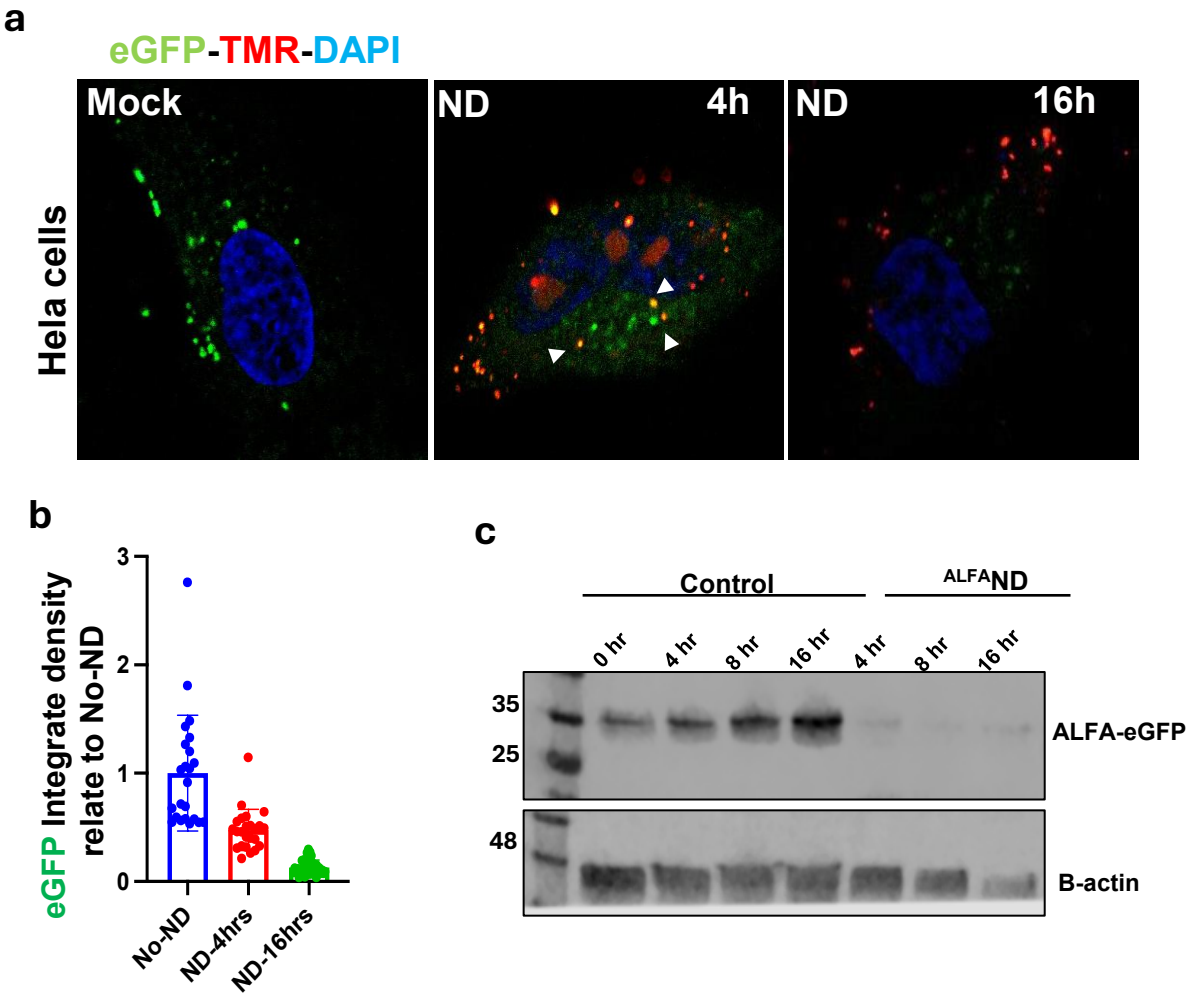
Extended Data Figure 1



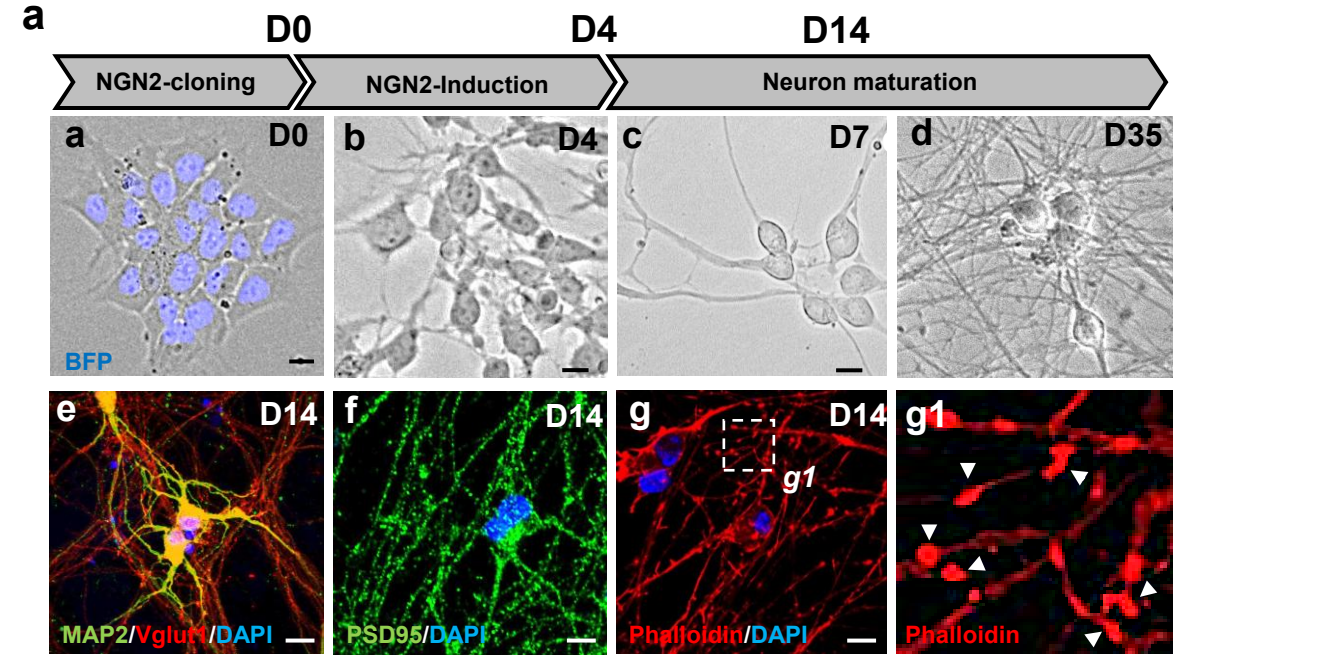
Extended Data Figure 1: ^{ALFA}ND efficiently internalizes and promotes target degradation.

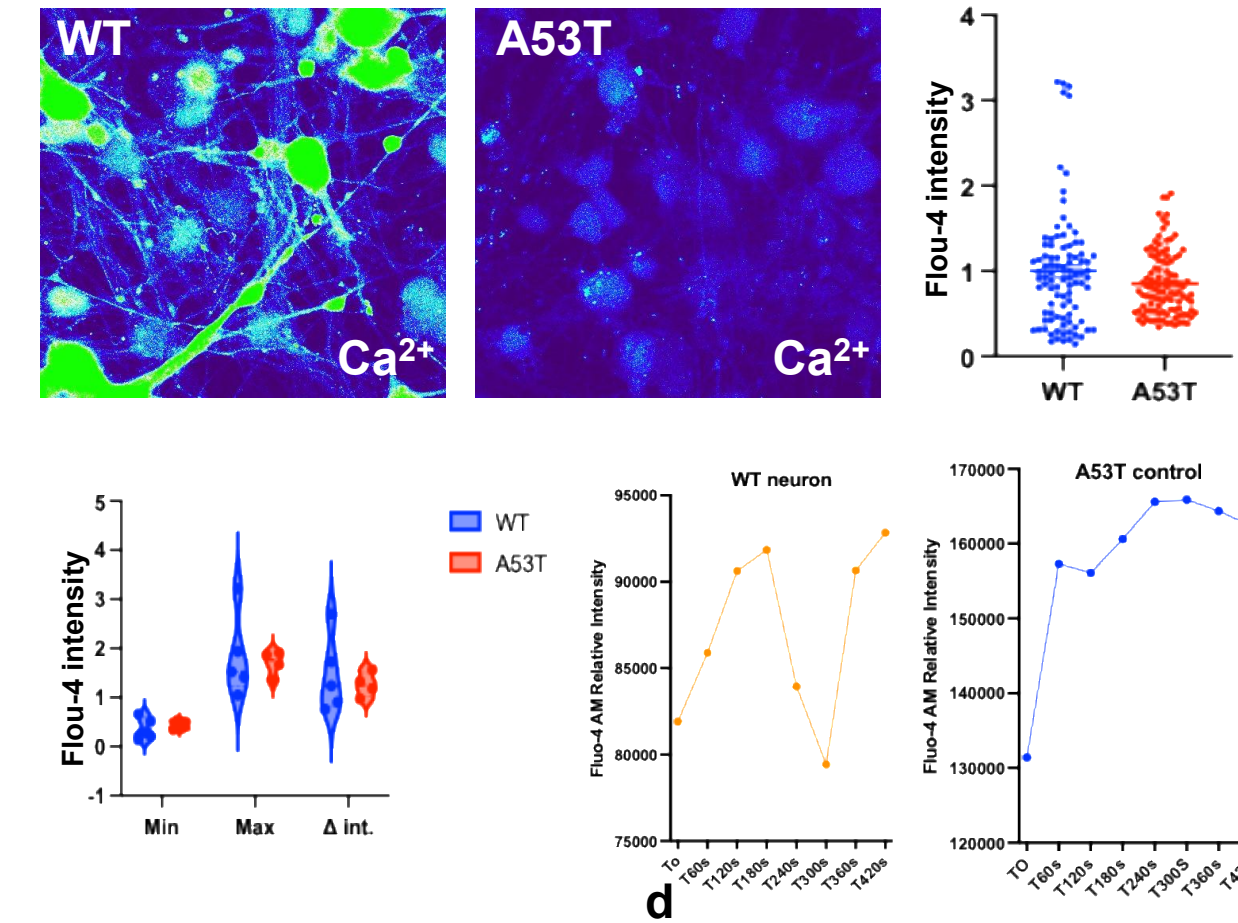
(a) Immunofluorescence staining of HeLa cells After 4 and 16 hours of ^{ALFA}ND treatment. ^{ALFA}ND significantly induced ALFA-eGFP degradation in Hela cells.

