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

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Key words: Genetic code expansion, Anap labeling, Stress granule, TDP-43

Abstract

We report a minimally disruptive labeling strategy for stress granule protein G3BP1 and ALS-linked protein TDP-43 using the fluorescent noncanonical amino acid Anap. By integrating genetic code expansion 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.

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[1], 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[2]. Alternatively, genetic code expansion (GCE) has emerged as a versatile labeling strategy to label proteins site-specifically in a minimally disruptive manner[3-8].

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-aminopropanoic acid) is especially attractive for live imaging. Anap is intrinsically fluorescent, exhibits polarity-sensitive emission spectra, and requires no post-incorporation modification[2, 5]. 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[9]. Chronically aberrant dynamics of stress granules with observed cytotoxicity have also been implicated as a common feature of ALS/FTD[10, 11]. TDP-43 cytoplasmic inclusion is a hallmark of ALS/FTD pathology[12] and is closely associated with dysregulation of RNA metabolism, ultimately leading to cellular defects[13]. Both proteins undergo dynamic and stress-

49 dependent redistribution[9], making them ideal candidates for minimally invasive, site-specific
50 fluorescent labeling.

51 **Results and Discussion**

52 To implement the site-specific Anap incorporation system, we selected and generated two
53 amber mutants, G3BP1^{F337TAG} and TDP-43^{V100TAG}, using a combination of structural and
54 functional criteria: exclusion from functional domains or localization signals, absence of disease-
55 associated mutations, lack of post-translational modification, and low predicted structural impact
56 by AlphaFold models. Incorporation of Anap was achieved via co-expression of an orthogonal
57 tRNA/synthetase pair in cells.

58 We first tested the feasibility of the Anap labeling system for G3BP1. In HeLa cells, G3BP1-
59 Anap localized diffusely in the cytoplasm under basal conditions, closely matching antibody
60 staining. Interestingly, a nuclear signal was detected with Anap but not with antibody, indicating
61 the presence of nuclear pools of G3BP1 inaccessible to antibody detection. Upon sodium
62 arsenite treatment, both the Anap and antibody signals colocalized within stress granules (Fig.
63 1A), validating the ability of Anap labeling to visualize the dynamics of G3BP1-driven stress
64 granule formation. To assess labeling fidelity, we compared the performance of G3BP1-Anap
65 with G3BP1-GFP using fluorescence recovery after photobleaching (FRAP). Following stress,
66 both labeled proteins localized to granules, but G3BP1-Anap exhibited significantly higher
67 fluorescence recovery (~53%) than G3BP1-GFP (~33%) (Fig. 1C, 1F). These results suggested
68 that Anap labeling better preserved the native mobility and biophysical properties of G3BP1
69 than the conventional GFP tag.

70 We next applied Anap labeling to TDP-43. Under basal conditions, the signal of TDP-43-Anap
71 overlapped with that of anti-TDP-43 antibody staining, predominantly within the nucleus (Fig.
72 1B). Following sodium arsenite treatment, TDP-43-Anap mislocalized to cytoplasmic inclusions,
73 validated by antibody staining. By contrast, TDP-43-YFP failed to recapitulate this cytoplasmic

74 mislocalization, instead forming prominent nuclear puncta under stress conditions (Fig. 1E),
75 suggesting that large C-terminal tags may distort native localization of the protein. We then
76 performed FRAP analysis to test the mobility of TDP-43-Anap in these cytoplasmic inclusions.
77 Fluorescence recovery of TDP-43-Anap reached ~45% within 20 seconds post-bleaching (Fig.
78 1D, 1G), consistent with liquid-like dynamics. This differs from previous reports of immobile
79 aggregates in nuclear localization signal (NLS)-deleted TDP-43[14], illustrating the value of Anap
80 labeling in preserving native protein properties, allowing real-time assessment of pathological
81 dynamics without requiring disruptive mutations.

