Ribosomal RNA methylation induced by MYC regulates translation

Martin Jansson (✉ martin.jansson@bric.ku.dk)

University of Copenhagen

Sophia Hafner

University of Copenhagen

Kübra Altinel

University of Copenhagen

Disa Tehler

University of Copenhagen

Nicolai Krogh

University of Copenhagen

Emil Jakobsen

University of Copenhagen <https://orcid.org/0000-0001-6290-4222>

Jens Andersen

University of Copenhagen

Kasper Andersen

University of Copenhagen

Erwin Schoof

University of Copenhagen

Patrice Ménard

University of Copenhagen

Henrik Nielsen

University of Copenhagen, Department of Cellular and Molecular Medicine <https://orcid.org/0000-0001-7143-2810>

Anders Lund (✉ anders.lund@bric.ku.dk)

University of Copenhagen <https://orcid.org/0000-0002-7407-3398>

Article

Keywords: ribozymes, mRNA, rRNA, MYC

Posted Date: August 6th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-48673/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Nature Structural & Molecular Biology on November 10th, 2021. See the published version at <https://doi.org/10.1038/s41594-021-00669-4>.

Ribosomal RNA methylation induced by MYC regulates translation

Martin D. Jansson^{1*}, Sophia J. Häfner¹, Kübra Altinel^{1†}, Disa Tehler^{1†}, Nicolai Krogh², Emil Jakobsen³, Jens V. Andersen³, Kasper L. Andersen¹, Erwin M. Schoof⁴, Patrice Ménard¹, Henrik Nielsen², and Anders H. Lund^{1*}

¹Biotech Research and Innovation Centre, University of Copenhagen, Denmark

²Department of Cellular and Molecular Medicine, University of Copenhagen, Denmark

³Department of Drug Design and Pharmacology, University of Copenhagen, Denmark

⁴Department of Biotechnology and Biomedicine, Technical University of Denmark, Lyngby, Denmark

[†]These authors contributed equally

*Corresponding authors

Abstract

Ribosomes are complex ribozymes that interpret genetic information by translating messenger RNA (mRNA) into proteins. Natural variation in ribosome composition has been documented in several organisms, and can stem from several different sources. A key question is whether specific control over ribosome heterogeneity represents a mechanism by which translation can be regulated. We used RiboMeth-seq to demonstrate that differential 2'-O-methylation of ribosomal RNA (rRNA) represents a considerable source of ribosome heterogeneity in human cells, and that modification levels at distinct sites can change dynamically in response to upstream signalling pathways. Ablation of one prominent methylation, induced by *MYC* oncogene expression, resulted in altered translation of select mRNAs and corresponding changes in cellular phenotypes. Thus, differential rRNA 2'-O-methylation can give rise to ribosomes with specialized function. This constitutes a broader mechanism where the specific regulation of rRNA modification patterns fine-tune translation.

Main

Given the complexity of the ribosome, heterogeneity in its composition can emanate from many potential sources. These include variations in the nucleotide sequences of ribosomal RNAs (rRNA) expressed from distinct loci, post-transcriptional rRNA modifications, as well as the complement of associated ribosomal proteins and their post-translational modifications¹⁻⁵. Whether this heterogeneity gives rise to ribosomes with specialized function is currently a matter of debate^{6,7}.

During ribosome biogenesis, ribose methylation at the 2'-O position occurs only at certain defined rRNA nucleotide positions, that generally cluster in highly conserved and functionally important parts of the ribosome⁸. In Eukarya, almost all 2'-O- methylations (2'-O-me) are added by the methyltransferase Fibrillarin, which is guided to specific sites by different C/D box snoRNAs (SNORDs), via base-pairing interactions with their target rRNA⁹. Studies in bacteria and yeast indicate 2'-O-me can influence ribosome biogenesis, stabilization of rRNA structures, and translational fidelity¹⁰⁻¹². 2'-O-me is the most abundant post-transcriptional modification in human rRNA, however little is known about the impact of differential methylation on ribosome function and translation³.

Results

Variation and dynamics in rRNA 2'-O-methylation

We previously used RiboMeth-seq (RMS)¹³ to map and quantify rRNA 2'-O-me in two human cancer cell lines³. To obtain a broader perspective, we profiled additional cell lines (Extended Data Fig. 1). A previous candidate at 28S:G3606 was verified, and a new 2'-O-me site at 28S:U2031 was detected in non-tumor derived fibroblasts, probably guided by SNORD123

(Extended Data Fig. 1a, 2a-c). In human rRNA, 113 2'-O-me sites (42 in 18S, 69 in 28S and 2 in 5.8S) have now been identified in analyses of various cell lines by RMS, mass spectrometry, or both^{4,14}.

Comparing the rRNA 2'-O-me signatures across multiple cell lines, it became apparent that substantial variation exists between levels at many sites (Extended Data Fig. 1). This raised the question of whether these differences are hard-wired and cell type specific or could arise due to the action of distinct regulatory pathways within the cell. As some of the largest methylation changes at particular sites were observed between tumor and non-tumor derived cell lines, such as HeLaS3 adenocarcinoma and BJ^{hTERT} fibroblasts for example (Extended Data Fig. 2d-g), we aimed to explore the potential impact of key cancer-relevant pathways on rRNA 2'-O-me levels.

The *MYC* proto-oncogene is a master transcription factor, promoting cell proliferation and also translation¹⁵. As a model for oncogene activation, we engineered BJ^{hTERT} fibroblasts to express *MYC* in an inducible manner (BJ^{MYC}). Following treatment with doxycycline for 72 or 120h, increased *MYC* mRNA and protein levels are observed (Extended Data Fig. 2h, i). As expected, oncogene expression in these non-tumor cells resulted in slightly elevated p53 protein levels, as well as mildly increased expression of cell cycle inhibitors p21 and p16 (Extended Data Fig. 2i, j). No cell cycle arrest was detected under these conditions however (Extended Data Fig. 2k). *MYC* expression resulted in upregulation of Nucleophosmin and Fibrillarin, both known target genes with established roles in ribosome biogenesis¹⁶ (Extended Data Fig. 2l). The 2'-O-me profiles of 18S and 28S rRNA in uninduced BJ^{MYC} cells were compared those following 72 and 120h of *MYC* induction (Fig. 1a, b and Extended Data Fig. 1b-d). Particularly robust changes at three sites were detected, 2'-O-me increased at 18S:C174, while levels

decreased at the newly identified site 28S:U2031, and at 28S:U2402 (Fig. 1a, b and Extended Data Fig. 2m, n).

As a second example of the notion that distinct cellular pathways can influence methylation at specific rRNA 2'-O-me sites, the RMS profiles of HCT116 and HCT116^{p53-/-} colorectal carcinoma cells were compared. Here, reduced methylation at two other positions was observed in the absence of the p53 tumor suppressor (Extended Data Fig. 2o-r).

Using unsupervised hierarchical clustering, we examined the RMS scores of 18S and 28S rRNA across the datasets (Fig. 1c). Around two thirds of all sites are close to fully methylated and generally invariant, while the remainder are fractionally methylated and exhibit a higher degree of variation between cell types and conditions. The most variable methylated sites tend to be poorly conserved with those occurring at equivalent positions in the yeast ribosome (Fig. 1c and Supplementary Table 1). Furthermore, the 2'-O-me profiles of the fibroblasts after MYC induction cluster alongside the cancer cell lines, for both the 18S and 28S rRNAs (Fig. 1c). Methylation levels at 18S:C174 and 28S:U2031 are among the principal determinants of variation in the 18S and 28S rRNA clusters respectively.

From *in silico* predictions, SNORD45C was proposed to guide methylation at 18S:C174 (Cm174), and also at 18S:A159, using two different antisense elements¹⁷ (Extended Data Fig. 2s). SNORD45A however, may also guide methylation at 18S:A159, as well as 18S:U172. The SNORD45 family members are processed from introns of their host gene *RABGGTB*, which interestingly has been implicated as a MYC transcriptional target¹⁸(Fig. 1d). Notably, expression of both SNORD45C and SNORD45A is elevated along with the host gene, following MYC induction in fibroblasts (Fig. 1e). So, in this case there appears to be a direct link between induction of SNORD45C by MYC and increased Cm174 levels. We therefore selected Cm174 as

the primary candidate for a proof of principle study. The results pertaining to manipulation of MYC expression, and to p53 status, both support the idea that discrete cellular pathways can influence rRNA methylation signatures.

Differential 2'-O-methylation of ribosomal RNA influences translation

Typical of many cancer cell lines, MYC expression is elevated in HeLaS3 and moreover, the proportion of ribosomes with Cm174 is similar to that of BJ^{MYC} 120h post MYC induction (Extended Data Fig. 3a-d). To investigate its function, we used CRISPR-Cas9 gene editing to abolish the MYC-responsive 2'-O-me at 18S:C174, by deletion of SNORD45C in HeLaS3.

Expression of SNORD45C was lost in the knock-out cells (HeLaS3^{45CKO}), with modest reductions in *RABGGTB* and SNORD45A (Extended Data Fig. 3e). RMS analysis showed that Cm174 was completely absent in HeLaS3^{45CKO} cells, while methylation at 18S:A159, 18S:U172 and other sites remained largely unaffected (Fig. 2a, b and Extended Data Fig. 3f, g). This confirms SNORD45C as the guide for Cm174 and implies that Am159 is predominantly guided by SNORD45A.

Loss of Cm174 did not affect global translation, as determined by both ³⁵S-methionine incorporation and by examining ribosome distribution in polysome gradients (Fig. 2c-e). Furthermore, the levels of rRNA processing intermediates were also unaltered, indicating no defects in rRNA biogenesis (Extended Data Fig. 3h).

To investigate whether Cm174 status might influence the translation of specific mRNAs, we performed ribosome profiling of HeLaS3 and HeLaS3^{45CKO} cells (Extended Data Fig. 3i-p). Transcripts were assigned to one of 5 categories depending on their differential regulation in HeLaS3^{45CKO} cells: those that displayed significant change in mRNA expression (transcription);

ribosome occupancy (translation); both transcription and translation together; opposite change in transcription and translation; or were unchanged. While the majority of transcripts were unaffected (8519), abrogation of Cm174 resulted in a large number of transcripts changing at the level of translation alone (2049), while few changes in transcription (103) were detected (Fig. 2f and Supplementary Table 2). 1072 transcripts displayed decreased translation (TL-DN) and 977 increased translation (TL-UP) in HeLaS3^{45CKO} (Fig. 2f). This predominantly translational regulation implicates modulation of ribosome function by Cm174 as a probable mechanism.

mRNAs in the TL-DN and TL-UP sets are associated with different gene ontology (GO) categories for biological processes (Fig. 2g, h). For example, cell cycle and mitosis related terms are significantly enriched in the TL-DN set. On the other hand, metabolism-related, oxidation-reduction and intracellular transport terms are overrepresented in the TL-UP set. Intriguingly, gene set enrichment analysis (GSEA) indicated that mRNAs representing genes bound by MYC were significantly more likely to exhibit reduced translation in HeLaS3^{45CKO} cells (Extended Data Fig. 3q). In contrast to the translationally regulated sets, no enriched GO categories were found associated with transcripts displaying reduced mRNA expression (TC-DN), and few for those with increased expression (TC-UP) (Extended Data Fig. 3r).

