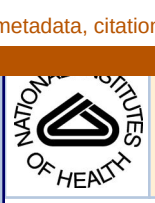

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28S rRNA is inducibly pseudouridylated by the mTOR pathway translational control in CHO cell cultures

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Abstract

The mTOR pathway is a conserved master regulator of translational activity that influences the fate of industrially relevant CHO cell cultures, yet its molecular mechanisms remain unclear. Interestingly, rapamycin specific inhibition of the mTOR pathway in CHO cells was found to down-regulate the small nucleolar RNA U19 (snoRNA U19) by 2-fold via translatome profiling. snoRNA U19 guides the two most conserved pseudouridylation modifications on 28S ribosomal RNA (rRNA) that are important for the biogenesis and proper function of ribosomes. In order to further understand the role of snoRNA U19 as a potential player in the mTOR pathway, we measured 28S rRNA pseudouridylation upon rapamycin treatments and/or snoRNA U19 overexpression conditions, thereby characterizing the subsequent effects on ribosome efficiency and global translation by polysome profiling. We showed that 28S rRNA pseudouridylation was increased by rapamycin treatment and/or overexpression of snoRNA U19, but only the latter condition improved ribosome efficiency towards higher global translation, thus implying that the mTOR pathway induces pseudouridylation at different sites along the 28S rRNA possibly with either positive or negative effects on the cellular phenotype. This discovery of snoRNA U19 as a new downstream effector of the mTOR pathway suggests that cell engineering of snoRNAs can be used to regulate translation and improve cellular growth in CHO cell cultures in the future.

Keywords

snoRNA U19; inducible pseudouridylation; mTOR pathway; translatome; CHO cells culture

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1. Introduction

Translational control via the mTOR pathway plays important role in regulating the growth and productivity of the industrially relevant CHO cells producing monoclonal antibodies (Courtes et al. 2013a; Dreesen and Fussenegger 2011); however, the underlying cellular mechanisms remain elusive. Experimentally, targeted inhibition of the mTOR pathway by rapamycin treatment in CHO cells down-regulated a number of genes, as such leading to reduced cellular growth (Courtes et al. 2013b). Interestingly, a recent study reported that growth arrest upon rapamycin treatment in human T-lymphocytes involves changes in expression of the small nucleolar RNA host gene (SNHG) GAS5 (Williams et al. 2011), which motivated us to explore the emerging role of small nucleolar RNA (snoRNAs) in the mTOR pathway regulation of CHO cells.

In mammalian cells, SNHGs belong to the family of 5'TOP mRNA (Smith and Steitz 1998) whose expression is regulated by the mTOR pathway (Meyuhas 2000), and they encode for snoRNAs in their intron regions (Ganot et al. 1997). snoRNAs are a group of non-coding RNAs responsible for posttranscriptional modification of ribosomal RNAs (rRNA) such as pseudouridylation, the most common and evolutionary conserved modification from yeast to human cells (Charette and Gray 2000). Insertion of pseudouridines generates more hydrogen bond donors in the rRNA structure, thus providing the potential to form new intra- and inter molecular interactions between partner proteins and mature ribosomes (Jack et al. 2011). Although the exact effect and function of pseudouridine modifications remain unclear, it is suggested that these modifications may serve to adjust rRNA structure for regulating the accuracy and efficiency as well as the biosynthesis of ribosomes (Piekna-Przybylska et al. 2008). In yeast, combinatorial depletions of five snoRNAs altered the structure of the large ribosomal subunit, which led to a shift of ribosome from polysome towards monosomes resulting in a 45% decrease of global translation output (King et al. 2003). Similarly, a substitution mutation of four snoRNAs guiding pseudouridylation modification in the loop and stem structures of the core H69 region known to interact with tRNAs (Yusupov et al. 2001), reduced amino acid incorporation rates *in vivo* and increased stop-codon readthrough activity (Liang et al. 2007). A mechanistic study on the same pseudouridylation sites of the H69 region further demonstrated that the lack of pseudouridylated residues led to conformational change of rRNA in the large subunit so as to assure correct positioning of critical rRNA bases involved in tRNAs accommodation (Baxter-Roshek et al. 2007).

