Supplemental Information

Supplemental Figure Legends:

Figure S1. The membrane protein Bap31 localizes to all ER domains, related to Figure 1.

(A) The localization of endogenous Bap31 is compared with that of the stably overexpressed membrane protein GFP-Sec61β using confocal microscopy in BSC1 cells. Bap31 was detected with specific antibodies by indirect immunofluorescence (left panel) and Sec61β by GFP fluorescence (middle panel). The right panel shows a merged image. Scale bar, 10 μm.

(B) The localization of endogenous Bap31 is compared with that of endogenous TRAPα using confocal microscopy in COS7 cells. Note that the localization of Bap31 does not change whether or not Sec61β is overexpressed. Scale bar, 10 μm.

Figure S2. The ER membrane proteins p180 and Climp63 localize to peripheral ER sheets, related to Figure 2.

(A) Schematic of Climp63, p180, and kinectin. All three proteins have a single trans-membrane segment (red) and an extended coiled-coil domain (blue), as determined by TMHMM and COILs programs. Approximate amino acid positions (AAs) are all based on the human protein sequences.

(B) The localization of p180-GFP, expressed at low level, is compared with that of endogenous Climp63 and calreticulin, using confocal microscopy in BSC1 cells. P180 was detected by GFP fluorescence (left panel) and Climp63 and calreticulin with specific antibodies by indirect immunofluorescence (second and third panels). The right most panel shows a merged image. Note that p180 localizes to peripheral ER sheets and is depleted from the tubular ER and nuclear envelope. Scale bar, 10 μm.

Figure S3. ER morphology changes and protein redistribution upon disassembly of polysomes, related to Figure 3.

(A) The localization of the translocon component TRAPα and of Climp63 was visualized after 15 min of treatment with puromycin. The right most panel shows a merged image. Note that the peripheral ER sheets occupy a significantly larger surface than in untreated cells (e.g. see Figure 1D). Scale bar, 10 μm.

(B) The localization of the endogenous membrane protein calnexin was compared with that of the stably overexpressed membrane protein GFP-Sec61β after 15 min of treatment with puromycin (PURO), using confocal microscopy in BSC1 cells. The right most panel shows a merged image. Scale bar, 10 μm.

(C) As in (B), but after 15 min treatment with cycloheximide.

(D) The localization of Climp63 and TRAPα is visualized after 15 min of treatment with the translation initiation inhibitor pactamycin. The right most panel shows a merged image. Note that both proteins localize to the ER tubules (boxed area). Scale bar, 10 μm.

(E) As in (D), but showing an example in which the peripheral ER sheets occupy a significantly larger area than in wild type cells. Scale bar, 10 μm.

Figure S4. Inhibition of translation by cycloheximide or puromycin, related to Figure 3.

COS7 cells were incubated with ^{35}S-methionine in the absence or presence of 200 μM cycloheximide or 200 μM puromycin. Some cells were analyzed after 15 min, others were washed with medium and re-incubated for 15 or 30 min with ^{35}S-methionine to test for the removal of the protein synthesis inhibitors. Total protein was analyzed by SDS-PAGE and autoradiography.

Figure S5. RNAi depletion of endogenous Climp63, kinectin, and p180, related to Figure 2.

(A) COS7 cells were transfected with siRNA oligonucleotides directed against Climp63, kinectin, p180 alone, in combination, or with control siRNA. The respective endogenous protein levels were then determined by immunoblotting. TRAPα was monitored as a loading control.

(B) The distribution of endogenous TRAPα and calreticulin was analyzed in COS7 cells. Four different line scans from the nuclear envelope to the cell periphery are shown in the bottom panel. The scans were normalized in length and fluorescence intensities. Note that TRAPα is concentrated close to the nucleus.

(C) As in (B), but after RNAi depletion of Climp63, kinectin, and p180. Note that TRAPα has moved out to the cell periphery.

Figure S6. ER sheet proliferation and segregation of ER proteins upon Climp63 overexpression, related to Figure 4.

