Supplemental Information

Directed evolution of a genetically encoded photocatalyst for temporally resolved proximity labeling of subcellular RNAs and proteins

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SUPPLEMENTARY FIGURES

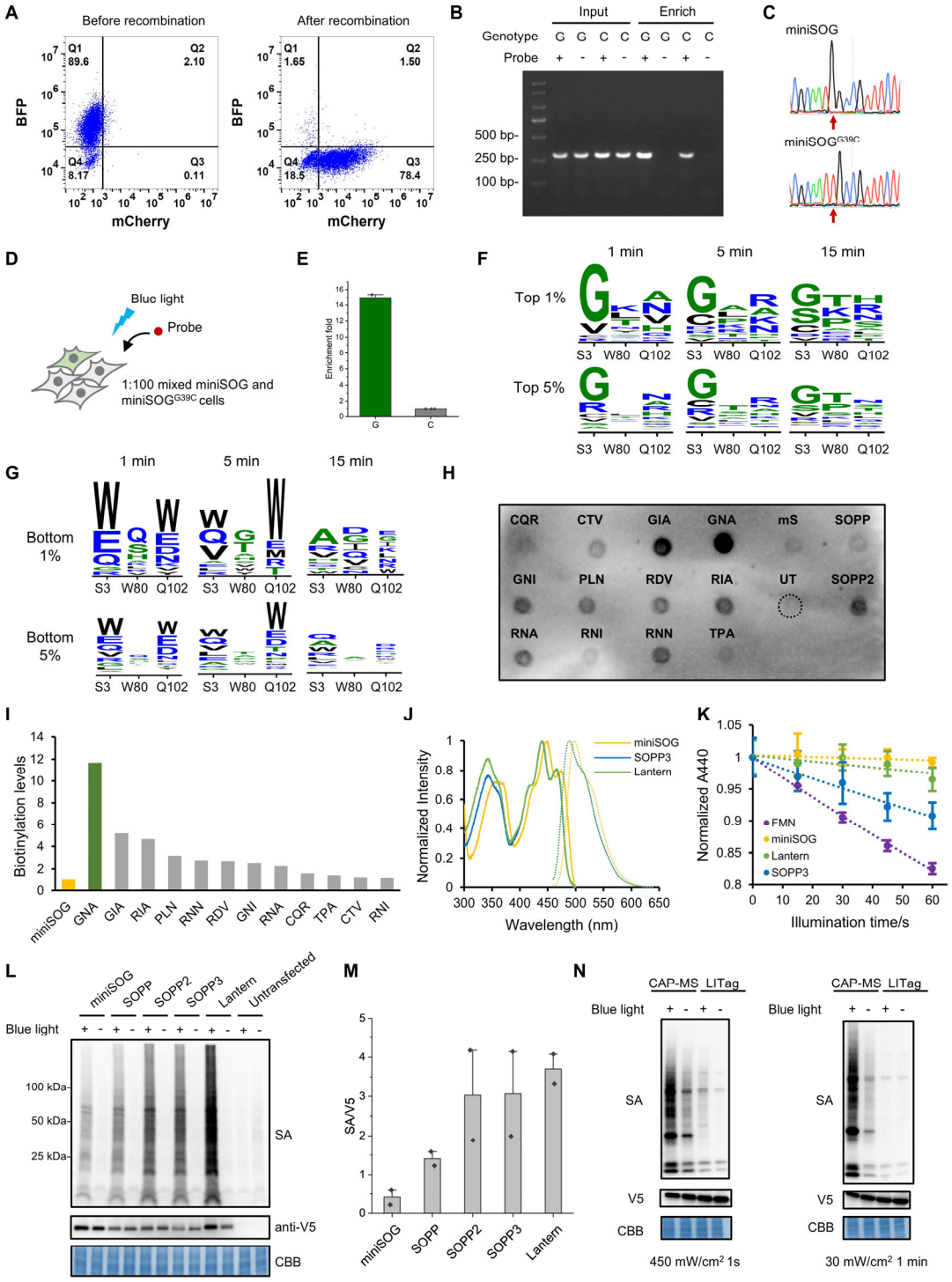

Figure S1. Directed evolution of Lantern. (A) After recombination, the expression of mCherry was turned on and the expression of BFP was turn off, indicating the successful insertion of the mutant library. **(B)** DNA gel electrophoresis analysis of the RT-PCR products prepared from pre- and post- enrichment RNAs. **(C)** Sanger sequencing results of recovered DNAs around the mutation site. **(D)** Schematic of the model selection using miniSOG and miniSOG^{G39C} mixed cells. **(E)** Enrichment fold of miniSOG and miniSOG^{G39C} calculated by the fold change of sequence abundance after selection. **(F)** Top enriched

amino acid residues in the first-round selection under 1, 5 and 15 min of irradiation. **(G)** Bottom enriched amino acid residues in the first-round selection under 1, 5 and 15 min of irradiation. **(H)** Streptavidin dot blot analysis of the RNA labeling results of 12 top enriched mutants. UT, untransfected cells. The winner mutant “GNA” was named as Lantern. **(I)** Quantitative greyscale analysis of **(H)**. **(J)** The absorption and fluorescence spectra of miniSOG, SOPP3 and Lantern. **(K)** Photobleaching curves of different photosensitizers upon blue-light irradiation. **(L)** Western blot analysis comparing the labeling results of miniSOG variants in HEK293T cells. Cells were transiently transfected with mitochondrial-matrix-targeting miniSOG variants, then labeled with 10 mM PA and 1-min irradiation. **(M)** Quantitation greyscale analysis of **(L**). Labeling efficiency was calculated as the ratio of streptavidin intensity relative to V5 intensity. The experiments were repeated twice. (**N)** Western blot analysis comparing the labeling results of CAP-MS and LITag. Cells were transiently transfected with H2B-fused Lantern (CAP-MS) or LOV* (LITag), then labeled with 1 mM 3-EA (CAP-MS) or 0.5 mM BP (LITag) under the same irradiation condition. Left, 450 mW/cm^2 irradiation for 1 s; right, 30 mW/cm^2 irradiation for 1 min.

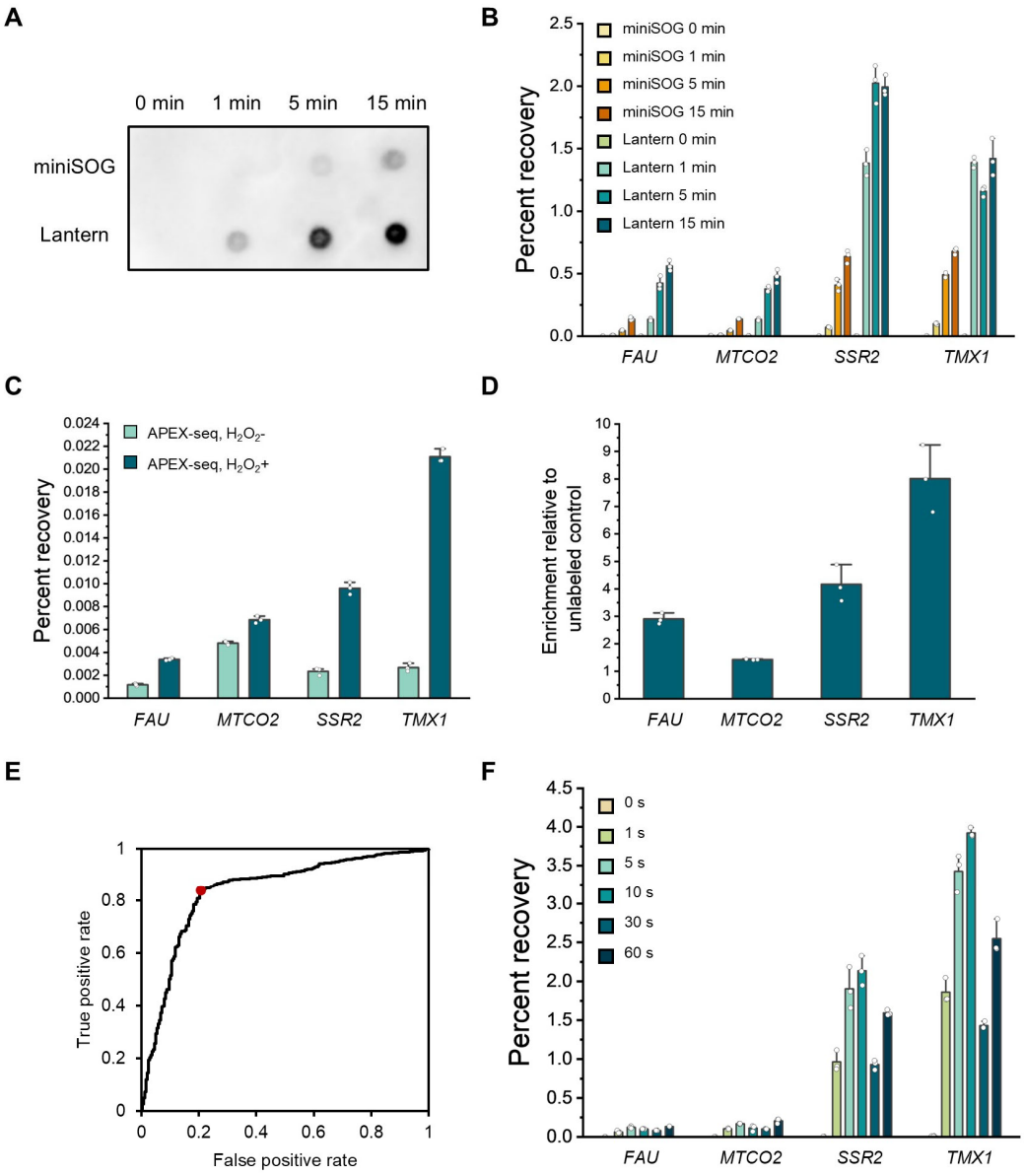

Figure S2. Profiling the ERM transcriptome with Lantern-mediated CAP-seq. (A) Streptavidin dot blot analysis of the RNA labeling results with ERM-miniSOG or ERM-Lantern. Cells were labeled with 10 mM PA and 1-15 min of irradiation. **(B-C)** RT-qPCR analysis revealing the enrichment of secretory genes over non-secretory genes with CAP-seq **(B)** or APEX-seq **(C)**. ERM-Lantern or ERM-miniSOG cells were labeled with 10 mM PA and 1-15 min of irradiation. Cells stably expressing APEX2-sec61β were labeled with 0.5 mM BP and 1 mM H_2O_2 for 1 min. *SSR2* and *TMX1* are secretory transcripts. *FAU* is a transcript that encodes a cytosol-localized protein. *MTCO2* belongs to the mitochondrial transcriptome. The percent recovery of each gene was calculated based on the relative RNA abundance before and after enrichment. Each qPCR reaction was conducted in triplicates. **(D)** RT-qPCR analysis revealing the relative enrichment folds of RNAs with APEX-seq. The secretory and non-secretory genes were selected as in **(B)**. The APEX

labeling was conducted as in **(C)**. Each qPCR reaction was conducted in triplicates. **(E)** ROC cutoff analysis of the ERM-Lantern enrichment dataset obtained with 1 min of irradiation. The red dot indicates the best performing threshold, corresponding to Log_2FC (Enrich vs. Input) = 0.925. Only protein-coding RNAs with $P_{adj} < 0.05$ were included in this analysis. **(F)** RT-qPCR analysis revealing the enrichment of secretory genes over non-secretory genes with Lantern-mediated CAP-seq. ERM-Lantern cells were labeled with 10 mM PA and 1-60 s of irradiation at a power of 450 mW/cm^2. The selection of secretory and non-secretory genes, and the calculation of percent recovery were as in **(B)**. Each qPCR reaction was conducted in triplicates.