Interestingly, thermodynamic and NMR based studies revealed that pseudouridylated residues can stabilize or destabilize an RNA hairpin structure when located at a stem loop junction or in single-stranded loop region respectively (Meroueh et al. 2000). Alternatively, defect in pseudouridylation was also observed to strongly delay rRNA processing thus modulating ribosome synthesis (Liang et al. 2009). Indeed, a number of individual snoRNAs guiding pseudouridylation at specific sites within core and highly conserved regions of rRNA, have proven to affect cellular growth phenotype in various organisms. For example, knockdown of snoRNA U19, the snoRNA that guides two most conserved pseudouridylation in 28S rRNA from yeast to human (Badis et al. 2003), induced a growth inhibition in yeast as compared to the wild type. Moreover, overexpression of snoRNA-42 in

bronchial epitheliums cells, a snoRNA found in high levels in lung tumors, increased cell growth and colony formation (Mei et al. 2012).

In this study, translatome analysis of CHO cells upon mTOR pathway inhibition by rapamycin allowed us to identify the non protein-coding SNHG4 hosting the equivalent of human snoRNA U19 as potential downstream effector in this signaling pathway. Thus, deciphering snoRNA U19 implication in gene expression regulation of CHO cell, in line with translational control of 5'TOP mRNAs through mTOR pathway is of great interest to understand new molecular mechanisms that contribute to regulating cellular growth. Toward this end, we overexpressed snoRNA U19 for characterization of its potential role in the mTOR pathway and its effects on pseudouridylation, ribosome efficiency and cellular growth phenotype. These preliminary evidences reveal that snoRNAs, alike other short non-coding RNAs such as miRNA, hold promising potential for improving CHO cell cultures.

2. Materials and Methods

2.1. Cell culture and rapamycin treatment

CHO-DG44 cell line was cultivated in a protein free and chemically defined property medium in shake flasks. All cell cultures were performed essentially as described in our previous work (Courtes et al. 2013a). For rapamycin treatment, 20 $ng.mL^{-1}$ was added on day 0 of cultures.

2.2. Translatome analysis

Translatome analysis was conducted by quantifying the ratio of rapamycin translatome over control translatome for each gene. Translatome data for the control condition were previously generated and validated by Courtes et al. (2013a), and translatome data under rapamycin treatment were similarly calculated as the ratio of polysome to monosome enriched pools based on the translatomic platform for CHO cell cultures. In brief, total RNA was extracted from cells and separated on 10–50% sucrose gradient. RNAs from 13 fractions were pooled together in pool A (polysome enriched) and pool B (monosome enriched) and purified via phenol-chloroform (Sigma-Aldrich) extraction before quantification on microarrays.

2.3. Overexpression of snoRNA U19

The insert containing snoRNA U19 was amplified from total CHO cells gDNA template with the forward (GGCGCTAGCTAACTTACAATCAGGCAAGTG) and reverse (GGCGGATCCCCTGATGGAGTCAGTTTTCTC) primers and was inserted into vector pcDNA™3.1/Hygro (Invitrogen). CHO cells were transfected using the Amaxa® Cell Line Nucleofector® Kit V (Lonza) according to the manufacturer instruction. Stable pools were selected with 300 $\mu g.mL^{-1}$ hygromycin (Clontech) twenty-four hours post transfection.

2.4. Quantification of snoRNA U19

Total RNA was extracted from 5×10^6 cells with Trizol (Invitrogen) as per the manufacturer's instructions. Equal volumes of total RNA samples were subjected to gDNA digestion with the RQ1 RNase-Free DNase (Promega) and were supplemented with 10 µL of

the internal control gene *thrB* of B. *Subtilis* (dilution 1:2000; Affymetrix). RNA samples were then polyadenylated by polyA polymerase (Ambion) according to the method developed by Ro et al. (2006). Equal volumes of polyadenylated RNA samples per day were subsequently reversed transcribed using the ImProm-II™ Reverse Transcription System (Promega).

