Biochemistry and Chemical Biology
Developmental Biology

Human eIF2A has a minimal role in translation initiation and in uORF-mediated translational control in HeLa cells

Reviewed Preprint
v2 • June 17, 2025
Revised by authors

Reviewed Preprint
v1 • January 17, 2025

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eLife Assessment

In this **valuable** study, Roiuk et al combined ribosome profiling and reporter assays to provide **compelling** evidence that eIF2A does not have a major impact on mRNA translation in HeLa cells. These findings are consistent with several recent publications that disaffirm the previously proposed role of eIF2A in directing protein synthesis under stress. Considering that stress-dependent perturbations in translation play a major role in homeostasis and several pathological states (e.g., cancer and neurological disorders), this work should be of broad interest to researchers studying regulation of gene expression, stress-adaptation, cancer and neurobiology.

https://doi.org/10.7554/eLife.105311.2.sa4

Abstract

Summary

Initiation of translation on eukaryotic mRNAs requires a 40S ribosome loaded with an initiator tRNA in order to scan for, and to identify, an initiation codon. Under most conditions, the initiator tRNA is recruited to the ribosome as part of a ternary complex composed of initiator tRNA, eIF2 and GTP. Although this function of recruiting the initiator tRNA was originally ascribed to another factor, eIF2A, it was later disproven and shown to belong to eIF2. Nonetheless, eIF2A is still considered a translation initiation factor because it binds the ribosome and shows genetic interactions with other initiation factors such as eIF4E. The exact function of eIF2A during translation initiation, however, remains unclear. Here we systematically test in HeLa cells, using ribosome profiling and luciferase reporter assays, the role of eIF2A in translation initiation, including translation of upstream ORFs that are either initiated with a AUG or near-cognate codons. Since eIF2A is thought to take over the function of eIF2 when eIF2 is inhibited, we also test conditions where the integrated stress response is activated, thereby leading to eIF2 inactivation. In none of our assays, however, could we

detect a role of eIF2A in translation initiation. It is possible that eIF2A plays a role in translation regulation in specific conditions that we have not tested here, or that it plays a role in a different aspect of RNA biology.

Introduction

For cells and organisms to grow and develop properly, they require a machinery that translates mRNAs in a finely-tuned manner. Alterations in the amount or activity of components of the translational machinery lead to severe pathological conditions (Tahmasebi et al., 2018). Of all the steps of protein production, translation initiation is considered to be the most precisely regulated and rate-limiting (Palmiter, 1975). Translation initiation under normal physiological conditions is well studied and occurs through a cap-dependent mechanism (Hinnebusch and Lorsch, 2012) that relies on the recruitment of the 40S ribosomal subunit preloaded with a set of initiation factors to the 5'-end of mRNAs. Correct positioning of the 40S at the 5'-end enables scanning, i.e. movement of the 40S in a 5' to 3' direction in search of the first initiation codon (AUG) within an optimal context (Pestova and Kolupaeva, 2002). The ribosome uses the CAU anticodon of the initiator Methionine tRNA ($tRNA_i^{Met}$) to identify the AUG start codon (Kozak, 1991). In the most common initiation pathway, $tRNA_i^{Met}$ is delivered to the 40S via the ternary complex which consists of eIF2 bound to GTP and the $tRNA_i^{Met}$ itself. However, several alternative initiation factors have been reported to possess the ability to also interact and potentially deliver initiator tRNA: eIF2D, the DENR/MCTS1 complex, and eIF2A (reviewed in (Grove et al., 2024)). We focus here on eIF2A, since its function is still elusive.

eIF2A was initially considered to be a functional analogue of prokaryotic IF2 (Shafritz and Anderson, 1970), however later this role was reassigned to the above-mentioned heterotrimeric factor eIF2 (α,β,ψ) (Levin et al., 1973). Unlike eIF2, eIF2A was proposed to recruit $tRNA_i^{Met}$ to the 40S in a GTP-independent but codon-dependent manner (Zoll et al., 2002), however this activity was later challenged (Dmitriev et al., 2010) by attributing it to eIF2D which co-purified with eIF2A in the initial study. Unlike eIF2, eIF2A is dispensable for organismal viability, as single knock-outs of *eIF2A* in model organisms such as *Saccharomyces cerevisiae* (Komar et al., 2005), *C. elegans* (Kim et al., 2018) and *M. musculus* (Anderson et al., 2021; Golovko et al., 2016) show little or no phenotype. However, double knock-outs of *eIF2A* and *eIF5B* exhibit severe growth defects both in yeast and in *C.elegans* (Kim et al., 2018; Zoll et al., 2002) and eIF2A is synthetic lethal with eIF4E in yeast (Komar et al., 2005). These genetic interactions suggest a role for eIF2A in translation initiation, however, the precise function of eIF2A is still not well defined, with contradictory results being reported. For example, eIF2A has been studied in the context of internal ribosome entry sites (IRES), where it was proposed to act both as a suppressor and an activator of IRES-mediated initiation. In yeast, eIF2A was reported to inhibit translation of URE2, GIC1 and PAB1 IRESes (Reineke et al., 2008; Reineke and Merrick, 2009). In Huh7 cells, knock-down of eIF2A was reported to hamper translation of a Hepatitis C virus (HCV) IRES reporter upon ER-stress (Kim et al., 2011), while a subsequent study detected no effect on the HCV-IRES reporter (Jaafar et al., 2016). Similarly, knock-out of eIF2A in HAP1 cells did not affect translation of either a HCV-IRES reporter or a EMCV-IRES reporter (Gonzalez-Almela et al., 2018), nor did it affect the infection rate of Sindbis virus (Sanz et al., 2019). Beyond IRES-mediated translation, eIF2A was reported to promote initiation from near-cognate start sites, initiated at leucine codons (CUG, GUG), by delivering elongator leucine $tRNA^{Leu}$ to the 40S subunit (Sendoel et al., 2017; Starck et al., 2012; Starck et al., 2016). Such eIF2A-driven non-AUG initiation events were proposed to play a crucial role in different aspects of cell physiology and disease progression: cellular adaptation during the integrated stress response (Chen et al., 2019; Starck et al., 2016), fine-tuning of mitochondrial function (Liang et al., 2014), tumour progression (Sendoel et al., 2017), and repeat-associated non-AUG translation in familial amyotrophic lateral sclerosis (Sonobe et al., 2018). Such non-AUG initiation by eIF2A implies that eIF2A should possess high affinity for leucine tRNAs, however, according to *in vitro* filter

binding assays, eIF2A binds inefficiently to elongator $tRNA^{Leu}$ compared to initiator $tRNA^{Met}$ (Kim et al., 2018), although, as mentioned above, it is questionable whether eIF2A has any tRNA binding capacity at all. Furthermore, loss of *eIF2A* in several systems did not recapitulate these effects on non-AUG initiation in either non-stressed or stress conditions (caused either by amino acid depletion or sodium arsenate treatment) (Gaikwad et al., 2024; Ichihara et al., 2021). In particular, one study (Gaikwad et al., 2024) found that eIF2A has little or no role in translation initiation and uORF mediated translation in yeast. Since in some cases mRNA translation in human cells can differ from mRNA translation in yeast, whether eIF2A also has such a minimal role in human cells remains to be clarified.

Due to these various discrepancies, we decided to study the role of eIF2A on mRNA translation and cellular fitness in human cells. For this, we generated *eIF2A* knock-out HeLa cells and applied ribosome profiling to analyze changes in mRNA translation. Overall, we find little contribution of eIF2A to cellular translation both under normal and stressed conditions in HeLa cells.

Results

eIF2A-KO HeLa cell lines have no proliferative defect or change in global translation rates

To study the impact of eIF2A on cellular translation, we used CRISPR/Cas9 to generate two independent eIF2A knockout ($eIF2A^{KO}$) HeLa cell lines, in which different exons of *eIF2A* were targeted. We confirmed the absence of eIF2A by immunoblotting (**Fig.1A**) and by genotyping the clones (**Suppl. Fig. 1A**). $eIF2A^{KO}$ lines have reduced levels of *eIF2A* mRNA, likely arising from nonsense-mediated decay, due to premature stop codons in the *eIF2A* mRNA (**Suppl. Fig. 1B**). The $eIF2A^{KO}$ cells exhibit no defect in cellular proliferation rates (**Fig.1** B), which agrees with eIF2A having a dispensable role in organismal viability (Anderson et al., 2021; Golovko et al., 2016; Kim et al., 2018; Komar et al., 2005). To test if the loss of *eIF2A* affects global translation rates, we performed an *O*-propargyl-puromycin (OPP)-incorporation assay, which labels newly synthesized peptides and allows subsequent detection with anti-puromycin antibody. OPP-incorporation, however, did not show any significant change in $eIF2A^{KO}$ cells compared to wild-type isogenic controls (**Fig. 1C-D**). In line with the OPP-incorporation assay we did not observe changes in polysome profiles of $eIF2A^{KO}$ cells compared to controls (**Fig. 1E-F**).

Several studies have reported that stress can upregulate eIF2A levels (Panzhinskiy et al., 2021; Starck et al., 2016), or cause eIF2A to shuttle from the nucleus into the cytosol (Kim et al., 2011). Interestingly, Grove et al. recently reported (Grove et al., 2023) that increased levels on eIF2A in an *in vitro* translation system can suppress global translation initiation by directly binding and sequestering 40S ribosomal subunits. Thus, an increase in cytosolic eIF2A, either due to increased total levels or due to shuttling out of the nucleus, could be a potential mechanism to suppress translation upon stress. To determine if either localization or global levels of eIF2A change in response to stress in HeLa cells, we performed subcellular fractionation and loaded on a gel nuclear and cytosolic fractions in proportion to their abundance in a cell (i.e. both fractions were lysed in equal volumes). In HeLa cells eIF2A is present in both the cytosol and the nucleus, with higher levels in the cytosol (**Suppl. Fig. 1C**). Nonetheless, stress caused by tunicamycin treatment did not affect the subcellular distribution of eIF2A (**Suppl. Fig. 1C-D**), nor overall eIF2A levels (**Suppl. Fig. 1F-G**). To assess the localization of eIF2A with an orthogonal approach, we performed cell immunostaining. Since the eIF2A antibodies we have are not suitable for immunostaining, we overexpressed N-terminally FLAG-tagged eIF2A in HeLa cells treated cells with either DMSO or 100μM sodium arsenite (SA) for 1 hour (condition used in (Kim et al., 2011)). This revealed that overexpressed eIF2A was excluded from the nucleus, and showed a cytosolic, speckled staining pattern, which was not affected by SA treatment (**Suppl. Fig. 1E**). These results are consistent with what has been observed for endogenous eIF2A in HAP1 cells

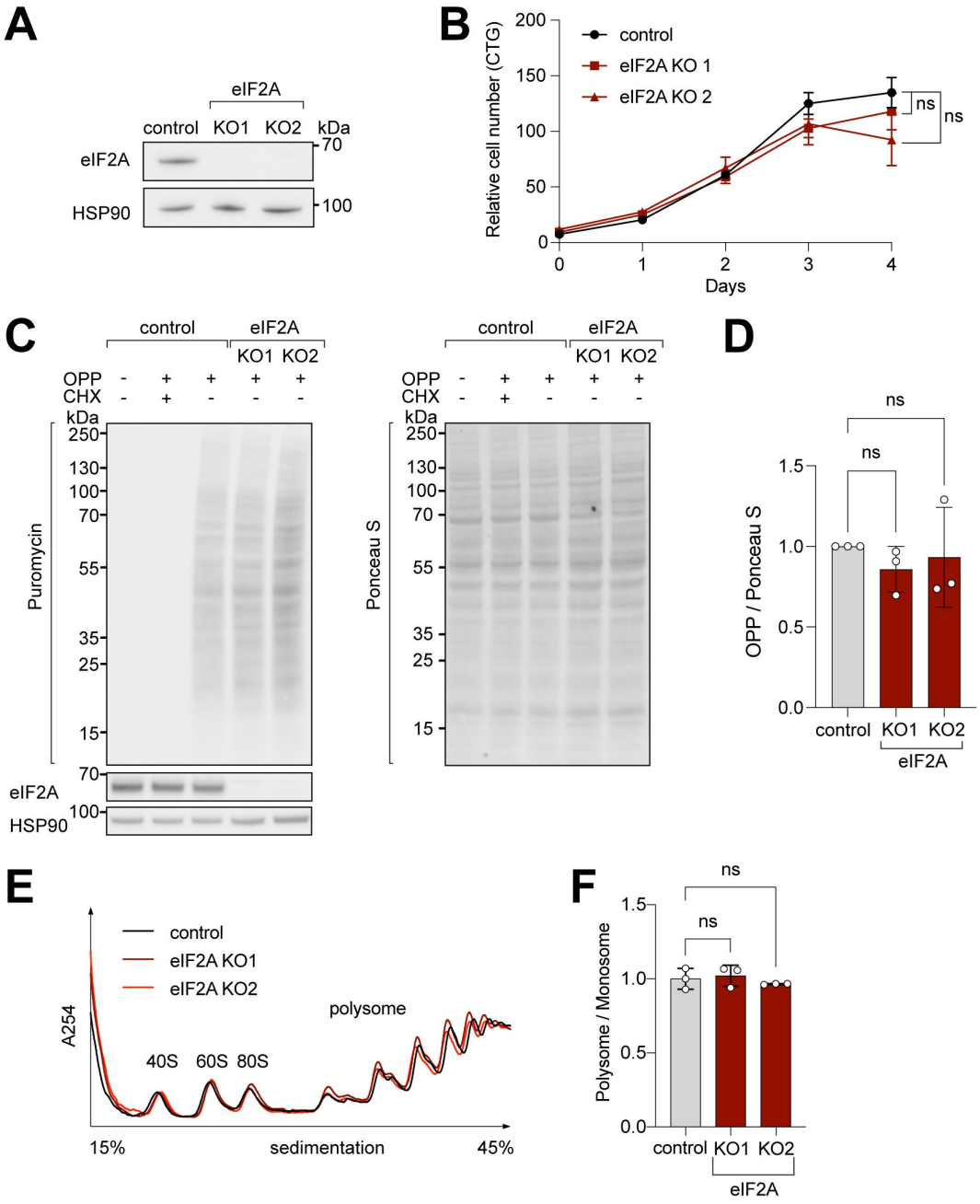


Figure 1

eIF2A has minimum effect on cell proliferation and global translation.

(A) Validation that *eIF2A* knockout cells have no eIF2A protein by immunoblotting. **(B)** Two independent eIF2A knockout HeLa cell lines have no proliferation defect, assayed by CellTiter Glo. Error bars: standard deviation. Significance by ordinary one-way ANOVA. **(C-D)** eIF2A knockout HeLa cells have no detectable change in global translation rates compared to control cells. (C) The translation rate was measured by immunoblotting to detect OPP incorporated by metabolic labeling (left), and normalized to total protein amount assayed by PonceauS (right). Three independent replicates are quantified in panel (D). CHX = cycloheximide treated sample to completely block global translation (negative control). Error bars: standard deviation. Significance by ordinary one-way ANOVA. **(E-F)** Polysome profiles of *eIF2A*^{KO} cells show little to no difference to profiles from control cells. Lysates from either control or eIF2^{KO} HeLa cells were separated on a sucrose gradient. One representative graph is shown in panel D. The polysome/80S ratio of three independent replicates is shown in panel F. Error bars: standard deviation. Significance by ordinary one-way ANOVA.

(Gonzalez-Almela et al., 2018; Sanz et al., 2017). To check if cellular stresses affects eIF2A levels in HeLa cells, we treated cells with different agents at concentrations and timeframes that were previously reported to alter eIF2A levels (Starck et al., 2016). However, we did not record significant changes in eIF2A levels with any of these treatments (**Suppl. Fig. 1F-G**). Nonetheless, we decided to test whether elevated levels of eIF2A would have the capacity to inhibit global translation levels *in vivo*. To this end we overexpressed eIF2A and quantified global translation rates by OPP incorporation but did not observe any significant change (**Suppl. Fig. 1 I-H**). In sum, our results indicate that loss of eIF2A as well as eIF2A ectopic overexpression in HeLa cells has little or no impact on global translation and cellular proliferation.

