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Supplemental Information

A Nuclear Stress Pathway that Parallels Cytoplasmic Stress Granule Formation

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Supplemental Tables

Stress	Mode of Action	nucSF/SG Formation
Sodium Arsenite (0.5 mM, 30 min)	ROS production	Yes/Yes (Fig. 1d)
Bortezomib (25 μM, 4 hrs)	Inhibits 26S Proteasome	Yes/Yes (Fig. 1g)
Serum/glucose-free media (overnight)	Nutrient/serum starvation	Yes/Yes (Fig. 1g)
Rocaglamide A (1 μM, 1 hr)	Inhibits eIF4A	No/Yes* (Fig. 1g)
Rocaglamide A (1 μM, 6 hr)	Inhibits eIF4A	No/Yes* (Fig. S1m)
Hydrogen peroxide (1 mM, 2 hrs)	Oxidative stress	No/Yes* (Fig. 1g)
Actinomycin D (2.5 μg/ml, 2 hrs)	Inhibits RNA transcription	No/No (Fig. S1a)
5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB; 25 μg/ml, 2 hrs)	Inhibits CTD kinases	No/No (Fig. S1b)
Hydroxyurea (2 mM, overnight)	Inhibits DNA synthesis	No/No (Fig. S1c)
Camptothecin (1 μg/ml, 1 hr)	Inhibits DNA topoisomerase I	No/No (Fig. S1d)
Cisplatin (1 μg/ml, overnight)	Crosslinks DNA	No/No (Fig. S1e)
Etoposide (50 μM, 4 hrs)	Inhibits DNA Topoisomerase II	No/No (Fig. S1f)
Olaparib (10 μM, overnight)	Inhibits PARP	No/No (Fig. S1g)
MG132 (10 μg/ml, 1 hr)	Inhibits 26S proteasome	Yes/Yes (Fig. S1h)
Sorbitol (0.4 M, 1 hr)	Osmotic stress	Yes^/Yes* (Fig. S1i)
NaCl (0.2 M, 1 hr)	Osmotic stress	Yes^/Yes* (Fig. S1j)
Heat Shock (45°C, 30 min)	Heat stress	No/Yes (Fig. S1k)
Heat Shock (45°C, 1 hr)	Heat stress (extreme)	Yes^/Yes (Fig. S1l)
TIA-1 overexpression	Aggregation of SG factors	No/Yes* (Fig. S1o-p)

Table S1. Summary of cellular stresses tested for nucSF and SG formation, Related to Figures 1 and S1. Formation of nucSF and cytoplasmic SGs was assessed in U2OS cells (see indicated panels in Figures 1 and S1). Yes = >50% of cells have >6 nuclear foci and/or SGs. *non-canonical SGs. ^ fewer nuclear foci, clustered at the nucleolar periphery.

Cell Line	Description	nucSF/SG formation
HeLa	Cervical carcinoma (f)	Yes/Yes (Fig. 1d)
U-2 OS	Osteosarcoma (f)	Yes/Yes (Fig. 1d)
MCF7	Breast cancer, derived from metastatic site (f)	Yes/Yes (Fig. S1a)
NTERA-2	Pluripotent testicular embryonal carcinoma (m)	Yes/Yes (Fig. S2b)
SH-SY5Y	Neuroblastoma (f)	Yes/Yes (Fig. S2c)
HCT116	Colorectal carcinoma (m)	Yes/Yes (Fig. S2d)
SW480	Colorectal adenocarcinoma (m)	Yes/Yes (Fig. S2e)
SW620	Colorectal adenocarcinoma, derived from metastatic site (m)	Yes/Yes (Fig. S2f)
HEK293	Embryonic kidney, adenoviral-transformed (f)	Yes/Yes (Fig. S2g)
RPE-1	Retinal pigmented epithelium, hTERT-immortalized (f)	Yes/Yes (Fig. S2h)

Table S2. Summary of cultured human cell lines tested for arsenite-induced nucSF and SG formation, Related to Figurse 1 and S2. Cells transiently expressing GFP-RepoMan were treated with arsenite (0.5 mM, 30 min), fixed and stained with anti-G3BP to assess formation of nucSF and SGs (see indicated panels in Figures 1 and S2). Yes = >50% of cells had >6 nuclear foci and/or SGs. (f) = female. (m) = male.

