High capacity of the endoplasmic reticulum to prevent secretion and aggregation of amyloidogenic proteins

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Appendix

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Supplementary Materials and Methods

Immunoblotting

Cells were harvested after one wash in PBS and lysed in either RIPA buffer (for denaturing conditions, 25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) or using the NativePAGE Sample Prep Kit (for native conditions, Thermo Fisher Scientific, BN2008) each supplemented with cOmplete Protease Inhibitor Cocktail (Roche) and benzonase. Protein concentrations were measured using a BCA assay (Pierce). Lysates were denatured in Laemmli sample buffer at 70 °C for 10 min. 20 μg of protein were resolved on 8% or 12% Bis-Tris gels (denaturing) or on 4-16% NativePAGE Novex Bis-Tris gels (Thermo Fisher Scientific, BN2008) and transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes in 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS)/methanol. To control for loading of proteins from conditioned media, PVDF membranes were stained using the Pierce™ Reversible Protein Stain Kit (Thermo Fisher Scientific). Membranes were blocked for 1 h in 5% low fat dry milk in TBS-T and then incubated with primary antibodies diluted in 5% low fat dry milk at 4 °C overnight. The following primary antibodies were used for immunoblotting at the indicated dilutions: BiP/GRP78 (1:1,000, Abcam #ab21685), calnexin (1:500, Enzo Life Sciences, #SPA860), Erlin-2/SPFH2 (1:1,000, Abcam, ab128924), GAPDH (1:1,000, Millipore #MAB374), GFP (1:1,000, Roche, #11814460001), GRP94 (1:1,000, Pierce, #MA3-016), HYOU1 (1:1,000, Abcam #ab134944), mCherry (1:1,000, Life Technologies, #M11217), Myc (1:200, mouse monoclonal produced in hybridoma cell line Myc-9E10), OS-9 (1:1,000, Abcam, #ab109510), PDIA6 (1:500, Abcam #ab154820), SEL1L (1:1,000, Sigma #S3699), α-tubulin (1:1,000, Sigma #T5168). Membranes were washed three times with TBS-T and then incubated

with HRP-conjugated secondary antibodies diluted in TBS-T (anti-mouse, 1:5,000, Sigma #A4416; anti-rat, 1:2,000, Sigma #A9037) or 5% milk (anti-rabbit, 1:20,000, Sigma #A9169) for 1.5 h at RT. After additional washing in TBS-T HRP substrate was added and chemiluminescence was quantified using a LAS 3000 image reader (Fujifilm).

SILAC and Sample Preparation for MS Analysis

SILAC media for light (L) or heavy (H) labeling were prepared as follows: DMEM with 4.5 g/L glucose and without arginine, lysine, and glutamine (PAA Laboratories #E15-086) was supplemented with 10% dialyzed FBS, Pen/Strep and stable glutamine (dipeptide Gln-Ala) (PAA Laboratories). Combinations of different isotopes of arginine (0.28 mM) and lysine (0.56 mM) were added, to L: Arg0 and Lys0 (Sigma) and to H: Arg10 and Lys8 (Cambridge Isotope Laboratories). HEK293T cells were cultured in either L or H media for at least 5 passages and efficient incorporation of amino acid isotopes was confirmed by MS.

SILAC-labeled HEK293T cells were transfected by lipofection with Fugene 6 (L: pcDNA3.1 and H: ER-β), harvested 48 h later and lysed in 1% Triton X-100 in PBS supplemented with cOmplete Protease Inhibitor Cocktail (Roche) and benzonase. After 1 h of end-over-end rotation at 4 °C, cell debris was removed by centrifugation at 2,000 xg for 5 min at 4 °C and lysates were transferred to fresh tubes. 1.5 mg protein (in a total of 1 ml) were incubated with 50 µl µMacs anti-Myc beads (Miltenyi Biotec # 130-091-123) at 4 °C on an end-over-end rotor overnight. Samples were then applied to µMACS 20µ columns that were equilibrated with 200 µl lysis buffer on a magnetic rack. Columns were washed once with 200 µl lysis buffer and then three times with 200 µl 0.1% Triton X-100/PBS and once with PBS. Proteins were eluted by addition of 70 µl hot sample buffer. Eluates from H and L samples were mixed 1:1 before separation on a NuPAGE Bis-Tris gradient gel (Invitrogen) according to the manufacturer's instructions. For