Ribosome profiling identifies no eIF2A-dependent transcripts

We next investigated whether loss of *eIF2A* causes a change in translation of specific mRNAs which might be overlooked when assaying total global translation. To do this, we performed ribosome profiling, which sequences the mRNA footprints protected by ribosomes, on control and *eIF2A*^{KO} cells. Normalization of the footprint counts for each mRNA relative to the abundance of that mRNA in total RNA yields an estimate of a transcript's translation efficiency. We used *eIF2A*^{KO} clone 1, since the exon targeted in this clone is further 5' than in clone 2, thereby yielding a short, truncated protein of only 24 amino acids (Suppl. Fig 1A). Correlation analysis revealed that both footprint counts and total RNA counts were highly reproducible across technical triplicates in both control and *eIF2A*^{KO} cells (**Suppl. Fig. 2A**). In addition, our data revealed the expected enrichment of footprints in the coding sequence versus UTRs, as well as distinct triplet periodicity, both hallmarks of ribosome profiling data (**Suppl. Fig. 2 B,C**). Metagene analysis showed similar global profiles in footprint densities within ORFs in wild type and *eIF2A*^{KO} samples, in agreement with the data presented above that eIF2A is dispensable for global mRNA translation (**Suppl. Fig. 2 B,C**). A per-gene analysis identified only 15 genes in addition to eIF2A whose translation efficiency changes in *eIF2A*^{KO} cells compared to control cells (**Fig. 2A**). This is very different from our previous footprinting studies where we found that loss of DENR leads to reduced translation of 517 mRNAs (Bohlen et al., 2020), loss of PRRC2A/B/C leads to altered translation of 109 mRNAs (Bohlen et al., 2023), and expression of 4E-BP leads to altered translation of 605 mRNAs (Roiuk et al., 2024), suggesting that in comparison eIF2A plays a minor role in translational regulation (**Fig. 2A**). We decided to validate some of these eIF2A-dependent candidates by immunoblotting, and to our surprise, amongst all the proteins we tested, only CCND3 showed reduced levels in one of the two *eIF2A*^{KO} clones (**Fig 2** B,C). We then quantified mRNA levels for all the genes that we tested (**Fig. 2D**). For NCAPH2, PPFIA1 and RPS6KB2, mRNA levels were either unchanged or reduced in the *eIF2A*^{KO} cells, indicating that translation efficiency for these genes was either unchanged or increased upon loss of eIF2A, which does not agree with the ribosome profiling data. For CCND3, protein levels were reduced in KO1 and mRNA levels were not, consistent with a drop in CCND3 translation in these cells, but this effect was not reproduced in eIF2A KO2 (**Fig. 2C-D**). Together, this indicates there is an enrichment for false-positives amongst the 15 genes identified by ribosome profiling. These results would be consistent with eIF2A having no effect on translation of any mRNA, with a few false-positives coming through in the transcriptome-wide ribosome footprinting, as is always the case. Nonetheless, to test further whether translation of any of these candidates is altered upon loss of eIF2A, we generated reporter constructs by cloning the 5'-UTRs of these candidates upstream of Renilla luciferase, and then testing their expression in the presence or absence of eIF2A. We used this approach multiple times in the past to identify mRNAs whose translation is dependent on initiation factors (Bohlen et al., 2023; Roiuk et al., 2024; Schleich et al., 2014). We succeeded in cloning 14 of the 15 5' UTRs of the transcripts predicted to be eIF2A-dependent. None of the reporters, however, showed a consistent drop in translation in eIF2A KO1, eIF2A KO2, and siRNA-mediated eIF2A knockdown cells (**Fig. 2E**, **Suppl. Fig. 3A-B**). Although we did not exclude a possible effect of eIF2A on translation via the 3'UTR or coding sequence of the 12 genes that we did not assay via immunoblotting (**Fig. 2B-D**), our results indicate that eIF2A has little or no effect on translation

of cellular mRNAs in HeLa cells under non-stressed conditions. Consistent with this, analysis of our ribosome footprinting data with anota2seq (Oertlin et al., 2019), also did not identify any transcripts with altered translation (**Suppl. Fig. 3C**).

Since eIF2A was proposed to deliver tRNA to the ribosome in a codon dependent manner, we tested if loss of eIF2A affects translation initiation on a reporter where the ribosome is directly positioned on the initiation AUG. To perform this, we cloned a 5' UTR reporter with a short 5'UTR (12 nt) where loading of the small ribosomal subunit should place the AUG in close proximity to the P-site (Gu et al., 2021). In addition, we tested a reporter bearing the EMCV IRES in the 5'-UTR, where initiation relies on close positioning of the 40S to the AUG (Davies and Kaufman, 1992). Translation of neither reporter, however, was affected by loss of eIF2A (**Suppl. Fig. 3D**). In sum, we were not able to find any transcript whose translation depends on eIF2A under non-stressed conditions in HeLa cells.

eIF2A does not contribute to uORF translation

Several studies have reported that eIF2A can deliver alternative initiator tRNAs to uORFs with near-cognate start codons (Sendoel et al., 2017; Starck et al., 2012; Starck et al., 2016). Based on these reports we tested if eIF2A depletion affects uORF translation in HeLa cells. First, we analysed if translation initiation or termination on endogenous, AUG-initiated uORFs is altered in *eIF2A*^{KO} cells. For this, we calculated a metagene profile of footprint reads at start and stop codons of all AUG-initiated uORFs, however we could not detect any significant differences in uORF translation (**Suppl. Fig. 4A,B**).

Since uORFs are elements that inhibit translation of the downstream main ORF (mORF), initiation factors that influence uORF translation also influence, as a consequence, translation of the downstream main ORF. Therefore, we checked if translation efficiency of the main ORF of uORF-bearing transcripts changes between eIF2A^{KO} and control cells. For this we looked at the translation efficiency of different sets of transcripts: 1) all transcripts, 2) transcripts possessing AUG-initiated uORFs, or 3) transcripts containing uORFs starting with a near cognate initiation codon – CUG, GUG or UUG (**Suppl. Fig. 4C**). Although essentially no genes show a significant change in translation efficiency when analyzed singly (**Fig. 2A**), this aggregate analysis might identify significant trends caused by small changes in groups of transcripts. This analysis revealed, however, that translation of transcripts with near-cognate uORFs did not differ from the global distribution in translation efficiency of all transcripts (**Suppl. Fig. 4C**). Transcripts with AUG-initiated uORFs, however, did have a very slight, but statistically significant increase in translation efficiency, compared to the whole dataset (log2(fold change) of −0.03 versus −0.05) (**Suppl. Fig. 4C**).

To validate this minor role of eIF2A in uORF translation we tested various luciferase reporters in eIF2A^{KO} cells. We placed Renilla luciferase under control of a 5' UTR with no uORF (LMNB1, as a negative control) or the same 5'UTR where we synthetically introduced a uORF with different start codons or Kozak sequences (**Fig. 3A**). In line with our ribosome profiling data, all of these luciferase reporters showed no significant difference in expression in *eIF2A*^{KO} or eIF2A overexpressing cells compared to controls (**Fig. 3A**, **Suppl. Fig. 4D**). Considering that this luciferase assay is a readout for translation of the main ORF, and not the uORF directly, we also performed an experiment where we directly visualised uORF peptide production. For this purpose, we generated fluorescent reporters carrying a uORF coding for the SINFEKL peptide which can be presented on the surface of cells expressing the H-2Kb Class I MHC complex (HEK293T-H2-Kb) where it can be detected with antibodies (Starck et al., 2016). To assess the impact of eIF2A on the production of this peptide, we generated a HEK293T-H2-Kb, *eIF2A*^{KO} cell line (**Fig. 3B**). Like *eIF2A*^{KO} HeLa cells, HEK293T-H2-Kb *eIF2A*^{KO} cells also did not display any defect in proliferation (**Suppl. Fig. 4E**) or global translation (**Suppl. Fig. 4F-G**). This revealed, however, no difference in translation of the uORF peptide when comparing *eIF2A*^{KO} to control cells, regardless of whether the uORF was initiated by a AUG codon or a near-cognate initiation

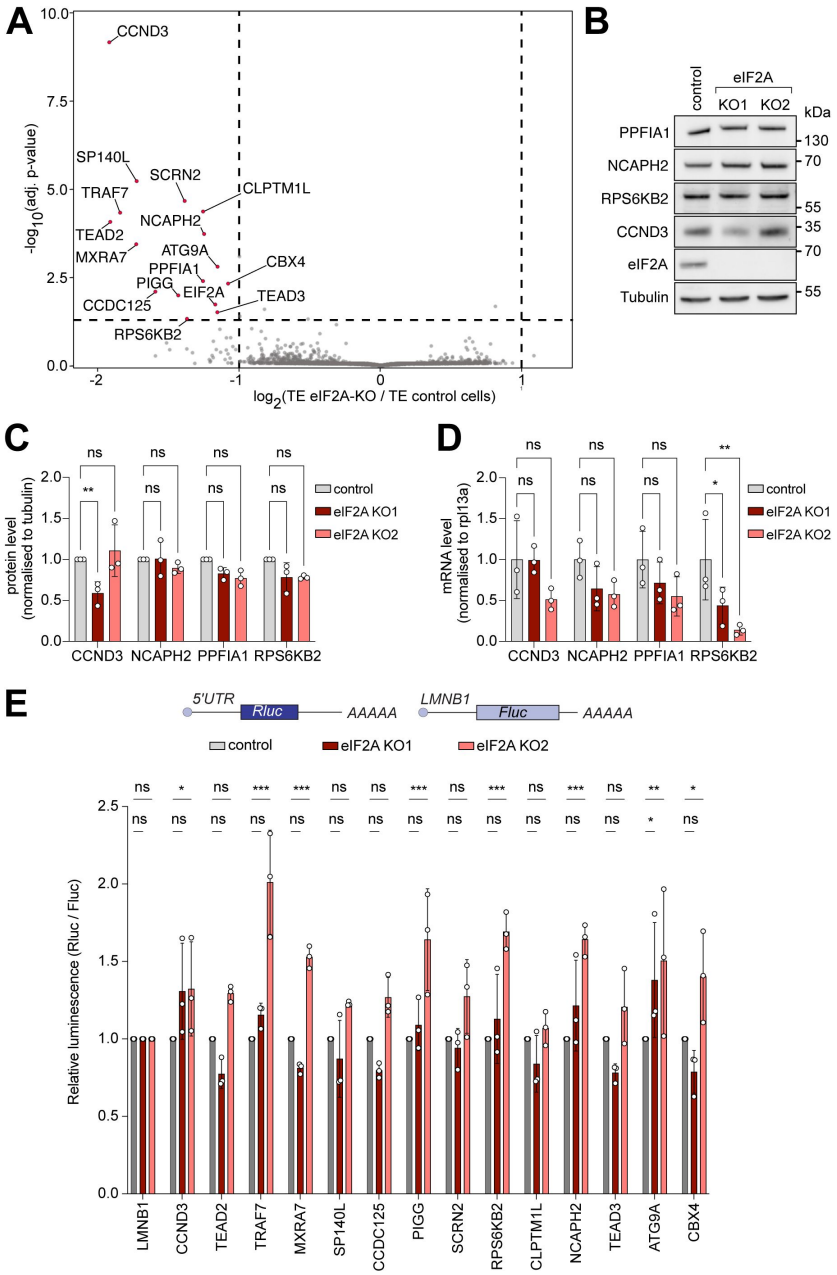


Figure 2

Ribosome profiling of eIF2A-KO lines finds little impact of eIF2A on translation.

(A) Ribosome profiling identifies a handful of mRNAs sensitive to eIF2A depletion. Scatter plot of log2(fold change of Translation Efficiency eIF2A^{KO}/ control) versus significance. Significant candidates with log2(fold change) < −1 are shown in red. Significance was estimated with the Wald test performed by the DESeq2 package. p-values are adjusted for multiple comparison. **(B-D)** Western blot validation of ribosome profiling results. Among the tested candidates, only CCND3 shows decreased protein levels in one eIF2A^{KO} clone. Representative blot in (B), of triplicates quantified in (C). mRNA levels of the corresponding transcripts are quantified and shown in (D). Significance by Dunnett's multiple comparison test ANOVA. error bar = st. dev., ns = not significant, * p < 0.05, ** p < 0.01 **(E)** Luciferase reporters harboring 5' UTRs of eIF2A-dependent transcripts do not show strong changes in expression upon loss of eIF2A. Reporters carrying the 5' UTRs of the indicated candidate genes were cloned upstream of Renilla Luciferase (RLuc) and co-transfected with a Firefly Luciferase (FLuc) normalization control. The negative control RLuc reporter and the FLuc normalization control carry the 5'UTR of Lamin B1 (LMNB1). Significance by Dunnett's multiple comparison test ANOVA. error bar = st. dev., ns = not significant, * p < 0.05, ** p < 0.01, ***<0.001

codon, as detected by flow cytometry (**Fig. 3D**). This differs to what we previously observed upon loss of PRRC2A/B/C proteins, which caused increased expression of the uORF SINFEKL peptide and reduced expression of the downstream main ORF (Bohlen et al., 2023).

No detectable role for eIF2A in translation when eIF2 is inhibited

eIF2A has been proposed to act as a backup pathway for tRNA delivery when eIF2 function is attenuated due to phosphorylation of its alpha subunit by one of the kinases of the integrated stress response (Kim et al., 2018; Kwon et al., 2017; Starck et al., 2016; Tusi et al., 2021). We therefore induced the integrated stress response and tested the impact of loss of eIF2A on several different translational changes that occur – the global reduction in translation levels, the formation of stress granules, and induction of the few target genes such as ATF4 which evade the global reduction in translation and instead are translationally induced (Andreev et al., 2015; Sidrauski et al., 2015). For this we treated cells with tunicamycin or sodium arsenite to phosphorylate eIF2α (eIF2S1) by two independent kinases – PERK or HRI – and estimated global translation by assessing polysome profiles. Both treatments led to suppression of translation (compared **Suppl. Fig. 5A-B** to **Fig. 1E**) however the magnitude of suppression was similar in *eIF2A*^{KO} and isogenic control HeLa cells (**Suppl. Fig. 5A-B**). This suggests that, as in non-stressed conditions, eIF2A has a minimal effect on global translation also when the integrated stress response is active. We next tested if *eIF2A*^{KO} cells have any defect in stress granule formation, which is a hallmark of translation inhibition upon eIF2α phosphorylation (Sidrauski et al., 2015). However, eIF2A^{KO} cells formed stress granules to the same degree as the isogenic control HeLa cells (**Suppl. Fig. 5C-D**). To test induction of target genes during the integrated stress response we selected tunicamycin treatment because sodium arsenite is too strong and completely shuts off all translation. We cloned luciferase reporters carrying 5'UTRs of genes that were previously shown to be translationally upregulated upon eIF2α phosphorylation (Andreev et al., 2015). We then transfected these into control or *eIF2A*^{KO} HeLa cells and treated the cells with either DMSO or 1 µg/ml tunicamycin for 16 hours. Tunicamycin treatment resulted in the same level of eIF2α phosphorylation in either cell line (**Suppl. Fig. 5E**). None of the reporters we tested – *ATF4*, *PPP1R15B*, *IFRD1* – had an induction defect in *eIF2A*^{KO} cells (**Fig. 4A**). This is in line with a previous report showing that in yeast, the ATF4 homolog GCN4 is induced properly during amino acid starvation in cells lacking eIF2A (Zoll et al., 2002).

The assays described above mainly assess bulk translation, and might have missed changes in translation of individual mRNAs. To test whether eIF2A affects translation of any mRNA when eIF2 is inactive, we performed ribosome profiling on control and eIF2A^{KO} HeLa cells treated with tunicamycin and compared these data to the data from non-stressed controls described above. The results showed good reproducibility across duplicates for both ribosome profiling and total RNA samples (**Suppl. Fig. 6A**). Surprisingly, by comparing translation efficiency for all mRNAs in *eIF2A*^{KO} versus control cells in the tunicamycin-treated condition, we did not find any transcript with significantly changed translation apart from eIF2A itself (**Suppl. Fig. 6B**). In line with this, we also did not find any significant changes in a metagene profile of footprints on all main ORFs in control and *eIF2A*^{KO} cells upon tunicamycin treatment (**Suppl. Fig. 6 C-D**).

Nonetheless, it is possible that the induction of particular transcripts in response to stress may be affected in eIF2A-knock-out cells compared to controls. To test it, we compared changes in translation efficiency between untreated and treated cells in both control and *eIF2A*^{KO} cells. This identified a small group of transcripts whose translation was more strongly induced in control cells than in *eIF2A*^{KO} cells (**Fig. 4B**). Statistical analysis identified 12 transcripts that were significantly induced in control cells (log2 fold change of TE (TM/untreated) >= 1, p-adj<0.05), but with blunted induction in *eIF2A*^{KO} cells (yellow dots, **Fig.4B**). Translational induction of genes in response to eIF2α phosphorylation is thought to occur mainly via their 5'UTRs. Therefore, to study systematically the induction of these 12 transcripts, we cloned their 5'-UTRs into luciferase reporters. We succeeded in cloning the 5'UTRs of 11 out of the 12 transcripts. Transfection of these

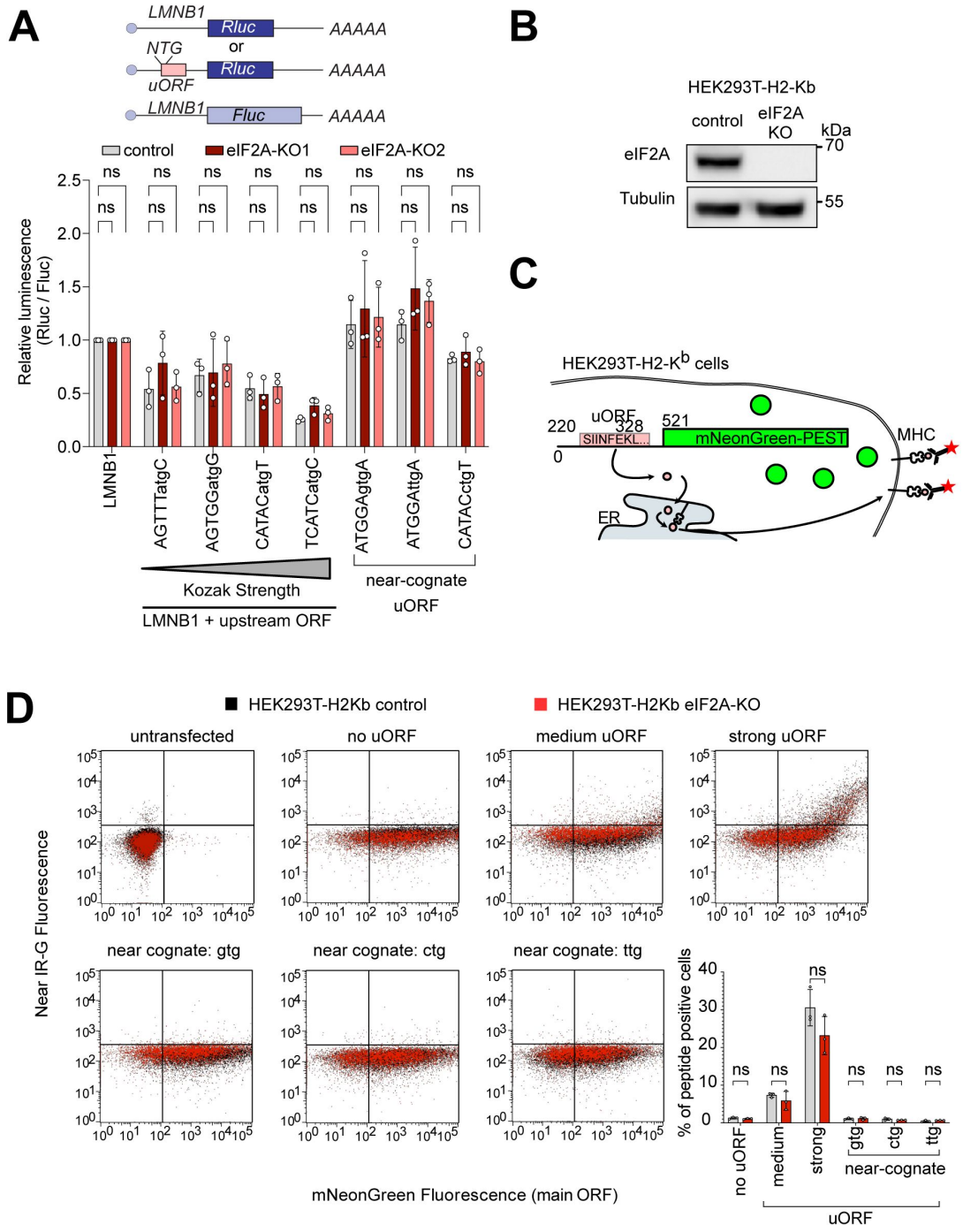


Figure 3

eIF2A has little or effect on uORF translation.