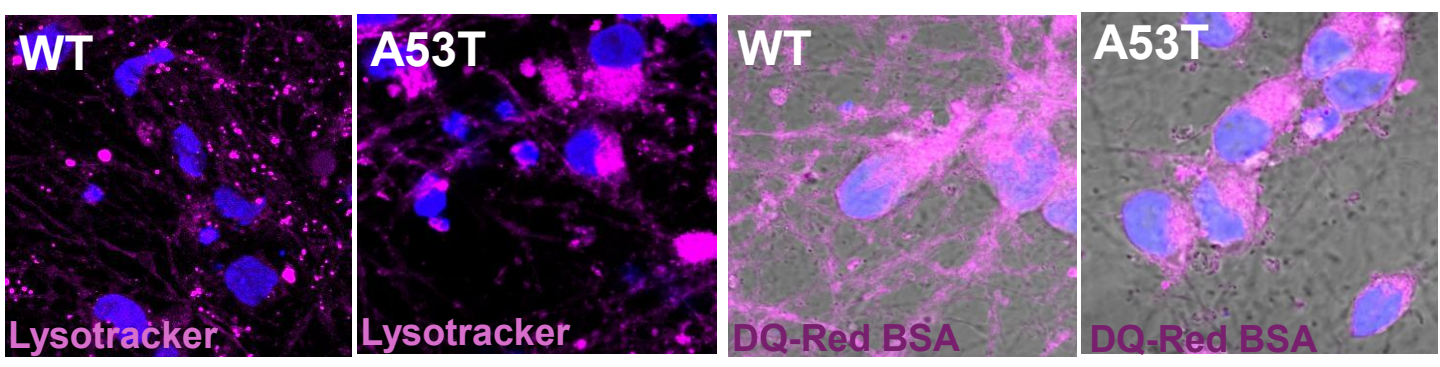
(b) Quantification of ALFA-eGFP degradation by immunoblotting in HeLa cells following 4 and16 hours treatment with 0.5 μM ^{ALFA}ND. Data is presented as mean ± SD.

(c) Immunoblot showing ALFA-eGFP degradation in HeLa cells after 4, 8 and 16-hour treatment with 0.5 μM ^{ALFA}ND.

Extended Data Figure 2







Extended Data Figure 2: Characterization of A53T α-syn iPSC-derived cortical neurons and their impaired intracellular Ca^{2+} dynamics.

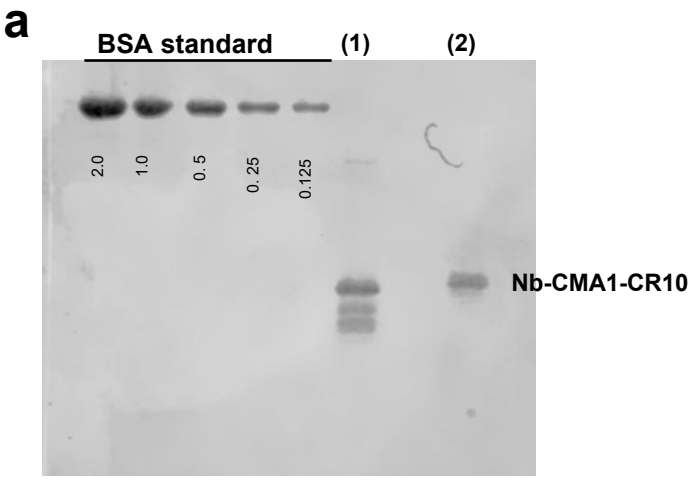
(a) Schematic and micrographs of the neuronal differentiation timeline (a-d) and characterization of mature neurons (e-h) expressing markers MAP2, Vglut1, PSD95, and displaying dendritic spines (phalloidin).

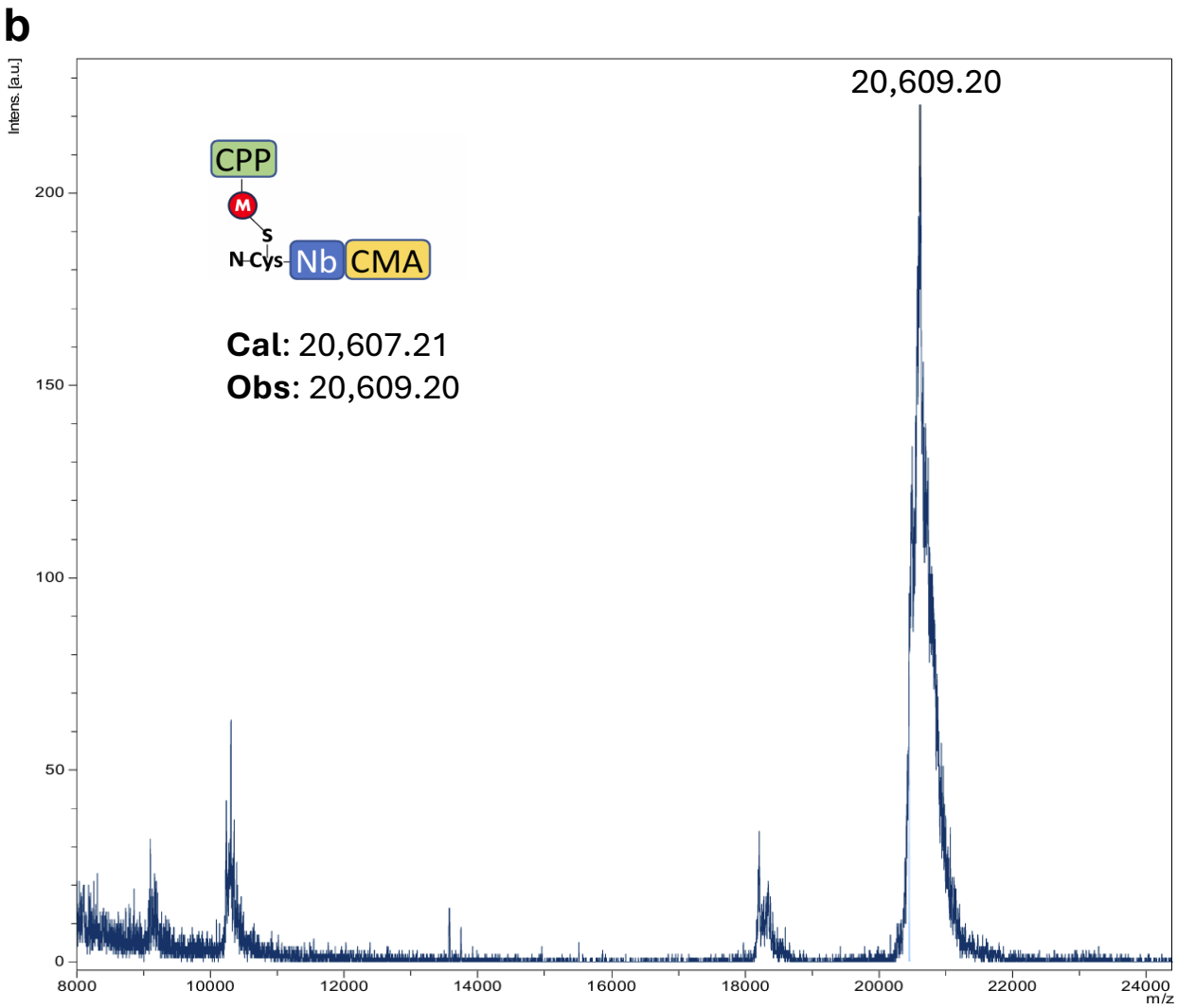
(b) Representative Fluo-4 AM staining images and quantification of WT and A53T neurons used to evaluate intracellular Ca^{2+} levels. Quantification of maximum Fluo-4 fluorescence intensity shows significantly reduced intracellular Ca^{2+} in A53T neurons compared to WT neurons. In addition, micrographs indicate the changes in calcium spectra in WT and A53T neurons upon Fluo-4 AM binds to Ca^{2+}.

(c) Representative confocal images of WT and A53T neurons labeled with LysoTracker.

(d) Representative confocal images of WT and A53T neurons labeled with DQ-Red BSA. A53T neurons display abnormal lysosomal distribution and reduced dendritic proteolysis, evidenced by somatic accumulation of DQ-Red BSA.

