Supplemental Materials

Mutant FUS Proteins that Cause Amyotrophic Lateral Sclerosis Incorporate into Stress Granules

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Figure and video legends

Figure S1. The FUS R495X mutation segregates with ALS in the index pedigree. The fALS pedigree F521 displays the cosegregation of the FUS c.1566C>T, R495X, over five (I-V) generations. DNA from IV:6 and V:2 (green) confirmed the presence of this mutation within the pedigree. Open and dark symbols respectively indicate unaffected and affected (suspected, probable and definite ALS-diagnosis) individuals; slash marks denote deceased individuals. Affected individuals are identified as generation:position within the family tree (counting from the left). Clinical information pertaining to these affected individuals appears in Table 1.

Figure S2. Fluorescence quantitation of cytoplasmic and nuclear GFP-hFUS expression in live HEK-293 cells. To assess the relative concentrations of GFP in the cytoplasm and nucleus for each cell, stacks of 60-100 thin slices (Δz = 0.2 μm for each slice) were acquired and processed to subtract the dark current image and to correct for pixel inefficiencies (see Methods). **(A)** Shown are live cell DIC images (left) and pseudocolor maximum intensity z-projections of GFP fluorescence from ten contiguous slices near the middle of the cell (right). Regions of interest (10×10 pixels) for nuclear, cytoplasmic, and background fluorescence quantitation are indicated. **(B)** The average fluorescence intensity in each region of cytoplasm (*c*) and nucleus (*n*) was compared to the residual background (*b*) for individual 0.2 μm slices. The cytoplasmic/nuclear ratio for each slice was calculated as $(c-b)/(n-b)$, and the ratios obtained from the ten contiguous slices near the middle of the cell (black boxes) were averaged to obtain a single value for each cell examined. Scale bar, 5 μm.

Figure S3. Transient transfection of GFP-FUS constructs into HEK-293 cells yields increased expression levels compared to induced cell lines. (**A**) GFP-FUS constructs were acutely transfected into

naive HEK-293 cells for 40 h as described previously (13), and stable HEK-293 cells were induced to express the GFP-FUS proteins with doxycycline for 40 h, after which cell lysates were prepared (Methods). Immunoblotting of these lysates (3 μg total protein/ lane) with anti-GFP and anti-GAPDH (loading standard) antibodies followed by a densitometry analysis revealed that transiently transfected cells express GFP-FUS at ~5-fold higher levels compared to the stable lines. (**B**) Fluorescence microscopy of the transiently transfected GFP-FUS HEK-293 cells described in (**A**) revealed prominent GFP-FUS cytoplasmic aggregates (green) for GFP-FUS(R521G, R495X, and G515X), whereas GFP-FUS(WT and H517Q) exhibited a relatively diffuse expression pattern in the nucleus (DAPI; blue). Scale bar, 10 μm.

Figure S4. Stress granule formation was induced in HEK-293 cells upon both sodium arsenite and thapsigargin treatment. (**A**) Immunofluorescence of stable GFP-FUS HEK-293 cells treated with sodium arsenite was performed as described in Fig. 2, except that the G3BP (red) stress granule marker was employed here. Confocal microscopy revealed similar results as in Fig. 2, in that GFP-FUS(R521G, R495X, and G515X) co-localized to stress granules that formed upon sodium arsenite treatment, whereas GFP-FUS(WT and H517Q) exhibited a predominately nuclear (DAPI, blue) expression pattern. (**B**) GFP-FUS(R521G, R495X, and G515X) also co-localized to TIAR-positive (red) stress granules as in (**A**) upon treatment with 10 μM thapsigargin for 2 h, whereas GFP-FUS(WT and H517Q) did not. (**C**) HEK-293 cell lines were plated into 96-well plates (5,000 cells/ well with n=8 wells/ condition) and induced with doxycycline for 24 h at 37°C (see Methods for culture conditions). Cells were treated with 0.5 mM sodium arsenite for 4 h, after which cell viability was quantified with the MTT cell proliferation kit-1 (Roche). Cell viability following this stress did not differ among naïve cells or those expressing GFP-FUS(WT, R521G and R495X), as determined by ANOVA (p>0.05). Similar results were obtained for cells exposed to sodium arsenite for 1 h and 2 h (data not shown). (**D**) HEK-293 cell lines were plated and induced as in (C). Cells were exposed to 0.5 mM sodium arsenite for 1 h, and cell viability was assessed 24 h later using the MTT assay as described in (C). Recovery of cells from this stress did not differ (p>0.05) among naïve cells or those expressing GFP-FUS(WT, R521G and R495X). Similar results were obtained when cell viability was quantified 48 h post sodium arsenite treatment (data not shown).