Finally, snoRNA U19 was quantified via qRT-PCR using the supermix SsoFast™EvaGreen® (Biorad), for 40 cycles at 95°C for 30 seconds and 60°C for 10 seconds in iQ5 cycler system (Biorad). Measured CT values were processed according to the standard Δ(CT) method (Livak and Schmittgen 2001) with the internal control *thrB* gene in order to account for possible loss of RNA during the various extraction and purification steps.

2.5. Quantification of pseudouridine in 28S rRNA

Pseudouridine was quantified in 28S rRNA in triplicate from mid-exponential growth phase for each experimental condition. 28S rRNA was purified from total RNA by size-exclusion chromatography as described in Chionh et al. (2013). Following concentration on a 10kD filter (Pall, YM-10), the RNA was hydrolyzed and dephosphorylated into ribonucleoside form (Chan et al. 2010) and proteins were removed by a second 10kD filtration. The ribonucleosides were then resolved by HPLC (Thermo Scientific Hypercarb reversed-phase column, 100 × 2.1 mm, 3 μm particle size) with a gradient of acetonitrile in water containing 0.1% formic acid at a flow rate of 0.2 $mL.min^{-1}$ at 65 °C: 0 – 5 min, 5%; 15 – 25 min, 5 – 28%; 25 – 75 min, 28 – 75%; 25 – 30 min, 75%. Synthetic pseudouridine (Berry & Associates) eluted at 12.7 min under these conditions. The HPLC system was directly coupled to an Agilent 6410 QqQ LC/MS mass spectrometer with an ESI source operating in positive ion mode: gas temperature, 350 °C; N_2 gas flow, 10 L/min; nebulizer pressure, 20 psi; and capillary voltage, 3500 V. Pseudouridine was detected by setting Q1 to transmit the parent ion with *m/z* 125 (unit resolution) and Q3 set to monitor the *m/z* = 191 and 125 product ions resulting from collision-induced dissociation (collision energy, 10 eV; fragmentor voltage, 80 V; dwell time 200 ms), as described previously (Chan et al. 2010; Dudley et al. 2005). An example of the LC-MS/MS chromatogram for these CID fragmentation products is shown in Fig. S1A. To account for sample variation, the signal for pseudouridine was normalized by dividing the integrated MS peak for *m/z* 245 → 191 by the integrated 260 nm absorbance peak for cytidine measured by an in-line UV detector.

3. Results and discussion

3.1. mTOR pathway regulates snoRNA U19 expression in CHO cells culture

CHO cells were treated with rapamycin on day 0 of cultures and the translatome of treated and control cultures (Courtes et al. 2013a) were compared on days 1, 2 and 3 where most of the mRNAs shift from polysomes to monosomes was previously observed (Courtes et al. 2013b). Interestingly, the translatome of the snoRNA host gene 4 (SNHG4), a non-protein coding host gene member of the 5'TOP genes family (Smith and Steitz 1998), was reduced by at least 4-fold consistently over the three days (Fig. 1A-left). It should be noted that although SNHG4 and several other snoRNA host genes including U17HG, gas5, U19HG,

