Quality Control of Integral Membrane Proteins by Assembly-dependent Membrane Integration

Matthias J. Feige[1,*] and Linda M. Hendershot[1,*]

[1] Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

* Correspondence should be addressed to matthias.feige@stjude.org or linda.hendershot@stjude.org

Supplemental Information

Inventory of Supplemental Information

- Figure S1: extends Figure 1 with additional experimental data
- Figure S2: extends Figure 2 with additional controls and experimental data
- Figure S3: extends Figure 3 with additional experimental data
- Figure S4: extends Figure 4 with additional experimental data
- Figure S5: extends Figure 5 with additional experimental data
- Supplemental Experimental Procedures: detailed description of the materials and methods used in this study
- Supplemental References: References for the Supplemental Experimental Procedures

Supplemental Figures

Figure S1

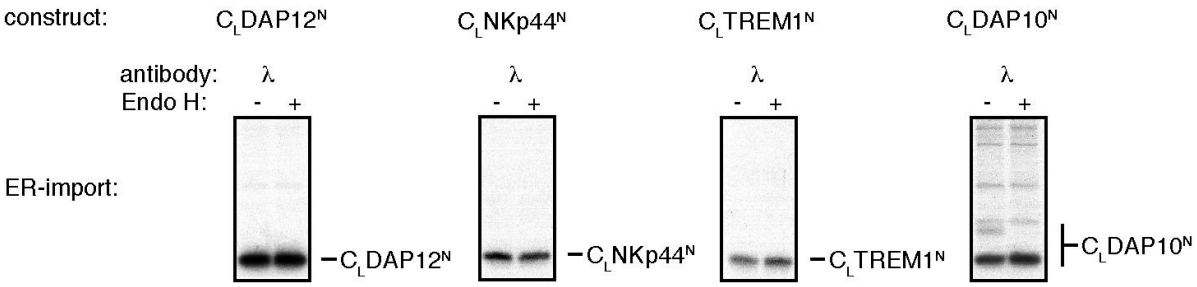
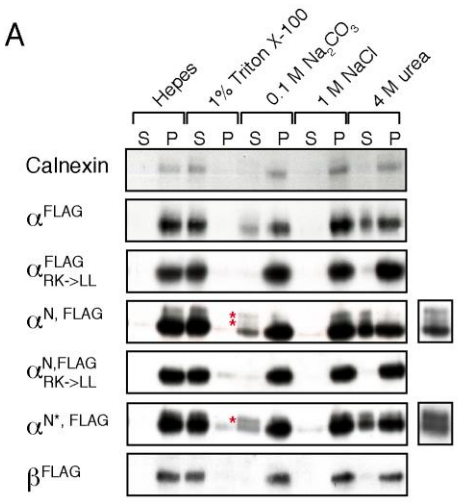
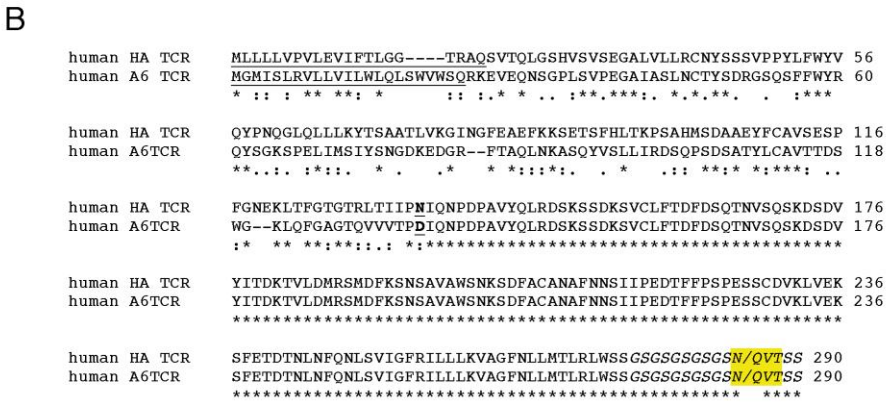


Figure S1, related to Figure 1: TM sequences of low hydrophobicity can enter the ER lumen

Experimental data to assess ER-import of different TM sequences (see also Figure 1). TM regions were derived from the indicated proteins (see Figure 1A) and cloned into the C_L-based reporter construct (see Figure 1B). Four microgram of each construct were used to transfect p60 dishes of COS-1 cells. Labeled lysates were immunoprecipitated with anti-mouse λ light chain antiserum and where indicated proteins were deglycosylated with EndoH prior to SDS-PAGE analysis. C-terminal glycosylation was used as an indicator of complete ER-import.

