ABSTRACT

The endoplasmic reticulum (ER) is a major membrane-bound organelle in all eukaryotic cells. This organelle comprises morphologically distinct domains, including the nuclear envelope and peripheral sheets and tubules. The tubules are connected by three-way junctions into a network. Several membrane proteins have been implicated in network formation; curvature-stabilizing proteins generate the tubules themselves, and membrane-anchored GTPases fuse tubules into a network. Recent experiments have shown that a tubular network can be formed with reconstituted proteoliposomes containing the yeast membrane-fusing GTPase Sey1 and a curvature-stabilizing protein of either the reticulon or REEP protein families. The network forms in the presence of GTP and is rapidly disassembled when GTP hydrolysis of Sey1 is inhibited, indicating that continuous membrane fusion is required for its maintenance. Atlastin, the ortholog of Sey1 in metazoans, forms a network on its own, serving both as a fusion and curvature-stabilizing protein. These results show that the reticular ER can be generated by a surprisingly small set of proteins, and represents an energy-dependent steady state between formation and disassembly. Models for the molecular mechanism by which curvature-stabilizing proteins cooperate with fusion GTPases to form a reticular network have been proposed, but many aspects remain speculative, including the function of additional proteins, such as the lunapark protein, and the mechanism by which the ER interacts with the cytoskeleton. How the nuclear envelope and peripheral ER sheets are formed remain major unresolved questions in the field. Here, we review reconstitution experiments with purified curvature-stabilizing proteins and fusion GTPases, discuss mechanistic implications and point out open questions.

KEY WORDS: Cell biology, Endoplasmic reticulum, Reconstitution, Reticulon, Atlastin, Lunapark

Introduction

All organelles have characteristic shapes, but how their morphology is generated is largely unknown. The endoplasmic reticulum (ER) is a particularly intriguing organelle, as it consists of morphologically distinct domains that change during differentiation and the cell cycle. In interphase, the ER consists of the nuclear envelope and a connected peripheral network of tubules and interspersed sheets (Baumann and Walz, 2001; Shibata et al., 2009; Terasaki et al., 1984; Voeltz et al., 2006). The tubules continuously form, retract and slide along one another, a process facilitated by associated molecular motors and dynamic changes of the cytoskeleton. During mitosis in metazoans, the nuclear envelope disassembles and peripheral ER tubules appear to be transformed into sheets (Lu et al., 2009; Wang et al., 2013). How the network is generated and maintained, and how its morphology changes during the cell cycle, is poorly understood.

The tubules themselves are shaped by two evolutionarily conserved protein families, the reticulons (Rtns) and REEPs (Yop1 in yeast) (Voeltz et al., 2006). These are ubiquitous membrane proteins that are both necessary and sufficient to generate tubules (Hu et al., 2008). They belong to the ten most-abundant membrane proteins in a cell and together occupy ~10% of the membrane surface of the reticular ER in *Saccharomyces cerevisiae* (Hu et al., 2008). The Rtns and REEPs stabilize the high membrane curvature seen in cross-sections of tubules and sheet edges. How these proteins generate and stabilize membrane curvature is uncertain, as there are no structures available, but they all contain pairs of closely spaced transmembrane (TM) segments and have an amphipathic helix (Fig. 1A).

Connecting tubules into a network requires membrane fusion, which is mediated by membrane-anchored GTPases, the atlastins (ATLs) in metazoans and Sey1 and related proteins in yeast and plants (Hu et al., 2009; Orso et al., 2009). Mammals have three closely related ATLs, with ATL1 being prominently expressed in neurons, and ATL2 and ATL3 more broadly in different cell types (Zhu et al., 2003). These proteins contain a cytoplasmic GTPase domain, followed by a helical bundle, a membrane-embedded region and an amphipathic helix (Fig. 1B). Until recently, the membrane-bound region was thought to comprise two closely spaced TM segments, but it may actually consist of two intramembrane hairpin loops (Betancourt-Solis et al., 2018), similar to those in the Rtns and REEPs. Crystal structures and biochemical experiments have led to a model in which ATL molecules localized to different membranes dimerize through their GTPase domains, and undergo a conformational change during the GTPase cycle, thereby pulling the two membranes together and fusing them (for a review, see Hu and Rapoport, 2016). Mutations in ATLs and REEPs can cause hereditary spastic paraplegia (Hübner and Kurth, 2014; Salinas et al., 2008; Westrate et al., 2015), a neurodegenerative disease that is characterized by length-dependent axonal dysfunction or degeneration.

