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RIBOSOME BIOGENESIS AND CONTROL OF TRANSLATION IN CANCER CELLS

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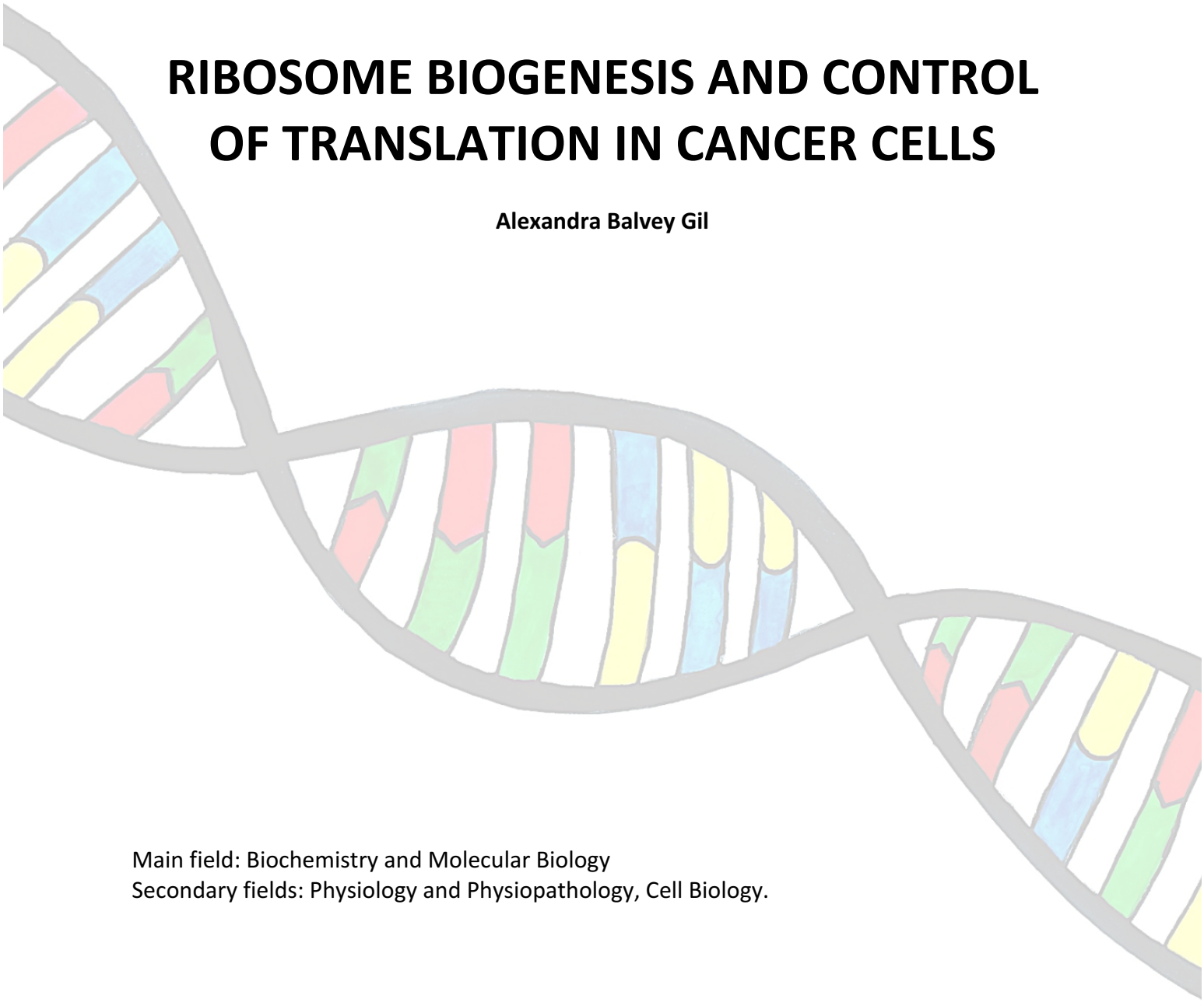

Main field: Biochemistry and Molecular Biology
Secondary fields: Physiology and Physiopathology, Cell Biology.

CONTENTS

ABSTRACT

Ribosomes are ribonucleoprotein complexes present in every cell type, their main function being the synthesis of proteins, ribosomal proteins included. Ribosome biogenesis is the process by which the different elements forming the two ribosomal subunits are synthesized and assembled. Since almost all cellular activities depend on the protein synthesis capacity of the cell, an Impaired Ribosome Biogenesis Checkpoint (IRBC) has evolved to monitor the proper execution of ribosome synthesis. This checkpoint allows the cell to identify alterations during the process of ribosome production, and, in case of any impairment, a p53-mediated response will be activated. This p53 upregulation is the result of the activation of a signaling cascade mediated by components of the ribosome, as we will see along this assignment.

The present work aims to overview the factors that lead to the activation of the Impaired Ribosome Biogenesis Checkpoint, as well as the main pathologies derived from its activation or suppression (ribosomopathies and cancer), and to study the impact of translation in a cancer setting. In this regard, two techniques used to study the engagement in translation of mRNAs (polysome profiling and ribosome profiling) will be introduced and described along this work. These techniques will be accompanied by illustrative examples that will enable their comprehension and will help at better understanding the role of ribosome biogenesis in cancer.

RESUM

Els ribosomes són complexes formats per proteïnes i àcids ribonucleics presents en tots els tipus de cèl·lules, i la seva funció principal és la síntesi de proteïnes, incloent les pròpies proteïnes ribosomals que els formen. La biogènesi ribosomal és el procés pel qual es sintetitzen els diferents elements que més tard conformaran les dues subunitats ribosomals. Donat que una gran part de l'activitat cel·lular depèn de la capacitat de síntesi proteica de la cèl·lula, aquesta ha desenvolupat, al llarg de l'evolució, un punt de control anomenat IRBC (*Impaired Ribosome Biogenesis Checkpoint*), que és capaç de monitoritzar alteracions en el procés de síntesi ribosomal i d'activar una resposta mediada per p53. Aquesta resposta serà

el resultat de l'activació d'una cascada de senyalització mediada per components del propi ribosoma, tal i com veurem al llarg d'aquest treball.

En la present revisió, s'introduiran quins són els factors que porten a activar el punt de control de la biogènesi ribosomal, així com les principals patologies que es deriven d'aquesta activació o inactivació (ribosomopaties i càncer), i també s'estudiarà l'impacte de la traducció en el marc del càncer. A més, s'introduiran dues tècniques utilitzades en l'estudi del grau de traducció de mRNAs (*polysome profiling* i *ribosome profiling*), que proporcionen informació sobre el nivell de traducció dels mRNA. Aquestes dues tècniques s'acompanyaran d'exemples il·lustratius que facilitaran la seva comprensió i que ens ajudaran a entendre millor quin és el paper de la biogènesi ribosomal en el càncer.

