Supplementary Materials

Optoproteomics elucidates the interactome of L-type amino acid transporter 3 (LAT3)

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Materials and Methods

General genetic procedures. Standard *E. coli* genetic techniques were performed. *E. coli* strains DH5α from Stratagene were used for plasmid propagation and isolation. Oligonucleotides were obtained from eOligo. Plasmid DNAs were purified using standard MiniPrep Kits from Invitrogen. AzF was purchased from ChemImpex. DNA sequencing was performed according to the ABI 3730 Capillary Sequencer protocol.

Plasmids for expression of suppressor tRNA, AzF-RS and amber mutations of LAT genes in mammalian cells. Plasmids for expression suppressor tRNA (Yam) and AzF-RS (pcDNA-RS) genes in mammalian cells were obtained from Dr. Thomas P. Sakmar at the Rockefeller University. The gene encoding human LAT3 was PCR amplified from the plasmid of pHIV-1SDmCMVhLAT3ireseGFPpre by designing oligonucleotides with restriction sites of 5' EcoRI and 3' XbaI. The PCR product was double-digested and inserted into a mammalian expression vector pCDNA3.1 containing HA tag at C-terminal to create pCDNA3.1-hLAT3-HA. The new construct was confirmed by DNA gel analysis and sequencing.

LAT-amber mutants. To create amber stop mutations at positions Y251, S262, K264, S267, F250, T252, Q263, A265, P266, L268, oligonucleotides were designed to introduce TAG codon by site-directed mutagenesis using Pfu Ultra polymerase. Mutant constructs were purified and confirmed by DNA sequencing.

Incorporation of AzF into LAT3 in Mammalian Cells. 5 x 10^5 HEK293T (ATCC, CRL-11268) were maintained in Dulbecco's modified Eagle's medium (DMEM) medium (4.5g/liter glucose, Gibco), 10% fetal bovine serum (Gibco), 2mM glutamine, 100 units/ml penicillin, and 100ug/ml streptomycin (Invitrogen) at 37 °C in a 5% CO_2 atmosphere at least for 2 hours before transfection. Cells were incubated transfected with plasmid DNA using Lipofectamine Plus (Invitrogen) according manufacture's protocol. For transfection in a 6-well culture plate, 1 μg of pcDNA3.1-LAT3-HA, 1 μg of Yam, and 0.1 μg of pcDNA-RS were mixed with 5 μl Lipofectamine and 2.5 μl plus reagent and added to cells at 70–80% confluence according to the manufacturer's protocol. After transfection, cells were fed with or without 1mM AzF. AzF was dissolved directly in DMEM at a stock concentration 10 mM and supplemented to the medium at a final 1mM concentration. After 24h, medium was changed (containing AzF). Cells expressing wt LAT3 or LAT3-AzF mutant were harvested 48 h post-transfection for crosslinking and other functional assays.

Photo-cross-linking LAT3-AzF mutants in live cells. HEK293T cells over-expressing LAT3-AzF mutants were washed with PBS 48 h post-transfection and treated with UV light. Two types of UV light sources have been tested: 1) 30min using the Philips TUV light source (15w, 365nm), 2) 10min using CL-1000L (Analytik Jena, 40w, 365nm). After UV light exposure the cells were scraped in with 100ul lysis buffer containing RIPA buffer and protease inhibitors cocktail and phosphatase inhibitor cocktail (Cell Signaling Technology, 100X) and sonicated at 4 ˚C for 15 cycles.

Western blot analysis. Lysates were analyzed by BCA protein assay kit. Samples were then diluted to normalize the protein concentration using 4X NuPAGE LDS sample buffer containing DTT and heated at 65°C for 5 minutes. The samples were then run on an SDS-PAGE gel (NuPage Novex 4-12% Bis-Tris Gel) and then transferred to a polyvinylidene difluoride (PVDF) membrane for immunoblotting. PVDF membrane was blocked in 5% BSA in 1X TBST for 1 hour at room temperature on a shaker followed by

incubation with primary and secondary antibodies diluted in 5% BSA in 1X TBST. For detection of HA tagged LAT3, PVDF membranes were blotted with a primary HA rAb antibody (company Cell Signaling Technology, mAb clone number, 1:1000) followed by incubation with a secondary anti-rabbit antibody (company, clone number, 1:5000) conjugated with horseradish peroxidase (HRP). GAPDH as equal amounts of total protein were detected using a primary GAPDH mAb (company abcam, mAb clone number, 1:2000) followed by a secondary anti-mouse antibody (company, clone number, 1:5000) conjugated with HRP. The membranes were then incubated in a chemiluminescent substrate (Thermo Scientific) to be exposed.