82 To determine whether TDP-43-Anap retains biological function, we expressed it in a TDP-43
83 knockout HeLa cell line (Fig. 1H) and tested cell viability under oxidative stress. Following 12.5
84 μM sodium arsenite treatment for 24 hours, the expression of TDP-43-Anap significantly
85 rescued cell survival, reaching levels comparable to wild-type TDP-43 overexpression (45% vs
86 46%; Fig. 1I). We further validated this finding in a mouse embryonic stem (ES) cell model with
87 an inducible TDP-43 knockout (iTDPKO). For mouse ES cells, TDP-43 KO alone was sufficient
88 to induce cell death. Here, either wild-type TDP-43 or TDP-43-Anap was expressed in iTDPKO
89 mouse ES cells, where TDP-43 was deleted following induction with 4-hydroxytamoxifen (4-HT).
90 Expression of TDP-43-Anap restored ES cell viability nearly to wild-type TDP-43 (47% vs 51%;
91 Fig. 1J). Together, these results confirmed that Anap incorporation preserves the biological
92 function of TDP-43.

93 Furthermore, to extend Anap labeling to neuronal systems, we applied this approach to label
94 both proteins in primary mouse cortical neurons. The neurons were co-stained with human-
95 specific anti-TDP-43 or human-specific anti-G3BP1 antibodies. Under basal conditions, the
96 signal of G3BP1-Anap colocalized with antibody staining in the cytoplasm and relocalized to
97 stress granule upon sodium arsenite treatment (Fig. 2A, 2B). 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 (Fig. 2C, 2D). 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 genetic code expansion provides a minimally disruptive strategy for tracking the dynamic behavior of G3BP1 and TDP-43 in live cells (Fig. 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.

Acknowledgements

This work was supported by grants from NIH (NS110098). We thank Dr. Peter Schultz from Scripps Research for providing the pAnap plasmid, Dr. Shawn Ferguson from Yale University for the TDP-43 knockout HeLa cell line, and Dr. Philip Wong from Johns Hopkins University for the iTDPKO mouse ES cell line. We also thank the members of Wang's lab for discussion and suggestions.

Author contributions

H.C. performed and analyzed most of the experiments. H.C.W. helped with plasmid preparation, western blot, and imaging. Y.N.L. provides cells and helped with data preparation. P.C. provides mouse primary cortical neurons. Z.F.Z helped with imaging. T.Z. helped culture iTDPKO mouse ES cells. H.C. and J.W. designed the studies and wrote the paper with inputs from other authors.

Methods and materials

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), chicken anti-βIII-tubulin (Tuj1; GeneTex, GTX85469), donkey anti-mouse Alexa Fluor 555 (Thermo Fisher, A-31570), donkey anti-rabbit Alexa Fluor 488 (Thermo Fisher, A-21202), donkey anti-chicken Alexa Fluor 647 (Thermo Fisher, A78952), and donkey anti-rabbit Alexa Fluor Plus 800 (Thermo Fisher, A32808).

Plasmids

147 pAnap plasmid was a gift from Peter Schultz's lab[2]. pCMV-G3BP1 was purchased from Sino
148 Biologics. pRK5-TDP-43, pEGFP-G3BP1, and pCMV/TO-TDP-43-YFP were conducted by our
149 lab. Site-directed mutagenesis was conducted using the NEB Q5 site-directed mutagenesis kit
150 (NEB, E0554). TAG substitutions were introduced by PCR amplification with Q5 Hot Start High-
151 Fidelity DNA Polymerase using primers designed with NEBaseChanger. Then the PCR products
152 were incubated with an enzyme mix consisting of a kinase, a ligase, and DpnI to rapidly
153 circularize the PCR products and remove the template DNA. And then the mix will be
154 transformed into DH5α cells.

