Supplementary Materials for

Ubiquitination of G3BP1 mediates stress granule disassembly in a stress-specific manner

Youngdae Gwon[1], Brian A. Maxwell[1], Regina-Maria Kolaitis[1], Peipei Zhang[1], Hong Joo Kim[1] and J. Paul Taylor[1,2*]

Correspondence to: jpaul.taylor@stjude.org

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Caption for table S1
Captions for movies S1 to S12
Figs. S1 to S6

Other Supplementary Materials for this manuscript include the following:

Table S1 (Excel format)
Movies S1 to S12

Table S1. (separate file) Integrated stress granule proteome and VCP adaptors. Table showing a list of proteins identified in stress granules and integrated stress granule proteome used in Fig. 5A. The table also contains a list of 35 VCP adaptors.

Movie S1. Impaired disassembly of heat shock-induced stress granules by TAK-243.
U2OS GFP-G3BP1 cells in the presence or absence of TAK-243 were incubated at 37°C for 2 min, 43°C for 30 min, and 37°C for 88 min, and GFP signals were monitored with 30-sec intervals. See Fig. 1, M and N.

Movie S2. Impaired disassembly of heat shock-induced stress granules composed of G3BP1 6KR or 10KR.
U2OS *G3BP1/2* dKO cells stably expressing GFP-G3BP1 WT, NTF2L 6KR, RRM 4KR, or NTF2L/RRM 10KR were incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min, and GFP signals were monitored with 30-sec intervals. See Fig. 2, F and G.

Movie S3. Impaired disassembly of heat shock-induced stress granules by loss of ATP.
U2OS GFP-G3BP1 cells were incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min, and GFP signals were monitored with 30-sec intervals. See fig. S3, D and E.

Movie S4. Autophagy-dependent clearance of stress granules generated by a short (30-minute) exposure to heat shock.
U2OS GFP-G3BP1 cells in the presence or absence of bafilomycin A1 were incubated at 37°C for 2 min, 43°C for 30 min, and 37°C for 88 min. GFP signals were monitored with 30-sec intervals. See Fig. 4A.

Movie S5. Autophagy-dependent clearance of stress granules generated by an intermediate (60-minute) exposure to heat shock.
U2OS GFP-G3BP1 cells in the presence or absence of bafilomycin A1 were incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min. GFP signals were monitored with 30-sec intervals. See Fig. 4B.

Movie S6. Autophagy-dependent clearance of stress granules generated by prolonged heat shock.
U2OS GFP-G3BP1 cells in the presence or absence of bafilomycin A1 were incubated at 37°C for 2 min, 43°C for 90 min, and 37°C for 148 min. GFP signals were monitored with 30-sec intervals. See Fig. 4C.

Movie S7. Impaired disassembly of heat shock-induced stress granules by CB-5083. U2OS GFP-G3BP1 cells in the presence or absence of CB-5083 were incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min, and GFP signals were monitored with 30-sec intervals. See Fig. 4D.

Movie S8. Impaired disassembly of heat shock-induced stress granules by gene silencing of VCP. U2OS GFP-G3BP1 cells were transfected with non-targeting siRNA or VCP siRNA and incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min, and GFP signals were monitored with 30-sec intervals. See Fig. 4E.

Movie S9. Impaired disassembly of heat shock-induced stress granules by ALS-associated VCP mutants.
U2OS GFP-G3BP1 cells were transfected with pmCherry-N1 empty vector, VCP WT-mCherry, VCP R155H-mCherry, or VCP A232E-mCherry. Cells were incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 88 min, and GFP signals of mCherry-positive cells were monitored with 60-sec intervals. See Fig. 4, G and H.

Movie S10. Limited redistribution of G3BP1 from stress granules by control knockdown. U2OS cells stably expressing photoactivatable G3BP1 (PAGFP-G3BP1) were transfected with non-targeting siRNA. 48 h post transfection, ROIs were activated by 405-nm laser and GFP signals were monitored with 200-ms intervals. See Fig. 4, I and J.

Movie S11. Limited redistribution of G3BP1 from stress granules by VCP knockdown. U2OS cells stably expressing photoactivatable G3BP1 (PAGFP-G3BP1) were transfected with VCP siRNA. 48 h post transfection, ROIs were activated by 405-nm laser and GFP signals were monitored with 200-ms intervals. See Fig. 4, I and J.

Movie S12. Impaired disassembly of heat shock-induced stress granules by FAF2 knockdown. U2OS GFP-G3BP1 cells were transfected with non-targeting siRNA or FAF2 siRNA and incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min, and GFP signals were monitored with 30-sec intervals. See Fig. 5, H and I.