Immunoprecipitation and trypsin digestion. Cell lysates were subjected to HA-resin (Sigma) for immunoprecipitation following manufacture's protocol, in brief, after incubation followed with stringent wash multiple times. Trypsin digestion was performed directly on HA-resin following the manufacture's protocols. Eluents were desalted and applied to LC-MS/MS analysis.

To prepare cell lysates, three lysis buffer (**Table 1**) needs to be made and stored at 4℃, and all lysis buffer needs to be supplemented with 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail immediately before use. Cell pellet was lysed with 10 mL cold LB1, and rotate on a roller at 4℃ for 10 min. Centrifuge at 4500 rpm for 5 min at 4℃, and remove supernatant. Then resuspend pellet with 10 mL cold LB2, and rotate on a roller at 4℃ for 5 min. Centrifuge at 4,500 rpm for 5 min at 4℃, and remove supernatant. Resuspend cell pellet in 300 µL cold LB3, and transfer to fresh eppendorf tubes for sonication. In order to maximise the sonication efficiency, sonicate no more than 20 million cell per 300 µL at 4℃ for 15 cycles with 30 sec on and 30 sec off. Split into multiple tubes if necessary. Keep sonicated cell lysates on ice and add 30 µL Triton X. Invert the tubes multiple times to mix it well. Then centrifuge the lysate at 18,000 ×g for 10 min at 4℃ to pellet the debris. Combine supernatant where applicable, and collect 10% of supernatant into separate tubes as input, and store at -80℃. Transfer the rest supernatant into fresh tubes and adjust the final volume to 1,000 µL with LB3. Mix the supernatant with 100 µL magnetic beads that have pre-bound with antibodies or IgG, and incubate on a rotator overnight at 4℃.

The next day, collect the beads, and transfer supernatant into fresh tubes as control. Wash the beads with 1 mL cold RIPA buffer (Table 1) for 10 times and flick the tube to ensure the beads are fully resuspended. Incubate for 1 min between washes, and briefly spin down before removing the RIPA buffer. Then, wash bead with freshly made COLD 100mM ammonium hydrogen carbonate (AMBIC) solution as quickly as possible for 2 times. After second wash, spin down the tubes at 960×g for 3 min and remove the residue of AMBIC buffer. Once being washed, beads can be snap frozen and stored at -20℃, or continue to trypsin digestion.

Trypsin (Trypsin Gold, MS grade, Promega) was reconstituted in freshly made 100 mM AMBIC buffer to reach the final concentration of 10 µg/mL. Add 20 µL trypsin solution into beads, and vortex samples for 15 sec every 2-3 min for the first 15 min to ensure the beads are fully resuspended and well covered in trypsin solution. Then incubate at 37℃ overnight without any further agitation. The next day, add another 10 µL of trypsin solution into each sample, and further digest for 4 hr at 37℃. Place samples in the magnetic rack to collect supernatant that contains digested peptides, and transfer to fresh tubes. Add 1.5 µL 100% formic acid (FA, Sigma) into each sample to acidify digested peptides at a final concentration of 5% (v/v). The samples can be stored at -20℃.

Supplementary Table 1. Recipe for RIME buffers

RIME Buffer	Recipe
Lysis Buffer 1 (LB1)	50 mM HEPES-KOH, (pH 7.5)
	140 mM NaCl
	1 mM EDTA
	10% (vol/vol) glycerol
	0.5% (vol/vol) NP-40
	0.25% (vol/vol) Triton X-100
Lysis Buffer 2 (LB2)	10 mM Tris-HCL (pH 8.0)
	200 mM NaCl
	1 mM EDTA
	0.5 mM EGTA
Lysis Buffer 3 (LB3)	10 mM Tris-HCl (pH 8.0)
	100 mM NaCl
	1 mM EDTA
	0.5 mM EGTA
	0.1% (wt/vol) sodium deoxycholate
	0.5% (vol/vol) N-lauroylsarcosine
RIPA Buffer for RIME	50 mM HEPES (pH 7.6)
	1 mM EDTA
	0.7% (wt/vol) sodium deoxycholate
	1% (vol/vol) NP-40
	0.5M LiCl

