Supplemental Information

Supplemental Materials and Methods

Expression and purification of TTR in *E. coli.*

Human E54K TTR and E54K monomeric TTR (M-TTR) cDNA sequences were amplified by PCR. The PCR products were then doubly digested with *Nde*I and *Sal*I and ligated with a *Nde*I/*Sal*I predigested pET-22b(+) vector (Novagen). The sequences of the inserted DNA segments were verified by DNA sequencing. E54K TTR and M-TTR were expressed in *Escherichia coli* BL21(DE3) using the pET-22b(+) system. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, when OD_{600nm} of cell culture reached approximately 0.6. Twenty-four hours after induction, cells were harvested by centrifugation. The cell pellets were resuspended in 20 mM phosphate (pH 7.0). After cell lysis by sonication, E54K TTR was detected in a soluble fraction. On the contrary, E54K M-TTR was expressed as inclusion body. The inclusion body was solubilized in a buffer containing 20 mM phosphate (pH 7.0) and 8 M urea. Protein purification was performed by

anion-exchange chromatography and reverse-phase high-performance liquid chromatography.

Circular Dichroism

Circular dichroism (CD) spectra were measured using a Jasco J-805 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The temperature of the measuring cell was maintained at 25°C by circulating water. Quartz cuvettes with path lengths of 1 and 10 mm were used for the far- and near-UV CD measurements, respectively. The buffer solutions contained 50 mM sodium phosphate, 100 mM KCl, and 1 mM ethylenediaminetetraacetic acid (pH 7.0). Protein concentrations of the samples for the CD spectra were 17-40 μM. Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of E = 18450. Samples for the urea-induced unfolding were prepared as described previously (Jiang et al., *Biochemistry*, 2001). Before CD measurements were performed, samples of E54K M-TTR and E54K TTR were incubated in urea at 25°C for 24 h and 96 h, respectively. The protein concentrations of the samples for the urea-induced unfolding were 29-31 μM.

Supplemental Figure Legends

Supplemental Figure 1. Secretion Pattern of wild-type TTR and TTR variants in native PAGE and non-reduced, non-boiled SDS-PAGE.

(A-D) Wild-type TTR, non-amyloidogenic TTRs (A-B) or amyloidogenic TTRs (C-D) were transiently transfected in CHO-K1 cells. Media and cell lysate samples were suspended in native sample buffer and not boiled prior to loading onto gels. Protein samples were analyzed by native PAGE on 5-20% gradient gels (BIO CRAFT, Japan) (A and C) or by non-reduced and non-boiled SDS-PAGE on 14% gels (B and D). Western blots were probed using anti-human TTR antibody (DakoCytomation, Denmark). The brackets or arrowheads indicate the TTR tetramer, dimer and monomer. * indicates wild-type TTR protein purified from human.

Supplemental Figure 2. Structural characterization of E54K TTR and E54K monomeric TTR (E54K M-TTR).

(A) Urea-induced equilibrium unfolding curves of E54K TTR (open squares) and E54K M-TTR (closed squares) measured by the CD ellipticities at 215 nm at pH 7.0 and 25°C.

(B-C) CD spectra of 17-40 μM E54K TTR (open squares) and 17-40 μM E54K M-TTR (closed squares) in (B) far- and (C) near-UV regions (pH 7.0 and 25°C).

Supplemental Figure 3. Secretion pattern of wild-type, non-amyloidogenic and amyloidogenic monomeric TTRs (M-TTRs) in native PAGE and non-reduced, non-boiled SDS-PAGE.

(A-D) Wild-type M-TTR, non-amyloidogenic M-TTRs (A-B) or amyloidogenic M-TTRs (C-D) were transiently transfected in CHO-K1 cells. Media and cell lysate samples were suspended in native sample buffer and not boiled prior to loading onto gels. Protein samples were analyzed by native PAGE on 5-20% gradient gels (BIO CRAFT) (A and C) or by non-reduced and non-boiled SDS-PAGE on 14% gels (B and D). Western blots were probed using anti-human TTR antibody (DakoCytomation). The brackets or arrowheads indicate the TTR tetramer, dimer and monomer. * indicates wild-type TTR protein purified from human.

Supplemental Figure 4. Secretion pattern of TTRs and monomeric TTRs (M-TTRs) in HepG2 cells.

Wild-type, D18G and V30M TTRs and M-TTRs were transiently transfected in HepG2 cells. Cells were maintained for 24 h in serum-free DMEM. Medium and cell lysate samples were reduced and boiled before loading. Samples were analyzed by Western blotting using anti-human TTR or anti-calnexin (CNX) antibodies. The arrowheads indicate the TTR monomer (M). * indicates wild-type TTR protein purified from human.

Supplemental Figure 5. The ER chaperone induction of cells transfected with wild-type and variant TTR constructs.

Hela cells were transfected with empty vector or the indicated TTR constructs. Cell lysates and media were recovered 48 h post-transfection, boiled and subjected to SDS-PAGE under reduced condition. Proteins were probed with anti-human TTR, -KDEL (BiP and GRP94), -PDI and -Actin antibodies. As positive control for ER chaperones induction, cells transfected with empty vector were treated for 16 h with 2 μg/ml tunicamycin 32 h post-transfection (right panels, lane 2).

Supplemental Figure 6. Effect of thyroxine (T_4) on wild-type TTR monomers in

media of various cell lines.

Wild-type TTR was transiently transfected in mammalian cell lines, CHO-K1, HeLa, BHK, and HEK293. Cells were maintained for 24 h in serum-free DMEM containing 0-10 μM T_4. Medium samples were non-reduced and non-boiled (a) or reduced and boiled (b) (left panels) while the cell lysates samples were reduced and boiled (right panels) before loading. Samples were analyzed by Western blotting using anti-human TTR or anti-calnexin (CNX) antibodies. The arrowheads indicate the TTR monomer (M). * indicates wild-type TTR protein purified from human.

Supplemental Figure 7. The dose-dependent effect of thyroxine (T_4) and diflunisal (DF) on the level of TTR monomers in the cell media.

