Supplemental Material

Static retention of the lumenal monotopic membrane protein torsinA in the endoplasmic reticulum

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This supplement includes brief supplemental methods, 4 supplemental figures and accompanying legends.

Supplemental Methods

In vitro translation

Template was generated by amplifying the coding region of interest using a 5' primer that contains the T7 promoter sequence and a 3' primer that anneals just beyond the stop codon. The resulting product was purified using the Qiagen PCR purification kit and eluted in 50 μL water. 2 μL of the product was then mixed with 0.2 μL T7 RNA Polymerase (Promega), 0.2 μL water, and 7.6 μL T1 mix (final concentration 40 mM HEPES, pH 7.6, 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 500 μM ATP, 500 μM UTP, 500 μM CTP, 100 μM GTP, and 500 μM 7-methyl diguanosine triphosphate). This transcription mix was incubated for 1 h at 37°C, then RNA was precipitated by adding 1.5 μL 5 M NaCl and 24 μL of 100% ethanol and incubating at -20°C overnight. The RNA was pelleted at 15,000 x g for 30 min at 4°C and resuspended in 10 μL of nuclease-free water. *In vitro* translation reactions were then set up as follows: 12.5 μL rabbit

reticulocyte lysate, 0.5 μL TnT reaction buffer (Promega), 0.5 μL amino acids – Met (Promega), 1 μL ^{35}S-methionine (Perkin Elmer), 5 μL of the freshly prepared transcript, 2 μl canine rough microsomes, and water to a 25 μL final reaction volume. Translation reactions were incubated for 1 h at 30°C. The reaction was stopped by boiling in SDS-PAGE sample buffer; samples were then separated by SDSPAGE and detected by autoradiography.

Linescan analysis

To analyze ER subdomain preference, cells expressing the indicated proteins were fixed and costained for CRT as a bulk ER marker. The distribution of red and green fluorescence was analyzed by linescan in Metamorph. Two lines per cell were drawn from the nuclear periphery to the cell edge, and a line in a cell-free region of the coverslip was drawn for background measurement. The average background intensities were subtracted and the intensities normalized to 1 before replotting the data in Microsoft Excel.

Supplemental Figure Legends

Figure S1. The NTD does not traverse the ER bilayer in an *in vitro* TMD reporter system. (A) Diagram of the Lep membrane insertion reporter construct with test domain in gray and bracketing glycosylation sites in red. If the test domain is inserted as a TMD, only one site is glycosylated (left); if it is not, both are glycosylated (right). (B) *In vitro* translations of Lep reporter constructs in the absence or presence of canine rough

microsomes (RMs) with the following test domains: the model TM segments Lep13A/6L, Lep18A/1L (Hessa et al., 2007); torsinA residues 21-43 (NTD) in N_{lum}-C_{cyto} (type I) or N_{cyto}-C_{lum} (type II) orientation. Unglycosylated, singly glycosylated, and doubly glycosylated bands are designated 0g, 1g, and 2g, respectively. While unglycosylated bands remain prominent, the membrane-inserted Lep13A/6L segment is also singly glycosylated, while the translocated Lep18A/1L segment is doubly glycosylated. The torsinA segment in either orientation was doubly glycosylated, similarly to the latter model segment, indicating that it is translocated into the ER lumen.