Since the washing buffer contains high concentration of salt, all samples need to be desalted before analysed by mass spectrometer. C18 ZipTip (Millipore) was used to perform the clean-up steps with

freshly made wetting/condition solution, equilibration/wash solution, and elution buffer (Table 2). Aliquot 10 µL elution buffer in fresh tubes with labels for each sample. Firstly, balance the C18 column with 10 µL condition solution twice and equilibrate with 10 µL equilibration solution twice. Secondly, load the peptides with C18 column and pipette up and down slowly for 20 times. Thirdly, wash C18 column with 10 µL wash solution for 4 times and discard wash solution. Finally, elute the bound peptides from C18 column in 10 µL elution buffer twice in a fresh collection tube, and the samples are now desalted.

The desalted peptides were dried in a centrifugal vacuum concentrator (5301, Eppendorf) for 10-30 min, and reconstituted in 20 µL loading buffer 3% acetonitrile (ACN) and 0.1% formic acid (FA). The samples can be stored at 4℃ for short term and at -20℃ for long term. For mass-spec analysis, 10 µL samples were loaded into either 96-well plate or PCR strip according to specification of the mass spectrometer.

Supplementary Table 2. Buffers for C18 ZipTip clean-up

Clean-up Buffer	Recipe
Wetting/Conditioning solution	100% acetonitrile
Equilibration/ Wash Solution	0.1% formic acid
Elution Buffer	0.1% formic acid, 60% acetonitrile

LC-MS/MS analysis

Liquid chromatography tandem mass spectrometry (LC-MS/MS) experiments were carried out at the Mass Spectrometry Core Facility at Natural History Museum of Paris using high-resolution TripleTOF® 6600 Quadrupole Time-Of-Flight (QTOF) and Q Exactive™ mass spectrometers. Samples prepared for MS with RIME or on-resin digestion method were reconstituted in loading buffer and were loaded into mass spectrometer with 10 µL. Each sample contained peptide mixture, and was separated by liquid chromatography (LC) based on hydrophobicity and then ionised by electrospray (ESI). Mass spectrometer detects the intensity of ion signals as a function of retention time, as well as precursor ions and product ions of peptides based on mass-to-charge (m/z) ratios.

All proteolytic digested samples were separated by nano-liquid chromatography (nano-LC) using Ultimate 3000 HPLC and autosampler system (Dionex). A total of 10 µL of reconstituted peptide solution were loaded onto Acclaim™ PepMap™ C18 micro-column (Thermo Fisher) 15 µL/min. Peptides were eluted with a linear gradient of H_2O: ACN (98:2, 0.1 % FA) to H_2O: ACN (64:36, 0.1 % FA) at 250 nL/min over 60 min with a nanoflow of 0.3 µL/min. Positive ions were generated by electrospray and captured by detector and analyser.

A survey scan m/z 350-1800 was acquired in the Orbitrap operated in data dependent mode (Resolution = 60,000 at m/z 400, with automatic gain control target of 1,000,000 ions counts). Up to the 20 most abundant ions (>5,000 ion counts) with charges over +2 were sequentially isolated and fragmented by collision induced dissociation (CID). M/z ratios selected for MS/MS were dynamically excluded for 30 seconds.