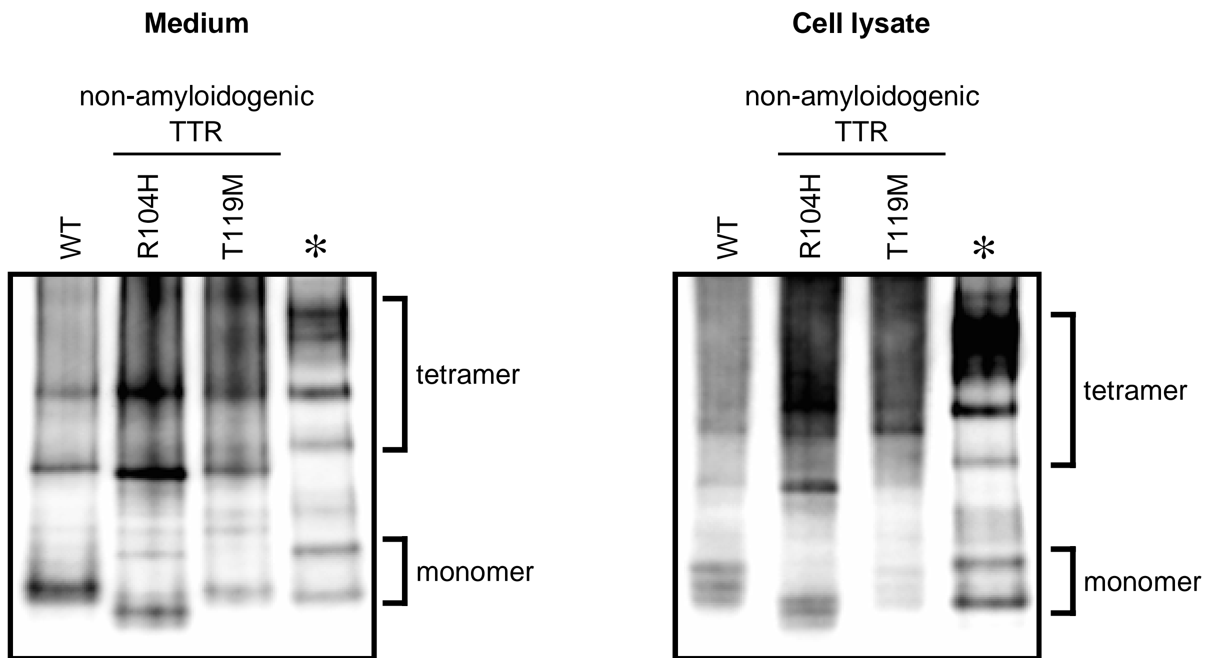
(A-G) Wild-type TTR (A), R104H TTR (B) or amyloidogenic TTRs (C-G) were transiently transfected in CHO-K1. Cells were maintained for 24 h in serum-free DMEM containing 0-10 μM T_4 (left panels) or 0-10 μM DF (right panels). Medium samples, which were non-reduced and non-boiled (a) or reduced and boiled (b), were analyzed by Western blotting. Proteins were probed with anti-human TTR antibodies. The arrowheads indicate the TTR monomer (M). * indicates wild-type TTR protein

purified from human. (A-D: bar graphs) Western blots of secreted TTR monomer were quantified by Image Gauge software (ver. 3.45, Fujifilm) and the ratio of monomer to total TTR in the media was calculated as follows:

Monomer (%) =100 x (non-reduced, non-boiled monomer/ reduced, boiled monomer at given T_4 or DF conc.)/ (non-reduced, non-boiled monomer/ reduced, boiled monomer at T_4 or DF = 0 μM).

Supplemental Figure 1

A



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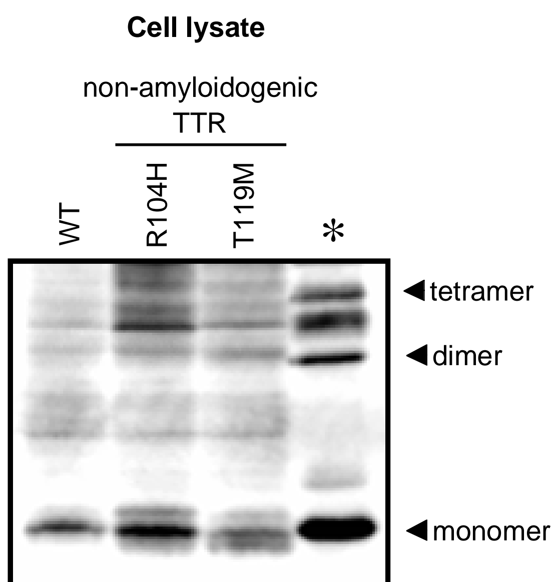
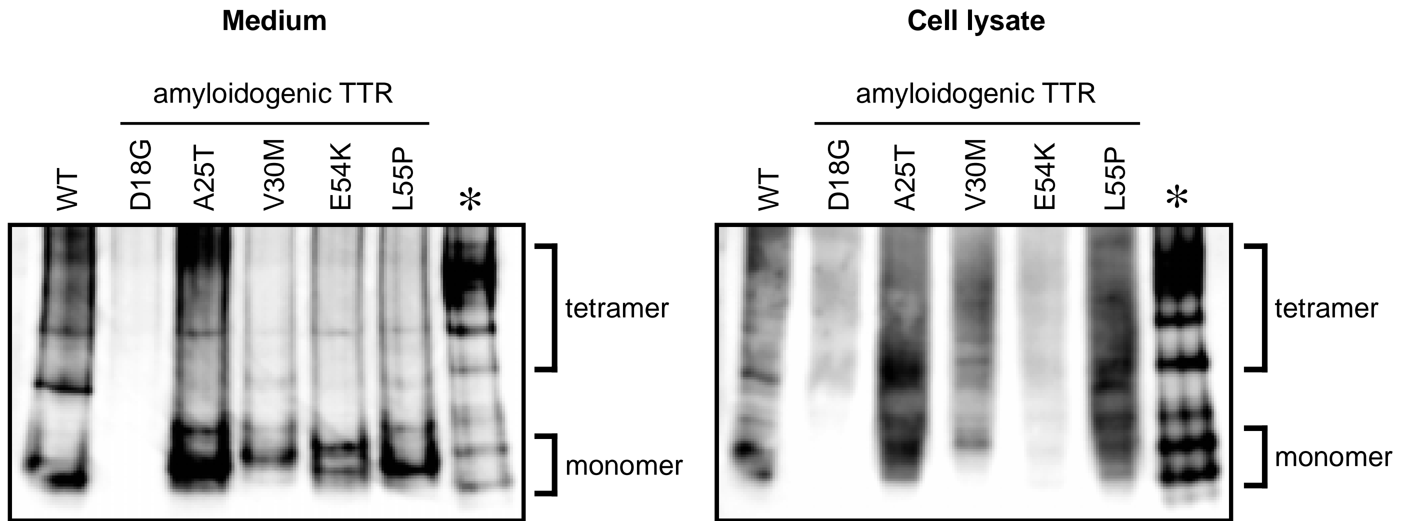