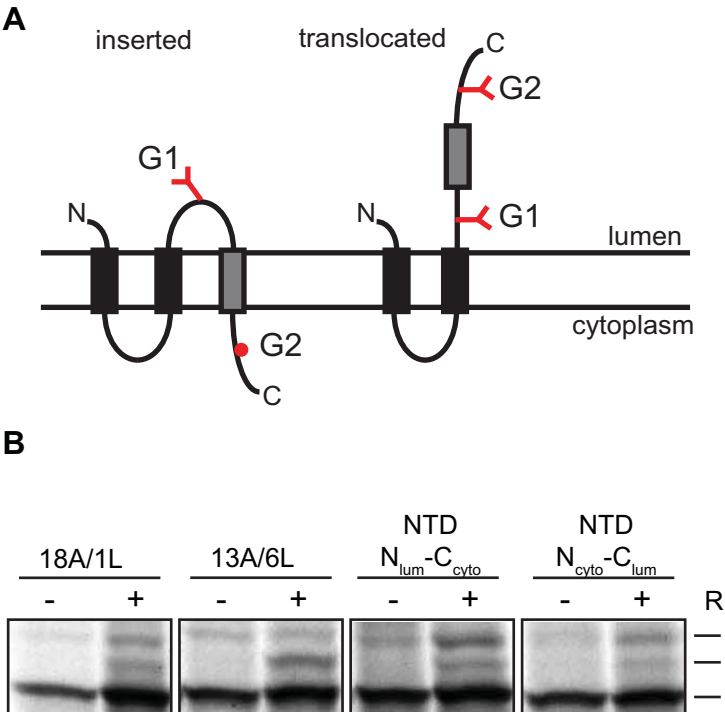
Figure S2. Increasing hydrophobicity of the NTD allows ER exit. Colocalization of the indicated TorsinA (1-67)-mGFP constructs with the intermediate compartment marker ERGIC-53. (1-67)-GFP (A) and (1-67) 2 x Leu- GFP (B) do not appreciably accumulate in the ERGIC-53 stained compartment. (1-67) 5 x Leu-GFP (D), (1-67)-LALALA-GFP (E), and to a lesser extent (1-67) 3 x Leu-GFP (C) do colocalize partially with ERGIC-53, indicating that these mutants are able to escape the ER. (1-67) 2 x Leu- GFP, 3 x Leu-GFP, and LALALA-GFP traverse the ER bilayer as transmembrane domains (Figure 5).

Figure S3. Effects of scanning mutagenesis on ER retention. All mutations were made in ATP-stabilized E171Q-torsinA-mGFP; similar results were seen with wild type torsinA-mGFP. (A) The intracellular localization of torsinA is unaffected by conservative substitutions throughout the NTD. (B) Nonconservative replacement of residues 26, 30, or 34 with Arg causes partial mislocalization to the ERGIC-53 stained intermediate compartment. A conservative Ala substitution at these positions does not have this effect.

(C) Nonconservative replacement of residues 24, 29, 31, or 38 with Arg has no effect on torsinA's ER localization. (D) Immunoprecipitation of NTD mutants from cell media. Mutation of residues 26, 30, or 34 to Arg leads to secretion of torsinA-mGFP into the cell medium. Conservative Ala substitutions at these positions did not cause secretion. Mutation of residues 24, 28, 29, 31, or 38 to Arg also did not cause secretion.

Figure S4. Linescan analysis of ER subdomain preference. (A-C) Representative confocal images of COS-7 cells costained for (A) endogenous CLIMP-63 and calreticulin (CRT); (B) torsinA-mGFP and CRT; (C) Δ26-43-torsinA-mGFP and CRT. Linescans of the relative intensities of the two signals are shown to the right; two linescans per image were performed, a background linescan was subtracted, and the intensities were normalized before plotting.

