GENERAL ARTICLE

Quantitative proteomics identifies proteins that resist translational repression and become dysregulated in ALS-FUS

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Abstract

Aberrant translational repression is a feature of multiple neurodegenerative diseases. The association between disease-linked proteins and stress granules further implicates impaired stress responses in neurodegeneration. However, our knowledge of the proteins that evade translational repression is incomplete. It is also unclear whether disease-linked proteins influence the proteome under conditions of translational repression. To address these questions, a quantitative proteomics approach was used to identify proteins that evade stress-induced translational repression in arsenite-treated cells expressing either wild-type or amyotrophic lateral sclerosis (ALS)-linked mutant FUS. This study revealed hundreds of proteins that are actively synthesized during stress-induced translational repression, irrespective of FUS genotype. In addition to proteins involved in RNA- and protein-processing, proteins associated with neurodegenerative diseases such as ALS were also actively synthesized during stress. Protein synthesis under stress was largely unperturbed by mutant FUS, although several proteins were found to be differentially expressed between mutant and control cells. One protein in particular, COPBI, was downregulated in mutant FUS-expressing cells under stress. COPBI is the beta subunit of the coat protein I (COP1), which is involved in Golgi to endoplasmic reticulum (ER) retrograde transport. Further investigation revealed reduced levels of other COP1 subunit proteins and defects in COPBI-related

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processes in cells expressing mutant FUS. Even in the absence of stress, COPBI localization was altered in primary and human stem cell-derived neurons expressing ALS-linked FUS variants. Our results suggest that Golgi to ER retrograde transport may be important under conditions of stress and is perturbed upon the expression of disease-linked proteins such as FUS.

Introduction

Protein translation is one of the highest energy-consuming processes in the cell (1). Under conditions of stress, translation is repressed and resources are redirected to mitigate stress as the cell works to re-establish homeostasis (1). Oxidative, hyperosmolar and endoplasmic reticulum (ER) stress have been shown to repress global translation through signaling events that induce phosphorylation of the alpha subunit of the translation initiation factor 2 (eIF2 α) (1,2). During stress-induced translational repression, polysomes disassemble and components of the translation machinery, mRNAs and RNA-binding proteins condense into cytoplasmic foci called stress granules (SGs) (3). SGs are membraneless organelles (4) that may be formed through liquid–liquid phase separation (LLPS), which refers to the partitioning of biological molecules into liquid and solid phases through weak non-covalent interactions (5). In recent years, multiple neurodegenerative disease-associated proteins were shown to incorporate into SGs (6), and, in some cases, alter the properties of these granules (7–15). As SGs are thought to function in mRNA triage, translation, signaling and cellular homeostasis (16), we hypothesized that incorporation of disease-linked proteins within SGs could have adverse effects on these processes (17).

Stress recovery and cell viability depend on proteins that resist translational repression and are actively translated under stress (1). Although a subset of transcription factors and heat-shock proteins were known to be activated and/or upregulated during stress (1,16), a comprehensive list of proteins that evade translational repression is lacking. Our knowledge of proteins that evade translational repression is limited by methods that can reliably detect newly synthesized, low-abundance proteins. To address this limitation, we employed a quantitative proteomics approach called BONLAC, which combines bioorthogonal noncanonical amino acid tagging (BONCAT) and stable isotope labeling by amino acids in culture (SILAC). BONLAC is ideally suited for quantifying low-abundance proteins and was used here to identify the actively translated proteome under conditions of arsenite stress (18). To gain insight into the effect of disease-linked proteins on stress-induced translational repression, we carried out these BONLAC studies in neuronal cells expressing wild type (WT) and a mutant form of FUS/TLS (fused in sarcoma/ translocated in liposarcoma, or FUS) that is associated with the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS) (19,20). A majority of ALS-linked mutations are positioned within the C-terminal nuclear localization sequence (NLS) of FUS, causing the protein to accumulate within the cytoplasm of cells (17). The degree to which mutant FUS mislocalizes to the cytoplasm directly correlates with disease pathogenesis in humans (21). For example, humans expressing NLS-truncating mutations (e.g. FUS R495X) present with particularly aggressive forms of ALS, often with juvenile onset (22,23). Indeed, recent FUS-ALS mouse models indicate that motor neuron degeneration is driven by excessive cytoplasmic FUS expression, rather than from a loss of nuclear expression (24–26). We reasoned that FUS represents a model protein for the current BONLAC study, as ALS-linked FUS variants robustly incorporate

into arsenite-induced SGs, whereas FUS WT does not (22,27,28). In fact mutant FUS has been shown to alter SG morphology and dynamics (7,12), raising the possibility that mutant FUS affects the functional properties of SGs as well. In support of this notion, the association of mutant FUS with adenomatous polyposis coli-containing RNA granules was shown to cause enhanced translation of select transcripts under homeostatic conditions (29).

The results of our BONLAC study revealed hundreds of proteins that are actively translated under conditions of stress-induced translational repression. These data provide new insight into translational repression and stress granule biology, as transcripts that tend to be depleted from SGs (30) encode proteins that were robustly synthesized under stress in our study. In addition to proteins involved in gene expression and stress response, our experiments also revealed the translation of proteins associated with neurodegenerative disease under stress. Although mutant FUS did not appear to grossly alter the protein expression profile under stress, our BONLAC analysis uncovered the differential expression of COPBI, which is a subunit of the coat protein I (COP1) complex involved in vesicular retrograde transport between the Golgi and ER and between Golgi cisternae (31). Further investigation revealed both stress-dependent and stress-independent effects of mutant FUS on the retrograde transport machinery and Golgi morphology. Even in the absence of stress, COPBI exhibited an altered localization pattern in primary neurons and human neurons derived from induced pluripotent stem cells (iPSCs) expressing different ALS-linked FUS variants. These findings implicate Golgi dysfunction and impaired retrograde transport in FUS-ALS pathogenesis and support the premise of targeting these pathways for therapeutic intervention (32–39).

Results

Selective protein synthesis under repressive stress conditions revealed through quantitative proteomics

Cells become highly selective with respect to gene expression during acute stress; however, the genes that are expressed and the mechanism(s) modulating their expression are incompletely defined. Here, BONLAC was used to label newly synthesized proteins under repressive stress conditions, and these proteins were subsequently identified by tandem mass spectrometry (MS/MS or MS2; Fig. 1A) (18). This dual labeling approach distinguishes between pre-existing (unlabeled) proteins and (labeled) proteins that are synthesized under stress. We reasoned that expression of an ALS-linked variant of FUS could influence protein expression under stress, as mutant FUS proteins incorporate into and alter the properties of SGs, presumed sites of translational repression (7,27,28). To test this hypothesis, inducible SK-N-AS human neuroblastoma cell lines were created that express untagged versions of either FUS WT or FUS R495X, a truncated form of FUS that robustly associates with SGs (7,22) (Fig. 1B; Supplementary Material, Fig. S1A and B). FUS mRNA expression was similar between FUS WT and R495X lines. Exogenous FUS proteins were expressed within approximately 2-fold between mutant and WT lines and at near-endogenous levels, although

Figure 1. Experimental conditions for the BONLAC study. **(A)** A schematic diagram of the BONLAC pipeline. FUS WT and R495X (RX) cells were exposed to SA stress for 35 min, after which cells were switched to media containing AHA-NH₃ (AHA), isotopically labeled amino acids for SILAC and SA for an additional 100 min 'labeling period'. FUS WT cells were grown in medium (orange) and FUS RX cells in heavy (blue) isotopically labeled media for two biological experiments; labels were reversed for the third experiment. FUS WT and R495X lysates were combined, and proteins synthesized under stress that contained the AHA label (pink) were subjected to copper catalyzed cycloaddition click chemistry with an alkyne-conjugated resin. Pre-existing, unlabeled proteins (gray) were removed and bound proteins eluted via proteolysis. Peptides were identified through tandem mass spectrometry (MS2) in FUS WT versus R495X cells, and unlabeled peptides that evaded the wash step were identified as such by the MS2 analysis. The MS1 scan was used for the SILAC quantitation of peptides that were detected in both FUS WT and R495X samples. **(B)** Representative immunofluorescence micrographs of inducible SK-N-AS cells expressing untagged FUS WT or R495X upon treatment with 0.2 mM SA probed with anti-FUS (red) and anti-G3BP (green) antibodies. Stress granule (G3BP, green) formation was detectable by 35 min and continued during the 100 min labeling period. Scale bar, 10 μm. **(C)** Western blot analysis of the cell lines in **(B)** shows an increase in eIF2α phosphorylation (anti-P-eIF2α) relative to total eIF2α levels (anti-eIF2α total). **(D)** Relative to total protein (silver-stained gel, left), levels of newly synthesized AHA-labeled protein from naïve SK-N-AS cells decrease with increasing concentrations of SA.

induced expression of FUS R495X was higher relative to FUS WT (Supplementary Material, Fig. S1C and D). Similarly, our previous isogenic Flp-In T-REx stable cell lines expressing GFP-tagged FUS variants also resulted in higher expression of mutant FUS relative to WT FUS, despite the transgenes being expressed from a single genomic locus (22). Enhanced expression of mutant

FUS may signify disruption of FUS autoregulatory function (40). Indeed, we detected relatively high levels of mutant FUS in ALS post-mortem brain tissues (41). Efforts to increase FUS WT expression resulted in cell toxicity (D. Baron and D.A. Bosco, unpublished results), consistent with other models of FUS WT overexpression (42,43).

