

Non-canonical amino acid incorporation enables minimally disruptive labeling of stress granule and TDP-43 proteinopathy

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This paper demonstrates that a genetic code expansion to tag two amyotrophic lateral sclerosis (ALS) proteins associated with stress granules is **useful** in an experimental context. The data are **solid** and demonstrate the feasibility of using ANAP-fluorescence for live cell imaging.

Abstract We report a minimally disruptive labeling strategy for stress granule protein, G3BP Stress Granule Assembly Factor 1 (G3BP1), and ALS-linked protein, TAR DNA-binding protein 43 (TDP-43), using the fluorescent non-canonical amino acid Anap. By integrating the genetic code expansion (GCE) with rational site selection, we achieved precise incorporation of Anap that preserves protein structure and function. In live cells and neurons, Anap labeling faithfully recapitulated localization, stress-induced dynamics, and recovery behavior, outperforming conventional fluorescent tags, and enabling physiologically relevant visualization of protein pathobiology.

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Introduction

Fluorescent protein labeling remains a cornerstone of live-cell biology, yet conventional techniques rely heavily on large fusion tags, such as auto-fluorescent tags (AFPs) or small-molecule-binding motifs (Crivat and Taraska, 2012), at limited positions (typically N- or C-terminal). These tags might potentially affect the structure, function, and even localization pattern of proteins, limiting their use for studying proteins with complex dynamics (Chatterjee et al., 2013). Alternatively, genetic code expansion (GCE) has emerged as a versatile labeling strategy to label proteins site-specifically in a minimally disruptive manner (Hao et al., 2024; Nikić et al., 2015; Nygaard et al., 2024; Bessa-Neto et al., 2021; Eddins et al., 2025; Arsić et al., 2022).

GCE employs an engineered orthogonal aminoacyl tRNA synthetase/tRNA pair to incorporate non-canonical amino acids (ncAAs) at desired positions of proteins according to reassigned codons, most commonly the amber stop codon (TAG), thereby introducing a single-residue substitution within the protein of interest. Among these ncAAs, L-Anap (3-(6-acetylnaphthalen-2-ylamino)-2-aminopropionic acid) is especially attractive for live-cell imaging. Anap is intrinsically fluorescent, exhibits polarity-sensitive emission spectra, and requires no post-incorporation modification (Chatterjee et al., 2013; Nygaard et al., 2024). Despite these advantages, GCE-based Anap labeling has rarely been systematically applied to track disease-relevant protein dynamics in live mammalian cells. In this study, we developed an Anap-based labeling platform optimized for minimally disruptive labeling of two

important proteins, G3BP1 and TDP-43, involved in membraneless organelles and neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).

G3BP1 is a core protein in stress granules, dynamic membraneless organelles related to stress response (Yang et al., 2020). Altered stress-granule dynamics have been associated with ALS/FTD (Kassouf et al., 2023; Van Nerom et al., 2024); however, whether stress granules directly drive neurodegeneration remains debated, as several studies suggest that stress granules primarily function as protective stress responses (Wolozin and Ivanov, 2019). TDP-43 cytoplasmic inclusion is a hallmark of ALS/FTD pathology (Neumann et al., 2006) and is closely associated with dysregulation of RNA metabolism, ultimately leading to cellular defects (Suk and Rousseaux, 2020). Conventional fluorescent protein tags have enabled visualization of TDP-43 and G3BP1 in living cells; however, these approaches can perturb the native biophysical properties of the proteins being studied. For example, GFP or other fluorescently tagged TDP-43 usually requires additional modifications, such as deletion of the nuclear localization signal (NLS) (Gasset-Rosa et al., 2019; Yan et al., 2025), to induce cytoplasmic inclusion formation. Such manipulations introduce non-physiological conditions that may alter the native trafficking and aggregation behavior of TDP-43. As for G3BP1, tags like GFP may also cause unexpected effects on the phase separation or other dynamics of the protein. In contrast, Anap-based GCE strategy allows the minimally perturbative labeling and visualization of protein localization and stress-induced redistribution while preserving native protein architecture and function of both proteins. Importantly, the approach provides a generalizable genetically encoded platform for quantitatively examining the behavior of ALS-associated proteins in living cells. By enabling faithful monitoring of protein trafficking and stress-granule dynamics without extensive protein engineering, Anap-based GCE can offer a powerful strategy for probing molecular-scale mechanisms underlying ALS-linked proteinopathies.

Results and discussion

To implement the site-specific Anap incorporation system, we selected and generated two amber mutants, G3BP1^{F337TAG} and TDP-43^{V100TAG}, using a combination of structural and functional criteria: exclusion from functional domains or localization signals, absence of disease-associated mutations, lack of post-translational modification, and low predicted structural impact by AlphaFold models. For G3BP1, the selected site was chosen to minimize interference with domains important for stress granule assembly, RNA binding, and protein-protein interactions. For TDP-43, the incorporation site was selected to avoid the major functional domains involved in RNA binding, nuclear localization, and aggregation-related behavior, thereby reducing the likelihood that Anap incorporation would perturb its native trafficking or function. More generally, we aimed to place the ncAA at positions likely to be solvent-accessible and tolerant of substitution, while avoiding highly conserved or functionally essential residues. Incorporation of Anap was achieved via co-expression of an orthogonal tRNA/synthetase pair in cells. To ensure that the fluorescence signal observed in our experiments was specifically derived from site-specific Anap incorporation rather than background fluorescence, we performed three control conditions. Specifically, we tested: (1) cells cultured with the addition of Anap, (2) cells expressing the Anap incorporation system with the addition of Anap, and (3) cells expressing both the TAG-mutated protein plasmid and the Anap incorporation system but without the addition of Anap. These control experiments were performed for both TDP-43 and G3BP1, and no observable fluorescence signal was detected under any of these conditions (Figure 1—figure supplement 1A and B).

