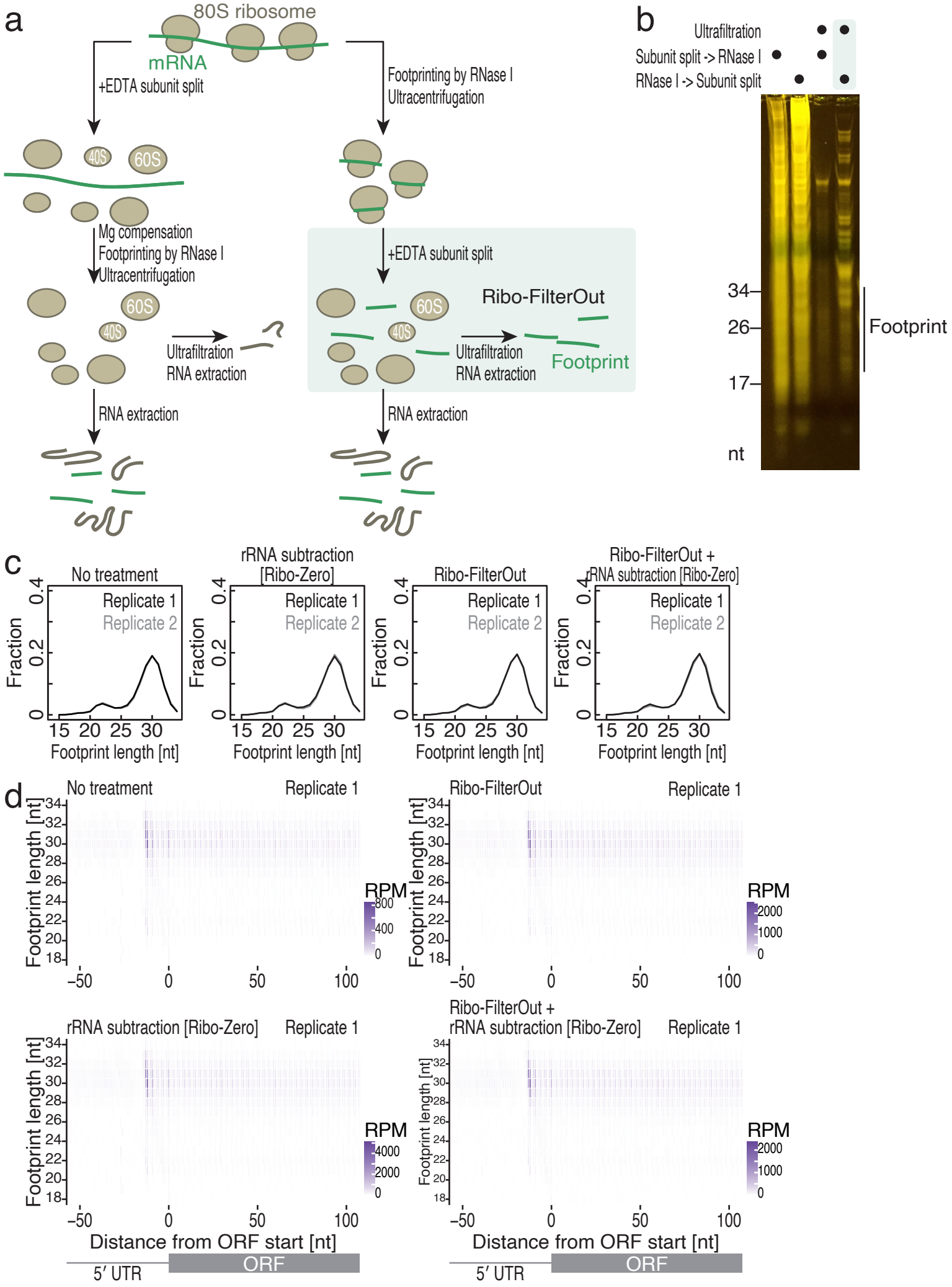
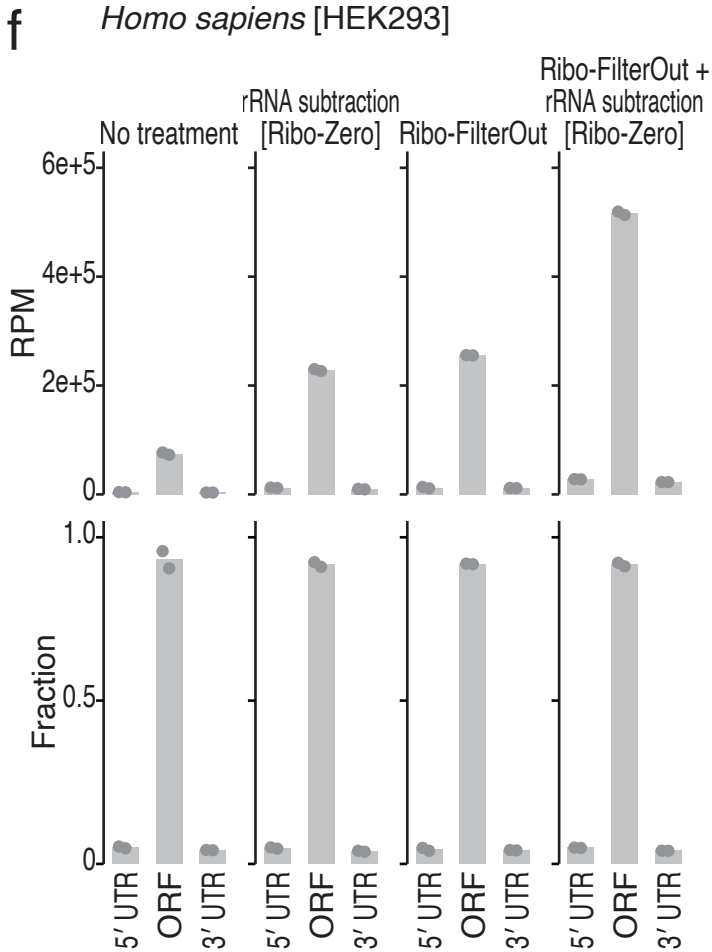
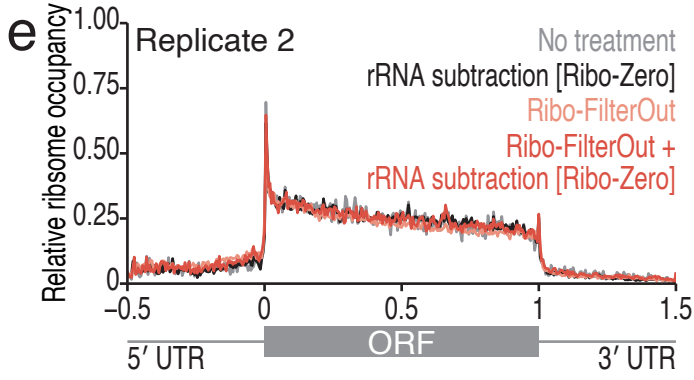




Supplementary Fig. 1 Tomuro, Mito *et al.* (2024)



Supplementary Fig. 1: Characterization of the Ribo-FilterOut technique.

(a) Schematic of the experiments in b. Right: mRNA-ribosome complexes were treated with RNase I, after which the ribosomal complex was isolated via ultracentrifugation. Ribosome footprints were separated from ribosome subunits by EDTA treatment and then isolated by ultrafiltration. Left: For the control, the lysate was pretreated with EDTA to release mRNA from ribosomes before RNase I digestion.

(b) SYBR Gold staining of the RNA fragments obtained from the experiments shown in a.

