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ABSTRACT

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MYC dysregulation is the most common alteration in cancers, driving several cellular processes that cooperatively promote aberrant cell growth and proliferation. Among them, the induction of protein synthesis has been demonstrated to have a functional significance in the process of tumor initiation and maintenance. MYC-driven tumors are therefore characterized by increased rates of Ribosome Biogenesis (RiBi), which sustain their overt anabolic demand. The role of MYC in RiBi is traditionally linked to its transcriptional activity, by upregulation of the RNA polymerases (Pol) I, II and III required for the transcription of ribosomes constituents, respectively the 47S precursor ribosomal RNA (pre-rRNA), ~80 ribosomal proteins (RPs) mRNAs and the 5S rRNA. With respect to RPs transcripts, the synthesis of their cognate proteins is ultimately controlled at the translational level by a *cis* regulatory motif located at their transcriptional start site called 5' oligopyrimidine tract (5'TOP) and defined by an invariant cytosine at position +1 followed by a stretch of pyrimidines of variable length and composition. Our laboratory demonstrated that the RNA binding protein LARP1, together with the free 40S ribosomal subunit, forms a complex that stabilizes 5'TOP mRNAs. As RiBi is a high energy consumption process, its regulation is strictly dependent on the energetic status of the cell, which is sensed by the master regulator of cellular metabolism, the mechanistic target of rapamycin (mTOR). mTOR acts to lower the protein synthesis rate and global metabolism upon nutritional limitations, whereas it re-establishes the anabolic rate of the cell in response to proliferative signals. Consistently, our laboratory showed that upon mTOR inhibition 5'TOP transcripts are preserved from degradation by the 40S-LARP1 complex in a translationally repressed state, generating a reservoir of anabolic power in the form of mRNA that cells can unleash when external conditions return permissive.

In light of the role of LARP1 in the post-transcriptional regulation of RP mRNAs and based on recent evidence pointing at the ability of MYC to promote translational efficiency of RPs, we sought to investigate the role of LARP1 on RP mRNAs translation in the context of oncogenic MYC.

To this aim, we discovered a novel regulatory axis between MYC and LARP1 sustained by oncogenic MYC levels in osteosarcoma, lymphoid and colorectal cancer cells. We showed that MYC, by inhibiting the expression of the tumor suppressor microRNAs miR-26a and miR-26b, relieves their targeting on LARP1 mRNA thus sustaining LARP1 expression. In turn, the increase in LARP1 expression appeared to be necessary for the execution of MYC oncogenic program with respect to the increased global protein

synthesis and cell proliferation rates. We reported indeed that ablating LARP1 under MYC overexpression leads to a strong reduction in RP mRNAs translational efficiency, thus impairing MYC-driven protein synthetic and proliferative capacity of the cell.

In conclusion, these observations are unraveling a novel molecular mechanism explaining how MYC oncogenesis sustains the ribosome biogenesis at the translational level.

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1. MYC role in cancer

1.1 The proto-oncogene MYC

Since its discovery in tumor-inducing chicken retrovirus, the proto-oncogene *MYC* has been well characterized in humans as a driver of tumorigenesis.

Considered as the master regulator of gene transcription, MYC expression and activity are tightly controlled in non-malignant cells, however its dysregulation can alter key cellular processes involved in tumor formation. By direct and indirect regulation of thousands of genes, MYC rewires biological programs such as cell cycle, metabolism, stemness, tumor microenvironment and immune response, to finally sustain the transformation of normal cells. In addition, the overexpression of MYC is necessary to maintain the malignant phenotype of a tumor, as it has been observed that its inhibition leads to tumor regression, a phenomenon referred to as “MYC addiction” (reviewed in Dang, 2012)

1.1.1 MYC structure

The transcription factor *MYC,* located at chromosome 8 q24.21, has two paralogues, *MYC-L* and *MYC-N.* For long time MYC has been considered responsible for all MYC-driven tumors, either solid and hematological, while L-MYC and N-MYC have been related to only small-cell lung cancer and neuroblastoma respectively, leading to the assumption that the three different isoforms could be involved in tissue-specific oncogenic pathways. Recently, however, genome sequencing analysis-based studies have led to the conclusion that L-MYC and N-MYC (Ohshima et al., 2017; Rickman et al., 2018) can be implicated in a wide variety of human cancers together with MYC (Schaub et al., 2018).

The activity of MYC as transcription factor is dependent on its dimerization to the protein MAX (Mathsyaraja et al., 2019). The binding to MAX however can be antagonized by the MXD/MNT/MGA family of proteins which compete with MYC for MAX binding. Opposite to MYC-MAX complex, the heterodimers MAX-MGA1 and MAX-MNT ultimately activate a transcriptional program leading to growth inhibition. Consistently, the balance between MYC and MGA1 or MNT protein expression is strongly shifted toward MYC in tumoral

settings (Schaub et al., 2018), in accordance with the role of MYC-MAX as inducer of cell growth. The interaction with MAX is mediated by a highly conserved region which comprises a helix-loop-helix and leucine zipper (HLH-LZ) domain in the carboxy-terminal part of the protein, shared by all the three MYC oncoproteins (Fig. I-1). The heterodimerization with MAX is indeed fundamental for the binding of MYC to the DNA sequence element known as E-box (CAC(G/A)TG), largely present in the promoters and/or enhancers of MYC-responsive genes. However, the binding of MYC to DNA can also occur at sequences different from the E-box, adding additional layers of complexity to the transcriptional regulation executed by MYC. The same HLH-LZ domain can serve as scaffold to the dimerization with the MIZ1 protein, resulting in a complex that mediates transcriptional repression (Walz et al., 2014). Conversely to the HLH-LZ domain, the N-terminal part of MYC protein family is unique to each isoform, except for short domains called MYC boxes (MB0, MBI, MBII, MBIIIa, MBIIIb, MBIV). These six homology domains mediate the association with other partners for a finer tuning of MYC activity. For instance, the MBI is responsible of MYC protein stability through the recruitment of kinases (glycogen synthase kinase 3ß (GSK3ß), cyclin-dependent kinases (CDKs), or kinases related to the MAPK signaling) and phosphatases (serine/threonine protein phosphatase 2A, PP2A) that regulate the phosphorylation status of MYC. Phosphorylation at Threonine 58 (T58), indeed, triggers MYC degradation by the ubiquitin-proteasome system (Farrell & Sears, 2014), while phosphorylation at Serine 62 (S62) stabilizes MYC protein (described in the paragraph “Regulation of MYC expression”). The MBII, instead, interacts with transformation/transcription associated protein (TRAPP), a scaffolding protein that recruits chromatin remodelers and therefore deputed to control chromatin accessibility. Finally, the MBIIIb binds the protein WDR5 which facilitates histone 3 Lysine 4 (H3K4) methylation and has been found to be a key determinant of MYC recruitment to chromatin (Thomas et al., 2015).

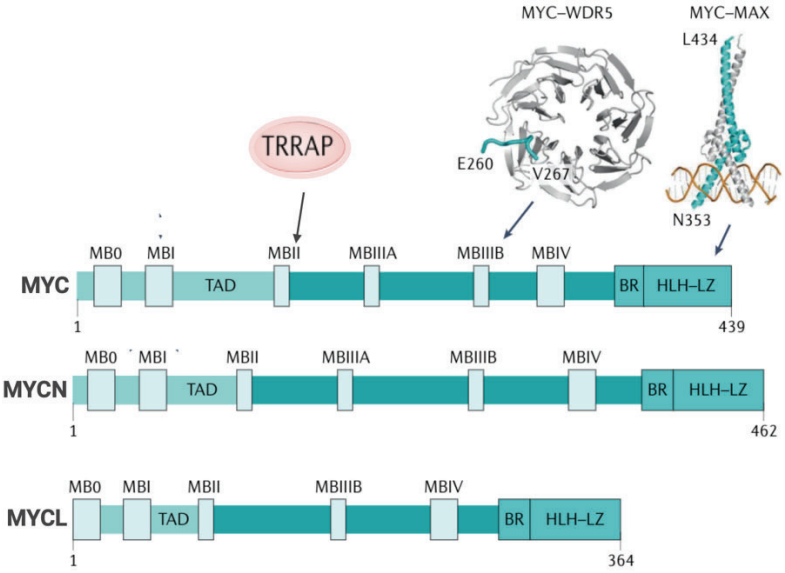

Fig. I-1 **| Structure of the MYC family** MYC, MYCN and MYCL share evolutionary conserved regions including including the MYC homology boxes (MBs), transactivation domain (TAD), basic region (BR) and helix–loop–helix–leucine zipper (HLH–LZ) domain. Here are depicted some interactions of these domains with interactors whose function has been described in the text. Adapted from (Lourenco et al., 2021)

1.1.2 Regulation of MYC expression

Mutations of the MYC family and of other components controlling MYC network is a common hallmark of human cancers. MYC is found overexpressed in almost 70% of tumors (Hoffman & Liebermann, 2008). Since MYC overexpression is a critical determinant of its oncogenic transcriptional activity, it is not surprising that its expression is regulated at multiple steps, including transcription, mRNA stability, translation and protein stability.

MYC transcription is regulated by proliferative signaling pathways that promote transcriptional initiation at distinct promoters of MYC such as P0, P1, P2, P3 and initiation regions (Chung & Levens, 2005). Among the signaling routes that sustain MYC transcriptional activation (Levens, 2008) there are growth factors (PDGF, EGF, CSF-1), hormones, PI3K/AKT, Ras/Raf, JAK/STAT, Wnt, Notch, interleukins and cytokines (J. Liu & Levens, 2006; Wierstra & Alves, 2008). In addition, MYC promoters are also subjected to a negative regulation by differentiation and anti-proliferative factors like c/EBPα, C/EBPß, Blimp-1, GATA-1, KLF11, IFN-λ, p21, p53 and TGF-ß (Wierstra & Alves, 2008). Once transcribed, MYC mRNA is rapidly transported to the cytoplasm and translated within the first 10 minutes approximately (Dani et al., 1984). The stability of the transcript

can be compromised by two microRNAs, miR-34b and miR34c, which bind to MYC 3'UTR and induce its degradation in response to DNA damage (Cannell et al., 2010; Cannell & Bushell, 2010).

Translation of MYC mRNA is instead regulated by multiple signaling cascades, among which mTOR Complex 1 (mTORC1), which is a master regulator of protein synthesis. With a half-life of only 20 minutes (Hann & Eisenman, 1984), MYC protein is subjected to a rapid turnover mediated by the ubiquitin-proteasome system (UPS). This mechanism relies on a network of kinases and phosphatases that, as mentioned before, influence the phosphorylation status of the MBI domain. MBI contains indeed two residues whose phosphorylation strongly impacts the overall protein stability, namely the Serine 62 (S62) and Threonine 58 (T58) (Lutterbach & Hann, 1994). Following cell growth stimulation, during early G1 phase, the Ras-activated kinase ERK and CDK2 stabilize MYC by phosphorylation on S62 (Sears et al., 2000; Tsai et al., 2012). Subsequently, while progressing through the cell cycle, MYC can become phosphorylated on T58 by active GSK-3ß in late G1 phase. T58 phosphorylation induces MYC poly-ubiquitinilation to lower MYC levels, by recruiting several E3 ubiquitin ligases such as Fbw7, Skp2, TRUSS, HectH9, TRIM32 and CHIP(Adhikary et al., 2005; Choi et al., 2010; Paul et al., 2013; Von Der Lehr et al., 2003; Yada et al., 2004). Phosphorylations at S62 and T58 residues have therefore opposing effects on the stability of MYC protein. This state of dual phosphorylation induces the activity of the prolyl isomerase Pin1 which induces the isomerization of Proline 63 (P63) from cis to trans (Farrell et al., 2023), increasing the susceptibility to the protein phosphatase 2A (PP2A) and therefore reducing MYC stability. PP2A is indeed a serine/threonine phosphatase that by dephosphorylation of S62 (Arnold & Sears, 2006) favors the destabilizing p-T58 status.

1.1.3 MYC as transcription factor

Despite its crucial role in many physiological and tumorigenic cellular pathways, MYC activity induces only moderate changes in total mRNA levels, however is responsible for the transcription of roughly 15% of cellular genes (Adhikary & Eilers, 2005; Cowling & Cole, 2006).

The binding of the MYC-MAX heterodimer occurs usually in chromatin regions with specific molecular marks defined by histone modifications like H3 lysine 4 trimethylation (H3K4me3) or H3 lysine 27 acetylation (H3K27ac) and the presence of the transcriptional machinery. MYC, in fact, requires prior activity of transcriptional factors to gain access

to already open promoters. Consistently, it is generally accepted that the role of MYC is to amplify the expression of already transcribed genes (reviewed in (Kress et al., 2015). The binding of MYC to the genome occurs in a stepwise process with MYC being recruited to open chromatin domains, such as promoters and enhancers, by chromatin-associated proteins or by the basal transcriptional machinery. This is followed by the association of MYC-MAX heterodimer with the DNA double-helix and which then scan the sequence until an E-box is reached (Lin et al., 2012; Nie et al., 2012; Sabò et al., 2014). Analysis of Encyclopedia of DNA elements (ENCODE) Project ChIP-seq data, however, showed that a large number of MYC binding sites are not enriched in E-boxes, thus meaning either that the binding of MYC is indirect or that specificity of MYC binding is not so strict (Neph et al., 2012a; Soufi et al., 2015). Tethering factors including early growth response 1 (EGR1), GA-binding protein-a chain (GABPA) and activating protein 1 (AP1) are believed to mediate the association of MYC with those promoters (Neph et al., 2012b; J. Wang et al., 2012). The absence or presence of E-boxes is instead what defines the affinity of MYC for a promoter. High-affinity MYC interactions occur in those regions of methylated/acetylated chromatin enriched in E-boxes, making the binding to MYC more probable even at physiological levels of MYC. On the contrary, the absence of E-boxes in the open chromatin region of a promoter, requires higher expression of MYC to increase the probability of the binding. In part, this dosage of MYC levels is what determines the physiological or oncogenic output of MYC activity. A study conducted in U2OS cells, indeed, showed that supra-physiological MYC levels directed the expression of novel target genes. This was true only for low-affinity genes, for which the intensity of MYC binding was correlated to dysregulation of their expression. High-affinity loci, instead, did not show any further increase in MYC occupancy and transcriptional activation. In this scenario, oncogenic MYC implements its physiological transcriptional landscape with the activation of a specific tumorigenic program (Walz et al., 2014).

Once recruited to the DNA, MYC can affect the epigenetic landscape in order to further stabilize chromatin accessibility. This ability is mediated by the interaction of MYC with histone modifiers such as ASHL2 and WDR5, which promotes trimethylation of H3 at Lys 4 (H3K4me3) (Thomas et al., 2015; Ullius et al., 2014). MYC can also promotes histone acetylation by the binding to TRRAP, which is considered a scaffold protein between transcription factors and histone acetyl transferases (HATs). TRRAP, indeed, although it lacks catalytic activity, is critical for transcriptional coactivation function as it redirects the HAT complexes STAGA and NuA4 to the specific MYC-regulated genes (Feris et al., 2019).

For the transcription to start, it is required the formation of a pre-initiation complex (PIC), formed by general transcription factors (GTFs) and the RNA polymerase, at the promoter. MYC can regulate the activity of all the three RNA polymerases (Pol I, Pol II and Pol III) found in metazoans. It is associated with elevated transcription levels of genes controlled by RNA polymerase II (Pol II). In addition, it controls the transcription of ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) by activating Pol I and III respectively (Oskarsson & Trumpp, 2005). As just mentioned, the interaction of MYC with specific GTFs is essential to exert its function. The transcription factors (TFs) associated to RNA Pol II PIC are TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (K. Gupta et al., 2016; Nogales et al., 2016). Once the transcriptional PIC is formed (Fig. I-2), the RNA Pol II must be phosphorylated at Serine 5 (S5) of its carboxy-terminal domain (CTD) in order to initiate the transcription. This is achieved by a MYC-dependent, but indirect, recruitment of CDK7 kinase. CDK7 is found complexed to the XBP helicase and TFIIH. The binding of CDK7-XBP-TFIIH complex to MYC interactors, such as TRAPP and TIP60 (a HAT of NuA4), therefore allows the positioning of CDK7 to MYC-regulated PIC (Cowling & Cole, 2007). Once initiated, RNA Pol II will execute an early elongation followed by a productive elongation. Early elongation consists in 20-50bp transcription downstream the transcription start site, necessary for the capping of early transcript (Jonkers & Lis, 2015). Cap addition confers stability to the mRNA and, after methylation of the incorporated cap, improves its translational engagement. This process takes place during Pol II pausing from early to productive elongation, when MYC recruits the mRNA capping enzymes (RNGTT) (Lombardi et al., 2016) and the RNA guanine-7 methyltransferase (RNMT) (Cole & Cowling, 2009) for methylation of the cap. Eventually, by direct or mediator complex subunit 23 (MED23) and bromodomain- containing protein 4 (BRD4)-mediated interaction with positive transcription elongation factor b (P-TEFb), MYC promotes the release of Pol II from pausing to support elongation(Moon et al., 2005). Mechanistically, the recruitment of pTEFb by MYC enables CDK9 to phosphorylate Pol II at Serine 2 (S2) on the CTD, triggering productive elongation (Rahl et al., 2010). Finally, MYC can also regulate Pol II transcription rates to ensure efficient splicing by the recruitment of the serine/threonine kinase NUAK1. In detail, NUAK1 locally represses the PP1-PNUTS phosphatase complex that dephosphorylates the CTD of Pol II, therefore controlling the travelling speed of Pol II (Cossa et al., 2020).

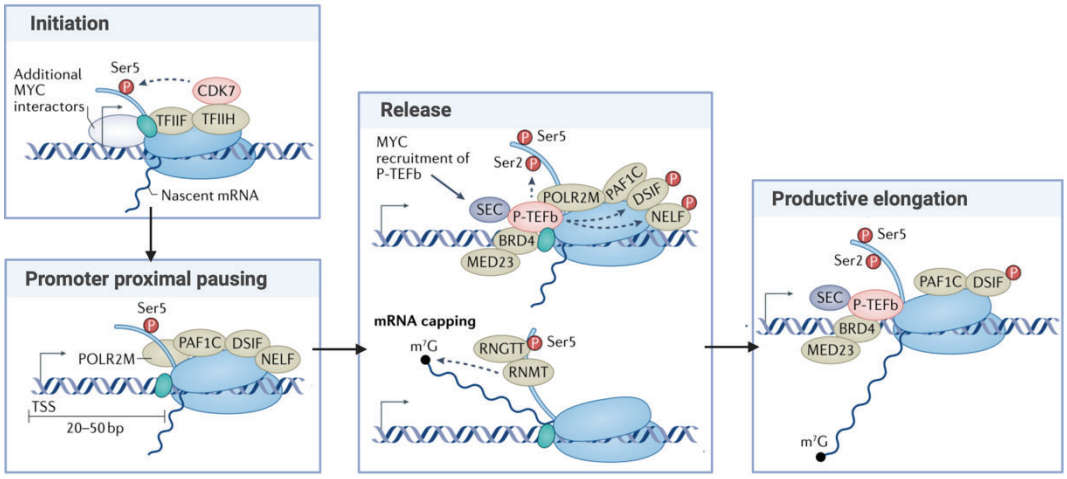

Fig. I-2 | **MYC interactions during transcription by RNA Pol II** During initiation, assembly of the transcriptional PIC facilitates an opened promoter. CDK7 then phosphorylates the Pol II CTD at S5, allowing Pol II to start the transcription. 20–50 bp downstream of the TSS, at the promoter proximal pausing site, RNA Pol II gets stalled to allow the capping of the nascent mRNA. P- TEF recruitment facilitates phosphorylation of the Pol II CTD at S2, thus generating a doubly phosphorylated Pol II molecule that can proceed through a productive elongation of the nascent mRNA. Adapted from (Lourenco et al., 2021)

On the other hand, MYC can also repress transcription of several tumor suppressor genes, mainly related to cell cycle progression. The mechanism of action is still based on the partnership with specific interactors like MIZ1 or SP1 (Staller et al., 2001; Wanzel et al., 2003). Other studies have also demonstrated that MYC can act as transcriptional inhibitor by inducing epigenetic modifications through the interaction with histone deacetylase 3 (HDAC3) (Kurland & Tansey, 2008).

In conclusion, intrinsically disordered regions of MYC enable the binding to diverse MYC interactors. Establishing such interactions is fundamental for MYC to exert its core function as transcription factor. Overall, MYC transcriptional regulatory mechanism mainly consists in modifying DNA accessibility by epigenetic alterations, increasing the binding of the transcriptional machinery to the promoter and enhancer, and to finally follow every step of the process. The ability of MYC to monitor all the different steps just described is ensured by the presence in the cell of distinct MYC molecules. Each of them binds distinct MYC interactors for the formation of complexes that independently, yet cooperatively, are involved in a different step of transcription. The existence of this “coalition model” was observed studying the tumorigenic potential of two MYC mutants, one with a deletion in MB0 and one in MBIII. When co-expressed in MYC-driven breast

cancer cell line and then injected in xenograft models, the two loss-of-function mutants were still able to induce tumorigenesis as the WT MYC. This was explained as the ability of each mutant to recruit the missing interactome of the other. Consistently, expression of only one of them, impaired cell transformation, meaning that the overall activity of MYC comes from the average of each MYC complex activity (reviewed in (Lourenco et al., 2021).

1.2 Oncogenic MYC

In vitro studies on murine fibroblasts showed that MYC overexpression was able to induce tumorigenesis. These studies set the stage for mouse transgenic models that proved the ability of MYC, in concomitance with other oncogenic events like K-Ras mutagenesis, to drive tumor formation (reviewed in (Dang, 2012).

Overexpression of MYC can be mediated by different mechanisms including genetic modifications, increased transcriptional activation of MYC gene and post-translational modifications (Fig. I-3).

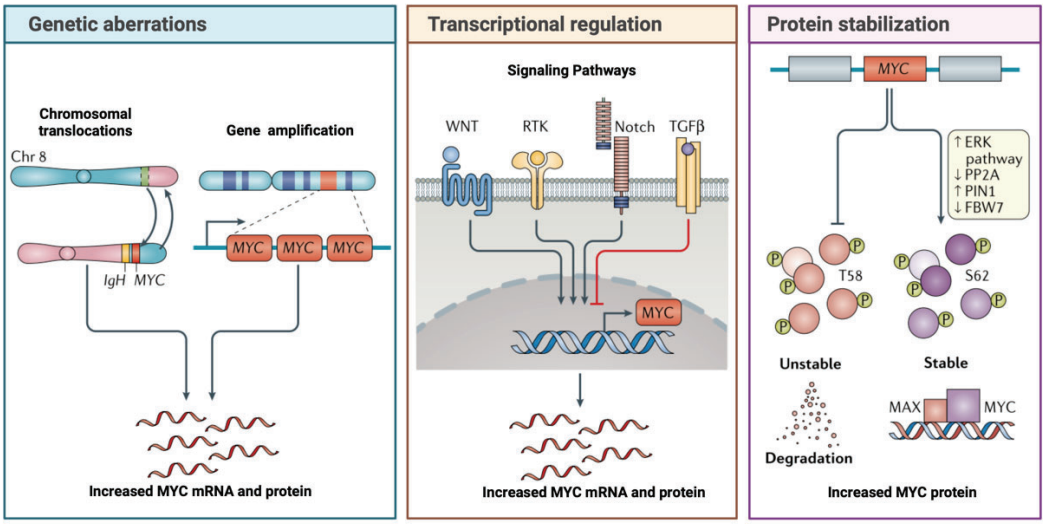

Fig. I-3 | **Mechanisms leading to MYC dysregulation** Genetic alterations and deregulated signaling pathway are upstream events that induce increased transcription of MYC, with consequent augment of protein levels. Post-translational modifications mediated by preferential phosphorylation of S62 over T58 promote MYC protein stability by blocking its degradation. Adapted from (Dhanasekaran et al., 2021)

Genomic amplification and translocation of MYC genes are the most diffused genetic alteration leading to increased MYC levels. A pan-cancer assessment of genomic alterations in 33 different types of cancers showed MYC to be amplified in 28% of them

(Schaub et al., 2018), mainly in solid tumors like breast and liver carcinomas. Aberrant expression of MYC is instead responsible for virtually all Burkitt's lymphomas. This is the result of a canonical translocation of the MYC transcriptional unit downstream the heavy immunoglobulin chain promoter locus (observed in 80% of cases) and to the κ or λ light chain promoter (occurring in 10% of cases) (Boxer & Dang, 2001). MYC expression can also be altered by retroviruses, which encodes for viral proteins able to activate MYC expression or that, most commonly, insert a viral enhancer upstream of MYC (Dudley et al., 2002).

Mutations that hyperactivates the transduction of proliferative signaling pathways such as SRC, tyrosine kinases receptors and Notch are also responsible for deregulated MYC expression (Weng et al., 2006). In addition, around 85% of human colorectal cancers are caused by inactivation of the tumor suppressor gene APC that leads to increased WNT-ß-catenin signaling (T. C. He et al., 1998). In non-malignant cells, the levels of cytosolic ß-catenin are regulated by a proteasomal degradation mechanism initiated by kinases glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1) together with the scaffold proteins APC and AXIN. The activation of the WNT signaling leads to the disassemble of the APC degradation complex and the consequent translocation of ß-catenin into the nucleus, where it binds to transcription factors from the T-cell factor/Lymphoid enhancer factor (TCF/LEF) family and drives the expression of genes involved in cell proliferation, migration, and embryonic development. Inactivation of APC, therefore, impairs ß-catenin degradation and supports its translocation to the nucleus and binding to TCF4, ultimately causing transcriptional activation of MYC (Clevers, 2006).

Finally, aberrant levels of MYC protein are found as a result of increased MYC stability. The activation of mitogenic pathway like ERK increases the stable p-S62 MYC isoform (Farrell et al., 2017), while mutations in T58 lead to constitutive phosphorylation at S62 (X. Wang et al., 2011). Likewise, mutations in phosphatase PP2A can also affect the phosphorylation status and stability of MYC (Reavie et al., 2013).

1.2.1 Cell-intrinsic processes driven by MYC

MYC sustains uncontrolled cell growth by increasing anabolic processes like ribosome biogenesis and protein synthesis (Rosenwald, 1996), and by rewiring the global metabolism of lipids, amino acids and purines to ultimately generate energy and building blocks (reviewed in (Dong et al., 2020). Among other mechanisms, MYC also regulates glucose and glutamine metabolism by increasing the expression of their respective

transporters, thus ensuring a sufficient uptake of metabolites to meet the oncogenic anabolic demand.

Aberrant cell proliferation also relies on the ability of MYC to force quiescent cells to re-enter the cell cycle and, by inhibiting cell-cycle checkpoint proteins like p15 or p21 (García-Gutiérrez et al., 2019), to boost cell cycle progression and prevent cellular senescence. Consequences of MYC (Kaczmarek et al., 1985) ability to bypass cell-cycle checkpoints and to block the DNA double-strand repair mechanism are, however, the induction of genomic instability and genomic damages, such as aneuploidy and polyploidy, DNA breaks and chromosomal translocation (Li et al., 2012). MYC also promotes entry into the S phase, during which it can activate DNA replication machinery and coordinate both transcription and DNA replication, in order to avoid replication stalls caused by head-to-head collisions between Pol II and DNA polymerase (Hamperl & Cimprich, 2016; Poli et al., 2016).

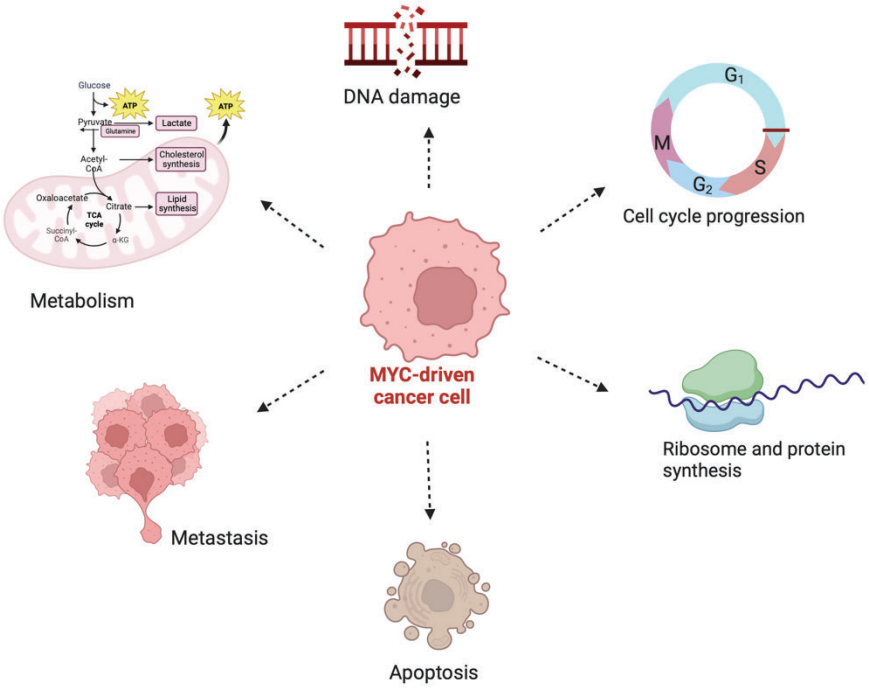

Fig. I-4 | **MYC activation of cell-intrinsic tumorigenic processes** A simplified resume of cell-intrinsic pathways activated by MYC to promote tumor initiation and maintenance. Triggering of DNA damage and activation of apoptosis requires a further balance between these events and the cellular context to ultimately promote cell survival. Adapted from (Dhanasekaran et al., 2021)

Of note, although in contrast to its proliferative role, MYC triggers apoptosis when overexpressed. One of the many mechanisms through which MYC can regulate apoptotic signaling is through the tumor suppressor p53 (TP53). p53 accumulates in the

nucleus and interacts with pro-apoptotic genes like *PUMA, NOXA, APAF-1,* and *BAX*, upregulating their expression (Wawryk-Gawda et al., 2014), but it also suppresses the expression of anti-apoptotic proteins like BCL-2, BCL-XL, and MCL-1. p53 is characterized by a short half-life, dictated by the binding of the E3 ubiquitin ligase MDM2 (or HDM2 in humans) deputed to mediate p53 degradation (Geyer et al., 2000). MYC dysregulation, however, sustains the expression p14ARF, an MDM2 inhibitor, thus allowing p53 release from MDM2 and hence p53 stabilization. This mechanism was observed in a study using primary murine embryonic fibroblasts (MEFs), where overexpression of MYC led to an arrest in cell growth and stimulated apoptosis by the p14ARF-mediated stabilization of p53 (p19ARF in mouse). However, the oncogenic potential of MYC reappeared 14 days after MYC induction, when cells resisting to apoptotic cell death restarted their proliferation program as a consequence of p53 mutations and p19ARF loss (Zindy et al., 1998).

In addition to its tumor initiation function, MYC also supports tumor maintenance, as described by *in vivo* studies where the suppression of MYC expression induces tumor regression (reviewed in (Gabay et al., 2014). Lowering MYC levels in MYC-driven tumors, indeed, leads to restoration of normal cell cycle checkpoints and mechanisms of DNA repair, that will account for proliferative arrest and differentiation, senescence and/or apoptosis. Likewise, loss of MYC helps the restoration of cell death signals like TGF-ß that elicits proliferative arrest and senescence (Van Riggelen, Müller, et al., 2010). However, the extent of regression is dependent on the origin and genetic context of the tumor. For example, in T-cell acute leukemia, inhibition of MYC causes proliferative arrest, senescence and differentiation leading to rapid tumor regression (Felsher & Bishop, 1999), while in epithelial-derived tumors, like hepatocellular carcinoma (HCC), inactivation of MYC reduces tumor growth however a population of dormant tumor cells still remains (Shroff et al., 2015).

Finally, MYC participates in tumor aggressiveness by controlling epithelial to mesenchymal transition (EMT) and metastasis through regulation of miR-9, which targets E-cadherin, and transactivation of Bmi-1, linked to EMT (Fig. I-4).