analysis of total protein, input lysates were directly mixed (1:1) and separated on a gradient gel. Gels were sliced, and proteins were reduced, alkylated, and digested within the gel as described (Ong & Mann, 2006). For analysis of total amounts of ER-β-mCh in cell lysates, cells were transfected as above. Cells were lysed in RIPA buffer and proteins were reduced with 1 mM DTT and denatured in 2% SDS at 96 °C for 5 min. Proteins were digested using the filter-aided sample preparation (FASP) method (Wisniewski et al., 2011) and fractionated using SAX microcolumns. After extraction, peptides were desalted using homemade columns containing C18 Empore disks or commercial OMIX96 C18 tips (Agilent Technologies). Peptides were eluted with 1% formic acid in 70% acetonitrile (ACN) and dried in a vacuum concentrator.

LC-MS/MS

Peptides eluted from desalting tips were dissolved in 5% (vol/vol) formic acid and sonicated for 5 min. Samples were analyzed on a Proxeon EASY-nLC 1000 nano-HPLC system (Thermo) coupled to a Q-Exactive orbitrap mass spectrometer (Thermo). Peptides were separated on spray-columns (ID 75 µm, 30 cm long, 8 µm tip opening, NewObjective) packed with 1.9 µm C18 particles (Reprosil-Pur C18-AQ, Dr Maisch GmbH) using a 2 h linear gradient between 5 % solvent A (0.2 % formic acid in water) and 30 % solvent B (0.2 % formic acid in ACN). Samples were loaded on the column by the nano-HPLC autosampler at a flow rate of 0.5 µL per minute. No trap column was used. The HPLC flow rate was set to 0.25 µL per minute during analysis. MS/MS analysis was performed with standard settings using cycles of 1 high resolution (70000 FWHM) MS scan followed by 10 MS/MS scans of the most intense ions with charge states of 2 or higher at a resolution setting of 17500 (FWHM).

Analysis of MS data

Protein identification and SILAC-based quantitation was performed with MaxQuant (version 1.3.0.5) (Tyanova et al., 2014) using default settings. The human sequences of UNIPROT (version 2012-06-14) were selected as the database for protein identification. MaxQuant used a decoy version of the specified UNIPROT database to adjust the false discovery rates for proteins and peptides below 1%. The protein OS-9 was identified in one replicate with SILAC ratios of 6.57 but could not be quantified by the MaxQuant algorithm in two other experimental replicates. However, this protein was also enriched in these replicates as judged based on the extracted ion intensities of light and heavy labeled peptides, which was confirmed by visual inspection of the mass spectra. OS-9 was thus included in the interactor set. Normalized ratios (H/L) and combined ratios (H/L) from three independent experiments as calculated by MaxQuant were used for analysis. Identified interactors were further analyzed using Perseus (1.5.2.12). For the label-free analysis of the proteome from ER-β-mCh-expressing cells, three independently prepared and measured samples were analyzed by MaxQuant. The percentage of transfected cells was calculated using fluorescence-activated cell sorting (FACS). Transfected cells were detached using TrypLE Express (Life Technologies) 48 h after transfection followed by suspension in PBS. Cells were kept on ice until analysis. Cells were analyzed using a BD FACSCalibur. FACS data were analyzed using the FlowJo software (Version 9.9). The fraction of mCherry-positive cells was calculated by generating a gate including all mCherry-positive cells and excluding the untransfected control cells.

Supplementary References

Tyanova S, Mann M, Cox J (2014) MaxQuant for in-depth analysis of large SILAC datasets. Methods Mol Biol 1188: 351-64

Wisniewski JR, Zielinska DF, Mann M (2011) Comparison of ultrafiltration units for proteomic and N-glycoproteomic analysis by the filter-aided sample preparation method. Analytical biochemistry 410: 307-9

