SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Growth Conditions

For all biochemical experiments, yeast strain BY4741 was transformed with pRP 1363, pRP 1556 or pRP 2071 and grown in –Ura minimal media with 2% glucose to an OD_{600} of 0.4-0.6 at 30°C. For stress granule purification, TIF4631-TAP tag strain from the yeast TAP tag library was transformed with pRP 1363 and grown in –Ura minimal media with 2% glucose to an OD_{600} of 0.4-0.6 at 30°C. For the microscopic validation of Mass Spectrometry hits, strains from the yeast GFP library were transformed with pRP 2132 and grown in –Ura minimal media with 2% glucose to an OD_{600} of 0.4-0.6 at 30°C. Human osteosarcoma U-2 OS cells (expressing GFP-G3BP1, mRFP-DCP1a) (Kedersha et al., 2008), maintained in DMEM, High Glucose, GlutaMAX with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1mM sodium pyruvate at 37°C/5% $C0_2$ were used for all biochemical experiments, stress granule purification, and validation of mass spectrometric hits.

Stressing cells and Cycloheximide treatment

Yeast: NaN_3 stress: Cells were treated with 0.5%NaN3 for 30min at 30°C (unless otherwise stated). Vanillin stress: Cells were treated with 50mM vanillin (2M stock made in DMSO) for 30min at 30°C**.** Treatment with Cycloheximide was performed with a final concentration of 100µg/ml Cycloheximide added at the same time as NaN_3.

Mammalian: $NaAs0_2$ stress: Cells were treated with 0.5mM $NaAs0_2$ for 1hr at 37°C/5% $C0_2$. Thapsigargin stress: Cells were treated with 1uM Thapsigargin in DMSO (Sigma) for 1hr at 37°C/5% $C0_2$. Heat shock stress: Cells were transferred to 43°C/5% $C0_2$ for 1hr. Treatment with Cycloheximide was performed with a final concentration of 100µg/ml Cycloheximide added at the same time as $NaAs0_2$.

Purification of yeast stress granule cores

For each sample, 1.8L of culture was grown in the appropriate selection medium and grown to OD_{600} 0.4-0.6. After NaN_3 stress, cells were pelleted at 3220Xg for 1min at RT in 50ml falcon tubes. Cell pellets were flash frozen in liquid nitrogen and stored at -80°C till ready for lysis. Cell pellets were resuspended in lysis buffer (50mM Tris HCl pH 7.4, 100mM Potassium Acetate, 2mM Magnesium Acetate, 0.5mM DTT, 50µg/ml Heparin, 0.5%NP40, 1:5000 Antifoam emulsion, 1 complete mini EDTA free protease inhibitor tablet/ 50ml of lysis buffer), such that cell pellet from 50ml of culture was resuspended in 0.5ml of lysis buffer. Resuspended cell pellets were transferred to 2ml microcentrifuge tubes. ~0.3ml of acid washed glass beads were added to each tube. Cells were lysed at 4C by 3 cycles of vortexing for 2min followed by 2min on ice. Lysate was spun at 850Xg for 2min and supernatant was taken. Stress granule core enriched fraction was obtained from this supernatant as in Figure S4A (all pellets were resuspended in Lysis Buffer). Cores were bound to Dynabeads conjugated with Rabbit IgG at room temperature for 15min. Dynabeads were then washed with lysis buffer 3 times, followed by 1 short wash with Lysis Buffer + 2M Urea, followed by 1 wash with Lysis Buffer + 300mM Potassium Acetate and another wash with Lysis Buffer. Dynabeads were then resuspended in 2X SDS PAGE loading buffer and boiled at 100°C for 10min. Supernatant was loaded onto a 4-12% Novex precast SDS PAGE gel. Gels were stained with Sypro Ruby to visualize protein. In gel trypsin digestion was performed for Mass Spectrometry as described in Shevchenko and Mann, 2006 (Shevchenko et al., 2006).