FLAG-Climp63 was expressed in COS7 cells at high levels and its localization was compared with that of endogenous calreticulin, using indirect immunofluorescence and confocal microscopy. The bottom row shows merged images. A complete series of z-sections (step size 0.25 μm) was taken with a confocal microscope. The first column shows the 3D reconstruction, the second shows an image taken near the bottom of the cell, and the last column shows an orthogonal view of the cell along the y-z axis shown by the blue line. Scale bar, 10 μm.

Figure S7. Overexpression of GFP-Climp63 induces stacked ER sheets, related to Figure 4.

(A) Thin-section electron micrograph of a COS7 cell overexpressing GFP-Climp63. Scale bar, 0.5 μm.

(B) As in (A), but with boxed area magnified.

Table S1. 25 most abundant ER membrane proteins in dog pancreas, related to Figure 2.

Dog pancreatic microsomes were treated with puromycin/high salt and alkali. Proteins were separated by SDS-PAGE and tryptic peptides were identified by mass spectrometry. Normalized spectral counts were determined by dividing the number of peptides by the total number of amino acids in a protein.

Table S2. 25 most upregulated ER membrane protein-encoding transcripts during B-cell differentiation (derived from Luckey et al., 2006), related to Figure 2.

Table S1. 25 most abundant ER membrane proteins in dog pancreas, related to Figure 2.

Rank	Normalized spectral count	Protein Name
1	0.36806	Bap31
2	0.27825	Calnexin
3	0.22566	Ribophorin II
4	**0.21252**	**p180**
5	0.21239	Dad1
6	0.20629	TRAPα
7	0.19868	TRAPδ
8	0.16850	Surfeit locus protein 4
9	**0.16611**	**Climp63**
10	0.15909	Uncharacerized XP_848469
11	0.14201	TRAPγ
12	0.13849	Ribophorin I
13	0.12395	Sec61α
14	0.12299	Tram1
15	0.11667	Signal peptidase complex subunit 3
16	0.11618	TMED6
17	0.10780	STT3
18	0.09735	Signal peptidase subunit 2
19	0.09375	Sec61β
20	0.09053	VAPB
21	0.08955	Magt1 (OST3/OST6 family)
22	0.08856	SRP Receptor β
23	0.08456	Malectin
24	0.08451	PIS1
25	0.08377	VAPA

**** Kinectin, *normalized spectral count 0.0468***

Table S2. 25 most upregulated ER membrane protein-encoding transcripts during B-cell differentiation (derived from Luckey et al., 2006), related to Figure 2.

Rank	Normalized transcript fold increase	Protein Name
1	110.8	Sphingosine-1-phosphate phosphatase 2
2	66.24	Derlin-3
3	**25.81**	**Climp63**
4	23.56	EDEM1
5	**19.17**	**p180**
6	19.16	EDEM2
7	18.32	TRAM2
8	15.59	TRAPα
9	14.78	SRP receptor β
10	13.94	Derlin-1
11	12.68	TRAPβ
12	12.43	Sec61α
13	11.61	Ire1
14	11.34	Ribophorin II
15	10.26	OST3
16	9.62	Sec63
17	9.59	TRAPδ
18	8.87	Signal peptidase subunit 1
19	8.77	IKK-interacting protein
20	8.42	SEL1 homolog
21	8.3	LASS6
22	8.26	Sec12
23	8.08	GPI mannosyltransferase 3
24	7.87	Asparagine-linked glycosylation protein 8
25	7.74	Wolframin

Supplemental Experimental Procedures

Mammalian DNA and siRNA constructs

Small interfering RNA (siRNA) experiments to deplete Climp63, p180, and kinectin were performed using 5'GGUGCAGUCUUUGCAAGCCACAUUU 3' (Invitrogen), 5' (GGCAGCAGUUGAGUGAAAU)dTdT 3'(Qiagen), and 5'(GCAGUUCCAUUCCCAGAUA)dTdT 3' (Qiagen) oligonucleotides, respectively.

FLAG-Climp63, myc-Rtn4a, HA-DP1 were described previously (Hu et al., 2009; Voeltz et al., 2006). P180-GFP was a gift from K. Ogawa-Goto. HA-Rtn4b was PCR amplified out of human cDNA using primers that included an N-terminal HA-tag, and inserted into pcDNA3.1D (Invitrogen).