Supplemental Figures

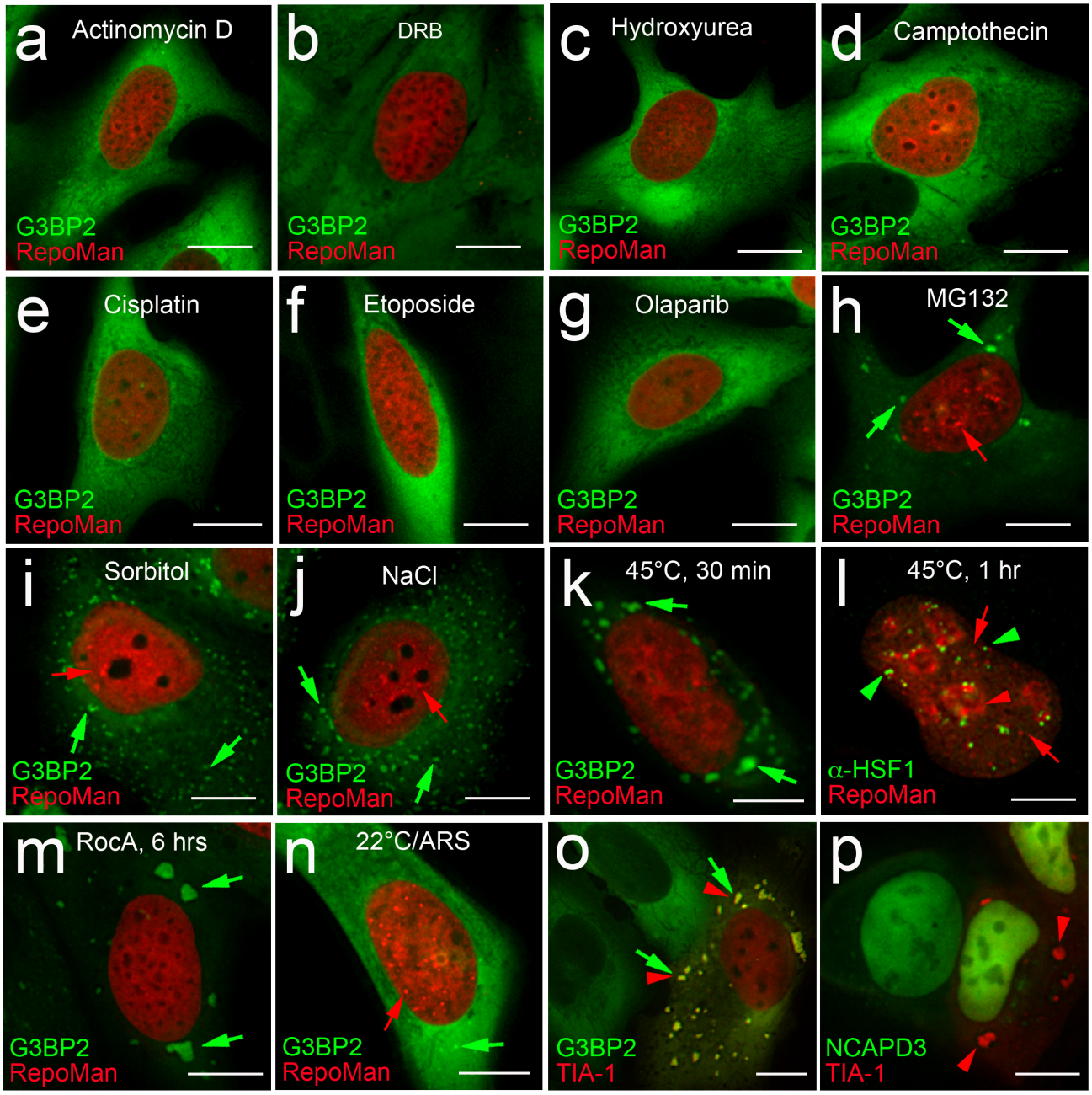

Figure S1. Stress-specific formation of nucSF and cytoplasmic SGs, Related to Figure 1. a-k, m-n. U2OS cells stably expressing GFP-G3BP2 (green) and transiently expressing mCh-RepoMan (red) were subjected to the treatment indicated at the top of each panel and imaged live to assess formation of nucSF (red arrows) and SGs (green arrows). See Table S2 for details of each treatment (concentration, time, mode of action). Cells exposed to heat shock were fixed prior to imaging (k-l). In response to more extreme heat shock (45°C for 1 hr), accumulation of mCh-RepoMan at small nucSF (red arrows) and the peri-nucleolar region (red arrowhead) in parental U2OS cells was observed (l). For this experiment, the cells were fixed and stained with anti-HSF1 (green) to mark heat shock-induced nuclear stress bodies (green arrowheads). o. Transient overexpression of mCh-TIA1 in U2OS cells stably expressing G3BP2 induced SGs containing both proteins (red arrowheads, green arrows) but did not trigger accumulation of NCAPD3 in nucSF (p). Scale bars are 5 μm.

