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# Ribosome biogenesis as a novel therapeutic target in breast cancer

Piero Lo Monaco

► **To cite this version:**

Piero Lo Monaco. Ribosome biogenesis as a novel therapeutic target in breast cancer. Cancer. Université de Lyon, 2020. English. NNT : 2020LYSE1009 . tel-02864766

**HAL Id: tel-02864766**

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N° d'ordre NNT :  
2020LYSE1009



**THESE de DOCTORAT DE L'UNIVERSITE DE LYON**  
opérée au sein de  
**l'Université Claude Bernard Lyon 1**

**Ecole Doctorale N° ED340**  
**Biologie Moléculaire Intégrative et Cellulaire**

**Spécialité de doctorat** : Oncologie  
**Discipline** : Biologie Cellulaire et Biologie Moléculaire

Soutenue publiquement le 21/01/2020, par :  
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**La biogénèse des ribosomes cible thérapeutique  
dans le cancer du sein**

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# Acknowledgements

First of all, I wish to thank all the members of my PhD thesis committee for agreeing to devote some of their valuable time to reading my work. The writing of this thesis was exciting and stimulating, but extremely tiring. I sincerely hope that reading will spare you the tiring side and interest you as much as it interested me, and that you will rather enjoy the exciting side.

I would like to express my heartfelt thankfulness to Fred and Jean-Jacques, who have followed me along this path, indulging the ups and downs of scientific research and always being able to hold the reins of the situation and the motivation to move forward despite the thousands of obstacles that the insidious world of biology hides in order to trap researchers. Their experience and perseverance have been essential.

No research work is a solitary one. Each idea is the result of an exchange with other people, whether this exchange occurred during the research project or in the past long before the PhD began, whether it took place in the laboratory with other members of the research group or outside, in the streets, in parks, in other cities or countries. My PhD lasted more than three years, and there are many people to thank for their help, their closeness, their understanding, their daily presence. I won't name anyone of them, because it would be unforgivable to forget someone, and I don't want to take that risk. If you're smiling as you read, you're probably one of those people. Thank you.

Cancer research has obvious practical applications, which even when not immediate are at least easy to guess. There is also a more abstract intellectual sense that consists in the contribution to collective knowledge. During the writing of this thesis, I found myself making considerations about epistemology and the nature of human understanding of the world. Obviously I won't go into details, because this is not a thesis in philosophy and because I'm very far from having the necessary skills and knowledge to structure a serious and experienced dissertation on the topic. However, I mention it rather as a pretext for remembering the obvious fact that a PhD is part of something larger. From a scientific point of view, it is part of a broader research project and a step towards greater collective knowledge. From a work or academic point of view, it is part of a more or less defined path that we commonly call a career. From a personal point of view, it is part of life, and reflecting on this aspect has led me to reconsider the meaning of my PhD. In more than three years, many things have changed and many have not, but even those that have not

are never really unchanged and constantly assume different meanings and roles from time to time, according to their relationships with every other aspect of life, according to the moment, according to the perspective. Everything changes, everything escapes us, but we manage, sometimes at the cost of enormous efforts, to draw something out from this fleeting change, to understand certain basic rules, or to believe that we understand them, then question them again and so on. Just like in scientific research. So what do we have different from the cells we observe under the lens of the microscope? Is it more difficult to grasp the sense of their continuous anxiety or the sense of ours?

Biology is the science of life. Science and life, both are extremely complicated, never simple and linear. Here, apart from the subject of my thesis, is something I would have learned anyway, whatever the subject might have been: a lesson on the complexity of any given thing and on the non-linearity of life.

## Résumé

Le cancer du sein est classé en plusieurs sous-types dont les caractéristiques cliniques varient considérablement. En particulier, le sous-type dit triple-négatif est associé à un mauvais pronostic et à une probabilité plus élevée de récurrence du cancer après traitement, un problème clinique majeur dans le traitement de cette maladie. Cela s'explique principalement par l'absence de thérapies ciblées spécifiques. Il est nécessaire d'identifier de nouveaux biomarqueurs prédictifs de la survie en fonction de la nature de la tumeur et de nouvelles cibles thérapeutiques, afin de développer de nouveaux médicaments et ainsi améliorer la prise en charge clinique des patientes atteintes d'un cancer du sein triple négatif.

Récemment, il a été démontré qu'une nouvelle cible peut être utilisée dans le traitement des cancers : le ribosome. Les ribosomes sont responsables de la synthèse des protéines cellulaires et jouent donc un rôle central dans la croissance et la prolifération cellulaire. La biogénèse des ribosomes est altérée dans la plupart des cancers, en raison de la dérégulation des principales voies oncogéniques et suppresseurs de tumeur, tels que MYC, p53 et PTEN, qui régulent l'activité de la ARN Polymerase I, l'enzyme responsable de la transcription de l'ARN ribosomique. Ainsi, la biogénèse des ribosomes apparaît aujourd'hui comme un processus essentiel dans la tumorigénèse et l'idée de cibler la biogénèse des ribosomes s'impose dans le domaine du traitement anti-tumoral.

L'inhibition de la biogénèse des ribosomes a été validée comme outil thérapeutique, à partir de trois composés qui inhibent l'activité de l'ARN Polymerase I (CX-5461, CX-3543 et BMH-21) qui ciblent sélectivement les cellules cancéreuses par rapport aux cellules saines. Leur activité anti-tumorale a été rapportée dans plusieurs types de cancer, y compris la leucémie, le lymphome, le myélome, l'ostéosarcome, le cancer de la prostate et le cancer de l'ovaire, mais l'avantage potentiel de cibler la biogénèse des ribosomes dans certains types de cancer du sein, notamment les triple-négatifs, n'a pas été étudié à ce jour. Bien que très peu de choses soient connues sur la biogénèse des ribosomes dans les cancers du sein triple-négatifs, il y a aujourd'hui un besoin urgent de nouveaux outils thérapeutiques et le potentiel de l'inhibition de la biogénèse des ribosomes doit être évalué.

Par ailleurs, la biogénèse des ribosomes dépend de nombreux facteurs de maturation qui représentent une nouvelle famille de cibles à explorer. Ici, le ciblage de la Fibrillarine est proposé. Cela pourrait être particulièrement pertinent, car la fibrillarine est souvent surexprimée dans les cancers du sein triple-négatifs et est associée à un mauvais pronostic et à un risque de récurrence. Ainsi, dans ce travail de thèse, l'inhibition de la biogénèse des

ribosomes est réalisée en ciblant soit sa première étape, par l'intermédiaire des inhibiteurs de l'ARN Polymerase I, soit l'effecteur Fibrillarine en aval, par ciblage génique.

Plusieurs mécanismes cytotoxiques ont été décrits dans divers modèles de cancer lors du traitement par des inhibiteurs de l'ARN Pol I et la réponse obtenue semble être propre à chaque modèle. À l'heure actuelle, comme l'effet sur les cellules est imprévisible, la caractérisation d'une telle réponse dans les modèles d'études n'est pas sans fondement, d'autant plus que les cancer du sein triple-négatifs sont très hétérogènes.

Les deux principaux objectifs de cette thèse sont donc :

1. Étudier la sensibilité de lignées de cancer du sein triple négatif à l'inhibition de la biogénèse des ribosomes par un traitement pharmacologique ciblant l'ARN Polymerase I ou bien par ciblage génétique de la Fibrillarine ;
2. Déterminer les mécanismes induisant l'effet cytotoxique de l'inhibition de la biogénèse des ribosomes dans ce modèle d'étude.

Dans ce travail, en utilisant un large panel de lignées de cellules cancéreuses représentant quatre sous-types différents des triple-négatifs, il est montré que l'inhibition de la biogénèse des ribosomes par les composés BMH-21 et CX-5461 induit une forte inhibition de la croissance cellulaire et une perte du phénotype tumorigène *in vitro*. Conformément à l'hétérogénéité du modèle, les lignées cellulaires triple-négatives présentent une large gamme de sensibilité allant de 10 nM à 500 nM, mais sans association avec les sous-types selon les systèmes de classification courants. Concernant les réponses cellulaires induites par ces traitements, un phénomène intéressant a été observé : bien que le BMH-21 provoque l'apoptose, aucun signe d'apoptose n'a été observé lors du traitement par CX-5461. De plus, les résultats de ce travail de thèse montrent que l'inhibition de la biogénèse des ribosomes au niveau post-transcriptionnel, en aval de l'ARN Polymerase I, par ciblage génique de la Fibrillarine, induit également une forte inhibition de la croissance cellulaire, associée à une réponse apoptotique. L'inhibition de la croissance induite par le ciblage de la biogénèse des ribosomes ne s'est pas avérée associée aux niveaux d'expression des facteurs impliqués dans la biogénèse des ribosomes ni aux mutations courantes des lignées cellulaires triple-négatives, ce qui reste un problème pour l'identification des tumeurs qui pourraient bénéficier d'avantage de ce genre de traitement. Dans l'ensemble, ces résultats suggèrent que l'inhibition de la biogénèse des ribosomes pourrait constituer une stratégie thérapeutique prometteuse pour le cancer du sein triple-négatif, en démontrant le potentiel clinique de cette approche et en positionnant la

Fibrillarine et la maturation des ARN ribosomiques comme de nouvelles cibles thérapeutiques intéressantes.

En parallèle de l'avancement de ce projet principal, des études supplémentaires ont été menées au cours de cette thèse, dont un travail dans le même esprit que le principal, visant à évaluer la pertinence de l'inhibition de la biogénèse des ribosomes dans le neuroblastome.

De plus, des collaborations dans d'autres projets scientifiques au sein de l'équipe de recherche ont permis la contribution à des travaux concernant la fonction des ribosomes dans plusieurs modèles d'intérêt clinique, ou bien d'approfondir des sujets de biologie fondamentale du ribosome tels que la fonction des modifications post-transcriptionnelles des ARN ribosomiques et leur contribution à l'hétérogénéité des ribosomes, selon le concept récent de "ribosomes spécialisés".



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# List of abbreviations

<b>5'ETS</b>	5' external transcribed spacer	<b>FACT</b>	Facilitates chromatin transcription
<b>5-FU</b>	5-fluorouracyl	<b>FBL</b>	Fibrillar
<b>5'TOP</b>	5' terminal oligopyrimidine tract	<b>FC</b>	Fibrillar center
<b>AKT</b>	Protein kinase B	<b>FGF</b>	Fibroblast growth factor
<b>ALL</b>	Acute lymphocytic leukemia	<b>G4</b>	G-quadruplex
<b>AML</b>	Acute myeloid leukemia	<b>GC</b>	Granular component
<b>AMP</b>	Adenosine monophosphate	<b>GDP</b>	Guanosine diphosphate
<b>AMPK</b>	AMP-dependent kinase	<b>GTP</b>	Guanosine triphosphate
<b>ARF</b>	Alternate reading frame tumor suppressor	<b>H1</b>	Histone 1
<b>ATR</b>	Ataxia telangiectasia and Rad3 related	<b>H2A</b>	Histone H2A
<b>BAX</b>	BCL2-associated X protein	<b>HBV</b>	Hepatitis B Virus
<b>BOP1</b>	Block of proliferation 1	<b>HDM2</b>	Human double minute 2
<b>BRCA1/2</b>	Breast cancer type 1/2 susceptibility protein	<b>HER2</b>	Human epidermal growth factor receptor 2
<b>CBP</b>	CREB-binding protein	<b>HMG</b>	High Mobility Group
<b>CBS</b>	Cystathionine beta synthase	<b>IGF</b>	Insulin-like growth factor
<b>CDK</b>	Cyclin-dependent kinase	<b>IGF-1R</b>	IGF-1 receptor
<b>CDKN1A</b>	Cyclin-dependent kinase inhibitor 1A	<b>IGS</b>	Intergenic spacer
<b>CHH</b>	Cartilage hair hypoplasia	<b>IHC</b>	Immunohistochemistry
<b>CK2</b>	Casein kinase 2	<b>IL-6</b>	Interleukine 6
<b>CRISPR</b>	Clustered regularly interspaced short palindromic repeats	<b>IP</b>	Immunoprecipitation
<b>Cryo-EM</b>	Cryo-electron microscopy	<b>IRES</b>	Internal Ribosome Entry Site
<b>CSB</b>	Cockayne syndrome B	<b>ITS1/2</b>	Internal transcribed space 1/2
<b>CRCL</b>	Centre de recherche en Cancerologie de Lyon	<b>KI67</b>	Antigen KI-67
<b>DBA</b>	Diamond-Blackfan anemia	<b>LAR</b>	Luminal androgen receptor
<b>DC</b>	Dyskeratosis congenita	<b>LKB1</b>	Liver kinase B1
<b>DEF</b>	Digestive organ expansion factor	<b>MAPK</b>	Mitogen-activated protein kinase
<b>DFC</b>	Dense fibrillar centre	<b>MDM2</b>	Mouse double minute 2
<b>DKC1</b>	Dyskerin	<b>MDS</b>	Myelodysplastic syndrome
<b>DNMT1/3</b>	DNA methyltransferase 1/3	<b>MKI67</b>	Marker of proliferation KI-67
<b>EC50</b>	Half-maximal effective concentration	<b>MLL</b>	Mixed lineage leukemia
<b>EGF</b>	Epidermal growth factor	<b>NAD+</b>	Nicotinamide adenine dinucleotide
<b>EIF6</b>	Eukaryotic initiation factor 6	<b>NOR</b>	Nucleolar organizer region
<b>ER</b>	Estrogen receptor	<b>NPM1</b>	Nucleophosmin
<b>ERK</b>	Extra-cellular signal-related kinase 1	<b>PARP</b>	Poly-(ADP-ribose) polymerase
		<b>PCAF</b>	P300/CBP-associated factor
		<b>PCNA</b>	Proliferatin cell nuclear antigen
		<b>PES1</b>	Pescadillo ribosomal biogenesis factor

<b>PI3K</b>	Phosphatidylinositol-4,5-bisphosphate 3-kinase	<b>TAF</b>	TBP-associated factor
<b>PIC</b>	Pre-initiation complex	<b>TBP</b>	TATA-binding protein
<b>Pol</b>	Polymerase	<b>TCGA</b>	The Cancer Genoma Atlas
<b>PPAN</b>	Peter pan gene homolog	<b>TCS</b>	Treacher Collins syndrome
<b>PR</b>	Progesterone receptor	<b>TFII</b>	Transcription factor for RNA Pol II
<b>pRB</b>	Retinoblastoma protein	<b>TFIII</b>	Transcription factor for RNA Pol III
<b>PTEN</b>	Phosphatase and tensin homolog	<b>TIF</b>	Transcription initiation factor
<b>PTRF</b>	Transcript release factor	<b>TIF6</b>	Translation initiation factor 6
<b>RB</b>	Retinoblastoma	<b>TNBC</b>	Triple negative breast cancer
<b>RISC</b>	RNA-induced silencing complex	<b>TOR</b>	Target of rapamycin
<b>RNP</b>	Rinonucleoprotein	<b>TP53</b>	Tumor protein p53
<b>RP</b>	Ribosomal protein	<b>TRAMP</b>	Thrombospondin-related apical membrane protein
<b>RPA</b>	RNA Pol I subunit	<b>TRRAP</b>	Transformation/transcription domain-associated protein
<b>RPL</b>	Ribosomal protein (large subunit)	<b>TTF</b>	Transcription termination factor 1
<b>RPS</b>	Ribosomal protein (small subunit)	<b>UBF</b>	Upstream binding factor
<b>rRNA</b>	Ribosomal RNA	<b>UMP</b>	Uridine monophosphate
<b>S6K</b>	Ribosomal protein S6 kinase	<b>UTR</b>	Untranslated region
<b>SDS</b>	Shwachman-Diamond syndrome	<b>VEGF</b>	Vascular endothelial growth factor
<b>SL1</b>	Selectivity factor 1		
<b>snoRNA</b>	Small nucleolar RNA		
<b>snoRNP</b>	Small nucleolar ribonucleoprotein		

## Preface

Cancer is a major health care issue: in 2015, about 90.5 million people had cancer and 8.8 million people died of cancer, accounting for 15.7% of deaths, and similar figures are estimated every year. In both sexes combined, lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death. In women, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death. According to the World Health Organization, breast cancer impacts 2.1 million women each year. Indeed, significant advances contributed to reduce breast cancer-related mortality and several treatments are available. Nevertheless, many patients still die of breast cancer: it is estimated that 627,000 women died from breast cancer in 2018. Such mortality may depend on the presence of a metastatic tumour, on the development of resistance to treatment or on the absence of effective treatments for particular subtypes of breast cancer. Thus, current studies aim to decipher the different mechanisms responsible for the aggressiveness, resistance and heterogeneity of breast cancers in order to develop new treatment options and to identify prognostic or predictive biomarkers.

Breast cancer is classified into several subtypes and clinical features vary substantially among different breast cancer subtypes. In particular, the so-called triple negative subtype is associated to a poor prognosis and a higher probability of cancer recurrence after treatment. This is mainly due to the current lack of specific targeted therapies. To improve the clinical outcome for patients affected by triple negative breast cancer, it is necessary to identify new biomarkers to predict survival according to the nature of their tumour, and ultimately to discover new therapeutic targets and develop new drugs adapted to each patient.

Recently, it has been demonstrated that a novel target can be used in anti-cancer therapy: the ribosome. The ribosome is an essential molecular machinery responsible for protein synthesis starting from an mRNA molecule, through the process of translation. Ribosome biogenesis is overactivated in most cancers. This is generally viewed as the obvious implication of the fact that a cancer cell needs a higher protein synthesis rate for its abnormally high proliferation and metabolic activity. The overactivation of ribosome biogenesis in cancer results from the deregulation and loss of coordination between the different pathways under the control of the major oncogenes and tumor suppressors such as c-Myc, p53 and PTEN and is now emerging as an essential process in tumorigenesis. The abnormal overproduction of ribosomes in cancer cells has led to the idea of inhibiting

ribosome biogenesis to selectively target cancer cells in clinical context. This strategy has historically been tested with RNA Pol I inhibitors such as actinomycin D, but not exploited, due to limited specificity and high toxicity of actinomycin D for healthy cells. It is only recently that this hypothesis has been brought back to clinical interest, as the inhibition of ribosome biogenesis has been validated as a therapeutic tool, on the basis of novel compounds that preferentially target cancer cells against healthy cells and display lesser toxicity in several reported cases. Thus, blocking ribosome production is nowadays a therapeutic approach with proven potential, and its scope must be explored.

So far, the anti-cancer activity of these compounds has been reported in several cancer diseases, including leukemia, lymphoma, myeloma, osteosarcoma, prostate and ovarian cancer, and two compounds achieved phase 2 clinical trials in patients with solid tumours and lymphomas. However, the value of this approach in breast cancer has not yet been explored and especially new strategies are urgently needed for the clinical context of triple negative breast cancer. For this reason, the first objective of this thesis is to put forward inhibition of ribosome biogenesis by targeting RNA Pol I as a potential anti-cancer treatment in the aforesaid context.

In addition, because the current demand from the clinical field is to identify new targets of therapeutic interest and because targeting RNA Pol I may not be effective for the treatment of specific cancers, other ways to block ribosome biogenesis with selectivity for cancer cells are investigated. In particular, data from my research group and others highlights the importance of fibrillarlin, that represents a tempting candidate to target in breast cancer. Indeed, Fibrillarlin is an essential protein required for ribosome biogenesis and its inhibition impairs ribosome production in human cells. From a translational point of view, it is interesting that fibrillarlin is overexpressed in several cancers including breast cancer, especially a large proportion of triple negative breast cancers, its expression being associated with poor prognosis and risk of recurrence. Therefore, patients carrying tumours with high levels of fibrillarlin could reasonably benefit from targeted therapy against this essential factor of ribosome biogenesis.

For these reasons, this thesis focuses on the inhibition of ribosome biogenesis by targeting RNA Pol I or, alternatively, fibrillarlin in triple negative breast cancer, with particular regard to the associated cytotoxic mechanisms accounting for anti-cancer properties.

# Introduction



# Chapter 1.

## Ribosome biogenesis at the core of cell functions

### 1. The nucleolus

#### Nucleolar morphology

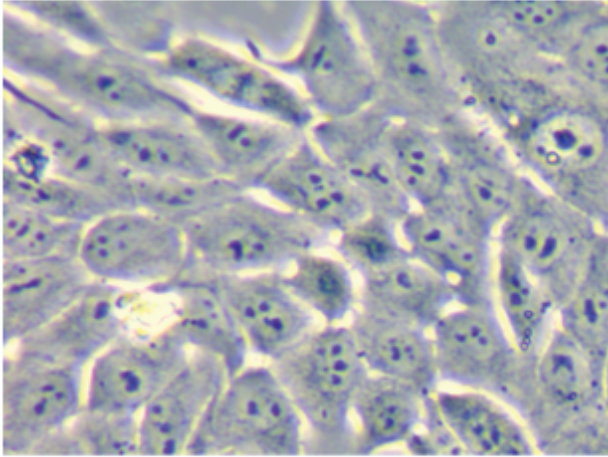
The nucleoli are structural and functional domains present within the nucleus of all eukaryotic cells. Lacking a delimiting membrane, nucleoli are not organelles but rather specific domains within the nucleus. The nucleolus was originally described in 1781, but a long time had to pass before it was known that the key biological function of nucleoli is ribosome subunit production, as first described in 1964 and well established ever since (Brown and Gurdon, 1964).

The nucleolus is one of the most highly dynamic cellular compartments: the structural and morphological features of nucleoli, such as size and shape, as well as their number within a nucleus, can vary greatly depending on cell type, cell cycle stage, culture conditions (Figure I A,B). In higher eukaryotes, nucleoli are disassembled during mitosis and cytokinesis, reappear as rapidly assembling structures at the end of cell division and completely form at the beginning of interphase (Andersen et al., 2005; Boisvert et al., 2007; Dundr et al., 2000; Leung et al., 2004). In cycling cells, the volume of the nucleoli increases between G1 and G2 phases and the number of subnuclear functional units, namely fibrillar centers, doubles in G2 phase (Junera et al., 1995).

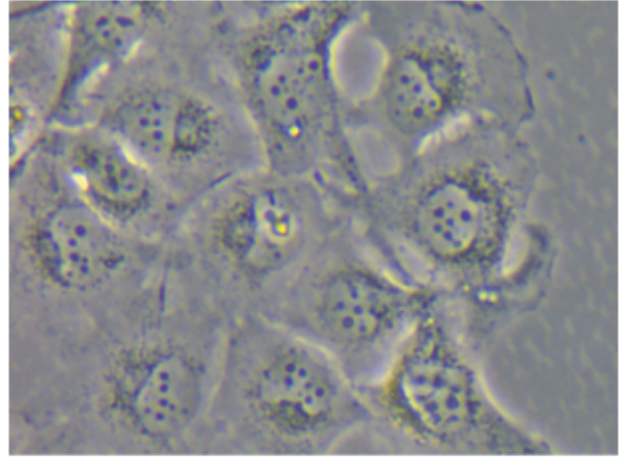
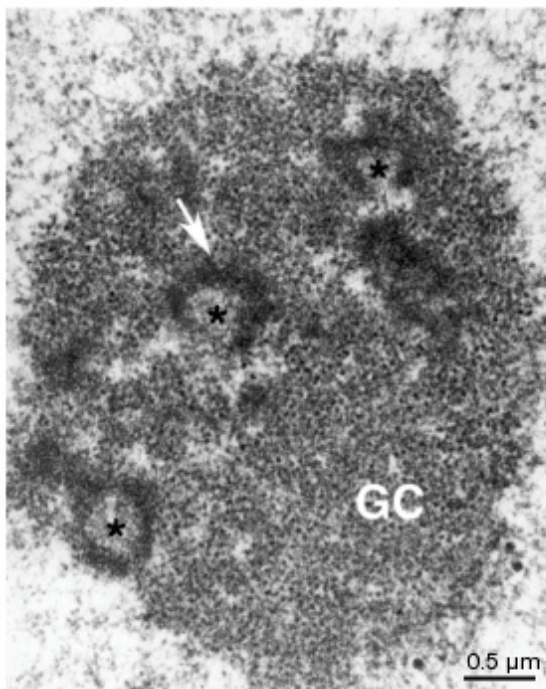
During the interphase, seen through a light microscope the nucleolus appears as a dense, large, roundish structure within the nucleoplasm and inner subnuclear structures are visible as minor spots (Love and Wildy, 1963): these are the fibrillar centres, one of the three nucleolar components. However, the morphology of the nucleolus has been characterized mainly by electron microscopy, the use of which has allowed detailed description at the ultrastructural level: in human nucleoli, three distinct components are constantly present, namely the fibrillar centres (FC), the dense fibrillar component (DFC) and the granular component (GC). The FCs, visible through light microscope as well, are light electron-dense structures varying in size, ranging from 0.1 to 1  $\mu\text{m}$ ; the DFC is a highly contrasted, compact structure formed by densely packed fibrils and located at the border of FC; finally, the GC consists of granules, 15-20 nm in diameter, surrounding the other two components in a loosely organized distribution (Figure I C). The chemical properties displayed by some among these factors allow the use of a histological silver-

**A**

MCF10-A

**B**

BT-549

**C**

### Figure I. Nucleolar structure

**A-B)** Nucleoli appear as dense roundish spots within the nuclei. A normal cell has two or three nucleoli on average, as in the case of the non-tumorigenic MCF-10A breast epithelial cells shown in A. In contrast, highly proliferating cells such as many types of cancer cells display a higher number of nucleoli, up to six in BT-549 breast cancer cells as shown in B. Both pictures were taken by a contrast phase microscope. **C)** Section of one nucleolus of a HeLa cell, prepared by conventional methods for electron microscopy, showing nucleolar organization including the three nucleolar components. Fibrillar centres are indicated by black asterisks, a dense fibrillar component is indicated by a white arrow, while a unique granular component surrounds the two fibrillar components. Picture adapted from Hernandez-Verdun et al., 2010.

based staining method in order to selectively mark them and thus visualize the nucleoli: in particular, the largest RNA polymerase I (RNA Pol I) subunit, UBF, nucleophosmin and nucleolin are selectively stained by a silver treatment. Thus, silver staining evidently marks nucleolus and has been for decades the standard method for the observation of nucleolar morphology (Derenzini et al., 2009; Hernandez-Verdun et al., 2010). The two fibrillar components, FC and DFC, constitute the structural-functional unit of the nucleolus: it is where ribosomal genes are located, together with all factors necessary for rDNA transcription, such that upon silver staining these structures appear as evidently stained spots.

Genes encoding ribosomal RNA (rDNA) are organized in a peculiar structure consisting in several hundreds of tandemly repeated copies, termed nucleolar organizer regions (NORs). In human, from a genomic point of view, NORs are distributed on five chromosomes, namely chromosome 13, 14, 15, 21, and 22, which bear about 400 copies. Anyways, one should always keep in mind that the gene copy number of ribosomal genes is extremely variable among humans (Stults et al., 2008). The nucleolus is either organized around a single NOR or alternatively several active NORs merge into a single nucleolus. In particular, in normal human cells there are two to three nucleoli per cell corresponding to six active NORs (Savino et al., 2001). Indeed, silver-based staining selectively marks NORs and for this reason it is commonly referred to as AgNOR staining.

Human nucleoli are generally surrounded by a shell of condensed chromatin that occasionally penetrates deeply into the nucleolar domain reaching the FCs; this so-called perinucleolar chromatin and FCs are thus contiguous. In sections, these invaginations are often visualized as nucleolar interstices (Scheer et al., 1993). In perinucleolar chromatin, nucleolus-associated domains were recently isolated, sequenced and characterized in human cells. They correspond to at least 4% of the total genome sequences (Németh et al., 2010) and are enriched in RNA-binding proteins and Pol III transcripts (Pollock and Huang, 2010).

Finally, worth of note, there is an intimate and evolutionary conserved relationship between nucleoli and the nuclear envelope. In yeast, nucleolus is extensively in contact with the nuclear membrane and the same vicinity is found in higher eukaryotes, where nucleoli are frequently located close to the nuclear envelope. In some cases in higher eukaryotes, even when the nucleolus is centrally located in the nucleus, it is directly connected to the nuclear membrane through one or more invaginations called "nucleolar

canals" (Hernandez-Verdun et al., 2010). The function of such topology will be discussed later on.

## **Functions of nucleolar components**

The nucleolus is the site where ribosomal genes are located and ribosome biogenesis mainly takes place. The nucleolus comprises an increasingly complex set of proteins, ribosomal RNA (rRNA) and hundreds of small nucleolar RNA (snoRNA) species. Each nucleolar compartment is characterized by a distinct biochemical composition, reflecting its function. FCs contain rDNA in a transcription-competent structure, some nascent pre-rRNA and most importantly transcription factors such as RNA Pol I, UBF, DNA topoisomerase I; DFCs contain nascent pre-rRNAs and factors involved in their modifications, including fibrillarin and dyskerin; GCs, which account for up to 75% of the nucleolar mass, contains pre-ribosomal particles and consist in the only compartment that is not AgNOR staining positive. This precise localization of factors and intermediates of ribosome biogenesis reflects the spacial distribution of biological functions involved in the process of ribosome biogenesis itself: the sites of active RNA Pol I transcription are localized at the interface between the FC and the DFC, newly transcribed rRNA molecules are specifically located in the DFC, where the early steps of pre-rRNA processing and maturation take place, whereas the late steps of ribosome biogenesis take place in the GC, where the rRNA molecules and ribosomal proteins are assembled together and form the two ribosome subunits. Indeed, these events occur in a gradient/vectorial spacial and functional organization (Cisterna and Biggiogera, 2010; Hernandez-Verdun et al., 2010).

Such correspondence between functional and spacial organizations makes it possible to visualize the three-dimensional structure of nucleolus. The three basic nucleolar components can be mapped by immunolabeling or using fluorescent proteins corresponding to a precise step in ribosome biogenesis. By using a confocal microscope, it has been observed that in cells where ribosome biogenesis is active, the FC and DFC are distributed in the foci in the internal part of the nucleoli surrounded at the nucleolar periphery by the GC (Hernandez-Verdun et al., 2010; Ochs, 1998)

Of note, the precise role of FCs is still under debate: according quantitative analysis based on a combination of immunocytochemistry and electron microscope, most of the RNA Pol I pool is accumulated at FCs (Scheer and Rose, 1984). Consistently with the previously mentioned interpretation of FC and DFC functions, it has been hypothesized that high concentration of Pol I seen in FCs corresponds to inactive enzyme. This is in line with the estimation that less than 10% of nucleolar RNA Pol I is engaged in transcription

(Dundr et al., 2002; Milkereit and Tschochner, 1998; Miller et al., 2001). Non-transcribing RNA Pol I would then be accumulated in FCs and transcribing RNA Pol I in DFCs: when an inactive ribosomal gene within the FC becomes activated, it automatically becomes a part of the DFC (Ochs, 1998; Raška et al., 2006a). Therefore, the presence of RNA Pol I in the FC should be reconsidered as indicating a sort of reservoir of the enzyme, in its inactive form, ready to be activated in case of need.

## **Evolution of nucleolar components**

Proteomics analyses of purified human nucleoli demonstrated the presence of more than 700 individual proteins and more than 4500 are likely to be comprised, and comparisons of these first data with the proteome from yeast demonstrated that 90% of human proteins have yeast homologues, indicating that the nucleolar proteome is largely conserved throughout evolution (Andersen et al., 2005; Scherl et al., 2002). Nonetheless, differences between yeast and human nucleolus exist in terms of number of components and occurrence of intranucleolar bodies; yeast nucleoli lack condensed perinucleolar chromatin and show extensive nuclear membrane attachment; in yeast, nucleoli are not disassembled during mitosis; finally, there is about a 10-fold difference in size between yeast and human nucleoli (Hernandez-Verdun et al., 2010).

A model for the evolution of nucleolar compartments was proposed, based on in-depth comparative analysis of nucleolar structure in several species: the authors propose that the acquisition of three nucleolar compartments is a recent evolutionary trait. In all eukaryotes but amniotes, the nucleolus is bipartite as it displays two compartments: a transcription-competent fibrillar component containing RNA Pol I, where transcription takes place, and a granular component, with nucleolar interstices eventually protruding into the nucleolus. In amniotes, both FCs and nucleolar interstices contain rDNA, although they differ by the exclusive presence in FCs of RNA Pol I and transcription-associated factors. By unambiguously defining the FC as a compartment containing RNA Pol I and associated factors, the authors claim that FCs are only detected in the amniote clade, with nucleolar interstices being transcriptionally inactive, the FC being transcription-competent and the DFC being transcriptionally active with transcription actually taking place. This distinction is not present in the vast majority of eukaryotes, including plants, all invertebrates and anamniote vertebrates, that all display two nucleolar compartments. So, the physical separation between transcriptionally inactive and transcription-competent chromatin demarcates nucleolar interstices and FCs, defining the nucleolar compartments in amniotes including humans. Interestingly, a striking correlation appears between the

transition from two to three nucleolar compartments and a remarkable increase in the size of the rDNA intergenic region (Thiry and Lafontaine, 2005).

The authors speculate that this evolutionarily recent trait, allowing to exclude one form of chromatin from localized regions of transcriptional activity and generating a pool of transcription-related factors already available on the spot, offers not only the potential for rapid responses to environmental stimuli, but also for supplemental regulatory events and extra functions, adding a further layer of complexity to the system (Thiry and Lafontaine, 2005). This hypothesis is in line with the multiple functions of nucleolar proteins, that have been demonstrated to go far beyond mere ribosome biogenesis, as mentioned later in this chapter.

### **Dependence from transcriptional activity**

Indeed, as previously mentioned, the morphology of the nucleolus depends on its activity: when ribosome biogenesis, in particular rRNA synthesis, physiologically does not occur, for instance at defined periods of differentiation and cell maturation as well as during mitosis and cytokinesis in higher eukaryotes, no nucleolar structure is present. In the same way, as reviewed by Boulon and colleagues, stresses that lead to blocked rRNA synthesis, such as specific pharmacological treatments or UV irradiation, cause fast and dynamic rearrangement of the nucleolar proteins. In all of these conditions, finely reviewed by Boulon and colleagues, transcription in the nucleolus is inhibited or decreased and disruption of nucleolar structure is observed, with segregation of nucleolar components. In most cases nucleolar disruption takes place and peculiar structures, called "nucleolar caps", are formed by proteins of the fibrillar center and the dense fibrillar component relocalized to nucleolar periphery, while granular component proteins commonly translocate to the nucleoplasm (Boulon et al., 2010; Montgomery et al., 1966; Moore et al., 2011; Reynolds et al., 1964; Shav-Tal et al., 2005).

In cancer cells, the area occupied by nucleoli within the cell nucleus has been measured and has been shown to be closely and directly related to rRNA transcription activity (Derenzini et al., 1998).

### **Nucleolar assembly**

As seen in the previous part, and reprised in the next ones, nucleolar morphology appears mainly built on the main nucleolar function, that is ribosome production; the structure and relative disposition of nucleolar components reflects the need to organize spatially and temporally the many steps of ribosome biogenesis. In general, the formation

of nuclear domains occurs through recruitment and accumulation of proteins by nucleating RNA transcripts, that can function as a scaffold (Shevtsov and Dundr, 2011). It has been described, as well, that RNA Pol I transcriptional activity is essential for nucleolar structure. This tight link is furtherly underscored by the process of nucleolar assembly, that make up a nucleolus starting from cellular conditions lacking this structure, such as higher eukaryotic mitosis. In this section, nucleolar assembly will be described in this perspective and the essential link between transcriptional activity and nucleolus will become even more evident.

Activation of rDNA transcription at the exit from mitosis precedes the assembly of a visible nucleolus. Nucleolar proteins can form high-concentration assemblies in the absence of rDNA, but this occurs without any spatiotemporal precision in the assembly process. Conversely, it has been demonstrated that rRNA directs spatiotemporal precision of nucleolar assembly. Thus, it crucially contributes to the organization of the nucleolus (Falahati et al., 2016).

Despite the pivotal role in organize the structure of the nucleolus, transcription itself is not sufficient to start the event but nucleolar assembly can start independently of rDNA transcription. In synchronized cells, inhibition of RNA Pol I transcription in late mitosis, when nucleogenesis normally occurs, does not impair the initial localization of nucleolin, fibrillarin and pre-rRNAs synthesized in the previous cell cycle to UBF-associated NORs, and the resulting structures are similar to normal nucleoli, even though they are smaller, interspersed and obviously transcriptionally inactive (Dousset et al., 2000). Apparently a paradox: transcription arrest means disassembly, reassembly does not mean transcription restart. This is because the formation of functional nucleoli is not governed solely by the resumption of rDNA transcription: other factors, such as CDK, intervene to regulate both transcription and processing, and this will be discussed in a following section.

Though transcription is repressed during metaphase, NORs that were active in the previous interphase keep displaying a different feature: at the cytogenetic level, they are characterized by under-condensation in comparison with the rest of the chromosome and the inactive NORs. This is because of extensive binding of UBF over the ribosomal gene repeat at the level of this chromosomal feature, sequestering Pol I machinery in nucleoli even if it is not active (Gébrane-Younès et al., 1997; Prieto and McStay, 2005). So, rDNA transcription factor UBF remains associated with competent NORs throughout mitosis, allowing propagation of transcription-competent rDNA genes throughout cell divisions.

## **Nucleolus as a hub of molecular interactions**

Evidence has been accumulated suggesting that the nucleolus at least partially obeys the rules of self-organization, that is, a system that is built on the transient and functional interactions of its constituents (Lewis and Tollervey, 2000). This was confirmed by a number of studies that will not be extensively discussed here, showing that impaired function or unbalanced expression of a single component of nucleolar proteome leads to disruption of nucleolar structure, even when its interactions with the other components are transient (Chen et al., 1999; Colau et al., 2004). This conclusion is compatible with a model of self-organization for nucleolar assembly.

Hence, the nucleolus exists as a discrete structure because certain proteins, some of which remain to be characterized, bind to the rDNA, forming a more or less stable core on which the complex set of nucleolar interactions and dynamic processes is built. The surrounding nucleoplasm continually exchanges with this nucleolar super-complex and the steady-state composition of the nucleolus is the result of the fact that the nucleolar residence time of non-nucleolar proteins, which do not find interacting partners in nucleoli, is one or more orders of magnitude shorter than that of nucleolar proteins. Indeed, nucleolar proteins are in a constant influx and outflux (Dundr et al., 2002). Thus, the nucleolus is a steady-state structure with its components in dynamic equilibrium with the nucleoplasm (Raška et al., 2006b, 2006a).

## **Non-ribosomal functions**

Although the nucleolus is primarily associated with ribosome biogenesis, several lines of evidence now show that it has additional functions. Some of these functions, such as regulation of mitosis, cell-cycle progression and proliferation, many forms of stress response and biogenesis of multiple ribonucleoprotein particles (Andersen et al., 2005; Boisvert et al., 2007)

In addition to ribosome biogenesis, the nucleolus has been associated with a number of further functions, shown or inferred from proteomic, genomic and functional studies. Several studies indicate that nucleoli can regulate multiple cellular processes by serving as 'depots' for sequestration of various proteins, with the nucleolar dynamic structure used as a specific compartment where proteins can be sequestered or excluded from, in order to regulate their function (Dundr et al., 2002). This is exemplified by the cell-cycle-specific nucleolar retention of telomerase (Wong et al., 2002) and ARF-mediated regulation of p53 through sequestration of Mdm2 into the nucleolus, as described later on in the context of nucleolar stress (Olson, 2004). It has been shown that such functions include the



regulation of mRNA and tRNA maturation and nuclear export and DNA replication and repair (Pederson, 1998, 2011). A recent study revealed the existence of nucleolar detention signals that mediate non-coding RNA-dependent protein immobilization in the nucleolus (Audas et al., 2012). Studies of nucleolar proteome indicate that nucleolus is also involved in the regulation of cell cycle (Couté et al., 2006; Scherl et al., 2002). Other important extra-ribosomal functions, until recently unexpected, have been exhaustively reviewed by Lindstrom (Lindström et al., 2018).

One essential function of the nucleolus, tightly related to ribosome biogenesis, makes it a key sensor of cellular stress, through a process known as ribosomal stress or nucleolar stress which be discussed below in much detail. Nucleolar stress enables coordination of ribosome production with other cellular processes such as cell cycle, cell death and cell survival (Boisvert et al., 2007; Grummt, 2013; James et al., 2014; Tsai and Pederson, 2014). (Andersen et al., 2005; Bahadori et al., 2018; Bensaddek et al., 2016; Moore et al., 2011)

## **2. Ribosome biogenesis**

Ribosome biogenesis is a complex, highly controlled and regulated process including several steps and involving an impressive number of factors, maintaining an estimated rate of thousands of ribosomes produced per minute in a growing eukaryotic cell. All of this is a highly energy-demanding process, making ribosome biogenesis one of the most complex and energy-consuming processes in the cell (Warner, 1999; Warner et al., 2001). Here, a very quick overview will be given, focusing on eukaryote ribosome biogenesis, but one should always consider that a certain degree of variability can be found between biological systems: for instance, many human ribosome biogenesis factors have not known yeast homologues, suggesting a further layer of complexity (Tafforeau et al., 2013). The eukaryotic ribosome is composed by four ribosomal RNA molecules and 80 ribosomal proteins, assembled into two subunits: the large subunit and the small subunit. The small subunit contains 18S rRNA and 33 ribosomal proteins, while the large subunit contains three rRNA molecules, namely 28S (25S in yeast), 5S and 5.8S rRNAs, and 47 ribosomal proteins. The nomenclature of ribosomal proteins has been recently refined by a new naming system, taking into considerations structural homology across life domains, in order to eliminate previously ambiguous discrepancies (Ban et al., 2014). However, the traditional naming system is still commonly used. A comparison between the two nomenclatures for ribosomal proteins is illustrated in Table 1.

**Table 1. New nomenclature for ribosomal proteins**

New name <sup>#</sup>	Taxonomic range <sup>*</sup>	Bacteria name	Yeast name	Human name	New name <sup>#</sup>	Taxonomic range <sup>*</sup>	Bacteria name	Yeast name	Human name
<b>Large subunit</b>					<b>Small subunit</b>				
uL1	B A E	L1	L1	L10A	bS1	B	S1	–	–
uL2	B A E	L2	L2	L8	eS1	A E	–	S1	S3A
uL3	B A E	L3	L3	L3	uS2	B A E	S2	S0	SA
uL4	B A E	L4	L4	L4	uS3	B A E	S3	S3	S3
uL5	B A E	L5	L11	L11	uS4	B A E	S4	S9	S9
uL6	B A E	L6	L9	L9	eS4	A E	–	S4	S4
eL6	E	–	L6	L6	uS5	B A E	S5	S2	S2
eL8	A E	–	L8	L7A	bS6	B	S6	–	–
bL9	B	L9	–	–	eS6	A E	–	S6	S6
uL10	B A E	L10	P0	P0	uS7	B A E	S7	S5	S5
uL11	B A E	L11	L12	L12	eS7	E	–	S7	S7
bL12	B	L7/L12	–	–	uS8	B A E	S8	S22	S15A
uL13	B A E	L13	L16	L13A	eS8	A E	–	S8	S8
eL13	A E	–	L13	L13	uS9	B A E	S9	S16	S16
uL14	B A E	L14	L23	L23	uS10	B A E	S10	S20	S20
eL14	A E	–	L14	L14	eS10	E	–	S10	S10
uL15	B A E	L15	L28	L27A	uS11	B A E	S11	S14	S14
eL15	A E	–	L15	L15	uS12	B A E	S12	S23	S23
uL16	B A E	L16	L10	L10	eS12	E	–	S12	S12
bL17	B	L17	–	–	uS13	B A E	S13	S18	S18
uL18	B A E	L18	L5	L5	uS14	B A E	S14	S29	S29
eL18	A E	–	L18	L18	uS15	B A E	S15	S13	S13
bL19	B	L19	–	–	bS16	B	S16	–	–
eL19	A E	–	L19	L19	uS17	B A E	S17	S11	S11
bL20	B	L20	–	–	eS17	A E	–	S17	S17
eL20	E	–	L20	L18A	bS18	B	S18	–	–
bL21	B	L21	–	–	uS19	B A E	S19	S15	S15
eL21	A E	–	L21	L21	eS19	A E	–	S19	S19
uL22	B A E	L22	L17	L17	bS20	B	S20	–	–
eL22	E	–	L22	L22	bS21	B	S21	–	–
uL23	B A E	L23	L25	L23A	bTHX	B	THX	–	–
uL24	B A E	L24	L26	L26	eS21	E	–	S21	S21
eL24	A E	–	L24	L24	eS24	A E	–	S24	S24
bL25	B	L25	–	–	eS25	A E	–	S25	S25
bL27	B	L27	–	–	eS26	E	–	S26	S26
eL27	E	–	L27	L27	eS27	A E	–	S27	S27
bL28	B	L28	–	–	eS28	A E	–	S28	S28
eL28	E	–	–	L28	eS30	A E	–	S30	S30
uL29	B A E	L29	L35	L35	eS31	A E	–	S31	S27A
eL29	E	–	L29	L29	RACK1	E	–	Asc1	RACK1
uL30	B A E	L30	L7	L7					
eL30	A E	–	L30	L30					
bL31	B	L31	–	–					
eL31	A E	–	L31	L31					
bL32	B	L32	–	–					
eL32	A E	–	L32	L32					
bL33	B	L33	–	–					
eL33	A E	–	L33	L35A					
bL34	B	L34	–	–					
eL34	A E	–	L34	L34					
bL35	B	L35	–	–					
bL36	B	L36	–	–					
eL36	E	–	L36	L36					
eL37	A E	–	L37	L37					
eL38	A E	–	L38	L38					
eL39	A E	–	L39	L39					
eL40	A E	–	L40	L40					
eL41	A E	–	L41	L41					
eL42	A E	–	L42	L36A					
eL43	A E	–	L43	L37A					
P1/P2	A E	–	P1/P2 (AB)	P1/P2 (αβ)					

<sup>#</sup>b: bacterial, e: eukaryotic, u: universal.

<sup>\*</sup>B: bacteria, A: archaea, E: eukaryotes.

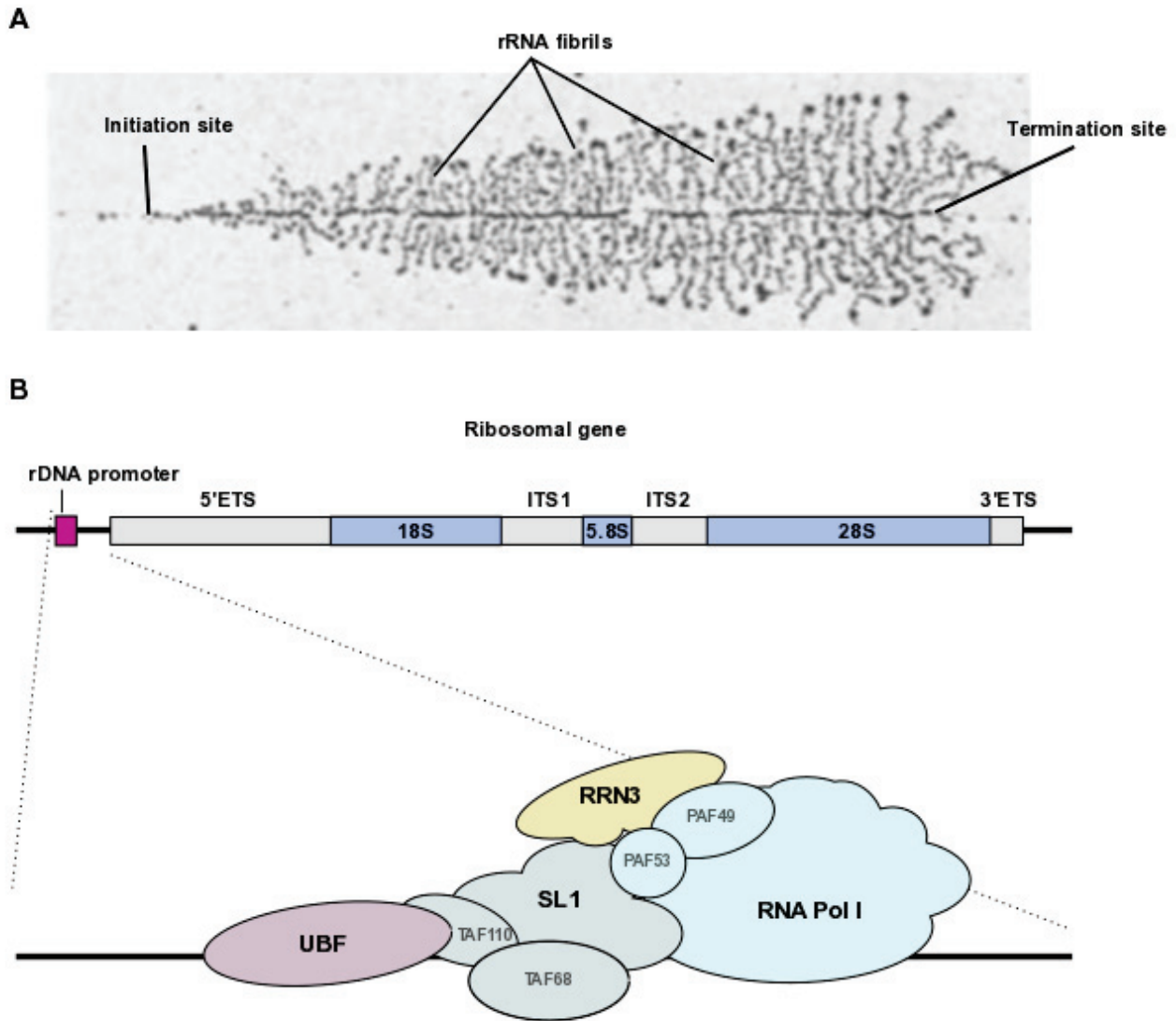
from Ban et al., 2014

In eukaryotes, ribosome biogenesis begins in the nucleolus. In mammals, rDNA clusters are characterized by multiple alternating modules of long intergenic spacer of approximately 30 kb and a precursor rRNA coding region of approximately 13 kb. Each gene is transcribed by the specialized RNA polymerase I to generate a single pre-rRNA transcript, 47S pre-rRNA molecule. This transcript is then processed and modified to generate three final molecules, namely 18S, 28S and 5.8S rRNAs, which together with 5S rRNA will form the RNA backbone structure of the functional ribosome. In yeast, 5S rRNA belongs to the same repeat cluster and is transcribed in the reverse direction; in human 5S rRNA does not belong to the nucleolar structure and is transcribed by RNA polymerase III from multiple genes in the nucleoplasm (Henras et al., 2015; Thomson et al., 2013). A number of ribosomal proteins and other factors associate with the nascent pre-rRNA transcript, forming an intermediate complex named 90S pre-ribosome, facilitating its folding and modification. This intermediate complex is subsequently cleaved into a pre-40S particle and a pre-60S particle through one out of several alternative processes, each featured by specific intermediates. The pre-40S particle already contains most small subunit ribosomal proteins and is exported to cytoplasm where maturation occurs (Schäfer et al., 2003). Conversely, the pre-60S particle interacts with a number of biogenesis factors in an ordered multi-step maturation process, and only later can it move to the cytoplasm (Nissan et al., 2002). Once in the cytoplasm, both pre-ribosomal particles are still inactive and further maturation is required for the formation of functional subunits. At this step, key functional domains of both small and large subunits are bound by factors inhibiting the association of translation factors or subunit joining, thus preventing premature translation process to be performed by non mature ribosomes (Bradatsch et al., 2012; Greber et al., 2012; Raychaudhuri et al., 1984; Strunk et al., 2012). Maturation might include a translation-like event to check integrity and ribosome function before the ultimate steps occur (Lebaron et al., 2012; Strunk et al., 2012; Thomson et al., 2013).

In conclusion, ribosome biogenesis is a coordination of multiple parallel processes, entailing separately the synthesis of ribosome components, then their subsequent cleavage, folding, modification, assembly into ribosomal subunits and final maturation in the cytoplasm. A more detailed description of each of these steps will be given in the following sections.

### **RNA Polymerase I transcription machinery**

The yeast RNA Pol I enzyme has a total mass of 589 kDa and consists of 14 subunits: the core includes the two largest subunits, A190 and A135, forming the DNA binding cleft,



## Figure II. Ribosomal gene and RNA Polymerase I transcriptional machinery

**A)** Structure formed by a transcriptionally active functional unit of a rDNA gene, as seen by electron microscopy. Transcription progresses from the initiation site (left) to the termination site (right), and multiple intermediate transcripts are produced at the same time. Picture adapted from Trendelenburg et al., 1983. **B)** Cartoon depicting one ribosomal gene on the top, including the rDNA promoter and the portions of the primary transcript that will constitute the mature 18S, 5.8S and 28S rRNAs (in blue). The sequence of the primary transcript also contains external transcribed spacers at the 5' and 3' ends (5'ETS and 3'ETS), as well as two internal transcribed spacers (ITS1 and ITS2). On the bottom, the main components of the pre-initiation complex are represented. TAF110 and TAF68 are two of the six subunits of SL1, PAF53 and PAF49 are two of the twelve subunits of RNA Pol I. The sketch represents the relative position of each component and its molecular interactions with the others.

plus five subunits present in all nuclear RNA polymerases (Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12), the AC40–AC19 heterodimer and A12.2 subunit that participates in RNA cleavage. Outside the core, the A49–A34.5, as revealed by crosslinking-based studies, interact other subunits, is capable of interacting with newly synthesized RNA and, importantly, provides a platform for initiation factors (Jennebach et al., 2012; Kuhn et al., 2007; Russell and Zomerdijk, 2006). In human, the active center of RNA Pol I is formed by the two largest subunits, RPA135 and RPA194, that associate and are responsible for catalytic activity, and most of subunits have been identified as functional homologs of subunits from yeasts. For instance, the human PAF53 and PAF49 subunits are homologs of the yeast A49 and A34.5 subunits, respectively (Hanada et al., 1996; Penrod et al., 2012). RNA Pol I is not a dynamic protein complex but rather a stable enzyme that can be recycled remaining intact through multiple cycles of transcription (Schneider and Nomura, 2004). The crystal structure of yeast RNA Pol I was resolved independently by two groups and provided further understanding of the relative position and functional roles of most of RNA Pol I components (Engel et al., 2013; Fernández-Tornero et al., 2013).

RNA Pol I is unique as its function is the transcription only of genes encoding rRNAs. The function of RNA Pol I is dependent on the composition and appropriate formation of a complex molecular machine involving a surprising number of factors, named the pre-initiation complex (PIC). The PIC is a platform of molecular interactions and only an overview of its core elements will be presented here, listing the most important factors required for transcription of rRNA (Figure II B). In mammals, two specific factors bind to the rDNA promoter and subsequently recruit other auxiliary factors leading to the assembly of the PIC: these are known as upstream binding factor (UBF) (Jantzen et al., 1990) and promoter selectivity factor (SL1 in humans, TIF-IB in mice) (Clos et al., 1986; Learned et al., 1986; Russell and Zomerdijk, 2005).

UBF is a protein belonging to a sequence-nonspecific protein class termed high mobility group (HMG) proteins, that bind DNA through HMG boxes, a protein motif which bends DNA. The binding of UBF to rDNA promoter through HMG boxes, involving about 140 bp into a single turn, results in a nucleosome-like structure called enhancosome (Bazett-Jones et al., 1994). In this conformation, UBF is able to activate RNA Pol I-dependent gene transcription in multiple ways. First, it recruits RNA Pol I itself to the rDNA promoter. Second, it stabilizes binding of SL1 factor. Third, UBF associates to the entire transcribed region, displaces nonspecific DNA binding proteins such as histone H1

and contributes to the active chromatin state of rDNA (Kuhn and Grummt, 1992; Sanij et al., 2008; Stefanovsky et al., 2006).

SL1 is a protein complex containing the TATA binding protein and five associated factors: it confers RNA Pol I promoter specificity mediating specific interactions between the rDNA promoter and RNA Pol I, thereby recruiting the latter to the former (Denissov et al., 2007; Gorski et al., 2007; Heix et al., 1997; Zomerdijk et al., 1994). Indeed, such interactions are mediated and not direct: SL1 alone has no sequence-specific DNA binding activity, and recruitment of RNA Pol I requires the interaction of SL1 with UBF, bound to rDNA, and PAF53, factor associated to RNA Pol I (Bell et al., 1988; Hanada et al., 1996). Importantly, a recent study revealed that PAF53 function is essential not only for optimal proliferation but even for viability, as attempts to eliminate its expression by using CRISPR/Cas9 technology were not successful (Rothblum et al., 2017). The structure of the yeast homolog of SL1 has been resolved and is conserved with human (Knutson et al., 2014).

Thus, UBF and SL1 transcription factors interact cooperatively, giving RNA Pol I promoter specificity and supporting its efficient recruitment and initiation of RNA Pol I-dependent transcription.

Another essential, evolutionarily conserved factor for RNA Pol I-dependent transcription is RRN3 (Rrn3p in yeast, TIF-IA in mouse (Bodem et al., 2000; Moorefield et al., 2000)): while the other previously described components recognize different regions in promoter DNA and confer RNA Pol I sequence specificity, RRN3 binding to Pol I is a prerequisite for enzyme recruitment to promoter-bound initiation factors, as shown in yeast models (Keener et al., 1998; Yamamoto et al., 1996). As already mentioned, in the whole RNA Pol I pool present in a nucleus, only a small fraction is transcription-competent: these competent RNA Pol I molecules were found to contain RRN3. RRN3 acts as a bridge between RNA Pol I and a committed promoter. RRN3 interacts directly with SL1 and, in addition, defines a distinct subpopulation of transcriptionally active and initiation-competent RNA Pol I (Cavanaugh et al., 2008; Miller et al., 2001). In yeast, Rrn3p protein is strongly associated with RNA Pol I by interacting as a monomer that extends along the back of RNA Pol I between subcomplexes AC40–AC19 and A14–A43 (Beckouet et al., 2008; Blattner et al., 2011; Peyroche et al., 2000; Schnapp et al., 1993). Recently, electron cryomicroscopy (cryo-EM) structures of the RNA Pol I–Rrn3p complex were resolved, confirming how these two components interact (Engel et al., 2016, 2017; Pilsl et al., 2016). Initiation-active Rrn3p dissociates from the template during or

immediately after Pol I has switched from initiation to elongation, consistently with the observation that, in yeast, a large fraction of cellular Rrn3p is not associated with RNA Pol I (Bier et al., 2004). Similarly, in human cells, approximately 10% of catalytically active RNA Pol I is associated with RRN3 (Miller et al., 2001). In both yeast and mammals, once RRN3 has bound RNA Pol I to activate transcription, it is inactivated and not only dissociates from RNA polymerase I, but is not capable of forming a stable complex with RNA polymerase I (Beckouet et al., 2008; Hirschler-Laszkiewicz et al., 2003). The function of RRN3 and its regulation have been extensively reviewed (Jin and Zhou, 2016). Recently, it emerged that RRN3 is also a DNA binding protein and that this property could be required for its transcription factor activity. However, the authors could not assess this possibility, as all attempts to produce a single amino acid mutation in the DNA binding domain generated an overall misfolding of the entire structure of the protein, and further studies will be necessary to unveil this aspect (Stepanchick et al., 2013).

Once the PIC is recruited to the rDNA promoter, RNA Pol I needs to dissociate from it for productive transcription elongation to begin, in a process known as promoter escape (Panov et al., 2006; Russell and Zomerdijk, 2005). This event coincides with the release of RRN3/TIF-IA from RNA Pol I (Hirschler-Laszkiewicz et al., 2003; Milkereit and Tschochner, 1998), as confirmed by the observation that covalent attachment of TIF-IA in mouse cells impairs rDNA transcription (Bierhoff et al., 2008). In mouse, the release of TIF-IA depends from the phosphorylation of two specific serine residues, promoted by casein kinase 2 (CK2), which is associated with the PIC (Bierhoff et al., 2008). In addition, UBF is also known to regulate promoter escape and transcription elongation through its association to the entire transcribed region and may contribute to the active chromatin state of rDNA (Panov et al., 2006). As a result of promoter escape, UBF and SL1 keep binding the rDNA promoter, facilitating further recruitment of a RNA Pol I transcription complex.

Proper transcription elongation by RNA Pol I is required for efficient pre-rRNA processing and pre-ribosome assembly and elongation may be rate-limiting for rRNA synthesis (Hung et al., 2017; Schneider et al., 2007). UBF regulates elongation by phosphorylation-dependent remodelling of the rDNA chromatin (O'Sullivan et al., 2002; Stefanovsky et al., 2006). SL1 has been proposed to promote the formation of a particular topology of transcribed rDNA genes favourable for productive elongation (Denissov et al., 2011). This results in a high density of RNA Pol I molecules, each progressively transcribing the same gene at a different site, giving rise to the distinctive structure, which

is visible through electronic microscope (Figure II A), resembling a Christmas tree, with a trunk corresponding to the rDNA and lateral branches corresponding to rRNA molecules at different stages of synthesis displaying different length, proportional to their distance from the promoter region, with terminal globular structures at their 5' end corresponding to processing machinery (Miller and Beatty, 1969; Mougey et al., 1993).

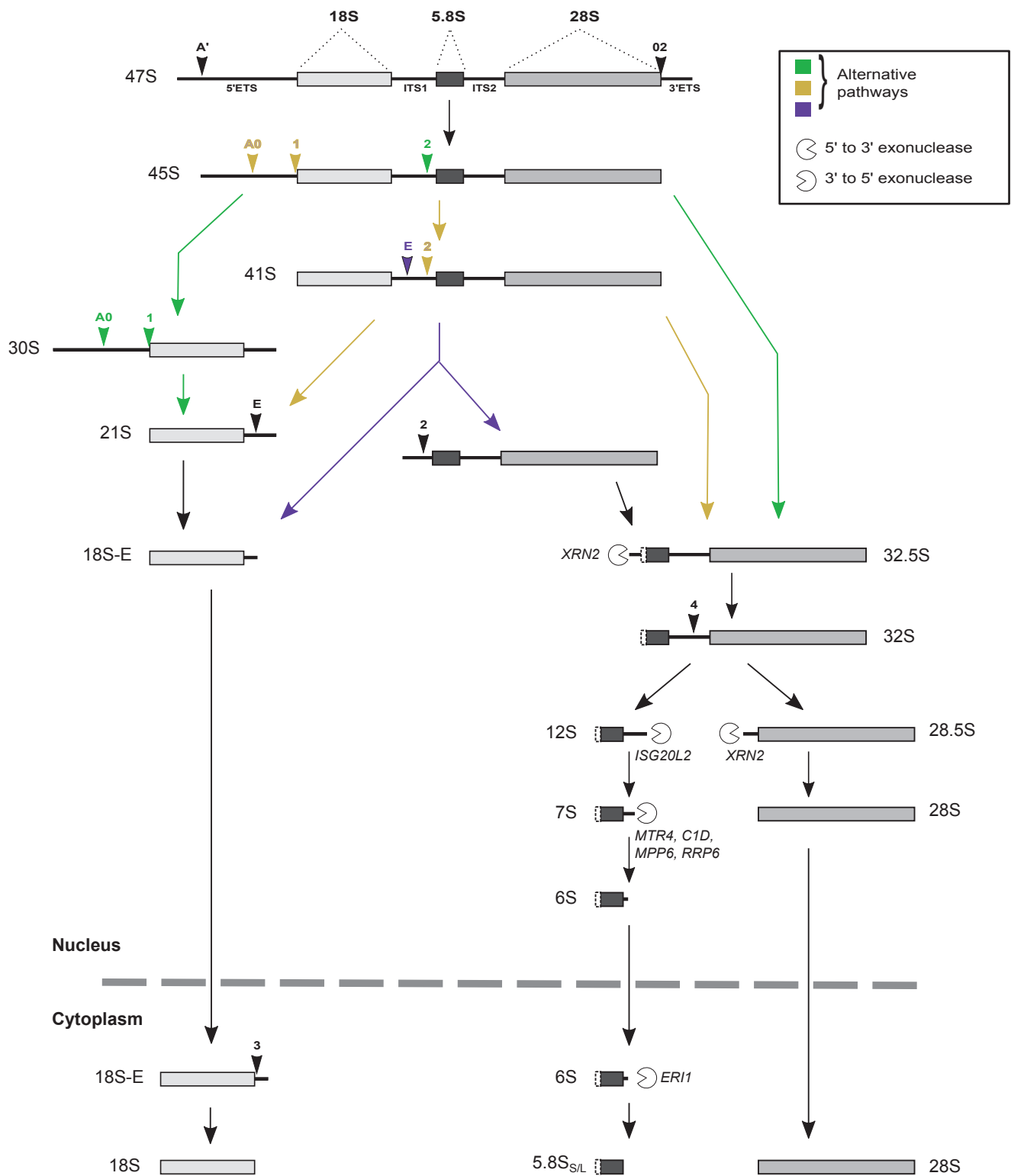
Transcription termination, that is the final step of transcription, occurs when RNA Pol I is paused by TTF-1, recruited at the level of a specific termination sequence downstream of the rDNA gene, and subsequently displaced by transcript release factor PTRF. Several mechanisms facilitate this process, ensuring accurate and efficient termination of transcription (Braglia et al., 2010, 2011; Jansa et al., 2001; Prescott et al., 2004).

To conclude, it must be said that a large number of additional proteins, playing roles in various processes, have been found to be associated with the Pol I transcription machinery, including the chromatin modifiers PCAF and G9a, the histone chaperones nucleolin, nucleophosmin and FACT, nuclear actin and myosin, proteins involved in DNA repair and replication such as topoisomerases I and II $\alpha$ , Ku70/80, PCNA, TFIID and CSB (Louvret and Percipalle, 2008). Assembly of Pol I and functional initiation complexes may proceed in a sequential manner via metastable intermediates, each with increasing stability as more subunits are added (Dundr et al., 2002; Grummt, 2003). This non-exhaustive list supports the current model stating that Pol I is recruited to the rDNA promoter as a massive multiprotein complex that acts as a scaffold to coordinate rRNA synthesis and maturation as well as chromatin modification and DNA repair.

## **Processing of pre-rRNA**

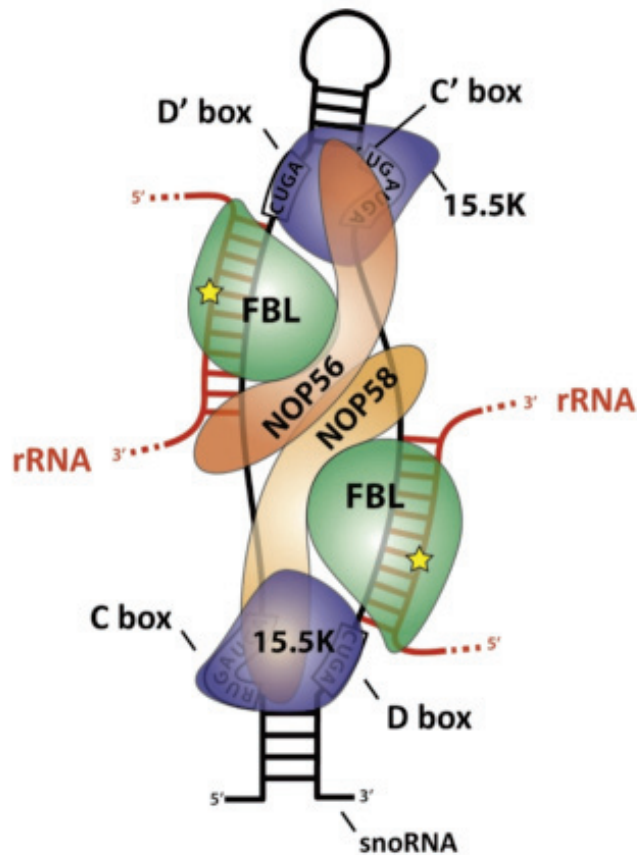
As a result of RNA Pol I-dependent transcription, a transcript named 47S rRNA precursor (pre-rRNA) is generated. In this precursor transcript, 18S, 5.8S and 28S rRNAs are embedded between spacer sequences including the 5' and 3' portions known as external transcribed spacers (ETS) and two internal transcribed spacers (ITS). The spacer sequences vary greatly in composition and length in different organisms, though the core elements in the mature rRNAs are highly conserved (Nazar, 2004). Co-transcriptionally, pre-rRNA undergoes an ordered series of cleavage, folding, chemical modification and ribosomal protein association steps, and this series is referred to as pre-rRNA maturation. It should be kept in mind that pre-rRNA maturation is an extremely complex process involving hundreds of nucleolar factors and only an overview will be given here, focusing on a few chosen aspects. In the first steps of pre-rRNA maturation, interacting factors associate to the nascent transcript leading to the formation of the so-called 90S





### Figure III. Overview of rRNA processing

Schematic view of the main pre-rRNA processing steps in human cells. The primary transcript 47S contains the embedded sequences of the mature 18S, 5.8S and 28S rRNAs, flanked by 5'ETS and 3'ETS and separated by ITS1 and ITS2. At each cleavage step, a coloured arrowhead marks the cleavage site and the subsequent products are represented downstream of an arrow of the same colour. Thus, alternative pathways are represented in different colours. 3' to 5' or 5' to 3' exonucleases are represented by crunching smileys with its jaws wide open. From the 32.5S intermediate on, two alternative forms exist differing in the 5' end of the 5.8S sequence, represented by a dashed-border white extremity. The last maturation steps of 18S and 5.8S rRNAs take place in the cytoplasm.



#### Figure IV. Structure of C/D box snoRNP complex

Schematic representation of the C/D box snoRNP complex, depicting the main features of the complex. The C and D boxes are depicted with their consensus sequence. The substrate ribosomal RNA (rRNA, red) forms an RNA:RNA duplex with the snoRNA along 10–21 nucleotides (nt). The catalytic site of fibrillarin (FBL) faces the fifth nucleotide downstream of the D or D' boxes (yellow star). The 15.5K protein specifically binds a k-turn motif. The NOP56:NOP58 heterodimer forms two long alpha helices that are placed across the snoRNA:rRNA complex and contribute to locking FBL in its active position.

processome, 90S particle or 90S pre-ribosome. The cryo-EM structure of the 90S pre-ribosome showed that, even before transcript release, pre-rRNA is already encapsulated and properly folded by at least 70 of interacting factors (Barandun et al., 2017; Cheng et al., 2017; Kornprobst et al., 2016; Sun et al., 2017).

The analysis of pre-rRNA processing was performed elucidating a number of intermediates and multiple alternative processing paths resulting from differently ordered cleavage events, that ultimately yield the mature rRNAs (Mullineux and Lafontaine, 2012; Tafforeau et al., 2013). These events have been studied extensively in eukaryotes, mostly in yeast and mammalian models, shedding light on common features and organism-specific differences, and the state-of-the-art about current understanding of pre-rRNA processing has been reviewed and progressively updated (Bohnsack and Bohnsack, 2019; Cerezo et al., 2019; Eichler and Craig, 1994; Fatica and Tollervey, 2002; Gerbi and Borovjagin, 2013; Granneman and Baserga, 2005; Henras et al., 2008, 2015; Tomecki et al., 2017). A

schematic overview of the main pre-rRNA processing steps in human cells is shown in Figure III.

The first region to be processed is the 5'ETS. Because of its fast kinetics, this event is often used as a proxy to detect and evaluate transcriptional activity of RNA Pol I (Popov et al., 2013). The pre-40S particles are released after 5'ETS removal and ITS1 cleavage at site A2. In yeast, especially upon favorable growth condition, cleavage in the ITS1 occurs co-transcriptionally without the formation of an intermediate 90S pre-ribosome (Osheim et al., 2004). In higher eukaryotes, no evidence of co-transcriptional release of pre-40S particles has been found. A large number of factors are required for processing of the 5'ETS and the small subunit. These snoRNPs function as RNA chaperones assisting early cleavages of the pre-rRNA (Dragon et al., 2002; Henras et al., 2008, 2015; Osheim et al., 2004; Phipps et al., 2011).

In particular, the U3 snoRNA plays a central role in 5'ETS processing and in the 18S rRNA maturation, by direct base-pairing with distant segments of the pre-rRNA and subsequently orchestrating the correct folding of the central core of 18S rRNA. These base-pairing interactions may occur through U3 snoRNA conserved A and A' boxes. The two 18S rRNA sequence elements interacting with U3 snoRNA are separated by more than 1 kb in the primary transcript sequence, so it has been hypothesized that binding of U3 to the upstream one may prevent it from establishing wrong base-pairing interactions prior to the transcription of its correct partner sequence on the transcript (Beltrame and Tollervey, 1995; Hughes, 1996; Marmier-Gourrier et al., 2011; Méreau et al., 1997; Sharma and Tollervey, 1999). Depletion of U3 snoRNA in yeast inhibits early endonucleolytic cleavage of the pre-rRNA at multiple sites in the 5'ETS and in the ITS1 (Hughes and Ares Jr, 1991). The role of U3 snoRNA seems similar in higher eukaryotes. For example, in *Xenopus*, mouse and human, and U3 is required for cleavage in the 5'ETS at the end of 18S rRNA sequence and within ITS1 (Borovjagin and Gerbi, 1999, 2000, 2005; Kass et al., 1990; Langhendries et al., 2016; Savino and Gerbi, 1990). It appears that the recruitment of the U3 snoRNA to the cleavage sites is mediated in part by nucleolin, that directly interacts with the pre-rRNA through its four central RNA-binding domain (Ginisty et al., 1998). As confirmed by the recently published structure of the 90S pre-ribosome, the U3 snoRNP is strategically positioned at the center of the particle to perform its multiple tasks during pre-rRNA folding and processing (Kornprobst et al., 2016).

U3 snoRNA does not carry out its function all alone as a free factor: it actually acts in a particular ribonucleoproteic complex, belonging to the family of C/D box snoRNP

complexes (Figure IV). In human, the essential components of the U3 snoRNP complex are fibrillarin, NOP56, NOP58, that are core proteins shared by all C/D box snoRNPs, and hU3-55K, the only U3-specific one (Watkins et al., 2004). The general structure, composition and function of C/D box snoRNPs have been discussed in a review (Watkins and Bohnsack, 2012).

Increasing evidence suggests that the interactions established between snoRNPs or other factors involved in maturation and the respective molecular partners are almost certainly modulated and energy-dependent (Brüning et al., 2018). U3 snoRNA hybridization to and dissociation from pre-rRNA makes no exception and factors possessing RNA helicase activities have been identified in yeast as potential candidates for this function: Imp3p and Imp4p, which have been found to bind the U3 snoRNA directly in vitro (Gérczei and Correll, 2004; Gérczei et al., 2009), Dhr1p, a putative helicase associated with U3 and present in 90S pre-ribosomes (Colley et al., 2000), and Has1p, suggested to be required for the release of U3 snoRNA from pre-ribosomal particles (Liang and Fournier, 2006).

As a result of U3 snoRNP-mediated cleavage events, pre-40S particles are produced. These particles contain 21S or 18S-E rRNA intermediates and most small subunit ribosomal proteins, establish very transient interactions and undergo little rearrangement before they are rapidly exported to the cytoplasm (Figure III). Major differences exist between yeast and mammals regarding the formation of the 3' end of the 18S rRNA. In human, a specific cleavage results in the intermediate 18S-E pre-rRNA, gradually shortened in the nucleus and the cytoplasm. After nuclear transport, NOB1 protein likely cleaves the 18S-E pre-rRNA and yields the mature 18S rRNA (Sloan et al., 2019). In yeast, the pre-40S particles containing the 20S pre-rRNA are directly exported to the cytoplasm where endonucleolytic cleavage by Nob1p forms the mature 18S rRNA (Fatica et al., 2003).

In parallel, the maturing pre-60S particles undergo extensive processing in the granular component of the nucleolus (Nissan et al., 2002). Formation of the 3' end of the 28S rRNA is a very early event that may participate in transcription termination in mammalian cells (Németh et al., 2013). The structure of pre-60S particles has been resolved by cryo-EM and contributed to the understanding of the major remodelling events before nuclear export, including rotation of the 5S RNP (see later), construction of the active centre and ITS2 removal (Fromm et al., 2017; Greber, 2016; Wu et al., 2016).

In mammalian cells, the efficiency of cleavage in the ITS1 at site 2 depends on a number of factors including U8 snoRNP, PES1, BOP1, NOL12 (see later) and RPs of the

large subunit such as RPL15 and RPL26. Following cleavage of the ITS1 in the early precursor, the 5' end of the 5.8S rRNA is tailored through two alternative processing pathways that yield a long and a short form (Figure III). This processing scheme first described for yeast appears now to be approximately conserved across evolution (Henras et al., 2015).