Figure S1

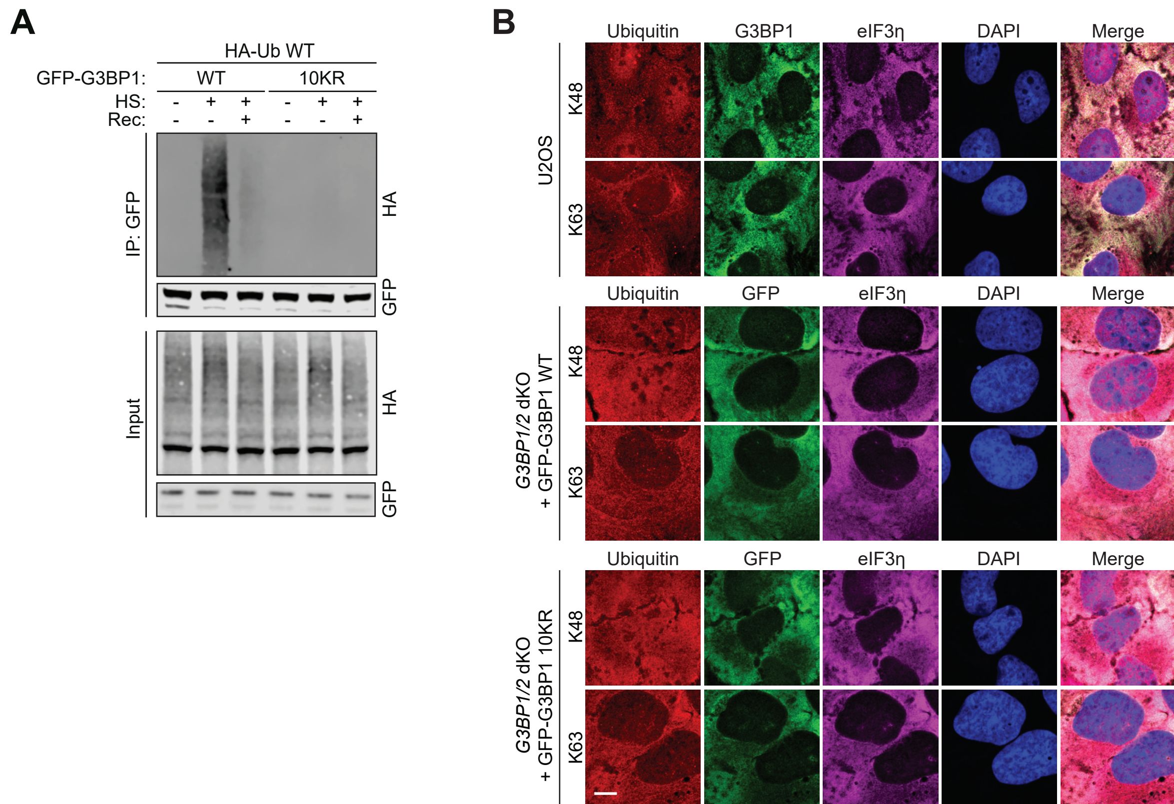
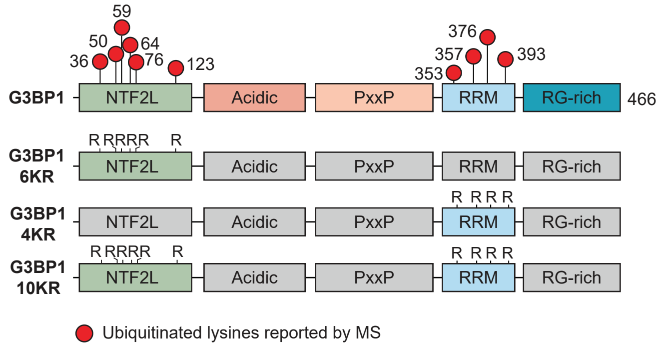
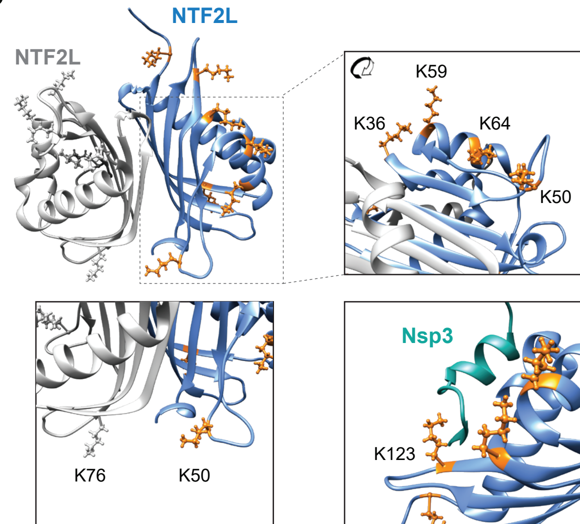
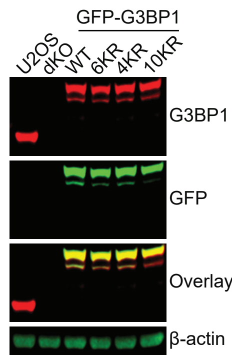


