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Pur-alpha regulates cytoplasmic stress granule dynamics and ameliorates FUS toxicity

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Abstract

Amyotrophic lateral Sclerosis is characterized by progressive loss of motor neurons in the brain and spinal cord. Mutations in several genes, including FUS, TDP43, Matrin 3, hnRNPA2 and other RNA binding proteins, have been linked to ALS pathology. Recently, Pur-alpha a DNA/RNA binding protein was found to bind to C9orf72 repeat expansions and could possibly play a role in the pathogenesis of ALS. When overexpressed, Pur-alpha mitigates toxicities associated with Fragile X tumor ataxia syndrome (FXTAS) and C9orf72 repeat expansion diseases in Drosophila and mammalian cell culture models. However, the function of Pur-alpha in regulating ALS pathogenesis has not been fully understood. We identified Pur-alpha as a novel component of cytoplasmic stress granules (SGs) in ALS patient cells carrying disease-causing mutations in FUS. When cells were challenged with stress, we observed that Pur-alpha co-localized with mutant FUS in ALS patient cells and became trapped in constitutive SGs. We also found that FUS physically interacted with Pur-alpha in mammalian neuronal cells. Interestingly, shRNA mediated knock down of endogenous Pur-alpha significantly reduced formation of

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cytoplasmic stress granules in mammalian cells suggesting that Pur-alpha is essential for the formation of SGs. Furthermore, ectopic expression of Pur-alpha blocked cytoplasmic mislocalization of mutant FUS and strongly suppressed toxicity associated with mutant FUS expression in primary motor neurons. Our data emphasizes the importance of stress granules in ALS pathogenesis and identifies Pur-alpha as a novel regulator of SG dynamics.

Keywords

ALS; FUS; TDP-43; Pur-alpha; Stress granules; RNA binding proteins; primary motor neurons; Motor neuron diseases; neurodegeneration; C9orf72; amyotrophic lateral sclerosis

Introduction

RNA-binding proteins have been implicated in several neurodegenerative diseases, such as polyglutamine expansion diseases and amyotrophic lateral sclerosis (ALS) [9, 22, 51, 52, 64]. Over the last decade, TDP-43, FUS, Matrin-3, VCP and several other RNA-binding proteins have been found to be linked with causing ALS pathogenesis suggesting that disease-causing mutations perturbing RNA metabolism might be central to causing ALS pathogenesis [26, 51, 52, 64, 68]. This is supported by recent studies showing that ALS-causing mutations in RNA binding proteins cause defects in RNA splicing, stability, transcription, mRNA processing, translation and transport, which may lead to gross functional impairments in several key biological pathways [3, 5–7, 10, 18–23, 25, 30, 31, 33, 34, 60, 61, 63, 65, 66].

Several RNA-binding proteins harboring a prion-like domain have been identified as components of cytoplasmic stress granules (SGs), which are ribonucleoprotein granules (RNP granules) [16, 46, 67, 71]. SGs are highly dynamic cytoplasmic structures known to be involved in regulating RNA homeostasis [59]. SGs form rapidly when cells are exposed to stress-causing agents such as heat, cold, infection or chemicals; these SGs protect cells by sequestering actively translated mRNA and proteins until stressful conditions are relieved. A majority of the RNA-binding proteins involved in ALS pathogenesis are sequestered into SGs, suggesting that these cytoplasmic structures are relevant to ALS [13, 16, 27, 28, 31, 32, 34, 35, 44, 67, 71, 73]. SGs are rapidly formed when cells are challenged with stress-causing agents and they rapidly disassemble when stressful conditions end. ALS-causing mutations in FUS impair SG dynamics by perturbing their assembly and disassembly processes. Similarly, disease-causing mutations in valosin-containing protein (VCP) produce constitutive SGs containing another ALS-causing protein, TDP-43 [16]. Interestingly, knocking down VCP impairs the ability of cells to turnover SGs without affecting their assembly [16]. RNA-binding proteins involved in ALS pathogenesis are important building blocks of SGs under physiological conditions. Pathogenic mutations in proteins such as TDP-43 and FUS perturb SG dynamics, driving misregulation of multiple cellular pathways involved in regulating neuronal function and survival. Despite tremendous progress towards identifying ALS-causing genes and their roles in disease pathogenesis, components involved in regulating SG assembly and disassembly are still largely unknown.

Here, we identified Pur-alpha as a novel component of cytoplasmic stress granules in ALS patient cells carrying disease-causing mutations in FUS. We found that Pur-alpha is sequestered into cytoplasmic stress granules along with mutant FUS and observed that Pur-alpha physically interacts with FUS in mammalian cells. Pur-alpha is a sequence-specific single-stranded DNA- and RNA-binding protein whose sequence element strongly resembles the C9ORF72 hexamer [12]. Pur-alpha deficiency has been linked to disorders in brain development [42]. Surprisingly, we discovered that Pur-alpha is required for the formation of SGs, as knocking down endogenous Pur-alpha prevents formation of SGs in mammalian cells. We were able to restore SG formation by exogenously expressing full length Pur-alpha in cells expressing shRNA against Pur-alpha. Importantly, knocking down Pur-alpha neither perturbed p-body formation nor influenced expression of FUS or G3BP (a component of stress granules). Furthermore, we demonstrate that ectopic expression of Pur-alpha blocks cytoplasmic mislocalization of FUS carrying a disease-causing mutation and suppresses FUS-medicated toxicity in primary motor neurons These observations uncover the role of Pur-alpha in regulating cytoplasmic SG dynamics and cytoplasmic mislocalization and toxicity in FUS-related neurodegeneration.

Materials and Methods

Culturing human lymphoblastoid cells

Human B-lymphoblastoid cells were obtained from Coriell Institute cell repository. ALS patient cells with defined endogenous FUS mutations R521C (ND14790) and R518G (ND14136) were utilized. Age and sex matched population control (ND00066) were used for comparison. All lymphoblastiod cell lines were cultured in suspensions with Advanced RPMI 1640 medium (Life Technologies 12633-012) containing 10% FBS and 1% Glutamax (Life Technologies 35050-061). Cell densities and passage number were constants. Lymphoblastoid cell cultures were not allowed to reach densities below 2×10^6 cells/ml.

Immunofluorescence (IF)

Following fixation with 4% paraformaldehyde, samples were permeabilized with 0.1% Triton X-100 in PBS. Samples were blocked with 0.1% PBST + 5% normal goat serum (NGS). Antibody dilutions were made in blocking solution (5% NGS in 0.1% PBST) and incubated overnight at 4°C on a rocker. After 12 hours of incubation with the primary antibody solution a series of washes was performed with 0.1% PBST (5x 10 minutes each). Secondary antibodies were diluted in blocking solution and incubated with samples at room temperature for 3 hours. Antibodies used were: anti-G3BP mouse (Becton Dickinson catalogue #611126), anti-G3BP rabbit (Proteintech #13057-2-AP) (1:500), anti-Pur-alpha mouse [41] (1:750), anti-GW182, and anti-FUS N-terminal (Bethyl laboratories #A300-302A). As secondary antibodies, we used anti-rabbit AlexaFluor 488 (Invitrogen #A11008), anti-mouse AlexaFluor 488 (Invitrogen # A11029), anti-rabbit AlexaFluor 546 (Invitrogen #A11035), anti-mouse AlexaFluor 546 (Invitrogen #A11030) and anti-rabbit AlexaFluor 647 (Invitrogen #21245), all at a dilution of 1:500. After the staining procedure was complete, DAPI was included in the final wash for 15 minutes. Finally, coverslips were mounted with ProLong Gold (Life Technologies #P36930) and allowed to cure overnight in the dark.