Maturation of the 5' end of the 5.8S rRNA is coordinated with formation of the 3' end of the 25S/28S rRNA. Elimination of the ITS2 is initiated by endonucleolytic cleavages (at site C2 in yeast, at site 4 in mammals) allowing 3' to 5' and 5' to 3' exonucleases (which, in human, include XRN2 and ISG20L2, as shown in Figure III), to respectively generate the mature 3' end of the 5.8S rRNA and the mature 5' end of the 25S/28S rRNA. The cleavage of ITS2 gives rise to the 12S and 28.5 rRNA species which are precursors to the mature 5.8S and the 28S rRNAs, respectively. The 5' end of the 28.5S rRNA is then trimmed to form the 28S rRNA. The processing of 5.8S rRNA 3' end from the 12S precursor is a multistep mechanism (Figure III). The very last step of the 5.8S rRNA 3' end maturation takes place in the cytoplasm (Henras et al., 2015).

The role of U8 snoRNA, specific to higher eukaryotes, is similar to the one played by the U3 snoRNA in small subunit maturation, except that U3 snoRNA acts at an early step of pre-rRNA processing, whereas U8 snoRNA acts at a late step (Langhendries et al., 2016). U8 snoRNA is required for normal pre-rRNA processing and 5.8S and 28S rRNA accumulation and the U8 snoRNA-mediated cleavage is an essential step for 60S subunit maturation. Depletion of the U8 snoRNA in *X. laevis* impairs endonucleolytic cleavages flanking the region containing 5.8S and 28S rRNAs and leads to the accumulation of abnormal precursors of both rRNAs, indicating that these processing events are somehow coupled (Peculis and Steitz, 1993). In *Xenopus*, the 5' sequence of U8 snoRNA is critical for such maturation (Peculis, 1997). X29, an RNA binding protein recruited to the ITS2 sequence, is a member of a conserved family of nuclear decapping proteins that function in regulating the level of U8 snoRNA and other nuclear RNAs with methylated caps (Ghosh et al., 2004; Peculis and Greer, 1998; Tomasevic and Peculis, 1999). In human cells, U8 snoRNA depletion was found to inhibit processing primarily in ITS2 and the 3'-ETS, evidenced by accumulation of multiple pre-rRNAs having retained 3'-ETS sequences and showing abnormal extension in 3' end and by reduced levels of large subunit rRNAs and their precursors. In addition, U8 depletion also affected processing in the 5'-ETS and within ITS1 (Langhendries et al., 2016).

A number of protein complexes have been described that associate to the pre-ribosomal particles. Many of these complexes may exist in cells as free complexes held together by protein-protein high-affinity interactions mediated by specific protein binding motifs such as WD40. At least 22 components of the pre-ribosomal particles have been reported to contain WD40 motif. For instance, the PeBoW complex, formed by Nop7p, Erb1p, Ytm1p in yeast and by Pes1, Bop1, WDR12 proteins in mammals (where it is also called the PeBoW complex) is required for pre-rRNA processing steps leading to the production of 5.8S and 25S/28S rRNAs (Grimm et al., 2006; Hölzel et al., 2005; Lapik et al., 2004; Lerch-Gaggl et al., 2002; Oeffinger et al., 2002; Pestov et al., 2001a; Rohrmoser et al., 2007; Strezoska et al., 2000, 2002). The specific functions of many of these ribosome biogenesis factors are not known in detail, but their involvement is underscored by several studies. Much effort has been made in the last decade to identify novel factors required for ribosome assembly and different high-throughput screens have found hundreds of proteins potentially involved in this process in human cells. It is unlikely that all the identified proteins are directly involved in mechanisms of ribosome biogenesis, as many of the observed effects may imply a role in regulation of these mechanisms. In addition, the presence of each factor in the pre-ribosomal particles should be demonstrated at the molecular level (Badertscher et al., 2015; Bohnsack and Bohnsack, 2019; Farley-Barnes et al., 2018; Tafforeau et al., 2013; Wild et al., 2010).

## **rRNA modifications**

Co-transcriptionally and during subsequent maturation, rRNAs are extensively modified by isomerization of specific nucleotides or addition of chemical groups at specific sites of the rRNA sequence (Natchiar et al., 2017; Sharma and Lafontaine, 2015).

In eukaryotes, the most abundant rRNA modifications are 2'-O-methylation of the ribose, which can occur on any nucleotide, and the isomerisation of uridine to pseudouridine, but many others exist, including acetylation, aminocarboxypropylation, methylation, and the inventory of rRNA modifications in yeast and human is constantly increasing.

Several studies provided systematic mapping of rRNA modifications by means of different independent methods (Birkedal et al., 2015; Maden, 2001; Marchand et al., 2016; Ofengand and Bakin, 1997; Piekna-Przybylska et al., 2008; Taoka et al., 2016; Yang et al., 2016).

A comparison between species shows that such a striking pattern is conserved throughout evolution and is likely to carry a biological function. Modifications localized at

the intersubunit surface are the most variable across species (human, Arabidopsis, representative organisms from eubacteria, archaea, eukaryotes) (Piekna-Przybylska et al., 2008).

Chemical modifications are generally thought to stabilize secondary and tertiary structures thereby fine-tuning the structure of the ribosome. For most modifications, the particular role of any single modification has not been understood yet. In general, it seems that modifications act in a cumulative manner, as the depletion of a single modification rarely impacts phenotype in yeast, while depletions of multiple snoRNAs tend to result in growth defects (Esguerra et al., 2008; Liang et al., 2009). It is noteworthy that rRNA modifications usually cluster at functionally important sites of the ribosome, such as the peptidyltransferase center and the decoding site, where they could facilitate efficient and accurate protein synthesis (Decatur and Fournier, 2002). Consistently with this, modifications in functional regions of the ribosome affect growth and translational efficiency in a cooperative way (Baudin-Baillieu et al., 2009; Esguerra et al., 2008; Liang et al., 2009).

A recent publication achieved the direct visualization of a significant number of rRNA modifications for the first time in human ribosome structure, even accounting for previously unreported modifications. This supports the idea that humans and other eukaryotes have evolved an extended additional shell of rRNA modifications around the functional centres, resulting in a class of modifications absent in bacteria (where 95% are in functional regions) (Natchiar et al., 2017).

2'-O-methylations and pseudouridines are introduced by two non-specific enzymes, the methyltransferase fibrillarin and the pseudouridylase dyskerin, whose site-specific activities are conferred by their association with one of two classes of snoRNAs, termed C/D box and H/ACA box snoRNPs, respectively, to form snoRNP complexes. C/D box and H/ACA box snoRNAs guide fibrillarin and dyskerin activities to target specific rRNA sites for 2'-O-methylation or pseudouridylation, respectively (Watkins and Bohnsack, 2012).

## **Synthesis of ribosomal proteins**

Ribosomal protein (RP) genes are regulated in a coordinated manner at the level of both transcription (Perry, 2007) and translation, being under the control of mTOR signalling (Cardenas et al., 1999). In yeast, inhibition of TOR pathway globally represses the transcription of most RP-encoding genes (Cardenas et al., 1999). Consistently, several transcription factors such as Sfp1p and Fhl1p directly associate with gene promoters (Jorgensen et al., 2004; Marion et al., 2004; Martin et al., 2004; Rudra et al., 2005;

Schawalder et al., 2004), activating their transcription under favorable conditions. All the mRNAs encoding RPs contain a translation repressing 5'TOP sequence at their 5' end, indicating a coordinated regulation (Fumagalli et al., 2009; Meyuhas, 2000). For example, TOR is known to promote 5'TOP mRNA translation (Mayer and Grummt, 2005).

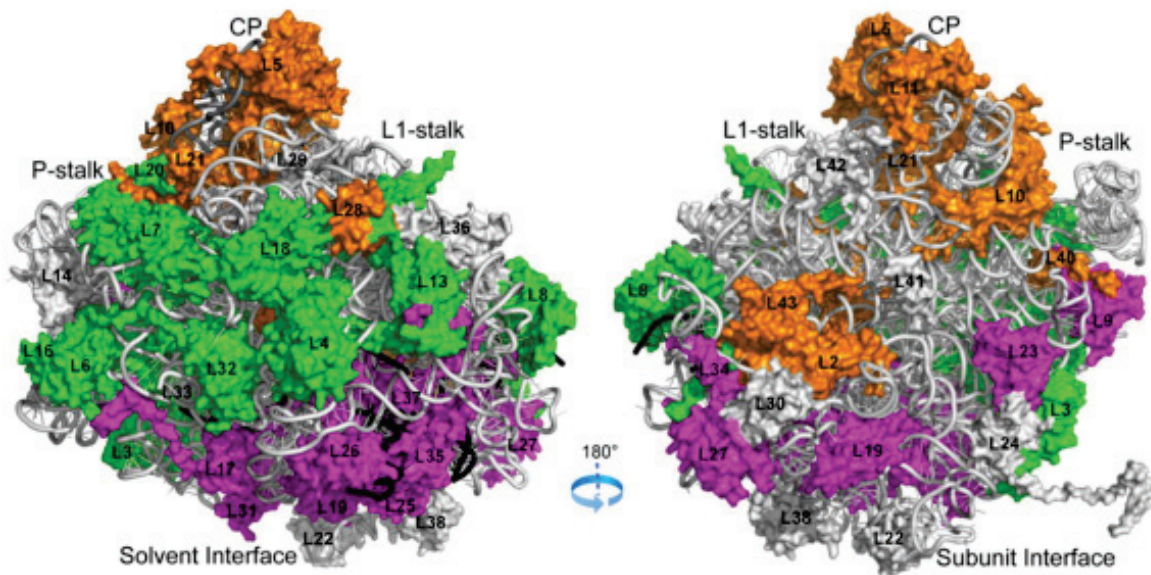
Insights about the regulation of expression and stability of RPs come from studies about their depletion. RPs are required for structural assembly, pre-rRNA processing and nuclear export of the pre-ribosomal particles (see below). Only a few RPs are dispensable for production of the mature 18S rRNA. Importantly, depletion of any RP blocks ribosome maturation at many specific steps of pre-rRNA processing resulting in the accumulation of different rRNA precursors. The only shared feature was that depletion impaired the production of ribosome (Gamalinda et al., 2014; Ohmayer et al., 2013; Pöll et al., 2009; Robledo et al., 2008). Interestingly, depletion of RPs in one of the two subunits caused, with a few exceptions, a decrease in all RPs belonging to the same subunit and a decrease in the corresponding subunit, fully assembled ribosomes and polysomes. The first observation may imply a very well orchestrated coordination between all ribosomal components, but the authors rather suggest that all RPs are produced in excess, imported into the nucleus and nucleolus, where they associate with rRNA and assemble into ribosomal subunits, then they are exported to the cytoplasm, or else, when not assembled into functional ribosomes, they get ubiquitinated and are rapidly degraded in the nucleolus (Ferreira-Cerca et al., 2007; Robledo et al., 2008). This is supported by at least three other independent studies in human cells. First, it has been demonstrated that RPs are degraded at the nuclear level and that the amount of RPs is by far larger than that required for the typical rate of ribosome subunit production. In general, when small subunit ribosomal proteins are depleted, a reduction in the amount of free 40S subunits, a great reduction in the amount of mature 80S ribosomes and an increase in the amount of free 60S subunits were observed. Similarly, depletion of large subunit ribosomal proteins lead to greatly reduced amount of the 60S subunit and a peak in 80S ribosomes (Lam et al., 2007). This was consistent with previous results from mass spectrometry-based proteomic analysis showing that ribosomal proteins are synthesized more rapidly than any other nucleolar proteins (Andersen et al., 2005). Finally, this hypothesis is supported by another study showing the presence of ubiquitin-dependent proteasome-mediated degradation in the nucleolus and its importance in several steps of ribosome biogenesis (Stavreva et al., 2006).



## Synthesis of 5S rRNA

In mammalian systems, 5S rRNA is not transcribed in the nucleolus nor by RNA Pol I. Contrary to yeast, where the rDNA encodes the 5S rRNA transcribed in the reverse direction, in human, the precursor to the 5S rRNA is synthesized by RNA Pol III from a cluster of tandemly repeated genes, mainly on chromosome 1 (bearing 100 to 150 copies) (Steffensen et al., 1975), but also present in clusters having smaller copy numbers (5 to 10) spread all over the genome in a total number of 200 to 300 copies (Sørensen and Frederiksen, 1991; Sørensen et al., 1991). During interphase, when ribosome biogenesis occurs entailing functional nucleolar structures, 5S rRNA genes are generally found within the perinucleolar region (Fedoriw et al., 2012). Like Pol I-dependent transcription, Pol III-dependent transcription requires the formation of an initiation complex consisting of several factors and involving specific molecular interactions, such as TFIIB which recruit the Pol III functional subunit, TFIIA, TFIIC<sub>2</sub>, TFIIC<sub>1</sub> (Oettel et al., 1997).

Once transcribed, 5S rRNA rapidly associates with RPL5, is imported into the nucleus and, in the nucleolus, assembles with RPL11 into a small ternary complex, termed the 5S RNP, that functionally and structurally acts as a single unit: it has been known for a long time that the 5S RNP can even be dissociated from the mature ribosome (Isoda et al., 1981) and the role played in stress response, that will be described later, is carried out by the ternary complex as a whole. Each of the three components of the 5S RNP functions to stabilize the others: RPL5 stabilizes 5S rRNA and RPL5 and RPL11 are required for each other's metabolic stability (Deshmukh et al., 1993; Langhendries et al., 2016; Lee and Nazar, 1997). In addition, RPL5 binding is required for 5S rRNA nuclear localization, and this activity is modulated by RPL5 phosphorylation: RPL5 carrying mutations that disrupt its interaction with 5S rRNA lead to defective localization of 5S rRNA and affect subsequent ribosomal assembly (Park and Bae, 1999; Rosorius et al., 2000). 5S RNP is incorporated into 90S pre-ribosomes, early in ribosome biogenesis (Zhang et al., 2007). The nucleolar localization of the 5S RNP and its assembly into preribosomes are performed by a specialized complex composed of Rpf2 and Rrs1 in yeast or Bxdc1 and hRrs1 in humans. Rpf2–Rrs1 guides the incorporation of the 5S RNP in preribosomes in the initial conformation prior to its rotation to form the central protuberance found in the mature large ribosomal subunit (Madru et al., 2015; Zhang et al., 2007). Two independent structural studies, published at the same time, resolved the Rpf2-Rrs1 complex bound to 5S RNP and provided a sequential model for 5S RNP recruitment to the pre-60S particle, showing that removal of Rpf2-Rrs1 is necessary for the rearrangements that drive pre-60S



**Figure V. Sequential assembly of ribosomal proteins into the large subunit**

Structure of the yeast large subunit of the ribosome. Early-acting (green), intermediate-acting (magenta), and late-acting (orange) RPLs are mapped onto the crystal structure of the 60S subunit (Protein Data Bank accession nos. 3U5D and 3U5E) and proteins acting at the same step of ribosome biogenesis tend to cluster within the same domains. This clearly suggests a correlation between the function of RPLs in ribosome biogenesis and their location in the ribosome structure. rRNAs and RPLs are shown in cartoon and surface representations, respectively. 5.8S, 25S, and 5S rRNAs are colored black, light gray, and dark gray, respectively. Picture from Gamalinda et al., 2014.

particle maturation (Asano et al., 2015; Kharde et al., 2015). In the mature ribosome, 5S RNP constitutes the central protuberance on the mature 60S subunit. Integration of central protuberance is a late step of large subunit maturation. The 5S RNP complex must undergo a semicircular movement to adopt its final position and form the central protuberance (Leidig et al., 2014).

**Assembly of ribosomal components**

During ribosome biogenesis, several processes take place simultaneously. Translocation, export to the cytoplasm and assembly of the ribosomal components are very intimately associated processes and it would be difficult to discuss them separately. Therefore, even though they will be the subject of three distinct sections, one should always keep in mind that the described events actually occur at the same time, are strongly associated and often may represent different aspects of the same event that are artificially reported as separated for sake of simplicity.

A major role attributed to ribosomal proteins is to stabilize rRNA structure in mature ribosome and play a chaperon-like role during ribosomal assembly or nuclear export

(Ferreira-Cerca et al., 2005, 2007; O'Donohue et al., 2010; Robledo et al., 2008). Ribosomal proteins act in a hierarchical order. The interaction network between proteins and RNA, at first somewhat loose and dynamic, gradually tightens during subunit assembly, and eventually forms stable ribosomal subunits. RPs probably act as chaperones assisting the proper folding of the nascent transcript. RPs are required in a hierarchical order, and depletion of eukaryotic RPs results in blockades at different pre-rRNA processing steps, in both humans and yeast. A subset of ribosomal proteins from the 40S ribosomal subunit associate early with the 5' end of the nascent pre-rRNA and are required for the initiation of cleavages at the 5' ETS and ITS1. A second set of ribosomal proteins is only necessary for ITS1 cleavage. This ordered process has been studied by checking the effects of specific ribosomal protein depletions on the accumulation of intermediates. Most of depletions affect pre-rRNA processing in a unique way, leading to the accumulation of different rRNA precursors and disruption of ribosome biogenesis at different steps (Choismel et al., 2007, 2008; Espinar-Marchena et al., 2016; Flygare et al., 2007; Léger-Silvestre et al., 2005; Robledo et al., 2008).

Ribosome assembly has been extensively studied in yeast and only more recently in mammalian systems. Therefore, it is not completely understood in human cells, and as usual in biology, further layer of complexity could operate in reality. Here, a very quick overview will be given and for further information the topic has been reviewed (Klinge and Woolford, 2019).

In yeast, the 40S structure is sequentially assembled: first, the body domain is formed, which contains the 5' end and central domains of 18S rRNA and primary and secondary binding RPs, followed by the head domain, which is composed of the major 3' end domain of 18S rRNA and secondary or tertiary binding RPs.

Most RPs of the small subunit associate with 35S pre-rRNA, albeit weakly; as 90S particles evolve to produce pre-40S particles, the integration of small subunit RPs within pre-ribosomal particles becomes more stable (Ferreira-Cerca et al., 2005, 2007; Mitterer et al., 2019). In yeast, interdependency between formation of large scale structural domains and pre-rRNA processing was also demonstrated for the large subunit (as shown in Figure V), with RPs assembled at close steps of ribosome biogenesis clustering in close domains in the final structure of the ribosome (Gamalinda et al., 2014).

## **Nuclear export of ribosome subunits**

In a previous section of this thesis, regarding nucleolar morphology, the intimate association between nucleolus and the nuclear membrane has been described. This

topology is commonly interpreted as an indication that nucleolar function is coupled with export through the nuclear membrane, consistently with a role in the export of ribosome subunits, that may occur at a high rate. Whether the tightness of this association and the number and extension of nucleolar canals (or other structures increasing the exchange surface or reducing the distance between nucleolus and nuclear envelope) do correlate with ribosome biogenesis rate or export rate of pre-ribosomal particles has not been studied yet. However, the current opinion is that this would be a manner to ensure efficiency in the export of pre-ribosomal particles from the nucleus to the cytoplasm (Hernandez-Verdun et al., 2010).

Following nucleoplasmic maturation steps, pre-60S and pre-40S ribosomes are eventually translocated to the cytoplasm where they undergo final maturation steps to form the mature 60S and 40S ribosome subunits and achieve translation competence (Fromont-Racine et al., 2003; Gerhardy et al., 2014; Nerurkar et al., 2015; Tschochner and Hurt, 2003; Zemp and Kutay, 2007).

The first export process to occur in the course of ribosome biogenesis is the exit of particles from the nucleolus. The discovery and analysis of Noc proteins revealed the first example of a transient interaction between ribosomal biogenesis factors being coupled with transport and maturation of pre-ribosomal particles. Noc proteins are dynamically associated with pre-ribosomal particles: a stable heterodimeric Noc1-Noc2 complex is present on early pre-60S particles in the nucleolus, whereas the Noc2-Noc3 complex is found on pre-60S particles both in the nucleolus and in the nucleoplasm (Milkereit et al., 2001). This suggests a model in which Noc1 and Noc3 are required at different steps of ribosome maturation and the exchange between Noc1 and Noc3 in the heterodimer determines the transport direction from the nucleolus to the nucleoplasm. A similar mechanism might function for the transport of pre-40S particles: a homolog complex formed by Noc4 and Noc14 is found to be associated with early pre-90S particles only before the cleavage that produces pre-40S particles, suggesting a role in maturation of the latter (Milkereit et al., 2003). However, whether another complex acts similarly to Noc1-Noc3 in the export of the large subunit, or whether these exchange mechanisms are energy-dependent or required to overcome retention signals is not known and no studies have elucidated this aspect ever since. By contrast, after these early studies, other mechanisms have emerged and have been extensively described. Only some examples will be illustrated here, illustrating a wide diversity in the involved mechanisms.

For instance, Rlp7p activity could be required for the release of the pre-60S particles from the granular component of the nucleolus. Rlp7p protein localizes to the granular component and a mutation in a single amino acid blocks both cleavage of the ITS2 and transport of pre-60S particles from the nucleolus to the nucleoplasm. When the pre-60S subunits leave the granular component and enter the nucleoplasm, Rlp7p dissociates from the particles. This suggests that not only the cleavage of ITS2 but also the transport of pre-60S particles is a function of Rlp7p protein (Dunbar et al., 2000; Gadal et al., 2002).

Mechanisms for nuclear export of ribosomal subunits mostly rely on binding factors that allow selective interaction with nuclear pore complexes and are then released in the cytoplasm. It is a facilitated diffusion process that relies on the energy-dependent small GTPase Ran system. The Ran-GTP to Ran-GDP ratio is high in the nucleus and low in the cytoplasm, because of two activities asymmetrically distributed across the nuclear membrane, that are the GTPase-activating protein (RanGAP) in the cytoplasm and of the nucleotide exchange factor (RanGEF) in the nucleus (Koepp and Silver, 1996; Pemberton and Paschal, 2005). In both yeast and higher eukaryotes, mutations have been reported and reviewed that helped to describe in detail the mechanisms driving nuclear export of pre-ribosomal particles through the nuclear pore complexes. First of all, mutations of components of the Ran GTPase cycle affect the export of pre-40S or pre-60S particles, suggesting that their export relies on this system. Secondly, only a specific subset of nucleoporins seem to be required for export of the pre-ribosomal subunits, namely Nup82p in yeast (Moy and Silver, 1999) and the proposed homolog Nup214-Nup88 complex in HeLa cells, suggesting an evolutionary conserved function (Bernad et al., 2006). Thirdly, several export receptors associate with the pre-ribosomal particles to facilitate their interaction with nucleoporins. Nuclear pore complexes expose a hydrophobic meshwork on the nuclear side of the pore, the role of which has been proposed to be acting as a selective barrier preventing the indiscriminate passage of non-specific large molecules through the pore. A number of export receptors have been identified and are specifically required for nuclear export of pre-40S or pre-60S particles, respectively.

One key maturation event in ribosome biogenesis is a conformational rearrangement that takes place within nuclear pre-40S pre-ribosomal particles. In yeast, a complex formed by Enp1p, Ltv1p and Rps3p proteins binds pre-40S particles. All three proteins can be phosphorylated and their phosphorylation increases affinity for 40S particles. This might be necessary for them to bind the pre-40S particles and make it adopt a structure

compatible with efficient passage through the nuclear pore. Consistently, depletion of Hrr25p, a casein kinase I isoform responsible for the phosphorylation of the complex, results in inhibition of nuclear export of pre-40S particles (Schäfer et al., 2006).

Of note, the process of nuclear export of pre-ribosomes has also been observed by electron microscopy (Delavoie et al., 2019).

### **Cytoplasmic steps of maturation**

After entering the cytoplasm, pre-60S particles are not directly incorporated into functional ribosomes: first, non-ribosomal components such as export receptors are sequentially removed in an energy-dependent and controlled fashion, entailing conformational rearrangements (Lo et al., 2010). Some of these factors facilitate export, while others, including Tif6, may prevent the premature interaction of the ribosome subunits (Lebreton et al., 2006; Pertschy et al., 2007). However, a detailed description of this aspect of ribosomal maturation would go beyond the scope of this text, and will not be discussed here. Of note, the removal of non-ribosomal components is coupled with the incorporation of the last ribosomal proteins, including Rpl24.

The inactivation of the cytoplasmic factors required for the removal of non-ribosomal components results in the nuclear accumulation of pre-60S components due to failure to recycle shuttling components back to the nucleus and in the lack of functionality of stalled nearly mature 60S subunits, unable to associate with 40S subunits to engage in translation because functional domains are still shielded by non-ribosomal factors (Henras et al., 2008).

Several small subunit ribosomal proteins are also implicated in the cytoplasmic maturation of yeast 20S pre-rRNA, corresponding to the mammalian 18S-E rRNA. For example, alterations to the C-terminal extension of Rps14p in yeast result in a massive accumulation of the 20S pre-rRNA in the cytoplasm. Because no nuclease domain is present within Rps14p protein, nor within Rpsop or Rps2p that seem to lie in the vicinity of the 3' end of 18S rRNA, it has rather been hypothesized that these ribosomal proteins are needed for proper conformation of 20S pre-rRNA for cleavage at its 3' end and/or recruitment of necessary factors (Ferreira-Cerca et al., 2005; Perreault et al., 2008). Several small subunit ribosomal proteins display a higher affinity for 18S rRNA than for 20S pre-rRNA. This suggests that a number of conformational rearrangements could occur in the very late maturation steps of the small subunit, with ribosomal proteins undergoing restructuring events. At least one example of this late mechanism has been described for Rps3p (Ferreira-Cerca et al., 2007). Following translocation through the nuclear pore, pre-

40S particles acquire the beak structure, an event linked with the final stable positioning of Rps3p within the 40S subunit. This conformational rearrangement requires the dephosphorylation of Rps3p, previously phosphorylated in the nucleus by Hrr25p (Schäfer et al., 2006).

In the cytoplasm the exported 20S pre-rRNA undergoes dimethylation of two adjacent adenine bases (A1779 and A1780 in yeast) at the very 3' end of 18S rRNA, catalysed by Dim1p in yeast (Brand et al., 1977; Lafontaine et al., 1995). These base modifications are conserved from bacteria to eukaryotes, suggesting an essential role. Remarkably, human homolog of Dim1 displays nuclear localization, indicating that this modification step occurs earlier in human cells, so the function is conserved whereas the modification process is not (Zorbas et al., 2015). The 18S rRNA dimethylations are believed to be required for optimal 40S ribosomal subunit function, as extracts from strains lacking these modifications are inactive in *in vitro* translation assays (Lafontaine et al., 1998).

The last pre-rRNA processing step leading to the mature 18S rRNA is the cytoplasmic endonucleolytic cleavage of the 20S pre-rRNA at site D, at the junction between the 3' end of 18S rRNA and ITS1, dependent on the Nob1p function in yeast (Fatica et al., 2003). A number of other candidate activities have been proposed to carry out this function, including Fap7p, Rio1p and Rio2p, as described by Henras and colleagues (Henras et al., 2008).

## **Ribosome heterogeneity**

The idea that the ribosome pool can be heterogeneous concerning the composition of ribosomes at the level of rRNA, ribosomal proteins, or other ribosome-associated factors is emerging, supported by increasingly convincing evidence throughout all kingdoms of life. The major functional implication is the possibility that ribosomes with different composition display specialized functions, for instance preferential translation of specific subset of mRNAs or localization to a specific cell compartment, and that these are differentially regulated in development or disease in a tissue- or cell-specific manner (Dalla Venezia et al., 2019; Dinman, 2016; Genuth and Barna, 2018a, 2018b; Guo, 2018; Xue and Barna, 2012).

At the protein level, there is not only variation in the post-translation modifications present on RPs in different contexts, that could modulate interactions of hundreds of ribosome-associated proteins (Simsek et al., 2017), but also variability coming from alternatively or differentially expressed tissue-specific paralogs of ribosomal proteins (Fisher et al., 1990; Lopes et al., 2010; Skaletsky et al., 2003), and tissue-specific RP

mRNA levels have been reported among mouse and human tissues, human hematopoietic cell types, human cancer cell lines (Guimaraes and Zavolan, 2016; Gupta and Warner, 2014; Kondrashov et al., 2011). In addition, differences in the RP composition of translating ribosomes have been described, with specific RPs present in substoichiometric amount, implying that some ribosomes do not contain all RPs (Shi et al., 2017).

Another incredibly productive source of variability may come from rRNA modifications. As well as other rRNA modifications, 2'-O-methylation has traditionally been regarded as a constitutive feature. Recent studies account for a different view of rRNA modification landscape, which appears to display a remarkable degree of variability, with particular sites that are differentially methylated in a cell-specific, tissue-specific or cancer-specific manner (Krogh et al., 2016; Marcel et al., 2015; Sharma et al., 2017; Taoka et al., 2016). With hundreds of modified sites (Natchiar et al., 2017), the extent of modulation via site-specific fractional modifications is potentially huge. Such differential modification could be derive from regulation of the activity of snoRNAs. Indeed, expression levels of snoRNAs have been found to be heterogeneous among different tissues (Castle et al., 2010) and could contribute to tumorigenesis (Su et al., 2014).

Recently, current evidence for the link between variability in rRNA modification and variability in ribosome functions have been reviewed (Penzo et al., 2016; Sloan et al., 2017).

Other sources of variability could be rRNA variants and the existence of two 5.8S rRNA forms. rDNA copy number vary between 50 and 1500 copies among individuals. Single nucleotide variants in the rRNAs of assembled and actively translating ribosomes. rDNA variants are evolutionarily conserved between mouse and human and differ in sites belonging to functional centres of the ribosome (Parks et al., 2018). Several rRNA variants exist and can be differentially expressed and regulated (Santoro et al., 2010; Tseng et al., 2008).

The 5.8S rRNA can be present in either a long or a short form in mature large subunits, with different 5' ends, generated by two alternative processing pathways (Henras et al., 2015). Yet, it is not known whether this variability may have functional implications.

The existence of ribosome heterogeneity supports the idea that specialized ribosomes exist in the cell with distinct functions (Dinman, 2016; Genuth and Barna, 2018a, 2018b; Guo, 2018; Xue and Barna, 2012). For example, differences in rRNA 2'-O-methylation and pseudouridylation have been shown to influence the intrinsic translational capacities (Bellodi et al., 2010; Erales et al., 2017; Jack et al., 2011; Yoon et al., 2006). Similarly,



human ribosomes containing RPS25 or RPL10 were observed to preferentially translate specific subsets of mRNAs involved in different functions, suggesting that the presence of specific RPs could act as a filter selecting mRNA molecules to translate (Shi et al., 2017).

In conclusion, increasing evidence indicate that different ribosome populations coexist. The biological relevance of such diversity is intriguingly coming to light.

### **Nucleolar surveillance pathway**

Cells have evolved multiple quality control pathways that recognize and target defective RNP particles for rapid degradation. For instance, yeast 18S rRNAs or 25S rRNAs bearing point mutations in the decoding center or in the peptidyltransferase center, respectively, are synthesized normally but display markedly decreased stability (LaRiviere et al., 2006). In the yeast nucleus, improperly processed pre-rRNAs are rapidly targeted by the nucleolar surveillance pathway, that may include several mechanisms. The best characterized mechanism is the TRAMP-exosome pathway. The pre-rRNAs are tagged for degradation through the addition of a short tail consisting of four to five adenosines at their 3' end by two related complexes, TRAMP4 and TRAMP5. Polyadenylation acts as a recruitment and stimulatory signal for the RNA exosome that turns over the RNA. The 3' to 5' degradation of the oligoadenylated pre-rRNAs is mainly carried out by Rpp6p (Andersen et al., 2008; Henras et al., 2015; Houseley et al., 2006). A similar quality control mechanism appears to participate in 5S rRNA processing, with several yeast exonucleases involved (van Hoof et al., 2000). The Ro protein, capable of binding a broad population of RNA Pol III transcripts, may target defective 5S rRNA for polyadenylation and subsequent degradation (Fulnecek and Kovarik, 2007; Kuai et al., 2004; O'Brien and Wolin, 1994).

Inactivation of the nucleolar surveillance pathway restores not only the accumulation of the targeted pre-rRNAs but also the production of the mature rRNAs in some cases, suggesting that nucleolar surveillance mechanism may also target pre-rRNAs whose processing is only kinetically delayed. How defective pre-rRNAs are recognized remains unclear. Similar mechanisms appear to exist in mammalian cells as well.

## **3. Regulation of ribosome biogenesis**

Nucleolar structure permits high transcription efficiency in order to meet the cell's metabolic activity and demand for ribosomes. It has been estimated that a cultured mammalian cell produces approximately 7500 subunits per minute, implying the synthesis of about 300,000 RPs and the transcription of 41 megabases of pre-rRNAs per minute (Lewis and Tollervey, 2000), and that a yeast cell produces around 2000 ribosomes per

minute (Warner, 1999). Obviously, this requires a considerable amount of energy. In one eukaryotic cell, RNA Pol I-dependent transcription represents nearly 60% of the total transcription in the cell, and 50% of Pol II-dependent transcription and 90% of mRNA splicing are devoted to ribosomal proteins (Warner, 1999; Warner et al., 2001). As rRNA synthesis is a predominant energy-consuming process, switching off transcription of rRNA genes is crucial for saving energy and maintain cellular homeostasis in scarcity conditions. In addition, rRNA synthesis represents the rate-limiting step in the whole process of ribosome biogenesis (Chédin et al., 2007). Even more generally, RNA Pol I seems to be a master regulator coordinating the activity of the other transcription machineries: an elegant genetic study in yeast showed that the expression of a constitutively active RNA Pol I leads not only to higher levels of rRNA synthesis but also to higher activity of RNA Pol II and RNA Pol III (Laferté et al., 2006). Indeed, the pool of ribosomes is carefully regulated by ribosome biogenesis and is adjustable to cellular needs. Therefore, it is not surprising that most signalling pathways responsible for regulating cell growth and proliferation are also directly involved in ribosome biogenesis regulation, with their downstream effectors converging at the RNA Pol I transcription machinery. For example, stimulation or activation of epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin and insulin-like growth factor (IGF) all revealed very rapid response with almost immediate effect on rRNA transcription in vivo (Hannan et al., 1998; James and Zomerdijk, 2004; Stefanovsky et al., 2001; Zhao et al., 2003). Among the signalling pathways involved in ribosome biogenesis regulation, c-MYC and the components of the AKT/PI3K/mTOR axis have emerged as major regulators (Drygin et al., 2010; Piazzini et al., 2019). In addition, AMP-dependent kinase (AMPK) is another essential sensor of the energy status in the cell, being activated upon high AMP/ATP ratio reflecting low nutritional state, and regulator of ribosome biogenesis (Grummt and Grummt, 1976). Attenuation of ribosome biosynthesis can occur at different levels, epigenetic rDNA activation, localization of nucleolar factors, modulation of RNA Pol I transcription or elongation, pre-rRNA processing and transport, synthesis of ribosomal proteins (Grummt, 2003, 2013; Grummt and Voit, 2010; Iadevaia et al., 2014; Moss et al., 2007; van Riggelen et al., 2010).

The fact that all major regulatory pathways control ribosome biogenesis and the diversity of these mechanisms confer robustness to the process of ribosome production and account for its importance in cellular functions.

**Table 2. Known post-translational modifications of components of the RNA Pol I transcriptional machinery and their function**

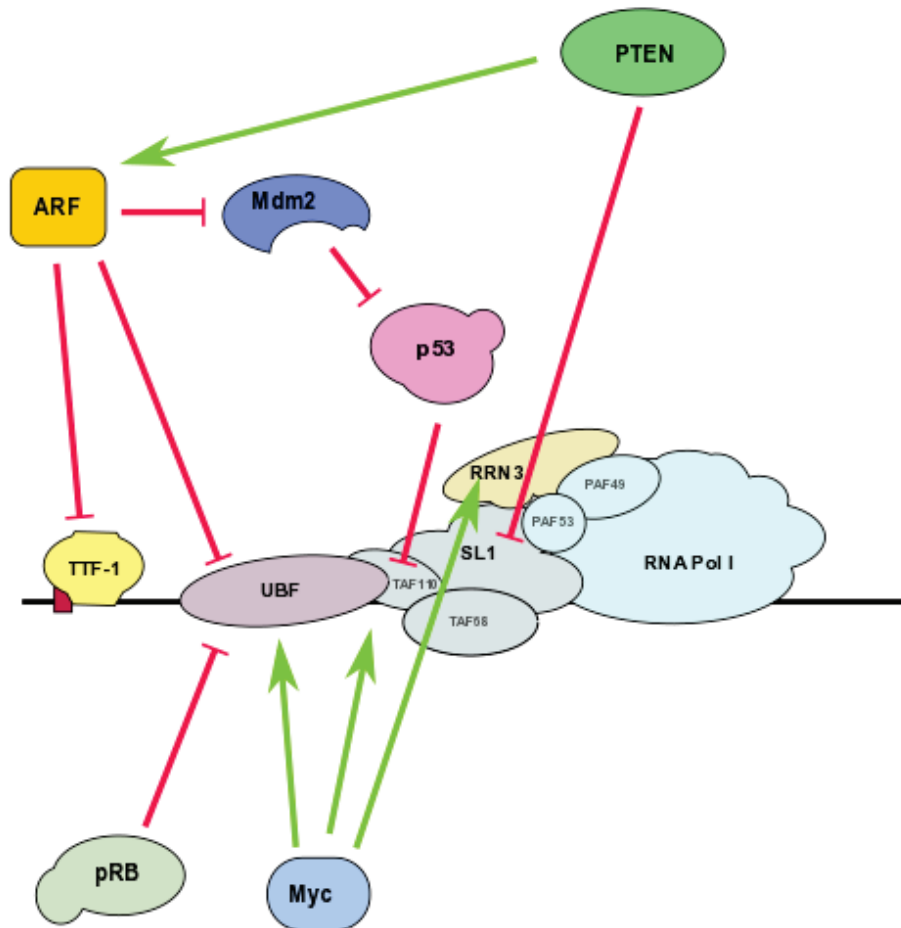
Machinery component	Modified site	Modification	Function	Responsible enzyme	Reference
TIF-IA/RRN3	Thr 200	Phosphorylation	Impairs interaction with PAF53	S6K	Mayer & Grummt, <i>Oncogene</i> , 2006
	Ser 44	Dephosphorylation	Inactivation and translocation to the cytoplasm	PP2A	Claypool, <i>Mol Biol Cell</i> , 2004 Mayer, <i>Genes Dev</i> , 2004
	Ser 170, Ser 172	Phosphorylation	Promotes elongation	Casein kinase 2	Bierhoff, <i>Mol Cell Biol</i> , 2008
	Ser 199	Phosphorylation	Inactivation and translocation to the cytoplasm	S6K	Nguyen & Mitchell, <i>PNAS</i> , 2013 Claypool, <i>Mol Biol Cell</i> , 2004 Mayer, <i>Genes Dev</i> , 2005
	Ser 633, Ser 649	Phosphorylation	Enhances TIF-IA	ERK, S6K	Zhao, <i>Mol Cell</i> , 2003
UBF	Ser 635	Phosphorylation	Impairs interaction with SL1	AMPK	Hoppe, <i>PNAS</i> , 2009
	Ser484	Phosphorylation	Stimulates interaction with SL1	S6K, Cdk4/cyclin D, Cdk2/cyclin E	Hannan, <i>Bioch et Bioph Acta</i> 2013 Voit, <i>EMBO</i> , 1999
	Ser 388	Phosphorylation	Stimulates rDNA transcription	Cdk2/cyclin A or E, ERK	Voit & Grummt, <i>PNAS</i> , 2001 Mariappan, <i>Am J Physiol Renal</i> , 2010
	Thr 117, Thr 201	Phosphorylation	Induces remodeling of chromatin	ERK	Stefanovsky, <i>Mol Cell</i> , 2006
	n.a.	Acetylation	Protects from pRb-mediated repression	CBP	Stefanovsky, <i>Biochemistry</i> , 2006 Pelletier, <i>Mol Cell</i> , 2000 Meraner, <i>NAR</i> , 2006 Hirschler-Laszkiwicz, <i>NAR</i> , 2001
SL1	Lys sites of TAF68	Acetylation	Stimulates recruitment of the PIC	PCAF	Muth, <i>EMBO</i> , 2001
	Lys sites of TAF68	Deacetylation	Inhibits recruitment of the PIC	SIRT1	Muth, <i>EMBO</i> , 2001
	TAF68	Deacetylation	Prevents interaction with UBF and rDNA promoter	SIRT7	Voit, <i>PLoS Genetics</i> , 2015
	Thr 852 of TAF110	Phosphorylation	Prevents interaction with UBF and rDNA promoter	Cdk1/cyclin B	Heix, <i>EMBO</i> , 1998 Kuhn, <i>J Mol Biol</i> , 1998
RNA Pol I	Thr 852 of TAF110	Dephosphorylation	Enhances interaction with UBF and rDNA promoter	Cdc14b	Voit, <i>PLoS Genetics</i> , 2015
	Lys 373 of PAF53	Acetylation	Inhibits transcription elongation	CBP	Chen, <i>Mol Cell</i> , 2013
	Lys 373 of PAF53	Deacetylation	Stimulates transcription elongation	SIRT7	Chen, <i>Mol Cell</i> , 2013

## **Post-translational modifications of RNA Pol I transcription machinery**

The activity of Pol I transcription factors is regulated by post-translational modifications (Table 2). This ties ribosome biogenesis to the major cellular pathways so that in normal cells ribosome biogenesis rate is tightly linked to nutritional availability and cell metabolic state (Goodfellow and Zomerdijk, 2012; Grummt, 2010; Lempiäinen and Shore, 2009; Sirri et al., 2008).

TIF-IA (also known as RRN3, as previously described) is the major target of signalling cascades that ultimately up or downregulate rDNA transcription (Grummt and Voit, 2010). TIF-IA can be phosphorylated at multiple sites and its phosphorylation pattern is regulated in response to external signals: phosphorylation of TIF-IA is considered a regulatory checkpoint for ribosome biogenesis, as the active form can interact with the RNA Pol I to recruit it at the PIC (Cavanaugh et al., 2002; Nguyen and Mitchell, 2013). For example, stress-induced phosphorylation of TIF-IA at threonine 200 impairs the interaction between TIF-IA and RNA Pol I thereby preventing the recruitment of the transcription machinery at the rDNA promoter (Mayer and Grummt, 2006). Likewise, nutritional stress, experimentally mimicked by inhibition of the mTOR pathway, inactivates TIF-IA by decreasing phosphorylation at serine 44 and increasing phosphorylation at serine 199, concurrently entailing its translocation to the cytoplasm (Claypool et al., 2004; Mayer et al., 2004). Extra-cellular Signal-Regulated (ERK) and S6K kinases associate with TIF-IA and phosphorylate serines 633 and 649 leading to a ten-fold activation of rRNA synthesis (Zhao et al., 2003). The activation of AMPK triggers phosphorylation of TIF-IA at serine 635, which prevents interaction with SL1 thereby inhibiting rRNA synthesis (Hoppe et al., 2009).

Like TIF-IA, both SL1 and UBF are subject to regulation via post-translational modifications, namely phosphorylation and acetylation (Chen et al., 2013; Heix et al., 1998; Hirschler-Laszkiewicz et al., 2001; Lin et al., 2006; Mahony et al., 1992; O'Mahony et al., 1992; Pelletier et al., 2000; Voit et al., 1999). In general, hyperphosphorylation of UBF activates RNA Pol I transcription, whereas hypophosphorylation is correlated with transcriptional silencing in quiescent cells. UBF can be phosphorylated at several specific serine and threonine residues, mainly located within the HMG domains, resulting in enhanced interaction of UBF with rDNA promoter (Hannan et al., 2003; Mahony et al., 1992; Mariappan et al., 2010; Pelletier et al., 2000; Stefanovsky et al., 2006, 2006, 2001; Voit and Grummt, 2001; Voit et al., 1992, 1999). Acetylation of UBF by the CREB-binding



**Figure VI. Regulation of RNA Pol I transcriptional machinery by the main oncogenes and tumor suppressors**

Cartoon depicting the negative or positive contribution of the main oncogenes and tumor suppressors to RNA Pol I activity through regulation of components of the translational machinery. Retinoblastoma protein (pRB) binds to UBF impairing its interaction with the rDNA promoter. ARF tumor suppressor inactivates both UBF and TTF-1; it also inhibits MDM2 activity thereby activating p53 function. PTEN disrupts SL1 complex and stabilizes ARF, which in turn stabilizes p53. p53 prevents the interaction between UBF and SL1 by interfering with TAF110 subunit of SL1. Myc enhances transcription directly by facilitating the recruitment of SL1 and indirectly by increasing the expression of UBF, SL1 and RRN3.

protein (CBP), originally identified as a histone acetyltransferase, protects UBF activity from inhibition by the tumor suppressor pRb (Meraner et al., 2006; Pelletier et al., 2000). TAF68, a subunit of SL1 required for SL1 binding to rDNA promoter, is acetylated by PCAF, another histone acetyltransferase, stimulating the recruitment of the PIC on the rDNA promoter (Muth et al., 2001).

In yeast, Sir2 links rRNA synthesis directly to the energy status of the cell. Sirtuins are NAD<sup>+</sup> dependent deacetylases able to sense the redox state of the cell and changes in sirtuin activity plays a central role in many processes as broad as gene expression, cell

survival under stress, cell cycle control, metabolic homeostasis (Kobayashi and Ganley, 2005). In mammals, seven sirtuins (SIRT1-7) are present, each characterized by different cellular location, substrates and functions (Houtkooper et al., 2012). In mouse, SIRT1 downregulates RNA Pol I transcription by counteracting PCAF-dependent acetylation of TAF68 (Muth et al., 2001). In human cell lines, SIRT7 is a nucleolar sirtuin associated with rDNA interacting with both UBF and RNA Pol I promoting cell growth and proliferation by enhancing rRNA transcription in response to favorable metabolic conditions. SIRT7 are low or even absent in non-proliferating cells (Ford et al., 2006; Grob et al., 2009; Tsai et al., 2012). PAF53, the mammalian homolog of the yeast Pol I subunit A49, is one of the SIRT7 substrates at its lysine 373 that can be acetylated by CBP and deacetylated by SIRT7. In the hypoacetylated state, PAF53-mediated interaction between RNA Pol I and rDNA is facilitated resulting in efficient transcription elongation (Chen et al., 2013). Thus, a dynamic balance between multiple post-translational modification patterns (mainly summarized in Table 2) are responsible for the regulation of several factors belonging to the RNA Pol I transcription machinery.

### **Regulation by tumor suppressors at multiple level**

To counteract alteration of ribosome biogenesis, mammalian cells have developed tumor suppressor based surveillance mechanisms that suspend cell proliferation in the event of uncontrolled ribosome production, thereby coordinating the two processes. The most important surveillance mechanisms rely on the function of the tumor suppressors p53, retinoblastoma protein (pRb) and phosphatase and tensin homolog (PTEN).

The major tumor suppressor p53 binds to SL1 and TFIIIB, inhibiting RNA Pol I- and Pol III-dependent transcription, respectively (Felton-Edkins et al., 2003; Zhai and Comai, 2000).

In addition, p53 controls the phosphorylation of pRb, modulating its functions. pRb, in its hypophosphorylated form, is able to bind E2F transcription factors, required for the transcription of a set of genes during the restriction point of the cell cycle (Levine, 1997). Apart from this function, in this form pRb inhibits both RNA Pol I- and Pol III-dependent transcription by binding UBF and TFIIIB, respectively. When phosphorylated, pRb binding function is not effective and its inhibitory effect is relieved. Thus, the tumor suppressors p53 and pRb are responsible for the transition from G1 to S phase of cell cycle and also regulate rRNA synthesis (Cavanaugh et al., 1995; Ciarmatori et al., 2001; Felton-Edkins et al., 2003; Hannan et al., 2000; Voit et al., 1997; White et al., 1996).

ARF, a positive regulator of p53 stabilization, as described later on, is also able to reduce the ribosome biogenesis rate by impairing the recruitment of UBF on the rDNA promoter and by interfering with rRNA processing (Ayrault et al., 2006; Bertwistle et al., 2004). In addition, ARF inhibits rRNA biogenesis modulating the RNA Pol I transcription factor TTF-I (Lessard et al., 2010).

Finally, PTEN represses RNA Pol I transcription by disrupting the SL1 complex (Zhang et al., 2005a).

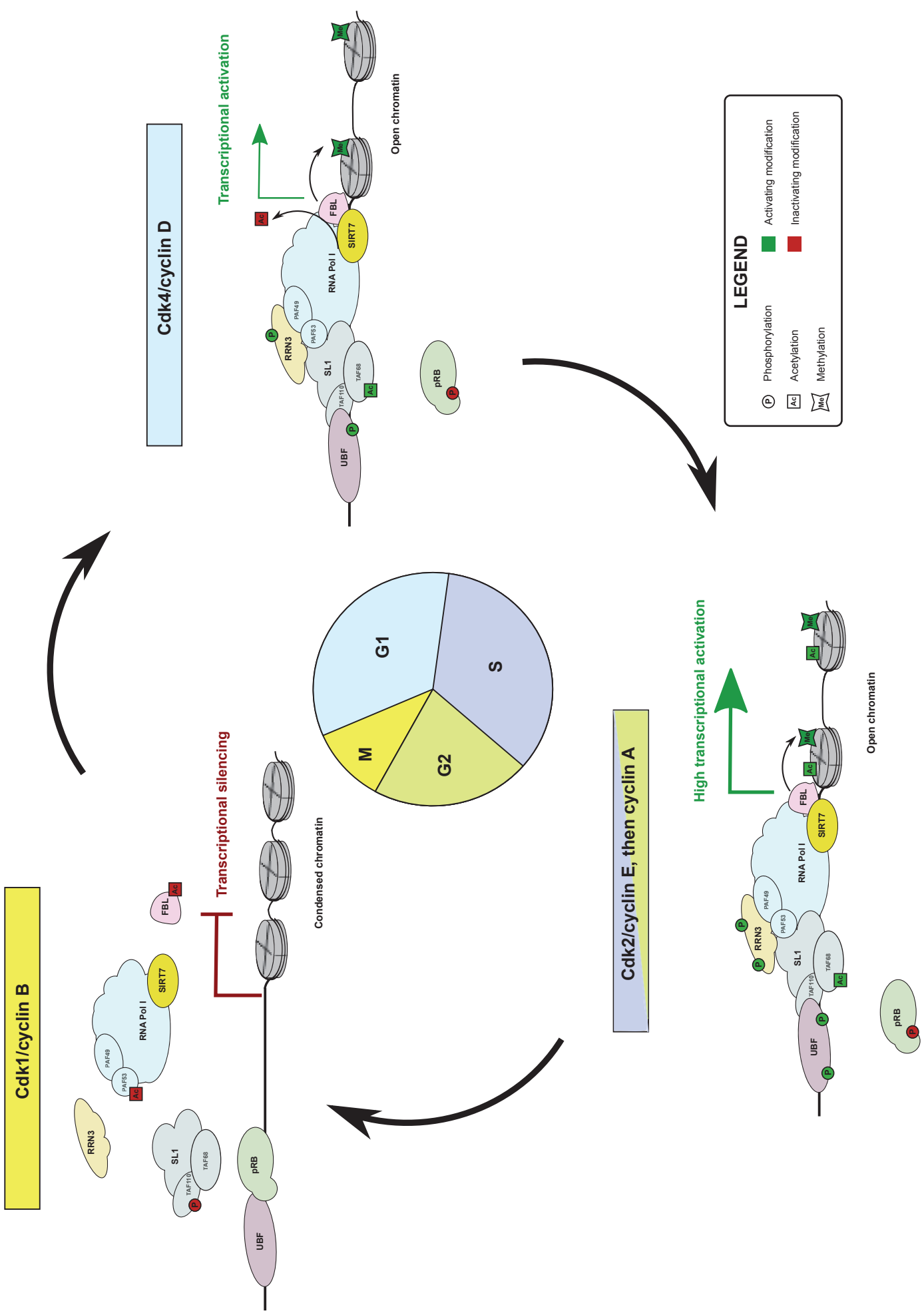
It is then clear that tumor suppressors play a role in negative regulation of ribosome biogenesis. The role of the main oncogenes and tumor suppressors in the regulation of RNA Pol I transcriptional machinery is represented in Figure VI.

### **MYC and ribosome biogenesis**

One of the major regulators of ribosome biogenesis is MYC, that has a widespread effect as it acts as a transcription factor and coordinates multiple steps of the process including the synthesis of rRNA and ribosomal proteins (Campbell and White, 2014; van Riggelen et al., 2010). First, it directly stimulates RNA Pol I activity, as it binds to specific elements in close proximity to the rDNA promoter, thereby facilitating the recruitment of SL1 required for RNA Pol I activity, and also recruits TRRAP factor, part of the histone acetyltransferase complex responsible for targeting acetylation of histones H3 and H4, thereby opening the DNA for transcription (Arabi et al., 2005; Frank et al., 2001; Grandori et al., 2005; McMahon et al., 2000). Second, in a similar way, it stimulates Pol III-dependent transcription of 5S rRNA through activation of TFIIB transcription factor and regulation of the chromatin state through recruitment of TRRAP and subsequent histone acetyltransferase activity (Felton-Edkins et al., 2003; Gomez-Roman et al., 2006; Kenneth et al., 2007). Third, it promotes RNA Pol II-dependent expression of multiple genes encoding ribosomal proteins and nucleolar factors, including C/D and H/ACA snoRNAs, fibrillarin, nucleolin, BOP1, dyskerin (Ginisty et al., 1998; Herter et al., 2015; Watson et al., 2002). In addition, by means of its various transcriptional targets, it indirectly upregulates RNA Pol I-dependent transcription by regulating the abundance of UBF, SL1 and TIF-IA, as well as most of the core components of the PIC (Poortinga et al., 2011).

Finally, target genes of c-Myc include essential regulators of cell cycle progression such as Cdks, cyclins and E2F transcription factors (Bretones et al., 2015; Schlosser et al., 2003). Coordinating all steps of ribosome biogenesis, the MYC gene plays a pivotal role in its regulation and links it to the proliferative state of the cell by coupling the entire process with cell cycle progression.

Figure VII - Regulation of RNA Pol I transcriptional machinery throughout the cell cycle





## **Figure VII. Regulation of RNA Pol I transcriptional machinery throughout the cell cycle (previous page)**

Cartoon summarizing the main molecular events regulating the transcriptional machinery required for RNA Pol I activity during the cell cycle, with particular regard for post-translational modifications of the machinery components and regulation at the epigenetic level. During mitosis, Cdk1/cyclin B phosphorylates TAF110, a subunit of SL1, preventing its interaction with UBF and thus the recruitment of SL1 on the rDNA promoter. At the same time, the PAF53 subunit of RNA Pol I is inactivated by CBP-mediated acetylation. In addition, pRB is found in its hypophosphorylated form and interacts with UBF by preventing its function and recruitment of the RNA Pol I transcriptional machinery. Epigenetic regulation is provided by the inactivating acetylation of FBL, preventing it from chromatin remodelling and thus leaving rDNA in a condensed chromatin state, associated with transcriptional silencing. At the exit from mitosis, UBF functional repression by pRB is relieved as pRB is hyperphosphorylated. UBF is also activated by Cdk4/cyclin D1-mediated phosphorylation. The TAF110 subunit of SL1 is dephosphorylated by Cdc14b phosphatase activity and can thus interact with UBF. RRN3 is activated by phosphorylation. SIRT7 deacetylase is activated and thus can deacetylate PAF53 subunit of RNA Pol I and FBL. This latter event leads to chromatin remodelling and transcriptional activation of the rDNA gene. During the progression of the cell cycle, UBF and RRN3 are furtherly activated by progressive phosphorylation first due to Cdk4/cyclin D1 activity, then to Cdk2/cyclin A or E activity. In addition, mTOR-dependent and ERK-dependent pathways activate RRN3 at multiple sites. At the epigenetic level, high transcriptional activation of rDNA genes is ensured by acetylation of H4 histone, peaking at the S phase. Activating modifications are marked in green, and inhibiting ones in red. See text for further details.

## **mTOR and ribosome biogenesis**

The mTOR pathway is a central regulator of metabolism and physiology: it integrates external stimuli such as nutrients and growth factors as well as internal ones such as the cellular energy status, to activate metabolic pathways that ultimately drive cell growth.

Like MYC, mTOR has been shown to coordinate the transcription of all three classes of RNA polymerases needed for ribosome biogenesis (Mayer and Grummt, 2006). Inhibition of mTOR function by rapamycin treatment or depletion leads to a number of important physiological changes typical of starved cells, including reduced nucleolar size (Zaragoza et al., 1998). Ribosome biogenesis is regulated by mTOR signalling pathway at multiple levels, mainly transcription of rRNA and translation of mRNAs coding for ribosomal proteins.

Downregulation of mTOR signalling causes a rapid and strong downregulation of rRNA transcription and pre-rRNA processing (Claypool et al., 2004; Hannan et al., 2003; Mahajan, 1994; Zaragoza et al., 1998; Zhang et al., 2005a). In particular, in a pivotal experiment, transcriptional repression observed after rapamycin treatment could be relieved upon overexpression of exogenous TIF-IA, indicating that such repression was mediated by TIF-IA function. In addition, transcriptional repression was rescued by

exogenous mTOR or S6K1, but not by a kinase-deficient mutant form of S6K1, indicating that S6K1 regulates TIF-IA activity (Mayer et al., 2004). Nowadays, it is well established that mTOR controls RNA Pol I-dependent transcription through modulation of phosphorylation of TIF-IA (Claypool et al., 2004; Mayer et al., 2004).

Like TIF-IA, UBF is regulated by mTOR signalling via S6K1 activity. S6K1-dependent phosphorylation of UBF facilitates the interaction with SL1 and the subsequent formation of the PIC (Hannan et al., 2003). UBF is also affected by mTOR signalling in an indirect manner, as mTOR signalling leads to a cyclin D1-dependent increase in Cdk4 activity and subsequent phosphorylation of pRb. In turn, pRb protein, that is otherwise associated with UBF limiting its availability, dissociates from UBF causing an increase in its availability (Nader et al., 2005). These are the most remarkable results underscoring the role of mTOR signalling in regulation of RNA Pol I transcription initiation complex formation.

The role of mTOR signalling in Pol III-dependent transcription was accounted by studies in both yeast and mammalian cells, where inhibition of mTOR signalling was first found to attenuate Pol III-dependent transcription (White, 2005; Zaragoza et al., 1998). Indeed, mTOR signalling promotes the recruitment of TFIIB and TFIIC transcription factors at the promoters of 5S rRNA genes (Mayer and Grummt, 2006).

Finally, the role of mTOR in the translational regulation of ribosomal proteins must be mentioned. A common feature shared by a large subset of mRNAs encoding many components of the translational machinery, especially ribosomal proteins, is the presence of a 5' terminal oligopyrimidine tract (5'TOP). TOP-containing mRNAs are inefficiently translated even in growing cells, because 5'TOP motif carries an inhibitory function. However, mTOR signalling relieves 5'TOP-mediated translational repression through activation of S6K1 and subsequent RPS6 phosphorylation, that is thought to increase the affinity of ribosomes for 5'TOP and thus facilitate translation of these mRNAs (Jefferies et al., 1997; Meyuhas, 2000; Raught et al., 2001). Thus, the synthesis of many components needed for ribosome biogenesis is coordinated by mTOR signalling and coupled with cell growth and cell proliferation.

### **Ribosome biogenesis throughout the cell cycle**

It has been mentioned above that the nucleolus displays variable features throughout the cell cycle. Reasonably, such variation is true also with regard to nucleolar functions at the molecular level.

Aside from regulation dependent on growth conditions, RNA Pol I activity oscillates during cell cycle progression. It is maximal at S and G<sub>2</sub> phase, stops during mitosis and progressively recovers since telophase and during G<sub>1</sub> phase (Klein and Grummt, 1999).

Mitotic silencing is caused by Cdk1/cyclin B-dependent phosphorylation of the SL1 subunit TAF110 at threonine 852 and by SIRT1-dependent deacetylation of TAF68, that together prevent interaction between SL1 and UBF and SL1 binding to the rDNA promoter (Heix et al., 1998; Kuhn et al., 1998; Voit et al., 2015). Despite its interaction with SL1 is impaired, UBF remains associated to rDNA within NORs that were transcriptionally active during the previous interphase and this is why they can be detected by AgNOR staining. However, SL1 is functionally inactivated and, accordingly, DFC disassembly reflects the repression of Pol I transcription.

At the end of mitosis, the same threonine on TAF110 is dephosphorylated by Cdc14b phosphatase, PCAF acetylates TAF68 subunit and SIRT7 deacetylates PAF53 subunit, thereby activating SL1 and relieving mitotic transcriptional shutdown (Chen et al., 2013; Voit et al., 2015). Concurrently, Cdk4/cyclin D1 and Cdk2/cyclin E phosphorylate UBF at serine 484 (Voit et al., 1999). This overlaps with the first events of nucleologenesis, starting from release of silencing of Pol I transcription, with nucleoli assembling at the level of active NORs.

Later, during the S phase, RNA Pol I-dependent transcriptional activity is at its peak, because Cdk2/cyclin E and Cdk2/cyclin A phosphorylate UBF at serine 388 facilitating its activity (Voit and Grummt, 2001) and this is reflected by gradual increase in nucleolar size from early G<sub>1</sub> to G<sub>2</sub>.

Recently, a new element was added to the list of cell cycle-dependent modifications: fibrillarin is acetylated at several lysine residues by CBP and deacetylated by SIRT7 (Iyer-Bierhoff and Grummt, 2019; Iyer-Bierhoff et al., 2018). Fibrillarin mediates methylation of histone H<sub>2</sub>A at glutamine 104, that correlates with activation of RNA Pol I (Tessarz et al., 2014). Acetylated fibrillarin displays a reduced histone methyltransferase activity, so SIRT7 enhances and CBP impairs such fibrillarin function, in line with their roles in cell cycle described above (Iyer-Bierhoff and Grummt, 2019; Iyer-Bierhoff et al., 2018).

In parallel, other mechanisms operate during the cell cycle. In resting cells, pRb is present in its hypophosphorylated form and inhibits UBF and TFIIB, this inhibiting RNA Pol I- and Pol III-dependent transcription (Cavanaugh et al., 1995; Ciarmatori et al., 2001; Felton-Edkins et al., 2003; Hannan et al., 2000; Voit et al., 1997; White et al., 1996). In G<sub>1</sub> phase, Cdk4 and Cdk6/cyclin D1 activity and, at the end of G<sub>1</sub> phase, Cdk2/cyclin E, trigger

phosphorylation of pRb and lead to its functional inactivation derepressing pRb-mediated inhibitory activity (Giacinti and Giordano, 2006; Mittnacht, 1998). Thus, progressive phosphorylation of pRb from early G1 phase to G2 phase mediates a gradual increase in rRNA synthesis rate.

Thus, a network of molecular actors contribute to the appropriate combination of post-translational modifications of factors involved in transcriptional machinery, regulating their functions in a cell cycle-dependent manner. The main molecular events that account for the regulation of the RNA Pol I transcriptional machinery throughout the cell cycle are summarized in Figure VII. All of these mechanisms account for the dynamics of nucleolar structure over cell cycle progression.

### **Epigenetic regulation of rDNA**

A long-term epigenetic mechanism regulates RNA Pol I transcription by determining the proportion of active rDNA genes and appropriately maintaining it throughout cell divisions. The ratio between active and silenced genes can be affected by differentiation, cellular senescence and malignant transformation (Sharifi and Bierhoff, 2018). Multiple mechanisms control the epigenetic state of ribosomal genes, including DNA methylation, histone modifications, change in chromatin state. Hypomethylation of rDNA promoters results in increased ribosome biogenesis and higher genomic instability in several types of cancer (Grummt, 2010; Grummt and Längst, 2013; Grummt and Pikaard, 2003; McStay and Grummt, 2008). Consistently, a euchromatic state of rDNA genes is favoured by factors that prevent repressive methylation of rDNA or promote its active demethylation, including the methyl-CpG binding MBD3, GADD45a and CHD7 proteins (Brown and Szyf, 2007; Schmitz et al., 2009; Zentner et al., 2010).

Epigenetic regulation is dependent on the balance between DNA methyltransferases and histone deacetylases, associated with transcriptional repression, on one hand and DNA demethylases, histone acetyltransferases and histone lysine demethylases on the other hand (Feng et al., 2010; Iyer-Bierhoff et al., 2018; Murayama et al., 2008; Tanaka et al., 2010).

Another emerging mechanism for epigenetic regulation of RNA Pol I-dependent transcription relies on the interplay between termination factor TTF-1, Pol I-transcribed non-coding RNAs and the chromatin remodeling complex NoRC. In addition to the main rDNA gene promoter, related sequence elements known as spacer elements are found within the IGS in several species (Goodfellow and Zomerdijk, 2012). Studies in mouse cells suggest that RNA Pol I can bind the space promoters of a subset of inactive rDNA genes to

produce short transcripts (pRNAs) involved in transcriptional silencing of the rRNA genes (Mayer et al., 2006; Santoro et al., 2010). TTF-1 is one among the key factors for the maintenance of a proportion of active rDNA genes in mammals. Its as a transcription terminator has been mentioned, but it can also associate with specific complexes and recruit them to the rDNA genes impacting chromatin conformation. For instance, TTF-1 mediates epigenetic silencing by binding near the rDNA promoter and recruiting the nucleolar remodelling complex (NoRC), which in turn recruits DNA methyltransferases DNMT1 and DNMT3 and histone-deacetylase activities such as the Sin3 complex, resulting in transcriptional repression (Santoro and Grummt, 2005; Santoro et al., 2002; Zhou et al., 2002). Interestingly, NoRC recruitment is dependent on the interaction between its subunit TIP5 and pRNAs (Mayer et al., 2006; Santoro et al., 2010). TTF-1 can also mediate transcriptional activation through interaction with CSB, a chromatin remodeler (Yuan et al., 2007). So, TTF-1 carries out two conflicting functions at the epigenetic level. Whether this is regulated and at what extent it could play a role in the global regulation of rRNA synthesis is not known.

Although epigenetic regulation is commonly regarded as a strategy of the cell for longer-term effects relative to post-translational modifications, an analysis of epigenetic marks in cells positioned at different phases of the cell cycle revealed that cell cycle-dependent oscillations are observed at the epigenetic level as well. The role of fibrillarin in regulating RNA Pol I activity through methylation of histone H2A and its cell cycle-dependent oscillation (Iyer-Bierhoff et al., 2018; Tessarz et al., 2014) has been described above. Moreover, the levels of acetylated histone 4 are highest in early S phase, coinciding with the peak of binding of UBF to the rDNA promoter; binding of the DNA methyltransferase DNMT1 is highest during mid-S phase; binding of DNMT3B comes later in G2. Also, DNA methylation state is not stable during the cell cycle, being lowest during early and late S phase, which is consistent with transcriptional activation (Brown and Szyf, 2008).

Recently, it has been reported that loss of UBF is accompanied by profound alterations of the epigenetic landscape, suggesting previously unknown roles in the regulation of rDNA genes at the epigenetic level, in addition to the contribution to the maintenance of chromatin in an active state by mere binding to the entire rDNA gene during transcription elongation (Hamdane et al., 2014; Moss et al., 2019).

## 4. Ribosome biogenesis as a sensor of stress

The prominent place of ribosome biogenesis in cell metabolism and its regulation by most major signalling pathways makes it a highly sensitive integrator of information. As previously mentioned, the nucleolus is a dynamic structure where a huge number of factors interact, and this feature makes it a convergence point for different cellular functions.

Proteomics and live cell fluorescence studies have revealed that rapid changes take place in the concentration and localization of typically nucleolar proteins, including those involved in rDNA transcription and pre-rRNA processing, chaperones and multitasking proteins, are produced in response to various stress stimuli, highlighting the high sensitivity of the nucleolus (Ahmad et al., 2009; Andersen et al., 2005; Bański et al., 2010; Boisvert and Lamond, 2010; Hiscox et al., 2010; Lam et al., 2010).

### Discovery of the nucleolar stress

In a previous section, the Bop1-Pes1-WDR12 ternary protein complex, required for maturation of the large subunit, was mentioned. Its role in ribosome biogenesis has not been fully understood yet, but there is a historical reason why Bop1-Pes1-WDR12 complex cannot be missing from a discussion regarding nucleolar functions: it was the first reported ribosome biogenesis factor whose disruption caused what has been called “nucleolar stress” (Pestov et al., 2001b).

In their experiments, Pestov and colleagues used Bop1 $\Delta$ , an amino-terminally truncated dominant negative mutant form of Bop1, resulting in inhibition of 60S ribosomal subunit formation (Strezoska et al., 2000). Unexpectedly, when they forced Bop1 $\Delta$  expression in mouse cells, they observed not only ribosome biogenesis inhibition but also a strong, yet reversible, cell cycle arrest. Indeed, the impairment of Bop1 function coincided with a decrease in G<sub>1</sub>-specific Cdk2 and Cdk4 kinase activities, a concomitant increase in the levels of p21 and p27 Cdk inhibitors, lack of phosphorylation of pRb protein and decreased expression of cyclin A. Together, all these events indicate a strong perturbation of cell cycle and inability of the cells to progress through the restriction point. Interestingly, inactivation of functional p53 could restore normal cell cycle, contrary to defective ribosome biogenesis, indicating that defective Bop1 function leads to p53-dependent cell cycle arrest. These findings showed for the first time that deficiencies in ribosome synthesis can be uncoupled from the cell cycle arrest and ascribed p53 a new role

as a mediator of the signalling link between ribosome biogenesis and the cell cycle (Pestov et al., 2001b).

Just a couple of years later, another key study provided meticulous demonstration that nucleolar disruption is necessary for p53 stabilization, enforcing the idea that the nucleolus is a sensor of stress. Indeed, many types of stress, including hypoxia, heat shock, growth factor deprivation, UV irradiation, were reported to cause the disruption of nucleolar structure with concomitant formation of nucleolar caps. In particular, by using UV beams in order to produce very localized damage in the cell, the study showed that nucleolar disruption was a prerequisite for p53 stabilization. In drug-induced stress conditions that do not specifically impact nucleolar structures or UV irradiation that does not specifically hit the nucleoli, p53 is not stabilized and the cell cycle remains unaffected. Thus, the nucleolus represents a link between stress signals and p53-mediated cellular response (Rubbi and Milner, 2003).

Ever since, evidence has accumulated at an incredible rate supporting the concept that aberrant rRNA processing and/or ribosome biogenesis cause “nucleolar stress”. Initially, the model stated that this would result in cell cycle arrest in a p53-dependent manner; however, this view has become much broader now and the widely accepted, current model states that nucleolar stress would lead to cell cycle arrest through multiple ways that might rely on p53 function or not and that eventually might induce cell death (Golomb et al., 2014; Grummt, 2013; Holmberg Olausson et al., 2012; Mayer and Grummt, 2005; Olson, 2004; Yang et al., 2018).

A number of later observations confirmed the role of the nucleolus and, in particular, ribosome biogenesis, in the regulation of stress response. Virtually, given that the process of ribosome biogenesis is extremely demanding and highly regulated, nucleolar stress can be triggered by any stress event, that would be rapidly reflected by notched ribosome biogenesis with subsequent unbalanced presence of ribosome components in the cell, whether it would be a reduction of rRNA transcription, a blockade of ribosome assembly or impaired production of one ribosomal component. It has been shown by several studies that enhanced rRNA transcription reduces p53 expression (Bursac et al., 2014; Deisenroth and Zhang, 2010; Golomb et al., 2014; Zhang and Lu, 2009) and that, on the contrary, functional inactivation of RNA Pol I (Donati et al., 2011a; Drygin et al., 2011; Peltonen et al., 2010; Shav-Tal et al., 2005), TIF-IA (Yuan et al., 2005), U3 or U8 snoRNAs (Langhendries et al., 2016) or several ribosomal proteins (Bursac et al., 2014), as well as perturbation of nuclear import/export directly impacting ribosome biogenesis (Bursać et

al., 2012), all lead to growth arrest and apoptosis with a clear disruption of nucleolar structure.

### **p53-dependent nucleolar stress**

At the mechanistic level, the induction of p53 stabilization has been extensively investigated and has become clear. In normal conditions, p53 protein levels are typically kept very low by robust regulation consisting of several mechanisms acting in parallel (Brooks and Gu, 2011a; Lee and Gu, 2010). The best characterized and also the most prominent one, as depicted by Figure VIII, is the activity of the E3 ubiquitin ligase Mdm2 (HDM2 in human, but Mdm2 is generally used referring to mammalian systems), that interacts with p53 protein and ubiquitinates it thereby promoting its proteasome-mediated degradation (Freedman and Levine, 1998; Freedman et al., 1999; Haupt et al., 1997; Honda et al., 1997). Proteasome-mediated degradation occurs in the cytoplasm and Mdm2-p53 interaction is required for the shuttling of both proteins from the nucleus (Xirodimas et al., 2001), although it has also been reported that an alternative, proteasome-independent and calpain-dependent mechanism takes place in the nucleus (Tao et al., 2013). Besides promoting p53 degradation, interaction with Mdm2 also inactivates p53 transcriptional activity. The two activities possessed by Mdm2 can be uncoupled, indicating that they rely on two different molecular events, as confirmed by structural studies (Nomura et al., 2017).

Several stress signals, especially the well studied case of DNA damage, cause perturbations of Mdm2-p53 interaction through post-translational modifications such as phosphorylation (Ashcroft et al., 1999; Maya et al., 2001; Meek, 1998; Pereg et al., 2005) or acetylation (Brooks and Gu, 2011b; Gu and Roeder, 1997) targeting p53 or Mdm2 thereby preventing their association. This leads to p53 protein stabilization with subsequent activation of p53-mediated cell response, entailing cell cycle arrest, DNA repair and, potentially, apoptosis or senescence, depending on the extent of the stress or damage and on cellular context.

In addition to post-translational modifications, other multiple routes to p53 activation exist, including ARF, nucleophosmin and nucleostemin, other members of the Mdm2 family (Mdmx) most of which are based on the modulation of Mdm2 binding to p53. For instance, ARF is a potent tumorsuppressor involved in maintaining nucleolar structure and limiting protein synthesis and acts as an upstream regulator of p53 signalling. ARF binds and relocalizes Mdm2 to nucleoli and prevents degradation of p53 leading to its activation, apoptosis and growth arrest (Weber et al., 1999). ARF accumulates upon



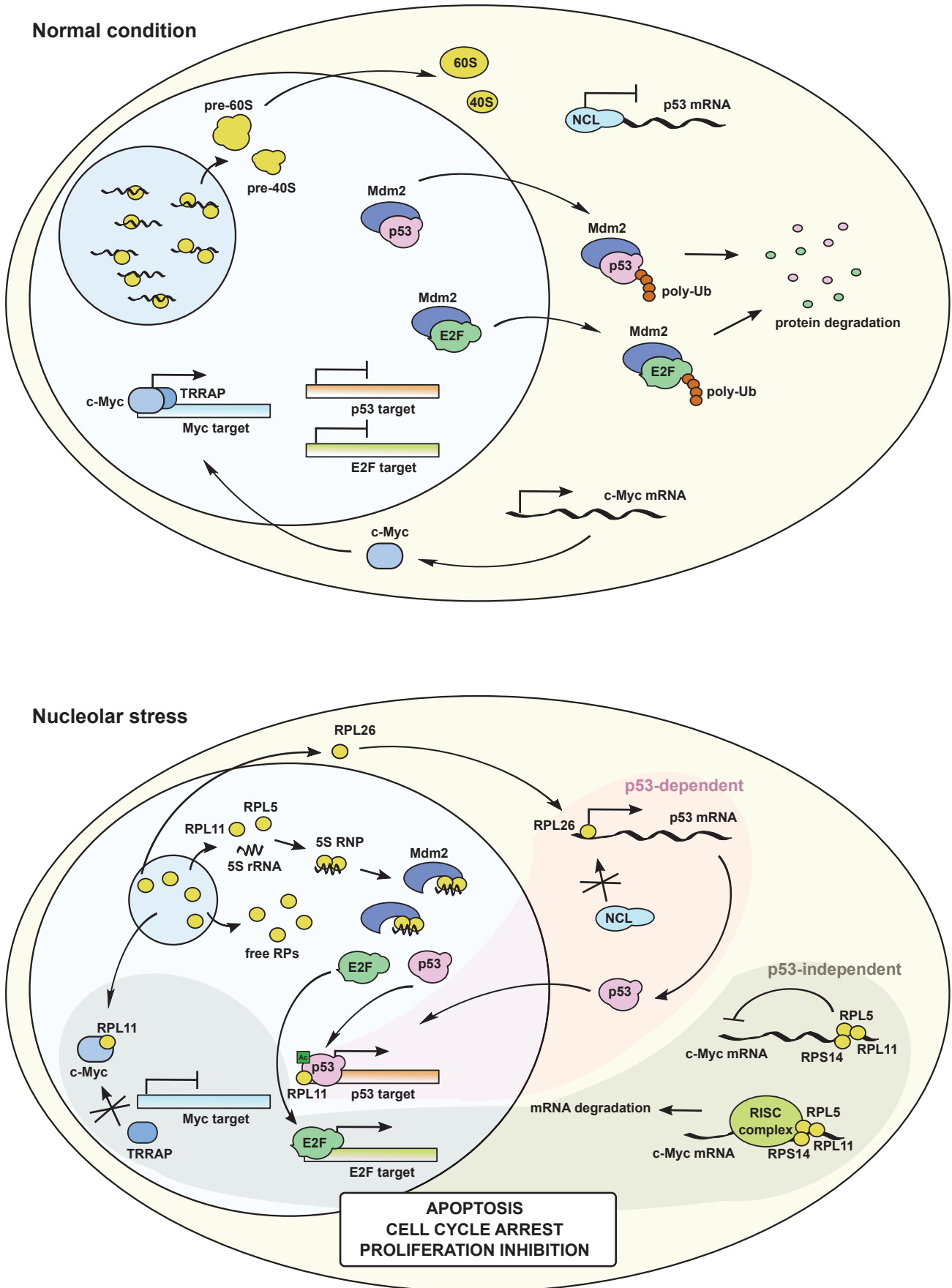
excessive mitogenic signalling, like c-Myc overexpression, representing a first line defence against hyperproliferation (Zindy et al., 1998). ARF plays multiple roles in the regulation of nucleolar stress and p53 stability (Maggi et al., 2014). Similarly, several proteins are positive or negative regulators of Mdm2-p53 interaction, either contributing to the maintenance of low p53 levels or inducing p53 stabilization, respectively (Holmberg Olausson et al., 2012; James et al., 2014; Wang and Jiang, 2012).