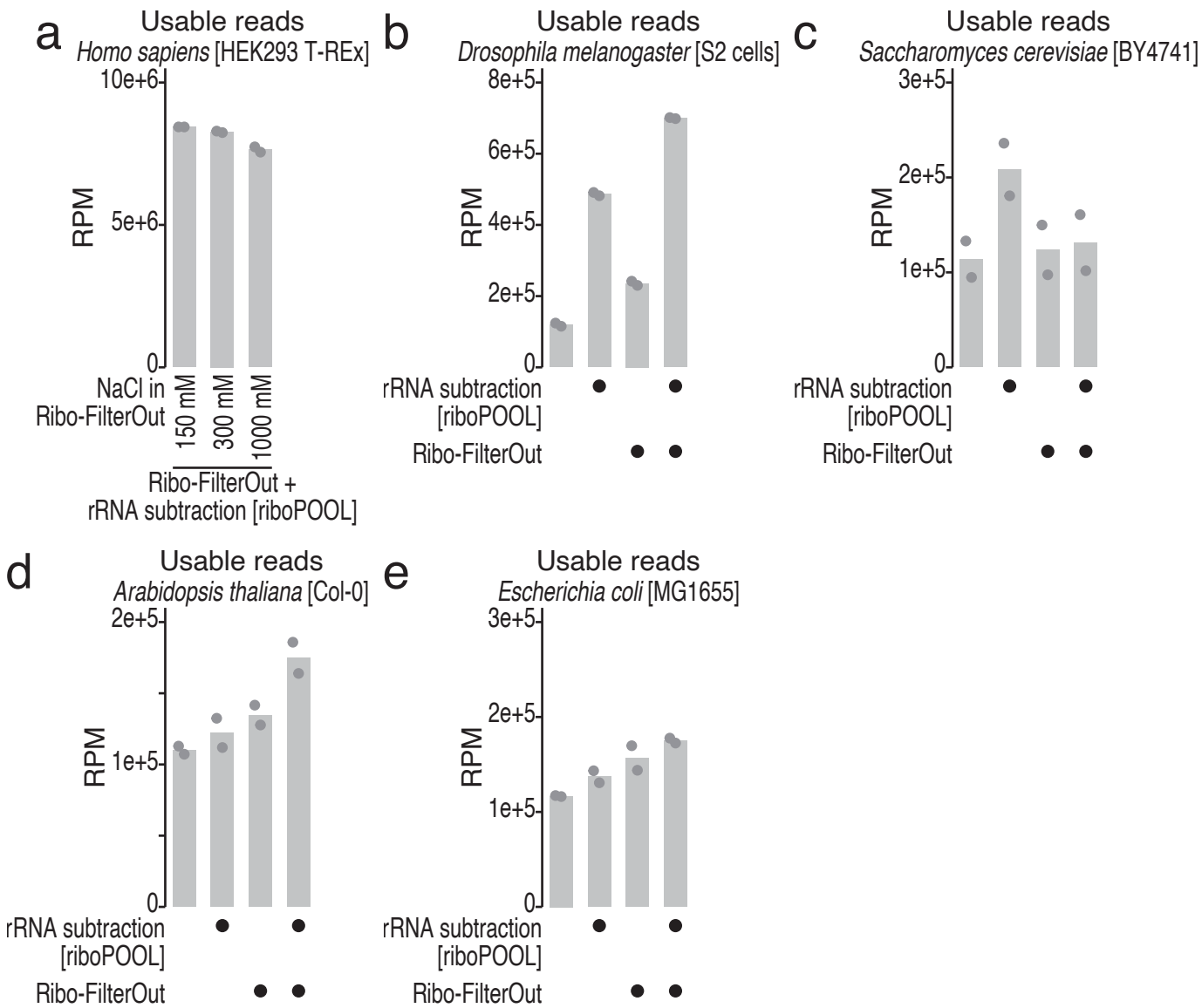
(c) The distribution of ribosome footprint length in ribosome profiling for the indicated conditions.

(d) Metagene plots of the 5' end of ribosome footprints around the start codon (the first position of the start codon was set to 0) along the footprint length. The color scales for read abundance are shown.

(e) Metagene plot of the relative ribosome footprint distribution (ribosome occupancy) along the 5' UTR, ORF, and 3' UTR. The length of the ORF was set to 1, whereas the lengths of the 5' UTR and 3' UTR were set to 0.5.

(f) Reads obtained from ribosome profiling were mapped to the indicated region of mRNAs (top). The relative distributions of the 5' UTR, ORF, and 3' UTR are shown (bottom). The mean (bar) and individual replicates ($n = 2$, points) are shown.

RPM, reads per million mapped reads. Source data are provided as a Source Data file.

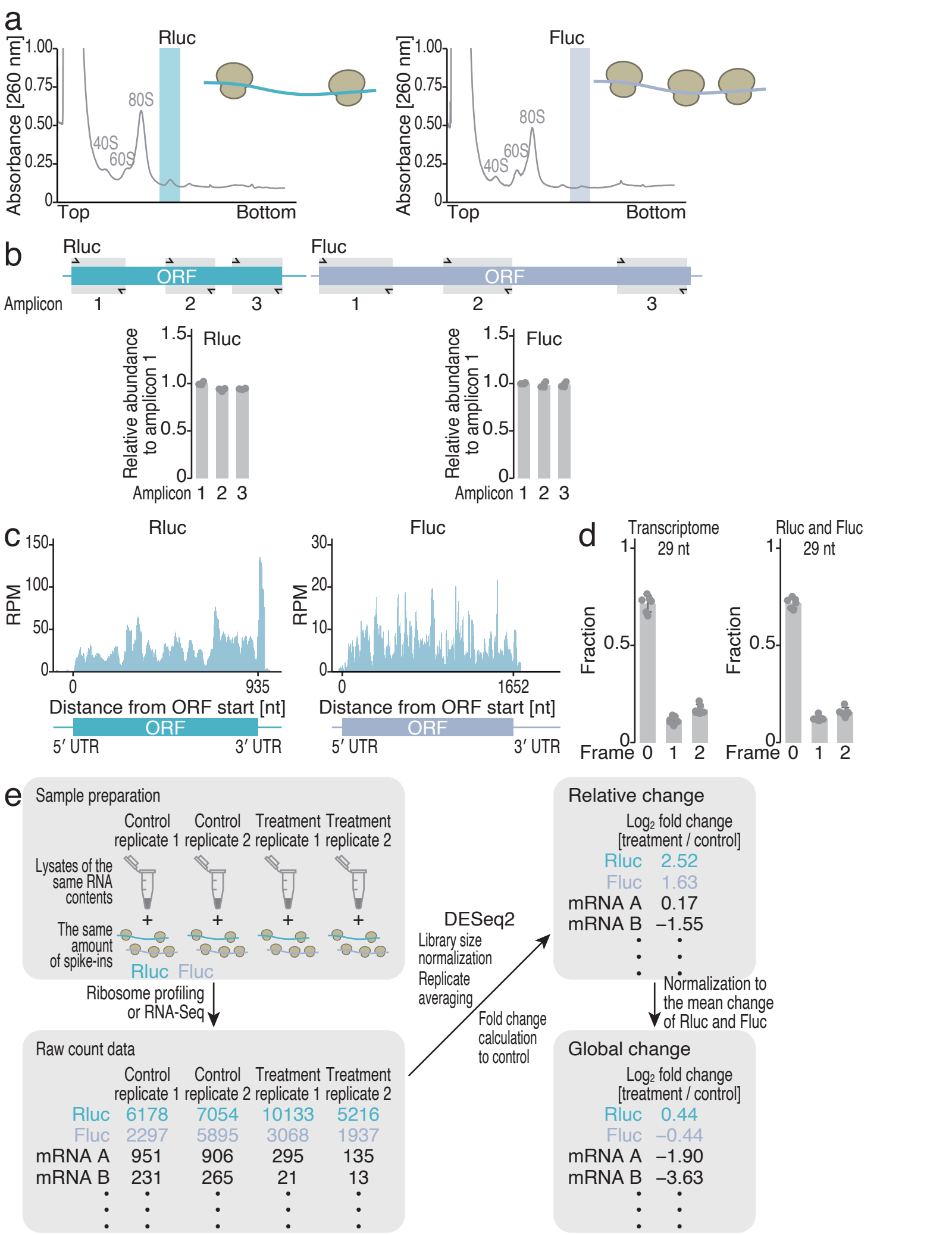


Supplementary Fig. 2: Application of Ribo-FilterOut to various species.

