluorescence analyses. At the functional point of view, animal models have been extensively used for the study of liver development. Interestingly, pioneering work in the field of translational regulation of RPs include the observation of a decreased ribosome loading of mRNAs encoding RPs in rat adult liver compared to fetal liver, which is in agreement with our results (Aloni, Peleg and Meyuhas, 1992). Interestingly, it is currently possible to adapt our experimental approach to characterize global protein synthesis as well as specific translational remodeling in mice model : several examples of *in vivo* measurement of global protein synthesis using puromycin incorporation assay or classical labelled amino-acid incorporation as well as polysome profiling performed from tissue samples are reported in the literature (Calonne *et al.*, 2019; Charif *et al.*, 2020). Such experiments would be very informative to determine if our conclusions are applicable to *in vivo* liver development.

2. Translation is globally repressed during hepatogenic differentiation

In this work, we characterized a global downregulation of protein synthesis during hepatogenic maturation and this is associated with a global reduction of TOP mRNAs TE (including many RP-encoding mRNAs). Taking our results altogether, it is reasonable to assume that this decreased protein abundance of TOP mRNA-encoded proteins and the global downregulation of protein synthesis are interconnected in a differentiated cell presenting a phenotype of global translational repression. Further analyses regarding (1) the molecular pathways responsible for the regulation of TOP mRNAs and (2) the physiological impact of a decreased protein synthesis during differentiation, are required to better understand the link between translational regulation and hepatogenic differentiation.

- *Dynamics of global protein synthesis regulation*

Using two iPSC differentiation protocols, we characterized a global downregulation of protein synthesis during hepatogenic differentiation. This observation is somehow unexpected considering that: (1) hepatocytes represent "metabolic factories" involved in protein, carbohydrate and lipid metabolisms and are characterized by a high metabolic rate; (2) several lines of evidence support that PSCs maintain a low basal translation rate (Tahmasebi, Amiri and Sonenberg, 2019); and (3) several studies have demonstrated that differentiation of PSCs induces global upregulation of protein synthesis (Sampath *et al.*, 2008; Easley *et al.*, 2010; Guzzi *et al.*, 2018). It is worth noting that those investigations of global protein synthesis regulation during PSCs differentiation were limited to pluripotency exit and lineage commitment rather than terminal differentiation toward a precise cell type. The results of puromycin-incorporation assays performed during the pluripotency exit and lineage commitment step in the hepatogenic differentiation (corresponding to the first 4 days of the

STD and HC3X models), while not significant, are in line with those observations (Fig 16.). Therefore, we propose that, during hepatogenic differentiation, protein synthesis regulation follows a two-step mode where early events of pluripotency exit would be accompanied by a global transient upregulation of protein synthesis, while later cell specification and maturation steps would induce translational repression. Additionally, some other examples of more advanced differentiation protocols that also yield decreased global protein synthesis are described in the literature. This is the case for cardiomyocyte and neural differentiation (Baser *et al.*, 2019; Pereira *et al.*, 2019).

Interestingly, in addition to the global upregulation of protein synthesis observed upon pluripotency exit in a model of mESCs embryoid body formation, RPs abundance and rRNA synthesis are repressed, which could constitute an anticipating mechanism for a later decrease in protein synthesis (Ingolia, Lareau and Weissman, 2011; Woolnough *et al.*, 2016). This supports a revisited model of global translational regulation of stem cell differentiation where the activation of the translation machinery previously documented is only a transient step that is not maintained throughout the complete differentiation process. Finally, the fact that this observation has been reported in differentiated cells of the endodermal (our results), mesodermal (Pereira *et al.*, 2019) and ectodermal (Baser *et al.*, 2019) lineages argues that this mechanism is not restricted to hepatocyte nor endodermal-derived differentiation. However, whether this mechanism is a hallmark of differentiation would require further analyses including characterization of bulk protein synthesis at several steps of different differentiation processes. Additionally, the variability in quantification of the puromycin-incorporation western blot prevented us to reach significance, we thus suggest to perform those additional experiments using more suitable methods for quantifications, such as the gold standard 35^{s}-methionine incorporation assay.

- *Role of the global translational repression in differentiation*

The first question raised in our investigations was the potential role of the global translational repression phenotype, including downregulation of global protein synthesis and reduction of TOP mRNAs translational efficiency, in differentiated cells. As above mentioned, global translational repression is somehow unexpected considering the functions of hepatocytes. Since the regulation of TOP mRNAs expression was reported to impact cell proliferation and growth (see introduction, 3.5.2.), it is tempting to speculate that the phenotype of global translational repression is required for transitioning from a proliferative stem cell toward quiescent differentiated cell. Cell proliferation is timely regulated during *in vivo* hepatogenic differentiation (see introduction 2.1.) and three phases can be distinguished with respect to the proliferative status of (future) hepatocytes: a first proliferative step during liver development, followed by mature adult hepatocytes with arrested cell cycle and finally, reactivated proliferation when liver regeneration is requested (see introduction, 1.4.). This proliferative profile is clearly paralleled by the evolution of ribosomal translational efficiency in rat liver that is repressed at the end of development and reactivated during regeneration (Aloni, Peleg and Meyuhas, 1992). In the *in vitro* hepatogenic models we studied, differentiated cells still proliferate until D4-D5 prior to reach a fully confluent monolayer until D20, indicating proliferation arrest (although, this should be confirmed by additional

experiments such as KI 67 staining or deoxynucleotide incorporation assays). Again, translation of TOP mRNAs encoding RPs follows the same trend with a global decrease upon differentiation. When the molecular mechanisms responsible for the downregulation of TOP mRNA translation in our models will be better characterized, it could be interesting to interfere with these mechanisms in order to test a putative link between the global decrease in protein synthesis rate and proliferation arrest in hepatogenic differentiation.

- *Translational status of hepato-specific genes*

Beside global protein synthesis regulation, our objective was also to evaluate the contribution of translational regulation for hepatocyte proteome acquisition. Current opinions about the impact of transcription versus translation in different contexts include the definition of differentiation as a highly dynamic process requiring cell adaptation before the establishment of a new transcriptional program, which therefore, heavily relies on translational regulation (Liu, Beyer and Aebersold, 2016; Tahmasebi, Amiri and Sonenberg, 2019). It should be noted that, while this definition may be adapted to pluripotency exit, it does not precisely correspond to a lengthy and progressive process required to fully differentiate cells *in vivo*. This is in agreement with the fact that those conclusions have been drawn based on limited models of differentiation (such as THP1 activation toward macrophages, 48h differentiation of C2C12 myoblasts toward myotubes or Nanog repression in ESCs) rather than a complete differentiation process from PSCs (Lu *et al.*, 2009; Kristensen, Gsponer and Foster, 2013). Beside this, considering differentiation as a process mainly regulated by translational regulation is not realistic since many genes encoding functional proteins of the differentiated cells are not or very modestly transcribed in stem cells. This idea is supported by our results since the scatter plots of Log2FC mRNA abundance in HP and total RNA samples in every comparison between differentiated cells and control iPSCs show that the large majority of mRNAs are distributed on the diagonal of the graph (Fig. 17) indicating that the magnitude of the transcriptional regulation is stronger than translational regulation. This suggests that the concept of translational regulation primarily regulating cell fate may be restricted to pluripotency exit rather than complete differentiation.