Figure S1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G1
N
N
G1
lumen
cytoplasm
G2
C
B
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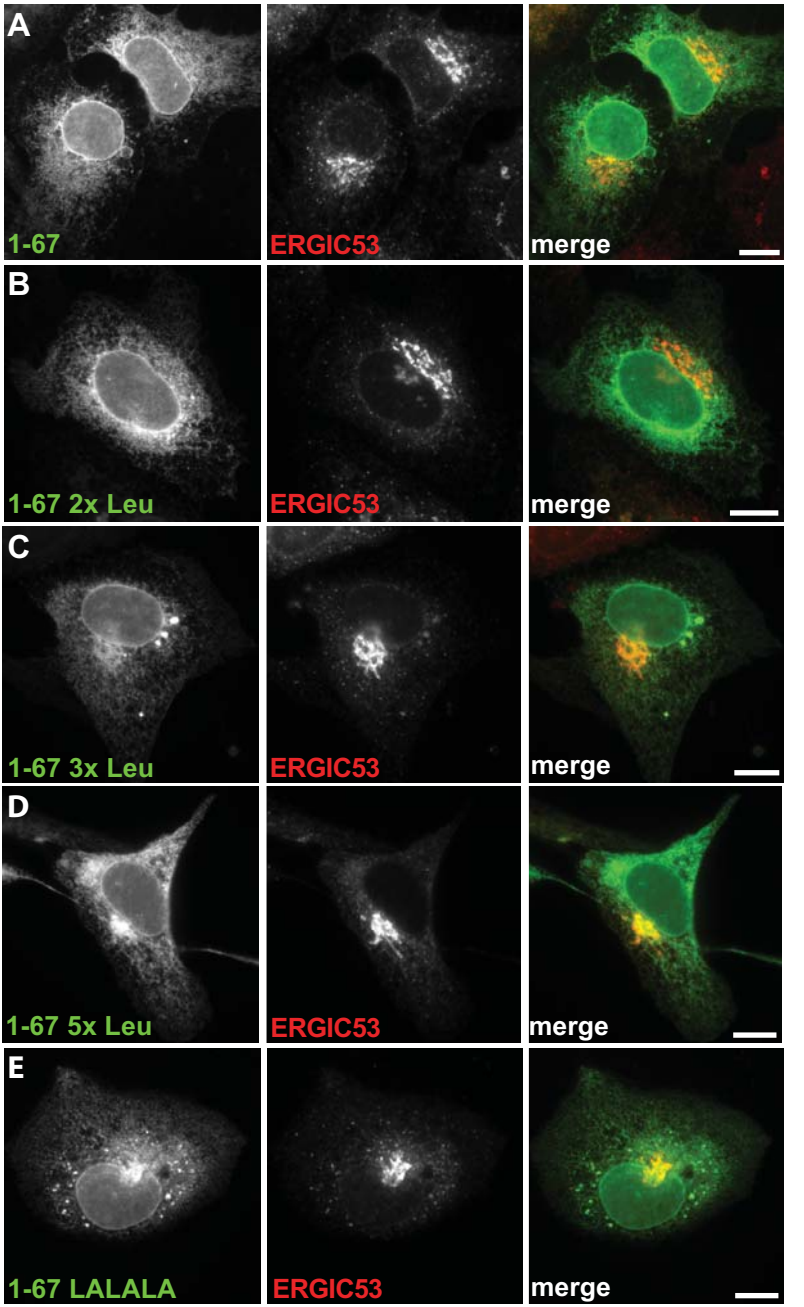
13A/6L
NTD
N_lum-C_cyto
NTD
N_cyto-C_lum
- + - + - + - +
RMs
2g
1g
0g

