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A liaison between mTOR signaling, ribosome biogenesis and cancer☆

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Abstract

The ability to translate genetic information into functional proteins is considered a landmark in evolution. Ribosomes have evolved to take on this responsibility and, although there are some differences in their molecular make-up, both prokaryotes and eukaryotes share a common structural architecture and similar underlying mechanisms of protein synthesis. Understanding ribosome function and biogenesis has been the focus of extensive research since the early days of their discovery. In the last decade however, new and unexpected roles have emerged that place deregulated ribosome biogenesis and protein synthesis at the crossroads of pathological settings, particularly cancer, revealing a set of novel cellular checkpoints. Moreover, it is also becoming evident that mTOR signaling, which regulates an array of anabolic processes, including ribosome biogenesis, is often exploited by cancer cells to sustain proliferation through the upregulation of global protein synthesis. The use of pharmacological agents that interfere with ribosome biogenesis and mTOR signaling has proven to be an effective strategy to control cancer development clinically. Here we discuss the most recent findings concerning the underlying mechanisms by which mTOR signaling controls ribosome production and the potential impact of ribosome bio-genesis in tumor development. This article is part of a Special Issue entitled: Translation and Cancer.

Keywords

mTOR; Ribosome biogenesis; Ribosomal protein; Cancer

1. Introduction

The complexity of life has been shaped by a large number of environmental and intrinsic factors, which have influenced the development of intricate biological mechanisms to adapt and to respond to external stimuli and internal signals [1]. Nutrient availability, diet

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composition, developmental programs and many other elements have molded different species by means of selective processes that have provided a unique fitness to their respective niches. Notably, the maintenance of unicellular organisms and higher metazoans requires the same basic needs of nourishment, growth and reproduction. Indeed, bacteria utilize many of the same complex biological mechanisms employed by mammalian cells. Based on these observations many fundamental cellular questions have been addressed in simple model organisms. Indeed these more simple systems initially taught us a number of basic phenomena required for the maintenance of all species, including that nearly all physiological activities of a living organism depend on its ability to convert genomic information into functional proteins. The importance of protein synthesis becomes even more obvious in proliferating cells, which require a continuous supply of structural and catalytic proteins in order to feed, grow and duplicate. Similarly, cancer cells, characterized by uncontrolled proliferative potential, rely on the sustained upregulation of anabolic pathways, including the synthesis of nascent proteins. At the center of the protein synthetic supply chain is the ribosome, composed of 4 non-coding RNAs (rRNA) and ~80 distinct ribosomal proteins (RPs) [2], a molecular machine responsible for the conversion of information encoded by nucleic acids into functional proteins [3]. Of note, the ability of a cell to increase the rate of protein synthesis upon physiological demand, is largely mediated at the level of ribosome biogenesis [4]. This response is accomplished by a complex integrated, and not yet completely resolved, molecular signaling network. Moreover, ribosome biogenesis itself is a huge anabolic investment, a highly coordinated multistep process, involving more than 200 molecular components [5, 6]. Ribosome biogenesis is spatially and temporally organized, with quality control checkpoints to ensure the fidelity of synthesis and assembly of ribosomal subunits at different stages of their generation [2]. All three RNA polymerases, I II and III, contribute to the production of nascent ribosomes, by transcribing the different structural and catalytic molecular components required for their assembly [7]. The 47S precursor rRNA is transcribed by RNA polymerase I in the nucleolus and then processed by a specific set of enzymes and small non-coding RNAs into mature 18S, 28S and 5.8S rRNA [8]. In contrast, 5S rRNA, synthesized by RNA polymerase III in the nucleus, must be first exported to the cytoplasm, processed, reimported to the nucleus and then to the nucleolus before being assembled into a nascent ribosomal subunit [9]. In contrast, RNA polymerase II is in charge of transcribing the subset of mRNAs that encode for RPs as well as the enzymes and small non-coding RNAs required for the processing of both the 40S and 60S ribosomal subunits [10]. RPs, themselves, are first translated in the cytoplasm and then like 5S rRNA shuttled to the nucleus and then to the nucleolus, where their specific association with nascent 47S rRNA is required for the processing and maturation of the 40S and 60S ribosomal subunits [11]. Given the considerable number of components and events involved in ribosomal biogenesis, it is clear that for such a complex process to efficiently respond to extrinsic demands, ribosome bio-genesis has to rely on the harmonic coordination among all different parts of the machinery, both in terms of stoichiometry and assembly. In this regard, over the last decade a growing body of evidence has supported the concept that multiple pathways modulate ribosome biogenesis in response to external stimuli [4] and that an impairment that leads to either hypo- or hyper-ribosome biogenesis is closely monitored by an intrinsic checkpoint, which is elicited when this delicate equilibrium is altered [12]. Moreover, most if not all of the enzymatic and structural

protein components that sustain ribosome biogenesis, are upregulated in cells, which overexpress or express deregulated protooncogenes including Myc, Ras, PI3K, AKT and the mammalian Target of Rapamycin (mTOR) [13–15]. Recent studies suggest that many of the signaling pathways utilized by these proto-oncogenes converge on mTOR, which acts as a key nexus to integrate their signals with the nutrient and energy status of the cell, in order to control cellular protein biosynthetic capacity. Novel observations have also revealed that RPs and rRNAs have unexpected extra-ribosomal functions, which monitor the status of the ribosomal apparatus in human pathological conditions including Diamond Blackfan Anemia (DBA), $5q^-$ Syndrome as well as other "Ribosomopathies" [16]. Indeed hypomorphic lesions in RPs or monoallelic deletions lead to the upregulation of p53, which is argued to be the cause of many of these pathologies, rather than the impairment of global protein synthesis [17]. Indeed, these same components may participate in monitoring hyperactivation of ribosome biogenesis, such as in the case of Myc or Ras driven tumors. In this review we will cover the most recent findings with respect to (i) mTOR-signaling in ribosome biogenesis, (ii) the mechanisms controlling RP synthesis during this response, (iii) targeting the mTOR pathway in cancer and (iv) the role of RPs and noncoding rRNAs in extra ribosomal cell cycle checkpoints in disorders characterized by RP deficiencies and the onset of cancer.

