Endoplasmatic reticulum shaping by generic mechanisms and protein-induced spontaneous curvature.

Erich Sackmann *

Physics Department, Technical University Munich, James Franck Str.1

D85747 Garching, Germany.

Email: sackmann @ph.tum.de

Footnote: This manuscript was presented at the membrane workshop of the KITPC from May 6 to June 1.

Abstract: We describe the function of the ER shaping molecules reticulon, atlastin spastin and REED as generators and stabilisers of tubular networks of the Endoplasmatic Reticulum membranes. The formation of tubular networks is controlled by the generic mechanisms of maximizing the area to volume ratio of ER membranes in the highly crowded cytoplasmic space. The elastic energy associated with the nanotube formation is minimized by spontaneous curvature inducing proteins that form complexes acting as hydrophobic wedges. The tubular junctions may be stabilized by reticulon forming two forceps twisted by 90°. The extension into the dendrites and axons are mediated by coupling of the tubes to the microtubules which is mediated by REEP. An intriguing function of the tubular ER network is the long range stimulation of the genetic expression by calcium waves activating Ca-mediated transcription factors as suggested by Park [Park 2008]. At the end I present models of tension driven membrane fusion, (i) of bud detachment via tension driven pore formation (instead of pinching and popping).

I. Introduction:

From the point of view of physics of membrane shapes the endoplasmatic reticulum is the most astonishing and versatile cellular organelle. This has become It forms a continuous closed surface which originates in the outer nuclear shell, extends into an assembly of collapsed, disk-like vesicles (called cisternae) from which tube like extensions emanate into the cytoplasm (see Figure 3). The cisternae and the outer nuclear membrane are densely studded with ribosomes and are called rough ER while the tube like fraction is free of ribosomes and is called smooth ER. The smooth ER is assumed to be formed by newly synthesized lipids and proteins. In the resting states of cells most of the ER forms cisterna while the tubular fraction is small. The tubules form numerous branches, in such a way that a continuous closed network is formed which encloses a single luminal space. It has become evident only recently, that he astonishing manifold of shapes, that the ER can form, is made possible by a new class of proteins, such as the family of reticulons discovered by the group of Tom Rapoport [Voeltz et al, 2006]..

The ER of different cells can differ remarkably. Secretory cells (as those of the pancreas) exhibit extended stacks of rough ER. Liver cells exhibit an extensive network with few ribosomes which is enriched in enzymes serving the metabolism, detoxification of

biosynthetic products and exogenous substances. Uniquely, muscle cells are packed with a specialized form of ER: the sarcoplasmic reticulum (SR), which serves as reservoir for Ca^{2+}.The tubular fraction of the ER is extremely extended in nerve cells where it protrudes into the tips of the dendrites and even the axon (see Figure 3 below and [Park 2008]. Due to the interconnection of the tubules by stable junctions, the local fluctuations in calcium concentration, caused by the numerous processes in the dendrites (which are controlled by second messengers), are rapidly equilibrated. Experiments by SH. Park and coworkers suggest that local fluctuations in calcium in the dendrites can propagated through the tubules with a speed of 30 μm/sec and travel from the tips of the dendrites to the nucleus within fractions of a second. This could result in the Ca-triggered genetic expression [Park 2008].

Before I describe our present knowledge of the basic processes of tubular network formation and introduce some physical concepts, I summarize basic processes of intracellular trafficking by vesicles to point out some analogies between this cellular process and ER shaping mechanisms.

II. A short intermezzo on intracellular trafficking by budding-fusion-fission chains of events,

One of the most fascinating cellular processes is the rapid material exchange between intracellular organelles, the import of nutrition by endocytosis and the export of waste or hormones by exocytosis which is mediated by vesicles (see Fig 1a). Central enigmas of these processes are (i) how the budding and fission of vesicles from one organelle (say the plasma membrane) and their fusion with the specific target membrane (say the cis-Golgi membrane) controlled or (ii) how the characteristic composition of the membranes and the lumen of each compartment is maintained during the material exchange (see also lecture by Patricia Bassereau, reference [Sorrera 2012] or the review [Sackmann 2006]).

Another open question is whether the actin cortex plays a role for fission. Indeed, coupling of the actin cortex to the coated pits could evoke a tension in the neck of the invagination and thus drive vesicle fission, for instance by pore formation (see model in Fig. 9). Recent experiments by Merrifield et al [Merrifield 2005] show that cortactin mediates the binding of F-actin to clathrin and enhances the efficiency of membrane scission at the coated pits drastically. Cortactin is activated via phosphorylation, by tyrosine kinases or serine/threonine kinases, in response to extracellular signals like growth factors

The processes associated with endocytosis processes have fascinated physicists since some 10 years as an example of the sorting of lipids and proteins by coupling of membrane curvature and phase separation (see [Andleman 1987], [Sackmann 1995]) or by the concept of hydrophobic matching between the lipid bilayer and the membrane bound part of integral membrane proteins ([Ben Shaul,1995], [Sackmann [2006]). With the discovery of the role of the BAR domains as curvature inducing agent [Gallop 2005] and the unconventional GTPase Dynamin as fission promoter [Stowell 1999] deeper insights into the molecular mechanisms controlling the budding and fission have been gained. In contrast the physical basis of lipid and protein sorting is not established well experimentally (see Lecture by Patricia Bassereau, Appendix B of Reference [Sackmann 2006] or The Supplementary Chapter 9 accessible over www.biophy.de)

The material import by cells (endocytosis) is mediated by caveolae and coated pits. Both are formed by local curvature induced by macromolecular adsorption but they transport materials to different targets. In the case of coated pits the spontaneous curvature is often assumed to be triggered by the adsorption of the coat protein clathrin [Bruinsma 1998] but it has been postulated that due to the stiffness of the clathrin network, the spontaneous curvature is mainly induced by adaptor proteins which expand the inner leaflet of the PM by insertion of their lipid anchors. Clathrin is recruited to the buds by binding to the adaptor proteins (see Figure 1b [Sackmann 2006]). Its role is most likely to stabilize the buds and to mediate their coupling to other cytoplasmic proteins guiding the transport of the coated vesicles. In 2001 it has indeed been shown that clathrin binds to actin via the specific actin binding protein HSP1. This dumbbell-shaped protein exposes two actin binding domains at a distance of about 60 nm and its central section binds to clathrin . It can thus mediate the embedment of coated vesicle into the actin cortex [Engqvist-Goldste 2001], [Kaksonen 2012] [Ferguson 2009].

Coated vesicles mediate also the transfer of vesicles from the ER to the trans Golgi complex and from the cis-Golgi to the PM. However the budding is mediated by the coat proteins COP1 and COP 2, respectively [Rothman 1996]. It is obvious that by using different coat proteins the transport vesicles obtain their specific signature which guides them to the right target organelle.

Fig 1b shows a simplified model of the budding process triggered by the recruitment of the adaptor proteins AP2 (possibly together with A180, [Ford 2001]) to the membrane micro-domains loaded with receptor (such as transferrin described in [Sackmann 2006]). Spontaneous curvature is induced by the insertion of lipid anchors which are exposed after activation of the adaptor by GTPases. Model membrane studies, as that shown in Fig 2, suggest that the budding is requires the formation of clusters of the receptor (such as transferrin). In Fig 2 the budding of vesicles composed of DMPC is triggered by lateral phase separation of the (thermo-sensitive) macro-lipid which is mediated by accordion-like lateral contraction of its hydrophilic chain above a transition temperature T_c. While in the expanded state the macrolipid is distributed homogeneously and the vesicle shape is only slightly deformed, it undergoes lateral phase separation at $T > T_c$ resulting in the formation of buds. The expansion of the cytoplasmic leaflet by insertion of hydrophobic tails drives also the budding of caveoli. The coat protein caveolin exposes two hydrophobic chains that form hairpin loops that seem to bind most readily in membranes containing cholesterol (see Sackmann 2006].

