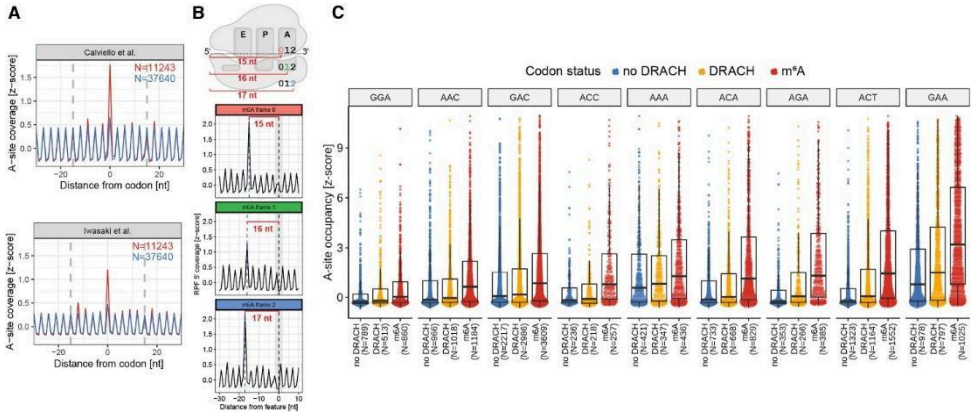
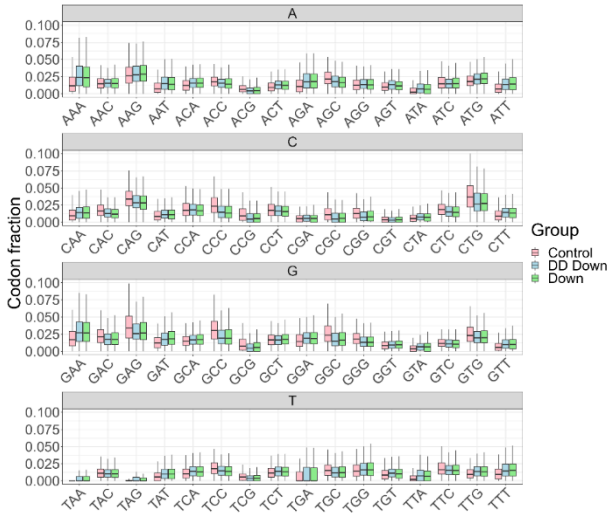
Reviewer 1

Q1

One further recent study that they should cite and discuss is (Linder, Bastian, et al. "tRNA modifications tune m6A-dependent mRNA decay." Cell 188.14 (2025): 3715-3727). Overall, in my view, the key conclusions of this manuscript are supported by the data and clearly deserve getting noticed by others in the field.

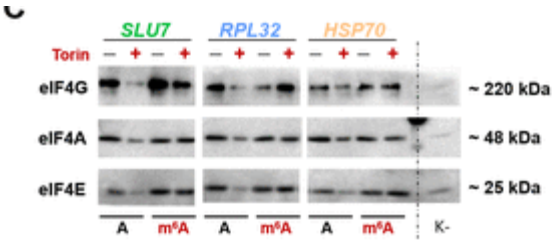
https://www.cell.com/cell/fulltext/S0092-8674(25)00415-5





