

## Expanded View Figures

### Figure EV1. UPF3A overexpression does not cause upregulation of NMD-targets.

- A Western blot analysis of unaltered HEK293 WT cells or with induced FLAG-UPF3A or FLAG-UPF3B expression. UPF3A, UPF3B, and FLAG levels were detected. Tubulin serves as control. Protein levels were quantified, normalized to tubulin expression, and shown as datapoints and mean ( $n = 4$ ). Fold-changes of relevant conditions are shown.
- B Skyline analysis of WT and UPF3A-overexpressing cells after whole proteome mass spec analysis. Quantifier intensities of UPF3A (left) and UPF3B (right) were normalized to actin (top) and tubulin (bottom) which were used as “loading controls”. Results are shown as datapoints and mean ( $n = 4$ ). The means were used to calculate the respective fold-changes.
- C Schematic overview of the analysis pipeline.
- D Heatmap of mean log<sub>2</sub> fold changes (log<sub>2</sub>FC) of previously reported UPF3A-responsive NMD targets (Fig 3C of Shum *et al*, 2016) as determined by DESeq2 using the indicated RNA-Seq data. The data from SMG7 KO with SMG6 KD (Data ref: Boehm *et al*, 2021) serve as positive control for NMD inhibition.
- E Volcano plot showing the differential transcript usage (via IsoformSwitchAnalyzeR) in RNA-Seq data of HEK293 and HeLa WT cells overexpressing UPF3A. Isoforms containing GENCODE (release 33) annotated PTC (red, TRUE), regular stop codons (blue, FALSE) or having no annotated open reading frame (gray, NA) are indicated. The change in isoform fraction (dIF) is plotted against the  $-\log_{10}$  adjusted  $P$ -value ( $P_{\text{adj}}$ ). Density plots show the distribution of filtered isoforms in respect to the dIF, cutoffs were  $|\text{dIF}| > 0.1$  and  $P_{\text{adj}} < 0.05$ .  $P$ -values were calculated by IsoformSwitchAnalyzeR using a DEXSeq-based test and corrected for multiple testing using the Benjamini–Hochberg method. OE = overexpression.

Source data are available online for this figure.

