Metabolic turnover and dynamics of RNA modifications by ^{13}C labelling

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Title: Metabolic turnover and dynamics of RNA modifications by ¹³C labelling

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Abstract

RNA methylation is essential for appropriate assembly of ribonucleoprotein complexes. Dynamics of RNA methylation is thus important, but we lack quantitative methods to comprehensively assess it. We developed ¹³C-dynamods, an isotopic labelling approach using ¹³C-methyl-methionine, to quantify the turnover of base modifications in newly synthesized RNA, which is effective in distinguishing modifications in mRNAs from those in ncRNAs. This approach detected the presence of N6,N6-dimethyladenosine (m62A) in mRNA and tRNA in mammalian cells, and uncovered distinct kinetics of N-6-methyladenosine (m6A) and N-7-methylguanosine (m7G) in mRNA. Moreover, by assessing RNA metabolism during T-cell activation, we showed how methylation dynamics is coordinated with ribonucleotide biosynthesis. Finally, by quantification of methylation turnover and ribonucleoside abundance, we uncover the post-transcriptional lability of m6A in response to metabolic stress. Thus, ¹³C-dynamods enables studies of the origin, maintenance and biological regulation of RNA modifications under steady-state and non-stationary conditions.

INTRODUCTION

RNA methylation is essential for appropriate assembly of ribonucleoprotein complexes, and recent years have uncovered the unforeseen importance of dynamic N6-methyl-adenosine (m6A) RNA methylation during cell development and of transfer RNA (tRNA) modifications in response to environmental stresses¹⁻⁶. Mass spectrometry (MS) is a highly accurate tool that is often combined with stable isotopes for absolute quantification of modified ribonucleosides⁷⁻¹². Yet, abundance quantification does not reveal RNA methylation activity and may insufficiently explain its dynamics in biological systems. Moreover, the identification of low-abundance modifications by MS methods is very challenging due to contaminations by abundant ribosomal RNA (rRNA) and tRNA species that are heavily modified^{12,13}. Therefore, **we lack of a comprehensive method to assess the kinetics of RNA methylations, especially to distinguish the modifications in mRNAs from those in non-coding (nc) RNAs.**

Stable isotope labelling of cultured cells is a well-established method to quantify metabolic activity¹⁴⁻¹⁶. Here, we have employed ¹³C-methyl-methionine labelling to quantify the turnover of base modifications (¹³C-dynamods) in polyadenylated and ncRNA. With ¹³C-dynamods, we measure the fraction of isotopologues (m+0, m+1, m+2) in modified ribonucleosides and show its specificity to monitor SAM-dependent RNA methylation. As expected, we find that the turnover of modified ribonucleosides is inherently linked to the half-life of the RNA itself, which is itself distinct for mRNA, rRNA and tRNA¹⁷⁻¹⁹. We examine the kinetics of RNA methylation turnover within and across RNA classes as well as in free ribonucleosides to obtain new insights on the origin and identification of mRNA modifications. We assess RNA methylation dynamics at metabolic steady-state, during T-cell activation and metabolic stresses linked to the co-substrates of RNA (de)methylation, which reveals the dynamic modulation of mRNA and tRNA modifications. The **sensitivity and quantitative nature of ¹³C-dynamods demonstrates its capacity for in-depth characterization of RNA methylation dynamics in multiple RNA classes of interest**, which offers complementary insights to the existing epi-transcriptomic methods.

29 **¹³C labelling of polyadenylated and ribosomal RNA modifications**

30 S-adenosylmethionine (SAM) is the direct substrate of RNA methylation reactions in
31 eukaryotic cells²⁰. To trace the incorporation of SAM into RNA, we cultured human 786O cells in
32 methionine-free DMEM medium supplemented with either naturally-labelled methionine ('Unlab') or
33 ¹³C-methyl-methionine and analysed the isotopologues (m+0, m+1, m+2) of modified and unmodified
34 ribonucleosides by tandem mass spectrometry (LC-MS/MS) (Fig. 1a,b). The m+0 isotopologue (e.g.
35 150.1 *m/z* for m6A) represents the mass of the analysed molecule where all atoms are present as the
36 most common isotope, whereas the m+1 isotopologue (e.g. 151.1 *m/z* for m6A) indicates the mass shift
37 due to ¹³C incorporation from the ¹³C-labelled methionine tracer or from the natural abundance of ¹³C,
38 N, H and O stable isotopes. Here, we analysed the isotopologues of N6-methyladenosine (m6A), N7-
39 methylguanosine (m7G), N1-methyladenosine (m1A), N6,6-dimethyladenosine (m62A), 2'-O-
40 methyladenosine (Am), 5-methylcytosine (m5C) and unmodified ribonucleosides from digested
41 polyadenylated (polyA+) RNA, large RNA (>200nt) and tRNA. We observed increased m+1 and
42 concomitant decreased m+0 ion counts in modified ribonucleosides from polyA+ and large RNA after
43 4 and 24 hours of ¹³C-methyl-methionine labelling, while the abundance of adenosine and guanosine
44 isotopologues was unaltered (Fig. 1c,d, Extended Data Fig. 1a). Conceptually, the isotopologue fraction
45 of each ribonucleoside is internally controlled for the amount of pre-existing ribonucleosides (m+0 and
46 naturally labelled m+1). Also, we determined that the ¹³C enrichment of intracellular methionine and
47 SAM reaches a 98-100% plateau within 30 minutes and remains constant thereafter (Extended Data
48 Fig. 1b). **Thus, the change in the heavy isotopologue fraction relative to the unlabelled condition**
49 **indicates SAM-dependent RNA methylation of newly synthesized RNA**, whose turnover we have
50 examined within and across RNA classes. In contrast to single methylated ribonucleosides, the m62A
51 modification exhibited an enrichment mostly of the m+2 isotopologue upon ¹³C labelling (Extended
52 Data Fig. 1a), and so the m+2 fraction was used to assess m62A dynamics in subsequent analyses.

