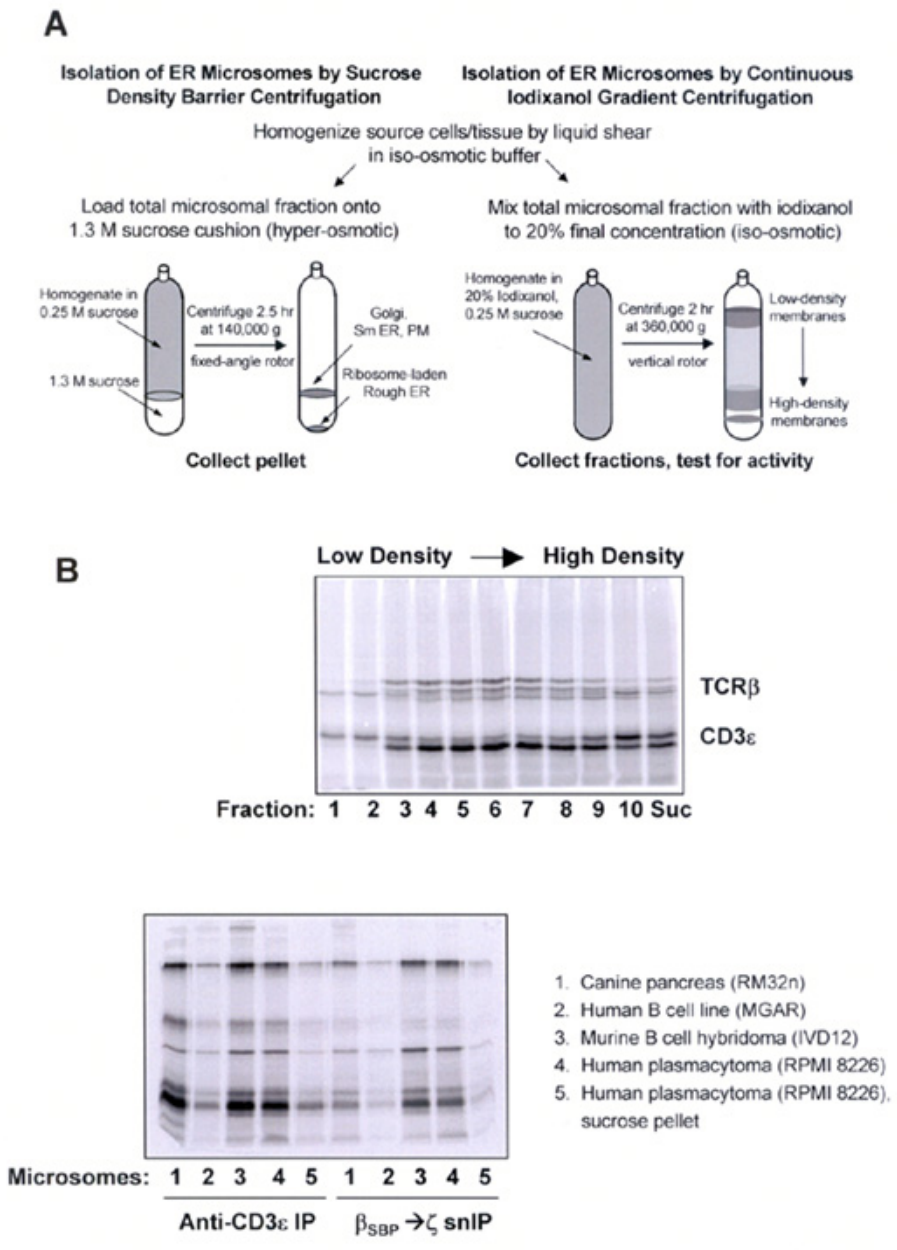

Figure S1. Preparation of ER microsomes from cultured cells by fractionation on self-generating iodixanol gradients

(A) Illustration of the traditional sucrose density barrier method versus continuous iodixanol gradients. Cellular homogenates are cleared of unbroken cells and nuclei by centrifugation at 10,000 g for 20 minutes. The supernatant contains membrane microsomes from intracellular compartments. The traditional sucrose density barrier

method (left side; Walter and Blobel, 1983) yields a pellet containing a ribosome-laden, high-density membrane fraction containing most of the active ER membranes. However, for plasma cell homogenates, this procedure did not yield high-activity ER microsomes. In contrast, fractionation on a self-generating, continuous density gradient formed by 20% iodixanol under high g-forces resulted in separation of membrane populations, allowing testing of individual fractions for the highest activity population.