UHG and U87HG do not translate proteins (Bortolin and Kiss 1998; Pelczar and Filipowicz 1998), they were still found to associate with ribosomes and also to shift from polysomes towards monosomes after rapamycin treatment (Makarova and Kramerov 2005; Smith and Steitz 1998). The presence of numerous stop-codons in their exons suggests the potential involvement of nonsense-mediated decay, a process known to require active translation (Maquat 1995), as a mechanism to dispose of the exon portions of these transcripts, or a possible increase of stop-codon read-through activity (Liang et al. 2007). The unknown snoRNA hosted in SNHG4 of CHO cells, was identified as equivalent to the human snoRNA U19 with 87% ClustalW (version 2.0.12; Larkin et al. 2007) alignment homology against the snoRNA database (www-snorna.biotoul.fr; Lestrade and Weber 2006; Fig. 1B). Such non-coding gene as SNHG4 is currently believed to be expressed solely for the purpose of expressing snoRNA U19 that possess biological role (Bortolin and Kiss 1998). Hence we explored how the mTOR pathway's inhibition in CHO cells affected the expression of snoRNA U19 by measuring its level upon rapamycin treatment. As a result, we observed a 2-fold decrease of snoRNA U19 quantity from day 1 to day 3 of rapamycin treatment, relative to the respective untreated control level (Fig. 1A-right). Note that snoRNA U19 is known to guide pseudouridylation of two most conserved pseudouridines from yeast to human (Bortolin and Kiss 1998) that are crucial for ribosome biogenesis and functioning in support of cellular growth (Badis et al. 2003). Based on the observation, we derived the hypothesis that the growth inhibition of the CHO cells due to mTOR pathway (upon rapamycin treatment) was mediated in part through a mechanism involving snoRNA U19. To test this hypothesis further, the snoRNA U19 was over-expressed in CHO cells in addition to rapamycin treatment condition.

3.2. mTOR pathway inducibly pseudouridylates 28s rRNA in CHO cell cultures

Overexpression of snoRNA U19 gave rise to a 25-fold increase in its expression level on average from day 1 to day 5 during cultures as compared to control cells (Fig. 2A). The interplay between mTOR pathway and snoRNA U19 in CHO cells was then characterized via a set of four experimental conditions comparing the effects of snoRNA U19 overexpression and rapamycin treatment at three different cellular levels including the direct pseudouridylation activity on 28S rRNA (Fig. 2B left panel), the subsequent intermediate impact on ribosome efficiency for global translation (Fig. 2B middle panel) and the resulting influence on cellular growth phenotype (Fig. 2B right panel). Firstly, snoRNA U19 overexpression and rapamycin treatment led to 6% and 11% increases (Pvalue < 0.05; $n = 3$) in global 28S rRNA pseudouridylation content (Ψ) respectively, while there was 15% increase for the combined effect (Pvalue < 0.05; $n = 3$). Importantly, since the measurement of 28S rRNA pseudouridylation was global (i.e. no distinction between specific pseudouridylation sites), the increased pseudouridylation by two variables was not contradictory: overexpressed snoRNA U19 presumably increased the pseudouridylation at its two specific sites (Bortolin and Kiss 1998) whereas rapamycin treatment could increase the pseudouridylation elsewhere amongst the ~100 sites in mammalian cells rRNAs (Ge and Yu 2013). Secondly, the ribosome distribution between monosomes and polysomes was profiled on biological triplicates, in order to assess the subsequent effects on global translation activity. As pointed by the two black arrows, rapamycin treatment induced a strong shift of ribosomes from the polysomes towards the 80S peak (monosome) as was

previously observed (Courtes et al. 2013b). At the opposite snoRNA U19 overexpression increased the polysome content towards higher polysome degree with a concomitant decrease of 80S peak, thereby conferring greater global translation efficiency. Lastly, cellular growth phenotype calculated in terms of exponential growth rate μ_{max} was decreased by 0.7-fold on average (from 0.61 to 0.45 day^{-1}) after rapamycin treatment and was insignificantly affected by snoRNA U19 overexpression. Based on these first pseudouridylation data in the field of CHO bioprocessing, it appears clear that the mTOR pathway has the potential to regulate the degree of such rRNA base modifications in order to fine-tune translation activity. It is interesting to note that despite the increase in global translation activity after snoRNA U19 overexpression, there was no apparent reverting effect on cellular growth, which could be due to the overwhelming inhibitory effect of rapamycin treatment and contribution of additional regulations.