1.2.2 miRNAs regulation by MYC

microRNAs (miRNAs) are short non-coding RNA sequences of 18-24nt length that are known to negatively regulate the stability and the expression of target mRNAs, therefore influencing the total levels of the cognate proteins.

Synthesis of miRNAs is directed by RNA pol II, resulting in the production of a primary transcript (pri-miRNA) that is processed by the double-stranded RNA (dsRNA)-specific endonuclease DROSHA in the nucleus (Fig. I-5) The activity of DROSHA is to excise the sequence of the miRNA to obtain a hairpin structure precursor called pre-miRNA. Exportin 5 (XPO5) therefore mediates the translocation of pre-miRNA to the cytoplasm where the mature double strand miRNA will be generated by the dsRNA-specific RNase III enzyme DICER. Final incorporation of the miRNA into the RNA-induced silencing complex (RISC) includes the association of the guide strand with the Argonaute (AGO) protein and degradation of the opposite strand (miRNA*) (Bartel, 2009; Hammond et al., 2000, 2001). The 5' end of the guide strand contains 7nt sequence stretches that recognize the target mRNA sequence, usually found in the 3'-untraslated region (UTR). As the targeting occurs through such a short sequence, a single miRNA can have multiple and non-exclusive targets (Fabian et al., 2010).

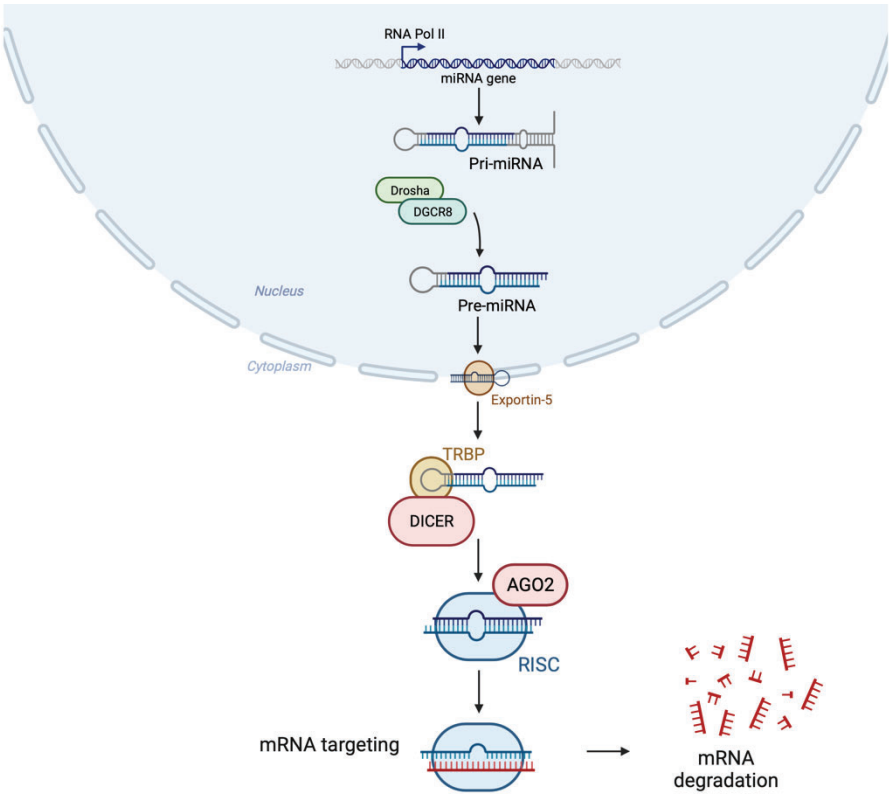

Fig. I-5 **| Process of microRNA maturation** Processing of microRNA from RNA Pol II-mediated transcription to the targeting of the complementary mRNA. More details in the text.

In tumor context, miRNAs can function either as onco-miRs or as tumor suppressor miRNAs, according to the cell-type. In this regard, MYC has been shown to regulate miRNAs expression either by direct binding to the promoter of specific miRNAs, either

by affecting general mechanisms of miRNAs processing regulation, thus altering global miRNAs expression. An example is given by the transcriptional induction of RNA binding proteins (RBPs) Lin28 and Lin28b that bind let-7 pre-miRNA thus impeding its processing by DICER (T. C. Chang et al., 2009). On the contrary, MYC can also enhance pri-miRNA processing by increasing the expression of DROSHA (X. Wang et al., 2013).

The first identified specific miRNAs directly upregulated by MYC were the miRNAs of the miR-17-92 cluster thar include: miR17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1 (L. He et al., 2005). Ectopic expression of this cluster showed to induce B-cell lymphomas in mice (Sandhu et al., 2013) and increase tumorigenesis of colorectal cancer (Dews et al., 2006), while conditional knockout in MYC-driven lymphomas increased cell death thus reducing tumorigenicity. miR-22 is another well-described MYC-induced miRNA that acts as an onco-miR by promoting EMT in breast cancer metastasis and targeting the tumor suppressor PTEN. Other MYC and MYC-N induced miRNAs are miR-378, miR-9, miR-130a and miR-214 (reviewed in (Jackstadt & Hermeking, 2014).

In 2008, instead, Chang et al. identified for the first time a wide spectrum of miRNAs whose expression was downregulated by direct binding of MYC to their promoters (T.-C. Chang et al., 2008). Importantly, they observed that expression of MYC-repressed miRNAs impaired lymphoma cells growth *in vivo.* Among them, miR-26a and miR-26b were found to be down-regulated in a plethora of human cancers like lymphomas, HCC and breast cancer. Distinctly, the same group demonstrated that miR-26a expression suppressed intestinal adenoma formation in $Apc^{min/+}$ mice, a model in which *Pten* dosage accelerates tumorigenesis (Zeitels et al., 2014). miR-26a role of tumor suppressor was mediated by the inhibition of anti-proliferative targets like Cyclin D2, MYC binding protein and EZH2. However, in other tumoral settings like glioma, lung cancer and T-cell lymphoblastic leukemia, miR-26 exerts an oncogenic activity by suppressing PTEN.

1.2.3 Tumor micro-environment responses

In addition to the above-mentioned cell-intrinsic mechanisms activated by MYC, there are other responses elicited by the oncoprotein that are inherent to the tumor microenvironment modulation and host immune response (Fig. I-6).

Indeed, MYC can support angiogenesis by regulating the expression of cytokines that act over vascular endothelial cells and by repressing the anti-angiogenic factor thrombospondin 1 (TSP1) (Giuriato et al., 2006).

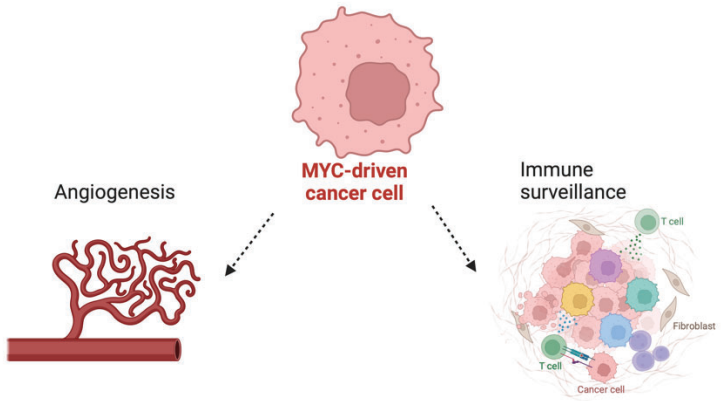

Fig. I-6 | **MYC regulation of tumor microenvironment response** Simplified representation of MYC ability to control tumor microenvironment in order to support cancer progression. MYC activation stimulates angiogenesis and enables cancer cells to evade and inhibit immune surveillance. Adapted from (Dhanasekaran et al., 2021)

In addition, MYC affects immune cells to sustain immune evasion of the tumor. Well-described mechanisms are the downregulation of MHC I antigen presentation complex that impedes immune cells activation and the modulation of several immune ligands and receptors like PD-L1 and CD47 (Bernards et al., 1986; Rakhra et al., 2010). They are known as immune checkpoints and their binding to T cells leads to T-cells exhaustion. Moreover, MYC induces the expression of cytokines to modify the immune cells response. By promoting the release of CCL-9, CCL-2 and IL-23, MYC inhibits activation of NK cells, B and T cells and promotes the conversion of M1 antitumor macrophages to M2 pro-tumoral ones. Additionally, CCL-9 activates mast cells that support angiogenesis while CCL-2 and IL-13 promote the recruitment of immunosuppressive macrophages to favor metastasis (Braun et al., 1992). The release of antitumoral cytokines like TGF-ß abrogates, instead, T cell responses (Van Riggelen, Müller, et al., 2010)

1.2.4 MYC targeting

The described role of MYC in oncogenesis makes it an attractive target for therapy. However, several technical and conceptual complications obstacle the discovery of a compound that can be efficiently used in cancer therapy, thus acknowledging MYC as the “undruggable target”.

Specifically, MYC is an intrinsically disordered protein with no catalytic domains. As of that, canonical small inhibitory molecules do not constitute a plausible strategy to inhibit MYC transcriptional activity. Also, the activity of MYC and the isoforms MYC-N and MYC-L are partially redundant so that the three of them should be targeted in order to obtain

the inhibition of MYC functions. Finally, MYC is involved in physiological processes like normal tissue regeneration, therefore its inhibition could also be controversial for non-cancer cells and homeostasis of normal tissues. Knockout of *Myc* in mice, indeed, has been shown not to be viable (Davis et al., 1993).

A first strategy for the targeting of MYC is the disruption of MYC-MAX heterodimers, achieved by 10058-F4 molecule which showed to reduce tumor growth and prolong survival in transgenic model of neuroblastoma (Zirath et al., 2013). An alternative is represented by Omomyc, a 90 aminoacid MYC mutant comprising the HLH-LZ domain mutated in four aminoacids known to impede MYC homodimerization. With a dimerization pattern altered, Omomyc is therefore able to heterodimerize with MAX and MYC itself thus constituting a specific competitor for MYC (Soucek et al., 1998).

The direct targeting of MYC by RNA-mediated interference and antisense oligonucleotides demonstrated a limited efficacy due to drug delivery problems (Devi et al., 2005). An alternative strategy to directly inhibit MYC transcription was therefore attempted by stabilizing G-quadruplexes structures, identified as repressive elements of the MYC promoter. CX-3543 stabilizer of G4 conformation reached phase II clinical studies but demonstrated to mediate its anti-tumor effect also by MYC-independent mechanisms (H. Xu et al., 2017).

In non-small-cell lung cancers, epigenetic modifiers like HDAC and histone methyl transferases have been used to repress MYC signalling while in a mouse model of liver cancer the use of anti-miR-17 oligonucleotides reduced tumor progression.

An additional strong MYC inhibitor was found by serendipity in an attempt to identify suppressors of the pTEFb recruited by MYC (Filippakopoulos et al., 2010). Bromodomain and extraterminal (BET) proteins binds acetylated promoters and promote the recruitment of co-factors like pTEFb to support MYC-induced transcription. Thieno-triazolo-1,4-diazepine JQ1 was developed to inhibit MYC transcriptional regulation by masking bromodomain acetyl-binding pocket of the BET subfamily BRD4 specifically, therefore impeding the recruitment of pTEFb. However, when used for treatment of multiple myeloma cells, it was observed that JQ1 was directly inhibiting MYC expression (Delmore et al., 2011). Currently, distinct BET inhibitors are being tested in clinical trials for their efficacy against hematopoietic and solid tumor. However, the limited specificity in targeting MYC transcription might be responsible for the toxicity levels observed in some of these already concluded trials (Postel-Vinay et al., 2019).

MYC levels can also be modulated by targeting MYC protein stability, through inhibition of ubiquitin-proteasome degradation and adjustment of its phosphorylation status toward

the more stable p-S62 isoform. Formation of a complex between MYC and Aurora-A kinase suppresses the association of FBXL3 ubiquitin ligase to MYC, therefore blocking its ubiquitination and promoting MYC stability (Dauch et al., 2016). In this context, inhibitors of Aurora kinase have demonstrated to induce MYC degradation without affecting MYC physiological levels in non-malignant cells (D. Yang et al., 2010).

Although different strategies have been approached to circumvent the mechanistic hurdles of targeting MYC, only one of the above-described is currently running in clinical trial phases, Omomyc.

2. mTOR and LARP1

2.1 mTOR

mTOR discovery dates back in 1994, in the context of studies aimed at finding the molecular target of the antibiotic rapamycin, known to act in a complex with the peptidyl-prolil-isomerase FKBP12. Defined as the “mechanistic target of rapamycin” ((Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995), since then mTOR has been profoundly studied and characterized due to its central role in fine tuning the cellular metabolism. In response to diverse intra-cellular and environmental cues, mTOR can sense the availability of cellular resources and modulate accordingly key biosynthetic and catabolic pathways. In light of this capacity mTOR is considered a master regulator of cellular metabolism.

2.1.1 mTORC1 and mTORC2 structure

mTOR is a 289kDa serine/threonine protein kinase belonging to the phosphatidylinositol 3-kinase (PI3K) family (Keith & Schreiber, 1995) that constitutes the catalytic component of two major complexes, the mTOR Complex 1 or mTORC1 and mTOR Complex 2 or mTORC2, with mTORC1 being the only one directly inhibited by rapamycin. Structurally, the two complexes share the core subunit mTOR as well as the *mammalian lethal with SEC13 protein 8* (mLST8), also known as GßL, which although originally discovered as mTORC1 constituent (D. H. Kim et al., 2003), is now considered dispensable for mTORC1 assembly and activity and indispensable for mTORC2 stability and function (Hwang et al., 2019) (Fig. I-7). Apart from that, mTORC1 and mTORC2 are different in their make-up, characterized by subunits that are unique to each of them. The scaffold protein *regulatory-associated protein of mTOR* (RAPTOR) is the distinctive molecular companion of mTORC1. RAPTOR binds to the N-terminus of mTOR and bridges the substrates of mTORC1 carrying the TOR binding motif (TOS) (Schalm et al., 2003). That RAPTOR is necessary to maintain proper mTORC1 signaling has been demonstrated by the observation that germline deletion of RAPTOR causes embryonic lethality (Guertin et al., 2006). It serves also as a scaffold for mTORC1 inhibitory molecules such as proline-rich AKT substrate 40 (PRAS40), which senses low levels of insulin and transmits this information to mTORC1 (Sancak et al., 2007) and the DEP-domain-containing mTOR-interacting protein (DEPTOR). DEPTOR is an mTORC1/2 inhibitor and as of that its expression is low in the majority of cancers to promote cell growth and

proliferation, with the exception of a subset of multiple myeloma cell lines, where reducing the expression of DEPTOR leads to apoptosis (Peterson et al., 2009).

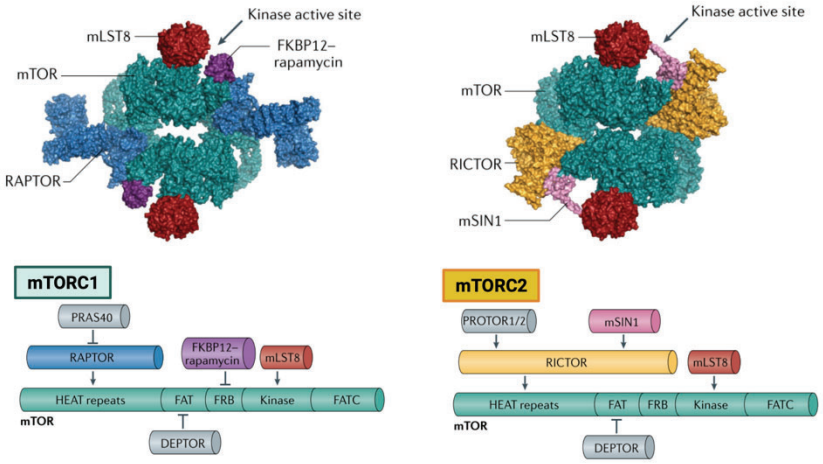

Fig. I-7 **| Structure of mTORC1 and mTORC2** mTOR N-terminal part is constituted of clusters Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A and TOR1 (HEAT) repeats, followed by a FRAP, ATM and TRRAP (FAT) domain, the FKBP12–rapamycin binding (FRB) domain; the catalytic kinase domain; and the C-terminal FATC domain. Other interactors specific of each complex are described in the text. Figure adapted from (G. Y. Liu & Sabatini, 2020)

On the other hand, the *rapamycin insensitive companion of mTOR* (RICTOR) molecularly defines the mTORC2. As for RAPTOR, it works as a scaffold for the mTORC1/2 inhibitor DEPTOR but it is also responsible for the binding of MAPK-interacting protein 1 (mSIN1), necessary for mTORC2 phosphorylation of Akt/PKB (Frias et al., 2006), and protein associated with rictor 1 or 2 (PROTOR 1/2) (Dos et al., 2004). Particularly, recent cryo-electron microscopy data have revealed that the mechanism behind the different sensitivity of mTORC1 and mTORC2 to rapamycin lays on their different structural composition, as the binding of RICTOR to mTOR blocks the binding site of the FKBP12-rapamycin complex, therefore explaining the insensitivity to rapamycin of mTORC2 (Chen et al., 2018).

2.1.2 Stimuli and signaling cascades converging on mTORC1 and mTORC2

As mentioned, mTOR is considered a central signaling node connecting upstream stimuli, such as growth factors, nutrients, oxygen levels and energy, and the activation of downstream processes to maintain the cellular metabolic homeostasis and to respond to sudden changes. The different structural components that constitute mTORC1 and mTORC2, however, determine the sensitivity to specific inputs and the execution of

distinct responses, which ultimately can crosstalk to integrate different signaling cascades.

Activation of mTORC1 is dictated by the coordination of two events: translocation of the cytosolic mTORC1 to the lysosomal compartment and activation of mTORC1 by the lysosomal GTPase Rheb, promoted respectively by nutrients and growth factors (Fig. I-8). Lysosomal localization of mTORC1 is assisted by nutrients, particularly amino acids, via the RAS-related GTP-binding proteins (Rags), which are small GTPases in the form of obligate heterodimers. In the active conformation, GTP-bound RagA or RagB associates to the GDP-loaded RagC or RagD, thereby promoting the binding to RAPTOR and therefore mTORC1 lysosomal translocation (Sancak et al., 2008). Once in the lysosome, GTP-bound active Rheb directly binds mTORC1 inducing the activation of its catalytic site (H. Yang et al., 2017). The nucleotide status of Rheb is regulated by growth factors, such as insulin. Binding of growth factors to their cognate receptors ultimately induces AKT-mediated phosphorylation and inhibition of the Tuberous Sclerosis Complex (TSC), which comprises TSC1, TSC2 and TBC1D7, and is a GTPase activating protein (GAP) over Rheb (Inoki et al., 2003; Tee et al., 2003) . Thus, TSC works as key negative regulator of mTORC1 by promoting the GDP-loaded status of Rheb. In particular, insulin mediates mTORC1 activation by multiple phosphorylation of TSC2 followed by its dislocation from the lysosomal membrane (Menon et al., 2014). Similarly, TSC2 inhibitory phosphorylation is mediated by the activation of the receptor tyrosine kinase (RTK) and the Ras signaling, which ultimately leads to MAP kinase-mediated phosphorylation of TSC2 (Ma et al., 2005). Other growth factors such as Wnt and the inflammatory cytokine TNFα, instead, activate mTORC1 by inhibiting TSC1 (Inoki et al., 2006; Lee et al., 2007).

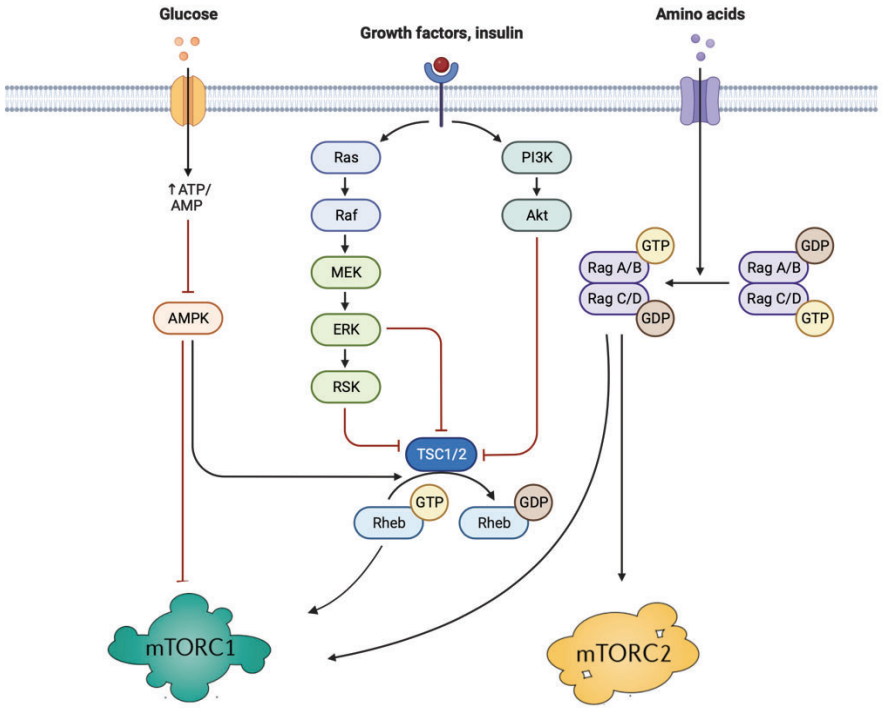

Fig. I-8 **| Upstream regulators of mTOR signaling** Simplified representation of upstream signaling pathways that impinge over mTORC1 and mTORC2. mTORC1 gets activated to promote anabolic cellular processes only in conditions of amino acids, insulin/growth factors, ATP and oxygen availability. mTORC2 is thought to be primarily regulated by growth factors. Figure created with Biorender.

In addition to growth factor cascade, RAPTOR and the TSC complex constitute the hub for energy and oxygen levels sensing. In conditions of oxidative stress and low ATP levels, indeed, AMPK inhibits mTORC1 by phosphorylating RAPTOR and by activating TSC2 (Gwinn et al., 2008) Interestingly, poor energetic conditions dictated by low glucose levels can also bypass the AMPK signaling to inhibit mTORC1. Efeyan et al. showed indeed that AMPK-deficient cells maintains their ability to inhibit mTORC1 upon glucose starvation by inhibiting Rag GTPases (Efeyan et al., 2013). Additionally, in response to low oxygen levels, hypoxia-inducible gene REDD1 is overexpressed, which in turn activates TSC1/2 independently of AMPK (Brugarolas et al., 2004). A final upstream signal sensed and integrated by mTORC1 is DNA damage. Under genomic stress p53 is stabilized with consequent expression of p53-target genes such as TSC and AMPK, resulting in mTORC1 inhibition.

The activation of Rheb by growth factors is therefore fundamental to trigger mTORC1 catalytic activity. Nevertheless, it must be complemented by nutrients-mediated activation of Rags. mTORC1 senses the availability of cytosolic amino acids mainly by

two GTPase activating proteins (GAPs) that ultimately affect RagA/B GDP/GTP status, GATOR1 and GATOR2 (GAP activity towards the Rags). The mechanisms by which they act are still incompletely understood, however GATOR1 is known to directly inhibit RagA/B and therefore mTORC1 (Shen et al., 2018) while GATOR2, by interacting with GATOR1, suppresses its activity and allows Rags-mediated transport of mTORC1 to the lysosome (Bar-Peled et al., 2013). When amino acids levels fall, GATOR2 can be bound and inhibited by SESTRIN2 and CASTOR, sensors of leucine and arginine respectively, thus favoring the inhibitory activity of GATOR1 towards mTORC1 (Chantranupong et al., 2016). Low levels of the by-product of methionine S-adenosylmethionine, instead, are sensed by SAMTOR which binds and activates GATOR1 (Gu et al., 2017). Sensing of amino acids levels, however, extends also to the intra-lysosomal compartment. In this case, the status of Rags is dictated by the interaction of the lysosomal v-ATPase with the pentameric complex Ragulator, responsible for tethering the Rags in the lysosomal membrane (Bar-Peled et al., 2013; Zoncu et al., 2011). The lysosomal amino acid transporter SCL-3849 works as a sensor of arginine levels in lysosomes. Its interaction with the v-ATPase-Ragulator complex favors the GTP-bound state of RagA/C for the recruitment and activation of mTORC1 by arginine (Jung et al., 2015). Finally, a non-canonical mechanism of aminoacids-mediated regulation of mTORC1 is activated by the folliculin and folliculin interacting protein complex (FLCN/FNIP) that works as a GAP for RagC/D so that in conditions of aminoacids replenishment sustains mTORC1 activation (reviewed in (Napolitano et al., 2022) .

Unlike mTORC1, activation of mTORC2 is dictated preminently by growth factors through mTORC2 component mSin1. The pleckstrin homology (PH) domain of mSin1 autoinhibits mTORC2 catalytic activity in the absence of insulin, while the binding of high levels of phosphatidylinositol-(3,4,5)-triphosphate (PIP3), produced by insulin- or serum-induced activation of PI3K, relieves mSin1 inhibition over mTORC2 (Gan et al., 2011).

Although they are structurally and functionally different, a crosstalk between mTORC1 and mTORC2 has been described. Insulin triggers mTORC1 activation by inhibition of TSC2. This mechanism is mediated by the insulin receptor substrate 1 (IRS-1) which recruits PI3K to the plasma membrane in order to promote PIP3 production from the phosphorylation of phosphatidylinositol-(4,5)-biphosphate (PIP2). This allows PDK1 to phosphorylate Akt at the Thr308 (Williams et al., 2000), crucial for its activation. Active Akt can therefore target and inhibit TSC2 to promote mTORC1 activation. In case of a sustained activation of mTORC1 by insulin, p70 S6 kinase 1 (S6K1), one of the two major substrates of active mTORC1, phosphorylates and inhibits IRS-1 (Um et al., 2004), as a

negative feedback loop necessary to mitigates mTORC1 activation. On the other hand, this mechanism also converges on mTORC2, since inhibition of IRS-1 impairs the production of PIP3 levels necessary to activate mTORC2. This is particularly evident in cells lacking the TSC1-TSC2 complex, where constitutive activation of mTORC1 desensitize mTORC2 from the IRS-1-mediated insulin signaling pathway. Overexpression of GAP-dead TSC2 in *Tsc2*$^{-/-}$ MEFs, however, showed to partially rescue mTORC2 activity thus suggesting a positive regulation of mTORC2 by TSC1-TSC2 complex, independent of the mTORC1-mediated feedback over IRS-1 (reviewed in (Huang & Manning, 2009). Additionally, active mTORC1 can phosphorylate and stabilize the negative regulator of IRS-1 Grb10, to ultimately inhibit mTORC2 (Ebner et al., 2017).

An additional layer of complexity at the intersection between mTORC1 and mTORC2 is AKT, being an upstream signal of the first and a direct target of the second. As mentioned, AKT phosphorylation at Thr308 by insulin-induced PDK1 is essential for AKT activation and phosphorylation of TSC2, while mTORC2-mediated Akt phosphorylation at Ser473 increases its activity towards specific targets (Sarbassov et al., 2005), although not toward TSC2 (Guertin et al., 2006).

2.1.3 Cellular processes regulated by mTOR

Activation of mTOR by the signaling pathways above-mentioned leads to a balanced activation of anabolic processes and inhibition of catabolic ones to eventually sustain cell growth (Fig. I-9).

mTORC1 is known to promote the synthesis of the cellular building blocks such as proteins, nucleotides and lipids and to sustain the energy demand of the cell (Saxton & Sabatini, 2017). The high energy-consuming process of protein synthesis is promoted by mTORC1 through phosphorylation of two targets: S6K1 and the eukaryotic initiation factor 4E-binding proteins (4E-BPs). Hypo-phosphorylated 4E-BP1 binds to the eukaryotic initiation factor 4E (eIF4E), impeding its association to the eukaryotic initiation factor 4G (eIF4G) and therefore impairing the formation of the eIF4F complex, necessary for the initiation of 5'cap-dependent translation. Consequently, mTORC1 inhibitory phosphorylation of 4E-BPs releases eIF4E to promote translation of mRNAs. Conversely, mTORC1-mediated phosphorylation of S6K1 at the hydrophobic residue Thr389 stimulates its kinase activity to further target and activate eIF4B, a positive regulator of 5' cap-dependent translation (Brunn et al., 1997; Hara et al., 1997). Generally, the relevance of mTORC1 in sustaining the protein synthetic capacity of the

cell was demonstrated by a 65% decrease in ^{35}S-Cys/Met incorporation into proteins observed upon acute mTORC1 inhibition. Particularly, this effect was mediated by the selective suppression of a specific class of transcripts, defined by the so-called TOP motif in their 5'UTR, which encodes for ribosomal components and translation factors. (Hsieh et al., 2012; Thoreen et al., 2012). This aspect will be discussed further on.

Along with proteins, mTORC1 also drives the biosynthesis of nucleotides to sustain the processes of DNA replication and rRNA synthesis. In this case, activation of the transcription factor ATF4 and its downstream target MTHFD2 leads to *de novo* purine synthesis (Ben-Sahra et al., 2016), while S6K1-mediated phosphorylation of CAD enzyme supports pyrimidine biosynthesis (Ben-Sahra et al., 2013a).

As the cell grows, lipids are needed for generation of new membranes. In this regard, mTORC1 supports the process of lipogenesis by promoting the transcriptional activity of two transcription factors involved in adipogenesis, the sterol regulatory element binding protein 1/2(SREBP1/2) and the peroxisome proliferator-activated receptor-γ (PPAR-γ). Under low sterol levels, mTORC1 inhibits SREBP1/2 inhibitor Lipin-1, therefore allowing transcription of genes for lipid and cholesterol synthesis. (Horton et al., 2002) Inhibition of mTORC1 by Rapamycin, instead, has been observed to decrease the activity of PPAR-γ, a transcription factor necessary for *de novo* adipogenesis and lipid homeostasis (J. E. Kim & Chen, 2004).

All the anabolic processes just mentioned require a considerable amount of energy availability. To sustain the high energy demand of growing cells, mTORC1 also increases the production of mitochondria to boost the generation of ATP, and this is executed by sustaining the formation of the YY1-PGC1α transcriptional complex, responsible for the expression of mitochondrial biogenesis transcripts. This was evident in skeletal muscle cells, where Rapamycin treatment inhibited the interaction between the transcription factor YY1 and its co-activator PGC1α, thus leading to reduced mitochondrial gene expression and oxygen consumption rate (Cunningham et al., 2007).

In order to optimize the utilization of the anabolic products generated, mTORC1 also inhibits a catabolic process such as autophagy by phosphorylating the early effectors of autophagy induction ULK1 and ATG13 (Ganley et al., 2009; Hosokawa et al., 2009) and by phosphorylation of UVRAG, responsible of lysosome trafficking and fusion to the endosome (Y. M. Kim et al., 2015). On the contrary, under mTORC1 inhibition, the cell enters in a "energy-save" mode for which necessary macro components such as amino acids are recovered from recycled proteins. In this case, shut down of mTORC1 removes the break in autophagosome formation and promotes lysosomal biogenesis to favor the

breakdown of proteins into amino acids (Settembre et al., 2012). This mechanism ensures mTORC1 reactivation after starvation and it has been described to be crucial to maintain cell viability in conditions of mTORC1 inhibition upon nutrient starvation. The major source of proteins utilized comes from ribosomal components, through the receptor for selective "ribophagy" NUFIP1 (nuclear fragile X mental retardation-interacting protein 1) (Wyant et al., 2018).

