Somasekharan et al., http://www.jcb.org/cgi/content/full/jcb.201411047/DC1

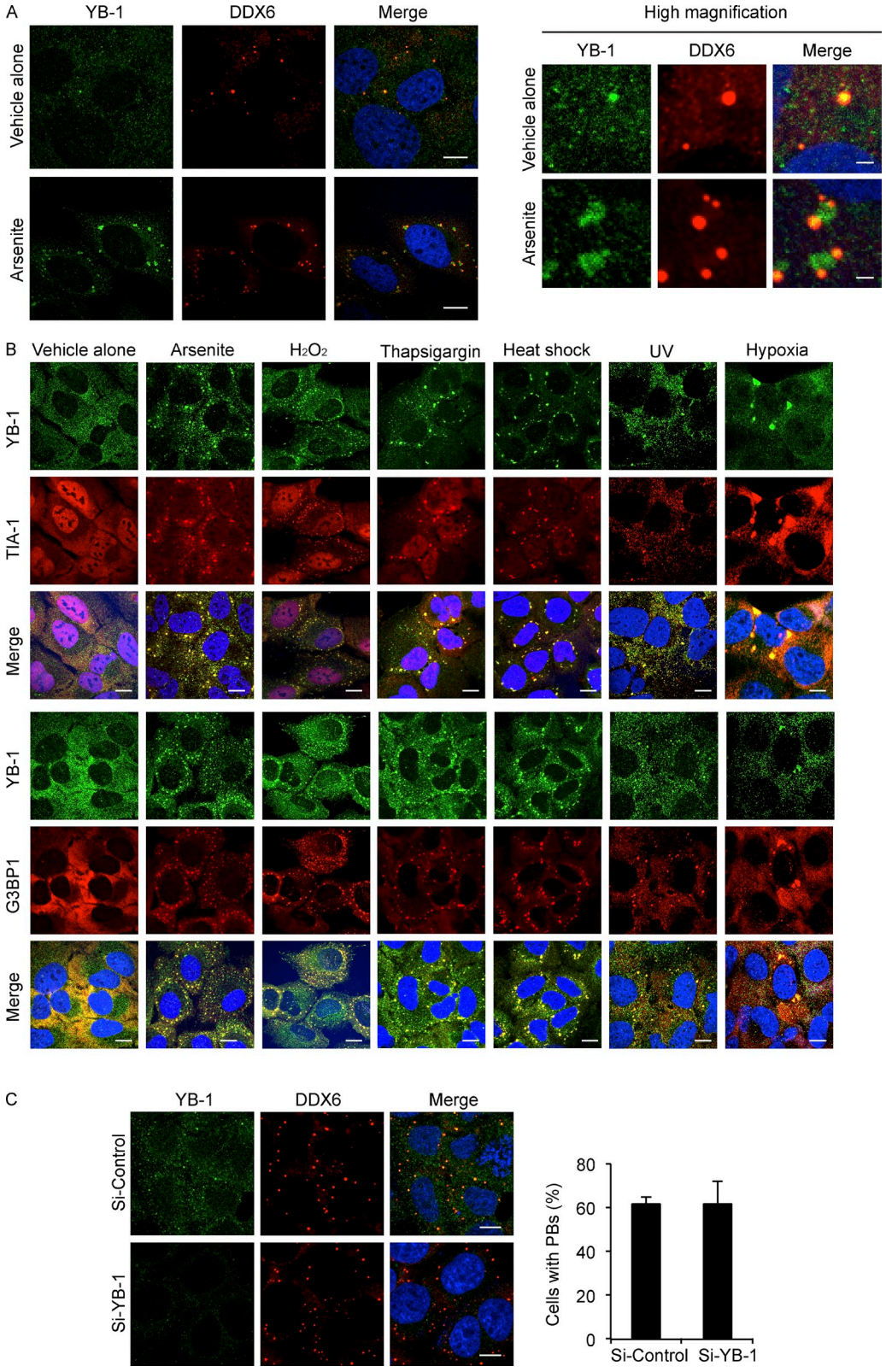


Figure S1. **YB-1 localizes to SGs under cell stress.** (A) U2OS cells treated with vehicle alone or 0.5 mM arsenite were subjected to IF using antibodies against the PB marker DDX6 and YB-1. 10-fold high-magnification images of representative areas from A are shown on the right. Bars: (left) 10 µm; (right) 1 µm. (B) U2OS cells were treated with vehicle alone or the indicated stress inducers (as described in the Materials and methods), and monitored for SG formation by IF using the indicated antibodies. Bars, 10 µm. (C) U2OS cells transfected with siControl or siYB-1 siRNAs were subjected to IF using the indicated antibodies. PBs were quantified using ImageJ software by counting the number of cells containing PBs divided by the total number of cells, and represented by a bar graph. Bars, 10 µm.