Primary antibodies

The following antibodies were used: Rabbit anti-calnexin (Santa Cruz), rabbit and chicken anti-calreticulin (Abcam), mouse anti-KDEL (Abcam), rabbit anti-Sec61β (Gorlich and Rapoport, 1993), rabbit anti-TRAPα (Gorlich et al., 1990) , mouse anti-Bap31 (Alexis), mouse anti-Climp63 (Alexis), rabbit anti-kinectin (Santa Cruz or Sigma-Aldrich), rabbit anti-p180 (Ogawa-Goto et al., 2007), rabbit anti-Rtn4a/b (He et al., 2004), mouse anti-FLAG (Sigma-Aldrich), rat anti-HA (Roche), and rabbit anti-Myc (Sigma-Aldrich).

Yeast strains and constructs

The following yeast strains were used: wildtype **BY4741** (*MATa his3Δ1 leu2Δ met15Δ ura3Δ*) and **BY4742** (*MATalpha his3Δ1 leu2Δ lys2Δ ura3Δ*); *opi1Δ* expressing endogenous Rtn1-GFP, **SSY531** (*RTN1-GFP::TRP1 dsRED-HDEL opi1::kan YEplac195; SSY532 RTN1-GFP::TRP1 dsRED-HDEL opi1::kan YEplac195-RTN1*, described previously in Schuck et al., 2009); and *sey1Δyop1Δ* expressing endogenous Rtn1-GFP, **ACY66** (*MATa leu2-3, -112 his 3-11,-15 trp1-1 ura3-1 ade2-1 sey1::hyg-MX4 yop1::kan-MX4 RTN1-GFP:HIS3-MX4*).

The following plasmids were used: YIplac201/TKC-DsRed-HDEL plasmid encoding ssRFP-HDEL (a gift from B. Glick); and previously described YCplac33-RTN1 (CEN) and YEplac195-RTN1 (2 μ) (Schuck et al., 2009).

Mass spectrometry of dog pancreatic microsomes

Dog pancreatic rough microsomes treated with puromycin and high salt were prepared as described previously (Neuhof et al., 1998; Walter and Blobel, 1983). Microsomes were treated with 0.1 M Na_2CO_3, pH 11.5 for 30 min on ice and then centrifuged at 100,000 x g for 15 min. Membrane pellets were washed and 100 μg of total protein were resolved by SDS-PAGE and analyzed by tandem mass spectrometry. The abundance of all identified proteins was ranked based on normalized spectral counting which considered protein length, i.e. the ratio of the number of identified peptides to the total amino acid sequence length for a given protein (Zybailov et al., 2006). The list was curated to include only resident ER membrane proteins.

Microarray data analysis

Published microarray datasets for mRNAs upregulated during B cell differentiation (Luckey et al., 2006) were analyzed by normalizing and filtering it using the DNA-Chip Analysis software (Li and Wong, 2001). The ratios of mRNA levels of plasma B-cells were compared to that of naive B-cells for each gene on the microarray, and the P-value for these ratios was required to be less than 0.05 using a standard Student's t-test. The resultant list was annotated for all ER protein-encoding transcripts using Gene Ontology, further curated for all transcripts encoding for *bona fide* membrane ER proteins, and ranked according to their fold increase using Microsoft Excel.

Image quantification of relative protein concentration in sheets and tubules

For each cell, the fluorescent image of the protein of interest was stacked onto the image of the control ER protein GFP-Sec61β or calreticulin. Regions of interest (ROI) outlining a portion of the peripheral sheets (*sh*) or tubular ER (*tube*) were drawn. An ROI to account for background/nonspecific staining (*bg*) was also drawn either in the cytoplasm or outside of the cell. The average fluorescence intensities of these three ROIs were measured for both the protein of interest (*P*) and GFP-Sec61β/calreticulin (*GFP*),

and the ratio of fluorescence intensity of the protein of interest in sheets to tubules, C_s, was calculated using the equation

$$C_s = \frac{(P_{sh} - P_{bg})/(P_{tube} - P_{bg})}{(GFP_{sh} - GFP_{bg})/(GFP_{tube} - GFP_{bg})}$$

The ratios of 7-40 cells for each condition were compiled and the means and standard errors were calculated using Microsoft Excel.