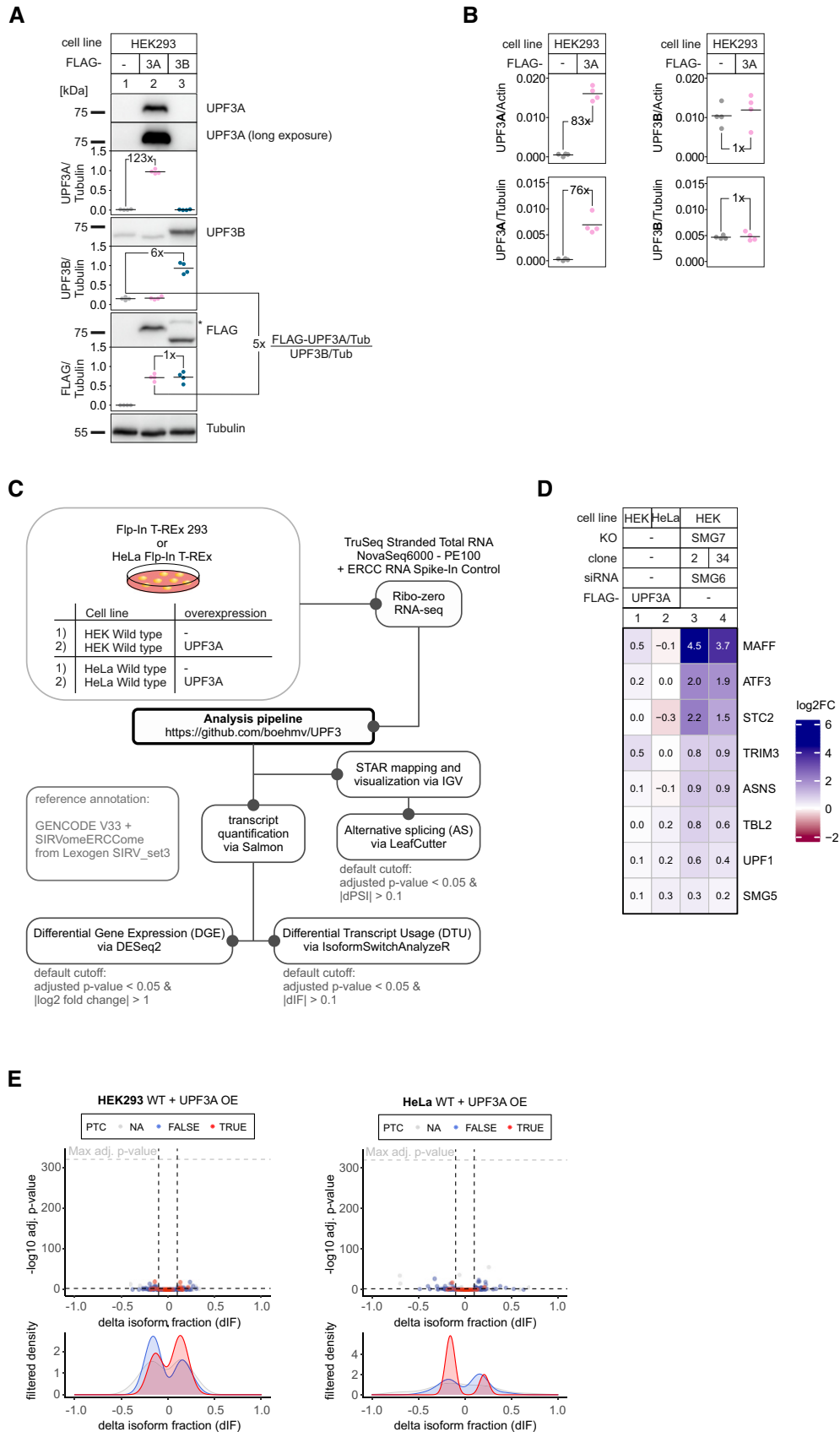


Figure EV1.

**Figure EV2. Light NMD inhibition after UPF3B KD in UPF3A KO cells.**

- A PCR amplification of targeted genomic locus of UPF3A for Sanger sequencing analysis.
- B, C The targeted exon region and anticipated PTC location following insertions (green) or deletions (red) are indicated for detected alleles of UPF3A in clone 14 (B) and 20 (C).
- D Western blot analysis of WT and UPF3A KO cells (clones 14 and 20) with or without expression of FLAG-tagged UPF3A rescue construct. UPF3A and UPF3B protein levels were detected, Tubulin serves as control ( $n = 1$ ).
- E Quantitative RT-PCR of the samples from (D). Expression of four targets with significant DGE in both UPF3A KO clones (ZFAS1, BCHE, AMOT, CTSZ) was normalized to C1orf43 reference. For PHF21B and DDIT3, DGE analysis showed significant downregulation in both UPF3A KO clones and upregulation in SMG7 KO + SMG6 KD and UPF3 dKO cells. Expression was normalized to EMC7 reference. Data points and means are plotted as log<sub>2</sub> fold change (log<sub>2</sub>FC) ( $n = 3$ ).
- F Schematic overview of the analysis pipeline.
- G, H Volcano plots showing the differential transcript usage (via IsoformSwitchAnalyzeR) in various RNA-Seq data. Isoforms containing GENCODE (release 33) annotated PTC (red, TRUE), regular stop codons (blue, FALSE) or having no annotated open reading frame (gray, NA) are indicated. The change in isoform fraction (dIF) is plotted against the  $-\log_{10}$  adjusted  $P$ -value ( $P_{adj}$ ). Density plots show the distribution of filtered isoforms in respect to the dIF, cutoffs were  $|dIF| > 0.1$  and  $P_{adj} < 0.05$ .  $P$ -values were calculated by IsoformSwitchAnalyzeR using a DEXSeq-based test and corrected for multiple testing using the Benjamini–Hochberg method.
- I Volcano plots showing the differential gene expression analyses from the indicated RNA-Seq datasets. The log<sub>2</sub> fold change is plotted against the  $-\log_{10}$  adjusted  $P$ -value ( $P_{adj}$ ).  $P$ -values were calculated by DESeq2 using a two-sided Wald test and corrected for multiple testing using the Benjamini–Hochberg method.

Source data are available online for this figure.