Quantification of isotopologue fractions showed a faster kinetics for m6A, m7G and m5C in polyA+ RNAs when compared to large RNA and tRNAs (Fig. 1e), in accordance with the faster lifecycle of mRNA in mammalian cells¹⁸. In contrast, the kinetics of m1A, m62A and Am methylation was similar between polyA+ and large RNA fractions (Fig. 1e), suggesting that a large portion of the signal for these modifications in the polyA+ fraction might derive from contaminating ribosomal RNA (rRNA). Of note, the isotopologues are analysed on all ribonucleosides from the same polyA+ pool (Extended Data Fig. 1c), so the purity of polyA+ enrichment is the same in all cases. This indicates that the relative abundance in mRNAs vs contaminating ncRNA is much higher for m6A, m7G and m5C compared to m1A, m62A and Am. Interestingly, we detected m62A in tRNA from 786O cells, and its kinetics was different when compared to large RNA (Fig. 1e and Extended Data Fig. 1c).

Methylation turnover for identification of bona fide mRNA modifications

To examine methylation turnover, we cultured cells for 24 hours with ¹³C-methyl methionine followed by replacement (chase) with naturally labelled methionine and analysed the isotopologues over time. In accordance with the preceding findings (Fig. 1e), we observed an exponential decay of the m+1 isotopologue fraction for m6A/m7G but not for Am/m1A/m62A in polyA+ RNA (Fig. 2a). Conversely, the modifications of total/large RNAs exhibited uniformly slow turnover (Fig. 2b), consistent with the high stability of rRNA and tRNA in growing cells¹⁷. To test the kinetic behaviour underlying methylation turnover, we examined the goodness-of-fit of a linear versus exponential regression of the isotopologue fraction. Analysis of residual errors showed that the linear regression ($m_{(t)} = m_{(0)} + kt$) fits well the turnover of m1A/m62A/Am but not m6A/m7G in polyA+ RNA, in which the ‘U-shaped’ curve supports a non-linear model ($m_{(t)} = m_{(0)} e^{(-kt)}$) (Fig. 2c, Extended Data Fig. 2a). Conversely, the linear regression fitted well the turnover of ncRNA modifications (Extended Data Fig. 2b,c). The turnover frequency determined for Am/m1A/m62A in polyA+ was similar to ncRNA modifications ($k = 0.031 \text{ hr}^{-1}$ on average), and significantly slower than m6A ($k = 0.244 \text{ hr}^{-1}$) or m7G ($k = 0.089 \text{ hr}^{-1}$) in polyA+ RNA (Fig. 2a,b). Thus, **the turnover of m1A/m62A/Am from polyA+ fractions is incompatible with the exponential turnover of mRNA modifications^{17,18}, confirming that it most likely originates from ncRNA contamination.**

The m7G modification was similar to less abundant than m1A/Am/m62A in polyA+ RNA (Fig. 2d), and the sensitivity of MS detection was lower for m7G than m1A/Am/m62A, as determined from equimolar injections of pure compounds (Extended Data Fig. 2d). Thus, **the distinct turnover of polyA+ modifications was not explained by analytical sensitivity or normalised levels alone**, since the levels of modified ribonucleosides were greater in large RNA compared to polyA+ RNA by a factor of 17/37/97/9 for Am/m1A/m62A/m7G, respectively, while m6A level was greater in polyA+ RNA by a factor of 2 (Fig. 2e). From the turnover frequency of ncRNA modifications, we estimated that a 50% contribution of ncRNA signal to the ribonucleoside pool in polyA+ RNA would lower the detected turnover of the bona fide m6A mRNA modification, with a turnover frequency of 0.242hr^{-1} , to that of m7G, with a turnover frequency of $\sim 0.09\text{hr}^{-1}$ (Extended Data Fig. 2e). That is, reliable turnover-based detection of a hypothetical mRNA modification that is more abundant by a factor of 17-97 in ncRNA relative to mRNA will require a maximal ncRNA contamination of 0.5% (50%/97) to 2.9% (50%/17), but even an optimal performance of available methods to purify polyA+ RNA commonly contains over 3% of contaminating ncRNA (Extended Data Fig. 2f)^{21,22}. Thus, **the ability to resolve bona fide mRNA modifications from methylation turnover is limited by the depletion efficiency of highly abundant rRNA modifications.**

As the turnover frequency of m6A was ~ 8 times faster in polyA+ RNA than ncRNA (Fig 2a,b), we reasoned that the ^{13}C labelling of free ribonucleosides derived from RNA degradation would consist mostly of degraded mRNA rather than ncRNA. **To interrogate mRNA modifications further with higher sensitivity, we examined methylation dynamics in the pool of free ribonucleosides derived from metabolic extracts.** We did not include the isotopologue analysis of m5C in these time-series experiments due to low abundance of its free ribonucleosides, and due to low MS sensitivity for m5C (Fig. 2d, Extended Data Fig. 2d,g), which compromises the quantification under conditions of partial ^{13}C labelling. We found that the Michaelis-Menten kinetics following ^{13}C labelling was similar for m6A, m7G and Am (Fig. 2f-h). Interestingly, the m62A modification also exhibited a non-linear, faster Michaelis-Menten kinetics than m1A or N1-methylguanosine (m1G) (Fig. 2f-h), which are known tRNA modifications and were present in the metabolic extracts at high levels (Extended Data Fig. 2g)²³.