Metagene read coverage analysis indicates that ribosome occupancy is globally reduced in TL-DN and increased in the TL-UP sets respectively, in accordance with altered ribosome density in the translationally-regulated transcripts (Fig. 2i). No major changes in the distribution of ribosomes over the coding sequence region (CDS) are apparent, although we noticed a tendency for increased ribosome density around the translation start-site in HeLaS3^{45CKO}, particularly for TL-UP transcripts (Fig. 2l). These observations are compatible with differences in either translation initiation or elongation rates.

Taken together, these findings indicate that the proportion of ribosomes methylated at 18S:C174 affects how efficiently different subsets of mRNAs are translated.

Cm174 affects cellular phenotypes

Consistent with decreased translation of mRNAs linked to cell division processes, proliferation, and the percentage of cells in S-phase, is reduced in HeLaS3^{45CKO} (Extended Data Fig. 4a-c). To exclude the possibility that clonal artefacts might contribute to this growth phenotype, we reinstated SNORD45C expression in the knockout background (HeLaS3-GFP^{45CKO+45C}). This completely rescued the decrease in cell proliferation, and crucially Cm174 levels were also restored (Fig. 3a-c and Extended Data Fig. 4d-f). Further analysis suggests the growth defect is due to SNORD45C and concomitant Cm174 loss, rather than any reduction in *RABGGTB* or *SNORD45A* expression, since these were not restored to wild-type levels in HeLaS3-GFP^{45CKO+45C} cells (Extended Data Fig. 4g).

To assess if the increased translation of metabolic-related genes gave rise to any phenotypes, we analyzed respiration and key parameters of mitochondrial function. Cells lacking Cm174 displayed higher basal and ATP-coupled respiration, suggestive of altered metabolism (Fig. 3d and Extended Data Fig. 4h-j). Reintroduction of SNORD45C fully reversed the altered metabolic phenotype (Fig. 3d).

We then set out to ascertain whether the observed translational regulation resulted in changes at the protein level, that might explain these cellular phenotypes. Quantitative tandem-mass-tag MS was used to analyze the proteomes of HeLaS3 and HeLaS3^{45CKO} cells (Fig. 4a, Extended Data Fig. 5a and Supplementary Table 3). Peptides derived from 7975 distinct proteins were detected in both conditions and used for protein abundance (PA) measurements (Data S2).

148 proteins were identified with significantly increased abundances (PA-UP) in HeLaS3^{45CKO} and 189 with reduced (PA-DN) abundances (Fig. 4a). Supporting the ribosome profiling data, GO analysis of the PA-UP and PA-DN sets revealed striking similarities in the overrepresented terms with those of the corresponding translationally regulated mRNA sets (Fig. 4b, c).

We then compared the protein abundances measured in both wild-type and HeLaS3^{45CKO} cells to ribosome occupancy or mRNA expression for equivalent protein-coding genes. The patterns observed were highly similar for the two cell lines and both correlations were statistically significant, although that between protein and ribosome occupancy was stronger (Fig. 4d, e and Extended Data Fig. 5b, c). This affirms, that globally, the ribosome footprint measurements are strongly predictive of protein level, and a more accurate indicator than mRNA levels alone. Examining those hits identified both by proteomic and ribosome profiling analyses, we found a robust association between protein abundance ratio and translation fold change (Fig. 4f). GO analysis of genes present in both PA-DN and TL-DN sets revealed cell division-related categories to be the most enriched, consistent with reduced cell proliferation in HeLaS3^{45CKO} (Extended Data Fig. 5d). The metabolic-related GO categories were not as well represented in hits common to both PA-UP and TL-UP sets however (Extended Data Fig. 5e).

In combination, these results show that absence of ribosomes methylated at 18S:C174 results in considerable phenotypic changes, and imply these arise due to the impact of differential translation on the levels of specific proteins.

Features of translationally altered mRNAs

We began searching for features, common among the mRNA transcripts comprising each respective set, that might explain their altered translation. Several studies have reported that

other potential sources of ribosome specialization may influence the preference for 5'cap- or IRES-dependent modes of translational initiation^{19,20}. To assess whether Cm174 status could affect IRES-mediated translation, we performed GSEA on the translationally regulated mRNAs using a list of high-confidence IRES-regulated genes, no significant enrichments were found (Extended Data Fig. 6a). Additionally, in reporter assay experiments, no differences in the activities of two cellular IRES elements were observed between HeLaS3^{45CKO} and parental cells (Extended Data Fig. 6b). Together, these results suggest that Cm174 has no major influence on IRES-dependent translation.

Next, we evaluated the codon compositions of mRNAs in the TL-DN, TL-UP and NS sets. Principal component analysis indicated a strong variance in the usage of elongation-related codons present in the CDSs of TL-DN and TL-UP transcripts (Fig. 5a). Comparison of the individual codon frequencies in these two sets revealed a striking two-pronged distribution pattern (Fig. 5b). In all, we identified 22 codons in TL-DN and 13 in TL-UP significantly enriched with log2-fold frequency changes >1 (Fig. 5b, c and Supplementary Table 4). Codons enriched in TL-DN tend to be AU-rich, whereas those in TL-UP are more GC-rich, this is especially apparent at the 3rd nucleotide position (AT3 or GC3) (Fig. 5d).

These patterns apply only to translationally-regulated transcripts, as no such differences in codon-usage are apparent between the differentially expressed mRNA sets (Extended Data Fig. 6c-e).

Despite large energetic differences between GC- and AU-rich codon-anticodon duplexes, binding and accommodation rates of aminoacyl-tRNAs and the complex stabilities at the ribosomal A-site appear to be remarkably similar *in vitro*²¹. This is due to evolution of intricate mechanisms in both ribosomes and tRNAs, that minimize differential thermodynamic

contributions of codon-anticodon interactions to maintain translation fidelity. The complex ribosome structural rearrangements associated with conformational changes and activation of Elongation Factor Thermo-unstable (EF-Tu or eEF1A in mammalian cells) during translation elongation constitutes one such example.

A revised energy-based interpretation of the genetic code was previously described²². Interestingly, codons with increased frequencies in TL-DN transcripts show a robust tendency to have thermodynamically weaker interactions with their cognate tRNAs, while those with increased frequencies in TL-UP tend to be stronger (Supplementary Table 4). Although the 3rd codon position carries less weight in this energy-based theoretical model, recent work has linked GC3 and AT3 codons with optimal and sub-optimal translation, as well as mRNA stability, respectively^{23,24}. Here though, we do not observe significant changes in mRNA levels associated with altered stability between the differentially translationally regulated transcripts carrying GC3/AT3 codons. In addition to the general GC/AT bias, all codons we find enriched in the TL-UP set are GC3 codons, while all but one in TL-DN are AT3 (Fig. 5b and Supplementary Table 4).

Moreover, there is considerable overlap between the TL-DN and TL-UP enriched codons with corresponding anti-codons previously shown to be utilized in translation of proliferation or differentiation associated genes, which appear to segregate according to GC/AT and also GC3/AT3 content^{24,25} (Extended Data Fig. 6f).

This suggests that certain factors contributing to functional ribosome specialization itself, in this case methylation of 18S:C174, can mediate differential translation of such codons, by modulating the capacity of the ribosome to compensate for differences in codon-anticodon interactions for instance.

Regulation of translation by rRNA 18S:C174 methylation

These observations are particularly interesting when considering the location of Cm174 within the ribosome tertiary structure (Fig. 6a). 18S:C174 is situated at the base of an internal loop of helix-8 (h8), a highly flexible region of the small ribosomal subunit that forms part of the intersubunit bridge-B8 (B8), together with helix-14 (h14)²⁶. While this area is poorly resolved in human ribosome structures that capture dynamics of the translation elongation cycle²⁷, h8 is evolutionarily well conserved (Extended Data Fig. 6g), and in bacteria is known to participate in substantial conformational changes during translation elongation^{28,29}. Furthermore, in *E. coli*, h8 is a hotspot for ribosomal ambiguity mutations (*ram*) that alter the helix structure and disrupt B8^{30,31}. *ram* in h8 result in translation errors characterized by a decreased stringency of decoding and an increased rate of GTP hydrolysis by EF-Tu, which plays a central role in translation elongation³⁰⁻³². It is therefore plausible that methylation status at C174 could affect ribosome function, albeit in a less drastic way than *ram* mutations, potentially by regulating the conformational flexibility of h8. We speculate that this in turn may modulate h8:h14 interaction, the dissociation of which is known to induce far-reaching ribosomal structural rearrangements culminating in domain closure, EF-Tu activation and subsequent decoding³⁰.

With this in mind, we examined the average ribosome occupancy, considered a measure of ribosome dwell time or pausing³³, in both HeLaS3^{45CKO} and parental cells. Transcripts comprising the TL-UP and TL-DN sets were compared (Fig. 5b). Curiously, the average ribosome occupancy rates over the first 5 codons appear higher in HeLaS3^{45CKO} for both sets. Over codons 6 to 40, TL-DN transcripts have higher occupancy in HeLaS3^{45CKO}, whereas this difference is not observed for the TL-UP set. This apparent queuing of ribosomes on TL-DN

transcripts in HeLaS3^{45CKO}, is consistent with slower decoding at AU-rich codons present with higher frequencies in this set.

Discussion

Several important conclusions can be drawn from our study, and these may conceptually enhance our understanding of how ribosome function can be regulated. Ribosome 2'-O-me sites can be generally grouped into two distinct categories, highly and fractionally methylated positions. There is little variation in 2'-O-me levels between different cell lines and conditions tested in regard to the highly-methylated sites, suggesting that these are important for ribosome biogenesis and optimal function. In contrast, much greater variability is seen in the fractional set, indicating that methylation levels at these sites may be less constrained, making them better suited for regulatory functions.

The existence of fractionally methylated positions demonstrates the presence of distinct pools of differentially 2'-O-methylated ribosomes within a specific cell population, and establishes that ribosome heterogeneity exists at the level of RNA modifications. In addition, the observation that methylation profiles can differ substantially between cell types suggests a role for 2'-O-me in regulating translation to meet the specific requirements of each cell type. Interestingly, the sites found to exhibit the greatest degree of variation in human cell lines are not conserved in yeast, though are present in murine ribosomes. This raises the possibility that regulation of ribosome function via differential 2'-O-me has become manifest more recently in evolutionary terms. However, there are also 15 2'-O-methylated nucleotides present in yeast rRNA that have no obvious counterparts in human or mouse cells. Overall the number of rRNA modifications has steadily increased during evolution together with increased ribosome size and

complexity³. While previous work in yeast demonstrated that depletion of clusters of ribosomal modifications had various effects on growth, ribosome biogenesis and translation efficiency, it was thought that individual 2'-O-me were of little consequence in terms of their impact on translation^{10,34}. Other studies though, suggest that disruption of single sites could potentially effect ribosome function, for example knockouts of several individual snoRNAs displayed phenotypes with respect to proliferation and drug sensitivity in yeast³⁵.