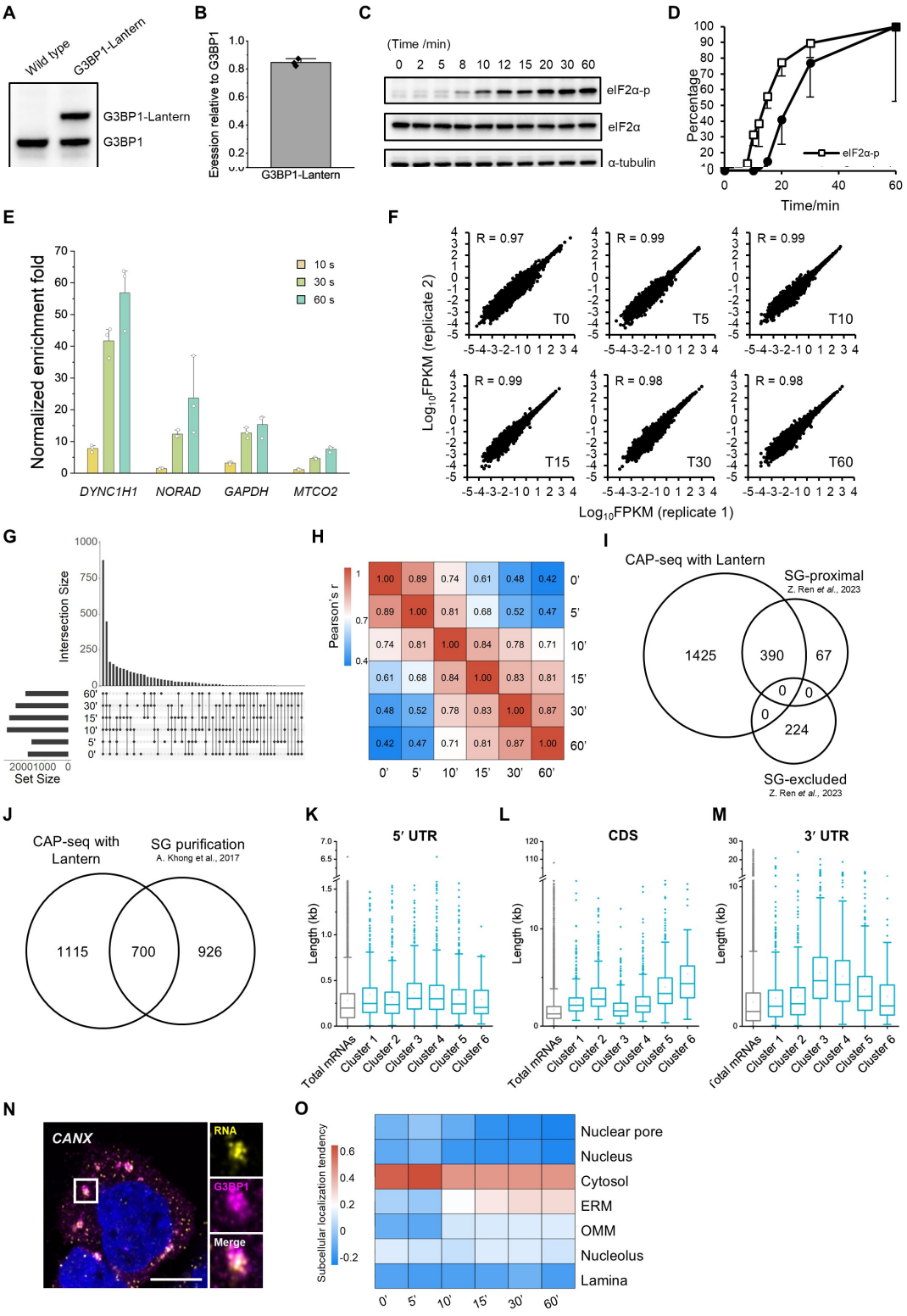

Figure S3. Tracking the dynamic G3BP1-proximal transcriptome during SG assembly. (A) Western blot analysis of the G3BP1 overexpression levels in the G3BP1-Lantern stable cell line. **(B)** Grey scale quantitation analysis of **(A)**. The mean and standard deviation values were calculated from three technical replicates. **(C)** Western blot analysis charactering the eIF2α phosphorylation levels during the SG assembly process. **(D)** Quantitation analysis of the eIF2α phosphorylation and SG growth during the SG assembly

process. The eIF2α phosphorylation blot was conducted in triplicate. The average granule size of each time point was calculated from imaging results of 20 cells. **(E)** RT-qPCR analysis of the enriched RNAs. The G3BP1-Lantern cells were labeled with 10 mM PA and 10-60 s of irradiation at 30 mW/cm^2. *DYNC1H1* and *NORAD* are two SG-enriched transcripts reported previously. *GAPDH* is a house-keeping gene normally localized in the cytosol. *MTCO2* belongs to the mitochondrial transcriptome. The normalized enrichment fold was calculated as the ratio of enrichment relative to the negative control omitting-light. Each reaction was conducted in triplicate. **(F)** FPKM values of the transcripts commonly detected by two replicates. Pearson's correlation coefficients between two replicates were calculated. **(G)** Upset plot showing the overlapping transcripts enriched at different time points. **(H)** Heatmap showing the Pearson's correlation coefficients between the Log_2FC (G3BP1 vs. Untargeted) values determined at different time points during the SG assembly. The total 3,289 G3BP1-associated transcripts enriched by at least one time point were included in this calculation. **(I)** Venn diagram comparing the SG transcriptome revealed by Lantern-mediated CAP-seq with the SG-proximal and SG-excluded datasets determined by miniSOG-mediated CAP-seq. **(J)** Venn diagram comparing the SG transcriptome lists identified by Lantern-mediated CAP-seq and by biochemical purification. **(K-M)** Box plot comparing the lengths of 5′ UTR **(K)**, CDS **(L)** and 3′ UTR **(M)** of cluster 1-6 RNAs. The box marks the first and third quartiles; whiskers indicate the minima and maxima; the central line represents the median. Transcript length features are referenced from Ensembl website. **(N)** Representative smFISH images of an ER marker gene *CANX*. Scale bar: 10 μm. **(O)** Heatmap showing the average subcellular localization tendency of G3BP1-interacting RNAs enriched at each time point. The subcellular localization tendency values were adapted from the enrichment fold of APEX-seq at each subcellular localization after *z*-score normalization.

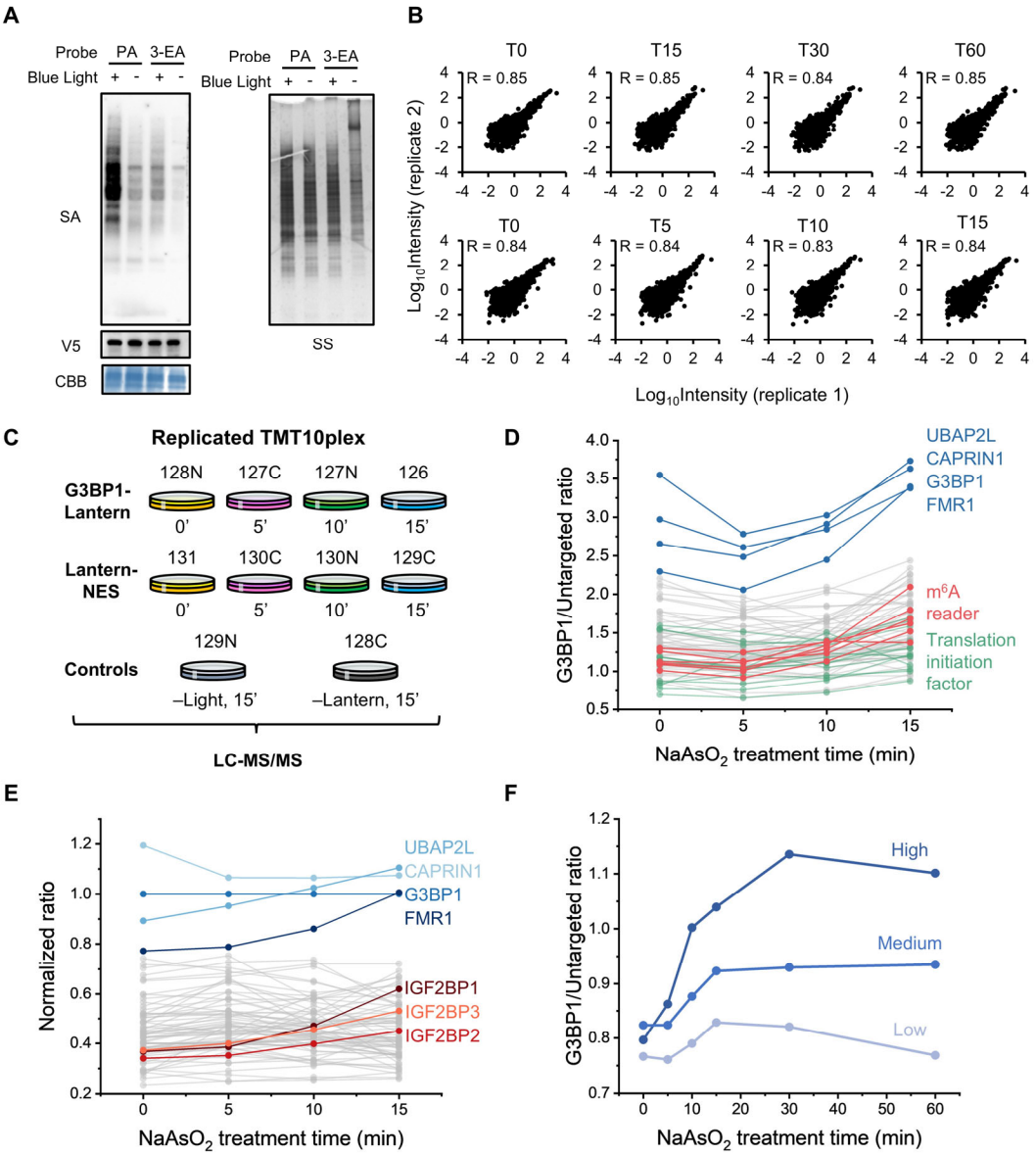

Figure S4. Profiling the dynamic G3BP1-proximal proteome during the SG assembly process with Lantern-mediated CAP-MS. (A) Western blot analysis of the labeling results in the G3BP1-Lantern cell line with 10 mM PA and 1 mM 3-EA under 30 s of irradiation at a power of 30 mW/cm^2. **(B)** Mass spectrometry intensity of proteins detected in the G3BP1-Lantern labeling groups. Pearson's correlation coefficients between two replicates were calculated. **(C)** Schematic of TMT-based quantitative proteomics to profile the G3BP1-interacting proteome at 0-, 5-, 10- and 15-min post stress. **(D)** Line plot showing the G3BP1/Untargeted ratios of SG proteins during the 0-15 min period of stress. **(E)** Line plot showing the normalized G3BP1/Untargeted ratios with respect to that of G3BP1 during the 0-15 min period of stress. **(F)** Line plot showing the G3BP1/Untargeted ratios of human RNAs with low, medium and high levels of m^{6}A modifications. Number of m^{6}A sites were referenced from GLORI (Liu *et al.*, 2023).