Purification of mammalian stress granule cores

GFP-G3BP U-2 OS cells were grown to 85% confluency. Cell culture media was replaced with fresh complete DMEM growth media 1hr prior to stress induction. Cells were stressed with sodium arsenite ($NaAs0_2$, 0.5mM) for 1hr at 37°C/5% $C0_2$. Following $NaAs0_2$ stress, media was aspirated and cells were washed two times with 5mL PBS and lysed in 3mL of lysis buffer. For cross-linked stress granule purification, cells were washed 2 x PBS and cross-linked

using formaldehyde (2%) in PBS for 10min at room temperature, and quenched with 1M glycine. Cells were harvested on ice in 15mL falcon tubes and lysed at 4°C by two cycles of sonication for 10s followed by 20s on ice. Cells were spun at 4°C for 5 minutes at 1000Xg and supernatant was taken. Stress granule core enriched fraction was obtained from this supernatant as in Figure S4B. Protein amount was quantified using BCA Protein Assay Kit (Thermo Scientific) according to manufacture's instructions. 150µg of total protein in lysis buffer was added to 0.5µg of rabbit anti-GFP antibody at a volume of 500µL and nutated overnight at 4°C. Cores were bound to equilibrated Protein A Dynabeads beads in a total volume of 700µL lysis buffer and nutated at 4°C for 4hrs. Dynabeads were then washed in 1mL, 3 x 5min in buffer 1 (20mM Tris HCl pH 8.0; 200mM NaCl), 1 x 5min in buffer 2 (20mM Tris HCl pH 8.0, 500mM NaCl), 1 x 5min in buffer 3 (10mM Tris HCl pH 8.0; 250mM LiCl; 1mM EDTA pH 8.0), 2 x 5min in TE (10mM Tris HCl pH 8.0; 1mM EDTA pH 8.0). Following the final wash, Dynabeads were resuspended into 200µL TE. Dynabeads were then resuspended in 2X SDS PAGE loading buffer and boiled at 100°C for 10min. Supernatant was loaded onto a 4-12% Novex precast SDS PAGE gel. On bead trypsin digestion was performed for Mass Spectrometric analysis.

Biochemical Characterization

Stress granule lysis buffer was prepared with NaCl, Urea and EDTA at respective concentrations, diluted 1:1 into 50µL of stress granule core enriched fraction, incubated at room temperature for 10min. To assess RNase sensitivity, 1µL of Ambion RNase cocktail (5U/ml RNase A and 400U/ml RNase T1) was added per 50µL of stress granule core enriched fraction and incubated at room temperature for 30min. To assay granule stability, cores were counted by microscopy (see below). For assaying 2% SDS sensitivity, a pelleting assay was used as 2% SDS could also disrupt GFP fluorescence. Briefly, stress granule core enriched fractions were diluted in Stress granule lysis buffer with 4% SDS to reach a final concentration of 2%. After 10min of incubation at room temperature, solution was spun at 18,000Xg for 10min and supernatant and pellet were run on

an SDS PAGE gel. Amounts of Pab1-GFP or GFP-G3BP in supernatant and pellet were assessed by western blot analysis.

To assess the possibility that cellular components could interact post lysis and localize to granules *in vitro*, cells carrying MCM4-GFP or Ded1-mCherry were stressed with NaN_3 for 30min. These cells were then lysed and their lysates were mixed at room temperature for 30min. Stress granule core enriched fractions were then prepared as described in Figure S4A. Cores were then visualized using Deltavision.

Microscopy and image analyses

All images were taken using a DeltaVision Elite microscope with a 100X objective using a PCO Edge sCMOS camera. At least 3 images were taken for each experiment comprising of 8 or 10 Z-sections each. ImageJ was used for all image analyses. For counting cores *ex vivo*, all images were Z-projected using maximum projection. The Z-projections were thresholded between 1000 and 30,000 counts. Then the “Analyze particles” feature was used to count the number of foci. For overlap between different markers for granules *ex vivo*, cores were identified as described above in channel1. Then background intensities were determined for the channel2 by measuring intensity in 20 different parts of the image with no discernable core. This was used to determine intensities above background (using 1% FDR). Intensities of channel2 at core positions (as determined by channel1) were obtained. A higher than background intensity at that position was interpreted as overlap.