1.1. mTOR and the control of ribosome biogenesis

In an effort to identify the underlying mechanism by which the antibiotic rapamycin inhibited cell growth, the Target of Rapamycin (TOR) was discovered in an elegant genetic study in *Saccharomyces cerevisiae*, where FKBP (FK506 binding protein), a proline/isoleucine isomerase, was recognized as a critical component of a rapamycin gain-of-function inhibitory complex with TOR1 and TOR2 [18]. Subsequently TOR1 was cloned and characterized as a phosphatidylinositol kinase in *Saccharomyces cerevisiae* [19]. Shortly after the mammalian homologue, mammalian Target of Rapamycin (mTOR) was purified and cloned in four other laboratories [20–23]. Although mTOR belongs to the phosphatidylinositol 3-kinase-related protein kinase, subsequent studies in mammalian systems showed that mTOR was a protein rather than a lipid kinase [24,25]. Since then considerable efforts have been invested in understanding the functions, mechanisms and contexts where this pivotal regulator of cellular metabolism exerts its effects. Many proteins have been found associated with mTOR, and distinct components define two mTOR protein complexes, mTOR Complex1 (mTORC1) and mTORC2 [26]. Common partners are found in both complexes, including mammalian lethal with SEC13 protein 8 (mLST8) [27] and the inhibitory protein DEP domain-containing mTOR-interacting protein (DEPTOR) [28], whereas specific components, differentiate the two complexes in terms of target specificity and sensitivity to external stimuli, such as Regulatory-associated protein of mTOR (RAPTOR) and Proline-rich AKT1 substrate 1 (PRAS40), in the case of mTORC1, or Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) and mSIN-1 in the case of mTORC2 [29]. Although it has been argued that rapamycin is selective for mTORC1 and not mTORC2, recent studies provide evidence that it can also bind mTORC2 when combined with one of the novel mTOR ATP-site competitive inhibitors [29].

The number of signaling pathways branching from mTORC1 and mTORC2, the cascade of events which control both complexes and the feedback mechanisms between effectors, have revealed an intricate network of regulatory events [30]. Seminal studies first in yeast and drosophila [31,32], then in mice, have demonstrated that mTOR is essential for cell growth and proliferation, as mTOR knockout mouse embryos fail to progress beyond the early step of pre-implantation, due to a defect in blastocyst inner-cell-mass proliferation and trophoblast differentiation [33,34]. Importantly, rapamycin administration to wild type blastocysts partially recapitulates the phenotype of $mTOR^{-/-}$ embryos, which suggested that loss of mTORC1 might be the culprit [33]. This was also supported by the effects of RAPTOR deletion, which recapitulated the phenotype of mTOR loss in mouse development [35]. The time at which mTORC1 comes into play during mouse development reflects a specific energetic need of the blastocyst, which at the early steps of embryogenesis is dependent on amino acids as an energy source. Of note, genetic ablation of RICTOR in the mouse unraveled a differential role for mTORC2, which is required at a later stage of gestation as embryos die at E10.5. In the same study Guertin et al. determined that the mLST8, although shared by both mTORC1 and mTORC2 complexes, has a more profound role in maintaining the functional and physical integrity of mTORC2, as confirmed by the finding that *mLST8*$^{-/-}$ mice die at the same stage as RICTOR null mice [35].

The two key substrates downstream of mTORC1 are the RP S6 kinases (S6K1/2) and the protein initiation factor 4E binding proteins (4E-BP1/2/3) [26]. Genetic ablation of the S6Ks or the 4E-BPs in mice have revealed specific roles in glucose and insulin homeostasis [36–39], adipogenesis [40,41] and neurogenesis [42–46]. Moreover biochemical and cell biology approaches have deepened our understanding on basic cellular processes mediated downstream of the S6Ks and the 4E-BPs. Here we have focused on the role of these downstream effectors in protein synthesis and ribosome biogenesis [30].

1.1.1. S6Ks: control of protein synthesis and ribosome biogenesis—

Phosphorylation of S6K1 on T389 by mTORC1 creates a docking site for the 3-phosphoinositide-dependent protein kinase 1 (PDK1), allowing phosphorylation at T229, resulting in S6K1 activation [47,48]. With respect to the control of translation it has been suggested by Holz et al. that S6K1 associates with eukaryotic Initiation Factor-3 (eIF3) at the 5′ m^7G cap of the mRNA, in an inactive state [49]. It is important to note that initiation of translation is thought to be the most rate-limiting step of protein synthesis [50]. Following mitogen or amino acid stimulation, mTORC1 is recruited by eIF3 to the site of translational initiation, which is composed of multiple components required for initiation of protein synthesis, including the 40S subunit, and is referred as the 43S Pre-Initiation Complex. Here mTORC1 activates S6K1, which in turn is argued to dissociate from the 5′ m^7G cap and phosphorylate key targets, including 40S RPS6, eIF4B, and programmed cell death protein 4 (PDCD4). EIF4B, in its phosphorylated state, binds eIF4A at the Pre-Initiation Complex and stimulates its mRNA helicase activity, which is important for efficient translation of mRNAs with a highly structured 5′ untranslated regions (5′UTRs) [50] (Fig. 1). However, subsequent studies have shown that mTORC1 is recruited to the lyso-some by the Ras-Related GTPases (RAG), where it is activated by the small GTPase Ras homologue enriched in brain (Rheb)

[51] (see, below). How lysosomal mTORC1 localization is integrated with the model of protein synthesis initiation proposed by Holz et al. [49], has yet to be elucidated.