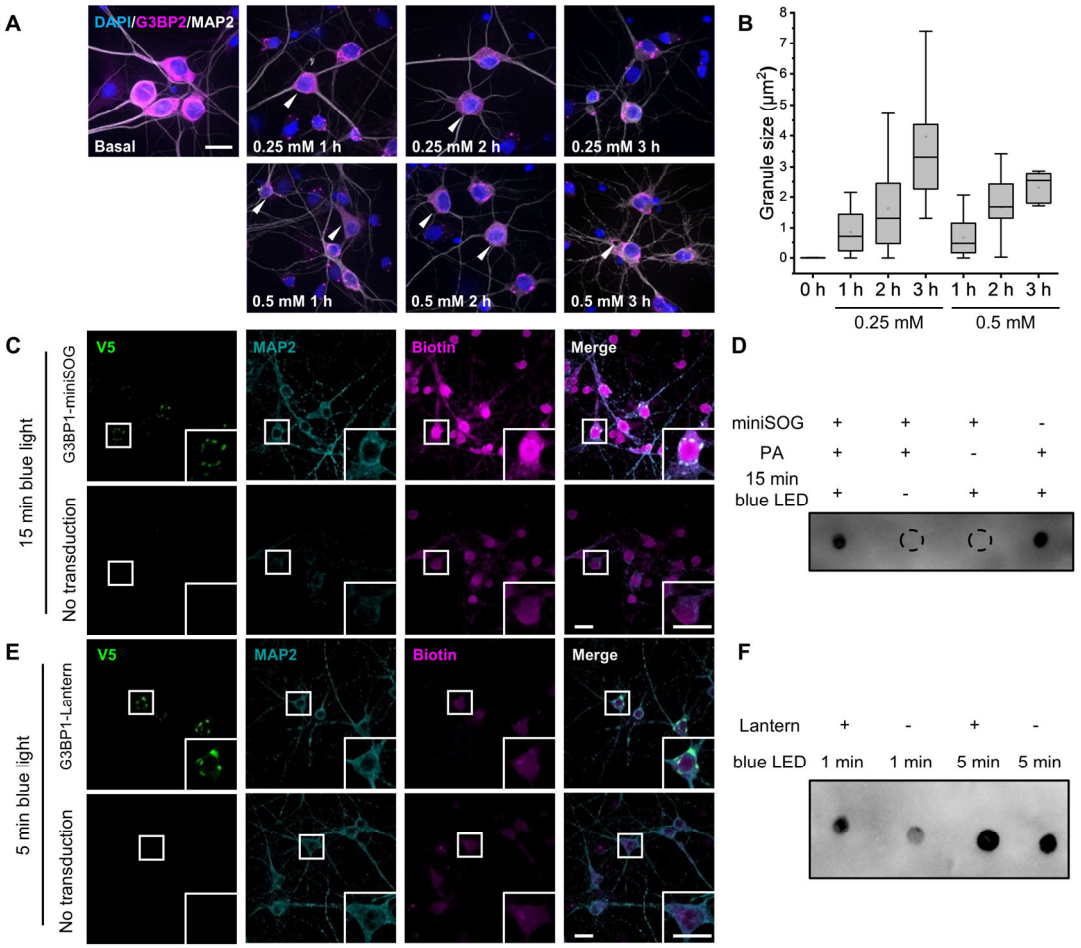

Figure S5. Optimization of the SG induction and CAP-seq labeling conditions in cultured neurons. (A) Representative immunofluorescence images showing the localization of G3BP1 in rat cortical neurons under different stress conditions. Scale bar is 20 μm. **(B)** Quantitation analysis of the SG size under different $NaAsO_2$ stimulation conditions. **(C)** Representative immunofluorescence images showing the labeling results with G3BP1-miniSOG in rat cortical neurons. Cells were labeled with 10 mM PA and with 15 min irradiation. Scale bar: 20 μm. **(D)** Streptavidin dot blot analysis of the enriched RNAs labeled with miniSOG under 15 min of irradiation. **(E)** Representative immunofluorescence images showing the labeling results with G3BP1-Lantern in rat cortical neurons. Cells were labeled with 10 mM PA and with 5 min of irradiation. Scale bar is 20 μm. **(F)** Streptavidin dot blot analysis of the enriched RNAs labeled with Lantern under 1 or 5 min of irradiation.

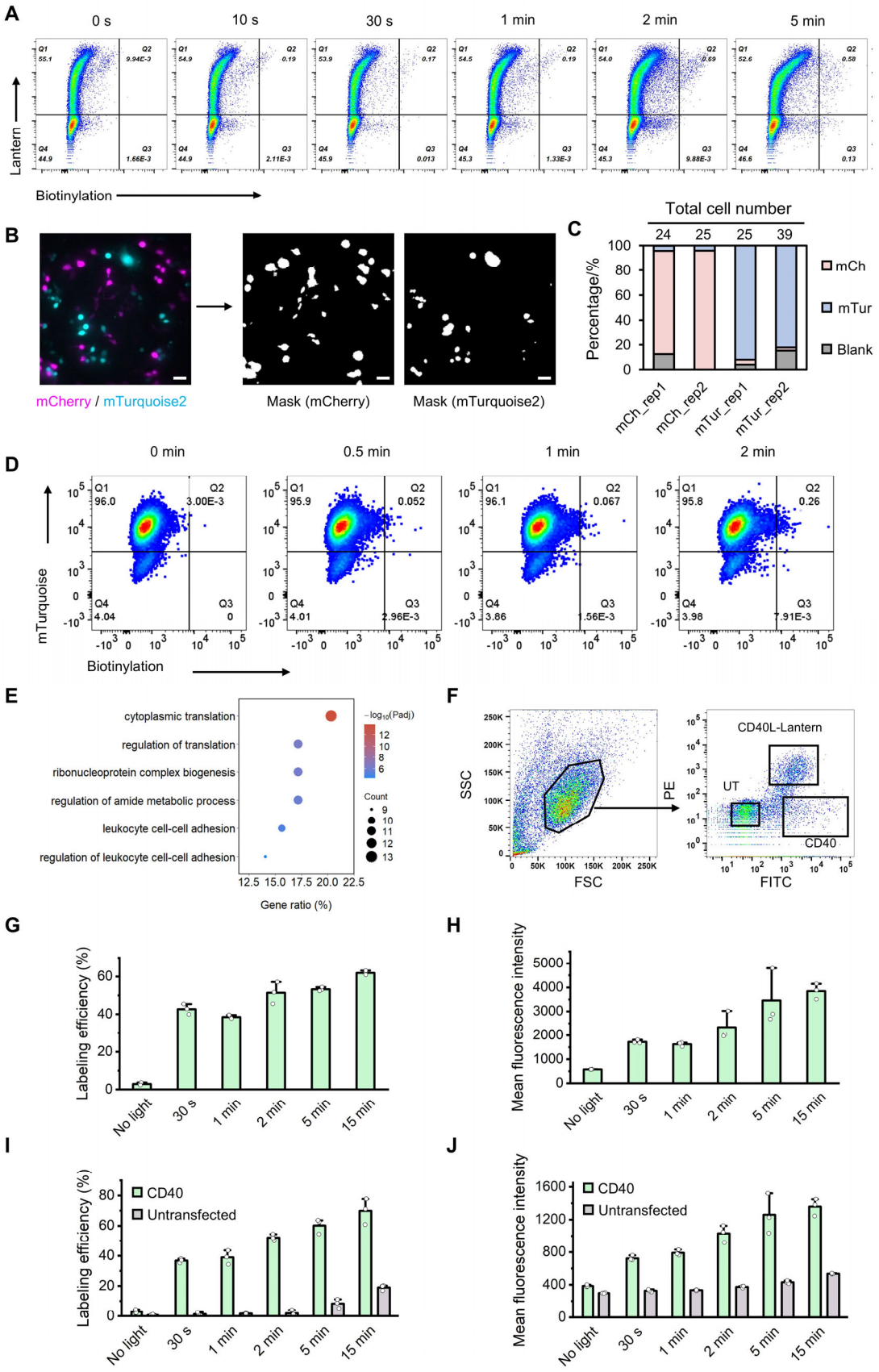

Figure S6. Spatially resolved cellular tagging with Lantern. (A) Flow cytometry results of Lantern-TM cells labeled with 2 mM Btn-NH_2 under 0-5 min of irradiation. **(B)**

Demonstration of DMD masks generated from fluorescence imaging of either the mCherry or mTurquoise2 channels. **(C)** Percentage distribution of different cell types in the sorted cell population. **(D)** Flow cytometry results of Lantern-TM MC38 cells labeled with 2 mM Btn-NH_2 under 0-2 min of irradiation. **(E)** Significantly enriched biological processes in the migrating cells revealed by gene ontology analysis. **(F)** Gate setup in the flow cytometry analysis to separate the CD40L-Lantern, CD40 and non-transfected cells. **(G-H)** Statistics of the flow cytometry results showing the *cis*-labeling efficiency and intensity. Efficiency, population frequency of cells with high APC signals **(G)**. Mean fluorescence intensity, average biotin signals of cells **(H)**. The experiment was repeated three times and results were calculated on average. **(I-J)** Statistics of the flow cytometry results showing the *trans*-Labeling efficiency **(I)** and mean fluorescence intensity **(J)** of CD40 cells. Efficiency, population frequency of cells with high APC signals. Mean fluorescence intensity, average biotin signals of cells. The experiment was repeated three times and results were calculated on average.

METHODS

Reagents

Reagent	Vendor	Catalog number
Antibodies		
Anti-G3BP rabbit IgG	Abcam	ab181150
Anti-G3BP mouse IgG	Abcam	ab6574
Anti-G3BP2	Abcam	ab86135
Anti-MAP2	Abcam	ab5392
Anti-TOMM20	Abcam	ab56783
Anti-Calnexin	Abcam	ab22595
Anti-DDX6	ABclonal	A16270
Anti-eIF2α	CST	5324
Anti-EIF2S1 (phosphor S51)	Abcam	ab32157
Anti-Flag	Sigma-Aldrich	F1804
Anti-V5	Biodragon	B1005
Anti-α-Tubulin	Biodragon	B1052
Goat anti-Mouse IgG (H+L), Alexa Fluor 488	Invitrogen	A-11029
Goat anti-Rabbit IgG (H+L), Alexa Fluor 488	Invitrogen	A-11034
Streptavidin, Alexa Fluor 637	Invitrogen	S21374
Goat anti-Mouse IgG (H+L), HRP	Biodragon	BF03001
Goat anti-Rabbit IgG (H+L), HRP	Biodragon	BF03008
Streptavidin, HRP	Thermo Scientific	21124
Chemicals and Enzymes		
DAPI	Invitrogen	D1306

Calcein-AM	Beyotime	C2012
Lipofectamine 3000	Invitrogen	L3000008
Polyethylenimine (PEI)	Sigma-Aldrich	408727
Opti-MEM	Gibco	31985062
Blasticidin	Selleck	S7419
HBSS	Gibco	14025092
HEPES (1 M)	Gibco	15630080
Live Cell Imaging Solution	Invitrogen	A14291DJ
Matrigel	Corning	356234
Poly-D-lysine (PDL)	Sigma-Aldrich	P7280
Laminin	Gibco	23017015
GlutaMAX Supplement	Gibco	35050061
Neurobasal Medium	Gibco	21103049
B-27 Supplement	Gibco	17504044
Sodium arsenite	Sigma-Aldrich	S7400-100G
DNA Assembly Mix Plus	LabLead	D0204P
Singlet Oxygen Sensor Green (SOSG)	Meilunbio	MA0326
Propargylamine (PA)	Accela	SY002930
Btn-NH_2	Biomatrik	246702
3-Ethynylaniline (3-EA)	Bidepharm	BD11069
N_3-PEG_3-Biotin	Click Chemistry Tools	AZ104
$CuSO_4 \cdot 5H_2O$	Aladdin	C112401-25g
BTTAA	Click Chemistry Tools	1236
THPTA	Click Chemistry Tools	1010
Sodium ascorbate	Aladdin	S105024-100g
0.5 M EDTA, pH 8.0	Life Technologies	AM9260G