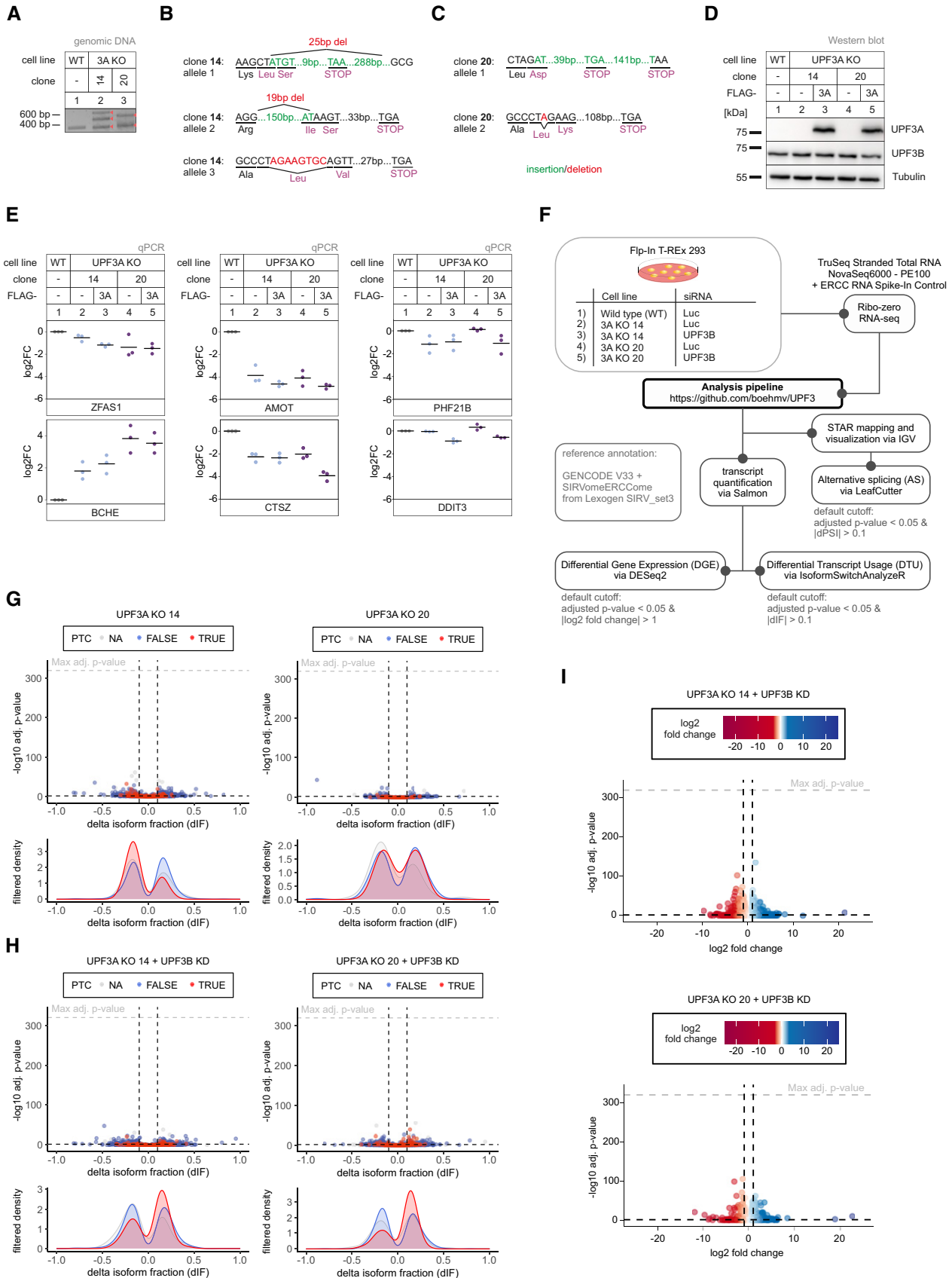


Figure EV2.

