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Review

# Weaving the Web of ER Tubules

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**How is the characteristic shape of an organelle generated? Recent work has provided insight into how the tubular network of the endoplasmic reticulum (ER) is formed. The tubules themselves are shaped by the reticulons and DP1/Yop1p, whereas their fusion into a network is brought about by membrane-bound GTPases that include the atlastins, Sey1p, and RHD3.**

Membrane-bound organelles in eukaryotic cells have characteristic shapes. Some are relatively spherical, whereas others consist of flat sheets or a tubular network. How organelles are shaped is a fundamental problem in cell biology, but surprisingly, until about a decade ago this question had not been addressed (Dreier and Rapoport, 2000; Hermann et al., 1998; Chen et al., 2003). The endoplasmic reticulum (ER) is particularly attractive for studying the morphogenesis of an organelle, as it contains distinct, but contiguous domains. The ER consists of the nuclear envelope and the peripheral ER, which in turn is composed of a polygonal network of tubules and interdispersed sheets (Baumann and Walz, 2001; Shibata et al., 2006). A recent study of the peripheral ER in yeast revealed that it is a complex mix of fenestrated sheets of various sizes connected by tubules (West et al., 2011). The peripheral ER is very dynamic; the tubules continuously form and disappear, and the sheets rearrange (Du et al., 2004; Griffing, 2010). In mammals, the tubular ER network extends throughout the entire cell, whereas in yeast and plant cells, it is located close to the plasma membrane and is referred to as "cortical ER" (Griffing, 2010; Prinz et al., 2000). In all eukaryotic cells, the ER plays an important role in key processes, such as the biosynthesis of secretory and membrane proteins, lipid synthesis, and $Ca^{2+}$ storage.

The relative amounts of ER sheets and tubules vary greatly among different cell types (Shibata et al., 2006). For example, sheets are prominent in "professional" secretory cells, such as pancreatic cells or B cells, and tubules predominate in adrenal cells. It is likely that peripheral ER sheets correspond to rough ER domains, which are regions of the ER membrane to which polysomes are bound. These ER domains are thus specialized in synthesizing secretory and membrane proteins. The tubular ER contains some bound ribosomes, but it is uncertain whether large polysomes can be accommodated on their highly curved surfaces. Instead, tubular structures may be the preferred site for lipid synthesis, $Ca^{2+}$ signaling, vesicle budding, and contact to other organelles, such as mitochondria and lipid droplets.

Recent results have provided important insight into how the different domains of the ER are generated and maintained, with most of the progress related to the mechanisms by which the tubular ER network is generated. Specifically, we now have some understanding of how the tubules themselves are formed and how they fuse to generate a three-dimensional network. Here we discuss these recent advances and point out unresolved issues.

## The Cytoskeleton

Many ER tubules are formed on the basis of their connection with the cytoskeleton. In mammalian cells, ER tubules are pulled out of membrane reservoirs by being attached to the tips of growing microtubules or by associating with molecular motors that move along microtubules (Du et al., 2004). This second mechanism, called sliding, is more prominent and significantly faster, and it occurs primarily along acetylated microtubules (Friedman et al., 2010). In yeast and plant cells, the ER is also dynamic, but the movements occur along actin filaments (Griffing, 2010; Prinz et al., 2000). Ultimately, however, the alignment of ER tubules with the cytoskeleton is not perfect. In addition, the ER network does not disassemble upon depolymerization of the actin filaments in yeast cells, and it collapses only with some delay upon depolymerization of microtubules in mammalian cells (Prinz et al., 2000; Terasaki et al., 1986). Finally, it is possible that ER tubules can be generated from vesicles, as observed in vitro (Dreier and Rapoport, 2000). Thus, the cytoskeleton is probably not required for maintaining the shape of ER tubules. Instead, as we will discuss below, curvature-stabilizing proteins play a critical role.