Analyses of evolution of transcripts translational efficiencies showed positive enrichment of several terms associated with different metabolic pathways including fatty acid, amino acid and glucose metabolism, cellular respiration and cellular detoxification. Interestingly, similar observations have been documented in a different model of hepatogenic maturation of HepaRG immortalized hepatic progenitors (Parent and Beretta, 2008). Indeed, micro-array results of polysome-bound and total mRNAs of differentiated HepaRG cells showed increased polysome-bound abundance of several mRNAs encoding proteins involved in lipid and drug metabolism such as the Fatty Acid Synthase (FASN), which we also found as differentially translated in this study. As hepatocytes are well known for their high metabolic activity involved in carbohydrate, lipid, protein and exogenous compounds (Liu *et al.*, 2017), our results thus support that the global translational reprogramming occurring during hepatogenic differentiation might contributes to the metabolic maturation of differentiated hepatocytes by inducing expression of the metabolic protein machinery.

Finally, regarding hepato-specific transcript translational regulation, while a significant

number of hepato-specific mRNA markers were excluded from the Anota2seq analysis, gene ontology analysis still highlighted several mRNAs encoding metabolic enzymes involved in cellular detoxification such as ADH6, ALDH3B1 or SULT1A2 as being translationally regulated. Whether a common specific mechanism of translational upregulation of these candidates is involved in hepatogenic differentiation remains an interesting open question. FGL1 is another transcript that is translationally induced during hepatogenic differentiation (Fig. 17). This hepatokine, initially identified as upregulated in regenerating liver (Hara *et al.*, 2001), is proposed to play an autocrine role regulating different aspects of hepatocyte biology such as proliferation, liver injury protection, and lipid metabolic crosstalk with adipocytes (Liu and Ukomadu, 2008; Li *et al.*, 2010; Demchev *et al.*, 2013). Regulation of FGL1 expression and its role in differentiation are currently not characterized while our data suggest that translational regulation may be involved in the expression of this protein during differentiation. Additionally, our results also showed consistent TE of some key hepatic genes in differentiated cells (fig. 17).

In order to go one step further in the understanding of translational regulation during hepatogenic differentiation, the identification of the mechanisms promoting translation of metabolic protein machinery are required. In agreement with this, an interesting perspective of this work would be to analyze the mRNA sequences of differentially translated transcripts. This could be achieved by characterizing their 5'UTR length and structuration as well as by searching for motif enrichment in 5'UTR and 3'UTR to retrieve potential RBP or miRNA responsible for translational control (McLeay and Bailey, 2010).

3. Regulation of TOP mRNAs during hepatogenic differentiation

During STD and HC3X differentiation, both quantitative proteomic and polysome profiling analyses showed a decrease in protein abundance and TE of the components of the translation machinery, including many RPs. Many components of the translation machinery are encoded by TOP mRNAs and analysis of the TE, mRNA and protein abundance of a recently identified group of "core TOP mRNAs" (Philippe *et al.*, 2020) indicated a global repression of translation throughout hepatogenic differentiation followed by a decrease in mRNA abundance in hepatocyte maturation. A central question raised by those observations concerns the identification of the regulating mechanisms of TOP mRNAs translation during differentiation. A precise understanding of the molecular mechanism of TOP mRNA translational regulation is lacking, but current hypotheses converge toward a model wherein LARP1 acts as a molecular switch downstream of mTOR serving both for translational repression and increase in mRNA stability of TOP mRNAs upon mTORC1 inhibition (See introduction, 3.5. (Berman *et al.*, 2020)). This section discusses the different hypotheses of TOP mRNA translational regulation mechanisms in hepatogenic differentiation.

- *mTOR pathway*

Our results indicate that mTORC1 is not differentially activated during hepatic specification or maturation in cells undergoing the STD or the HC3X protocol (Fig. 19). The fact that both protocols showed similar mTORC1 activation status is somehow unexpected

since (1) mTORC1 is a well-known sensor of amino acid deprivation; (2) HC3X differentiation medium is substantially supplemented with amino acids; (3) induction of CYP3A4 expression upon stem cell hepatogenic differentiation or amino acid-induced maturation of HepG2 cells is inhibited by rapamycin (Boon *et al.*, 2020). Interestingly, this latter result is in line with previous observations of rapamycin-treated fetal rat livers presenting differentially expressed genes linked to "xenobiotics by cytochrome P450", "fatty acid metabolism" or "tryptophan metabolism" (Gruppuso, Boylan and Sanders, 2011). Additionally, further investigations by the same group showed that mTORC1 signaling has a differential effect on TOP mRNAs translational regulation in late fetal versus adult hepatocytes (Gruppuso *et al.*, 2008; Boylan *et al.*, 2015). Indeed, while rapamycin is able to inhibit translation of TOP mRNAs in adult hepatocytes, this effect is not observed in fetal hepatocytes and groups of differentially translated genes in fetal or adult hepatocytes differ. Altogether, these results allow us to hypothesize that, although not stimulated during differentiation, basal mTORC1 activity is required for hepatocyte differentiation. Additionally, mTORC1-mediated translational control in fetal hepatocytes is not classical and does not control TOP mRNAs.

- *LARP1*

We also characterized a strong downregulation of LARP1 protein abundance both during hepatic specification and maturation (Fig. 19). This result should be first confirmed *in vivo* by immunofluorescence analyses in fetal versus adult livers. Following Occam's razor principle, the most straightforward hypothesis explaining the mechanisms of TOP mRNA translational regulation would be that, in differentiated hepatocytes, LARP1 phosphorylation status makes it a promoter of TOP mRNA translation. According to this model, downregulation of LARP1 protein abundance would be responsible for a decrease in the translation of TOP mRNAs. This situation would correspond to previously described biological contexts associated with proliferation arrest where LARP1 depletion induces a global decrease in protein synthesis and reduction of TOP mRNA translational efficiency (Burrows *et al.*, 2010; Tcherkezian *et al.*, 2014). Interestingly, those results have been generated on cancer cell models (HeLa and HEK293T, respectively), while the role of LARP1 protein abundance has been shown as a negative prognosis marker for different cancers (Xie *et al.*, 2013; Mura *et al.*, 2015; Ye *et al.*, 2016; Xu *et al.*, 2017). None of those 3 studies investigated the impact of LARP1 depletion on the translation of TOP mRNAs, but LARP1 depletion induces reduction of growth and proliferation of cancer cells and promotes cancer stem cell-like features in OVCAR3 (an ovarian adenocarcinoma cell line). Thus, decrease in LARP1 protein abundance would impact the translational efficiency of TOP mRNAs, resulting in proliferation arrest of the differentiated hepatocytes. To test this hypothesis, it would be of interest to restore LARP1 protein abundance level during differentiation by lentivirus-mediated transduction of a LARP1 overexpression plasmid and to characterize the translational efficiency of a synthetic TOP mRNA by dual luciferase assay (i.e. an overexpressed luciferase mRNA bearing a TOP sequence used as a readout of the TE of this family of transcripts). This would allow us to confirm the link between LARP1 protein abundance and TOP mRNA translation and further indicate the consequence of TOP mRNA translational repression during hepatocyte differentiation.

It is important to note that investigations of LARP1 functions are currently limited by the fact that LARP1 phosphorylation sites and their regulatory function(s) are not identified but are supposed to strongly impact the function of the protein, resulting in the ability of LARP1 to repress translation, induce translation, or stabilize TOP mRNAs. In our model, such regulations would further complicate the straightforward hypothesis of LARP1-depletion mediated reduction in TOP mRNAs TE. It can be reasonably assumed that this is not the case for mTORC1 regulation since our results did not show differential activation of the pathway during differentiation. However, a role for other kinases susceptible to regulate LARP1 cannot be excluded. As an example, Cyclin-Dependent Kinase 1 (CDK1) has recently been described as a LARP1 kinase controlling global protein synthesis and TOP mRNA translation (Haneke *et al.*, 2020). Thus, whether additional post-translational regulations of LARP1 play a role in the regulation of TOP mRNAs remains unknown.