Plasmids

WT FUS, FUS R518K and FUS R521C plasmid constructs are described previously [27, 55, 70]. Since these disease-causing mutations in FUS cause ALS via a similar mechanism i.e. disruption of C-terminal nuclear localization signals (NLS), we used these mutations interchangeably in our manuscript. pBK-CMV HA-Pur-alpha was generated as described [41]. For shRNA knockdown of endogenous human Pur-alpha, shRNA pGFP-C-shLenti (4 unique 29mer target-specific shRNAs) and scrambled shRNA control (Origene TL310036V) were used. For direct visualization of Pur-alpha in unfixed primary motor neuron cultures, we generated an mCherry-tagged Pur-alpha using pmCherry-C1 (Clontech) as a backbone for subcloning Pur-alpha into XhoI and EcoRI sites. To induce expression of FUS using doxycycline, we used the TET-inducible system. The FUS constructs epB-TT-RFP-FLAG-FUS WT and epB-TT-GFP-FLAG-FUS R521C were kindly provided by Dr. Alessandro Rosa.

Generation of deletion constructs

Using the pBK-CMV HA-Pur-alpha WT plasmid as a template, we generated gBlocks Gene Fragments (Integrated DNA Technologies) with flanking unique restriction enzymes sites surrounding the sequence we would use to ligate a new portion of the gene with a sequence missing at either the C-terminal end or N-terminal end. Constructs were analyzed with diagnostic digests, then sequenced in both forward and reverse directions to verify that gBlock fragments containing the deleted portion of the gene were integrated in frame.

Western Blotting

Total lysates were generated from 35,000 cells, denatured using β-mercaptoethanol and Laemmli sample loading buffer. Proteins were separated on Nupage 4–12% PAGE gels (Life Technologies) and were transferred to nitrocellulose membranes using the semidry iBlot transfer system. Membranes were labeled with primary antibodies overnight at room temperature. For visualization, membranes were labeled with LI-COR fluorescent secondary antibodies (Dylight 680 and Dylight 800). The LI-COR Odyssey fluorescence scanner was used to capture images of membranes.

Quantitative PCR

Total RNA was extracted with Trizol (Invitrogen). Total RNA (2 μg) was reverse transcribed using the SuperScript III First-Strand Synthesis System kit (Invitrogen). Gene expression was measured by quantitative real-time PCR using the 7900 HT Fast Real-Time PCR System (Applied Biosystems). The level of each transcript was measured with the threshold cycle (Ct) method. Values were normalized to the mean GAPDH levels.

Assessment of Neuronal Viability

Neuronal viability was examined by MAP-2 immunofluorescence. Degenerated/dead neurons show a striking loss of MAP-2. Motor neuron-enriched cultures were prepared from E13.5 Sprague Dawley rat embryos (Charles River Laboratories) and transfected using Lipofectamine 2000 (Life Technologies) on day 7 of culture. Cell cultures were transfected in duplicate with a) GFP, b) WT FUS-GFP, c) WT FUS-GFP +Pur-alpha-mCherry, d)

R521G FUS-GFP, e) R521G FUS-GFP + Pur-alpha-mCherry, and f) Pur-alpha-mCherry. At 72–90 hours post-transfection, cells were fixed with 4% paraformaldehyde in sucrose in PBS for 20 min at room temperature. Cells were labeled with anti-MAP2 antibody (1:500, Millipore) and AlexaFluor 647 goat anti-rabbit secondary antibody (1:500, Life Technologies). GFP and mCherry fluorescence were visualized using FITC and TRITC channels, whereas MAP-2 was visualized in the far red channel. Z stacks of images were captured sequentially to eliminate bleed-through of the fluorophores. Loss of MAP-2 fluorescence was taken as a measure of cell death. Neuronal viability was expressed as a percentage of MAP-2 positive cells. Statistical analysis was performed using Student's t-test.

Propidium iodide (PI) staining

To examine FUS mediated neuronal death in the presence/absence of Pur-alpha overexpression, motor neurons were transfected as before. 96 hours post transfection cells were processed for immunocytochemistry to label GFP (1:1000, Millipore), HA (1:500, Abcam) and MAP-2 (1:1000, Millipore). Before mounting the coverslips with antifade, cells were exposed to 10 μM propidium iodide (Molecular Probes, Eugene, OR, USA) for 20 min. The PI enters dead cells with loss of membrane integrity and exhibits a red fluorescence (maximum emission at 620 nm). The number of dead cells was measured as percentage of propidium iodide positive cells to total number of transfected neurons identified by MAP-2. Student T test was performed between groups. Error bars represent ± SEM (**P<0.01).

TUNEL assay

Primary neuronal cells were transfected on DIV 9 with mCherry tagged FUS (FUS-WT and FUS-R521C) and either GFP tagged Pur-alpha shRNA1 construct or control shRNA construct using lipofectamine 2000 (Invitrogen). 750 ng of each plasmid constructs were used for all the experiments. 96 hours post transfection, neuronal viability was determined using Click-iT TUNEL Alexa Fluor 647 Imaging assay according to the manufacturer's instructions (Molecular Probes). Cells were fixed in 4% v/v paraformaldehyde/sucrose for 15 min, permeabilized with 0.25% triton 100 for 10 minutes, and then labeled by TUNEL and counterstained with DAPI. Cells were visualized using Olympus FV1000 microscope (Olympus). The fraction of TUNEL-positive cells as a percentage of total number of transfected neurons was determined for each condition. A minimum of 30 neurons was scored for each condition and data presented as mean and standard error from two independent experiments.

Stress granule induction and quantification

To induce stress granules, cells were treated with 0.5 mM sodium arsenite (Ricca Chem Co #7140-16) in DMEM for 1 hour 30 minutes at 37°C. Immunofluorescence was performed as previously described using a SG marker anti-G3BP antibody. Images were taken on an Olympus FluorView1000 microscope using a 60X objective. Stacks of images were captured from three different fields for each experimental group. Maximum projections were created using ImageJ and were then coded. All images were given to an unbiased analyst who was instructed to count distinct structures using the ImageJ cell counter. Prior to starting the quantification of SGs, the average size of G3BP-positive SGs was determined.

G3BP-positive granules within two SD of the mean size of puncta were included in the analysis. For each field of view, the number of DAPI-positive cells was quantitated as a total number of cells. Next, the analyst was told to count the number of cells containing >1 G3BP-positive puncta (n=~300 cells/group). The number of cells containing SGs was divided by the total number of cells to give a percentage of cells containing SGs. For the GFP-shRNA Pur-alpha groups, one of the inclusion criteria was that the cell expressed GFP. Statistical analysis was performed on three independent quantifications per group.

Primary neurons 9 DIV (Days in vitro) were transfected with FUS-R521G GFP alone or with Pur-alpha mCherry using lipofectamine 2000.48 hours post transfection cells were treated with 0.5 mM sodium arsenite for 150 minutes at 37°C. Cells were then processed for immunocytochemistry and labeled with antibodies for GFP (1:1000, Millipore), stress granule marker (SG) G3BP1 (1:500, Proteintech). Cells were counterstained with DAPI and were visualized using Alexa Fluor 647 tagged secondary antibody. All Images were captured using Olympus FV 1000 (60X oil immersion objective and 2.5 optical zoom) confocal microscope. SG number was quantified using Image J (Version 1.50b) after background correction. Data are represented as total number of G3BP1 positive SGs per cell and differences were analyzed by Student's t test.

