Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Uncovering the Pathological Functions of Ser404 Phosphorylation by Semisynthesis of Phosphorylated TDP-43 Prion-Like Domain

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Experimental Materials and Methods

Protein expression and purification

The DNA sequences of wTDP PLD was inserted into pET22b plasmid through restriction endonuclease NdeI and XhoI to obtain the plasmid for expressing wTDP PLD-CH. The wTDP PLD-CH plasmid was truncated by PCR with designed primer, and then ligated by T4 ligase to get the plasmid to express tTDP PLD-CH. All plasmids were transfected into BL21 codon plus E. coli cells. The expression of proteins was initiated by 0.5 mM IPTG under 37℃ after the turbidity of E. coli cells achieved 0.8. The E. coli cells could express proteins for 4 hours after IPTG treatment. Then the cells were harvested and lysed by ultrasonication in 50 mM phosphates buffer (pH 9.5) containing 150 mM NaCl. After centrifugation, the pellet was collected and dissolved in the 6 M GuHCl containing 50 mM phosphates (pH 7.4). The solution was centrifuged, and the supernatant was collected and purified by Ni NTA column. The final elution of Ni NTA purification was purified by RP-HPLC (Shimadzu, LC-6AD) with proteonavi column purchased in Shiseido Company. The collected solutions containing targeted proteins from RP-HPLC were lyophilized to get the wTDP PLD-CH and tTDP PLD-CH powder.

In this article, the identification of all proteins was confirmed by low resolution ESI-MS (Thermo Scientific, Ultimate 3000 + MSQ Plus) and RP-HPLC (Shimadzu, LC-2010A HT) with Shiseido analytic proteonavi C4 column. Since the m/z in low resolution are only accurate to one digit after the decimal point, there will be 1-3 deviation in final experimental molecular weight of proteins after deconvolution of ESI mass spectrum.

Sequence of TDP-43

MSEYIRVTEDENDEPIEIPSEDDGTVLLSTVTAQFPGACGLRYRNPVSQCMR
GVRLVEGILHAPDAGWGNLVYVVNYPKDNKRKMDETDASSAVKVKRAVQKT
SDLIVLGLPWKTTEQDLKEYFSTFGEVLMVQVKKDLKTGHSKGFGFVRFTEY
ETQVKVMSQRHMIDGRWCDCKLPNSKQSQDEPLRSRKVFVGRCTEDMTED
ELREFFSQYGDVMDVFIPKPFRAFAFVTFADDQIAQSLCGEDLIIKGISVHISNA
EPKHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMG
GGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQNQ
GNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSGSGFNGGFGSSM
DSKSSGWGM

Sequence of wTDP PLD-*CH*

MHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMGG
GMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQNQG
NMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSGSGFNGGFGSSMD
SKSSGWGM-*CLEHHHHHH*

Sequence of tTDP PLD-*CH*

MHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMGG
GMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQNQG
NMQREPNQAFGSGNNSYSGSNSGAAIGWGS-*CLEHHHHHH*

Sequence of wTDP PLD

MHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMGG
GMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQNQG

NMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSGSGFNGGFGSSMD
SKSSGWGM

Sequence of tTDP PLD

MHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMGG
GMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQNQG
NMQREPNQAFGSGNNSYSGSNSGAAIGWGS

Peptide synthesis

The peptide pS404 TDP 388-414 A388C was manually synthesized by standard Fmoc-based solid-phase peptide synthesis (Fmoc SPPS). 2-chlorotrityl chloride resin, Fmoc-protected amino acids were purchased from GL Biochem (Shanghai) Ltd. Other chemicals were purchased from Sigma-Aldrich. The peptide was cleaved from resin by cleavage cocktail (TFA/Phenol/TIS/H2O/EDT/Me2S/NH4I=81:3:5:5:2.5:2:1.5) for 3 hours at room temperature. Peptide solutions were precipitated in cold diethyl ether and centrifuged to get crude products. Crude peptides were further purified by RP-HPLC (Shimadzu, LC-6AD) with C18 column. The collected solutions with purified peptide were lyophilized to get peptide powder. The identification of pure peptide was confirmed by ESI-MS (Thermo Scientific, Ultimate 3000 + MSQ Plus) and RP-HPLC (Shimadzu, LC-2010A HT) with YMC analytic C18 column.

Protein S-cyanylation

wTDP PLD-CH or tTDP PLD-CH (5 mg) was dissolved in solution (1 ml) of CH_3CN and H_2O with ratio of 1:1 containing 0.1% TFA. Then, N-cyano-4-dimethylaminopyridinium tetra-fluoroborate (CDAP, 10 eq) was added into the solution. After 15 min reaction at 37℃, the solution was filtered and purified by RP-HPLC (Shimadzu, LC6-AD) with preparative C18 column. The collected solutions with purified wTDP PLD-CN or tTDP PLD-CN were lyophilized to get protein powder.

Protein hydrazinolysis

wTDP PLD-CN or tTDP PLD-CN (5 mg) was dissolved in solution (5 ml) of CH_3CN and H_2O with ratio of 1:1. Then, N,N-diisopropylethylamine (DIEA, 0.2%) was added into the solution and was reacted for 5 min. Then hydrazine (0.4%) was added to initiate hydrazinolysis. After 30 min reaction at 37℃, the solution was filtered and purified by RP-HPLC (Shimadzu, LC6-AD) with preparative C18 column. The collected solutions with purified wTDP PLD-$NHNH_2$ or tTDP PLD-$NHNH_2$ were lyophilized to get protein powder. The reaction yield was quantitated by peak area integration of HPLC trace.

Protein thioester synthesis

tTDP PLD-$NHNH_2$ (6 mg) was dissolved in 6 M GuHCl with 0.2 M phosphate (pH 3.0, 0.25 ml) and incubated in ice salt bath at -15℃ for 10 min. $NaNO_2$ (25 eq) was added into the solution and quickly mixed. After 15 min reaction at -15℃, 6 M GuHCl with 0.2 M phosphate (pH 7.0, 0.6 ml) containing 0.1 M 4-Mercaptophenylacetic acid (MPAA) was added into the solution. After 0.5 h reaction at room temperature, the solution was filtered and purified by RP-HPLC (Shimadzu, LC6-AD) with preparative C18 column. The collected solutions with purified tTDP PLD-MPAA were lyophilized to get protein powder.

NCL

pS404-containing peptide (pS404 TDP 388-414 A388C, 6 mg) and MPAA (8 mg) were dissolved in 6 M GuHCl with 0.2 M phosphate (pH 7.0, 0.6 ml) and pH was adjusted to pH 6.0. tTDP PLD-MPAA (6 mg) was dissolved into the solution. After overnight reaction at 37℃, the solution was filtered and purified by RP-HPLC (Shimadzu, LC6-AD) with preparative C18 column. The collected solutions with purified pTDP PLD A388C were lyophilized to get protein powder.

Desulfurization

pTDP PLD A388C, wTDP PLD-CH or tTDP PLD-CH (3 mg) was dissolved in 6 M GuHCl with 0.2 M phosphate (pH 7.0, 0.6 ml). 1 M tris(2-carboxyethyl) phosphine (TCEP, 0.2 ml), 2-Methyl-2-propanethiol (40 µl) and 0.1 M VA-044 (20 µl) were added into the solution and fully mixed. After overnight reaction at 37℃, the solution was filtered and purified by RP-HPLC (Shimadzu, LC6-AD) with preparative C18 column. The collected solutions with purified pTDP PLD, wTDP PLD-AH or tTDP PLD-AH were lyophilized to get protein powder.