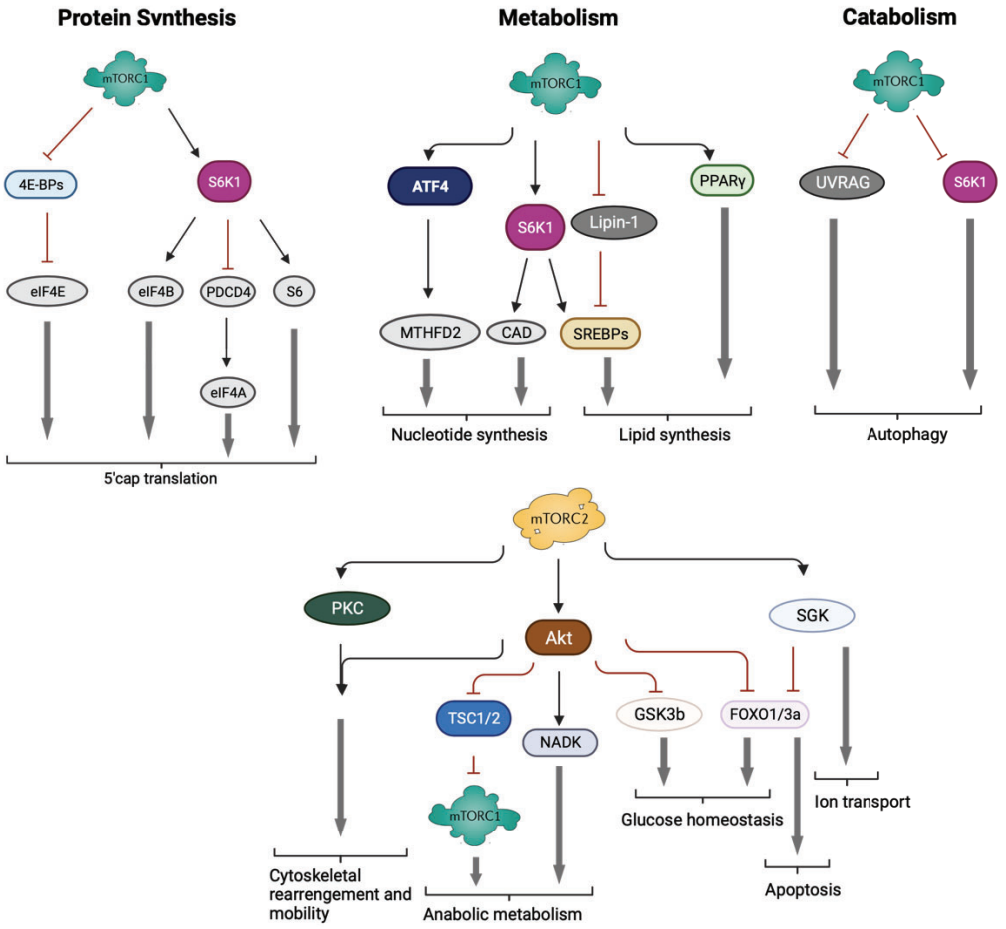

Fig. I-9 | **Downstream pathways activated by mTORC1 and mTORC2** Active mTORC1 triggers a downstream anabolic program for the production of proteins, nucleotides, lipids, and other macromolecules. Simultaneously, inhibits catabolic processes such as autophagy. mTORC2 cross-talks with mTORC1, regulates cytoskeleton organization, promotes a metabolic program of resistance to nutritional stress and suppress apoptosis, as well as support ion transport. Figure adapted from (G. Y. Liu & Sabatini, 2020)

The activity of mTORC2, instead, is mainly circumscribed to cellular cytoskeleton organization (Fig. I-9). Responsible of this effect is the mTORC2 target phosphorylation site on protein kinase Cα (PKCα), although the mechanistic is not completely understood

(Larsson, 2006). Regulation of cytoskeletal organization have been demonstrated to affect also cancer cells motility and metastatic potential with multiple evidences showing that impairment of mTORC2 signaling negatively affects cancer cells metastasis (Morrison Joly et al., 2017; Schmidt et al., 2018). Other targets of mTORC2 are the ion-transporter regulator serum- and glucocorticoid-induced protein kinase 1 (SGK1)(García-Martínez & Alessi, 2008) and AKT. As mentioned above, AKT promotes the cellular response to insulin and proliferation, as well as it represents a crosstalk between mTORC1 and mTORC2 (Sarbassov et al., 2005). Additionally, mTORC2-mediated phosphorylation of AKT is indispensable for AKT-targeting of forkhead-box FOXO1/3a transcription factor, which coordinate transcriptional programs of cellular resistance to nutritional stress (Webb & Brunet, 2014). Other targets of AKT, not necessarily dependent on mTORC2, are the glycogen synthase kinase 3b (GSK3b), which promotes cancer cells fitness by suppression of apoptosis and regulation of glucose homeostasis and mSIN1, self-inhibitor of mTORC2 (Humphrey et al., 2013).

2.1.4 mTORC1 in cancer

As observed when discussing the role of MYC as tumor driver, cancer cells are characterized by aberrant growth and proliferation, which is sustained by large amounts of proteins, lipids, nucleotides and energy availability. In order to meet the increased demand of anabolic compounds, cells undergo a metabolic reprogramming that is driven by hyperactive mTOR as well as metabolic alterations that ultimately impact mTOR signaling (reviewed in (Mossmann et al., 2018). The crosstalk between sustained mTOR activity and oncogenic metabolism creates an inter-dependency that ensures tumor survival even under stressful conditions.

Hyperactivation of mTOR signaling is observed in 80% of cancers and is usually driven by mutations in the upstream signaling pathways that converge on mTOR, rather than mutations of the mTOR kinase itself (reviewed in (Menon & Manning, 2009). Among them, PI3K/Akt-activating mutations, gain of function mutation in Ras signaling and loss of tumor suppressor phosphatase and tensin homolog (PTEN), which is a phosphatase for PIP3. All of them result in augmented levels of PIP3 which directly activates mTORC2 and sustains mTORC1 activity by AKT-mediated phosphorylation of TSC2. AKT, in addition, leads to phosphorylation and dissociation of PRAS40 from RAPTOR, thus relieving an inhibitory constraint on mTORC1 activity (Haar et al., 2007). Similarly, RAS-related kinases ERK and RSK phosphorylates TSC2 and PRAS40 to induce Rheb and RAPTOR activation (reviewed in (L. C. Kim et al., 2016).

The resulting hyperactivation of mTOR mediates increased uptake of amino acids by inducing the expression of the transporter for essential amino acid arginine (Abdelmagid et al., 2011; Lu et al., 2013) or by promoting glutaminolysis for the generation of glutamine (Csibi et al., 2014), necessary as nitrogen and carbon donor in anaplerotic replenishment of tricarboxylic acid cycle (TCA) (L. Yang et al., 2017). On the other way around, colorectal, liver and lung cancer overexpress the transporter for the essential branched chain amino acids (BCAAs) leucine, isoleucine and valine, resulting in increased mTORC1 activation (reviewed in (Mossmann et al., 2018).
One of the hallmarks of cancer cells is the switch from oxidative phosphorylation to aerobic glycolysis. Although this feature represents an inefficient way for tumor cells to obtain energy in form of ATP, it is preferred as it represents a source of carbon units for anabolic processes (Heiden et al., 2009). In this context, aberrant activation of mTORC1 ensures the proper refurbishment of glucose by increasing glucose transporters expression and glycolytic enzymes (Düvel et al., 2010).
In addition, mTORC1 sustains nucleotides generation by promoting the pentose phosphate pathway (PPP) and enzymes involved in purine and pyrimidine metabolism, as well as it supports lipids production by stimulating SREBP1/2 transcription factor (reviewed in (Mossmann et al., 2018).

Apart from the vast impact over metabolism and protein synthesis, mTOR also affects the tumor microenvironment by promoting two fundamental processes for the establishment of tumors and their maintenance: angiogenesis and tumor evasion. Generation of new blood vessels is promoted by mTORC1-mediated increase in translation of HIF transcription factor mRNA, which promotes vascular endothelial growth factor (VEGF) secretion, and by mTORC2-mediated boost of endothelial cells proliferation (Guba et al., 2002; Viñals et al., 1999). Immune evasion, instead, is developed by tumoral cells through several mechanisms that ultimately suppress the ability of immune cells to recognize and kill tumor cells. Hyperactive AKT-mTORC1 signaling induces PD-L1 expression in cancer cell membranes to inhibit CD8+ T cell cytotoxic activity (Lastwika et al., 2016), while mTORC2 impacts the differentiation of Th2 CD4+ T cells (Heikamp et al., 2014).

2.1.5 mTOR inhibitors

Considering the role just described of mTOR in tumorigenesis and tumor maintenance, huge work has been invested to identify molecules capable of inhibiting mTOR activity.

mTOR inhibitors fall mainly in two classes: the allosteric inhibitor rapamycin and its derivatives, which block only mTORC1, and the ATP site-competitive mTOR inhibitors that block the catalytic activity of both mTORC1 and mTORC2.

"Rapalogs" (rapamycin analogs) such as everolimus are clinically approved drugs for the treatment of various cancers but their efficacy strongly depends on the tumor and / or mTOR mutations (Kwiatkowski & Wagle, 2015). This small range of application is justified when considering the ability of mTOR to drive tumoral responses through a wide variety of effectors and that mTOR inhibitors can also induce compensatory pathways. Also, rapalogs inhibit only partially the 4E-BPs phosphorylation depending on the cell context, thus failing to inhibit pro-survival mechanisms dictated by mTORC2 (Tabernero et al., 2008).

The mechanism of action behind second generation of inhibitors such as Torin1, PP242, Ku0063794, instead, relies on competition with ATP to occupy the catalytic site of the mTOR enzyme (Thoreen et al., 2009). Although they inhibit both mTORC1 and mTORC2 complexes, by now, they represent mainly an advantage in research application rather than in clinical. This is mainly due to inconsistent responses in clinical trials and the limit of drug toxicity, as well as the insurgence of resistance mechanisms after prolonged treatment given by mTOR-indipendent activation of AKT (Janku et al., 2018).

2.2 LARP1

The RNA binding protein LARP1 is a highly conserved protein first described in *Drosophila Melanogaster* required for oogenesis, spermatogenesis, segregation of mitochondria and cell cycle progression (Blagden et al., 2009; Chauvet et al., 2000; Ichihara et al., 2007; Zhang et al., 2019). Nonetheless, recent findings have connected this protein also to ribosome biogenesis and protein synthesis processes in light of its role as a nexus between mTORC1 and 5'TOP mRNAs expression (reviewed in (Berman et al., 2020).

2.2.1 LARP1 family and structure

LARP1 belongs to the family of La-related proteins, which all share an "La module" composed of two RNA binding domains, the "La motif" (LaM) and an "RNA recognition motif" (RRM)(Bousquet-Antonelli & Deragon, 2009). Five subfamilies of LARP proteins exists (LARP1, LARP3, LARP4, LARP6 and LARP7) and in humans the duplication of LARP1 and LARP4 genes has led to the generation of LARP1B and LARP4B genes, for a total of 7 LARP subfamilies (Deragon, 2021) (Fig. I-10). LARP3 is the ancestral nuclear

La protein and, as the nuclear LARP7, protects a subset of mRNAs characterized at the 3' terminus by an oligo(U) sequence (Markert et al., 2008; Teplova et al., 2006). All the other LARPs are instead cytoplasmatic with diversified functions. LARP1 and LARP4 only share the ability to stabilize mRNAs with long 3' poly(A) tail, thanks to a sequence in the La module called "PAM2 motif" that recognizes the MLLE domain in the poly(A) binding protein (PABP) (Aoki et al., 2013; R. Yang et al., 2011).

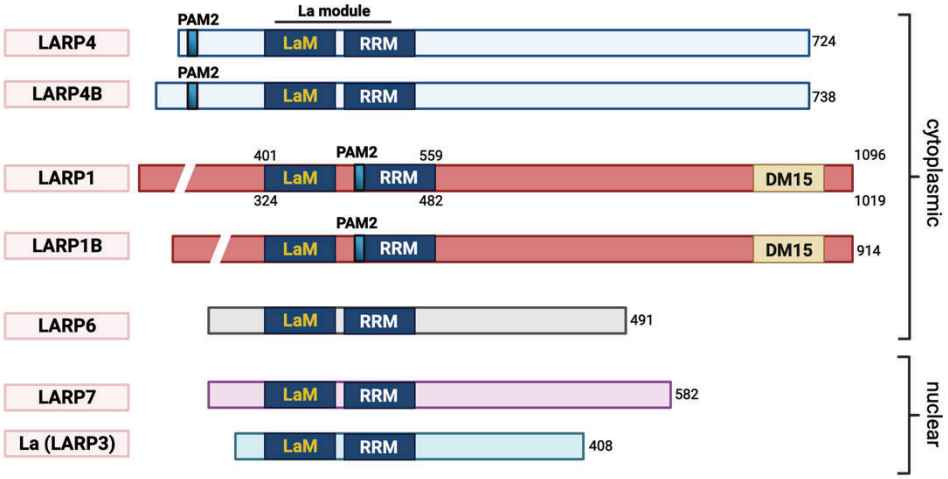

Fig. I-10 **| Schematic representation of LARP human proteins** Functional modules of LARP proteins are represented and the numbering of amino acids. For further details, see the text. Figure adapated from (Mattijssen et al., 2021)

With respect to LARP1, the two paralogues genes found in human genome are the canonical LARP1 gene located at chromosome 5q33.2 encoding for a 1096 amino acid protein and LARP1B at 4q28 encoding for a 914 amino acid protein (reviewed in (Bousquet-Antonelli & Deragon, 2009) . However, LARP1 is the most expressed paralogue in a wide array of tissues and at a large extent the current knowledge about the molecular events assisted by these two paralogues is centered on LARP1. In addition, two variants of LARP1 have been described, produced by two alternative promoters that generate two LARP1 isoforms, differing only for the first exon. A short-isoform (SI) of 1019 amino acids, that lacks the first 77 residues, and a 1096 long isoform (LI), with no clear function ascribed to this N-terminal variant (Schwenzer et al., 2021). All isoforms encoded by LARP1, as well as LARP1B, are characterized at the C-terminus by an RNA binding domain termed "DM15", which differentiate LARP1 and LARP1B from the other LARP members and is responsible for the specific recognition of the TOP motif of 5'TOP mRNAs (Tcherkezian et al., 2014).

5’TOP mRNAs are a class of transcripts characterized by an invariant cytosine at the Trascriptional Start Site (TSS) followed by an oligopyrimidine sequence (Meyuhas & Kahan, 2015). This family of transcripts mainly encode for the components of the protein synthetic machinery, including almost all the ribosomal proteins and many translation factors, and their expression in terms of cognate proteins is finely regulated by mTORC1. The TOP motif, indeed, acts as a potent translational regulatory element that has been shown to sense the multiple signaling metabolic cascades transmitted to mTORC1 (Fonseca et al., 2014). Pharmacological inhibition of mTOR (Jefferies et al., 1994; Terada et al., 1994), as well as deprivation of amino acids (Damgaard & Lykke-Andersen, 2011; Tang et al., 2001), growth factors (Stolovich et al., 2002), oxygen (Miloslavski et al., 2014) and hormones (Patursky-Polischuk et al., 2009) inhibit mTORC1 and consequently the 5’TOP mRNAs translation, therefore restraining the biosynthesis of the constituents of the translational machinery. In the search for molecular players that could connect the metabolic status of the cell and ribosome biosynthesis, LARP1 emerged as the link between mTORC1 signaling and 5’TOPs translation. Indeed, LARP1 is a phospho-target of mTORC1 (as described below) and a selective binder of the TOP element (reviewed in (Berman et al., 2020; Stavraka & Blagden, 2015)

As commented, the binding to the TOP motif is mediated by the DM15 domain of LARP1. The crystal structure of the complex DM15 domain-TOP motif resolved by the Berman’s group showed that the DM15 motif can bind the 5’ 7-methylguanosine cap (m^7G) and the first few nucleotides of TOP motif thanks to a cap-binding pocket generated by a region of the DM15 able to assume a 3_{10}helix conformation (Lahr et al., 2017). Recently, by *in vitro* assays, the same group observed that the recognition of the TOP motif is not exclusive of the DM15 region. The La module can indeed bind the TOP motif in a cap-independent manner while also binding poly(A) tail, either of the same transcript leading to its circularization, either of two different mRNAs to form multimeric complexes (Al-Ashtal et al., 2021).

Finally, it is important to underline that although LARP1 is generally referred to as a 5’TOP RNA binding protein, the analysis of its RNA interactome in HeLa cells revealed that 5’TOP mRNAs were not the most abundant targets, accounting for only the 4% of the 3000 mRNAs of its RNA interactome (Mura et al., 2014).

2.2.2 LARP1 regulation by mTORC1

As mentioned before, TOP mRNAs translation is extremely sensitive to mTORC1 activity. Eventually, this last decade of studies has revealed two major players belonging to the phospho-proteome of mTOR regulating 5'TOP translation. Knockout of eIF4E-BP1 and 2 desensitizes the translational inhibition of 5'TOP upon mTOR inhibition, however the mechanistical evidence of how this occurs in the cell is still elusive (Thoreen et al., 2012) as well as how pervasive the absence of 4E-BPs is on the non-TOP translatome (Dowling et al., 2010). Parallel studies have identified LARP1 as the 5'TOP RNA binding protein able to selectively connect mTOR signaling and their translational status (Tcherkezian et al., 2014).

The first evidence of LARP1 as a substrate of mTORC1 came from two independent proteome-wide phosphoscreen studies (Hsu et al., 2011; Yu et al., 2011). By quantifying the phospho-peptides changes in a short time window of mTOR pharmacological inhibition (30 minutes) or by genetic upregulation of mTORC1 signaling, they revealed LARP1 as a novel mTOR phosphorylation target, in addition to the canonical 4E-BP1 and 2 and S6K1. Successively, a new phospho-proteomic study showed the direct phosphorylation of LARP1 by mTORC1 at serines in position 689 and 697, with respect to the 1019 amino acid LARP1 isoform(Kang et al., 2013). Hong *et al.* confirmed LARP1 as a direct substrate of mTORC1 *in vitro* and, by using a GST-LARP1 fragment carrying the double mutation on S689A and T692A, they observed that the LARP1 phosphorylation was lost, thus indicating that at least one of these two sites are phosphorylated by mTORC1 (Hong et al., 2017). Recently, Jia *et al.*, by dissecting the LARP1 protein into several different clusters of putative mTORC1 phosphorylation sites, identified 26 new rapamycin-sensitive phospho-residues on LARP1. Some of them, including the previously described S689, S697 and T692, were important for LARP1 association with RAPTOR in condition of RNAse A treatment, however it is not known whether this interaction is altered when the ribonucleoprotein complexes are maintained intact (no RNAse A treatment). Others, such as S747, T768, S770, S772, S774, S776, T779, S784, T788, S791 were important for LARP1 association to the 5' UTR of the 5'TOP mRNA RPS6 *in vitro* (Jia et al., 2021).

However, how does LARP1 impact 5'TOP mRNAs translation in response to mTOR signaling? With a study aimed at clarifying the mechanism behind mTORC1 ability to differentially regulate both global and selective translation (Hsieh et al., 2012; Thoreen et al., 2012), the Roux laboratory identified LARP1 as a novel RBP whose association to the 5' cap was subordinated to mTOR activity. More importantly, they observed that

knockdown of LARP1 was partially mimicking the effect of mTORC1 inhibition over protein synthesis, by reducing the assembly of actively translating ribosomes, namely polysomes, while increasing the amount of non-translating 80S monosome. In contrast to mTORC1 inhibition, however, LARP1 depletion showed to reduce the engagement in translation specifically of 5' TOP mRNAs, while the levels of non-5'TOP in polysomes remained unaltered (Tcherkezian et al., 2014). This study recognized for the first time the downstream role of LARP1 in mTORC1-mediated targeted regulation of 5'TOP mRNAs, however several evidences were not reproduced by other studies. Efforts from other groups have followed to better identify the implications of LARP1 in 5'TOP mRNAs translational regulation and some controversies have arisen around it. The observation that mTORC1 inhibition leads to an accumulation of RP mRNAs in non-polysomal fractions under the guidance of LARP1 while depletion of LARP1 rescues the translation of these transcripts in the same mTOR inhibitory context (Fonseca et al., 2015), have encouraged the interpretation of LARP1 as a negative regulator of RP mRNAs, downstream of mTORC1. The efforts made in our laboratory have implemented that information and offered a different view over the regulation of LARP1 by mTORC1 and its regulation of 5'TOP mRNAs. In a first study, we demonstrated that the LARP1 protein selectively binds and stabilizes 5'TOP mRNAs in a complex with the small ribosomal subunit 40S. The formation of the so-called "40S-LARP1" complex is a necessary event for the mTORC1-mediated control of 5'TOP mRNAs. Acute inhibition of mTORC1, indeed, stimulates the formation of the complex, leading to their accumulation with the non-polysomal pool of ribosomes in a stable conformation. However, in a following work, we also identified the biological implication of this mechanism. Poor nutritional and energetic conditions represent an unfavorable situation for the high-energy demanding process of cellular growth. Therefore, limitation of anabolic and energy-consuming processes, such as ribosome biogenesis and protein synthesis, is a survival strategy that the cell activates by means of mTORC1 inhibition. According to this logic, we observed that chronic restriction of mTORC1 signaling, mediated either by pharmacological inhibition of mTORC1 or serum/amino acids depletion, reduces protein synthesis. However, in contrast to the view of LARP1 as negative regulator of RP mRNAs expression, we observed that its depletion did not rescue protein synthesis under mTORC1 inhibition. On the contrary, chronic inhibition of mTORC1 leads to 5'TOP mRNAs levels preservation only in the presence of LARP1. The 40S-LARP1 complex associated with 5'TOP mRNAs in non-polysomal fractions protects them from ribophagy-mediated mRNAs degradation. Actually, although the translocation to the 40S and 80S

non-translating fractions inhibits 5'TOP mRNAs translation, we proved this procedure to be programmed to resume cell growth once the stimuli that inhibit mTORC1 are halted. Indeed, the pool of transcripts translationally inactive created by the 40S-LARP1 complex represents a reservoir of ribosome biogenesis- and protein synthesis-related mRNAs that will be rapidly translated once mTORC1 is reactivated. Such ability to secure the cellular anabolic potential, conferred by the 40S-LARP1 complex, assures cell resistance to restrictive energetic and nutritional conditions (Fuentes et al., 2021). This model is also in line with recent findings by Ogami et al. according to which LARP1 preserves the long-poly(A)-tailed TOP mRNAs under chronic mTOR inactivation to facilitates accelerated reconstitution of translation once mTORC1 is reactivated (Ogami et al., 2022).

Nonetheless, a recent work from other LARP1-specialized researchers have proposed a reconciling model of mTORC1-mediated regulation of 5'TOP mRNAs by LARP1. (Berman et al., 2020). In this model, under mTORC1 inhibition the dephosphorylated LARP1 would be able to bind the 5'cap of TOP mRNAs, through the DM15 motif. In doing so, it would compete with eIF4E for the 5' cap thus impeding eIF4F translation initiation complex formation. Upon mTORC1 activation, instead, phosphorylation of the DM15 domain leads to its dissociation from the 5' cap while, in parallel, the La module keeps binding the poly(A) tail to circularize the mRNA and facilitate rapid translational recovery.

2.2.3 LARP1 in cancer

LARP1 is found overexpressed in a wide variety of cancers when compared to the respective normal tissues. In cervical cancer, levels of LARP1 protein as measured by immunohistochemistry positively correlate with the disease progression from pre-invasive to invasive stages. In hepatocellular carcinoma (HCC), the overexpression of LARP1 correlates with a 35% increased risk of death, with tumor size and overall survival rate. In prostate cancer, instead, knockdown of LARP1 has shown to attenuate tumor migration (Kato et al., 2015), while in colorectal cancer it has been shown to cover its oncogenic role by promoting tumor cells proliferation (Ye et al., 2016). Further demonstration of LARP1 as a as promoter of cell proliferation, migration and invasion has been also achieved in non-small cell lung cancer (Z. Xu et al., 2017). Summing up, all the clinicopathological data derived from the above-mentioned studies shows that high levels of LARP1 protein in a tumor are associated with more aggressive cancer growth and worse patient outcome.

Although accruing evidences are pointing at elevated LARP1 levels as a bad prognostic marker in many different cancers, the mechanistic role of LARP1 in cancer initiation and maintenance has not been fully investigated. A study from the Blagden laboratory showed that the overexpression of LARP1 in HeLa cells leads to increased cell migration, invasion, EMT and *in vivo* tumorigenesis (Mura et al., 2014). Using the same cell line, they also showed that the interactome of LARP1 is not confined to the protein translation-related family of 5'TOP mRNAs but extends to a broader set of transcripts involved in actin remodeling, focal adhesion and extracellular matrix interactions (Mura et al., 2014).

3. Ribosome biogenesis and protein synthesis

3.1 Ribosome Biogenesis

By translating mRNAs into proteins, the ribosome is the cellular machinery in charge of decoding the genetic information. For years the ribosome has been considered a relatively “simple machine”, executing its function with low control and selectivity for the transcripts engaged in translation. Over the last decade, a blooming of technological advances has moved the field forward by disclosing a whole underworld of translational programs operated by specific ribosomal mechanisms. In this regard the discovery of ribosome heterogeneity with respect to its protein composition has positioned the translational machinery at a regulatory node in executing the genetic program of the cell (reviewed in (Genuth & Barna, 2018).

As the ribosomes represent the protein synthetic factories of the cell, growing cells strongly rely on the process of ribosome biogenesis (RiBi). It is estimated, indeed, that mammalian cells contain from 1 to 10 million ribosomes, with a production rate of 3000 ribosomes per minute in highly dividing cells like tumoral ones (M. Wang et al., 2015). Nevertheless, RiBi is a complex and multi-step process, characterized by high energy expenditure, whose activation is tightly controlled by growing and energetic signaling pathways.

3.1.1 Ribosome components

Ribosomes are constituted by two independent subunits that join in the presence of an mRNA to form the 80S ribosome, as defined by its sedimentation rate in Svedberg unit (S). The small ribosomal subunit 40S is composed of 33 ribosomal proteins (RPs) and the 18S ribosomal RNA (rRNA), while the 60S large ribosomal subunit is made up of 47 RPs and 3 rRNAs, the 28S, 5S and 5.8S (Fig. I-11). The major functional domains of the ribosome essential for mRNA translation are distributed between the two subunits. The 40S subunit harbors the decoding center, designed to match the mRNA codons with the cognate tRNA anticodons. In addition to this, the 40S subunit, associated with a specific set of eukaryotic translational initiation factors (eIF), forms the 43S Pre-Initiation Complex (PIC) that has the ability to unwind and scan the mRNA to find the AUG start codon (48S complex). The peptidyl transferase center (PTC) of the 60S subunit instead catalyzes the peptide bond formation within the nascent polypeptide chain that emerge from the ribosome through the peptide exit tunnel (PET) (Klinge & Woolford, 2018).

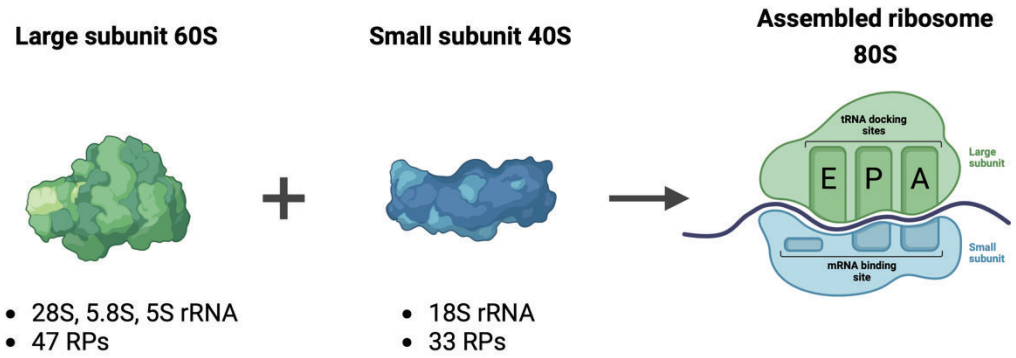

Fig. I-11 **| Ribosome components** rRNAs and RPs are assembled to form the small and large subunits of ribosomes. Binding to the mRNA allows the formation of a functional 80S monosome that allows protein synthesis. The tRNA aminoacyl site (abbreviated A) serves to bind upcoming tRNA loaded with a new amino acid. The peptidyl site (abbreviated P) catalyzes the peptide bond formation and hold the tRNA with the growing polypeptide chain. The exit site (abbreviated E) releases the empty tRNA. Figure created with Biorender.

Because of their relevance in mRNA translation, these domains have been highly conserved during evolution and are referred to as the core ribosomal structure. However, several studies conducted over recent years, have evidenced heterogeneity in ribosomal composition, both at the RP and rRNA levels (reviewed in (Genuth & Barna, 2018). Measuring the abundance of 15 out of the 80 core RPs in actively translating ribosomes from mouse embryonic stem cells (mESCs), Shi *et al*. observed that six RPs were sub-stoichiometric while four of them were present in only 60%-70% of ribosomes, thus concluding that translating ribosomes can indeed lack at least one of the core RPs in a single cell type. The same study also elucidates the implications of ribosome heterogeneity in regulation of translation, such as preferential selectivity towards a particular subgroup of mRNAs (Shi et al., 2017). As an example, translating ribosomes enriched for the large ribosomal protein RPL10a, increase the translational output of a cluster of transcripts involved in processes associated with cell growth, while these ribosomes appear to deplete the loading of transcripts that express opposing processes such as stress response and cell death. Same principle was observed for RPS25-enriched ribosomes, where the pool of translated transcripts is specific and not overlapping with RPL10a-enriched ribosomes. Accordingly, a previous study observed that mutations in the large ribosomal protein RPL38, selectively regulating the translation of the developmental Hox mRNAs, lead to defects in tissue patterning of the embryo while global translational is unaltered (Kondrashov et al., 2011). Heterogeneity of

ribosomes is observed also at the levels of rRNA. Variation in rDNA copy number and sequence variation of rRNAs have been observed between human individuals, as well as intra-variation in rRNA alleles with tissue-specific expression in mice which are functionally incorporated into ribosomes (Parks et al., 2018). Interestingly, the core ribosomal structure can also interact with an outer shell of proteins other than RPs that could confer specific functions. Such "ribo-interactome" can comprise mRNA binding proteins, mRNA/tRNA modifiers, RNA helicases, and regulators of cell cycle and metabolism such as the glycolytic enzyme PKM2 which is preminently enriched in ribosomes associated with the endoplasmic reticulum (Simsek et al., 2017). Overall, these findings point the ribosome as an active post-transcriptional regulator of mRNAs expression and could underlie the clinical manifestations of human pathologies associated with the mutations in ribosome components. The so-called "ribosomopathies", such as *5q*$^{-}$ syndrome and the Diamond-Blackfan anemia (DBA), are indeed disorders with haploinsufficient expression of RPs or RiBi-related proteins that affect only a specific subset of cell types/tissues, commonly the hematopoietic system. In this regard, ribosome heterogeneity explains how a mutation affecting ribosomes expressed in all the cells ultimately manifests a disease phenotype only in a certain tissue type. As mentioned, ribosomes can express a preference for particular mRNAs. Mutations affecting ribosomes will therefore influence the intrinsic ribosomal preference resulting in an altered proteome for which only some cell types are sensitive (reviewed in (Kampen et al., 2020)

3.1.2 Ribosome Biogenesis

As mentioned, the production of ribosomes is a multi-step process that requires the involvement of hundreds of non-ribosomal factors, the ribosome biogenesis factors (RBFs), which serve as scaffold, rRNA processing, modification and chaperone factors (reviewed in (Dörner et al., 2023). In eukaryotes RiBi is compartmentalized, occurring between the nucleolus, the nucleus and the cytoplasm.

Ribosome synthesis starts with the transcription of a polycistronic pre-rRNA in the nucleolus by RNA polymerase I (Baßler & Hurt, 2019). Hundreds of rDNA tandem repeats are indeed organized in nucleolar organizing regions (NORs) (Caburet et al., 2005), which are densely packed in the fibrillar center (FC) of the nucleolus and are present in acrocentric chromosomes 13, 14, 15, 21 and 22 (Birch & Zomerdijk, 2008; Boisvert et al., 2007; Gibbons et al., 2015) (Fig. I-12). Importantly, not all the NORs are

active in a cell and their activation may be tissue-specific. Each rDNA unit is defined by an intergenic space (IG) of 30kb and a rRNA coding region of 13kb. The rRNA coding region contains the sequences for 18S, 5.8S and 28S expression, separated by two internal transcribed spacer sequences (ITS1 and ITS2), and flanked by 5' and 3' external transcribed spacer sequences (5' and 3' ETS) that contain regulatory sequences such as promoter and enhancers from which the transcription can start (Goodfellow & Zomerdijk, 2013).