An interesting experiment that could provide further information on the status of LARP1 regulation of TOP mRNAs would be RNA-immunoprecipitations (RNA-IP). Indeed, Fonseca and colleagues successfully showed a decrease in TOP mRNA binding by LARP1 upon mTORC1 inhibition by RNA-IP (Fonseca *et al.*, 2015). We thus aimed at replicating this result but, importantly, we added a non-TOP mRNA as a negative control (data not shown). Unfortunately, the assay turned out to be non-TOP mRNA specific, since we observed a similar increase of pulled-down mRNA for both TOP mRNAs and negative controls. Of note, careful investigation of the literature led us to find a pre-print publication of the same group including negative controls, that confirms our observations (Fonseca *et al.*, 2018). It is likely that the ability of LARP1 to bind PABP via its PAM2-l motif is responsible for the non-specificity of the assay. This could potentially be elegantly bypassed by setting up a specific version of the RNA-IP that integrates a PABP competitive binding inhibitor in order to displace PABP from poly-A tails. Poly(A)-SPOT ON, a chemically-modified RNA based inhibitor of PABP has been developed to bind PABP with higher affinity than poly-A tails *in vitro* (Barragán-Iglesias *et al.*, 2018). Thus, ambitious technical optimization of such a strategy could be very beneficial in the field of TOP mRNAs translational regulation and LARP1 activity.

- *EIF4F complex assembly*

Our investigations of mTORC1 pathway activation led us to assess the formation of EIF4F complex assembly during differentiation. A preliminary single replicate of this experiment confirmed that mTORC1 is not differentially activated since 4EBP1 binding to EIF4E remains constant throughout differentiation. Oppositely, differentiated cells displayed a reduced EIF4F complex assembly that is associated with a strong decrease in EIF4G1 protein abundance.

EIF4F complex assembly has also been reported to impact translation of TOP mRNAs. This is based on the observation that EIF4G1 knockdown selectively inhibits translational efficiency of TOP mRNAs (Thoreen et al., 2012). It could actually be argued that this effect is mediated by differential affinity of EIF4E for the mRNA cap, which depends on the +1 nucleotide (Thoreen et al., 2012). Indeed, although EIF4E recognizes all nuclear-encoded mRNAs, the binding affinity varies depending on cap-proximal nucleotides, with the lower affinity corresponding to mRNAs with a +1 cytidine, as observed in a relatively low proportion of transcripts (Keys and Sabatini, 2017; Tamarkin-Ben-Harush et al., 2017). Reduction of

EIF4F complex availability would impact more strongly the translation of transcripts characterized by a lower affinity with EIF4E.

Therefore, another hypothesis explaining the reduction of translational efficiency of TOP mRNAs upon hepatogenic differentiation would be that the decrease in EIF4G1 protein abundance make it less available for EIF4F complex assembly. Thus, the reduction in EIF4F complex would be responsible for the global decrease in protein synthesis associated with a selectively more pronounced decrease of translational efficiency of TOP mRNAs. Again, testing this hypothesis by overexpressing EIF4G1 in the *in vitro* hepatogenic differentiation models would be necessary.

4. Conclusion and key perspectives

In this work, we concluded that *in vitro* iPSCs derived hepatocyte-like is a more suitable model for the study of translational regulation than hBM-MSCs. By characterizing the global protein synthesis rate and performing polysome profiling analyses of cells undergoing hepatic specification or hepatocyte maturation, we showed that *in vitro* hepatogenic differentiation displays a repressed translational phenotype including global repression of protein synthesis and decrease in RPs abundance. We therefore propose a revisited model of global protein synthesis regulation of stem cell differentiation occurring in two steps where a primary upregulation of translation is followed by a global repression. This phenotype is accompanied by a repression of TOP mRNAs translation, a class of mRNAs encoding many components of the translation machinery including nearly all RPs.

Although the precise mechanism(s) regulating translation of TOP mRNAs is currently lacking, our results allow to propose two non-exclusive hypotheses of such translational regulation in our models. First, our results showed a decrease in LARP1 protein abundance during differentiation. Since LARP1 is a key RBP controlling TOP mRNAs translation and stability, this could result in repression of TOP mRNA translation. Second, EIF4G1, the scaffold protein of the EIF4F complex involved in cap-dependent translation, also presents a decreased protein abundance upon differentiation. This could result in decreased cap-binding capacity of the complex on TOP mRNAs due to the TOP sequence defined by a cytidine as first nucleotide.

Together with the global decrease in protein synthesis, our results also show translation induction of mRNAs encoding various axes of metabolism including fatty acid, amino acid and glucose metabolism, cellular respiration and cellular detoxification, in agreement with the global metabolic maturation required for hepatocyte differentiation. This indicates that translational regulation might participates in the metabolic maturation in these models of differentiation.

Importantly, while this project generated several interesting sets of omics data, the requirement for extensive time-consuming technical optimization limited the possibilities to further dive into the results produced. Thus, this project opens doors for different types of perspectives that will hopefully further add value to the time and money invested in this research question. Practically, it would be first interesting to extend the conclusion of our translatomic experiments by further *in silico* analyses. Indeed, the comparison of translatomic data from the STD and HC3X experimental conditions would allow us to investigate whether

translational regulation plays a role in the acquisition on the improved hepatocyte maturity phenotype observed in HC3X progeny when compared with the STD progeny. Additionally, in order to identify other potential hallmarks of mRNAs translationally regulated in our conditions, a motif enrichment analysis could be performed on the set of translationally regulated mRNA in order to decipher a potential role for regulation through specific sequence in the 5' or 3'UTR of mRNAs during hepatogenic differentiation. This has already been undergone in the lab using the MeMe suite and is the goal of a master project (McLeay and Bailey, 2010). Finally, the sequencing depth used in our translatomic analysis has been designed to allow the extension of the mapping step at the transcript level. Using those data could help us to decipher a potential role for alternative splicing on translational regulation during hepatogenic differentiation, as this has been previously done in the literature (Floor and Doudna, 2016). Secondly, it would be interesting to further strengthen and validate the results described in this manuscript. On one hand, we could further confirm the puromycin-incorporation assay results by additional experiment that are more suitable for quantification, such as 35^{s}-methionine incorporation assay in order to validate our two-step model of global protein synthesis regulation. On the other hand, an important perspective is indeed the validation of our results *in vivo*. Characterization of the abundance of RPs, LARP1 or EIF4G1 by immunohistochemistry in embryonic, fetal or adult livers would allow to determine to which extent our results are applicable *in vivo*. Third, to investigate further the role of TOP mRNA translational regulation and its regulating mechanisms, the setup of cell biology tools in our models, such as protein overexpression by lentiviral-mediated transduction or the use of luciferase reporter as readout for translational efficiency are of major importance.