D-Biotin	Invitrogen	B20656
Glycogen	Thermo Scientific	R0551
TRIzol	Invitrogen	15596018
Yeast tRNA	Invitrogen	15401029
DNase I	NEB	M0303S
PowerUp SYBR Green	Applied Biosystems	A25742
Dynabeads MyOne Streptavidin C1	Invitrogen	65002
SUPERaseIN RNase Inhibitor	Invitrogen	AM2694
SuperScript IV reverse transcriptase	Invitrogen	18090050
5 M Betaine	Sigma-Aldrich	B0300
Phanta Max Super-Fidelity DNA Polymerase	Vazyme	P505
Bst 3.0 DNA polymerase	NEB	M0374M
Q5 High-Fidelity 2x Master Mix	NEB	M0492
VAHTS DNA Clean beads	Vazyme	N411
Dextran sulfate	Sigma-Aldrich	D6001
Formamide	Sigma-Aldrich	F9037
20 × SSC	Invitrogen	AM9770
Ribonucleoside Vanadyl Complex (RVC)	NEB	S1402S
Salmon Sperm DNA Solution	Invitrogen	15632011
E. coli tRNA	Roche	10109541001
Fluoromount-G Anti-Fade Mounting Medium	SouthernBiotech	0100-35
BSA	Sangon Biotech	A500023-0100
cOmplete Proteinase Inhibitor	Roche	4693032001
Phosphatase Inhibitor Cocktail II	Selleck	B15001
Clarity Western ECL Substrate	Bio-Rad	1705060
Clarity MAX Western ECL Substrate	Bio-Rad	1705062

Streptavidin agarose beads	Pierce	20353
Urea	Sigma	U1250
Iodoacetamide (IAA)	Sigma	T7408
Dithiothreitol (DTT)	Sigma	D9163
1 M TEAB	Sigma	T7408
Sequencing-grade trypsin	Promega	V5111
Materials and Equipment		
Electroporation cuvette, 0.1 cm gap	BioRad	1652083
Immun-Blot PVDF Membrane	Bio-Rad	1620177
Immobilon-Ny + membrane	Millipore	INYC00010
Pierce C18 Spin tips & Columns	Thermo Scientific	87784
PR160L-440 nm	Kessil LED	-
MicroPulser Electroporator	BioRad	1652100
Critical Commercial Assays		
VAHTS Universal DNA library Prep Kit	Vazyme	ND607
NEBNext Ultra II RNA Library Prep Kit for Illumina	NEB	E7770
TruePrep DNA Library Prep Kit V2 for Illumina	Vazyme	TD501
TruePrep Index Kit V2 for Illumina	Vazyme	TD202
ProtoScript II First Strand cDNA Synthesis Kit	NEB	E6560L
RNA Clean & Concentrator Kit	Zymo	R1019
TMT 10plex Isobaric Label Reagents and Kits	Thermo Scientific	90110
Pierce High pH Reverse Phase Peptide Fractionation Kit	Pierce	84868

Plasmid construction

Name	Vector	Features
Landing Pad Cell Line		
tdMCP-miniSOG	AttB	attB-tdMCP-miniSOG-IRES-mCherry-24×MS2
tdMCP-miniSOG^{G39C}	AttB	attB-tdMCP-miniSOG^{G39C}-IRES-mCherry-24×MS2
HEK293T and MC38 Expression Plasmids		
Mito-Lantern	pcDNA3.1	pCMV-Mito-V5-Lantern
Lantern-Sec61β	pLX304	pCMV-V5-Lantern-sec61β
G3BP1-Lantern	pLX304	pCMV-V5-G3BP1-Lantern
Lantern-NES	pLX304	pCMV-V5-Lantern-HaloTag-NES
Lantern-MAVS	pLX304	pCMV-V5-Lantern-MAVS
Lantern-TM-mTurquoise	pLX304	pCMV-mTurquoise-P2A-ss-Lantern-TM
Lantern-TM-mCherry	pLX304	pCMV-mCherry-P2A-ss-Lantern-TM
Lantern-TM	pLX304	pCAG-mTurquoise-P2A-ss-Lantern-TM
Lantern-CD40L	pLX304	pCMV-dTomato-P2A-CD40L-Lantern-Flag
CD40	pLX304	pCMV-CD40-EGFP
Rat Cortical Neuron Expression Plasmids		
G3BP1-Lantern	FSW	phSyn-V5-rG3BP1-Lantern
Lantern-NES	FSW	phSyn-V5-Lantern-NES

Oligonucleotides

Name	Sequence (5′ to 3′)
Mutant Library Construction	
SSM-S3W80_F	CGGCATCTACGCGGATTCTAGAGAAAAGNNKTTTGTGATTACCGATCCG
SSM-S3W80_R	CGCATCGGTTGCAGATGCAGTAAGTTMNNAAATTTCTTGCCGCTTTTCGTATAGTT
SSM-Q102_F	TTACTGCATCTGCAACCGATGCG
SSM-Q102_R	GCGGATCAGCTTGGTACGATATCCTAGCCATCCAGMNNCACACCA
OEPCR-F	CGGCATCTACGCGGATTCTAGA
OEPCR-R	GCGGATCAGCTTGGTACGATATCCTA
qPCR Primers	
SSR2_F	GTTTGGGATGCCAACGATGAG
SSR2_R	CTCCACGGCGTATCTGTTCA
TMX1_F	ACGGACGAGAACTGGAGAGA
TMX1_R	ATTTTGACAAGCAGGGCACC
FAU_F	TCCTAAGGTGGCCAAACAGG
FAU_R	GTGGGCACAACGTTGACAAA
GAPDH_F	TGTCAAGCTCATTTCCTGGTAT
GAPDH_R	CTCTCTTCCTCTTGTGCTCTTG
MTCO2_F	AACCAAACCACTTTCACCGC
MTCO2_R	CGATGGGCATGAAACTGTGG
DYNC1H1_F	GCTGAACGTGAACGATGGGA
DYNC1H1_R	TAACCCGCGTAGGCAATGAA
NORAD_F	AGGCGTGTTGCCATTTTTGT
NORAD_R	ACACTACACCAACTCAAACTGC
mTurquoise_F	CCACTACCAGCAGAACACCC

mTurquoise_R	TTGGGGTCTTTGCTCAGCTT
mCherry_F	CTGAAGGGCGAGATCAAGCA
mCherry_R	GGGCTTCTTGGCCTTGTAGG

Mammalian cell line culture

HEK293T, U-2 OS and MC38 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and maintained at 37°C with 5% CO_2.

Stable cell lines are created via lentiviral transduction. To produce lentivirus, HEK293T cells were cultured in 6-well plates, and transfected at ~90% confluence using Lipofectamine™ 3000 reagent. A 200 µL transfection system was prepared for cells from each well, in which the lentiviral vector pLX304 (1 µg), was mixed with two packaging plasmids, dR8.91 (1 µg) and pVSV-G (0.7 µg), together with 6 µL P3000™ and 6 uL Lipofectamine™ 3000. The transfection mix was incubated at room temperature for 15 min before added to cells. The culture medium was changed to 1 mL DMEM free of serum during the transfection. Transfection reagents were replaced with 2 mL complete culture medium 6-8 h post transfection. 48h after transfection, the culture medium containing lentivirus was collected and filtered through a 0.45 µm filter.

The landing pad cell line was constructed as previously described[1]. HEK293T cells were infected with ~0.1 multiplicity of infection (MOI) when cell confluence reached 60%. BFP was used as a marker for successful insertion of the attP site and Bxb1 recombinase[1]. After 48 h, BFP positive cells were sorted by flow cytometry and seeded one cell per well in 96-well plates. Mono-clonal cells were cultured for at least 2 weeks, then were validated by transfection with attB plasmids. The selected clone was expanded and employed for mutant library construction. Cell culture medium was supplemented with 2.5 µg/mL blasticidin.

For the construction of cell lines stably expressing Lantern, 1 mL of the virus suspension was mixed with 1 mL of fresh cell culture medium, then added to HEK293T cells at ~30% confluence cultured in six-well plates. Next, 48 h after lentivirus transfection, the culture medium was exchanged to fresh complete medium. For cell selection, HEK293T cell lines were cultured in 5 µg/ml of blasticidin-containing culture medium for at least 7 days. The positive rate of Lantern expression was measured by immunofluorescence.

To control G3BP1 overexpression levels in the G3BP1-Lantern cell line, cells exhibiting moderate green fluorescence (the fluorescence from Lantern) were sorted using flow cytometry. Overexpression levels of G3BP1 in each monoclonal cell strain were assessed by Western blot with an anti-G3BP1 antibody, and a strain demonstrating 55% overexpression of G3BP1 (the expression levels of G3BP1-Lantern are 55% of the endogenous G3BP1 expression levels) was chosen for transcriptome and proteome identification experiments.

Primary culture of rat cortical neurons

Neonatal Sprague Dawley rats (within 24 h post-birth) were selected for cortical

neurons dissection. Dissected cortical tissue was cut into approximately 1 mm^3 blocks, washed three times with a mixed buffer solution of 1× Hank's Balanced Salt Solution (HBSS) and 10 mM HEPES, and then digested with trypsin-EDTA solution (0.25%) at 37°C for 15 min. Upon completion of digestion, cells were washed three times with the mixed buffer solution of HBSS + 10 mM HEPES, thoroughly dispersed in 15 mL of glia plating medium (GPM, DMEM with 10% (v/v) FBS, penicillin and streptomycin) and filtered through a 70 μm filter. Then the cells were plated onto 0.12 mm glass coverslips or Petri dishes and cultured with GPM for 3-6 h before switching to neural cell culture medium (Neurobasal medium with 2% (v/v) B-27 supplement, 1% (v/v) GlutaMAX supplement, penicillin and streptomycin). Glass coverslips were pre-incubated overnight at 37°C with 20 μg/mL poly-D-lysine (PDL), followed with another overnight incubation with 10 μg/mL mouse laminin. Petri dishes were pre-incubated overnight with 50-100 μg/mL PDL at 37°C. The day of rat dissection was designated as DIV0 (0 days *in vitro*), and thereafter, half of the spent culture medium was replaced with fresh neural cell culture medium every 3 days.

Directed evolution of Lantern

The mutant library was constructed via degeneracy codon mutagenesis. In brief, mutations were introduced by PCR with primers containing NNK triplet at the target sites, using the miniSOG sequence as the template. Fragments containing mutations were ligated via overlap extension PCR. Vector fragments are prepared by PCR using the attB-tdMCP-miniSOG^{G39C}-IRES-mCherry-24×MS2 plasmid as the template. Then, 300 ng insert fragment (363 bp) and 1.6 μg vectors (7.16 kb) were ligated with DNA Assembly Mix Plus (LabLead) in a 100 μL reaction system. The ligation products were purified with DNA Clean & Concentrator Kit (ZYMO) and eluted with 20 μL nuclease-free water.