Excitingly, we show that discrete rRNA 2'-O-me sites can actually respond dynamically to distinct upstream signaling pathways. Furthermore, as demonstrated in the case of Cm174, site-dependent rRNA 2'-O-me levels can influence translation of mRNAs associated with functionally distinct categories. Importantly, this resulted in altered cellular phenotypes. The enrichment for MYC target genes among mRNAs with reduced translation in HeLaS3^{45CKO}, alludes to a tantalizing hypothetical mechanism in which MYC may regulate the translation of a selection of its own transcriptional targets, by altering cellular ribosome pools through modulation of specific 2'-O-methylations.

The study of SNORD45C knockout cells, however, represents an artificial situation. Importantly though, it facilitates dissection of Cm174 function from the pleiotropic effects of MYC expression (Fig. 7a), which result in increased ribosome biogenesis together with regulation of various translation factors as well as considerable transcriptional changes¹⁵. In a biological setting, we expect that SNORD45C induction by MYC would occur in concert with an increase in ribosome number and other changes promoting or regulating global translation (Fig. 7b). In this sense, the production of specialized ribosomes can act concomitantly with changes in ribosome abundance, which alone can influence translation of distinct mRNAs³⁶. As such, an additional newly synthesized ribosome pool, harboring Cm174, would act to more efficiently

translate proliferation-related mRNAs and reinforce a MYC-activated program. Yet, the other pools of ribosomes lacking Cm174 would also still be present for some time, and efficiently translate other mRNA classes involved in maintenance of different cellular processes.

It is intriguing to contemplate a role for rRNA modifications, or indeed other means of ribosome specialization, in regulating translation of specific mRNAs based on their codon compositions. Indeed, there may be a role for Cm174 in fine-tuning ribosome structural rearrangements related to balancing energetic differences, and those intrinsic to GC3/AT3 codons, in tRNA-mRNA interactions during translation elongation. Further in-depth studies would be required to fully substantiate this however.

The dynamics of the MYC responsive 2'-O-me sites are not rapid, with changes occurring over time scales in line with turnover of the cellular ribosome pool³⁷. Thus, this type of regulation may be more suited to tuning translation in long term cellular programs, such as during cell differentiation. Also, the possibility exists that in diseases such as cancer, mechanisms generating differentially 2'-O-methylated ribosomes are positively selected for in order to produce ribosome populations favoring translation of disease-promoting mRNAs. It will be of great interest to determine the exact mechanisms by which different regulatory 2'O-me sites impact specific aspects of ribosome structure and function to elicit effects on translation. Likewise, elucidating the distinct consequences that the diverse signals converging on the ribosome have, and how they differently influence modification of its function, will prove exciting. Overall, our findings promote the idea that the core ribosome itself is a direct translational regulator and point to 2'-O-methylation of rRNA being a potentially powerful means to tune ribosome function and impact translation.

Fig. 1 | MYC expression induces dynamic changes in 2'-O-methylation of human ribosomal rRNA. **a**, Comparison of RiboMeth-seq (RMS) scores representing fraction of 18S rRNA molecules 2'-O-methylated at each site in BJ^{MYC} cells, where MYC expression is induced for 120h or not induced. Points denote mean RMS score ($n=3$) per site. Horizontal and vertical error bars indicate $\pm$ s.d. for each condition. Sites exhibiting significant change are labelled with nucleotide position (magenta). **b**, RMS scores for 2'-O-me at 18S:C174 in BJ^{MYC} cells, without MYC induction or with MYC induction for 72 or 120h. Columns indicate mean RMS score ($n=3$) for each condition. Error bars represent $\pm$ s.d. P values are shown for each pairwise

336 comparison, indicated by brackets. **c**, Heatmap showing unsupervised clustering of RMS scores
337 at 2'-O-me sites (columns) in 18S (top panel) and 28S (bottom panel) rRNAs from the multiple
338 cell lines indicated (rows). Color scale represents degree of methylation from 0 (low, blue) to 1
339 (high, red). Dendrograms define identified clusters. Directly below each RMS heatmap,
340 conservation of human 2'-O-me sites is indicated for equivalent nucleotide positions known to
341 be methylated in mouse and yeast (purple = present, black = absent). **d**, Schematic showing
342 location of SNORD45 family members in snoRNA host gene *RABGGTB*. MYC transcription
343 factor is depicted by the cyan oval. **e**, Expression of *RABGGTB* and SNORD45 family members
344 -C and -A in BJ^{MYC} cells where MYC expression is not induced, or induced for 72 or 120h.
345 Columns indicate mean ($n=3$) fold-change relative to the control condition, normalized to
346 snRNA U6 expression, as determined by RT-qPCR analysis. Error bars indicate 95% confidence
347 intervals. *P* values are shown for pairwise comparisons, indicated by brackets.

Fig. 2 | Ribosome 2'-O-methylation at 18S:C174 regulates translation of specific mRNAs. a,
Comparison of RiboMeth-seq (RMS) scores in 18S rRNA from HeLaS3 and HeLaS3^{45CKO} cells.

351 Points denote mean RMS score ($n=3$) per site. Horizontal and vertical error bars indicate $\pm$ s.d.
 352 for each condition. Sites exhibiting significant change are labelled with nucleotide position
 353 (magenta). **b**, RMS scores for 2'-O-me at 18S:C174 in HeLaS3 and HeLaS3^{45CKO} cells. Columns
 354 indicate mean RMS score ($n=3$) for each condition. Error bars represent $\pm$ s.d. P value is shown
 355 above brackets. **c**, Polysome gradient profiles in HeLaS3 (WT, black) and HeLaS3^{45CKO} (45CKO,
 356 red) cells. Representative ($n=3$) profiles are shown. **d**, Global protein synthesis rates in HeLaS3
 357 and HeLaS3^{45CKO} cells assessed by [³⁵S] methionine/cysteine incorporation. Representative ($n=3$)
 358 autoradiograph of PAGE gel with Western blot for protein loading control below is shown. **e**,
 359 Quantification of [³⁵S] methionine/cysteine incorporation from $n=3$ independent experiments, as
 360 in (D). Columns indicate mean relative to control (HeLaS3). Error bars represent $\pm$ s.d. and P
 361 value is shown above brackets. **f**, Transcriptome-wide analysis of changes in mRNA expression
 362 and ribosome footprint levels from ribosome-profiling data, comparing HeLaS3 and HeLaS3^{45CKO}
 363 cells. Histogram (left) giving the number of mRNA transcripts, found to be significantly
 364 regulated in each category ($P^{adj} < 0.05$). Log2 fold-change scatter plots (right) with mRNA
 365 transcripts colored according to indicated regulation type. Total number of sequenced transcripts
 366 analyzed is given by "n". Number of transcripts up- or down-regulated in 45CKO relative to WT
 367 cells in the translation alone set is also shown (TL-UP, TL-DN respectively). **g**, Gene ontology
 368 analysis of mRNA transcripts in TL-DN set. Top 10 GO categories (FDR < 0.05) for biological
 369 process are labelled. Number of genes overlapping with each GO category is indicated by the
 370 color scale gradient (count). **h**, As (g) for TL-UP set. **i**, Metagene analysis plots of ribosome
 371 footprint coverage across mRNAs in each category for HeLaS3 (WT, black) and HeLaS3^{45CKO}
 372 (KO, red) cells, with extreme outliers removed. ALL, TL-DN, TL-UP represent all,
 373 translationally down-regulated or up-regulated transcripts respectively. Coding regions (between

START and STOP) for each transcript are scaled to 100nt. 5' and 3' untranslated regions (+/- 30nt) are unscaled. Coverage plotted is the RPKM normalized mean of libraries ($n=3$) in each case.

Fig. 3

Fig. 3 | Ribosome methylation at 18S:174 affects cellular phenotypes. **a**, Cell proliferation in HeLaS3^{GFP} (WT-GFP, black), HeLaS3^{45CKO-GFP} (45CKO-GFP, red) and HeLaS3^{45CKO-GFP+45C} (45CKO-GFP+45C, red, dashed) cells, measured by crystal violet assay at specified times. Points

382 represent mean of independent experiments ($n=3$). Error bars represent $\pm$ s.d. P values, indicated
 383 by brackets are shown for day 3. Significant changes in proliferation ($p<0.05$), between 45CKO-
 384 GFP and WT-GFP or 45CKO-GFP+45C, were observed at all timepoints except at day 1. **b**,
 385 Comparison of RMS scores representing fraction of 18S rRNA molecules 2'-O-methylated at
 386 each site in HeLaS3^{45CKO-GFP} and HeLaS3^{45CKO-GFP+45C} cells (left). Points denote mean RMS score
 387 ($n=3$) per site. Horizontal and vertical error bars indicate $\pm$ s.d. for each condition. Sites
 388 exhibiting significant change are labelled with nucleotide position (magenta). **c**, RMS scores for
 389 2'-O-me at 18S:C174 in HeLaS3^{45CKO-GFP+45C} and HeLaS3^{45CKO-GFP} cells. Columns indicate mean
 390 RMS score ($n=3$) for each condition. Error bars represent $\pm$ s.d. P value is shown above brackets.
 391 **d**, Respiration and key parameters of mitochondrial function in HeLaS3^{GFP}, HeLaS3^{45CKO-GFP} and
 392 HeLaS3^{45CKO-GFP+45C} cells. Basal respiration, ATP synthesis, proton leak and uncoupled
 393 respiration are measured by oxygen consumption rate (OCR) following injections with
 394 compounds modulating cellular respiration. Columns indicate mean OCR ($n=4$) for each
 395 condition. P values are shown for each comparison, indicated by brackets. Error bars represent $\pm$
 396 s.d.
 397

Fig.4

Fig. 4 | Ribosome methylation status at 18S:174 influences the cellular proteome. a, Protein abundance changes in HeLaS3^{45CKO} (45CKO) compared to HeLaS3 (WT) cells. Log2 abundance ratio (45CKO/WT) and corresponding -Log10 *P*_{adj} values are shown for all proteins detected in mass spectrometry analysis, represented by points. Proteins showing significant abundance change between conditions are colored (magenta). “n” denotes total number of proteins detected in both conditions. Number of proteins enriched in each condition is given (Enr WT, Enr KO). **b,** Gene ontology analysis of proteins in PA-DN set. Top 10 GO categories (FDR<0.05) for biological process are labelled. Number of genes overlapping with each GO category is indicated by the color scale gradient (count). **c,** As (b), for PA-UP set. **d,** Correlation between mRNA translation and protein abundance in HeLaS3 (WT) cells. Points represent mean ribosome protected fragment (RPF) count per million mapped reads (CPM) for each transcript (*n*=3, from ribosome profiling) and corresponding mean protein abundance for the same gene (*n*=4, from MS analysis). Color scale indicates plotting density. “n” denotes number of genes/proteins.

Spearman's ρ and associated P value are shown. **e**, As (d), for HeLaS3^{45CKO} (45CKO) cells. **f**, Correlation between mRNA translation and protein abundance for those genes with significant changes in both ribosome profiling and MS analysis of HeLaS3^{45CKO} (45CKO) and HeLaS3 (WT) cells. Points represent Log2FC in translation and log2FC in protein abundance ratio. "n" denotes number of genes. Spearman's ρ and associated P value are shown.

