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Supplemental Information

Active Protein Neddylation or Ubiquitylation Is Dispensable for Stress Granule Dynamics

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Markmiller et al Fig S1 - related to Figure 1

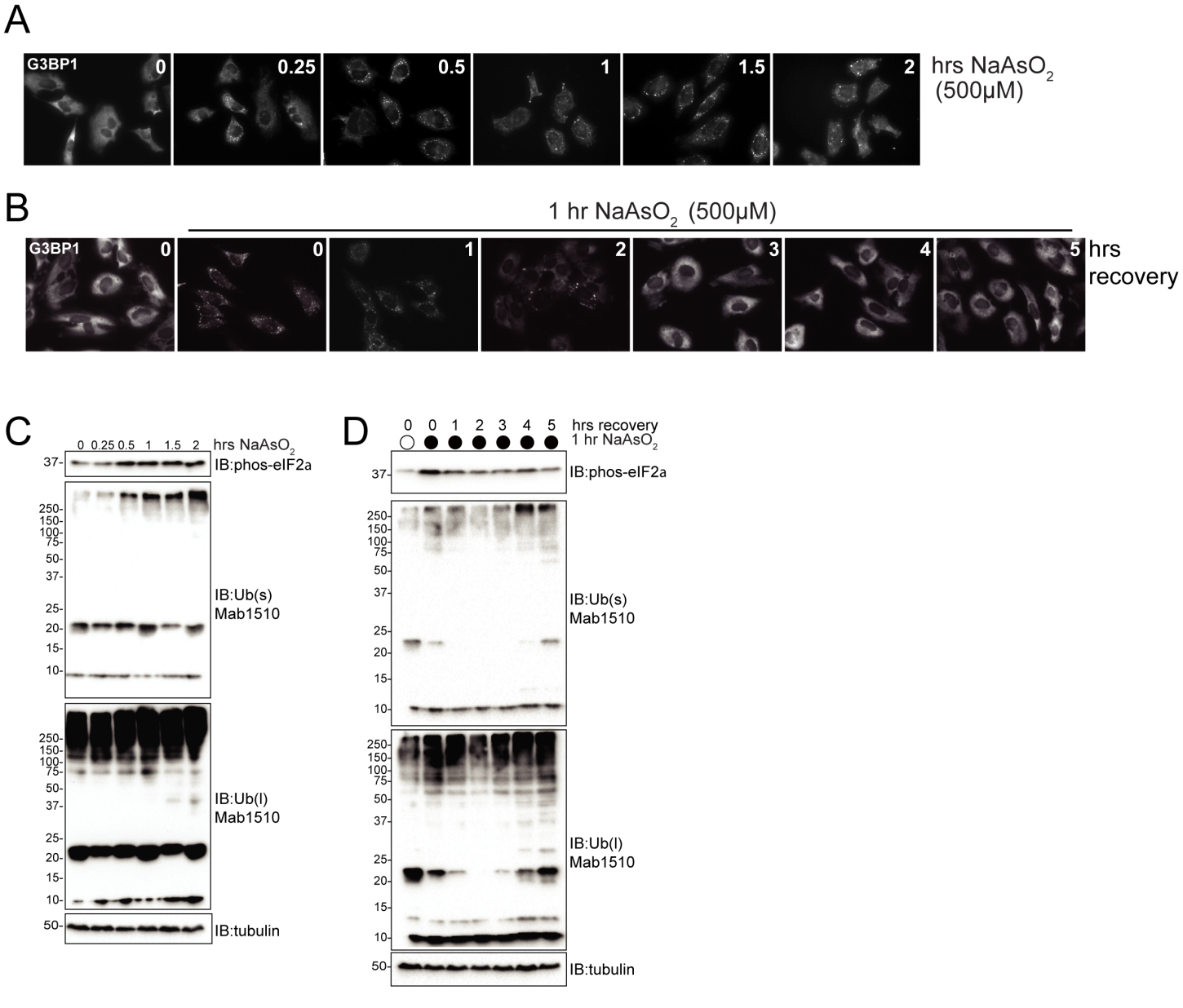

Figure S1 – Sodium arsenite treatment induces stress granule formation and global protein ubiquitylation. Related to Figure 1.