(B) Signal peptide cleavage and glycosylation activities of iodixanol fractions from human plasmacytoma cells. mRNAs encoding A6 TCRβ and CD3ε were co-translated in 25 μl *in vitro* translation reactions with 2 μl of each membrane fraction at 100 A_{280nm}. Higher MW TCRβ bands reflect glycosylation, while lower MW CD3ε bands reflect signal peptide cleavage. As illustrated here, the highest processing activities were found in fractions of intermediate density (4-8). Microsomes prepared by sucrose density barrier centrifugation (Suc) exhibit lower activity, similar to the densest fractions (9 and 10) from the iodixanol gradient.

(C) Comparison of ER microsomes isolated by the two different methods in terms of TCR-CD3 assembly efficiency. Microsomes were isolated on continuous iodixanol gradients from three different cell lines: a human EBV-transformed B cell line (MGAR), a murine B cell hybridoma (IVD12) and a human plasmacytoma cell line (RPMI 8226). These preparations were compared to microsomes isolated on sucrose density gradients from the plasmacytoma cell line (RPMI 8226) and canine pancreas (RM32n). All preparations were adjusted to 100 $A_{280\ nm}$, and 2 μl were added to 25 μl *in vitro* translation reactions with mRNAs encoding TCRα, $TCR\beta_{SBP}$, CD3γ, δ, ε and ζ chains. While canine preparations exhibited consistently higher translocation, signal peptide cleavage and glycosylation activities (not shown), densitometry measurements of proteins precipitated in a $TCR\beta_{SBP}\rightarrow\zeta$ snIP experiment indicated that murine B cell hybridoma (IVD12; lane 3) and human plasmacytoma (RPMI 8226; lane 4) microsomes exhibited the highest assembly efficiency. Direct comparison of microsomes isolated from the human plasmacytoma cell line by the two methods demonstrated substantially higher assembly activity with membranes isolated on continuous iodixanol gradients.

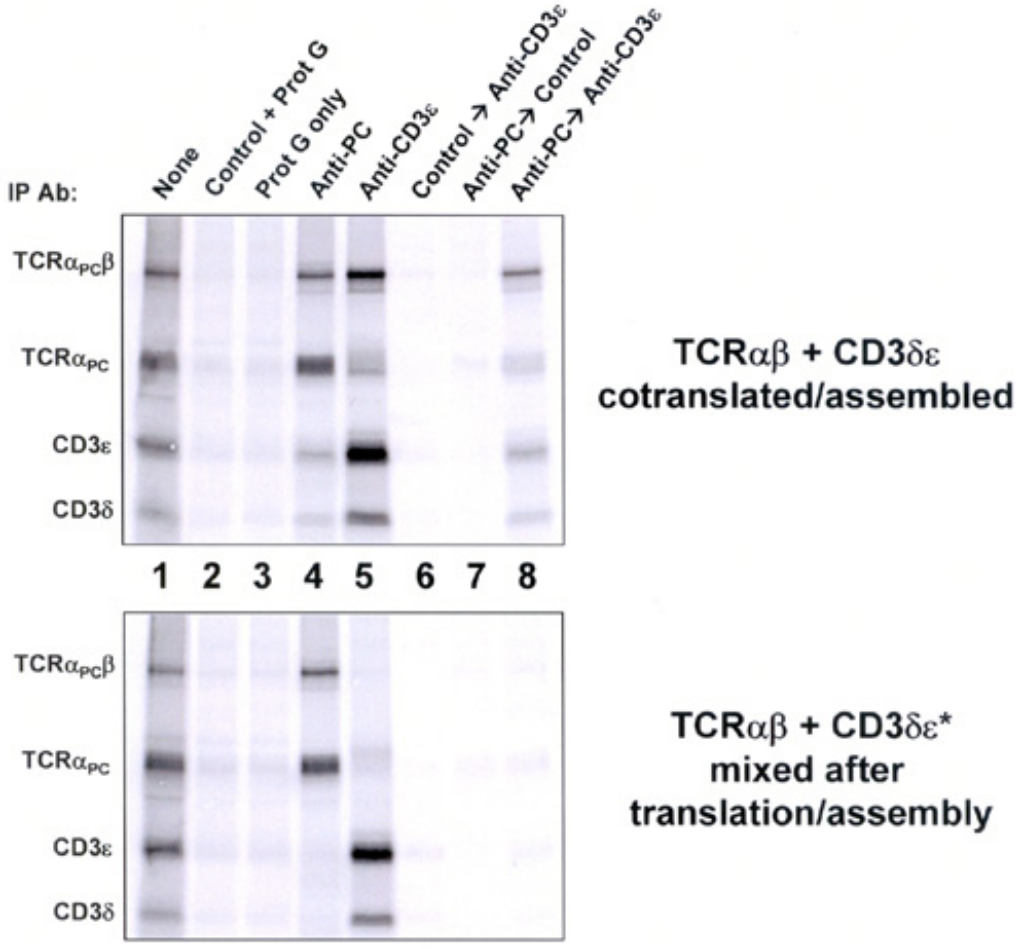

Figure S2. TCRαβ-CD3δε association occurs in a co-translational fashion and not during or after solubilization.