3.3. mTOR pathway controls cellular growth via inducible pseudouridylation in CHO cell cultures

To summarize our data, a hypothetical model can be suggested to describe the interplay between mTOR pathway and snoRNA U19 in CHO cells (Fig. 3). Of its various ranges of regulatory mechanisms for cellular growth, the mTOR pathway seems to involve pseudouridylation modification of rRNAs by orchestrating the expression level of snoRNAs, which belong the 5'TOP gene family. On the one hand, under adverse growth conditions (mimicked by rapamycin treatment), the mTOR pathway is inhibited, possibly up-regulating snoRNAs that guide pseudouridylations (increase of Ψ) with negative impact on cellular growth (decrease of μ_{max}). Such negative effects from pseudouridylation were also observed under similar circumstances. For example, it was recently demonstrated that extracellular stimuli such as starvation and heat shock triggered the introduction of additional pseudouridines in yeast spliceosomal snRNA U2 (Wu et al. 2011) which negatively impacted pre-mRNA splicing, causing a growth defect. In that study, the starvation trigger was likely to inhibit the mTOR pathway comparably to the rapamycin treatment in our work. Moreover, the mTOR pathway was also shown to regulate the expression of the snoRNA host gene GAS5 that has a deleterious effect on cell growth upon rapamycin treatment (Mourtada-Maarabouni et al. 2010; Williams et al. 2011). In the same light, the snoRNA host gene ZFAS1 was also observed to have an inhibitory effect on