**Figure EV3. No NMD inhibition by UPF3A in the absence of UPF3B.**

- A, B The targeted exon region and anticipated PTC location following insertions (green) or deletions (red) are indicated for detected alleles of UPF3B in KO clone 90 (A) and 91 (B).
- C Schematic overview of the analysis pipeline.
- D, E Read coverage of RSRC2 (D) and SRSF2 (E) from the indicated RNA-seq sample data with or without UPF3A siRNA treatment shown as Integrative Genomics Viewer (IGV) snapshot. The canonical and NMD-sensitive isoforms are schematically indicated below.
- F, G Volcano plots showing the differential gene expression analyses from the indicated RNA-Seq datasets. The log<sub>2</sub> fold change is plotted against the -log<sub>10</sub> adjusted *P*-value (*P*<sub>adj</sub>). *P*-values were calculated by DESeq2 using a two-sided Wald test and corrected for multiple testing using the Benjamini–Hochberg method.
- H Western blot analysis of HEK293 and HeLa WT and UPF3B KO cells (clone 90 for HEK293 cells) with or without expression of FLAG-tagged UPF3A rescue construct (*n* = 1). UPF3A, UPF3B and FLAG protein levels were detected, Tubulin serves as control. The asterisk indicates unspecific bands.
- I Quantitative RT–PCR of the HEK293 and HeLa samples from (H). For RSRC2 and SRSF2 the ratio of NMD isoform to canonical isoform was calculated. ZFAS1 expression was normalized to C1orf43 reference. Data points and means are plotted as log<sub>2</sub> fold change (log<sub>2</sub>FC) (*n* = 3).

Source data are available online for this figure.

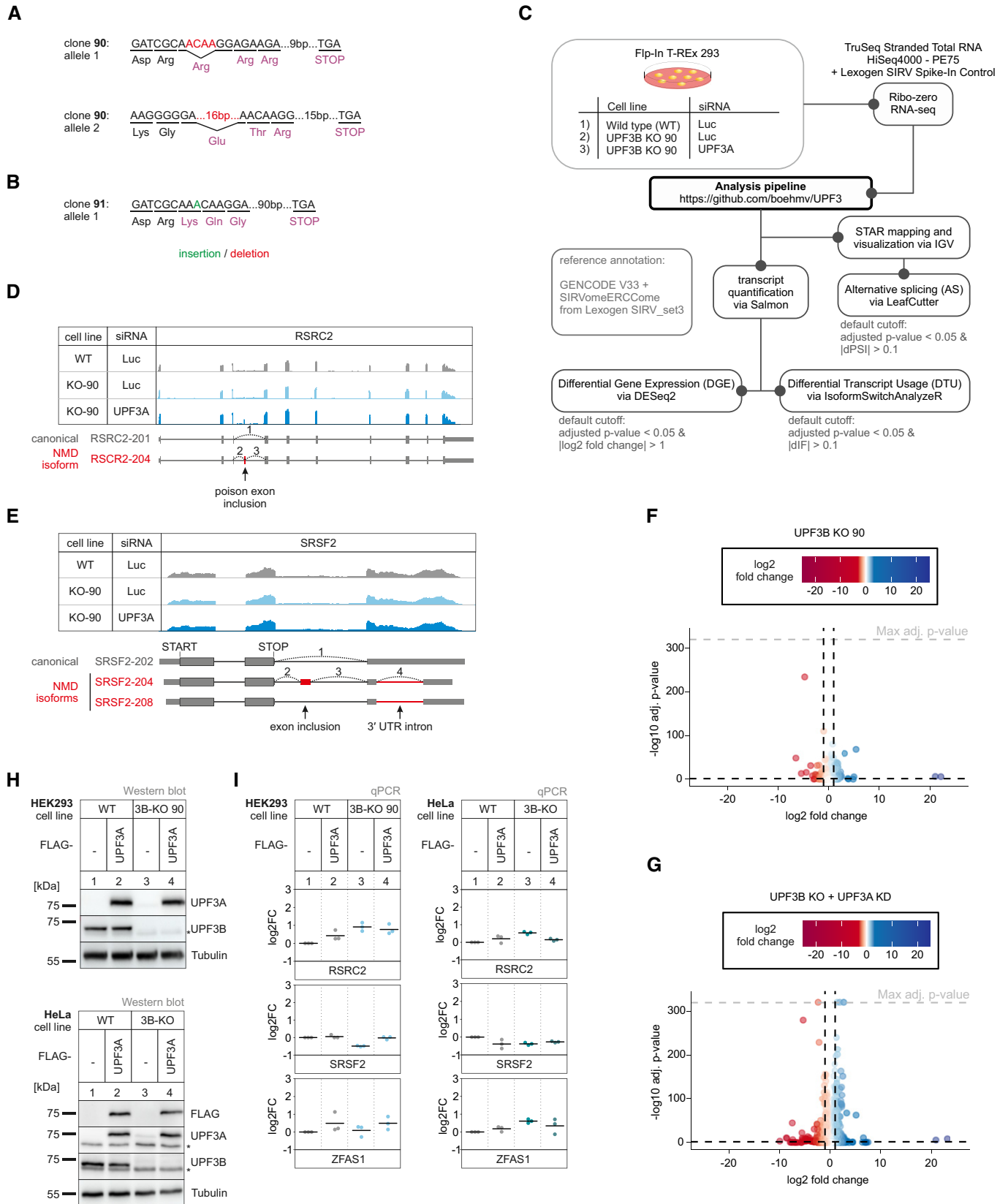


Figure EV3.