Figure S5. Incorporation of FUS into stress granules is not mediated by the GFP tag. (**A**) Transiently transfected (40 h) untagged-GFP did not co-localize to TIAR-positive (red) stress granules upon treatment with sodium arsenite (described in Fig. 2) within HEK-293 cells, but rather exhibited a relatively diffuse nuclear and cytoplasmic expression pattern under these conditions. (B) Untagged FUS (R521G and R495X) proteins, but not WT, were incorporated into stress granules upon transient transfection (16 h here, compared to 40 h in Fig. S3) of the respective construct into HEK-293 cells followed by arsenite treatment. Endogenous FUS did not incorporate into stress granules, as illustrated by the image of non-transfected (NT) HEK-293 cells after arsenite treatment. FUS expression was detected by immunofluorescence (Methods) with the anti-FUS antibody (Bethyl Labs, #A300-293A; 1:400). Scale bar, 10 μm (A and B). (**C**) Western analysis (Methods) of cell lysates prepared from the experiment described in (B). Duplicate experiments are shown for each cell line, and the ratio of FUS/GAPDH reflects the average of the densitometry measurements for these experiments.

Fig1video1.avi. Mobile cytoplasmic granules containing GFP-FUS(G515X). Stable HEK-293 cells expressing GFP-FUS(G515X) induced by doxycycline as in Fig. 1 C were imaged by time-lapse microscopy at 37°C using a spinning disk confocal microscope (CSU10B; Solamere Technology Group) to detect green fluorescence. Frames were acquired every 1 sec for 60 sec and displayed at 10 fps. Scale bar, 10 μm.

Fig3videos2-5.avi. Localization of GFP-FUS variants in response to arsenite treatment. Stable HEK-293 cells expressing GFP-FUS variants were imaged by time-lapse microscopy at 37°C as above, and sodium arsenite (0.5 mM) was applied at the indicated times. Frames were acquired every 20 sec for 20-40 min and displayed at 10 fps (with elapsed time in hh:mm indicated). Representative snapshots appear in Fig. 3. Shown are cells expressing G515X (Fig3video2.avi), R495X (Fig3video3.avi), R521G (Fig3video4.avi), and WT FUS (Fig3video5.avi). Scale bars, 10 μm.

Fig4videos6-10.avi. Localization of GFP-FUS variants in response to heat shock and subsequent recovery. Stable HEK-293 cells expressing GFP-FUS variants were imaged by time-lapse microscopy as above, and the temperature was raised from 37°C to 42.5°C at the indicated times. Frames were acquired every 20 sec for 13-30 min and displayed at 10 fps (with elapsed time in hh:mm indicated). Recovery back to

37°C was captured for cells expressing G515X, R495X, and R521G, and representative snapshots appear in Fig. 4. Shown are cells expressing G515X (Fig4video6.avi), R495X (Fig4video7.avi), R521G (Fig4video8.avi), H517Q (Fig4video9.avi), and WT FUS (Fig4video10.avi). Scale bars, 10 μm.