The fusion of membrane tubules generates three-way junctions, which are small, triangular sheets with negatively curved edge lines (Shemesh et al., 2014). It has been suggested that these junctions are stabilized by the lunapark protein (Lnp, also known as LNPK) (Chen et al., 2012, 2015; Shemesh et al., 2014), a conserved membrane protein containing two closely spaced TM segments and several other domains (Fig. 1C).

Peripheral ER sheets might be generated by the Rtns and REEPs stabilizing the highly curved membrane edges. Consistent with this model, overexpression of these proteins in *S. cerevisiae* and mammalian cells favors the formation of tubules over sheets (Shibata

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**REVIEW**

**Reconstituting the reticular ER network – mechanistic implications and open questions**

Ning Wang and Tom A. Rapoport*

Howard Hughes Medical Institute and Department of Cell Biology, Harvard Medical School, 240 Longwood Ave, Boston, MA 02115, USA.

*Author for correspondence (tom_rapoport@hms.harvard.edu)

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Reconstituted systems containing purified proteins have been instrumental in understanding the mechanism of many biological processes, including transcription (Ge et al., 1996; Sayre et al., 2010; Voeltz et al., 2006). Conversely, sheets become more abundant at the expense of tubules when these proteins are absent (Shibata et al., 2010; Voeltz et al., 2006) or when phospholipid synthesis is increased (Shibata et al., 2010). In higher eukaryotes, sheet formation is stimulated by coiled-coil-containing membrane proteins, such as p180 (also known as RRB1), kinecin (also known as KTN1) and CLIMP-63 (also known as CKAP4), with the latter forming luminal bridges between the apposing membranes (Shibata et al., 2010).

How the nuclear envelope is formed remains unclear. Experiments in Xenopus egg extracts have suggested that both ATL- and ER SNARE-mediated membrane fusion steps are required to form the double membrane around chromatin (Wang et al., 2013), but how exactly membranes bind and fuse on the surface of chromatin at the end of mitosis remains to be elucidated.

Reconstructed systems containing purified proteins have been instrumental in understanding the mechanism of many biological processes, including transcription (Ge et al., 1996; Sayre et al., 1992), replication (Waga and Stillman, 1994; Yeeles et al., 2015), protein translocation (Akimaru et al., 1991; Brundage et al., 1990; Görlich and Rapoport, 1993) and ER-associated protein degradation (ERAD) (Baldrige and Rapoport, 2016). This Review focuses on the ongoing efforts to reconstitute the ER. Our goal is to establish reconstituted systems that recapitulate the formation of different ER morphologies. As a first step, we concentrated on the formation of a tubular ER network. In this Review, we will first discuss the reconstitution of membrane tubules by the curvature-stabilizing proteins of the Rtn and REEP families, then cover the reconstitution of membrane fusion by the ATL/Sey1 GTPases, and finally the formation of a reticular membrane network by co-reconstitution of the curvature-stabilizing proteins and fusion GTPases. At the end of this Review, we point out open questions and future directions.

**Tubule formation by reconstituted Rtns or REEPs**

Our initial reconstitution experiments focused on the role of the Rtns and REEPs in tubule formation (Hu et al., 2008). Yeast Yop1 (the single REEP family member in *S. cerevisiae*) and Rtn1 were expressed in *Escherichia coli* or *S. cerevisiae* and purified to near homogeneity in detergent. After mixing with phospholipids, the detergent was removed and the samples were analyzed by negative-stain electron microscopy (EM). At early time points (a few hours) during detergent removal, small particles were observed. These might be small vesicles, but their small size and the lack of an obvious luminal space raises the possibility that they are micelles containing a mixture of protein, lipids and detergent. Ultimately, after longer times of detergent removal, membrane tubules were observed that reached several hundred nanometers in length, indicating that the Rtns and REEPs alone can generate the high membrane curvature that is characteristic of tubules when viewed in cross-section. The tubules had a luminal space, but were much narrower than ER tubules observed in yeast cells (17 nm versus 30 nm). This can be explained by the significantly higher protein-to-lipid ratio (by a factor of ~20). Indeed, when Rtn or REEPs were overexpressed in yeast, plant or mammalian cells, ER tubules became so narrow that luminal proteins were forced to move to other areas of the ER (Hu et al., 2008; Tolley et al., 2008). Interestingly, ER tubules with a diameter of ~20 nm are common in neuronal axons (Terasaki, 2018), indicating that narrow tubules can be physiological. However, it is unknown whether these tubules contain a particularly high concentration of curvature-stabilizing proteins.

