Supplemental Material

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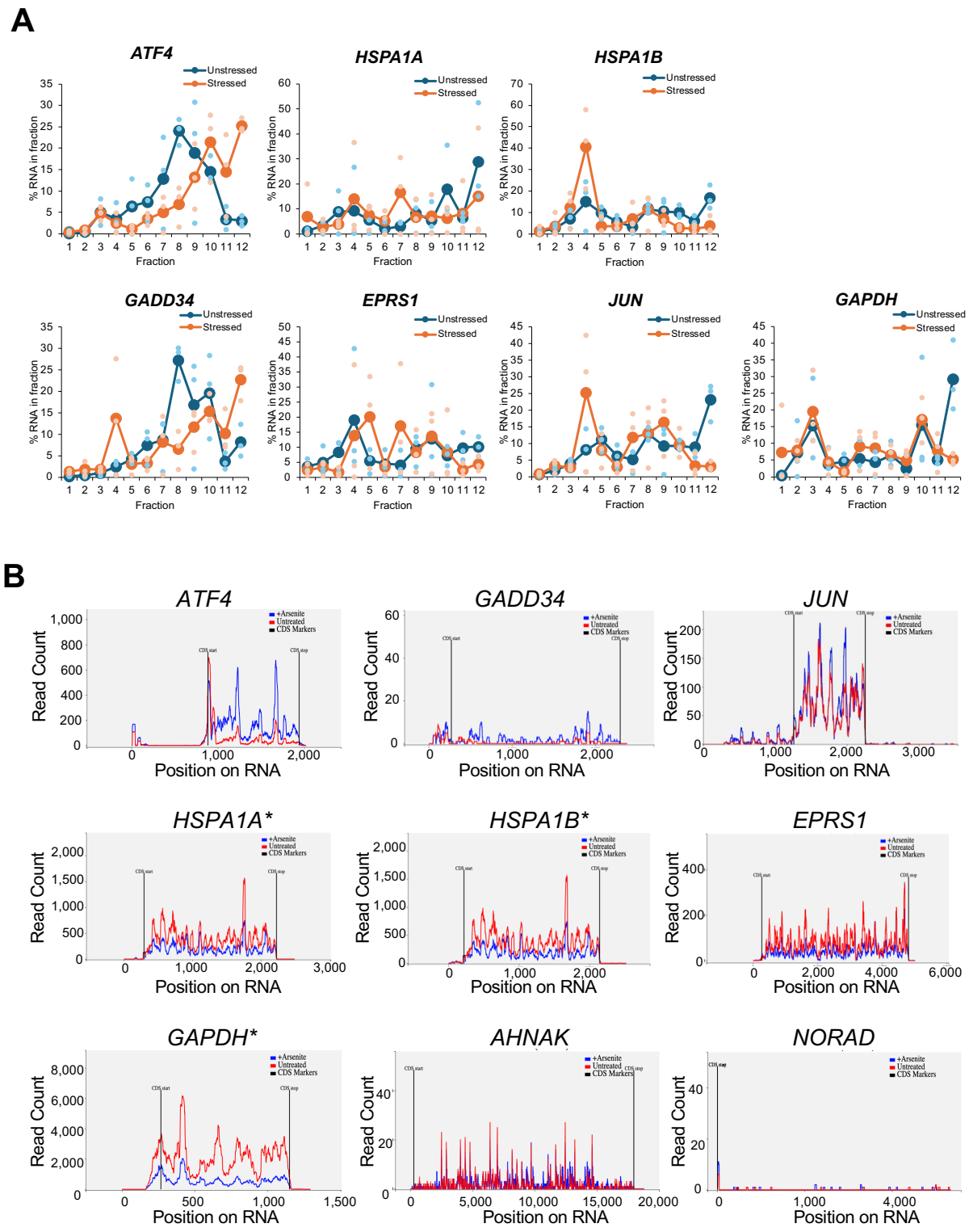
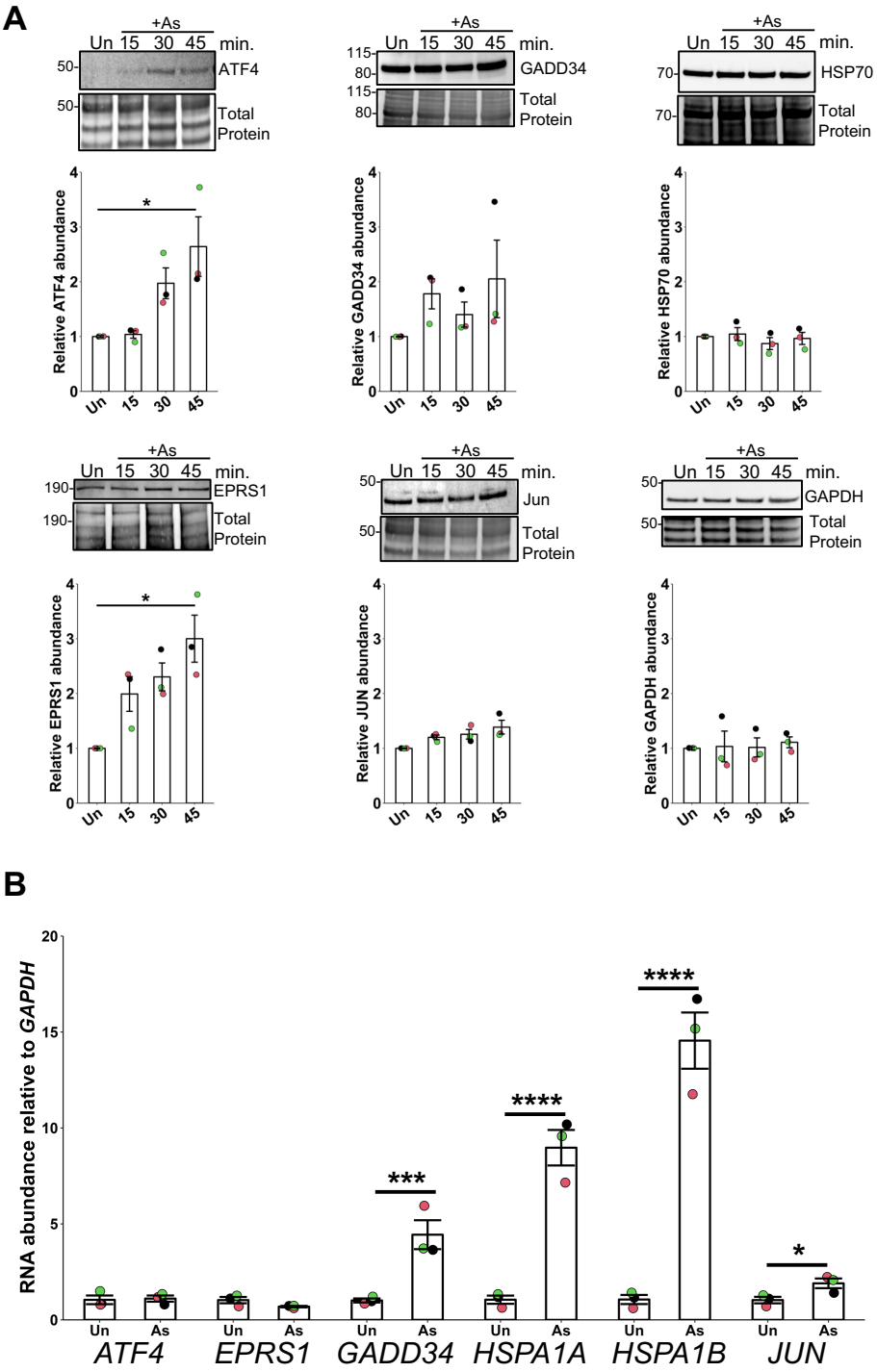
References Cited in Supplemental