Figure S2





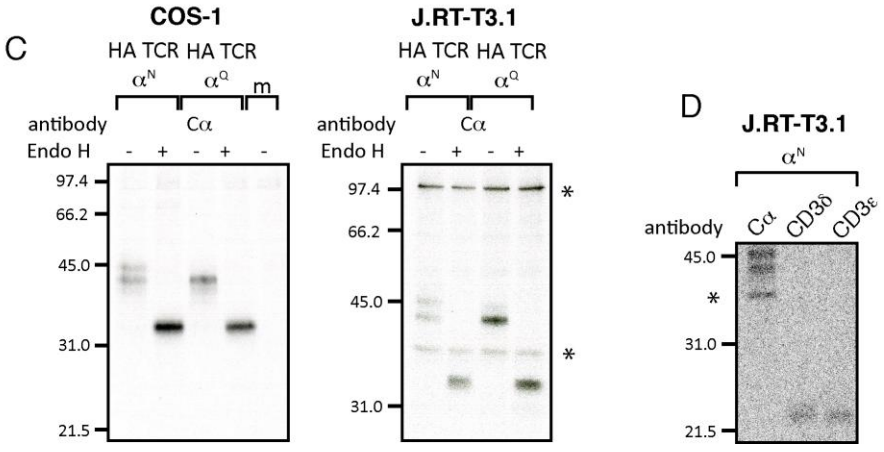


Figure S2, related to Figure 2: The TCR α-chain is mislocalized into the ER lumen

(A) Membrane integration test for various TCR constructs. Cells were broken by Dounce homogenization and the ER was isolated as described (Lai et al., 2012). ER preparations from cells expressing the indicated constructs were incubated in Hepes buffer alone or containing 1% Triton X-100, 1 M NaCl, or 4 M urea or in water containing 0.1 M Na_2CO_3 prior to centrifugation to separate fully soluble proteins (S) from membrane-anchored ones that pellet under these conditions (P). Proteins glycosylated at the C-terminal reporter site are indicated with a red asterisk. Note that $\alpha^{N,\ FLAG}$ populates three glycospecies (longer exposure of the sodium carbonate soluble fraction shown on the right), whereas $\alpha^{N*,\ FLAG}$ only populates two glycospecies (see Figure S4A for details about the three glycosypecies and α^{N*}). Again a longer exposure of the sodium carbonate soluble fraction is shown on the right. Note that whenever the TM basic residues are present for any of the α-chain constructs, these proteins can be partially extracted with sodium carbonate and urea. The former argues for improper membrane integration, the latter for retention in the membrane by a proteinaceous component. Note that the C-terminally glycosylated species (marked with a red asterisk) are enriched in the soluble fractions with sodium carbonate in comparison to the not C-terminally glycosylated species. In the case of Calnexin, an integral membrane protein control, the endogenous protein was monitored. For all the other constructs, a total of 24 microgram of each construct were co-transfected with six microgram BiP DNA in three p100 dishes of COS-1.

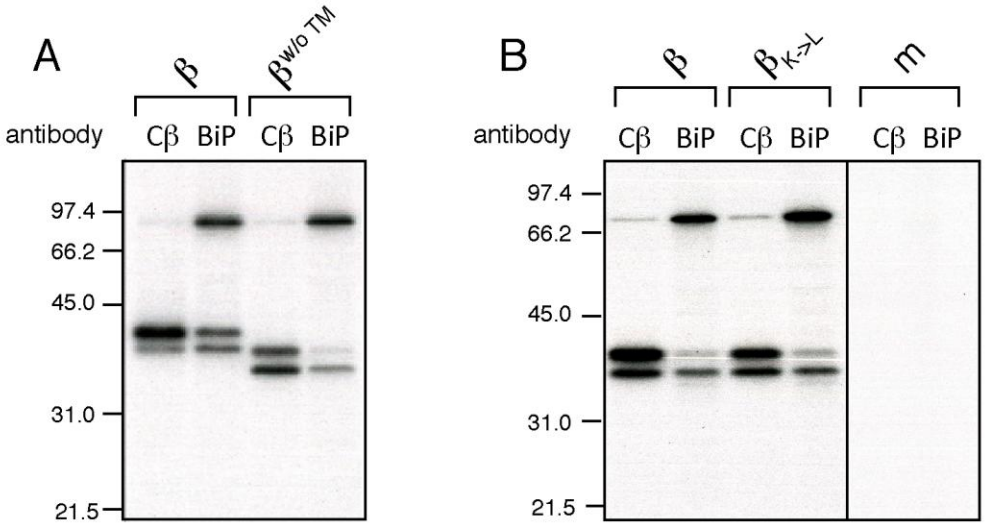
(B) Sequence alignment (www.ebi.ac.uk/Tools/msa/clustalw2/) of a human hemagglutinin-specific (HA) TCR α-chain with the human A6 TCR α-chain.