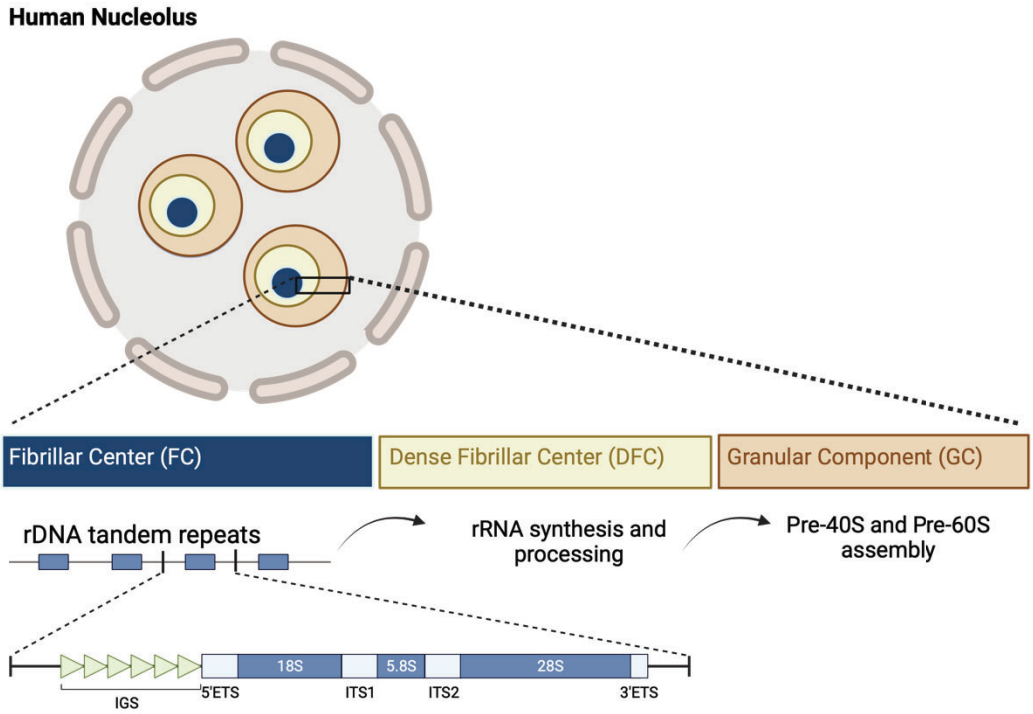

Fig. I-12 **| Organization of nucleolus and rDNA** Human nucleolus consisting of three functional subcompartments: fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC). rDNA tandem repeats are transcribed in the DFC to generate the 47S precursor rRNA. Assembly of ribosomal proteins for the formation of pre-40S and pre-60S subunits generates the GC. Schematic of rDNA is also represented. Figure created with Biorender.

RNA Pol I generates a polycistronic precursor rRNA termed 47S that as early as is transcribed, is surrounded by ribosome assembly factors (RAFs) recruited by the 5' ETS. This early phase of ribosome assembly takes place in the dense fibrillary center (DFC) of the nucleolus and is followed by further recruitment of other proteins among which 20 of the final 33 ribosomal proteins that will constitute the 40S particle (reviewed in (Hurt et al., 2023). Altogether, this network of factors forms the blueprint for the small subunit

and is therefore referred to as the small subunit (SSU) processome. It serves as three-dimensional scaffold to support the correct folding and maturation of 18S rRNA and constitutes the granular component (GC) of the nucleolus.

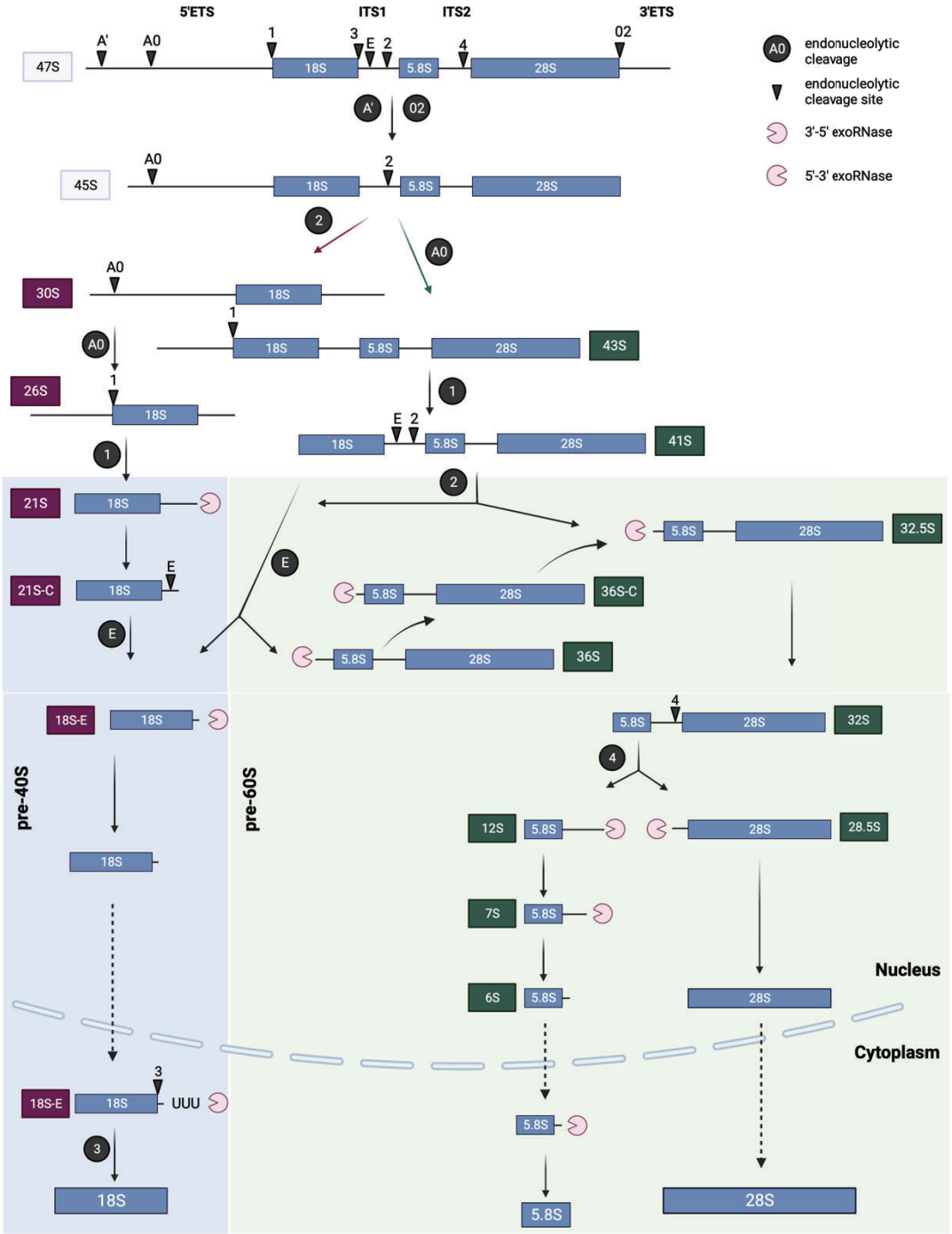

Fig. I-13 | **47S pre-rRNA processing in human cells** Transcribed by the RNA pol I, the 47S pre-rRNA encodes three rRNAs out of four. The sequences of 18S, 5.8S$_{S/L}$ and 28S are flanked by external (5'-ETS, 3'ETS) and internal transcribed spacers (ITS1, ITS2), which are progressively cut by endo- and exonucleases. Depending on the relative kinetics of endoribonucleolytic

cleavage, various rRNA processing pathway may occur. In the main maturation pathway (pre-rRNAs processed in violet), the pre-45S is cleaved at site 2. Cut at site A0 is usually less common (pre-rRNA processed in green are therefore less abundant). Adapted from (Aubert et al., 2018)

Maturation to 18S rRNA involves the trimming of the 47S pre-rRNA at cleavage sites contained in both ETS and ITS (Fig. I-13). First, two exo-nucleolytic cleavages at site A' in 5'ETS and site 2 in 3'ETS rapidly convert the 47S into 45S pre-rRNA. Two alternative routes then follow. The endo-nucleolytic cleavage of 45S pre-rRNA at site 2 of the ITS1 will generate the 30S pre-rRNA, precursor of 18S, and the 32.5S pre-rRNA containing both 5.8S and 28S. Removal of the 5'ETS and sequential trimming of the 3' end will lead to the maturation of the 30S to the 18S-E precursor. Alternatively to the cut in the ITS1, the 45S pre-rRNA can be trimmed at site A0 generating the 43S pre-rRNA, whose remaining 5'ETS is successively removed for the formation of 41S pre-rRNA. In this case, two further cleavages at ITS1 will separate the 18S-E precursor rRNA from the 36S, precursor of 32.5S pre-rRNA. The 18S-E pre-rRNA still contains an 80-nt stretch of ITS1 that will be further shortened by poly-(A)-specific ribonuclease (PARN). Together with PARN release, the majority of RAFs that form the SSU processome are dismantled to finally form the pre-40S. Subsequently, recruitment of late nuclear pre-40S factors to the nuclear pores allows the transport of the pre-40S particle from the nucleus to the cytoplasm. Here, the remaining pre-40S factors are dissociated, the missing ribosomal proteins of the small subunit (S-proteins) are incorporated and a last endonucleolytic cleavage operated by NOB1 separates the ITS1 from the 18S to finally obtain the mature small 40S subunit (Hurt et al., 2023).

Maturation of 5.8S and 28S rRNA for the formation of the large ribosomal subunit stems from the 32.5S precursor, which recruits pre-60S assembly factors and ribosomal proteins to initiate the assembly of the 60S large ribosomal subunit. Here, the remaining ITS1 stretch is removed by the exonuclease XRN2 which forms the 5' end of the 5.8S rRNA, while an endo-nucleolytic cleavage at ITS2 separates the precursor 28.5S. 28S rRNA is ultimately formed by XRN2 activity at its 5'end in the nucleolus. Final steps of 5.8S maturation involve instead a sequence of cleavages at its 3'end that terminates in the cytoplasm with the generation of the short or long 5.8S rRNA ($5.8S_{S/L}$), which are two forms of the mature 5.8S rRNA that differ for the length of the 5'end (reviewed in (Klinge & Woolford, 2018).

The 5S rRNA is instead encoded by 200 hundred rDNA repeats located in chromosome 1 (Gibbons et al., 2015)and is transcribed by RNA polymerase III (RNA Pol III) in the nucleoplasm (Fedoriw et al., 2012). Maturation of 5S rRNA is mediated by the exonuclease Rexo5 in Drosophila, where it cleaves 2-3 nt at the 3' end (Gerstberger et al., 2017). In humans, REXO5 action over 5S rRNA has not been confirmed but since this protein is mainly localized in the nucleolus, it suggests that 5S maturation is mainly nucleolar (Gerstberger et al., 2017; Silva et al., 2017). The 5S rRNA is then assembled with the ribosomal protein of the large subunit RPL5 (Kressler et al., 2012). This complex is then transported to the nucleolus where it binds to the RPL11 to further be incorporated into the 60S precursor.

Notably, the complex 5S rRNA/RPL5/RPL11 constitutes a critical checkpoint in the process of RiBi. It has been showed, indeed, that upon ribosome biogenesis impairment the complex 5S rRNA/RPL5/RPL11 binds the E3 ubiquitin protein ligase HDM2 preventing the ubiquitinylation and degradation of tumor suppressor p53 (Domostegui et al., 2021; Donati et al., 2013; Fumagalli et al., 2009; Sasaki et al., 2011) (Fig. I-14). This regulatory mechanism was termed Impaired Ribosome Biogenesis Checkpoint (IRBC) from the event that redirects the 5S rRNA/RPL5/RPL11 (the Impaired Ribosome Biogenesis Checkpoint complex or IRBC complex) to HDM2 (reviewed in (Pelletier et al., 2017). However, it was successively observed that the IRBC response can also arise upon hyperactivation of RiBi. In our laboratory it was showed indeed that MYC overexpression increases the binding of HDM2 by the IRBC complex with consequent stabilization of p53 and reduction in tumoral cell proliferation (Domostegui et al., 2021; Morcelle et al., 2019).

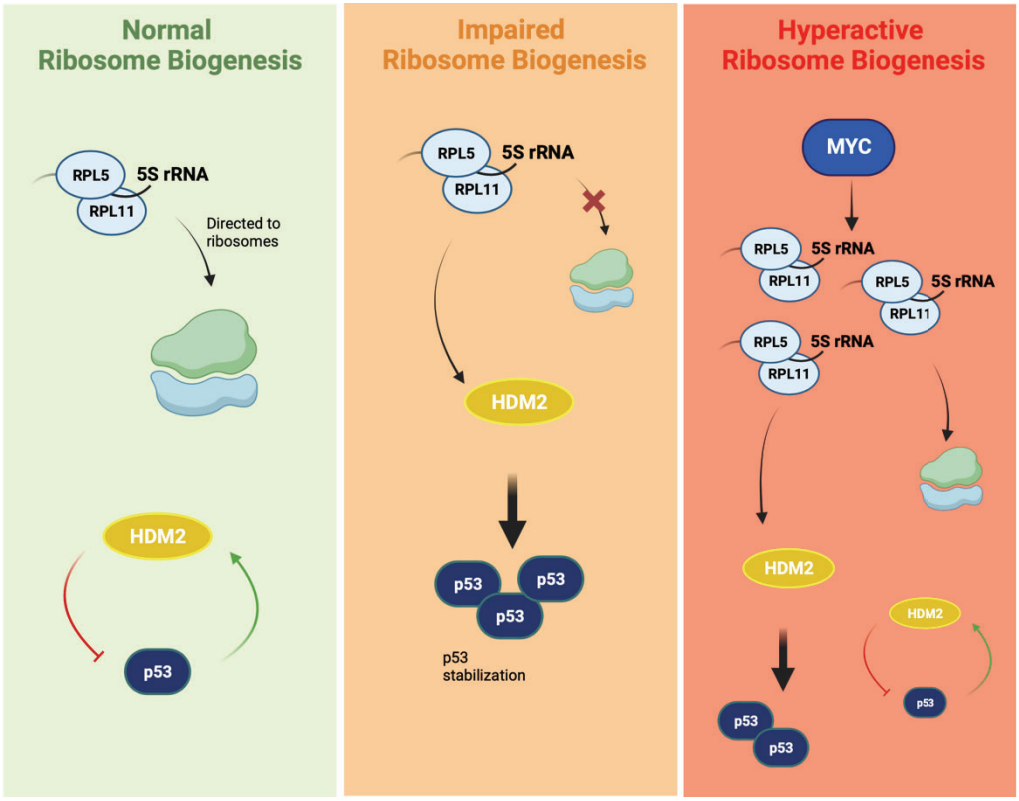

Fig. I-14 **| The impaired ribosome biogenesis checkpoint** Under normal cell growth conditions, the nascent 5S rRNA associated to RPs L5 (RPL5) and L11 (RPL11) is incorporated into newly synthetized ribosomes in the nucleolus. Under these conditions, the E3 ubiquitin-protein ligase HDM2 mediates proteasomal degradation of p53. Upon impairment of RiBi, the nascent RPL5–RPL11–5S rRNA precursor complex is redirected from the incorporation into ribosomes to the HDM2, leading to p53 stabilization. This step identifies the conversion of RPL5–RPL11–5S rRNA precursor to IRBC complex. MYC proto-oncogene deregulation induces an excessive production of the nascent RPL5–RPL11–5S rRNA complex, leading to its incorporation into ribosomes as well as to its accumulation. The latter fraction is responsible for inhibition of HDM2 activity towards p53. Adapted from (Pelletier et al., 2017)

Finally, RP-encoding genes are distributed all across the genome (Kenmochi et al., 1998) and are transcribed in the nucleoplasm by RNA Pol II. Subsequently, RPs mRNAs, characterized by the 5'TOP motif in their 5' UTR, are exported into the cytoplasm where they are translated and then re-imported into the nucleus (Fig. I-15).

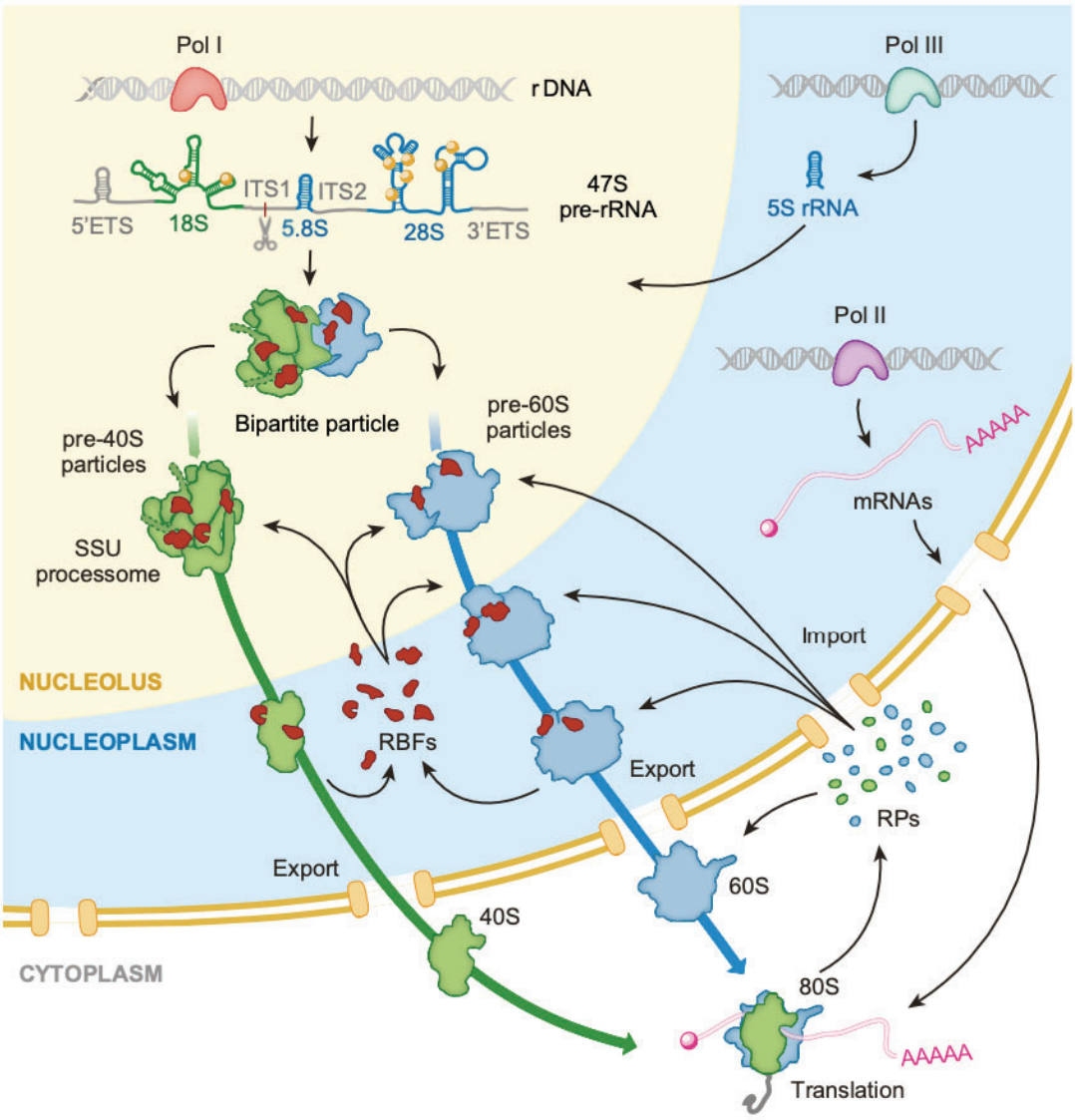

Fig. I-15 | **Overview of the eukaryotic ribosome assembly pathway** The RNA polymerase I transcribes the 47S pre-rRNA in the nucleolus, while the 5S rRNA is transcribed by RNA polymerase III. RPs that will be part of the small and large ribosomal subunits, as well as RBFs, are transcribed by the RNA polymerase II, translated in the cytoplasm and actively imported into the nucleolus to form the SSU processome as well as pre-60S particles. After the processing described in the text, mature 40S and 60S RPs are transported in the cytoplasm where they can join to form the 80S monosome. Adapted from (Baßler & Hurt, 2019)

According to the current knowledge, the production of RPs is not coordinated with the synthesis of rRNAs (Granneman & Tollervey, 2007), for which these proteins are produced in exceeding amounts and are therefore destined to proteasomal degradation. Because of this, RPs nuclear translocation occurs 2-3 minutes after synthesis (Golomb

et al., 2012). Once in the nucleolus, their half-life increases up to 6 hours but its only when incorporated into mature ribosome that the average half-life reaches 30 hours (Boisvert et al., 2012). Stability of RPs represents only one of the multiple levels at which the expression of ribosomal proteins, and therefore RiBi, is regulated. In particular, post-transcriptional regulations of 5'TOP mRNAs by mTORC1 is a critical node of RP synthesis to provide the structural components of ribosomes according to the cellular metabolic demand (discussed in the paragraph 3.1.5).

3.1.3 RiBi in cancer

By taking the responsibility for synthesizing almost all cellular components, ribosomes are necessary to support cell growth. This becomes even more evident for tumoral cells, in which oncogenic signals coordinate different metabolic pathways to sustain the increase in cell growth and proliferation. In this regard, upregulating the biogenesis of new ribosomes is a suitable strategy often exploited by tumors to sustain the overt anabolic demand. This evidence was obtained in 2008 by Barna et al. using the *Eμ-Myc/+* transgenic mice, a model of increased protein synthesis and cell growth supported by the oncogenic stimulus of MYC overexpression (Barna et al., 2008). In this study, they showed that uncontrolled protein synthesis by cap-dependent translation bypasses the IRES-dependent translation of Cdk11 during mitosis, leading to increased centrosome numbers and genomic instability. However, crossing the *Eμ-Myc/+* mice with a heterozygous mice for RPL24, a model of RP haploinsufficiency that re-establishes normal levels of protein synthesis in the context of hyperactive MYC, was sufficient to suppress genomic instability and to mitigate tumor burden, with a marked increase in the overall survival. This study links the protein synthetic output controlled by the oncogenic program driven by MYC with the tumorigenic capacity as well as the implications that dysregulated translation has over cancer formation. In agreement with this model, pharmacological inhibition of RNA PolI activity by CX5461 recapitulated the overall survival obtained by genetic mitigation of protein synthesis in Eμ-Myc lymphoma (Bywater et al., 2012). In addition to this, many studies have described the overexpression of ribosomal proteins in specific tumor types. An example is given by increased expression of RPL29, RPS8, RPL12, RPL23A, RPL27 and RPL30 which are found overexpressed in different tumor types, or by RPS3A which is able to induce NIH3T3 mouse fibroblasts transformation and tumor formation in nude mice (reviewed in (Van Riggelen, Yetil, et al., 2010). Interestingly, the detection of enlarged nucleoli in cells has been used for over 200 years to distinguish normal cells from cancer ones

(Pianese, 1896), being the nucleoli the site of ribosome biogenesis (Lam & Trinkle-Mulcahy, 2015).

The total number of ribosomes, however, is not the only determinant of the connection between ribosomes and cancer. Qualitative alterations in ribosome composition can affect the genomic program by selective translation of transcripts that have more affinity for the "specialized" ribosome. In addition, it has been observed that altered ribosomal composition correlates to increased cancer susceptibility. For this reason, the concept of "cancer ribosomes" has been proposed in the field as the presence of ribosomes whose structure exists only in cancer cells and is responsible for their dysregulated translational program (Y. Xu & Ruggero, 2020). The oncogenic impact of heterogenous ribosomal structure is also evidenced by ribosomopathies, human diseases caused by mutations in RPs and characterized by elevated incidence of different types of tumors such as myelodysplastic syndrome, acute myeloid leukaemia, colon carcinoma and osteogenic sarcoma (Ajore et al., 2017; Ellis & Gleizes, 2011). Another example is provided by the R98S mutation in RPL10 observed in patients with T cell acute lymphoblastic leukaemia (Girardi et al., 2018). The mutation is associated with increased expression of BCL2 and hyperactivation of JAK-STAT oncogenic signaling. However, modifications at the level of rRNA such as methylation and pseudo-uridylation are also commonly deregulated in cancer (Genuth & Barna, 2018).

3.1.4 RiBi regulation by MYC

The generation of new ribosomes in large amounts is a strategy that cancer cells exploit to sustain their growth. It is therefore not surprising that oncogenes such as MYC and mTORC1 control the Ribosome Biogenesis at different steps of the process.

MYC regulates RiBi by increasing the transcription of multiple factors involved in the process, including the rRNAs as well as the mRNAs encoding RPs, RAFs and nucleus-cytoplasm transporters. As seen in chapter 1, MYC exerts its transcription factor activity by either remodeling chromatin status and/or by recruiting other factors relevant for the PIC formation. Indeed, MYC upregulates the transcription of rRNAs 5S and tRNAs by stimulating the RNA pol III activity, while it increases the transcription of the 47S precursor by directly binding to the E-boxes of rDNA loci (Arabi et al., 2005; Grandori et al., 2005; Shiue et al., 2009). By chromatin immunoprecipitation it has been observed that MYC binding to rDNA is accompanied by the co-presence of TRAPP, thus increasing

acetylation and the open status of chromatin (Arabi et al., 2005; Grandori et al., 2005). MYC also promotes rRNA expression by recruiting the essential RNA pol I cofactors SL1 (selectivity factor) and UBF (upstream binding transcription factor). SL1 facilitates the RNA Pol I PIC formation, while UBF stimulates the transition from initiation to elongation (Panov et al., 2006). By means of RNA pol II-dependent transcription, MYC also promotes transcription of RP genes, as described by Morcelle et al. using an osteosarcoma cell line in which overexpression of MYC was accompanied by increased levels of newly synthesized RPs (Morcelle et al., 2019). MYC positively regulates also the transcription of genes whose encoded proteins are involved in the processing of rRNA precursor and in the nuclear-cytoplasmic shuttling of mature ribosomal subunits. Among them, nucleolin (NCL) and nucleophosmin (NPM1). NCL binds to the 47S pre-rRNA and is required for its cleavage into 18S, 5.8S and 28S rRNA (Ginisty et al., 1998), NPM1 instead is required in multiple processes such as rRNA maturation, RPs stability and transport of ribosomal subunits into the cytoplasm, reasons for which mutations or loss of NPM1 inhibits the cytoplasmic levels of 40S and 60S subunits accompanied by decreased protein synthesis (Maggi et al., 2008) (Fig. I-16).

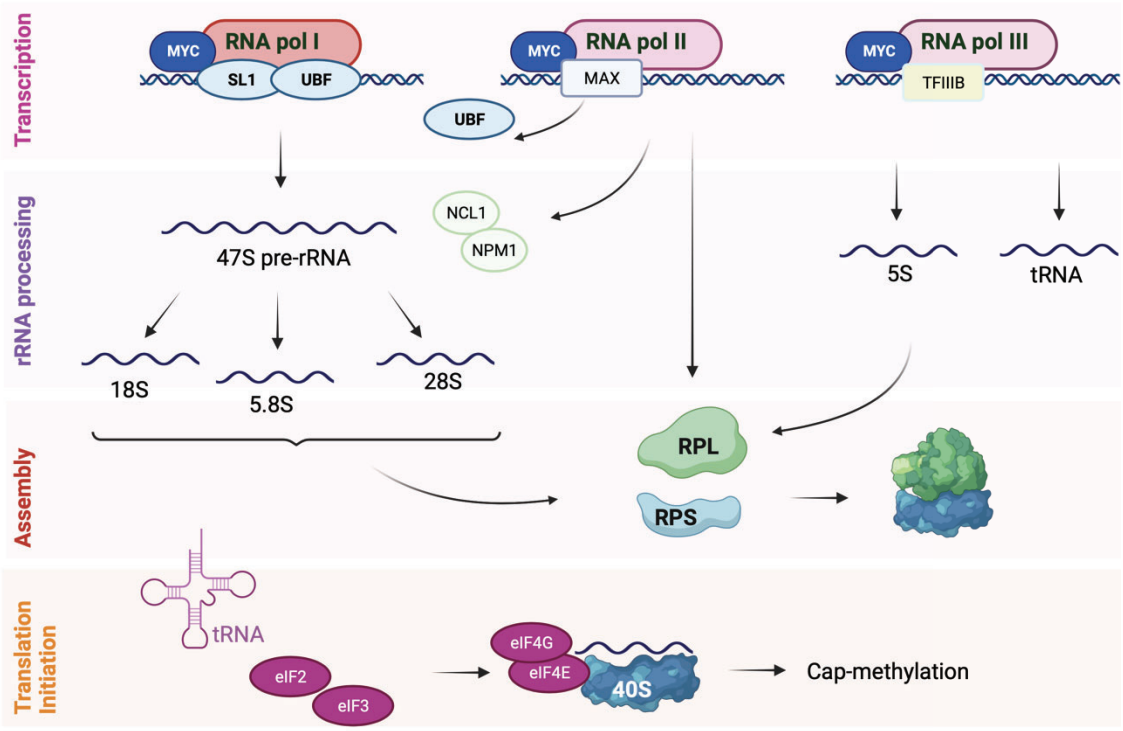

Fig. I-16 **| MYC controls RiBi at multiple steps** MYC regulates the expression of several RPs, rRNAs, tRNAs and factors required for rRNA processing, ribosome assembly and export of the mature form to the cytoplasm. In details, MYC promotes RNA Pol I-mediated transcription of 5.8S, 18S and 28S rRNAs, which requires upstream binding transcription factor (UBF) and selectivity factor (SL1). MYC activates Pol III-mediated transcription of 5S rRNA and transfer RNA (tRNA) and the dimerization with MAX stimulates the transcription of protein components by the RNA Pol II. Among them RPs, Nucleolin (NCL) and nucleophosmin (NPM1), which are involved in rRNA processing and export, UBF that functions as a cofactor for RNA pol I-dependent transcription as well as eukaryotic initiation factors that control the initiation of translation. Finally, the activity of MYC promotes cap methylation at the 5' end of some mRNAs. Adapted from (Van Riggelen, Yetil, et al., 2010)

Additionally, a growing body of evidences has shed light also on the transcription-independent roles of MYC in the control of protein synthesis. The first observation showed that in response to MYC induction several cyclin-dependent kinases (CDKs) increase their protein expression levels without changes in their mRNAs levels. This led to the discovery that MYC promotes cap methylation at the 5' end of these mRNAs, thus promoting their efficient translation (Cole & Cowling, 2008). A following work confirmed the impact of MYC on translational by observing a widespread coordination between MYC-induced changes at the mRNA level and the corresponding translation efficiency (Elkon et al., 2015). However, the mechanism still need to be elucidated, leaving the open possibility that it can be mediated directly by MYC (as in the case of induced cap-methylation) or indirectly by MYC-responsive regulators such as microRNAs or RBPs.