Supplemental Figure 1

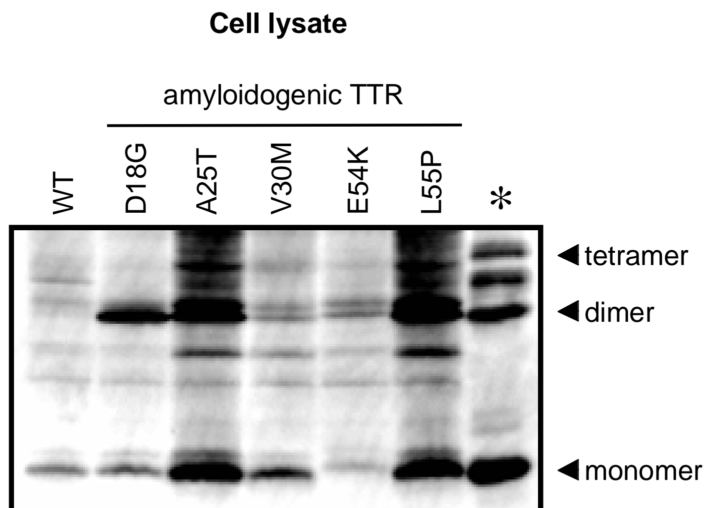
C

Native PAGE

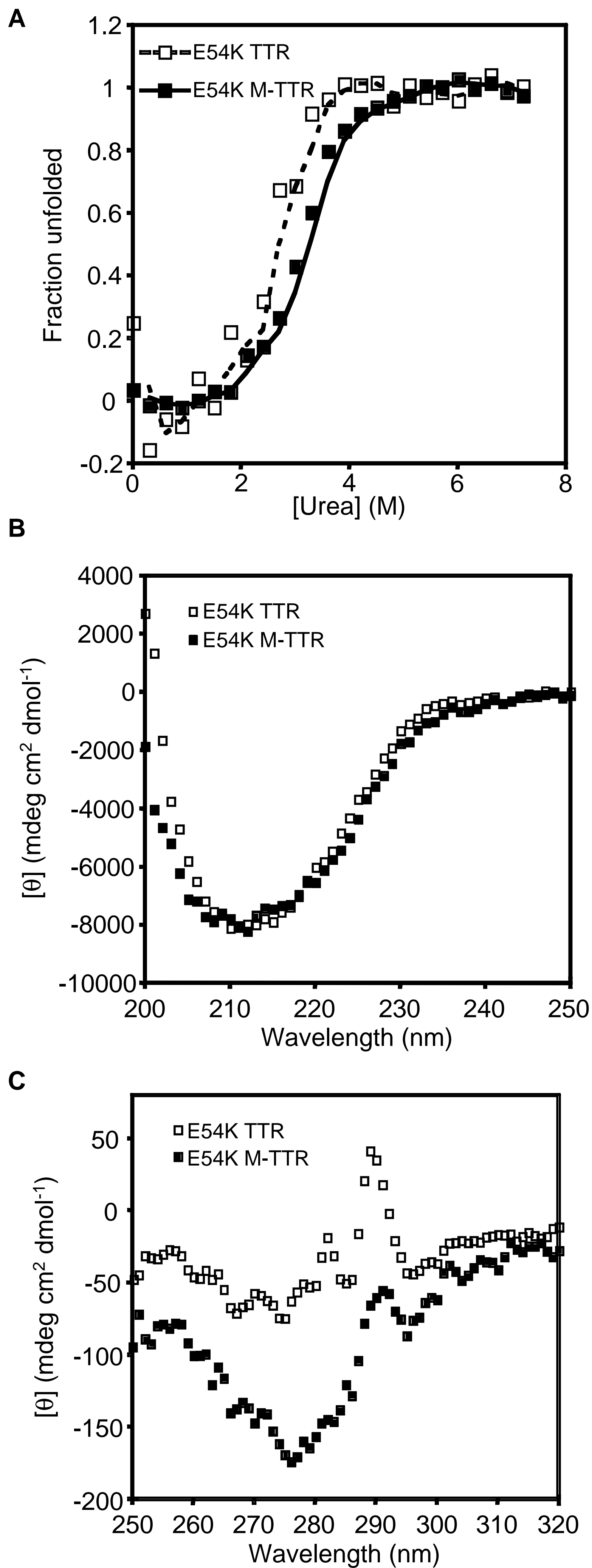


D

SDS-PAGE (non-reduced and non-boiled)



Supplemental Figure 2



Supplemental Figure 3

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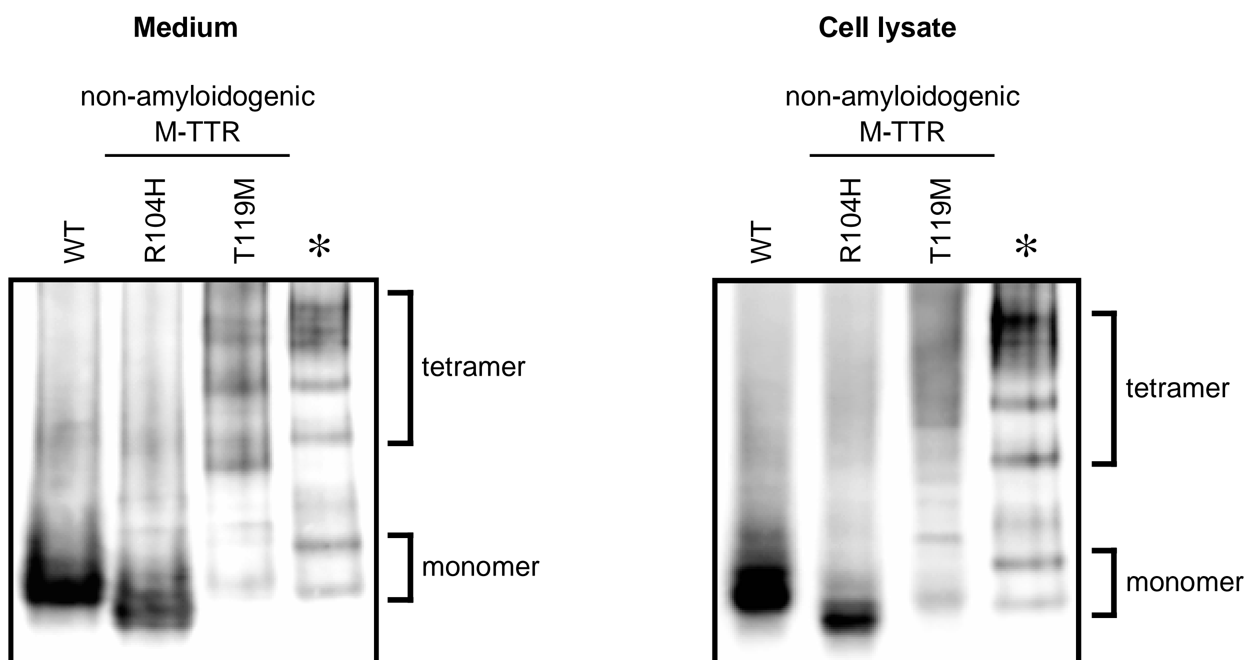