How ER membranes associate with the cytoskeleton or molecular motors remains elusive. The integral ER protein STIM1 has been proposed to interact with the tips of growing microtubules (Grigoriev et al., 2008). It harbors a motif in its cytoplasmic tail with which it interacts with EB1, a microtubule plus-end tracking protein. However, because STIM1 only moves to the plasma membrane upon $Ca^{2+}$ depletion from the ER, it remains unclear whether it is involved in the constitutive dynamics of the ER. The mechanism by which ER membranes attach to molecular motors is entirely unknown. Some proteins implicated in maintaining ER morphology (REEP1, Climp-63,

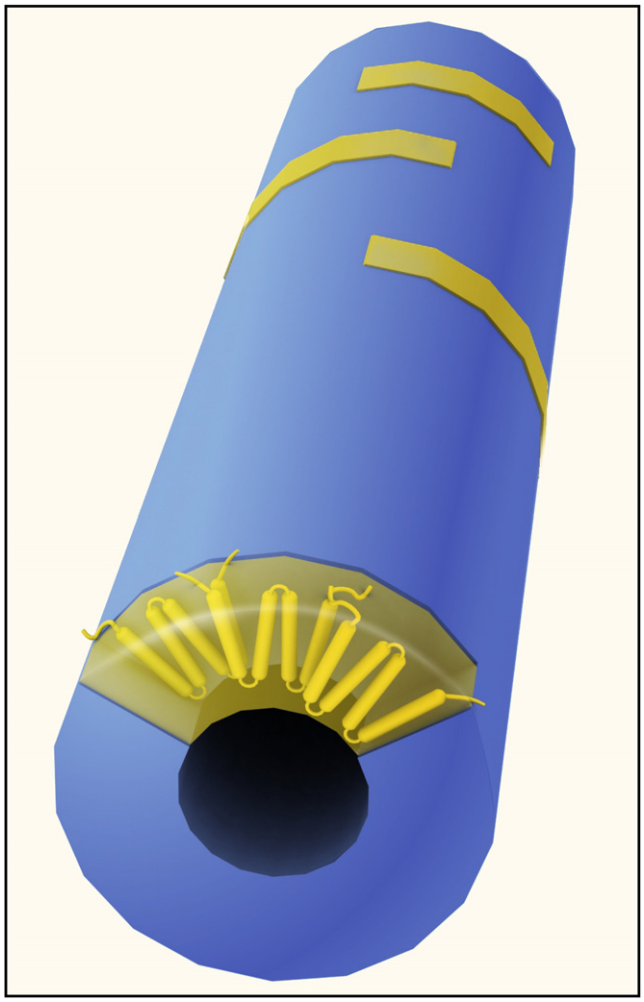

**Figure 1. The Reticulons and DP1/Yop1p Shape ER Tubules**
The cut-away view shows the double hairpin structure of the proteins proposed to form a wedge. Several of these molecules would form an arc-like structure around the tubule (indicated by yellow strips).

p180, kinectin; Shibata et al., 2010) have microtubule-binding domains, but their significance remains to be clarified.

## Curvature-Stabilizing Proteins

ER tubules are characterized by high membrane curvature in cross-section, as their diameter is relatively small (~30 nm in yeast and ~60 nm in mammals). Thus, there must be mechanisms that stabilize this high-energy state. To identify proteins involved in stabilizing membrane curvature and ER tubule formation, an in vitro assay was used, in which the formation of an ER network could be recapitulated, starting with small vesicles derived from *Xenopus leavis* eggs (Dreier and Rapoport, 2000). Network formation is inhibited by reagents that modify cysteines in membrane proteins. The target of these reagents is reticulon 4a (Rtn4a), confirmed by the inhibitory effect of antibodies on network formation (Voeltz et al., 2006). Rtn4a belongs to the reticulon family, members of which are probably found in every eukaryotic cell. The conserved region is a domain of ~200 amino acids, which contains two relatively long hydrophobic sequences (30–35 amino acids) that each sit in the membrane as a hairpin, with little exposure to the lumenal side of the membrane. The reticulons interact with members of another protein family, called DP1 or REEP5 in mammals and Yop1p in *Saccharomyces cerevisiae* (Voeltz et al., 2006). The DP1/Yop1p family is again ubiquitous. Although not sequence related to the reticulons, these proteins also contain a conserved domain with two hydrophobic hairpins in the membrane.