To evaluate the overlap between Ded1-mCherry and a GFP tagged protein, Z-projections were created by summing all Z-sections for both channels. A single Freehand line was drawn for each cell, such that a) most Ded1 mCherry foci were traversed by the line and b) nuclei and vacuoles were not intersected. An intensity profile was generated using ImageJ and the “peak finder” plugin in MATLAB was used to determine peak positions. A similar profile was created for the green channel for this same line and peak positions were identified such that they were above the levels of autofluorescence (as determined by the intensity in

the GFP channel for different exposure times for BY4741 under stress). An overlap was called if for a peak position in the mCherry channel, there existed a peak in the GFP channel within 200nm (resolution power of the setup).

STORM (Stochastic Optical Reconstruction Microscopy) (Rust et al., 2006; van de Linde et al., 2011) was performed on a Nikon Eclipse Ti system using a 1.49NA 100X objective lens. The images were captured using a Hamamatsu Orca Flash 4.0 sCMOS camera at 30ms exposure in 2x2 binning mode (for Nyquist sampling of the PSF).. Alexa-647 labeled anti-GFP antibody, excited using a 640 nm Agilent laser, was used to visualize GFP-G3BP and Alexa-647 labeled oligo(dT) probes were used to visualize poly(A+) RNAs. The density of active molecules was controlled using a 405nm laser which reactivates the molecules from their off state. The sample and the buffer were prepared as per Nikon's protocol. Image reconstruction was done using the Thunderbolt plugin for ImageJ (Ovesný et al., 2014).

3D STORM was performed using the above setup in conjunction with the Double-Helix (DH) SPINDLE module. The DH module is a 4f relay system that sits between the microscope and the camera. The module employs a phase plate in its Fourier plane that engineers the point spread function (PSF) of the system such that the image of each point emitters is converted to two well separated lobes. The center of the two lobes corresponds to the transverse position (x, y) of the molecule and the orientation between the lobes corresponds to the axial (z) position (Agrawal et al., 2012). The reconstruction was performed using the Double-Helix's patented dense emitters algorithm to resolve overlapping emitters (Barsic et al., 2014).

Processed 3D STORM images were analyzed using Imaris (Imaris X64 v 8.0.1, Bitplane Inc). Briefly, for identifying cores: small regions of interest (ROI) around a granule were defined and surfaces were identified within ROI after setting the threshold to 0.03 and splitting touching objects using a seed diameter of 0.06μm. Further a quality cutoff of 0.0165 and a minimum voxel number of 40 was applied (these values were optimized by using the cytoplasm as a negative control to avoid identifying single-molecules or other irrelevant cellular structures

as possible cores). For identifying stress granule boundaries: surfaces were identified within the same ROI as above after setting a threshold of $5X10^{-4}$ and surface smoothing = 0.150µm. Minimum voxel cutoff was adjusted so as to pick out the largest identified surface in the region of interest.

SIM (Structured Illumination Microscopy) was performed using the Nikon N-SIM microscope system run in 3D SIM mode with a Nikon 100X objective at a 1.49NA. Images were acquired using an Andor iXon DU897 EM-CCD camera. GFP-G3BP was visualized by exciting the sample using a 488nm laser at a gain of 300. Samples were mounted using ProLong Diamond Mounting media incubated overnight at room temperature. Sample was excited for 600ms/ image. Image reconstruction was performed in Nikon NIS Elements. Yeast cells carrying Pab1-GFP were fixed with 4% Paraformaldehyde for 15min at room temperature prior to visualizing by SIM. Yeast stress granules were sized using SIM images by determining the intensity profile along a straight line passing through a stress granule. The minimum intensity value was set to zero, and a Gaussian curve was fit to the resulting intensity profile using MATLAB. Granule size was calculated as the distance along the straight line (in nm) corresponding to 95% of the area under the Gaussian curve.