Thus, while the turnover analysis of the polyA⁺ fraction couldn't be used to validate lowly abundant modifications due to rRNA contamination (Fig. 2e), the turnover of Am and m62A in the free pool suggests that these modifications indeed partly derive from mRNA. This was supported by the normalized levels of Am and m62A in the free pool being more similar to those of polyA⁺ than rRNA (Extended Data Fig. 2h). **These results confirm the presence of Am in mRNA²⁴ and suggest m62A is a potential mRNA modification, whereas m1A, which has been studied in mRNAs^{25,26}, is very rare on mRNA.**

RNA methylation dynamics during T-cell activation

Upon stimulation with T-cell agonists, T cells undergo significant changes in gene expression, signalling and metabolism within hours to support proliferation²⁷. We examined RNA methylation turnover in human primary CD8⁺ T lymphocytes subjected to 4 and 24 hours of *in vitro* activation. **In agreement with our earlier observations, m6A and m7G in polyA⁺ RNA generally exhibited a faster turnover than ncRNA modifications (Fig. 3a-c).** Interestingly, the turnover of m7G was accelerated in activated T-cells relative to the respective non-activated state, although the variance was high between T-cells from different human healthy donors (Fig. 3a). The turnover of large and tRNA modifications was uniformly accelerated in activated T-cells (Fig. 3b,c). Yet, the methylation turnover varied between tRNA modifications in both T-cell states (Fig. 3c), suggesting a dynamic modulation of each modification at the post-transcriptional level.

The turnover of m6A in polyA⁺ was only slightly faster in activated than non-activated T-cells (Fig. 3a), despite the quiescent state that characterizes resting T-cells²⁷. Ribonucleotide triphosphates (rNTPs) are synthesized either via salvage or *de novo* synthesis of purines and pyrimidines, and proliferating cells synthesize rNTPs mostly *de novo*²⁸. To examine the contribution of anabolic pathways to newly synthesized RNA during T-cell activation, we traced the activity of rNTPs biosynthesis using 3-¹³C-based serine, which contributes with two carbons to nascent purines (yielding m+2 purine isotopologues)²⁹. Indeed, the contribution of purine biosynthesis was higher in activated CD8⁺ T-cells across RNA classes (Fig. 3d). **Thus, the high m6A turnover in polyA⁺ RNA is**

decoupled from the activity of *de novo* rNTP production in non-activated T-cells. In contrast, the methylation turnover of ncRNA classes is associated with increased activity of rNTP production in T-cell activation, likely reflecting an increased transcription and ribosomal biogenesis to support cell growth³⁰.

Sensitivity of mRNA and ncRNA modifications to metabolic stress

The maintenance and reversibility of DNA/RNA methylation requires SAM and other substrates derived from the central carbon metabolism³¹. We applied ¹³C-dynamods to obtain insights into methylation dynamics upon metabolic stresses linked to RNA (de)methylation. **Deprivation of serine or glutamine inhibited mainly the turnover of large RNA modifications (Fig. 4a)**, while the normalised levels of large RNA ribonucleosides were unaltered upon stress (Extended Data Fig. 4a). This suggests that reduced methylation turnover most likely results from a reduced transcription of rRNA. Interestingly, **glutamine deprivation increased m6A levels in polyA+ RNA (Fig. 4b) even though it did not significantly affect its turnover after 4hr (Fig. 4a)** or in a chase experiment (Extended Data Fig. 4b). We confirmed that actinomycin (ActD), a pan inhibitor of eukaryotic transcription, completely inhibited the methylation turnover of m6A and m7G in polyA+ RNA (Extended Data Fig. 4b), in accordance with the co-transcriptional deposition of m6A in mRNA and of most rRNA modifications^{32,33}. In contrast to glutamine deprivation, the ActD treatment decreased m6A levels in polyA+ RNA over time (Extended Data Fig. 4c), recapitulating initial findings of RNA destabilization by m6A RNA methylation³⁴. Conversely, m7G levels in polyA+ RNA were unaltered under glutamine deprivation or ActD (Extended Data Fig. 4c). These data demonstrate the transcriptional sensitivity of ncRNA modifications and post-transcriptional lability of m6A in mRNA in response to metabolic stress.

DISCUSSION

Isotopic labelling of cultured cells is a well-established method to quantify metabolic activity, but its application to RNA modifications has been limited. Here, we demonstrate that quantification of methylation turnover with dynamic ¹³C labelling informs on the distinct dynamics of polyA+ and

ncRNA modifications, and their sensitivity to T-cell activation or metabolic perturbations of mammalian cells. **This approach involves several advances in the application of MS techniques to quantify RNA modifications.** First, in contrast to approaches using sequential or multiplexed labelling of ribonucleosides to distinguish between pre-existing and newly synthesized RNA^{35,36}, the ‘heavy’ isotopologue fraction derived from ¹³C-methyl-methionine is internally controlled for the amount of unlabelled, pre-existing ribonucleosides and is specific to modified ribonucleosides. Second, in contrast to nucleotide labelling approaches in which nucleotide recycling within a cell may lead to ineffective chase with unlabeled nucleotides^{37,38}, a new SAM molecule is required for each methylation cycle in dynamods, adding to the specificity and versatility of experimental designs³⁸. Third, in contrast to approaches using multiplexing or spiking from isotopically labelled cultures to monitor turnover^{8,11,35}, the dynamic ¹³C labelling patterns directly inform on flux or turnover frequency in hr⁻¹^{14,16}, because the direct substrate (SAM) of the targeted reaction (RNA methylation) is close to 100% labelled (Extended Data Fig. 1b). Moreover, SAM needs not to be fully labelled for comparisons of dynamic ¹³C labelling between modifications at early time points as its enrichment is expected to be the same across SAM-dependent RNA modifications. Finally, combined measurements of methylation turnover and ribonucleoside levels allows dynamods to also inform on the lability, and thus possible reversibility, of RNA modifications under non-steady state conditions.