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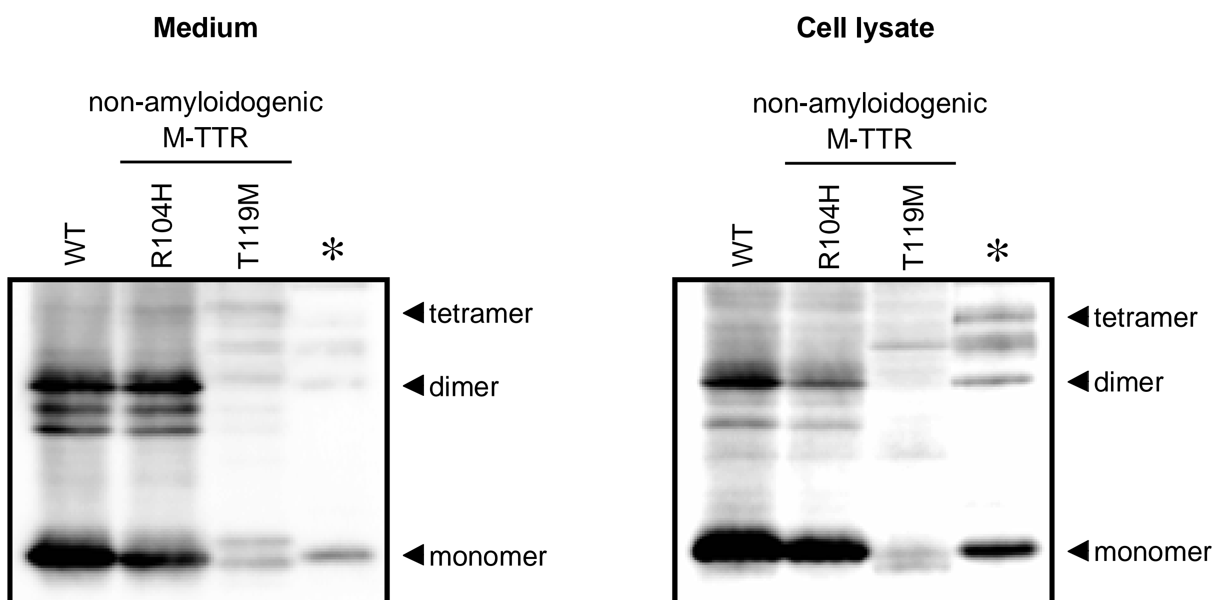
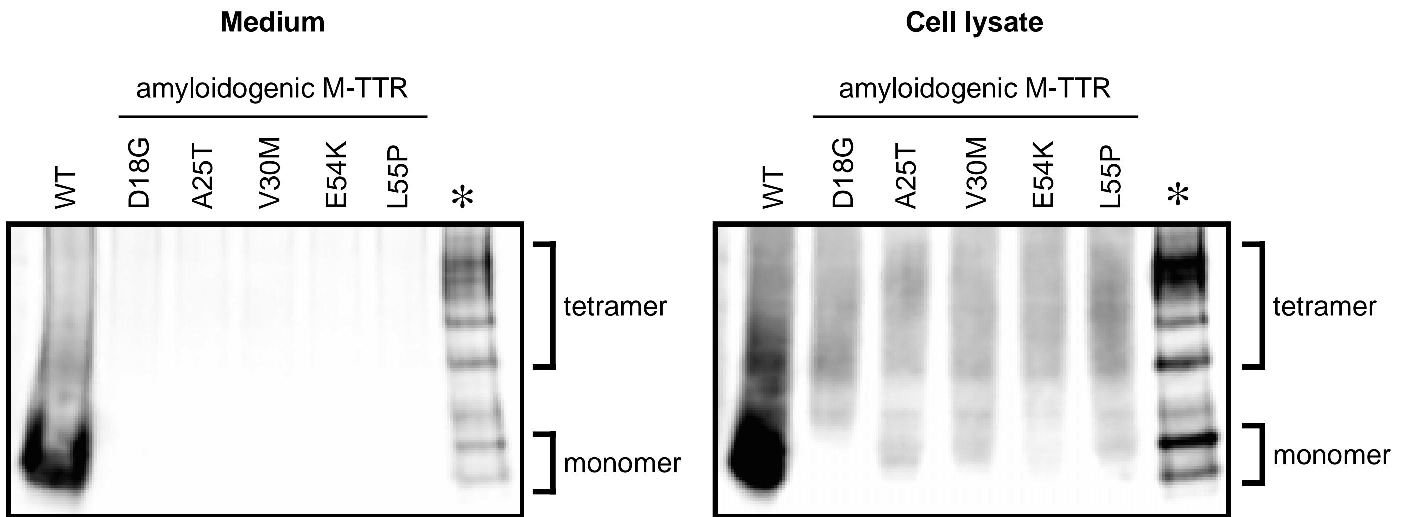