**Figure EV4. Genes upregulated in UPF3 dKO cells are high confidence NMD targets.**

- A Schematic overview of the analysis pipeline.
- B nVenn Diagrams showing the overlap of upregulated (upper panel) or downregulated genes (lower panel) in the UPF3 dKO cell lines 1 and 2, both with and without a supportive UPF3B KD. Log<sub>2</sub> fold change < -1 (downregulated) or > 1 (upregulated) and adjusted *P*-value (*P*<sub>adj</sub>) < 0.05. DGE = Differential Gene Expression.
- C nVenn Diagram showing the overlap of upregulated genes in the two UPF3 dKO clones and previously analyzed SMG7 KO cells with SMG6 KD (Data ref.: Boehm *et al*, 2021) as control for cells with inhibited NMD. The overlap demonstrates high-confidence NMD targets. Cut-offs: log<sub>2</sub>FoldChange > 1 and adjusted *P*-value (*P*<sub>adj</sub>) < 0.05. DGE = Differential Gene Expression.
- D Volcano plots showing the differential transcript usage (via IsoformSwitchAnalyzerR) in various RNA-Seq data. Isoforms containing GENCODE (release 33) annotated PTC (red, TRUE), regular stop codons (blue, FALSE) or having no annotated open reading frame (gray, NA) are indicated. The change in isoform fraction (dIF) is plotted against the -log<sub>10</sub> adjusted *P*-value (*P*<sub>adj</sub>). Density plots show the distribution of filtered isoforms in respect to the dIF, cutoffs were |dIF| > 0.1 and *P*<sub>adj</sub> < 0.05. *P*-values were calculated by IsoformSwitchAnalyzerR using a DEXSeq-based test and corrected for multiple testing using the Benjamini–Hochberg method.