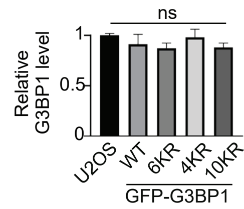
Fig. S1. G3BP1 undergoes K63-linked ubiquitination in response to heat stress. (**A**) Immunoblot of HEK293T cells transfected with HA-Ub and either GFP-G3BP1 WT or NTF2L/RRM KR (10KR) mutant constructs. Cells were exposed to no stress, heat shock (43°C, 1 h), or heat shock plus recovery (43°C, 1 h; 37°C, 30 min). Cell extracts were captured with magnetic beads conjugated with GFP antibody for IP and resulting beads were analyzed by immunoblot. (**B**) Control images corresponding to Figure 1I. Scale bar, 10 µm.

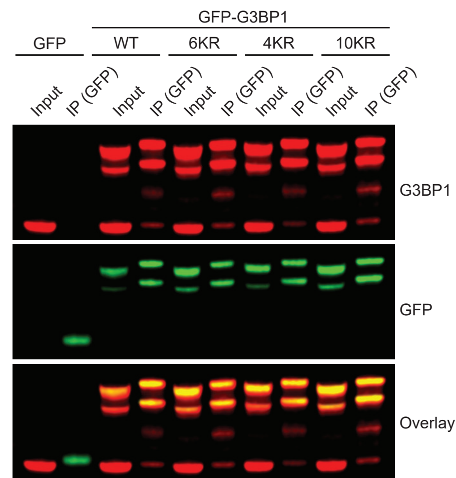
Figure S2

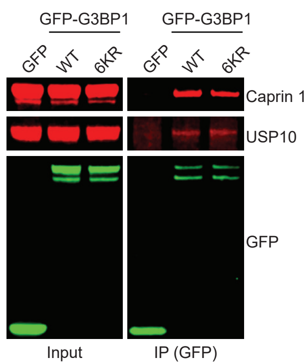


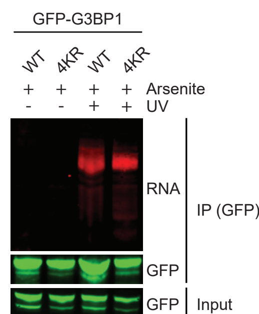












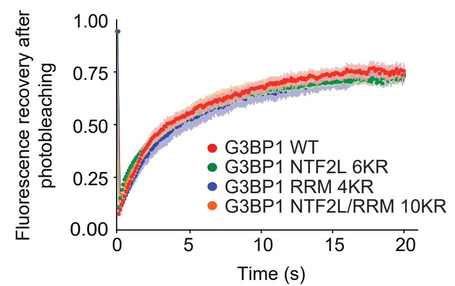


Fig. S2. Generation of stable cell lines expressing G3BP1 mutant proteins; ubiquitination does not influence G3BP1 mobility during stress granule assembly. (**A**) G3BP1 domain organization labeled with lysine residues on which ubiquitination has been reported. In G3BP1 6KR and 4KR mutants, 6 lysines in the NTF2L and 4 lysines in the RRM domain are mutated to arginine residues, respectively. In the G3BP1 10KR mutant, 10 lysines in the NTF2L and RRM domains are mutated to arginine residues. (**B**) Dimeric structure of G3BP1 NTF2L domain enlarged with K36, K50, K59, and K64 cluster (upper right), K50 and K76 from each NTF2L domain (lower left), and K123 and hydrophobic grove where nsP3 protein of Old World alphaviruses makes a contact (lower right). (**C-D**) Immunoblot of U2OS G3BP*1/2* dKO cells transfected with GFP-G3BP1 WT, 6KR, 4KR, or 10KR. Quantification of immunoblots from three biological replicates is shown in (D). Error bars indicate s.e.m. ns, not significant, ANOVA with Tukey's test. (**E**) Dimeric interaction of GFP-G3BP1 WT and KR mutants with endogenous G3BP1. HEK293T cells were transfected with pEGFP-C3 or indicated GFP-G3BP1 constructs. Cell extracts were captured with magnetic beads conjugated with GFP antibody for IP and resulting beads were analyzed by immunoblot. (**F**) Interaction of GFP-G3BP1 NTF2L 6KR mutant with caprin 1 and USP10. HEK293T cells were transfected with pEGFP-C3, GFP-G3BP1 WT, or NTF2L 6KR constructs. Cell extracts were captured with magnetic beads conjugated with GFP antibody for IP and resulting beads were analyzed by immunoblot. (**G**) Crosslinking of RNA to both G3BP1 WT and RRM KR mutant. HEK293T cells were transfected with GFP-G3BP1 WT or RRM 4K mutant. Cells were exposed to no stress or oxidative stress (0.5 mM sodium arsenite, 1 h) and protein-RNA complexes were crosslinked with UV. Cell extracts were captured with magnetic beads conjugated with GFP antibody for IP and resulting beads were analyzed by immunoblot. (**H**) FRAP of GFP-G3BP1 within U2OS G3BP*1/2* dKO cells transfected with GFP-G3BP1 WT, 6KR, 4KR, or 10KR and exposed to heat shock (43°C, 1 h). FRAP of GFP-positive puncta is plotted (G3BP1 WT n = 13, G3BP1 6KR n = 15, G3BP1 4KR n = 13, G3BP1 10KR n = 15). Error bars indicate s.e.m.