Results

Pur-alpha is a component of cytoplasmic stress granules (SGs)

Given the protective role of Pur-alpha in neurodegenerative diseases such as FXTAS and C9orf72 expanded repeat ALS, we examined the role of Pur-alpha in FUS-related neurodegeneration. We determined the subcellular distribution of Pur-alpha in FUS-related neurodegeneration under native and stress conditions. We utilized human lymphoblastoid cell lines from ALS patients carrying disease-causing mutations in FUS and age/sex matched control lines obtained from the Coriell Institute. Under native conditions, Pur-alpha is diffusely distributed in the cytoplasm and the nucleus (Figure 1a). However, when cells were challenged with sodium arsenite, we found that Pur-alpha localized with the cytoplasmic stress granule marker G3BP, suggesting that Pur-alpha is a component of SGs (Figure 1a). We also examined the subcellular distribution of Pur-alpha in ALS patient cells carrying the disease-causing mutation FUS R518G and found that Pur-alpha was localized to SGs under stress conditions. We quantified the number of cells showing SGs in control and ALS patient cells (Figure 1b). We found that ALS patient cells carrying disease-causing mutations (FUS R518G or FUS R521C) contained significantly more SGs than control cells (Figure 1b), indicating that pathological mutations in FUS caused an increase the number of cytoplasmic SGs. Furthermore, we quantified the size of SGs in control cells as well as two ALS patients' cells but we did not see any significant difference in the size of SGs between the FUS R521C and controls. However, we found a significant difference in the SG-size in the FUS 518G as compared to controls (Figure 1c). We quantified the percentage of cells containing SGs (G3BP-positive) under stress or native conditions. We found that FUS R518G-expressing cells had equal number of Pur-alpha positive SGs as compared to controls suggesting, that Pur-alpha incorporates into SGs equally in FUS R518G expressing cells and controls (Figure S1a). Interestingly, we observed that FUS R518G- or R521C-

expressing cells showed significantly higher number of FUS-positive SGs than controls (Figure S1b). Given that FUS carrying ALS-linked mutations (FUS R518G or R521C) become mislocalized to the cytoplasm, it was not surprising to observe significantly more FUS-positive SGs in ALS-patient cells than control cells.

We also validated our findings in a mammalian neuroblastoma cell line (N2a) and in primary motor neurons overexpressing tagged Pur-alpha, and found that Pur-alpha co-localized with endogenous G3BP under stress conditions (Figure S2 and S3). These observations suggest that Pur-alpha is a novel component of SGs in ALS patient cells carrying disease-causing mutations as well as in age/sex matched controls, mammalian neuronal cells (N2a) and mammalian primary motor neurons. Next, we asked if Pur-alpha co-localization with SGs is specific to sodium arsenite treatment or whether Pur-alpha is a component of SGs under other forms of stress. In addition to sodium arsenite treatment, we used two alternate forms of stress-causing agents heat (42°C) and hydrogen peroxide (H_2O_2). We found that Pur-alpha also co-localized with SGs under these stress conditions (Figure S4 and S5). In general, we observed that heat stress was a more robust inducer of SGs, producing more Pur-alpha positive SGs than H_2O_2 stress. Altogether, these observations suggest that Pur-alpha is an integral component of SGs under three independent forms of stress.

Pur-alpha is sequestered with mutant FUS in ALS patient cells carrying disease-causing FUS mutations and becomes trapped in constitutive SGs

It has been recently shown that ALS-causing mutations in FUS impair SG dynamics by both delaying their assembly and disrupting their turnover [8, 59, 69]. These observations are further supported by the fact that under pathological conditions stalled SGs trap important proteins and mRNAs essential for performing cellular functions, which in turn might lead to cellular dysfunction and degeneration [16]. To determine if Pur-alpha is sequestered with FUS-positive puncta in the cytoplasm under stress conditions, we challenged ALS patient cells expressing FUS R521C and FUS R518G, as well as cells from age/sex matched controls, with sodium arsenite and examined the subcellular distribution of FUS and Pur-alpha proteins. As shown previously, WT FUS is predominantly nuclear whereas FUS carrying ALS-causing mutations is distributed in both the cytoplasm and nucleus [50, 72]. We found that under stress conditions, Pur-alpha co-localizes with FUS in ALS patient cells carrying pathogenic mutations (Figure 2). In control cells, we occasionally observed a small fraction of WT FUS in the cytoplasm; however, we did not find WT FUS localizing with Pur-alpha in the cytoplasm under stressed or unstressed conditions. In contrast to WT FUS, we found that FUS R521C and FUS R518G were frequently incorporated into cytoplasmic SGs in ALS patient cells carrying disease-causing mutations (Figure 2). Furthermore, we found that Pur-alpha and FUS are both components of SGs in mammalian neuronal cells (N2a) expressing FUS R521C under stress conditions (Figure S6). Interestingly, SGs containing Pur-alpha and FUS do not disassemble at the same rate as in control cells when stress conditions are relieved (Figure S15). These observations suggest that Pur-alpha and FUS are trapped in constitutive SGs under pathological conditions and that they are not released after the stress conditions end.

Pur-alpha physically interacts with FUS in mammalian neuronal cells

To further examine the relationship between FUS and Pur-alpha, we asked whether these two RNA binding proteins physically interact. We transfected stable mammalian neuroblastoma (N2a) cell lines expressing FLAG-FUS (WT or R521C) with a vector expressing wild type Pur-alpha fused to an HA tag on the amino-terminal portion (HA-Pur-alpha) (Figure 3). FUS and Pur-alpha interaction was analyzed by an immunoprecipitation assay using anti-HA or anti-FLAG antibodies. We found that pulling down Pur-alpha with anti-HA antibody also brings down FUS, as evident from Western blots probed with ant-FLAG (Figure 3a). Similarly, we immunoprecipitated FUS using an anti-FLAG antibody and probed with anti-HA (for Pur-alpha) and found the same pattern of interaction between FUS and Pur-alpha (Figure 3b). We also tested whether ALS-causing mutations in FUS alter the physical interaction between FUS and Pur-alpha. We found that disease-causing mutant FUS retains the ability to interact with Pur-alpha in neuronal cells. These results suggest that Pur-alpha and FUS physically interact, and that disease-causing mutations in FUS do not affect interaction with Pur-alpha in a mammalian neuronal cell line.

Pur-alpha is essential for the formation of cytoplasmic stress granules

To understand the role of Pur-alpha in regulating cytoplasmic stress granules, we depleted endogenous Pur-alpha using an shRNA approach in the human embryonic kidney 293 T (HEK293T) cell line. We used 4 different shRNAs targeted against multiple functional regions of Pur-alpha to deplete endogenous Pur-alpha. After transfecting HEK293T cells with Pur-alpha shRNA for 24 hours, we challenged the cells with sodium arsenite. We found that knocking down endogenous Pur-alpha significantly impaired the ability of cells to form G3BP-positive cytoplasmic SGs under stress conditions (Figure 4a and c). In addition we used TIAR as a second marker for SGs to validate these findings and found that knocking down Pur-alpha blocks the formation of TIAR-positive SGs (Figure S7). Furthermore, we generated HEK293T cells stably expressing shRNAs and measured the reduction of Pur-alpha by qPCR and Western blot. All 4 shRNAs designed against Pur-alpha depleted endogenous Pur-alpha mRNA and protein at different levels (Figure 4b and S8a). In addition to sodium arsenite treatment, we also challenged the HEK293T cells stably expressing shRNA against Pur-alpha with heat (42°C) and found that knocking down endogenous Pur-alpha significantly impaired the ability of cells to form SGs under these conditions (Figure S9). Intriguingly, we found that knocking down Pur-alpha with shRNA significantly reduced mRNA levels of known SG components such as G3BP1, FMR1, and TIAL1 (Figure S8 b, c, and d). However, knocking down Pur-alpha with shRNA did not reduce G3BP1 protein levels (Figure S10). Interestingly, knocking down endogenous Pur-alpha did not alter FUS protein and RNA levels (Figure S11).

To determine if depleting endogenous Pur-alpha can disrupt processing bodies (p-body), we stained the HEK293T cells expressing Pur-alpha shRNA with anti-GW182 (a p-body marker) after challenging with stress. We did not observe any change in the p-body number or size in Pur-alpha depleted cells compared to control cells expressing scrambled shRNA, suggesting that depleting endogenous Pur-alpha does not affect p-body formation (Figure S12). These observations indicate that Pur-alpha is essential for the formation of cytoplasmic stress granules, but not for p-bodies, under stress conditions. We also

investigated whether knocking down endogenous Pur-alpha has any influence on subcellular distribution of endogenous FUS. We found that knocking down endogenous Pur-alpha does not change nuclear localization of endogenous FUS (Figure S13).