Supplemental Figures

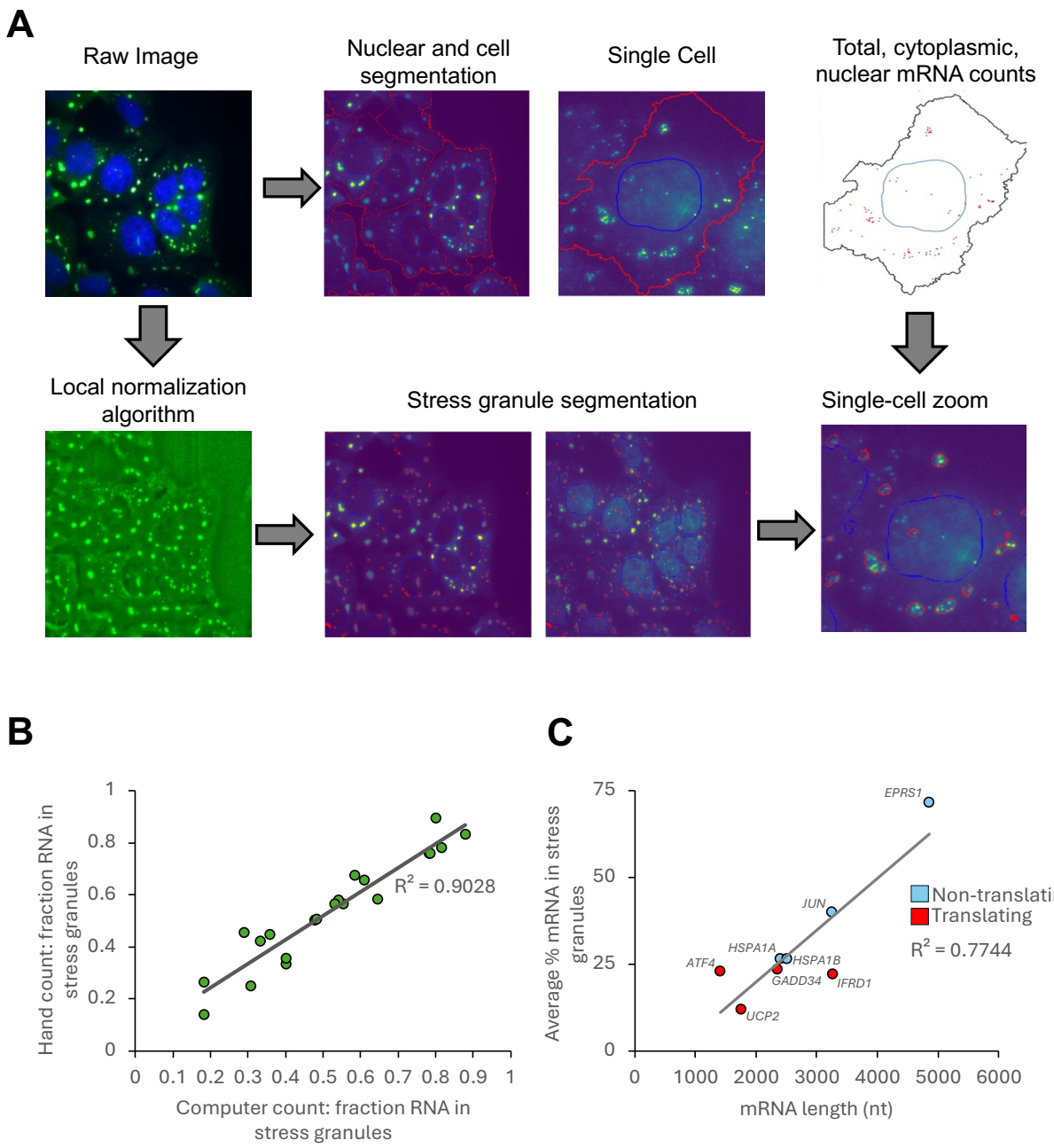


Supplemental Figure S1. Distribution of candidate stress-induced and constitutive RNAs in polysome fractions. (A) Total RNA was isolated from fractions (related to Fig. 1) and RT-qPCR was performed. Line graphs represent the average percent of mRNA sedimenting with each fraction with results from each independent replicate shown as small dots. Samples from stressed cells (arsenite, 250 μM, 45 minutes) shown in orange and unstressed in blue. (B) Single transcript comparison plots of ribosome-protected fragments for RNAs of interest from previously published ribosome profiling data (Andreev et al. 2015). Plots made in

TripsViz with blue representing fragments in arsenite treatment (40 μM, 30 minutes) and red representing fragments in untreated cells. Start and stop codons signified by black vertical lines labeled CDS start and CDS stop. Asterisk (*) by genes indicates unambiguous reads were allowed.