Circularity of granules was assessed using ImageJ.

SIM or STORM analysis of granules using an oligo(dT) probe was done as follows: Stress granule in situ hybridization using an oligo(dT) probe was performed as previously described (Kedersha et al., 2002). Oligo(dT) probes (30mer) were synthesized with an Alexa-647 5'-modification. In brief, cells were grown in 35mm glass bottom dishes, stressed for 1hr, washed once with PBS and fixed at room temperature for 10 min using 3% paraformaldehyde, 0.1% gluteraldehyde. Samples were reduced in 0.1% sodium borohydride in PBS for 10 min at room temperature and washed three times with PBS. Samples were permeablized for 15 min. with 0.2% Triton X-100 in PBS. Samples were washed twice in 2x SSC (300mM NaCl, 30mM sodium citrate, pH 7.0), 15% formamide. Samples were hybridized overnight at 37°C in a mixture containing 10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.02% BSA, 40 ug *E.coli* tRNA,

2x SSC, 15% formamide and 5ng of oligo(dT) probe. Cells were washed twice in 2x SSC, 15% formamide at 37°C. Samples were washed twice in PBS and re-fixed at room temperature for 10 min using 3% paraformaldehyde, 0.1% gluteraldehyde.

To test the presence of poly(A+) RNA in granule cores *in vitro*, stress granule core enriched fractions were prepared from either yeast or mammalian cells and hybridized with an Alexa647 labeled oligo(dT) probe overnight at 4°C by adding 1µg of total probe to each stress granule core preparation. To clear unbound probe following hybridization, granule cores were pelleted at 18,000xg for 10 min. The pellet was resuspended in lysis buffer and imaged on the Deltavision. Staining yeast and mammalian stress granule core enriched lysates with nucleic acid stains were performed by mixing in either 100µM (final concentration) of SYTO17 or 1X (final concentration) SYBR Gold (for yeast) at room temperature for 20min. In lysate immunofluorescence was performed by addition of conjugated primary antibody (pre-incubated with secondary for 1hr at room temperature in 3% BSA, PBS) to a stress granule core enriched fraction at a final concentration of 1:50. Samples were nutated at room temperature for 1hr, concentrated by centrifugation at 14,000RPM for 15min at 4°C. The pellet was resuspended into lysis buffer and imaged.

RNA extraction and oligodT Northern analysis

RNA was isolated from 'stress granule core enriched fraction' from NaN_3 and unstressed cells by Phenol – Chloroform extraction. All extracted RNA was run on a 1.25% formaldehyde agarose gel. RNA was transferred onto a Nitrocellulose membrane and hybridized with an αP^{32} labeled oligodT probe overnight at room temperature. Membrane was exposed to a phosphorimager screen. Image was obtained using a Typhoon scanner.

Nanosight Particle Size Analysis

Stress granule core enriched fractions were collected and diluted in lysis buffer (yeast, 1:20 and mammalian 1:25). Particles were analyzed using the

NanoSight Nanoparticle Tracking Analysis system NS300 (Malvern) with syringe pump, a 488 nm laser, and a sCMOS camera. Five videos of 60 seconds were collected for each sample using the 488nm laser and analyzed by NTA 3.0 software. A total of 363,471 and 78,286 valid tracks were collected for yeast and mammalian cells, respectively. Normalization was performed for each run by dividing by total valid tracks in each run.