In summary, multiple ways exist to activate stress response through p53 stabilization. Nonetheless, it is worth noting that stress can elicit a cellular response in p53-independent manner as well, and this will be discussed in the next section. Below, only p53-dependent mechanisms contributing to nucleolar stress will be taken into account, both because of historical reasons and for sake of clarity. Indeed, p53-dependent mechanisms were discovered earlier. Furthermore, the diversity of p53-independent mechanisms is difficult to ascribe to a common pattern, in contrast to more mechanistically uniform manners of p53-dependent nucleolar stress, involving several factors that play similar roles.

Activation of p53 in response to aberrant nucleolar activity is mainly brought by proteins that disrupt Mdm2-p53 complex (Kurki et al., 2004; Sherr and Weber, 2000; Zhang et al., 2003). The general principle of nucleolar stress is well represented by the observation that in the absence of active rRNA transcription, the precise stoichiometry of ribosomal subunit assembly is perturbed, leaving specific ribosomal proteins free to accumulate in the nucleoplasm (Figure VIII) and interact with Mdm2, preventing p53 from degradation and activating its functions (Deisenroth and Zhang, 2010; Zhang and Lu, 2009; Zhou et al., 2012, 2015).

Early studies about nucleolar stress revealed that RPL11 and RPL5 play a prominent role in p53 stabilization following inhibition of ribosome biogenesis (Dai and Lu, 2004; Lohrum et al., 2003; Zhang et al., 2003). Then, numerous studies were published on ribosomal proteins reported to exert an effect on p53 stabilization, including RPL5, RPL6, RPL22, RPL23, RPL26, RPL37 from the large subunit and RPS3, RPS7, RPS14, RPS15, RPS20, RPS25, RPS26, RPS27, RPS27A RPS27L from the small subunit, and the list is likely to grow (Bursac et al., 2014; Cao et al., 2017; Deisenroth et al., 2016; Golomb et al., 2014; Liu et al., 2016; Nicolas et al., 2016; Zhang and Lu, 2009; Zhou et al., 2015). Nevertheless, given that RPs are highly basic and among the most abundant proteins, and thus can non-specifically interact with other proteins, studies reporting interaction of overexpressed RPs with the acidic domain of Mdm2 must be interpreted with caution.

**Figure VIII - Main mechanisms of nucleolar stress**



### **Figure VIII. Main mechanisms of nucleolar stress (previous page)**

Cartoon summarizing the main molecular events regulating p53 levels and cell proliferation in normal conditions and those triggered upon nucleolar stress, leading to cell proliferation inhibition, cell cycle arrest and apoptosis. In normal conditions, p53 protein abundance is mainly regulated by its stability: p53 protein does not accumulate in the nucleus, because Mdm2 activity interacts with it, mediates its translocation to the cytoplasm and targets it for poly-ubiquitination and proteasome-mediated degradation. In addition, p53 expression is translationally repressed by nucleolin (NCL). Similarly, Mdm2 negatively regulates E2F protein abundance. In this context, p53 target genes (including E2F and its own target genes) are not expressed. On the contrary, proliferation is sustained by c-Myc transcriptional activity, which requires interaction between c-Myc and TRRAP. When ribosome biogenesis is inhibited, ribosomal proteins (RPs) are imported into the nucleus but do not find ribosomal RNAs to interact with and assemble into pre-ribosome subunits. Therefore, RPs accumulate within the nucleoplasm and interact with non-ribosomal molecular partners. RPL5, RPL11 and 5S rRNA form the 5S RNP complex, that prevents Mdm2 interaction with p53 and E2F proteins resulting in their stabilization. In parallel, RPL26 displaces NCL from p53 mRNA thereby relieving translational repression and stimulating p53 protein expression. In this context, p53 and E2F target genes are expressed. This is further stimulated by RPL11, both acting as a transcriptional factor together with p53 on the promoter of the target genes and also stimulating p53 acetylation, which promotes p53 transcriptional activity. Thus, cell cycle arrest is activated and, possibly, cell death. RPL5, RPL11 and RPS14 form a complex for translational repression of c-Myc mRNA and for its RISC-mediated degradation, while RPL11 alone binds c-Myc preventing its interaction with TRRAP. Thus, the multiple Myc target genes are no longer expressed and cell proliferation is inhibited. See text for further details.

Indeed, RPL5 and RPL11 seem to have a key role. Contrary to knockdown of most ribosomal proteins, that can itself activate nucleolar stress due to disrupted ribosome biogenesis, this is not true for RPL5 or RPL11: when one of these proteins is abrogated, nucleolar stress is impaired and p53-mediated response is not properly elicited, suggesting that p53 activation upon nucleolar stress is predominantly mediated by RPL5 and RPL11 (Fumagalli et al., 2012; Teng et al., 2013). This hypothesis is strongly supported by the observation that depletion of any one of the ribosomal proteins able to regulate p53 levels together with depletion of either RPL5 or RPL11 has no effect on p53 levels indicating that p53 stabilization upon nucleolar stress is dependent on the presence of these two proteins (Nicolas et al., 2016). Furthermore, upon nucleolar stress only RPL5 and RPL11 accumulate in the non-ribosomal fraction, where they are found to specifically bind Mdm2, as demonstrated by co-immunoprecipitation (Bursać et al., 2012). The only other ribosomal protein reported to co-immunoprecipitate with Mdm2 is RPL26 (Zhang et al., 2010). So, despite ambiguous results regarding RPL26, it is reasonable to ascribe RPL5 and RPL11 a special role in nucleolar response. This speculation is even more reasonable after the discovery that RPL5 and RPL11 together with 5S rRNA form a trimeric ribosomal complex, named 5S RNP (see section about 5S rRNA synthesis, as well as Figure VIII), with this conformation making RPL5 and RPL11 able to escape the degradation machinery through mutual protection, accounting for their high stability relative to other ribosomal

proteins (Bursać et al., 2012), and the fact that when ribosome biogenesis is inhibited free trimeric 5S RNP accumulates in the nucleoplasm, before binding to Mdm2 with consequent upregulation of p53 levels (Sloan et al., 2013). The 5S RNP is a part of the 60S ribosome subunit and its integration into the subunit is an essential step of ribosome biogenesis. The positioning of the 5S RNP in ribosome structure may make it more prone to sense disruption of ribosome integrity (Klinge et al., 2011). The extraribosomal interaction between RPL5, 5S rRNA, p53 and Mdm2 was first reported in 1994 (Marechal et al., 1994), but this finding did not attract much interest until it was discovered that RPL11 and RPL5 had a role in p53 stabilization through Mdm2 binding (Dai and Lu, 2004; Lohrum et al., 2003; Zhang et al., 2003). Nowadays, it is established that each of the three components of the 5S RNP is necessary for the exerted inhibitory function and the activation of nucleolar stress (Bursać et al., 2012; Donati et al., 2013; Pelava et al., 2016; Sloan et al., 2013). Consistently with this, transient depletion of 5S rRNA by knockdown-mediated inactivation of TIF-III, required for its RNA PolIII-dependent transcription (see section), abrogated nucleolar stress and subsequent p53 activation (Donati et al., 2013; Sloan et al., 2013). This highlights 5S rRNA as an essential regulator of nucleolar stress and reinforces the idea that RPL5, RPL11 and 5S rRNA share a common function: in fact, neither RPL5, RPL11 nor 5S rRNA alone are able to suppress Mdm2, but these three components need each other for such function. The importance of 5S RNP complex as a key player of nucleolar stress has been convincingly supported by a mouse model: mice expressing a mutant Mdm2 unable to bind RPL5 and RPL11 retain normal response to DNA damage but display impaired p53 activation upon nucleolar stress (Macias et al., 2010; Zhou et al., 2012).

Several proteins including NPM1, PPAN, ARF and nucleostemin, can intervene through multiple ways, directly or indirectly, in the Mdm2-p53-RP axis, but this thesis will not be focused on all possible mechanisms. In order to give a flavour of the complexity at issue, only some examples will be mentioned. For instance, RPL26 regulates p53 levels simultaneously in at least two ways: by preventing its Mdm2-mediated degradation as previously described and by associating with p53 mRNA, thereby relieving nucleolin-mediated repression and enhancing its translation (Chen et al., 2012; Takagi et al., 2005). Moreover, RPL26 is also a target of Mdm2 activity for proteasome-mediated degradation (Ofir-Rosenfeld et al., 2008). Besides its role in p53 stabilization through interaction with Mdm2, RPL11, provided that it is neddylated, can also enhance p53 transcriptional activity (Figure VIII) by binding p53 target genes, typically *CDKN1A* (p21) and BAX, to recruit the

p300/CBP acetyltransferases and by promoting p53 acetylation (Mahata et al., 2012). In addition, the release of RPL11 from the nucleolus is regulated by PICT1, a nucleolar protein overexpressed in tumor, able to sequester it and prevent its interaction with Mdm2 in the nucleoplasm (Sasaki et al., 2011).

### **p53-independent nucleolar stress**

The ability of RPs to bind other proteins, mRNA molecules or DNA promoters thereby controlling protein stability and acting as transcriptional or translational regulators is not specific to p53 protein, p53 mRNA or p53 target genes, but it is a general behaviour and p53 is not the only factor involved. Indeed, nucleolar stress elicits a cellular response also in cells in which p53 is not functional. In recent years, emerging evidence has revealed the existence of additional mechanisms that are p53-independent and involve RPs and other multifunctional nucleolar proteins, such as ARF, NPM1, PPAN, nucleostemin, adding a further layer of complexity to the relationship between ribosome biogenesis and cell proliferation. The extremely diverse p53-independent mechanisms of nucleolar stress have been the matter of several extensive reviews and will not be described in detail for every known aspect (Donati et al., 2012; Holmberg Olausson et al., 2012; James et al., 2014; Russo and Russo, 2017; Yang et al., 2018).

One should consider that a number of regulatory events constantly take place in the cells, keeping the homeostasis of ribosome biogenesis. For instance, the oncoprotein c-Myc is a crucial transcription factor which controls the expression of numerous genes involved in cell growth and proliferation (Dang, 1999) and its activity is tightly controlled. Upon nucleolar stress, RPS14, RPL5 and RPL11 form a complex with c-Myc mRNA binding at its 3'UTR and act in a cooperative manner to repress c-Myc expression at the translational level, leading to downregulation of c-Myc target genes such as nucleolin and E2F2 (Dai et al., 2007; Zhou et al., 2013). In addition, RPL11 and RPS14 recruit miR-24 or miR-130a and miR-145, respectively, to c-Myc mRNA leading to its RISC-mediated degradation. RPL11 also directly binds to c-Myc protein repressing its transcriptional activity (Challagundla et al., 2011; Dai et al., 2007; Wang et al., 2015; Zhou et al., 2013). These interactions, summarized in Figure VIII, all result in a negative feedback loop working for stable cell proliferation independently of p53, that can block cell cycle and proliferation in case of impaired ribosome biogenesis.

Another p53-independent axis is the direct activation of genes that are normally targeted by p53 as a transcriptional factor. Bypassing p53-mediated regulation, this mechanism is able to promote cell cycle arrest or apoptosis regardless of p53 function. For

example, E2F-1 is a transcriptional factor among the target genes of p53, required for the S phase of the cell cycle and for apoptosis. E2F-1 positively regulates some crucial pro-apoptotic genes including p73, Apaf-1, PUMA, Noxa, Hrk/DP5, and Bim (Ginsberg, 2002; Stiewe and Pützer, 2000). E2F-1 protein stability is controlled by Mdm2 that binds and protects it from ubiquitin-dependent degradation (Zhang et al., 2005b) Upon nucleolar stress, RPL11 interacts with Mdm2, thus promoting E2F-1 release and subsequent degradation (Donati et al., 2011b). Unsurprisingly, low levels of E2F-1 expression are associated with inhibition of cell proliferation (Dimova and Dyson, 2005).

Evidence indicates that several ribosomal proteins are able to act as transcription factors or regulators of protein stability. For example, RPL3 possesses extra-ribosomal functions as a transcriptional regulator, controlling cell cycle progression, apoptosis in response to drug-induced nucleolar stress, and its own expression by a negative feedback mechanism (Cuccurese et al., 2005). Upon nucleolar stress, RPL3 targets p21 and CBS (cystathionine- $\beta$ -synthase) genes with opposite regulatory effect. RPL3 accumulates in the nucleoplasm and interacts with phosphorylated transcription factor Sp1 forming a protein complex that binds to the promoter of the cyclin-dependent kinase inhibitor p21 gene and activates it at the transcriptional level to induce G1 arrest and apoptosis (Pagliara et al., 2016). In parallel, RPL3 prolongs the half-life of p21 protein by controlling the interaction between p21 and its negative regulator MDM2 (Russo et al., 2013). The ability to activate p21 transcription also renders RPL3 a regulator of DNA repair upon nucleolar stress induced by DNA damage (Esposito et al., 2014; Russo et al., 2013, 2016). In addition, RPL3-Sp1 interaction is essential to displace Sp1 from the CBS gene promoter thus inhibiting CBS transcription (Pagliara et al., 2016; Russo et al., 2016) and RPL3 negatively affects CBS protein by targeting it for degradation thus leading to a mitochondria-dependent cascade and eventually resulting in caspase activation (Pagliara et al., 2016). To recapitulate, RPL3 controls p21 and CBS functions both at transcriptional and protein level, promoting the former and repressing the latter, with subsequent activation of apoptosis.

Like RPS14, RPL3, RPL5 and RPL11, other free ribosomal proteins released upon nucleolar stress may play similar multiple regulatory roles though other yet undiscovered mechanisms.

An increasing number of nucleolar proteins, known to relocalize upon nucleolar stress, appear to be involved in regulation of p53-independent response. For example, in conditions of nucleolar stress, NPM1 is induced and translocates from the nucleolus to the

cytoplasm where it complexes with Bax, a crucial effector of mitochondrial apoptosis, preventing Bax translocation to mitochondria, required for its function. Thus, NPM1-Bax interaction prevents apoptosis and favors cell cycle arrest in cell decision between the two processes (Lo et al., 2013)

NPM1 also interacts with ARF, already mentioned for its p53-dependent function (Korgaonkar et al., 2005). ARF enforced expression induces cell cycle arrest and/or triggers apoptosis in cells lacking functional p53. ARF binds and antagonizes the transcriptional function of c-Myc and E2F1 oncogenes required for cell cycle progression in absence of p53. In addition, both c-Myc and E2F-1 induce ARF expression. This is an example of p53-independent negative feedback mechanism. ARF negatively controls the cell growth independently of p53 also by activating ATM/ATR/CHK signalling pathways following exposure to genotoxic drugs (Eymin et al., 2006).

In conclusion, a surprisingly complex and rich network of nucleolar factors has emerged and is still emerging, that mediate nucleolar stress response and contributing to the cell's decision between cell cycle arrest or cell death.

### **Autophagy induced upon nucleolar stress**

Evidence has been collected which links ribosome biogenesis and, in general, the nucleolus, to autophagy. In a context of impaired ribosome biogenesis, eventually implying the presence of substantial amounts of non-functional ribosomes, autophagy could be a compensation mechanism to confront low levels of functional ribosomes and recycle ribosome pool (Pfister, 2019). It has already been demonstrated that mature ribosomes can be selectively degraded through autophagy upon nutrient starvation (Kraft et al., 2008), so it is not unreasonable to speculate that a similar process could occur in pathological contexts. For example, mutations resulting in impaired RPS19 expression affect autophagy in DBA patient cells and red blood cells of zebrafish embryos (Heijnen et al., 2014).

The concept that autophagy may be a consequence of nucleolar stress, as a resistance mechanism, paves the way to potential therapeutic strategies such as combination of treatments inducing nucleolar stress with inhibition of autophagy in order to increase selectivity and reduce toxicity at once (Pfister, 2019).

## **Chapter 2.**

# **Ribosome biogenesis and cancer**

So far, the process of ribosome biogenesis has been described as it appears in physiological context.

Indeed, situations have been mentioned where the function of ribosome biogenesis is disrupted, especially when describing experimental sets that interfere the normal behaviour of the cell in order to acquire information about its biology. Eventually, among the discussed processes, nucleolar stress is the one that moves farthest from a normal situation, but even so it is still part of a programme executed by a healthy cell in particular conditions and its implementation is not pathological, if carried out correctly.

A connection can be hypothesized between ribosome and cancer. Because cancer is characterized, by definition, by uncontrolled growth and hyperproliferation, and these features rely on improved rate of protein synthesis, likely implying improved an improved rate of ribosome production, it is tempting to speculate that the overactivation of ribosome biogenesis is a condition for tumorigenesis and cancer progression. Dysregulation of translational machinery as well as rRNA synthesis has been reported in many cancers, but it has not been ascertained that increased ribosome biogenesis is a general mechanism underlying all cancer biology. In other words, despite current data, dysregulation of ribosome biogenesis, at present, is not considered a hallmark of cancer.

Yet, a number of pathological situations exist, that trace a line of evidence between ribosome biogenesis and cancer onset, suggesting a causal link. In these diseases, genetic abnormalities or the constant activation of normal physiological responses leads to alterations in the process of ribosome biogenesis, entailing profound changes in the behaviour of the cells. The alteration may consist in either an impairment of ribosome biogenesis, like in a category of diseases termed ribosomopathies, or rather an overactivation of ribosome biogenesis, like in chronic inflammation. Intriguingly, although ribosomopathies and chronic inflammations are intrinsically different in the alterations they display with respect to ribosome biogenesis, they share a common feature, that is increased susceptibility to develop a cancer. In ribosomopathies, ribosome biogenesis is reduced; in chronic inflammation, ribosome biogenesis is upregulated; both alterations lead to higher cancer risk. This suggests that something in the correct regulation of ribosome biogenesis prevents cancer, whereas dysregulation promotes or contributes to cancer, and this link cannot be ignored, as discussed below.



# 1. Ribosomopathies

Ribosomopathies are a heterogeneous category of congenital disorders in which genetic abnormalities lead to defects in ribosome biogenesis and function. At the genetic level, ribosomopathies are characterized by defects in RPs, rRNA processing or ribosome assembly factors. At the level of the phenotype, a remarkable tissue-specificity exist. This means that despite the fact that every cell relies on ribosome function, some tissues, typically highly proliferating ones, appear to be affected more than others and in particular manners (Freed et al., 2010; Narla and Ebert, 2010). However, in some cases it remains unclear at which extent defects in ribosome biogenesis contribute to the phenotype of the disease and classification of all of these disorders as ribosomopathies may be inappropriate (Aspesi and Ellis, 2019). On the contrary, other diseases are collectively considered as bona fide ribosomopathies, because the analysis of mutational spectrum linked to their phenotype has identified multiple genes that are tied together mechanistically, giving confidence that specific yet distinct defects in ribosome biogenesis play a role in disease occurrence. For example, Diamond-Blackfan anaemia (DBA) can be caused by any mutation among RPS7, RPS10, RPS15A, RPS17, RPS19, RPS24, RPS26, RPS27, RPS28, RPS29, RPL5, RPL11, RPL15, RPL18, RPL26, RPL27, RPL31, RPL35, RPL35A or TSR2 (Aspesi and Ellis, 2019; Bohnsack and Bohnsack, 2019), with TSR2 encoding a direct binding partner of RPS26 (Gripp et al., 2014). Experimental depletion of all ribosomal proteins mutated in DBA always impaired ribosome biogenesis while affecting either subunit and disturbing rRNA processing at different levels, indicating that the rate of ribosome production rather than a specific step in ribosome biogenesis is critical in patients with DBA (Robledo et al., 2008). Thus, the disease phenotype of DBA is due to impaired ribosome biogenesis and DBA is undoubtedly considered a ribosomopathy.

In dyskeratosis congenita (DC), it is now clear that telomerase is the primary affected activity responsible for disease pathology, but ribosome function is also dysregulated and contributes to the most severe X-linked forms of the disease because of a mutation on DKC1 gene coding for dyskerin protein, which is involved in rRNA modification (Dokal, 2011; Mason and Bessler, 2011). Therefore, DC is considered a ribosomopathy in its X-linked forms (X-DC).

## **Congenital ribosomopathies**

The best characterized ribosomopathies include DBA, X-DC, Schwachmann-Diamond syndrome (SDS), cartilage hair hypoplasia (CHH), Treacher Collins syndrome (TCS) and

5q minus syndrome (Freed et al., 2010; Nakhoul et al., 2014; Narla and Ebert, 2010). In addition, a number of diseases have been included among the suspected ribosomopathies, like alopecia and neurological and endocrinopathy syndrome, aplasia cutis congenita, Bowen-Conradi syndrome, congenital asplenia, leukoencephalopathy, but these diseases will not be discussed here (Aspesi and Ellis, 2019).

RP mutations have been identified in about 50% of DBA patients, with loss-of-function mutations in RPS19, RPL5, RPL11 and RPS10 being the most recurrent (Aspesi and Ellis, 2019). Inactivating mutations of SBDS gene have been identified in 90% of SDS patients. The protein encoded by this gene is required during cytoplasmic maturation of pre-60S subunit for the release of EIF6 factor. In SDS patients, EIF6 is not released resulting in accumulation of stalled pre-60S ribosomes (Finch et al., 2011; Woloszynek et al., 2004). CHH is caused by mutations in a single gene, RMRP, involved in the cleavage of pre-rRNA at the ITS1 (Goldfarb and Cech, 2017; Thiel et al., 2005). DKC1 mutations have been identified in 25% of DC patients (Mason and Bessler, 2011). The 5q minus

syndrome is a subtype of myelodysplastic syndrome (MDS) with dysplastic cells in the bone marrow harboring deletions on the long arm of chromosome 5, that contains the RPS14 gene (Ebert, 2011).

Phenotypic traits associated to ribosomopathies are diverse: DBA symptoms include anemia, macrocytosis, reticulocytopenia, selective decrease or absence of erythroid precursors in bone marrow, frequently thumb abnormalities, cardiac defects and development of blood or solid tumors (Engidaye et al., 2019); patients affected by 5q minus syndrome display, in addition to general MDS symptoms, severe macrocytic anemia, frequently elevated platelets with hypolobulated micromegakaryocytes and a relatively low rate of progression to acute myeloid leukemia (AML) compared with other types of MDS (McManus, 2005); SDS patients are characterized by exocrinepancreatic insufficiency, ineffective hematopoiesis commonly resulting in neutropenia (with consequent susceptibility to develop serious infections), an increased risk of leukemia, and frequently display anemia, thrombocytopenia, short stature, and skeletal abnormalities (Ganapathi and Shimamura, 2008); X-DC is characterized by mucocutaneous abnormalities like skin hyperpigmentation, oral leukoplakia, and nail dystrophy, as well as bone marrow failure and high risk of developing leukemia, solid tumors, and pulmonary fibrosis (Dokal, 2011); CHH is a form of short-limbed dwarfism resulting from skeletal dysplasia, also characterized by hypoplastic hair, immune dysfunction, and an increased predisposition to malignancies, especially non-Hodgkin lymphoma and basal cell

carcinoma (Ganapathi and Shimamura, 2008; Mäkitie et al., 1995; Mckusick et al., 1965); TCS is associated with characteristic craniofacial developmental defects (Aljerian and Gilardino, 2019).

Thus, each ribosomopathy is associated with a number of specific features consisting in remarkably tissue-specific abnormalities, described as cellular hypoproliferative defects. Potential explanations for tissue-specific phenotypes despite the critical importance of ribosome biogenesis and function in all living cells have been provided by recent reviews (Gerst, 2018; Mills and Green, 2017; Sulima and De Keersmaecker, 2018).

Only recently, after the introduction of therapies able to mitigate the previously lethal effects of ribosomopathies and improve life-span, a peculiar aspect of ribosomopathies turned out, that initially has been regarded as a paradox: early in life, affected patients present hypoproliferative defects; later in life, patients have an elevated risk of developing hyperproliferative phenotypes and subsequently cancer. Ribosomopathy patients have a 2.5- to 8.5-fold higher cancer risk and for some cancer types the risk can be up to 200-fold higher (De Keersmaecker et al., 2015).

TCS is the only ribosomopathy without bone marrow failure or particular association with cancer onset. So, from the above list, two symptoms emerge as typical of ribosomopathies: hematopoietic dysfunction and increased incidence of cancer. The former aspect has been described in a recent review (Sulima and De Keersmaecker, 2018). The latter has been reviewed (Penzo et al., 2019; Sulima et al., 2019a) and will also be discussed here.

## **Somatic ribosomopathies**

In addition to inherited ribosomopathies, further evidence raises the question about the relationship between ribosome defects and cancer: somatic ribosomopathies are found in cancer. Genomic analyses on cancer patient samples and cell lines found that RP deletions are displayed in almost a half of cases. Most of the identified deletions are heterozygous, as homozygous loss is lethal with rare exceptions, such as the non-essential RPL22. Hence, RP haploinsufficiency is connected with elevated cancer incidence (Ajore et al., 2017; Guimaraes and Zavolan, 2016; Shenoy et al., 2012; Sulima and Keersmaecker, 2017; Vlachos, 2017).

Experiments in animal models, especially zebrafish and transgenic mouse, support the idea that heterozygous loss of RP genes has an oncogenic role (Amsterdam et al., 2004; Morgado-Palacin et al., 2015; Rao et al., 2016). A tumor suppressor role for RPL5 is supported by the fact that its downregulation accelerates tumor formation in xenografted

breast cancer cell lines (Fancello et al., 2017). A differential response upon genetic perturbation of ribosome biogenesis has been observed in human cells: loss of RPL5 inhibits cell proliferation in normal cells. This suggests that RPL5 has a tumor suppressor role only in particular genetic background, like the one found in breast cancer (Teng et al., 2013).

Similarly to cancer-specific mutations in genes encoding ribosomal proteins, somatic rDNA gene copy number losses have recently been described in a variety of tumor types in which pathways stimulating ribosome biogenesis, like mTOR, are upregulated. Remarkably, tumor cells with rDNA copy loss are more sensitive to DNA damaging agents and RNA Pol I inhibitors (Udugama et al., 2018; Wang and Lemos, 2017; Xu et al., 2017a).

### **Nucleolar stress in ribosomopathies**

An extremely large number of extra-ribosomal functions have been reported for several ribosomal proteins, including regulation of cell cycle, apoptosis, cell migration and invasion, and importantly maintenance of genomic integrity (Xu et al., 2016; Zhou et al., 2015). Ribosome defects typically result in freely available RPs, some of which are involved in p53 stabilization through the process of nucleolar induction. Above in this thesis, the role of several ribosomal proteins, especially RPL5 and RPL11, in the nucleolar stress response has been described in detail.

A role for nucleolar stress in physiopathology of ribosomopathies has been proposed. In particular, the case of DBA has been characterized at a molecular level (Ellis, 2014; Fumagalli and Thomas, 2011). Because erythroid precursors from DBA patients have a pro-apoptotic phenotype associated with elevated p53 levels (Perdahl et al., 1994) and such phenotype could be reversed by reduced levels of p53 (Danilova et al., 2008; Dutt et al., 2011; Fumagalli et al., 2009; Jaako et al., 2011; McGowan et al., 2011), it is likely that p53 activation plays a critical role in determining the pathological phenotype. Besides, lenalidomide, a common drug used to treat both DBA and 5q-syndrome, was shown to inhibit p53 function (Wei et al., 2013). Indeed, because of the reduced amount of specific ribosomal proteins in DBA, p53 may be induced through nucleolar stress caused by impaired biogenesis. Tissue-specificity would be due to differentially high sensitivity to reactive oxygen species ROS levels in erythroid lineage, lowering the threshold for induction of nucleolar stress and subsequent apoptosis, resulting in tissue hypoplasia (Ellis, 2014). However, DBA patients carrying mutations in RPL5 and RPL11, key players of nucleolar stress, have been reported. Indeed, these patients tend to have more severe developmental defects (Gazda et al., 2008). This finding suggests that other events besides

accumulation of RPL5 and RPL11 are capable of triggering p53 activation in response to RP mutations. On the other hand, RPL5 and RPL11 mutations may explain the higher cancer incidence in these patients, as this loss-of-function might predispose to cancer due to inability to activate p53 tumor suppressor by RPL5- and RPL11-dependent nucleolar stress (Molavi et al., 2019). For potential RPL5- and RPL11-independent mechanisms of nucleolar stress, see the section about ribosome as a sensor of stress. In any case, it is clear that the unscheduled activation of p53 is responsible for perturbations in tissue homeostasis that cause the development of ribosomopathies.

### **Oncogenic mechanisms in ribosomopathies**

Apart from the induction of nucleolar stress, defects in ribosome biogenesis and composition could impact in different other ways: through the production of altered ribosomes that display differential translational capacities, or through dysregulation of translation-independent extra-ribosomal functions of ribosomal proteins, impacting cellular functions that have been directly or indirectly linked to ribosome, such as proteasome-mediated degradation, redox homeostasis, glycolysis and serine/glycine metabolism (Kampen et al., 2019a; Mills and Green, 2017; Narla and Ebert, 2010; Sulima et al., 2019b). All of these processes may be oncogenic. Here, some examples will be given.

One possible oncogenic mechanism deriving from defects in ribosome biogenesis or function is specific alteration of mRNA of translation. As described in a previous section, the absence of a specific ribosomal protein from the ribosome may result in altered properties, such as decreased translational fidelity or preferential translation of specific subsets of genes. The potential connection between differential ribosome composition and cancer has been extensively reviewed (Bastide and David, 2018; Bustelo and Dosil, 2018; Penzo et al., 2016; Sulima et al., 2017), as well as the connection between ribosome composition and tissue-specific phenotypes in ribosomopathies (Farley and Baserga, 2016). For instance, in chronic lymphocytic leukemia, three recurrent mutations of the RPS15 gene impact specific aspects of translation, promoting near-cognate amino-acid misincorporation or stop-codon read-through (Bretones et al., 2018); in T-ALL, the same phenotype can be promoted by a specific mutation of RPL10 (Sulima et al., 2014). In addition, the same mutation is associated with constitutive IRES-mediated overexpression of the anti-apoptotic factor BCL2 in leukemia cells (Kampen et al., 2019b). IRES-mediated changes in translation are not specific to contexts with mutated RPL10. It has been reported that DBA-associated mutations of RPS19 and RPL11 reduce IRES-mediated translation of specific genes involved in differentiation, in both mouse models and patient

samples (Horos et al., 2012), although other mechanisms may operate (see below). Similarly, DKC1 mutations in X-DC and altered FBL expression in cancer, resulting in ribosomes containing differentially pseudo-uridylated or 2'-O-methylated rRNAs, respectively, are linked to altered translation of IRES-containing mRNAs, including those encoding for tumor suppressors p53 and CDKN1B, anti-apoptotic factors BCL2L1 and XIAP, angiogenic growth factor VEGF or IGF-1R, and also impair translational accuracy, with reduced fidelity and increased frequency of ribosomal frameshift (Bellodi et al., 2010; Jack et al., 2011; Marcel et al., 2013; Rocchi et al., 2013; Sharma et al., 2017; Yoon et al., 2006). In SBDS, specific mutations affect ribosome capacity of translating C/EBP mRNA, leading to impaired hematopoiesis and increasing 202-fold the risk to develop AML (In et al., 2016).

All this information depicts a model in which defects in ribosome biogenesis and subsequently in ribosome function produce potentially broad changes in translational activity.

An additional mechanism could rely on non-ribosomal functions of ribosomal proteins. For example, several proteins are negative regulators of c-MYC expression, establishing a feedback regulation loop (Molavi et al., 2019). RPL11 binds the promoter region of c-MYC target genes to repress transcription (Dai et al., 2007); RPL5 and RPL11 can bind c-MYC mRNA and promote its RISC-mediated degradation (Challagundla et al., 2011; Liao et al., 2014); a similar feedback loop has been described for RPS14 (Zhou et al., 2013). Being that deregulation of c-MYC is a critical event in oncogenesis, disruption of feedback regulation through ribosomal proteins could contribute to this process in ribosomopathies.

## **From hypoproliferation to hyperproliferation**

In conclusion, much evidence has been accumulated about potential mechanisms promoting cancer in ribosomopathies. The matter is to explain the paradoxical progression from hypoproliferative disease phenotypes, obviously produced by impaired ribosome functions, toward hyperproliferative phenotypes, that come later in life of patients. A model was proposed that states that causes of both hypoproliferative and hyperproliferative phenotypes coexist since the beginning of the disease, but the hyperproliferative phenotype takes advantage in the long term because of accumulation of damages that the cells are not anymore able to counter. At the beginning of the disease, ribosomal lesions do entail oncogenic mechanisms but also defective growth and extensive oxidative stress that is toxic and impairs cell growth and proliferation. Thus, despite

existing tumor-promoting changes, their effect is overcome by impaired proliferation. As time passes, oxidative stress leads to DNA damage and eventually secondary mutations that can alleviate toxicity associated with defective ribosome functions, that would undergo positive selective pressure, allowing a transition from a hypoproliferative into a hyperproliferative state (Kampen et al., 2019a).

A slightly different formulation of the same principle is the one proposed in a recent review, stating that the unbalanced production of ribosome components, typical of ribosomopathies, induces a constant activation of nucleolar stress leading to p53 stabilization and generalized propensity for p53-mediated cell cycle arrest. This would exert selective pressure for p53 loss of function and subsequently allow for cell survival and rescue cell proliferation (Penzo et al., 2019). This model, being still based on selective pressure in particular genetic context, may be regarded as a special case of the aforementioned one.

For this model to hold true, proliferating tissues observed in most ribosomopathies should display high levels of p53 due to its stabilization induced by nucleolar stress and cancers associated with ribosomopathies should preferentially accumulate p53 loss-of-function mutations. As mentioned above, high levels of p53 protein are found in cells from DBA patients (Dutt et al., 2011). As demonstrated by immunostaining, this is true also for SDS (Elghetany and Alter, 2002), DC (Fok et al., 2017), 5q minus syndrome (Dutt et al., 2011) and TCS (but one must keep in mind that TCS is not associated to higher cancer onset) (Jones et al., 2008; Kadakia et al., 2014). With regard to the genetic profile of cancers, a study considering over 10,000 patient cancer samples and cell lines correlated haploinsufficiency for ribosomal protein genes with loss-of-function mutations in TP53 (Ajore et al., 2017); furthermore, 5q minus syndrome is specifically associated with TP53 negative leukemia (Jädersten et al., 2011; Saft et al., 2014; Scharenberg et al., 2017). Finally, haematopoietic stem cell populations in patients with SDS revealed clonal haematopoiesis with an associated high level of p53 mutations. These cells are not cancer cells but are highly proliferating and accumulation of p53 mutations is in line with a model of selective pressure for p53 loss-of-function (Xia et al., 2018). This tendency was also confirmed in animal models of tumorigenesis: mouse models of hepatocellular carcinoma and hepatoblastoma have revealed features that mimic ribosomopathies, including deregulation of RP expression and accumulation of unprocessed rRNA precursors. In this context, even though p53 is stabilized, compensating mechanisms prevent cell death and promote survival, like the up-regulation of the pro-survival protein Bcl-2 or the

cytoplasmic sequestration of p53 protein, that may cooperate to drive tumor growth and survival (Kulkarni et al., 2017). This indicates that cancer cells may become insensitive to p53 activation, in line with the idea that inactivation of nucleolar stress response is a critical step in cancer progression.

In conclusion, all these findings are consistent with the idea that in ribosomopathies a selective pressure is established, leading to selection of mutations that enable the cell to bypass the growth limitation imposed by p53-dependent nucleolar stress and, ultimately, promoting cancer.

## **2. Chronic inflammation**

A further indication that dysregulated ribosome biogenesis is oncogenic comes from another heterogeneous category of diseases, having as common feature the constant activation of the inflammatory response. Indeed, it has been established that a close relationship exists between inflammation and the development of many types of human cancers (Coussens and Werb, 2002; Mantovani et al., 2008). It is well established that a number of factors released during the inflammatory response are related to tumorigenesis. In particular, a major role in tumorigenesis is played by interleukin 6 (IL-6) (Grivennikov et al., 2009; Park et al., 2010). So, like ribosomopathies, inflammation processes are associated with a higher propensity to develop cancer.

Here, the case of chronic inflammation will be reported. One of the most widespread and clinically relevant examples of tissue lesion associated with an increased risk of cancer is chronic liver inflammation, responsible for eighty percent of all hepatocellular carcinoma reported worldwide (Libbrecht et al., 2005). Interestingly, as recently brought to attention (Derenzini et al., 2017; Penzo et al., 2019), this category of pathologies is also characterized by dysregulated nucleolar structure. This has been observed in epithelial cells of colon mucosa with chronic ulcerative disease (Brighenti et al., 2014), in pancreatic acinar cells from patients with chronic pancreatitis (Macchini, 2016) and in hepatocytes of livers with chronic inflammatory diseases driven by hepatitis B virus (HBV) viral infection (Trerè et al., 2003). Of note, HBV has been shown to upregulate both RNA Pol I and III activity (Aufiero and Schneider, 1990; Wang et al., 1998) and this results in enlarged nucleoli independently from proliferative activity of the cells (Lee et al., 1997). All these conditions, which share the chronic nature of inflammatory response, are characterized by both elevated nucleolar activity and an increased propensity to develop cancer, and in the case of HBV-driven chronic inflammation the extent of nucleolar hypertrophy is even a



predictive parameter for cancer risk, supporting a model in which enhanced nucleolar activity is a driver of tumorigenesis.

A series of epidemiologic observations and population-based studies furtherly supported a potential connection between inflammatory conditions and cancer onset. There is evidence that a regular use of the non-steroidal anti-inflammatory drug aspirin (acetylsalicylic acid) is associated with a reduced longterm risk for overall cancers (Thun et al., 2012). Statistically, this result is mainly due to a lower incidence of the gastrointestinal tract cancers, especially colorectal cancers (Cao and Chan, 2016). Because the link between inflammatory response and tumorigenesis is well known and accepted, this finding was not strikingly surprising. However, for the purposes of this section (and, it could be said, all of this thesis) an element is of particular interest: the cancer relieving effect of aspirin has actually been shown to be accompanied by downregulation of nucleolar functions in cancer and non-cancer cell lines, and in mouse liver *in vivo* (Brighenti et al., 2016). In particular, at the mechanistic level, aspirin is able to counteract the pro-tumorigenic effects of the inflammatory cytokine IL-6 by preventing IL-6-mediated downregulation of p53 expression. This effect is obtained through reduction of c-Myc mRNA transcription, which is notoriously a major regulator of ribosome biogenesis process, although in this case the regulatory pathway is not the standard one (it relies on down-regulation of RPS6 expression which, in turn, slows down the rRNA maturation process, thus reducing the ribosome biogenesis rate) (Brighenti et al., 2016). On the contrary, when IL-6 is not countered by aspirin, rRNA transcription is increased, leading to downregulation of p53 protein expression. Such decrease endows the cells with phenotypical changes characteristic of epithelial-mesenchymal transition, resulting in increased cell invasiveness, decreased response to cytotoxic stress and, ultimately, the acquisition of cancer features (Brighenti et al., 2014; Trerè et al., 2003).

Together, all these findings suggest that nucleolar morphology, hence nucleolar functions, may have a direct role in tumorigenesis. Certainly, there is a correlation between nucleolar features and cancer pathology: this has been known for a very long time and has been commonly used by pathologists for clinical evaluation of sample tissues in order to have an indication of the proliferative properties of the sample. Nevertheless, whether the relationship between the two is of causal nature is not clearly established yet. Indeed, it is reasonable to address the question and explore the topic more in detail, as it will be attempted in next section.

### 3. Cancer

Cancer is a generic term to designate hundreds of diseases sharing hallmark traits progressively acquired throughout tumor development, including in particular extensive and uncontrolled cell proliferation.

Changes in ribosome biogenesis rate correlate with the development of cancer and a similar correlation has been reported between the deregulation of genes involved in ribosome biogenesis and cancer, suggesting that overexpression of rRNA could be an initiating step in tumorigenesis.

This is not surprising, because ribosome biogenesis and cell growth and proliferation are coordinated processes, mostly regulated in tight association: consequently, the disruption of one or more of the steps that control ribosome biogenesis has been associated with alterations of cell growth.

It is not difficult to explain these observations: in proliferating cells, the shorter the cell cycle, the greater the ribosome biogenesis rate. It is clear that such concurrence is not a coincidence: fast-dividing malignant cells require enhanced ribosome production to meet the increased demand for protein synthesis to sustain their elevated metabolism and proliferation. This intuitive consideration about correlation between ribosome biogenesis rate and cell proliferation is not only sustained by experimental observation, but are also corroborated by other characteristics of cancer cells including the altered expression of oncogenes and tumor suppressors.

In the last decade, new and unexpected roles have emerged that place deregulated ribosome biogenesis and protein synthesis at the core of cancer etiology (Bustelo and Dosil, 2018; Bywater et al., 2013; Derenzini et al., 2017; Montanaro et al., 2008; Pelletier et al., 2018; Ruggero, 2012a).

#### **Nucleolar morphology and function in cancer**

The most direct way to evaluate the ribosome biogenesis rate in proliferating cells is nucleolar morphology, the latter reflecting the rate of the former. For a long time, since the observation that nucleolar morphology is associated to tumor, an increased nucleolar size was considered a hallmark of cancer cells. Despite the debate about the reliability of nucleolar size as a diagnostic factor is still currently ongoing, the link between nucleolar functions and morphology, including size, and tumor pathology has been extensively studied and general correlation between particular nucleolar features and increased cell proliferation is widely accepted concept. In particular, two features are of outstanding

relevance: number and size, defining the degree of nucleolar hypertrophy. The area occupied by nucleoli in proliferating cells is directly related to rRNA transcription activity and correlates with the rapidity of cell proliferation in cancer tissues (Derenzini et al., 1998, 2000; Trerè et al., 1991). In aggressive human breast cancer cell lines, the average number of FC/DFC modules increases from four to six and overall rRNA production by 20% (Beghin et al., 2009; Belin et al., 2009). In addition, the presence of very enlarged nucleoli in cancer tissues is frequently associated with a poor survival (Trerè et al., 2004), endowing nucleolar hypertrophy with an independent prognostic and predictive value in several cancer types (Derenzini et al., 2009).

Of note, the perinucleolar compartment is another nucleolar feature that has been linked to cancer. The presence of the perinucleolar compartment has been correlated with the metastatic potential of several tumor-derived cell lines and is independent prognostic value for breast cancer (Kamath et al., 2005; Norton et al., 2008).

So cancer cells, characterized by increased cell proliferation, display increased nucleolar size, reflecting increased rate of ribosome biogenesis. In line with this, increased Pol I and Pol III activity is a signature of cancer (Ruggero and Pandolfi, 2003; White, 2008). From a clinical perspective, high rate of ribosome biogenesis has been found to have prognostic value in some cancers: for example, overexpression of pre-45S is strongly associated with poor prognosis in alveolar rhabdomyosarcoma (Williamson et al., 2006), colorectal cancer (Tsoi et al., 2017) and several studies have reported a correlation between alterations in the RP expression pattern and a poor prognosis (Bee et al., 2006; Guimaraes and Zavolan, 2016; Huang et al., 2006; Kobayashi et al., 2006).

Consistently with a role of ribosome biogenesis, several components required for rRNA transcription, processing and maturation are dysregulated in cancer. For example, UBF is overexpressed in hepatocellular carcinoma (Huang et al., 2002). The expression of snoRNA genes is altered in a growing list of cancers including brain, breast, cervical, liver, lung and prostate cancers, contributing to cell transformation, tumorigenesis and metastasis (Langhendries et al., 2016; Su et al., 2014; Thorenoor and Slaby, 2015; Williams and Farzaneh, 2012) and, therefore, snoRNAs are promising circulating biomarkers (Mannoor et al., 2012).

Similarly, fibrillarin is overexpressed in primary and metastatic breast cancer, prostate cancer and squamous cell cervical carcinoma (Choi et al., 2007; Koh et al., 2011; Marcel et al., 2013; Su et al., 2014) and high level of fibrillarin is also generally associated with poor survival in primary breast tumors (data from our group).

It has been emerging in recent years that the change in ribosome biogenesis in cancer is not only quantitative, with a higher amount of ribosomes produced, but also qualitative, with unexpected pre-rRNA intermediates accumulated in the cells, reflecting alternative pre-rRNA processing pathways taking place, as well as rRNA molecules that are differentially modified leading to altered translational capacities, but how changes in pre-rRNA intermediate dynamics could impact ribosome production and function remains hypothetical (Beghin et al., 2009; Belin et al., 2009; Marcel et al., 2013).

In particular, fibrillarin, involved in 40S ribosome subunit maturation and rRNA post-transcriptional modification through its methyltransferase activity, is overexpressed in breast cancer because of the loss of p53 expression. As a result of fibrillarin overexpression, rRNA 2'-O-methylation is elevated (Marcel et al., 2013). So, reciprocally, depletion of fibrillarin has been proposed to cause an increase in p53 expression levels and this response was suggested to not only be mediated by the nucleolar stress but also by an increase in the cap-independent translation of the p53 mRNA (Marcel et al., 2013; Su et al., 2014). Changes in the rRNA 2'-O-methylation pattern have also been linked to cancer and differences in the extent of rRNA modification at several sites have been observed in different cancer cell lines (Krogh et al., 2016). The precise biological role of these modifications in tumorigenesis, as well as other modulation of ribosome composition, is currently investigated by an increasing number of studies suggesting that ribosomes in cancer cells could be qualitatively different from those in normal cells, with intrinsically different translational activity (Kampen et al., 2018; Marcel et al., 2015; Penzo et al., 2019; Stumpf and Ruggero, 2011; Sulima et al., 2017).

All together, these studies indicate that ribosome biogenesis is altered at multiple levels in cancer.

### **Mechanisms underlying upregulation of ribosome biogenesis in cancer**

In a previous section, a flavour of the diversity of mechanisms involved in the regulation of ribosome biogenesis was given, with particular attention for the major pathways that, importantly, happen also to control cell growth and proliferation (Drygin et al., 2010; Sulić et al., 2005). As a result of the disruption of these regulation mechanisms, for example the activation of downstream effectors independently from extracellular signalling events, cancer cells acquire self-sufficiency and unlink ribosome biogenesis rate from nutritional and metabolic state of the cell. Indeed, it is becoming more and more evident that ribosome biogenesis is generally increased in cancer cells due to the perturbation of pathways responsible for cell proliferation. Dysregulation of ribosome

biogenesis has been found in a growing list of cancer types and each type of cancer activates ribosome biogenesis through distinct processes, with the direct modulation of Pol I activity being the most common. It is noteworthy that the most frequently amplified oncogene (MYC), the gene with the highest rate of mutation (TP53), the most often deleted tumor suppressor gene (RB) and the pathway most often upregulated (PTEN/PI3K/AKT/mTOR) in cancers all directly impact RNA Pol I transcription. Most molecular mechanisms have already been described in detail above in this thesis and will not be recalled unless specifically needed.

Ribosome biogenesis was shown to be positively regulated by oncogenes and negatively regulated by tumor suppressors. In several cancers, loss of function of tumor suppressors pRb and p53 was shown to lead to an increase in RNA Pol I transcription mainly due to derepression at the level of rDNA promoter, where both p53 and pRb interact with UBF and SL1 (Budde and Grummt, 1999; Hannan et al., 2000; Trerè et al., 2004; Zhai and Comai, 2000). Similarly, tumor suppressor ARF was shown to inhibit rRNA synthesis at the level of UBF binding, of transcription termination through interaction with TTF-1, and indirectly by contributing to p53 stabilization. Therefore, loss of ARF expression may enhance ribosome biogenesis in multiple ways (Ayrault et al., 2006; Lessard et al., 2010; Maggi et al., 2014). Finally, as already mentioned, functional PTEN represses RNA Pol I transcription by disrupting SL1 complex, so deletion or mutation of PTEN in cancer could stimulate rRNA transcription (Zhang et al., 2005a).

A number of signalling pathways typically activated in response to mitogens and growth factors, but constantly activated in cancer cells, including the PI3K/AKT pathway, converge to upregulate the MYC gene function (Zhu et al., 2008). MYC is frequently deregulated in cancer, and overexpression of MYC oncogene and increased ribosome biogenesis have been reported in several solid tumors and hematological malignancies (Gomez-Roman et al., 2006; van Riggelen et al., 2010; White, 2005). Because MYC regulates all the steps of ribosome biogenesis at multiple levels, the generally increased ribosome biogenesis rate in cancer is mainly a consequence of the overexpression of MYC (Mayer and Grummt, 2006). In a model of MYC-driven cancer, both the mRNA and protein abundance of UBF, TIF-1A and POLR1B, the second largest subunit of Pol I, were all highly elevated in premalignant cells compared to normal cells (Bywater et al., 2012).

Similarly, mitogenic and growth factor signalling activates mTOR, another key modulator of ribosome biogenesis (Mayer and Grummt, 2006). Cancer cells are frequently characterized by dysregulation of MYC and/or mTOR functions and are consistently

associated with dysregulation of ribosome biogenesis and increase in protein synthesis (Hsieh and Dang, 2016; Hsieh et al., 2012).

Finally, the overactivation of RNA Pol I activity in cancer can be due to epigenetic changes that increase the proportion of transcriptionally active rDNA genes, like in prostate and cervical cancer (Uemura et al., 2012; Zhou et al., 2016), or to genomic alterations such as rDNA copy number amplification and loss, linked to tumor genetic context, nucleolus activity, and proliferation (Derenzini et al., 2009; Wang and Lemos, 2017).

### **Ribosome biogenesis as a driver of tumorigenesis**

Current evidence has been outlined about the association between ribosome biogenesis, consequent nucleolar morphology, and cancer phenotype. Although elevated ribosome activity might simply correlate with increased protein synthesis resulting from dysregulated proliferation of cancer cells, recent studies rather suggest that increased RNA Pol I-dependent transcription is a critical step in tumorigenesis (Drygin et al., 2014).

Several reports have proposed an active role for ribosome biogenesis and evidence has been accumulated that upregulation of ribosome biogenesis may be responsible for tumorigenesis, thus being not only required but even sufficient to drive malignant transformation. As mentioned in a previous section, strong indications come from nonmalignant lesions associated with increased ribosome biogenesis, in which higher cancer risk is observed and such risk is correlated to the extent of the increase in ribosome biogenesis. Here, some further examples will be given of evidence or strong indication that tumorigenesis is dependent on the overactivation of ribosome biogenesis.

The first evidence dates back to 2008. In a mouse model of MYC-driven cancer, E $\mu$ -MYC transgenic mice, the overexpression of MYC oncogene leads to B lymphoma and is associated with enhanced ribosome biogenesis. When this genetic background is perturbed by crossing these mice with heterozygous RPL24<sup>+/-</sup> or RPL38<sup>+/-</sup> mice, not only ribosome biogenesis was restored to normal levels but also MYC-driven oncogenesis is suppressed, significantly delaying the onset of B lymphoma. This indicates that the oncogenic potential of MYC is due to its effect on ribosome biogenesis (Barna et al., 2008).

Another indication comes from an experiment, in which the depletion of only TIP5, a negative epigenetic regulator of rDNA, not only enhanced ribosome biogenesis but also induced transformation of NIH3T3 cells (Guettg et al., 2010). In a very similar manner, functional inactivation of MTG16a protein, a negative regulator of rDNA transcription that can counter its MYC-driven activation, not only induces an increase in transcription in

human breast epithelial cells but also endows cells with morphological features typical of breast cancer cells (Rossetti et al., 2010). However, this finding must be taken with caution: although TIP5 depletion or MTG16a inactivation lead to a cancer phenotype, this might be mediated by other yet unknown functions of TIP5 or MTG16a rather than by their effect on enhanced ribosome biogenesis. For example, the disruption of epigenetic silencing of rDNA is known to lead to genome instability (Diesch et al., 2014; Grummt, 2010; Grummt and Längst, 2013; Grummt and Pikaard, 2003; McStay and Grummt, 2008).

In 2012, a study about a nucleolar isoform of netrin-1 ( $\Delta$ N-netrin-1), found exclusively in cancer cells but not in normal cells, provided a further evidence.  $\Delta$ N-netrin-1 binds to components of the RNA Pol I transcriptional machinery and promotes rDNA transcription, leading to increased ribosome biogenesis. The presence of  $\Delta$ N-netrin-1 in the nucleolus was required for cancer cell proliferation and for tumorigenesis in xenograft experiments (Delloye-Bourgeois et al., 2012).

One more key study in E $\mu$ -MYC transgenic mice determined that MYC-driven B lymphoma is strictly dependent on rRNA synthesis rate. In particular, impairment of RNA Pol I-dependent transcription, through either functional inactivation of UBF, TIF-IA or treatment with CX-5461, a molecule targeting SL1 binding to the rDNA promoter, almost totally suppressed cancer onset and provided further evidence that overactivation of ribosome biogenesis is crucial for tumorigenesis. The same effect was demonstrated in a murine model of AML1-ETO-driven cancer (Bywater et al., 2012). This study showed that the oncogenic potential of MYC strictly requires the upregulation of rRNA synthesis, independently from its function in regulating ribosome levels, protein translation, and proliferative growth. Importantly, it also showed that cancer cells can be dependent on high rates of rRNA synthesis and that, unexpectedly, such dependence can be exploited for specific targeting of cancer cells by pharmacological treatment. Indeed, restoring RNA Pol I activity to that found in normal cells leads to cell death (see below) (Bywater et al., 2012).

There is experimental evidence that not only the rate of ribosome biogenesis, but other steps of the process can be required for tumorigenesis, supporting the idea that the entirety of the process itself contributes to tumorigenesis, rather than solely its first step. Again, evidence supporting this idea comes from a study in a MYC-driven cancer model, more precisely MYC-driven neuroblastoma in zebrafish. In this model of neuroblastoma, like in human neuroblastoma, the nucleolar factor DEF has a key role in the processing of the small subunit. The authors found that loss of function of DEF in the form of

haploinsufficiency significantly decreased the penetrance of MYCN-driven neuroblastoma, via decreased cell proliferation and increased cell apoptosis, whereas gain of function robustly accelerated the onset of the disease and increased its penetrance through increased cell proliferation (Tao et al., 2017).

Finally, evidence exists also regarding the importance of qualitative alterations of ribosome biogenesis. A recent study found that, in acute myeloid leukemia, the AML1-ETO fusion oncoprotein impacts ribosome biogenesis by promoting C/D box snoRNP component formation, thereby altering the level of rRNA 2'-O-methylation in a site-specific manner. In this context, overexpression of components of C/D box snoRNPs, as well as the resulting altered 2'-O-methylation, are required for the tumorigenic potential of the AML1-ETO-driven cancer, indicating that tumorigenesis is mediated by these ribosome biogenesis factors (Zhou et al., 2017). As previously discussed, one explanation is that such alteration in ribosome biogenesis may change the pattern of translated mRNAs, thereby contributing to tumorigenesis. After all, this is precisely one of the features found in Eμ-MYC RPL24+/- mice, with changes in the translation of specific subsets of mRNAs (Barna et al., 2008). Of note, this could be due to qualitative changes in ribosome biogenesis, like the ones observed in AML1-ETO-driven system, but also to mere quantitative changes in the amount of ribosomes in the cell: when the number of ribosomes is limiting, specific mRNAs with low affinity could be out-competed by those with high affinity (Lodish, 1974).

Furtherly speaking of mechanisms by which increased ribosome biogenesis could contribute to tumorigenesis, on the light of its role in surveillance mechanisms controlling cell cycle progression, the most obvious one is that upregulated ribosome biogenesis reduces p53 levels: by constantly consuming free RPs to form ribosomes, it reduces their binding to Mdm2 with consequent increase in proteasome-mediated p53 degradation (Donati et al., 2011a). As previously described, this is the mechanistic basis of the anti-cancer effect of aspirin, that counteracts tumorigenic potential of IL-6 by reducing the ribosome biogenesis rate which in turn leads to restored p53 function (Brighenti et al., 2016). Furthermore, upregulated ribosome biogenesis may inhibit cell differentiation, potentially contributing to tumorigenesis (Hayashi et al., 2014; Zhang et al., 2014).

In conclusion, coming back to the question raised at the beginning of this section, of whether ribosome biogenesis may be responsible for tumorigenesis, evidence is starting to be accumulated that, at least in some cases like MYC-driven cancer, the answer is yes. For this reason, in these contexts and potentially others yet to be investigated, targeting ribosome biogenesis may be envisaged as an effective therapeutic strategy.



## **Chapter 3.**

# **Ribosome biogenesis as a therapeutic target**

### **1. Druggability of ribosome biogenesis**

Until recently, targeting ribosomes or ribosome biogenesis to develop anticancer therapeutic strategies was not seriously envisaged for several reasons. The main reason is that it was assumed that translation and ribosome biogenesis are essential functions, so that their inhibition would indistinctively kill normal and cancer cells. However, surprisingly, evidence has been accumulated demonstrating that cancer cells are more sensitive than normal cells to treatments that inhibit ribosome function or production. This raises the possibility of effectively targeting ribosome biogenesis as a therapeutic strategy.

The basic idea is that tumor cells are addicted to increased ribosome production and increased translational activity. It is largely assumed that cancer cells become addicted to ribosomes due to their enhanced need for protein production to sustain their dysregulated growth. Consistently with this, a number of inhibitors of translation have been introduced for their anti-cancer effect, such as inhibitors targeting the translation initiation machinery including mTOR inhibitors and eIF4F complex inhibitors. This shows that a function as essential as translation can be targeted resulting in the selective killing of cancer cells while sparing normal cells, supporting the concept that cancer cells are addicted to ribosome functions (Boussemart et al., 2014; Drygin et al., 2014; Hsieh et al., 2012; She et al., 2010).

The rationale for targeting ribosome biogenesis in cancer cells takes the steps from the analogous general observation that cancer cells produce more ribosomes and are thus likely to be more vulnerable to a reduction in ribosome production than healthy cells. A number of previously established observations already suggested enhanced ribosome biogenesis in cancer cells: for instance, alterations of nucleolar morphology observed in cancer cells have been known for more than a century. As previously described, several studies have elucidated that nucleolar structure and morphology are built on the activity of ribosome biogenesis, so now we know that the alterations observed in cancer cells directly show alteration of ribosome biogenesis and likely reflect the overactivation of ribosome production. Indeed, today we know that increased ribosome biogenesis is a general trend in cancer cells (Ruggero, 2012a, 2013; Ruggero and Pandolfi, 2003; Whittaker et al., 2010), which is important for cell transformation and tumorigenesis (Barna et al., 2008;

Montanaro et al., 2012; Pelletier et al., 2018), although it remains unclear why some tumor cells rely more than others on ribosome production and protein synthesis.

Importantly, the abnormal oncogenic signalling of cancer cells prevents the efficient feedback control that coordinates the synthesis of rRNAs and ribosomal proteins, and eventually elicits a stress response such as nucleolar stress. Recently, it has been proposed that inhibition of nucleolar stress represents an emerging hallmark of cancer and thus the nucleolus would be a potential target for cancer treatment. In this interpretation, cancers displaying enhanced rDNA transcription and ribosome biogenesis would be vulnerable to the induction of a nucleolar stress response by agents that target the nucleolus (Quin et al., 2014). This was proposed on the basis that upregulation of rRNA transcription in human cancer cell lines in vitro, and a regenerating rat liver model in vivo, decreased the p53 response to cytotoxic stress (Donati et al., 2011a, 2012). Similarly, it has been reported that mutant mice defective for induction of nucleolar stress (because of mutations in MDM2 protein preventing its interaction with RPL5 or RPL11, that is a key event in nucleolar stress) exhibited earlier onset and more frequent tumors when crossed with E $\mu$ -MYC transgenic mice compared to MDM2 wild-type mice (Macias et al., 2010). These findings support a model where, under conditions of hyperactivated rDNA transcription, nucleolar components that mediate p53 signalling (such as RPs) are constantly consumed by the process of ribosome biogenesis, preventing p53 activation. Hence, the need to reactivate anti-proliferative nucleolar stress response through the inhibition of ribosome biogenesis.

This question complies with a major strategical issue in anti-cancer treatment: the need to activate cell death response in a non genotoxic (DNA damaging) manner. Indeed, there is currently a significant number of approved chemotherapeutics that work by damaging DNA and rely on the resulting activation of p53 and other stress response pathways for their therapeutic activity. A major problem with such chemotherapeutics, like cytotoxic drugs or  $\gamma$ -irradiation, is that they generally induce DNA damage and p53 activation in both the tumor cell population and the surrounding normal cells. In other words, they target cancer cells preferentially but not specifically, entailing potentially serious secondary effects, in this way building up strict pharmacologic limit on the utilization of these anticancer medications .

Report here some reviews about Mdm2-p53 system as an anti-cancer target (Pei et al., 2012).

Several distinct methods for nongenotoxic stress have been proposed, but many of them exhibit serious limitations and do not completely solve the issue of specificity with

regard to cancer cells. For example, RG7112, the first selective small molecule non-genotoxic wild type p53 activator, an MDM2 antagonist recently advanced in clinical testing, acts by disrupting the interaction between p53 and MDM2 but to provide its benefit RG7112 had to be administered at its maximum tolerated dose, with serious side effects. In addition, RG7112 mechanism of action is not selective in that it indistinctly activates wild type p53 in cancer and normal cells (Patnaik et al., 2015, 2015). Therefore the need for novel therapeutic approaches for cancer specific non genotoxic p53 activation.

There is at least another limitation to therapeutic strategies relying on genotoxic stress: in some contexts, genotoxic induction of p53 cannot be efficiently exploited. Genetic rearrangements were shown to drive resistance to standard treatment by suppressing the activation of p53 in response to genotoxic stress. This is, for example, the case of the mixed lineage leukemia (MLL) in aggressive acute leukemias, where standard treatment based on cytarabine and doxorubicin fail to induce p53 activation even when administered at high doses. A targeted treatment capable to induce anti-proliferative effect in cells that are not sensitive to standard genotoxic treatments would obviously provide strong therapeutic benefit.

As previously mentioned, a physiological cellular mechanism able to elicit an anti-proliferative effect in a non genotoxic manner could be exploited by anti-cancer therapies: nucleolar stress activated by inhibition of ribosome biogenesis.

In addition to the general non genotoxic activation of p53 response, the inhibition of ribosome biogenesis would boast further advantages. A recent review recapitulates the advantages of targeting ribosomal biogenesis in order to induce p53 activation in anti-cancer therapy (Penzo et al., 2019). These inhibitors can spare resting cells due to the long half-life of their ribosomes (Nikolov et al., 1983; Stoykova et al., 1983), making them selective for rapidly proliferating cells and suitable to effectively target cancer cells. Further, in the case of already published studies about known inhibitors of rRNA synthesis, which will be discussed later in detail, it appears that they do not only block cell cycle but also induce cell death, especially in cells with high ribosome biogenesis rate. There is direct relationship between the level of p53 stabilization induced by rRNA synthesis-inhibiting drugs and the cell ribosome biogenesis rate (Scala et al., 2016). All of this makes inhibitors of ribosome biogenesis particularly suitable for cancer treatment, because they are selective for cancer cells, that are both highly proliferative and addicted to ribosome production, and also, in the case of known inhibitors, because they lead to selective cell death enabling a more effective destruction of cancer cells.

So, apart from the conceptual defense of inhibiting ribosome biogenesis to prevent tumor growth and progression, unexpected evidence has been recently found supporting the feasibility of such a strategy: systematic studies about the mode of action of several known anti-cancer treatments, some of which have even been used for decades, have revealed that ribosome biogenesis is actually impacted by many of them at various levels, eventually being the main pharmacologically targeted process, stilling most of doubts about the concrete possibility to effectively target ribosome biogenesis in a direct pharmacological manner. In particular, RNA Pol I-dependent transcription can be strongly inhibited by platinating agents cisplatin and oxaliplatin, topoisomerase II poisons doxorubicin and mitoxantrone, Streptomyces-derived chemotherapeutics actinomycin D and mitomycin C, and antifolate methotrexate. The list includes mTOR inhibitor rapamycin, alkylating agent melphalan and topoisomerase I poison camptothecin having less pronounced, yet still significant, effects (Burger et al., 2010). Mechanistic studies will be needed to elucidate if these drugs do specifically target rRNA synthesis or rather impact it indirectly as a consequence of cellular response. For example, the latter is likely to be the case for mTOR inhibitors such as rapamycin because it is well established that mTOR signalling pathway regulates ribosome biogenesis and rapamycin has already been shown to inhibit RNA Pol I-dependent transcription by inactivating TIF-IA factor (Mayer and Grummt, 2006; Mayer et al., 2004). In some other cases, the effect on RNA Pol I activity was already known, as for actinomycin D or cisplatin that had been characterized at a mechanistic level. Actinomycin D will be discussed later on. Cisplatin forms covalent adducts in DNA structure, with subsequent DNA damage response. This has traditionally been regarded as the main mode of action of platin-based chemotherapeutic drugs (Riddell, 2018). In addition, cisplatin functions by sequestering UBF, because UBF displays picomolar affinity for intrastrand cisplatin–DNA crosslinked complexes and it is hijacked from its main functions (Chao et al., 1996; Jordan and Carmo-Fonseca, 1998; Treiber et al., 1994; Zhai et al., 1998). Consistently with this, sensitivity of cancer cells to cisplatin was shown to correlate with UBF expression levels (Huang et al., 2002). So, the effect exerted by some drugs on RNA Pol I activity was already known, but because the cellular targets for the therapeutic efficacy are often not unambiguously identified, systematic screening contributed to reconsider impact on ribosome biogenesis as a major mode of action rather than a secondary effect. In the case of cisplatin, this finding has been crucial, because drug resistance and numerous undesirable side effects such as severe kidney problems, allergic reactions, decrease immunity to infections, gastrointestinal

disorders, hemorrhage, and hearing loss especially in younger patients, raised the need to develop and use other platinum-containing anti-cancer drugs such as carboplatin or oxaliplatin (Dasari and Bernard Tchounwou, 2014). A later study confirmed that platinum-containing drugs such as cisplatin, oxaliplatin and phenanthriplatin all inhibit ribosome biogenesis (Bruno et al., 2017). With regard to oxaliplatin and phenanthriplatin, in contrast with cisplatin treatment, no evidence of DNA damage was found, indicating that inhibition of ribosome biogenesis could be the main mode of action and not an off-target effect. Importantly, sensitivity to oxaliplatin is associated to high ribosome biogenesis and translation rate in cancer cells. Oxaliplatin, unlike other platinum-based compounds, kills cancer cells mainly through nucleolar stress rather than via DNA damage (Bruno et al., 2017). A proteomic study, published at the same time, claimed that DNA damage response is elicited together with nucleolar disruption and nucleolar stress upon treatment with any platinum-based drug, including but this conclusion was based merely on proteomic data and no direct observation to check DNA damage response at a cellular or molecular level (Ozdian et al., 2017). At the clinical level, oxaliplatin is widely used to treat colorectal cancer but also gastric and pancreatic metastatic cancer (Garrido-Laguna and Hidalgo, 2015; Stintzing, 2014). It is also being tested for use in treating hematological, ovarian, breast and non-small cell lung cancers (Dilruba and Kalayda, 2016; Mounier et al., 2013).

Actinomycin D, or Dactinomycin, is another drug historically used in chemotherapy that for a long time has been known to impair rRNA synthesis (Iapalucci-Espinoza and Franze-Fernández, 1979; Perry and Kelley, 1970). Actinomycin is a DNA intercalator that preferentially binds guanosine-rich sequences (Hollstein, 1974; Sobell, 1974; Sobell et al., 1971). At low doses, it is a semiselective inhibitor of Pol I transcription elongation, because it intercalates into promoter regions of rDNA, which are enriched in guanosine-rich sequences, blocking the progression of the RNA Pol I complex (Fetherston et al., 1984; Sobell, 1985). For a long time, actinomycin D has been one of the most potent anti-cancer agents known and it is effective in the treatment of the kidney malignancy Wilm's tumor and Ewing's sarcoma (Jaffe et al., 1976; Malogolowkin et al., 2008). Only recently, low doses of actinomycin have been proposed for the treatment of high-risk chronic lymphocytic leukemia and neuroblastoma. In models of these two diseases, actinomycin D has been shown to induce cell cycle arrest, p53-independent cell death and, in the case of p53-negative neuroblastoma, activation of autophagy (Cortes et al., 2016; Merkel et al., 2012). Despite this, clinical application has always been problematic due to its limited specificity and its indiscriminate toxicity towards cells.

In conclusion, these data prompted the idea that targeting ribosome production may be a promising clinical approach, paving the way for the development of innovative anti-cancer drugs and feeding current debate about the topic of ribosome biogenesis as a target (Brighenti et al., 2015; Carotenuto et al., 2019; Hannan et al., 2013; Hein et al., 2013; Pickard and Bierbach, 2013; Quin et al., 2014; Villicaña et al., 2014; Vlatković et al., 2014).

## **2. Novel RNA Pol I inhibitors**

It becomes evident that RNA Pol I-dependent transcription and nucleolar integrity are significant determinants for the various processes required for the expansion of malignant cells and give convincing evidence to target of RNA Pol I function as a therapeutic strategy. Accordingly, a scientific rush has begun in search of new therapeutic drugs fulfilling this property. Recently, novel classes of compounds have been identified expressly for their ability to target RNA Pol I activity. A number of compounds from these studies were characterized, mainly CX-3543 (also known as quarfloxin), CX-5461 and BMH-21 (Bywater et al., 2012; Drygin et al., 2009, 2011; Peltonen et al., 2010). Another high throughput screening study has identified two further molecules, but it is still too early to conclude on their anti-tumorigenic potential (Scull et al., 2019).

### **CX-3543**

CX-3543 (also known as quarfloxin) is a small molecule originally identified as a fluoroquinolone derivative that specifically binds G-quadruplex (G4) structures (Duan et al., 2001). A first attempt to use it as a therapeutic drug intended to exploit its interaction with G4 structures to target transcription of MYC oncogene, due to enrichment of its promoter region with these structures (Chen et al., 2014). On the contrary, CX-3543 was rather found to be preferentially concentrated in the nucleoli of cancer cells where it binds rDNA, which is more highly enriched in G4 structures. A search algorithm had identified 14 putative G4-forming sequences in rDNA region. CX-3543 was found to disrupt 13 of the 14 nucleolin/G-quadruplex complexes (Drygin et al., 2009), and the interaction between G4 structures and nucleolin to form G4-nucleolin complexes is an absolutely required event for RNA Pol I-dependent transcription (Cong et al., 2012; Rickards et al., 2007). The immediate result of disruption of these complexes is a redistribution of the nucleolin into the nucleoplasm, occurring before inhibition of rRNA synthesis and showing that nucleolin relocalization is not a consequence of the former. The same effect was observed independently from functionality of p53, both in p53-positive A549 lung carcinoma cells and p53-null Saos-2 osteosarcoma cell line, confirming that nucleolin relocalization to the

nucleoplasm, that is known to be elicited by a number of drug-induced stress responses, is not dependent from p53-mediated stress response but more likely a direct effect of CX-3543 (Drygin et al., 2009).

Thus, CX-3543 disrupts nucleolin binding on rDNA region causing its redistribution and preventing the formation of RNA Pol I initiation complex. This leads to selective inhibition of RNA Pol I-dependent transcription and induces nucleolar stress resulting in stabilization of p53 followed by activation of apoptotic program and, ultimately, cell death. Interestingly, this response is induced in p53-null cell line HL-60 as well, consistently with a potentially p53-independent caspase activation (Drygin et al., 2009).

The same study reported CX-3543-mediated triggering of DNA damage response, but excluded a direct primary effect of the drug because such response appears quite late after treatment and, importantly, is abrogated by inhibiting caspase activity, indicating that the displayed DNA damage is driven by apoptosis and most likely due to double-stranded DNA breaks introduced by caspase-activated DNase (Drygin et al., 2009).

CX-3543 antiproliferative activity was tested on a panel of 80 cell lines including representatives of many cancers and yielding a remarkable variability. No correlation was found with action profiles of any known compound, suggesting a unique mode of action. Anti-cancer activity was assessed in murine xenograft models of several human cancers, including breast cancer: tumor growth was inhibited with negligible effect on animal body weight. No resistance was observed following treatment interruption and resumption and no cancer cells (pancreatic and colorectal) resistant to CX-3543 could be isolated, despite numerous attempts (Drygin et al., 2009).

CX-3543 has completed phase 1 and 2 trials in patients with advanced solid tumors and lymphomas (NCT00955786) and neuroendocrine/carcinoid tumors (NCT00780663), respectively.

Because the effect of low doses of actinomycin D, inhibiting ribosome biogenesis, had been tested with encouraging results for neuroblastoma treatment (Cortes et al., 2016), and because preclinical biodistribution studies in rodents revealed that CX-3543 accumulates in tissues derived from the neural crest, that give rise to carcinoid/neuroendocrine tumors, it has been tested for the treatment of a subset of neuroblastomas, namely MYCN-amplified ones, in parallel with another RNA Pol I inhibitor, namely CX-5461. In all tested p53-positive neuroblastomas, increased caspase activity and p21 induction were detected upon treatment; in contrast, the response of all tested neuroblastomas carrying p53 mutations did not entail apoptosis but rather cell cycle

arrest, with an accumulation in the G2/M phase of the cell cycle. In addition, DNA damage was found in treated cells, but the chosen 24 h long treatment condition does not allow to discriminate eventual direct effect of CX-3543 or CX-5461 molecules on DNA integrity from indirect apoptosis-driven DNA damage in cells undergoing apoptosis (Hald et al., 2019).

Intriguingly, the effect of CX-3543 and CX-5461 on rRNA synthesis was evaluated in neuroblastoma cells and no reduction in rRNA synthesis rate was observed at drug doses capable of activating apoptotic programme or cell cycle arrest; an effect was detected upon 10-fold increase of treatment doses. In parallel, MycN levels were evaluated as well, revealing that this oncoprotein was downregulated only in p53-positive genetic context (Hald et al., 2019). This is consistent with previous findings indicating that CX-3543 and CX-5461 mediate destabilization of MycN or c-Myc proteins (Lee et al., 2017; Niemas-Teshiba et al., 2017). As previously mentioned, a plausible mechanism for CX-3543 may involve G4 structures present in promoter region of c-MYC gene (Chen et al., 2014).

Together, these data suggest that inhibition of ribosome biogenesis in neuroblastoma should be tested as a treatment option for MYC-driven neuroblastoma (characterized by MYCN gene amplification, high expression of MYCN and c-MYC signature genes, enhanced ribosome biogenesis) treatment neuroblastoma patients expressing high levels of MycN and/or c-Myc, more sensitive to treatment with these compounds (Hald et al., 2019).

CX-3543 has also been proposed to treat parasitic diseases such as trypanosomiasis and malaria. In the case of trypanosomiasis, cell death in *Trypanosoma brucei* was due to rapid inhibition of Pol I transcription, making RNA Pol I transcription inhibitors suitable to develop new approaches to treat the associated disease (Kerry et al., 2017). On the other hand, when CX-3543 was tested as an anti-malarial drug, despite effectively binding G4 structures, having a rapid initial rate of kill and being active against several developmental stages in the life cycle of *Plasmodium falciparum* malaria parasite, no inhibition of rRNA transcription was detected upon treatment with CX-3543, contrary to human cells and trypanosome (Harris et al., 2018).

In conclusion, with regard to cancer, initially CX-3543 has been mainly characterized in A549 lung carcinoma cells. It has been tested to address specific biological questions, such as p53-dependence of cellular response, but not fully characterized in Saos-2 osteosarcoma cell line, as well as in HCT116 colorectal cells. Finally, in pancreatic carcinoma MIA PaCa-2 cells and MDA-MB-231 or MCF7 breast adenocarcinoma cells for



mouse xenograft models (Drygin et al., 2009). Recently, MYC-driven neuroblastoma have been added to the list, and a genetically diverse panel of cell lines has been characterized to a certain extent (Hald et al., 2019).