Putative ER-import sequences (www.cbs.dtu.dk/services/SignalP/) are underlined. The first amino acid of the constant domain is bold and underlined and the engineered C-terminal reporter site is in italics and shaded in yellow, the linker/C-terminus is in italics only.

(C) Glycosylation analysis of radiolabeled HA TCR α-chain constructs in COS-1 or Jurkat J.RT-T3.1 cells as indicated. HA TCR α^N possesses a C-terminal reporter site, HA TCR α^Q is the control with an Asn to Gln exchange (see (B), m: mock transfection). Four microgram DNA were transfected in COS-1 cells for each p60, 40 microgram in Jurkat cells for $4x10^6$ cells. Labeled lysates were immunoprecipitated with anti-TCR Cα-specific antibody and treated with Endo H as indicated. Black asterisks indicate non-specific bands in the Jurkat panel.

(D) Expression levels of overexpressed α^N in comparison to the endogenous CD3δ and CD3ε chains in Jurkat J.RT-T3.1 cells by metabolic labeling. 40 microgram of α^N DNA were transfected for $4x10^6$ cells. The lysate was split evenly and immunoprecipitated with the indicated antibodies. A non-specific band in J.RT-T3.1 cells is marked with a black asterisk. Note that α^N and CD3ε each contain four methionines, the residue that is predominantly labeled with EasyTag™ EXPRESS35S Protein Labeling Mix, whereas CD3δ only contains one methionine. Thus, the relative levels of CD3δ will be even higher than deduced from the relative intensity of the bands.

Figure S3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βw/o TM
antibody
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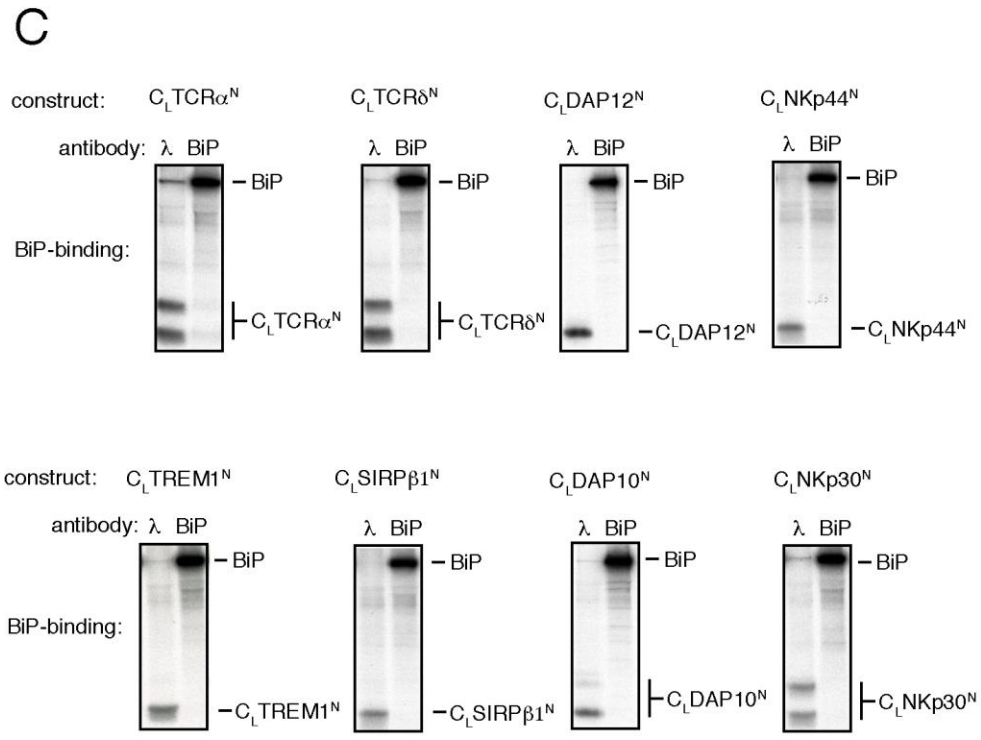
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antibody: λ BiP
BiP-binding:
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CLNKp30N

Figure S3, related to Figure 3: BiP binds to the TM region of ER-lumenal α-chains

(A) Metabolic labeling shows that independent of the presence (β) or absence of its TM region ($\beta^{w/o\ TM}$) the β-chain binds to BiP. Metabolically labeled lysates from COS-1 cells expressing the indicated constructs were divided equally and immunoprecipitated with the indicated antibodies.