We first tested the feasibility of the Anap labeling system for G3BP1. In HeLa cells, G3BP1-Anap localized diffusely in the cytoplasm under basal conditions, closely matching antibody staining. Interestingly, a nuclear signal was detected with Anap but not with antibody, indicating the presence of nuclear pools of G3BP1 inaccessible to antibody detection. Upon sodium arsenite treatment, both the Anap and antibody signals colocalized within stress granules (Figure 1A), validating the ability of Anap labeling to visualize the dynamics of G3BP1-driven stress granule formation. In addition, to independently validate protein expression, we performed western blot analysis in a G3BP1 knockout U2OS cell line, confirming the expression of G3BP1-Anap (Figure 1H). To assess labeling fidelity, we compared the performance of G3BP1-Anap with G3BP1-GFP using fluorescence recovery after photobleaching (FRAP). Following stress, both labeled proteins localized to granules, but G3BP1-Anap exhibited significantly higher fluorescence recovery (~53%) than G3BP1-GFP (~33%) (Figure 1C and F). These results suggest that G3BP1-Anap displays higher mobility compared with

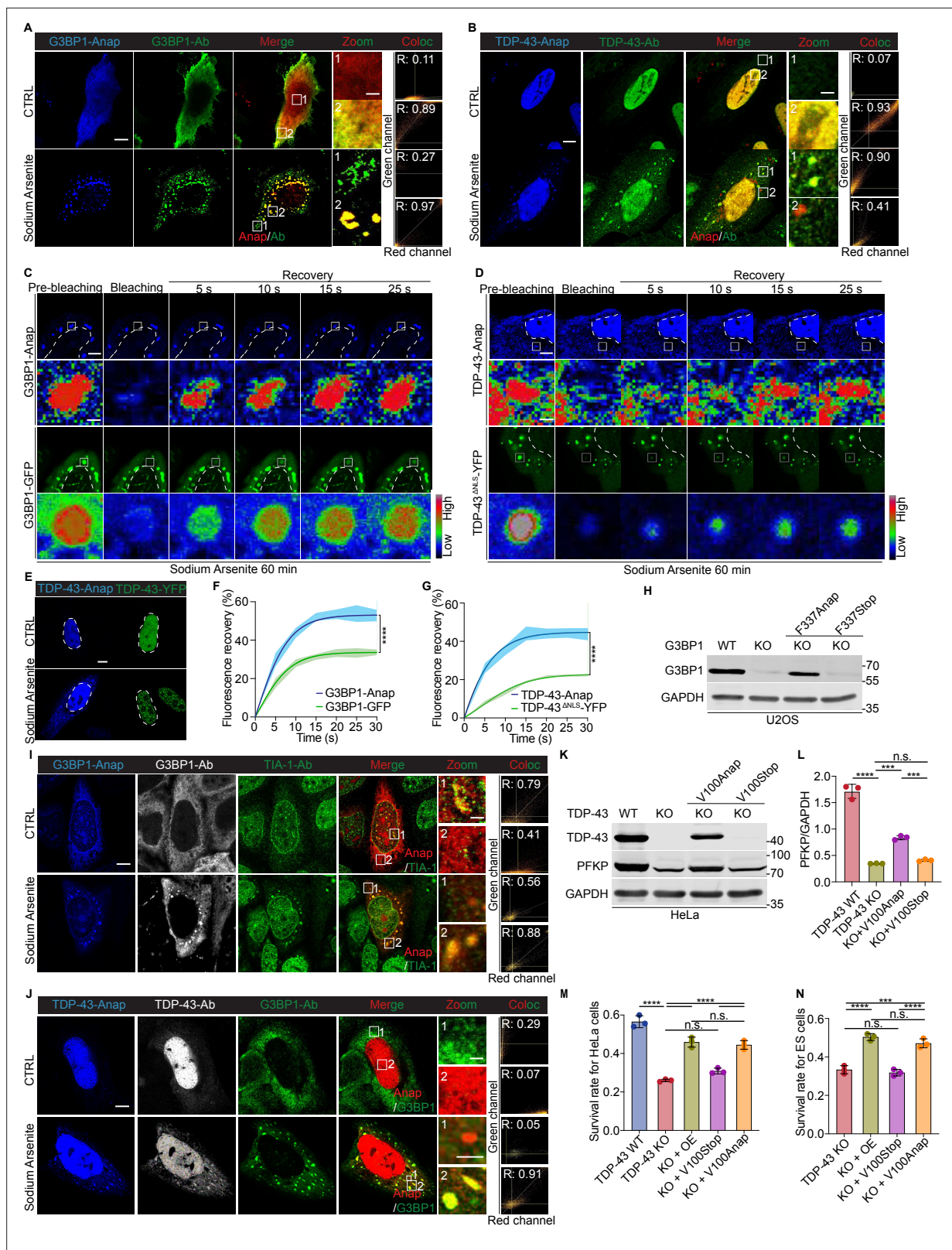


Figure 1. Anap-based labeling enables visualization of TDP-43 and G3BP1. **(A, B)** HeLa cells expressing G3BP1-Anap and TDP-43-Anap under basal conditions or 250 μ M sodium arsenite treatment. Anap and antibody signals are shown in blue and green, respectively; for merged channels, Anap was pseudo-colored red. Scale bars: 10 μ m (overview), 3 μ m (zoom). **(C, D)** Fluorescence recovery after photobleaching (FRAP) of G3BP1-Anap, G3BP1-GFP, and TDP-43-Anap, following 250 μ M sodium arsenite treatment. One granule from each of three independent cells was selected and photobleached