Due to the limited selectivity of its mode of action, it cannot be excluded that the anti-cancer effect observed upon treatment with CX-3543 is, at least partially, due to repression of MYC oncogene rather than inhibitory effect on rRNA synthesis (Chen et al., 2014). Indeed, a 2018 study proposing CX-3543 as a potential drug for the treatment of colon cancer, still argued that the observed anti-proliferative effect was due to reduced expression of c-Myc but no evidence was given that rRNA synthesis was not impacted (Yao et al., 2018). This is a crucial point that further studies will need to take into account.

### **CX-5461**

In 2011, a cell-based assay differentiating between RNA Pol I- and RNA Pol II-driven transcription was developed and used to screen for compounds that selectively inhibit RNA Pol I activity relative to RNA Pol II activity. This resulted in the discovery of a class of molecules that specifically inhibit RNA Pol I-mediated rRNA synthesis (Drygin et al., 2011). Initially, this selective effect was observed in HCT116 colorectal cancer cell lines and confirmed in melanoma A375 and pancreatic carcinoma MIA PaCa-2 cell lines, with differences up to two degrees of magnitudes between the concentrations needed to exert an effect on RNA Pol I relative to RNA Pol II. This striking selectivity is not comparable with actinomycin D, also known to inhibit RNA Pol I at very low doses. Indeed, high doses of actinomycin D, contrary to high doses of CX-5461, greatly affect RNA Pol II-dependent transcription. The mode of action was characterized showing that CX-5461 inhibits RNA Pol I via disruption of the SL1-rDNA complex (Drygin et al., 2011). The effect of CX-5461 on other Pol I-specific binding factors such as UBF is most likely to be a consequence of the effect on SL1, because the binding of SL1 to rDNA promoter is required for stabilization of UBF and subsequent recruitment of pre-initiation complex prior to RNA Pol I binding (Friedrich et al., 2005).

The antiproliferative effect of CX-5461 treatment was assessed in a large panel of cell lines, revealing a surprising variability among them and most importantly between normal cell lines and cancer cell lines: all tested normal cell lines were much less sensitive to CX-5461. Of note, such variability is not due to a reduced uptake of CX-5461, as rRNA synthesis is reduced in a dose-dependent manner in all cell lines. This provided the first convincing proof of concept that cancer cells can be dependent on high rates of RNA Pol I transcription, and that this dependency can be pharmacologically targeted by selectively

inhibiting RNA Pol I activity: normal cells can tolerate reductions in rRNA synthesis without antiproliferative effect whereas the cancer cells cannot. That could be the reason why CX-5461 can be orally administered in murine xenograft models without changes in body weight nor other obvious toxicity (so, with a favorable safety profile) (Bywater et al., 2012; Drygin et al., 2011).

Another result worth of note coming from the analysis of antiproliferative effect of CX-5461 was that variability among cancer cell lines is not dependent from p53 mutational status. This is clinically relevant because p53 is frequently mutated in many types of cancer.

Early studies also provided preliminary information about the cellular mechanism underlying the antiproliferative effect of CX-5461. Contrary to the semi-selective RNA Pol I inhibitor actinomycin D, CX-5461 was not found to trigger apoptosis in melanoma and pancreatic cells. Absence of apoptosis was clearly demonstrated by means of several independent tests evaluating both early and late apoptosis. This was unexpected because the inhibition of rRNA synthesis in p53-positive context is thought to trigger nucleolar stress resulting in caspase activation and apoptosis. Instead, CX-5461 induces autophagy in the two cancer cell lines. This was demonstrated by increased production of LC3B-II autophagy marker and increased acidic vesicular organelles associated with autophagy (Drygin et al., 2011). The process of autophagy is known to play a role in the survival of cancer cells, as it prevents genotoxic stress and DNA damage, regulating the redox state, and overactivation of autophagy in cancer cells is a drug-resistance mechanism (Levy et al., 2017; Li et al., 2017). Therefore, autophagy inhibitors such as chloroquine and hydroxychloroquine have been tested for cancer therapy (Mariño et al., 2014; Yang et al., 2011). Cancer cells are continuously subjected to autophagy inducing stress (Chen and Debnath, 2010; Chen and Karantza, 2011), and the authors argue that this may explain differential sensitivity of cancer cells, making them more susceptible to certain types of metabolic stress such as inhibition of rRNA synthesis. Importantly, it has already been demonstrated that autophagy plays a role in resistance to oxaliplatin in hepatocellular carcinoma (Du et al., 2012) and in resistance to cisplatin in ovarian, esophageal and lung cancer (He et al., 2015; Wang and Wu, 2014; Wu et al., 2015; Yu et al., 2014).

In the same study, in addition to autophagy in response to treatment with CX-5461, the authors found that CX-5461 is able to induce senescence. This was inferred by detecting a slight increase in b-galactosidase staining (Drygin et al., 2011).

In a following crucial study, which has been mentioned in a previous section about ribosome biogenesis as a driver of tumorigenesis, CX-5461 was used in a B-lymphoma model and displayed the capacity of selectively killing cancer cells. In contrast with  $\gamma$ -irradiation, that indistinctively kills normal and cancer cells, CX-5461 administered at therapeutic doses in a mouse model does not trigger apoptosis nor cell cycle arrest in normal cells and does not impact body weight. In this model, sensitivity to CX-5461 proved to be strictly p53-dependent, with an 180-fold decrease in p53 mutant or null context, and concomitant activation of apoptotic signaling in responsive cells (Bywater et al., 2012). This is not consistent with the behaviour previously observed in cancer cell lines, irrespective of the p53 status and not relying on apoptosis as a mode of action (Drygin et al., 2011).

After the discovery of CX-5461 and its efficacy as an anti-cancer compound, and especially after the key demonstration that not only B-lymphoma cells display enhanced rRNA synthesis but also their viability was dependent from it and they were selectively killed by inhibition of RNA Pol I, CX-5461 was approved for clinical trial and entered Phase 1 for advanced solid tumors in Canada (NCT02719977) (Hilton et al., 2018) and Phase 1 for advanced hematological malignancies in Australia (ACTRN12613001061729).

In the meanwhile, CX-5461 has been exploited in an increasing number of studies (performed by the same research group and by others) in several cancer types and its mode of action at a cellular level has been characterized in more or less detail in different models. The antiproliferative effect of CX-5461 treatment was confirmed in ovarian cancer (Cornelison et al., 2017; Xu et al., 2017b; Yan et al., 2017), prostate cancer (Rebello et al., 2016), MYCN-amplified neuroblastoma (Hald et al., 2019; Niemas-Teshiba et al., 2017; Taylor et al., 2019), multiple myeloma (Lee et al., 2017), acute myeloid leukemia (Hein et al., 2017), acute lymphoblastic leukemia (Negi and Brown, 2015a, 2015b), osteosarcoma (Li et al., 2016). The cellular responses triggered by CX-5461 vary greatly among the evaluated models, so that the available data do not depict a unique response, as apoptosis, senescence, cell cycle arrest or autophagy can be induced, and no unambiguous criteria have been found yet to predict how cells would behave upon treatment (Table 3). The same is true for sensitivity to CX-5461, irrespective of the elicited response: no general genetic contexts have been identified that could particularly benefit from treatment with CX-5461, except for specific cases. Among these specific cases, sensitivity has been associated with rDNA copy number loss in ATRX-negative and ALT-positive cancer cell lines (Udugama et

al., 2018) and with BRCA1/2 mutations or other mutations in the DNA damage repair pathway in breast cancer cell lines (Xu et al., 2017b).

The promising potential of the new drug aroused so much enthusiasm that some groups proposed to use it in other pathological contexts associated to dysregulation of rRNA synthesis, that may benefit from its targeting. So, CX-5461 was used as an antiviral compound due to its impact on viral replication, for instance to treat infection with drug-resistant strains of human cytomegalovirus (Westdorp and Terhune, 2018), or designed and characterized a CX-5461-based derivate (named RAM-589.555) for treatment of multiple sclerosis (Achiron et al., 2017).

Of note, different studies reported remarkable sensitivity to CX-5461 in chemoresistant models, making RNA Pol I inhibitors even more attractive from a clinical perspective (Cornelison et al., 2017; Rebello et al., 2016).

In addition, in order to improve therapeutic benefit, some of these studies investigated rational dual treatments with CX-5461 and other approved drugs in the perspective of effective combinatorial strategies. In particular, CX-5461 was successfully combined with the PI3K/AKT/mTOR inhibitor everolimus in MYC-driven B lymphoma (Devlin et al., 2016), where AKT signalling is required for rRNA synthesis and tumor progression (Devlin et al., 2013); with the PIM kinase (modulator of translation) inhibitor CX-6258 in advanced prostate cancer (Rebello et al., 2016); with doxorubicin in osteosarcoma (Li et al., 2016); with checkpoint inhibitor UCN-01 and several MAPK pathway inhibitors in acute lymphoblastic leukemia (Negi and Brown, 2015b).

Furtherly, two groups focused on the improvement of drug delivery or pharmacokinetics to enhance therapeutic potential. In one study, CX-5461 effect was improved through coupling with a nanoparticle-based pharmaceutical carrier (mesoporous nanoparticle enveloped by polyethylene glycole, polydopamine and aptamer AS-1411 with promoted nucleolar accumulation) (Duo et al., 2018). Another study proposed a copper-based formulation within liposomes to enhance low solubility of CX-5461 molecule at physiological pH, which is a major pharmacological challenge (Leung et al., 2018).

**Table 3. Reported cytotoxicity mechanisms observed upon treatment with RNA Pol I inhibitors in several models**

Inhibitor	Reference	Pathology	Cell lines / Models	Apoptose	Cell cycle arrest	Autophagy	Senescence	Necrosis
CX-5461	Drygin, <i>Cancer Res</i> , 2011	Solid tumors	50 cell lines + 5 non-transformed + HCT116, MIA PaCa-2, A375	PARP1, Casp3/7/9, TUNEL. No apoptosis with CX-5461		LC3BII by FACS, AVO	b-Gal staining	No necrosis by LDH release
	Bywater, <i>Cancer Cell</i> , 2012	Lymphoma	E $\mu$ -Myc lymphoma	FACS, Casp3 by WB				
	Negi, <i>Oncotarget</i> , 2015a	Acute lymphoblastic leukemia (ALL)	ALL cell lines	Annexin V, PARP1, Casp3 by WB	G2/M arrest			
	Negi, <i>Oncotarget</i> , 2015b	ALL	ALL cell lines		G2/M cell cycle arrest			
	Devlin, <i>Cancer Discovery</i> , 2016	Lymphoma	E $\mu$ -Myc lymphoma					
	Quin, <i>Oncotarget</i> , 2016		BJ-hTert + shp53 + Ras	Yes, both in p53+/+ and p53-/- cell lines	G2/M arrest. Loss of S-phase in BJ but not BJ-shp53. G1 arrest less strong in p53-shRNA cells.		Positive for bGal in p53+/+ and -/-	
	Hald, <i>Oncogene</i> 2019	Neuroblastoma	Neuroblastoma cell lines	Yes, in wt-TP53	Cell cycle arrest in mut-TP53			regional necrosis in treated tumoral tissues
	Xu, <i>Nature Comm</i> , 2017	Colorectal cancer, ovarian cancer, osteosarcoma, breast cancer	HCT116, DLD1, U2OS, panel of 50 breast cancer cell lines	apoptosis in BRCA1/2 negative context				
	Cornelison, <i>Clin Cancer Res</i> , 2017	Ovarian cancer	Cell lines	Yes	G2/M cell cycle arrest			
	Rebello, <i>Clin Cancer Res</i> , 2016	Prostate cancer	Prostate cancer cell lines, PDX	no subG1 accumulation	G2/M cell cycle arrest			
	Lee, <i>British J Haem</i> , 2017	Multiple myeloma	myeloma cell lines	Yes, through Annexin V, PARP and caspase activation, in P53 positive or negative				
	Hein, <i>Blood</i> , 2017	Acute myeloid leukemia	Leukemia cell lines	yes	G2/M cell cycle arrest			
	Li, <i>Oncotargets and therapy</i> , 2016	Osteosarcoma	OS cell lines	no	G2/M cell cycle arrest	yes		

Inhibitor	Reference	Pathology	Cell lines / Models	Apoptose	Cell cycle arrest	Autophagy	Senescence	Necrosis
CX-3543	<i>Drygin, Cancer Res, 2009</i>	Solid tumors	NCI60 + 20 cell lines.	Shown in A549 and HL60-p53-/- PARP by WB, Casp3 by IF				
	<i>Hald, Oncogene 2019</i>	Neuroblastoma	Neuroblastoma cell lines	Yes, in wt-TP53	Cell cycle arrest in mut-TP53			
BMH-21	<i>Peltonen, Cancer Cell, 2014</i>	Melanoma, colon cancer	A375 cells, HCT116 cells	subG1	Potently inhibited S phase			
	<i>Colis, Oncotarget, 2014</i>	Melanoma, osteosarcoma	A375 cells, U2OS cells	subG1				
	<i>Wei, Cell Reports, 2018</i>	Melanoma, colon cancer	A375 cells, HCT116 cells					
	<i>Low, The Prostate, 2019</i>	Prostate cancer	Prostate cancer cell lines, xenograft mouse models	p53 target genes activated				
	<i>Fu, Oncology, 2017</i>	Ovarian cancer	SKOV3 cells	Yes, caspase activation	G2/M cell cycle arrest			
	<i>Musso, Biochimica et Biophysica Acta, 2018</i>	Lymphoma	SUDHL4	subG1	G2/M cell cycle arrest			

A box is coloured in green if there are indications that the mechanism takes place; in red, if there are indications that it does not; in gray, if there are not available data.

## **BMH-21**

BMH-21 was identified for its capacity to induce p53 expression among a chemical library consisting of 40 000 small molecule compounds. A cell-based assay for p53-dependent expression of a fluorescent reporter was established and revealed a set of lead compounds that were novel and, partially, structurally related. The most potent compound was BMH-21, able to induce p53 and p53 target genes at nanomolar concentrations, as compared to other cells active at low micromolar concentrations. Remarkably, some of the lead compounds were able to induce p53 target genes in a p53-independent manner. Like other selected lead compounds, BMH-21 was shown to intercalate with DNA without eliciting DNA damage response. The antiproliferative effect was assessed in different cell lines and this indicated higher resistances of the normal cells relative to cancer cells, making it suitable for anti-cancer treatment (Peltonen et al., 2010). So, BMH-21 displayed anti-proliferative properties and induced p53 without causing DNA damage: once again, a p53-inducing drug was discovered that acts in a non genotoxic manner. Absence of DNA damage was then confirmed in A375 melanoma cells and U2OS osteosarcoma cells by contrasting BMH-21 with a series of BMH-21 derivatives that differ for the addition of chemical groups. All these molecules are still DNA intercalators but several times less potent than BMH-21 and display acquired ability to induce DNA damage at far lesser concentration (Colis et al., 2014). This confirms that BMH-21 features unique properties that specifically induce p53 without DNA damage.

Some years later, the same group demonstrated that BMH-21 acts via binding GC-rich sequences at the level of rDNA thereby potently and rapidly repressing RNA Pol I-dependent transcription and leading to nucleolar stress and, ultimately, apoptosis. Importantly, a screen on cell lines from various cancer types revealed that average sensitivity in cancer cell lines is about two degrees of magnitude higher than in normal cells. BMH-21 anti-cancer activity was assessed in xenograft mouse models of human melanoma A375 and colon cancer HCT116 cells, with dose-dependent anti-proliferative effect, suppression of tumor growth and no evident changes in mice weight or organ histology, indicating that BMH-21 is well tolerated (Peltonen et al., 2014).

Further investigation showed that BMH-21 causes loss of the RNA Pol I catalytic subunit RPA194 and dissociation of the transcriptional machinery of from rDNA, resulting in a concomitant decrease in rRNA synthesis. In particular, RPA194 protein levels were strongly reduced upon treatment with BMH-21 and such reduction was specific to RPA194, as protein levels of a number of other components of the transcription machinery were

unchanged. The effect of BMH-21 on RPA194 levels varies among cancer cell lines, with higher sensitivity being correlated with the extent of RPA194 reduction and with the inhibition of rRNA synthesis, indicating that the anti-proliferative effect of BMH-21 is mediated by the loss of RPA194 catalytic activity (Peltonen et al., 2014).

In the same study, the authors demonstrated that RPA194 degradation depends on ubiquitin-mediated targeting to the proteasome (Peltonen et al., 2014). This suggests that in cells efficiently treated with BMH-21 a mechanism could be at work analogous to the specific, ubiquitin-mediated degradation of the large subunit of RNA Pol II, Rpb1, in response to stalled Pol II transcription upon stress induction (Wilson et al., 2013). A following study focused on the fate of the other catalytic subunit of RNA Pol I, RPA135, upon treatment with BMH-21. RPA135 protein levels were mildly affected, but it was reported that RPA135 nucleolar localization decreased over time, concurrent with its relocation into the cytoplasm. An interplay between RPA135 and RPA194 was shown to operate, with RPA135 being necessary to protect RPA194 from degradation and RPA194 being necessary for RPA135 nucleolar localization. In addition, interestingly, it appears from a number of experiments that RPA194 is degraded only if belonging to transcriptionally active RNA Pol I complexes, with impairment of transcription elongation being the triggering molecular event of the effect of BMH-21 (Wei et al., 2018). Again, this supports a model that mirrors the one formulated for clearance of stalled RNA Pol II transcription machinery (Wilson et al., 2013).

These results clarified the mode of action of BMH-21 at the mechanistic level and ascribed BMH-21 to a unique class of anti-cancer drugs promising for the treatment of cancer also in p53-negative context.

Recently, transcriptome analysis of cells treated with BMH-21 was carried out, revealing that BMH-21 induce a dramatic perturbation on cellular functions, with 3316 affected genes including those related to cell-cycle arrest, DNA replication stress, p53 signalling and, unexpectedly, those implicated in carboxylic acid synthesis and tRNA aminoacylation, confirming the uniqueness of BMH-21 (Espinoza et al., 2019).

Since its discovery, BMH-21 has been tested in a number of cancer models, including melanoma and colon cancer cells (Peltonen et al., 2014; Wei et al., 2018), ovarian cancer cells (Fu et al., 2017), prostate cancer cells (Low et al., 2019), MYC-driven lymphoma cells (Musso et al., 2018).

In all the tested systems, BMH-21 has been reported to inhibit cell viability and, when evaluated, tumor growth, through inhibition of rRNA synthesis, nucleolar stress



accumulation of p53 protein. However, the mode of action has not been well characterized yet (Table 3). In one case, phosphorylation of p53 at serine 15 has been reported, which enhances p53 function as a transcription factor leading to caspase-dependent activation of apoptosis (Fu et al., 2017). This is the only study demonstrating caspase activity and a clear apoptotic response upon treatment with BMH-21.

In the first studies, unexpectedly, despite that BMH-21 was originally identified for its ability to induce p53 activation, the authors also observed growth repression in TP53 null and mutant cell lines (Peltonen et al., 2014). However, at present, BMH-21 mode of action in p53-negative context has not been especially examined yet, and further investigation urges as loss of p53 function is a common feature among cancers.

Recently, a group developed a novel platinum-based RNA Pol I selective inhibitor by chemically modifying cisplatin through binding to an analogue of BMH-21. This novel monofunctional platinum-based agent, named P1-B1, displayed anti-cancer activity as much as 17-fold greater than the clinical drug cisplatin, even in cisplatin-resistant non-small cell lung cancer cells. P1-B1 displayed significant nucleolar accumulation, selectively inhibited Pol I transcription, and induced nucleolar stress, leading to cell cycle arrest and apoptosis (Zhang et al., 2018).

## **Other compounds**

A number of other compounds have been identified that selectively target RNA Pol I, but yet without a deep and detailed characterization of anti-cancer properties. A short list will be presented here, accompanied by an overview for each compound.

A high-throughput screening for compounds that selectively produce nucleolar disruption led to the identification of an acridine derivative (CID-765471) (Morgado-Palacin et al., 2014). The identified molecule turned out to have already been tested under the name BMH-22 (Peltonen et al., 2010). Similar to BMH-21, this molecule disrupts nucleolus by triggering selective degradation of RPA194 subunit of RNA Pol I and leading to RPL11-dependent p53 activation in absence of DNA damage. Two additional compounds, aminacridine and ethacridine, were found to operate in a similar manner (Morgado-Palacin et al., 2014).

Hernandonine destabilizes RPA194 catalytic protein in a proteasome-dependent manner. It induces apoptosis in p53-dependent or independent manner making it a suitable for a potential cancer treatment through oral administration. The study identifies another structurally new molecule, oxoaporphine, for RNA Pol I degradation (Chen et al., 2019).

Ellipticine targets the preinitiation complex at the rDNA promoter, stabilizes G-quadruplex structures by disrupting the SL-1 binding to the rDNA promoter and causes DNA damage (Andrews et al., 2013).

The antimalarial drug amodiaquine was shown to inhibit rRNA transcription in a dose-dependent manner. Amodiaquine triggers degradation of the catalytic subunit of RNA Pol I and subsequent nucleolar stress, without DNA damage (Espinoza et al., 2019).

In another high throughput screening study, two small molecule inhibitors, Ribosome Biogenesis Inhibitor 1 and 2 (RBI1 and RBI2), were identified. Cell viability analysis indicates that both RBI1 and RBI2 potently inhibit the growth of A375 malignant melanoma cells *in vitro*. Furthermore, acute, transient treatment with RBI2 inhibits anchorage-independent cell growth and proliferation (Scull et al., 2019)

A peptide mimicking a highly conserved region of 22 amino acids in the RNA Pol I subunit RPA43, responsible for the interaction with TIF-IA factor, is capable of inhibiting rRNA transcription both *in vitro* and *in vivo*. In normal cells, this peptide reversibly inhibits cell proliferation, while it causes cell death in tumor cells through both apoptosis and necrosis, reminding the selectivity observed in some of the previously described models (Rothblum et al., 2014)

Finally, a potential strategy proposed to discriminate between highly proliferating normal cells and cancer cells, which is a major limitation of most anti-cancer treatment nowadays, relies upon the function of TIF-IA in particular genetic context. In normal cells, low intracellular uridine levels do not suppress rRNA synthesis because a kinase activity, LKB1, promotes TIF-IA nuclear import to maintain TIF-IA function in the nucleolus. In LKB1 mutant context, low levels of uridine suppress rRNA synthesis and lead to cell death. This discovery suggests the potential of developing a novel treatment concept to specifically eliminate LKB1-mutant tumor cells using molecules that inhibit the *de novo* synthesis of UMP, such as the already clinically available leflunomide, inducing cell death preferentially in LKB1-mutant cells (Liu et al., 2015). Because several mechanisms operate within a cell to maintain cellular functions under stress and prevent cell death, and because ribosome biogenesis is central in sensing stress, this concept deserves to be further studied, as other cancer-associated genetic contexts could expose cancer cells to higher sensitivity to particular conditions, and such conditions could be expressly induced in anti-cancer treatment to take advantage of differential behaviours between cancer cells and normal, albeit highly proliferating, cells.

### **3. Targeting ribosome biogenesis downstream of RNA Pol I**

In addition to inhibition of ribosome biogenesis at a transcriptional level, targeting RNA Pol I activity, hence the rate-limiting step of rRNA biogenesis, Burger and colleagues also assessed other steps of the process: by monitoring the effects of chemotherapeutics on the processing of pre-rRNA, they have shown that early stages of processing can be affected by camptothecin, and kinase inhibitors DRB (5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole), flavopiridol and roscovitine, whereas the late stages were affected by nucleoside analog 5-fluorouracil, translation inhibitors cycloheximide and homoharringtonine, proteasome inhibitors MG132 and bortezomib, and to a lesser extent by the topoisomerase II poison etoposide and microtubule inhibitor vinblastine (Burger et al., 2010). Once again, it is difficult to tell whether there is a direct effect on pre-rRNA processing or rather it appears as a consequence of other mechanistic targets. For instance, incorporation of 5-fluorouracil in pre-rRNA has been shown to interfere with pre-rRNA processing (Ghoshal and Jacob, 1997). In any case, these studies enforced the idea that inhibiting ribosome biogenesis can have anti-cancer effect but raised the need to develop specific inhibitors of rRNA synthesis or processing in order to avoid off target effects.

#### **Targeting pre-rRNA cleavage**

Among the hundreds of factors involved in a process as complex as ribosome biogenesis, some promising candidates have been identified for pharmacological targeting.

A recent study suggested to target post-transcriptional steps in pre-rRNA processing, by using an anti-sense pharmacological technology to silence U3 or U8 snoRNA expression, in order to block ribosome biogenesis and elicit p53 activation (Langhendries et al., 2016). U3 and U8 which interact with the prerRNA through the formation of Watson-Crick base-pairing interaction, are essential for early and late pre-rRNA processing reactions leading to the formation of small and large ribosomal subunits, respectively. Using a mouse xenograft model, it was recently shown that aggressive cancer cells in which the expression of U3 or U8 was silenced lost their tumorigenic potential once implanted into the flanks of nude animals. When tumors formed, these were not only smaller but also more heterogeneous in composition as a consequence of healthy tissue repopulating the microenvironment. Increased tumor heterogeneity, as established by positive emission tomography that relies on the use of a metabolic tracer, is considered to be a positive clinical parameter owing to the presence of healthy tissues. U3 and U8 would

be the first case of factors required for maturation steps of ribosome biogenesis to be specifically targeted to counter tumor growth (Langhendries et al., 2016).

As mentioned in a previous section of this thesis, U3 and U8 snoRNAs belong to the class of C/D box snoRNAs and play their function within a C/D box snoRNP complex, containing NOP56, NOP58 and Fibrillarin. In order to inhibit pre-rRNA processing, the function of any component necessary for the formation of this complex may be targeted. Consistently with this, siRNA-mediated knockdown of each of these three proteins resulted in the reduced growth of MCF7 xenografts (Su et al., 2014).

A recent review remarked that the amplification of the 19q13 cytogenetic band, including the FBL gene, coding for Fibrillarin, correlated to cell viability and resistance in pancreatic cells as well as to a trend toward a shorter survival in pancreatic cancer patients (El Hassouni et al., 2018). Indeed, targeting FBL has a great potential, due to its pivotal role in ribosome biogenesis, its correlation to improved survival rate at low expression in breast cancer patients and its expression controlled by p53 (Marcel et al., 2013; Sharma et al., 2017). Fibrillarin controls the amount of ribosome production through various molecular mechanisms, including rDNA histone methylation, pre-rRNA processing, and pre-rRNA modification (Rodriguez-Corona et al., 2015). Therefore, targeting such a master regulator of ribosome biogenesis could be a promising approach to interfere with the production of ribosomes.

## **Targeting ribosome modifications**

In a previous section, the concept of ribosome heterogeneity was introduced, with a mention to specialized ribosomes. In particular, changes in ribosome composition could be associated with cancer phenotype, leading to the concept of cancer ribosomes (Bastide and David, 2018; Sulima et al., 2017). The development of drugs directly targeting specific ribosome variants, such as cancer ribosomes, can seriously be envisaged as several antibiotics bind to highly specific ribosomal structures and selectivity their binding is often provided by very subtle differences at the structural (Myasnikov et al., 2016; Vázquez-Laslop and Mankin, 2018). Ideally, only the cancer ribosomes would be targeted without affecting wild-type ribosomes. This is not a limitation, as it has been shown that resistance to antibiotics often relies on high selectivity of antibiotics towards extremely specific structural features in prokaryotes, sparing cells that display, or lack, a single modification (Poehlsgaard and Douthwaite, 2005) and there is no reason to believe that the same would not hold true in eukaryotic systems. So, such high binding selectivity makes perturbation of ribosome modifications a potential strategy to target ribosome function. In particular,

recent structural analysis of prokaryotic and eukaryotic ribosomes demonstrated that interaction selectivity of factors that bind to the ribosome is often provided by subtle structural differences (Garreau de Loubresse et al., 2014; Jenner et al., 2013; Lin et al., 2018; Myasnikov et al., 2016; Pellegrino et al., 2018; Prokhorova et al., 2017; Svidritskiy et al., 2013; Vázquez-Laslop and Mankin, 2018). This provides the proof-of-concept that it may be possible to develop antibiotic-like molecules to target ribosomes accumulating in cancer cells, provided that they feature structural differences, even slightly ones, from those of healthy cells.

### **Potential targets from the yeast model**

In yeast, a few pharmacological inhibitors of ribosome assembly have been identified through systematic screening of a large set of substances using a microscopy-based screen scoring ribosomal subunit export defects. As much as 128 compounds were found to inhibit maturation of either the small or the large ribosomal subunit or both, at various levels and with a broad spectrum of rRNA processing defects. Many of the identified substances were previously shown to interfere with growth of tumor cells, are currently being investigated in clinical trials, or have already been used for clinical cancer treatment, but a number of molecules constitute a new class of potential novel drugs targeting ribosome biogenesis (Awad et al., 2019). The first compound to be characterized was diazaborin, which inhibits the formation of the large ribosomal subunit at a late stage. In particular, diazaborine inhibits cleavage at position A3 of the 27S pre-rRNA, leading to preribosome particles retention in the nucleolus after drug treatment (Pertschy et al., 2004). Further studies showed that diazaborine targets the activity of AAA-ATPase Drg1, required for the release of Rlp24 from pre-60S particles, allowing the progression of cytoplasmic maturation (Kappel et al., 2012; Loibl et al., 2014; Pertschy et al., 2007). This research was advanced mainly on experimental purpose, providing for the first time a comprehensive set of inhibitors to study ribosome biogenesis by chemical inhibition of individual maturation steps. For example, diazaborin has been used to study the dynamics of pre-60S maturation (Zisser et al., 2018) At the same time, the process of ribosome maturation was thus established as promising druggable pathway for chemical intervention. Another preliminary study in yeast have revealed the therapeutic potential of targeting Midasin (also Rea1 or Mdn1), AAA+ ATPase involved in the maturation of 60S subunit. A triazinoindole-based inhibitor has been already developed (Kawashima et al., 2016). However, being all of this research carried out in yeast model, it is not known whether the inhibitory effect of all of these drugs would work in human cells with the same

efficacy, considering that despite overall process is conserved throughout eukaryotes considerable differences exist at specific steps of ribosome biogenesis.

## **Chapter 4.**

# **Triple negative breast cancer**

Breast cancer is the most common cancer in women worldwide and represents the first cause of cancer-related death in women. On the other hand, a sharp reduction in breast cancer-related mortality has been observed in the last decade thanks to the introduction of screening programs. In addition, breast cancers are today one of the best characterized cancers and this has allowed the development of effective targeted therapies. Despite these advances, some subtypes of breast cancer are still poorly understood and dedicated treatment protocols remain ineffective. Therefore, a better understanding of the biology of breast cancer is urgently needed in order to identify novel adapted therapeutic options. Here, first of all, the classification of breast cancers and current treatments will be described. Then clinical features of a specific subtype, triple negative breast cancer (TNBC), will be outlined. Finally, a state of the art of current knowledge about the role of ribosome biogenesis in TNBC will conclude this section.

### **1. Classification of breast cancer types**

The biology of breast cancer is now better and better characterized. It is now well known that breast cancer is not a single disease but a group of diseases that have different clinical, biological and molecular features. The classification of breast cancer is essential in the clinic for the management of patients. Indeed, it allows the prediction of patient survival as well as the selection of patients who would respond to a specific type of treatment. Currently, there is not a unique, unambiguous classification system, and classification criteria are constantly refined. In the early 1990s, a classification based on anatomico-pathological criteria was developed (Ellis et al., 1992; Elston and Ellis, 1991). Instead, molecular classification of breast cancer has been developed since 2010 and continues to be refined today. Such different classifications of clinical relevance will be detailed in this section.

#### **Molecular classification of breast cancers**

The anatomopathological classification makes it possible to highlight the heterogeneity of breast cancers. On the other hand, this type of classification does not make it possible to identify potential therapeutic targets. Over the past decade, much effort has been focused on improving the classification of breast cancer through molecular approaches to gain a better understanding of the heterogeneity at the molecular level of

this disease and to identify new therapeutic targets. Three types of classification will be addressed in this section: the molecular classification according to the gene expression profile by transcriptomic analyses; classification according to receptor status by immunohistochemistry (IHC); and the recent classification of the triple negative breast cancer (TNBC) subtype. Since the beginning of the 21st century, advanced molecular approaches such as DNA microarrays and new methods for data analysis in the field of biostatistics and bioinformatics have made it possible to stratify tumors according to their gene expression profile. In this context, the first published study revealed a remarkable heterogeneity at the molecular level among mammary tumors (Perou et al., 2000). The use of DNA chips in about 40 samples provided a list of genes whose expression varies significantly between different tumors but remains unchanged between different samples of the same tumor. Based on this list, the authors identified four molecular subtypes associated with differential gene expression of the estrogenic signaling pathway: luminal, basal-like, HER2+ and normal-like subtypes. These are characterized, respectively: by a strong expression of a group of genes that are typical of mammary gland-like epithelial cells, such as estrogen receptor (ER) or cytokeratin (CK) 8 and 18 (luminal subtype); by overexpression of genes typical of basal epithelial cells of the mammary gland such as laminin or CK5, CK6 and CK17 (basal-like subtype); by an amplification of the human epidermal growth factor receptor 2 (HER2) gene and other genes located in the same region of chromosome 17 (HER2+ subtype); by a strong expression of markers of basal cells and adipocytes and a weak expression of genes characteristic of luminal cells (normal-like subtype).

This initial classification according to gene expression has been validated by three different studies with a larger number of samples (Sørli et al., 2001, 2003; Sotiriou et al., 2003). These three studies also showed that the luminal subtype was heterogeneous and could be furtherly stratified into at least two groups, namely luminal A and luminal B groups, the latter characterized by increased expression of genes involved in proliferation, such as MKI67 (Marker of proliferation KI-67), and by a poor prognosis (Sørli et al., 2003). Among the subtypes defined by this molecular classification, tumors classified as HER+ and basal-like are associated with the worst survival of patients (Sørli et al., 2001, 2003).

Another classification system, based on protein expression, has been made possible in the wake of the development of tissue micro-array technology, allowing the analysis by IHC of slides from various tumors, thus validating gene signatures at the protein level. A



first study focused on the expression of 13 biomarkers by IHC, including ER, progesterone receptor (PR), HER2, luminal and basal CKs, on more than 100 tumors, and found that the same intrinsic subtypes previously defined through transcriptomic analysis could be also distinguished by IHC: luminal A, luminal B, HER2+ and basal-like subtypes (Callagy et al., 2003). Therefore, this study identified markers detectable by IHC and reflecting the molecular classification derived from transcriptomic breast cancer analyses, providing easily accessible histological criteria working as a proxy for stratification based on gene expression profiles.

Nowadays, the three most widely used stratification markers are ER, PR and HER2 receptors, whose expression profiles define three distinct broad groups: luminal, HER2+ and triple negative.

The luminal subtype is identified by the overexpression of the hormone receptors ER and PR and the absence of overexpression of the HER2 receptor. Within luminal subtype, luminal A and B subtypes are still distinct, as luminal B tumors are characterized by a higher levels of KI-67 proliferation markers. The luminal subtype represents 70% of tumors and is associated with the best survival among the three groups (Cheang et al., 2009; Nielsen et al., 2004).

The HER2 subtype is identified by the overexpression of HER2 and the absence of overexpression of ER and PR. This subtype represents approximately 15% of breast cancers and is associated with poor survival compared to Luminal A and Luminal B tumors (Nielsen et al., 2004; Vallejos et al., 2010).

Finally, the triple negative subtype is characterized by the absence of overexpression of the three receptors ER, PR and HER2. It represents 15% of breast cancers and patients with this tumor subtype have a poor prognosis. Patients with TNBC display a higher risk of cancer progression during the 5 years after diagnosis compared to patients with other subtypes of breast cancer, meaning that TNBC is a rapidly progressing disease (Dent et al., 2007).

Basal-like cancers are also characterized by the absence of overexpression of these three receptors at the protein level (Nielsen et al., 2004). For this reason, basal-like tumors are often misclassified as triple negative tumors: it has been evaluated that 78.6% of triple negative tumors actually correspond to basal-like tumors and conversely 68.5% of basal-like tumors are triple negative (Prat et al., 2013).

## **Classification of the triple negative subtype**

The triple negative subtype is surprisingly heterogeneous and there is clinical need to improve its stratification thereby unveiling potential therapeutic targets. In order to characterize such diversity, an innovative study analyzed two independent series of breast cancer based on gene expression profiling followed by analysis of the enriched biological functions and thus classified the triple negative subtype into 6 classes (Lehmann et al., 2011).

The identified classes of TNBC are: basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor. Each of the listed subtypes is characterized by an enrichment of signaling pathways related to different functions: related to the cell cycle and cell division, in particular proliferation-related genes, such as MKI67, in the basal-like 1 subtype; related to growth factors, such as EGF or IGF-1R, in the basal-like 2 subtype; related to immunity, such as genes involved in cytokine signaling pathways, antigen presentation, and antigen recognition response, in the immunomodulatory subtype; related to mobility and cell differentiation, such as genes linked to epithelial to mesenchymal transition, with a decrease in the expression of CDH1 and an increase in the expression of mesenchymal markers, in the mesenchymal subtype; similarly related to mesenchymal cell phenotype but with an additional enrichment of the signaling pathways involved in growth factors, angiogenesis and stem cells in the mesenchymal stem-like subtype; and related to hormone regulation and androgen and estrogen metabolism, such as androgen receptor and its downstream targets, in the luminal androgen receptor (LAR) subtype.

As a conclusion, it must be pointed out that the classification of breast cancers is clinically important, as it determines the prognosis (according to the grade, the stage and the subtype of the cancer) of the patients. These classifications have enabled the development of new targeted treatments and make it possible to predict their effectiveness. In particular, depending on the status of ER, PR and HER2 receptors, clinicians are able to prescribe appropriate treatment. For example, patients who overexpress hormone receptors would benefit from anti-hormonal therapy targeting the ER signaling pathway more than patients whose HER2 is amplified. This is exactly the clinical problem for the treatment of TNBC, as described in the next section.

## **2. Treatment of TNBC**

The goal of cancer treatment is ideally to be able to remove the tumor or metastases or, at least, to slow the development of the tumor, and in the worst cases to reduce the risk of recurrence of the disease or metastases. Treatment of breast cancer patients depends on different prognostic factors related to the tumor, including anatomic-pathological, histological and molecular criteria. Other factors are also taken into account, which depend on the patient, such as age, menopause and/or overall health. Based on these factors at the time of diagnosis, adapted treatments are chosen for anti-cancer therapy.

### **Treatment of breast cancer**

Breast cancer treatments can be divided into two groups: localized treatments and systemic treatments. Localized treatments are surgery and radiotherapy that target the tumor locally. Radiotherapy uses ionizing radiation applied at a specific point to target cancer cells while sparing healthy tissues and nearby organs. Ionization induces free radicals that cause cell death by causing breaks in DNA strands. Only proliferative cells are radiosensitive, which accounts for the specificity of this treatment since cancer cells are hyperproliferative compared to healthy cells.

Systemic treatments are drug treatments such as chemotherapy or hormone therapy delivered orally or by blood. In systemic treatments, anti-cancer drugs circulate throughout the body and reach cancer cells at different locations, even when they were not detected at diagnosis. These treatments are often administered in three situations: to reduce tumor size before surgery (neoadjuvant treatments); to avoid recurrence, especially in invasive cancers (adjuvant treatment); or in the presence of metastatic breast cancer, to reach cancer cells at different locations. There are three types of systemic treatments: chemotherapy, hormone therapy and targeted therapies.

Chemotherapy consists in the use of molecules, alone or in combination in order to reduce risk of resistance and enhance cytotoxic effects, inhibiting cell division and inducing cell death in cancer cells, generally characterized by high proliferation. Chemotherapeutic drugs target highly proliferating cells, including cancer cells but also some healthy cells, such as blood cells, thereby inducing undesirable side effects.

Hormone therapy inhibits hormone functions by preventing secretion or blocking the action of hormones that stimulate proliferation of cancer cells. This treatment is offered to patients with hormone-sensitive tumors, like those who express ER and PR, usually corresponding to the luminal subtypes.

Targeted therapies specifically target proteins directly involved in tumorigenesis. Several targeted therapies are used in breast cancer, including HER2 inhibitors and the mTOR inhibitor everolimus.

### **Clinical features of TNBC and therapeutic options**

Targeted therapies are effective in that they are conceived to display reduced side effects, but rely on mechanisms that cannot always be usable because of lack of identified specific molecular targets. TNBC is a typical example of breast cancer problematic to treat: patients with TNBC do not benefit from hormone therapy (such as tamoxifen) nor therapies designed to target HER2 (such as trastuzumab or Herceptin), because of the loss of target receptors. Although these patients respond to chemotherapeutic agents better than other subtypes of breast cancer, being highly proliferative, prognosis remains.

TNBCs are associated with a four-fold increase in the risk of distant metastases, especially to the lungs and the central nervous system, with an estimated 15-30% of TNBC patients developing brain metastases. This aggressive metastatic behaviour, together with a high rate of cancer recurrence, contributes to the overall shortened survival of patients with TNBC compared with other breast cancers. In addition, prognosis is furtherly worsened due to the fact that targeted therapies are ruled out in these patients (Yao et al., 2016).

For these reasons, patients with TNBC are usually treated similarly as patients presenting other breast cancers. The European Society for Medical Oncology (ESMO) states that cytotoxic chemotherapy is the standard of care for the treatment of TNBC and that the choice of the regimen should be taken depending on the context, after taking into account disease-related and patient-related factors. Surgery and radiotherapy are employed routinely in a similar way as with other types of breast cancer, and chemotherapy in combination with these localized treatments is the standard mode for TNBC.

Several studies have been attempting for many years to identify a single targetable pathway in TNBC, but a major obstacle is TNBC heterogeneity. Comprehensive genomic studies have provided extensive data on the mutational landscape of breast cancer, but have failed to identify tumor mutations typical of TNBC. Some mutations seem to characterize particular subpopulations of TNBC conferring them higher sensitivity to chemotherapeutic treatment, with greater pathological complete response. For instance, the coupling of platinum-induced DNA damage and deficiencies in BRCA-associated DNA repair. Indeed, some targeted therapies have been implemented and tested that could be

more effective in particular genetic contexts, including the use of PARP inhibitors in BRCA-mutated TNBC, where they can lead to cell death because of the inability of the cell to repair DNA damage. However, several mechanisms of resistance have already been reported. The case of the LAR subtype of TNBC must be taken into particular consideration, because it is the class of TNBC for which promising results for androgen receptor-targeted therapy are being most robustly provided. In other cases, like tyrosine kinase inhibitors, selectivity for TNBC is not clear. Other proposed treatments, like the lipid-lowering statins, have been shown to preferentially target TNBC compared with other breast cancers, but the understanding of the molecular basis for such preference is critically lacking. Here some partial examples were given of ongoing studies for targeted therapies in TNBC, and many others are in place, aiming to target pathways as diverse as immune checkpoint modulators, epigenetic regulators, apoptosis effectors, cyclin-dependent kinases, PI3K/AKT/mTOR axis, cell surface growth factor receptors (Al-Mahmood et al., 2018; Denkert et al., 2017; Nakhjavani et al., 2019; Yao et al., 2016).

All in all, the knowledge about TNBC has undoubtedly increased during the last years, and some of the clinical trials are encouraging. Nevertheless, none of the new therapies have reached phase 3 trials, and chemotherapy is still the only validated therapy option for TNBC patients in clinical context. Certainly, the molecular characterization and consequent classification of TNBC have been essential to direct research efforts toward potential targets and will be essential to identify, if not a common druggable target, at least a TNBC subpopulation that could benefit from new targeted therapies. In conclusion, TNBC is today a cancer whose treatment remains limited compared to other subtypes of breast cancer.

### **3. Ribosome biogenesis and breast cancer**

In general, early tumorigenesis in invasive breast cancer is associated with amplification or upregulation of basal components of the RNA Pol I transcription machinery, as reported in a majority (> 66%) of invasive breast carcinoma from TCGA database (Rossetti et al., 2016). A study suggests that increased rRNA synthesis due to RRN3 upregulation, by affecting mammary epithelial morphogenetic processes and sustaining breast cancer cell proliferation, can contribute to breast cancer initiation and progression (Rossetti et al., 2016). Therefore, overactivation of ribosome biogenesis through upregulation of RRN3 may be sufficient to drive cell transformation of mammary epithelial cells. A similar potential role of overactivated ribosome biogenesis in cell

transformation has been reported upon depletion of a negative epigenetic regulator of rDNA (Rossetti et al., 2010). Although these experiments showed that increased ribosome biogenesis is pivotal for breast cancer cell proliferation, it is not clear whether it is a cause or a consequence of malignant cell transformation. However, like in other models described above, ribosome biogenesis could play an essential role in breast cancer and even be sufficient for tumorigenesis.

Not only the basal components of RNA Pol I transcription machinery are upregulated in breast cancer, but also factors required for maturation of rRNA, like C/D and H/ACA snoRNA classes and components of the associated complexes such as FBL, NOP56 and NOP58 (Su et al., 2014).

Little information has been accumulated about the extent or the status of ribosome biogenesis in TNBC, hence about potential specific contribution of ribosome biogenesis to the cancer phenotype in the triple negative subtype. Of note, the most frequently mutated genes in TNBC include TP53, PTEN and RB (Yao et al., 2016). After p53-negative status, mutations involving genes of the mTOR signalling pathway are the commonest in TNBCs, and PI3K regulation components such as PI3KCA, Akt1 or PTEN are frequently lost or mutated. Consequently, mTOR pathway is activated in TNBC. This generally occurs through genomic alterations in the PTEN locus resulting in low PTEN protein expression (Marty et al., 2008). As extensively described so far all along this thesis, mTOR pathway and p53 and RB tumor suppressors all regulate ribosome biogenesis at multiple levels. Therefore, it is reasonable to imagine that TNBC is associated with dysregulated ribosome biogenesis.

A recent functional and genomic study found that ribosome biogenesis is overactivated in a metastatic TNBC model and that it is associated with an increase in aggressiveness. Four populations of the MDA-MB-231 human breast cancer line, were assessed for their metastatic potential and classified accordingly. Then, primary tumors formed after inoculation of cells into xenograft mouse model were characterized at the level of gene expression. It was found that the most aggressive metastatic cells were associated to the upregulation of gene sets related to RNA metabolism or ribosome biogenesis. However, the authors found no evidence for increased expression of genes involved in cell proliferation, that could explain the enhanced ribosome biogenesis, and suggest that ribosome biogenesis could play a direct role in metastatic dissemination independently of effects on cell proliferation (Johnstone et al., 2018).

Another study focusing on TNBC raised the possibility that ribosome biogenesis and mTOR signalling could work together to endow TNBC with resistance to anti-cancer treatment, so together could they be targeted in order to impair their synergy. The study is based on the observation that despite PI3K/Akt/mTOR pathway overactivation is very frequent in TNBCs making PI3K/mTOR or mTOR inhibitor good candidates for treating the disease, clinical efficacy of these drugs in patients with TNBC has been limited due to acquired resistance and survival of a cancer stem cell-like population. The results indicate that mTOR inhibition leads to the activation of Notch 1 resulting, in turn, in an accumulation of cancer stem cells accompanied by a differential expression of a number of genes, including MYC (Bhola et al., 2016). As a reminder, the MYC oncogene is responsible for the coordination of all three RNA polymerases, needed for ribosome biogenesis (Mayer and Grummt, 2006). As a conclusion of their study, the authors suggest to develop treatments targeting, together with mTOR signalling, Notch 1 pathway in order to prevent the formation of cancer stem cells and the resistance to mTOR treatment alone (Bhola et al., 2016). However, other second targets could be contemplated in order to limit the expansion of a cancer stem cell population. Despite it is part of the results of the study, MYC-dependent regulation of ribosome biogenesis is not suggested as a potential target.

To conclude, the status of ribosome biogenesis in TNBC and its contribution to the aggressive phenotype of this breast cancer subtype, accounting for its poor clinical outcome, have not been much investigated yet, and the available information comes from experiments carried out in limited models that are not representative of the diversity found in TNBC. Being that TNBC is a heterogeneous disease and that ribosome biogenesis has been shown to play a role in an increasing number of cancers with promising clinical implications, a systematic characterization of TNBC with regard to the status of ribosome biogenesis will be needed to reveal the potential relevance of a targetable pathway in the development of anti-cancer treatment adapted to TNBC.

## Rationale and objectives

Ribosome biogenesis is altered in most cancers, due to the deregulation of the major oncogenic and tumor suppressor pathways, such as MYC, p53 and PTEN, which regulate RNA Pol I activity. Thus, ribosome biogenesis is now emerging as an essential process in tumorigenesis and the idea of targeting ribosome biogenesis is getting a foothold in the field of anti-cancer treatment.

Recently, the inhibition of ribosome biogenesis has been validated as a therapeutic tool, based on three compounds that inhibit RNA Pol I activity (CX-5461, CX-3543 and BMH-21) that preferentially target cancer cells against healthy cells. Anti-cancer activity has been reported in several cancer types, including leukemia, lymphoma, myeloma, osteosarcoma, prostate and ovarian cancer. Today, the potential benefit of targeting ribosome biogenesis in TNBC has not been explored. Although very little is known about ribosome biogenesis in TNBC, there is urgent need for new therapeutic tools in order to overcome the lack of efficient and targeted strategies, a major clinical problem in the treatment of this disease, and the potential of inhibiting ribosome biogenesis must not be missed.

For this reason, the relevance of RNA Pol I inhibitors for the treatment of TNBC deserves to be evaluated. In addition to the use of RNA Pol I inhibitors, the effectiveness of which is yet under evaluation in patients, alternative strategies for the inhibition of ribosome biogenesis must be investigated. Here, the potential targeting of fibrillarin is proposed. This could be particularly relevant, as fibrillarin is often overexpressed in TNBC and is associated with poor prognosis and risk of recurrence. So, in this work, the inhibition of ribosome biogenesis is achieved by targeting either its first step, through RNA Pol I inhibitors, or the downstream effector FBL, by gene targeting.

Several cytotoxic mechanisms have been described in other models upon treatment with RNA Pol I inhibitors and the elicited response seems to be strictly model-specific. At present, because the effect on the cells is unpredictable, a characterization of such response in TNBC is not unfounded.

Therefore, the two main objectives of this thesis are:

1. To study the sensitivity to inhibition of ribosome biogenesis in TNBC by either treatment with RNA Pol I inhibitors or FBL by gene targeting, demonstrating the relevance of ribosome biogenesis in TNBC and positioning FBL and rRNA maturation as new targets of therapeutic interest;



2. To determine the mechanisms inducing the cytotoxic effect of ribosome biogenesis inhibition in TNBC.

# Results

# **Article 1: Inhibition of ribosome biogenesis at multiple levels inhibits triple negative breast cancer tumor growth**

(in preparation)

# **Inhibition of ribosome biogenesis at multiple levels inhibits triple negative breast cancer tumor growth**

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## **Abstract**

Triple negative breast cancer (TNBC) represents a clinical burden characterized by a poor prognosis, resistance to treatment and high level of relapse. In addition, there is a lack of biomarkers and targeted therapies for this breast cancer subtype. High protein synthesis rate and ribosome production are hallmark of cancer, due to high metabolic and proliferation demand in tumor cells. Ribosome biogenesis is increased in tumors due to dysregulation of several oncogenic and tumor suppressive pathways that controls ribosome homeostasis in normal cells. Here we show that inhibiting ribosome biogenesis with RNA Pol I inhibitors inhibits growth of triple negative breast cancer cells regardless of their subtype, with EC<sub>50</sub> ranging from 10 to 500 nM. In addition, we show that inhibiting ribosome biogenesis at the post-transcriptional stage by targeting C/D box snoRNP factor Fibrillarin also represent an efficient approach to repress TNBC cells growth. Ribosome biogenesis elicited several cytotoxic responses including apoptosis and autophagy despite p53 pathway deficiency. Importantly, the impact of ribosome biogenesis inhibition was independent of the mTOR pathway. These results suggest that inhibiting ribosome biogenesis might be a promising therapeutic strategy for TNBC.

## Introduction

Triple negative breast cancers (TNBC) are characterized by the absence of overexpression of the estrogen receptor (ER), progesterone receptor (PR) and HER2. TNBC represents 15-20 % of breast cancer in women and is associated with a poor prognosis and high relapse rates (Dent et al., 2007). This unfavorable clinical presentation in part due to limited therapeutic options which are based on conventional chemotherapy, and to the limited response rate to these conventional neoadjuvant chemotherapy treatments (Liedtke et al., 2008). TNBC are also characterized by a high heterogeneity as reflected by the Lehmann classification into 6 subtypes (Lehmann et al., 2011). Importantly, a set of models TNBC cell lines could be assigned to each of the 6 subtypes and represent relevant model for pre-clinical studies (Lehmann et al., 2011).

The significant effort of the community to develop targeted therapy is reflected by numerous clinical trials investigating a wide range of targets including cell surface proteins (hormone and growth factor receptors), intracellular targets (apoptotic factors, signaling pathways), nuclear regulatory factors (stress response regulators, epigenetic regulators) and immune response targets (PD-1, CTLA-4, neuropilin-2). Favorable response rates are being reported and make targeted therapy a promising approach, despite the molecular heterogeneity of TNBC (Nakhjavani et al., 2019).

Increased ribosome biogenesis is a general trend in cancer cells (Ruggero, 2012b, 2013; Ruggero and Pandolfi, 2003; Whittaker et al., 2010), which is important for cell transformation and tumorigenesis (Barna et al., 2008; Montanaro et al., 2012; Pelletier et al., 2018). rDNA transcription is regulated through several signaling pathways that coordinate ribosome production with the cell physiological status and access to nutrients, including cyclins, growth factor pathways, mTOR pathway and major oncogenes and tumor suppressors including c-Myc and p53 (Campbell and White, 2014; Ciarmatori et al., 2001; Felton-Edkins et al., 2003; Grummt and Grummt, 1976; Mayer and Grummt, 2006; Piazzini et al., 2019; van Riggelen et al., 2010; White, 2005). In breast cancer, increased ribosome biogenesis is evidenced by changes in nucleolar morphology (Ceccarelli et al., 2000; Derenzini and Trerè, 1991). Direct contribution of ribosome production in mammary tumorigenesis is supported by studies showing that knockdown of MTG16a, a tumor suppressor which negatively regulate rDNA transcription repressor, leads to acquisition of transformed phenotype in human mammary epithelial cells. Conversely, knockdown of factors essential for ribosome production including RRN3 (Rossetti et al., 2016) or components of the C/D box snoRNP complex (Su et al., 2014) decrease the

tumorigenic properties of MCF7 cells. Alteration or upregulation of specific components of the RNA Pol I machinery was also reported in a majority (> 66%) of invasive breast carcinoma from TCGA database (Rossetti et al., 2016).

Targeting ribosome biogenesis as a strategy to therapeutically treat cancer as recently received increased interest following publication of proof of concept that pharmacological inhibition of RNA Pol I with small molecules CX-3543, CX-5461 and BMH-21 induce a selective anti-tumoral response (Bywater et al., 2012; Drygin et al., 2009; Peltonen et al., 2014). The anti-cancer activity of these compounds has been reported in various cancer types including leukemia, lymphomas and myelomas (Bywater et al., 2012; Hein et al., 2017; Lee et al., 2017; Negi and Brown, 2015b), prostate (Low et al., 2019), osteosarcoma (Li et al., 2016), ovary (Cornelison et al., 2017) and neuroblastoma (Hald et al., 2019). Inhibition of RNA Pol I with these compounds induced nucleolar stress response which resulted variable cellular cytotoxic effects including cell cycle arrest, apoptosis, senescence and autophagy. The type of cytotoxic response elicited by RNA Pol I inhibition appears to be pathology or cellular context dependent. For example, CX-5461 was shown to induce apoptosis in BJ-hTert or lymphoma (Bywater et al., 2012; Quin et al., 2016) but not in MiaPaCa-2 or HCT116 cell lines (Drygin et al., 2011). The molecular status of the cell was also shown to contribute to the cell response, as illustrated by studies on c-Myc activation in lymphoma and neuroblastoma (Bywater et al., 2012; Hald et al., 2019), p53 mutational status (Drygin et al., 2009; Quin et al., 2016) and BRCA1/2 mutational status (Xu et al., 2017b). Despite these dataset, no molecular profile emerged that would allow prediction of cell and tumor response to these RNA Pol I inhibitors, and more broadly to ribosome biogenesis inhibition.

There is currently no evidence indicating whether ribosome biogenesis inhibition might represent an efficient therapeutic strategy in TNBC. In this study, using a large panel of TNBC cell lines representing four different TNBC subtypes, we show that RNA Pol I inhibition by BMH-21 and CX-5461 compounds induces strong growth inhibition and loss of *in vitro* tumorigenic phenotype. TNBC cell lines displayed a large range of sensitivity ranging from 10 nM to 500 nM, although without association with the TNBC subtype. While BMH-21 induced apoptosis, no evidence of apoptosis was observed upon CX-5461 treatment. In addition, we show that inhibition of ribosome biogenesis at a post-transcriptional level, downstream of RNA Pol I, by knockdown of ribosomal RNA processing factor Fibrillarin, also induced a strong growth inhibition, associated with apoptotic response. The growth inhibition induced by ribosome biogenesis targeting was

not associated with expression levels of ribosome biogenesis factors nor with common mutations of TNBC cell lines. Altogether, these data suggest that inhibiting ribosome biogenesis might be a promising therapeutic strategy for TNBC.

## Results

### **There is no specific ribosome biogenesis signature in TNBC cell lines**

Given that TNBC is a very heterogeneous disease, we chose a set of TNBC cell lines representative of several TNBC subtypes as described by Lehman et al. (Lehmann et al., 2011). The panel included nine cell lines representative of the diversity found throughout TNBC, of which two belong to the basal-like 1 (BL-1) subtype, two to the basal-like 2 (BL-2) subtype, two to the mesenchymal (M) subtype and three to the mesenchymal stem-like subtype (MSL). In addition, two luminal breast cancer cell lines were included in the panel to take into account potential TNBC-specific features (see Materials and Methods section). First, in order to quantitatively characterize cell lines with respect to ribosome production, total RNA isolation was carried out from each cancer cell line and rRNA level was evaluated after separation through electrophoresis in agarose gel. The amount of rRNA per cell was found highly variable among cell lines, with an average amount higher in TNBC than in luminal cell lines (Figure 1A, Figure S1A). Such variability in ribosome production was also observed by FISH analysis targeting the nascent short-lived 5'ETS-containing pre-rRNA, the early precursor of 18S, 28S and 5.8S rRNA species, in five out of nine TNBC cell lines (Figure 1B). No association was observed between ribosome production and TNBC subtype (Figure 1A). To further characterize possible ribosome biogenesis pattern in TNBC cells, we decided to extend the study to multiple genes coding factors involved in different steps of ribosome biogenesis and to evaluate their mRNA expression levels. Using medium throughput RT-qPCR, mRNA levels of 21 ribosome biogenesis genes including RNA Pol I, RNA Pol I complex factors, and rRNA processing factors were determined and grouped by hierarchical clustering. This revealed a remarkable heterogeneity of the expression of ribosome biogenesis factors among cell lines (Figure 1C). Three clusters of genes exhibiting distinct variation among the cell lines were observed. Cluster 1, characterized by the largest variability and higher expression levels compared to the reference RNA (see Materials and Methods), mainly consists of genes involved in rRNA maturation, whereas Cluster 3 mainly contains genes coding for components of the RNA Pol I transcription machinery. Expression of cluster 3 genes is relatively consistent among the different cell lines, suggesting that the RNA Pol I transcription machinery exhibit similar level among the

TNBC cell lines. Cluster 2 contains only one gene, whose expression is not particularly variable (Figure 1C). Hierarchical clustering also shows that the 21 genes signature did not allow discrimination between the different TNBC subtypes, meaning that there is no specific ribosome biogenesis signature among the different TNBC cell lines. Thus there is no evident signature of ribosome biogenesis characteristics in TNBC cell lines.

### **TNBC cell lines are sensitive to inhibition of RNA Pol I**

In order to evaluate the antiproliferative potential of inhibiting ribosome biogenesis in TNBC, we used CX-5461 and BMH-21, two small molecules that selectively inhibit RNA Pol I-dependent transcription (Figure 2A) (Drygin et al., 2011; Peltonen et al., 2014). First, we verified that CX-5461 and BMH-21 inhibit rRNA synthesis by systematic observation of nucleolar size which is reduced upon RNA Pol I inhibition. Phase contrast observation of treated cells confirmed reduced nucleolar size upon CX-5461 and BMH-21 treatment (Figure S2A). Inhibition of pre-rRNA was also evaluated by FISH analysis using probes for the detection of the short-lived 5'ETS segment and ITS1 region, showing that pre-rRNA signal is almost abolished upon treatment (Figure 2B, Figure S2B). Next, impact of RNA Pol I inhibitor on cell proliferation was monitored using live cell imaging technology (IncuCyte) that evaluates cell viability of cultured cells by measuring confluence of cells attached to the well at multiple time points. We observed a clear growth inhibition, although with variable sensitivity to CX-5461 and BMH-21, depending on the cell line (Figure 2C, Figure S2C-D). RNA Pol I inhibition was also deleterious for colony formation capabilities of the TNBC cell lines, supporting an anti-tumoral effect upon ribosome biogenesis inhibition (Figure 2D). Next, we aimed to evaluate the sensitivity of the TNBC panel. For this purpose, we treated cells with a concentration range of either CX-5461 or BMH-21 spanning from 1 nM to 1  $\mu$ M to calculate the respective half-maximal effective concentration ( $EC_{50}$ ) relative to cell proliferation. This experiment revealed a 15-fold difference and an almost 4-fold difference between the cell lines less sensitive and most sensitive to CX-5461 and BMH-1, respectively (Figure 3A,C, Figure S2C-D). Sensitivity to CX-5461 or to BMH-21 was not found to significantly correlate with the mutational status of any of the most common breast cancer-associated oncogenes or tumor suppressors (Figure 3B,D, Figure S3A-B), nor with the expression levels of any of the analyzed genes involved in ribosome biogenesis or with rRNA levels (Figure S4). This indicated that sensitivity to RNA Pol I inhibitor is not dependent on the rate of ribosome production. Of



note, sensitivity to CX-5461 was not correlated to BMH-21 sensitivity, reflecting two different modes of action (Figure 3E).

### **Inhibition of rRNA synthesis induces p53-independent cell death**

Inhibition of ribosome biogenesis is well known to activate p53 functions through a process known as nucleolar stress, leading to cell cycle arrest and, potentially, cell death. All the analyzed TNBC cell lines, except for CAL51 cells, lack functional p53, reflecting the high p53 mutation rate in TNBC (see first gene column in Figure 3B,D and (Hollestelle et al., 2010; Lehmann et al., 2011). However, p53-independent mechanisms have been described that activate cell cycle arrest and apoptosis upon nucleolar stress (James et al., 2014; Russo and Russo, 2017). In order to know if apoptosis is activated as a response to RNA Pol I inhibition, TNBC cells were treated with CX-5461 or BMH-21 and a caspase activation assay was carried out. This revealed that caspases are activated upon treatment with BMH-21 but not with CX-5461. Of note, the relative level of caspase activation in CAL51 cells, sensitive to BMH-21, is two-fold higher than in MDA-MB-157 and HS578T cells that are more resistant to BMH-21 (Figure 3F), suggesting that apoptosis might be a significant mechanism in cytotoxicity of BMH-21.

Because cell cycle arrest is a main response activated upon nucleolar stress, we assessed the effect of treatment with RNA Pol I inhibitors on the distribution of cells in cell cycle phases. The analysis showed that treatment with either CX-5461 or BMH-21 generally leads to an accumulation of cells in G<sub>2</sub> phase, in a dose-dependent manner (Figure 3G). The extent of the effect on the cell cycle was variable among cell lines and did not seem to correlate with differential sensitivity to RNA Pol I inhibitors (see CAL51 and HS578T cells).

These results show that cell cycle arrest at G<sub>2</sub> phase is induced upon inhibition of rRNA synthesis through treatment with CX-5461 and BMH-21 in TNBC cell lines and that p53-independent response is elicited upon treatment with BMH-21, entailing the activation of caspase-dependent mechanisms, at variable extent depending on cell lines.

### **Inhibition of mTOR**

Because the mTOR pathway is often deregulated in TNBC (Marty et al., 2008; TCGA, 2012) and mTOR has been shown to coordinate ribosome biogenesis at multiple steps (Mayer and Grummt, 2006), we reasoned that differential mTOR pathway activation among TNBC cell lines might contribute to response to rRNA synthesis inhibition. In order to test such possibility, we first characterized TNBC cell lines with regard to mTOR

activation. Western blot analysis showed that RPS6, a major substrate of mTOR pathway kinase activity, is hyperphosphorylated in most of TNBC cells, and that the most important upstream activators of mTOR pathway, Akt and ERK1/2, both display deregulated activation (Figure 4A and Figure S5A). However, phosphorylated RPS6 levels did not correlate with response to RNA Pol I inhibition. Next, we compared EC<sub>50</sub> values relative to CX-5461 and BMH-21 with those relative to mTOR inhibitors used in anti-cancer treatment, such as albesilib, buparsilib and INK-128, and found no correlation between sensitivity to RNA Pol I inhibition and sensitivity to mTOR pathway inhibition (Figure 4B-G). Finally, we reasoned that concurrent targeting of RNA Pol I and mTOR signaling could enhance antiproliferative effect. To test this hypothesis, three TNBC cell lines, namely HS578T, HCC1937 and MDA-MB-157, were chosen for combined treatment targeting rRNA synthesis and mTOR signaling. HS578T and HCC1937 cells displayed the highest mTOR activation level; HS578T cells were the most resistant to RNA Pol I inhibitors, MDA-MB-157 and HCC1937 the most sensitive to CX-5461 among TNBC cell lines. The antiproliferative effect of CX-5461 was slightly improved in combination with INK-128 or rapamycin in HCC1937 and MDA-MB-157 cells (as much as 5-10% relative to treatment with CX-5461 alone) and only mildly improved in HS578T cells (as much as 20%) and similar results were obtained upon combination of BMH-21 and mTOR inhibitors (Figure 4H-J, Figure S5B-E), suggesting that both pathways do not functionally interact.

Altogether, these results indicate that mTOR pathway is not a primary driver of ribosome biogenesis in TNBC cell lines and that targeting mTOR pathway concurrently with rRNA transcription is not a viable strategy to reduce cell proliferation of TNBC cells.

### **FBL knockdown impairs proliferation of TNBC cells and induces cell death**

Our results showed that TNBC cell line proliferation can be variably reduced through inhibition of ribosome biogenesis, by targeting rRNA transcription, the first step of the process, and that this causes an increase in cell death in a p53-independent manner. Because some post-transcriptional steps of ribosome biogenesis are also essential for ribosome production, we wondered if inhibition of ribosome biogenesis at later steps of rRNA maturation can lead to similar effects. To this end, we focused on FBL, a major ribosome biogenesis factor that is required for the cleavage of the pre-rRNA as part of the C/D box snoRNP complex (Watkins and Bohnsack, 2012). We previously showed that FBL knock-down inhibits pre-rRNA cleavage and 18S rRNA maturation, and decrease ribosome production in human cells (Erales et al., 2017).

To evaluate the consequence of FBL knockdown on TNBC cells, we chose MDA-MB-231 and BT-20 cells and derived cell lines each containing a doxycycline-inducible cassette for the expression of a short hairpin RNA (shRNA) targeting either FBL (shRNA-FBL) or a control non-silencing shRNA (shRNA-NS) (see Materials and Methods). As expected, when the expression of shRNA-FBL was induced, FBL protein level was strongly reduced, in both MDA-MB-231 and BT-20 cell lines, as shown by Western blot analysis (Figure 5A, Figure S6A). Also, as expected, ribosome production was affected by FBL knockdown, with a reduction in rRNA amount compared with total RNA and a specific reduction of 18S rRNA amount compared with 28S rRNA (Figure S6B-C). In both cell lines, cell proliferation was reduced upon shRNA-mediated FBL knockdown compared to non-induced cells and induced shRNA-NS control cells (Figure 5B,E, Figure S6D-F). This observation was consistent with previous results obtained in luminal MCF7 cells (Su et al., 2014). In addition, we assessed the capacity of MDA-MB-231 cells to form spheroid structures and colonies at low density, two parameters associated with tumorigenic potential. Spheroid formation was impaired upon FBL knockdown, with an impact on spheroid size (Figure 5C). In addition, peripheral cells did not organized as in control spheroids, indicating that cells had lost some capabilities to organize themselves in three dimensions. Finally, colony formation assay revealed that FBL knockdown strikingly decreases the number of formed colonies (Figure 5D). Altogether, these observations show that targeting FBL bears an anti-tumorigenic potential in TNBC cells.

To investigate the causes of reduced proliferation, we first tested whether FBL knockdown could induce cell death, by labeling cells with the viability marker SYTOX Green. Cell death strikingly increased upon FBL knockdown in MDA-MB-231 cells and moderately increased in BT-20 cells, while cell proliferation was reduced (Figure 5E-F, Figure S6F-G).

These results show that FBL knockdown impairs cell proliferation, colony formation capabilities and spheroid formation, three parameters associated with tumorigenic potential, in both MDA-MB-231 and BT-20 cells, and that the decreased cell proliferation is associated to increased cell death.

Next, we extended the study to the cell lines from the TNBC panel. FBL protein levels were evaluated by Western blot analysis. This revealed detectable albeit moderate differences in FBL protein expression among cell lines. Worth of note, no significant difference was observed between normal and cancer cells, nor between non-TNBC and TNBC cancer cells, likely due to the proliferative state of the non-transformed cell lines

(Figure 6A, Figure S7A). FBL was knocked down by use of short interfering RNA (siRNA) specifically targeting FBL mRNA (siRNA-FBL) or a control sequence (siRNA-CT). Western blot analysis confirmed that FBL protein levels were reduced upon siRNA-FBL transfection, albeit at different levels among the cell lines (Figure S7B). The effect of FBL knockdown on cell proliferation was then evaluated and proved to be variable among cell lines (Figure 6B-C). Importantly, this difference could not be explained by a difference in FBL knockdown (see for example CAL51 and MDA-MB-157 cells). Thus, we concluded that cell lines are differentially sensitive to FBL knockdown.

Differential sensitivity to FBL knockdown could be explained by differential initial FBL expression. However, FBL protein expression levels were not found correlated with sensitivity to FBL knockdown (Figure 6D). Next, we interrogated whether inhibiting rRNA maturation had a comparable effect to inhibiting rRNA synthesis. FBL-sensitivity was shown not to significantly correlate with sensitivity to CX-5461 or BMH-21, although a tendency could be observed with CX-5461 (Figure 6E-F). Finally, sensitivity to FBL knockdown was not related to TNBC subtype (Figure S7C) nor with any genetic mutation characterized in the TNBC cell lines (data not shown).

### **FBL knockdown activates p53-independent apoptosis in TNBC cells**

Once demonstrated that FBL knockdown impairs cell proliferation in TNBC cell lines and several cellular functions associated to tumorigenicity in MDA-MB-231 and BT-20 cells, with concurrent increase in cell death, we focused on potential cell death mechanisms upon FBL knockdown.

Caspase activation assay revealed that siRNA-mediated FBL knockdown elicits a caspase-mediated response. The extent of such response is cell line-dependent, spanning from 1.5-fold increase in HS578T cells to 10-fold increase in BT-549 cells, compared to untreated cells (Figure 6G-K). However, differential extent of caspase activation is not sufficient to explain the variable FBL-sensitivity among TNBC cell lines. For instance CAL51 and MDA-MB-157 are very resistant and very sensitive to FBL knockdown, respectively, although they activate caspase at similar relative levels.

In conclusion, as these cell lines prevalently carry inactivating mutations of p53, the observed increase in caspase activation relies on a p53-independent mechanism. Therefore, targeting ribosome biogenesis by functional inactivation of FBL impacts cell proliferation and viability through p53-independent and caspase-dependent mechanisms.

## Discussion

TNBC represents today a clinical problem, being associated with a poor prognosis and high probability of cancer recurrence after treatment (Dent et al., 2007). This is in part due to limited therapeutic options and to the limited response rate to conventional chemotherapy treatments (Liedtke et al., 2008). In addition, TNBC is characterized by a remarkable heterogeneity (Lehmann et al., 2011), making it even more difficult to identify new markers for patient stratification and to find novel targets for the development of specific treatments. To improve the clinical outcome for patients affected by TNBC, it is urgent to identify biomarkers and to discover new therapeutic targets.

Recently, ribosome biogenesis has been proposed as a target for anti-cancer treatment. In particular, its inhibition by RNA Pol I inhibitors CX-5461 and BMH-21 proved to be effective in several cancers, including lymphoma, melanoma, osteosarcoma, prostate and ovarian cancers, in both p53-positive or negative genetic backgrounds (Bywater et al., 2012; Drygin et al., 2011; Fu et al., 2017; Li et al., 2016; Low et al., 2019; Peltonen et al., 2014).

In this study, we show that treatment with RNA Pol I inhibitors CX-5461 and BMH-21 could be effective for TNBC. Indeed, CX-5461 and BMH-21 at nanomolar concentrations cause a reduction in cell proliferation and tumorigenic potential in TNBC cell lines and induce p53-independent responses leading to cell death. In order to confirm such results *in vivo* and fully validate the effectiveness supporting CX-5461 and BMH-21 as compounds for a targeted anti-cancer strategy in TNBC, experiments in xenograft models will be needed.

From a clinical perspective, it is crucial to be able to predict the efficacy of a treatment depending on the features of a disease, in order to stratify patients and identify those who are likely to benefit from treatment.

In this study, we have not found any predictive criteria that could possibly contribute to stratify TNBC heterogeneity: sensitivity to CX-5461 and BMH-21 was not found to correlate with basal rRNA levels, expression levels of factors involved in ribosome biogenesis nor with any gene mutation commonly found in TNBC. However, the lack of significant correlation between sensitivity to treatment with RNA Pol I inhibitors and other clinically exploitable factors should not be discouraging. First, although our study was performed on a rather large number of cell lines compared to other studies, our data is based a population of too limited size to reach statistical significance. Yet, our results show some tendencies that could underlie biological relevance and guide future research on a

larger data set to achieve statistical robustness. Secondly, for our analysis we took into account the mRNA expression of factors involved in ribosome biogenesis, but not their expression at the protein level, yet. Because the expression of such factors could be regulated at the translational level, this deserves a further investigation. Third, we need to furtherly characterize the mechanisms of action of these inhibitors in TNBC cells. Indeed, it has been shown that sensitivity to BMH-21 is not merely associated to the basal expression level of its target RPA194, but rather with the extent of its decrease upon treatment, irrespective of the basal RPA194 protein expression (Peltonen et al., 2014). This would not constitute a clinically relevant factor, but would be a further advance for the understanding of the mechanisms that regulate the mode of action of BMH-21 that could shed light and pave the way for the finding of predictive factors. The same is true for CX-5461. CX-5461 has been shown to act by disrupting the multi-subunit SL1 factor binding to the rDNA promoter (Drygin et al., 2011). To our knowledge, the contribution of potentially different basal expression of SL1 among cancers or cancer subtypes has not been assessed yet. Analysis of SL1 composition, protein levels and binding to the rDNA promoter could help to understand the mode of action of CX-5461 at the molecular level and potentially provide insights for finding predictive criteria of sensitivity to CX-5461.

Finally, although previous studies have reported that sensitivity to CX-5461 is decreased in p53 mutant or null context (Bywater et al., 2012), and increased in BRCA1/2 deficient context (Xu et al., 2017b), in our analysis we did not find significant correlations with the mutational status of BRCA1 or p53, nor with any gene mutation commonly found in TNBC. Another important factor to considerate could be MYC status, as it has been described that MYC overexpression can be sufficient to sensitize human fibroblasts, normally resistant, to RNA Pol I inhibition and that overexpression of MYC correlates significantly with high sensitivity to CX-5461 in ovarian cancer cell lines (Hannan et al., 2013). However, we do not have available data about MYC status in TNBC to test this possibility. Of note, both CX-5461 and BMH-21 are DNA intercalator specifically targeting G-quadruplex DNA structures enriched in the rDNA gene promoters. The rDNA gene copy number is surprisingly variable among humans (Stults et al., 2008) and whether and how this might affect sensitivity to RNA Pol I inhibitors in patients is not at all understood. This could be a further factor to eventually take into consideration at the clinical level. Indeed, sensitivity could be related to the differential expression of other factors that were not included in our analysis. A screening taking into account transcriptomic and translatomic data among TNBC cell lines could highlight interesting correlations. In this regard, an

additional bioinformatic analysis will be performed on a data set of mRNA expression in the TNBC cell lines obtained using exon-array analysis.

So, although predictive criteria have not been found yet, our results point to the need to the extend data set for the identification of predictive criteria. In any case, a step has been done toward the validation of ribosome biogenesis as a therapeutic target in TNBC.