Hydrolysis of protein hydrazides

wTDP PLD-$NHNH_2$ or tTDP PLD-$NHNH_2$ (6 mg) was dissolved in 6 M GuHCl with 0.2 M phosphate (pH 3.0, 0.25 ml) and incubated in ice salt bath at -15℃ for 10 min. 0.5 M $NaNO_2$ (24 µl) was added into the solution and quickly mixed. After 15 min reaction at -15℃, 6 M GuHCl with 0.2 M phosphate (pH 7.0, 0.6 ml) containing 0.1 M 4-Mercaptophenylacetic acid (MPAA) was added into the solution. After 1 h reaction at room temperature, pH of the solution was adjusted to 9-10. After 12 h reaction at room temperature, the solution was filtered and purified by RP-HPLC (Shimadzu, LC6-AD) with preparative C18 column. The collected solutions with purified wTDP PLD and tTDP PLD were lyophilized to get protein powder.

ThT assay

The protein powders were dissolved in DMSO to get 1 mM protein stock. 400 µM protein stocks were prepared by diluting 1 mM protein stocks in DMSO. For ThT kinetic assay, 1 µl of 400 µM protein stocks was added into 99 µl of 50 µM ThT solution to monitor the real-time ThT kinetics. For ThT time point assay, 10 µl of 400 µM protein stocks was added into 990 µl 1X PBS. The protein solutions were shake at 300 rpm, 37℃. 90 µl protein solutions was added with 10 µl of 500 µM ThT stock to monitor the ThT fluorescence at indicated time point. The excitation and emission wavelengths of ThT fluorescence were 440 nm and 483 nm, respectively. The ThT assays were conducted by a microplate reader (BioTek synergy 4). All the experiments were conducted at least three times.

Transmission electron microscopy (TEM) analysis

The protein stock (0.4 mM) of wTDP PLD and pTDP PLD were diluted into 4 µM solutions by 1 X PBS buffer, respectively. And 4 µM protein solutions were shake under 300 rpm and 37℃ to promote the protein aggregation. Under indicated time point, the protein solutions (8 µl) were loaded on copper grid with carbon support films for 3 times and stained with tungstophosphoric acid. Samples were imaged on a Hitachi H-7650B TEM after dried overnight. All the experiments were conducted at least three times.

Cell culture

N2a cells were propagated in 44.5% dulbecco's modified eagle medium (DMEM) and 44.5% alpha modification medium (alpha MEM) with 10% FBS, 1% Penicillin-Streptomycin Solution. All mediums and FBS were purchased from Gibco. N2a Cells were cultured in a sterile incubator containing 5% CO2 at 37℃.

MTT assay for wild type N2a cells

Cells in exponential growth phase were harvested and seeded in a 96-well plate at a concentration of 6000 cell/well. The cells were cultured for 24 h at 37℃. Then the medium was removed and 100 μl opti MEM containing monomeric or aggregated wTDP PLD and pTDP PLD at gradient concentrations (0, 0.5, 1, 1.5, 2 μM) was added into each well, respectively. After 24 h incubation, 20 μl of 5 mg/ml MTT ((3-(4, 5-DimethylthiazoL-2-yl)-2, 5-diphenyltetrazolium bromide) was supplied into each well and the plate was cultured at 37℃ for another 4 h. After that, the medium was removed and 150 μl DMSO was added to each well to dissolve the purple precipitation. The 570 nm absorption of the solution was measured in a microplate reader (BioTek synergy 4). To obtain aggregated wTDP PLD and pTDP PLD, the protein stock (0.4 mM) of wTDP PLD and pTDP PLD were diluted into 4 μM solutions by sterile opti-MEM in biological safety cabinet, respectively. And 4 μM protein solutions were shake under 300 rpm and 37℃ to promote the protein aggregation. After 8 h shaking, the aggregated wTDP PLD and pTDP PLD were obtained. All the experiments were conducted at least three times.

MTT assay for N2a cells transfected with TDP-43 plasmid

Cells in exponential growth phase were harvested and seeded in a 96-well plate at a concentration of 6000 cell/well. The cells were cultured for 24 h at 37℃ and then transient transfected with WT/S404D/S404E TDP-43 EGFP plasmid or only transfection reagents as control group. The cells were transiently transfected by lipofectamine 3000 reagent kit (Thermo Fisher). After 24 h of transfection, all cells were treated with 800 μg/ml G418 for screening. the MTT assay was done after screening for 3 d. All the experiments were conducted at least three times.

For control group, N2a cells did not carry pEGFP-N3 plasmid, therefore N2a cells in control group were almost dead after G418 screening. For experimental group, N2a cells that were successfully transfected with WT/S404D/S404E TDP-43 EGFP plasmid could survive after G418 screening for resistance of pEGFP-N3 plasmid. Therefore, we could utilize MTT to evaluate the cell proliferation rate of N2a cells transfected with WT/S404D/S404E TDP-43 EGFP plasmid and avoid the influence brought by rapid proliferation of N2a cells without transfection of WT/S404D/S404E TDP-43 EGFP plasmid.

Supplementary Figures

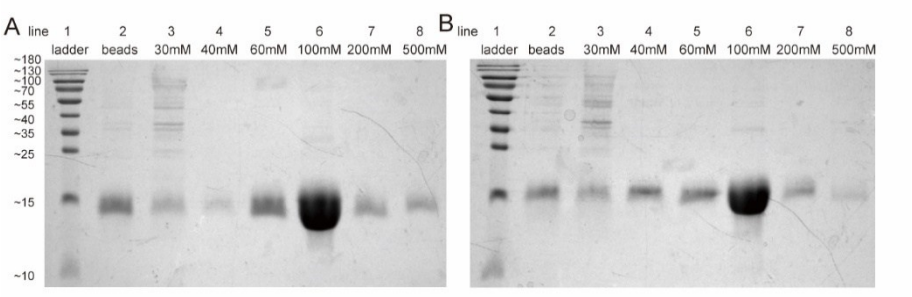

Fig. S1 SDS PAGE of purification traces of tTDP PLD-CH **(A)** and wTDP PLD-CH **(B)**, line1 is protein ladders, line2 is Ni-NTA beads after incubation with *E coli* inclusion body solution (6 M GuHCl, 50 mM Phosphate, pH7.0) for 2 h, line 3-8 are elution under different imidazole concentration.

