

# eIF3 engages with 3'-UTR termini of highly translated mRNAs

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## eLife Assessment

This **valuable** study shows previously unappreciated binding of the eukaryotic translation initiation factor 3 (eIF3) to the poly(A) tail proximal portion of 3' untranslated regions (UTRs) of mRNAs that are efficiently translated in neuronal progenitors. The authors' conclusions are supported by **solid** experimental evidence which is based on several orthogonal systems biology approaches. This article is of considerable interest to the broad spectrum of biomedical researchers interested in studying post-transcriptional regulation of gene expression.

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## Abstract

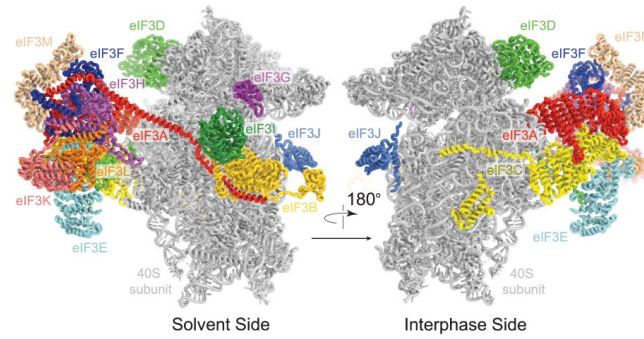
Stem cell differentiation involves a global increase in protein synthesis to meet the demands of specialized cell types. However, the molecular mechanisms underlying this translational burst and the involvement of initiation factors remains largely unknown. Here, we investigate the role of eukaryotic initiation factor 3 (eIF3) in early differentiation of human pluripotent stem cell (hPSC)-derived neural progenitor cells (NPCs). Using Quick-irCLIP and alternative polyadenylation (APA) Seq, we show eIF3 crosslinks predominantly with 3' untranslated region (3'-UTR) termini of multiple mRNA isoforms, adjacent to the poly(A) tail. Furthermore, we find that eIF3 engagement at 3'-UTR ends is dependent on polyadenylation. High eIF3 crosslinking at 3'-UTR termini of mRNAs correlates with high translational activity, as determined by ribosome profiling, but not with translational efficiency. The results presented here show that eIF3 engages with 3'-UTR termini of highly translated mRNAs, likely reflecting a general rather than specific regulatory function of eIF3, and supporting a role of mRNA circularization in the mechanisms governing mRNA translation.

## Introduction

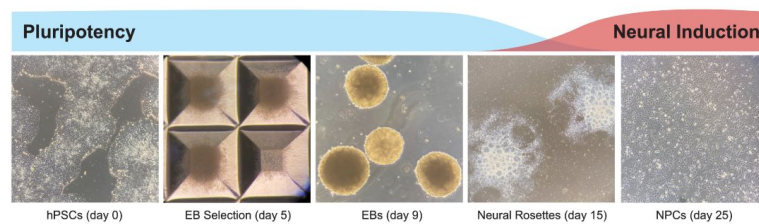
Stem cells are a group of diverse cells that are characterized by their ability to self-renew and differentiate into multiple cell types. This remarkable plasticity enables them to contribute to tissue development, maintenance and repair. In their quiescent state, stem cells present low protein synthesis levels, conserving energy and resources while maintaining their undifferentiated state. However, upon receiving differentiation signals, they exhibit a global increase in protein synthesis that results in a drastic change in the proteome composition to meet the demands of newly specialized progenitor cells (Baser et al., 2019 [↗](#); Blanco et al., 2016 [↗](#); Saba et al., 2021 [↗](#); Sampath et al., 2008 [↗](#); Signer et al., 2014 [↗](#); Zismanov et al., 2016 [↗](#)). Since initiation is translation's rate-limiting step, it must be highly regulated to maintain quiescence and to promote the global increase in translation that occurs during the initial steps of stem cell differentiation. Despite its importance, the roles that initiation factors play in quiescent and newly differentiated stem cells are poorly understood.

Eukaryotic initiation factor 3 (eIF3) is the largest of all initiation factors in eukaryotes and plays a pivotal role in the initiation of translation. In humans, eIF3 is composed of 13 subunits and helps position ribosomes at the start codon of mRNAs (Jackson et al., 2010 [↗](#)) (**Figure 1A** [↗](#)). However, recent discoveries have highlighted several non-canonical functions of eIF3 that extend beyond its general role in translation initiation. For instance, photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) experiments (Hafner et al., 2010 [↗](#)) performed in human embryonic kidney (HEK) 293T cells uncovered regulatory roles of eIF3 in the translation of a specific pool of mRNAs (Lee et al., 2015 [↗](#)). These studies also revealed that eIF3 subunit d (EIF3D) activates the translation of *JUN* mRNA by binding to its 5' cap (Lee et al., 2016 [↗](#)). Intriguingly, the pool of mRNAs regulated by eIF3 varies dramatically across different cell types and physiological conditions. PAR-CLIP experiments performed in Jurkat T cells showed that robust T cell activation requires the direct interaction of eIF3 with the mRNAs encoding for the human T cell receptor subunits  $\alpha$  and  $\beta$ , a phenomenon that was also observed in primary T cells (De Silva et al., 2021 [↗](#)). Additionally, eIF3 modulates the translation of mRNAs critical for adaptation and survival under stress conditions such as nutrient deprivation (Lamper et al., 2020 [↗](#)). Beyond its established functions in translation initiation, eIF3 has been shown to also be involved in translation elongation (Lin et al., 2020 [↗](#); Wagner et al., 2020 [↗](#)) and termination (Beznosková et al., 2013 [↗](#); Valášek, 2012 [↗](#); Valášek et al., 2017 [↗](#)). These findings indicate that eIF3 serves distinct regulatory roles depending on specific cellular environments.

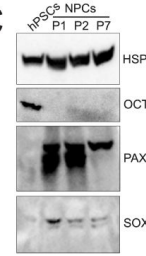
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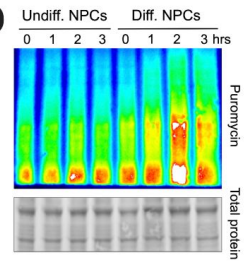
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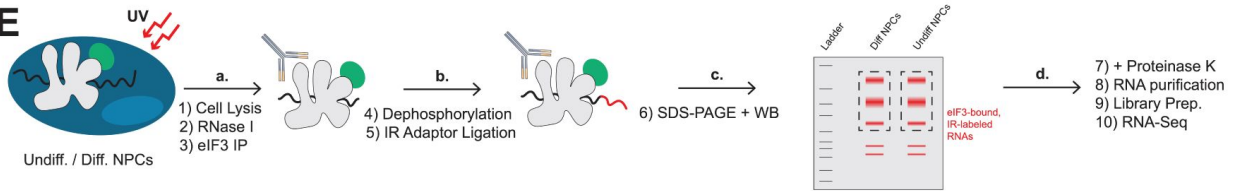
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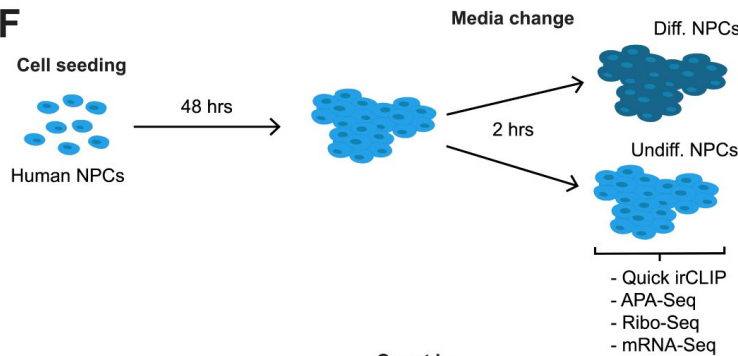
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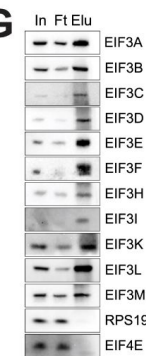
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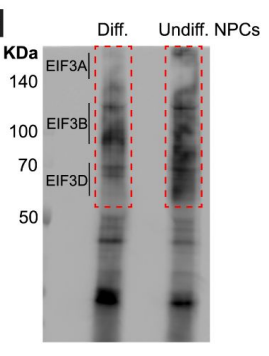
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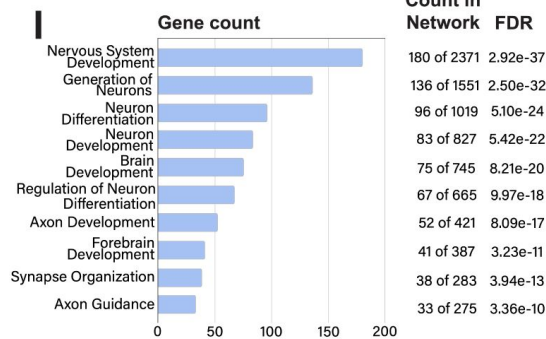
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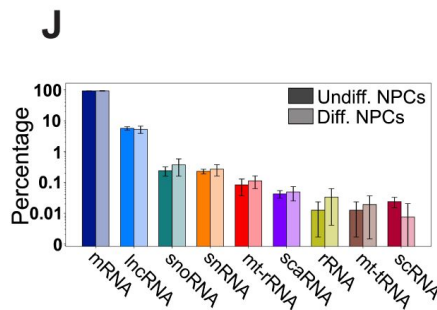
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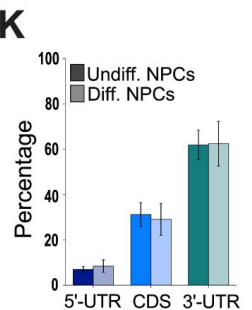
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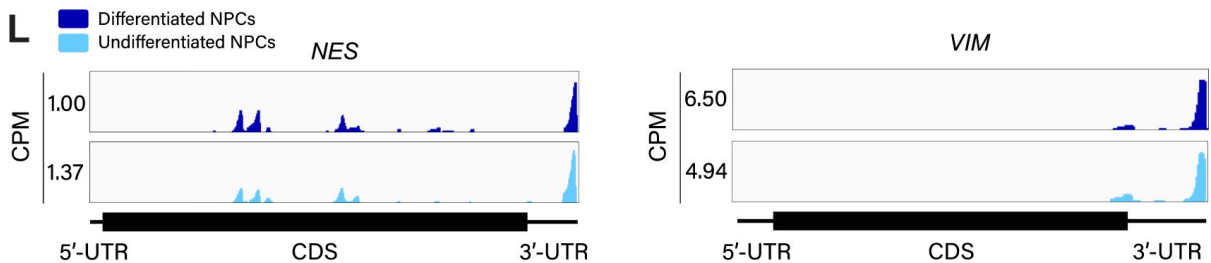
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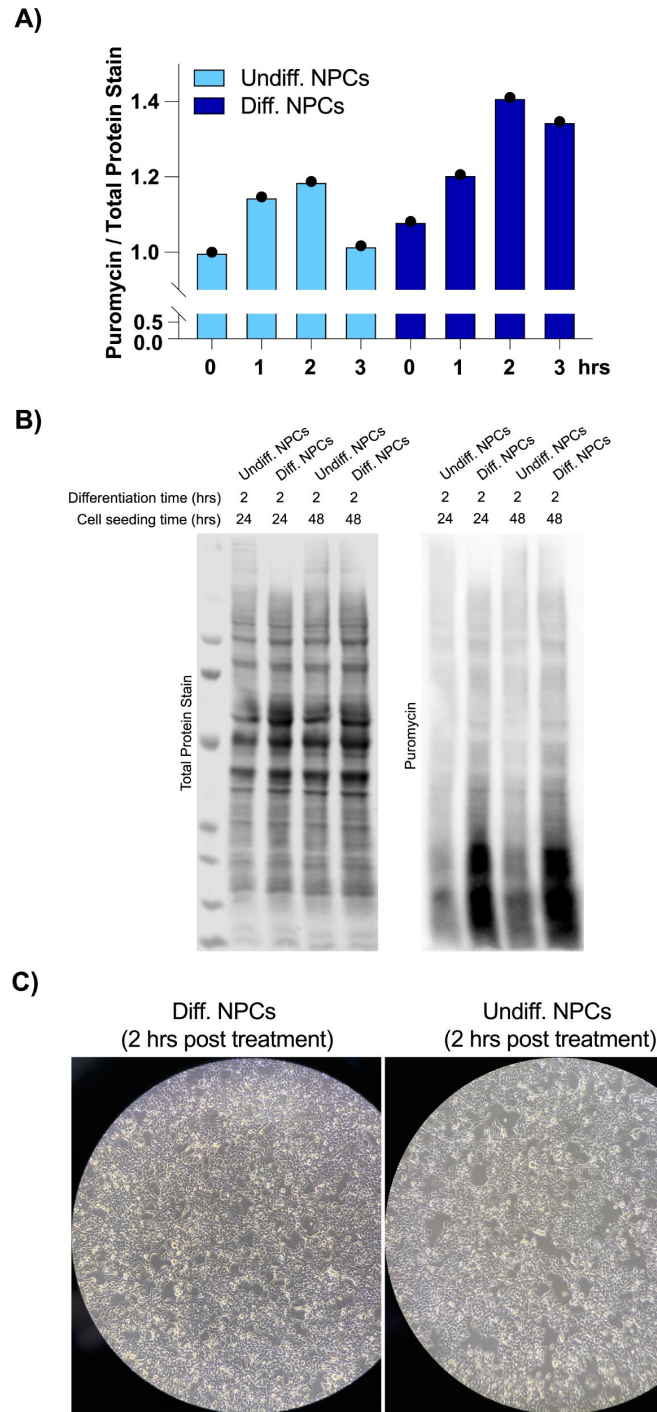


**Figure 1.**

**Analysis of Quick irCLIP of eIF3 to RNAs in undifferentiated and differentiated neural progenitor cells.**

**A)** Structure of the human eIF3 complex as part of the 48S initiation complex. PDB: 6ZMW (Brito Querido et al., 2020 [DOI](#)). **B)** Generation of NPCs from hPSCs by embryoid body (EB) and neural rosette selections. **C)** Western blots of neural markers Pax6 and Sox1 and pluripotent marker Oct4 from hPSCs and hPSC-derived NPCs (NPCs corresponding to passages 1, 2 and 7 are shown). **D)** Western blots of puromycin-treated (15 min) NPCs. Total protein stain is shown as loading control. Differentiated NPCs were treated with forebrain neuron differentiation medium for the indicated time points. Undifferentiated NPCs were treated with NPC medium for the indicated time points. The ratios of puromycin signal to total protein levels can be found in **Figure 1-figure supplement 1A** [DOI](#). **E)** Schematic of Quick-irCLIP. **F)** Schematic of the NPC treatment performed in the Quick-irCLIP, APA-Seq, Ribosome profiling, and mRNA-Seq experiments. Treatment consisted of change of media and incubation at 37 °C for 2 hrs. Differentiation media was used for differentiated (Diff.) NPCs and Basal media for undifferentiated (Undiff.) NPCs. **G)** Immunoprecipitation samples of assembled eIF3 complexes from undifferentiated NPCs. 5% inputs (In), flowthroughs (Ft), and eluates (Elu) are shown. Western blot for subunit EIF3G is not shown because we could not identify an effective antibody for its detection. EIF3J, not shown, is usually dissociated from the eIF3 complex upon immunoprecipitation. However, we detected EIF3G by mass spectrometry in the IPs (Supplementary file 1). **H)** Infrared (IR) image of IR dye-labeled, eIF3 UV-crosslinked RNA transcripts from Diff. and Undiff. NPCs. Regions marked with red boxes, which correspond to subunits EIF3A through EIF3D, were excised from the blot. **I)** Biological Function enrichment determined using the STRING database for the undifferentiated NPC biological replicates of the EIF3A/B/C/D Quick-irCLIP libraries, using the top 500 mRNA hits from the crosslinking analysis. **J)** Categories of RNAs crosslinked to eIF3 in undifferentiated and differentiated NPCs, for 3 replicates plotted in log-scale with standard deviation error bars. **K)** mRNA regions that crosslink to eIF3 in undifferentiated and differentiated NPCs. **L)** Crosslinking of eIF3 across the transcripts of *NES* and *VIM* mRNAs in differentiated and undifferentiated NPCs. Read coverage is provided in Counts Per Million (CPM).

Here, we derived neural progenitor cells (NPCs) from human pluripotent stem cells (hPSCs) and committed them to a forebrain neuron fate to study the potential roles of eIF3 in regulating the early differentiation-dependent global increase in protein synthesis. NPCs are considered more committed to the neural lineage than NSCs (Oikari et al., 2016 [DOI](#)) and hence are likely to present higher protein synthesis levels than quiescent NSCs (Baser et al., 2019 [DOI](#)). Despite their commitment, NPCs maintain the self-renewal and multipotent characteristics of NSCs and therefore are widely used to study stem-like properties *in vitro*. Our study focused on identifying the mRNAs that crosslink to eIF3 during the translational burst observed upon NPC differentiation. Instead of identifying specific regulatory roles for eIF3, we discovered that eIF3 predominantly crosslinks to the 3' untranslated regions (3'-UTRs) of mRNAs. Using next-generation sequencing to map polyadenylation events in NPCs, we found that eIF3 crosslinking at 3'-UTRs occurs at the termini of multiple mRNA isoforms, adjacent to polyadenylated (polyA) tails. Transcriptome-wide analysis further demonstrated that increased eIF3 crosslinking at 3'-UTR termini correlates with an increased level of ribosome footprint (RPF) levels on mRNAs. Interestingly, the binding of eIF3 to 3'-UTR termini was observed only in polyadenylated transcripts and is independent of interactions between eIF3 and polyA-binding proteins, as inferred from eIF3 immunoprecipitation experiments. These findings show that high levels of active translation drive eIF3 engagement at mRNA 3'-UTR termini. Given eIF3's known role in associating with 5'-UTRs to promote translation initiation, our observation of eIF3 interactions with 3'-UTR ends points to an unexpected role for eIF3 in mRNA circularization, potentially facilitating communication between the 5' and 3' ends of actively translated mRNAs.



**Figure 1-figure supplement 1.**

**NPCs treated with differentiation media or kept in undifferentiated media (2-hour treatment).**

**A)** Levels of protein synthesis in undifferentiated and differentiated NPCs at different time points, as quantified by puromycin incorporation. Puromycin signal was normalized to total protein levels, which were measured with total protein stain. This is quantification of **Figure 1D**. **B)** A second set of experiments showing changes in levels of protein synthesis in undifferentiated vs. differentiated NPCs. **C)** Cellular morphology of the NPCs using dark field microscopy.

Figure 1-figure supplement 2.

**Venn Diagram of genes identified by Quick-irCLIP (differentiated NPCs) and PAR-CLIP (HEK293T and activated Jurkat T cells).**

The top ~ 200 mRNA transcripts from the three libraries ranked by total reads mapped to a given gene are compared (See Materials and Methods).