Figure 2. Analysis of proteins that are actively translated during stress. **(A)** Venn diagram for the number of proteins identified in FUS WT (dark gray; 436) or R495X (light gray; 481) SK-N-AS cells over three independent BONLAC experiments. The 362 proteins that were consistently detected in both FUS WT and R495X cells were used for subsequent DAVID analysis (Supplementary Material, Dataset S2) and were compared to published SG transcriptomics results [see (C)–(F) below and Supplementary Material, Dataset S1]. **(B)** A comparison of the NSAF scores in WT versus FUS R495X samples over three independent BONLAC experiments. **(C)** Venn diagram depicting overlap of transcripts identified by BONLAC and transcripts identified as depleted in the stress granule transcriptome ($P = 5.44 \times 10^{-10}$). **(D)** Venn diagram depicting overlap of transcripts identified by BONLAC and transcripts identified as enriched in the stress granule transcriptome ($P = 0.9998$). **(E)** Boxplot of NSAF scores for corresponding transcripts that are depleted, neither depleted nor enriched or enriched in SGs. **(F)** Scatter plot of NSAF scores versus SG enrichment shows a moderate inverse correlation ($R = -0.45$) between proteins that are synthesized under stress and the localization of the corresponding transcript within SGs.

For the BONLAC experiments, FUS WT and R495X cells were grown in medium- and heavy-isotope-labeled media for two biological replicates (Fig. 1A), respectively, and the SILAC labeling was reversed for the third. To ensure that only proteins synthesized under translationally repressive stress conditions were labeled, FUS WT and R495X cells were exposed to arsenite stress for 35 min prior to the labeling period, allowing for G3BP-positive stress granule assembly (Fig. 1B; Supplementary Material, Fig. S1B) and eIF2 α phosphorylation to occur (Fig. 1C). There did not appear to be any difference in the levels of eIF2 α phosphorylation between FUS WT and R495X cells under these conditions. Protein translation was substantially repressed as evidenced by reduced L-azidohomoalanine (AHA, a methionine analogue)

incorporation (Fig. 1D). To maximize the duration of the labeling period (100 min) without compromising cell health, relatively low levels of sodium arsenite (SA; 0.20 mM) were used here. The MTT cell viability assay confirmed that cells remained viable over the time course of the arsenite exposure (Supplementary Material, Fig. S1E). During the labeling period, cells were cultured under stress with both AHA and isotopically labeled amino acids for BONCAT and SILAC, respectively (Fig. 1A). Consequently, AHA (Supplementary Material, Fig. S1F) and either medium or heavy isotopically labeled amino acids were incorporated into proteins that were translated under stress. AHA-containing proteins were enriched through ‘click-chemistry’ and proteolyzed (into peptides) for subsequent tandem MS analysis (Fig. 1A) (18).

Table 1. Proteins exhibiting the most robust synthesis under stress-induced translational repression

Gene	Alternate gene name	Protein name/description	NSAF score
H4	HIST1H4A	Histone H4	1.214
PPIA	PPIA	Peptidyl-prolyl cis-trans isomerase A	0.770
H2B1L	HIST1H2BL	Histone H2B type 1-L	0.698
GRP78	HSPA5	78 kDa glucose-regulated protein	0.509
MYL6	MYL6	Isoform smooth muscle of myosin light polypeptide 6	0.424
ATPB	ATP5B	ATP synthase subunit beta, mitochondrial	0.382
VIME	VIM	Vimentin	0.373
RL27	RPL27	60S ribosomal protein L27	0.360
DDX5	DDX5	Probable ATP-dependent RNA helicase DDX5	0.350
ROA2	HNRNPA2B1	hnRNPs A2/B1	0.334
RL30	RPL30	60S ribosomal protein L30	0.235
GRP75	HSPA9	Stress-70 protein, mitochondrial	0.222
RS3A	RPS3A	40S ribosomal protein S3a	0.217
CH10	HSPE1	10 kDa heat shock protein, mitochondrial	0.216
ATPA	ATP5A1	ATP synthase subunit alpha, mitochondrial	0.210
SMD3	SNRPD3	Isoform 2 of small nuclear ribonucleoprotein Sm D3	0.208
J3QRS3	MYL12A	Myosin regulatory light chain 12A	0.198
KAP0	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit	0.192
TCPE	CCT5	T-complex protein 1 subunit epsilon	0.190
S10AA	S100A10	Protein S100-A10	0.186
RS14	RPS14	40S ribosomal protein S14	0.179
CH60	HSPD1	60 kDa heat shock protein, mitochondrial	0.178
RS5	RPS5	40S ribosomal protein S5	0.176
IF5A1	EIF5A	Eukaryotic translation initiation factor 5A-1	0.173
TCPG	CCT3	T-complex protein 1 subunit gamma	0.165
COF1	CFL1	Cofilin-1	0.163
HSP7C	HSPA8	Heat shock cognate 71 kDa protein	0.161
ANXA2	ANXA2	Annexin A2	0.153
NDKB	NME2	Isoform 3 of nucleoside diphosphate kinase B	0.150
TCPH	CCT7	T-complex protein 1 subunit eta	0.149
TCPQ	CCT8	T-complex protein 1 subunit theta	0.147
MDHM	MDH2	Malate dehydrogenase, mitochondrial	0.145
RS3	RPS3	40S ribosomal protein S3	0.144
ANXA1	ANXA1	Annexin A1	0.142
HMGB1	HMGB1	High mobility group protein B1	0.140
ROA1	HNRNPA1	Isoform A1-A of hnRNP A1	0.137
RS25	RPS25	40S ribosomal protein S25	0.136
HNRPK	HNRNPK	Isoform 3 of hnRNP K	0.134
RS10	RPS10	40S ribosomal protein S10	0.133
RS24	RPS24	Isoform 2 of 40S ribosomal protein S24	0.131
HS90B	HSP90AB1	Heat shock protein HSP 90-beta	0.131
TERA	VCP	Transitional ER ATPase	0.130
SPRC	SPARC	SPARC	0.129
TCPB	CCT2	T-complex protein 1 subunit beta	0.125
DPYL3	DPYSL3	Isoform LCRMP-4 of Dihydropyrimidinase-related protein 3	0.123
SKP1	SKP1	S-phase kinase-associated protein 1	0.123
RSSA	RPSA	40S ribosomal protein SA	0.122
NPM	NPM1	Isoform 2 of Nucleophosmin	0.121
RSMB	SNRPB	Isoform SM-B of Small nuclear ribonucleoprotein-associated proteins B and B'	0.120
TAGL2	TAGLN2	Isoform 2 of Transgelin-2	0.118

Proteins are a subset (top 50) of [Supplementary Material, Dataset S1](#), ranked according to their NSAF scores (highest to lowest). Gene, alternative gene and protein names are shown.

Proteins were considered as synthesized under stress if the corresponding isotopically labeled peptides were reproducibly detected in two of three BONLAC experiments, within either FUS WT or R495X cells. There were 555 proteins that met these criteria. Of these 555 proteins, 362 were detected in both cell lines, 74 were overrepresented (found in two of three BONLAC experiments) in FUS WT samples but not FUS R495X samples and 119 were overrepresented in FUS R495X samples ([Fig. 2A](#); [Supplementary Material, Dataset S1](#)). We reasoned that the

362 proteins synthesized in both FUS WT and R495X cells, independent of FUS genotype, were the most likely to participate in the 'normal' translational response to stress and hence the most informative for the identification of pathways that either mitigate or exacerbate stress. These 362 proteins were ranked according to the sum of their normalized spectral abundance factor (NSAF) across all six conditions (three biological experiments, each including FUS WT and R495X cells under stress). NSAF is a measure of the total spectral