Supplemental Figure 3

C



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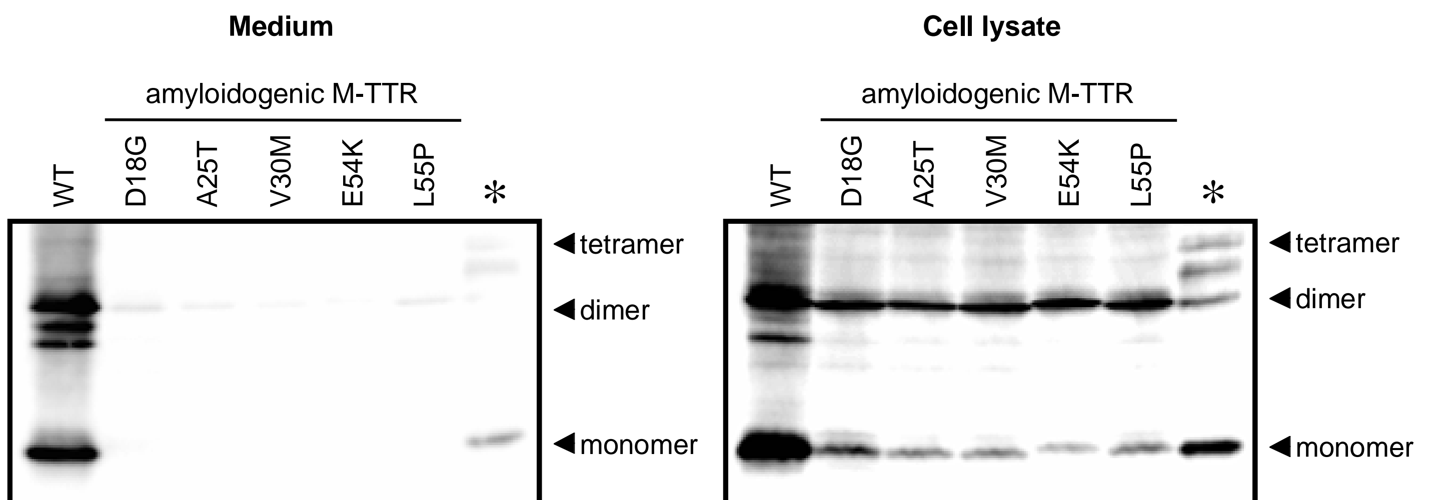
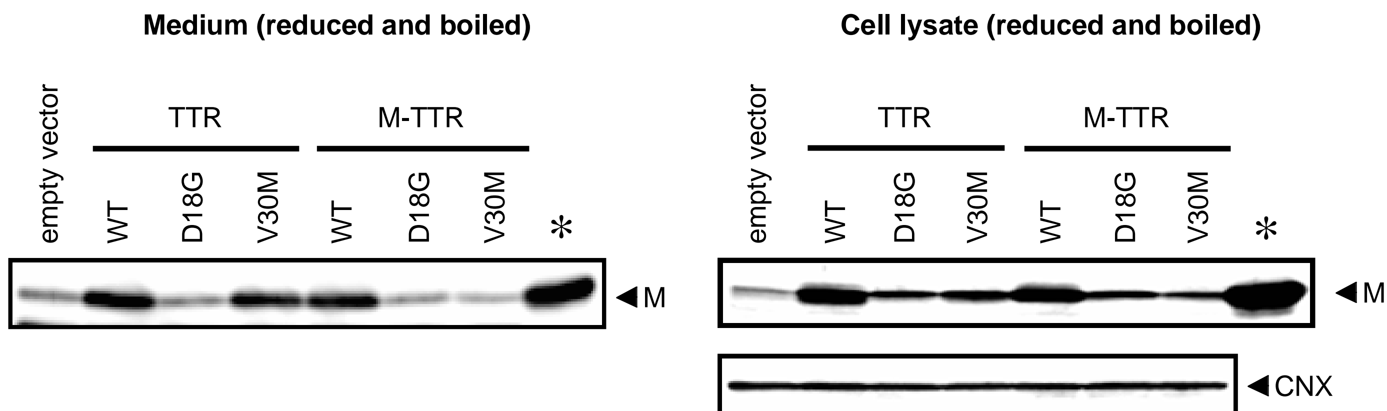
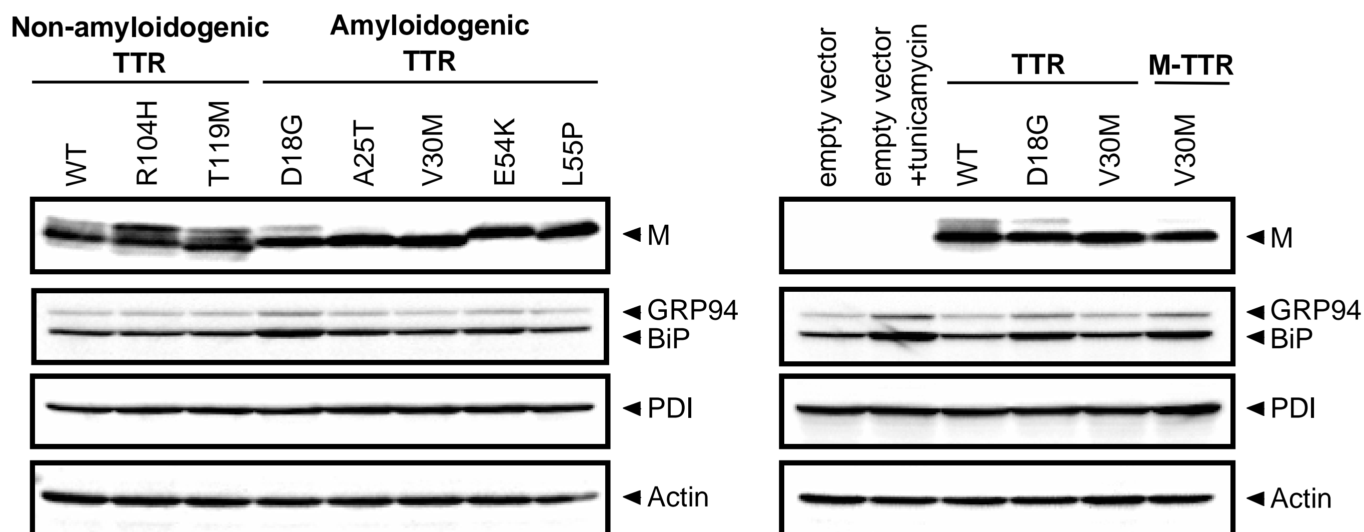
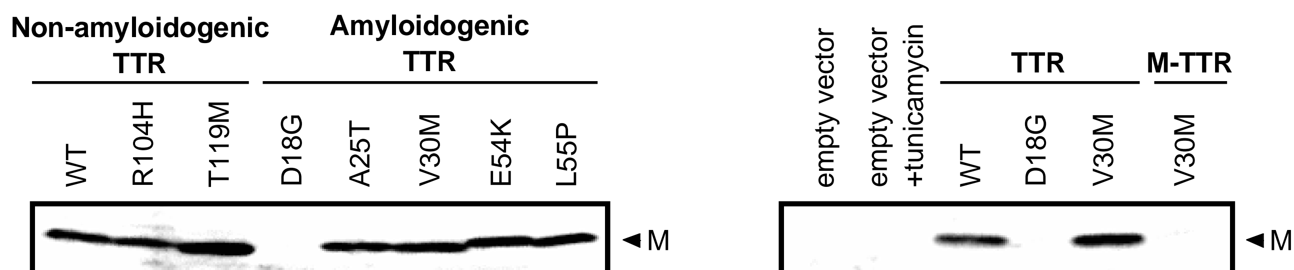