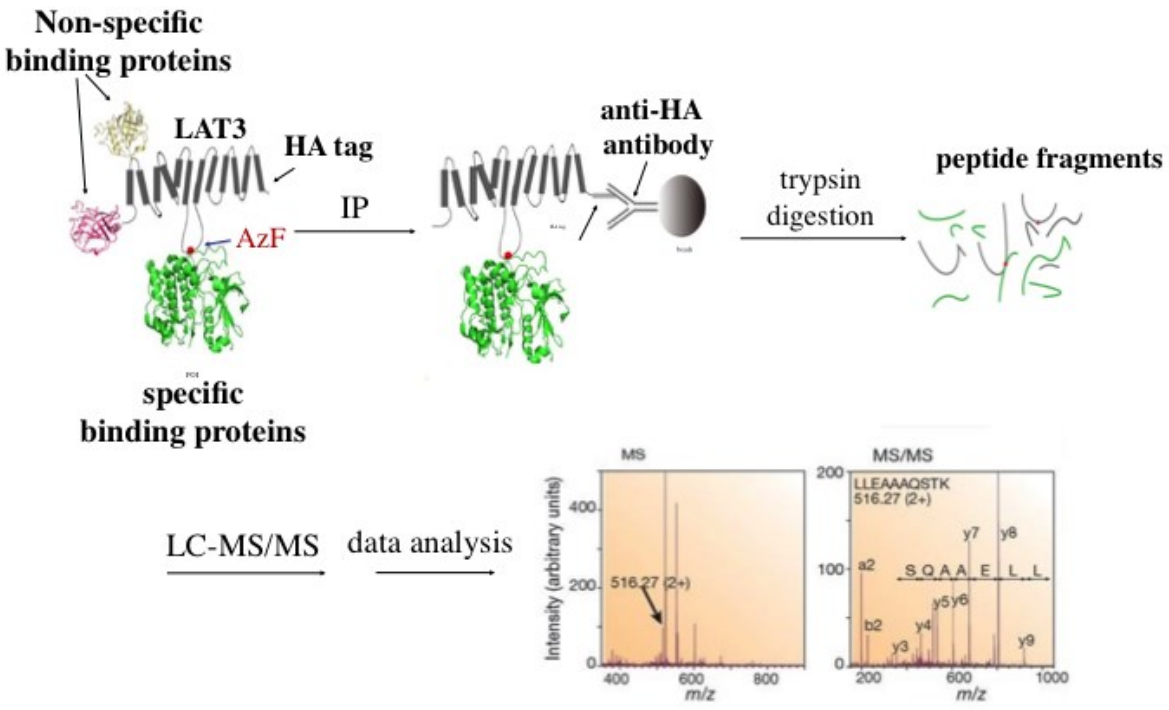
Proteome Discoverer 2.2 (Thermo Fischer Scientific) and Mascot (Matrix Science) were used to collect and analyse MS data. The result data were converted to peak list files to query at Mascot search engine for MS/MS ions match in the SwissProt Database (uniprot_SwissProt_Human_2018-04.fasta) with a FDR (false discovery rate) <1%. The parameters used to search for protein were allowed up to two missed cleavages for trypsin; one fixed modification (carbamidomethyl of Cys); one variable modifications for oxidation of Met; mass tolerance of 10 ppm for the precursor ion and mass tolerance of 0.8 Da for the fragment ion. The search results included a list of identified peptides for further analysis. Scaffold 4 (Proteome Software) was used to combine and compare search results to identify biological relevance, display spectrum details and counts and create comprehensive lists of target proteins classified by their molecular function or involvement in biological processes.

Protein sequences were downloaded from Uniprot and database search was carried out using in-house Mascot software (www.matrixscience.com) with following parameters: Homo sapiens (taxonomy), carbamidomethylation (fixed modification), oxidation of Methionine and deaminated (NQ) as variable modifications. We define the high confidence partners based on two criteria: 1) peptides with high identification probability (≥95%), 2) peptides appearing in all five biological replicates (Supp Datasets 1-2). Enzyme was Trypsin with 2 missed cleavage. MS and MS/MS tolerance were set to 0.1 Da and 0.2 Da respectively. Protein hits from Mascot ($p < 0.05$) were analysed with Scaffold (version Scaffold_4.10.0, Proteome Software Inc., Portland, OR) to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm [1] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm [2]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Protein FDR was estimated to be lesser than 0.1%.

