ONLINE METHODS

Purification of recombinant CSPα wild-type and mutant proteins

For recombinant protein expression, CSPα^{WT}, CSPα^{L115R}, CSPα$^{L116\Delta}$ and SSPα (L^{115}LTSSYSSSSLSSSFNSSSGKSKP^{138}; underlined = C to S substitutions) were cloned into a modified pGEX-KG vector. Glutathione S-transferase (GST) was followed by a thrombin-cleavage site and a Tobacco Etch Virus (TEV) protease cleavage site, followed by the cDNA of the CSPα variant. Transformed BL21(DE3) *E. coli* were grown at 37 °C. Protein expression was induced with 0.8 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD 0.6-0.8 at 20 °C for 18 hours. Bacteria were pelleted and then lysed in phosphate buffered saline (PBS; pH 7.4) containing 2 mM dithiotheitol (DTT), 0.1 mg/ml hen egg lysozyme, 1 mM phenylmethylsulfonylfluoride (PMSF), EDTA-free protease inhibitor cocktail (Thermo Fisher), and 20-25 units/ml DNase I. Cells were broken by sonication (30 pulses of 0.5 second at 50% amplitude), and insoluble material was removed by centrifugation for 30 min at 7000 average centrifugal force (g_{av}) at 4 °C. Proteins in the supernatant were affinity-purified using glutathione Sepharose resin (GE Healthcare) incubation overnight at 4 °C, followed by several washes with PBS containing 1 mM DTT. Bound GST-protein was approximated by SDS-PAGE followed by Coomassie brilliant blue R-250 staining, in comparison with bovine serum albumin (BSA) standards. The GST-protein bound to resin was cleaved overnight by TEV protease at 1:300 molar ratio (protease : protein) at 4 °C. The final buffer for storage at 4 °C (short-term) or -30 °C (long-term) was PBS containing 2 mM DTT, 337 mM total NaCl, 0.01% Triton X-100, supplemented with EDTA-free protease inhibitor cocktail (Thermo Fisher). Despite exposure to air throughout this procedure, CSPα^{WT}, CSPα^{L115R}, CSPα$^{L116\Delta}$ had a stable amber-brown color while SSPα was clear in solution. Protein concentrations post-TEV cleavage were estimated by: a) BSA standards compared via SDS-PAGE followed by Coomassie staining, 2) NanoDrop spectrophotometry (Abs 280 nm) using a Take3 (BioTek) plate reader, and 3) Bicinchoninic acid (BCA) assay (Thermo Scientific).

Heterologous expression of CSPα wild-type and mutant proteins in mammalian cells

Human embryonic kidney 293T cells (HEK293T; American Type Culture Collection) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Rocky Mountain Biologicals) and L-glutamine/penicillin/streptomycin (Thermo Fisher Scientific). Cells were thawed from initially frozen stocks every 15 days to maintain a lower passage number, and tested for mycoplasma contamination crudely by DAPI staining. Cells were transfected at ~60% confluency with pCMV5 plasmid encoding N-terminally Myc-epitope tagged CSPα^{WT}, CSPα^{L115R}, CSPα$^{L116\Delta}$ and SSPα (Myc-tag and linker sequence = MEQKLISEEDLNEFGQGAQGQL), using calcium-phosphate precipitation method (*1*). 48 h post-transfection, cells were lysed in: 1) 0.1 N HCl containing 0.1% Triton X-100 and EDTA-free protease inhibitor cocktail (Thermo Fisher), for disruption of Fe-S clusters measuring

palmitoylation and for chemical depalmitoylation; or 2) PBS containing 0.1% Triton X-100, supplemented with EDTA-free protease inhibitor cocktail (Thermo Fisher) for immunoprecipitation followed by UV-Vis spectrometry and measurement of [$Fe^{2+/3+}$] and [S^{2-}].