3.1.5 RiBi regulation by mTORC1

RiBi is the most energy demanding anabolic process of a growing cell. RPs are among the most abundant cellular proteins (Geiger et al., 2012) and rRNAs constitute more than 80% of total nucleic acids in growing cells (Lane & Fan, 2015). The high amounts of rRNAs and RPs produced in a cancer cell reflects a huge investment in terms of energy, which justifies why this process is turned on and off according (1) to the availability of resources as well as (2) to the energetic status of the cell, the sensor of which converge on mTORC1 as seen in chapter 2.

mTORC1 controls RiBi by means of two key downstream targets, the RPS6 kinases (S6K1/2) and the initiation factor 4E binding proteins (4E-BP1/2/3) (Fig. I-17). S6K1 activation is mediated by a first phosphorylation at T389 by mTORC1 followed by a

subsequent phosphorylation at T229 by PDK1(Pullen et al., 1998). Once activated, its kinase activity triggers a series of events that promotes global protein synthesis as well as purine, pyrimidine and RP biosynthesis, thus boosting RiBi at different levels. S6K1 promotes translation by phosphorylating the translation initiation factor eIF4B. p-eIF4B binds eIF4A at the translational pre-initiation complex (PIC), stimulating its helicase activity and thus promoting efficient translation (Sonenberg & Hinnebusch, 2009). In addition, eIF4A can be recruited to the PIC by eIF4G only under mitogenic signaling, otherwise it is sequestered by PDCD4 hampering translation initiation. However, the phosphorylation of PDCD4 at Serine 67 by S6K1 mediates PDCD4 ubiquitination and degradation, thus releasing eIF4A and promoting its binding to eIF4G (Dorrello et al., 2006) (More details about the process of cap-dependent translation will be given in next chapter). The activity of S6K1 extends also to the step of translational elongation. By phosphorylating the eEF2 kinase (eF2K), mTORC1 restores the activity of the eukaryotic elongation factor-2 (eEF2), which catalyzes the GTP-dependent tRNA translocation step during elongation. In addition to the positive regulation over S6K1, mTORC1 supports protein synthesis by relieving the inhibitory control of eIF4E-BP1/2 on the cap-dependent translation initiation. Briefly, this mechanism consists of the assembly of a multimeric complex termed eIF4F at the 7-methyl-Guanosine (m^7G) cap of the transcript. eIF4E-BPs, in their hypo-phosphorylated form, competes with eIF4G for the association with eIF4E, a component necessary for the assembly of the eIF4F complex, thus hampering the formation of the 43S complex. Upon mTORC1 activation, a hierarchical phosphorylation at multiple sites of the eIF4E-BPs weakens the affinity of eIF4E for eIF4E-BPs, thus allowing eIF4E association with eIF4G and the formation of the 43S translational PIC and therefore initiating translation (Gingras et al., 1998, 2001). Upregulation of protein translation is then paralleled by an increase in the synthesis of pyrimidines and purines to sustain rRNA production. Activation of S6K1 by mTORC1, indeed, phosphorylates a key enzyme involved in the *de novo* pyrimidine biogenesis of carbamoyl-phosphate synthetase 2 aspartate transcarbamylase and di-hydro-orotase (CAD)(Ben-Sahra et al., 2013).

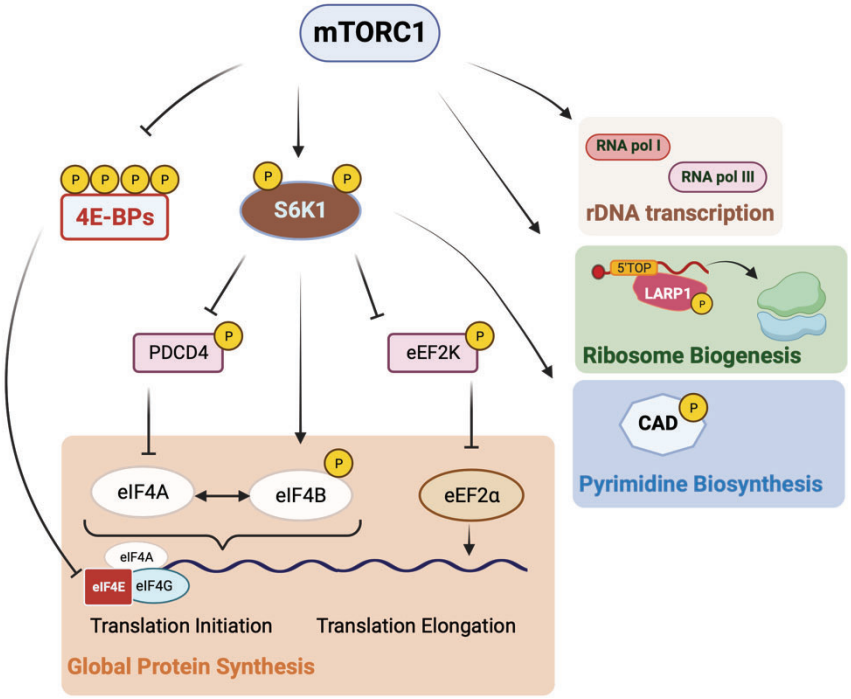

Fig. I-17 **| mTORC1 regulation of RiBi** mTORC1 activates a signaling pathway cascade that globally supports RiBi by increasing global protein synthesis, rDNA transcription and nucleotide synthesis which in turn sustains rRNA production. Detailed explanation in the text. Adapted from (Gentilella et al., 2015)

The mechanisms of mTORC1 to support rRNAs synthesis, however, are not limited to the phosphorylation of its two main targets. Under favorable growing conditions, mTORC1 activates the transcription initiation factor 1A (TIF-1A) of the RNA pol I complex to promote 47S rDNA transcription (Mayer et al., 2004) and inactivate the inhibitor of RNA pol III Maf1 by phosphorylation at Serine 75, thus facilitating the transcription of 5S rDNA and tRNAs (Shor et al., 2010). Furthermore, mTORC1 regulates RiBi by coordinating the translation of RP-encoding mRNAs, belonging to the 5'TOP family of transcripts. As seen in paragraph 2.2.2, during the last decade, LARP1 has been identified by our group and by others as the main mTORC1 downstream player responsible for this type of regulation (Gentilella et al., 2017). Moreover, a recent study from our laboratory, has advanced the understanding of the biological implications of 5'TOP mRNAs binding by LARP1 over RiBi and protein synthesis. We observed indeed that the selective degradation of ribosomes consequent to chronic mTORC1 restriction, a process termed ribophagy, is responsible for the decrease in non-TOP mRNAs levels. Interestingly, 5'TOP mRNAs were immune to this destabilization, so that their levels

remained unchanged upon prolonged mTORC1 inhibition. In this condition, indeed, the formation of the 40S-LARP1 complex is induced to protect the 5'TOP mRNAs from ribophagic degradation in a translationally-inactive state, in order to preserve the cellular anabolic potential in the form of mRNAs. Importantly, once mTORC1 is reactivated, these transcripts are readily translated. This strategy adopted by the cell serves to facilitate a rapid reestablishment of the anabolic power. Re-activation of mTORC1 signaling leads, indeed, to a rapid increase in the assembly of new ribosomes with consequent stimulation of protein synthesis rate. This fast adaptation of the cell to upstream stimuli is guaranteed by the pool of transcripts preserved by the 40S-LARP1 complex, which are enriched for mRNAs encoding for the 40S and 60S RPs, RiBi assisting factors and translation initiation and elongation factors, all belonging to the 5'TOP family of transcripts. Moreover, characterization of the 40S-LARP1-enriched mRNAs revealed the presence of others 5'TOP transcripts which are noncoding RNAs important for rRNA modifications occurring during ribosome maturation (Fuentes et al., 2021) (Fig. I-18)

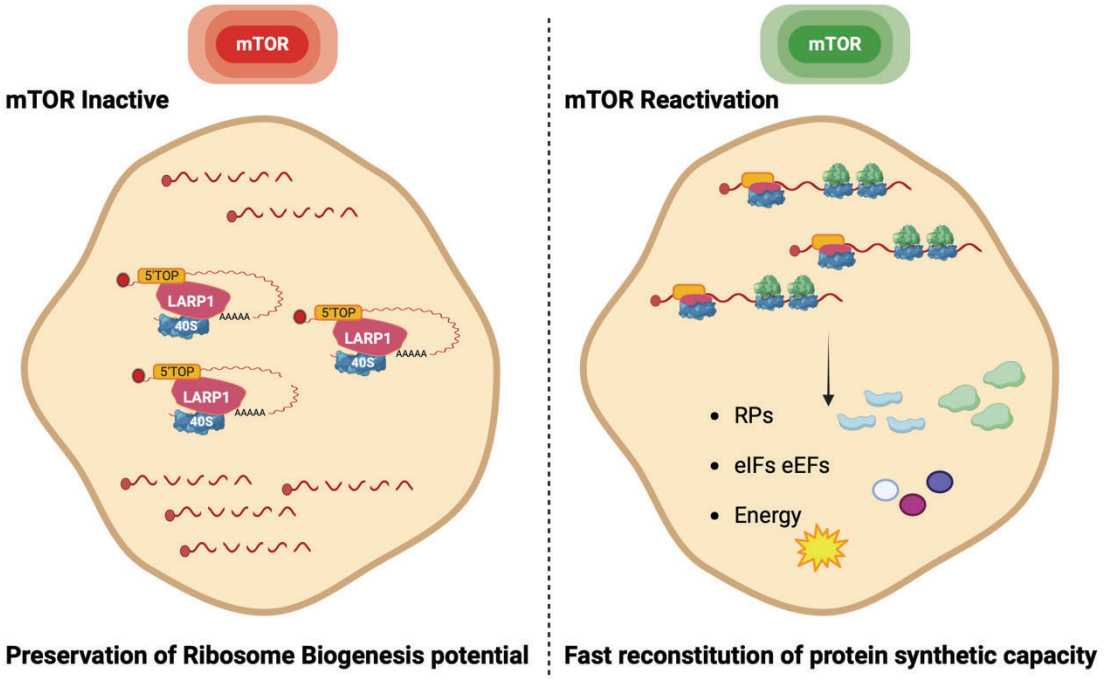

Fig. I-18 **| Mechanism of 40S-LARP1 complex regulation of 5'TOP mRNAs under mTORC1 signaling** Upon mTOR inhibition, the 40S-LARP1 complex protects 5'TOP mRNAs from ribophagy-mediated degradation, a fate to which are destined non-5'TOP mRNAs. Upon reactivation of mTOR signaling by anabolic signaling, the pool of 5'TOP mRNAs protected by 40S-LARP1 is rapidly translated to face the anabolic stimuli. Adapted from (Fuentes et al., 2021)

3.2 Protein synthesis in cancer

In the previous chapter the role of ribosome biogenesis in the context of translational regulation has been discussed. Cancer cells, in particular, increase the availability of ribosomes to reinforce the protein synthetic machinery and therefore to bolster the expression of catalytic and structural components necessary for cell growth. In this paragraph, mechanisms of translational regulation by oncogenic signaling will be introduced. In addition, it is worth to note that the translational landscape reprogrammed on specific mRNAs reshapes the cellular proteome to sustain malignancy. The way by which tumoral cells modify the translational program and, on the other way around, how the oncogenic translational fingerprint sustains tumorigenic mechanisms is a phenomenon referred to as “plasticity” of tumor translation (reviewed in (Fabbri et al., 2021))

3.2.1 Translation machinery

Translation is a cyclic process constituted of 4 phases: initiation, elongation, termination and ribosome recycling. Translating an mRNA into a protein requires not only ribosomes, but several other players such as tRNAs, amino acids and translation factors (namely eukaryotic initiation factors (eIFs), elongation factors (eEFs) and release factors (eRFs)). Two main modalities of initiation are known, cap-dependent and cap-independent. Cap-dependent translation involves the assembly of the eIF4F complex on the 7-methylguanosine cap at the 5’ end of the mRNA to be translated (Fig. I-19). eIF4F is constituted by the cap-binding protein eIF4E, the RNA helicase eIF4A, responsible for unwinding mRNA secondary structures, and the eIF4G scaffolding protein, which interacts with the poly(A)-binding protein (PABP) located at the 3’ end of the mRNA. Once the eIF4F complex is bound to the cap, it recruits the 43S pre-initiation complex (PIC) to form the 43S initiation complex + activated mRNA. 43S PIC formation involves first the assembly of the ternary complex, comprising the GTP-bound eIF2 and the initiator methionyl tRNA ($tRNA_i^{Met}$), with the following association of 40S small ribosomal subunit and initiation factors eIF3, eIF1, eIF1A and eIF5. Once the 43S initiation complex is assembled, the the mRNA is scanned in direction 5’ → 3’ to find the AUG start codon encoding for methionine. Recognizing the AUG induces a series of reactions over the eIFs generativng the 48S complex that allow the recruitment and placing of the 60S large ribosomal subunit (Jackson et al., 2010). The 80S monosome is now ready to proceed with the elongation. The elongation step consists in the sequential addition of amino acids to a nascent polypeptide chain, based on the sequential configuration of the

codons in the open reading frame on the mRNA (Knight et al., 2020). Once the ribosome reads a stop codon through the action of eRF1 (which can recognize all the three stop codons UAA, UAG, UGA), the GTPase eRF3 mediates the release of the polypeptide chain and translation is terminated. The resulting post-termination complex (post-TC) is then recycled to allow the mRNA and ribosomes to re-initiate a new cycle of translation (Hellen, 2018).

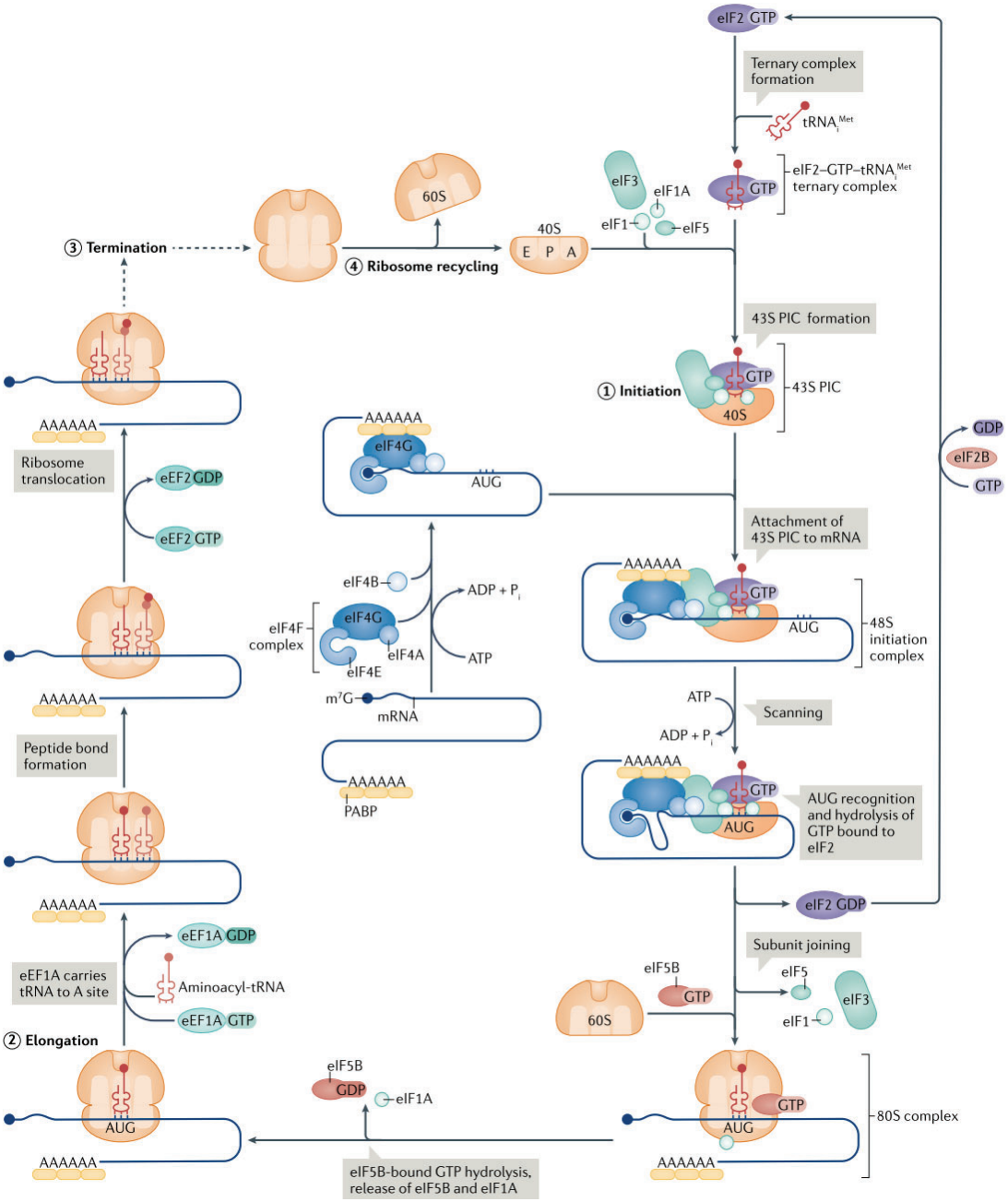

Fig. I-19 | **Steps of cap-dependent mRNA translation** The 4 phases of translation are represented (initiation, elongation, termination and ribosome recycling) with the translational factors involved in each step. More detailed explanation in the text. Figure from (Fabbri et al., 2021)

Cap-independent translation differs instead for the recruitment of the 43S pre-initiation complex directly to an internal region at the 5'UTR of the mRNA, independently of an eIF4F complex. Internal ribosome entry sites (IRES) elements are indeed complex structures at the 5'UTR which were first described in viral mRNAs that were uncapped but efficiently translated (Jang et al., 1988; Pelletier & Sonenberg, 1988). Recruitment of the 40S small ribosomal subunit in the form of 43S PIC is mediated by IRES trans-acting factors (ITAFs), whose function is still not completely understood but can vary from structural stabilizer of the IRES element to anchors for other proteins (reviewed in (King et al., 2010). Interestingly, the availability of ITAFs is cell line-dependent and strongly determines the activity of the IRES itself, as observed by the IRES of MYC, 20-fold more active in He-La cells than in MCF7 cells (Stoneley et al., 2000).

3.2.2 Translation-regulated cancer proteome

Oncogenic insults can have a major impact on the translational machinery to modify the repertoire of mRNAs engaged in translation. The regulation of cancer-related genes at the level of translation can occur through multiple mechanisms. On the one hand, increasing the expression of initiation and elongation translation factors supports higher rates of protein synthesis (Truitt et al., 2015), however enhancing the activity of specific factors is the most prevalent strategy through which common dysregulated oncogenic pathways control selective translation (Fig. I-20). Indeed, some mRNAs encoding for growth factor receptors, survival proteins or oncogenes such as MYC, are characterized by secondary structures like G-quadruplexes or hairpins in their 5'UTRs, that impede their translation in a normal cellular setting (Kozak, 1986; Pelletier & Sonenberg, 1985). In cancer, however, the eIF4F complex activity is enhanced, especially for the activity of the eIF4A helicase that unwind the mRNA and therefore resolves these structures allowing a correct ribosome scanning (Koromilas et al., 1992). Another evidence about the relevance of helicases activity comes from the finding that the DEAD box RNA helicase DDX3 is responsible for the translation of a network of tumorigenic mRNAs and interestingly, its coding gene *DDX3X* has been found mutated in 36% of WNT molecular subgroup medulloblastoma (Northcott et al., 2017; Oh et al., 2016). However, oncogenic stresses can also mediate a cap-independent translation for those mRNAs containing an internal ribosome entry site (IRES) structure. Approximately 10% of mammalian mRNAs contain an IRES(King et al., 2010), most of them encoding for proteins with a role in cancer such as pro-survival (BCL-2, MYC, cyclin D1 and XIAP) (Y. Shi et al., 2015), antiapoptotic and pro-angiogenic factors (VEGF). That this type of translation initiation is

usually activated upon stress conditions commonly experienced by cancer cells such as hypoxia, DNA damage and amino acid starvation, concomitantly to cap-dependent translation inhibition, is indicative of the plasticity of cancer cells. Likewise, the R98S mutation of RPL10 confers a survival advantage to T-cells of acute lymphoblastic leukaemia by promoting IRES-mediated translation of BCL-2 (Kampen et al., 2019).

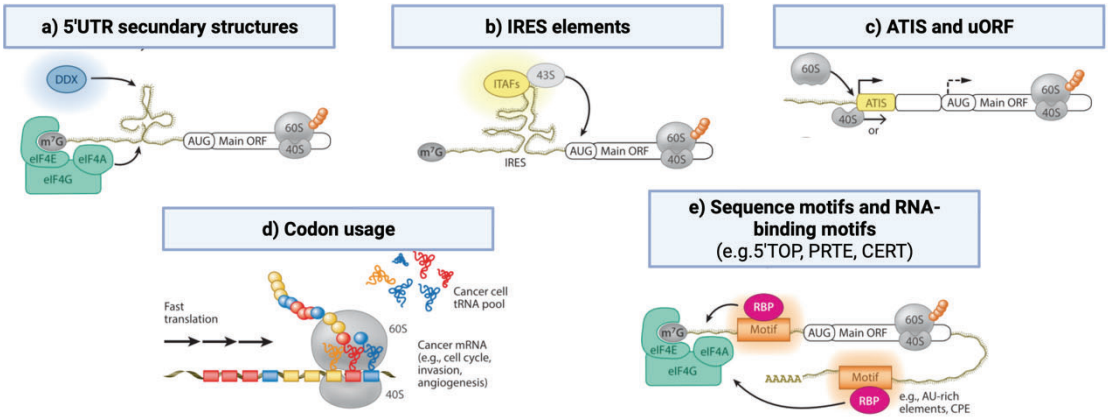

Fig. I-20 | **mRNAs structures and motifs involved in translation regulation a)** 5'UTR secundary structures such as G-quadruplexes and **b)** IRES elements control the translation of specific mRNAs involved in tumorigenesis. **c)** ATISs sequences upstream the main ORF can lead to the expression of usually repressed tumor suppressor (dashed arrows indicate a less efficient translation initiation at the main AUG). **d)** Expression of a specific repertoire of cancer tRNAs favors the translation of pro-tumorigenic mRNAs. **e)** Sequence motifs and RNA-binding motifs can guide cap-dependent translation of oncogenic transcripts. Figure adapted from (Y. Xu & Ruggero, 2020)

Another example of cancer-specific translational mechanism is given by the usage of an alternative translation initiation site (ATIS) that tumor cells can take advantage of. ATIS are located in the 5'UTR of oncogenic mRNAs, as part of an upstream open reading frame (uORF) that usually inhibits the translation of the downstream ORF, therefore inhibiting the expression of the oncoprotein. Mechanistically, ribosomes translating the uORF can get stalled and create a barrier to the scanning of the 43S PIC or can dissociate once translated the uORF, therefore impeding the usage of the main ORF (reviewed in (Hinnebusch et al., 2016). Cancer cells, however, can bypass the inhibition mediated by the uORF to promote oncoprotein expression. A complete knowledge of all the genes whose expression is sustained by this mechanism is still missing, but the most known example is PD-L1, an immune check-point ligand that diminishes the T-cell response and whose expression is enhanced by high levels of MYC (see paragraph

1.2.3). The mechanism responsible, at least in part, for bypassing the PD-L1 uORF is the phosphorylation of eIF2α, a component of the ternary complex and therefore of the 43S PIC (Y. Xu et al., 2019). eIF2α phosphorylation at Serine 51 by the RNA-dependent protein kinase (PKR) and PKR like endoplasmic reticulum kinase (PERK) decreases the ability to recognize the start codon, therefore promoting a leaky scanning of the uORF that allows the ribosome to reach the main ORF(Wek, 2018). Similarly, phosphorylation of eIF2α allows the leaky scanning of two uORFs in the ATF4 mRNA thus reaching the canonical ORF and inducing its increased expression. Cancer cells usually activate this mechanism under hypoxia, amino acid deprivation, oxidative and genotoxic stress (Vattem & Wek, 2004). The transcriptional activity of ATF4, indeed, reprograms cellular metabolism to make cancer cells resistant to the stress and allow their survival. On the other way around, mutations of upstream initiation codons can create new uORFs to repress tumor suppressors expression. This is the case for the mutational events in the 5' UTR of mRNAs encoding for tumor suppressors like p16, p14 and p27 that predispose patients to melanoma and pituitary adenoma (L. Liu et al., 1999; Occhi et al., 2013).

Cancer cells can also mediate the selective translation of a specific network of mRNAs by exploiting the specific codon composition of these transcripts. By increasing the expression of distinct tRNAs, indeed, tumor cells preferentially sustain the elongation of mRNAs enriched for the corresponding codons, such as those encoding for proteins involved in cellular proliferation and metastasis (Goodarzi et al., 2016). This type of regulation through the tRNAs, however, can also occur by the tRNA epitranscriptome modifications. Uridines at position 34 (U34) of tRNAs such as $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA^{Gln}$ can be modified by acetylation, methylation or thiolation (Barbieri & Kouzarides, 2020). In melanoma, for example, the PI3K-mTORC2 pathway promotes the upregulation of the enzymes involved in tRNAs modifications to support HIF1α-encoding mRNA translation and therefore sustain glycolytic metabolism (Rapino et al., 2018).

Finally, mRNAs with sequence-specific elements in their 5' UTRs can pervade the translatome signature of cancer cells. mRNAs encoding for RPs and translation factors are characterized by a TOP motif in their 5' UTR that makes their translation sensitive to mTORC1. Mechanistically, mTORC1 regulates the translation of these transcripts by the RNA binding protein LARP1. Unfavorable growth conditions for the cancer cell inhibit mTORC1 and therefore all those anabolic processes, like RiBi and protein synthesis, preserving the anabolic potential of the cell from ribophagy, a specific degradation of translating ribosomes occurring when resources are limiting. Consequently, mTORC1 inhibition thanks to LARP1 restrains the translation of 5' TOP mRNAs and concomitantly

stabilizes them. This post-transcriptional regulatory mechanism allows rapid resume of the translation machinery expression once nutritional and energetic conditions return favorable (Fuentes et al.). However, the TOP element is not the only type of sequence that grants specific post-transcriptional mRNA regulations. Pyrimidine-rich translational element (PRTE) is a “TOP-like” motif found in pro-invasive transcripts such as YBX1 and metastasis-associated 1 (MTA1), whose regulation is still mediated by mTORC1 signaling (Hsieh et al., 2012). In addition, PRTE-mRNAs selective translation is responsible for increased expression of a rate limiting enzyme of nucleotides biosynthesis in MYC-driven tumorigenesis (Cunningham et al., 2014). Truitt et al. also showed that cytosine-enriched regulator of translation (CERT) represents another example of sequence that translationally mark a pool of mRNAs with pro-tumorigenic features. They observed that CERT transcripts encode for antioxidant proteins glutathione and ferritine, important to face the increase in reactive oxygen species (ROS) occurring during cell transformation and tumor progression. The CERT “tag” is preferentially enriched in 81% of eIF4E targets increased by oncogenic transformation, thus showing a selective translation of these transcripts in an eIF4E-dependent manner (Truitt et al., 2015).

3.2.3 Role of protein synthesis in cancer

The translational reprogramming observed during oncogenic transformation results in the expression of a “cancer proteome” that promotes tumor aggressiveness through different mechanisms. One of these is the epithelial-to-mesenchymal transition (EMT) that is the process in which epithelial cells lose their polarity and cell-cell adhesions, gaining migratory and invasive properties. Translation initiation has a well-defined role in EMT as it has been observed that TGFß-MNK axis signals to the translation initiator factor eIF4E. Mouse embryonic fibroblasts expressing a mutant of eIF4E (S209A), insensitive to phosphorylation by MNK, reduce metastatic spread thus showing the impact of translation initiation on cancer invasiveness (Robichaud et al., 2015). However, EMT is also associated with the acquisition of a de-differentiated stem-like state, characterized by low proliferation rate and reduced protein synthesis (Blanco et al., 2016; P. B. Gupta et al., 2019). Therefore, it does not surprise that hematopoietic stem cells display high levels of hypo-phoshorylated 4E-BPs to reduce mTORC1 signaling (Signer et al., 2016) as well as in skeletal muscle cells high levels of eIF2α limit the availability of the ternary complex for translation initiation (Zismanov et al., 2015). Maintenance of the stemness status, however, implies a reduced global protein synthesis rate

accompanied by selective translation of stemness-related mRNAs. In breast cancer, specific 5' UTR sequences present in NANOG, SNAIL and NODAL mRNAs allowed their selective translation under global protein synthesis repression in a p-eIF2α-dependent manner, ultimately supporting the stemness fate(Jewer et al., 2020). In the same way, p-eIF2α promotes the leaky scanning of uORFs for the expression of stemness related transcripts *Uspx9* and *Chd4,* important for mouse muscle stem cells self-renewal, while promoting global inhibition of translation (Zismanov et al., 2015).

Importantly, proteomic remodeling affects (and is reciprocally affected by) the environment surrounding the tumor cells, in order to support the process of angiogenesis and regulate tumor immunity. Under hypoxia, reduced mTORC1 activation impairs eIF4E-mediated cap-dependent translation. Tumor cells therefore supply this mechanism with alternative ones which sustains tumor progression. IRES-dependent translation activated upon hypoxia drives the expression of mRNAs encoding the neoangiogenic factors VEGF, BCL-2 and HIF1α in endothelial cells, thus allowing the generation of new vasculature (Braunstein et al., 2007). Also, selective cap-dependent translation has been observed to be activated under hypoxia and eIF4E inhibition. Uniacke *et al.* showed indeed the formation of a complex constituted by the hypoxia-inducible factor 2α (HIF-2α), RBM4 and a homologue of eIF4E, eIF4E2, which drives the cap-dependent translation of the epidermal growth factor receptor (EGFR) and other mRNAs with a specific RNA hypoxia response element (rHRE) (Uniacke et al., 2012).

Immune cells, instead, have a double role in the tumor micro-environment (TME), as they can induce immune tolerance or activate an anti-tumor response. This differential behavior can be controlled by several mechanisms and one of them is translation. A recent study (Wolf et al., 2020) demonstrated that naïve T-cells are characterized by a homeostatic metabolism and quiescence that is not inert as commonly thought, but it is instead characterized by unstable proteins with high turnover rates that allow the maintenance of this status. An example is the half-life of the MHC-I complex, responsible for antigen presentation, whose proteins are constantly renewed in the cell surface, but also transcription factors. If on one side this mechanism is high energy-consuming, on the other it allows a rapid protein turnover, thus granting responsiveness of the T-cell to activating stimuli. The same study also showed that T-cell are poised to rapidly activate a glycolytic metabolism in just few minutes thanks to the maintenance of mRNAs encoding for glycolytic enzymes in a translationally repressed state. The majority of these repressed mRNAs contains TOP motifs and are inhibited in a mTOR-dependent manner. Finally, translational mechanisms contribute to T-cell responsiveness with idle ribosomes

that, upon T-cells activation, increase their translational output up to 4-fold (Wolf et al., 2020). In a different biological paradigm, translation can also modulate immune evasion. Tumor cells express the immune checkpoint protein PD-L1 to interact with the PD-1 receptor in T-cells and mediate their inactivation. Mechanistically, the expression of PD-L1 is promoted by INF-γ -mediated activation of the eIF4F complex which sustains the expression of the trancriptional factor STAT1. STAT1, ultimately, starts the transcription of PD-L1 (Cerezo et al., 2018). In addition, a study using MYC^{Tg}/$KRAS^{G12D}$ mouse model showed that p-eIF2α relieved the repression of PD-L1 expression by uORF-dependent translation in a MYC-dependent manner (Y. Xu et al., 2019).

RATIONALE OF THE STUDY

RATIONALE OF THE STUDY

Oncogenic MYC is frequently found deregulated in a vast variety of human cancers by genetic, epigenetic or post-transcriptional mechanisms (Schaub et al., 2018). This can occur as a consequence of mutations directly on MYC genomic locus (Boxer & Dang, 2001) or as the result of deregulated upstream pathway ultimately impinging on MYC expression, as in the case of hyperactive WNT in colorectal cancer (Brannon et al., 2014)(Clevers, 2006).

Overexpression or constitutive activation of MYC leads to the acquisition of a coordinated network of mechanisms that ensures the ability of MYC to promote tumor initiation and progression (Gabay et al., 2014). Such responses vary from dysregulation of tumor microenvironment (TME) and host immune response to the induction of cell-intrinsic processes that promote cell growth and proliferation (reviewed in (Dhanasekaran et al., 2021). To this end, MYC sustains anabolic processes like Ribosome Biogenesis (RiBi) and protein synthesis, necessary for the availability of building blocks in the cell (Van Riggelen, Yetil, et al., 2010).

RiBi is a complex multistep process that is regulated by MYC mainly by its transcriptional activity, through the upregulation of RNA Pol I, II and III required for the transcription of ribosomes constituents, respectively the 47S pre-rRNA, ~80 ribosomal proteins (RPs) mRNAs and the 5S rRNA. However, recent evidences support the existence of a post-transcriptional role for MYC in modulating the expression of RiBi-related transcripts. A coordination in regulation of mRNAs levels and their translation has been observed, but which player downstream MYC could connect transcription and translation is still not known (Elkon et al., 2015).