(**A**) Synthetic reporters harboring uORFs with different start codons and initiation contexts do not show dependence on eIF2A. The sequence context of the uORF start codons is indicated: either AUG or a near-cognate start codon (GTG, TTG, CTG) was used. Significance by Dunnett's multiple comparison test ANOVA, error bar = st. dev. ns = not significant. (**B**) Validation that HEK293T-H2-K^{b} *eIF2A*^{KO} cells have no eIF2A protein by immunoblotting. (**C**) Schematic diagram illustrating the setup to simultaneously detect a small peptide produced by a uORF and fluorescent mNeonGreen encoded by the main ORF. The short peptide SIINFEKL is presented on the cell surface by MHC-I and detected using a monoclonal antibody. (**D**) *eIF2A* knockout does not cause a drop in uORF translation. In the graph to the right, the percent of uORF-positive cells relative to all mNeonGreen cells is quantified. Significance by unpaired, two-sided, t-test. ns = not significant.

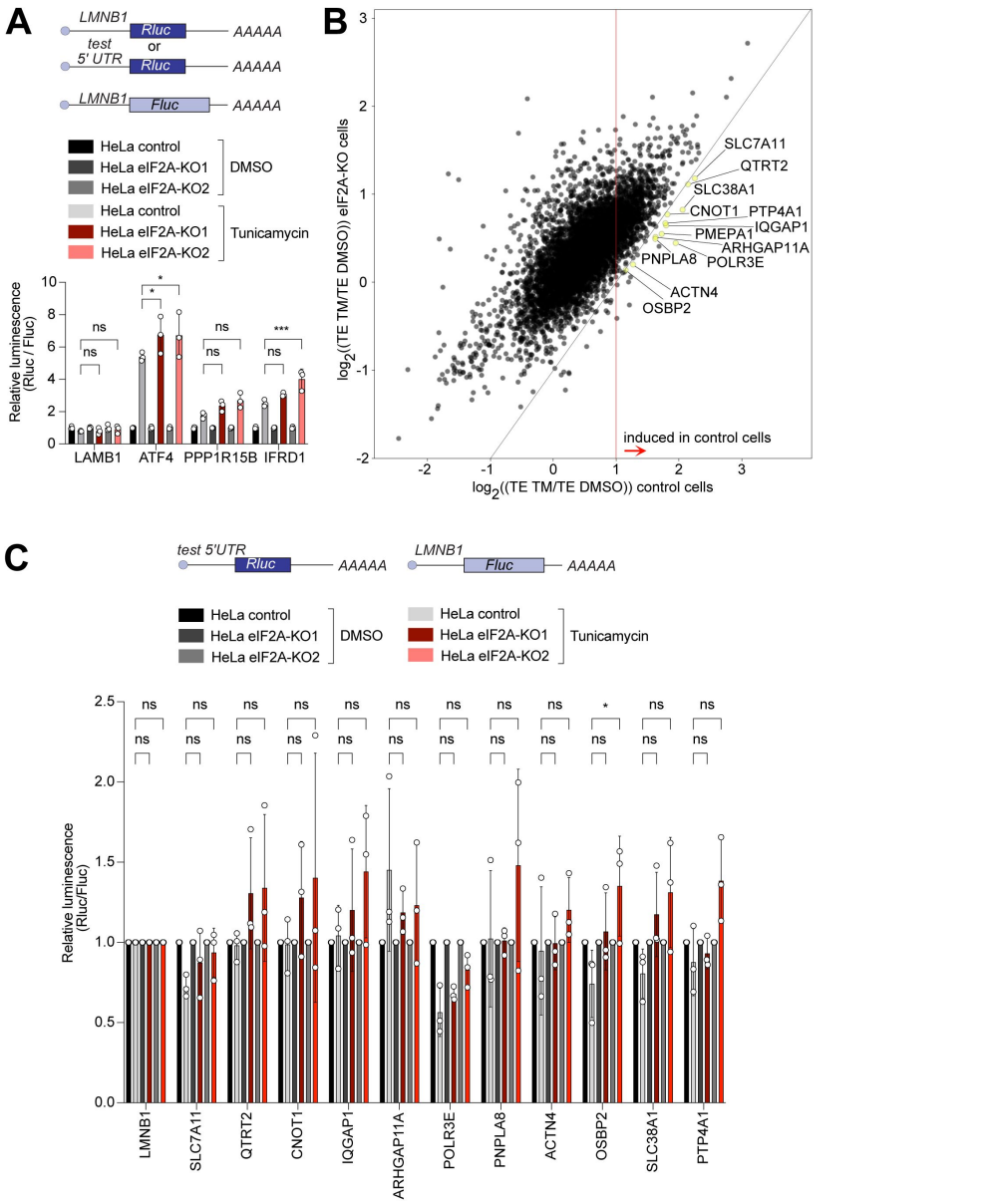


Figure 4

eIF2A has a minor impact on translation during the integrated stress response.

(**A**) Loss of eIF2A does not blunt induction of target genes of the integrated stress response. Reporters carrying the 5′ UTRs of the indicated candidate genes were co-transfected with a Firefly Luciferase (FLuc) normalization control reporter into eIF2A^{KO} and control HeLa cells and treated for 16 hours either with DMSO or 1µg/ml tunicamycin (TM). Significance by Dunnett's multiple comparison test ANOVA. error bar = st. dev., ns = not significant, * $p < 0.05$, ***<0.001 (**B**) Ribosome profiling identifies 12 mRNAs that are significantly induced upon tunicamycin treatment (1 ug/ml) in control cells but not eIF2A^{KO} cells. Scatter plot of log2(fold change) of Translation Efficiency TM/DMSO for control cells on the x-axis versus eIF2A^{KO} cells on the y-axis. mRNAs that are statistically significantly induced with log2(fold change)>1 in control cells but not in eIF2A^{KO} cells are shown in yellow and marked by gene name. Significance was estimated with the Wald test performed by DESeq2 package. p-values are adjusted for multiple comparison. (**C**) Transfection of luciferase reporters harboring 5′ UTRs of eIF2A-dependent transcripts do not show impaired induction in eIF2A^{KO} cells upon tunicamycin treatment. 5′ UTRs of eIF2A-dependent transcripts from panel B were cloned upstream of Renilla luciferase and co-transfected with a Firefly Luciferase (FLuc) normalization control reporter into control or EIF2A^{KO} HeLa cells with subsequent treatment for 16 hours either with DMSO or 1ug/ml tunicamycin (TM). Significance by Dunnett's multiple comparison test ANOVA. error bar = st. dev., ns = not significant, * $p < 0.05$.

reporters and co-treatment with tunicamycin, however, did not show any impaired induction in *eIF2A*^{KO} cells compared to control cells (**Fig. 4C**). This suggesting that either the induction in translation of these transcripts relies on features outside of their 5'-UTRs, or these 12 transcripts are false-positives from the ribosome footprinting.

Finally, we assessed if there is any impact of eIF2A loss on translation of uORF-containing transcripts upon tunicamycin treatment. First, we looked at the metagene profiles for footprints on all uORFs aligned to their start or stop codons, however this did not show any significant changes, apart from a minor faster transition from initiation to elongation in eIF2A^{KO} cells (**Suppl. Fig. 7 A-B**), which is the opposite of what one would expect. Next, we analyzed how the translation efficiency of transcripts containing either AUG- or near-cognate-initiated uORFs changes upon loss of eIF2A, compared to all transcripts, however we did not find any significant changes in either case (**Suppl. Fig. 7C**). Lastly, we tested the synthetic reporters containing uORFs with different start codons or Kozak sequences, described above, in the presence of tunicamycin, but found no differences in translation in eIF2A^{KO} cells compared to controls (**Suppl. Fig. 7B**). In sum, overall, we find a very minor, or no contribution of eIF2A on translation upon stress in HeLa cells.

Discussion

Although eIF2A was discovered prior to eIF2α, its role in translation still remains unclear, with a broad range of different functions attributed to it (reviewed in (Komar and Merrick, 2020)). In this study, we aimed to systematically assess the impact of eIF2A on translation regulation in HeLa cells. Our data show that more eIF2A is localized to the cytosol than the nucleus. Despite a previous report that eIF2A shuttles out of the nucleus in response to cellular stresses (Kim et al., 2011), we observed no change in its distribution between these compartments upon stress, consistent with previous findings in HAP1 cells (Gonzalez-Almela et al., 2018; Sanz et al., 2017). Recently, eIF2A was reported to act as a global suppressor of translation, affecting all mRNAs (Grove et al., 2023). This would result in increased global translation in *eIF2A* knock-out cells. However, we did not observe any change in bulk translation, as measured by OPP-incorporation assays, upon loss or increased expression of eIF2A. In line with no impact of eIF2A on global translation, we also did not detect any proliferation defect of eIF2A^{KO} cells, which fits with a dispensable role of eIF2A in other species (Anderson et al., 2021; Golovko et al., 2016; Kim et al., 2018; Komar et al., 2005). This is further supported by our ribosome profiling data, which showed that only a few transcripts, if any, changed in their translation upon loss of eIF2A, while the vast majority of the translatome remained unaffected. Given that eIF2A was reported to modulate translation initiation through elements in the 5'-UTR (e.g. uORFs, repeats etc.) we tested whether cloning the 5'-UTRs of affected transcripts into luciferase reporters would reveal eIF2A dependence, however, this was not the case. This suggests that if certain transcripts are affected by the lack of eIF2A this is not due to their 5' UTRs and may rely on features in their CDS or 3'-UTRs.

The tRNA-binding properties of eIF2A remain a topic of debate. While initial studies reported that eIF2A could bind tRNA^{Met} in a GTP-independent manner (Kim et al., 2018; Zoll et al., 2002), the purification protocols used in these studies were later shown to result in eIF2D contamination, which was subsequently identified as the tRNA^{Met} binding factor, whereas eIF2A showed no such affinity (Dmitriev et al., 2010). Despite the contested tRNA-binding capabilities of eIF2A, several subsequent studies still reported a switch of tRNA delivery from eIF2α to eIF2A upon cellular stresses, when eIF2 activity is attenuated due to phosphorylation by one of the four ISR kinases (Kim et al., 2018; Kwon et al., 2017; Starck et al., 2016; Tusi et al., 2021). Taking this into consideration, we carried out ribosome profiling upon tunicamycin treatment, which results in eIF2α phosphorylation through PERK kinase. Surprisingly, also in this condition we did not find any transcript whose translation is significantly affected when comparing eIF2A^{KO} to control cells. However, we did find some transcripts whose induction was blunted in eIF2A^{KO} cells. Since no

transcript showed reduced translation in eIF2A^{KO} cells compared to control cells in either the control or the tunicamycin condition, these must be transcripts which had mildly, but not significantly increased translation in the non-stressed condition and mildly, but not significantly reduced translation in the tunicamycin condition, leading to a significant difference when comparing the two treatment conditions. To further dissect if this defect in induction arises from elements within the transcript 5'-UTRs, we cloned their 5'UTRs into luciferase reporters, however, we found no significant difference between eIF2A^{KO} and control cells. Given that the more straight-forward analysis comparing translation efficiency in eIF2A^{KO} versus control cells in the tunicamycin condition revealed no transcripts with altered translation, we think the most simple explanation is that also in the stressed condition where eIF2 function is suppressed, eIF2A does not impact translation in HeLa cells.

Several reports linked eIF2A function to uORF translation. Although our ribosome profiling data indicates that there are no obvious defects in translation of transcripts with uORFs, we nonetheless decided to test the impact of eIF2A on synthetic uORF reporters. We used reporters with uORFs initiated by either AUG or by near-cognate start codons, thereby testing the role of eIF2A in leaky scanning, reinitiation, and near-cognate initiation. Our data, however, show that none of the tested reporters was affected by eIF2A loss or overexpression in both non-stressed and stressed conditions, indicating that eIF2A is dispensable for uORF translation in HeLa cell lines. We did not detect increased translation (i.e. footprints) of any other initiation factor that can potentially deliver tRNAs in *eIF2A* knock-outs (**Suppl. Fig. 8**), however we cannot exclude that the functional consequence of eIF2A loss is masked by another protein with redundant function.

Overall our findings are fully in agreement with recent reports showing little or no effect on translation of eIF2A loss in yeast or human HEK293-T cells (Gaikwad et al., 2024; Ichihara et al., 2021). Ichihara and colleagues generated eIF2A knockout HEK293-T cells and compared their translation to parental control cells via ribosome profiling. This revealed only 1 mRNA with reduced translation efficiency in non-stressed conditions and 4 mRNAs with reduced translation efficiency in cells treated with arsenite where eIF2 is inhibited (Ichihara et al., 2021). Thus, the lack of impact of eIF2A on mRNA translation does not appear to be specific to HeLa cells.

Historically, eIF2A was linked to translation because it was purified with other initiation factors and it shows synthetic lethality with eIF4E (Komar et al., 2005). Even though some of the initial tests with eIF2A showed no activity in reconstituted translational extracts (Merrick and Anderson, 1975), subsequent reports attributed different translational functions to eIF2A. Given that eIF2A contains an RNA-binding domain, it is possible that eIF2A plays a role in RNA trafficking or mRNA decay. The synthetic lethality between eIF2A and eIF4E (Komar et al., 2005) is interesting in this regard since eIF4E is also involved in the nuclear export of transcripts with 4E-SE elements. This raises the possibility that eIF2A might contribute to mRNA regulation beyond translation initiation. For instance, the last 50 amino acids of eIF2A are highly similar to PYM (Diem et al., 2007), a protein that binds to the 40S ribosomal subunit and to Y14, an exon-junction complex protein.

In this study we used predominantly HeLa cells, with some tests in HEK293T cells. Thus, we cannot rule out that in other cell types eIF2A might have a function in translation initiation. eIF2A knockout mice have reduced abundance of B-lymphocytes and dendritic cells in the thymic medulla, as well as lipid metabolism defects (Anderson et al., 2021). A recent study reported that mutation of *eIF2A* in Drosophila melanogaster via a MiMic transposon insertion into the second exon of *eIF2A* is lethal for the organism (Lowe and Montell, 2022). This is the first example of a lethal phenotype for eIF2A-loss in an organism, although this result needs to be confirmed with a clean knockout. The same study found that insertion of a different, piggyback transposon into the second intron resulted in viable, but infertile males due to failed sperm individualization, supporting the idea of cell-type specific function of eIF2A (Lowe and Montell, 2022). Whether

these phenotypes in Drosophila are due to a role of eIF2A in translation initiation, however, remains to be investigated. Overall our results support the idea that eIF2A plays a minor, or no role in regulating translation initiation in human HeLa and HEK293T cells.

Materials & methods

Cell lines, culture conditions, and treatments

Cell lines were cultured in DMEM (Gibco 41965039) supplemented with 10% fetal bovine serum (FBS) (Sigma, S0615) and 100 U/ml Penicillin/Streptomycin (Gibco 15140122). Cell splitting was done with a quick PBS wash and treatment with Trypsin-EDTA (Gibco, 25200056). All cell lines were tested negative for mycoplasma and authenticated using SNP typing. For the indicated experiments, cells were treated either with DMSO, or with 100 µM Sodium arsenite (Sigma, S7400-100G), 1µg/ml poly (I:C) (Tocris Bioscience, 4287/10), 1 µg/ml lipopolysaccharides LPC (Sigma, L2630) or 1 µg/ml tunicamycin (PanReac AppliChem, A2242,0005) for the indicated periods of time. For siRNA mediated knock-down, cells were reverse-transfected during seeding with 1.5 µl of 20 µM siRNA mix and 9 µl Lipofectamine RNAiMax reagent (Invitrogene 13778075). 72 hours post transfection, cells were re-seeded in 96-well format to perform luciferase assays. Sequences of siRNAs used in this study are provided in Suppl. Table 1.

Generation of knockout cell lines and targeted CRISPR-Cas9 screen

eIF2A knock-out HeLa and HEK293T-H-Kb cell lines were generated using CRISPR-Cas9 with sgRNA sequences designed using CHOPCHOP software (Labun et al., 2019), and listed in Supp. Table 2. Oligos coding for the sgRNA sequences were cloned into pX459V2.0 (Doench et al., 2016) via the Bbs1 site, then wildtype HeLa or HEK293T-H-Kb cells were transfected with the plasmids using Lipofectamine 2000 in a ratio of 2:1 reagent:DNA (Life Technologies, 11668500). 24 hours post-transfection, transfected cells were selected with medium containing 1.5 µg/ml puromycin (Sigma-Aldrich, P9620). After three days, surviving cells were shifted into normal medium (1x DMEM, 10% fetal bovine serum, 1% Penicillin/Streptomycin) and regrown to confluence. Single clones were selected by serial dilution into 96 well plates. Loss of protein in expanded single clones was tested with anti eIF2A antibodies by immunoblotting and clones were confirmed by genotyping.