INTEGRATING EDUCATIONAL FIELDS

The elaboration of this work has been supported by the integration of three different disciplines, in order to rationally approach and interrelate the educational contents imparted over the Pharmacy degree, to finally apply the acquired knowledge to develop a subject of interest in the pharmaceutical field.

Biochemistry and molecular biology is the main field of this assignment. It has allowed to describe the processes and pathways of ribosome biogenesis and its impairment, as well as the methods used for its study. This field has also contributed to explaining and linking the molecular mechanisms by which ribosomopathies and cancer occur upon ribosome biogenesis impairment.

Physiology and physiopathology provided the knowledge to recognize the distinct signs and symptoms of ribosome biogenesis impairment, as well as understanding and identifying the differences between two diseases underlying ribosome biogenesis impairment.

Cellular Biology has enabled to understand the physiological attributes of ribosomes and the metabolic processes by which ribosome biogenesis occurs. This discipline has also helped at understanding through which signalling pathways the ribosome biogenesis impairment affects the progression of cell cycle.

INTRODUCTION

What are ribosomes?

Ribosomes are the ribonucleoprotein complexes responsible of decoding the genetic information into proteins and accountable for the production of the cellular biomass. This basic cellular function is essential for life[1], explaining why ribosomes are universally distributed thorough the prokaryote and eukaryote kingdoms. Indeed, they belong to the most ancient collection of cellular machineries evolved in life.

Both in eukaryotes and prokaryotes, ribosomes are found within the cytoplasm. Prokaryotic ribosomes (70S) are more simple than eukaryotic ribosomes (80S), containing fewer ribosomal proteins and fewer rRNAs, resulting in smaller subunits. However, eukaryotic cells also contain 70S ribosomes, which are found inside mitochondria and, in plantae, inside chloroplasts, as a result of the symbiosis of eukaryotic cells with prokaryotic organisms several million years ago. In eukaryotes, ribosomes can also be found attached to the endoplasmic reticulum (ER), translating mRNAs that encode for proteins destined to a number of different cellular functions, for example, the ones that will transit through the secretory pathway[2]. Importantly, a single mRNA molecule can be translated by many ribosomes that independently associate and decode the nucleotide sequence, simultaneously generating multiple copies of the same protein from just one mRNA template. This structure is termed polysome and is often found associated with the ER membranes.

As stated before, ribosomes are formed by about 80 proteins, termed Ribosomal Proteins (RPs), which in turn require other ribosomes for their synthesis. The amount of newly synthesized RPs determines the rate at which new ribosomes are formed and vice-versa, the number of available ribosomes affects the pool of RPs produced. This mutual influence leads us to look in more details the process by which new ribosomes are formed, also known as ribosome biogenesis.

Ribosome biogenesis

Ribosome biogenesis is a critical, greatly elaborated and well-coordinated cellular process in the life cycle of a cell, required for a correct cellular activity and function. It starts in the nucleolus, a special compartment within the nucleus[3], and requires four different kinds of rRNAs, about 80 RPs and the activity of three RNA polymerases, alongside a considerable number of accessory factors and small nucleolar RNAs (snoRNAs).

Ribosomal Proteins (RPs), are a family of RNA binding proteins and constitute a core component of the ribosome, playing a leading role in mRNA translation. The multiple roles of RPs span from stabilizing rRNAs and promoting their correct folding for the assembly of ribosomal subunits, to mediating the catalytic activity required in protein synthesis. However, over the last years, several studies indicate that RPs are also able to perform extraribosomal functions beyond protein synthesis, such as DNA damage repair (and hence the maintenance of genomic stability), regulation of apoptosis, cell proliferation, cell cycle arrest and cell migration and invasion[4,5]. In this context, RPs interact with non-ribosomal components of the cell and trigger a special cellular program that could eventually result into pathological consequences, as we will see later.

The generation of new ribosomes is extremely demanding in terms of energy expenditure and cellular resources. It has been recently noted that ribosome biogenesis has a specific checkpoint that controls the proper execution of the whole process. Indeed, the perturbation at many steps of the ribosomal synthesis determines a series of events that trigger a stress response (also known as Impaired Ribosome Biogenesis Checkpoint or IRBC), characterized by a loss of nucleolar integrity and a block in the cell proliferation[6,7].
As ribosome activity is the main determinant in the production of cellular biomass, it is not surprising that the entire process is often hijacked and boosted by tumor cells to more rapidly provide structural and catalytic components of the cell. Both impairment and hyperactivation of ribosome biogenesis have been associated with deregulation of cell cycle and proliferation, and surprisingly, the same cellular process in the two different contexts above mentioned could eventually lead either to an oncogenic or a tumor suppressive response, respectively.

Steps of ribosome biogenesis

Ribosomes are classified according to the sedimentation coefficient expressed in Svedbergs (S). Eukaryotic ribosomes are larger that prokaryotic ribosomes and this is also reflected by their coefficient of sedimentation. Mammalian ribosomes indeed have a coefficient of 80S, and a diameter of 22nm. They are formed by two ribonucleoprotein subunits: the large subunit (60S) catalyzes the formation of peptide bonds, and the small subunit (40S) is accountable for decoding mRNA sequences into aminoacid chains. Four rRNAs (18S, 28S, 5.8S and 5S) and about 80 ribosomal proteins (RPs) are part of the ribosome structure.

The rate of rRNA transcription is a limiting factor in the production of ribosomes[5]. The process starts in the nucleolus, with the transcription of the polycistronic precursor 45/47S pre-rRNA by the RNA polymerase I[6]. The 45/47S pre-rRNA is later modified and processed by small nucleolar RNAs (snoRNAs) and other protein cofactors, including RPs, giving rise to the three rRNAs: 18S, 28S and 5.8S. Meanwhile, outside the nucleolus but still within the nucleoplasm, a second rRNA molecule, the 5S, is transcribed by the RNA polymerase III.

These steps are paralleled by the transcription of RP mRNAs by RNA polymerase II, followed by their translation in the cytoplasm. At this stage, the different RPs are imported through the nucleolar pores in the nucleus, where they associate with the 45/47S pre-rRNA and process it into the two subunits, the 40S and the 60S. Once the ribosomal subunits are formed, they exit the nucleus through the nuclear pores and enter the cytoplasm, ready to start executing their function.