### **Starting to unveil mechanisms of response variability in TNBC**

The inhibition of ribosome biogenesis through molecules that target RNA Pol I activity has been shown to exert an anti-proliferative effect in cancer cells. However, the reported elicited cellular responses and cytotoxic mechanisms are variable among the studied pathological contexts and the induced response seem to be strictly model-specific and dependent from the genetic background. As already highlighted, TNBC is an overly heterogeneous disease. For this reason, a characterization of such response in TNBC is of great importance.

In our study, we assessed caspase activation levels and cell cycle arrest upon inhibition of ribosome biogenesis. We observed different responses depending on the specific treatment and cell line. In particular, a certain degree of variability in the levels of caspase activation is found among TNBC cell lines upon treatment with BMH-1 treatment. This had been previously observed only in ovarian cancer cells (Fu et al., 2017), while other studies only reported accumulation of cells in sub-G1 (Musso et al., 2018; Peltonen et al., 2014, 2014) or transcriptional activation of p53 target genes (Wei et al., 2018) upon treatment with BMH-21. Regarding the cell cycle, BMH-21 had been reported to induce accumulation in G2 phase in lymphoma and ovarian cancer cells (Fu et al., 2017; Musso et al., 2018) and not to impact G2 phase but rather inhibit the S phase in melanoma cells (Peltonen et al., 2014), suggesting that the effect on cell cycle is cell type-dependent. In our study, the increase of caspase activity in TNBC was accompanied by cell cycle arrest at G2/M phase upon treatment with BMH-21 in a dose-dependent manner. The extent of the effect seemed to be related to the effect on caspase activity, with cells activating caspases at the highest levels displaying also the most prominent effect on the cell cycle, and cells with relatively low or even no detected caspase activity displaying negligible effects on the cell cycle, consistently with a coupling between apoptosis and cell cycle arrest. In contrast with BMH-21, CX-5461 did not induce apoptosis. This behaviour was unpredictable, because apoptotic cell death was found upon treatment with CX-5461 in ovarian cancer cells (Cornelison et al., 2017), myeloma cells (Lee et al., 2017), leukemia and lymphoma cells (Bywater et al., 2012; Negi and Brown, 2015a; Quin et al., 2016), colon cancer cells (Xu et

al., 2017b) and neuroblastoma (Hald et al., 2019), but not in prostate cancer (Rebello et al., 2016), osteosarcoma (Li et al., 2016), melanoma or pancreatic carcinoma cells (Drygin et al., 2011). So, no increase in caspase activity was observed upon treatment of TNBC cell lines with CX-5461. In contrast, treatment with CX-5461 induced cell cycle arrest in TNBC cell lines, with accumulation of cells in G2 phase. Like in the case of BMH-21, the extent of the effect of CX-5461 on the cell cycle was roughly dose-dependent. Our data now adds TNBC as a cellular context in which CX-5461 does not induce apoptosis. Importantly, this study is only the second to clearly report apoptosis as a response to BMH-21. These results highlight differences in the modes of action of various inhibitors of ribosome biogenesis, consistently with previously published studies (Xu et al., 2017b).

### **Contribution of mTOR pathway**

In order to improve therapeutic benefit, CX-5461 has been successfully combined with the PI3K/AKT/mTOR inhibitor everolimus in MYC-driven B lymphoma (Devlin et al., 2016); with CX-6258, inhibitor of the modulator of translation PIM kinase, in advanced prostate cancer (Rebello et al., 2016); with doxorubicin in osteosarcoma (Li et al., 2016); with checkpoint inhibitor UCN-01 and several MAPK inhibitors in acute lymphoblastic leukemia (Negi and Brown, 2015b). On the contrary, to our knowledge, BMH-21 was never tested in combination with other drugs and our study is the first to explore this strategy.

In our study, we reasoned that differential mTOR pathway activation among TNBC cell lines might contribute to response to rRNA synthesis inhibition, because the mTOR pathway has been shown to coordinate ribosome biogenesis at multiple steps (Mayer and Grummt, 2006) and it is often deregulated in TNBC (Marty et al., 2008; TCGA, 2012).

Our results showed that mTOR pathway activation levels did not correlate with response to RNA Pol I inhibition in TNBC and that co-targeting of RNA Pol I activity and mTOR resulted in a mildly additive effect on cell proliferation, contrary to the other tested cell lines where the effect was negligible. Thus, our results suggest that mTOR only plays a limited role in resistance to treatment with CX-5461 or BMH-21 in TNBC. We did not check whether mTOR pathway is perturbed by inhibition of ribosome biogenesis, as shown in acute lymphoblastic leukemia, where it confers resistance to CX-5461 (Negi and Brown, 2015b). Further studies are actually ongoing to evaluate the activation of mTOR pathway upon inhibition of ribosome biogenesis to have a more complete view of the cellular effects of treatment with RNA Pol I inhibitors.



## **Validation of FBL as a therapeutic target**

While targeting RNA Pol I is arguably a logical approach to inhibit ribosome biogenesis, many other ribosome maturation steps and the factors responsible for them are essential for ribosome production (Tafforeau et al., 2013) and thus constitute potential targets. The idea of specifically targeting ribosome biogenesis at the level of maturation rather than transcription of rRNA has been first tested only recently: the interest of targeting U3 and U8 snoRNAs, required for rRNA maturation (Langhendries et al., 2016), as well as the components of C/D box snoRNPs, including FBL, NOP56 and NOP58, in breast cancer cells (Su et al., 2014) have been demonstrated.

Our results show that inhibiting ribosome biogenesis by targeting FBL function has an effective anti-tumorigenic potential, as assessed by means of several methods, including colony formation assay and spheroid formation, using especially MDA-MB-231 cells, and with promising indications for other TNBC cell lines. We showed that FBL knockdown impacts cell proliferation and elicits p53-independent caspase activity at variable extent in a cell line-dependent manner.

Here, we propose the targeting of FBL as an anti-cancer therapeutic strategy. A major advantage of FBL is that its overexpression is strongly associated with TNBC disease, implying that patients with FBL-overexpressing TNBC could benefit from treatments targeting FBL function (Nguyen Van Long, 2019). However, nor the FBL expression levels or the effectiveness of the protein depletion upon knockdown did seem to be predictive for response so far in TNBC cell lines.

Finally, FBL is one of the component of the C/D box snoRNP, and impact of U3/U8 or NOP56 and NOP58 knockdown should also be considered to further demonstrate the interest of targeting this complex to inhibit tumor cell growth in TNBC.

In conclusion, this work highlights the potential of targeting FBL in TNBC.

## **Materials and Methods**

### **Cell lines and cell culture**

Breast cancer cell lines (BT-474, BT-549, CAL51, HCC1937, HCC70, HS578T, MDA-MB-157, MDA-MB-231, MDA-MB-468, MCF7, SUM149PT) were purchased from ATCC. MDA-MB-231 and BT-20 cell lines expressing an inducible shRNA were generated by lentiviral infection. Lentiviral particles were produced using the pTRIPZ-shRNA-NS and pTRIPZ-shRNA-FBL-351067 vectors, which were acquired from Open Biosystems (Dharmacon). Upon lentiviral infection, cell populations were selected using puromycin at

2 µg/mL for 14 days. Cells were grown under selection at 1µg/mL and cultured without puromycin 24h before any experiment. shRNA expression was induced by doxycycline treatment for 4 or 7 days at 1µg/mL. Induction efficiency was monitored by evaluating the tRFP expression marker, by observation under fluorescent microscope or by flow cytometry. Change in FBL levels was verified by Western blot analysis.

All cell lines were maintained in 2D culture under 5% CO<sub>2</sub> atmosphere at 37°C. BT-474 and MDA-MB-468 cells were maintained in RPMI1640 Glutamax supplemented with 10% foetal bovine serum (Sigma); BT-549, HCC70 and HCC1937 cells in RPMI1640 Glutamax supplemented 10% foetal bovine serum, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1 mM sodium pyruvate; CAL51, HS578T and MDA-MB-157 cell in DMEM Glutamax supplemented with 10% foetal bovine serum; MDA-MB-231 cells in DMEM/F12 Glutamax supplemented with 10% foetal bovine serum; MCF7 cells in MEM (Eagle) L-glutamine (-) (Thermofisher) supplemented with 10% foetal bovine serum, 1% glutamax (Gibco), 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate and 0.1 mM non-essential amino-acids; SUM149PT cells in Ham's F-12 L-glutamine (+) medium supplemented with 5% foetal bovine serum, 10 mM HEPES, 1 µg/mL hydrocortisone, 5 µg/mL insulin. All culture media were supplemented with 1% penicillin/streptomycin (Gibco). All cells were replated every three to seven days and experiments were always carried out using cells with a passage number lesser than 30.

## Chemicals

CX-5461 (SelleckChem) was resuspended into 50 nM sodium phosphate buffer, as described in (Drygin et al., 2011). BMH-21 (Sigma-Aldrich) was resuspended into sodium acetate buffer composed as follows: 35 mM NaOAc, 162.8 mM NaCl, 1.75 mM EDTA, pH 5.0, as described in (Peltonen et al., 2014).

## siRNA transfection

For siRNA experiments, cells were transfected with either a mix three siRNA duplexes targeting FBL gene (namely 5'-GUCUUCAUUUGUCGAGGAA-AdTdT-3'; 5'-UGGAGGACACUUUGUGAUUUUdTdT-3'; and 5'-CUGUCAGGAUUGCGAGAGAdTdT-3'), or a control siRNA that does not target any human sequence (negative control siRNA duplex; Eurogentec). Cells were transfected using the INTERFERin<sup>®</sup> siRNA reagent (Polyplus) according to the manufacturer's instructions. Two or three days after siRNA

transfection, depending on the cell line, cells were replated into a 96-well culture plate to assess cell viability through IncuCyte® live imaging technology.

### **Cell proliferation and cell death assay**

Cells were plated into 96-well plates at a density adapted for every cell line. RNA Pol I inhibitors were added in triplicate and the cells were incubated for 96 h days. Cell viability was evaluated through IncuCyte® live imaging technology. The experiment was repeated two to four times per compound per cell line. and results are presented as % viability as compared to the control.

Cell death-specific marker SYTOX Green was added to the culture medium two hours prior to the time point of interest, following the manufacturers' instructions, and the number of dead cells was assessed and normalized relative to cell confluence in each culture well.

### **Cell cycle analysis by High Content Analysis**

Cells were seeded in 96-well plates and treated with RNA Pol I inhibitors as indicated. After 24 h, cells were fixed with 4% formaldehyde, permeabilized with 0.5 % Triton X-100. Cells were stained with Hoechst 33342 at 0.5 µg/mL in 1X PBS. For quantification by high content screening approach, plates were imaged on a Perkin Elmer High content analysis system Operetta CLS with a water x20 objective. Image analysis and quantifications were performed with the Columbus imaging software (Perkin Elmer). Data were processed in Microsoft Excel. Total Hoechst signal per nucleus was measured and nuclei were classified to establish intensity range of each cell cycle phase (G<sub>1</sub>, S-Phase, G<sub>2</sub>-M). The fraction of cells in each phase was determined automatically with Columbus software.

### **Fluorescent in situ hybridization**

Cells were plated either on coverslips at 50 000 cells per well in 24-well plates either at 1000 cells per well in 96-well plates, and grown and treated as indicated. Cells were fixed with 4% formaldehyde and incubated overnight in 70% ethanol. Hybridization was carried out in 10% formamide, SSC 1X, 0.5 µg/µL yeast tRNA, 0.25 µg/µL BSA, in presence of Ribonucleoside Vanadyl Complex RNase inhibitor, and 0.5 ng/µL of each fluorescent probe. Hybridization was carried out overnight at 37°C, and washed with 2X SSC, 10 % formamide. Nuclei were stained with Hoechst 33342 at 0.5 µg/mL, and cells were mounted with Fluoromount G. The probes were custom synthesized (Eurognetec) as follows: 5'-ETS1b FITC-AGACGAGAACGCCTGACACGCACGGCAC; 5'-ITS1 AlexaFluor® 555-

CCTCGCCCTCCGGGCTCCGTTAATGATC; 28S rRNA AlexaFluor® 647-CCCGTTCCCTTGGCTGTGGTTTCGCTAGATA. Cells were imaged using a Zeiss AxioObserver M2 with a x63 PlanApochromat objective. For quantification by high content screening approach, plates were imaged on a Perkin Elmer High content analysis system Operetta CLS with a water x20 objective. Image analysis and quantifications were performed with the Columbus imaging software (Perkin Elmer). Data were processed in Microsoft Excel.

### **Colony formation assay**

Cells were plated at 1000 cells per well in 6-well plates, in their corresponding medium. Cells were grown for 10 days. For shRNA-expressing cells, cells were cultured for 3 days in presence of doxycycline at 0.5 µg/mL before plating and cultured in presence of 0.5 µg/mL doxycycline during the colony formation assay as indicated. For treatment with Actinomycin D, CX-5461 and BMH-21, cells were allowed to attach and initiate growth for 3 days before being treated at the indicated concentration. After 10 days, cells were fixed with 4 % formaldehyde and stained with crystal violet.

### **Spheroid formation**

For spheroid formation assay, 2500 cells/well were placed in round-bottom ultra-low adhesion 96-well plates in their respective medium completed with 0.0625% Matrigel<sup>®</sup> (BD Biosciences) and cultured up to 8 days. Images were collected using a Zeiss AxioObserver with x10 objective. Phase contrast images and red channel fluorescent images to visualize tRFP expression were collected.

### **Western blot**

Twenty-five micrograms of total protein lysates were run on a 12% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 3% nonfat milk in TBST. The antibodies used were the following: anti-fibrillarin (Abcam, ab166630, 1/500), anti-β-actin (Sigma, A5541, 1/2000), anti-H3 (Abcam, ab1791, 1/1000), anti-mTOR (Cell Signaling Technology, 1/1000), anti-phospho-mTOR (Cell Signaling Technology, 1/1000), anti-RPS6 (Bethyl Laboratories, A300-557A, 1/1000), anti-phospho-RPS6 (Bethyl Laboratories, A300-584A, 1/1000), anti-ERK1/2 (Cell Signaling Technology, 1/1000), anti-phospho-ERK1/2 (Cell Signaling Technology, 1/1000), anti-Akt (Cell Signaling Technology, 1/1000), anti-phospho-Akt (Cell Signaling Technology, 1/1000). Antibodies were incubated for over night at 4 °C in 2.5% milk-TBST. Proteins were

detected by chemiluminescence using an anti-rabbit or anti-mouse peroxidase-conjugated antibody (Cell Signaling Technology, #7074 or #7076, 1/10 000), and Clarity ECL substrate (Bio-Rad), or by immunofluorescence using an anti-rabbit or anti-mouse fluorescent StarBright antibody diluted 1/5000. Images were collected on a ChemiDoc MP Imaging System (Bio-Rad), the signal analyzed using the Bio-Rad ImageLab software.

### **RNA isolation**

RNA was extracted from pellets of constant number of counted cells. TriPure reagent was added to pellets for cell lysis, followed by 5 minute long incubation at RT. Next, chloroform was added, samples mixed and centrifuged at 12000 g for 15 min at 4 °C. The aqueous phase was collected and mixed with 0.8 volumes of isopropanol, then mixed and centrifuged again at 12000 g for 15 min at 4 °C. The obtained pellet was washed twice with 70% ethanol and finally resuspended in pure water. 18S/28S rRNA and rRNA/total RNA ratios were calculated through TapeStation analysis.

### **Caspase activation assay**

Caspase activation was monitored using the Caspase-Glo 3/7 luciferase assay (Promega) according to manufacturer's instruction.

### **Gene expression analysis**

About 200 ng of total RNAs were reverse transcribed using PrimeScript Reverse Transcriptase (Takara) according to the manufacturer's instructions. Medium throughput RT-qPCR was performed as previously described (Nguyen Van Long et al., 2018). Briefly, cDNAs were pre-amplified during a multiplex PCR using primers of the 21 genes and Pre-Amp Master Mix (Fluidigm) followed by an Exonuclease I treatment (New England BioLabs). After 1:5 dilution in 1X Tris-EDTA buffer (Promega), gene expression was quantified by medium throughput real-time quantitative PCR using the HD Biomark system (Fluidigm) coupled with Master Mix 2X EvaGreen (BioRad) and the GE 96.96 Dynamic Array DNA Binding Dye Sample & Assay Loading reagent kit (Fluidigm). Relative fold-changes (FC) were calculated using the 2-DDCT method by the Fluidigm Real-Time PCR Analysis software (v 3.1.3, Fluidigm) and normalized with Xpress Ref Universal Total RNA (Qiagen) as sample control. The median Ct value of five housekeeping genes were used (*β-actin*, *GAPDH*, *HPRT1*, *PGK1* and *PPIA*). For each cell line, the three biological replicates were analysed in triplicate twice.

## Statistical analysis and graphics

All *P*-values corresponded to two-tailed *P*-values. A *P*-value < 0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism v7.0a softwares (GraphPad Software, Inc). For statistical correlation, non-parametric analyses were used and *P*-values and Spearman *r*-values are indicated for each graph. For comparison of sensitivities according to mutational status, Mann-Whitney test was carried out and *P*-values are indicated for each graph. Graphical representations were depicted using either GraphPad Prism software.

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## Figure legends

### Figure 1. Ribosome biogenesis in TNBC.

**A)** Normalized values of ribosomal RNA content as measured by analysis of RNA agarose gel. A representative example of RNA gel is shown in Figure S1A. Cultured cells were counted and a constant cell number of each cell line was used for trizol-based total RNA extraction. Cells are divided according to their subtype, indicated by the colour code. Mean and error bars are relative to three independent replicates. **B)** Normalized values of pre-rRNA levels as measured by fluorescent in situ hybridization analysis using a probe targeting 5'ETS, the 5' end of the pre-rRNA, that is cleaved and degraded early in rRNA biosynthesis. **C)** Fluidigm data relative to TNBC cell lines. Clustering trees of genes and cell lines is showed on the left and at the top of the heatmap, respectively. On the bottom, TNBC subtype is indicated next to cell line names. On the right, clustering groups are indicated as Cluster 1-3. The colour map (blue to red) refers to the abundance of mRNA relative to a standard RNA, as described in Materials & Methods.

### Figure 2. RNA Pol I inhibitors target nucleolus and impact cell growth

**A)** Molecular structures of CX-5461 and BMH-21. **B)** FISH results obtained by using probes targeting either 5'ETS or ITS1b in untreated HCC1937 cells and HCC1937 cells treated with 700 nM CX-5461 or 700 nM BMH-21 for 24 h or actinomycin D for 6 h. **C)** Cell proliferation curves obtained by treating four different TNBC cell lines with wide concentration ranges of CX-5461 or BMH-21, spanning from 1 nM to 1  $\mu$ M. Curves were obtained from Incucyte image analysis. **D)** Results of colony formation assay in five different TNBC cell lines 10 days either after treatment with 200 nM CX-5461 or 300 nM BMH-21 or left untreated. Actinomycin D was used as a negative control of clonogenicity.

### Figure 3. TNBC cell lines are differentially sensitive to RNA Pol I inhibitors

**A)**  $EC_{50}$  values relative to the effect of treatment with CX-5461 on cell proliferation curves. Cell lines are divided between non-TNBC (left) and TNBC (right) and sorted by  $EC_{50}$  value. **B)** Mutational status of the same panel of cells relative to a number of oncogenes and tumor suppressors commonly mutated in breast cancer. Each column represents a gene, with colour indicating mutational status (green: wild type, red: mutated, grey: data not available). **C)** Same as A but relative to BMH-21. **D)** Same as B but relative to BMH-21. **E)** Graph plotting  $EC_{50}$  values relative to CX-5461 with those relative to BMH-

21 in TNBC cell lines. **F)** Caspase activation assay in five different TNBC cell lines upon treatment with 200 nM CX-5461 or 300 nM BMH-21 for 24 h or treatment with actinomycin D or staurosporin for 6 h. Actinomycin D and staurosporin are used as positive control for induction of caspase activity. Values of caspase activation are normalized against untreated cells for each cell line. **G)** Portion of cells in G<sub>1</sub>, S and G<sub>2</sub> phase of the cell cycle upon treatment of five different TNBC cell lines with CX-5461 or BMH-21, each at two different concentrations.

#### **Figure 4. mTOR pathway activation status and targeting in TNBC**

**A)** Phosphorylation status of RPS6 in breast cancer cell lines. The histogram shows the ratio between phospho-RPS6 levels and total RPS6 levels in each cell line, evaluated through image analysis of the blot at the top of the figure. **B-G)** EC<sub>50</sub> values relative to CX-5461 and BMH-21 plotted against EC<sub>50</sub> values relative to alpasilib, buparsilib and INK-128 in TNBC cell lines. *P*-values and Spearman *r*-values relative to a non parametric test are indicated for each data set. **H-J)** Relative cell proliferation in HCC1937, MDA-MB-157 or HS578T cells either untreated or treated with CX-5461 alone, INK-128 alone or a combination of CX-5461 and INK-128 at the same concentrations as alone. Values were normalized against untreated cells.

#### **Figure 5. shRNA-mediated FBL knockdown impairs tumorigenicity of TNBC cell lines**

**A)** Western blot analysis showing FBL protein levels upon doxycycline-mediated shRNA induction in two MDA-MB-231 cell lines expressing shRNA targeting FBL (FBL-1 and FBL-2) and one cell line expressing shRNA-NS. **B)** Cell proliferation upon shRNA induction with doxycycline. **C)** Spheroid formation of MDA-MB-231 cells upon doxycycline induction of shRNA-NS or shRNA-FBL at day 2 and day 6 after seeding. Induced cells appear coloured because of concurrent expression of red fluorescent protein. **D)** Colony formation assay upon shRNA induction with doxycycline. **E-F)** Cell proliferation and cell death relative to non induced shRNA in MDA-MB-231 cells. Shown data for cell proliferation and cell death are representative of at least four independent experiments.

#### **Figure 6. siRNA-mediated FBL knockdown impacts TNBC cell proliferation and elicits caspase activity**

**A)** Histogram showing FBL protein levels in TNBC cell lines relative to the average expression level. Protein levels were calculated by image analysis of the Western blot

shown in Figure S6A as a ratio between FBL signal and total protein staining signal. **B-C)** Cell proliferation upon siRNA-FBL transfection relative to siRNA-CTRL transfection in luminal (B) and TNBC (C) cell lines. Cells are sorted by decreasing relative proliferation. **D)** Relative cell proliferation upon siRNA-FBL transfection plotted against relative FBL protein level (same values as histogram in A). **E-F)** Relative cell proliferation upon siRNA-FBL transfection plotted against EC<sub>50</sub> values relative to CX-5461 (E) or BMH-21 (F). **G-K)** Caspase activation assay in five different TNBC cell lines upon either siRNA-FBL or siRNA-CTRL transfection. Treatment with actinomycin D or staurosporin was used as a positive control for induction of caspase activity. Values of caspase activation are normalized against untreated cells for each cell line.

### **Figure S1. Ribosome biogenesis in TNBC.**

**A)** Representative gel showing total RNA levels extracted from a constant cell number of each cell line. Cultured cells were counted and the same number of cells was used for trizol-based total RNA extraction. Cells are divided according to their subtype, indicated by the colour code.

### **Figure S2. Cell proliferation upon treatment with RNA Pol I inhibitors**

**A)** Pictures of cells treated with CX-5461 or BMH-21 compared to untreated cells. Nucleoli appear as dense roundish spots in untreated cells, while they are clear and ring-shaped (MDA-MB-231) or strongly reduced (SUM149PT and HS578T) in treated cells. **B)** Amount of 5'ETS as measured by FISH analysis upon treatment with either CX-5461 or BMH-21 in four cell lines. Values are normalized against untreated cells for each cell line. **C-D)** Representative proliferation curves showing behaviour of different cell lines upon treatment with concentration ranges spanning from 1 nM to 1  $\mu$ M CX-5461 (C) or BMH-21 (D). Each curve is representative of at least two independent replicates.

### **Figure S3. Sensitivity to RNA Pol I inhibitors and mutational status**

**A-B)** Dot plot graphs comparing EC<sub>50</sub> values relative to CX-5461 (A) or BMH-21 (B) between TNBC cells carrying wild type genes and TNBC cells carrying inactivating mutation of the same genes. No correlations are statistically significant. *P*-values from Mann-Whitney test are indicated for data set.

### **Figure S4. Sensitivity to RNA Pol I inhibitors and expression of ribosome biogenesis genes**

**A-B)** EC<sub>50</sub> values relative to CX-5461 (A) or BMH-21 (B) plotted against gene expression levels relative to a selected set of ribosome biogenesis genes. No correlations are statistically significant. *P*-values and Spearman *r*-values relative to a non parametric test are indicated for each data set.

### **Figure S5. mTOR pathway activation status and targeting in TNBC (supplemental)**

**A)** Western blot analysis showing activation (phosphorylation) status of mTOR, ERK1/2 and Akt in breast cancer cell lines. **B)** Relative cell proliferation in HCC1937, MDA-MB-157 or HS578T cells upon treatment with either CX-5461 at different concentrations, INK-128 at different concentrations or a combination of the two. **C)** Relative cell proliferation in HCC1937, MDA-MB-157 or HS578T cells upon treatment with either BMH-21 at different concentrations, INK-128 at different concentrations or a combination of the two. **D)** Relative cell proliferation in MDA-MB-157 or HS578T cells upon treatment with either CX-5461 at different concentrations, rapamycin at different concentrations or a combination of the two. **E)** Relative cell proliferation in MDA-MB-157 or HS578T cells upon treatment with either BMH-21 at different concentrations, rapamycin at different concentrations or a combination of the two. In B-E values were normalized against untreated cells for each data set.

### **Figure S6. shRNA-mediated FBL knockdown impairs tumorigenicity of TNBC cell lines (supplemental)**

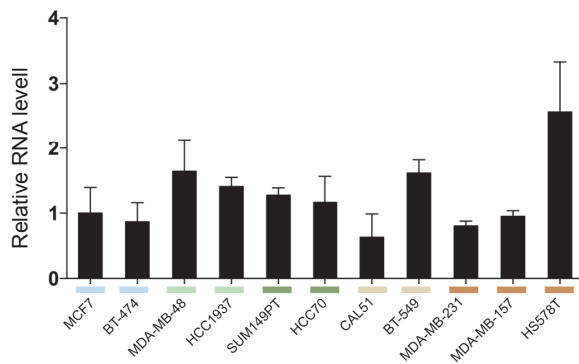
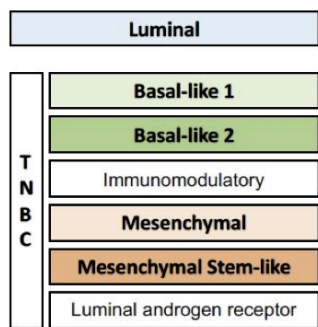
**A)** Western blot analysis showing FBL protein levels upon doxycycline-mediated shRNA induction in BT-20 cell lines expressing shRNA targeting FBL or shRNA-NS. **B-C)** rRNA/total RNA ratio and 18S/28S rRNA ratio upon shRNA-induced FBL knockdown in MDA-MB-231 cells. **D)** MDA-MB-231 cell proliferation upon shRNA induction with doxycycline. This is another representation of the same data shown in Figure 5E. **E)** BT-20 cell proliferation upon shRNA induction with doxycycline. **F)** BT-20 cell proliferation at 96 h upon induction of shRNA (same data as E). **G)** Cell death relative to non induced shRNA in BT-20 cells. Shown data for cell proliferation and cell death are representative of at least two independent experiments.

**Figure S7. siRNA-mediated FBL knockdown impacts TNBC cell proliferation**

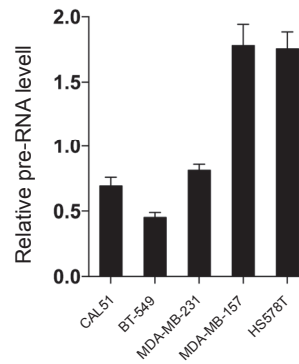
**A)** Western blot analysis showing FBL protein levels among TNBC. Protein levels calculated as a ratio between FBL signal and total protein staining signal in each cell line. **B)** Western blot analyses showing FBL protein levels upon transfection of either siRNA-FBL or siRNA-CTRL in breast cancer cell lines. **C)** Cell proliferation upon siRNA-FBL transfection relative to siRNA-CTRL transfection in TNBC cell lines. This represents the same data as Figure 6C but sorted by TNBC subtype.

**Figure 1**

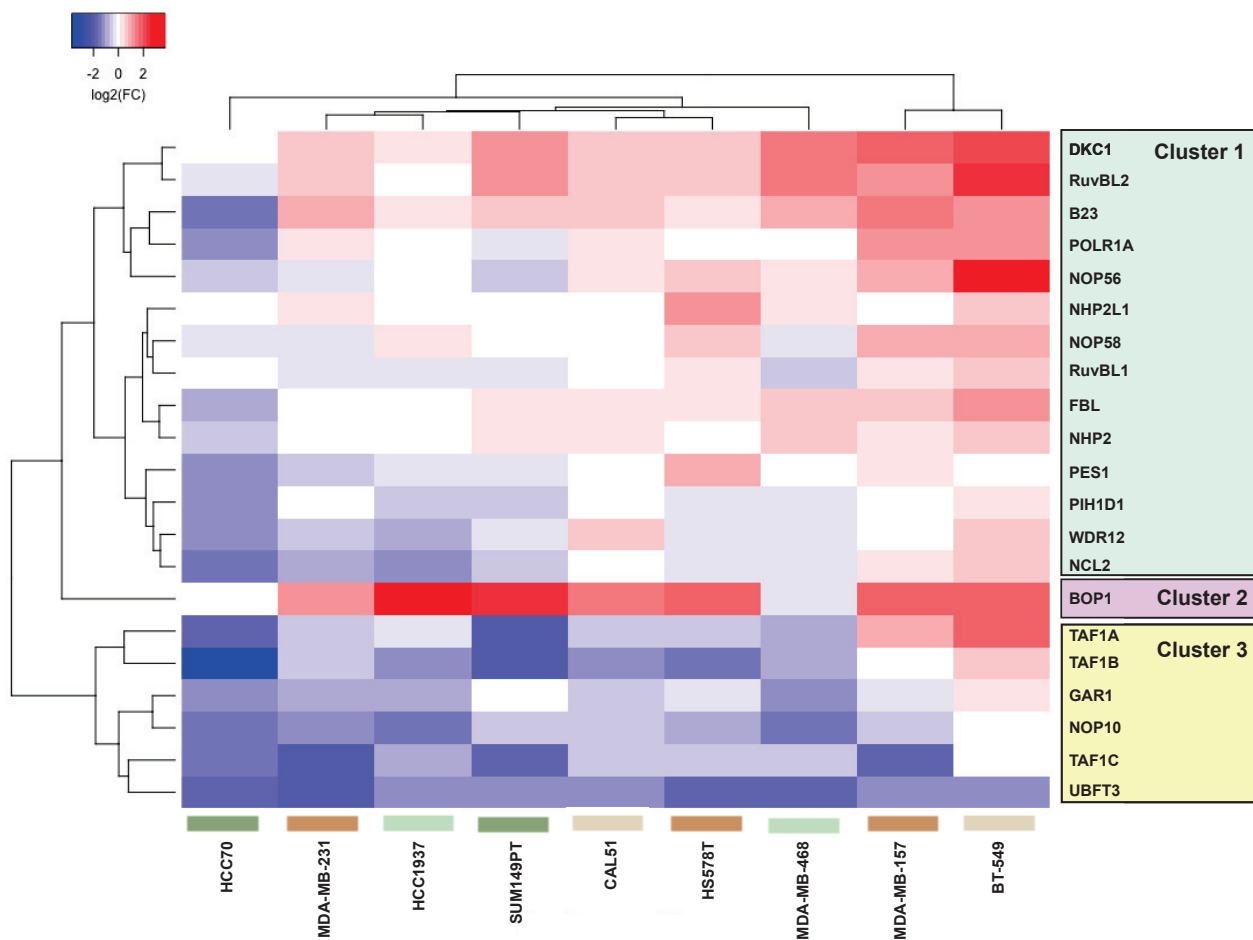
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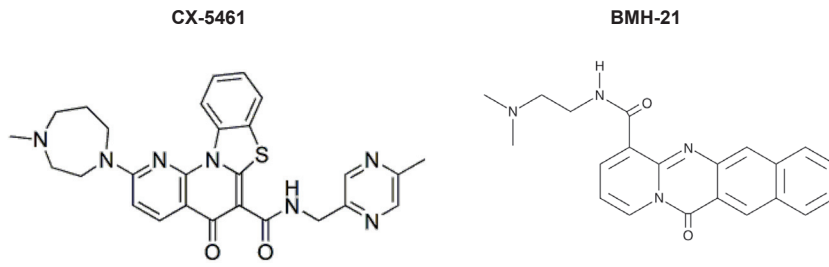


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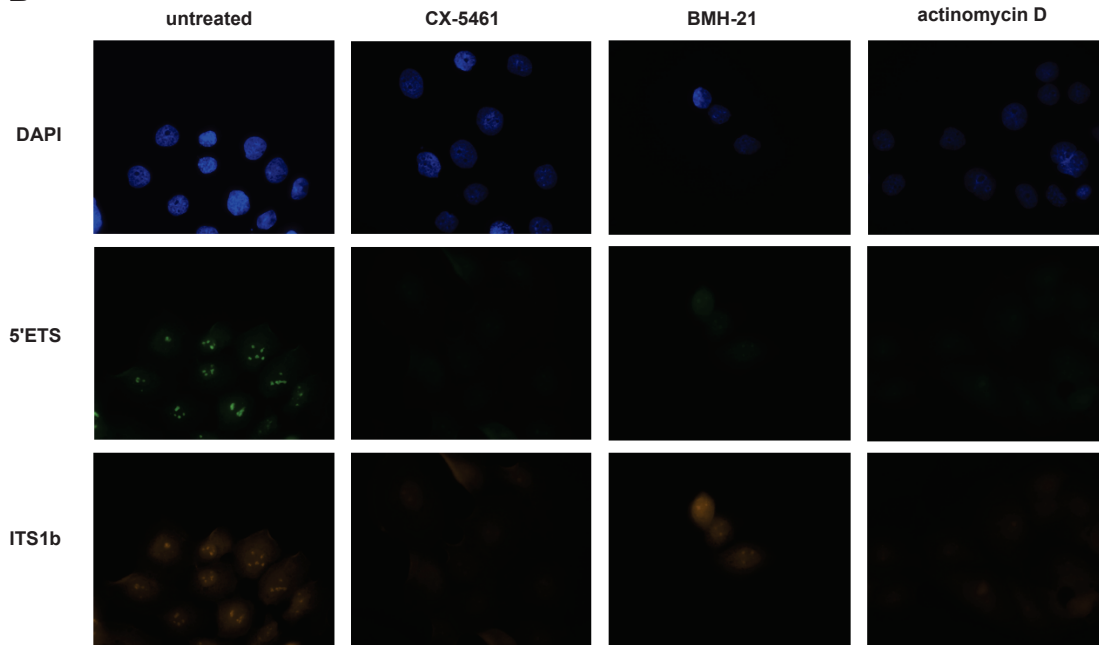


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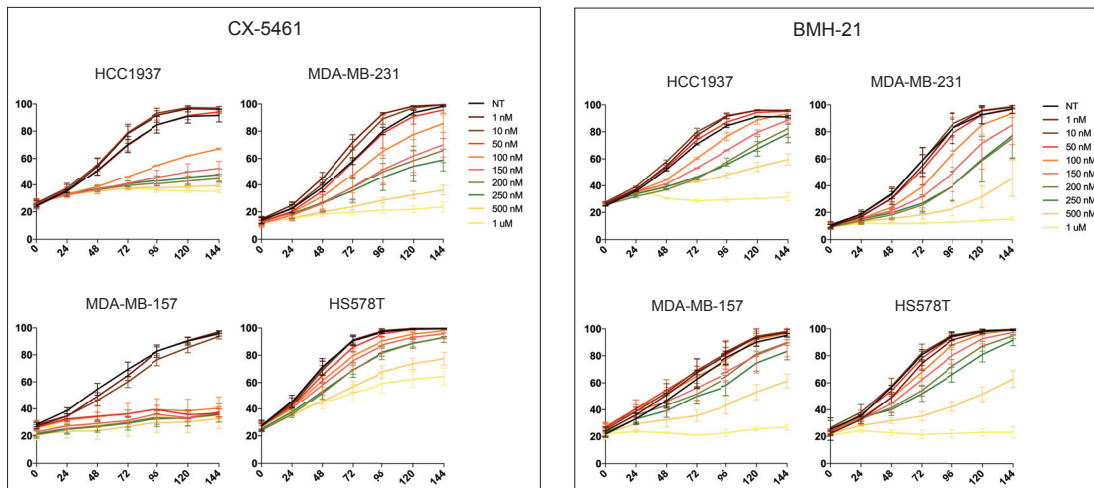
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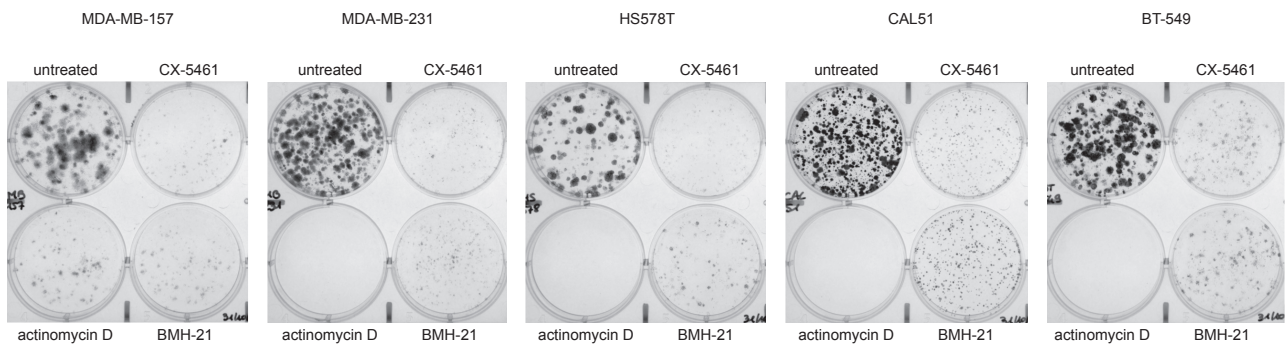
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**C**



**D**

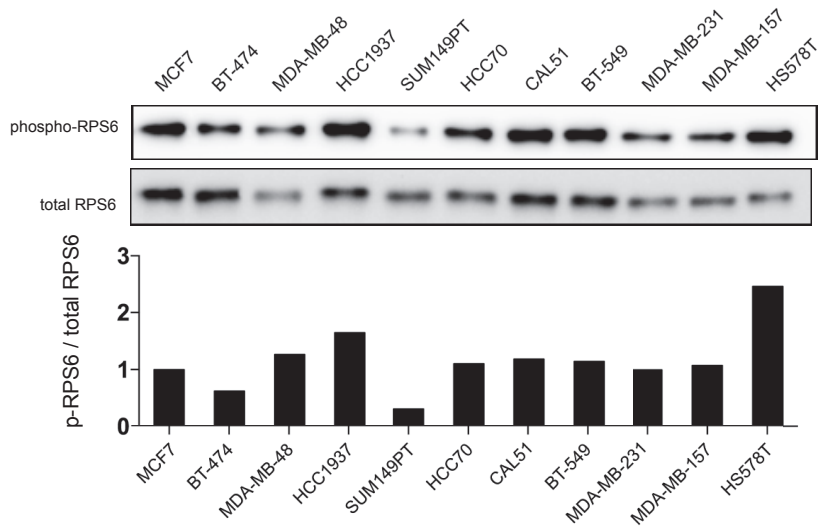




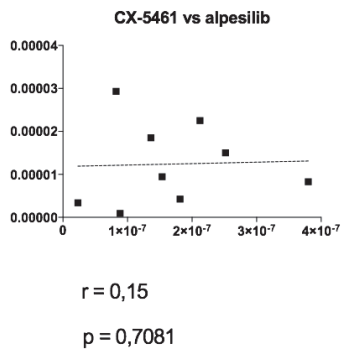


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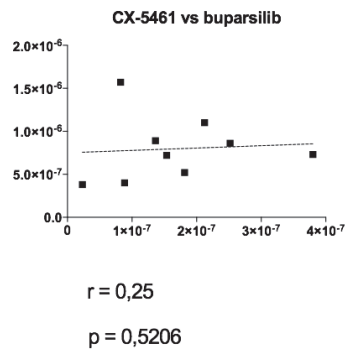
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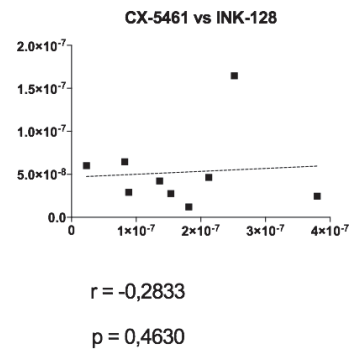
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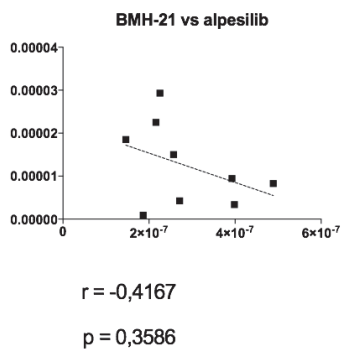
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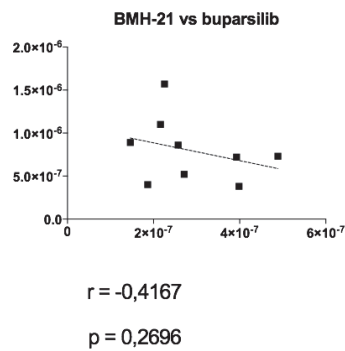
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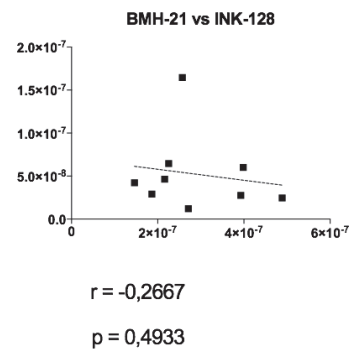
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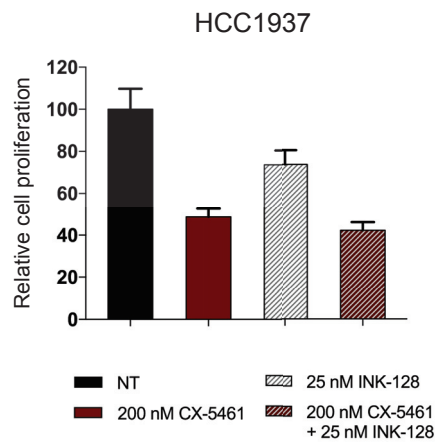
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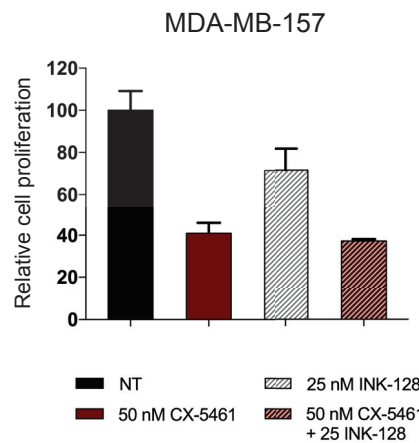
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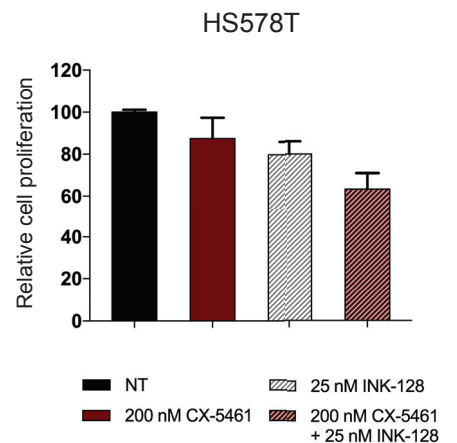
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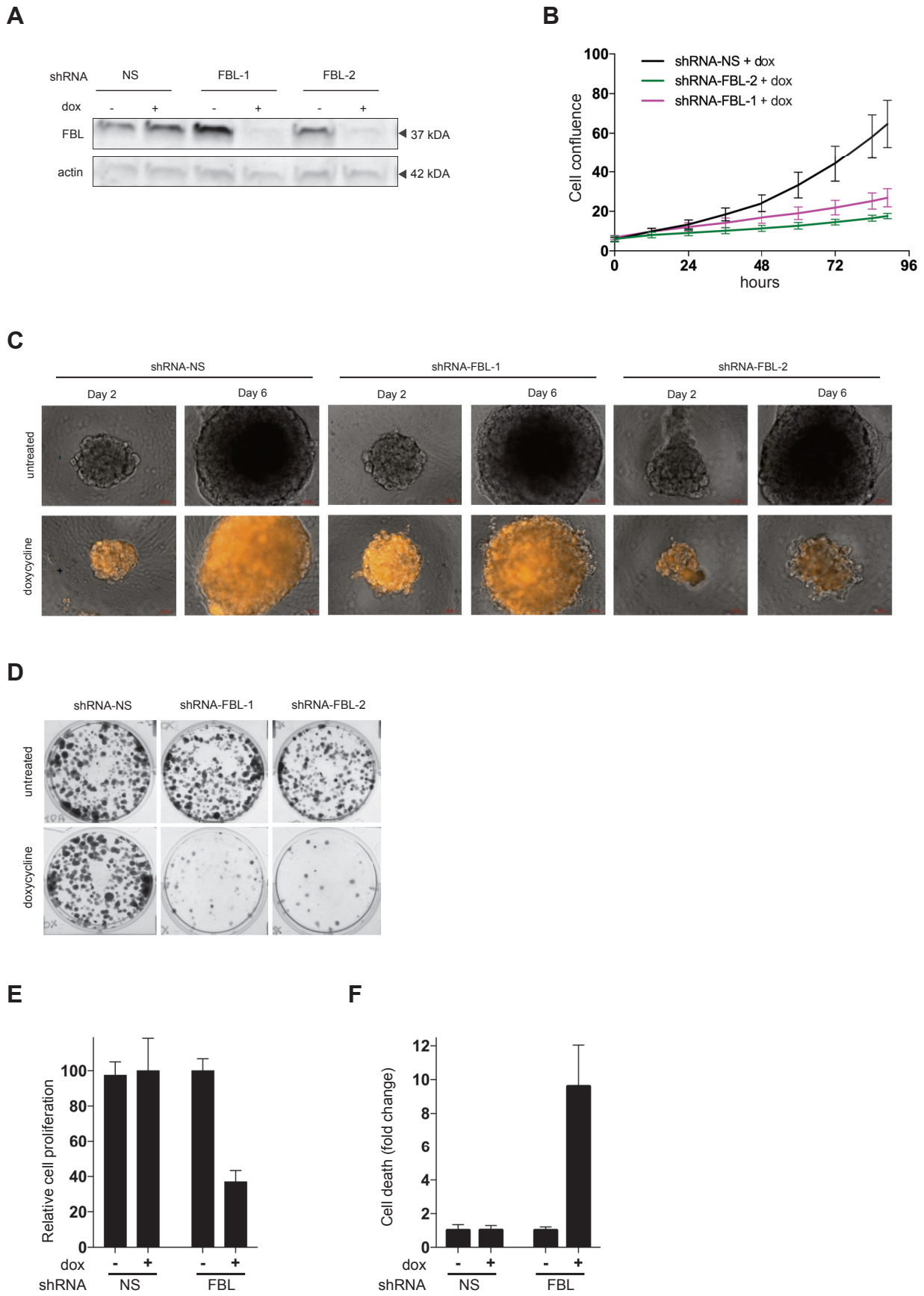
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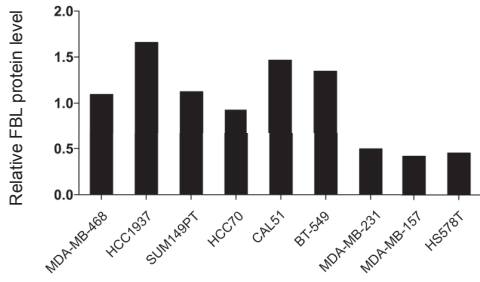


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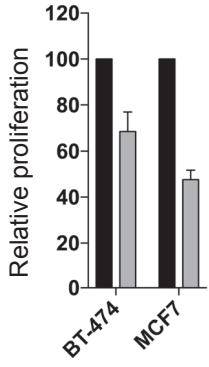


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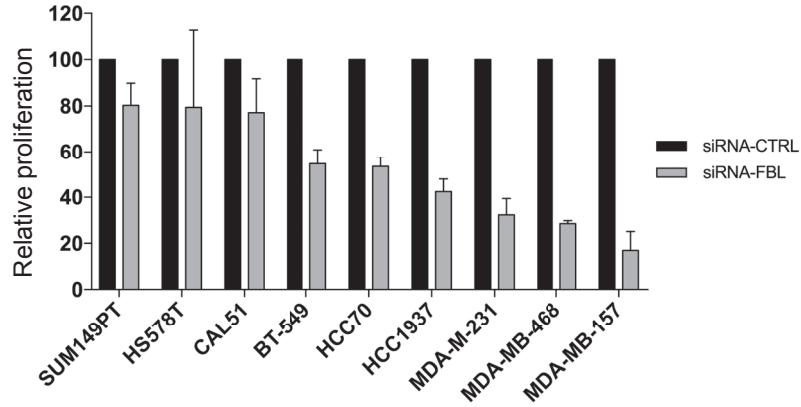
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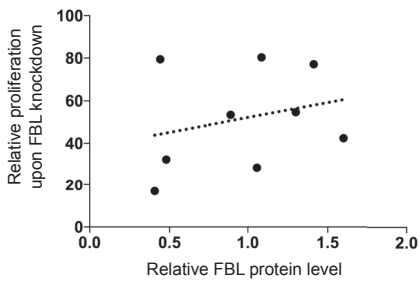
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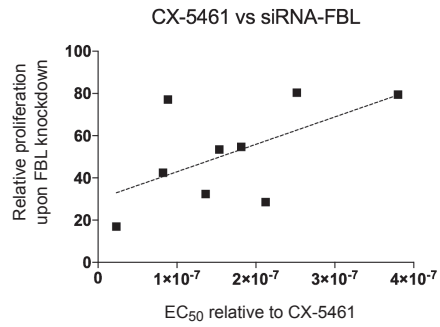
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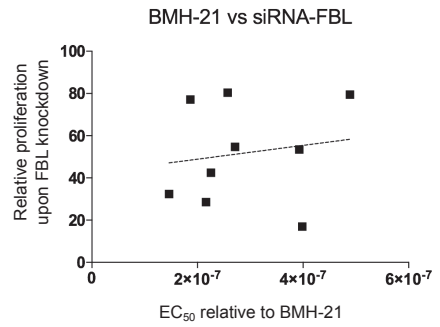
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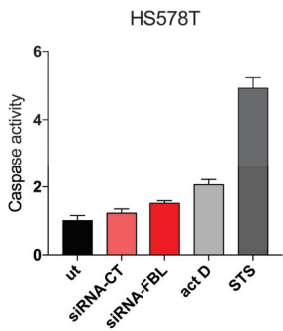
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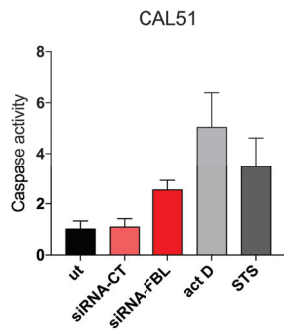
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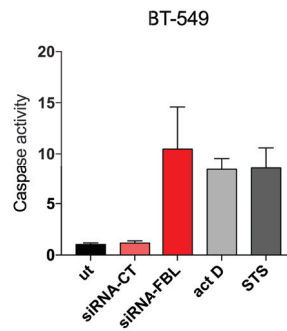
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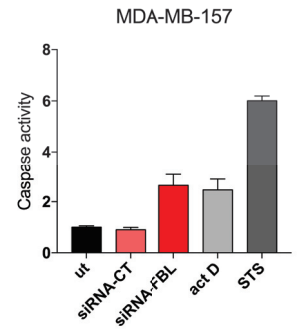
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**I**



**J**



**K**

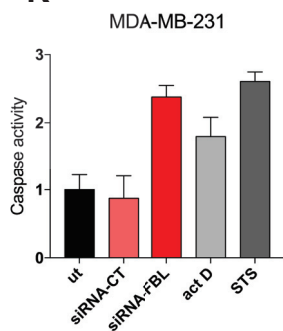
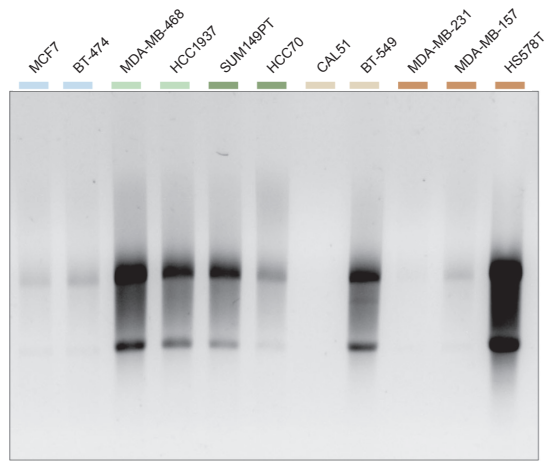
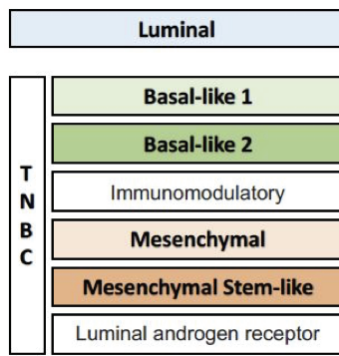


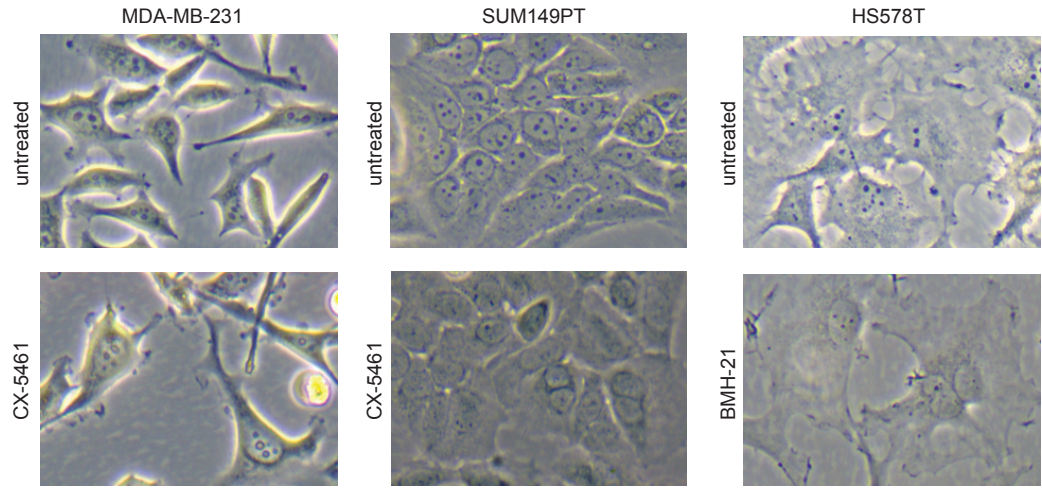
Figure S1

A



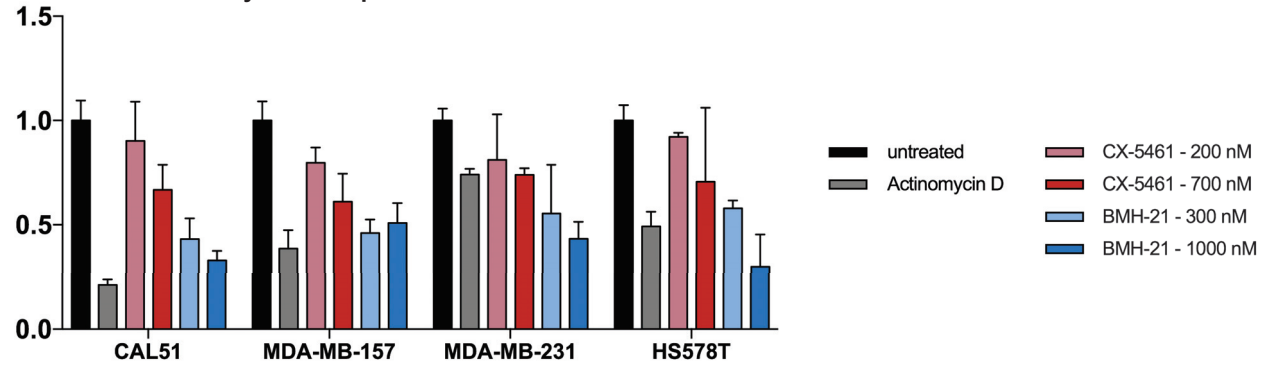
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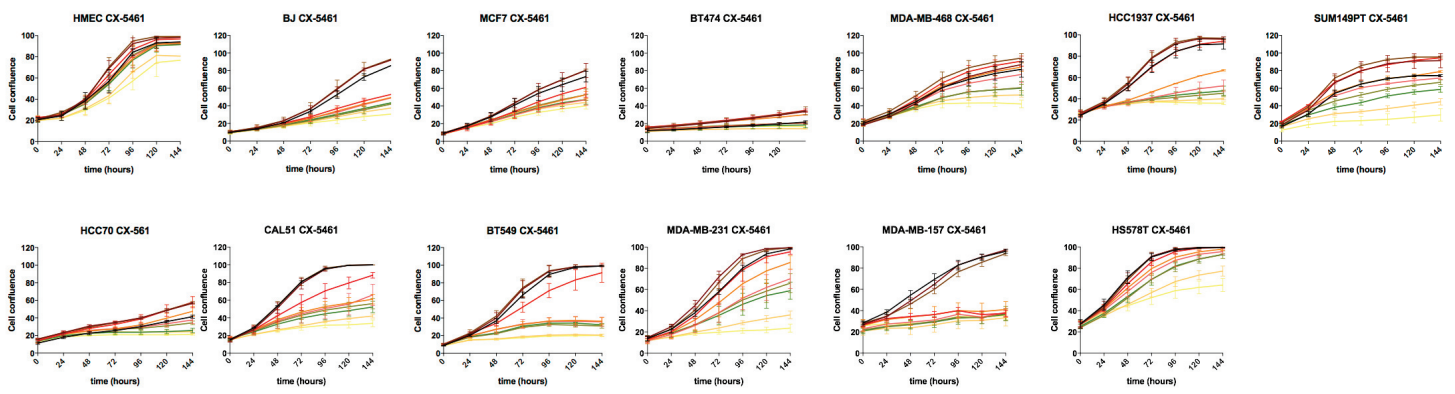


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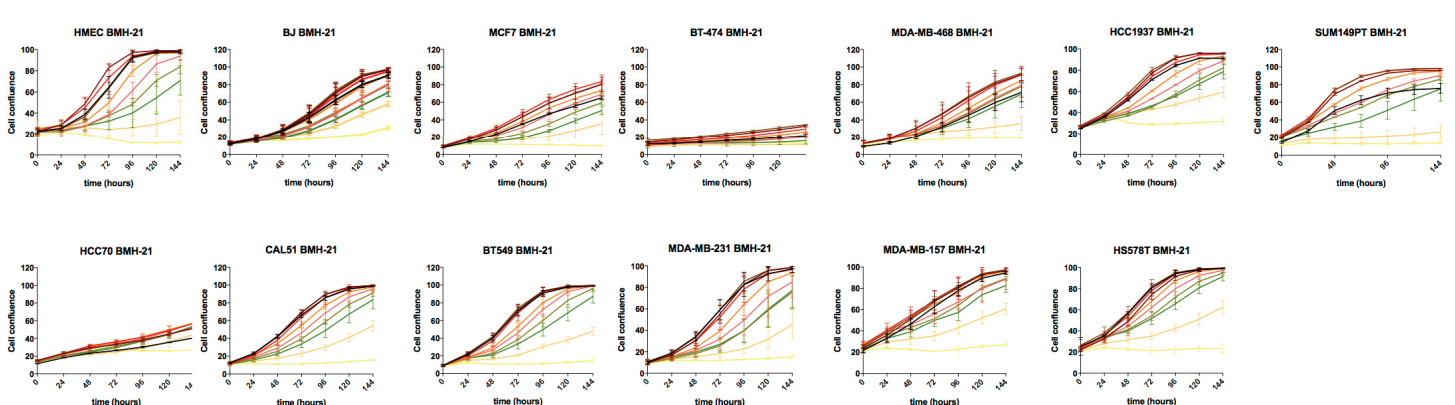
**rRNA synthesis upon treatment with RNA Pol I inhibitors**



**C**

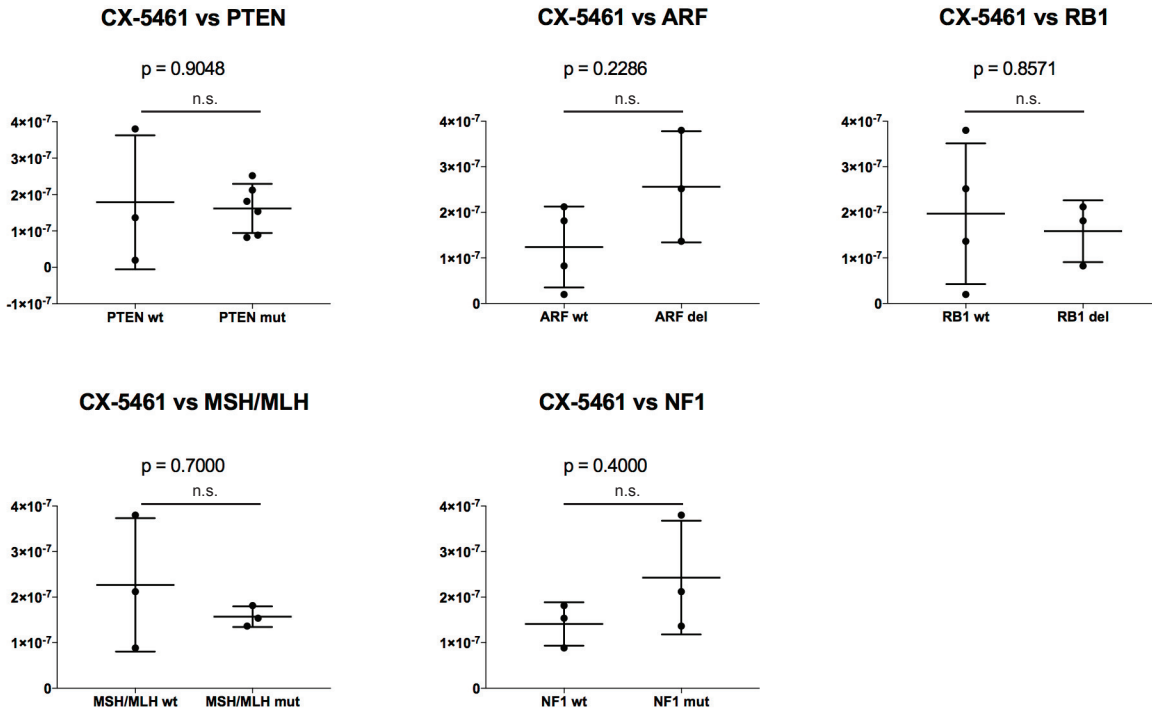


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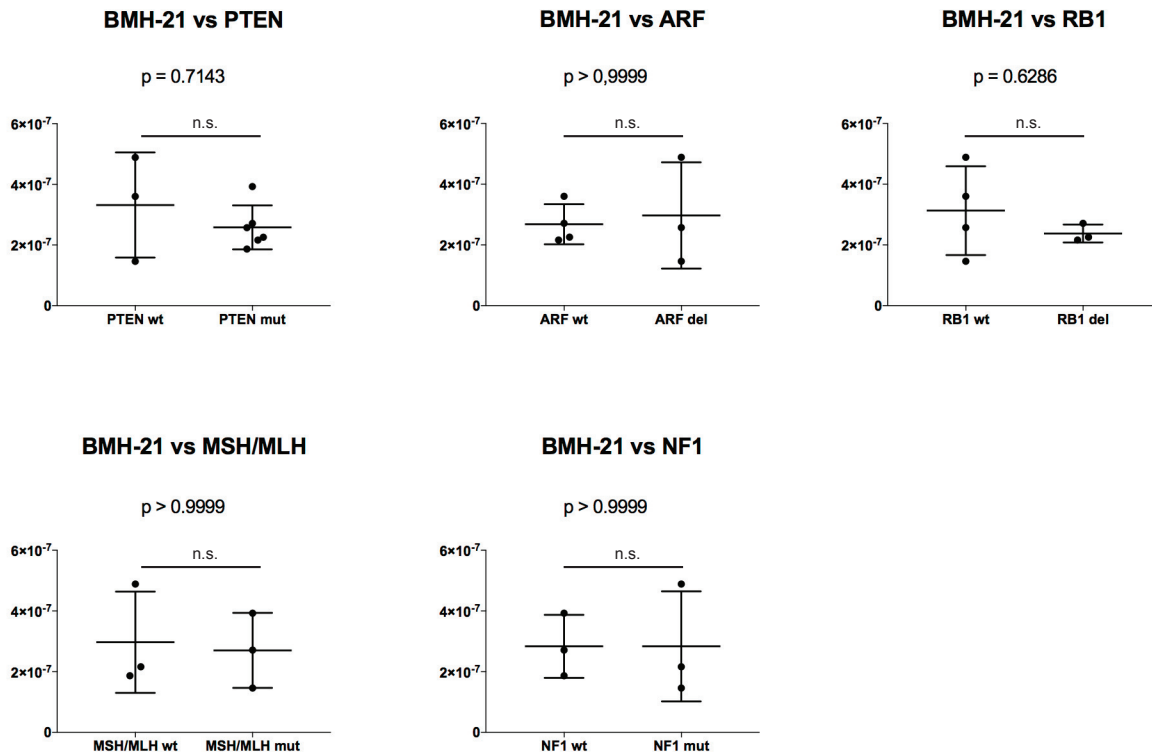


**Figure S3**

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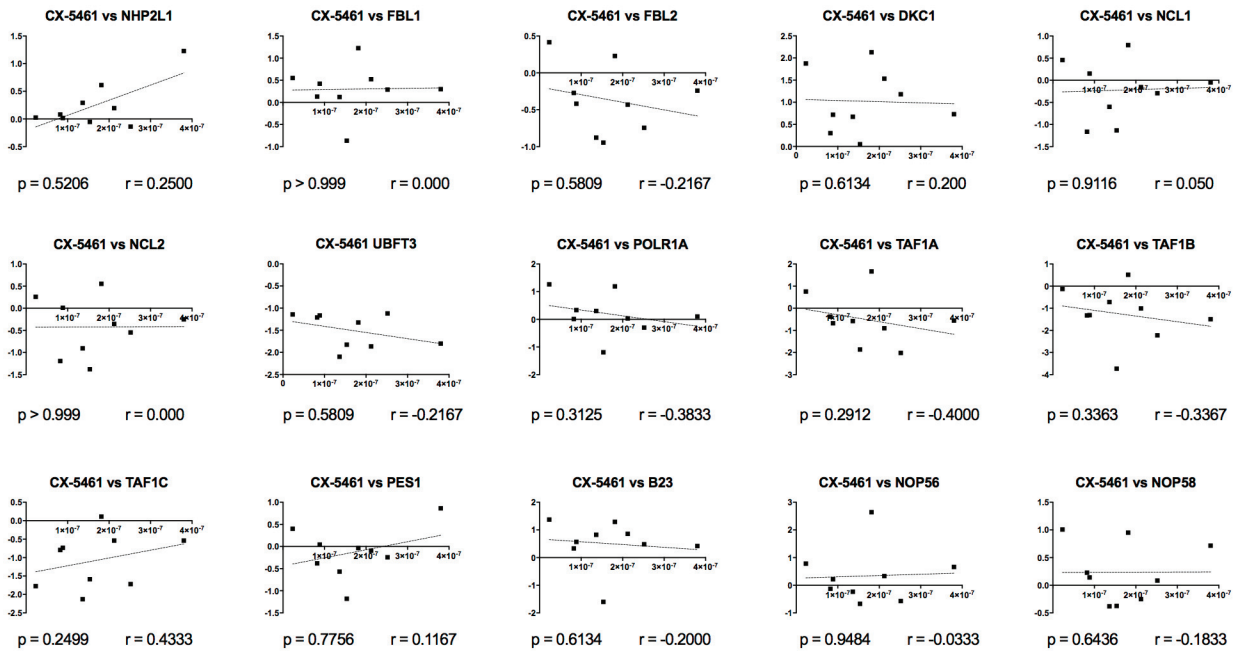


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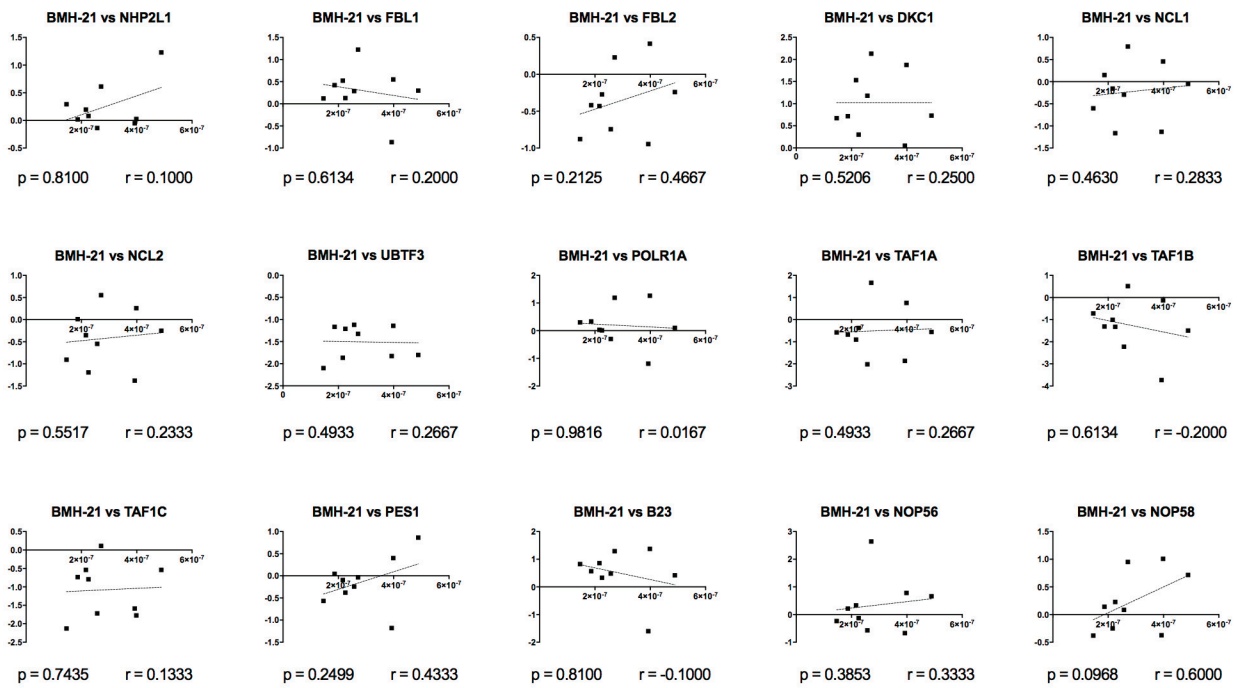


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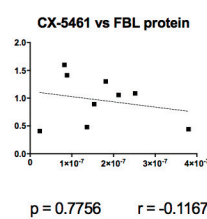
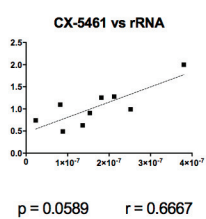
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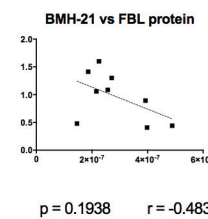
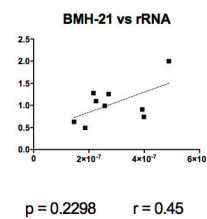
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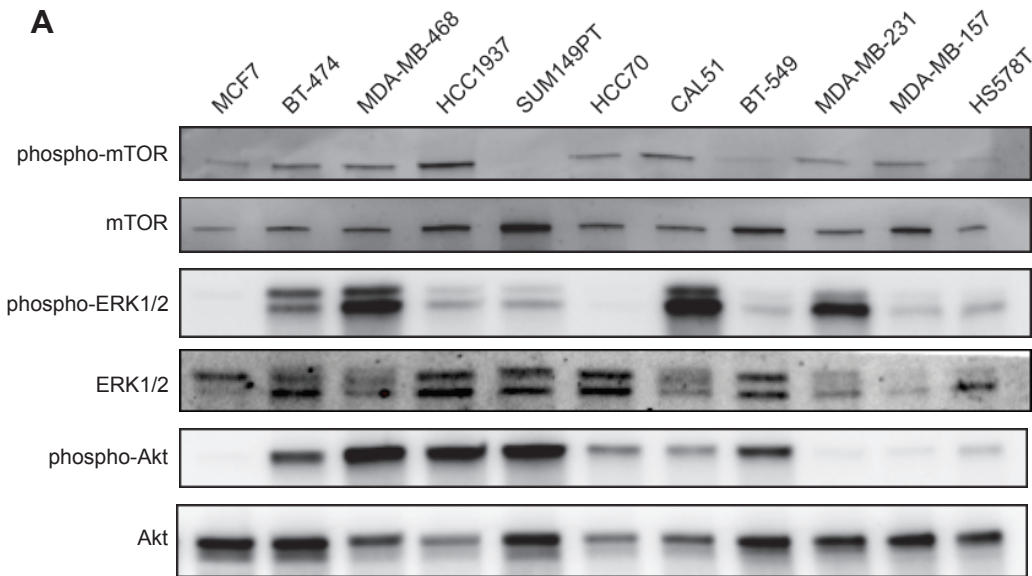
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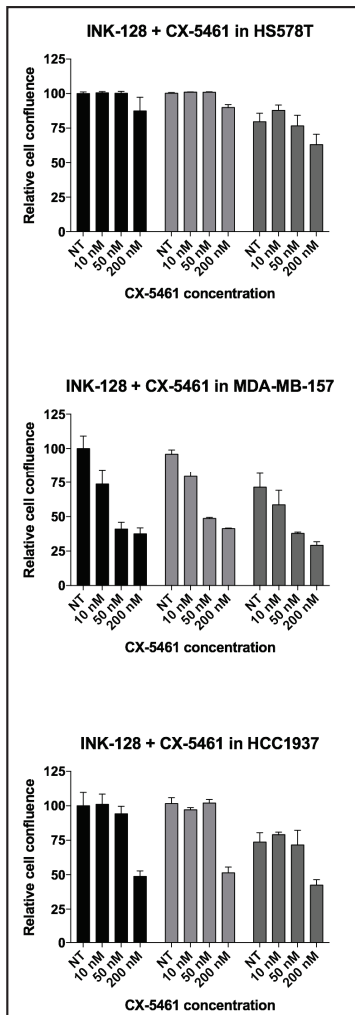


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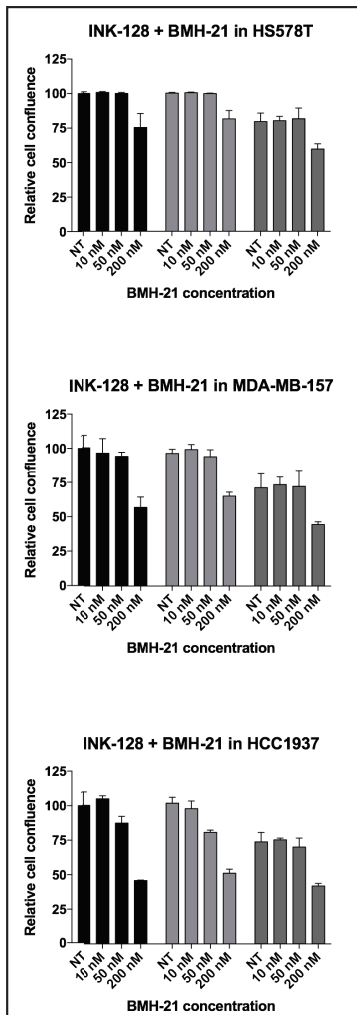
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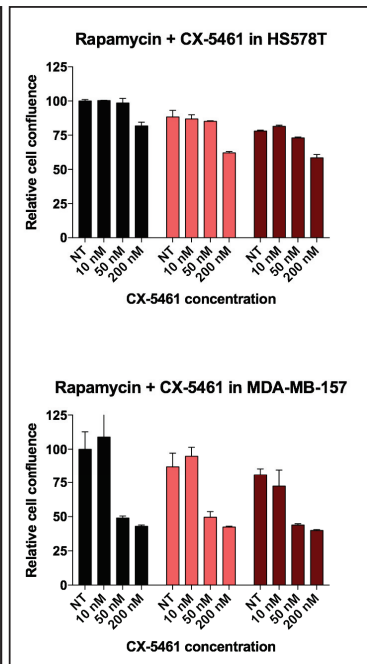
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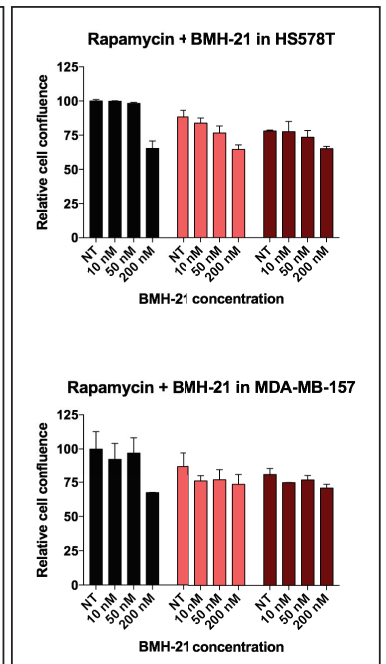
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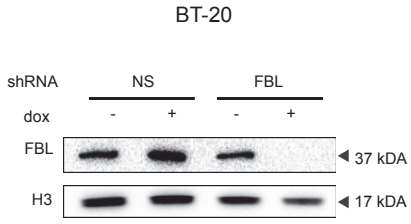
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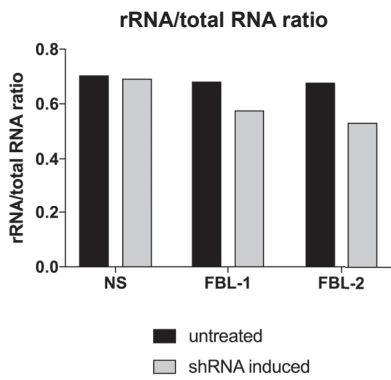
■ no rapamycin  
 ■ 10 nM rapamycin  
 ■ 25 nM rapamycin

**Figure S6**

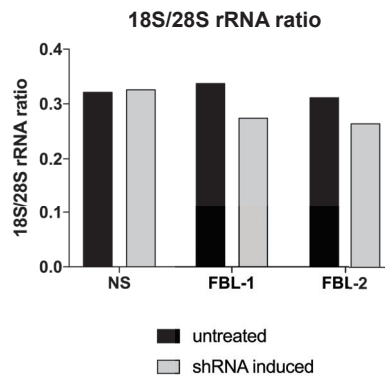
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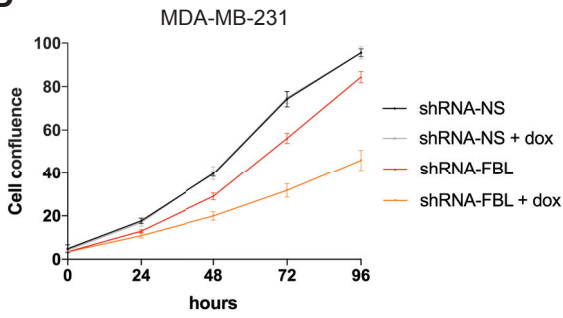
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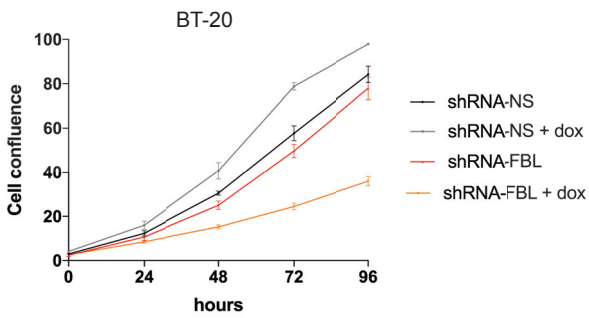
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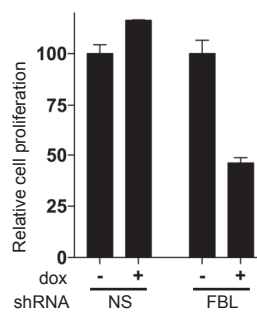
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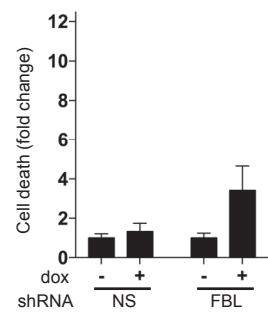
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**F**

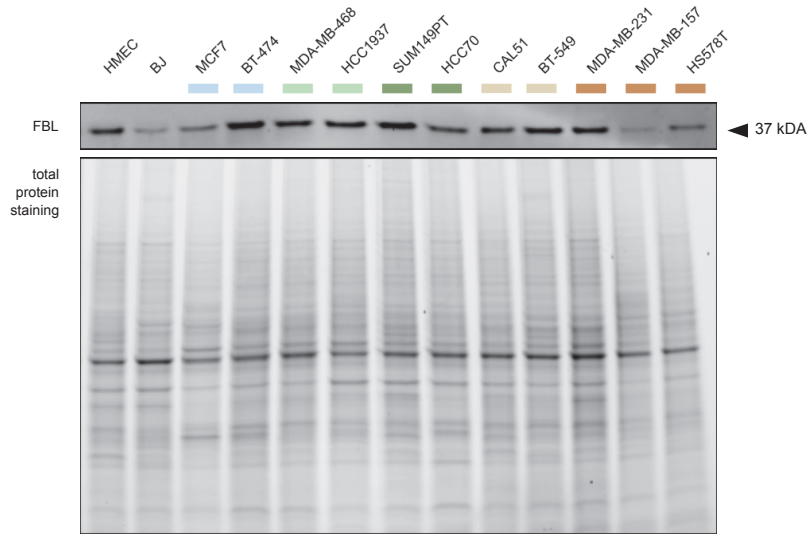


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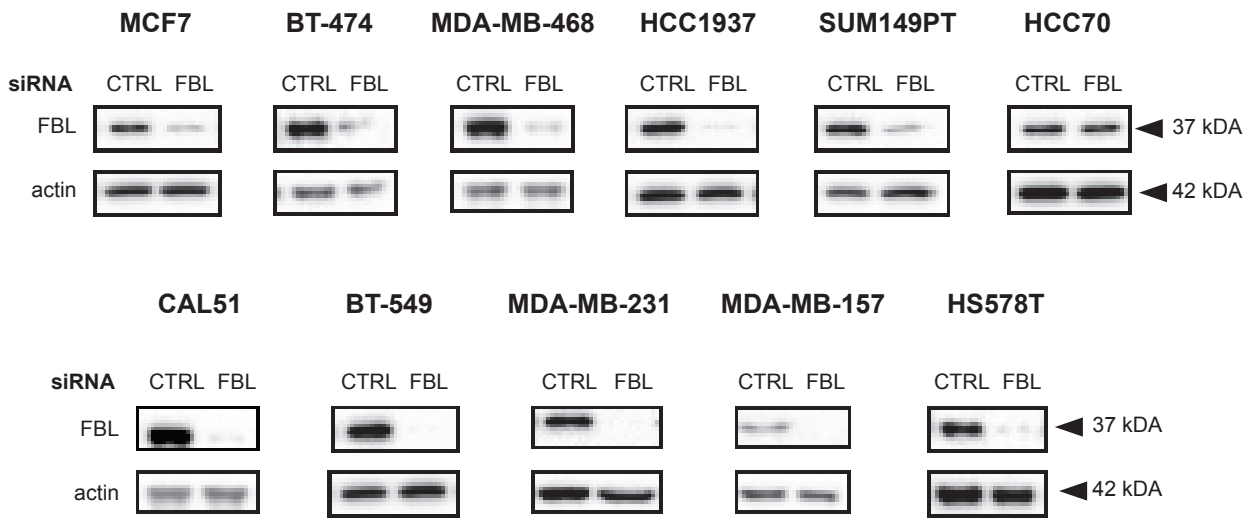


**Figure S7**

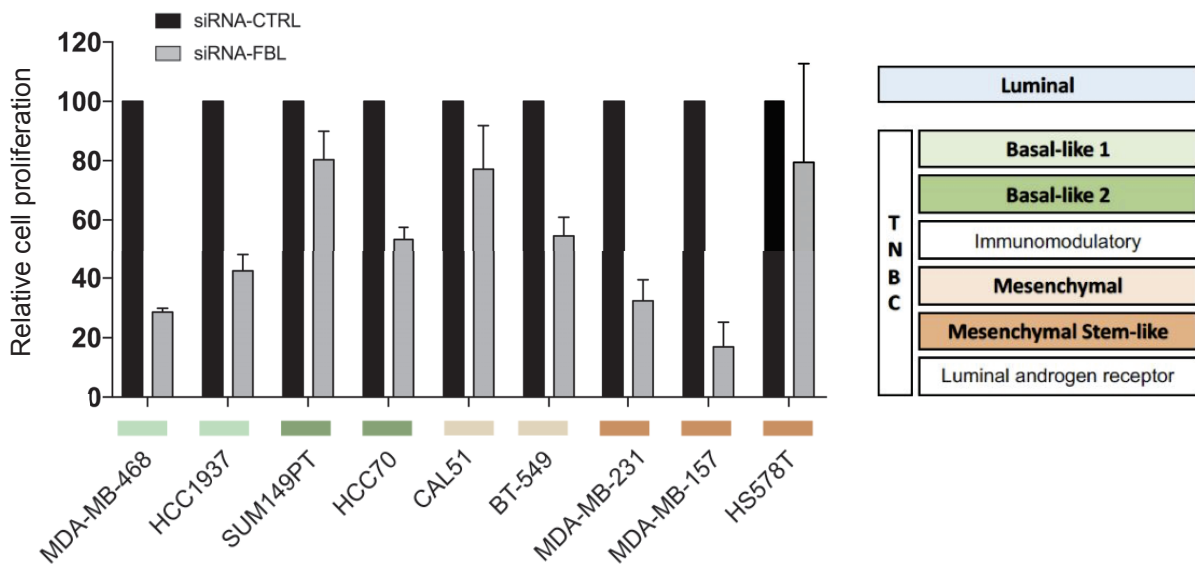
**A**



**B**



**C**



# Appendix to Article 1

The objective of my thesis project is to show that inhibition of ribosome biogenesis by targeting RNA Pol I and FBL has an anti-cancer effect in TNBC and to study the cytotoxic mechanisms involved. To this end, several experiments were carried out, in which cells were treated with CX-5461 or BMH-21, or rather underwent FBL knockdown, and a number of parameters were assessed. Most of these results were included into the manuscript presented above. For practical reasons, some of the experiments could not be included into a manuscript for publication. Nevertheless, some of them were selected to be shown beyond the publication format, because they may represent significant observations and are promising for future research on the topic. In particular, preliminary data will be shown that account for our work to evaluate the effect of the inhibition of ribosome biogenesis on the cell cycle.