Supplemental Figure 4

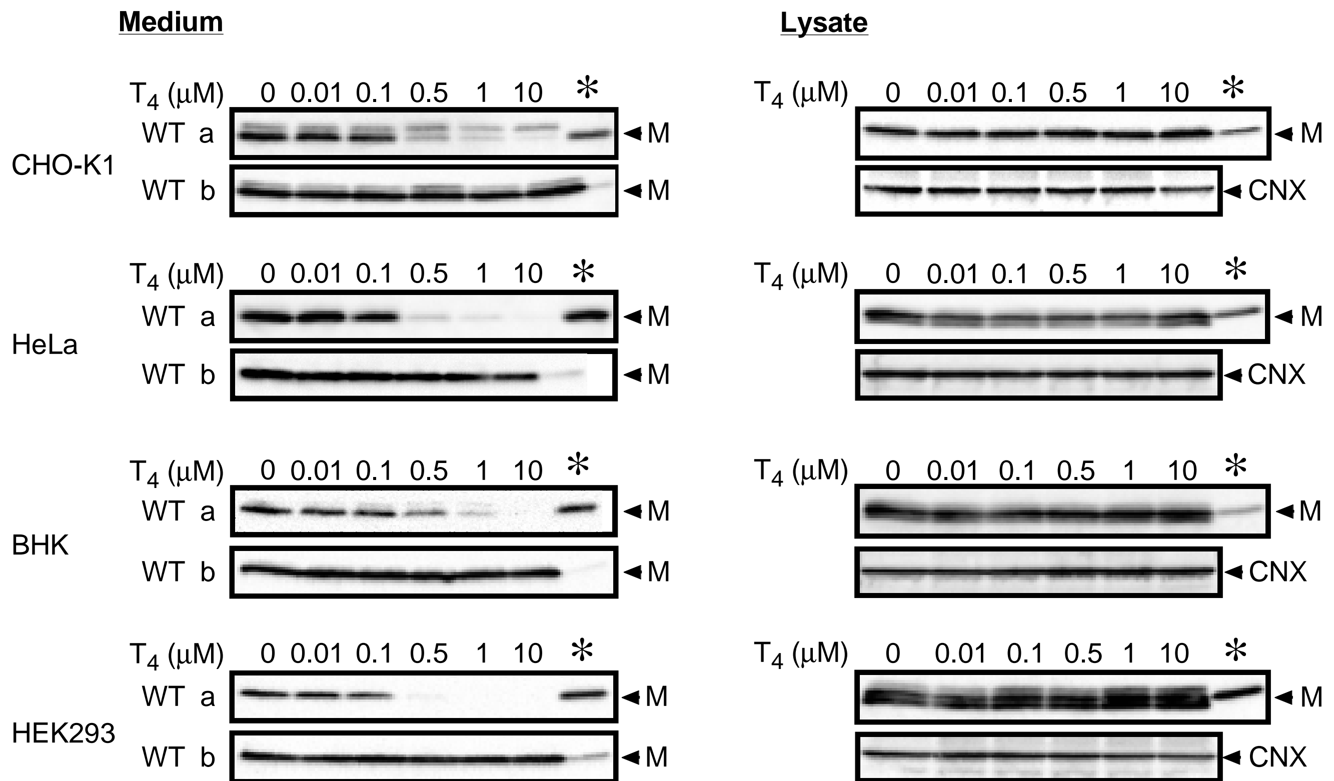


Supplemental Figure 5



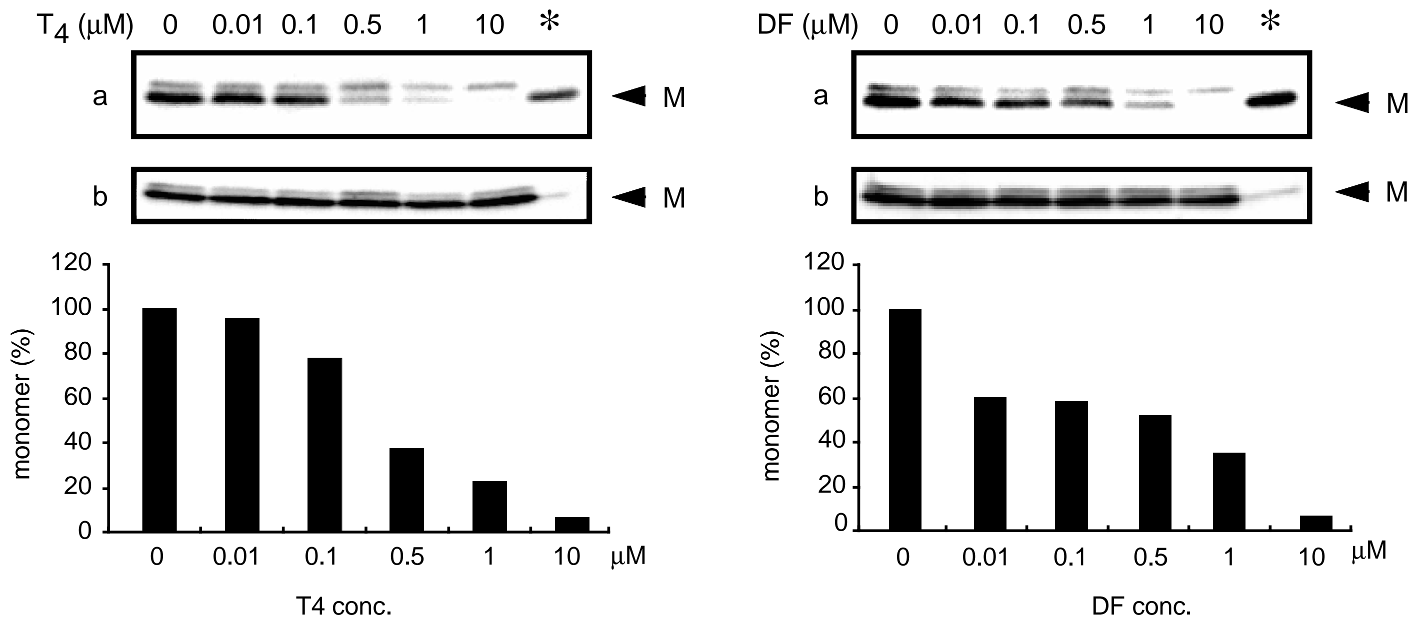


Supplemental Figure 6



Supplemental Figure 7

A