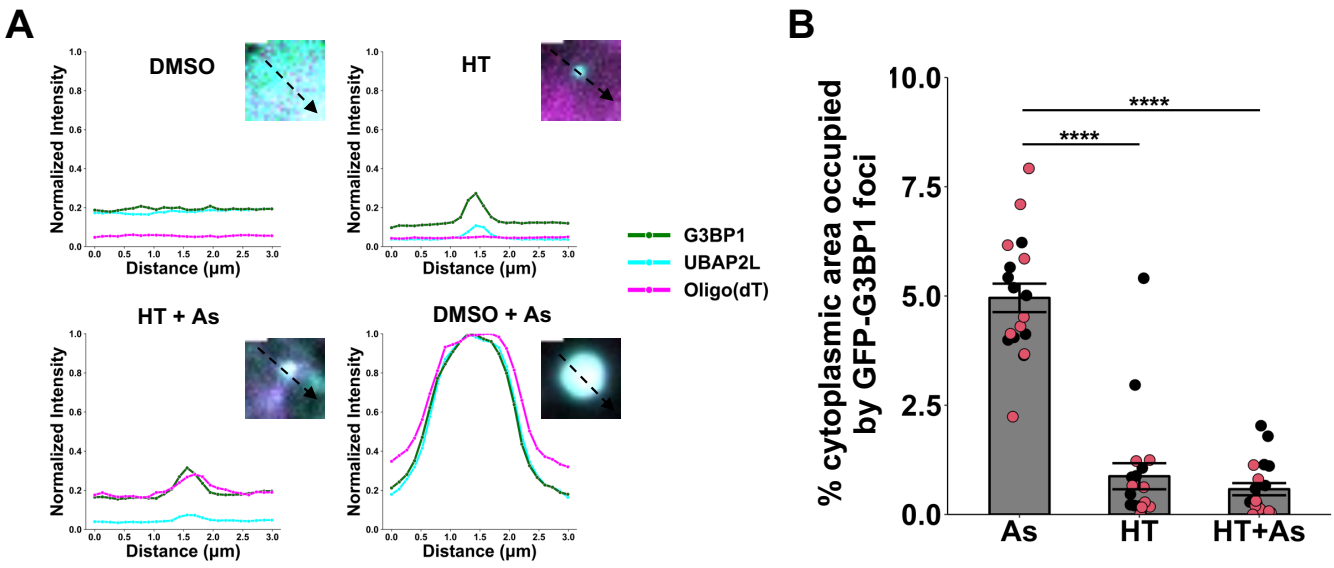


Supplemental Figure S2. Protein and RNA abundance of candidate stress-induced and constitutively expressed genes. (A) Western blots of stress-induced and constitutively expressed genes in unstressed (Un) or arsenite-stressed (As) U-2 OS cells for 15, 30, or 45 minutes (min.) related to Fig. 1. Graphs below are average relative abundances normalized to total protein stain +/- s.e.m.; values from independent replicates (n=3) are signified by dots on the graphs. (B) RT-qPCR of stress-induced gene mRNAs in unstressed (Un) or arsenite (As) stressed (250 μM, 45 minutes) conditions. Data represents average mRNA abundance +/- s.e.m. relative to the constitutive mRNA *GAPDH*. Values from individual replicates (n=3) are signified by dots on graphs. One-way ANOVA tests were done to determine significance between unstressed and stressed conditions; * $p<0.05$, *** $p<0.005$, **** $p<0.001$.