(B) COS-1 cells were transfected with β-chain constructs containing (β) or devoid of its single TM Lys residue ($\beta_{K->L}$) and analyzed as in (A). The m indicates mock transfected cells. The two bands are two naturally occurring glycoforms of the β-chain (Lee, 1998). Apparently the less-glycosylated form is preferably bound by BiP, stabilized by BiP and becomes more prominent than in the absence of BiP (compare to Figure 2B).

(C) BiP binding of the different C_L-based reporter constructs was performed as in (A). For details of the constructs see Figure 1.

Two microgram of DNA for both BiP and the different constructs were co-transfected for each p60 of COS-1 in (A), (B) and (C).

Figure S4

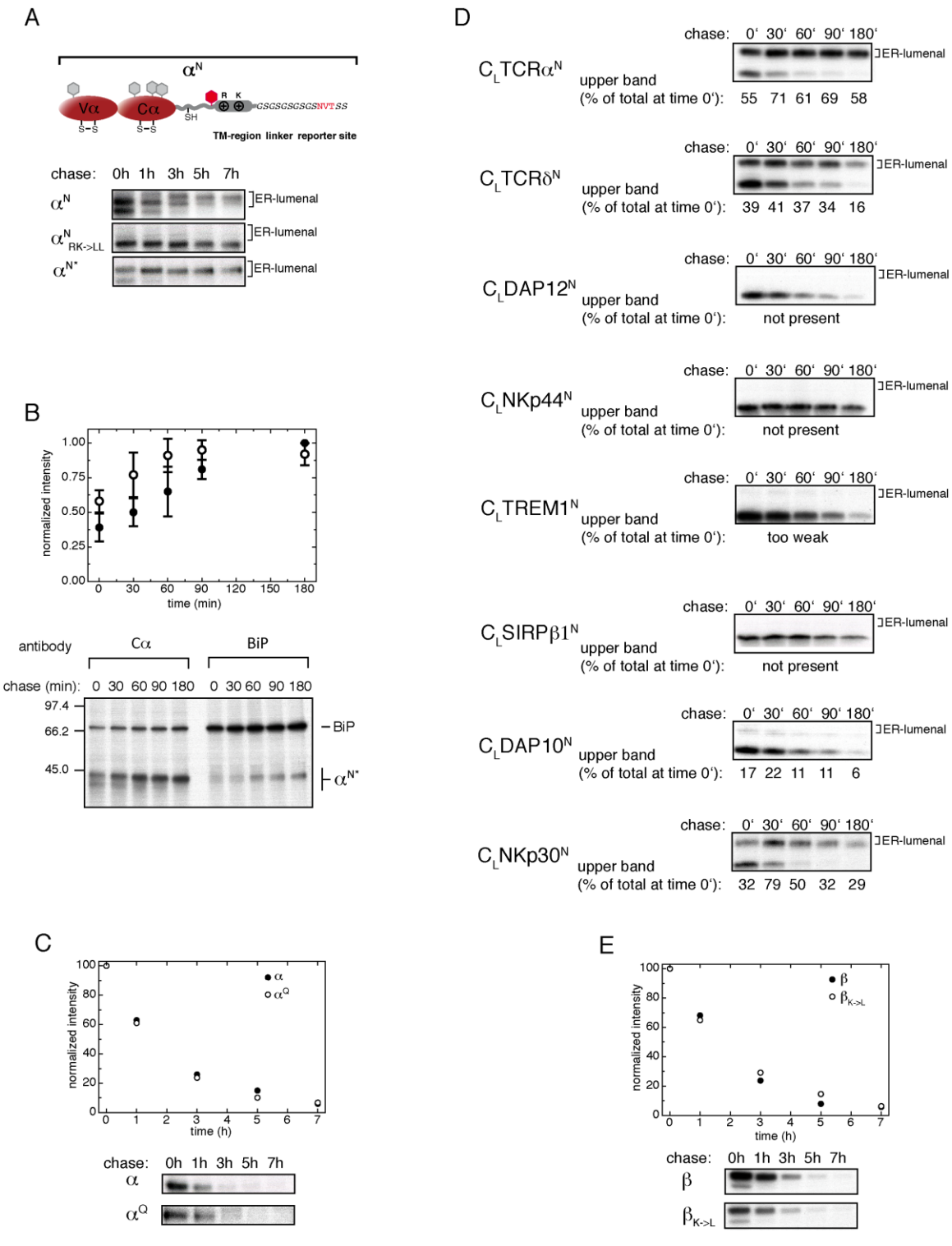

Figure S4, related to Figure 4: Degradation of the TCR α-chain correlates with its localization into the ER lumen