Since assembly in the ER occurs in a co-translational fashion, we included controls in many of the experiments in which the relevant components were translated separately and mixed prior to solubilization. The purpose of these mixing controls was to exclude the possibility that components interacted during or following solubilization, in addition to permitting assessment of the background for the IP procedure. The experiment shown here illustrates that the individual components (TCRαβ and CD3δε) were present in the

mixing control (lower panel), but did not interact. In addition, a series of other controls were included in this experiment to document that mixing controls are also appropriate for assessing the overall background of the IP procedure: isotype control antibody and protein G beads (lane 2), protein G beads alone (lane 3), replacement of either primary or secondary antibody in a two-step IP by an isotype control antibody (lanes 6 and 7). Equal amounts of material were present in all IP procedures shown on each gel (lanes 2-8) since samples were split from a single reaction after translation and assembly.

For the upper panel, a single reaction containing TCRα_{PC}, TCRβ, CD3δ and CD3ε was split after translation and assembly, and the membrane fractions were solubilized in SDS for direct analysis (lane 1) or in 1% digitonin for IP (lanes 2-8). For the lower panel, TCRα_{PC}β and CD3δε were translated and assembled in separate tubes, then mixed and split as above. All components were present in both reactions, as demonstrated by direct analysis of membrane lysates (lane 1), and by single-step IP for TCR (lane 4) or CD3 (lane 5) components. Comparison of the upper and lower panels demonstrates that TCR and CD3 polypeptides were only co-precipitated in the single-step IPs when TCRαβ and CD3δε had been co-translated in a single reaction (lanes 4 and 5). The two-step snIP (TCRα_{PC}→CD3ε snIP) confirmed this conclusion since the TCRαβ-CD3δε complex was only recovered from the co-translation reaction, but not the mixing control (lane 8, compare upper and lower panels). Comparable levels of background were observed for the co-translation experiment and the mixing control when control antibodies were used (lanes 2, 6 and 7), indicating that the mixing controls are also appropriate for assessing the overall background of the IP procedure.