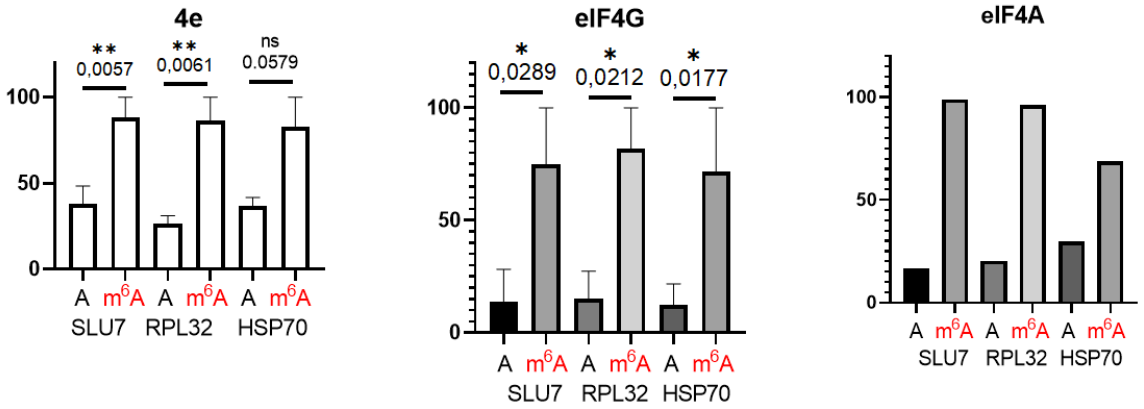
1. **А есть ли возможность через Plastid посмотреть фазирование именно по A-сайту? В контексте предложенной статьи это позволило бы оценить задержку рибосомы в момент декодирования.**
2. **Может дополнительно сгруппировать кодоны по их вхождению в DRACH-мотив?**

Q2.



1. Going through the manuscript in order has raised the following questions and concerns: Figure 1 and associated text – The western image in Figure 1C is not of a compelling quality and there is no quantification or information on reproducibility.

Качество и правда низкое. Может унести в Suplement?



2. Also, could probings for eIF3 and maybe some of the other m6A readers be added?

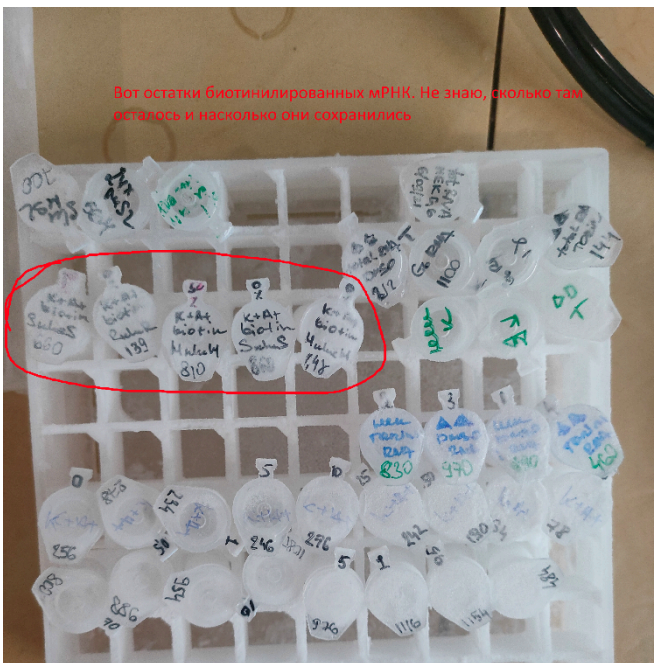
eIF3: в статье Meyer (https://pmc.ncbi.nlm.nih.gov/articles/PMC4695625/) наиболее сильный сигнал связывания с метилированной мРНК давали субъединицы **c, d, i**,. При этом сам PAR-CLIP они делали антителами на **eIF3a**.

Можно попробовать **DAP5** (https://pmc.ncbi.nlm.nih.gov/articles/PMC5520850/)

Про m6A : они лежат в морозилке в 245 вместе с остальными антителами (~90%), в прозрачном зип-пакете (мышиные, Abcam, расфасованы по ПЦР-пробиркам). Идеально работают на **1:2000**, но можно пробовать и **1:4000**. Все забивки делать строго на 7,5% BSA. На молоке результат будет плохой, картинка получается аховая.

Для транскрипции: Насколько я помню, всё должно лежать в железном штативе в кельвинаторе в 243 (вероятность ~75%), если там нет — то в морозилке 245 (~25%).