Next, given that knockdown of Pur-alpha inhibited the formation of SGs, we asked if ectopic expression of Pur-alpha has any influence on the formation and disassembly of cytoplasmic SGs in mammalian cells. Using sodium arsenite treatment, we performed a time-course of the induction of SGs to monitor the formation of SG at multiple time points (10, 20, 30, 45 and 60 minutes) post stress. We found that exogenous expression of Pur-alpha led to the formation of SGs after 20 minutes of sodium arsenite treatment, whereas controls at 20 minutes of sodium arsenite treatment did not show the presence of SGs. These results suggest that ectopic expression of Pur- alpha promotes the formation of SGs in mammalian neuronal cells (Figure S14). Since Pur-alpha may accelerate SG dynamics, we tested whether overexpression of Pur-alpha could influence the SG disassembly process. We performed a recovery assay where mammalian neuronal cells were stressed with 0.5 mM sodium arsenite for 1 hour. We then removed the stress causing agent, washed the cells and replaced with medium, allowing them to recover for 2 hours and 30 minutes. Using this recovery assay, we observed that overexpression of Pur-alpha facilitated the disassembly of SGs (Figure S15). These observations indicate that Pur-alpha is important for both assembly and disassembly of cytoplasmic SGs.

Mapping functional domain (s) of Pur-alpha required for the cytoplasmic SG assembly

The Pur-alpha protein contains several functional domains with distinct biological functions. To determine which domain of Pur-alpha is required for SG assembly, we generated a series of Pur-alpha constructs with deletions in their functional domains (Figure 5b). We found that the Pur-alpha mutant constructs were expressed correctly when transfected into HEK293 cells (Figure 5a). We next asked if ectopic expression of WT Pur-alpha can restore SG assembly in HEK293T cells stably expressing shRNA against Pur-alpha. We found that reintroducing WT Pur-alpha significantly restored SG formation in HEK293T cells expressing shRNA against Pur-alpha (Figure 5a and 5c). These findings indicate that SG assembly is specifically perturbed by knocking down endogenous Pur-alpha levels. Using a bioinformatic predictive algorithm [54] to identify potential functional domains, we found Pur-alpha contains a putative prion-like domain (Figure S16). We reintroduced Pur-alpha with deletions in different functional domains to the HEK293 cells stably expressing shRNA against Pur-alpha. The majority of the mutant Pur-alpha constructs showed a subcellular distribution comparable to that of WT Pur-alpha, except the construct with the deletion in the first N-terminal 85 amino acids. Most of the Pur-alpha mutant constructs were able to restore, with different propensity, SG formation in HEK2932 cells stably expressing Pur-alpha shRNA (Figure 5a). Interestingly, we observed that deleting 160 amino acids from the C-terminus of Pur-alpha spanning the repeat region, psycho domain, C-terminal glutamine-rich and glutamate-rich domains, impaired the ability of Pur-alpha to restore the formation of SGs in HEK293T cells expressing Pur-alpha shRNA, suggesting that this region is critical for the formation of SGs in HEK293T cells.

Expression of Pur-alpha can block cytoplasmic accumulation of mutant FUS in primary motor neurons

Cytoplasmic mislocalization of FUS carrying disease-causing mutations is a hallmark of disease, as observed in ALS patients and in several experimental models of FUS-related neurodegeneration [50, 56, 72]. We found that blocking cytoplasmic mislocalization of mutant FUS strongly suppressed neurodegenerative phenotypes in a fly model of ALS [55, 56]. We investigated whether Pur-alpha expression can affect cytoplasmic mislocalization of FUS in primary motor neurons expressing FUS R521G. We expressed GFP-tagged FUS WT or GFP-tagged FUS R521G with or without mCherry-tagged Pur-alpha in primary motor neurons. As expected, predominant nuclear localization of WT FUS was seen, whereas FUS R521G was distributed in both the cytoplasm and the nucleus. Interestingly, we found that expression of Pur-alpha significantly blocked cytoplasmic mislocalization of FUS R521G in primary motor neurons (Figure 6). Furthermore, we quantified the number of cells containing FUS localized to the nucleus or the nucleus and cytoplasm. Only about 20% of primary motor neurons expressing FUS R521G had FUS localized to the nucleus. However, Pur-alpha expression in the FUS R521C background restored nuclear localization of FUS in approximately 80% of cells (Figure 6b). Our data suggest that Pur-alpha expression is sufficient to prevent cytoplasmic mislocalization of mutant FUS in primary motor neurons.

Pur-alpha expression can suppress toxicity associated with mutant FUS in primary motor neurons

Next, we asked whether expression of Pur-alpha can mitigate toxicity associated with mutant FUS expression in primary motor neurons. We expressed GFP-tagged FUS WT or GFP-tagged FUS R521G with or without mCherry-tagged Pur-alpha in primary motor neurons. We found that expression of FUS R521G, but not FUS WT, disrupted dendritic integrity in primary motor neurons, as evident from anti-MAP2 staining (Figure 7a). Interestingly, expression of Pur-alpha significantly restored viability in primary neuronal cells as compared to FUS WT and control (Figure 7b). To further evaluate the effects of Pur-alpha on primary neurons expressing FUS R521G, we quantified the dendritic morphology and length. Pur-alpha expression mitigated FUS R521C dendritic morphology and ameliorated average dendritic length to control levels (Figure 7c and d). In addition, we measured the soma size and found that primary neurons expressing FUS R521C had reduced soma size, which was increased with Pur-alpha overexpression (Figure S17). We performed Propidium Iodide staining of primary neuronal cells expressing human FUS and found that expression of Pur-alpha significantly suppressed cell death associated with expression of FUS R521G (Figure 7e). In order to further delineate the role of Pur-alpha in regulating FUS-mediated toxicity, we depleted endogenous Pur-alpha in primary neurons expressing WT or mutant version of FUS and performed TUNEL assay. We found that knocking down endogenous FUS significantly enhanced FUS mediated toxicity suggesting that endogenous Pur-alpha plays an important role in regulating FUS toxicity (Figure 7f). Finally, we reasoned if Pur-alpha mediated amelioration of FUS toxicity involve SGs in primary neuronal cells. We expressed FUS R521G in primary neuronal cells and challenged them with sodium arsenite and found that a robust induction of cytoplasmic stress granule formation as expected. We compared the total number of cytoplasmic stress granules (G3BP positive) in cells expressing FUS R521G alone with cells expressing FUS R521G and Pur-

alpha together. Two key observations emerged from our abovementioned experimental approach. First, we found that expression of Pur-alpha significantly reduced the total number of cytoplasmic stress granules in primary neuronal cells expressing FUS R521G as compared to cells expressing FUS R521G alone (Figure S18a, b). These observations suggest that cytoplasmic stress granules are cleared much faster when Pur-alpha is overexpressed in mutant FUS expressing cells. Second, we observed that ectopic expression of Pur-alpha significantly reduced the number of FUS- containing SGs (Figure S18c). These observations suggest that Pur-alpha expression is sufficient to suppress toxicity associated with human FUS expression and reduce total number of SGs as well as FUS-positive SGs in neuronal cells.