Material and methods

1. List of reagents

Table 1

Reagent	Manufacturer	Reference
Recombinant human EGF	Peprotech	AF-100-15
Recombinant Human FGF-basic	Peprotech	100-18B
Recombinant Human HGF	Peprotech	100-39
Recombinant Human Oncostatin M	Peprotech	300-10
DMEM-LG	Gibco	11885084
Rat tail collagen	Corning	354236
Matrigel hESC-Qualified Matrix	Corning	354277
DMEM/F-12(1:1)	Gibco	31330-038
mTESR PLUS kit	StemCells	5825
Y27632 dihydrochloride	Axon medchem	1683
Accutase	StemCells	7920
Penicillin-Streptomycin	Gibco	15140-122
Matrigel Growth Factor Reduced	Corning	354230
DMEM LG	Gibco	31885-023
MCDB 201 Water	US biological	C4000-05
L-Ascorbic acid	Sigma	A8960
ITS	Gibco	41400-045
LA-BSA	Sigma	L9530
B-mercaptoethanol	Gibco	31350-010
Dexametasone	Sigma	D2915
MEM-NEAA	Gibco	11140-035
MEM-AA	Gibco	11130-051
Glycin	CarlRoth	HN07.1
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
DMSO	CarlRoth	A994.1
Cycloheximide	Sigma	01810
EDTA-free complete protease inhibitor cocktail	Roche	11873580001
RNAse inhibitor	Applied	4469082

	Biosystems	
Tri-Reagent Solution	Invitrogen	AM9738

2. Cell culture

2.1. Culture surface coating

Culture of Stem Cells initially required a proper culture substrate including the presence of feeder cells such as cell-cycle-arrested mouse embryonic fibroblasts (MEF) (Hayashi and Furue, 2016). In this work, we used a feeder-free cell culture system consisting in the coating of cell culture surface by different culture substrates containing extra-cellular matrix components.

Cell culture surfaces used for differentiated hBM-MSCs were coated with 5µg/cm^2 of rat tail type-1 collagen diluted at 50µg/ml in 0.02M acetic acid. Dishes were coated by 2h incubation at RT followed by air drying of the surface and storage at 4°C. For hiPSCs culture maintenance, cell culture surfaces were coated with hESCs qualified-matrix Matrigel diluted in DMEM/F-12 following the lot-specific dilution factor provided by the manufacturer. Coating was made by 1h incubation at 37°C immediately followed by cell seeding. For hiPSCs differentiation experiments, dishes were coated with 1/62.5 Growth Factor reduced Matrigel diluted in DMEM/F-12 using a similar protocol.

2.2. hBM-MSCs stem cells culture maintenance

hBM-MSCs originating from different healthy donors used in this work were obtained in cryotubes from the Clinical Cellular Therapy Research Laboratory (LTCC), Jules Bordet Institute (Belgium). hBM-MSCs were thawed and routinely cultured using DMEM low glucose (DMEM-LG) supplemented with 1% penicillin-streptomycin (pen-strep) and 10% fetal bovine serum (FBS). Cell passaging was made by Trypsin-EDTA (0.05%) cell detachment followed by seeding 5000 cell/cm^2 (corresponding to approx. 1/5-1/8 passage depending on the donor). Cells were kept in culture for only 7 passages (from initial cell isolation). Experiments were performed on 3 biological replicates, unless specified otherwise.

2.3. hBM-MSCs *in vitro* hepatogenic differentiation protocol

In vitro hepatogenic differentiation of hBM-MSCs consisted in seeding 10 000 cell/cm2 on type-1 collagen coated dishes until 85% confluency, corresponding to the D0 control condition. For Differentiated conditions, medium was then replaced by 1% penicillin-streptomycin Iscove's Modified Dulbecco's Medium (IMDM) supplemented with corresponding cytokines cocktails (see Table 2). Control cells were cultured in parallel with IMDM supplemented with 1% FBS and 1% pen-strep.

Table 2: hBM-MSCs differentiation medium supplements

Days of differentiation	Supplement
D0-D2	20ng/ml EGF 10ng/ml FGF2
D2-D12	20ng/ml HGF 10ng/ml FGF2 0.61mg/ml Nicotinamide 1x ITS
D12-D22	20ng/ml OSM 1µM Dexamethasone 1x ITS

2.4. hiPSCs stem cells culture maintenance

BJ1 HC3X iPSCs inducible for HNF1A, FOXA3 and PROX1 (Boon *et al.*, 2020) were cultured on hESCs qualified-matrix Matrigel coated surfaces in mTESR PLUS medium supplemented with 1% penicillin-streptomycin. Cells were passaged every 5-6 days by accutase detachment and plated with 1/10 dilution in medium culture supplemented with 10µM ROCK inhibitor Y27632 for the first 24h prior to replacement with fresh medium.

2.5. hiPSCs hepatogenic differentiation protocols

Standard and HC3X hepatogenic protocols were conducted as described in (Boon *et al.*, 2020). 52.10^3 cells/cm^2 were seeded on Growth Factor reduced Matrigel coated surfaces in culture medium supplemented with 10µM ROCK inhibitor Y27632 for the first 24h. Cells were then cultivated in regular culture medium for an additional 24h until colonies reach 80% confluency. Standard differentiation was conducted by incubating cells with Liver Differentiation Medium (LDM, see Table 3. for complete formulation) supplemented with the corresponding cytokine cocktails (see Table 4). For HC3X differentiation, LDM-AA was prepared by supplementing 100ml of LDM with 16ml of MEM-NEAA and 8ml of MEM-AA prior pH7.2 adjustment. LDM-AAGLY was prepared by supplementing LDM-AA with 20g/l glycin. LDM, LDM-AA, and LDM-AAGLY were then supplemented with the corresponding cytokine cocktails (Table 4). Medium were replaced every other day during the 20 days of differentiation for both protocols.

Table 3: Liver differentiation medium (LDM) composition

Reagent	Final concentration
DMEM LG	57%
MCDB Water pH 7.2	40%
Penicillin streptomycin	1%
L-Ascorbic Acid	0.1µM
ITS	0.25x

LA-BSA	0.25x
β-mercaptoethanol	50μM
Dexamethasone	1μM

Table 4: hiPSCs differentiation medium supplements

Days of differentiation	Differentiation type	Medium	Supplement
D0-D2	STD	LDM	50ng/ml Activin A 50ng/ml WNT3a 0.6% DMSO
D2-D4	STD	LDM	50ng/ml Activin A 0.6% DMSO
D4-D8	STD	LDM	50ng/ml BMP4 0.6% DMSO
D8-D12	STD	LDM	20ng/ml aFGF 0.6% DMSO
D12-D20	STD	LDM	20ng/ml HGF 2% DMSO
D4-D8	HC3X	LDM	50ng/ml BMP4 0.6% DMSO 5μg/ml Doxycycline
D8-D12	HC3X	LDM	20ng/ml aFGF 0.6% DMSO 5μg/ml Doxycycline
D12-D14	HC3X	LDM-AA	20ng/ml HGF 2% DMSO 5μg/ml Doxycycline
D14-D20	HC3X	LDM-AAGLY	20ng/ml HGF 5μg/ml Doxycycline

3. General molecular biology techniques

3.1. RT-qPCR analysis

Total RNA samples were extracted from control and differentiating cells using Reliaprep RNA Miniprep System (Promega Z6010) following manufacturer's instructions. RNA was reverse transcribed using GoScript reverse transcriptase with random primers (Promega A2791). cDNA was then analyzed by real-time qPCR using a GoTaq qPCR Master Mix (Promega, A6002) on a ViiA 7 Real-Time PCR System (Thermo Fisher). Used primers are listed in Table 5. Differentiation marker expression was calculated as a relative expression

normalized on housekeeping gene UBE3C using the $2^{-\Delta Ct}$ method.