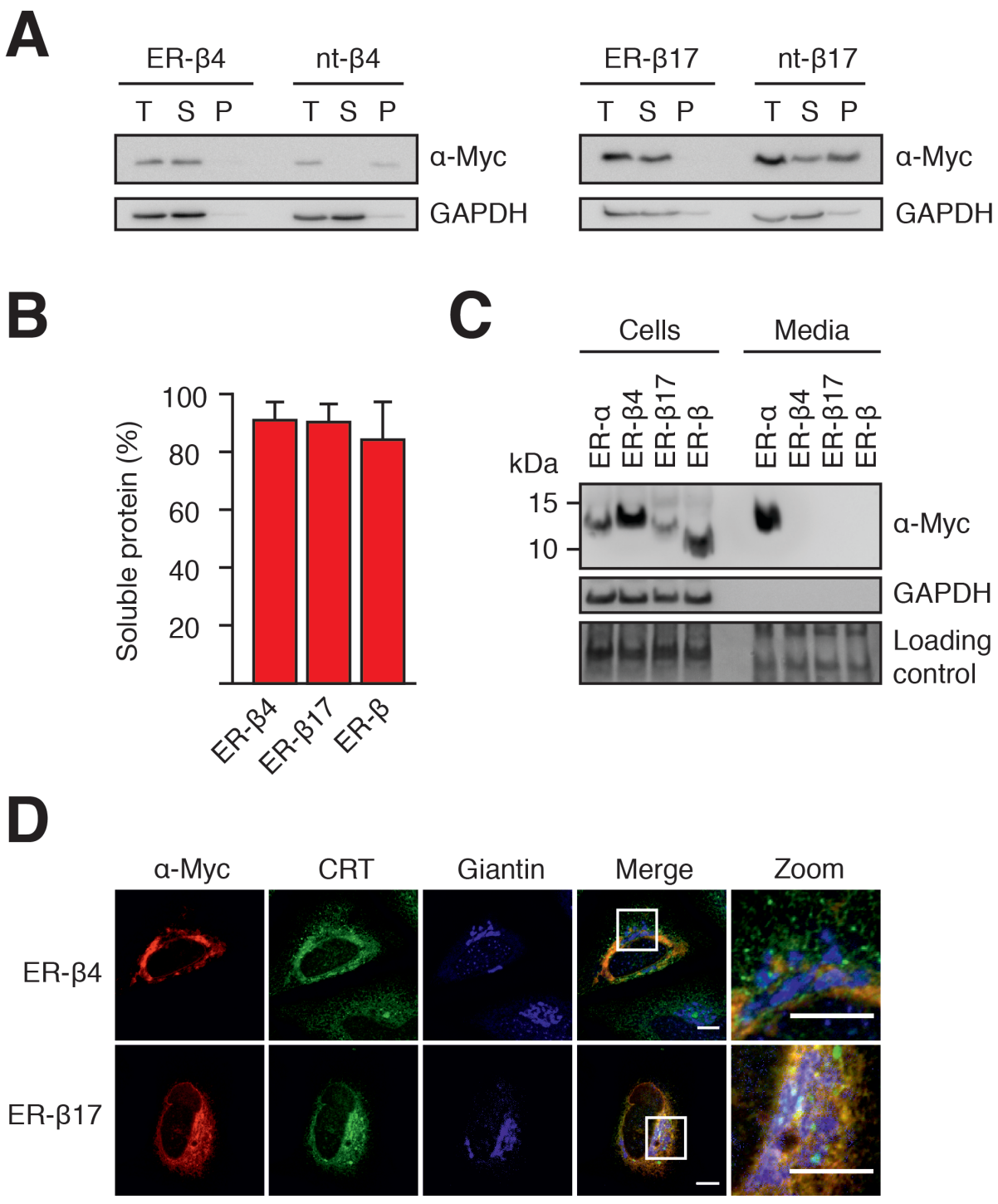

Figure S1 - ER-targeted β-proteins ER-β4 and ER-β17 are also more soluble than their cytosolic counterparts and are retained in the ER.

A Solubility of ER-β4, ER-β17 and their non-targeted (nt) counterparts was analyzed 48 h after transfection by fractionation of lysates and immunoblotting with anti-Myc antibody. T: total lysate, S: soluble fraction, P: pellet fraction.

B Comaprison of soluble fraction of ER-β4, ER-β17 and ER-β in A and Fig. 2C. Error bars represent SD from three independent experiments.

C HEK293T cells were transfected with ER-α, ER-β4, ER-β17 or ER-β. Cells and media were collected separately. Proteins from media samples were concentrated by TCA precipitation and equal fractions of total protein from media and cell samples were analyzed by immunoblotting. The PVDF membrane was stained to provide a loading control for media samples. Protein levels were analyzed by immunoblotting with anti-Myc antibody. GAPDH served as a loading control and to demonstrate absence of cell leakage.