Other results could not be included for strategical reasons, in particular within a translational research project that was developed at the same time as my thesis. In previous experiments, targeting of FBL was obtained by functional inactivation through gene silencing technology, thereby reducing FBL protein levels. In contrast, this project aims to validate the feasibility of inhibiting FBL by pharmacological approach targeting FBL protein activity through disruption of specific molecular interactions. In this context, much preliminary work was accumulated that still deserves further development in order to make a scientific publication on its own. Thus, complementary experiments need to be implemented, or previous data need to be confirmed, to highlight the biological relevance of this work. Here, in the second part of this section, some preliminary experiments will be described to give a flavour of this exciting research.

## 1. Preliminary data on cell cycle and autophagy induction upon inhibition of ribosome biogenesis

### Induction of p21

In nucleolar stress, transient expression of the p21<sup>Waf1/Cip1</sup> (p21) cyclin-dependent kinase inhibitor, encoded by *CDKN1A* gene, either triggers momentary G1 cell cycle arrest or leads to senescence or apoptosis. p21 is one of the main functional targets of p53, but recent studies showed that p21 expression can be induced in p53-independent manner during nucleolar stress in contexts lacking functional p53 (James et al., 2014; Russo and Russo, 2017).

Most of TNBC cell lines lack functional p53. Because p21 is known to be activated upon p53-independent stress, we evaluated p21 protein levels upon treatment with two different concentrations of CX-5461 or BMH-21 in TNBC cell lines, in order to study their effect on the regulation of cell cycle through a first experiment. Western blot analysis showed that p21 was induced only in CAL51 cells, bearing functional p53, as a result of p53 activation. In MDA-MB-231 cells, p21 is detected in all conditions, independently of treatment with RNA Pol I inhibitors, and the same is true for HS578T cells, albeit very faintly. In the remaining tested cell lines, p21 signal is not detected (Figure A1A). Similar results were obtained upon FBL knockdown, with clear p21 induction in CAL51 cells but not in other cell lines (Figure A1B). Thus, except for the only case of p53-positive TNBC cell line, p21 protein levels appear unchanged upon inhibition of ribosome biogenesis.

These results suggest that p21 is not induced in p53-deficient TNBC cell lines upon treatment with RNA Pol I inhibitors or FBL knockdown. However, the lack of detection or a very faint signal detection of p21 proteins in most cell lines may be due to a limited sensitivity of the method. Therefore, further studies are needed to confirm that p21 is unchanged in these conditions. For instance, because p21 is induced at the transcriptional level, quantitative analysis of *CDKN1A* mRNA could reveal potential transient induction difficult to detect at the protein level by Western blot analysis.

### **Set-up of flow cytometry analysis**

In parallel, flow cytometry-based analysis of the cell cycle was set up in order to evaluate potential effect on the cell cycle upon nucleolar stress in TNBC cell lines. In particular, the experiment was carried out using MDA-MB-231 cells expressing shRNA-FBL upon doxycycline treatment, leading to FBL knockdown, as described in the manuscript of the article to which this results are attached.

A preliminary experiment showed that the proportion of cells in G<sub>1</sub>, S and G<sub>2</sub> phases of the cell cycle are unchanged upon FBL knockdown (Figure A1C-D).

Although this experiment must be repeated and results confirmed, notably because the non induced shRNA-NS sample was degraded (it did not display the expected standard flow cytometry profile as shown in Figure A1D), this suggests that FBL knockdown does not lead to cell cycle arrest with concurrent accumulation of cells in G<sub>1</sub> phase. This is not consistent with previously published results showing that the inhibition of ribosome biogenesis at multiple levels results in such accumulation, due to the activation of a cell cycle checkpoint. However, this experiment was carried out only once, while more

replicates will be needed to confirm the preliminary result. In perspective, flow cytometry analysis will be eventually extended to the other TNBC cell lines of the panel, in order to investigate the effect of the inhibition of ribosome biogenesis, either through FBL knockdown or treatment with RNA Pol I inhibitors, on the cell cycle and potentially identify variable responses accounting for variable sensitivity among cell lines.

### **Induction of autophagy upon treatment with RNA Pol I inhibitors**

Inhibition of ribosome biogenesis through treatment with CX-5461 has been reported to induce autophagy but not apoptosis in osteosarcoma, pancreatic carcinoma and melanoma cells (Drygin et al., 2011; Li et al., 2016). Furthermore, pharmacological inhibition of autophagy in combination with standard chemotherapy has been already proved to be an effective strategy to improve treatment in TNBC cell lines by overcoming multidrug resistance mechanisms (Chittaranjan et al., 2014). Because our study revealed that apoptosis is not induced by treatment of TNBC cells with CX-5461 and because autophagy may be a general response mechanism upon inhibition of ribosome biogenesis, we aimed to assess the possibility that autophagy is induced upon treatment with CX-5461 and BMH-21 in TNBC cells.

In order to know if autophagy is induced, TNBC cells were treated with CX-5461 or BMH-21 and an immuno-fluorescence analysis was carried out using an antibody targeting an autophagy marker, LC3B. Because autophagy is a circular flux mechanism and perturbations of autophagy could alter the cycling flow rate without changes in the steady state, cells in all conditions were either treated with chloroquine autophagy inhibitor or not. Chloroquine, by impairing the recycling of LCB3, leads to its accumulation at a rate that is dependent from the flow rate of the autophagy flux. Thus, both steady state LC3B amount and its accumulation could be evaluated upon treatment with RNA Pol I inhibitors. This experiment revealed that LC3B steady-state levels are increased in CAL51, BT-549 and MDA-MB-157 cells upon treatment with RNA Pol I inhibitors (Figure A2A, plain colour bars). Concurrent treatment with chloroquine lead to a furtherly increased LC3B detection, also in MDA-MB-231 and HS578T cells that were roughly not sensitive to simple treatment with RNA Pol I inhibitors (Figure A2A, dashed colour bars). The extent of such changes was generally dose-dependent. These results suggest that autophagy is induced in TNBC cell lines in response to inhibition of ribosome biogenesis.

## **2. Pharmacological approach to target FBL function**

As previously described, FBL plays an essential role in the maturation of pre-rRNA, by mediating rRNA cleavage at the 5'ETS and ITS1 site and directing site-specific 2'-O-methylation of rRNA. In particular, FBL does not carry out these functions as a free factor, but within a complex containing NOP56, NOP58 and 15.5K core proteins as well as C/D box snoRNAs, named C/D box snoRNP complex. The formation of this complex requires the interaction of FBL with partners NOP56 and NOP58. To target such interaction thereby preventing C/D box complex formation and FBL function in pre-rRNA maturation, an inhibitory peptide approach is being tested in the group.

### **In silico analysis identifies inhibitory peptides targeting FBL-NOP56 interaction**

A study to evaluate the potential for pharmacological targeting of the FBL-NOP56 interaction was carried out in silico, in collaboration with B. Villoutreix (Inserm / Paris Diderot). This study characterized the interaction surfaces between FBL and NOP56, based on recently published structures that reveal the assembly of the snoRNP complex (Cheng et al., 2017), and provided a list of peptides that may interact with FBL or NOP56 preventing this specific protein-protein interaction. Such peptides would disrupt protein-protein interactions through molecular mimicry of the interacting surface of either FBL or NOP56. The sequence of the identified peptides cannot be disclosed because of patent rights.

### **Cell proliferation can be reduced through inhibitory peptides targeting FBL function**

In order to facilitate the penetration of inhibitory peptides into the cell, we synthesized the identified peptides coupled to TAT sequence, known to optimize internalization through the cell membrane. In addition, a set of peptides was covalently coupled to a fluorophore, to study the behaviour of the peptides and their dynamics in cellular systems.

In early experiments, three peptides were tested, namely F1, F2 and F3, mimicking in three distinct ways the molecular surface of FBL protein interacting with NOP56. I treated MDA-MB-231 cells with each of them, in their fluorophore-coupled (F1-F, F2-F, F3-F) or uncoupled form. Initially, cells were treated with peptides directly added into growth medium at high concentrations (up to 10  $\mu$ M). I did not report any effect on cell proliferation, and no specific intracellular fluorescence was detected (data not shown). I reasoned that a problem of inefficient internalization through the cell membrane or

instability within the cell could be the cause of the lack of fluorescence detection. To overcome a potential problem of internalization, I used an electroporation technology (Nucleofector) that produces transient destabilization of cell membrane structures to allow the transfer of small or large molecules into the cell. I carried out electroporation of MDA-MB-231 cells, concurrently treating them with either F1, F2 or F3 peptides or their fluorophore-coupled forms F1-F, F2-F, F3-F peptides. I observed a reduced cell number after electroporation with F3 and 3F-3 peptides, with a more pronounced effect for F3, relative to untreated cells and cells treated with other peptides (Figure A3A-G). Observation through fluorescence microscopy showed that F3-F, contrary to F1-F, is found within the cells and tends to accumulate at specific spots, corresponding to the nucleoli (Figure A3H-I). Live imaging technology (IncuCyte) was used to monitor cell proliferation after peptide electroporation and confirmed that F3 and F3-F have an inhibitory effect, reducing cell proliferation as much as 35-40%. Moreover, the effect of F3 seems to be dose-dependent, as it is displayed at 5  $\mu$ M but not 2  $\mu$ M concentration (Figure A3J-K).

Together, these results show that inhibitory peptides mimicking FBL protein to target FBL-NOP56 molecular interaction can reduce cell proliferation. Further studies will be needed to confirm that FBL-NOP56 interaction is disrupted in the presence of inhibitory peptides *in vitro* and *in vivo*, to characterize peptides with respect to molecular dynamics and stability.

In perspective, these studies will possibly validate the use of inhibitory peptides to impair cell proliferation through the inhibition of FBL function and will eventually pave the way for the development of a pharmacological approach targeting ribosome biogenesis in anti-cancer treatment.

## Materials and methods

### Western blot analysis

Western blot analysis was performed as described in the Materials and Methods of Article 1. Anti-fibrillarin (Abcam, ab166630, 1/500), anti-p21 (Cell Signaling Technology®, #2947, 1/1000) and anti- $\beta$ -actin (Sigma, A5541, 1/2000) antibodies were used. Proteins were detected by chemiluminescence using peroxidase-conjugated secondary antibodies and Clarity ECL substrate (Bio-Rad).



## **Flow cytometry analysis**

For each condition, cells were counted and one million cells were collected, spun, washed twice with 1X PBS, finally fixed and permeabilized with 75% ethanol. Next, cells were resuspended in 300  $\mu$ L of 1  $\mu$ g/mL DAPI and incubated 30 min at RT protected from light. DAPI-stained cells were analyzed using flow cytometry through BD LSR Fortessa technology, and cell cycle data were further analyzed using FlowLogic (Miltenyi) with the Watson pragmatic model for evaluation of cell cycle distribution.

## **Autophagy analysis by High Content Analysis**

Cells were seeded in 96-well plates and treated with RNA Pol I inhibitors and chloroquine as indicated. After 24 h, cells were fixed with 4% formaldehyde, permeabilized with 0.5 % Triton X-100. Immuno-fluorescence analysis was carried out with anti-LC3B (Cell Signaling Technology<sup>®</sup>, #3868, 1/1000) and anti-tubulin (Merck Millipore, CPO6, 1/1000) antibodies. For quantification by high content screening approach, plates were imaged on a Perkin Elmer High content analysis system Operetta CLS with a water x20 objective. Image analysis and quantifications were performed with the Columbus imaging software (Perkin Elmer). For autophagy analysis, cytoplasmic LC3B staining was measured. Data were processed in Microsoft Excel.

## **Electroporation analysis**

Cells were electroporated by using the Amaxa<sup>®</sup> Cell Line Nucleofector<sup>®</sup> kit following the manufacturer's instructions. For each condition, one million cells were counted and resuspended into 100  $\mu$ L of the provided buffer and electroporation was carried out by using the X-093 in-built protocol. After electroporation, cells were directly taken into growth medium and seeded into a 6-well culture plate.

## **Statistical analysis and graphics**

All *P*-values corresponded to two-tailed *P*-values. A *P*-value < 0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism v7.0a softwares (GraphPad Software, Inc). For comparison of sensitivities according to MYC mutational status, Mann-Whitney test was carried out and *P*-values are indicated for each graph. Graphical representations were depicted using either GraphPad Prism software.

## Figure legends

### Figure A1. Effect of inhibition of ribosome biogenesis on cell cycle

**A)** p21 protein levels in five TNBC cell lines upon treatment with two different concentrations of CX-5461 or BMH-21, compared to untreated cells. Cells were treated with a concentration of CX-5461 or BMH-21 corresponding to either the respective EC<sub>50</sub> or two-fold higher concentration, for 48 h. **B)** p21 protein levels in five TNBC cell lines upon FBL knockdown. Cells were transfected with either siRNA-FBL or siRNA-CTRL and harvested after 48 h. **C)** Graph showing the proportion of cells in G<sub>1</sub>, S and G<sub>2</sub> phase of the cell cycle upon treatment of MDA-MB-231 cells with doxycyclin in either a shRNA-NS or a shRNA-FBL inducible system. The proportions were calculated by integrating a flow cytometry data set, of the type shown in D. **D)** Example of flow cytometry profile used to calculate proportions shown in C. The G<sub>1</sub> phase peak is coloured in red, the G<sub>2</sub> phase peak in green and the S phase in yellow.

### Figure A2. Effect of RNA Pol I inhibitors on autophagy-inhibitory

**A)** LC3B levels in five TNBC cell lines upon treatment with RNA Pol I inhibitors combined or not with chloroquine autophagy inhibitor. Cells were treated for 24 h with either CX-5461 or BMH-21, each at two different concentrations, and signal detection levels were analyzed as described in Materials and Methods.

### Figure A3. Cell proliferation upon treatment with peptide targeting FBL

**A-G)** Representative pictures of MDA-MB-231 cells electroporated with either F1, F2, or F3 peptide, or rather the corresponding fluorophore-coupled forms F1-F, F2-F, or F3-F. Pictures were taken by a phase contrast microscope 48 h later and cell density was evaluated. **H-I)** Representative pictures of MDA-MB-231 cells electroporated with either F1-F or F3-F fluorophore-coupled peptides. Pictures were taken 6 h after electroporation. **J)** MDA-MB-231 cell proliferation upon electroporation with either vehicle, F1 peptide, F3-F peptide or two different concentrations of F3 peptide. **K)** MDA-MB-231 cell proliferation upon electroporation with F1, F3-F or two different concentrations of F3 peptide, relative to untreated cells, 96 h after treatment. This represents the same data as J.

**Figure A1**

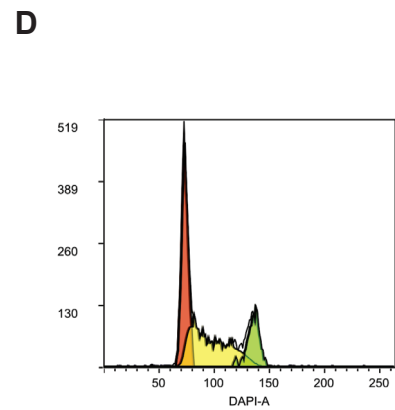
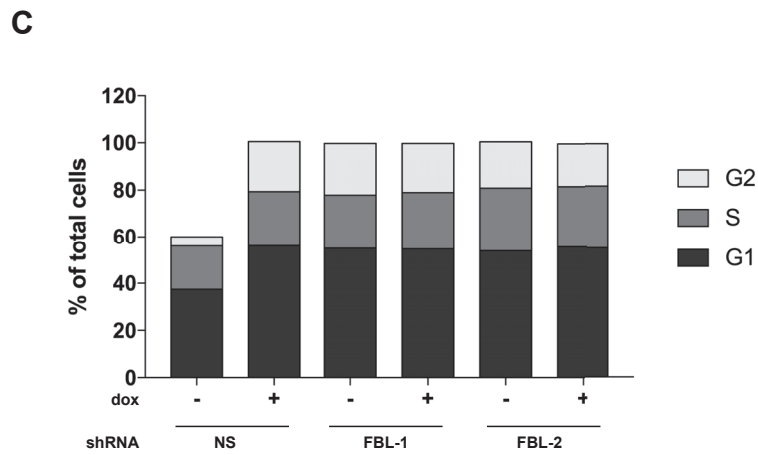
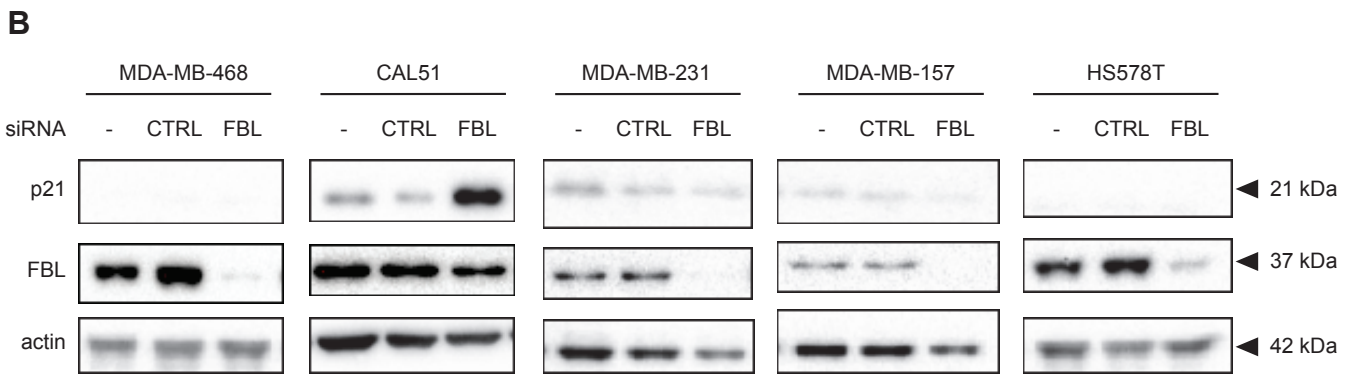
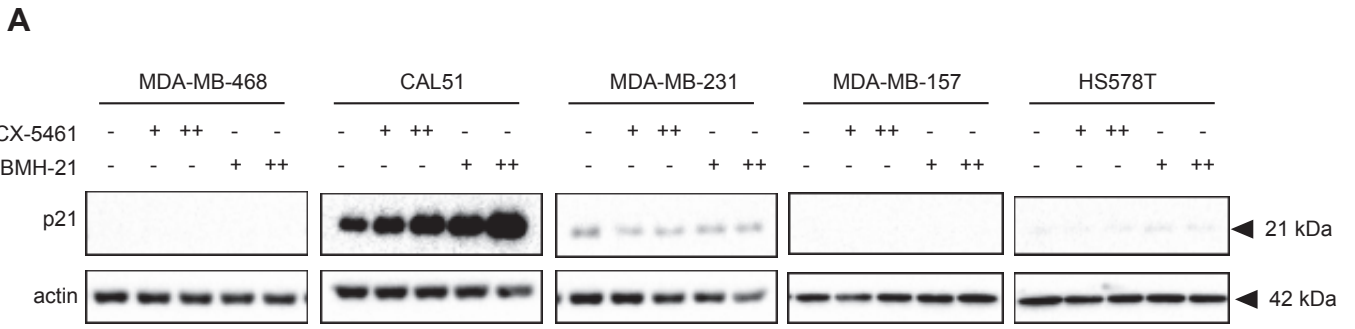


Figure A2

A

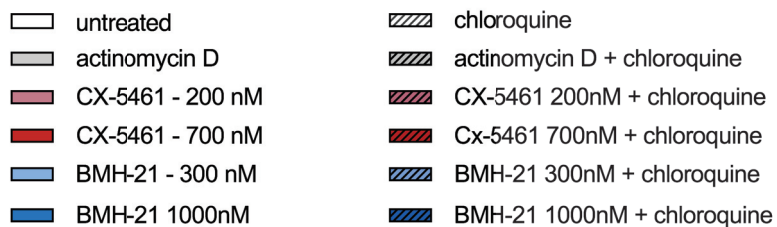
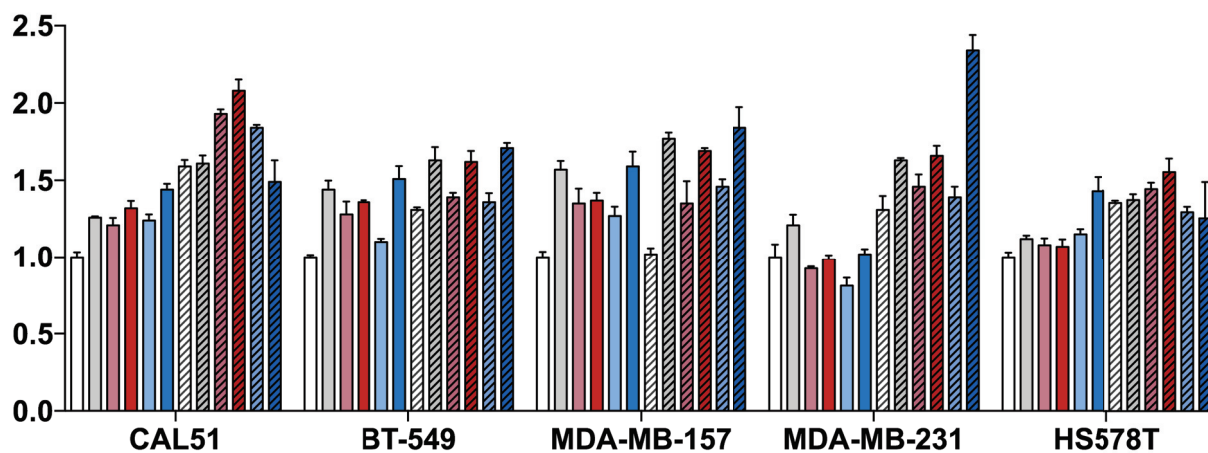
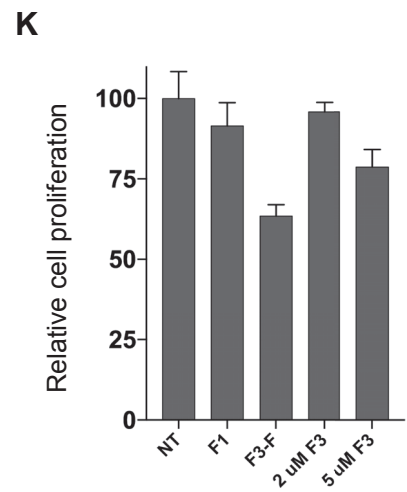
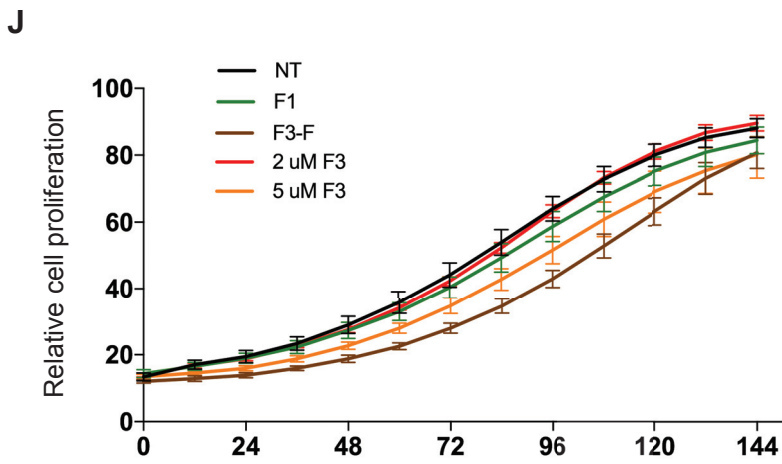
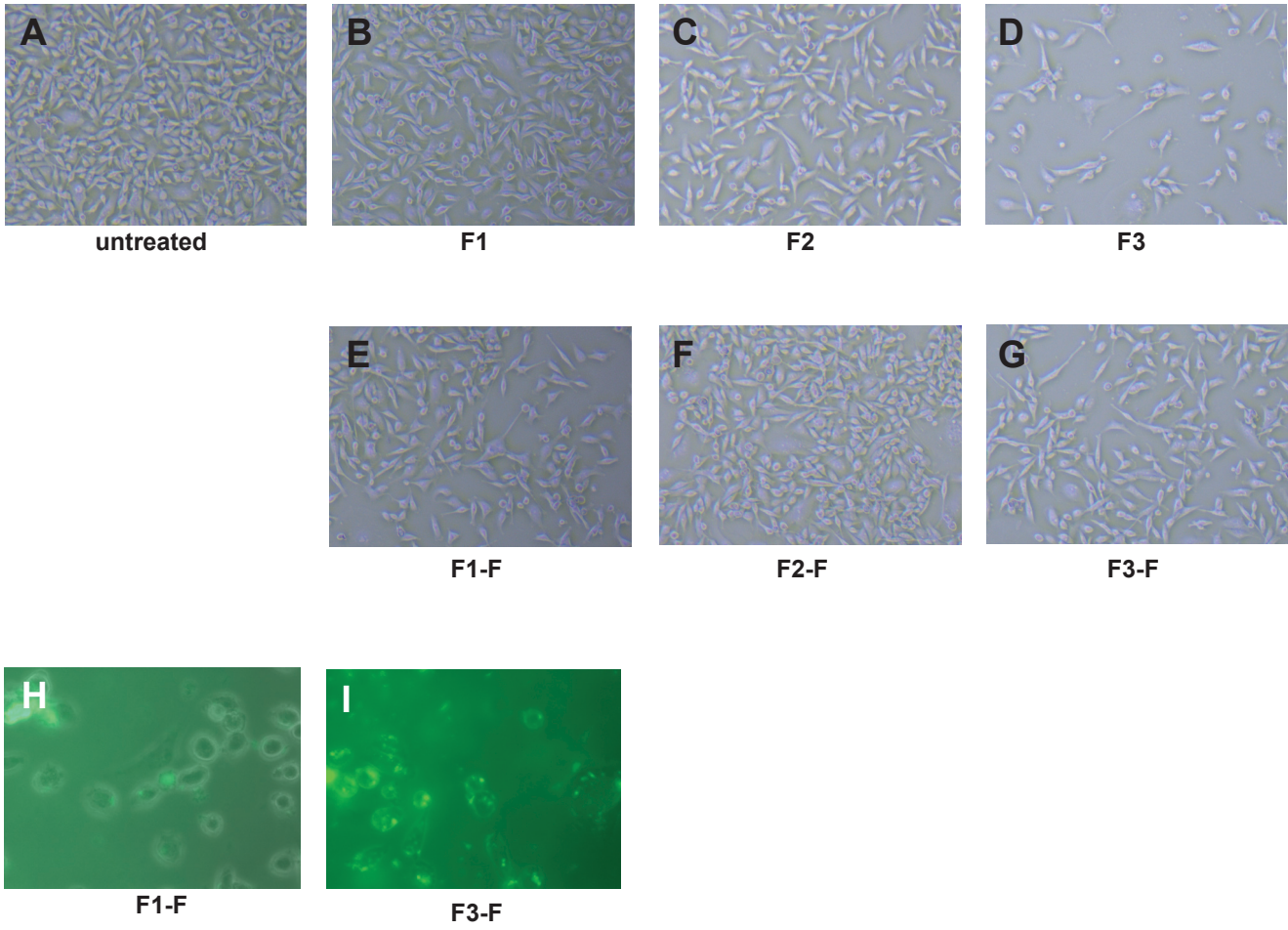


Figure A3



## Other results

In parallel with the progress of the core project, additional studies were conducted during this thesis, including work in the same spirit as the main one on TNBC, to evaluate the relevance of inhibiting ribosome biogenesis in neuroblastoma, recently characterized for sensitivity to inhibition of ribosome biogenesis in a MYCN-driven model.

In addition, participation in other scientific projects within the research group have made it possible to contribute to research about ribosome function in several models of clinical interest, or to investigate topics related to pure research on ribosome biology such as the function of post-transcriptional modifications of ribosomal RNAs and their contribution to ribosome heterogeneity, according to the recent concept of "specialized ribosomes". In the following sections, these contributions will be presented.

Further side work resulted in the writing and publication of a review article about the role of rRNA 2'-O-methylation in translational control, with particular regard to the contribution of 2'-O-methylation to ribosome heterogeneity and its potential functional implications. I contributed to the article in question in terms of bibliographic research, theoretical reflection, scientific speculation, manuscript outline and writing. This work is part of an ongoing radical change in the view of the ribosome, from a platform dedicated only to translation to a direct actor of the process of translation endowed with regulative capacities that we are only starting to unveil. The published version of the article is included in this thesis.

A second article still focuses on the topic discussed in the review article, the role of rRNA 2'-O-methylation. In this manuscript, currently in preparation, the variability of rRNA 2'-O-methylation profiles with single site resolution was analyzed for the first time in a large set of tumor samples. This showed that the variability of rRNA at the level of 2'-O-methylation among tumor samples is restricted to a number of positions in humans, and such variability is differentially associated with breast cancer features. While stable positions displaying a low variability might be required to maintain the core structure and function of the ribosome, the variant positions displaying high variability might be the source of regulatory events. The discovery of 2'-O-methylation profiles associated to particular cancer types supports the idea that cancer cells could carry ribosomes different in their composition, thereby resulting in altered functions. In perspective, this could be taken into account for potential targeting in anti-cancer treatment. The manuscript is included as an attachment.

## **Inhibition of ribosome biogenesis in patient-derived neuroblastoma cell lines**

Neuroblastoma is a childhood tumor of the peripheral sympathetic nervous system, accounting for more than 7% of malignancies in patients younger than 15 years and about 15% of all pediatric cancer-related deaths. The clinical hallmark of neuroblastoma is its heterogeneity, with a highly variable clinical course ranging from spontaneous regression to fatal progression. Although substantial improvement in the efficacy of treatment has been achieved during the past decades regarding certain well-defined subsets of patients, the outcome for children with a high-risk clinical phenotype has improved only modestly and long-term survival is still less than 40% (Maris, 2010).

The major oncogenic pathways generally involved in neuroblastoma progression do not seem to be deregulated with the exception of *MYCN* in a specific subset: amplified *MYCN* is found in roughly 20-25% of primary tumors and is strongly correlated with advanced stage disease and treatment failure, defining a highly aggressive subtype (Huang and Weiss, 2013; Maris, 2010; Niemas-Teshiba et al., 2017). In neuroblastoma, N-myc protein (encoded by *MYCN* gene) is responsible for the enhanced expression of a large set of genes involved in ribosome biogenesis and protein synthesis (Boon et al., 2001). In other cases of high-risk neuroblastoma, *MYCN* is not amplified but the *MYCN* homolog c-Myc protein is frequently found to be highly expressed (Westermann et al., 2008). Like N-myc, c-Myc is well known to activate ribosome biogenesis at multiple levels (van Riggelen et al., 2010). Consistently with this, highly proliferative neuroblastoma is associated with enlarged and increased numbers of nucleoli, indicative of increased rRNA synthesis (Niemas-Teshiba et al., 2017). Moreover, the overexpression of at least two factors directly involved in ribosome biogenesis has been associated with poor outcome. In particular, DEF, a pre-rRNA processing factor required for the maturation of the small subunit, is highly expressed in human neuroblastoma and its depletion in *MYCN*-driven neuroblastoma cell lines decreased cell growth and induced apoptosis, while its forced overexpression enhanced cell proliferation (Tao et al., 2017). Similarly, high expression levels of PES1, that plays a role in both ribosome biogenesis and neural development, are found in neuroblastoma cases characterized by *MYCN* gene amplification and clinically unfavorable stage. In neuroblastoma cell lines, PES1 knockdown suppressed tumor cell growth and induced apoptosis (Nakaguro et al., 2015).

On the basis of these observations, inhibition of ribosome biogenesis has been proposed as a therapeutic tool to target *MYCN*-amplified neuroblastoma proliferation. This has been tested by treating neuroblastoma cell lines with low doses of actinomycin D, inducing cell death *in vitro* and tumor progression *in vivo* (Cortes et al., 2016), and proved to be a suitable therapeutic strategy using CX-5461 and CX-3543 at nanomolar concentrations, with both compounds reducing rRNA synthesis and inducing p53 signaling or cell cycle arrest depending on the *TP53* gene mutational status (Hald et al., 2019).

Interestingly, autophagy has been reported to be activated upon treatment with chemotherapeutic drugs such as vincristine, doxorubicin and cisplatin temozolomide, and is likely to be a chemoresistance mechanism in neuroblastoma. Consistently with this, combination of the same drugs with an autophagy inhibitor results in reduced chemoresistance (Belounis et al., 2016). Importantly, autophagy induction has also been observed upon treatment of neuroblastoma cell lines with actinomycin D at low doses, known to selectively inhibit ribosome biogenesis in p53-negative genetic background, suggesting that autophagy may be a p53-independent response mechanism activated upon nucleolar stress in this disease (Cortes et al., 2016).

In order to better characterize the effect of inhibition of ribosome biogenesis in neuroblastoma cell lines through treatment with RNA Pol I inhibitors, we established a collaboration with V. Combaret's research group at the CRCL, Lyon, aimed to evaluate the association between sensitivity to RNA Pol I inhibitors, including BMH-21 that has never been tested in neuroblastoma, and *MYCN* mutational status. In addition, this work is carried out on a set of cell lines derived from patient tumors by V. Combaret's group. Here, the first results of this project, to which I contributed, are presented.

## Results

Given that neuroblastoma is a heterogeneous disease, but its most aggressive subtype is driven by *MYCN* amplification leading to enhanced ribosome biogenesis, we selected a panel of neuroblastoma cell lines carrying either a single-copy of the *MYC* oncogene or its amplification. In order to evaluate the effect of treatment with RNA Pol I inhibitors on cell proliferation, we treated neuroblastoma cell lines with a concentration range of CX-5461 or BMH-21 spanning from 1 nM to 1  $\mu$ M and we evaluated cell proliferation through MTS assay. The  $EC_{50}$  values relative to sensitivity to CX-5461 and BMH-21 were found to be variable among cell lines, with approximately two-fold difference and four-fold difference between the most sensitive and the most resistant cell lines relative to CX-5461 or BMH-



21, respectively (Figure A4A,C). In this data set, cell sensitivity was not correlated with *MYCN* gene status (Figure A4B,D), contrary to previously published results concerning CX-5461 in neuroblastoma (Hald et al., 2019). Currently, work is ongoing to confirm our first result by another method. Indeed, Hald and colleagues did not assess cell viability by using the same assay. In addition, further characterization of the genetic features of neuroblastoma cell lines could provide a potential explanation for the observed *MYCN*-independent variability. Work is in progress for the characterization of the status of *ALK* and *hTERT* in order to furtherly stratify the tumor response and for characterization of apoptotic and autophagic response. Most importantly, these results are encouraging because they confirm that neuroblastoma cell lines are sensitive to treatment with CX-5461 at nanomolar concentrations and provide for the first time information about the effectiveness of BMH-21 as a therapeutic drug for the treatment of this disease. In perspective, we also aim to evaluate the relevance of targeting fibrillarlin in neuroblastoma, similarly to what we have done in TNBC (see Article 1 and Appendix to Article 1).

## **Materials and Methods**

### **Neuroblastoma cell lines**

Except for IMR32 cell line, all patient-derived neuroblastoma cell lines and information about *MYCN* mutational status were provided by V. Combaret's group from the CRCL, Lyon.

### **MTS assay**

MTS assay estimates cell proliferation by evaluating the metabolic activity of cells. For MTS assay, CellTiter 96<sup>®</sup> AQueousOne Solution Cell Proliferation Assay kit (Promega, G3580) was used by following the manufacturer's instructions. Absorbance values at 490 nm were then read through Tecan<sup>®</sup> Infinite 200 Pro technology and data analyzed through GraphPad Prism v7.0a (GraphPad Software, Inc).

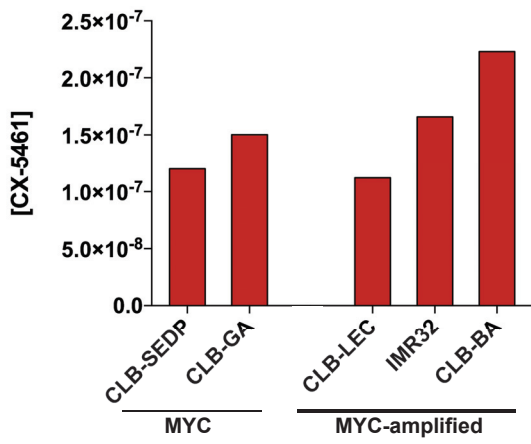
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### Figure A4. Sensitivity of neuroblastoma cell lines to RNA Pol I inhibitors

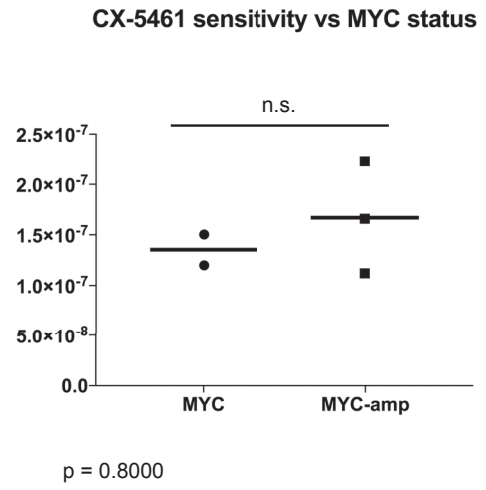
**A)**  $EC_{50}$  values relative to the effect of CX-5461 on neuroblastoma cell proliferation, as measured through MTS assay 96 h after treatment with a wide concentration range of CX-5461 spanning from 1 nM to 1  $\mu$ M. Cell lines are grouped by mutational status relative to the MYCN gene, with non-amplified MYCN (left) and MYCN-amplified (right) sorted also by  $EC_{50}$  value. **B)** Dot plot graph comparing  $EC_{50}$  values relative to CX-5461 between neuroblastoma cell lines with normal MYCN gene and MYCN-amplified ones. *P*-value is shown. **C)** Same as A but relative to BMH-21. **D)** Same as B but relative to BMH-21

Figure A4

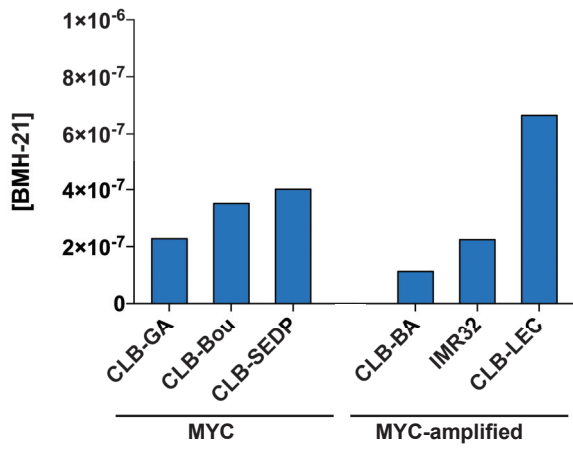
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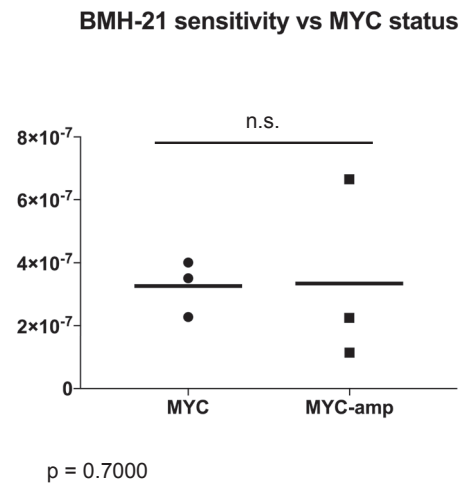
B



C



D



# Ribosome fishing

## General context

The concept of specialized ribosomes is emerging, supported by an increasing amount of experimental evidence. The ribosome population can vary much in terms of its composition and the source of such heterogeneity can come from variability in rRNA sequence and post-transcriptional modifications, from differential stoichiometry of specific ribosomal proteins, from post-translational modifications of ribosomal proteins potentially affecting their functions and interactions with other ribosome-associated factors. Notably, a recent study showed that heterogeneity resulting from ribosomal protein composition endows ribosomes with different selectivity for translating specific pools of transcripts (Shi et al., 2017). This was shown by generating mouse models endogenously expressing specific ribosomal proteins (RPL10A or RPS25) coupled with a FLAG tag. These two ribosomal proteins had been shown to be present in substoichiometric amount in the ribosome population, suggesting that ribosomes can either contain or not some specific ribosomal proteins. Tagged ribosomes were subsequently purified from monosomal and polysomal fractions of actively translating ribosomes, by FLAG-specific affinity purification and ribosome profiling was carried out, revealing enrichment or depletion of specific subsets of mRNAs (Shi et al., 2017). In these experiments, the authors could isolate ribosomes characterized by a specific composition in ribosomal proteins by exploiting the availability of technologies able to distinguish and separate ribosomes with a specific composition in ribosomal proteins from a mixed ribosome pool. This is not the case for all features contributing to ribosome heterogeneity. In particular, up to the present, no methods have been developed for the isolation of ribosomes carrying specific post-transcriptional modifications on their rRNA components, yet.

Until recently, 2'-O-methylation was considered to be a constitutive and universal modification, leading to the alteration of each site in every ribosome of the cell. Several recent technical and conceptual breakthroughs have challenged this view and the number of reported cases of functional implications of heterogeneity in 2'-O-methylation is constantly growing. In the group, we focus on rRNA 2'-O-methylation and, in line with other groups worldwide, we have been accumulating evidence for a similar mechanism of selective mRNA translation. For instance, in the past we have been progressively demonstrating that 2'-O-methylation plays a role in regulating translation of selected

mRNAs, first by correlative observations between altered 2'-O-methylation profiles and translation reporter assays in breast cancer models (Belin et al., 2009; Marcel et al., 2013), and second in models in which expression of the rRNA methyltransferase FBL was knocked down (Erales et al., 2017; Marcel et al., 2013). Importantly, alterations in translational regulation observed in previously described cellular assays could be attributed to ribosomes, by means of an *in vitro* cell-free translation assay, in which purified ribosomes were the only variable parameter. Ribosomes exhibiting an altered 2'-O-methylation profile displayed similar translational activity on mRNA carrying a GAPDH or actin 5'-UTR compared to control ribosomes, but decreased ability to initiate translation from the dicistrovirus IRES elements (Erales et al., 2017). Of note, a similar observation was made with ribosomes carrying altered levels of pseudouridylation, further strengthening the central role of rRNA modifications in ribosome functioning and translational control (Penzo et al., 2015). However, all the experiments demonstrating an intrinsically altered translational activity in ribosomes differentially modified at the rRNA level are built on *in vitro* cell-free assays. In order to ascertain whether specific translational activities are controlled by rRNA modifications and potentially identify target mRNAs *in vivo*, the development of methods able to distinguish and separate differentially modified ribosomes from an indistinct ribosome pool will be needed.

Currently, the major limitation to the development of such methods is the current lack of technical tools to target a specific rRNA modification within a functional ribosome. During my thesis, I reasoned that if differentially modified ribosomes preferentially translate distinct pools of mRNAs, establishing a number of functional interactions between particular mRNAs and particular profiles of rRNA modification, then affinity-based purification of specifically translated mRNAs followed by analysis of ribosome composition could be used as a proxy to reveal such interactions. In particular, for this purpose I focused on the specific complex formed by a translated mRNA, the associated nascent polypeptide chain synthesized from the mRNA and the ribosome mediating the translation process (nascent polypeptide chain complex, NPC complex). I designed two methods that aim to isolate this specific functional complex: one is based on immunoprecipitation (IP), another on specific mRNA elements. Here, I will present some preliminary results about the first method, because not only they may have a promising potential for further technical development, but also could suggest unexpected aspects of ribosome biology.

# Results

## Immunoprecipitation of the NPC complex

As a preliminary proof of concept, I used an experimental cellular system in which the expression of a tagged protein is induced upon treatment with doxycyclin. In particular, I used modified HeLa cells over-expressing a tagged form of FBL, FLAG-FBL, with the FLAG tag on its N-terminal end. In this context, the target NPC is the nascent FLAG-FBL synthesized by the ribosome from the FLAG-FBL mRNA template. I took advantage from this system because large amounts of ribosomes can be easily purified from HeLa cells, because the FLAG-FBL protein is over-expressed likely leading to a large quantity of the targeted NPC complex, because the anti-FLAG antibody is extremely specific and it had been previously tested for IP experiments. In addition, to prevent potential loss of material, cells underwent treatment with emetine prior to harvest, which targets all active ribosomes locking them into a configuration that remains tightly bound to the mRNA molecule. I purified ribosomes from HeLa cells overexpressing FLAG-FBL and I carried out IP by using an anti-FLAG antibody (FLAG-IP), thereby confirming that ribosomes can be immuno-precipitated (Figure A5A). Total RNA was extracted from the immuno-precipitated samples revealing that, as expected, FLAG-IP is selective for samples in which FLAG-FBL is overexpressed and its mRNA is translated (Figure A5B). Importantly, the amount of RNA obtained through IP is sufficient to carry out RiboMeth-Seq analysis, an extremely sensitive method for the quantitative mapping of 2'-O-methylation at each position of rRNA sequence, providing information on 2'-O-methylation profiles and potential 2'-O-methylation variations. RiboMeth-Seq analysis was carried out to test its feasibility from samples obtained through immuno-precipitation of purified ribosomes. In this case, we found that no particular variation in 2'-O-methylation profile can be associated to FLAG-IP-ed ribosomes in this model (Figure A6). Nevertheless, this result provides a proof of concept that IP-ed ribosomes possibly carrying out specific molecular functions can be analyzed with regard to their 2'-O-methylation profile.

## IP of the RNC complex reveals potential interactions with non-specific mRNAs

To characterize the immuno-precipitated sample, RT-qPCR analysis was carried out. This showed that FLAG-FBL mRNA and 18S rRNA are enriched in the FLAG-containing sample compared to the input sample, collected before FLAG-IP (Figure A5C-E). This is consistent with a selective isolation of a complex formed by the translating ribosome, the

translated FLAG-FBL mRNA, and the resulting nascent polypeptide with a FLAG sequence at its N-terminus. However, unexpectedly, other mRNAs were found to be enriched in RNA extracted FLAG-IP-ed samples, including *GAPDH*,  *$\beta$ -actin* and *IGF1R* mRNAs (Figure A5F-H). We speculated that such unexpected presence of mRNA species other than FLAG-FBL mRNA could be due to specific unknown functions of FLAG-FBL protein, that FLAG-FBL could carry out independently of its own translation. For example, FLAG-FBL protein could interact with ribosomes translating other mRNAs, or rather directly interact with other mRNAs, that are subsequently IP-ed by an anti-FLAG antibody.

In order to know whether the presence of unexpected mRNAs species in FLAG-IP-ed RNA is associated to FLAG-FBL functions, another experimental system was used, in which, instead of FBL, the green fluorescent protein (GFP) is covalently attached to a FLAG tag. The advantage of such system, in this context, is that not only the expression of GFP is easily detectable but also, importantly, GFP is not involved in any ribosome-related function. HeLa cells were transfected with an expression vector coding for FLAG-GFP and the expression of GFP was checked by fluorescence microscopy (Figure 7A), then FLAG-IP was carried out, followed by total RNA extraction. Again, FLAG-IP is selective for samples in which FLAG-GFP is expressed (Figure A7B). RT-qPCR analysis revealed that, as expected, FLAG-GFP mRNA is enriched upon FLAG-IP compared to the input sample, while 18S rRNA is present at comparable amount (Figure A7C-D). Other mRNAs, such as *FBL*,  *$\beta$ -actin* and *IGF1R*, are still present upon FLAG-IP. However, IGF1R is the only one enriched compared to the input sample (Figure A7E-G). These results suggest that upon FLAG-IP the specific mRNA enrichment, but not the presence of the unexpected mRNAs, is associated with FLAG-FBL.

## **Discussion of these results**

Although, at present, it cannot be excluded that the choice of the FLAG tag leads to a bias in the system and the use of another tag would clarify this, our results suggest that upon FLAG-IP the specific mRNA enrichment, but not the presence of the unexpected mRNAs, is associated with FLAG-FBL. FLAG-FBL and FLAG-GFP mRNAs or proteins could interact differentially with ribosomes translating other mRNAs, or directly with other mRNAs. Nevertheless, because GFP is unlikely to bind mRNA, we speculate that the first possibility is more probable. In this case, the mRNA species found in FLAG-IP-ed RNAs after FLAG-GFP expression could be considered non-specific, whereas those found in the FLAG-FBL overexpressing system would be due to some RNA binding activity of

FBL protein. Another biologically interesting possibility is that ribosomes engaged in translation are able to interact with other factors, including mRNAs or even other ribosomes translating other mRNAs, in a context of coordinated gene regulation. In this case, the differential enrichment observed in the two systems could reflect that ribosomes engaged in the translation of specific mRNAs are able to interact differentially with other factors, for example ribosomes translating *FBL* mRNA could preferentially interact with ribosomes translating *IGF1R* mRNA. Finally, it must be kept in mind that these experiments provide only enrichment factors, that are relative quantities: there is no means to compare mRNA species to each other. This is crucial, because if the "unexpected" mRNAs, even if enriched upon FLAG-IP, are present in negligible amount relative to the mRNA of interest, thus relative to the RNC complex of interest, then their presence would not overly disturb the analysis of the IP-ed ribosomes. To answer this question, an absolute quantification of mRNA species would be needed. In the course of the thesis, we could not implement further experiments. However, the digital qPCR technology is being implemented in the laboratory and could be used to test some of the proposed hypotheses. In any case, altogether, these results raise several questions, and further investigations to answer them could be exciting and open intriguing possibilities of higher levels of complexity in gene expression regulation.

## Materials and methods

### Cell lines

HeLa cell lines expressing an inducible gene encoding FLAG-FBL were generated by lentiviral infection. The vectors used for lentiviral infection were originally acquired from Open Biosystems (Dharmacon), then modified in the laboratory. Upon lentiviral infection, cell populations were selected using puromycin at 2 µg/mL for 14 d. Cells were grown under selection at 1 µg/mL. shRNA expression was induced by doxycycline treatment for 3 d at 1 µg/mL. Induction efficiency was monitored by evaluating the tRFP expression marker, by observation under fluorescent microscope or by flow cytometry. The expression of FLAG-FBL was verified by Western blot analysis.

### Western blot

Western blot analysis was performed as described in the Materials and Methods of Article 1, using Monoclonal ANTI-FLAG<sup>®</sup> M2 antibody (Sigma-Aldrich, F1804, 1/1000), a



peroxidase-conjugated anti-mouse secondary antibody (Sigma) and Clarity ECL substrate (Bio-Rad).

### **Ribosome purification**

Ribosomes were purified as previously described (Belin et al., 2009). Briefly, cytoplasmic fractions were obtained by mechanical lysis of cells with a Dounce cell lysis buffer (10 mM Tris·HCl pH 7.4, 0.5 MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTE) supplemented with RNaseOUT™ Ribonuclease inhibitor (Invitrogen) and cOmplete™ protease inhibitor cocktail (Merck). The obtained lysate was centrifugated at 12 000 g for 10 min to pellet nuclei and mitochondria. Cytoplasmic fractions were loaded onto a 1 M sucrose cushion in a buffer containing 50 mM Tris·HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 25 mM KCl, and 2 mM DTT, and centrifuged for 2 h at 240 000 g. The pellet containing the ribosomes was resuspended into a buffer containing 50 mM Tris·HCl pH 7.4, 5 mM MgCl<sub>2</sub>, and 25 mM KCl.

### **Immunoprecipitation**

Prior to immunoprecipitation, Dynabeads™ magnetic beads (ThermoFisher, 10004D) were conjugated with anti-FLAG® M2 antibody (Sigma-Aldrich, F1804) by diluting the antibody to 5 µg/mL in 1X PBS-Tween 0.1% and incubating it with beads (25 µL per condition) for 10 min at RT upon gentle agitation. Then, beads were collected using a magnetic support and resuspended into a buffer containing 50 mM Tris·HCl pH 7.4, 5 mM MgCl<sub>2</sub>, and 25 mM KCl. After a 30 min incubation of samples with 25 µL non-conjugated beads (pre-clearing step), the supernatant was transferred into a new tube for IP, 25 µL beads conjugated with anti-FLAG antibody were added and samples were incubated overnight at 4 °C upon gentle agitation. After IP, beads were washed three times in non-stringent conditions, with a washing buffer containing 50 mM Tris·HCl pH 7.4, 5 mM MgCl<sub>2</sub>, and 150 mM KCl, transferring the supernatant into a new tube at each wash. Samples were directly used for total RNA isolation.

### **Total RNA isolation**

Total RNA isolation was carried out using RNeasy Mini Kit (Qiagen) and RNA samples were finally eluted into 30 µL ultra-pure water. The RNA amount was quantified by Nanodrop spectrophotometer (ThermoFisher).

## **RT-qPCR**

Retrotranscription was carried out from 50 ng of total RNA using PrimeScript Reverse Transcriptase (Takara) according to the manufacturer's instructions. Next, qPCR was carried out using Light Cycler 480 II instrument (Roche) coupled with the Light Cycler 480 SYBR Green I Master Mix (Roche) and RNA quantities were calculated using the  $2^{-\Delta\Delta CT}$  method.

## **RiboMethSeq analysis**

RiboMethSeq analysis was carried out starting from at least 150 ng RNA per sample. RiboMethSeq is based on protection of phosphodiester bonds against alkaline hydrolysis conferred by replacement of the 2'OH by a methyl group. The 3'-downstream nucleotide is thus absent at the 5' end in the collection of RNA fragments. Partial alkaline hydrolysis of total RNA samples is followed by deep-sequencing and allows 5' end counting for every fragment. The calculated score represents the level of methylation of a given nucleotide in the ribosomal population. RiboMethSeq was essentially carried out as previously described (Marchand et al., 2016).

## Figure legends

### Figure A5. Immunoprecipitation of ribosomes translating FLAG-FBL mRNA

**A)** Western blot analysis of samples obtained through immunoprecipitation. Ribosome samples were purified from HeLa cells overexpressing FLAG-FBL under the control of a doxycycline-inducible cassette, through a 25 mM KCl-containing sucrose cushion, then immunoprecipitated with beads conjugated with anti-FLAG antibody. Input samples correspond to ribosome samples before the incubation with the anti-FLAG antibody. **B)** Total amount of RNA (ng) extracted from immunoprecipitated ribosome samples. Prior to immunoprecipitation, ribosome samples were purified from normal HeLa cells (wt) or from non induced (-) or induced (+) HeLa cells carrying an inducible cassette for the overexpression of FLAG-FBL. Mock samples were obtained by incubating ribosome samples with non-conjugated beads. Mean and SD values are relative to three independent experiments. **C-H)** Quantitative detection of specific RNA species contained in the immunoprecipitated ribosome samples. Input samples correspond the ribosome samples before immunoprecipitation. The RNA samples from B were retrotranscribed and qPCR was carried out by using primers targeting, respectively, *FLAG-FBL* mRNA, *FBL* mRNA, 18S rRNA, *GAPDH* mRNA,  $\beta$ -actin mRNA or *IGF1R* mRNA species. Values are given in arbitrary units and are not normalized because of lack of an internal control, due to the intrinsic nature of the experiment.

### Figure A6. RiboMeth-Seq analysis of immunoprecipitated ribosomes

**A-C)** C-score values relative to the 2'-O-methylated sites within the 18S, 5.8S and 28S rRNA sequences, respectively. For each site, the value obtained from the input ribosome sample and the one obtained from the immunoprecipitated ribosome sample are shown. Shown data is representative of three independent experiments. **D)** C-score values relative to each 2'-O-methylated site in ribosomes from the input sample plotted against C-score values relative to the same 2'-O-methylated sites in ribosomes immunoprecipitated using an anti-FLAG antibody in a system overexpressing FLAG-FBL.

### Figure A7. Immunoprecipitation of ribosomes translating FLAG-GFP mRNA

**A)** Image of HeLa cells 24 h after transfection with an expression vector containing a gene encoding FLAG-GFP. The picture was taken through a fluorescence microscope. **B)** Total amount of RNA (ng) extracted from immunoprecipitated ribosome samples. Prior to

immunoprecipitation, ribosome samples were purified from HeLa cells expressing FLAG-GFP after transfection of an expression vector. The mock sample was obtained by incubating ribosome samples with non-conjugated beads. **C-H)** Quantitative detection of specific RNA species contained in the immunoprecipitated ribosome samples. Input samples correspond the ribosome samples before immunoprecipitation. The RNA samples from B were retrotranscribed and qPCR was carried out by using primers targeting, respectively, 18S rRNA, *GFP* mRNA, *FBL* mRNA,  $\beta$ -*actin* mRNA or *IGF1R* mRNA species. Values are given in arbitrary units and are not normalized because of lack of an internal control, due to the intrinsic nature of the experiment.

**Figure A5**

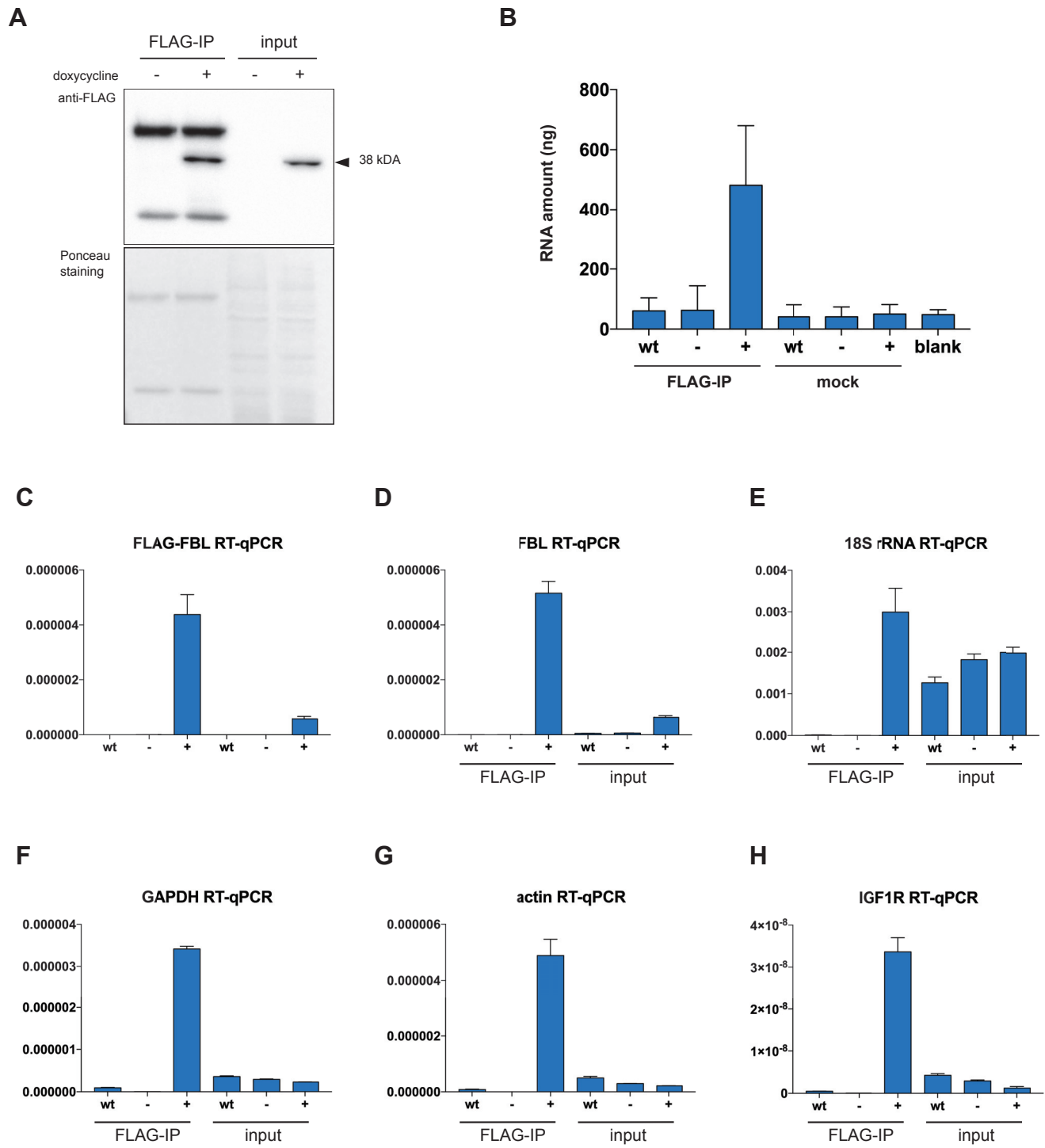


Figure A6

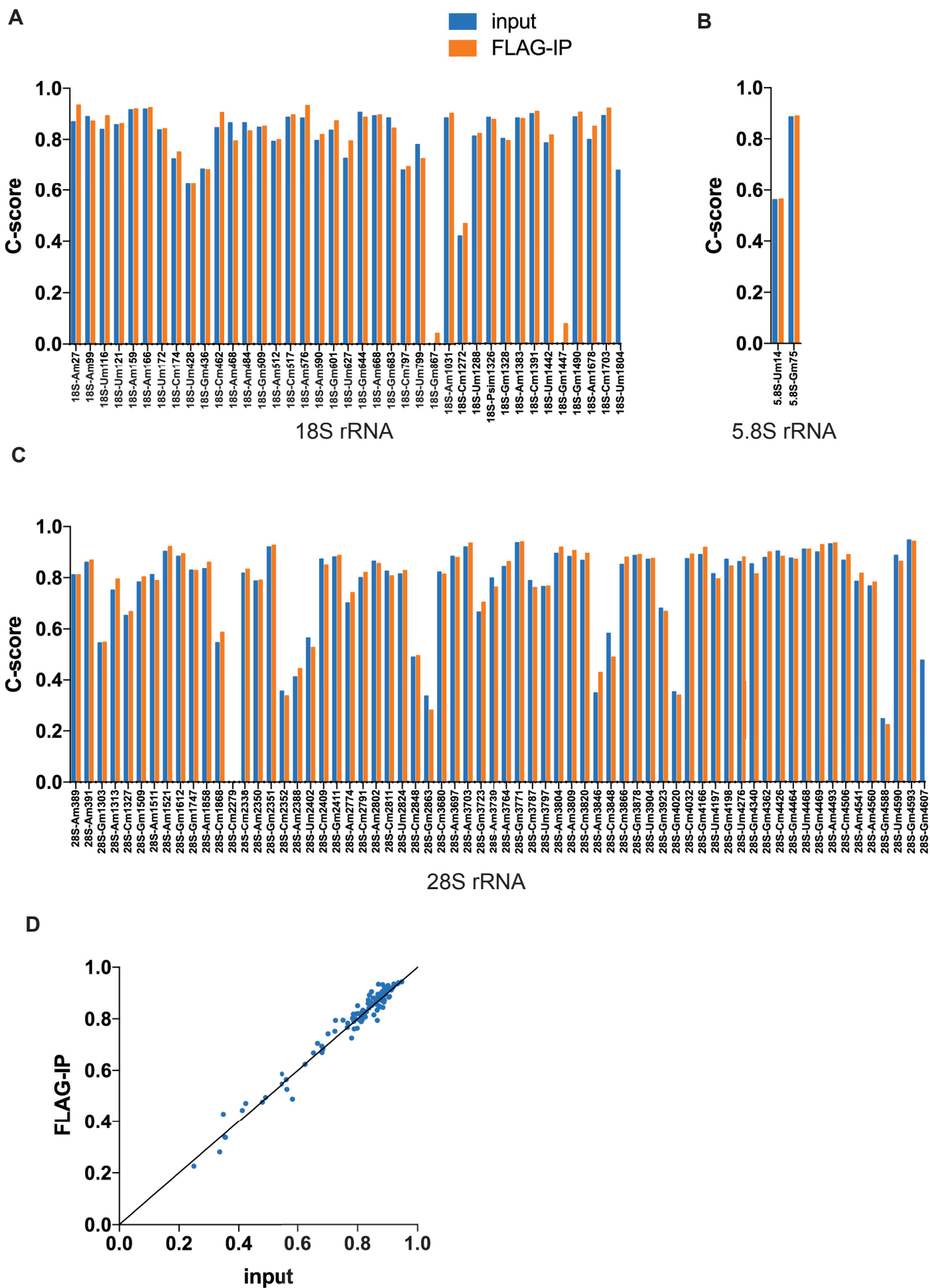
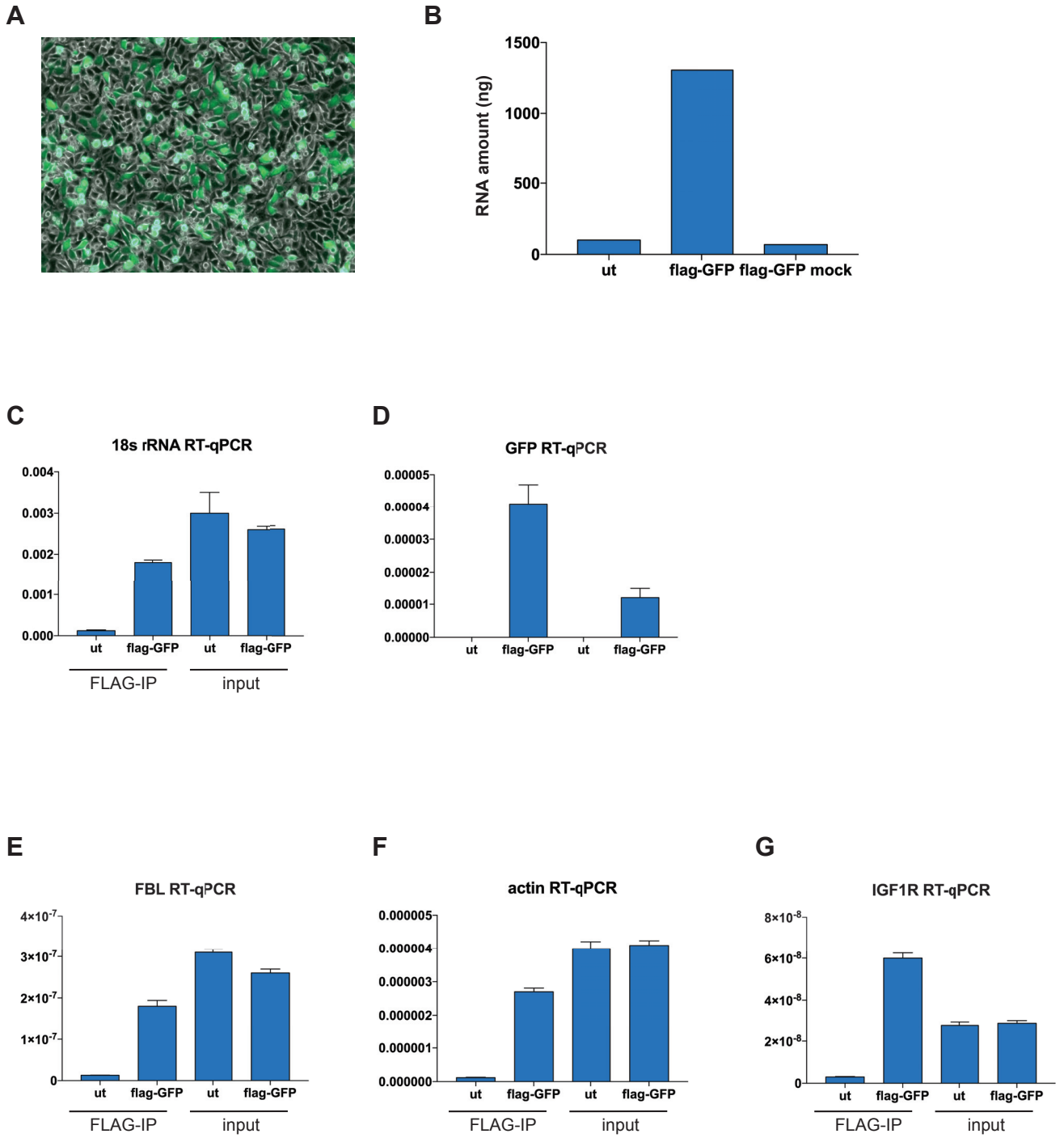


Figure A7



# Discussion



TNBC represents today a clinical problem, being associated with a poor prognosis and high probability of cancer recurrence after treatment (Dent et al., 2007). This is in part due to limited therapeutic options, as by definition TNBC lacks the main molecular targets exploited to specifically treat breast cancer so that conventional chemotherapy remains the standard therapeutic strategy, and to the limited response rate to such conventional chemotherapy treatments (Liedtke et al., 2008). In addition, TNBC is characterized by a remarkable heterogeneity (Lehmann et al., 2011), making it even more difficult to identify new markers for patient stratification and to find novel targets for the development of specific treatments. To improve the clinical outcome for patients affected by TNBC, it is urgent to identify biomarkers to predict survival according to the nature of their tumour, and to discover new therapeutic targets.