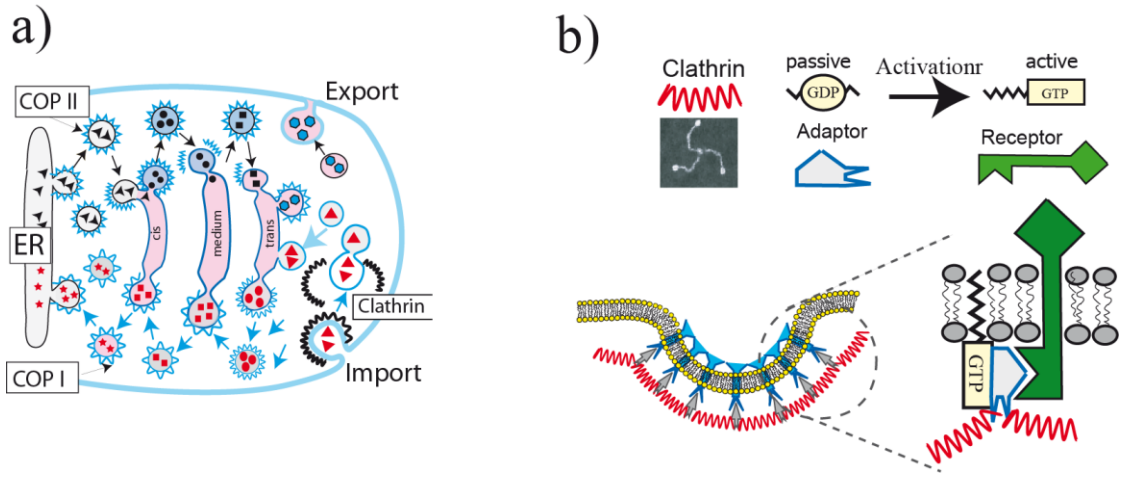


Figure 1: (a) Schematic view of bi-directional material transport mediated by vesicles. Proteins (such as insulin) synthesized in the ER are transported via the three Golgi sub-systems cis, medium and trans to the plasma membranes (PM) and secreted by exocytosis. Each step is associated with a budding-fission-fusion sequence of events. In the Golgi many proteins are conditioned for their function (such as the transformation of pro-insulin to insulin). The budding is generally assumed to be induced by the adsorption of coat proteins. As illustrated in (b) some spontaneous curvature is also triggered by the membrane anchoring of the adaptor proteins recognizing specific membrane bound proteins (such as transferrin receptors). Each organelle has its own set of coat proteins and associated adaptors. The transfer from the ER to the cis Golgi and in the reverse direction is mediated by the coat proteins COPII and COP I, respectively. The import via the PM is mediated by clathrin and its adaptors.

(b) The top shows on the left side an electron micrograph of a ternary complex of clathrin, the triskeleon, which assembles into the coat. The knobs at the end bind to the adaptor. The adaptor binds to the membrane by electrostatic forces, specific anchoring to phosphoinositides (PIP-(4.5)P2) and by fatty acid anchors (which are exposed after activation by GTPases). It recognizes also membrane bound receptors (such as transferrin receptors shown in the image at the bottom. (For structural details see review by [Edeling 2006]

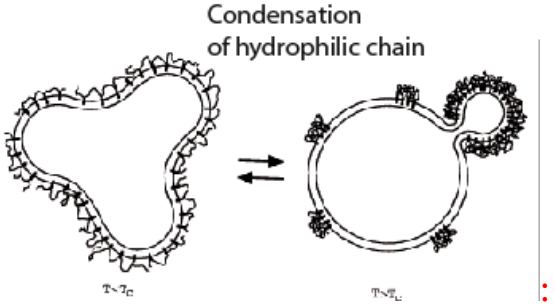


Figure 2: Induced membrane budding of vesicle composed of DMPC and doped with the macro-lipid N-isopropylacrylamide (NIPAM). In the low temperature state the hydrophilic chains are extended. The macro-lipid is distributed continuously, resulting in a soft shape change of the liposome. Budding occurs only if the hydrophilic chain collapses and forms clusters by lateral phase separation. Reference: J. Simon et al Chem. Phys. Lipids 76 241 (1995)

The second essential step of the budding-fission-fusion sequence is the formation of narrow necks between the bud and the bulk of the plasma membrane exhibiting radii of $r_{neck} \approx$ 30 nm. In the direction perpendicular to the axis of the neck, the radius of negative curvature can be even smaller (~15nm see [Sackmann 2006]). The bending energy associated with the neck formation in lipid bilayers is small and is generally neglected. This is likely no longer true for receptor loaded buds with membrane penetrating protein trunks. This could be one reason for the introduction of a new class of helper proteins such as endophilin and amphyphysin (see lecture by Patricia Bassereau and [Sorrea 2012]). These proteins exhibit protein domains (called BAR-domains) which form banana shaped dimers. From the two ends of each BAR monomer protrude pieces of amphiphatic helices which are tethered to the dimer through flexible peptide sequences (see [Gallup 1996] Figure 8). In this way the long axes of the helical segments can orient in arbitrary directions with respect to the long axis of the banana. For that reason, the banana shaped complexes can form a helical collar around the cylindrical neck, thus enforcing a positive curvature (see Glossary and [Kozlov 1999], [Stowell 1999], [Ford 2001]). We will discuss possible mechanisms of vesicle fission at the end.

III. **Generic and specific driving force of tubule formation- Analogies to tubular networks of microemulsions**.

Figure 3a: shows a schematic view of the Endoplasmatic Reticulum of nerve cells. The ER consists of two fractions: first, the rough ER which is studded with ribosomes (the biosynthesis machines) and forms disc shaped vesicles (called cisternae) which are penetrated by passages (worm holes) and second networks of interconnected tubules. Cisternae with worm holes can be formed by heterogeneous lipid membranes composed of territories of positive and negative spontaneous curvature [Gozdz 1999]. The bending energy of a disc-shaped lipid membrane, with zero curvature of the two flat regions, of disc radius R and a radius r of the rim can be easily estimated from the mean curvature $2 < C >= R^{-1} + r^{-1}$ by the bending elasticity model. For r<<R one obtains $\Delta G_{disc} \approx \pi^2 \kappa R / r$. This result shows that the rim energy is the smaller the larger the discs. Typical values are: R≈ 500 nm and r≈ 25 nm [Lei 2009], resulting in a bending energy of $\Delta G_{disc} \approx 5\,10^4 k_B T$ (for κ=50 k_BT).

During cell division (mitosis) the nuclear envelope has to be transiently dissolved. High resolution electron microscopy tomography experiments showed that this occurs in the pro-metaphase of the cell cycle by the partitioning of the lipid protein bilayer fraction of the nuclear envelope into the ER. Despite of this process, the bulk of the ER remains mainly organized as extended cisternae from the pro-metaphase until the teleophase when both cells start to form their nuclear membrane again. Only a very small fraction remains organized as tubules. In contrast, during interphase, when the cells prepare for the new division by synthesizing new materials including lipids and membrane proteins, the ER forms a network of small cisternae interconnected by a network of tubules [Lei Lu 2009]. In the second part of the lecture I discuss the physical basis of this drastic change in the topology of the ER.

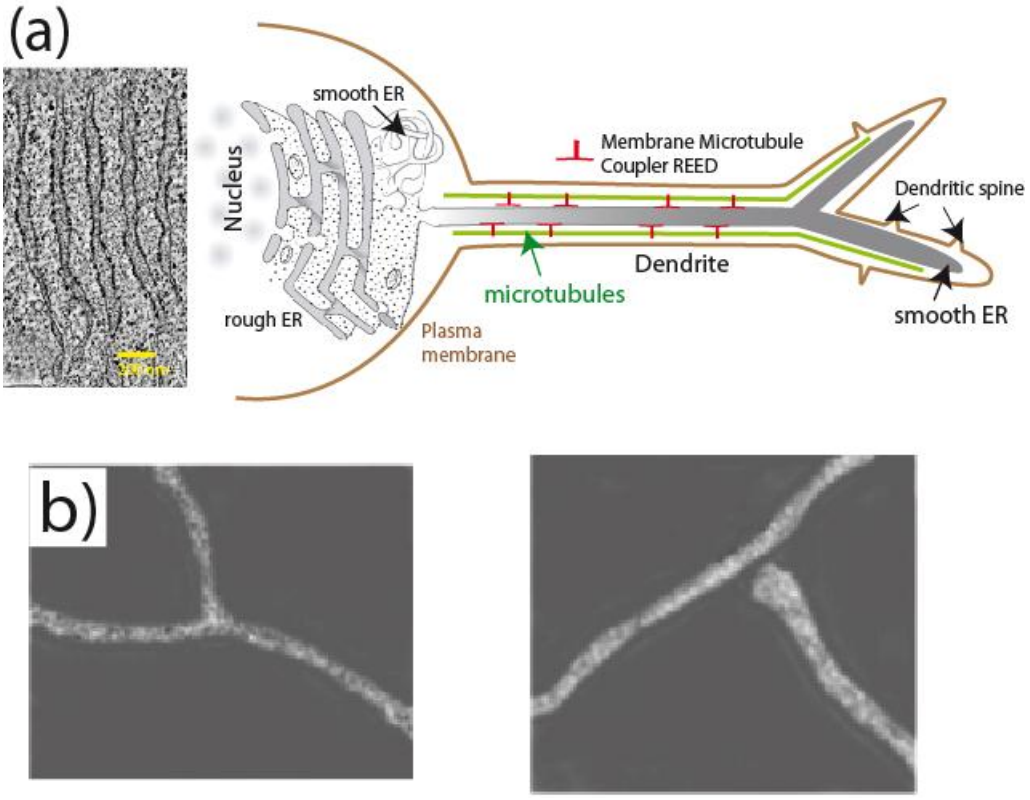


Figure 3: To the morphogenesis of the ER (a) Model of tubular ER network extending from the cell body into cellular protrusions such as axons and dendrites (Image redrawn following [Park 2008]). c) Bottom: electron micrograph of tubular network formed by the amphiphile. Image reproduced from [Safran 2000] with the permission of the author.