(A) A schematic at the top of the panel indicates constitutive glycosylation sites (grey hexagons), a cryptic glycosylation site that is modified post-translationally very late in the chase period (red hexagon), and basic residues in the TM region of the α-chain. COS-1 cells expressing the indicated constructs were subjected to pulse-chase analyses and labeled lysates were immunoprecipitated with anti-Cα antibody. Examination of the α^{N} construct reveals that α^{N} is more stable than the wt α-chain (compare to Figure 4D) and that post-translational glycosylation takes place at a cryptic glycosylation site. The site is part of an Asn-Leu-Ser sequence close to the membrane that seems to be masked when the α-chain is integrated into the membrane. As the pulse-chase experiment for $\alpha^{N}_{RK->LL}$ shows, this cryptic site only becomes glycosylated when α^{N} can enter the ER lumen. Mutation of this site to QLS, the resulting protein denominated as α^{N*}, abolishes this posttranslational glycosylation.

(B) Pulse-chase experiments were conducted to compare the complete entry of the α-chain into the ER with its binding to BiP *via* its TM region. Open circles show the amount of C-terminally glycosylated, completely ER-lumenal α^{N*}. Closed circles show the amount of α^{N*} co-immunoprecipitating with BiP (n=4±SD). A representative autoradiograph is shown below the quantification. Note that only the C-terminally glycosylated, completely ER-lumenal species of α^{N*} co-immunoprecipitates with BiP whereas the less glycosylated species does not co-immunoprecipitate with BiP(compare to Figure 3), thus revealing the ability to be co-immunoprecipitated with BiP as another assay for ER entry of the TCR α-chain TM region.

(C) Pulse-chase experiment of the wt α-chain (α) compared to the construct with an engineered C-terminal QVT-site (α^{Q}) (see Figure 2B). Quantifications of the bands by phosphorimager analysis are shown above the autoradiographs. Both α and α^{Q} are degraded at the same rate, demonstrating that it is the glycosylation of the C-terminal reporter site that stabilizes α^{N} (see (A)).

(D) Changes in the amount of the ER-lumenal species were assessed over time for the indicated C_L-based reporter constructs to monitor post-translational ER-import. Quantifications of the ER-lumenal species ("upper band") were performed at the indicated chase times by phosphorimager analysis and normalized to the total amount of signal at t=0' of the chase.

(E) Replacement of the single TM Lys residue in the β-chain ($\beta_{K->L}$) does not affect its stability as assessed by a pulse-chase experiment. Quantifications of the bands by phosphorimager analysis are shown above the autoradiographs.

In each experiment, four microgram DNA were transfected for each p60 of COS-1 except for (B), where two microgram of α^{N*} were co-transfected with three microgram of BiP.