155 **Cell culture**

156 Mammalian cell lines were cultured at 37°C and 5% CO_2 in humidified incubators. HeLa cells
157 (ATCC) were cultured in DMEM/F12 (Corning, 10-013-CV) supplemented with 10% FBS (Gibco,
158 A5256801). The TDP-43 knockout HeLa cell line was a gift from Dr. Shawn M. Ferguson[15].
159 Primary cortical neurons were prepared as previously described [16]. Briefly, the cortex of the
160 mouse embryo was separated into HBSS on ice, then digested into single-cell suspension with
161 0.25% trypsin and 0.1mg/ml DNase I at 37°C for 20 min. Dissociated cells were washed twice
162 and resuspended in plating media (DMEM supplemented with 10% FBS) before seeding onto
163 poly-D-lysine (Gibco, A3890401)-coated plates. Cells were cultured for 3-4 hours in plating
164 medium to settle down, and then the medium was replaced with maintenance medium
165 (neurobasal medium (Gibco, 21103049)) supplemented with 2% B-27 supplement
166 (Gibco,17504044), 1% GlutaMax (Gibco, 35050061), and 1% penicillin/streptomycin (Gibco,
167 15140163). The iTDPKO inducible mouse embryonic stem ES cell line was a gift from Wong's
168 lab[17]. Cells were maintained on attachment factor (Gibco, S-006-100) coated plates in 2i media
169 containing half of DMEM/F12 and half of Neurobasal media complemented with L-Glutamine
170 (Gibco, 25030081), B-27 supplement, N2 supplement (Gibco, 17502048), BSA (Gibco,
171 15260037), 1% penicillin/streptomycin (Gibco, 15140163), PD0325901 (MedChemExpress,

172 391210-10-9), CHIR99021(MedChemExpress, 252917-06-9), monothioglycerol(Millipore
173 Sigmal, M6145), and mlif(Millipore Sigma, ESG1107).

174 **Transfection**

175 HeLa cells were transfected using Lipofectamine 2000 reagent (ThermoFisher, 11668030). Cells
176 were seeded 24 h before transfection, and plasmid DNA was mixed with Lipofectamine 2000 at
177 a ratio of 1 μg DNA:2 μL reagent in Opti-MEM medium (Thermo Fisher, 31985070). After 15 min
178 incubation at room temperature, the complexes were added to the cells. Six hours post-
179 transfection, the medium was replaced with fresh growth medium supplemented with or without
180 20 μM L-Anap, and cells were incubated overnight before further experiments.

181 Mouse primary cortical neurons and inducible TDP-43 knockout (iTDPKO) mouse ES cells were
182 transfected using Lipofectamine 3000 (Thermo Fisher, L3000015) according to the
183 manufacturer's instructions. For neurons, first, the total DNA was diluted and mixed with p3000
184 reagent in Opti-MEM media, and the Lipofectamine 3000 was diluted in Opti-MEM media
185 separately. Second, both diluted DNA and Lipofectamine 3000 were mixed and incubated at
186 room temperature for 15 minutes. The final mix would then be added to cells seeded on poly-D-
187 lysine-coated plates at DIV5. After incubation overnight, the media were half-replaced with fresh
188 neurobasal media with 10μM Anap, and the neurons were incubated for two additional days (the
189 media were half-replaced each day to keep the Anap supply). Differently, for mouse ES cells,
190 the DNA-lipo3000 mix was added directly into the cell suspension, and the suspension was
191 added to attachment factor-coated plate. After overnight incubation, the media for ES cells was
192 replaced with fresh 2i media supplemented with 10μM Anap and any further treatments.

193 **Anap labeling and immunofluorescence in cell fixation**

194 6h post-transfection, the cells were cultured in medium supplemented with L-Anap for another
195 20-24 h. Cells were then washed three times with DPBS to remove excess Anap and incubated
196 in fresh medium for 1 h before treatment with 250 μM sodium arsenite for anther 1h. After the

197 treatment, the cells were washed with DPBS three times and then fixed with 4%
198 paraformaldehyde (PFA; Millipore Sigma, 158127) for 15-20 mins at room temperature. Fixed
199 cells were permeabilized and blocked in immunofluorescence blocking buffer (Cell Signaling
200 Technology, 12411) containing 0.1% Triton X-100 (Millipore Sigma, X100) for 45 min.
201 Immunostaining was performed using anti-TDP-43 and anti-G3BP1 antibodies in both HeLa
202 cells and primary cortical neurons, with anti-Tuj1 antibody included as a neuronal marker.
203 Images were acquired using a Leica SP8 confocal microscope.