The formation of tubular networks allows cells to synthesize new membranes, while minimizing the expansion of the cell as the following consideration shows. The area to

volume ratio A/V depends sensitively on the shape of the organelles. For tubular shapes with radius r, spherical shapes of Radius R and cisterna forming prolate ellipsoids of rim radius ρ and thickness δ, respectively, the change of the ratio is

$$\frac{A}{V} \approx \frac{1}{3R} \qquad \frac{A}{V} \approx \frac{2}{r} \qquad \frac{A}{V} \approx \frac{3}{2\delta}$$

Clearly, the shape with the largest area at a given inner volume V, is the tubule, followed by the cisternae. Any change of the ER-membrane area in the highly crowded cytoplasmic space would cause a drastic increase in hydrostatic pressure unless the cell volume is increased simultaneously. By the formation of tubular networks the increase in volume is minimized. As noted above, another biological driving force for tubule formation is the requirement to bring the ER close to the dendrites of neurons or even to the tip of axons (see [Park 2008] and [Park 2010].

The network of interconnected tubules is reminiscent of the cubic phases or tubular networks frequently observed in micro-emulsions formed by amphiphiles in aqueous solutions. Transitions between lamellar phases (similar to cisternae), tubular networks, cylinders or spheres can be mediated by simply varying the spontaneous curvature [Safran 2002]. As shown in Figure 3b the tubules formed by monolayers of amphiphiles can also form junctions, very similar to the ER-tubules. However, the spontaneously formed junctions are constantly broken and reformed which is mainly a consequence of the entropy associated with the restriction of tube conformations by the junctions [Safran 2000].

It is well known that by varying the concentrations and types of lipids as well as the area-to-volume ratio lipid bilayers could in principle also form branched tubular network, coexisting with mother vesicles (see [Käs 1991]). More recently it has been shown that within giant vesicles filled with polymer mixtures (polyethylene glycol and dextran) long tubules can form if the polymer mixture undergoes phase separation [Li 2012]. All these shape changes mediated by generic mechanism cannot be reliably controlled and the spontaneously formed junctions must be stabilized. The question, how nature achieves this goal will be addressed in the following.

IV. The ER-shaping proteins: A short characterization of their structure and function.

We consider first the elastic energy associated with the three networks. The mechanical energy to bend a plate of area 1x1 μm (consisting of about 10^6 lipid molecules) into a tube of radius r~50 nm is of the order of $\Delta G_{ela} \approx \kappa A / r^2$ and the tube would be 6μm long. The bending modulus of the envelope of intracellular organelles (containing about 30 mole % cholesterol) is $\kappa \approx 2\,x10^{-19} J$ [Lipowsky and Sackmann 1995, Ch.5] resulting in elastic energy costs of $\Delta G_{ela,tube} \approx 2x10^4 k_B T$. The bending energy of spherical shapes does not depend on the radius and is $\Delta G_{ela,sphere} = 8\pi\kappa$ or about 12 k_BT. As noted above, the bending energy of the cisternae, which is determined by the rim, is of the order 10^4 k_BT.

Sharp edges with radii of curvature of about 25 nm in pure lipid bilayers are associated with a substantial bending energy since the hydrophobic moiety of the lipid bilayer is strongly distorted. This can be reduced by the formation of defects in the liquid crystalline order of the bilayer (see [Sackmann 1995]). As noted above the bilayer thickness in the radial direction of the bent is larger than that of the flat parts of disc shaped-vesicles. Typically the radii of the necks between cells is R <50 nm and the radius of the negatively curved transition between the bud and the bulk of the plasma membrane is r ~30 nm. The total energy of the neck is

$$\Delta Gela \propto \frac{2\pi^2 \kappa r}{R} \approx \frac{36x10^{-19}\,3x10^{-8}}{5x10^{-8}} J \approx 500 k_B T$$

The area of the neck is of the order of $A = 2\pi R\pi r \approx 2.2x10^{-14} m^2$. It contains about $2x10^{-14}/0.65x10^{-18} \approx 3x10^4$ lipids and the bending energy per lipid molecules is thus rather small about 0.02 k_BT. Bending energy costs were obviously not the main reason for the evolutionary pressure enforcing the introduction of a new class of ER shaping proteins. New proteins were most likely introduced to control the topology of the ER and the budding, fission and fusion processes in a robust way, such as through cell signaling processes controlling the activity of the ER-shaping by phosphorylation-dephosphorylation reactions. Another important function is the stabilization of the long membrane tubes by coupling to the microtubules bundles.

The role of the superfamily of reticulons as ER-shaping proteins was discovered by Tom Rapoport [Voeltz 2006] and coworkers. They showed that addition of these proteins to suspensions of vesicles (extracted from cells) induce the self-organization of extended tubular networks. They further showed that the reticulons share an unusual, membrane penetrating and hairpin-shaped peptide sequence and postulated their role as curvature inductors. Reticulons gained great interest in connection with recent research on the hereditary spastic paraplegia (HSP): an inherited disease whose main feature is a weakness and spontaneous contractions (spasticity) in the lower limbs. It turned out that more than 50% of the hereditary spastic paraplegia (HSP) cases result from mutations in three proteins involved in shaping the ER, namely **atlastin-x** (also known as SPG3A), **spastin** (SPG4), **REEP-x** (= receptor expression enhancing protein x or SPG31) and members of the **reticulon** protein family, also called **DP1/Yop1p** (see Glossar, [Voeltz 2006] and [Park 2010]). Mutations in the multifunctional protein spastin, account for 40 % of the cases. In the following we first summarize the main features of the reticulons.

Spastin is a huge protein (of 616 amino acids) performing many functions, some of which are indicated in the image below. It exhibits a hydrophobic domain that can form hairpin-like membrane anchors (see Figure 4 below). The C-terminal harbors an AAA-ATPase that can form a hexamer complex which acts as mechanical device for severing microtubules (see Glossar and [Blackstone 2010]). The monomers bind to microtubules via a specific binding motif (MTB). Finally, the hydrophobic domains (HR) serve the complex formation with the other ER shaping proteins. Thus, spastin exhibits a binding site for members of the atlastin proteins which mediate the coupling of the tubules to microtubules.

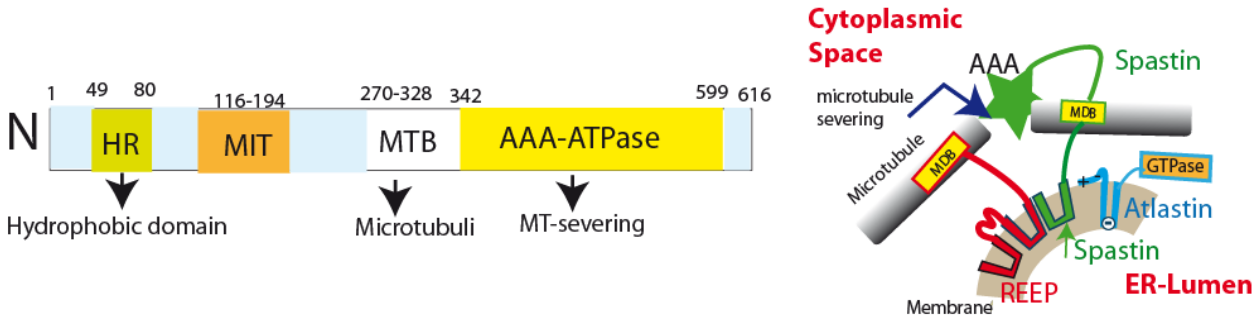
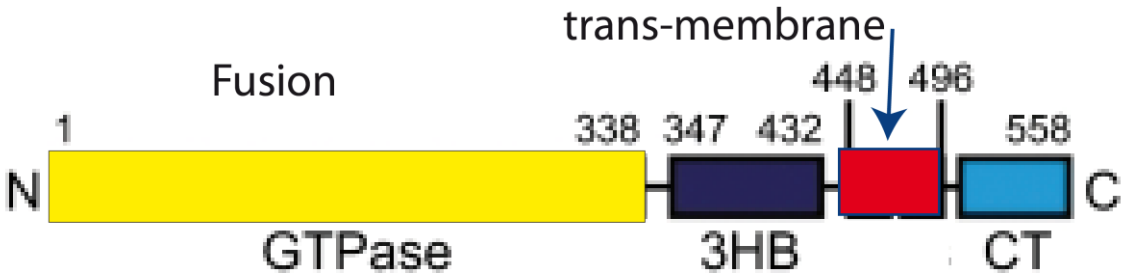


Figure 4: Left: Domain structure of the multifunctional protein spastin. The functions of the domains are indicated and will be described below. Right: summary of supramolecular complex of ER shaping proteins formed by complex formation between the hydrophobic domains of the ER shaping proteins.