Yeast Mass Spectrometric Analysis

Tryptic peptides were analyzed using a Waters nanoAcquity UPLC coupled directly to a linear ion trap (LTQ)-Orbitrap hybrid mass spectrometer (Thermo Scientific). The desalted, vacuum-dried peptide mixture was resuspended in 0.1% formic acid in water and 8 uL of the mixture was injected onto a nanoAcquity 1.7 um particle size, 75 um x 250 mm BEH130 C18 analytical column (Waters). A 150 min, linear, one-dimensional reversed-phase UPLC gradient implementing a flow rate of 300 nL/min was used to elute peptides for electrospray ionization-aided MS/MS analysis (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile). Accurate mass data were acquired on intact peptide ions using 60,000 resolution (at 400 m/*z*) in the Orbitrap (MS) and were followed by data-dependent, low resolution MS/MS analysis on the top 6 most abundant peptide ions per scan cycle in the linear ion trap. Charge state rejection was used to reject MS/MS scans on precursor ions with +1 or unassigned charge states. Dynamic exclusion was turned on with the following parameters: repeat count of 1, repeat duration of 30 s, exclusion list length of 500 ions and exclusion duration of 180 s.

Raw mass spectral data were converted to mzxml format using ReAdW (v4.3.1), searched using the Mascot search algorithm (Matrix Science) and further processed using IsoformResolver (Meyer-Arendt et al., 2011). All data were searched against the Uniprot yeast proteome database (release-2013_04) forward and reversed protein databases with the following search criteria: trypsin enzyme specificity, 2 possible missed cleavages, a single fixed modification (carbamidomethyl Cys), two variable modifications (conversion of N-terminal Gln

to pyro-Glu, oxidized Met), ± 20 ppm precursor ion mass tolerance, ± 0.5 Da fragment ion mass tolerance, and 1% false discovery rate based on MOWSE score. IsoformResolver processing required a minimum peptide length of 6 amino acids and a minimum of 1 peptide reported per protein. Spectral counts (the number of MS/MS spectra assigned to peptides for a given protein) were used as a relative measure of protein abundance and evaluated for significance of differential expression using a G-test as in Old et al. 2005 (Old et al., 2005).

Mammalian Mass Spectrometric Analysis

Immunoprecipitated samples on magnetic beads were washed with 0.1 M ammonium bicarbonate (ABC), and resuspended in 100 µL of 0.1 M ABC. The samples were, then reduced with 20 mM TCEP at room temperature for 45 min, and alkylated with 50 mM iodoacetamide at room temperature for 30 min in darkness. The samples were trypsinized with 5 µg of trypsin at 37 °C for 6 hours and proteolysis reaction was quenched by acidification using formic acid. The tryptic peptides were desalted using C18 spin columns and speed-vac to dryness. The tryptic peptides were resuspended in 0.1 M ABC/0.05% deoxycholic acid, and loaded onto 30 KDa MWCO spin filters, pretreated with 50 µg of BSA. The peptides were eluted by centrifugation. Deoxycholic acid was removed by phase-transfer using ethyl acetate, as previously described (Yeung and Stanley, 2010), speed-vac to dryness, and stored at -80 °C.

Samples were resolved by UPLC in the direct injection mode using a Waters nanoACQUITY system. Samples were resuspended in 10 µL of buffer A (0.1% formic acid/water), of which 2 µL (20% of total) was directly loaded onto BEH130 C18 analytical column (130 Å, 1.7 µm, 75 µm x 250 mm), equilibrated in 97% buffer A and 3% buffer B (0.1% formic acid/acetonitrile) at 0.3 µL/min for 10 min, followed by elution with 3-8% buffer B between 0-2 min, 8-32% buffer B between 2-131 min, and 32-85% buffer B between 131-140 min (0.3 µL/min). MS/MS was performed using an LTQ Orbitrap Velos, scanning MS between 400–1800 m/z (1 × 106 ions, 60,000 resolution) in FT, and selecting the 10 most intense MH22+ and MH33+ ions for MS/MS in LTQ with 180 s dynamic

exclusion, 10 ppm exclusion width, repeat count = 1. Maximal injection time was 500 ms for FT precursor scans with one microscan, and 250 ms for LTQ MS/MS with one microscan and AGC 1 × 104. The normalized collision energy was 35%, with activation Q=0.25 for 10 ms.