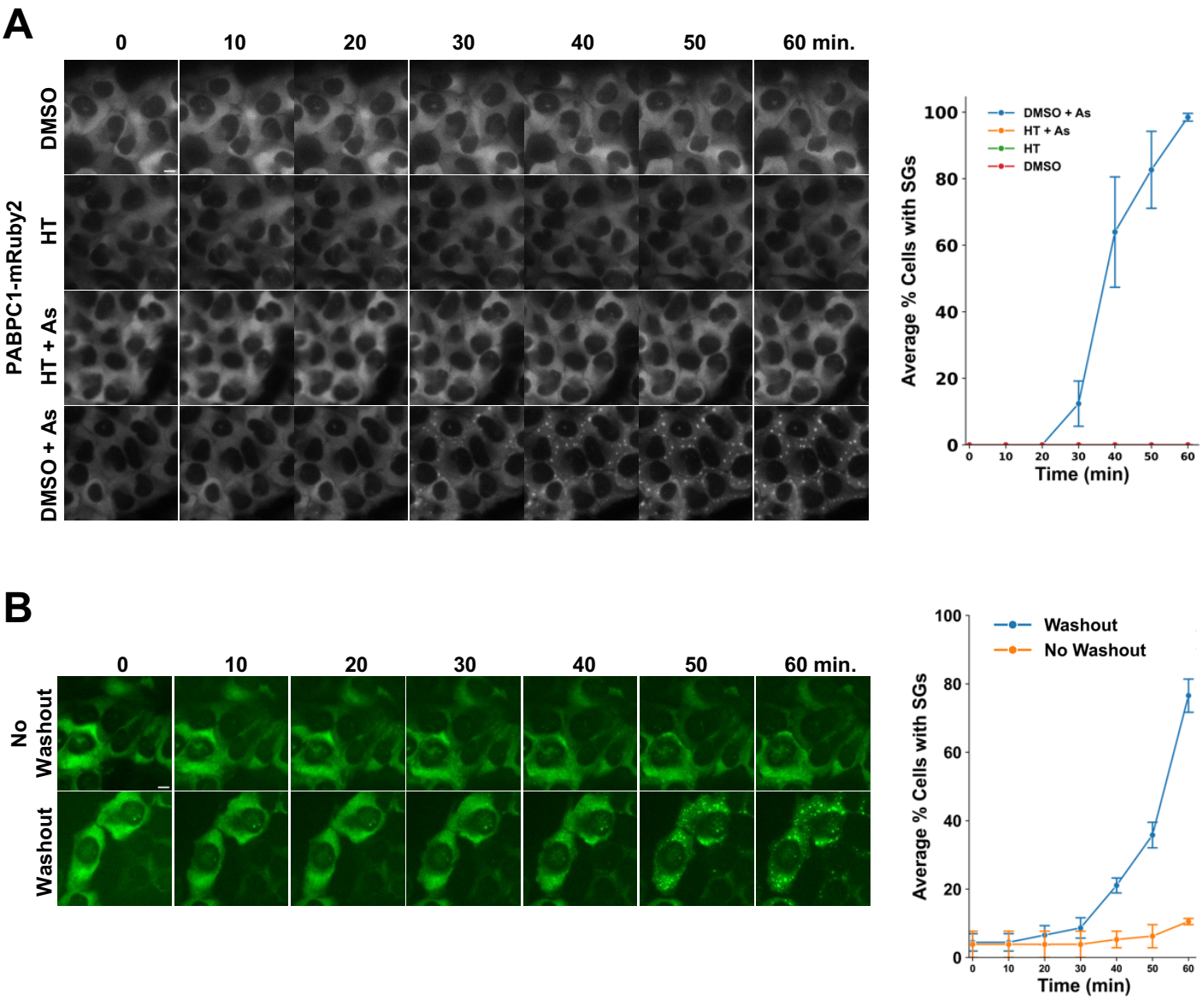


Supplemental Figure S3. smFISH analysis pipeline for quantifying the fraction of RNA in stress granules. (A) Pipeline for computationally analyzing smFISH data and localization to stress granules. The nucleus (outlined in blue), cell (outlined in red), and stress granules (outlined in red) are segmented, then the number of RNAs in the cytoplasm, in stress granules, and in the nucleus was quantified for each cell. The fraction of RNA in stress granules was determined by dividing the number of RNAs in stress granules by the number of cytoplasmic RNAs. Representative image shown (stress granules in green, RNA in yellow, nuclei in blue). (B) Hand count vs. computational analysis count to validate pipeline for analyzing percent mRNA in stress granules. The percent of cytoplasmic mRNA localized to stress granules of three candidate mRNAs (*GADD34*, *JUN*, and *EPRS1*) was counted by hand in ImageJ/Fiji in each cell (n=21 cells). The percent of cytoplasmic mRNA localized to stress granules in the same cells was calculated using FISH-Quant/BigFISH (Imbert et al. 2022) and plotted against the hand-counted cells. Dots on the graph represent individual cells

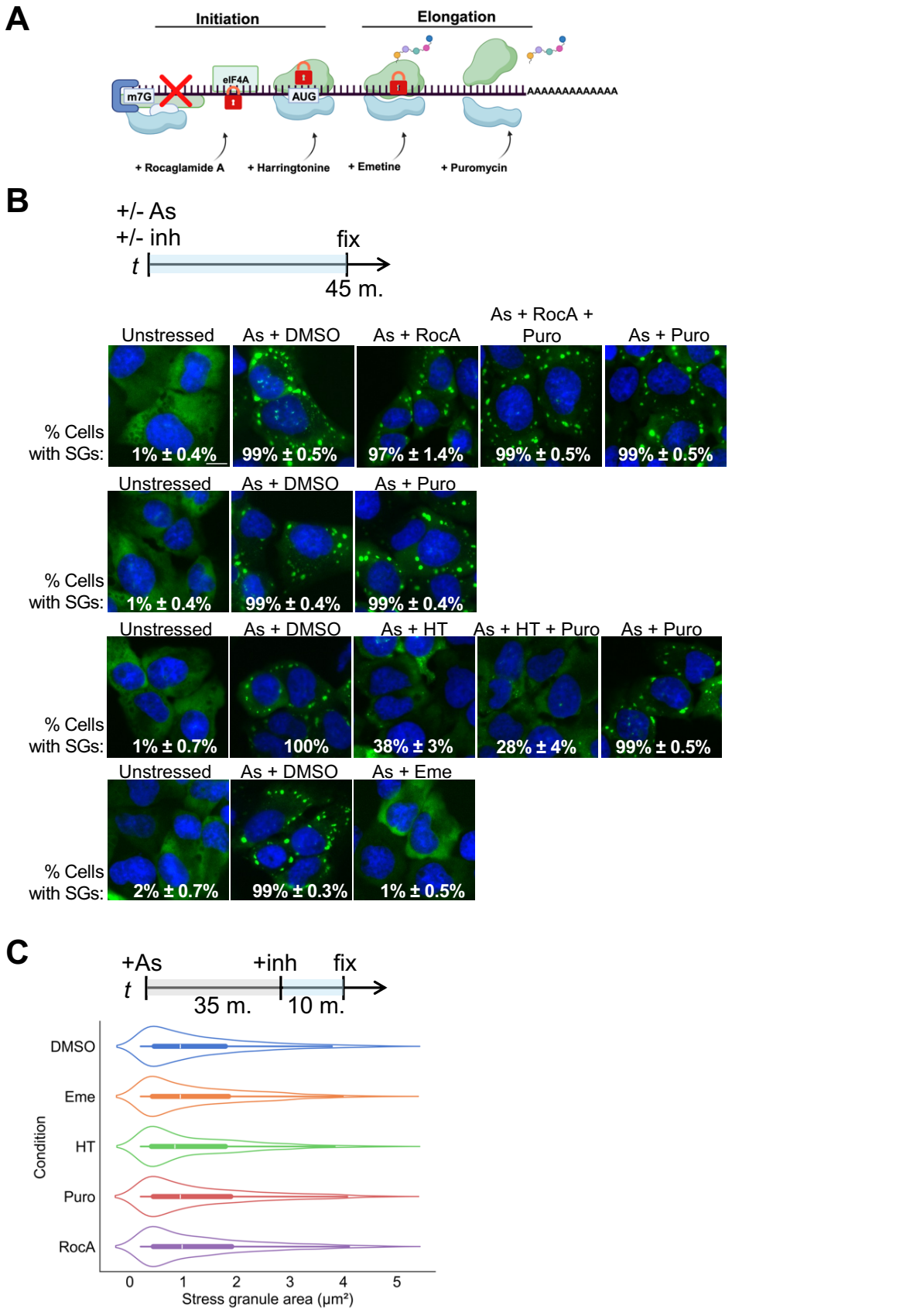
and a linear regression was done (shown as a line) to calculate R^2 value. (C) mRNA length (nucleotides; nt) of stress-induced gene mRNAs and percent mRNA localization to stress granules calculated from Fig. 2 for *ATF4*, *GADD34*, *JUN*, *EPRS1*, *HSPA1A*, and *HSPA1B*, and Figure 6 for *UCP2* and *IFRD1*. Dots indicate each mRNA with blue representing non-translating mRNAs and red representing mRNAs that are translationally upregulated during stress. Linear regression was done (shown as a line) to calculate R^2 value. Related to Fig. 2, 4, 5, 6, and 7.