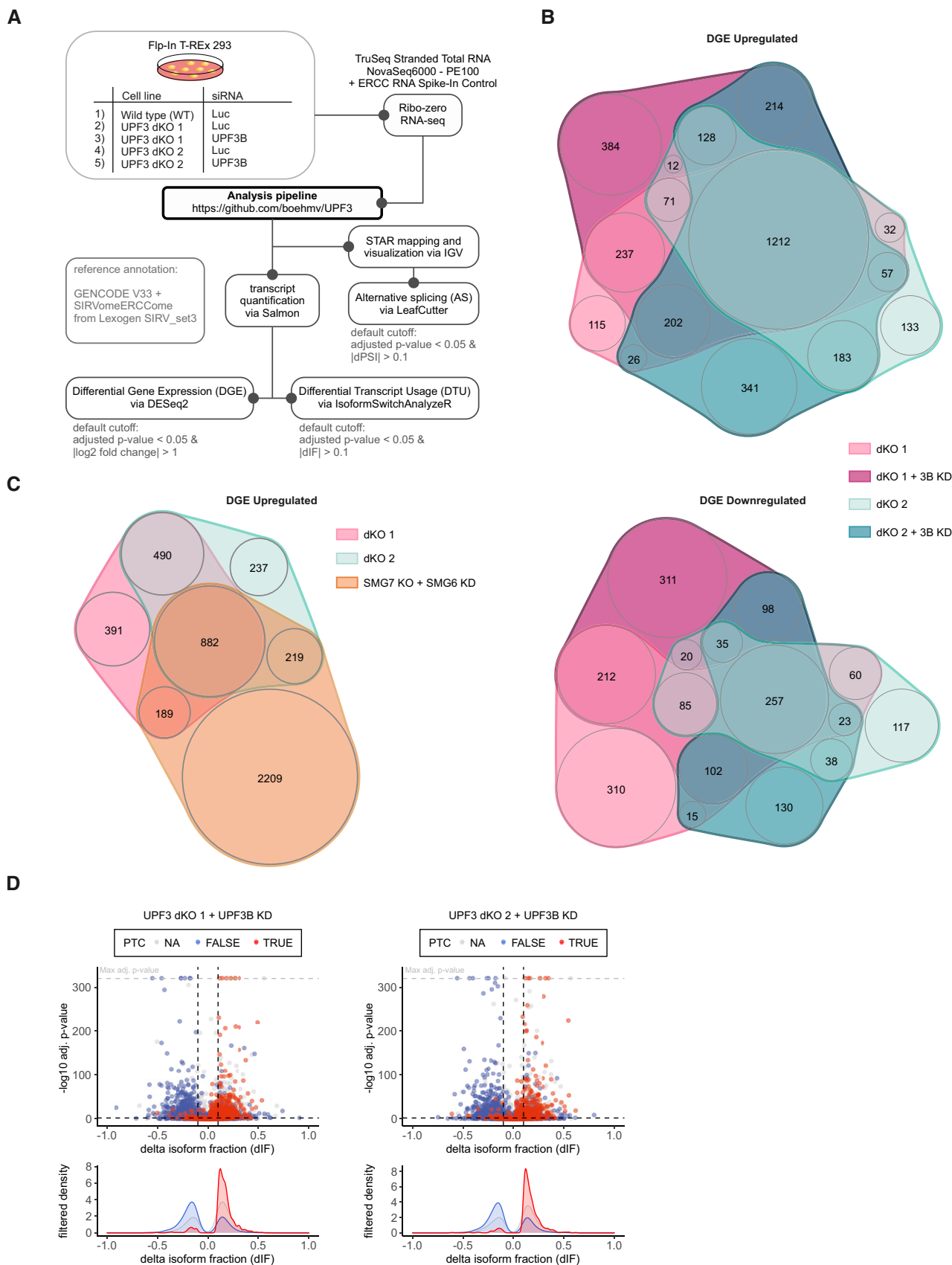


Figure EV4.



**Figure EV5. Single mutations do not destroy UPF3B NMD functionality in HEK293 and HeLa cells.**

- A Western blot analysis of WT and UPF3B KO clone 90 cells with Luciferase and UPF3A KD, respectively. Monitored expression of globin WT and PTC39 reporters and the indicated UPF3B rescue constructs. Rescue construct protein levels were detected with anti-FLAG and anti-UPF3B (AK-141) antibodies. Tubulin serves as control ( $n = 1$ ). The asterisk indicates an unspecific band.
- B End-point RT-PCR detection of RSRC2 transcript isoforms in the samples from (A). The detected RSRC2 isoforms are indicated on the bottom, the NMD-inducing included exon is marked in red (e = exon). Relative mRNA levels of RSRC2 isoforms were quantified from bands of agarose gels ( $n = 3$ ).
- C Northern blot analysis of globin reporter and xrFrag. Ethidium bromide stained 28S and 18S rRNAs are shown as controls. Lanes 5 and 6 were mirrored because of a pipetting error. Quantification results are shown as data points and mean ( $n = 3$ ).
- D Western blot analysis of HeLa WT and UPF3B KO cells with Luciferase and UPF3A KDs respectively. Monitored expression of the FLAG-tagged UPF3B rescue construct shown in (Fig 6D). Rescue construct protein levels were detected with anti-FLAG and anti-UPF3B (AK-141) antibodies. Tubulin serves as control ( $n = 1$ ). The asterisk indicates an unspecific band.
- E Quantitative RT-PCR of the samples from (D). For RSRC2 and SRSF2, the ratio of NMD isoform to canonical isoform was calculated. GAS5 expression was normalized to EMC7 reference. Data points and means are plotted as log<sub>2</sub> fold change ( $n = 3$ ).
- F Overview of TurboID-mediated proximity labeling of UPF3B WT and UPF3B mutant construct binding partners in UPF3B KO clone 90 with additional UPF3A KD. Transient UPF3B interactors are marked with biotin via TurboID catalysis. Biotinylated proteins are subsequently enriched with streptavidin beads.
- G–J Volcano plots of mass spectrometry-based analysis of streptavidin-enriched biotinylated proteins in the respective comparison of conditions. (G) FLAG-TurboID-UPF3B against FLAG-TurboID control, (H) FLAG-TurboID-UPF3B  $\Delta$ UPF2 interaction (K52D/RR56EE) against FLAG-TurboID-UPF3B, (I) FLAG-TurboID-UPF3B  $\Delta$ EJC interaction ( $\Delta$ 421–434) against FLAG-TurboID-UPF3B, (J) FLAG-TurboID-UPF3B  $\Delta$ middle domain ( $\Delta$ 147–256) against FLAG-TurboID-UPF3B, all in UPF3B KO + UPF3A KD cells. Points labeled in purple indicate NMD factors; points labeled in turquoise indicate EJC components ( $n = 3$  biologically independent samples).