The reticulons and DP1/Yop1p localize preferentially to the tubular ER and to the highly curved edges of sheets (Shibata et al., 2010; Voeltz et al., 2006). Deletion of these proteins in yeast or their depletion in mammalian cells converts most ER tubules into sheets, indicating that they are required for tubule formation. One of these proteins, if present in adequate amounts, is sufficient to maintain normal ER morphology in yeast (Voeltz et al., 2006). The deletion of the reticulons and Yop1p causes only a moderate growth defect in *S. cerevisiae*, indicating that yeast cells can manage with only a small amount of tubules. However, similar experiments in *Caenorhabditis elegans* result in a marked reduction of embryonic viability (Audhya et al., 2007), suggesting that the tubular ER plays a more important role in higher organisms. The overexpression of the reticulons or DP1/Yop1p leads to long tubules, which become resistant to the collapse that normally follows the depolymerization of microtubules, indicating that these proteins can stabilize ER tubules (Shibata et al., 2008). The reconstitution of purified members of the reticulon and DP1/Yop1p families into proteoliposomes transforms the vesicles into tubules (Hu et al., 2008). Thus, these proteins are both necessary and sufficient for tubule formation.

The reticulons and DP1/Yop1p might stabilize the high curvature of ER tubules seen in cross-section by utilizing two cooperating mechanisms, hydrophobic insertion (wedging) and scaffolding (Shibata et al., 2009) (Figure 1). The hydrophobic insertion mechanism is based on the two hydrophobic hairpins in these proteins, which are proposed to form a wedge-like structure that displaces the lipids preferentially in the outer leaflet of the lipid bilayer, thus causing local curvature. The scaffolding mechanism assumes that the reticulons and DP1/Yop1 form arc-like oligomers that mold the lipid bilayer into tubules (Figure 1). Arc-like scaffolds, rather than rings or spirals that wrap around tubules, are consistent with the observation that the reticulons also localize to sheet edges (Shibata et al., 2010). Because arcs are not entirely encircling a tubule, they would not block the long-distance diffusion of other membrane proteins, explaining the high mobility of these proteins observed in fluorescence recovery after photobleaching (FRAP) experiments. Calculations show that arc-like oligomers would have to occupy only ~10% of the total membrane surface to generate near perfect tubules, in agreement with estimates on the abundance of the curvature-stabilizing proteins in *S. cerevisiae* (Hu et al., 2008). Oligomers of the reticulons and DP1/Yop1p have indeed been identified by crosslinking, gradient centrifugation, and FRAP experiments, and mutants with oligomerization defects do not exclusively localize to tubules and are unable to generate tubules in vitro (Shibata et al., 2008). The membrane-embedded hairpin structures appear to mediate the intermolecular interactions.

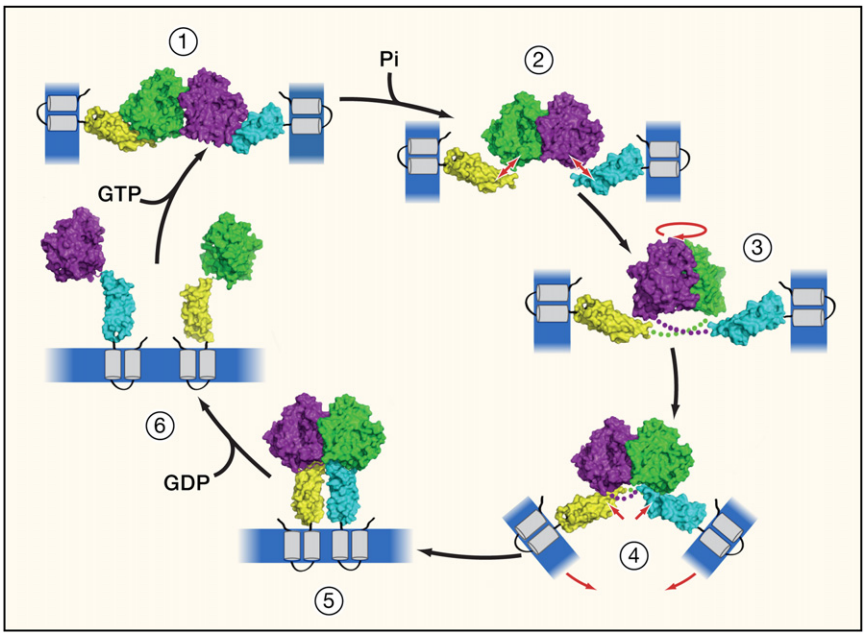


**Figure 2. The Fusion Reaction Mediated by the Atlastins**

The mechanism of membrane tethering and fusion is based on two crystal structures (state 1 [PDB 3QOF] and state 5 [3QNU]) and biochemical experiments. The atlastin (ATL) molecule in one membrane is colored with its GTPase domain in green and the helix bundle in yellow. The ATL molecule in the apposing membrane is colored with its GTPase domain in purple and the helix bundle in cyan. Upon GTP binding, the two ATL molecules dimerize and tether the membranes. GTP hydrolysis then causes conformational changes that pull the membranes together. Finally, GDP is released to reset the fusion machinery.