Image quantification of protein distribution after RNAi treatment

RNAi-depleted cells were immunostained for endogenous Climp63, TRAPα and calreticulin, and the spatial distribution of raw 16-bit images of TRAPα and calreticulin were analyzed using the Metamorph's linescan function. Four linescans per cell were drawn from the nuclear envelope to the cell periphery, and relative pixel positions along the X or Y axis were normalized from 0 to1, respectively. After subtraction of average background (bg), fluorescence intensities were normalized to 1 and plotted against the normalized position using Microsoft Excel.

Image quantification of Climp63 and Rtn4b overexpression

COS7 cells transiently overexpressing FLAG-Climp63 were immunostained and images of 176 cells were taken at the same exposure time. Relative expression levels were estimated by tracing the entire outline of the ER and measuring the average fluorescence intensities for each cell; the morphology of the peripheral ER was also noted. The correlation of expression levels to ER morphologies was done by binning the data into five groups based on their average fluorescence levels using Microsoft Excel. COS7 cells overexpressing HA-Rtn4b were immunostained with HA- and Climp63-antibodies and images of 60 random cells were taken. The relative Rtn4b expression levels and resultant morphologies were determined as above. For each cell the Climp63 and HA-Rtn4b images were thresholded above background levels and converted into binary images. The resultant pixel area coverage was measured for each image to calculate the total amounts of sheets and tubules, respectively. Relative sheet size was determined by dividing the Climp63 positive area by the total peripheral ER area (the

sum of the Climp63 and HA-Rtn4b positive pixel areas). The correlations of Rtn4b expression levels to ER morphologies and sheet size were calculated using Microsoft Excel as above.

EM image quantification of the luminal width of sheets and nuclear envelopes

Thin-section electron micrographs of differently treated COS7 or S2R+ cells stained with osmium tetraoxide to visualize ER membranes were taken at 18,500 or 23,000x magnification. For each cell, the distance between the two membranes of peripheral sheets or the nuclear envelope was measured every ~50-100 nm using ImageJ. Peripheral sheets were defined as ER membranes that extended continuously for at least 0.5 μm. Measurements were then averaged for each cell, and the means and standard errors were calculated for each sample using Microsoft Excel.

Theoretical modeling of sheet vs. tubule generation

Here we derive the major equations used in the analysis of the system configurations. We use the same notations as in the main text.

Stretching energy of the edge

The edge energy is determined by the curvature-producing proteins, which generate the cylindrical membrane curvature of radius R=15nm out of the flat membrane, and directly interact with each other along the edge surface. The overall interaction between the edge proteins can have several contributions from such factors as the interaction mediated by membrane deformations (Campelo et al., 2010; Campelo et al., 2008), steric repulsion mediated by the in-plane flexibility and the related in-plane undulations of the arc-like proteins, Van der Waals interactions, and possibly, electrostatic interactions. We assume that the resultant energy of these interactions is accounted by an effective line tension, γ_0, which determines the energy per unit length of the edge. The distribution of the proteins along the edge is characterized by the translational entropy, which also contributes to the free energy of the edge. The sum of these two contributions to the free energy, related to one arc-like protein, is given by

$$f_s = \gamma_0 (l_a + l) - k_B T \cdot \ln\left(\frac{l}{l_d}\right), \quad (1)$$

where l is the distance between the arc-like proteins along the edge, l_a is the width of one arc-like protein in the membrane plane, and l_d is a length characterizing discreteness of the protein distribution whose value does not influence the results of the analysis. Minimization of the energy (Eq.1) gives an optimal distance between the proteins along the edge,

$$l_0 = \frac{k_B T}{\gamma_0}. \quad (2)$$

Based on previous estimates (Hu et al., 2008), the optimal distance is taken as $l_0 = 40\text{nm}$. Under the assumption that the thickness of a protein arc is about $l_a = 4\text{nm}$, such an optimal distance corresponds to about 10% coverage of the edge surface by the curvature-stabilizing proteins.