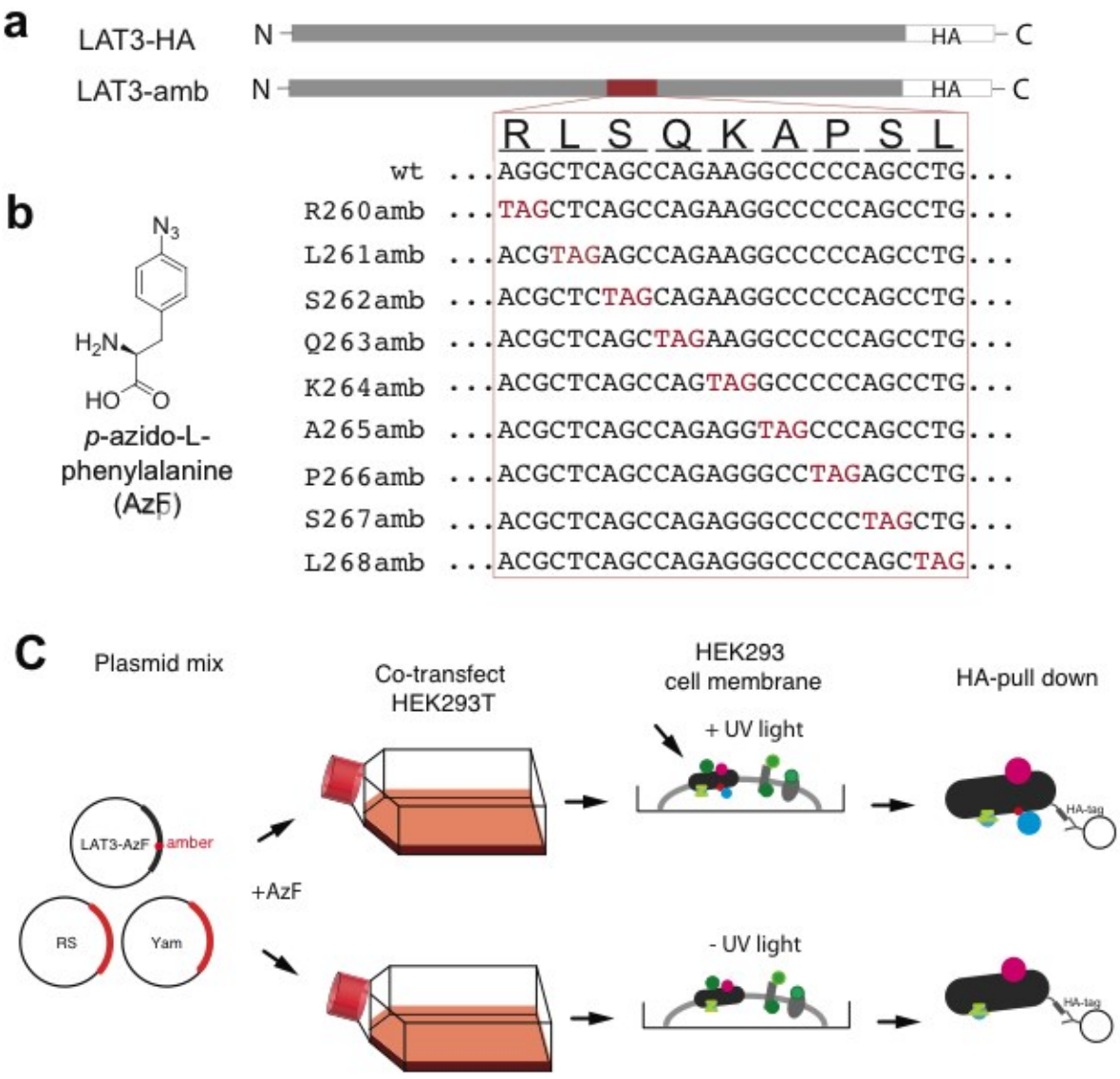
References:

1. Keller A et al *Anal. Chem.* 2002; 74(20):5383-92)
2. Nesvizhskii AL et al *Anal. Chem.* 2003;75(17):4646-58

Supplementary Figures

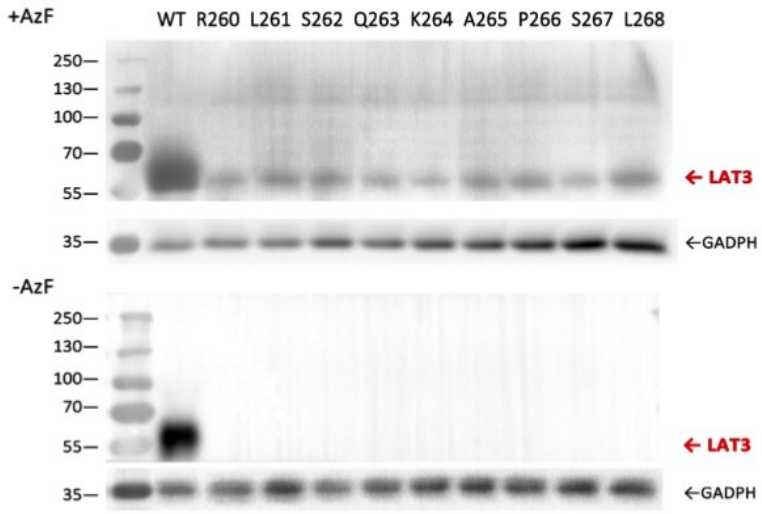


Supplementary Figure 1. Schematic workflow of detection of interactive proteins in optoproteomics. After lysis, protein complexes of LAT3 and associated proteins (non-specific and specific binding) formed and were enriched in immunoprecipitation with anti-HA tag, complexes were then subject to stringent washes, trypsin digestion, clean-up and acidification for LC-MS/MS mass spectrometric and data analysis.