The role of S6K1 in m^7G cap-dependent translation is not limited to eIF4B. EIF4A, who interacts with both eIF4G and PDCD4, is an indirect target of S6K1. When cells are devoid of a mitogenic input, PDCD4 binds to eIF4A hampering its association with eIF4G. Dorrello *et al*. have demonstrated that mitogen stimulation leads to PDCD4 degradation, which is triggered by S6K1-mediated phosphorylation at serine 67 (S67). Phosphorylation at this site leads to the recruitment of E3-ubiquitin ligase SCF^{TRCP} to PDCD4, followed by PDCD4 ubiquitination and degradation [52]. This releases eIF4A, allowing it to bind to eIF4G at the Pre-Initiation Complex and promote the activation of protein synthesis [52]. S6K1 also plays an important role in the elongation phase of protein synthesis, which mediates the rate of translation of an mRNA, directly affecting the amount of nascent protein over time. The activity of the eukaryotic elongation factor-2 (eEF2), which catalyzes the GTP-dependent tRNA translocation step during elongation, is suppressed by eEF2 kinase (eF2K) phosphorylation. In turn, in a rapamycin-sensitive manner, activated S6K1 can phosphorylate and inhibit eEF2K, increasing the rate of eEF2-mediated translation [53] (Fig. 1).

One of the major downstream anabolic targets of the mTORC1 pathway is increased ribosome biogenesis. It is clear that S6K1 can contribute at the level of RP synthesis due to its general impact on translation. However, a number of studies show that it can directly impact rRNA synthesis. At one level it was recently shown that S6K1 can mediate increased pyrimidine biosynthesis, through phosphorylation of a novel substrate, the carbamoyl-phosphate synthetase 2 aspartate transcarbamylase, and dihydroorotase (CAD) complex, which catalyzes the first three steps of de novo pyrimidine biosynthesis [54,55]. In an anabolically growing cell the biggest consumer of pyrimidines is rRNA synthesis (Fig. 1) [56]. Moreover, Meyer *et al.* have shown that the mTORC1/S6K1 axis, through an undefined mechanism, activates transcription initiation factor 1A (TIF-1A), an indispensable component of the RNA polymerase I complex, which drives rRNA transcription under favorable growth conditions [57]. In a separate study Hannan *et al.*, have used reporter constructs that mirror the transcriptional activity of rDNA locus, and have demonstrated that proliferating cells require mTORC1 and S6K1 activity to boost the demand for increased rDNA transcription, and that this event is tightly linked to the phosphorylation of Upstream Binding Factor (UBF) at the rDNA locus [58]. Finally, S6K1/2 were shown to be required for the transcription of a number components involved in ribosome biogenesis in a starvation-re-feeding experimental paradigm in mouse liver, although the underlying mechanism that controls this regulatory loop has not been defined [59]. Thus, mTORC1 through S6K1/2 can impact ribosome biogenesis at multiple levels of RP and rRNA production. However, which of the two biosynthetic pathways has a more profound impact on ribosome biogenesis? While direct evidence has linked S6K activity and rRNA synthesis, this link is less clear for 5′TOP mRNA translation. Although 5′TOP translational regulation does not appear to be altered in an *S6K1/2*$^{-/-}$ genetic background [38], the many protein synthetic mechanisms in which S6K1 is implicated may impact 5′TOP mRNA translation, since it is accountable for about 20% of total cellular mRNAs.

1.1.2. 4E-BPs: control of translation and ribosome biogenesis—5′ m^7G cap-dependent translation is the most utilized mechanism of protein synthesis initiation and is shared by the vast majority of cellular mRNAs [50]. The early steps of this process involve the assembly of a multiprotein complex, termed eIF4F, at the 5′ m^7G cap of the mRNA. EIF4E plays an essential role in the complex by independently binding both the 5′ m^7G cap and eIF4G, the latter serving as a scaffold for the binding of the 40S ribosomal subunit, eIF4A, the MAP kinase signal-integrating kinases (MNK1/2) and the Poly A Binding Protein (PABP) [50]. In contrast, the ability of eIF4E to bind to eIF4G is dictated by the 4E-BPs, which compete for the binding of 4E at the same site that eIF4G binds, ablating the assembly of the eIF4F complex and inhibiting mRNA translation. However, both the phosphorylation status and the abundance of the 4E-BPs dictate their ability to suppress translation [50]. When hyper-phosphorylated by mTORC1 the binding of the 4EBPs to eIF4E is disrupted [60,61]. mTORC1 controls 4EBPs activity by multiple site hierarchical phosphorylation of at least five residues that are essential for relieving the inhibitory binding of 4E-BP to eIF4E [62–64]. Intriguingly, mTORC1 signaling sustains its impact on global protein synthesis by exerting opposite effects on its two most characterized substrates, the S6Ks and the 4E-BPs, an activator and an inhibitor of this response, respectively. Importantly, 4E-BP1/2 double knockout MEFs grow at the same rate as the parental wild type MEF, however their rate of growth, unlike their wild type counterpart, is not effected by pharmacological or genetic inhibition of mTORC1 [65]. Since ribo-some biogenesis defects are mirrored by inhibition of cell proliferation, this suggests that in 4E-BP1/2 deficient cells, ribosome production remains unaltered. Indeed acute inhibition of mTOR, by ATP-site competitive inhibitors, severely compromises ribosome production. In this regard it should be noted that rapamycin, an allosteric inhibitor of mTOR binds at the FKBP/rapamycin-binding (FRB) domain, just upstream of the kinase domain [66]. Thus although it is a potent inhibitor of the S6K1 T389 phosphorylation, it does not completely suppress or sustain 4E-BP dephosphorylation [67–69].