Being a high energy-demanding process, RiBi also responds to the master regulator of cellular metabolism mTORC1. Conventionally, the kinase activity of mTORC1 has been known to contribute to RiBi and protein synthesis processes by phosphorylating its two main targets, S6K and 4E-BPs, which in turn cooperate to activate mRNA translation initiation and elongation, pyrimidine biosynthesis and rDNA transcription (reviewed in (Gentilella et al., 2015). During the last decade, however, the RNA binding protein LARP1 has emerged as a new mTORC1 target regulating the expression of mRNAs encoding for RPs. These transcripts are characterized by a TOP element in their 5'UTR that works as a post-transcriptional regulatory motif influencing their stability and translation. All the 5'TOP mRNAs are indeed recognized by LARP1 that, in complex with the small ribosomal subunit 40S, regulates the stabilization of these transcripts according

to mTORC1 signaling (Gentilella et al., 2017). This aspect has been recently tackled and elucidated by our group.

Under mTORC1 inhibition, the 40S-LARP1 complex preserves 5'TOP mRNAs in a pool of translationally-inactive transcripts, protecting them from ribophagy-mediated degradation. The generation of such reservoir of RP-encoding mRNAs, already loaded with the 40S ribosomal subunit, constitutes a ready-to-use anabolic source that the cell can rapidly exploit once mTORC1 is reactivated and the translational break is released. By virtue of this mechanism, the 40S-LARP1 complex confers an advantage to the cell in terms of protein synthetic capacity, as LARP1 depleted cells showed impaired recovery of protein synthesis upon mTORC1 reactivation(Fuentes et al., 2021). In this way, the 40S-LARP1 complex constitutes a post-transcriptional mechanism of RiBi and protein synthesis regulation downstream mTORC1.

In this study we set out to investigate in the context of oncogenic MYC the role of the 40S-LARP1 complex over RiBi and protein synthesis and to address its relevance in MYC-driven cell proliferation.

OBJECTIVES

OBJECTIVES

The main goal of this thesis is to investigate whether the 40S-LARP1 complex constitutes a post-transcriptional regulatory node of MYC oncogenic signaling in the control of MYC-driven anabolic processes and cell proliferation. To this end, we established the following specific objectives:

1. To decipher whether LARP1 depletion affects MYC-driven cell proliferation

2. To evaluate the impact of LARP1 in the ability of MYC to sustain RiBi and protein synthesis

3. To explore a potential regulation of MYC on LARP1 expression

MATERIALS AND METHODS

MATERIALS AND METHODS

Cell culture

U2OS cells stably transfected with a doxycycline-inducible two-vector system encoding human MYC gene were kindly provided by M. Eilers laboratory. P493-6 human lymphoblastoid cells, modified to carry a tetracycline (Tet)-repressible allele of MYC, were kindly provided by J. Kluiver laboratory. HCT116 and RKO human colorectal carcinoma cell lines were obtained from the American Type Culture Collection. DLD-1, SW403, LS174T cells engineered to express a tamoxifen-inducible dominant negative form of TCF4 (which consists in the ß-catenin-binding domain of TCF4 fused to a modified hormone-binding domain of the estrogen receptor (nTCF-ERT2)) were kindly provided by E. Batlle laboratory. All the cell lines mentioned were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA). For all studies, cells were incubated at 37°C, 5% CO2 and 90-95% of relative humidity.

Generation of U2OS T/O MYC LARP1 KO

U2OS T/O MYC cells were transfected with pX458 plasmid (Addgene catalog number 48138) containing CRISPR-Cas9–targeting exon 5 of the LARP1 gene using Lipofectamine 2000 transfection reagent (Invitrogen). As the pX458 plasmid contains green fluorescent protein, single-cell sorting was performed 48 hours after transfection using MoFlo Astrios Cell Sorter (Beckman), and individual clones were cultivated and subsequently analyzed for LARP1 expression by immunoblotting (Fig.R2a), leading to the isolation of the LARP1 KO clone #2.

Generation of shLARP1 P493-6 cells

The stable P493-6 cell line expressing for the shRNA against LARP1 (shLARP1) or a non-targeting shRNA (shSCR) was generated as in (Gentilella et al., 2017). Briefly, 5µg of each shRNA were co-transfected with 2.5µg of lentivirus packaging plasmids pLP1, pLP2, and pLP/vesicular stomatitis virus-G (pLP/VSV-G) into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen). Virus supernatants were collected 48 hours after transfection, filtered (0.45µm filter) to remove cellular debris and precipitated by ultra-centrifugation to concentrate the viral particles. 50µl of virus concentrate was then used to infect P493-6 cells in growing medium. Briefly, cells were pellet down and medium volume reduced to 500µl, 50µl of virus and polybrene at 10µg/ml were added.

The suspension of cells was incubated at 37°C, 5% CO2 for 4hours and then complete medium was added. Cells were then splitted 6 times to dilute the viral component before being sorted for GFP positive signal by fluorescence activated cell sorting (FACS) BD FACSAria. The GFP-positive pool of cells obtained was then screened by immunoblot for the reduction of LARP1 levels as shown in fig.R4.

Cell proliferation

$5x10^5$ U2OS T/O MYC cells were plated in 12-well plate and treated with doxycycline 2µg/ml. At the indicated timepoints, cells were washed twice and crystal violet solution (Sigma- Aldrich) was added to each well and incubated for 10 min at RT. The crystal violet solution was then removed and cells were washed twice with water. Staining was dissolved by a 15% solution of acetic acid agitating the plates on an orbital shaker until the color of each well was uniform, with no areas of dense coloration at the bottom. Absorbance at 595nm was measured in a microplate reader. Each experimental condition was performed in triplicate.

$3.5x10^6$ P493-6 cells were seeded for tetracycline treatment (100ng/ml). 72hours after, cells were washed twice with growing medium to remove tetracycline traces and $2x10^5$ cells were seeded in 12-well plates. Number of cells overtime was determined by Countess 3 Automated Cell Counter (Thermofisher).

Transfection procedures

siRNA, microRNA mimics and miRCURY LNA miRNA inhibitors (Qiagen) transfections were performed following manufacturer's instructions in Opti-MEM medium (Life Technologies) using Lipofectamine RNA-iMAX (Life Technologies). Transfections were performed during the indicated hours at a final concentration of 20 or 50 nM. For each treatment the amount of siRNA transfected was maintained constant between samples, by using siNS where required. The following siRNAs were used: non-silencing (NS) control (GCAUCAGUGUCACGUAAUA) was purchased from Sigma Aldrich (in the text referred to as non-targeting (NT)), siMYC was used as a mix of siMYC#1 (GCUUGUACCUGCAGGAUCU) and siMYC#2 (CGAUGUUGUUUCUGUGGAA) purchased from Sigma Aldrich, siLARP1 was used as a mix of siLARP1#1 (GAATGGAGATGAGGATTGC) and siLARP1#2 (GCGCCAGATTGAATACTACTTC). MISSION microRNA mimic hsa-miR-26a (TTCAAGTAATCCAGGATAGGCT)and hsa-miR-26b (TTCAAGTAATTCAGGATAGGT) (Sigma) used, 25nM of each used when transfected in combination.

Protein analysis

Cell protein extracts for western blot analysis were prepared by using a RIPA buffer (1% SDS lysis buffer (Tris pH7.4 50mM, SDS 1%) supplemented with the protease inhibitor cocktail (SIGMA), phosphatase inhibitor cocktail 3 (SIGMA) and Protease inhibitor (Roche). After lysis, cell lysates were incubated 30' on ice followed by centrifugation at 13,000 rpm for 15 minutes. Protein concentrations were determined for supernatants by the BCA assay (Pierce). 20 µg of total protein extracts were resuspended in protein loading buffer (Tris.HCl 375 mM, SDS 9%, glycerol 50% and bromophenol blue) and after treatment at 95°C for 10 minutes, proteins were separated on 10% SDS–polyacrylamide gels by electrophoresis, and transferred to PVDF membranes. Blots were stained with amido black solution (0.1% Amido Black 10B dye, 10% acetic acid, 25% isopropanol) to confirm equal loading and transfer of proteins, then blocked with 5% dry-fat milk in TBS-T and finally probed with the indicated antibodies (see Table M1). Immunoblots were developed using secondary horseradish peroxidase-coupled antibodies (Polyclonal swine anti-rabbit and polyclonal rabbit anti-mouse, Agilent, CA, USA) and an enhanced chemiluminescence kit (GE Healthcare).

ANTIBODY	ORIGIN	BRAND	REFERENCE
LARP1	R	Bethyl	A302-087A
LARP1	M	Santa Cruz Biotechnology	sc-515873
MYC	R	Abcam	Y69
MYC	R	Santa Cruz Biotechnology	sc-764
ß-Actin	M	Santa Cruz Biotechnology	sc-8432
α-Tubulin	M	Sigma	T6074
RPL5	R	Bethyl	A303-933A
RPL11	M	Invitrogen	37-3000
RPS6	R	Santa Cruz Biotechnology	sc-74459
RPS19	R	Abcam	ab123290

Table M1 **| List of antibodies**

RNA Analysis

Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (10-20 µg) was resolved on denaturing Urea PolyAcrylamide gel (6M urea) electrophoresis, stained with SYBR Gold (Invitrogen) and transfered to Hybond-N+ nylon membranes (GE Healthcare), after which UV-crosslinking by ultraviolet irradiation followed. After checking for the stability of the RNA by an UV transilluminator, the membrane was blocked in Church phosphate buffer (7% SDS, 1mM EDTA) at 55°C for 30' and probed overnight with 30ng/ml miR-26a probe (AGCCTATCCTGGATTACTTGAA, hybridization temperature 42°C), miR-26b (ACCTATCCTGAATTACTTGAA, hybridization temperature 37°C) or U6 normalizer (37°C). The blot was then rinsed three times for 5 min with 2X SSC 0.1% SDS at 55°C, then hybridized with streptavidin-HRP at 28°C for 30 min. The membrane was washed four times with PBS 1X, 0.5%SDS at 28° C for 5 min. The membrane was subjected to enhanced chemiluminescence reaction (GE Healthcare).

Real-Time PCR

Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was obtained from RNA by using random hexamers and M-MLV Reverse Transcriptase from Invitrogen following manufacturer's instructions. Quantitative PCR analysis was performed in triplicate on the Roche LightcCycler 96 detection system (Roche, Basel, Switzerland) using RNA-specific hydrolysis probes at 125 nM final concentration, in combination with the same primer pairs used for SYBR green qPCRs at 500 nM and the PrimeTime master mix (IDT). For the luciferase normalization, cells were harvested in an equal volume of cold phosphate-buffered saline (PBS), and 1/10 of the cell suspension was assayed for genomic DNA (gDNA) concentration, which we verified reflecting the cell number in the culturing conditions tested. The remaining volume of cells was centrifuged, and cell pellet was resuspended in TRIzol and spiked with an amount of Firefly Luciferase mRNA (Promega, L456A) proportional to the gDNA content, according to the ratio 10pg of Firefly mRNA per microgram of gDNA. The samples were processed as described above, and the levels of RPs or ß-Actin mRNAs were normalized to luciferase mRNA. The sequences of primers and probes used are reported in tables M2 and M3. All the reactions were performed in triplicate.

TARGET	FORWARD	REVERSE
LARP1	CGGGCAGAAGAAGAAAGGAAAC	ACGTAGGTGGCAGACTCAGAC
RPL5	GGTGTGAAGGTTGGCCTGAC	GGCACCTGGCTGACCATCAA
RPS6	TCTTGACCCATGGCCGTGTC	GCGGCGAGGCACTGTAGTAT
RPL11	TCCACTGCACAGTTCGAGGG	AAACCTGGCCTACCCAGCAC
ß-Actin	CTACAATGAGCTGCGTGTGGC	CTACAATGAGCTGCGTGTGGC
Firefly Luciferase	ACAGATGCACATATCGAGGTG	GATTTGTATTCAGCCCATATCG

Table M2 **| List of RT-qPCR primer sequences**

TARGET	PROBE
LARP1	CTAGACACATACCTGCCAATCGCGGAGA
RPL5	CTGGCCCGCAGGCTTCTCAATAGGTTT
RPS6	CCTGTTACAGACCAAGGAGAACTGGAG
RPL11	TATGACCCAAGCATTGGTATCTACGGCCT
ß-Actin	CAGATTTTCTCCATGTCGTCCCAGTTGGTGACGAT
Firefly Luciferase	GGAATACTTCGAAATGTCCGTTCGGTTGG

Table M3**| List of RT-qPCR probes sequences**

Luciferase assay

HCT116 cells were transfected using Lipofectamine™ RNAiMAX Transfection Reagent (invitrogen). Cells were plated at a density of 1×10^5 cells/well in a 12-well plate 24 h prior to transfection. 50nM siNT or miR-26a/b miRNA mimics (sequences from Sigma) were transfected as described above. 24 hours after, 1µg of psiCHECK™-2 Vector (Promega) was transfected using FuGENE® HD Transfection Reagent (promega) and following manufacturer's instructions. Cell extracts were subsequently prepared and assayed using the Dual-Luciferase® Reporter Assay System (Promega) and fluorescence was detected using Victor multilabel plate reader (HTDS). Renilla Luciferase activity was normalized to Firefly control one. Each experimental condition was performed in triplicate.

Polysome profile analysis

$2x10^6$ U2OS T/O MYC parental and LARP1 KO cells were plated in 150mm dish and treated with 2µg/ml doxycycline for 48 hours. For experiments with P493-6 cells, $4x10^6$ cells were seeded and treated with tetracycline for 72h, washed twice to remove tetracycline and then collected at the indicated timepoints. Cycloheximide (CHX) was added to the medium at a concentration of 100 µg/ml at 37°C for 5 min before collection. Cells were washed twice with cold PBS supplemented with CHX, scraped on ice, and pelleted by centrifugation at 3000 rpm for 3′ in cold. Cell pellets were resuspended in 220 µl of hypotonic lysis buffer [1.5 mM KCl, 2.5 mM MgCl2, 5 mM tris HCl (pH 7.4), 1 mM dithiothreitol (DTT), 1% sodium deoxycholate, 1% Triton X-100, and CHX (100 µg/ml)] supplemented with mammalian protease inhibitors (Sigma-Aldrich) and RNase inhibitor (NEB) at a concentration of 100 U/ml and left in ice for 10'. Cell lysates were cleared of debris and nuclei by centrifugation for 5′ at 13,000 rpm. Protein concentrations were determined by BCA assay, and equal amount of polysomal lysate (800 to 1000ng, depending on the experiment) was loaded on 10 to 50% sucrose linear gradients generated with a BIOCOMP gradient master and containing 80 mM NaCl, 5 mM MgCl2, 20 mM tris HCl (pH 7.4), 1 mM DTT, and RNase inhibitor (10 U/ml). Gradients were centrifuged on a SW40Ti rotor for 2 hours and 55 min at 35,000 rpm and then analyzed on a BIOCOMP gradient station and collected in 13 fractions ranging from light to heavy sucrose. Fractions were supplemented with SDS at a final concentration of 1% and placed for 10 min at 65°C. To each fraction was added 1 ng of firefly luciferase mRNA. Fractions were then used to purify RNA or protein according to the analysis of interest, respectively RT-qPCR or dot blot.

RT-qPCR of total mRNAs from sucrose fractions was performed by a first purification of RNA by means of phenol-chloroform extraction and precipitation with isopropanol, followed by reverse transcription. mRNA quantification was normalized to firefly mRNA.

For fot blot analysis, 180µl of each fraction were resuspended in 20µl of protein loading buffer, treated at 95°C for 5' and then loaded in Bio-Dot apparatus (Biorad). Vacuum was applied to allow the transfer of protein to PVDF membrane, which was subsequently stained with amido black solution, then blocked with 5% dry-fat milk in TBS-T and finally probed with the LARP1 antibody (Bethyl). Immunoblots were developed using secondary horseradish peroxidase-coupled antibodies (Polyclonal swine anti-rabbit Agilent, CA, USA) and an enhanced chemiluminescence kit (GE Healthcare).

De novo protein analysis

For Click-iT de novo synthesis analysis, $1.8x10^5$ U2OS T/O MYC parental and LARP1 KO cells were plated in 6-well plates and treated with doxycycline 2µg/ml for 24 hours. CHX treatment was used as negative control. AHA labeling was performed for 1h and AHA-labeled proteins were chemically processed according to manufacturer's protocol to ultimately perform "click" reaction with biotin-alkyne–conjugated proteins according to manufacturer's instructions. Click-iT® Protein Reaction Buffer Kit (Invitrogen) used.

Cell cycle analysis

$5x10^4$ U2OS T/O MYC parental and LARP1 KO cells were plated in 12-well plates and treated with doxycycline 2µg/ml for 48 hours. After treeatment cells were trypsinized, washed twice with cold PBS and fixed with cold 70% ethanol solution. Cells were resuspended in a propidium iodide solution (PBS, 0.1% NP40, RNAse A 20 µg/mL, propidium iodide 40 µg/mL) for 30' at 37°C and stored at -20°C for 15 days maximum. FACS analysis was performed using FACSCanto II (BD biosciencies, CA, USA) using BD FACSDiva™ software (BD biosciences). Cell cycle data were analyzed using ModFit LT software (Verity Software House) and analysis of subdiploid phases was carried out with FlowJo™ Software Version 10.9.0 (BD biosciencies) according to (Riccardi & Nicoletti, 2006)

Statistical analysis

Statistical analysis was performed using GraphPad Prism V9.0. Data are presented as means ± SEM. Comparisons were performed with a two-way analysis of variance (ANOVA) multiple comparisons test. Statistical signficance was considered for p values (P) below 0.05 ($p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$)

Figures in the introduction section, R-7b, R-15a and D-1 were all created with Biorender

RESULTS

RESULTS

1. Oncogenic MYC requires LARP1 expression to sustain cell proliferative capacity

According with the role of MYC as a master regulator of ribosome biogenesis, we previously verified that inducing oncogenic levels of MYC results in a progressive increase of ribosomal proteins (RPs) expression both at the RNA and protein level (Morcelle et al., 2019). Given the pivotal role of LARP1 in preserving and translating the mRNA regulon for ribosome biogenesis including RPs (Fuentes et al., 2021; Gentilella et al., 2017), we sought to investigate whether the transcriptional program driven by oncogenic MYC requires the assistance of LARP1 to have it executed. In this regard, to mimic the oncogenic activation orchestrated by MYC, we utilized U2OS osteosarcoma cells, characterized by low endogenous MYC levels, and carrying a tetracycline-inducible (T/O) transgene enforcing MYC over expression upon doxycycline administration (Walz et al., 2014). To this end we measured the proliferative capacity by crystal violet staining of U2OS cells in normal growing condition (-dox) or upon MYC induction (+dox) and as a function of LARP1 expression (Walz et al., 2014). To reduce endogenous LARP1 levels, we transfected U2OS T/O MYC cells with an siRNA against LARP1 transcript or with a non-targeting siRNA sequence (siNT) that served as control. Western blot analysis confirmed a strong reduction of LARP1 protein (Fig. R-1a).

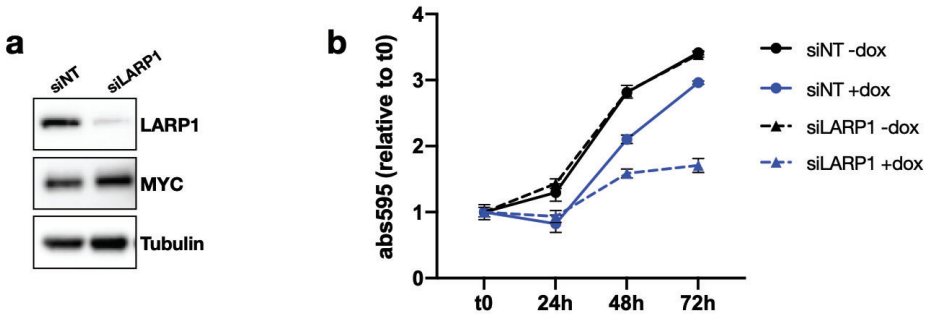

Fig. R-1 **| LARP1 knockdown affects MYC-induced cell proliferation in U2OS cells a)** Protein lysates of U2OS T/O MYC cells transfected for 24 hours with a non-targeting siRNA (siNT) or an siRNA against LARP1 mRNA (siLARP1) were immunoblotted with anti-LARP1, anti-MYC and anti-α-tubulin antibodies. **b)** Proliferation of U2OS T/O MYC cells transfected for 24h with the indicated siRNAs and further treated with doxycycline for the indicated timepoints was measured by crystal violet staining as described in Materials and Methods.

24 hours after transfection cells were maintained in normal growing medium or supplemented with doxycycline to induce the over expression of MYC. Measurement of crystal violet staining over time revealed that in absence of MYC induction depletion of LARP1 (siLARP1 -dox) does not affect cell number when compared to its control (siNT -dox). Importantly, almost a 50% decrease in cell proliferation was observed in siLARP1 cells upon MYC overexpression (siLARP1 +dox), as compared to the doxycycline-treated control cells (siNT +dox), indicating that a genetic interaction between MYC and LARP1 does exist in an acute knockdown setting (Fig. R-1b).

To corroborate this result, we sought to investigate this interaction upon a chronic depletion of LARP1. To this end we generated by Crispr-Cas9 an isogenic U2OS T/O MYC cell line knockout for LARP1 (clone #2, Fig. R-2a). We repeated the cell survival/proliferation assay by comparing parental (Par) and LARP1 KO (KO) U2OS T/O MYC in low MYC (-dox) and high MYC expression (+dox) and confirmed the reduction in survival/ proliferation of LARP1 KO cells under MYC-induction compared to parental cells (Fig. R-2b).

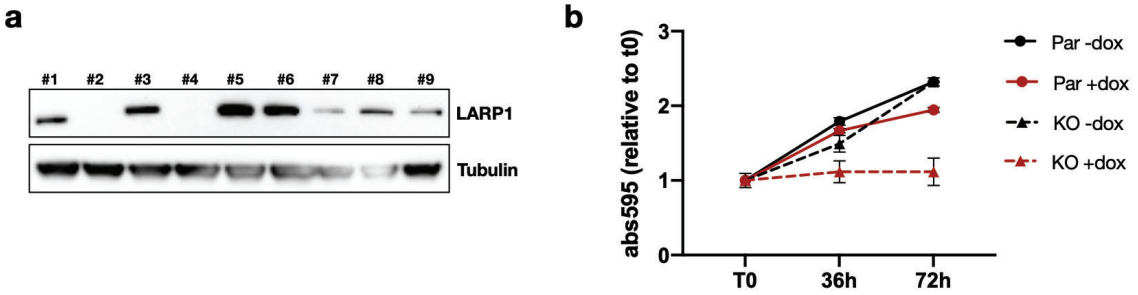

Fig. R-2 | **LARP1 stable depletion affects MYC-driven cell proliferation in U2OS T/O MYC cells a)** Screening by western blot analysis of LARP1 levels in the U2OS T/O MYC LARP1 KO clones generated by Crisr-Cas9 as described in Materials and Methods. α-Tubulin as loading control. **b)** Proliferation of U2OS T/O MYC parental and LARP1 KO cells treated with doxycycline for the indicated timepoints was measured by crystal violet staining as described in Materials and Methods.

As previously described (Morcelle et al., 2019; Walz et al., 2014), a small decrease in proliferation is detected upon MYC induction, and this is most likely due to cell-intrinsic anti-oncogenic responses triggered by oncogenic MYC (Hemann & Lowe, 2006). Considering that reduction of cell proliferative rate was worsen by LARP1 ablation, we hypothesized an impairment in cell cycle progression. However, propidium iodide

staining and fluorescence cell sorting (FCS) revealed that upon MYC over expression LARP1 KO cells maintained their ability to progress through the cell cycle as the parental cells, as revealed by the increase in S-phase entry and in tetraploid accumulation (Fig. R-3a). Intriguingly, in LARP1 KO cells this was paralleled by a remarkable enrichment of hypodiploid cells (Fig. R-3b), usually associated with apoptosis, that could explain the reduction in cell numbers observed in this setting (Fig. R-1b, R-2b).

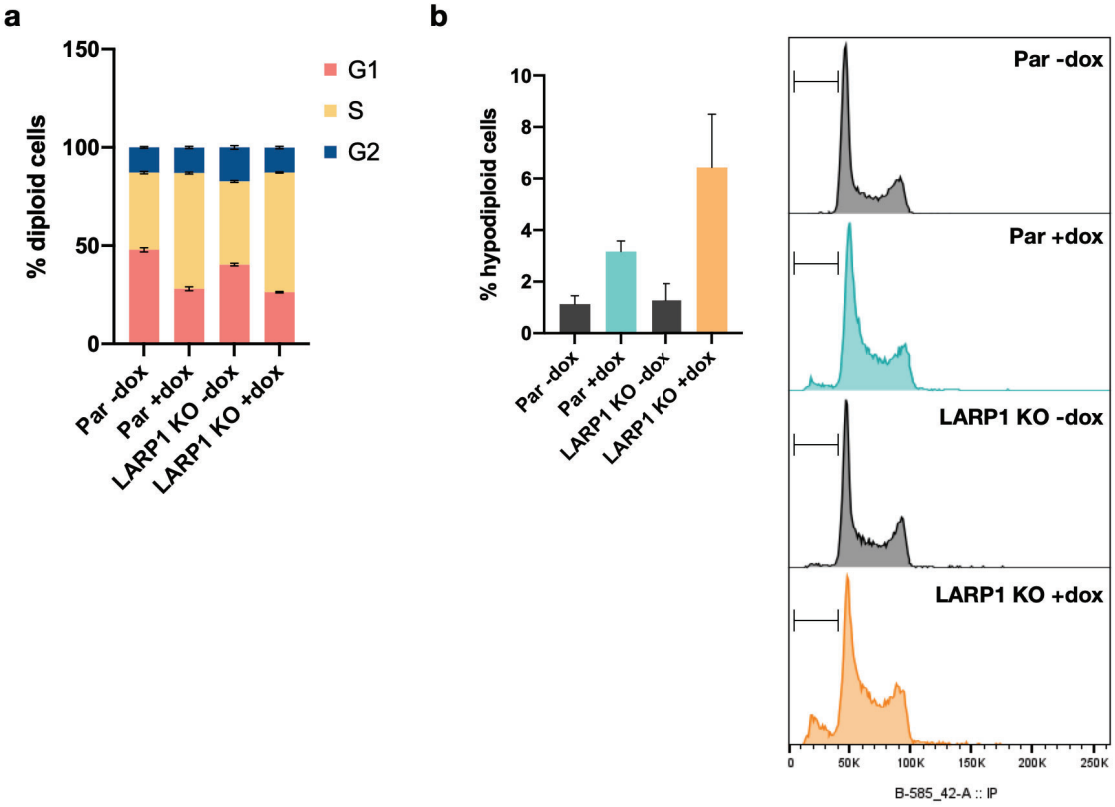

Fig. R-3 | **Effect of LARP1 depletion over cell cycle progression in U2OS cells. a)** Cell cycle distribution of U2OS T/O MYC parental and LARP1 KO cells in normal growing condition (- dox) or upon 48 hours of MYC over expression (+ dox) **b)** Quantification of hypodiploid U2OS T/O MYC parental and LARP1 KO cells treated as in a).

To further confirm the relevance of LARP1 in a MYC oncogenic setting, we took advantage of a different cell system suitable to dissect this functional interaction. P493-6 cells are Epstein-Barr virus–immortalized human B cells that carry a tetracycline (tet)-repressible allele of MYC and represent a model of B-cell lymphoma. Different from U2OS, the proliferation of P493-6 cells depends on MYC over expression as previously reported (Schuhmacher et al., 1999). In these cells, tetracycline administration was shown to reduce the levels of MYC protein ultimately leading to cell cycle arrest

(Schuhmacher et al., 1999). Re-expression of MYC by removal of the tetracycline rescues the proliferation rate in a time window of 48-72 hours.
We therefore generated stable P493-6 cells in which the levels of LARP1 were reduced by means of an shRNA directed against its transcript. LARP1 protein ablation was confirmed by western blot in comparison to non-infected cells and a stable control cell line expressing for a non-targeting shRNA (shScr, Fig. R-4).

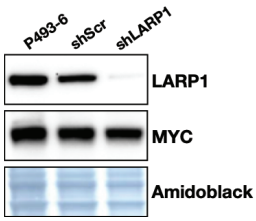

Fig. R-4 | **Generation of stable P493-6 cell line depleted for LARP1 levels** Protein lysates of P493-6 cells and P493-6 stable cells transduced with a lentivirus expressing a non-targeting shRNA (shScr) or an shRNA against LARP1 analyzed by western blot against LARP1 and MYC proteins. Amido black staining served as loading control.

To evaluate the proliferative impact of LARP1 expression under an oncogenic MYC stimulus, cells were first cultured with tetracycline to blunt MYC expression as well as cell proliferation (t0, Fig. R-5a,b). Re-expression of MYC by culturing the cells in medium devoid of tetracycline showed in P493-6 shLARP1 cells a reduction in their proliferative rate when compared to their not-targeting control, starting from 96 hours of tet withdrawal, thus confirming the observation obtained in U2OS cells about the role of LARP1 for cell proliferation upon MYC oncogenic levels.

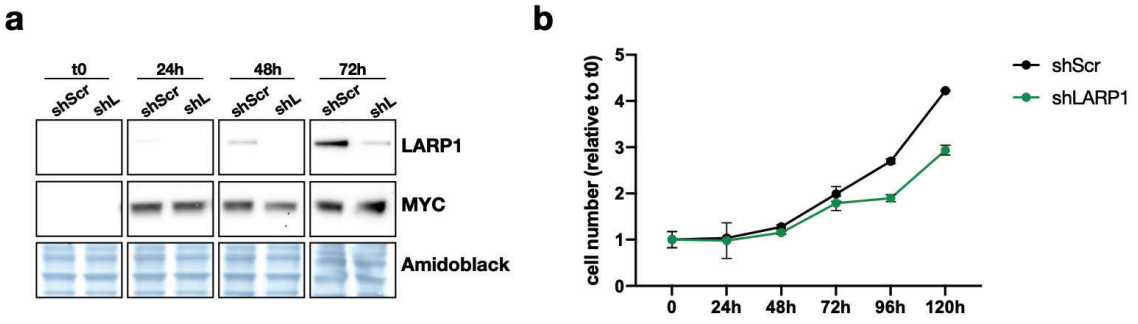

Fig. R-5 **| Depletion of LARP1 impairs MYC-induced cell proliferation in P493-6 cells a)** Protein lysates assayed for LARP1 and MYC expression from shScr and shLARP1 P493-6 cells treated with tetracycline for 72h (t0) and devoid of tetracycline for the indicated timepoints. Amidoblack as loading control. **b)** cell number was determined in the same samples from a).

2. Oncogenic MYC sustains LARP1 expression to translate ribosomal protein mRNAs and boost protein synthesis

Given the positive role of LARP1 in sustaining cell proliferative rates in a MYC oncogenic setting, we rationalized the possibility for MYC to upregulate LARP1 expression. Measurement of LARP1 mRNA and protein in U2OS T/O MYC cells confirmed the increase in LARP1 expression upon MYC induction (Fig. R-6a,b).