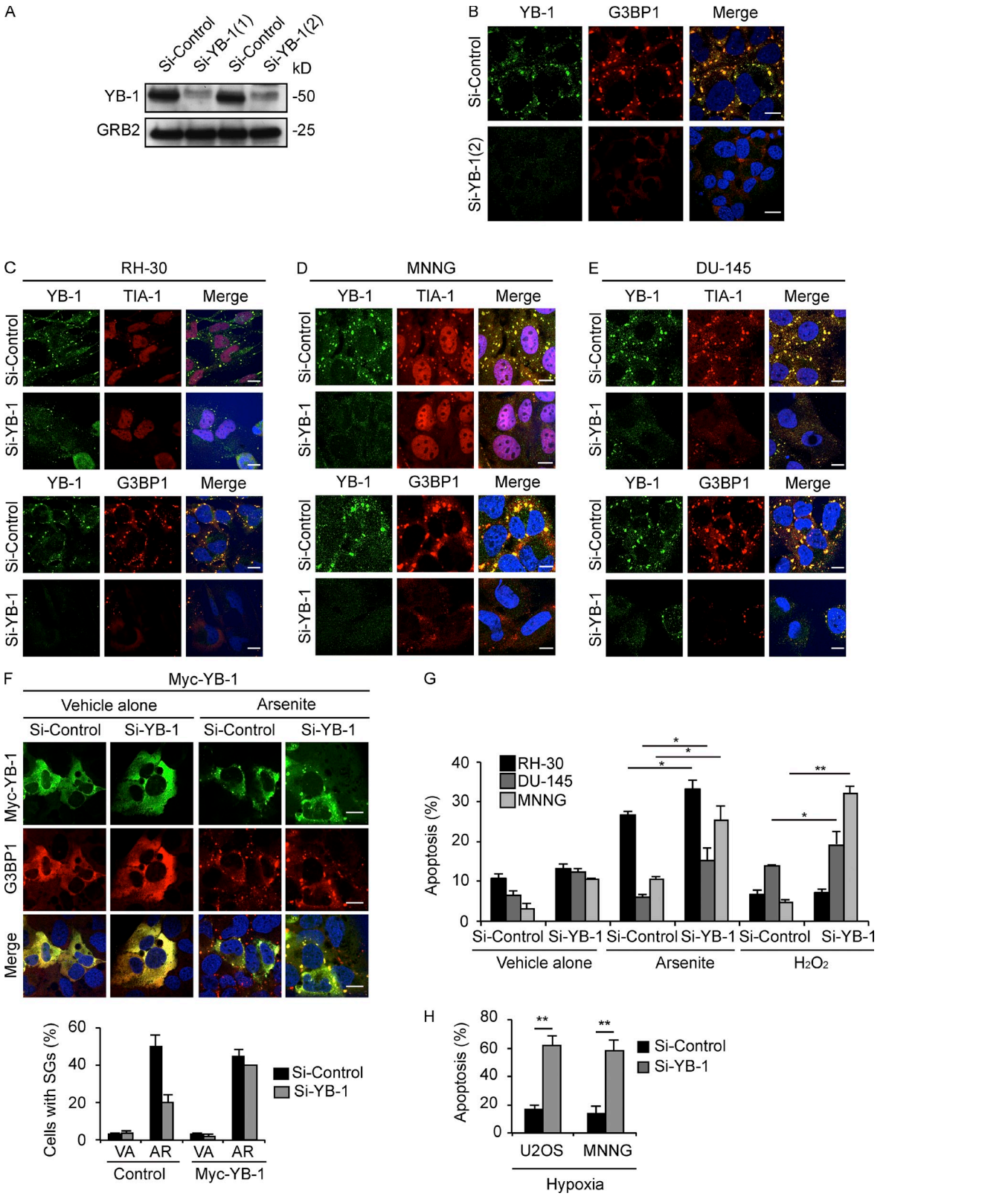


Figure S2. **YB-1 promotes SG formation.** (A) U2OS cells were transfected with control siRNAs (siControl) or two independent siRNAs (siYB-1 (1) and (2)) targeting YB-1. Cell lysates were prepared and immunoblotted using the indicated antibodies. (B) The same cells with or without YB-1 kd using siYB-1 (2) were treated with vehicle alone or arsenite (0.5 mM) and monitored for SG formation by IF with anti-G3BP1 antibodies. (C–E) RH-30 (C), MNNG (D), and DU-145 (E) cells transfected with siControl or siYB-1 siRNAs were treated with vehicle alone or arsenite (0.5 mM), and monitored for SG formation by IF using the indicated antibodies. (F) siControl or siYB-1 U2OS cells were transfected with Myc-YB-1 (2 µg), then the cells were treated with vehicle alone or 0.5 mM arsenite, and monitored for SG formation. SGs were quantified using ImageJ software by counting the number of cells containing SGs divided by the total number of cells, and represented by a bar graph. (G and H) RH-30, MNNG, and DU-145 cells transfected with siControl or siYB-1 siRNAs were treated with vehicle alone (VA), arsenite (AR; 0.5 mM), or H_2O_2 (0.5 mM) for 5 h (G) or subjected to hypoxia (1% O_2) for 24 h (H). They were then stained with Annexin V-FITC and the percentage of apoptosis was quantified by FACS using FACSCalibur. Mean values ± SD (error bars) are shown for three independent experiments. *, $P < 0.05$; **, $P < 0.01$. Bars, 10 µm.