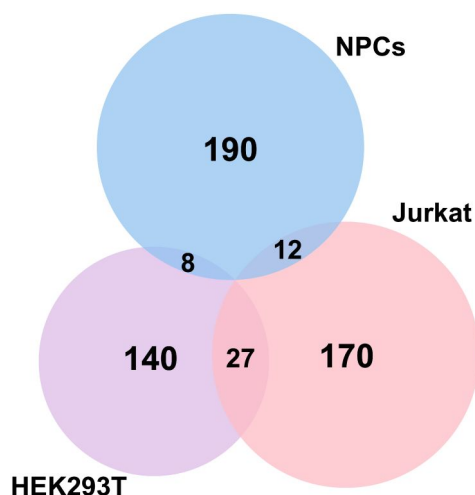
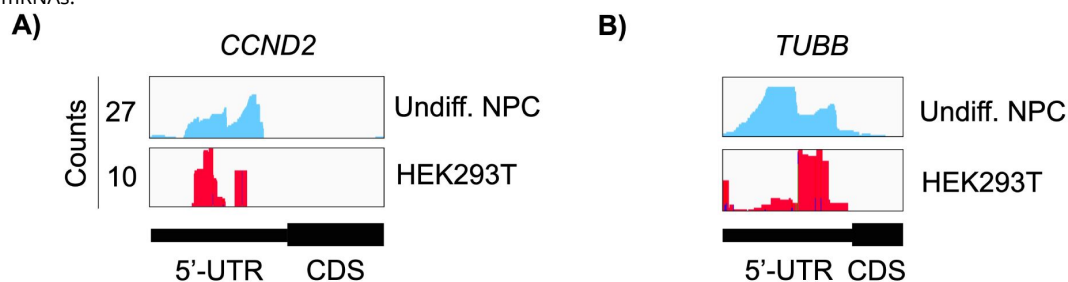
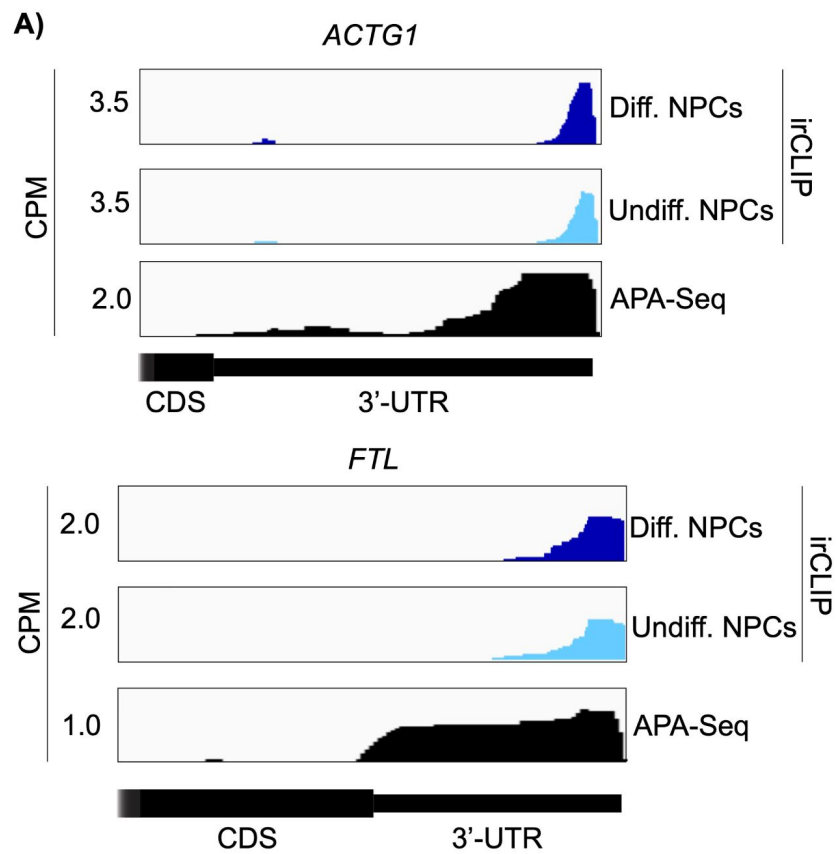


Figure 1-figure supplement 3.

**Crosslinking of eIF3 in undifferentiated NPCs and in HEK293T cells.**

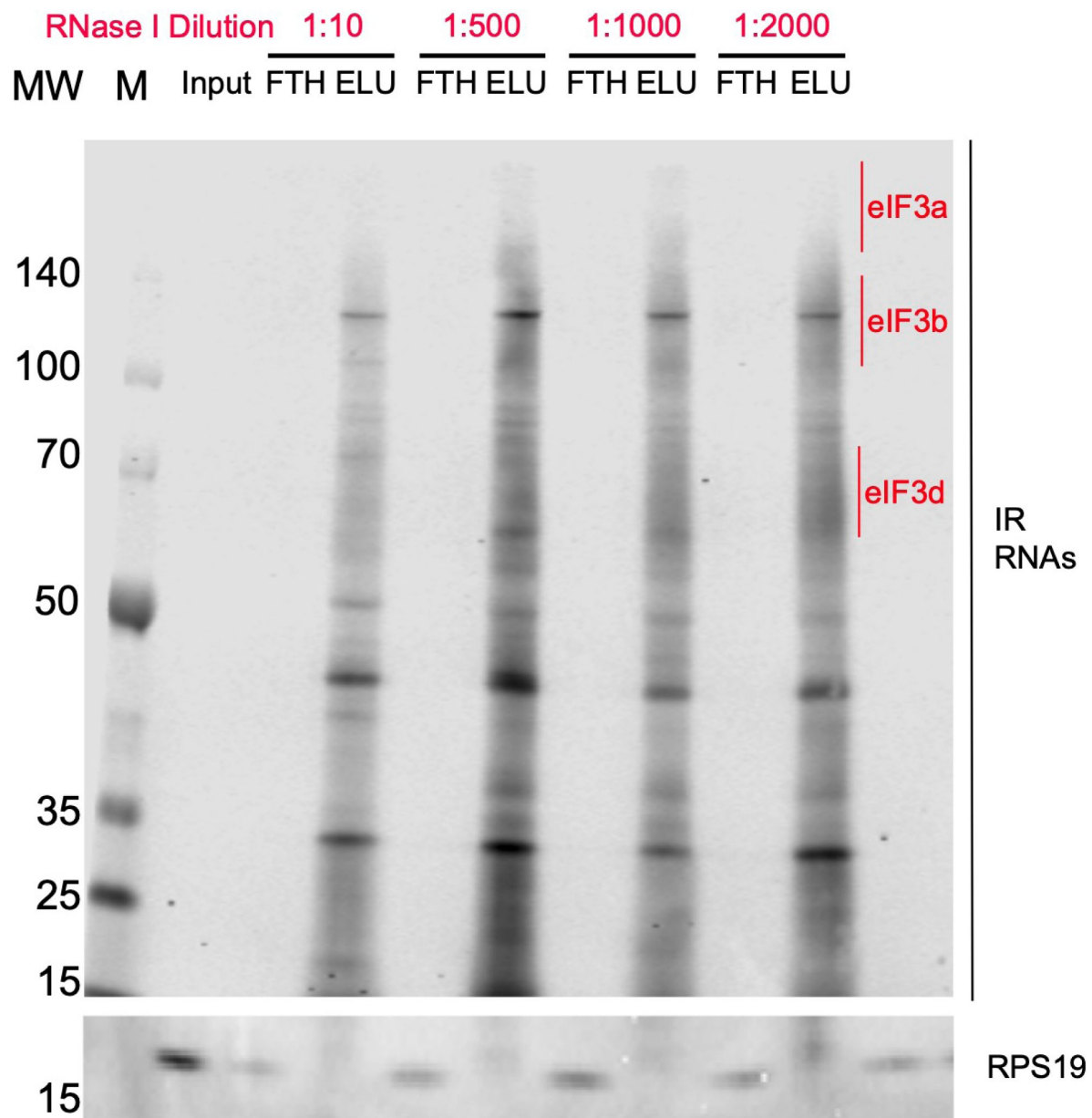
Crosslinks in undifferentiated NPCs are in blue, and in HEK294T cells in red. Shown are the 5'-UTR regions of **A) CCND2** and **B) TUBB** mRNAs.





**Figure 1-figure supplement 4.**

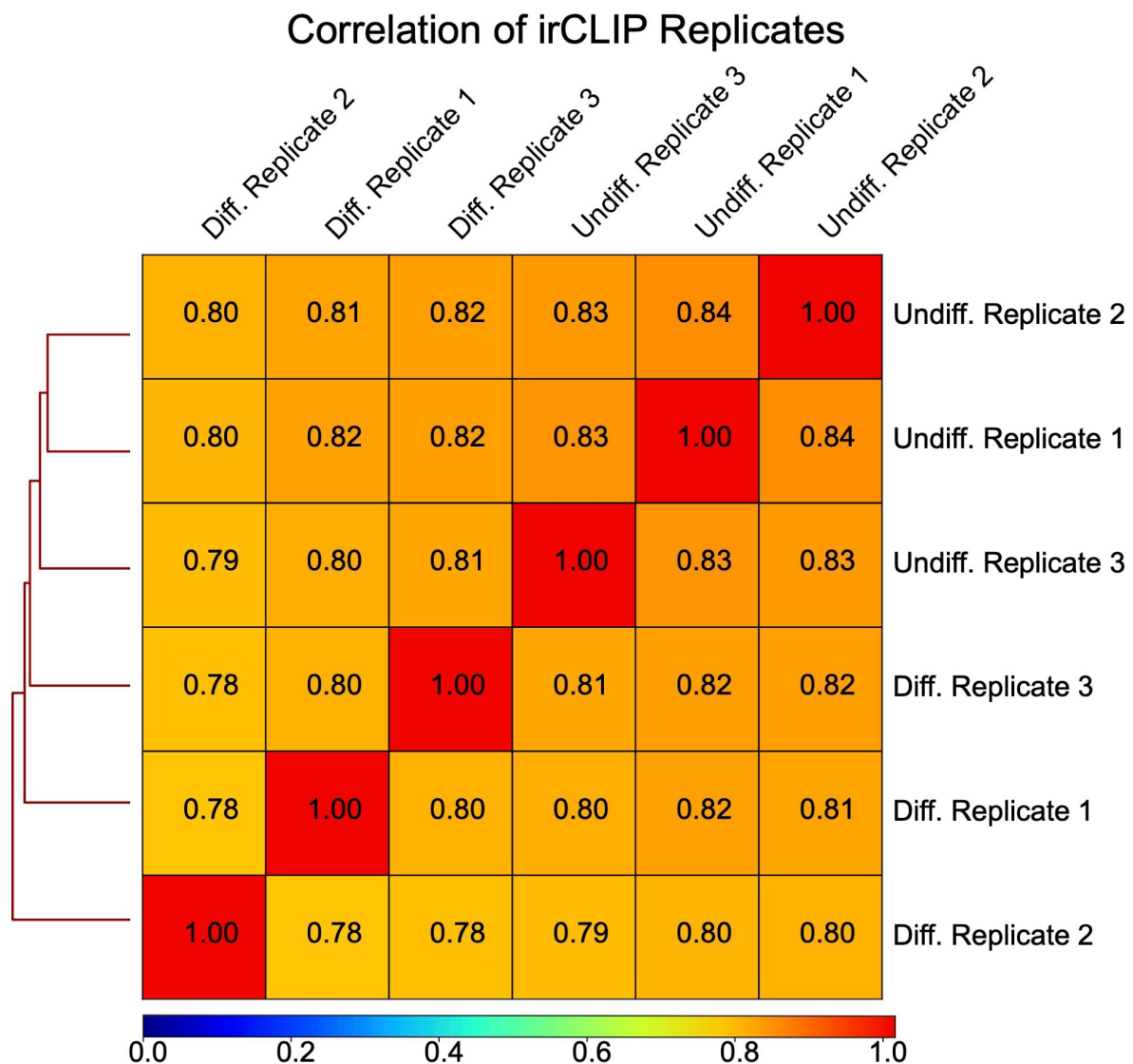
Crosslinking of eIF3 in differentiated and undifferentiated NPCs across the 3'-UTR regions of *ACTG1* and *FTL* mRNAs. APA-Seq data is also shown for both transcripts.



**Figure 1-figure supplement 5.**

#### **Titration of RNase I for the Quick-irCLIP experiment.**

Crosslinked samples were treated with RNase I for 3 minutes and subject to anti-eIF3B IP. Shown are 5% input of the lysate, flowthrough (FTH) and elution (ELU) for each concentration of RNase I. Molecular weight markers (MW, M) are shown to the left.



**Figure 1—figure supplement 6.**

#### **Correlation of Quick-irCLIP replicates.**

Spearman correlation coefficients (Rho) of irCLIP bioreplicates (n=3) for differentiated (average Rho: 0.7855, standard deviation: 0.0092) and undifferentiated NPCs (average Rho: 0.834, standard deviation: 0.0058) demonstrate high reproducibility.

## Results

### Generation of hPSC-derived NPCs

We differentiated hPSCs toward NPCs by embryoid body (EB) formation followed by neural rosette selection, giving rise to NPCs after ~3 weeks (**Figure 1B**). Successful NPC differentiation was assessed by western blotting. NPCs cultured for several passages (up to passage 7) presented an increase in the expression of neural markers Pax6 and Sox1 and the loss of the pluripotent marker Oct4 (**Figure 1C**).

### Early NPC differentiation results in a global increase in protein synthesis

To find the translational burst that occurs during the initial steps of stem cell differentiation, we differentiated NPCs toward a forebrain neuron fate using a dual-SMAD inhibition protocol (Chambers et al., 2009) and collected them at different time points that ranged from 1 hour to several days. In order to study active translation levels, prior to cell collection, we treated cells for a short time with puromycin, a tRNA analog that gets incorporated into the C-terminus of elongating nascent chains, releasing them from translating ribosomes. By immunoblotting with puromycin antibodies, puromycin-containing nascent peptides can be detected, reporting on active levels of protein translation. Our western blotting results show that NPCs treated for 2 hours with forebrain neuron differentiation medium present a significant increase in puromycin incorporation into nascent chains compared to NPCs treated for the same amount of time with medium that keeps them in their undifferentiated state (**Figure 1D**, **Figure 1-figure supplement 1A-1B**), indicating a differentiation-dependent global increase in protein synthesis.

### eIF3 crosslinks to 5'- and 3'-UTRs of neurologically-relevant mRNAs

To identify the RNA transcripts that interact with eIF3 during the observed translational burst in differentiated NPCs, we performed Quick-irCLIP (Kaczynski et al., 2019) (**Figure 1E**). We selected the aforementioned 2-hour treatment timepoint and immunoprecipitated eIF3 complexes from early differentiated NPCs that had started the differentiation pathway toward forebrain neurons (differentiated NPCs) and NPCs that we kept in their progenitor state for the same amount of time (undifferentiated NPCs) (**Figure 1F**). Differentiation treatment does not result in major cellular morphology changes (**Figure 1-figure supplement 1C**). After UV crosslinking the cells, we treated cell lysates with RNase I, followed by eIF3 immunoprecipitation, dephosphorylation of the protein-bound transcripts, IR adaptor ligation, and RNA-protein complex visualization via SDS-PAGE. We assessed the success of eIF3 immunoprecipitation by western blotting (**Figure 1G**) and mass spectrometry (Supplementary file 1). The previous PAR-CLIP studies performed with the eIF3 complex in HEK293T and Jurkat cells identified eIF3 subunits EIF3A, EIF3B, EIF3D and, to a lesser extent, EIF3G as the four subunits of the complex presenting significant amounts of RNA crosslinks (De Silva et al., 2021; Lee et al., 2015). Consequently, here we excised the RNA smears that appeared in the regions of subunits EIF3A/B/C/D (from ~170 kDa to ~65 kDa) (**Figure 1H**).

Using the STRING database (Szklarczyk et al., 2019) for the top 500 transcripts that crosslink to eIF3, we observed highly similar sets of mRNAs in undifferentiated and differentiated NPCs (449 in common). We observe a significant enrichment in neurologically-relevant biological processes, such as 'generation of neurons', 'neuron differentiation', 'neuron projection development', and 'axon development' (**Figure 1I**). To determine which top eIF3-binding transcripts are common between NPCs and the previous Jurkat T cell (De Silva et al., 2021) and HEK293T cell (Lee et al., 2015) PAR-CLIP studies, we compared the top ~200 eIF3-crosslinked transcripts between the

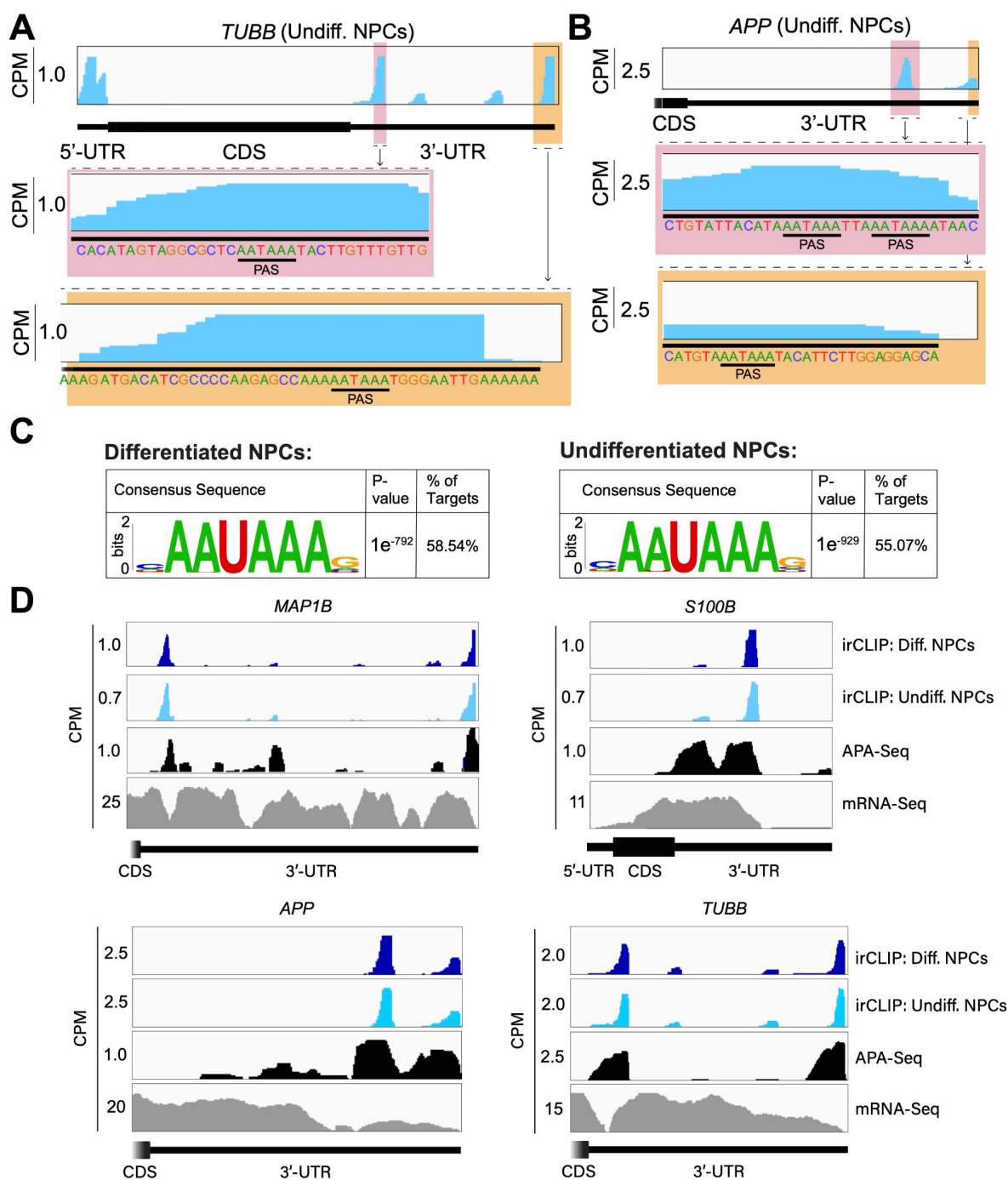
three cell lines. We found that only 12 are common between NPCs and Jurkat T cells and only 8 are common between NPCs and HEK293T, with no common hits between the 3 cell types (**Figure 1–figure supplement 2**).