Supplemental Figure S4. Analysis of GFP-G3BP1 punctae in fixed cells. (A) Intensity line-scans of fixed images in Fig. 3F of GFP-G3BP1, UBAP2L, and oligo(dT) with insets showing foci; scale bar: 1 μm. Observable foci were scanned in all three channels for each condition. Intensities were normalized to the highest intensity in the DMSO + arsenite (As) sample. Colors correspond to colors in Fig. 3F with oligo(dT) in magenta, UBAP2L in cyan, and GFP-G3BP1 in green. (B) The percent of cytoplasmic area that is occupied by foci marked by GFP-G3BP1 in fixed cells in the presence or absence of arsenite (As) and/or harringtonine (HT) is shown. Cells with GFP-G3BP1 punctae were analyzed using the equation 100% x (total stress granule area/cytoplasm area); n=18-19 cells quantified from n=2 independent replicates. Related to Fig. 3.

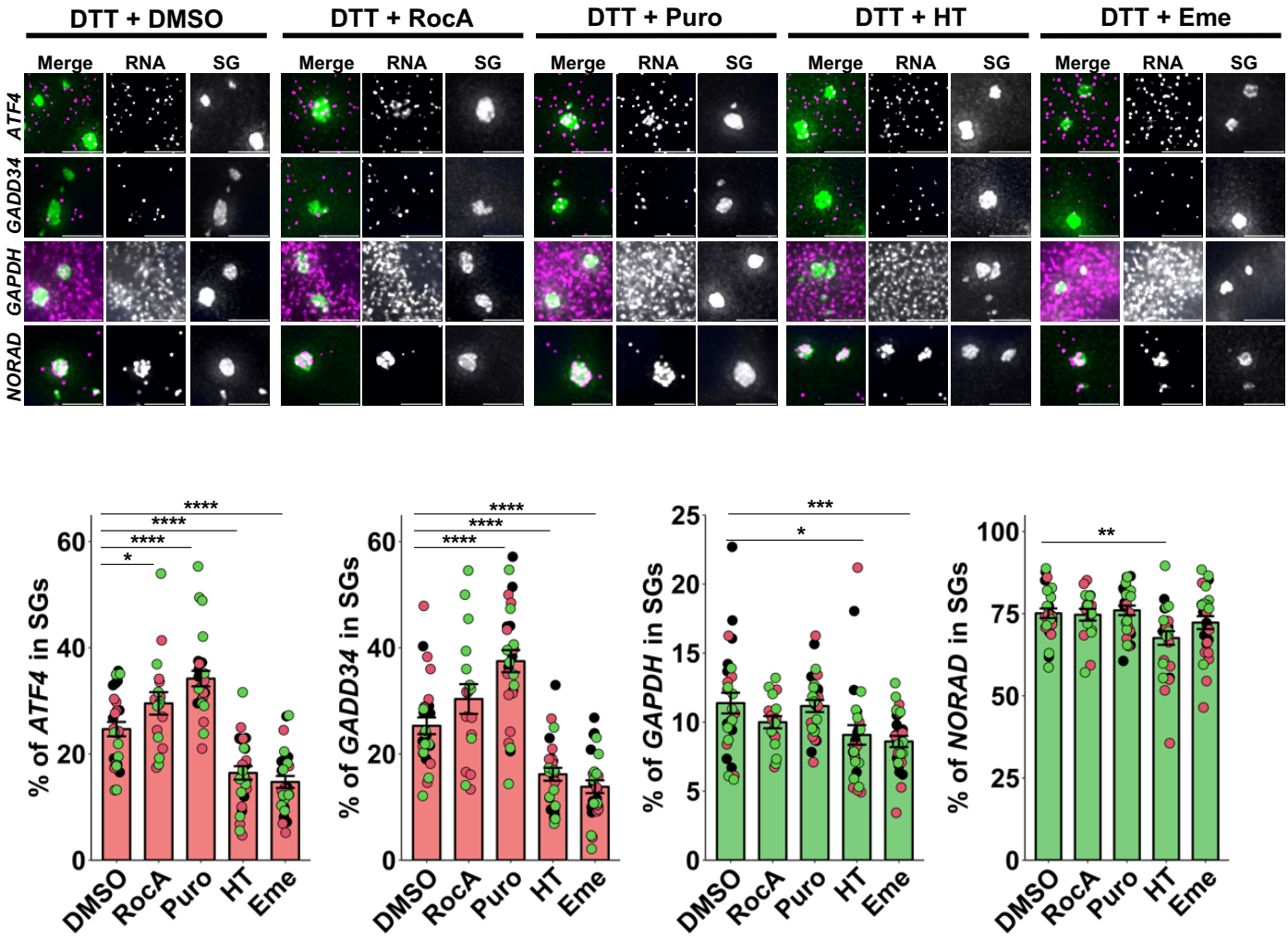


Supplemental Figure S5. A single initiating ribosome reversibly blocks stress granule assembly. (A) Harringtonine (HT) inhibits stress granule formation in live cells during arsenite (As) stress as visualized with the stress granule marker protein mRuby2-PABPC1. U-2 OS cells were pretreated with DMSO (0.1%) or HT (2 µg/mL) for 30 minutes followed by live-cell imaging at 40X magnification with or without arsenite (250 µM) for 60 minutes (min). *Left*: Images were taken every minute and representative images every 10 min. of U-2 OS cells stably expressing mRuby2-PABPC1 (grey) are shown. Scale bar: 10 µm. *Right*: The average +/- s.e.m. of the percent of cells in a frame positive for stress granules (n=3). (B) U-2 OS cells expressing GFP-G3BP1 were pretreated with HT for 30 minutes. After pre-treatment, arsenite was added to HT-containing media ("No Washout") or HT-containing media was removed and replaced with arsenite-containing media ("Washout"). Representative images of GFP-G3BP1-expressing U-2 OS cells shown at left with average percent cells with stress granules (SGs) +/- s.e.m. from n=2 independent replicates at right. Related to Fig. 3.



Supplemental Figure S6. Impact of translation inhibitors on stress granule formation. (A) Schematic diagram illustrating the effects of rocaglamide A, harringtonine, emetine, and puromycin treatment on mRNA association with ribosomes. (B) U-2 OS cells stably expressing GFP-G3BP1 (green) were stressed with

arsenite and co-treated with rocaglamide A, harringtonine, emetine, or puromycin. After 45 min., cells were fixed and stained with Hoescht to visualize nuclei (blue). Representative images (n=3, scale bar: 10 μm) of stress granule formation in unstressed or arsenite (As, 250 μM) stressed cells with DMSO (0.1%), rocaglamide A (RocA, 1 μM), rocaglamide A and puromycin (Puro, 2 μg/mL), puromycin (2 μg/mL), harringtonine (HT, 2 μg/mL), harringtonine (2 μg/mL) and puromycin (2 μg/mL), or emetine (Eme, 45 μM). Percentages represent the average +/- s.e.m. percent of cells in a frame (n=9 frames per condition, 3 independent replicates) that contain stress granules. (C) Stress granule area in fixed cell samples treated with a 10-minute pulse of translation inhibitors beginning at 35 minutes post-arsenite stress. The area of individual stress granules was determined from *ACTB*/*IFRD1* smFISH images from n=3 independent replicates. The area from 2,099 stress granules from DMSO, 2,090 from rocaglamide A (RocA), 1,853 from puromycin (Puro), 1,814 from harringtonine (HT), and 1,703 from emetine (Eme) was quantified. Related to Fig. 4, 5, 6, and 7.