Atlastin-1 The protein (558 amino acids) consists of three parts shown in the image below: a N-terminal domain (amino acids 1-338), a middle domain (347-432) which is followed by the hydrophobic, membrane spanning part (448-496) and a C-terminal domain (497-558) [Byrnes 2011], [Bian et al 2011]. The membrane spanning part consists of two hydrophobic segments of 20 amino acids separated by a negative peptide. Each of the hydrophobic segments could form a α-helix of 3.5 nm length. They match the hydrophobic thickness of the ER-bilayer and can orienting normal to the membrane plane. It could thus not act as curvature inductors. The atlastins can form complexes with the other ER-shaping proteins (as shown in Fig 4). The main function of atlastin is to mediate fusion of the ER membranes which is mediated by their N-terminal domain. It belongs to the family of dynamin-related GTPases which is also involved in the division of cell organelles. The fusenogetic function of atlastin is discussed below (see Figure 9)



REEP-1 to REEP-6**:** Both proteins of the REEP family expose two hydrophobic domains which can form two hairpin-like domains acting as membrane anchors (see Figure 6 below). However, the isoforms exhibit remarkable differences which determine their functions [see Park 2010]. REEP- 5 and REEP-6 exhibit two, about equally long hydrophobic domains which can form two hairpin-like membrane anchors. The REEP-1 to -4 harbor also two membrane anchors but one of these is much shorter than the other. The major difference

of the two groups of isoforms is the exposure of a microtubule binding domain MBD by the shorter isoforms REEP-1 to REEP-4.

***Reticulons**:* Major ER- shaping protein involved in generating of highly curved tubules and the stabilization of junctions. All members exhibit a highly conserved section comprising 190 amino acids in the C-domain. Ubiquitous family members (200 kDa) are: Rtn4a/NogoA and Rtn4b/NogoB. The two hydrophobic segments exhibit 36 and 32 amino acids. A minimum of two amino acids is needed to form a hairpin. Therefore the maximum length of the hairpins would be l ~ 2.7 nm and l~ 2.3 nm, if one assumes that two amino acids are required for the formation of the turn. The hydrophobic thickness of the bilayer is about 3nm. They could both form hairpin-like membrane spanning shapes with the N-and C-ends pointing in the cytoplasmic space and the turns of the hairpins penetrating into the polar region of the luminal membrane leaflet. A remarkable feature is the polybasic sequence of the Clear evidence has been provided that the N-terminal domain forms indeed a hairpin [Voeltz 2006], [Kiseleva 2007]. The orientation of the domain close to the C-end is not so clear and it could also penetrate into the lumen.

Molecular model of *reticculon function as curvature inducing proteins*

The function of reticulons as driving element of tubule formation has been established in several publications [Voeltz 2006]. Most direct evidence is provided by Figure 5d showing that, the reticulon Rtn4a/NogoA is only distributed in the tubular fraction of the ER. This follows from the observation that red and green labeled ER structures are clearly separated.

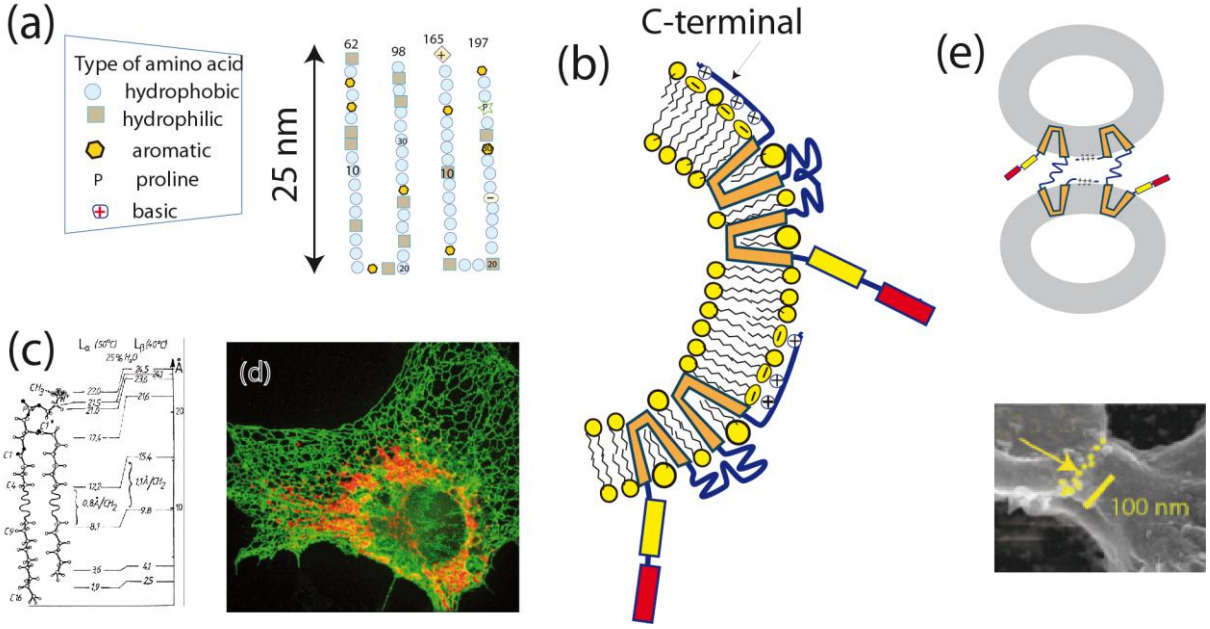


***Figure 5:** Structure and function of reticulons.*
(a) Primary structure of the two hydrophobic domains close to C-terminal. The 36 (and 32) amino acids attributed to the hydrophobic segments could form α-helices of 5.4 nm (and 4.6 nm) total lengths or a hairpin conformation with arms of about 2.5 nm (and 2.3 nm) length.

The thickness of the hydrophobic part of the fluid lipid bilayer (that is in the L_α-phase) is about 2.6 nm (see (c)). Note that the two hydrophobic domains are separated by a hydrophilic sequence of 65 amino acids and that the C-end exposes poly basic residues. It can thus adsorb to the cytoplasmic membrane surface which contains about 10mole % acidic lipids.
(b) Molecular model of membrane binding of the hydrophobic domains (see text.
(c) Molecular structure of phospholipid shown for comparison.
(d) Transformation of sheet-like into tubular ER by transfection of cells (cell lines COS7) with reticulon Rtn4a/NogoA-Myc (labeled green). The sheet like fraction of the ER is labeled with the specific marker Climp-63 (rot).The lack of yellow areas very strongly suggests that reticulon is only present in the tubular fraction of the ER. The image is a gift from unpublished work by the group of Tom Rapoport, Similar images are found in [Voeltz 2006].
(e)Possible model of linking of vesicles by reticulons (see text below). The bottom shows a electron micrograph of two connected vesicles The reticulons are visualized by antibody labeling. Figure reproduced for the lecture from [Karatekin 2003].

Figure 5a shows the primary structure of the hydrophobic domains. The longest segment close to the C-end could form α-helical hairpins with arms of 2.7 nm length. This is about equal to hydrophobic thickness of a bilayer composed of DPPC (which exhibits 16 CH_2 groups, see (c)). The membrane of the ER is composed of 40% DPPC and contains 40 % unsaturated lipids. The hydrophobic thickness could thus be smaller. This could give rise to a hydrophobic mismatch which could be accommodated by tilting the two arms with respect to the membrane normal. The wedge like structure of the protein could in principle induce a spontaneous curvature. The turn of the hairpin exhibit amino acids with hydrophilic or aromatic residues which both interact strongly with the semi-polar end of the lipid molecules at the luminal monolayer. Another remarkable feature of the peptide chain is the presence of three basic residues at the segment close to the membrane surface (shown in (b)). This structural feature is expected to enforce the membrane binding of the hydrophobic section of reticulons since the negatively charged lipids of the membranes are accumulated at the outer leaflet of the ER-membrane, as indicated in Figure 5b.

The reticulons could fulfill two other important tasks. One function of the reticulons could be to mediate the formation of complexes with the other proteins (such as spastin and REEP-4) by interaction between the hydrophobic domains. In this way complex membrane bound machines could be formed which control the binding and scission of microtubules similar to that shown in Figure 6. A second possible function is the stabilization of junctions between tubules as will be shown below. Finally, owing to the long hydrophilic segments separating the two the two hydrophobic hairpins, the reticulons could even link different vesicles. Some experimental evidence for such a role is the finding that the reticulons accumulate at the interface between two small vesicles of of chains of vesicles (see

Molecular model of ER shaping by REEP-X (REEP: receptor expression enhancing protein-1)

These ER-shape controlling proteins are structurally related to the reticulons of the DP1/Yop1p family. They are essential for the curvature induced formation of ER tubular

networks and its association with the microtubules. In REEP-null mutants the tubular network is abolished. As suggested by the amino acid sequence of the hydrophobic domains (see Figure 6a) the hydrophobic domains of the REEP protein-4 could form two hairpin-like α-helices which penetrate half through the lipid bilayer. The peptide sequence connecting the hairpins exposes a polybasic domain which could enforce the membrane binding of the protein. A second possible role of the REEP-4 proteins is to mediate the formation of supra-molecular functional complexes with other ER-shapers, in particular the fusion mediator atlastin and the MT severing protein spastin. According to Park et al [Park 2010] the function of REEP-x is closely connected with their capacity to form complexes with the spastin. This protein exhibits a single hydrophobic domain which could form a hairpin of 1.2 nm length and thus penetrate half the membrane. Most remarkably, the helical segments exhibit two positive amino acids at about the same position. A closer inspection of the REEP-4 protein shows that the hydrophobic domain at the C-end exhibits two negative charged, which are located at the same distance from the membrane surface as the positive charges of spastin. Therefore the complex between REEP-4 and spastin can be stabilized by salt bridges.