Figure S1 Bosco *et al.*

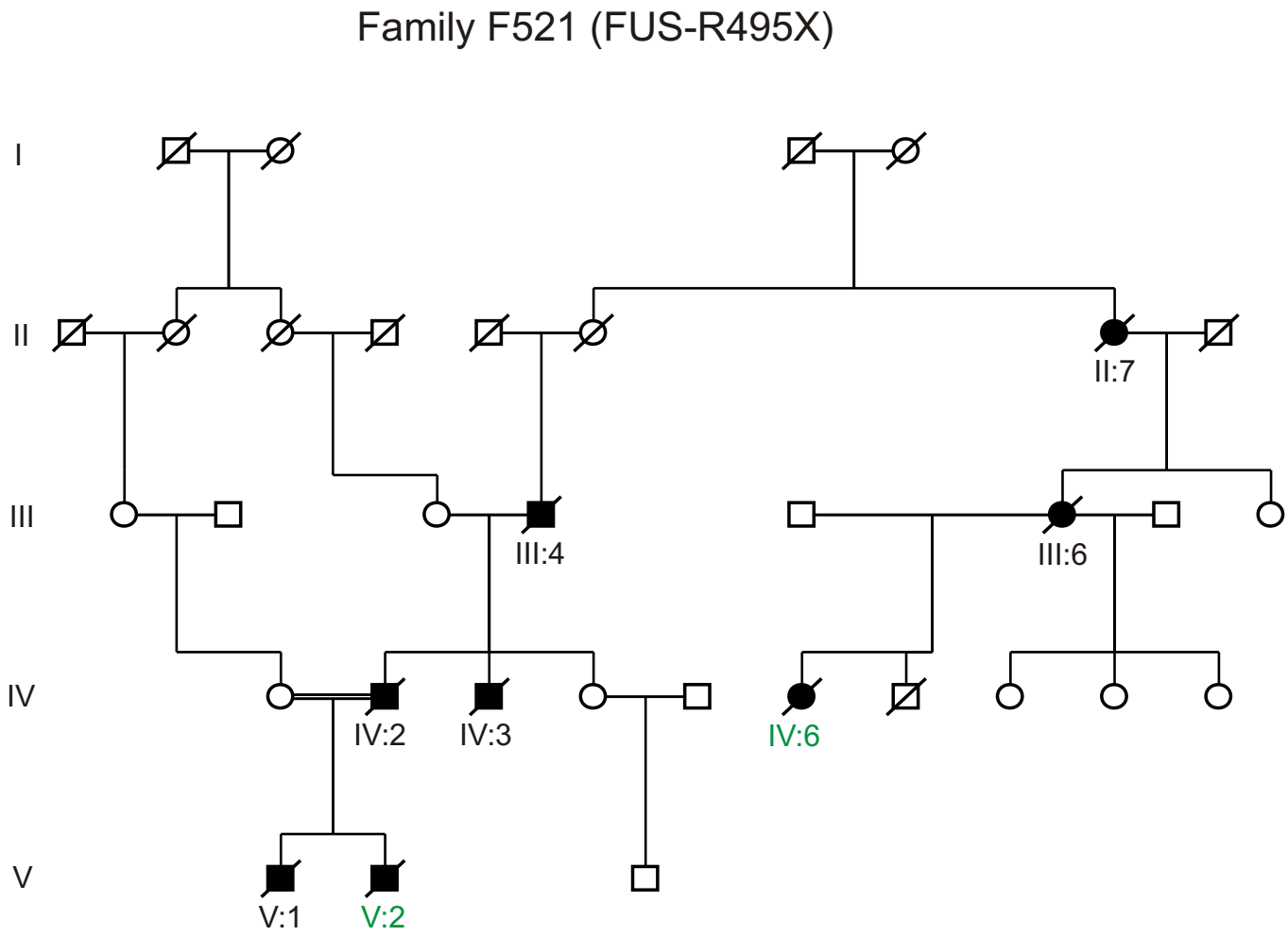