In an attempt to identify mRNAs that may escape rapamycin inhibition, Hsieh *et al.* discovered 144 translationally regulated transcripts that were more selectively suppressed by ATP site inhibitors [70]. Strikingly, the majority of this subset of mRNAs falls in a family of mRNAs, which include the RPs, termed 5′ Terminal Oligopyrimidine tract (5′ TOP) mRNAs, characterized by a polypyrimidine tract at their 5′ m^7G cap transcriptional start site [71] and under the translational control of mTORC1 [72,73]. Employing an analogous approach involving a different mTOR ATP-site competitive inhibitor, coupled to ribosome profiling, Thoreen *et al.* came to a similar conclusion [74]. Of note, in both studies, overexpression of dominant-negative form of 4E-BP [70] or 4E-BP deletion [74] relieved the translational inhibition of the mTOR ATP-site competitive inhibitors. This observation led to the conclusion that suppression of the 4E-BP branch of mTORC1 signaling pathway is a permissive prerequisite for RP translation and for the production of ribosomes (see below).

1.1.3. Other mTOR-dependent mechanisms—Unlike the 47S rRNA precursor, which gives rise to 28S, 18S and 5.8S rRNA, 5S rRNA is transcribed in the nucleus by RNA polymerase III. Maybe not unexpectedly mTORC1 has been directly implicated in the

regulation of RNA polymerase III. By employing a phosphoproteome approach to cells treated with an mTOR ATP site competitive inhibitor, Maf1, was identified as a target of mTORC1 [75]. Maf1 is a known repressor of RNA polymerase III transcription, and its phosphorylation at S75 by mTORC1 at 5S rDNA and tRNA loci, relieves this inhibitory loop and stimulates transcription. Interestingly mTORC1 is tethered to RNA polymerase III promoters by interacting with TFIIIC [76]. Although ribo-some biogenesis appears to be largely regulated by the mTORC1, rather than mTORC2, it should be noted that Zinzalla *et al.* have coupled mTORC2 activation with the physical interaction of mature ribosomes [77]. In proliferating cells mTORC2 promotes AKT signaling and, as a consequence, activated AKT is known to sustain survival pathways. This mechanism is of particular importance in cancer settings harboring either PI3K gain-of-function mutations or PTEN loss-of-function mutations [77]. In this regard, AKT signaling not only activates ribosome bio-genesis via mTORC1 (see below), but it is also required for RNA polymerase II transcriptional elongation and for cooperation with c-Myc, a master regulator of all three RNA polymerases [78].

1.2. RP synthesis and ribosome biogenesis

For ribosome biogenesis, the fact that ~80 distinct RPs are required for the maturation of the two subunits and that they can represent up to 20% of the total mRNA in the cell, underscores an intimate interdependence between ribosome biogenesis and protein synthesis. Given the central importance of protein synthesis, it is reasonable to hypothesize that translation of RP mRNAs should occupy a hierarchical layer of common control distinct from other mRNAs. As described above, this assumption has been validated by the identification of the 5′TOP at the transcriptional start sites of RP mRNAs [71,79]. In earlier studies it was demonstrated that addition of rapamycin to mitogen stimulated cells selectively inhibited 5′TOP mRNA translation [72]. Moreover, further studies employing either a wild-type or mutated 5′TOP demonstrated that the effects of rapamycin on translation were dictated by an intact 5′TOP and that this effect was mediated downstream of mTORC1 by S6K1, based on results obtained with either a rapamycin resistant or a dominant negative allele of the kinase [72]. However, it was later found that mouse embryo fibroblasts from mice deficient for S6K1/2, were not affected in 5′TOP mRNA translation, suggesting compensatory mechanisms [38]. Mutation of RPS6 phosphorylation sites demonstrated that altering one of the multiple substrates of S6K1/2 was also permissive for 5′TOP translational regulation, however other substrates were not considered [80]. As mentioned above two independent groups have shown by global ribosome profiling that, upon acute mTOR inhibition, 4E-BPs, through its interaction with eIF4E are critical for 5′TOP translation [70,74]. The inhibitory role of 4E-BPs on the assembly of eIF4G1 at the m^7G cap of mRNAs appears to be sufficient to inhibit the early step of 5′TOP translation. However, the interactions between the 4E-BPs and eIF4E, and eIF4G1 and eIF4E, are common players utilized by a wide spectrum of cell transcripts for translational initiation. Moreover, although their role in 5′TOP translation is necessary, it may not assure specificity. In addition, the 5′TOP mRNAs are highly abundant transcripts and their selective inhibition of translation by the 4E-BPs, could be explained by the number of reads in the ribosome profiling analyses, as previously discussed [81]. Consistent with such an explanation, overexpression of 4E was reported to have no effect on 5′TOP translation [82].