Fig. 5

Fig. 5 | Translationally regulated mRNAs display distinct codon-usage patterns. **a**, Principal component analysis of codon usage frequency in mRNA transcripts exhibiting increased (TL-UP, red), decreased (TL-DN, blue), and not-significantly altered (NS, grey) translation in

422 HeLaS3^{45CKO} compared to HeLaS3 cells. **b**, Codon usage frequencies in mRNA transcripts
423 exhibiting increased (TL-UP) or decreased (TL-DN) translation in HeLaS3^{45CKO} cells. Median
424 frequency of each codon is plotted in each case. Codons are color-coded by amino acid as
425 indicated. Codons changing with Log2 fold-change (Log2FC) >1, minimum frequency of 0.02
426 and $P^{adj} < 0.001$ are annotated by codon sequence. **c**, Examples of individual codons displaying
427 altered usage frequencies in each translation regulation category. Glutamic acid (gaa), alanine
428 (gcc), leucine (tta), leucine (ctg), isoleucine (ata) and arginine (cgc) and are shown. P^{adj} values
429 for comparisons are indicated by brackets. **d**, Fraction of G or C nucleotides present in codons
430 comprising mRNA transcripts in each translation regulation category; down-regulated (TL-DN,
431 blue), not-significantly regulated (NS, grey) or up-regulated (TL-UP, red). Comparisons for
432 entire codons (codon) or first, second and third codon positions (pos 1-3) are shown separately.
433 P^{adj} values for comparisons are shown above brackets.
434

Fig. 6

Fig. 6 | rRNA methylation at 18S:174 influences decoding of AT and GC-rich codons. a,

Structure of human ribosome indicating location of C174 (purple) at base of h8 (green) in human

rRNA. PyMol graphic based on cryo-EM ribosome structure modelled by Khatter *et al.* 18S

(dark grey) and 28S (light grey) rRNAs are indicated. E-site tRNA (red) is also shown for

orientation. b, Average ribosome occupancy or dwell time in HeLaS3 (WT, black) and

HeLaS3^{45CKO} (KO, red) cells. Metagene analysis of mRNA transcripts displaying down-regulated

(TL-DN, top panel), or up-regulated (TL-UP, bottom panel) translation in HelaS3^{45CKO} are shown. “n” denotes number of transcripts analyzed following removal of extreme outliers.

Fig. 7 | Roles of differential ribosome 2'-O-methylation in translational regulation. a, Working model based on Cm174 loss in SNORD45C knockout cells. Schematic illustrating how translation of specific mRNAs can be altered depending on presence or absence of rRNAs methylated at 18S:C174 (Cm174, cyan). rRNAs (red) are 2'-O-methylated (yellow) at specific positions guided by SNORDs during ribosome biogenesis. Differentially modified rRNAs are

451 incorporated, together with ribosomal proteins, into pre-ribosomal subunits (large subunit dark
452 grey, small subunit light grey) in the nucleus. SNORD45C is present in HeLaS3 (top), and absent
453 in HeLaS345CKO which lack Cm174 (bottom). Loss of Cm174 results in altered translation of
454 mRNAs in the cytoplasm, compared to the situation where 50-60% of ribosomes carry Cm174,
455 as indicated. Distinct functional classes of translationally regulated mRNAs are exemplified by
456 mRNA x (cell cycle and division related, green), mRNA y (metabolic related, black) and mRNA
457 z (other functional classes, blue). Altered translation, dependent on Cm174 status, results in
458 increased or decreased cell proliferation. **b**, Proposed hypothetical model describing how
459 differential rRNA 2'-O-methylation regulates translation in a biological situation. Illustrated by
460 induction of proliferative signaling or oncogene activation involving MYC expression. As (a),
461 though in this case, increased ribosome biogenesis driven by MYC, results in multiple
462 heterogenous ribosome types being produced. Both differential rRNA 2'-O-methylation or
463 potentially other means of ribosome specialization may occur in concert. MYC expression also
464 influences expression levels of certain functional classes of translationally regulated mRNAs.
465

Methods

Cell culture

The following cell lines used were used: HeLaS3 cervical adenocarcinoma; HCT116 and HCT116^{p53^{-/-}} colorectal carcinoma³⁸; DLD1 colorectal carcinoma; BJ^{hTERT} immortalized fibroblasts. HeLaS3 and BJ^{hTERT} were the parental lines for HeLaS3^{45CKO}, HeLaS3-GFP^{WT}, HeLaS3-GFP^{45CKO}, HeLaS3-GFP^{45CKO+45C} and BJ^{MYC} cells respectively. All cells were grown at 37°C, in 5% CO₂, as adherent monolayer cultures on Nunclon™Δ Polystyrene dishes/plates (Nunc). Cells were maintained in DMEM (HeLaS3 and derivatives; and BJ^{MYC}) with GlutaMAX™ and Pyruvate (ThermoFisher), McCoy's 5A (HCT116), or RPMI-1640 (DLD1). All media were supplemented with 10% fetal bovine serum.

Generation of BJ^{MYC} cells

The coding sequence of *MYC* (NM_002467.4) was cloned from cDNA derived from BJ^{hTERT}. This was inserted into pLVX-TetOne-Puro (Takara Bio) using the In-Fusion Cloning system (Takara Bio) to give pLVX-TetOne-Puro-MYC. Lentivirus was produced according to the Lenti-X Tet-One Inducible Expression Systems protocol (Takara Bio). BJ^{hTERT} cells were transduced with virus and selected with 0.25µg/mL Puromycin (Gibco) for 8d. The resistant mixed clonal population was then expanded. Puromycin selection was removed at least 72h before any experiments were performed.

Generation of SNORD45C knockout cells

HeLaS3^{45CKO} clones were generated using a CRISPR-Cas9 gene editing approach. In order to remove the SNORD45C, sequences for sgRNAs were cloned into pX335 and pX458 plasmids³⁹.

Each plasmid was co-transfected into HeLaS3 cells using Lipofectamine 3000 (ThermoFisher). Single cells positive for GFP and Crimson fluorescence were sorted into 96-well plates by FACS. After 2 weeks, clonal colonies were screened for successful deletion using PCR, verified by DNA sequencing, and expanded.

Generation of SNORD45C overexpression and control cells

SNORD45C was cloned into intron1 from host gene RPL23 replacing the endogenous snoRNA. The RPL23 intron containing SNORD45C was then inserted into a 2-exon EGFP sequence derived from pGINT (provided by Cristian Bellodi), which was used to replace the EGFP from the AAVS1-targeting vector pAAV-PuroCAG-EGFP, obtained from the Vallier lab⁴⁰. To generate the overexpression HeLaS3-GFP^{45CKO+45C} cells, the artificial EGFP-SNORD45C host gene was inserted into the AAVS1 locus of HeLaS3^{45CKO} using zinc-finger nucleases (according to the protocol from Bertero et al.). To generate the HeLaS3-GFP^{45CKO} and HeLaS3-GFP^{WT} control cells, the 2-exon EGFP without SNORD45C was similarly inserted in HeLaS3^{45CKO} and HeLaS3 respectively. The rescue and control cells were FACS-sorted for EGFP expression and sequenced to ensure correct insertion. SNORD45C expression was verified by RT-qPCR.

RNA isolation

Large scale and small-scale total RNA preparations were performed using QIAzol (Qiagen) or the Direct-zol RNA MiniPrep kit (Zymo Research) respectively, according to the manufacturer's instructions.

RiboMeth-seq

RiboMeth-seq library construction and sequencing were performed as previously described^{3,13}. Triplicate libraries were produced for each cell line or condition analysed grown to ~70–80% confluence before harvest. 4–5 µg of total RNA or purified 18S and 28S subunit rRNA (combined at 1:2.6 weight ratios to give approximate 1:1 molar ratio) were used for input. Note, we have observed no major differences in RMS scores for canonical 2'-O-me sites between libraries constructed from total RNA (as in the latest version of the RMS protocol) or purified subunit rRNA. RNA was partially degraded in alkaline at denaturing temperatures. The 20–40nt fragments were purified by polyacrylamide gel electrophoresis and linkers added using a system relying on a modified *Arabidopsis* tRNA ligase joining 2',3' cyclic phosphate and 5'-phosphate ends. The libraries were sequenced on the Ion Proton platform using Ion PI™ Chip Kit v3 (Life Technologies).

RiboMeth-seq Data treatment

Data was analysed as previously reported³. Briefly, sequencing reads were mapped to a corrected human rRNA reference sequence. To facilitate comparison with other studies, we have used the human rRNA sequence numbering according to snoRNABase⁴¹ throughout this study. An alignment table of these rRNA sequences is provided in Krogh and Jansson *et al*, 2016³. The RiboMeth-seq score (RMS score) represents the fraction of molecules methylated at each nucleotide position, and is calculated by comparing the number of read-end counts at the queried position to six flanking positions on either side. Quantifications are performed on 39 and 68 methylation sites in 18S and 28S rRNAs respectively, for which both RMS plus mass spectrometry evidence exists, and are reliably detected in at least one of cell lines examined in this study. For comparison plots of RMS signatures between two cell lines or conditions, sites

exhibiting significant change, in pairwise comparisons ($p < 0.05$, two-tailed unpaired Welch's t -test and ≥ 0.2 difference in RMS score), are labelled with nucleotide position and p values given in bar plots for specific sites. Heatmap representations were produced using the heatmap.2 function in R by unsupervised clustering. RMS data has been deposited to Gene Expression Omnibus (GEO), accession: *GSE153476*. Note, we have previously published 2 of 11 RMS datasets included in this study, the data for HeLaS3 (WT) and HCT116 (WT) cells³ is accessible from Gene Expression Omnibus (GEO), accession: *GSE76393*.

Evolutionary conservation of 2'-O-methylated rRNA nucleotides

Lists of validated 2'-O-methylated positions in 18S and 28S rRNAs, as described for human³, yeast¹³ and mouse⁴² were collated and compared. Conserved sites at equivalent nucleotide positions were determined by examination of the respective location in secondary structures of rRNA from each organism. The equivalent sites were then categorized as being present or absent in each organism. For Visualization and comparison, positions were rank ordered according to the RMS score unsupervised clustering analysis performed on multiple human cell lines. Heatmap representations were produced using the heatmap.2 function in R.

rRNA mass spectrometry analysis

rRNA fragments for MS analysis were isolated and subsequently digested with RNase A or T1 as previously described^{13,43}. Digested RNA fragments were subjected to MALDI-TOF MS analyses on an Autoflex Speed MS instrument (Bruker Daltonics) using reflector and positive ion mode.

Western blotting and antibodies

Western blotting was performed as previously described⁴⁴. The following antibodies were used: MYC D84C12 Rabbit mAb #5605 (Cell Signaling Technology); FBL sc-25397; p53 sc-126; MDM2 sc-56154 ; p16 CDKN2A sc-1661; GAPDH sc-25778 (Santa Cruz Biotechnology); p21 CDKN1A 556431 (BD pharmingen); Vinculin V9131 (Sigma).