D Transfected HeLa cells were fixed and stained with anti-Myc (red), anti-calreticulin (green) and anti-giantin (blue) antibodies, followed by fluorescently labeled secondary antibodies and analysis by confocal microscopy. Scale bars represent 10 μm.

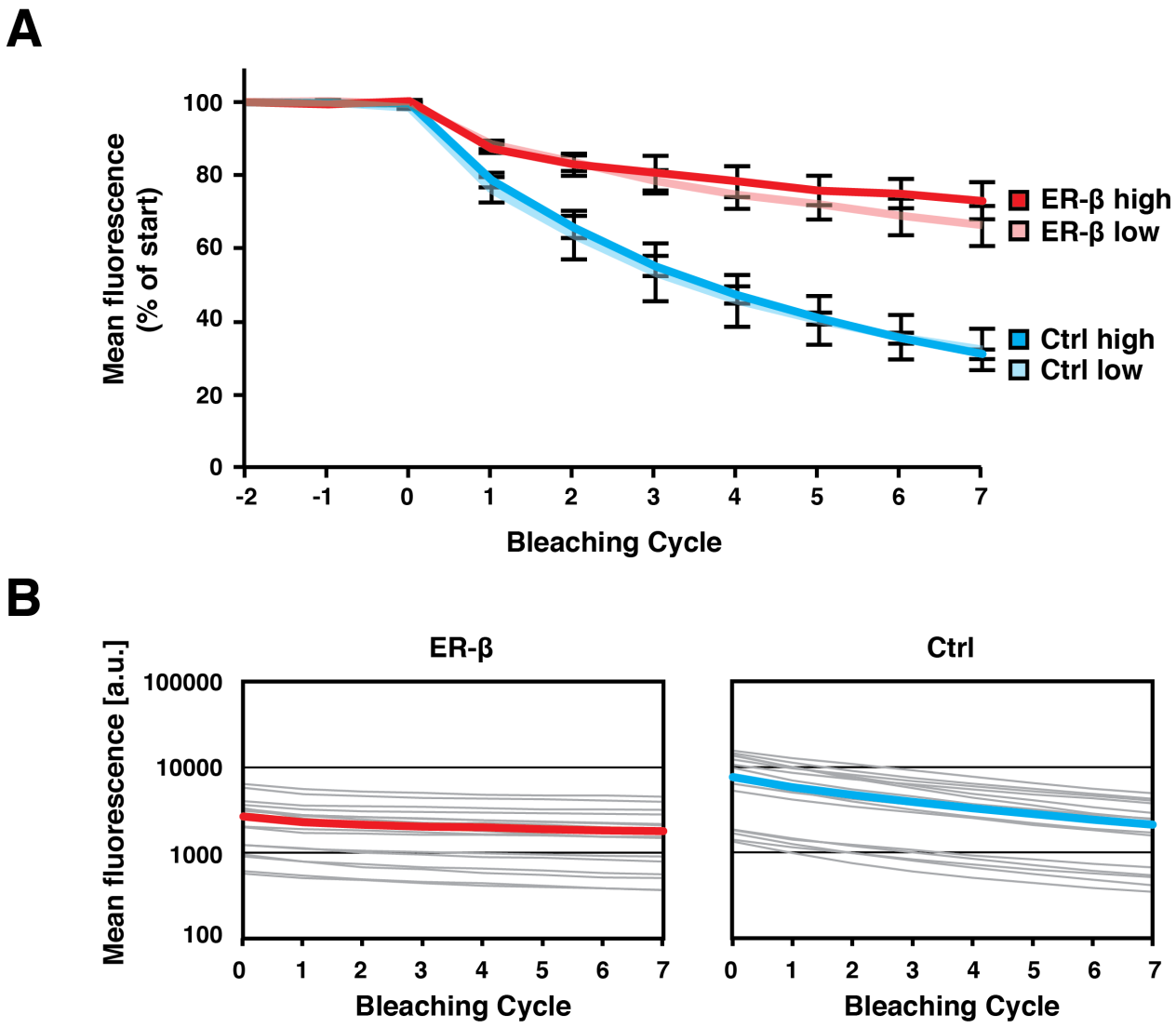

Figure S2 – ER-β-mCh mobility is not significantly affected by expression levels

A Fluorescence loss in photobleaching (FLIP) was performed in HEK293T cells 48 h after transfection with ER-β-mCh or ER-mCh (Ctrl). After recording three images by confocal microscopy, small areas within the ER of cells (bleaching region) were repeatedly bleached using a 561 nm laser and images were recorded after each bleaching cycle (every approx. 22 s). Relative changes in mean fluorescence of total cells were plotted for the cells with the highest

and lowest measured signal intensity of ER-mCh (Ctrl) and ER-β-mCh in each experiment. Error bars represent SD from three independent experiments.