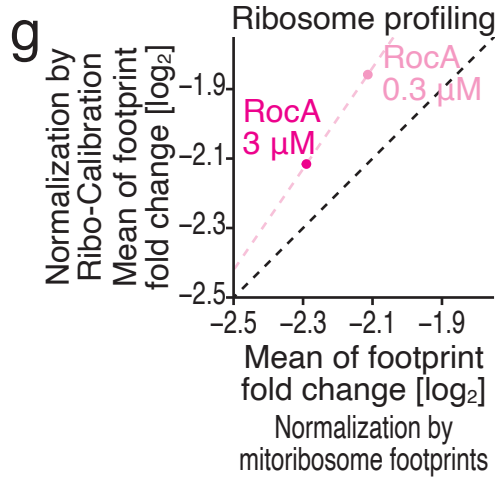
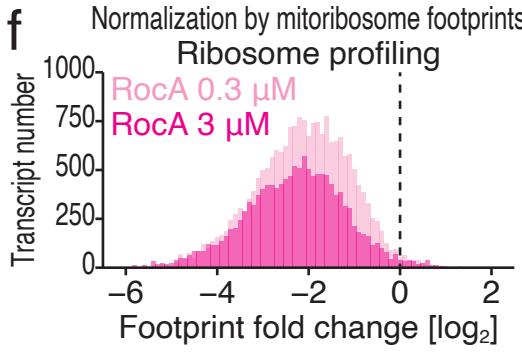
(a) Test of salt concentrations in the resuspension buffer of the ribosome pellet after sucrose cushion centrifugation. The amount of usable reads is shown.

(b-e) Combination of the standard rRNA subtraction method (riboPOOL) with Ribo-FilterOut for fly S2 cells (b), yeast (c), plants (d), and bacteria (e). The amount of usable reads is shown.

The mean (bar) and individual replicates ($n = 2$, points) are shown. RPM, reads per million mapped reads. Source data are provided as a Source Data file.



Supplementary Fig. 3 Tomuro, Mito *et al.* (2024)



Supplementary Fig. 3: Characterization of the method for global translation measurement by Ribo-Calibration.

(a) Sucrose density gradient ultracentrifugation for the separation of ribosomal complexes. Rluc mRNA (left) and Fluc mRNA (right) were translated *in vitro* with RRL. Highlighted fractions were isolated for spike-ins for ribosome profiling.

(b) RT-qPCR analyses of Rluc and Fluc mRNAs complexed with ribosomes. Three different amplicons were designed for each transcript (top). The data were calibrated with titrated amounts of bare mRNAs used for *in vitro* translation as standards. The mean (bar), s.d. (error), and individual data (n = 3, points) are shown.

(c) Ribosome footprint coverage on RLuc mRNA (left) and Fluc mRNA (right). RPM, reads per million mapped reads.

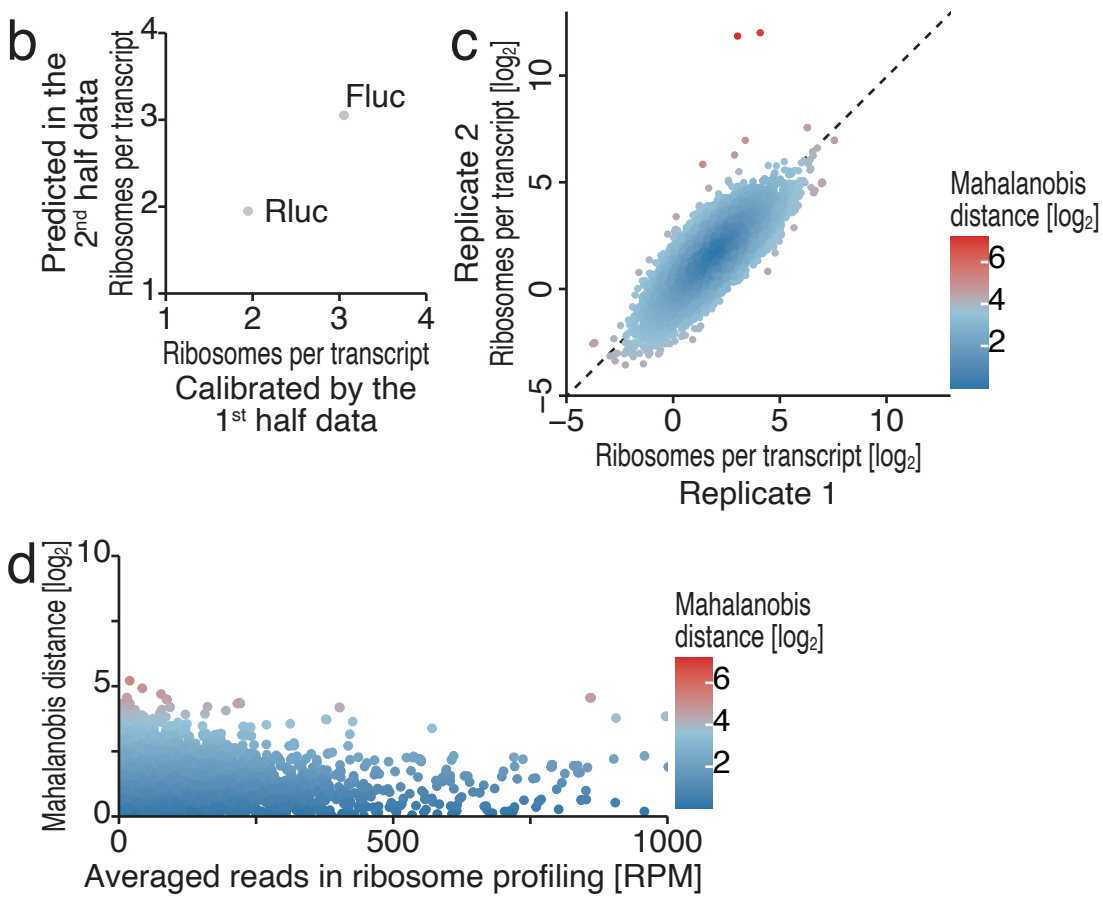
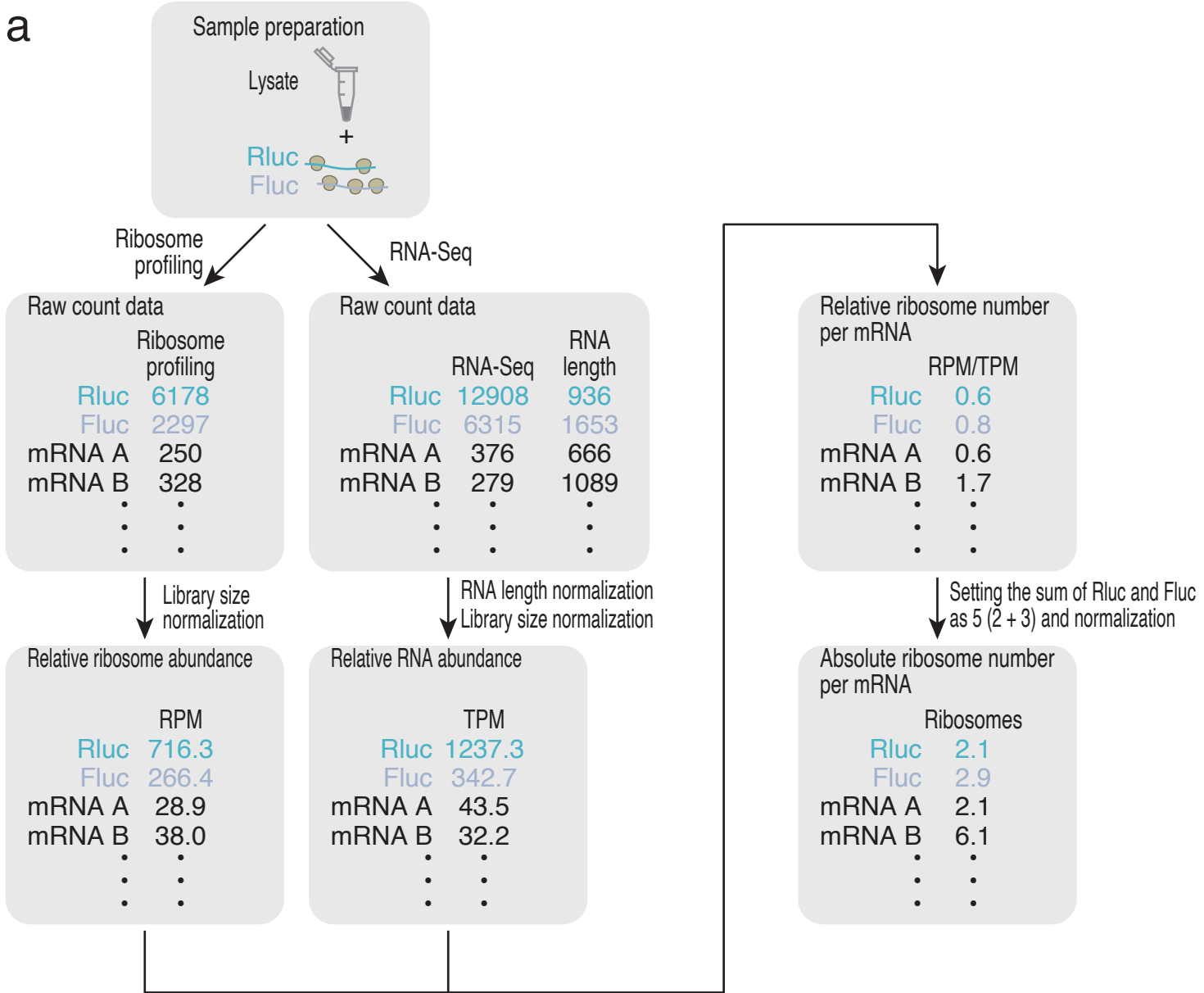
(d) Fraction of the frame position where the 5' ends of the reads are located. Twenty-nine nucleotide footprints were analyzed. The mean (bar), individual data (n = 6; 2 replicates for DMSO, 2 replicates for 0.3 μ M RocA treatment, and 2 replicates for 3 μ M RocA treatment, points), and s.d. (error) are shown.