Reliable study of mRNA modifications with mass spectroscopy is challenging due to the high abundance of heavily modified ncRNAs that can contaminate the polyA+ fraction. To address this challenge, we exploited the different decay rates; mRNA decay is fast, with a median half-life from 40 min to 9 hours in mammalian cells^{17,39,40}, while rRNA and tRNA exhibit half-lives of 60-70 hours in growing fibroblasts¹⁷. Since the turnover of SAM-dependent RNA modifications is affected mainly by the RNA half-life, monitoring the turnover could largely distinguish mRNA from ncRNA modifications. This analysis indicates that signal of Am/m1A/m62A in polyA+ RNA is predominantly derived from contaminant ncRNAs rather than mRNAs. Based on methylation turnover, the presence of these modifications could in theory be detected with ncRNA contaminations of up to 0.5-2.9% in the polyA+ fraction, since Am/m1A/m62A levels were higher in ncRNAs by a factor of 17-37-97,

186 respectively. In contrast, m7G levels were only ~9 times more abundant in large relative to polyA+
187 RNA, so a ~6% of ncRNA contamination would be required to fully account for the slower m7G
188 turnover detected in polyA+ RNA. Also, the turnover of m7G was sensitive to metabolic stresses in
189 large RNA but not polyA+ RNA, and this distinct response argues for the signal in the polyA+ fraction
190 being independent from ncRNA. Thus, **the distinct kinetics of coding RNA modifications suggests**
191 **that m7G deposition on nascent RNAs is temporally delayed relative to the co-transcriptional**
192 **m6A deposition**, as reported elsewhere^{41,42}.

193 Previous reports identified m5C and Am as mRNA modifications^{24,43}, and more recently also
194 m1A^{25,26}. While we could not reliably determine the Am and m62A modifications in the polyA+ as
195 being ‘mRNA modifications’ based on methylation turnover, we could do so by measuring them in the
196 free ribonucleoside pool. **Our analysis of free ribonucleosides showed a similar Michaelis-Menten**
197 **kinetics for the turnover of Am, m6A and m7G, consistent with these modifications being derived**
198 **from rapidly-decaying mRNAs**. This was supported by the normalized levels of free modified
199 ribonucleosides being more similar relative to those of polyA+ than ncRNA. In contrast, the turnover
200 of free m1A and m1G ribonucleosides were significantly slower, indicating that these modifications are
201 predominantly derived from a different ncRNA class, likely tRNAs, where they are present at high
202 levels²³. This aligns with the recent conclusions that m1A might be restricted to a handful of
203 mRNAs^{13,25}. Finally, despite the low sensitivity of m5C detection in chase experiments, the
204 quantification of m5C isotopologues at 24 hours is consistent with it m5C being derived from mRNA⁴³.

205 The m62A modification is thought to be present primarily on the 18S rRNA of mammalian
206 transcriptomes^{44,45}, but has not been described in mammalian tRNA or mRNA. While m62A has been
207 detected in bacterial tRNA⁴⁶, care must be taken for abundant m62A-modified rRNA fragments that co-
208 purify with tRNA⁴⁷, which is hardly resolved by standard MS quantifications. The slower turnover of
209 m62A in tRNA relative to large RNA suggests its bona fide m62A signal in tRNA. Alternatively, if
210 m62A was derived from a contaminant rRNA fragment, it would need to be deposited in a slower
211 fashion into rRNA fragments as compared to nascent/mature rRNA, which is unlikely. Interestingly,
212 the turnover of free m62A ribonucleosides exhibited a different Michaelis-Menten kinetics than

m1A/m1G, and the normalized levels (m62A/A) were low in tRNA. **Together, these evidences suggest that m62A is likely present in certain mammalian mRNAs and tRNAs,** which merits further investigation. Of note, single methylation intermediates of m62A have been detected *in vitro*⁴⁸, but we did not detect m+1 isotopologues for m62A above natural abundance. As SAM was 98-100% ¹³C-labelled within 30 minutes, the time resolution of our experiments does not capture the sequential methylation of adenosine into m62A. We conclude that the turnover of RNA modifications can serve a readout to survey candidate modifications in RNA subclasses and thus complement the sequencing-based mapping of RNA modifications^{13,49,50}.

Altered methylation turnover can result from changes in maintenance rates, co-transcriptionally or at the post-transcriptional level. Here, we used analysis of methylation turnover to inform on the dynamics of RNA modifications during T-cell stimulation. We found that the turnover of m7G in polyA+ RNA was increased upon stimulation, albeit with high variability among T-cell primary samples. It has been reported that S-adenosyl-homocysteine hydrolase (SAHH), which hydrolyses S-adenosyl-homocysteine (SAH) and thus alleviates its feedback inhibition on RNA methyltransferases, is upregulated in T cell activation and boosts m7G cap methylation during increased gene expression⁵¹. Whether the nuclear/cytoplasmic (de)capping of m7G^{41,42} or internal m7G⁵² is dynamically modulated during T-cell activation should be examined in more detail. Moreover, the turnover of m6A was equally fast during T-cell activation despite the fact that *de novo* purine biosynthesis was minimally active in the naïve-like state, as determined using ¹³C-labelled serine. This suggests that **CD8+ T-cells initially rely on the salvage and later on the *de novo* pathways to generate nucleotides required to transcribe polyA+ RNA after their stimulation.**

A non-stationary condition may affect the afferent (i.e. transcription) or efferent (i.e. demethylation or degradation) pathways of turnover, which can alter the ¹³C labelling and/or levels of modified ribonucleosides. We observed inhibited turnover of ncRNA methylation under serine deprivation, which is in line with the expected inhibition of mTOR activity and thereby of rRNA biogenesis⁵³. In contrast, **glutamine deprivation increased m6A levels without increasing m6A turnover in polyA+ RNA, which can't be explained by increased co-transcriptional deposition of**

m6A in mRNAs, and instead suggests post-transcriptional dynamics of m6A, such as by decreased demethylation. We did not quantify the conversion of ¹³C-methyl-groups into N6-hydroxymethyladenosine and N6-formyl-adenosine in the cell lines examined⁵⁴. Thus, related developments or orthogonal approaches are needed to address if post-transcriptional dynamics of m6A by metabolic stress conditions are due to RNA demethylation or preferential degradation of unmodified RNAs. **As glutamine is the main carbon source of α-ketoglutarate, a co-substrate of RNA demethylases^{5,55}, it is plausible that its depletion could inhibit RNA demethylation.** This supports the notion that the reversibility of m6A in mRNA is likely context-dependent⁵⁶.