Extended Data Figure 3



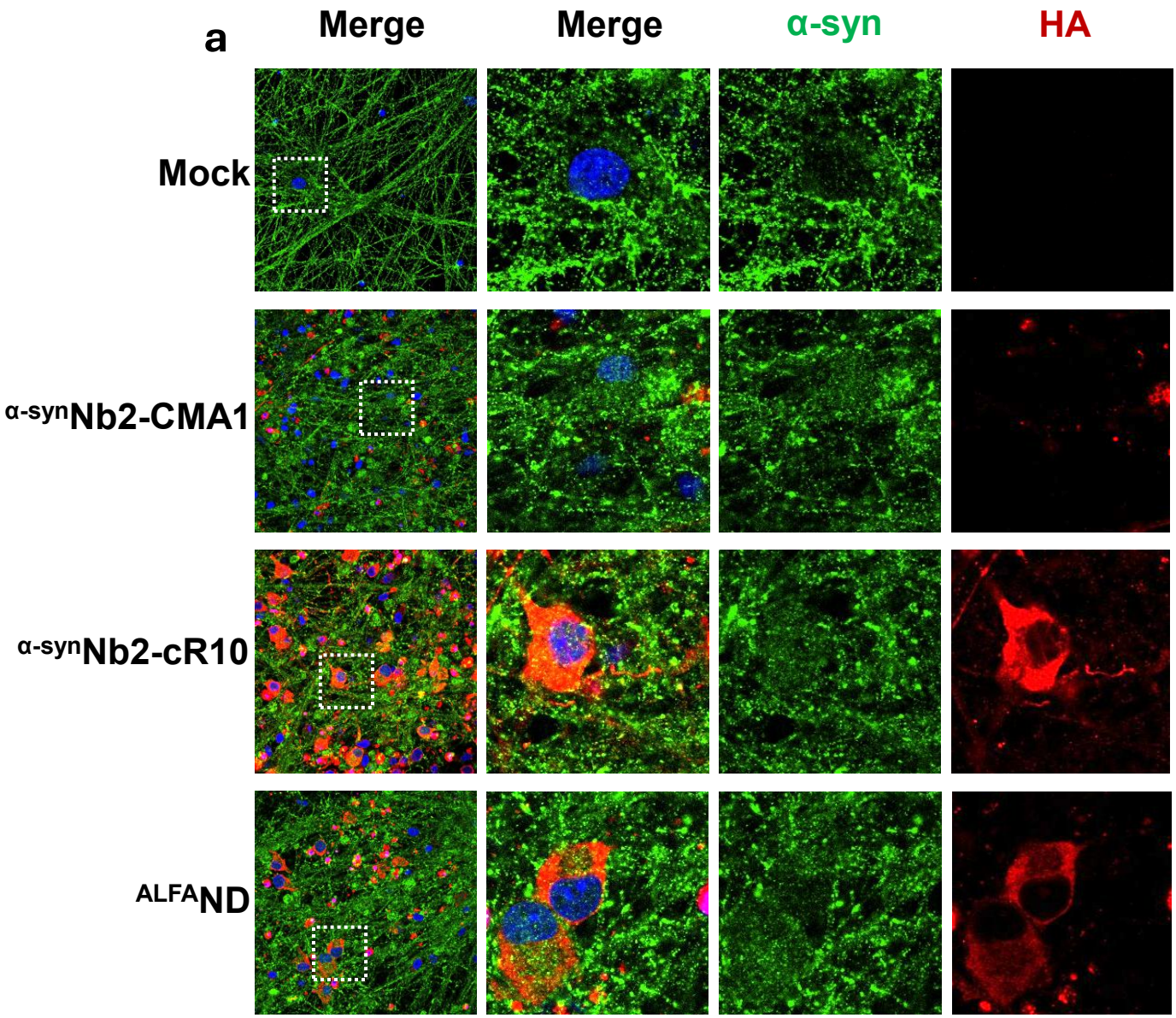


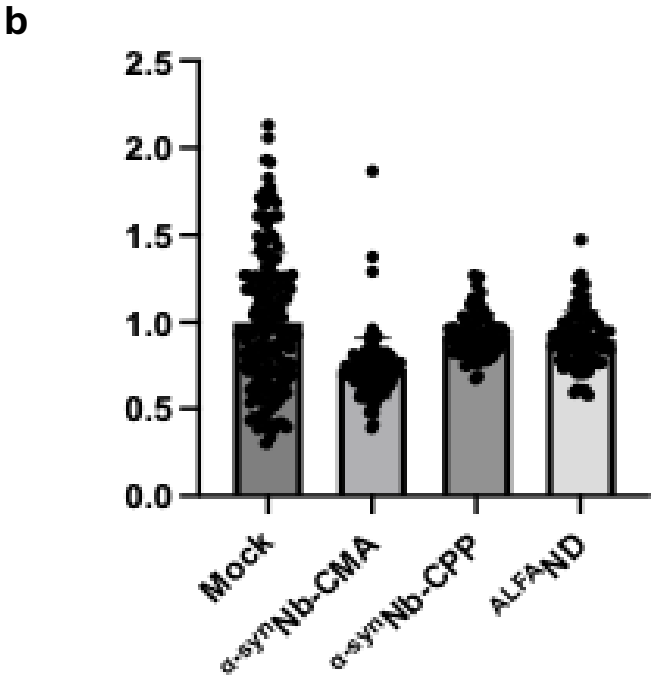
Extended Data Figure 3: Generation of lysosome-targeting nanodegrader ND21. ^{Syn}Nb2–CMA1 was conjugated to the cell-penetrating peptide cR10 via a cysteine–maleimide reaction to generate cR10–^{Syn}Nb2–CMA1 (ND21).

(a) Coomassie-stained SDS–PAGE analysis of the maleimide–cR10 conjugation reaction prior to purification (lane 1) and the size-exclusion chromatography–purified cR10–SynNb2–CMA1 product (ND21; lane 2).

(b) MALDI–TOF mass spectrometry analysis confirming the molecular mass of ND21.

Extended Data Figure 4

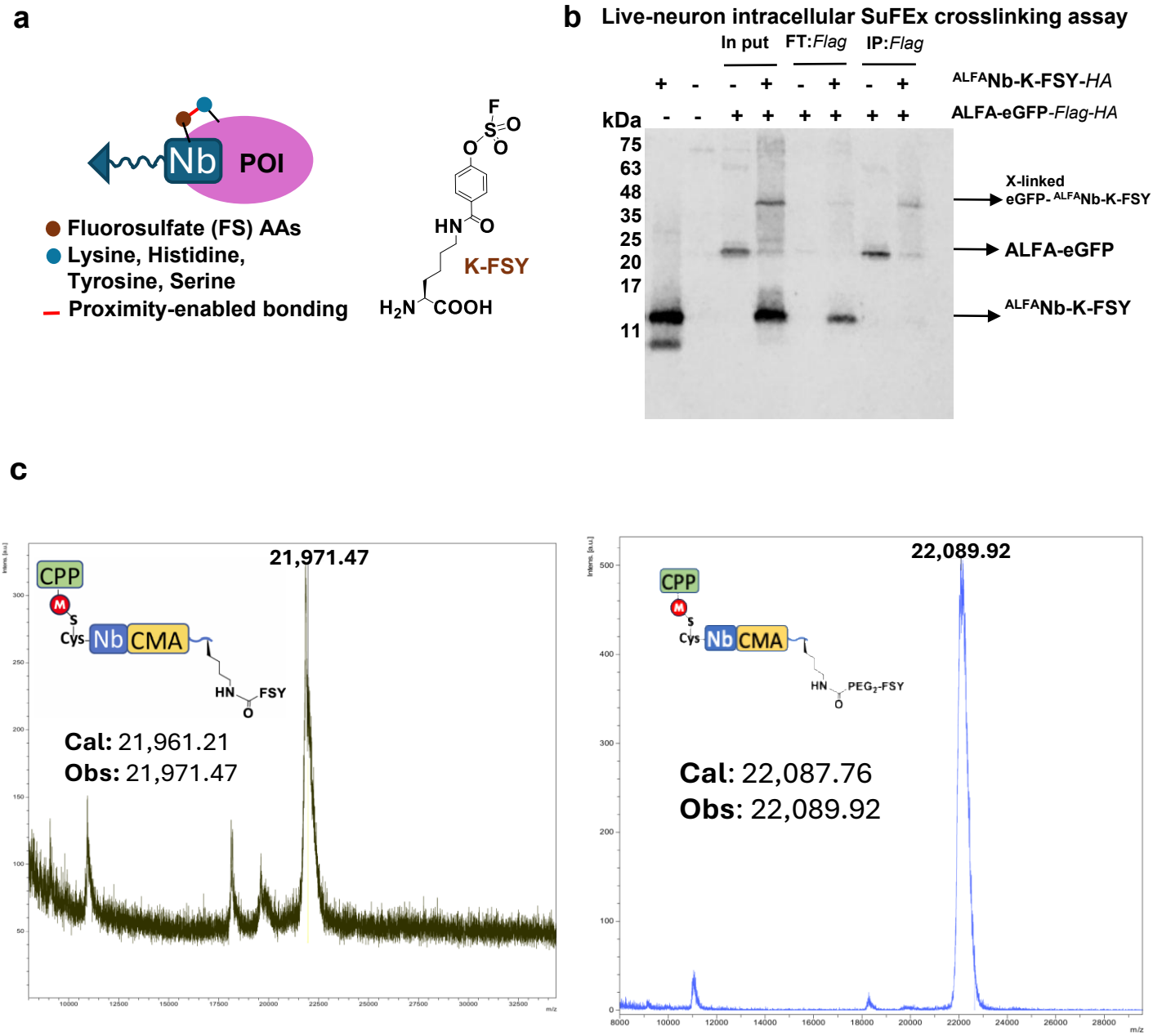




Extended Data Figure 4: Specificity controls for nanodegrader-mediated α-synuclein clearance.

(a) Immunofluorescence analysis of A53T α-Syn iPSC-derived neurons treated with control constructs demonstrates that cell penetration, target recognition, and lysosomal targeting are each required for ND activity. The construct lacking the cell-penetrating peptide ($^{\alpha\text{-syn}}$Nb–CMA) shows minimal neuronal uptake, whereas CPP-conjugated constructs ($^{\alpha\text{-syn}}$Nb-CPP and ^{ALFA}ND) efficiently internalize into neurons. The non–α-Syn-binding control (^{ALFA}ND) displays diffuse intracellular distribution without co-localization with α-Syn.
(b) Quantification of α-Syn levels reveals no significant reduction in α-Syn abundance following treatment with any control construct, confirming that efficient degradation requires the combined presence of the α-Syn nanobody, CPP-mediated delivery, and CMA-directed lysosomal targeting.