Figure S2.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1-67
ERGIC53
merge
B
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ERGIC53
merge
C
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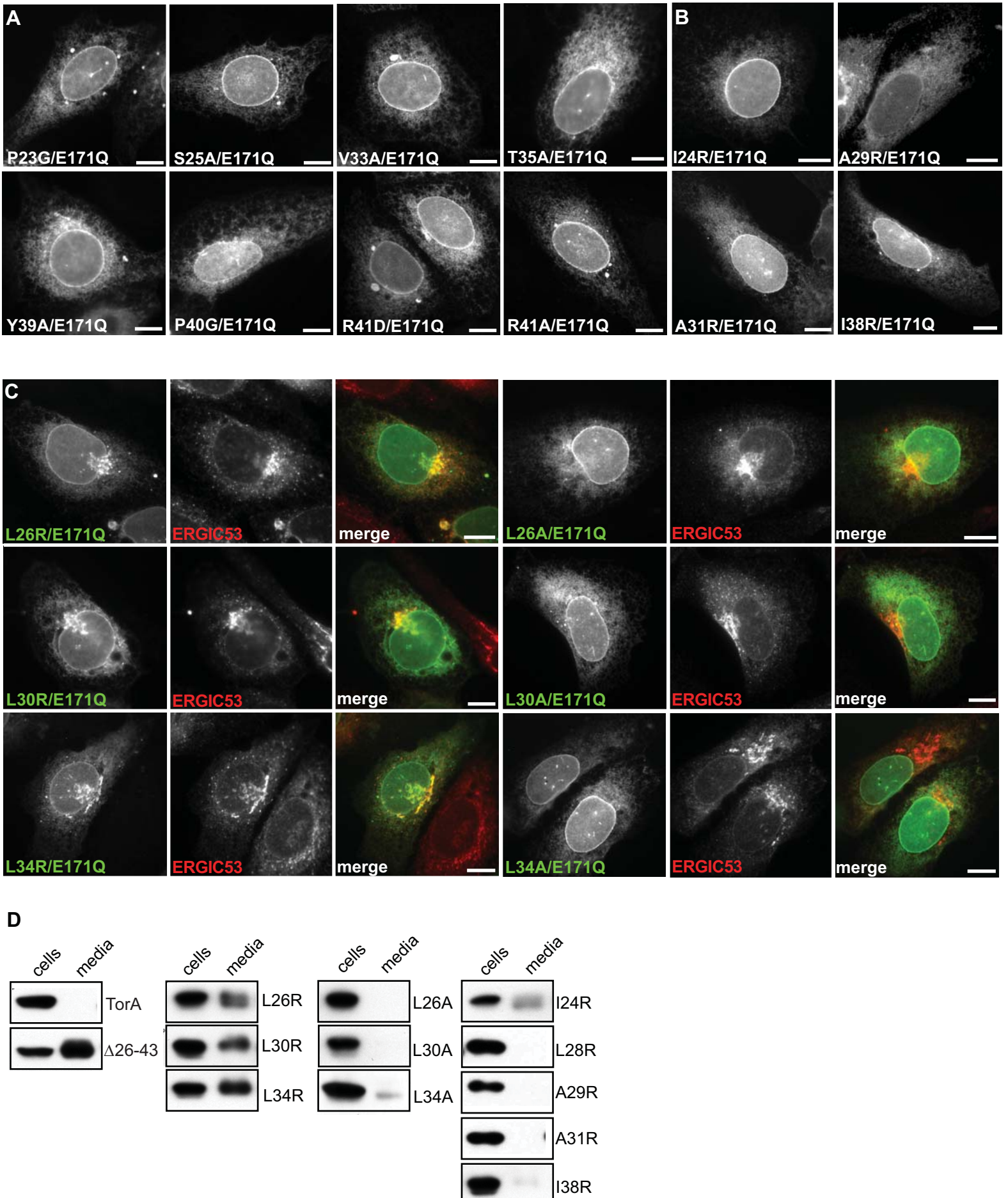
ERGIC53
merge
D
1-67 5x Leu
ERGIC53
merge
E
1-67 LALALA
ERGIC53
merge