Our study establishes quantification of methylation turnover and abundance with ¹³C-dynamods to examine the various forces driving the dynamics of RNA methylations in the major classes of RNAs. We gain particularly valuable insights into dynamic RNA modifications under non-steady state conditions, such as during T-cell activation or upon metabolic stress. These insights could be further explored by MS and sequencing-based methods that provide information on particular RNAs, and by isolating RNA species from cellular compartments, for instance to assess nuclear vs. cytoplasmic deposition of mRNA modifications, or to scrutinize the reversibility of m6A in cytoplasmic polyA+ RNA^{19,57}.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.A.G., and J.U.; Methodology, P.A.G, and V.E.; Investigation, P.A.G., and I.P.F.; Formal Analysis, P.A.G., V.E. and M.S.D.S.; Writing – Original Draft, P.A.G., and J.U.; Writing –

Review & Editing, P.A.G., I.P.F., J.I.M., R.S.J., and J.U.; Funding Acquisition, J.U.; Resources, P.A.G., I.P.F., J.I.M. and J.U.; Supervision, P.A.G., and J.U.

METHODS

Cell culture and metabolic labelling. 786O cells were obtained from the Crick Cell Services and cultured at 37°C with 5% CO₂ in high glucose DMEM medium (ThermoFisher Scientific, #61965026) supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific, #21875034). CD8⁺ T lymphocytes were cultured in RPMI-1640 (ThermoFisher Scientific, #21875034) supplemented with 10% heat-inactivated FBS (ThermoFisher Scientific #10270106), 1% penicillin/streptomycin (Sigma-Aldrich, #P4333) and 30 U/ml IL-2 (Roche, #11011456001). For ¹³C labelling experiments, 786O cells were grown in high glucose DMEM medium without glutamine, methionine and cystine (ThermoFisher Scientific, #21013024) supplemented with 10% dialyzed FBS (ThermoFisher Scientific, #26400044), 2 mM glutamine, 0.1 mM cystine and 0.2 mM ¹³C-methyl-methionine (CK Isotopes Limited). For labelling T-cells or 786Os in stress conditions, we used custom RPMI and DMEM media (media preparation lab at the Crick) lacking glucose, glutamine, serine and methionine (and otherwise equivalent formulation as commercial versions) supplemented with 11 mM glucose, 2 mM glutamine, 0.4 mM serine (or 3-¹³C-serine) and 0.2 mM ¹³C-methyl-methionine (or methionine). 786O cells were grown until 50-60% confluence before switching to ¹³C-labelled medium for the indicated time periods. 786O cells were maintained at 50-60% confluence (or 30-40% in chase experiments) and washed with PBS before switching to ¹³C-labelled medium (or regular medium in chase) for the indicated time periods. Total CD8⁺ T-cells isolated from healthy donor were centrifuged, washed with PBS, and 10-20 million cells were activated in ¹³C-labelled or unlabelled medium for the indicated time periods.

Isolation and activation of CD8⁺ T-cells. PBMCs were obtained from healthy donors from Cambridge Bioscience or NHSBT (Addenbrooke's hospital, Cambridge UK), and approved the Human Biology Research Ethics Committee at University of Cambridge (application no: HBREC.2018.11). The PBMC isolation was performed within 8-12 hours after blood collection, and T lymphocytes were isolated with MACS Miltenyi kits (total CD8⁺ T cells: 130-096-495 or CD8 MicroBeads: 130-045-201 plus FcR

blocking:130-059-901) following manufacturer's instructions. Cells were cultured in RPMI-1640 (ThermoFisher Scientific, #21875034) supplemented with 10% heat-inactivated FBS (ThermoFisher Scientific #10270106), 1% penicillin/streptomycin (Sigma-Aldrich, #P4333) and 30 U/ml IL-2 (Roche, #11011456001). Total CD8⁺ T-cells were activated with aCD3/CD28 beads (1:1 beads-to-cell ratio; Gibco #11132D) for 4 hours and 24 hours after isolation. Cell number and viability were measured by ADAM-MC automated cell counter (NanoEnTek) in RPMI medium.

RNA purification. At the conclusion of metabolic labelling, the medium was aspirated and extraction of total RNA was performed with the mirVana isolation kit according to manufacturer's instructions (ThermoFisher Scientific, #AM1560). Large (>200nt) RNAs were purified by adding 1/3 volume of 100% ethanol to the aqueous phase recovered from the organic extraction before loading into the filter cartridge of the mirVana Kit. Small (<200nt) RNAs were purified by collecting the total filtrate, addition of 2/3 volume of 100% ethanol and loading into the filter cartridge. Purified small RNAs were subjected to size selection using 15% TBE-Urea gel electrophoresis (ThermoFisher Scientific #EC6885BOX), tRNAs sliced and recovered from the gel in 200 mM, 50 mM KOAc, 1 mM EDTA with shaking overnight at 16°C. Polyadenylated RNA was purified from total/large RNA via two rounds of polyA tail hybridization with Oligo-dT magnetic Dynabeads (ThermoFisher Scientific, #61002).