growth (Askarian-Amiri et al. 2011). In parallel, the mTOR pathway down-regulates the expression of snoRNAs such as U19 whose pseudouridylation activity would otherwise contribute to enhancing ribosome structure and function towards increased growth (Baxter-Roshek et al. 2007). On the other hand, under favorable growth conditions, the mTOR pathway would inverse its regulation and foster positive pseudouridylation to take place. It should be noted that since the mTOR pathway has been known to be a master regulator (Ma and Blenis 2009), other mechanisms unidentified in this work could be involved in cellular growth regulation, which awaits further investigation in future. Thus, the current results provide preliminary evidence that snoRNAs are novel downstream effector of the mTOR pathway in CHO cells, which could be considered as potential cell engineering targets to improve CHO cell cultures. Furthermore, since the total number of such snoRNAs is estimated to be up to 1,000 (Rearick et al. 2011), it will be of great interest to perform a high-throughput profiling of snoRNAs under bioprocessing relevant conditions using specially designed microarrays

as developed by Ge et al. (2010). Such snoRNAs profiling will lead to a greater understanding of their implication in gene expression regulation in CHO cells that will certainly help devising strategies for enhancing culture performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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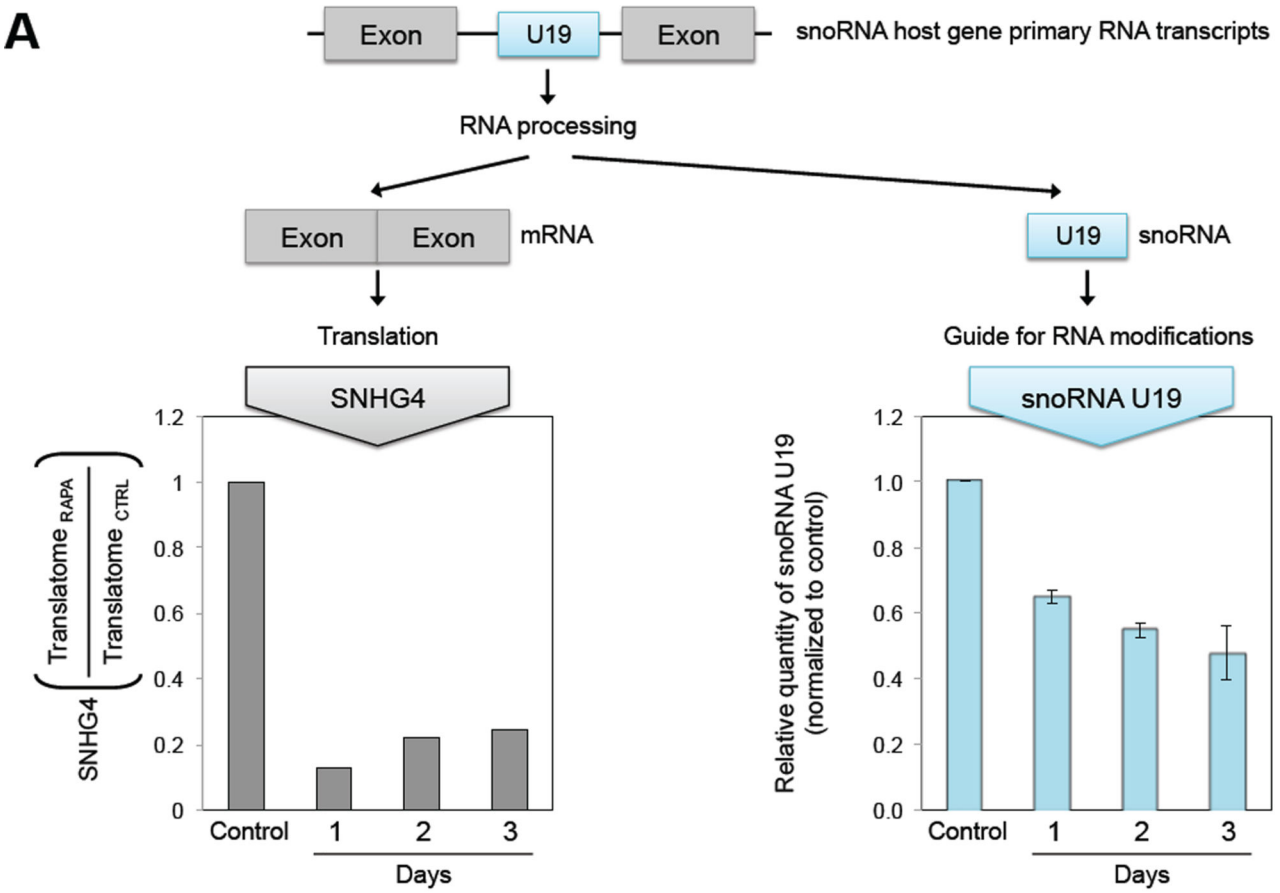