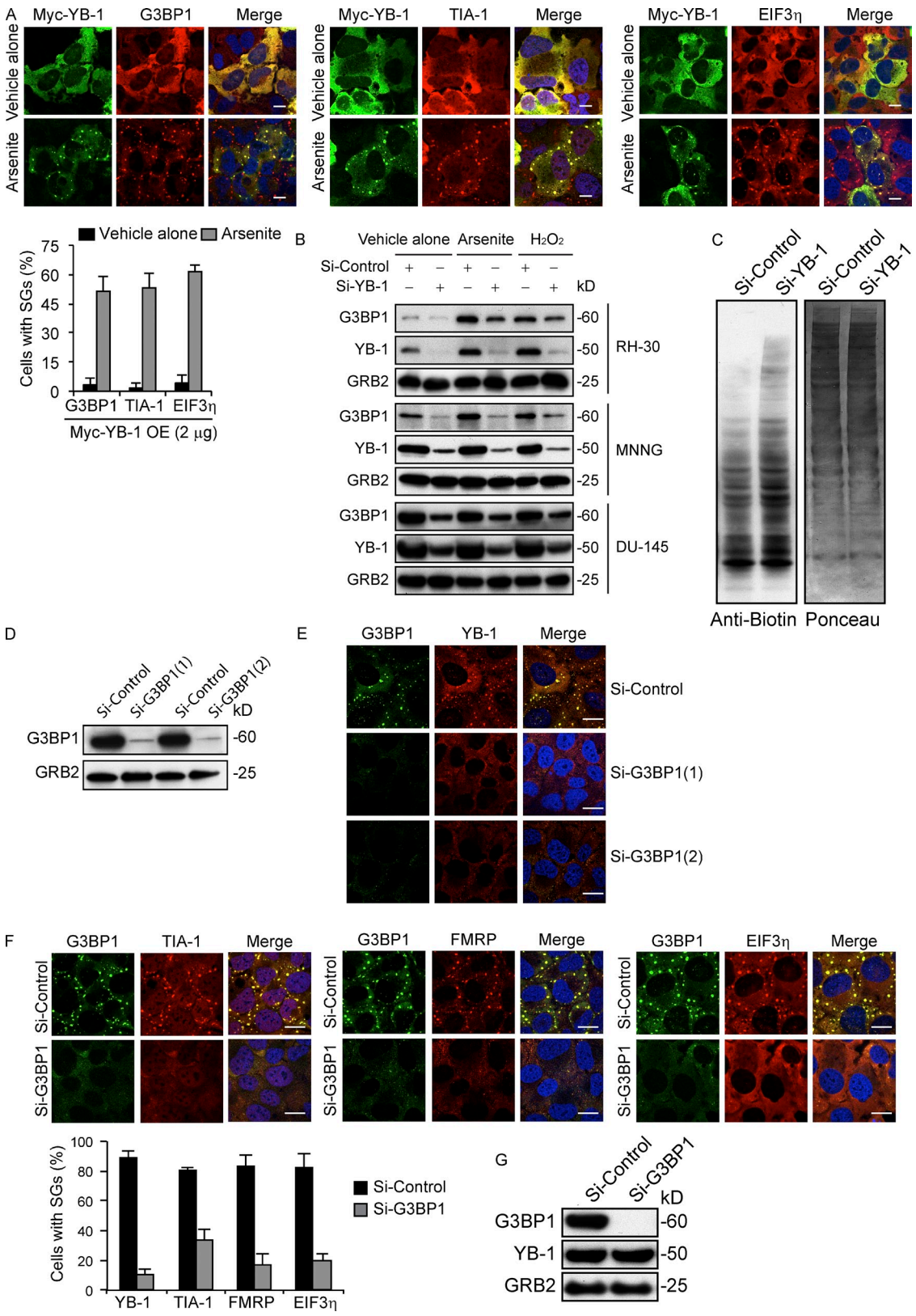


Figure S3. **YB-1 regulates G3BP1 protein expression.** (A) U2OS cells transfected with Myc-YB-1 (2 μg) were treated with vehicle alone or arsenite (0.5 mM) and monitored for SG formation. Quantification of SGs was performed as in Fig. S2 F. (B) RH-30, MNNG, and DU-145 cells transfected with siControl or siYB-1 siRNAs were treated with vehicle alone, arsenite (0.5 mM), or H_2O_2 (0.5 mM) for 1 h, and cell lysates were immunoblotted using the indicated antibodies. (C) U2OS cells transfected with siControl or siYB-1 siRNAs were methionine-depleted in methionine-free DMEM for 30 min. Both cell types were then pulsed with the AHA methionine analogue for 1 h to capture newly synthesized proteins. Total lysates prepared from AHA-labeled siControl or siYB-1 cells were subjected to click reactions with biotin-alkyne as described in the Materials and methods and immunoblotted with anti-biotin antibodies. Equal loading was determined by Ponceau staining. (D) U2OS cells were transfected with control siRNAs (siControl) or two independent siRNAs (siG3BP-1 (1) and (2)) targeting G3BP1. Cell lysates were prepared and immunoblotted using the indicated antibodies. (E) The same cells with or without G3BP1 kd using siG3BP1 (1) and (2) were treated with vehicle alone or arsenite (0.5 mM) and monitored for SG formation by IF with anti–YB-1 antibodies. (F) The same cells with or without G3BP1 kd using siG3BP1 (2) were treated with vehicle alone or arsenite (0.5 mM) and monitored for SG formation by IF with anti–TIA-1, anti-FMRP, and anti-EIF3η antibodies. Quantification of SGs was performed as in Fig. S2 F. (G) Lysates were extracted from the same cells with or without G3BP1 kd using siG3BP1 (2) and subjected to immunoblot analysis using anti-G3BP1, anti-YB-1, and anti-GRB2 antibodies. Error bars indicate SD. Bars, 10 μm.