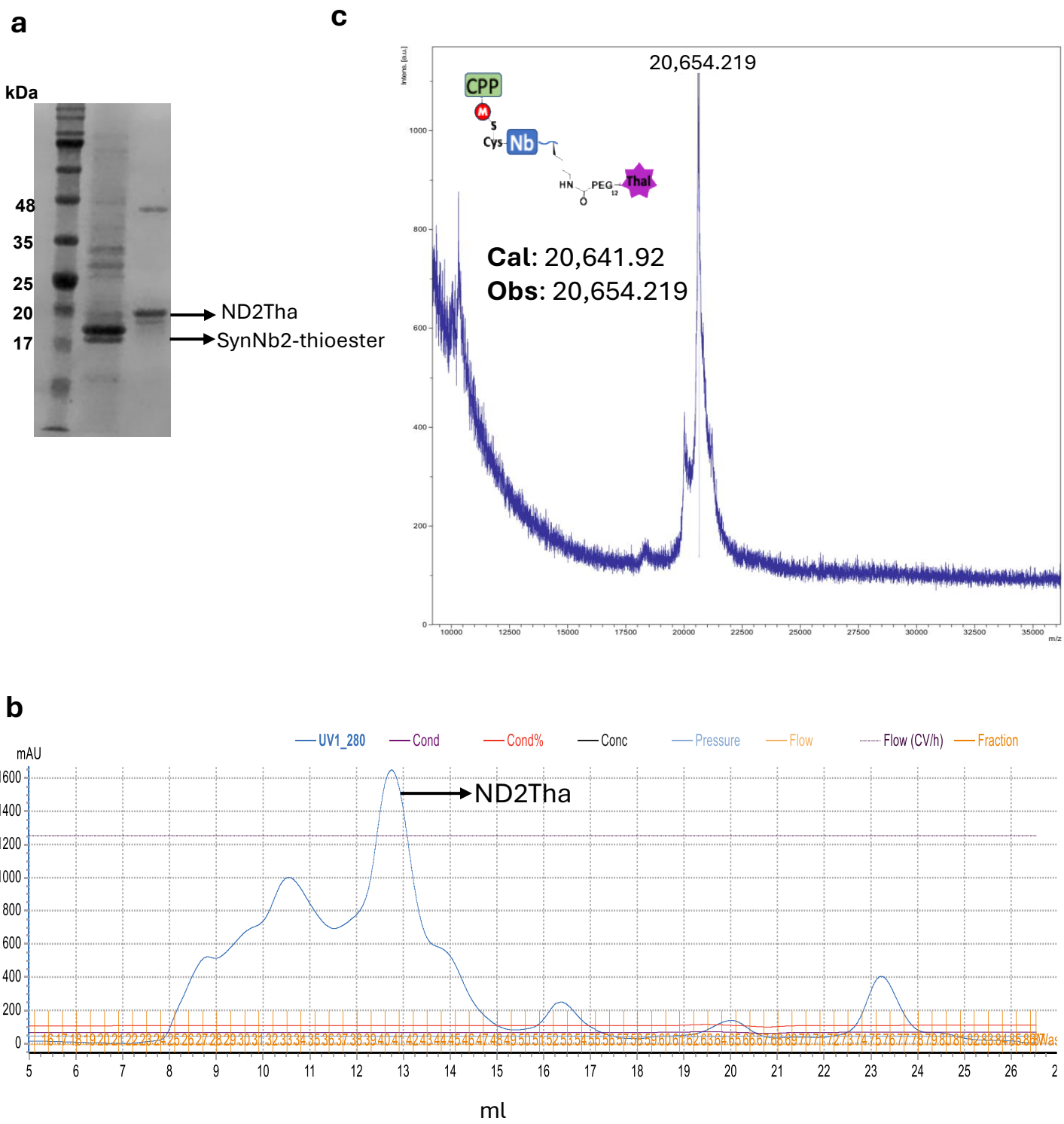
Extended Data Figure 5



Extended Data Figure 5: Design, generation and functional validation of covalent nanodegraders.

(a) Schematic of proximity-enabled SuFEx-mediated covalent cross-linking between nanobody-K-FSY and protein of interest (POI). The ^{ALFA}Nb-K-FSY conjugate was generated by EPL-mediated ligation of ^{ALFA}Nb-thioester with an N-ter Cysteine peptide bearing unnatural SuFEx-reactive lysine (K-FSY, FSY denotes the aryl fluorosulfate). (b) Proximity-enabled intracellular SuFEx crosslinking assay in live neurons. To validate proximity-enabled covalent target engagement in neurons, we performed a live-cell intracellular SuFEx crosslinking assay by delivering cR10–^{ALFA}Nb–K-FSY into ALFA-tagged EGFP expressing human neurons, followed by co-immunoprecipitation to detect covalent nanobody–target adducts. Live neurons expressing ALFA–eGFP were treated with ^{ALFA}Nb-K-FSY for 2 h. Cell lysates were collected and subjected to anti-FLAG immunoprecipitation (IP) using Dynabeads, followed by immunoblotting with anti-HA antibody to detect both ^{ALFA}Nb-K-FSY and ALFA–eGFP. Co-detection of the HA-reactive bands in the lysate (input) and IP fractions demonstrates successful cross-linking between ALFANb–K-FSY and ALFA–eGFP. FT, flow-through fraction after incubation with FLAG beads. (c) MALDI-ToF mass spectra of ND21F and ND21P2F. (d) Immunoprecipitation of α-synuclein followed by Western blotting detected associated ND21P2F in the precipitates. (e) Time-course cross-linking of purified, recombinant A53T α-Syn with ND21F analyzed by Coomassie-stained SDS–PAGE. Reactions were performed with A53T α-Syn and ND21F at 1:2 or 1:4 (A53T α-Syn:ND21F, molar) and were incubated at 37 °C in NaPi (pH 7.5) for 1, 4, 17, and 20 hr. prior to quenching and electrophoresis.

Extended Data Figure 6



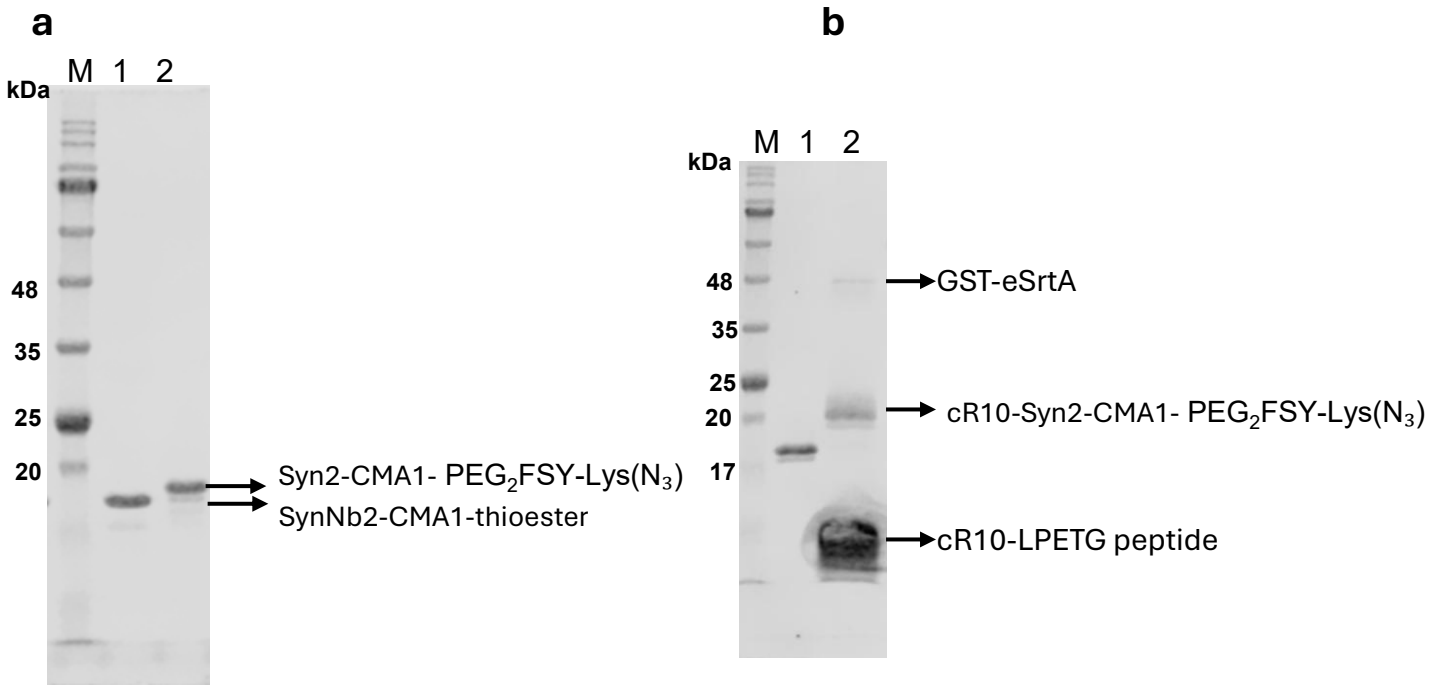
Extended Data Figure 6: Generation of proteasome-targeting α-Syn nanodegrader

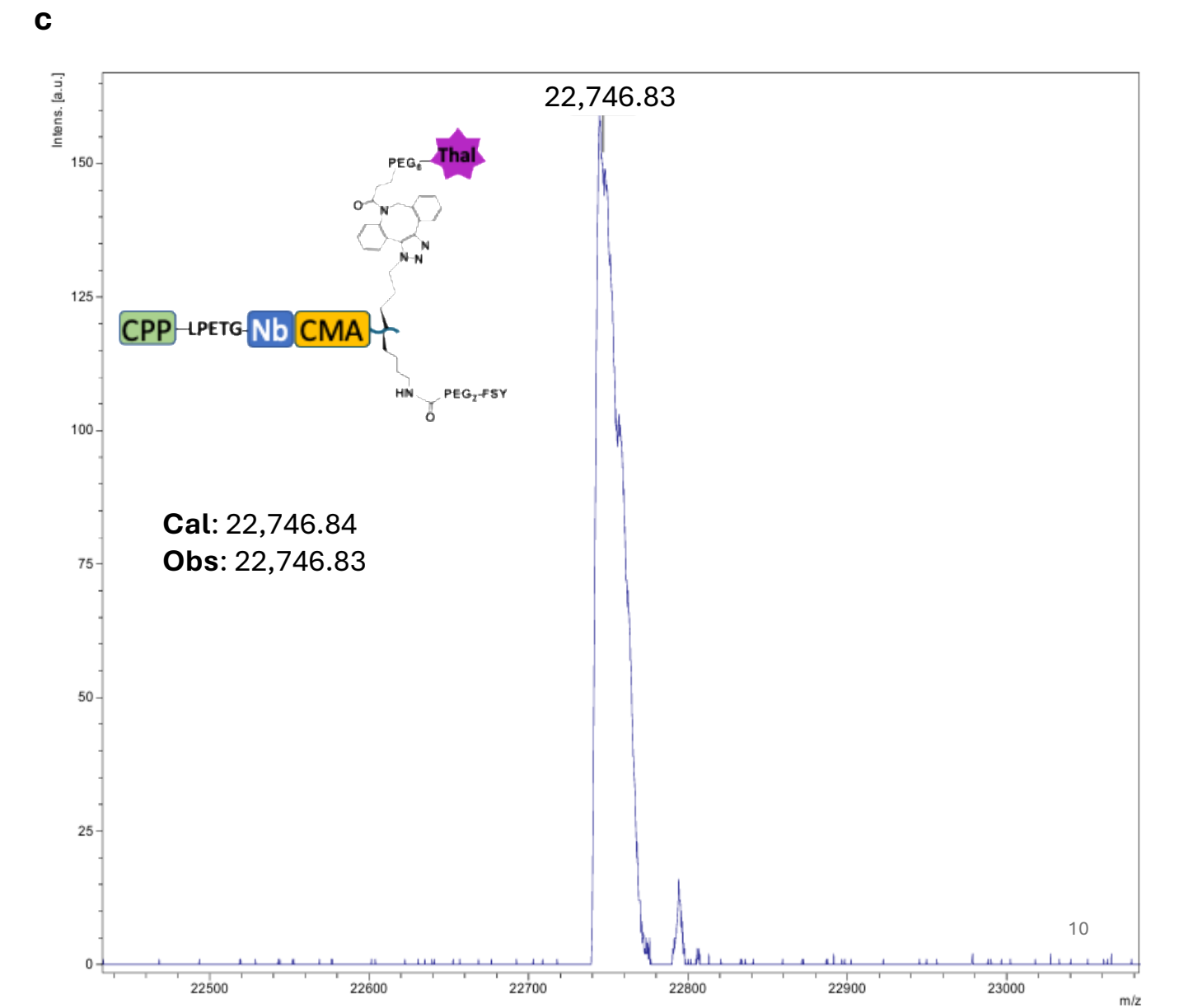
(a) Coomassie-stained SDS–PAGE of the successfully ligation of N-terminal cysteine peptide containing PEG_{12} linker and a Thalidomide moiety to SynNb2.