Figure S5

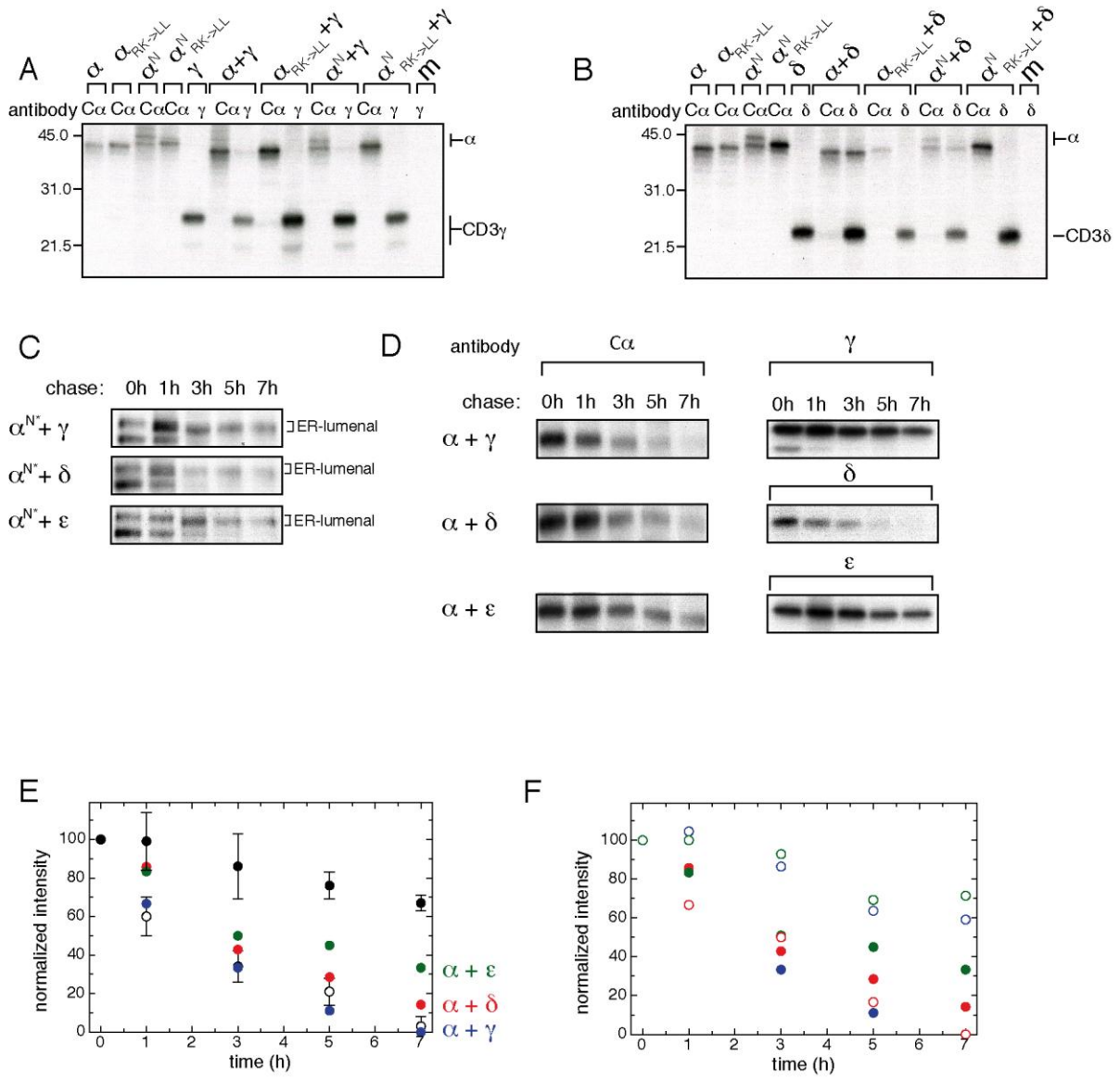

Figure S5, related to Figure 5: CD3δ and ε retain the α-chain in the ER membrane and stabilize it against degradation

(A) Co-immunoprecipitation experiments were performed on each of the different radiolabeled α-chain constructs with the CD3γ chain. Replacement of TM basic residues in the α-chain by Leu is indicated (RK->LL).

(B) The same experiment was performed and analyzed for CD3δ.

(C) Pulse-chase experiments to follow the ER import of α^{N*} over time in the presence of the indicated CD3 subunits. C-terminally glycosylated, ER-lumenal species are indicated.

(D) Pulse-chase experiments were conducted on the wt α-chain expressed alone or in the presence of different CD3 subunits as indicated. Either the α-chain or the individual CD3 subunits were immunoprecipitated as indicated. Quantifications are shown in (F) and (G).

(E) Quantification of α-chain degradation in the presence of either CD3γ (blue), CD3δ (red) or CD3ε (green) as indicated. As a comparison, α-chain degradation in the presence of either pSVL (open circles) or CD3δ and ε (closed circles) is shown (data taken from Figure 5G, n=3±SD).

(F) Comparison of the stabilities of the α-chain in the presence of either CD3γ (blue, closed circles), CD3δ (red, closed circles) or CD3ε (green, closed circles) (taken from (E)) with the stabilities of the CD3 subunits CD3γ (blue, open circles), CD3δ (red, open circles) or CD3ε (green, open circles).

In (A) and (B) two plus two microgram of DNA were co-transfected for each p60 of COS-1 cells. In (C) and (D), two microgram of α^{N*} or α were co-transfected with four microgram or two plus two microgram of the indicated CD3 subunits.