Figure S2 Bosco *et al.*

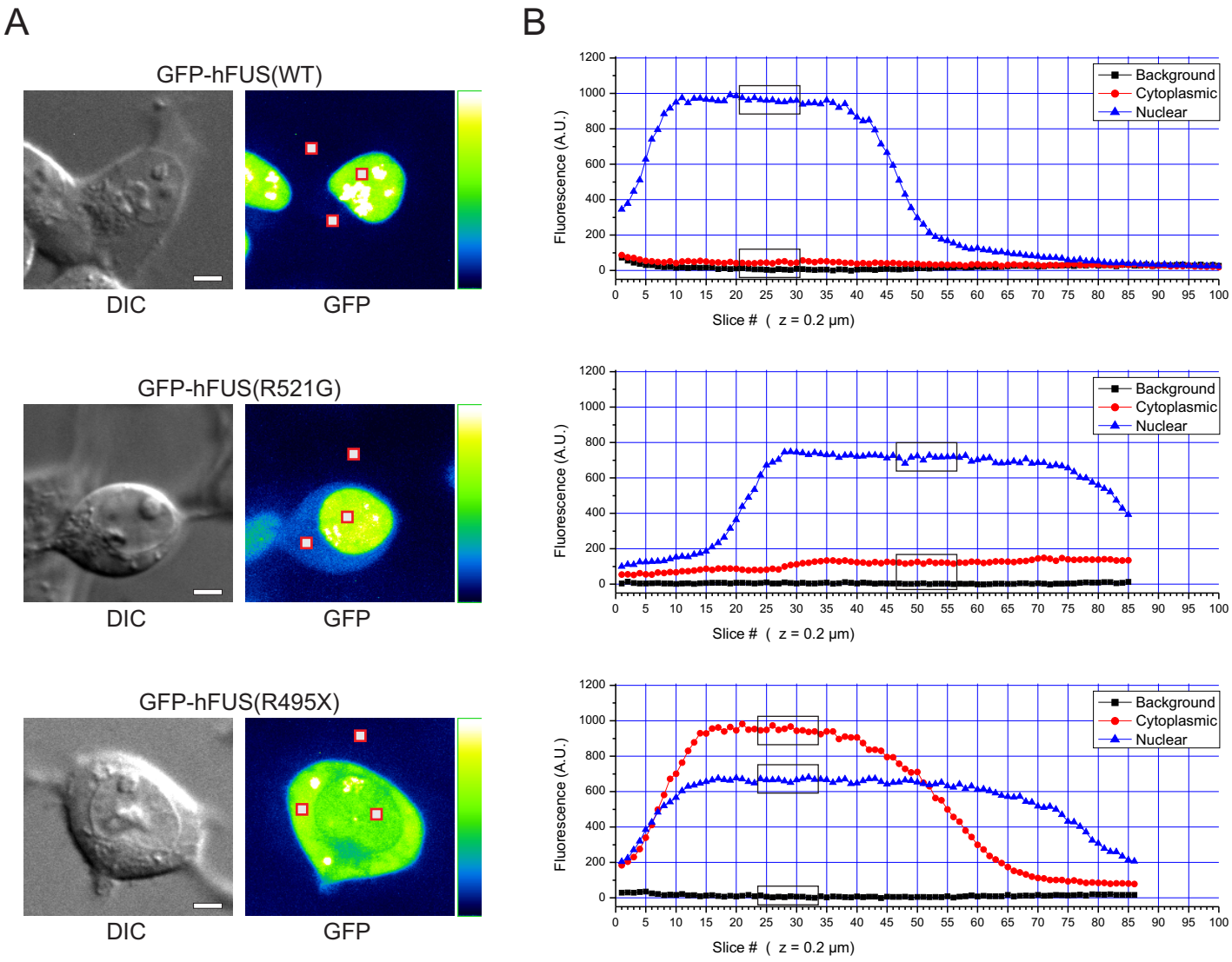