Figure S3

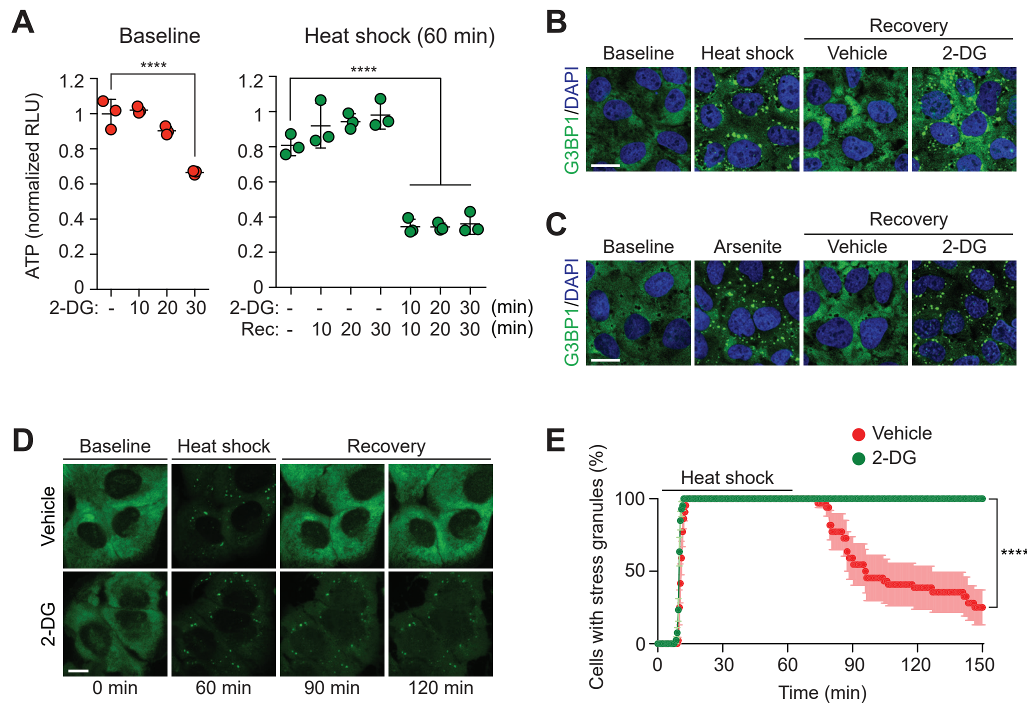


Fig. S3. ATP is required for the disassembly of stress granules. (**A**) ATP assay of U2OS cells exposed to no stress (left), heat shock (43°C, 1 h) or indicated duration of 37°C recovery (right), in the presence or absence of 200 mM 2-deoxy-D-glucose (2-DG) from three biological replicates. Assay shows reduced cellular ATP contents by 2-DG during recovery after heat shock. Error bars indicate s.e.m. ****$P< 0.0001$, ANOVA with Tukey's test. (**B**) Fluorescent imaging of U2OS cells exposed to no stress, heat shock (43°C, 1 h), or heat shock plus recovery (43°C, 1 h; 37°C, 1 h) in the presence or absence of 200 mM 2-DG. Images show impaired disassembly of stress granules with decreasing cellular ATP. Scale bar, 20 µm. (**C**) Fluorescent imaging of U2OS cells exposed to no stress, oxidative stress (0.5 mM sodium arsenite, 1 h) or recovery with culture media (1 h) in the presence or absence of 200 mM 2-DG. Images show impaired disassembly of stress granules with decreasing cellular ATP. Scale bar, 20 µm. (**D-E**) Live-cell imaging of U2OS cells stably expressing GFP-G3BP1. Cells were incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min, and GFP signals were monitored with 30-sec intervals. 200 mM 2-DG or the same volume of dissolving media were added to cells 62 min after monitoring, when the temperature returned to 37°C. Images show impaired disassembly of heat shock-induced stress granules. Representative still images are shown in (D). The percentage of cells with ≥ 2 stress granules from two biological replicates is plotted in (E) (vehicle $n = 28$, 2-DG $n = 43$). Scale bar, 10 µm. Error bars indicate s.e.m. ****$P< 0.0001$, Mantel-Cox test. See also Movie S3.