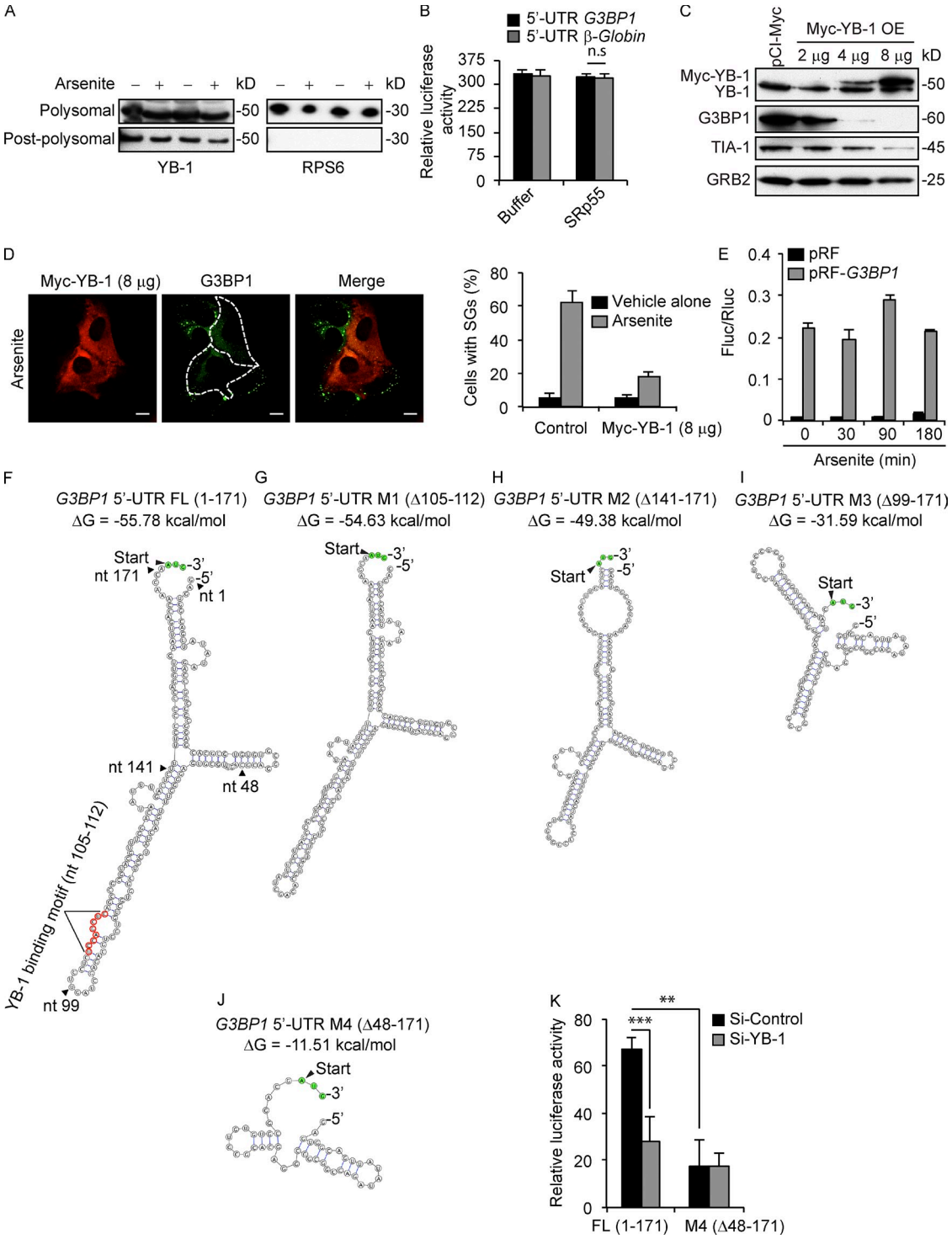


Figure S4. **YB-1 translationally regulates G3BP1.** (A) Lysates prepared from vehicle alone or arsenite (0.5 mM)-treated U2OS cells were separated into polysomal and postpolysomal fractions using sucrose gradient centrifugation, and the fractions were immunoblotted by using antibodies against YB-1 and RPS6. (B) Constructs containing the 5′ UTR of *G3BP1* or *β-Globin* fused in frame to a *Luciferase* gene were used for in vitro coupled transcription translation assays. Buffer control or recombinant SRp*55* protein was added to the assay mixture and luciferase activity was measured. (C) Lysates prepared from U2OS cells transfected with Myc-YB-1 (2, 4, and 8 μg) were immunoblotted using antibodies to YB-1, G3BP1, TIA-1, and GRB2. (D) U2OS cells transfected with Myc-YB-1 (8 μg) were treated with arsenite (0.5 mM) for 1 h. Myc (red) and SG formation (green) were monitored by IF using the indicated antibodies. Broken outlines represent two Myc-YB-1–expressing cells from the left panel that failed to form SGs. The percentage of cells with SGs in the control and Myc-YB-1–transfected cells, treated with vehicle alone or arsenite (0.5 mM), was quantified as described in Fig. S2 F, and represented by a bar graph on the right. Bar, 10 μm. (E) Analysis of the 5′ UTR of *G3BP1* mRNA for IRES-mediated translation. U2OS cells were transiently