Source data are available online for this figure.

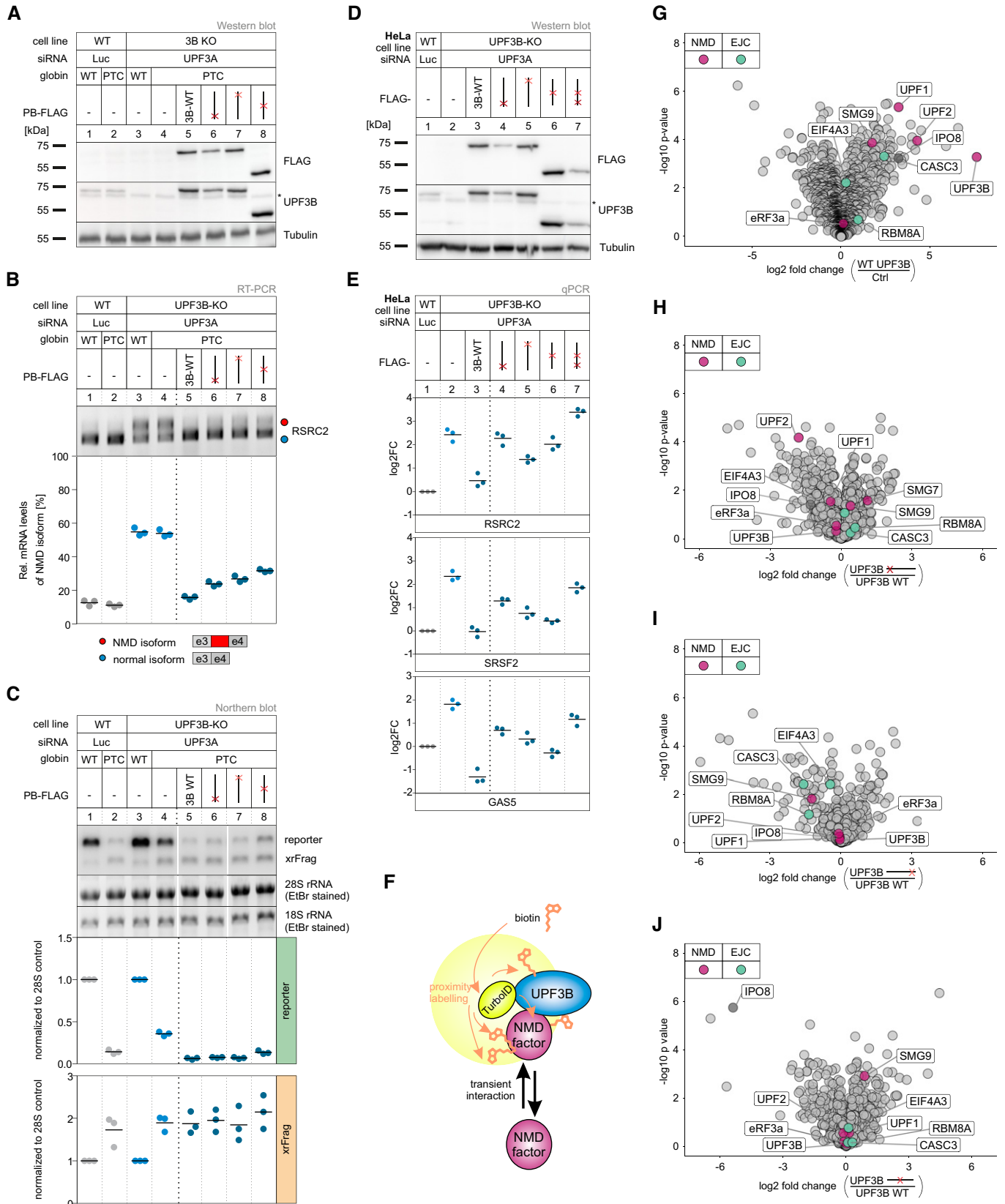


Figure EV5.