LC-MS/MS analysis of ribonucleosides. Purified RNA (100-250 ng) was digested into ribonucleosides using one unit of nuclease P1 (Sigma-Aldrich, #N8630-1VL) in 25 µl of buffer 25 mM NaCl, 2.5 mM ZnCl₂ and 10mM NaCH₃COO pH 5.3 and incubated for 2 hours at 37°C. Subsequently, NH₄HCO₃ (100 mM) and 5 units of alkaline phosphatase (CIP) (NEB, #M0525S) were added and the sample incubated for 2 hours (or 20 min, with Quick CIP) at 37°C. Formic acid was added at 0.1% v/v in a final volume of 50 µl and samples were filtered (0.22 µm, Millipore) and 15-20 µl analysed in duplicate by LC-MS. Ribonucleosides were resolved with a C18 reverse phase column (100 x 2.1 mm, 3 µm particle size, Chromex Scientific, #F18-020503) and eluted with a gradient of 0.1% v/v formic acid (solvent A) and 80% acetonitrile in 0.1% formic acid (solvent B) at a flow rate of 0.2 ml/min and 40°C: 100% solvent A for 3 min, 12% solvent B for 12 min, and 100% solvent B for 2 min after which the column was re-equilibrated with 100% solvent A for 3 min (20 min total run time). The

ribonucleoside separation was performed using U3000 HPLC (Thermo Scientific) and the detection by a TSQ Quantiva Triple Quadrupole mass spectrometer (TSQ Quantiva, Thermo Scientific) controlled by the Xcalibur software version 4.0.27 (Thermo Scientific). The HPLC was coupled to the TSQ Quantiva using a HESI (heated electrospray) ion source (Thermo Scientific) operating in positive ionization mode with the following parameters: capillary voltage, 3500V; sheath gas flow, 7.35 l/min; gas temperature, 325C. The first and third quadrupoles (Q1 and Q3) were stringently fixed to 0.2 units of resolution and set to detect the mass of the precursor ribonucleoside ion (Q1) and of the base and ribose product ions (Q3). The ribonucleosides were identified by comparison of the retention time and detected mass transitions to commercially available standards. The collision energies were experimentally defined based on the fragmentation pattern of each ribonucleoside standard and chosen based on the maximum intensity of the base product; the ribose ring was used only as a qualifying transition. The retention time, mass transitions (m/z) and collision energies of each ribonucleoside were: adenosine, ~4.1 min, 268.1 $\rightarrow$ 136.1 m/z , 20 V; guanosine, ~5.4 min, 284.1 $\rightarrow$ 135.0 m/z , 35.5 V; cytidine, ~1.4 min, 244.2 $\rightarrow$ 112.05 m/z , 12 V; m6A, ~8.7 min, 282.1 $\rightarrow$ 150.1 m/z , 20 V; m1A, ~1.8 min, 282.1 $\rightarrow$ 150.1 m/z , 20V; m7G, ~2.3 min, 298.05 $\rightarrow$ 166.1 m/z , 20V; m5C, ~1.8 min, 258.2 $\rightarrow$ 126.1, m/z , 13V; m62A, ~11.9 min, 296.2 $\rightarrow$ 164.1 m/z , 22V. Each mass transition above corresponded to the $m+0$ isotopologue, and increased by one ($m+1$), two ($m+2$) and three ($m+3$) units for detection of the other isotopologues *e.g.* m62A: 297.2 $\rightarrow$ 165.1 ($m+1$), 298.2 $\rightarrow$ 166.1 ($m+2$), 299.2 $\rightarrow$ 167.1 ($m+3$). The dwell time for each transition was 30 ms for a duty cycle of 930 ms (31 transitions), and 8 to 20 data points per chromatographic peak were obtained for 'short' and 'long' peaks, respectively. A mix of ribonucleoside standards containing 0.5, 1, 5, 10, 50, 100, 500 fmol, 1, 5, 10, 50 or 100 pmol of each ribonucleoside was run in parallel after the biological samples for absolute quantifications, a subset of which is shown in Extended Data Fig. 2d. Data were recorded using the Xcalibur 3.0.63 software (ThermoFisher Scientific) and analysed using Skyline (version 19.1)⁵⁸.

LC-MS analysis of free metabolites. LC-MS analysis was performed using a Dionex UltiMate LC system (ThermoFisher Scientific) with a ZIC-pHILIC column (150 mm x 4.6 mm, 5 μ m particle, Merck Sequant), as described previously⁵⁹. A 15 min elution gradient of 80% Solvent A (20 mM ammonium

carbonate in Optima HPLC grade water, Sigma Aldrich) to 20% Solvent B (acetonitrile Optima HPLC grade, Sigma Aldrich) was used, followed by a 5 min wash of 95:5 Solvent A to Solvent B and 5 min re-equilibration. Other parameters were as follows: flow rate, 300 $\mu\text{L}/\text{min}$; column temperature, 25°C; injection volume, 10 μL ; autosampler temperature, 4°C. All metabolites were detected across a mass range of 70-1050 m/z using a Q Exactive Orbitrap instrument (ThermoFisher Scientific) with heated electrospray ionization and polarity switching mode at a resolution of 70,000 (at 200 m/z). MS parameters were as follows: spray voltage 3.5 kV for positive mode and 3.2 kV for negative mode; probe temperature, 320°C; sheath gas, 30 arbitrary units; auxiliary gas, 5 arbitrary units. Parallel reaction monitoring (PRM) was used at a resolution of 17,500 to confirm the identification of metabolites; collision energies were set individually in HCD (high-energy collisional dissociation) mode. Data were recorded using the Xcalibur 3.0.63 software and analysed using Tracefinder 4.1 (ThermoFisher Scientific) according to the manufacturer's workflows.

Quantification of methylation turnover. The isotopologue fractions were defined as the total ion counts of the $m+1$ isotopologue (except for $m+2$ in $m62A$) relative to the total ion counts of the $m+0$ plus $m+1$ isotopologues. The kinetics of isotopologue fractions and goodness-of-fit were determined using the Curve Fitting toolbox of Matlab R2020a (MathWorks) either by a linear regression [$f(x) = p1 \cdot x + p2$] or an exponential fit: a one-term function in the chase experiments [$f(x) = a \cdot \exp(b \cdot x)$, Levenberg-Marquardt algorithm] and a two-term function to fit the isotopologue fractions of free ribonucleosides [$f(x) = a \cdot \exp(b \cdot x) + c \cdot \exp(d \cdot x)$, Levenberg-Marquardt algorithm].