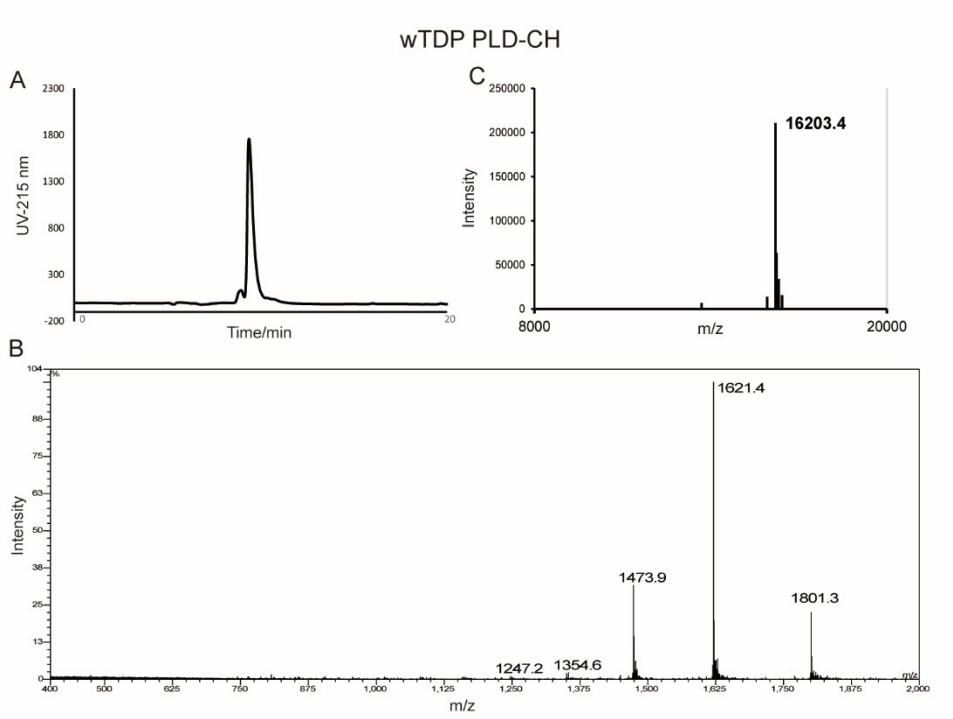

Fig. S2 (A) Analytical RP-HPLC trace of wTDP PLD-CH (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of wTDP PLD-CH. **(C)** Mass spectrum of wTDP PLD-CH after deconvolution of ESI mass spectrum. wTDP PLD-CH Calculated Mass: 16202.3; Experimental Mass: 16203.4±3.0.

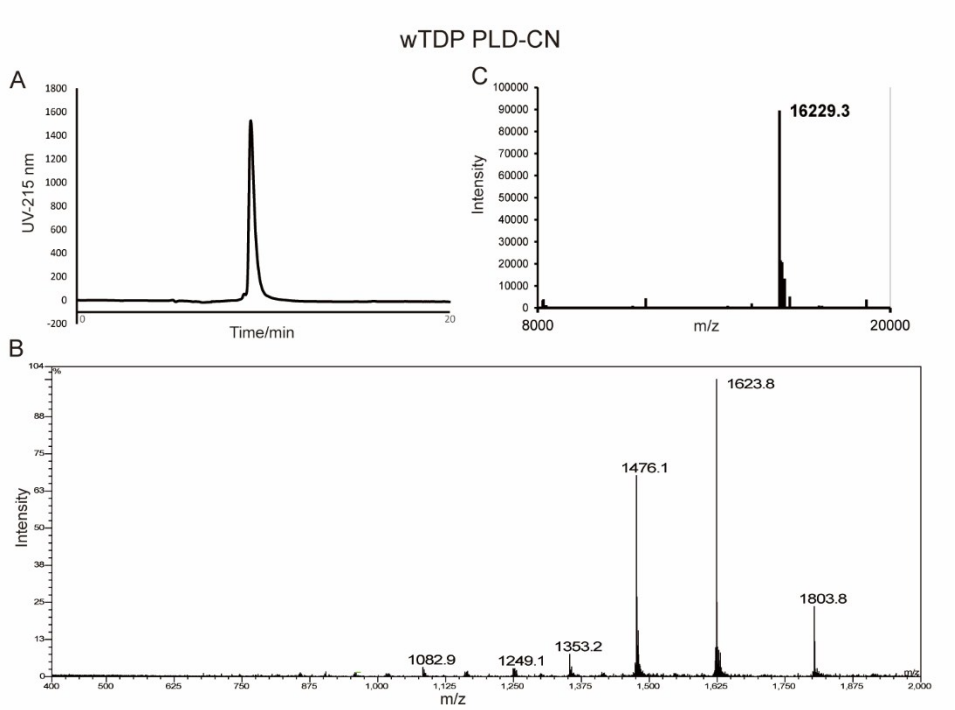

Fig. S3 (A) Analytical RP-HPLC trace of wTDP PLD-CN (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of wTDP PLD-CN. **(C)** Mass spectrum of wTDP PLD-CN after deconvolution of ESI mass spectrum. wTDP PLD-CN Calculated Mass: 16228.3; Experimental Mass: 16229.3±3.0.

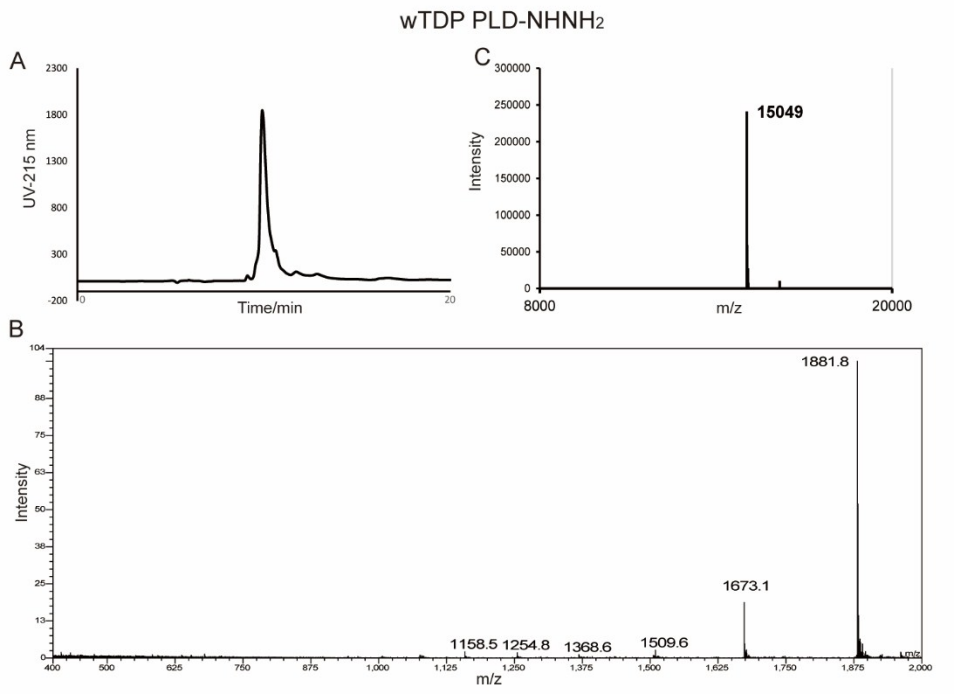

Fig. S4 (A) Analytical RP-HPLC trace of wTDP PLD-$NHNH_2$ (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of wTDP PLD-$NHNH_2$. **(C)**

Mass spectrum of wTDP PLD-$NHNH_2$ after deconvolution of ESI mass spectrum. wTDP PLD-$NHNH_2$ Calculated Mass: 15048.1; Experimental Mass: 15049.0±3.0.