Supplementary Figure 2. Constructs, expression and UV treatment of LAT3-AzF mutants. a. LAT3-HA and LAT3-amb mutant constructs. **b.** The amber stop codon point mutation (TAG) is introduced into the LAT3-HA construct at R260, L261, S262, Q263, K264, A265, P266, S267, and L268 site. **c.** After co-transfection of site-specific amber mutant of LAT3 with the orthogonal pair of tRNA and AzF-RS, culture the cells for 48 hours in the presence of AzF. Before lysing the cells, two batches of cells were either treated for photo-crosslink with UV light (365nm) (+UV) or without UV (-UV), followed by immunoprecipitation enrichment with anti-HA tag.

a.



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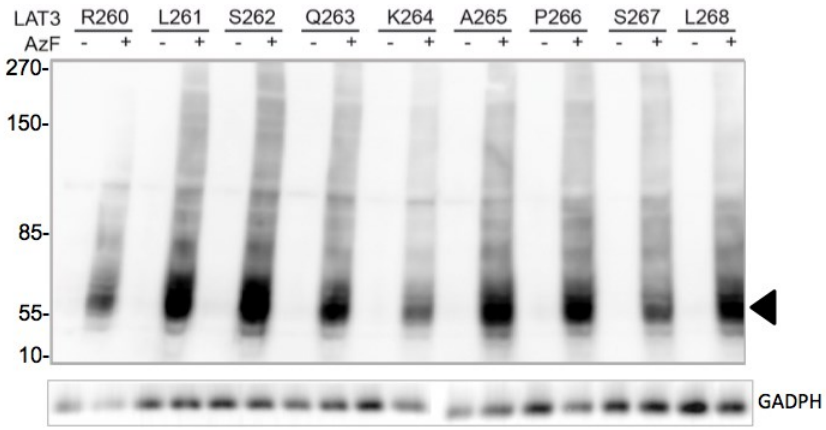
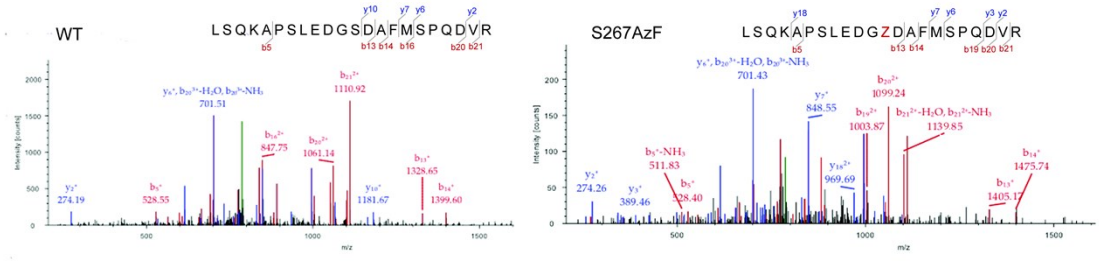
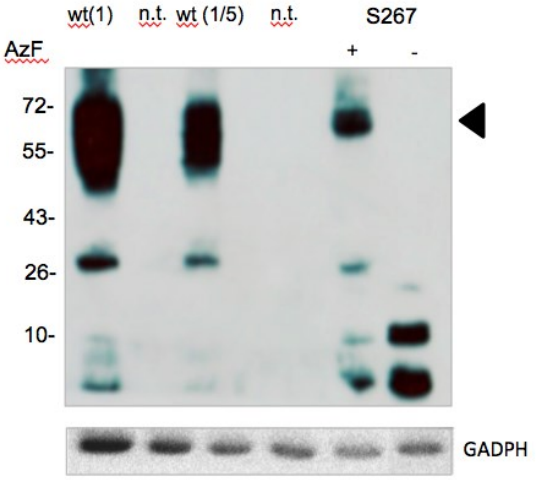


c.