From the studies of Damgaard et al., it is clear that 5′ TOP translation may require other molecular components [83]. They demonstrated that when cells are placed in unfavorable growth conditions, such as amino acid deprivation, translation of RPs are inhibited by means of RNA binding proteins TIA-1 and TIAR, through a GCN2-mediated mechanism [83]. Again, mTORC1 inhibition may operate as a permissive mechanism in this setting, as a constitutively active Rheb mutant rescued the amino acid-mediated translational repression [83] (Fig. 2). Of note co-knockdown of TIA-1 and TIAR by Sabatini's group did not rescue the pharmacological inhibition of mTORC1 over 5′TOP translation, excluding an implication of these players and potentially suggesting that other actors might come into play when mTOR activity is halted [74].

Other potential candidates have also recently arisen. In an attempt to identify targets of microRNA-10a, Orum *et al.* showed that this non-coding RNA retains the ability to bind in a non-canonical manner to the RP mRNAs just downstream of the 5′TOP motif and to stimulate their translation [84]. Competitive inhibition or overexpression of microRNA-10a either diminished or sustained RP synthesis, respectively and consequently de novo synthesis of ribosomes and global protein synthesis. In line with these observations, the same experimental setting reflected the ability of mir10a to induce oncogenic transformation [84]. However, a better characterization on the underlying mechanisms is required to understand the role of mTORC1 signaling in mir-10a-regulated ribosome biogenesis and transformation, potentially offering a novel therapeutic window. In this regard mir-10a expression appears to counteract the effect of amino acid starvation over RP translation, and it has been hypothesized that mir-10a association with the 5′TOP competes for the binding site of TIA-1 and TIAR [85] (Fig. 2). However, it should be noted that others showed that depletion of Drosha or Dicer, two enzymes required for microRNA maturation, had no effect on 5′ TOP mRNA translation [86]. Again, this observation suggests that the underlying network connecting translational regulation of RPs, mTOR signaling and microRNA might be more complex than originally thought.

Finally, in a recent phospho-proteome screen for mTORC1 candidate substrates another target emerged that could potentially connect 5′TOP translation to mTOR signaling, the RNA binding protein LARP1, belonging to the La-related family of proteins [87,88]. Two studies have pointed out a specific role of LARP1 in 5′TOP biology. While LARP1 has a basal affinity for the poly A of all mRNAs, Aoki *et al.* have observed that only 5′ TOP mRNAs steady state levels are affected by LARP1 expression [89]. It is still debated whether LARP1 interacts directly with poly A tail or utilizes PABP as a specific companion to associate with poly A+ transcripts. In this regard Tcherkezian *et al.* have verified that LARP1 is associated with eIF4E utilizing the 5′ m^7G cap bound to sepharose in the presence of RNAse, by its co-immunoprecipitation in the eIF4F complex. Moreover the interaction correlated positively with mTORC1 activation state [90]. Stable depletion of LARP1 in HEK293 cells resulted in less association of 5′TOP mRNAs with polysomes when compared to control cells [90]. This data suggests that LARP1 could be a translational activator of 5′TOP mRNAs. However, LARP1 depletion is also accompanied by a drop in 5′TOP mRNA levels, an effect that Tcherkezian *et al.* did not observe [90]. Hence it is still unresolved which of the two mechanisms prevails and whether the change in polysomal

distribution is a consequence of the fall in 5′TOP mRNA total levels (Fig. 2). Despite this lack of knowledge, the direct interaction of LARP1 with raptor positions it in a unique branch of the mTORC1 signaling pathway, compatible with the observation that rapamycin is able to inhibit 5′TOP translation. However, uncoupling the 5′TOP- vs. non-5′TOP-specific functions of LARP1 will be critical in understanding the mechanisms that mediate RP synthesis and ribosome biogenesis. Moreover, the importance of nascent 5′TOP translation may also play a critical role in cell cycle checkpoint, which monitors the status of ribosome biogenesis, as described below.

1.3. mTOR pathway and cancer

In most solid tumors the mTORC1 pathway has been found to be up-regulated, consistent with the number of tumor suppressors monitoring this signaling pathway [30]. The molecular network upstream mTORC1 is an intricate complex of multiple signaling components that integrate extracellular signals and intracellular cues. Growth factors such as IGF and insulin, which signal through either PI3K and/or RAS, eventually converge on and inhibit a negative regulator of mTORC1 the Tuberous Sclerosis Complex (TSC1/2-TBC1D7) [91]. Inhibition is mediated by TSC2 phosphorylation at distinct sites by either PKB/Akt or the mitogen activated protein kinase (MAPK) and ribosomal S6 kinase (RSK), respectively [26]. TSC1/2 act as a tumor suppressor complex, which is mutated in a rare multisystem, autosomal dominant disorder in both children and adults, characterized by the formation of benign tumors that in rare cases can lead to metastatic tumors of the lung [92]. Downstream the GTPase stimulating activity of TSC2, drives the small GTPase Rheb into the inactive GDP-bound state, preventing it from directly binding and activating mTORC1 [26]. Rheb is constitutively localized to the lysosomal membrane, whereas mTORC1, through raptor is recruited to this location by the RAG GTPases. Thus activating mutations in key components of the Ras/MAPK or PI3K/Akt pathways drives constitutive mTORC1 signaling. In this regard, many tumors associated with either PI3K gain-of-function mutations or PTEN loss-of-function mutations, stimulate the AKT/mTORC1 regulatory axis [93]. Accordingly, an important numbers of PI3K/Akt and mTORC1 pathway inhibitors have been developed and investigated as cancer therapeutics [94]. In the recent years, the allosteric mTORC1 inhibitor rapamycin (everolimus) has been approved for advance kidney cancer, subependymal giant cell astrocytoma (SEGA), pancreatic neuroendocrine tumor and for breast cancer in post-menopausal women with advanced hormone-receptor positive, HER2-negative type cancer, in conjunction with aromatase inhibitor. As in the case of the latter cancer indication, combination therapies including everolimus and/or dual mTOR inhibitors show promise in cancer trials [94,95]. However, as with other targeted-therapies, drug resistance develops with time and the aim now becomes to detect the cancer cell's oncogenic addiction profile, to circumvent resistance [96]. Among other ribosomal-biogenesis regulating molecules, the RNA binding protein LARP1 was recently shown to promote the stability of mTOR mRNA and sustain downstream mTOR signaling, a mechanism that appears to correlate with cancer progression as shown in human specimens from cervical and non-small cell lung cancers [97]. Similar evidence has been also observed in hepatocellular carcinoma [98]. The anabolic and catabolic processes regulated by mTORC1 span from protein, ribosome, lipid and nucleotide biosynthesis to autophagy and cell survival, respectively. Moreover it appears that many tumor types are addicted to high