Supplemental Experimental Procedures

Construct design

For the A6 TCR, individual constructs were amplified from synthetic TCR genes optimized for human expression (Geneart, Regensburg, Germany) and cloned into the pSVL vector (GE Healthcare, Pittsburg, PA) *via* XhoI/BamHI for expression in COS-1 cells. Chimeric constructs of the λ light chain C_L domain and the α-/β-chain transmembrane (TM) region were generated using the previously described λ light chain C_L domain construct with an ER import sequence (Hellman et al., 1999). Its XhoI restriction site was deleted *via* site directed mutagenesis. Sequences coding for the different α-/β-chain TM regions were synthesized (Geneart, Regensburg, Germany) and cloned into pSVL *via* XhoI/BamHI with an internal XbaI restriction site before the $(GS)_5$ linker and the TM region. Subsequently, the λ light chain C_L domain was inserted *via* XhoI/XbaI thus inserting it before the linker preceding the TM regions and using its authentic ER import sequence. TM sequences of the other immunoreceptors were inserted into the C_L-α^N construct by site directed mutagenesis. For expression in Jurkat cells, constructs were cloned into the pcDNA 3.1 vector (Invitrogen, Grand Island, NY). For western blot experiments, a double FLAG-tag was cloned to the C-terminus of the respective constructs separated by a $(GS)_2$ linker. The human HA TCR was synthesized (Geneart, Regensburg, Germany) with its authentic mRNA sequence and cloned into pSVL or pcDNA3.1 as described above for the A6 TCR. A hamster BiP construct in the pMT vector was used (Lee et al., 1999). Site directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All constructs were sequenced.

Cell culture

COS cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and a 1% (v/v) antibiotic-antimycotic solution (25 µg/ml amphotenicin B, 10,000 µg/ml streptomycin, and 10,000 units of penicillin; Cellgro/Mediatech, Manassas, VA) (complete DMEM) at 37°C and 3% CO_2. COS transfections were carried out for 24h using GeneCellin (BioCellChallenge, Toulon, France) according to the manufacturer's protocol.

Jurkat J.RT-T3.1 cells (Saito et al., 1987) were grown in RPMI supplemented with 10% (v/v) FBS, 2 mM L-glutamine and antibiotic-antimycotic solution (complete RPMI) at 37°C and 5% CO_2. Transfections were carried out with the NEON electroporation system (Invitrogen, Grand Island, NY) for 24h according to the manufacturer's protocol.

Amount of transfected DNA for transient transfections

In Figure 1B four microgram DNA of each construct were transfected. In Figure 2, for each experiment in COS cells, four microgram DNA of each construct were transfected, in Jurkat cells 40 microgram. In Figure 3, two microgram of each construct were co-transfected with two microgram of BiP DNA. In Figure 4 four microgram DNA of each construct were transfected. Were indicated, two microgram of $Hrd1_{C291S}$ were co-transfected. In Figure 5A two microgram DNA of each construct / empty pSVL vector were co-transfected. In Figure 5B two

microgram of α^{N*} were co-transfected with four microgram of empty pSVL, individual CD3 chains or two plus two microgram of two CD3 chains. In Figure 5C, two microgram of α^{N*} were co-transfected with increasing equal amounts of CD3 δ and ε (p100 dishes were used in this experiment). In Figures 5D, 5E and 5F two microgram of the α-chain constructs were co-transfected with four micrograms of each CD3 chains or two plus two microgram of CD3 δ and ε. For COS cells, p60 dishes were used and for Jurkat cells $4x10^6$ cells if not stated otherwise.

Metabolic labeling, pulse-chase experiments and immunoprecipitations

For metabolic labeling, cells were washed twice with PBS, then starved for 30 min in complete DMEM (COS) or RPMI (Jurkat J.RT-T3.1) without Met and Cys and subsequently labeled for 30 min with 100 µCi/p60, 300 µCi/p100 dish (COS-1) or 200 µCi/$4x10^6$ cells (J.RT-T3.1), respectively, of EasyTag™ EXPRESS35S Protein Labeling Mix (Perkin Elmer, Waltham, MA). Pulse-chase experiments were performed analogously, only that after the labeling period, cells were washed twice with ice cold PBS and then chased in complete medium supplemented with additional 2 mM of cold Met and Cys. If present, MG132 (Sigma-Aldrich, St. Louis, MO) was added at 10 µM 2.5 h before the pulse, during the starving period of 30 min and during the subsequent pulse and chase. Prior to lysis, cells were washed twice in ice cold PBS. Generally, cell lysis was performed in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1.0% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM PMSF, 1x Roche