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Figure 1. ^{13}C labelling of RNA modifications across RNA classes. **a**, The ^{13}C -dynamods workflow shows the tracing of RNA methylation: cells are cultured with ^{13}C -methyl methionine, the RNA is isolated, digested to ribonucleosides and subjected to LC-MS/MS analysis. The isotopologues detected for m6A are shown. **b**, Representative chromatogram of unmodified and modified ribonucleosides from total RNA. **c-d**, The m+0 and m+1 isotopologues of modified and unmodified ribonucleosides (representative chromatograms) in polyA+ (**c**) and large RNA (**d**). **e**, Quantification of the isotopologue fractions of each ribonucleoside in polyA+, large and tRNA under unlabelled ('Unlab') conditions, after 4 and 24 hours of culture with ^{13}C -methyl-methionine. m6A, N6-methyladenosine; m7G, N7-methylguanosine; m1A, N1-methyladenosine; m62A, N6,6-dimethyladenosine; Am, 2O-methyladenosine; m5C, 5-methylcytosine; m1G, N1-methylguanosine; m2G, N2-methylguanosine. SAM, s-adenosylmethionine; SAH, s-adenosylhomocysteine; homoCys, homocysteine. Error bars represent standard deviation of three to four biological replicates, with the exception of two replicates for tRNA modifications. In all cases, each replicate is the average of two technical replicates. * denotes $P < 0.005$, ¥ denotes $P < 0.05$, # denotes $P = 0.059$, of a two-sided Student's t-Test comparing samples as indicated.

FIGURE 2

Figure 2. Kinetic profiling of RNA modifications in polyA+, ncRNA and free ribonucleosides. **a-b**, Isotopologue fractions during the ‘chase’ of ^{13}C -labelled modifications with naturally labelled methionine for 0, 1, 3, 6 and 16 hours, in polyA+ (a) and total/large RNA (b). **c**, Residuals of a linear vs. exponential regression of isotopologue fractions in polyA+ RNA in the chase experiment. **d**, Normalized ion counts of modified ribonucleosides relative to the ion counts sum of all ribonucleosides, shown for polyA+ and large RNA. **(e)** Ratio of normalised ion counts between polyA+ and large RNA, as determined in (d). **f**, Isotopologue fraction of free ribonucleosides analysed from metabolic extracts. **g-h**, Goodness-of-fit of a linear vs. exponential fit of the isotopologue fractions in free modified ribonucleosides. SSE, sum of squared errors. m6A, N6-methyladenosine; m7G, N7-methylguanosine; m1A, N1-methyladenosine; m62A, N6,6-dimethyladenosine; Am, 2O-methyladenosine; m5C, 5-methyl-cytosine; m1G, N1-methylguanosine; A, adenosine; G, guanosine. Error bars represent 90% confidence intervals in (a-b), or standard deviation in (d, f) of at least three biological replicates with exception of Am (two replicates). In all cases, each replicate is the average of two technical replicas. * denotes $P < 0.005$, $\ddagger$ denotes $P < 0.05$, of a two-sided Student’s t-Test comparing m6A and m62A to m1A, respectively.

FIGURE 3

Figure 3. RNA methylation dynamics during CD8+ T-cell activation. **a-c**, Isotopologue fractions of modified ribonucleosides in polyA+ (**a**), large (**b**) and tRNA (**c**) in CD8+ T-cells labelled with ^{13}C -methyl-methionine for 4 and 24 hours and stimulated with CD3/CD28 antibodies for the same time period. **d**, Quantification of the m+2 fraction from 3- ^{13}C -serine. Error bars represent standard deviation of T-cell samples from three healthy donors. In all cases, each replicate is the average of two technical replicas. * denotes $P < 0.005$, $\text{\textyen}$ denotes $P < 0.05$, ns denotes not significant, of a two-sided Student's t-Test comparing non-activated to activated T-cells at 24 hours or comparing modified ribonucleosides as indicated.

FIGURE 4

Figure 4. RNA methylation dynamics under metabolic stress. **a**, Isotopologue fractions of modified ribonucleosides in polyA+ and large RNA following ^{13}C -methyl-methionine labelling of 786O cells cultured with complete (ctrl), glutamine- (NoQ), or serine- (NoSer) deprived DMEM medium for 4 hours. **b**, Normalised levels of m6A relative to adenosine in polyA+ RNA from 786O cells cultured with ctrl, NoQ or NoSer DMEM medium for 4 hours. Error bars represent standard deviation of three or more biological replicates, with exception of m5C under NoQ and m6A under NoSer (two replicates). In all cases, each replicate is the average of two technical replicas. * denotes $P < 0.005$, $\text{\textyen}$ denotes $P < 0.05$, ns denotes not significant, of a two-sided Student's t-Test comparing samples as indicated.

Extended Data Fig 1. a, The m+0 and m+1 isotopologues of m1A, m62A, Am, C and m5C (representative chromatograms) in polyA+ and large RNA **b**, ^{13}C labelling of intracellular methionine and SAM following labelling with ^{13}C -methyl-methionine for the indicated time periods. **c**, Representative bioanalyzer traces of polyA+ and tRNA samples. m1A, N1-methyladenosine; m62A, N6,6-dimethyladenosine; Am, 2O-methyladenosine; C, cytidine; m5C, 5-methylcytidine. Error bars represent 90% confidence intervals of three biological replicates in b.

Extended Data Fig.2

Extended Data Fig 2. a-c, Goodness-of-fit of a linear vs. exponential fit of isotopologue fractions determined in the chase experiment for polyA+ (a), and large RNA (b-c); SSE, sum of squared errors. **d**, Ion counts of equimolar analysis of pure ribonucleosides. **e**, Projected isotopologue fraction of a bona fide mRNA modification as measured in polyA+ RNA, with different degrees of ncRNA contamination. The effect on the isotopologue fraction over time was estimated from combinations of the m6A turnover in mRNA (0.244hr⁻¹) with that of ncRNA (0.031hr⁻¹, on average). **f**, Bioanalyzer traces of the polyA+ samples used in the chase experiments. **g**, Normalized ion counts of modified ribonucleosides relative to the ion counts sum of all ribonucleosides, in the free ribonucleoside pool. **h**, Ratio of normalised ion counts between free ribonucleosides and the different RNA classes. Error bars represent standard deviation of at least three biological replicates. In all cases, each replicate is the average of two technical replicas.