Figure 1 continued on next page

Figure 1 continued

for FRAP analysis. Regions of interest (ROI) signal intensities are displayed in rainbow RGB (red-high, blue-low). Scale bars: 5 μm (cells), 1 μm (ROI). (E) Comparison of HeLa cells expressing TDP-43-Anap and TDP-43-YFP under basal conditions or 250 μM sodium arsenite treatment. Here, Anap labeling and YFP labeling yield a blue signal and a yellow to green signal, respectively. (F, G) Relative fluorescence recovery at each time point after photobleaching for G3BP1-Anap, G3BP1-GFP, TDP-43-Anap and TDP-43^{ΔNLS}-YFP. Here, error bars with filled area are used for data presentation. FRAP recovery curves were compared using two-way ANOVA. $n=3/\text{group}$. (H) Immunoblotting of wild-type and Anap-labeled G3BP1. The mouse anti-G3BP1 antibody was used. KO: G3BP knockout; F337Stop/Anap: the expression of G3BP1^{F337TAG} via Anap labeling without or with the addition of Anap. (I) Colocalization of G3BP1-Anap with TIA-1. The mouse anti-G3BP1 antibody and rabbit anti-TIA-1 antibody were used. Anap, G3BP1-Ab, and TIA-1-Ab signals are shown in blue, gray, and green, respectively; for merged channels, Anap was pseudo-colored red. Scale bars: 10 μm (overview), 3 μm (zoom). (J) Colocalization of TDP-43-Anap with G3BP1. The mouse anti-G3BP1 antibody and rabbit anti-TDP-43 antibody were used. Anap, TDP-43-Ab, and G3BP1-Ab signals are shown in blue, gray, and green, respectively; for merged channels, Anap was pseudo-colored red. Scale bars: 10 μm (overview), 3 μm (zoom). (K) Immunoblotting of wild-type TDP-43, TDP-43-Anap, and PFKP proteins in TDP-43 knockout HeLa cells. The rabbit anti-TDP-43 antibody and rabbit anti-PFKP antibody were used. KO: TDP-43 knockout; V100Stop/Anap: the expression of TDP-43^{V100TAG} via Anap labeling without or with the addition of Anap. (L) The expression levels of PFKP in TDP-43 knockout HeLa cells expressing wild-type TDP-43 or TDP-43-Anap. One-way ANOVA was used to compare the levels among groups. $n=3/\text{group}$. (M) Survival of TDP-43 knockout HeLa cells expressing TDP-43-Anap after treatment with 12.5 μM sodium arsenite for 24 hr. Calcein AM staining was used to quantify cell survival, and the relative survival rate was calculated as the ratio of sodium arsenite-treated to untreated cells for each group. OE: overexpression of TDP-43. One-way ANOVA was used to compare levels among groups. $n=3/\text{group}$. (N) Survival of inducible TDP-43 knockout (iTPDKO) mouse embryonic stem (ES) cells expressing TDP-43-Anap. Here, Cell Counting-Lite 2.0 Luminescent Cell Viability Assay Kit was used to detect the survival rate of ES cells. TDP-43 knockout was induced by 4-HT (300 ng/ml) for 5 days. The relative survival rate = 4-HT induction/DMSO for each group. One-way ANOVA was used to compare levels among groups. $n=3/\text{group}$. Colocalization threshold analysis was performed in Fiji/ImageJ to calculate the Pearson correlation coefficient (R) for each region of interest (A, B, I, J). The X and Y axes represent the fluorescence intensity values of the red and green channels, respectively. When signals are colocalized, pixels with high intensity in one channel correspond to high intensity in the other, forming a diagonal distribution. In contrast, non-colocalized signals cluster along the axes. A higher R value indicates a greater degree of colocalization. Scale bar, 3 μm . All quantitative data (F, G, L, M, N) are shown as mean $\pm$ SEM. *** $p<0.001$; **** $p<0.0001$; n.s., not significant.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. Excel file containing data used for quantitative analysis in **Figure 1F, G, L, M and N**.

Source data 2. PDF file containing original western blots for **Figure 1K and H**.

Source data 3. Original files of western blots for **Figure 1K and H**.

Figure supplement 1. Controls for Anap-based labeling of TDP-43 and G3BP1 in HeLa cells.

G3BP1-GFP, indicating that Anap labeling may provide a less perturbative approach for monitoring G3BP1 dynamics. Additionally, we examined the colocalization of G3BP1-Anap with TIA-1, another established stress granule marker. Under stress conditions, G3BP1-Anap largely colocalized with TIA-1 within stress granules. Interestingly, under basal conditions, the nuclear signal of G3BP1-Anap, which was not detected by antibody staining, appeared to partially colocalize with TIA-1 in several condensate-like structures (**Figure 1I**).

We next applied Anap labeling to TDP-43. Under basal conditions, the signal of TDP-43-Anap overlapped with that of anti-TDP-43 antibody staining, predominantly within the nucleus (**Figure 1B**). Following sodium arsenite treatment, TDP-43-Anap mislocalized mostly to the cytoplasmic inclusions stained by TDP-43 antibody, with a noticeable difference that a few puncta showed Anap signal only, and antibody gave a more dispersed signal. By contrast, TDP-43-YFP failed to recapitulate this cytoplasmic mislocalization, instead forming prominent nuclear puncta under stress conditions (**Figure 1E**), suggesting that large C-terminal tags may distort native localization of the protein. We then used YFP-tagged NLS-deleted TDP-43 (TDP-43^{ΔNLS}-YFP) as a reference and performed FRAP analysis to compare the mobility of TDP-43-Anap and TDP-43^{ΔNLS}-YFP. Fluorescence recovery of TDP-43-Anap reached ~45% within 20 s after photobleaching, consistent with liquid-like dynamics. In contrast, TDP-43^{ΔNLS}-YFP showed only ~22% recovery, suggesting more solid-like dynamics (**Figure 1D and G**). These results are consistent with previous reports describing relatively immobile aggregates formed by TDP-43^{ΔNLS} [Yan et al., 2025](#) and illustrate the advantage of Anap-based labeling, which preserves native protein properties and enables real-time assessment of protein dynamics without introducing disruptive mutations. To further investigate the relationship between TDP-43-Anap-positive cytoplasmic inclusions and stress granules, we performed co-immunostaining with a G3BP1 antibody. Under stress conditions, most TDP-43-Anap-positive cytoplasmic inclusions colocalized with G3BP1-positive stress granules. However, a small subset of puncta contained TDP-43-Anap but lacked detectable G3BP1, suggesting that not all mislocalized TDP-43 is incorporated into stress granules under

oxidative stress. These observations raise the possibility that additional mechanisms may contribute to the formation of TDP-43-positive cytoplasmic puncta (**Figure 1J**).