Recently, ribosome biogenesis has been proposed as a target for anti-cancer treatment. In particular, its inhibition by RNA Pol I inhibitors CX-5461, CX-3543 and BMH-21 proved to be effective in several cancers, including lymphoma, melanoma, osteosarcoma, prostate and ovarian cancers, in both p53-positive or negative genetic backgrounds (Bywater et al., 2012; Drygin et al., 2009, 2011; Fu et al., 2017; Li et al., 2016; Low et al., 2019; Peltonen et al., 2014). However, very little data is available about the role of ribosome biogenesis in TNBC, its possible contribution to tumorigenesis and the relevance of targeting ribosome biogenesis in TNBC.

In this study, we show that targeting ribosome biogenesis could be effective for treating TNBC and that treatment with RNA Pol I inhibitors CX-5461 and BMH-21 at nanomolar concentrations leads to p53-independent cell response potentially causing cell death. Our results raise a number of issues from a clinical and molecular point of view, in particular concerning the remarkable variability among TNBC cell lines and the selectivity compared to normal cells, and lack of criteria to predict TNBC response to ribosome biogenesis inhibition. The contribution of mTOR pathway and the relevance of co-targeting mTOR pathway together with ribosome biogenesis have been assessed and appears not to have any clinical interest in this pathology. In addition, this study consolidate the idea that rRNA maturation holds great potential as a therapeutic target to inhibit ribosome biogenesis in TNBC. Finally, from a broader biological perspective, our results stimulate more basic questions about the role of ribosome biogenesis in cancer and its regulation in relation with ribosome activity and translational control of cellular functions.

Each of these questions will be discussed in the following paragraphs.

## **Inhibition of RNA Pol I activity represents a potential strategy to target TNBC**

In this study, we show that the inhibition of ribosome biogenesis through treatment with RNA Pol I inhibitors CX-5461 and BMH-21 could be effective for treating TNBC. Indeed, treatment of TNBC cell lines at nanomolar concentrations causes a reduction in cell proliferation and induces p53-independent cell responses leading to cell death, including cell cycle arrest and the activation of apoptosis. Importantly, apart from cell proliferation, two other cellular functions associated with tumorigenic potential, namely colony formation and spheroid formation capabilities, are shown to be strikingly impacted by RNA Pol I inhibitors in both MDA-MB-231 and BT-20 cells and preliminary experiments in other TNBC cell lines carried out in the laboratory indicate the same tendency.

Altogether, our results indicate that, at least *in vitro*, CX-5461 and BMH-21 are able to counter the aggressive phenotype of TNBC cell lines. In order to confirm such results *in vivo* and fully validate the effectiveness supporting CX-5461 and BMH-21 as compounds for a targeted anti-cancer strategy in TNBC, experiments in xenograft models will be needed and are currently being planned in our laboratory.

### **Need to identify predictive criteria for TNBC stratification**

From a clinical perspective, it is crucial to be able to anticipate the efficacy of a treatment depending on the features of a disease. In particular, there is need to describe cellular responses activated upon anti-cancer treatment inhibiting ribosome biogenesis in TNBC cell lines, for two reasons. First, in order to understand the mode of action and characterize it at the mechanistical level. Second, from a translational point of view, in order to stratify patients and identify those who are likely to benefit from treatment.

In this study, we have not found any predictive criteria that could possibly contribute to stratify TNBC heterogeneity: sensitivity to CX-5461 and BMH-21 was not found to correlate with basal rRNA levels, expression levels of factors involved in ribosome biogenesis nor with any gene mutation commonly found in TNBC. However, the lack of significant correlation between sensitivity to treatment with RNA Pol I inhibitors and other clinically exploitable factors should not be discouraging. First, although our study was performed on a rather large number of cell lines compared to other studies, our data is based a population of too limited size to reach statistical significance. Yet, our results show some tendencies that could underlie biological relevance, albeit not statistically robust, and studies on a larger data set could be useful to confirm such tendencies and possibly to

identify predictive criteria in order to anticipate clinical response. Secondly, for our analysis we took into account the mRNA expression of factors involved in ribosome biogenesis, but not their expression at the protein level, yet. Because the expression of such factors could be regulated at the translational level, this deserves a further investigation. Third, we need to furtherly characterize the mechanisms of action of these inhibitors in TNBC cells. Indeed, it has been shown that sensitivity to BMH-21 is not merely associated to the basal expression level of its target RPA194, but rather with the extent of its decrease upon treatment, irrespective of the basal RPA194 protein expression (Peltonen et al., 2014). This would not constitute a clinically relevant factor, but would be a further advance for the understanding of the mechanisms that regulate the mode of action of BMH-21 that could shed light and pave the way for the finding of predictive factors. The same is true for CX-5461. CX-5461 has been shown to act by disrupting the multi-subunit SL1 factor binding to the rDNA promoter (Drygin et al., 2011). To our knowledge, the contribution of potentially different basal expression of SL1 among cancers or cancer subtypes has not been assessed yet. Analysis of SL1 composition, protein levels and binding to the rDNA promoter could help to understand the mode of action of CX-5461 at the molecular level and potentially provide insights for finding predictive criteria of sensitivity to CX-5461.

Finally, in our analysis we did not find statistically significant correlations between sensitivity to RNA Pol I inhibitors and any gene mutation commonly found in TNBC. Previous studies have reported that sensitivity to CX-5461 is decreased in p53 mutant or null context (Bywater et al., 2012), and increased in BRCA1/2 deficient context (Xu et al., 2017b). Conversely, our results in TNBC cell lines do not highlight any trend with regard to the mutational status of BRCA1 or p53. Another important factor to considerate could be MYC status, as it has been described that MYC overexpression can be sufficient to sensitize human fibroblasts, normally resistant, to RNA Pol I inhibition and that overexpression of MYC correlates significantly with high sensitivity to CX-5461 in ovarian cancer cell lines (Hannan et al., 2013). However, we do not have available data about MYC status in TNBC to test this possibility. Of note, both CX-5461 and BMH-21 are DNA intercalator specifically targeting G-quadruplex DNA structures enriched in the rDNA gene promoters. The rDNA gene copy number is surprisingly variable among humans (Stults et al., 2008) and whether and how this might affect sensitivity to RNA Pol I inhibitors in patients is not at all understood. This could be a further factor to eventually take into consideration at the clinical level. Indeed, sensitivity could be related to the differential expression of other factors that were not included in our analysis. A screening taking into account

transcriptomic and translomic data among TNBC cell lines could highlight interesting correlations. In this regard, an additional bioinformatic analysis will be performed on a data set of mRNA expression in the TNBC cell lines, obtained by T. Dubois (Curie Institute, who provided us with the cells lines) using exon-array analysis.

In conclusion, in this study we prove that TNBC are sensitive to treatment with RNA Pol I inhibitors in a nanomolar concentration range. From a clinical perspective, a key question is to identify what endows cancer cells with higher sensitivity to inhibition of ribosome production, so to provide for predictive criteria in order to identify the tumor subtypes that may therapeutically respond and patients that could benefit from such therapeutic strategies. Such criteria have not been found yet, and our results point to the need to the extend data set for the identification of predictive criteria, possibly including genes related to multi-drug resistance and other clinically exploitable factors. In any case, a step has been done toward the validation of ribosome biogenesis as a therapeutic target in TNBC.

### **Starting to unveil mechanisms of response variability in TNBC**

TNBC is an overly heterogeneous disease, which has been classified into several subtypes each with distinct molecular characteristics and underlying biology (Lehmann et al., 2011). TNBC heterogeneity is a clinical issue, as it is the major obstacle to the identification of a single targetable pathway for treatment. The inhibition of ribosome biogenesis through molecules that target RNA Pol I activity has been shown to exert an anti-proliferative effect in cancer cells. However, the reported elicited cellular responses and cytotoxic mechanisms are variable among the studied pathological contexts and include cell cycle arrest, apoptotic, autophagy, necrosis, senescence, in either p53-dependent or independent manner (see Table 3 in the Introduction). Thus, the induced response seem to be strictly model-specific and dependent from the genetic background. Currently, no features have been clearly and unambiguously identified as main contributors to cell behaviour upon treatment with RNA Pol I inhibitors. For this reason, a characterization of such response in TNBC is of great importance.

In our study, we assessed caspase activation levels and cell cycle arrest upon inhibition of ribosome biogenesis. We observed different responses depending on the specific treatment and cell line. In particular, a certain degree of variability in the levels of caspase activation is found among TNBC cell lines upon treatment with BMH-1 treatment. This had been previously observed only in ovarian cancer cells (Fu et al., 2017), while other studies only reported accumulation of cells in sub-G1 (Colis et al., 2014; Musso et al., 2018;

Peltonen et al., 2014) or transcriptional activation of p53 target genes (Wei et al., 2018) upon treatment with BMH-21. Regarding the cell cycle, BMH-21 had been reported to induce accumulation in G2 phase in lymphoma and ovarian cancer cells (Fu et al., 2017; Musso et al., 2018) and not to impact G2 phase but rather inhibit the S phase in melanoma cells (Peltonen et al., 2014), suggesting that the effect on cell cycle is cell type-dependent and that distinct cell cycle checkpoints are impacted. In our study, the increase of caspase activity in TNBC was accompanied by cell cycle arrest at G2/M phase upon treatment with BMH-21 in a dose-dependent manner. The extent of the effect seemed to be related to the effect on caspase activity, with cells activating caspases at the highest levels displaying also the most prominent effect on the cell cycle, and cells with relatively low or even no detected caspase activity displaying negligible effects on the cell cycle, consistently with a coupling between apoptosis and cell cycle arrest. In contrast with BMH-21, CX-5461 did not induce apoptosis. This behaviour was unpredictable, because apoptotic cell death was found upon treatment with CX-5461 in ovarian cancer cells (Cornelison et al., 2017), myeloma cells (Lee et al., 2017), leukemia and lymphoma cells (Bywater et al., 2012; Negi and Brown, 2015a; Quin et al., 2016), colon cancer cells (Xu et al., 2017b) and neuroblastoma (Hald et al., 2019), but not in prostate cancer (Rebello et al., 2016), osteosarcoma (Li et al., 2016), melanoma or pancreatic carcinoma cells (Drygin et al., 2011). So, no increase in caspase activity was observed upon treatment of TNBC cell lines with CX-5461. In contrast, treatment with CX-5461 induced cell cycle arrest in TNBC cell lines, with accumulation of cells in G2 phase. Like in the case of BMH-21, the extent of the effect of CX-5461 on the cell cycle was roughly dose-dependent.

The difference between the responses elicited by CX-5461 and BMH-21 is not surprising, as the two drugs have been shown to operate through distinct molecular mechanisms. Our data now adds TNBC as a cellular context in which CX-5461 does not induce apoptosis. Importantly, this study is only the second to clearly report apoptosis as a response to BMH-21.

In addition, we investigated autophagy induction upon nucleolar stress in TNBC. Autophagy may be a consequence of nucleolar stress (Pfister, 2019), and has been identified as a chemoresistance mechanism in neuroblastoma and pancreatic ductal adenocarcinoma (Belounis et al., 2016; Kinsey et al., 2019). These studies pave the way to potential therapeutic strategies such as combination of treatments inducing nucleolar stress with inhibition of autophagy in order to increase selectivity and reduce toxicity (Pfister, 2019). Inhibition of ribosome biogenesis through treatment with CX-5461 had

been reported to induce autophagy but not apoptosis in osteosarcoma, pancreatic carcinoma and melanoma cells (Drygin et al., 2011; Li et al., 2016). Here, our results indicate that autophagy is activated after treatment of TNBC cell lines with CX-5461 and also report for the first time that autophagy might be induced upon treatment with BMH-21. Interestingly, the extent of autophagy activation upon treatment with RNA Pol I inhibitors is dose-dependent and also seems to be related to the effect observed on the cell cycle and, in the case of BMH-21, on caspase activity, with cells displaying little effect on the cell cycle (MDA-MB-231 and HS578T cells) or no effect on caspase activity (MDA-MB-231 cells) being roughly the less sensitive to the induction of autophagy by RNA Pol I inhibitors (compare Figure 3F-G and Figure A2). It should be noted that we have analyzed only one marker of autophagy, LC3B accumulation, which by itself does not demonstrate autophagy induction. Additional experiments will be necessary such as use of pH sensitive fluorescent probes which mark autophagolysosomes.

TNBC cell lines displayed high caspase activity upon FBL knockdown, indicating that inhibiting ribosome biogenesis at downstream rRNA processing steps also induces apoptosis. Yet, the caspase induction upon FBL knockdown was not correlated to the response displayed upon treatment with BMH-21. The variability of caspase activation among cell lines and dependence on the modality of inhibition of ribosome biogenesis suggests that different mechanisms are activated in cell lines upon targeting of different steps of ribosome biogenesis. Moreover, the fact that upon FBL knockdown caspase activity is increased in resistant cell lines (like CAL51 cells), without resulting in reduced cell proliferation, whereas in other cells (like MDA-MB-157) increased caspase activity strongly impair cell proliferation, suggests some genetic context enables cell lines to pass through caspase activation sparing them from cell death although a response mechanism is elicited.

Of note, senescence is another cell response that has been reported in solid tumor cell lines and non-transformed BJ fibroblasts treated with CX-5461 (Drygin et al., 2011; Quin et al., 2016). In the course of this work we noted the presence of large polynucleated cells containing vacuoles, all signs of senescence. Presence of senescent cells will be searched using well established markers such as senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal), p16, and 53BP1. This will further describe the cell response in TNBC and widen the scope of mechanisms potentially responsible for cell line-dependent response.

In conclusion, our results highlight differences in the modes of action of various inhibitors of ribosome biogenesis. Treatment with BMH-21 and FBL knockdown leads to

caspase activation, indicating an apoptotic response, but not at the same extent in the same cell lines. Treatment with CX-5461 has not been associated to caspase activation, but only to cell cycle arrest. Of course, further studies are needed to confirm the presence or absence of apoptosis through detection of different apoptotic markers, to understand the role and the potential importance of induced autophagy and shed light to the features that make a cell line sensitive or resistant to these treatments.

Our results confirm the well-known heterogeneity of TNBC extending it to the function of ribosome biogenesis and showing that although increased ribosome biogenesis is not a characteristic signature of TNBC cell lines, ribosome biogenesis can be targeted to inhibit cell proliferation with variable effects. Sensitivity to inhibition of ribosome biogenesis is characterized by a remarkable degree of variability and such variability cannot be explained by differential activation of ribosome biogenesis at multiple levels. Our results show that the anti-proliferative effect observed upon treatment of TNBC with CX-5461 or BMH-21 is of variable extent, with up to 15-fold change in sensitivity between the less and the most sensitive cell lines. This is consistent with previous studies, that reported wide differences among the tested breast cancer cell lines, including a limited number of examples from the TNBC subtype (Drygin et al., 2011; Peltonen et al., 2014). Of note, in the case of CX-5461, the sensitivity of the tested TNBC cell lines relative to each other is the same reported by previous studies, despite that a distinct method was used for the evaluation of the anti-proliferative effect.

### **A normal versus cancer selectivity issue**

A major characteristics that anti-cancer treatments are roughly required to have to be approved and be successfully used in clinics is their selectivity for cancer cells with low levels of cytotoxicity for normal cells. The most attractive feature of the recently discovered classes of RNA Pol I inhibitors is that they seem to selectively target cancer cells, with observed cytotoxicity and cell death in treated cancer cells at concentrations of the drugs that do not exert any cytotoxicity or trigger cell cycle arrest without inducing cell death in healthy cells (Bywater et al., 2012; Drygin et al., 2011; Peltonen et al., 2014).

These results have been interpreted as the proof that cancer cells, at least in specific cancer models, are addicted to ribosome biogenesis and that the process of ribosome biogenesis is essential for tumorigenesis in those cancers. In order to test this hypothesis in our TNBC model, we included in our analysis two normal immortalized cell lines, namely HMEC mammary epithelial cells and BJ fibroblasts. We found that HMEC cells are very resistant to inhibition of ribosome biogenesis by CX-5461, contrary to BJ, and that both

HMEC and BJ cells display sensitivity to BMH-21 similar to breast cancer cells. This strikingly contrasts with previously published data, showing that BJ cells do not suffer any anti-proliferative effect upon treatment with CX-5461, despite this causes effective inhibition of ribosome biogenesis (Drygin et al., 2011). We do not have any explanation for this incongruence. Further analysis of the nucleolar stress response might bring some light onto this question.

### **Validation of FBL as a therapeutic target**

While targeting RNA Pol I is arguably a logical approach to inhibit ribosome biogenesis, many other steps of ribosome maturation are essential for ribosome production, and so are the factors that contribute to these steps. This was illustrated in several studies including a systematic siRNA screen of nucleolar proteins in HeLa cells (Tafforeau et al., 2013). These steps represent potential targets.

The idea of specifically targeting ribosome biogenesis at the level of maturation rather than transcription of rRNA has been first tested only recently: the interest of targeting U3 and U8 snoRNAs, required for rRNA maturation (Langhendries et al., 2016), as well as the components of C/D box snoRNPs, including FBL, NOP56 and NOP58, in breast cancer cells (Su et al., 2014) have been demonstrated.

Our results show that inhibiting ribosome biogenesis by targeting downstream of RNA Pol I through the impairment of FBL function has an effective anti-tumorigenic potential, as assessed by means of several methods, including colony formation assay and spheroid formation, using especially MDA-MB-231 cells, and with promising indications for other TNBC cell lines. We showed that FBL knockdown impacts cell proliferation and elicits p53-independent caspase activity at variable extent in a cell line-dependent manner. This work highlights the potential of targeting FBL in TNBC, that is thus validated as a target for anti-cancer therapies. Preliminary data using mouse xenograft of MDA-MB-231 cells expressing shRNA-FBL showed a slower tumor growth. These studies will need to be completed, and we planned to use the MDA-MB-231 and BT-20 cells used in this work, as well as HMEC-hTert cells expressing shRNA-FBL, recently derived in the laboratory. Altogether, these data will validate FBL as potential anti-cancer target.

Here, we propose the targeting of FBL as an anti-cancer therapeutic strategy. A major advantage of FBL is that its overexpression is strongly associated with TNBC disease, implying that patients with FBL-overexpressing TNBC could benefit from treatments targeting FBL function (Nguyen Van Long, 2019). However, nor the FBL expression levels or the effectiveness of the protein depletion upon knockdown did seem to be predictive for



response so far in TNBC cell lines. Currently, we have not found any predictive criteria that could possibly contribute to stratify TNBC heterogeneity in a clinically relevant manner. Similarly to the case of RNA Pol I inhibitors, we stress the need to extend our study to a larger data set including further information about the genetic context of TNBC cell lines, to possibly identify factors of interest based on their differential expression among cell lines that could explain different sensitivity to FBL knockdown.

In addition, although we also showed that sensitivity to FBL knockdown is not correlated with sensitivity to RNA Pol I inhibitors, we highlight that the three cell lines most sensitive to FBL knockdown happen to be particularly sensitive to CX-5461 and two out of the three cell lines most resistant to FBL knockdown are also the two TNBC cell lines most resistant to CX-541. Although this correlation is not statistically significant, this could underlie some biological pattern. As FBL knockdown and CX-5461 mechanistically target two steps of the same process, it is tempting to speculate that unknown factors conferring resistance to the former play also a biologically relevant role in resistance to the latter. So, this observation could potentially guide deeper analysis. Further studies extending the set of cell lines would be needed to support or rather exclude this hypothesis.

Finally, FBL is one of the component of the C/D box snoRNP, and impact of U3/U8 or NOP56 and NOP58 knockdown should also be considered to further demonstrate the interest of targeting this complex to inhibit tumor cell growth.

In conclusion, our results indicate that ribosome biogenesis can also be targeted at the level of a downstream essential component, FBL, that is close to be validated as a target for anti-cancer therapies.

### **Contribution of mTOR pathway**

In order to improve therapeutic benefit, anti-cancer drugs are frequently combined into rational dual treatments in the perspective of reducing drug concentrations and subsequent potential side effects, as well as exploiting potential synergistic effects. In particular, CX-5461 has been successfully combined with the PI3K/AKT/mTOR inhibitor everolimus in MYC-driven B lymphoma (Devlin et al., 2016), where AKT signalling is required for rRNA synthesis and tumor progression (Devlin et al., 2013); with CX-6258, inhibitor of the modulator of translation PIM kinase, in advanced prostate cancer, where this strategy overcomes the chemoresistance of metastatic tumors *in vivo* (Rebello et al., 2016); with doxorubicin in osteosarcoma where the anti-cancer effect of doxorubicin is synergistically enhanced (Li et al., 2016); with checkpoint inhibitor UCN-01 and several MAPK inhibitors in acute lymphoblastic leukemia, where cell cycle arrest at G2 phase and

activation of ERK1/2 upon treatment with CX-5461 are resistance mechanisms, so that abrogation of the cell cycle checkpoint and inhibition of ERK1/2 activation potentiate the cytotoxic effect of CX-5461 (Negi and Brown, 2015b). On the contrary, to our knowledge, BMH-21 was never tested in combination with other drugs and our study is the first to explore this strategy.

In our study, we reasoned that differential mTOR pathway activation among TNBC cell lines might contribute to response to rRNA synthesis inhibition, because the mTOR pathway has been shown to coordinate ribosome biogenesis at multiple steps (Mayer and Grummt, 2006) and it is often deregulated in TNBC (Marty et al., 2008; TCGA, 2012). For this reasons, our hypothesis was that a combination of mTOR inhibitors and RNA Pol I inhibitors could enhance the anti-proliferative effect of each other. Our results showed that mTOR pathway activation levels did not correlate with response to RNA Pol I inhibition in TNBC and that co-targeting of RNA Pol I activity and mTOR in HS578T cells resulted in additive, albeit not striking, effect on cell proliferation, contrary to the other tested cell lines where the effect was negligible. Because HS578T cells display stronger mTOR pathway activation and also higher resistance to RNA Pol I inhibitors compared the other cells, our results suggest that mTOR could play a role in resistance to treatment with CX-5461 or BMH-21 in TNBC, but with a limited contribution. Thus, from our results, it appears that mTOR pathway does not contribute to cell response to ribosome biogenesis inhibitors. We did not check whether mTOR pathway is perturbed by inhibition of ribosome biogenesis, as shown in acute lymphoblastic leukemia, where it confers resistance to CX-5461 (Negi and Brown, 2015b). Further studies are actually ongoing to evaluate the activation of mTOR pathway upon inhibition of ribosome biogenesis to have a more complete view of the cellular effects of treatment with RNA Pol I inhibitors.

### **Ribosome biogenesis as a hallmark of cancer: we are not there yet**

Recently, it has been proposed that inhibition of nucleolar stress represents an emerging hallmark of cancer and thus the nucleolus would be a potential target for cancer treatment (Quin et al., 2014). This is in line with the flourishing concept of targeting ribosome biogenesis in cancer, based on its dysregulation caused by oncogenic signaling or loss of tumor suppressors and other surveillance mechanisms in cancer cells.

In this study, we show that targeting ribosome biogenesis could be effective for treating TNBC and that treatment with RNA Pol I inhibitors or FBL knockdown leads to p53-independent cell response potentially causing cell death, but we also show that ribosome biogenesis is not specifically enhanced in TNBC. Moreover, sensitivity to

inhibition of ribosome biogenesis is variable and such variability does not seem related to differential activation of ribosome biogenesis. Indeed, high levels of ribosome biogenesis do not confer particular sensitivity to its inhibition and are not predictive of the behaviour of cancer cells. In fact, our results show that also the mRNA expression levels of genes involved in ribosome biogenesis fail to do the task and do not correlate with sensitivity to RNA Pol I inhibitors or to functional inactivation of FBL. This may reflect the fact that the activity of RNA Pol I machinery components is mainly regulated at the post-translational level, so that the activation of ribosome biogenesis is not controlled at the transcriptional level. This is in line with the observation that most of the RNA Pol I pool is accumulated within the nucleoli in the appropriate ready-to-go conformation, but transcriptionally inactive, constituting a sort of reservoir rapidly activated in case of need (Dundr et al., 2002; Milkereit and Tschochner, 1998; Miller et al., 2001; Scheer and Rose, 1984). In addition, the absence of correlation between sensitivity to the inhibition of ribosome biogenesis and its basal levels in the cells extends even farther, as also the main cellular regulators of ribosome biogenesis seem not to be relevant to explain variable sensitivity. Indeed, no correlation is found with the loss of the main tumor suppressor functions nor with the activation of mTOR pathway.

In the case of breast cancer, it has been observed that overactivation of ribosome biogenesis is associated with tumor progression (Belin et al., 2009) and could be a driver of tumorigenesis in mammary epithelial cells (Rossetti et al., 2016). Conversely, our results show that this may not hold true in TNBC. This is not surprising, as TNBC is overly heterogeneous and the deregulation of ribosome biogenesis could be associated to more than one driver, or different drivers among tumors.

In conclusion, currently the relevance of ribosome biogenesis in a specific cancer cannot be predicted, hence cannot the response to its inhibition be anticipated and, from a clinical perspective, efficacy of treatment estimated at this point. However, the idea of targeting ribosome biogenesis as a therapeutic tool has just started to make its way: further studies will hopefully characterize a "ribosome biogenesis status" in different cancer types and identify useful factors to predict the behaviour of a cancer upon targeted therapies.

## **Uncoupling ribosome biogenesis from ribosome function**

As extensively described in the Introduction of this thesis, ribosome biogenesis is a tightly regulated process entailing numerous steps and involving a large number of factors. This requires coordination between multiple pathways and activities involved in the same functions. In cancer cells, ribosome biogenesis can be dysregulated at multiple levels, but

especially increased RNA Pol I activity is generally considered as a signature of cancer (Ruggero and Pandolfi, 2003; White, 2008). RNA Pol I-dependent rRNA synthesis is a rate-limiting step of ribosome biogenesis and is able to act as a master regulator of cellular functions (Chédin et al., 2007; Laferté et al., 2006). Therefore, it is reasonable to hypothesize that an increased ribosome biogenesis is associated to increased activity of factors involved in ribosome biogenesis at multiple steps of the process. Indeed, in breast cancer, FBL expression and ribosome biogenesis rate both have been found to be deregulated and to contribute to tumorigenesis (Belin et al., 2009; Marcel et al., 2013).

Our results suggest that this may not hold true as a general assumption. Because FBL is an effector of ribosome biogenesis and genes involved in ribosome biogenesis tend to be regulated in a coordinated manner, we speculated that FBL levels would correlate with ribosome biogenesis and, possibly, with the amount of rRNA produced in TNBC cell lines. However, no correlation was found between the amount of rRNA and the expression of FBL, suggesting that ribosome biogenesis could be unlinked from ribosome activity. Of course, the actual rate of ribosome biogenesis should be confirmed by the use of more sensitive and quantitative methods such as EU incorporation in pulse chase experiments. Nevertheless, our results already suggest that there may not be any correlation between the expression of an essential factor for ribosome biogenesis and ribosome production. This may reflect additional roles of factors involved in ribosome biogenesis, such as FBL, and ribosome biogenesis itself, other than the mere activity of the ribosome, that is only the final product of the process, in its contribution to tumorigenesis.

This hypothesis could even be pushed farther, involving the role and regulation of protein synthesis in cancer and reconsidering its link with ribosome biogenesis. It is today widely accepted that higher levels of ribosome biogenesis correlate with the development of cancer because malignant cells require enhanced ribosome production to fulfill the increased demand for protein synthesis needed in turn for the increased metabolism and cell proliferation, hallmarks of cancer cells. This implies that generally ribosome biogenesis and cell growth and proliferation are coordinated processes, mostly regulated in tight association. However, this assumption may not hold true in cancer cells, as the coordination of cellular processes is ensured by mechanisms that are frequently deregulated. Cancer cells may produce high amounts of ribosomes irrespective of their need for enhanced protein synthesis. Indeed, it has been reported that breast cancer cells displaying the same protein synthesis rate do not necessarily have the same ribosome biogenesis rate (Belin et al., 2009), suggesting that ribosome biogenesis may play

additional roles other than the mere production of high amounts of ribosomes in the contribution to the cancer phenotype. A recent study classified different populations of MDA-MB-231 cells depending on their metastatic capacity and aggressiveness, then characterized gene expression profiles of derived metastases obtained in xenograft mouse models. It was found that the most aggressive metastatic cells were associated to the upregulation of gene sets related to RNA metabolism or ribosome biogenesis, but no evidence was found for increased expression of genes involved in cell proliferation. The authors suggested that ribosome biogenesis could play a direct role in metastatic dissemination independently of effects on cell proliferation (Johnstone et al., 2018).

Our study shows that the amount of ribosomes per cell varies greatly among TNBC cell lines, unlike their cell proliferation activity, suggesting that a higher amount of ribosomes is not necessary for cancer phenotype, whereas ribosome biogenesis rate could be crucial for cancer cell survival. In any case, less ribosomes do not imply less translational activity: ribosomes in particular types of cells could be endowed with quantitatively improved or qualitatively altered translation, contributing to cancer phenotype. Also, it should be considered that the amount of ribosomes is a steady-state level that it could not be necessarily correlated with ribosome biogenesis rate: ribosome stability could vary among different cellular systems and a cell could produce and degrade large amounts of ribosomes while displaying low levels of ribosomes at each moment.

Obviously, these speculations should be proven by appropriate experimental evidence. Further investigations about the relationship between translation rate and ribosome biogenesis rate in cancer cells could answer many of the raised questions. For example, the quantitative evaluation of protein synthesis rate through metabolic labeling or non-isotopic labeling (puromycin labeling, AHA-click chemistry) in parallel with the evaluation of rRNA synthesis rate through pulse chase could be a first step to answer the question. This may become a critical parameter for understanding and exploiting ribosome biogenesis and protein synthesis in anti-cancer treatment.

# Attachments

# **2'-O-Methylation of Ribosomal RNA: Towards an Epitranscriptomic Control of Translation?**

Review

# 2'-O-Methylation of Ribosomal RNA: Towards an Epitranscriptomic Control of Translation?

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Received: 17 August 2018; Accepted: 27 September 2018; Published: 3 October 2018



**Abstract:** Ribosomal RNA (rRNA) undergoes post-transcriptional modification of over 200 nucleotides, predominantly 2'-O-methylation (2'-O-Me). 2'-O-Methylation protects RNA from hydrolysis and modifies RNA strand flexibility but does not contribute to Watson-Crick base pairing. The contribution of 2'-O-Me to the translational capacity of ribosomes has been established. Yet, how 2'-O-Me participates in ribosome biogenesis and ribosome functioning remains unclear. The development of 2'-O-Me quantitative mapping methods has contributed to the demonstration that these modifications are not constitutive but rather provide heterogeneity to the ribosomal population. Moreover, recent advances in ribosome structure analysis and in vitro translation assays have proven, for the first time, that 2'-O-Me contributes to regulating protein synthesis. This review highlights the recent data exploring the impact of 2'-O-Me on ribosome structure and function, and the emerging idea that the rRNA epitranscriptome is involved in translational control.

**Keywords:** 2'-O-methylation; ribosomal RNA; fibrillar; snoRNP; ribosome heterogeneity; mRNA translation

## 1. Introduction

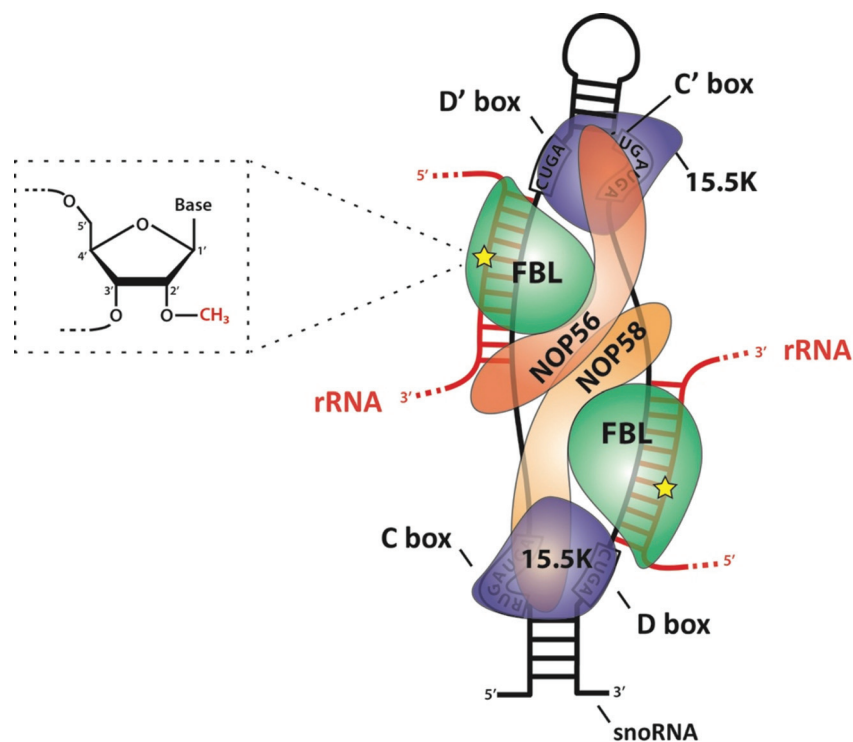
Chemical modification is a major mechanism that regulates the properties and functions of biological molecules. RNAs are no exception and modifications are found in all classes of RNA and in all living organisms. To date, at least 171 types of modifications have been identified (Modomics database, <http://modomics.genesilico.pl>) [1]. Ribosomal RNA (rRNA) bases undergo several types of modifications either through the addition of chemical groups, such as methyl- or acetyl-groups, or through the isomerization of uridine into pseudouridine ( $\psi$ ). The ribose is also subjected to modifications by the addition of a methyl group at its 2' position (2'-O-methylation, 2'-O-Me). Moreover, several nucleotides are subjected to multiple sequential modifications. The number of modified nucleotides increases from 33 in bacteria to 112 in yeast and over 210 in humans, a tendency that parallels not only the growing size and complexity of the ribosome but also the increased complexity of translational programs [2–4]. Deciphering the molecular roles and biological functions of these modifications is one of the greatest challenges of modern biology. This review focuses on recent progress regarding rRNA 2'-O-methylation and its contribution to the functioning of human ribosomes and messenger RNA (mRNA) translation.

## 2. Adding 2'-O-Methylation to Eukaryotic rRNA: How, Where, and When?

2'-O-Methylation is added to rRNA post-transcriptionally by ribonucleoprotein (snoRNP) complexes containing C/D box family small nucleolar RNAs (snoRNAs), thus termed C/D snoRNP complexes, in which the snoRNA defines the nucleotide to be methylated by sequence complementarity with rRNA (Figure 1). snoRNA-mediated guidance is also used to specify  $\psi$  sites (see [5]). This differs



from stand-alone enzymes that directly ensure substrate specificity and represents the main mechanism of base modification. However, there are some exceptions, for instance, the acetylation of two bases of the yeast 18S rRNA, the modification of which is carried out by *Kre33*, and substrate specificity guided by snoRNAs snR4 and snR45 [6]. The assembly and composition of C/D snoRNP complexes have been reviewed extensively and is simply summarized herein [2,7–9]. In addition to one C/D box snoRNA, C/D snoRNPs contain the methyltransferase fibrillarin (FBL, Nop1 in yeast), the RNA binding protein 15.5K (also named NHP2L1, and Snu13 in yeast), and the heterodimer of two closely related proteins NOP56 and NOP58. Methyltransferase fibrillarin uses S-adenosyl methionine (SAM) as the methyl donor. C/D box snoRNAs are defined by two conserved sequences named C (RUGAUGA, where R is a purine) and D boxes (CUGA), which are usually present in duplicate (the second set is marked C' and D') and flank an antisense sequence to the targeted rRNA. C/D box snoRNPs are structured in a manner that positions the catalytic site of FBL directly opposite the nucleotide to be methylated, within the duplex formed by the guiding snoRNA and the targeted rRNA, precisely five nucleotides upstream of a D box (or D') [10–12]. The archaeal C/D sRNP complex is the main model used to study this structure, due in part to the difficulty in assembling the eukaryotic snoRNP complex in vitro. Some controversy remains as to how the complex is specifically organized. Several studies have revealed how rRNA/snoRNA base pairing, 15.5K (named L7ae in archaea), and the NOP56/NOP58 dimer (Nop5 homodimer in archaea) are instrumental in positioning the FBL catalytic site and the methyl donor SAM within close proximity of the 2'-OH group to be methylated [12–15].



**Figure 1.** Schematic representation of the C/D box snoRNP complex, depicting the main features of the complex according to both biochemical data and structural studies using an archaeal complex. The C and D boxes are depicted with their consensus sequence. The substrate ribosomal RNA (rRNA, red) forms an RNA:RNA duplex with the snoRNA along 10–21 nucleotides (nt). The catalytic site of fibrillarin (FBL) faces the fifth nucleotide downstream of the D or D' boxes (yellow star). The 15.5K protein specifically binds a k-turn motif. The NOP56:NOP58 heterodimer (which is a homodimer in archaea) forms two long alpha helices that are placed across the snoRNA:rRNA complex and contribute to locking FBL in its active position. rRNA: ribosomal RNA; snoRNA: small nucleolar ribonucleoprotein.

In humans, C/D box snoRNAs (or SNORD) are 70–120 nucleotides (nt) long and are mainly encoded within introns of coding genes, and processed from lariat introns upon mRNA splicing. Since many snoRNAs are encoded by genes associated with ribosomal biology, such as ribosome biogenesis factors or ribosomal proteins, it is expected that this genomic organization promotes co-regulatory mechanisms, although this remains to be demonstrated. C/D box snoRNP particles are assembled via a multi-step process, involving chaperone and helicase activities carried out by complexes such as R2TP [16–19], which ensures the loading of 15.5K, NOP58, NOP56, and FBL proteins onto the C/D box snoRNA. During this process, 15.5K will specifically bind to a k-turn structure within the C/D box. C/D box snoRNP assembly takes place in part within the Cajal bodies (one of the preeminent nuclear domains involved in small non-coding RNA maturation) before the complex migrates to the nucleolus where it operates at an early stage of ribosome biogenesis, likely within or at the edge of the dense fibrillar component.

Evaluating the timing of 2'-O-Me has been a long-standing issue. Early studies demonstrated that 2'-O-Me is a nuclear event [20]. More recently, in an elegant series of isotope labeling experiments, data fitting and mathematical modeling, Tollervy's laboratory provided the first dataset showing that a large portion of 2'-O-Me occurs co-transcriptionally in yeast, with the 18S rRNA precursor being almost fully modified before its release from the RNA polymerase I (Pol I) complex [21]. Quantitative site-specific analysis comparing chromatin-associated RNA with mature rRNA further confirmed the general principle established by labeling assays [22]. In addition, it revealed that in yeast, even at a very early stage, before the 18S rRNA is cleaved from the pre-rRNA, all but one position of the 18S rRNA (18S-Am100) displayed over 80% of their final level of modification. Accordingly, the 25S rRNA sites are less methylated in the RNA Pol I-associated pre-rRNA [22]. Thus, the current knowledge on the timing of 2'-O-Me in yeast cells supports a general model in which rRNA is modified at a very early stage of ribosome biogenesis and is likely to be completed before rRNA is fully assembled as ribosomal pre-subunits. It remains to be determined whether such a model applies to human rRNAs, which display partially methylated nucleotides (see below).

In most cases, a 2'-O-Me position is modified by a unique C/D box snoRNA so that single SNORD/2'-O-Me associations can be defined. However, there are a few cases of combinatorial associations, such as C/D snoRNAs modifying several sites (e.g., SNORD50A modifies 28S-Cm2848 and 28S-Gm2863 in humans) or 2'-O-Me positions modified by more than one C/D snoRNA (e.g., 18S-Am668 by SNORD36A and SNORD36B, both encoded by the *RPL7A* gene in humans). How such SNORD combinations contribute to regulating 2'-O-Me remains obscure.

### 3. 2'-O-Methylation as a Source of Heterogeneity: All Ribosomes Are Not Created Equal

In line with other RNA chemical modifications, detecting and measuring 2'-O-Me remains challenging [23]. 2'-O-Me detection methods have previously been extensively reviewed and are only summarized herein [23–26]. Detection of 2'-O-Me is based essentially on two properties of 2'-O-methylated RNA. First, 2'-O-Me replaces the 2'-OH group, thus rendering methylated RNA resistant to hydrolysis compared to unmethylated RNA. Second, 2'-O-Me tends to block retro-transcription of RNA into complementary DNA (cDNA) when the reaction is performed under non-optimal conditions (e.g., low deoxy-nucleoside triphosphate (dNTP) concentration). Both properties have been used for site-by-site analysis and were exploited only recently for global mapping in a single experiment using RNA-sequencing based methods [16,19]. All RNA-sequencing based approaches provided qualitative mapping to identify 2'-O-Me positions and revealed novel perspectives in ribosome biology but also in the field of epitranscriptomics. Of note, the method called Nm-seq appears to be one of the most sensitive methods and allows the detection of 2'-O-Me even from scarce amounts of RNA, including mRNA and non-coding RNA [27]. This is achieved by means of iterative cycles of oxidative-elimination-dephosphorylation reactions, which sequentially eliminate 2'-unmodified nucleotides from 3' to 5' and leave the 2'-O-methylated sites intact. In the final step, dephosphorylation is prevented so that only RNA fragments with a 2'-O-methylated 3' end possess a 3'-OH at their 3'

end, and can be ligated for library preparation. Consequently, 2'-O-Me sites generate a positive signal and not a lack of signal, in contrast to the methods based on the inhibition of retro-transcription (RT) reaction or of hydrolysis. Among these novel mapping methods, RiboMeth-seq has emerged as the only method that consistently detects all known 2'-O-Me sites in yeast and human rRNAs [22,28–30]. Most importantly, RiboMeth-seq is the only available method for the quantitative evaluation of 2'-O-Me levels at each position, thus providing information on potential 2'-O-Me variations and providing 2'-O-Me profiles [22,29]. At present, two different protocols have been published. The first one was reported by Nielsen's group and the second alternative protocol, which uses very low amounts of starting material, was reported by Motorin's group [22,28–31].

The repertoire of 2'-O-Me was recently updated by Krogh and colleagues and contains a list of 106 validated sites, as well as two additional strong candidate sites that currently lack clear evidence by mass spectrometry [28,30]. These 106 2'-O-methylated nucleotides were considered validated using several approaches: (1) biochemically [24]; (2) RiboMeth-seq; (3) mass spectrometry (if new or uncertain); and (4) verifying that at least one guide C/D box snoRNA had been attributed to the corresponding nucleotide environment. This list is expected to grow as nucleotides that are modified either at a low level or only in specific biological contexts have not yet been validated by several independent techniques. At present, several novel candidate nucleotides have been published using different high confidence 2'-O-Me mapping techniques [27,32,33]. In addition, a set of 14 novel 2'-O-Me positions were exposed based on rRNA:snoRNA interactions and unveiled by cross-linking immunoprecipitation (CLIP) with antibodies against FBL, NOP58, and NOP56 [34]. It is critical that the RNA-modification databases are updated because new 2'-O-Me sites (and other modifications) are being discovered and validated.

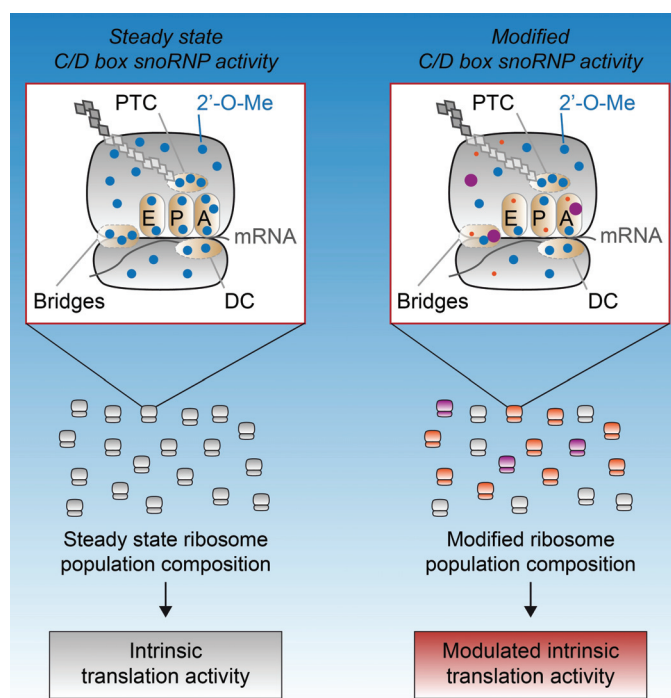
2'-O-Me was considered to be a constitutive and universal modification, leading to the alteration of each site in every ribosome of the cell. Such a dogma stemmed from the methods used that were unable to detect small differences in the level of modification [24,35], and from the observation that in yeast, one of the historical 2'-O-Me models, rRNA display complete methylation at almost all sites in all ribosomes. Yet, evidence of fractional methylation of 5.8S-Um14 was provided in early studies based on nuclease fragmentation of 5.8S rRNA in mammalian tissue and HeLa cells [36,37] and more recently in yeast at the 18S-Am100 position using DNazyme-based hydrolysis and LC-UV-MS/MS analysis [38]. This view of the rRNA 2'-O-Me landscape has largely evolved based in part on RiboMeth-seq data that showed that some specific positions in yeast rRNA are partially methylated [22,29]. This was further supported by large-scale MS-based analyses and mung bean nuclease assays followed by RP-HPLC [39,40].

Whether 2'-O-Me profiles of yeast rRNAs can vary under various physiological conditions remains to be explored. In humans, evidence of fractional 2'-O-Me was obtained using a semi-quantitative method based on the inhibition of the RT-reaction followed by qPCR, and revealed that 2'-O-Me levels at some sites varied among rRNAs from breast cancer cells at various stages of tumorigenesis and tumor progression [41,42]. Recent RiboMeth-seq based studies also demonstrated that methylation is partial (i.e., <80%), around a third of the 106 sites in exponentially growing cultured cells [28,30,31,34]. Having a number of partially methylated sites implies that the pool of ribosomes is heterogeneous in composition and that subpopulations of differentially methylated ribosomes co-exist (i.e., ribosomes that lack methylation at several sites). Hence, the complete set of 2'-O-Me is not required for rRNA assembly in a human ribosome as was initially assumed based on studies in yeast. This finding was corroborated by small interfering RNA (siRNA) results, which showed that FBL knockdown resulted in the production of ribosomes displaying up to 50% less 2'-O-Me [30]. Importantly, these data demonstrate that cells tolerate the production of partially methylated ribosomes (Figure 2). Overall, 2'-O-Me contributes to shaping a wide variety of ribosomes, thus creating ribosomal heterogeneity.

Mechanistically, the regulation of 2'-O-Me profiles is uncertain. siRNA knockdown studies unexpectedly revealed that changes in the FBL level result in a site-specific decrease in 2'-O-Me instead of a global and homogeneous decrease across all 2'-O-Me sites [30,31]. This indicated that the

mechanisms regulating 2'-O-Me act in a site-by-site manner rather than globally. A likely regulatory mechanism resides in the modulation of the level of methylation at corresponding sites through changes in the expression of some C/D snoRNAs, depending on physiopathological conditions. Indeed, several examples have been reported in cancer, including significant changes in SNORD expression in leukemia and lung cancer [43–46]. Some of these were shown to contribute to tumorigenesis, such as SNORD50 in lymphoma and prostate cancer or SNORD78 in lung cancer [47]. However, their impact on modulating 2'-O-Me levels at the associated rRNA site remains to be addressed. Although these examples illustrate putative variations of 2'-O-Me via changes in snoRNA expression, the first analyses comparing 2'-O-Me profiles and snoRNA expression levels in cultured cells using omics approaches failed to establish any global correlation [28,31], thus suggesting that C/D box snoRNA levels may not be the primary 2'-O-Me regulatory mechanism. Additional mechanisms should be considered, such as the variable catalytic efficacy of individual C/D box snoRNPs. Post-translational modifications of FBL or its partners [48] could also contribute to this regulation, together with the structure of the snoRNA itself, since *in vitro* methylation assays revealed that D and D' boxes can display variable catalytic efficacy and that the complementary sequence and its flanking sequences also influence the activity of the complex [12,15,49]. Another possibility is that additional factors might regulate the core snoRNP complex and its function. For instance, it was shown that uL13 protein (also named RPL13a) contributes to rRNA 2'-O-Me and can be pulled down with FBL and the snoRNA U15 [50]. Finally, it should be noted that the nucleolus is a very dense nuclear domain in which the probability of molecular interactions can be either reduced due to competitive binding to the same substrate or facilitated by a molecular crowding effect [51,52]. Whether this particular nucleolus environment influences the probability of snoRNPs finding their target and regulating 2'-OMe pattern remains to be explored.

The demonstration that 2'-O-Me is not constitutively added in all ribosomes and that cells tolerate the production of partially modified ribosomes represents major evidence of ribosome plasticity through composition variation. It is now critical to determine whether 2'-O-Me-mediated ribosomal heterogeneity takes place in normal and pathological tissues. Interestingly, genetically encoded variations in ribosome composition have been reported for ribosomal proteins, but they remain to be identified in rRNA [53,54].



**Figure 2.** Model of 2'-O-methylation (2'-O-Me) profile modulation and its consequence on the intrinsic translational activity of ribosomes. In this model, we represent two possible states: a steady state and

a modified state. Following a change in C/D box snoRNP activity (e.g., change in FBL or SNORD expression) during ribosome biogenesis, 2'-O-Me levels change in a site-specific manner as revealed in [30,31] (unchanged level: blue dots; decreased level: red dots; increased level: purple dots). 2'-O-Me is altered in key functional regions of the ribosome, including the inter-subunit bridges, the decoding center (DC), and the peptidyl-transferase center (PTC), the latter being located close to the transfer RNA (tRNA) binding sites (A: aminoacyl-tRNA binding site; P: peptidyl-tRNA binding site; and E: exit site) [25,26]. The ribosomal population changes in its composition and variety (gray, orange, and purple ribosomes). When tested in an in vitro cell-free translation assay, these ribosomal populations exhibit a differential translational activity [25]. mRNA: messenger RNA; snoRNA: small nucleolar ribonucleoprotein; SNORD: C/D box snoRNA gene.

#### 4. 2'-O-Methylation Contribution to Ribosome Structure and Function

A key, yet unresolved issue is determining to what extent the presence or absence of 2'-O-Me modulates rRNA folding, dynamics, and interactions with RNAs (including rRNA, mRNA, tRNA) and ribosomal proteins. 2'-O-Me is one of the RNA modifications that does not directly alter the Watson-Crick laws governing base pairing, making its contribution to RNA structure and function even more complex to anticipate. In addition to protecting RNA from hydrolytic cleavage, a well-known consequence of 2'-O-Me is the stabilization of the nucleotide conformation (i.e., the C3'-endo puckering of the ribose moiety), which is favored in RNA strands due to the OH group [55]. In addition, 2'-O-Me restricts the rotational freedom of 3'-phosphate, thus potentially restricting RNA strand conformation and flexibility. Similar consequences apply to the RNA duplex. Indeed, the 2'-O-methylated RNA duplex displays hydration patterns, helix winding, and base tilting that are slightly different from non-2'-O-methylated RNA duplexes and indicate that 2'-O-Me may influence RNA helix flexibility [56,57].

There are no published follow-up studies focusing on the role of 2'-O-Me on RNA conformation, such as single strand rRNA or rRNA:protein complexes, that are found within ribosomes, and the field has thus not been updated for several years. Fortunately, recent progress in structural analysis of entire ribosomes has enabled the visualization of some rRNA modifications and unraveled their involvement in molecular interactions. Steitz's laboratory reported a crystal structure of bacterial ribosome at a 2.3–2.5 Å resolution revealing several rRNA modifications including 2'-O-Me, and showed that they establish contact with ribosome ligands between the ribosomal subunits and within rRNAs [58]. For example, 2'-O-Me contributes to hydrophobic interactions between nucleotides, and 2'-O-methylated nucleotides, such as 23S-Gm2251 and 23S-Um2552, establish contacts with the CCA-end of tRNAs located both in A and P sites. Notably, these modifications are highly conserved across species, suggesting that they play an important role in shaping ribosome structure. There has also been a dramatic improvement in the resolution of the structure of large complexes using cryo-electron microscopy (cryoEM), which has granted access to the structure of human and other eukaryotic ribosomes [59–61]. Using improved cryoEM and refinement methods during image processing, Klaholz's laboratory obtained structures at a resolution of up to 2.5 Å for human ribosomes and visualized 130 rRNA modifications including 57 2'-O-Me [61]. This study further demonstrated the role of 2'-O-Me in local and global stabilization of the rRNA structure, particularly in more flexible rRNA regions, such as bulges, hairpin loops, and rRNA-specific structural elements, such as k-turn or A-minor motifs. Overall, these studies have supported a contribution of 2'-O-Me and other rRNA modifications to the stabilization rRNA folds and the establishment of interactions not provided by non-modified nucleotides within functionally important regions of the ribosome. However, determining the structural impact of 2'-O-Me by comparing ribosomes carrying either methylated or non-methylated nucleotides will be essential. In yeast, footprinting of rRNA previously demonstrated that removal of 2'-O-Me and pseudouridylation alters the accessibility of rRNA [62]. Thus, data on 2'-O-Me localization within the ribosome and on their contribution to rRNA structure strongly support that 2'-O-Me contributes to ribosome function.

The role of 2'-O-Me on ribosome functioning and mRNA translation was initially explored in yeast in a series of studies in which both 2'-O-Me and  $\psi$  were selectively removed from key regions of the ribosome, such as the subunit interface, the peptidyl-transferase center, and the decoding center by introducing mutations into the corresponding snoRNA guide [62–65]. Similar observations were made for 2'-O-Me and  $\psi$  individually, and led to the conclusion that the lack of chemical modifications in at least two to three positions was necessary to observe an impact on cell growth and on the protein synthesis capacity of yeast cells. This observation led to the belief that individual 2'-O-Me sites were not essential to ribosome functioning. Nevertheless, additional data in yeast has indicated that 2'-O-Me contributes to regulating translational activity of the ribosomes, and may selectively affect translation rather than regulating bulk protein synthesis. Indeed, it was shown that 2'-O-Me impacted translation reliability and reproducibility and appeared to modify yeast phenotype when cells were exposed to antibiotics, stresses, or poor growth conditions [66,67]. These data suggested that 2'-O-Me might regulate translation of a subset of mRNAs, a hypothesis that is now supported by studies in metazoan models.

While single SNORD knockout had no or little impact on yeast cells, knocking down the expression of single snoRNAs had severe consequences on zebrafish embryonic development, suggesting that complex metazoan genetic programs require the full potential of ribosome 2'-O-Me [68]. Yet, this conclusion may have been hasty since lack of 2'-O-Me was verified at only one site (U26 target) and further experiments were not carried out to exclude snoRNA host gene deficiency. The role of 2'-O-Me in regulating translation of selected mRNAs has been progressively demonstrated, first by correlative observations between altered 2'-O-Me profiles and translation reporter assays in breast cancer models [41,42], and second in models in which expression of the rRNA methyltransferase FBL was knocked down [30,42,50,69]. Interestingly, modulation of FBL expression impacted mRNA translation initiated from internal ribosome entry sites (IRES). Internal ribosome entry site elements are highly structured RNA sequences that are able to recruit the small ribosomal subunit and initiate translation in a process that short-cuts the cap structure at the 5' end of mRNAs. This mode of initiation is highly stimulated under stress when cap-dependent translation is reduced or inhibited, such as in response to heat shock, hypoxia, or viral infection. This concerns many cellular mRNAs that are key to cell behavior and contain IRES elements, including growth factors and receptors (e.g., *IGF1R*), apoptosis regulators (e.g., *XIAP*), oncogenes (e.g., *c-Myc*), and tumor suppressors (e.g., *TP53*) [70]. Using reporter assays, several independent studies established that modulation of FBL expression differentially impacted translation from various IRES elements, suggesting that 2'-O-Me might regulate mRNA selection by the ribosome. Of note, altering the pseudouridylation content of ribosomes also led to changes in IRES-dependent translation that differed from the one observed for 2'-O-Me, and further support that the two types of rRNA modifications modulate translation, although with distinct regulatory outcomes. In addition to IRES-containing mRNAs, a ribosome profiling analysis demonstrated that FBL modulation affects the translation of several other mRNAs, thus expanding the subset of mRNAs the translation of which can be regulated by 2'-O-Me [30]. Interestingly, mRNA translation was either increased or decreased in response to 2'-O-Me profile modulation, indicating that 2'-O-Me contributes to both stimulatory and inhibitory mechanisms [71]. These data suggest that modulation of FBL expression, and therefore of 2'-O-Me profile, likely impacts several regulatory mechanisms that remain to be identified [30].

Importantly, alterations in translational regulation observed in previously described cellular assays could be attributed to ribosomes. This was shown by means of an in vitro cell-free translation assay, in which purified ribosomes were the only variable parameter [30,72]. While ribosomes exhibiting an altered 2'-O-Me profile displayed similar translational activity on mRNA carrying a GAPDH or actin 5'-UTR compared to control ribosomes, they exhibited a decreased ability to initiate translation from the dicistrovirus IRES elements [30]. Thus, 2'-O-Me modulates the intrinsic ability of ribosomes to initiate translation in a 5'-UTR-dependent manner (Figure 2). Of note, a similar observation was made with ribosomes carrying an altered  $\psi$  content, further strengthening the

central role of rRNA modifications in ribosome functioning and translational control [73]. Further exploration of *in vitro* cell-free assays [72,74] should help decipher which steps of the translation process are controlled by rRNA chemical modifications and identify target mRNAs. Altogether, these studies demonstrate that 2'-O-Me represents a novel molecular support for mRNA translation and contributes to the functional specialization of the ribosome. Deciphering how 2'-O-Me and other RNA modifications regulate translation will be a challenge in the coming years. Indeed, the precise molecular mechanisms governing the translational efficacy of a given mRNA remains partially understood. mRNAs are associated with many factors (cap-binding complex, RNA binding proteins, microRNAs) that modulate the recruitment of the 40S and 60S subunits. The ribosomal subunits play a significant regulatory role in this process, at least through subunit-bound factors. For example, the 40S-associated eIF3 "canonical" translation initiation complex was shown to carry a selective preference toward particular mRNA 5'-UTR sequences, thus modulating the translational efficacy of mRNA subsets [75]. Some 2'-O-Me rRNA may modulate the ribosome-bound proteomes that will either favor or restrict the recruitment of specific mRNA subsets. Another possibility is that 2'-O-Me regulates the direct interaction of the mRNA complexes with rRNA as suggested for mRNAs on which translation can be initiated in a cap-independent manner [70].

### 5. Perspective: Towards an rRNA-Based Epitranscriptomic Control of mRNA Translation

rRNA 2'-O-methylation was considered to be a constitutive chemical modification that optimized the functioning of higher eukaryotic ribosomes. Consequently, its role was underexplored in studies aiming at deciphering mechanisms involved in translational regulation. Several recent technical and conceptual breakthroughs have challenged this view and drawn attention to these mysterious modifications: first, the "specialized ribosome" concept, which has fueled new interest in ribosome functioning; and second, rRNA modification detection by omics-based mapping methods and by high-resolution structural techniques. Based on recent technical advances, new evidence supports this novel view of 2'-O-Me in different fields. Indeed, variability in 2'-O-Me and in the expression of 2'-O-methylation factors (FBL, C/D box snoRNAs) and in their contribution to particular cellular phenotypes have been investigated in various physiological contexts, including brain development [76,77] and stem cells [78], as well as pathological contexts, such as cancer [47,79,80] and genetic diseases [81].

In the context of translation, ribosomal diversity induced by 2'-O-Me heterogeneity stands to reason. Translation relies on a delicate molecular process that ensures not only the proper decoding of mRNA and peptide assembly but also the accommodation of a large variety of mRNAs. Consistently, it is not surprising that such a process relies on a variety of ribosomes. Recent publications on rRNA 2'-O-methylation [30], as well as rRNA pseudouridylation [73] and ribosomal proteins [82], have paved the way for further demonstrations of this novel concept.

Within chromatin, epigenetic regulatory mechanisms rely primarily on chemical modifications of both histones and DNA that modulate the use of the underlying DNA sequence. It is very tempting to establish a parallel with mRNA translation, and thus to propose that rRNA modifications represent a molecular support of an rRNA-mediated epitranscriptomic regulation of mRNA translation. The variation in 2'-O-Me profiles, the identification of potential hyper-sensitive 2'-O-Me sites [30,31], and the contribution of 2'-O-Me to the intrinsic ribosomal activities make 2'-O-Me an excellent candidate for rRNA epitranscriptomics. However, further studies should attempt to elucidate 2'-O-Me sites that promote—individually or in combination—translational regulation events of particular mRNAs. Adapting the composition of ribosome subpopulations would represent another means of regulating subfamilies of mRNAs, contributing to particular cellular pathways at the translational level.

Undoubtedly, recently developed tools to quantitatively analyze 2'-O-Me profiles, to visualize their role in the ribosome structure, and to evaluate translational regulation by ribosome profiling and *in vitro* translation will be instrumental in breaking the ribosome code.

**Author Contributions:** P.L.M. and F.C. contributed manuscript outline and overviewed the project; P.L.M., V.M., J.-J.D. and F.C. equally contributed to the writing.

**Funding:** P.L.M. is supported by a PhD fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche of the French government. F.C. is a CNRS research fellow. V.M. and J.-J.D. are Inserm research fellows. J.-J.D.'s laboratory is supported by Inserm, CNRS, Université Claude Bernard Lyon 1, Centre Léon Bérard, Agence Nationale pour la Recherche (RIBOMETH ANR-13-BSV8-0012-01), Programmes d'Actions Intégrées de Recherche (PAIR Sein, RiboTEM, ARC\_INCa\_LNCC\_7625), Ligue Nationale contre le Cancer, Ligue contre le Cancer comité départemental (Drôme, Rhône, Isère, Puy-de-Dôme, Allier) and Fondation ARC pour la Recherche sur le Cancer (2016120486).

**Acknowledgments:** We thank F. Lafôret for the original preparation of Figure 1, and Brigitte Manship for editing the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# **Co-existence of stable and variant rRNA 2'-O-Methylation sites in human breast tumours**

(in preparation)

**OBJECTIVE: Letters to Nature, PNAS (format letter), Nat com, Nature cancer**

**TITLE PAGE (title = 90 characters with space)**

**Co-existence of stable and variant rRNA 2'-O-Methylation sites in human breast tumours**

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**Keywords:** ribosomal RNA (rRNA), 2'O-methylation (2'OMe), ribosome structure, specialized ribosome, breast cancer

**Word count:**

**Abstract (Nature 200-300 words with references/ usually 150 words for other journals)**

Emerging evidence suggests that the human ribosome plays a key role in regulating translation through changes in chemical modification levels such as ribosomal RNA 2'-O-ribose methylation (rRNA 2'OMe), and thereby might contribute in setting particular phenotypes such as hallmarks of cancer cells {Marcel:2013jl, Eralles:2017iz, Monaco:2018fp, Genuth:2018kb, Helm:2017eg}. Here we have analysed the rRNA 2'OMe in a large series of 195 human primary breast tumours using the RiboMETH-seq approach {Birkedal:2015jf, Marchand:2017fr, Eralles:2017iz}. We demonstrated that rRNA 2'OMe varies between human samples. Moreover, analysis of large-scale human series showed that the rRNA molecules can tolerate the absence of 2'OMe, however at some particular but restricted number of positions in humans. We identified the co-existence of stable and variant 2'-O-ribose methylated positions along the human rRNA molecules. Differential association of rRNA 2'OMe with breast cancer features, evolution and structure/function characteristics suggest the stable positions showing a low inter-variability might be required to maintain the core structure and thus the function of the ribosome, while the variant positions displaying high inter-variability might be the source of regulatory events. Data issued from rRNA 2'OMe profiling of a large-scale human sample series reunites two divergent visions of the ribosome and sustain the emerging role of the ribosome as regulator of translation in cancer.



## **Main text (1500 words)**

Changes in ribosome composition at both ribosomal protein and RNA (rRNA) levels is an emerging mechanism that contributes in translational regulation and phenotype establishment, including in cancer {Genuth:2018kb, DallaVenezia:2019de, Xue:2012el}. The human ribosome is a ribonucleoprotein complex in which the 4 rRNAs (5S, 5.8S, 18S and 28S) are chemically modified {Monaco:2018fp, Genuth:2018kb, Natchiar:2018gd}. The human rRNAs exhibit more than 210 chemical modifications out of which the 2'O-ribose methylation (thereafter 2'O-methylation) is one of the most abundant modifications with about 106 described rRNA 2'O-methylated positions {Monaco:2018fp, Helm:2017eg, Natchiar:2018gd}. Originally the chemical modifications of the rRNAs were first identified in bacteria and yeast and were thought to be compulsory to maintain the highly conserved structure of the rRNAs on which the ribosome activity relies, through their involvement in RNA:RNA and RNA:protein interactions {Decatur:2002wf, Monaco:2018fp}. However, this idea has been highly challenged in the past few years. Thanks to the recent resolution of technological issues, including the development of RNA-seq based approaches dedicated to the profiling of rRNA 2'O-methylation {Helm:2017eg, Genuth:2018kb, Marchand:2016ey, Krogh:2016cu} or the improvement of cryo-EM resolution {Khatter:2015fs, Natchiar:2017ij, Natchiar:2018gd}, rRNA 2'O-methylation has been characterized in human cell lines, however in a few number. rRNA 2'O-methylation has been analysed in the ovarian HeLa {Incarnato:2016cz, Krogh:2016cu, Eroles:2017iz, Natchiar:2017ij} and PA1 cell lines {Zhu:2017io}, in the colorectal HCT-116 cells {Sharma:2017fi, Krogh:2016cu}, and in the human embryonic kidney 293 cells {Gumienny:2017fi}. Alterations of rRNA 2'O-methylation have also been described in cellular models modelling tumour initiation and progression of solid and haematological cancers {Su:2014ha, Marcel:2013jl, Gachet:2018gy, Zhou:2017io}. These studies demonstrated that the frequency of the methyl groups added at the 2' position of the ribose could be different between the 106 nucleotide positions, suggesting the existence of several ribosome populations exhibiting different rRNA 2'O-methylation profiles

within a single cell population. In parallel, we and others reported an association between variation in rRNA 2'O-methylation and modulation of translation efficiency of particular mRNA subsets {Monaco:2018fp}. Modulation of rRNA 2'O-methylation in different cancer cellular models, has indeed been shown to regulate the translation of some cellular Internal Ribosome Entry Sites (IRES)-containing mRNAs, such as IGF1R or FGFR family members, or of mRNAs encoding proteins involved in oxidative phosphorylation {Belin:2009kk, Marcel:2013jl, Gachet:2018gy, DSouza:2018fu, Su:2014ha}. Moreover, using cell-free *in vitro* translation assays, we recently demonstrated that modulation of rRNA 2'O-methylation directly affects IRES-dependent translation initiation of Cricket paralysis virus (CrPV) mRNA {Erales:2017iz}. However at present, due to the lack of analyses of large-scale human series, the fact that rRNA 2'O-methylation can vary in real-life situation, both between the 106 rRNA 2'O-methylated positions and/or between human samples, remains a matter of discussion {Monaco:2018fp, Genuth:2018kb}.

We hypothesized that the high heterogeneity of breast tumours {Prat:2015fr, Perou:2000if} might be accompanied by a corresponding diversity in rRNA 2'O-methylation that can now be evaluated thanks to RiboMETH-seq {Helm:2017eg, Marchand:2016ey, Birkedal:2015jf}. This approach relies on the protection of the phosphodiester bond located at the 3' of the 2'O-methylated nucleotide from alkaline hydrolysis. The protection is measured by counting the 5' end reads using RNA-seq and allows the determination of the frequency of 2'O-methylation at a given rRNA position using the surrounding nucleotides to estimate the value corresponding to the total rRNA molecules (i.e., C-score) {Birkedal:2015jf}. Since rRNA 2'O-methylation has never been characterized on large series of human samples, we developed RiboMETH-seq-dedicated quality controls based on numerous metrics (see Methods). Using both human RNA reference samples combining human and foetal organs of different origins and analysed in five technical replicates, and 20 primary breast tumour samples analysed in duplicates, we showed a strong correlation in the 5' end raw counts along the rRNA molecules and in the C-scores among the technical replicates (Extended Fig. 1-2),

demonstrating that RiboMETH-seq is a reproducible and robust approach even when sequencing delicate biological material such as human frozen biopsies.

We then investigated the variability of rRNA 2'O-methylation in a series of 195 primary breast tumours using RiboMETH-seq (Extended Table 1). The C-score was computed at the 106 individual rRNA 2'O-methylated positions for each of the 195 breast tumours. Unsupervised analysis revealed variability in the C-scores (Fig. 1). The hierarchical clustering first showed that, for a given tumour, the C-scores at the 106 rRNA 2'O-methylated positions exhibit a high variability (Fig. 1). In a given human sample and depending on the rRNA 2'O-methylated position, the C-score can vary from 0 to 0.98, meaning that all the rRNA molecules are not 2'O-methylated at the given position in that particular human sample (C-score = 0), that all the rRNA are 2'O-methylated at the given position (C-score = 1), or that the sample displays a mix of 2'O-methylated and un-2'O-methylated rRNA molecules at the given position ( $0 < \text{C-score} < 1$ ). This corresponded to what previously reported in cellular models {Belin:2009kk, Marcel:2013jl, Sharma:2017fi, Eroles:2017iz, Natchiar:2017ij, Krogh:2016cu}, indicating that like in cell lines, all rRNA molecules are not fully and equally 2'O-methylated at the 106 positions in a single human sample.

The hierarchical clustering also showed that, for a given rRNA 2'O-methylated position, the C-scores display two distinct profiles when comparing the 195 human tumours (Fig. 1 and Extended Data Fig. 3a). In one hand, some rRNA 2'O-methylated positions exhibit low inter-variability in their C-scores between the 195 tumour samples although they represent the high diversity of breast cancer subtypes. In the other hand, some rRNA 2'O-methylated positions exhibit high inter-variability in their C-scores, that is however found for a limited number of positions. In particular, three rRNA 2'O-methylated positions (28S-Am1313, 28S-Cm2352 and 28S-Gm3723) exhibited huge inter-variability, the C-scores ranging from 0 to  $> 0.90$  and the minimal C-score being observed in different tumours (Fig. 1 and Extended Data Fig. 3a). Position-by-position comparison of the  $\partial\text{C-score}$  (C-score max – C-score min) between the 195 breast tumours and the 5 technical replicates of human RNA reference

samples indicates the high inter-variability in C-scores corresponds to a biological variability rather than a technical variability (Extended Data Fig. 3b). To clearly distinguish the two classes of rRNA 2'O-methylated positions showing either a low or high inter-variability in their 2'O-methylation frequency, linear regression based on the C-score inter-variability measured by using interquartile range (IQR) was performed (Extended Data Fig. 3c). This analysis identifies 60 rRNA 2'O-methylation positions (56.6%) which exhibit the most stable C-scores between the 195 primary breast tumours, and 46 positions (43.4%) which exhibit the most variable C-scores (Fig. 2a and Extended Data Fig.4). These data demonstrate that rRNA 2'O-methylation varies between human samples and suggest the co-existence of two classes of rRNA 2'O-methylated positions in humans.

To assess the biological significance of the co-existence of two classes of rRNA 2'O-methylated positions displaying either the most "stable" 2'O-methylation frequency between human samples (thereafter termed "stable" positions) or the most "variable" 2'O-methylation frequency ("variant" positions), we evaluated the association between variations in C-score at the 106 rRNA 2'O-methylated positions and biological or clinical characteristics of primary breast tumours (Fig. 2b-c). No "stable" rRNA 2'O-methylated position shows association with any of the available characteristics of breast tumours. In contrast, we identified 4 variant rRNA 2'O-methylated positions whose C-score shows significant difference with breast cancer subtype, oestrogen and progesterone status, as well as tumour grade (Fig. 2c). For example, C-scores of the 18S-Gm1447, 28S-Gm1303, 28S-Gm4588 positions decrease in the triple-negative breast cancer (TNBC) compared to the luminal tumours, and C-score of the 18S-Am576 position significantly increases in TNBC compared to luminal tumours (Fig. 2b-c). Similar changes in rRNA 2'O-methylation were observed for the 18S-Gm1447 and 18S-Am576 positions in luminal and TNBC cancer cell lines, reinforcing the human data (Extended Data Fig. 5). Moreover, comparison of C-scores at the 106 rRNA 2'O-methylated positions in a series of 9 benign and 10 malignant mammary tumours revealed that decrease in C-score at 18S-Am576 is an hallmark of malignant tumours (Fig.2d). Finally, clustering breast cancer tumours on the basis of their C-scores at the 106 rRNA 2'O-methylated

positions tends to discriminate patients displaying the poorest overall survival and to identify patients who didn't benefit from the conventional treatment protocol usually used for patient diagnosed with small tumour size (Extended Data Fig. 6). Overall, these data indicate that the variant rRNA 2'O-methylated positions, but not the stable ones, display variation in their 2'O-methylation frequency that is correlated with particular mammary malignant features.