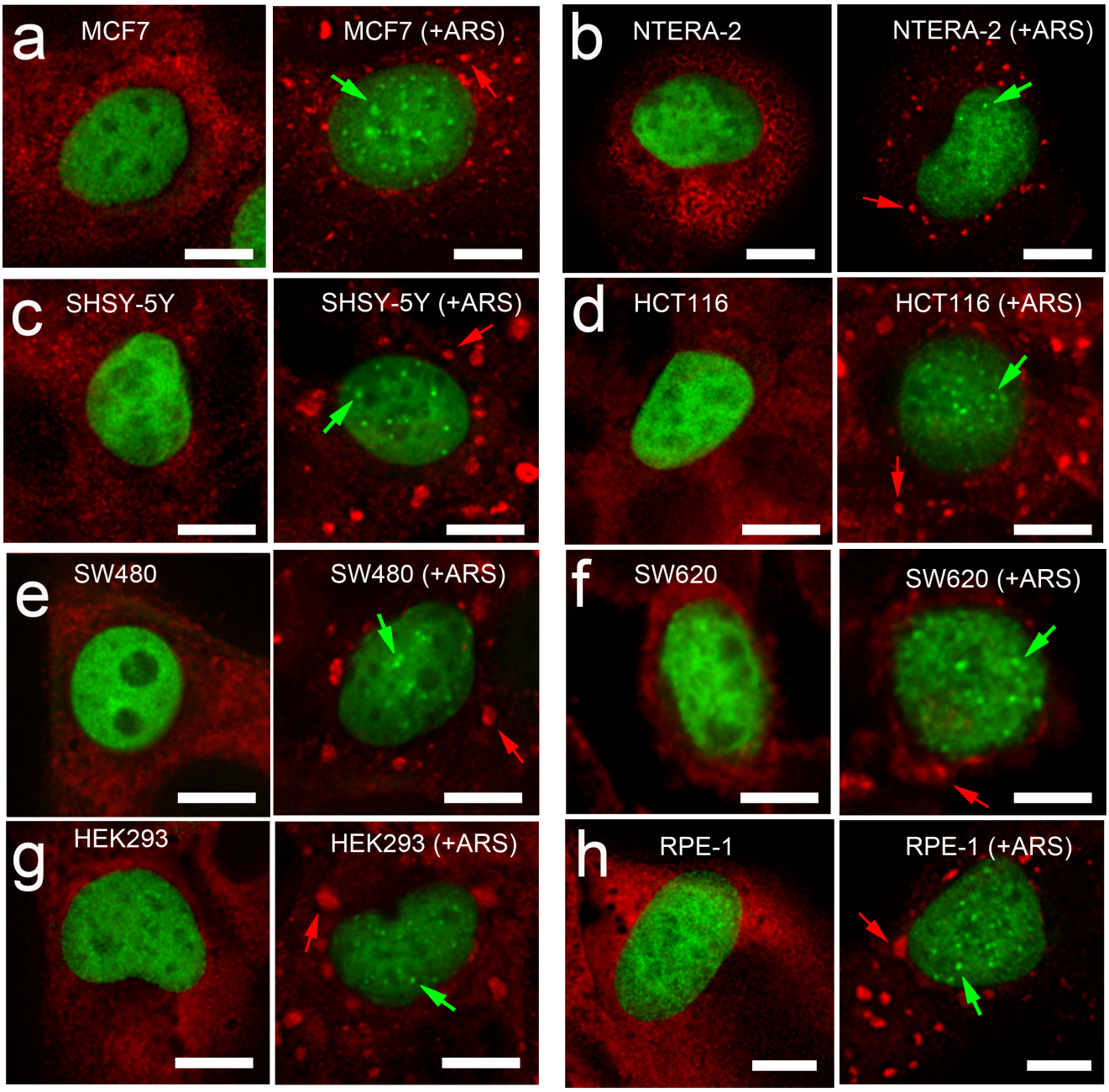

Figure S2. Arsenite stress induces formation of nucSF and cytoplasmic SGs in a range of cultured cell lines, Related to Figure 1. a-h. The cell lines indicated at the top of each panel were transiently transfected with GFP-RepoMan and either left untreated or treated with 0.5 mM arsenite for 30 min prior to fixation (+ARS). The fixed cells were stained with anti-G3BP (red). Green arrows point to nucSF while red arrows point to SGs. Scale bars are 5 μm.