rates of ribosome biogenesis and protein synthesis. Seminal studies by Ruggero and collaborators have demonstrated that restoring physiological levels of protein synthesis capacity in an *Eμ-Myc*-lymphoma mouse model, by crossing these mice with *RPL24*$^{+/-}$, strongly reduces lymphomagenesis and extend the lifespan and disease-free survival of the *Eμ-Myc* mice [99]. 5′ m^{7}G cap-dependent translation, which is usually suppressed during mitosis, is abnormally upregulated in *Eμ-Myc* B cell lymphocytes, compromising the physiological switch to m^{7}G cap-independent translation, which is critical for progression through mitosis. Indeed, an important regulator of cell cycle progression, Cdk11, which is translated only during mitosis by means of an IRES element present in its mRNA, was shown to be translated at lower levels in *Eμ-Myc* B cell lymphocytes, potentially contributing to mitotic catastrophe [99]. Finally, the genetic background of RP haploinsufficiency (*RPL24*$^{+/-}$ or *RPL38*$^{+/-}$) or rapamycin treatment were shown to reestablish normal levels of the 5′ m^{7}G cap-dependent translation during mitosis in the *Eμ-Myc*-lymphoma mouse model, highlighting the importance of ribosome biogenesis, protein synthesis and mTORC1 in the cancer phenotype. Of note, in a similar experimental paradigm, where lymphomagenesis is driven instead by an oncogenic allele of *AKT*, the importance of the upregulation of m^{7}G cap-dependent translation has further underscored, as inhibition of the eIF4E/4E-BP branch of mTORC1 signaling pathway suppressed lymphomagenesis [100].

Attacking the liaison between two master regulators of ribosome biogenesis, c-MYC and mTOR, has demonstrated to be a promising strategy for treating c-MYC-driven tumors. Mitigation of 4E-BP1 phosphorylation, which is a hallmark of c-MYC-driven lymphomas, by mTOR ATP site competitive inhibitors or by exploiting a dominant nonphosphorylatable allele of 4E-BP1, has shown lethality not only in mouse model of lymphoma but also myelomas where c-MYC is hyperactivated [101]. As discussed earlier, based on the inhibition of 4E-BP1 phosphorylation/signaling, these studies have also provided a rationale for understanding the differential response of tumors to allosteric as compared to ATP site competitive mTOR inhibitors, the former not durable in terms of maintaining 4E-BPs in the dephosphorylated state. The model of c-MYC-induced tumorigenesis has demonstrated that the role of ribosome biogenesis is not only confined in sustaining protein synthetic capacity, as explained in the last section.

1.4. Ribosome biogenesis checkpoints

Ribosome content determines the growth capacity and the proliferative potential of the cell. This implies that the cell is able to monitor the ribosome biogenesis and evaluate whether the protein synthesis capacity is adequate to face demand. The first *in vivo* evidence of a crosstalk between ribosome biogenesis and cell cycle checkpoints was identified in a mouse model of liver regeneration. Following hepatectomy, the hepatocytes of mice in which both alleles of an essential RP, RPS6, had been deleted, were unable to de-differentiate and re-enter a round of cell cycle progression to replace the lost liver mass [102]. Later studies demonstrated that deletion of RPS6 in the liver led to the stabilization of p53, which was associated with the failure of hepatocytes to reenter the cell cycle [103]. Parallel studies also showed that deletion of one allele of RPS6 in the whole embryo was sufficient to halt mouse development at day E5.5, a blockade that was relieved when the *RPS6*$^{-/+}$ mice were crossed

into a *p53*$^{-/-}$ mouse background, allowing embryos to develop to E12.5 stage [104]. A hypomorphic mouse strain of RPS24, with milder upregulation of p53 during embryogenesis, as compared to RPS6 heterozygous mice, is able to survive to adulthood, although displaying a complex scenario of defective developmental phenotypes. Again, absence of p53 rescued the majority of embryonic aberrant phenotypes, suggesting that congenital malformations are not only due to an insufficient protein synthesis capacity [99], but also to the stabilization of p53 [105]. The importance of this checkpoint is also evidenced in the survival of mice carrying *Eu-Myc*-driven B cell lymphomas, the same model used by Barna *et al.* [99], where its abrogation increased lymphomagenesis [106]. It is difficult to rationalize, though, the contribution of protein synthesis versus the p53-driven ribosome biogenesis checkpoint in the tumor development as Barna *et al.* observed no differences in tumor-free survival, when they crossed the *p53*$^{-/-}$ strain into an L24$^{+/+}$ or L24$^{+/-}$ background [99].