Both in differentiated and undifferentiated NPCs, eIF3 predominantly (>90%) crosslinks to mRNAs over other non-coding RNAs such as lncRNAs and snoRNAs (**Figure 1J**). Within mRNA regions, eIF3 primarily interacts with 3'-UTRs and, to a lesser extent, with CDS regions and 5'-UTRs (**Figure 1K**). Our Quick-irCLIP experiment in NPCs identified several eIF3 crosslinks located at 5'-UTR regions that were also observed in the previous eIF3 PAR-CLIP experiment performed in HEK293T (Lee et al., 2015), such as *CCND2* and *TUBB* mRNAs (**Figure 1–figure supplement 3**). The distinctive 'pan-mRNA' pattern observed in the eIF3 PAR-CLIP performed in Jurkat T cells (De Silva et al., 2021) is not observed in NPCs. Notably, here we observe an enrichment of eIF3 crosslinks at 3'-UTRs of transcripts encoding neurologically-relevant proteins, such as *VIM* and *NES* (**Figure 1L**), as well as highly abundant mRNAs, such as *ACTG1* and *FTL* (**Figure 1–figure supplement 4**). Overall, we observe similar eIF3 crosslinking levels to transcripts in differentiated and undifferentiated NPCs (*VIM* and *NES* are shown as examples in **Figure 1L**).

## eIF3 3'-UTR crosslinking events map to 3'-UTR termini of multiple isoforms, upstream of poly(A) tails

In many instances, we observe eIF3 crosslinking peaks at the termini of annotated 3'-UTRs, adjacent to the poly(A) tail and where the polyadenylation signal (PAS; canonical PAS sequence = AAUAAA) is located (**Figure 2A**, *TUBB*). However, in addition to ends of annotated 3'-UTRs, we also observe the same type of crosslinking pattern across multiple regions within 3'-UTRs (**Figure 1I**, *VIM*). Looking at the nucleotide sequence of the regions within 3'-UTRs where eIF3 crosslinks, we observe that most of them contain PAS sequences as well (**Figure 2B**, *APP*). Hypergeometric optimization of motif enrichment (HOMER) motif discovery analysis (Heinz et al., 2010) of the eIF3 crosslinks that map to 3'-UTR regions revealed that in differentiated and undifferentiated NPCs 58% and 55% of the 3'-UTR eIF3 crosslinks, respectively, map to regions that are extremely enriched in the canonical PAS sequence AAUAAA (**Figure 2C**, Supplementary file 2, Supplementary file 3). We also find other enriched 3'-UTR motifs but none of them has either the level of eIF3 enrichment nor the statistical significance as AAUAAA (**Figure 2–figure supplement 1**). Overall, these data indicate eIF3 crosslinks near 3'-UTR termini of multiple isoforms, upstream of poly(A) tails in NPCs. In neurons, 3'-UTR isoforms are known to play key roles in mRNA localization and expression in different neuronal compartments (Taliaferro et al., 2016; Tushev et al., 2018) and alternative polyadenylation is highly prevalent in the nervous system, leading to the expression of multiple mRNA isoforms with different 3'-UTR lengths (Miura et al., 2013).

To test our hypothesis that the observed eIF3 crosslinking pattern reflects the expression of bona fide alternative 3'-UTRs in NPCs, we performed alternative polyadenylation next-generation sequencing (APA-Seq) in undifferentiated NPCs. Given that we observe similar eIF3 crosslinking levels in undifferentiated and differentiated NPCs, we did not perform APA-Seq in differentiated NPCs. APA-Seq creates sequencing libraries by amplifying the region upstream of a polyadenylation event, allowing the identification of all 3'-UTR mRNA isoforms being expressed for any given gene. Comparing our Quick-irCLIP and APA-Seq experiments, we observe that the eIF3 peaks located in 3'-UTRs map to the same location as APA peaks, indicating that the predominant eIF3 crosslinking peaks in 3'-UTRs identified by Quick-irCLIP localize to 3'-UTR termini, adjacent to the poly(A) tail. For instance, the eIF3 crosslinking peaks located in the 3'-UTR of the *MAP1B* mRNA correspond to APA-Seq peaks (**Figure 2D**). The aforementioned cases of *NES* and *VIM* mRNAs, where we observed eIF3 crosslinks in their 3'-UTRs, also have APA-Seq peaks at the same locations as the irCLIP peaks (**Figure 2–figure supplement 2**). As another example, our irCLIP experiment shows that eIF3 crosslinks to both the 5'- and 3'-UTRs of the *S100B* mRNA, which encodes for a calcium-binding protein highly expressed in brain cells and that is commonly



**Figure 2.**

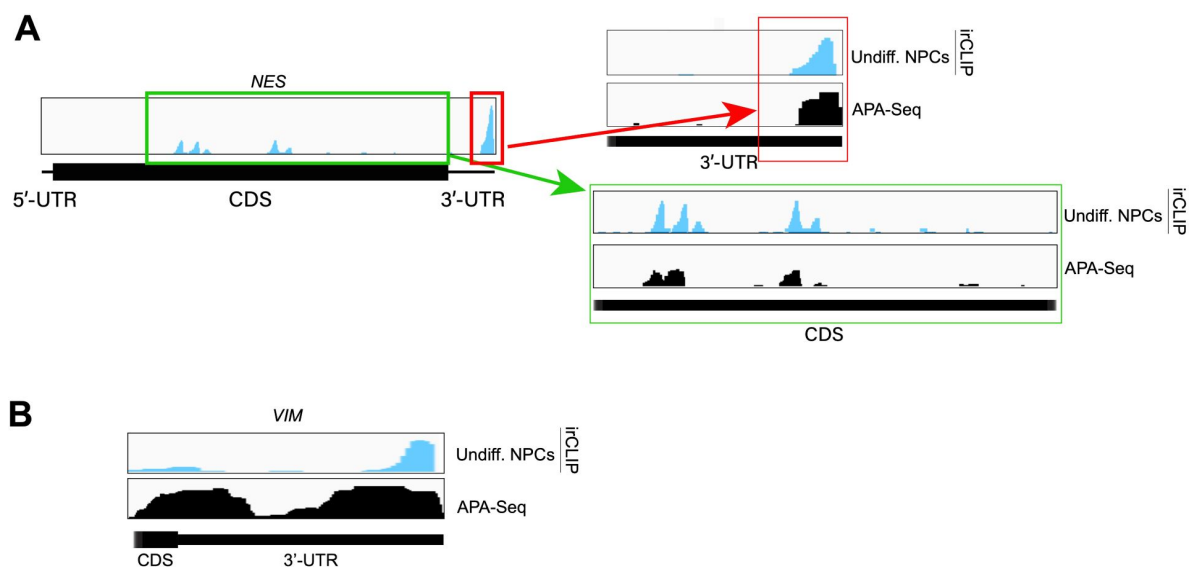
Crosslinking of eIF3 to mRNA 3'-UTRs adjacent to the poly(A) tail. A) Crosslinking of eIF3 across the *TUBB* mRNA in undifferentiated NPCs. A zoomed-in view of *TUBB* mRNA 3'-UTR terminus with the eIF3 crosslinks is shown. The polyadenylation signal (PAS) is marked. B) Crosslinking of eIF3 across the *APP* mRNA 3'-UTR in undifferentiated NPCs. A zoomed-in view of the two 3'-UTR regions presenting eIF3 crosslinks are shown as well as their PAS sequences. C) Sequence logo of the eIF3 crosslinks located at 3'-UTRs in differentiated and undifferentiated NPCs. D) Crosslinking of eIF3, APA-Seq peaks, and mRNA-Seq across the *MAP1B*, *S100B*, *TUBB* and *APP* mRNAs in differentiated and undifferentiated NPCs.

| Undifferentiated NPCs:      |                                     |         |             |              | Differentiated NPCs:        |      |                         |         |             |              |                 |
|-----------------------------|-------------------------------------|---------|-------------|--------------|-----------------------------|------|-------------------------|---------|-------------|--------------|-----------------|
| * - possible false positive |                                     |         |             |              | * - possible false positive |      |                         |         |             |              |                 |
| Rank                        | Motif                               | P-value | log P-value | % of Targets | % of Background             | Rank | Motif                   | P-value | log P-value | % of Targets | % of Background |
| 1                           | AAUAAA                              | 1e-929  | -2.141e+03  | 55.07%       | 16.79%                      | 1    | AAUAAA                  | 1e-792  | -1.825e+03  | 58.54%       | 17.12%          |
| 2                           | GGGAAAAAAAAA                        | 1e-41   | -9.637e+01  | 10.17%       | 5.56%                       | 2    | U <u>GU</u> U <u>GU</u> | 1e-33   | -7.728e+01  | 30.66%       | 22.53%          |
| 3                           | U <u>GU</u> U <u>GU</u> U <u>GU</u> | 1e-36   | -8.349e+01  | 11.92%       | 7.19%                       | 3    | GGGAAAAAAAAA            | 1e-33   | -7.659e+01  | 9.67%        | 5.08%           |
| 4                           | CUGUG <u>GU</u> U <u>GU</u>         | 1e-34   | -8.003e+01  | 28.97%       | 21.93%                      | 4    | UUUU                    | 1e-29   | -6.890e+01  | 6.22%        | 2.84%           |
| 5                           | AGGAGGAGGAG                         | 1e-34   | -7.862e+01  | 2.86%        | 0.92%                       | 5    | GAGGAGGAGGAG            | 1e-25   | -5.854e+01  | 2.71%        | 0.84%           |
| 6                           | UUUUUGUAGAAA                        | 1e-23   | -5.352e+01  | 1.55%        | 0.42%                       | 6    | UUUGUACAGU              | 1e-24   | -5.709e+01  | 3.06%        | 1.05%           |
| 7                           | AGCCAAAGU <u>GU</u> AC              | 1e-18   | -4.152e+01  | 0.99%        | 0.23%                       | 7    | CC <u>GU</u> CC         | 1e-18   | -4.284e+01  | 4.61%        | 2.27%           |
| 8                           | AGGGAGGAGAG                         | 1e-17   | -4.025e+01  | 3.62%        | 1.87%                       | 8    | GU <u>GU</u>            | 1e-18   | -4.157e+01  | 7.65%        | 4.56%           |
| 9                           | GGG                                 | 1e-17   | -3.977e+01  | 4.94%        | 2.85%                       | 9    | UGCCU <u>GU</u> U       | 1e-17   | -3.986e+01  | 0.81%        | 0.11%           |
| 10                          | CCU <u>GU</u> CCC                   | 1e-16   | -3.911e+01  | 1.31%        | 0.40%                       | 10   | GGGCGCGAGC              | 1e-16   | -3.727e+01  | 0.90%        | 0.16%           |
| 11                          | AGUG <u>GU</u> U                    | 1e-16   | -3.827e+01  | 0.99%        | 0.25%                       | 11   | CC <u>GU</u> U          | 1e-15   | -3.489e+01  | 0.26%        | 0.01%           |
| 12                          | CC <u>GU</u> U                      | 1e-16   | -3.779e+01  | 10.86%       | 7.73%                       | 12   | CGA <u>GU</u>           | 1e-13   | -3.194e+01  | 1.76%        | 0.62%           |
| 13                          | GAGC <u>GU</u> U                    | 1e-15   | -3.614e+01  | 0.49%        | 0.06%                       | 13   | GU <u>GU</u> U          | 1e-12   | -2.949e+01  | 0.95%        | 0.23%           |
| 14                          | UU <u>GU</u> U                      | 1e-15   | -3.503e+01  | 6.25%        | 4.00%                       | 14   | CGG <u>GU</u> U         | 1e-12   | -2.875e+01  | 0.19%        | 0.00%           |
| 15                          | CAU <u>GU</u> U                     | 1e-14   | -3.340e+01  | 2.45%        | 1.17%                       | 15   | UG <u>GU</u> U          | 1e-12   | -2.875e+01  | 0.19%        | 0.00%           |
| 16                          | GUA <u>GU</u> U                     | 1e-13   | -3.023e+01  | 0.16%        | 0.00%                       | 16   | AAAAAAAA                | 1e-12   | -2.842e+01  | 1.42%        | 0.48%           |
| 17                          | AUCG <u>GU</u> U                    | 1e-12   | -2.863e+01  | 0.37%        | 0.04%                       | 17 * | AGAG <u>GU</u> U        | 1e-11   | -2.690e+01  | 0.33%        | 0.02%           |
| 18                          | CU <u>GU</u> U                      | 1e-12   | -2.764e+01  | 3.11%        | 1.75%                       | 18 * | CCGGAGA                 | 1e-10   | -2.507e+01  | 2.23%        | 1.03%           |
| 19 *                        | UG <u>GU</u> U                      | 1e-11   | -2.760e+01  | 1.32%        | 0.52%                       | 19 * | GU <u>GU</u> U          | 1e-10   | -2.426e+01  | 1.76%        | 0.74%           |
| 20 *                        | UC <u>GU</u> U                      | 1e-10   | -2.500e+01  | 0.58%        | 0.14%                       | 20 * | UGAG <u>GU</u> U        | 1e-10   | -2.419e+01  | 0.24%        | 0.01%           |
| 21 *                        | CGAAGAG                             | 1e-10   | -2.495e+01  | 1.13%        | 0.43%                       | 21 * | CAUG <u>GU</u> U        | 1e-9    | -2.158e+01  | 0.95%        | 0.30%           |
| 22 *                        | UC <u>GU</u> U                      | 1e-10   | -2.331e+01  | 0.32%        | 0.04%                       | 22 * | UCAG <u>GU</u> U        | 1e-8    | -1.965e+01  | 0.17%        | 0.01%           |
| 23 *                        | UUUGUGGGG                           | 1e-9    | -2.211e+01  | 0.12%        | 0.00%                       | 23 * | AAAGUGUC                | 1e-7    | -1.755e+01  | 0.21%        | 0.02%           |
| 24 *                        | GU <u>GU</u> U                      | 1e-9    | -2.210e+01  | 0.69%        | 0.21%                       | 24 * | CC <u>GU</u> U          | 1e-7    | -1.614e+01  | 0.14%        | 0.01%           |
| 25 *                        | CU <u>GU</u> U                      | 1e-9    | -2.159e+01  | 0.23%        | 0.02%                       | 25 * | CA <u>GU</u> U          | 1e-6    | -1.546e+01  | 0.85%        | 0.32%           |
| 26 *                        | AAAGGAGG                            | 1e-9    | -2.128e+01  | 2.91%        | 1.75%                       | 26 * | GGUGGGAGG               | 1e-5    | -1.278e+01  | 0.12%        | 0.01%           |
| 27 *                        | CAAGCAGACA                          | 1e-8    | -1.964e+01  | 1.01%        | 0.42%                       | 27 * | GG <u>GU</u> U          | 1e-5    | -1.278e+01  | 0.12%        | 0.01%           |
| 28 *                        | CA <u>GU</u> U                      | 1e-8    | -1.906e+01  | 0.26%        | 0.04%                       | 28 * | CCAGCAGACA              | 1e-5    | -1.255e+01  | 0.74%        | 0.29%           |
|                             |                                     |         |             |              |                             | 29 * | AAAGGAGG                | 1e-5    | -1.232e+01  | 0.17%        | 0.02%           |

Figure 2-figure supplement 1.

Motif enrichment of eIF3 3'-UTR crosslinks for undifferentiated and differentiated NPCs.

Motifs were identified with HOMER (v4.11).



**Figure 2-figure supplement 2.**

### Examples of eIF3 crosslinking to 3'-UTR regions of mRNAs.

**A)** Crosslinking of eIF3 in undifferentiated NPCs as indicated across the 3'-UTR region of *NES* mRNA. APA-Seq data is also shown. **B)** Crosslinking of eIF3 in undifferentiated NPCs as indicated across the 3'-UTR region of *VIM* mRNA. APA-Seq data is also shown.

used as a glial marker (Brockes et al., 1979 [↗](#); Ferri et al., 1982 [↗](#); Ludwin et al., 1976 [↗](#)). Within *S100B*'s 3'-UTR, we observe 2 major eIF3 crosslinking peaks at the 3'-UTR termini of 2 distinct *S100B* mRNA isoforms expressed in NPCs (**Figure 2D** [↗](#)).