Table 5: RT-qPCR primer sequences

Gene	Forward primer	Reverse primer	Source
HNF1a	ACACCTCAACAAGGGCACTC	TGGTAGCTCATCACCTGTGG	(Boon et al., 2020)
FOXA3	ATTCTCTCTGGCATGGGTTG	AAATTCCCCACACCCTAACC	
PROX1	TCACCTTATTCGGGAAGTGC	GGAGCTGGGATAACGGGTA	
GATA4	TCCAAACCAGAAAACGGAAG	CTGTGCCCGTAGTGAGATGA	
HNF4A	ACTACGGTGCCTCGAGCTGT	GGCACTGGTTCCTCTTGTCT	
AAT	AGGGCCTGAAGCTAGTGGAT	TCCTCGGTGTCCTTGACTTC	
ALB	ATGCTGAGGCAAAGGATGTC	AGCAGCAGCACGACAGAGTA	
CYP3A4	TTCCTCCCTGAAAGATTCAGC	GTTGAAGAAGTCCTCCTAAGCT	
POU5F1	ACATCAAAGCTCTGCAGAAAGAACT	CTGAATACCTTCCCAAATAGAACCC	
UBE3C	TTTCCCATTGCTAATGGCC	CTGATACAGCCATATCAAACGT	GetPrime #2079621
HNF1B	GCAAAAGAACCCCAGCAAGG	CAGAGGGTTCAGGCTGTGAG	
FGL1	ATTGTGACATGTCCGATGG	TTCATAGTCTTTCCATCCTCTG	GetPrime #1840886
RPL21	AAACATGGAGTTGTTCCTTTGG	AGTACCCATTCCCTTGATGTC	GetPrime #1889390
EEF2	TCTTCAAGGTGTTTGATGCG	CCAGTTTGATGTCCAGTTTCTC	GetPrime #1955377
RPL13	TCCGGAACGTCTATAAGAAGG	ATACGGAGACTAGCGAAGG	GetPrime #2087768
RPS6	GAGAATGAAGGAGGCTAAGG	GAAGTAGAAGCTCGCAGAG	GetPrime #1861997
EIF3F	TGCAGAGGATGTACTGTCTG	GGTACTTGGTTAACCAGGCT	GetPrime #1884548
ACTB	AGAAGGATTCCTATGTGGGC	TACTTCAGGGTGAGGATGC	GetPrime #2013505

3.2. Western Blot analysis

Control and differentiating cells at indicated timepoints were rinsed twice in ice-cold PBS prior to cell scraping in lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 15% Glycerol, 1% Triton X-100, 2% SDS, 1x Complete protease inhibitor cocktail, 25mM Na_3VO_4, 250mM 4-nitrophenylphosphate, 250mM glycerophosphate, 125mM NaF, 0.17u/µl Supernuclease). Lysates were incubated 10min at 12°C with medium agitation and cleared by centrifugation at 16 000xg for 10min at 12°C. Protein concentration was measured in supernatant using Pierce 660 nm Protein Assay Reagent (ThermoFischer Scientific, 22660) according to manufacturer's instructions. 10-50µg of proteins were resolved in NuPage 4-12% bis-tris gels (Invitrogen, NP0321BOX) prior to transfer onto PVDF membrane. Primary antibodies, secondary infrared dye-coupled antibodies were diluted in Intercept (PBS) Blocking Buffer (Li-Cor Biosciences, 927-70001) prior to immunodetection using an Odyssey Infrared Imager (Li-Cor Biosciences) (see Table 6 for complete list of antibodies and their dilution).

Table 6: Antibodies

Antibody	Dilution	Supplier	Reference
Anti-RPL13A	1/1000	Cell Signaling	#2765

Anti-RPS5	1/1000	Santa Cruz	sc-390935
Anti-LARP1	1/1000	Abcam	ab86359
Anti-β-Actin	1/20000	Sigma	T5168
Anti-Puromycin	1/5000	Merck Millipore	MABE343
Anti-RPS6	1/1000	Cell Signaling	2217
Anti-mTOR	1/1000	Cell Signaling	2972
Anti-P-mTOR(S2448)	1/1000	Cell Signaling	2971
Anti-4EBP1	1/2000	Cell Signaling	9644
Anti-4EBP1(T37/46)	1/1000	Cell Signaling	2855
Anti-S6K1	1/1000	Cell Signaling	9202
Anti-P-S6K1(T389)	1/1000	Cell Signaling	9205
Anti-P-RPS6	1/1000	Cell Signaling	2215
Anti-EIF4E	1/1000	Santa Cruz	SC9976
Anti-EIF4G1	1/1000	Santa Cruz	SC133155
Anti-EEF2	1/1000	Abcam	ab40812
Goat anti-Rabbit IgG	1/10000	Li-Cor Bioscience	926-32211
Goat anti-Mouse IgG	1/10000	Li-Cor Bioscience	926-32210
Goat anti-Mouse IgG	1/10000	Li-Cor Bioscience	926-68070

4. Periodic Acid Schiff (PAS) staining

PAS staining was performed using the Sigma-Aldrich PAS kit (395B-1KT). Control and differentiating hBM-MSCs at the end of the differentiation process (D22) were trypsinized and seeded on collagen-coated coverslips at 45000 cells/cm^2. After overnight attachment, cells were washed 3x with ice cold PBS and fixed using 4% paraformaldehyde for 20min. Cells were stained by 10min incubation with 1% periodic acid, washed 3x with ddH_2O and incubated 15min with Schiff reagent. Coloration was developed by 10 1min-washes using tap water. Coverslips were mounted on slides using Mowiol and left to harden overnight at 4°C prior to observation with phase contrast microscope.

5. Translation experiments

5.1. Puromycin-incorporation assay

Puromycin-incorporation assay (Schmidt *et al.*, 2009) was achieved by treating cells with

5µg/ml puromycin in culture medium for 10min prior to lysis and western blot analysis as described in dedicated western blot section. Western Blot Analysis using anti-puromycin antibody. Cells left untreated, or cells treated with 20µg/ml cycloheximide 30min before puromycin treatment were used as negative controls.

5.2. Polysome fractionation

The polysome fractionation protocol was adapted from (Gandin *et al.*, 2014). 12x150 cm or 2x150 cm plate of control or differentiated cells (for hBM-MSCs and hiPSCs, respectively) were incubated with 0.1mg/ml cycloheximide for 5min in control or differentiation medium. Cells were then rinsed twice in 0.1mg/ml cycloheximide ice-cold PBS, scrapped in 500µl of polysome lysis buffer (5mM Tris-HCl pH7.4, 1.5mM KCl, 2.5mM $MgCl_2$, 0.1mg/ml cycloheximide, 100units/ml RNAseIN, 1x EDTA-free Complete protease inhibitor cocktail, 2mM DTT, 0.5% Triton X-100, 0.5% deoxycholate) and incubated on ice for 10min. Lysates were cleared by centrifugation at 16,000g for 7min at 4°C and 10-15 OD (Abs 260 nm) were loaded on a continuous 10-50% sucrose density gradient prepared in a buffer containing 20mM HEPES pH 7.6, 100mM KCl, 5mM $MgCl_2$, 0.1mg/ml cycloheximide, 1x EDTA-free Complete protease inhibitor cocktail and 100 unit/ml RNAseIN. Gradients were centrifuged at 35,000 RPM for 3h in a SW41Ti rotor at 4°C. Gradients were then fractionated in 24 fractions from the top to the bottom using a Foxy Jr. fraction collector (Teledyne ISCO) with simultaneous measurement of absorbance at 254 nm using a UA-6 cell (Teledyne ISCO). Graphs of absorbance at 254 nm along the sucrose density gradient were then manually retraced and numerized. For each experimental condition, ImageJ software was used to calculate the area under the curve of polysomal signal which was normalized by area under the curve of total signal in order to identify the percentage of polysome area. For hBM-MSCs Polysome profiles, 250µl of fractions were used for Tri-reagent RNA extraction according to manufacturer's instructions followed by agarose gel electrophoresis in the presence of Ethidium Bromide. Similarly, 200µl of each fraction was used for Trichloroacetic acid/Acetone protein precipitation: samples were mixed with 1.6ml of ice-cold 100% acetone and 200µl of 100% trichloroacetic acid (TCA) prior to incubation at -20°C for 1h. Sample were then centrifuged at 18,000xg for 15min at 4°C, supernatants were discarded and pellets were incubated with 1ml ice-cold acetone supplemented with 0.3g/ml DTT for 15min at 4°C. Samples were centrifuged at 18,000xg for 15min at 4°C and supernatant was discarded. Pellets were air-dried for 2-3min and resuspended in Western blot Sample buffer prior to Western blot Analysis as described in dedicated section for Western blot.