transfected with control pRF bicistronic vector or pRF-*G3BP1* construct with full-length *G3BP1* 5′ UTR cloned into the polylinker region between the *Rluc* and *Fluc* codons. Luciferase activity was analyzed after 24 h and expressed as the ratio of Fluc/Rluc to indicate the relative level of IRES activity compared with cap-dependent translation. (F–J) RNA secondary structure analysis of the full-length 5′ UTR of *G3BP1* (F) and its deletion mutants (G–J) from Fig. 3 E generated using VARNAGUI software (http://varna.lri.fr), and with melting temperatures calculated using RNAfold (http://rna.tbi.univie.ac.at). The start codon (AUG) is highlighted in green and 5′ and 3′ ends are indicated. A previously reported putative YB-1 binding site (nt 105–112; Paranjape and Harris, 2007) is denoted by red circles. Arrows in F, i.e., nt 1, 48, 99, and 141, represent nucleotides of the full-length *G3BP1* 5′ UTR, where deletions were performed to generate the indicated mutants. (K) SiControl or siYB-1 kd U2OS cells were transfected with constructs containing full-length (FL, 1–171) or mutant (M4, Δ48–171) 5′ UTR sequences of *G3BP1* fused in frame to a *Luciferase* gene. Luciferase activity was measured after 24 h of transfection and relative luciferase activity is represented by a bar graph. Mean values ± SD (error bars) are shown for three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