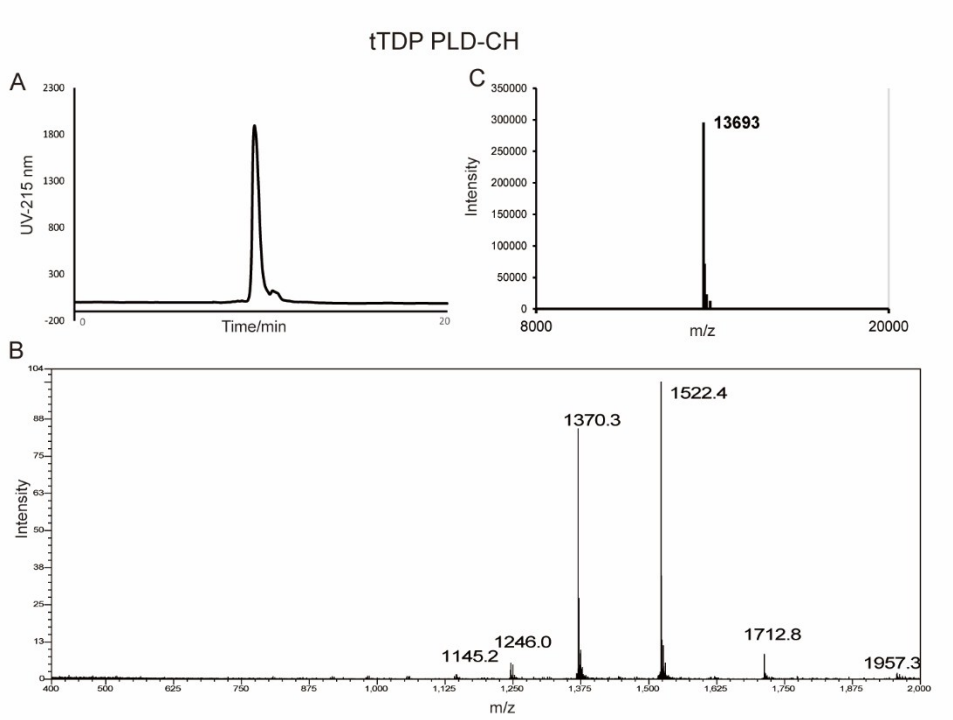

Fig. S5 (A) Analytical RP-HPLC trace of tTDP PLD-CH (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of tTDP PLD-CH. **(C)** Mass spectrum of tTDP PLD-CH after deconvolution of ESI mass spectrum. tTDP PLD-CH Calculated Mass: 13692.7; Experimental Mass: 13693.0±3.0.

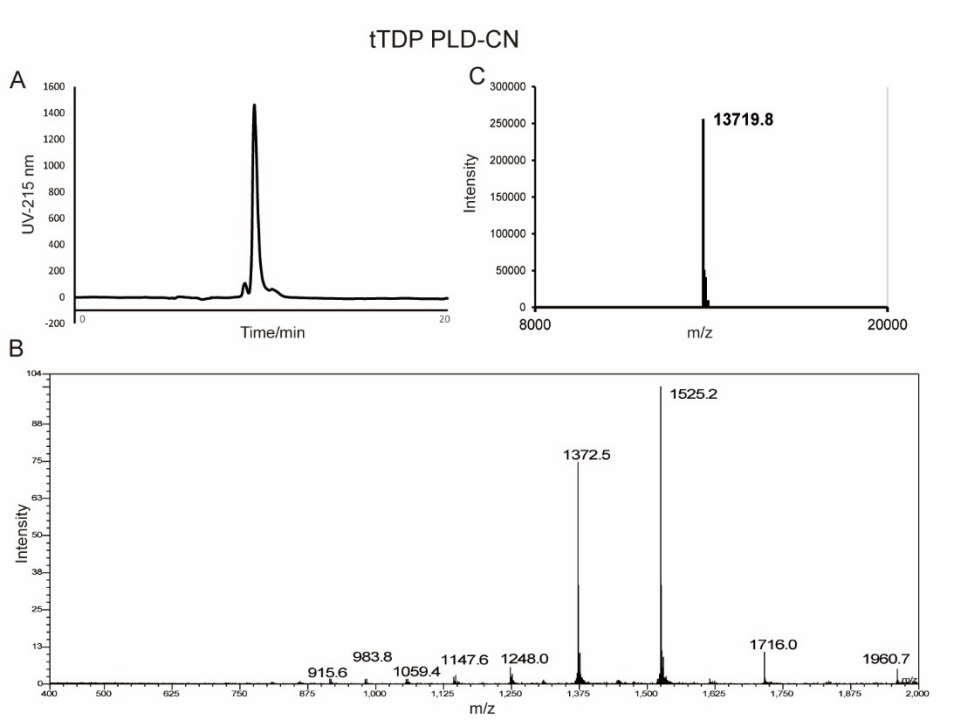

Fig. S6 (A) Analytical RP-HPLC trace of tTDP PLD-CN (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of tTDP PLD-CN. **(C)** Mass spectrum of tTDP PLD-CN after deconvolution of ESI mass spectrum. tTDP PLD-CN Calculated Mass: 13717.8; Experimental Mass: 13719.8±3.0.

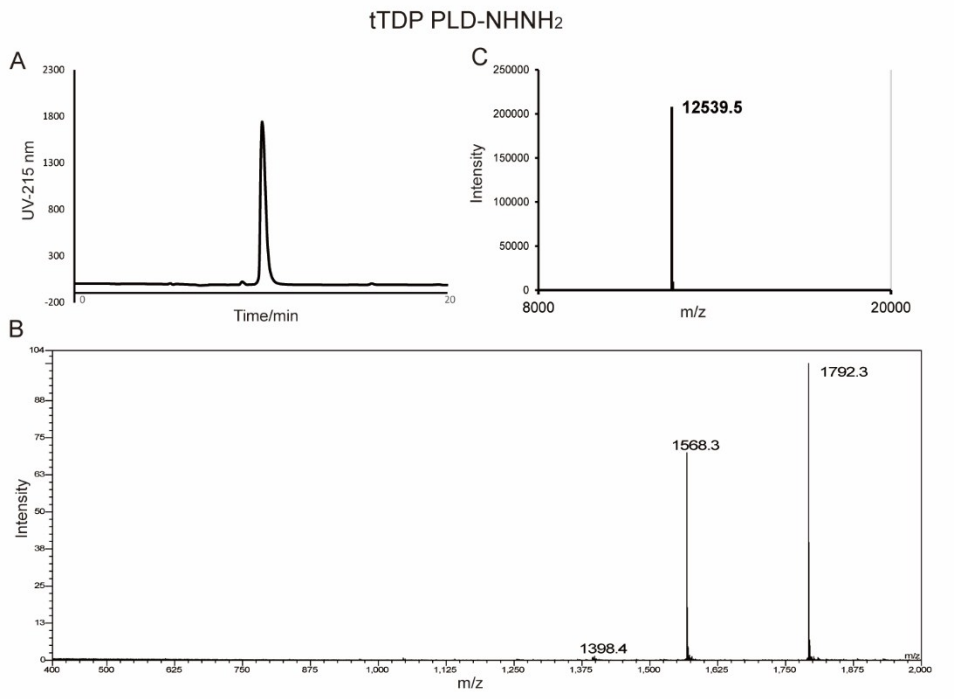

Fig. S7 (A) Analytical RP-HPLC trace of tTDP PLD-$NHNH_2$ (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of tTDP PLD-$NHNH_2$. **(C)**

Mass spectrum of tTDP PLD-$NHNH_2$ after deconvolution of ESI mass spectrum. tTDP PLD-$NHNH_2$ Calculated Mass: 12538.5; Experimental Mass: 12539.5±3.0.