Figure 3. Newly synthesized COPBI protein is reduced in mutant FUS expressing cells under stress. (A, B) Western blot analysis of immunoprecipitated COPBI from arsenite stressed SK-N-AS FUS WT and R495X cells. (A) Input lanes show similar levels of COPBI protein in cell lysates, whereas AHA-labeled COPBI protein is below the limit of detection in the whole cell lysate (the shadow of a band at the top of the image corresponds to a protein of higher molecular weight than COPBI) (left). Anti-COPBI antibody (top) confirms that similar levels of COPBI were isolated from the FUS WT and R495X lysates (right). Immunoblotting the same blot with a streptavidin secondary antibody against biotin-conjugated AHA-labeled proteins (bottom) shows this band contains less newly synthesized COPBI protein in FUS R495X cells under arsenite (SA) stress. (B) Quantification of (A), $n = 3$ independent biological experiments (Student's *t*-test, $^{**}P = 0.002$). (C, D) Western analysis of neurons derived from transgenic FUS R495X (RX) mice and NTG littermates. (C) Representative Western blot with anti-COPBI and anti-TUBB3 (Tuj1; loading control) antibodies. (D) Quantification of (C), 3 biological experiments. (E–G) Western analysis of other COPI subunits. (E) Representative Western blot of NTG and FUS R495X neurons with anti-COPA, COPGI and Tuj1 antibodies. (F, G) Quantification of (E), 3 or 4 biological experiments for (F) and (G), respectively. (H) Quantitative PCR analysis of COPBI mRNA levels in NTG and FUS R495X neurons (4 biological experiments). (D, F–H) Data were analyzed by two-way ANOVA with Tukey post hoc testing for multiple comparisons, and error bars reflect standard error of the mean. Statistically significant comparisons are represented by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. All significant comparisons are shown.

counts derived from the MS2 data for a protein normalized to the number of amino acids in that protein. This normalization factor takes into account that larger proteins will naturally produce a greater number of spectral counts (44). As such, we considered proteins with high cumulative NSAF scores as robustly and reproducibly synthesized under stress. The cumulative NSAF scores for proteins detected in the FUS WT and mutant samples were highly correlative (Fig. 2B), indicating that global protein synthesis under stress was similar in WT and mutant FUS expressing cells. The top 50 NSAF ranked proteins from this analysis are shown in Table 1. Consistent with the expected effects of arsenite stress, the top proteins include the ER chaperone immunoglobulin heavy chain-binding protein (BiP), a member of the heat-shock protein-70 family encoded by GRP78 that plays a role in the unfolded protein response and the protein-folding enzyme cyclophilin A encoded by PPIA. Histone and cytoskeletal proteins also have high cumulative NSAF scores, implicating factors involved with transcription and transport, respectively, in the cellular stress response.

Neurodegenerative-disease-associated proteins are actively translated under repressive stress conditions

Next, this list of 362 proteins was analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (45,46). As FUS underwent doxycycline-induced expression, it was excluded from the DAVID analysis. Most of the statistically significant terms were related to mRNA processing and protein synthesis and turnover (Supplementary Material, Dataset S2). As expected, 'stress response' ($P = 0.02$, Bonferroni corrected) was also represented by the proteins in this list. Somewhat unexpectedly, statistically significant keywords also included 'disease mutation' ($P = 1.73 \times 10^{-5}$), 'neurodegeneration' ($P = 8.42 \times 10^{-5}$) and 'ALS' ($P = 0.043$) (Supplementary Material, Dataset S2). The extent of these enrichments may have been underestimated, since Ewing's sarcoma breakpoint region 1 (EWSR1), heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 and cyclophilin A have been linked to ALS and related disorders (47–49) but were not yet annotated as such by DAVID. Ranked by highest to lowest cumulative NSAF score, ALS-linked proteins translated under stress include: hnRNP A1, valosin-containing protein (VCP), profilin 1, TAR DNA-binding protein 43 (TDP-43), EWS, Sequestosome-1 and matrin-3 (MATR3) (48,50). A Western analysis was performed for several proteins with high cumulative NSAF scores and/or an association with ALS, including BiP, ATP synthase subunit beta (ATP5B), TDP-43 and EWS (Supplementary Material, Fig. S2). A significant increase in steady-state protein levels under stress was detected only for EWS (Supplementary Material, Fig. S2D). This is likely because proteins could not accumulate to levels detectable by Western within the 100 min labeling period (Fig. 1A), and/or that protein expression was not upregulated, per se, but rather expression was maintained. TDP-43 steady-state levels were significantly decreased under stress (Supplementary Material, Fig. S2C), consistent with the metastable nature of this protein (51) and reports that TDP-43 partitions into the insoluble fraction under stress (52,53). That TDP-43 is actively translated under repressive stress conditions (Supplementary Material, Datasets 1 and 2) and yet is known to partition into the insoluble fraction may be relevant to neurodegenerative disease pathogenesis, as TDP-43 aggregation is detected within the central nervous system (CNS) in the vast majority of ALS cases as well as in other neurodegenerative disorders (54).

Figure 4. COPBI dispersion and Golgi fragmentation in mutant FUS expressing primary neurons with and without stress. (A, B) Representative immunofluorescence micrographs of NTG control and FUS R495X (RX)-expressing neurons probed with antibodies against the stress granule marker TIAR, FUS and COPBI, and counterstained with DAPI to highlight the nucleus. For clarity, TIAR, FUS and COPBI signals are shown individually in black and white micrographs (left). Pseudo-colored images of FUS (red), COPBI (green) and DAPI (blue) overlaid at low and high magnification highlight COPBI dispersion in unstressed R495X neurons, as well as in stressed NTG and R495X neurons. Dashed boxes denote cells that are shown at high magnification (right). Low magnification scale bar, 20µm; high magnification scale bar, 5µm. (C) Significantly more M511Nfs neurons exhibited a disperse COPBI phenotype [as exemplified in (A) and (B)] than M511Nfs*Cor neurons in the absence of stress (* $P < 0.05$ by two-way ANOVA with Tukey post hoc testing). Additional significant comparisons include all unstressed versus all stressed conditions (not shown for clarity). (D,E) Representative Western blot and quantification of FUS levels in M511Nfs*Cor and M511Nfs MNs from three independent differentiations. (* $P < 0.05$ by Student's *t*-test).

Genes translated during stress correspond to transcripts that are depleted from SGs

We also considered whether there is a relationship between proteins that are synthesized under stress and the localization of the corresponding transcripts within SGs. Transcripts with poor translation efficiency have previously been reported to localize to SGs (30,55). However, whether genes that are depleted from SGs are actually translated during stress remained unknown.

Here, we compared NSAF values for the list of 362 proteins that are synthesized under stress to the previously identified SG transcriptome, which was determined from SGs formed in response to arsenite stress (30). Of the 362 proteins, 328 were also detected at sufficient levels (>1 fragments per kilobase of transcript per million; FPKM) in the SG transcriptome (Supplementary Material, Datasets S1). Many translated genes ($n = 94$) were identified as depleted transcripts in the SG transcriptome ($P = 5.44 \times 10^{-10}$; Fig. 2C). Conversely, fewer translated genes

Figure 5. Enhanced COPBI dispersion is detected in human iPSC-derived neurons expressing endogenous levels of ALS-linked FUS. (A, B) Representative immunofluorescence micrographs of iPSC-derived neurons expressing the ALS-linked FUS M511 fs variant or derived from the CRISPR-Cas9 corrected line (M511 fs*cor). All neurons are TUJ-1 positive (not shown). Neurons were probed with antibodies against FUS and COPBI, and counterstained with DAPI to highlight the nucleus. FUS and COPBI signals are shown individually in black and white micrographs (left). Pseudo-colored images of FUS (red), COPBI (green) and DAPI (blue) overlaid at low and high magnification are shown at the right. Dashed boxes denote cells that are shown at high magnification (right). Low magnification scale bar, 15 μ m; high magnification scale bar, 5 μ m. C. Significantly more R495X neurons exhibited a disperse COPBI phenotype [as exemplified in (A) and (B)] than NTG neurons in the absence of stress (** $P < 0.01$ by two-way ANOVA with Tukey post hoc testing). Additional significant comparisons include all unstressed versus all stressed conditions (not shown for clarity). (D) Representative immunofluorescence micrographs for unstressed and stressed neurons probed with anti-COPBI (green) and anti-GM130 (red). Scale bar, 10 μ m. (E) Representative Western blot and quantification of FUS levels in M511Nfs*Cor and M511Nfs MNs from three independent differentiations (* $P < 0.05$ by Student's t-test).

($n = 28$) overlap with transcripts that are enriched in SGs ($P = 0.9998$; Fig. 2D). Further, a moderate correlation between SG-depleted transcripts and higher NSAF scores was observed (Fig. 2E and F). We also uncovered an inverse correlation between NSAF score and transcript length (Supplementary Material, Fig. S3A), consistent with a prior finding that SG-depleted transcripts tend to be smaller (30). Proteins with high NSAF

scores do not simply correspond to transcripts with inherently high translation efficiency (Supplementary Material, Fig. S3B) (55), suggesting there is some degree of selectivity with regard to their expression under stress. Taken together, transcripts that are depleted from SGs are actively translated during stress, consistent with the model that SGs are assemblies of translationally repressed mRNA granules.