## Atlastin GTPase-Mediated Homotypic ER Fusion

Once ER tubules are formed, they need to be connected into a network, which requires the fusion of identical membranes, a process called homotypic fusion. Much progress has been made in understanding this reaction, which turns out to be quite different from heterotypic fusion mediated by SNAREs or viral proteins.

In metazoans, the homotypic fusion of ER membranes appears to be mediated by the atlastins (ATLs), a class of GTPases that belong to the dynamin family (Hu et al., 2009; Orso et al., 2009). Some species, such as *Drosophila melanogaster*, *C. elegans,* and *Danio rerio*, contain only one ATL gene, but many organisms express several isoforms (Rismanchi et al., 2008). The ATLs are anchored in the membrane by a hairpin of two closely spaced transmembrane segments, exposing both the N-terminal GTPase domain and the C-terminal tail to the cytosol. As in the case of the reticulons and DP1, the hairpin localizes the ATLs specifically to the tubular ER, perhaps by sensing the high curvature of tubules. All isoforms of ATLs interact with different isoforms of the reticulons and DP1 inside the membrane (Hu et al., 2009). A role for the ATLs in ER fusion is suggested by the observation that the depletion of ATLs leads to long, nonbranched ER tubules in tissue culture cells (Hu et al., 2009) and ER fragmentation in *Drosophila* (Orso et al., 2009), which may be caused by insufficient fusion between the tubules. Nonbranched ER tubules are also observed when dominant-negative ATL mutants, which have reduced GTPase activity or lack either the cytosolic or transmembrane regions, are expressed (Hu et al., 2009). In addition, antibodies to ATL inhibit ER network formation in *Xenopus* egg extracts (Hu et al., 2009). Finally, and most convincingly, proteoliposomes containing purified *Drosophila* ATL undergo GTP-dependent fusion in vitro (Orso et al., 2009).

Mutations in human ATL1, the isoform that is abundant in neuronal tissues, cause hereditary spastic paraplegia (HSP) (Zhao et al., 2001), a disease characterized by progressive spasticity and weakening of the lower limbs due to the axonal degeneration of the longest corticospinal motor neurons (Salinas et al., 2008). The depletion of ATL1 in primary neuron cultures leads to axon shortening (Zhu et al., 2006), mimicking the pathological condition, and the deletion or depletion of ATL in *Drosophila* and zebrafish causes neuronal defects (Fassier et al., 2010; Lee et al., 2009). Interestingly, mutations in two other ER proteins also frequently cause HSP (in spastin, a microtubule-severing ATPase, and in REEP1, a member of the DP1/Yop1p protein family). Mutations in the genes encoding ATL, spastin, and REEP1 collectively account for over 50% of all HSP cases (Salinas et al., 2008), suggesting that ER morphology defects may be a major cause of the disease. Defects in the function of these proteins might be particularly manifest in neurons with long axons, which rely to the greatest extent on an extended ER network. Although other HSP mutations affect genes that are not obviously related to ER morphology, they could still affect the ER indirectly, for example, by altering the cytoskeleton.