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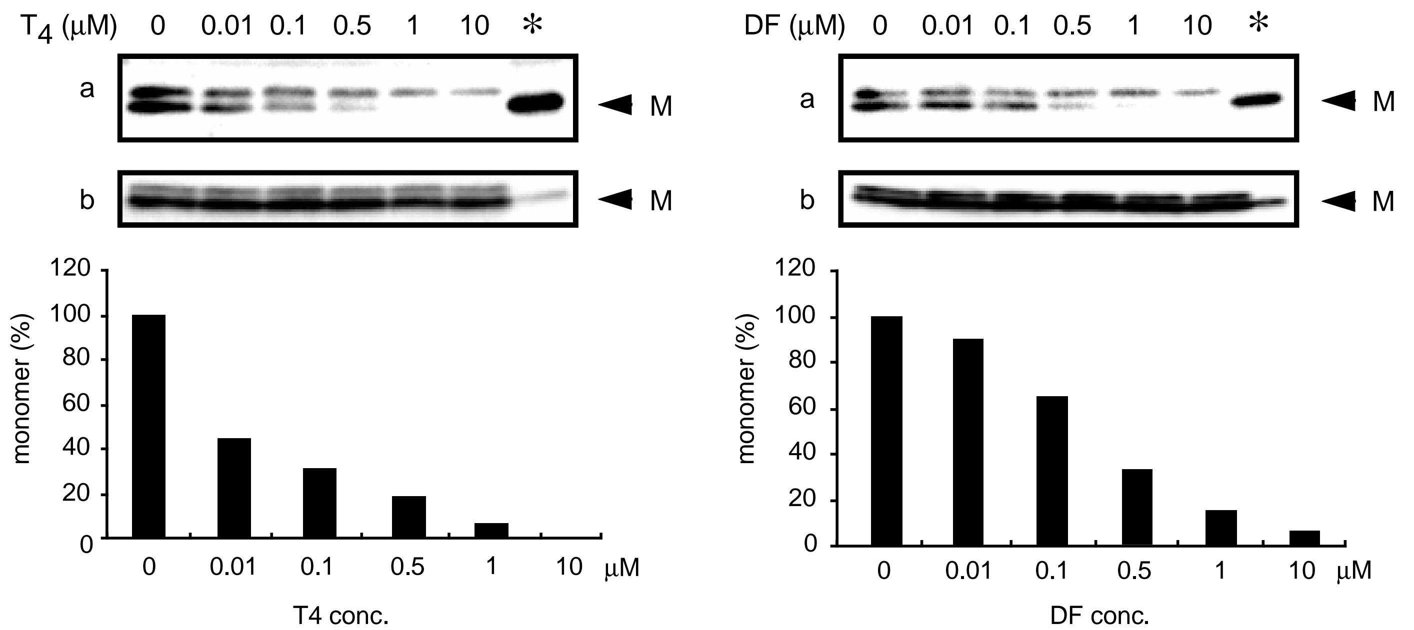

Supplemental Figure 7

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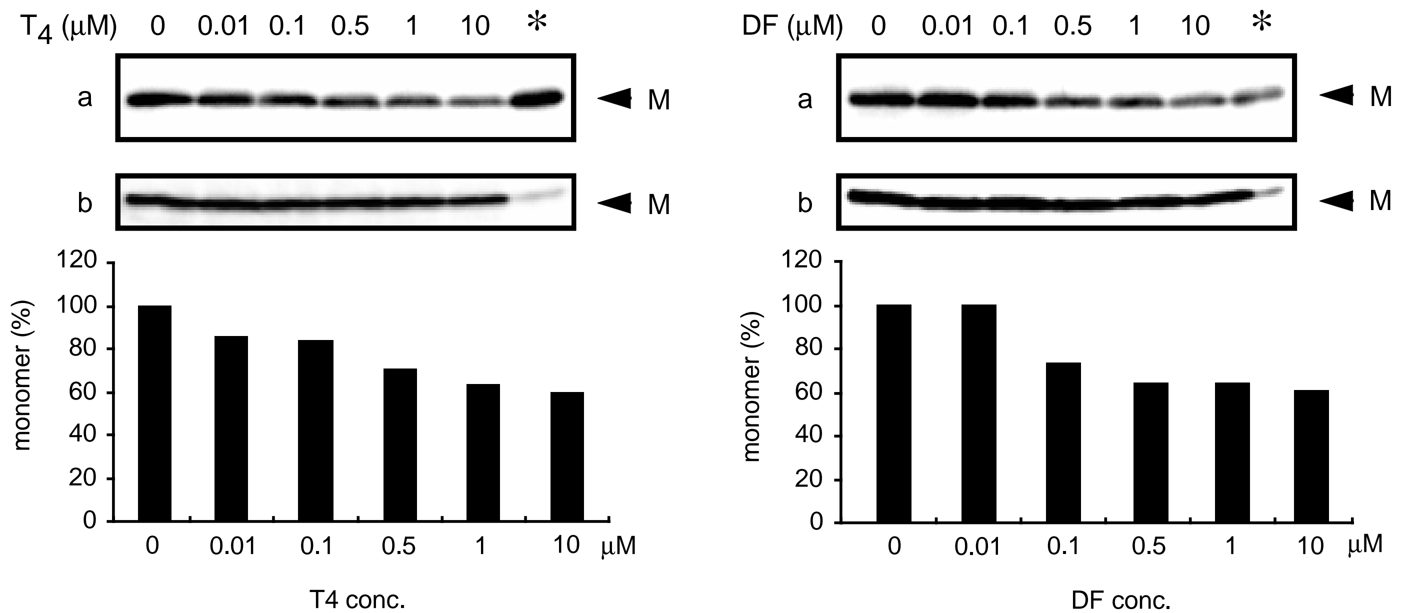