RT-qPCR

Reverse transcription reactions were performed using the TaqMan™ Reverse Transcription Reagents (ThermoFisher), according to the manufacturer's instructions. Quantitative real-time PCRs were performed using TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (ThermoFisher) or Fast SYBR™ Green Master Mix (ThermoFisher), on a StepOne™ Real-Time PCR System (ThermoFisher) using automatic threshold detection. The following TaqMan™ Gene Expression Assays were used: ACTB, Hs01060665_g1; CDKN1A, Hs00355782_m1; CDKN2A, Hs02902543_mH; FBL, Hs01070449_m1; MYC, Hs00153408_m1; NPM1, Hs02339479_g1 (ThermoFisher). Primers sequences used in SYBR green qPCR reactions, are given (Supplementary Table 5). For individual experiments, reactions were performed in triplicate or duplicate per target gene for each condition. For a biological replicates ($n=3$), the mean Ct values from individual experiments were normalised to the control condition and standardised as previously described⁴⁵. Statistical significance was tested using two-tailed unpaired Welch's t test.

Polysome profiling

Cells at 70%-80% confluency were incubated with 100 µg/mL cycloheximide (Sigma Aldrich) for 3min and harvested by scraping in PBS containing 100 µg/mL cycloheximide. Cells were lysed at 4°C for 10min in Lysis buffer (20mM Tris-HCl, 150mM KCl, 5mM MgCl₂) supplemented with 0.5% NP40 (Igepal CA-630, Sigma Aldrich), 2mM DTT, 100µg/mL cycloheximide, Protease Inhibitor Cocktail (cOmplete EDTA free, Roche), and murine RNase inhibitor (NEB). The cell lysate was cleared by centrifugation at 12,000g for 15min at 4°C. Cleared lysates were normalized according to NanoDrop UV spectrophotometer (ThermoFisher) measurements and layered onto a 7% - 47% (w/v) linear sucrose gradient (Sigma BioUltra) in polysome buffer. Gradients were centrifuged at 35,000g for 3h at 4°C in an ultracentrifuge with the SW40ti rotor head (Beckman). Fractions of 1 mL were harvested from the top while continuously measuring A₂₆₀ using the BioLogicP system (BioRad). Absorbance spectra were plotted against time (from 4-16mins) to generate the polysome profiles.

³⁵S Metabolic labelling assay

For each of three biological replicates, HeLaS3 and HeLaS3^{45CKO} were seeded at equal density, in 6-well plates, in triplicate. 24h after, media was replaced with methionine and cysteine deficient media, supplemented with 33µCi of EasyTag EXPRESS Protein Labeling Mix (containing both ³⁵S-L-methionine and ³⁵S-L-cysteine, Perkin Elmer), and plates returned to the incubator for 1h. The cells were then washed and harvested by scraping into 1mL ice-cold PBS, pelleted, snap-frozen and stored at -80°C until further use. Lysis, and quantification of ³⁵S incorporation by densitometry, were performed as previously described ⁴⁶. Vinculin protein

levels were used to normalise ^{35}S incorporation measurements in each gel lane. Statistical significance was tested using two-tailed unpaired Welch's t test.

Northern blotting analysis of rRNA processing

Total RNA (7.5 μg) was separated on a formaldehyde denaturing 1% agarose gel and transferred to a BrightStar-Plus membrane (Ambion) using capillary blotting followed by UV cross-linking. The probes (10pmol each) were radiolabelled with [γ - ^{32}P]-ATP using T4 PNK (Thermo Scientific) and hybridized to the membrane overnight in hybridization buffer (4x Denhardt's solution, 6x SSC, 0.1% SDS) at T_m of the probe -10°C. The membrane was subsequently washed 4 times in 3x SSC supplemented with 0.1% SDS followed by exposure to a PI screen, scanned on a Typhoon scanner (GE Healthcare). Gel images were analysed using Fiji software.

Ribosome Profiling

Ribosome Profiling was performed essentially as previously described ⁴⁷, following the protocol given in TruSeq Ribo Profile Mammalian (Illumina), with minor modifications. Three individual replicates for each of the two cell lines were collected. A single 15cm dish corresponding to one replicate was harvested at a time. For each replicate, cell media was aspirated and cells washed with ice-cold PBS. No cycloheximide pre-treatment was performed. After thorough removal of the PBS, the dish was fully immersed in liquid nitrogen and placed on dry ice. For cell lysis, 1mL of 1x Mammalian Lysis Buffer (Illumina) containing 100 $\mu\text{g}/\text{mL}$ cycloheximide was added dropwise to the dish which was then placed on wet ice. Cells were then scraped off to the lower portion of the dish and allowed to thaw in the lysis buffer. Lysate was homogenised by pipetting and triturated ten times through a 25-gauge needle. The lysate was then transferred to a DNA

LoBind 1.5mL microfuge tube (Eppendorf) and incubated on ice for 5min. The lysate was cleared by centrifugation at 20000g, 4°C for 10min and the supernatant transferred to a fresh microfuge tube. Aliquots were prepared for each replicate, flash-frozen in liquid nitrogen and stored at -80°C until further use. The steps detailed in TruSeq Ribo Profile Mammalian protocol (Illumina) were followed to generate total RNA and ribosome protected fragment (RPF) RNA-seq libraries corresponding to the 3 individual replicates from each of the two cell lines. For RPF libraries, following nuclease digestion, monosomes were purified using illustra MicroSpin S-400 HR Columns. Ribo-Zero™ Gold Kit (Illumina) was used to deplete ribosomal rRNA. The libraries prepared from total RNA or RPF for both conditions were pooled and sequenced on a NextSeq® 500 System (Illumina).

Ribosome Profiling data analysis

The sequencing data was demultiplexed using Illumina bcl2fastq. Quality of the sequencing files were controlled with fastqc. Adaptor sequences were removed with cutadapt. Reads derived from RPF and total RNA were aligned to human rRNA and tRNA sequences with bowtie2 (v2.2.9) and the mapped reads discarded. The remaining reads were aligned to GRCh38.p12 (Ensembl v.97) with STAR. Reads mapping to Human Genome Organisation (HUGO) approved genes were used for downstream analyses.

RPF read lengths were analysed for trinucleotide periodicity using Ribotaper⁴⁸. RPF reads with lengths between 29 to 34 nucleotides were selected and the optimal P-site offset was defined as position 12 from 5' read ends. RPF read alignment files were filtered with samtools to retain only 29 to 34nt read lengths, no read length filtering was applied to the total RNA alignment files. A single canonical transcript representing each protein-coding gene was selected from the

GRCh38, v97 Ensembl annotation file (Supplementary Table 6). FeatureCounts was used to generate counts of primary reads mapping to exons of these transcripts for both total RNA and RPF. RPF reads with ribosome P-site positions mapping within transcript coding-region sequences (CDS) were again counted using FeatureCounts, and along with the mRNA exons-mapped reads, used for further measurements of differential translation and mRNA expression. Ribosomal Investigation and Visualization to Evaluate Translation (RIVET)⁴⁹, was used for translation and expression analysis of the representative transcripts (similar results were obtained for gene-level analysis). No fold-change cut-offs were directly applied, in order to additionally detect more subtle changes in translation. Regulated transcripts were therefore nominally identified by statistical significance. Translation regulation categories were defined according to RIVET, based on mRNA expression and ribosome occupancy, derived from normalized total RNA read counts or RPF read counts mapping to protein-coding mRNA transcripts, respectively (Supplementary Table 2). Plots from the resulting RIVET output files were generated using the ggplot2 package in R. The RNA sequencing data has been deposited to GEO, accession: *GSE153476*.

Gene ontology and gene-set enrichment analysis

All Gene ontology (GO) analyses of ribosome profiling and proteomic data was performed using the WebGestalt using the over-representation test against GO biological process database⁵⁰. Gene-set enrichment analysis (GSEA) was performed using GSEA software (version 4.0.3) {Subramanian:ByC22jeV}. Genes for analysis were pre-ranked according to Log2FC in translation from RIVET analysis ($P^{adj} < 0.05$) and compared to the following gene sets: i) DANG_BOUND_BY_MYC (from Molecular Signatures Database), ii) a custom gene set was

compiled (containing 112 distinct human genes) from those previously described to contain experimentally validated IRES elements or associated with polysomes following cap-dependent translation inhibition ⁵¹.

Metagene analysis

For RPF coverage metagene analyses, bam files containing exon-mapped reads for each library were converted to normalised (reads per kilobase per million, RPKM) single nucleotide resolution coverage bigwig files, with bamCoverage from the deepTools suite ⁵². WiggleTools ⁵³ (Ensembl) and wigToBigWig (Encode, kentUtils) were then used to merge these and create mean coverage files per condition. These were input to deepTools computeMatrix, together with an annotation file containing the exon coordinates for the selected mRNA transcripts. A count matrix was then generated for mean RPF coverage over the coding regions (CDS), scaled to size 100, for all transcripts. For further analysis, the scaled coverages of transcripts comprising the different translationally regulated categories were extracted from this matrix and plotted using custom R scripts. For average ribosome occupancy, mean coverage files per condition were created similarly. Here though, counts per million mapped reads (CPM) normalisation was performed and offset applied using bamCoverage, so as to use only the nucleotide position representing the ribosome P-site for each read as the signal (see Ribosome Profiling data analysis, above). The P-site coverage files were input to computeMatrix and a count matrix generated for -30 to +330 or -330 to +30 nucleotides, relative to the CDS start or end site respectively for each transcript (unscaled). The resulting counts at each position were divided by the total RPF count in CDS for each corresponding transcript to give the average ribosome occupancy per nucleotide position in each transcript. The mean values at each equivalent

695 nucleotide position relative to the translation start site were plotted, no smoothing was applied.
696 For P-site CPM the same matrices were used, but the counts at each position were not divided by
697 CDS RPF count per transcript. For plotting, extreme outliers ($>3\times$ interquartile range) were
698 removed. Plots were produced using ggplot2 in R.

699

700 **Cell proliferation assay by crystal violet staining.**

701 Cells were seeded in triplicate at equal density in 12-well plates. At each timepoint, cells were
702 washed and then fixed with 4% paraformaldehyde. Subsequently crystal violet staining was
703 performed for 30min at room temperature followed by three washes in PBS. The staining was
704 dissolved in 10% acetic acid, and absorbance (600nm) measured using a GloMaxMulti Detection
705 plate reader (Promega).

706

707 **EdU incorporation assay and flow cytometry analysis**

708 For each of three biological replicates, HeLaS3 and HeLaS3^{45CKO} were seeded in 6cm dishes in
709 triplicate at densities resulting in equal confluence (approximately 70%) at harvest. 48h later,
710 cells were pulsed with 30 μ M EdU for 40min and then harvested by trypsinisation and washed 3x
711 in PBS with 1% BSA. EdU detection was performed using Click-iT EdU Alexa Fluor 647 Flow
712 Cytometry Assay Kit (ThermoFisher) according to manufacturer's instructions, and
713 incorporation assessed by flow cytometry analysis of 20,000 cells per condition. Statistical
714 significance of cell cycle phase (G1, S, G2/M) data was determined using ordinary two-way
715 ANOVA. For propidium iodide staining, BJ^{MYC} harvested cells were harvested, washed, fixed in
716 70% ethanol, washed again and counted before incubation in FxCycle™ PI/RNase Staining

Solution (ThermoFisher). Analysis of all FACS data was performed using the FlowJo software package (Tree Star).