B Mean fluorescence of all individual cells transfected with ER-β-mCh (left panel) or ER-mCh (Ctrl) that were analyzed in (A). The average decay is indicated in bold.

Name	Amino acid sequence	Source
ER-α	MAESHLLQWLLLLLPTLCGPGTACEQKLISEEDLGMYGKLNDLLEDLQEVLKNLHKNWHGGKDNL HDVDNHLQNVIEDIHDFMQGGGSGGKLQEMMKEFQQVLDELNNHLQGGKHTVHHIEQNIKEIFHH LEELVHR	Dolfe et al. 2015
ER-β	MAESHLLQWLLLLLPTLCGPGTACEQKLISEEDLGMQISMDYNIQFHNNGNEIQFEIDDSGGDIEIEI RGPGGRVHIQLNDGHGHIKVDFHNDGGELQIDMH	Dolfe et al. 2015
ER-β4	MAESHLLQWLLLLLPTLCGPGTACEQKLISEEDLGMQISMDYQLEIEGNDNKVELQLNDSGGEVKL QIRGPGGRVHFNVHSSGSNLEVNFNNDGGEVQFHMH	Dolfe et al. 2015
ER-β17	MAESHLLQWLLLLLPTLCGPGTACEQKLISEEDLGMQISMDYEIKFHGDGDNFDLNLDDSGGDLQL QIRGPGGRVHVHIHSSSGKVDFHVNNDGGDVEVKMH	Dolfe et al. 2015
ER-α-mCh	MAESHLLQWLLLLLPTLCGPGTACEQKLISEEDLGMYGKLNDLLEDLQEVLKNLHKNWHGGKDNL HDVDNHLQNVIEDIHDFMQGGGSGGKLQEMMKEFQQVLDELNNHLQGGKHTVHHIEQNIKEIFHH LEELVHRGPVATMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKV TKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVTVTQDSSL QDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEV KTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK	This study
ER-β-mCh	MAESHLLQWLLLLLPTLCGPGTAA*EQKLISEEDLGMQISMDYNIQFHNNGNEIQFEIDDSGGDIEIEI RGPGGRVHIQLNDGHGHIKVDFHNDGGELQIDMHGPVATMVSKGEEDNMAIIKEFMRFKVHMEG SVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLS FPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSER MYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYE RAEGRHSTGGMDELYK	This study
ER-mCh	MAESHLLQWLLLLLPTLCGPGTAAEQKLISEEDLMVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEF EIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFK WERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPEDG ALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHS TGGMDELYKKDEL	This study
nt-α	MCEQKLISEEDLGMYGKLNDLLEDLQEVLKNLHKNWHGGKDNLHDVDNHLQNVIEDIHDFMQGG GSGGKLQEMMKEFQQVLDELNNHLQGGKHTVHHIEQNIKEIFHHLEELVHR	Olzscha et al. 2011
nt-β	MCEQKLISEEDLGMQISMDYNIQFHNNGNEIQFEIDDSGGDIEIEIRGPGGRVHIQLNDGHGHIKVD FHNDGGELQIDMH	Olzscha et al. 2011
nt-β4	MCEQKLISEEDLGMQISMDYQLEIEGNDNKVELQLNDSGGEVKLQIRGPGGRVHFNVHSSGSNLE VNFNNDGGEVQFHMH	Olzscha et al. 2011
nt-β17	MCEQKLISEEDLGMQISMDYEIKFHGDGDNFDLNLDDSGGDLQLQIRGPGGRVHVHIHSSSGKVD FHVNNDGGDVEVKMH	Olzscha et al. 2011

*In Movie 1 and Figure EV2 A,B an ER-β-mCh construct was used that contained C in position 24 to match ER-α-mCh.

Supplementary Table S1. Sequences of model α- and β-proteins.