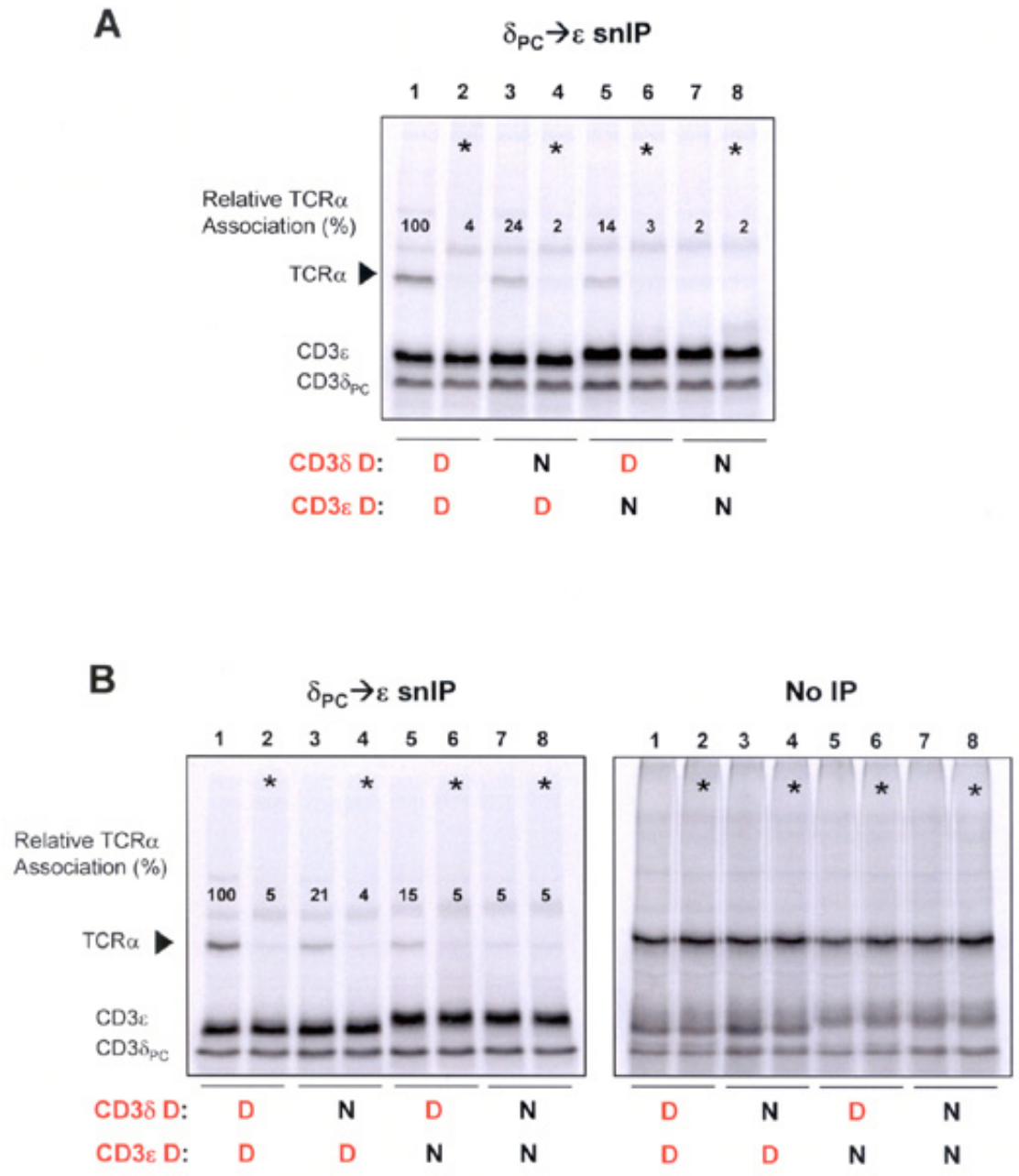

Figure S3. ***In vitro*** **assembly experiments are highly reproducible.**

The complexity of the experiments described in this report raises the question of whether the results are reproducible, in particular when a quantitative assessment of co-precipitated components is performed. We have therefore repeated a key experiment and

performed densitometry analysis of the co-precipitated TCRα chain. In addition, membrane lysates were analyzed in parallel in one of these experiments in order to document that all components were present in each reaction.

A key result from our studies of TCRα-CD3δε assembly – that single asparagine (N) substitution of either aspartic acid (D) residue in the CD3δε TM regions reduced association, while double substitution abrogated assembly (Fig. 2F) – was reproduced with additional controls. Reactions and mixing controls (*) for each combination of CD3 TM mutants were subjected to $\delta_{PC}\rightarrow\varepsilon$ snIP to isolate assembled CD3δε heterodimers and analyze TCRα (arrow) association, as before (A and left panel of B). Quantification of co-precipitated TCRα (from densitometry measurements) shows a high degree of reproducibility between these two experiments and Figure 2F.

Supplemental Methods

Preparation of ER microsomes from cultured cells

Human plasmacytoma RPMI 8226 cells (ATCC) were maintained in suspension in 2 L roller bottles with DMEM, 10% fetal calf serum, and were grown to a density of 1.5 – 2.0 x 10^6 cells/ml on the last day before collection. Approximately 10-20 x 10^9 cells were pelleted and washed twice with ice-cold iso-osmotic buffer (10 mM HEPES/KOH, pH 7.5, 0.25 M sucrose). All subsequent steps were performed at 4°C. Cells were resuspended in 5 ml/g hypo-osmotic buffer (10 mM HEPES/KOH, pH 7.5) and swelled on ice for 10 minutes to facilitate lysis. Cells were returned to iso-osmotic conditions (0.25 M sucrose) by addition of 0.60 M sucrose and homogenized in 20 ml aliquots using an overhead motor-driven Teflon Duall pestle and a 30 ml-capacity glass tube (Kontes Glass Co). Protease Inhibitor Cocktail P8340 (Sigma) was added and crude homogenates were centrifuged at 10,000 g for 20 minutes to pellet nuclei and larger organelles. Pellets were re-processed to homogenize remaining cells, and 10,000 g supernatants were pooled and centrifuged at 150,000 g for 20 minutes to pellet total microsomal fractions. Pellets were resuspended in iso-osmotic buffer.

For sucrose density-barrier fractionation, the total microsomal suspension was centrifuged through a 1.3 M sucrose cushion in iso-osmotic buffer as described (Walter and Blobel, 1983), and pellets were resuspended in membrane buffer (0.25 M sucrose, 50 mM HEPES/KOH, pH 7.5, 1 mM DTT) using a Dounce homogenizer to an OD of approximately 100 at A_{280nm} and stored at -80°C. For fractionation on self-generating iodixanol gradients, the total microsomal suspension was mixed with Opti-Prep (NycoMed) 60% iodixanol stock solution to yield a final concentration of 20% iodixanol and loaded into Beckman OptiSeal (362181) tubes. Suspensions were centrifuged at 65,000 rpm in a Beckman VTi65.1 vertical rotor for 2 hours at 4°C with no brake. Gradients were unloaded from the bottom of the tubes in 10 x 1 ml fractions, which were diluted with iso-osmotic buffer and pelleted by centrifugation as before. Pellets were resuspended in membrane buffer and stored as above. Fractions exhibiting the highest

translocation, glycosylation and signal peptide cleavage activities in test translations (see supplemental Fig. S1) were pooled and stocks were stored at -80°C in 100 µl aliquots.