Preparation of cell lysates with RIPA buffer

Cells were seeded at a density of 500.000 cells per 6-well. Following a treatment, cells were washed briefly with PBS, and then lysed with 120µl of RIPA buffer supplemented with 20U of Benzonase (Merk Millipore, 70746-3), protease (Sigma, 4693159001) and phosphatase (Sigma, 4906837001) inhibitors. Cells were collected by scraping and the lysate was clarified by centrifugation at 4°C for 10 minutes at 20.000g. The protein concentration was measured by Pierce BCA (Life technologies, 23224, 23228). Samples were balanced to equal protein concentration and mixed with 5x Laemmli buffer (1/5 of the final volume). Samples were incubated at 95°C for 5 minutes and then loaded on an SDS-PAGE gel.

Western blotting

Cell lysates were separated on SDS-PAGE gels, and transferred to a nitrocellulose membrane with 0.4 µm pore size (Amersham, 10600002) by wet transfer. To block unspecific binding, membranes were blocked in 5% skim milk / PBST for 1 hour. Membranes were then probed by overnight incubation in primary antibody solution (5% BSA / PBST) at 4°C. On the following day membranes were washed three times 15’ in PBST and incubated in secondary antibody (1:10000 in 5% skim milk / PBST) for 2 hours at room temperature. To remove unbound secondary antibodies,

membranes were washed three times for 15 minutes in PBST. Finally, chemiluminescence was detected with ECL reagents (Thermo Schientific, 32109) and imaged with a Biorad ChemiDoc imaging system. Antibodies used for immunoblotting are listed in Suppl. Table 3.

Cloning

Firefly (pAT1620) and Renilla luciferase (pAT1618) under control of LMNB1 5'UTR were described previously (Schleich et al., 2017). The 5'UTRs of eIF2A-dependent candidate transcripts, as well as integrated stress responsive 5'UTRs, were PCR amplified from HeLa cDNA with the oligos indicated in Suppl. Table 4. PCR products were gel purified, digested with HindIII and Bsp119I, and subsequently cloned into pAT1618 via the same sites. If the 5' UTR length was below 120 nucleotides, it was directly oligo cloned into pAT1618 via HindIII and Bsp119i. The cloned 5' UTR variants are listed in Suppl. Table 5 with the indicated transcript ID. Reporters with uORFs with different Kozak strengths were generated and described previously (Bohlen et al., 2023). The reporters with uORFs starting with near cognate start codons were generated for this study by substituting the AUG-uORF generated in (Bohlen et al., 2023) with the near-cognate one via oligo-cloning using the Kpn2I and BshT1 sites with oligos listed in Suppl. Table 4. The EMCV-IRES reporter was generated and described previously in (Roiuk et al., 2024). The short 5'-UTR reporter was produced by oligocloning via SacI and Bsp119I sites in pAT1618. All generated constructs were verified by sanger sequencing.

Ribosome profiling

Control or eIF2^{KO} HeLa cells were seeded at 1.2 million cells per 15 cm dish in 20 ml of growth medium two days before harvesting. The following day, cells were treated either with DMSO or 1 µg/ml tunicamycin for 16 hours. After treatment, cells were quickly washed with ice-cold 1xPBS supplemented with 10 mM MgCl2 and 800 µM Cycloheximide. After the wash, all residual solution was removed by gentle taping of the 15cm dish on its side, followed by cell lysis with 150 µl of the following buffer: 0,25 M HEPES pH 7.5, 50 mM MgCl2, 1 M KCl, 5% NP40, 1000 µM Cycloheximide. Cells were scraped into an Eppendorf tube and the lysate was clarified by centrifugation at 15.000xg for 10 minutes at 4°C. The concentration of lysate was estimated using a nanodrop spectrophotometer, measuring RNA content against a water-blanked control. 150 ul of lysate was used for the total RNA preparation with the RNeasy kit (Qiagen, cat. No. 74106). The remaining lysate was used for treatment with RNaseI (100 Unit per 120µg of lysate) on ice for 30 minutes. Following digestion, the lysates were loaded on a 15-65% sucrose gradient, which was prepared in advance with the use of a Biocomp Gradient Master. The lysate was ultracentrifuged for 3 hours at 35000 rpm in a Beckman Ultracentrifuge with a SW40Ti rotor. To collect the 80S fraction the gradient was separated on a Biocomp Gradient Profiler system. The collected 80S fractions were used for RNA extraction with acid-phenol. Briefly, the volume of the sample was adjusted to 700µl with 10mM Tris pH 7.0 and mixed with 750 µl of prewarmed acid phenol. Sample-phenol mix was incubated at 65C with constant shaking at 1400 rpm for 15 minutes, followed by incubation on ice for 5 minutes. The sample was subsequently centrifuged at 20000g for 2 minutes and the supernatant was transferred into a new tube, containing 700µl of acid phenol. After 5 minutes of incubation at room temperature, the sample was spun at 20000g for 2 minutes and the supernatant was transferred into the tube with 600 µl of chloroform. The sample was then mixed by vortexing and centrifuged at 20000g for 2 minutes. The supernatant fraction was transferred into a new tube and mixed with equal volume of isopropanol, 2µl of Glycoblue (Invitrogene AM9516) and 1/10 volume 3M NaAc pH5.2. To precipitate RNA, the sample was incubated overnight at −80C. The following day, the sample was centrifuged at 4C for 30 minutes at 14000 rpm, followed by a 70% Ethanol wash. The RNA-pellet was resuspended in RNase-DNase free water. The integrity of all RNA samples was analysed on a Bioanalyser. To size-select footprints, RNA extracted from the 80S peak was run on a 15% Urea-Polyacrylamide gel and fragments of 25-35 nucleotides were purified from the gel. For this, the gel pieces containing the footprints were broken into small pieces with gel smasher tubes. 0.5 ml of 10 mM Tris pH 7 were added to the smashed gel pieces and the suspension was incubated at 70°C for 10 minutes with shaking. The

mix was briefly centrifuged and the supernatant was used for RNA precipitation by isopropanol. Purified footprints were phosphorylated by means of T4 PNK (NEB) for 1 hours at 37°C in PNK buffer supplemented with 10 mM ATP. After this, the footprints were again precipitated and purified using isopropanol. To estimate the quality of footprints, RNA was run on an Agilent Bioanalyzer small RNA chip, followed by library preparation with the Next-Flex small RNA v.4 kit protocol (Perkin Elmer, NOVA-5132-06), in accordance to manufacture recommendations. Total RNA libraries were prepared using the Illumina TruSeq Stranded library preparation kit. The quality of libraries were checked on a Bioanalyser with the use of a High sensitivity DNA kit (Agilent, 5067-4626). The libraries were sequenced on an Illumina Next-Seq 550 system.

Data Analyses of Ribosome profiling

Reads were trimmed from adaptors and randomized nucleotides derived from use of the Nextflex kit with cutadapt software. By use of bowtie2 the reads aligning to tRNA or rRNA were removed. All remaining reads were mapped to the human transcriptome (Ensemble transcript assembly 94) and genome (hg38) using BBMap software, with multiple mapping allowed. The reads mapping to the coding sequences were quantified with lab-based software written in C (*https://github.com/aureliotteleman/Teleman-Lab*). For each transcript the value of reads per kilobase of coding sequence was estimated and only transcripts with values more or equal of 2.5 were used for the subsequent analysis. Metagene profiles were built with the custom-made software written in C. For the metagene profile of uORF stop codons, only transcripts with a space of more than 50 nucleotides between uORF and main ORF were used. The DESeq2 software package was used to calculate log2 fold-changes and adjusted p values for the difference in translation efficiency (defined as ribosome footprints / total RNA) in control versus eIF2A knockout cells. DESeq2 was run with the design = ~assay + condition + assay:condition.

OPP incorporation assay

A total of 0.5 million control or eIF2A^{KO} HeLa cells were seeded in six-well plates a day prior to treatment. On the next day, cells were labelled by incubation with 20 μM OPP reagent (Jena Bioscience NU-931-05) for 30 min. Negative control sample was pre-treated with 100μg/ml cycloheximide, and the cycloheximide was maintained during OPP labelling. Following labelling, the cells were lysed, the concentration of samples was measured and equalized, and the incorporation of OPP was estimated via western blot using anti-puromycin antibodies.

Dual-luciferase translation reporter assay

Cells were seeded in 96-well plate format at a density of 8000 cells per well. The following day, cells were transfected with Lipofectamine 2000 with 100 ng of Renilla luciferase plasmid and 100 ng of Firefly luciferase plasmid per well. In case of tunicamycin (TM) treatment, 6 hours post transfection, medium was exchanged with fresh medium supplemented either with either DMSO or 1μg/ml TM. After 16 hours the luciferase assay was carried out using the Promega Dual-Luciferase assay system (Promega, E1910) following manufacturer's instructions.

Immunofluorescent detection of overexpressed Flag-eIF2A

15,000 control HeLa cells were seeded in 12-well format on poly-lysine coated 12-mm cover slips. The following day, cells were transfected with plasmid coding for eIF2A-Flag using Lipofectamine 2000 in a ratio 1:2 DNA:reagent (Life Technologies, 11668500). One day later, cells were treated with or without sodium arsenite (100μM, 1h) and then washed with PBS and fixed by incubation for 15 minutes in 4% formaldehyde in PBS. Cells were permeabilized by incubation for 10 minutes in 0.2% Triton-X-100 in PBS and blocked for 1 hour in 0.25% BSA in PBS. Following blocking, cells were incubated overnight at 4C in primary antibodies. On the following day, the cells were washed twice with blocking buffer and incubated with fluorophore-conjugated secondary antibodies for 1

hour. Finally, cells were shortly stained with 5 µg DAPI (Applichem A1001) and mounted on glass slides using Vectashield (Vector Labs H-1000). The distribution of Flag-eIF2A was analysed on a confocal microscope (Leika TCS SP8) using a 63x objective.

Cell proliferation assay

For cell proliferation assays, cells were seeded into 96 well plates at a density of 1000 cells per well in 100 µl of medium, 8 wells per condition. A total of 5 plates were seeded for the sequential sample collection. The proliferation curve was built by harvesting one plate every 24 hours and performing the Cell Titer Glo (Promega, G7572) according to the manufacturer's instructions.

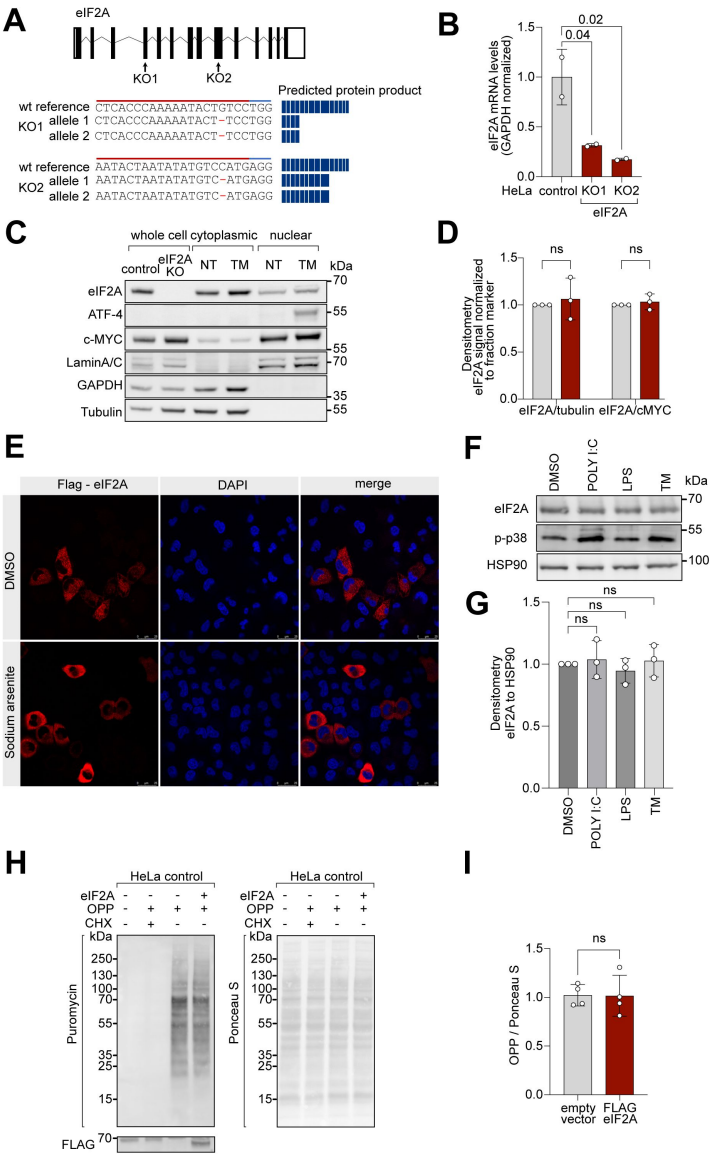
Subcellular fractionation

On the day prior to the experiment, cells were seeded in 10cm dish format, 1.5-2 million cells per dish. The next day, cells were treated for 2 hours either with 1µg/ml Tunicamycin or DMSO, followed by a quick wash with PBS and harvesting by trypsinization. The collected cells were pelleted by gentle centrifugation at 4C (3000g 5 minutes). The supernatant was removed, and the cell pellet was quickly washed twice with PBS. Cells were resuspended with 200µl of 1x Hypotonic Buffer (20mM Tric-HCl, pH7,4; 10mM NaCl, 3mM MgCl) by gently pipetting up and down. The resuspended cells were incubated on ice for 15 minutes, followed by addition of 1/20 of the suspension volume of 10% NP40. Cells with NP40 were vortexed for 10 seconds at highest speed and centrifuged at 3000g for 10 minutes at 4C. The supernatant was moved into a new tube and marked as the cytosolic fraction, while the pellet was washed twice with PBS, moved into a new tube and resuspended in 200 ul of Cell Extraction Buffer (10mM Tris,pH 7.4, 2mM Na_3VO_4, 100mM NaCL, 1% Triton X-100, 1mM EDTA, 10% glycerol, 1mM EGTA, 0.1% SDS. 1mM NaF, 0.5% deoxycholate, 20mM $Na_4P_2O_7$). The pellet was incubated on ice for 30 minutes with occasional vortexing, followed by centrifugation at 14000g at 4C for 30 minutes. The supernatant was collected and marked as the nuclear fraction.

Simultaneous detection of a uORF/oORF peptide and mainORF mNeonGreen The HEK293T-K^B *eIF2A*^{KO} cell line was generated the same way as HeLa-*eIF2A*^{KO} cells. Two days prior to the experiment, cells were seeded in 6 well format. The following day, cells were transfected with empty vector, or with plasmids encoding mNeonGreen-PEST carrying either an oORF-less 5'UTR, or a 5'UTR containing a uORF coding for "SIINFEKL" with either AUG or near-cognate start codons. The AUG codon was placed in a Kozak context predicted to be either 'medium' or 'strong'. 24 hours post-transfection, cells were washed with PBS, collected by trypsinization and resuspended and incubated for 10 minutes in blocking buffer (1% BSA in 1xPBS). After blocking, cells were washed once more with PBS and stained for 30 minutes in the dark at 4C with 0.25 ug/ml monoclonal Antibody OVA257-264, which detects the SIINFEKL-peptide bound to H-2Kb (Life technologies 25-5743-82). Following staining, cells were washed three times with blocking buffer to remove unbound antibodies, resuspended with 300 ul of blocking buffer and analysed with a Guava® easyCyteTM flow cytometer running Guava Soft 3.3

Quantitative RT-PCR

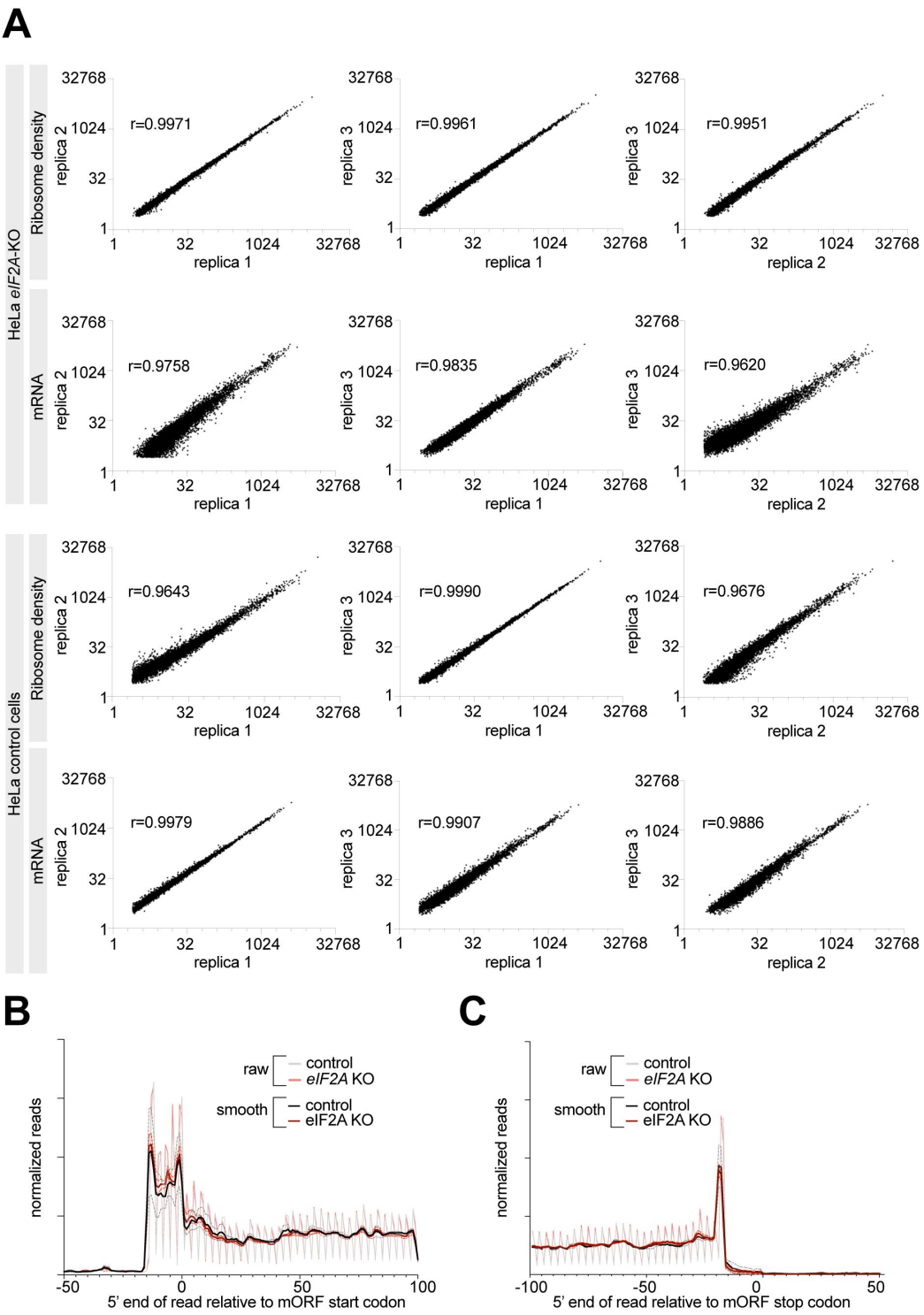
Total RNA from either control of eIF2A^{KO} HeLa cells was extracted using RNase Mini spin columns (Qiagen, cat. no. 74106). Reverse transcription (RT) of 1ug of total RNA with random hexamer and oligo-dT+ primers using Maxima H minus reverse transcriptase was performed to generate cDNA. The amplification efficiency of all Q-RT-PCR primer pairs was checked using serial dilution of a sample. Quantitative RT-PCR was run on a QuantStudio3 instrument with primaQUANT SYBRGreen low ROX master mix. RNA levels were normalized to the levels of either *GAPDH* or *RPL13A* mRNA. Sequences of oligos used for Q-RT-PCR are provided in Supplemental Table 6.



