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Supplemental Information

Endoplasmic Reticulum Proteostasis Influences the Oligomeric State of an Amyloidogenic Protein Secreted from Mammalian Cells

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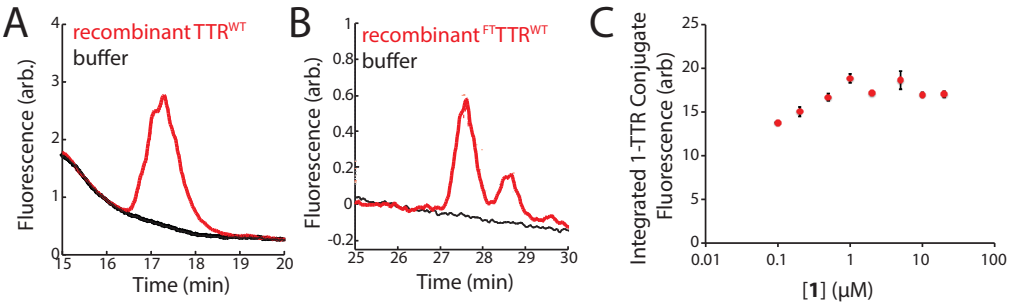
SUPPLEMENTAL FIGURES



D

Mass Diff (vs. $^{FT}TTR^{WT}$)	Modification	Predicted Site of Modification	Relative Abundance (%)
+199	+SO_3 and +Cys	Tyr and Cys10	38 +/- 4
+80	+SO_3	Tyr	31 +/- 5
+119	Cys	Cys10	12 +/- 2
+160	2 x SO_3	Tyr	8 +/- 2
Parent			6 +/- 3

Figure S1 (Supplement to Figure 1).

A. Plot showing the compound **1**-TTR conjugate fluorescence of recombinant TTR^{WT} (200 nM) in cell culture media separated by anion exchange chromatography. Compound **1** (10 μM) was added to unconditioned media containing recombinant TTR^{WT} for 16 h prior to analysis by anion exchange chromatography.

B. Plot showing the compound **1**-TTR conjugate fluorescence of recombinant $^{FT}TTR^{WT}$ (200 nM) in cell culture media separated by anion exchange chromatography. Compound **1** (10 μM) was added to unconditioned media containing recombinant $^{FT}TTR^{WT}$ for 16 h prior to analysis by anion exchange chromatography.

C. Plot showing the compound **1**-TTR conjugate fluorescence of $^{FT}TTR^{WT}$ in conditioned media. Compound **1** at the indicated concentration was added to conditioned media containing $^{FT}TTR^{WT}$ for 16 h prior to analysis by anion exchange chromatography.

D. Table showing modifications of $^{FT}TTR^{WT}$ identified by mass spectrometric analysis of TTR immunopurified from media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{WT}$. The mass difference from parent, modification, predicted modification site and relative abundance are shown. These modified TTRs integrate into heterotetramers to comprise the 8 distinct peaks identified by compound **1**-TTR conjugate fluorescence in UPLC anion exchange chromatograms, as shown in **Fig. 1D**.