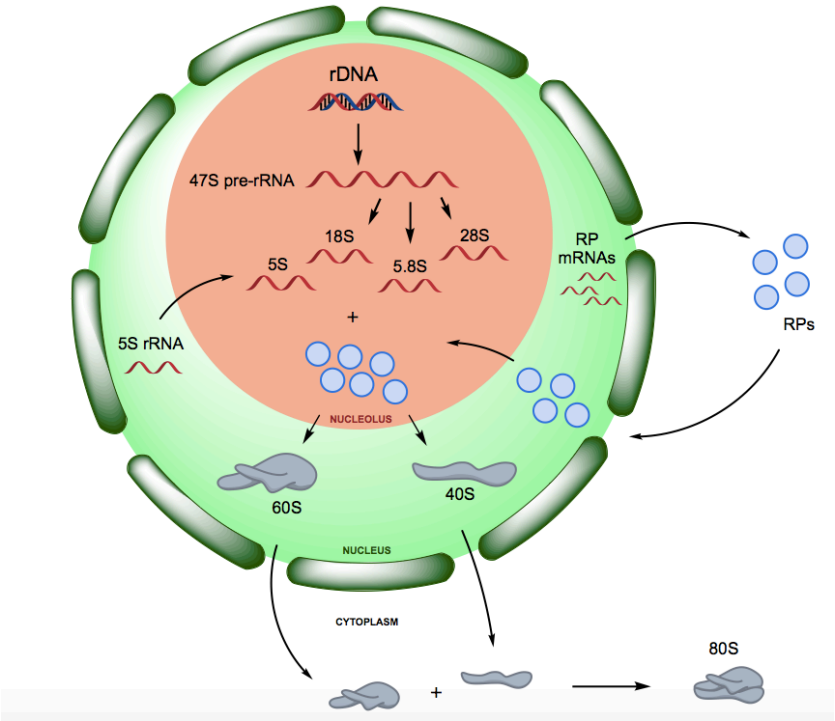

Figure 1: Simplified diagram of ribosome biogenesis process.

The various rRNAs and ribosomal proteins are produced in equimolar amounts and their synthesis is tightly regulated by a variety of cellular parameters.

Translation process

Once the ribosomal subunits are released to the cytoplasm, they are ready to initiate the translation process. Protein biosynthesis is a complex process, consisting of three major steps: initiation, elongation and termination[7]. Eukaryotic initiation step is the most complex phase of the translation process, and can occur via multiple ways. Cap-dependent translation is the most general mechanism of translation initiation[1]. Eukaryotic initiation factors (eIFs) are a set of proteins required for the recruitment of the 40S subunit on the 5’ end of the mRNAs. The resulting 43S complex scans the 5’ untranslated region (5’UTR) until finding the start codon (AUG). Once the AUG codon is pinpointed and paired with the anticodon tRNA at the P-site, the 60S subunit is recruited to form the 80S ribosome complex. The formation of this complex allows to proceed to the elongation phase.

However, there is an alternative mode of translation initiation, named Internal Ribosome Entry Site (IRES)-mediated translation[1], which allows the translation of mRNAs in a cap-independent manner. This mechanism spares the need of 5' end recognition, as well as the mRNA scanning process, by directly recruiting the 40S subunit nearby the codon where the translation must be initiated.

In some physiological and pathophysiological conditions, IRES-dependent translation is stimulated, supporting a robust translation of mRNAs when the cap-dependent pathway is compromised or suppressed. Diverse studies over the last years have found that many mRNAs containing IRES elements within their 5'UTR actually encode proteins involved in stress protection and apoptosis[7], leading to the thought of IRES-dependent translation as an important regulator of cell-fate decisions under a variety of conditions.

P53 activation and the Impaired Ribosome Biogenesis Checkpoint

The role of p53 as a tumor suppressor in humans has been largely studied since its discovery; it has been attributed multiple functions such as DNA repair and maintenance of the genomic stability, two features that assigned to p53 the role of "guardian of the genome". As a result of its stabilization, p53 can operate and arrest the cell cycle, or induce a programmed cell death (apoptosis). In addition to the above mentioned roles, upon a defined biological context, p53 expression can determine the induction of senescence or a switch in cellular metabolism. A crucial step in the activation of p53 is its stabilization. Under normal conditions, p53 is efficiently ubiquitinated and degraded, resulting in its short half life. In contrast, following exposure of cells to stress, the levels of p53 increase, predominantly due to its reduced degradation[8]. Of the several ubiquitin ligases known to be involved in the degradation of p53, a central role is played by Hdm2 (human double minute 2, also known as Mdm2)[6,9,10,11]. In unstressed cells, p53 levels and its transcriptional activity are negatively regulated by Hdm2[9]. At the same time, Hdm2 is a transcriptional target of p53, leading to the generation of a negative feedback loop that keeps p53 under control[10].

The first evidence for the existence of a cell cycle checkpoint in higher eukaryotes that responds to impaired ribosome biogenesis came from studies in out laboratory. The gene

encoding ribosomal protein RPS6 was conditionally deleted in the liver of adult mice and, following partial hepatectomy, hepatocytes failed to re-enter the cell cycle and proliferate[4]. Subsequent studies demonstrated that this hindrance in proliferation is due to the activation of a p53-dependent checkpoint that is triggered by a specific pre-ribosomal complex constituted by the ribosomal proteins RPL11, RPL5 and the 5S rRNA. In normal growing conditions, this complex is incorporated into the pre-60S subunit, giving rise to a complete 60S subunit, whereas upon a ribosome biogenesis insult, it is redirected to bind and inhibit Hdm2, resulting into the stabilization of p53 levels and its transcriptional activity[14,15,16]. This checkpoint was recently termed Impaired Ribosome Biogenesis Checkpoint (IRBC)[12].

5S rRNA is also involved in the regulation of another component of the p53 pathway; the transcriptional regulator HdmX. In normal conditions, the formation of the Hdm2-HdmX heterodimer represses the transcriptional activity of p53 and promotes its polyubiquitinization and degradation. The binding of RPL11 to Hdm2, besides blocking its E3-ligase activity, also facilitates HdmX degradation, strengthening p53's hyperactivation. However, when HdmX is bound to 5S rRNA, it becomes unaffected by RPL11-Hdm2 degradation[9].

Ribosome biogenesis in cancer

The fact that mRNA expression patterns change when the translational machinery becomes quantitatively and/or qualitatively altered provides us an opening to address how defects in ribosome biogenesis could be directly implicated in cancer. Transformation of normal cells into cancer cells is a pretty complex process. It requires the dysregulation of oncogenes and/or tumor suppressors' activity, through multiple mechanisms, resulting in the impairment of the cellular proliferation and survival response, an alteration in metabolism and a promotion of invasion into contiguous tissue.

Among all the possible mechanisms by which a normal cell transforms into a cancer cell, ribosome biogenesis plays an important role. This is not surprising, considering that ribosome synthesis constitutes the anabolic engine for the biomass production and hence for the cell

growth and proliferation. Many key proto-oncogenes (such as c-Myc) and tumor suppressors (such as p53) have been found to regulate this process, as we previously introduced.

c-Myc and the components of the PI3K-mTORC1 signaling pathway stand out among these key factors, as mentioned above. Deregulation of Myc activity is one of the most frequent oncogenic lesions underlying human cancers[13]. With respect to Myc, it controls the expression of several components of the protein synthetic machinery (including RPs, initiation factors of translation, RNA polymerase I and ribosomal DNA transcription, RNA polymerase II and III). All these events point at a pathological role for Myc in tumorigenesis, through the stimulation of ribosome biogenesis. Indeed, uncontrolled proliferation, a hallmark of all cancers, requires a continuous supply of structural and catalytic cellular components to generate the cell mass required for replication. As ribosomes are the centers for production of cellular biomass, it is not surprising that many cancer promote ribosome biogenesis during the process of tumorigenesis.