5.3. Cap-binding assay

The cap-binding assay (CBA) protocol was adapted from (Tahmasebi *et al.*, 2016). Briefly, at indicated timepoints, control or differentiating cells were washed twice with ice-cold PBS followed by cell scraping in CBA lysis buffer (50mM MOPS-KOH pH7.4, 100mM KCl, 0.02mM NaN_3, 0.5mM EDTA, 1% NP40, 1% Na Deoxycholate, 1x Complete protease inhibitor cocktail, 25mM Na_3VO_4, 250mM 4-nitrophenylphosphate, 250mM

glycerophosphate, 125mM NaF). Lysates were incubated in lysis buffer for 10min and centrifuged at 16,000xg for 10min at 4°C. Protein concentration in lysates was measured by Pierce Protein Assay and 500µg of proteins were resuspended in a final volume of 1ml of CBA Washing Buffer (CBA-WB, 50mM MOPS-KOH pH7.4, 100mM KCl, 0.02mM NaN_3, 0.5mM EDTA) (for this step, CBA-WB was supplemented with 1x Complete protease inhibitor cocktail, 25mM Na_3VO_4, 250mM 4-nitrophenylphosphate, 250mM glycerophosphate, 125mM NaF) and incubated 30min at 4°C with 50µl of 7-methyl guanoside (M7GTP)-coated agarose beads under mild agitation. Beads were washed three times by 1ml of CBA-WB. Elution was performed by incubating beads in CBA elution buffer (CBA-EB, corresponding to CBA-WB supplemented with 0.2mM M7GTP) for 15min at 4°C under agitation. Eluates were collected and analyzed by Western blot (following the protocol described in the dedicated section) for detection of EIF4E, EIF4G1 and 4EBP1.

6. Polysome profiling

This section describes the use of polysome fractionations prepared for further RNA-seq or qPCR analysis.

6.1. Preparation of fraction pools for mRNA analysis

Polysome fractions were further analyzed by RNA-seq or RT-qPCR, which both required different sample preparation protocols. For RNA-seq, fractions containing HP were identified based on the Polysome profile and similar volumes were collected and pooled as a 500µl "HP RNA Sample" (for the majority of experimental conditions HP were distributed from fraction 12 to 22 thus 62.5µl were collected in those 8 fractions). In addition, for each experimental condition, a volume of total polysome lysate corresponding to 1.5 OD at 254 nm (equivalent to 10% of the input sample engaged in polysome fractionation) was diluted in a final volume of 500µl gradient buffer and constituted the "Total RNA Sample". For RT-qPCR validations, 100µl of fractions were pooled 3 by 3 to prepare pooled fractions A to H prior to addition of 1ng of exogenous Renilla Luciferase RNA as spike-in RT-qPCR control.

6.2. Column-based RNA extraction from fractions

For RNA extractions, we used an adapted version of the Reliaprep RNA Miniprep System (Promega Z6010). HP RNA Samples, Total RNA Samples or Pooled fractions were mixed with 1 volume of Lysis Buffer (LBA) and 0.7 volume of isopropanol prior to proceeding the rest of the extraction with the manufacturer's instructions.

6.3. RNA-seq

RNA-seq library preparation was achieved using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB). Sequencing libraries were validated using NGS Kit on the Agilent 5300 Fragment Analyzer (Agilent

Technologies) and quantified by using Qubit 4.0 Fluorometer. 2x150 Paired-End sequencing was then performed using Illumina NovaSeq 6000 instrument according to manufacturer's instructions. Quality of raw sequencing data was validated prior to sequence trimming and alignment on ENSEMBL *Homo sapiens* reference genome using Trimmomatic V.0.36 and STAR aligner v.2.5.2b. Unique gene hit counts were calculated by using feature Counts from the Subread package v.1.5.2. Data are available on Gene Omnibus repository under accession GSE173106.

6.4. RT-qPCR on fractions pools

Half of the RNA extracted from pooled fractions was reverse transcribed using GoScript reverse transcriptase with random primers (Promega A2791) according to manufacturer's instructions. cDNA were diluted 1/25 and analyzed by RT-qPCR using a GoTaq qPCR Master Mix (Promega, A6002) on a ViiA 7 Real-Time PCR System (Thermo Fisher). For each gene of interest, the relative abundance fold change of the gene of interest over spike-in luciferase was first calculated (data for fractions A and H, generally presenting aberrant results, were removed at this step). For each pool of fractions of an experimental condition, relative distribution of mRNA was then calculated by normalizing relative abundance fold change for this fraction to the sum of relative abundance fold change in all fractions of this experimental condition. Finally, the sum of relative distribution of mRNA in pooled fractions F and G (those containing HP) was used as an RT-qPCR-derived TE.

7. Label-free mass spectrometry analysis

For proteomic analysis, cells were lysed as mentioned in the section dedicated to western blot. Lysates were prepared by a modified filter-aided sample preparation (FASP) protocol (Wiśniewski *et al.*, 2009; Distler *et al.*, 2014) using modified trypsin (Promega V511A 11439101) on microcon filters (Millipore Microcon 30 MRCFOR030 Ultracel PL-30). The digest was analyzed using nano-LC-ESI-MS/MS tims TOF Pro (Bruker) coupled with an UHPLC nanoElute (Bruker). For peptide separation, a 25cm C18 column with integrated CaptiveSpray insert (Aurora, Ionopticks) was used using 0.1% formic acid H_2O as liquid chromatography mobile phase A and 0.1% formic acid Acetonitrile as phase B. The digest (1 µl) was injected, and the organic content of the mobile phase was increased linearly from 2% B to 15 % in 60 min, from 15 % B to 25% in 30 min, from 25% B to 37 % in 10 min and from 37% B to 95% in 5 min. Data acquisition on the tims TOF Pro was performed using Hystar 5.1 and timsControl 2.0. tims TOF Pro data were acquired using 160 ms TIMS accumulation time, mobility (1/K0) range from 0.7 to 1.4 Vs/cm². Mass-spectrometric analyses were carried out using the parallel accumulation serial fragmentation (PASEF) acquisition method (Meier *et al.*, 2015). One MS spectrum followed by six PASEF MSMS spectra per total cycle of 1.16 s. Two injections per sample were done. Data analysis was performed using PEAKS Studio X Pro with ion mobility module and Q module for label-free quantification (Bioinformatics Solutions Inc., Waterloo, ON). Protein identification parameters were set to 15ppm for parent mass error tolerance and 0.05 Da as fragment mass error tolerance. The peak lists were searched against the Homo Sapiens taxonomy with isoforms from UNIREF 100. Peptide spectrum matches and

protein identifications were normalized to less than 1.0% false discovery rate. For the quantitation, mass error and ion mobility tolerance were set respectively to 15 ppm and 0.08 1/k0. ANOVA was used as the significance testing method. Modified peptides were excluded and only proteins with at least two peptides were used for the quantitation. Total ion current was used to calculate the normalization factors.

8. Bioinformatic analysis

All analysis presented in this work were made using statistical programing language R.