Extended Data Fig 3. a, Normalised abundance of modified ribonucleosides in large RNA as indicated, from 786O cells cultured with complete (ctrl) or serine- (NoSer) deprived DMEM medium for 4 hours. **b**, Isotopologue fractions of m6A and m7G in polyA+ RNA determined from the chase experiment in 786O cells cultured with ctrl, glutamine-deprived (NoQ) DMEM medium or treated with actinomycin D (ActD, 10 μ M). **c**, Levels of m6A and m7G in polyA+ under stress conditions as indicated, normalised relative to the ctrl condition in the chase experiment. Error bars represent standard deviation of at least three biological replicates (a) or two biological replicates per time point (b-c). In all cases, each replicate is the average of two technical replicas. * denotes $P < 0.005$, ‡ denotes $P < 0.05$, ns not significant, of a two-sided Student's t-Test comparing stress to ctrl conditions.

Figures

Figure 1

13C labelling of RNA modifications across RNA classes. a, The 13C-dynamods workflow shows the tracing of RNA methylation: cells are cultured with 13C-methyl methionine, the RNA is isolated, digested to ribonucleosides and subjected to LC-MS/MS analysis. The isotopologues detected for m6A are shown.

b, Representative chromatogram of unmodified and modified ribonucleosides from total RNA. c-d, The m+0 and m+1 isotopologues of modified and unmodified ribonucleosides (representative chromatograms) in polyA+ (c) and large RNA (d). e, Quantification of the isotopologue fractions of each ribonucleoside in polyA+, large and tRNA under unlabelled ('Unlab') conditions, after 4 and 24 hours of culture with ^{13}C -methylethionine. m6A, N6-methyladenosine; m7G, N7-methylguanosine; m1A, N1-methyladenosine; m62A, N6,6-dimethyladenosine; Am, 2O-methyladenosine; m5C, 5-methylcytidine; m1G, N1-methylguanosine; m2G, N2-methylguanosine. SAM, s-adenosylmethionine; SAH, s-adenosylhomocysteine; homoCys, homocysteine. Error bars represent standard deviation of three to four biological replicates, with the exception of two replicates for tRNA modifications. In all cases, each replicate is the average of two technical replicates. * denotes $P < 0.005$, ¥ denotes $P < 0.05$, # denotes $P = 0.059$, of a two-sided Student's t-Test comparing samples as indicated.

Figure 2

Kinetic profiling of RNA modifications in polyA+, ncRNA and free ribonucleosides. a-b, Isotopologue fractions during the 'chase' of ^{13}C -labelled modifications with naturally labelled methionine for 0, 1, 3, 6 and 16 hours, in polyA+ (a) and total/large RNA (b). c, Residuals of a linear vs. exponential regression of isotopologue fractions in polyA+ RNA in the chase experiment. d, Normalized ion counts of modified ribonucleosides relative to the ion counts sum of all ribonucleosides, shown for polyA+ and large RNA. (e)

Ratio of normalised ion counts between polyA+ and large RNA, as determined in (d). f, Isotopologue fraction of free ribonucleosides analysed from metabolic extracts. g-h, Goodness-of-fit of a linear vs. exponential fit of the isotopologue fractions in free modified ribonucleosides. SSE, sum of squared errors. m6A, N6-methyladenosine; m7G, N7-methylguanosine; m1A, N1-methyladenosine; m62A, N6,6-dimethyladenosine; Am, 2O-methyladenosine; m5C, 5-methyl-cytosine; m1G, N1-methylguanosine; A, adenosine; G, guanosine. Error bars represent 90% confidence intervals in (a-b), or standard deviation in (d, f) of at least three biological replicates with exception of Am (two replicates). In all cases, each replicate is the average of two technical replicas. * denotes $P < 0.005$, ¥ denotes $P < 0.05$, of a two-sided Student's t-Test comparing m6A and m62A to m1A, respectively.

Figure 3

RNA methylation dynamics during CD8+ T-cell activation. a-c, Isotopologue fractions of modified ribonucleosides in polyA+ (a), large (b) and tRNA (c) in CD8+ T-cells labelled with 13C-methyl-methionine for 4 and 24 hours and stimulated with CD3/ CD28 antibodies for the same time period. d, Quantification of the m+2 fraction from 3-13C-serine. Error bars represent standard deviation of T-cell samples from three healthy donors. In all cases, each replicate is the average of two technical replicas. * denotes $P < 0.005$, ¥ denotes $P < 0.05$, ns denotes not significant, of a two-sided Student's t-Test comparing non-activated to activated T-cells at 24 hours or comparing modified ribonucleosides as indicated.

Figure 4

RNA methylation dynamics under metabolic stress. a, Isotopologue fractions of modified ribonucleosides in polyA+ and large RNA following ^{13}C -methyl-methionine labelling of 786O cells cultured with complete (ctrl), glutamine- (NoQ), or serine-(NoSer) deprived DMEM medium for 4 hours. b, Normalised levels of m6A relative to adenosine in polyA+ RNA from 786O cells cultured with ctrl, NoQ or NoSer DMEM medium for 4 hours. Error bars represent standard deviation of three or more biological replicates, with exception of m5C under NoQ and m6A under NoSer (two replicates). In all cases, each replicate is the average of two technical replicas. * denotes $P < 0.005$, ¥ denotes $P < 0.05$, ns denotes not significant, of a two-sided Student's t-Test comparing samples as indicated.