Figure 1.

Identification of snoRNA U19 in the mTOR pathway response to rapamycin treatment in CHO cells. (A) snoRNAs encoded in snoRNA host genes introns are separated from exons during splicing of the primary RNA transcripts and guide rRNA modifications which are crucial for the correct functioning of ribosomes. Upon rapamycin treatment, the mature SNHG4 mRNA (grey-left) and its hosted snoRNA (green-right) were down-regulated as normalized to the untreated control cells. SNHG4 was quantified as the intensity ratio of rapamycin translatome over control translatome measured by microarrays and snoRNA U19

was quantified by qRT-PCR. Standard deviations were calculated for n=2 or 3. (B) ClustalW alignment between CHO snoRNA encoded in SNHG4 and human snoRNA database identified snoRNA U19. Alignment legend: “*”: identical, “.”: conserved substitution and “.”: semi-conserved substitution.

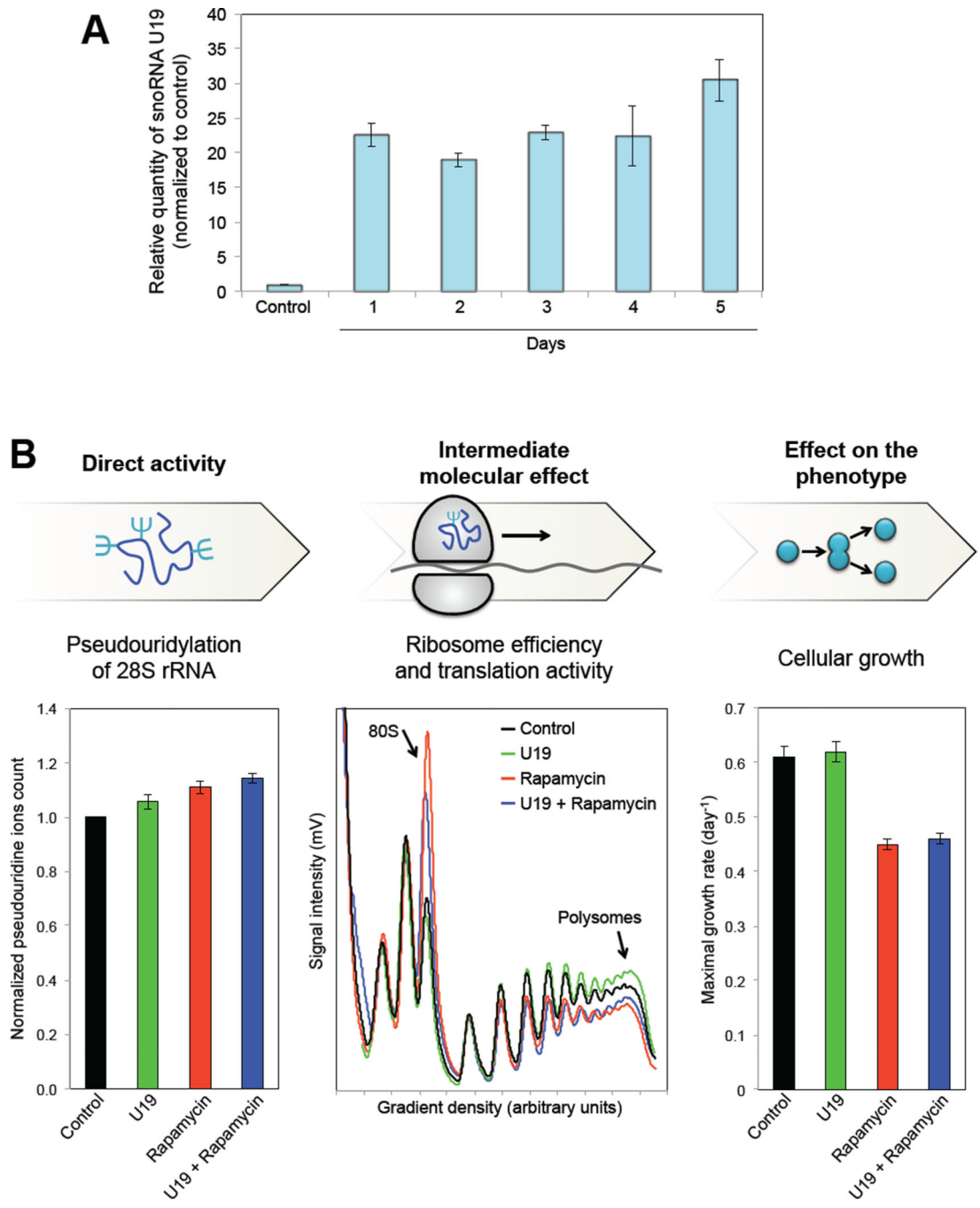

Figure 2.

Functional characterization of snoRNA U19 interplay with mTOR pathway in CHO cells. (A) Overexpression of snoRNA U19 in CHO-U19 pools relative to control pools. (B) The impact of snoRNA U19 was characterized at three subsequent levels: (1; left panel) direct activity on the pseudouridylation of 28S rRNA, (2; middle panel) intermediate molecular effect on ribosome efficiency and (3; right panel) effect on the cellular growth phenotype. Global 28S rRNA pseudouridylation (Ψ) values were obtained from mass spectrometry peak area normalized to the cytidine content. The ribosome efficiency was assessed by polysome

profiling. Black arrows locate the area of interest for interpretation with the 80S and polysome peaks respectively, where a shift of ribosomes from polysomes towards 80S peak indicates a decrease of global translation activity. Cellular growth values (μ_{max}) were calculated from day 1 to day 4 of the exponential growth phase as the slope of the straight trend-line on logarithmic scale. The control cell line M250-9 with (red) our without (black) rapamycin treatment was compared to the engineered pool overexpressing snoRNA U19 with (blue) or without (green) rapamycin treatment. Error bars show ± standard deviation on three biological replicates.