A) HeLa cells were treated with sodium arsenite (500μM) over the indicated time course. Cells were fixed and stress granules were visualized using G3BP1 immunofluorescence at the indicated times.

B) HeLa cells were either untreated or treated with sodium arsenite (500μM) for 1 hour. The sodium arsenite-containing media was replaced with fresh media without sodium arsenite and cells were allowed to recover for the indicated times. Stress granules were visualized using G3BP1 immunofluorescence.

C,D) Hela whole cell lysates corresponding to the sodium arsenite (500μM) treatment (C) and recovery (D) were separated by SDS-PAGE and immunoblotted as indicated. s and l denote short and long exposures, respectively.

Markmiller et al Fig S2 - related to Figure 3

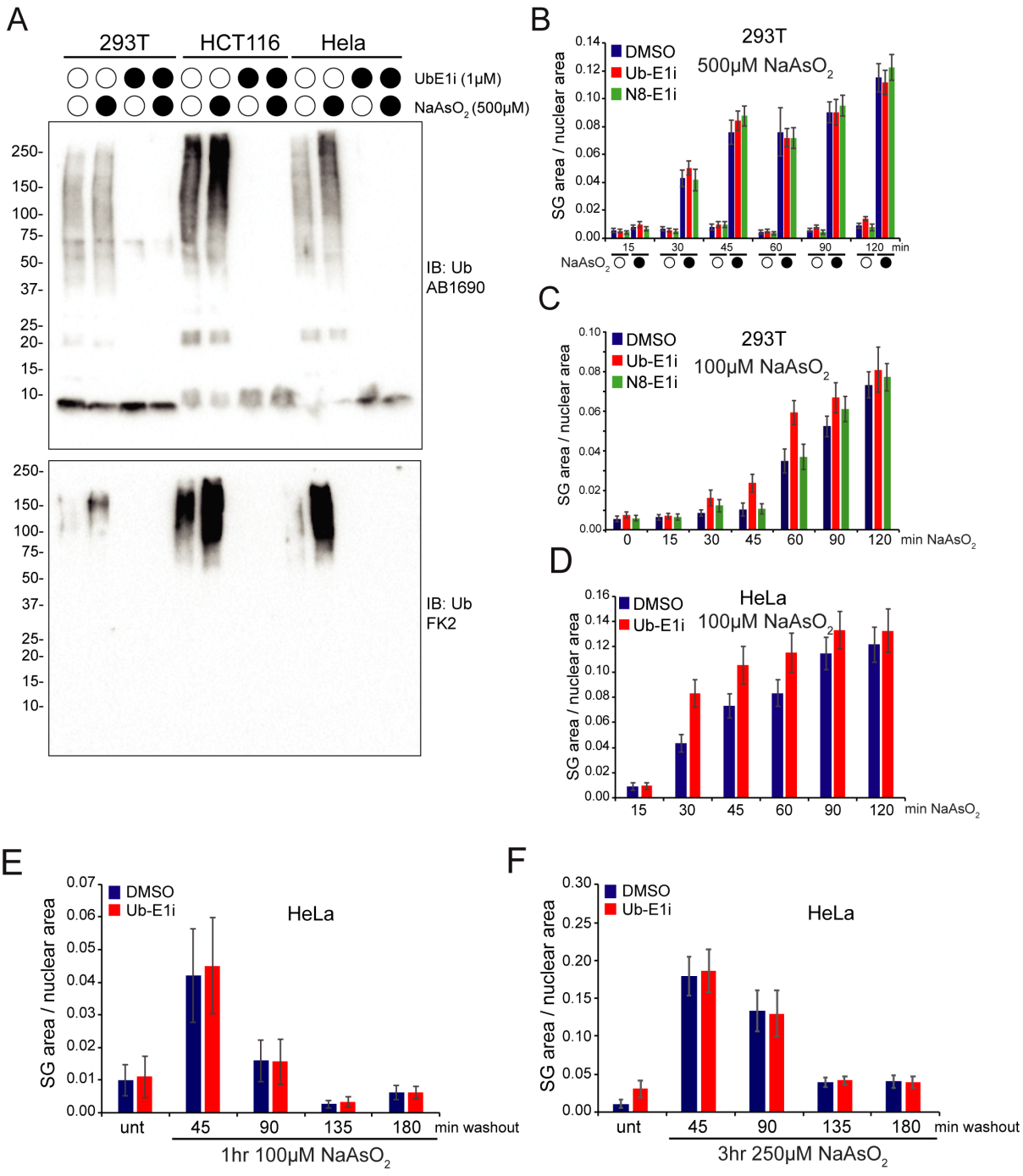

Figure S2 – Stress granule dynamics are unaffected by inhibition of protein ubiquitylation or neddylation. Related to Figure 3.

A) The indicated cells were treated with Ub-E1i (1μM) for 60' prior to sodium arsenite (500μM) addition for 60'. Untreated cells are indicated by open circles. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

B) 293T cells expressing GFP-tagged G3BP1 were pre-treated for 90' with DMSO (blue bars), Ub-E1i (red bars), or N8-E1i (green bars) followed by treatment with (black circles) or without (white circles) sodium arsenite (500μM) for the indicated times. Cells were fixed and imaged at the indicated times.

C) 293T cells expressing GFP-tagged G3BP1 were pre-treated for 90' with DMSO (blue bars), Ub-E1i (red bars), or N8-E1i (green bars) followed by treatment with sodium arsenite (100μM) for the indicated times. Cells were fixed and imaged at the indicated times.

D) Hela cells were pre-treated with DMSO or Ub-E1i for 90' followed by treatment with sodium arsenite (100μM) for the indicated times. Cells were fixed and SGs were localized using G3BP1 immunofluorescence.

E) Hela cells were pre-treated with DMSO or Ub-E1i for 90' followed by treatment with sodium arsenite (100μM) for 60' at which time the sodium arsenite and the Ub-E1i was washed out and cells were fixed at the indicated times following sodium arsenite washout. SGs were localized using G3BP1 immunofluorescence.

F) Hela cells were pre-treated with DMSO or Ub-E1i for 90' followed by treatment with sodium arsenite (250μM) for 3 hours at which time the sodium arsenite and the Ub-E1i was washed out and cells were fixed at the indicated times following sodium arsenite washout. SGs were localized using G3BP1 immunofluorescence. For panels B-F, stress granule and nuclear area was quantified using custom image analysis scripts for the indicated time points and treatments. Error bars depict standard deviation of the mean.