Recent evidence suggests that the RPL5-RPL11-Hdm2-p53 pathway may monitor excessive ribosome biogenesis to prevent tumorigenesis[9]. c-Myc, as the regulator of the 45/47S pre-rRNA, 5S rRNA and all RP mRNA transcription, becomes directly associated to ribosome biogenesis. Several experiments carried out using mouse models of B-cell lymphoma predisposition overexpressing c-Myc[13] demonstrated how c-Myc is capable of upregulating ribosome biogenesis in this tumor context. An RPL24-haploinsufficiency background, that reduces the ribosome production and the protein synthetic capacity of B-cell to a premalignant state, dramatically reduces the tumorigenic burden determined by c-Myc upregulation. Importantly, Macias et al. demonstrated that c-Myc driven B-cell lymphomagenesis was further boosted in a genetic context impaired for the IRBC response. The most plausible hypothesis is that RPL5-RPL11-Hdm2-p53 pathway monitors the excessive ribosome biogenesis in order to counteract tumorigenesis[14]. All these studies prompted the field to consider the upregulation of ribosome biogenesis as an oncogenic event.

Ribosomopathies

A category of anemic syndromes like Diamond Blackfan Anemia (DBA), $5q^-$ syndrome and Schwachman Diamond Syndrome (SDS), characterized by bone marrow failure, growth retardation and cancer later in life, are associated with haploinsufficient mutation in RPs haploinsufficiency. For this last reason, they are also categorized as Ribosomopathies. Surprisingly, despite the congenital mutation is common to all tissues, only highly proliferating tissues are impaired by altered ribosome biogenesis determined by the RP mutation.
Studies on animal models of defective ribosome biogenesis raised evidences suggesting that activation of p53 IRBC-dependent underlies the pathogenesis of the human DBA, $5q^-$ syndrome and SDS[15]. Conditional deletion of RPS6 in mouse bone marrow can recapitulate the features of DBA. Intriguingly, p53-/- background can rescue most of the phenotypes associated with DBA, underscoring the role of p53 checkpoint[16].
Importantly, accumulation of nuclear p53 has been found in marrow biopsy samples from these patients, suggesting the basis for the failure of erythropoiesis in these diseases.

Any mutation affecting components involved in ribosome biogenesis will potentially lead to either a reduction of the ribosome number, or to aberrant ribosomes, directly affecting the protein translation process[15]. In case of having a reduction in the number of ribosomes, there's a competition between different kinds of mRNAs. In this situation, TOP mRNAs come into play. mRNAs containing a 5'TOP sequence at the 5'UTR region have a higher affinity for specific translational machinery than the ones lacking it. We must remember that these mRNAs usually codify for almost all RPs and many translational factors, as stated before. Qualitative defects in ribosomes might affect the translation of specific target mRNAs[15]. Thus, it is reasonable to assume that specific mutations that lead to a reduced number of functional ribosomes in the cell would alter not only the rate of total protein synthesis, but also the patterns of translated mRNAs.

In ribosomopathies, the most affected tissues will be the ones with a high cell division rate (associated with a high ribosome production rate), such as bone marrow. Of course, the degree of damage in ribosome synthesis caused by any mutation affecting a component involved in ribosome biogenesis will depend on the relative expression levels of that

component, its requirement in the ribosome biogenesis process or the severity of the mutant allele. Studies on human erythroid progenitor cells from DBA, $5q^-$ syndrome and SDS patients evinced a p53 activation after the depletion of RPs[1,15].

As it was mentioned before, DBA is a congenital disease that leads to macrocytic anemia, caused by the reduction of erythroid precursors in bone marrow[15,17]. About half of these patients suffer from malformations in other organs. The same mechanism underlying this pathology also predisposes not only these patients, but $5q^-$ syndrome and SDS patients as well, to develop acute myeloid leukemia (AML) and solid malignancies[18].

Ribosomal Proteins and 5'TOP translation

Given the central importance of maintaining a functional and robust translation machinery, it is reasonable to believe that the translation of RP mRNAs must be carried out in an organized and hierarchical manner[19]. The finding of 5'TOP nucleotide consensus at the transcriptional start sites of RP mRNAs supported this hypothesis. The broad category of 5'TOP mRNAs are characterized by containing a 5' terminal oligopyridimidine (5'TOP) sequence at the 5'UTR region. Several studies in vertebrates found that many RPs, as well as many genes involved in translation, actually are encoded by 5'TOP mRNAs.

Several studies have addressed the link between the translation of 5'TOP mRNAs and the mTOR pathway, such as upon mTOR inhibition[20], or under aminoacid deprivation[21]. Recently, the RNA binding protein LARP1, a protein belonging to the La-related family of proteins[19], was successfully co-immunoprecipitated with eIF4F complex, which was previously believed to be involved in the 5'TOP mRNA regulation and mTOR network. Other studies in HEK293, where LARP1 was depleted, corroborated that the levels and translation of 5'TOP mRNAs are subject to the expression of LARP1[22,23]. The same study also showed how levels of 5'TOP mRNAs dropped in the absence of LARP1, as well as their association with polysomes. At the same time, other studies have demonstrated how the mammalian target of rapamycin complex 1 (mTORC1) is also able to drive the translation of RP mRNAs, among its countless other functions[24,25]. Recent genome-wide studies show that 5'TOP mRNAs constitute a considerable share of the mTORC1 translatome. In light of these results, it was

hypothesized that emergent 5'TOP mRNA translation could play a critical role in the cell cycle checkpoint controlling ribosome biogenesis.

OBJECTIVES

A key step of Ribosome Biogenesis is the synthesis of ribosomal proteins (RPs). The availability of RPs in the cell determines the rate at which new ribosomes are produced. Moreover, by means of an increase in ribosome biogenesis, many external stimuli that prompt the cell to proliferate, can rapidly control the production of biomass required for cell duplication, by stimulating the translation of RP mRNAs and hence the production of more ribosomes. In order to understand how RP mRNAs translation is regulated upon different anabolic conditions, the following objectives have been outlined:

1 – Overview of the approaches utilized to study mRNA translation: polysome profiling and ribosome profiling analyses.

2 – Analysis of ribosomal protein mRNA translation in cancer cells and analysis of ribosome biogenesis.

MATHERIALS AND METHODS

The present work is mainly based on the collection of bibliographic information extracted from PubMed and Scopus databases. The initial search terms in the first screening were *ribosome biogenesis* and *cancer*. This initial selection provided a set of scientific articles that leaded to other articles, by looking at the citations within their bibliography. This step was repeated as much as necessary, and the obtained information was occasionally complemented with new searches over specific topics. This easy and rapid method not only provided a wide source of information to learn about the topic, but also allowed to come up with studies specifically addressing our subject matter.