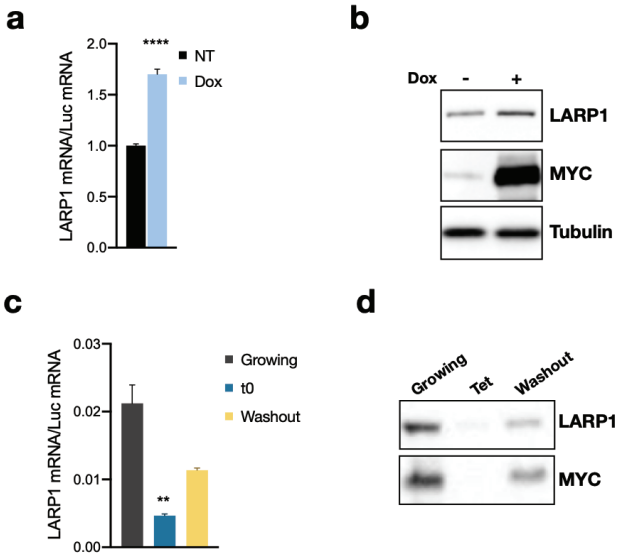

Fig. R-6 | **Oncogenic MYC controls LARP1 expression a)** LARP1 mRNA quantified by RT-qPCR and normalized by cell number (Luciferase mRNA) in U2OS T/O MYC cells treated with doxycycline for 48 hours **b)** Protein lysates from U2OS cells treated as in a) were assayed by western blot with anti-LARP1, anti-MYC and anti-Tubulin antibodies **c)** LARP1 mRNA quantified by RT-qPCR and normalized by cell number (Luciferase mRNA) in P493-6 cells grown in normal medium, after 72h tetracycline treatment (t0) and 48h after washout of the drug (Washout) **d)** Protein lysates of P493-6 cells treated as in c) were assayed by western blot with anti-LARP1 and anti-MYC antibodies.
Statistical comparisons were performed with a two-way analysis of variance (ANOVA) multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

In agreement with this, the reduction of MYC expression in P493-6 cells after 72 hours of tetracycline administration was accompanied by a concomitant decrease of LARP1 transcript and protein. Moreover, the recovery of MYC expression by depriving tetracycline from the medium was paralleled by the rescue of LARP1 mRNA and protein (Fig. R-6c,d).

Altoghether, the results above confirmed that MYC stimulates LARP1 expression to sustain cell proliferation. We sought to uncover the molecular mechanism that support

this phenotype. In this regard, we reasoned that, in addition to the described role in preserving the cellular anabolic capacity under restrictive metabolic constraints, LARP1 could confer a translational priority to RP mRNAs and the whole 5'TOP family in a MYC oncogenic setting in order to increase the ribosome production and hence to sustain the high anabolic demand. To address whether LARP1 is necessary for MYC to activate RPs expression, we used the U2OS T/O MYC cells which were already described to boost RPs synthesis upon doxycycline treatment (Morcelle et al., 2019). In line with the hypothesis, depletion of LARP1 prior to MYC over-expression mitigated the RPs upregulation, as shown by the levels of RPL11 (L11), RPL5 (L5), RPS6 (S6) and RPS19 (S19), suggesting that LARP1 is critical for MYC-driven production of ribosomal components (Fig. R-7)

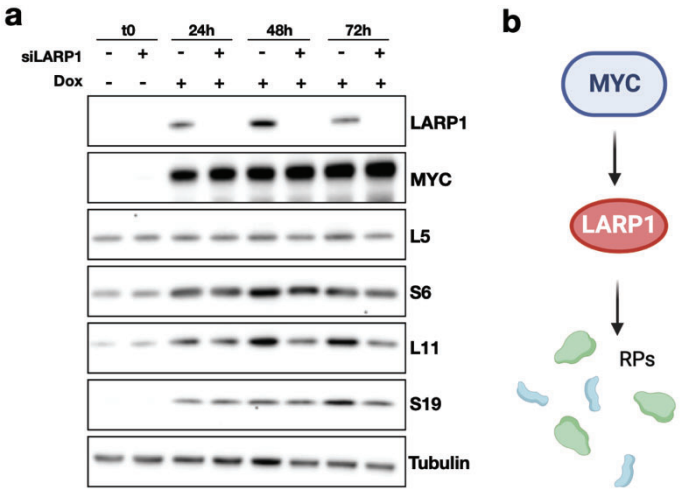

Fig. R-7 | **LARP1 sustains MYC-induced RPs synthesis a)** U2OS T/O MYC cells knocked-down for LARP1 24h prior to doxycycline treatment administered for the indicated time. Protein lysates were assayed by western blot with antibodies against LARP1, MYC and Ribosomal Proteins L5, S6, L11, S19 and α-Tubulin for normalization control **b)** Schematic representation of MYC-driven LARP1 expression to support RPs synthesis.

According with the roles of MYC as transcriptional activator of RP genes and LARP1 as stabilizer for RP mRNAs, this last evidence opened the possibility that the up regulation of LARP1 by MYC over expression is required to sustain the RP synthesis, according to the model of 40S-LARP1 complex to turn ON and OFF the family of 5'TOP mRNAs. Polysome profile analysis of U2OS T/O MYC cells evidenced that the increase of LARP1 in whole cells extracts observed in parental cells corresponds to an augmented

association of LARP1 protein with the small and high polysomes as well as a higher mean polysome size (Fig. R-8).

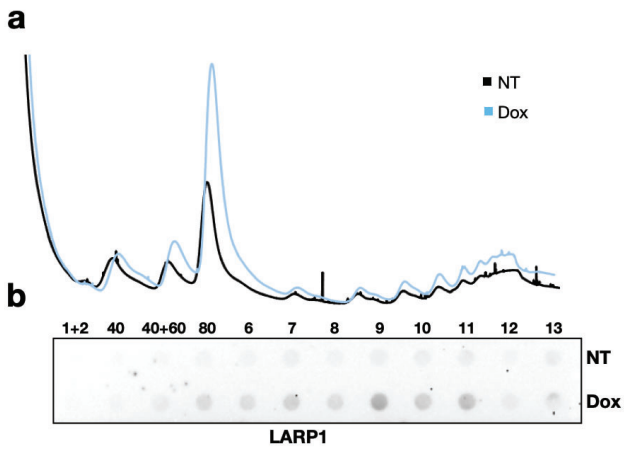

Fig. R-8 | **LARP1 co-sedimentation with non-polysomal and polysomal fractions in MYC-over expressing U2OS cells a)** U2OS T/O MYC parental cells were treated with doxycycline for 48h. Polysomal lysates were resolved by polysome profiling on 10%-50% linear sucrose gradients as described in Materials and Methods. **b)** 20% volume of sucrose gradient fractions as in Fig.R8a was subjected to dot blot analysis with an anti-LARP1 antibody.

Importantly, in LARP1 KO cells MYC over expression did not increase the actively-translating ribosomes (namely polysomes), as observed in parental cells upon MYC induction (Fig. R-9a,c).

Activation of the translational activity could help to rationalize the up-regulation of Ribosomal Protein levels upon MYC overexpression (Fig. R-7). The increased association of LARP1 with small and higher polysomes suggested that RP mRNAs are translationally activated by MYC over expression. To test this possibility, we measured the translational engagement of RP transcripts by RT-qPCR on sucrose gradients fractions. Measurement of RPL11 and RPS6 mRNAs in parental cells showed MYC overexpression to induce a huge increase of these 5'TOP mRNAs in translating polysomes, in agreement with the boost of RP proteins previously observed (Fig. R-9b). In addition, a slight increase of 5'TOP mRNAs levels was detected also in non-polysomal 40S containing fractions, mirroring the involvement of the 40S-LARP1 complex. Interestingly, this distribution was characteristic of 5'TOP mRNAs. Non-TOP mRNAs such as ß-Actin mainly associated with large polysomes, most likely due to the increase in protein synthetic capacity of the cells.

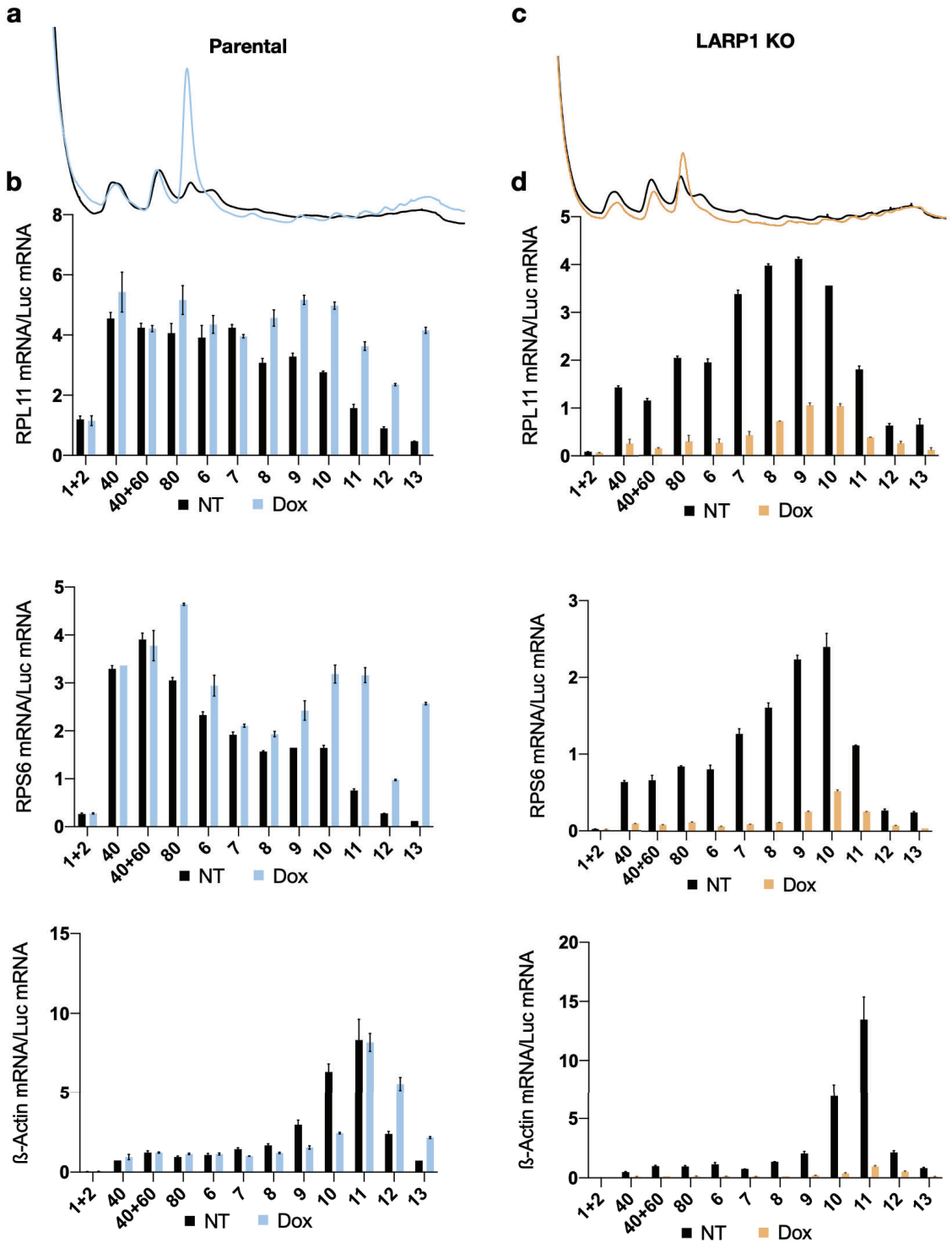
a
Parental
b
RPL11 mRNA/Luc mRNA
RPS6 mRNA/Luc mRNA
β-Actin mRNA/Luc mRNA
1+2
40
40+60
80
6
7
8
9
10
11
12
13
NT
Dox
c
LARP1 KO
d

Fig. R-9 **| Effect of LARP1 depletion over RP mRNAs translational engagement in U2OS cells** U2OS T/O MYC parental (**a**) and LARP1 KO (**c**) cells were treated with doxycycline for 48h. Polysomal lysates were resolved by polysome profiling as described in Materials and Methods**.** RPL11, RPS6 and β-actin mRNAs distributions across the gradient were evaluated in each fraction by real-time qPCR as described in Materials and Methods for parental (**b**) and LARP1 KO (**d**) cells.

In line with our hypothesis, measurement of 5'TOP mRNAs levels in sucrose fractions derived from LARP1 KO cell upon the same conditions revealed that the absence of LARP1, although did not affect RP mRNAs levels and distribution in untreated cells, dramatically reduced their content under MYC overexpression (Fig. R-9d). Overall, these results suggest the role of LARP1 in promoting both RP mRNAs translational efficiency and stability under MYC induction.

To corroborate this finding, we tested the mechanistic model in P493-6 cells in which LARP1 parallels MYC expression (Fig. R-6c,d). We first monitored the changes in RP mRNAs in these cells according to MYC levels. Along with LARP1, tetracycline-mediated MYC suppression (T0) drastically reduced RPs and ß-Actin mRNA levels. However, reconstituting MYC expression 48 hours after removal of tetracycline (washout) showed the recovery of only RPs mRNAs expression, while maintaining the levels of the non-5'TOP mRNA ß-Actin almost unchanged (Fig. R-10).

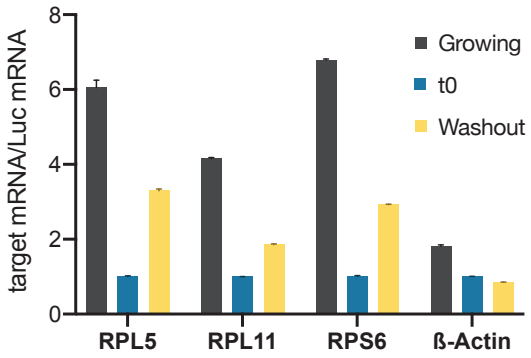

Fig. R-10 **| MYC-driven RP mRNAs levels parallel LARP1 expression** Ribosomal Proteins L5, L11 and S6 and ß-Actin mRNAs quantified by RT-qPCR and normalized by cell number (Luciferase mRNA) P493-6 cells grown in normal medium, after 72h tetracycline treatment (t0) and 48h after washout of the drug.

To better decipher the implications of LARP1 in this mechanism, we studied by sucrose gradient fractionation the polysome/non-polysome distribution of these cells in the same

experimental setting (Fig. R-11a). As expected, we observed a drastic decrease in large polysomes after MYC inhibition (T0), which are partially rescued 48 hours after the washout of tetracycline. Measuring the levels of RPL5 and RPS6 5'TOP mRNAs, we observed a homogenous distribution between non-polysomal and polysomal fractions under growing conditions, paralleled by a similar distribution of LARP1 protein. Upon MYC inactivation (t0), 5'TOP mRNAs along with LARP1 protein underwent a massive decrease as well as non-TOP mRNA ß-Actin. Importantly, upon reconstitution of MYC expression (washout), 5'TOP mRNAs and LARP1 protein recovered their levels with translating polysomes. The non-TOP mRNA ß-Actin, however, failed to rescue its levels, at least at this early time point.

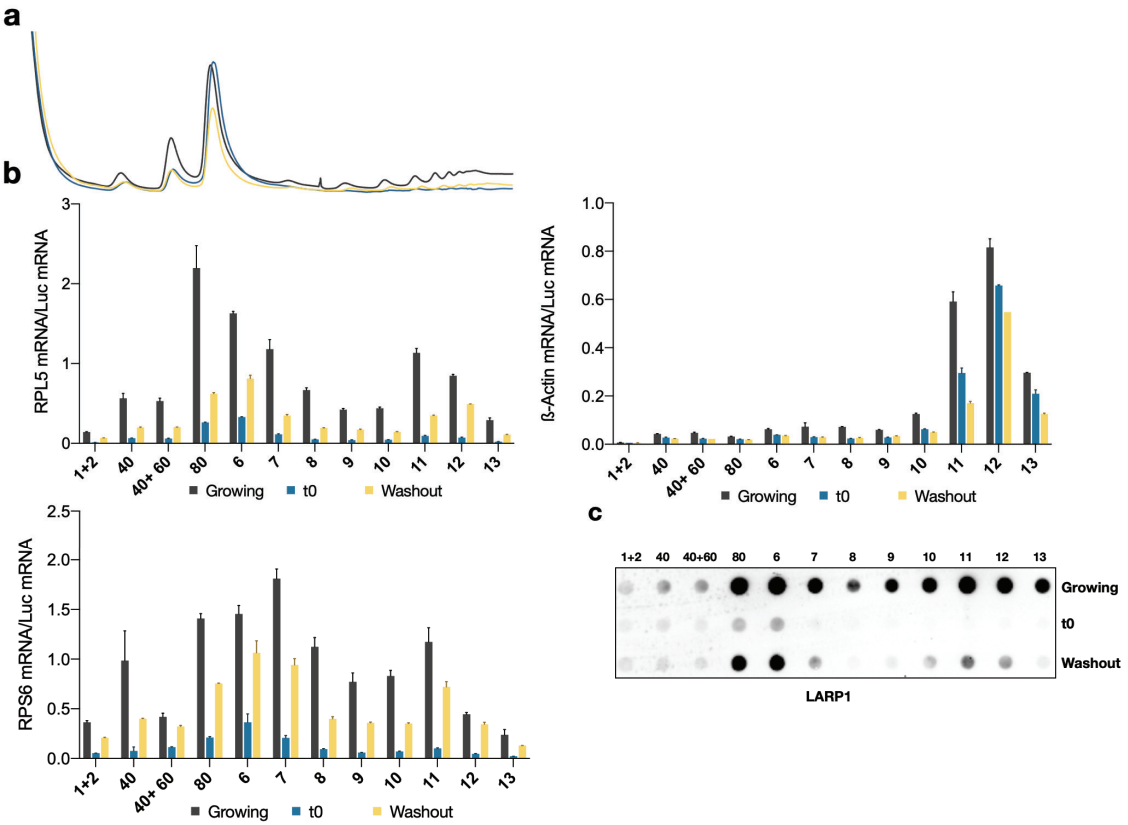

Fig. R-11 | **Polysome Profile analysis of RP mRNAs and LARP1 protein in P493-6 cells a)** P493-6 parental cells grown in normal medium, after 72h tetracycline treatment (t0) and 48h after washout of the drug. Polysomal lysates were resolved by polysome profiling as described in Materials and Methods **b)** RPL5, RPS6 and β-actin mRNAs distributions across the gradient were evaluated in each fraction by real-time qPCR as described in Materials and Methods **c)** 20% volume of sucrose gradient fractions as in Fig.R11a was subjected to dot blot analysis with an anti-LARP1 antibody.

The findings above suggest that by controlling LARP1 expression MYC can stabilize RP mRNAs and promote their translation upon MYC-dependent proliferation. Consistent with this, the total levels of RPS6 and RPL11 mRNAs in shLARP1 cells were not reconstituted even 96h after MYC re-induction, while having little to no effect on ß-Actin mRNA. Moreover, LARP1 expression appeared to stabilize the basal levels of RP mRNAs, an effect that is not detected for the non-TOP mRNA ß-Actin (Fig. R-12).

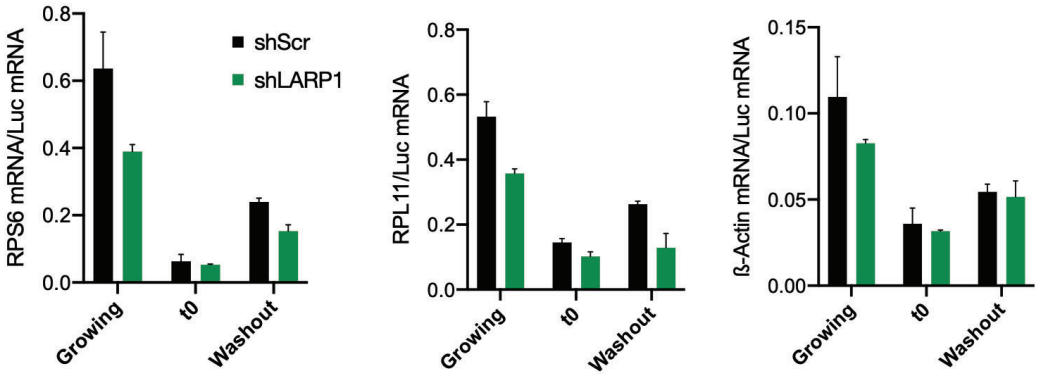

Fig. R-12 | **Effect of LARP1 depletion in P493-6 cells over RP mRNAs levels** Ribosomal Proteins S6, L11 and ß-Actin mRNAs quantified by RT-qPCR and normalized by cell number (Luciferase mRNA) in stable P493-6 shScr and shLARP1 cells grown in normal medium, after 72h tetracycline treatment (t0) and 96 hours after washout of the drug.

That oncogenic MYC determined an up-regulation of RP mRNAs translation in a LARP1-dependent manner suggested an increase in ribosome biogenesis and protein synthesis. We reasoned that this MYC-LARP1 functional interaction should be reflected at the level of global protein synthesis. To test this possibility, nascent proteins were labeled with the methionine analog AHA (azyde-homoalanine) amenable for orthogonal click chemistry with an alkyne-biotin moiety, that allows the detection of newly synthesized proteins as a readout of protein synthesis rate. 24 hours after doxycycline-induced MYC over expression, nascent proteins of U2OS T/O MYC parental and KO were labeled for 60 minutes with AHA. The global protein synthesis rate was almost doubled in U2OS parental cells, correlating also with the increase in polysomal size observed in figure R-13. Strikingly, this was not the case of LARP1 KO cells, confirming that LARP1 is needed to sustain the MYC anabolic program.

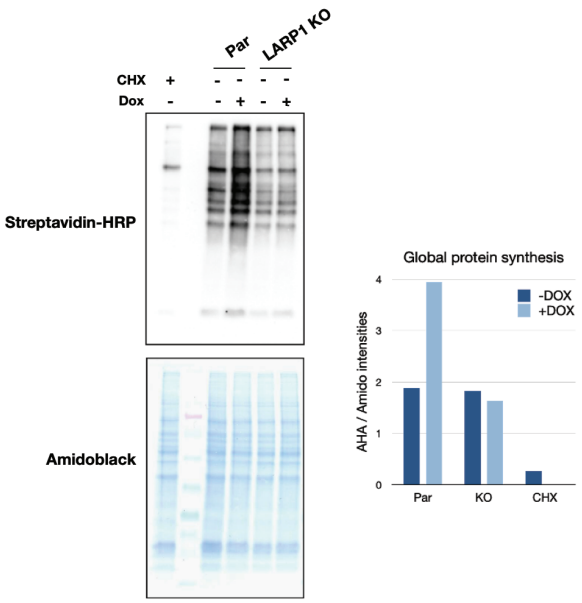

Fig. R-13 **| LARP1 sustains MYC-induced protein synthesis** U2OS T/O MYC parental and LARP1 KO cells were treated with doxycycline for 24h and incubated with the methionine analog AHA as indicated in methodology. Amido black served as normalization control.

3. miR-26 expression controlled by MYC mediates LARP1 expression and cell proliferative capacity

To determine the mechanism by which MYC supports LARP1 expression, we first hypothesized that LARP1 could be a transcriptional target of MYC, however metadata analysis of ChIP-seq dataset carried out in P493-6 cells in low and high MYC expression didn't support this possibility. We then sought for a MYC transcriptional target responsible to connect MYC to LARP1. A specific category controlled by MYC in P493-6 cells is constituted by micro-RNAs or miRNAs, the expression of which is transcriptionally repressed by high MYC levels, among which the miR26a and miR26b (T.-C. Chang et al., 2008). Interestingly, we performed an *in silico* analysis of the ~3.5 Kb LARP1 3'UTR and observed an enrichment for highly conserved interspersed regions containing clusters of miRNA binding sites. Among them, we detected two putative targets of miR-26a and miR-26b, representative members of the oncosuppressor miR-26 family, differing in the mature miRNA sequence by 2 nucleotides only (Fig. R-14).

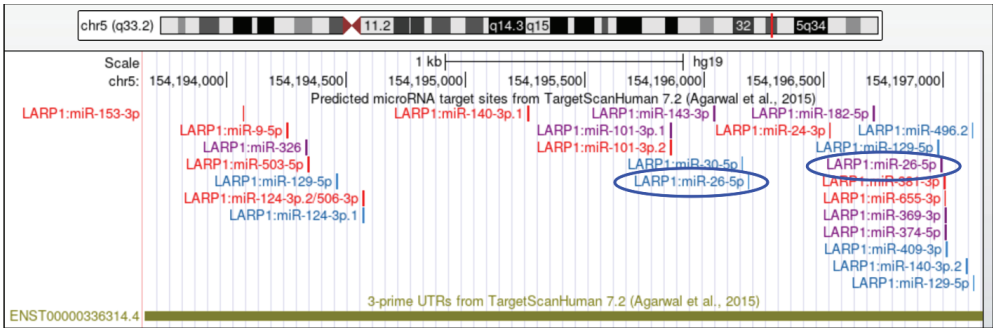

Fig. R-14 **| LARP1 3'UTR is enriched in microRNAs binding sites** Results of microRNA binding sites prediction by TargetScan in LARP1 3'UTR. Upstream and downstream binding sites for miR-26 are circled in blue.

We therefore tested the hypothesis that MYC might support LARP1 levels by silencing miR-26 (Fig. R-15a). To address this question, we first checked that the MYC-mediated inhibition of miR-26 in P493-6 cells observed by Chang et al. was reproduced also in our MYC-inducible U2OS cell system. Northern blot analysis of total mRNA levels confirmed the reduction in miR-26a and miR-26b expression expected upon doxycycline treatment (Fig. R-15b). To test the activity of miR-26 over LARP1 expression, we reconstituted miR-26 levels in MYC-induced U2OS cells by delivering exogenous miR-26a and miR-26b molecules. In line with our hypothesis, the presence of the miR-26 mimic impaired the ability of MYC to increase LARP1 expression. In an opposite approach, transfecting U2OS T/O MYC cells with a miR-26 inhibitor led to a rescue of LARP1 protein expression under low MYC levels, thus showing that LARP1 is under the control of miR-26 (Fig. R-15c).

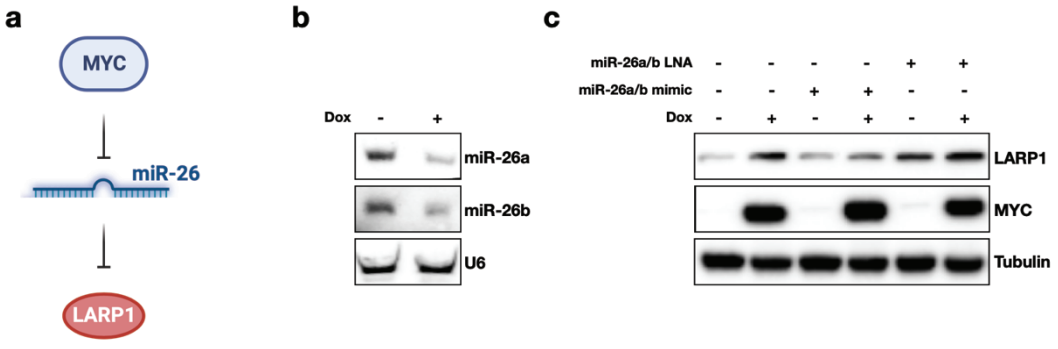

Fig. R-15 **| miR-26 controls LARP1 expression in U2OS cells a)** Schematic representation of the regulatory axis linking MYC, miR-26 and LARP1 **b)** U2OS T/O MYC cells treated for 72h with doxycycline. Total RNA was analyzed by Northern blot and hybridized with biotinylated probe directed against miR26-a, miR-26b and U6 nucleolar RNA as normalizer. **c)** U2OS T/O MYC cells transfected with miR-26a and miR26b mimic or inhibitor (LNA) overnight and then treated with doxycycline for 48h. Protein lysates assayed by western blot with antibodies against LARP1, MYC and tubulin as loading normalizer.

This evidence suggested the existence of a MYC–miR26–LARP1 axis according to which MYC supports LARP1 expression by inhibiting miR-26 expression. Phenotypically, this was further verified by cell proliferation assay in the presence of miR-26 mimic to test whether miR-26a and miR-26b reconstitution recapitulated LARP1 knockdown in the context of MYC overexpression as observed in Fig. R-1b. Like LARP1 knockdown, the expression of the miR-26 mimic alone did not affect U2OS cells proliferation rates, however it reduced cell numbers under MYC-induction, thus phenocopying LARP1 KO cells of figure Fig. R-2b. To verify that this effect was LARP1-driven, we replenished miR-26 levels in the LARP1 KO isogenic cells and observed no differences upon MYC overexpression as compared to its control (siNT, Fig. R-16)

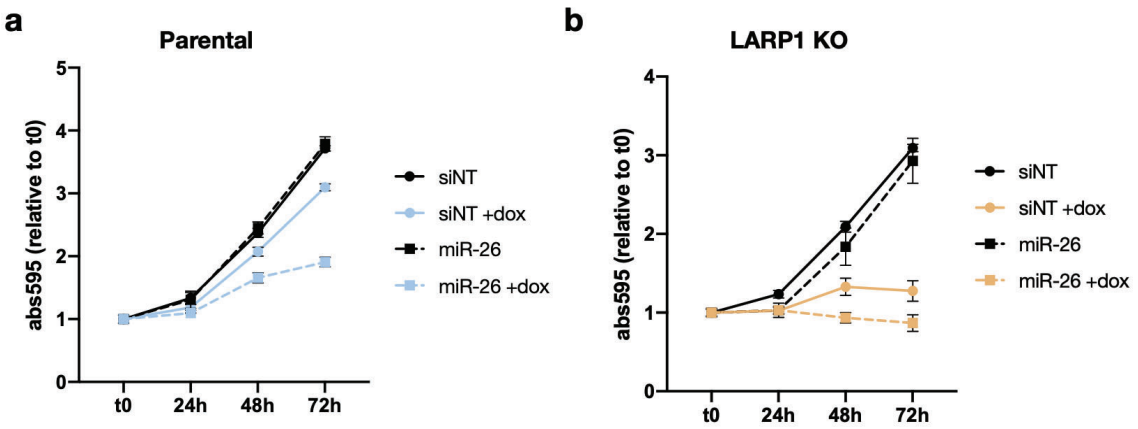

Fig. R-16 | **miR-26 targeting of LARP1 affects cell proliferation upon MYC over expression a)** Proliferation of U2OS T/O MYC parental cells transfected with miR-26a+miR-26b 24 hours prior to doxycycline treatment was measured for the indicated timepoints with crystal violet assay as described in Materials and Methods **b)** Proliferation of U2OS T/O MYC LARP1 KO cells treated as in a) measured by crystal violet assay

4. MYC-miR-26-LARP1 axis in colorectal cancer cells

MYC-driven tumorigenesis is a hallmark of many cancers, including colorectal (Schaub et al., 2018). CMS2 colorectal cancer subtype are characterized by hyperactive ribosome biogenesis and sustained MYC levels downstream of Wnt– β-Catenin/TCF4 axis (Guinney et al., 2015). We sought to investigate the existence of this connection between MYC-miR26-LARP1 in a system characterized by high levels of MYC such as colorectal cancer cell lines (Taylor et al., 1992), namely HCT116 and RKO. To verify that LARP1 expression is regulated by MYC, we suppressed MYC expression either by RNA interference or by pharmacological means. To this end, we used the bromodomain inhibitor JQ1, which suppresses the selective transcription of a set of genes including MYC (Delmore et al., 2011). RT-qPCR determination of LARP1 mRNA and western blot analysis of LARP1 protein confirmed that inhibition of MYC induced a drastic decrease of LARP1 expression (Fig. R-17).