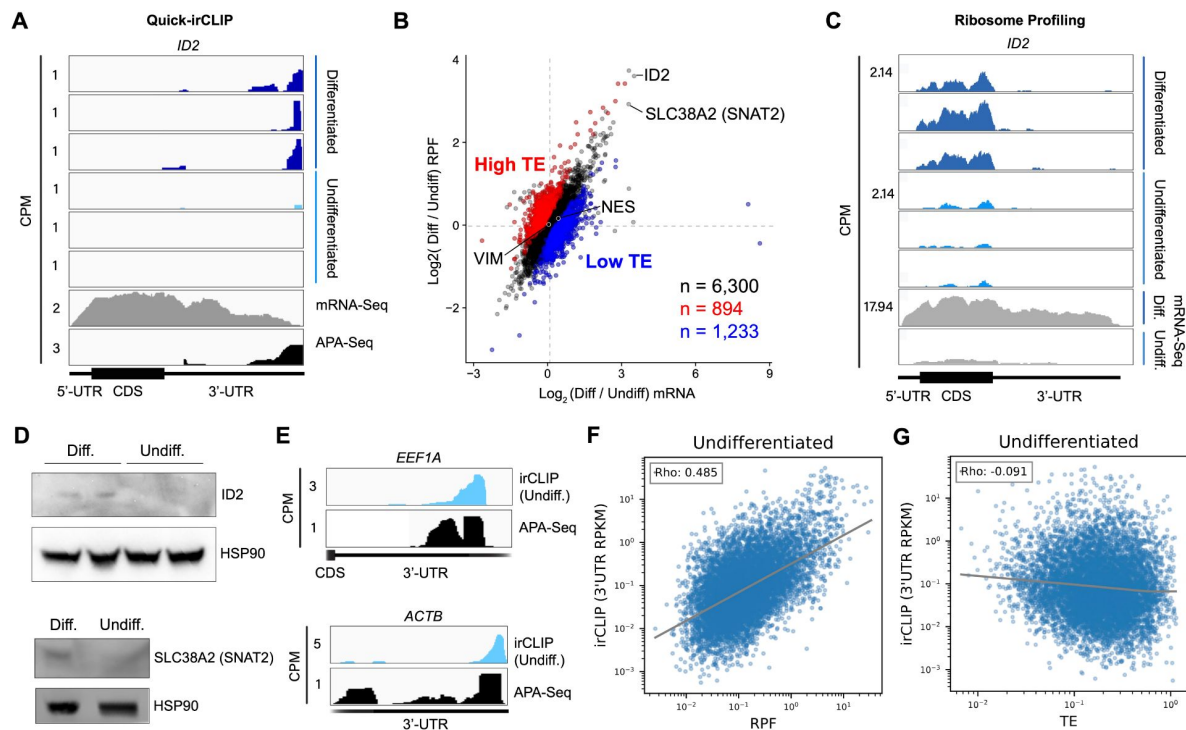
Interestingly, we found that eIF3 exhibits different crosslinking levels to the 3'-UTR termini of various 3'-UTR isoforms that are similarly expressed. For instance, our APA-Seq data reveals two major *S100B* mRNAs with similar expression levels. However, eIF3 preferentially crosslinks to the distal 3'-UTR isoform rather than the proximal 3'-UTR isoform (**Figure 2D** [↗](#)). Conversely, in the case of *APP*, which also expresses 2 major mRNA isoforms, eIF3 predominantly binds to the 3'-UTR terminus of the proximal isoform. Finally, similar to the case of *MAP1B* mRNA, in *TUBB* mRNA the extent of eIF3 crosslinking correlates with mRNA isoform expression, indicating that eIF3 binds to both major *TUBB* mRNA isoforms to a similar extent (**Figure 2D** [↗](#)). Overall, these results show that the eIF3 crosslinking events that we observe in 3'-UTRs often occur at 3'-UTR termini of multiple mRNA isoforms, upstream of poly(A) tails.

## eIF3 engages with 3'-UTR termini upon increased levels of protein synthesis

Our Quick-irCLIP experiment showed that eIF3 interacts with the 3'-UTR termini of mRNAs expressed in both differentiated and undifferentiated NPCs. Interestingly, eIF3 interacts with the mRNA encoding the transcription regulator ID2 only upon NPC differentiation (**Figure 3A** [↗](#)). ID2 is involved in migrating NPC differentiation into olfactory dopaminergic neurons (Havrdá et al., 2008 [↗](#)). Therefore, it is possible that the observed eIF3 engagement at its 3'-UTR terminus in differentiated NPCs is due to *ID2*'s mRNA translation activation upon differentiation. This would suggest that eIF3 interactions with 3'-UTR termini are involved with active translation levels for a given transcript.

To test whether eIF3 engagement at 3'-UTR termini correlates with translation activity at a transcriptome-wide level, we performed ribosome profiling, a technique that is based on deep sequencing of ribosome-protected mRNA fragments (RPFs) (Ingolia et al., 2011 [↗](#)), in undifferentiated and differentiated NPCs. Here, we used a low-bias ribosome profiling approach that takes advantage of the enzymatic activities of reverse transcriptase (RT) from eukaryotic retroelements to reduce the technicalities associated with ribosome profiling (Ferguson et al., 2023 [↗](#)). In addition, this method uses P1 nuclease instead of the commonly used RNase I and replaces RNA ligation with Ordered Two-Template Relay (OTTR) (Upton et al., 2021 [↗](#)). P1 nuclease digestion not only preserves monosome-protected mRNA fragments, which are the commonly used fragments in ribosome profiling experiments, but also mRNA fragments protected by collided ribosomes (disomes), which report on ribosome stalling and provide a screenshot of ribosome quality control (RQC) pathways (Ferguson et al., 2023 [↗](#); Meydan and Guydosh, 2020 [↗](#)).

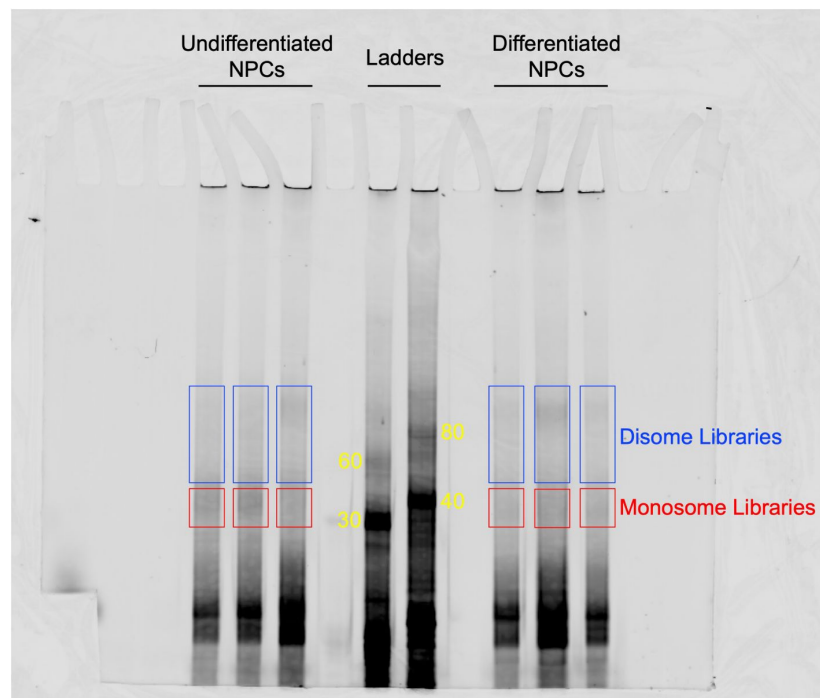
Here, we deep sequenced monosome- and disome-protected fragments from undifferentiated and differentiated NPCs (**Figure 3-figure supplement 1** [↗](#), **Figure 3-figure supplement 2** [↗](#)). We observe that about a third (monosome) and about a fifth (disome) of the libraries mapped to mRNAs (**Figure 3-figure supplement 2A** [↗](#)), similar to previous reports (Ferguson et al., 2023 [↗](#)). P1 nuclease digestion results in monosome footprints of 32 - 40 nts in length, observed in both differentiated and undifferentiated NPCs (**Figure 3-figure supplement 2B** [↗](#)). We also observe two populations of footprints in the disome library as previously reported upon P1 digestion, with the larger one corresponding to the “true disome” population (i.e., ≥ 60 nt disome footprints shown in **Figure 3-figure supplement 2C** [↗](#)), composed of colliding ribosomes and populations of two translating ribosomes in close proximity to each other, and to the “sub-disome” population (i.e., < 60 nt disome footprints shown in **Figure 3-figure supplement 2C** [↗](#)), proposed to correspond to 80S monosomes in close proximity to a scanning 40S ribosomal subunit during translation initiation or to a 40S ribosomal subunit from which a 60S ribosomal subunit was recently dissociated from during translation termination (Ferguson et al., 2023 [↗](#)). In agreement with the



**Figure 3.**

### Ribosome profiling of undifferentiated and differentiated NPCs, and comparisons to eIF3 Quick irCLIP crosslinking.

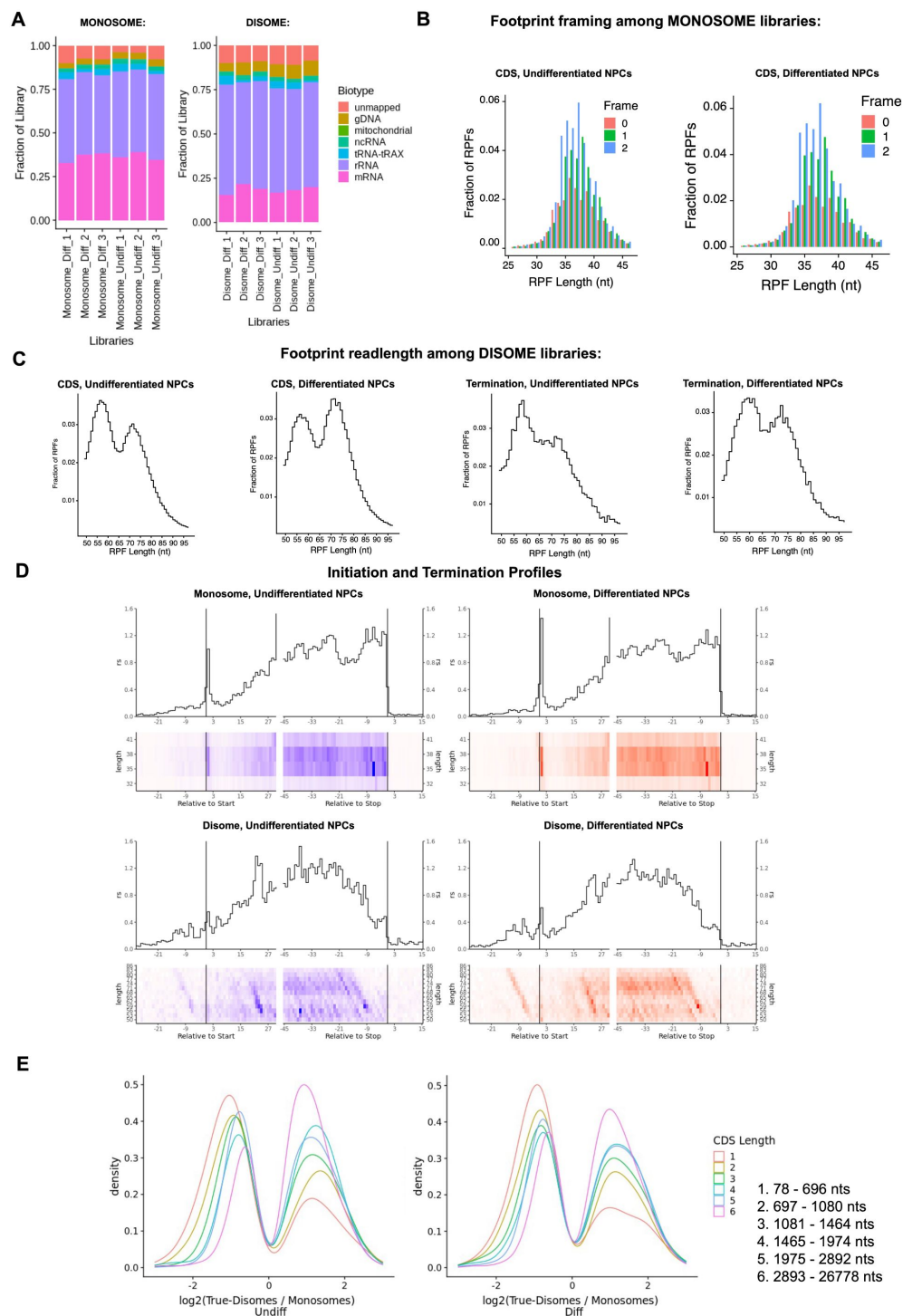
**A**) Crosslinking of eIF3 across the *ID2* mRNA in undifferentiated and differentiated NPCs. APA-Seq and mRNA-Seq data are also shown. **B**)  $\log_2$  fold change of ribosomal footprints and  $\log_2$  fold change of mRNA transcript levels upon NPC differentiation. Transcripts with a higher TE in differentiated NPCs compared to undifferentiated NPCs (high TE) are indicated in blue. Transcripts with a lower TE in differentiated NPCs compared to undifferentiated NPCs (low TE) are shown in red. The number of samples within each condition is shown. **C**) Ribosomal footprints across the *ID2* mRNA in undifferentiated and differentiated NPCs. mRNA-Seq data are also shown. **D**) Western blots of ID2, SLC38A2 (SNAT2) and Hsp90 in undifferentiated and differentiated NPCs. **E**) Crosslinking of eIF3 to the 3'-UTR of the *EEF1A* and *ACTB* mRNAs in undifferentiated NPCs. APA-Seq data is also shown. **F**) Comparison of irCLIP reads in mRNA 3'-UTRs to ribosome protected fragments (RPF) in undifferentiated NPCs. **G**) Comparison of irCLIP reads in mRNA 3'-UTRs to translation efficiency (TE) in undifferentiated NPCs. Outliers exceeding the TE's 99th percentile were removed, to make the log-log plot more readable. In panels **F**) and **G**), the Spearman correlation of average irCLIP 3'-UTR RPKM with mean RPF or TE across replicates (n=3) is shown.



**Figure 3–figure supplement 1.**

TBE 8% Urea-PAGE of the OTTR reaction products of cDNA libraries constructed from undifferentiated and differentiated NPCs.

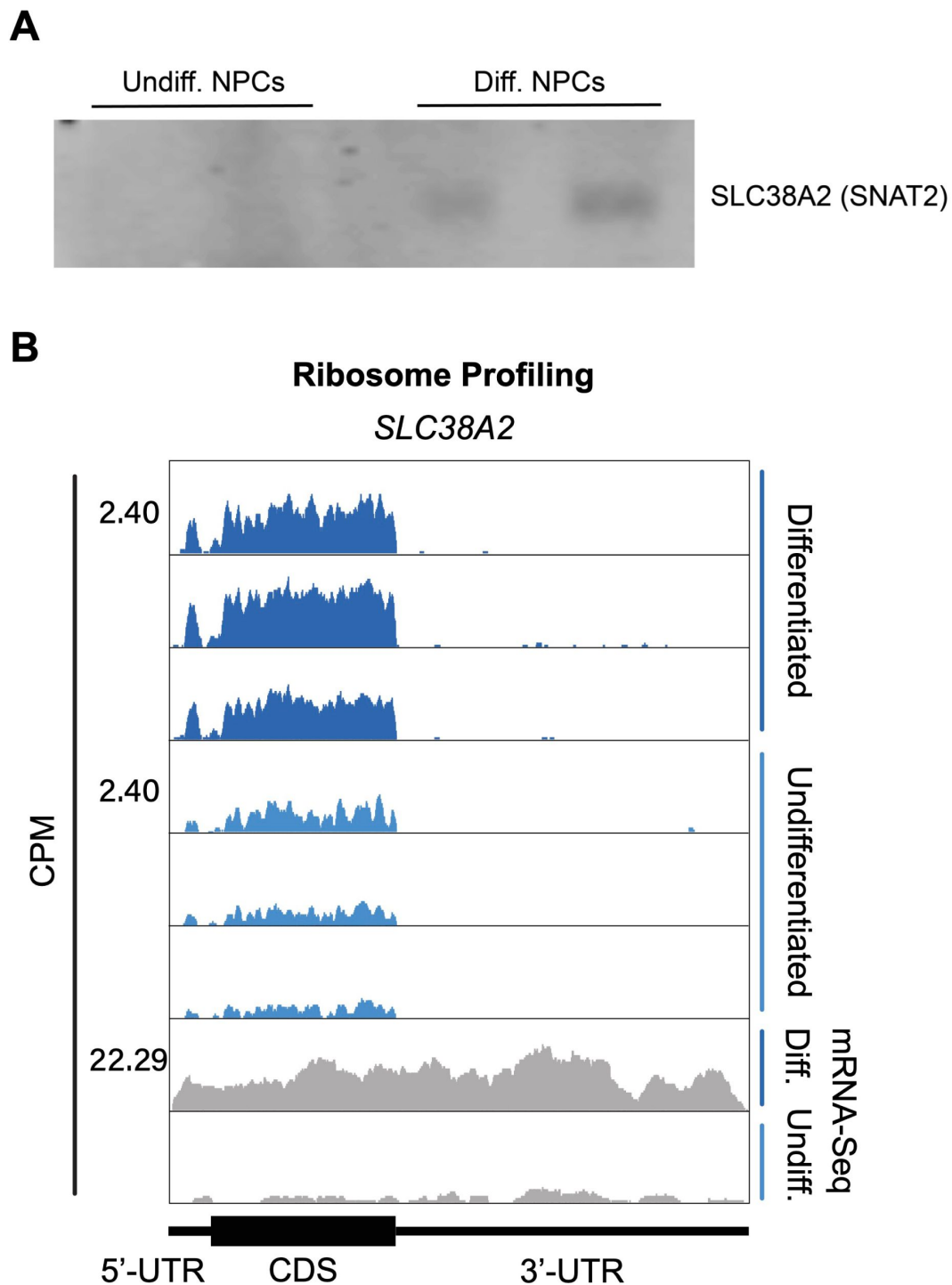
OTTR was also performed with control samples that yield marker products of 30, 40, 60, 80 nts in size (marked in yellow) so they could be used as references for gel excision for monosome and disome libraries. Gel excisions performed are shown in red (monosome) and blue (disome) rectangles.



**Figure 3-figure supplement 2.**

### Ribosome profiling of undifferentiated and differentiated NPCs.

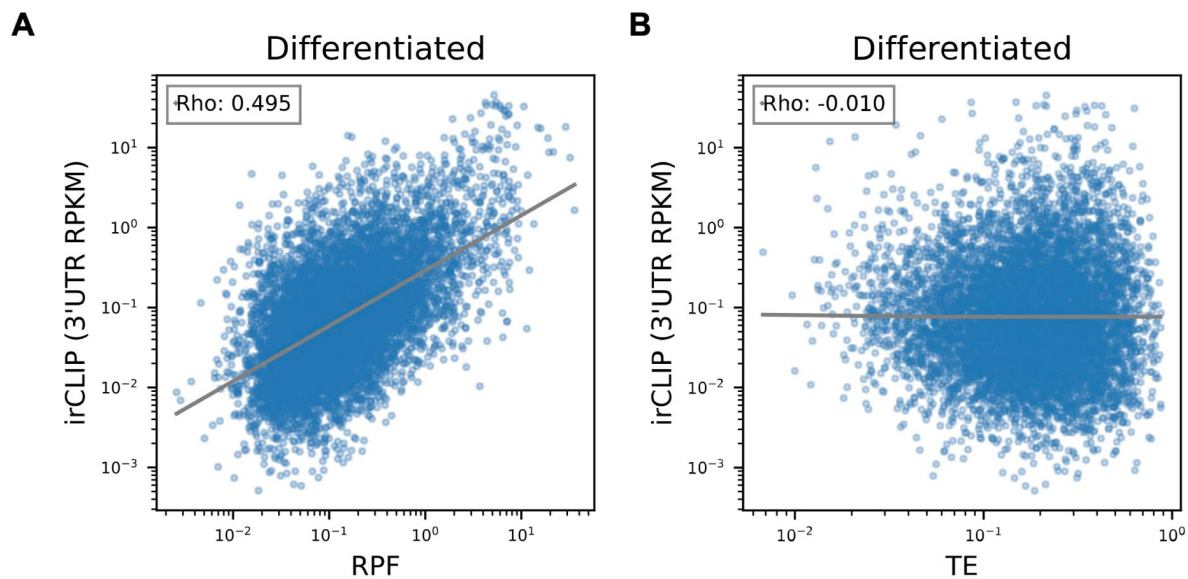
**A)** Fraction of sequencing reads mapped to each transcript type from monosome and disome profiling from P1 nuclease digested NPCs (undifferentiated and differentiated) and OTTR library cDNA synthesis. **B)** Framing among monosome libraries in undifferentiated and differentiated NPCs. **C)** Readlength of footprints aligned to CDS or terminating codons among disome libraries for undifferentiated and differentiated NPCs. **D)** A-site corrected initiation and termination profiles for genes with sufficient coverage ( $\geq 1$  read per codon) for monosome and disome libraries. **E)** DESeq2  $\log_2$  fold change in CDS occupancy for disome libraries with respect to monosome libraries, stratified by CDS length in nucleotides.