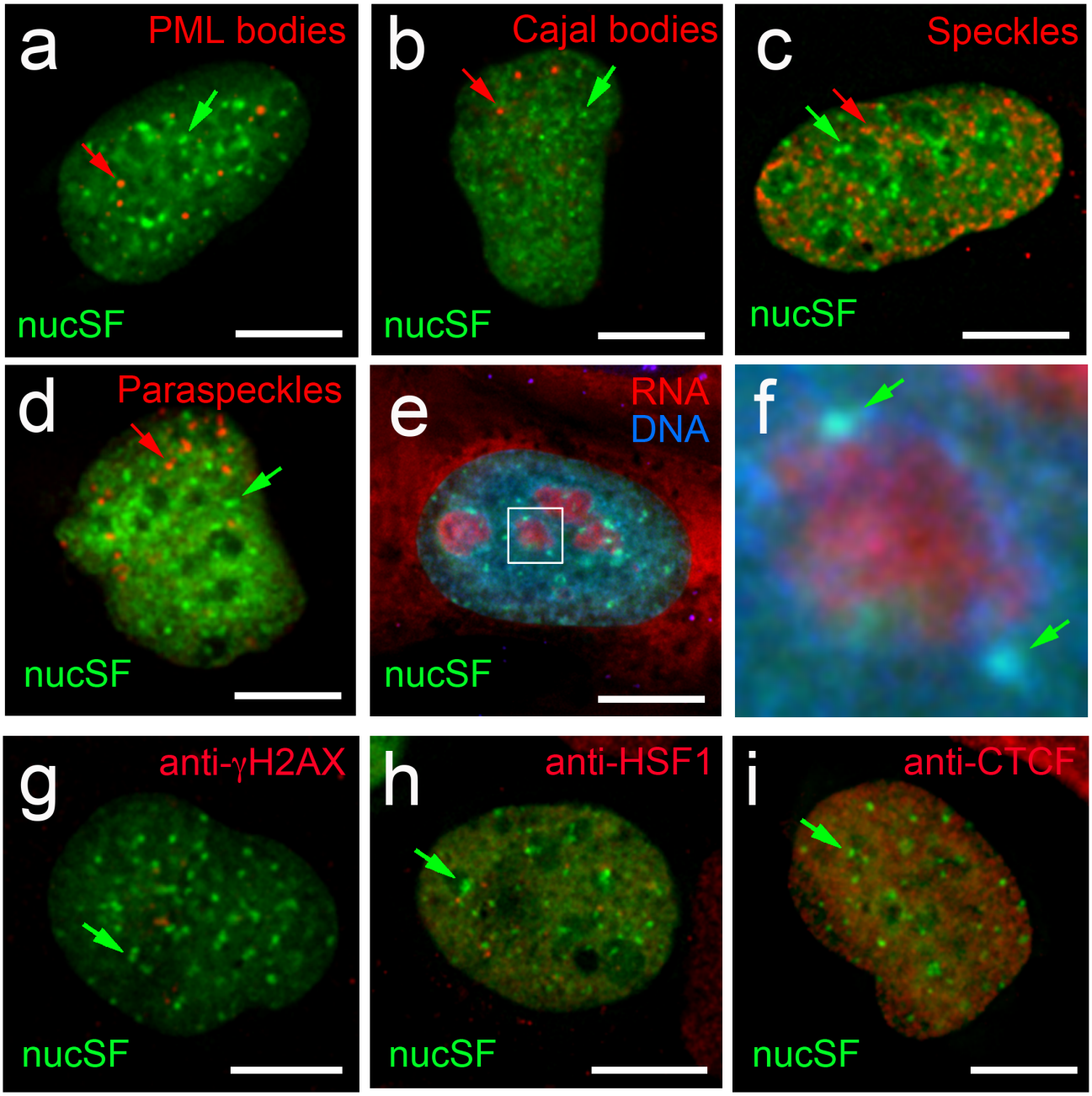

Figure S3. Arsenite-induced nucSF marked by accumulation of GFP-RepoMan in U2OS cells are distinct from other known nuclear bodies, Related to Figure 2. No overlap is detected between nucSF (GFP-RepoMan; green arrows) and nuclear organelles such as PML bodies (a; anti-PML; red arrows), Cajal bodies (b; anti-Coilin; red arrows), splicing speckles (c; anti-Y12; red arrows) or paraspeckles (d; mCh-PSP1; red arrows). Increased Hoechst 33342 (DNA; blue) but not Pyronin Y (RNA; red) staining is observed in nucSF (e; f is expanded view of the region delineated by the white box). The arsenite treatment use d (0.5 mM for 30 min) did not induce accumulation of either γH2AX (g; marker for sites of double-strand DNA breaks) or HSF1 (h; marker for heat shock-induced nuclear stress bodies). i. No localized accumulation of the chromatin insulator factor CTCF was observed in arsenite-stressed cells. Scale bars are 5 μm.