Nfs1 knockdown and rescue by Nfs1 overexpression

For knockdown and rescue experiments, HEK293T cells expressing CSPα variants were transfected with pIKO.1P shNFS1_1 shRNA (Addgene plasmid #102963) without or in combination with FUW-NFS1 expressing a knockdown-resistant version of NFS1 due to silent mutations (cloned from Addgene plasmid # 102977); both constructs are described and characterized in detail by the Possemato lab in (*2*). Cells were lysed after 72 h and measurements were made by quantitative immunoblotting.

Mouse lines and primary neuron culture

Primary neurons from CSPα knockout ($CSP\alpha^{-/-}$) mice were used as a clean background for expression of CSPα wild-type and mutant proteins. This mouse line was generated by and obtained from Dr. Thomas C. Sudhof's lab (*3, 4*), and is available from Jackson Laboratory (B6;129S6-Dnajc5tm1Sud/J; stock #: 006392). Animal husbandry and the experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Weill Cornell Medicine.

Neonates from $CSP\alpha^{+/-}$ to $CSP\alpha^{+/-}$ breeding pairs were genotyped before performing neuron culture from $CSP\alpha^{-/-}$ pups. Cortical neurons were cultured essentially as described previously (*5*). Cortices were dissected in ice-cold HBSS, dissociated by trypsinization (0.05% trypsin-EDTA for 10 min at 37 °C), triturated with a siliconized pipette, and plated onto poly-L-lysine coated 24-well plastic dishes or coverslips. Plating medium (DMEM, Thermo Fisher) supplemented with 5 g/L glucose, 0.2 g/L $NaHCO_3$, 0.1 g/L transferrin, 0.25 g/L insulin, 0.3 g/L L-glutamine, and 10% fetal bovine serum was replaced with growth medium (DMEM, Thermo Fisher) containing 5 g/L glucose, 0.2 g/L $NaHCO_3$, 0.1 g/L transferrin, 0.3 g/L L-glutamine, 5% fetal bovine serum, 2% B-27 or N21-MAX supplement, and 2 μM cytosine arabinoside 2 days after plating. Cultured neurons were transduced with lentiviral particles and harvested for experiments as described in figure captions.

Lentivirus production and transduction

N-terminally Myc-epitope tagged $CSP\alpha^{WT}$, $CSP\alpha^{L115R}$, $CSP\alpha^{L116\Delta}$ and SSPα (Myc-linker as in pCMV5 vector described above) were cloned into lentiviral FUW vector (*6*). HEK293T cells were co-transfected with the vector plasmid, the HIV-1 lentiviral packaging constructs pRSVREV and pMDLg/pRRE (*7*), and the vesicular stomatitis virus-G expression plasmid pHCMVG (*8*). The virus-containing culture supernatant was collected 48 h post-transfection and was either added directly

to medium of cells to transduce them, or was concentrated by centrifugation at 50,000 g_{av} for 90 min. The viral pellet was resuspended in neuronal medium (at 1/10 of the precentrifugation volume). All lentiviruses used in a single experiments were prepared together. Neurons were infected and harvested for experiments at times described in figure captions.

X-ray fluorescence (XRF)

The X-ray fluorescence experiments were carried out at the F3 station of CHESS (Cornell University, Ithaca, NY). The source of the F3 beamline is the radiation from a bending magnet in the 5.3 GeV Cornell Electron Storage Ring (CESR). Monochromatic X-rays of 12 keV were used to excite the fluorescence from the samples. Ten microliters of protein solution were pipetted into a quartz capillary (1 mm inside diameter) and mounted on the goniometer. X-ray fluorescence of the sample was collected by an energy dispersive detector: Hitachi Vortex-ME4 Silicon Drift Detector (SDD).

Limited proteolysis

Limited trypsin digestion was used to detect oligomerization in each protein's native *in vitro* state. Purified protein (10 mg/ml) was exposed to increasing concentration of trypsin (0.01-1 mg/ml) for 20 minutes at 4 °C, followed by immediate inhibition of trypsin with addition of PMSF to 1 mM. To clarify the effect of CSPα oligomerization on trypsin-susceptibility, CSPα oligomers were disrupted, 1) before trypsinization, by incubation with 0.1 N HCl for 20 min at room temperature, and then neutralized with 1 M sodium cacodylate to pH 7.0, or 2) after limited proteolysis, with 0.1 N HCl for 20 min at room temperature. Samples were boiled for 20 minutes in Laemmli sample buffer, followed by SDS-PAGE and immunoblotting.