Raw data from mass spectrometry were processed using MaxQuant/Andromeda (ver 1.5.0.12) (Cox and Mann, 2008; Cox et al., 2011) and searched against Uniprot human database (downloaded on Jan 2014, 88,479 entries) (UniProt Consortium, 2015) with common contaminant entries. The search used trypsin specificity with maximum two missed cleavages, included carbamidomethylation on Cys as a fixed modification, and N-terminal acetylation and oxidation on Met as variable modifications. Andromeda used seven ppm maximum mass deviation for the precursor ion, and 0.5 Da as MS/MS tolerance, searching eight top MS/MS peaks per 100 Da. False discovery rates were set to 0.01 for both protein and peptide identifications, with seven amino acid minimum peptide length, and two minimum total peptides.

Bioinformatics and Statistical analysis

Cytoscape v2.8.2 was used to identify known physical interactions between and create interaction maps for stress granule proteins, P body proteins or random sets of yeast genes (Biogrid tab file: BIOGRID-ORGANISM-Saccharomyces_cerevisiae-3.1.79.tab2.txt) (Stark et al., 2006). Proteins with predicted Prion-like domains were identified using PLAAC (Lancaster et al., 2014), Prionscan (Espinosa Angarica et al., 2014) or as in Michelitsch and Weissman (Michelitsch and Weissman, 2000). mRNA binding activity was determined using the SGD database (yeastgenome.org) (Cherry et al., 2012) and the published database in Castello et al. (Castello et al., 2012) for yeast and mammalian proteins respectively.

The plugin GeneMANIA was used to identify known physical interactions and create interaction maps or random gene sets for mammalian stress granule proteins (Beck et al., 2011; Warde-Farley et al., 2010). We wrote a Perl script to create sets of random yeast and mammalian genes. The plugin BiNGO 2.44 was

used to identify enriched GO categories. Mammalian proteins with prion-like domains were identified using PLAAC (Lancaster et al., 2014) and only proteins with a positive coreSCORE were used in the analysis. Statistical analysis was conducted using hypergeometric distribution to determine the chance overlap between two lists. Based on this distribution, p-values for greater than chance overlap between two lists was determined. Unpaired student's t test was used to determine significant changes. Error bars represent standard deviation.

Stress Granule Disassembly Experiments

All yeast strains harboring a temperature sensitive mutation in Mcm2 or Rvb2 were cultured and grown as stated in the section "Growth Conditions". *rvb2-1* and its corresponding WT cells were grown at 25°C till log phase and then shifted to 35°C 1hr prior to stress. *mcm2-1* and its corresponding WT cells were grown at 30°C till log phase and then shifted to 35°C 1hr prior to stress. Cells were then stressed with 0.5% NaN_3 for 30min at 35°C. Following NaN_3 stress, cells were pelleted at 3220xg for 1min, washed once in pre-warmed media, resuspended in pre-warmed media and returned to 35°C. Aliquots were then drawn at various time points for microscopy. Granules/ cell were manually counted from microscopy images.

WT or *cct4-1* cells carrying Pab1-GFP were grown to log phase at 30°C and then shifted to 37°C. After 40min, cells were stressed with 0.5% NaN_3 for 30min and imaged. Cells were then washed in media, and resuspended in media and returned to 37°C. After 60min, cells were imaged again, and fraction of cells carrying stress granules was determined.

U-2 OS cells were plated (25k cells/well) in 4-well chamber slides and grown in complete DMEM growth at 37°C/5% CO_2. Knockdown was performed using 5nmol siRNAs cocktail and transfected using INTERFERin (Polyplus) according to manufacture's instructions. Cells were transfected at 30-35% confluency. Cells were grown for 48hrs at 37°C/5% CO_2, stressed with 0.5mM $NaAsO_2$ for 1hr. For recovery, cells were washed twice in normal media and permitted to recover for various times prior to collection and fixation.

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