(b) Size exclusion chromatogram of the purified ND2Tha.

(c) MALDI-ToF mass spectra of ND2Tha.

Extended Data Figure 7





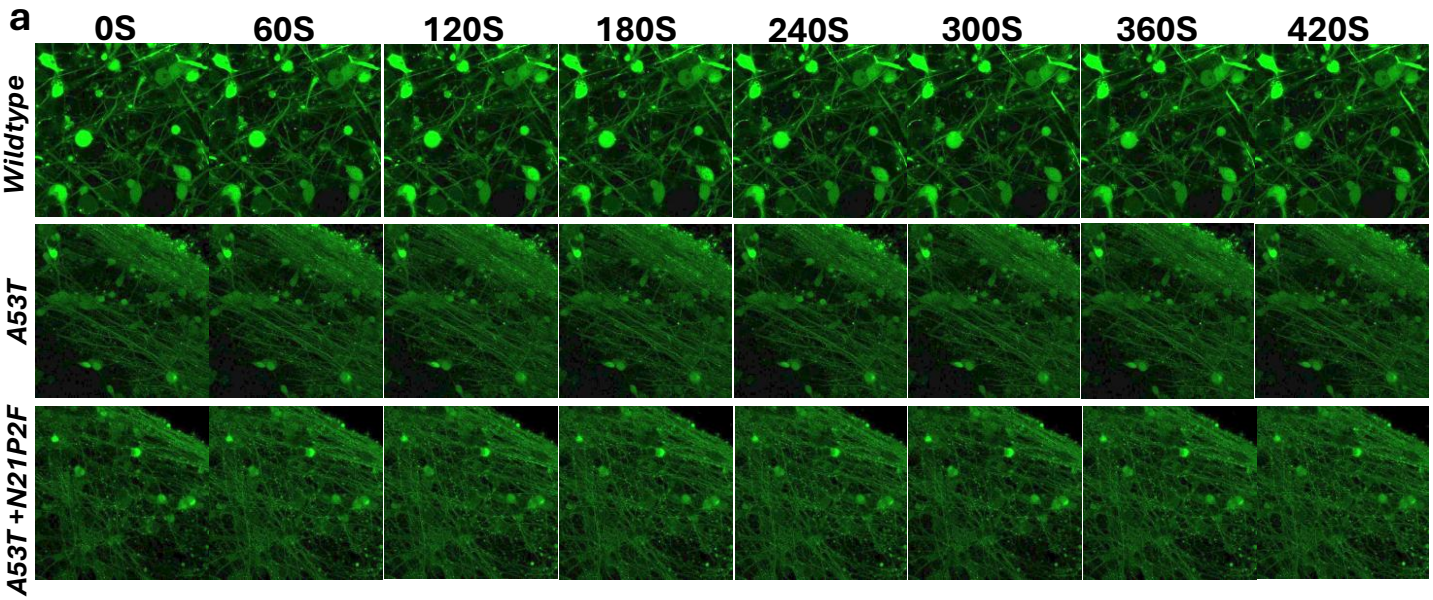
Extended Data Figure 7: Generation of dual-proteolytic α-syn nanodegraders.

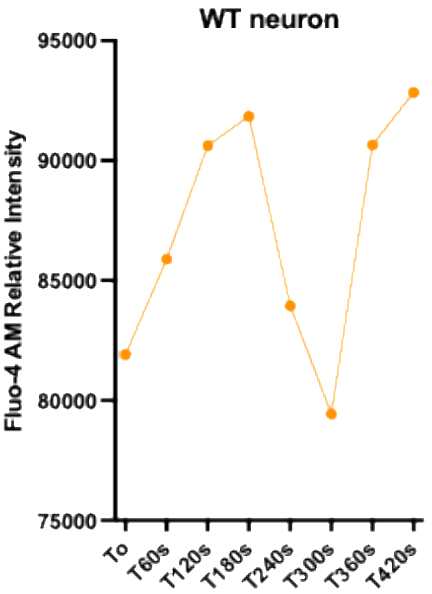
(a) Coomassie-stained SDS–PAGE of the EPL ligation of N-terminal cysteine peptide containing both K-PEG_2-FSY and azido-Lysine (Lys(N_3)) with SynNb2-CMA1-thioester

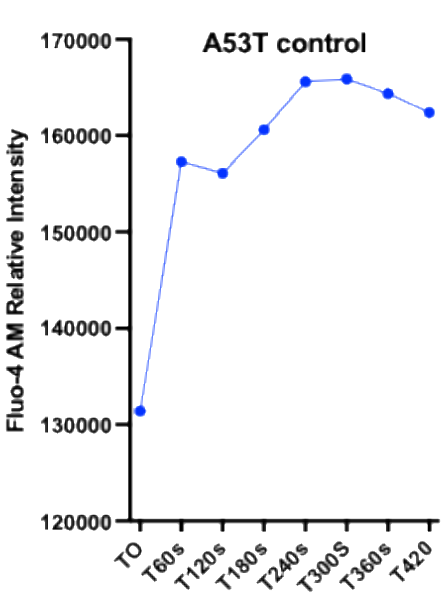
(b) Coomassie-stained SDS–PAGE of SrtA-mediated ligation of cR10-LPETG peptide with Syn2-CMA1- PEG_2FSY-Lys(N_3)

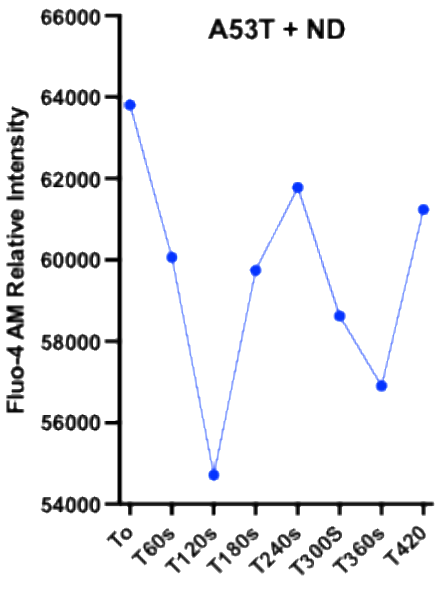
(c) MALDI-ToF mass spectra of ND21P2F-Thal