The DNA ligation products were transformed into *Escherichia coli* Stbl3 cells via electroporation. To prepare electro-competent cells, bacteria were inoculated in 100 mL Super Optimal Broth (SOB) medium (containing 20 g/L trptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) and incubated at 37°C until OD_{600} reached ~1. The bacteria culture was cooled on ice for 15-30 min, then was centrifuged at 2,000 × *g* at 4°C for 10 min. The supernatant was discarded. Cells were washed with 100 mL ice-cold sterile water and then pelleted by centrifugation at 2,000 × *g* at 4°C for 10 min. Cells were washed with 50 mL ice-cold 10% (v/v) glycerol and then pelleted by centrifugation at 2,000 × *g* at 4°C for 10 min. Cells were washed again with 5 mL ice-cold 10% glycerol and then pelleted by centrifugation at 2,000 × *g* at 4°C for 10 min. Finally, cells were resuspended in 250 μL ice-cold 10% glycerol, gently mixed with 20 μL DNA ligation products, and incubated on ice for 15 min. Thereafter, 55 μL cell suspension was removed to a pre-cooled electroporation cuvette (BioRad, 0.1 cm gap), and electroporated using MicroPulser Electroporator (BioRad) with one pulse in the EC1 mode. The electroporation was repeated 5 times. Bacteria were incubated in 10 mL SOC medium (the

SOB medium supplemented with 20 mM glucose) at 37°C for 1h, with shaking at 110 rpm. Subsequently, cells were pelleted, resuspended in 1 mL SOB medium, and plated onto LB plates supplemented with 70 μg/mL ampicillin. After incubation at 37°C overnight, bacteria colonies were counted and collected for plasmid extraction.

To construct the HEK293T mutant library, landing pad cells were seeded in a 10-cm dish. When the confluence reached 80%, landing pad cells were transfected with 13.5 μg purified attB plasmids encoding the mutant library using Lipofectamine 3000. 3-4 days post transfection, cells demonstrating both mCherry positive and BFP negative were selected by FACS and expanded for proximity labeling activity selection.

For the first round of selection, the cell library was seeded in 10-cm dishes. When the confluence reached 90%, cells were incubated with 5 mM propargylamine (PA) in HBSS at 37°C for 5 min, and irradiated with 30 mW/cm^2 blue light for 1 min, 5 min, and 15 min. Then cells were washed once with PBS, and lysed with 4 mL TRIzol reagent. Total RNAs were extracted, clicked with N_3-PEG_3-biotin, and purified with RNA Clean & Concentrator Kit (ZYMO). 180 μg purified RNAs were enriched with 18 μL streptavidin beads following typical CAP-seq procedures as described below. Enriched RNAs were dissolved in 10 μL nuclease-free water. 800 ng Input RNAs and 7 μL Enrich RNAs were reverse transcribed with ProtoScript II Reverse Transcriptase using 10 pmol gene-specific primer targeting the IRES sequence downstream of the miniSOG gene in a 20 μL reaction system. 5 μL of the reverse transcription (RT) product was employed as the template in a 50 μL PCR system, amplified for 35 cycles with Phanta Max Super-Fidelity DNA Polymerase (Vazyme). PCR products were analyzed by agarose gel electrophoresis, and the 363 bp band was purified by E.Z.N.A. Gel Extraction Kit (Omega). For each sample, 30 ng purified RT-PCR product was utilized for NGS library construction with VAHTS Universal DNA Library Prep Kit (Vazyme), and sequenced on the Illumina NovaSeq 6000 platform in the PE150 mode.

For the second round of selection, 150 ng RT-PCR product of RNAs enriched from the cells labeled with 1 min irradiation was ligated with 800 ng vector fragments using DNA Assembly Mix Plus (LabLead) in a 50 μL reaction system. The ligation product was purified and transformed into *Escherichia coli* Stbl3 cells via electroporation for plasmid amplification. To construct the mutant library in HEK293T, landing pad cells were seeded in a 6-cm dish, and transfected with 5 μg purified plasmid when the cell confluence reached 80%. 3 days after transfection, cells demonstrating both mCherry positive and BFP negative were selected by FACS and expanded for proximity labeling activity selection. The cell library was seeded in 10-cm dishes, and labeled with 5 mM PA and 1 min irradiation at 30 mW/cm^2. The experiments were conducted in two replicates. For each replicate, 180 μg purified RNAs were enriched with 18 μL streptavidin beads following the typical CAP-seq RNA affinity enrichment procedure. The RT-PCR analysis was conducted as described above. 15 μg RT-PCR products were utilized for NGS library construction and sequenced on the Illumina NovaSeq 6000 platform in the PE150 mode.

Recombinant protein expression

Escherichia coli BL21(DE3) cells were transformed with pET21a plasmids encoding the sequences of miniSOG, SOPP3 or Lantern. Bacteria were grown in 500 ml LB medium supplemented with 70 μg/ml ampicillin at 37°C. When the optical density (OD_{600}) reached 0.5, 0.5 mM IPTG was added to introduce protein expression and bacteria continued to grow at 18°C for 20 h. Bacteria were gathered by centrifugation (4,000 ×*g* for 30 min). Then, the cells were re-suspended in 20 mL binding buffer (50 mM Tris-HCl, 300 mM NaCl, pH7.5) and lysed by ultrasonication on ice. Supernatant was collected after centrifugation at 20,000 ×*g* for 30 min at 4°C, and was subsequently incubated with 1.5 ml Ni-NTA Agarose beads. The slurry was mixed on a rotator for 30 min at 4°C, before being loaded into a column. Excess liquid was removed by gravity flow. Ni-NTA beads were then washed with 20 ml wash buffer (50 mM Tris-HCl, 300 mM NaCl, pH7.5, 30 mM imidazole) and recombinant protein was eluted from beads with 10 ml elution buffer (50 mM Tris-HCl, 300 mM NaCl, pH7.5, 200 mM imidazole). The purified protein was dialyzed against phosphate-buffered saline (PBS) containing 1 mM dithiothreitol (DTT) overnight and concentrated by ultrafiltration. The protein sample was aliquoted and stored at −80°C.

Spectroscopic measurements

Absorbance spectra of minSOG, SOPP3 and Lantern were measured with a UV-3600i Plus UV-VIS-NIR Spectrophotometer (SHIMADZU), subtracting the absorbance of a blank PBS solution, and then scaled to a range between 0 and 1 by maximum-minimum normalization. The fluorescence emission spectra were measured with a Fluorescence Spectrophotometer F-7000 (HITACHI) with an excitation wavelength of 440 nm, and scaled to a range between 0 and 1 by maximum-minimum normalization.

Singlet oxygen quantum yield measurements

The Singlet Oxygen Sensor Green (SOSG) probe was used for $^1O_2$ detection. To prepare a 5 mM stock solution, 100 μg SOSG powder was dissolved with 25 μL methanol. Then the SOSG solution was diluted with PBS to a concentration of 3 μM. A PBS-based 30 μM FMN solution was prepared, and its absorbance at 440 nm was measured by NanoDrop 2000c (Thermo). Protein solutions of miniSOG, SOPP3 and Lantern were diluted with PBS to the same absorbance at 440 nm with 30 μM FMN. Equal volumes of SOSG and photocatalyst solutions were mixed, and irradiated with blue light (440 nm) at 75 mW/cm^2 for 0, 15, 30, 45 and 60 s. The $^1O_2$ production was analyzed by measuring the fluorescence of SOSG oxidation products (Ex 504 nm, Em 535 nm). The absorbance at 440 nm was also recorded to analyze the photobleaching of photocatalysts.

Lantern-mediated RNA labeling (CAP-seq) in live cells

For dot blot or RT-qPCR analysis, cells were seeded in 6-well plates. When the confluence reached 90%, cells were washed once with PBS, incubated with 1 mL 10 mM pre-warmed PA in HBSS at 37 °C for 5 min, and irradiated with blue light at a power of 30 mW/cm^2. The irradiation time was set according to experimental requirements. Subsequently, cells were washed once with PBS and lysed immediately with 1 mL TRIzol reagent.

For next-generation sequencing (NGS) analysis, cells were seeded in 10-cm dishes. When the confluence reached 90%, cells were washed once with PBS, incubated with 4 mL 10 mM pre-warmed PA in HBSS at 37 °C for 5 min, and irradiated with blue light at a power of 30 mW/cm^2 for 30 s (SG transcriptome profiling) or 1 min (ERM transcriptome profiling). Subsequently, cells were washed once with PBS and lysed immediately with 4 mL TRIzol reagent. For the induction of stress granules, cells were treated with 0.5 mM $NaAsO_2$ in complete medium at 37 °C for 5-60 min, and exchanged to 10 mM PA in HBSS with 0.5 mM $NaAsO_2$ 5 min before the end of stress treatment.

The second-scale labeling experiments were conducted with cells cultured in 35-mm dishes, following the same procedure for the labeling in 6-well plates, except for using an intense light source with a power of 450 mW/cm^2.

Rat cortical neurons were cultured in 6-cm dishes for dot blot and qPCR analysis, and in 10-cm dishes for next-generation sequencing. For virus infection, lentivirus was diluted in culture medium according to a pre-determined MOI at DIV3. 20 h after infection, cells were exchanged to fresh neuronal cell culture medium, and cultured until DIV8 for labeling. For unstimulated cell labeling, cells were incubated with 10 mM PA in Tyrode's buffer at 37°C for 5 min, followed by blue light LED irradiation at 30 mW/cm^2. For CAP-seq labeling under arsenite stress, cells were treated with complete neuronal cell culture medium containing 0.25 mM $NaAsO_2$, incubated at 37°C for 1 h or 3 h. The culture medium was replaced with Tyrode's buffer containing 10 mM PA and 0.25 mM $NaAsO_2$ 5 min before the end of stress treatment. All the lentivirus used for neuron infection was prepared by OBiO Technology.

Immunofluorescence

Cells were seeded on glass coverslips in 24-well plates at a density of ~70,000 cells per well. Glass coverslips were pretreated with -Corning Matrigel matrix diluted in DMEM (1:100) at 37 °C overnight and washed with PBS once before use. After 24 h, cells were treated with 0.5 mM $NaAsO_2$ or the photocatalytic proximity labeling procedure. Thereafter, cells were washed with PBS once and fixed with 4% (m/v) paraformaldehyde in PBS for 15 min at room temperature, followed with a 15-min permeabilization with 0.1% (v/v) Triton X-100 in PBST (PBS containing 0.05% (v/v) Tween-20) at 4°C. For the imaging of labeling signals, a 150 µl mixture of click reaction reagents was added to each well, containing 50 µM N_3-PEG_3-biotin, 1 mM $CuSO_4$, 2 mM THPTA and 1 mg/ml sodium ascorbate and

incubated at room temperature for 30 min. After the click reaction, cells were rinsed with PBS twice and then blocked with 3% BSA in PBST for 1 h at room temperature. Subsequently, cells were incubated with primary antibodies for 1 h at room temperature. After washing three times with PBST for 10 min each time, cells were incubated with secondary antibodies, streptavidin-Alexa Fluor 637 (1:1,000 dilution), and DAPI (1:1,000 dilution) for 30 min at room temperature. After washing three times with PBST for 10 min each time, immunofluorescence images were collected with an inverted fluorescence microscope (Nikon-TiE) equipped with a spinning disk confocal unit (Yokogawa CSU-X1) and a scientific complementary metal-oxide semiconductor camera (Hamamatsu ORCA-Flash 4.0 v.2). The imaging equipment was controlled with a customized software written in LabVIEW v.15.0 (National Instruments).