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

205 For Anap labeling, HeLa cells were seeded on 35-mm glass-bottom dishes (Cellvis, D35-20-
206 1.5H), transfected, and incubated with L-Anap following the same protocol used for fixed-cell
207 imaging. After removal of excess Anap, cells were cultured in fresh medium for 2-3 hours before
208 live cell imaging. For GFP/YFP tagging, cells expressing the tagged proteins were directly ready
209 for live cell imaging. Before FRAP, cells expressing Anap-labeled or GFP/YFP-tagged TDP-43
210 or G3BP1 were treated with 250 μM sodium arsenite for 1 hour. Next, regions of interest (ROIs)
211 corresponding to protein signals were selected for FRAP analysis using a Leica SP8 confocal
212 microscope.

213 **Cell survival tests**

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

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

221 **Immunoblotting**

222 Cells were washed three times with PBS and lysed in ice-cold RIPA buffer [50 mM Tris-HCl (PH
223 7.5), 150 mM NaCl, 1% NP40, 0.1% SDS, 100 mM NaF, 0.5% sodium deoxycholate, 17.5 mM
224 beta-glycerophosphate, 1 mM PMSF, and protease inhibitor cocktail (1:200, Millipore Sigma,
225 P8340)]. Lysates were sonicated and clarified by centrifugation (12,000 rpm, 20 min, 4 °C), and
226 supernatants were collected. Protein concentrations were determined using the BCA assay
227 (Thermo Fisher, Cat. No. 23225). Equal amounts of protein were resolved by SDS-PAGE and
228 transferred to membranes, which were blocked with 5% BSA and incubated with primary
229 antibodies overnight at 4 °C. After three washes with TBST, membranes were incubated with
230 fluorescence-conjugated secondary antibody dilution at room temperature for 2h. Blots were
231 scanned and imaged by LI-COR Odyssey M scanner.