**Figure 3-figure supplement 3.**

**SLC38A2 expression in differentiated and undifferentiated NPCs.**

**A)** Western blots of SLC38A2 for additional replicates of differentiated and undifferentiated NPCs. Gel loading was normalized by total protein amounts determined by Bradford assay. **B)** Ribosomal footprints across the SLC38A2 mRNA in undifferentiated and differentiated NPCs. mRNA-Seq data are also shown.



**Figure 3-figure supplement 4.**

#### **Correlation of Quick-irCLIP with Ribosome Profiling and Translational Efficiency in Differentiated NPCs.**

**A)** Comparison of irCLIP reads in mRNA 3'-UTRs to ribosome protected fragments (RPF) in differentiated NPCs. **B)** Comparison of irCLIP reads in mRNA 3'-UTRs to translation efficiency (TE) in differentiated NPCs. Outliers exceeding the TE's 99th percentile were removed, to make the log-log plot more readable. The Spearman correlation of average irCLIP 3'-UTR RPKM with mean RPF or TE across replicates (n=3) is shown.

translational burst that we identified (**Figure 1D** [↗](#)), our monosome profiles show an increase in RPFs around the start codon in differentiated NPCs, indicating an increase of initiating ribosomes in differentiated NPCs with respect to the undifferentiated conditions (**Figure 3-figure supplement 2D** [↗](#)). We observed a global increase in ribosome traffic after differentiation as evidenced by the increase in true-disome footprints among CDS and terminating codons (**Figure 3-figure supplement 2C** [↗](#)). By DESeq2 analysis, which does not consider transcript length in read-count normalization, we found in both differentiated and undifferentiated NPCs an increase in true-disome CDS occupancy, relative to monosome, correlates with CDS length (**Figure 3-figure supplement 2E** [↗](#)). This was expected since longer coding sequences are more likely to have more translating ribosomes per transcript than shorter ones.

Our ribosome profiling experiment indicates that for most mRNAs ( $n = 6,300$  of a total of 8,427, i.e. ~75%), translation efficiency levels (RPF counts / mRNA counts) remain unchanged upon NPC differentiation (**Figure 3B** [↗](#), Supplementary files 4-6). *ID2* is one of the transcripts with the highest increase in both RPFs and mRNA counts upon NPC differentiation (**Figure 3B** [↗](#)). Specifically, we observe a >5-fold RPF count increase upon NPC differentiation (**Figure 3C** [↗](#)), indicating a significant burst in *ID2* mRNA translation activity. However, we also observe that NPC differentiation results in a significant increase in *ID2* mRNA transcript levels (~ 15-fold, **Figure 3C** [↗](#)). Western blots of *ID2* and a second protein SLC38A2 (SNAT2) with increased RPFs on its mRNA in differentiated vs. undifferentiated NPCs are in agreement with the ribosome profiling data (**Figure 3D** [↗](#), **Figure 3-figure supplement 3** [↗](#)). Additional western blots for SLC38A2 can be found in **Figure 3-figure supplement 3** [↗](#). Notably, this observed stepwise increase in *ID2* mRNA levels and translation is well corroborated by our observed increase in eIF3 crosslinking to *ID2* mRNA's 3'-UTR termini, suggesting a connection for eIF3 in maintaining and promoting *ID2* translation upon differentiation beyond the 5'-UTR.

We next looked at the most actively translated transcripts in NPCs by sorting the ribosome profiling hits based on their amount of RPFs. We noticed that the most actively translated transcripts, such as *EEF1A* and *ACTB*, also present significant eIF3 peaks at their 3'-UTR termini (**Figure 3E** [↗](#)). Together with the *ID2* mRNA results, these data suggest that eIF3 interacts with 3'-UTR ends of mRNAs being actively translated.

To test whether the observed increase in eIF3 crosslinking to 3'-UTRs is linked to an increase in ribosome translation at a global level, we performed DESeq2 on the counted 3'-UTR eIF3 irCLIP reads to genes with 3'-UTRs longer than 50 nts. Indeed, regardless of whether we examined undifferentiated or differentiated NPCs, we found genes which had an increase in 3'-UTR irCLIP crosslinking tended to have an increase in ribosome occupancy (**Figure 3F** [↗](#), **Figure 3-figure supplement 4A** [↗](#)). By contrast, the increase in 3'-UTR irCLIP crosslinking correlated less well with increases in translational efficiency (**Figure 3G** [↗](#), **Figure 3-figure supplement 4B** [↗](#)). Overall, our ribosome profiling data combined with our eIF3 irCLIP data suggest that, in both undifferentiated and differentiated NPCs, there is a global increase in eIF3 engagement at 3'-UTR termini that correlates with an overall increase in protein synthesis.

## eIF3 interactions with 3'-UTR termini requires polyadenylation but is independent of poly(A) binding proteins

eIF3 crosslinking at 3'-UTR termini suggests that it must be in close proximity to the poly(A) tail and poly(A) tail binding proteins (PABPs). To determine whether the presence of eIF3 at 3'-UTR ends requires polyadenylation, we looked at histone mRNAs. Canonical histone mRNAs are the only known cellular non-polyadenylated mRNAs in eukaryotes due to their cell-cycle dependency and their need to be degraded rapidly. On the other hand, several variant histone mRNAs are not cell-cycle dependent and are polyadenylated. In non-polyadenylated canonical histone mRNAs (*H2AC1*, *H3C1*, and *H4-16*), we see no traces of eIF3 crosslinking at 3'-UTR termini nor APA events, as expected (**Figure 4A** [↗](#), **Figure 4-figure supplement 1A** [↗](#)). However, for the variant

polyadenylated histone mRNAs (*H3-3B* and *H2AZ1*), we observe eIF3 crosslinks that localize with APA-Seq peaks (**Figure 4A** [↗](#), **Figure 4-figure supplement 1A** [↗](#)). We also carried out a global comparison of the eIF3 Quick-irCLIP and APA-Seq crosslinking peaks, and observed that eIF3 crosslinking to the 3'-UTR termini had a positive association with polyadenylation (Methods). These data indicate that eIF3 only interacts with 3'-UTR termini of mRNAs that are polyadenylated, and thus should be in close proximity to the poly(A) tail.

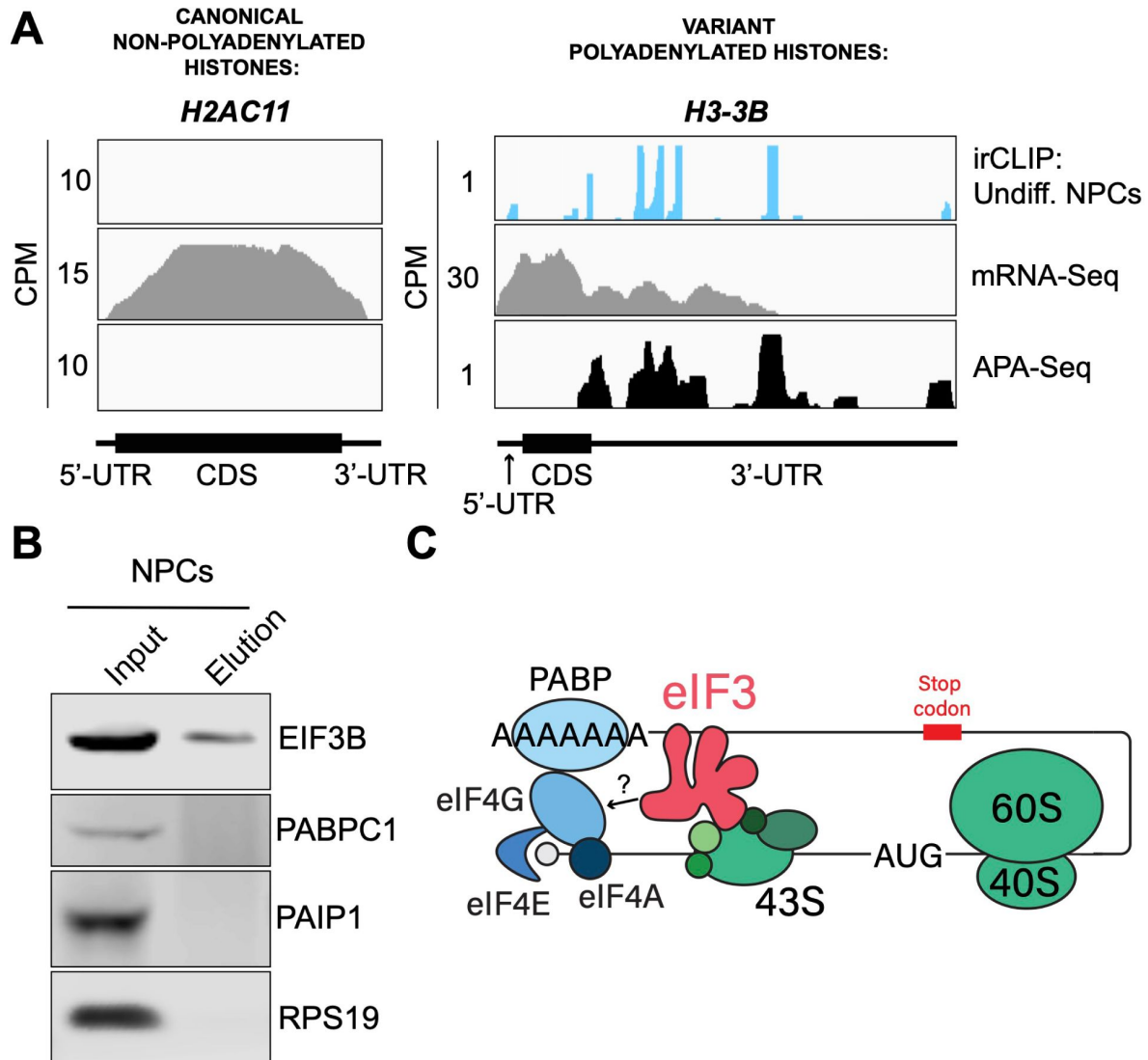
Our observations that eIF3 interacts with 3'-UTR termini in highly translated mRNAs support a model in which these mRNAs are circularized and their 5' and 3' ends are in close proximity. Since we observe eIF3 crosslinks adjacent to the poly(A) tail, it is conceivable that this eIF3-mediated mRNA circularization is stabilized through interactions between eIF3 and poly(A)-binding proteins. eIF3 has been reported to interact with the poly(A)-binding protein interacting protein 1 (PAIP1) in HeLa cells through the EIF3G subunit to promote closed loop formation (Martineau et al., 2008 [↗](#)). As in our Quick-irCLIP experiment, we immunoprecipitated assembled eIF3 complexes using an anti-EIF3B antibody from UV-crosslinked undifferentiated NPCs and immunoblotted against PAIP1 and against the major cytoplasmic isoform of the PABPs (PABPC1). Our results indicate that despite eIF3 being in close proximity to the location of the PABPs, these do not elute with it (**Figure 4B** [↗](#)). Immunoprecipitation of eIF3 complexes from UV-crosslinked HEK293T gave the same results (**Figure 4-figure supplement 1B** [↗](#)). It is possible that the eIF3 interactions with poly(A)-binding proteins are transient and eIF3 immunoprecipitation results in the disruption of these complexes.

Therefore, to maintain the integrity of these potentially transient interactions, we treated NPCs with the protein-protein crosslinker dithiobis(succinimidyl propionate) (DSP) before cell collection and eIF3 immunoprecipitation. DSP is a thiol-cleavable crosslinker so eluates can be treated with dithiothreitol (DTT) to cleave the disulfide bond in the spacer arm of DSP as a way to separate eIF3 from its polypeptide interactors. Our results show that even when protein-protein transient interactions are covalently linked by DSP treatment, PABPC1 and PAIP1 do not elute with eIF3 in NPCs (**Figure 4-figure supplement 1C** [↗](#)). The same experiments performed in HEK293T gave the same results (**Figure 4-figure supplement 1C** [↗](#)). Overall, these results suggest that eIF3 engagement with 3'-UTR termini in highly translated transcripts requires polyadenylation but is independent of interactions with poly(A)-binding proteins.

## Discussion

Here, we identified the transcripts that interact with eIF3 in hPSC-derived NPCs. NPCs that have undergone differentiation toward forebrain neurons exhibit a global increase in protein synthesis that we identified to occur 2 hours post differentiation treatment. Our Quick-irCLIP experiment shows that eIF3 predominantly crosslinks to 3'-UTRs in NPCs. By performing APA-Seq, we discovered that these eIF3 3'-UTR crosslinks map to 3'-UTR termini of multiple mRNA isoforms, adjacent to poly(A) tails and where the polyadenylation signal is located. Interestingly, we have also identified eIF3 irCLIP peaks within mRNA coding regions such as in *NES* mRNA (**Figure 2-figure supplement 2A** [↗](#)) that colocalize with APA-Seq peaks, indicating that different nestin protein isoforms are expressed in NPCs. Therefore, in addition to providing mRNA isoform-specific expression information, our APA-Seq experiment could potentially be used to investigate the expression of specific protein isoforms in NPCs.

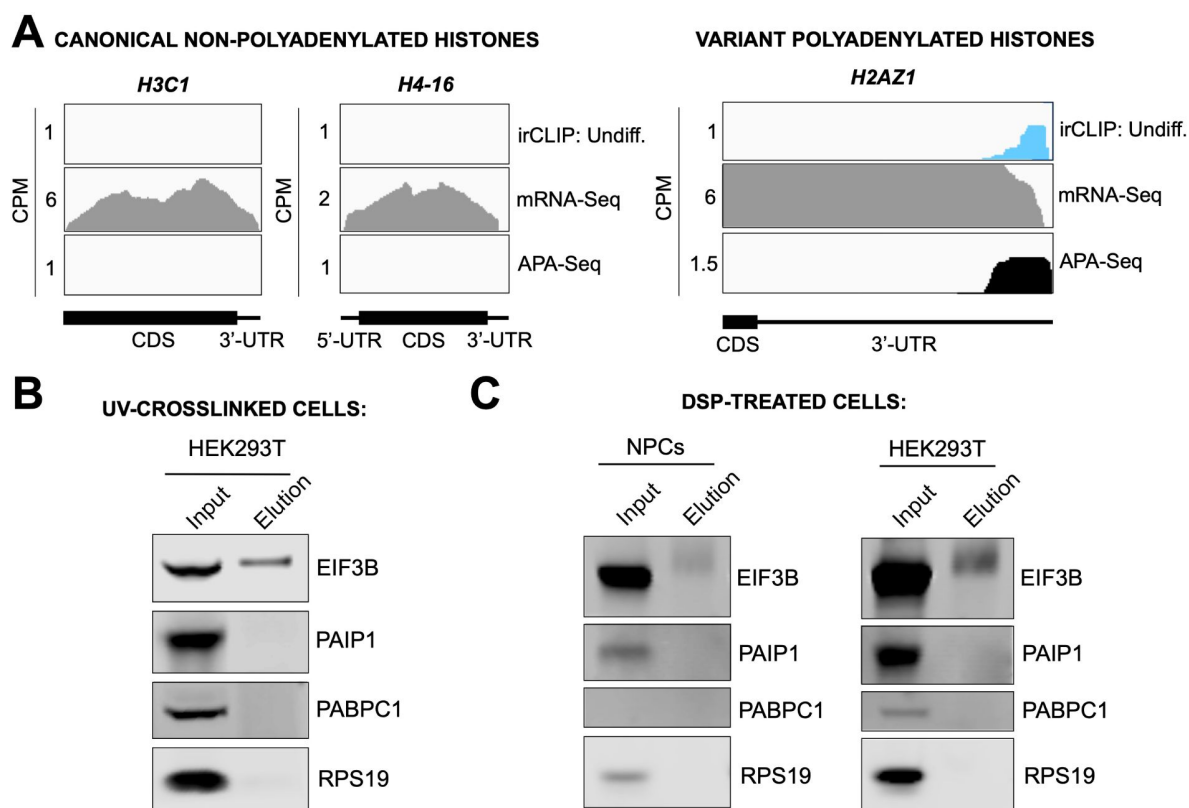
Here, we also observe that early NPC differentiation results in eIF3 engagement with the 3'-UTR terminus of the mRNA encoding the transcription regulator *ID2*. By ribosome profiling, we determined that this increase in eIF3 engagement on *ID2* mRNA is correlated with an increase in the levels of active transcription and translation of *ID2* (**Figure 3** [↗](#)). At a global level, we also observe that differentiated NPCs present higher levels of eIF3 crosslinks to 3'-UTRs and higher levels of active translation with respect to undifferentiated NPCs (**Figure 3F** [↗](#)), suggesting that



**Figure 4.**

**Crosslinking of eIF3 to polyadenylated mRNAs and model for mRNA circularization.**

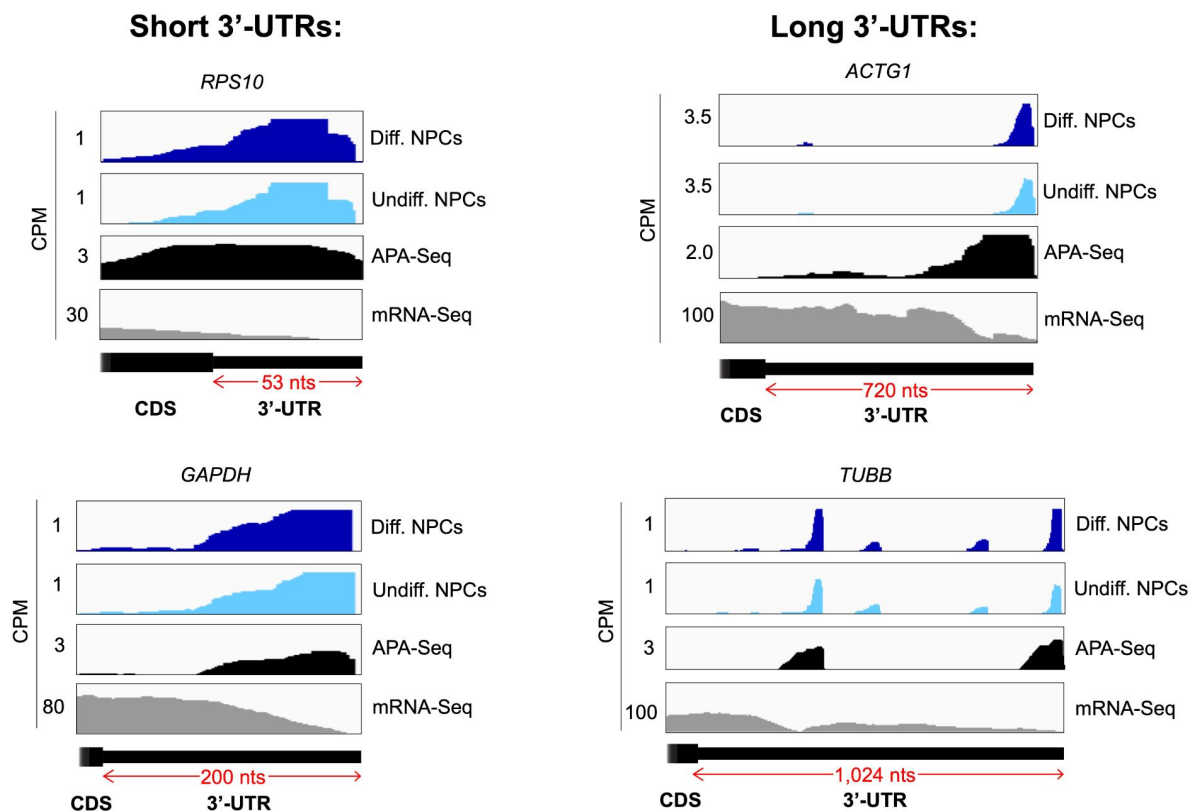
**A)** Crosslinking of eIF3 across the canonical non-polyadenylated histone *H2AC11* mRNA and the variant polyadenylated histone *H3-3B* mRNA in undifferentiated NPCs. APA-Seq and mRNA-Seq data for these regions are also shown. **B)** Western blots of EIF3B immunoprecipitations from undifferentiated NPCs. **C)** Model for eIF3 contribution to mRNA circularization in highly translated transcripts.