Immunoprecipitation of CSPα wild-type and mutant proteins from mammalian cells

Immunoprecipitation was used to isolate CSPα variants expressed in HEK293T cells or in CSPα$^{-/-}$ primary neurons, and to isolate the endogenously expressed CSPα in WT or ANCL fibroblasts, in order to detect Fe-S clusters by UV-Vis spectroscopy and to measure [$Fe^{2+/3+}$] and [S^{2-}]. Cells expressing myc-epitope tagged CSPα variants or endogenous CSPα were lysed in PBS containing 0.1% Triton X-100 and supplemented with EDTA-free protease inhibitor cocktail (Thermo Fisher). Post-nuclear supernatant was incubated for 2 h at 4 °C with the anti-Myc monoclonal antibody or with anti-CSPα rabbit polyclonal antibody for another 1 h following the addition of protein-G Sepharose or protein-A Sepharose (GE Healthcare), respectively. Protein bound to antibody-beads was washed 5 times with lysis buffer at 4 °C, and the beads were split into two portions: 1) 90% of the beads were "eluted" into 50 µl of PBS by proteolysis (0.5 mg/ml trypsin) for 20 min at room temperature, followed by trypsin-inhibition by addition of PMSF to 1 mM, and the supernatant was immediately used for UV-Vis spectroscopy or to measure [$Fe^{2+/3+}$] and [S^{2-}], 2) 10% of the beads were eluted in 0.1 N HCl for 10 min at room temperature (to disassemble Fe-S clusters/oligomers), followed by boiling for 10 min in Laemmli

sample buffer. Where [$Fe^{2+/3+}$] and [S^{2-}] ions bound per protein molecule was calculated, the myc-IP eluate was separated on SDS-PAGE and quantitatively immunoblotted alongside a known amount of recombinant SSPα using CSPα antibody, in order to estimate the protein yield from immunoprecipitation.

CSPα ubiquitination

Ubiquitination level of myc-tagged CSPα variants in HEK293T cells was assessed by immunoprecipitation of CSPα followed by immunoblot against poly-ubiquitin (clone FK1), or against the myc-tag to visualize precipitated CSPα (clone 9E10). For assessing the effect of acid treatment on ubiquitinated CSPα, immunoprecipitated protein was treated with 0.1 N HCl at room temperature for 20 minutes before immunoblotting.

Analysis of SNARE-complex assembly in primary neurons

Co-immunoprecipitation (co-IP), was used to quantify SNAP-25 and Synt-1 bound to Syb-2 in primary neurons from CSPα$^{-/-}$ mice either expressing CSPα^{WT}, CSPα^{L115R}, CSPα$^{L116\Delta}$ or SSPα, or following pharmacological iron-chelation. Post-nuclear supernatant in lysis buffer (PBS containing 0.1% Triton X-100) supplemented with EDTA-free protease inhibitor cocktail (Thermo Fisher) was incubated for 2 h at 4 °C with anti-Syb-2 polyclonal antiserum, or with plain rabbit serum for sham IP. Samples were incubated at 4 °C for additional 1 h after adding protein-A Sepharose slurry (GE Healthcare), followed by 5 washes with 1 ml lysis buffer, and then elution into Laemmli sample buffer by boiling for 20 min (which also disassembles the SNARE-complexes). Co-IP'd proteins and 5% of the input lysate were separated on SDS-PAGE and quantitatively immunoblotted using Syb-2, Synt-1 and SNAP-25 monoclonal antibodies.