Figure 6. Golgi to ER transport is delayed in mutant FUS expressing cells. (A) Representative immunofluorescence micrographs of FUS WT and R495X SK-N-AS cells transfected with the retrograde transport reporter, VSVGts045-KDEL-R-YFP, at various time points throughout the transport assay. Cells were probed with anti-GFP (green) and anti-GM130 (red) antibodies, to amplify the YFP signal and highlight the Golgi, respectively. Note the high degree of overlap between the GFP/YFP and Golgi signals at the start of the assays and still at 20 min for FUS R495X cells. Scale bar, 12 μ m. (B) The percent (%) co-localization between GM130 and GFP/YFP (VSVG) was quantified for 3 biological experiments, revealing a statistically significant difference between FUS WT and R495X cells at the 20 min time point (** $P < 0.01$ by two-way ANOVA with Tukey post hoc testing). Data was collected at either 40 or 45 min; data were grouped for this time point, as the results were similar. Additional significant comparisons were uncovered between the 0, 20 and 40/45 min time points but were not labeled for simplicity.

Reduced expression of COPBI in mutant FUS-expressing cells under stress

In addition to assessing global protein synthesis using NSAF scores as described above (Table 1; Supplementary Material, Dataset S1), SILAC ratio quantitation is a second type of analysis that can be performed with data from the BONLAC experiment (18). SILAC ratio quantitation is used to compare peptide abundances between two samples (18) and was used here to compare peptide (corresponding to newly synthesized protein) abundances between FUS WT and R495X cells under stress. Briefly, the extracted ion chromatogram (obtained from the MS1 scan) for each detectable isotopically labeled peptide was compared between FUS R495X and WT cells; a peptide must be present in both WT and mutant FUS samples in order for the protein to be quantified (18). This analysis did not reveal statistically significant differential expression between FUS WT and R495X cells. This was likely a consequence of the acute (100 min labeling period) translational repression induced by SA, which resulted in peptides that were at or below the signal-to-noise threshold for the mass spectrometer. In other words, many newly synthesized proteins were present in low abundance, which did not preclude their identification (i.e. there was sufficient information to confidently assign protein identifications, as discussed above), but limited the SILAC ratio quantitation. As an alternative approach, we considered proteins that were differentially expressed between FUS WT and R495X cells in all three BONLAC experiments and focused on those that changed by at least 1.5-fold in at least two experiments. The rationale for this approach is that a 1.5-fold change in expression can generally be validated by other biochemical methods (56), and ≥ 1.5 -fold differences in expression are more likely to be biologically relevant. Using this criterion, five proteins consistently exhibiting reduced expression in FUS R495X compared to FUS WT samples were identified: the DNA-directed RNA polymerase II subunit RPB1, ornithine aminotransferase, coatomer subunit beta, the 40S ribosomal protein S16 and spermidine synthase (Supplementary Material, Dataset S3).

In light of evidence linking loss of COPI function with neurodegeneration (32,33,35,37–39,57), we focused our validation efforts on the coatomer beta subunit (COPBI or β -COP) protein, which is a component of the COPI complex. The COPI complex functions in retrograde vesicular trafficking between the Golgi apparatus and ER (31). Differences in COPBI levels were not apparent at steady state (Supplementary Material, Fig. S4A and B). To enhance detection of newly synthesized COPBI, COPBI was immunoprecipitated from AHA-labeled and arsenite-stressed cells expressing either FUS R495X or WT (29), and simultaneously probed by Western blot analysis with antibodies against AHA and COPBI. This approach revealed significantly less newly synthesized COPBI in stressed FUS R495X cells compared to FUS WT (Fig. 3AB). Differences in newly synthesized COPBI protein were dependent on stress, as this experiment in the absence of stress revealed the same amount of newly synthesized COPBI in both lines (Supplementary Material, Fig. S4C and D).

We then sought to determine whether COPBI was affected by mutant FUS in *bona fide* neurons. To this end, COPBI levels were quantified in primary cortical neurons derived from transgenic mice expressing the human FUS ALS-linked R495X transgene (58). A human-specific FUS antibody was used to confirm FUS R495X transgene expression (59). Despite exogenous expression of the R495X transgene, total levels of FUS were within approximately 1.5-fold between mutant and control neurons (Supplementary Material, Fig. S5). As for SK-N-AS cells, a significant reduction of COPBI expression was detected in FUS R495X neurons compared to neurons from non-transgenic (NTG) littermates under stress (0.5 mM SA for 2 h); in the absence of stress COPBI levels were the same between genotypes (Fig. 3C and D). In contrast to SK-N-AS cells, significant differences in COPBI levels were discernible at steady state in neurons, suggesting a more pronounced effect of mutant FUS on COPBI in *bona fide* neurons than in immortalized cells. We also considered whether other components of COPI were reduced in stressed neurons, as the COPI complex is comprised of seven subunits within the B-subcomplex (α -, β' - and ϵ -COP) and F-subcomplex (β -, γ -, δ - and ζ -COP) (31); altered expression of

one subunit could affect the stability and expression of other subunits. As observed for COPBI, COPA levels were significantly reduced in stressed FUS R495X neurons (Fig. 3E and F). Interestingly, dysregulation of COPA is also linked to the juvenile motor neuron disease, spinal muscular atrophy (SMA) (38). Levels of COPGI were likewise reduced in stressed FUS R495X neurons; however, this change did not reach statistical significance (Fig. 3E and G).

Next, we investigated the mechanism for differential COPBI expression in cells expressing mutant FUS compared to controls. First, we considered whether mutant FUS affected the localization of COPBI in SGs and found no difference between control and FUS R495X neurons with respect to COPBI-transcript within SGs (Supplementary Material, Fig. S4E), although we cannot exclude the possibility that COPBI mRNA is processed differently in mutant FUS-containing SGs. COPBI mRNA was also assessed by quantitative real-time PCR (qPCR). Whereas differences in COPBI mRNA levels were not observed in SK-N-AS lines (Supplementary Material, Fig. S4F), COPBI mRNA was significantly lower in stressed FUS R495X neurons relative to unstressed NTG neurons (Fig. 3H). A trend toward reduced COPBI mRNA for the other conditions without a concomitant reduction in steady-state protein (Fig. 3D) is noted; however, these differences were not statistically significant. Taken together, reduced COPBI mRNA likely contributes to the reduced levels of the translated protein in FUS R495X neurons under stress.

Expression of mutant FUS induces Golgi fragmentation and delayed vesicular transport

Having established that mutant FUS leads to reduced expression of COPBI under stress, we probed for concomitant changes to COPBI localization and/or Golgi morphology using immunofluorescence microscopy. In unstressed SK-N-AS cells, immunostaining with the Golgi marker, GM130, revealed the Golgi as a relatively compact, juxtanuclear assembly. As expected, COPBI and GM130 signals largely co-localized (31,60) in both FUS WT and R495X cells (Supplementary Material, Fig. S6A). Upon exposure to SA, there was a striking dispersion of both COPBI and GM130 signals in agreement with the recent report by Catara et al. (61). Dispersion or fragmentation of Golgi-associated factors occurs in response to various types of stress and may represent a cellular mechanism for transducing stress signals to the nucleus (62). Despite this stress-induced dispersion, COPBI and GM130 signals remained largely co-localized with each other (Supplementary Material, Fig. S6B and C), whereas COPBI signals did not appear to co-localize with SGs (Supplementary Material, Fig. S6D).

In the absence of stress, there appeared to be more mutant FUS-expressing SK-N-AS cells with dispersed COPBI than WT cells, although this difference did not reach statistical significance (Supplementary Material, Fig. S6B and C). Therefore, we assessed whether the same phenotype could be observed in primary neurons. Compared to SK-N-AS cells, the COPBI staining pattern was more heterogeneous in neurons under unstressed conditions (Fig. 4A). For example, the COPBI signal was (i) compact, juxtanuclear and to one side of the nucleus (similar to SK-N-AS cells); (ii) compact and juxtanuclear but spread around the perimeter of the nucleus; or (iii) less compact and extended into the neurites. Despite this heterogeneity, the dispersed COPBI pattern in unstressed R495X expressing cells was obvious and was present in twice as many FUS R495X neurons compared to

controls (Fig. 4A and C). Again, COPBI dispersion was present in virtually all arsenite-treated neurons, regardless of genotype (Fig. 4B–D). As in SK-N-AS cells, mutant-FUS positive SGs did not co-localize with COPBI (Fig. 4B), and there was a high degree of overlap between COPBI and GM130 signals (Fig. 4D).