Вот остатки биотинилированных мРНК. Не знаю, сколько там осталось и насколько они сохранились



для pull-down:

1. Стрептавидин-сефарозу (70 мкл) промывал 10 раз PBS (по 700 мкл). Блокировку проводил в течение ночи в PBS, содержащем 7–10% FBS, гепарин (1С/мл), 200 мкг/мл гликогена и 200 мкг/мл ДНК лосося.
   Затем смолу промывал 10 раз PBS и уравновешивал в буфере для трансляции (20 мМ Hepes–KOH, pH 7,6; 2 мМ DTT; 1 мМ $Mg(OAc)_2$; 10 мМ KOAc).
2. 1 мкг биотинилированной мРНК инкубировал с 100 мкл клеточного лизата в присутствии 10 мкг суммарной РНК E. coli (и ингибитора РНказ) в течение 30–40 мин при 30 °C.
3. В каждую точку добавлял по 100 мкл суспензии подготовленной смолы (из расчета 10 мкл исходного сорбента на точку) и инкубировал на ротамиксе в течение 1 часа.
4. Потом промывки около 10 раз буфером PBS с повышенной ионной силой (до 250 мМ KCl) и добавлением 0,2 мМ VRC.
5. Элюировал БДО

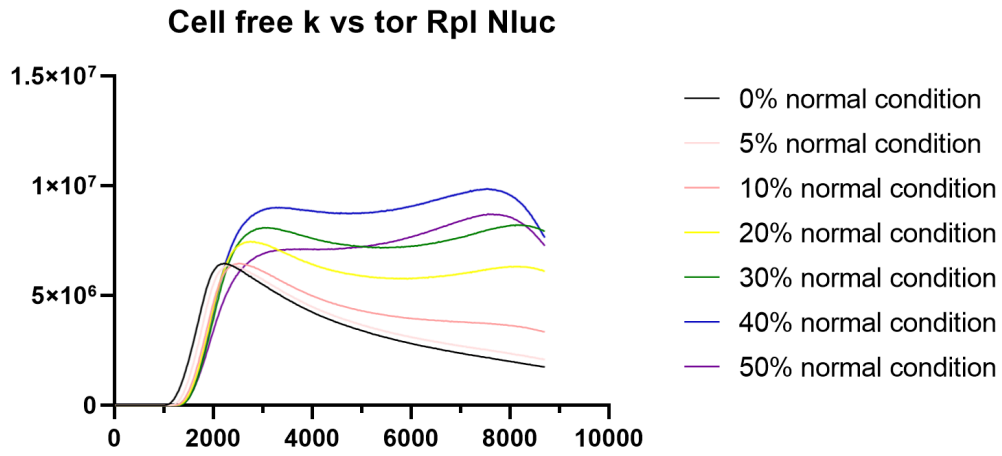
Q3.

Figure 2 and associated text – findings are consistent with quantitative data on m6A methylation of these mRNAs in another study, but could this be verified for the reporters used here?

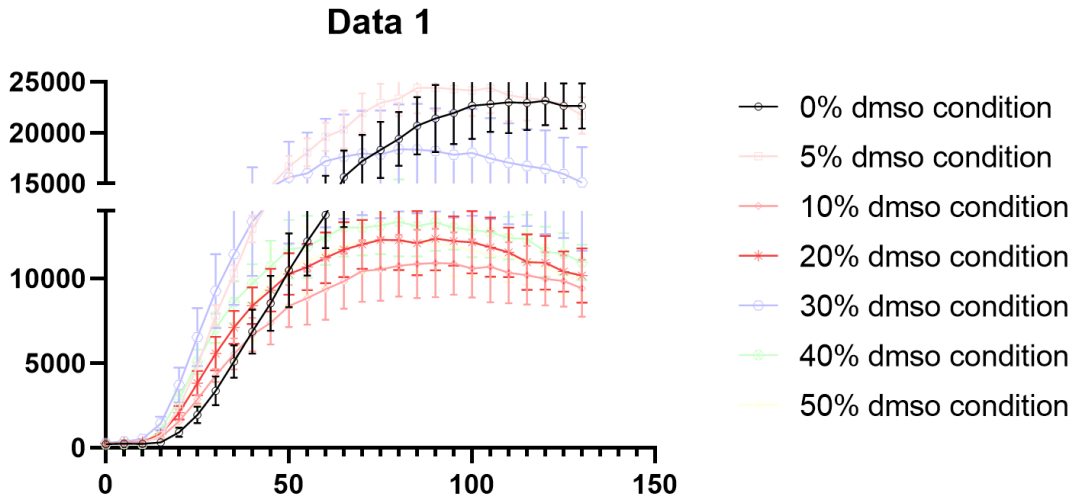
Q4.