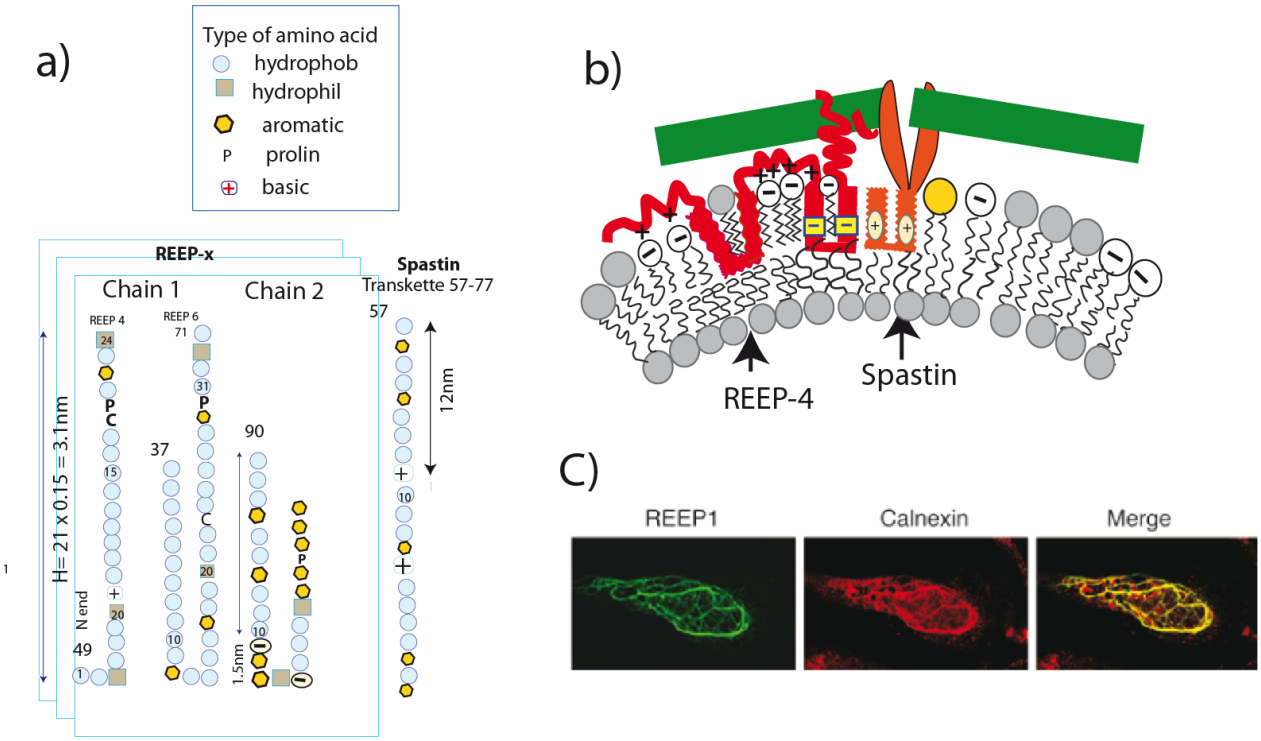


Figure 6: Structural features of REEP-x family of ER-shaping proteins. (a) Primary structure of membrane binding domains. Note that the chains C1 of REEP-1-4 and REEP- 6 differ in length by 9 amino acids, while the shorter chain C2 exhibits the same length. The chain number 2 contains two basic residues. As shown in (b) the charged residues of the chains of REEP-4 and spastin are located at about the same distance from the luminal membrane surface and could form complexes stabilized by salt bridges. (b) Schematic view of complex formed by electrostatic interaction between the C2-chain of REEP-4 and the hydrophobic hairpin of spastin. Since REEP-4 exhibits a microtubule binding sequence the two proteins can form a nano-machine for the cleavage of microtubules [Park 2010]. (c) Demonstration of the assembly of ER-tubules with microtubules mediated by REEP-1. REEP-1 is labeled with

GFP and the ER-membranes by calcineurin, an integral protein of the ER membrane with chaperon function. The yellow color indicates the regions were the ER tubules and microtubules overlap. The microfluorescence images were reproduced from [Park 2010].

As suggested by Figure 6b the aggregation of REEP-4 and atlastin could be mediated by the electrostatic interaction between the negative residues of the REEP chain and the basic AAs of the spastin chain. Note that the membrane anchor of spastin and the shorter hairpin segment of REEP-4 exhibit about the same length and that the position of the basic and acidic amino acids are located at about the same distance between enabling the formation of two salt bridges. The compensation of electric charges in the hydrophobic domain of membranes is important. The energy cost for the transfer of a single isolated positive charge into the hydrophobic region of the membrane would be of the order of several 20-30 k_BT which would prevent the penetration of the hydrophobic segments into the bilayer. However, if the aspargic acid is dissociated a salt bridge could form. The energy of a salt bridge buried in protein folds is typically of the order of $w \approx$- 15 kJ/M (or $30k_BT$ per bridge) and this energy gain would thus be more than suffcient to compensate for the self- energy of the positive lysine charge in a hydrophobic environment, which is about 20-30 k_BT.

***Molecular organization of the microtubule severing protein spastin*.**

Spastin exists in a long and a short isoform: Both contain the C-domain with the microtubule severing AAA-ATPase followed by the microtubule binding domain (MTB) which forms a triple-helical structure. The membrane binding domains of the N-terminal are missing.in the shortened form (M87, where M stands for missing segments) which therefore resides in the cytoplasm. As shown in Figure 6a the hydrophobic domain is rather short and could form a hairpin composed of two α-helices of 1.2 nm length. They can penetrate halfway through the lipid bilayer of the ER membrane and induce the spontaneous curvature. Spastin fulfills many functions, mostly together with the other members of ER shaping proteins (see Figure 6). The hydrophobic domains could mediate the complex formation of spastin with atlastin, REEP and reticulon.

Junction formation by reticulons.

A major question is how nature solved the problem of generating stable junctions between tubules with radii of about 50 nm. In this case one deals with a complex surface topology where membranes meet with differently oriented curvatures. Figure 7a shows a model how reticulons could help to facilitate this saddle point topology. The hydrophilic segment separating the two hairpin consist of 67 amino acids which would be sufficient to generate a minimum radius of curvature of R~ 10 nm at the edge of the junction. One of the two forks could thus insert into the horizontally and the other into the vertically oriented tube while the hydrophilic segment forms a random coil. The spontaneous curvature could then again be induced by complex formation with the other ER shapers/