8.1. Polysome-profiling RNA-seq data analysis

For translatomic analysis, TMM-Log2 transformed counts were firstly analyzed by Principal Component Analysis using R package PCATools in order to identify potential outliers samples prior to analysis using R package Anota2seq (Oertlin *et al.*, 2019) with custom settings (minSlopeTranslation = -1, maxSlopeTranslation = 2, minSlopeBuffering = -2, maxSlopeBuffering = 1, maxPAdj = 0.25, selDeltaPT = log2(1.5), selDeltaTP = log2(1.5), selDeltaP = 0, selDeltaT = 0). Heatmaps were generated using R package gplot.

8.2. Validation of RNA-seq results

Both mRNA Log2FC and Log2TE FC results obtained by Anota2seq were compared with similar results obtained by RT-qPCR by calculating the Pearson Correlation Coefficient in order to validate the omic approach.

8.3. Ontology analysis

Gene set enrichment analysis was done on gene lists ranked on Log2DeltaPT (for translatomic results, referred to as Log2Translation efficiency Fold Change, Log2TE FC in this publication) or Log2FC (for transcriptomic and proteomic results) using R package ClusterProfiler (Yu *et al.*, 2012). Similarly, over-representation analysis was similarly done on the “Translation” group of gene identified by Anota2Seq.

8.4. Statistical analysis

Unless stated otherwise, quantitative results of at least 3 biological replicates were analyzed by ANOVA followed by Tuckey HSD post Hoc test for pairwise comparisons. For each comparison, P values <0.05 were considered statistically significant and encoded as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

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Appendix

Table 7: GSEA Results for D0 vs D12 comparisons

Comparison	Ontology	ID	Description	NES	pvalue	mRNA
D0D12	KEGG	hsa00071	Fatty acid degradation	1.50	4.48E-02	ACSL5/ADH6/ACADL/HADHA/ ACAA1/ALDH7A1/ACOX3/ ACAT1/ECI1/ALDH2/GCDH/ HADHB/ALDH9A1/ADH5/ ACADVL/ECI2/CPT1C/ACAT2/ ACAA2/ACSL1/HADH/ECHS1
D0D12X	KEGG	hsa00071	Fatty acid degradation	2.01	1.03E-04	ACSL6/ACSL5/ACADL/ADH6/ HADHA/ACADVL/GCDH/ACAA1/ ALDH7A1/ACOX3/EHHADH/ ACOX1/ACADSB/CPT2/ACSL4/ ACAT1/ACSL1/CPT1A/HADHB/ ALDH9A1
D0D12	KEGG	hsa00061	Fatty acid biosynthesis	2.00	1.16E-03	FASN/ACSL5/ACACA/MECR/ACSF3
D0D12X	KEGG	hsa00061	Fatty acid biosynthesis	1.94	8.77E-04	FASN/ACSL6/ACSL5/ACACA/ ACSF3/MECR/ACACB
D0D12	BP	GO:0008652	cellular amino acid biosynthetic process	1.45	2.40E-02	CAD/NOXRED1/ACCS/MTHFD1/ NAT8L/SRR/GLUD2/PAH/MTR/ ALDH18A1/PHGDH/ASL/CTH/ GOT1/SHMT1/OAT/PYCR2/ SLC25A12/PSPH/THNSL2/AASS/ APIP/SLC1A3
D0D12X	BP	GO:0008652	cellular amino acid biosynthetic process	1.79	8.39E-04	ACCS/CAD/GAD1/MTR/ MTHFD1/NOXRED1/PAH/ AASS/CBS/MRI1/RIMKLB/ GLUD2/SRR/CTH/ASL/ SLC1A3/PHGDH/GLS/ ALDH18A1/MTRR/OAT/ BCAT1/SHMT1/PSPH/ BHMT/NAT8L/THNSL2/ ASNS/SLC25A12/ABAT/ ATP2B4/GOT2/GGT1/ BCAT2/GLUL/PYCR2/ GOT1/PYCR1
D0D12	KEGG	hsa00010	Glycolysis / Gluconeogenesis	1.75	1.55E-03	ALDH3B1/ACSS1/ADH6/ PKLR/PFKM/ALDH7A1/ FBP1/HK1/PGM1/PKM/ HK2/ENO1/PCK2/PGK1/ AKR1A1/ALDH2/PFKP/ PDHB/PDHA1/ALDH9A1/ ADH5

D0D12X	KEGG	hsa00010	Glycolysis / Gluconeogenesis	1.43	2.90E-02	ALDH3B1/ADH6/FBP2/ HK1/PFKM/HK2/PFKP/ ALDH7A1/PCK2/ENO2/ DLD/PKM/FBP1/ALDH9A1/ PDHA1/PGK1/PFKL/ AKR1A1/PGM1/ALDH3A1/ ENO1/GPI
D0D12	BP	GO:0015990	electron transport coupled proton transport	1.76	4.35E-04	CYTB/COX1/ND4
D0D12X	BP	GO:0015990	electron transport coupled proton transport	1.72	1.25E-03	ND4/COX1/CYTB
D0D12	BP	GO:0016482	cytosolic transport	1.35	2.02E-02	CLTC/MAGEL2/RAB41/ PRKN/CORO7/GBF1/ HEATR5B/UBE2O/ SNX32/WASHC2A/ GCC2/DOP1B/MON2/ MSN/PTPN23/DNAJC13/ DCTN1/WDR81/RDX/ WASHC2C/PIK3C3/SGSM2/ RILP/EZR/HEATR5A/ CLTCL1/GAK/SNF8/ HOOK2/WASHC1/AP4M1/ MYO1D/VPS11/PLEKHJ1/ RAB9B/STX16/KIF1A/ FAM160A2
D0D12X	BP	GO:0016482	cytosolic transport	1.71	3.19E-04	CLTC/HEATR5B/MAGEL2/ GBF1/CORO7/UBE2O/ WASHC2A/DOP1B/ RAB41/MSN/DCTN1/ KIF1A/MON2/ANKFY1/ WASHC2C/HEATR5A/ DNAJC13/SGSM2/WDR81/ PRKN/HOOK2/GCC2/ PTPN23/FAM160A2/ GAK/STX16/RAB9B/ EZR/VPS11/PIK3C3/ MYO1D/DOP1A/SNX32/ AP1G1/WASHC1/RILP/ CLTCL1/BAIAP3/SPAG9/ EEA1/VPS52/TRAPPC10/ BECN1/RIC1/RDX/AP4M1/ WDR91/KIF1B/ERC1/ VPS13C/VPS53/PREPL/ PIKFYVE/VPS35/KIF16B/ VTI1A

D0D12	BP	GO:0002181	cytoplasmic translation	-1.92	1.55E-04	RPL17/CPEB2/RPL35A/ RPL8/RPL38/RPS29/ EIF2S3/RPL19/RPS21/ DPH5/EIF3H/RPL36/ LIN28A/RPS28/RPSA/ RPL39/RPL32/RPL10A/ RPL11/RPL15/EIF4B/ RPL18/RPL26/RPL29/ RPL9/RPL30/EIF3E/ EIF3F/RPL31/EIF3L/ RPL18A/RPL36A/RPS23/ RPL6/RPL13A/RPLP0/ RPLP1
D0D12X	BP	GO:0002181	cytoplasmic translation	-2.50	1.51E-04	DPH5/EIF3M/RPL41/ EIF2S3/ZNF385A/ EIF4B/RWDD1/EIF3L/ RPL24/LIN28A/EIF3H/ RPL19/RPS26/EIF3F/ RPSA/RPL17/RPL8/ EIF3E/RPL15/RPL10A/ RPL35A/RPS28/RPL6/ RPL18/RPL9/RPS21/ RPL38/RPL11/RPL18A/ RPL36/RPL32/RPL26/ RPL29/RPL39/RPL30/ RPLP0/RPS29/RPL31/ RPL13A/RPS23/ RPL36A/RPLP1