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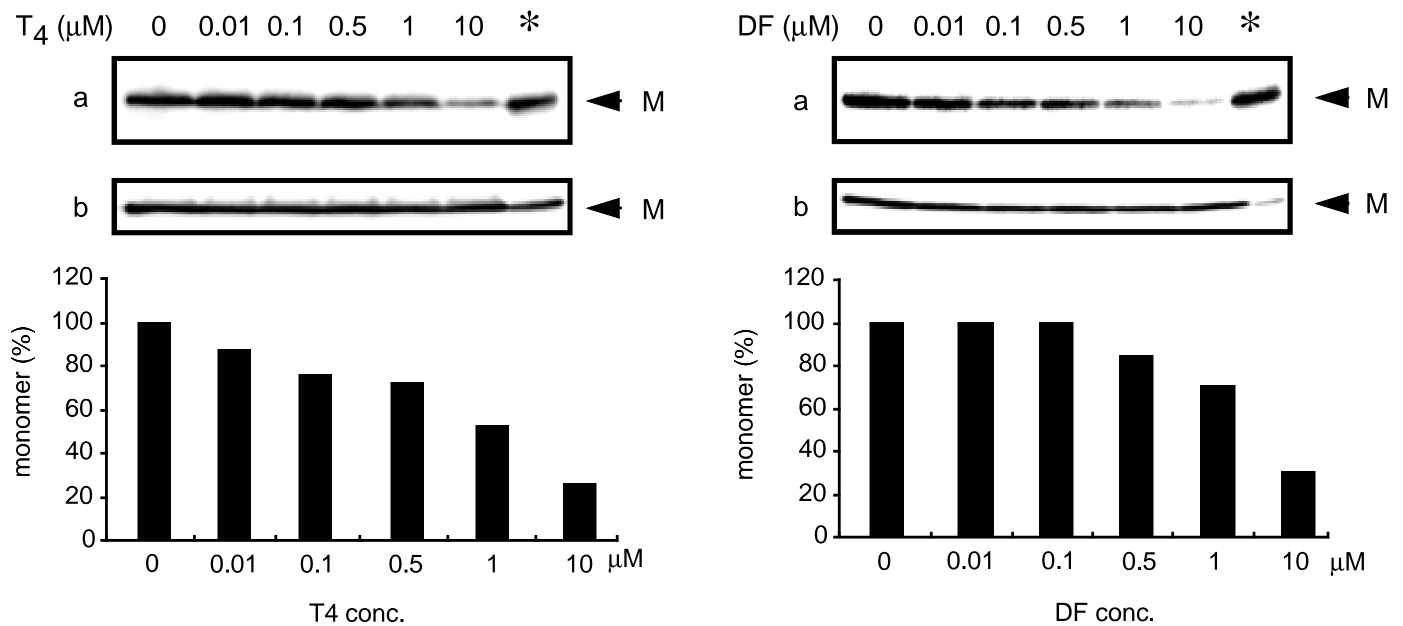
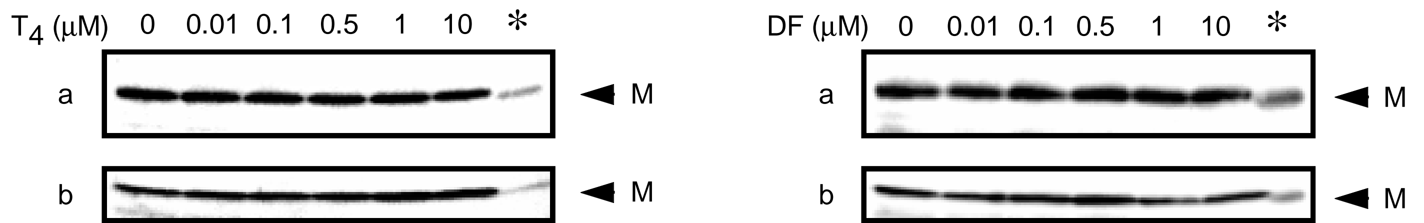


Supplemental Figure 7

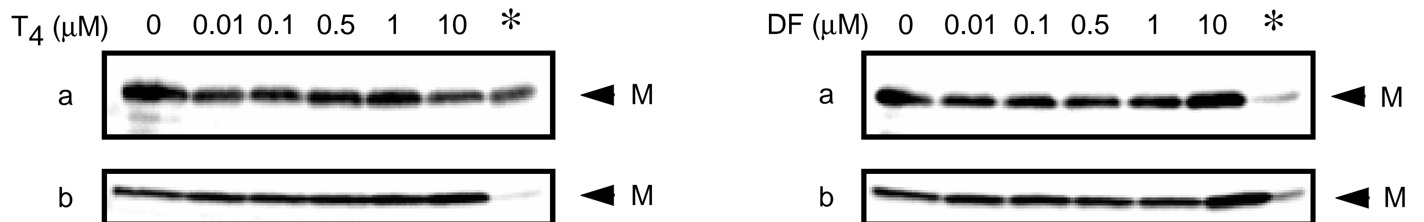
E

E54K TTR



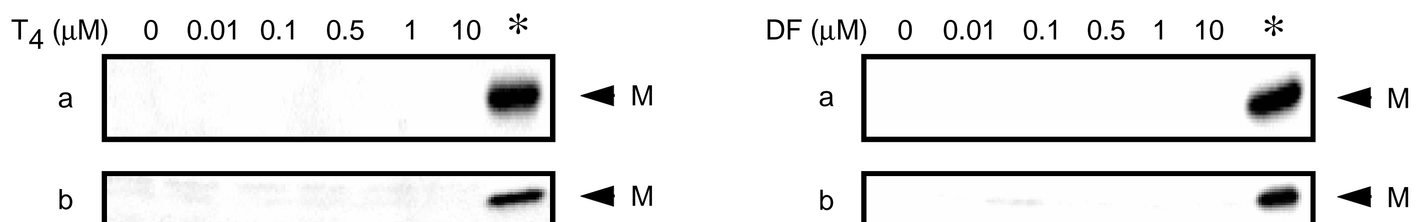
F

A25T TTR



G

D18G TTR



Supplemental Table 1

TTR sequences and states after recombinant overexpression in E.coli

TTR sequence	state	TTR sequence	state
WT TTR	Soluble[1]	WT M-TTR	Soluble[1]
R104H TTR	Soluble[2]	R104H M-TTR	Soluble[4]
T119M TTR	Soluble[1]	T119M M-TTR	Soluble[1]
D18G TTR	Inclusion bodies[1]	D18G M-TTR	Inclusion bodies[4]
A25T TTR	Soluble[3]	A25T M-TTR	Inclusion bodies[4]
V30M TTR	Soluble[1]	V30M M-TTR	Inclusion bodies[1]
E54K TTR	Soluble[4]	E54K M-TTR	Inclusion bodies[4]
L55P TTR	Soluble[1]	L55P M-TTR	Inclusion bodies[1]

[1]P. Hammarstrom, et. al., Biochemistry (2003)
[2]Y. Sekijima, et. al., Amyloid (2006)
[3]Y. Sekijima, et. al., Lab. Invest. (2003)
[4]Sato, et. al., present study

Supplemental Table 2

The ΔG and m-values of urea-induced unfolded curves of E54K TTR and E54K M-TTR

	ΔG (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})
E54K TTR	4.55 ± 0.17	1.76 ± 0.42
E54K M-TTR	4.17 ± 0.77	1.35 ± 0.13