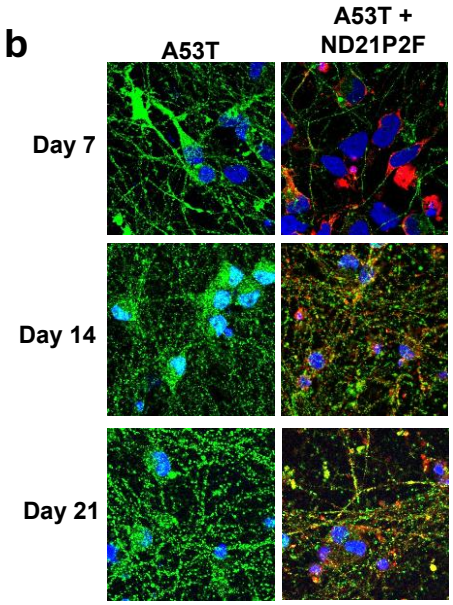
Extended Data Figure 8

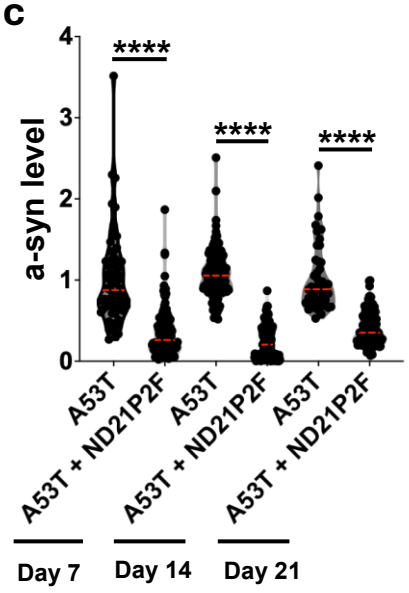








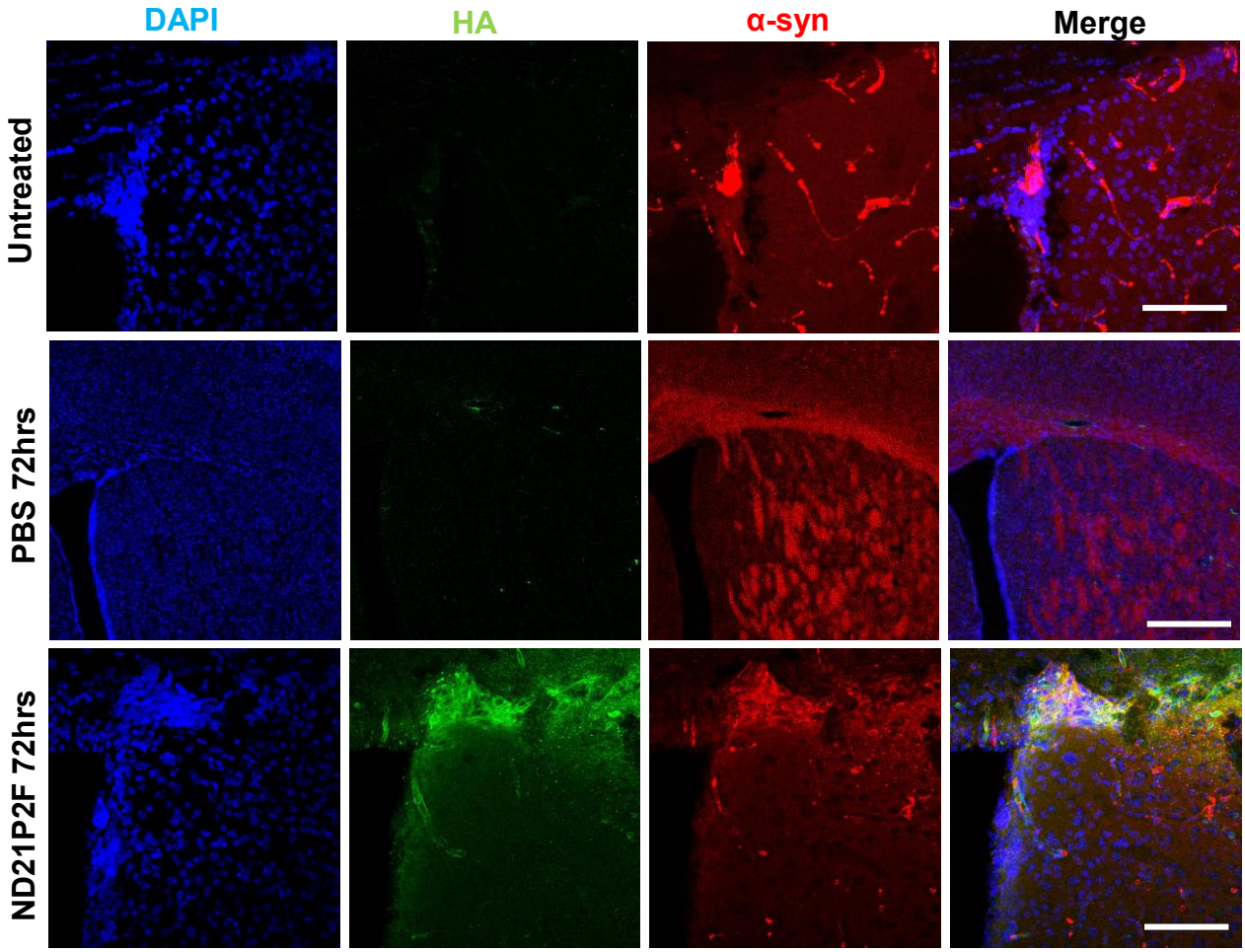




	Degradation %
Day 7	64.6%
Day 14	76.8%
Day 21	61.3%

Extended Data Figure 8: *In vitro* functional rescue and specificity of ND21P2F in human iPSC-derived A53T α-synuclein neurons.
(a) Representative time-lapse fluorescence images (top) and corresponding quantification (bottom) of Fluo-4 AM signals showing intracellular Ca^{2+} dynamics in wild-type neurons, untreated A53T α-syn neurons, and A53T α-syn neurons treated with ND21P2F. Images were acquired at the indicated time points (0–420 s).
(b) Representative immunofluorescence images of α-synuclein (α-Syn, green) in untreated A53T neurons and ND21P2F-treated A53T neurons (100 nM, 24 h, red) at days 7, 14, and 21 post-differentiation of human iPSCs into neurons. Nuclei are shown in blue (DAPI)
(c) Quantification of α-Syn levels in A53T neurons at days 7, 14, and 21 post-differentiation following ND21P2F treatment (100 nM, 24 h), normalized to untreated A53T controls. Degradation efficiencies at each time point are indicated. Data are presented as mean ± s.d.; statistical significance was determined relative to untreated A53T controls (****$p < 0.0001$).

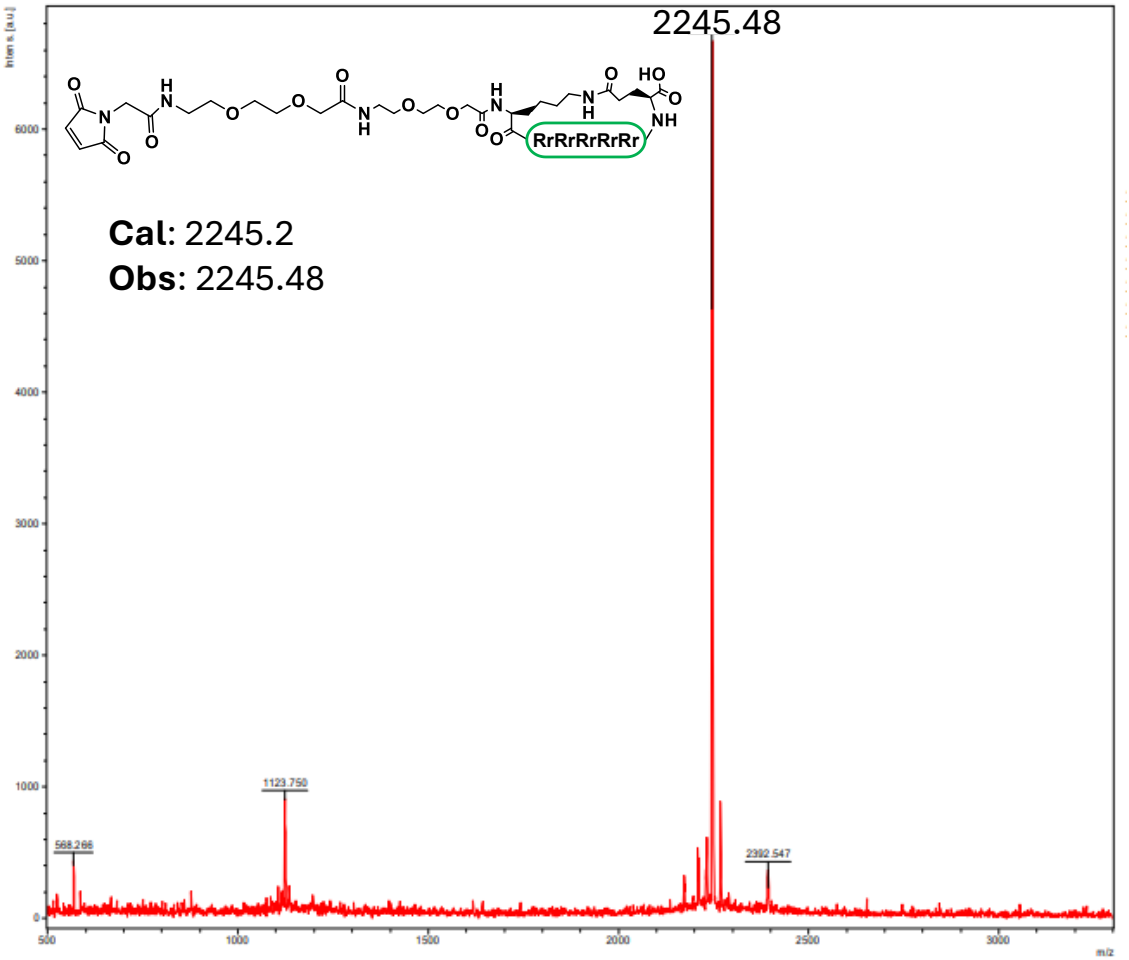
Extended Data Figure 9



Extended Data Figure 9: *In vivo* characterization of ND21P2F: stability, target selectivity, and tissue distribution.
Representative confocal images of coronal sections at 72 hours post-injection. HA-tag–positive ND (green) remains localized at the injection site with noticeable diffusion. In contrast, the untreated and PBS treated mice show no HA immunoreactivity. Nuclei are shown in blue (DAPI). Scale bar, 50 μm

Extended Data Figure 10

a



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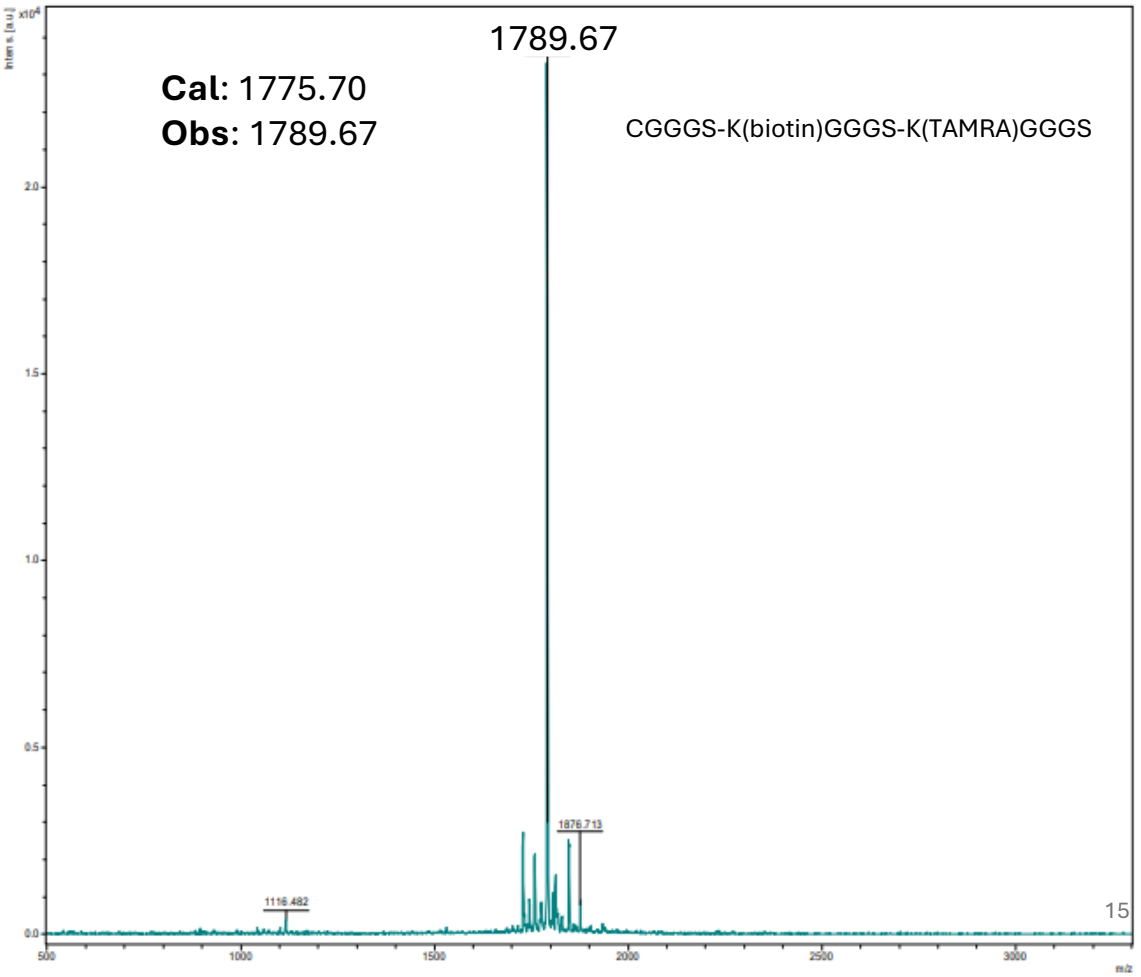