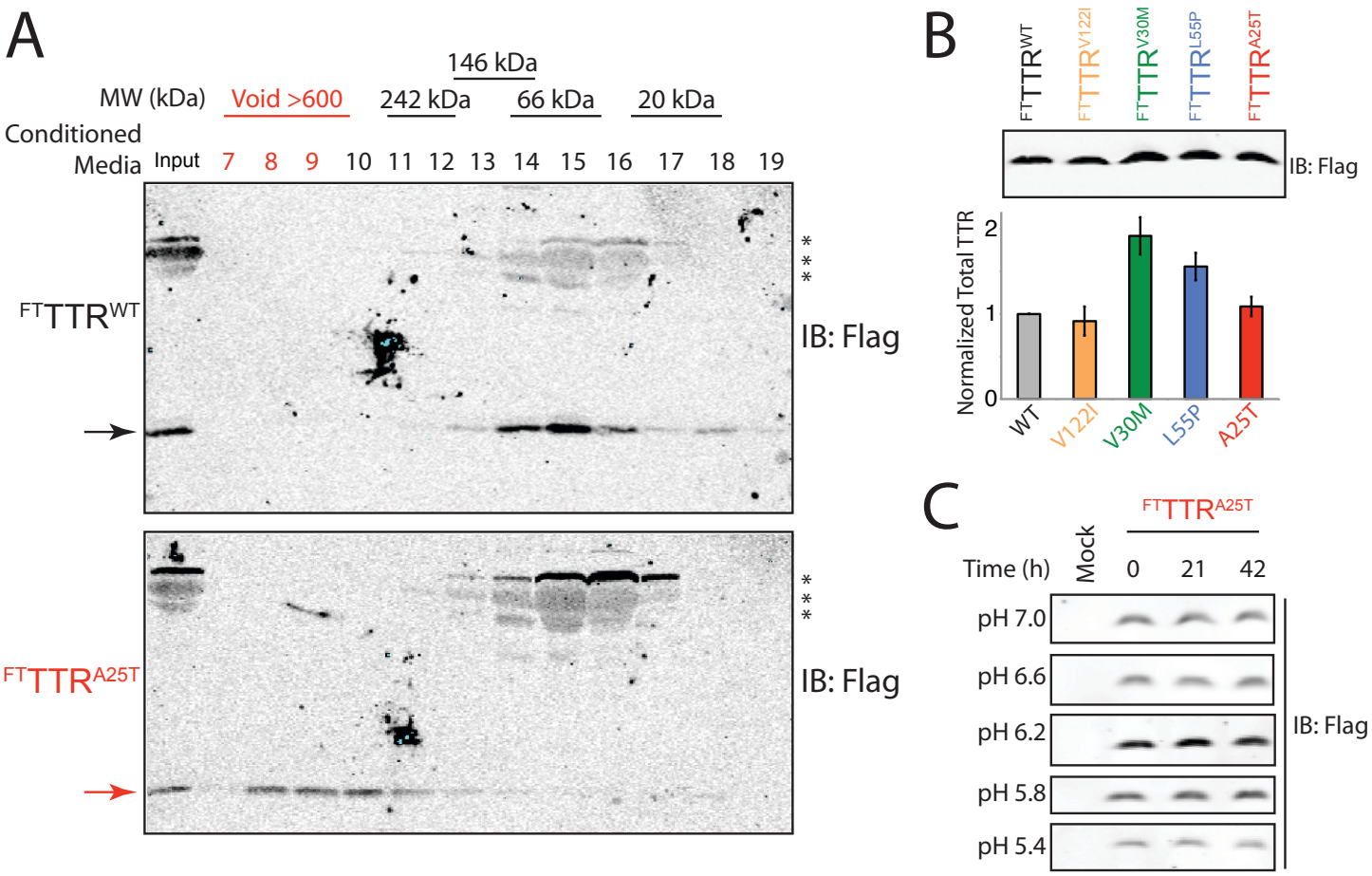


Figure S2 (Supplement to Figure 2).

A. Immunoblot of fractions collected from media conditioned on HEK293 cells expressing $^{FT}TTR^{WT}$ (top) or $^{FT}TTR^{A25T}$ (bottom) separated by gel filtration chromatography. The migration of molecular weight standards are shown above. The arrows show ^{FT}TTR. The asterix (*) identifies non-TTR background bands that cross-react with this antibody in conditioned media. The migration of $^{FT}TTR^{WT}$ is identical to that observed for recombinant $^{FT}TTR^{WT}$ (Chen et al., 2014).

B. Representative SDS-PAGE immunoblot and quantification of total TTR levels in media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{WT}$ (black), $^{FT}TTR^{V122I}$ (orange), $^{FT}TTR^{V30M}$ (green), $^{FT}TTR^{L55P}$ (blue), or $^{FT}TTR^{A25T}$ (red). The graph shows relative amounts of the different TTR variants normalized to $^{FT}TTR^{WT}$. Error bars show SEM for n=3.

C. Immunoblot of $^{FT}TTR^{A25T}$ incubated in conditioned media at 37 °C for the indicated time. The pH of the media was adjusted as indicated to induce protein unfolding. Note that the $^{FT}TTR^{A25T}$ signal is not lost during this incubation at any pH, indicating that no proteolysis is observed.