Over the last decade, a large effort has been invested in understanding and characterizing both the insults and the molecular players that trigger the p53 stabilization in response to impaired ribosome biogenesis (see [107–109]). Interfering at any step of ribosome production can potentially activate the control mechanisms that regulate this process. The potential role to p53 was first shown in cell culture studies where a dominant negative mutant of Bop1, a critical component in the maturation of 28S and 5.8S rRNA, induces p53-mediated cell cycle arrest [110]. Subsequently, perturbation of rRNA synthesis by pharmacological agents such as actinomycin D [111], 5-fluorouracil (5-FU) [112] and mycophenolic acid (MPA) [113] or depletion of other essential components required for rRNA synthesis, such as of TIF-1A, a cofactor of RNA polymerase I [114] and depletion of RPs [103,105,115] all appear to pheno-copy Bop1 mutant overexpression with respect to the stabilization of p53. However, not all RPs are equipotent, with respect to the activation of p53. Ablation of either RPL11 or RPL5, inhibits the production of the large ribosomal subunit, as seen for other large RPs, albeit without effecting p53 stabilization [12,116] Moreover, as shown in co-depletion studies, the role of RPL11 and RPL5 is dominant over other RPs whose depletion stabilizes p53 levels. A selective role for RPL5 and RPL11 emerged with seminal observations from a number of laboratories (see [107–109]), which have only been clarified over the last 5 years. From a series of studies, this checkpoint has been identified as a nascent preribosomal complex made up of RPL5, RPL11 and non-coding 5S rRNA, which upon ribosome impairment is redirected from assembly into 60S ribosomes to the binding and inhibition of the E_3-ligase, human double minute 2 (Hdm2), leading to p53 stabilization, cell cycle arrest and apoptosis [103,115–118]. Unexpectedly, impairing the synthesis of any single RP of either the 40S or 60S ribosomal subunit did not alter the production of the other subunit [12,102,103,115,116]. In the case of impaired 40S ribosome biogenesis this effect leads to the apparent translational upregulation of RPL5 and RPL11 mRNA, required to produce sufficient precursor RPL5/RPL11/5S rRNA complex to bind to Hdm2 in the face of continued 60S ribosome biogenesis and a sharp decrease in global translation [103]. In contrast, impaired 60S ribosome biogenesis leads to the apparent inhibition of RPL5 and RPL11 mRNA translation, since in the absence of 60S ribosome biogenesis, there is seemingly sufficient precursor RPL5/RPL11/5S rRNA complex to bind to Hdm2 [103]. As discussed earlier, the pathological consequences of activating this

pathway have been shown to be causal in two hemato-logical disorders, Diamond Blackfan Anemia and *5q-* syndrome, characterized by heterozygous loss of function mutations in RP genes or a monoallelic deletion of RPS14, respectively [17,109]. Although in mouse models of Diamond Blackfan Anemia and *5q-* syndrome the anemia is rescued by deletion of p53, this finding is controversial in zebra fish models of Diamond Blackfan Anemia [119,120]. Importantly boosting mTOR activity by leucine or arginine administration partially rescues the erythropoiesis in these animals, potentially through the up-regulation of 5′TOP mRNA translation, suppressing the effects of p53 stabilization [119,121].

Critically, in parallel while elucidating the components involved in mediating the p53 response to impaired ribosome biogenesis, others showed that this same checkpoint may be also activated under conditions that drive hyperactivation of ribosome biogenesis, in oncogenic Eμ-Myc driven lymphomas [106]. In this case persistent Eμ-Myc overexpression, may lead to increased levels of the precursor RPL5/RPL11/5S rRNA complex, beyond those required for 60S ribosome biogenesis, triggering inhibition of Hdm2, p53 stabilization and suppression of tumor progression [106]. Consistent with this finding, inhibition of ribosome biogenesis, at a point downstream of the formation of the precursor RPL5/RPL11/5S rRNA complex, leads to its liberation and almost the complete squelching of Hdm2, driving selective Eμ-Myc lymphoma cell death [122]. These studies are consistent with earlier hypothesis that the “Achilles heel” of c-Myc-driven tumors is ribosome biogenesis and protein synthesis [4,99]. In this case, it is worth speculating that the precursor RPL5/RPL11/5S rRNA complex may be a constituent of what is termed the intrinsic tumor suppressor response [123].

1.5. Conclusions and perspectives

Attacking a fundamental cellular process like protein synthesis, based on structural differences between eukaryotes versus prokaryotes, has been demonstrated to be a powerful strategy for bacteria in sustaining their biological niche. For the cancer cell to sustain its biological niche it is dependent on aid from anabolic pathways, with protooncogene deregulation often associated with a metabolic switch that boosts global protein synthetic capacity. Many studies have demonstrated the central role of mTOR in controlling ribosome content of the cell and this property, has turned it into appealing target for adjuvant therapy. The immunosuppressant drug rapamycin and rapalogs have had success in cancer, having been approved over the last 6 years by FDA in the treatment of renal cell carcinoma, subependymal giant cell astocytomas, pancreatic neuroendocrine tumors and ER positive, HER2 negative breast cancers. However they have had limited effectiveness in other cancers, potentially dependent on their incomplete inhibition of mTORC1, such as signaling to 4E-BP1, a master regulator of protein synthesis [124,125], and the potentiation of mTORC2 signaling. This has led to develop a new generation of compounds designed to better inhibit mTORC1 and mTORC2, the ATP-site competitive inhibitors [126]. Preclinical studies have demonstrated, as a proof of concept, that mTOR active site inhibitors are powerful tools in treating cancers where mTOR activity is upregulated. However, whether they will have the clinical efficacy of the rapalogs is awaited [70].