Metabolic analysis of oxygen consumption rate

Cellular oxygen consumption rate (OCR) was measured using a Seahorse 96XF analyzer, utilizing the Mitochondrial stress test according to the manufacturer's protocol. Cells were plated 24h before the experiment. HeLaS3, HeLaS3-GFP^{WT} and HeLaS3^{45CKO+45C} were seeded at 10,000 cells/well, while HeLaS3^{45CKO} and HeLaS3-GFP^{45CKO} were seeded at 14,000 cells/well (seeding densities were optimised to ensure equal confluence after 24h, and that OCR measurements were within the linear detection rate, data not shown). 1h before the experiment, the cells were washed and media exchanged into Seahorse assay medium (DMEM with 3mg/L phenol red and 5.5mM glucose, pH 7.4) and kept at 37 °C. 4 measurements were performed for each condition: baseline, oligomycin A (final concentration 1 µM), FCCP (final concentration 0.2 µM), and antimycin A/rotenone (final concentration 1.0 and 0.5 µM, respectively). Concentrations of oligomycin A, FCCP, and antimycin A/rotenone were optimized with dose/response experiments to ensure optimal conditions for the assay (data not shown). OCR measurements were analysed similarly to previously described⁵⁴. Each condition was performed with 16 technical replicates in each experiment, the data are presented as the mean of four individual experiments. Statistical significance was tested using repeated measures one-way ANOVA for experiments with three sample groups, or two-tailed unpaired Welch's *t* test for two groups.

Reporter assays for IRES-dependent translation

740 HeLaS3 and HeLaS3^{45CKO} cells were seeded in a 96-well plate and 24h later co-transfected with
741 firefly luciferase reporter constructs pIRES-c-myc or pBIC-943-IGF1R⁵⁵(IRES-dependant),
742 along with pRLTK renilla luciferase control (5'cap-depenant), using Lipofectamine 3000
743 (ThermoFisher). Luciferase assays were performed 24h post-transfection. In each experiment,
744 Firefly and Renilla luciferase activities were measured for quadruplicate transfections with the
745 Dual-Glo Luciferase Assay System (Promega) using the GloMax-Multi Detection System
746 (Promega). Ratios of Firefly to Renilla luciferase activity were then calculated to assess 5'cap vs
747 IRES-mediated translation initiation in each condition. Statistical significance was tested using
748 two-tailed unpaired Welch's *t* test.

749

750 **Codon-usage analysis**

751 Coding sequences for transcripts mapped in ribosome profiling analysis, were retrieved from
752 Ensembl (GRCh38.p12, v97). After exclusion of the start codon the codon usage frequency and
753 GC content were calculated using the uco and GC functions from the seqinr R package. The
754 codon usage frequencies or GC content were then grouped according to the regulation categories
755 defined in the analysis of the ribosome profiling data and compared between conditions.
756 Principal Component Analysis (PCA) was executed with R function prcomp, with the transcripts
757 as observations and codons as variables. In order to evaluate which codons contributed the most
758 to the different codon usage profiles the mean and median of each codon was calculated for the
759 above-mentioned categories. Log2 fold-changes were calculated from the means of the different
760 groups. P-values for individual codons and GC content were determined by Wilcoxon rank sum
761 test (unpaired, two-sided) and adjusted for multiple testing using Bonferroni correction. Codons
762 with Log2 fold change in frequency >1, $P^{\text{adj}} < 0.001$ were selected for further analysis. Codons

enriched in each translation category were then classified as “proliferation”, “differentiation” or “other” according to prior a study focused on tRNAs²⁵. Codons were designated as having strong, weak or intermediate interactions with their cognate tRNAs, as defined in previous analysis²² (Supplementary Table 4).

Proteomic mass spectrometry analysis

HeLaS3 and HeLaS3^{45CKO} cells were grown to ~70% confluency. A single 1°Cm dish was harvested for each of four biological replicates in each case. Cells were washed, collected by scraping into PBS, pelleted by centrifugation at 100g for 4min, and the supernatant removed. Samples were flash-frozen and stored at -80°C until further use. Lysate preparation and digestion was similarly to that previously described⁵⁶. Briefly, cell pellets were lysed using 300 µL of Guanidinium Hydrochloride lysis buffer (6M GndHCl, 100mM Tris-HCl pH8.5, 5Mm TCEp, 10mM CAA). Samples were sonicated on high for 5x 30sec in a Bioruptor sonication water bath (Diagenode) at 4°C. After determining sample protein concentration with Bradford (Sigma), 10µg of each was taken forward for digestion. Samples were diluted 1:3 with 10% Acetonitrile, 50 mM HEPES pH 8.5, LysC (MS grade, Wako) was added in a 1:50 (enzyme to protein) ratio, and samples were incubated at 37°C for 4hrs. Samples were further diluted to 1:10 with 10% Acetonitrile, 50 mM HEPES pH 8.5, trypsin (MS grade, Promega) was added in a 1:100 (enzyme to protein) ratio and samples were incubated overnight at 37°C. Enzyme activity was quenched by adding 2% trifluoroacetic acid (TFA) to a final concentration of 1%. Prior to TMT labeling, the peptides were desalted on in-house packed C18 Stagetips⁵⁷. For each sample, 2 discs of C18 material (3M Empore) were packed in a 200µL tip, and the C18 material activated with 40µL of 100% Methanol (HPLC grade, Sigma), then 40µL of 80% Acetonitrile, 0.1%

786 formic acid. The tips were subsequently equilibrated 2x with 40μL of 1%TFA, 3% Acetonitrile,
787 after which 10ug of sample was loaded using centrifugation at 4,000x rpm. After washing the
788 tips twice with 100ul of 0.1% formic acid, the peptides were eluted into clean 500μL Eppendorf
789 tubes using 40% Acetonitrile, 0.1% formic acid. The eluted peptides were concentrated in an
790 Eppendorf Speedvac (ThermoFisher), and re-constituted in 50mM HEPES (pH8.5) for TMT
791 labeling⁵⁸. Labeling was done according to manufacturer's instructions, and subsequently,
792 labeled peptides were mixed 1:1:1:1:1:1:1:1:1:1 (11-plex), acidified to 1% TFA and
793 Acetonitrile concentration brought down to <5% using 2% TFA. Prior to mass spectrometry
794 analysis, the peptides were fractionated using an offline ThermoFisher Ultimate3000 liquid
795 chromatography system using high pH fractionation (5mM Ammonium Bicarbonate, pH 10) at
796 5ul/min flowrate. 30μg of peptides were separated over a 120min gradient (5% to 35%
797 Acetonitrile), while collecting fractions every 120sec. The resulting 60 fractions were pooled
798 into 44 final fractions and vacuum concentrated to dryness. Fractions were resuspended in 1%
799 TFA, 2% Acetonitrile for MS analysis. Analysis was performed on an Orbitrap Fusion mass
800 spectrometer. For each fraction, peptides were loaded onto a 2cm C18 trap column
801 (ThermoFisher 164705), connected in-line to a 5°Cm C18 reverse-phase analytical column
802 (Thermo EasySpray ES803) using 100% Buffer A (0.1% Formic acid in water) at 750bar, using
803 the Thermo EasyLC 1200 HPLC system, and the column oven operating at 45°C. Peptides were
804 eluted over a 60min gradient ranging from 10% to 60% of 80% acetonitrile, 0.1% formic acid at
805 250 nL/min, and the Orbitrap Fusion instrument (Thermo Fisher Scientific) was run in an SPS
806 MS3 top speed method. Full MS spectra were collected at a resolution of 120,000, with an AGC
807 target of 2×10^5 or maximum injection time of 50ms and a scan range of 380–1500 m/z. The MS2
808 spectra were obtained in the ion trap operating at rapid speed, with an AGC target value of

1×10⁴ or maximum injection time of 50ms, a normalised CID collision energy of 35 and an intensity threshold of 5e3. Dynamic exclusion was set to 60sec, and ions with a charge state <2, >6 or unknown were excluded. From the resulting MS2 scan, 10 precursors were selected for SPS-MS3 analysis, fragmented with a normalised HCD collision energy of 65, and ions collected for a maximum of 86ms or AGC target of 1e5. Resulting MS3 spectra were collected at 50,000 resolution and scan range of 100-500 for reporter ion quantification. MS performance was verified for consistency by running complex cell lysate quality control standards, and chromatography was monitored to check for reproducibility. All MS spectra were searched in Proteome Discoverer 2.4 (ThermoFisher), using the SEQUEST algorithm against the human proteome Uniprot database (containing its reversed complement and known contaminants). Spectral matches were filtered to false discovery rate (FDR) <0.01, using the target-decoy strategy combined with linear discriminant analysis. Proteins were quantified only from peptides with an Average Reporter S/N Threshold of 10, and co-isolation specificity of 0.75. Statistical analysis of protein abundance changes was performed using the Background Proteins Hypothesis Test (Proteome Discoverer 2.4), which relies on robust variance and standard deviation calculations (Supplementary Table 3). For our data this test was more stringent (at FDR<0.1) than ANOVA testing (at FDR<0.05). The mass spectrometry data have been deposited to the ProteomeXchange Consortium, via the PRIDE repository with the dataset identifier: *PXD019449*.

Data availability

The datasets generated during the current study have been deposited in appropriate public databases, and accession numbers given in the appropriate Methods section.

Code availability

All the procedures but those for sequencing reads cleaning were performed using Python, R scripts and available bioinformatic tools as outlined in the Methods section. Any custom code is available upon reasonable request.

Acknowledgements

The Lund lab is supported by grants from the Danish Council for Independent Research (Sapere Aude program 418 3-00179B); the Novo Nordisk Foundation (NNF18OC0030656); the Lundbeck Foundation (R198-2015-174) and the Danish Cancer Society (R204-A12532).

Author Contributions

M.D.J. and A.H.L. conceived the study. M.D.J., S.J.H., N.K., E.J., J.V.A. and P.M. designed and performed the experiments. K.A., M.D.J. and D.T. of RNA analysed sequencing data and other computational analyses. E.M.S. and M.D.J. acquired and analysed proteomic data. K.L.A. and H.N. provided methodological and conceptual contributions. M.D.J wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

852

853 **Additional information**

854 **Extended data** is available for this paper (**Extended Data Figures 1-6**).

855 **Supplementary information** is available for this paper (**Supplementary Tables 1-6**).