Figure S4

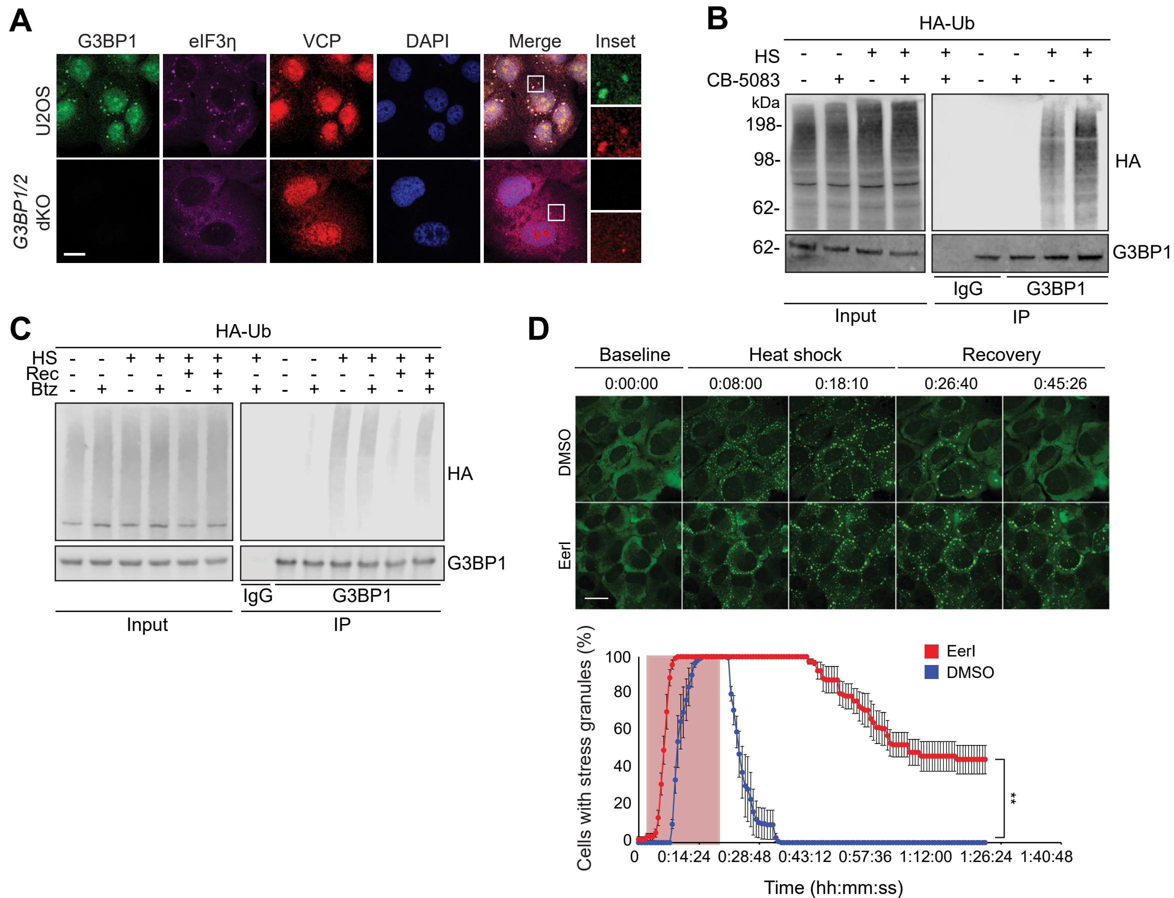
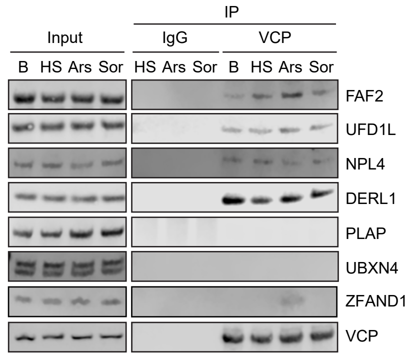