Figure 3 and associated text – Can it be made clearer what specific criteria/ thresholds were used to define the three gene groups? Is this based on a specific fold-change and if so, why? Discussion – The whole set of findings is certainly consistent with an effect of m6A on translation initiation, but given the other published observations showing effects on elongation and mRNA stability, should this be directly tested for key experiments done here? At least, this issue should be more directly discussed as a potential limitation of the present work.

Ну, **элонгацию** посмотреть не можем. Единственное — привести график динамики накопления и сказать, что кривые позже выходят; возможно, это эффект элонгации, но не точно



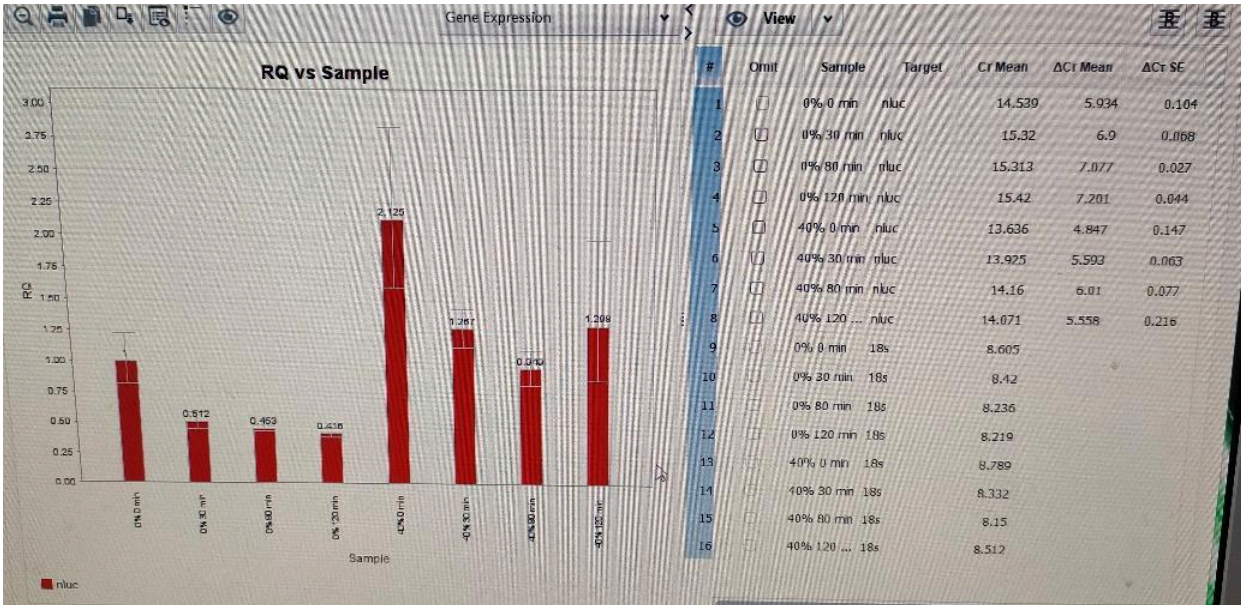
С другой стороны, в клетках действительно виден эффект на трансляцию/ стабильность