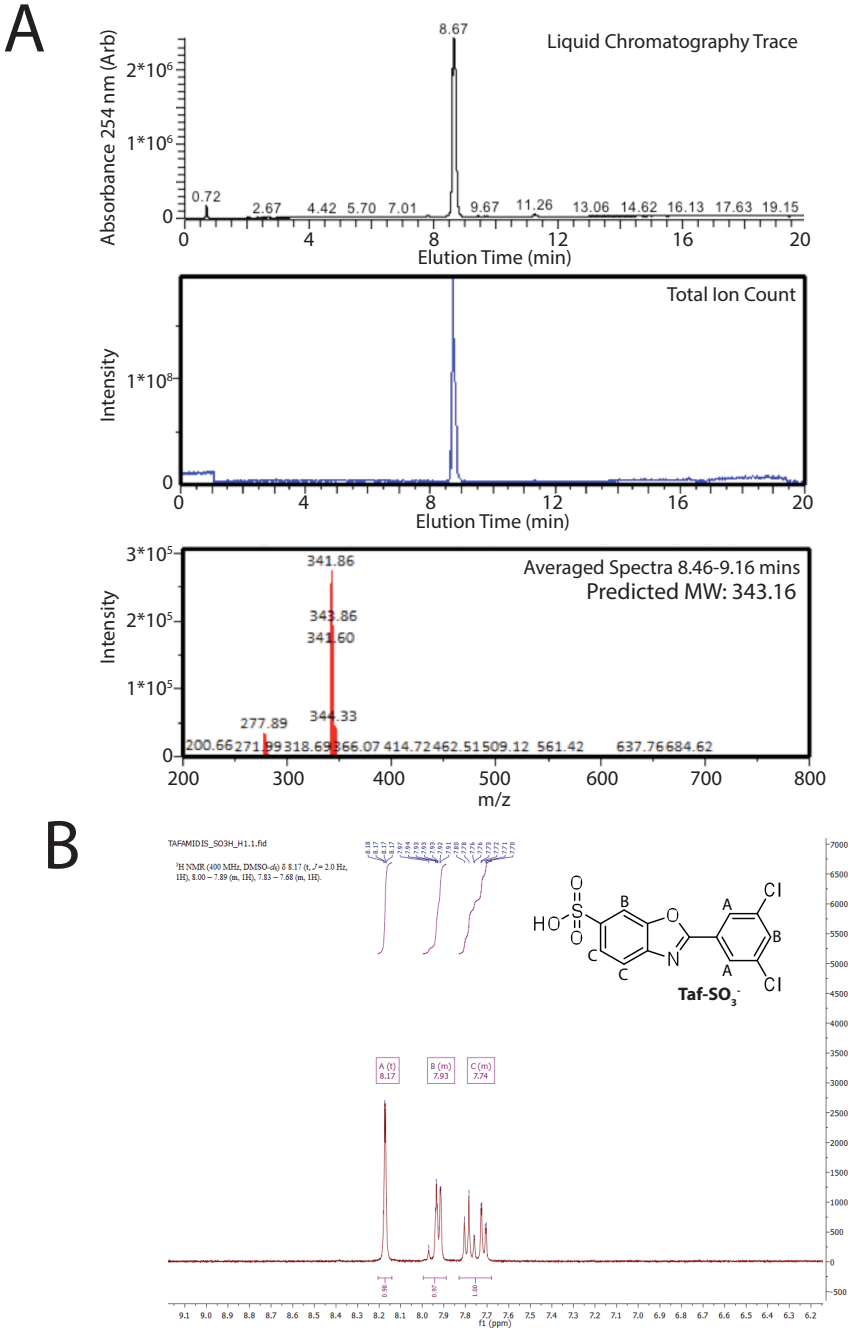


Figure S3 (Supplement to Figure 4)

A. Liquid chromatography (LC) trace (top), total ion count (middle), and mass spectrometry analysis (bottom) of Taf-SO_3^- analyzed by LC-MS. The LC-MS method is as follows: 1) 10% acetonitrile (ACN) / 90% H_2O + 0.1% NH_4OH for 2 min, 2) linear gradient to 90% ACN / 10% H_2O + 0.1% NH_4OH over 10 min, 3) 90% ACN / 10% H_2O + 0.1% NH_4OH for 8 min. Mass spectrometry analysis was performed in negative mode scanning molecular weights between 200-800 Da.