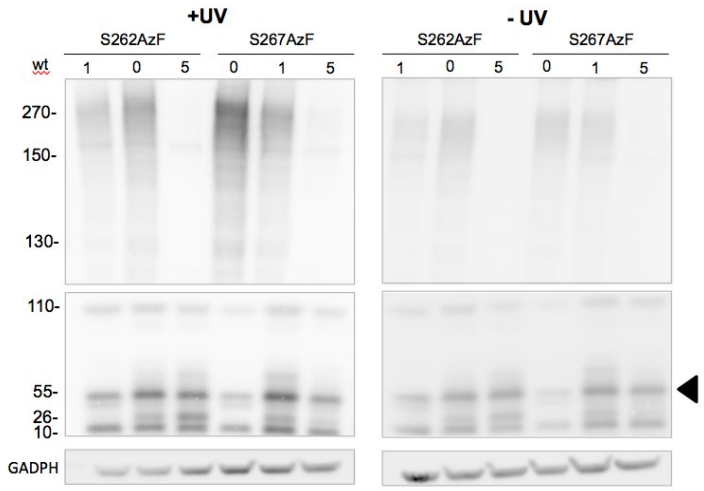


C. **Supplementary Figure 3. A.** Expression LAT3-AzF mutants. Upper panel, the amber stop codon point mutation (TAG) is introduced into the LAT3-HA construct at R260, L261, S262, Q263, K264, A265, P266, S267, and L268 site. After co-transfection of site-specific amber mutant of LAT3-amb with

the orthogonal pair of tRNA and AzF-RS, culture the cells for 48 hours in the presence of AzF (+AzF), western blotting against HA tag was performed. LAT3 appeared at the expected molecular weight of ~55kDaa. Transfected cells without the AzF in the media were tested in parallel (-AzF), as expected no full length LAT3 were detected due to the presence of stop codon in protein translation. GADPH was applied as the loading control.



Supplementary Figure 4. Expression of LAT3-S267AzF. The amber stop codon point mutation (TAG) is introduced into the LAT3-HA construct at S267 site. After co-transfection of site-specific amber mutant of LAT3-amb with the orthogonal pair of tRNA and AzF-RS, culture of the cells for 48 hours in the presence of AzF (+AzF) or absence of AzF (-AzF), western blotting against HA tag was performed. LAT3 appeared at the expected molecular weight of ~55 kDa. Significant truncated proteins were only detected in the case of S267 (-AzF) condition. Wild-type LAT3 at two different loading quantities (1 and 1/5 loading) compared. GAPDH was applied as the loading control.



Supplementary Figure 5. WT LAT3 competes with the UV crosslinking. Western blotting of HEK293 cells transfected with LAT3-S262AzF or LAT3-S267AzF in the absence or presence of LAT3-GFP (wt) construct and detected by anti-HA antibody. Samples were treated with UV (+UV) or without UV (-UV). The numbers indicate the ratios of transfected gene dosages between wt and the mutant LAT3 (0:1, 1:1, 5:1). LAT3 and truncate proteins were also analyzed. LAT3 shows at ~55kDa. GADPH was applied as the loading control. Complexes with molecular weight higher than 130 kDa were decreasing in a dose-dependent manner when wt LAT3 is present.

Supplementary Tables

Supplementary Table 3. 18 common elements in "LAT3-S262+UV", "LAT3-S262-UV", "LAT3-S267-UV" and "LAT3-S267+UV":

Accession Number	Protein Name	MW (kDa)
ROA2_HUMAN	Heterogeneous nuclear ribonucleoproteins A2/B1	37
HNRH1_HUMAN	Heterogeneous nuclear ribonucleoprotein H	49
DHX9_HUMAN	ATP-dependent RNA helicase A	140
HNRPC_HUMAN	Heterogeneous nuclear ribonucleoproteins C1/C2	33
ROA1_HUMAN	Heterogeneous nuclear ribonucleoprotein A1	38
FUS_HUMAN	RNA-binding protein FUS	53
HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprotein K	50
HNRPU_HUMAN	Heterogeneous nuclear ribonucleoprotein U	90
ROA0_HUMAN	Heterogeneous nuclear ribonucleoprotein A0	30
EF1A1_HUMAN (+1)	Elongation factor 1-alpha 1	50
U5S1_HUMAN	116 kDaa U5 small nuclear ribonucleoprotein component	109
SF3B1_HUMAN	Splicing factor 3B subunit 1	145
HNRH3_HUMAN	Heterogeneous nuclear ribonucleoprotein H3	36
HNRPF_HUMAN	Heterogeneous nuclear ribonucleoprotein F	45
HNRH2_HUMAN	Heterogeneous nuclear ribonucleoprotein H2	49
DDX3X_HUMAN	ATP-dependent RNA helicase DDX3X	73
XRCC5_HUMAN	X-ray repair cross-complementing protein 5	82
EIF3F_HUMAN	Eukaryotic translation initiation factor 3 subunit F	37