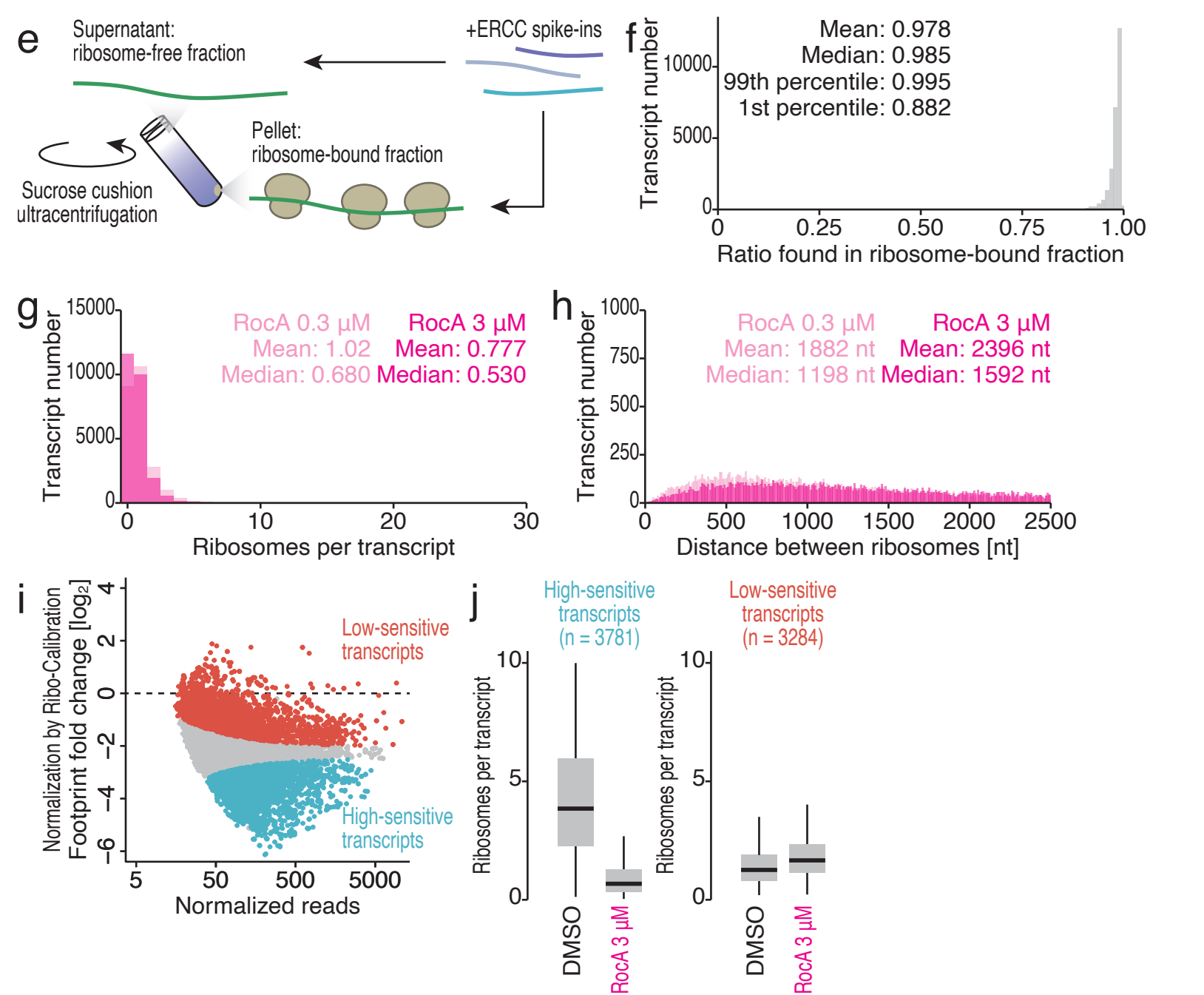
(e) Schematic of sample handling and data calculation for the measurement of global translation changes. The use of the same amount of lysate and spike-ins of mRNA-ribosome complexes was essential.

(f) Histograms of ribosome footprint fold changes after 0.3 or 3 μ M RocA treatment normalized to the mitochondrial footprint.