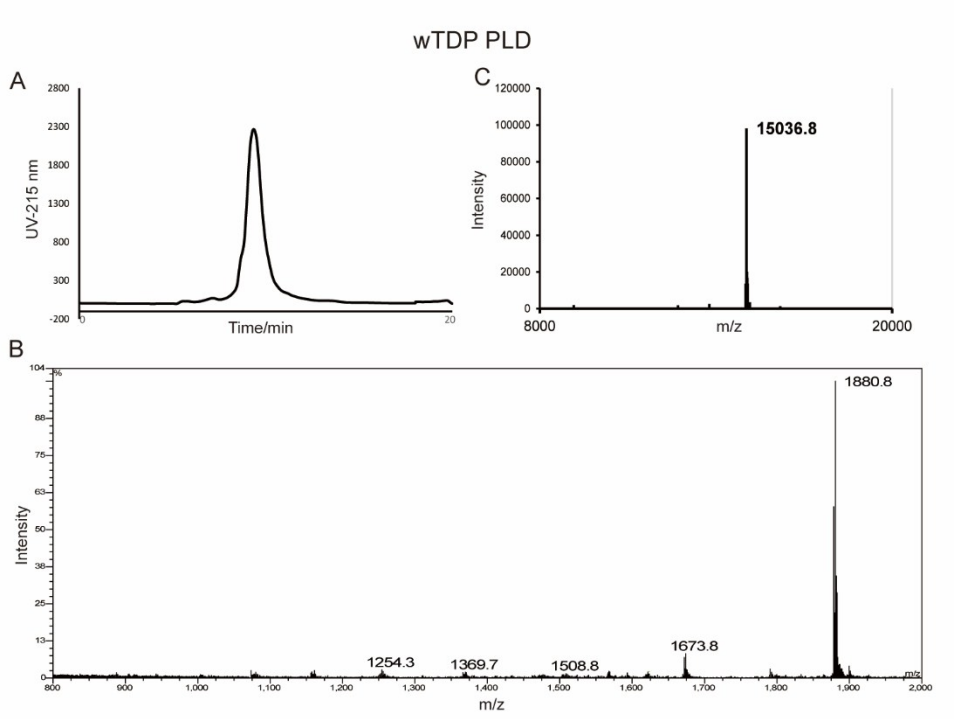

Fig. S8 (A) Analytical RP-HPLC trace of wTDP PLD (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of wTDP PLD. **(C)** Mass spectrum of wTDP PLD after deconvolution of ESI mass spectrum. wTDP PLD Calculated Mass: 15034.1; Experimental Mass: 15036.8±3.0.

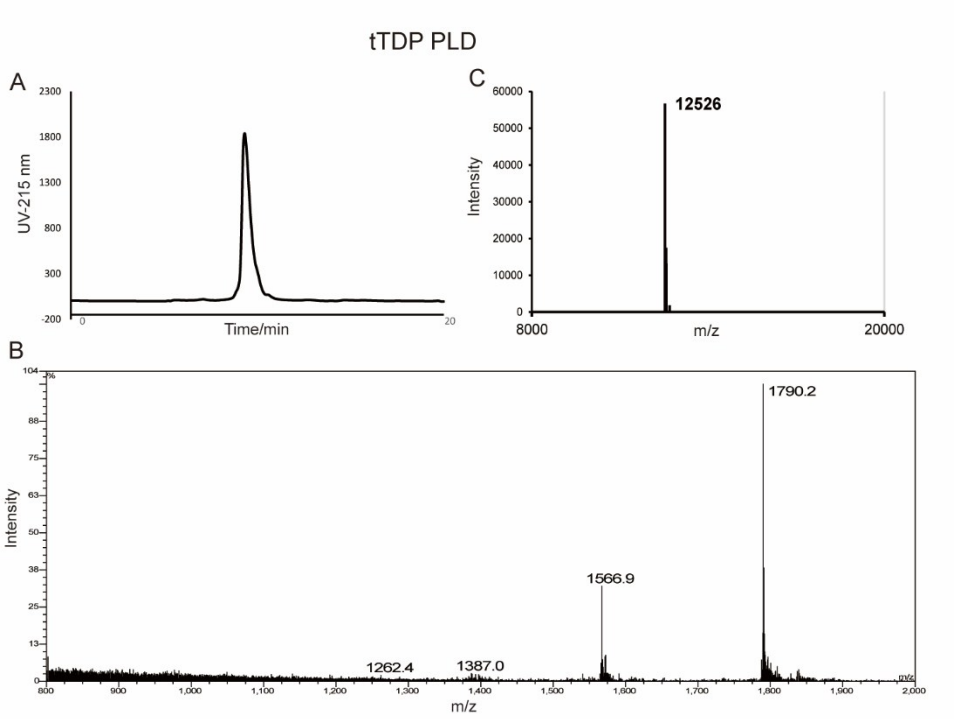

Fig. S9 (A) Analytical RP-HPLC trace of tTDP PLD (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of tTDP PLD. **(C)** Mass spectrum of tTDP PLD after deconvolution of ESI mass spectrum. tTDP PLD Calculated Mass: 12524.4; Experimental Mass: 12526.0±3.0.

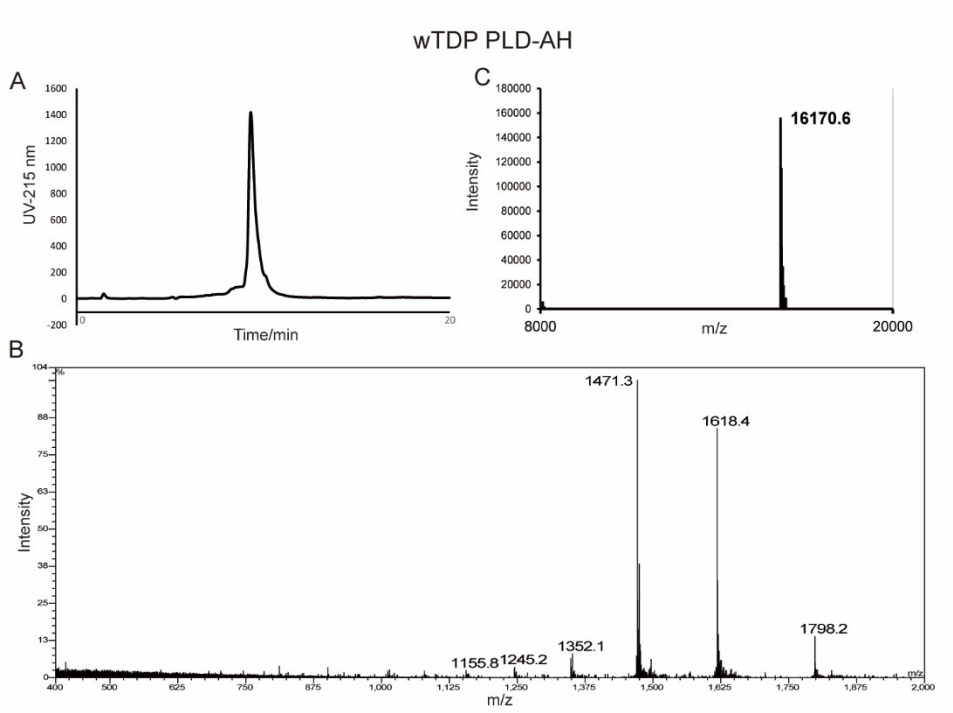

Fig. S10 (A) Analytical RP-HPLC trace of wTDP PLD-AH (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of wTDP PLD-AH. **(C)** Mass spectrum of wTDP PLD-AH after deconvolution of ESI mass spectrum. wTDP PLD-AH Calculated Mass: 16170.3; Experimental Mass: 16170.6±3.0.