The reconstitution experiments are consistent with results obtained with more physiological systems. For example, antibodies to an Rtn isoform (Rtn4a) block the formation of a tubular ER network in Xenopus egg extracts (Voeltz et al., 2006). Although the exact mode of action of the antibodies remains unclear, those experiments provided the first evidence that the Rtns are involved in tubule formation. Further evidence was provided by experiments with intact cells. Deletion of the Rtns and Yop1 in *S. cerevisiae*, and depletion of the proteins in mammalian cells, resulted in the conversion of ER tubules into sheets (Anderson and Hetzer, 2008; Voeltz et al., 2006; Wang et al., 2016); and overexpression of these proteins led to long tubules (Voeltz et al., 2006; Wang et al., 2016). Finally, all these proteins localize to tubules or sheet edges and avoid the low curvature areas of sheets (Shemesh et al., 2014; Shibata et al., 2010; Voeltz et al., 2006; Wang et al., 2016). Targeting to the high-curvature regions appears to be mediated by the TM hairpins, which can also be found in all the other proteins that specifically localize to these areas, such as ATLs, Sey1 and Lnp. Taken together with the reconstitution experiments, these results indicate that the Rtns and REEPs stabilize high membrane curvature.

The mechanism by which these proteins induce and stabilize high membrane curvature remains unclear. It has been proposed that they utilize two cooperating mechanisms, hydrophobic insertion (wedging) and scaffolding (Fig. 1D) (reviewed in Shibata et al., 2010).
The hydrophobic insertion mechanism postulates that the two hydrophobic TM hairpins in these proteins, together with the amphipathic helix, 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 Rns and REEPs form arc-like oligomers that mold the lipid bilayer into tubules (Fig. 1D). Arc-like scaffolds, rather than rings or spirals that wrap around tubules, are consistent with the observation that the Rns also localize to sheet edges and that the diffusion of ER proteins along tubules is not restricted (Shibata et al., 2008, 2010; Shemesh et al., 2014). Structural information on the Rns and REEPs are required to test these ideas.

### Membrane fusion in systems reconstituted with ATL or Sey1

The fusion of ER membranes could also be recapitulated with purified proteins. *Drosophila* ATL was successfully purified after expression in *E. coli* and reconstituted with phospholipids into proteoliposomes; the subsequent addition of GTP resulted in the fusion of these vesicles, as initially demonstrated by lipid mixing and size increase of the vesicles (Orso et al., 2009), and later by content mixing (Liu et al., 2012). The lipid composition of the membranes does not seem to play a major role (Orso et al., 2009). GTPase-competent ATL molecules need to be present in both vesicles for their fusion to happen (Orso et al., 2009). Similar results were obtained with Sey1, also purified after expression in *E. coli* (Anwar et al., 2012; Powers et al., 2017). These results indicate that these GTPases alone can mediate the homotypic fusion of membranes.

The reconstitution experiments are again in agreement with experiments performed in physiological systems, as the depletion of ATLs or the expression of dominant-negative mutants leads to ER tubule fragmentation and the appearance of long, unbranched ER tubules in tissue culture cells (Hu et al., 2009; Wang et al., 2016) or *Drosophila melanogaster* (Orso et al., 2009). These phenotypes are likely caused by insufficient fusion between the tubules. Similarly, in *Xenopus* egg extracts, the addition of a dominant-negative ATL mutant or of non-hydrolysable GTP analogs prevents ER network formation (Wang et al., 2013). Surprisingly, these reagents also cause a pre-formed ER network to disintegrate into smaller membrane structures within a few minutes (Wang et al., 2013), indicating that continuous ATL function is required to maintain the integrity of an ER network.