B. Proton NMR for Taf-SO_3^-.

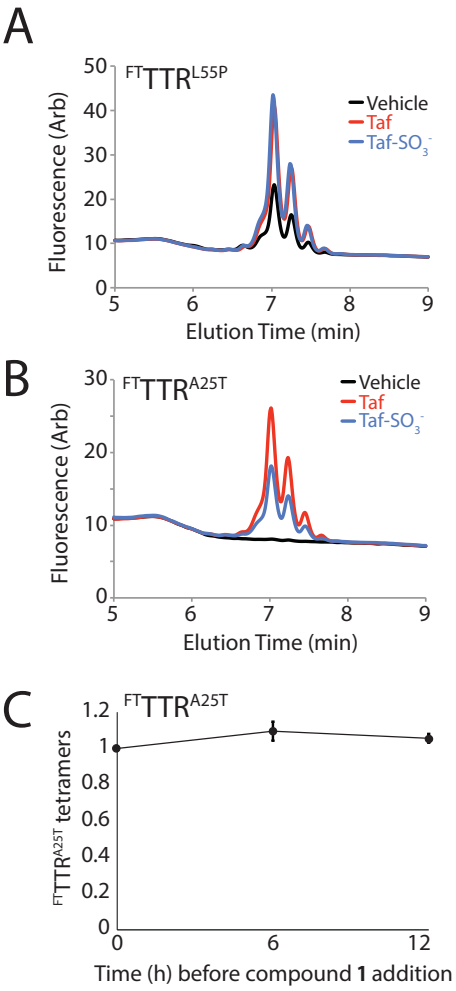


Figure S4 (Supplement to Figure 5).

A. Representative plot showing the compound **1**-TTR conjugate fluorescence of media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{L55P}$ in the presence of vehicle (black), Taf (10 μM; red), or Taf-SO_3^- (10 μM; blue). Compound **1** (10 μM) was added to conditioned media for 16 h prior to analysis by anion exchange chromatography.

B. Representative plot showing the compound **1**-TTR conjugate fluorescence of media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{A25T}$ in the presence of vehicle (black), Taf (10 μM; red), or Taf-SO_3^- (10 μM; blue). Media was conditioned for 16 h prior to collection. Compound **1** (10 μM) was added to conditioned media for 16 h prior to analysis by anion exchange chromatography.

C. Plot showing relative tetramer levels (measured by compound **1**-TTR conjugate fluorescence UPLC assay) of media conditioned on HEK293 cells expressing $^{FT}TTR^{A25T}$ in the presence of Taf-SO_3^- (10 μM). The x-axis shows the time media was incubated prior to addition of compound **1** (14.4 μM).