This section also introduces a classical technique that I was able to attend at the Laboratory of Cancer Metabolism (IDIBELL): the polysome profiling. In this regard, I have assisted many steps of the work performed by thesis director with respect to polysome profile analysis of ribosomal protein mRNAs. This technique is used to study the differential translational output of the mRNAs of interest in two or more conditions, and it has been part of many experiments performed in the scientific articles discussed in this work. The protocol of this technique, disclosed in the upcoming section, has been acquired from Faye and coworkers' review[26] and adapted to the gradient fractionation system of the lab.

Polysome profiling protocol

Polysome profiling is a technique used to investigate translational changes of the transcriptome under different conditions. Basically, this technique consists in the preparation of cell lysates in a way that preserves the association of ribosomes with the mRNAs that are in the process of translation at the time of lysis. By means of ultracentrifugation on a sucrose gradient, that enables the separation of ribosomes associated to mRNAs (polysomes) as a function of their number, a 260nm OD profile of the ribosomal separation is obtained, and a collection of the non-polysomal vs polysomal fractions is then operated. Fractions are then processed for RNA analysis. The principle assumed by this technique is as follows: the more ribosomes are associated to a transcript, the more the mRNA is translated. Cycloheximide (CHX), which blocks the steps of translational initiation and elongation, is used just before lysis

to “freeze” the positioning of the ribosomes on the mRNAs. In addition, standard precautions are needed in the execution of the protocol to protect RNA against degradation by RNases.

1. **Preparation of solutions**. In this step, basic solution and two sucrose solutions with different concentrations (10% and 50%) are prepared. The day of the experiment, fresh polysomal lysis buffer is prepared.

2. **Preparation of sucrose gradient**. In this step, an automated gradient machine is used. The tubes are placed in a fitting rack on a steady surface, allowing the layering of the sucrose solutions. Two syringes are filled with the two different sucrose solutions, respectively. Afterwards, a specific volume of the sucrose solutions (starting with the 10% solutions and following with the 50% solution) are added to the bottom of the tubes. It is important to avoid bubbles. The 10% sucrose solution stays at the top of the tube, due to its lower density, whereas the 50% sucrose solution stays at the bottom. Gradients are formed by the gradient master machine. The tubes are kept at 4ºC for 30 minutes.

3. **Cell lysis**. The day before the experiment, cells are plated in order to achieve an optimal cell density the following day. The day of the experiment, cells are incubated in CHX + media for 5 minutes at 37ºC and 5% CO_2 to arrest and stabilize polysomes. Quickly after, media is removed and the cell plates are placed on ice and washed with ice cold phosphate buffered saline (PBS) supplemented with CHX. Additional 10 ml of PBS mixed with CHX is added to the plates before proceeding to cell scraping. The total volume of the plates is transferred to a single centrifuge tube, previously placed on ice.

4. **Ultracentrifugation**. Samples are subjected to centrifugation at 4ºC at high speeds in order to obtain supernatants containing the cytoplasmic extracts enriched of polysomes. A protein determination is performed and equal amounts of protein extracts are loaded onto sucrose gradients. The gradients are then ultracentrifuged at 35,000 rpm for 3 hours at 4ºC.

5. **Gradient fractionation**. This step requires the set-up of the fractionation instrument and the “blanking” of the spectrophotometer set at 260 nm with water. Once the parameters are set, the instrument collects the ultracentrifuged samples starting from the upper part of the

gradient (10% sucrose) and proceeding till the bottom of the tube (50% sucrose). While fractionating the gradient into 1ml aliquots, the sucrose solution is read by the spectrophotometer which generate a profile of rRNA absorbance across the gradient. A sample from each fraction is stored for following RNA analysis.

6. **Isolation of RNA from sucrose fractions**. Equal amounts of phenol:chloroform:isoamyl alcohol are added to each sucrose fraction to remove proteins. After several centrifugations and removal of the phenolic phase, the fractions are added with one volume of Isopropanol and left overnight at -20ºC for precipitation. The following day, RNA pellet is obtained by centrifugation, washed, air dried, and finally resuspended with RNase-free water.
The RNA derived from each fraction is then analyzed by Northern Blot or RT-qPCR analyses according to specific protocols, and relative amounts of the mRNAs of interest can be determined.

RESULTS AND DISCUSSION

How to measure the translational rate of an mRNA?

Over the last fifteen years, global gene expression analysis, by means of microarray and RNA-seq approaches, has allowed to characterize unbiasedly the expression profile in a huge number of biological contexts, either physiological and pathological. This approach has been of fundamental importance to broaden the point of view of researchers from the single protein to the network of proteins. However, the assumption done in global RNA-based measurement is that the mRNA levels parallel the amounts of cognate protein synthesized in the cell. So do the changes observed in the transcriptome composition when comparing two different conditions. Although this principle holds true for the major part of the transcriptome, several subsets of mRNAs do not always correlate with the protein levels, due to regulatory mechanisms that control their translation. Many recent studies have demonstrated in different systems the importance of translational regulation as a layer of control of gene expression that can quickly convert genetic information into proteins. In this regards the need of determining the translation profile of a transcriptome has prompted the field to develop techniques that could address this biological problem. Here, two gold standard techniques that allow to determine the level of translation of mRNAs will be described. After a brief insight into the polysome profiling protocol, this technique, together with the ribosome profiling, will be discussed in the following sections, supported by two illustrative studies that will help to fully understand these experimental approaches for the translatome study.

Polysome Profiling

Polysome profiling is a standard technique used to separate cellular transcripts as a function of the number of ribosomes attached to them, by means of a sucrose gradient[27]. The initiation process is a limiting step in mRNA translation, therefore, the degree of associated ribosomes for a specific mRNA will give us an idea of the translation rate of the mRNAs of interest. By preserving the associations of actively translating ribosomes, the technique allows to capture mRNA translation and to distinguish between highly translated and poorly translated transcripts[26]. The sucrose gradient centrifugation enables separating polysomes

from non-polysomal fraction. The latter is resolved into free mRNA and RNPs fraction and the free 40S, free 60S subunits, and 80S monosomes (Figure 2). Downstream of polysomal fractionation, RTq-PCR and Northern Blot are two standard techniques that are usually implemented to monitor and quantify the distribution of different mRNAs within the gradient. When coupled with immunoblot analysis, polysome profiling will also enable the study of proteins and complexes associated with the process of translation.