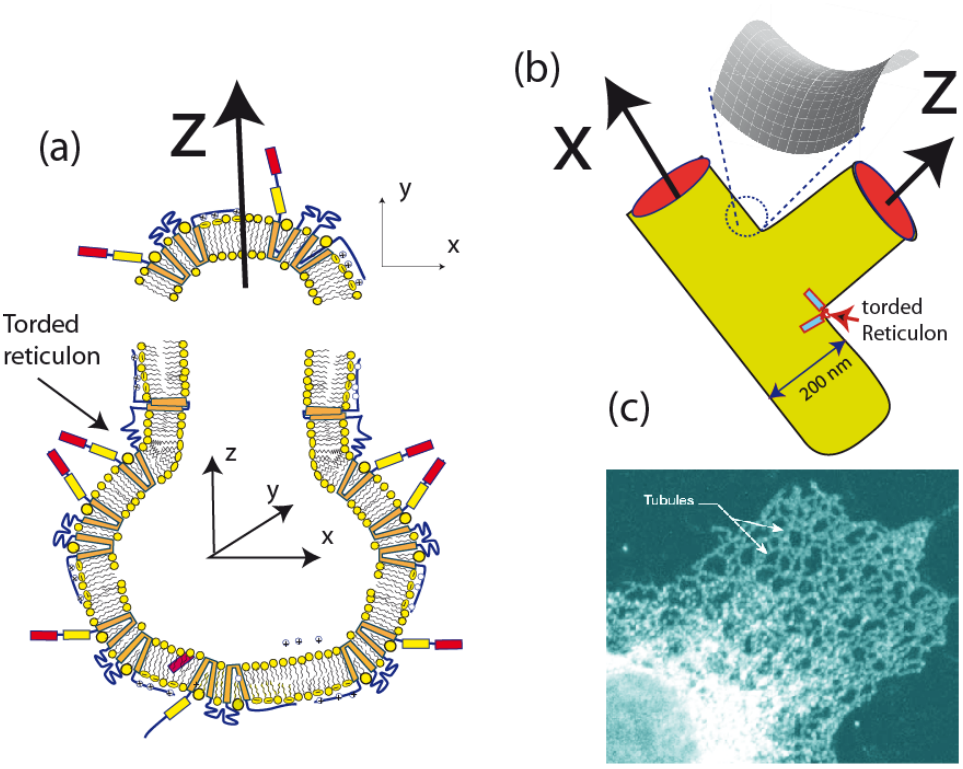


Figure 7. Model of reticulon mediated stabilization of junctions where two tubes of different orientation meet. Owing to the long hydrophilic connection between the two hairpins one of these segments could penetrate into the horizontally and the other in the vertically oriented tube. (c) Fluorescence micrograph of fibroblast like COS7 cells showing the tubular network with the reticulons labeled with fluorescent antigens. Figure 7b was reproduced from the work of Park and Blackstone [Park 2010] with permission by the authors.

Atlastin the membrane fusion protein*: *Fusion of ER-membranes by atlastin and SNARE/SNAP complexes share common features.

A major function of atlastin is the fusion of ER-membranes] [Orso 2009], [Bian et al 2011]. The ongoing fusion of separated segments of the ER is essential for the generation of the continuous membrane system shown in Figure 3. Similar to the reticulons, membrane binding of atlastin is mediated by two hydrophobic domains at the C-terminal (residues 48-96 which are long enough to form a hairpin of α-helices which penetrate the lipid bilayer. The segment at the turn of the hairpin contains a charged peptide (see Figure 9).

Atlastin belongs to a new class of fusion-mediating proteins which triggers fusion of identical organelles (a process called homotype fusion). The process is driven by a different energy source than the fusion of secretory vesicles with the target membranes, such as the presynaptic fusion with the plasma membrane. The presynaptic process is mediated by members of the SNARE/SNAP protein family. The fusion is generally assumed to be

triggered by the formation of a tight complex between the extracellular domains of SNARE and SNAP and their strong binding to the membrane (see Figure 9a). In contrast the fusion mediated by atlastin is powered by the hydrolysis of GTP, providing typically 30kJM^{-1}. The ER also harvests SNARE proteins, but they serve the transport of recycled vesicles to the ER. The role of atlastin as fusion mediator was demonstrated by the finding that removal of the 25 amino acid long stretch of the C-terminal domain results in the formation of fused lumbs of ER-assemblies (see [Orso 2009]). A prerequisite of fusion is the formation of dimers by complex formation between the N-terminals (with the GTPase function).

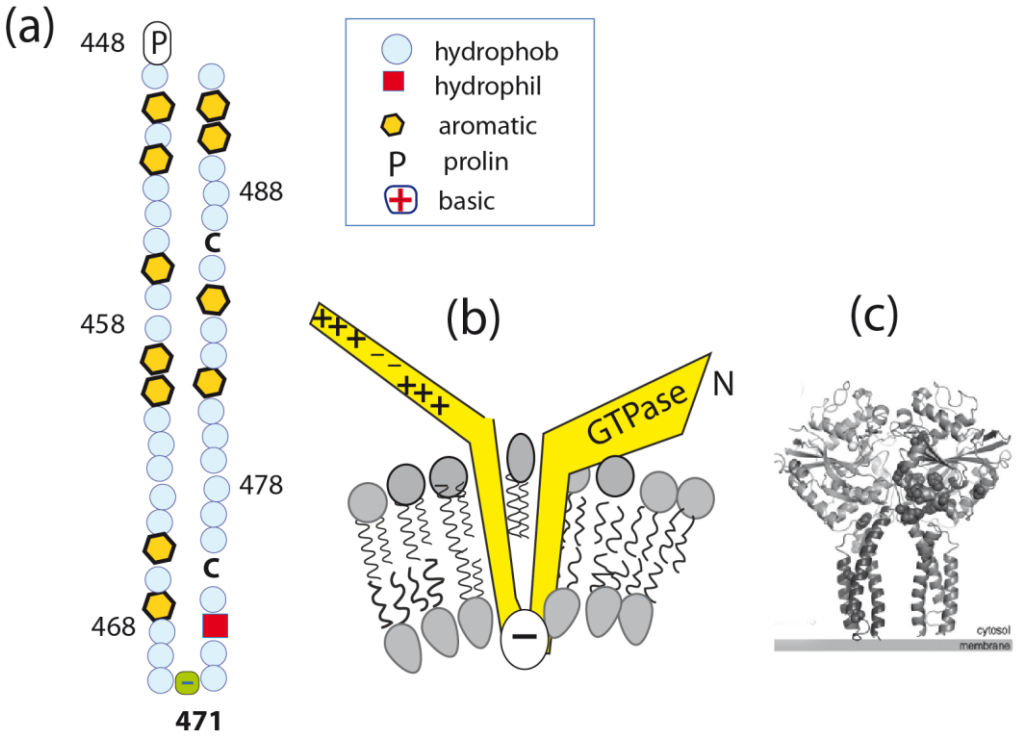


Figure 8: (a) molecular model of atlastin in the non-hydrolyzed state of GTP after [Byrnes 2011]. (b) Hairpin like conformation of the membrane anchoring domains. These are composed of segments comprising 24, mostly hydrophobic, domains. The lengths of the hairpins are about 1.8 nm. Their turns containing hydrophilic (black squares) and aromatic AAs (hexagons) could just penetrate into the semi-polar region of the bottom monolayer. (c) Schematic model of membrane binding of atlastin. Image reproduced from [Bian et al 2011.

A molecular model of the fusion mediated by SNARE/SNAP and Atlastin is shown below. It is assumed that the fusion process in both cases occurs in two steps called hemi-fusion: first the two monolayers facing the outer medium of both organelles are disrupted, resulting in the formation of a diaphragm consisting of a bilayer and the two inner monolayer while in the second step the diaphragm formed by the residual bilayer disrupts. In Figure 9 we present a model of the fusion process based on the idea that it is driven by membrane tension gradients, as first proposed by Sam Safran and coworkers [Kuhl 2001]. The local tension is

generated by the tight binding of the SNARE/SNAP complex and the subsequent adhesion of the two membranes mediated by the adsorption of the complex to the membrane surfaces. The gain in adhesion energy per unit area is a measure for the lateral tension, which is also called spreading pressure [Bruinsma 2000].

The model of the SNARE/SNAP mediated process is based on numerous experimental studies. In particular it appears rather well established that the fusion proceeds via hemifusion. A key role for this pathway is played by the peptide sequence penetrating the bilayer which exposes a hydrophilic tail into the luminal space and could act as a spring (see [Langosh 2001], [Sackmann 2006]). In the case of atlastin the positively charged C-terminal penetrates into the cytoplasmic space and could bind to the negative lipids by electrostatic forces. A common feature with the SNARE/SNAP system could be that the conformational change of the GTPase induced by GTP hydrolysis results in the attraction of the two membranes (see top of Figure 9b). Since the hemifusion process requires a membrane penetrating segment, the fusion by atlastin mediated process could proceed by direct pore formation, whereby the polybasic peptide loop could stabilizes the sharp curvature at the edge of the pore.

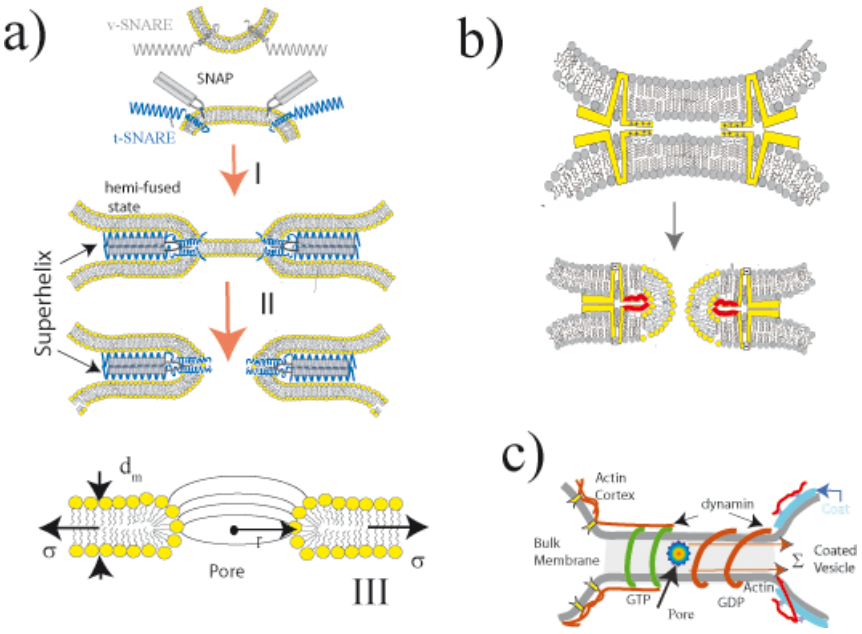


Figure 9: (a) Molecular models of membrane fusion mediated by SNARE/SNAPas described in the text (reproduced from [Sackmann 1996)]) (b) Model of atlastin-mediated fusion by direct pore formation, triggered by GTP hydrolysis. (c) The bottom shows a model of the tension gradient generated by the local activation of dynamin as explained in the text below. Note that the coat proteins are connected to actin filaments which could also contribute to the lateral tension mediated by the cell envelope.