Determination of interaction of CSPα with SNAP-25 or CIA machinery proteins

Interaction between CSPα and SNAP-25 was studied by co-IP of CSPα with SNAP-25. From iNs and from primary CSPα$^{-/-}$ neurons lentivirally expressing CSPα variants, either before or after pharmacological iron chelation, SNAP-25 or myc-tagged CSPα were IP'd as described above, using SNAP-25 monoclonal antibody or 6E9 anti-myc monoclonal antibody, or no IgG for sham IP, and protein-G sepharose (GE Healthcare). Co-IP'd proteins were eluted by boiling for 20 minutes in Laemmli sample buffer and separated on SDS-PAGE in parallel with 5% of input lysate, followed by quantitative immunoblotting using non-mouse polyclonal antibodies to the shown proteins (SNAP-25, CIA machinery proteins, and CSPα).

Chemical depalmitoylation

Palmitoylation of CSPα variants was assessed by chemical cleavage of Cys-palmitoyl thioester linkages with hydroxylamine. Lysates were incubated overnight at 4 °C with either 0.5 M Tris (as control), or 0.5 M hydroxylamine (from

50% w/v stock in H_2O; EMD Millipore). Molecular mass-shift between palmitoylated and depalmitoylated protein was analyzed by SDS-PAGE separation followed by quantitative immunoblotting.

Fe-S cluster disruption with L-cysteine, DTT, dithionite, H_2O_2 and HCl

For acid-disruption of Fe-S clusters, protein was incubated with 0.1 N HCl at room temperature for 10-20 min (as indicated in figure captions). The characteristic amber color of recombinant $CSP\alpha^{WT}$, $CSP\alpha^{L115R}$, and $CSP\alpha^{L116\Delta}$ was eliminated immediately after addition of HCl, indicating sensitivity of Fe-S clusters to acid treatment. Any aggregates in the now-colorless protein was pelleted for UV-Vis measurements on the supernatant. For pH-sensitive assays (e.g. trypsinolysis), the total protein solution was first neutralized using 1 M cacodylate. For SDS-PAGE, any acid-precipitated protein was re-solubilized in Laemmli sample buffer before separation.

For other redox-sensitivity assays, either the recombinant proteins were incubated with 1 mM L-cysteine for 2 hours at room temperature, or HEK293T post-nuclear lysates (in PBS containing 0.1% Triton X-100) were incubated with increasing concentrations of L-cysteine (0, 0.5, 1, 5, 25 mM), DTT (0, 1, 10, 100, 500 mM), sodium dithionite (0, 0.5, 1, 5, 25 mM), or H_2O_2 (0, 0.5, 1, 5, 25 mM) for 20 min at room temperature, before solubilization in Laemmli sample buffer and SDS-PAGE separation.

UV-Visible absorbance spectrum

100 µl of protein solution (20 mg/ml purified protein or ~2 mg/ml immunoprecipitated protein) was loaded into a UV-permeant 96-well clear bottom plate (Corning). The absorbance spectra (200-600 nm) were recorded using a Synergy H1 Hybrid Reader (BioTek). For acid treatment, protein was incubated with 0.1 N HCl for 10 min at room temperature. Any precipitate was centrifuged down, and supernatant was used for recording UV-Vis absorbance as above.

Determination of iron content

Iron content was measured using the ferrozine colorimetric method (*9, 10*). 250 µl of 20 µM recombinant protein or ~18 µM immunoprecipitated protein (determined by immunoblotting next to recombinant protein) was incubated with 250 µl 6% HNO_3 at 98 °C for 16 h. The samples were centrifuged at 18,000 g_{av} and the supernatant was then combined with iron-detection reagent containing sodium ascorbate (30 mM final), ferrozine (1 mM final) and ammonium acetate (300 mM final). A color change from clear to purple indicated iron ions. 180 µl of the solution were loaded into a UV-permeant 96-well clear bottom plate (Corning), and absorbance was measured at 550 nm against an $FeCl_3$ standard curve using a Synergy H1 Hybrid Reader, and normalized to the protein amount.