232 **Image analysis and statistical analysis**

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

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284 **Figure legend**

285 **Fig. 1: Anap labeling of TDP-43 and G3BP1 in HeLa cells.**

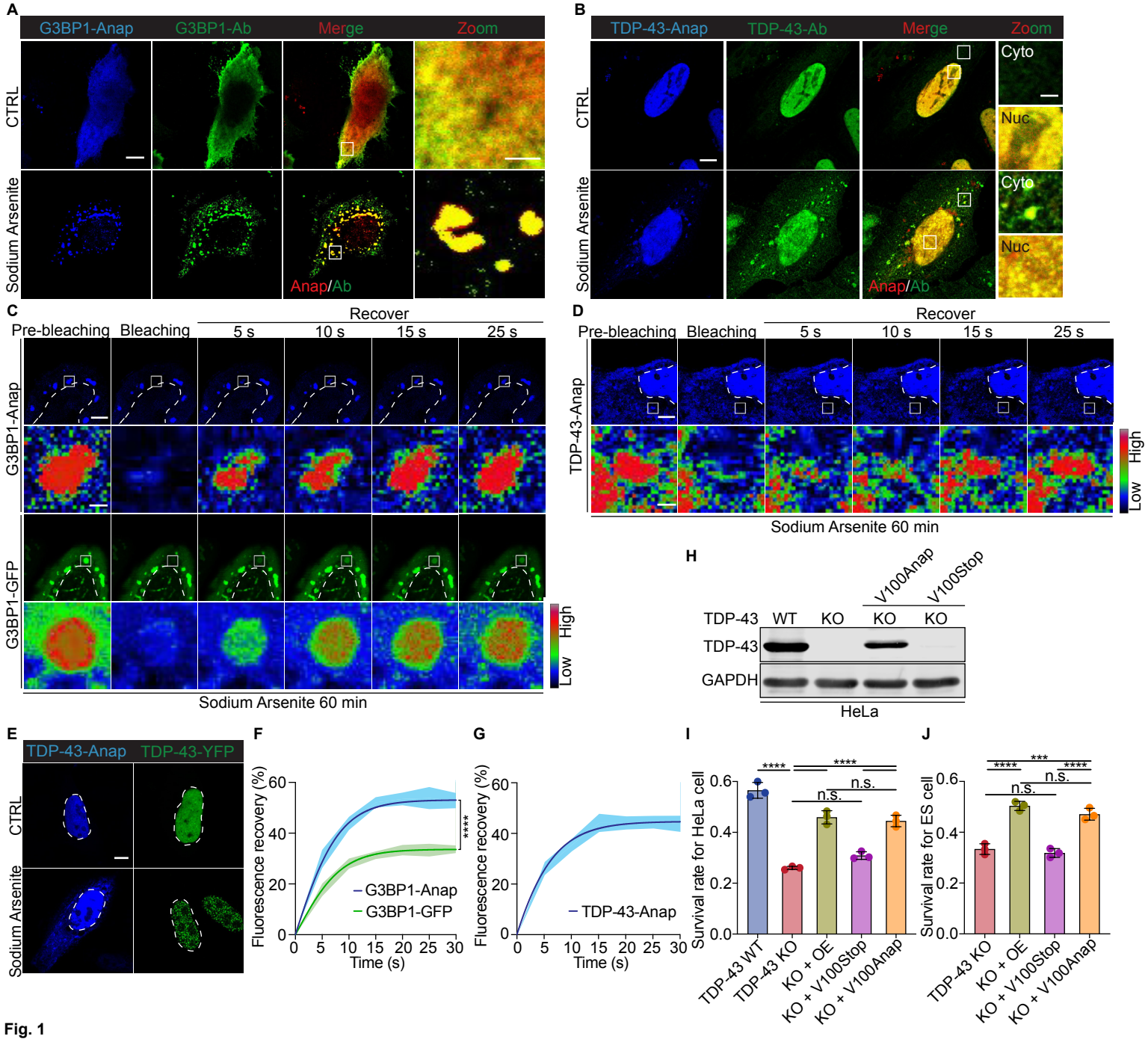
286 A, B. HeLa cells expressing G3BP1-Anap and TDP-43-Anap under basal conditions or 250 μM
287 sodium arsenite treatment. Anap and antibody signals are shown in blue and green,
288 respectively; for merged panels, Anap was pseudo-colored red. Scale bars: 10 μm (overview), 3
289 μm (zoom). C, D. FRAP of G3BP1-Anap, G3BP1-GFP, and TDP-43-Anap, following 250 μM
290 sodium arsenite treatment. ROI signal intensities are displayed in rainbow RGB (red–high, blue–
291 low). Scale bars: 5 μm (cells), 1 μm (ROI). E. Comparison of HeLa cells expressing TDP-43-
292 Anap and TDP-43-YFP under basal conditions or 250 μM sodium arsenite treatment. Here,
293 Anap labeling and YFP labeling yield a blue signal and a yellow to green signal, respectively. F,
294 G. Relative fluorescence recovery of each time point after photobleaching for G3BP1-Anap,
295 G3BP1-GFP, and TDP-43-Anap. H. Immunoblotting of wild-type TDP-43 and TDP-43-Anap. The
296 Rabbit anti-TDP-43 antibody was used. KO: TDP-43 knockout; V100Stop/Anap: the expression
297 of TDP-43^{V100TAG} via Anap labeling without or with the addition of Anap. I. Survival of TDP-43
298 knockout HeLa cells expressing TDP-43-Anap after being treated with 12.5 μM sodium arsenite
299 for 24h. Here, the calcien EM staining was used to detect the survival rate of cells, and the
300 relative survival rate= sodium arsenite treatment/ nontreatment for each group. OE:
301 overexpression of TDP-43. J. Survival of iTDPKO inducible mouse ES cells expressing TDP-43-
302 Anap. Here, cell counting-Lite 2.0 Luminescent cell viability assay kit was used to detect the
303 survival rate of ES cells. TDP-43 knockout was induced by 4-HT (300ng/ml) for 5 days. The
304 relative survival rate= 4-HT induction/DMSO for each group. All quantitative data (F, G, I, J) are
305 shown as mean ± SEM. ***P = 0.0001; ****P < 0.0001; n.s., not significant.