A critical caveat for the introduction of active site inhibitors in the clinic is represented by the many functions assisted by mTOR signaling in the physiological contexts, which are crucial for the homeostasis of different tissues and organs [30]. Moreover, selectively targeting the signaling to ribosome biogenesis and protein synthesis versus other metabolic functions of mTOR could potentially open a new window of intervention for mitigating global protein synthesis in tumor cells and, on the other hand, limiting general toxicity to normal tissues. To this end, isolation and characterization of the translational modulators of RP synthesis is a novel and promising approach, which could disclose new targets for pharmacological intervention. RP mRNA translation is controlled by mTOR pathway through the 5′TOP motif. A global analysis of 5′TOP-binding partners and a drug screen for inhibitors of 5′TOP translation, could be key strategies to employ in order to gain more insights into tumors addicted to ribosome biogenesis.

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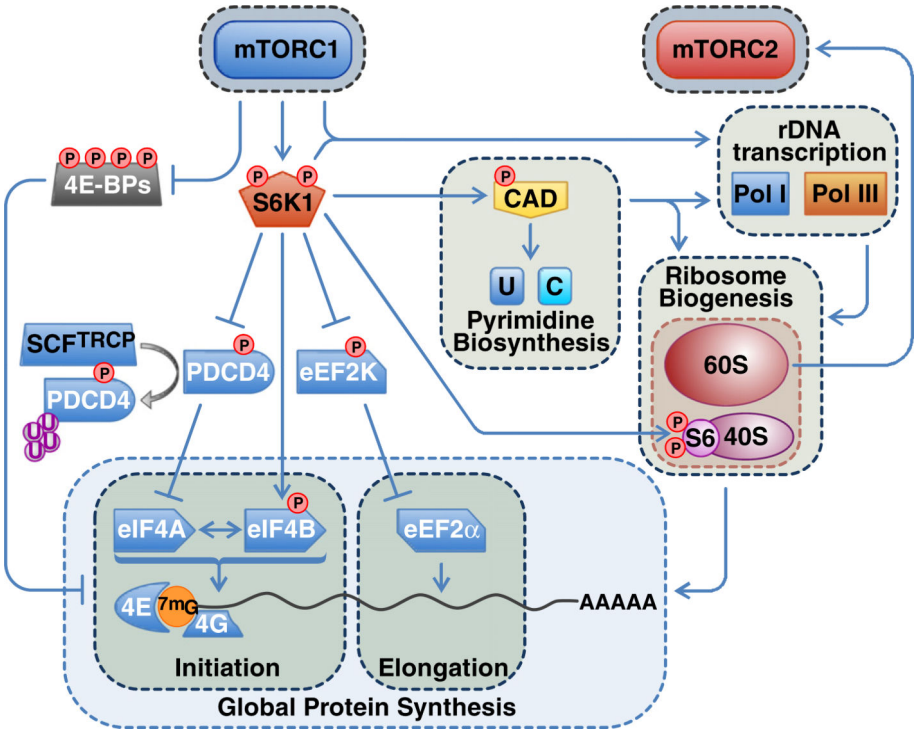

Fig. 1.

Schematic representation of mTOR pathways and ribosome biogenesis. mTORC1 phosphorylates and activates S6K1 which in turn positively regulates mRNA translation initiation, elongation, pyrimidine biosynthesis and rDNA transcription. mTORC1 phosphorylation on 4E-BPs relieves the inhibitory effect on translation initiation (see text). Straight lines and dotted lines indicate direct or indirect functional interactions respectively.

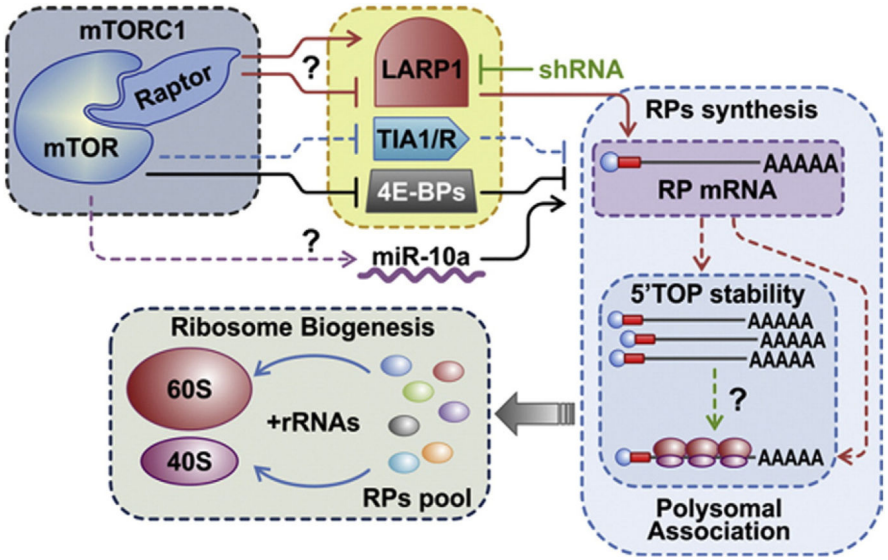

Fig. 2.

mTORC1 signaling to 5′TOP translation. Translational mediators of 5′TOP translation controlled by mTORC1: (a) LARP1 affects the steady state and the polysomal association of 5′ TOPs. (b) TIA1 and TIAR are translational inhibitors of 5′TOPs. (c) 4E-BPs are negative effectors of 5′TOP translation. (d) mir-10a stimulates translation of RPs mRNAs. Straight lines and dotted lines indicate direct or indirect functional interactions respectively.