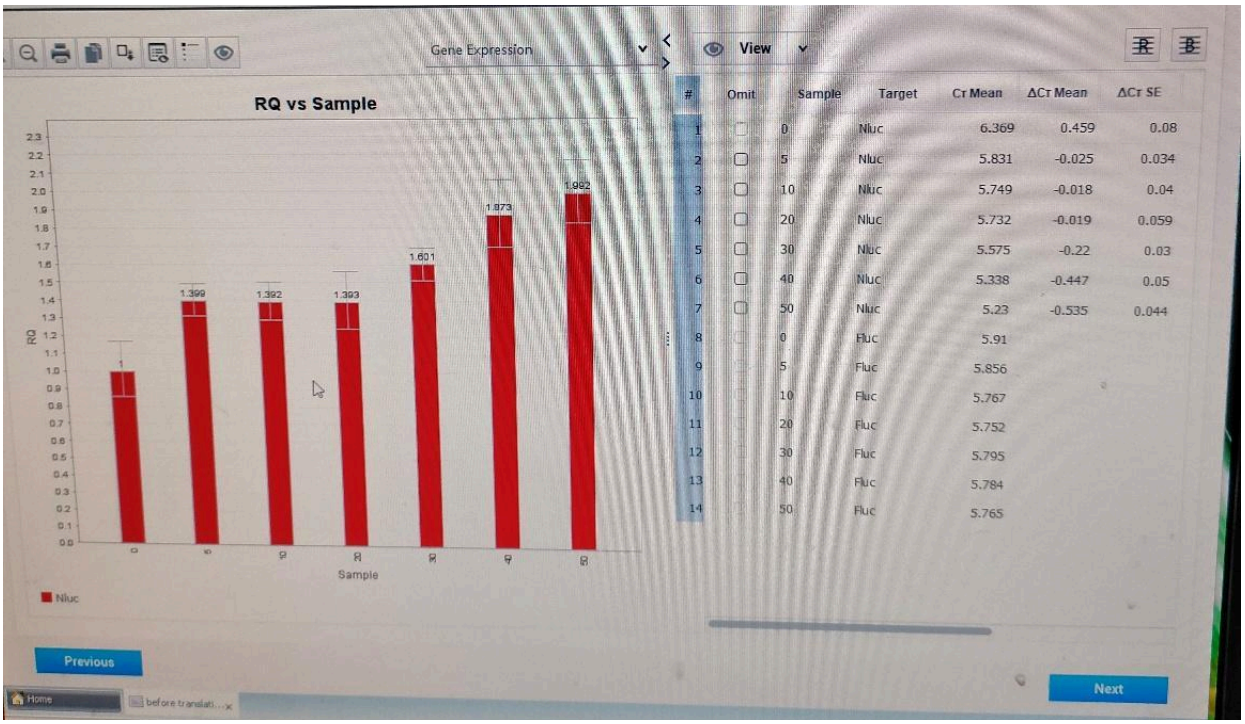


Ну, или можно сказать, что действительно есть ограничения: умеем мерить трансляцию только по конечной точке...

Про стабильность

Если говорить про бесклетку, то всё нормально: РНК условно такая же по стабильности, как неметилированная





Можно еще привести стабильность которую в свое время делали для pnl2.2.

Q5.

Minor issues: Figure 1 legend – modify statement on replication, error bars and statistics to make it clearer that it applies to panels A and B. In the main text, Figure 2B is described ahead of Figure 2A. Duplication near end of sentence in “However, SLU7 mRNA translation efficiency was insensitive to mTOR inhibition in wild-type HEK293T cells, but was reduced upon mTOR inhibition to mTOR inhibition in KD cells.” Reference

Figure S1B in this sentence: “Consistently, the total level of protein synthesis, measured by azidohomoalanine incorporation, was approximately 40% lower in the KD cells compared to the WT under normal conditions.” Reference Figure 3C instead in “We did not detect any significant RBP target enrichment for this group of mRNAs (Figure 2C).”

Reviewer 2

This is an interesting manuscript that changes the way we look at the relationship between m6A modification of transcripts and their resistance to mTOR inhibition. The main claim is that m6A modifications anywhere on a transcript help make its translation resistant to mTOR inhibition. This would be an important finding.