To determine whether TDP-43-Anap retains biological function, we expressed it in a TDP-43 knockout HeLa cell line (**Figure 1K**) and tested cell viability under oxidative stress. Following 12.5 μ M sodium arsenite treatment for 24 hr, the expression of TDP-43-Anap significantly rescued cell survival, reaching levels comparable to wild-type TDP-43 overexpression (45% vs 46%; **Figure 1M**). We further validated this finding in a mouse embryonic stem (ES) cell model with an inducible TDP-43 knockout (iTDPKO). For mouse ES cells, TDP-43 KO alone was sufficient to induce cell death. Here, either wild-type TDP-43 or TDP-43-Anap was expressed in iTDPKO mouse ES cells, where TDP-43 was deleted following induction with 4-hydroxytamoxifen (4-HT). Expression of TDP-43-Anap restored ES cell viability nearly to wild-type TDP-43 (47% vs 51%; **Figure 1N**). We also evaluated TDP-43-dependent RNA splicing activity by examining the expression of PFKF, a well-established target that undergoes cryptic exon inclusion upon loss of TDP-43 function (**Rothstein et al., 2025**). As shown in **Figure 1K and L**, expression of TDP-43-Anap in TDP-43 knockout HeLa cells restored PFKF expression, indicating that the Anap-labeled protein retains functional RNA splicing activity. These results demonstrate that TDP-43-Anap is capable of functionally compensating for endogenous TDP-43, supporting that the incorporation of Anap does not substantially disrupt the protein's biological function.

Furthermore, to extend Anap labeling to neuronal systems, we applied this approach to label both proteins in primary mouse cortical neurons. The neurons were co-stained with human-specific anti-TDP-43 or human-specific anti-G3BP1 antibodies. Under basal conditions, the signal of G3BP1-Anap colocalized with antibody staining in the cytoplasm and relocalized to stress granules upon sodium arsenite treatment (**Figure 2A and B**). Notably, nuclear Anap signal was again observed in neurons, suggesting additional pools of G3BP1 not captured by antibody staining.

TDP-43-Anap localized to the neuronal nucleus under basal conditions and showed strong colocalization with anti-TDP-43 antibody. Interestingly, antibody staining appeared more diffusely cytoplasmic than Anap, suggesting improved signal specificity of Anap labeling with direct genetic incorporation. Under oxidative stress, both Anap and antibody signals colocalized within cytoplasmic inclusions (**Figure 2C and D**). These findings demonstrated that the Anap system for G3BP1 and TDP-43 performs robustly in neuronal environments, a key setting for ALS/FTD research.

In summary, our results demonstrated that Anap-based GCE provides a minimally disruptive strategy for tracking the dynamic behavior of G3BP1 and TDP-43 in live cells (**Figure 3**). In G3BP1, Anap faithfully reported stress granule assembly and preserved native mobility, unlike GFP fusions that impaired dynamics. In TDP-43, Anap labeling maintained nuclear localization under basal conditions and revealed liquid-like behavior of cytoplasmic inclusions during stress, in contrast to the aberrant nuclear puncta produced by YFP-tagged TDP-43. Critically, Anap-labeled TDP-43 retained biological activity, rescuing cell survival in TDP-43-deficient HeLa and stem cells. Moreover, we have generated stable TDP-43-Anap cell lines that exhibited consistent expression, protein localization, and stress-induced aggregation, providing stable cell models for TDP-43 research.

By enabling high-fidelity visualization of both stress granule dynamics and TDP-43 aggregation in live cells and primary neurons, Anap labeling bridges a critical gap between structural preservation and functional readout. The ability to monitor native protein behavior without perturbation provides a unique opportunity to study early events in ALS/FTD progression, such as stress granule maturation, protein cytoplasmic mislocalization, and aggregate fluidity, at a resolution inaccessible with conventional tagging approaches.

Materials and methods

General information

Oligonucleotide synthesis was performed by IDT, and Sanger sequencing of DNA plasmids and PCR products was performed by Quintara. L-ANAP (trifluoroacetate salt) (15436) used in this study was purchased from Cayman. For immunostaining, the following primary and secondary antibodies were used: mouse anti-human G3BP (BD Biosciences, 611126), rabbit anti-TDP-43 (Proteintech, 10782-2-AP), mouse anti-TDP-43 (human-specific, monoclonal; Proteintech, 60019-2-Ig), rabbit anti-human TIA-1 (MBL Life Science, RN014P), chicken anti- β -tubulin (Tuj1; GeneTex, GTX85469), donkey anti-mouse Alexa Fluor 555 (Thermo Fisher, A-31570), donkey anti-rabbit Alexa Fluor 488 (Thermo Fisher,