**Figure 4-figure supplement 1.**

### Mechanism of eIF3 binding to mRNA 3'-UTR elements.

**A)** Crosslinking of eIF3 to histone mRNAs in undifferentiated NPCs. Shown are canonical non-polyadenylated histone H3C1 and H4-16 mRNAs and the variant polyadenylated histone H2AZ1 mRNA. **B)** Western blots for EIF3B immunoprecipitation samples from UV-crosslinked HEK293T cells. **C)** Western blots for EIF3B immunoprecipitation samples from DSP-treated undifferentiated NPCs and HEK293T.



**Figure 4-figure supplement 2.**

### Crosslinking of eIF3 to mRNA 3'-UTRs of various lengths in NPCs.

Crosslinking patterns of eIF3 in differentiated and undifferentiated NPCs across *RPS10*, *GAPDH*, *ACTG1*, and *TUBB* mRNAs are shown. The length of each 3'-UTR is indicated. APA-Seq and mRNA-Seq data are also shown.

these two phenomena are correlated. In addition, we observe that the highest levels of eIF3 crosslinking at 3'-UTR ends are observed on the most actively translated mRNAs (e.g. *EEF1A* and *ACTB*, **Figure 3E**). Overall, these data suggest that actively translated mRNAs engage eIF3 at their 3'-UTR termini, likely reflecting eIF3's general role in translation. However, the underlying molecular mechanisms of the eIF3 engagement with the 3'-UTR terminus remain to be determined.

Our APA-Seq experiment performed in NPCs maps all polyadenylation events and it infers mRNA expression at isoform level. We found several instances where eIF3 CLIP peaks that colocalize with polyadenylation events have different peak heights with respect to those of the APA-Seq experiment. For instance, our APA-Seq data suggests that the proximal and distal 3'-UTR isoforms of the *S100B* mRNA are expressed at similar levels in NPCs (**Figure 2D**). However, we observe higher eIF3 engagement levels with the distal isoform than with the proximal isoform. Given that our ribosome profiling experiment indicates eIF3 engagement at 3'-UTR termini is observed on actively translated mRNAs, our eIF3 irCLIP and APA-Seq datasets could potentially be used in combination to determine active translation levels at mRNA isoform level, a feature that cannot be determined by ribosome profiling alone since RPF counts cannot be mapped to multiple mRNA isoforms.

eIF3 is an initiation factor that is usually associated with 5'-UTRs, where it organizes interactions between other initiation factors and the 40S ribosomal subunit during translation initiation. Its presence at the very end of 3'-UTRs of actively translated mRNAs supports the mRNA circularization model, where the 5' and 3' ends of highly translated mRNAs are proposed to be brought in close proximity to each other by the interactions of multiple proteins that bridge 5'-UTRs with 3'-UTRs (Wells et al., 1998). It is possible that our observation of eIF3 at 3'-UTR termini is due to the recycling of eIF3 from 3'-UTRs to 5'-UTRs for successive rounds of translation. However, this would require eIF3 association with translating ribosomes until translation termination and subsequently scanning through the entire length of 3'-UTRs either by direct interactions with mRNAs or through binding with post-termination 40S ribosomal subunits scanning through 3'-UTRs until the poly(A) tail is encountered. If that were the case one would expect to observe higher eIF3 levels at the ends of short 3'-UTRs than at those of long 3'-UTRs, and we observe no evidence of this (i.e. **Figure 4-figure supplement 2**).

Furthermore, recent results indicate that eIF3 associates with elongating ribosomes but it dissociates after ~ 60 codons from the start codon, before termination (Lin et al., 2020). Alternatively, given the large size of eIF3 (800 KDa in humans), it is possible that during mRNA circularization eIF3 interactions with 3'-UTR termini are accomplished by a specific eIF3 module while performing its canonical role as a scaffold protein during translation initiation (**Figure 4C**). In the closed loop model of translation it is proposed that the 5' and 3' ends of translated mRNAs are connected via the multiprotein-RNA interactions composed of the mRNA 5' cap - eIF4E - eIF4G - PABP - poly(A) tail (Wells et al., 1998), which are conserved across phylogeny. The disruption of these interactions results in a decrease in translation (Borman et al., 2000; Imataka et al., 1998; Kahvejian et al., 2005; Tarun and Sachs, 1996). mRNA circularization promoted by the canonical closed loop model has been observed *in vitro* by atomic force microscopy (Wells et al., 1998). Circular polysomes have also been observed on the rough endoplasmic reticulum (Christensen et al., 1987). These studies remain the only structural evidence for the model to date.

Since these studies, additional interactions between proteins regularly associated with 5'-UTRs and proteins commonly associated with 3'-UTRs have been discovered. For example, EIF3H has been shown to interact with METTL3, which binds to N6-methyladenosine (m6A) modified sites near the stop codon, an interaction proposed to enhance translation, indicating a role in looping highly translated mRNAs (Choe et al., 2018). Another eIF3 subunit, EIF3G, has also been reported to be involved in mRNA circularization. Studies performed in HeLa cells showed EIF3G

coimmunoprecipitates with PAIP1, suggesting a role in bridging 5'-UTRs with poly(A) tails (Martineau et al., 2008 [↗](#)). Here, we immunoprecipitated the eIF3 complex from NPCs and HEK293T using an anti-eIF3B antibody but did not observe PAIP1 or other poly(A)-associated proteins in the eIF3 eluates. It is possible that these divergent results are due to the fact that HeLa extracts present high levels of EIF2 $\alpha$  phosphorylation, which are not observed in other cell extracts such as HEK293T (Aleksashin et al., 2023 [↗](#)), suggesting that protein translation is highly dysregulated in HeLa cells and therefore the mechanisms of translation regulation might be significantly different than in other cells. Furthermore, the EIF3G-PAIP1 interaction is reported to be RNA independent, while our observations reveal an interaction of eIF3 with 3'-UTRs of polyadenylated mRNAs, so it is also possible that these are two independent mechanisms. Future studies will be needed to identify the proteins that may contact eIF3 when it resides at the end of the 3'-UTR adjacent to the poly(A) tail.

It is worth noting that the eIF3 3'-UTR termini crosslinking pattern was not observed in the two previous studies that used PAR-CLIP to identify the eIF3 mRNA-binding sites in HEK293T cells (Lee et al., 2015 [↗](#)) and Jurkat cells (De Silva et al., 2021 [↗](#)). Quick-irCLIP uses a short UV wavelength (254 nm) to crosslink RNA to proteins, whereas PAR-CLIP is performed at 365 nm and requires the cellular internalization of 4-thiouridine (4SU). These different UV wavelengths lead to different chemical susceptibilities for amino acid-nucleotide crosslinks (Ascano et al., 2012 [↗](#)). eIF3 is a large multisubunit complex so it is possible that different UV wavelengths capture transcript interactions through different eIF3 modules containing different amino acid propensity to crosslink to mRNAs at the two wavelengths. Using a different UV crosslinking method with respect to the previous work performed in HEK293T and Jurkat is a caveat when it comes to comparative studies such the one reported in Figure 1–figure supplement 2, in which eIF3-crosslinked mRNAs in NPCs are nearly disjoint from those crosslinked to eIF3 in HEK293T and Jurkat cells. However, it is striking that we were still able to identify some common 5'-UTR eIF3 binding sites (**Figure 1–figure supplement 3** [↗](#)).

Overall, our results provide new insights into the broad repertoire of roles that eIF3 plays during translation initiation. Besides its canonical role as a scaffold protein for initiation complex assembly (Jackson et al., 2010 [↗](#)) and its non-canonical roles in translation activation/repression by direct mRNA interactions at 5'- and 3'-UTRs (De Silva et al., 2021 [↗](#); Lee et al., 2015 [↗](#)), our study reveals a role in which eIF3 interacts with 3'-UTR termini of highly translated mRNAs and suggests eIF3 assists in the communication between 5' and 3' mRNA ends, supporting the mRNA circularization model to promote efficient recycling of ribosomes and translation factors for successive rounds of translation.

## Materials and methods

### Maintenance and propagation of human pluripotent stem cells (hPSCs)

The surface of all cultureware used for the maintenance and propagation of human pluripotent stem cells (WTC-11 line) was coated with Corning Matrigel (Fisher Scientific) prior to cell seeding. hPSCs were maintained under feeder-free conditions in complete mTeSR Plus medium (Stem Cell Technologies) and passaged using Gentle Cell Dissociation Reagent (Stem Cell Technologies).

### Generation of hPSC-derived neural progenitor cells (NPCs)

NPCs were generated from hPSCs using the embryoid body protocol (Stem Cell Technologies) following the manufacturer's protocol. At day 17, hPSC-generated NPCs (passage 1) were maintained and propagated using complete STEMdiff™ Neural Progenitor Medium (Stem Cell Technologies). Cells from passages 1 to 6 were cryopreserved.

## Maintenance and propagation of hPSC-derived NPCs

The surface of all cultureware used for the maintenance and propagation of hPSC-derived NPCs was coated with Corning Matrigel (Fisher Scientific) prior to cell seeding. Maintenance and propagation of hPSC-derived NPCs was performed following manufacturer's instructions. Briefly, NPCs were maintained using complete STEMdiff™ Neural Progenitor Medium (Stem Cell Technologies) for 7-9 days, with medium change every other day. NPCs were passaged using Accutase (Stem Cell Technologies) and seeded onto Matrigel-coated cultureware at an initial concentration of  $1.25 \times 10^5$  cells/mL.

## Differentiation treatment of hPSC-derived NPCs and identification of the global increase in protein synthesis

For differentiation studies, hPSC-derived NPCs were seeded at an initial concentration of  $5 \times 10^5$  cells/mL in STEMdiff™ Neural Progenitor Medium (Stem Cell Technologies) and allowed to sit at 37 °C for 48 hrs. After 48 hrs, the medium was changed to complete STEMdiff™ Forebrain Neuron Differentiation medium (Stem Cell Technologies) (for differentiated NPCs) or to fresh complete STEMdiff™ Neural Progenitor Medium (for undifferentiated NPCs) for 2 hrs at 37 °C and collected by washing with 1 mL PBS and scraping, followed by brief centrifugation. Cells were stored at -80 °C until further use. For protein synthesis assays, cells were treated with 20 mM puromycin for 15 min at 37 °C prior to collecting, and Western blotting was performed with an anti-puromycin antibody (Abcam, ab315887; 1:1,000 dilution) and imaged using Li-COR Odyssey CLx.

## eIF3 immunoprecipitation

Fifty µL of slurry Dynabeads Protein G (Invitrogen) were washed twice with 1 mL PBS + 0.01% Tween 20. Then, 25 µL of anti-eIF3B antibody (Bethyl, A301-757A) were added to washed beads and incubated at room temperature for 40 min with mixing by gentle rotation. For IPs with anti-eIF3B antibody performed to look for potential interactions with polyA-binding proteins, the anti-eIF3B antibody was crosslinked to Dynabeads Protein G with (bis(sulfosuccinimidyl)suberate) (BS3) (ThermoFisher Scientific) following the manufacturer's protocol. For cell lysis, NPCs were lysed using lysis buffer (50 mM Hepes-KOH, pH 7.5, 150 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM EDTA, 0.5% Nonidet P-40 alternative, 0.5 mM DTT, 1 Complete EDTA-free Proteinase Inhibitor Cocktail tablet per 10 mL of buffer). For IPs performed to look for potential eIF3B transient interactions with polyA-binding proteins, prior to collection, NPCs and HEK293T were treated with the protein-protein crosslinker dithiobis[succinimidyl]propionate (DSP) (1 mM) in PBS for 30 min at room temperature, and reaction was quenched by the addition of 10 mM Tris-HCl, pH 7.5 for 15 min at room temperature. Cells were then collected and lysed as described above. The cell pellets resuspended in lysis buffer were incubated on ice for 10 min, passed through a 18G needle four times, and centrifuged at 13,000g for 10 min at 4 °C. Twenty µL of lysate was saved as 5% input and the remaining lysate was loaded to washed beads conjugated to anti-eIF3b antibody and incubated at 4 °C for 2 hrs with gentle rotation.

Next, the beads were washed three times with lysis buffer. Then, eIF3-bound RNAs were eluted from the beads with 50 µL 1X NuPAGE LDS sample buffer and boiled at 70°C for 5 min. Samples were loaded onto a 4-12% Bis-Tris gel (Invitrogen), transferred to a nitrocellulose membrane, which was blotted against the desired proteins.

## Quick-irCLIP

Quick-irCLIP was performed as previously described (Kaczynski et al., 2019 [DOI](#)). NPCs subjected to Quick-irCLIP were prepared as follows. NPCs were seeded at an initial concentration of  $5 \times 10^5$  cells/mL and incubated at 37 °C for 48 hrs in STEMdiff™ Neural Progenitor Medium. Then, the medium was changed to either fresh STEMdiff™ Neural Progenitor Medium (undifferentiated

cells) or STEMdiff™ Forebrain Neuron Differentiation medium (differentiated NPCs) and incubated at 37 °C for 2 hrs. Cells were then crosslinked by irradiating them on ice with 0.15 J/cm<sup>2</sup> of 254 nm UV light.

Then, cells were collected by scraping in 1 mL PBS followed by centrifugation at 150x *g* for 5 min and stored at -80 °C until further use. Next, NPC pellets were allowed to thaw on ice for 10 min. In the meantime, 100 µL of slurry Dynabeads (Invitrogen) were washed twice with 1X PBS + 0.01% Tween 20. Then, 25 µL of anti-EIF3B antibody (Bethyl, A301-757A) were added to the washed beads solution and incubated at room temperature for 40 min with gentle rotation. Then, thawed NPCs were lysed by the addition of 1 mL of lysis buffer (50 mM Hepes-KOH, pH 7.5, 150 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM EDTA, 0.5% Nonidet P-40 alternative, 0.5 mM DTT, 1 Complete EDTA-free Proteinase Inhibitor Cocktail tablet per 10 mL of buffer) and allowed to sit on ice for 10 min. The resuspended pellet was then passed through a 18G needle four times, and centrifuged at 13,000x *g* for 10 min at 4 °C. Lysate protein concentration was determined by Bradford assay to determine the amount needed to obtain 0.5 mg of protein. Then, lysate with 0.5 mg per sample was transferred to a new Eppendorf tube. Ten µL of RNase I (Fisher Scientific) (1:1,000 dilution) and 2 µL of Turbo DNase (Fisher Scientific) were added to the lysate and allowed to mix at 37 °C for 3 minutes while shaking at 1100 rpm. We chose a 1:1,000 dilution of RNase I because it is sufficient to yield significant RNA smears where the RNA-binding eIF3 subunits EIF3A, EIF3B and EIF3D are located (**Figure 1–figure supplement 5** [↗](#)). Samples were then immediately placed on ice for 3 min followed by centrifugation at 18,000x *g* for 10 min at 4 °C. Supernatants were then transferred to new tubes. At this point, washed beads conjugated to anti-EIF3B antibody were washed twice with 1X PBS + 0.01% Tween 20 and washed once with lysis buffer. Then, RNase-treated lysates were loaded to conjugated beads and incubated at 4 °C for 2 hrs with gentle rotation. Beads were then washed twice with high-salt wash buffer (50 mM Hepes-KOH, pH 7.5, 500 mM KCl, 0.5% Nonidet P-40 alternative, 0.5 mM DTT, 5 mM MgCl<sub>2</sub>, 1 Complete EDTA-free Proteinase Inhibitor Cocktail tablet per 10 mL of buffer), and then with PNK wash buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 0.2% Tween-20). Beads were subsequently resuspended in 20 µL dephosphorylation reaction mix (1X PNK buffer, pH 6.5, 5 units of T4 polynucleotide kinase (New England Biolabs), 0.5 µL RNaseOut Ribonuclease inhibitor (Fisher Scientific)) and incubated for 20 min at 37 °C while shaking at 1100 rpm. Next, beads were washed once with PNK buffer, then washed once with high-salt wash buffer, and then washed again once with PNK buffer.

The following steps describe the ligation of the adaptor previously conjugated to an IR dye. Briefly, 20 µL of ligation reaction mix was prepared (1X ligation buffer, 5 units of T4 RNA ligase I (New England Biolabs), 0.5 µL RNaseOut Ribonuclease inhibitor, 1.5 µL of 10 mM infrared adaptor oligonucleotide, 4 µL polyethylene glycol 400). PNK buffer was removed from the beads and then 20 µL of ligation reaction mix were added to the beads and allowed to incubate at 16 °C while shaking at 1100 rpm overnight.