Supplemental Figure S7. Ribosome association reduces *ATF4* and *GADD34* mRNA localization to stress granules during ER stress. U-2 OS cells stably expressing GFP-G3BP1 were stressed with DTT (2 mM) for 50 minutes followed by addition of DMSO (0.1%), rocaglamide A (RocA, 1 μM), puromycin (Puro, 2 μg/mL), harringtonine (HT, 2 μg/mL), or emetine (45 μM). smFISH was performed on fixed cells to determine localization of RNAs encoding stress-induced genes that are translationally up-regulated (red) or constitutively expressed genes (green). Top: representative images are shown with RNA in magenta and stress granules (SG) in green as labeled with GFP-G3BP1. Scale bar: 5 μm. Bottom: The average +/- s.e.m. of the percent of cytoplasmic RNA localized to stress granules per cell (n=27; n=18 for rocaglamide A treatment) from three independent replicates (denoted as green, red, and black circles with individual circles representing a single cell; n=2 for rocaglamide A treatment). One-way ANOVA tests were done to determine significance between DMSO condition and other treatments; *p<0.05, **p<0.01, ***p<0.005, ****p<0.001. Related to Fig. 4 and 5.

Supplemental Video Captions

Supplemental Video S1. Harringtonine pretreatment blocks stress granule formation. Representative video related to Fig. 3D & E. GFP-G3BP1 (green) in U-2 OS cells pre-treated with DMSO (0.1%) or harringtonine (HT; 2 µg/mL) for 30 minutes followed by live-cell imaging at 40X magnification with or without arsenite (As; 250 µM) every minute (min.) for 60 minutes. Top Left: DMSO only; Top Right: HT pretreatment followed by arsenite; Bottom Left: HT only; Bottom Right: DMSO pre-treatment followed by arsenite. Scalebar: 10 µm.

Supplemental Video S2. Harringtonine pretreatment blocks stress granule formation marked by mRuby2-PABPC1. Representative video (related to Fig. 3 and Supplemental Fig. S5) of mRuby2-PABPC1 (grey) in U-2 OS cells pre-treated with DMSO (0.1%) or harringtonine (HT, 2 µg/mL) for 30 minutes followed by live-cell imaging at 40X magnification with or without arsenite (As; 250 µM) every minute (min.) for 60 minutes. Top Left: DMSO only; top right: HT pretreatment followed by arsenite; bottom left: HT only; bottom right: DMSO pre-treatment followed by arsenite. Scalebar: 10 µm.

Supplemental Video S3. Lactimidomycin pretreatment blocks stress granule formation. Related to Fig. 3G, representative video of U-2 OS cells stably expressing GFP-G3BP1 pre-treated with lactimidomycin (LTM, 50 µM) for 30 minutes followed by live-cell imaging with or without arsenite every minute for 60 minutes. Top Left: DMSO only; top right: LTM pretreatment followed by arsenite; bottom left: LTM only; bottom right: DMSO pre-treatment followed by arsenite. Scalebar: 10 µm.

Supplemental Video S4. Harringtonine reversibly inhibits stress granule formation. Related to Supplemental Fig. S5 and Fig. 3, U-2 OS cells stably expressing GFP-G3BP1 were pretreated with HT for 30 min., then arsenite was added to the HT-containing media (“No washout”, at left) or the HT-containing media was removed and replaced with media containing arsenite (“Washout”, at right). Cells were imaged every minute for 60 minutes. Scalebar: 10 µm.

Supplemental Table Captions

Supplemental Table S1. Oligonucleotide sequences of uORF-luciferase reporter constructs, related to Fig. 7.

Supplemental Table S2. Oligonucleotide sequences for smFISH probes used to detect *ATF4*, *GADD34*, *HSPA1A*, *HSPA1B*, *JUN*, *EPRS1*, *GAPDH*, *NORAD*, *IFRD1*, *UCP2*, *ACTB*, *AHNAK*, and Luciferase. Related to Fig. 2, 4, 5, 6, 7 and Supplemental Fig. S3 and S7.

Supplemental Table S3. qPCR primers used to detect *ATF4*, *GADD34*, *HSPA1A*, *HSPA1B*, *JUN*, *EPRS1*, and *GAPDH*, related to Supplemental Fig. S2.

Supplemental Table S4. Source data for all figures.

References Cited in Supplemental

Andreev DE, O'Connor PBF, Fahey C, Kenny EM, Terenin IM, Dmitriev SE, Cormican P, Morris DW, Shatsky IN, Baranov PV. 2015. Translation of 5' leaders is pervasive in genes resistant to eIF2 repression. *Elife* **4**: e03971.

Imbert A, Ouyang W, Safieddine A, Coleno E, Zimmer C, Bertrand E, Walter T, Mueller F. 2022. FISH-quant v2: a scalable and modular tool for smFISH image analysis. *RNA* **28**: 786–795.