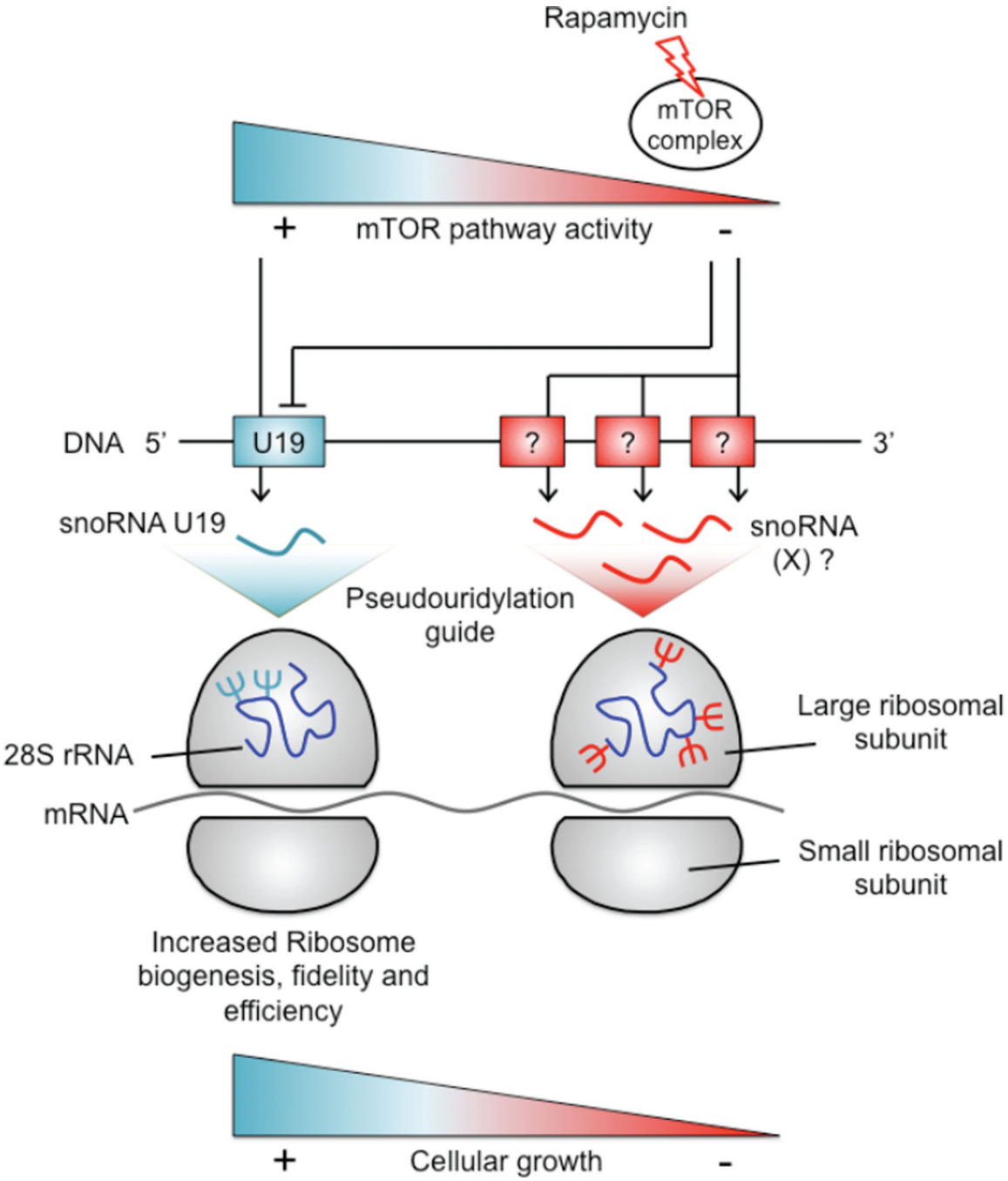

Figure 3.

Hypothetical model for mTOR pathway control of growth via inducible pseudouridylation in CHO cells. In addition to its various known mechanisms, the mTOR pathway is proposed to differentially activate pseudouridylation sites that may have positive or negative impact on cellular growth depending on the stress conditions.