For small deviations of the inter-protein distance l from l_0, the energy (Eq.1) can be presented by

$$f_s = \frac{1}{2} \cdot k_B T \cdot \left(\frac{l - l_0}{l_0}\right)^2. \quad (3)$$

The total stretching energy of the edge which includes N_c proteins is given by

$$F_s = \frac{1}{2} k_B T N_c \left[\frac{L_e - N_c (l_0 + l_a)}{N_c l_0}\right]^2, \quad (4)$$

where L_e is the total length of the edge. In the relaxed state, corresponding to a vanishing stretching energy, $F_s = 0$, the total length of the edge is simply proportional to the number of the edge proteins,

$$L_e = N_e \cdot (l_0 + l_a). \quad (5)$$

The "osmotic pressure" energy of the sheet-promoting proteins

The sheet-promoting proteins are prohibited from entering the curved regions of the edges and tubes, and are restricted to the flat regions of the system. While not going into the specific interactions between these proteins, we assume that the net effect of these interactions is that each protein is associated with a net occupancy area, b, on the projected surface of the sheets. The energy contribution of adding N_s such proteins to the system is entropic in nature, and may be evaluated using the standard Florry-Huggins approach, by approximating the system to a lattice with M occupancy sites of size b

$$F_p = -k_B T \cdot \ln\left(\frac{M!}{(M-N_s)!\cdot N_s!}\right) . \quad (6)$$

The number of lattice sites, M, is proportional to the projected flat area of the system A_{flat}, which is related to the total edge length (including the tubes) by $A_{flat} = \frac{1}{2}(A_{tot} - a \cdot L_e)$, where a is the membrane area absorbed by a unit length of the edge. Since the edge is cylindrical with radius $R = 15nm$, $a = \pi \cdot R \approx 50\text{nm}$.

In the case of low concentrations of the sheet-forming proteins in the sheet plane, where the available flat area greatly exceeds the total area occupied by the proteins, $A_{flat} \gg N_s\beta$, the energy contribution from the sheet-promoting proteins becomes

$$F_p = k_B T \cdot N_s \cdot \ln\left(2 \cdot \frac{N_s b}{A_{tot} - aL_e}\right) . \quad (7)$$

The bending energy of the edge

While interaction of the curvature-producing proteins with the membrane generates the edge by compensating for the energy of its bending in the direction transverse to the sheet plane, bending of the edge in the sheet plane requires additional energy. The in-plane bending of the edge can be characterized by the curvature, c_e, of the edge line. The local value of this energy related to unit length of the edge, f_B, is given by

$$f_B = \frac{1}{2} \cdot \kappa_m \cdot c_e^2 \cdot a = \frac{1}{2} \cdot B \cdot c_e^2 , \quad (10)$$

where $\kappa_m = 20k_BT$ is the membrane bending modulus, and B is the effective in-plane bending modulus of the edge, $B \approx 1000k_BT \cdot nm$.

The total in-plane bending energy of the edge, F_B, is determined by integration of f_B along the edge line.

$$F_B = \oint f_B dL_e$$

Minimization of this energy is equivalent to computations performed by Markin, (1981).

Calculation of the edge length L_e

For systems composed mainly of large sheets, the contribution from the in-plane bending energy of the edge, F_B, is small and may be neglected. Therefore, to find the total length of the edge, the sum of the edge stretching energy and "osmotic pressure" energy has to be minimized with respect to L_e.

$$F_{edge} = F_s + F_p \qquad (8)$$

For the case where the area absorbed by the edges is much smaller than the total membrane area, $L_e \cdot a \ll A_{tot}$, the result of this minimization is

$$L_e = N_c \cdot l_0 \cdot \left(1 + \frac{l_a}{l_0} - N_s \cdot \frac{a \cdot l_0}{A_{tot}}\right) \qquad (9)$$