Figure S3.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P23G/E171Q
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V33A/E171Q
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B
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P40G/E171Q
R41D/E171Q
R41A/E171Q
A31R/E171Q
I38R/E171Q
C
L26R/E171Q
ERGIC53
merge
L26A/E171Q
ERGIC53
merge
L30R/E171Q
ERGIC53
merge
L30A/E171Q
ERGIC53
merge
L34R/E171Q
ERGIC53
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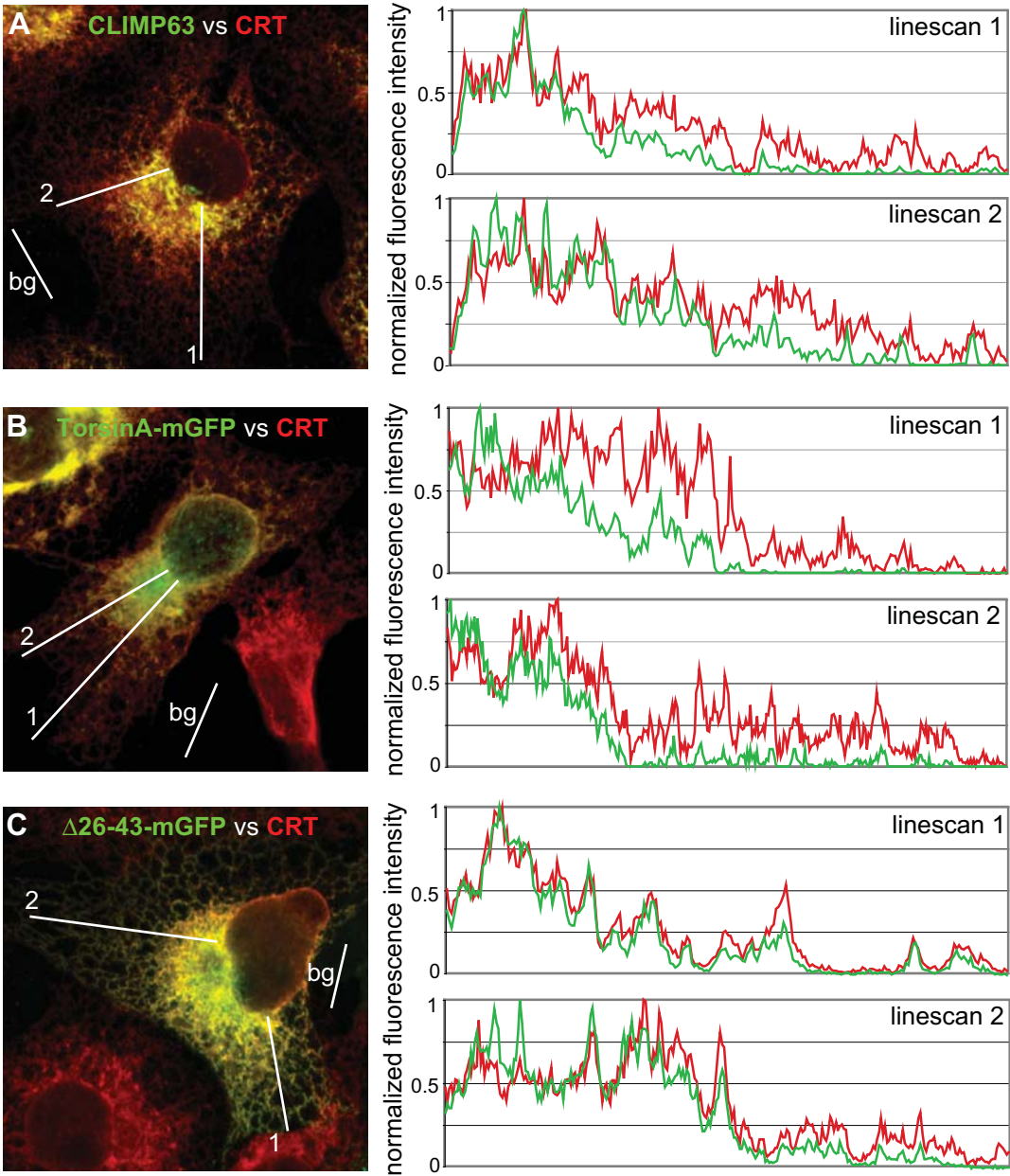
A31R
I38R

Figure S4.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CLIMP63 vs CRT
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0
linescan 1
linescan 2
B
TorsinA-mGFP vs CRT
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1
bg
C
Δ26-43-mGFP vs CRT
2
bg
1