Supplementary Table 4. 7 common elements in "LAT3-S262+UV", "LAT3-S267-UV" and "LAT3-S267+UV":

Accession Number	Protein Name	MW (kDa)
HNRPL_HUMAN	Heterogeneous nuclear ribonucleoprotein L	64
HNRDL_HUMAN	Heterogeneous nuclear ribonucleoprotein D-like	46
U520_HUMAN	U5 small nuclear ribonucleoprotein 200 kDaa helicase	244
PRKDAC_HUMAN	DNA-dependent protein kinase catalytic subunit	469
ADT2_HUMAN	ADP/ATP translocase 2	32
PRP8_HUMAN	Pre-mRNA-processing-splicing factor 8	273
PRP19_HUMAN	Pre-mRNA-processing factor 19	55

Supplementary Table 5. 5 common elements in "LAT3-S262-UV", "LAT3-S267-UV" and "LAT3-S267+UV":

Accession Number	Protein Name	MW (kDa)
CPSF6_HUMAN	Cleavage and polyadenylation specificity factor subunit 6	59
CPSF7_HUMAN	Cleavage and polyadenylation specificity factor subunit 7	52
ANFY1_HUMAN	Rabankyrin-5	128
RS3A_HUMAN	40S ribosomal protein S3a	29

EIF3B_HUMAN	Eukaryotic translation initiation factor 3 subunit B	92

Supplementary Table 6. 6 common elements in "LAT3-S267-UV" and "LAT3-S267+UV":

Accession Number	**Protein Name**	**MW (kDa)**
HNRL1_HUMAN	Heterogeneous nuclear ribonucleoprotein U-like protein 1	95
TBA1A_HUMAN (+1)	Tubulin alpha-1A chain	50
U2AF2_HUMAN	Splicing factor U2AF 65 kDaa subunit	53
TCPZ_HUMAN	T-complex protein 1 subunit zeta	58
HNRPM_HUMAN	Heterogeneous nuclear ribonucleoprotein M	77
RL18_HUMAN	60S ribosomal protein L18	21

Supplementary Table 7. 3 common elements in "LAT3-S262+UV" and "LAT3-S267+UV":

Accession Number	**Protein Name**	**MW (kDa)**
HNRPR_HUMAN	Heterogeneous nuclear ribonucleoprotein R	70
VIME_HUMAN	Vimentin	53
YLPM1_HUMAN	YLP motif-containing protein 1	241

Supplementary Table 8. 2 common elements in "LAT3-S262-UV" and "LAT3-S267+UV":

Accession Number	**Protein Name**	**MW (kDa)**
PTBP1_HUMAN	Polypyrimidine tract-binding protein 1	57
H32_HUMAN	Histone H3.2	15

Supplementary Table 9. 2 elements included exclusively in "LAT3-S262-UV":

Accession Number	**Protein Name**	**MW (kDa)**
TBA1B_HUMAN	Tubulin alpha-1B chain	50
SMD1_HUMAN	Small nuclearribonucleoprotein Sm D1	13

Supplementary Table 10. 2 elements included exclusively in "LAT3-S267+UV":

Accession Number	**Protein Name**	**MW (kDa)**
SC16A_HUMAN	Protein transport protein Sec16A	252
XRCC6_HUMAN	X-ray repair cross-complementing protein 6	69

Supplementary Table 11. 9 elements included exclusively in "LAT3-S262+UV":

Accession Number	Protein Name	MW (kDa)
MATR3_HUMAN	Matrin-3	95
ROA3_HUMAN	Heterogeneous nuclear ribonucleoprotein A3	40
ILF3_HUMAN	Interleukin enhancer-binding factor 3	95
RS5_HUMAN	40S ribosomal protein S5	23
HNRPQ_HUMAN	Heterogeneous nuclear ribonucleoprotein Q	70
DDX5_HUMAN	Probable ATP-dependent RNA helicase DDX5	69
K2C1_HUMAN	Keratin, type II cytoskeletal 1	66
RL7A_HUMAN	60S ribosomal protein L7a	30
FSIP2_HUMAN	Fibrous sheath-interacting protein 2	392