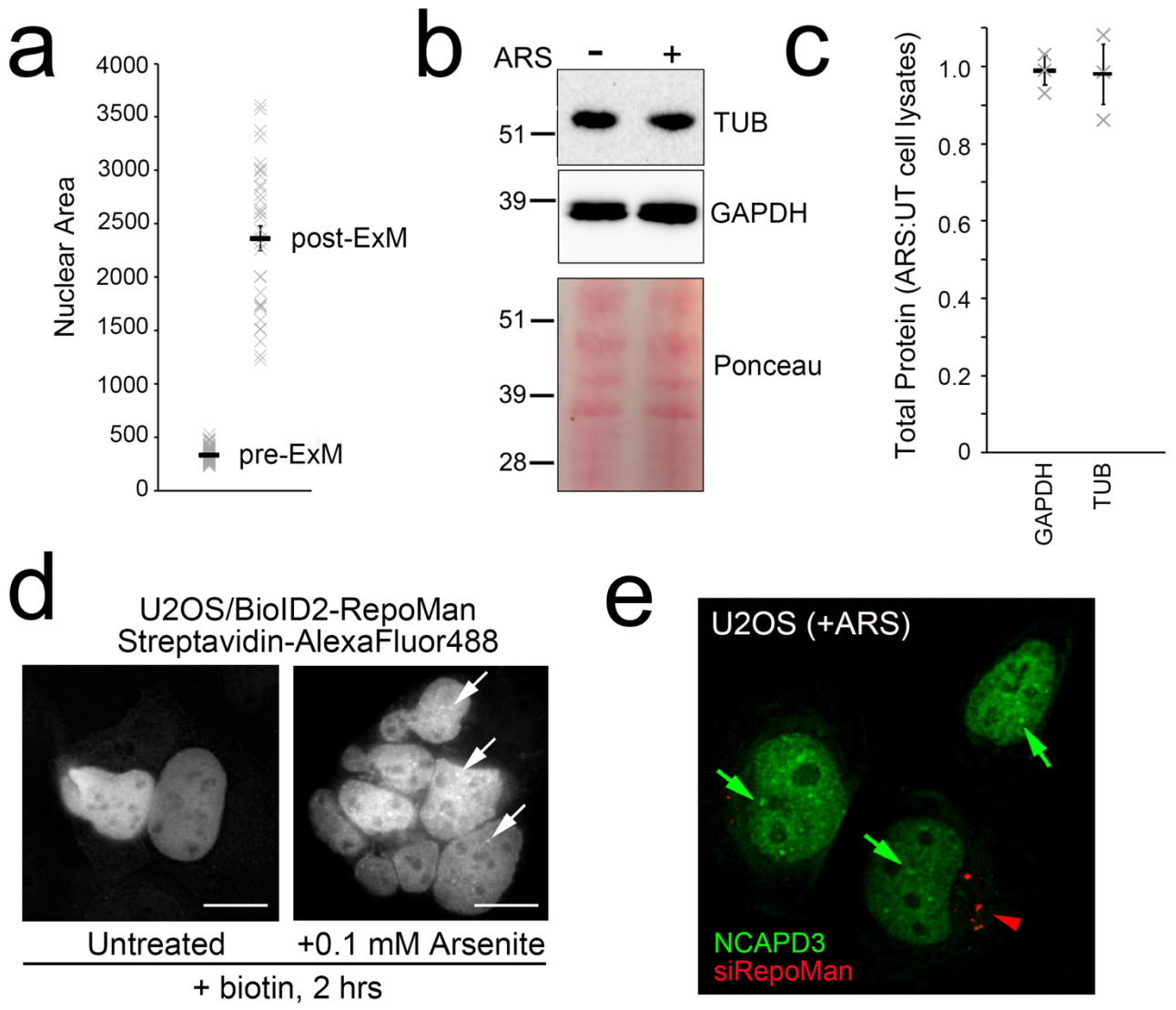

Figure S4. Control experiments, Related to Figures 2-4. a. Estimation of ExM expansion factor based on measurement of Hoechst 33342-stained nuclear area in pre- and post-expansion cells. Results shown here are for 92 pre-ExM and 36 post-ExM cells measured in 4 separate experiments. The average expansion factor was 7.1. b. Western blot assessing protein levels of GAPDH and alpha-tubulin (TUB) in equivalent total protein amounts (50 μg) of U2OS whole cell lysates with and without arsenite stress (0.5 mM for 30 min). Ponceau S staining of the relevant region of the blot is shown. c. Graph showing the results of 3 independent experiments (X) and the mean ± SE (-). For each experiment, GAPDH and TUB levels were quantified, and then normalized by the level of total stained protein in each lane (measured on a Coomassie-stained gel run in parallel). d. Coverslips included in the dishes of cells harvested for the BioID experiment (U2OS cells transduced to transiently express BioID2-RepoMan) were removed for fixation and streptavidin staining to confirm accumulation of RepoMan at arsenite-induced nucSF (arrows). e. U2OS cells stably expressing GFP-NCAPD3 were treated with Cy5-tagged siRNA targeting RepoMan for 48 hrs prior to arsenite stress. Red arrowhead indicates cell that has taken up the siRNA and green arrows indicate NCAPD3 accumulation in nuclear foci. Scale bars are 5 μm.