To further interrogate the effects of mutant FUS on COPBI localization, we assessed the COPBI dispersion phenotype in human iPSC-derived neurons that were generated using a protocol for motor neuron differentiation (Supplementary Material, Fig. S7). A patient iPSC line harboring a heterozygous frame-shift mutation corresponding to amino acid 511 in FUS (M511Nfs) was corrected with CRISPR-Cas9, thereby generating an isogenic control line (M511Nfs*cor) (63). The sequence of M511Nf is changed at position M511 to DGFQG which effectively eliminates the FUS NLS. As expected, FUS M511Nfs mislocalizes to the cytoplasm in untreated cells (Fig. 5A). Higher magnification, single-plane immunofluorescence images further demonstrate that mutant FUS mislocalizes to the cytoplasm and throughout the neurites of iPSC-derived neurons (Supplementary Material, Fig. S7D and E). Also as expected, mutant FUS assembles into granules in arsenite-treated neurons (Fig. 5B). A significantly higher percentage of TUJ1-positive neurons exhibited dispersed COPBI signal in unstressed mutant FUS neurons compared to neurons derived from the isogenic control (Fig. 5C). Exposure to SA produced this phenotype in virtually all cells (Fig. 5C). It is also noted that the endogenous levels of FUS are higher in M511Nfs cultures relative to M511Nfs*cor (Fig. 5D and E), consistent with higher levels of mutant FUS expression in our stable SK-N-AS lines (Supplementary Material, Fig. S1C and D) and within patient derived CNS tissues (41).

These observations prompted us to assess whether COPI function was also affected by mutant FUS expression. To this end, we transfected the thermoreversible folding mutant of the vesicular stomatitis virus G-protein (VSVG) fused to the KDEL receptor (KDELr) into our SK-N-AS lines. The KDELr constitutively cycles between the Golgi and ER, retrieving escaped ER-proteins and transporting them from the Golgi back to the ER (65). This assay is an established method for assessing Golgi to ER transport (35,65). An anti-GFP antibody was used to amplify the YFP signal for the analysis, revealing that VSVGts045-KDELr-YFP predominately co-localized with the Golgi marker GM130 in both FUS WT and R495X lines at the permissive temperature of 32°C (Fig. 6A and B). A temperature shift to 40°C causes VSVGts045 to become misfolded and retained within the ER, resulting in less Golgi-localized VSVGts045 (35,65). Indeed, by ~40 min, very little VSVGts045-KDELr-YFP co-localized with GM130 in both lines (WT: 5.0%; RX: 4.1%; Fig. 6B). As expected, the equilibrium of VSVGts045-KDELr-YFP shifted away from the Golgi by 20 min in FUS WT cells (65), as evidenced by reduced GM130/VSVG co-localization (28.5%; Fig. 6B). In contrast, there was a significant lag in FUS R495X cells, in which more VSVG/GM130 remained co-localized (42.6%; Fig. 6B) after 20 min. This lag in VSVGts045-KDELr-YFP transport away from the Golgi supports the premise that mutant FUS affects COPBI-related functions in the context of vesicular transport. It is noted that SGs were not expected to affect this process, as this assay is carried out in the presence of cycloheximide, which inhibits SG formation. Indeed, <5% of cells from this assay exhibited SGs as assessed by G3BP staining (not shown). It is noted that toxicity associated with transfection of the VSVGts045-KDELr-YFP construct in neurons precluded the trafficking studies in those cells.

Discussion

Protein translation is one of the highest energy-consuming processes in the cell. Under conditions of stress, translation is repressed and resources are redirected to mitigate stress as the cell works to re-establish homeostasis (1). Various types of stress including arsenite, oxidative, hyperosmolar and ER stress have been shown to repress global translation through signaling events that induce phosphorylation, and thus inhibition, of the translation initiation factor 2 (eIF2 α) (1,2). While short-term or moderate degrees of translational repression are protective, chronic translational repression is pathogenic (66). For example, multiple models of neurodegenerative disease exhibit heightened activation of the integrated stress response (ISR), which in turn causes phosphorylated eIF2 α (P-eIF2 α)-dependent translational repression (13,67–70). Small molecules that target the ISR and alleviate translational repression also ameliorate disease-related phenotypes *in vivo* (67,68,70). Therefore, elucidating the factors that modulate translational repression could have a significant impact on therapeutic development for neurodegenerative diseases.

During stress-induced translational repression, polysomes disassemble and components of the translation machinery accumulate within SGs (16). SGs are membraneless organelles formed through weak non-covalent interactions that drive LLPS. An unbiased transcriptomics analysis of cells exposed to arsenite stress revealed that transcripts with poor translation efficiency localize to SGs (30,55). By comparing the NSAF scores from the current study to this previously identified SG transcriptome (30), we uncovered a significant correlation between proteins that are robustly synthesized (i.e. high NSAF scores) and a depletion of the corresponding transcripts from SGs (Fig. 2). These results are consistent with a model whereby gene expression under stress is influenced by the association (or lack thereof) of transcripts with SGs. Based on evidence that FUS binds thousands of RNAs (71–73), and that mutant FUS alters the properties of SGs (7,12) as well as other assemblies formed via LLPS (74,75), we hypothesized that the association of mutant FUS with SGs could, in turn, alter gene expression. The current BONLAC/MS study was designed to address this possibility by directly comparing the actively translated proteomes in mutant versus WT FUS expressing cells under stress. Our results indicate that ALS-linked FUS has a targeted, rather than a global, effect on protein synthesis under stress. For example, proteins that were considered robustly synthesized and present at relatively high abundance (i.e. proteins with high NSAF scores; Table 1) were unaffected by mutant FUS expression. Further, the COPBI transcript did not appear enriched within mutant FUS-containing SGs (Supplementary Material, Fig. S4E), arguing against a mechanism whereby mutant FUS sequesters and represses COPBI within SGs. However, we suspect the effect of mutant FUS on gene expression could be underestimated in our BONLAC study, as there were subsets of proteins that were enriched in either FUS WT or R495X (Fig. 2A) but could not be quantified because the corresponding peptides were not present in both samples. Moreover, reduced expression of COPA was detected in primary neurons (Fig. 3E and F), whereas differential expression of COPA had not been reproducibly detected by BONLAC in SK-N-AS cells. This outcome could have been due to differences in neurons versus immortalized cells and may also reflect limitations of the BONLAC method. These limitations could be mitigated by using models of chronic stress, potentially *in vivo* (67), thus allowing for differentially expressed proteins to accumulate to high enough levels for comparative quantitation.

The pathways and processes that must remain active during stress for cell survival have not been fully defined. Despite the aforementioned limitations of the SILAC ratio quantitation (for assessing differences between mutant and WT FUS cells), BONLAC/MS very reliably identified 362 proteins that were actively synthesized under conditions of stress-induced translational repression, irrespective of FUS genotype. An advantage of a SILAC/MS approach is that it directly detects the actual translated protein, as opposed to an indirect assessment based on ribosome density (55,76). Our study detected active synthesis of the expected chaperones and folding enzymes under conditions of stress (Table 1) (1,76). Rather unexpectedly, we also uncovered the active synthesis of proteins associated with neurodegeneration, including ALS, under stress. We speculate that these proteins have a functional role in stress response, justifying their production during a period of extreme energy conservation (1). Indeed, TDP-43 has been implicated in the cellular stress response (77,78) and was actively synthesized here under arsenite stress. However, levels of soluble TDP-43 were also significantly reduced, consistent with the metastable nature of TDP-43 (51–53) and prior observations that newly synthesized proteins are especially prone to misfolding and degradation (79,80). These metastable proteins may contribute to a vicious cycle of chronic translational repression and pathological protein accumulation that characterize neurodegenerative disorders (51,67). At this time, it is not clear whether these proteins are upregulated or if their synthesis is maintained, under stress. Because of the vast difference in protein synthesis under homeostatic versus translational repressive conditions (Fig. 1D), our BONLAC protocol will require optimization for direct comparison of unstressed and stressed conditions (i.e. signals generated in the unstressed condition can overwhelm signals from the stressed condition in the mass spectrometer). We note that other studies have uncovered effects of mutant FUS on translation in the absence of externally applied stress (29,69,74,81).