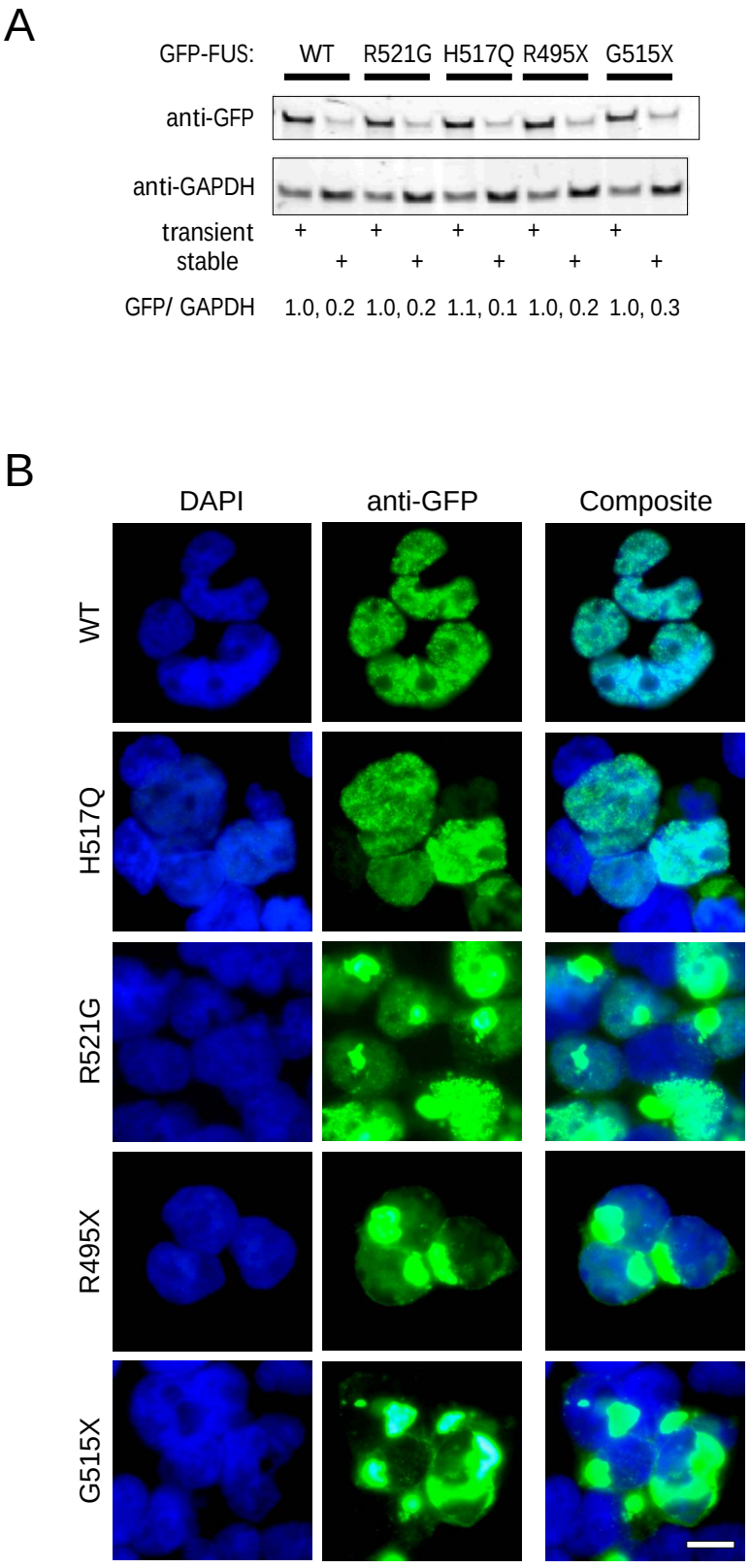