Suppl. Figure 1

Loss of eIF2A does not perturb cell proliferation and global translation.

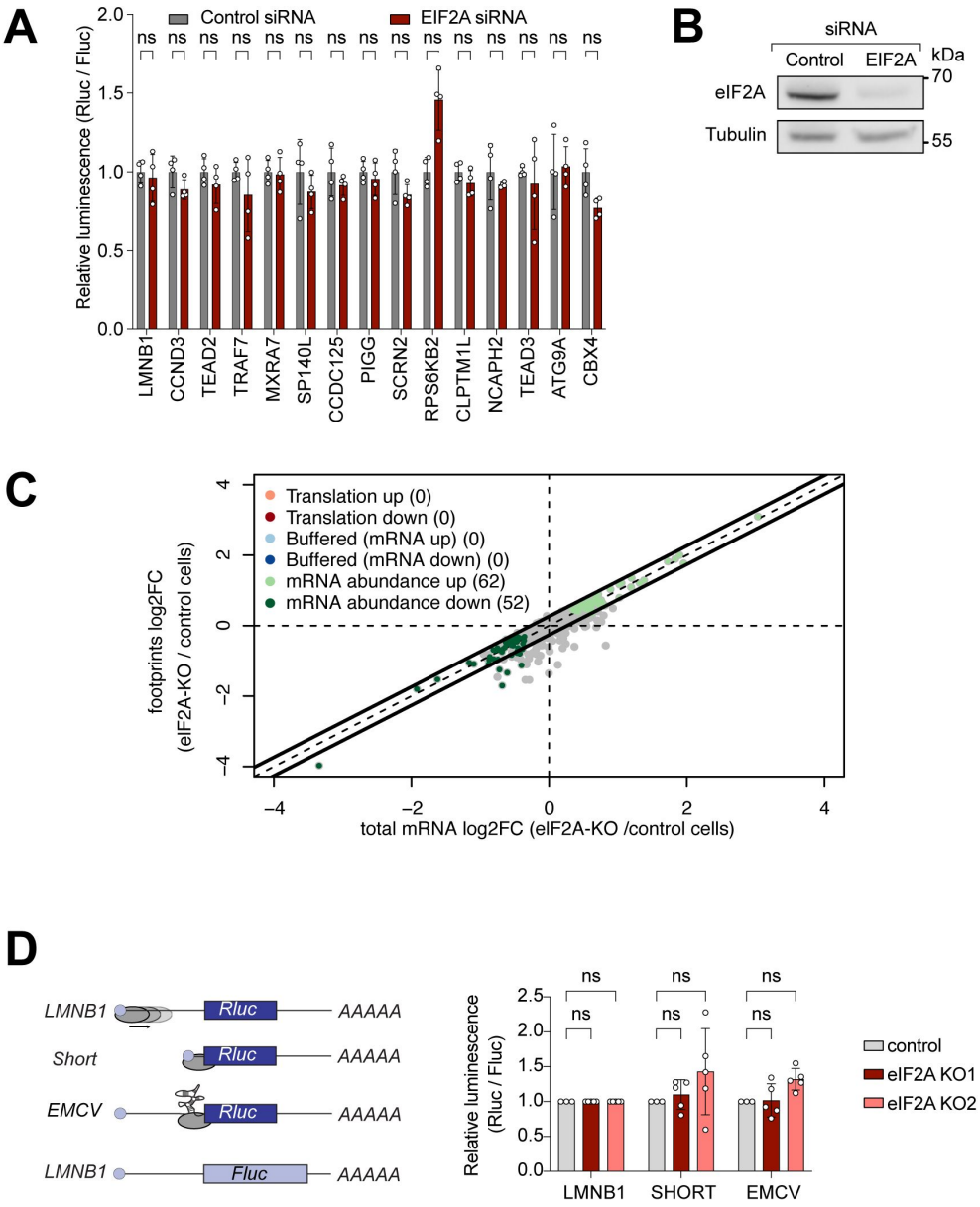
(**A**) Genotyping of *eIF2A*^{KO} cell lines. Two eIF2A knockout lines were generated by targeting two separate coding exons. Shown is the resulting mutation at the DNA level, and a schematic of the predicted protein product. (**B**) mRNA levels of *eIF2A,* quantified by qRT-PCR, are reduced in the eIF2A^{KO} lines. Error bars: standard deviation. Significance by ANOVA with Dunnett's multiple comparison test. **(C-D)** Subcellular localization of eIF2A, detected by immunoblotting cytosolic and nuclear fractions or whole cell lysate (WCL), does not change upon treatment with tunicamycin (TM). Cells were treated with DMSO or Tunicamycin (1 µg/µl) for 2 hours. (C). Representative immunoblot (C) of three independent replicates quantified in (D). Nuclear and cytosolic fractions were lysed in equal volumes to reflect the relative contribution of the two compartments in a cell. Error bars: deviation. Significance by multiple unpaired, t-test. (**E**) Overexpressed FLAG-eIF2A shows predominantly cytoplasmic localization, assessed by immunofluorescent staining with anti-FLAG antibodies. Cells transfected with plasmid expressing eIF2A-FLAG were treated for 1 hour either with DMSO or 100 µM Sodium arsenite (SA). **(F-G)** eIF2A levels in HeLa cells are not affected by poly (I:C) (1µg/ml), LPC (1 µg/ml) or tunicamycin (1µg/ml) for 3 hours. Phospho-p38 signal is used as a positive control for the treatment. Representative immunoblot (F) or three independent replicates quantified in (G). Error bars: standard deviation. Significance by ANOVA with Dunnett's multiple comparison test. **(H-I)** Global protein translation levels in HeLa cells do not change upon Flag-eIF2A overexpression, assessed by immunoblot against OPP (left) and normalized to total protein amount assayed by PonceauS (right). Representative blots in (H), of four independent replicates quantified in (I). Error bars: standard deviation. Significance by unpaired, two-sided, t-test. All panels: ns = not significant.



Suppl. Figure 2

Ribosome profiling of control and eIF2A^{KO} HeLa cells.

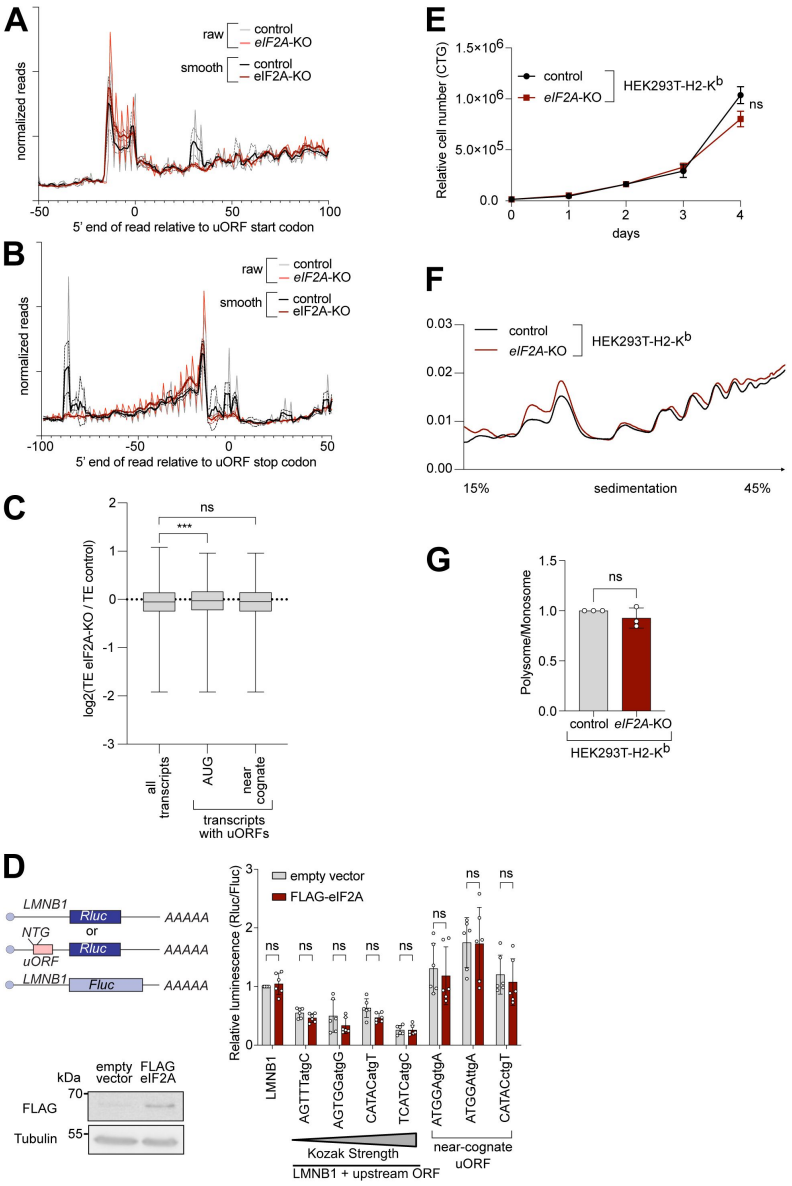
(A) Reproducibility between replicates of Ribosome profiling and total-mRNA libraries is shown. Three biological replicates were generated for control and eIF2A^{KO} HeLa cells each. The Pearson's coefficient (r) is shown for each compared pair. **(B-C)** Metagene profiles of footprints aligned to either the start codon (B) or the stop codon (C) of all main Open Reading Frames, for control and eIF2A^{KO} HeLa cells. "Smooth" curves were generated by averaging read counts with the sliding window of 3 nt. The dotted lines indicated standard deviation between three replicates.



Suppl. Figure 3

Loss of eIF2A does not affect translation of multiple different types of reporters.

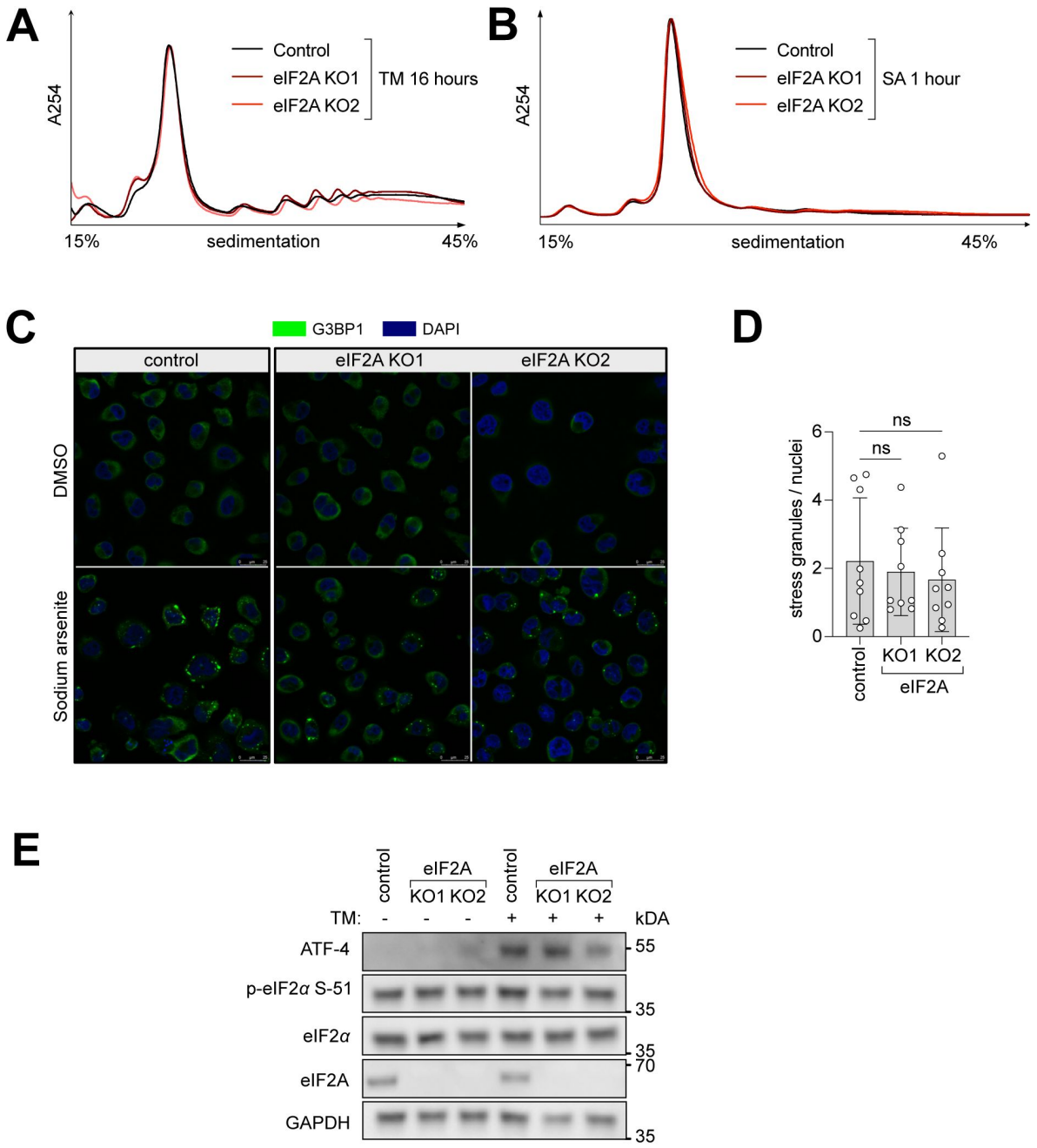
(A) Luciferase reporters harboring 5′ UTRs of transcripts predicted to be eIF2A-dependent from ribosome footprinting, do not show significantly reduced translation upon siRNA mediated knockdown of *eIF2A*. The 5′ UTRs of the indicated genes were cloned upstream of Renilla Luciferase (RLuc) and co-transfected with a Firefly Luciferase (FLuc) normalization control reporter. Negative control RLuc reporter and the FLuc normalization control carry the 5′UTR of lamin B1 (LMNB1). Significance by multiple unpaired t-test, ns = not significant. Error bars represent standard deviation. **(B)** Western blot control for efficiency of siRNA-mediated knockdown of *eIF2A*. **(C)** Transcripts shown in **Fig. 2A** were reanalyzed with anota2seq package (Oertlin et al., 2019). Scatter plot of log2 fold-change of total RNA eIF2A^{KO}/ control (x axis) versus log2 fold-change of footprints eIF2A^{KO}/ control (y axis) is shown. Significant changes are detected in mRNA levels, while loss of eIF2A does not perturb translation. **(D)** Transfection of luciferase reporters designed to place the ribosome directly on top of the initiation AUG do not show reduced translation in eIF2A^{KO} cells compared to controls. A 5′UTR containing the EMCV IRES or a short 5′ UTR of only 12 nt was cloned upstream of Renilla Luciferase (RLuc) and co-transfected with a Firefly Luciferase (FLuc) normalization control. Negative control RLuc reporter and the FLuc normalization control carry the 5′UTR of Lamin B1 (LMNB1). Significance by ANOVA with Dunnett's multiple comparison test. error bar = st. dev., ns = not significant.



Suppl. Figure 4

Knockout of *eIF2A* has no effect on uORF translation.