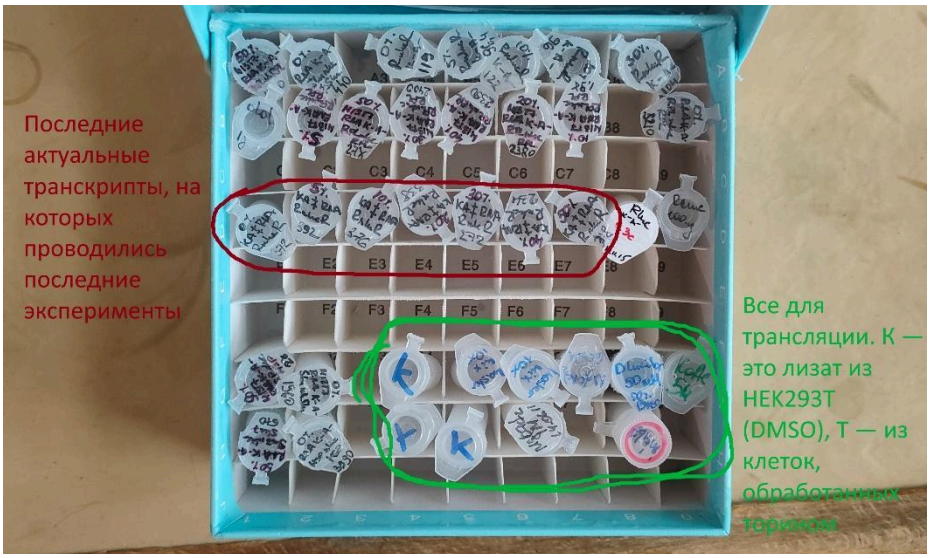
I feel that the manuscript can be divided into two parts. One experimental part consisting of figures 1, 2, and 5 seems reasonably solid to me, and shows the functional impact of altered m6A levels on translation. The other part is a more 'correlative' part consisting of figures 3 and 4 which I believe are currently not solid as described in my Major Issues below. That said, addressing these issues would simply involve re-analyzing the data, so I think this should be easily fixed.

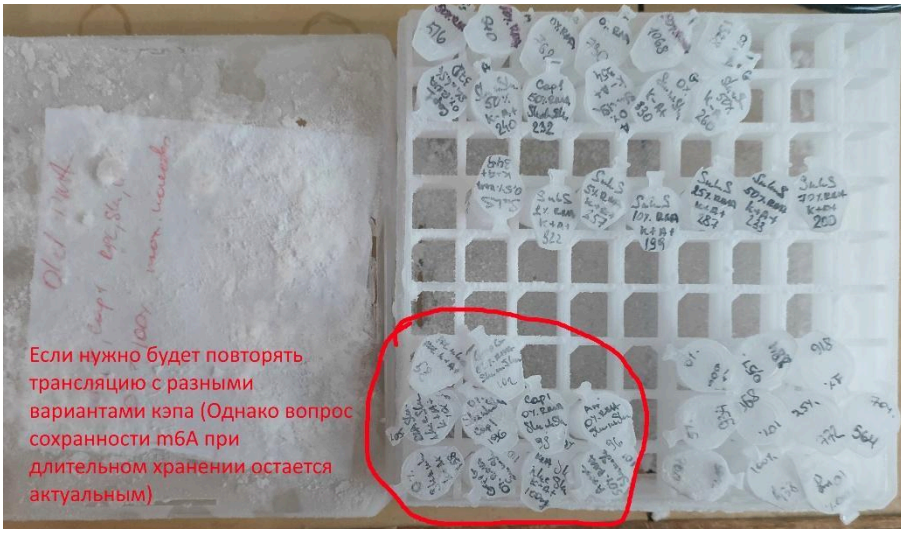
Another major issue is whether the results described here relate to 'cap-independent' translation or 'eIF4E-independent translation', which should be clarified, perhaps with some additional experiments.

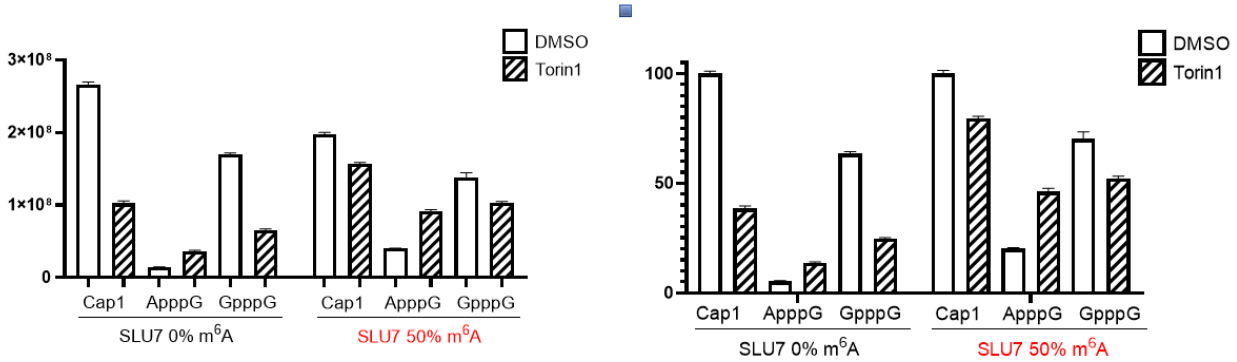
Q1.