The molecular mechanism of ATL function has been elucidated from crystal structures of the cytosolic domain (Bian et al., 2011; Byrnes and Sondermann, 2011) and biochemical studies (Bian et al., 2011; Byrnes and Sondermann, 2011; Liu et al., 2012, 2015). These have led to a plausible model for ATL-mediated membrane fusion, which begins with the binding of GTP to ATL monomers (Fig. 2; step 1). The monomers then rapidly form a dimer, with the bound GTP molecules buried at the interface. When GTP is hydrolyzed by the dimer, the two helical bundle domains associate with one another, generating a tight dimer in the transition state (step 2). This interaction likely pulls the two membranes together so that they can fuse. The C-terminal amphipathic helix may facilitate membrane fusion, likely by perturbing the phospholipid bilayer (Liu et al., 2012). Finally, phosphate (P) and GDP are released in a sequential manner, causing the dissociation of the dimer into monomers (step 3). All these reactions can occur with ATL molecules located either in different or the same membranes (trans- and cis-interactions, respectively), but membrane tethering and fusion happen only with trans-interactions (Liu et al., 2015). The formation and disassembly of cis-dimers is thus a futile cycle.

ATLs also form trans-dimers in a futile manner (Liu et al., 2015; Saini et al., 2014). Experiments with reconstituted proteoliposomes have shown that multiple GTPase cycles are required for a successful fusion event, as the transition state of trans-dimers often converts them back into monomers without having caused fusion (Liu et al., 2015). Other experiments have indicated that the efficiency of tethering and fusion increases with the density of ATL molecules in the two membranes (Liu et al., 2015). ATL molecules appear to associate with one another through their TM segments. In fact, the TM segments cannot be replaced by those from other proteins, and point mutations in these regions can reduce the efficiency of fusion (Liu et al., 2012). Recent experiments suggest that the TMs form two intramembrane hairpin loops, rather than two segments that completely span the membrane (Betancourt-Solis et al., 2018), which may explain their unique role in fusion. Taken together, it appears that a successful fusion event requires the cooperation of multiple trans-dimerization events: as one dimer reaches the transition state, other dimers forming nearby help to maintain the tethered state and add to the pulling force exerted on the opposing membranes. The futile formation of trans-dimers suggests that a fraction of three-way junctions in cells may actually consist of tethered, rather than fused, tubules, and may explain, at least in part, why the ER network rapidly disassembles upon inhibition of the GTPase activity of ATL (Wang et al., 2013).

### Reconstitution of a reticular ER network

The next step in reconstituting the ER is to generate a reticular ER network. We therefore tested whether using a curvature-stabilizing protein, an Rn or REEP protein, and a fusion GTPase, either ATL or Sey1, is sufficient (Powers et al., 2017). To this end, we first purified full-length *S. cerevisiae* Yop1 and Sey1 after expression in *E. coli* and reconstituted both proteins together into proteoliposomes. Essentially all molecules were incorporated into the vesicles with their cytosolic domains on the outside, as shown by protease protection experiments. The reconstituted vesicles converted into a reticular network upon addition of GTP, which could be visualized with a hydrophobic fluorescent dye in a light microscope (Fig. 3) or
of tubules may preferentially occur at free ends, as the tip of a membrane tubule already corresponds to half a vesicle. However, such free ends are infrequent in mammalian cells and in Xenopus extracts (Friedman et al., 2010; Wang et al., 2013); most tubules are anchored at three-way junctions, and free ends are often associated with the tip of a growing microtubule or molecular motor (Friedman et al., 2010). Fusion of the tips of ER tubules with other tubules continuously generates new three-way junctions (Lee and Chen, 1988) and thus increases the number of polygons in the tubular network. This process is counteracted by ‘ring closure’ (Lee and Chen, 1988), a process in which three-way junctions merge and thus abolish polygons. The dynamics of the ER network is different from that of mitochondria, which maintain their structure by a balance of fusion and fission reactions (Shaw and Nunnari, 2002).

The Rtns and REEPs have been shown to form oligomers, which could contribute to tubule formation (Shibata et al., 2008). However, whether their oligomeric state is different in tubules and vesicles and affected by the fusion GTPases remains to be tested. The roles of the amphipathic helices of the curvature-stabilizing proteins and GTPases in network formation also needs to be clarified.