306 **Fig. 2: Anap labeling of TDP-43 and G3BP1 in neurons.**

307 A, C. Primary mouse cortical neurons expressing G3BP1-Anap and TDP-43-Anap under basal
308 conditions or 250 μM sodium arsenite treatment. Cells were stained with anti-G3BP1 (human-
309 specific) or anti-TDP-43 (human-specific) antibodies with chicken anti-Tuj1 as a neuron marker.
310 Signals: Anap (blue, pseudo-colored red in merged images), antibody (green), Tuj1 (gray).
311 Scale bar, 10 μm. B, D. The colocalization level of each region of interest for G3BP1 and TDP-
312 43. Colocalization threshold analysis in Fuji ImageJ was used to analyze the R value of each
313 region (a higher R value means a higher colocalization level). Scale bar, 1 μm.

314 **Fig. 3: Schematic of the Anap labeling system for G3BP1 and TDP-43 using genetic code**
315 **expansion.**

316 Briefly, two plasmids were required to express the protein with site-specific Anap incorporation,
317 one for Anap incorporation and one for the mutated protein of interest (TAG introduction). As the
318 plasmids were transfected into cells, the orthogonal Anap-tRNA synthetase would charge the
319 Anap to its cognate tRNA, and the tRNA would incorporate the Anap site-specifically into the
320 protein of interest in response to the TAG stop codon. Cells expressing Anap-labeled TDP-43
321 and G3BP1 were subsequently imaged by confocal microscopy, either after fixation or in live-
322 cell conditions.


A
G3BP1-Anap
G3BP1-Ab
Merge
Zoom
CTRL
Sodium Arsenite
Anap/Ab
B
TDP-43-Anap
TDP-43-Ab
Merge
Zoom
Cyto
Nuc
CTRL
Sodium Arsenite
Anap/Ab
C
Recover
Pre-bleaching
Bleaching
5 s
10 s
15 s
25 s
G3BP1-Anap
G3BP1-GFP
High
Low
Sodium Arsenite 60 min
D
Recover
Pre-bleaching
Bleaching
5 s
10 s
15 s
25 s
TDP-43-Anap
High
Low
Sodium Arsenite 60 min
H
V100Anap
V100Stop
TDP-43
WT
KO
KO
KO
TDP-43
GAPDH
HeLa
E
TDP-43-Anap
TDP-43-YFP
CTRL
Sodium Arsenite
F
Fluorescence recovery (%)
Time (s)
G3BP1-Anap
G3BP1-GFP
****
G
Fluorescence recovery (%)
Time (s)
TDP-43-Anap
I
Survival rate for HeLa cell
****
****
n.s.
n.s.
TDP-43 WT
TDP-43 KO
KO + OE
KO + V100Stop
KO + V100Anap
J
Survival rate for ES cell
***
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n.s.
n.s.
TDP-43 KO
KO + OE
KO + V100Stop
KO + V100Anap