Below, a previously proposed model of hemifusion by the SNAP/SNARE system is discussed (see [Sackmann 2006]). The formation of the bilayer diaphragm is a continuous process which can occur by hydrodynamic flow of the inner lipid monolayers towards the rim

of the diaphragm. It has been proposed that hemifusion can be caused by lipid packing density gradients generated by tensions arising far from the hemifusion zone. Such tensions can be caused by adhesion mediated by Ca-ions or by light osmotic swelling of one of the vesicles [Kuhl 2000] and [Held-Safran 2002]. A similar model has been applied to explain the fusion mediated by the SNARE/SNAP protein pair (see Figure 9 [Chermomordik 1995], [Langosch 2001]). The fusenogenes have to fulfill two tasks. First, they must bring the membrane into close contact and second they must provide the driving force for the fracture of the diaphragms separating the juxtaposed bilayers. The strong adhesion of the two membranes by the collar of SNARE/SNAP complexes is well established. The open question is, what drives the fracture of the diaphragm separating the two luminal spaces.

One likely possibility (shown in figure 9a) is that the driving force for the opening of passages between coupled membranes is provided by the free energy W gain by adsorption of the extracellular SNARE–SNAP domains to the membranes, where W is a measure for the binding energy of the SNARE/SNAP complex. This creates a tension gradient between the SNARE/SNAP covered region and the bilayer of the diaphragm. The tensiondifference is of the order $\delta\sigma = \sigma_0 - W$, where σ_0 is the lateral tension of one or both of the bulk membranes [Karatekin 2003]. The fracture of the membranes in the diaphragm can be described in terms of the formation of pores in bilayers. The free energy of this process can be expressed as $G_f = G_0 + 2\pi\Gamma r - 2\pi r L\sigma_e$, where r is the radius of the hole, σ_e is the effective membrane tension and Γ is the line tension (the energy cost per unit length to generate a hole through the hydrophobic film). The third term on the right side accounts for the gain in energy due to the relaxation of the membrane tension by the growth of the opening. For the first step of the fusion process, the retraction of the initially adhering monolayers (resulting in the hemi-fused state) an additional energy gain term g_{ad} (measured in energy per unit area) has to be added. It accounts for the gain in energy by coupling of the hydrophobic brushes of the two monolayers. One can easily see that the free energy first increases with the radius r of the pore, reaches a maximum and decreases again. The retraction of the inner and the outer monolayers occurs spontaneously at the critical radius $r \geq r^*$. The critical radius r* and the activation energy ΔG^* are obtained by minimizing the free energy leading to (see [Sackmann2006]).

$$r^* = \frac{\Gamma}{\delta\sigma + g_{ad}} \qquad \Delta G^* = \frac{\Gamma^2}{\delta\sigma + g_{ad}}$$

These equations leads to the following conclusion: at a given membrane tension the two fusion steps (the formation and fracturing of the bilayer diaphragm) can be controlled by different mechanisms. The first step leading to the hemi-fusion state is facilitated by the adhesion of the hydrophobic surfaces of the monolayers associated with the energy gain g_{ad}. Conversely, the fracture of the bilayer diaphragm is promoted by the tails of the trans-membrane domains of the SNAREs and SNAPs penetrating in the lumina of the vesicles (see Figure 9a). Insertion of experimental data in the above equations yields a critical radius of r*~1 nm for the second step of the fusion process [Sackmann 2006]. This corresponds to the area covered by only 4 lipid molecules. This small value of r* may be the reason why fusion of the membrane under physiological conditions is only possible by an additional pulling force which is mediated by the membrane penetrating peptide sequences of SNAP and

SNARE. As demonstrated in several papers, removal of these segments or their replacement by lipid anchors abolishes the hemifuison [McNew 2000], [Langosch 2001]. Therefore it has been postulated that the hydrophobic peptide sequence can stretch by a factor of two and act like a spring, generating a pulling force on the bilayer.

A final note:Fission can be triggered by lateral tension-induced pore formation in nanotubes.

The GTPase dynamin (and other members of this superfamily of membrane shaping proteins) play an essential part in endocytosis by catalyzing the fission of clathrin-coated vesicles from the plasma membrane. Convincing evidence has been provided that the fission of the neck mediated by dynamin is triggered by the GTP hydrolysis of the dynamin associated GTPase (see [Stowell 1999] and the Glossary for a summary of the dynamin domain structure and function). Two mechanism of the fission process have been discussed: the pinchase and the poppase model. The former model postulates that the neck is contracted resulting in the pinching-off of the vesicle [Kozlov 1999]. The latter model is based on the assumption that the membrane of the neck is elongated by tangential forces generated by the ring like arrangements of the dynamin. Electron microscopy studies show indeed that the dynamin aggregates form helical arrangements around the neck and that the pitch of the helix is increased by the energy liberated by the GTPase hydrolysis. However, it does not provide an explanation why and how the membrane breaks. It is not clear how the tube of 25 nm diameter can be contracted much more. There are two softer mechanisms which can cause the destabilization of the nanotubes.

- One is suggested by model membrane studies with phospholipid vesicles

containing high cholesterol contents (similar to the cholesterol content of plasma membranes). Buds formed by slight thermal expansion of the vesicle area are unstable in the presence of > 40 mole % and detach form the mother vesicle. Schmidt et al [Schmidt 1999] provided some evidence that the destabilization of the neck could be mediated by lipids exhibiting highly unsaturated arachidonic acid chains. The fourfold unsaturated fatty acid in all cis-conformation are not stretched as chains with one or two double bonds but can form hook-like shapes. They could destabilize the membrane by accumulating within the neck similar to cholesterol. However, recent studies provided evidence that the generation of the arachidoyl-phosphatidyl-phosphatidic acid was due an artifact [Gallop 2005]. On the other side, older studies of the fission of COP coated vesicles from Golgi organelles in cell free systems showed that coated pits readily detached from the Golgi membrane in the presence of coenzyme A (CoA) with bound fatty acids [Ostermann 1993]. This is an amphiphatic molecule with a large head group that is supposed to destabilize membranes. In summary, fission by amphiphatic solutes is certainly possible. However, it could not be controlled by cell signals.

- The second mechanism is based on the tension induced pore formation.

This is an activated process since it is associated with a high activation energy required for the formation of a hydrophilic ring around the rim of the holes. We assume that fission occurs if the radius of the pore ρ exceeds a critical value ρ^* and growth spontaneously. We consider the dynamin covered neck of radius R between the bulk membrane stiffened by the actin

cortex and the coated vesicle. Pore formation is well known to be accelerated if the membrane tension Σ is increased or if the line tension Γ is reduced by amphiphatic solutes. An excess membrane tension could be generated by the stretching of the helical dynamin assemblies along the tube axis triggered by a local GTPase hydrolysis as shown in Figure 9b. The energy cost for pore formation is given by:

$$\Delta G = 2\pi\rho\Gamma - 2\pi RL\Sigma$$

were L is the length of the neck The first term accounts for the energy required for the pore formation, where Γ is the line tension of the rim. The second term stands for the energy gain after the tension is relaxed within the neck by the fission. As in the case of the fracture of the diaphragm of the hemi-fusion state (see Figure 9a), the energy costs increase with ρ until a critical value is reached above which it grows spontaneously until the neck breaks. The critical radius is given by

$$\rho^* \approx \frac{RL\Sigma}{\Gamma}$$

The line tension of pure lipid bilayers is $\Gamma \approx 10\,pN$ [Karatekin 2003]. For a neck of L=1μm and R=30 nm, a moderate excess membrane tension of $\Sigma \approx 1\,\mu N/m$ would be sufficient to generate an unstable pore of radius $\rho^* \approx 10nm$ and thus promote vesicle fission. Fission of the neck could also be facilitated by reducing the line tension Γ. Γ could for instance be reduced by the short amphiphatic helix which is coupled to the N-terminus of endophilin through flexible peptide segments (see [Gallop 2005]).