Table 8: GSEA Results for D0 vs D20 comparisons

Comparison	Ontology	ID	Description	NES	pvalue	mRNA
D0D20	BP	GO:0006067	ethanol metabolic process	1.61	2.55E-02	ALDH3B1/ADH6/SULT1A2/ SULT1A1
D0D20X	BP	GO:0006067	ethanol metabolic process	1.66	1.58E-02	ALDH3B1/ADH6/SULT1A2
D0D20	BP	GO:0016999	antibiotic metabolic process	1.92	1.87E-04	ALDH3B1/CYBB/AKR1C2/ ADH6/COX2/ACLY/ HDAC6/SULT1A2/ FTCD/ABCC2/ MTHFD1L/NAGK/ SULT1A1/KDM3A/ ACO1/NOXA1/PCK2/ CYBA/AKR1C3/GPX1/ AKR1C1/STAR/AKR1A1/ ACO2/AMDHD2/PRDX5
D0D20X	BP	GO:0016999	antibiotic metabolic process	1.55	8.12E-03	ALDH3B1/AKR1C2/ ADH6/COX2/SULT1A2/ FTCD/ACLY/ABCC2/ HDAC6/KDM3A/ AKR1A1/NOXA1/ SULT1A1/NAGK/ RAC2/RENBP/ACO1/ CYBA/ACO2/GPX1/ PRDX2/PCK2/STAR/ AKR1C1
D0D20	KEGG	hsa00061	Fatty acid biosynthesis	1.60	1.64E-02	FASN/ACSL5/ACACA/ ACSF3/MECR/ACACB

D0D20X	KEGG	hsa00061	Fatty acid biosynthesis	1.61	3.39E-02	FASN/ACSL5/ACACA/ACSF3
D0D20	KEGG	hsa00020	Citrate cycle (TCA cycle)	1.54	3.69E-02	ACLY/PC/OGDHL/OGDH/ ACO1/PCK2/ACO2/ SDHA/IDH3G/IDH2/ FH/MDH2/PDHA1
D0D20X	KEGG	hsa00020	Citrate cycle (TCA cycle)	1.54	2.77E-02	ACLY/OGDHL/PC/SDHA/ ACO1/OGDH/ACO2/ PDHA1/PCK2/IDH3G/ SUCLG1/IDH2/FH
D0D20	KEGG	hsa00190	Oxidative phosphorylation	1.50	5.97E-03	COX2/ND2/ATP6V1E2/ TCIRG1/COX3/ND4/ ATP6V0A4/CYTB/ ATP6V0A1/COX1/ NDUFA11/NDUFS3/ SDHA/ND4L/UQCRQ/ COX7B/ND1/NDUFS8/ UQCRC1/ATP5MC1/ COX5B/COX7A2/ NDUFS7/NDUFB9/ ATP5F1D/ATP5PD/ ATP6/NDUFB2/ATP6V1A/ NDUFS6/NDUFS5/NDUFB7/ NDUFV1/NDUFA12/ NDUFB1/ATP5MF/ NDUFA13/NDUFA10/ ATP5F1A/NDUFA3/ COX6B1/NDUFV2/ NDUFB8/NDUFV3/ NDUFA8/NDUFB11/ ATP5PF/NDUFA7/ ATP8/ATP6V0C/ NDUFA9/NDUFA6/ NDUFS2/NDUFA2/ ATP5F1C/ATP6V1C2/ NDUFB4/ATP6V0E2/ ATP5MC3/NDUFB10/ ATP6V1H/UQCR10/ NDUFS1/ATP6V1F/ NDUFB6/NDUFS4/ COX8A/ATP5MG/ ATP5ME/LHPP
D0D20X	KEGG	hsa00190	Oxidative phosphorylation	1.38	2.34E-02	ND2/ND4/COX2/ND4L/ CYTB/COX3/COX1/ ND1/ATP6/NDUFA11/ UQCRQ/ATP8/NDUFS3/ COX7A2/TCIRG1/SDHA/ NDUFB8/NDUFS8/ UQCRC1/COX7B/ ATP5PD/NDUFS4/ ND6/NDUFB7/ NDUFB6/ATP6V0A1/ NDUFS5/NDUFA13/ NDUFA8/NDUFA3/ ATP5MC1/COX4I1/ COX5B/COX7A2L/ ATP6V1A/ATP5F1C/ ATP6V1H/NDUFB2/ ATP5PF/COX5A/NDUFA9/ NDUFS7/COX6A1/COX6B1/

						ND5/NDUFB1/NDUFA10/ NDUFA12/NDUFV1/ ATP5F1A/UQCR11/ATP5PO
D0D20	BP	GO:0002181	cytoplasmic translation	-1.80	4.33E-04	EIF4B/RPS26/DPH5/RPL8/ CPEB4/EIF2S3/CPEB3/ RPL38/RPL35A/RPS28/ DENR/EIF3H/RPL19/ RPS21/ZNF385A/RPL29/ EIF2S3B/EIF3F/RPL10A/ RPS29/RPL15/RPL11/ RPL18/RPSA/RPL39/ RPL18A/EIF3L/RPL30/ RPL32/RPL9/CPEB2/ EIF3E/RPL6/RPL31/ RPL36A/RPL13A/RPL26/ RPS23/RPLP0/RPLP1
D0D20X	BP	GO:0002181	cytoplasmic translation	-2.20	2.61E-04	DPH7/RPL17/RPL19/ CPEB3/EIF3L/CPEB4/ RPL41/ETF1/EIF3H/ RPL18/RPS26/EIF4B/ DENR/RPL35A/RPL8/ RPSA/RPL18A/RPL10A/ RPL29/EIF3E/RPL15/ EIF2S3B/RPL11/RPL9/ RPL38/RPS21/RPS29/ RPL6/UNK/RPL39/ RPS28/RPL31/RPL30/ CPEB2/RPL13A/RPL32/ RPS23/RPL26/LIN28A/ RPLP0/RPL36A/ZNF385A/ RPLP1
D0D20	KEGG	hsa04550	Signaling pathways regulating pluripotency of stem cells	-1.68	5.37E-04	WNT2B/FZD4/FGFR1/ NRAS/FGFR3/GSK3B/ PIK3R1/MAPK14/IGF1R/ FGFR4/APC2/SMAD5/ WNT5A/ACVR1C/ PCGF6/BMPR2/ BMI1/ACVR1/FZD1/ KRAS/PCGF2/ONECUT1/ FZD6/SKIL/FGFR2/ INHBE/FZD7/BMPR1A/ AXIN2/KLF4/FZD3/FZD5/ ACVR2B/IL6ST/WNT3/ WNT9A/ACVR1B/ ACVR2A/LIFR/HESX1
D0D20X	KEGG	hsa04550	Signaling pathways regulating pluripotency of stem cells	-1.72	3.94E-04	PIK3R3/PIK3CA/KAT6A/ AXIN1/MAPK14/ PCGF6/GSK3B/SMAD5/ SMAD2/FZD7/ACVR2B/ BMPR1A/FZD5/PIK3CB/ PCGF5/KRAS/ONECUT1/ APC2/FZD3/FGFR2/PIK3R1/ AKT3/KLF4/FZD1/IL6ST/ ACVR1B/ACVR1/ ACVR2A/PCGF2/BMI1/ LIFR/INHBA/HESX1/ WNT9A/INHBE