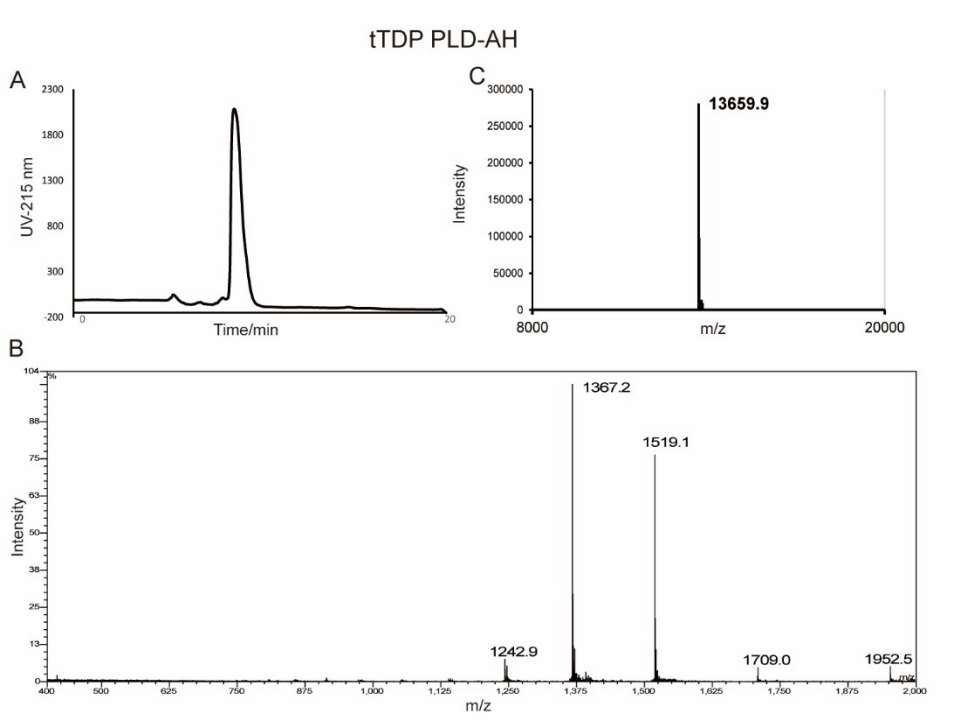

Fig. S11 (A) Analytical RP-HPLC trace of tTDP PLD-AH (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of tTDP PLD-AH. **(C)** Mass spectrum of tTDP PLD-AH after deconvolution of ESI mass spectrum. tTDP PLD-AH Calculated Mass: 13660.70; Experimental Mass: 13659.9±3.0.

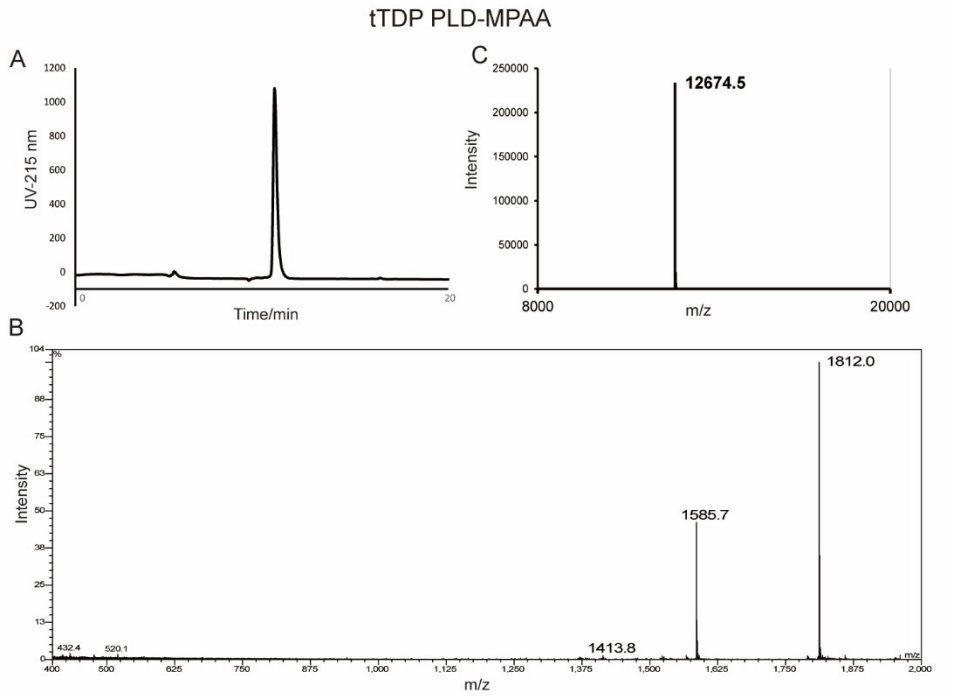

Fig. S12 (A) Analytical RP-HPLC trace of tTDP PLD-MPAA (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA.

All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of tTDP PLD-MPAA. **(C)** Mass spectrum of tTDP PLD-MPAA after deconvolution of ESI mass spectrum. tTDP PLD-MPAA Calculated Mass: 12674.4; Experimental Mass: 12674.5±3.0.

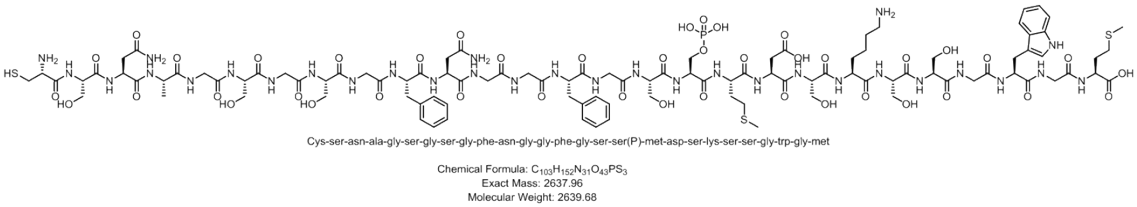

Fig. S13 Molecular structure of pS404 TDP 388-414 A388C

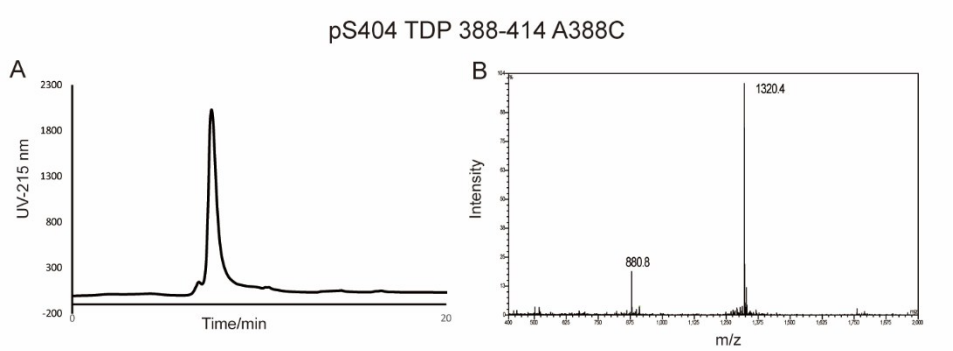

Fig. S14 (A) Analytical RP-HPLC trace of pS404 TDP 388-414 A388C (0-20 min: 35%-55% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of pS404 TDP 388-414 A388C, Calculated Mass: 2639.7; Experimental Mass: $[M]^{3+}$ 880.8±0.2, $[M]^{2+}$ 1320.4±0.2.