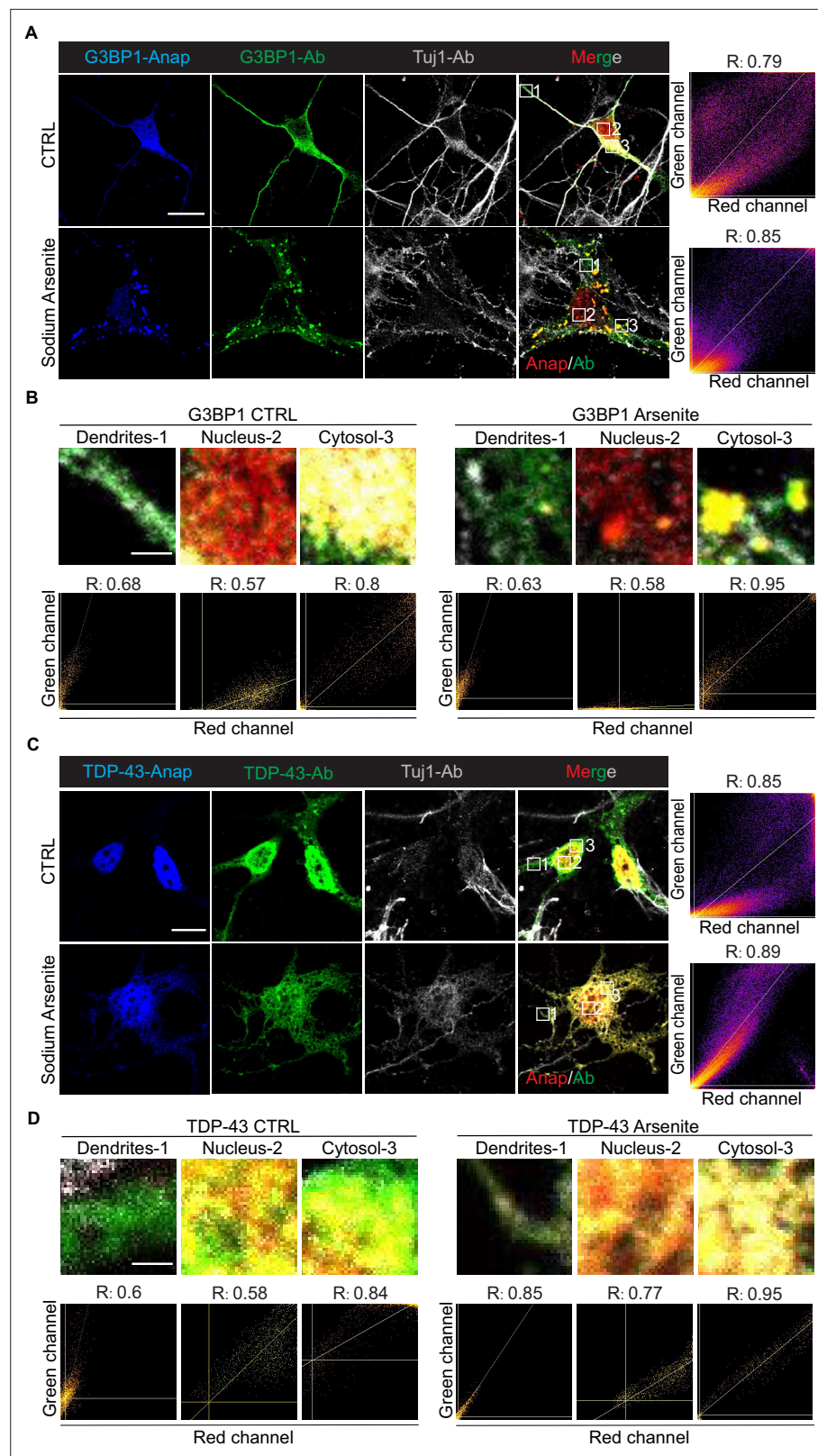


Figure 2. Anap labeling of TDP-43 and G3BP1 in neurons. **(A, C)** Primary mouse cortical neurons expressing G3BP1-Anap and TDP-43-Anap under basal conditions or 250 μ M sodium arsenite treatment. Cells were stained with anti-G3BP1 (human-specific) or anti-TDP-43 (human-specific) antibodies with chicken anti-Tuj1 as a neuron marker. Signals: Anap (blue, pseudo-colored red in merged images), antibody (green), Tuj1 (gray). Scale bar, 10 μ m.

Figure 2 continued on next page

Figure 2 continued

(B, D) Colocalization levels for each region of interest for G3BP1 and TDP-43. Colocalization threshold analysis was performed in Fiji/ImageJ to calculate the Pearson correlation coefficient (R) for each region of interest. The X and Y axes represent the fluorescence intensity values of the red and green channels, respectively. When signals are colocalized, pixels with high intensity in one channel correspond to high intensity in the other, forming a diagonal distribution. In contrast, non-colocalized signals cluster along the axes. A higher R value indicates a greater degree of colocalization. Scale bar, 1 μ m.