Discussion

Recently, RNA granules have been implicated in many human diseases, including neurodegeneration, cancer, autoimmune diseases and infectious diseases [2, 4, 14, 15, 17, 58, 74, 76]. RNA granules (stress granules (SGs), processing bodies (p-bodies), nuclear paraspeckles, neuronal granules etc.) can be classified based on their constituents, subcellular localization (cytoplasmic, nuclear, axonal, etc.), origin (e.g. germ cells or neurons), dynamic behavior and predicted functions (mRNA storage/decay or stress response, etc.) [1, 2]. SGs are physiological structures formed to combat adverse conditions such as infection, oxidative stress, heat and cold) [2, 15]. Recent studies have shown that several RNA-binding proteins implicated in human neurodegenerative diseases are constituents of SGs [48]. Most RNA-binding proteins contain a prion-like domain or low complexity domain, and these proteins are sticky and highly aggregation-prone. Incorporation of RNA-binding proteins and RNAs into cytoplasmic SGs is a highly organized, reversible process. Disease-causing mutations in RNA-bindings proteins (RBPs) might serve to “seed” irreversible aggregation of RBPs and RNAs. These SGs may form or may lead to formation of constitutive SGs. These SGs may form irreversible interactions among SG components that could disrupt SG dynamics, functions of other SG components, and global RNA metabolism, all which would be expected to have deleterious effects on neurons. Our data showing that FUS and Pur-alpha become trapped into cytoplasmic SGs under stress conditions supports this model. These notions have been further supported by evidence that mutations in the RNA-binding protein TIA1 (a component of SGs) can cause defects in RNA splicing and cellular stress which result in Welander distal myopathy [36, 49].

Components of RNA granules have been shown to modify neurodegenerative phenotypes associated with fragile X mental retardation and TDP-43 mediated ALS in Drosophila [24, 47]. It has been recently shown that the expression of ALS-causing protein TDP-43 can upregulate phosphorylated eIF2 alpha levels in Drosophila brains, suggesting a chronic translation arrest stage. In addition, drugs that target the phosphorylated version of eIF2 could suppress neurodegeneration associated with TDP-43 related pathologies [47]. These findings suggest that strategies to ameliorate defects in RNA granule dynamics might serve as potential therapeutic interventions for human neurodegenerative diseases, particularly ALS.

Pur-alpha is a DNA/RNA binding protein involved in regulating multiple aspects of transcription, tumor suppression and RNA transport granules [42, 75]. Pur proteins are highly conserved from bacteria to mammals with only a two amino acid difference between human and murine Pur-alpha [40, 42]. The human Pur proteins family consists of four members; Pur-alpha, Pur-beta, and two isoforms of Pur-gamma [11, 12, 57]. The pur-alpha proteins have been shown to bind purine-rich nucleic acids [11, 12, 38]. There are several functional domains in the Pur-alpha proteins and each domain of the pur-alpha protein is involved in regulating multiple aspects of cellular functions such as transcription, DNA replication, and cell growth [42]. Pur-alpha has an N-terminal glycine-rich domain, a central DNA-binding domain, a prion-like domain and C-terminal glutamine-rich and glutamate-rich domains [42]. Furthermore, heterozygous knockouts of endogenous Pur-alpha in mice have been shown to cause neurological symptoms such as severe tremor and spontaneous seizures indicating the importance of this protein in the mouse nervous system [45].

Recently, mutations have been identified in Pur-alpha that lead to profound neurodevelopmental delay, learning disabilities, neonatal hypotonia, seizures and encephalopathy, suggesting the neuronal functions of Pur-alpha in humans [37, 53]. Pur-alpha plays an important role in the pathogenesis of fragile X tremor ataxia syndrome (FXTAS) and C9orf72 related neurodegeneration in ALS. Pur-alpha colocalizes with FMRP (fragile X mental retardation protein 1 homolog) in the dendrites of rat hippocampal neurons, and the Drosophila homolog of human Pur-alpha interacts with rCGG-containing repeats in the FMR1 gene in a fly model of FXTAS [39, 43]. Interestingly, ectopic expression of Pur-alpha can suppress FXTAS-related neurodegeneration in Drosophila. Similarly, Pur-alpha binds to expanded GGGGCC repeats, and expression of Pur-alpha is sufficient to suppress neurodegenerative phenotypes associated with expanded GGGGCC repeat-mediated neurodegeneration in a Drosophila model of ALS [77]. However, the exact mechanisms of Pur-alpha-mediated suppression of neurodegenerative phenotypes are still not clear.

Our current studies, though highly intriguing, raise many key questions that remain to be answered. It is not yet clear which molecular pathway(s) are regulated by Pur-alpha. Since Pur-alpha is a multi-functional protein, it is possible that it might regulate multiple cellular pathways. A mechanistic understanding of the role of Pur-alpha in relation to other RNA binding proteins is another area of investigation for future studies. Our data show that Pur-alpha expression can cause redistribution of mutant FUS to the nucleus, indicating that Pur-alpha could be involved in nuclear-cytoplasmic shuttling of FUS or other RBPs. The molecular interactions facilitating transport of FUS to the nucleus are still unknown. It is unclear whether the interaction between FUS and Pur-alpha is dependent on RNA. Since both of these proteins are involved in regulating different aspects of RNA metabolism, it is reasonable to believe that FUS and Pur-alpha interaction is RNA-dependent, however it is still not known which functional domains of Pur-alpha or FUS are involved in mediating their physical interaction. Of note, it has been demonstrated that Pur-alpha interacts with FMRP and other RBPs involved in mRNP formation in an RNA-dependent manner [62]. Similarly, RNA-binding ability of FUS is critical for regulating FUS-mediated toxicity, subcellular distribution and incorporation into SGs [27]. Furthermore, it is not clear whether the Pur-alpha and FUS interaction occurs directly or their interaction is mediated by

complexing with other RBPs. We observed that Pur-alpha contains a predicted prion-like domain, which is known to function in protein-protein interaction. In addition, Pur-alpha has been shown to bind to proteins and is responsible for Pur Binding Protein (PurBPs) transport into the nucleus during neuronal development [78]. Therefore, Pur-alpha could function directly in transporting RBPs to and from the nucleus. Under pathological conditions, we found that Pur-alpha becomes trapped in cytoplasmic SGs potentially inhibiting its normal function. Pur-alpha is an RNA binding protein; it is still unknown if the RNA-binding ability of Pur-alpha is required for transport of FUS or other RBS to the nucleus. Given that SGs are degraded by autophagy [16, 67], it is possible that Pur-alpha is involved in regulating the autophagy pathway, directly or indirectly. Since autophagy is a druggable pathway, further studies might help in developing therapeutic interventions for neurodegenerative conditions involving perturbed SG dynamics. While our manuscript was under review process, it came to our attention that a study has just been published on line that reported incorporation of Pur-alpha in SGs [29].

Our data suggest that Pur-alpha is a novel component of SGs in ALS patient cells, mammalian neuronal cells and primary motor neurons. We demonstrate that knocking down endogenous Pur-alpha can impair the abilities of cells to form SGs under stress conditions, indicating that Pur-alpha is an essential component of SGs. Interestingly; we observed that Pur-alpha expression can block the cytoplasmic accumulation of mutant FUS protein and suppress toxicity associated with FUS carrying the disease-causing mutation R521G in primary motor neurons. In conclusion, our data demonstrate a role of Pur-alpha in FUS-mediated neurodegeneration and suggest that Pur-alpha expression could help in suppressing toxicity in mammalian neurons through ameliorating the number of SGs.