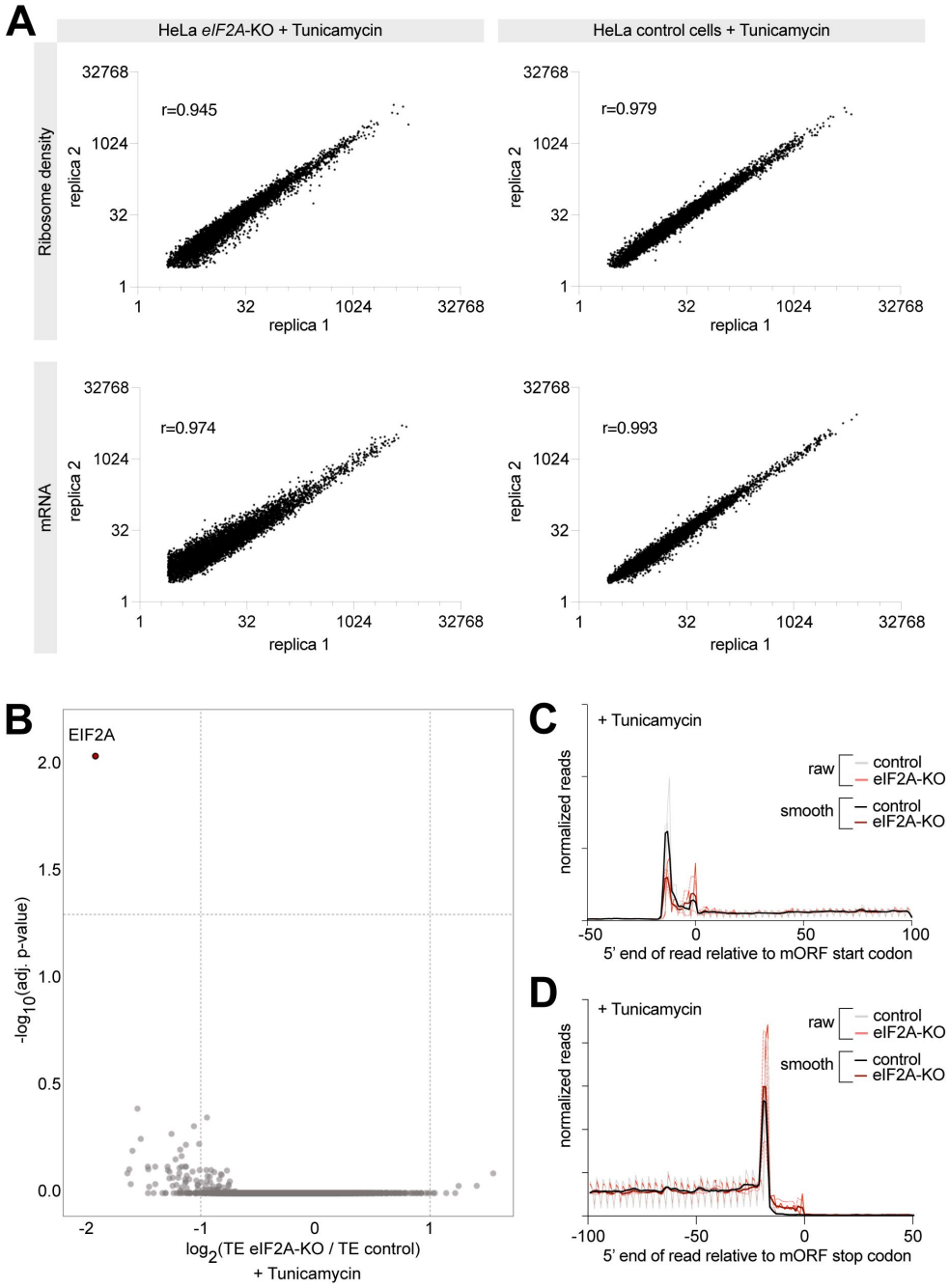
(A-B) Metagene profiles of footprints relative to either the start codon (A) or stop codon (B) of uORFs transcriptome-wide, for control or eIF2A^{KO} HeLa cells. "Smooth" curves were generated by averaging footprint counts with a sliding window of 3nt. The dotted lines indicated standard deviation between three replicates. (**C**) eIF2A has little impact genome wide on translation of mRNAs with uORFs. Comparison of the change in translation efficiency between eIF2A^{KO}and control HeLa cells for three different groups of mRNAs: all transcripts, transcripts with AUG-initiated uORFs, or transcripts with uORFs that start with a near-cognate codon. Significance by ANOVA with Dunnett's multiple comparison test. ns = not significant, *** p<0.001 (**D**) Activity of synthetic reporters harboring uORFs with different start codons and initiation contexts is not altered upon eIF2A-overexpression. The sequence context of the uORF start codons is indicated: either AUG or a near-cognate start codon (GTG, TTG, CTG) was used. Overexpression of eIF2A was validated by western blot against the FLAG-tag. Significance by Dunnett's multiple comparison test ANOVA, error bar = st. dev. ns = not significant. (**E**) Proliferation of eIF2A^{KO} or control HEK293T-H2-K^{b} cells by CellTiter Glo. Error bars: standard deviation. Significance by unpaired, two-sided, t-test. ns = not significant. **(F-G)** Global cellular translation, assayed via polysome profiles, shows little difference between eIF2A^{KO} and control HEK293T-H2-K^{b} cells. Lysates from either eIF2A^{KO} or control HEK293T-H2-K^{b} cells were separated on sucrose gradients. One representative graph is shown in (F). The polysome/80S ratio of three independent replicates is shown in (G). Error bars: standard deviation. Significance by unpaired, two-sided, t-test. ns = not significant.



Suppl. Figure 5

The Integrated Stress Response suppresses translation equally well in control and *eIF2A*^{KO} HeLa cells.

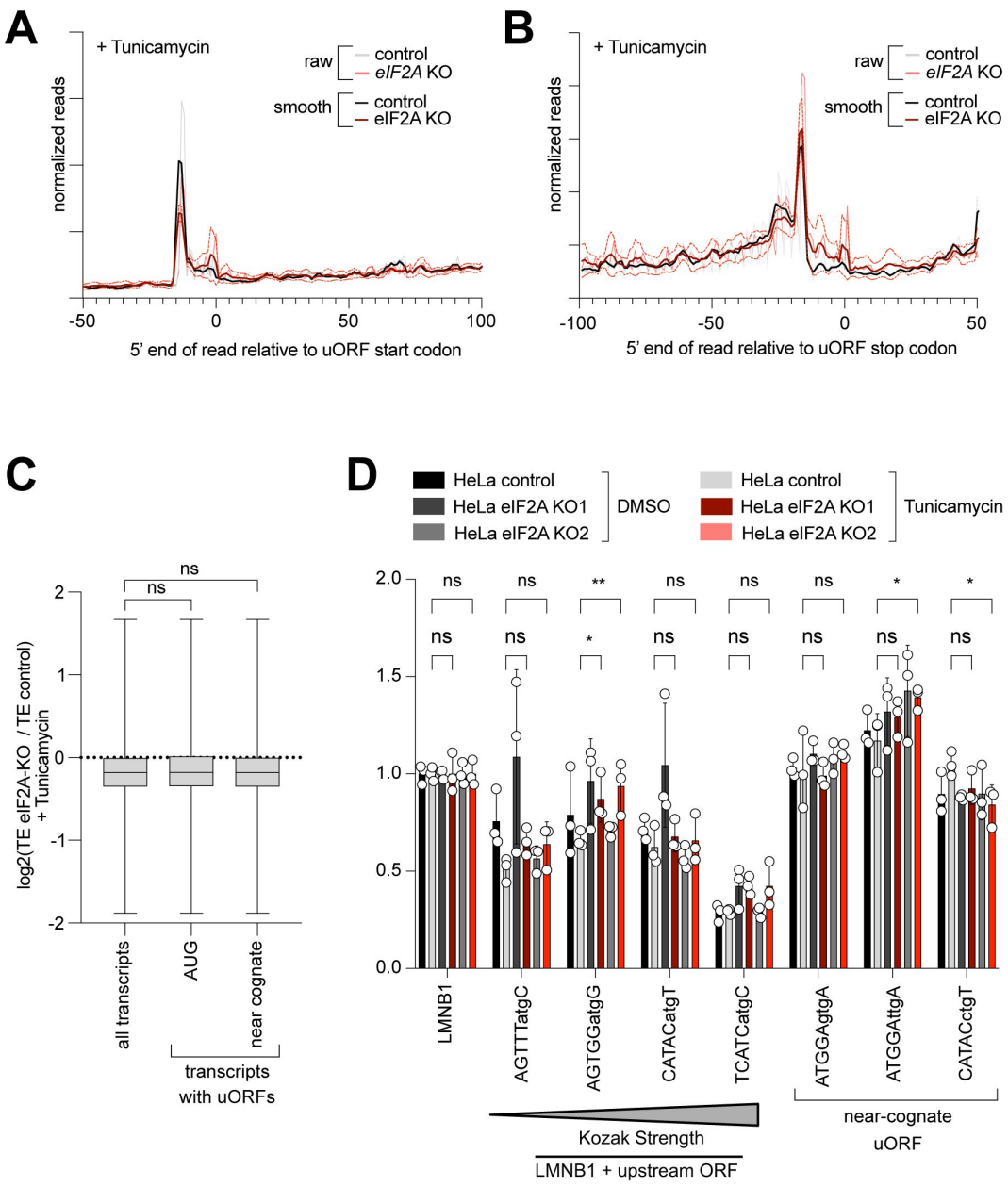
(A-B) Polysome profiles of eIF2A^{KO} versus control HeLa cell lines show hardly any differences upon stress caused either with (A) 1 µg/ml tunicamycin (TM) for 16 hours or (B) 100µM sodium arsenite (SA) for 1 hour. Profiles were aligned by the height of the 80S peak. **(C-D)** eIF2A knockout cells form stress granules to the same degree as the parental control HeLa cell line. Control or eIF2A^{KO} HeLa cells were treated for 2 hours with 100 µM sodium arsenite and stained for G3BP1. (C) Representative images. (D) The number of stress granules, normalised to the number of nuclei in each field of view is shown. Significance by ANOVA with Dunnett's multiple comparison test. ns = not significant. **(E)** *eIF2A*^{KO} and control HeLa cells phosphorylate eIF2α to the same degree in response to tunicamycin (1µg/mL for 16 hours). ATF-4 is used as a marker for downstream activation of the integrated stress response.



Suppl. Figure 6

Ribosome profiling of tunicamycin treated *eIF2A*^{KO} and control HeLa cells.

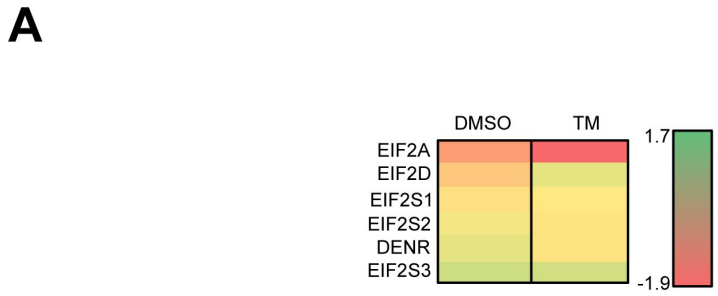
(**A**) Reproducibility between replicates of Ribosome profiling or total-mRNA libraries from eIF2A^{KO} or control HeLa cells treated with tunicamycin. Two biological replicates were generated for each genotype. The Pearson's coefficient (r) is shown for each comparison. (**B**) Ribosome profiling identifies zero transcripts affected by eIF2A knockout in tunicamycin treated condition. Scatter plot of log2(fold change) of Translation Efficiency comparing eIF2A^{KO} to control HeLa cells, both treated with tunicamycin, on the x-axis, versus significance on the y-axis. Significant candidates with log2(fold change) < −1 are shown in red. Significance was estimated with the Wald test performed by DESeq2 package. p-values are adjusted for multiple comparison. **(C-D)** Metagene profiles of footprints aligned to either the start codon (C) or the stop codon (D) of all main Open Reading Frames, for control and eIF2A^{KO} HeLa cells, both treated with tunicamycin. "Smooth" curves were generated by averaging read counts with the sliding window of 3 nt. The dotted lines indicate standard deviation between three replicates.



Suppl. Figure 7

Translation of uORF-bearing transcripts is not affected upon loss of eIF2A in tunicamycin treated cells.

(A-B) Metagene profiles of footprints relative to either the start codon (A) or stop codon (B) of uORFs transcriptome-wide, for control or eIF2A^{KO} HeLa cells, both treated with tunicamycin. "Smooth" curves were generated by averaging footprint counts with a sliding window of 3nt. The dotted lines indicated standard deviation between three replicates. (**C**) eIF2A has little impact genome wide on translation of mRNAs with uORFs in cells treated with tunicamycin. Comparison of the change in translation efficiency between eIF2A^{KO} and control HeLa cells, both treated with tunicamycin, for three different groups of mRNAs: all transcripts, transcripts with AUG-initiated uORFs, or transcripts with uORFs that start with a near-cognate codon. Significance by ANOVA with Dunnett's multiple comparison test. ns = not significant. (**D**) Synthetic reporters harboring uORFs with different start codons and initiation contexts do not show dependence on eIF2A also when the integrated stress response is activated, thereby suppressing eIF2 function. Either control or eIF2A^{KO} HeLa cells were transfected with the indicated reporters and then treated with 1µg/mL tunicamycin for 16 hours prior to assaying luciferase activity. The sequence context of the uORF start codons is indicated: either AUG or a near-cognate start codon (GTG, TTG, CTG) was used. Significance by Dunnett's multiple comparison test ANOVA, error bar = st. dev. ns = not significant. * $p < 0.05$, ** $p < 0.01$.



Suppl. Figure 8

Expression of initiation factors reported to possess $tRNA_i^{Me}$ binding activities.

(A) Heat map showing the log2 fold change of translation efficiency ($eIF2A^{KO}$/control cells) of different initiation factors reported to possess binding capacity to $tRNA_i^{Met}$. Changes in DMSO and Tunicamycin treated samples are shown.

Acknowledgements

We thank the DKFZ Genomics Core Facility for next-generation DNA sequencing.

Additional files

Supplementary Table 1

Supplementary Table 2

Supplementary Table 3

Supplementary Table 4

Supplementary Table 5

Supplementary Table 6

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Reviewer #1 (Public review):

Summary:

Beyond what is stated in the title of this paper, not much needs to be summarized. eIF2A in HeLa cells promotes translation initiation of neither the main ORFs nor short uORFs under any of the conditions tested.

Strengths:

Very comprehensive, in fact, given the huge amount of purely negative data, an admirably comprehensive and well-executed analysis of the factor of interest.

Weaknesses:

The study is limited to the HeLa cell line, which is now addressed and clearly stated by the authors.

https://doi.org/10.7554/eLife.105311.2.sa3

Reviewer #2 (Public review):

Summary

Roiuk et al describe a work in which they have investigated the role of eIF2A in translation initiation in mammals without much success. Thus, the manuscript focuses on negative results. Further, the results, while original, are generally not novel, but confirmatory, since related claims have been made before independently in different systems with Haikwad et al study recently published in eLife being the most relevant.

Despite this, we find this work highly important. This is because of a massive wealth of unreliable information and speculations regarding eIF2A role in translation arising from series of artifacts that began at the moment of eIF2A discovery. This, in combination with its misfortunate naming (eIF2A is often mixed up with alpha subunit of eIF2, eIF2S1) has generated a widespread confusion among researchers who are not experts in eukaryotic translation initiation. Given this, it is not only justifiable but critical to make independent efforts to clear up this confusion and I very much appreciate the authors' efforts in this regard.

Strengths

The experimental investigation described in this manuscript is thorough, appropriate and convincing.

Weaknesses

No major weaknesses as the authors have improved their presentation.

https://doi.org/10.7554/eLife.105311.2.sa2

Reviewer #3 (Public review):

Summary:

This is a valuable study providing solid evidence that the putative non-canonical initiation factor eIF2A has little or no role in the translation of any expressed mRNAs in cultured human (primarily HeLa) cells. Previous studies have implicated eIF2A in GTP-independent recruitment of initiator tRNA to the small (40S) ribosomal subunit, a function analogous to canonical initiation factor eIF2, and in supporting initiation on mRNAs that do not require scanning to select the AUG codon or that contain near-cognate start codons, especially upstream ORFs with non-AUG start codons, and may use the cognate elongator tRNA for initiation. Moreover, the detected functions for eIF2A were limited to, or enhanced by, stress conditions where canonical eIF2 is phosphorylated and inactivated, suggesting that eIF2A provides a back-up function for eIF2 in such stress conditions. CRISPR gene editing was used to construct two different knock-out cell lines that were compared to the parental cell line in a large battery of assays for bulk or gene-specific translation in both unstressed conditions and when cells were treated with inhibitors that induce eIF2 phosphorylation. None of these assays identified any effects of eIF2A KO on translation in unstressed or stressed cells, indicating little or no role for eIF2A as a back-up to eIF2 and in translation initiation at near-cognate start codons, in these cultured cells.

The study is very thorough and generally well executed, examining bulk translation by puromycin labeling and polysome analysis and translational efficiencies of all expressed

mRNAs by ribosome profiling, with extensive utilization of reporters equipped with the 5'UTRs of many different native transcripts to follow up on the limited number of genes whose transcripts showed significant differences in translational efficiencies (TEs) in the profiling experiments. They also looked for differences in translation of uORFs in the profiling data and examined reporters of uORF-containing mRNAs known to be translationally regulated by their uORFs in response to stress, going so far as to monitor peptide production from a uORF itself. The high precision and reproducibility of the replicate measurements instil strong confidence that the myriad of negative results they obtained reflects the lack of eIF2A function in these cells rather than data that would be too noisy to detect small effects on the eIF2A mutations. They also tested and found no evidence for a recent claim that eIF2A localizes to the cytoplasm in stress and exerts a global inhibition of translation. Given the numerous papers that have been published reporting functions of eIF2A in specific and general translational control, this study is important in providing abundant, high-quality data to the contrary, at least in these cultured cells.

Strengths:

The paper employed two CRISPR knock-out cell lines and subjected them to a combination of high-quality ribosome profiling experiments, interrogating both main coding sequences and uORFs throughout the translatome, which was complemented by extensive reporter analysis, and cell imaging in cells both unstressed and subjected to conditions of eIF2 phosphorylation, all in an effort to test previous conclusions about eIF2A functioning as an alternative to eIF2.