Transparent Methods

Plasmids and antibodies

The pEGFP(C1)-RepoMan and pmCherry(C1)-RepoMan plasmids were previously described (Trinkle-Mulcahy et al., 2006) and are available through Addgene (plasmids #44212 and 46274). For BioID experiments, RepoMan was fused to the BioID2 tag sequence (Addgene plasmid #74223; (Kim et al., 2016)) and subcloned into the pLVX-IRES-mCherry vector (Takara Bio). Coding sequences for G3BP2 and NCAPD3 were amplified from donor constructs (obtained from ThermoFisher) and subcloned into the pEGFP-C1 expression plasmid. All cloning was confirmed by DNA sequencing (StemCore Laboratories, Ottawa Hospital Research Institute). pmCherry-PSP1 and pmCherry-TIA1 were gifts from Drs. Archa Fox and Derrick Gibbings, respectively. Antibodies that recognize HP1γ, H3K9me3, HSF1, Coilin, SNRPB (Y12), ADCY2 and NCAPD3 were obtained from Abcam. Alpha-tubulin and GFP antibodies were from Millipore Sigma. The G3BP antibody was from BD Biosciences, the CTCF antibody from New England Biolabs and the PML antibody from Santa Cruz Biotechnology. The TIA1 antibody was a gift from Dr. Jocelyn Côté. All HRP- and fluorophore-conjugated secondary antibodies and streptavidin were from ThermoFisher.

CRISPR/Cas 9 gene editing

To knock GFP in frame upstream of Exon 1 in endogenous RepoMan, guide RNAs were designed (ThermoFisher), annealed and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (Ran et al., 2013)**) (**Addgene plasmid #62988) using BbsI. The guide sequences were #1 (FWD CACCGTCGAATCACGTTCATCTTGC; REV AAACGCAAGATGAACGTGATTCGAC), #2 (FWD CACCGATCACGTTCATCTTGCTGGT; REV AAACACCAGCAAGATGAACGTGATC) and #4 (FWD CACCGTGAATTGGCATCCATCTAAG; REV AAACCTTAGATGGATGCCAATTCAC). A linear donor/repair template (GeneArt Strings; ThermoFisher) was designed with 800 bp left and right arms that anneal to the RepoMan gene locus near the initiating methionine flanking the GFP sequence and a linker region (5 x alanine). Silent mutations were introduced into the PAM sequences in the donor template targeted by the guide RNAs. The linear template was subcloned into pCR-Blunt (ThermoFisher), using EcoRI, for amplification. HEK293 cells were transfected with a 3:1 ratio of the circular Cas9/gRNA plasmid to the linear donor/repair template, using polyethylenimine (PEI) transfection reagent. Cells were selected by puromycin for 24 hrs and recovered in normal media. GFP-based cell sorting (uOttawa Flow Cytometry Core Facility) was used to sort 1 cell per well in a 96-well plate. Clonal lines were expanded for analysis by imaging and Western blot. RNAi-mediated knockdown of RepoMan was carried out as previously described (Trinkle-Mulcahy et al., 2006).

Cell culture

The cell lines used in this study (detailed in Table S2) were all obtained from ATCC and grown in Dulbecco's modified Eagles' medium (DMEM; Wisent Bioproducts Inc.) supplemented with 10% fetal calf serum and 100 U/mL penicillin and streptomycin (Wisent Bioproducts Inc). For overnight glucose/serum starvation, cells were incubated in glucose-free DMEM (ThermoFisher). Stable cell lines were generated as previously described and maintained in media supplemented with G418 (Trinkle-Mulcahy et al., 2007). For hypoxia experiments, cells were maintained at 1% O_2 in a H35 HypOxyStation® hypoxia chamber (Whitley). For BioID experiments, 20 μM biotin was added to the media for 2 hrs prior to harvesting. All drug treatments are summarized in Table S1. All chemicals were purchased from Millipore Sigma, with the exception of Olaparib (SelleckChem) and NaCl (Fisher Scientific).