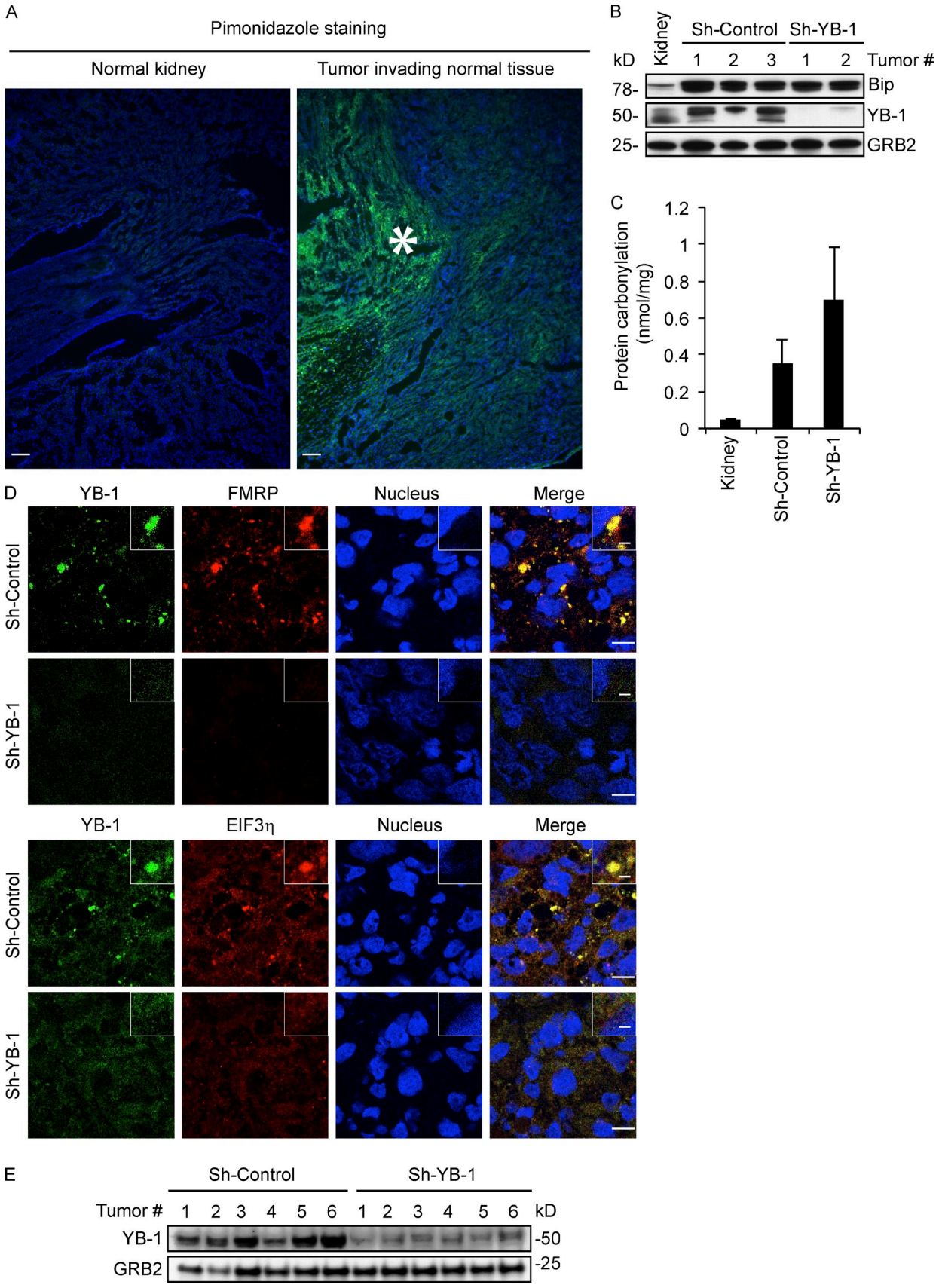
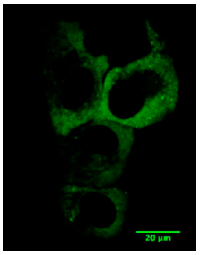
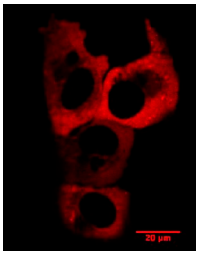