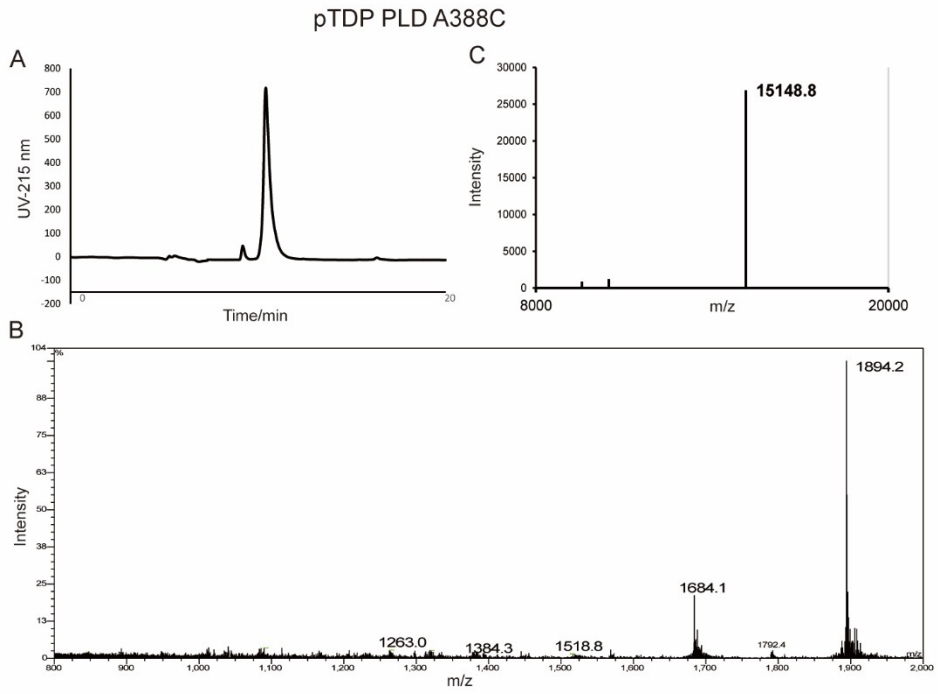

Fig. S15 (A) Analytical RP-HPLC trace of pTDP PLD A388C (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA.

All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of pTDP PLD A388C. **(C)** Mass spectrum of pTDP PLD A388C after deconvolution of ESI mass spectrum. pTDP PLD A388C Calculated Mass: 15146.1; Experimental Mass: 15148.8±3.0.

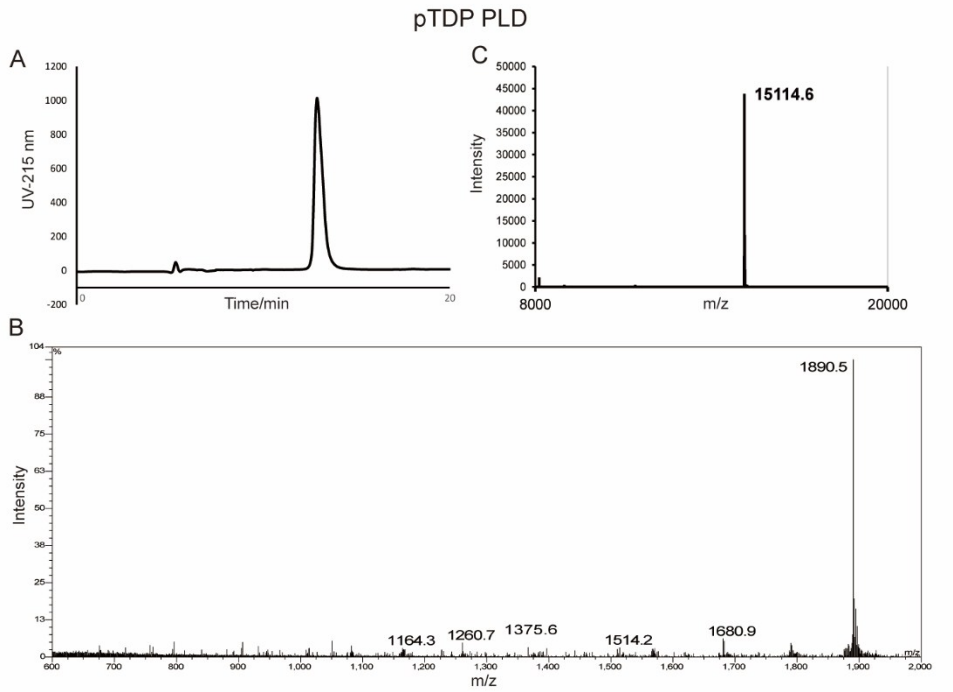

Fig. S16 (A) Analytical RP-HPLC trace of pTDP PLD (0-20 min: 45%-62% solution B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). **(B)** ESI mass spectrum of pTDP PLD. **(C)** Mass spectrum of pTDP PLD after deconvolution of ESI mass spectrum. pTDP PLD Calculated Mass: 15114.1; Experimental Mass: 15114.6±3.0.

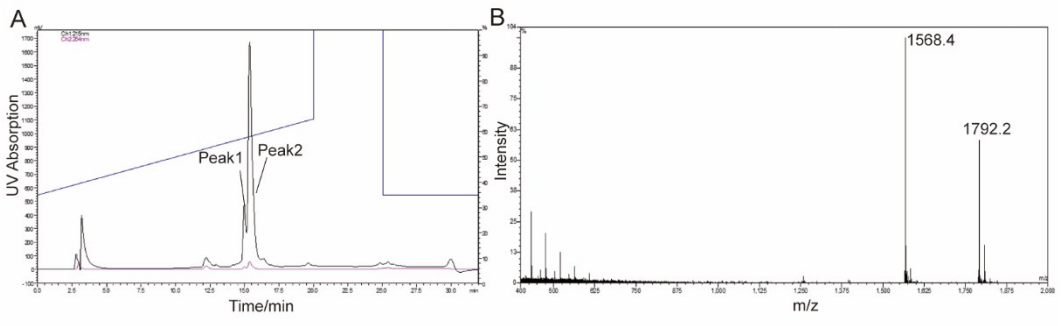

Fig. S17 (A) Analytical RP-HPLC trace of tTDP PLD-CN hydrazinolysis after 30 min reaction (0-20min: 35%-65% solution. **(B)** ESI mass of peak2, which was identified as tTDP PLD-$NHNH_2$, peak1 was identified as tTDP PLD-CN.