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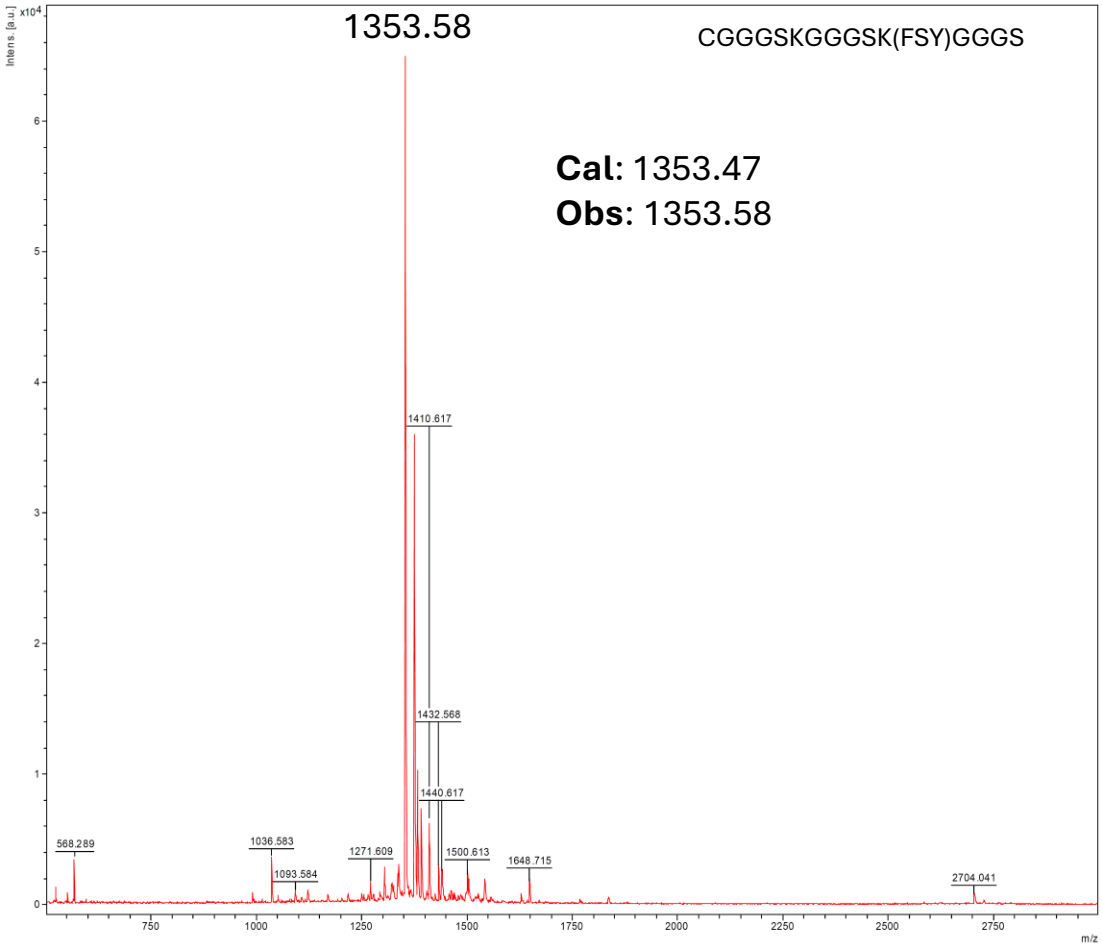
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Obs: 1353.58
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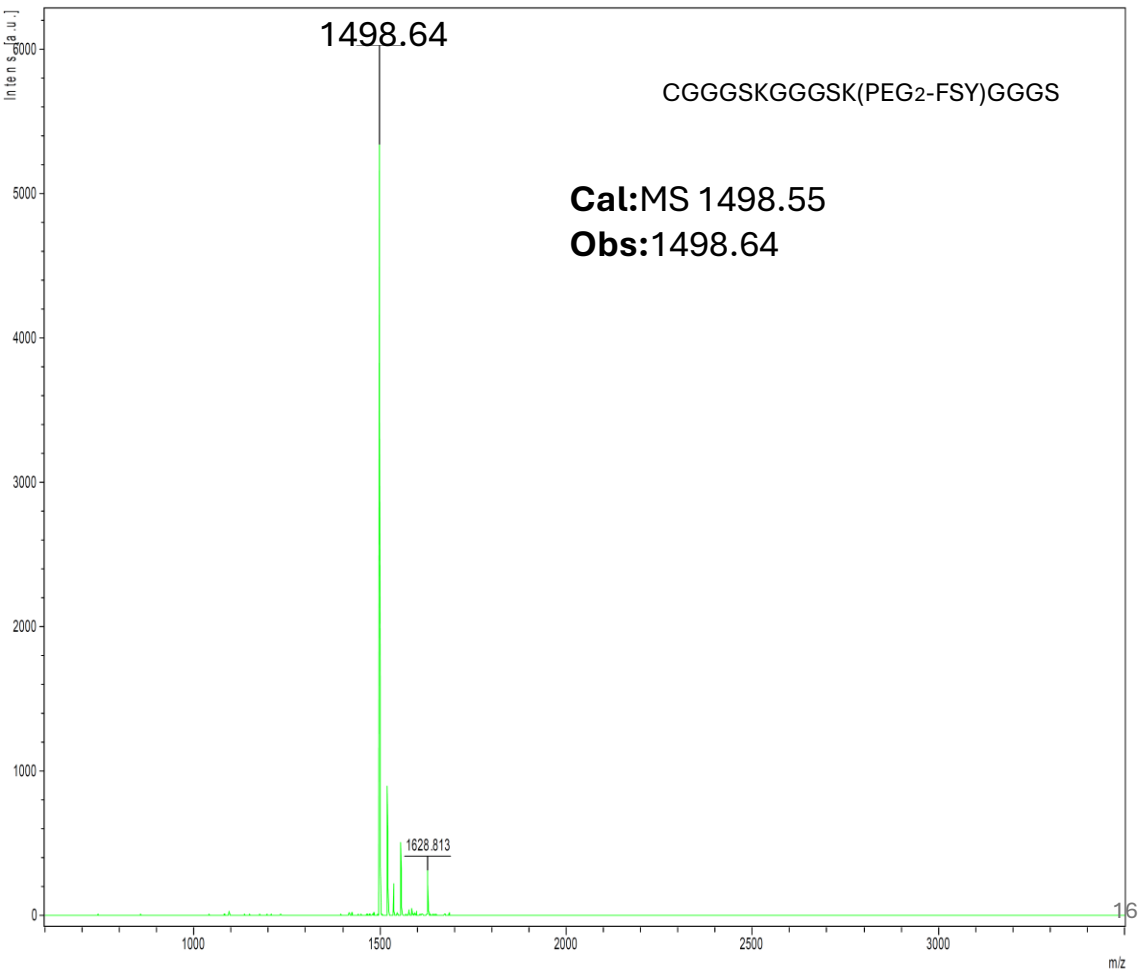
2704.041
m/z