Do the results described here relate **to 'cap-independent'** translation or **'eIF4E-independent translation'**, which are not the same thing because there are other cap-binding proteins besides eIF4E? The experiments are a bit mixed, sometimes addressing cap-dependence (with cap-analogs, e.g. Fig 1A) and sometimes addressing the other (with Torin, Fig. 1B, Fig 2, etc). e.g. On page 8 the authors conclude "All in all, we conclude that m^6A methylation along the mRNA reduces its sensitivity to cap-dependent translation inhibition". The experiments shown in Fig 2, however, address **eIF4E-dependent** translation because they are done +/- Torin.

It would be good to tease apart these two things - does m6A promote eIF4E-independent translation or cap-independent translation? This can be done, for instance, by studying the effect of m6A on in vitro transcribed mRNA with an **A-cap** (or another cap analog) versus a normal **G-cap and** testing this in translation extracts, and by transfecting into cells (as in Fig 2C).







Q2.

Fig 3A - the way that the transcript groups are defined is strange. For instance, the "mTOR-insensitive group" should be transcripts that do not change in translation efficiency upon torin treatment in WT cells. These would be transcripts with a logFC(Torin/DMSO) around zero in the WT cells - the red dots should be a stripe centered around 0 on the x-axis that goes all the way from top to bottom (ie should

include the green dots as well as the grey dots in between). Likewise, the 'mTOR sensitive group" should include the grey dots on top of the blue dots. And 'm6A -dependent" means their translation drops upon loss of m6A, so this should be defined as logFC(KD DMSO/WT DMSO) < 0 - I don't see how the Torin/DMSO ratio is relevant for defining a m6A-dependent set (as currently done on the graph) ? Currently, the 'm6a-dependent' group in green is transcripts whose translation drops in the KD cells, which have little/no m6A, upon Torin treatment. This means they are mTOR-dependent.

This affects all downstream analysis with these groups (e.g. Fig 4).

Q3.

For Fig 4, it would be good to do the analysis the other way around - group mRNAs according to their m6A levels, and see what happens to their translation upon TOR inhibition in WT cells. i.e. Are m6A levels predictive? Is this effect gone in the KD cells?

Q4

Minor Issues:

1. Fig 1A - it's unclear if the cap analog was added into the translation extract, or used to 'cap' the in vitro transcribed mRNA? From the results text, it sounds like it was added into the translation extract ("to compete for eIF4E binding", which would not be the case if it were directly on the mRNA) ?

Это вопрос 1

How does the experiment in Fig 1A look like if one compares RLU for in vitro transcribed mRNA that carries an A-cap (or another cap analog) versus a normal G-cap?

Q5.

Fig 1C - how do you control/normalize for the amount of mRNA pulled down in each sample? e.g. if the m6A affects stability of the mRNA in the translation extract, this would affect the interpretation of the results?