856

857 **References**

- 858 1. Kurylo, C. M. *et al.* Endogenous rRNA Sequence Variation Can Regulate Stress Response
859 Gene Expression and Phenotype. *Cell Rep* **25**, 236–248.e6 (2018).
- 860 2. Emmott, E., Jovanovic, M. & Slavov, N. Ribosome Stoichiometry: From Form to
861 Function. *Trends in biochemical sciences* **44**, 95–109 (2019).
- 862 3. Krogh, N. *et al.* Profiling of 2'-O-Me in human rRNA reveals a subset of fractionally
863 modified positions and provides evidence for ribosome heterogeneity. *Nucleic acids*
864 *research* **44**, 7884–7895 (2016).
- 865 4. Taoka, M. *et al.* Landscape of the complete RNA chemical modifications in the human
866 80S ribosome. *Nucleic Acids Research* **520**, 640 (2018).
- 867 5. Parks, M. M. *et al.* Variant ribosomal RNA alleles are conserved and exhibit tissue-
868 specific expression. *Sci Adv* **4**, eaao0665 (2018).
- 869 6. Ferretti, M. B. & Karbstein, K. Does functional specialization of ribosomes really exist?
870 *RNA* **25**, 521–538 (2019).
- 871 7. Dinman, J. D. Pathways to Specialized Ribosomes: The Brussels Lecture. *Journal of*
872 *molecular biology* **428**, 2186–2194 (2016).
- 873 8. Sloan, K. E. *et al.* Tuning the ribosome: the influence of rRNA modification on eukaryotic
874 ribosome biogenesis and function. *RNA Biol* 0–16 (2016).
875 doi:10.1080/15476286.2016.1259781
- 876 9. Kiss-László, Z., Henry, Y. & Bachellerie, J. P. Site-specific ribose methylation of
877 preribosomal RNA: a novel function for small nucleolar RNAs. *Cell* (1996).
- 878 10. Liang, X.-H., Liu, Q. & Fournier, M. J. rRNA modifications in an intersubunit bridge of
879 the ribosome strongly affect both ribosome biogenesis and activity. *Molecular cell* **28**,
880 965–977 (2007).
- 881 11. Polikanov, Y. S., Melnikov, S. V., Söll, D. & Steitz, T. A. Structural insights into the role
882 of rRNA modifications in protein synthesis and ribosome assembly. *Nat. Struct. Mol. Biol.*
883 **22**, 342–344 (2015).
- 884 12. Baudin-Baillieu, A. *et al.* Nucleotide modifications in three functionally important regions
885 of the *Saccharomyces cerevisiae* ribosome affect translation accuracy. *Nucleic acids*
886 *research* **37**, 7665–7677 (2009).
- 887 13. Birkedal, U. *et al.* Profiling of ribose methylations in RNA by high-throughput
888 sequencing. *Angew. Chem. Int. Ed. Engl.* **54**, 451–455 (2015).
- 889 14. Krogh, N. & Nielsen, H. Sequencing-based methods for detection and quantitation of
890 ribose methylations in RNA. *Methods (San Diego, Calif)* **156**, 5–15 (2018).
- 891 15. van Riggelen, J., Yetil, A. & Felsher, D. W. MYC as a regulator of ribosome biogenesis
892 and protein synthesis. *Nature reviews* **10**, 301–309 (2010).
- 893 16. Collier, H. A. *et al.* Expression analysis with oligonucleotide microarrays reveals that
894 MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proceedings*
895 *of the National Academy of Sciences of the United States of America* **97**, 3260–3265
896 (2000).
- 897 17. Makarova, J. A. & Kramerov, D. A. Analysis of C/D box snoRNA genes in vertebrates:
898 The number of copies decreases in placental mammals. *Genomics* **94**, 11–19 (2009).
- 899 18. Zeller, K. I. *et al.* Global mapping of c-Myc binding sites and target gene networks in
900 human B cells. *Proceedings of the National Academy of Sciences of the United States of*
901 *America* **103**, 17834–17839 (2006).

19. Shi, Z. *et al.* Heterogeneous Ribosomes Preferentially Translate Distinct Subpools of mRNAs Genome-wide. *Molecular Cell* (2017). doi:10.1016/j.molcel.2017.05.021
20. Jack, K. *et al.* rRNA pseudouridylation defects affect ribosomal ligand binding and translational fidelity from yeast to human cells. *Molecular cell* **44**, 660–666 (2011).
21. Wohlgemuth, I., Pohl, C. & Rodnina, M. V. Optimization of speed and accuracy of decoding in translation. *The EMBO journal* **29**, 3701–3709 (2010).
22. Grosjean, H. & Westhof, E. An integrated, structure- and energy-based view of the genetic code. *Nucleic acids research* **44**, 8020–8040 (2016).
23. Forrest, M. E. *et al.* Codon and amino acid content are associated with mRNA stability in mammalian cells. *PLoS ONE* **15**, e0228730 (2020).
24. Hia, F. *et al.* Codon bias confers stability to human mRNAs. *EMBO reports* **20**, e48220 (2019).
25. A Dual Program for Translation Regulation in Cellular Proliferation and Differentiation. *Cell* **158**, 1281–1292 (2014).
26. Liu, Q. & Fredrick, K. Intersubunit Bridges of the Bacterial Ribosome. *Journal of molecular biology* **428**, 2146–2164 (2016).
27. Behrmann, E. *et al.* Structural snapshots of actively translating human ribosomes. *Cell* **161**, 845–857 (2015).
28. Villa, E. *et al.* Ribosome-induced changes in elongation factor Tu conformation control GTP hydrolysis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 1063–1068 (2009).
29. Paci, M. & Fox, G. E. Centers of motion associated with EF-Tu binding to the ribosome. *RNA Biol* **13**, 524–530 (2016).
30. Fagan, C. E. *et al.* Reorganization of an intersubunit bridge induced by disparate 16S ribosomal ambiguity mutations mimics an EF-Tu-bound state. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 9716–9721 (2013).
31. McClory, S. P., Leisring, J. M., Qin, D. & Fredrick, K. Missense suppressor mutations in 16S rRNA reveal the importance of helices h8 and h14 in aminoacyl-tRNA selection. *RNA* **16**, 1925–1934 (2010).
32. Hoffer, E. D., Maehigashi, T., Fredrick, K. & Dunham, C. M. Ribosomal ambiguity (ram) mutations promote the open (off) to closed (on) transition and thereby increase miscoding. *Nucleic acids research* **47**, 1557–1563 (2019).
33. Schuller, A. P., Wu, C. C.-C., Dever, T. E., Buskirk, A. R. & Green, R. eIF5A Functions Globally in Translation Elongation and Termination. *Molecular cell* **66**, 194–205.e5 (2017).
34. Liang, X.-H., Liu, Q. & Fournier, M. J. Loss of rRNA modifications in the decoding center of the ribosome impairs translation and strongly delays pre-rRNA processing. *RNA* **15**, 1716–1728 (2009).
35. Esguerra, J., Warringer, J. & Blomberg, A. Functional importance of individual rRNA 2'-O-ribose methylations revealed by high-resolution phenotyping. *RNA* **14**, 649–656 (2008).
36. Mills, E. W. & Green, R. Ribosomopathies: There's strength in numbers. *Science (New York, N.Y)* **358**, eaan2755 (2017).
37. Wolf, S., Sameshima, M., Liebhaber, S. A. & Schlessinger, D. Regulation of ribosomal ribonucleic acid levels in growing, 3H-arrested, and crisis-phase WI-38 human diploid fibroblasts. *Biochemistry* **19**, 3484–3490 (1980).

38. Bunz, F. *et al.* Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science (New York, N.Y)* **282**, 1497–1501 (1998).
39. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, N.Y)* **339**, 819–823 (2013).
40. Bertero, A. *et al.* Optimized inducible shRNA and CRISPR/Cas9 platforms for in vitro studies of human development using hPSCs. *Development* **143**, 4405–4418 (2016).
41. Lestrade, L. snoRNA-LBME-db, a comprehensive database of human H/ACA and C/D box snoRNAs. *Nucleic acids research* **34**, D158–D162 (2006).
42. Hebras, J., Krogh, N., Marty, V., Nielsen, H. & Cavaillé, J. Developmental changes of rRNA ribose methylations in the mouse. *RNA Biol* **17**, 150–164 (2020).
43. Douthwaite, S. & Kirpekar, F. Identifying modifications in RNA by MALDI mass spectrometry. *Meth. Enzymol.* **425**, 3–20 (2007).
44. Jansson, M. D., Damas, N. D., Lees, M., Jacobsen, A. & Lund, A. H. miR-339-5p regulates the p53 tumor-suppressor pathway by targeting MDM2. *Oncogene* **34**, 1908–1918 (2015).
45. Willems, E., Leyns, L. & Vandesompele, J. Standardization of real-time PCR gene expression data from independent biological replicates. *Anal. Biochem.* **379**, 127–129 (2008).
46. Cunningham, J. T., Pourdehnad, M., Stumpf, C. R. & Ruggero, D. Investigating Myc-dependent translational regulation in normal and cancer cells. *Methods in molecular biology (Clifton, N.J)* **1012**, 201–212 (2013).
47. Ingolia, N. T., Ghaemmaghami, S., Newman, J. R. S. & Weissman, J. S. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science (New York, N.Y)* **324**, 218–223 (2009).
48. Calviello, L. *et al.* Detecting actively translated open reading frames in ribosome profiling data. *Nature methods* **13**, 165–170 (2016).
49. Ernlund, A. W., Schneider, R. J. & Ruggles, K. V. RIVET: comprehensive graphic user interface for analysis and exploration of genome-wide translomics data. *BMC Genomics* **19**, 809 (2018).
50. Liao, Y., Wang, J., Jaehnig, E. J., Shi, Z. & Zhang, B. WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic acids research* **47**, W199–W205 (2019).
51. Weingarten-Gabbay, S. *et al.* Comparative genetics. Systematic discovery of cap-independent translation sequences in human and viral genomes. *Science (New York, N.Y)* **351**, (2016).
52. Ramírez, F. *et al.* deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic acids research* **44**, W160–5 (2016).
53. Zerbino, D. R., Johnson, N., Juettemann, T., Wilder, S. P. & Flicek, P. WiggleTools: parallel processing of large collections of genome-wide datasets for visualization and statistical analysis. *Bioinformatics* **30**, 1008–1009 (2014).
54. Zhang, J. *et al.* Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. *Nat Protoc* **7**, 1068–1085 (2012).
55. Belin, S. *et al.* Dysregulation of ribosome biogenesis and translational capacity is associated with tumor progression of human breast cancer cells. *PLoS ONE* **4**, e7147 (2009).

- 991 56. Kulak, N. A., Pichler, G., Paron, I., Nagaraj, N. & Mann, M. Minimal, encapsulated
992 proteomic-sample processing applied to copy-number estimation in eukaryotic cells.
993 *Nature methods* **11**, 319–324 (2014).
- 994 57. Rappsilber, J., Mann, M. & Ishihama, Y. Protocol for micro-purification, enrichment, pre-
995 fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* **2**, 1896–
996 1906 (2007).
- 997 58. Thompson, A. *et al.* Tandem mass tags: a novel quantification strategy for comparative
998 analysis of complex protein mixtures by MS/MS. *Anal. Chem.* **75**, 1895–1904 (2003).
999

Figures

Figure 1

MYC expression induces dynamic changes in 2'-O-methylation of human ribosomal rRNA. a, Comparison of RiboMeth-seq (RMS) scores representing fraction of 18S rRNA molecules 2'-O-methylated at each site in BJMYC cells, where MYC expression is induced for 120h or not induced. Points denote mean RMS score (n=3) per site. Horizontal and vertical error bars indicate $\pm$ s.d. for each condition. Sites exhibiting significant change are labelled with nucleotide position (magenta). b, RMS scores for 2'-O-me at 18S:C174 in BJMYC cells, without MYC induction or with MYC induction for 72 or 120h. Columns indicate mean RMS score (n=3) for each condition. Error bars represent $\pm$ s.d. P values are shown for each pairwise comparison, indicated by brackets. c, Heatmap showing unsupervised clustering of RMS scores at 2'-O-me sites (columns) in 18S (top panel) and 28S (bottom panel) rRNAs from the multiple cell lines indicated (rows). Color scale represents degree of methylation from 0 (low, blue) to 1 (high, red). Dendrograms define identified clusters. Directly below each RMS heatmap, conservation of human 2'-O-me sites is indicated for equivalent nucleotide positions known to be methylated in mouse and yeast

(purple = present, black = absent). d, Schematic showing location of SNORD45 family members in snoRNA host gene RABGGTB. MYC transcription factor is depicted by the cyan oval. e, Expression of RABGGTB and SNORD45 family members -C and -A in BJMYC cells where MYC expression is not induced, or induced for 72 or 120h. Columns indicate mean (n=3) fold-change relative to the control condition, normalized to snRNA U6 expression, as determined by RT-qPCR analysis. Error bars indicate 95% confidence intervals. P values are shown for pairwise comparisons, indicated by brackets.