Weaknesses:

No major issues were observed as the authors have provided additional evidence of the extent of ISR induction by tunicamycin. The discussion was also expanded to address concerns stemming from the previous version of the manuscript.

[Editors note: Reviewers and editors concluded that the authors revised the article in a satisfactory manner and no further concerns were raised]

https://doi.org/10.7554/eLife.105311.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Beyond what is stated in the title of this paper, not much needs to be summarized. eIF2A in HeLa cells promotes translation initiation of neither the main ORFs nor short uORFs under any of the conditions tested.*
>
> *Strengths:*
>
> *Very comprehensive, in fact, given the huge amount of purely negative data, an admirably comprehensive and well-executed analysis of the factor of interest.*
>
> *Weaknesses:*
>
> *The study is limited to the HeLa cell line, focusing primarily on KO of eIF2A and neglecting the opposite scenario, higher eIF2A expression which could potentially result in an*

> *increase in non-canonical initiation events.*

We thank the reviewer for the positive evaluation. As suggested by the reviewer in the detailed recommendations, we will clarify in the title, abstract and text that our conclusions are limited to HeLa cells. Furthermore, as suggested we will test the effect of eIF2A overexpression on the luciferase reporter constructs, and will upload a revised manuscript.

> ***Reviewer #2 (Public review):***
>
> *Summary*
>
> *Roiuk et al describe a work in which they have investigated the role of eIF2A in translation initiation in mammals without much success. Thus, the manuscript focuses on negative results. Further, the results, while original, are generally not novel, but confirmatory, since related claims have been made before independently in different systems with Haikwad et al study recently published in eLife being the most relevant.*
>
> *Despite this, we find this work highly important. This is because of a massive wealth of unreliable information and speculations regarding eIF2A role in translation arising from series of artifacts that began at the moment of eIF2A discovery. This, in combination with its misfortunate naming (eIF2A is often mixed up with alpha subunit of eIF2, eIF2S1) has generated a widespread confusion among researchers who are not experts in eukaryotic translation initiation. Given this, it is not only justifiable but critical to make independent efforts to clear up this confusion and I very much appreciate the authors' efforts in this regard.*
>
> *Strengths*
>
> *The experimental investigation described in this manuscript is thorough, appropriate and convincing.*
>
> *Weaknesses*
>
> *However, we are not entirely satisfied with the presentation of this work which we think should be improved.*

We thank the reviewer for the positive evaluation. We will revise the manuscript according to the reviewer's suggestions made in the detailed recommendations.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *This is a valuable study providing solid evidence that the putative non-canonical initiation factor eIF2A has little or no role in the translation of any expressed mRNAs in cultured human (primarily HeLa) cells. Previous studies have implicated eIF2A in GTP-independent recruitment of initiator tRNA to the small (40S) ribosomal subunit, a function analogous to canonical initiation factor eIF2, and in supporting initiation on mRNAs that do not require scanning to select the AUG codon or that contain near-cognate start codons, especially upstream ORFs with non-AUG start codons, and may use the cognate elongator tRNA for initiation. Moreover, the detected functions for eIF2A were limited to, or enhanced by, stress conditions where canonical eIF2 is phosphorylated and inactivated, suggesting that eIF2A provides a back-up function for eIF2 in such stress conditions. CRISPR gene editing was used to construct two different knockout cell lines that were compared to the parental cell line in a large battery of assays for bulk or gene-specific translation in both unstressed conditions and when cells were treated with inhibitors that induce eIF2 phosphorylation. None of these assays*

identified any effects of eIF2A KO on translation in unstressed or stressed cells, indicating little or no role for eIF2A as a back-up to eIF2 and in translation initiation at near-cognate start codons, in these cultured cells.

The study is very thorough and generally well executed, examining bulk translation by puromycin labeling and polysome analysis and translational efficiencies of all expressed mRNAs by ribosome profiling, with extensive utilization of reporters equipped with the 5'UTRs of many different native transcripts to follow up on the limited number of genes whose transcripts showed significant differences in translational efficiencies (TEs) in the profiling experiments. They also looked for differences in translation of uORFs in the profiling data and examined reporters of uORF-containing mRNAs known to be translationally regulated by their uORFs in response to stress, going so far as to monitor peptide production from a uORF itself. The high precision and reproducibility of the replicate measurements instil strong confidence that the myriad of negative results they obtained reflects the lack of eIF2A function in these cells rather than data that would be too noisy to detect small effects on the eIF2A mutations. They also tested and found no evidence for a recent claim that eIF2A localizes to the cytoplasm in stress and exerts a global inhibition of translation. Given the numerous papers that have been published reporting functions of eIF2A in specific and general translational control, this study is important in providing abundant, high-quality data to the contrary, at least in these cultured cells.

Strengths:

The paper employed two CRISPR knock-out cell lines and subjected them to a combination of high-quality ribosome profiling experiments, interrogating both main coding sequences and uORFs throughout the translatome, which was complemented by extensive reporter analysis, and cell imaging in cells both unstressed and subjected to conditions of eIF2 phosphorylation, all in an effort to test previous conclusions about eIF2A functioning as an alternative to eIF2.

Weaknesses:

There is some question about whether their induction of eIF2 phosphorylation using tunicamycin was extensive enough to state forcefully that eIF2A has little or no role in the translatome when eIF2 function is strongly impaired. Also, similar conclusions regarding the minimal role of eIF2A were reached previously for a different human cell line from a study that also enlisted ribosome profiling under conditions of extensive eIF2 phosphorylation; although that study lacked the extensive use of reporters to confirm or refute the identification by ribosome profiling of a small group of mRNAs regulated by eIF2A during stress.

We thank the reviewer for the positive evaluation. We will revise the manuscript according to the recommendations made in the detailed recommendations. Regarding the two points mentioned here:

(1) The reason eIF2alpha phosphorylation does not increase appreciably is because unfortunately the antibody is very poor. The fact that the Integrated Stress Response (ISR) is induced by our treatment can be seen, for instance, by the fact that ATF4 protein levels increase strongly (in the very same samples where eIF2alpha phosphorylation does not increase much, in Suppl. Fig. 5E). We will strengthen the conclusion that the ISR is indeed activated with additional experiments/data as suggested by the reviewer.

(2) We agree that our results are in line with results from the previous study mentioned by the reviewer, so we will revise the manuscript to mention this other study more extensively in the discussion.

> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations for the authors):***
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> *(1) I suggest to state (already in the abstract, but perhaps also even in the title, definitely in the rest of the paper) that this analysis is limited to the HeLa cell line.*

As suggested, we have now specified in both the title and the abstract that the work is done in HeLa cells.

> *(2) In my view, it is a pity that the authors - given the tools are available - did not check the impact of high eIF2A levels on expression of individual mRNAs under normal and stress conditions. I am not suggesting to repeat ribo-seq in this setup, it would be too much to ask for, but re-examining some of the many reporters the authors generated with eIF2A overexpressed may point to some function, e.g. increased number of non-canonical initiation events (non-AUG-initiated)? If anything, the use of HeLa and the primary focus on eIF2A KO neglecting the prospective impact of eIF2A overexpression should be mentioned as two main limitations of this study.*

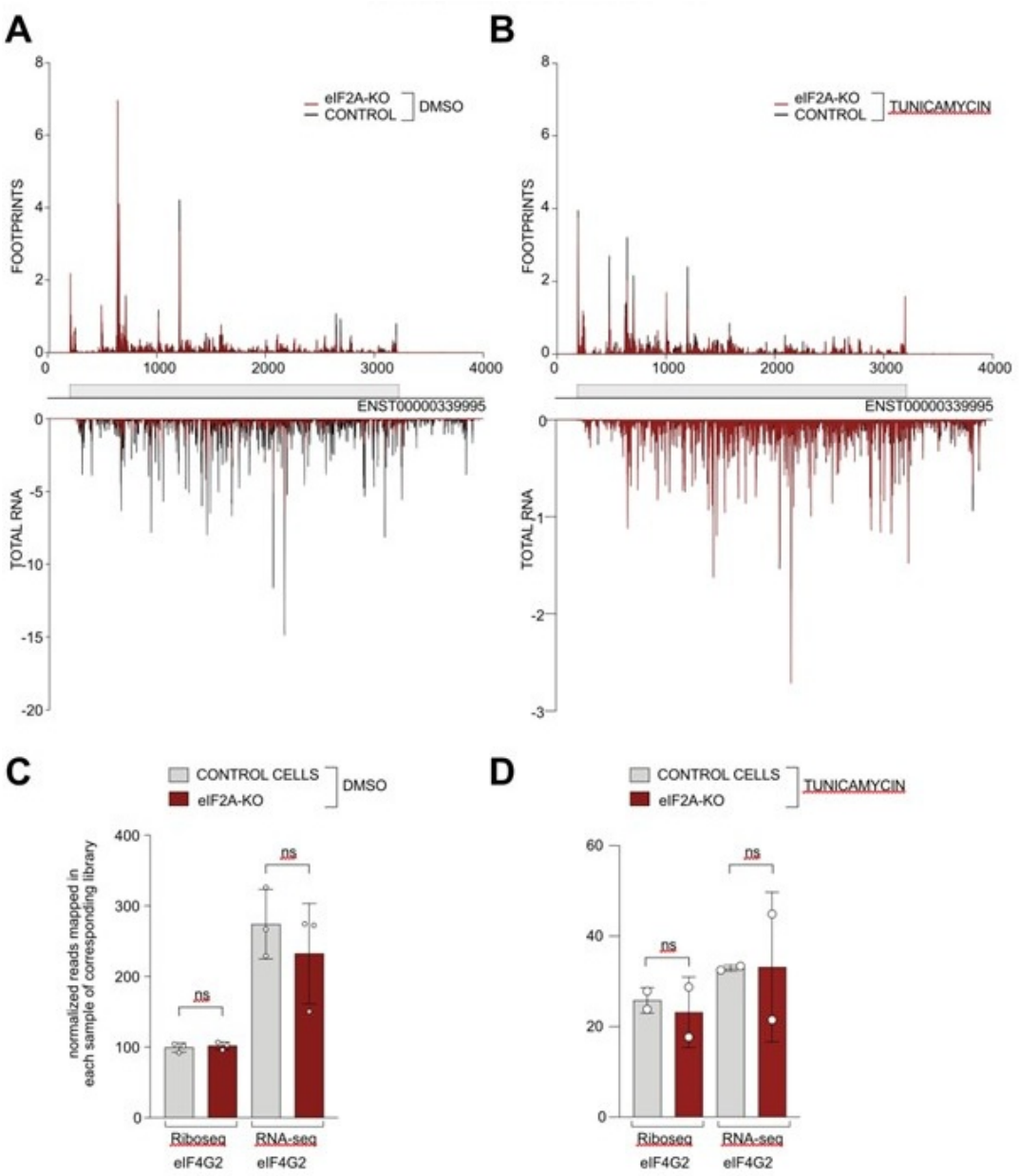
We thank the reviewer for the good suggestion to test our synthetic reporters with eIF2A overexpression. New Suppl. Fig. 4G now shows that overexpression of eIF2A does not affect translation of synthetic reporters carrying an ATG start codon in different initiation contexts, or carrying near-cognate start codons, in agreement with a lack of effect on translation which we previously observed with loss of eIF2A.

> *(3) Ribo-seq with eIF2A. Did the authors focus on ORFs that are known, or whose isoforms are known, to be non-AUG initiated? Would the loss of eIF2A decrease FPs in their CDSes under at least some conditions?*

We have now assessed the read distribution on the eIF4G2 transcript in both the control and tunicamycin conditions (Author response image 1). In our hands, eIF4G2 is one of the best examples of non-AUG initiation in human cells, since the main coding sequence starts with GTG and the CDS is well translated. Nonetheless, we do not observe any significant changes in read distribution (panels A-B) or overall translation efficiency of eIF4G2 upon eIF2A loss (panels C-D).

Author response image 1.

(A-B) Average reads occupancy on the eIF4G2 (ENST0000339995) transcript in DMSO treated (panel A, n=3) or tunicamycin treated samples (panel B, n=2) derived from either control (black) or eIF2A-KO (red) HeLa cells. Reads counts were normalized to sequencing depth and averaged between either 3 (DMSO-treated) or 2 (tunicamycin-treated) replicates. Graphs were then smoothened with a sliding window of 3 nt. (C-D) The total number of reads mapping to the eIF4G2 CDS, normalized to library sequencing depth per replica was quantified. No significant difference between control and eIF2A-KO cells was observed in either DMSO treated (panel C) or tunicamycin treated (panel D) cells. Significance by unpaired, two-sided, t-test. ns = not significant.



Thank you for giving me the opportunity to review this article.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *While some of our suggestions below may be considered subtle, in our opinion they are important and it would be good if the authors consider them for their revision, we also have a couple of technical suggestions.*
>
> *(1) Abstract.*
>
> *The authors failed to identify the role of eIF2A in translation initiation and have provided compelling evidence that eIF2A is not involved in recognition of non-AUG codons as start codons nor in recruitment of initiator tRNA during stress conditions which are two activities most commonly misattributed to eIF2A. However, they have not exhausted all possible potential functions of eIF2A, see below, it is also possible that eIF2A may have a role not yet suggested by anyone and it may function in translation initiation in special*

> *circumstances that have not been tested yet. The authors indeed discuss such possibility in the Discussion section. Given that there is genetic evidence (that is unaffected by biochemical impurities) linking eIF2A to other initiation factors (5B and 4E), we are not yet convinced that eIF2A does not have any role in translation initiation and therefore we find the last sentence of the abstract premature. We suggest to soften this statement into something like this: whether eIF2A has any role in translation remains unknown, it may even have a role in a different aspect of RNA Biology.*

We agree with the reviewer. We changed the last sentence of the abstract to read as follows:

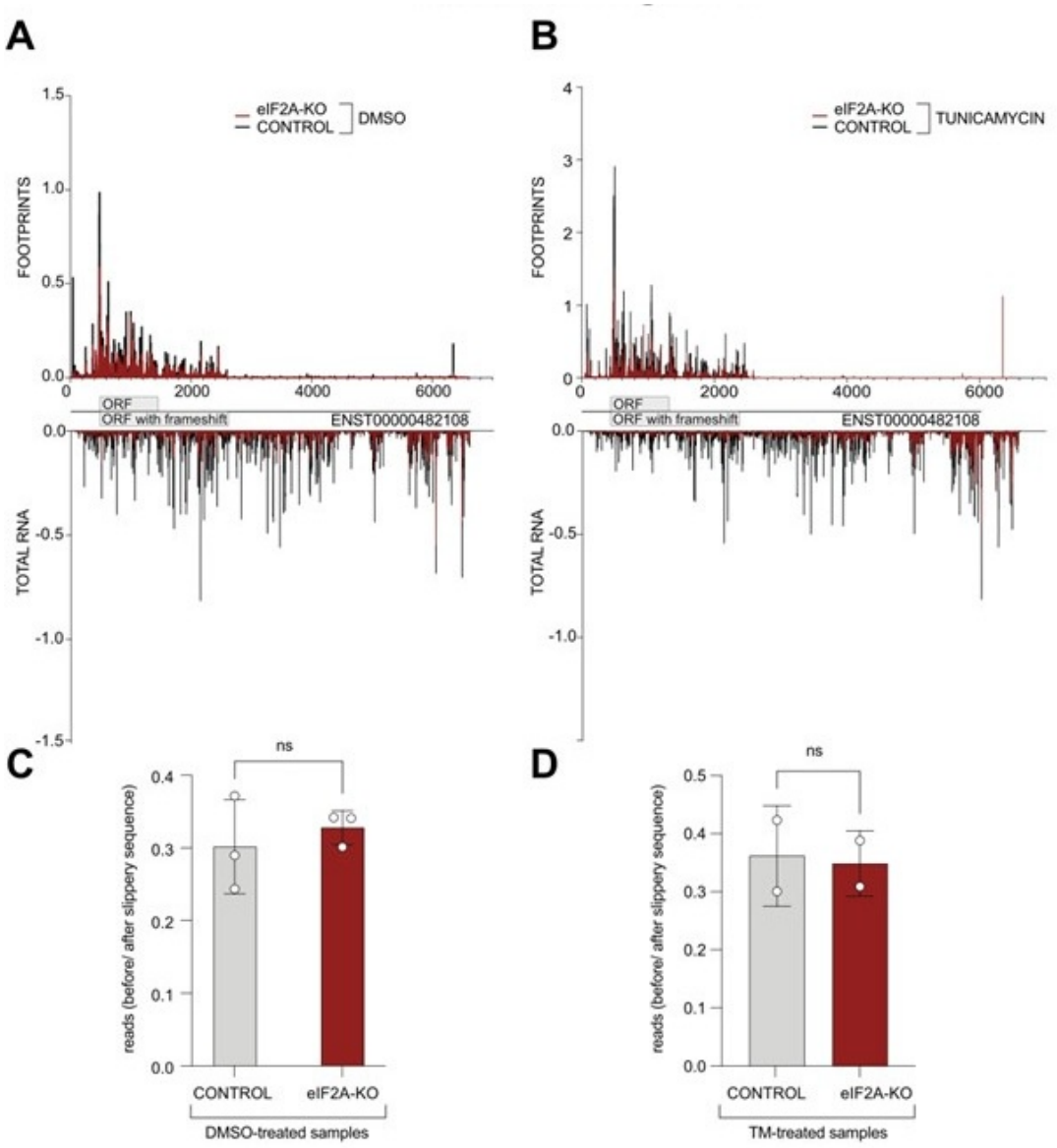
"It is possible that eIF2A plays a role in translation regulation in specific conditions that we have not tested here, or that it plays a role in a different aspect of RNA biology."