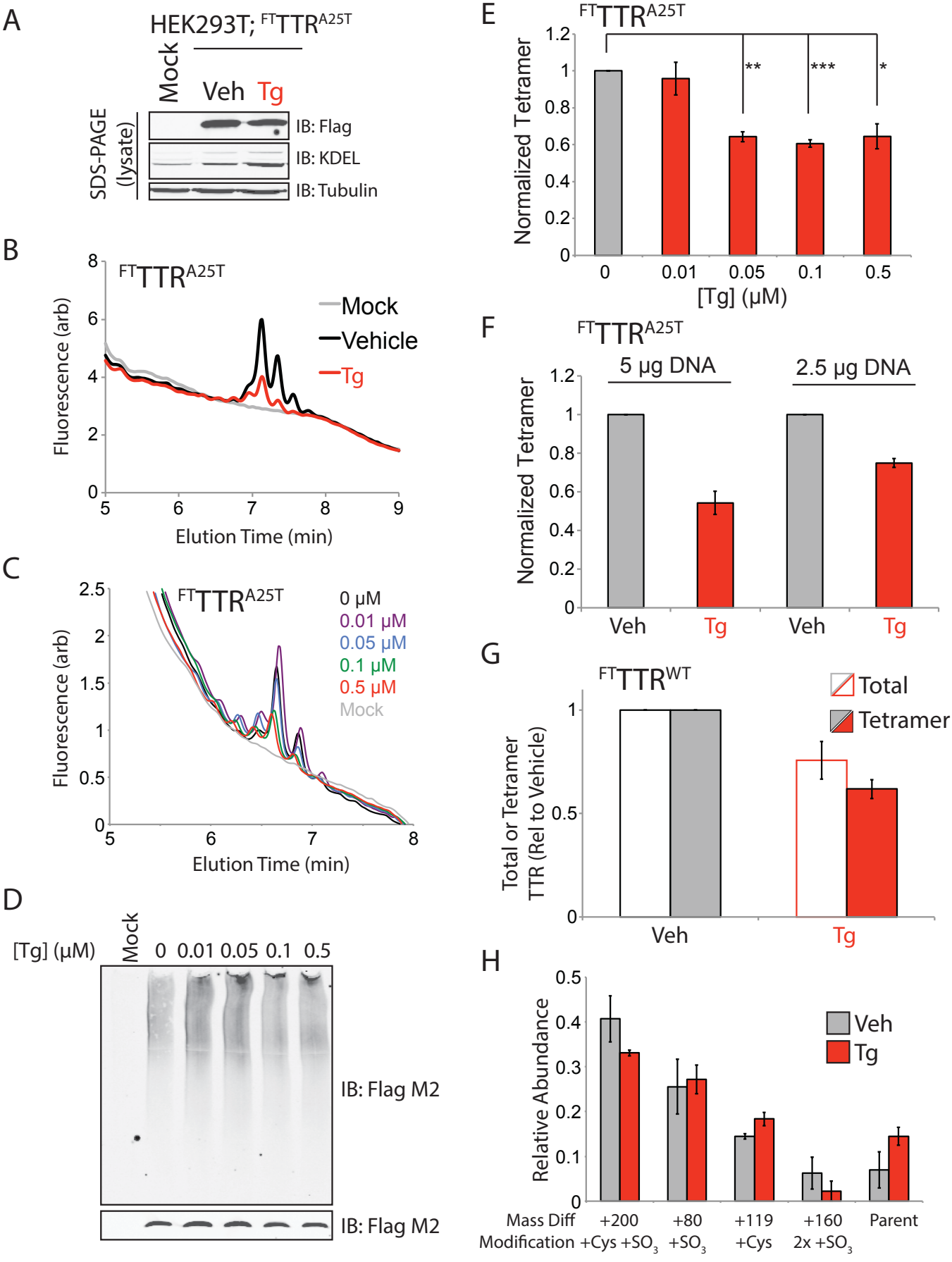

A
HEK293T; FTTTRA25T
Mock
Veh Tg
SDS-PAGE (lysate)
IB: Flag
IB: KDEL
IB: Tubulin
B
FTTTRA25T
Mock
Vehicle
Tg
Fluorescence (arb)
Elution Time (min)
C
FTTTRA25T
0 μM
0.01 μM
0.05 μM
0.1 μM
0.5 μM
Mock
Fluorescence (arb)
Elution Time (min)
D
[Tg] (μM)
Mock
0 0.01 0.05 0.1 0.5
IB: Flag M2
IB: Flag M2
E
FTTTRA25T
Normalized Tetramer
**
***
*
[Tg] (μM)
F
FTTTRA25T
5 μg DNA
2.5 μg DNA
Normalized Tetramer
Veh
Tg
Veh
Tg
G
FTTTRWT
Total
Tetramer
Total or Tetramer TTR (Rel to Vehicle)
Veh
Tg
H
Relative Abundance
Veh
Tg
Mass Diff
Modification
+200 +Cys +SO3
+80 +SO3
+119 +Cys
+160 2x +SO3
Parent

Figure S5 (Supplement to Figure 6)

A. Immunoblot of lysates prepared from HEK293 cells expressing $^{FT}TTR^{A25T}$ treated with vehicle or Tg (0.5 μM). The Tg-dependent increase in BiP is shown in the KDEL immunoblot panel.

B. Representative plot showing the compound **1**-TTR conjugate fluorescence of media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{A25T}$ conditioned in the presence of vehicle (black) or Tg (0.5 μM; red). Media was conditioned the presence of Taf-SO_3^- (10 μM). Compound **1** (10 μM) was added to conditioned media for 16 h prior to analysis by anion exchange chromatography.

C. Representative plot showing the compound **1**-TTR conjugate fluorescence of media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{A25T}$ conditioned in the presence of vehicle (black) or increasing Tg concentration, as indicated. Media was conditioned the presence of Taf-SO_3^- (10 μM). Compound **1** (10 μM) was added to conditioned media for 16 h prior to analysis by anion exchange chromatography.

D. Representative CN-PAGE and SDS-PAGE immunoblot of media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{A25T}$ conditioned in the presence of vehicle (black) or increasing Tg concentration, as indicated. Media was conditioned the presence of Taf-SO_3^- (10 μM).