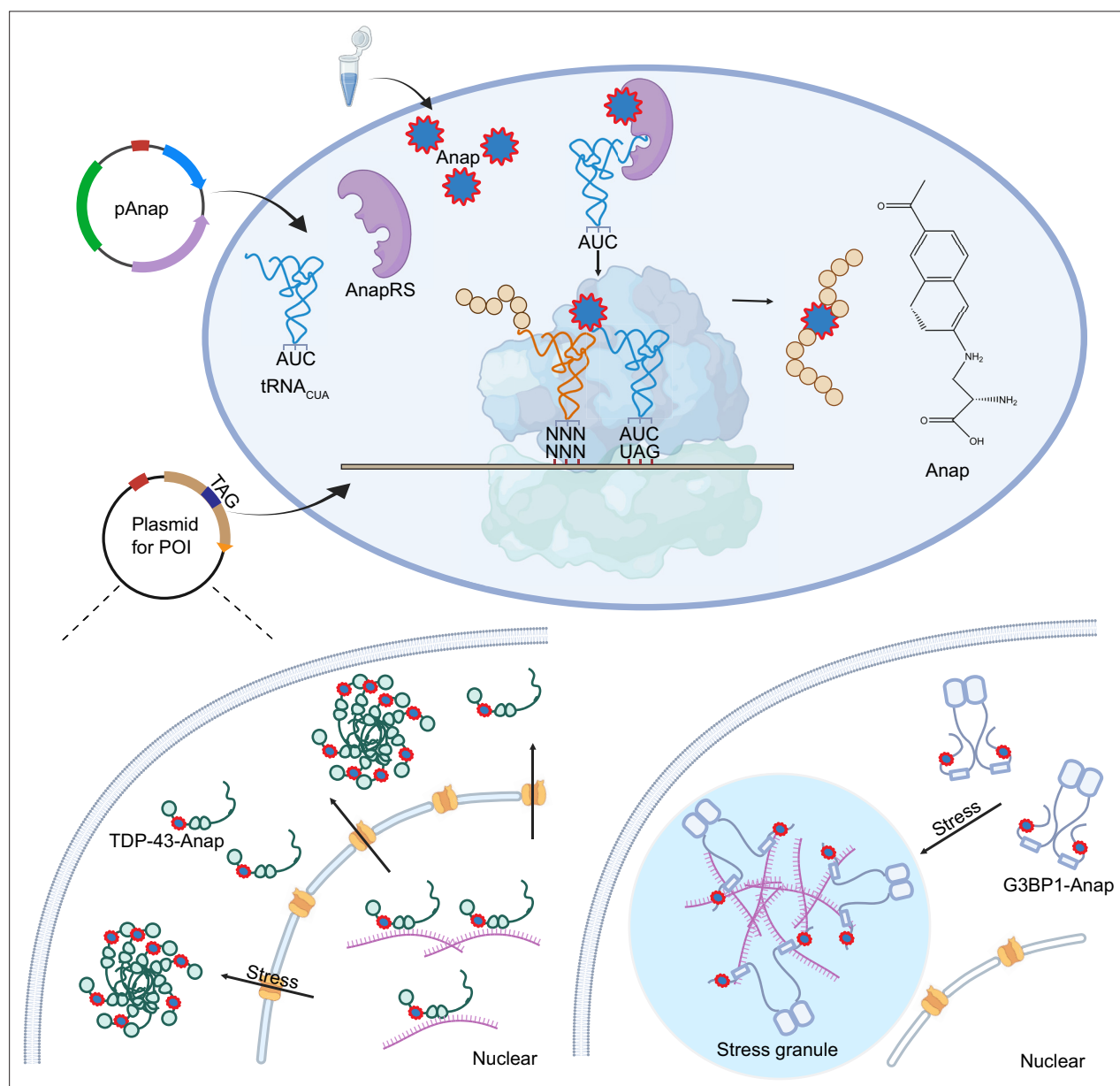


Figure 3. Schematic of the Anap labeling system for G3BP1 and TDP-43 using genetic code expansion. Briefly, two plasmids were required to express the protein with site-specific Anap incorporation, one for Anap incorporation and one for the mutated protein of interest (TAG introduction). After the plasmids were transfected into cells, the orthogonal Anap-tRNA synthetase would charge Anap, a fluorescent amino acid, onto its cognate tRNA, and the tRNA would incorporate Anap site-specifically into the protein of interest in response to the TAG stop codon. Cells expressing Anap-labeled TDP-43 and G3BP1 were subsequently imaged by confocal microscopy, either after fixation or in live-cell conditions. Under stress conditions, TDP-43-Anap redistributes to the cytoplasmic inclusions, and G3BP1-Anap assembles into stress granules.

A-21202), donkey anti-chicken Alexa Fluor 647 (Thermo Fisher, A78952), and donkey anti-rabbit Alexa Fluor Plus 800 (Thermo Fisher, A32808).

Plasmids

pAnap plasmid was a gift from Peter G. Schultz's lab ([Chatterjee et al., 2013](#)). pCMV-G3BP1 was purchased from Sino Biologicals. pRK5-TDP-43, pEGFP-G3BP1, and pCMV/TO-TDP-43-YFP were generated by our lab. pLVX-Puro-TDP-43-WT was a gift from Shawn Ferguson's lab ([Roczniak-Ferguson and Ferguson, 2019](#)) and reconstructed into pLVX-Puro-TDP-43-ΔNLS. Site-directed mutagenesis was conducted using the NEB Q5 site-directed mutagenesis kit (NEB, E0554). TAG substitutions were introduced by PCR amplification with Q5 Hot Start High-Fidelity DNA Polymerase using primers designed with NEBaseChanger. Then the PCR products were incubated with an enzyme mix consisting of a kinase, a ligase, and DpnI to rapidly circularize the PCR products and remove the template DNA. And then the mix will be transformed into DH5α cells.

Cell culture

Mammalian cell lines were cultured at 37 °C and 5% CO₂ in humidified incubators. HeLa cells (ATCC) were cultured in DMEM/F12 (Corning, 10–013-CV) supplemented with 10% FBS (Gibco, A5256801). The TDP-43 knockout HeLa cell line was a gift from Shawn M. Ferguson ([Roczniak-Ferguson and Ferguson, 2019](#)) and the G3BP knockout U2OS cell line was a gift from J. Paul Taylor ([Yang et al., 2020](#)). Primary cortical neurons were prepared as previously described ([Chen et al., 2021](#)). Briefly, the cortex of the mouse embryo was separated into HBSS on ice, then digested into a single-cell suspension with 0.25% trypsin and 0.1 mg/ml DNase I at 37 °C for 20 min. Dissociated cells were washed twice and resuspended in plating medium (DMEM supplemented with 10% FBS) before seeding onto poly-D-lysine (Gibco, A3890401)-coated plates. Cells were cultured for 3–4 hr in plating medium to allow attachment, and then the medium was replaced with maintenance medium (Neurobasal medium (Gibco, 21103049)) supplemented with 2% B-27 supplement (Gibco, 17504044), 1% GlutaMax (Gibco, 35050061), and 1% penicillin/streptomycin (Gibco, 15140163). The inducible TDP-43 knockout (iTDPKO) mouse embryonic stem (ES) cell line was a gift from Philip Wong ([Chiang et al., 2010](#)). Cells were maintained on an attachment factor (Gibco, S-006–100) coated plates in 2i medium containing half of DMEM/F12 and half of Neurobasal medium supplemented with L-glutamine (Gibco, 25030081), B-27 supplement, N2 supplement (Gibco, 17502048), BSA (Gibco, 15260037), 1% penicillin/streptomycin (Gibco, 15140163), PD0325901 (MedChemExpress, 391210-10-9), CHIR99021 (MedChemExpress, 252917-06-9), monothiolglycerol (Millipore Sigma, M6145), and mLIF (Millipore Sigma, ESG1107). All cell lines used in this study were routinely tested and confirmed to be negative for mycoplasma contamination using the Mycolor One-step Mycoplasma Detector Kit (Vazyme). Cell line authentication was performed by short tandem repeat analysis.

Transfection

HeLa cells were transfected using Lipofectamine 2000 reagent (Thermo Fisher, 11668030). Cells were seeded 24 hr before transfection, and plasmid DNA was mixed with Lipofectamine 2000 at a ratio of 1 μg DNA: 2 μL reagent in Opti-MEM medium (Thermo Fisher, 31985070). After 15 min incubation at room temperature, the complexes were added to the cells. Six hours post-transfection, the medium was replaced with fresh growth medium supplemented with or without 20 μM L-Anap, and cells were incubated overnight before further experiments.