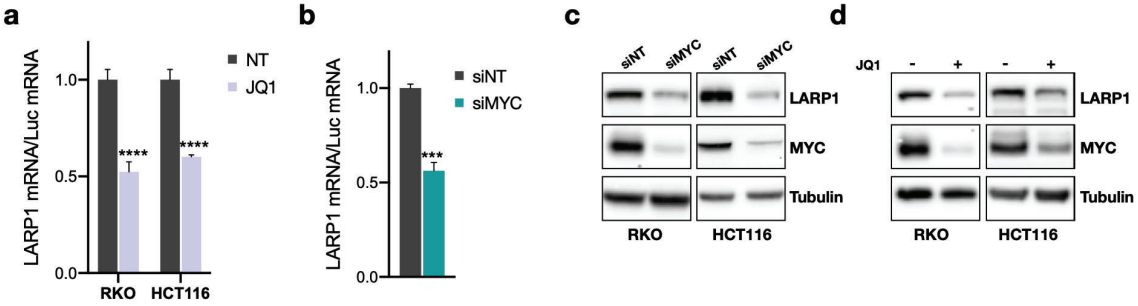

Fig. R-17 **| Oncogenic MYC sustains LARP1 levels in colorectal cancer cells a)** LARP1 mRNA quantified by RT-qPCR and normalized by cell number (Luciferase mRNA) in RKO and HCT116 cells treated for 48h and 72h respectively with the pharmacological inhibitor of MYC expression JQ1 (1μM) **b)** LARP1 mRNA quantified by RT-qPCR and normalized by cell number (Luciferase mRNA) in HCT116 cells knocked-down for MYC. **c)** Protein lysates of RKO and HCT116 cells knocked-down for MYC for 72h were assayed by western blot with anti-LARP1, anti-MYC and anti-Tubulin antibodies. **d)** Protein lysates of RKO and HCT116 cells treated as in a) were assayed by western blot with anti-LARP1, anti-MYC and anti-Tubulin antibodies
Statistical comparisons were performed with a two-way analysis of variance (ANOVA) multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

To check that also in this system miR-26 regulation by MYC inversely correlates with LARP1 expression, we first inhibited MYC expression in HCT116 and RKO cells by JQ1 for 72 hours and confirmed by northern blot analysis that miR-26a levels inversely correlated with MYC expression (Fig. R-18a). Importantly, transfecting miR-26a and miR-26b alone or in combination proved to decrease endogenous LARP1 levels at a similar extent than cells transfected with an siRNA against LARP1 (Fig. R-18b).

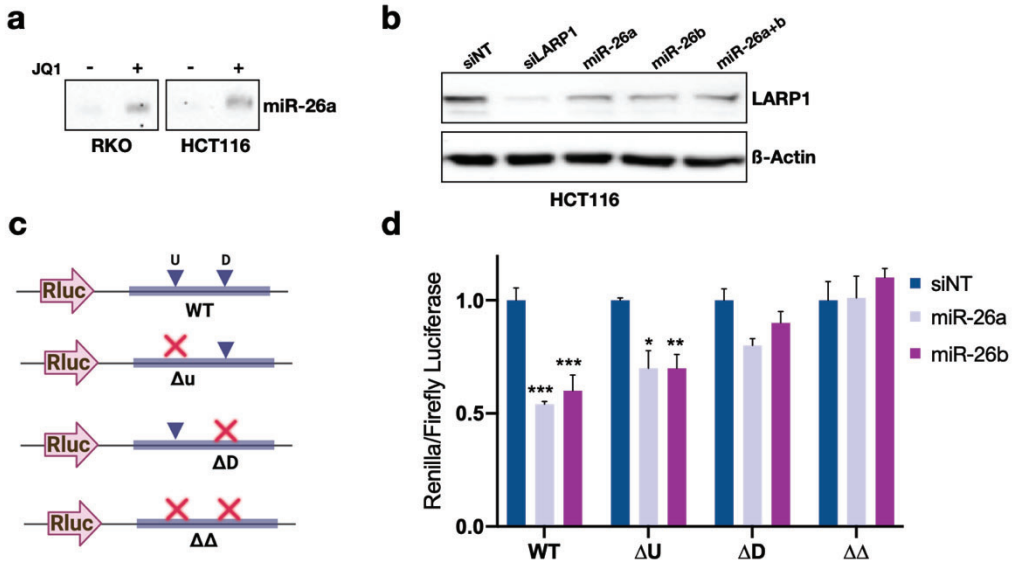

Fig. R-18 | **miR-26 inhibits LARP1 expression in HCT116 cells a)** RKO and HCT116 cells treated for 72h with 1µM JQ1. Total RNA was analyzed by Northern blot and blotted with biotinylated probe directed against miR26-a **b)** HCT116 parental cells transfected with siRNA directed against LARP1 or miR26-a and -b mimic molecules for 48 hours. Equal amounts of protein lysates were analyzed by western blot and immunoblotted for anti-LARP1 and anti-ß-Actin **c)** Schematic representation of the LARP1 3'UTR reporter constructs used in d) **d)** Dual luciferase assay of HCT116 parental cells transfected with miR26-a and -b mimic molecule at 20nM followed by the introduction of psiCHECK vector encoding for Renilla Luciferase fused to LARP1 3'UTR wild type or mutated in the upstream and/or downstream miR26 target site.

To finally verify the direct targeting of miR-26 on LARP1 3'UTR in its two putative binding sites (hereafter called upstream and downstream miR26 binding sites), we generated a Renilla luciferase reporter plasmid in which the Renilla coding sequence is fused to the full length LARP1 3'UTR (3.5kb) (Fig. R-18c). We monitored the effect of miR-26 on LARP1 3'UTR after transfecting miR26a/b or a control sequence (siNT). The strong reduction of Renilla activity in cells transfected with miR26-a and miR26-b was partially rescued by deletion of one of the two miR-26 binding sites, while the deletion of both the upstream and downstream binding sites in the LARP1 3'UTR fully desensitized Renilla expression to miR-26 expression (Fig. R-18d).

Taken together, these results confirmed the existence of the MYC-miR-26-LARP1 axis also in these cells. In Morcelle et al., the relevance of MYC in promoting cell growth was demonstrated by observing in HCT116 and RKO cells that MYC depletion leads to a significant reduction in cell number, accompanied by a decrease in total RPs mRNAs.

Considering the regulatory role of LARP1 over RP mRNAs, we tested whether a co-depletion of LARP1 and MYC levels would worsen the decrease in cell proliferation observed under MYC ablation alone. To this end, we used HCT116 LARP1 KO cells already available in the laboratory and inhibited MYC by RNA interference. However, cell proliferation assay showed no relevant changes (Fig. R-19), suggesting that a system with constitutive high levels of MYC expression is not dependent on LARP1 to promote uncontrolled tumor growth.

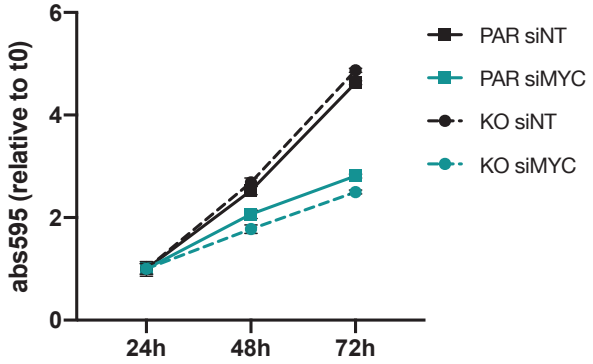

Fig. R-19 | **MYC-driven HCT116 cell growth independent of LARP1 levels**
Proliferation of HCT116 parental and LARP1 KO cells knocked-down in MYC levels (50nM) for the indicated timepoints was measured by crystal violet staining as described in Materials and Methods.

Finally, as a significant percentage of colorectal cancers are initiated by inactivation of the APC tumor suppressor, which leads to deregulation of pro-proliferative WNT signaling and MYC activation, we sought to investigate whether MYC-LARP1 regulation is dependent on the aberrant activation of WNT. To address this question, we used three CRC cell lines (DLD-1, LS174T and SW403 cells) expressing a tamoxifen-inducible dominant-negative TCF4 transcription factor, which revert the aberrant transcriptional program orchestrated by hyperactive WNT (Morral et al., 2020). Gratifyingly, we observed that shutting down the WNT signaling by the addition of tamoxifen for 24 and 48 hours reduced MYC levels in all the three cell lines, accompanied by a robust downregulation of LARP1 protein levels in SW403 and a modest decrease in DLD-1 and LS174T cells (Fig. R-20). These preliminary results suggested that MYC-LARP1 axis could be involved already at early stages of tumor initiation, thus opening a scenario in which LARP1 presence might be necessary not only for MYC-mediated protein synthesis and cell proliferation but also tumorigenesis.

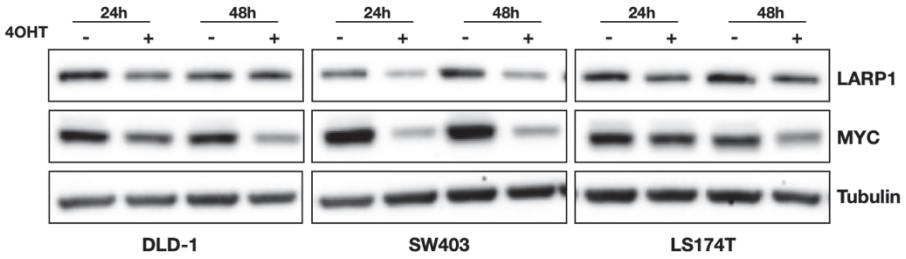

Fig. R-20 | **WNT signaling modulates LARP1 through MYC** Western blot analysis of DLD-1, SW403 and LS174T cell extracts treated with 4-hydroxytamoxifene for the indicated time. Protein lysates immunoblotted with anti-LARP1, anti-MYC and anti-tubulin as loading control.

DISCUSSION

DISCUSSION

One of the hallmarks of cancer cells is aberrant cell growth and proliferation. In this context, the role of protein synthesis has been well established with the very first evidence of a link between cell growth and protein synthesis observed in drosophila. A class of mutants haploinsufficient for translational components, known as *minutes*, indeed displays smaller body size and an overall decrease in protein synthesis of 30% (Boring et al., 1989).

The proto-oncogene MYC, which is one of the most common genetic alterations in cancer, globally controls protein synthesis by regulating the expression of translational components. Importantly, the role of MYC-induced protein synthesis in the setting of cancerous lesions has been widely investigated. Starting from evidences in drosophila, where the growth advantage given by MYC overexpressing cells was lost in *minutes* genetic background (Moreno & Basler, 2004), the impact of MYC-driven translational capacity over cell growth and proliferation was studied also in mammals. Using mice heterozygous for Rpl24 or Rpl38, which display smaller body size and decreased protein synthesis rate compared to the wild-type counterpart, the Ruggero laboratory observed the effect of suboptimal translational activity in a context of MYC tumorigenesis, by crossing the minutes mice with Eμ-Myc mice, in which the aberrant Myc expression in B-Cells gives rise to lymphomagenesis (Barna et al., 2008). In the genetic settings of Eμ-Myc/+;L24$^{+/-}$ and Eμ-Myc/+;L38$^{+/-}$, they observed that the reduction in protein synthesis rate delayed abnormal MYC-induced lymphomagenesis restoring both cell growth as well as cell division of transformed B-Cell to normal levels. Importantly, the significance of protein synthesis in the context of MYC-driven tumorigenesis was underlined by the dramatic increase in overall survival of Eμ-Myc/+;L24$^{+/-}$ and Eμ-Myc/+;L38$^{+/-}$ genetic backgrounds.

MYC-driven tumors are therefore characterized by increased expression of ribosomal proteins that properly refurbish the protein synthetic machinery. Although regulation of RPs expression has been traditionally thought to be exerted by its activity as transcription factor, recent evidence point at the ability of MYC to promote translational efficiency of RPs in osteosacoma cells (Elkon et al., 2015). In addition, they described mTOR inhibition to abolish MYC-induced translation of RPs, thus suggesting that a MYC-mTOR axis should regulate this response. Nowadays the results obtained by Elkon could be rationalized in light of our findings according to which mTORC1 inhibition induces the

stabilization of RP mRNAs by the 40S/LARP1 complex in a pool of translationally inactive transcripts (Fuentes et al., 2021), thus justifying the decrease in RPs expression.
Here, we proposed LARP1 as the missing link between MYC and regulation of RPs expression at the translational level, exploring its impact on the higher protein synthetic capacity of MYC oncogenesis. We focused primarily on the regulation that MYC exerts on LARP1 to execute its oncogenic program rather than exploring the interplay with the mTORC1 signaling, which has been partly tackled by previous studies (Pourdehnad et al., 2013)

MYC and LARP1

The first evidence that LARP1 is part of the genetic program activated by MYC comes from the finding that its expression is indeed sustained by MYC oncogenic levels. Measurement of LARP1 mRNA and protein in MYC-inducible U2OS osteosarcoma cell line confirmed that LARP1 is under the control of MYC (Fig. R-6a,b). Importantly, in P493-6 lymphoid cells we observed the reversibility of this regulation in response to MYC modulation, with high MYC-expressing cells, characterized by high LARP1 levels, being able to lower LARP1 protein upon tetracycline-mediated suppression of MYC and, on the other way around, low-MYC expressing cells able to re-constitute LARP1 expression upon re-expression of MYC after tetracycline removal (Fig. R-6c,d). In addition, we showed MYC-driven sustainment of LARP1 levels also in HCT116 and RKO colorectal cancer cells by acute knockdown of MYC paralleled by a reduction in LARP1 transcript and protein (Fig. R-17). In line with this result, Desi et al. have recently observed a positive feedback loop between MYC and LARP1 (Desi et al., 2022). In their work, they showed LARP1 protein to interact with the 3'UTR of MYC mRNA to promote its expression, although the mechanism behind this regulation was not defined. On the other side, MYC was shown to bind to an enhancer sequence in the promoter of LARP1 to ultimately sustain its levels, however a systematic transcriptional study on LARP1 promoter was missing to draw this conclusion. In line with our results, they observed MYC knockdown to reduce LARP1 protein levels in HCT116 and DLD-1 colorectal cancer cells, although we haven't been able to verify the reciprocal regulation of LARP1 on MYC (data not shown). In addition, overexpression of MYC in DLD-1 cells characterized by lower MYC levels than HCT116, was followed by an increase in LARP1. Although we don't exclude that LARP1 can be sustained by a transcriptional loop of MYC on LARP1 promoter, we haven't verified this possibility by metadata analysis of ChIP-seq dataset carried out in P493-6 cells upon low-MYC and high-MYC settings. In this

regard it is important to underline that in aberrantly MYC-expressing cell lines, like HCT116, MYC acts as a global non-specific potentiator of transcriptionally active genes. We therefore deciphered an alternative mechanism to uncover LARP1 regulation by MYC.

A study conducted in P493-6 cells demonstrated the ability of MYC to regulate miRNAs expression by inhibiting their transcription or the one of the miRNAs host genes (T.-C. Chang et al., 2008). Interestingly, most of the MYC-repressed miRNAs displayed tumor-suppressor activities. In particular, in a following study, the same group clarified the tumor-suppressor role of miR-26a in colorectal cancer by generating a mouse model of CRC tumorigenesis in which the overexpressing miR-26a resulted in a clear reduction in tumor size and migratory phenotype (Zeitels et al., 2014). miR-26a targets identified to be responsible for the anti-proliferative activity of the miRNA were genes related to cell cycle control, mytosis and DNA replication. However, a following work revealed a novel tumor suppressor activity for miR-26a/b in prostate cancer by the targeting of LARP1 mRNA (Kato et al., 2015). They showed by luciferase reporter assay that an 8-nucleotide seed sequence from LARP1 3'UTR responds to miR26a/b. In agreement with this, our results showed in HCT116 cells that the full 3,5 Kb 3'UTR of LARP1 is able to sense miR26a/b levels only when the two miR26 binding sites are maintained intact (Fig. R-18c,d). Likewise, the protein levels of LARP1 were reduced upon miR-26a/b overexpression in the PC3 prostate cancer cell lines they used, therefore validating our findings in HCT116 and U2OS T/O MYC cells (Fig. R-18b, R-15c).

Here, we interrogated whether a functional interaction between MYC regulation of miR-26 and miR-26 targeting of LARP1 mRNA could exist to ultimately affect LARP1 expression. We proved that MYC modulation of miR-26a/b levels is valid also in U2OS T/O MYC cells (Fig. R-15b), where we observed by Northern blot analysis reduced expression of the two miRNAs 26a and 26b upon MYC-overexpression. We surmised that such impairment of miR-26 expression could account for the increase in LARP1 levels observed upon doxycycline treatment. In accordance with this, in fig. R-15c we observed that rescuing miR-26 levels upon MYC overexpression by transfecting a miR-26a/b mimic sequence impaired the ability of MYC to sustain LARP1 levels. On the other way around, inhibiting the high basal levels of miR-26 in untreated cells induced LARP1 protein levels similar to MYC overexpression condition. We therefore described for the first time a MYC-miR-26-LARP1 axis in U2OS osteosarcoma cell line and HCT116 colorectal cancer cells, as observed by the decreased levels of miR-26a upon acute knockdown of MYC (Fig. R-18a) and the depletion of LARP1 levels upon miR-26a/b

transfection in those cells (Fig. R-18b). Considering that miR-26 regulation by MYC was first described in P493-6 cells, we suggest that this axis is also responsible for MYC-driven regulation of LARP1 in these cells, but further confirmations will address this point.

Is LARP1 affecting MYC oncogenic program?

The evidence that MYC supports LARP1 expression suggests that LARP1 serves to execute its oncogenic program. Indeed, there are several evidences showing a pro-tumorigenic role for LARP1 that could help to rationalize its regulation by MYC. A first correlation emerged from in silico database analysis that showed LARP1 being overexpressed in the majority of epithelial tumors compared to their non-malignant counterpart (Rhodes et al., 2004). In addition, the role of LARP1 in promoting tumorigenic features has been studied in prostate cancer (Kato et al., 2015), where it was shown to promote malignant cell proliferation and migration and in HeLa cells, where his loss was responsible for arrest in cell cycle progression, increased apoptosis and delayed tumor migration (Mura et al., 2014). Our findings about LARP1 being a critical player in MYC-induced cell proliferation are partly in agreement with this evidence. In P493-6 cells, characterized by proliferation rates which are strictly dependent on MYC expression, we showed LARP1 ablation to decrease the proliferative capacities of MYC-overexpressing cells (Fig. R-5b), although we did not investigate deeper whether a block of cell cycle progression and/or apoptosis could be involved in this phenotype.

In addition, acute LARP1 knockdown (Fig. R-1b) or stable knock-out in U2OS T/O MYC cells (Fig. R-2b) strongly decreased cell proliferation. In this cellular system, MYC activation was already described to lead to a small reduction in cell proliferation in parental cells in a previous work from our laboratory (Morcelle et al., 2019). This response was observed to be accompanied by the activation of an IRBC-mediated stabilization of p53 to prevent MYC-mediated oncogenic stress, but the mechanism behind reduced cell number was not clear. Depletion of p53 upon MYC overexpression was indeed still inducing a decrease in cell number but was also inducing more genomic instability and tetraploidy. Here, we observed that LARP1 ablation was worsening this phenotype only upon MYC overexpression, accompanied by an increase in the number of sub-diploid cells, usually associated with apoptosis. A further confirmation of apoptosis activation was not pursued, but this type of response could justify the strong decrease in cell number observed in LARP1 KO upon MYC overexpression. Importantly, that the impairment in cell proliferative capacity was detected only upon MYC overexpression suggested that, rather than an intrinsic LARP1-related mechanism, reduced cell

proliferation was due to a defective activation of MYC oncogenic program as a consequence of the absence of LARP1. Therefore, increasing LARP1 expression is necessary for oncogenic MYC to sustain aberrant proliferation.

In further support of this hypothesis, we observed that overexpressing miR-26 in U2OS T/O MYC Parental cells upon MYC overexpression phenocopied the reduction in cell number observed in LARP1 KO cells. However no additional effects were observed in the same setting in LARP1 KO cells (Fig. R-16). From one hand this evidence confirms the tumor-suppressor role of miR-26 already demonstrated in vivo in CRC (Zeitels et al., 2014) and in vitro in prostate cancer (Kato et al., 2015). On the other hand, it shows the direct implication of LARP1 targeting in this response, as overexpression of miR-26 in LARP1 KO cells does not have any effect in MYC-induced U2OS cells.

Although we proved that the MYC-LARP1 regulatory axis does exist also in colorectal cancer HCT116 cells, we did not observe such proliferative relationship in these cells (Fig. R-19). We found indeed that the reduction in cell proliferation induced by acute knockdown of MYC, and already described in (Morcelle et al., 2019), was not worsen in a LARP1 KO genetic background, meaning that in a system already addicted to MYC overexpression, MYC is epistatic over LARP1. Indeed, absence of LARP1 prior to MYC downregulation does not confer any additional decrease in survival and/or proliferation. It is possible that a mechanism of adaptation to chronic absence of LARP1 was already established so that the reduction in cell proliferation observed upon MYC depletion is modulated by other MYC-dependent proliferative signals. Indeed, no differences in terms of cell proliferation can be appreciated when comparing cell number of parental and LARP1 KO cells under siNT condition, in contrast to the finding in (Desi et al., 2022) where acute LARP1 depletion decreased cell proliferation of CRC cells. Nonetheless, it is important to underline that HCT116 and RKO colorectal cancer cells are the result of a MYC-driven oncogenesis already established. On the contrary, U2OS T/O are characterized by low MYC levels so that its inducible overexpression better mimics the initial molecular steps of MYC oncogenesis (Walz et al., 2014). Similarly, P493-6 cells have the advantage of being dependent of MYC expression and, as for U2OS T/O MYC, the reactivation of MYC expression recapitulates the establishment of MYC oncogenic program. The observation that in U2OS T/O MYC and P493-6 cells LARP1 becomes necessary only upon MYC over-expression led us to speculate that LARP1 might be fundamental for the cell to respond to the different anabolic stimuli activated by MYC induction. HCT116 cells, on the contrary, are cells already adapted to the elevated

metabolic signature conferred by MYC, in which the contribution of LARP1 to oncogenesis potentially already occurred in the initiation step.

LARP1 regulates MYC-induced RP mRNAs expression

The finding that LARP1 ablation reduces MYC-induced cell proliferation, is indicative of an impaired activation of MYC-controlled molecular events at a post-transcriptional level. One of the best known oncogenic effects of MYC induction is the global increase in protein synthesis. In line with this, we observed that MYC-inducible U2OS T/O MYC cellular system was characterized by increased global protein synthesis rate upon MYC overexpression (Fig. R-13). The implication of LARP1 in this response resulted clear when observing that LARP1 ablation impaired the ability of MYC to increase protein synthesis in U2OS MYC T/O LARP1 KO cells. The same result was also confirmed by observing that LARP1 KO cells had little to no differences in polysomal size in response to MYC over expression, in contrast to parental cells characterized by an increase in polysomes (Fig. R-9a,c).

One of the mechanisms through which MYC supports protein synthesis is an increase in ribosome biogenesis to feed the protein synthetic machinery. That MYC-induced U2OS cells increase their global protein synthesis is therefore in line with previous findings from our group showing that MYC overexpression boosts RPs synthesis in this cell line (Morcelle et al., 2019). Consistently, by RT-qPCR of sucrose gradients we found that the translational engagement of RP mRNAs is increased in these cells upon MYC overexpression (Fig. R-9b). The absence of LARP1, instead, caused a drastic drop in the association of these transcripts with polysomes (Fig. R-9d), in line with the impaired increase in protein synthesis observed in fig. R-13. Measurement of RPs levels by western blot, indeed, confirmed the loss of RPs boost upon MYC induction when LARP1 is knocked down (Fig. R-7).

The observation that LARP1 is necessary to sustain RPs expression and cellular protein synthesis rate under MYC signaling adds a new piece of evidence in support of the multifaceted roles of LARP1 as both activator of 5'TOP mRNAs translation (Tcherkezian et al., 2014) and 5'TOP inhibitor (Fonseca et al., 2015), depending on the metabolic cellular context. We observed indeed that the newly synthesized LARP1 protein (Fig. R-8b), expressed upon the stimulus of MYC, is mirroring the distribution of 5'TOP mRNAs in sucrose fractions, thus suggesting that its binding to the transcripts does not affect negatively their translation. On the contrary, based on our recent work according to which the 40S/LARP1 complex stabilizes 5'TOP mRNAs, we hypothesize that LARP1

expression is needed in this system to sustain RP mRNAs stability. This result is in line to what we observed at the level of cell proliferation: U2OS parental and LARP1 KO cells do not show any particular difference in cell proliferative capacity or protein synthesis. It's only upon the MYC oncogenic stimulus that the presence of a metabolic player like LARP1 makes a difference. When its expression is suppressed, indeed, the cell cannot face the anabolic boost induced by MYC.

In support of this hypothesis, we observed that the levels of RP mRNAs in P493-6 lymphoid cells followed MYC expression, being high while growing in normal conditions and drastically reduced upon tetracycline-mediated MYC down regulation (Fig. R-10). Contrary to the evidence from (Wu et al., 2008), where inhibition of MYC was irreversibly ablating RP mRNAs expression, in our system removal of tetracycline almost led to a rescue of MYC levels as well as RP mRNAs. Interestingly, however, the non-TOP mRNA ß-Actin failed to recover upon MYC re-expression, thus suggesting that this differential response between 5'TOP and non-TOP mRNAs could be dependent on LARP1. Indeed, 5'TOP transcripts distribute homogenously along sucrose fractions (Fig. R-11b) mirroring the same distribution of LARP1 (Fig.R11c). Non-TOP ß-Actin transcript instead, populates mainly polysomal fractions and upon MYC reactivation its levels are not rescued as for 5'TOP mRNAs, indicating a hierarchical translational activation of the components involved in ribosome biogenesis.

If the re-population of sucrose fractions by 5'TOP mRNAs that we observe upon MYC reactivation is dependent on LARP1 as we suggest, we could also predict that in P493-6 shLARP1 cells this event will be impaired.

In (Morcelle et al., 2019), our group also observed that MYC inhibition in HCT116 CRC cells induced a strong decrease in RP mRNAs and to a larger extent a reduction in newly synthesized RPs. Consistently with the evidence from (Elkon et al., 2015) supporting the involvement of MYC in translational regulation of RPs and in light of the findings described here, we could rationalize the decrease in newly generated RPs observed upon MYC silencing in HCT116 cells. We could speculate indeed that the reduction of LARP1 levels following MYC knockdown is responsible for the decrease in RPs transcript levels and translational engagement. This hypothesis, however, would collide with the insensitivity to chronic LARP1 ablation observed in terms of cell proliferation in fig. R-19. Another possibility is that MYC inactivation simply leads to inhibition of mTOR-mediated phosphorylation of 4E-BPs, therefore impairing RP mRNAs translation (Pourdehnad et

al., 2013). Here, however, we did not study the possible implications of mTOR pathway over MYC-LARP1 axis.

An additional open question in this study is represented by the observation that regulation of LARP1 lays also under the WNT signaling pathway. Expression of tamoxifen-inducible dominant-negative TCF4 transcription factor in LS174T, DLD-1 or SW403 CRC cell lines showed indeed to downregulate MYC and LARP1 levels (Fig. R-20). Now, it would be interesting to investigate whether the decrease in LARP1 protein observed upon WNT signaling inhibition is a consequence of MYC downregulation or whether these are parallel pathways. However, linking LARP1 to WNT signaling could allow us to study a possible connection between LARP1 and a WNT-mediated cellular signature such as stemness. In a recent study (Morral et al., 2020) showed indeed that prolonged WNT inhibition in LS174T and SW403 cells leads to a decline in intestinal stem cells gene expression and an increase in markers of tumor cell differentiation. Interestingly, this particular setting induced by WNT blockade was characterized by a significant reduction in protein synthesis rates and pre-rRNAs. We might therefore speculate that also in this context the role of LARP1 in regulating 5'TOP mRNAs might be involved in the reduced protein synthetic capacity observed in these cells upon WNT (and LARP1) inhibition, which would ultimately influence the stemness or differentiated profile of these cells.

To conclude, we have showed that LARP1 is fundamental for MYC-driven increase in RPs translation and protein synthetic capacity. This represents a novel piece of information in the field of LARP1 regulation, since its regulatory role over 5'TOP mRNAs has always been connected to mTORC1 signaling. Here, instead, we identified a LARP1 post-transcriptional regulation of 5'TOP mRNAs linked to the oncogenic program activated by MYC. In particular, we observed LARP1 to promote MYC-induced RPs mRNAs stability and expression, which further allowed an increase in global protein synthesis with the ultimate support of cell proliferation.
Further studies will be needed to confirm that LARP1 depletion leads to an impairment of ribosome biogenesis process, by measuring newly synthesized RPs and rRNA. In addition, it is also in our interest to investigate the role of LARP1 in MYC-driven tumor initiation. It has been already shown that P493-6 cells are capable to form tumors in immunodeficient SCID mice (Gao et al., 2007) which are dependent on MYC, since tetracycline oral administration inhibits tumor formation. In line with this work and with

the decreased cell proliferative rate observed in P493-6 cells upon LARP1 ablation (fig. R-5b), we would expect shLARP1 cells to impair MYC-driven tumorigenesis.

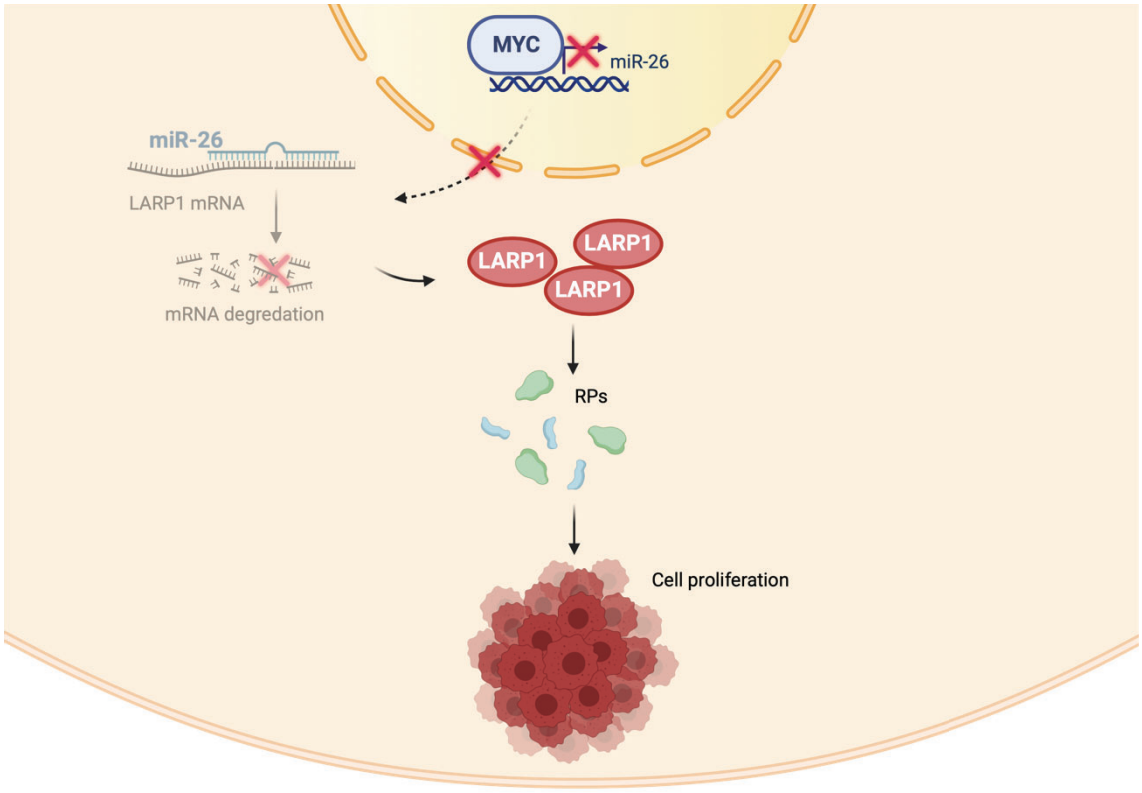

Fig. D-1 **| Working model presented in this thesis** MYC sustains LARP1 levels by inhibition of miR-26 targeting. In MYC-inducible cell systems, the upregulation of LARP1 is converted into increased ribosomal protein synthesis and cell proliferation.

CONCLUSIONS

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From the work developed in this thesis, we have reached the following conclusions:

1. Oncogenic MYC promotes LARP1 expression

2. Mechanistically, MYC over expression downregulates miR-26a/b levels thus releasing the transcriptional silencing of LARP1 operated by those miRNAs

3. In cellular models that mimic MYC oncogenesis, such as U2OS T/O MYC and P493-6, activation of LARP1 by oncogenic MYC is necessary to sustain MYC-driven boost of Ribosomal Proteins production and global protein synthesis.

4. LARP1 upregulation by MYC sustains cell proliferation in U2OS T/O MYC and P493-6 cells

5. In cellular models of MYC-driven oncogenesis such as HCT116 and RKO colorectal cancer cells, MYC is epistatic over LARP1

6. In Colorectal Cancer cell models WNT signaling pathway sustain both MYC and LARP1 expression.

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