Fig. 1

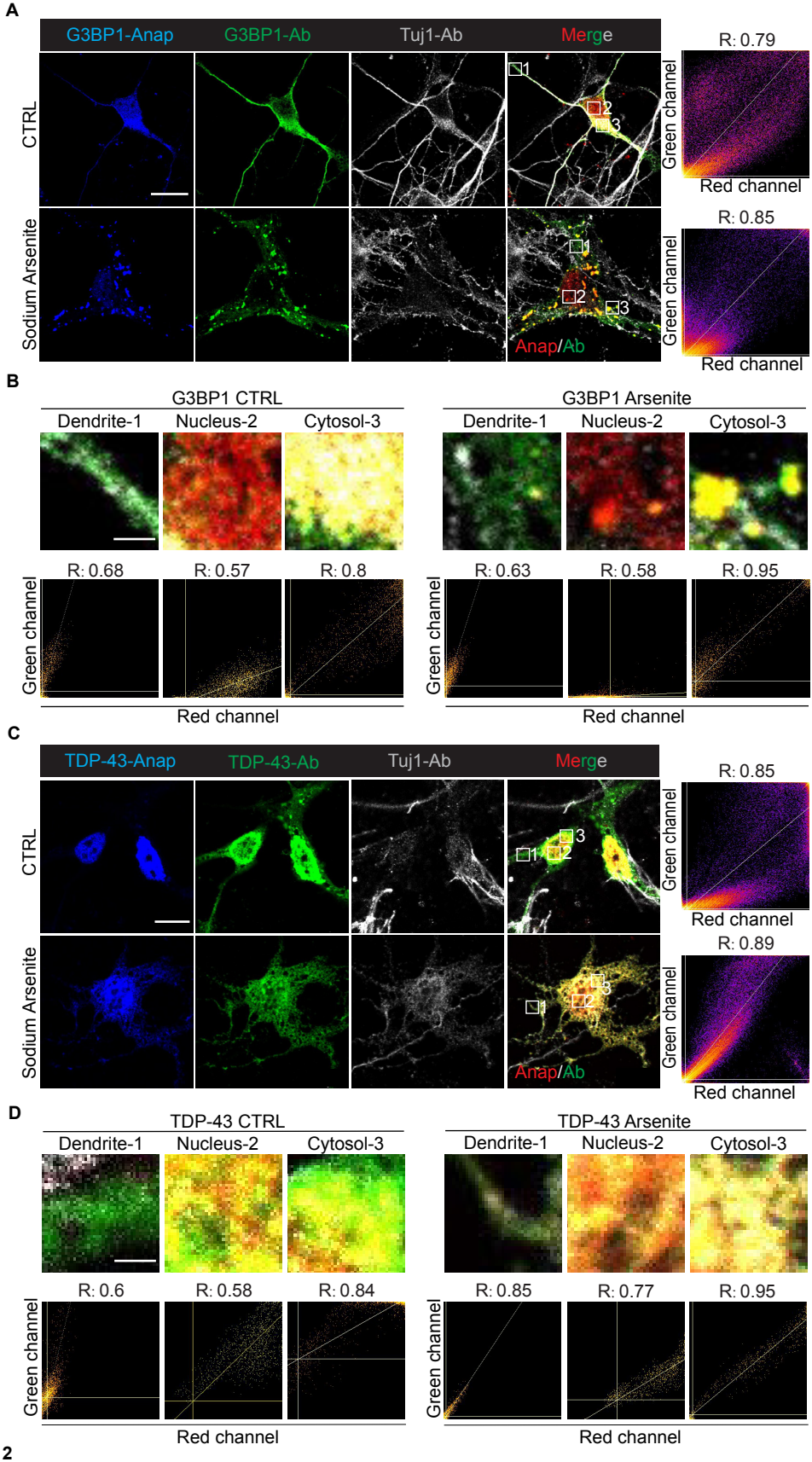
A
G3BP1-Anap
G3BP1-Ab
Tuj1-Ab
Merge
CTRL
Sodium Arsenite
R: 0.79
R: 0.85
Green channel
Red channel
Anap/Ab
B
G3BP1 CTRL
G3BP1 Arsenite
Dendrite-1
Nucleus-2
Cytosol-3
R: 0.68
R: 0.57
R: 0.8
R: 0.63
R: 0.58
R: 0.95
C
TDP-43-Anap
TDP-43-Ab
R: 0.85
R: 0.89
D
TDP-43 CTRL
TDP-43 Arsenite
R: 0.6
R: 0.58
R: 0.84
R: 0.85
R: 0.77
R: 0.95