(g) Comparison of the changes in global translation caused by RocA treatment calculated from Ribo-calibration and mitoribosome footprints.

Source data are provided as a Source Data file.





Supplementary Fig. 4: Characterization of the method for the absolute calibration of ribosome number by Ribo-Calibration.

(a) Schematic of sample handling and data calculation for the absolute quantification of ribosome numbers on ORFs. Ribosome profiling and RNA-Seq must be performed using the same lysate supplemented with purified mRNA-ribosome complexes.

(b) To validate the performance of Ribo-Calibration for the absolute quantification of ribosome numbers on ORFs, we divided the data into halves, used the first half for calibration of the data, and then predicted the ribosome numbers on Rluc and Fluc in the second half.

(c) Scatter plot of ribosome numbers on ORFs in replicates, scoring the reproducibility with the Mahalanobis distance (shown in the color scale).

(d) Scatter plot of the Mahalanobis distance (shown in c) and average ribosome footprint reads from replicates. The color scales for the Mahalanobis distance are shown. RPM, reads per million mapped reads.

(e) Schematic of isolating ribosome-bound and ribosome-free mRNAs by sucrose cushion ultracentrifugation. The spike-in RNA controls defined by the External RNA Controls Consortium (ERCC) were added to the purified mRNAs for the downstream RNA-Seq experiments.

(f) Histograms of the mRNA ratio found in the ribosome-bound fraction.

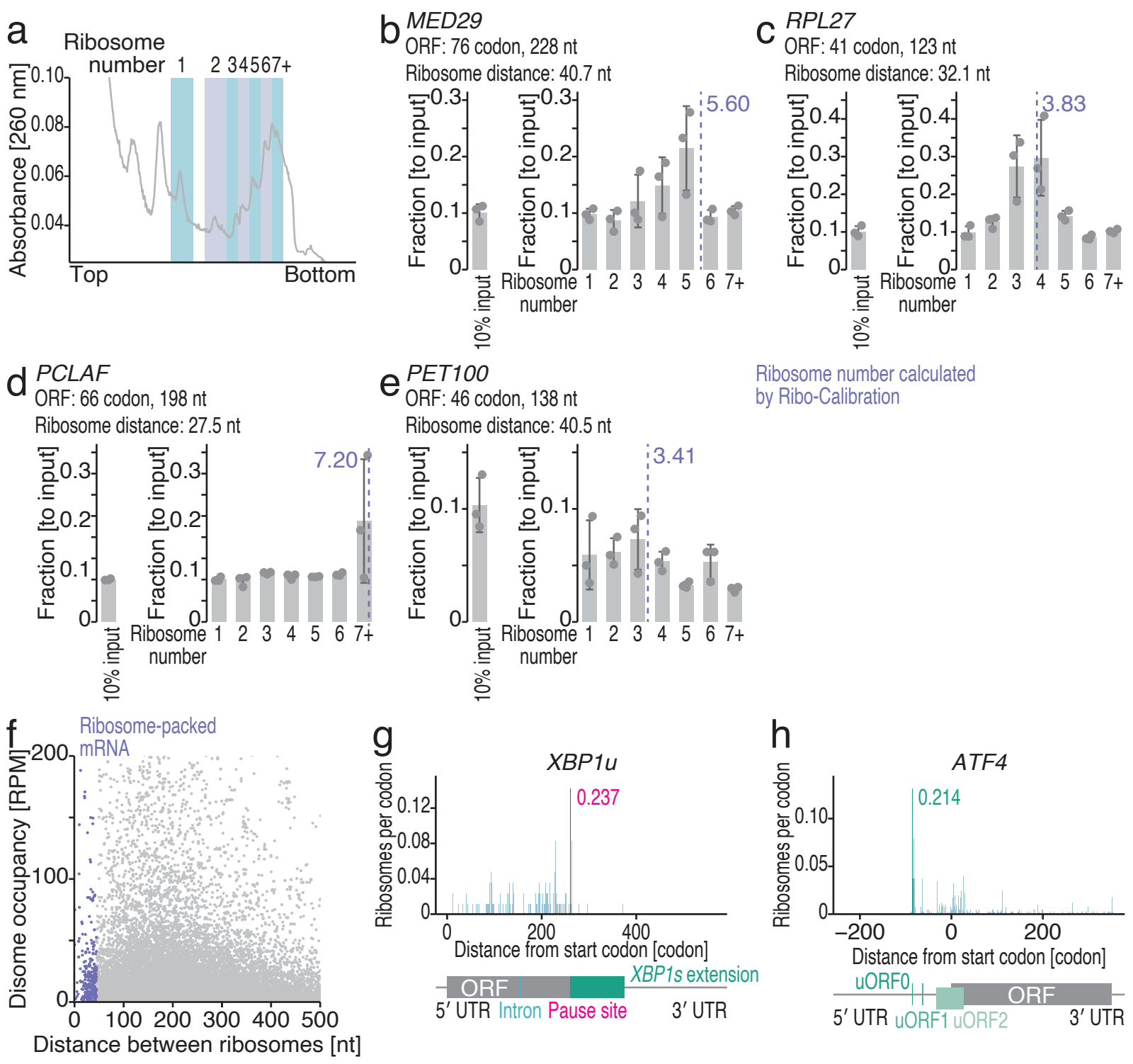
(g) Histograms of the absolute ribosome number on transcripts with 0.3 or 3 μ M RocA treatment.

(h) Histograms of the distance between ribosomes with 0.3 or 3 μ M RocA treatment.

(i) MA (M, log ratio; A, mean average) plot of the ribosome footprint fold change with 3 μ M RocA treatment normalized by Ribo-Calibration spike-ins. Transcripts with significant changes ($FDR < 0.05$) are highlighted.

(j) Box plots of the absolute number of ribosomes for RocA high-sensitivity transcripts (left) and low-sensitivity transcripts (right).

In box plots, the median (centerline), upper/lower quartiles (box limits), and $1.5\times$ interquartile range (whiskers) are shown. Source data are provided as a Source Data file.



Supplementary Fig. 5: Validation of ribosome numbers on ORFs obtained by Ribo-Calibration.