Figure 2

Ribosome 2'-O-methylation at 18S:C174 regulates translation of specific mRNAs. a, Comparison of RiboMeth-seq (RMS) scores in 18S rRNA from HeLaS3 and HeLaS345CKO cells. Points denote mean RMS score (n=3) per site. Horizontal and vertical error bars indicate $\pm$ s.d. for each condition. Sites exhibiting significant change are labelled with nucleotide position (magenta). b, RMS scores for 2'-O-me at 18S:C174 in HeLaS3 and HeLaS345CKO cells. Columns indicate mean RMS score (n=3) for each condition. Error bars represent $\pm$ s.d. P value is shown above brackets. c, Polysome gradient profiles in HeLaS3 (WT, black) and HeLaS345CKO (45CKO, red) cells. Representative (n=3) profiles are shown. d, Global protein synthesis rates in HeLaS3 and HeLaS345CKO cells assessed by [35S] methionine/cysteine

incorporation. Representative (n=3) autoradiograph of PAGE gel with Western blot for protein loading control below is shown. e, Quantification of [35S] methionine/cysteine incorporation from n=3 independent experiments, as in (D). Columns indicate mean relative to control (HelaS3). Error bars represent $\pm$ s.d. and P value is shown above brackets. f, Transcriptome-wide analysis of changes in mRNA expression and ribosome footprint levels from ribosome-profiling data, comparing HelaS3 and HelaS345CKO cells. Histogram (left) giving the number of mRNA transcripts, found to be significantly regulated in each category ($P_{adj} < 0.05$). Log2 fold-change scatter plots (right) with mRNA transcripts colored according to indicated regulation type. Total number of sequenced transcripts 366 analyzed is given by "n". Number of transcripts up- or down-regulated in 45CKO relative to WT cells in the translation alone set is also shown (TL-UP, TL-DN respectively). g, Gene ontology analysis of mRNA transcripts in TL-DN set. Top 10 GO categories ($FDR < 0.05$) for biological process are labelled. Number of genes overlapping with each GO category is indicated by the color scale gradient (count). h, As (g) for TL-UP set. i, Metagene analysis plots of ribosome footprint coverage across mRNAs in each category for HelaS3 (WT, black) and HelaS345CKO (KO, red) cells, with extreme outliers removed. ALL, TL-DN, TL-UP represent all, translationally down-regulated or up-regulated transcripts respectively. Coding regions (between START and STOP) for each transcript are scaled to 100nt. 5' and 3' untranslated regions (± 30 nt) are unscaled. Coverage plotted is the RPKM normalized mean of libraries (n=3) in each case.

Figure 3

Ribosome methylation at 18S:174 affects cellular phenotypes. **a**, Cell proliferation in HeLaS3GFP (WT-GFP, black), HeLaS345CKO-GFP (45CKO-GFP, red) and HeLaS345CKO-GFP+45C (45CKO-GFP+45C, red, dashed) cells, measured by crystal violet assay at specified times. Points represent mean of independent experiments (n=3). Error bars represent $\pm$ 382 s.d. P values, indicated by brackets are shown for day 3. Significant changes in proliferation ($p < 0.05$), between 45CKO384 GFP and WT-GFP or 45CKO-GFP+45C, were observed at all timepoints except at day 1. **b**, Comparison of RMS scores representing fraction of 18S rRNA molecules 2'-O-methylated at each site in HeLaS345CKO-GFP and HeLaS345CKO-GFP+45C cells (left). Points denote mean RMS score (n=3) per site. Horizontal and vertical error bars indicate $\pm$ s.d. for each condition. Sites exhibiting significant change are labelled with nucleotide position (magenta). **c**, RMS scores for 2'-O-me at 18S:C174 in HeLaS345CKO-GFP+45C and HeLaS345CKO-GFP cells. Columns indicate mean RMS score (n=3) for each condition. Error bars represent $\pm$ s.d. P value is shown above brackets. **d**, Respiration and key parameters of mitochondrial function in HeLaS3GFP, HeLaS345CKO-GFP and HeLaS345CKO-GFP+45C cells. Basal respiration, ATP synthesis, proton leak and uncoupled

respiration are measured by oxygen consumption rate (OCR) following injections with compounds modulating cellular respiration. Columns indicate mean OCR (n=4) for each condition. P values are shown for each comparison, indicated by brackets. Error bars represent $\pm$ s.d.

Figure 4

Ribosome methylation status at 18S:174 influences the cellular proteome. a, Protein abundance changes in HeLaS345CKO (45CKO) compared to HeLaS3 (WT) cells. Log2 abundance ratio (45CKO/WT) and corresponding -Log10 Padj values are shown for all proteins detected in mass spectrometry analysis, represented by points. Proteins showing significant abundance change between conditions are colored (magenta). “n” denotes total number of proteins detected in both conditions. Number of proteins enriched in each condition is given (Enr WT, Enr KO). b, Gene ontology analysis of proteins in PA-DN set. Top 10 GO categories (FDR<0.05) for biological process are labelled. Number of genes overlapping with each GO category is indicated by the color scale gradient (count). c, As (b), for PA-UP set. d, Correlation between mRNA translation and protein abundance in HeLaS3 (WT) cells. Points represent mean ribosome protected fragment (RPF) count per million mapped reads (CPM) for each transcript (n=3, from ribosome profiling) and corresponding mean protein abundance for the same gene (n=4, from MS analysis). Color scale indicates plotting density. “n” denotes number of genes/proteins. Spearman’s rho and associated P value are shown. e, As (d), for HeLaS345CKO 412 (45CKO) cells. f, Correlation between mRNA translation and protein abundance for those genes with significant changes in both ribosome profiling and MS analysis of HeLaS345CKO (45CKO) and HeLaS3 (WT)cells. Points represent Log2FC in translation and log2FC in protein abundance ratio. “n” denotes number of genes. Spearman’s rho and associated P value are shown.

Figure 5

Translationally regulated mRNAs display distinct codon-usage patterns. a, Principal component analysis of codon usage frequency in mRNA transcripts exhibiting increased (TL421 UP, red), decreased (TL-DN, blue), and not-significantly altered (NS, grey) translation in HeLaS345CKO compared to HeLaS3 cells. b, Codon usage frequencies 422 in mRNA transcripts exhibiting increased (TL-UP) or decreased (TL-DN) translation in HeLaS345CKO cells. Median frequency of each codon is plotted in each case. Codons are color-coded by amino acid as indicated. Codons changing with Log2 fold-change (Log2FC) >1, minimum frequency of 0.02 and Padj < 0.001 are annotated by codon sequence. c, Examples of individual codons displaying altered usage frequencies in each translation regulation category. Glutamic acid (gaa), alanine (gcc), leucine (tta), leucine (ctg), isoleucine (ata) and arginine (cgc) and are shown. Padj values for comparisons are indicated by brackets. d, Fraction of G or C nucleotides present in codons comprising mRNA transcripts in each translation regulation category; down-regulated (TL-DN, blue), not-significantly regulated (NS, grey) or up-regulated (TL-UP, red). Comparisons for entire codons (codon) or first, second and third codon positions (pos 1-3) are shown separately. Padj values for comparisons are shown above brackets.

Figure 6

rRNA methylation at 18S:174 influences decoding of AT and GC-rich codons. a, Structure of human ribosome indicating location of C174 (purple) at base of h8 (green) in human rRNA. PyMol graphic based on cryo-EM ribosome structure modelled by Khatter et al. 18S (dark grey) and 28S (light grey) rRNAs are indicated. E-site tRNA (red) is also shown for orientation. b, Average ribosome occupancy or dwell time in HeLaS3 (WT, black) and HeLaS345CKO (KO, red) cells. Metagene analysis of mRNA transcripts displaying down-regulated (TL-DN, top panel), or up-regulated (TL-UP, bottom panel) translation 442 in HeLaS345CKO are shown. “n” denotes number of transcripts analyzed following removal of extreme outliers.

Figure 7

Roles of differential ribosome 2'-O-methylation in translational regulation. a, Working model based on Cm174 loss in SNORD45C knockout cells. Schematic illustrating how translation of specific mRNAs can be altered depending on presence or absence of rRNAs methylated at 18S:C174 (Cm174, cyan). rRNAs (red) are 2'-O-methylated (yellow) at specific positions guided by SNORDs during ribosome biogenesis. Differentially modified rRNAs are incorporated, together with ribosomal proteins, into pre-ribosomal 451 subunits (large subunit dark grey, small subunit light grey) in the nucleus. SNORD45C is present in HeLaS3 (top), and absent in HeLaS345CKO which lack Cm174 (bottom). Loss of Cm174 results in altered translation of mRNAs in the cytoplasm, compared to the situation where 50-60% of ribosomes carry Cm174, as indicated. Distinct functional classes of translationally regulated mRNAs are exemplified by mRNA x (cell cycle and division related, green), mRNA y (metabolic related, black) and mRNA z (other functional classes, blue). Altered translation, dependent on Cm174 status, results in increased or decreased cell proliferation. b, Proposed hypothetical model describing how differential rRNA 2'-O-methylation regulates translation in a biological situation. Illustrated by induction of proliferative

signaling or oncogene activation involving MYC expression. As (a), though in this case, increased ribosome biogenesis driven by MYC, results in multiple heterogenous ribosome types being produced. Both differential rRNA 2'-O-methylation or potentially other means of ribosome specialization may occur in concert. MYC expression also influences expression levels of certain functional classes of translationally regulated mRNAs.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Janssonetal2020NSMBSupplementaryinformation.pdf](#)
- [SupplementaryTable1.xlsx](#)
- [SupplementaryTable2.xlsx](#)
- [SupplementaryTable3.xlsx](#)
- [SupplementaryTable4.xlsx](#)
- [SupplementaryTable5.xlsx](#)
- [SupplementaryTable6.xlsx](#)
- [FigureS1.jpg](#)
- [FigureS2.jpg](#)
- [FigureS3.jpg](#)
- [FigureS4.jpg](#)
- [FigureS5.jpg](#)
- [FigureS6.jpg](#)