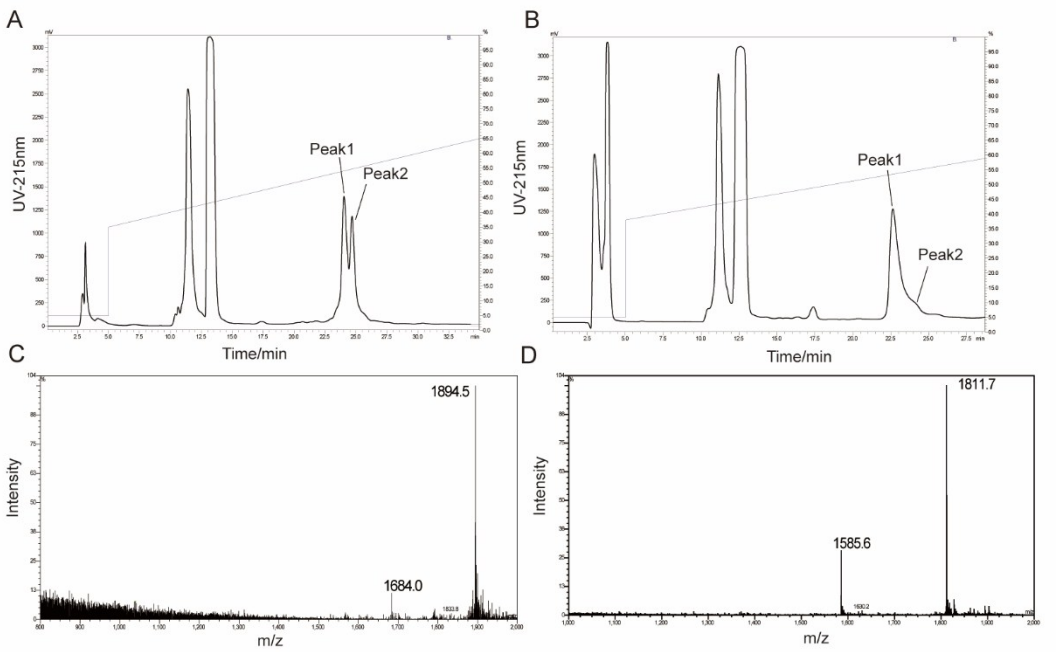

Fig. S18 (A) Analytical RP-HPLC trace of NCL between tTDP PLD-MPAA and pS404 TDP 388-414 A388C after 1 h reaction (5-35 min: 40%-65% solution B). **(B)** Analytical RP-HPLC trace of NCL between tTDP PLD-MPAA and pS404 TDP 388-414 A388C after 5 h reaction (5-35 min: 40%-65% solution B), A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA, All solvents are RP-HPLC grade. **(C)** ESI mass of peak1, which was identified as pTDP PLD A388C. **(D)** ESI mass of peak2, which was identified as tTDP PLD-MPAA.

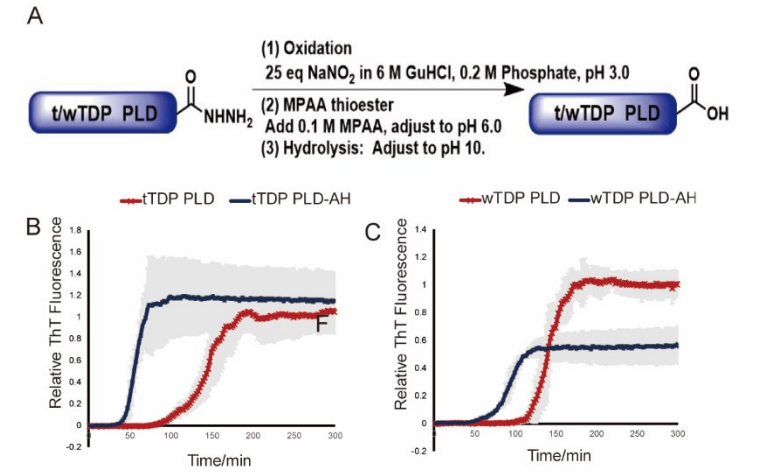

Fig. S19 (A) Method of synthesizing t/wTDP PLD from t/wTDP PLD-$NHNH_2$. **(B)**ThT kinetic assay for tTDP PLD-AH and tTDP PLD. **(C)** ThT kinetic assay for wTDP PLD-AH and wTDP PLD. For ThT kinetic assay, lyophilized protein powders were dissolved in DMSO to obtain 0.4 mM stock solutions. Proteins (4 μM) aggregated in 1X PBS containing 50 μM ThT. n=3.

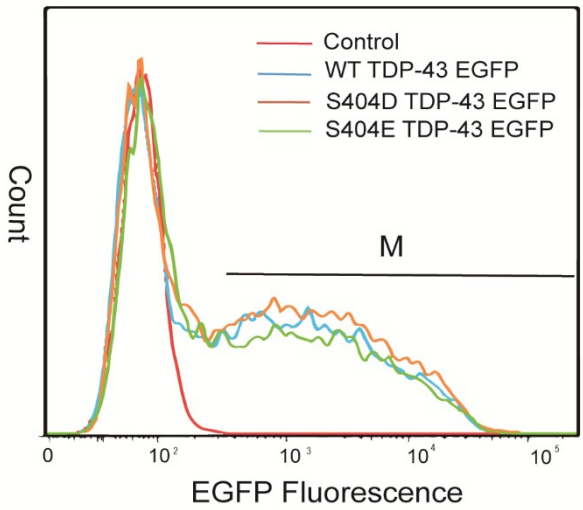

Fig. S20 Flow cytometry of N2a cells transfected with full-length TDP-43. N2a cells were transiently transfected with WT/S404D/S404E TDP-43 EGFP plasmid. The control group was wild type N2a treated with transfection reagent without any plasmid. After 24 h of transfection, all cells were harvested and measure EGFP fluorescence by flow cytometry (BD Biosciences).

Table S1. Hydrazinolysis conditions of wTDP PLD-CN.

Entry	Condition	Solution	Time	Yield
1	0.5% NH_2NH_2,	1 M GuHCl, 33 mM phosphate, pH 10	30 min	N*
2	0.2% NH_2NH_2	CH_3CN/H_2O	30 min	N
3	0.4% NH_2NH_2	CH_3CN/H_2O	30 min	30%
4	0.5% NH_2NH_2	CH_3CN/H_2O	30 min	40%
5	0.1% Ethylenediamine for 5 min 0.4% NH_2NH_2	CH_3CN/H_2O	30 min	68%
6	0.1% Piperidine for 5 min 0.4% NH_2NH_2	CH_3CN/H_2O	30 min	70%
7	0.1% DIEA for 5 min 0.4% NH_2NH_2	CH_3CN/H_2O	30 min	75%
8	0.2% DIEA for 5 min 0.4% NH_2NH_2	CH_3CN/H_2O	30 min	85%

Note: The wTDP PLD-CN concentration in each condition was 1 mg/ml. N, wTDP PLD-$NHNH_2$ was not detected in analytical RP-HPLC and ESI-MS. *, wTDP PLD-CN precipitated in indicated condition. The reaction yield was calculated by peak area integration of analytical RP-HPLC trace.