Chromatin Immunoprecipitation

GFP-RepoMan knock-in HEK293 cells (untreated or arsenite-stressed) were formaldehyde-fixed, scraped into PBS, pelleted and frozen in liquid N_2. Native chromatin immunoprecipitation (ChIP) was performed as previously described (Brand et al., 2008), with ChIPed DNA purified by phenol-chloroform extraction and precipitated with ethanol, and GAPDH, ADCY2 and PPP2R2C DNA sequences (de Castro et al 2017) amplified with PerfeC TaSYBRGreen SuperMix (Quanta Biosciences) using a Rotor-Gene Q real-time PCR system (QIAGEN). ChIPed DNA quantity was calculated compared with a genomic DNA standard curve using Rotor-Gene Q Series Software 1.7 (QIAGEN) with $R2 > 0.96$. Drosophila spike-in chromatin was used as an internal standard. GFP-Trap_A resin (Chromotek) was used to capture GFP-RepoMan and Protein G Sepharose Fast Flow beads (ThermoFisher) as a negative control. Four experiments (2 x 15 cm dishes untreated vs. arsenite each) were analyzed.

RT-qPCR

Total cellular RNA was extracted from cells and reverse-transcribed, and transcripts were amplified as previously described (Chamousset et al., 2010). For qPCR, 4 µL of each cDNA reaction was analyzed in duplicate using Power Up SYBR Green Master Mix (ThermoFisher) and a CF96 Touch™ Real-Time PCR Detection System (BioRad). The comparative C_T method (Schmittgen and Livak, 2008) was used to compare relative levels of mRNA in arsenite-stressed versus untreated U2OS cells, using GAPDH as an internal standard (see Fig. S4). Custom qPCR primers for GAPDH, ADCY2 and PPP2R2C were as previously described (de Castro et al 2017), with three biological replicates analyzed for each.

Metabolic labeling

Stable isotope labeling with amino acids in cell culture (SILAC) for label-based quantitative MS was carried out as previously described (Trinkle-Mulcahy et al., 2008). Briefly, cells were grown for 7-10 passages in high glucose DMEM containing either L-arginine and L- lysine (Light), or the isotopes L-arginine$^{13}C/^{15}N$ and L-lysine$^{13}C/^{15}N$ (Heavy). SILAC media was prepared by supplementing high glucose DMEM minus Arg/Lys/Leu/Met (AthenaES) with 10% dialyzed FBS (ThermoFisher) and the appropriate amino acids, mixing well and filtering through a 0.22 µm filter (Millipore). For the BioID experiment, Heavy isotope-encoded cells were exposed to arsenite stress. This labeling was flipped for the AP/MS experiment, with Light-encoded cells exposed to arsenite stress.

Preparation of cell extracts and affinity purification

For standard Western blots and AP/MS experiments, whole cell extracts were prepared by scraping cells into ice-cold RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, protease inhibitors), sonicating and clearing by centrifuging at 2800g for 10 min at 4 °C. For BioID experiments, whole cell lysates were prepared in high salt (500 mM NaCl) RIPA buffer. Total protein concentrations were measured using the Pierce BCA Protein Assay Kit (ThermoFisher).

GFP-RepoMan complexes were captured from cell extracts as previously described (Prévost et al., 2013), with equal amounts of total protein extract for each condition incubated with GFP-Trap_A beads (Chromotek) at 4°C for 1 hour. Following an initial wash with RIPA buffer, beads from the control and experimental pulldowns were combined for additional washes (to minimize variability in downstream processing) and bound proteins eluted with 1% SDS. For BioID experiments, the salt concentration in the lysates was first reduced to 250 mM by adding an equal volume of "no salt" (0 mM NaCl) RIPA buffer, and then equal amounts of total protein extract for each condition incubated with Streptavidin-agarose beads (ThermoFisher) at 4°C for 4 hours. Following an initial wash with RIPA buffer, beads from the control and experimental pulldowns were combined

for additional washes (to minimize variability in downstream processing) and bound proteins eluted with 2% SDS/30 mM biotin.

The eluted proteins were reduced and alkylated by treatment with DTT and iodoacetamide, respectively. Sample buffer was then added and the proteins resolved by electrophoresis on a NuPAGE 10% BisTris gel (Thermo Fisher). The gel was stained using SimplyBlue Safestain (Thermo Fisher) and the entire lane cut into five slices. Each slice was cut into 2 × 2 mm fragments, destained, and digested overnight at 30°C with Trypsin Gold (ThermoFisher).

Mass spectrometry and data analysis

An aliquot of each tryptic digest was analyzed by LC-MS/MS on an Orbitrap Fusion Lumos system (Thermo Scientific) coupled to a Dionex UltiMate 3000 RSLC nano HPL. The raw files were searched against the Human UniProt Database using MaxQuant software v1.5.5.1 (http:/www.maxquant.org) (Tyanova et al., 2016) and the following criteria: peptide tolerance = 10 ppm, trypsin as the enzyme (two missed cleavages allowed), and carboxyamidomethylation of cysteine as a fixed modification. Variable modifications are oxidation of methionine and N-terminal acetylation. Heavy SILAC labels were Arg10 (R10) and Lys8 (K8). Quantitation of SILAC ratios was based on razor and unique peptides, and the minimum ratio count was 2. The peptide and protein FDR was 0.01. The BioID and AP/MS datasets (minus common environmental contaminants as per http://maxquant.org and proteins identified via the decoy database) are provided in Table S3.