Affinity purification of biotinylated RNAs

Total RNAs were extracted and purified with the TRIzol reagent following the manufacturer's protocol. Briefly, each 1 mL homogenized sample was mixed with 200 uL chloroform, centrifuged at 12,000 × *g* at 4°C for 15min, then the upper aqueous phase was pipetted out and subjected to RNA precipitation by adding an equal volume of isopropanol. After incubation at -20°C for at least 20 min, RNAs were pelleted by centrifugation at 12,000 × *g* at 4°C for 15min. The RNA pellet was washed with 1 mL 75% ethanol and dissolved in 50 μL RNase-free water. The purified RNA sample were treated with 0.5-1 μL DNaseI at 37 °C for 30 min, and then incubated with click reagents. Each 50 μL RNA (~ 50 μg) was diluted to a 62.5 μL click system, containing 0.1 mM N_3-PEG_3-biotin, 2 mM THPTA, 0.5 mM $CuSO_4$ and 5 mM sodium ascorbate. After 10 min incubation at room temperature with vigorously shaking, RNAs were purified with RNA Clean & Concentrator Kit (ZYMO) and eluted with 50 μL pre-warmed nuclease-free water.

For dot blot and RT-qPCR analysis, 10-30 μg of purified RNA was enriched with 10 μL of Dynabeads MyOne Streptavidin C1. For profiling the ERM transcriptome under 1 min irradiation, 180 μg purified RNAs were enriched with 40 μL C1 beads. For profiling the SG transcriptome, 180 μg purified RNAs were enriched with 50 μL C1 beads. For profiling the ERM transcriptome under 1 s irradiation, 150 μg purified RNAs were enriched with 40 μL C1 beads.

The C1 beads were washed three times with 200 μL B&W buffer (5 mM Tris pH 7.5, 1 M NaCl, 0.5 mM EDTA, 0.1% (v/v) Tween-20), followed with one wash with 200 μL solution A (0.1 M NaOH, 0.05 M NaCl in nuclease-free water) and once with 200 μL solution B (0.1 M NaCl in nuclease-free water). The beads were then incubated with 200 μL blocking buffer (1 mg/mL BSA, 1 mg/mL Yeast-tRNA in B&W buffer) at 25 °C for 2 h with gently rotating. Thereafter, pre-blocked beads were washed three times with 200 μL B&W buffer and resuspended in 2× B&W buffer (10 mM Tris pH 7.5, 2 M NaCl, 1 mM EDTA, 0.2% (v/v) Tween-20). Equal volumes of beads suspension and RNA solution were mixed, and

incubated at room temperature for 45 min with vigorously shaking. The supernatant was discarded, and the beads were washed three times with 200 µL B&W buffer, twice with 200 µL Urea buffer (4 M Urea, 0.1% (w/v) SDS in PBS), and twice with 200 µL PBS. The beads were finally re-suspended in 50 µL Elution buffer (95% formamide, 10 mM EDTA, 1.5 mM *D*-biotin), heated at 50 °C for 5 min and then at 90 °C for 5 min. The eluted RNAs were then mixed with 1 mL TRIzol reagent, and purified following the same protocol as described above. To promote the precipitation of dissolved RNAs, 20 µg glycogen was added to the isopropanol precipitation system, and then were incubated at -20 °C overnight before pelleting the RNAs with centrifugation. The sediment was washed with 1 mL 75% (v/v) ethanol and dissolved in 10 µL of pre-warmed nuclease-free water.

RNA dot blot analysis

After the CAP-seq labeling and affinity purification procedure, enriched RNAs were re-dissolved in 10 µL ultrapure water. Equal volume of purified biotinylated RNA was loaded onto Immobilon-Ny + membrane and crosslinked to the membrane by an ultraviolet crosslinker. The membrane was blocked with 3% BSA in PBST (PBS containing 0.05% Tween-20) at room temperature for 1 h and incubated with 0.5 µg/mL streptavidin-HRP in PBST at room temperature for 1 h. The membrane was washed three times with PBST for 10 min each time, incubated in Clarity Max Western ECL Substrate and then imaged on a ChemiDoc MP imaging system (Bio-Rad).

RT-qPCR analysis of enriched RNA

For each sample, 0.3 µg total RNAs pre-enrichment (“Input”) and 3 µL enriched RNAs were reverse transcribed with and ProtoScript II Reverse Transcriptase using random primers in a 10 µL reaction system, following the manufacturer’s protocol. The cDNAs were aliquoted into four tubes (for four genes) as templates for qPCR. The templates were mixed with PowerUp SYBR Green Master Mix and primers, and then quantified by ABI StepOne Plus system. C_t values were averaged from three replicate measurements. Negative controls were treated in the same manner as the sample, and were used here to calculate enrichment fold: $2^{\Delta Ct\_control - \Delta Ct\_label}$, where $\Delta C_t = C_t^{Enrich} - C_t^{Input}$. The recovery yield was calculated using the following formula:

$$\text{Recovery yield} = 2^{-\Delta Ct} \times \frac{\text{mass of Input RNAs}}{0.3 \times \text{mass of RNAs used for enrichment}} \times 100$$

cDNA library construction for next-generation sequencing

The RNA concentration was measured by Qubit RNA HS Assay Kit. 100 ng RNA samples pre- and post- enrichment were utilized for NGS library construction. Concentrations of RNAs enriched from the labeled G3BP1-Lantern cells and from the unlabeled cells were below the detection limit, and hence 5 µL RNA samples were used

for NGS library construction.

For profiling the SG transcriptome, rRNAs were removed with Ribo-off rRNA Depletion Kit following the manufacturer's protocol. The purified RNAs were dissolved in 5 μL nuclease-free water.

NEBNext Ultra II RNA Library Prep Kit for Illumina was used for NGS library construction. RNAs were fragmentated to ~300 nt before first strand synthesis. Finally, the DNA libraries were sequenced on the Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads.

Next-generation sequencing data analysis

For the analysis of mutant library selection results, adaptors were firstly removed from the raw sequencing reads with Trimmomatic (v0.36). Then, reads were mapped to the DNA sequence of miniSOG using bowtie2 (v2.3.4.3). Reads with MAPQ <= 30 were discarded. Codons mapped to targeted mutation sites were extracted, converted to the corresponding amino acids. For each pair of reads, amino acids at three mutation sites were combined and counted as one mutant genotype. Only the paired reads successfully mapped to all the three mutation sites were kept for counting. Next, genotypes were filtered by counts >= 10 in the Input library, and the enrichment folds were calculated by the following formula:

$$Log2FC = \log_2\left(\frac{Counts^{Enrich} \times Total\ Counts^{Input}}{Total\ Counts^{Enrich} \times Counts^{Input}}\right)$$

For the analysis of subcellular transcriptome data, sequencing reads were firstly processed with Cutadapt (v.1.18) to remove adaptors. Then the reads from HEK293T samples were mapped against the human genome assembly GRCh38 (hg38) with hisat2 (v.2.1.0). Using the gene annotation (v.87) downloaded from Ensembl, number of reads mapped to each gene was counted with HTSeq (v0.7.2) using the option "–stranded no". Reads from samples of rat cortical neurons were mapped against the rat genome assembly Rnor_6.0 downloaded from Ensembl, and counted with the Ensembl gene annotation 104. Then, differential expression analysis was carried out by DESeq2 (v.1.16.1).

To define the list of ERM-proximal RNAs, protein coding genes with adjusted *P* value (P_{adj}) < 0.05 were utilized for receiver operating characteristic (ROC) analysis. True positive RNAs were combined from the GOCC-secretome and the HPA-secretome defined by the previous CAP-seq work[2]. False positive RNAs were defined by genes in the MitoCarta v.2.0 database and also not included in the GOCC- or HPA-secretome. In the DESeq2 analysis, we conducted both the Enrich-versus-Input and Enrich-versus-Control calculations, and found that the Enrich-versus-Input calculation generated more significantly enriched genes (P_{adj} < 0.05), possibly due to poor sequencing quality of enriched RNA from the unlabeled cells. A cutoff of $\log_2$FC (Enrich versus Input) > 0.925 and a cutoff of $\log_2$FC (Enrich versus Input) > 1.014 were determined for the 1-min and the 1-s labeling results respectively.

To define the list of OMM-proximal RNAs, we employed DESeq2 analysis comparing 1) Enrich and Input samples from the labeled Lantern-MAVS cells; 2) Enrich samples from the labeled Lantern-MAVS cells and the omitting-light unlabeled control cells. Genes enriched with $\log_2$FC > 1 and P_{adj} < 0.05 in both calculations were defined as OMM-proximal RNAs.To define the list of G3BP1-associated RNAs in HEK293T cells, DESeq2 analysis was employed to compare the Enrich and Input samples from the G3BP1-Lantern cells, and the Enrich samples from G3BP1-Lantern cells and Lantern-NES cells. Genes enriched with $\log_2$FC > 0.3 and P_{adj} < 0.05 in both calculations were defined as G3BP1-associated RNAs.

To define the list of G3BP1-associated RNAs in rat cortical neurons, we employed DESeq2 analysis comparing 1) Enrich and Input samples from the neurons infected with G3BP1-Lantern; 2) the Enrich samples from the neurons infected with G3BP1-Lantern and neurons infected with Lantern-NES; 3) Enrich samples from the neurons infected with G3BP1-Lantern and wild type neurons. Genes enriched with $\log_2$FC > 0.3 and P_{adj} < 0.05 in all the three calculations were defined as G3BP1-associated RNAs.

In RNA feature analysis, transcript lengths of human transcriptome were downloaded from Biomart Ensembl. According to the Ensembl 87 annotation, the longest stable transcripts were selected for analysis. The 5′UTR, CDS and 3′UTR lengths of rat transcripts were downloaded from Biomart Ensembl. The longest stable transcripts were selected for analysis based on the Ensembl 104 annotation. The m^{6}A sites data were from the previous GLORI work[3]. RNA feature analysis was conducted with Origin 2023b.

For the analysis of spatial transcriptome during cancer cell migration, sequencing reads were firstly processed with Cutadapt (v.1.18) to remove adaptors. Then the reads were mapped against the mouse genome assembly GRCm39 with hisat2 (v.2.1.0). Using the gene annotation (v.107) downloaded from Ensembl, number of reads mapped to each gene was counted with HTSeq (v0.7.2) using the option “–stranded no”. Then, differential expression analysis was carried out by DESeq2 (v.1.16.1). Transcripts with average counts less than 10 were excluded. Transcripts with $\log_2$FC (Near/Far) > 0 and *p* value < 0.05 were defined as up-regulated genes, and transcripts with $\log_2$FC (Near/Far) < 0 and *p* value < 0.05 were defined as down-regulated genes.

Single-molecule fluorescence *in situ* hybridization

Primary probes for targeted mRNAs were designed by Oligostan52 following the supplemented protocol with FLAP X complementary sequence conjugated at the 3′-end of each probe (see Supplementary Table 8). Primary probes targeted to a mRNA of interest were dissolved to 100 μM in Tris-EDTA (TE) buffer, mixed at equal volume, and then diluted in TE buffer to a final concentration of 20 μM. Secondary probes conjugated with Alexa Fluor 647 was dissolved at 10 μM by TE buffer.