Next, beads were washed once with PNK buffer, twice with high-salt wash buffer, and once more with PNK buffer. Beads were then transferred to new Eppendorf tubes and resuspended in PNK buffer. Meanwhile, 500 mL of 1X NuPAGE MOPS-SDS buffer were prepared and 500 µL antioxidant (Fisher Scientific, cat#: NP0005) were added fresh before loading buffer use. PNK buffer was then removed from beads and samples were resuspended with 13 µL nuclease-free water, 2 µL sample reducing agent (Fisher Scientific, cat#: NP0004), and 5 µL 4X NuPAGE protein loading buffer. Samples were then incubated at 80 °C for 5 min, briefly centrifuged, and loaded onto a 4-12% SDS NuPAGE Bis-tris gel. The gel was run for 50 min at 180 V. Protein-RNA complexes were then transferred to a nitrocellulose membrane for 90 min at 30 V and then were visualized using a near infrared imager (Li-COR Odyssey CLx). The membrane was then put in a light-protected box. A grayscale image of the membrane was printed on acetate film, the membrane was wrapped in a plastic wrap and then the membrane and grayscale image were aligned using the ladder as reference.

Using a razor blade per sample, a box corresponding to the molecular weight range of eIF3 subunits EIF3A, EIF3B, EIF3C, and EIF3D (140 to 65 kDa) was cut into small pieces and transferred to a new Eppendorf tube. Proteins in the cut box were then digested by adding 10  $\mu$ L Proteinase K (20  $\mu$ g/ $\mu$ L) and 200  $\mu$ L PK buffer (100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM EDTA) and incubated at 37 °C for 20 min while shaking at 1100 rpm. Then, 200  $\mu$ L PK-Urea buffer (100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM EDTA, 7 M urea) and incubated for an additional 20 min while shaking at 37 °C at 1100 rpm. Supernatants were then transferred to phase lock heavy columns along with 400  $\mu$ L neutral phenol-chloroform and incubated for 5 min at 30 °C while shaking at 1100 rpm. Samples were then centrifuged for 5 min at 18,000x *g* at room temperature. The aqueous upper layers were transferred to new low-binding 1.5 mL tubes and centrifuged for 1 min at 18,000x *g* at room temperature. Samples were then transferred to new low-binding 1.5 mL tubes and 0.75  $\mu$ L glycogen (RNA grade, Fisher Scientific, cat#: FERR0551), 40  $\mu$ L 3 M sodium acetate (pH 5.5), and 1 mL 100% ethanol were added per sample. Samples were briefly vortexed and precipitated overnight at -20 °C.

The next day, samples were centrifuged for 20 min at 18,000x *g* at 4 °C, supernatants removed, leaving approximately 50  $\mu$ L of the RNA pellet. Then, 1 mL of 80% ethanol was added, without overly agitating the pellet. Supernatants were carefully removed and pellets were air-dried at room temperature. Pellets were then resuspended in 7  $\mu$ L nuclease-free water and transferred to new PCR tubes. Sequencing libraries were created following the protocol described in the SMARTer® smRNA-Seq Kit for Illumina user manual (Takara Bio).

## Total RNA cDNA library preparation

RNA samples were extracted from differentiated NPCs or undifferentiated NPCs treated for 2 hrs with STEMdiff™ Forebrain Neuron Differentiation medium (Stem Cell Technologies) for differentiated NPCs, or with fresh complete STEMdiff™ Neural Progenitor Medium for undifferentiated NPCs, using Trizol reagent (Thermo Fisher). cDNA libraries were prepared using NEBNext® ultra II Directional RNA Library Prep Kit for Illumina (NEBNext rRNA Depletion Kit v2).

## Quick-irCLIP Computational Analysis

Quick-irCLIP and Total RNA cDNA libraries were sequenced on an Illumina NovaSeq S1 150PE platform. Cutadapt (Martin, 2011 [DOI](#)) (version 3.5), with a minimum length of 20 nucleotides, was used to remove 3' adapter sequences. Strand-specific alignments were generated using HISAT v.2.2.1 (Kim et al., 2019 [DOI](#)) and hg38. Duplicate reads were removed with Picard (v2.21.9, <https://broadinstitute.github.io/picard/>) MarkDuplicates, followed by peak calling with HOMER (v4.11) findPeaks and a FDR cutoff of 0.01 (Heinz et al., 2010 [DOI](#)). Bedtools intersect (v2.29.2) was used to identify statistically significant peaks (BH-adjusted p-value < 1.0e-04) present in all replicates, then motifs were generated with HOMER findMotifsGenome. Peak statistics were tabulated using custom Python scripts. For coverage graphs, bams were CPM (Counts Per Million) normalized and converted to BigWigs with Deeptools (v3.5.1) (Ramírez et al., 2016 [DOI](#)) then visualized with IGV. Figures without irCLIP and APA-seq replicate tracks represent the union of all replicate reads, prepared with Samtools (v1.17) (Danecek et al., 2021 [DOI](#)) merge and CPM normalization. To assess the consistency of irCLIP bioreplicates (n = 3), the Spearman Correlation coefficient was calculated using deduplicated, CPM normalized BigWigs and DeepTools plotCorrelation. The average correlation coefficients were 0.7855 (standard deviation: 0.0092) for differentiated NPCs and 0.834 (standard deviation: 0.0058) for undifferentiated NPCs, indicating high reproducibility (**Figure 1–figure supplement 6** [DOI](#)). Additionally, to investigate the relationship between eIF3 3'-UTR crosslinking and translation (**Figure 3F** [DOI](#), **Figure 3–figure supplement 4A** [DOI](#)), the Spearman correlation coefficient was calculated between each transcript's average irCLIP RPKM and mean TE across replicates (n=3) for differentiated and undifferentiated cells. The Spearman correlation was also determined for irCLIP RPKM and average RPF coverage, normalized for coding sequence (CDS) length. In the case of TE values, outliers exceeding the TE's 99th percentile were removed, to make the log-log plots more readable.

## Comparison of eIF3 Quick-irCLIP results in NPCs with eIF3 PAR-CLIP results in Jurkat and HEK293T cells

Since the eIF3 PAR-CLIP experiments done in Jurkat and HEK293T cells were performed to identify the transcripts that interact with individual eIF3 subunits, first we obtained the RNAs that are common interactors of eIF3 subunits EIF3A (328 RNA-EIF3A reported clusters), EIF3B (264 RNA-EIF3B reported clusters), and d (356 RNA-EIF3D reported clusters) in HEK293T (Lee et al., 2015 [DOI](#)). The reason we only picked these subunits for this comparison study is because in our Quick-irCLIP experiment we only isolated the RNAs bound to these subunits. This rendered a list of 175 RNA-eIF3 common clusters between subunits EIF3A, EIF3B, and EIF3D in HEK293T. We then picked the top 400 transcripts that interact with activated Jurkat cells (De Silva et al., 2021 [DOI](#)) and performed the same analysis to find the common RNAs that bind to subunits EIF3A, EIF3B, and EIF3D, giving a total of 209 transcripts. Next, we identified the common transcripts between the two studies by comparing the 175 common in HEK293T with the 209 common in activated Jurkat cells, giving a total of 27 transcripts. Finally, we picked the top 210 transcripts (common in the 3 replicates) identified by eIF3 Quick-irCLIP in differentiated NPCs and compared them to the 175 common RNAs in HEK293T cells (resulting in 8 NPC-HEK293T common RNAs) and to the 209 common RNAs in activated Jurkat cells (resulting in 12 NPC-Jurkat common RNAs). Performing the same analysis with the top 210 common transcripts that bind eIF3 in undifferentiated NPCs gave the same results.

## Alternative Polyadenylation (APA)-Seq

Total RNA was extracted using Trizol Reagent from NPCs that were seeded at an initial concentration of  $5 \times 10^5$  cells/mL and incubated at 37 °C for 48 hrs in STEMdiff™ Neural Progenitor Medium. After 48 hrs, the media was changed to fresh STEMdiff™ Neural Progenitor Medium and NPCs were incubated 37 °C for 2 hrs and collected. Total RNA was then extracted (Trizol reagent) from NPCs. 3' mRNA-Seq libraries were prepared from 500 ng total RNA using QuantSeq REV kits (Lexogen) according to the manufacturer's protocol.

## APA-Seq Computational Analysis

APA-Seq and Total RNA cDNA libraries were sequenced on an Illumina NovaSeq S1 150PE platform. Cutadapt (version 3.5) (Martin, 2011 [DOI](#)) with a minimum length of 20 nucleotides was used to remove 3' adapter sequences. RNA-seq reads were aligned using Hisat2 v.2.2.1 (Kim et al., 2019 [DOI](#)) and mapped to the hg38 reference genome. SAMtools (Danecek et al., 2021 [DOI](#)) was used to remove PCR duplicates and filter uniquely mapped reads. Peaks were called with HOMER (v4.11) (Heinz et al., 2010 [DOI](#)) findPeaks at an FDR cutoff of 0.01.

## Statistical analysis of irCLIP eIF3 crosslinking and APA-Seq

We performed a statistical analysis to assess the dependency of eIF3 3'-UTR engagement on polyadenylation. For precision, non-overlapping 3'-UTRs were selected from transcripts expressed in the mRNA-Seq dataset (TPM > 1). APA-Seq and irCLIP peaks, consistent in all three biological replicates within these 3'-UTRs, were analyzed using a Fisher's Exact test. The analysis yielded a p-value of  $9.993 \times 10^{-43}$  and an odds ratio of 5.616, indicating a significant positive association between eIF3 3'-UTR binding and polyadenylation. To address the fact that APA-Seq and irCLIP were performed separately and each method cannot be used to distinguish between distinct mRNA isoforms, we focused the analysis on reproducible peaks within non-overlapping 3'-UTRs of expressed transcripts.

## Ribosome profiling

## Preparation of NPC lysates

Ribosome profiling was performed as previously described (Ferguson et al., 2023 [DOI](#); McGlinchy and Ingolia, 2017 [DOI](#)), with the following modifications. Briefly, NPCs were seeded in four Matrigel-coated 15-cm dishes per condition and allowed to reach 70-80% confluency in STEMdiff™ Neural Progenitor Medium (Stem Cell Technologies). Then, they were treated for 2 hrs with STEMdiff™ Forebrain Neuron Differentiation medium for differentiated NPCs, or STEMdiff™ Neural Progenitor Medium for undifferentiated NPCs. NPCs were treated with 100 µg/mL cycloheximide for 5 min prior to collection and washed with ice-cold PBS containing 100 µg/mL cycloheximide. PBS was discarded and 1.2 mL Lysis Buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 100 µg/mL cycloheximide, 1 % v/v Triton X-100, 25 U/mL Turbo DNase I) was added and cells were scraped, transferred to an Eppendorf tube, and allowed to sit on ice for 15 min. Cells were then passed 10 times through a 26 gauge needle and lysate was clarified by centrifugation (10 min, 20,000x g, 4 °C). Supernatants were recovered and stored at -80 °C.

## RNA quantification of NPC lysates

RNA concentration of lysates diluted in 1X TE buffer was quantified by using Quant-iT RiboGreen RNA kit (ThermoFisher) through direct comparison with a 0.0 - 1.0 ng/µL rRNA standard curve (ThermoFisher).

## P1 nuclease treatment of NPC lysates and ribosome pelleting

The pH of lysates was adjusted to 6.5 for optimal P1 nuclease activity by adding RNase-free 300 mM Bis-Tris pH 6.0 per 100 µL of cell lysate. Then, 450 Units of P1 Nuclease (100 U/µL, New England Biolabs) were added per 30 µg of RNA and lysates were incubated at 37 °C for 1 hr with gentle rotation. In the meantime, sucrose density gradients were prepared by dissolving 1M D-sucrose in 10 mL Polysome Buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 100 µg/mL cycloheximide) and 900 µL were added to 13 x 56 mm polycarbonate thick wall tube (Beckman Coulter). Then, 300 µL of digested lysates were carefully added to the top of the sucrose gradient and centrifuged at 100,000 RPM for 1 hr at 4 °C in a pre-chilled 4 °C TLA 100.3 rotor. The sucrose cushion was then aspirated and 30 µL nuclease-free water were added to the ribosome pellet, allowing it to sit on ice for 10 min. The ribosome pellet was disrupted by pipetting and 300 µL TRIzol (ThermoFisher) were added and the entire volume was transferred to a pre-chilled tube, vigorously vortexed and stored at -80 °C.

## mirRICH small RNA enrichment from pelleted ribosomes

A modified TRIzol (ThermoFisher) RNA extraction (Choi et al., 2018 [DOI](#)) was performed by first adding chloroform, vortexing, centrifuging, and isolating the aqueous phase as described by the manufacturer. RNA was precipitated by adding 100% isopropanol and incubating on ice for 15 min. RNA was pelleted by centrifugation at max speed for 15 min at 4 °C. Supernatant was carefully removed and pellets were allowed to air-dry at room temperature for 2 hrs. Dried pellets were then resuspended in 10 µL nuclease-free water, vortexed, and briefly centrifuged and incubated at room temperature for 5 min. Resuspended pellets were vortexed and briefly centrifuged again and 9 µL of the eluate liquid was removed and purified by RNA & Clean Concentrator-5, eluted in 25 µL nuclease-free water, and quantified by Nanodrop.

## cDNA size selection, elution, and quantification

The protocol from Ferguson et al. (Ferguson et al., 2023 [DOI](#)) was followed for the OTTR reaction. OTTR reaction products were resuspended in bromophenol blue formamide loading dye. OTTR cDNAs were resolved by electrophoresis in 0.6X TBE 8% Urea-PAGE and visualized by fluorescence

using the 5' Cy5-dye. Gel excision and elution was performed as described (Ferguson et al., 2023 [↗](#)). To quantify cDNA by qPCR and determine the number of PCR cycles needed for library amplification, we followed a previously described method (McGlinchy and Ingolia, 2017 [↗](#)).

## Ribosome profiling computational analysis

Ribosome profiling and Total RNA cDNA libraries were sequenced on an Illumina NovaSeq 150PE platform. The adapters were trimmed, the unique molecular identifier (UMI) was appended to the FASTQ entry header, and reads shorter than 20 were excluded using Cutadapt, as described (Ferguson et al., 2023 [↗](#)):

```
cutadapt -m 2 -a GATCGGAAGAGCACAGTCTGAACTCCAGTCAC
```

```
$tmpvar.fastq |
```

```
cutadapt -u 7 --rename='{id} UMI={cut_prefix}' - | cutadapt -u 1 --  
rename='{id}_{comment}_primer={cut_suffix}' - | cutadapt -m 20 -q 10 -
```

Human references described here were used as in (Ferguson et al., 2023 [↗](#)).

Briefly, human rRNA sequences NR\_023379.1, NR\_146151.1, NR\_146144.1, NR\_145819.1, NR\_146117.1, NR\_003285.3, NR\_003286.4, X12811.1, and NR\_003287.4 from NCBI and ENST00000389680.2 and ENST00000387347.2 from Ensembl were used as the rRNA reference. The human tRNA sequences and references were prepared as described (Holmes et al., 2022 [↗](#)). ncRNA from Ensembl and lncRNA from Gencode were concatenated. The primary assembly of the human gDNA from NCBI, GRCh38, was used. 18,640 NCBI RefSeq MANE v0.95 transcripts were used, and the 5'-UTR, CDS, and 3'-UTR lengths were parsed from the GenBank entries using a custom script. The 13 mitochondrial mRNA sequences from Ensembl were also included in the mRNA reference, but typically excluded from analysis.

Contaminating reads were removed with a sequential analysis pipeline as described (Ferguson et al., 2023 [↗](#)). Briefly, the trimmed reads were first mapped to the rRNA reference (bowtie -v 3 -a --best --norc), then tRNA reference by tRAX (Holmes et al., 2022 [↗](#)), then ncRNA reference (bowtie -v 3 -a --best --norc), and then mtDNA reference (bowtie -v 2 -a --best --norc). The remaining reads were then mapped to the mRNA references (bowtie -v 2 -m 200 -a --norc) and sorted (samtools sort -n). Remaining reads were aligned to the gDNA reference (bowtie -v 2 -a --best) before mapping unaligned reads to a reference of size-selecting oligos and adapter oligos (bowtie -v 2 -a --best).

To measure CDS occupancy we used Bedtools intersect (v2.25.0) to exclude reads which aligned outside of the +15th to -10th codon of the CDS, counted reads using RSEM (rsem-calculate-expression --alignments --strandedness forward --seed-length 20 --sampling-for-bam). Gene counts were then normalized and analyzed by DESeq2 (Love et al., 2014 [↗](#)). Three replicates were produced per condition. DESeq2 normalized (median-of-ratios) gene-level counts for each replicated condition were averaged. mRNA sequencing libraries were processed by an identical pipeline and normalized and analyzed by DESeq2. To compare ribosome profiling and irCLIP datasets, the irCLIP reads were re-processed by the same pipeline for ribosome profiling libraries, but only the irCLIP read counts to the 3'-UTR region of genes were analyzed by DESeq2.

For initiation, termination, and solitary codon occupancy profiles, only genes with  $\geq 50$  nt of annotated 5'-UTR,  $\geq 450$  nt of annotated CDS,  $\geq 50$  nt of annotated 3'-UTR, and  $\geq 1$  reads per codon were considered. Alignments were counted based on read length and either 5' or 3' position relative to the first base of a codon of interest (e.g., start codon), and rescaled by average number of reads per codon for a given gene.

Rescaled counts for each read length and relative position were summed and divided by the number of genes under consideration. For visualization, read lengths were binned (e.g., read lengths of 58, 59, and 60 nt were binned to 58 – 60 nt) and summed by rescaled counts. A-site codon offsets for P1 nuclease footprints were assigned based on the frame of the 5' end of the read, as described in (Ferguson et al., 2023 [DOI](#)).

## Data availability statement

Data underlying this article are publicly available. irCLIP-seq and RNA-seq data are deposited under GEO accession GSE246727. Sequencing data from the APA-seq experiment are available as GEO accession GSE246786. Ribosome profiling data are accessible as SRA project ID PRJNA1029246. Scripts for data processing are available upon request.

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## Additional information

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### Competing interests

L.F. is a named inventor on patent applications filed by the University of California describing biochemical activities of RTs used for OTTR.

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## Joint Public Review:

Reviewers thought that the authors addressed some, but not all the concerns raised in the previous round of a review.

**Strengths:** The authors employed a battery of next-generation sequencing and crosslinking techniques (e.g., Quick-irCLIP, APA-Seq, and Ribo-Seq) to describe a previously unappreciated binding of eIF3 to the 3'UTRs of the mRNAs. It is also shown that eIF3:3'UTR binding occurs in the vicinity of poly(A) tail of mRNAs that are actively translated in neuronal progenitor cells derived from human pluripotent stem cells. Collectively, these findings provide evidence for the role of eIF3 in regulating translation from the 3'UTR end of the mRNA.