Of significance, we uncovered a novel relationship between mutant FUS and COPBI. COPBI levels were consistently lower in stressed cells expressing mutant FUS (Fig. 3). Neither WT nor mutant FUS has been found to directly bind COPBI mRNA (72,73,82,83), although FUS expression influences COPBI splicing (83). Therefore, it is conceivable that mutant FUS affects COPBI splicing under stress, culminating in reduced COPBI mRNA levels in mutant-FUS expressing primary neurons (Fig. 3H). We noticed that COPBI mRNA was reduced in control neurons under stress and R495X neurons without stress, although these differences did not reach statistical significance (Fig. 3D). Possibly, neurons are able to compensate for lower levels of COPBI transcript under these conditions by stabilizing the COPBI protein and/or increasing translation of the transcript, but such compensatory mechanisms are compromised in FUS R495X neurons under stress. Alternatively, mutant FUS expression could expedite the turnover of COPBI protein. While the exact mechanism for mutant FUS-induced dysregulation of COPBI has yet to be defined, these initial findings provide new and potentially important insights into the role of the Golgi to ER retrograde transport pathway under stress and during disease. That COPBI and other COPI subunits are actively translated under stress implies a role for retrograde transport in cellular stress response. A possible role may be to accommodate increased numbers of misfolded proteins escaping the ER during stress. Although COPI and Golgi to ER retrograde transport were not implicated in ALS before this study, this pathway has been linked to several other forms of neurodegeneration, including the juvenile-onset motor neuron disease SMA (33,35,38,57), spinal cerebellar

neurodegeneration (32,37) and Purkinje cell degeneration (39). In the case of SMA, survival motor neuron protein binds COPA (38). COPA overexpression rescues SMA-induced phenotypes (35), while knockdown of COPA induces SMA-relevant phenotypes such as reduced neurite outgrowth of motor neurons (35). Our study revealed that COPBI and COPA were both significantly reduced in mutant-FUS expressing neurons under conditions of stress (Fig. 3). Further investigation showed that COPBI and the Golgi signals become dispersed upon expression of mutant FUS, even in the absence of stress (Figs 4 and 5). The reason for this morphological change is unclear, but may indicate that expression of mutant FUS is sufficient to induce some degree of cellular stress. In fact, others have hypothesized that the Golgi is part of an ISR pathway. For instance, the Golgi may function in both sensing and transducing stress signals to other organelles, including the nucleus, in a manner that could ultimately favor apoptosis if the stress is not alleviated (36,62). Indeed, Golgi fragmentation has also been observed in cells exposed to DNA-damaging agents (84) and within post-mortem CNS tissues from both animal models and humans with neurodegenerative disease (34,36). Our observations that ALS-linked FUS effects COPBI and the Golgi both in the absence and presence of stress reinforce the vulnerability of the retrograde transport to factors that drive neurodegenerative disease pathogenesis.

Materials and Methods

Cloning and cell line construction

The pLenti CMV-TO-Puro DEST constructs containing full-length untagged human FUS WT was created as follows: PCR amplified full-length FUS WT flanked by KpnI and XhoI sites (3' and 5', respectively) was cloned into the pENTR4 no ccDB (686-1) vector (Addgene, 17424) at the aforementioned sites using standard sub-cloning protocols to generate the plasmid pENTR4-untagged FUS WT. This construct was then cloned into the expression vector pLenti CMV/TO Puro DEST (670-1) (Addgene, 17293) using Gateway cloning with LR Clonase II (Invitrogen, 11791-100) according to the manufacturer's instructions, and the QuikChange II Mutagenesis kit (Stratagene; 200523) was used to convert FUS WT to FUS R495X. SK-N-AS cells stably expressing the tetracycline repressor (TetR) were created with pLenti CMV-TetR Blast (716-1) (Addgene, 17492) and were then transduced with pLenti-CMV-TO-Puro DEST containing FUS WT or R495X. Stable, inducible SK-N-AS TetR WT and R495X lines were selected for with 0.5 μ g/ml Puromycin over 2 weeks.

Generation of FUS isogenic iPSC lines

The FUS M511Nfs iPSC line was described previously (63). To correct the mutation, CRISPR guides were designed using the Zhang Lab CRISPR Design Tool (crispr.mit.edu). Guides were selected that had high predicted activity and had off-targets with at least 3 bp mismatches in coding regions and at least 2 bp mismatches in non-coding regions. Guides were cloned into pSpCas9n(BB)-2A-GFP (pX461, Addgene #48140) using standard protocols. A 200 bp single-stranded oligodeoxynucleotide with the WT sequence was used to correct the FUS mutation. The transfection was performed using the NEON Transfection System (Invitrogen) using a single 1400 V, 20 ms pulse. Cells were plated in mTeSR1 with 10 μ M ROCK inhibitor (DMSO International). Forty-eight hours later, cells were sorted on a BD FACS Aria SORP and the top 25% GFP-positive cells were collected and plated for expansion. Single-cell clones were then expanded for

PCR genotyping. The presence of the mutation in the mutant line and the CRISPR/Cas9-induced correction were confirmed by Sanger sequencing.

Drug treatments

The following stocks were prepared and stored as indicated: 50 mg/ml doxycycline (Sigma, D9891) in water (-80°C), 1 M SA (Sigma, 71287) in water (-20°C), 3 mM cytosine β -D-arabinofuranoside hydrochloride (AraC, Millipore Sigma, C6645), 500 mM L-Azidohomoalanine (AHA; Invitrogen, C10102) in DMSO (-20°C) and 100 mg/ml cycloheximide (Sigma, C7698) in Methanol (-20°C). All dilutions were made with culture media.

Cell culture

SK-N-AS TetR WT and R495X lines were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, 11965118) supplemented with 10% (v/v) heat inactivated tetracycline-tested fetal bovine serum (Millipore Sigma, F6178), 2 mM L-glutamine (Gibco, 25030) and 1% (v/v) penicillin and streptomycin solution (Gibco, 15140), 15 μ g/ml Blasticidin (Cellgro, 30-100-RB) and 0.5 μ g/ml puromycin (Invitrogen, A11138-03). Cells were passaged into culture dishes and allowed to adhere for 18–24 h, after which exogenous FUS expression was induced with 1 μ g/ml doxycycline for 24 h for all experiments unless stated otherwise.

Primary cortical neuron cultures were prepared from transgenic human FUS R495X mice (58) and NTG littermates as follows. Cortices from embryonic day (E)14–15 embryos were isolated and meninges removed in ice-cold Hanks buffered saline solution (MediaTech, 21-023-CV). Individual embryos were kept separated on ice in HibernateE solution (BrainBits, HE-Lf) until FUS genotyping was verified (within 3 hrs). Tail or hind leg samples from each embryo were collected for genotyping with the KAPA Mouse genotyping kit according to the manufacturer's instructions (KAPA Biosystems, KK7352). Embryos with the same genotype were pooled and cells were dissociated for 12 min in 0.05% Trypsin (Invitrogen, 25300-054) at 37°C . Cells were diluted in Dulbecco's modified Eagle medium (Invitrogen, 11965118) containing 10% fetal bovine serum (Millipore Sigma, F4135) and passed through a 40 μ m nylon cell strainer (Falcon, 352340) before pelleting at 1 K rpm for 5 min. Cells were resuspended in Neurobasal media (Invitrogen, 21103049) supplemented with 1% glutamax (Invitrogen, 35050-061), 1% pen-strep (Invitrogen, 15140122) and 2% B27 (Invitrogen, 0080085-SA) and plated at 2×10^5 cells/ml on poly-ornithine (Millipore Sigma, P4957) coated plates or coverslips. Neurons were maintained under standard culture conditions (37°C , 5% CO_2) and fed every 3–4 days. Cultures were treated with 0.5 μ M AraC at day in vitro (DIV) 2–3 to prevent the growth of non-neuronal cells. All experiments using primary neurons herein were performed at DIV8.

iPSCs were maintained and differentiated into motor neurons using a similar protocol as (85). This protocol produces 40–60% Islet1/2-positive neurons and 80–90% TUJ1-positive cells. The normal karyotype and purity of all iPSC lines used herein were confirmed (Cell Line Genetics). The differentiation protocol is illustrated in [Supplementary Material, Fig. S7A](#). Specifically, iPSCs were maintained in Matrigel (Corning, CB-40230) coated 6-well plates in mTeSR1 media (StemCell Technologies, 85850) and passaged every 4–6 days with Accutase (Corning, MT25058CI) following manufacturer's instruction. Pluripotency markers OCT4 and SOX2 were examined by immunofluorescence staining to verify stem cell identity.