Fig. 2

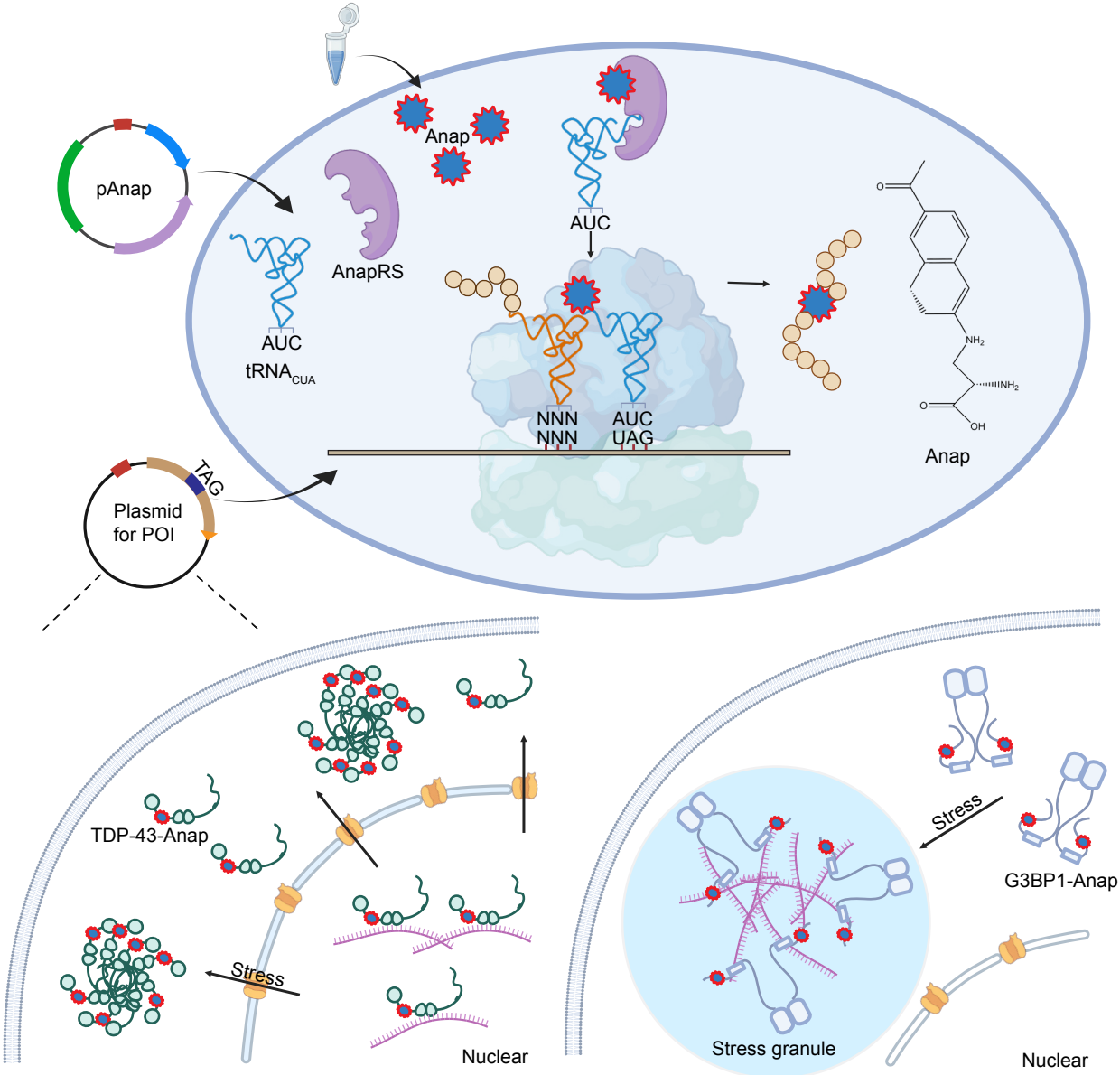
pAnap
Anap
AnapRS
AUC
tRNA$_{CUA}$
AUC
NNN
NNN
AUC
UAG
Anap
Plasmid
for POI
TAG
TDP-43-Anap
Stress
Nuclear
Stress
G3BP1-Anap
Stress granule
Nuclear

Fig. 3