Supplementary Material

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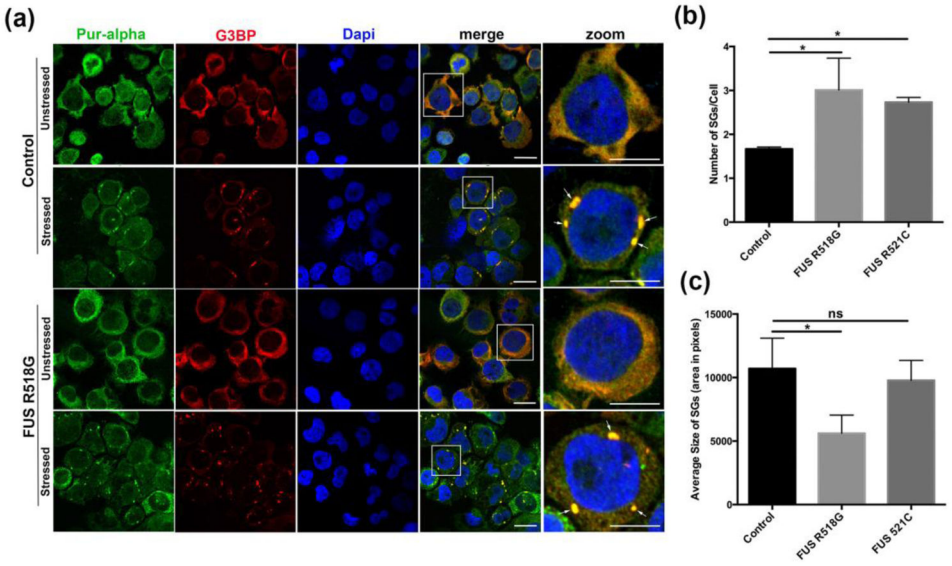

Figure 1. Pur-alpha is a novel component of cytoplasmic stress granules in both control and ALS patient cells

(a). Control lymphoblastoid cells were stained with anti-Pur-alpha (green) and a stress granule marker anti-G3BP (Red). In unstressed conditions both Pur-alpha and G3BP remain diffuse in the cytoplasm. Under stress conditions (0.5 mM sodium arsenite) both Pur-alpha and G3BP aggregate to form cytoplasmic puncta that colocalize with each other. ALS patient lymphoblastoid cells carrying FUS R518G mutations are found to have diffuse Pur-alpha staining as well as G3BP localizing primarily to the cytoplasm. Upon treatment with sodium arsenite Pur-alpha and G3BP colocalize in cytoplasmic stress granules. Scale bars represent 10 μm. Arrows indicate areas of colocalization. (b). Number of G3BP positive SG were quantified per cell in control, ALS patient Lymphoblastiod cells with FUS R518G, and FUS R521C mutations. (c). Total area of G3BP positive SGs were determined using imageJ by subtracting background to an constant threshold. Total area was divided by the number of SGs to give a relative area in pixels. N=~100 cells/genotype. Averages from 3 fields at 60x were considered in the analysis. ANOVA with Tukey's Post hoc analysis was applied. (*$p < 0.05$).

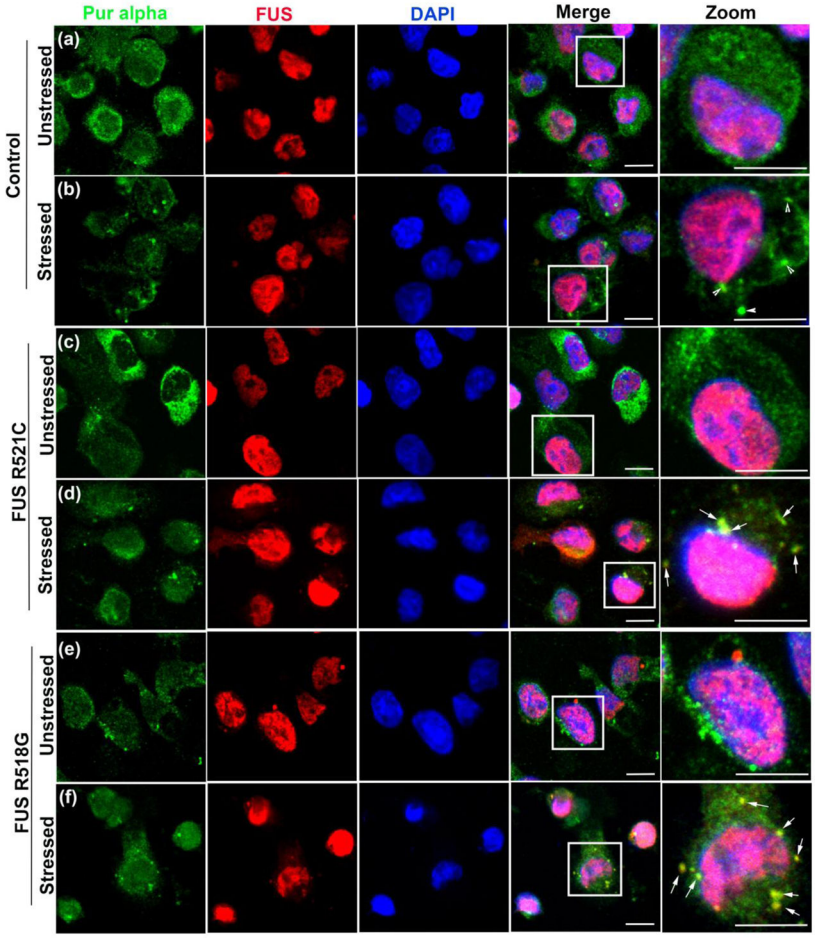

Figure 2. Pur-alpha co-localizes with ALS-linked versions of FUS in cytoplasmic aggregates

(a). Control lymphoblastoid cell were stained for Pur-alpha and FUS under unstressed conditions. Pur-alpha staining is diffuse in the cytoplasm and FUS localizes primarily in the nucleus. (b). Under stressed (0.5 mM sodium arsenite) conditions Pur-alpha aggregates in the cytoplasm and these puncta are not positive for WT FUS. (c) and (e). FUS ALS patient cells carrying R521C and R518G mutations in unstressed conditions show Pur-alpha staining throughout the cytoplasmic compartment. (d) and (f) Under stress conditions Pur-alpha and FUS aggregate in the cytoplasm. These cytoplasmic puncta colocalize. Arrows indicate areas of colocalization of FUS and Pur-alpha in the cytoplasm. Scale bars represent 10 μm.

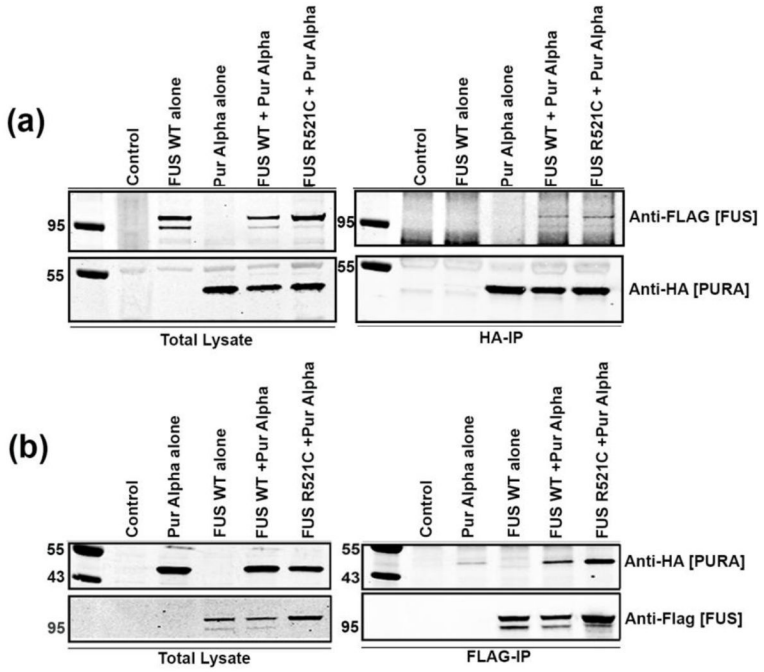

Figure 3. Pur-alpha physically interacts with FUS in mammalian cell cultures

N2A cells stably carrying TET-inducible FLAG-FUS WT and FLAG-FUS R521C constructs were treated with 300 ng/ml of doxycycline to induce FUS expression and were transfected with HA-Pur-alpha. (a). Anti-HA7 was used to immunoprecipitate HA-Pur-alpha. Pur-alpha levels and induced FUS expression levels in total lysate are shown. In the immunoprecipitation (IP), Pur-alpha levels were enriched and FLAG-FUS constructs co-precipitated. (b). FLAG-FUS constructs were again induced with doxycycline and transfected with HA-Pur-alpha. Total lysates indicate Pur-alpha and FUS input levels. IP was performed with anti-FLAG, and FLAG-FUS levels were enriched by IP. HA-Pur-alpha co-precipitated with FLAG-FUS.