Markmiller et al Fig S3 - related to Figure 3

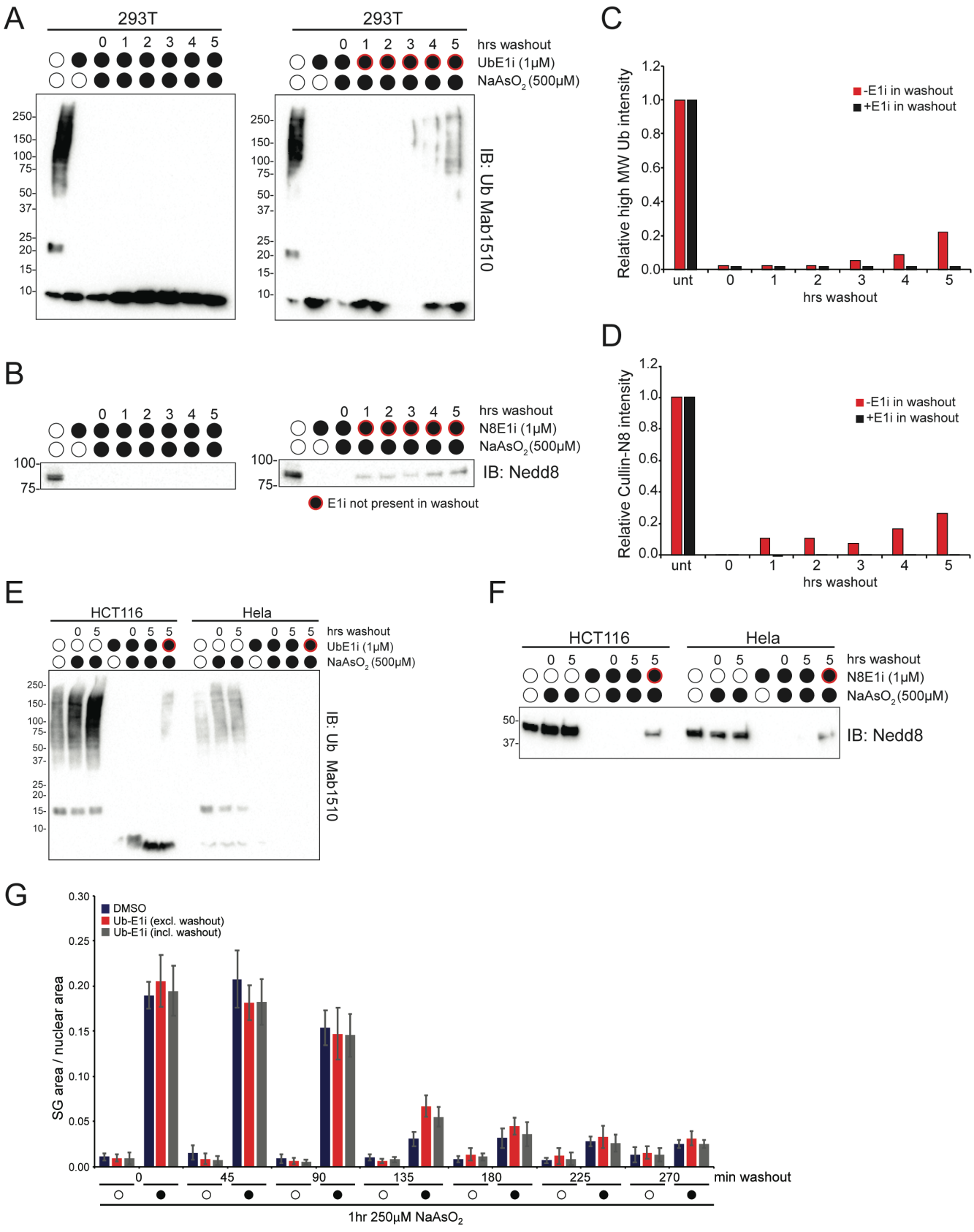

Figure S3 – Recovery dynamics after removal of UAE or NAE inhibitors. Related to Figure 3.

A,B) 293T cells were treated with (A) Ub-E1i (1μM) or (B) N8-E1i (1vM) for 60' prior to sodium arsenite (500μM) addition for 60'. The sodium arsenite was then washed out and cells were collected at the indicted times after washout. Solid black circles denote that the Ub-E1i or N8-E1i was included in the washout media and black circles with a red outline denote that the Ub-E1i of N8-E1i was omitted from the washout media. Untreated cells are indicated by open circles. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

C,D) Quantification of the (C) high molecular weight (MW) ubiquitin immunoreactivity detected above the free ubiquitin band in the entire lane or (D) the neddylated cullin immunoreactivity from indicated samples on the SDS-PAGE gels depicted in panels A or B.

E,F) HCT116 or Hela cells were treated with (E) Ub-E1i (1μM) or (F) N8-E1i (1μM) for 60' prior to sodium arsenite (500μM) addition for 60'. The sodium arsenite was then washed out and cells were collected at the indicted times after washout. Solid black circles denote that the Ub-E1i or N8-E1i was included in the washout media and black circles with a red outline denote that the Ub-E1i of N8-E1i was omitted from the washout media. Untreated cells are indicated by open circles. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

G) Hela cells were pre-treated with DMSO (blue bars) or Ub-E1i (red and grey bars) for 90' followed by treatment with sodium arsenite (250μM) for 60' at which time the sodium arsenite and the Ub-E1i was washed out (red bars) or the sodium arsenite was washed out but the Ub-E1i remained in the media for the duration of the washout period (grey bars). Cells were fixed at the indicated times following sodium arsenite washout. SGs were localized using G3BP1 immunofluorescence. The stress granule and nuclear area was quantified using custom image analysis scripts for the indicated time points and treatments. Error bars depict standard deviation of the mean.