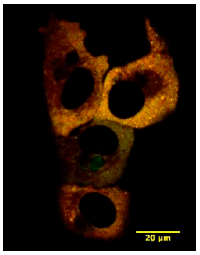
Figure S5. **Down-regulation of YB-1 affects in vivo SG formation.** (A) Staining of hypoxic regions in normal kidney (left) and tumor invading normal tissue (right) using the pimonidazole hypoxia marker (green staining). The asterisk shows specific staining of pimonidazole in tumor tissues. Bars, 400 µm. (B) Lysates isolated from normal kidney and primary implantation site tumor samples from shControl and shYB-1 cells were subjected to immunoblot analysis using antibodies against Bip, YB-1, and GRB2. (C) Carbonylated proteins were measured as described in the Materials and methods in lysates from normal kidney, shControl, and shYB-1 tumor tissues (n = 5 per tumor group). (D) Viable regions of shControl and shYB-1 kd primary implantation site tumors were cryosectioned, and SGs were monitored with IF using the indicated antibodies. Insets show fivefold higher magnification of representative areas. Bars: (main panels) 10 µm; (enlarged insets) 1 µm. (E) Lysates extracted from primary implantation site tumor tissues (n = 6 per tumor group) from D were subjected to immunoblot analysis using anti–YB-1 and anti-GRB2 antibodies.



Video 1. **Localization of GFP-YB-1 in SGs.** U2OS cells were cotransfected with GFP-YB-1 and RFP-G3BP1. After 24 h of transfection, cultures were placed in a 37°C chamber equilibrated with humidified air containing 5% CO_2. Before live cell imaging, the media was changed to fresh DMEM containing 0.5 mM arsenite. Time-lapse images were taken with a microscope (Axio Observer Z1; Carl Zeiss) using a 40× objective lens and analyzed by AxioVision software (Carl Zeiss). Images were captured at every 2 min for 1 h, and the movie was generated with the time-lapse series using ImageJ software.



Video 2. **Localization of RFP-G3BP1 in SGs.** U2OS cells were cotransfected with GFP-YB-1 and RFP-G3BP1. After 24 h of transfection, cultures were placed in a 37°C chamber equilibrated with humidified air containing 5% CO_2. Before live cell imaging, the media was changed to fresh DMEM containing 0.5 mM arsenite. Time-lapse images were taken with a microscope (Axio Observer Z1; Carl Zeiss) using a 40× objective lens and analyzed by AxioVision software (Carl Zeiss). Images were captured at every 2 min for 1 h, and the movie was generated with the time-lapse series using ImageJ software.



Video 3. **Merge showing colocalization of GFP-YB-1 and RFP-G3BP1 in SGs.** U2OS cells were cotransfected with GFP-YB-1 and RFP-G3BP1. After 24 h of transfection, cultures were placed in a 37°C chamber equilibrated with humidified air containing 5% CO_2. Before live cell imaging, the media was changed to fresh DMEM containing 0.5 mM arsenite. Time-lapse images were taken with a microscope (Axio Observer Z1; Carl Zeiss) using a 40× objective lens and analyzed by AxioVision software (Carl Zeiss). Images were captured at every 2 min for 1 h, and the movie was generated with the time-lapse series using ImageJ software.

Reference

Paranjape, S.M., and E. Harris. 2007. Y box-binding protein-1 binds to the dengue virus 3′-untranslated region and mediates antiviral effects. *J. Biol. Chem.* 282:30497–30508. http://dx.doi.org/10.1074/jbc.M705755200