Reconstitution of Lnp and its implications

Another protein that is important for ER network formation is Lnp, a conserved membrane protein containing two closely spaced TM segments, two coiled-coil regions and a Zn\(^{2+}\)-finger domain involved in dimerization (Fig. 1C) (Wang et al., 2018). The mammalian protein also has a domain that is phosphorylated during mitosis. The protein was originally discovered in a genetic screen in S. cerevisiae for ER morphology defects and recognized as a counteracting factor of the fusion GTPase Sey1 (Chen et al., 2012). It was later proposed to stabilize three-way tubular junctions (Chen et al., 2015; Shemesh et al., 2014). However, although Lnp localizes preferentially to three-way junctions of the ER, it is not essential for their formation. Indeed, mammalian cells lacking Lnp have more ER sheets, but the tubular network does not completely disappear, particularly remaining at the periphery of the cells (Wang et al., 2016; Zhou et al., 2018).

To better understand the function of Lnp, we purified Xenopus or human Lnp after expression in E. coli, before mixing the protein with lipid and removing the detergent (Wang et al., 2018). Surprisingly, negative-stain EM showed that the resulting structures were stacked membrane discs (bicelles) (Fig. 4A), as confirmed by EM cryomicrography. Mutagenesis indicated that the Lnp protein is incorporated into the membrane discs in both orientations; the discs are linked with one another through the cytosolic domains of Lnp (Fig. 4B). The domain phosphorylated during mitosis is important for trans-interactions between Lnp molecules, and a phosphomimetic Lnp mutant showed reduced bicelle stacking (Wang et al., 2018). Inactivation of Lnp during mitosis is also suggested by experiments with Xenopus egg extracts: treatment with dominant-negative Lnp fragments converts the tubular ER into a network of small sheets that are connected by short tubules, which resembles the ER morphology seen during mitosis (Wang et al., 2016).

We have proposed that Lnp localizes preferentially to three-way junctions because it engages in trans-interactions when junctions come into close proximity within a 3D network (Wang et al., 2018). Three-way junctions are small, triangular membrane sheets and offer a better geometry for trans-interactions between multiple Lnp molecules than tubules. Lnp-containing three-way junctions may not be able to undergo efficient membrane fusion because Lnp could prevent ATL from entering the junctional sheets or because

### Fig. 3. Generation of a tubular membrane network with purified proteins.

Purified Yop1, a curvature-stabilizing protein from S. cerevisiae that belongs to the REEP family, was mixed with purified Sey1, the fusion GTPase in S. cerevisiae. Phospholipids, including Rhodamine-labeled phosphoethanolamine (PE) were added, and the detergent was subsequently removed to generate proteoliposomes. The vesicles were incubated without or with GTP (left and right panel, respectively). The samples were placed on a coverslip and visualized in a fluorescence microscope. Scale bars: 20 μm. The figure is reproduced from Powers et al., (2017) with permission from Springer Nature.

**What is the mechanism of ER network formation?**

Both the in vivo and in vitro results indicate that the ER network corresponds to a steady-state of continuous membrane fusion and fragmentation. Fusion is mediated by the GTPases ATL or Sey1 and fragmentation is caused by the curvature-stabilizing proteins of the Rtn and REEP/Yop1 families, which, in the absence of ATL activity, appear to prefer the higher membrane curvature of small vesicles to that of tubules. In a steady-state network, fusion mediated by the GTPases appears to be faster than the breakage of tubules or the shedding of small vesicles mediated by the curvature-stabilizing proteins, explaining why tubule fission is rarely observed in vivo (Friedman et al., 2010). Under normal conditions, the disassembly
state that requires continuous GTP hydrolysis by ATL/Sey1. The resulting network represents a dynamic curvature-stabilizing protein, that is, a member of the Rtn or REEP system required to form a reticular ER network consists of a reconstitution experiments discussed here show that the minimum network (Wang et al., 2016). It is also consistent with the observation that in \textit{S. pombe}, Lnp and Sey1 have opposing effects on ER morphology (Chen et al., 2012). The postulated mechanism in which Lnp counteracts ATL is consistent with the structure of the Rtn or REEP proteins, which will shed light on how these proteins induce high membrane curvature. The possible role of oligomers formed by these proteins and the significance of their TM hairpins and amphipathic helices also need to be clarified. Further studies are also needed on whether a physical interaction between the curvature-stabilizing proteins and the fusion-mediating GTPases is required for network formation.