E. Graph showing normalized TTR tetramers in the conditioned media described in **Fig. S5C,D**. Normalized tetramers were calculated by dividing the relative tetramer levels by the total amount of TTR in each sample measured by SDS-PAGE. This normalization is necessary to account for the reduced total $^{FT}TTR^{A25T}$ levels observed in Tg-treated cells. Error bars show SEM for n=3. *p<0.05, **p<0.01, ***p<0.005.

F. Graph showing normalized TTR tetramers in the conditioned media prepared on HEK293 cells transfected with 5 or 2.5 μg DNA and treated with vehicle (grey) or Tg (0.5 μM; red), as indicated. Normalized tetramers were calculated by dividing the relative tetramer levels by the total amount of TTR in each sample measured by SDS-PAGE. This normalization is necessary to account for the reduced total $^{FT}TTR^{A25T}$ levels observed in Tg-treated cells. Error bars show SEM for n=2.

G. Graph showing total (open bars; measured by SDS-PAGE) or tetrameric (closed bars; measured by integrating compound **1**-TTR conjugate fluorescence from UPLC) TTR in media conditioned for 16 h on HEK293 cells expressing $^{FT}TTR^{WT}$ in the presence of vehicle (grey) or Tg (0.5 μM; red). Media was

conditioned in the presence of Taf-SO_3^- (10 μM). All data are shown relative to vehicle-treated controls. Error bars show SEM for n=6.

H. Graph showing the posttranslational modifications of $^{FT}TTR^{WT}$ immunopurified from media conditioned on HEK293T cells expressing $^{FT}TTR^{WT}$ and treated in the presence of vehicle (grey) or Tg (0.5 μM; red). Modifications of $^{FT}TTR^{WT}$ were determined using the same LC-MS approach described in **Fig. 1E**.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies

The antibodies used for immunoblotting were monoclonal mouse M2 anti-FLAG (Sigma), monoclonal mouse anti-tubulin (Sigma) and polyclonal rabbit anti-TTR antibody (Dako).

Cell Culture, Lysates, Plasmids, and Transfections

HEK293 cells used in this study were purchased from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine, penicillin/streptomycin, and 10% fetal bovine serum, as previously described (Shoulders et al., 2013). All TTR point mutations were incorporated into the previously described $FLAG_2$.TTR.pcDNA3.1 vector through site-directed mutagenesis. Transient transfections of ^{FT}TTR variants were performed by calcium phosphate transfection using established procedures (Shoulders et al., 2013). Whole cell lysates were prepared in Lysis Buffer (20 mM HEPES [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1% Triton X-100 supplemented with EDTA-free protease inhibitors [Roche]). Protein concentration in lysates was normalized using the Bio-Rad protein assay.

Characterization of TTR modifications

$^{FT}TTR^{WT}$ was immunopurified from cell-conditioned media by overnight incubation with protein A resin covalently conjugated to anti-TTR antibody at 4 ^{O}C, as previously described (Sekijima et al., 2003). The beads were washed four times with 10 mM Tris pH 8.0 100 mM NaCl 0.05% saponin and two times with 10 mM Tris pH 8.0 100 mM NaCl. $^{FT}TTR^{WT}$ was then eluted overnight using 100 mM triethylamine pH 11.5 with gentle shaking. For LC-MS analysis, eluates were separated using a 5 µm ID 300 Å pore size C8 RP-HPLC column (Agilent) over a 15%/min linear acetonitrile gradient and analyzed on an 1100 MSD SL mass spectrometer (Agilent). Deconvolution parameters were set to 10 kDa ≤ MW ≤ 20 kDa, q ≤ 50, and minimum 6 peaks for identification.

Recombinant TTR Aggregation Assay

Recombinant TTR^{WT} (7.2 µM tetramer) in 10 mM Tris pH 7.5 100 mM NaCl 1 mM EDTA was incubated for 1 h with the indicated equivalent of Taf-SO_3^- or Taf (1 equivalent = 7.2 µM molecule). The protein was then diluted

1:1 into 50 mM Acetate pH 4.4 100 mM NaCl 1 mM EDTA resulting in a final tetramer concentration of 3.6 μM. The sample was then incubated for 72 h at room temperature. Aggregation was measured by turbidity at 330 and 400 nm, as previously described (Razavi et al., 2003; Sekijima et al., 2005).

SUPPLEMENTAL REFERENCES

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