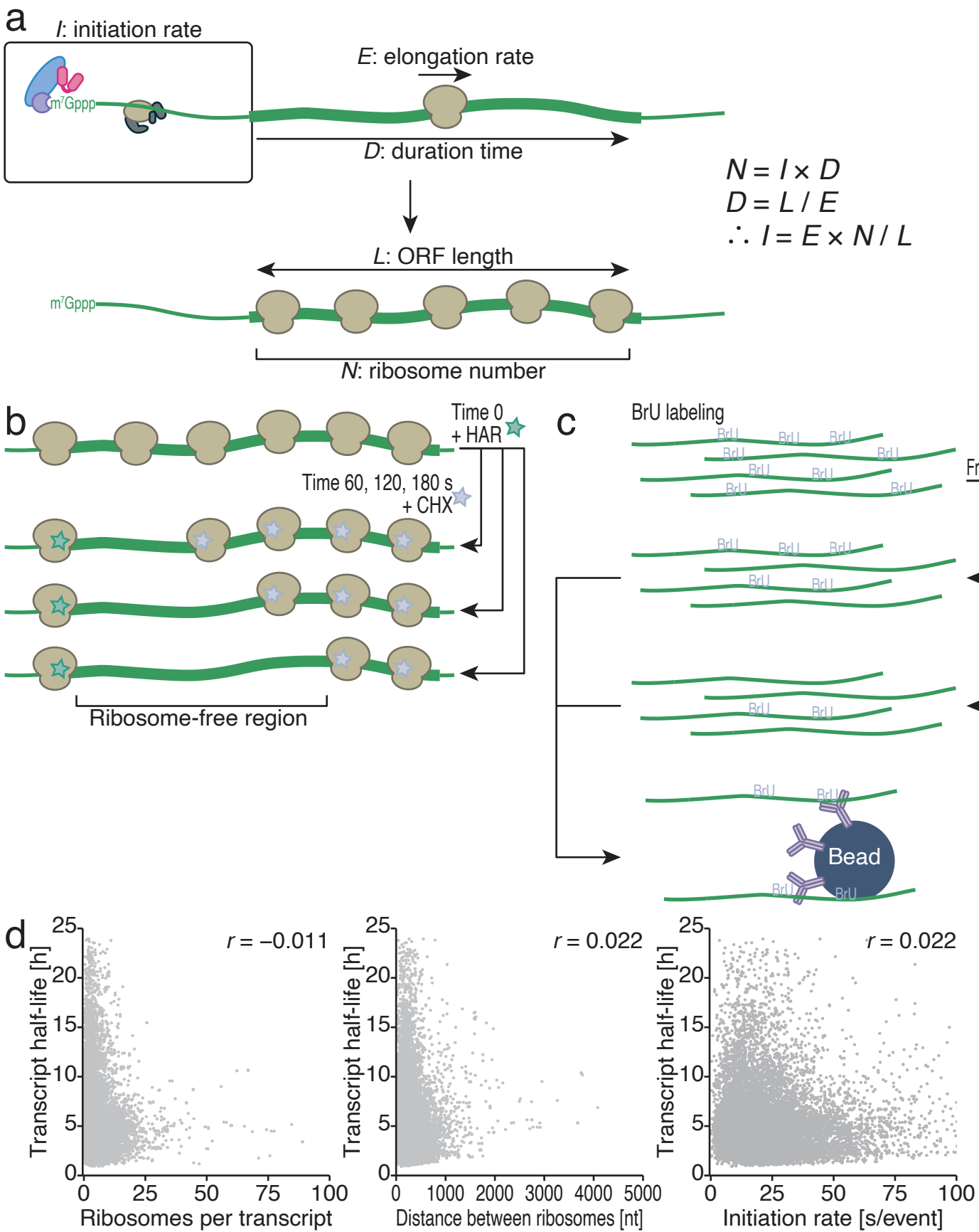
(a) Sucrose density gradient ultracentrifugation for the separation of ribosomal complexes from HEK293 T-REx cells, highlighting the fractions used for downstream RT-qPCR analyses.

(b-e) RT-qPCR analysis of the indicated mRNAs in the polysome fractions according to the number of ribosomes on the transcripts. Ten percent of the input lysate was used for the standard. The mean (bar), s.d. (error), and individual data (n = 3, points) are shown.

(f) Scatter plots of the distance between ribosomes and disome occupancy. RPM, reads per million mapped reads.

(g and h) Distribution of ribosome footprints along the indicated genes. The A-site position of the reads is depicted. The calibrated ribosome numbers on the pause site in *XBPIu* and uORF0 in *ATF4* (including ribosomes on codons before and ahead) are highlighted.

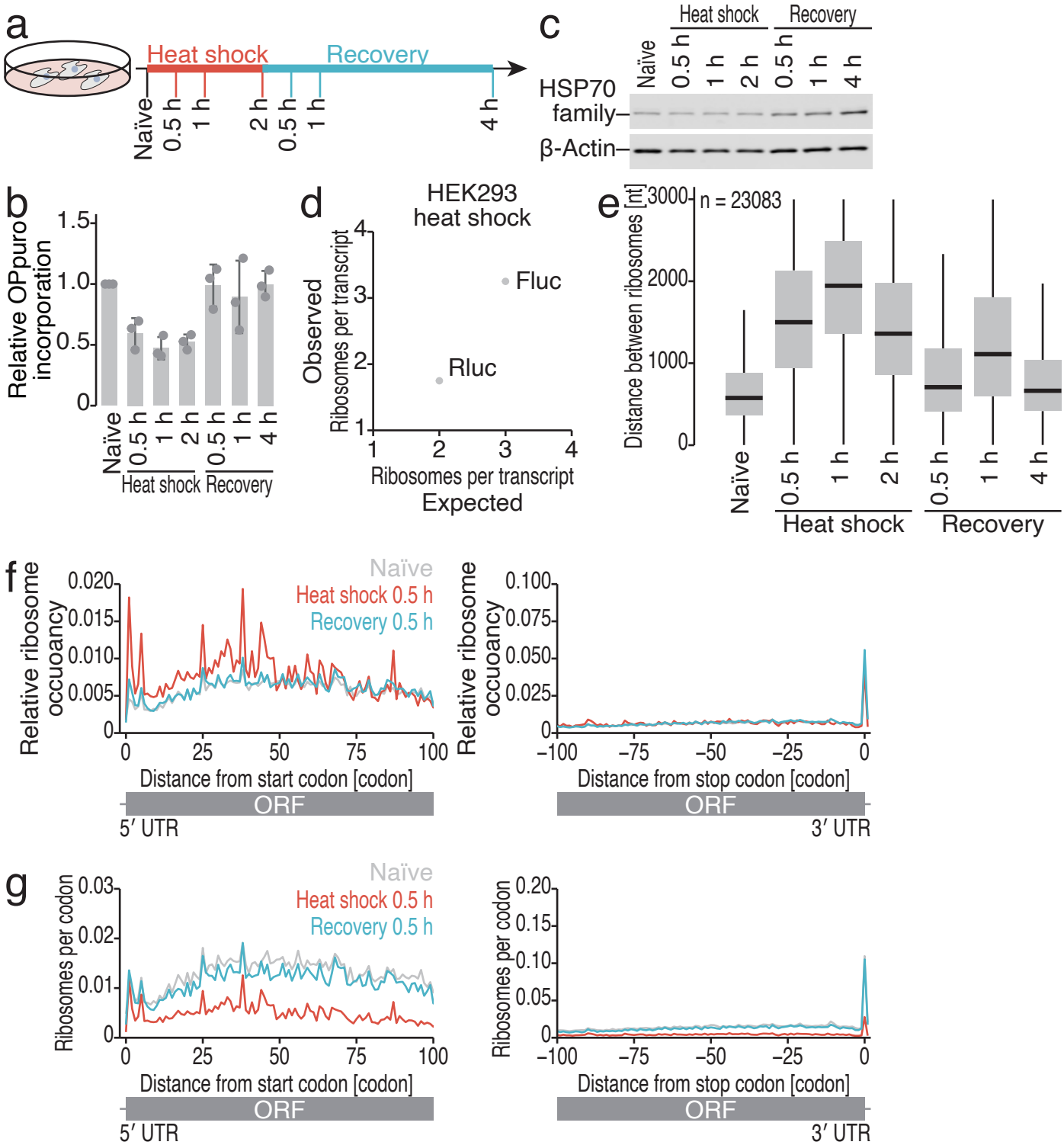
Source data are provided as a Source Data file.



Supplementary Fig. 6: Characterization of ribosome run-off assay and BRIC-Seq.

- (a) Schematic representation of the relationships among the number of ribosomes on the ORF (N), the duration of ribosome residence on the ORF (D), the length of the ORF (L), the translation elongation rate (E), and the translation initiation rate (I).
- (b) Schematic of the ribosome run-off assay with harringtonine (HAR) chase. After a short incubation with HAR, ribosomes on the transcripts were fixed with cycloheximide (CHX).
- (c) Schematic of BRIC-Seq with bromouridine (BrU). After the labeling of cellular mRNA with BrU, the cells were incubated in medium without BrU over the time course. mRNAs were isolated with an anti-BrdU antibody.
- (d) Comparison of transcript half-life and the absolute number of ribosomes on transcripts (left), the distance between ribosomes (middle), and the translation initiation rate (right). r , Pearson's correlation coefficient.