Cells stably expressed G3BP1-Lantern fusion protein were cultured to ~50%

confluence on glass coverslips. Glass coverslips were pre-coated with 20% Corning Matrigel matrix diluted in DMEM (1:100) at 37°C overnight. Following a 60-min treatment of 0.5 mM $NaAsO_2$, cells were washed once with PBSM (PBS with 1 mM $MgCl_2$), fixed with 3.2% (w/v) paraformaldehyde in PBSM for 10 min at room temperature, and washed with cold PBSM in the presence of 10 mM glycine. Thereafter, cells were washed once with PBS and permeabilized on ice for 20 min with PBSM containing 0.1% Triton X-100 and 2 mM vanadyl ribonucleoside complex (VRC). After washed by PBSM, cells were then incubated at room temperature for 15 min by prehybridization-30 buffer (30% formamide in 2× SSC). Cells were stained overnight at 37°C with hybridization buffer containing 10% (v/v) dextran sulfate, 30% formamide, 2 × SSC, 2 mM VRC, 10 μg/ml Salmon Sperm DNA Solution, 10 μg/ml E. coli tRNA, 10 μg/mL BSA and 110 nM (~200 ng) primary probe mix. The next day, cells were washed twice with prehybridization-30 buffer, each time incubating at 37°C for 20 min. Cells were post-fixed in 1% (w/v) paraformaldehyde in PBSM for 5 min at room temperature, followed by rinsing twice with 2 × SSC and incubation in prehybybridization-10 (10% (v/v) formamide in 2× SSC) for 10 min at 37°C. Cells were stained with hybridization buffer containing 10% (v/v) dextran sulfate, 10% formamide, 2× SSC, 2 mM VRC, 10 μg/ml Salmon Sperm DNA Solution, 10 μg/ml E. coli tRNA, 10 μg/ml BSA and 10 pM secondary probe for 3 h at 37°C. Secondary probes containing Alexa Fluor 647 conjugated at the 3′- and 5′- termini of the FLAP X sequence (see Supplementary Table 8) were synthesized by Invitrogen and stored in TE buffer as 100 μM stock solution. Stained cells were washed twice with prehybybridization-10, each time incubating at 37°C for 10 min, and then were washed once with 2× SSC. Finally, cells were counterstained with DAPI (1:1,000 diluted in 2 × SSC) for 10 min at room temperature. The coverslips with stained cells were mounted on a glass slide in the presence of Fluoromount-G Anti-Fade.

For immunofluorescence-combined smFISH, cells were incubated with anti-V5 (for HEK293T cells, 1:500 diluted) or anti-G3BP2 (for rat cortical neurons, 1:200 diluted) primary antibodies in PBSM with 2 mM RVC at room temperature for 1 h. Then the cells were washed 3 times with PBSM, each time incubating at room temperature for 5 min with gentle shaking. Thereafter, cells were incubated with goat anti-rabbit Alexa Fluor 488 (1:1,000 diluted) and DAPI (1:1,000 diluted) in PBSM with 2 mM RVC for 30 min, followed with washing with PBSM for 3 times, and mounted on glass slides in Fluoromount-G Anti-Fade.

For smFISH imaging, a 60 × oil immersion lens with 1.5 × magnifying was used to acquire confocal image stacks, with a step size of 0.4 μm along the z-axis. The scan range was set as 6 μm for HEK293T cells and 6-10 μm for rat cortical neurons, depending on depth of endogenous G3BP2 signal. The script for quantitation of smFISH images was written in MATLAB (R2021a, V9.10.0.1602886). Briefly, the cell boundary was manually outlined from the maximum intensity projection of the G3BP2 image stack. G3BP2-positive regions were identified as those pixels with intensities higher than a manually determined

threshold in the maximum intensity projection image of the G3BP2 channel. The RNA puncta were identified from the FISH images by selecting those pixels with intensities at least 2-fold higher than the average intensity. RNA puncta less than 12 or 15 pixels (corresponding to 0.063 or 0.078 μm^2, depending on the target mRNA) were discarded from further analysis. The extent of RNA colocalization with SG was quantified as the ratio between the number of RNA puncta falling within G3BP2-positive regions and the total number of RNA puncta for a given cell. We also applied the following formula to calculate the level of RNA enrichment in SG:

$$\text{Levels of enrichment} = \frac{\text{pixels(RNA inside SG)/SG area}}{\text{pixels(RNA outside SG)/(whole cell} - \text{SG area)}}$$

Lantern-mediated protein (CAP-MS) labeling in live cells

For the comparison of different miniSOG variants, HEK293T cells were cultured in 6-well plates. When the cell confluency reached 80%, the culture medium was exchanged to 1 mL DMEM containing 1% (v/v) FBS, and cells were transfected with transfection reagents (2 μg plasmids and 3 μL polyethylenimine (PEI) in 200 μL opti-MEM™ reduced serum medium). After incubation at 37°C for 4-6 h, the transfection reagents were exchanged to 2 mL complete medium. 16-20 h after transfection, cells were washed once with 1 mL PBS, incubated with 1 mL HBSS containing 10 mM PA at 37°C for 5 min, and then irradiated with blue light at a power of 30 mW/cm^2 for 1 min.

For the comparison of LITag and Lantern-mediated CAP-MS, HEK293T cells were cultured in 35-mm dishes and were transfected as described above. For LITag labeling, cells were washed once with 1 mL PBS, incubated with 1 mL Live Cell Imaging Solution™ containing 0.5 mM biotin-phenol (BP) at 37°C for 30 min, and then irradiated with 30 mW/cm^2 blue light for 1 min or with 450 mW/cm^2 blue light for 1 s. For CAP-MS labeling, cells were incubated with 1 mL complete medium containing 1 mM 3-EA at 37°C for 1 h, then were washed once with 1 mL PBS, immersed in 1 mL HBSS containing 1 mM 3-EA, and then irradiated with 30 mW/cm^2 blue light for 1 min or with 450 mW/cm^2 blue light for 1 s.

For optimizing the labeling conditions for SG proteome identification, HEK293T cells that stably express G3BP1-Lantern, Lantern-NES or G3BP1-miniSOG were cultured in 6-well plates. When the cell confluency reached 90%, cells were incubated at 37°C either with 10 mM PA in HBSS (1 mL) for 5 min or with 1 mM 3-EA in complete medium (1 mL) for 1 h. The complete medium containing 3-EA was exchanged to 1 mM 3-EA in HBSS (1 mL) before irradiation.

When the irradiation was completed, cells were scrapped off and resuspended in 1 mL PBS, centrifuged at 4°C and 300 × *g* for 3 min with 'soft' mode, and lysed with 200 μL RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% (v/v) NP-40, 1% (m/v) sodium

deoxycholate, 2% (m/v) SDS) containing 1 × cOmplete Proteinase Inhibitor Cocktail (Roche) with sonication on ice. Each 100 μL cell lysate was mixed with 800 μL of cold methanol (pre-incubated at -80°C for at least 30 min), incubated at -80°C overnight. Thereafter, samples were centrifuged at 4,000 × *g* at 4°C for 10 min. The protein pellet was dissolved in 100 μL 0.5% (m/v) sodium dodecyl sulfate (SDS) solution. The protein concentration was measured with Pierce™ BCA protein Assay kit, and samples were diluted to equal concentrations. Subsequently, 50 μL click reaction reagents were added to each 100 μL protein solution, which containing 300 μM N_3-PEG_3-biotin, 2 mM $CuSO_4$, 4 mM BTTAA and 1.5 mg/mL sodium ascorbate. The click reaction was conducted at room temperature for 1 h with shaking. Protein samples were mixed with 5 × protein loading buffer, followed by boiling at 95 °C for 10 min, and then analyzed by gel electrophoresis and Western blot.

Gel electrophoresis and Western blot analysis

The samples were loaded to a 4–20% gradient SDS-PAGE gel and analyzed by electrophoresis. For Coomassie Brilliant Blue staining, the gel was incubated overnight with 0.25% (m/v) Coomassie Brilliant Blue G250 in 10% (v/v) acetic acid and 20% (v/v) isopropanol at room temperature, then was incubated with destaining solution (10% (v/v) acetic acid and 30% (v/v) methanol) until the background was clear enough for photography. For Western blot analysis, the protein gel was transferred to a PVDF membrane under 230 mA for 1 h. The membrane was blocked with blocking buffer (5% BSA in TBST) at room temperature for 60 min and then incubated with Streptavidin-HRP (1:5,000 dilution) in TBST at room temperature for 1 h. For anti-V5 Western blot, the membrane was incubated with mouse anti-V5 primary antibody (1:5,000 dilution) at room temperature for 60 min, followed by incubating anti-mouse secondary antibody conjugated with HRP (1:5,000 dilution) for 0.5 h. The membrane was washed by TBST three times after each step of incubation, 5 min for each time. The membrane was incubated with Clarity Western ECL Substrate and then imaged by ChemiDoc Imaging System (Bio-Rad).

Mass spectrometry sample preparation

Cells stably expressing G3BP1-Lantern or Lantern-NES were cultured in 10-cm dishes in complete medium. At approximately 90% confluency, cells were incubated with 1 mM 3-EA in complete medium for 60 min at 37°C. Oxidative stress was induced by adding 0.5 mM $NaAsO_2$ to the cell culture medium for 5, 10, 15, 30 and 60 min. Thereafter, cells were washed once with 1 mL PBS, incubated in 4 mL HBSS containing 1 mM 3-EA and 0.5 mM $NaAsO_2$, then were irradiated with blue light at 150 mW/cm^2 for 30 s. Cells were collected and lysed on ice in 600 μL RIPA lysis buffer with sonication. Proteins in the cell lysate were precipitated with 4.8 mL pre-chilled methanol with incubation at -80°C overnight.

Following centrifugation at 4,500 × *g* for 10 min at 4°C, pelleted proteins were washed

twice with cold methanol, dissolved in 600 μL 0.5% (m/v) SDS solution, and reacted with biotin-conjugated azide in a 900 μL solution containing 100 μM N_3-PEG_3-biotin, 0.67 mM $CuSO_4$, 1.34 mM BTTAA and 0.5 mg/mL sodium ascorbate. The click reaction was conducted at room temperature for 1 h with vigorous shaking. Thereafter, proteins were precipitated once again by adding 7.2 mL cold methanol and incubated at -80°C overnight. Protein pellets were washed twice with cold methanol (-80°C) and dissolved by 800 μL 0.5% (m/v) SDS solution.

The protein concentration was measured with Pierce™ BCA protein Assay kit. 4 mg proteins from the G3BP1-Lantern cells and wildtype HEK293T cells, 0.4 mg proteins from the Lantern-NES cells were used for enrichment. For each enrichment group, 50 μL streptavidin agarose beads were washed once with 1 mL PBS, then was incubated with the protein solution for 3 h at room temperature with gentle rotation. Subsequently, the agarose beads were centrifuged at 3,000 × *g* for 2 min. The supernatant was removed, and the beads were washed with 1 mL 0.5% SDS in PBS for 10 min with gentle rotation, followed by washed with 1 mL PBS for 6 times. The beads were then resuspended in 500 μL 6 M urea and 10 mM dithiothreitol (DTT) in PBS, and incubated at 60°C for 15 min. Then, 25 μL 400 mM iodoacetamide (IAA) solution was added, followed with an incubation at 25°C for 30 min in dark. Agarose beads were washed twice with 1 mL 100 mM triethylammonium bicarbonate (TEAB) buffer and resuspended in 200 μL 100 mM TEAB. 1 μg sequencing-grade trypsin was then added for protein digestion, with shaking at 1,200 rpm and 37°C for 16 h. Thereafter, the released peptides in supernatant were collected by centrifugation at 15,000 × *g* for 10 min.