> *(2) Recently eIF2A has been implicated in ribosomal frameshifting, see Wei et al 2023 DOI: 10.1016/j.celrep.2023.112987*
>
> *Could authors look into PEG10 mRNA ribosome profile to see if there are detectable statistically significant changes in footprint density downstream of frameshift site between WT and eIF2A Kos? It is likely that the coverage will be insufficient to give a definitive answer, but it is worth checking, it would be a pity to miss it.*

We thank the reviewer for this suggestion. We have now looked at the distribution of ribosome footprints on the PEG10 transcript variant that is expressed in HeLa cells (ENST00000482108) and indeed observe coverage downstream of the annotated stop codon, consistent with a frameshifting event that results in an extended protein isoform being translated. Visual assessment of the read distribution between the main ORF and the "ORF extension" does not show a substantial difference between control and eIF2A knock-out cells (Author response image 2A-B). Additionally, we quantified the ratio of reads mapping to the PEG10 ORF upstream of the slippery site versus those mapping downstream, extending into the predicted longer protein. Nonetheless, we could not detect significant changes between control and eIF2A-KO cells in either tested condition (Author response image 2C-D).

Author response image 2.

(A-B) Average reads occupancy on the PEG10 (ENST00000482108) transcript in DMSO treated (panel A, n=3) or tunicamycin treated samples (panel B, n=2) derived from either control (black) or eIF2A-KO (red) HeLa cells are shown. Reads counts were normalized to sequencing depth and averaged between either 3 (DMSO-treated) or 2 (tunicamycin-treated) replicates. Graphs were then smoothened with a sliding window of 3 nt. (C-D) The ratio of reads mapping to the ORF upstream of the slippery site to reads mapping to the predicted extended protein downstream to the slippery site is shown. Reads counts were normalized to the sequencing depth. Neither DMSO treated samples (panel C) nor tunicamycin treated samples (panel D) had a significant difference between control and eIF2A-KO cells. Significance by unpaired, two-sided, t-test. ns = not significant.



(3) Introduction

Given the volume of unreliable claims regarding eIF2A in the literature and the overall confusion it is very difficult (may even be impossible) to write a clear coherent introduction into the topic. Nonetheless, there are few points that need to be taken into account.

The authors state that eIF2A is capable to recruit initiator tRNA citing Zoll et al 2002. This activity was later shown to be a biochemical artefact (which was most likely reproduced by Kim et al 2018), eIF2A fraction was contaminated with eIF2D which does bind tRNAs in GTP-independent manner. eIF2A purified from RRL separates from initiator tRNA binding activity, see Dmitriev et al 2010 DOI: 10.1074/jbc.M110.119693. This point is also relevant to the second paragraph of Discussion, it should be acknowledged that it has been shown previously that eIF2A does not bind the initiator tRNA.

We appreciate the advice provided by the reviewer. We have modified both the introduction and the 2nd paragraph of the discussion to reflect that the tRNA-binding activity is due to

contaminating eIF2D rather than eIF2A.

> *In many cases the authors describe certain claims as facts even though they refute them themselves. For example*
>
> *"Such eIF2A-driven non-AUG initiation events were shown to play a crucial role in different aspects of cell physiology and disease progression: cellular adaptation during the integrated stress response (Chen et al., 2019; Starck et al., 2016)" While non-AUG initiation events do play crucial roles in different aspects of cell physiology (reviewed in Andreev et al 2023 doi: 10.1186/s13059-022-02674-2) eIF2A has nothing to do with it as the authors show themselves. Therefore different language should be used, e.g.. "eIF2A has been suggested (or proposed or reported) to be responsible for non-AUG initiation events that were shown to play ..."*
>
> *The word "shown" is used in many other instances for the claims that the authors refute. "Shown" is only appropriate for strong evidence that leaves little doubt.*

We agree with the reviewer and made the suggested changes in the text.

> *(4) Supplementary Fig. 1.*
>
> *Panel C is used to argue that eIF2A has a higher concentration than in the nucleus, perhaps it is worth explaining how this conclusion was drawn. If levels in cytoplasm are comparable to GAPDH and Tubulin but less than c-Myc in nucleus does it really mean that there is less eIF2A in the nucleus than in cytoplasm? This is not obvious to us. Also, presumably WCL stands for Whole Cell Lysate, it would be nice to introduce this abbreviation somewhere.*

To compare levels of eIF2A in the nuclear and cytosolic fractions, we lysed the two fractions in equal volumes of buffer (i.e. the cytosolic fraction was extracted in 200 µl of hypotonic buffer, and the nuclear fraction was extracted in 200 µl of cell extraction buffer). This assures that per microliter of lysate we have the same number of "cytosols" or nuclei. Hence, equal intensity bands in the cytosolic and nuclear fractions would mean that half of the protein is in the nucleus and half is in the cytosol. We originally described this in the Methods section, but now also mention it in the Results and in the figure legend.

We replaced WCL with "whole cell" in the figure.

> *(5) The differential translation analysis is described very briefly "To obtain values of translation efficiency, log2 fold changes, and adjusted p values the DESeq2 software package was used". Was TE calculated based on ribosome footprint to RNA-seq ratios? How exactly DESeq2 was used here? TE measured in this way spuriously correlates with RNA-seq values, see Larsson et al 2010 DOI: 10.1073/pnas.1006821107, perhaps it would be worse assessing differential translation with anota2seq (Oertlin et al 2019 doi: 10.1093/nar/gkz223.)? Anota2seq avoids calculating the ratios and enables comprehensive analysis of differential translation including detection of buffered translation which might be the case here while avoiding artefacts that may arise from varying RNA levels.*

We now specified in more detail in the Methods section how we analyzed the data. Indeed, the DeSeq2 was used on translation efficiency values, which we calculated as the ratio of ribosome footprints to RNA-seq.

As suggested, we have now also performed the analysis using anota2seq (Suppl. Fig. 3C) and this analysis identified zero transcripts that are translationally regulated, in agreement with our analysis.

> *(6) Section "eIF2a-inactivating stresses do not redirect tRNA delivery function to eIF2A."*
>
> *The description of ISR mechanism is a bit inaccurate. Strictly speaking eIF2alpha phosphorylation does not inactivate it eIF2alpha. It results in formation of a very stable eIF2*GDP*eIF2B complex, thus severely depleting eIF2B which serves as a GEF for eIF2. This in turn reduces the ternary complex (eIF2*GTP*tRNAi) concentration since there is no free eIF2B to exchange GDP for GTP. Without getting into much detail, we think it would be more accurate to say that eIF2alpha phosphorylation leads to ternary complex depletion instead of saying that stress inactivates eIF2alpha.*

We agree with the reviewer - we were trying to use simple, compact wording. We have now reworded the section title to "No detectable role for eIF2A in translation when eIF2 is inhibited" and rephrased the subsequent text to be correct.

> *Also the subtitle uses eIF2a with small a that stands for alpha which potentially could lead to substantial confusion since in this case the difference between eIF2alpha and eIF2A is only in capitalisation of the last letter, many text-mining engines such as modern LLMs may not be able to pick the differences. Perhaps it would be better to refer to eIF2alpha by the HGNC approved name of its gene - eIF2S1 to avoid further confusions. For clarity it may be stated at the beginning that eIF2S1 is commonly known as eIF2alpha.*

We thank the reviewer for this point. We have removed all instances of eIF2a (with lowercase a) from the manuscript to avoid this source of confusion. In the first instance of eIF2a we also added the official HGNC gene name. However, we prefer to use eIF2a instead of eIF2S1 because people outside the translation field tend to know the subunit as eIF2a, and we think it is important that also people outside the translation field read this manuscript, since some of the questionable papers on eIF2A come from labs working at the interface between translation and other fields.

> *Minor*
>
> *Introduction*
>
> *(7) "uses the CAT anticodon" change CAT to CAU*

We corrected CAT to CAU

> *(8) "In the canonical initiation pathway", change "canonical" to "most common", canonical is somewhat a judgemental statement that originates in theology. Same applies to numerous occurrences of "canonical AUG", simply using "AUG" would be simpler and more accurate as you will avoid giving impression that there are "non-canonical AUGs".*

Done.

> *(9) "eIF2A was initially considered to be a functional analogue of prokaryotic IF2 (Merrick and Anderson, 1975), however later this role was reassigned to the above-mentioned heterotrimeric factor eIF2 (a,b,g) (Levin et al., 1973)." - there is a chronological contradiction within this sentence, the initial consideration is attributed to 1975 while its later reassignment to 1973.*

We are grateful to the reviewer for spotting this mistake. There was a citation problem; we fixed it and now cite the correct paper for the initial discovery of eIF2A to PMID 5472357

(Shafritz et al 1970).

> *(10) "On the other hand, studies on the role of eIF2A on viral IRES translation have arrived at conflicting results." Remove "On the other hand" since conflicting results have been mentioned above. In fact the entire sentence is somewhat redundant given prior "For example, eIF2A has been studied in the context of internal ribosome entry sites (IRES), where it was found to act both as a suppressor and an activator of IRESmediated initiation."*

We have rewritten the paragraph to make it more coherent.

> *(11) Fig. 1. C-D. is using CHX abbreviation for cycloheximide, this need to be mentioned on the legend or elsewhere in the text. Otherwise CHX may not be clear for a reader uninitiated in ribosome profiling.*

We now mention in the figure legend that CHX stands for cycloheximide and indicate that it was used as a negative control to block translation.

> *(12) Page 7, section "Ribosome profiling reveals a few eIF2Adependent transcripts"*
>
> *In this section you describe ribosome profiling experiments and identify few transcripts whose translation seems to be changing based on ribosome profiling data. Then you attempt to verify them using gene expression reporters and reasonably suggest that these are false positives. In essence this section argues that there are no eIF2A-dependent transcripts, therefore the title of this subsection is misleading, it makes sense to rename it so that it better reflects the content of this section.*

We agree and have renamed the section to "Ribosome profiling identifies no eIF2Adependent transcripts"

> *(13) Page 8, top. Rephrase "To do this, we performed ribosome profiling on control and eIF2AKO cells, which sequences the mRNA footprints protected by ribosomes."*

Fixed.

> *(14) Page 10, bottom. "Several studies have reported that eIF2A can delivery alternative initiator tRNAs to uORFs with nearcognate start codons". Change "delivery" to "deliver".*

Thanks for spotting it. We corrected to “deliver”

> *(15) Page 13 "This suggests that, as in non-stressed conditions, eIF2A has a minimal effect on global translation also when eIF2a activity is low." - rephrase to avoid impression that eIF2alpha activity is low in normal conditions, also please see comment #6 above.*

We fixed this sentence to read: “This suggests that, as in non-stressed conditions, eIF2A has a minimal effect on global translation also when the integrated stress response is active.”

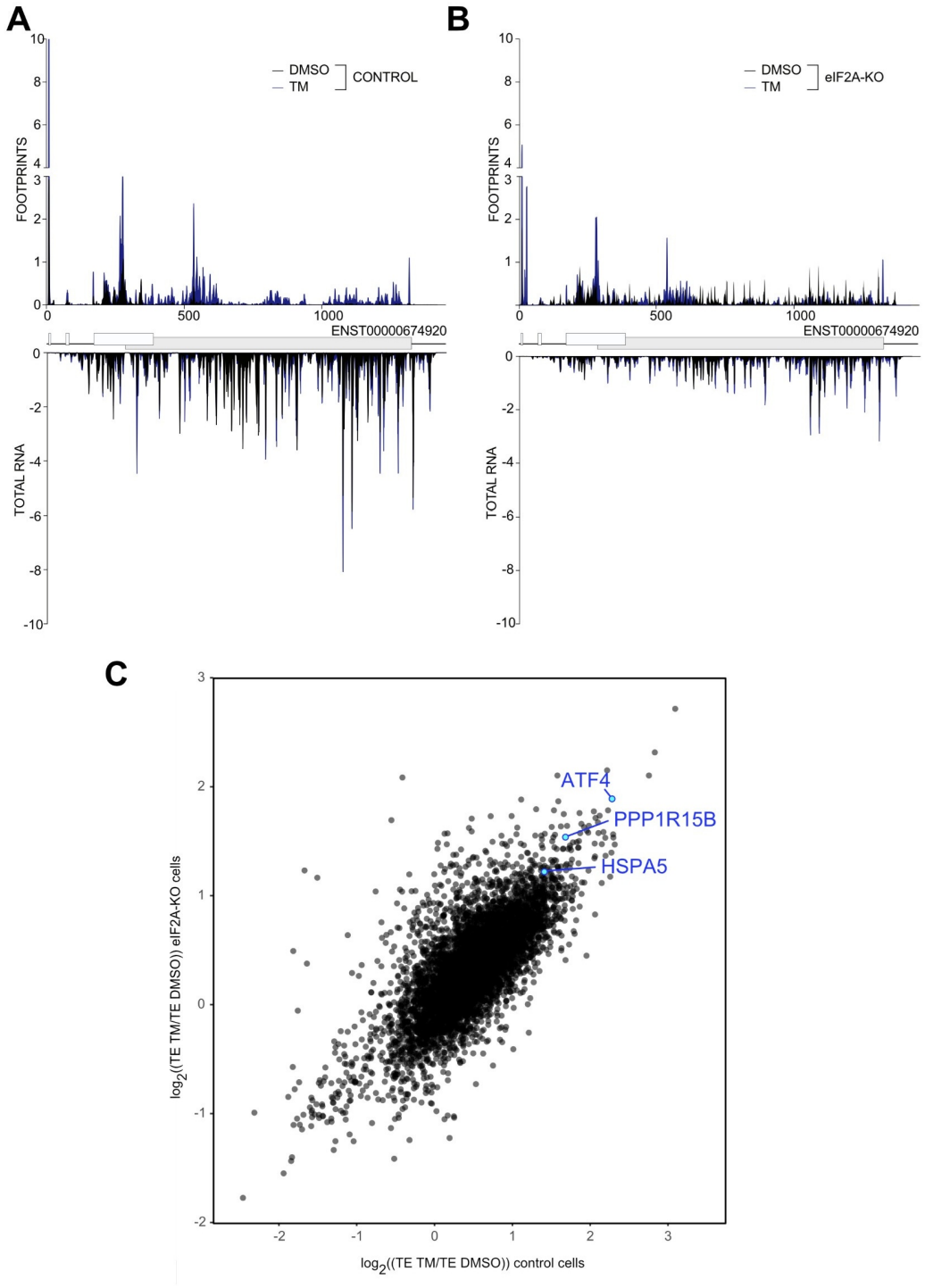
> ***Reviewer #3 (Recommendations for the authors):***
>
> *- The experimental data in Fig. S5E do not support the claim of increased eIF2 phosphorylation on TM treatment; although, comparing Fig. S5A with Fig. 1B supports a marked reduction in bulk translation and the reporter data in Fig. 4A show the expected induction of the uORF-containing reporters by TM. Because these are the conditions employed for ribosome profiling in stress conditions shown in Fig. 4B, it would be*

> *reassuring to document TM-induced translational efficiencies of ATF4 and the other known mRNAs resistant to eIF2 phosphorylation in the ribosome profiling data, including gene browser images of the replicate experiments. If the induction of TEs by TM for such mRNAs was not robust, it would be valuable to repeat the analysis using arsenite (SA) treatment, which produces a greater inhibition of bulk translation.*

Unfortunately, the eIF2alpha antibody is not very good and also detects the nonphosphorylated protein, causing high background and poor apparent induction in response to tunicamycin. The fact that the ISR was activated is visible from the induction of ATF that was assessed by western blot in the Suppl. Fig. 5E. To ensure that our ribosome profiling libraries also recorded the activation of ISR we built single gene plots for ATF4 both in control and HeLa eIF2A-KO cell. As shown in Author response image 3 A&B in both cell lines tunicamycin treatment led to the induction of ATF4. This can also be seen by the 4-fold induction in ATF4 translation efficiency in response to tunicamycin in both WT and eIF2A-KO cells (Author response image 3C). Additionally, we checked that another marker induced by tunicamycin, HSPA5, is also translationally upregulated in both cell lines, as well as the downstream target of ATF4 – PPP1R15B. (Author response image 3C).

Author response image 3.

(A-B) Average read occupancy on the ATF4 (ENST00000674920) transcript in DMSO treated (n=3) or tunicamycin treated samples (n=2) derived from either control (panel A) or eIF2A-KO (panel B) HeLa cells are shown. Read counts were normalized to sequencing depth and averaged between either 3 (DMSO-treated) or 2 (tunicamycin-treated) replicates. Graphs were then smoothened with a sliding window of 3 nt. (C) Scatter plot of log2(fold change) of Translation Efficiency TM/DMSO for control cells on the xaxis versus eIF2AKO cells on the y-axis. The induction of ATF4 as well as the downstream target PPP1R15B are shown. The upregulation of HSP5A translation, the other hallmark of ER-stress induced by tunicamycin treatment is shown.



> *- It should be pointed out in the text that in both published studies being cited here of cells lacking eIF2A, that by Gaikwad et al. on a yeast eIF2A deletion mutant, and that by Ichihara et al. on human HEK293 CRISPR KO cells, the analyses included stress conditions in which eIF2 phosphorylation is induced (amino acid starvation or SA treatment, respectively), as was conducted here.*

Good point - we added this information into the introduction:

"Furthermore, loss of eIF2A in several systems did not recapitulate these effects on non-AUG initiation in either non-stressed or stress conditions (caused either by amino acid depletion or sodium arsenate treatment) (Gaikwad et al., 2024; Ichihara et al., 2021)."

> *- The Ichihara et al. (2021) study just mentioned reached some of the same conclusions for HEK cells obtained here by conducting ribosome profiling in untreated and SA-treated cells, finding only 1 mRNA (untreated) or four mRNAs (SA-treated cells) that showed significantly reduced TEs in the eIF2A knockout vs. parental cells. It seems appropriate for the authors to expand their treatment of this prior work by summarizing its findings in some detail and also noting how their study goes beyond this previous one.*

We have added a paragraph to the discussion pointing out that our data agree fully with Ichihara et al. (2021), and that Ichihara et al. (2021) also found only very few mRNAs that change in TE upon loss of eIF2A in either non-stressed or stressed conditions.

https://doi.org/10.7554/eLife.105311.2.sa0