Source data are provided as a Source Data file.

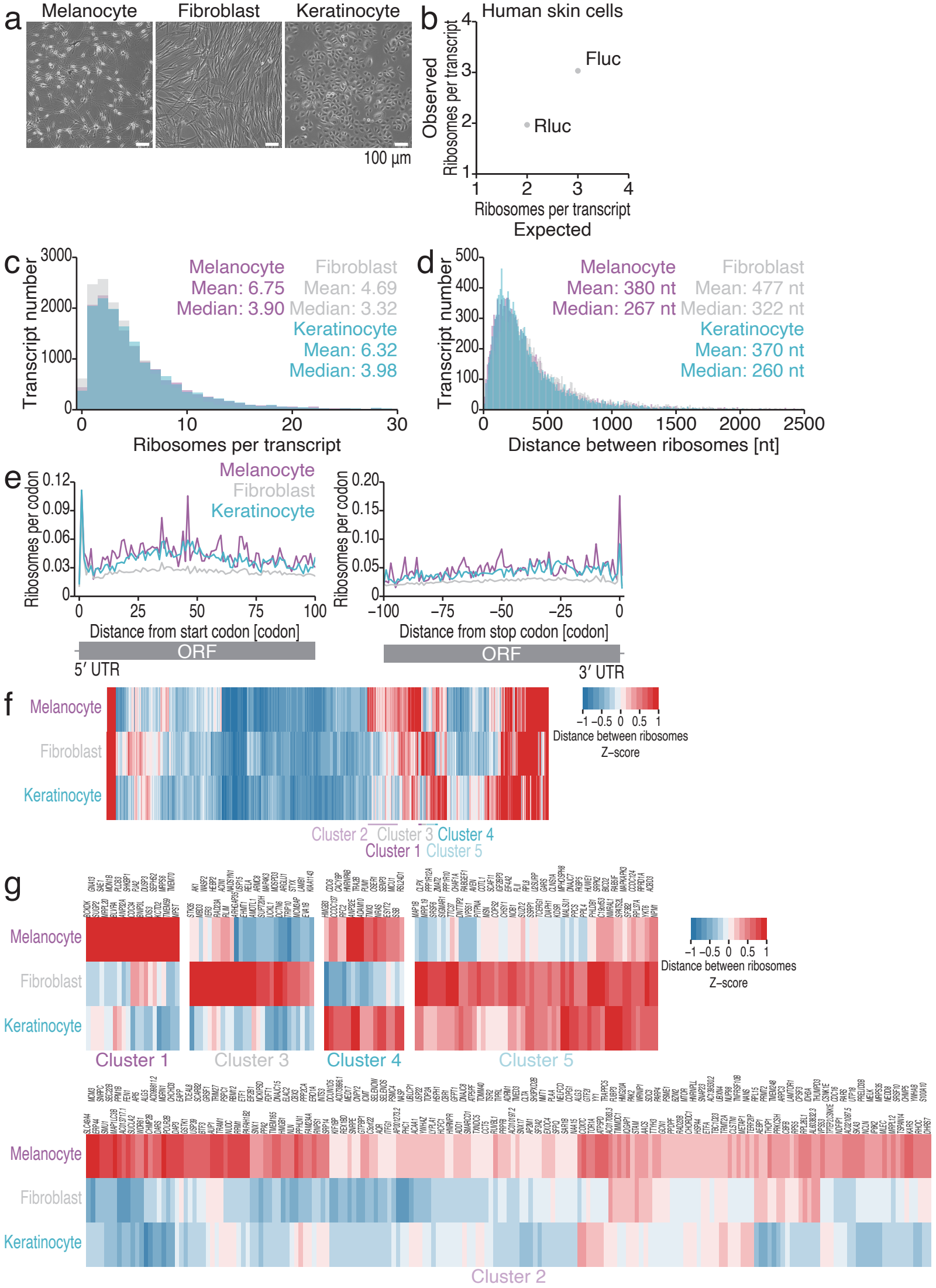


Supplementary Fig. 7: Characterization of heat shock and recovery in HEK293 cells.

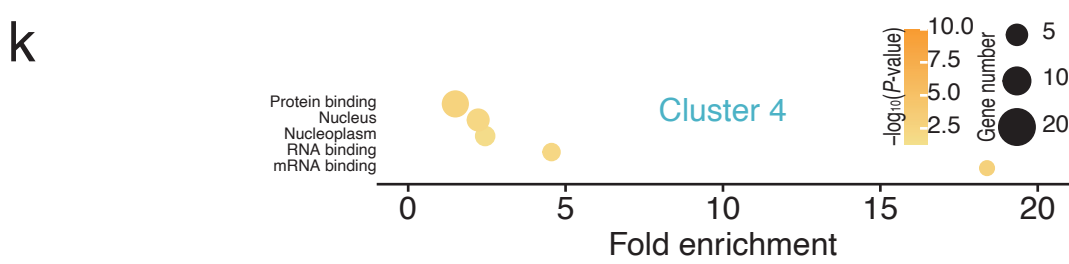
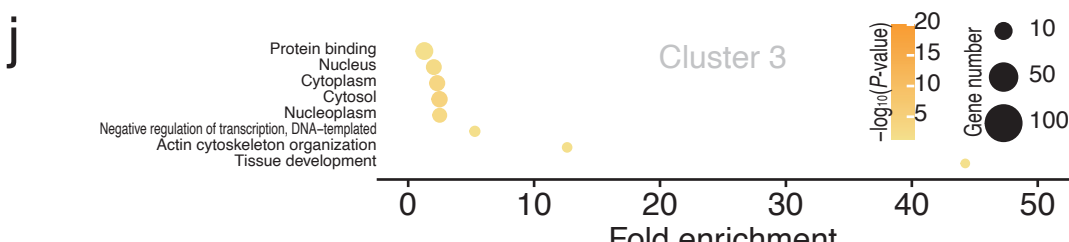
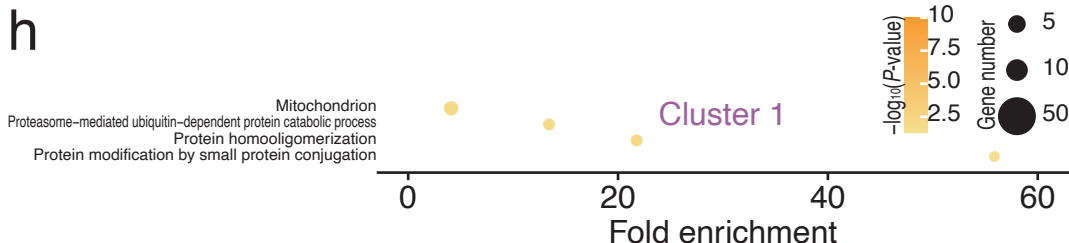
- (a) Schematic of the experiments over the time course.
- (b) Relative OP-puro incorporation during heat shock and recovery. The mean (bar), s.d. (error), and individual data (n = 3, points) are shown.
- (c) Western blotting of the indicated proteins. The anti-HSP70 antibody used in this study detected both heat-inducible HSP70 family proteins and constitutively expressed HSP70 (Hsc70) family proteins.
- (d) Comparison of expected and observed ribosome numbers on Rluc and Fluc transcripts used for Ribo-Calibration.
- (e) Box plots of the distance between ribosomes during heat shock and recovery.
- (f) Metagene plots with relative ribosome occupancy at each position around start codons (left, the first position of the start codon was set to 0) and stop codons (right, the first position of the stop codon was set to 0) under the indicated conditions.
- (g) Metagene plots with calibrated ribosome numbers at each position around start codons (left, the first position of the start codon was set to 0) and stop codons (right, the first position of the stop codon was set to 0) under the indicated conditions.