Determination of inorganic sulfur content

Inorganic sulfur content was determined using the methylene blue colorimetric assay (*11*). Briefly, 200 µl of 40 µM recombinant protein or ~16 µM immunoprecipitated protein (determined by immunoblotting next to recombinant protein) was incubated with 600 µl of 1% zinc acetate and 50 µl of 7% NaOH for 15 minutes at room temperature. The samples were centrifuged at 4200 g_{av} and 150 µl of 0.1% N,N-dimethyl-*p*-phenylenediamine in 5 M HCl was added to the bottom of the tube, followed by addition of 150 µl of 10 mM $FeCl_3$. The samples were then centrifuged for 10 min at 18,000 g_{av}. A color change from light pink to blue revealed methylene blue formation, indicating sulfide concentration. Sulfur content was measured by absorbance at 670 nm against a sodium sulfide standard curve using a Synergy H1 Hybrid Reader, and normalized to the protein amount.

Pharmacological iron-chelation

For pharmacological iron-chelation, deferiprone (L1; 200 µM; Biotang), deferoxamine mesylate (Dfx; 200 µM; Calbiochem), or 0.1% DMSO as vehicle control were added to the respective culture media of primary cortical neurons, fibroblasts, or iNs. When these media were changed, the fresh media contained identical concentration of the respective iron-chelator or vehicle.

Direct conversion of patient fibroblasts to induced neurons

Fibroblasts from an affected 30-year-old female carrying the L116∆ mutation were obtained from Dr. Milen T. Velinov, and an age-and-sex matched unaffected fibroblast line was frozen, thawed and cultured in parallel to maintain similar passage numbers. Fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum supplemented with L-glutamine, penicillin, and streptomycin. For direct conversion to induced neurons, fibroblasts were transduced by concentrated lentiviral particles in order to express *Ascl1*, *Brn2* and *Myt1l* and *NeuroD1* (BAM+ND1) via the rtTA induction system, using doxycycline (*12*). Virus containing medium was removed after 24 h and replaced with N3 medium (*13*) containing doxycycline (2 µg/ml). Every 3-5 days (depending on medium-acidity), half the medium was changed to fresh N3 medium with doxycycline. Time course experiments, such as cycloheximide chase and iron-chelator treatment, were performed in the N3 medium in the presence of doxycycline.

Immunofluorescence in neurons and iNs, and lipofuscin fluorescence in iNs

For, immunofluorescent labeling of $CSP\alpha^{-/-}$ cortical neurons expressing myc-tagged CSPα variants, cells on coverslips were washed with PBS + 1 mM $MgCl_2$ and fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were permeabilized for 5 min in PBS containing + 0.1% Triton X-100, and blocked for 20 min in PBS + 5% BSA (blocking buffer). Coverslips were then incubated in primary antibodies – mouse anti-myc (9E10, DSHB; 1:500) and rabbit anti-Map2 (ab5622, Millipore; 1:1000) overnight at 4 °C. Following 5 washes, cover slips were incubated with anti-mouse

Alexa 488 and anti-rabbit Alexa 546 (Life Technologies) in blocking buffer containing DAPI, for 1-2 hours, followed by 5 PBS washes, and mounting on slides in Fluormount G (Thermo Fisher). Images were acquired on Nikon H550L microscope.

In the iNs, MAP-2 staining (m1406, Sigma; 1:250) was performed and visualized as above.

Lipofuscin fluorescence was detected in fixed iNs at Ex480 (BP30), Dm505 (LP), Em535 (BP40) using Nikon Eclipse Ts2-FL Inverted Fluorescence Microscope.

Lipofuscin ELISA

iNs from each well of a 6 well dish were solubilized in 100 µl PBS with 0.1% Triton X-100 for 1 h at 4 °C. Post nuclear supernatant collected by centrifugation at 1000 g_{av} for 15 min was used for the assay. These samples were analyzed for lipofuscin content using human lipofuscin competitive ELISA (MyBiosource; MBS7217173). Following the colorimetric reaction, optical density at 450 nm was read from the standard and the sample wells using Synergy H1 Hybrid Reader (BioTek).