To further characterize the two classes of rRNA 2'O-methylated positions, we first compared their co-occurrence during evolution. An enrichment analysis showed an increased number of the variant rRNA 2'O-methylated positions, paralleled with a decreased number of the stable ones, in humans compared to the other life three branches (Fig. 3a). It suggests an association with appearance of the variant rRNA 2'O-methylated positions and evolution, characterized by an increasing number of fine-tuning gene expression mechanisms. We then mapped the stable and variant rRNA 2'O-methylated positions on the human ribosome structure {Khatter:2015fs, Natchiar:2017ij, Natchiar:2018gd}. It reveals an interesting distribution in space, where the decoding center (DC), the peptidyl-transferase center (PTC) and the polypeptide exit tunnel (PET) are mostly decorated with stable 2'O-methylated positions, surrounded by some variant positions found in 1 or 2 nucleotide layers away from the catalytic sites (Fig. 3b-c). Moreover, 13 rRNA 2'O-methylated positions are found in various binding pockets of the A-, P- and E-site tRNAs, indicating that they possibly interact with the tRNAs in classical and various translocated states. These tRNA binding sites are mostly constellated with stable 2'O-methylated positions, with the exception of 4 variant 2'O-methylated positions, 18S-Um627, 28S-Am3739, 28S-Gm4340 and 28S-Cm4426. The 28S-Am3739 nucleotide is located at the hairpin loop of helix H69, whose 2'O-methylation may influence P-site tRNA binding. The 28S-Gm4340 is located at the CCA end region of the E-site tRNA that provides favourable van der Waals contacts to the tRNA when 2'O-methylated {Natchiar:2017ij}. In another region of the ribosome, the two stable 28S-Cm3787 and 28S-Gm3771 positions are involved in base pairing at edge of helix H71 where termination factors eRF1 and ABCE1 bind. We finally mapped on the structure of the human ribosome the 4

variant rRNA 2'O-methylated positions whose variation in their 2'O-methylation frequency associates with biological and clinical features of breast cancer. Their mapping showed that they are located at the periphery of the PTC of the ribosomes, and they would be accessible for various ribosomal factors associating with ribosomes during translation (Fig. 3c-f). These data suggest that stable rRNA 2'O-methylated positions are mainly involved in conserved catalytic activities of the ribosome, while the variant rRNA 2'O-methylated positions may contribute to regulating interactions of the ribosome with different ribosomal factors involved in translational control.

Altogether our data demonstrated that rRNA 2'O-methylation varies in humans although with distinct intensity depending on the rRNA 2'O-methylated position. First, it appears that rRNA 2'O-methylation varies between the 106 rRNA 2'O-methylated positions in a single human sample ( $0 < \text{C-score} < 0.98$ ). It indicates that the rRNA molecules exist naturally as molecules not fully and equally 2'O-methylated at the 106 positions within a single human sample. Such observations have already been reported in cell line population however in few number of models {Belin:2009kk, Natchiar:2017ij, Sharma:2017fi, Krogh:2016cu, DSouza:2018fu, Marcel:2013jl}, and mainly in response to the alteration in expression of one of the components of the rRNA 2'O-methylation complex {Catez:2019fz, Natchiar:2018gd, Monaco:2018fp, deLoubresse:2014jx}. Future technical breakthrough will be required to determine whether such observation results from the intra-tumoral heterogeneity and/or heterogeneity in chemically modified rRNA molecules between and/or within a single cell. Our data also showed, and for the first time, that rRNA 2'O-methylation frequency can differ between the human tumours, although not randomly but rather depending on some biological and clinical features. rRNA 2'O-methylation at some given positions are indeed associated with breast cancer subtype and tumour grade, demonstrating that the inter-variability of rRNA 2'O-methylation is the reflect of the inter-tumoral heterogeneity. Our data also suggest that rRNA 2'O-methylation may help in the near future to improve cancer patient management. Interestingly, the rRNA 2'O-methylated positions which are not fully 2'O-methylated in a

single human sample and the ones which display strong inter-variability between human samples, are mostly overlapping in these breast cancer samples.

Analysing rRNA 2'O-methylation in a large panel of human samples, as well as in series of benign/malignant mammary tumours and in breast cancer cell lines, demonstrated that two classes of rRNA 2'O-methylated positions co-occurred depending on their ability to tolerate or not the absence of chemical modifications. Structure/function analysis, disparate appearance through evolution and differential association with biological characteristics not only support the existence of these two classes of rRNA 2'O-methylated positions but also suggest that they can have distinct impact on the ribosome function and on the biology of the tumour. The most "stable" rRNA positions, which were 2'O-methylated at a high frequency, are necessary to sustain the core function of the ribosome as expected {Parks:2018be}. In contrast, the existence of "variant" rRNA positions, which can exhibit or not 2'O-methylation depending on the biological context, suggests these sites may be optional to the core ribosome function. Among the variant rRNA 2'O-methylation positions, the 18S-Gm1447 displays the highest variability in rRNA 2'O-methylation among tumours with the identification of human tumours and breast cancer cell lines that lack 2'O-methylation at this particular nucleotide. 18S-Gm1447 is in the close vicinity of the uS10 ribosomal protein, which has been shown to bind to HCV IRES, regulate translation initiation of short 5'UTR containing mRNAs and contribute in resolving stalled ribosomes {Haimov:2017kv, DiGiuseppe:2018bb, Malygin:2013kp, Matsuo:2017da, Sundaramoorthy:2017dl}. Thus, absence or presence of rRNA 2'O-methylation at 18S-Gm1447 might finely modulate uS10 activity. These variant rRNA 2'O-methylated positions may finely regulate the intrinsic ribosome activities and thus be a source of ribosome plasticity.

The discovery of the co-existence of compulsory and optional rRNA 2'O-methylation at some given positions reunites two divergent visions of the ribosome {Decatur:2002wf, Genuth:2018kb, Xue:2012el}. From the first identification of rRNA chemical modifications in bacteria and yeast models, the ancestral vision claims that chemical modifications are

compulsory to the fine structuration of the ribosome on which its functions relied. In contrast, the emerging view of the modern ribosome claims that chemical modifications can be modulated to provide plasticity to the ribosome structure on which its regulatory effects relied and thus to extend the functions of the ribosome to translational regulation in humans and more generally in mammals. The co-existence of distinct but complementary classes of rRNA chemical modifications leading to ribosome plasticity will undoubtedly lead to a deeper and more complete understanding of rRNA chemical modifications in ribosome function and in translational regulation with direct impacts in the understanding of cell identity's determination both in physio- and pathological conditions.



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## **Extended Data Information**

Extended Data information are given in a separate document.

## **Acknowledgements**

This research project was funded by Institut National du Cancer (PLBio2019, MARACAS.v2.0 and PRT-K17, EMT-CoNCEPT), Fondation pour la recherche ARC (20161204686, MARACAS), PAIR Sein program (ARC\_INCa\_LNCC\_7625, RiboTEM), ITMO Cancer (18CN044-00, RESIST), SIRIC program (INCa-DGOS-Inserm\_12563, LyRICAN) and Synergie Lyon Cancer Foundation. H.P and F.NVL were a recipient of a fellowship from Ligue Nationale Contre le Cancer (LNCC). F.NVL was a recipient of a fellowship from the Fondation pour la Recherche Médicale (FRM). P.LM was a recipient of a fellowship from French Research Minister. SK.N, F.C and B.K are supported by Centre National de la Recherche Scientifique (CNRS). J-J.D and V.Marcel are supported by Institut National de la Santé et de la Recherche Médicale (Inserm). A.D and J-C.B are supported by Breast Cancer Now fellowship (2012MaySF127). The Tayside Tissue Bank is supported by the Chief Scientist Office (CSO), NHS and Dundee University.

## **Authors Information**

The authors declare no conflict of interest.

## **METHODS (Nature: 3000 words. Present: 1715 words)**

**Human breast tumour samples.** A series of 195 primary breast tumours, including biological materials and clinical data (last update 2016), have been collected from 1997 to 2011 and maintained by the Tayside Tissue Bank (TTB, Dundee, Scotland, UK) that received ethical approvals (REC Reference 07/S1402/90). Tumour collection corresponded to snap-frozen materials gathered after macrodissection by breast cancer pathologists of biopsies performed at diagnosis{Bourdon:2011bl}. Total RNA were extracted from frozen tissues by TTB services as already described{Bourdon:2011bl}. All biopsies were issued from untreated female patients without metastasis at diagnosis or family history. Hormonal receptors (oestrogen ER and progesterone PR) and HER2/ErbB2 receptors status were determined by immunohistochemistry on FFPE sections{Bourdon:2011bl}. These three markers allowed to identify the different breast cancer subtypes: ER+ PR+/- HER2-, considered as equivalent to the luminal subtype; ER+/- PR+/- HER2+, equivalent to the HER2-amplified subtype (HER2+); and ER- PR- HER2-, as the triple-negative subtype (TNBC). The current median follow-up of this series of 195 primary breast tumours series was of 12.3 years. This series exhibited expected clinical characteristics (Extended Data Table 1), in particular regarding association of overall survival with tumour size ( $P < 0.0001^{***}$ ), lymph node invasion ( $P = 0.0079^{**}$ ), grade ( $P = 0.0151^*$ ) and breast cancer subtypes ( $P = 0.0569$ ).

A series of 19 mammary samples has been collected between 2006 and 2007 (Institut Gustave Roussy, France) using fine-needle aspiration after suspicion of breast lesion{Andre:2009bj} (Ethical approvals reference). Diagnosis of malignant tumours (n=10) was based on the identification of breast cancer cells either on the core-needle biopsy or surgical specimen while diagnosis of benign tumours (n=9) was based on the lack of cancer cells together with the lack of radiological change after at least 3 month's follow-up.

**Breast cancer cell lines.** Four breast cancer cell lines were maintained in 2D culture under 5% CO<sub>2</sub> atmosphere at 37°C. The MCF7 and BT474 cell lines considered as representative of the luminal breast cancer subtype were maintained respectively, in MEM(Eagle) L-glutamine (-) (Thermofisher) supplemented with 10% foetal bovine serum (Sigma), 1% penicillin/streptomycin (Gibco), 1% glutamax (Gibco), 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate and 0.1 mM non-essential amino-acids or in RPMI1640 Glutamax supplemented with 10% foetal bovin serum and 1% glutamax. The MDA-MB-468 and MDA-MB-231 cell lines considered as representative of the triple-negative breast cancer subtype were maintained respectively, in RPMI1640 Glutamax or DMEM/F12 Glutamax, the two media being supplemented with 10% foetal bovine serum and 1% glutamax.

**RiboMETH-seq.** Frequency of rRNA 2'O-methylation at the 106 rRNA 2'O-methylated positions was determined in biological samples by RiboMETH-seq, the only approach allowing absolute quantification of rRNA 2'O-methylation frequency to date{Krogh:2018iu, Birkedal:2015jf, Marchand:2016ey}. Presence of 2'O-methylation protects the phosphodiester bond located at the 3' of the 2'O-methylated nucleotide from alkaline hydrolysis. Thus, the presence of 2'O-methylation at the given nucleotide  $n$  induces a decrease in RNA fragments starting at the nucleotide  $n+1$  while it increases the one finishing at the nucleotide  $n-1$ , allowing to calculate a 2'O-methylation frequency at the corresponding nucleotide position (or C-score){Birkedal:2015jf}.

RiboMETH-seq was performed as previously described using Illumina technology{Marchand:2016ey, Marchand:2017fr}. Briefly, total RNA was first subjected to partial alkaline hydrolysis to generate RNA fragments ranging from 50 to 100 bp. Libraries were then prepared using NEBNext Multiplex Small RNA Library Prep Set (Illumina) and multiplexed. Sequencing were performed using HiSeq sequencer (Illumina).

**Bioinformatical pre-processing.** After sequencing, FastQ files were pre-processed, aligned and C-scores were computed from raw counts as already described{Marchand:2017fr}. Briefly, Trimmomatic v0.36 was run to remove adapter sequences from the reads and to discard reads shorter than 17 nucleotides. The remaining reads were aligned to a human rRNA sequence (NR\_046235) using Bowtie 3. 5' end reads were counted at each rRNA nucleotide position and used to compute a 2'O-methylation score, the C-score, as previously described{Birkedal:2015jf}. The C-score is defined as the frequency of 2'O-methylation at a given position using the surrounding (i.e., 6 nucleotides located just upstream and downstream of the nucleotide position  $n$ ) to estimate the value corresponding to total 2'O-methylation of all molecules at the position.

To assess the performance of the C-Score, we computed three different versions of the C-score (Extended Data Fig. 1A). We varied the window size of the neighbouring nucleotides (6, 4, or 2) and used either the arithmetic mean or the median raw counts as estimator of the total 2'O-methylation. By considering each score value as threshold, we computed the sensitivity and specificity of the different C-score versions and plotted the ROC (*Receiver Operating Characteristics*) curve (Extended Data Figure 1A). In the present study, a C'-score that was computed using a 6 nucleotides window and a normalization to the median raw counts of neighbouring nucleotides was found as best performer. This C'-score was termed C-score throughout the manuscript.

Numbering of rRNA 2'O-methylated position was assigned according to the numbering in the snoRNA-LBME-db to facilitate comparison between studies. A table is given with the correspondence of the nomenclature used in the present study and in Natchiar and colleagues (Extended Data Table 2){Natchiar:2017ij}.

**Quality control.** We performed exhaustive quality controls (QC) to assess the technical reproducibility and robustness of the RiboMETH-seq protocol and to ensure the high quality

of the data for further downstream analyses. To this end, we used first a human RNA reference sample as a calibrated source of rRNA, which corresponds to the commercial Human XpressRef Universal Total RNA (Qiagen) prepared from 20 different human adult and foetal normal major organs. Five of these reference samples were processed in parallel. Second, we processed twice independently 20 primary breast tumours randomly chosen to obtain a set of technical duplicates from relevant biological tissue.

We computed Spearman correlations between replicates in terms of raw counts and C-scores at all genomic positions of the rRNA sequences as well as exclusively at the 106 2'-O-methylated positions. To assess the reproducibility of the data, we calculated the QC metrics for the five replicates of the human XpressRef Universal Total RNA as well as for the 10 tumour duplicates (Extended Data Fig. 1B-C and 2). All replicates showed a high technical reproducibility and very strong correlations between each other, emphasizing the reproducibility and robustness of the RiboMETH-seq protocol.

**Analysis of the breast cancer series data.** Among an initial series of 214 primary breast tumours, 195 primary breast tumour samples passed the QC criteria (representing 8.8% of the series) and were thus retained for the downstream analyses (Extended Data Table 1). In our first exploratory, unsupervised data analysis, we performed hierarchical clustering and principal component analysis (PCA) using the C-scores at the 106 individual rRNA 2'-O-methylated positions of the 195 samples. Both methods allow to detect similarities between samples and potentially to define groups among them. Hierarchical clustering creates a heatmap (i.e., a coloured matrix, that represents a grid of values where the rows correspond to the samples and the columns to the rRNA 2'-O-methylated positions). Thus, each rectangle in a heatmap represents one C-score at a given rRNA 2'-O-methylated position of a sample and is coloured according to the numerical value in the matrix, with a range of colours proportionate to the C-score. Samples/positions are ordered according to the dendrogram, a



tree-like structure, and successively paired together based on the degree of similarity. Dendrograms allow thus to identify subgroups (clusters of both breast tumours exhibiting the most similar rRNA 2'O-methylation profiles and rRNA 2'O-methylated positions exhibiting the most similar frequency) across the 195 primary breast tumours.

PCA creates a low-dimensional representation of the samples, which contains as much of the variance in the original data set as is possible. The PCA plot shows the first two principal components and the amount of variance explained by each component.

**rRNA 2'O-methylation profiles.** rRNA 2'O-methylation profiles were shown as either box-and-whiskers plots, line charts or histograms. To summarize the data, we constructed box-and-whiskers plots (boxplot) for each of the 106 rRNA 2'O-methylated positions. The ends of the box are the lower (Q1), and upper (Q3) quartiles (i.e., C-score delimiting 25% and 75% of the population), so the box spans the interquartile range (IQR). The horizontal line inside the box represents the median C-score at a given rRNA 2'O-methylated position. The lines outside the box (top and bottom whiskers) extend to the highest and lowest observations the 97.5% and 2.5th. Line charts and histograms represent the mean and standard deviation of C-scores across the samples at each rRNA 2'O-methylated positions.

**Classification of the rRNA 2'O-methylated positions into variant and stable ones.** We observed that the variability of the C-Scores across samples was depended on the rRNA 2'O-methylated position. The total variability observed at a given 2'O-methylated position is a composition of measurement error, biological noise and intrinsic biological variation. Although the first two terms should be independent of the 2'O-methylated position, this may not be the case for the intrinsic variation. We thus hypothesized that the total variability at a specific 2'O-methylated position beyond a certain threshold is mainly driven by its intrinsic biological variation. To define this threshold, we compared the IQR of the C-scores at the

106 rRNA 2'O-methylated positions from the 195 breast tumor samples. IQR values were sorted in ascending order and linear regressions were performed by successively adding IQR values, starting with the 20 lowest IQR values. Linear regression encompassing a maximum of 2'O-methylated positions with the lowest slope was retained. The threshold was defined as the maximum distance of the IQR from the regression line. 2'O-methylated positions having IQR values below the threshold will be assigned as "stable" while sites having IQR values above will be assigned as "variant".

### **Association of rRNA 2'O-methylation with biological and clinical characteristics.**

Between-group comparisons were performed using a Chi-2's or Fisher's exact test for categorical data. Comparison of rRNA 2'O-methylation frequency between two groups of breast tumours was performed using non-parametric Mann-Whitney tests. Significant association was considered when  $P$ -value  $< 0.05$  and  $\Delta C$ -score (C-score condition 1 – C-score condition 2)  $> 0.05$ . Results were summarized using bubble plots where  $-\log_{10}(P$ -values) are represented by circle size, absolute difference in C-score between the two conditions of interest by line thickness, and colours indicate either a decrease (pink) or an increase (blue) in C-score. Survival curves for overall survival (OS) with associated log-rank tests were generated using the Kaplan Meier method. OS corresponded to the timing from the date of diagnosis to either the date of death or last follow-up for censored patients. Survival median has been estimated using the inverted Kaplan-Meier method.

**Statistics and graphics.** All  $P$ -values corresponded to two-tailed  $P$ -values. A  $P$ -value  $< 0.05$  was considered as statistically significant. Statistical analyses were performed using either R studio or GraphPad Prism v7.0a softwares (GraphPad Software, Inc). Graphical representations were depicted using either R studio or GaphPad Prism software.

**rRNA 2'O-methylation mapping.** The rRNA 2'O-methylated positions have been mapped on the human ribosome resolved by cryo-EM{Natchiar:2017ij} and pictures have been drawn using the PyMol software.

**Data availability.** Deposit FastQ and/or raw counts as dataset in GEO ?

**Methods reference (continuous numbering regarding Reference section)**

## Figure Legends (Nature: 100 words/legends)

**Figure 1. Stability and variability of rRNA 2'O-methylation frequency between breast tumours.** Frequency of rRNA 2'O-methylation (i.e., C-score) was determined at the 106 rRNA 2'O-methylated positions using RiboMETH-seq{Birkedal:2015jf, Marchand:2016ey} in a series of 195 human primary breast tumours. Data are presented as a hierarchical clustering where the C-score of each position (column) for each tumour (row) is represented (colour scale). A C-score of 0.6 means that 60% of the rRNA molecules are 2'O-methylated at this position (i.e., sub-stoichiometry). Dendrogram representing similarities in the breast tumours on the basis of their rRNA 2'O-methylation profiles (see right panel) or in the rRNA 2'O-methylation frequency at a given sites between tumours (see top panel). A zoom on the rRNA 2'O-methylated positions whose C-scores showed the strongest variation among tumours (i.e., inter-variability) is given.

**Figure 2. Biological relevance of variant rRNA 2'O-methylation frequency in breast cancer.** **a**, C-score variation among the 195 human primary breast tumours at each of the 106 rRNA 2'O-methylated positions were ordered by increasing interquartile range (IQR). Based on linear regression, two classes of rRNA 2'O-methylated positions were identified: positions whose C-scores are the most stables (red); and positions whose C-scores are the most variants (blue). **b**, Example of significant variations in rRNA 2'O-methylation frequency at the 18S-Gm1447 position between luminal, HER2+ and TNBC breast tumours. **c**, Summary of the four variant rRNA position whose 2'O-methylation frequency were significantly different regarding breast cancer subtypes, hormonal and HER2 receptor, and tumour grade. ER: oestrogen receptor; PR: progesterone receptor; Luminal: ER+ PR+/- HER2-; HER2+: ER- PR- HER2+; TNBC: ER- PR- HER2-; G: grade. **d**, Comparison of rRNA 2'O-methylation profiles in a series of 9 benign (green) and 10 malignant (orange) tumours. A

zoom present the significant variation of rRNA 2'O-methylation frequency at the variant 18S-Am576 position in malignant compared to benign mammary tumours.

**Figure 3. Structural properties of stable and variant rRNA 2'O-methylated positions.** **a**, Pie charts present the number and the percentage of stable and variant rRNA 2'O-methylated positions through evolution. Universal: sites conserved from bacteria to humans; eukaryotes: sites conserved from yeast to humans; humans: sites only described in humans. **b-c**, Mapping of the two classes of rRNA 2'O-methylated positions on the human ribosome (b) and at the peptidyl-transferase center (PTC, c){Natchiar:2017ij, Khatter:2015fs}. **d-f**, Structural environments of the variant rRNA 2'O-methylated positions significantly associated with breast cancer features. Red: stable sites; blue: variant sites.

## Supplementary Tables

**Supplementary Table 1. Characteristics of breast tumours.**

		<b>Human breast tumour series</b> (n = 195)
<b>Age (years)</b>	Median (min – max)	59.0 (28.0 – 90.0)
<b>Invasive grade</b>	No data	3
	Grade 1	12 (6.3 %)
	Grade 2	49 (25.5 %)
	Grade 3	131 (68.2 %)
<b>Tumor size (mm)</b>	n Median (min – max)	192 28.0 (2.0 – 110.0)
<b>Number of invaded lymph node</b>	n Median (min – max)	195 1.00 (0.0 – 41.0)
<b>Invaded lymph node</b>	No (n = 0) Yes (n ≥ 1)	88 (45.1 %) 107 (54.9 %)
<b>ER status</b>	No data Negative Positive	1 69 (35.6 %) 125 (64.4 %)
<b>PR status</b>	No data Negative Positive	1 110 (56.7 %) 84 (43.3 %)
<b>HER status</b>	No data Negative Positive	13 113 (62.1 %) 69 (37.9 %)
<b>Breast cancer subtype</b>	No data ER+ PR+/- HER2- ER+/- PR+/- HER2+ ER- PR- HER2-	13 73 (40.1 %) 69 (37.9 %) 40 (22.0 %)
<b>TP53 mutation status</b>	No data Wild-type Mutant	13 127 (69.8 %) 55 (30.2 %)

## Extended Data Figure Legends

**Extended Data Figure 1. Technical variation of rRNA 2'O-methylation profiling in human RNA reference sample.** **a**, Frequency of rRNA 2'O-methylation (or C-score) was determined at the 106 rRNA methylated positions using an innovative RNA-seq based method, the RiboMETH-seq{Birkedal:2015jf, Marchand:2016ey}, in five independent technical replicates of human RNA reference sample. Performance of the C-score was assessed by computing ROC curve using six different versions of the C-score calculated with both variable numbers of neighbouring nucleotides and either the arithmetic mean or the median raw counts (table, right side). Compared to the original description of C-score{Birkedal:2015jf}, we determined a C'-score (termed C-score throughout the manuscript) based on the median raw counts and the 6 neighbouring positions as the most robust measure of rRNA 2'O-methylation frequency using human RNA reference. **b**, Quality control of RiboMETH-seq was performed by comparing correlation (top) and profiles (bottom) of both 5' end raw counts (left) and C-scores (right) of the longest 28S rRNA, between the five technical replicates of the human RNA reference sample. Strong reproducibility and robustness was observed using these two approaches (5' end raw counts:  $r_2 > 0.96$ ; C-scores:  $r_2 > 0.92$  at the non 2'O-methylated positions and  $r_2 > 0.88$  at the 106 2'O-methylated positions). **c**, Boxplot presents C-score variation among the five technical replicates of human RNA reference at each of the 106 rRNA 2'O-methylated positions ordered by increasing rRNA molecules and nucleotide positions. About 75% of rRNA 2'O-methylated positions exhibited only minor variation in their C-score across the five technical replicates ( $\partial C\text{-score} = C\text{-score max} - C\text{-score min}$ ,  $\partial C\text{-score} < 0.069$ ), the other sites having a  $\partial C\text{-score}$  ranging from 0.070 to 0.2047. These data showed the strong technical reproducibility and robustness of the RiboMETH-seq approach.

**Extended Data Figure 2. Technical variation of rRNA 2'O-methylation profiling in 20 human primary breast tumours.** **a**, Example of quality control of RiboMETH-seq performed in two technical duplicates of human primary breast tumours that show the highest (top) and the lowest (bottom) duplicate quality. Correlation (top) and profiles (bottom) of 5' end raw counts of 5.8S, 18S and 28S rRNAs were compared between the technical duplicates. While a  $r_2$  correlation coefficient ranging from 0.64 to 0.85 and depending on the considered rRNA was determined in the "lowest duplicate quality" sample, a strong correlation with  $r_2 > 0.99$  was observed for the three rRNAs in the "highest duplicate quality" human breast sample. **b**, The  $r_2$  correlation coefficient for the three rRNAs between technical duplicate of the 20 human breast tumours was shown as a cumulative frequency. The lowest, but nevertheless

showing strong  $r^2$  correlation coefficient, was observed for 5.8S possibly due to the fact it corresponds to the smallest rRNA molecule (i.e. 157 bp compared to 1875 or 5035 bp for 18S and 28S, respectively) and thus being associated with only small number of raw data. More than 50%, 85% and 65% of technical duplicates showed a  $r^2 > 0.8$  for 5.8S, 18S and 28S rRNA respectively, further demonstrating the strong reproducibility and robustness of the RibomETH-seq approach even in delicate biological material such as human frozen biopsies.

**Extended Data Figure 3. Variability of rRNA 2'O-methylation frequency between the 195 human primary breast tumours.** **a**, Boxplot presents C-score variation among the 195 human primary breast tumours at each of the 106 rRNA 2'O-methylated positions ordered by increasing rRNA molecule and nucleotide positions. Median C-scores vary depending on the rRNA 2'O-methylated position, the rRNA 2'O-methylation frequency showing strong variation across human primary breast tumours with C-score ranging from 0 to about 0.95 for some sites. These data show for the first time the high variability of rRNA 2'O-methylation frequency between human samples. **b**, Histogram presents the  $\partial$ C-score (C-score max – C-score min) at each rRNA 2'O-methylated position across the 195 human primary breast tumours (black) and the five technical replicates of human RNA reference sample (white).  $\partial$ C-score of human breast samples were higher than the  $\partial$ C-score of human RNA reference sample at all the rRNA 2'O-methylated positions, indicating that variation in rRNA 2'O-methylation frequency observed between human breast tumours corresponds to biological variation rather than technical issues. **c**, Plot represents the interquartile range (IQR) of C-scores calculated using the 195 human primary breast tumours at each of the 106 rRNA 2'O-methylated positions ordered by increasing IQR. Linear regression-based approach allowed the identification of two classes of rRNA 2'O-methylated positions: the ones with the most stable C-score; and the ones with the most variant C-score. Overall, these data indicated that 60% of the rRNA 2'O-methylated positions correspond to chemically modified nucleotides with conserved rRNA 2'O-methylation frequency in humans, while 40% of them have the ability to be 2'O-methylated or not.

**Extended Data Figure 4. Examples of variability in rRNA 2'O-methylation frequency at three rRNA positions between breast cancer tumours.** The plot presents C-score for each of the 195 human primary breast tumours at three rRNA 2'O-methylated positions that showed either the smallest IQR (**a**, 28S-Am3703), an intermediate IQR (**b**, 28S-Cm2811) or the highest IQR (**c**, 18S-Gm1447). Interquartile range (IQR) is represented on the right. These data illustrated the two classes of stable and variant rRNA 2'O-methylated positions with distinct degrees of variability between human breast tumour samples.



**Extended Data Figure 5. Association of rRNA 2'O-methylation profiles with biological and clinical breast cancer characteristics.** **a**, Principal component analysis (PCA) identified 4 clusters of breast tumours based on their rRNA 2'O-methylation profiles that correspond to the ones identified using hierarchical clustering approach (Fig. 1). **b**, Kaplan-Meier curve depicts the overall survival of patients clustered on the basis of their rRNA 2'O-methylation profiles. Although no significant association was observed, a tendency was observed, the patients carrying breast tumours characterized by a blue rRNA 2'O-methylation profile display the poorest overall survival. **c-d**, Cumulative histogram represents the composition of each of the 4 clusters for tumour grade (c) and breast cancer subtype (d). Significant differences in repartition among the 4 clusters were observed regarding these two parameters. The blue group showing the poorest overall survival was enrichment in triple-negative breast cancer (TNBC) and in tumours of high grade compared with the other groups. Regarding the fact that tumours in the blue groups exhibited the lowest C-score at most of the rRNA 2'O-methylated positions, these data suggested that global decrease in rRNA 2'O-methylation might be associated with breast tumour aggressiveness. Luminal: ER+ PR+/- HER2-; HER2+: ER- PR- HER2+; TNBC: ER- PR- HER2-. **h**, Kaplan-Meier curve depicts the overall survival of patients grouped on the basis of both their tumour size at diagnosis and their rRNA 2'O-methylation profiles. A significant difference in overall survival was observed due to the tumour size at diagnosis, the patients carrying small breast tumours exhibiting a better outcome than the ones carrying large tumours. However, the use of rRNA 2'O-methylation profiles allows the identification of patients carrying small tumour size but displaying outcome as bad as patients bearing large tumour size. These data indicate that rRNA 2'O-methylation profiles might help in identifying patients who didn't benefit from the conventional treatment protocol used for the "small tumour size". rRNA 2'O-methylation profiling might thus be used in the near future to improve breast cancer patient management.

**Extended Data Figure 6. Variability of rRNA 2'O-methylation frequency between breast cancer cell lines.** **a**, rRNA 2'O-methylation was profiled in luminal breast cancer cell lines (MCF-7 and BT474) and triple-negative breast cancer cell lines (MDA-MB-468 and MDA-MB-231). Mean C-score of two biological replicates issued from the different cell lines of each subtypes is presented at the 106 rRNA 2'O-methylated positions. The most stable (red) and the most variant (blue) rRNA 2'O-methylated positions are shown as identify in the series of 195 breast tumours (Fig. 2a). The most variant rRNA 2'O-methylated positions identified in the series of 195 primary breast tumours displayed also a strong variability in these breast cell lines. The rRNA 2'O-methylated positions, whose frequency was significantly associated with malignant mammary tumours, breast cancer subtypes and tumour grades, are indicated

by a black arrow. **b**, Variation of rRNA 2'O-methylation frequency at the positions associated with clinical and biological characteristics of breast tumours are shown in the different cell lines. While C-score is not associated with breast cancer cell line subtypes at 28S-Gm1303 and 28S-Gm4588 rRNA 2'O-methylated positions, C-score is strongly coherent among the two different subtypes of breast cancer cell lines at 18S-Gm1447 and 18S-Am576 positions. rRNA 2'O-methylation is almost absent in the triple-negative breast cancer (MDA-MB-468 and MDA-MB-231) cell lines at 18S-AmGm1447, while it is 2'O-methylated, even if at sub-stoichiometric level, in luminal breast cancer (MCF-7 and BT474) cell lines. In contrast, a decrease in rRNA 2'O-methylation frequency at 18S-Am576 position is observed in the luminal breast cancer cell lines compared to the triple-negative cell lines.

### **Extended Data References**



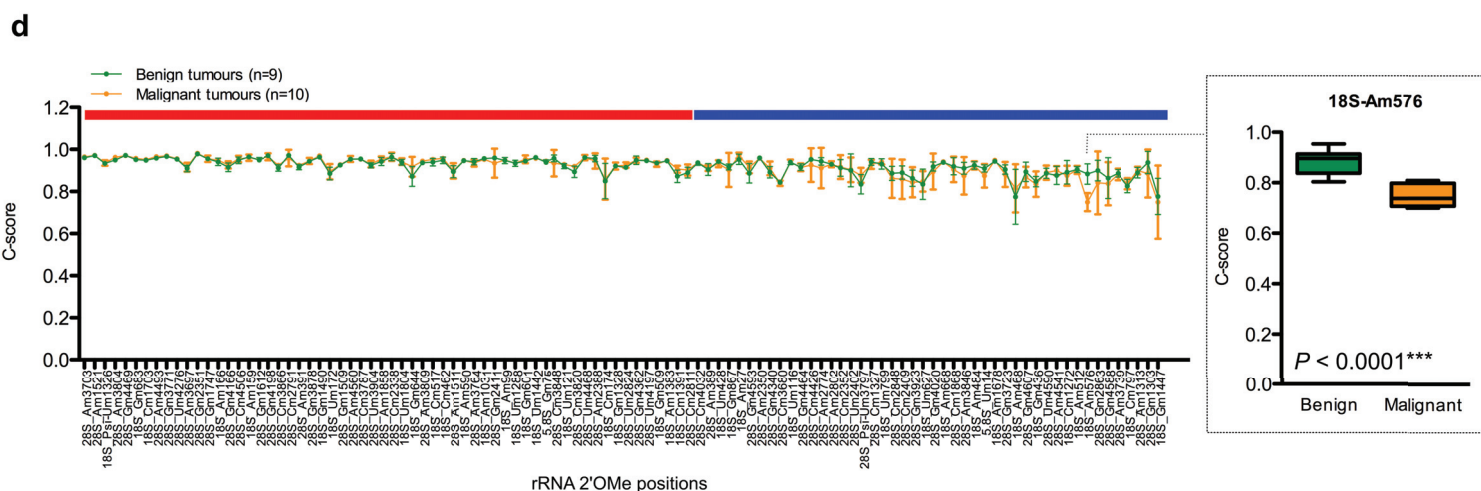
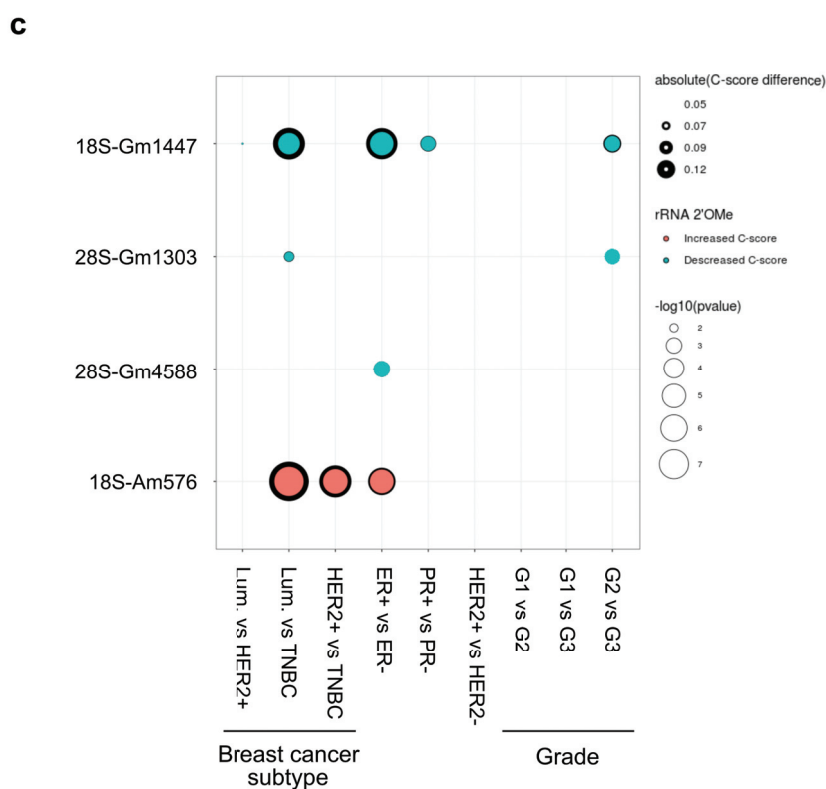
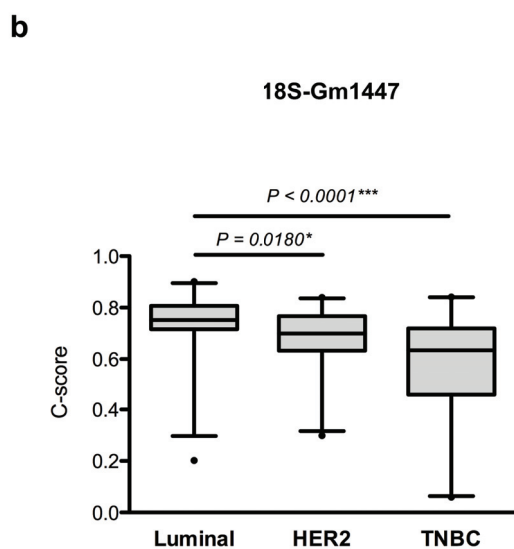
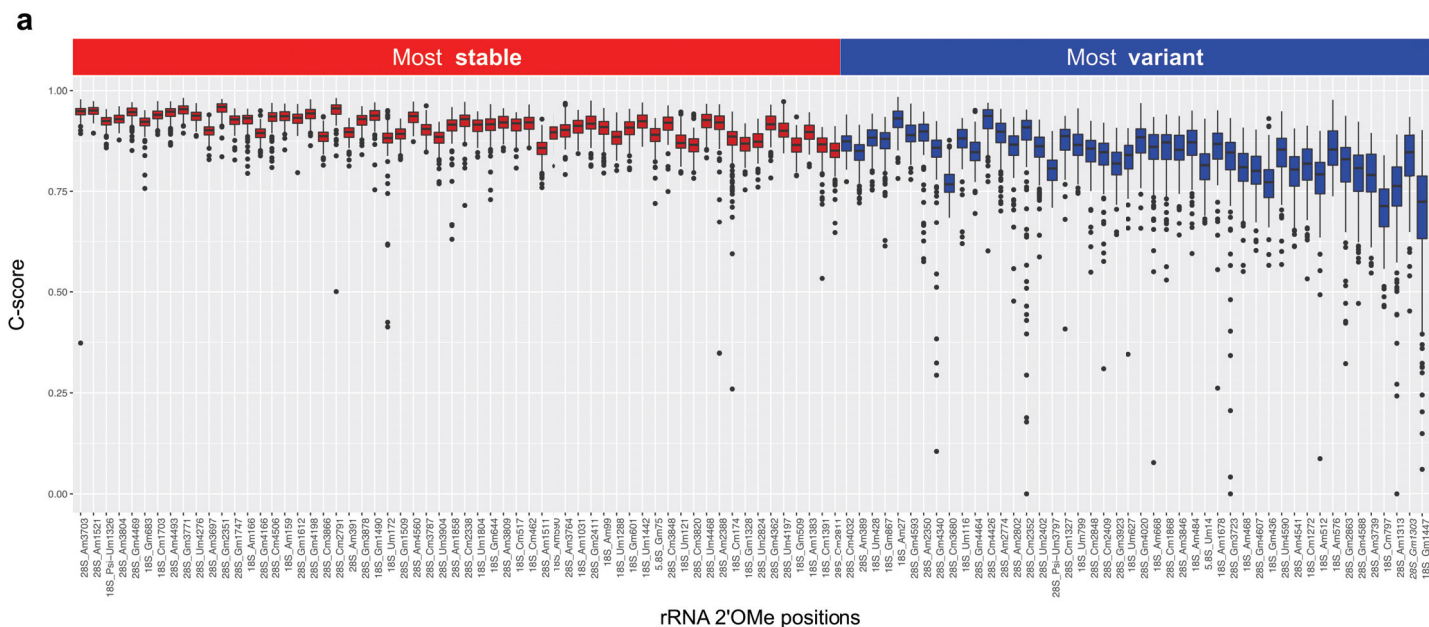


Figure 2

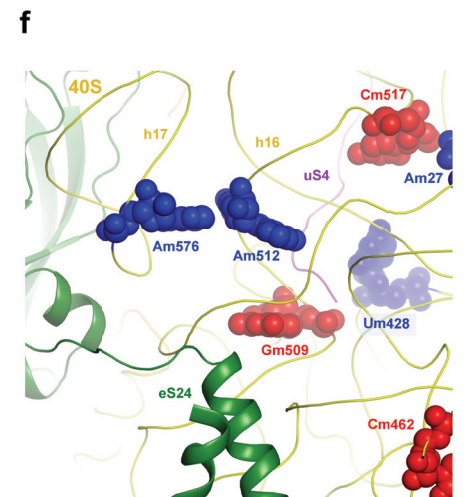
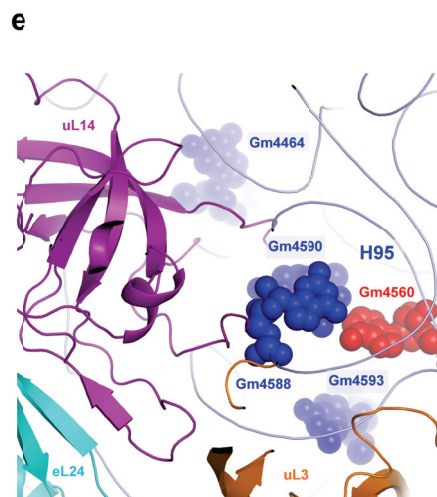
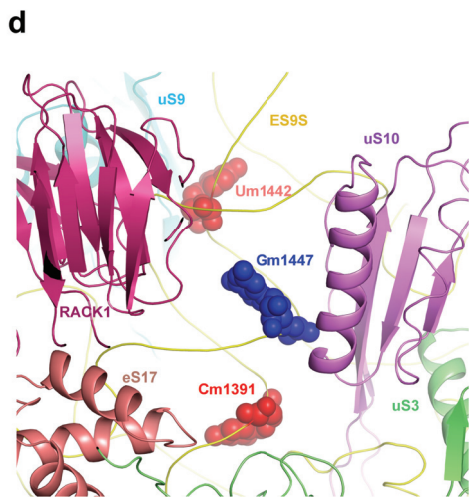
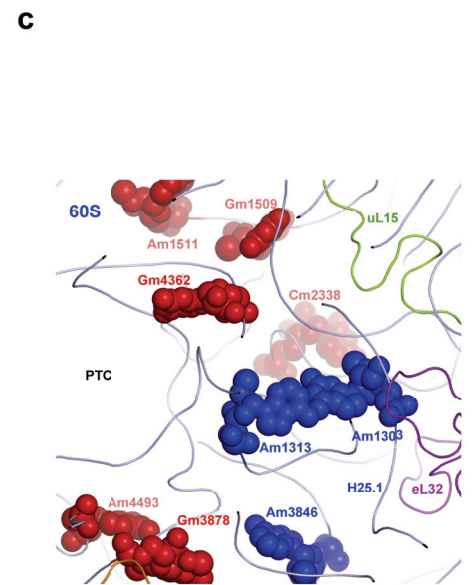
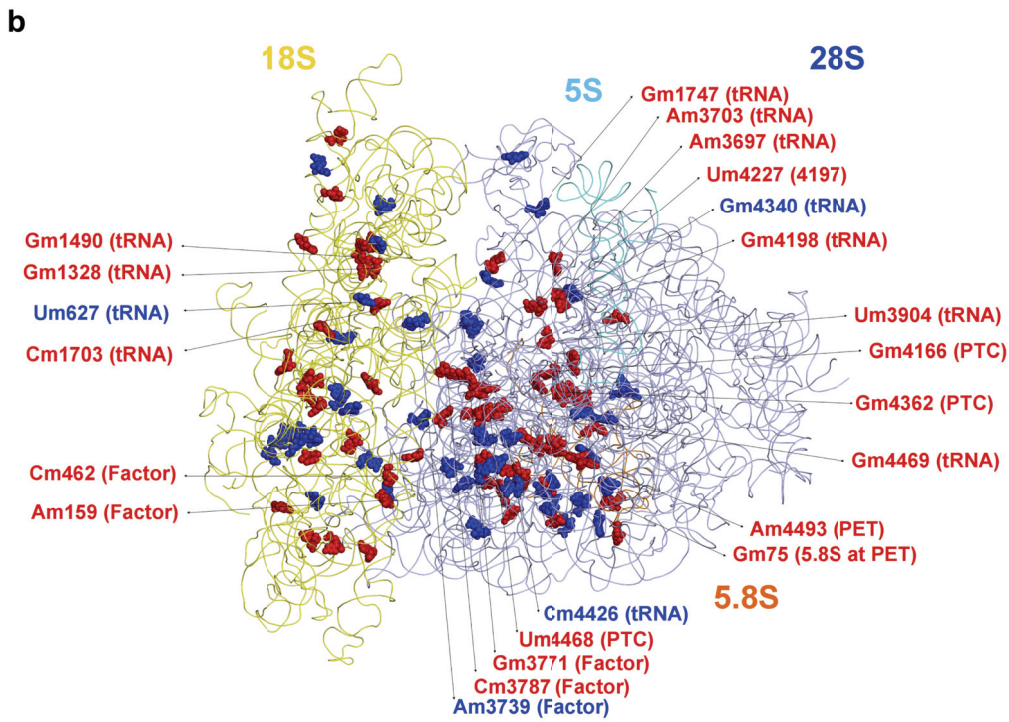
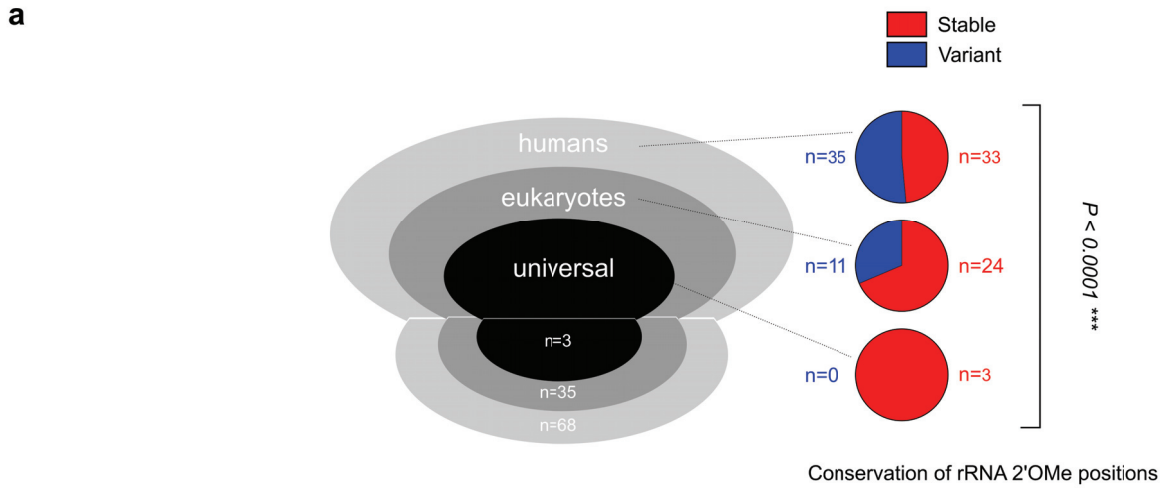
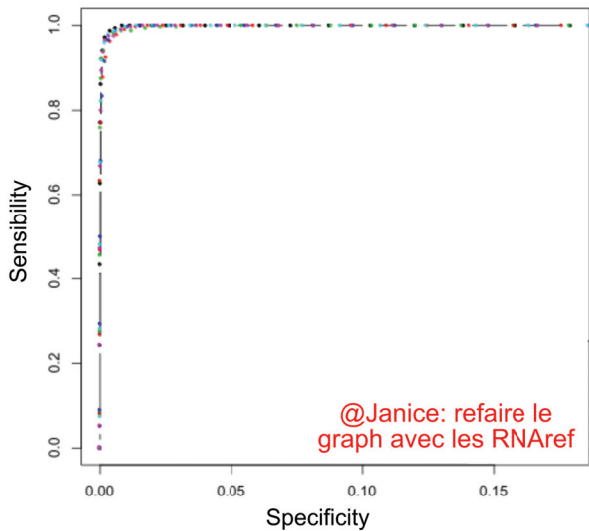


Figure 3

**a**



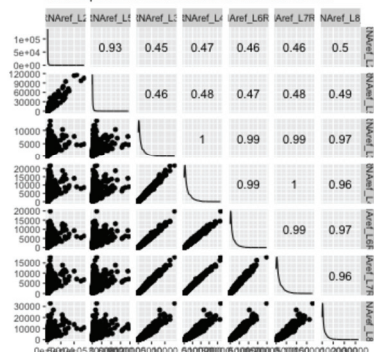
Method for average counts	Number of neighboring positions	C-score
mean	6	C-score M6
	4	C-score M4
	2	C-score M2
median	6	C-score Med6
	4	C-score Med4
	2	C-score Med2

(Birkedal et al, 2015)

**b**

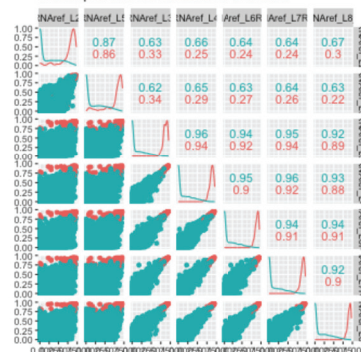
**28S raw counts**

Scatterplot and correlations of raw counts 5/3'

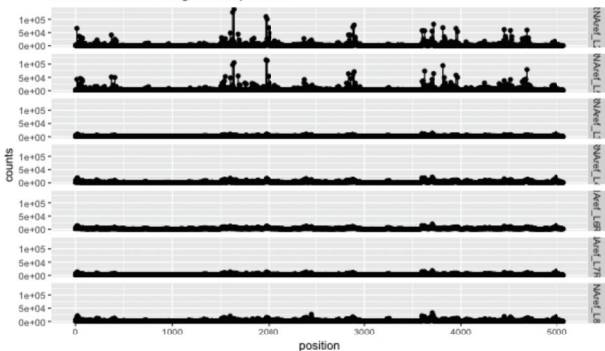


**28S C-scores**

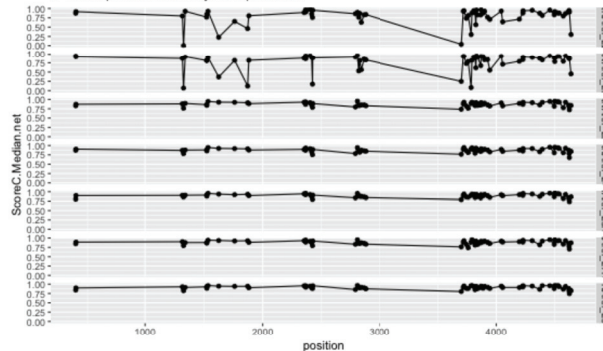
Scatterplot and correlations of C-scores



Raw counts 5/3' vs. genomic position

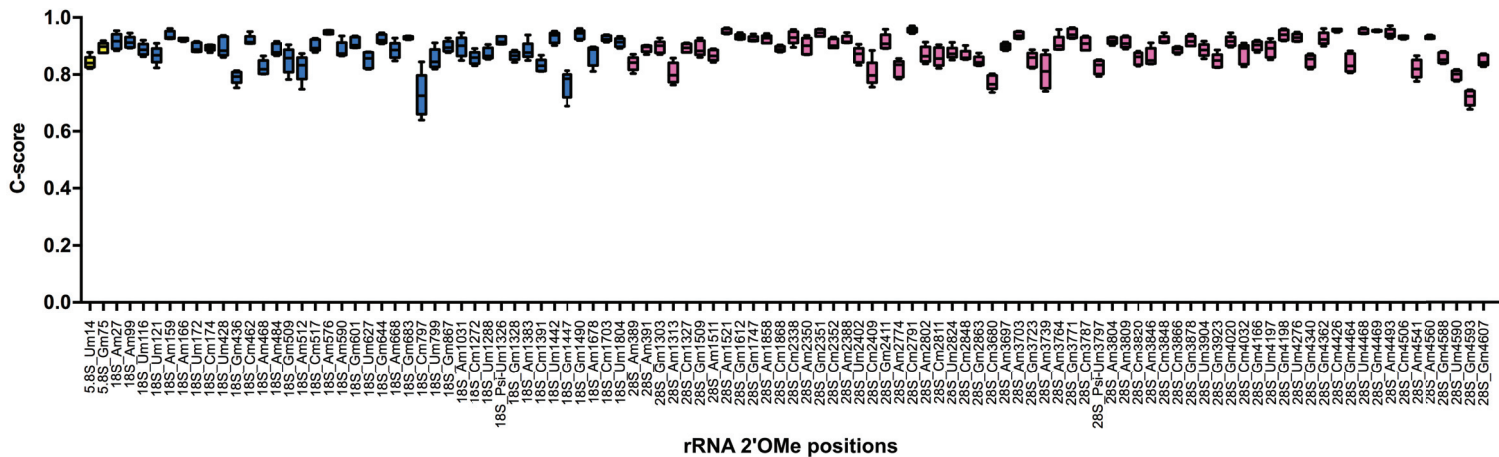


C-Score profiles of methylated positions



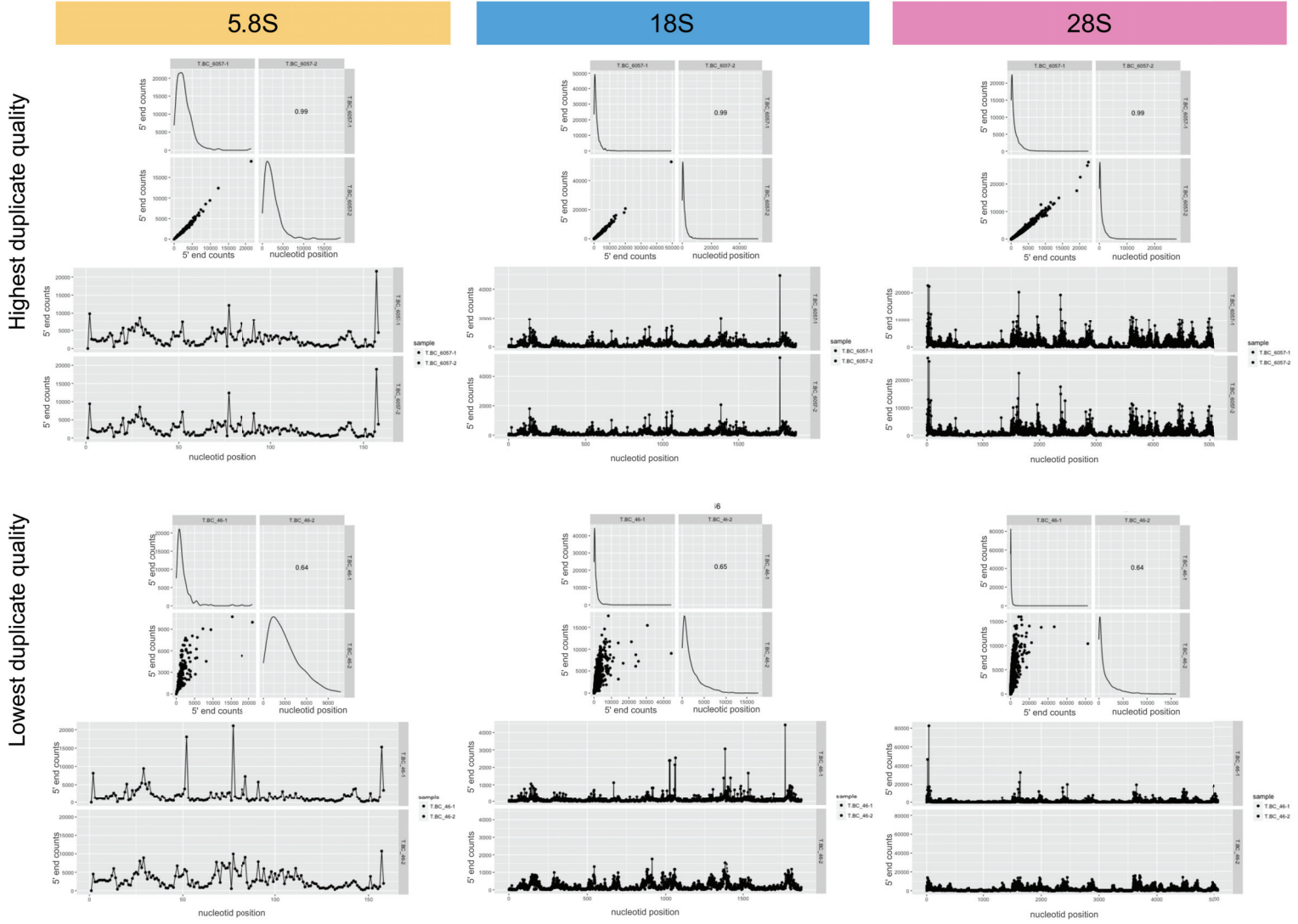
@Janice: seulement les 5 ARN ref d'intérêt + pas de légende au dessus

**c**

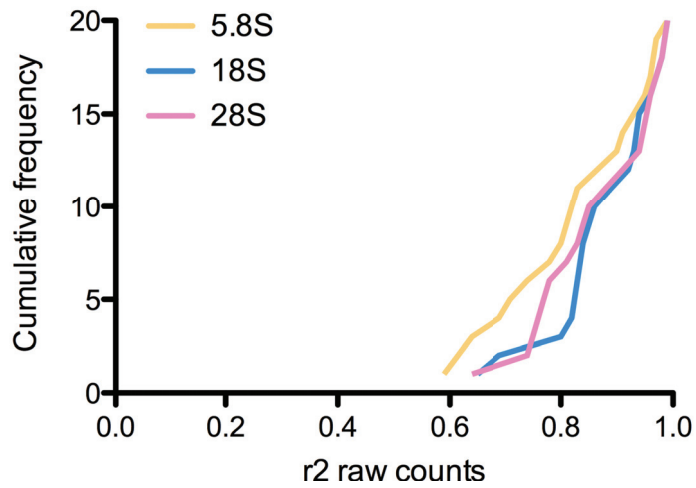


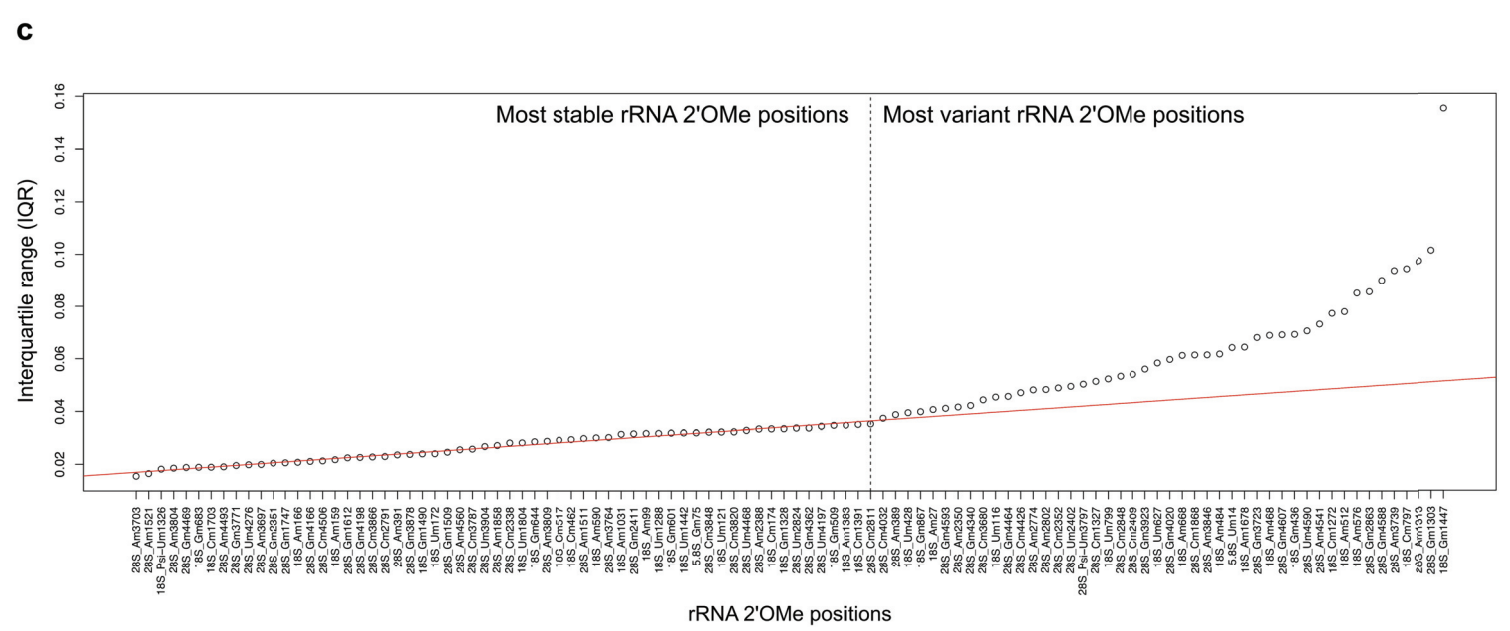
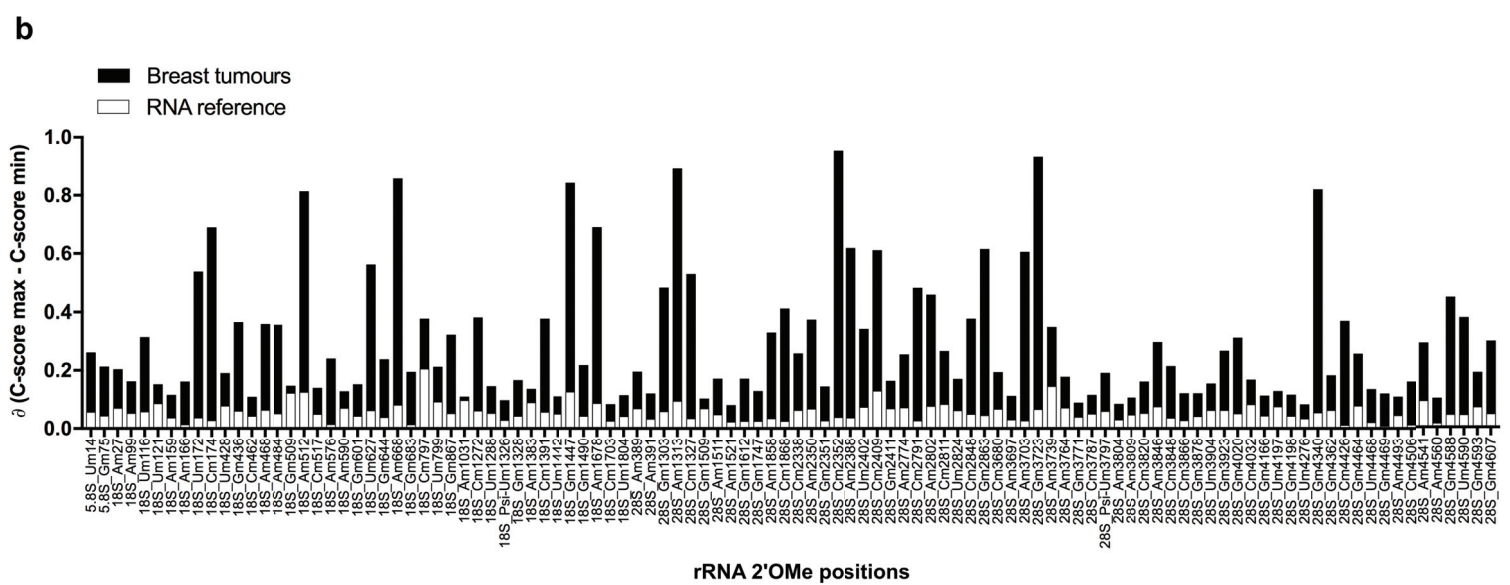
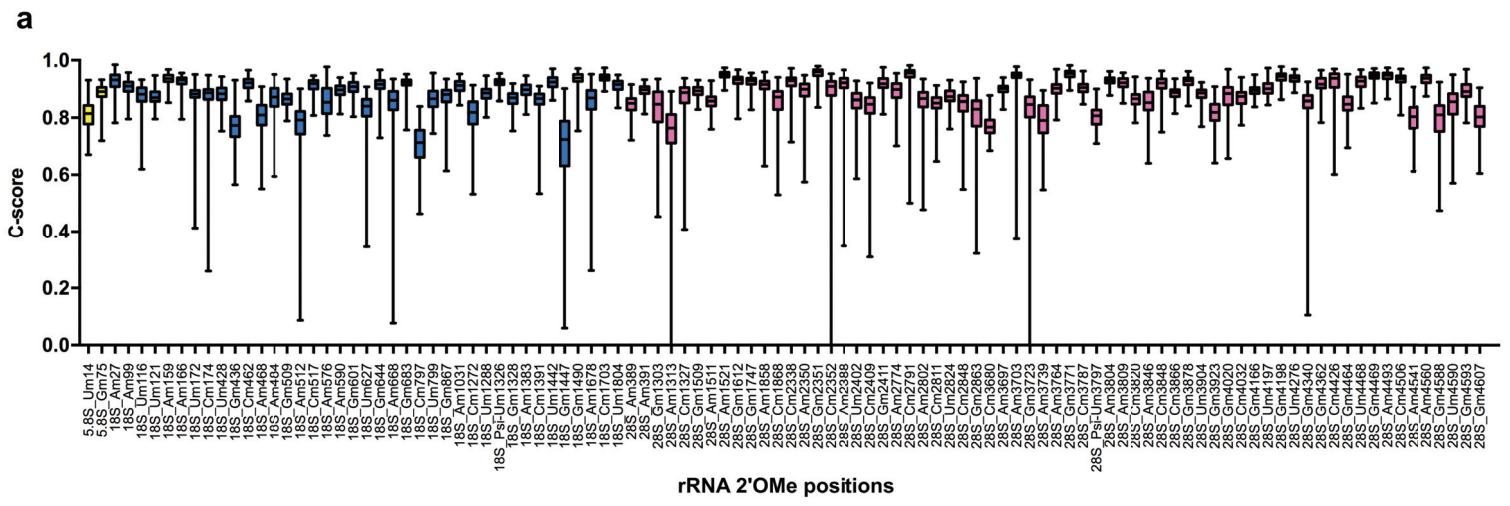
Supplementary Figure 1

**a** @Janice: enlever les légendes des graphs + axes + "samples"



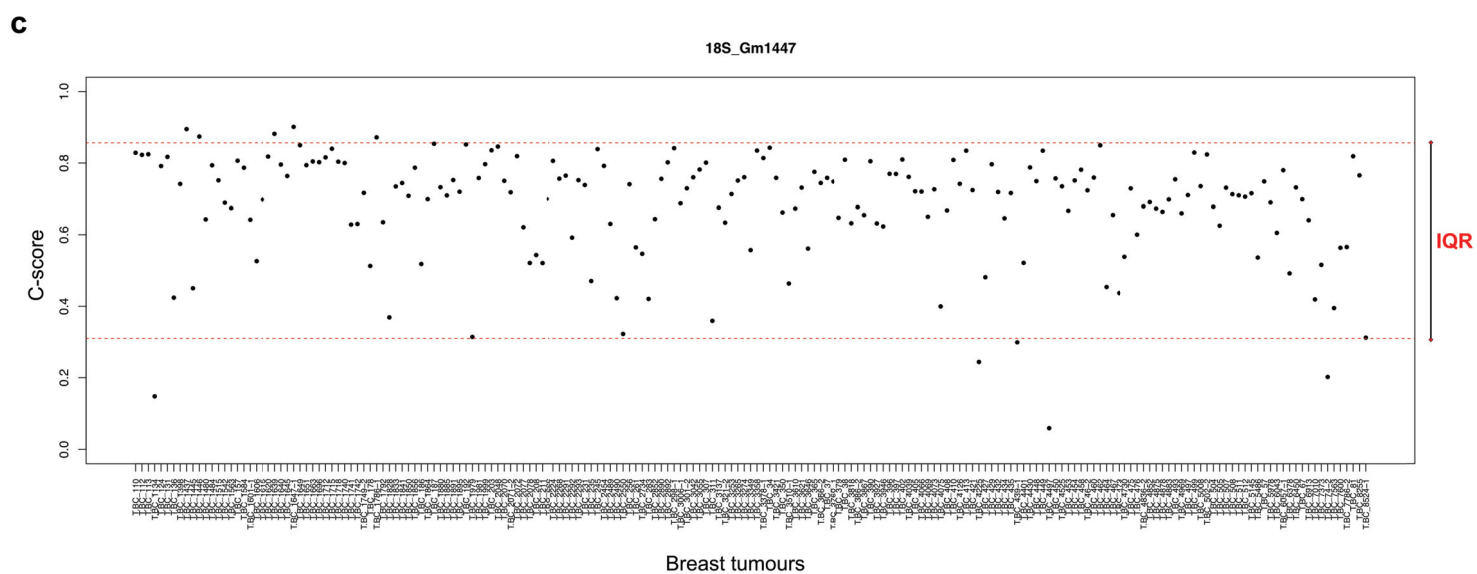
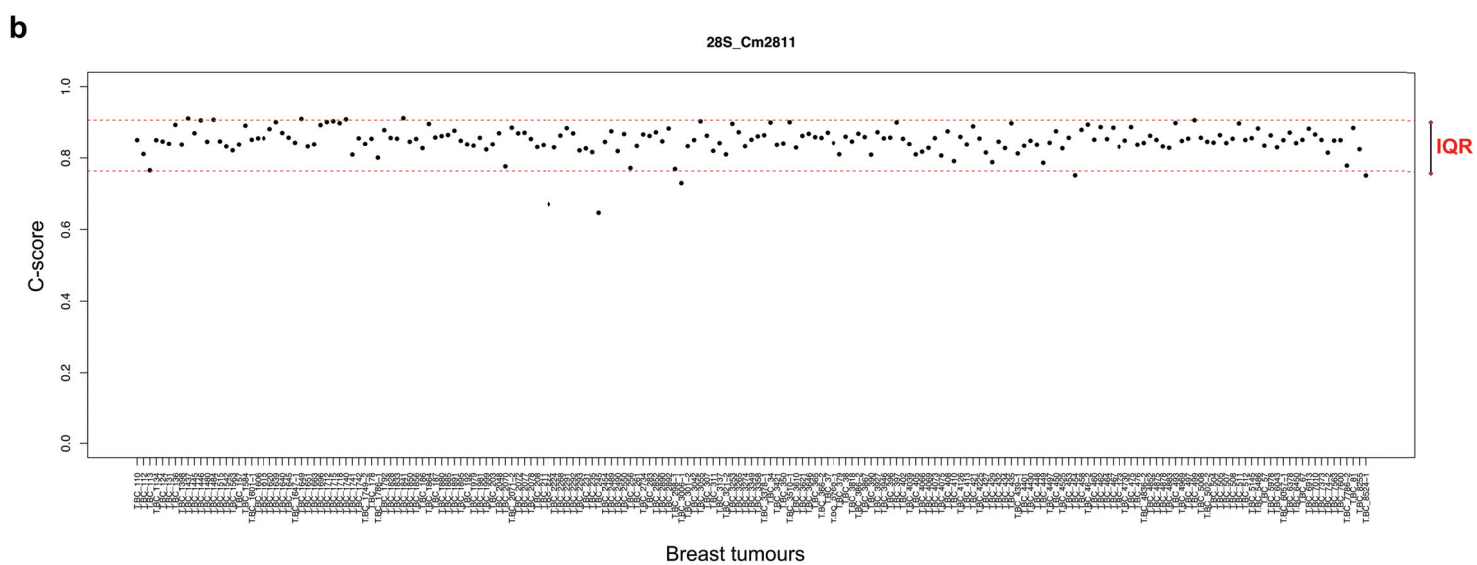
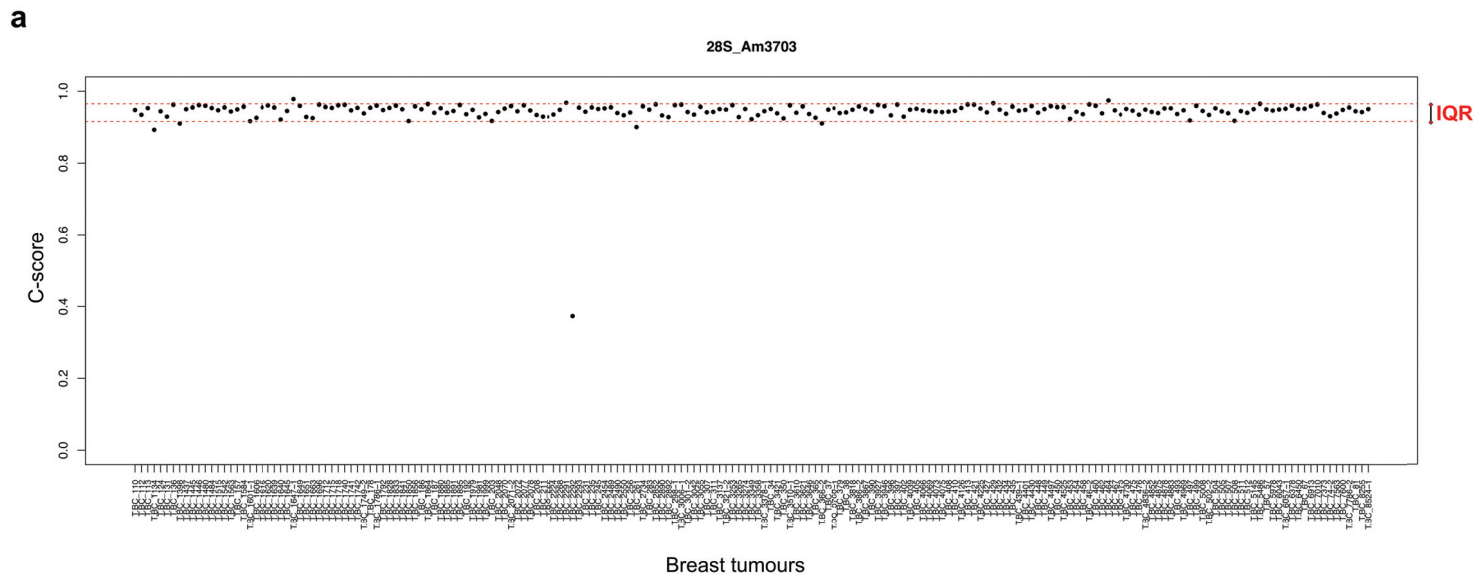
**b**

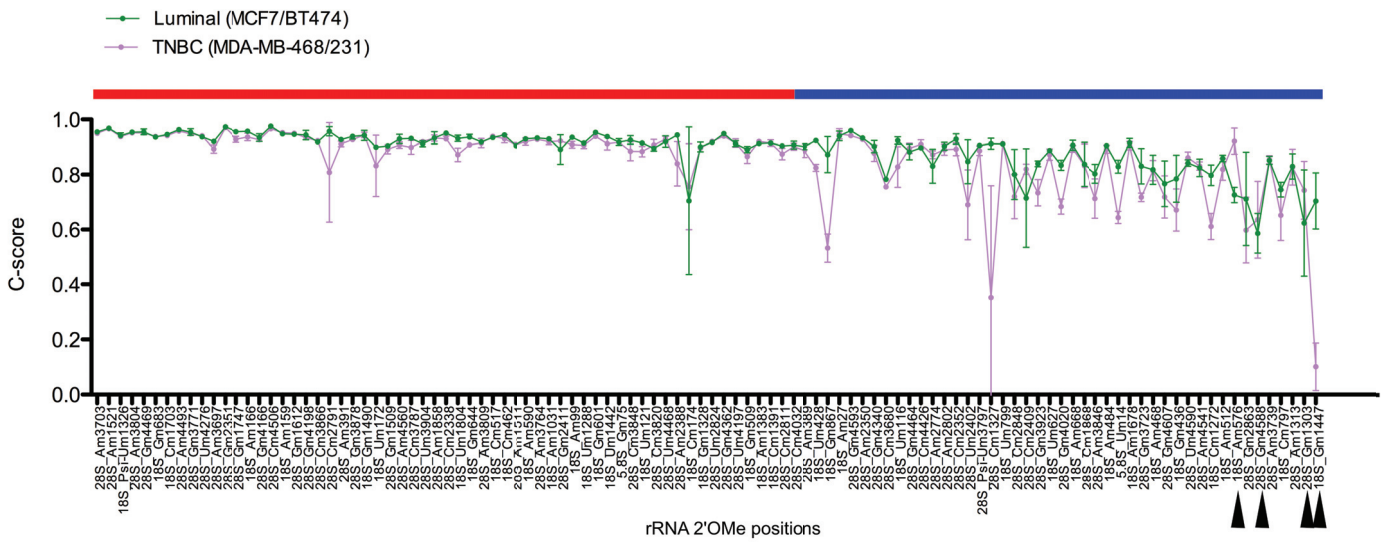
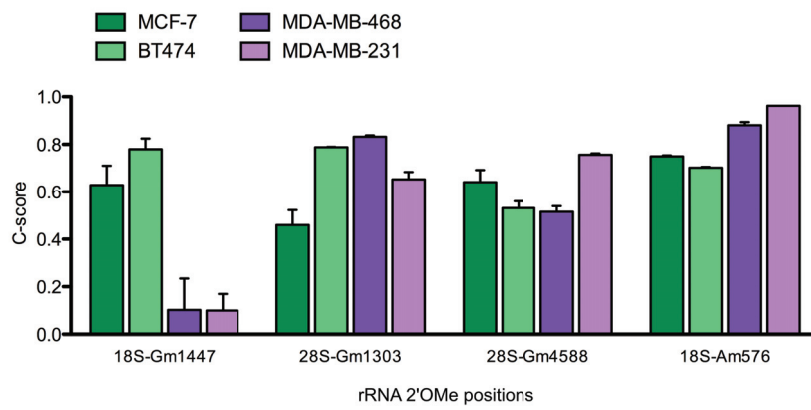




Supplementary Figure 3





**a****b**



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