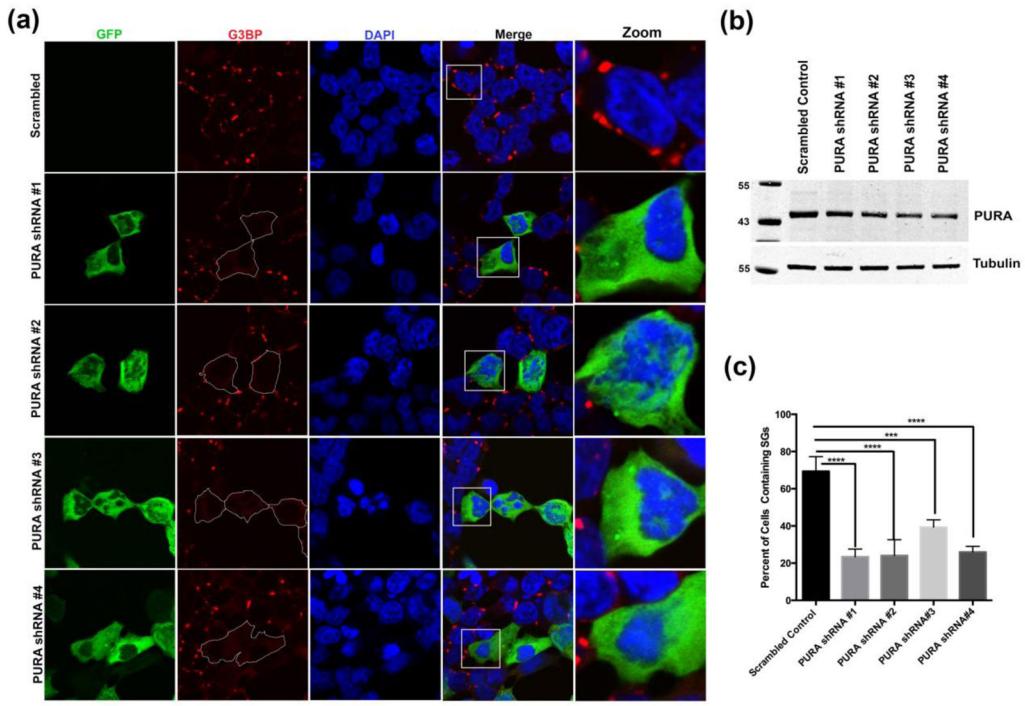

Figure 4. Knockdown of Pur-alpha with shRNA disrupts SG assembly in mammalian cell culture

HEK293T cells expressing four independent human Pur-alpha shRNA-GFP constructs were stressed with sodium arsenite to determine the effect of knocking down Pur-alpha on SG dynamics. (a). In control HEK293T cells transfected with scrambled shRNA, cytoplasmic SGs (G3BP-Red) formed under sodium arsenite stress (0.5mM for 90 mins). In HEK293T cells transiently expressing four different Pur-alpha shRNA-GFP (Green) constructs (Origene), G3BP-positive SGs did not assemble in the cytoplasm. (b). HEK293T cell stably expressing Pur-alpha shRNA were used to generate total lysates (35,000 cells/well). Proteins in total lysates were resolved by SDS PAGE, Western blots were probed with anti-Pur-alpha and anti-tubulin. Pur-alpha protein levels were substantially reduced in Pur-alpha shRNA samples compared to scrambled shRNA controls. (c) Quantification of the percentage of cells containing SGs (G3BP-positive) under stress conditions. In cells expressing Pur-alpha shRNA, the formation of SGs was significantly inhibited. ANOVA was performed with Tukey's multiplecomparison. Error bars represent ± SEM. (P=0.001***, 0.0001****).

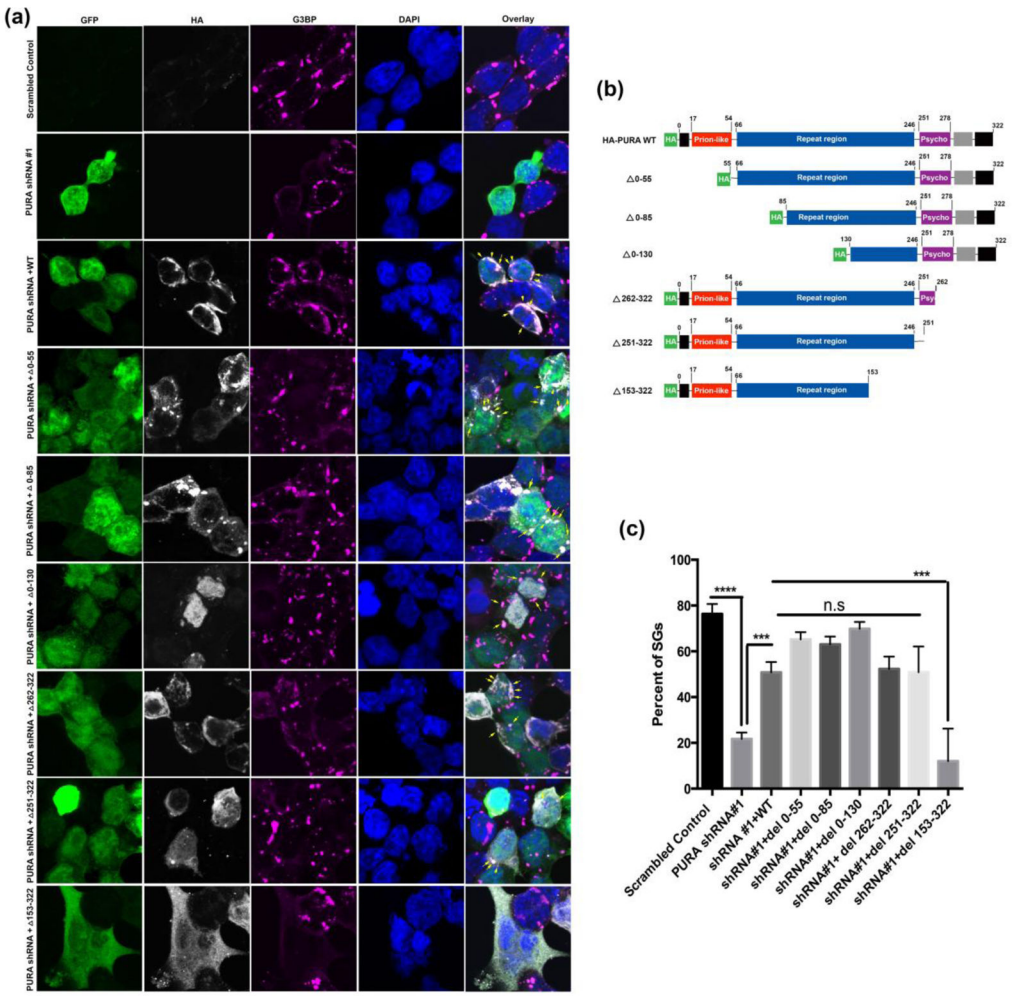

Figure 5. Pur-alpha functional domains required for SG assembly and incorporation

HEK293T cell expressing human Pur-alpha shRNA were transfected with deletion constructs to determine the functional domain required to restore SG formation. (a). Immunofluoresence of Pur-alpha shRNA GFP #1 (green), HA-Pur-alpha deletion constructs (white), G3BP (magenta), DAPI (blue). Arrows indicate SGs positive for both Pur-alpha and G3BP. (b) Schematic diagram to demonstrate the functional domains systematically deleted to map the domain required for SG formation and incorporation (c). Quantification of the number of cells containing SGs. Expression of Pur-alpha shRNA cause a significant reduction in the total percentage of cells containing SGs. Co-expression of Pur-alpha WT and other mutations in the presence of Pur-alpha shRNA restored SG formation. Pur-alpha del 153–322 mutant does not restore SG formation. ANOVA was performed with Tukey's multiple comparison. Error bars represent ± SEM. (P=0.001***, 0.0001****).