## Mechanism of ATL-Mediated Fusion

Insight into ATL-mediated fusion was obtained by determining crystal structures of the N-terminal part of human ATL1 (Bian et al., 2011; Byrnes and Sondermann, 2011), which includes the GTPase domain, a linker region, and a three-helix bundle. One crystal structure was obtained in the presence of GDP and high concentrations of inorganic phosphate (Figure 2, state 1). The structure shows a dimer in which the GTPase domains with their bound nucleotides face each other. The helical bundles of the two ATL molecules point in opposite directions, suggesting that in the full-length proteins, the molecules would sit in apposing membranes. This structure thus likely corresponds to a pre-fusion state, in which the two membranes are tethered but not yet fused. A second crystal structure has been obtained in the presence of GDP (Figure 2, state 5). Although the GTPase domains are arranged as in the pre-fusion state, the helical bundles of the two ATL molecules have undergone a major conformational change. They are now parallel to one another, and the C termini come so close that they have to sit in the same membrane. This conformation thus likely corresponds to

a post-fusion state. Interestingly, the two ATL molecules cross each other: the helix bundle of one molecule interacts with the GTPase domain of the other. The transition from the pre- to the post-fusion conformation was confirmed by trypsin protection and crosslinking experiments and occurs not only with the cytosolic domain of human ATL but also with the full-length *Drosophila* protein reconstituted into proteoliposomes (Bian et al., 2011).

Based on the structures and biochemical experiments, it was proposed that the first step in the fusion reaction is the GTP-dependent dimerization of ATL molecules sitting in apposing membranes (Figure 2, transition to state 1). Nucleotide-dependent dimerization has been verified experimentally and is indeed a common feature of dynamin-like GTPases. Following GTP hydrolysis and phosphate release, the helical bundle of each ATL molecule would be dislodged from its own GTPase domain (state 2). The linker region would then allow the GTPase domains to rotate freely (state 3). When rotated by 180°, each GTPase domain could capture the helical bundle of the partner molecule (state 4), which would pull the apposing membranes together so that they can fuse (state 5). In principle, it is possible that the GTPase domains are stationary and the helical bundles move instead, but it is difficult to see how this would occur when the bundles are anchored in membranes. The final step would be release of GDP, which would dissociate the ATL dimers (state 6) and set the stage for the next round of fusion. The proposed model is consistent with the location of mutations that cause HSP, as many of them would be expected to disrupt dimer formation or disturb the conformational change deduced from the crystal structures (Bian et al., 2011).

A comparison of the pre- and post-fusion conformations of ATL indicates that the gain in energy is much smaller than observed in fusion reactions caused by SNAREs or viral proteins. This suggests that domains not included in the crystal structures may contribute to ATL-mediated fusion. In fact, deletion of the C-terminal tail following the two transmembrane segments drastically reduces fusion in vitro and causes HSP in humans (Bian et al., 2011). The tail contains a conserved, predicted amphipathic helix that may destabilize the lipid bilayer and thus facilitate fusion. Indeed, point mutants in the hydrophobic face of the amphipathic helix abolish fusion, and synthetic peptides, but not peptides carrying the mutations, stimulate fusion (T. Liu, X. Bian, S. Sun, X. Hu, T.A.R., and J.H., unpublished data). It is also likely that the two transmembrane segments play a role, perhaps by mediating oligomerization. This is supported by the observation of higher oligomers with full-length ATL (Zhu et al., 2006; Rismanchi et al., 2008), by the fact that the expression of the membrane-embedded region plus the C-terminal tail acts as a dominant-negative mutant (Hu et al., 2009), and by the finding that the transmembrane segments cannot be replaced by unrelated hydrophobic sequences (unpublished data). Perhaps, these oligomers allow multiple ATL molecules in each membrane to simultaneously undergo the proposed conformational change. Finally, it should be noted that the experiments so far have not entirely excluded the possibility that the ATLs only mediate hemifusion, rather than the fusion of both leaflets of the lipid bilayer, and that other factors contribute to the fusion reaction in vivo.

ATL-mediated homotypic fusion differs from heterotypic viral and SNARE-mediated fusion, in that nucleotide triphosphate hydrolysis directly drives the reaction. In SNARE-mediated fusion, nucleotide hydrolysis by the NEM-sensitive fusion factor (NSF) is used to reset the fusion machinery by disassembling SNARE complexes, and viral fusion does not require nucleotide hydrolysis at all. On the other hand, the mechanism proposed for ATLs may also apply to the mitofusins (Fzo1p in *S. cerevisiae* and fuzzy onion in *Drosophila*). These are proteins that mediate the homotypic fusion of the outer membrane of mitochondria (Hermann et al., 1998; Chen et al., 2003). Like the ATLs, the mitofusins are GTPases of the dynamin family that contain two closely spaced transmembrane segments and a cytoplasmic tail (which is longer than in the ATLs). Thus, it is possible that membrane-bound GTPases of the dynamin family may be generally involved in the homotypic fusion of organelles.