It remains puzzling that there are so many different Rtn and REEP proteins, even within a single cell. Several members of these protein families have large cytosolic domains of unknown function. Presumably, these domains bind other proteins and might diversify the function of the Rtns and REEPs. In addition, several Rtn-like proteins have been discovered, including Ts1 in \textit{S. pombe} (Zhang and Oliﬁrenko, 2014; Zhang et al., 2010) and its homologs in other organisms, the ER autophagy receptor FAM134 (also known as RETREG1) (Khaminets et al., 2015) and ARL6IP1, which recruits an inositol 5-phosphatase to the ER (Dong et al., 2018). Whether these proteins induce high membrane curvature and whether such an effect is required for their functions remains to be investigated.

Another unresolved issue concerns the interaction between the ER and the cytoskeleton. In mammalian cells, ER tubules are often generated by the force generated by either microtubule-associated molecular motors or by polymerizing microtubule tips (Lee and Chen, 1988; Prinz et al., 2000; Terasaki et al., 1986; Waterman-Storer and Salmon, 1998). The proteins that mediate the interaction with motors or microtubules have not yet been identiﬁed. In \textit{S. cerevisiae}, ER tubules are formed along actin ﬁlaments (Estrada et al., 2003). Although the cytoskeleton is required for the generation and distribution of the ER network, ultimately the alignment of ER tubules with the cytoskeleton is not perfect. In addition, the ER network does not collapse upon depolymerization of actin ﬁlaments in yeast (Prinz et al., 2000), and it retracts only with some delay upon depolymerization of microtubules in mammalian cells (Terasaki et al., 1986). In vitro, an ER network can be generated in the absence of an intact cytoskeleton (Dreier and Rapporport, 2000). Interestingly, p180 and CLIMP-63, the membrane proteins implicated in the generation of peripheral ER sheets in mammalian cells, contain microtubule-binding domains (Klopfenstein et al., 1998; Ogawa-Goto et al., 2007), but their precise function remains unclear.

Peripheral ER sheets seem to be present in all cells, although some may actually be fenestrated (Pulka et al., 2012) or composed of dense clusters of three-way tubular junctions (Nixon-Abell et al., 2016). The abundance of peripheral ER sheets varies among cells and changes during the cell cycle. Although controversial (Pulka et al., 2012), in some mammalian tissue culture cells (Lu et al., 2009) and in \textit{Xenopus} extracts (Wang et al., 2013), ER tubules convert into sheets during mitosis. Sheets are generally thought to correspond to rough ER (Shibata et al., 2006), that is, regions containing membrane-bound ribosomes. The membranes of the rough and smooth ER are continuous, and yet the ribosome-studded area is kept segregated. How such a distinct rough ER domain is formed remains unclear. The concentration of sheets in one area of the cell requires intact polysomes and the presence of the sheet-promoting membrane proteins p180, kinecin and CLIMP-63 (Shibata et al., 2010). Perhaps, these membrane proteins link different polysomes to form a rough ER domain. How peripheral ER sheets are stacked on top of each other in highly secretory cells also remains to be investigated. Serial sectioning EM has demonstrated that the sheets are connected by twisted membrane surfaces,
resembling a parking garage in which the different levels are connected by helicoidal ramps (Terasaki et al., 2013). However, the proteins required for sheet stacking are currently unknown.

Finally, the mechanism of nuclear envelope formation remains one of the most challenging unresolved issues. Experiments with Xenopus extracts have suggested that two membrane-fusion steps are required, one mediated by ATL to generate an ER network and a second mediated by ER SNAREs and NSF to form the nuclear envelope (Wang et al., 2013). However, the role of ER SNARE-mediated fusion is based on dominant-negative reagents and thus requires further studies in vitro and in vivo. The formation of a double membrane around chromatin in Xenopus egg extracts does not require nuclear pores or lamins (Newport et al., 1990; Sullivan et al., 1993). How membrane fusion is coordinated with chromatin decondensation at the end of mitosis (Ramadan et al., 2007), and how it is confined to the surface of chromatin are also important questions for the future. Ultimately, the reconstitution of nuclear envelope formation with purified components will be required to understand its molecular mechanism.

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