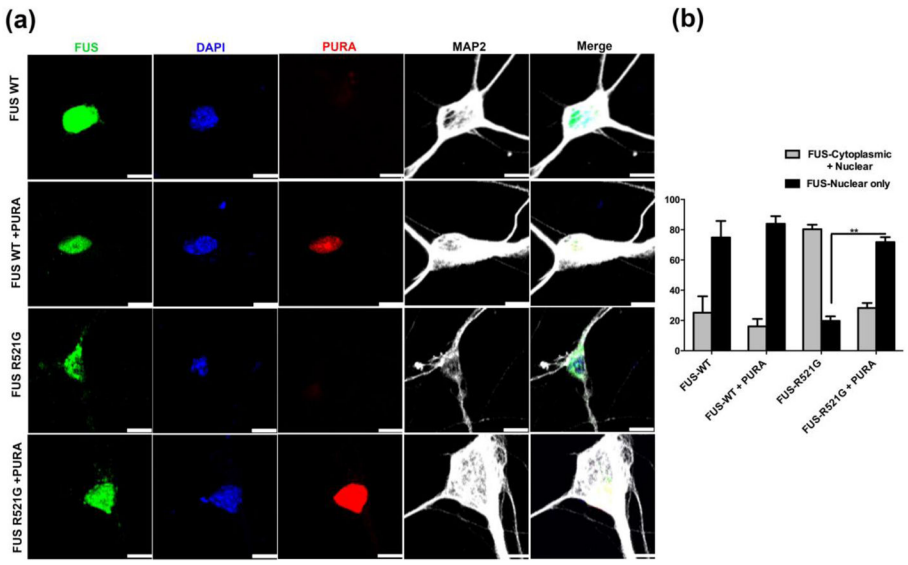

Figure 6. Pur-alpha co-expression rescues mutant FUS mislocalization in primary motor neurons

(a) Representative confocal images of primary motor neurons expressing GFP-FUS-WT and GFP-FUS-R521G alone or coexpressed with mCherry-Pur-alpha. Cytoplasmic mislocalization of FUS-R521G was evident in motor neurons whereas coexpression with Pur-alpha prevented this mislocalization. (b) Bar graph shows the mean percentage of neurons with FUS localized in the nucleus alone or nucleus + cytoplasm under different condition. The percentage of neurons with FUS-R521G in the nucleus alone was significantly increased upon co-expression with Pur-alpha. Student T test were prerformed. Error bars represent ± SEM. (**P=0.01) Scale= 10 μm.

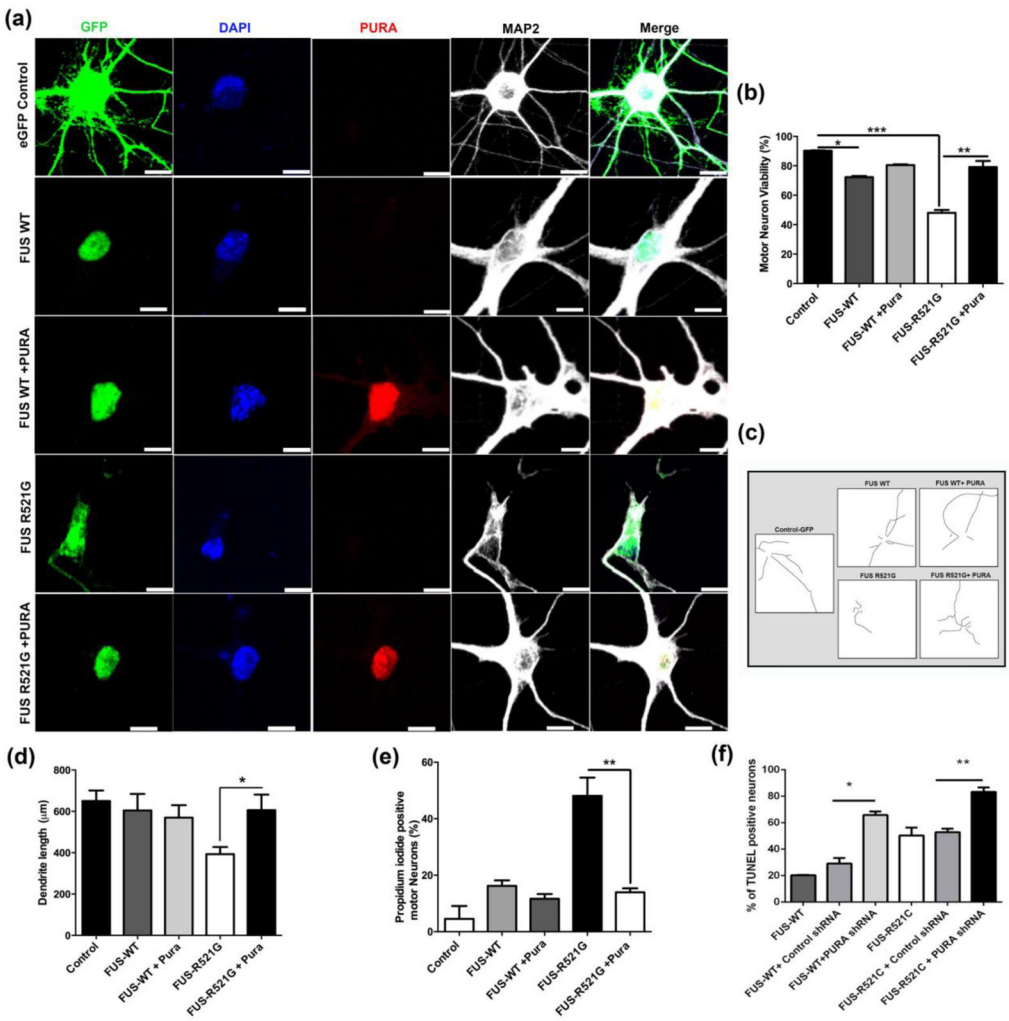

Figure 7. Pur-alpha co-expression rescues mutant FUS toxicity in primary motor neurons

(a) Representative confocal images show loss and fragmentation of MAP-2 immunofluorescence in GFP-FUS-R521G transfected motor neurons. In primary motor neurons expressing both mCherry-Pur-alpha and GFP-FUS R521C MAP-2 fragmentation was not seen. (b) Bar graph shows the mean viability of motor neurons. FUS-R521G toxicity was significantly attenuated by Pur-alpha co-expression. (c). Representative dendrite tracings generated using image J. (d). Ectopic expression of Pur-alpha in mutant FUS expressing primary motor neurons rescues dendritic loss. Average dendritic length of motor neurons measured with neurite tracer ImageJ. (*P<0.05, **P=0.01, ***P=0.001). Data are shown as mean ± SEM. Scale= 10 μm. (e) Primary motor neurons expressing FUS R521G show positive Propidium iodide staining indicating cell death that was significantly rescued by overexpression of Pur-alpha. (f) Knocking down endogenous Pur-alpha significantly exacerbated human FUS toxicity in primary neurons. Percentage of TUNEL positive neurons in FUS WT and FUS R521C expressing neurons with and without PURA knockdown were analyzed. Percentage of TUNEL positive neurons were drastically

increased in both FUS-WT and FUS-R521C conditions upon PURA shRNA1 expression ($P < 0.05$) compared to control shRNA.