Markmiller et al Fig S4 - related to Figure 4

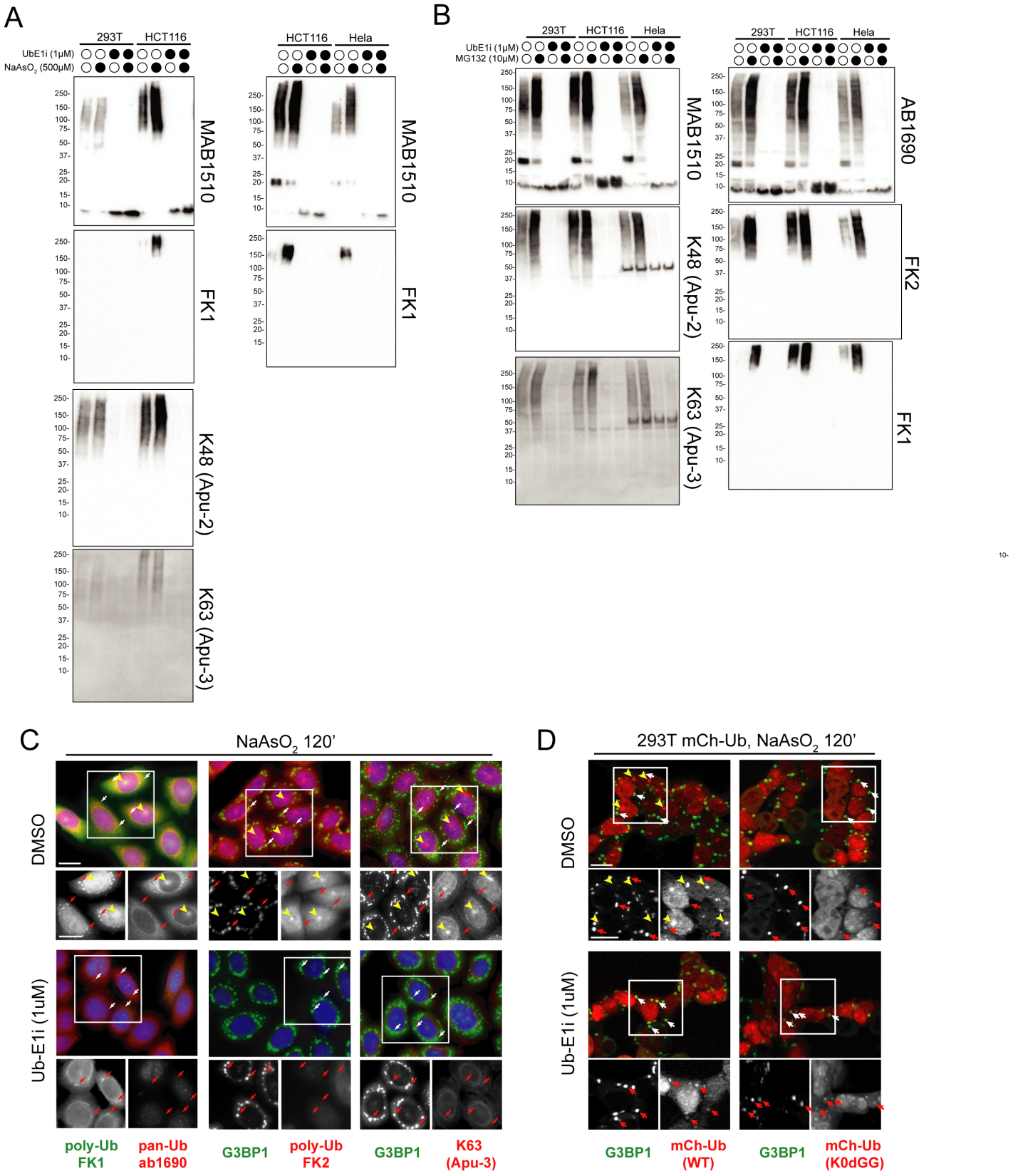

Figure S4 – Characterization of ubiquitin antibodies by denaturing SDS-PAGE. Related to Figure 4.
A) The indicated cell lines were either untreated of treated with Ub-E1i (1μM) for 1 hour. Cells were then either treated of untreated with sodium arsenite (500μM) for 1 hour. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.
B) The indicated cell lines were either untreated of treated with Ub-E1i (1μM) for 1 hour. Cells were then either treated of untreated with MG132 (10μM) for 4 hours. Cells treated with MG132 and Ub-E1i were treated with both inhibitors for the entire time course. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.
C) Immunofluorescence staining of HeLa cells treated with sodium arsenite (250μM) for 120' prior to fixation. Cells were stained using an antibody that recognizes both free and conjugated forms of ubiquitin (ab1690, shown in red in left panel) an antibody with a preference for polyubiquitin (FK1, shown in green in left panel), antibodies against G3BP (shown in green in middle and right panels), an antibody with a preference for polyubiquitin (FK2, shown in red in middle panel) or a linkage-specific antibody against K63-linked polyubiquitin chains (Apu-3, shown in red in right panel).
D) Immunofluorescence staining of 293T cells stably expressing either mCherry-tagged wild type ubiquitin (mCh-Ub-WT) or mCherry-tagged ubiquitin in which all internal lysine residues were mutated to arginine and the c-terminal diglycine residues was removed (mCh-Ub-K0ΔGG). Cells were pretreated with DMSO or Ub-E1i (1μM) for 90', followed by treatment with sodium arsenite (250μM) for 120' prior to fixation. Cells were stained using an antibody against G3BP1 (green). Co-localization of ubiquitin signal with G3BP1-positive stress granules is indicated by arrows, while G3BP1-negative perinuclear foci are indicated by solid yellow arrowheads.