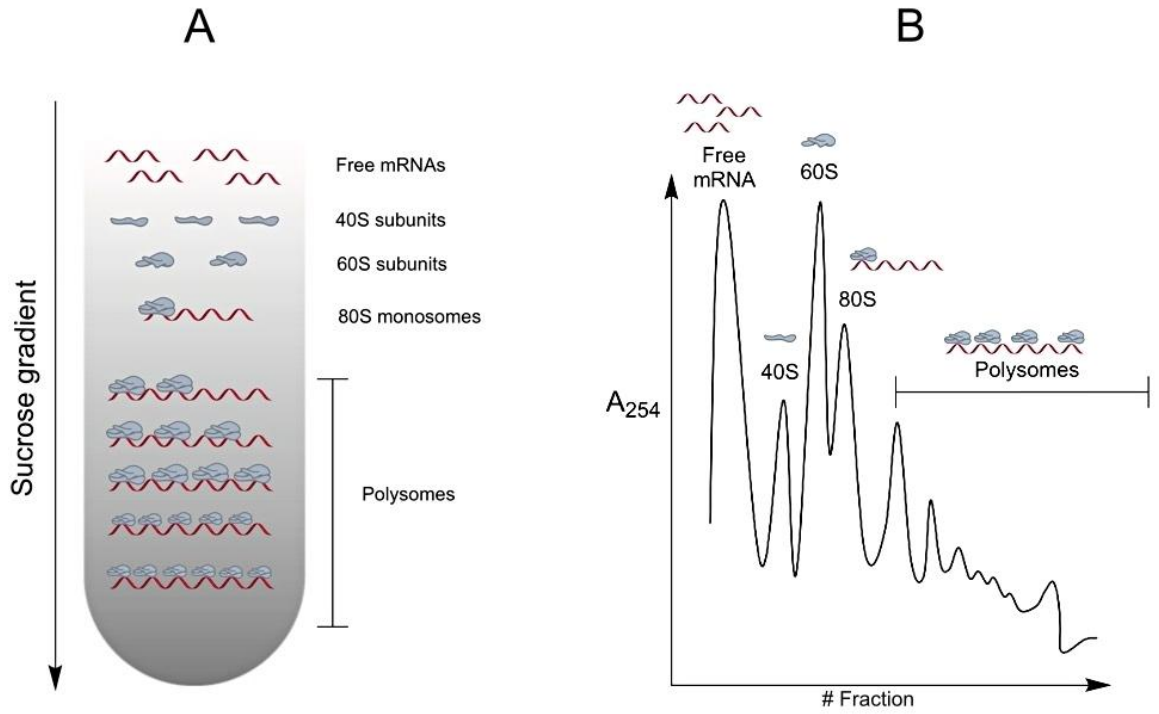

Figure 2: General overview of ultracentrifuged sample through a linear 10-50% sucrose density gradient (A) and gradient fractionation at 254nm absorbance (B).

This technique is widely used for initial screenings of translational changes in cultured cells and tissues, as well as tracking the translational status of specific mRNAs whose identity is known[27]. For instance, Damgaard and coworkers[28] used polysome profiling to demonstrate that the RNA binding proteins TIA-1 and TIAR can associate to 5'TOP mRNAs by binding to their 5' element when cells are cultured in absence of amino acids. The authors show that this association determines a release of these transcripts from polysomes and hence, a reduction in the synthesis of the cognate proteins. The team quantified the levels of three specific mRNAs containing the 5'TOP sequence (RPL23a, RPL12/36 and PABPC1) in the polysomal fractions upon aminoacid starvation. β-actin and calmodulin 2 mRNAs (lacking 5'TOP sequence but containing a binding site for TIA-1 and TIAR at their 3' end) were used as

controls. Figure 3 shows the results from northern blots of sucrose gradient polysomal fractionations.

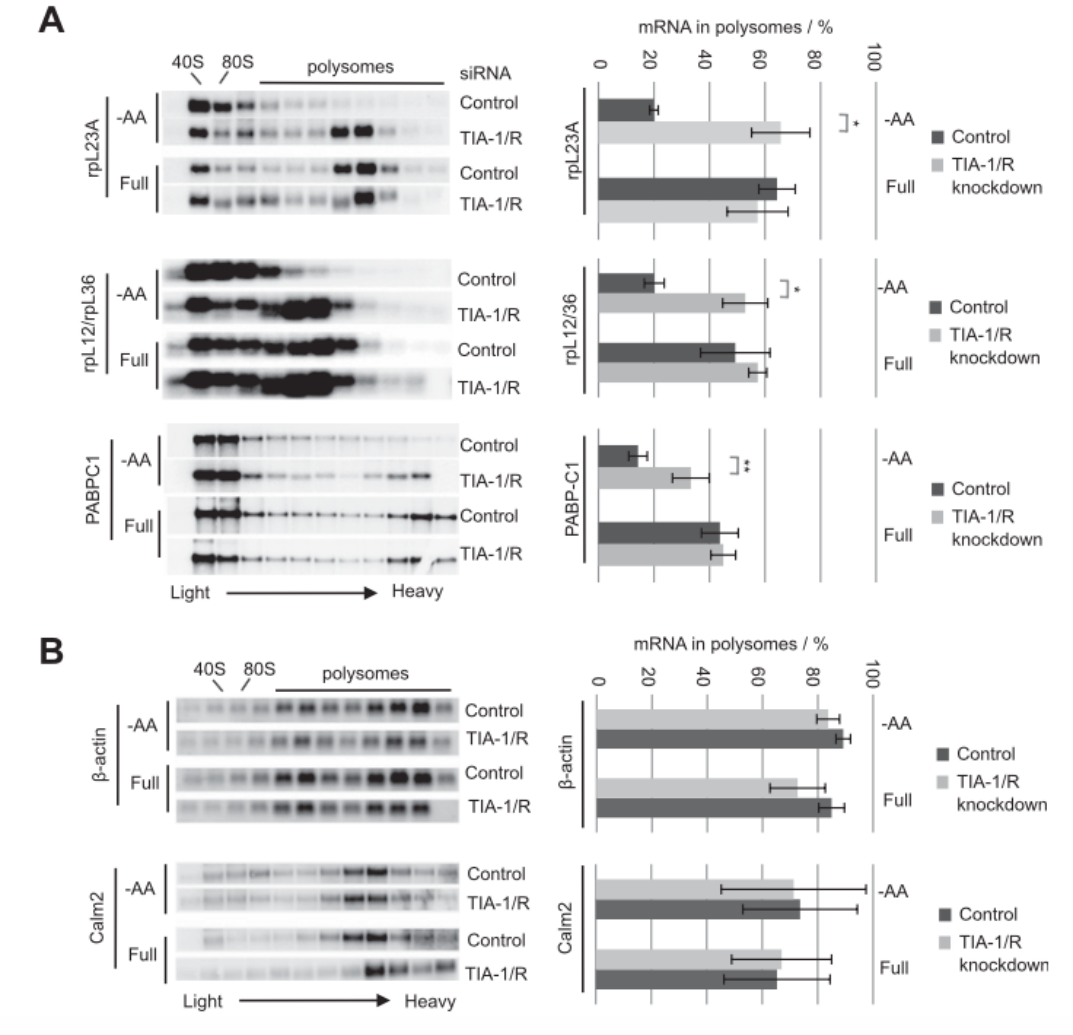

Figure 3: Northern blots from sucrose gradient polysomal fractions of RPL23a, RPL12/36 and PABPC1 mRNAs upon exposure to full medium (Full) and amino acid starvation (–AA) medium, in the presence of siNT (control) and siTIA-1/R knockdowns (A); equivalent northern blots from β-actin and calmodulin-2 mRNAs (B). Figure from Daamgard et al.[28].