According to (Eq.9), the total edge length increases with the number of the edge proteins N_c and decrease with the number of the sheet proteins N_s, as expected from the qualitative consideration (see the main text). It is convenient to normalize the edge length L_e by the circumference of the disc of the same total area, $L_0 \approx \sqrt{\frac{A_{tot}}{2} \cdot \frac{1}{2\pi}}$, and use the parameter, $\Gamma = \frac{L_e}{L_0}$, which, according to (9) is equal for small absorbed areas to

$$\Gamma = \frac{N_c \cdot l_0}{\sqrt{2\pi \cdot A_{tot}}} \cdot \left(1 + \frac{l_a}{l_0} - N_s \cdot \frac{a \cdot l_0}{A_{tot}}\right)$$

Supplemental References

Campelo, F., Fabrikant, G., McMahon, H.T., and Kozlov, M.M. (2010). Modeling membrane shaping by proteins: focus on EHD2 and N-BAR domains. FEBS Lett *584*, 1830-1839.

Campelo, F., McMahon, H.T., and Kozlov, M.M. (2008). The hydrophobic insertion mechanism of membrane curvature generation by proteins. Biophys J *95*, 2325-2339.

Gorlich, D., Prehn, S., Hartmann, E., Herz, J., Otto, A., Kraft, R., Wiedmann, M., Knespel, S., Dobberstein, B., and Rapoport, T.A. (1990). The signal sequence receptor has a second subunit and is part of a translocation complex in the endoplasmic reticulum as probed by bifunctional reagents. J Cell Biol *111*, 2283-2294.

Gorlich, D., and Rapoport, T.A. (1993). Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. Cell *75*, 615-630.

He, W., Lu, Y., Qahwash, I., Hu, X.Y., Chang, A., and Yan, R. (2004). Reticulon family members modulate BACE1 activity and amyloid-beta peptide generation. Nat Med *10*, 959-965.

Hu, J., Shibata, Y., Voss, C., Shemesh, T., Li, Z., Coughlin, M., Kozlov, M.M., Rapoport, T.A., and Prinz, W.A. (2008). Membrane proteins of the endoplasmic reticulum induce high-curvature tubules. Science *319*, 1247-1250.

Hu, J., Shibata, Y., Zhu, P.P., Voss, C., Rismanchi, N., Prinz, W.A., Rapoport, T.A., and Blackstone, C. (2009). A class of dynamin-like GTPases involved in the generation of the tubular ER network. Cell *138*, 549-561.

Li, C., and Wong, W.H. (2001). Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A *98*, 31-36.

Luckey, C.J., Bhattacharya, D., Goldrath, A.W., Weissman, I.L., Benoist, C., and Mathis, D. (2006). Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. Proc Natl Acad Sci U S A *103*, 3304-3309.

Markin, V.S. (1981). Lateral organization of membranes and cell shapes. Biophys J *36*, 1-19.

Neuhof, A., Rolls, M.M., Jungnickel, B., Kalies, K.U., and Rapoport, T.A. (1998). Binding of signal recognition particle gives ribosome/nascent chain complexes a competitive advantage in endoplasmic reticulum membrane interaction. Mol Biol Cell *9*, 103-115.

Ogawa-Goto, K., Tanaka, K., Ueno, T., Tanaka, K., Kurata, T., Sata, T., and Irie, S. (2007). p180 is involved in the interaction between the endoplasmic reticulum and microtubules through a novel microtubule-binding and bundling domain. Mol Biol Cell *18*, 3741-3751.

Voeltz, G.K., Prinz, W.A., Shibata, Y., Rist, J.M., and Rapoport, T.A. (2006). A class of membrane proteins shaping the tubular endoplasmic reticulum. Cell *124*, 573-586.

Walter, P., and Blobel, G. (1983). Preparation of microsomal membranes for cotranslational protein translocation. Methods Enzymol *96*, 84-93.

Zybailov, B., Mosley, A.L., Sardiu, M.E., Coleman, M.K., Florens, L., and Washburn, M.P. (2006). Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae. J Proteome Res *5*, 2339-2347.