Mouse primary cortical neurons and inducible TDP-43 knockout (iTDPKO) mouse ES cells were transfected using Lipofectamine 3000 (Thermo Fisher, L3000015) according to the manufacturer's instructions. For neurons, first, the total DNA was diluted and mixed with p3000 reagent in Opti-MEM medium, and the Lipofectamine 3000 was diluted in Opti-MEM medium separately. Second, both diluted DNA and Lipofectamine 3000 were mixed and incubated at room temperature for 15 min. The final mix would then be added to cells seeded on poly-D-lysine-coated plates at DIV5. After incubation overnight, the medium was half-replaced with fresh neurobasal medium with 10 μM Anap, and the neurons were incubated for two additional days (the medium was half-replaced each day to keep the Anap supply). Differently, for mouse ES cells, the DNA-lipo3000 mix was added directly into the cell suspension, and the suspension was added to an attachment factor-coated plate. After overnight

incubation, the medium for ES cells was replaced with fresh 2i medium supplemented with 10 μ M Anap and any further treatments.

Anap labeling and immunofluorescence in cell fixation

Six hours post-transfection, the cells were cultured in medium supplemented with L-Anap for another 20–24 hr. Cells were then washed three times with DPBS to remove excess Anap and incubated in fresh medium for 1 hr before treatment with 250 μ M sodium arsenite for another 1 hr. After the treatment, the cells were washed with DPBS three times and then fixed with 4% paraformaldehyde (PFA; Millipore Sigma, 158127) for 15–20 min at room temperature. Fixed cells were permeabilized and blocked in immunofluorescence blocking buffer (Cell Signaling Technology, 12411) containing 0.1% Triton X-100 (Millipore Sigma, X100) for 45 min. Immunostaining was performed using anti-TDP-43 and anti-G3BP1 antibodies in both HeLa cells and primary cortical neurons, with anti-Tuj1 antibody included as a neuronal marker. Images were acquired using a Leica SP8 confocal microscope.

Anap labeling and GFP/YFP tagging in live-cell imaging

For Anap labeling, HeLa cells were seeded on 35 mm glass-bottom dishes (Cellvis, D35-20-1.5H), transfected, and incubated with L-Anap following the same protocol used for fixed-cell imaging. After removal of excess Anap, cells were cultured in fresh medium for 2–3 hr before live-cell imaging. For GFP/YFP tagging, cells expressing the tagged proteins were directly ready for live-cell imaging. Before FRAP, cells expressing Anap-labeled or GFP/YFP-tagged TDP-43 or G3BP1 were treated with 250 μ M sodium arsenite for 1 hr. Next, regions of interest (ROIs) corresponding to protein signals were selected for FRAP analysis using a Leica SP8 confocal microscope.

Cell survival tests

Cell viability was assessed using Calcein AM staining (Invitrogen, C1430). HeLa cells were treated with 12.5 μ M sodium arsenite for 24 hr, followed by incubation with 3 μ M Calcein AM. Fluorescence from viable cells was measured using a Synergy H1 Hybrid Multi-Mode Plate Reader (BioTek) at excitation/emission wavelengths of 485/535 nm.

For mouse ES cells, the conditional knockout of TDP-43 was induced by 4-hydroxytamoxifen (4-HT, Millipore Sigma, H7904-5MG) treatment (300 ng/ml) for 5 days. Cell viability was assessed using the Cell Counting-Lite 2.0 Luminescent Cell Viability Assay Kit (Vazyme, DD1101-02).

Immunoblotting

Cells were washed three times with PBS and lysed in ice-cold RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS, 100 mM NaF, 0.5% sodium deoxycholate, 17.5 mM beta-glycerophosphate, 1 mM PMSF, and protease inhibitor cocktail (1:200, Millipore Sigma, P8340)]. Lysates were sonicated and clarified by centrifugation (12,000 rpm, 20 min, 4 °C), and supernatants were collected. Protein concentrations were determined using the BCA assay (Thermo Fisher, Cat. No. 23225). Equal amounts of protein were resolved by SDS-PAGE and transferred to membranes, which were blocked with 5% BSA and incubated with primary antibodies overnight at 4 °C. After three washes with TBST, membranes were incubated with fluorescence-conjugated secondary antibody dilution at room temperature for 2 hr. Blots were scanned and imaged using a LI-COR Odyssey M scanner.

Image analysis and statistical analysis

All images were processed and analyzed by Fiji/ImageJ software, and the colocalization of signals was analyzed by the colocalization threshold analysis plugin in ImageJ. Experiments requiring quantification were repeated at least three times independently. The FRAP results were analyzed by ImageJ to quantify the fluorescence intensity at each time point. The average relative intensities (normalized to pre-bleaching intensity) of each time point were analyzed in Prism 10 software. For survival tests, the average relative rates (normalized to the control group) for each group were analyzed in Prism 10 software, and the one-way analysis of variance (ANOVA) was used to compare the significance between each group. All data were presented as means $\pm$ SEM.

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Author contributions

Hao Chen, Conceptualization, Resources, Data curation, Software, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing – original draft, Writing – review and editing; Haocheng Wang, Formal analysis, Validation, Visualization; Yu-Ning Lu, Data curation, Validation, Visualization, Writing – review and editing; Peng Chen, Tao Zhang, Resources, Writing – review and editing; Zhongfan Zheng, Resources, Visualization, Writing – review and editing; Jiou Wang, Conceptualization, Resources, Supervision, Funding acquisition, Investigation, Methodology, Writing – original draft, Project administration, Writing – review and editing

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Additional files

Supplementary files

MDAR checklist

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files; source data files have been provided for **Figure 1**.