However, just like other techniques, polysome profiling also has its limitations. This complex and arduous method requires specialized equipment that may not be accessible to every laboratory. Moreover, it demands a large sample size, adding an extra difficulty to the experiments based on low abundance of tissue samples, such as cancer tissue biopsies[27].

Ribosome Profiling

Ribosome Profiling (also called Ribosome Footprinting) was recently developed by Nicholas Ingolia and Jonathan Weissman at UCSF[29] and enables determining the engagement in translation at a global level of the whole transcriptome of a cell and the position of ribosomes at near nucleotide resolution on each transcript[27]. Similarly to polysome profiling, this genome wide technique allows monitoring cellular translation process and predicting protein abundance.

The protocol of ribosome profiling requires the preparation of a library of the RNA fragments occupied by ribosomes. An enzyme with RNAse activity is added to the cell extracts, previously prepared as for polysome profiling. Upon controlled reaction condition, every RNA sequence that is not protected by ribosome occupancy, is exposed to RNAse activity and is then degraded. Under this setting, the ribosome protection generates footprints in the whole transcriptome representing site of translation or stallment. Once the RNA footprints are purified, these are converted into a cDNA library that is then subjected to deep sequencing, thus enabling the determination of the amount of each protected fragment and their exact position along the mRNA sequence. (Figure 4).

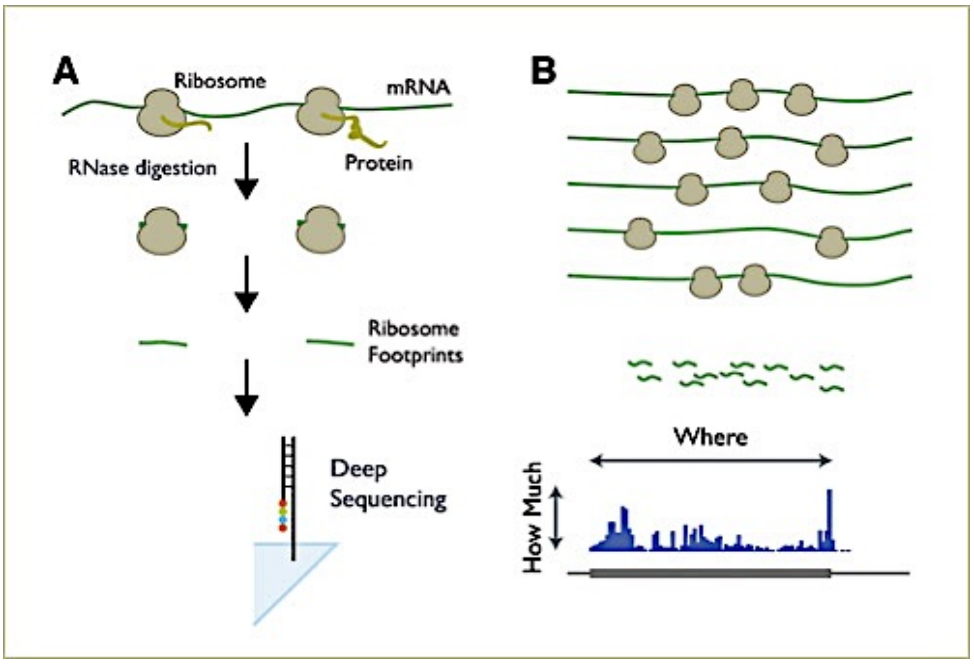

Figure 4: General overview of ribosome profiling process. Samples are treated with RNase to digest unprotected mRNA. Ribosome protected fragments (RPFs) are isolated and size-fractionated and eventually sequenced by RNA-seq. Figure taken from [29].

This powerful approach has led to the discovery of novel coding transcripts, new protein isoforms and the accurate determination of elongation and decoding speeds, among many other features and functions. Furthermore, the possibility of discerning the initiation and elongation events allowed the discovery of countless new uORFs and alternative start codons involved with ribosomes[30]. This is the case of a study by Hsieh and coworkers[31] in PC3 cells, where ribosome profiling was used to compare the effect of two mTOR inhibitors (rapamycin and PP242) over a 3h treatment period through the sequencing of RPFs (ribosome protected fragments). The method enabled the determination of mRNA abundance (RNA-seq reads), ribosome occupancy (RPF reads) and translational efficiency (RPF reads/RNA-seq reads). Data treatment revealed a significant higher number of target mRNAs selectively decreased at the translational level upon PP242 treatment compared to rapamycin treatment, as shown in figure 5, meaning a higher effectiveness for mTOR inhibition. Moreover, ribosome profiling revealed the presence of two regulatory motives within the 5'UTR sequence of mTOR-sensitive mRNAs: 68% of them contained a 5'TOP sequence, against 63% containing PRTE (pyrimidine-rich translational element). When looking for 5'TOP and PRTE together within the same 5'UTR, it was found that 89% of the target mRNAs contained both sequences, making the presence of one or preferably both sequences a strong predictor for mTOR responsiveness.

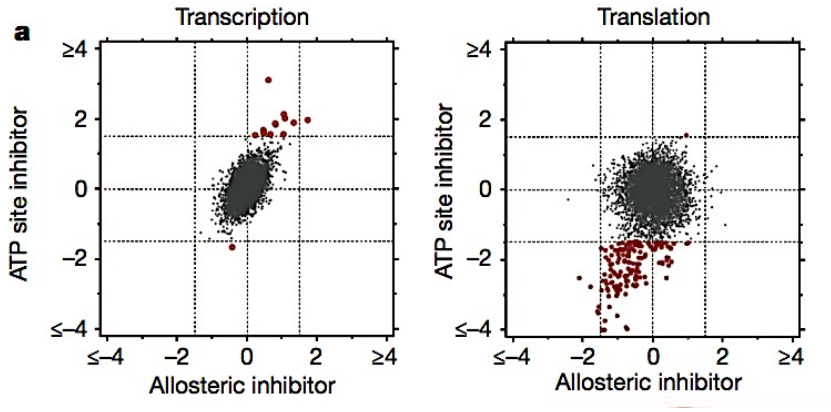

Figure 5: Comparison between RNA levels (left) and translational efficiency (right) upon treatment with PP242 (ATP site mTOR inhibitor) against rapamycin (allosteric mTOR inhibitor) in PC3 cells. Figure taken from [31].

Finally, translationally regulated mTOR-sensitive mRNAs were classified according to their function. Among all the different functions, protein synthesis and cell invasion/metastasis were the predominant classes of translationally regulated mRNAs, elucidating the cellular invasive features of human prostate cancer cells that hyperactivated mTOR controls at the translational level.