In summary, fission could well occur both by dynamin mediated lateral tensions and by solute accumulating at the neck. In particular, the above equation predicts that the activation energy of pore formation could be drastically reduced by amphiphatic solutes, such as fatty acids, or by peptide segments such as the amphiphatic helix of endophilin. The above model of fission shows that both generic mechanisms could work together or compete with each other.

Acknowledgement: The problem of ER shaping proteins has been brought to my attention by Guenther Woehlke (Physics Department E22 TUM). I am most grateful for helpful criticismss by Tom Rapoport who pointed out some important shortcomings in the original manuscript. The lecture notes were partially written at ITTP during my stay at the membrane conference in May 2012 and as guest at the Lehrstuhl for Angewandte Physik of the Ludwig Maximilian University. Moreover, very helpful comments by Sam Safran from Weizmann Institute on the stabilization of the tubular junctions are gratefully acknowledged.

References.

[Andleman 1987] (a) Leibler, S., Andleman, D. (1987). "Ordered and curved meso-structures in membranes and amphiphilic films". Journal de Physique **48:** 2013 -2018. (b) Kawakatsu, T., et al. (1993) Phase transitions and shapes of two component membranes and vesicles I: strong segregation limit J. Phys. II France **3**: 971-997

[Ben Shaul 1995] Ben-Shaul A. (1995) Molecular theory of chain packing elasticity and lipid protein interaction in lipid bilayers
Handbook of Biological Physics vol 1A, ed R Lipowsky and E Sackmann (Amsterdam: Elsevier)

[Bian et al 2011] Xin Bian et al. (2011) Structures of the atlastin GTPase provide insight into homotypic fusion of endoplasmic reticulum membranes PNAS **108:** 3976–3981,

[Bruinsma 1998] Mashl, R., Bruisnma R. (1998) Spontaneous-curvature theory of clathrin-coated membranes. Biophys. Journal. **74:** 2862–72.

[Bruinsma 2000] Bruinsma, R., Behrisch A., Sackmann, E. (2000) Adhesive switching of membranes: Experiment and theory. Phys. Rev. E **61:** 4253-4267

[Byrnes 2011] Byrnes, LI. Sonderman H. (2011) Structural basis for the nucleotide-dependent dimerization of the large G protein atlastin-1/SPG3A)
Proc Natl Acad Sci USA **108:** 2216–2221.

[Chermomordik 1995] Chermomordik, L. *et al* (1995) The hemifusion intermediate and its conversion to complete fusion: regulation by membrane composition. Biophys. J. **69:** 922–929.

[Edeling 2006] Edeling, MA. et al. (2006) Life of clathrin coat: insights from clathrin and AP structures. Nature Rev. Mol. Cell Biol. 7. 32-44

[Engqvist-Goldste 2001] Engqvist-Goldstein, Å., (2001) The actin-binding protein Hip1R associates with clathrin during early stages of endocytosis and
promotes clathrin assembly in vitro. J. Cell Bio. 154: 1209-1224.
,
[Ferguson 2009] Ferguson, S., et al. Coordinated Actions of Actin and BAR Proteins Upstream of Dynaminat endocytic clathrin-coated pits. developmental cell **17:** 811–822,

[Ford 2001] Ford MG et al. (2001). "Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes". Science **291:** 1051–5.

[Gallop 2005] Gallop J. et al (2005) Endophilin and CtBP/BARS are not acetyl transferases in endocytosis or Golgi fission. Nature **438:** 675-678

[Gozdz 1999] Gozdz WT., Gompper G. (1999) Shapes and Shape transformations of two-component membranes of complex topology. Phys.
Rev. E **59:** 4305-4316.

[Hed-Safran 1999] Hed, G., Safran S.A. (2003) Initiation and dynamics of hemifusion in lipid bilayers. Biophys. J. **85**: 381–389

[Hu 2011] Hu, J. et al. (2009) A Class of Dynamin-like GTPasesInvolved in the Generationof the Tubular ER Network. Cell **138:** 549–561.

[Käs 1991] Käs, J., Sackmann, E. (1991) Shape transitions and shape stability of giant phospholipid vesicles in pure water induced by area-to-volume changes. Biophys. J. **60:** 825-844.

[Kaksonen 2012] Kaksonen, M. et al (2012) Harnessing actin dynamics for clathrin-mediated endocytosis. Nature Rev. Mol. Cell Biol. **7**: 404-414

[Karatekin 2003] Karatekin, E. et al (1990) Cascades of transient pores in giant vesicles: line tension and transport. Biophys. Journal **84:** 1734-1749

[Kiseleva 2007] Kiseleva, E., et al. (2007) Reticulon 4a/NogoA locates to regions of high membrane curvature and may have a role in nuclear envelope growth. J. Structural Biol. **160:** 224–23.

[Klemm 2011], Klemm Bian X et al. (2011)Structures of the atlastin GTPase provide insight into homotypic fusion of endoplasmic reticulum membranes.
Proc Natl Acad Sci U S A. 108:3976-81.

[Knorr et al 2012] Knorr, R., et al. (2012) Curvature of double membrane organelles generated by changes in membrane size and composition. Plos One **7**: e32753

[Kozlov 1999] Kozlov, MM. (1999) Possible mechanism of "Pinchase" action. Biophys. J. **77:** 604–616.

[Kuhl 2001] Safran S, Kuhl T and Israelachvili J. (2001) Biophys. J. **81**: 659–65

[Langosch 2001] Langosch D. et al (2001) "Dimerisation of the glycophorin A trans-membrane segment in membranes probed with the ToxR transcription activator". J. Mol. Biol. **311:** 709–21

[Lei Lu 2009] Lei Lu et al. [2009] Cisternal organization of the endoplasmic reticulum during mitosis. Mol. Biol. Cell **20:** 3471-3480.

[Li 2011] Lia Yanhong et al (2011) Membrane nanotubes induced by aqueous phase separation and stabilized by spontaneous curvature. PNAS **108:** 4731–4736

[McNew 2000] McNew J. et al (2000) Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. J. Cell Biol. **150**: 105–17

[Merrifield 2005] Merrifield CH. et al. (2005) Coupling between clathrin-coated-pit invagination, Cortactin recruitment, and membrane scission observed in live cells
Cell. 121: 593–606.

[Morin-Leisk 2011] Morin-Leisk, J. et al. (2011) An intramolecular salt bridge drives the soluble domain of GTP-bound atlastin into the postfusion conformation J. Cell Biol. **195:** 4 605-615 J. Cell Biol. Vol. 195 No. 4 605–615

[Orso 2009] Orso G, et al. (2009) Homotypic fusion of ER membranes requires the dynamin-like GTPase Atlastin. Nature **460:**978–983.

[Osterman 1993] Ostermann, J. et al (1993) Stepwise assembly of functionally active transport vesicles. Cell. 75: 1015-1025

[Park 2008] Park, SH. et al. (2008) The endoplasmic reticulum as an integrator of multiple dendritic events. Neuroscientist **14:** 68-77

[Park 2010] Park, SH. (2010) Hereditary spastic paraplegia proteins REEP1, spastin, and atlastin-1 coordinate microtubule interactions with the tubular ER network. Journal of Clinical Investigation**. 120:** 1097-1110 .

[Park 2010] Park, SH., Blackstone C. (2010) Further assembly required: construction and dynamics of the endoplasmic reticulum network. EMBO Rep. **11:** 515-21.

[Rothman 1996] Rothman, JE. Wieland, FT. (1996). Protein sorting by transport vesicles. Science **272**, 227-234

[Sackmann 2005] Sackmann E 1995 Physics of Vesicles Handbook of Biological Physics, Chapter V in Vol. 1A, ed. R Lipowsky and E Sackmann (Amsterdam: Elsevier)

[Safran 2000] (a) Tlusty T. et al (2000) Topology, phase instabilities, and wetting of microemulsion networks Phys. Rev Letters **84:**1244.
(b) [Safran 2002] Zilman, AG., Safran S. (2002) Thermodynamics and structure of self-assembled networks. Phys Rev E **66:** 051107

[Safran 2001] Safran, SA. (2001) Polymer-induced membrane contraction, phase separation, and fusion via Marangoni flow. Biophys. J. **81:** 2001 659–666.

[Schmidt 1999] Schmidt, A. et al. (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature.**401:** 133-41.

[Simson 1998] Simson, R. et al. (1998) Membrane bending modulus and adhesion energy of wild-type and mutant cells of Dictyostelium. Biophys. J. **74:** 514-522

[Sorrea 2012] Sorrera, B. et al. (2012) Nature of curvature coupling of amphiphysin with membranes depends on its bound density. Proc. Natl. Acad. Sci. USA 109: 173–178

[Stowell 1999] Stowell M.H.B. (1999) Nucleotide-dependent conformational changes in dynamin: evidence for a mechanochemical molecular spring. Nature Cell Biol. **1**: 27 - 32

[Voeltz 2006] Voeltz, GK. et al. (2006) A Class of membrane proteins shaping the tubular endoplasmic reticulum. Cell **124**: 573–586.