complete protease inhibitor tablets w/o EDTA (Roche Applied Science, Indianapolis, IN)). In the case of BiP co-immunoprecipitation experiments, cell lysis was carried out in NP40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40 substitute, 0.5% sodium deoxycholate, 0.1 mM PMSF, 1x Roche complete protease inhibitor tablets w/o EDTA) supplemented with 10 U/ml Apyrase (Sigma-Aldrich, St. Louis, MO). For CD3 co-immunoprecipitation experiments, Digitonin lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Digitonin (Sigma-Aldrich, St. Louis, MO), 0.1 mM PMSF, 1x Roche complete protease inhibitor tablets w/o EDTA) was used. Immunoprecipitations were performed with commercially available antibodies against the TCR Cα domain (mouse monoclonal anti-human, TCR1145, Thermo Fisher Scientific, Rockford, IL), Cβ domain (mouse monoclonal anti-human, TCR1151, Thermo Fisher Scientific, Rockford, IL), CD3γ (rabbit monoclonal anti-human, 3256-1, epitomics, Burlingame, CA), CD3δ (rabbit monoclonal anti-human, 3762-1, epitomics, Burlingame, CA), CD3ε (rabbit monoclonal anti-human, ab32186, abcam, Cambridge, MA) and the mouse lambda antibody light chain (goat antiserum, 1060-01, Southern Biotechnology, Birmingham, AL). A previously described antiserum against rodent BiP (Hendershot et al., 1995) was used. Antibody concentrations were used according to the manufacturer's protocol.

Prior to immunoprecipitation, cell lysates were centrifuged for 15 min, 20.000 g, 4°C, then antibody was added and rotated o.n. at 4°C. Subsequently CaptivA™ PriMAB proteinA agarose (Repligen Bioprocessing, Waltham, MA) was added for 1 h at 4°C under rotation. Immunoprecipitated proteins were washed three times

with RIPA buffer, NP40 washing buffer (50 mM Tris/HCl, pH 7.5, 400 mM NaCl, 0.5% Nonidet P40 substitute, 0.5% sodium deoxycholate) (for BiP co-immunoprecipitations), or, whenever CD3 co-immunoprecipitation experiments were performed, Digitonin wash buffer (50 mM Tris/HCl, pH 7.5, 400 mM NaCl, 1% Digitonin) and eluted with Laemmli buffer for 5 min at 95°C. For EndoH (NEB, Ipswich, MA) deglycosylation experiments, proteins were eluted and subsequently treated according to the manufacturer's protocol. Samples were run on 10% or 14% SDS-PAGE gels, signals were amplified with Amplify (GE Healthcare, Pittsburg, PA) and subsequently gels were dried and either used in autoradiography or phosphorimager analysis. Phosphorimager scans were carried out on a Storm 860 scanner (GE Healthcare, Pittsburg, PA) and quantified with the ImageQuant TL software (GE Healthcare, Pittsburg, PA). For Figure 5C, data were quantified from film with the ImageQuant TL software due to the low signal intensity.

Subcellular fractionation and western blot

Subcellular fractionation was essentially performed as published (Lai et al., 2012). For each construct, 5-10x10^6 cells were used. Cells were removed from the dishes by trypsinization, proteolysis was stopped by addition of complete DMEM and cells were harvested by centrifugation for 5 min at 500 g at 4°C. Subsequently, cells were washed two times with PBS and one time in

homogenization buffer (25 mM Hepes/KOH, pH 7.2, 125 mM KCl). Then, cells were resuspended in 2 ml homogenization buffer supplemented with 10 U/ml Apyrase (Sigma-Aldrich, St. Louis, MO) and 1x Roche complete protease inhibitor tablets w/o EDTA and homogenized with a glass/teflon Dounce homogenizer. The solution was spun for 10 min, 500 g at 4°C. The supernatant of this step was divided into five equal aliquots and each spun for 10 min at 16,000 g at 4°C. Each pellet was resuspended in either 100 µl 10 mM Hepes/KOH, pH 7.4, or in 10 mM Hepes/KOH, pH 7.4 supplemented with either 1% Triton X-100, with 1 M NaCl or with 4 M urea or in 0.1 M Na_2CO_3 in water. Samples were rotated for 1 h at 4°C and then centrifuged at 10,000 g for 5 min at 4°C. The supernatants of this step were designated as “soluble” fractions, the insoluble fractions as “pellet”. The pellet was resuspended in 100 µl RIPA buffer and to all samples Laemmli buffer was added before boiling. Samples were separated on SDS-PAGE gels, transferred to PVDF membranes and blotted with anti-Calnexin antibody (rabbit polyclonal, produced in our lab) or with anti FLAG-antibody (rabbit polyclonal, F7425, Sigma, St. Louis, MO) according to the manufacturer’s protocol.

Supplemental References

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