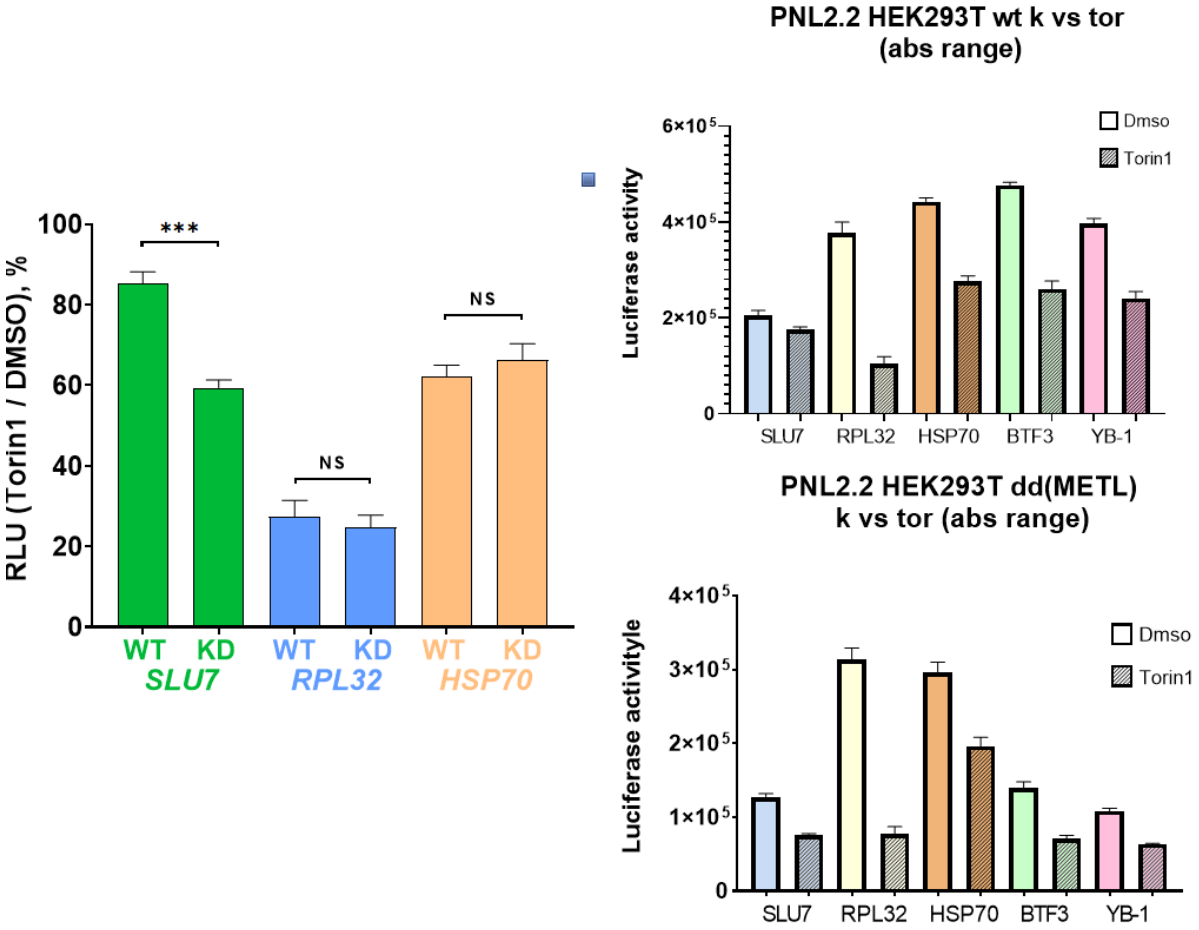
Можно использовать тот же ответ, что и предыдущему рецензенту по поводу стабильности

Q6.

Fig 2B - The authors should show (e.g. in a supplemental figure) the separate values of the luciferase activity in the Torin and the DMSO condition, because a lot of important information goes lost when showing only the Torin/DMSO ratio, as currently shown in

the figure. (e.g. does the SLU7 reporter ratio drop in the KD condition because it goes up in the DMSO or because it goes down in the Torin condition?)

В supplement можно включить сырые данные. Вопрос лишь в том, делать это только для данного рисунка или для каждого?



Q7.

"Consistently, the total level of protein synthesis, measured by azidohomoalanine incorporation, was approximately 40% lower in the KD cells compared to the WT under normal conditions." should cite Suppl. Fig. 1B.

5. Page 8 " Following the Torin1 treatment, the global translation was further reduced by 3.5-fold in the KD cells and by 2.5-fold in the WT cells." A reduction of more than 100% (ie 1-fold) is not mathematically possible.

6. Fig 5 - it's confusing to label the experiment where the mRNAs are transfected into cells "ex vivo" compared to translation extracts... Most clear would be to label panel A with "in translation extracts" and panel B "in cells".