## Homotypic ER Fusion in Yeast and Plants

Yeast and plant cells do not possess ATLs, but a similar GTPase, called Sey1p in *S. cerevisiae* and Root Hair Defective 3 (RHD3) in *Arabidopsis thaliana*, may have an analogous function. All eukaryotic organisms appear to have either ATL or Sey1p homologs, and no species has both. Sey1p was originally identified as a genetic interaction partner of Yop1p (hence the name *s*ynthetic *e*nhancement of *YOP1*) (Brands and Ho, 2002), immediately suggesting that it plays a role in formation of the tubular ER. Sey1p has the same topology as ATL, that is, an N-terminal GTPase domain, a predicted helical bundle (which is significantly longer than in ATLs), two closely spaced transmembrane segments, and a C-terminal tail that includes an amphipathic helix. The GTPase domain contains the signature motifs that are typical for this subfamily of dynamin-like proteins. Like the ATLs, Sey1p interacts with the curvature-stabilizing proteins and localizes to the tubular ER (Hu et al., 2009). Mutations in the plant homolog RHD3 cause ER morphology defects (Zheng et al., 2004), similar to those seen after depletion of ATLs. Surprisingly, in yeast the deletion of *SEY1* alone does not abolish the tubular ER or result in ER fragmentation (K. Anwar, R. Klemm, A. Condon, M. Zhang, G. Ghirlando, J.H., T.A.R., and W.A.P., unpublished data); it requires the absence of both Sey1p and one of the curvature-stabilizing proteins Rtn1p or Yop1p to disrupt the tubular ER (Hu et al., 2009). Normal ER morphology can be re-established by expression of wild-type Sey1p, but not by Sey1p defective in GTP binding. Human ATL can partially replace Sey1p. A direct role for Sey1p in ER fusion is suggested by experiments in which the fusion of ER membranes is assessed after mating of haploid yeast cells and by the observation that proteoliposomes containing purified Sey1p undergo GTP-dependent fusion in vitro (unpublished data). All these results suggest that Sey1p functions like ATL to mediate homotypic ER fusion. However, the lack of an ER morphology defect in a *sey1* deletion mutant indicates that there must be an alternative fusion mechanism in yeast. This process might involve ER SNAREs, which had previously been implicated in homotypic ER fusion (Patel et al., 1998). Recent results indicate that cells lacking Sey1p and the ER SNARE Ufe1p have severe ER morphology defects and that there is a strong genetic interaction between *SEY1* and *UFE1* or *USE1*, which encodes another ER

SNARE (unpublished data). It is unclear why mammalian and plant cells do not have a back-up fusion mechanism, but perhaps the alternate process occurs only in the cortical ER, which is much more abundant in yeast.

## Perspective

Despite significant progress over recent years, much remains to be learned. One immediate goal is a molecular understanding of how ATL and Sey1p catalyze homotypic ER fusion. The molecular mechanism of membrane fusion remains a general unresolved issue, but the GTP dependence of ATL-dependent fusion offers the opportunity to analyze intermediate stages of the reaction, something that is not easily done for viral or SNARE-mediated fusion. The role of ATL and Sey1p in vivo also needs further investigation. For example, there may be a link between these GTPases and lipid droplets. Many questions also concern the reticulons and DP1/Yop1p. Are the models for how they shape ER tubules correct? What is the significance of their interaction with ATL and Sey1p? Why are there so many isoforms of these proteins, which often have long segments unrelated to their proposed role in curvature stabilization? Are they regulated during the cell cycle?

Concerning the more general question of how other ER domains are generated, the surface has only been scratched. Recent work has provided first insight into how peripheral ER sheets might be generated (Shibata et al., 2010), but the exact function of the potential sheet-promoting proteins (Climp-63, p180, kinectin) remains to be elucidated, and the mechanism by which sheets are stacked on top of each other is totally unknown. Exciting research topics also include whether and how ER fission occurs, how the nuclear envelope is shaped, and how ER membranes interact with the cytoskeleton and molecular motors. One might hope that answering these questions will also have an impact on the understanding of the morphology of other organelles.

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