Partially and spontaneously differentiated colonies were manually removed prior differentiations. Confluent iPSCs were dissociated to single cells with 0.5 mM EDTA and transferred to suspension flasks at 150 000 cells/cm² density in day 0 differentiation medium containing basal medium [1:1 neurobasal medium (Invitrogen, 21103049): DMEM/F12 medium (Corning, MT-15-090-CV), B27 (Invitrogen, 17504044)/ N2 (Invitrogen, 17502-048) supplements, glutamax (Invitrogen, 35050-061) and 20 µM ascorbic acid (Sigma, A4403)]; 10 µM ROCK inhibitor (Y-27632; BD Bioscience, BDB562822); dual Smad inhibitors: 10 µM SB431521 (Tocris, 1614), 3 µM CHIR99021 (Sigma, SML1047), 100 nM LDN193189 (Sigma, SML0559). To initiate neural induction and spinal motor neuron patterning during a 15-day differentiation process, small molecule cocktails as follows were supplemented to the basal medium. Day 2: dual Smad inhibitors; 1 µM retinoic acid (RA, Sigma, R2625). Day 4: dual Smad inhibitors; RA; 1 µM Smoothed agonist (SAG, EMD Millipore, 566661). Day 7: RA; SAG. Day 9: RA; SAG; 10 µM DAPT (Tocris, 2634). Day 11: RA; SAG; DAPT; 10 ng/ml BDNF (Peprotech, 450-02). Day 13: RA; SAG; DAPT; BDNF; 10 ng/ml GDNF (Peprotech, 450-10). On day 15, EBs were dissociated with activated papain (5 units/ml; Worthington, LS003126) in the presence of 10 µg/ml DNase I (Worthington, LS006355). Undissociated EBs and clumps were filtered with cell strainer before centrifugation. Cells were spun down at 700 rpm for 4 min and resuspended in motor neuron medium containing neurobasal medium, B27, glutamax, ascorbic acid, 10 ng/ml each BDNF, GDNF, IGF-1 (Peprotech, 100-11), CNTF (Peprotech, 450-13) and 1 µg/ml laminin (Invitrogen, 23017-015). Cells were plated at 150 000/cm² density on plates or coverslips pre-coated with 25 µg/ml poly-D-Lysine (Sigma, P7405), 25 µg/ml poly-ornithine (Sigma, P3655), 3–5 µg/ml laminin and 5 µg/ml fibronectin (Sigma, F1141). Cultures were maintained in motor neuron medium for weeks and media was half-changed every 3 days. Cultures were treated with 1 µM AraC at DIV 7 to prevent the growth of non-neuronal cells only for neurons used for Western blot analysis of FUS.

Unless stated otherwise, SK-N-AS cells and neurons were treated with 0.2 mM and 0.5 mM SA, respectively, for 2 hrs at 37°C to induce arsenite stress and translational repression. The viability of SK-N-AS cells exposed to arsenite stress was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) reagent (Invitrogen, M-6494) as described (56).

Western blotting

Western blot analyses were performed as described (7) except in some cases stain-free gels (Biorad, 1610182) or silver staining (Biorad 161-0449) was used as noted according to the manufacturer's instructions to assess protein loading. Primary antibodies were used as follows: 1:1000 for mouse anti-Tubulin (Sigma, T9026), 1:1000 for rabbit anti-FUS (7), 1:2000 rabbit anti-eIF2α (Cell Signaling, 9722S), 1:1000 rabbit anti-phos-eIF2α (Cell Signaling, 9721S), 1:2000 mouse anti-GAPDH (Sigma, G8795), 1:500 for mouse anti-EWS (Santa Cruz sc-28327), 1:1000 for rabbit anti-Bip (Abcam Ab21685), 1:1000 for mouse anti-ATP5B (Abcam Ab14730), 1:1000–1:3000 for rabbit anti-COPB1 (Genetex GTX22899), 1:500 for mouse anti-COPA (Santa Cruz sc-398099), 1:1000 for rabbit anti-COPG1 (Proteintech 12393-1-AP), 1:1000 for mouse anti-TDP43 (Encor MCA-3H8) and 1:5000 for mouse anti-Tuj1 (TUBB3) (Biolegend, 801201). Blots were incubated with primary antibodies overnight at 4°C and secondary antibodies were used as described (7). For blots with AHA-labeled proteins modified with biotin, streptavidin IR800 secondary (Licor 926-

32230) was used at 1:5000 for 2 h at ambient temperature. Blots were visualized with an Odyssey Infrared Imager (LiCor, Model 9120) and densitometry measurements performed with the Odyssey Software (LiCor, V3.0).

Immunofluorescence microscopy and analyses

Immunofluorescence was performed as described (7). Primary antibodies were used as follows: 1:200 for mouse anti-FUS [Santa Cruz Biotechnology, sc-373698 (H6)], 1:500 for rabbit anti-FUS (Bethyl lab, A300-293A), 1:500–1:1000 for rabbit anti-COPB1 (Genetex, GTX22899), 1:500 for goat anti-TIAR (Santa Cruz Biotechnology, sc-1749), 1:1000 rabbit anti-GFP conjugated to Alex Fluor-488 (Invitrogen A-21311), 1:500 mouse anti-GM130 (BD Transduction labs, 610822) and 1:1000 for rabbit anti-G3BP (Proteintech, 130-57-2AP). For iPSCs and neurons differentiated from these cells, the following additional antibodies were used: 1:500 rabbit anti-FUS (Proteintech, 11570-1-AP), 1:1000 mouse anti-Sox2 (R&D Systems, MAB2018-SP), 1:1000 mouse anti-Oct4 (R&D Systems, MAB17591-SP) and 1:500 mouse anti-Islet1/2 (Developmental Studies Hybridoma Bank, 39.4D5) SK-N-AS and neurons were exposed to primary antibody for 1 and 3 h at ambient temperature, respectively. Secondary antibodies were diluted 1:500–1:1000 in PBSAT (1X PBS/ 1% BSA/ 0.1% Tween-20) and incubated for 1 h at ambient temperature. Secondary antibodies from Jackson ImmunoResearch Labs were used as described (7). Cy5-conjugated Tuj1 (Biolegend, 657405) was applied to neurons at 1:400 for 1 h at ambient temperature following secondary antibody incubations. Cells were stained with 34 ng/ml DAPI in distilled water, and coverslips were mounted with ProLong Gold anti-fade reagent (Invitrogen, P36930).

All fluorescence images were collected with a Leica DMI 6000B inverted fluorescent microscope with a PL FL L 40x/0.60, APO 63x/1.40 or APO 100x/1.30 objective and a Leica DFC365 FX camera using AF6000 Leica Software v3.1.0 (Leica Microsystems). Stacked images were acquired at 63× or 100× with a 0.25 µm step size and, unless otherwise noted, presented as a maximum projection. Maximum projected images were used for all of the quantitative analyses. Within an individual experiment, all images were acquired using the identical microscope settings. In some cases, images were adjusted post-acquisition between conditions but were never adjusted between control and FUS R495X cells within the same treatment condition. The DAPI channel was adjusted as necessary to observe the nucleus. To assess COPB1 dispersion, COPB1 signal was qualitatively assigned as disperse or compact in a blinded manner. Between 35 and 50 cells were assessed per line and per condition for three (SK-N-AS) or four (primary neurons) independent biological experiments. The co-localization module in Metamorph software version 7.6.3 (Molecular devices) was used for all co-localization analyses. Cell boundaries were defined by anti-TIAR immunofluorescence using the conditions described above. Co-localization was reported as an average of 35–50 cells per experiment, for three independent biological experiments. Dead cells or cells with pyknotic nuclei were excluded from our quantitative analyses.

Quantitative PCR analysis

RNA was extracted with the Aurum Total RNA mini kit (Biorad, 732-6820) for SK-N-AS cells and Trizol (Invitrogen, 15596026) for primary neurons according to the manufacturer's instructions. RNA concentrations were determined with a Nanodrop (Thermo Fisher Scientific). One microgram of RNA per sample

was converted to cDNA using iScript Reverse Transcription Supermix (Biorad, 170-8840) according to the manufacturer's instructions. PCR reactions contained 50 ng RNA, SYBR probes (Biorad) for COPB1 and B2M and 5× iTaq Universal SYBR Green Supermix (Biorad, 172-5120). The Ribosomal 18S SYBR and FUS probes were also used for SK-N-AS cells. A CFX384 Touch Real-Time PCR Detection System (Biorad) was set to the following program: 95°C for 2 min, 40 cycles of 95°C for 5 s and 60°C for 30 s and a melt curve of 65–95°C (0.5°C increments per 5 s). Differential RNA expression was calculated by the comparative Ct method using Bio-Rad CFX Manager 3.1 software with sample threshold cycle (Ct) values normalized to 18S and/or B2M reference gene Ct values. Samples were run in triplicate per experiment, and qPCR data is reported as an average of at least three independent biological experiments.

BONLAC and BONCAT analyses

The BONLAC study was carried out using the SK-N-AS TetR WT and R495X lines described above using a modified version of the protocol established by Zhang *et al.* (18). Briefly, cells were starved in -Met, -Arg, -Lys media for 40 min, followed by a 35 min incubation in starvation media +0.2 mM SA to induce arsenite stress. Cells were cultured for another 100 min, referred to as the 'labeling period', in the same media supplemented with 2 mM AHA + 4 mM Arg and 8 mM Lys medium or heavy isotopically labeled amino acids (Athena Enzyme Systems, 0440-, 0441-, 0442- and 0443-100). FUS WT cells were grown in light-isotope labeled media and FUS R495X cells in heavy-isotope labeled media for two biological replicates, and the SILAC labeling was reversed for the third. Cells were washed and collected in 1X PBS, then 5% of the sample was pelleted and lysed in nuclear lysis buffer (7) for total protein determination by the bicinchoninic acid assay (Thermo Scientific Pierce, 23277). The remaining cell suspension was processed for labeled protein isolation on Click-it resin (Invitrogen C10416) according to the manufacturer's instructions, except the reaction volumes and reagents were increased 3-fold to facilitate capture of labeled proteins; proteins synthesized under stress that contained the AHA label were subjected to copper catalyzed cycloaddition click chemistry with an alkyne-conjugated resin. For each BONLAC experiment, 800–1000 g each of SK-N-AS FUS WT and R495X cell lysate in 2.4 ml was incubated with the Click-it resin (Invitrogen, C10416) for 20 h. The clicked reaction was applied to desalting columns (Grace Davison Discovery Science, 255100) and subjected to trypsin (Promega, V5113) digestion for 12–18 h at 37°C. Peptides were eluted from the resin and dried with a Speed Vac (Savant Instruments).