d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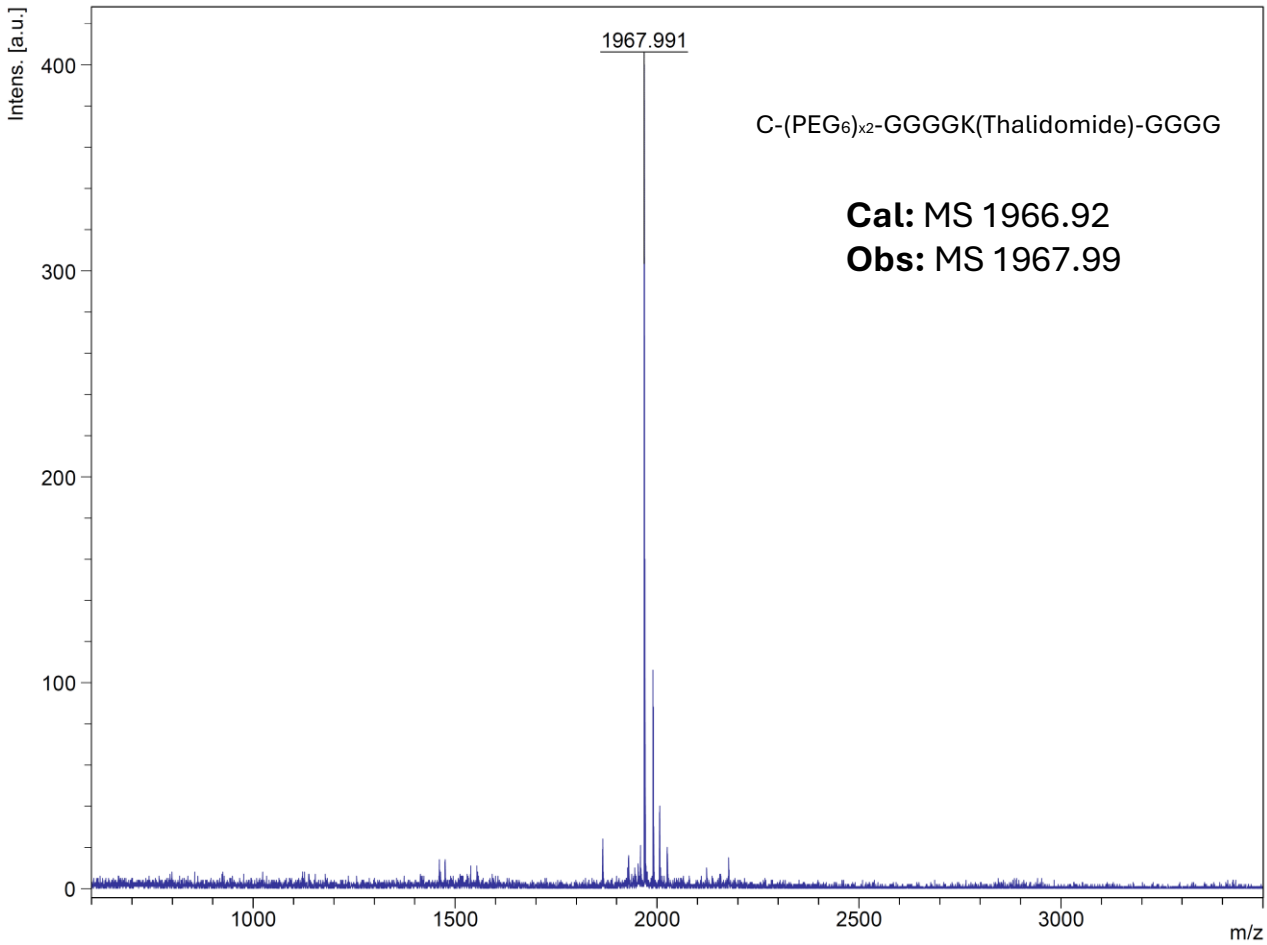
e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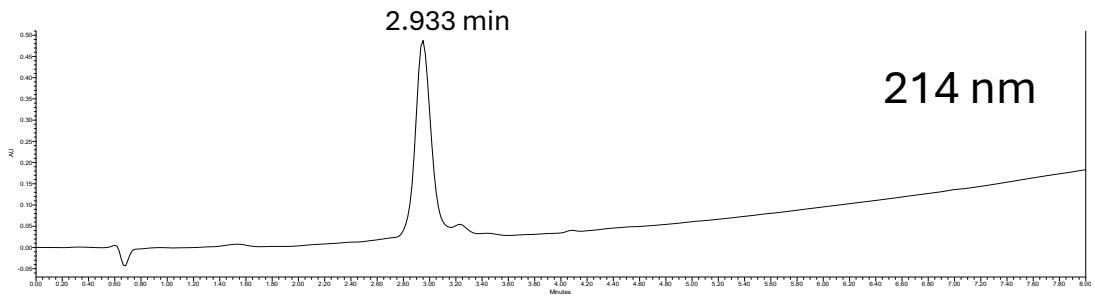
m/z

f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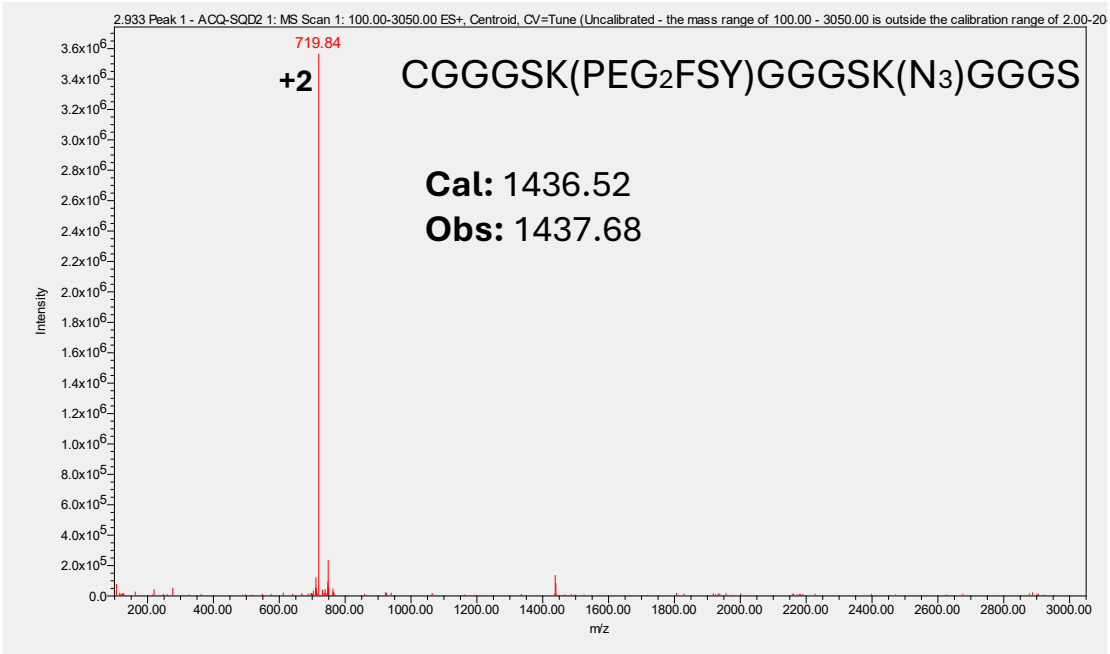
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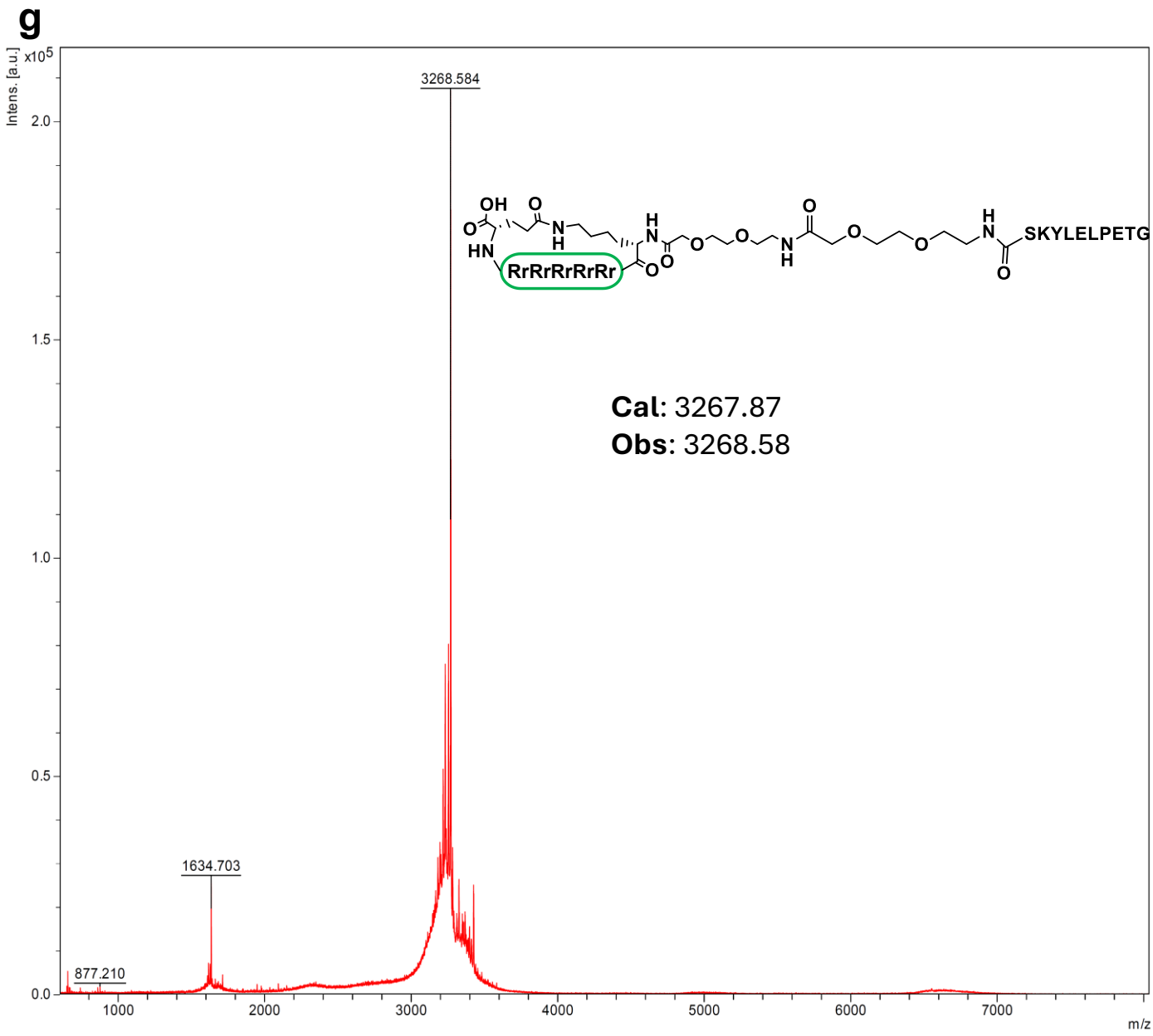
Intensity
m/z



Extended Data Figure 10: Mass spec analysis of synthetic peptides

(a) MALDI-ToF mass spectra of Maleimide-cyclic R10 (cR10) peptide.

(b) MALDI-ToF mass spectra of N-terminal cysteine peptide containing biotin and tetramethylrhodamine (TMR).

(c) MALDI-ToF mass spectra of N-terminal cysteine peptide containing proximity-reactive lysine (K-FSY).

(d) MALDI-ToF mass spectra of N-terminal cysteine peptide containing proximity-reactive lysine (K-PEG_2-FSY).

(e) MALDI-ToF mass spectra of N-terminal cysteine peptide containing both PEG_{12} and Thalidomide.

(f) MALDI-ToF mass spectra of N-terminal cysteine peptide containing both K-PEG_2-FSY and Lys-azide.

(g) MALDI-ToF mass spectra of cR10-LPETG peptide