Table S1 – Alterations to the proteome and ubiquitin-modified proteome upon arsenite treatment and washout. Related to Figure 1.

Table of identified proteins and diGLY-modified peptides and associated SILAC (H:L) ratios from HCT116 cells treated with sodium arsenite (500μM) for 0', 20', 45', or treated for 45' followed by a 3hr washout. Heavy labeled cells were arsenite treated. The diGLY-modified lysine residue is indicated in the peptide sequence column as K#.

Table S2 – List of annotated stress granule-localized proteins and SILAC ratios for SG proteins after arsenite or Ub-E1i treatment. Related to Figures 1 and 2.

The Known_SG_prots tab is the curated list of previously determined SG-localized proteins used for all proteomic analyses of known SG proteins. The SG_prot_digly_Arsen_Fig_1E tab is the SILAC ratios for all quantified known SG proteins (from the first tab) in the arsenite treatment experiment that is depicted in figure 1E. The SG_prot_digly_UbE1i_Fig_2G tab is the SILAC ratios for all quantified known SG proteins (from the first tab) in the Ub-E1i treatment experiment that is depicted in figure 2G.

Table S3 – Alterations to the proteome and ubiquitin-modified proteome upon UAE inhibition. Related to Figure 2.

Table of identified proteins and diGLY-modified peptides and associated SILAC (H:L) ratios from HCT116 cells treated with either the Ub-E1i (1μM) alone or the Ub-E1i in combination with MG132 (10μM) for 4 hours. Heavy labeled cells treated with the Ub-E1i were mixed with untreated light-labeled cells and heavy-labeled cells treated with the Ub-E1i and MG132 were mixed with light-labeled cells treated with MG132 alone. The diGLY-modified lysine residue is indicated in the peptide sequence column as K#.