Even though ribosome profiling is presented as a powerful technique, it is not exempt of limitations. As with polysome profiling, specialized equipment is required, and the complex methodology demands a laborious and intensive work as much as solid extensive bioinformatics.

Polysome Profiling of Ribosomal Protein mRNAs upon mTOR inhibition

In many tumor types the mTOR pathway is hyperactivated. The number of anabolic processes that mTOR controls have made it an attractive therapeutic target that is now exploited in the clinic[32]. In this regard, the effects of mTOR inhibitors on cell proliferation are mediated by blocking many steps of ribosome biogenesis capacity of the cell. RPs synthesis is mainly controlled at the translational level, and mTOR signaling is in charge of sustaining RP mRNA translation by virtue of the 5'TOP sequence element that almost all RPs possess at the transcriptional start site (TSS). One of the research interests of the Laboratory of Cancer Metabolism (LMC) is unraveling the mechanism by which mTOR controls the translation of 5'TOP mRNAs and how the inhibition of this pathway can be efficiently exploited to attack tumor spreading and relapse. During my stay at the LMC I have been following the work of my thesis director, who showed me how the engagement in translation of an RP mRNA in a cancer cell line upon TOR inhibition is measured.

Cells from HCT116 human colon adenocarcinoma cell line were cultured in full medium and in full medium supplemented with the mTORC1 allosteric inhibitor rapamycin, at the concentration of 40nM for 16h. Cells were harvested and polysomal lysates prepared according to the protocol described above. Equal amounts of polysomal lysates were applied to sucrose linear gradients and ultracentrifuged, then fractionated with a Gradient

Fractionation Unit and the abundance of rRNA was determined by measuring the absorbance at 260nm with a spectrophotometer (Figure 6).

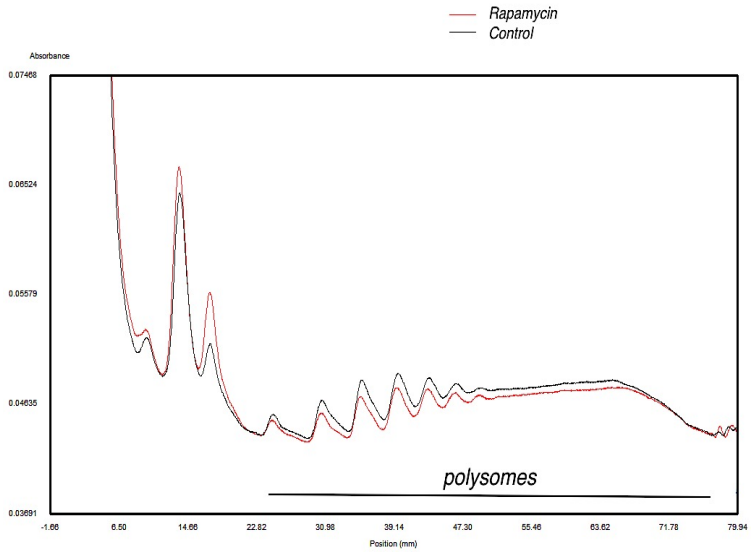

Figure 6: Polysome profiles of HCT116 cell lysate (black) and HCT116 rapamycin-treated cells (red).

Every sucrose gradient was fractionated in 12 samples and each fraction was added with an exogenous Luciferase RNA that served as normalization control. RNA was extracted from all fractions and subjected to retro-transcription. Distribution of a 5'TOP mRNA (RPL11) and a non-5'TOP mRNA (GAPDH) was determined by qPCR and the amounts of RPL11 or GAPDH cDNAs normalized to luciferase cDNA spike were calculated in each fraction. As shown in Figure 7, RPL11, which is distributed in a proportion of 40%/60% non-polysomes/polysomes respectively in control cells, is redistributed in favor of the non-polysomal part of the gradient upon rapamycin treatment, evidencing the predicted translational inhibition of 5'TOP mRNA when mTORC1 signaling is pharmacologically blocked. A non-5'TOP mRNA such as GAPDH didn't show such redistribution upon TOR inhibition, but only a sharp drop in large polysomes, presumably due to general effect of rapamycin on global protein synthesis rate.

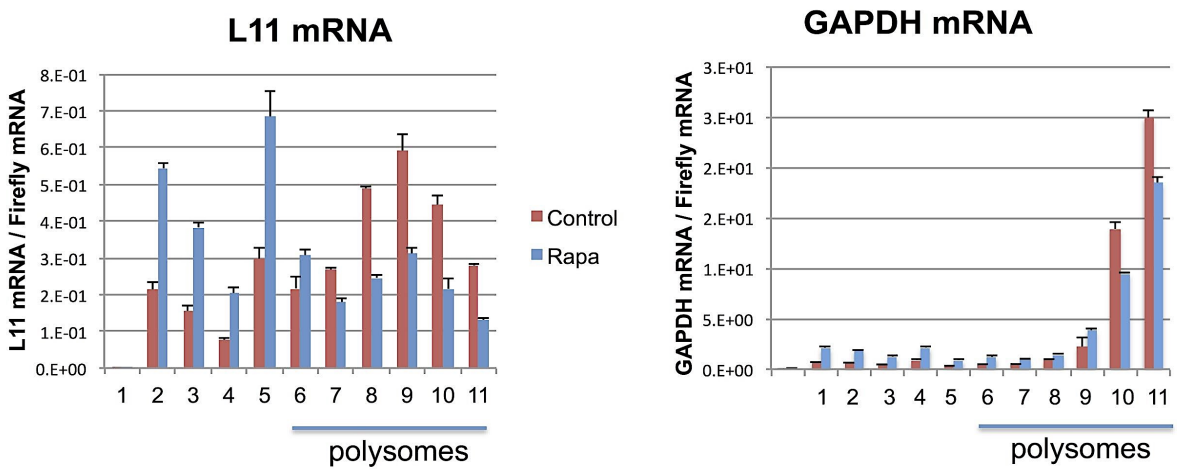

Figure 7: RT-qPCR analysis from gradients shown in Figure 6. RPL11, GAPDH and luciferase cDNAs were determined in each fraction and the ratio RPL11/Luciferase (left panel) or GAPDH/Luciferase (right panel) were calculated.

CONCLUSIONS

1 – Polysome profiling and ribosome profiling are two techniques that allow the study and analysis of the translatome in a different degree. While polysome profiling is used for initial screenings of translational changes and for tracking the translational status of already known mRNAs, ribosome profiling offers the possibility of constructing RNA libraries from ribosome protected fragments, providing plenty of information about the translation process and leading to the discovery of new elements and sequences.

2 – Inhibition of mTOR signaling pathway using rapamycin reduces the translation of 5'TOP mRNAs in HTC116 cancer cells. As almost all RPs (including RPL11) contain this 5'TOP sequence, this effect negatively impacts the synthesis RPs and hence of ribosomes, and consequently slow down the proliferation rate of cancer cells. This effect can be measured by using the polysome profiling technique.

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