In preparation for mass spectrometry, peptides were reconstituted in 20 µl of 5% acetonitrile, 0.1% (v/v) trifluoroacetic acid. Samples were analyzed on a Waters nanoAcquity UPLC configured to a Thermo Scientific Q Exactive quadrupole-Orbitrap hybrid mass spectrometer. In brief, technical replicates of 4.5 µl each were directly loaded at 4.0 µl/min onto a fused-silica precolumn (100 µm ID) with Kasil frit custom packed with 2 cm of 5 µm, 100 Å C18AQ particles (Michrom Bioresources). Peptides were separated and eluted from a fused-silica analytical column (75 µm ID) custom packed with 25 cm of 3 µm, 100 Å C18AQ particles (Michrom Bioresources) terminating to a pulled-tip emitter. Electrospray ionization voltage (1.4 kV) was applied using a zero dead volume internal electrode junction located between the precolumn and analytical column. Peptides were eluted at 300 µl/min using a linear gradient from 95% solvent A (0.1% formic acid in water) to 35% solvent B (0.1% formic acid

in acetonitrile) over 150 min. Data-dependent acquisition was performed where each full MS scan (300–1750 *m/z*) was acquired at a resolution setting of 70 000 followed by 10 MS/MS scans using higher-energy collisional dissociation (27% NCE) at a resolution of 17 500 and using an isolation width of 1.6 Da. Other acquisition parameters were utilized to minimize data redundancy and maximize peptide identifications as described (86). Raw data files were processed with Proteome Discoverer (Thermo, version 1.4) prior to searching with Mascot Server (version 2.5) against the Uniprot_Human database. Search parameters utilized were fully tryptic with up to two missed cleavages with parent mass tolerances of 10 ppm and fragment mass tolerances of 0.05 Da. A fixed modification of carbamidomethyl cysteine and variable modifications of N-terminal acetylation, N-terminal pyroglutamination, methionine oxidation and AHA-alkyne of methionine were considered. SILAC labels of lysine ($^{13}\text{C}_6$; $^{13}\text{C}_6^{15}\text{N}_2$) and arginine ($^{13}\text{C}_6$; $^{13}\text{C}_6^{15}\text{N}_4$) were used to determine relative protein abundance changes after determining precursor extracted ion chromatogram (XIC) peak areas using Proteome Discoverer. Both identification and quantitative data from Mascot server and Proteome Discoverer were loaded into the Scaffold Viewer (Proteome Software; version 4.5.3) for assessment of protein identification probabilities and display of SILAC ratio.

Peptide identifications were accepted with greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction (87). Protein identifications were accepted if they were established at greater than 99.9% probability and contained at least two unique identified peptides with at least one having isotope incorporation; isotope incorporation was used to confirm the protein was synthesized under stress during the labeling period. For each BONLAC experiment, this led to a false discovery rate of 0.4–0.9%. Protein probabilities were assigned by the Protein Prophet algorithm (88). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were ranked according to their NSAF (44) values. NSAF is a measure of the total spectral counts derived from the MS2 data for a protein normalized to the number of amino acids in that protein; this normalization factor takes into account that larger proteins will naturally produce a greater number of spectral counts (44).

SILAC ratio quantitation was accomplished using the Q+ feature of the Scaffold Viewer (Proteome Software, Inc; Version 4.8.3), wherein the appropriate labels (Heavy or Medium) were assigned to either the FUS WT or R495X sample. Specific settings for the quantitation included the use of non-exclusive peptides, a blocking level of unique peptides, no normalization between samples and no spectrum quality filter. T-tests were used for statistical analysis of the relative peptide intensities between mutant FUS and WT FUS samples (i.e. the extracted ion chromatogram obtained from the MS1 scan for each detectable isotopically labeled peptide was compared between FUS R495X and WT samples).

Gene set enrichment analyses were carried out using DAVID Bioinformatics Resources v6.8 (45,46) using the default settings and criteria described in the text.

BONCAT was performed for COPBI immunoprecipitation analysis as follows. AHA-labeled SK-N-AS WT and R495X cell lysates were prepared with and without arsenite stress as described above for the mass spectrometry BONLAC study (except isotopically labeled amino acids were excluded) and lysed in Click lysis buffer (50 mM Tris-HCl/ 1% SDS w/v). Lysate (750 µg/ml) was applied to Sure beads (Biorad, 1614013) that had

been charged with 5–5.5 µg anti-COPB1 (Bethyl Labs, A304-724A) in 200 µl PBS-T for 12–18 h at 4°C. After sufficient washing, the sample was eluted from the beads by applying 60 µl of Click lysis buffer for 15 min at 70°C with intermittent vortexing. The eluted sample was clicked to biotin (Invitrogen, B10185) using a protein Click-it kit (Invitrogen, C10276) according to the manufacturer's instructions and subjected to Western blot analysis as described above.

The statistical significance of overlap between genes identified by BONLAC and genes depleted or enriched in SGs that were identified by Khong *et al.* (30) was calculated using a two-population proportion test in R (alternative = 'greater'), thereby giving the probability that one would observe the given amount of overlap, or more, by chance. All scatter plots and R-values were created using Tableau v. 10.0. In the case of Figure 2F and Supplementary Material, Fig. S3B the data were fit to an exponential function. Box plots were created in GraphPad Prism and significance was computed using a Student's t-test.

Fluorescence in situ hybridization

Primary neurons were prepared for fluorescence in situ hybridization (FISH) by washing with 1X RNase-free PBS and fixing with fresh 4% paraformaldehyde (Fisher Scientific, F79-500) diluted with RNase-free water (Corning, 46-000-CM) for 30 min at ambient temperature. FISH was accomplished using the QuantiGene ViewRNA ISH Cell Assay Kit (Thermo Fisher Scientific, QVC0001) according to the manufacturer's instructions except cells were dehydrated after fixation with 2 min incubations in 50%, 70% and 100% ethanol at ambient temperature followed by a second addition of 100% ethanol and then stored at –20°C for 5 days before processing. The Cy3-labeled COPB1 probe was designed by Thermo Fisher Scientific (Probe ID: VB1-3037421-VC). Post-FISH immunofluorescence was accomplished using the protocol and microscope described above. For the microscopy and image analysis, the experimental cover slips were thresholded to the negative controls (cells lacking the probe or treated with RNase). The remaining COPB1 mRNA signals were enlarged by 2-fold using the dilate morphology filter in Metamorph software version 7.6.3 (Molecular Devices) to facilitate visualization. Cells with COPB1 mRNA co-localized with G3BP-positive SGs were reported as a percentage of cells containing COPB1 mRNA. Co-localization data from 25–65 cells per line was collected for two independent biological experiments.

VSVG trafficking assay

The VSVGts045-KDEL-R-YFP plasmid used to assess retrograde transport away from the Golgi was generously provided by Drs Elliot Androphy and Sara Custer (Indiana University School of Medicine). VSVGts045-KDEL-R-YFP was transiently transfected into SK-N-AS TetR WT and R495X cells using Lipofectamine 2000 (Invitrogen, 11668019) following the manufacturer's instructions. Cells were immediately placed at 32°C and exogenous FUS expression was induced 5 h later. Twenty hours thereafter, cells were treated with 20 µg/ml cyclohexamide, and the temperature was increased to 40°C (65). Coverslips were fixed with 4% PFA at various time points for the immunofluorescence procedure described above, where cells were labeled with GM130 to visualize the Golgi and GFP to enhance the YFP signal. For the microscopy and image analysis, VSVG-transfected cells with moderate YFP/GFP expression were identified; the YFP/GFP signal was used to define a region around the cell. The

percentage of GM130 signal that was co-localized with YFP/GFP was calculated using the co-localization module in Metamorph software version 7.6.3 (Molecular devices) for 20–30 cells per experiment, for three independent biological experiments.

Statistical analyses

Data presented herein were graphed and analyzed using GraphPad Prism version 7.00 (GraphPad Software, La Jolla California). Statistical tests and results are stated in the figure legends.

Supplementary Material

Supplementary Material is available at HMG online.

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