References

- Arsić A, Hagemann C, Stajković N, Schubert T, Nikić-Spiegel I. 2022. Minimal genetically encoded tags for fluorescent protein labeling in living neurons. *Nature Communications* **13**:314. DOI: <https://doi.org/10.1038/s41467-022-27956-y>, PMID: 35031604
- Bessa-Neto D, Beliu G, Kuhlemann A, Pecoraro V, Doose S, Retailleau N, Chevrier N, Perrais D, Sauer M, Choquet D. 2021. Bioorthogonal labeling of transmembrane proteins with non-canonical amino acids unveils masked epitopes in live neurons. *Nature Communications* **12**:6715. DOI: <https://doi.org/10.1038/s41467-021-27025-w>, PMID: 34795271
- Chatterjee A, Guo J, Lee HS, Schultz PG. 2013. A genetically encoded fluorescent probe in mammalian cells. *Journal of the American Chemical Society* **135**:12540–12543. DOI: <https://doi.org/10.1021/ja4059553>, PMID: 23924161
- Chen P, Jing H, Xiong M, Zhang Q, Lin D, Ren D, Wang S, Yin D, Chen Y, Zhou T, Li B, Fei E, Pan BX. 2021. Spine impairment in mice high-expressing neuregulin 1 due to LIMK1 activation. *Cell Death & Disease* **12**:403. DOI: <https://doi.org/10.1038/s41419-021-03687-8>, PMID: 33854034
- Chiang P-M, Ling J, Jeong YH, Price DL, Aja SM, Wong PC. 2010. Deletion of TDP-43 down-regulates Tbc1d1, a gene linked to obesity, and alters body fat metabolism. *PNAS* **107**:16320–16324. DOI: <https://doi.org/10.1073/pnas.1002176107>, PMID: 20660762
- Criat G, Taraska JW. 2012. Imaging proteins inside cells with fluorescent tags. *Trends in Biotechnology* **30**:8–16. DOI: <https://doi.org/10.1016/j.tibtech.2011.08.002>, PMID: 21924508
- Eddins AJ, Gangarde YM, Singh A, Jana S, Zheng Y, Alexander ND, Reitsma JM, Cooley RB, Karplus PA, Mehl RA. 2025. Quantitative protein labeling in live cells by controlling the redox state of encoded tetrazines. *Journal of the American Chemical Society* **147**:23625–23634. DOI: <https://doi.org/10.1021/jacs.5c04605>, PMID: 40560690
- Gasset-Rosa F, Lu S, Yu H, Chen C, Melamed Z, Guo L, Shorter J, Da Cruz S, Cleveland DW. 2019. Cytoplasmic TDP-43 de-mixing independent of stress granules drives inhibition of nuclear import, loss of nuclear TDP-43, and cell death. *Neuron* **102**:339–357. DOI: <https://doi.org/10.1016/j.neuron.2019.02.038>, PMID: 30853299
- Hao M, Ling X, Sun Y, Wang X, Li W, Chang L, Zeng Z, Shi X, Niu M, Chen L, Liu T. 2024. Tracking endogenous proteins based on RNA editing-mediated genetic code expansion. *Nature Chemical Biology* **20**:721–731. DOI: <https://doi.org/10.1038/s41589-023-01533-w>, PMID: 38302606
- Kassouf T, Shrivastava R, Meszka I, Bailly A, Polanowska J, Trauchessec H, Mandrioli J, Carra S, Xiroidimas DP. 2023. Targeting the NEDP1 enzyme to ameliorate ALS phenotypes through stress granule disassembly. *Science Advances* **9**:eabq7585. DOI: <https://doi.org/10.1126/sciadv.abq7585>, PMID: 37000881
- Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, Mackenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM-Y. 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**:130–133. DOI: <https://doi.org/10.1126/science.1134108>, PMID: 17023659
- Nikić I, Kang JH, Girona GE, Aramburu IV, Lemke EA. 2015. Labeling proteins on live mammalian cells using click chemistry. *Nature Protocols* **10**:780–791. DOI: <https://doi.org/10.1038/nprot.2015.045>, PMID: 25906116
- Nygaard A, Zachariassen LG, Larsen KS, Kristensen AS, Loland CJ. 2024. Fluorescent non-canonical amino acid provides insight into the human serotonin transporter. *Nature Communications* **15**:9267. DOI: <https://doi.org/10.1038/s41467-024-53584-9>, PMID: 39463388
- Roczniak-Ferguson A, Ferguson SM. 2019. Pleiotropic requirements for human TDP-43 in the regulation of cell and organelle homeostasis. *Life Science Alliance* **2**:e201900358. DOI: <https://doi.org/10.26508/lsa.201900358>, PMID: 31527135
- Rothstein JD, Keeley O, Warlick C, Miller TM, Ly CV, Glass JD, Coyne AN. 2025. Sporadic ALS induced pluripotent stem cell derived neurons reveal hallmarks of TDP-43 loss of function. *Nature Communications* **16**:7092. DOI: <https://doi.org/10.1038/s41467-025-62482-7>, PMID: 40753166
- Suk TR, Rousseaux MWC. 2020. The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. *Molecular Neurodegeneration* **15**:45. DOI: <https://doi.org/10.1186/s13024-020-00397-1>, PMID: 32799899

- Van Nerom M**, Ahmed J, Lazar T, Meszaros A, Galand Q, De Malsche W, Van Lindt J, Pancsa R, Maes D, Tompa P. 2024. C9orf72-linked arginine-rich dipeptide repeats aggravate pathological phase separation of G3BP1. *PNAS* **121**:e2402847121. DOI: <https://doi.org/10.1073/pnas.2402847121>, PMID: 39621905
- Wolozin B**, Ivanov P. 2019. Stress granules and neurodegeneration. *Nature Reviews. Neuroscience* **20**:649–666. DOI: <https://doi.org/10.1038/s41583-019-0222-5>, PMID: 31582840
- Yan X**, Kuster D, Mohanty P, Nijssen J, Pombo-García K, Garcia Morato J, Rizuan A, Franzmann TM, Sergeeva A, Ly AM, Liu F, Passos PM, George L, Wang SH, Shenoy J, Danielson HL, Ozguney B, Honigmann A, Ayala YM, Fawzi NL, et al. 2025. Intra-condensate demixing of TDP-43 inside stress granules generates pathological aggregates. *Cell* **188**:4123–4140. DOI: <https://doi.org/10.1016/j.cell.2025.04.039>, PMID: 40412392
- Yang P**, Mathieu C, Kolaitis R-M, Zhang P, Messing J, Yurtsever U, Yang Z, Wu J, Li Y, Pan Q, Yu J, Martin EW, Mittag T, Kim HJ, Taylor JP. 2020. G3BP1 is a tunable switch that triggers phase separation to assemble stress granules. *Cell* **181**:325–345.. DOI: <https://doi.org/10.1016/j.cell.2020.03.046>, PMID: 32302571