Figure S3 Bosco *et al.*



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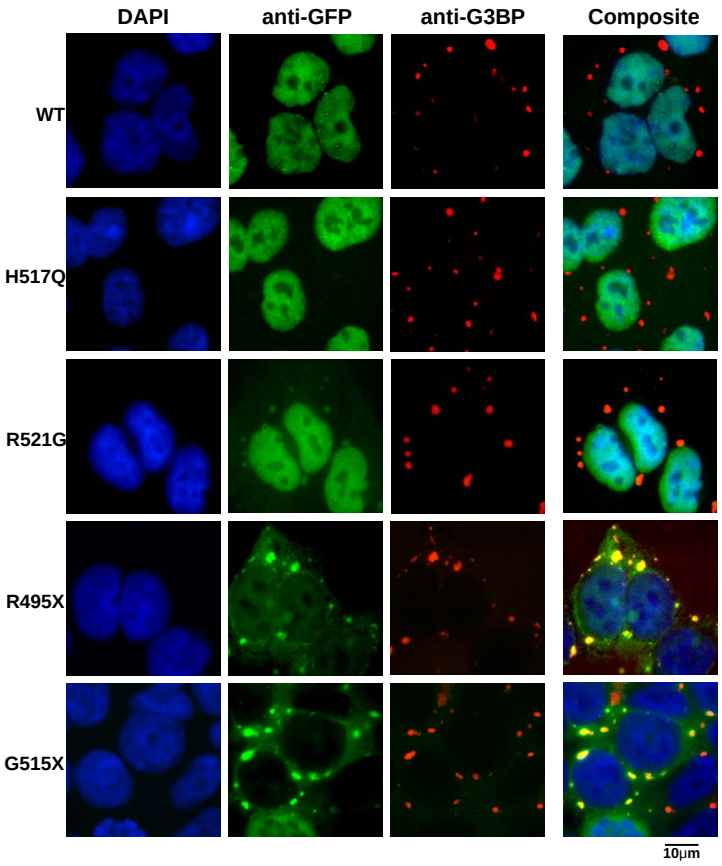
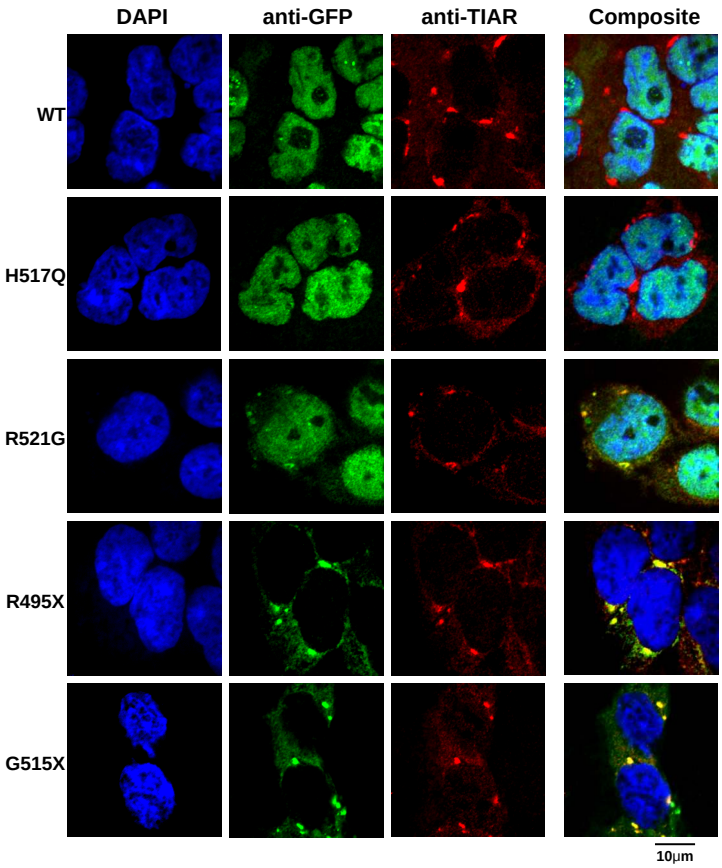
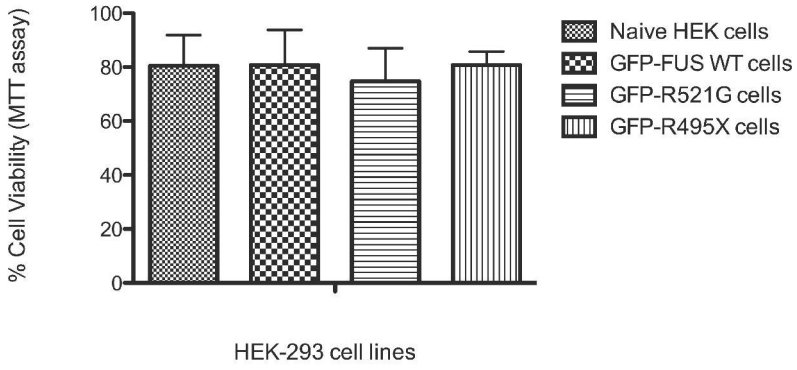
A. Sodium arsenite treatment / G3BP marker



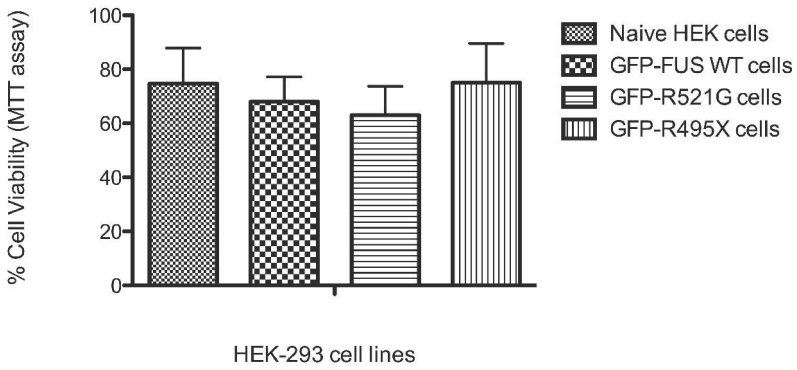
B. Thapsigargin treatment / TIAR marker



C. Viability of HEK-293 cells after 4-h arsenite treatment



D. Recovery of HEK-293 cells 24 h after 1-h arsenite treatment



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