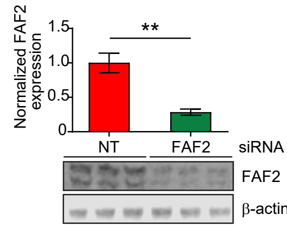
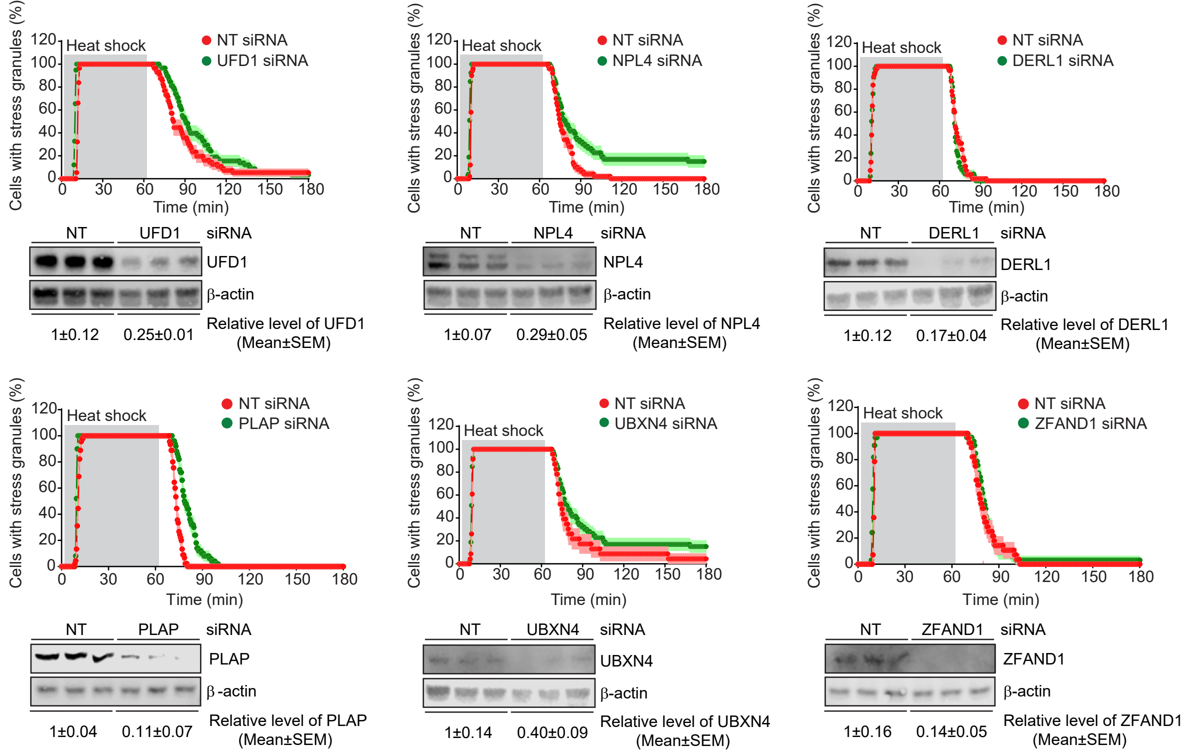