In box plots, the median (centerline), upper/lower quartiles (box limits), and 1.5× interquartile range (whiskers) are shown.

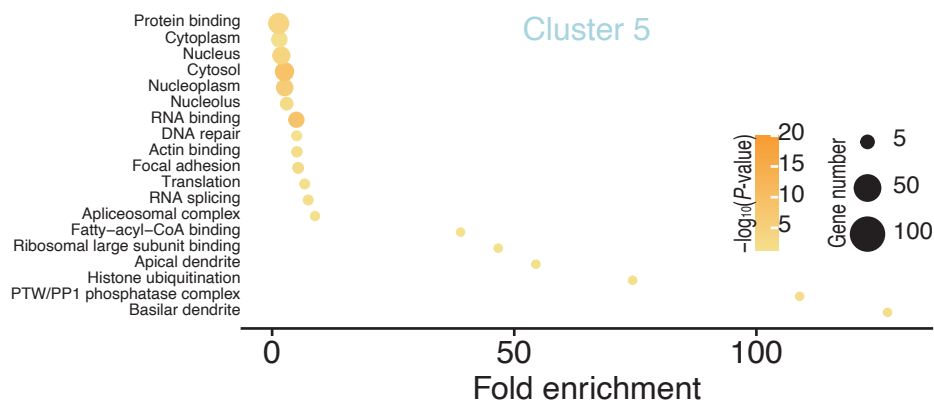
Source data are provided as a Source Data file.



Supplementary Fig. 8 Tomuro, Mito *et al.* (2024)



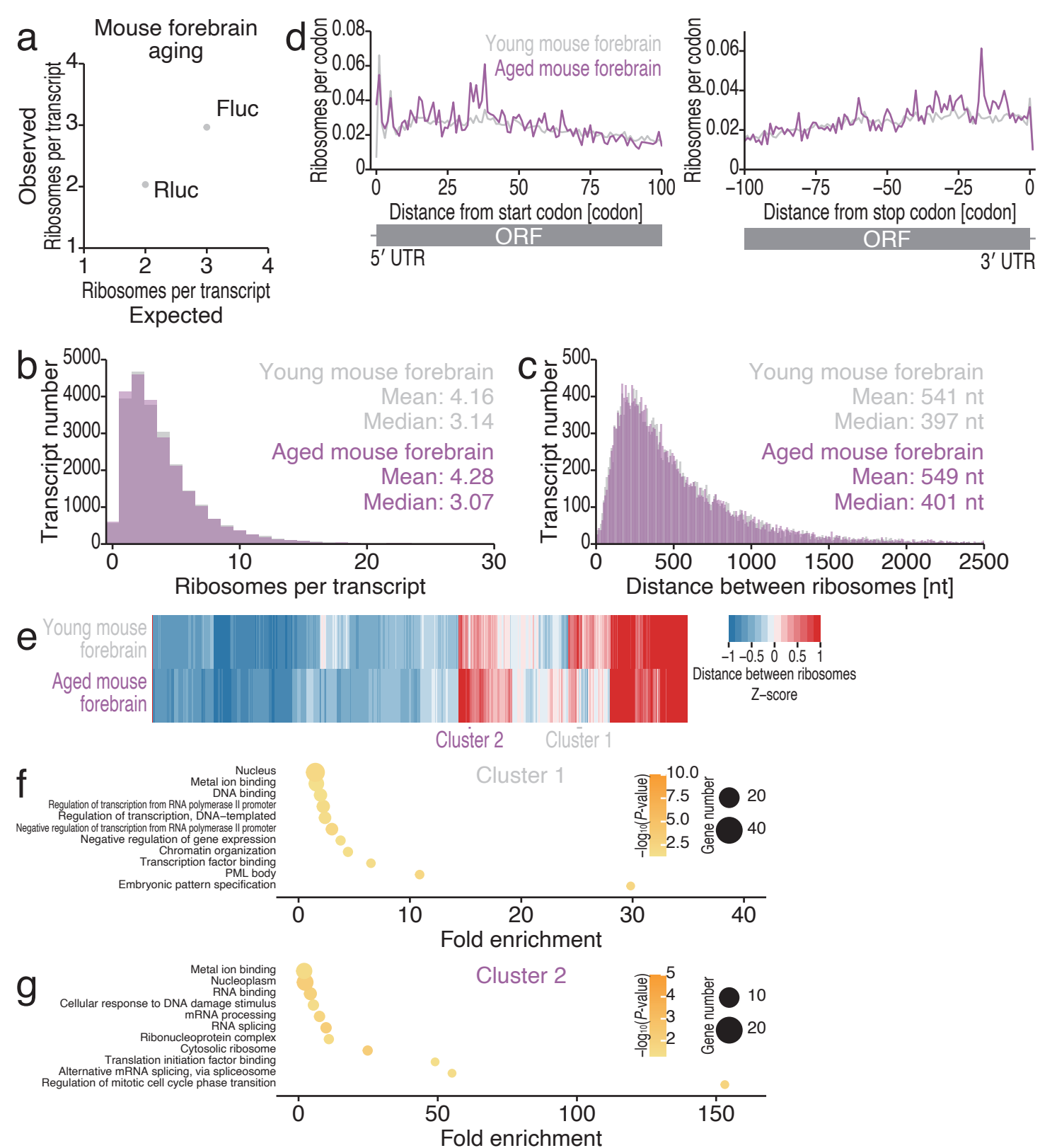
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Supplementary Fig. 8: Characterization of ribosome profiling with Ribo-Calibration in skin cells.

- (a) The morphology of the indicated skin cells. Scale bar, 100 μm .
- (b) Comparison of expected and observed ribosome numbers on Rluc and Fluc transcripts used for Ribo-Calibration.
- (c) Histograms of the absolute ribosome number on transcripts in the indicated skin cells.
- (d) Histograms of the distance between ribosomes in the indicated skin cells.
- (e) Metagene plots with calibrated ribosome numbers at each position around start codons (left, the first position of the start codon was set to 0) and stop codons (right, the first position of the stop codon was set to 0) in the indicated skin cells.
- (f-g) Heatmap of the distance between ribosomes in the indicated skin cells. The color scales for the Z scores are shown.
- (h-l) GO terms enriched in the gene clusters defined in f. The color scales for significance and the size scales for the number of genes in each category are shown.

Source data are provided as a Source Data file.



Supplementary Fig. 9: Characterization of ribosome profiling with Ribo-Calibration in mouse forebrains.

(a) Comparison of expected and observed ribosome numbers on Rluc and Fluc transcripts used for Ribo-Calibration.

(b) Histograms of the absolute ribosome number on transcripts in the young and aged mouse forebrains.

(c) Histograms of the distance between ribosomes in the forebrains of young and aged mice.

(d) Metagene plots with calibrated ribosome numbers at each position around start codons (left, the first position of the start codon was set to 0) and stop codons (right, the first position of the stop codon was set to 0) in the young and aged mouse forebrains.

(e) Heatmap of the distance between ribosomes in young and aged mouse forebrains. The color scales for the Z scores are shown.

(f-g) GO terms enriched in the gene clusters defined in e. The color scales for significance and the size scales for the number of genes in each category are shown.

Source data are provided as a Source Data file.