TMT 10 plex Mass Tag Labeling Kits and Reagents was used for quantitative proteomics identification. Peptide samples were first dried by rotary evaporator (1,500 × g, 45°C, 4–6 h) and desalted by Pierce C18 tips 100 μL (Thermo, 87784). The desalted samples were re-dried as descripted before. Re-dried samples were labeled by different TMT reagents (126: G3BP1-Lantern cells treated with 60 min and 15 min (the 0-15 min profiling group) $NaAsO_2$; 127 N: G3BP1-Lantern cells treated with 30 min and 10 min $NaAsO_2$; 127 C: G3BP1-Lantern cells treated with 15 min (the 0-60 min profiling group) and 5 min $NaAsO_2$; 128 N: G3BP1-Lantern cells treated with 0 min $NaAsO_2$; 128 C: omitting Lantern; 129 N: omitting light; 129 C: Lantern-NES cells treated with 60 min and 15 min (the 0-15 min profiling group) $NaAsO_2$; 130 N: Lantern-NES cells treated with 30 min and 10 min $NaAsO_2$; 130 C: Lantern-NES cells treated with 15 min (the 0-60 min profiling group) and 5 min $NaAsO_2$; 131: Lantern-NES cells treated with 0 min $NaAsO_2$), then combined, dried and stored at −80°C.

Thereafter, the peptide sample was fractionized by Pierce High pH Reverse Phase Peptide Fractionation Kit. Then the samples were combined as '1+5', '2+6', '3+7' and '4+8'. Combined samples were dried again by rotary evaporator (1,500 × g, 45°C, 4–6 h), and then identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Liquid chromatography-tandem mass spectrometry

Peptides were separated using a loading column (100 μm × 2 cm) and a C18 separating capillary column (75 μm × 15 cm) packed in-house with Luna 3 μm C18(2) bulk packing material (Phenomenex, USA). The mobile phases (A: water with 0.1% formic acid and B: 80% acetonitrile with 0.1% formic acid) were driven and controlled by a Dionex Ultimate 3000 RPLC nano system (Thermo Fisher Scientific). The LC gradient was held at 2% for the first 8 min of the analysis, followed by an increase from 2 to 10% B from 8 to 9 min, an increase from 10 to 44% B from 9 to 183 min, an increase from 44 to 99% B from 123 to 188 min, and a stable gradient of 99% from 188 min to 199 min.

For the samples analyzed by Orbitrap Fusion LUMOS Tribrid Mass Spectrometer, the precursors were ionized using an EASY-Spray ionization source (Thermo Fisher Scientific) source held at +2.0 kV compared to ground, and the inlet capillary temperature was held at 320°C. Survey scans of peptide precursors were collected in the Orbitrap from 350 to 1,600 Th with an AGC target of 400,000, a maximum injection time of 50 ms, RF lens at 30%, and a resolution of 120,000 at 200 m/z. Monoisotopic precursor selection was enabled for peptide isotopic distributions, precursors of z = 2–7 were selected for data-dependent MS/MS scans (with a resolution of 60,000) for 3 s of cycle time, and dynamic exclusion was set to 15 s with a ±10 ppm window set around the precursor mono-isotope.

In HCD scans, an automated scan range determination was enabled. An isolation window of 1.6 Th was used to select precursor ions with the quadrupole. Product ions were collected in the Orbitrap with the first mass of 110 Th, an AGC target of 50,000, a maximum injection time of 118 ms, HCD collision energy at 38%, and a resolution of 60,000.

Mass spectrometry data analysis

All raw data were processed within the MaxQuant software package (version 1.6.1.0). Data were searched against the UP000005640 proteome database from Uniprot (79052 human proteins in total). Half-tryptic termini and up to 1 missing trypsin cleavages were allowed. Carbamidomethylation at cysteine (+57.0215 Da) and isotopic modifications (+28.0313 and +34.0631 Da for light and heavy labeling, respectively) at lysine/N-terminal were set as fixed modifications. Oxidation at methionine (+15.9949 Da) and acetylation of N-terminal (+42.0106 Da) were set as variable modifications. Each of the biological replicates was analyzed separately. Contaminants and proteins identified as reverse hits were removed. Proteins with unique peptides < 2 or TMT reporter ion intensity = 0 were also removed. We normalized the TMT reporter ion intensity of each detected protein with the median intensity of all detected proteins. Subsequently, we calculated the ratios of normalized ion intensity detected in the G3BP1-Lantern samples treated with 60-min stress over the omitting-Lantern, omitting-light and NES controls. To determine the list of SG proteins, cut-off ratios of +/- Lantern, +/- light and G3BP1/NES were set as 1.1, and only

proteins commonly enriched by two replicates are determined as SG proteins.

CAP-CELL labeling of the mTurquoise and mCherry co-culture system

HEK293T cells were transfected with Lantern-TM co-expressing mTurquoise or mCherry separately. Once the transfection was completed, cells expressing mTurqoise and mCherry were mixed together at a ratio of 1:1, seeded onto pre-coated 14-mm coverslips, and cultured in complete culture medium at 37°C overnight. Then the cells were immersed in the Live Cell Imaging Solution (Invitrogen) containing 2 mM Btn-NH_2. Before labeling with a DMD-controlled 445 nm laser (5 mW/cm^2), the mTurquoise or mCherry expression patterns were captured with an upright fluorescence microscope. DMD masks were determined by manually defined thresholds. Each view was labeled with 445 nm for 2 min, and 10 views were labeled for each sample.

Then the cells were washed once with PBS, digested with trypsin-EDTA (0.05%) solution, and collected by centrifugation at 700 × *g* for 2 min. The cells were stained with Streptavidin-Alexa Fluor™ 647 (1:500 dilution) and Calcein-AM (1:400 dilution) at room temperature for 30 min. After washing with PBS for twice, the cells were analyzed by BD FACSAria™ III. Cells with both strong Calcein-AM and AlexaFluor 647 fluorescence were sorted into PCR tubes (50 cells per tube) containing 2 μL lysis buffer (0.5% (v/v) Triton X-100, 1 U SUPERaseIn™ RNase Inhibitor (Invitrogen, 40 U/μL), 0.1 U DNase I (NEB)). After cell sorting, the PCR tubes were incubated at 20°C for 10 min for DNA digestion. Then 0.1 μL of Oligo dT (100 μM), 1 μL of dNTP (10 mM each) and 0.08 μL of EDTA (25 mM) were added to the reaction system, and the mixture was incubated at 65°C for 10 min, followed with incubation at 72°C for 3 min to inactivate the DNase and denature RNA secondary structures. Thereafter, add 0.35 μL of SuperScript IV reverse transcriptase (Invitrogen, 200 U/μL), 0.25 μL of SUPERaseIn™ RNase Inhibitor (Invitrogen, 40 U/μL), 2 μL of 5 × SSIV buffer, 0.5 μL of 0.1 M DTT, 2 μL of 5 M Betaine and 0.6 μL of Ultrapure water. The reaction was incubated at 50°C for 50 min for reverse transcription, followed with 80°C 10 min to inactivate the reverse transcriptase.

After reverse transcription, 2 μL reaction solution was pre-amplified for 20 cycles with Phanta Max Super-Fidelity DNA polymerase (Vazyme) using gene-specific primers targeting the *mTurquoise*, *mCherry* and human *GAPDH* genes. Then 1 μL PCR product was diluted by 16 folds, mixed with PowerUp SYBR Green Master Mix and primers, and then quantified by ABI StepOne Plus system. C_t values were averaged from four replicate measurements. The enrichment fold of the *mTurquoise* or *mCherry* gene was calculated by: $2^{-\Delta Ct}$, where $\Delta C_t = C_t^{mTurquoise/mCherry} - C_t^{GAPDH}$.

CAP-CELL labeling of migrating cancer cells

The MC38 cells stably expressing Lantern-TM were seeded onto 14-mm coverslips pre-coated with Corning Matrigel matrix diluted in DMEM (1:100) at 37 °C overnight. A

culture insert (Ibidi) was placed at the center of the coverslip to create a 500 μm cell-free gap. After overnight culturing at 37°C, the insert was removed and cells were cultured for another 24 h. During the DMD-controlled CAP-CELL labeling, cells were immersed in the Live Cell Imaging Solution (Invitrogen) containing 2 mM Btn-NH_2. A ~200 μm band near the wound border ("Near") and a band at least 300 μm away from the border ("Far") were labeled respectively, with 1-min irradiation of 445 nm laser (85 mW/cm^2).

When the labeling was completed, cells were digested, stained with Streptavidin-Alexa Fluor™ 647 and sorted as described above. After cell sorting, the cell lysis and reverse transcription were conducted as described above. Thereafter, the Sherry2 protocol[4], was adopt for NGS library construction. 10 μL tagmentation mix (containing 0.05 μL TTE mix V50, 4 μL 5 × TD buffer, 0.2 μL SUPERaseIn™ RNase Inhibitor (Invitrogen, 40 U/μL), 4 μL 40% PEG8000 and 0.5 μL 10 mM ATP) was added to 10 μL reverse transcription product, then the reaction was incubated at 55°C for 30 min for RNA-cDNA hybrids tagmentation. Subsequently, the reaction system was supplemented with 0.05 μL Bst 3.0 DNA polymerase (NEB), 20.5 μL Q5 High-Fidelity 2x Master Mix (NEB), and 0.45 μL ultrapure water, incubated at 72°C for 15 min, and then at 80°C for 5 min. After that, 6 μL primer mix (1.5 μL N50X and 1.5 μL N70X primer from the TruePrep Index Kit V2 (Vazyme), and 3 μL Q5 High-Fidelity 2x Master Mix) was added. The reaction mixture was amplified for 21 cycles, and purified with 35 μL VAHTS cleanup beads. Finally, the DNA libraries were sequenced on the Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads.

Selective irradiation with digital micromirror device (DMD)

For DMD-controlled selective irradiation, experiments were performed on a lab-made upright fluorescence microscope equipped with a x25, 1.10 NA water objective lens (Nikon, N25x-APO-MP), scientific CMOS cameras (Hamamatsu ORCA-Flash 4.0 v2), laser lines (Coherent OBIS 405 nm, 488 nm, 561 nm) and a digital micromirror device (Texas Instruments, DLPC910+DLP6500). The DMD control solution was provided by Fldiscovery (F9120 DDR 0.65 1080P). Masking sheets loaded on the DMDs were made by ImageJ Fiji (version 2.1.0) and Matlab (version 23.1).

CAP-CELL labeling of the CD40/CD40L-guided cell-cell interactions

HEK293T cells were transfected with tdtomato-P2A-CD40L-Lantern and CD40-EGFP respectively. Transfected HEK293T cells were digested with trypsin-EDTA solution (0.05%) for 1 min, collected and counted. 4×10^5 of CD40L-Lantern cells were mixed with 2×10^5 of CD40-EGFP cells, then incubate with 100 μM Btn-NH_2 probe in 1 mL PBS solution containing 10 mg/mL BSA at 37 °C for 15 min. After blue light irradiation (30 mW/cm^2) in a 6 well plate, the cells were collected by centrifugation for 2 min at 700 × *g*, then wash with PBS for 3 times. Then cells were stained with Streptavidin-Alexa Fluor™ 647 (1:500) at room temperature for 30 min. After washing twice, cell mixtures were analyzed by BD

LSRFortessa™ Cell Analyzer.

References

1. Matreyek, K.A., Stephany, J.J., Chiasson, M.A., Hasle, N. & Fowler, D.M. An improved platform for functional assessment of large protein libraries in mammalian cells. *Nucleic Acids Research* (2019).
2. Wang, P. et al. Mapping spatial transcriptome with light-activated proximity-dependent RNA labeling. *Nature Chemical Biology* **15**, 1110-1119 (2019).
3. Liu, C. et al. Absolute quantification of single-base m(6)A methylation in the mammalian transcriptome using GLORI. *Nat Biotechnol* **41**, 355-366 (2023).
4. Di, L. et al. Rapid and sensitive single-cell RNA sequencing with SHERRY2. *BMC Biol* **20**, 213 (2022).