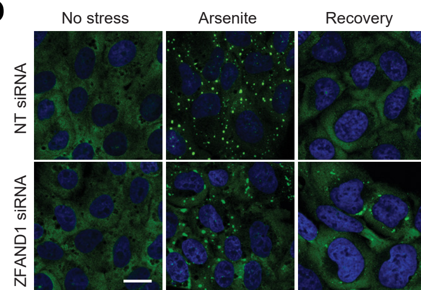
Fig. S4. Impairing VCP function perturbs stress granule dynamics. (**A**) Fluorescent imaging of U2OS and U2OS G3BP*1/2* dKO cells exposed to heat shock (43°C, 1 h). Scale bar, 20 µm. (**B**) Immunoblot of HEK293T cells transfected with HA-tagged ubiquitin, treated with DMSO or CB-5083 (1 h) and exposed to heat shock (43°C, 1 h). Cell extracts were captured with magnetic beads conjugated with G3BP1 antibody for IP and resulting beads were analyzed by immunoblot. Blots suggest VCP-mediated decoupling of ubiquitin chains anchored to G3BP1 in response to heat shock. (**C**) Immunoblots of HEK293T cells transfected with HA-tagged ubiquitin and exposed to no stress, heat shock (43°C, 1 h), or heat shock plus recovery (43°C, 1 h; 37°C, 1 h) in the presence or absence of bortezomib (Btz). Cell extracts were captured with magnetic beads conjugated with G3BP1 antibody for IP and resulting beads were analyzed by immunoblot. Blots suggest that ubiquitinated G3BP1 is targeted to the proteasome upon recovery. (**D**) Live-cell imaging of U2OS cells stably expressing GFP-G3BP1 and treated with DMSO or eeyarestatin I (EerI, 56 µM) for 30 min prior to imaging. Cells were exposed to heat shock (43°C, 18 min), then allowed to recover for indicated times. GFP signals were monitored with 40-sec intervals. The percentage of cells with ≥ 2 stress granules is plotted. Scale bar, 20 µm. Error bars indicate s.d. $^{**}P < 0.01$, Mantel-Cox test.

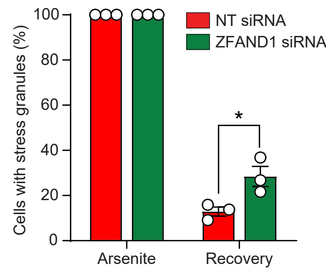
Figure S5

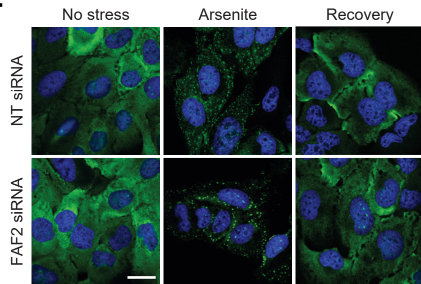












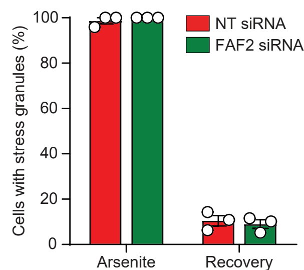


Fig. S5. FAF2 links ubiquitinated G3BP1 to VCP. (**A**) Immunoblot of U2OS cells exposed to no stress, heat shock (43°C, 1.5 h), oxidative stress (0.5 mM sodium arsenite, 1.5 h), or osmotic stress (0.4 M sorbitol, 1.5 h). Cell extracts were captured with magnetic beads conjugated with VCP antibody for IP and resulting beads were analyzed by immunoblot. Blots assess interaction between VCP and stress granule-resident adaptors listed in Figure 5A. (**B**) Immunoblot of U2OS cells transfected with non-targeting (NT) siRNA or FAF2 siRNA for 48 h. Blots show knock-down efficiency of FAF2 siRNA as used in Figure 5. Quantification of immunoblots from three biological replicates are plotted. Error bars indicate s.e.m. $**P< 0.01$, two-tailed t test. (**C**) Graphs show results from fluorescent imaging of U2OS cells stably expressing GFP-G3BP1 and transfected with non-targeting siRNA or siRNAs of indicated genes (all stress granule-resident VCP adaptors). Cells were incubated at 37°C for 2 min, 43°C for 60 min, and 37°C for 118 min, and GFP signals were monitored with 30-sec intervals. The percentage of cells with ≥ 2 stress granules from three technical replicates is plotted. (NT siRNA n = 56, UFD siRNA n = 58), (NT siRNA n = 50, NPL4 siRNA n = 53), (NT siRNA n = 55, DERL1 siRNA n = 50), (NT siRNA n = 45, PLAP siRNA n = 50), (NT siRNA n = 23, UBXN4 siRNA n = 53), (NT siRNA n =28 , ZFAND1 siRNA n = 33). Immunoblots below each graph demonstrate knockdown of indicated genes; each condition is shown in triplicate. Blots were measured with densitometry and relative expression levels are shown below each blot. (**D-E**) Fluorescent imaging of U2OS cells exposed to no stress, oxidative stress (0.5 mM sodium arsenite, 1 h) or recovery with culture media (1 h) after transfection of non-target (NT) siRNA or ZFAND1 siRNA. Scale bar, 20 μm. Quantification of cells with stress granules from three biological replicates is shown in (E). Error bars indicate s.d. $*P< 0.05$, ANOVA with Tukey's test. (**F-G**) Fluorescent imaging of U2OS cells exposed to no stress, oxidative stress (0.5 mM sodium arsenite, 1 h) or recovery with culture media (1 h) after transfection of non-target (NT) siRNA or FAF2 siRNA. Scale bar, 20 μm. Quantification of cells with stress granules from three biological replicates is shown in (G). Error bars indicate s.d. No significant differences by ANOVA with Tukey's test.