Fluorescence microscopy

Cells seeded on No. 1.5 coverslips were fixed for 10 min at room temperature in 3.7% (wt/vol) paraformaldehyde (PFA) in CSK buffer (1mM PIPES pH 6.8, 100mM NaCl, 300mM sucrose, 3mM MgCl2, 2mM EDTA). Following a 10 min permeabilization with 1% Triton X-100 in PBS, nonspecific binding sites were blocked by incubation in PBS with 1% BSA and 0.1% Tween-20. For immunostaining, cells were incubated with the appropriate primary antibody, followed by incubation with the appropriate fluorophore-conjugated secondary antibody. Coverslips were prepared for imaging by mounting in Vectashield liquid mounting media (Vector Labs).

For live imaging, cells were cultured in 35 μm optically clear polymer-bottom μ-dishes or 4-well μ-slides (ibidi) and growth medium replaced with Phenol Red-free CO_2 independent medium (ThermoFisher). If desired, DNA was stained by incubating the cells for 20 min at 37°C in medium containing 0.25 μg/ml Hoechst No. 33342 (Millipore Sigma). Images were acquired using a DeltaVision CORE widefield fluorescence system fitted with a 60× NA 1.4 PlanApochromat objective (Olympus), CoolSNAP charge-coupled device (CCD) camera (Roper Scientific) and environmental chamber. The microscope was controlled and images processed by SoftWorX acquisition and deconvolution software (GE Healthcare). All images are single, deconvolved optical sections. Photobleaching experiments were carried out as previously described (Prévost et al., 2013)

Expansion microscopy

Expansion microscopy was carried out as per the X10 protocol (Truckenbrodt et al., 2019). Briefly, cells seeded on coverslips were fixed and stained as described above, followed by overnight incubation at room temperature with Acryloyl-X anchoring reagent (ThermoFisher) diluted to 0.1 mg/ml in PBS. The gelation solution was prepared by mixing 1.335g DMAA and 0.32 g sodium acrylate (both from Millipore Sigma) with 2.85 g ddH2O, vortexing and purging O_2 by bubbling with N_2 for 40 min at room temperature. A solution of 0.036 g/ml of potassium persulfate was prepared in ddH2O, 0.3 ml added to 2.7 ml of gelation solution and O_2 purged by bubbling with N_2 for 15 min on ice. Gelation chambers were prepared by sandwiching the

stained coverslip, cell side up, between two coverslips and adding spacer coverslips along the sides. 2 µl of TEMED was added to 0.5 ml of the gel solution and vortexed, 100 µl of gel solution was pipetted on top of the cells using a pre-chilled pipette tip and a 22 x 22 mm coverslip placed on top. Gels were placed in a humidified chamber and allowed to polymerize for 3 hours at room temperature. The gels were removed from the coverslips and placed in 35 µm culture dishes for overnight incubation at 50°C with 8 U/ml Proteinase K (Millipore Sigma) prepared fresh in digestion buffer (50mM TRIS, 800mM Guanidine HCl, 1mM EDTA and 0.5% (v/v) Triton X-100 in ddH2O, pH 8.0). To swell the gels, they were transferred to 10 cm culture dishes and 10 ml of deionized water containing 1.7 µg/ml Hoechst 33342 (Millipore Sigma) was added. After 10 min, this was replaced with deionized water for a further 50 min incubation. To image cells post-expansion, circular sections were punched out of the gel using the top of a P1000 pipette tip and transferred to 8-well chambered coverslips (ibidi). Multiwavelength Z-stacks were acquired using a Zeiss LSM880 confocal laser scanning microscope with a 63x/1.4 NA Plan-Apo objective in AiryScan mode (Huff, 2015). 3D Surfaces volume rendering was carried out using Imaris software (Bitplane). To calculated the expansion factor, pre- and post-expansion cells were imaged and nuclear area measured using SoftWorX (Figure S4a).

Supplemental References

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., Zhang, F., 2013. Genome engineering using the CRISPR-Cas9 system. Nature Protocols 8, 2281–2308.

Brand, M., Rampalli, S., Chaturvedi, C.-P., Dilworth, F.J., 2008. Analysis of epigenetic modifications of chromatin at specific gene loci by native chromatin immunoprecipitation of nucleosomes isolated using hydroxyapatite chromatography. Nature Protocols 3, 398–409.