**Weaknesses:** In addition to these clear strengths of the article, some weaknesses were observed pertinent to the lack of mechanistic data. It was therefore thought that the experiments aiming to dissect the mechanisms of eIF3 binding to 3'UTRs and their impact on translation warrant future studies. Finally, establishing the impact of the proposed eIF3:3'UTR binding mechanism of translational regulation on cellular fate is required to further support the biological importance of the observed phenomena. It was found that this should also be addressed in the follow up studies.

<https://doi.org/10.7554/eLife.102977.2.sa1>

#### **Author response:**

The following is the authors' response to the current reviews.

The authors agree with the reviewers that future studies are needed to dissect the mechanisms of eIF3 binding to 3'UTRs and their impact on translation, and the impact of this binding on cellular fate.

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The following is the authors' response to the original reviews.

#### **eLife Assessment**

*This valuable study reveals extensive binding of eukaryotic translation initiation factor 3 (eIF3) to the 3' untranslated regions (UTRs) of efficiently translated mRNAs in human pluripotent stem cell-derived neuronal progenitor cells. The authors provide solid evidence to support their conclusions, although this study may be enhanced by addressing potential biases of techniques employed to study eIF3:mRNA binding and providing additional mechanistic detail. This work will be of significant interest to researchers exploring post-transcriptional regulation of gene expression, including cellular, molecular, and developmental biologists, as well as biochemists.*

We thank the reviewers for their positive views of the results we present, along with the constructive feedback regarding the strengths and weaknesses of our manuscript, with which we generally agree. We acknowledge our results will require a deeper exploration of the molecular mechanisms behind eIF3 interactions with 3'-UTR termini and experiments to identify the molecular partners involved. Additionally, given that NPC differentiation toward mature neurons is a process that takes around 3 weeks, we recognize the importance of examining eIF3-mRNA interactions in NPCs that have undergone differentiation over longer periods than the 2-hr time point selected in this study. Finally, considering the molecular complexity of the 13subunit human eIF3, we agree that a direct comparison between Quick-irCLIP and PAR-CLIP will be highly beneficial and will determine whether different UV crosslinking wavelengths report on different eIF3 molecular interactions. Additional comments are given below to the identified weaknesses.

#### **Public Reviews:**

##### **Reviewer #1 (Public review):**

##### *Summary:*

*The authors perform irCLIP of neuronal progenitor cells to profile eIF3-RNA interactions upon short-term neuronal differentiation. The data shows that eIF3 mostly interacts with 3'-UTRs - specifically, the poly-A signal. There appears to be a general correlation*

*between eIF3 binding to 3'-UTRs and ribosome occupancy, which might suggest that eIF3 binding promotes protein*

**Strengths:**

*The study provides a wealth of new data on eIF3-mRNA interactions and points to the potential new concept that eIF3-mRNA interactions are polyadenylation-dependent and correlate with ribosome occupancy.*

**Weaknesses:**

*(1) A main limitation is the correlative nature of the study. Whereas the evidence that eIF3 interacts with 3'-UTRs is solid, the biological role of the interactions remains entirely unknown. Similarly, the claim that eIF3 interactions with 3'-UTR termini require polyadenylation but are independent of poly(A) binding proteins lacks support as it solely relies on the absence of observable eIF3 binding to poly-A (-) histone mRNAs and a seeming failure to detect PABP binding to eIF3 by co-immunoprecipitation and Western blotting. In contrast, LC-MS data in Supplementary File 1 show ready co-purification of eIF3 with PABP.*

We agree the molecular mechanisms underlying the crosslinking between eIF3 and the end of mRNA 3'-UTRs remains to be determined. We also agree that the lack of interaction seen between eIF3 and PABP in Westerns, even from HEK293T cells, is a puzzle. The low sequence coverage in the LC-MS data gave us pause about making a strong statement that these represent direct eIF3 interactions, given the similar background levels of some ribosomal proteins.

*(2) Another question concerns the relevance of the cellular model studied. irCLIP is performed on neuronal progenitor cells subjected to neuronal induction for 2 hours. This short-term induction leads to a very modest - perhaps 10% - and very transient 1-hour-long increase in translation, although this is not carefully quantified. The cellular phenotype also does not appear to change and calling the cells treated with differentiation media for 2 hours "differentiated NPCs" seems a bit misleading. Perhaps unsurprisingly, the minor "burst" of translation coincides with minor effects on eIF3-mRNA interactions most of which seem to be driven by mRNA levels. Based on the ~15-fold increase in ID2 mRNA coinciding with a ~5-fold increase in ribosome occupancy (RPF), ID2 TE actually goes down upon neuronal induction.*

We agree that it will be interesting to look at eIF3-mRNA interactions at longer time points after induction of NPC differentiation. However, the pattern of eIF3 crosslinking to the end of 3'-UTRs occurs in both time points reported here, which is likely to be the more general finding in what we present.

*(3) The overlap in eIF3-mRNA interactions identified here and in the authors' previous reports is minimal. Some of the discrepancies may be related to the not well-justified approach for filtering data prior to assessing overlap. Still, the fundamentally different binding patterns - eIF3 mostly interacting with 5'-UTRs in the authors' previous report and other studies versus the strong preference for 3'-UTRs shown here - are striking. In the Discussion, it is speculated that the different methods used - PAR-CLIP versus irCLIP - lead to these fundamental differences. Unfortunately, this is not supported by any data, even though it would be very important for the translation field to learn whether different CLIP methodologies assess very different aspects of eIF3-mRNA interactions.*

We agree the more interesting aspect of what we observe is the difference in location of eIF3 crosslinking, i.e. the end of 3'-UTRs rather than 5'-UTRs or the pan-mRNA pattern we

observed in T cells. The reviewer is right that it will be important in the future to compare PAR-CLIP and Quick-irCLIP side-by-side to begin to unravel the differences we observe with the two approaches.

**Reviewer #2 (Public review):**

*Summary:*

*The paper documents the role of eIF3 in translational control during neural progenitor cell (NPC) differentiation. eIF3 predominantly binds to the 3' UTR termini of mRNAs during NPC differentiation, adjacent to the poly(A) tails, and is associated with efficiently translated mRNAs, indicating a role for eIF3 in promoting translation.*

*Strengths:*

*The manuscript is strong in addressing molecular mechanisms by using a combination of nextgeneration sequencing and crosslinking techniques, thus providing a comprehensive dataset that supports the authors' claims. The manuscript is methodologically sound, with clear experimental designs.*

*Weaknesses:*

*(1) The study could benefit from further exploration into the molecular mechanisms by which eIF3 interacts with 3' UTR termini. While the correlation between eIF3 binding and high translation levels is established, the functionality of these interactions needs validation. The authors should consider including experiments that test whether eIF3 binding sites are necessary for increased translation efficiency using reporter constructs.*

We agree with the reviewer that the molecular mechanism by which eIF3 interacts with the 3'UTR termini remains unclear, along with its biological significance, i.e. how it contributes to translation levels. We think it could be useful to try reporters in, perhaps, HEK293T cells in the future to probe the mechanism in more detail.

*(2) The authors mention that the eIF3 3' UTR termini crosslinking pattern observed in their study was not reported in previous PAR-CLIP studies performed in HEK293T cells (Lee et al., 2015) and Jurkat cells (De Silva et al., 2021). They attribute this difference to the different UV wavelengths used in Quick-irCLIP (254 nm) and PAR-CLIP (365 nm with 4-thiouridine). While the explanation is plausible, it remains a caveat that different UV crosslinking methods may capture different eIF3 modules or binding sites, depending on the chemical propensities of the amino acid-nucleotide crosslinks at each wavelength. Without addressing this caveat in more detail, the authors cannot generalize their findings, and thus, the title of the paper, which suggests a broad role for eIF3, may be misleading. Previous studies have pointed to an enrichment of eIF3 binding at the 5' UTRs, and the divergence in results between studies needs to be more explicitly acknowledged.*

We agree with the reviewer that the two methods of crosslinking will require a more detailed head-to-head comparison in the future. However, we do think the title is justified by the fact that we see crosslinking to the termini of 3'-UTRs across thousands of transcripts in each condition. Furthermore, the 3'-UTR crosslinking is enriched on mRNAs with higher ribosome protected fragment counts (RPF) in differentiated cells, Figure 3F.

*(3) While the manuscript concludes that eIF3's interaction with 3' UTR termini is independent of poly(A)-binding proteins, transient or indirect interactions should be tested using assays such as PLA (Proximity Ligation Assay), which could provide more insights.*

This is a good idea, but would require a substantial effort better suited to a future publication. We think our observations are interesting enough to the field to stimulate future experimentation that we may or may not be most capable of doing in our lab.

**Reviewer #3 (Public review):**

*Summary:*

*In this manuscript by Mestre-Fos and colleagues, authors have analyzed the involvement of eIF3 binding to mRNA during differentiation of neural progenitor cells (NPC). The authors bring a lot of interesting observations leading to a novel function for eIF3 at the 3'UTR.*

*During the translational burst that occurs during NPC differentiation, analysis of eIF3-associated mRNA by Quick-irCLIP reveals the unexpected binding of this initiation factor at the 3'UTR of most mRNA. Further analysis of alternative polyadenylation by APAseq highlights the close proximity of the eIF3-crosslinking position and the poly(A) tail. Furthermore, this interaction is not detected in Poly(A)-less transcripts. Using Riboseq, the authors then attempted to correlate eIF3 binding with the translation efficacy of mRNA, which would suggest a common mechanism of translational control in these cells. These observations indicate that eIF3-binding at the 3'UTR of mRNA, near the poly(A) tail, may participate to the closed-loop model of mRNA translation, bridging 5' and 3', and allowing ribosomes recycling. However, authors failed to detect interactions of eIF3, with either PABP or Paip1 or 40S subunit proteins, which is quite unexpected.*

*Strength:*

*The well-written manuscript presents an attractive concept regarding the mechanism of eIF3 function at the 3'UTR. Most mRNA in NPC seems to have eIF3 binding at the 3'UTR and only a few at the 5'end where it's commonly thought to bind. In a previous study from the Cate lab, eIF3 was reported to bind to a small region of the 3'UTR of the TCRA and TCRB mRNA, which was responsible for their specific translational stimulation, during T cell activation. Surprisingly in this study, the eIF3 association with mRNA occurs near polyadenylation signals in NPC, independently of cell differentiation status. This compelling evidence suggests a general mechanism of translation control by eIF3 in NPC. This observation brings back the old concept of mRNA circularization with new arguments, independent of PABP and eIF4G interaction. Finally, the discussion adequately describes the potential technical limitations of the present study compared to previous ones by the same group, due to the use of Quick-irCLIP as opposed to the PAR-CLIP/thiouridine.*

*Weaknesses:*

*(1) These data were obtained from an unusual cell type, limiting the generalizability of the model.*

We agree that unraveling the mechanism employed by eIF3 at the mRNA 3'-UTR termini might be better studied in a stable cell line rather than in primary cells.

*(2) This study lacks a clear explanation for the increased translation associated with NPC differentiation, as eIF3 binding is observed in both differentiated and undifferentiated NPC. For example, I find a kind of inconsistency between changes in Riboseq density (Figure 3B) and changes in protein synthesis (Figure 1D). Thus, the title overstates a modest correlation between eIF3 binding and important changes in protein synthesis.*

We thank the reviewer for this question. Riboseq data and RNASeq data are not on absolute scales when comparing across cell conditions. They are normalized internally, so increases in for example RPF in Figure 3B are relative to the bulk RPF in a given condition. By contrast, the changes in protein synthesis measured in Figure 1D is closer to an absolute measure of protein synthesis.

*(3) This is illustrated by the candidate selection that supports this demonstration. Looking at Figure 3B, ID2, and SNAT2 mRNA are not part of the High TE transcripts (in red). In contrast, the increase in mRNA abundance could explain a proportionally increased association with eIF3 as well as with ribosomes. The example of increased protein abundance of these best candidates is overall weak and uncertain.*

We agree that using TE as the criterion for defining increased eIF3 association would not be correct. By “highly translated” we only mean to convey the extent of protein synthesis, i.e. increases in ribosome protected fragments (RPF), rather than the translational efficiency.

*(4) Despite several attempts (chemical and UV cross-linking) to identify eIF3 partners in NPC such as PABP, PAIP1, or proteins from the 40S, the authors could not provide any evidence for such a mechanism consistent with the closed-loop model. Overall, this rather descriptive study lacks mechanistic insight (eIF3 binding partners).*

We agree that it will be important to identify the molecular mechanism used by eIF3 to engage the termini of mRNA 3'-UTRs. Nevertheless, the identification of eIF3 crosslinking to that location in mRNAs is new, and we think will stimulate new experiments in the field.

*(5) Finally, the authors suspect a potential impact of technical improvement provided by QuickirCLIP, that could have been addressed rather than discussed.*

We agree a side-by-side comparison of eIF3 crosslinks captured by PAR-CLIP versus QuickirCLIP will be an important experiment to do. However, NPCs or other primary cells may not be the best system for the comparison. We think using an established cell line might be more informative, to control for effects such as 4-thiouridine toxicity.

#### **Recommendations for the authors:**

##### **Reviewer #1 (Recommendations for the authors):**

*(1) The Western blot signals for SLC38A2 and ID2 are close to the membrane background and little convincing. Size markers are missing.*

We agree these antibodies are not great. They are the best we could find, unfortunately. We have included originals of all western blots and gels as supplementary information. It's important to note that the Riboseq data for ID2 and SLC38A2 are consistent with the western blots. See Figure 3C and Figure 3-figure supplement 3B.

*(2) Figure 1 - Figure Supplement 1 appears to present data from a single experiment. This is far less than ideal considering the minor differences measured.*

Thanks for the comment. This is a representative experiment showing the early time course. We have added a second experiment with two different treatments that show the same pattern in the puromycin assay, in Figure 1-figure supplement 1.

*(3) Figure 3F: One wonders what this would look like if TE was plotted instead of RPF. Figure 3 - Figure Supplement 4 seems to show something along those lines. However, the*

*data are not mentioned in the main results section are quite unclear. Why are data separated into TE high and low? Doesn't TE high in differentiated cells equal TE low in undifferentiated cells?*

This is an interesting question. Note that in Figure 3B,  $n=6300$  genes show no change in TE upon differentiation, compared to a total of  $n=2127$  that show a change in TE, with most of those changes not very large. We have now replotted Figure 3F comparing irCLIP read counts in 3'-UTRs to RPF read counts, which shows a significant positive correlation, regardless of whether we look at undifferentiated or differentiated NPCs (See Figure 3F and a new Figure 3-figure supplement 4A). We also compare irCLIP reads in 3'-UTRs to TE values, which show no correlation (See Figure 3G and Figure 3-figure supplement 4B).

Figure 3-figure supplement 4 was actually a response to a previous round of review (at PLOS Biology) to a rather technical question from a reviewer. We think this figure and associated text should be removed. Instead, we now include supplementary tables with the processed RPF and TE values, for reference (Supplemental files 4-6). We omitted these in the original submission when they should have been included. We also abandoned comparing undifferentiated and differentiated NPCs, and instead look directly at irCLIP reads vs. RPFs or TE, regardless of NPC state, as noted above (Figure 3F, G, and Figure 3-figure supplement 4).

*(4) Figure 3C: The data should be plotted on the same y-axis scale. This would make a visual assessment of the differences in mRNA and RFP levels more intuitive.*

Thanks for this suggestion. We have rescaled the plots as requested.

#### **Reviewer #2 (Recommendations for the authors):**

*(1) The quality of the Western blots in several figures is quite poor. Notably, Figure 1C seems to be a composite gel, as each blot appears to come from a different gel. Additionally, in Supplementary Figure 1A, there is only a single data point, yet the authors indicate that this image is representative of multiple assays. The lack of error bars in this figure raises a question vis-a-vis the reproducibility of the experiments.*

Thanks for the comments. We now include all the original gels as supplementary information. As noted above, the antibodies for ID2 and SLC38A2 are not great, we agree. And as we noted above, the Riboseq data for ID2 and SLC38A2 are consistent with the western blots.

*(2) For the top 500 targets of undifferentiated and differentiated NPCs in the Quick-irCLIP assay, the manuscript does not clarify how many targets are common and how many are unique to each condition. This information is important for understanding the extent of overlap and differentiation-specific interactions of eIF3 with mRNAs. Providing this data would strengthen the interpretation of the results.*

There are 449 of the top 500 hits in common between undifferentiated and differentiated NPCs. We have now added this information to the text, to add clarity.

*(3) The manuscript does not provide detailed percentages or numbers regarding the overlap between iCLIP and APA-Seq peaks. Clarifying this overlap, particularly in terms of how many of the APA sites are also targets of eIF3, would bolster the understanding of how these two datasets converge to support the authors' conclusions.*

This is a difficult calculation to make, due to the fact that APA-Seq reads are generally much longer than the Quick-irCLIP reads. This is why we focused instead on quantifying the

percent of Quick-irCLIP peaks (which are more narrow) overlap with predicted polyadenylation sequences, in Figure 2-figure supplement 1.

**Reviewer #3 (Recommendations for the authors):**

*(1) Perform Quick-irCLIP in HEK293 cells to infer technical limitations and/or to generalize the model. The authors will then compare again eIF3 binding site in Jurkat, HEK293, and NPC.*

This is an experiment we plan to do for a future publication, given that we would want to repeat both Quick-irCLIP and PAR-CLIP at the same time.

*(2) Select mRNA candidates with high or low TE changes and analyze eIF3 binding and RPF density and protein abundance along NPC differentiation to support the role of eIF3 binding in stimulating translation.*

We agree looking at time courses in more depth would be interesting. However, this would require substantial experimentation, which is better suited to a future study. Furthermore, now that we have moved away from comparing undifferentiated NPCs and differentiated NPCs when examining TE and RPF values (Figure 3 and Figure 3-figure supplement 4), we think the results now support a more general mechanism of translation reflected in the irCLIP 3'-UTR vs. RPF correlation, independent of NPC state.

*(3) Analyze the interaction of eIF3 with eIF4G and other known partners. This will really provide an improvement to the manuscript. The lack of interaction between eIF3 and the 40S is quite surprising.*

We agree more work needs to be done on the mechanistic side. These are experiments we think would be best to carry out in a stable cell line in the future, rather than primary cells.

*(4) Perform Oligo-dT pulldown (or cap column if possible) and analyze the relative association of PABP, eIF3, and eIF4F on mRNA in NPC versus HEK293. This will clarify whether this mechanism of mRNA translation is specific to NPC or not.*

Thanks for this suggestion. We are uncertain how it would be possible to deconvolute all the possible ways to interpret results from such an experiment. We agree thinking about ways to study the mechanism will keep us occupied for a while.

*(5) Citations in the text indicate the first author, whereas the references are numbered!*

Our apologies for this oversight. This was a carryover from previous formatting, and has been fixed.

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