Figure S6

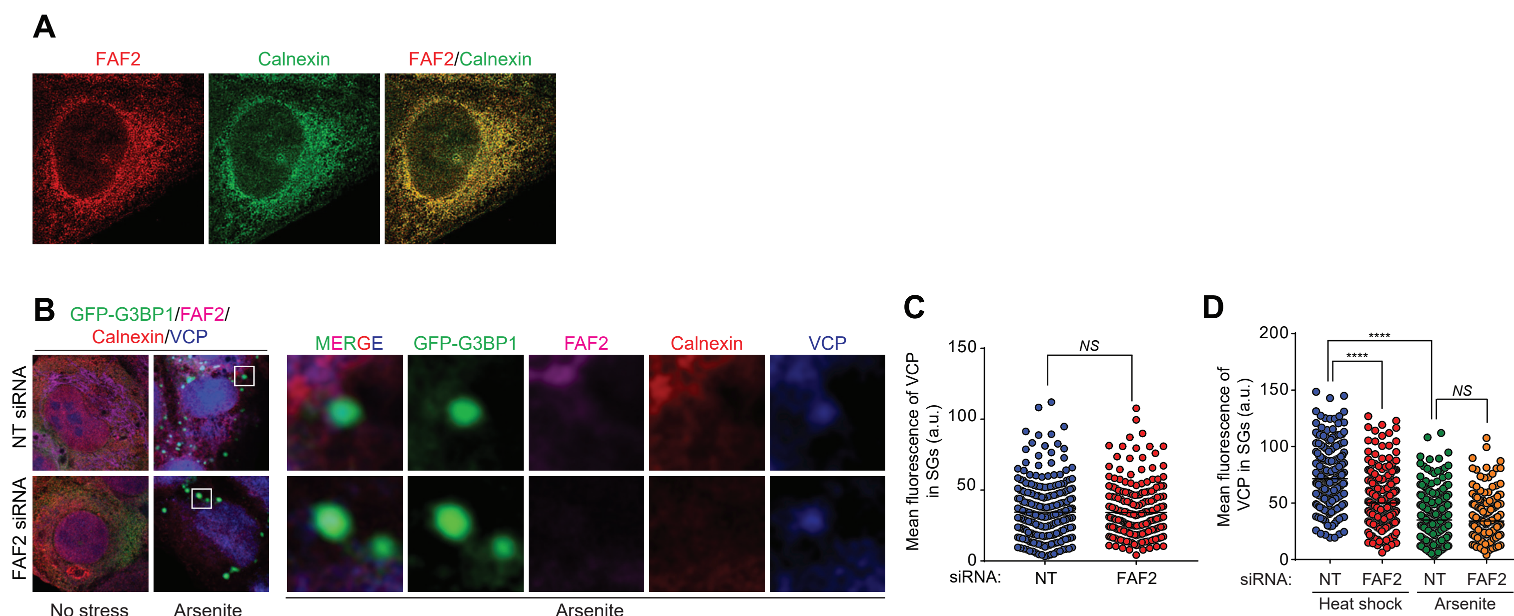


Fig. S6. FAF2, an ER-resident VCP adaptor, links ER to G3BP1 for disassembly of stress granules upon heat shock. (**A**) ER localization of FAF2. Unstressed U2OS cells were fixed and stained with FAF2 and calnexin antibodies for immunofluorescence. (**B-C**) Imaging of U2OS cells stably expressing GFP-G3BP1 and exposed to no stress or 0.5 mM sodium arsenite, fixed, and stained with indicated antibodies for immunofluorescence. Fluorescent intensities of VCP in stress granules from three biological replicates are plotted in (C) (NT siRNA n = 286, FAF siRNA n = 234). Analysis shows that recruitment of VCP to arsenite-induced stress granules is not regulated by FAF2. Error bars indicate s.e.m. ns, not significant, two-tailed t test. (**D**) Combined plots of Figure 5K and Figure S6C for comparison of VCP recruitment to stress granules. Error bars indicate s.e.m. ns, not significant, ****P< 0.0001, ANOVA with Tukey's test.