Measurement of SNAP-25 turnover

Cycloheximide chase was performed in patient-derived induced neurons at the indicated time points at 0.1 g/L (Sigma) as previously described (*14*). For turnover measurements with pharmacological iron-chelation, iNs were first treated for 72 h with the iron-chelators, and cycloheximide chase was performed in the presence of iron-chelators.

SDS-PAGE and quantitative immunoblotting

For SDS-PAGE, 10-15% Laemmli gels (10.3%T and 3.3%C) were used to separate proteins on Bio-Rad apparati. No reducing agents were used in buffers unless specifically noted. Proteins were transferred onto nitrocellulose (pore-size = 0.45 µm; GE Healthcare) and blocked with 5% w/v fat-free dry milk in tris buffered saline, pH 7.5 supplemented with 0.1% Tween 20 (TBS-T). Immunoblotting was performed by incubating the blocked membranes with primary antibodies in blocking buffer for 8-16 hours. Following 5 washes with TBS-Tween 20 (0.1%). Blots were incubated with secondary antibodies (goat anti-rabbit conjugated to IRDye 600RD or 800CW; LI-COR) at 1/5000 in blocking buffer for 1-3 hours. Immunoblots were washed 5x with TBS-T and dried, then scanned on Odyssey CLx (LI-COR) and quantified using Image-Studio software (LI-COR).

For ATP5G immunoblots, following the transfer, nitrocellulose membranes were dried and fixed for 15 min at room temperature in 0.2% glutaraldehyde in PBS (*15*). Membranes were then washed 3x with TBS-T and treated as above.

Antibodies

Myc tag (Mouse = 9E10 ascites and culture supernatant of hybridoma, DSHB; Rabbit = C3956, Sigma), GFP (mouse = JL8, Clontech), CSPα (Rabbit = R807 serum; ab1576, Millipore), SGT (Rabbit = CHAT33 serum), Hsc70 (Mouse = cl. 3C5, SYSY; Rabbit = A903 serum), Nfs1 (Mouse = cl. B-7; sc-365308, Santa Cruz), CIAPIN1 (Rabbit = ab154904, Abcam), NUBP2 (Rabbit = 15409-1-AP, Proteintech), CIA1 (Rabbit = NBP1-84474, Novus Bio), CIA2b (Rabbit = 20108-1-AP, Proteintech), ISCU (Rabbit = NBP2-14998, Novus Bio), SNAP-25 (Mouse = cl. 71.1, SYSY; Rabbit = P913 serum), Syb-2 (Mouse = cl. 69.1, SYSY; Rabbit = P939 serum), Synt-1 (Mouse = cl. 78.2, SYSY; Rabbit = 438B serum), Map2 (Mouse = m1406, Sigma; Rabbit = ab5622, Millipore), ATP5G (Rabbit = ab181243, Abcam), VDAC1 (Mouse = cl. N152B/23, Neuromab), Tim23 (Mouse = cl. 32/Tim23, BD Bioscience), Ubiquitin (Mouse = cl. FK1; Enzo), α-tubulin (Mouse = cl. 12G10, DSHB; Rabbit = ab18251, Abcam), β-Actin (Mouse = A1978, Sigma).

Statistical analyses

Each “n” consisted of reagents produced in parallel (e.g., purified proteins, plating of heterologous cells, lentivirus production, and culturing neurons from a single litter) and experiments performed in parallel (e.g. transfection or lentiviral infection, immunoprecipitation, sample collection for ELISA or immunoblotting etc.). For blinding, band or well identities were coded for the person who performed raw biochemical measurements (not the experimenter), and the coverslips/slides were randomized and coded for the experimenter before performing microscopy. Experiments were quantified using parametric as well as non-parametric statistical tests, as indicated in each figure caption. Nonparametric analyses were performed by Mann-Whitney-Wilcoxon *U*-test in case the sample distributions were not normal. Time-course experiments such as cycloheximide chase and Iron-chelator treatment were analyzed by 2-way ANOVA for comparing the overall curve and by Tukey’s multiple comparisons post-test to compare data at each time-point independently.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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